

# Progenesis QI for proteomics User Guide

Analysis workflow guidelines for HDMse and MSe data

# THE SCIENCE OF WHAT'S POSSIBLE.

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## Introduction

This user guide takes you through a complete analysis of 9 LC-MS runs with 3 groups (3 replicate runs per group) using the unique Progenesis QI for Proteomics workflow. It starts with LC-MS data loading then Alignment, followed by Peak Detection that creates a list of interesting peptide ions (peptides) which are explored within Peptide Ion Stats using multivariate statistical methods then onto Protein identity and Protein Stats.

To allow ease of use the tutorial is designed to start with the restoration of an Archived experiment where the data files have already been loaded. The document covers all the stages in the workflow, initially focusing on the Automatic Processing of the data then the use of the individual analysis stages.

If you are using your own data files please refer to Appendix 1 (page 75) then start at page 6.

## How to use this document

You can print this user guide to help you work hands-on with the software. The complete user guide takes about 60 to 90 minutes. This means you can perform the first half focused on LC-MS run alignment and analysis then complete the second half of analysis exploring comparative differences and Protein identity at a convenient time.

If you experience any problems or require assistance, please contact us at <a href="mailto:support@nonlinear.com">support@nonlinear.com</a>

# How can I analyse my own runs using Progenesis QI for proteomics?

You can freely explore the quality of your LC-MS data using Data Import and then licence your own LC-MS runs using this evaluation copy of Progenesis QI for proteomics. Instructions on how to do this are included in a section at the end of the user guide document.

# LC-MS Data used in this user guide

For the purposes of this data set the MS<sup>E</sup> parameters were set to 250:125 instead of the default settings as defined in Appendix 1 (page 75). This was done to reduce the time taken to demo the data analysis.

# Workflow approach to LC-MS run analysis

Progenesis QI for proteomics adopts an intuitive **Workflow** approach to performing comparative LC-MS data analysis. The following user guide describes the various stages of this workflow (see below) focusing mainly on the stages from Alignment to Report.



Stage	Description	Page
Import Data	LC-MS Import Data: Selection and review of data files for analysis	6
	Automatic Processing: setting up steps for automatic processing	7
	After Automatic Processing: how to work with auto analysed data	14
Licensing	<b>Licensing</b> : allows licensing of individual data files when there is no dongle attached (Appendix 3)	15
Review Alignment	Review Alignment: review of automatic and manual LC-MS run alignment	16
Filtering	<b>Filtering</b> : defining filters for peaks based on Retention Time, m/z, Charge State and Number of Isotopes.	23
	Review Normalisation: exploring LC-MS normalisation	26
Experiment Design Setup	<b>Experiment Design Setup</b> : defining one or more group set ups for the analysed aligned runs	29
Review Peak Picking	<b>Review Peak Picking</b> : review and validate results, edit peak detection, tag groups of peaks and select peaks for further analysis	32
Peptide Ion Statistics	<b>Peptide Ion Statistics</b> : performing multivariate statistical analysis on tagged and selected groups of peptide ions	45
Identify Peptides	Identify Peptides: managing export of MS/MS spectra to, and import of peptide ids from Peptide Search engines	49
QC Metrics	QC metrics: quality control charts for experimental/analysed data	52
Refine Identifications	Refine Identifications: manage filtering of peptide ids	54
	<b>Resolve Conflicts</b> : validation and resolution of peptide id conflicts for data entered from Database Search engines	56
Review Proteins	Review proteins: review protein and peptide identity and data export	60
Protein Statistics	Protein Statistics: multivariate statistical analysis on proteins	72
Report	Reporting: generate a report for proteins and/or peptides	73

Waters

# **Restoring the Tutorial**

Open Progenesis QI for proteomics and download the Compressed (.zip) Tutorial Archive file from the 'User guide and tutorial' link shown below, placing it in a **new folder** on your desktop. Before restoring the tutorial in the software **you must** first right click on the (.zip) file and extract it to the same folder.

Now restore the uncompressed tutorial archive file. To do this, first locate the **Progenesis QI.p Tutorial HDMSe.Progenesis QIP Archive** file using the **Open** button and press Open.

OP Progenesis QI for proteomics		
File		
Experiments		nonlinear
•		A Waters Company
Perform analysis Combine analysed fractions		New to Progenesis QI for proteomics?
Recent experiments	Search P	Here are some resources to help you get started with Progenesis QI for proteomics:
😋 🗢 📦 « Progene 🕨 Progenesis QI.p v4 HDMSe	Tutorial • • Search Progenesis QLp v4 HD •	<u>The Progenesis QI for proteomics</u> workflow
Organize 🔻 New folder	8≡ ▾ 🗔 💿	User guide and tutorial data
	Name Date modif	Frequently-asked questions  Quickly go to an ion map location Want to quickly validate your sample running by zooming to a known ion?      500 Go To Location
Retwork -	< III → Experiments and Archives (*.Prc ▼ Open ▼ Cancel	Jump to a specific m/z and RT using the <u>Go To Location tool</u> in the top-left corner of the ion maps.
Other experiments	4.0.6381.30896	Latest blog posts • Just because it's natural doesn't mean it's safe • Stay in the fast lane with Progenesis at 45%5 500 Indiananolis

This opens the 'Import from archive' dialog.

Select the **Create a new experiment** option and select the folder in which you placed the archive, using Browse.

OP Import Experiment from Archive					
Import experiment from archive After importing the experiment from this archive, any changes to the experiment will be saved to the location below, not back to the archive.					
Replace an existin	g experiment				
Experiment to repla	ace: Progenesis QLp Tutorial HDMSe 🔹				
Oreate a new expension	Create a new experiment				
Experiment name:	Progenesis QI.p Tutorial HDMSe				
Save to folder:	and Demo Suites\Progenesis QI.p Tutorial HDMSe Browse				
	Import				

Then press Import.

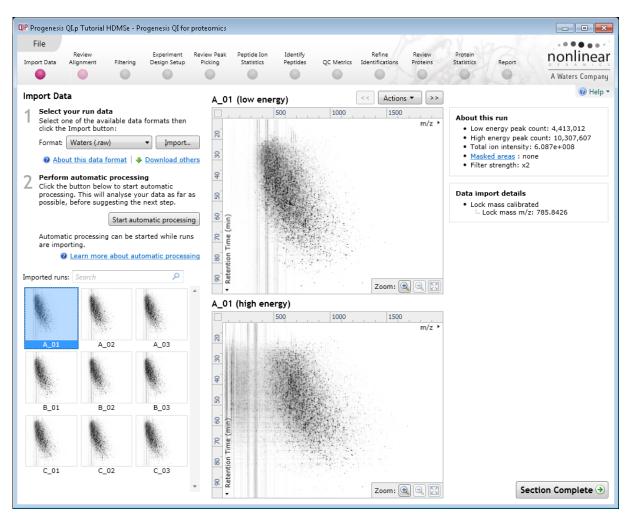
Loading: Progenesis QI.p Tutorial HDMSe			

**Note**: use the **Replace an existing experiment** option if you want to over-write an existing version of the tutorial.

Tip: at each stage in the software there are links to more information and help on the website.

# Stage 1: Import Data and QC review of LC-MS data set

The tutorial will now open at the Import Data stage (see below).



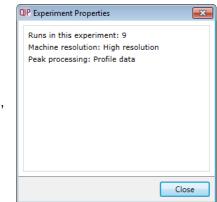
Each data file appears as a 2D representation of the run.

**Note**: the **Experiment Properties** are available from the File menu. These were selected when the experiment was created (see Appendix 1, page 75). The tutorial data is profile data.

*Tip*: the **'Mask areas for peak picking'** facility, accessed by right clicking on the run thumbnail) allows you to examine and exclude areas (usually early and/or late in the LC dimension (Retention Time)) that appear excessively noisy due to capture of data during column regeneration. This is not required for this data set.

**Note**: use the **Remove this run** to remove run(s) from the current experiment.

Now start the automatic processing.





# Stage 2A: Automatic Processing of your data

The Automatic Processing of your data can be set up and started before the import of your data has been completed by clicking on **Start automatic processing.** 

Note: for this tutorial the data has been imported already.

Perform automatic processing Click the button below to start automatic processing. This will analyse your data as far as possible, before suggesting the next step.				
	Start automatic processing			
are importing.	can be started while runs			
	Click the button below processing. This will a possible, before sugg Automatic processing are importing.			

Setup of processing steps in the Analysis Workflow, up to and including Identify Peptides, can be performed in the Automatic Processing wizard, these include:

- Select an alignment reference
- Automatic alignment of all runs to a reference run
- Automatic peak picking for peptide ion detection
- Define an Experiment design
- Perform peptide and protein identification (for MS<sup>E</sup> data only, as DDA data will require external database searching)
- Choosing a Quantitation method to be applied for abundance analysis

In this tutorial example you have 9 HDMSe LC-MS runs, so the automatic identification of peptides is available as part of the automatic processing.

As the runs have already been imported, click **Start automatic processing** to setup the Automatic processing wizard.

💵 Start automatic processing	
Select an alignment reference To compensate for drifts in retention time, all runs in the experiment must be aligned to a single reference run.	
How do you want to choose your alignment reference?	
Assess all runs in the experiment for suitability	
Output Set the most suitable run from candidates that I select	
🔘 Use this run:	
A 01 -	
For information on choosing the alignment reference, and why you might want to select your own candidates, please see the online guidance.	
server your own candidates, prease see the <u>omme garantee</u> .	_
< Back Next > Cancel	

Progenesis QI for proteomics provides three methods for choosing the alignment reference run:

### 1. Assess all runs in the experiment for suitability

long time for a large number of runs.

This method compares every run in your experiment to every other run for similarity, then selects the run with the greatest similarity to all other runs as the alignment reference. If you have no prior knowledge about which of your runs would make a good reference, then this choice will normally produce a good alignment reference for you. This method, however, can take a

2. Use the most suitable run from candidates that I select

This method asks you to make a selection of reference candidates; the automatic algorithm then chooses the best reference from this subset of runs.

This method is appropriate when you have some prior knowledge of your runs suitability as references:

i.e when all the candidate runs are pooled samples or when all the candidates are from a condition that displays the largest set of common peptide ions.

### 3. Use this run

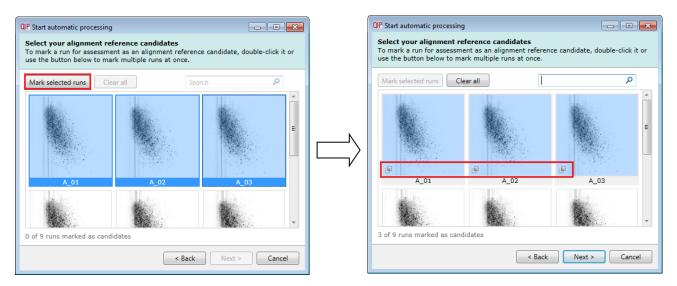
This method allows you to manually choose the reference run.

Manual selection gives you full control, but there are a couple of risks to note:

- If you choose a pending run which subsequently fails to load, alignment will not be performed.
- If you choose a run before it fully loads, and it turns out to have chromatography issues, alignment will be negatively affected (for this reason we recommend that you let your reference run fully load and assess its chromatography before loading further runs).

For this tutorial we will select the second option.

Select a subset of your runs as 'alignment reference candidates' and click **Mark selected runs.** An icon appears on the bottom left of each run to indicate that it is selected as a candidate.



On clicking **Next** you will be asked if you want to align your runs automatically.

The default is for automatic alignment, click **Next**.

QP Start automatic processing	- • •
Automatic alignment After selecting the experiment's alignment reference, the software car automatically align all runs.	n also
After the alignment reference is chosen, do you want to start automatic a	lignment?
< Back Next >	Cancel

The next page of the processing wizard will ask you if you want to **Perform peak picking** and allow you to set appropriate parameters.

QP Start automatic processing	- • •
Peak picking Peak picking is the process by which we locate the peptide ions and peaks in your samples.	their isotopic
After the automatic alignment is finished, do you want to start automat Perform peak picking	ic peak picking? Set parameters
< Back Next :	Cancel

For the purposes of this User guide we will use the default settings for peak picking except in the case of defining Retention Time limits where we will limit the peak picking to between 15 and 90 min.

Note: for more details on setting Peak Picking parameters refer to the section on Filtering (page 23)

Click on **Set parameters** and select the fourth tab to set **Retention time limits** for the detection. The default limits are displayed in the unticked boxes and detection will use these values whenever the box(s) are unticked.

QIP Peak Picking Parameters	<b>×</b>	Q	Peak Picking Parameters	s			×
Runs for peak picking Peak picking limi Choose runs for peak picking -	ts Maximum charge Retention time limits		Runs for peak picking	Peak picking limit	s Maximum charge	Retention tim	e limits
You can tick or un-tick each run to control which will be used by the peak picking algorithm. Although any run which is left un-ticked will not affect the peotide ion outlines, it will still have outlines added to it and will be available in the experiment design setup. Learn more about why you might not want to select all runs.	Ø Run         Ø A1         Ø A2         Ø A3         Ø C1         Ø C2         Ø C3		You can set the minimu maximum retention tim picking. Ions that elute after these values will t	ne for peak before or	✓ Ignore ions before ✓ Ignore ions after		minutes
	Start peak picking Cancel				Start pea	k picking	Cancel

Enter values of 15 and 90 min and tick the boxes as shown above.

Click **OK** to return to the Automatic Processing Wizard and click **Next** to Define an Experiment design.

QIP Start automatic pro	cessing	- • •
Experiment design Experiment designs experimental condit	allow you to group and compare	e your samples according to their
By defining an experi calculated automatic		al measures such as ANOVA can be
🔽 Set up an expe	iment design	
Enter a name fo	the experiment design:	
ABC		
Load the criteria	for grouping runs from this file:	
		Browse
Group runs by:	<no groups="" valid=""></no>	Ŧ
What file formats ar	e supported?	
	< Ba	ck Next > Cancel

To handle the grouping of your run data you can make use of **sample tracking information** that has been stored in a spread sheet at the time of sample collection and/or preparation.

**Note**: if a spreadsheet file of your data is not available you can create your experiment designs after the automatic analysis of the runs

For this example there is a **QIP\_Conditions.spl** file available with the Experiment Archive you restored at the beginning of this tutorial exercise.

Give the experiment design a name (i.e A B C) and then use the **Browse** function to locate the **QIP\_Conditions.spl** file.

OP Start automatic pr	ocessing	- • •		
Experiment design Experiment designs allow you to group and compare your samples according to their experimental conditions.				
By defining an exper calculated automatic	iment design in advance, statistical measures such as AN :ally.	OVA can be		
📝 Set up an expe	riment design			
Enter a name fo	or the experiment design:			
ABC		<b>(</b>		
Load the criteria	a for grouping runs from this file:			
10 Suites\Proge	enesis QI.p_utorial for HDMSe_v3.0\QIP_Conditions.SPL	Browse		
Group runs by:	VERSION	•		
	VERSION			
	Condition			
	Location			
	User Index			
	Index			
What file formats are supported?				
	< Back Next >	Cancel		

To use this approach select the **Import design from file** option from the New Experiment Design dialog. Then locate the QIP\_Conditions file and select what to **Group runs by**, for example: **Condition**.

Note: you can create additional experiment designs following the completion of automatic processing.

Select Conditions and then click Next.

For MS<sup>E</sup> fragmentation data you can set up the peptide identification to be performed automatically.

**Note**: if the software has detected MS<sup>E</sup> data then this option will be ticked by default.

QP Start automatic processing	QIP Enter search parameters	_ 0 🗾
Identify peptides Get identifications for the peptides in your samples using their MS <sup>s</sup> fragmentation data.	Enter the search parameters Select your FASTA file containing peptide and protein id SWISSPROT-1.0	entifications:
After peak picking has completed, do you wish to automatically identify peptides? Use MS <sup>4</sup> data from my runs to identify peptides <u>Set parameters</u>	Enter the search parameters to use: Common search parameters Digest reagent: Trypsin Missed cleavages: Max protein mass: 250 Modifications: Carbamidomethyl C Oxidation M Add/remove modifications Constructions Search tolerance parameters Solor Ion matching requirements Admin tools Change the digest reagents and modifications that are re-	
< Back Next > Cancel	Modi	Cave parameters

Click on Set Parameters. The default Databank is for Swissprot-1.0 (which is a locked example).

To create a new Databank from a Fasta file click on **Edit** and then create a new one using the example FASTA file that is available with the Experiment Archive you restored at the beginning of this tutorial exercise (**Tutorial\_nd\_DB.fasta**).

For a new Databank click **Add.** Then give it name (i.e. Tutorial DB), select the parsing rules (UNIPROT) and specify the location of the FASTA file, see the example below.

QIP D	atabank Editor		<b>X</b>
8	Name	Name:	Tutorial DB
â	SWISSPROT-1.0	Parsing rules:	
	Tutorial DB	Location:	
		Location:	C:\Users\andy.borthwick\Documents\Custo
	Add Remove		
			Save Cancel

The new Data bank will appear in the left panel now click Save to return to the Search parameters.

If your databank is not already displayed then select it from the drop down list.

### Check the Common search parameters

The default settings are displayed:

**Digest reagent:** is set as Trypsin. Alternative Digest reagents are available from the list and additional ones can be added to the list using the **Reagent editor...** 

Missed cleavages: is set as 1.

### Maximum protein mass: is set at 250kDa

**Modifications:** are set Carbamidomethyl C (Fixed) and Oxidation M (Variable). More modifications are available from the list and additional ones can be added to the list using the **Modification editor...** 

### Ion matching requirements: are set at

Fragments/peptide: 3, Fragments/protein: 7 and Peptides/protein: 1

### Click Save parameters and then Next.

OP Enter search param	eters	
Enter the search p Select your FASTA f	arameters ile containing peptide and protein identific	cations:
Tutorial DB		▼ Edit
Enter the search par	rameters to use:	
<ul> <li>Common sear</li> </ul>	ch parameters	
Digest reagent:	Trypsin	•
Missed cleavages:	· · · · ·	1 max
Max protein mass:	250	kDa 🔹
Modifications:           Search tolerand           Ion matching		
Fragments/peptide:	3	or more
Fragments/protein:	7	or more
Peptides/protein:	1	or more
Admin tools Change the digest n	eagents and modifications that are availa Modificatio	ble for peptide searches. on editor Reagent editor Save parameters

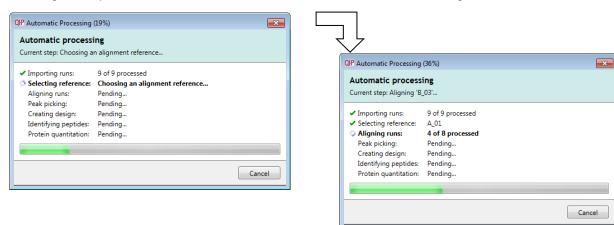
QIP Start automatic processing	OP Start automatic processing
Protein Quantitation If you've included a known amount of a calibrant protein in each of your samples, you can calculate the absolute amounts of each identified protein.	Protein Quantitation If you've included a known amount of a calibrant protein in each of your samples, you can calculate the absolute amounts of each identified protein.
Quantitation method:	Quantitation method:
Relative Quantitation using Hi-N	Relative Quantitation using Hi-N
Number of peptides to measure per protein (N):	Absolute Quantitation using Hi-N         • Requires a calibrant protein to calculate absolute amounts         • Uses the most abundant N peptides         • Allows comparison between proteins within a run         Relative Quantitation using Hi-N         • Uses the most abundant N peptides         • Allows comparison between proteins within a run
	Relative Quantitation using non-conflicting peptides • Uses only peptides which have no conflicting protein identifications • Allows comparison of a single protein across runs
Employ protein grouping, i.e. hide proteins whose peptides are a subset of another protein's.	Relative Quantitation using all peptides • Uses all peptides identified as part of a protein • Allows comparison of a single protein across runs protein's.
< Back Finish Cancel	< Back Finish Cancel

The **Protein Quantitation** dialog opens displaying the default method, **Relative Quantitation using Hi-N** which uses up to 3 peptides per protein to compute the relative amount of each 'identified' protein.

Use the drop down to reveal the alternative methods for protein quantitation

Select the Default option and click Finish.

The Alignment process starts with the automatic selection of A\_01 as the alignment reference



Once Alignment completes Peak Picking commences followed by Design and Identification.

OP Automatic Processing (44%) Automatic processing Current step: Analysing	
	QP Automatic Processing (73%)
<ul> <li>✓ Importing runs: 9 of 9 processed</li> <li>✓ Selecting reference: A_01</li> <li>✓ Aligning runs: 8 of 8 processed</li> <li>✓ Peak picking: Picking</li> </ul>	Automatic processing Current step: Searching A_02
Creating design: Pending Identifying peptides: Pending Protein quantitation: Pending	<ul> <li>✓ Importing runs: 9 of 9 processed</li> <li>✓ Selecting reference: A_01</li> <li>✓ Aligning runs: 8 of 8 processed</li> </ul>
Cancel	<ul> <li>✓ Peak picking: 46893 peptide ions found</li> <li>✓ Creating design: Created</li> <li>✓ Identifying peptides: Searching</li> <li>Protein quantitation: Pending</li> </ul>
	Cancel

Finally the Automatic Processing completes with the Quantitation being performed

OP Processing Complete	
Automatic process Time taken: 19 minutes 3	• •
<ul> <li>Importing runs:</li> <li>Selecting reference:</li> <li>Aligning runs:</li> <li>Peak picking:</li> <li>Creating design:</li> <li>Identifying peptides:</li> <li>Protein quantitation:</li> </ul>	
	Close QC Metrics 🔿

As the whole process proceeds you get information on what stage has been performed and also the % of the process that has been completed.

When Processing completes, depending on what stages you selected to perform, the Wizard displays what stage the workflow will open at. In this example it will open at QC Metrics.



You can either:

- Continue with the analysis, as the Processing dialog is not displaying any warnings, and review QC Metrics. In which case you can go to page 52
- Open the analysis at QC Metrics and immediately return to the Review Alignment stage by clicking on it in the Workflow to review alignment quality (page 16)
- Alternatively you can **Close** the dialog. This will not move you to a later stage in the workflow but instead allow you to navigate through all the stages yourself or jump from stage to stage.

QP Progenesis	QI.p Tutorial H	IDMSe - Proge	enesis QI for pro	teomics									
File Import Data	Review Alignment	Filtering	Experiment Design Setup	Review Peak Picking	Peptide Ion Statistics	Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report	nonli	inear Company
Import Dat	Import Data A_01			<< ] Actions • ] >> ]				🕜 Help 🔻					

**Note**: if processing fails to complete successfully there are a number of suggested strategies you can use to proceed with your analysis. These are out lined in Appendix 2 (page 80)

**Please Note:** the time taken to automatically process this data through all the steps including Peptide Identification takes approximately 45 minutes (as shown on the previous page). This is dependent on the specification of your PC.

Note: this does not include the time to load the data

You can explore and re-perform the steps, sequentially and/or as part of the automatic processing as described in this guide.

For this dataset the alignment and detection from the automatic processing was of good enough quality to not require any further amendments. However, in the course of exploring the data you may choose to edit and re-perform the alignment and or the peak detection as described in this document.

You will be warned that you are about to lose the analysis as you 'unlock' a step and reanalyse.

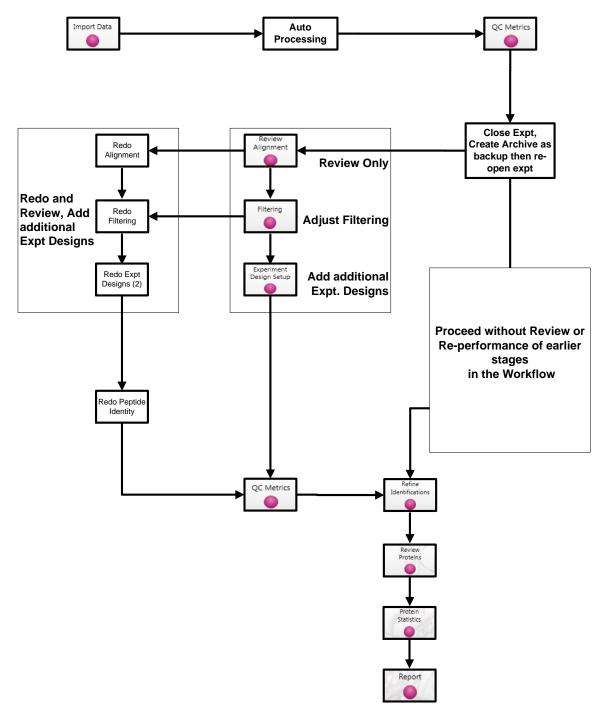
An example of the time it takes to perform each step is outlined in Appendix 10 (page 102).

The next stage in this document gives you a graphical view of how to proceed with your analysis following automatic processing.

# Stage 2B: After Automatic Processing

When Processing completes, depending on what stages you selected to perform, the Automatic Processing Dialog displays (on the right) what stage the workflow will open at.

The flow chart gives you an overview of the various steps you can take to proceed with your automatically processed data.

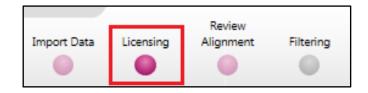


In this example, as the data is HDMSe (Waters) it is going to open at QC Metrics if you chose to perform all the steps in the Automatic Processing Wizard so you can either:

- Continue with the analysis, as the Processing dialog is not displaying any warnings, review the analysis at QC Metrics before proceeding with Refine Identifications. In which case you can go to page 52.
- If warnings are displayed or on Review of the Quality Metrics you require to review/redo earlier stages in the analysis then proceed to pages 16 and Appendices 1 (page 75) and 2 (page 80).

# Stage 3: Licensing

This stage in the analysis workflow will **only** appear if you are using 'Unlicensed' data files to evaluate the software and have no dongle attached.



If you have performed an analysis using Automatic Processing without a valid dongle or do not have the appropriate code to licence your runs, if you close Progenesis QI for proteomics you will be warned that the analysis will be lost.

port Data Licensing Alignment Filtering Design Se		A Waters Compa
ongle License Runs		
This installation is currently restricted to analyse icensed runs only.	Run name Lice	
To license vour runs, vou need an evaluation or	C:\Users\andy.borthwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlice	ensed 🔽
ease licence code which can be obtained from	C:\Users\andy.bothwick\Documents\Customer Data\Progenesis Q1.p v3.0 Tutorials and Demo Suites\Pr Unlice	ensed 🔽
a sales representative.	C:\Users\andy.borthwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlice	ensed 🔽
Once licensed, your runs can be analysed on	C:\Users\andy.bothwick\Documents\Customer Data\Progenesis Q1.p v3.0 Tutorials and Demo Suites\Pr Unlice	ensed 🔽
any installation of the software. The licence is	C:\Users\andy.bothwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlice	ensed 🔽
automatically included when archiving an experiment.	C:\Users\andy.bothwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlice	ensed 🔽
	C:\Users\andy.borthwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlice	ensed 🔽
f your runs have been licensed on another computer, click here to make the licences	$\label{eq:c:Users} C: \label{eq:Question} C: \label{eq:Question} Users \label{eq:Question} C: eq:Question$	ensed 🔽
available on this computer.	C:\Users\andy.borthwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlice	ensed 🔽
f you have one, you can <u>open a licence file</u> to nstall.	QIP Unable to save experiment	
f you have just installed a dongle, <u>click here</u> .	You cannot save the analysis without a valid license. If you close now your analysis will be lost.	
	OK Cancel	
	Run licence code: Use	Licence Code

**Note:** although the analysis will be lost (Alignment Peak Picking etc) the experiment file and all the 'loaded pre-processed runs are retained'. This allows you to reopen the experiment, when you have a licenced code and/or dongle available, and redo the analysis steps without having to reload the data.

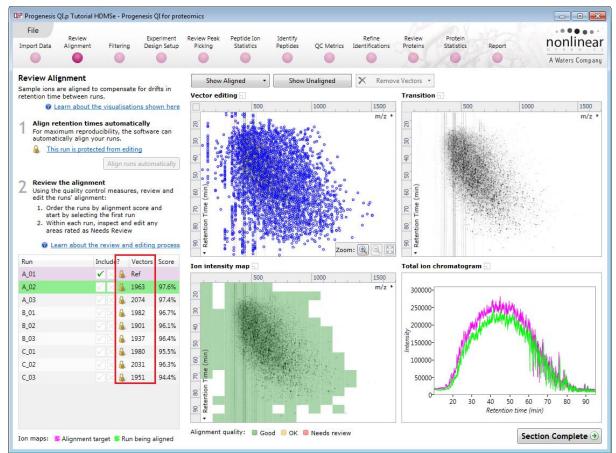
For more details on managing the licencing of your data refer to Appendix 3 (page 82)

If you are using the tutorial archive, this page will not appear as the data files are licensed.

# **Stage 4: Review Alignment**

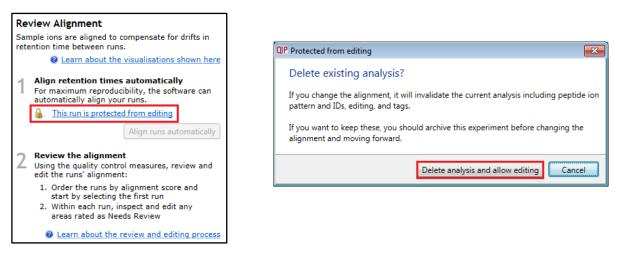
At this stage Progenesis QI for proteomics **Review Alignment** opens displaying the alignment of the runs to the Reference run ( $A_01$ ).

Having performed the analysis automatically there will be icons next to each run in the vectors table indicating that the run is protected from editing.



In the course of reviewing the quality of alignment, you may decide that the alignment requires editing.

To do this click on the **This run is protected from editing** link above the 'greyed out' **Align runs automatically** button.



**Note**: as you click on the link, you will be warned that you are discarding the current analysis (all steps beyond alignment).

Details on editing alignment are described in Appendix 5 (page 82)

### Layout of Alignment

To familiarize you with Progenesis QI for proteomics Alignment, this section describes the various views used in the alignment of the LC-MS runs

To setup the display so that it looks similar to the one below:

• In the Run table click on Run B\_02 to make it current. You will now be looking at the alignment of B\_02 to A\_01 in the Unaligned view. Now drag out an area to review on the **Ion intensity map**. The other 3 views will update to reflect the new focus.

Run	Include?	Vectors	Score
A_01	🖌 🖂 🌡	Ref	
A_02	🗸 🗙 🌡	1963	97.6%
A_03	X X 4	2074	97.4%
B_01	X 🕹	1982	96.7%
B_02	🗹 🗙 🌲	1901	96.1%
B_03	< X 🌡	1937	96.4%
C_01	🗹 🗙 🌲	1980	95.5%
C_02	X 🕹	2031	96.3%
C_03	🗹 🗙 🔒	1951	94.4%



**Vector Editing (Window A)**: is the main alignment area and displays the area defined by the current **focus** rectangle shown in Window C. The current run is displayed in green and the reference run is displayed in magenta. Here is where you can review in detail the vectors and also place the manual alignment vectors when required.

**Transition (Window B)**: uses an **alpha blend** to animate between the current and reference runs. Before the runs are aligned, the peptide ions appear to move up and down. Once correctly aligned, they will appear to pulse. During the process of adding vectors, this view can be used to zoom in and also navigate thus helping with accurate placement of manual vectors.

**Ion Intensity Map (Window C)**: shows the **focus** for the other windows. When you click on the view the blue rectangle will move to the selected area. The focus can be moved systematically across the view using the cursor keys. The focus area size can be altered by clicking and dragging out a new area with the mouse. This view also provides a visual quality metric for the Alignment of the runs, from green through yellow to red.

**Total Ion Chromatogram (Window D)**: shows the current **total ion** chromatogram (green) overlaid on the Reference chromatogram (magenta). As the peptide ions are aligned in the **Vector Editing** view the chromatograms become aligned. The retention time range displayed is the vertical dimension of the Focus Grid currently displayed in the **Ion Intensity map** (Window C).

### **Reviewing quality of alignment vectors**

After **Automatic alignment** the number of vectors and Quality Scores will be updated on the **Runs** panel and the vectors will appear (in blue) on the view.

Where the alignment has worked well, the alignment views will look as below with the Ion Intensity Map showing green indicating good quality alignment and the Transition view showing peptide ions pulsing slightly but not moving up and down.



To simulate poor alignment following automatic alignment, place a single manual vector on the Vector editing view (Window A).

### Placing an 'incorrect vector' to simulate miss alignment in Retention Time

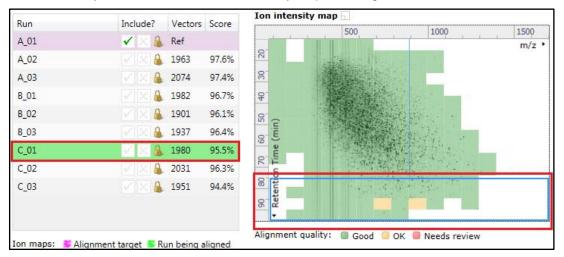
This section of the user guide is provided as a **demonstration of the alignment process**, it is **not** required for the normal analysis workflow of this data.

**Note**: if you do explore the alignment process the final steps in this section show you how to return the alignment to the state following automatic alignment

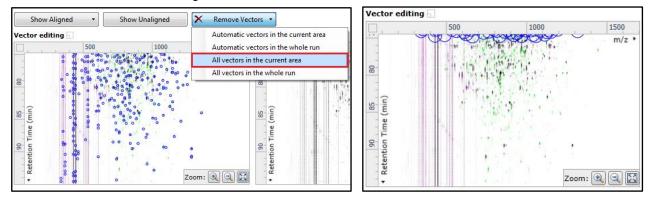
To edit the alignment of your data you must first unlock the analysis. To do this click on the link **This run is protected from editing** and then click **Delete analysis and allow editing** 



First click on run **C\_01** in the Runs table to make it current. Then remove the automatic vectors from 75 min onwards. To do this you must click on the lon intensity map and drag out an area as shown.



This resets all the other views. Now click **Remove Vectors** and select **All vectors in the current area** all the vectors in the Vector editing view will be removed.



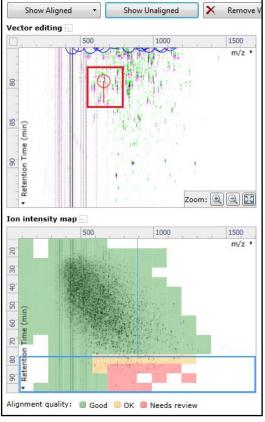
Now click on the Vector editing view and drag the view slightly upwards causing the two runs to go out of synch. Then release the mouse button.

By doing this a single manual vector will appear with a length corresponding to the 'drag'.

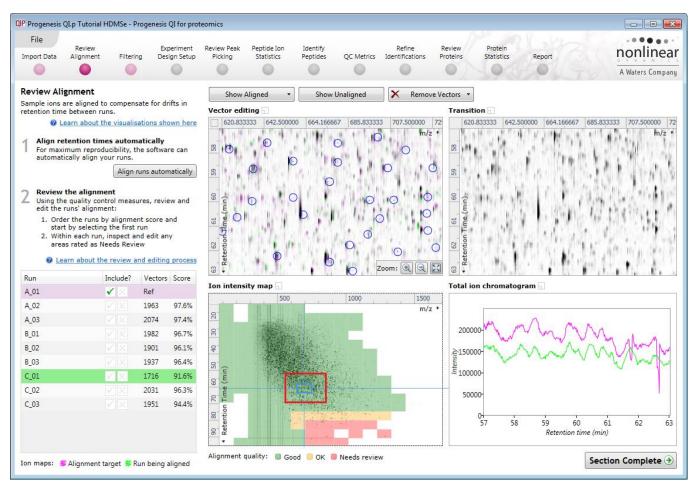
**Note**: the manual vector is **red**, to distinguish it from the automatic vectors (blue)

The effect of adding this incorrect manual vector is to reduce the Alignment score and also cause a significant proportion of the Alignment quality squares to turn red on the Ion Intensity Map.

Finally click on Show Aligned.



Using the **Simulated** miss-aligned example, you can explore the review process for alignment. The alignment looks as below with a region of poor alignment (highlighted in red).

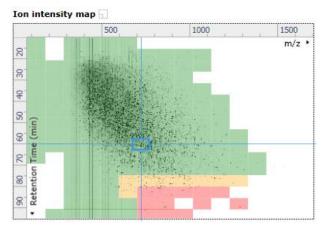


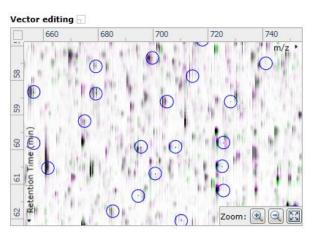
Note: now a smaller area in the 'green' well aligned region

# **Reviewing Quality of Alignment**

At this point the quality metric, overlaid on the Ion Intensity Map as coloured squares, acts as a guide drawing your attention to areas of the alignment. These range from Good (Green) through OK (Yellow) to Needs review (Red). Drag out a 'Focus' area that corresponds to one of the coloured squares. Three example squares are examined here.

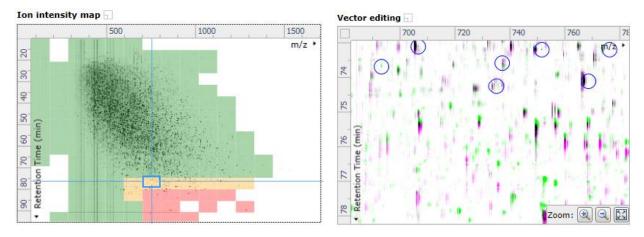
For a 'green' square, the majority of the data appears overlapped (black) indicating good alignment. When viewed in the Transition view the data appears to pulse.



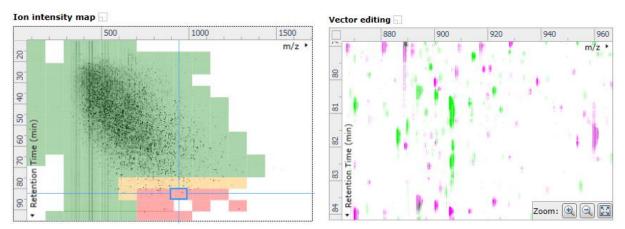


Waters

For a 'yellow' square some of the data appears overlapped (black) indicating OK alignment. When viewed in the Transition view some of the data appears to pulse.

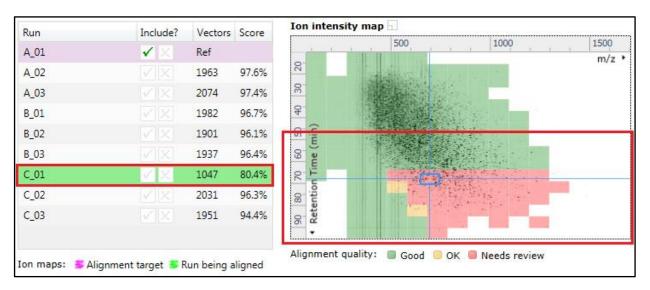


For a 'red' square little if any of the data appears overlapped (black) indicating questionable alignment. When viewed in the Transition view little data appears to pulse.



**Note**: the coloured metric **should be used as a guide**. In cases where there are a few 'isolated' red squares this can also be indicative of 'real' differences between the two runs being aligned and should be considered when examining the overall score and surrounding squares in the current alignment.

The weighted average of the individual squares gives the overall percentage score for each run's alignment.



**Note**: a marked red area combined with a low score clearly indicates a 'misalignment' and may require some manual intervention (see Appendix 4, page 83).

The alignment quality of this tutorial data set does not require any manual intervention so before going to the next section make sure you have removed all manual vectors and re-performed the Automatic alignment.

To do this for C\_01 first select 'All vectors in the whole run' from the Remove Vectors and then click Align runs automatically.



Having aligned the runs automatically, click Section Complete to move to Filtering.

# Stage 5A: Filtering

The Peak picking Parameters dialog opens if Picking has not been performed. If it has been performed, move to section 5B.

### **Peak Picking Parameters**

The Peak Picking Parameters dialog opens, showing all the runs in the current experiment and a tick against each run. This is the default setting, where the peak picking algorithm uses information from all of the runs to contribute to the pattern of peptide ion outlines.

QIP Peak Picking Parameters		QIP Peak Picking Parameters	×
Runs for peak picking Peak picking limi	ts Maximum charge Retention time limits	Runs for peak picking Peak picking limits Maximum charge Retention time limits	_
Choose runs for peak picking — You can tick or un-tick each run to control which will be used by the peak picking algorithm. Although any run which is left un-ticked will not affect the peptide ion outlines, it will still have outlines added to it and will be available in the experiment design setup. Learn more about why you might not want to select all runs.	<ul> <li>✓ Run</li> <li>✓ A_01</li> <li>✓ A_02</li> <li>✓ A_03</li> <li>✓ B_01</li> <li>✓ B_02</li> <li>✓ B_03</li> <li>✓ C_01</li> <li>✓ C_02</li> </ul>	Sensitivity         You can adjust the sensitivity of the peak picking algorithm using these different methods. Each sensitivity method examines the intensities of groups of MS peaks to judge whether they are likely to form part of an ion or whether they represent noise and so should be ignored. Peaks that are rejected as noise will not be used to build ion outlines.         Image: Mathematic descent form part of an ion or whether they represent noise and so should be ignored. Peaks that are rejected as noise will not be used to build ion outlines.	
	♥ C_03       Start peak picking     Cancel	Chromatographic peak width The chromatographic peak width gives the length of time over which an ion has eluted. If you sta a minimum peak width, any ion that has eluted over a shorter period will be rejected. Minimum width: 0 minutes Start peak picking Cancel	- -

*Tip*: It may be appropriate **only** to pick peaks that are present in a limited number of your runs. In which case un-tick the runs that you do **NOT** want to contribute to the peptide ion detection pattern. This may be important when one or more of the runs appear noisy due to non-optimal chromatography or sample handling.

**Note**: peptide ions outlines will be added to 'un-ticked' runs; although these runs will not contribute to the peak picking pattern.

*Tip*: depending on run quality, a suggested minimum number of ticked runs should include at least one replicate of each experimental condition.

The sensitivity of the detection can be controlled by adjusting settings under the **Peak picking limits** tab.

Note: for all 3 Sensitivity modes a Chromatographic
peak width (Retention time window) for the peaks can be
set by applying a minimum retention window or peak
width in minutes.

Chromatographic peak width					
The chromatographic peak width gives the length of time over which an ion has eluted. If you set a minimum peak width, any ion that has eluted over a shorter period will be rejected.	Apply a minimum peak width				
	Minimum width:	0.5	minutes		

For the runs in this user guide we will use the default settings for the Automatic method and NOT apply a minimum peak width.

The third tab allows you to set the **Maximum charge** of the ions, which will be detected. The default setting is a charge state of 20.

	ers		
Runs for peak picking	Peak picking limits	Maximum charge	Retention time limits
Runs for peak picking Maximum allowa You can set the maxi of ions to be detecte charge greater than be rejected.	ble charge	Maximum charge	

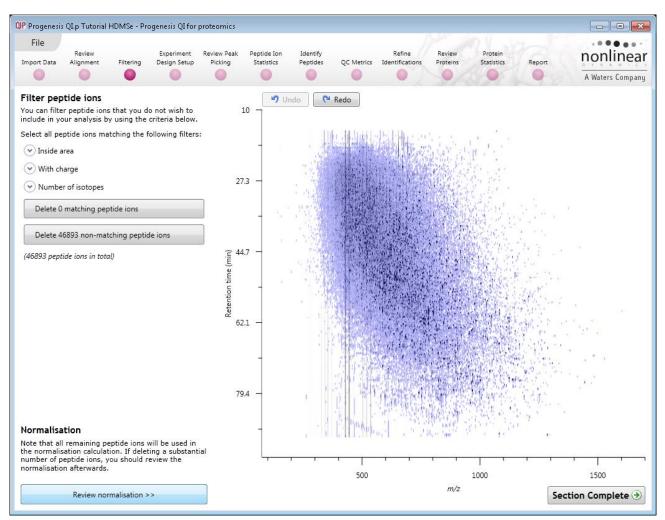
Finally, you can set **Retention time limits** for the detection. Current limits are displayed in the unticked boxes and detection will use these values whenever the box(s) are unticked.

OP Peak Picking Paramete	ers			<b>—</b> ×
Runs for peak picking	Peak picking limit	s Maximum charge	Retention tim	e limits
Retention time li	mits			
You can set the minim maximum retention ti picking. Ions that elut after these values will	me for peak te before or	✓ Ignore ions before	e 15	minutes
arter these values will	be ignored.	Ignore ions after	90	minutes
		Start pe	ak picking	Cancel

For this dataset we will use the RT settings as shown above 15 and 90 minutes.

Press Start peak picking to start the detection process.

On completion of analysis, the Filtering stage will open displaying the number of peptide ions detected, in this example 46893.



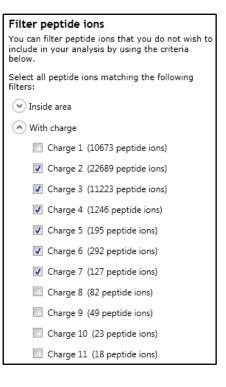
If required you can remove peptide ions based on position, charge state, number of isotopes or combinations of these peptide ion properties.

As an example, we will filter the peptide ions based on 'charge state'.

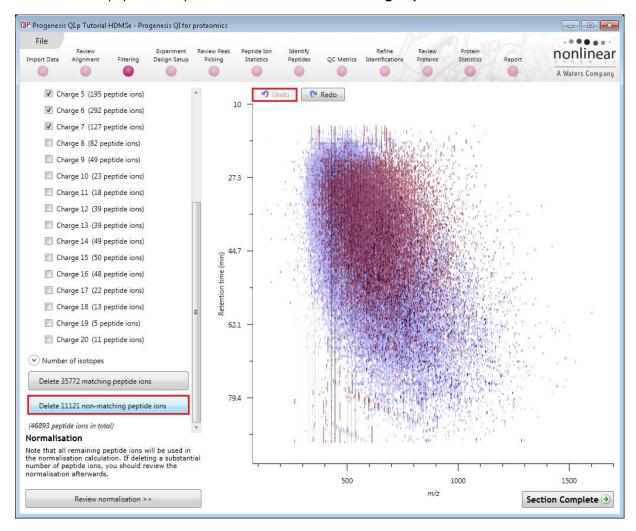
When **With charge** is selected the number of peptide ions present at each charge state is displayed, these can be selected accordingly. In this case we will retain peptide ions with a charge state of 2 to 7.

*Tip*: when filtering on one property of the peptide ions i.e. with charge, make sure you have 'collapsed' the other filters as expanded filters are applied concurrently.

Filter peptide ions						
You can filter peptide ions that you do include in your analysis by using the						
Select all peptide ions matching the f	Filter peptide ions					
💌 inside area	You can filter peptide ions that you do not wish to include in your analysis by using the criteria below.					
With charge						
✓ Number of isotopes	Select all peptide ions matching the following filters:					
	💌 Inside area					
	With charge					
	Charge 1 (10673 peptide ions)					
	Charge 2 (22689 peptide ions)					



Hence all peptide ions with a charge state of 1 or 8 and above will appear red on the main view as you hold the cursor over the appropriate delete button.



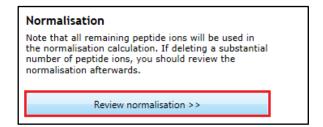
To remove these peptide ions press Delete 11121 Non Matching Peptide ions.

You can use the **Undo** button to bring back deleted peptide ions, however, when you move to the next section you will lose the capacity to undo the filter. Before moving on from filtering, you can review the normalisation of the experiment.

*Tip*: When you have reached the filtering stage, it is good practice to close the experiment and save an archive. This can be used to restore the unfiltered state if the filtering you have performed is too 'stringent'.

# Stage 5B: Reviewing Normalisation

Normalisation review is accessed from the button at the bottom left corner of the filtering page.



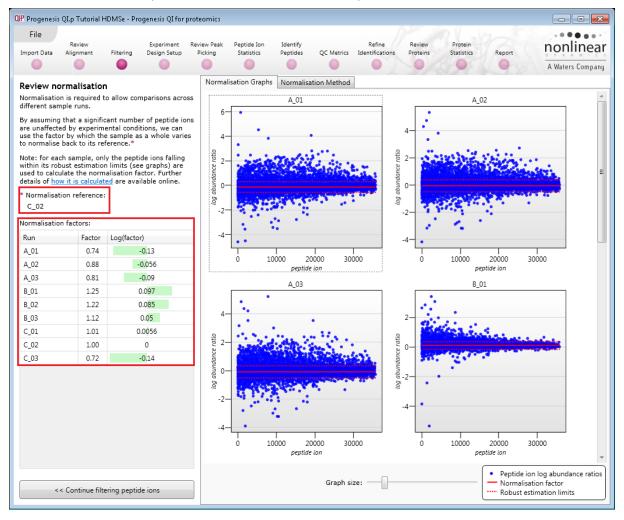
If you have filtered out a number of peptide ions from the original detection pattern then the normalisation will update.

Recalculating normalisation								

The **Review Normalisation** page will open displaying plots for the normalisation of all the peptide ions on each run.

This page in the workflow **does not** allow you to alter the Normalisation of your data but provides you with individual views for each run showing the data points used in the calculation of the normalisation factor for the run.

Normalisation factors are reported in the table to the left of the plots.

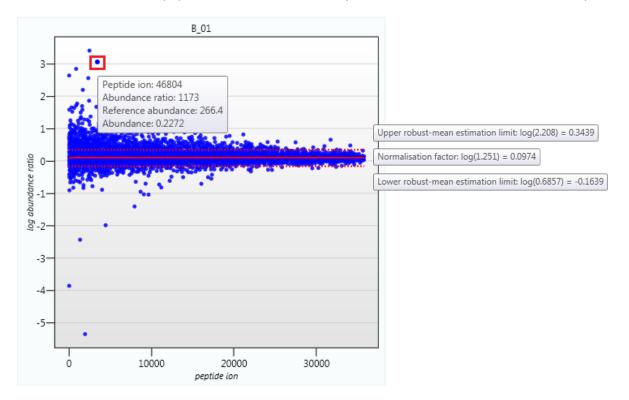


### **Calculation of Normalisation Factor:**

Progenesis QI for proteomics will automatically select one of the runs that is 'least different' from all the other runs in the data and then set this to be the 'Normalising reference'. The run used, is shown above the table of Normalisation factors (in this example C\_02).

For each sample run, each blue dot shows the log of the abundance ratio for a different peptide ion (normalisation target abundance/run abundance).

The details for individual peptide ions can be viewed as you hold the cursor over the dots on the plot.



On the graph the peptide ions are shown ordered by ascending mean abundance. The normalisation factor is then calculated by finding the mean of the log abundance ratios of the peptide ions that fall within the 'robust estimated limits' (dotted red lines). Peptide ions outside these limits are considered to be outliers and therefore will not affect the normalisation.

Finally, if you do **not** wish to work with normalised data then you can **use the raw abundances** by switching off the normalisation.

UP Progenesis QLp Tutorial HDMSe - Progenesis QL for proteomics												
File Import Data	Review Alignment	Filtering	Experiment Design Setup	Review Peak Picking	Peptide Ion Statistics	Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report	nonlinear A Waters Company
Normalisatic different sar By assuming are unaffect use the facto	Review normalisation Normalisation is required to allow comparisons across different sample runs. By assuming that a significant number of peptide ions are unaffected by experimental conditions, we can use the factor by which the sample as a whole varies to normalise back to its reference."				on Graphs No o all proteins o all proteins o a set of house ny normalisation	ekeeping prote						

**Note**: once you have identified the peptide ions, you can then apply the **Normalise to a set of house keeping proteins** by using this option to locate and select the peptide ions.

For this experiment, you should leave the **Normalise to all proteins** option selected.

Now return to filtering by clicking on the button on the bottom left of the screen

	7-
<< Continue filtering peptide ions	

For this example, we **DO NOT** do any additional Filtering so click on **Section complete**.

# Stage 6: Experiment Design Setup for Analysed Runs

At this stage in the workflow you can setup one or more experimental designs for your data.

There are two basic types of experimental designs:

**Between-subject design**: here samples from any given subject appear in only one condition. (i.e. control versus various drug treatments). The ANOVA calculation assumes that the conditions are independent and applies the statistical test that assumes the means of the conditions are equal.

Progenesis QI.p Tutorial HDMSe - Progenesis QI f	or proteomics									
File Review Experim port Data Alignment Filtering Design :	Picking	Peptide Ion Statistics	Identify Peptides	0	Refine Identifications	Review Proteins	Protein Statistics	Report	<u> </u>	Waters Com
hich experiment design type do y		C	reate	0-0 0-0 0-0 Have yo	<b>Vithin-subj</b> u taken samp	les from a giv	ren			
appear in only one condition? Then use the between-subject design.	A		Delete Remove	Then us	under differe e the within-s	ubject desig	n.	Before	During	After
To set up this design, you simply group the runs according to the condition (factor level) of the samples. The	to the condition e samples. The			Note: you must have a sample from every subject for every condition to use a within-subject design.				x X1	X2	X3
ANOVA calculation assumes that the conditions are independent and therefore gives a statistical test of whether the means of the conditions are all equal.	c	C1	Delete Remove Remove	type of experim	nple, you woul design for a tir ent where eve npled at each	ne series ry subject ha	Dationt	Y Y1	Y2	Y3
	Add condition	C3	Remove	software (factor l also whi	p this design, y e not only whic evel) each run ch subject it c	h condition belongs to bu ame from. Tl	he	Z Z1	Z2	Z3
				measure	e will then per es ANOVA. ard ANOVA is n					
				because assumpt repeate differen reduced conditio	the data viola ion of indepen d measures AN ces can be elir as a source of n differences ( more powerfu	tes the ANOV dence. With a OVA individua ninated or between (which helps t	A a Il			
				thought paired-s compari	nin-subject des of as an exten amples t-test t son between n d measures.	sion of the to include	1			

**Within-subject design**: here samples have been taken from a given subject under different conditions (i.e. the same subject has been sampled over a period of time or after one or more treatments). Here a standard ANOVA is not appropriate as the data violates the ANOVA assumption of independance. Therefore by using a *repeated measures* ANOVA, individual differences can be eliminated or reduced as a source of between condition differences. This within-subject design can be thought of as a extension of the paired samples t-test, including comparison between more than two repeated measures.

Additional information on how to apply the Within-subject Design is in Appendix 5 page 88

This experiment contains 2 conditions: Control and Treated and uses the **Between-subject design** to group the analysed runs to reflect the Biological conditions in the original study.

To create a new **Between-subject Design** hold the cursor over this option and click to open the dialog.

QIP Create New Experiment Design									
Enter a name for the experiment design:									
A B C									
How do you want to group the runs?									
Group the runs manually									
Copy an existing design:									
O Import criteria from a file: Browse									
Group runs by: <pre><ru></ru></pre>									
What file formats are supported?         Create design         Cancel									

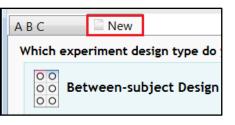
Give the new experiment design a name and then click Create design.

QP Progenesis QI.p Tutorial HDMSe - Progenes	sis QI for proteomics				- • •
		Peptide Ion Identify Statistics Peptides	Refine Review QC Metrics Identifications Proteins	Protein Statistics Report	nonlinear
ABCI × New	•	• •		1011111	A Waters Company
Setup conditions Setup the conditions that you want to com (e.g., control, drug A, etc), and then assig your samples to the correct condition.	pare below	elected Runs to Condition	Search B_02	B_03	
A A.C	Delete 11 Remove 12 Remove	18.8 C			
A.C.	13 <u>Remove</u>	C_01	C_02	C_03	
				Section	n Complete 🏵

### To create a new condition

- 1. Select the runs for the condition by clicking on the required icon in the **Runs** panel, as shown.
- 2. Press the 'black triangle' next to the Add Selected Runs to Condition button on the main toolbar.
- 3. Select Add to new condition... from the drop down menu.
- 4. A new condition will appear in the **Conditions** panel on the left.
- 5. Rename the condition (e.g. C) by over typing the default name
- 6. Repeat steps 1 to 5 until all the runs are grouped into conditions.

An alternative way to handling the grouping of this set and other larger (and more complex) experimental designs is to make use of **sample tracking information** that has been stored in a spreadsheet at the time of sample collection and/or preparation.



For this example there is a **QIP\_Conditions.spl** file available in the Experiment Archive you restored at the beginning of this tutorial exercise.

To use this approach select the **Import design from file** option from the New Experiment Design dialog. Then locate the QIP\_Conditions file and select what to **Group by**, for example: **Condition**.

QIP Create New Experiment Design	n 💌	
Enter a name for the experime	ent design:	
АВ	(-)	
How do you want to group the	runs?	
Group the runs manually		
Copy an existing design:	A B C 🔹	
Import criteria from a file:	ial_HDMSe\QIP_Conditions.SPL Browse	ļ
Group runs by:	Condition	
	VERSION	H
What file formats are support	Condition Location	
	User	H
	Index	

When **Create design** is pressed the new tab refreshes to allow you to adjust the conditions.

QIP Progenesis QI.p Tutorial HDMSe - Progenesis QI for proteomics												
File Import Data	Review Alignment	Filtering	Experiment Design Setup	Review Peak Picking	Peptide Ion Statistics	Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report	nonlinear
	•	•		•	•	•		•				A Waters Company
ABC I	× 🗋 New											🕜 Help 🔻
(e.g., contro	onditions that yo ol, drug A, etc),	and then as	ompare below sign each of	Runs A	dd Selected Run	s to Condition	•	6.00	Q		6.02	
your sample	s to the correct	condition.	Delete		C_01			C_02			C_03	
			A_01 <u>Remove</u>									
			A_02 <u>Remove</u>									
			A_03 <u>Remove</u>									
B			<u>Delete</u>									
			B_01 <u>Remove</u>									
			B_02 Remove									
			B_03 Remove									
Add conditi	<u>ion</u>											
												Section Complete $ e  e  e  e  e  e  e  e  e  e  e  e  e  $

Use **Delete** on the Conditions panel to remove conditions that are not required in this particular design.

Note: On deleting a condition the runs will reappear in the Runs window.

Note: both designs are available as separate tabs.

To move to the next stage in the workflow, Review Peak Picking, click Section Complete.

# Stage 7: Review Peak Picking and editing of results

The purpose of this stage in the Workflow is to review the list of peptide ions using the visual tools provided and edit peptide ions if required.

The review stage has 5 display modes: 1D, 2D, 3D, Drift Time and Peptide ion Details controlled by the tabs on the bottom left of the display and the expander bar to the right of the table. Each display has multiple views to allow comparative exploration of the detected peptide ions on the aligned LC-MS runs.

### Exploring analysed data using the Data displays

To set up the display as shown below, click on the Go To Location on the top right of the zoomed run view (right). Enter 906.700 m/z and 38.686 min and click **Go**. Then click on/or inside the peptide ion boundary to re-focus the view and table.

**Window A**: shows the list of peptide ions ranked by the p value for the one way **Anova** using the current Experiment Design (A B C).



### Note: a value of 'Infinity' in the Fold column indicates 'Presence/Absence'



Progenesis QI for Proteomics User Guide

Window B: displays the Mass spectrum for the current peptide ion on the selected Run (in window D). Hold the cursor over the peak to indicate m/z and intensity

Window C: displays the Chromatogram for the current peptide ion on the selected Run (in window D). Hold the cursor over the peak to indicate Retention time and intensity.

Window D: displays the details of the currently selected run. By default the selected run is an Aggregate view of all the aligned runs.

Details of individual runs can be viewed by using the 'Run' link and selecting the run you wish to view.

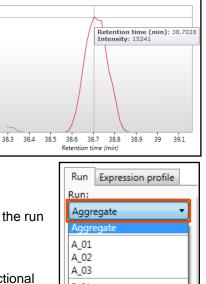
The peptide ion editing tools are located in this window (see page 41 for functional explanation).

Clicking on the Expression Profile tab in Window D shows the comparative behaviour of the peptide ion across the various biological groups based on group average normalised volume. The error bars show +/- 3 standard errors.

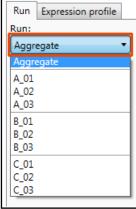
Window E: shows where the current peptide ion is located on the LC-MS run by means of the cross hairs.

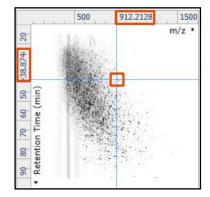
To change the current location, click on the image of the run (note: the retention time and m/z values update as you move the cursor around this view).

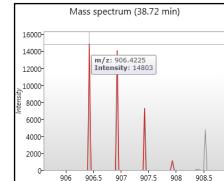
Note: doing this updates the focus of all the other windows.



909







Chromatogram (m/z = 906.4239)

16000 14000

12000

,10000

8000

6000 4000 2000-

Run Expression profile

ArcSinh Normalised Abundar

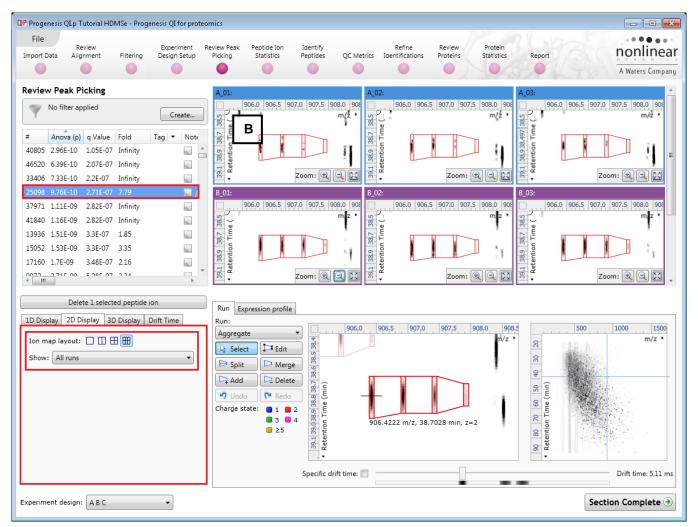
You can also drag out an area (blue square) on this view that will re-focus the other windows.



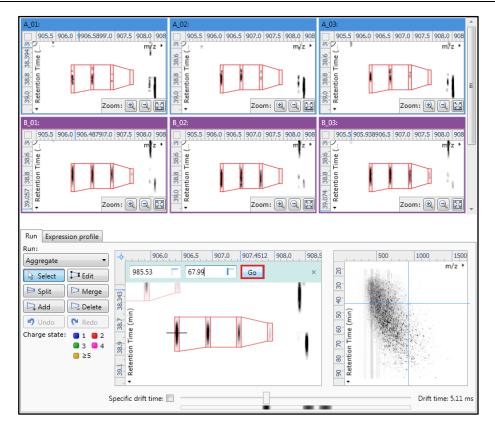
### The 2D Display

Windows A, D and E: perform the same functions across all 4 display modes.

In the 2D Montage mode, Window B displays a montage of the current peptide ion across all the aligned LC-MS runs.



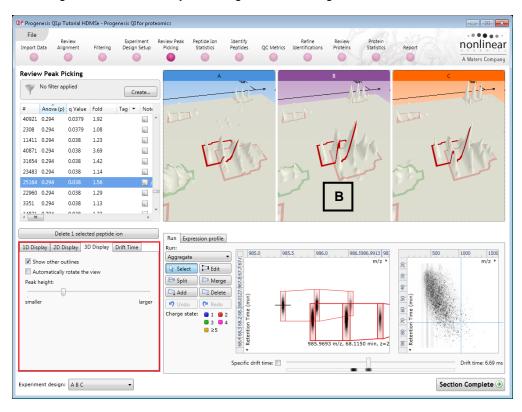
The appearance of the Montage (window B) is controlled by the panel on the bottom left of the display.



Using the the various views in the 2D display one can examine the peptide ion detection in detail to validate the correct detection of even fully overlapping peptide ions as shown above. (i.e Go To Location 985.53m/z and 67.99min)

### The 3D Display

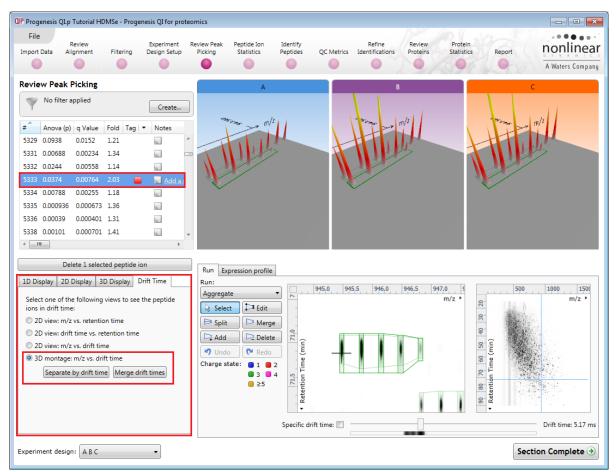
Window B changes into a 3D view by selecting the 3D Montage tab on the bottom left of the display.



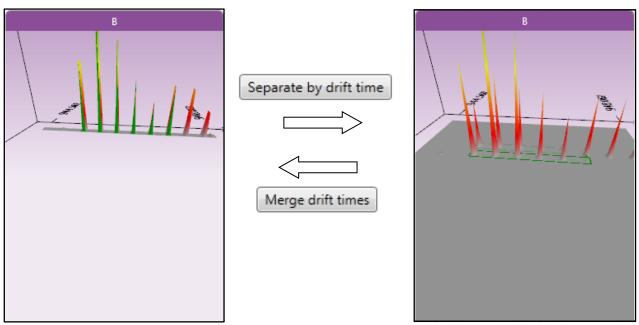
The views can be set to **Automatically Rotate** or you can rotate them manually by clicking and dragging them with the mouse.

### **Drift Time Display**

When the Drift Time tab (**F**) is selected in the bottom left of the display Window B changes into a 3D view displaying the Drift Time dimension (to examine this example order table on # and select 5333.



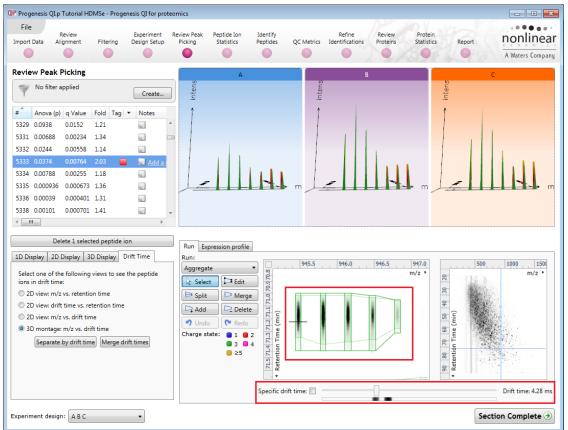
To view the drift time dimension for the current peptide ion, click on **Separate by drift time** on the Drift Time tab. This will expand the view showing the drift time separation between the detected peptide ions (below right)



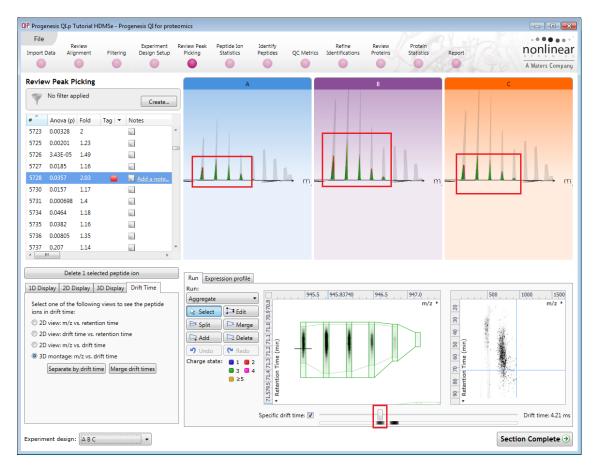
The left hand view displays the merged Drift Times.

**Note**: you can manipulate the orientation of the views by clicking on them and dragging the display to the required orientation. You can also zoom in and out of the panels by using the 'scroll' wheel on your mouse

**Note**: you can step through the specific **Drift Times** (measured in milli seconds) for the current peptide ion by clicking on the **Specific drift time** tick box at the bottom of the display.



The 'crosshairs' on the peptide ion in the Run view identifies it as the current peptide ion in the table.



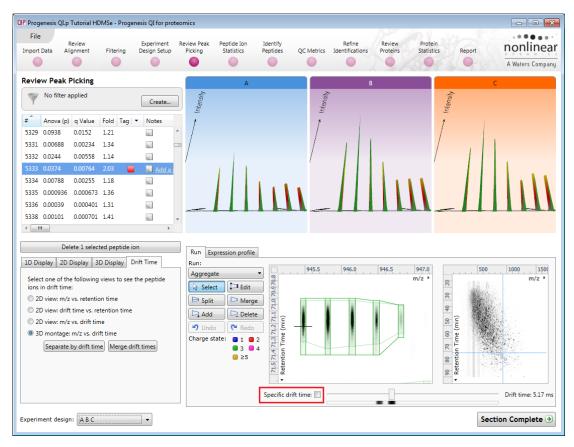
Waters

As you move the slider over the intense areas, indicated below, all the views update to the corresponding drift time.

	Alignment Filter	ing Design Setup	Review Peak Picking	Peptide Ion Statistics	Identify Peptides QC I	Refine Metrics Identifications	Review Protein Proteins Statistics	Report	A Waters Compar
Anova           228         0.0357           730         0.0157           731         0.0066           734         0.0464           735         0.0382           736         0.0080           737         0.207           738         0.672           739         0.00357           740         0.0528           741         0.0278	(p) Fold Tag ( 2.03 1.17 1.17 1.18 1.16 5 1.35 1.14 1.06	Add a note		A	][1].	m. <b>-</b>	B	m	c
D Display 21 Select one of 1 ons in drift tir 2D view: m 2D view: dr 2D view: m 3D montag	elete 1 selected pep D Display 3D Displ the following views to re: /z vs. retention time if time vs. retention /z vs. drift time e: m/z vs. drift time e: by drift time Me	ay Drift Time	Run Exp Run: Aggregati Select Split Add	Edit Merge Delete Redo	e (min)	945.5 . 946.0 .	946.5 947.0 m/z	200 20 20 20 20 20 20 20 20 20 20 20 20	1000 1500 m/z *

Note: the crosshairs will remain on the original peptide ion in the table as you explore the Specific Drift times

When you un-tick the 'Specific drift time, tick box the 3D views will return to showing the Merged Views for the current peptide ion in the table.



### Using Quick Tags to locate examples of Drift Time

In the previous section, describing how to view Drift Time, you may have noticed the presence of a red 'Tag' in the table next to the peptide ion that we examined. Progenesis QI for proteomics allows you to assign tags based on the properties of detected peptide ions either through the manual sorting of the table or making use of the 'Quick Tags'. These tags can be used to filter the list of displayed data in order to aid exploration of the data.

To create a Quick tag for all peptide ions demonstrating separation by Drift time, right click on the table. Select **Quick Tags** then **Separated by drift time.** 

In the new tag dialog either accept or overtype the tag name.

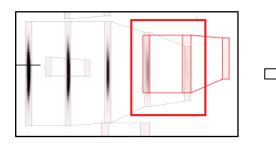
Create new tag			×
Separated by drift time	ОК	Cance	el

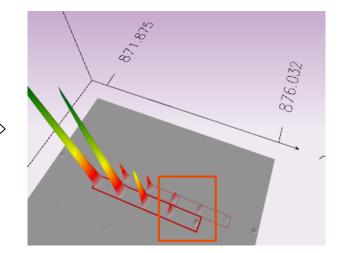
Review	w Peak Pi	icking							Mass spe
Y	No filter ap	plied			Create				Mass spe
#	Anova (p)	Fold	Tag 💌	Notes			80000-		1
5726	3.43E-05	1.49		12		*	70000-		
5727	0.0185	1.16	_	10.		_	60000-		
572° 573		ed by drift	time	Ad	<u>d a note</u>		,50000-		
573	New tag	J		ĥ			(tisu.		
573	Quick Ta	ags		۲.	Anova p	-valu	ıe		1
573 🗳	Edit tags	5			Max fold	cha	nge		- 1
5736	0.00805	1.35			Modifica				
5737	0.207	1.14		1.5	No MS/N				
5738	0.672	1.06			No prote				
5739	0.00352	1.19					drift time	l by drift ti	
5740	0.0528	1.23		4	uchtine	- T	a separated		5.5

When the tag is created it will appear against those peptide ions that meet the criteria for the creation of the tag, in this case:

It tags peptide ions that overlap in both m/z and retention time but do not show an overlap in the drift time dimension i.e. those peptide ions that drift time has separated

For example the peptide ions below is overlapping at the same m/z and RT but are separated in drift time





Now filter the table so that it currently only displays a list of peptide ions containing the **separated by drift time** tag.

Click on **Create** on the filter panel above the table.

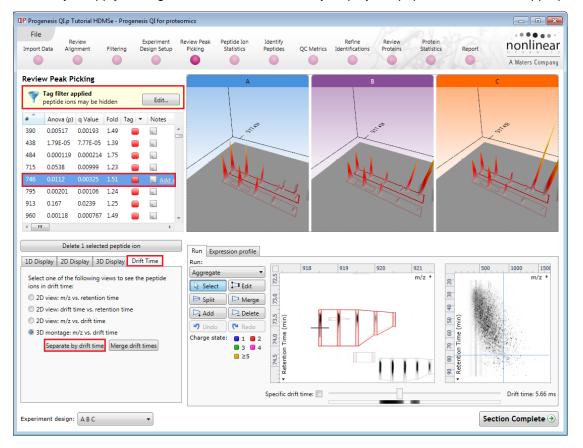
<b>T</b>	No filter applied	Create
----------	-------------------	--------

This will open a Tag filter dialog, in this example, displaying that you have created assigned the **Separated by drift time** tag to 683 peptide ions in your experiment.

To display only those peptide ions containing this tag drag the **Separated by drift time** tag on to the **Show** panel and click OK.

QIP Filter the peptide ions		ſ	QP Filter the peptide ions	
Create a filter Show or hide peptide ions based on a selection of their tags. Move tags to th to create the filter. For more guidance, please see the <u>online reference</u> .	e appropriate boxes		Create a filter Show or hide peptide ions based on a selection o to create the filter. For more guidance, please se	
Available tags:  Separated by drift time (683 peptide ions)  separ	ve at least one of		Available tags:	Show peptide ions that have all of these tags: Show peptide ions that have at least one of these tags: Hide peptide ions that have any of these tags:
Clear the filter OK	Cancel		<u>Clear the filter</u>	OK Cancel

When you apply the tag filter the table will now only display the peptide ions with the appropriate tag(s).



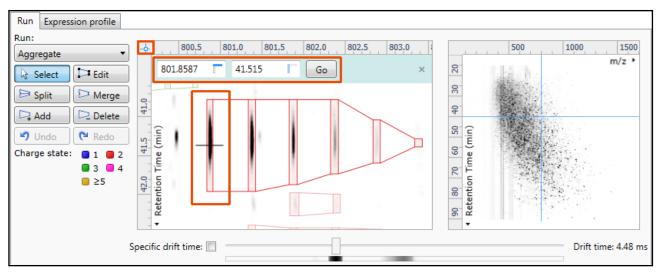
**Note**: with this **Tag filter applied** you can easily review the effect of Drift time separation for the peptide ions.

To remove the filter click on Edit, above the table, and Clear the filter followed by OK.

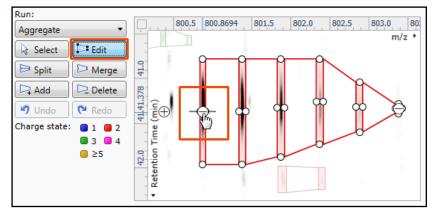
### Editing of peptide ions in the View Results stage

As an example of using the editing tools which are located on the left of the LC-MS Run view, we will remove and add back the 'monoisotopic peak' for the detected peptide ion selected below. A peptide ion can be selected from the 'Peptide ions' list or located using the various views.

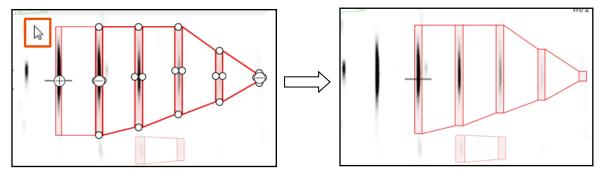
1. Locate the peptide ion at approx 801.86 m/z and 41.5 min using the **Go To Location** tool (top left of zoomed ion map), right click and zoom out and click the cross hairs on the monoisotopic peak to set the zoom.



2. Select the **Edit** tool and click on the peptide ion (in the Run view) to reveal the 'edit handles'. You can zoom in more by dragging an area around the peptide ion of interest.

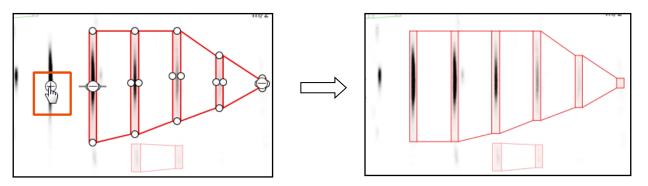


3. Click on the 'minus' handle over the monoisotopic peak to remove it.

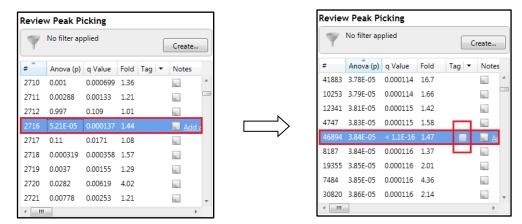


- 4. Click outside the boundary of the peptide ion to update the view.
- 5. To add a peak to an existing peptide ion, ensure that **Edit** is selected then click inside the peptide ion to reveal the handles.

6. Click on the 'plus' handle on the peak to add it.



- 7. Then click outside the peptide ion to update the view.
- 8. Note: If you are not satisfied with the editing use the Undo button and retry.
- 9. **Note**: that a tag is automatically added to the edited peptide ion in the table and the peptide ions id. number is changed to the next available one at the end of the list.



The other tools: **split**, **merge**, **add and delete** behave in a similar fashion and their use can be combined to achieve the desired results.

**Note**: if you have been exploring the editing of peptide ions then a quick way of reversing the edit is to repeatedly press **Undo**.



Edit Tags	<b>X</b>
🕴 🎦 New tag 🛛 alje Rename tag	🗙 Delete tag 🐒 Merge tags
Tag name	
Separated by drift time	Delete tag
Edited	
	OK Cancel

Then right click on the table and select **Edit tags**. Then select the automatically added **Edited** tag and press Delete tag.

### Selecting and tagging peptide ions for Peptide Ion Statistics

There are a number of ways to 'refine' your 'Ranked List' of analysed peptide ions before examining them with the Statistical tools in **Peptide Ion Statistics**. These make use of simple 'Selection' and 'Tagging' tools that can be applied to the various groupings created in Stage 6 (page 29). An example is described below.

First expand the 'Peptide ions' table to show all the details by double clicking on the 'Splitter Control' to the right of the Review Peptide ions table.

Then order on Abundance and select all peptide ions with an Abundance >  $2x10^4$ .

Y	No filter ap	plied		Create	ļ
#	Anova (p)	q Value	Fold	Tag 💌 Not	
18180	5.19E-05	0.000137	1.35	·	
21698	5.2E-05	0.000137	1.54		
35011	5.2E-05	0.000137	6.99	14	I
28122	5.2E-05	0.000137	2.58		
2716	5.21E-05	0.000137	1.44		
10738	5.21E-05	0.000137	1.59	10.	
26113	5.22E-05	0.000137	10.2		
15956	5.22E-05	0.000137	1.91	14	
12382	5.24E-05	0.000137	2.98		
34456	5.24E-05	0.000137	2.68	·	

Right click on the highlighted peptide ions and select 'New Tag'. Give the Tag a name. i.e.	'Most Abundant'.
---	------------------

Ť	Picking					Peptides	QC Metrics	Ide	ntifications	Proteins	Statistics	Report	1101	111116	ea
No filte	Picking		-	•					•	0	1806	0	A Wat	ers Com	panj
Ť															
	r applied													Create	
Anova	(p) q Value	Fold	Tag 👻 Note	is	Highest Mean	Lowest Mean	m/z	z	Mass	RT (mins)	RT window (mins)	Abundance	Intensity	Max CV	(
												2.01E+04			^
			E E												
	63 0.000252	1.47	d by drift time	dd a note											
07 0.0002	05 0.0002		· ·	ote	В	Α	478.2701	2	954.526	43.915	0.577	2.01E+04	4.24E+04	4.97	
94 0.0001	_	New tag			В	Α	575.3202	3	1722.939	43.458	0.634	2E+04	3.28E+04	6.83	
41 0.016	0.0041	Quick Ta	-	•	A	С	369.6911	2	737.368	52.018	0.978	2E+04	4.16E+04	11.2	
31 0.0269	0.006	P Edit tags			В	С	698.3269	2	1394.639	28.748	1.13	2E+04	3.2E+04	4.35	
48 0.0006	24 0.00053	1.43	100		В	Α	657.8667	2	1313.719	39.572	0.514	2E+04	2.55E+04	6.63	
.95 0.0164	0.00423	1.19	102		А	С	490.2539	2	978.493	40.783	0.749	2E+04	4.11E+04	7.79	Ŧ
Display 2	D Display   3D	Display Dr	ift Time	Run: Aggrega Selec Split Add	Edit	60 59 58		783.	0 783.5		4.5 785.0 m/z • 65 09 02 00 00 00 00 00 00 00 00 00 00 00 00	Retention Time (min)     Oos	1000	15 m/z	
						Specific drift	time:			_	-			me: 4.00	_
eriment des			•									Se	ction Cor	npiete	3

Create new tag

**Note**: there is already a red tag present that was assigned to those peptide ions that are **Separated by drift time**, which you created in the previous section.

On clicking **OK** the Tag is added to the peptide ions highlighted in the table (signified by a coloured square, green in this example).

Review	v Peak Pi	cking								
<b></b>	No filter applied									
#	Anova (p)	q Value	Fold		Tag 💌	Notes				
5812	7.27E-05	0.000164	1.35			Add				
7071						Add				
10087	0.00421					Adc				
2978			1.47			Add				
4507			1.33			Adc				
5794	0.00013	0.000224	1.5			10				
5341	0.016	0.00415	1.26			12				
9231	0.0269	0.006	1.11			12				
9848	0.000624	0.00053	1.43			12				
7195	0.0164	0.00423	1.19			12				
•				_!!!						
	Delete	4338 select	ed pepti	ide i	ons					

Now right click on any peptide ion in the table and select **Quick Tags** this will offer you a number of standard tag options. Select **Anova p-value....** Then set the threshold as required and adjust default name as required and click **Create Tag**.

	w Peak P	-						
Y	No filter ap	oplied						
#	Anova (p)	Fold	Tag 💌	Notes		Highest Mean	Lowest Mean	m/z
4759	0.000598	1.45		a.		В	Α	693.35
7699	0.00	4 40	1.20.12	-	a note	В	А	521.27
6040	0.01	Separated k Most Abun		ie		А	С	1005.5
9375	0.00	MOSt ADUN	uani			В	Α	430.74
5226	0.00	New tag				n	٨	706.2
6640	0.13	Quick Tags		•	Anov	a p-value		
	0.00	Edit tags			Max f	old change		
6745		-		-	Modi	fication		
5070	0.0376	1.16		10	No M	S/MS data		
6330	0.0604	1.13		a.	No pr	otein ID		
4415	0.000459	1.56		D.	Separ	ated by drift tim	e	
4310	5.44E-05	1.56		la.	Identi	fied and separat	ed by drift time	
8226	0 1 9 3	1 13		-		B	<u> </u>	528.0

OP New Quick Tag	×
Where a peptide ion has:	
Anova p-value: ≤ ▼ 0.05	
Apply the following tag:	
Anova p-value ≤ 0.05	
Create tag Ca	ncel

Once this tag appears against peptide ions in the table right click on the table again and create another Quick Tag, this time for peptide ions with a **Max fold change**  $\geq 2$ 

OP New Quick Tag
Where a peptide ion has: Max fold change: 2
Apply the following tag:
Max fold change ≥ 2
Create tag Cancel

The table now displays peptide ions with multiple tags.

The tags can be used to quickly focus the table on those peptide ions that display similar properties.

-	No filter applied	
Υ.		Create

Y	No filter ap	plied		Cre	eate
#	Anova (p)	q Value	Fold	Tag 💌	Not
20113	0.214	0.0291	1.14		4
33205	0.0229	0.00533	460	-	la.
15957	0.895	0.0985	1.05		la.
22392	0.00303	0.00137	1.18		la.
29311	0.00716	0.0024	83.3		
19066	0.000405	0.00041	2.33	=	4
20437	0.458	0.0551	1.04		12
29501	9.41E-05	0.000186	1.79		10
21696	0.000417	0.000416	1.75		10
22210	0.026	0.00584	1.43		10.

For example: to focus the table on displaying those peptide ions that are **Most Abundant** click on **Create** on the filter panel above the table.

Drag the tag on to the panel Show peptide ions that have all of these tags and press OK.

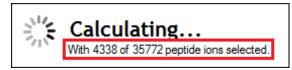
IP Filter the peptide ions	×	QP Filter the peptide ions	
Create a filter Show or hide peptide ions based on a selection boxes to create the filter. For more guidance, p		Create a filter Show or hide peptide ions based on a selection boxes to create the filter. For more guidance, p	
Available tags: Most Abundant (4338 peptide ions) Anova p-value ≤ 0.05 (20887 peptide ions) Max fold change ≥ 2 (7863 peptide ions) Separated by drift time (683 peptide ions)	Show peptide ions that have all of these tags: Show peptide ions that have at least one of these tags: Hide peptide ions that have any of these tags:	Available tags: Available tags: Max fold change 2 2 (7863 peptide ions) Separated by drift time (683 peptide ions)	Show peptide ions that have all of there tags: Most Abundant (4338 peptide ions) Show peptide ions that have at least of these tags: Hide peptide ions that have any of there ags:
<u>C</u> lear the filter	OK Cancel	Clear the filter	OK Can

To move to the next stage in the workflow, Peptide Ion Statistics, click Section Complete.

# Stage 8: Peptide Ion Statistics on Selected Peptide ions

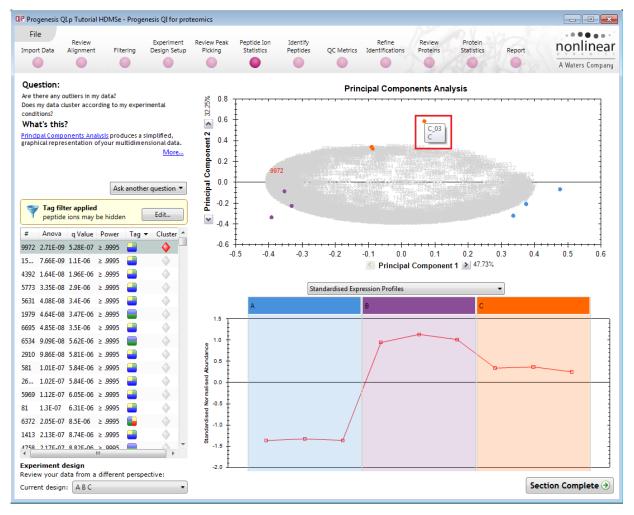
The user guide now describes the functionality of the Multivariate Statistics.

Peptide Ion Statistics opens calculating the Principal Components Analysis (PCA) for the active 'tag' in this case the **Most Abundant** peptide ions.



As an example we will start by examining the behaviour of the **Most Abundant** peptide ions from the previous stage, **Review Peak Picking**.

The statistical analysis of the selected data is presented to you in the form of interactive graphical representation of answers to questions asked of the analysed data.



**Note**: the LC-MS runs (samples) are displayed as solid coloured circles on the plot. To identify the runs, a tooltip is displayed when the cursor is held over each circle.

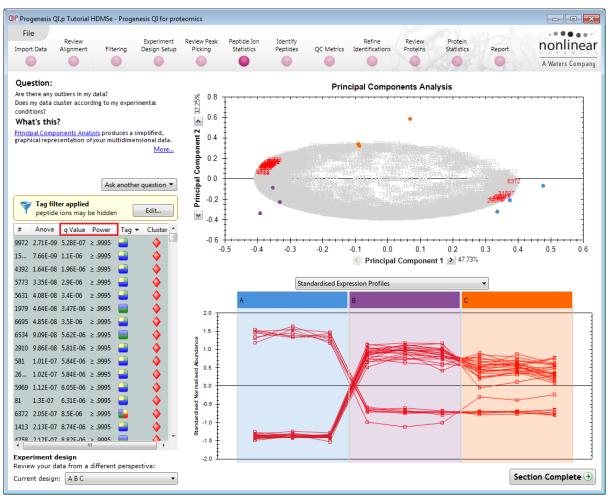
#### **Principal Component Analysis (PCA)**

In **Peptide Stats** the first statistically based question asked of the data takes the form of a Quality Control assessment:

Are there any outliers in my data? And does my data cluster according to my experimental conditions?

It answers this question by:

'Using Principal Components Analysis (PCA) to produce a simplified graphical representation of your multidimensional data'.



PCA can be used to determine whether there are any outliers in the data and also look at how well the samples group. The groupings that can be observed on the 2D PCA plot can be compared to your experimental conditions and conclusions can be drawn regarding possible outliers in your data. Selecting peptide ions in the table will highlight the peptide ions on the 'Biplot' and their expression profiles will appear in the lower panel.

Note: the Table in the Stats view contains additional columns:

**q value**: tells us the expected proportion of false positives if that peptide ion's p-value is chosen as the significance threshold.

**Power**: can be defined as the probability of finding a real difference if it exists. 80% or 0.8 is considered an acceptable value for power. The Power Analysis is performed independently for each peptide ion, using the expression variance, sample size and difference between the means.

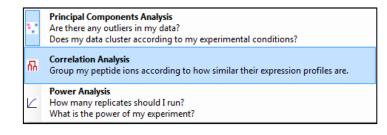
Also, for a given power of 80% we can determine how many samples are required to ensure we find a difference if it actually exists.

**Note**: Power analysis is discussed in Appendix 6 (page 90)

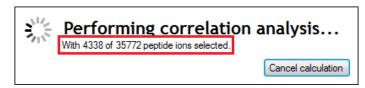
### **Correlation Analysis**

QIP Filter the peptide ions × Create a filter Show or hide peptide ions based on a selection of their tags. Move tags to the appr boxes to create the filter. For more guidance, please see the <u>online reference</u>. With the tag filter still set to display only the top 4338 Most Abundant peptide ions, we are going to explore Available tags: Show peptide ions that have all of these the Correlation Analysis of these peptide ions. tags: Anova p-value ≤ 0.05 (20587 peptide ions) Most Abundant (4338 peptide ions) Max fold change ≥ 2 (7863 peptide ions) Separated by drift time (683 peptide ions) Show peptide ions that have at least one of these tags: Hide peptide ions that have any of these tags: To set up the Correlation Analysis using this filtered data set click on Ask another question (above the table) OK Cancel  $\underline{C}$ lear the filter

A selection of 3 tools will appear in the form of questions.



Select the second option to explore 'peptide ion correlation based on similarity of expression profiles'



This time the statistically based question(s) being asked is:

'Group my (selected) peptide ions according to how similar their expression profiles are'

The question is answered by:

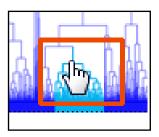
'Using Correlation analysis to evaluate the relationships between the (selected) peptide ions' expression profiles'.

The answer is displayed graphically in the form of an interactive dendrogram where the vertical distance, between each peptide ion can be taken as indicative of how similar the expression profiles of each cluster of peptide ions are to each other.



**Correlation Analysis** enables the grouping of peptide ions together according to how similar their expression profiles are.

**For example**: To highlight groups of peptide ions demonstrating **different expression profiles** click on a 'node' for a branch of the Dendrogram (as shown above) while holding the mouse button, hold down the **Ctrl** key and then click on another node as shown.



If you have selected 2 nodes then there will be two expression profile graphs

**Note**: by highlighting a group of peptide ions with similar expression the peptide ions are identified with the same colour of cluster flag in the table. This allows the table to be sorted on cluster and tagged accordingly

*Tip*: when reviewing the tags (see above) if you are not applying a new filter then use the **Cancel** button to return to the main view, this prevents unnecessary recalculation of your data.

Before moving to the Identify Peptides stage in the Workflow, first return to the PCA display and clear all tag filters **Clear all Tag filters**.

To move to the next stage in the workflow, Identify Peptides, click Section Complete.

# Stage 9: Identify peptides

Progenesis QI for proteomics is designed to perform peptide identifications either directly or by allowing you to export MS/MS peak lists in formats which can be used to perform peptide searches by various search engines. The resulting identifications can then be imported back into Progenesis QI for proteomics, using a number of different file types, and matched to your detected peptide ions.

File	omics							
Review Experiment Alignment Filtering Design Setup	Review P Picking					Refine ntifications	Review Proteins	Protein Statistics Report A Waters Com
dentify Peptides elect your peptide identification method:	Featu	ires						Wo filter applied Create
Q Ion Accounting	#	Identifications	m/z	Charge	Retention time	Drift time	Tag 🛛 💌	
Mascot Version: 1.0	• 1	0	828.4192	2	58.67	4.90		
PLGS (*.xml)	03	0	822.4425	2	69.31	4.76		
Version: 1.0	04	0	962.7957	3	76.54	4.97	-	
SEQUEST (dta & out files) Version: 1.0	05	0	894.4744	3	67.50	4.48		
SEQUEST (dta & pepXml files)	0 6	0	964.1703	3	63.92	5.45		
Version: 1.0 Phenyx	07	0	763.7625	3	69.69	4.00		
Version: 4.0.6344.40563	08	0	823.4391	2	75.96	5.04		
Spectral Library Search Version: 4.0.6344.40563	09	0	920.1447	3	74.49	4.48	-	
Modifications: 🚺 🖤 Carbamidomethyl C								IIII m/
C Oxidation M     Add/remove modifications     Search tolerance parameters     Ion matching requirements     Search for identifications     Identifications								60.4         59.3         58.2         57.4           4         Retention Time (min)         57.4
Oxidation M     Add/remove modifications     Search tolerance parameters     Ion matching requirements     Search for identifications	Fragm	ent matches	for:					66-4 59-3 58-2 Retention Time (min)
Oxidation M     Add/remove modifications     Search tolerance parameters     Ion matching requirements     Search for identifications     Identifications will be assigned to the relevant features automatically.	Intensity (counts)	5-	for:		03 035 0	4 0.45	0.5 0.55	66-4 59-3 58-2 Retention Time (min)

For this example we are using the direct method **lon Accounting**.

**Note:** Following the full automatic processing, described in Stage 2 of this guide, the Identify Peptides page currently displays the full list of the detected peptide ions in your experiment and some of their attributes, including the number of **Identifications** (as shown on page 51). If search results exist these can be cleared by clicking **Clear all identifications**, this will allow you to re-perform the search.

Entering Search Parameters	
Littering Search Farameters	Identify Peptides Select your peptide identification method:
Firstly you need to select the FASTA file containing peptide and protein identifications.	Q Ion Accounting
	Help For MS <sup>*</sup> , HDMS <sup>*</sup> and SONAR data
SWISSPROT-1 is provided with the installation of the software.	1 Enter the search parameters Select your FASTA file containing peptide and protein identifications:
To add new Databanks in the form of FASTA files click on <b>Edit</b> to open the Databank editor	SWISSPROT-1.0 Edit SWISSPROT-1.0 STANDARD_SPACED Common search parameters
Note: the SWISSPROT-1.0 is locked	<ul> <li>Search tolerance parameters</li> <li>Ion matching requirements</li> </ul>

QIP Databank Editor		X
	Name:	SWISSPROT-1.0
B SWISSPROT-1.0	Parsing rules:	STANDARD_SPACED  -
	Location:	sprot.fas
Add Remove		
		Save Cancel

For a new Databank you need to give it name, select the parsing rules and specify the location of the FASTA file, see the example below.

QIP (	Databank Editor		<b>—</b> X—
۵	Name	Name:	Tutorial DB
â		Parsing rules:	UNIPROT -
	Tutorial DB	Location:	> v4 HDMSe Tutorial\Tutorial_nd_DB.fasta
	Add Remove		
			Save

The new Data bank will appear in the left panel now click **Save** to return to the Search parameters.

If your databank is not already displayed then select it from the drop down list.

#### Expand the Common search parameters

The default settings are displayed:

**Digest reagent:** is set as Trypsin. Alternative Digest reagents are available from the list and additional ones can be added to the list using the **Reagent editor...** 

Missed cleavages: is set as 1.

Maximum protein mass: is set at 250kDa

**Modifications:** are set Carbamidomethyl C (Fixed) and Oxidation M (variable). More modifications are available from the list and additional ones can be added to the list using the **Modification** editor...

Identify Peptides
Select your peptide identification method:
🔍 Ion Accounting 🗸 🗸
Help
For MS <sup>1</sup> , HDMS <sup>1</sup> and SONAR data
1 Enter the search parameters Select your FASTA file containing peptide and protein identifications:
Tutorial DB
Enter the search parameters to use:
Common search parameters
$\bigcirc$ Search tolerance parameters
✓ Ion matching requirements
Common search parameters
Digest reagent: Trypsin
Missed cleavages: 1 max
Max protein mass: 250 kDa 🔹
Modifications: 💽 💟 Carbamidomethyl C E 💟 Oxidation M
Add/remove modifications

Having selected the Databank then set the Search tolerance parameters and ion matching parameters as shown to the right.

When all the runs are ready for searching, click **Search for identifications**.

Depending on the search parameters and the number of runs the **Ion Accounting** search can take some time.

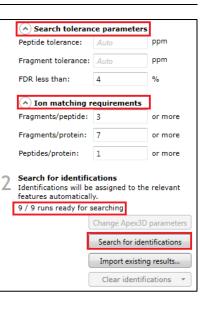
Once the Ion Accounting is complete, peptide ions with identifications are identified with a solid grey symbol and the number of identifications appears in the next column.

OP Progenesis QLp Tutorial HDMSe - Progenesis QI for pro	eomics									
File Review Alignment Filtering Experiment Design Setup	Review Pea Picking	k Peptide Ion Statistics	Iden Pepti		QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report	A Waters Company
Identify Peptides Select your peptide identification method:	Peptid	e ions (1085	6 identi	ified)					W No filter ap	Greate     Create
Q Ion Accounting	# ]	dentifications	m/z	Charge	Retention	time Drift time	Tag 🔻			
Help	• 1	1 ;	828.4192	2	58.67	4.90				A
For MS <sup>t</sup> , HDMS <sup>t</sup> and SONAR data	• 3	3 1	822.4425	2	69.31	4.76				
Enter the search parameters     Select your FASTA file containing peptide and	● 4	1 !	962.7957	3	76.54	4.97	-			
protein identifications:	• 5	1 ;	894.4744	3	67.50	4.48				
Tutorial DB    Edit	● 6	1 !	964.1703	3	63.92	5.45				
Enter the search parameters to use:	• 7	2	763.7625	3	69.69	4.00				
Common search parameters	● 8	1 <b>QIP</b> Impo	rt search r	esults				×		
Max protein mass: 250 kDa ▼ Modifications: Carbamidomethy Carbamidomethy Add/remove modifications Search tolerance parameters ✓ Ion matching requirements	1654.82		11					ОК	DLSEAST 2'28 C'85	
7 Search for identifications	Fragme	nt matches fo	r: DLSEAS	SVYAEY					1.42	
Identifications will be assigned to the relevant features automatically.     9 / 9 runs ready for searching     Change Apex3D parameters     Search for identifications     Import eviction result     * Admin tools	0 ntensity 0 ntensity 0	KP	200 30	A  E		s v v v v v v v v v v v v v v v v v v v		A + E + F		<u>sto</u> pk 1 0 1500 1600 1700 1800
Change the digest reagents and modifications that are available for peptide searches. Modification editor Reagent editor										Section Complete 🤿

Details for the current peptide ion identifications are displayed in the table below and the Fragment ions for the current identification are displayed in the bottom panel.

Note: if you want to perform the search with a new set of parameters then first select Clear all identifications

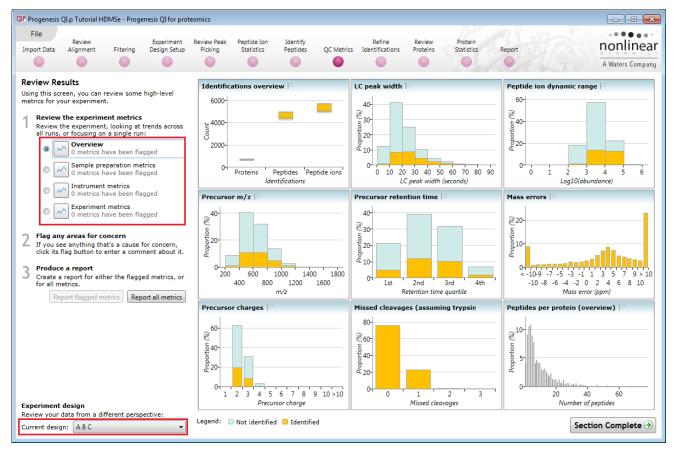
Having performed the process of peptide identification you can review the overall quality of your analysis by using the various batch-based quality metrics in the next section, by clicking on QC Metrics on the workflow.



## Stage 10: QC Metrics

Progenesis QI for proteomics includes a number of batch-based QC metrics which you can examine to increase confidence in, or identify issues with, your data. These views are presented at the "QC Metrics" page. By setting up experimental groupings that correspond to your batch metadata, you are able to investigate whether there are any systematic effects associated with your processing. This gives you confidence that your conclusions are not affected by technical biases within the course of the experiment.

The charts are updated with changes in your active experimental design, allowing you to examine the effects of all stages of your processing if you wish through using different batch divisions.



#### **Metrics**

There are two sets of metrics. Firstly, the overview page provides nine at-a-glance summary measurements which cover the experiment as a whole. Then, the remainder of the pages provide more detailed information and bring in the batch-by-batch detail, some of which builds on the overview charts:

- Sample preparation metrics highlighting issues or problems with the preparation of your samples: Missed cleavages, modifications and abundance dynamic range
- Instrument metrics highlighting whether your chromatography column and mass spectrometer are configured and performing correctly: Mass accuracy, abundance dynamic range (again), precursor charges, MS1 scan rates
- Experiment metrics concerning the identified proteins and peptides in your experiment, allowing you to pick out any outlying runs or conditions:

Proteins, peptides, peptides per protein, % of peptide ions identified, proteins per condition

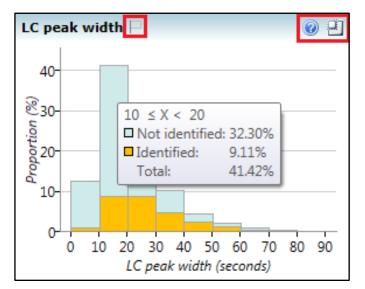
More detailed information on the QC metrics is available on the FAQ pages.

#### Interpretation and use

It's worth noting that there is no simple 'right' or 'wrong' answer as to whether your data are of high enough quality.

**Note**: QC measurements are designed to flag up potential issues for investigation, hence there must be an assignable cause to the variation observed to render QC practical.

Furthermore, the QC metrics will be rebuilt if you delete and re-do any stages leading up to them (for example, clearing identifications and re-searching with changed identification parameters, or altering the alignment). Hence, if you archive your experiment before and after your changes, or export the charts before and after, you can see the effects of your changes upon the quality measurements – a very useful method for assessing your interventions.



**Note**: that the overview metrics can each be expanded by clicking on the top-right icon in the sub-window. Additionally, hovering over a column will bring up a tooltip containing quantitative information on the results.

You can also tag metrics of interest or concern with a comment for your records, which is saved with the experiment. To do this, click the empty flag icon in the tab header for the given metric (or in the tile header on the overview screen):

Missed cleavages	PTMs 📃	Abundance dynamic range 🖂	Identifications overview	$\square$	() U
					-

This will pop up a dialog allowing you to enter a comment describing why this metric has been flagged.

#### **Reporting**

You can export a report for your own records, or to pass on to another member of your team:

3	Produce a report Create a report for either the flagged metrics, or for all metrics.
	Report flagged metrics Report all metrics

#### **Report all metrics:**

Generates a report containing all metrics shown in the application (overview, sample preparation metrics, instrument metrics and experiment metrics). This may be useful for documentation purposes, or to verify the quality of your experiment.

#### Report flagged metrics:

Generates a report of only metrics you have flagged, along with the message you provided. This may be useful for giving to a technician or other team member, to highlight areas of the experiment that need improvement.

In order to review, and refine the quality of the **Peptide Search** results click on the next stage in the workflow, **Refine Identifications**.

# Stage 11: Refine Identifications

In this example we are going to apply a number of filters to 'refine' the quality of the Databank search.

**Note:** before removing any identifications, make sure there are **no** tag filters applied at the Identify peptides stage.

As an **example** the following section describes how sequential filtering of the Peptide results can be performed using the following thresholds described below:

- Remove identifications with a Score less than 4
- Remove identifications where less than 2 hits were returned
- Remove all identifications where the Protein Description **Contains** the following: 'Putative', 'Probable', 'Like', 'Potential' and 'Predicted'

To perform these filters, on the Batch detection options panel, set the Score to less than 4, then **Delete matching search results**.

nport		Review	Filterin	Experime g Design Se		Review Picki	ng	Peptide Ior Statistics	Pe	entify ptides	QC Metrics		efine ifications	Revie Protei		R	eport			nonline
				-									•				1011		TR	A Waters Comp
	e Identif		ns include	e unwanted or		Bate	ch deleti	ion criteria	Score []	ess than	•	4			Sequence	Length	less than	•		
	ant results,							L				4	_		Sequence	-				
	pecify a se								Hits	ess than	•					Charge	less than	•		
				nter the prope s you want to	rty				Mass	ess than	•				Se	equence	contains	•		
	elete.			o you mane co		Abe	olute m	ass error (		acc than	•				۵	cession	contains	•		
D	elete the i	unwanter	l identifi	cations			orace m	lass error (												
т	delete the			don't want, d	lick				m/z [	ess than	•				Des	cription	contains	•		
e	ther:							Retention	Time	ess than	•				Modif	ications	contains	•		
		latching S lighted ID		sults, to delete	e									Valata au	-		Delete		and and k	Deset the suit of
				h Results, to highlighted										Jelete mat	tching search resul	ts	Delete non-	matching se	arch results	Reset the criteri
	delete ti	ne IDs tha	t are not	nignlighted			#	Score	Hits	m/z	RT(mins)	Charge	Mass	Mass ern	Sequence	Ac	cession	Modifi	cations	
	eset the c						156	8.60	9	830.46			1658.91		Sequence	Acc P4		Would	cations	Heat shock prote
				tifications to nd then return	n to		156	8.60	9	830.46			1658.91		INEPTAAA	P4				Heat shock prote
	ep 1 above						158	3.69	1	543.98		-	1628.91		QALSETVD	G C4				Putative unchara
_	N. Classe						158	3.73	1	543.98			1628.91		TTLTEINGP	05	A9Y0			Putative unchara
Y	No filter a	ipplied		Create			161	5.71	1	618.84	50.83	2	1235.67	-8.39	S FDNGLRFIVR	Q Q5	A202			Putative unchara
							161	0.00	1	618.84	50.83	2	1235.67	42.52	GLSYSKDE	🕥 C4	YIX5			Putative unchara
	Total Hits	m/z	RT (min	Charge Tag			161	8.59	4	618.84	50.83	2	1235.67	13.06	S TLASDGIA	🕥 Q5	A516			ADP_ATP carrier
	7	828.41!	58.67	2	<u> </u>	1	163	8.11	9	697.37	56.25	2	1392.72	7.98	ALDADVVS	P8	2610			5-methyltetrahy
	11	822.442	69.31	2		1	164	4.90	5	673.37	55.95	2	1344.73	-1.64	LQIDELMK	🎯 C4	8LLY			Putative unchara
	9	962.795	76.54	3 🧧		1	165	5.32	1	965.48	51.10	2	1928.95	8.09	AVSSGMV	🌒 C4	YEB6			Glycyl-tRNA syn
	8	894.474	67.50	3		1	165	8.10	8	965.48	51.10	2	1928.95	11.92	VWLDPNE	🎯 C4	YQK4			60S ribosomal p
	8	964.17(	63.92	3		1	166	4.56	3	821.96	50.91	2	1641.9(	11.73	LASESTLPV	🌒 C4	YSQ4			Putative unchara
	11	763.762	69.69	3			166	3.54	1	821.96	50.91	2	1641.9(	4.06	S NLLIQHGR	🕥 C4	YNH7			Periodic tryptop
	7	823.439	75.96	2			167	9.09	6	887.48	62.54	2	1772.95	14.40	GISELGIYP	🎯 Q5	9UR7			ATP synthase su
	19	920.144	74.49	3 🧧		1	171	8.82	9	882.96	66.34	2	1763.91	7.87	SEEFSSPD	🎯 Q5	A5V6			Pyruvate dehydr
L	10	657.367	36.18	2		1	175	5.98	1	875.97	58.59	2	1749.9:	-7.70	LEEIVSSIEK	🌒 Q5	9K93			Hexokinase-like
3	9	828.449	45.13	2		1	175	5.98	1	875.97	58.59	2	1749.9:	-7.70	LEEIVSSIEK	🌒 Q5	9RR7			Likely hexokinas
-	13	776.411	51.90	2		<b>V</b>	175	5.98	1	875.97	58.59	2	1749.93	-7.70	LEEIVSSIEK	🎯 C4	YMD4			Putative unchara
<i>'</i>	6	740.478	79.13	2			175	5.98	1	875.97			1749.93		LEEIVSSIEK	🌒 Q5				Likely hexokinas
7 9	-	845.76(	70.78	3	-		176	8.81	1	718.39	55.08	2	1434.77	15.75	FTQAGSEV	🌒 Q5	9UR7			ATP synthase sul
	8																			

**Note**: the search results matching the filter criteria turn pink and the total is displayed at the bottom of the table (in this example: 2012 matching out of 14732)

 OP Delete 2012 search results?
 83

 OP Delete 2012 search results?
 83

 OP Delete 2012 peptide search results?
 83

**Note**: a dialog warns you of what you are about to delete Click **Yes**.

Now click Reset the criteria to clear the previous threshold then apply the next filter 'Hits: less than 2'.

Tip: always click Reset the Criteria after each deletion.

File																		
nport Dat		eview inment	Filtering		xperiment esian Setup	Review I Pickir		Peptide Ion Statistics	Identify Peptides	OC Metri	Refi s Identific		Review Proteins	Protein Statistics		Report		nonli
•	(		•		•	0	-	•	•	•			•	•		0		A Waters (
efine l	dentifi	cations				Batc	h delet	ion criteria										
		ntificatior vou can r						Sc	ore less than	•				Sequence	Lengt	h less than	•	
		t of delet							lits less than	•	2				Charg	e less than	•	
In the	e batch o	leletion ci	riteria, en	ter the	property			м	ass less than	•				Se	quenc	contains	•	
delet		et of iden	tifications	s you w	ant to			ass error (pr		_						n contains	•	
Dala	ta tha u	nwanted	idantifi	cation	-	ADS	oute n											
To de	lete the				• vant, click			1	m/z less than	•				Des	criptic	contains	•	
eithe		atching S	earch Per	ulte te	delete			Retention Ti	me less than	•				Modifi	catior	ns contains	•	
t	he highli	ghted ID:	5									Dele	ete match	ing search result	s (	Delete nor	n-matching search res	ults Reset the c
		on-matchi e IDs that																
							#		te 4945 search	results?					8	cession	Modifications	
		iteria to other bato			ns to		1		te is is search	(country)						ALV6		40S ribosom
delet	e, click R	eset the			return to		3		Are you sure	you want to	o permanent	ly delete	e 4945 pe	otide search resu	ilts?	6W54		40S ribosom
step	1 above.					-	3									YCM1		Putative und
- N	o filter ap	plied		_	]		4	4								ADM7		Glyceraldeh
T				C	reate		5									YCU4	[14] Carbamidomet	hyl C 60S ribosom
Т	otal Hits	m/7	RT (min	Charge	Tag		6	4					Yes	No		ADM7		Glyceraldeh
7	- Car They		58.67	2	E A		7									AAU7		Eukaryotic ti
11				2			7	8.12 9						ANVDGFLV	_	29P940		Triosephosp
9				3			8	8.27 7	02011					TIAECLAEE	_	25AG43	[5] Carbamidometh	, ,
9		902.79: 894.474		3			9	5.05 1	- PEORA			57.41 13		CQLKWNS	-	25AIC3	[1] Carbamidometh	,
8				3			9	9.25 9				57.41 5.		SIVTLDVKP	_	25A652		Putative und
				-			9	9.25 9				57.41 5.		SIVTLDVKP		78590		Elongation f
11		763.762		3			11	8.95 9				12.72 1		AVGGEVG	-	25AJF7		60S ribosom
7				2			11	4.26 1				12.7: -4		ANAWKIV		259ZX6		U3 small nu
19				3			13	8.79 9				54.88 16		SINPNYTP	-	25A1E2		Pyruvate de
1 10	)	657.367		2			17	4.31 1				50.81 -0		GNIANFFV	-	259NC1		Likely mitoc
39		828.449		2			17	4.31 1				50.8: -0		GNIANFFV	-	4YCZ9		Putative und
7 13	;	776.411	52.50	2	•		17 17	4.50 1				50.81 9.		NLVSKDQY	-	25AEF3		Putative und
96		740.47{		2			1/ 17	8.62 5 8.62 5						TLAETAQE		259YM0 4YR96		Putative und
0 8			70.78	3		_	1/	8.62 5	//6.4.	1 51.90		50.81 10	0.90	TLAETAQE	20	41K90		Putative und
					F.	-												

Now in the Description first enter 'Like' and delete matching search results. Then enter the 'regular expression': **regex: Puta|Prob|Pote|Pred** and delete matching search results.

Progenesis	s QI.p Tutorial H	DMSe - Prog	genesis QI for	protec	omics									2
File									Refine					
mport Data	Review Alignment	Filtering	Experime Design Se		Review Peak Picking	Peptide Ion Statistics	Identify Peptides	QC Metric		Revi ns Prote		Report		nonlinea
	•		•				•		•			No.8		A Waters Compa
efine Ide	entifications				Batch dele	tion criteria								
	ide identificatio sults, you can i					Sc	ore less than	•			Sequenc	e Length less than	•	
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	the unwanted te the identifica			ck		n	n/z less than	•			De	scription contains	<ul> <li>regex: Puta Pr</li> </ul>	ob Pote Pred
either:						Retention Ti	me less than	•			Mod	ifications contains	•	
	lete Matching S e highlighted ID		its, to delete							Delete	atching search res		n-matching search results	Reset the criteria
	lete Non-match lete the IDs tha									Delete ma	atching search resi		n-matching search results	Reset the criteria
					a	P Delete 2150 s	earch results?				8	Accession	Modifications	
	the criteria to ify another bat				<b>V</b> 1	_						S Q5ALV6		40S ribosomal pr
delete,	click Reset the			to	<b>√</b> 3	? Are you	i sure you wan	it to perman	ently delete 21	i0 peptide	search results?	Q96W54		40S ribosomal pr
step 1 a	above.				<b>V</b> 3	-						S C4YCM1		Putative unchara
🐨 No f	filter applied				☑ 4							Q5ADM7		Glyceraldehyde 3
Т			Create		✓ 5							C4YCU4	[14] Carbamidomethyl C	60S ribosomal pr
# Tota	al Hits m/z	RT (min_C	harge Tag		✓ 6					Yes	No	Q5ADM7		Glyceraldehyde 3
1 7	828.41		narge rag		7						-	Q5AAU7		Eukaryotic transla
3 11					7	8.12 9				10.45	ANVDGFLV.			Triosephosphate
4 9	962.795	76.54 3			<ul><li>✓ 8</li><li>✓ 9</li></ul>	8.27 7				14.82	TIAECLAEE	Q5AG43	[5] Carbamidomethyl C	40S ribosomal pr
5 8					<b>⊘</b> 9	9.25 9 9.25 9				1 5.92 1 5.92	SIVTLDVKP SIVTLDVKP			Putative unchara
5 8	964.17(	63.92 3			▼ 9 ▼ 11	9.25 9 8.95 9					AVGGEVG	05AJF7		Elongation factor
7 11	763.762	69.69 3			<ul> <li>✓ 11</li> <li>✓ 13</li> </ul>	8.79 9				2 15.90 8 16.34	SINPNYTP	OSA1E2		60S ribosomal pr Pyruvate decarbo
, 11 B 7	823.439	75.96 2			15	8.62 5				16.95	TLAETAQE	Q5A1E2		Putative unchara
) / ) 19	920.144	74.49 3			17	8.62 5				1 16.95	TLAETAQE	C4YR96		Putative unchara
11 10	657.361	36.18 2			<ul><li>✓ 1/</li><li>✓ 19</li></ul>	8.26 6				4 16.07	ASLVPGTV	O9P834		60S ribosomal pr
11 10	828.449		-		20	8.07 7				{ 10.07	SINPDEAV	Q9F834 P46587		Heat shock prote
15 9	776.41	45.25 2	-		20	8.49 9				1 10.08	AGLKPEVP	O13430		Elongation factor
17 13 19 6	740.41				24	7.85 9				4 5.42	AGEKPETT	O59931		60S ribosomal pr
20 8	740.478 845.766	79.13 2			26	8.49 5				12.90	TLLEAIDAIE.	-		Elongation factor
20 8	845.700	/0./6 3		*	-						-			

Having applied all the filters there will be 5299 search results remaining

To validate the Peptide search results at the protein level select Resolve Conflicts (bottom left).

# **Resolving Conflicts**

This stage allows you to examine the behaviour of the identified peptides and choose to resolve any conflicts for the various peptide assignments at the protein level.

The Resolve Conflicts stage is now accessed the bottom left of the Refine Identifications stage.

The number of conflicts you have to resolve will depend on the scope and stringency of the filters you apply at the **Refine Identifications** stage.

Details on performing conflict resolution are available in Appendix 7 (page 91)

For this guide: we will NOT resolve the conflicts.

The following pages describe the handling of Protein grouping and Protein Quantitation. Options for these are available at both Resolve Conflicts and Review Proteins

Note: the default settings are to Use Protein Grouping and Relative Quantitation using HiN

### **Protein Grouping**

QP Protein quantitation options
Quantitation method:
Relative Quantitation using Hi-N
Number of peptides to measure per protein (N): 3
Use protein grouping .e. hide proteins whose peptides are a subset of another protein s.
OK Cancel

Where proteins are identified containing the same peptides then they are effectively indistinguishable aside from score. Also where one protein contains only peptides that represent a subset of another protein's peptides, the protein with fewer peptides can be subsumed into that with the greater number.

With protein grouping switched on (default setting) protein groups and the additional members are indicated by a bracketed number located after the Accession number. Taking **Glutathione reductase (C4YR40)** as an example, we can look at the other group members by changing the protein options.

Although Conflict resolution has not been performed this protein has no conflicting peptides.

Accession	Peptides	Unique	Conflict:	Score	Tag	*		#	Σ	中	Score	Hits	Correlation	Mass	Mass error (p	RT (mins)	Charge	e Tag	<ul> <li>Abundance</li> </ul>	С ^
C4YEG9	12	8	5	115				1001	٠	3	6.41	7	0.289	1100.643	-1.19	42.3	2		6.57E+04	Ε
C4YNX7	12	12	0	120			V	1284	٠	2	5.73	2	0.336	1092.59	8.18	42.2	2		5.74E+04	
🎯 C4YR40 (+2) 🕠	o 12	12	0	77.4			<b>V</b>	6168	•	0	6.72	9	0.986	1195.694	0.919	50.1	2		1.75E+04	
O42766 C4YR4	40 - Glutath	ione redu	ictase GN=	CAWG_04539 P	PE=4 SV	=1	1	6639	0	1	6.51	7	0.832	1393.662	9.94	41.5	2		1.15E+04	-
•						_	•													P
Peptide ion views						Pe	ptic	le ion	s of	con	nflicting p	roteii	n							
						Pe	ptic	le ion	s of	con	nflicting p	roteii	n							
Accession I	Peptides U	nique (	Conflict: P	rotein Score	P		#	Σ	e	] Sc	ore Hi	s C	orrelation N	Aass N	lass error (p R	(T (mins)	Charge	Tag	<ul> <li>Abundance</li> </ul>	Conflic
۰ []	11				4	۲							III							4
E Refine Ident	tification	s Prot	tein option	S														Se	ction Complete	

Use protein grouping e. hide proteins whose peptides are a subset of another protein's.
Aass Mass error (p RT (mins) Charge Tag ▼ Abundance Col ▲
100.643 -1.19 42.3 2 6.57E+04
774.877 2.12 70.1 2 4.91E+03 . 972.551 1.69 54 2 1.11E+04 .
393.662 9.94 41.5 2 1.15E+04
F
s Mass er <mark>er (p</mark> RT (mins) Charge Tag ▼ Abundance Confl ▲
.643 -1.19 42.3 2 🔲 6.57E+04 2
.877 2.12 70.1 2 📕 4.91E+03 2
551 1.69 54 2 🚺 1.11E+04 2 🛫
1 7 9 3

Note: when the grouping is switched off the other group members appear in panel E showing conflicts with the other group members and no Unique peptides

Before moving to **Review Proteins** it is worth understanding the available options to handling Protein Quantitation in Progenesis QI for proteomics.

	Relative Quantitation using Hi-N
	Number of peptides to measure per protein (N): 3
The next section in this guide describes the use of alternatives to the default method shown to the right.	Use protein grouping .e. hide proteins whose peptides are a subs

QIP Protein quantitation options Quantitation method:

Note: If you are using the default method, you can go directly to the Review Protein stage page 60 using the workflow.

×

• 3 🔷

Cancel

OK

### **Protein Quantitation options**

There are 4 options with which to control how the Protein Quantification is performed by Progenesis QI for proteomics.

The default option that will be applied is **Relative Quantitation using Hi-N**.

If you have selected one of the other options during the setup of the Auto Processing of your data (Stage 1) then this option will be applied.

(	Quantitation method:
ļ	Relative Quantitation using Hi-N
	Absolute Quantitation using Hi-N  Requires a calibrant protein to calculate absolute amounts Uses the most abundant N peptides Allows comparison between proteins within a run
	Relative Quantitation using Hi-N  Uses the most abundant N peptides Allows comparison between proteins within a run
	Relative Quantitation using non-conflicting peptides • Uses only peptides which have no conflicting protein identifications • Allows comparison of a single protein across runs
	Relative Quantitation using all peptides • Uses all peptides identified as part of a protein • Allows comparison of a single protein across runs

Hi-N in Progenesis QI for proteomics is an implementation of Hi-3 as described by Silva *et al.* [References]. After peptide and protein identification, the abundance of each peptide is calculated from all its constituent peptide ions.

For each protein, the N most abundant peptides (N being set according to the user selection) have their **abundances averaged** to provide a reading for the protein signal.

The ranking of peptide abundance is based on the integrated value across all the runs, allowed by the accurate alignment and lack of missing values. This gives added confidence in the peptide selection, taking all runs into account to make the ranking robust.

OP Protein quantitation options
Quantitation method:
Relative Quantitation using Hi-N
Number of peptides to measure per protein (N): 3
Use protein grouping i.e. hide proteins whose peptides are a subset of another protein's.
OK Cancel

The averaged abundance readings not only make possible the **relative quantitation** of the same protein across all runs to be determined but also allow, with the inclusion of a known amount of a calibrant protein in each run, this to be converted to an **absolute** reading for protein amount.

To generate values for absolute quantitation enter the accession number and amount for the calibrant.

OP Protein quantitation options	QP Protein quantitation options
Quantitation method:	Quantitation method:
Absolute Quantitation using Hi-N	Absolute Quantitation using Hi-N
Number of peptides to measure per protein (N):	Number of peptides to measure per protein (N):
Calibrant accession:	Calibrant accession: P83773
Amount (fmol):	Amount (fmol): 50
Use protein grouping i.e. hide proteins whose peptides are a subset of another protein's.	Use protein grouping i.e. hide proteins whose peptides are a subset of another protein's.
OK Cancel	OK Cancel

The absolute amounts, based on the calibrant used, are reported at the Review Proteins stage as additional columns (one for each condition) following the protein description.

Review Proteins Using this screen, you can find the proteins of interest in your experiment.	Vo filter applied Create			<b>()</b> H	elp '
<ul> <li>Set the quantitation options</li> </ul>	Description	Amount (fmol) - A	Amount (fmol) - B	Amount (fmol) -	٩.
If you've not already done so, choose between relative and absolute quantitation,	Glyceraldehyde 3-phosphate dehydrogenase GN=TDH3 PE=3 SV=1	480	805	856	*
use of Hi-N, protein grouping and more.	Glycerol-3-phosphate dehydrogenase GN=GPD2 PE=3 SV=1	80.1	141		L
Protein options	Glycerol-3-phosphate dehydrogenase_ mitochondrial GN=GUT2 PE=3 SV=1	30.5	33.7	33.3	1
	Glycine cleavage system H protein GN=CAWG_00084 PE=4 SV=1	27.3	37.6	31.9	G

The ranking of peptide abundance is based on the integrated value across all the runs, allowed by the accurate alignment and lack of missing values. This gives added confidence in the peptide selection, taking all runs into account to make the ranking robust. These Hi-N methods allow the relative and absolute comparison of proteins within the same run.

**Note**: When there are **peptide conflicts** (a peptide is shared between two proteins, for example) it is important to assign the signal correctly for absolute quantitation. To do this, Progenesis QI for proteomics carries out a two-step process. Firstly, Hi-N is carried out only on the N most abundant **unique** (non-conflicting) peptides of the proteins concerned. This provides a ratio estimate for the two proteins based only on unshared peptides. The abundance of any shared peptides is then divided and allotted in this ratio between the two proteins, and the full Hi-N calculation is then applied using the divided values for conflicted peptides.

Naturally, if the conflicted peptides are not among the N most abundant in either protein initially, then this will not cause any difference in the result.

**Relative Quantitation** can also be performed comparing a single protein across all the runs using only the unique or non-conflicting peptides. Select the third method from the drop down

Using non conflicting peptides

OP Protein quantitation opt	ions 💌
Quantitation method:	
Relative Quantitation us	ing non-conflicting peptides 🔹 👻
Use protein grouping is of another protein's.	e. hide proteins whose peptides are a subset
	OK Cancel

The relative Quantitation can also be performed using all peptides.

OP Protein quantitation options
Quantitation method:
Relative Quantitation using all peptides
Use protein grouping i.e. hide proteins whose peptides are a subset of another protein's.
OK Cancel

**Note**: if you have performed conflict resolution then there will be no difference between these methods.

Make sure that the Protein quantitation method is set to **Relative Quantitation** using Hi-N (where N=3) and Use protein grouping is ticked.

Now move to the **Review Proteins** section by clicking on the icon on the workflow at the top of the screen.

# **Stage 12: Review Proteins**

The **Review Proteins** stage opens displaying details for all proteins. You can now create tags at the level of the proteins. Right click on the table and create Quick Tags for proteins with an Anova p value  $\leq 0.05$  and Max Fold change  $\geq 2$ .

Progenesis QLp Tutorial HDMSe - Progenesis QI for p     File     Review     Review     Alignment Filtering Design Setu;	Review Peak Pe	ptide Ion itatistics	Identify Peptides QC	Refine Metrics Identificat		view teins	Protein Statistics	Report			nonline
	-			• •					Contraction of the second	TX	A Waters Compa
Review Proteins Using this screen, you can find the proteins of nterest in your experiment.	W No filte	r applied	Create	earch	٩						(i) Hel
Set the quantitation options	Accession	Peptides	Unique peptides	Confidence score	Anova (p)	q Value	Tag 💌	Max fold change	Highest Mean	Lowest Mean	Description
If you've not already done so, choose between relative and absolute guantitation, use of Hi-N,	P87219	3	3	22.9	2.09E-06	4.46E-06		4.1	С	A	Sorbose reductase SO
protein grouping and more.	P43066	3	3	24.8	2.45E-06	4.46E-06		1.78	В	A	D-arabinitol 2-dehydr
Protein options	Q59W63	2	1	11.1	2.64E-06	4.46E-06		1.35	A	С	Ribosomal RNA assen
	Q7Z8E8	3	3	21.8	4.02E-06	5.31E-06		3.36	С	Α	Cell surface hydropho
Create a shortlist to review	🔮 Q9P8Q7	8	7	20.5	4075 05	5.31E-06		2.01	В	A	Isocitrate lyase GN=I0
<ul> <li>In the table, sort and <u>filter the proteins</u> based on their measurements, to generate a shortlist</li> </ul>	C4YNC2 (+1	L) 9	9	No tags to assign		5.31E-06		1.51	В	A	Phosphoenolpyruvate
for further review.	Q5AEB8	4	3	New tag		1.05E-05		1.41	В	A	Proteasome compone
How are the measurements calculated	C4YNI1	3	2	Quick Tags	•	Ano	/a p-value		В	A	1_3-beta-glucan syntl
To sort the table by a given value, simply click the relevant column header.	C4YFV8	2	2	Edit tags		Max	fold change	h	В	A	Glycine cleavage syste
	C4YHP3	5	5	Add to Clip Galle	ry	Mod	ification		В	A	Carnitine O-acetyltrar
For each protein of interest, review its peptide	P42800	4	4	26	1.46E-05	Sequ	ience		в	A	Inositol-3-phosphate
measurements and correlations:	Q59LQ6	1	1	8.07	1.6E-05	Pept	ide tags cor	tain	A	B	Protein translation fac
View peptide measurements	Q59US5	3	3	24.7	1.7E-05	1.05E-05		1.27	B	A	O-acetylhomoserine (
You can also double-click to review a protein.	© 094039	25	25	260		1.05E-05		1.78	B	A	Transketolase 1 GN=
	4	25	25	111	1.755-05	1.052-05		1.70	D	A	Fransketolase i GN=
Export data for further processing By exporting your data to external tools, there's no limit to your analysis.	Selected p		lsocitrate ly	/ase GN=ICL1	PE=3 \$	5V=1					
Export to pathways tool			А		_					с	
Export protein measurements Export peptide measurements Export peptide ion measurements	ArcSinh Normalised Abu		M				) M			*	1
xperiment design eview your data from a different perspective: urrent design: ABC	₹ 3.0 =	notoine die	played: 516							S	ection Complete

As an example let us explore Isocitrate Lyase.

First filter the table to show only proteins showing a significant 2 fold or greater change.

Fil		QLp Tutorial H Review Alignment	IDMSe - Proger Filtering	nesis QI for prot Experiment Design Setup		Peptide Ion Statistics	Identify Peptides QC	Refine Metrics Identificati		view teins	Protein Statistics	Report			nonlinea
															A Waters Compar
Using			find the prote t.	ins of		<b>ter applied</b> Is may be hidd	en Edit	Search		٩					🙆 Help
1 5	Set the o	quantitation	options		Accession	Peptides	Unique peptides	Confidence score	Anova (p)	q Value	Tag 💌	Max fold change	Highest Mean	Lowest Mean	Description
			done so, choos quantitation, u		Q9UVL1	3	3	26.5	6.22E-07	3.14E-06	۲	2.45	Α	с	Non-histone chron
		rouping and		ac or min,	C4YL44	2	2	12.8	7.96E-07	3.14E-06	۷	2.19	В	Α	Extracellular matrix
			Prote	ein options	P87219	3	3	22.9	2.09E-06	4.46E-06	۷	4.1	с	Α	Sorbose reductase
					Q7Z8E8	3	3	21.8	4.02E-06	5.31E-06	۲	3.36	с	Α	Cell surface hydrop
		shortlist to		ains based	🔇 Q9P8Q7						۵				Isocitrate lyase GN
0	In the table, sort and <u>filter the proteins</u> based on their measurements, to generate a shortlist for further review. <sup>(2)</sup> How are the measurements calculated?		P42800	4	4	26	1.46E-05	1.05E-05	۷	2.7	В	A	Inositol-3-phospha		
ţ			c calculated?	C4YF25	1	1	6.57	0.000156	3.35E-05	۲	2.25	Α	в	Bud site selection p	
			given value, s		Q59U83	1	1	5.68	0.000884	8.72E-05	۷	4.41	с	A	Ribonucleoside-dip
		ant column h		imply click	C4YRH4	6	6	35.4	0.0011	9.77E-05	۷	2.12	В	Α	NAD(P)H-depende
	Douiou +	the proteins			C4YE92	2	2	13.4	0.00124	0.000105		2.65	С	Α	6_7-dimethyl-8-rib
) F	For each	protein of int	erest, review	its peptide	Q5ALX8	5	5	51.3	0.00202	0.000143		2.05	с	A	Adenine phosphor
п	neasurer	ments and co			Q9HFQ6	1	1	14.6	0.00406	0.000228		2.22	С	Α	60S acidic ribosom
		View	/ peptide meas	urements	Q59NN8	1	1	6.72	0.0117	0.000489		3.46	Α	В	Hsp70 nucleotide e
Y	íou can a	also double-c	lick to review	a protein.				-	0.0140	0.00050	-	2.21	~		lose a los espe
4 в	By export	ting your dat o your analys		ools, there's	Selected View peptide r			ase GN=ICL1	PE=3 S	5 <b>V</b> =1					
		Ex	port to pathwa	ays tool			А				В			С	
		Expo	rt protein mea rt peptide mea peptide ion me	surements	ArcSinh Normalised Ab 2 2 0 7 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2		Mari				M			1 <b>4</b> 1	
evie			ifferent perspe	ective:	₹ 3.0 ∃	proteins dis	played: 14							Sec	tion Complete (

The table indicates that this protein is most highly expressed in Condition B, 2.01 fold over the lowest condition (A).

To view the corresponding peptide measurements for the current protein either double click on the protein in the table or use the **View peptide measurements** beside table and ensure that Show **Peptides** is selected.

File Review Experiment port Data Alignment Filtering Design Setup	Review Pick			Identify Peptides	QC Metrics	Refine Identification:	Review s Proteins	Protein Statistics	Report			nonli	nea
							•					A Waters C	ompar
Return to list of proteins	₽ Ne	ext protein											Help Help
view selected protein view the selected protein's identified peptides and idate their expression patterns.		ession: <u>Q9P8Q7</u>	-	e GN=IC	L1 PE=3 SV=1					9	No filter approximation	pplied Crea	te
	ŝ	Identifier	Ions	Score	Correlation	Anova (p)	Max Fold Change	Highest Mean	Lowest Mean	Tag	Abundance	Neutral Mass	Ret
Choose the level of detail View the properties and expression profiles of	•	53.57_1729.8822n	1	7.423	0.962	0.000364	1.52	В	А		2.818E+04	1729.8822	53.
either peptides or individual peptide ions:	•	64.49_1503.8215n		7.388	0.928	2.89E-05		В		-	1.808E+04	1503.8215	64.
Show:      Peptides     Peptide ions	•	63.34_1322.6874n		7.246	0.967	2.6E-06		В			1.38E+04	1322.6874	63.
Tip: you can also double-click a peptide to select and view its component ions.	0	57.38_1715.9187n	1	5.670	0.949	0.000466	1.41	В	А		5.349E+04	1715.9187	57.
	0	57.38_1715.9197n	1	5.670	0.894	0.00266	1.13	В	А		2.296E+04	1715.9197	57.
Compare expression profiles Select peptides in the table to show their	0	53.57_1729.8549n	1	7.423	0.989	0.000385	7.4	В	Α	-	3627	1729.8549	53.
expression profiles in the chart below.	0	38.07_1302.7206n	1	6.440	0.992	5.4E-06	2.08	В	Α	-	5535	1302.7206	38.
Select all peptides	0	38.09_1302.7213n	1	6.440	0.862	0.0814	2.09	В	Α		634.7	1302.7213	38.
Correlation values for the expression profiles can	0	38.29_1302.6459n	1	6.440	0.933	0.00223	1.32	В	А		4071	1302.6459	38.
also be seen in the table.	0	47.52_1312.7092n	1	6.960	0.978	1.07E-05	2.9	В	Α	-	7303	1312.7092	47.
Resolve any quantitative outliers Tag any peptide ions whose expression profile is an outlier for this protein.	•	34.10 1489.7484n	1	7.207	0.990	3.43E-05 III	3.21	В	A	-	8935	1489.7484	34.I )+
<ul> <li>Output for this protein</li> <li>O Learn about tagging and filtering</li> </ul>					Stand	lardised Expre	ssion Profiles		•				
You can then review their identifications in more detail at the <u>Refine Identifications</u> step.		A				В			с				
	Star						¢ŧ						
periment design view your data from a different perspective:	- 2	1											

The solid icon in  $\Sigma$  column indicates that the peptide contributes to protein measurements.

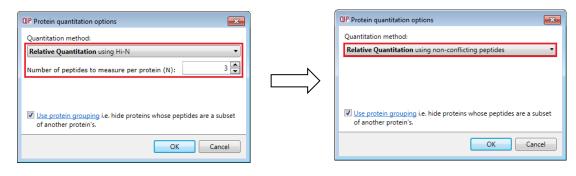
**Note**: a correlation score (between -1 and 1) for each peptide is available to facilitate the validation of peptide expression.

You can control what peptides are used in Protein Quantitation by using the **Protein options...** at the protein level of **Review Proteins** 

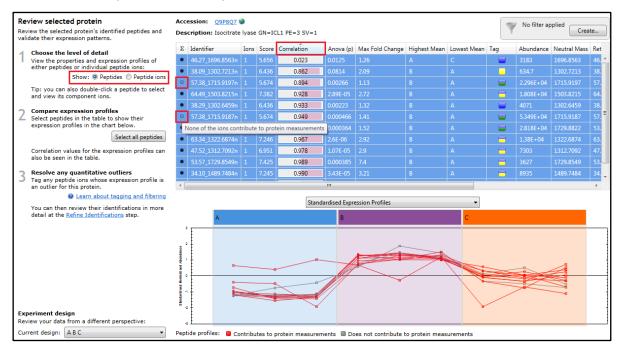
Usir	view Proteins ng this screen, you can find the proteins of rest in your experiment.			<b>r applied</b> may be hidd	en Edit
1	Set the quantitation options		Accession	Peptides	Unique peptides
11	If you've not already done so, choose betwe relative and absolute quantitation, use of Hi-		Q9UVL1	3	3
	protein grouping and more.		C4YL44	2	2
	Protein option	ns	🔇 Q9P8Q7	8	7
			P87219	3	3
2	Create a shortlist to review In the table, sort and filter the proteins base	A	Q7Z8E8	3	3
	on their measurements, to generate a short		P42800	4	4
	for further review.		C4YF25	1	1
	How are the measurements calculated and the second seco	tea?	059U83	1	1

**Note**: the default quantitation method is based on the Relative Quantitation using Hi-N, in this case n=3 so the top 3 most abundant peptides are used to determine the relative abundance for each protein. Where there are multiple charge states the combined abundance of the charge states is used.

Changing to **Relative Quantitation using non conflicting** peptides will utilise all non conflicting peptides in the abundance calculation. (as shown below)



**Note**: doing this will update the peptide table to show open symbols against conflicting peptides which will not contribute to the quantitation of the protein.



In this example if you view the data at the level of the peptide ions then the two open symbols correspond to the 2 charge states of the conflicting peptide ion

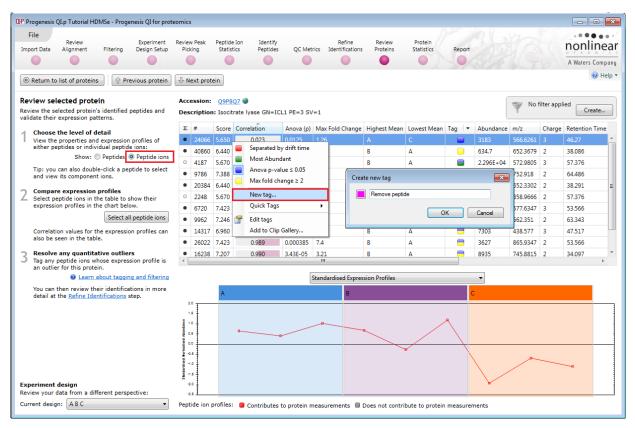
Review selected protein	Accession	<u>Q9P</u>	98 <u>07</u>	٥						S No filter applie	ed
Review the selected protein's identified peptides and validate their expression patterns.	Descriptio	n: Isoc	citrate	lyase GN=	ICL1 PE=3	SV=1				Y	Create
Choose the level of detail	west Mean	Tag	• A	Abundance	m/z	Charge	Retention Time (mins)	Mass error (ppm)	Drift time (ms)	Peptide Sequence	Modifications
View the properties and expression profiles of		8	1	L.808E+04			64.486	11.30	4.28	S ASADIFGSNLLAVAR	
either peptides or individual peptide ions:		-	8	3935	745.8815	2	34.097	4.79	4.21	AYGQTVQQPEIEK	
Show: O Peptides O Peptide ions			5	5.349E+04	858.9666	2	57.376	11.18	4.90	HQKWSGATYIDGLLK	
Tip: you can also double-click a peptide to select and view its component ions.			2	2.296E+04	572.9805	3	57.376	11.76	3.31	HQKWSGATYIDGLLK	

#### Returning to the Peptides view of the table

Rev	view selected protein	Acc	ession: <u>Q9P8Q7</u>	٥								No filter ap	nlied	
	iew the selected protein's identified peptides and date their expression patterns.	Des	cription: Isocitrate	lyase	GN=IC	CL1 PE=3 SV=1					Y	ino inter op	Creat	te
	Choose the level of detail	Σ	Identifier	Ions	Score	Correlation	Anova (p)	Max Fold Change	Highest Mean	Lowest Mean	Tag	Abundance	Neutral Mass	Ret
1	View the properties and expression profiles of either peptides or individual peptide ions: Show:  Peptides Peptide ions	٠	46.27_1696.8563n	1	5.656	0.023	0.0125	1.26	Α	С		3183	1696.8563	46. 🔺
		٠	38.09_1302.7213n		6.436	0.862						634.7	1302.7213	38.
		0	57.38_1715.9197n		5.674	0.894	0.00266	1.13	В	А		2.296E+04	1715.9197	57.
	Tip: you can also double-click a peptide to select and view its component ions.	•	64.49_1503.8215n		7.382	0.928	2.89E-05	2.72	В	А		1.808E+04	1503.8215	64.

If you order the Peptide table using the **Correlation** column then hold down the Ctrl key and then click on the first peptide (with the lowest correlation) you can remove them from the expression profiles.

As an example: the removal of a poorly correlated peptide from a protein requires you to first create a tag (at the peptide ion level for the corresponding peptide ions of the peptide you wish to remove (see below).



Once the tag is created, return to the peptide level, to identify additional, poorly correlated peptides. For each additional peptide, double click on it and then right click on the corresponding peptide ion to select and add the **Remove peptide** tag, the tag appears in the tag column.

Rev			ession: cription		07 🧐 rate lyase GN=IC	L1 PE=3	SV=1					W No	filter appl	ied Create	
	Choose the level of detail	Σ	#	Score	Correlation	Anova	(p) Max Fold Change	Highest Me	an Lowest Mean	Tag 💌	Abundance	m/z	Charge	Retention Time	
1	View the properties and expression profiles of	0	2248	5.670	0.949	0.0004	56 1.41	В	Α		5.349E+04	858.9666	2	57.376	*
	either peptides or individual peptide ions:	0	4187	5.670	0.894	0.			A	-	2.296E+04	572.9805	3	57.376	
	Show:  Peptides	٠	14317	6.960	0.978	1.	Separated by drift tim Most Abundant	e	А	-	7303	438.577	3	47.517	'n
	Tip: you can also double-click a peptide to select and view its component ions.	٠	6720	7.423	0.962	0.	Anova p-value ≤ 0.05		А		2.818E+04	577.6347	3	53.566	
_	·	•	26022	7.423	0.989	0.	Max fold change $\geq 2$		А	-	3627	865.9347	2	53.566	
2	Compare expression profiles Select peptide ions in the table to show their	٠	24066	5.650	0.023	0.	Remove peptide		С		3183	566.6261	3	46.27	1
	expression profiles in the chart below.		12029	6.440	0.992	5.			А	-	5535	435.2475	3	38.068	E
	Select all peptide ions	•	20384	6.440	0.933	0.	New tag		А		4071	652.3302	2	38.291	
	Correlation values for the expression profiles can	٠	40860	6.440	0.862	0.	Quick Tags	•	A		634.7	652.3679	2	38.086	
	also be seen in the table.	•	9962	7.246	0.967	2. 🖀	Edit tags		Α	-	1.38E+04	662.351	2	63.343	1
С	Resolve any quantitative outliers						Add to Clip Gallery								-
3	Tag any peptide ions whose expression profile is an outlier for this protein.	•				_								Þ	

Now at the Peptides level, set up a Tag Filter to display only the poorly correlated peptide ions in this example 2 (with the **Remove peptide** tag).

File Review Import Data Alignmen		Experiment Design Setup	Review Pick			Identify Peptides	QC Metrics	Refine Identification	s Proteins	Protein Statistics	Report			A Waters C	nea
@ Return to list of pro															🖉 Help
		evious protein		ext protein	_										
Review selected providence of the selected provi	rotein otein's identified		Acce	ession: <u>Q9P8Q7</u> cription: Isocitrat	-	e GN=IC	L1 PE=3 SV=1						filter applied des may be h		it
eview selected p eview the selected pr alidate their expression	rotein otein's identified n patterns.		Acce	ession: <u>Q9P8Q7</u>	e lyas		L1 PE=3 SV=1 Correlation	Anova (p)	Max Fold Change	Highest Mean	Lowest Mean	Y pept		nidden Edi	
eview selected p	rotein btein's identified on patterns. of detail es and expression	peptides and	Acce	ession: <u>Q9P8Q7</u> cription: Isocitra	e lyase Ions			Anova (p) 0.00266	Max Fold Change	Highest Mean B	Lowest Mean	Y pept	ides may be l	nidden Edi	_

Then click on Refine Identifications on the workflow.

Select the peptides showing in the left hand table and then right click on one of the highlighted rows in the right hand table and click **Delete selected peptide(s)**.

File mport Data	Review Alignment	Filtering	Experiment Design Setup	Review Picki		Peptide Ion Statistics		Identify Peptides	QC Metri	cs Ide	Refine ntifications	Revie Protei		Re	eport			nonline
				•					•				•	-				A Waters Comp
efine Ide	ntifications			Bate	ch delet	ion criteria												
	de identification sults, you can r					:	Score	less than	•				Sequence	e Length	less than	•		
	a set of dele						Hits	less than	•					Charge	less than	•		
values fo	atch deletion c or a set of iden						Mass	less than	•				S	equence	contains	•		
delete.				Abs	olute n	nass error (	ppm)	less than	•				A	ccession	contains	•		
	the unwanted e the identifica						m/z	less than	•				De	cription	contains	•	regex: Puta P	rob Pote Pred
either:						Retention	Time	less than	•				Modi	fications	contains	•		
the • Dele	ete Matching S highlighted ID ete Non-match ete the IDs tha	s ing Search R	esults, to									Delete ma	atching search resu	ilts	Delete no	n-matching	search results	Reset the criteria
		-	-		#	Score	Hit	s m/z	RT(mins	Charge	e Mass	Mass err	Sequence	Acc	ession	Mod	ifications	
To specif	he criteria to fy another bate	ch of identific			4171	7.02	8	446.25	39.90	2		3.57	YPIEELK	C4)				Isoleucyl-tRNA syr
delete, d step 1 a	lick Reset the	Criteria and t	then return to		4175	7.69	8	744.40	48.93	2	1486.7{		QPTVAIGA	S C41				Triosephosphate i
otop I o					4175	7.69	8	744.40	48.93	2	1486.78		QPTVAIGA	Q9I				Triosephosphate i
	ilter applied		Edit		4177	7.86	9	968.81	64.49	3	2903.41		VGAVGEN	S C4				Elongation factor
<ul> <li>peptic</li> </ul>	de ions may be	hidden [	Contin	<b>v</b>	4179 4179	8.22 8.22	9 9	773.73 773.73	60.39 60.39	3	2318.1( 2318.1(		DKAPYSDE	P43				Fatty acid synthas
Total	Hits m/z	RT (min Cha	arge Tag 💌		41/9	8.13	9 8	489.28	48.56	3	2318.1t 976.55		MGVPYAIVK	C4 05				Fatty acid synthas
187 7	572.98(	57.38 3			4185	8.13	8 8	489.28	48.56	2	976.55		MGVPYALVK	Q3 05/ 05/				60S ribosomal pro
4061 3		46.27 3			4185	7.14	0 5	409.20	57.38	2	1715.92		YEVNPLLID	O5/				60S ribosomal pro CTP synthase GN=
					4187	5.67	2	572.98	57.38	3			peptide(s)	-	P807			Isocitrate lyase GN
					4204	7.01	2	567.27	29.38	2	1132.5:		SHLEDEEFK		APLO			Glucose-6-phosph
					4205	7.35	9	586.85	52.50	2	1171.69		ALEGLTISQ	C4)				Phenylalanyl-tRNA
					4210	7.55	9	729.87	47.57	2	1457.73		EAFSLFDK	Q5				Calmodulin GN=C
					4214	7.49	5	563.96	43.53	3	1688.8(		SKYEEEILE	G C41				Fatty acid synthas
					4214	7.49	5	563.96	43.53	3	1688.8t	13.99	SKYEEEILE	🕥 P43	3098			Fatty acid synthas
					4218	8.25	9	545.65	49.68	3	1633.92	2.67	AVVVIGDS	Q5/	A900			40S ribosomal pro
					4228	8.27	5	549.30	75.94	3	1644.87	21.14	STIAECLAEE	🕥 Q5	AG43	[5] Carban	nidomethyl C	40S ribosomal pro
			+	٠														÷.

Now return to **Review Proteins**, double click on Isocitrate Lyase (Q9P8Q7) and go to the **Peptides** view. Clear the filter, to view the remaining peptides, and then click **Select all peptides** for Isocitrate Lyase.

Note: the poorly correlated peptides are no longer present.

IP Progenesis QLp Tutorial HDMSe - Progenesis QI for prote File Import Data Alignment Filtering Design Setup	Review Peak Pep	tistics Pe	entify ptides QC N	Refine Ietrics Identificatio	Review Proteins	Protein Statistics	Report			AWaters	MIC
	I Next protein					- A -			1.17		Help
Review selected protein Review the selected protein's identified peptides and validate their expression patterns.	Accession: 09 Description: Iso		SN=ICL1 PE=3	SV=1				P	No filter a	pplied Cre	ate
Choose the level of detail	Σ Identifier	Ions S	Score Correlati	on Anova (p)	) Max Fold Change	Highest Mean	Lowest Mean	Tag	Abundance	Neutral Mass	Reten
View the properties and expression profiles of	• 64.49_1503.	3215n 1 7	.388 0.9	64 2.89E-05	2.72	В	А		1.808E+04	1503.8215	64.48
either peptides or individual peptide ions:	• 34.10_1489.	7484n 1 7	.207 0.9	94 3.43E-05				8	8935	1489.7484	34.0
Show: O Peptides Peptide ions	<ul> <li>57.38_1715.</li> </ul>	9187n 1 5	0.670 0.9	53 0.000466	1.41	В			5.349E+04	1715.9187	57.3
Tip: you can also double-click a peptide to select and view its component ions.	• 47.52_1312.	7092n 1 é	0.960 <b>0.9</b>	84 1.07E-05	2.9	В		8	7303	1312.7092	47.5
C	• 53.57_1729.	3822n 1 7	.423 0.9	59 0.000364	1.52				2.818E+04	1729.8822	53.5
2 Compare expression profiles Select peptides in the table to show their	• 53.57_1729.	3549n 1 7	.423 0.9	89 0.000385					3627	1729.8549	53.5
expression profiles in the chart below.	• 38.07_1302.	7206n 1 6	5.440 <b>0.9</b>	88 5.4E-06	2.08				5535	1302.7206	38.0
Select all peptides	• 38.09_1302.	7213n 1 6	6.440 0.8	62 0.0814	2.09				634.7	1302.7213	38.0
Correlation values for the expression profiles can	• 38.29_1302.	5459n 1 6	5.440 <b>0.9</b>	50 0.00223					4071	1302.6459	38.2
also be seen in the table.	• 63.34_1322.	5874n 1 7	.246 0.9	89 2.6E-06	2.92				1.38E+04	1322.6874	
Resolve any quantitative outliers Tag any peptide ions whose expression profile is an outlier for this protein.	•			m							
Learn about tagging and filtering				Standardised Exp	ression Profiles		•				
You can then review their identifications in more detail at the <u>Refine Identifications</u> step.	А			В			с				
Experiment design Review your data from a different perspective:	Brendrici and American American										
Current design: A B C	Peptide profiles:	Contribute	s to protein me	easurements 🔳 D	oes not contribute	to protein meas	surements				

Now return to the protein level by clicking on Return to list of proteins (top left).

Before creating tags for proteins containing modified peptides, reset the Quantitation method to **Relative Quantitation using Hi-N** using the **Protein options...** 

Now right click on the Protein table and select **Modification** from the **Quick tags**.

Usin	<b>riew Proteins</b> Ing this screen, you can find the proteins of rest in your experiment.	No filter a	opplied	Create	arch	Q			
1	Set the quantitation options	Accession	Peptides	Unique peptides	Confidence score	Anova (p)	q Value	Tag 💌	Max f
	If you've not already done so, choose between relative and absolute guantitation, use of Hi-N,	🔇 Q9P45	A	hus < 0.0E	31	1.31E-06	3.42E-06	-	1.95
	protein grouping and more.	C4YRH	Anova p-va Max fold cł		30.5	1.47E-06	3.42E-06	•	1.75
	Protein options	S P87219		lange 2 2	22.9	2.09E-06	3.83E-06		4.1
		P4306	New tag		24.7	2.45F-06	3.83E-06	-	1.78
2	Create a shortlist to review	🔇 Q59W	Quick Tags	•	Anova p-valu	ie	3E-06	•	1.35
~	In the table, sort and <u>filter the proteins</u> based on their measurements, to generate a shortlist	🔇 Q7Z8E 🚰	Edit tags		Max fold cha	nge	2E-06		3.36
	for further review.	Q9P8C	Add to Clip	Gallery	Modification		2E-06		2.01
	We have the measurements calculated?	C4YNC2 (+1)	9	9	Sequence		3E-06	•	1.51
	To sort the table by a given value, simply click the relevant column header.	Q5AEB8	3	3	Peptide tags	contain	JE-06	•	1.41

QP Protein quantitation options

Use protein grouping i.e. hide proteins whose peptides are a subset of another protein's.

OK

×

•

3

Cancel

To find those proteins containing peptides with Carbamidomethylated cysteine and Oxidated methionine residues create Quick tags for each modification as shown below.

QP New Quick Tag	QIP New Quick Tag
Where any peptide of a protein has Modification with: Carbamidomethyl C	Where any peptide of a protein has Modification with: Oxidation M
Can I use wildcards?	Can I use wildcards?
Apply the following tag:           Modification with Carbamidomethyl C	Apply the following tag: Modification with Oxidation M
Create tag Cancel	Create tag Cancel

To reduce the table to displaying only these proteins with modified peptides (on cysteines and or methionines) use a tag filter to focus on these proteins by placing the appropriate tags in the **Show proteins that have at least one of these tags:** 

<b>create a filter</b> how or hide proteins based on a selection of thei nore guidance, please see the <u>online reference</u> .	r tags. Move tags to the appropriate boxes to create the filter. For
vailable tags:	Show proteins that have all of these tags:
Anova p-value ≤ 0.05 (377 proteins)	
Max fold change ≥ 2 (15 proteins)	
	Show proteins that have at least one of these tags:
	Modification with ox (25 proteins)
	Modification with Carbamidomethyl C (207 protein)
	Hide proteins that have any of these tags:

The proteins table will now only display those proteins containing the modified peptides.

Progenesis QI.p Tutorial HDMSe - Progenesis QI for prote	omics									
File Review Filtering Experiment Design Setup		Peptide Ion Statistics	Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report		A Waters Compar
Review Proteins Ising this screen, you can find the proteins of nterest in your experiment.		<b>ter applied</b> is may be hid	den Ed	dit	h	Q				🕜 Help
Set the quantitation options	core Anova (p	o) q Value	Tag 🔹	Max fold change	e Highest Mean	Lowest Mean	Description			
If you've not already done so, choose between relative and absolute quantitation, use of Hi-N,	0.000449	5.58E-05	4	1.23	В	A	Lactoylglutat	hione lyase GN=GLO1 P	E=4 SV=1	-
protein grouping and more.	0.000477	5.87E-05	4	1.26	Α	с	Ribosome bio	ogenesis protein YTM1 (	GN=YTM1 PE=3 SV=1	
Protein options	0.0005	6.01E-05	٨	1.76	С	А	Pyruvate deca	arboxylase GN=PDC11 F	PE=3 SV=1	
	0.000536	6.39E-05	4	Anova p-value ≤	0.05		Threonine alo	dolase GN=GLY1 PE=3 S	V=1	L
Create a shortlist to review In the table, sort and filter the proteins based	0.000548	6.46E-05		Modification with		nyl C	60S ribosoma	al protein L10 GN=RPL1	0 PE=4 SV=1	
on their measurements, to generate a shortlist	0.000558 6.52E-05 🕘 Modification with ox				Aldehyde dehydrogenase_ mitochondrial GN=ALD5 PE=3 SV=1					
for further review.      O How are the measurements calculated?	0.00058	6.71E-05		1.19	в	A	Tyrosyl-tRNA	synthetase GN=TYS1 P	E=4 SV=1	
	0.000615	6.94E-05	4	1.23	В	Α	Fatty acid syn	nthase alpha subunit red	luctase GN=CAWG_02796 PE=	4 SV=1
To sort the table by a given value, simply click the relevant column header.	0.000701	7.63E-05	4	1.16	В	Α	Adenylyl-sulf	ate kinase GN=MET14 P	E=3 SV=1	
Review the proteins	0.000726	5 7.77E-05	٩	1.4	В	Α	Heat shock p	rotein SSA2 GN=SSA2 P	E=1 SV=3	
For each protein of interest, review its peptide	0.000732	2 7.77E-05	4	1.45	В	Α	Peroxiredoxin	TSA1 GN=TSA1 PE=2	SV=1	
measurements and correlations:	0.00074	7.78E-05	4	1.31	Α	С	Inorganic pho	osphate transporter PHC	084 GN=CAWG_00289 PE=4 S	V=1
View peptide measurements	0.000748	7.79E-05	4	1.35	В	С	Glycogen pho	osphorylase_ muscle for	m GN=PYGM PE=1 SV=3	
You can also double-click to review a protein.	0.000764	7 705 05	4	1 77	n	~	COC 11	i i na na na na na na	111 CAL CANAG 00000 DE	101.1
Export data for further processing By exporting your data to external tools, there's no limit to your analysis.	Selected		5		-phosphate	e dehydrog		N=TDH3 PE=3 S		
Export to pathways tool				A			В		С	
Export protein measurements Export peptide measurements Export peptide ion measurements	ArcSinh Normalised Ab. 13.0 13.0 12.5 13.0 12.5 11.5 11.5		i	\$			黛		<b>\$</b>	
cperiment design	11.5 −L									
eview your data from a different perspective: urrent design: A B C	Quantifiable	proteins di	splayed: 2	215					Sect	tion Complete

Note: hold the cursor over the tags for a description of the proteins current tags.

Note: the Sequence Quick tag can be used to locate Proteins containing peptides with specific motifs.

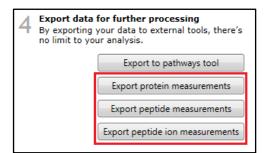
### **Stage 13: Exporting Protein Data**

Data can be exported in a csv file format. You can either export the **protein, peptide or peptide ion measurements** using the options in the File Menu or use the buttons under Step 4 both available at the **Review Proteins** stage.

As an example of Data export use the Tag filtered set from the previous section for only proteins that have Oxidised Methionine residues.

First set the tag filter as shown below. Then select **Export Protein Measurements**.

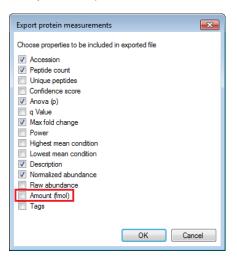
QIP Filter the proteins	×
Create a filter Show or hide proteins based on a selection of th create the filter. For more guidance, please see t	
Available tags:	Show proteins that have all of these tags:
<ul> <li>Anova p-value ≤ 0.05 (377 proteins)</li> <li>Modification with Carbamidomethyl C (207 p</li> <li>Max fold change ≥ 2 (15 proteins)</li> </ul>	Modification with ox (25 proteins) Show proteins that have at least one of these tags: Hide proteins that have any of these tags:
< <u> </u>	
Clear the filter	OK Cancel

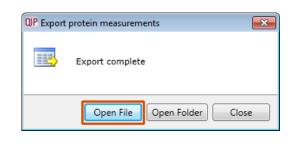


	Save
	Close
	Export peptide ion measurements
	Export peptide measurements
	Export protein measurements
	Export to pathways tool
	Import additional protein data
	Import protein accessions as tag
	Export mzIdentML for PRIDE submission
	Experiment properties
	Show Clip Gallery
×	Exit

The Export Protein Measurements dialog opens. Select the required fields and click OK. **Save** the file and then open the exported data file using the dialog that opens.

Note: if you have performed Absolute Quantification then the 'Amount' field will be available.





#### Excel will open displaying the exported protein measurements

	Α	В	С	D	E	F	G	н	I	J	К	L	м	N
1						Normalize	ed abundar	nce						
2						Α			В			С		
3	Accession	Peptide count	Anova (p)	Max fold o	Description	A_01	A_02	A_03	B_01	B_02	B_03	C_01	C_02	C_03
4	Q59WG3	19	0.000314	1.393401	Cell division control protein 48 GN=CDC48 PE=3 S	86.24353	87.17949	91.08677	111.5148	121.0896	116.0209	132.0787	120.0937	116.3959
5	Q5A1E2	19	0.0005	1.758111	Pyruvate decarboxylase GN=PDC11 PE=3 SV=1	435.0347	497.2753	502.0868	871.9053	823.0102	774.5956	924.5156	873.5239	723.7899
6	P46587	34	0.000726	1.397047	Heat shock protein SSA2 GN=SSA2 PE=1 SV=3	375.5511	398.4686	402.7144	522.4383	581.7589	539.7556	471.7949	484.4027	430.7232
7	Q9Y725	5	0.002622	1.256656	Mannose-1-phosphate guanyltransferase 1 OS=C	76.40372	78.92648	78.565	65.18226	61.05924	59.88363	64.74136	65.45224	56.97125
8	Q59Z65	5	0.00325	1.240083	Proteasome component PRE2 GN=PRE2 PE=3 SV=	30.56759	28.58105	27.96844	35.07647	37.75143	35.20454	32.74933	32.10559	35.20223
9	Q5ADM7	20	0.004251	1.785351	Glyceraldehyde 3-phosphate dehydrogenase GN	403.5391	535.228	500.3161	945.2801	744.4608	723.8168	929.069	901.9938	738.2057
10	Q59LW3	13	0.007127	1.92947	DNA-directed RNA polymerase GN=RPA190 PE=3	60.2368	53.86807	54.15851	93.36036	61.27316	60.6289	117.7418	112.8507	94.06668
11	Q59N00	15	0.009217	1.182696	40S ribosomal protein S3 GN=RPS3 PE=4 SV=1	441.9013	470.9894	455.5954	429.7327	457.4683	434.3039	398.926	400.6996	357.4652
12	C4YSV1	6	0.020075	1.197908	60S ribosomal protein L25 GN=CAWG_05170 PE=4	272.6004	300.9294	289.5693	305.139	286.347	288.0886	256.0056	257.4126	220.8407
13	P30575	18	0.022152	1.716485	Enolase 1 GN=ENO1 PE=1 SV=1	324.7802	442.0579	420.8157	768.6545	535.8539	532.1353	747.3102	727.1159	564.164
14	C4YQM1	4	0.022618	1.133014	40S ribosomal protein S15 GN=CAWG_02780 PE=4	105.5955	110.6672	105.9253	107.0582	111.1507	106.368	99.06681	99.30479	88.10059
15	C4YHD6	7	0.031191	1.083622	NADPH-cytochrome P450 reductase GN=CAWG_0	66.75509	71.7708	67.65506	71.68309	70.55012	69.71814	65.1287	66.85358	63.61291
16	C4YTC4	9	0.035013	1.175327	Glutaminyl-tRNA synthetase GN=CAWG_05411 P	134.8308	152.6384	139.4802	133.1898	120.7449	119.4546	125.331	124.5444	113.3847
17	C4YRA2	9	0.038891	1.156385	Eukaryotic translation initiation factor 2 gamma	57.26128	57.90527	58.5101	72.41611	63.72131	64.69966	64.1488	66.72412	59.7353
18	P41797	27	0.055294	1.245744	Heat shock protein SSA1 GN=SSA1 PE=1 SV=2	214.9863	169.441	163.2123	219.5388	234.095	228.5849	192.5273	196.454	173.6869
19	Q5A516	12	0.059456	1.180136	ADP_ATP carrier protein GN=PET9 PE=3 SV=1	432.9449	488.3769	468.724	492.2498	502.708	484.1944	441.257	440.7576	371.3597
20	Q5ABS1	7	0.062009	1.068077	Ubiquinol-cytochrome c reductase complex 14 kl	103.7973	106.2137	112.2433	117.2751	113.9978	112.9196	109.89	112.2287	112.5602
21	Q5A900	9	0.079168	1.248033	40S ribosomal protein S2 GN=RPS21 PE=3 SV=1	215.19	238.9594	215.9384	202.5111	172.9856	161.4182	222.1283	221.0237	182.2419
22	Q59M82	20	0.090605	1.126564	Clathrin heavy chain GN=CHC1 PE=4 SV=1	138.8952	146.8042	143.4408	147.8561	156.1469	142.4808	139.2993	136.7068	120.3174
23	C4YR46	37	0.099624	1.121701	Elongation factor 3 GN=CAWG_04545 PE=4 SV=1	342.2206	377.7598	362.8722	394.0138	426.415	394.2084	385.4636	376.6352	334.1258
24	Q5ACQ0	11	0.137876	1.118271	Long-chain-fatty-acid-CoA ligase 4 GN=FAA4 PE=	136.8234	143.6114	136.3706	137.0233	122.838	121.6096	128.34	131.1448	113.2382
25	P16017	19	0.236273	1.193481	Elongation factor 1-alpha GN=TEF1 PE=1 SV=1	979.0251	961.5015	928.8668	1007.179	711.4171	688.2377	991.2982	1042.33	838.8821
26	Q59ZX4	14	0.314636	1.114032	60S ribosomal protein L4-B GN=RPL4B PE=4 SV=1	539.0506	572.9628	547.2041	567.1365	515.6268	493.9599	536.2936	521.8626	431.2247
27	C4YHV4	11	0.829049	1.040838	40S ribosomal protein S6 GN=CAWG_03657 PE=4	275.2221	300.9816	285.3822	325.9705	285.1748	278.2593	307.5032	296.7165	250.2884

Note: where there are multiple group members the other accession numbers are also exported.

At each stage in the Work flow there are a number of Export and Import options available from the **File** Menu. This includes the option to **Import Additional Protein Data** which can be used to increase the Protein metadata and also be used to sort the existing tabular data.

QIP P	rogenesis QI.p Tutorial HDMSe - Progenesis QI for prote	e
	File	_
	Save	mics
	Close	Pe S
	Export peptide ion measurements	_
	Export peptide measurements	
	Export protein measurements	Acc
	Export to pathways tool	۲
	Import additional protein data	0
	Import protein accessions as tag	
	Export mzIdentML for PRIDE submission	٢
	Experiment properties	
	Show Clip Gallery	
×	Exit	
_	the relevant column neaden	

## Exporting Protein Data to Pathways Tool(s)

Using Progenesis, you can export protein lists to pathway analysis tools to help you understand your data in a wider biological context.

Currently Progenesis QI for proteomics supports the export to:

**IMPaLA**: which aggregates and queries many other pathway analysis tools including KEGG and Reactome)

PANTHER: classifies proteins for high-throughput analysis.

**Ingenuity Pathways Analysis (IPA):** Explores experimental data by identifying relationships, mechanisms, functions, and pathways of relevance.

**Note**: Plugins for these tools are provided as standard.

**Note: Access to IPA** is dependent on having a local licence for IPA. Details of exporting data to IPA are described in Appendix 9 (page 99)

As an example of exporting to a Pathways tool first use the tag filtering to 'focus' on the set to export. Then click **Export to pathways tool**, select **Panther** and then the test to be performed.

QIP Progenesis QI.p Tutorial HDMSe - Progenesis QI for pro	teomics										
File Review Experiment Import Data Alignment Filtering		Peptide Ion Statistics	Identify Peptides QC	Refin Metrics Identifica		eview oteins	Protein Statistics	Report			Waters Company
Review Proteins Using this screen, you can find the proteins of interest in your experiment.	Wo filt	er applied	Create	earch	Q						
1 Set the quantitation options	Accession	Peptides	Unique peptides	Confidence score	Anova (p)	q Value	Tag 💌	Max fold chan	ge Highest Mean	Lowest Mean	Description
If you've not already done so, choose between relative and absolute quantitation, use of Hi-N,	S C4YJJ0	8	7	70.5	3.83E-07	3.09E-06	-	1.37	В	Α	Aspartate-ser
protein grouping and more.	Q9UVL1	2	2	26.5	6 225-07	3 005-06		2.45	A	С	Non-histone
Protein options	C4YL44	QIP Export Pa	thways Informatio	on				<b>X</b>	В	A	Extracellular i
	Q9P457	Select a p	athways tool						В	A	Cu-binding n
2 Create a shortlist to review In the table, sort and filter the proteins based	P87219		athways tool from ng the links below	n the list below. Yo	u can find	out more	or downlo	ad new	с	A	Sorbose redu
on their measurements, to generate a shortlist	P43066	plugins usi	ing the links below	•.					В	A	D-arabinitol 2
for further review. How are the measurements calculated?	Q59W63	Which path	nways tool do you	want to use?					A	С	Ribosomal RI
-	Q7Z8E8	Q7Z8E8 Panther Classification System						-	с	Α	Cell surface h
To sort the table by a given value, simply click the relevant column header.	S Q9P8Q7	Q9P8Q7 IMPaLA: Integrated Molecular Pathway Level Analysis IPA: Ingenuity Pathway Analysis							В	A	Isocitrate lyas
Review the proteins	C4YNC2		assification System						в	Α	Phosphoenol
For each protein of interest, review its peptide	Q5AEB8								В	Α	Proteasome c
measurements and correlations:	C4YNI1								В	Α	1_3-beta-glu
View peptide measurements	C4YFV8								В	Α	Glycine cleav
You can also double-click to review a protein.	4								-		· · · · ·
4 Export data for further processing By exporting your data to external tools, there's no limit to your analysis.	Selected									C	
Export to pathways tool Export protein measurements Export peptide measurements Export peptide ion measurements	ArcSinh Normalised Ab. 002 000 000 000 000 000 000 000 000 000		1 <b>0</b> 1		< Back	Ne	κt >	Cancel		C ≱≢t	
Experiment design Review your data from a different perspective:	~										÷
Current design: A B C	Quantifiable	proteins dis	splayed: 516							Section	Complete 🏵

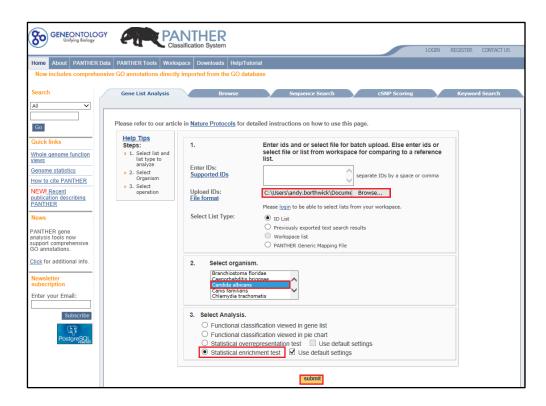
Select either Statistical over-representation test or Statistical enrichment test.

Make sure the **Open Panther in my browser** is ticked and then click **Export proteins to file.** Save file with appropriate name.

When Panther opens locate the file, select the appropriate organism (Candida albicans) and tick Statistical enrichment test.

QIP Export Pathways Information
Configure your export Choose which identifications to export and the type of analysis you want to perform.
Select the type of analysis to perform:
Statistical enrichment test
For statistical enrichment testing, choose two experimental conditions that you would like to compare.
Baseline: Comparison:
🗖 A 🔹 🖛 🖛
If greater, gives a <b>negative</b> fold change. If greater, gives a <b>positive</b> fold change.
To perform the pathway analysis, save the protein data to a file and select that file for the Upload IDs option on the Panther search page.
Open Panther in my browser
Sack Export proteins to file Close

4	a for further processing your data to external tools, there's our analysis.								
	Export to pathways tool								
	Export protein measurements								
	Export peptide measurements								
	Export peptide ion measurements								



#### Click submit.

Analysis results list is returned:

8		ENEONTO Unifying Biolo	Classification System		LOGIN RE	EGISTER COM	ITACT US			
Hom				lelp/Tutorial						
PA	Now includes comprehensive GO annotations directly imported from the GO database PANTHER PATHWAY LIST Convert List to: [-Select- V] Send list to: [-Select- V]									
	lay: 3 1-30 c		s per page <u>Refine Search</u> (1) <u>2</u> <u>3</u> ] Number of mapped ids found 76 <u>IDs not found (21</u>	<u>0)</u>						
clr	all	Pathway Accession	Mapped IDs	Pathway Name	Components	Subfamilies	Associated Sequence			
	1.	<u>P00039</u>	CANAL Gene=CGD=CAL0000821 UniProtKB=Q5ACM9	Metabotropic glutamate receptor group III pathway	<u>12</u>	<u>95</u>	<u>456</u>			
	2.	<u>P04398</u>	CANAL Gene=CGD=CAL0000896 UniProtKB=Q59XU5 CANAL Gene=CGD=CAL0002090 UniProtKB=P43063	p53 pathway feedback loops 2	<u>32</u>	<u>75</u>	<u>427</u>			
	3.	P02728	CANAL Gene=CGD=CAL0003247 UniProtKB=Q5A8A6	Arginine biosynthesis	<u>9</u>	<u>22</u>	165			
	4.	<u>P02773</u>	CANAL Gene=CGD=CAL0001034 UniProtKB=Q59W67	<u>S-adenosylmethionine</u> biosynthesis	2	<u>17</u>	<u>74</u>			
	5.	<u>P00008</u>	CANAL Gene=CGD=CAL0005137 UniProtKB=O42825	Axon guidance mediated by Slit/Robo	<u>14</u>	<u>40</u>	<u>191</u>			
	6.	P02787	CANAL Gene=CGD=CAL0005340 UniProtKB=Q59P52	Vitamin B6 metabolism	<u>19</u>	<u>12</u>	<u>57</u>			
	7.	<u>P02742</u>	CANAL Gene=CGD=CAL0005300 UniProtKB=Q5A7K0	Tetrahydrofolate biosynthesis	<u>11</u>	<u>21</u>	<u>114</u>			
	8.	<u>P00006</u>	$\label{eq:calibratic} CANAL   Gene=CGD=CAL0000006   UniProtKB=P41797\\ CANAL   Gene=CGD=CAF0007389   UniProtKB=Q988C9\\ CANAL   Gene=CGD=CAL0001208   UniProtKB=P46587\\ CANAL   Gene=CGD=CAL0000442   UniProtKB=Q5AAU7\\ \end{array}$	<u>Apoptosis signaling</u> pathway	<u>72</u>	<u>188</u>	<u>865</u>			
	9.	P00056	CANAL Gene=CGD=CAL0000896 UniProtKB=Q59XU5	VEGF signaling pathway	<u>25</u>	<u>87</u>	<u>434</u>			
	10.	<u>P00021</u>	CANAL Gene=CGD=CAL0000896 UniProtKB=Q59XU5 CANAL Gene=CGD=CAL0001346 UniProtKB=O42766	FGF signaling pathway	<u>26</u>	<u>219</u>	<u>1072</u>			
	11.	<u>P00037</u>	CANAL Gene=CGD=CAL0000821 UniProtKB=Q5ACM9	Ionotropic glutamate receptor pathway	<u>29</u>	<u>82</u>	<u>385</u>			
	12.	<u>P00026</u>	CANAL Gene=CGD=CAL0005122 UniProtKB=Q59M82	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	27	<u>184</u>	<u>948</u>			

Click to explore returned analysis.

**Note**: the success of a Pathways analysis is dependent on the organism under study being available to search using **Impala** and **Panther**.

Clear any applied tag filters then move to the next section/stage.

### Stage 13: Exporting identifications for submission to PRIDE

If you are submitting your raw data and results to PRIDE using the **mzIdentML** Complete submission process (described in section 2A on the PRIDE site) then it is now possible to export the identification results as mzIdentML which can be checked in **Pride Inspector tool** as part of the submission process.

**Note**: this only supports identifications obtained from searches performed in Progenesis QI for proteomics version 4.0 or later, using the search methods Mascot or Ion Accounting.

Export Identifications is carried out at the Review Proteins stage of the workflow. Click on the **File** menu and select **Export mzldentML for PRIDE submission...** 

A dialog will appear allowing you to include the protein sequence or not in your export. Choosing to export the sequence will allow the display of the coverage map in PRIDE Inspector.

Enter a file name. Progenesis will save two files the mzIdentML as a .mzid file and also a corresponding .mgf file containing the spectra.

Open PRIDE Inspector and locate and select the mzid file to import.

PRIDE Inspector 2.5.2     Open Export Help		
Quick Start	Try Examples	
Open Ider	Select mzML/mzXML/mzid/PRIDE xml Files  Look In: Progenesis QLp v4 HDMSe Tutorial  QLp_Tutorial A_Loaded_HDMSe.Analysis	
Review Pr	HDMSe 2 fold Significant.DerivedFromSearch880843c2-3cd5-4413-87be-21797717403d.mgf         ML           HDMSe 2 fold Significant mzid         Image: State	
		More examples
Feedback	Files of Type:	
🖂 Give Us Your Fe	edback	$\cap$
When use PRIDE Inspector	please cite:	

Click open.

Waters

You will then be asked if you want to load the corresponding spectrum files. Click yes and locate the .mgf file.

QUT P	rogenesis qup rutonal HDMSe - Progenesis Qufor pro
	File
	Save
<b>1</b>	Close
	Export peptide ion measurements
	Export peptide measurements
	Export protein measurements
	Export to pathways tool
	Import additional protein data
	Import protein accessions as tag
	Export mzIdentML for PRIDE submission
	Experiment properties
	Show Clip Gallery
×	Exit
_	the relevant column neader

010 -

Export mzIdentML for PRIDE submission
Choose properties to be included in exported file
Protein sequence
OK Cancel

A dialog opens and allows you to **Add spectra files.** Add the corresponding .mgf file and click **Set** 

S Load spectrum files				💽 🕄 Help
🧪 HDMSe 2 fold Sig	File Name	Size (M)	Туре	Remove
0	HDMSe 2 fold Significant.Deriv	44.0278	MGF	×
	Add spectra files			
			Cancel	Set

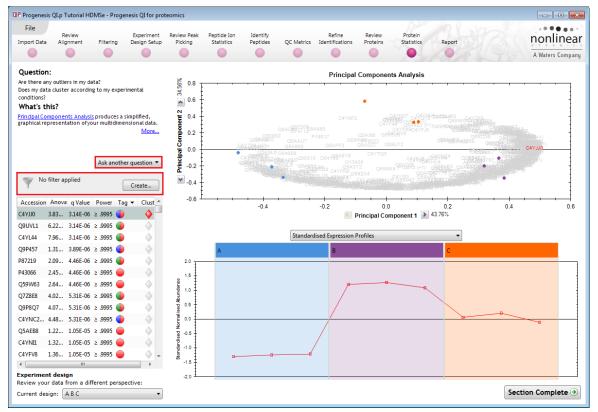
**PRIDE Inspector** will open allowing you to check the data.

an Expert Help									
en <u>E</u> xport <u>H</u> elp		Y Y							
🕠 Assays	Overview Protein Peptide	Spectrum Sumi	mary Charts						
🧪 HDMSe 2 fold Signific 🕮 🗙	Protein List					🛓 Update Prote	ein Details 🛛 🍸 Dec	by Filter 💡 🝟	
	Protein Group ID	Protein	rotein #PSMs		#Distinct Peptides #		#PTMs	#PTMs	
	ProteinGroup_165_C4YPV	56	56			0			
	ProteinGroup_168_C4YFJ6	97 97		9		3			
	ProteinGroup_293_Q5A940	26		5		0			
	ProteinGroup_458_Q5AJY5		9		2		0		
	ProteinGroup_406_Q59PZ4	Q59PZ4	11		3		1		
	ProteinGroup_251_C4YG2	C4YG28	31		6		0		
	ProteinGroup_194_Q5AH0	<u>Q5AH07</u>	45		8		2		
	ProteinGroup_515_C4YF48	C4YF48	4		1		0		
	ProteinGroup_412_P53696	P53696	10		3		1		
	ProteinGroup_241_Q9P84	<u>Q9P844</u>	56		6		0		
	ProteinGroup_260_C4YLN	C4YLN5	44		6		3		
	ProteinGroup_61_Q5ADU2	Q5ADU2	11	9	15		2		
	ProteinGroup_129_Q5AAU	Q5AAU7	61		10		2		
	Peptide LLNVEVPLR	Ranking 1	Delta m/z 0.0031	Charge 2	Precursor m/z 526.8299	9	173	Stop 181	
	LLNVEVPLR	1	0.0031	2	526.8299	9	173	181	
	LLNVEVPLR	1		2	526.8299	9	173	181	
Assay Summary	LLNVEVPLR	1	0.0031	2	526.8299	9	173	181	
<ul> <li>Spectra found</li> </ul>	LLNVEVPLR	1	0.0031	2	526.8299	9	173	181	
Proteins found					_				
Protein Group found	Spectrum Fragmentation T	Table Sequence			Selected	I PTM Fi	t 📕 Fuzzy Fit 📕	Overlap	
	Accession: Q5AH0	7						ovenap	
<ul> <li>Peptides found</li> </ul>		atched, 8 distinct), 11	12/478 amino	acids (23.	4% coverage)				
Mod: UNIMOD:4								-	
Mod: UNIMOD:35	MLRSASRISR	HRLYQSLSSQ	_		AAASSGPENR	PLVTPLGRHP	QKYSTPAPGF	6	
Widd. Olymod.35	GPTTFTEVLD	DVNITWDKND		_	KIRHFTINFG	PQHPAAHGVL	RLILELHGEE	11	
Imported MS/MS Annotations	IVRSDPHVGL	LHRGTEKLIE			FDRLDYVSMM	TNELVFALAV	EK <mark>llnvevpl</mark>	10	
	<b>R</b> AKYIRTLFG	EITRILNHCM			GLTPFLWGFE	EREKLMEFYE	RVSGARLHTA	2	
	YFRPGGVSQD	LPAGLLDDIY			DEVEELCTDN	RIWKDRTIGV		3	
	YSLSGVMLRG	SGIPFDIRKS	20		FDIAVGINGD	CYDRYLIRMA	EFRQSLRIF	31	
	QCINDIPEGP	<b>VK</b> VEDYKISP		4KEDM	EALIHHFLLF	TKGYAVPQGE	TYTAIEAPKG	42	

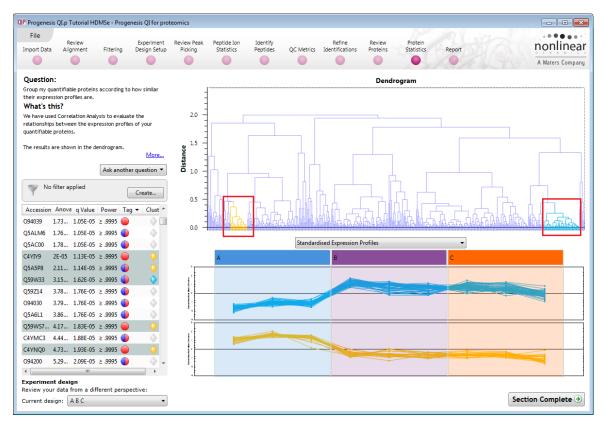
Once checked you can submit the results data to PRIDE, along with your raw data, as part of your submission process, following the guide lines provided on the PRIDE site.

# **Stage 14: Protein Statistics**

Protein Statistics opens with a Principal Components Analysis (PCA) for all the proteins displayed.



The Multivariate Stats can now be applied to all or subsets of proteins as determined by the current Tag filters. Allowing you to identify similar paterns of expression using the Correlation Analysis. Click on 2 of the branches (holding the **Ctrl** key down) to see differing patterns of expression.



Now move to the **Report** section to report on Proteins and /or peptides.

## Stage 15: Reporting

The **Report Design** stage allows you to select what views you want to include in a report based on the list of **currently selected proteins.** 

**Note**: this facility is used to generate Html reports on a limited selection of Proteins in your data. Creating a report on all the data in your experiment can take a long time

QIP Filter the proteins	
Create a filter Show or hide proteins based on a selection of their the filter. For more guidance, please see the <u>online</u>	
Available tags:	Show proteins that have all of these tags:
<ul> <li>Anova p-value ≤ 0.05 (377 proteins)</li> <li>Modification with Carbamidomethyl C (207 pro</li> <li>Modification with ox (25 proteins)</li> </ul>	Max fold change ≥ 2 (15 proteins)  Show proteins that have at least one of these tags:  Hide proteins that have any of these tags:
<u>C</u> lear the filter	OK Cancel

As an example we will create a report for **only** the proteins showing a Max Fold change of greater than 2.

- 1. First reduce the proteins to report on by selecting the 'Max fold change ≥ 2' tag. In this example it reduces the number of proteins in the table to 15.
- 2. Expand the various Report Design options (by default they are all selected)
- 3. Un-tick as shown below
- 4. Click Create Report

This opens a dialog to allow you to save the report, after which it will be opened in the form of a web page.

OP Progenesis	QI.p Tutorial H	DMSe - Prog	genesis QI for p	roteomics								- • •
File										k		
Import Data	Review Alignment	Filtering	Experiment Design Setu		Peptide Ion Statistics	Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report	nonlinear
												A Waters Company
Protein repor	t Peptide rep	ort										
Report on	your prote	ins			Structure th	e report						
Generate a r	report containir		eins of interest		Enter a report t							
experiment.					Max Fold Incre	ased Proteins						
	e what to repo				Select the secti	ons to includ	e in the repor	t:				
	he list below, fi s you want to i				Vervie	w run						
		<u>     Learn a</u>	about tagging a	and filtering	🗸 🔲 Data pro							
Custon	nise the repor	rt				-	lods					
📕 🖌 Enter a	title for your r include in it us	eport and s	elect the section	ons you	🕑 🔲 Experim							
want to	include in it us	sing the con	icrois to the rig	inc.	\land 🔽 Protein	report						
	filter applied			Edit	Include tables	showing prote	ein abundance	es and peptides i	dentified for	each protein		
rote	ins may be hide	den		cont	Protein							
Accession	Anova (p)	Fold	Tag 💌 🛙	escription	Peptide	e tables						
Q9UVL1	6.22E-07	2.4	🍈 N	lon-histone	\land 🔽 Protein	Details						
C4YL44	7.96E-07	2.2	🌗 E	xtracellular i	Reports the ful	l details of ev	ery protein w	hich matches yo	ur current filt	er		
P87219	2.09E-06	4.1	•	orbose redu	🔽 Tags							
Q7Z8E8	4.02E-06	3.4	•	ell surface h	Express	sion profile						
Q9P8Q7	4.07E-06	2.0	-	ocitrate lya:	マ 🔲 Peptide	ion table						
P42800	1.46E-05	2.7	-	nositol-3-ph	Peptide	ion details						
C4YF25	0.000156	2.3		ud site selec	<u> </u>							
Q59U83	0.000884	4.4	-	ibonucleosi	Create report							
C4YRH4 C4YE92	0.0011	2.1 2.7	-	IAD(P)H-der								
Q5ALX8	0.00124	2.7		_7-dimethyl .denine pho								
Q9HFQ6	0.00202	2.1	-	OS acidic rib								
Q59NN8	0.0117	3.5	-	lsp70 nucleo								
Q59WG0	0.0149	3.2	-	lit family pro								
Q59Z58	0.151	10.6		rotoplast se								
<												
Experiment	design: A B C		-	·								

Click on the **Accession No**. in the proteins section of the Report and this will take you to the Assigned peptides for this protein

M	Max Fold Increased Proteins															
Expe	Experiment: Progenesis QI.p Tutorial HDMSe															
Rep	ort cr	eated: 0	3/08/20	017 10:	16:2	0										
Pro	otein	5														
Dent																
Prot	ein gr	lding option Duping Antitation	Group s				Hi-3									
Acce	ession	Peptides		Anova p)*	Fold	d Tags	Descript	ion				1	Average Nor Abundances	rmalised		
<u>Q9</u> <u>Q5</u>	<u>Q9</u> F	<u>28Q7</u>											A B			]
	7 pept											-				
<u>P42</u>	Seque	nce	lon	e Score	Hits /	Mass	Charge	Tags C	Conflicts	Modifications	In quantitation	Drift time (ms)	, i i i i i i i i i i i i i i i i i i i		C C	
<u>P87</u> Q7	ASAE AYG	Accessi	on Q9P8	Q7												
	HQK HQK	Descripti	on Isocitra	ate lyase	GN=IC	CL1 PE=3	SV=1									
<u>Q9</u> <u>C4</u>	LFHE TDSE		es 7 (6) re 67.87													
<u>C4</u>	TDSE		va 4.07e-0 Id 2.01	006												
	VLVF VLVF		🛑 Anova 📄 Max fo													
	VLVF		Max TO		ezz											
L	WSG	. 1		А			В			C		]				
		ArcSinh Normalised Abundance 5 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7		1¢1			101									
		3.0 J	4								Þ					

Having closed the report it can be reopened by double clicking on the saved html file.

Note: you can also copy and paste all or selected sections of the report to Excel and/or Word.

**Note:** there are separate panels for reporting on Proteins and Peptides.

## Appendix 1: Stage 1 Data Import and QC review of LC-MS data set

You can use your own data files, either by directly loading the raw files (Waters, Thermo, Bruker, SCIEX and Agilent) or, for other Vendors, convert them to mzXML or mzML format first.

To create a new experiment with your (Waters) files: open Progenesis QI for proteomics and click **New**, bottom left of the **Experiments** page and give your experiment a name. Then select data type, the default is 'Profile data'.

**Note**: if you have converted or captured the data as centroided then select Centroided data and enter the Resolution for the MS machine used.

QIP Create New LC-MS Experiment	<b>×</b>	
Create a new label-free experiment named:		
Progenesis QI.p Tutorial HDMSe		
Data type		
Profile data		
Centroided data		
Resolution (full width at half maximum) 50000	]	
Machina tura		
Machine type		
High resolution mass spectrometer	•	
Experiment folder		tion mass spectrometer
Save experiment in the same folder as the run data		APT G2/G2-S, AB SCIEX TripleTOF, Agilent QTOF, Bruker Maxis, Thermo LTQ Orbitrap
Choose an experiment folder	Thermo LTC	Iontrap in Enhanced mode.
	Low resolut	ion ion trap
		Bruker HCT Ultra, Thermo LTQ XL
Create experiment	Thermo FT-	ICR

Click **Create experiment** to open the LC-MS Data Import stage of the workflow.

Select the 'Import Data file format', in this example they are Waters/SYNAPT data

Then locate your data files using Import...

GIP Progenesis File Import Data	Review Alignment	Filtering	Experiment Design Setup	r proteomics Review Peak Picking	Peptide Ion Statistics	Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report	A Waters Company
Automa Imported ru	vour run de one of the average of th	ailable data ton: w) 0.6355.256 ff) 0.6305.256 0.06409.313 0.6409.313 0.6409.313 0.6305.255 s 0.6305.255 s 0.6305.255 r 1.62R (.raw) 0.6305.257	matic tata as far tstep. 166 c processi 199 tic processi 197 2 132	as ng	N	o rui		ave b				i Help ▼

	Import Waters .RAW Data
	Select your runs The data for each run is stored in a separate .RAW folder, usually all within the same containing folder.
Locate and select all the .RAW folders (A_01 to C_03).	Find runs in folder: D:\Progenesis v3.0 HDMSe Tutorial\Q p_Tutorial HDMSe v3.0 Browse A_01.raw A_02.raw A_02.raw B_01.raw B_01.raw B_01.raw B_02.raw C_01.raw C_02.raw C_03.raw
	< <u>B</u> ack <u>N</u> ext> Cancel
	Import Waters .RAW Data Lock mass calibration All of your runs contain lock mass calibration information. Please provide the calibration m/c.
	Image: Verticity of the set of the se
	Note: If you have already calibrated your data externally, you should not perform calibration here.
On importing, the lock mass calibration is read and presented on this dialog	
You can, if required, alter the lock mass calibration at this step.	
	< <u>B</u> ack <u>N</u> ext> Cancel

For MSe, HDMSe and SONAR data formats the Ion accounting workflow is selected as default if your computer has a GPU.

Click Next. You can either choose to calculate optimal thresholds using an appropriate FASTA file for your data set or set the Thresholds manually.

Import Waters .RAW Data	Import Waters .RAW Data
Enable Ion Accounting workflow To identify peptides in your MS≢/HDMS≢/SONAR data, you will need to enable the Ion Accounting workflow.	Optimise peptide identification Specify the peak intensity thresholds to use when filtering your spectra prior to searching with Ion Accounting.
Select whether to run the Ion Accounting workflow.	How do you want to specify the threshold intensities?
Run the lon Accounting workflow and associated data analysis	<ul> <li>Calculate optimal thresholds using a representative FASTA file</li> </ul>
	FASTA file: E:\HDMSe Tutorial\Tutorial_nd_DB.fasta Browse
	Parsing rules: UNIPROT
	Specify threshold intensities manually
	Low energy: 250 counts
	Elevated energy: 150 counts
< <u>Back</u> Next > Cancel	How does the automatic calculation of thresholds work?      KBack Next> Cancel

If you choose to determine the thresholds automatically then Progenesis determines appropriate thresholds by sampling each run and finding the thresholds that yield the most protein identifications in the sample area. For each run, Progenesis performs the following steps:

- It finds the 5-minute retention time window that contains the highest total intensity. •
- It extracts the ions within this window and performs multiple Ion Accounting searches, each one using a different set of threshold values.

• Finally, it selects the thresholds that resulted in the largest number of protein identifications and applies those to the whole run. If more than one set of thresholds results in similar numbers of identifications (within 10% of the maximum), it will choose the highest thresholds as a way of optimising system performance.

**Note**: for HDMSe the settings are 150 and 30 and for MSe the settings are 250 and 150 for the Low and Elevated energies respectively.

Having selected how to handle the thresholds you will get the option to specify your own elution limits, the default is Start and End of the run, accept or make changes as necessary.

Import	mport Waters .RAW Data							
	Elution limits for the Ion Accounting workflow Specify elution limits if the defaults are not appropriate for your workflow.							
	you choose to spe le start time.	cify your own elution lin	nits, the end time m	ust be at least 5 n	ninutes after			
	Elution start:	<start of="" run=""></start>	minutes					
	Elution end:	<end of="" run=""></end>	minutes					
			< <u>B</u> ack	<u>N</u> ext >	Cancel			

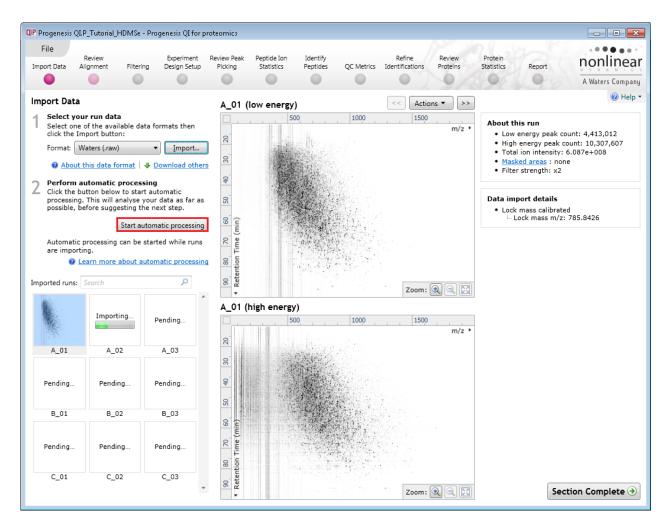
Ready to import			
Please review the information below be	fore starting the import pro	Cess.	
Your runs are ready to be imported. Ple	ase review the options belo	DW.	
1 run selected for import.			
Lock mass calibration: Yes Lock mass m/z: 785.8426			
Processing parameters: Threshold mode: Automatic FASTA file: E:\HDMSe Tutorial\Ti FASTA parsing rules: UNIPROT	utorial_nd_DB.fasta		
	< Back	Import	Cancel

A summary of the loading parameters is provided before you click Import

On loading the selected runs your data set will be automatically examined and the size of each file will be reduced by a 'data modelling routine', which reduces the data by several orders of magnitude but still retains all the relevant quantitation and positional information.

Note: For a large number of files this may take some time.

Note: you can start the automatic processing before the loading has completed.



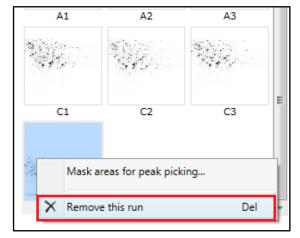
For details of setting up the steps in the automatic processing wizard return to Stage 2A page 7.

#### **Review Chromatography**

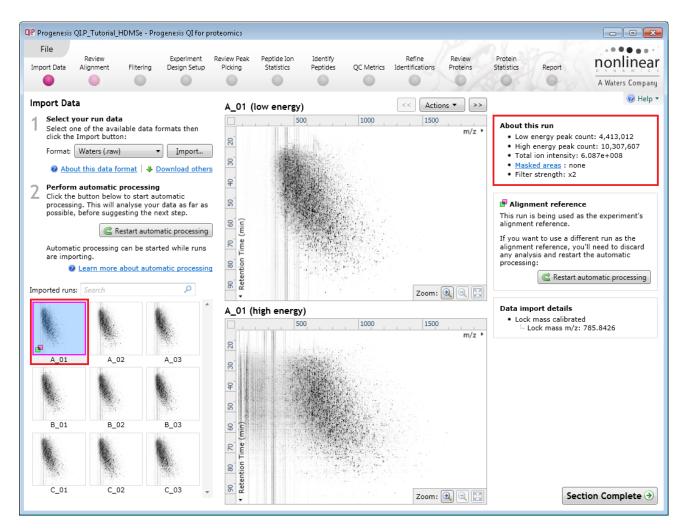
Each data file appears as a 2D representation of the run. If you created a **profile** experiment, at this stage you will be warned if any of the data files have been 'centroided' during the data acquisition and conversion process, as files must be of one format or the other.

You can delete run(s) by left clicking on the run in the list.

**Note**: you can also multi-select runs to remove by holding down the Ctrl key.



At the Import Data stage you can examine the quality of the imported runs using the 2D representation of the runs



Note: details of the current run appear on the top right of the view.

Once you have reviewed the imported runs click on **Review Alignment** on the workflow or **Section Complete** to move forward to the Review Alignment Stage.

Note: you will be offered the automatic alignment if you have not performed it automatically already.

Now move to the next stage in the workflow (page 7 in this user guide).

## **Appendix 2: Stage 1 Processing failures**

During automatic processing if a stage fails to complete successfully or only partially completes, the automatic processing dialog will warn you of the problem. Depending on the type of failure this may or may not allow the automatic processing to complete.

For example, a run that fails to automatically align will trigger a warning, although analysis will continue; however, the automatic processing dialog will prompt you to 'drop-off' at the **Review Alignment** stage on completion to investigate the problem.

QIP Processing Complete		×
Automatic process Time taken: 3 minutes 9 s	ing complete (with warnings).	
<ul> <li>Importing runs:</li> <li>Selecting reference:</li> </ul>	7 of 7 processed C1	
Aligning runs:	6 of 6 processed 1 run failed to align - continuing without it	
<ul> <li>Peak picking:</li> </ul>	14624 peaks found	
<ul> <li>Creating design:</li> </ul>	Created	
<ul> <li>Protein quantitation:</li> </ul>	Relative Quantitation using Hi-3	
	Close Identify Peptides	۲

**Note**: in this example the run that failed to align will not contribute to the peak picking and will be excluded at the alignment stage (a cross appears in the include column).



You can either remove the run from the experiment at the **Import Data** or add it back in at the **Review Alignment** stage once the alignment of the run has been corrected.

As another example, runs that import successfully but with warnings at the **Import Data** stage will cause a flag in the readout to notify you of the potential quality issue.

If some runs in a data set fail to import (but not all), the automatic processing will continue informing you that one or more runs have failed to import.

QIP Processing Complete	IP Processing Complete							
Automatic processing complete (with warnings). Time taken: 3 minutes 40 seconds								
A Importing runs:	7 of 7 processed 1 failed to import							
<ul> <li>Selecting reference:</li> </ul>	C1							
<ul> <li>Aligning runs:</li> </ul>	5 of 5 processed							
<ul> <li>Peak picking:</li> </ul>	14624 peaks found							
<ul> <li>Creating design:</li> </ul>	Created							
Protein quantitation:	Relative Quantitation us	ing Hi-3						
		Close Identify Peptides	•					

In this case you can remove the runs at Import Data and if appropriate replace them with additional runs.

**Note**: adding additional runs will then be aligned and peak picking should be re-done to include data from the added runs in the generation of the aggregate.

An example of a problem that would halt the automatic processing would be the failure to successfully import all the potential reference candidates, (for example: while importing, you specified the selection of the alignment reference to be made from several runs before they were fully imported and set the processing underway, and they later failed to import owing to problems with the runs).

QP Processing Complete		×
Automatic processi Time taken: 19 seconds	ng failed.	
A Importing runs:	6 of 6 processed 1 failed to import	
× Selecting reference:	All reference candidates failed to import	
Aligning runs:	Unable to start.	
Peak picking:	Unable to start.	
Protein quantitation:	Unable to start.	
	Close	Import Data 🌖

In this case, the processing dialog would halt and prompt you to select another reference.

## Appendix 3: Licensing runs (Stage 3)

When setting up a **New experiment** if you are evaluating Progenesis QI for proteomics with unlicensed runs then the licensing page will open after **Import Data section**.



If you already have a programmed dongle attached to your machine then the License Runs page will not appear.

To use this page to License your Runs you must first either obtain an 'Evaluation' Licence Code from a Sales Person or purchase a licence code directly.

Each code will allow you to license a set number of runs.

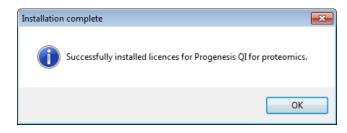
The runs in your experiment will be listed as shown below.

To activate license(s) for the selected runs enter the code in the space provided and click **Use Licence code**.

Note: you will need an internet connection to use this method.

File Review Experiment mport Data Licensing Alignment Filtering Design Set	up Picking Statistics Peptides QC Metrics Identifications Proteins Statistics Report	Waters Comp
Dongle License Runs		
This installation is currently restricted to analyse licensed runs only.	Run name Licence state	License this run
To license your runs, you need an evaluation or	C:\Users\andy.borthwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlicensed	V
lease licence code which can be obtained from	C:\Users\andy.borthwick\Documents\Customer Data\Progenesis Q1.p v3.0 Tutorials and Demo Suites\Pr Unlicensed	V
a sales representative.	C:\Users\andy.borthwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlicensed	
Once licensed, your runs can be analysed on	C:\Users\andy.borthwick\Documents\Customer Data\Progenesis Q1.p v3.0 Tutorials and Demo Suites\Pr Unlicensed	<b>V</b>
any installation of the software. The licence is	C:\Users\andy.borthwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlicensed	
automatically included when archiving an	C:\Users\andy.bothwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlicensed	<b>V</b>
experiment.	C:\Users\andy.borthwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlicensed	
If your runs have been licensed on another computer, click here to make the licences	C:\Users\andy.bothwick\Documents\Customer Data\Progenesis QI.p v3.0 Tutorials and Demo Suites\Pr Unlicensed	
available on this computer.	C:\Users\andy.bothwick\Documents\Customer Data\Progenesis Q1.p v3.0 Tutorials and Demo Suites\Pr Unlicensed	<b>V</b>
If you have one, you can <u>open a licence file</u> to install. If you have just installed a dongle, <u>click here</u> .		
	Run licence code: xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx	ice Code

A message confirming successful installation of your licences will appear.

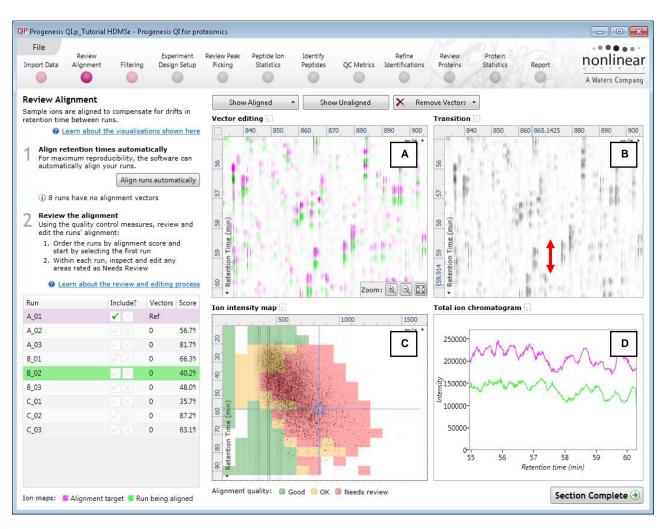


Click OK, the view will update and Alignment, the next stage in the workflow, will open with the licensed files.

## Appendix 4: Manual assistance of Alignment

### Approach to alignment

To place manual alignment vectors on a run (B\_02 in this example):



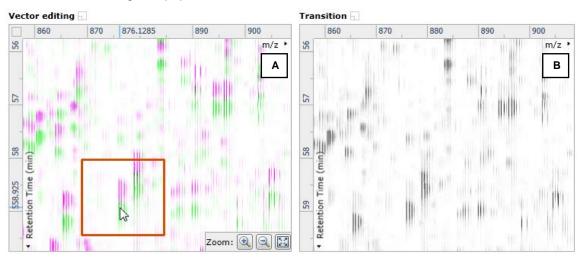
- 1. Click on Run B\_02 in the **Runs** panel, this will be highlighted in green and the reference run (A\_01) will be highlighted in magenta.
- 2. You will need to place approximately 5 10 **alignment vectors** evenly distributed from top to bottom of the whole run (RT range).
- 3. First drag out an area on the **Ion Intensity Map** (C), this will reset the other 3 windows to display the same 'zoomed' area

**Note**: the peptide ions moving back and forwards between the 2 runs in the **Transition** window (B) indicates the misalignment of the two runs.

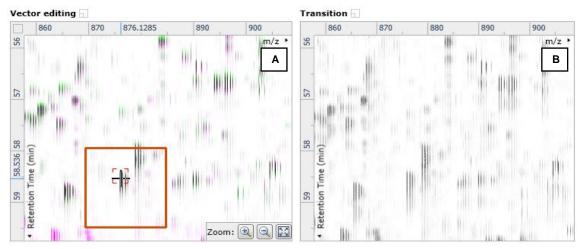
**Note:** the lon Intensity Map gives you a colour metric, visually scoring the current alignment and an overall score is placed next to the Vectors column in the table. With each additional vector added this score will update to reflect the 'changing' overall quality of the alignment. The colour coding on the lon intensity Map will also update with each additional vector.

**Note**: The **Total Ion Chromatograms** window (D) also reflects the misalignment of the 2 runs for the current Retention Time range (vertical dimension of the current Focus grid in the **Ion Intensity Map** window).

4. Click and hold on a green peptide ion in Window A as shown below.

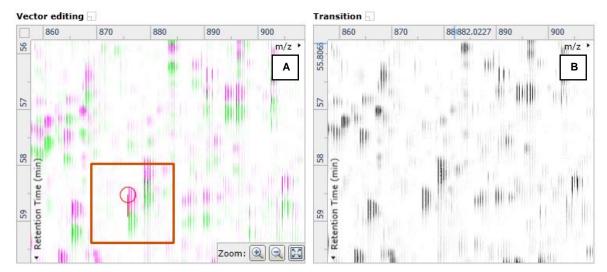


5. As you are holding down the left mouse button (depending on the severity of the misalignment), the alignment vector will automatically find the correct lock. If not, drag the green peptide ion over the corresponding magenta peptide ion of the reference run. The red box will appear as shown below indicating that a positional lock has been found for the overlapping peptide ions.



*Tip*: while holding down the mouse button hold down the **Alt** key. This will allow smooth movement of the cursor as the **Alt** key allows you to override the 'automatic alignment' performed as you depress the mouse button.

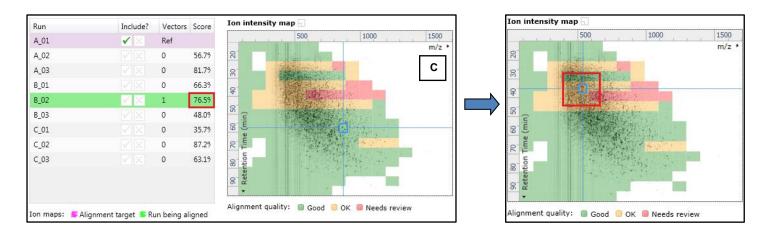
6. On releasing the left mouse button the view will 'bounce' back and a red vector, starting in the green peptide ion and finishing in the magenta peptide ion will appear.



**Note**: an incorrectly placed vector is removed by right clicking on it in the **Vector Editing** window and selecting delete vector.

- Vector editing Transition 860 875.9839 900 880 900 870 890 860 870 890 56 m/z • 56 m/z · в Α 57 22 59 58.536 58 58 Retention Time (min) (mim) Retention Time 59 • Zoom:
- 7. Now click **Show Aligned** on the top tool bar to see the effect of adding a single vector.

8. With the placement of a single manual vector the increase in the proportion of the **Ion Intensity Map** (C) showing green is reflected in the improved alignment score in the table. Now click in the Ion Intensity Map to relocate the focus in order to place the next manual vector.



9. Adding an additional vector will improve the alignment further as shown below.



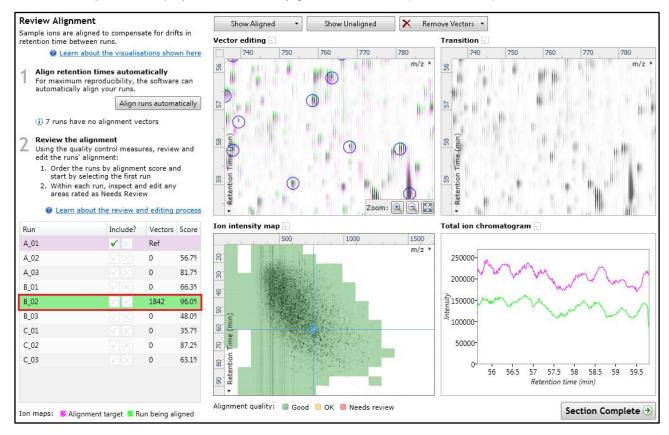
- 10. The shift in the Retention Time (RT) is as a result of incorrect running of the chromatography. In many of these cases if the Automatic Alignment fails to generate a good alignment then removing all the alignment vectors for this run and placing a single manual vector to act as a 'seed' for the Automatic Alignment algorithm maybe all that is required to generate a good alignment.
- 11. In the case of the example shown above placing a small number of vectors from the top to the bottom of the run is sufficient to markedly improve the alignment.



 At this point you would redo the automatic alignment of this image by selecting automatic alignment. Note: if you are focusing only on the alignment of one run, then un-tick the other runs in the alignment dialog.

Automatic Al	ignment	<b>•</b>
Choose whic	ch runs to automatically align:	
Run	Notes	Vectors
🗖 A_02	This run has not been automatically aligned	0
🗖 A_03	This run has not been automatically aligned	0
🗖 B_01	This run has not been automatically aligned	0
✓ B_02	run has user vectors	5
🗖 B_03	This run has not been automatically aligned	0
🗖 C_01	This run has not been automatically aligned	0
C_02	This run has not been automatically aligned	0
🗖 C_03	This run has not been automatically aligned	0
		OK Cancel

13. On pressing OK the Automatic Alignment will run for the selected run. On completion the table and views will update to display the automatically generated vectors (shown in blue).



14. Repeat this process for all the runs to be aligned.

The number of manual vectors that you add at this stage is dependent on the misalignment between the current run and the Reference run.

#### Note: In many cases only using the Automatic vector wizard will achieve the alignment.

*Tip*: a normal alignment strategy would be: to run the automatic alignment first for all runs, then order the alignments based on score. For low scoring alignments remove all the vectors and place 1 to 5 manual vectors to increase the score then perform automatic alignment. Then review the improved alignment score.

Also the 'ease' of addition of vectors is dependent on the actual differences between the LC-MS runs being aligned.

To review the vectors, automatic and manual, return to page 18

## Appendix 5: Within-subject Design

To create a **Within-subject Design** for your data set select this option on the **Experiment Design Setup** page and enter the name of the design.

In this example there are 3 Subjects (i.e. patients A, B and C) who have been individually sampled: Before(1), During (2) and After (3) treatment

File nport Data	Review Alignment	Filtering	Experiment Design Setup	Review Peak Picking	Peptide Ion Statistics	Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protein Statistie		port	D	onlin Waters Cor
B C Which ex	A B	Ne lesien tvo		ant to use	for this ex	periment?								
OO OO Do samp appear in	etween-su les from a gi n only one co between-sub	ubject De	esign hen A			Delete	0-0 Have y subjec	Within-sub ou taken samp t under differe ise the within-	ples from a g ent condition	iven 15?		Before	During	After
To set up the runs (factor le	this design, according to evel) of the sa alculation ass	you simply the condition amples. Th	group on e	Ente	eate New Experi r a name for th	ne experiment d	lesign:		×	use	Patient X	X1	X2	X3
therefore	ns are indepe e gives a stat the means of qual.	istical test o		How		After Treatment o group the run s manually			<b>~</b>	s	Patient Y	Y1	Y2	<b>Y3</b>
			Add	conditi	Copy an existir	ng design:	C	reate design	Cancel	ut he ted	Patient Z	Z1	Z2	Z3
							becaus assump repeat differe reduce conditi create The wi though paired- compa	dard ANOVA is a e the data viol tion of indepe d measures Al nces can be eli d as a source o on differences a more powerf thin-subject de t of as an exte samples t-test rison between d measures.	ates the ANO ndence. With NOVA individu iminated or f between (which helps ful test). esign can be nsion of the to include	te VA a Jal				

When the design page opens use the **Add Subject** and **Add Condition** buttons to create the matrix that fits your experimental design, over typing the names as required.

Then Drag and drop the Samples on to the correct 'cell' of the matrix.

	eomics iew Peak Peptide Io icking Statistics		Refin QC Metrics Identifica		Protein Statistics Report	nonlinear
A B C A B Before During an	d After Treatm	nent I × 🖻	New	1		A Waters Company
Setup conditions and subjects Setup the conditions and subjects for your experiment design on the right, and then assign each of your samples to the correct subject/condition cell		Before	During	After	Add Condition	
in the grid. 1. Add a column for each condition. 2. Add a row for each subject. 3. Drag each of your samples to the correct location in the grid. Filter samples: P	Patient A	A_01	A_02	A_03		
C_02	Patient B	B_01	B_02	B_03		
C_03	Patient C	C_01	Select Sample	Select Sample		
	Add Subject				-	
					S	ection Complete 🏵

You can create additional Experimental Designs using the New tab

All of these Experimental Designs are available at the later stages of the workflow with the exception of **Identify Peptides**, and **Refine Identifications** (including Resolve Conflicts).

## Appendix 6: Power Analysis (Peptide Ion Stats)

Power analysis is a statistical technique that is used to gauge how many replicates are needed to reliably see expression differences in your data. It is available through the Peptide Ion Stats section of the workflow.

To perform a power analysis of the data click on **Ask another question** at the top of the table in the Peptide Ion Stats screen. A selection of 3 tools will appear in the form of questions.

v	<b>Principal Components Analysis</b> Are there any outliers in my data? Does my data cluster according to my experimental conditions?
ለዝ	<b>Correlation Analysis</b> Group my quantifiable proteins according to how similar their expression profiles are.
	Power Analysis How many replicates should I run? What is the power of my experiment?

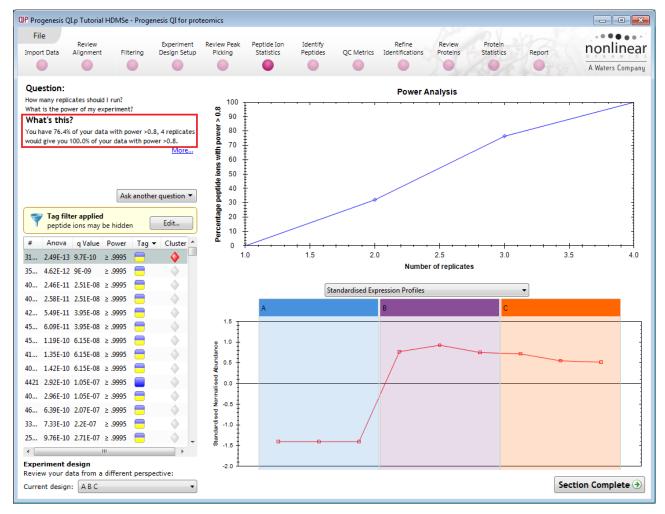
#### Select the option

'How many replicates should I run and what is the power of my experiment?'

It answers this question by informing you:

'How many replicates you need so that at least 80% of your peptide ions with a power >0.8'

Using the Significant p<0.05 peptide ions (20587), as an example, view the power analysis.



This is displayed graphically showing that 76.4% of the 20587 peptide ions have a power of 80% or that 4 replicates would give you 100% of your data with power > 0.8.

- The power of a statistical test reflects our confidence in the experimental data's ability to find the differences that do actually exist
- The power is expressed as a percentage, where 80% power is an accepted level, therefore allowing you to assess the number of sample replicates that would be required to achieve a power of 80%.

## **Appendix 7: Resolve Conflicts**

This stage allows you to examine the behaviour of the identified peptides and choose to resolve any conflicts for the various peptide assignments at the protein level.

The Resolve Conflicts stage is now accessed at the bottom left of the Refine Identifications stage.

The number of conflicts you have to resolve will depend on the scope and stringency of the filters you apply at the **Refine Identifications** stage.

Note: the default Protein options for protein grouping and Protein quantitation are set as shown

QIP Protein quantitation options	×
Quantitation method:	
Relative Quantitation using Hi-N	•
Number of peptides to measure per protein (N):	3 🔹
Employ protein grouping, i.e. hide proteins whose peptides are a subset of another protein s.	3
OK Car	icel

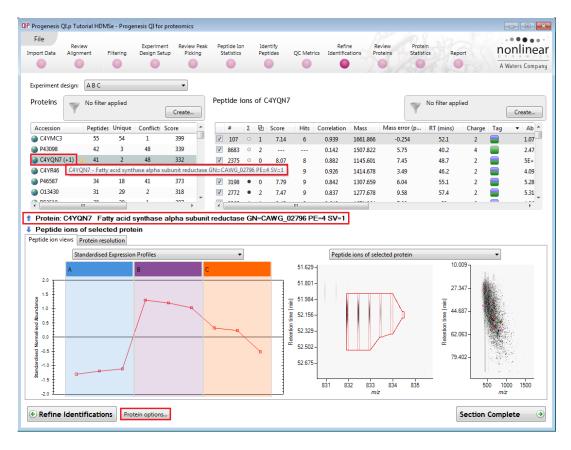
This means that if you choose **not** to resolve the conflicts then proteins, to be considered for quantitation, require at least one unique peptide (see Unique column).

# For this guide: we will not resolve the conflicts but click on Section Complete to go to Review Proteins (page 60).

(For more details on Protein Grouping page 56 and Protein Quantitation options go to page 58).

# If you wish/require to resolve conflicts then the remainder of this appendix provides some guidance and explanation of performing this process in Progenesis QI for proteomics.

With **Group similar proteins** selected the additional members are indicated by a bracketed number located after the Accession number.

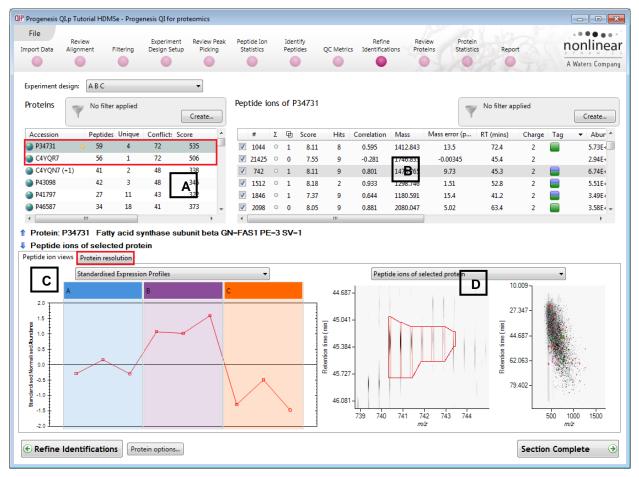


The number of the **Peptides** used for quantitation is displayed in the **Unique** column.

The **Resolve Conflicts** stage provides a number of interrelated graphical and tabular views to assist you in the validation of the peptides that have been assigned to proteins and also to review the relevance of the data returned from the search.

With Resolve Conflicts open order the data in the Proteins table A on the basis of Conflicts.

Note: the look of the tables (with regards to ordering) in the following pages may vary slightly.



Select the first protein in list A (in this case it has 72 conflicts) the panel to the right B lists the peptides for this protein and the conflicting protein for each peptide.

Panel C shows the expression profile(s) for the peptide(s) selected in list B

Panel D shows the details for the selected peptide.

Now click on the Protein Resolution in Panel C to display the proteins that are conflicting.

The lower left panel E displays the Conflicting proteins for the peptide ion highlighted in panel B this includes the current protein in panel A as indicated by the orange ball to the right of the accession.

Accession	Peptides	; Unique	Conflict	Score	Tag			#	Σ	中 Sco	re Hi	its Correlatio	on Mass	Mass error (p	RT (mins)	Charge	e Tag	<ul> <li>Abundance</li> </ul>	e Cor	nflict
P34731	59	4	72	535		μ.	V	1174	0	1 8	02 8	3 0.861	1303.682	2 12.5	60.8	2		7.59E+04		2
C4YQR7	56	1	72	506			<b>V</b>	1044	0	1 8	12 8	3 0.595	1412.843	13.5	72.4	2		5.73E+04	1	1
P43098	42	3	48	A 339			<b>V</b>	21425	0	0 7	54 9	9 -0.281	1746.833	3 00345	45.4	2		2.94E+03	:	1
C4YQN7 (+1)	41	2	48	A 332			<b>V</b>	742	0	1 8	13 9	9 0.801	1478.765	B <sup>00345</sup> 9.75	45.3	2		6.74E+04	1	1
P41797	27	11	41	322		-		1512	0	1 8	19 2	2 0.933	1298.746	5 1.51	52.8	2		5.51E+04	1	1
					4		•													1
Protein: P347: Protein: C4YQ	QR7 Fat Protein res	ty acid	synthas	e beta sut		drat	ase (	GN=C	AWO	_		SV=1								
Protein: C4YQ ptide ion views F Conflicting pro	OR7 Fat Protein reso oteins fo	ty acid	synthas	e beta sut	ounit dehy	drat	ase ( Peptid	GN=C	AW (	C4YQR	7			F						
Protein: C4YQ otide ion views F Conflicting pro Accession F	OR7 Fat Protein reso oteins fo	ty acid	synthas	e beta sut	punit dehy	drat P	eptid	e ion Σ	AWO	_		SV=1	Mass 1	<b>F</b> Mass err <del>te (pm - f</del>	T (mins)	Charge	Tag	<ul> <li>Abundance</li> </ul>	Conflic	ct:
Protein: C4YQ otide ion views F Conflicting pro Accession F	<b>QR7 Fat</b> Protein reso oteins fo	ty acid	synthas	2 E	ounit dehy	drat P	ase ( Peptid	e ion Σ	AWC 5 of 10	C4YQR	7		Mass 1478.765		T (mins) 45.3	Charge 2	Tag •	<ul> <li>Abundance</li> <li>6.74E+04</li> </ul>	Conflic 1	
Protein: C4YQ otide ion views F Conflicting pro Accession F @ P34731 •	<b>QR7 Fat</b> Protein reso oteins for Peptides U	ty acid	s <b>ynthas</b> e ion 74 Conflict: P	2 E Protein Score	punit dehy	P t	eptid	e ion Σ	AWC 5 of 1 (1)	C4YQR Score	Hits	Correlation		Mass err <del>or (p</del>			Tag •			_
Protein: C4YQ otide ion views F Conflicting pro Accession F @ P34731 •	Protein reso oteins for Peptides U 59	ty acid	e ion 74 Conflict: P 72 53	2 E Protein Score	Pep	P t	eptid #	GN=Ca le ion: Σ 2 • 14 ○	AWC s of @ 1	C4YQR Score 8.13	Hits 9	Correlation 0.789	1478.765	Mass err <del>or (p.m. 1</del> 9.73	45.3	2	Tag •	6.74E+04	1	
Protein: C4YQ otide ion views F Conflicting pro Accession F @ P34731 •	Protein reso oteins for Peptides U 59	ty acid	e ion 74 Conflict: P 72 53	2 E Protein Score	Pep	P	ase ( Peptid # √ 74 √ 104	GN=C	AWC 5 of 1 1 2	C4YQR Score 8.13 8.12	Hits 9 8	Correlation 0.789 0.629	1478.765 1412.843	Mass err <del>er (p.n. 1</del> 9.73 13.5	45.3 72.4	2 2	Tag •	6.74E+04 5.73E+04	1	
Protein: C4YQ ptide ion views F Conflicting pro Accession F	Protein reso oteins for Peptides U 59	ty acid	e ion 74 Conflict: P 72 53	2 E Protein Score	Pep	drat	ase ( Peptid ≇ √ 74 √ 104	e ion: Σ 2 • 4 0 12 0	AWC 5 of 1 1 2	C4YQR Score 8.13 8.12 7.54	Hits 9 8 9	Correlation 0.789 0.629 0.819	1478.765 1412.843 1746.872	Mass erre <del>r (p 1</del> 9.73 13.5 22.3	45.3 72.4 45.4	2 2 3	Tag ·	6.74E+04 5.73E+04 3.71E+04	1	ct:

The Accession and description for the 2 proteins highlighted in Panels A and E are shown in the middle margin. As most of the peptide ions are conflicting between the 2 closely related proteins one simple way to resolve these conflicts is to favour the protein with the higher score and greater number of non-conflicting peptides.

One way to do this is to right click on the lower scoring protein in panel E which only has one unique peptide and turn off all its peptides

Conflicting	g pi	roteins f	or pept	ide ion 7	742	
Accession		Peptides	Unique	Conflict	Protein Score	
🎯 P34731	0	59	4	72	535	1
🔇 C4YQR7		56	1	72	506	<b>v</b>
				Turn off a	all peptides	

All the peptides are now switched off in panel B and all the entries for the lower scoring protein are set to zero. The higher scoring protein now has 56 non-conflicting peptides and only 4 remaining conflicts

Accession	Peptides	Unique	Conflict:	Score	Tag 🔻	^		#	Σ	中 Scor	e Hits	Correlation	Mass	Mass error (p	RT (mins)	Charge	e Tag	<ul> <li>Abundance</li> </ul>	e Co	nflict
P34731	59	56	4	535			V	1174	0	1 8.	2 8	0.861	1303.682	12.5	60.8	2		7.59E+04		1
C4YQR7	0	0	0	0			<b>V</b>	1044	0	1 8.	2 8	0.595	1412.843	13.5	72.4	2		5.73E+04		0
P43098	42	3	48	233			V	21425	0	0 7.	4 9	-0.281	1746.833	-0.00345	45.4	2		2.94E+03		0
C4YQN7 (+1)	41	2	48	<sup>332</sup> A			<b>V</b>	742	•	1 8.	3 9	0.801	1478.765	9.7 <b>:B</b>	45.3	2		6.74E+04		0
P41797	27	11	41	822		-	V	1512	0	1 8.	92	0.933	1298.746		52.8	2		5.51E+04		0
					F.		4													•
Protein: P3473 Protein: C4YQ ptide ion views P	R7 Fatt rotein reso	y acid s	synthase	e beta subi	unit dehyd	irata:	se G	SN=CA	WG			/-1		— <b>Г</b>	╒┨──					
Protein: C4YQ ptide ion views P Conflicting pro	R7 Fatt rotein reso teins for	y acid	synthase e ion 742	e beta subi	E	irata:	se G	SN=CA	of (	C4YQR7			Aass N		F	Charge	[ag	<ul> <li>Abundance</li> </ul>	Conflic	rtı
Protein: C4YQ otide ion views P Conflicting pro Accession P	R7 Fatt rotein reso teins for eptides Un	y acid s lution peptide nique	s <b>ynthase</b> e ion 742 Conflict: P	e beta subu 2 rotein Score	E Pept	irata:	se G ptid	e ions Σ	WG	C4YQR7 Score		Correlation N		1ass error (p R	T (mins)	Charge 2	ſag ·	Abundance     674F+04	Conflic	ct:
Protein: C4YQ ptide ion views P Conflicting pro Accession P 9 P34731 •	R7 Fatt rotein reso teins for eptides Un	y acid	synthase e ion 742	e beta subu 2 rotein Score	E Pept Ø 8.13	irata:	se G	e ions Σ	of (	C4YQR7	Hits C	Correlation N	478.765			Charge 2	Tag ·	<ul> <li>Abundance</li> <li>6.74E+04</li> <li>5.73E+04</li> </ul>	Conflic 0	cte G
Protein: C4YQ ptide ion views P Conflicting pro Accession P 9 P34731 •	R7 Fatt rotein reso teins for eptides Un 59	y acid s lution peptide nique C	e ion 742 Conflict: P 4 53	e beta subu 2 rotein Score	E Pept	irata:	se G ptid # 74	e ions Σ 2 0	of (	C4YQR7 Score 8.13	Hits C	Correlation M		1ass error (p R 9.73	<b>T (mins)</b> 45.3	Charge 2 2 3	Fag ·	6.74E+04	0	ct: G
Protein: C4YQ ptide ion views P Conflicting pro Accession P 934731 •	R7 Fatt rotein reso teins for eptides Un 59	y acid s lution peptide nique C	e ion 742 Conflict: P 4 53	e beta subu 2 rotein Score	E Pept Ø 8.13	irata:	se G ptid # 74: 104	e ions 2 0 4 0	of (	C4YQR7 Score 8.13 8.12	Hits C 9 8	Correlation N 1 1 1	478.765 412.843	1ass error (p R 9.73 13.5	45.3 72.4	Charge 2 2 3 2	Fag ·	6.74E+04 5.73E+04	0	cte G G
Protein: C4YQ ptide ion views P Conflicting pro Accession P	R7 Fatt rotein reso teins for eptides Un 59	y acid s lution peptide nique C	e ion 742 Conflict: P 4 53	e beta subu 2 rotein Score	E Pept Ø 8.13	irata:	<b>se G ptid #</b> 74: 104 554	e ions 2 0 4 0 2 0	of (	C4YQR7 Score 8.13 8.12 7.54	Hits C 9 8	Correlation N 1 1 1 1	478.765 412.843 746.872	Mass error (p   R 9.73 13.5 22.3	45.3 72.4 45.4	Charge 2 2 3 2 2 3	Fag ·	6.74E+04 5.73E+04 3.71E+04	0 0 0	ct: G G G

To resolve the remaining conflict(s) first order the conflicts in panel B and select the top one (which may still be selected) Panel B will display the peptides for this protein and the number of conflicts for each peptide. Panel E will also update to show the conflicting protein.

Accession	Peptide	es Unique	Conflict	Score	Tag	<b>*</b> ^		#	Σ	中 Sco	re Hi	ts Correlatio	n Mass	Mass error (p.	RT (mins)	Charge	e Tag	<ul> <li>Abundance</li> </ul>	e Co	onflict
P34731	59	56	4	535				2185		0 5	88 2	0.732	1529.861	3.14	45	3		5.05E+04		1
C4YQR7	0	0	0	0			<b>V</b>	1253	0	2 5	88 2	-0.154	1529.827	-19.2	44.9	2		7.22E+04		1
P43098	42	3	48	339			V	18207	0	1 6	03 2	0.427	1115.554	-6.25	32.4	2		6.15E+03		1
C4YQN7 (+1)	41	2	48	332			<b>V</b>	1174	0	1 8	02 E	0.861	1303.682	12.5	60.8	2		7.59E+04		1
P41797	27	11	41	322		-		1044	0	1 8	12 8	0.595	1412.843	13.5	72.4	2		5.73E+04		0
	III					F.	•													•
Protein: P3473 Protein: C4YQ ptide ion views	<b>P9 60</b> Protein re	S ribose solution	omal pro	tein L35 GN		G_02	808 PE	=4 S	<b>V</b> =1											
Protein: C4YQ otide ion views	<b>P9 60</b> Protein re	S ribose solution	omal pro	tein L35 GN		G_02	808 PE	=4 S	<b>V</b> =1	C4YQP9										
Protein: C4YQ tide ion views P Conflicting pro	<b>PP9 60</b> Protein res	S riboso solution or peptio	omal pro	tein L35 GN		G_02	808 PE	=4 S	<b>V</b> =1		Hits	Correlation	Mass 1	Mass error (p	RT (mins)	Charge	Tag	<ul> <li>Abundance</li> </ul>	Confli	ct:
Protein: C4YQ tide ion views P Conflicting pro Accession P	<b>PP9 60</b> Protein res	S riboso solution or peptio	omal pro	tein L35 GN 85 Protein Score	I=CAW	G_02	808 PE	E <b>=4 S</b> e ion Σ	V=1 s of ⊕	C4YQP			Mass 1 1529.827	Mass error (p	RT (mins) 44.9	Charge 2	Tag	<ul> <li>Abundance</li> <li>7.22E+04</li> </ul>	Confli 1	
Protein: C4YQ itide ion views P Conflicting pro Accession P P34731 •	2 <b>P9 60</b> Protein res Diteins fo Peptides	S ribose solution or peptie Unique	de ion 21 Conflict: F	tein L35 GN 85 Protein Score	<b>J-CAW</b> C	G_02	808 PE Peptid	E=4 S e ion Σ	V=1 s of ⊕	C4YQP Score	Hits	0.890				Charge 2 3	Tag		Confli 1 1	(
Protein: C4YQ tide ion views P conflicting pro Accession P P34731 • C4YQP9	Protein res Deteins for Peptides 59	S ribose solution or peptie Unique	de ion 21 Conflict: F	tein L35 GN 85 Protein Score 35	<b>1=CAW</b> Per ▼ 5.88	G_02	808 PE Peptid # 125	e ion Σ 33 0 35 0	V=1	C4YQP9 Score 8.61	Hits 2	0.890 0.813	1529.827	-1.89	44.9	2	Tag	7.22E+04	1	(
Protein: C4YQ titide ion views P Conflicting pro Accession P P34731 • C4YQP9	Protein resolution for the protein for the proteins for the protein for the pr	S ribose solution or peptic Unique 56 1	de ion 21 Conflict: F 4 5: 2 10	tein L35 GN 85 Protein Score 35	Pe	G_02	808 PE Peptid # 125	e ion Σ 33 0 35 0	V=1	C4YQP Score 8.61	Hits 2 	0.890 0.813	1529.827 1529.861	-1.89 20.5	44.9 45	2		7.22E+04 5.05E+04	1 1	6
Protein: C4YQ otide ion views P Conflicting pro Accession P	Protein resolution for the protein for the proteins for the protein for the pr	S ribose solution or peptic Unique 56 1	de ion 21 Conflict: F 4 5: 2 10	tein L35 GN 85 Protein Score 35	Pe	G_02	808 PE Peptid # 125	e ion Σ 33 0 35 0	V=1	C4YQP Score 8.61	Hits 2 	0.890 0.813	1529.827 1529.861	-1.89 20.5	44.9 45	2		7.22E+04 5.05E+04	1 1	(

Favouring the protein with the higher score, but this time resolve the conflict by switching off (or unassigning) the peptide in panel F for the protein with the lower score. By doing this the other 3 panels update to show the change in conflicts.

Accession	Peptide	es Unique	Conflic	t: Score	Tag	•		#	Σ	Ð	Score	Hit	s Correlatio	n Mass	Mass error (	o RT (mins	s) Cha	rge Ta	ag	Abundance	e Cor	nflict 🔨
P34731	59	57	3	535			V	2185	5 0	0	5.88	2	0.732	1529.86	51 3.14	45	3	-		5.05E+04		0
🔇 C4YQR7	0	0	0	0				1253	3 0	2	5.88	2	-0.154	1529.82	-19.2	44.9	2			7.22E+04	1	1
P43098	42	3	48	339			1	1820	7 0	1	6.03	2	0.427	1115.55	-6.25	32.4	2			6.15E+03	1	1
C4YQN7 (+1)	41	2	48	332			1	1174	0	1	8.02	8	0.861	1303.68	32 12.5	60.8	2			7.59E+04	1	1
P41797	27	11	41	322		-	V	1044	0	1	8.12	8	0.595	1412.84	43 13.5	72.4	2			5.73E+04	(	• • 0
Peptide ion views	Protein res	L	de ion 1	253			Pepti	de io	ns of	f C4'	YQP9											
Conflicting pro	oteins fo	or peptio	de ion 1	253			Pepti	de io	ns of	f C4	YQP9											
Accession F	Peptides	Unique	Conflict:	Protein Score	Pe	pt	#	2	Σœ	b So	core	Hits	Correlation	Mass	Mass error (p	RT (mins)	Charge	Tag	•	Abundance	Conflic	t: Per
🎯 P34731 🛛 🍳	59	57	3 5	535	☑ 5.88	3	<b>V</b> 1	253	° 2		8.61	2	0.968	1529.827	-1.89	44.9	2		7	.22E+04	1	3
C4YQP9	2	1	1 1	16.9	✓ 8.61		<b>V</b> 3	58	• 1		8.32	2	0.968	1168.708	13.4	49.9	2		1	.1E+05	0	١
S C4YQR7	0	0	0 (	)	5.88	3	2	185 (	0 0					1529.861	20.5	45	3		5	.05E+04	0	١
•					,	Þ.	•															Þ

Repeat this process until there are no conflicts remaining for the current protein in Panel A.

Now repeat using a similar approach for the next protein in Panel A, here the situation is similar.

Accession	Peptides	Unique	Conflict	: Score	Tag	<b>*</b> ^		#	Σ	色 Sco	re H	its Correlatio	on Mass	Mass error (p	RT (mins)	Charge	Tag	<ul> <li>Abundance</li> </ul>	Conflic
P34731	59	59	0	535				107		1 7	.14 (	6 0.934	1661.866	-0.254	52.1	2		1.07E+05	2
C4YQR7	0	0	0	0			<b>V</b>	8683	0	2		0.089	1507.822	5.75	40.2	4		2.47E+04	2
🌏 P43098 🛛 🧿	<b>4</b> 2	3	48	339			V	2375	0	0 8	.07 8	B 0.911	1145.601	7.45	48.7	2		5E+04	1
C4YQN7 (+1)	41	2	48	332			<b>V</b>	2772	٠	2 7	.47 9	9 0.868	1277.678	9.58	57.4	2		5.31E+04	1
P41797	27	11	41	322		-	<b>V</b>	2928	0	0 8	.13 9	9 0.939	1414.678	3.49	46.2	2		4.09E+04	1
٠ [	III					F.	•					m							•
Protein: P4309	98 Fatty	acid s	ynthase	subunit alp	ha GN⊧	FAS	2 PE=	3 SV	=1										
							~			00700		V 1							
Protein: C4YQ	N7 Fat	ty acid	synthas	e alpha sub	ounit ree	ducta	ise Gr	N=CA	wG	_02796	PE=4 S	V=I							
Protein: C4YQ			synthas	e alpha sub	ounit rea	ducta	ise Gr	N=CA	wG	_02796	PE=4 5	V=I							
	Protein reso	olution			ounit ree					_02796 C4YQN		V=1							
Protein: C4YQ eptide ion views P Conflicting pro	Protein reso	peptid	e ion 10						s of			Correlation	Mass I	Mass error (p	RT (mins)	Charge 1	ſag	▼ Abundance	Conflict:
Protein: C4YQ eptide ion views P Conflicting pro	Protein reso Diteins for	peptid	e ion 10 Conflict:	7		ej [	Peptid	e ion Σ	s of @	C4YQN	7		Mass 1 1661.866	Mass error (p	RT (mins) 52.1	Charge 1 2	ſag	<ul> <li>Abundance</li> <li>1.07E+05</li> </ul>	Conflict:
Protein: C4YQ eptide ion views P Conflicting pro Accession	Protein reso Diteins for Peptides	peptid	e ion 10 Conflict: 48	7 Protein Score	P	  e  14	Peptid #	e ion Σ 7 °	s of @	C4YQN Score	7 Hits	Correlation				-	ſag		
Protein: C4YQ eptide ion views P Conflicting pro Accession P43098	Protein reso Diteins for Peptides 42	Peptid Unique 3	e ion 10 Conflict: 48	7 Protein Score 339 332	P	e [ 14   14	Peptid #	e ion Σ 7 0 33 0	s of 1 2	C4YQN Score 7.14	7 Hits 6	Correlation 0.939	1661.866	-0.254	52.1	-	ſag	1.07E+05	2 🧃
Protein: C4YQ eptide ion views P Conflicting pro Accession P43098 C4YQN7 (+1)	Protein reso oteins for Peptides 42 41	Peptid Unique 3	e ion 10 Conflict: 48 48	7 Protein Score 339 332	₽ ✓ 7. ✓ 7.	e [ 14   14	Peptid # 10	e ion Σ 7 0 33 0 75 0	s of 1 2 0	C4YQN Score 7.14	7 Hits 6 	Correlation 0.939 0.142	1661.866 1507.822	-0.254 5.75	52.1 40.2	2 4	[ag	1.07E+05 2.47E+04	2 🧃
Protein: C4YQ eptide ion views P Conflicting pro Accession P43098 C4YQN7 (+1)	Protein reso oteins for Peptides 42 41	Peptid Unique 3	e ion 10 Conflict: 48 48	7 Protein Score 339 332	₽ ✓ 7. ✓ 7.	l 'ei 14 14 68	Peptid # 10 868	e ion Σ 7 0 33 0 75 0 28 0	s of 1 2 0 0	C4YQN Score 7.14  8.07	7 Hits 6  8	Correlation 0.939 0.142 0.882	1661.866 1507.822 1145.601	-0.254 5.75 7.45	52.1 40.2 48.7	2 4 2	fag	1.07E+05 2.47E+04 5E+04	2 🧃

#### Resolution of conflicts for this protein

Accession	Peptides	Unique	Conflict	Score	Tag	•		#	Σ	9	Score	Hits	Correlation	Mass	Mass error (p	RT (mins)	Char	ge Ta	g 🔻 Abundai	nce C	onflict
P34731	59	59	0	535			V	107			1 7.14	6	0.934	1661.866	- 1.254	52.1	2		1.07E+05		1
C4YQR7	0	0	0	A <sup>0</sup>			V	868	3 0		2		0.089	1507.822	.75 B	40.2	4		2.47E+04		1
) P43098 🔹	o 42	40	2	339				237	5 0	) (	0 8.07	8	0.911	1145.601		48.7	2		5E+04		0
C4YQN7 (+1)	0	0	0	0			V	277	2 •		2 7.47	9	0.868	1277.678	9.58	57.4	2		5.31E+04		0
P41797	27	11	41	322		-	V	292	8 0	) (	0 8.13	9	0.939	1414.678	3.49	46.2	2		4.09E+04		0
						F		_					III								
Drotoin: D/30	08 Fath	r acid s	ynthase	subunit al	pha GN	=FAS	2 PE	=3 S	V=1	L											
1 10tenii. F4J0	JUIAU																				
			- ase GN⊧	TAL1 PE=	3 SV=1					L											
Protein: Q5A0	)17 Trar	nsaldol	ase GN⊧							1					Г	_					
	17 Trar Protein reso	Isaldol		E			Pept	ide ic	ons c	of Q	)5A017				[	F					
Protein: Q5A0 ptide ion views	017 Trar Protein reso	blution Polution	e ion 10	E			÷.			_	25A017 Score	Hits (	Correlation	Mass N	Mass error (p	F RT (mins)	Charge	Tag	✓ Abundance	Confl	ict:
Protein: Q5A0 ptide ion views F Conflicting pro	D <b>17 Tran</b> Protein reso Dteins for Peptides	blution Polution	e ion 10 Conflict:	7 <b>E</b>		Pel		¢		_	-	Hits 0		Mass N	Mass error (p 8.95	F RT (mins) 52.1	Charge 2	Tag	<ul> <li>Abundance</li> <li>1.07E+05</li> </ul>	Confl 1	ict:
Protein: Q5A0 btide ion views F Conflicting pro Accession 9 P43098 •	D <b>17 Tran</b> Protein reso Dteins for Peptides	n <b>saldol</b> olution peptid Unique	e ion 10 Conflict: 2	7 Protein Score		Pej 14		¢	ο :	_	Score		0.980 1					Tag			
Protein: Q5A0 btide ion views F Conflicting pro Accession P43098 • C4YQN7 (+1)	Protein reso Deteins for Peptides 42	nsaldol olution peptid Unique 40	e ion 10 Conflict: 2 0	7 Protein Score		Pej 14	: 	# 107	Σ Ο Ο	Ð 1	Score 8.68		0.980	1661.866	8.95	52.1		Tag	1.07E+05	1	
Protein: Q5A0 ptide ion views F Conflicting pro Accession P43098 • C4YQN7 (+1)	DIT Tran Protein reso Deteins for Peptides 42 0	Diution peptid Unique 40 0	e ion 10 Conflict: 2 0	7 Protein Score 339 0	e I ☑ 7	Pej 14		# 107 524 536	Σ Ο Ο	型 1 0	Score 8.68 8.44	9 8	0.980 1 0.958 1 0.968 1	1661.866 1345.749	8.95 29.6	52.1 67.6	2 2	Tag	1.07E+05 1.62E+05	1 0	
Protein: Q5A0 ptide ion views Conflicting pro Accession	DIT Tran Protein reso Deteins for Peptides 42 0	Diution peptid Unique 40 0	e ion 10 Conflict: 2 0	7 Protein Score 339 0	e I ☑ 7	Pej 14 14 .68	* * * *	# 107 524 536 664	Σ [	型 1 0	Score 8.68 8.44 8.75	9 8 9	0.980 1 0.958 1 0.968 1 0.958 1	1661.866 1345.749 1880.959	8.95 29.6 11.7	52.1 67.6 52.9	2 2 2 2	Tag	1.07E+05 1.62E+05 9.65E+04	1 0 0	

#### Finally move to the remaining conflict in panel B, and favour the higher scoring protein

Accession	Peptides	Unique	Conflict	: Score	Tag	•	#	ŧ	Σ	④ Scor	e Hits	s Correlation	Mass	Mass error (p.	RT (mins)	Charg	e Tag	<ul> <li>Abundance</li> </ul>	e Conflic
P34731	59	59	0	535			V 1	107	•	1 7.1	4 6	0.934	1661.866	-0.254	52.1	2		1.07E+05	0
C4YQR7	0	0	0	0	•		▼ 8	683		2		0.089	1507.822	5.75	40.2	4		2.47E+04	1
P43098 o	42	41	1	339			☑ 2	375	0	0 8.0	7 8	0.911	1145.601	7.45	48.7	2		5E+04	0
C4YQN7 (+1)	0	0	0	0	•		2	772	•	2 7.4	79	0.868	1277.678	9.58	57.4	2		5.31E+04	0
P41797	27	11	41	322		-	2	928	0	0 8.1	3 9	0.939	1414.678	3.49	46.2	2		4.09E+04	0
					,	•	٠												
Protein: P4309 Protein: Q5A50 ptide ion views Pt	Q8 40S	riboso							1										
Protein: Q5A50	Q8 40S Protein reso	<b>riboso</b> olution	mal prot	tein S4 GN=		PE=	4 SV=1			Q5A5Q8									
rotein: Q5A50 tide ion views Pr onflicting prot	Q8 40S Protein reso teins for	riboso	mal prot e ion 86	tein S4 GN=		PE=	4 SV=1	ions		Q5A5Q8 Score	Hits	Correlation 1	Mass N	lass error (p	RT (mins)	Charge	Tag	✓ Abundance	Conflict:
rotein: Q5A50 ide ion views Pr onflicting prot Accession I	Q8 40S Protein reso teins for	riboso	e ion 86 Conflict:	tein S4 GN= .83	RPS4A	PE=	<b>4 SV=1</b> Peptide	ions Σ	of ⊕		Hits (		Mass N 1507.822	lass error (p 29	RT (mins) 40.2	Charge 4	Tag	<ul> <li>✓ Abundance</li> <li>2.47E+04</li> </ul>	Conflict:
rotein: Q5A50 ide ion views Pr onflicting prot Accession 1 ) P43098 •	Q8 40S Protein reso teins for Peptides	riboso olution r peptid Unique	e ion 86 Conflict:	tein S4 GN= 83 Protein Score	RPS4A Pe	PE=	•4 SV=1 Peptide #	ions Σ ○	of 12	Score		0.334 1				-	Tag		
Protein: Q5A5C tide ion views Pron onflicting prot Accession 1 P43098 0 Q5A5Q8	Q8 40S Protein reso teins for Peptides 42	riboso olution r peptid Unique 41	e ion 86 Conflict: 1	tein S4 GN= 83 Protein Score 339	RPS4A Pe	PE= F	4 SV=1 Peptide # 8683	ions Σ ο	of 12	Score		0.334 1	1507.822	29	40.2	-	Tag	2.47E+04	1 (
Protein: Q5A5C tide ion views Pron onflicting prot Accession 1 P43098 0 Q5A5Q8	Q8 40S Protein reso teins for Peptides 42 13	riboso olution r peptid Unique 41 5	e ion 86 Conflict: 1	tein S4 GN= 83 Protein Score 339 116	RPS4A Pe	PE= F	4 SV=1 Peptide # ₩ 8683 ¥805	ions Σ ο	of 1	Score  7.95	 2	0.334 1 0.895 0.453	1507.822 831.466	29 2.55	40.2 33.9	4	Tag	2.47E+04 1.76E+04	1 (
Protein: Q5A50 tide ion views Pr onflicting prot Accession I	Q8 40S Protein reso teins for Peptides 42 13	riboso olution r peptid Unique 41 5	e ion 86 Conflict: 1	tein S4 GN= 83 Protein Score 339 116	RPS4A Pe	• [	4 SV=1 Peptide # 8683 √ 8683 √ 1435	ions Σ ο	of 1 2 2	Score  7.95 8.25	 2 9	0.334 1 0.895 0.453 -0.051 1	1507.822 831.466 980.496	29 2.55 15	40.2 33.9 28.4	4 2 2	Tag	2.47E+04 1.76E+04 1.14E+05	1 1 0

Adopting a similar approach to the next protein favouring the protein with the highest score as each conflict is examined.

Accession	Pep	tides Uniq	ue Confl	ct: Score	Tag	•		#	Σ	ф <u>9</u>	core H	lits Correlatio	on Mass	Mass error (p	RT (mins)	Charge	Tag	<ul> <li>Abundance</li> </ul>	e Cor	flict ^
C4YQR7		0 0	0	0	0			2053		0	8.43	6 0.973	1674.7	4 10.1	33.2	2		4.21E+04	4	
P43098	4	2 42	0	339	) 🌔		<b>V</b>	12379	0	1	7.96	9 0.862	1525.74	7 8.28	57.5	3		1.36E+04	3	
🎯 C4YQN7 (+1	1)	0 0	0	0			$\checkmark$	14073	0	0	7.77	3 0.907	1606.74	9 5.3	40.5	2		1.68E+04	3	
P41797	o 2	7 11	41	322	2 🕧		<b>V</b>	1181	0	1	8.43	5 0.918	1674.7	6 21.6	33 2	-		8.15E+04	י ל י	
P46587	3	4 18	41	373	3 🥝	∆⊤		17676	0	0	8.58	5 0.605	1786.98	9 3.48	523	B		4.37E+03	V 3	-
•	_		_			ſ	•	_	_	_	_		_			5				
Protein: P4	41797 I	leat sho	k protei:	n SSA1 G	SN=SSA1	PE=1	SV=2								_					
Protein: P4	16587 1	leat sho	k protei	n SSA2 G	N=SSA2	PE=1	SV=3													
Trotem. 1 -	10307 1	lout onlot																		
Peptide ion view	_																			
	/s Protei	n resolution					Pepti	e ion	s of I	P465	37						1			
Peptide ion view	<sub>/s</sub> Protei protein	n resolution	ide ion 2	2053					s of	P465 Scor		Correlation	Mass	Mass error (p	RT (mins)	Charge Ti		<ul> <li>Abundance</li> </ul>	Conflict	8 ^
Peptide ion view Conflicting	<sub>/s</sub> Protei protein	n resolution s for pept	ide ion 2 Conflict:	2053		Pep	<sup>Pepti</sup>		Ф		e Hits	Correlation 0.947	Mass 1674.74	Mass error (p 10.1	RT (mins) 33.2	Charge T	9	<ul> <li>Abundance</li> <li>4.21E+04</li> </ul>	Conflict 4	
Peptide ion view Conflicting Accession	protein Protein Peptid	n resolution s for pept es Unique	ide ion 2 Conflict: 41	2053 Protein Sco	ore	Рер 3.43	<sup>Рерні</sup> Е #	Σ 3 0	@ 0	Scor	e Hits 3 6					Charge Ti 2 3				
Peptide ion view Conflicting Accession	rotein Protein Peptid 34	n resolution s for pept es Unique 18	ide ion 2 Conflict: 41 41	2053 Protein Sco 373	ore	Рер 3.43 3.43	Pepti E #	Σ i3 0 79 0	@ 0	Scor 8.4	e Hits 3 6 6 9	0.947	1674.74	10.1	33.2	2		4.21E+04	4	
Peptide ion view Conflicting Accession P46587 P41797	protein Peptid 34 9 27	n resolution s for pept es Unique 18	tide ion 2 Conflict: 41 41 34	2053 Protein Sco 373 322	ore V (	Pep 3.43 3.43 3.38	Peptial E #	Σ 3 0 79 0 73 0	0 1 0	Scor 8.4 7.9	e Hits 3 6 6 9 7 3	0.947 0.805	1674.74 1525.747	10.1 8.28	33.2 57.5	3		4.21E+04 1.36E+04	4 3	
Peptide ion view Conflicting Accession P46587 P41797 P10591	Protein protein Peptid 34 • 27 14	n resolution s for pept es Unique 18	tide ion 2 Conflict: 41 41 34 7	2053 Protein Sco 373 322 146	ore v a	Pep 3.43 3.43 3.38 5.9	Pepti 1 E # 2 2 2 123 V 123 V 140	Σ 3 0 79 0 73 0 1 0	0 1 0	Scor 8.4 7.9 7.7	e Hits 3 6 6 9 7 3 3 5	0.947 0.805 0.913	1674.74 1525.747 1606.749	10.1 8.28 5.3	33.2 57.5 40.5	2 3 2		4.21E+04 1.36E+04 1.68E+04	4 3 3	

In this case the first peptide for protein (P41797) has 4 conflicting proteins in panel E. Resolve the conflict in favour of the protein with the higher score (P46587) by unticking the peptide ion in panel B then move on to the next conflicting peptide ion in Panel B (which has 3 conflicts) and resolve the conflict in favour of the protein with the higher score.

Acces	ession	Peptic	les Uniqu	e Conflic	t: Score	Tag 🔻	A	#		Σ	中 Sco	re H	its Correlatio	on Mass	Mass error (p.	RT (mins	) Charg	e Tag	<ul> <li>Abundance</li> </ul>	e Confli	ct ^
C4Y	YQR7	0	0	0	0	•		20	53		0 8	.43	5	1674.74	10.1	33.2	2		4.21E+04	1	Ľ
🌒 P43I	3098	42	42	0	339	0		12	379	0	1 7	.96	9 0.862	1525.747	8.28	57.5	3		1.36E+04	2	
C4Y	YQN7 (+1)	) 0	0	0	0	•		140	073	0	0 7	.77	3 0.905	1606.749	5.3	40.5	2		1.68E+04	2	
🌒 P41:	1797	<u> </u>	12	25	314			11	81	0	1 8	.43	5 0.919	1674.76	21.6	33.2	3		8.15E+04	2	
P46	6587	34	18	27	373		-	176	576	0	0 8	.58	5 0.604	1786.989	3.48	52.3	2		4.37E+03	2	۰.
•		m				- F		•					III								•
Prot	tein: P4	1797 He	at shocl	c protein	SSA1 GN=	SSA1 PE=	=1 SV	<b>/=2</b>													
													1 01/ 2								
	tein: P10	0592 He	at shocl	c protein	SSA2 OS=	Saccharo	myce	es cere	evis	lae	GN=SS	AZ PE	=13V=3								
Prot				c proteir	SSA2 OS=	Saccharo	myce	es cere	evis	iae	GN=SS	SAZ PE	=15V=5								
Prot eptide	e ion views	0592 He Protein re proteins f	esolution			Saccharo		es cere					=1 SV=3								
Prot eptide Conf	e ion views	Protein re proteins f	esolution or pepti	de ion 2		Saccharo							Correlation	Mass 1	Mass error (p	RT (mins)	Charge	Tag	<ul> <li>Abundance</li> </ul>	Conflict:	*
Prot eptide Conf Acce	e ion views flicting p	Protein re proteins f	esolution or pepti	de ion 2 Conflict:	053		Pe	eptide i		of I	P10592			Mass 1 1606.749	Mass error (p 5.3	RT (mins) 40.5		Tag	<ul> <li>Abundance</li> <li>1.68E+04</li> </ul>	Conflict:	•
Prot eptide Conf Acce	e ion views flicting p cession 246587	Protein re proteins f Peptides	esolution or pepti Unique	de ion 2 Conflict: 27	053 Protein Score	Pept	Pe	eptide i #	ions Σ	of I	P10592 Score	Hits	Correlation					_			^
Prot eptide Conf Acce 2 P4	e ion views flicting p cession 46587	Protein reproteins f Peptides 34	esolution or pepti Unique 18	de ion 2 Conflict: 27	053 Protein Score 873 814	Pept	Pe	eptide i # 14073	ions Σ	of I	P10592 Score 7.77	Hits 2	Correlation 0.810	1606.749	5.3	40.5	2	_	1.68E+04	2	•
Prote eptide Conf Acce 9 P4 9 P4 9 P1	e ion views flicting p cession 46587 41797	Protein reproteins f Peptides 34	or pepti Unique 18 12	de ion 2 Conflict: 27 : 25 : 0 (	053 Protein Score 873 814	Pept 8.43 8.43	Pe V	eptide i # 14073 12379	ions Ω	of   @ 0 1	P10592 Score 7.77 7.96	Hits 2 8	Correlation 0.810 0.885	1606.749 1525.747	5.3 8.28	40.5 57.5	2 3	_	1.68E+04 1.36E+04	2	*
Prote eptide Conf Acce 2 P4 2 P4 2 P1 2 O5	e ion views flicting p cession 46587 41797 <	Protein reproteins f Peptides 34	or pepti Unique 18 12 0 7	de ion 2 Conflict: 27 25 0 2 2 0	053 Protein Score 373 314 336	Pept 8.43 8.43 8.38	Pe V V	eptide i # 14073 12379 2053	ions Ω Ο Ο	of   @ 0 1	P10592 Score 7.77 7.96 8.18	Hits 2 8 3	Correlation 0.810 0.885 0.930	1606.749 1525.747 1674.74	5.3 8.28 10.1	40.5 57.5 33.2	2 3 2	_	1.68E+04 1.36E+04 4.21E+04	2 2 1	• III •

Alternatively you can review the other conflicting proteins. In this case P10591 and P10592 are from a similarly named protein but a different species so you can right click and turn off all the peptides.

Then reviewing the remaining conflicts between P41797 and P46587 they have 19 remaining conflicts. To resolve these in favour of P46587 you can highlight all the remaining conflicted peptides in panel B and click on the tick box to untick them, this will remove them from P41797.

Accession	Peptides	Unique	Conflict	Score	Tag	<b>*</b> ^		#	Σ	日 Scor	e Hit	s Correlation	n Mass	Mass error (p.	RT (mins)	Charg	e Tag	<ul> <li>Abundance</li> </ul>	Con	nflict
C4YQR7	0	0	0	0				1400		1 8.6	i3 8	0.970	2166.046	6 13.8	59.7	3		7.11E+04	1	L
P43098	42	42	0	339	•		<b>v</b>	690		1 8.5	i8 8	0.822	1283.706	5 11.4	68.8	2		1.09E+05	1	L
C4YQN7 (+1)	0	0	0	0	•		$\mathbf{v}$	297		1 8.0	6 4	0.913	1680.838	8 16	52.8	2		1.61E+05	1	
P41797 o	o 27	12	19	314	<b>(</b>		<b>V</b>	179	٠	3 8.4	6 9	0.634	1472.798	8 9.86	49.2	2		9.33E+04	0	)
P46587	34	19	20	373		-	V	6149	0	1 7.9	3 9	0.977	1230.697	7 -0.111	35.4	3		1.95E+04	0	)
						P	٠.													,
Protein: P4179 Protein: P4658 ptide ion views	87 Heat	shock																		
Protein: P4658	87 Heat Protein reso	shock lution	protein	SSA2 GN=		E=1 \$	SV=3	e ions	of	P46587										
Protein: P4658 tide ion views P Conflicting pro	87 Heat Protein reso	shock lution peptide	protein	SSA2 GN=		'E=1 \$	SV=3	e ions Σ	of ⊕	P46587 Score	Hits	Correlation	Mass	Mass error (p	RT (mins)	Charge	Tag	<ul> <li>Abundance</li> </ul>	Conflict	t:
Protein: P4658 tide ion views P Conflicting pro	87 Heat Protein reso Dteins for Peptides Un	shock lution peptide	protein	SSA2 GN= 7 rotein Score	SSA2 P	E=1 S	8 <b>V=3</b> Peptide	Σ			Hits 4		Mass 1 1680.838	Mass error (p 16	RT (mins) 52.8	Charge 2	Tag	<ul> <li>Abundance</li> <li>1.61E+05</li> </ul>	Conflict	t:
Protein: P4658 tide ion views P Conflicting pro Accession P P46587	87 Heat Protein reso Deteins for Peptides Un 34	shock lution peptide	protein : e ion 29 onflict: P	SSA2 GN= 7 rotein Score 3	SSA2 P	2 <b>E=1</b> \$	SV=3 Peptide #	Σ 7 Ο		Score		0.961					Tag			t:
Protein: P4658 tide ion views P Conflicting pro Accession P P46587	87 Heat Protein reso Deteins for Peptides Un 34	shock lution peptide nique C 19	protein se ion 29 onflict: P 20 37	SSA2 GN= 7 rotein Score 3	SSA2 P Pe Ø 8.00	PE=1 \$	SV=3 Peptide # ☑ 297	Σ 7 0 54 0		Score 8.06	4	0.961 0.703	1680.838	16	52.8	2	Tag	1.61E+05	1	t
Protein: P4658 bide ion views P Conflicting pro Accession P P46587	87 Heat Protein reso Deteins for Peptides Un 34	shock lution peptide nique C 19	protein se ion 29 onflict: P 20 37	SSA2 GN= 7 rotein Score 3	SSA2 P Pe Ø 8.00	E=1 \$	SV=3 Peptide # ☑ 297 ☑ 3036	Σ 7 0 54 0 2 0	1 0	Score 8.06 8.63	4	0.961 0.703 0.921	1680.838 2166.023	16 2.92	52.8 59.7	2	Tag	1.61E+05 3.02E+03	1	
Protein: P4658 bide ion views P Conflicting pro Accession P	87 Heat Protein reso Deteins for Peptides Un 34	shock lution peptide nique C 19	protein se ion 29 onflict: P 20 37	SSA2 GN= 7 rotein Score 3	SSA2 P Pe Ø 8.00	E=1 \$	SV=3 Peptide # ♥ 297 ♥ 3036 ♥ 163	Σ 7 0 54 0 2 0 9 0	1 0	Score 8.06 8.63 8.59	4 8 7	0.961 0.703 0.921 0.925	1680.838 2166.023 1590.767	16 2.92 13	52.8 59.7 45.8	2 2 2	Tag	1.61E+05 3.02E+03 7.77E+04	1 1 1	

The display will update to show one remaining conflict for P46587. Click on this protein in panel A.

Accession	Peptide	s Unique	Conflict:	Score	Tag	•		#	Σ	④ Sc	ore Hi	ts Correlatio	n Mass	Mass error (p	RT (mins)	Charge	e Tag	<ul> <li>Abundance</li> </ul>	Conflict
🔇 C4YQR7	0	0	0	0				181		8	.61 2	2 0.956	1292.682	1.35	49.9	2		3.02E+05	1
P43098	42	42	0	339			<b>V</b>	659	•	0	.08 5	5 <b>0.868</b>	2534.26	4.08	70.8	2		4.29E+04	0
🔇 C4YQN7 (+1)	0	0	0	0				156	٠	3	.61 9	0.963	1658.914	15.6	56.3	2		1.98E+05	0
P41797	12	12	0	153			$\checkmark$	3049	•	1	.61 8	0.982	1658.916	16.8	56.3	3		5.29E+04	0
🌚 P46587 🛛 🥥	o 34	33	1	373		-	1	20	•	0	.08 80.8	0.918	2534.277	10.5	70.8	3		1.96E+05	0
•	III					F						III							÷.
Protein: Q5AD	M7 GI	ycerald	•						TDH:	3 PE=3	SV=1								
Protein: Q5AD	OM7 Gl	yceralde	ehyde 3-	phosphate		roge	nase	GN=		3 PE=3									
Protein: Q5AD eptide ion views P Conflicting pro	OM7 GI Protein res	yceralde olution r peptid	e <b>hyde 3</b> -	phosphate		roge	nase	GN=	ns of	Q5ADA		Correlation	Mass N	Mass error (p	RT (mins)	Charge 1	Tag	<ul> <li>Abundance</li> </ul>	Conflict:
Protein: Q5AD eptide ion views P Conflicting pro	OM7 GI Protein res	yceralde olution r peptid	e <b>hyde 3</b> -	phosphate 1 rotein Score	e dehyd	roge	nase Pepti	GN= de io	ns of	Q5ADA	7	Correlation 0.883	Mass M 1292.682	Mass error (p	RT (mins) 49.9	Charge 1 2	Tag	<ul> <li>✓ Abundance</li> <li>3.02E+05</li> </ul>	Conflict:
Protein: Q5AD eptide ion views P Conflicting pro Accession P	OM7 GI Protein res Oteins fo Peptides	yceralde olution r peptid Jnique (	e <b>hyde 3</b> - e ion 18 <sup>-</sup> Conflict: P	phosphate	e dehyd Pe	roge	Pepti #	GN= de io	ns of E	Q5ADA Score	17 Hits					Charge 2 2	Tag		Conflict:
Protein: Q5AD eptide ion views P Conflicting pro Accession P P46587 9	OM7 GI Protein res Dteins fo Peptides 34	yceralde olution r peptid Jnique 33	e ion 18 Conflict: P 1 37	phosphate	e dehyd Pe Ø 6.6	roge	Pepti #	GN= de ion 1 81	ns of E	Q5ADA Score 8.44	17 Hits 5	0.883	1292.682	15.7	49.9	2	Tag	3.02E+05	1 📢
Protein: Q5AD eptide ion views P Conflicting pro Accession P P46587 9	OM7 GI Protein res Dteins fo Peptides 34	yceralde olution r peptid Jnique 33	e ion 18 Conflict: P 1 37	phosphate	e dehyd Pe Ø 6.6	roger ept 1	Pepti # 1 32	GN= de ion 201 0 6 0	ns of 8 0 0	Q5ADA Score 8.44 9.13	17 Hits 5 9	0.883 0.872	1292.682 2885.364	15.7 5.15	49.9 76.6	2	Tag	3.02E+05 4.07E+04	1 (
Peptide ion views P Conflicting pro Accession P P46587 •	OM7 GI Protein res Dteins fo Peptides 34	yceralde olution r peptid Jnique 33	e ion 18 Conflict: P 1 37	phosphate	e dehyd Pe Ø 6.6	roge	Peptie # 1 32 2 18	GN= de ion 201 0 6 0	ns of 8 0 0 0 0 0	Q5ADA Score 8.44 9.13 8.05	17 Hits 5 9	0.883 0.872 0.969	1292.682 2885.364 2889.489	15.7 5.15 1.97	49.9 76.6 63.9	2	Tag	3.02E+05 4.07E+04 2.4E+05	

The strategies that can be used to resolve conflicts can include differences in Mass error as well. However, if there is no difference for a given peptide then either resolution in favour of the protein with the highest protein score or unticking the peptide for both conflicting proteins is an option.

**Note:** the abundances will need to be recalculated as a result of performing Conflict resolution. This is achieved by clicking on the Recalculate abundances, which appears during Conflict resolution

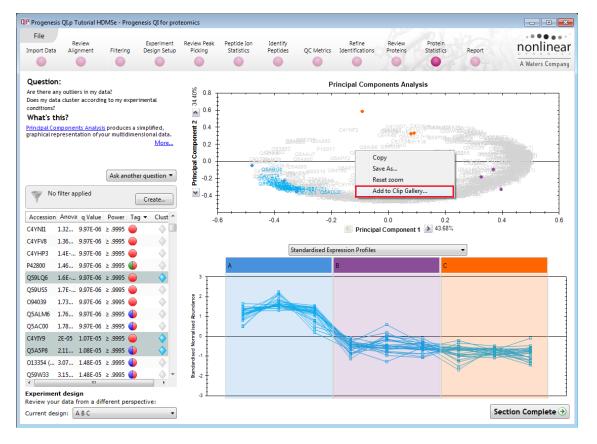
•		Þ	•
€ Refine Identifications	Protein options	Recalculate al	bundances

## Appendix 8: Using Clip Gallery to Save and Export Pictures and Data

At every stage of the Progenesis QI for proteomics workflow the views and data tables can be added to the Clip Gallery.

The saved images of the Views and the tables are retained as part of the experiment and are stored accordingly. This facility allows you to capture (high resolution) images that can be used in the development of specific reports and/or used as part of the process of publishing your experimental findings.

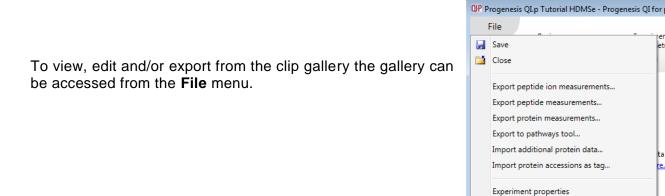
As an example of using the Clip Gallery, at the **Protein Statistics** view while displaying the PCA plot right click on the **Biplot** View and select **Add to Clip Gallery**...



This will open a dialog displaying what is to be saved and allows you to alter the title and provides a description of the image for later reference.

OP Add to Clip Gallery
Enter a title and description for this clipping:
Principal Components Analysis
PCA plot of all protein data All quantifiable proteins in experiment
Add to Clip Gallery Cancel

Enter details as required and click Add to Clip Gallery



Selecting an image in the gallery makes available an **Actions** menu that allows you to manipulate the output of the image.

Show Clip Gallery

× Exit

QP Clip Gallery		
Clip Gallery	Item size:	· D Search
Actions Edit Copy Ima Copy Title Copy Des Export Im Delete	ription	Data analysis performed using: Progenesis QI www.nonlinear.com
PCA plot of all protein data All quantifiable proteins in experiment	Progenesis QI for proteomics logo A high-resolution image that you can use in presentations and posters to show the software was used in your research.	Progenesis QI for proteomics analysis badge A high-resolution image that you can use in presentations and posters to show the software was used in your research.
		Export all Close

**Note**: there is also the capacity to **Export all...** the images in the experiments clip gallery which creates a list of files in a folder of your choice where the file name is based on the image title.

**Note**: right clicking on a table and adding it to the Clip Gallery allows you to export the current content to Excel.

## Appendix 9: Exporting protein identities to IPA

**IPA (Ingenuity Pathway Analysis)**: is a commercial web-based software application for the analysis, integration and interpretation of data derived from any experiments that generate gene and chemical lists with expression data, such as metabolomics and proteomics.

Before exporting data to IPA, make sure that you can launch IPA using the default browser on your system. The plug-in relies on the browser to upload the Progenesis-exported data to IPA.

Note: the use of IPA requires a licensed copy, for licensing details please visit the IPA website.

First select the Protein data to export to the pathways tool using tag filtering to 'focus' the set to export.

Click Export to pathways tool and select IPA and click Next.

QIP Progenesis QI.p Tutorial HDMSe - Progenesis QI for prot	eomics	
File	leview Peak Peptide Ion Identify Refine Review Protein Picking Statistics Peptides QC Metrics Identifications Proteins Statistics	Report A Waters Company
<section-header><section-header><text><text><text><text><text><text><text><text><text><text></text></text></text></text></text></text></text></text></text></text></section-header></section-header>	No filter applied Create	DTNAMICS
Export protein measurements Export peptide measurements Export peptide ion measurements	4         15.0	I∳I
Experiment design Review your data from a different perspective: Current design: A B C	∢ Quantifiable proteins displayed: 512	Section Complete 🥑

Progenesis QI for Proteomics User Guide

Select the appropriate identifier type used for proteins in the experiment.

**Note**: for proteins with different identifier types, filter the protein list to contain only compounds with single identifier type at a time, then perform the export operation and merge the resulting lists in IPA.

Finally select the way you want to export the compounds to IPA.

QIP Export Pathways Information
<b>Configure your export</b> Choose which identifications to export and the type of analysis you want to perform.
Select the identifier type used for proteins in the experiment:
UniProt/Swiss-Prot Accession 🔹
UniProt/Swiss-Prot Accession
GenPept GI Number
For expression data, choose two experimental conditions that you would like to compare.
Baseline: Comparison:
🗖 A 🔹 🕇 🗖 B 🔹
If greater, gives a <b>negative</b> fold change. If greater, gives a <b>positive</b> fold change.
< Back Export proteins to IPA Close

If you are performing over-representation analysis or other types of analysis that do not require the protein expression data then you can make use of the **Create a list** option to export data to IPA.

If you require to perform enrichment analysis or other types of analyses that use protein expression data, select the option to **Upload expression dataset** to IPA.

OPP Export Pathways Information
<b>Configure your export</b> Choose which identifications to export and the type of analysis you want to perform.
Select the identifier type used for proteins in the experiment:
UniProt/Swiss-Prot Accession 🔹
Select the way you want to export the proteins to IPA:
Upload expression dataset
For expression data, choose two experimental conditions that you would like to compare. Baseline: A Comparison: A C A If greater, gives a <b>negative</b> fold change. C A B C C
< Back Export proteins to IPA Close

**Note**: You need to select the type of protein identifier used by at least one protein whose expression data you want to export. If there are proteins with different identifier types, you will be able to select additional identifier types in IPA after the dataset is uploaded.

The operation exports data from a single observation at a time - select the two experimental conditions (taken from the current **experiment design as set in Progenesis QI for proteomics** you wish to compare as that observation).

Click Export compounds to IPA.

	apps.ingenuity.c ♀ ← 🚔 Ċ 🛛 🏧 Ingenuity – Ingenuity Login 🗙 🗤 voritesIoolsHelp		<mark>×</mark> ۲
INGEN	NUITY		^
Welcome! P	lease login	Contact Customer Support	
Email	smith@work.com	Customer Support Phone: 650.381.5111	
Password	•••••	Hours: 6am - 5pm (PST) Monday - Friday (excluding holidays)	
	Remember my password	support@ingenuity.com	
	LOG IN	For Product and Sales related inquiries contact:	
	Sign Up   Forgot Password	650.381.5056 sales@ingenuity.com	
		-	~
		>	<u>۲</u>

If you have access to a licenced copy of IPA then log in

IPA will open displaying the imported data from Progenesis QI for Proteomics.

<u>F</u> ile <u>E</u> dit	t <u>V</u> iew <u>W</u> indow	<u>H</u> elp						Provide Feedback   Support Janucz Nykiel Close IP.			
Genes and Chemicals Diseases and Functions Pathways and Tox Lists											
NEW ¥ Enter gene names/symbols/IDs or chemical/drug names here see							SEA				
Dataset Upload - New Dataset 2016-05-20 10:29 AM											
1. Select File Format   More Info					Data Upland Workflow						
2. Conta	ins Column Header:	O Yes	No					Data Upload Workflow			
2.044	Identifier Type:	UniDeat	/Swiss-Prot Accession	▼ Specify	the identifier type fou	and the allow of the same		Use Dataset Upload to import your dataset file into IPA.			
5. Select	identifier Type:	UniProt	/SWISS-Prot Accession	<ul> <li>Specify</li> </ul>	the identifier type rou	nd in the dataset.		Once uploaded, many different analysis options exist including Biomarker Filter, Molecular Tox and Core Analyses. Review the different type of analyses and see which one best fits			
4. Array	platform used for expe	riments: Not spe	ecified/applicable	<ul> <li>Select r</li> </ul>	elevant array platform	as a reference set for d	lata analysis.				
5. Use th	e dropdown menus to	specify the colum	n names that contain id	entifiers and observatio	ns. For observations, s	elect the appropriate ex	xpression value tvr	your needs.			
								1. To upload a dataset file, click here.			
Raw Da	ata (512) \ Dataset Sum	mary (1)						1. To upload a dataset me, <u>circk fiele</u> .			
	(out) ( butaset suit	(1) (1) (1)						W Open			
	ID -	Observation 1	▼ Ignore ▼	- Ignore -	Ignore 👻	Ignore 👻	Ignore	Look In: 🗀 Multiple Rank. 👻 🚱 🚰			
							- griere	Batch (mult-timepoint)			
		Exp Fold Chan	<b>•</b>					Affy_with_p-value_and_fold.txt			
1	P34731	-1.00902155389087	1					LL_with_Fold and_Normalized.txt			
2	C4YQR7	1.03618874042727									
3	C4YMC3	-1.1630436093987									
4	C4YQN7	-1.14248088552437									
5	P43098	-1.00167260838215									
6	C4YR46	1.11144195146305						File (jame:			
7	O13430	1.0005161363124						Files of Type: All Files			
8	P46587	1.16697510036399						Constant The Factor -			
9	P82610	1.14143675382931						Open Cancel			
10	P46598	1.06004923965961 -1.10613636924535									
	Q96VB9 P41797	1.50747573107542						2. Select the dataset file from your computer and click the			
12	P28877	-1.20624862059398						Open button.			
13	C4YK39	1.05494661189734									
	Q59KZ1	1.03999813845519						<ol><li>Select Flexible format for the file format from the</li></ol>			
16	C4YL05	-1.0312270752554						dropdown menu.			
17	P46273	1.31165982609058									
18	C4YIL8	1.26909493012736						4. Select an Identifier Type from the dropdown menu. IPA			
19	O94039	1.55325257659893						supports many identifiers and symbols and will attempt to quess at the type of identifier in your dataset file. To			
	013287	1.38535524141575						override the selection, uncheck the option and simply select			
						the most appropriate one. If more than one type of identifier					
								exists in your dataset, select all appropriate ones.			
								GenBank 💌			

You can now explore your protein expression data using the tools available in IPA. Instructions on how to manage and explore your imported data set are provided by IPA.

## **Appendix 10: Waters Machine Specification**

This appendix provides information on the approximate time(s) taken at each stage and the total time taken to analyse a set of 9 (Phase 1) HDMSe runs on a Waters Demo Spec PC.

Machine Spec: LenovoProcessor: Intel® Xeon® CPU E5-1630 v3 @ 3.70GHz 12coreK40c GPU cardRAM: 64.0 GB

System Type: 64-bit Operating System

File Folder Size: Each file folder (.RAW): 40.9 Gig

Analysis Stages:		Per file	Total	
Import Data:	Loading of Raw data per file	10min	1hr 12min	for 9 files
	Apex Background processing	18min (max)	2hr 42min	for 9 files
	(re-opening at Import Data)		20s	
Alignment:	Automatic alignment of data		3min 45s	
	(re-opening at Alignment)		10s	
Peak Detection:	Automatic Detection of data		9min 25s	
	(re-opening at Peak Detection)		10s	
Identify Peptides:	Performing MS <sup>E</sup> Search		13min 15s	
	(re-opening at Identify Peptides)		10s	
Total Analysis Time:	Excluding Background Apex Processing	9	1hr 38min	
	Including Apex processing		2hr 51min	
Restoring:	Tutorial Archive		3.5min	

### References

Silva, JC, Gorenstein, MV, Li, G-Z, Vissers, JPC and Geromanos. Molecular and Cellular Proteomics (2006); 5 : 144-156 Absolute Quantification of Proteins by LCMS<sup>E</sup>