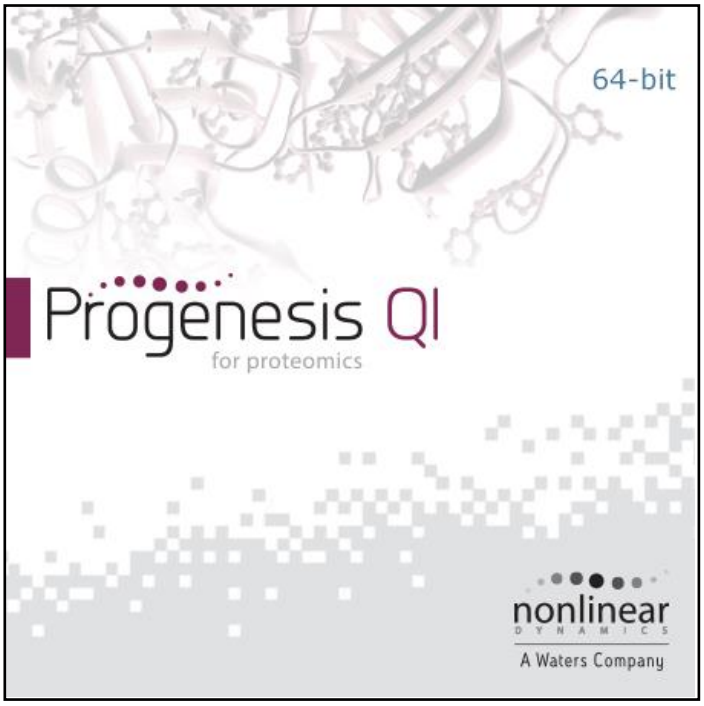


Progenesis QI for proteomics User Guide

Analysis workflow guidelines for HDMse and MSe data

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

Contents

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 76) 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 support@nonlinear.com

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

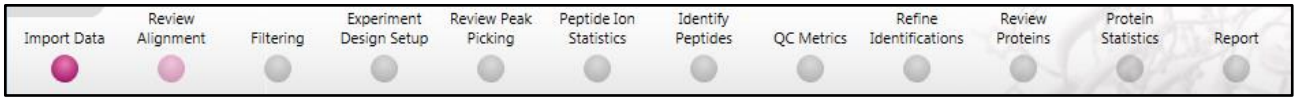
You can freely explore the quality of your LC-MS data using Data Import and then licence your own LC-MS runs using this evaluation copy of Progenesis QI for proteomics. Instructions on how to do this are included in a section at the end of the user guide document.

LC-MS Data used in this user guide

For the purposes of this data set the MS^E parameters were set to 250:125 instead of the default settings as defined in Appendix 1 (page 76). This was done to reduce the time taken to demo the data analysis.

Workflow approach to LC-MS run analysis

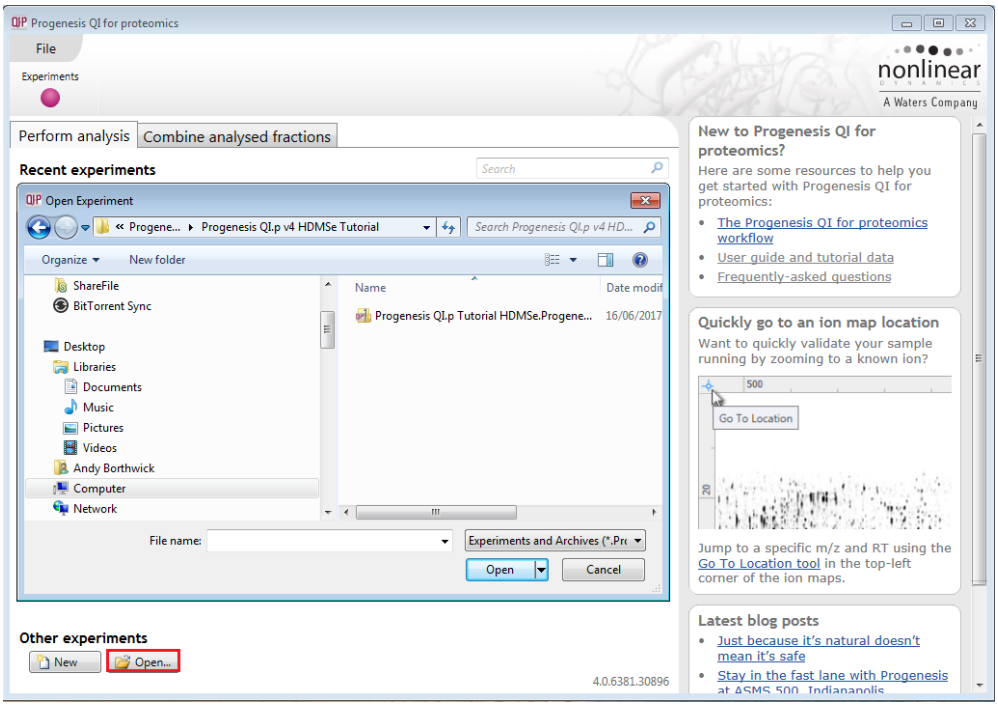
Progenesis QI for proteomics adopts an intuitive **Workflow** approach to performing comparative LC-MS data analysis. The following user guide describes the various stages of this workflow (see below) focusing mainly on the stages from Alignment to Report.



Restoring the Tutorial

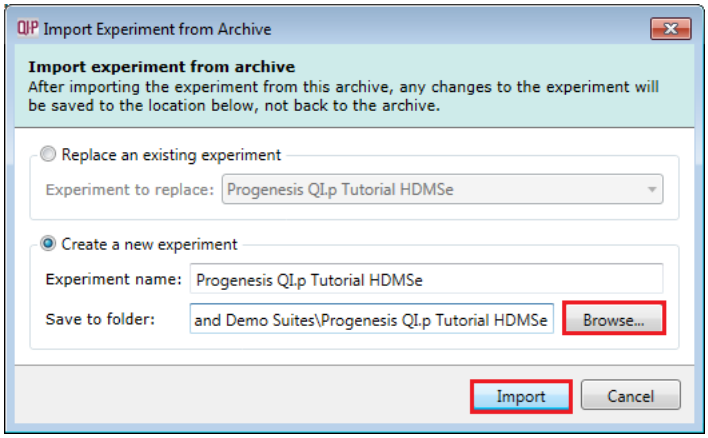
Open Progenesis QI for proteomics and download the Compressed (.zip) Tutorial Archive file from the 'User guide and tutorial' link shown below, placing it in a **new folder** on your desktop. Before restoring the tutorial in the software **you must** first right click on the (.zip) file and extract it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

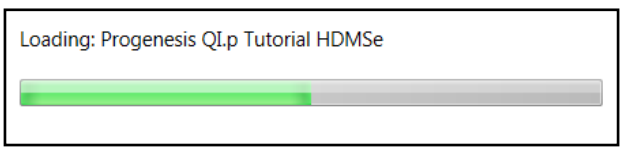


This opens the 'Import from archive' dialog.

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



Then press **Import.**

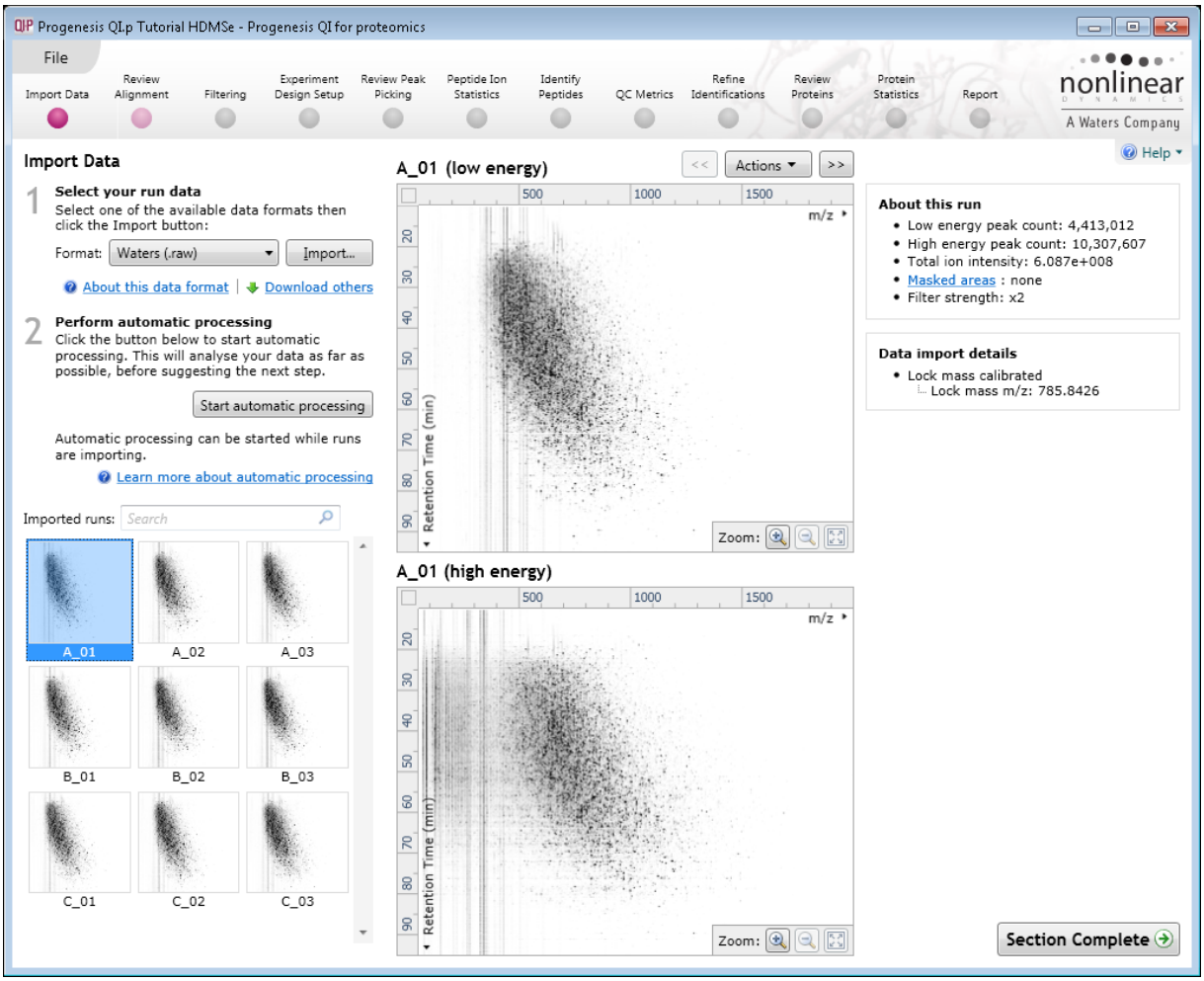


Note: use the **Replace an existing experiment** option if you want to over-write an existing version of the tutorial.

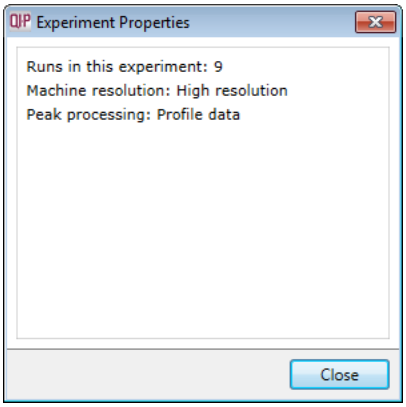
Tip: at each stage in the software there are links to more information and help on the website.

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.



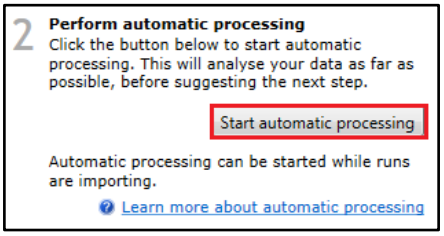
Note: the **Experiment Properties** are available from the File menu**.** These were selected when the experiment was created (see Appendix 1, page 76). The tutorial data is profile data.

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.**

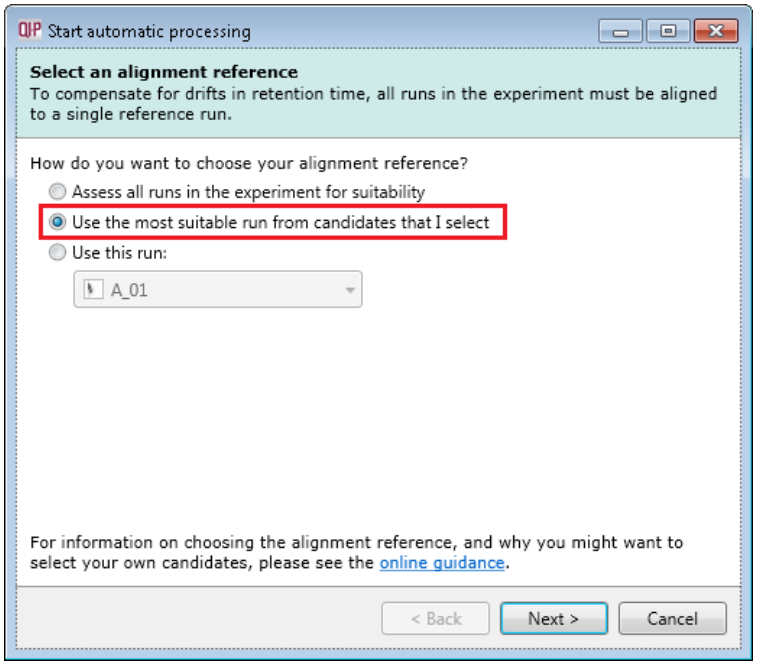
Note: for this tutorial the data has been imported already.

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^E 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.



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

1. **Assess all runs in the experiment for suitability**
   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 long time for a large number of runs.

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**

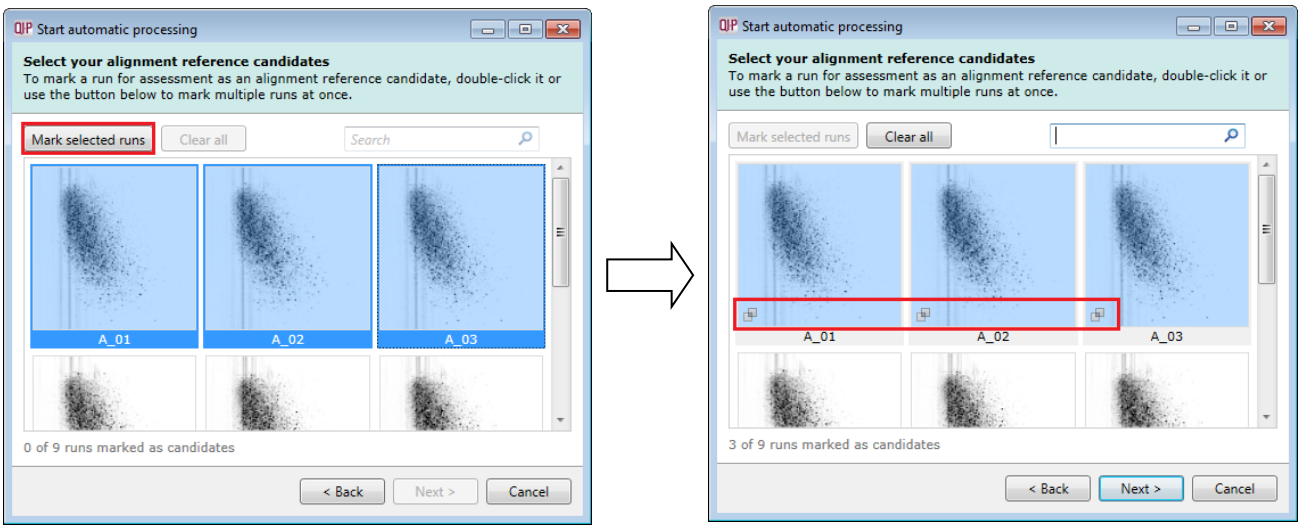
This method allows you to manually choose the reference run.

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).

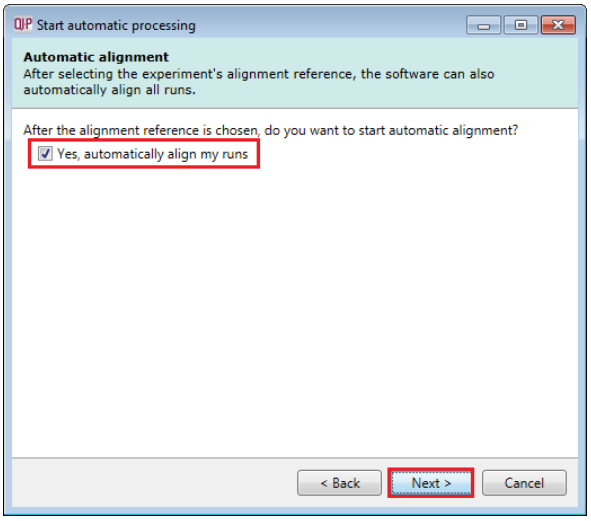
For this tutorial we will select the second option.

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.

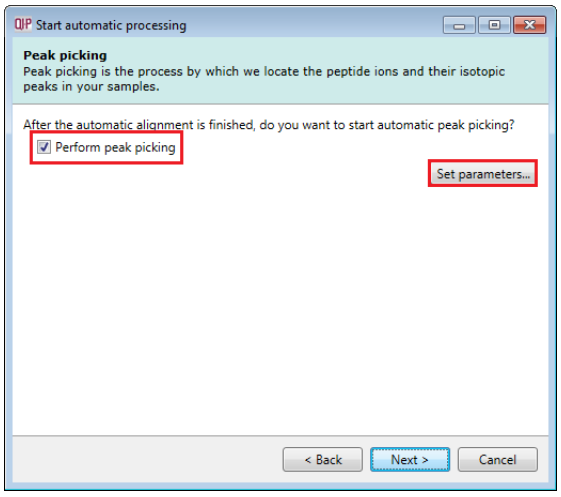


On clicking **Next** you will be asked if you want to align your runs automatically.

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



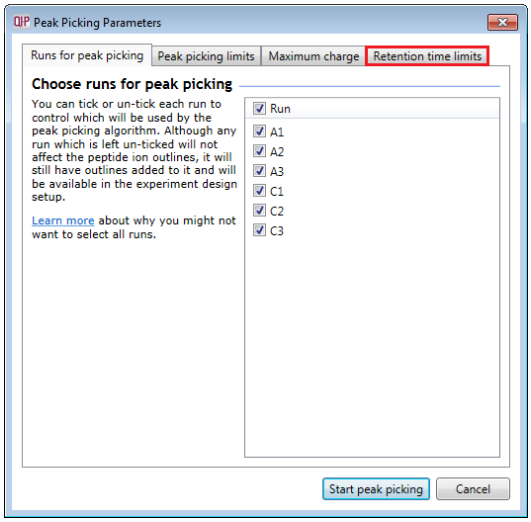
The next page of the processing wizard will ask you if you want to **Perform peak picking** and allow you to set appropriate parameters.

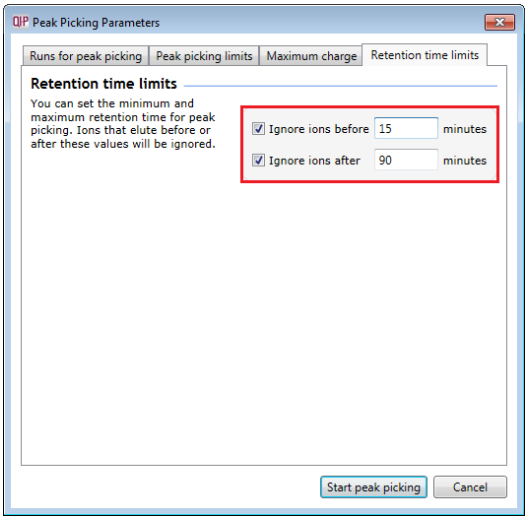


For the purposes of this User guide we will use the default settings for peak picking except in the case of defining Retention Time limits where we will limit the peak picking to between 15 and 90 min.

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

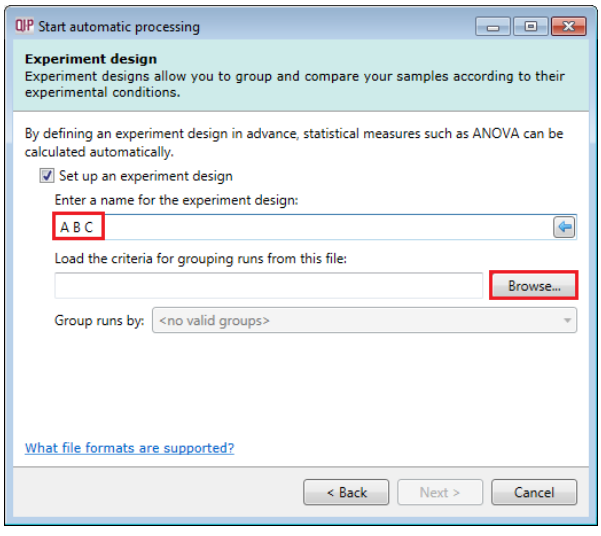
Click on **Set parameters** and select the fourth tab to set **Retention time limits** for the detection. The default limits are displayed in the unticked boxes and detection will use these values whenever the box(s) are unticked.





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.

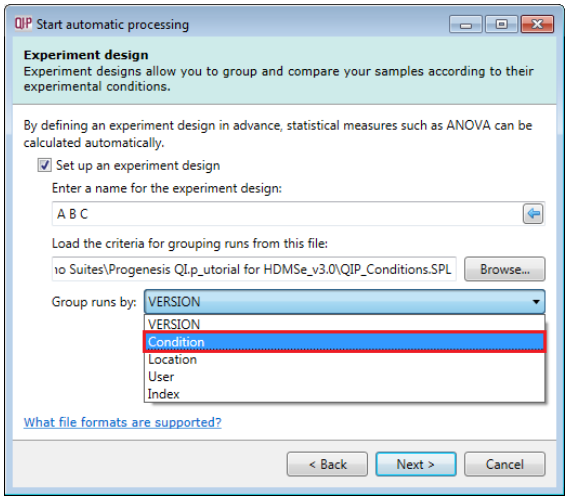


To handle the grouping of your run data you can make use of **sample tracking information** that has been stored in a spread sheet at the time of sample collection and/or preparation.

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.



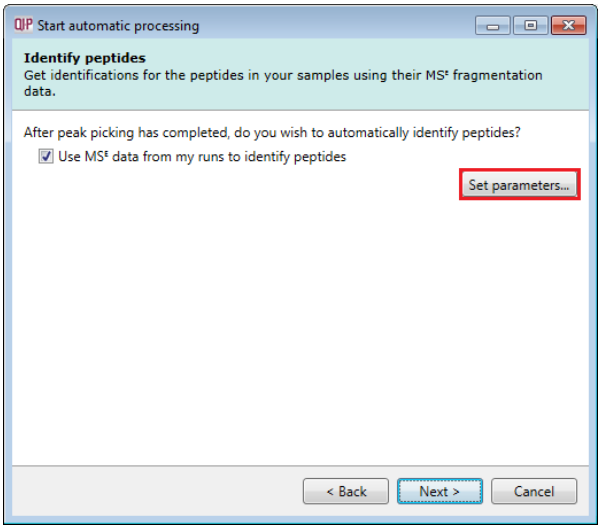
To use this approach select the **Import design from file** option from the New Experiment Design dialog. Then locate the QIP_Conditions file and select what to **Group runs by**, for example: **Condition**.

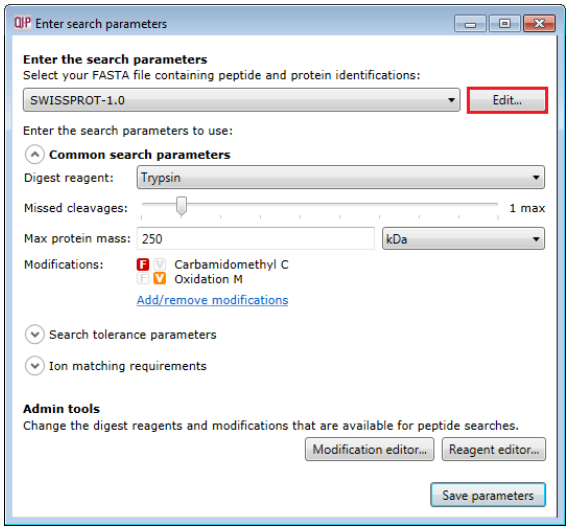
Note: you can create additional experiment designs following the completion of automatic processing.

Select Conditions and then click **Next**.

For MS^E fragmentation data you can set up the peptide identification to be performed automatically.

Note: if the software has detected MS^E data then this option will be ticked by default.

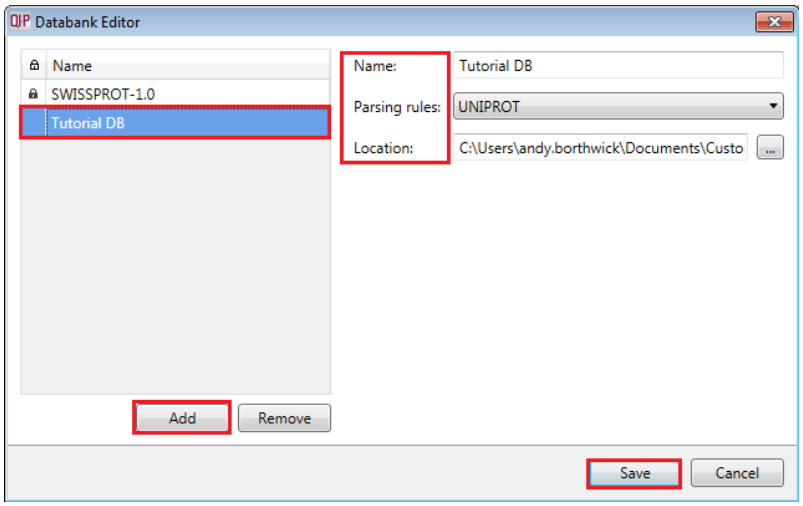




Click on **Set Parameters**. The default Databank is for Swissprot-1.0 (which is a locked example).

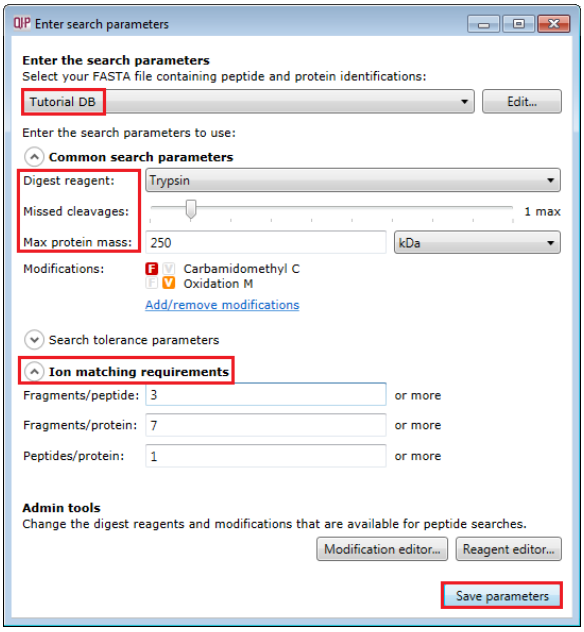
To create a new Databank from a Fasta file click on **Edit** and then create a new one using the example FASTA file that is available with the Experiment Archive you restored at the beginning of this tutorial exercise (**Tutorial_nd_DB.fasta**).

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.



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...**

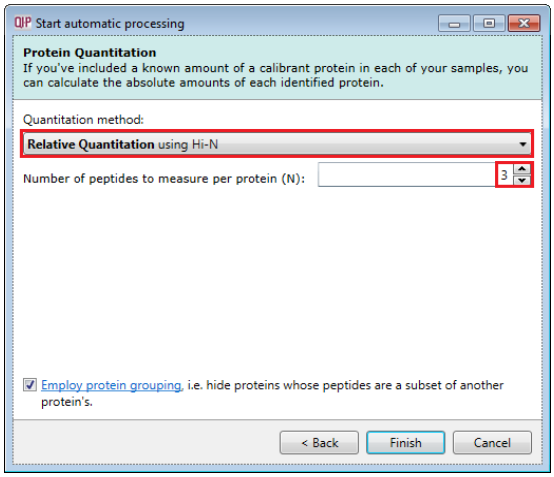
Missed cleavages: is set as 1.

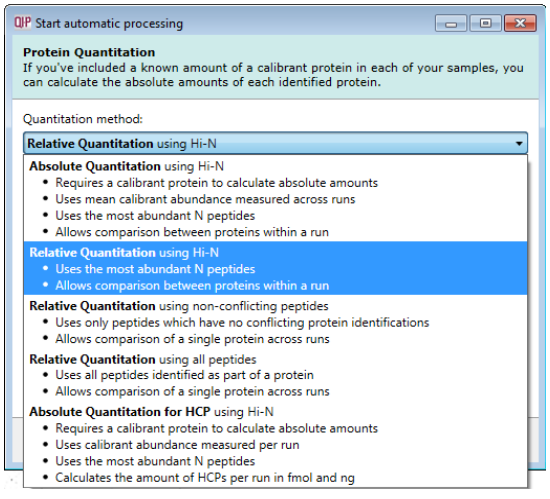
Maximum protein mass: is set at 250kDa

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.**



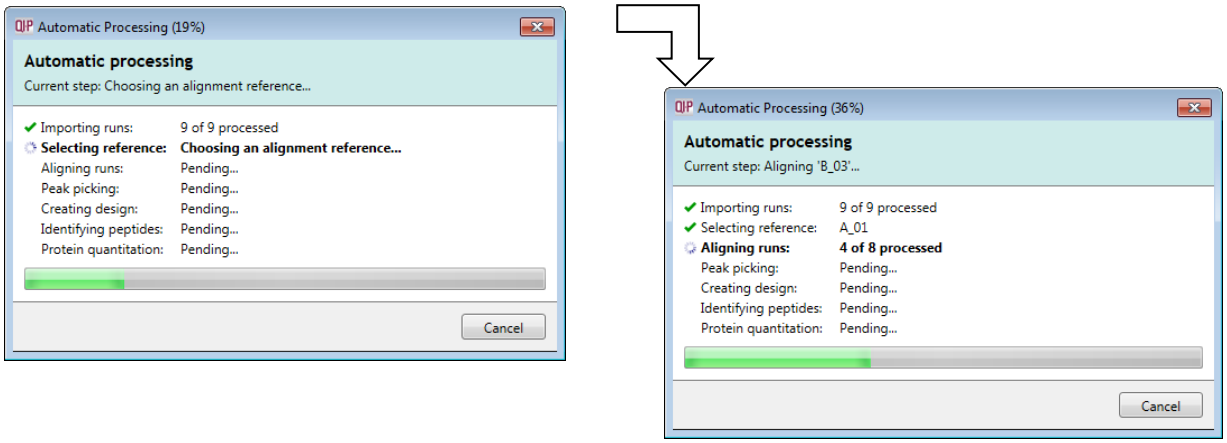


The **Protein Quantitation** dialog opens displaying the default method, **Relative Quantitation using Hi-N** which uses up to 3 peptides per protein to compute the relative amount of each 'identified' protein.

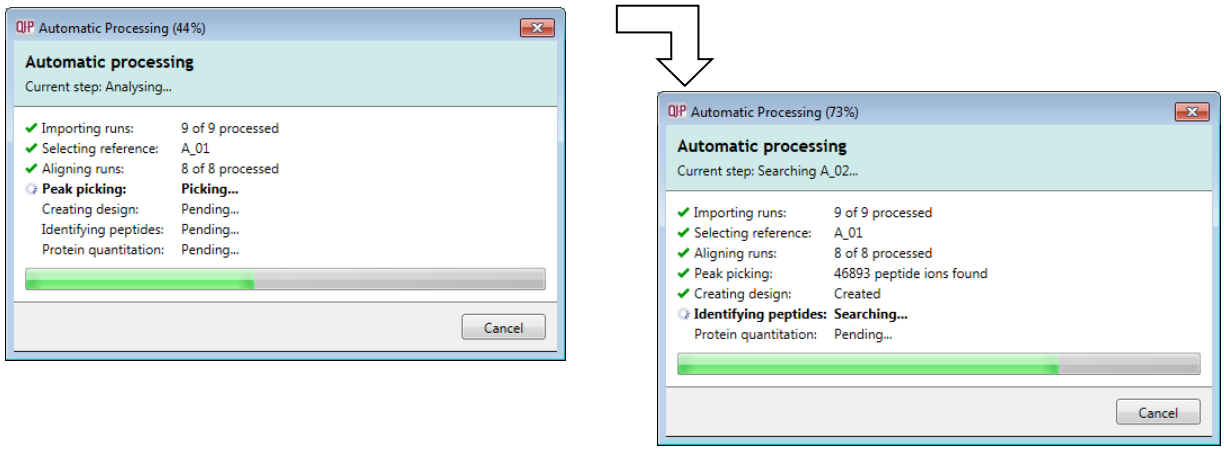
Use the drop down to reveal the alternative methods for protein quantitation

Select the Default option and click **Finish.**

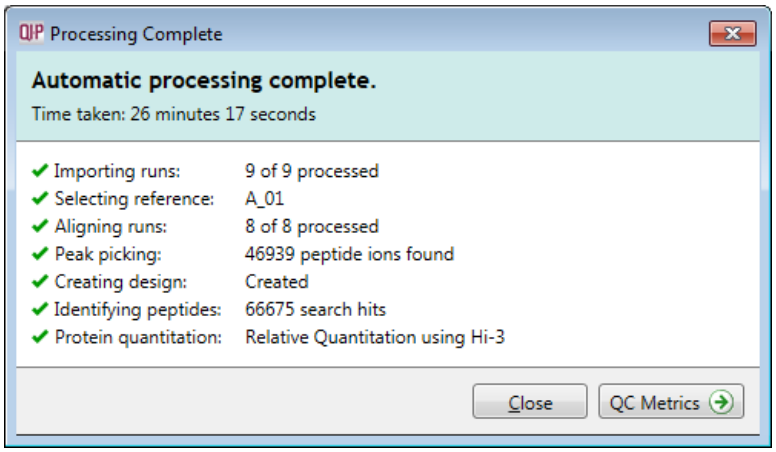
The Alignment process starts with the automatic selection of A_01 as the alignment reference



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



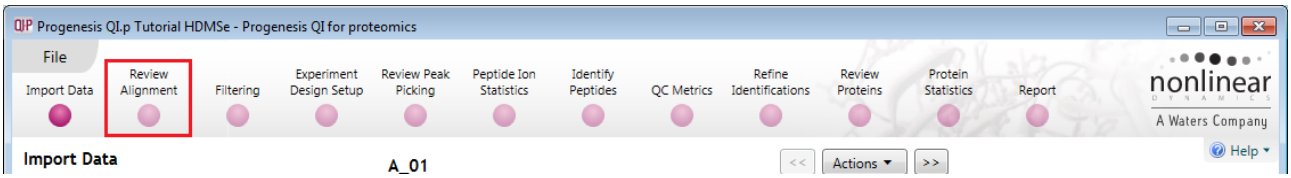
Finally the Automatic Processing completes with the Quantitation being performed



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.



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 81)

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.

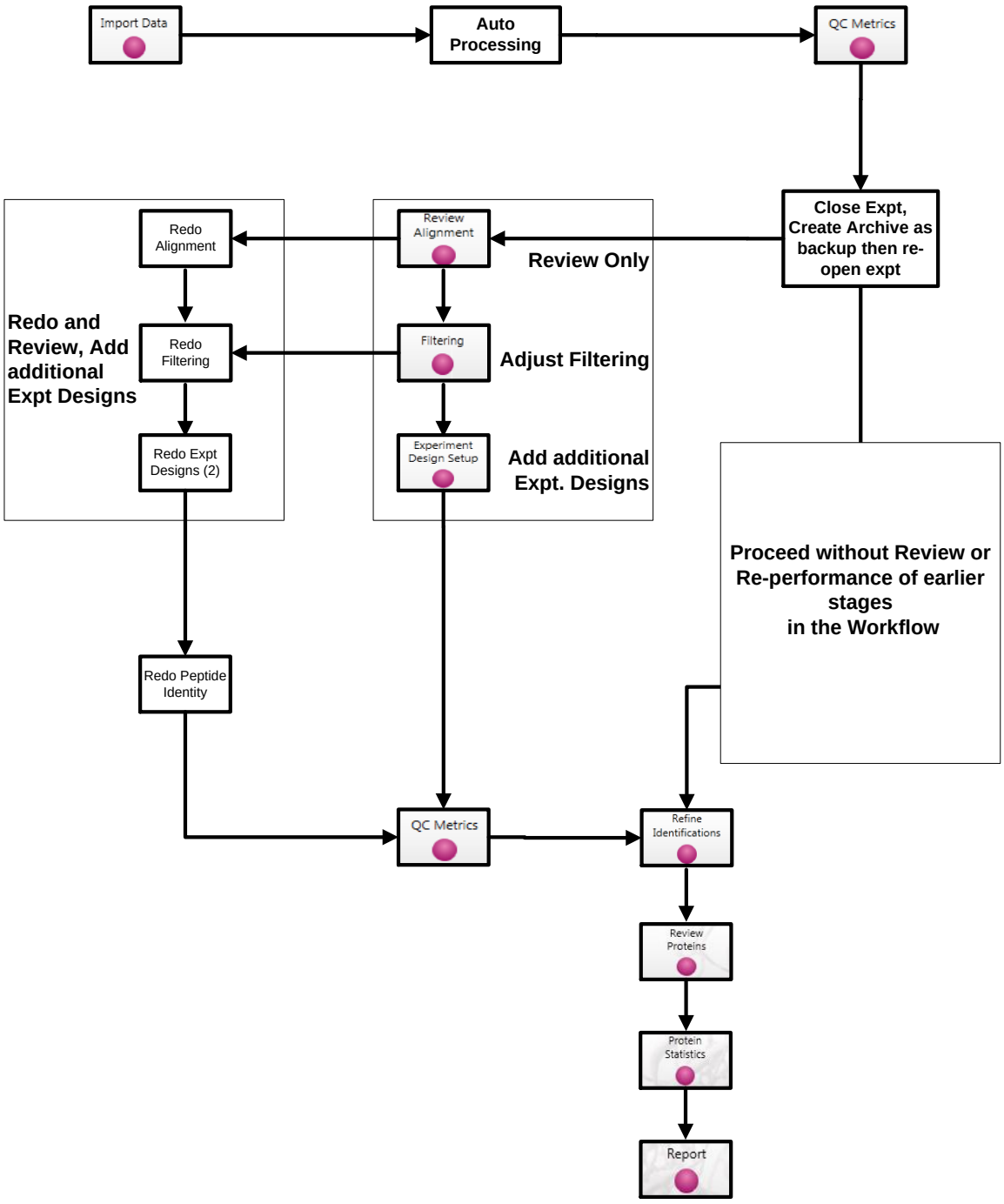
An example of the time it takes to perform each step is outlined in Appendix 10 (page 103).

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:

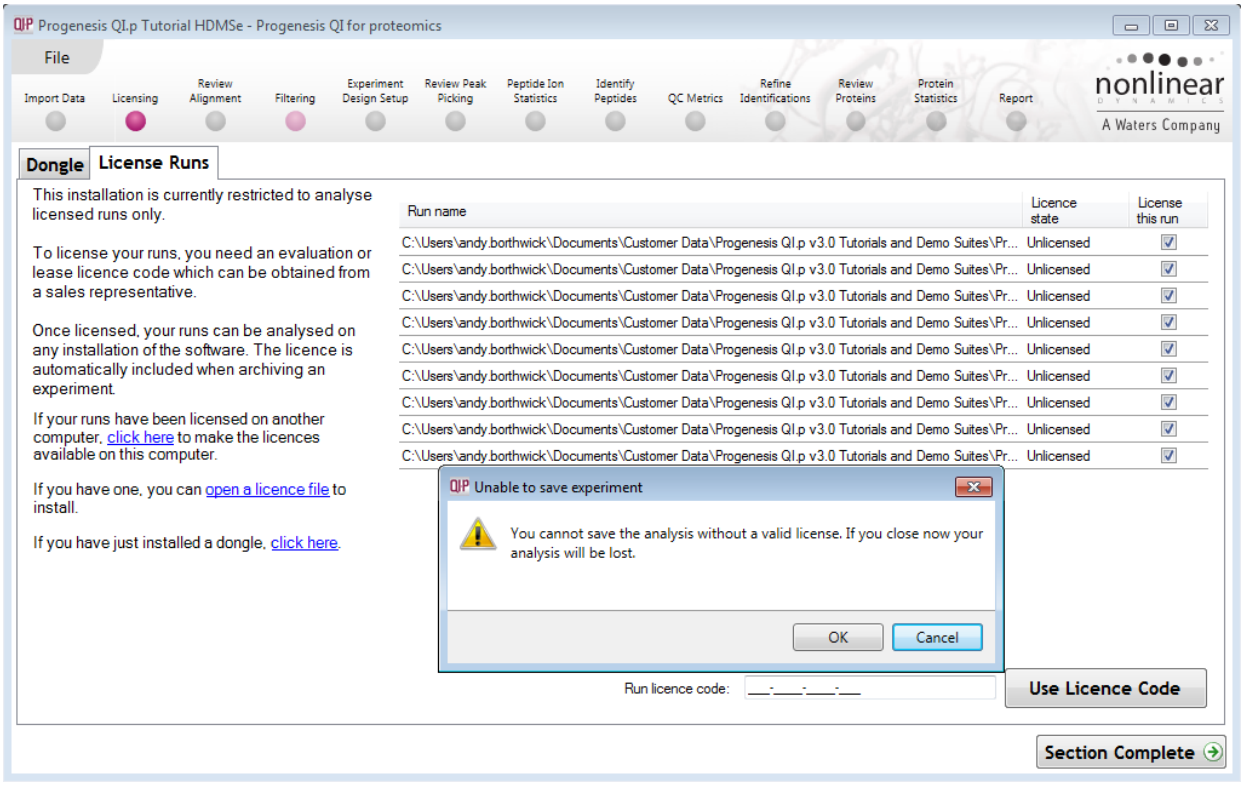
- Continue with the analysis, as the Processing dialog is not displaying any warnings, review the analysis at QC Metrics before proceeding with Refine Identifications. In which case you can go to page 52.

- 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 76) and 2 (page 81).

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.



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 83)

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

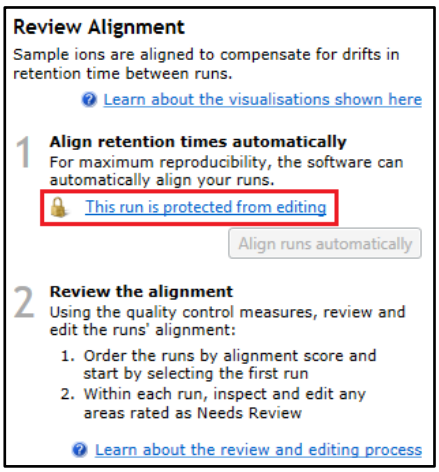
Stage 4: Review Alignment

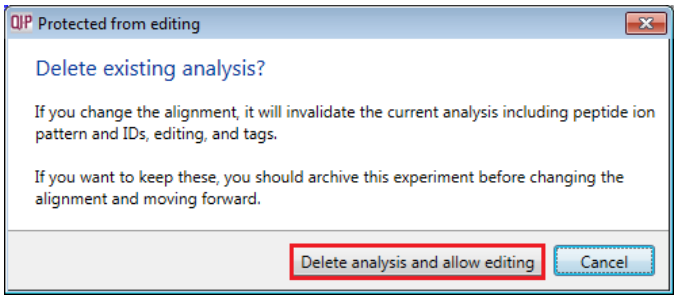
At this stage Progenesis QI for proteomics **Review Alignment** opens displaying the alignment of the runs to the Reference run (A_01).

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 4 (page 84)

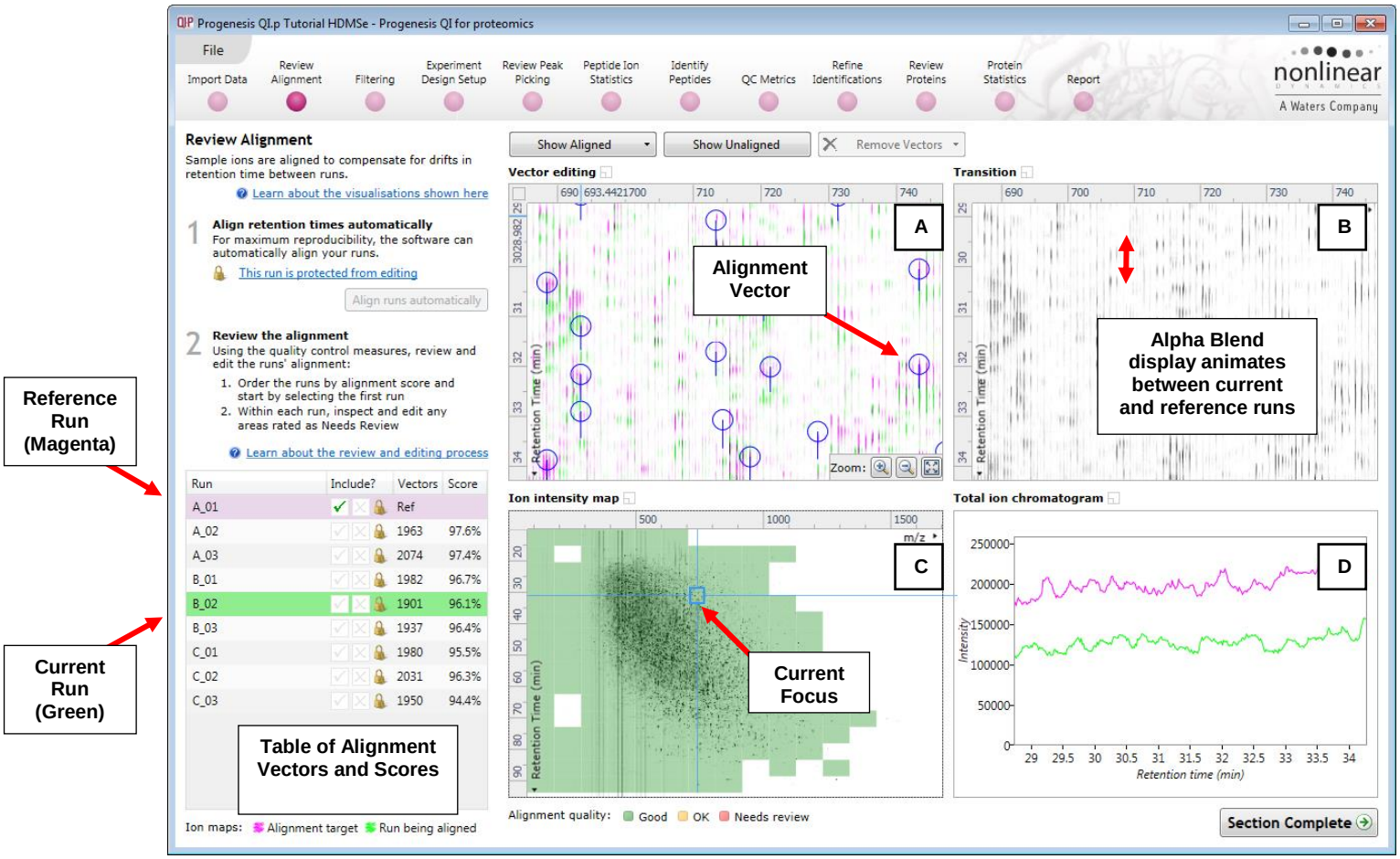
Layout of Alignment

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

Run	Include?	Vectors	Score
A_01		Ref	
A_02		1963	97.6%
A_03		2074	97.4%
B_01		1982	96.7%
B_02		1901	96.1%
B_03		1937	96.4%
C_01		1980	95.5%
C_02		2031	96.3%
C_03		1950	94.4%

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.



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.

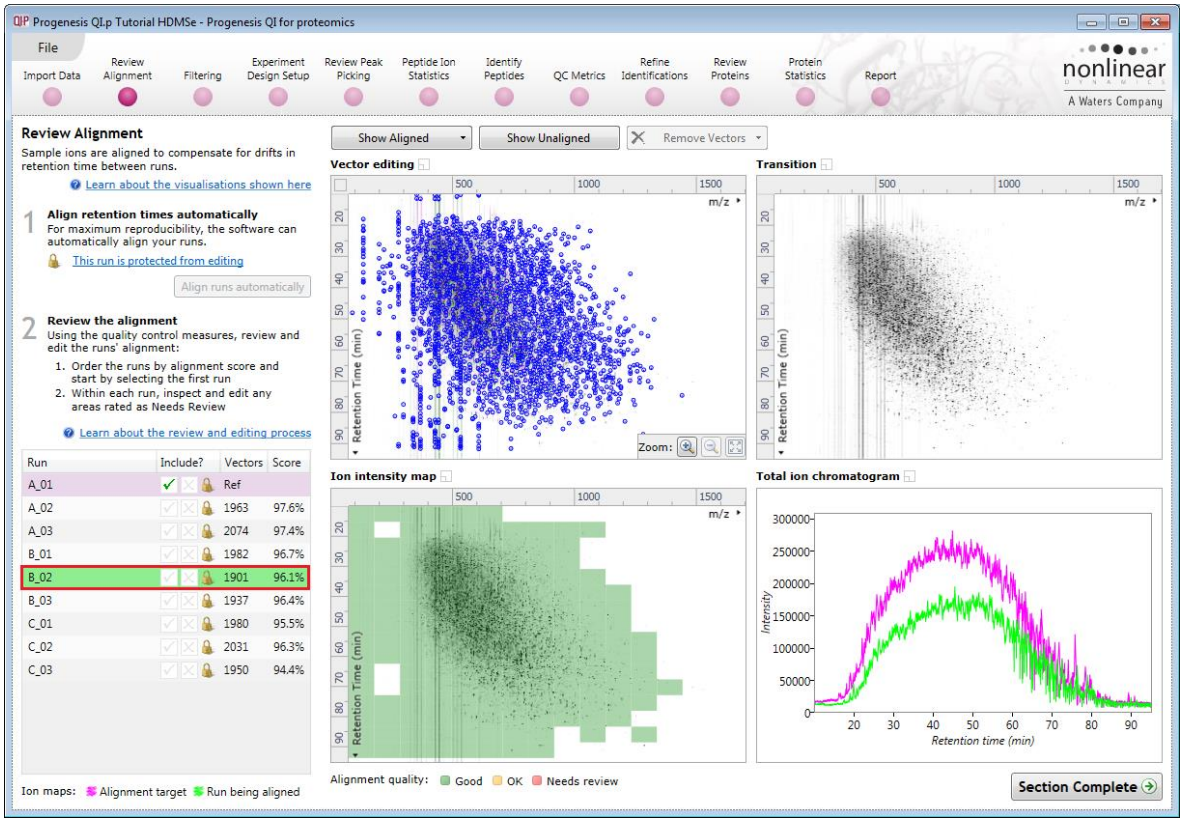
Ion Intensity Map (Window C): shows the **focus** for the other windows. When you click on the view the blue rectangle will move to the selected area. The focus can be moved systematically across the view using the cursor keys. The focus area size can be altered by clicking and dragging out a new area 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.



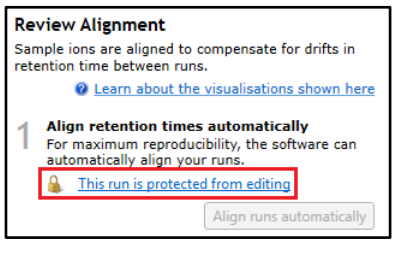
To simulate poor alignment following automatic alignment, place a single manual vector on the Vector editing view (Window A).

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

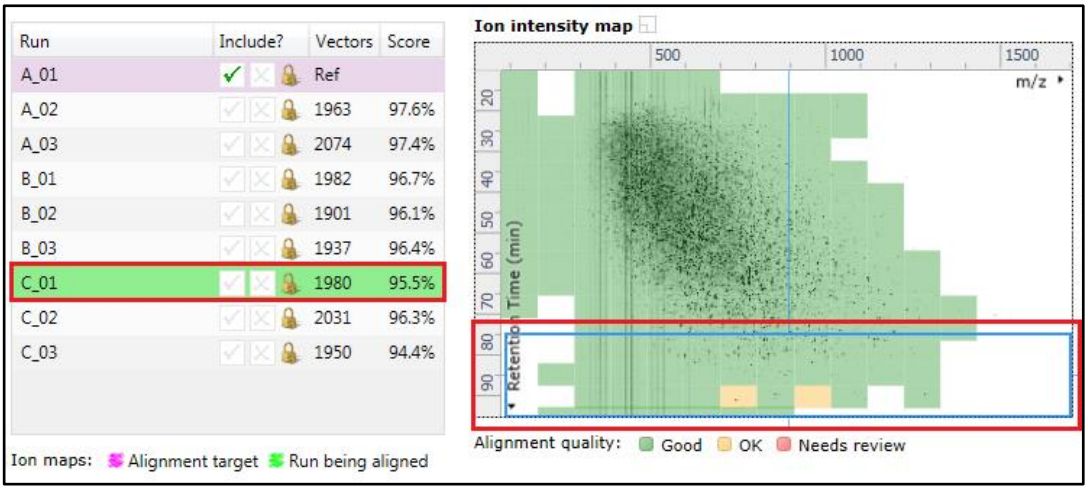
This section of the user guide is provided as a **demonstration of the alignment process,** it is **not** required for the normal analysis workflow of this data.

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

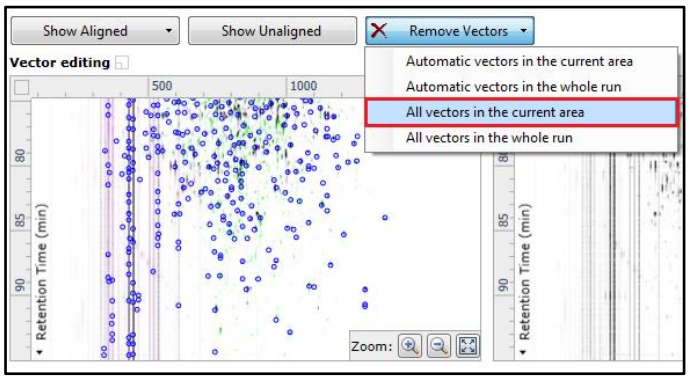
To edit the alignment of your data you must first unlock the analysis. To do this click on the link **This run is protected from editing** and then click **Delete analysis and allow editing**

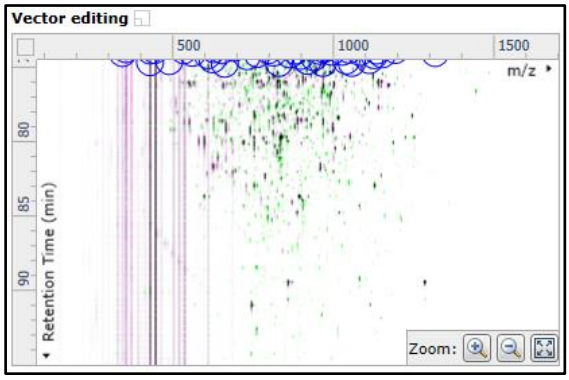


First click on run **C_01** in the Runs table to make it current. Then remove the automatic vectors from 75 min onwards. To do this you must click on the Ion intensity map and drag out an area as shown.



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.





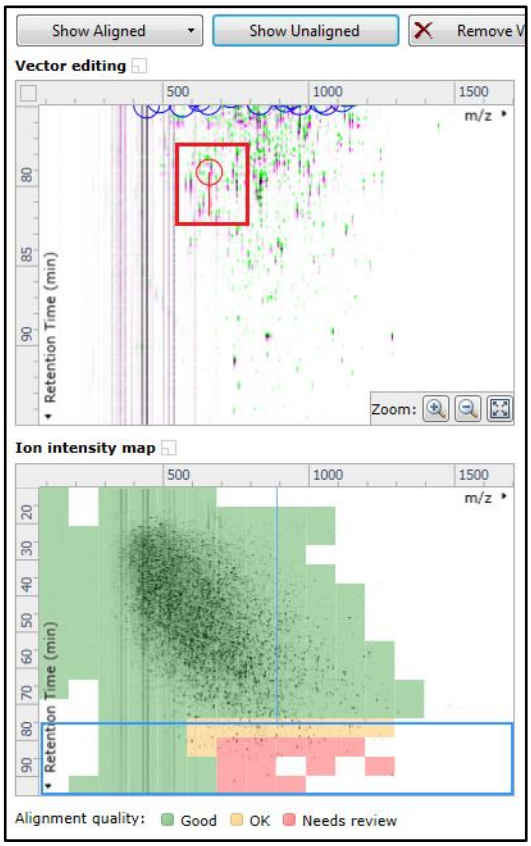
Now click on the Vector editing view and drag the view slightly upwards causing the two runs to go out of synch. Then release the mouse button.

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

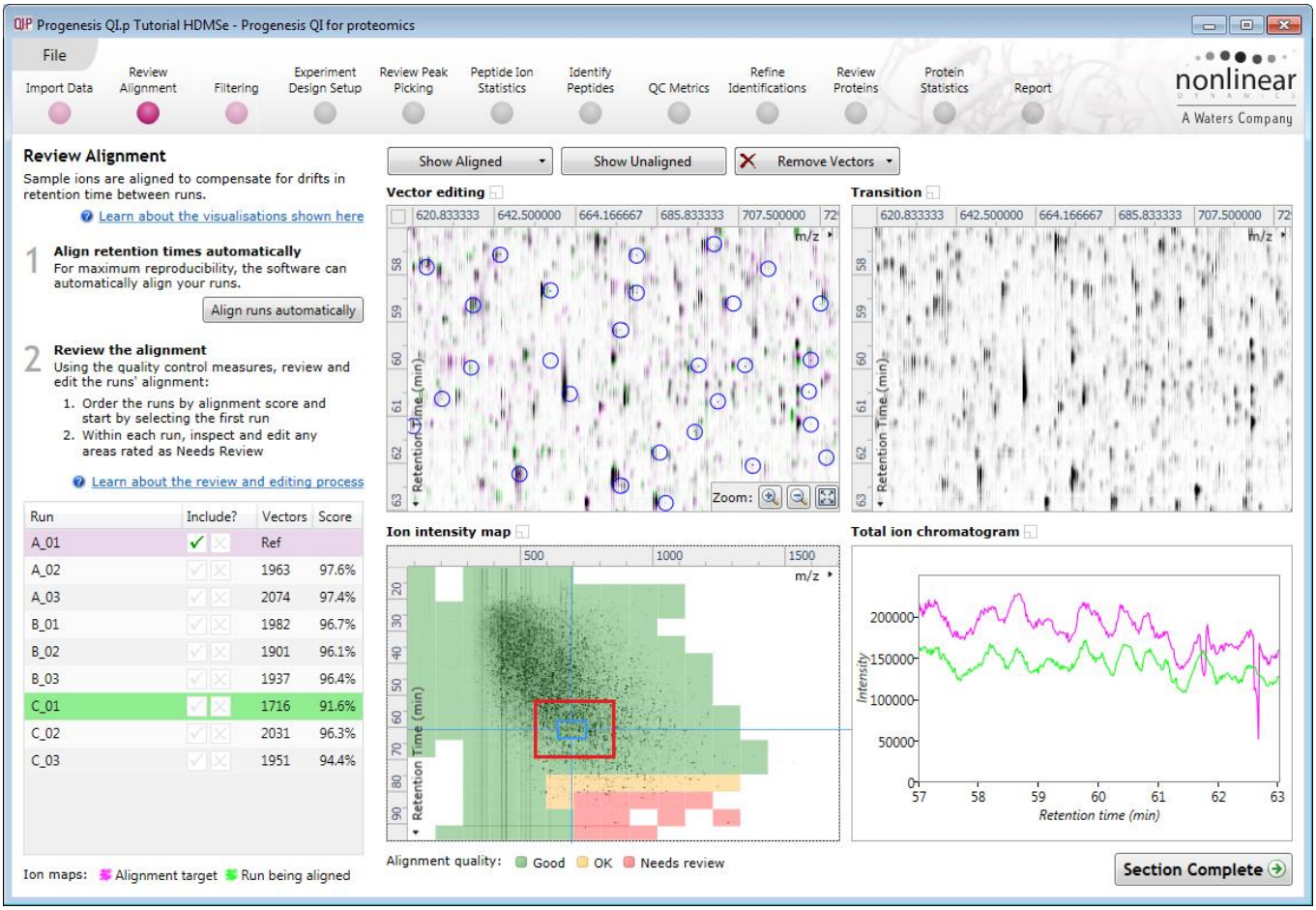
Note: the manual vector is **red**, to distinguish it from the automatic vectors (blue)

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).

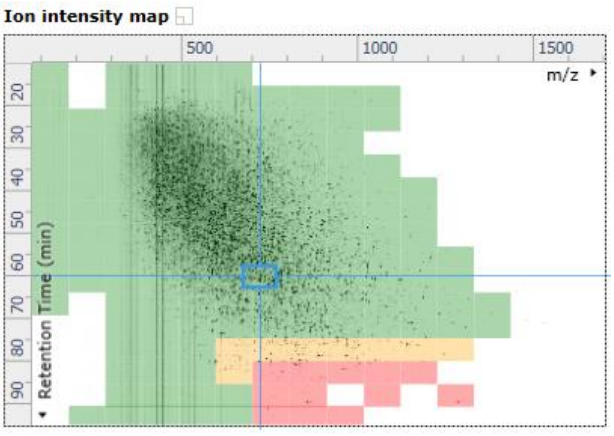


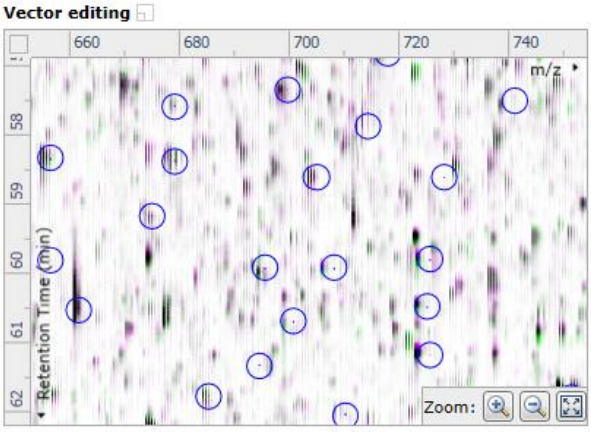
Note: now a smaller area in the 'green' well aligned region

Reviewing Quality of Alignment

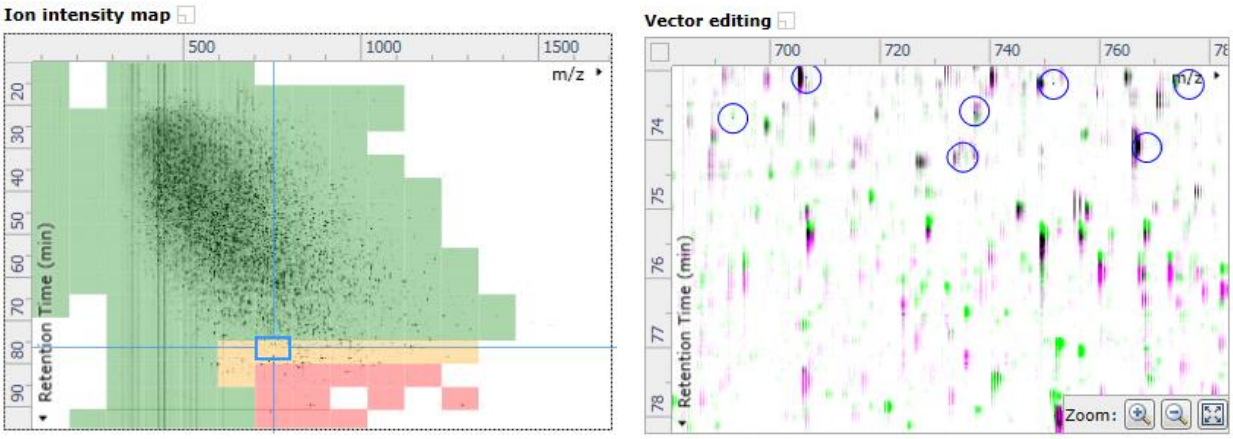
At this point the quality metric, overlaid on the Ion Intensity Map as coloured squares, acts as a guide drawing your attention to areas of the alignment. These range from Good (Green) through OK (Yellow) to Needs review (Red). Drag out a 'Focus' area that corresponds to one of the coloured squares.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

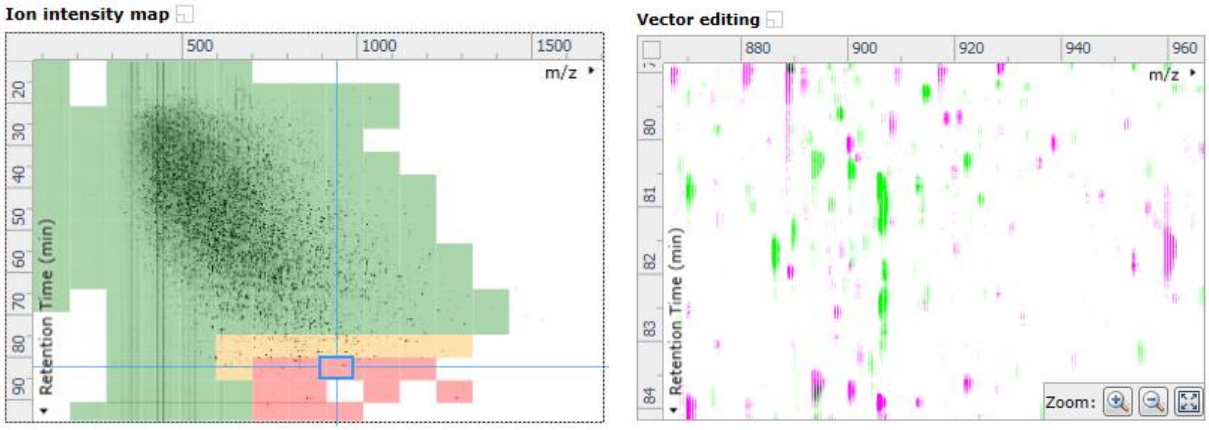




For a ‘yellow’ square some of the data appears overlapped (black) indicating OK alignment. When viewed in the Transition view some of the data appears to pulse.

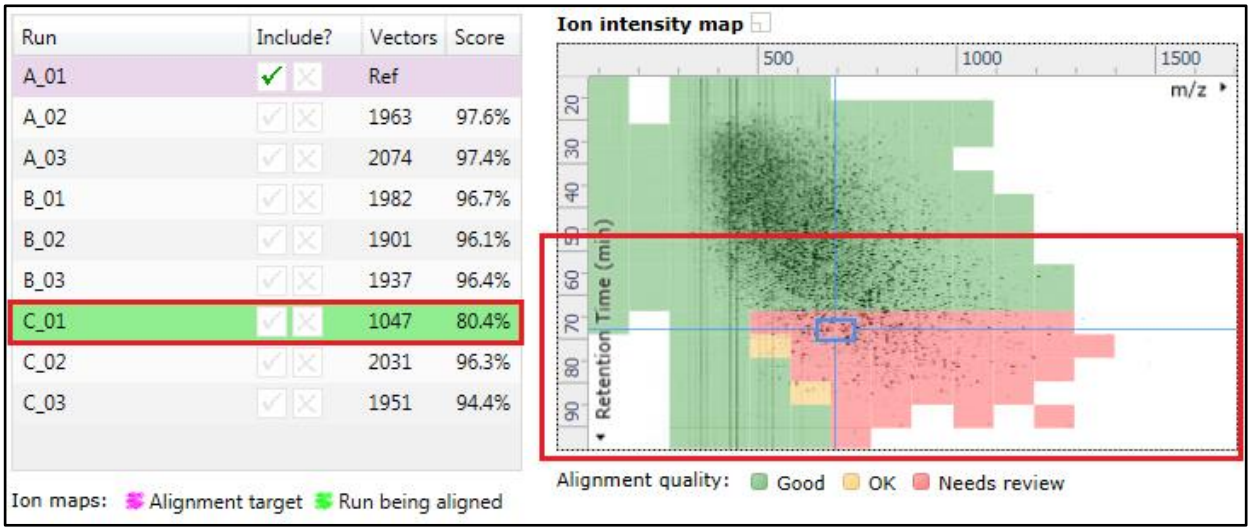


For a ‘red’ square little if any of the data appears overlapped (black) indicating questionable alignment. When viewed in the Transition view little data appears to pulse.



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 84).

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.

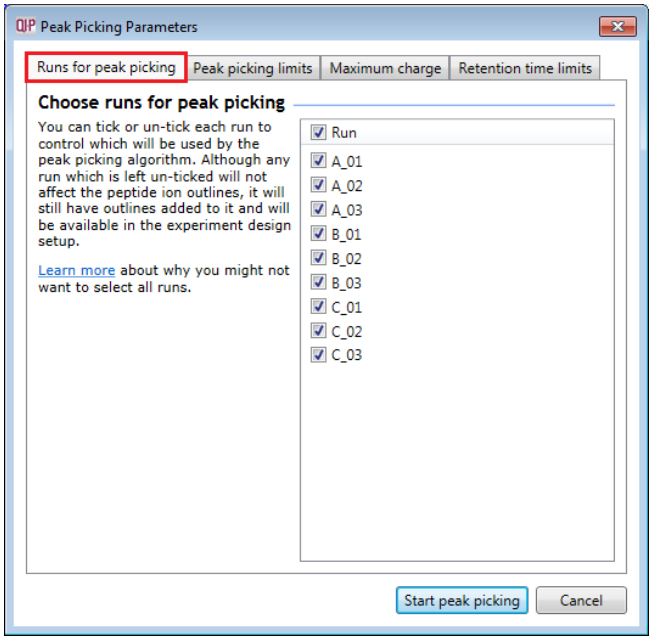
Having aligned the runs automatically, click **Section Complete** to move to Filtering.

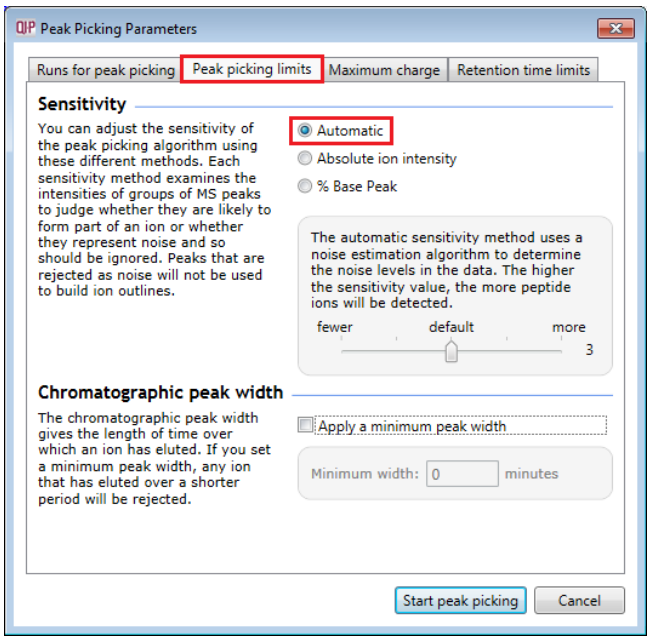
Stage 5A: Filtering

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.





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.

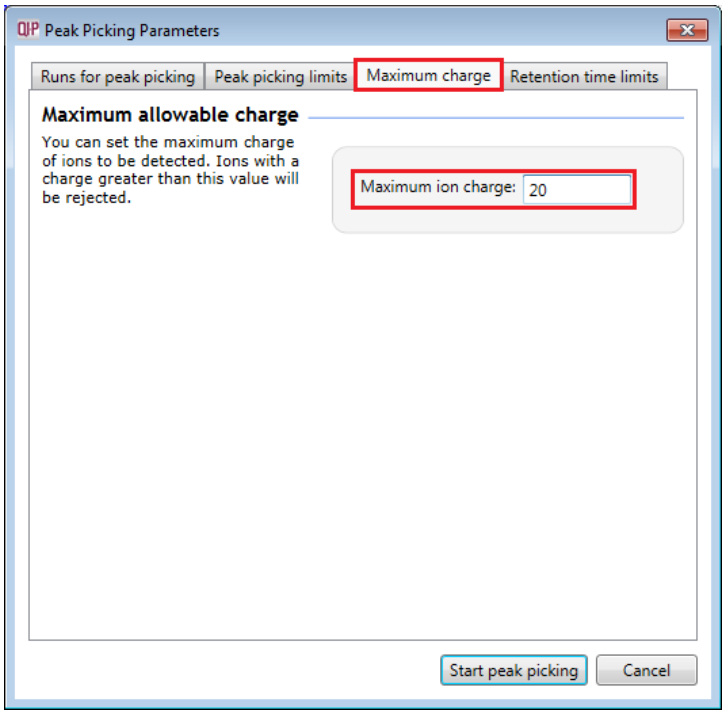
Tip: depending on run quality, a suggested minimum number of ticked runs should include at least one replicate of each experimental condition.

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

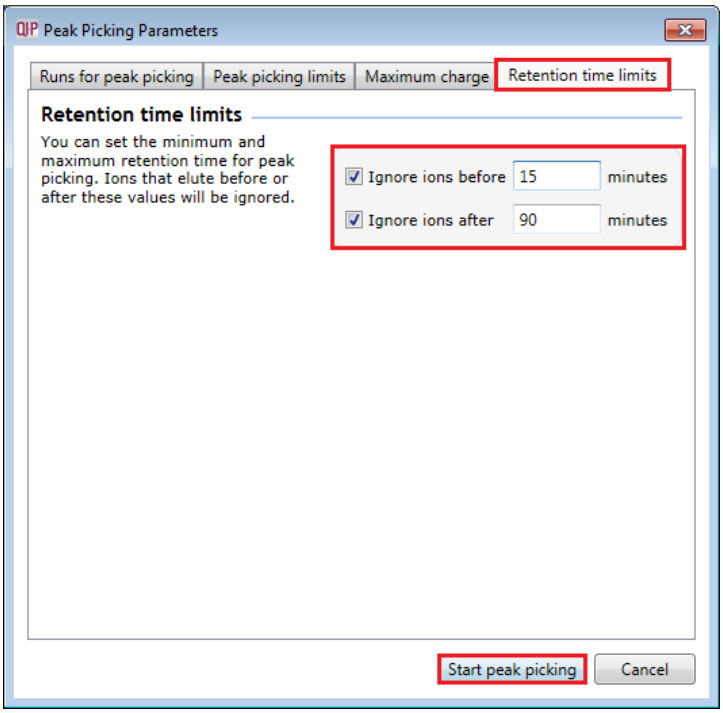
Note: for all 3 **Sensitivity** modes a Chromatographic peak width (Retention time window) for the peaks **can** be set by applying a minimum retention window or peak width in minutes.

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.



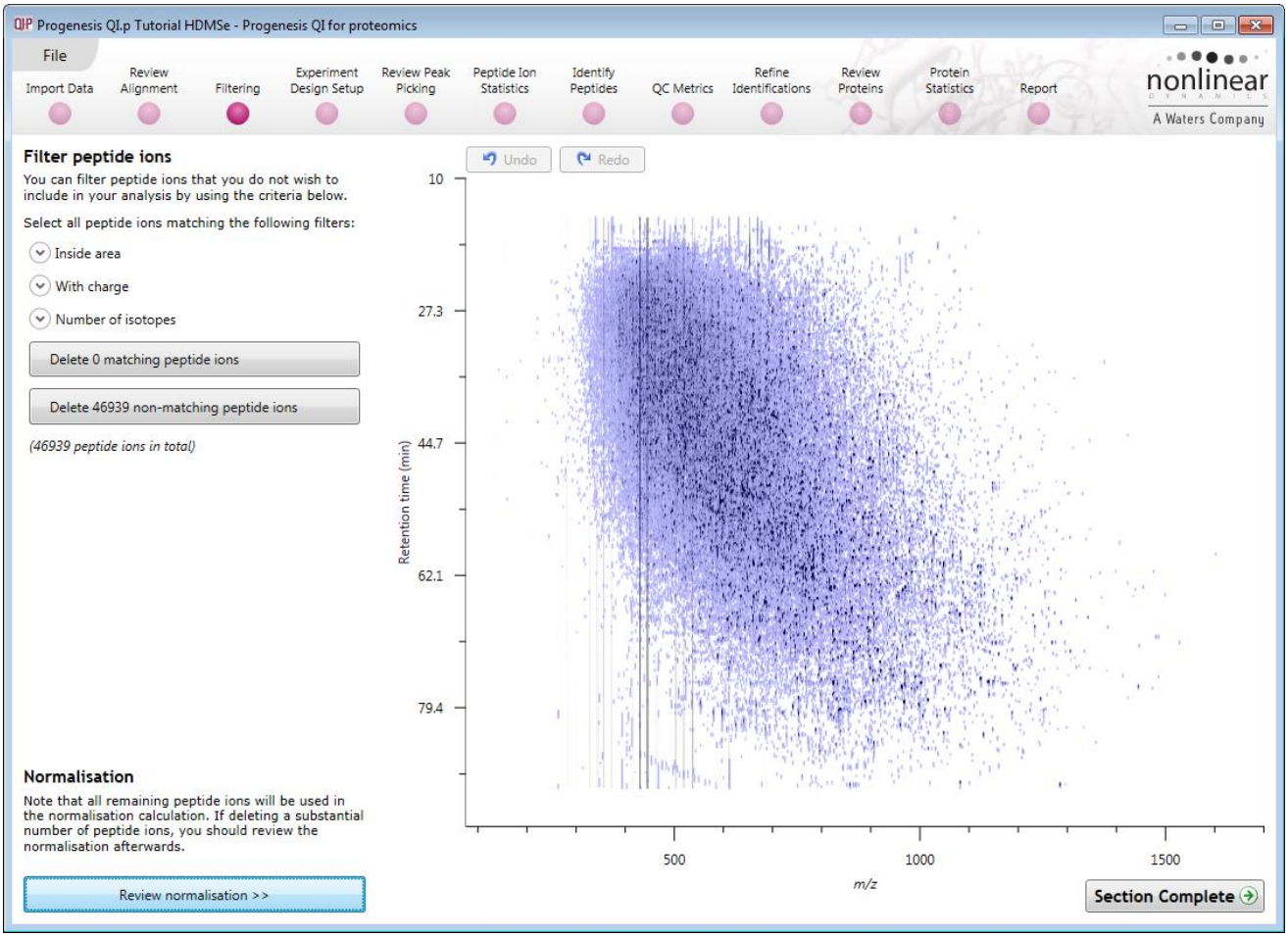
Finally, you can set **Retention time limits** for the detection. Current limits are displayed in the unticked boxes and detection will use these values whenever the box(s) are unticked.



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.

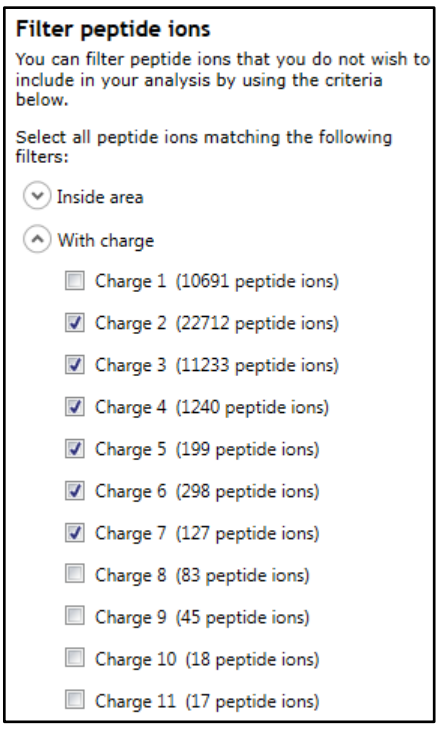
On completion of analysis, the Filtering stage will open displaying the number of peptide ions detected, in this example 46939.



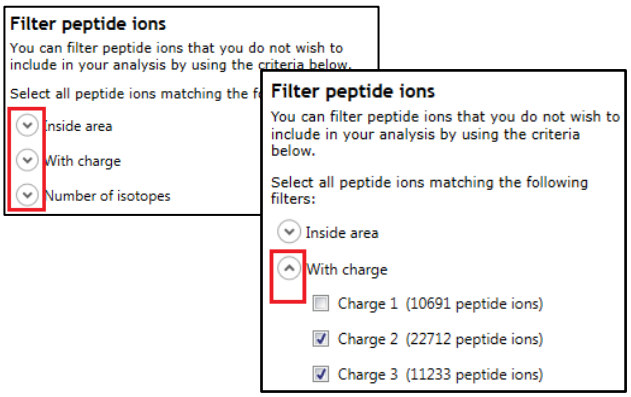
If required you can remove peptide ions based on position, charge state, number of isotopes or combinations of these peptide ion properties.

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.

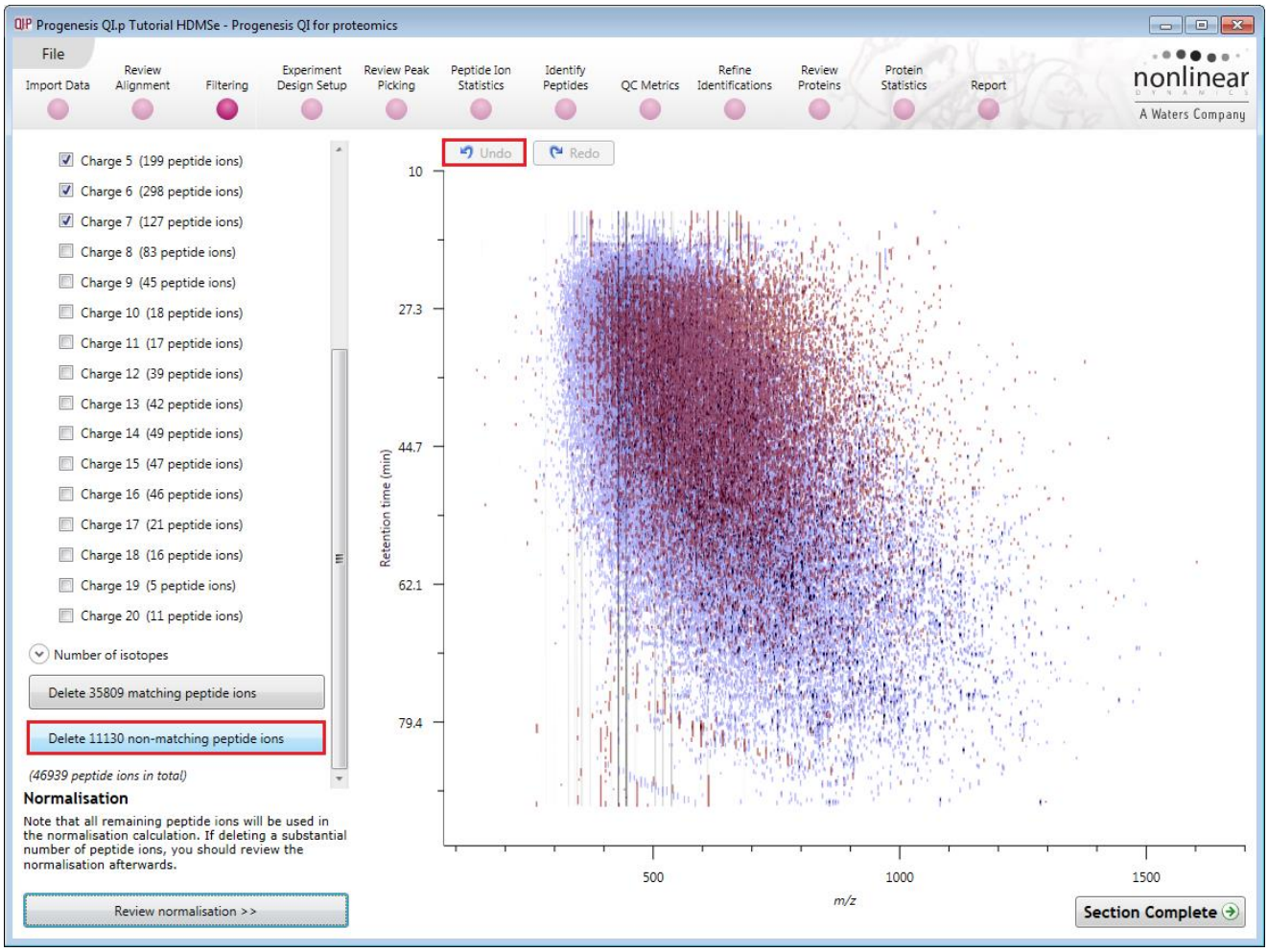


Tip: when filtering on one property of the peptide ions i.e. with charge, make sure you have 'collapsed' the other filters as expanded filters are applied concurrently.



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 11130 Non Matching Peptide ions.**



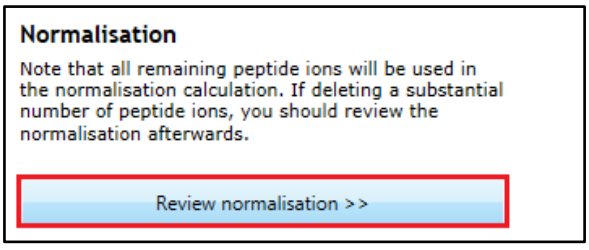
You can use the **Undo** button to bring back deleted peptide ions. For this tutorial we will proceed with the 35809 matching peptide ions.

Before moving on from filtering, you can review the normalisation of the experiment.

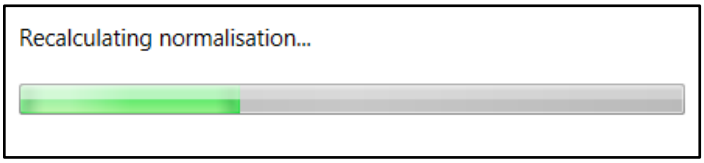
Tip: When you have reached the filtering stage, it is good practice to close the experiment and save an archive. This can be used to restore the unfiltered state if the filtering you have performed is too 'stringent'.

Stage 5B: Reviewing Normalisation

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

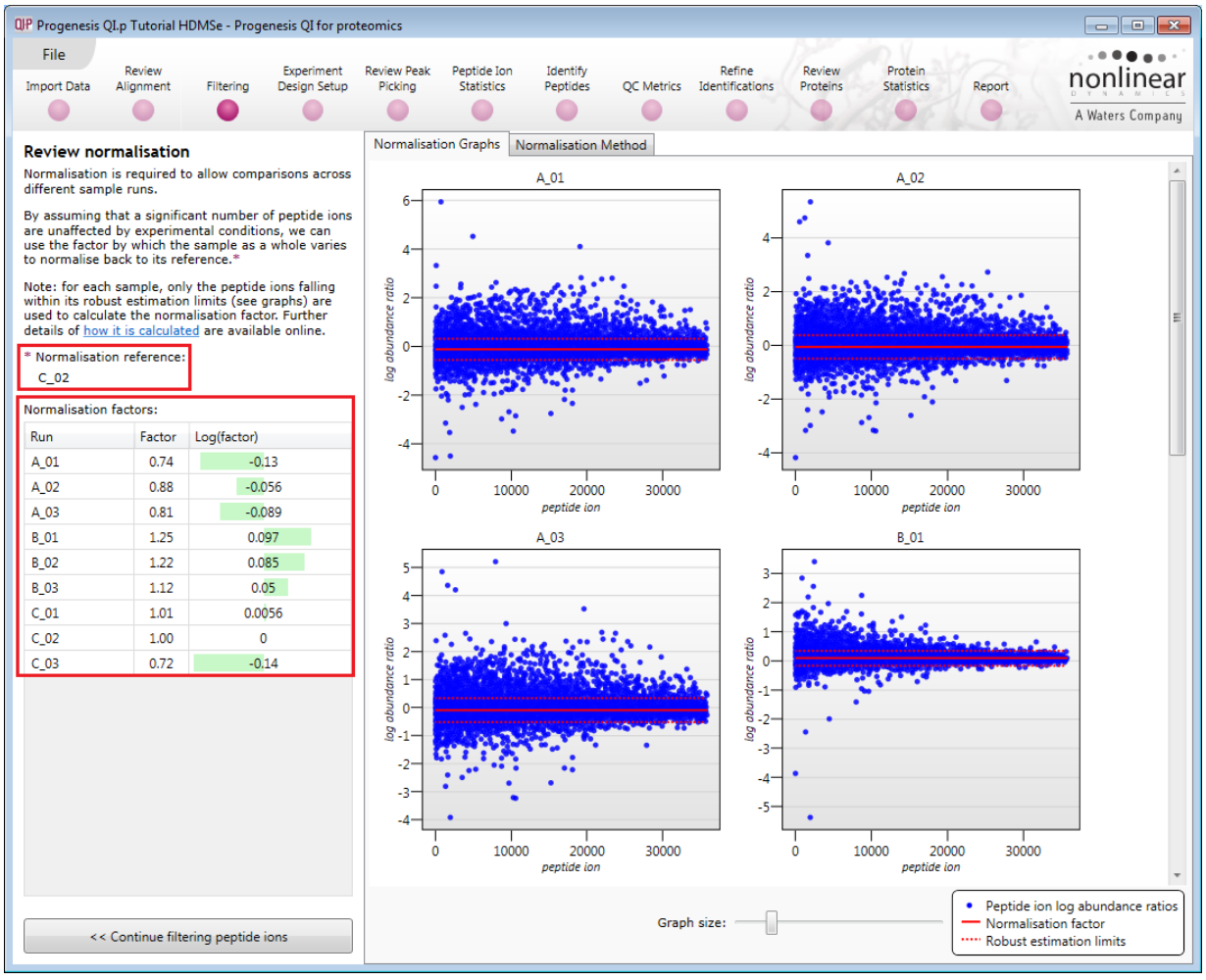


If you have filtered out a number of peptide ions from the original detection pattern then the normalisation will update.



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.



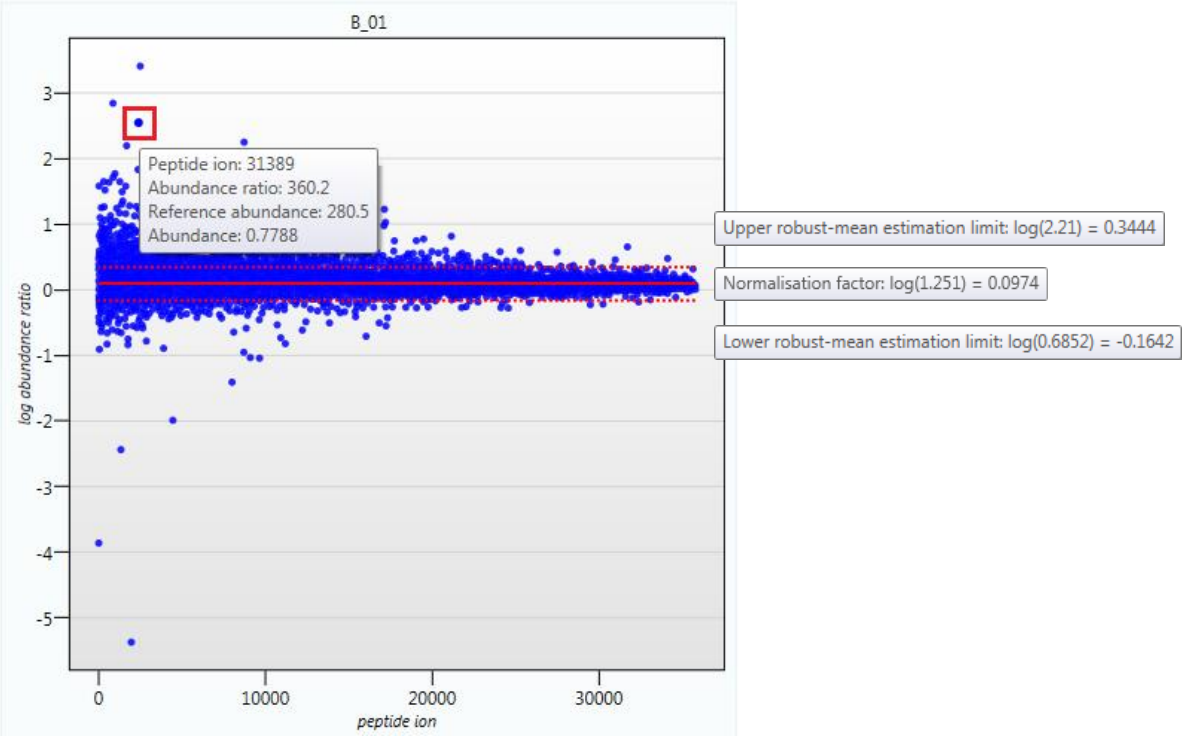
Normalisation factors are reported in the table to the left of the plots.

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).

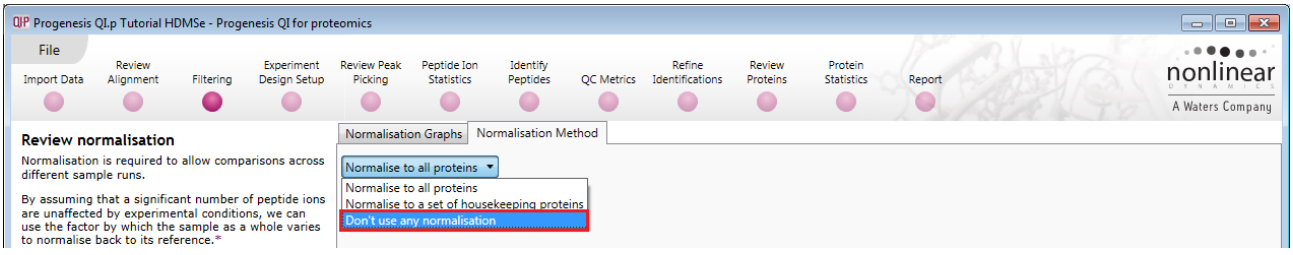
For each sample run, each blue dot shows the log of the abundance ratio for a different peptide ion (normalisation target abundance/run abundance).

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.

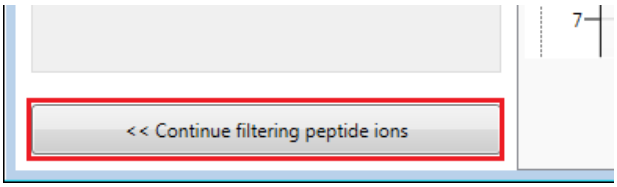
Finally, if you do **not** wish to work with normalised data then you can **use the raw abundances** by switching off the normalisation.



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



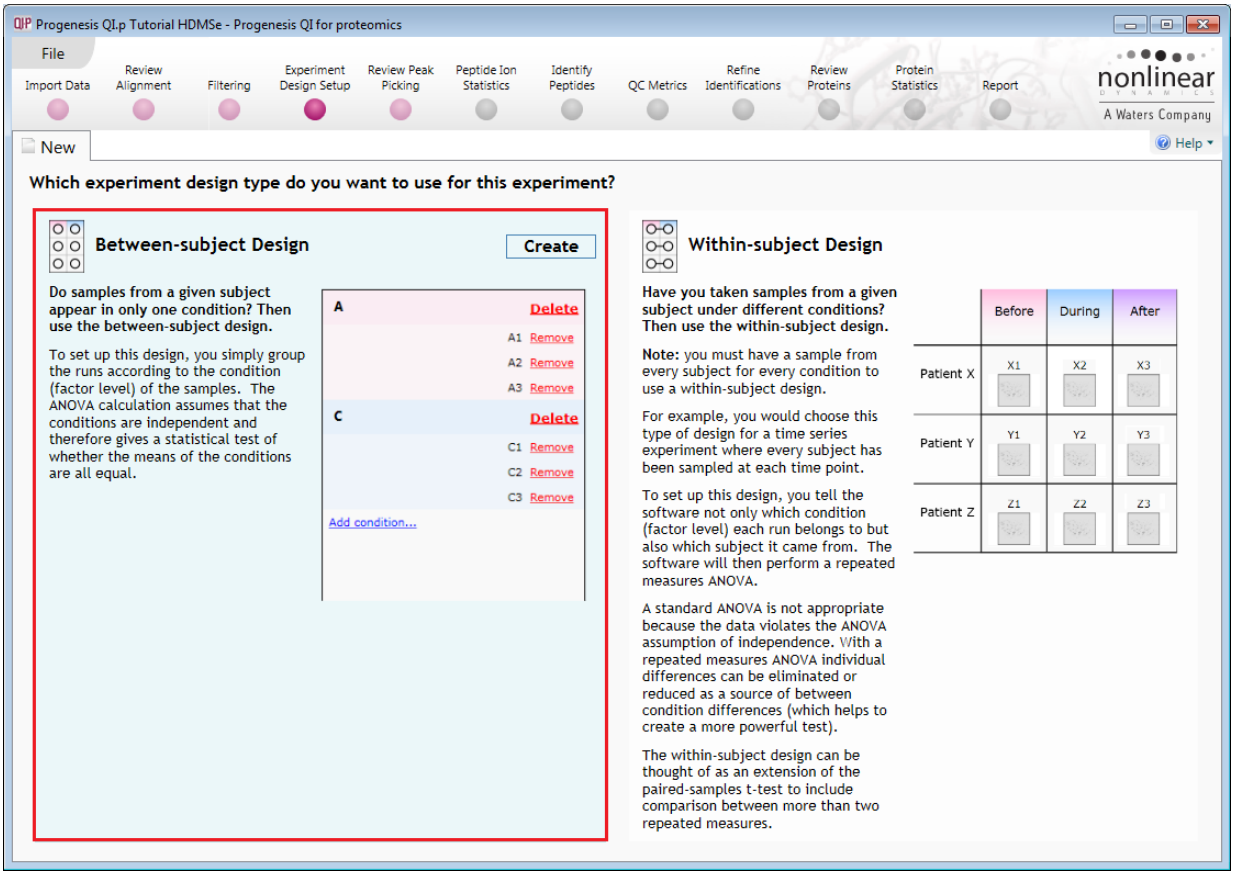
For this example, we **DO NOT** do any additional Filtering so click on **Section complete**.

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.

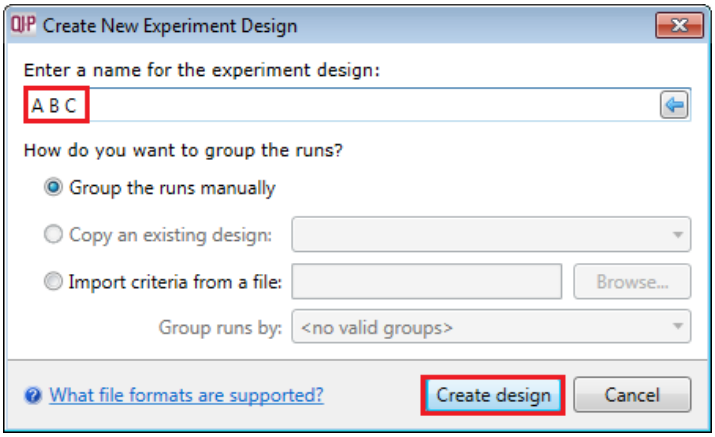


Within-subject design: here samples have been taken from a given subject under different conditions (i.e. the same subject has been sampled over a period of time or after one or more treatments). Here a standard ANOVA is not appropriate as the data violates the ANOVA assumption of independance. Therefore 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.

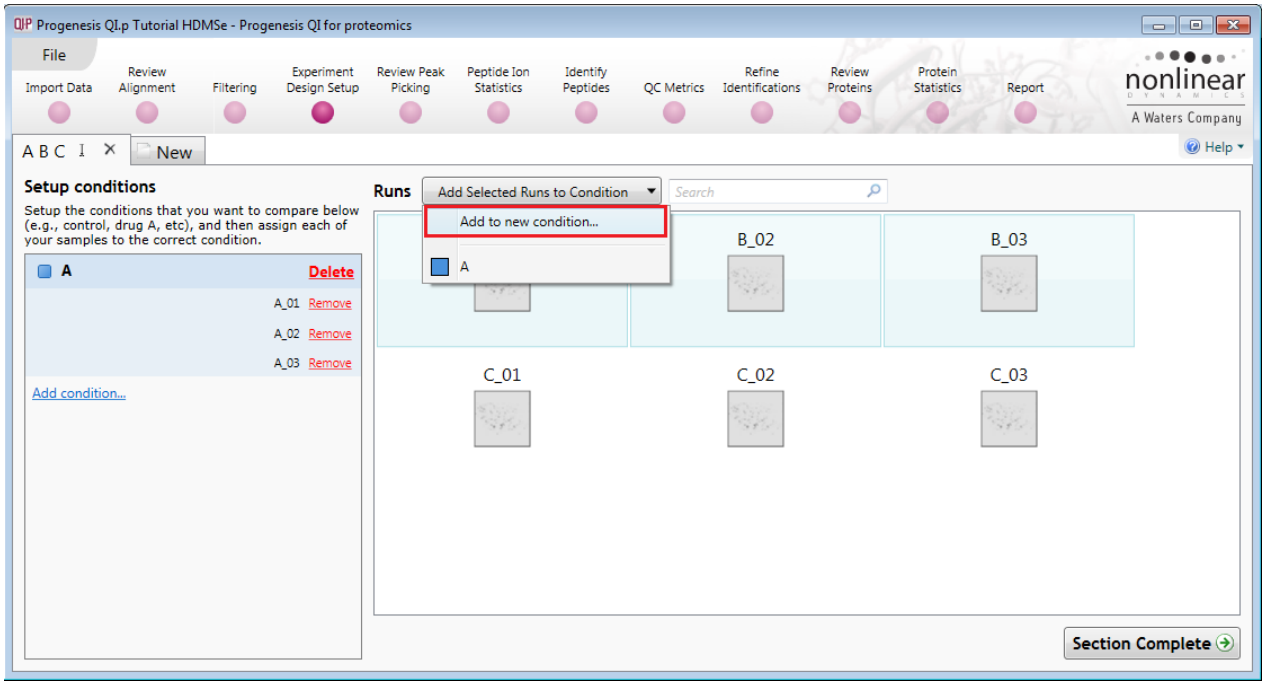
Additional information on how to apply the Within-subject Design is in Appendix 5 page 89

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.



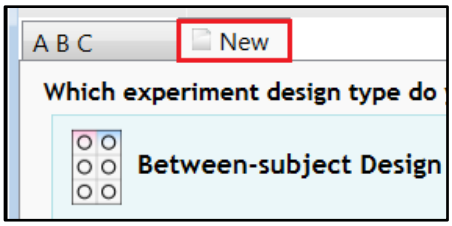
Give the new experiment design a name and then click **Create design**.



To create a new condition

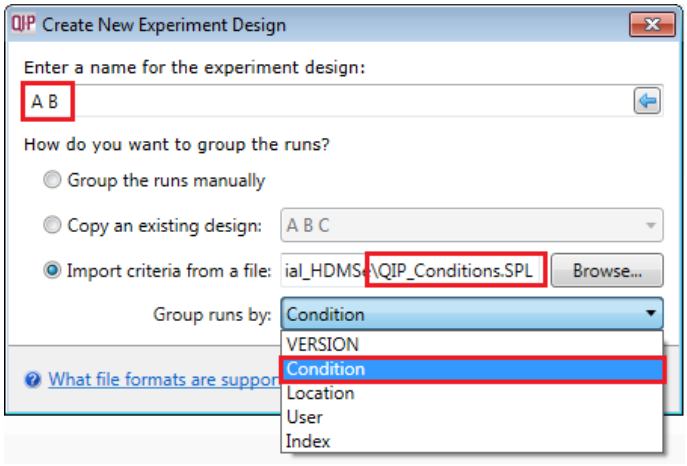
1. Select the runs for the condition by clicking on the required icon in the **Runs** panel, as shown.
2. Press the ‘black triangle’ next to the **Add Selected Runs to Condition** button on the main toolbar.
3. Select **Add to new condition**… from the drop down menu.
4. A new condition will appear in 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.



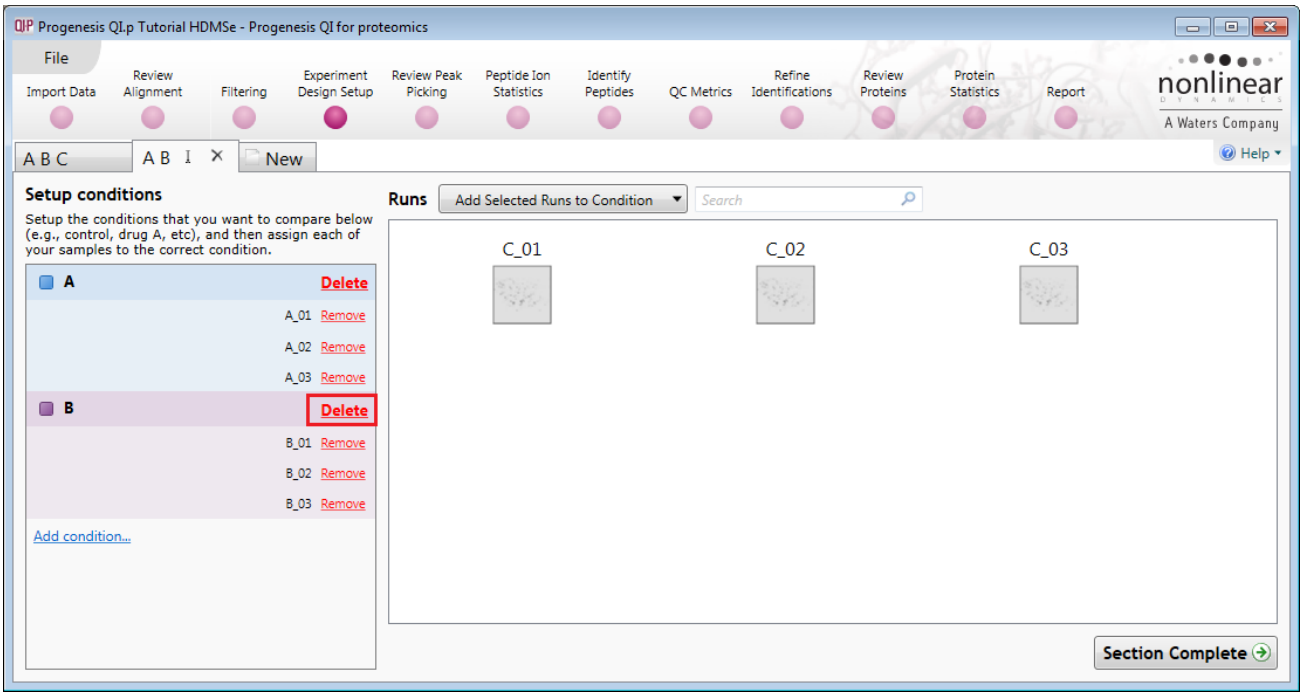
For this example there is a **QIP_Conditions.spl** file available in the Experiment Archive you restored at the beginning of this tutorial exercise.

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



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

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.

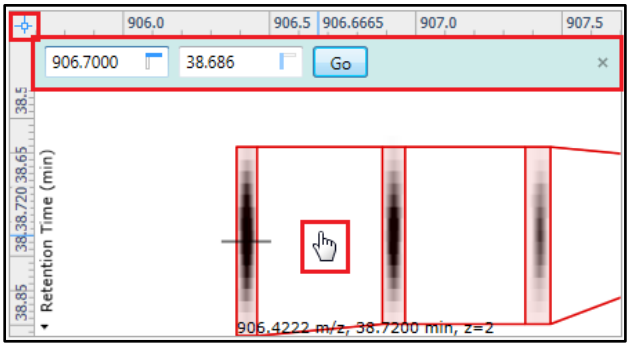
To move to the next stage in the workflow, **Review Peak Picking**, click **Section Complete.**

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.

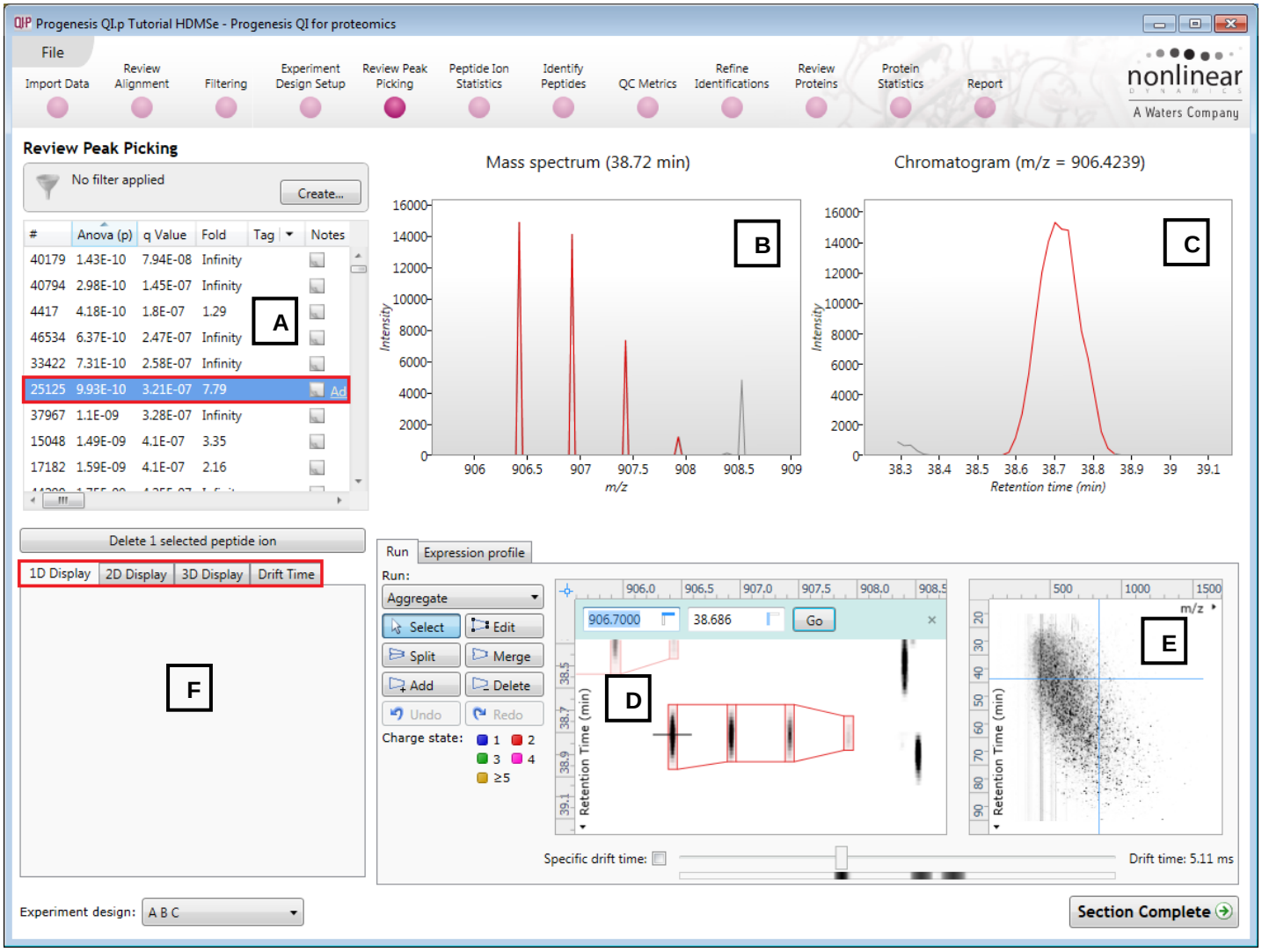
Exploring analysed data using the Data displays



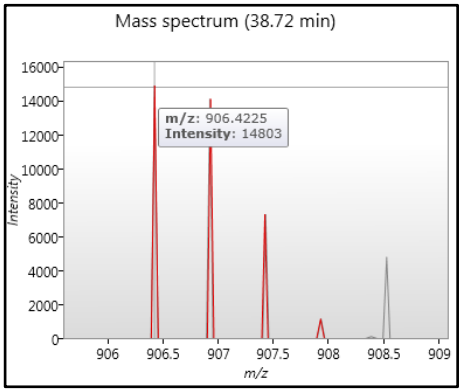
To set up the display as shown below, click on the Go To Location on the top right of the zoomed run view (right). Enter 906.700 m/z and 38.686 min and click **Go**. Then click on/or inside the peptide ion boundary to re-focus the view and table.

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).

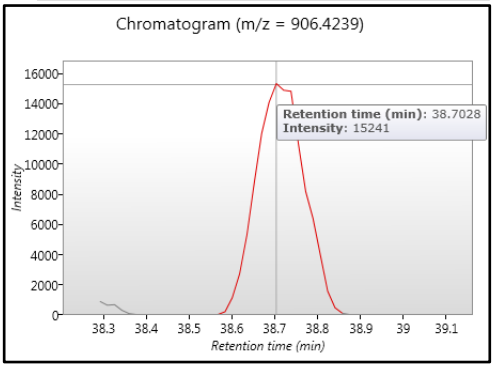
Note: a value of 'Infinity' in the **Fold** column indicates 'Presence/Absence'



The 1D Display

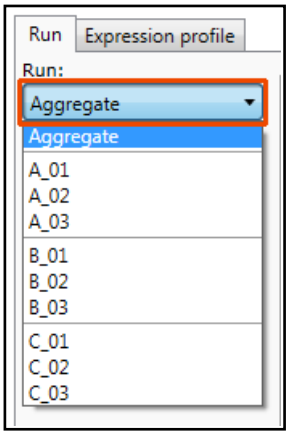


Window B: displays the Mass spectrum for the current peptide ion on the selected Run (in window D). Hold the cursor over the peak to indicate m/z and intensity



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.

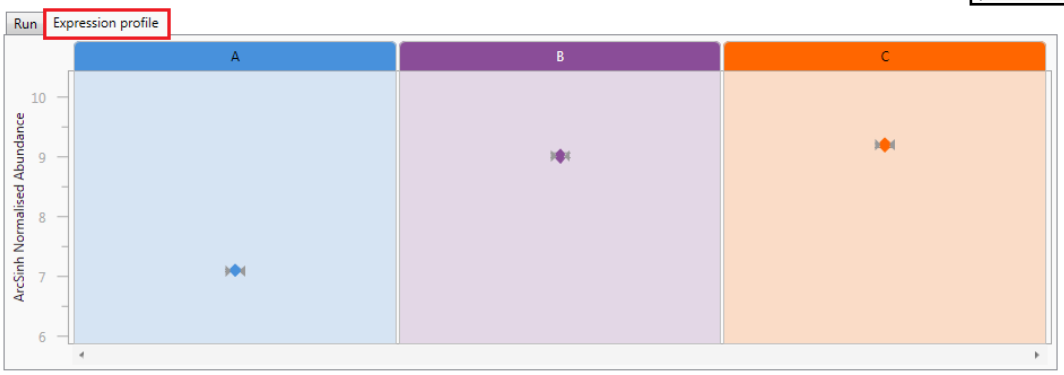
Window D: displays the details of the currently selected run. By default the selected run is an Aggregate view of all the aligned runs.

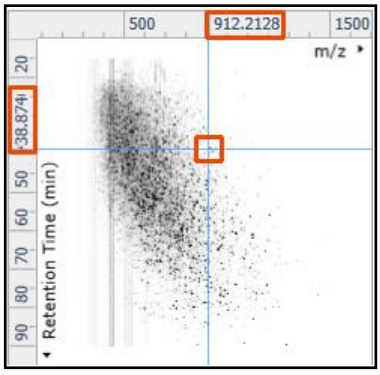


Details of individual runs can be viewed by using the 'Run' link and selecting the run you wish to view.

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.



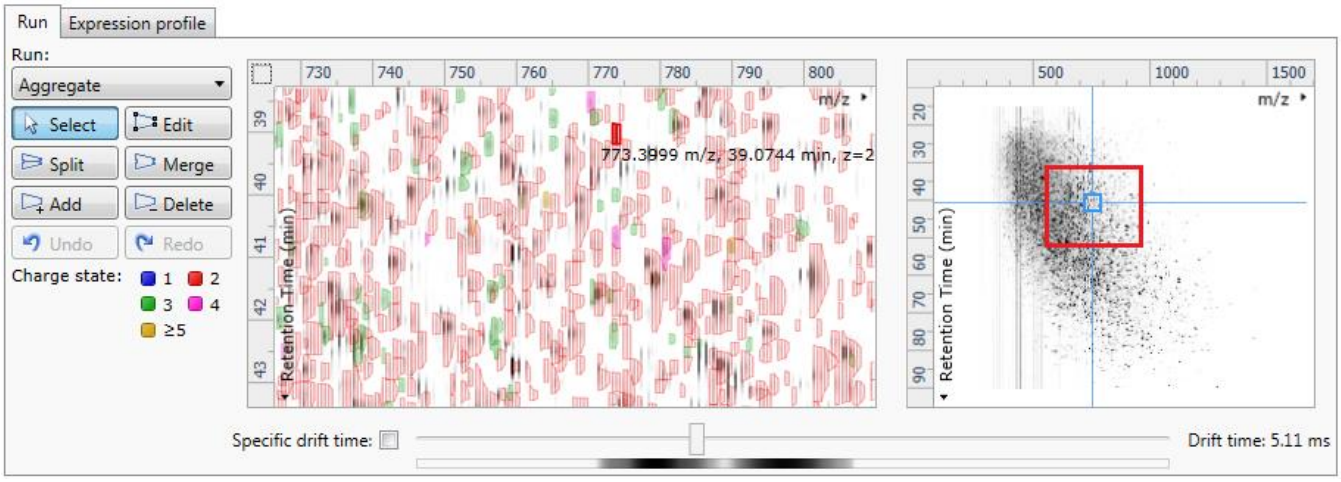


Window E: shows where the current peptide ion is located on the LC-MS run by means of the cross hairs.

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.

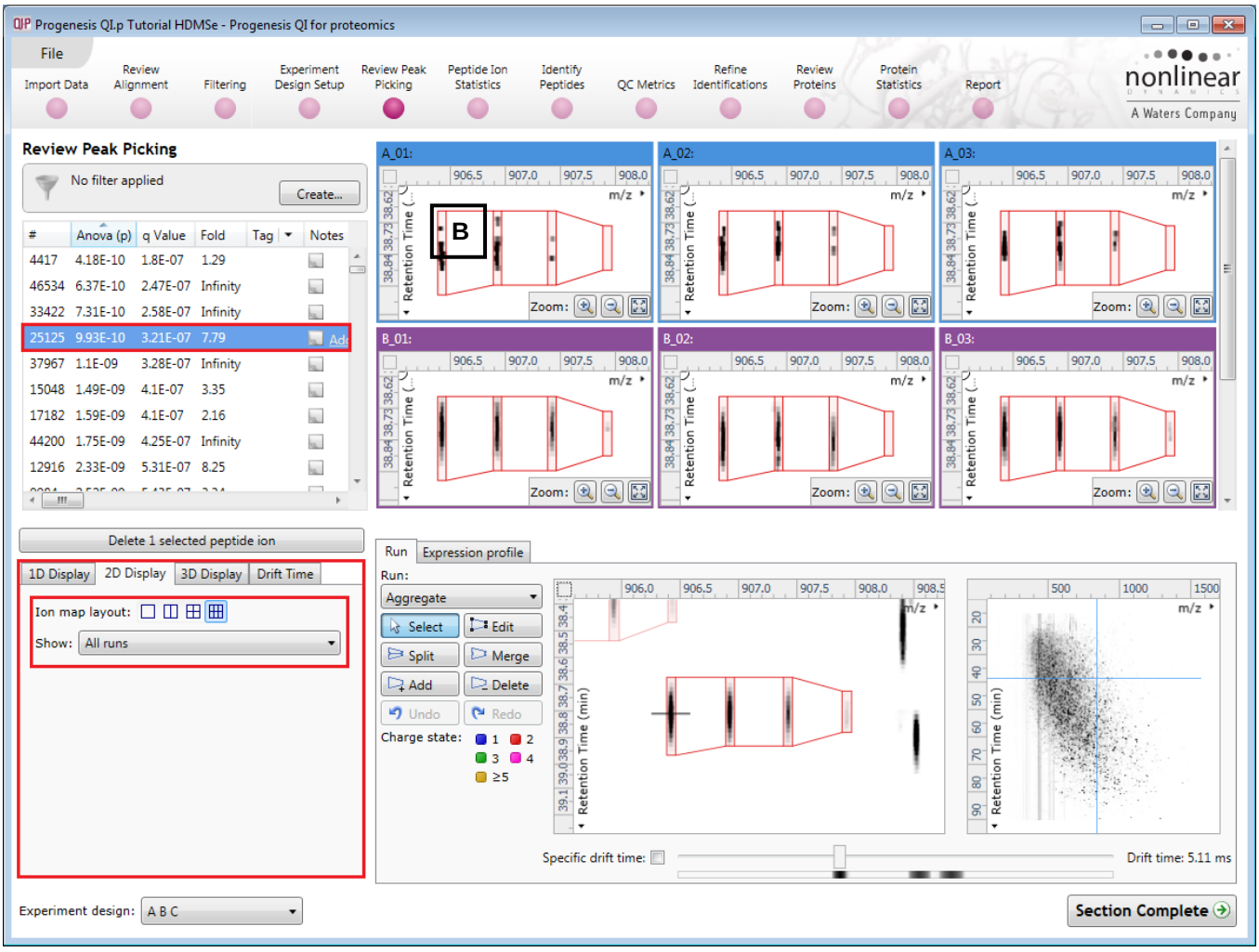
You can also drag out an area (blue square) on this view that will re-focus the other windows.



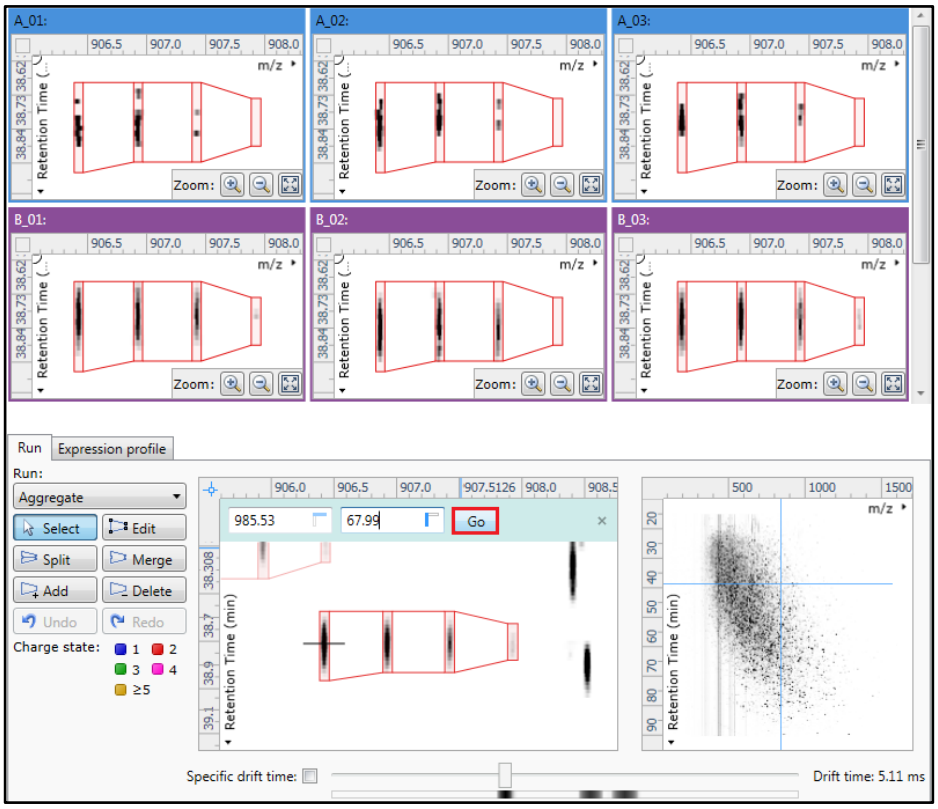
The 2D Display

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.



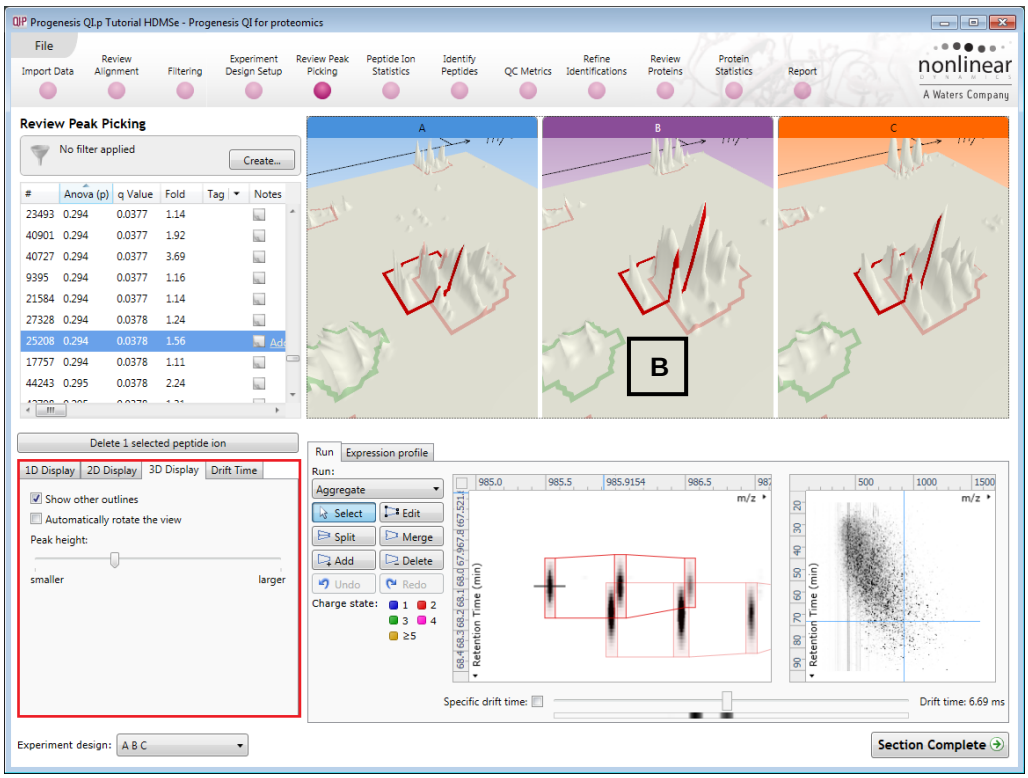
The appearance of the Montage (window B) is controlled by the panel on the bottom left of the display.



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 below. (i.e Go To Location 985.53m/z and 67.99min)

The 3D Display

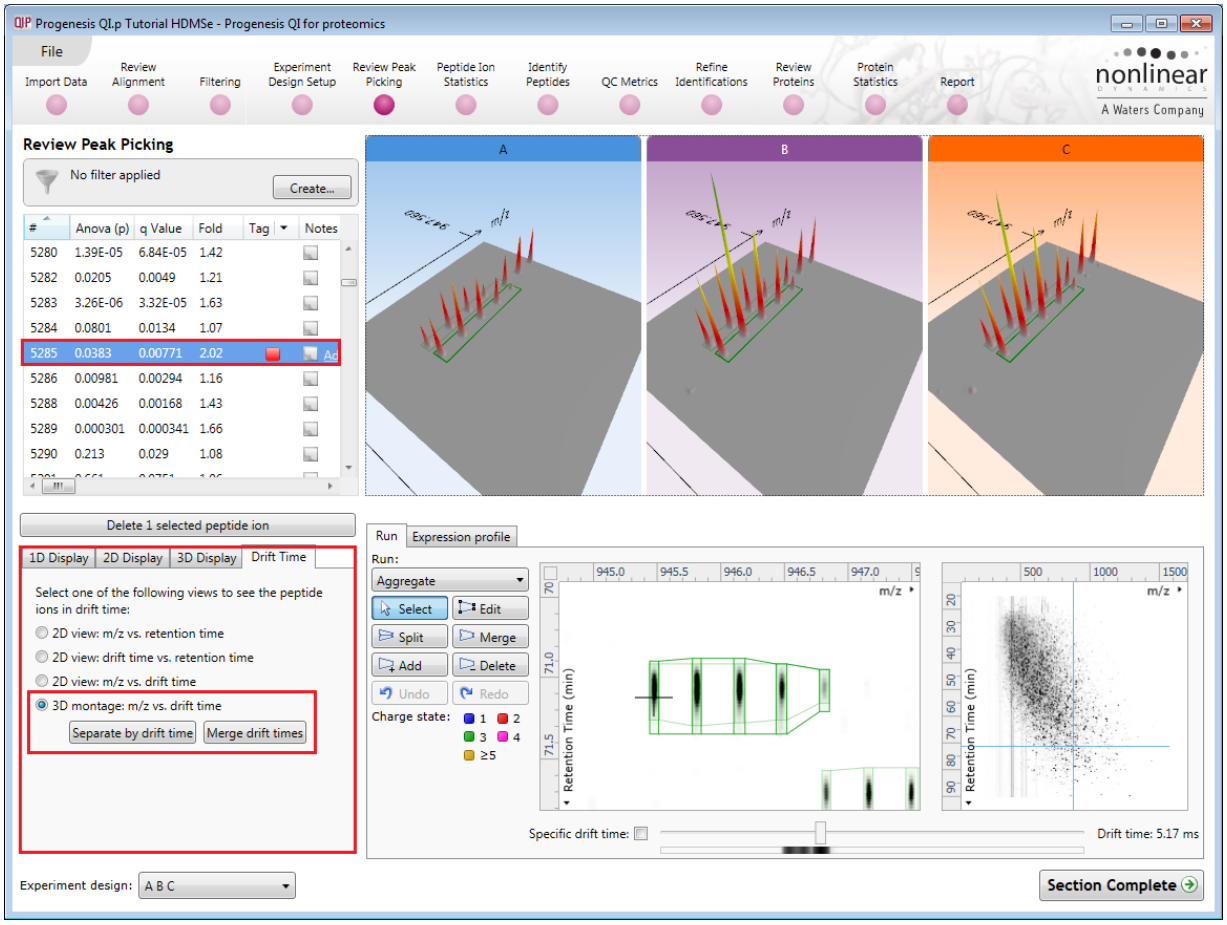
Window B changes into a 3D view by selecting the 3D Montage tab on the bottom left of the display.



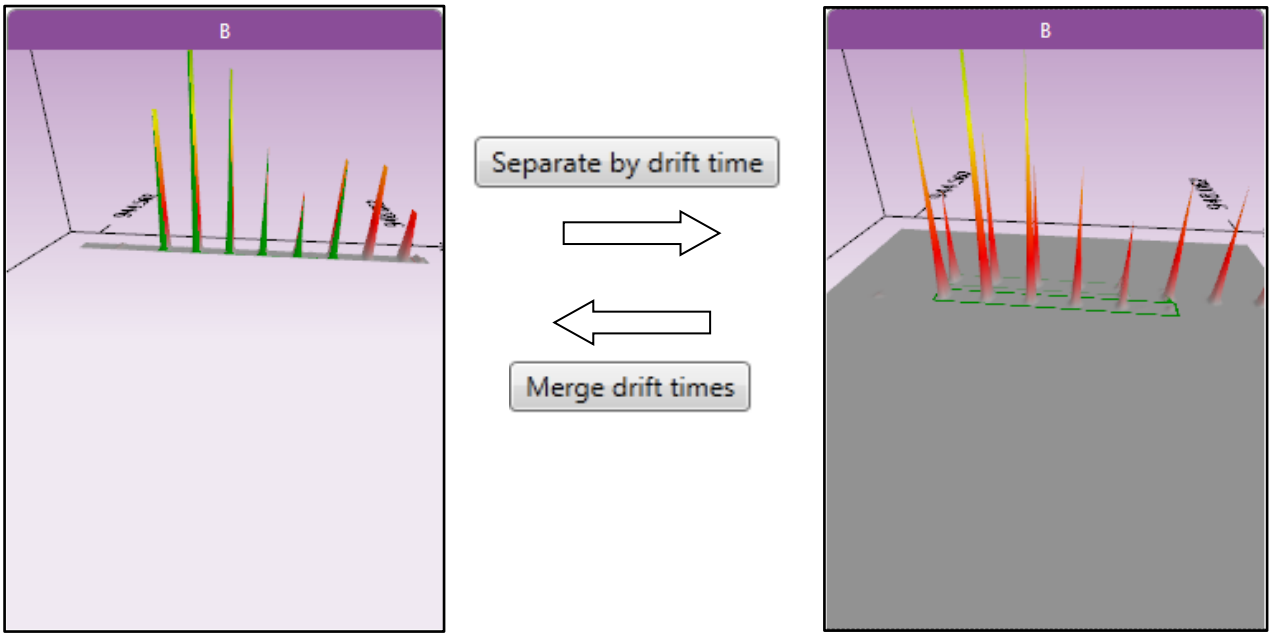
The views can be set to **Automatically Rotate** or you can rotate them manually by clicking and dragging them with the mouse.

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 5285.



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)

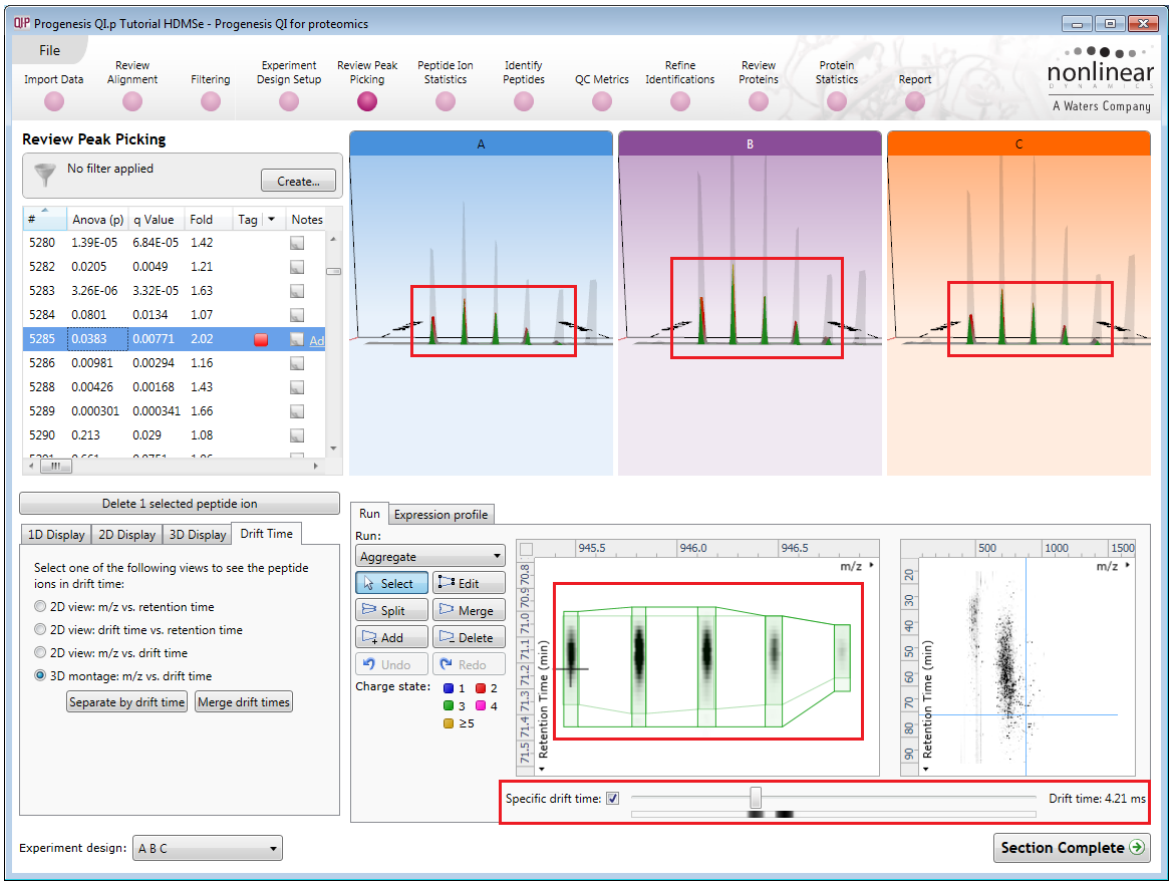


The left hand view displays the merged Drift Times.

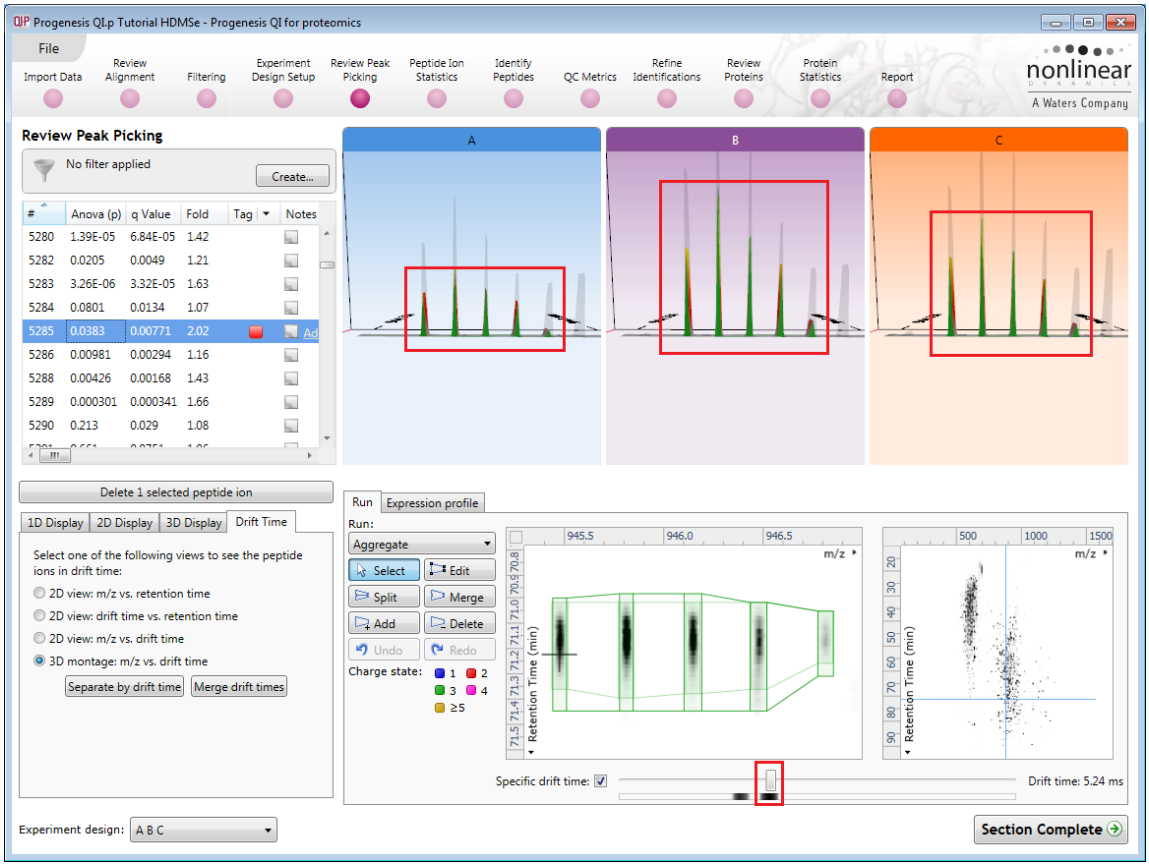
Note: you can manipulate the orientation of the views by clicking on them and dragging the display to the required orientation. You can also zoom in and out of the panels by using the 'scroll' wheel on your mouse

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.

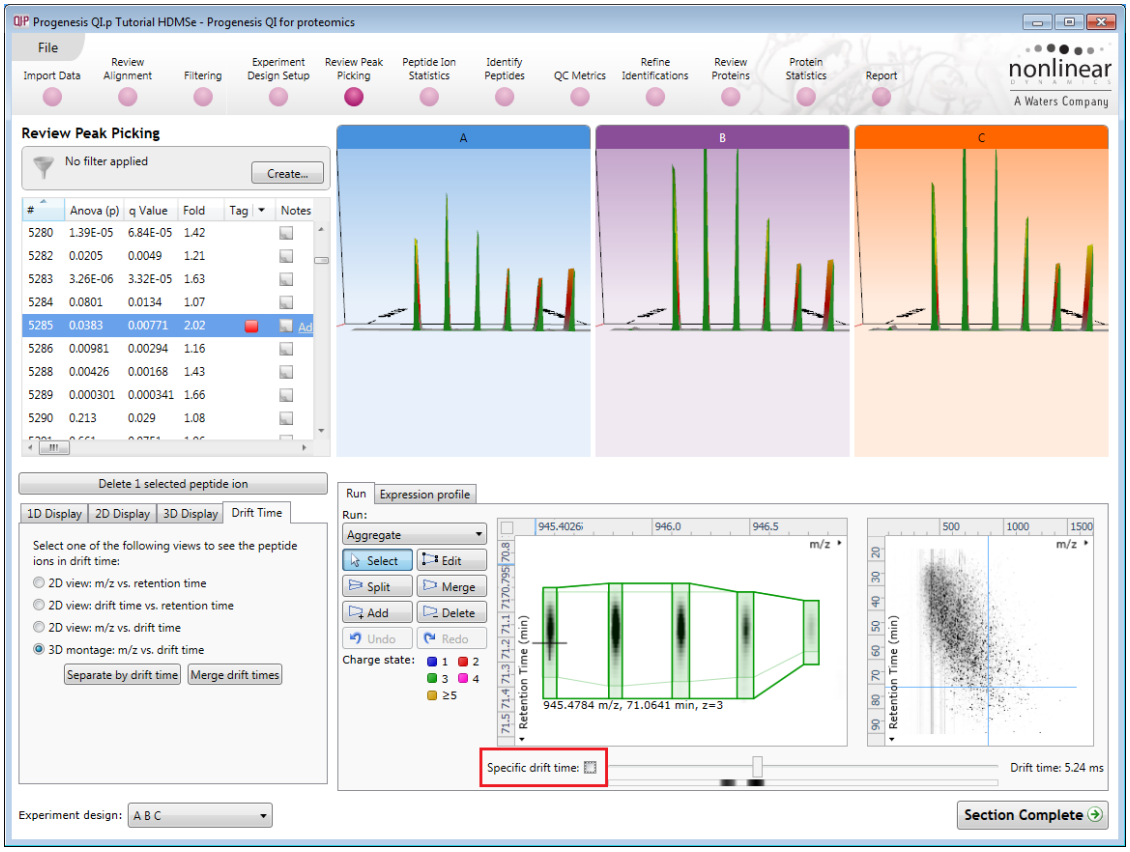


As you move the slider over the intense areas, indicated below, all the views update to the corresponding drift time.



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

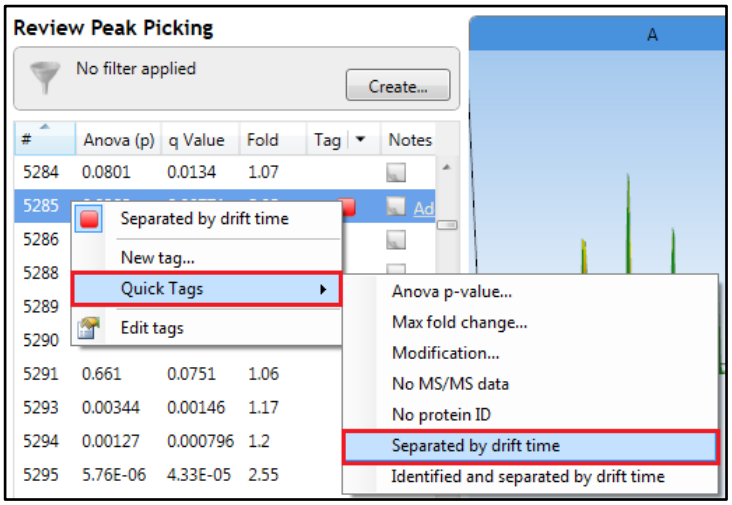
When you un-tick the 'Specific drift time, tick box the 3D views will return to showing the Merged Views for the current peptide ion in the table.



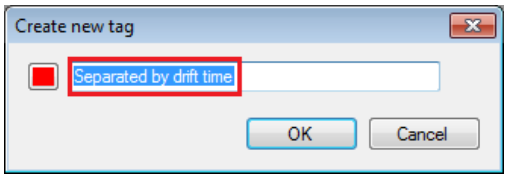
Using Quick Tags to locate examples of Drift Time

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.**



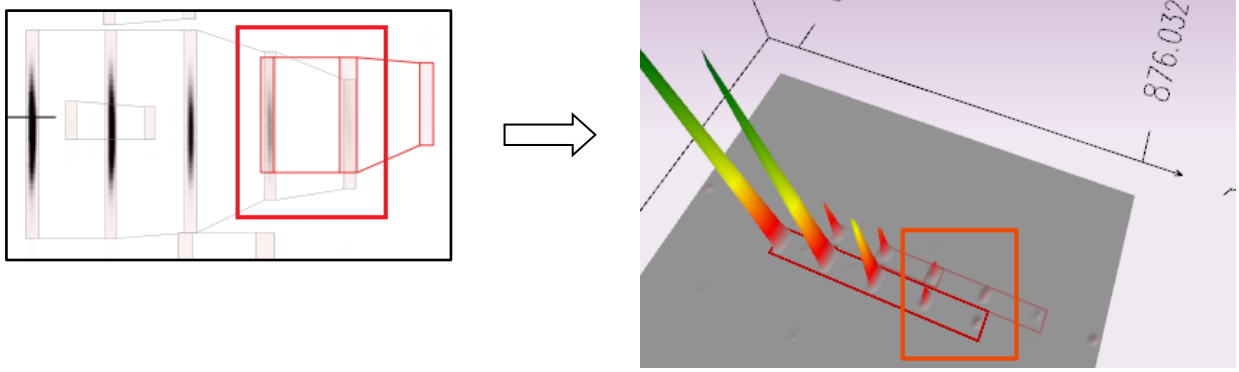
In the new tag dialog either accept or overtype the tag name.



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**

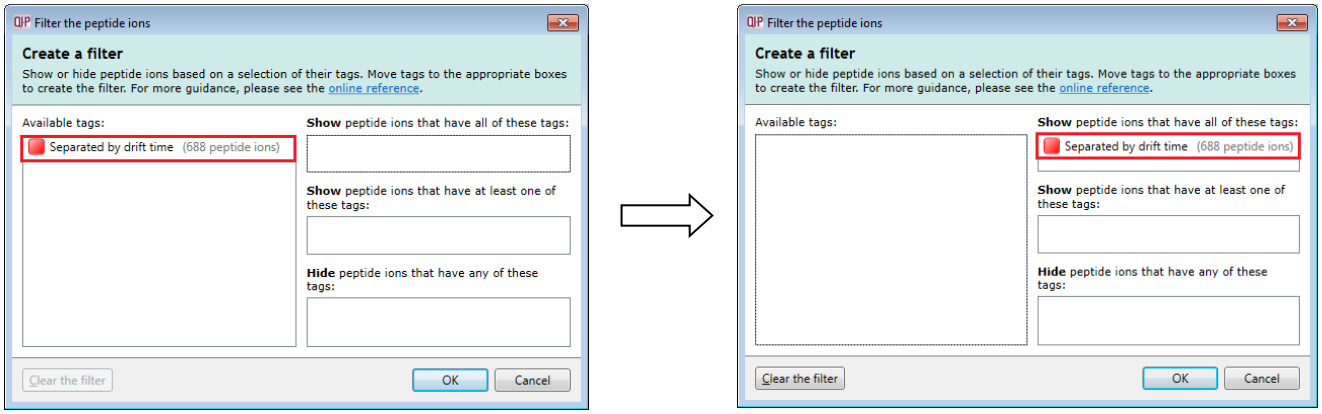


Now filter the table so that it currently only displays a list of peptide ions containing the **separated by drift time** tag.

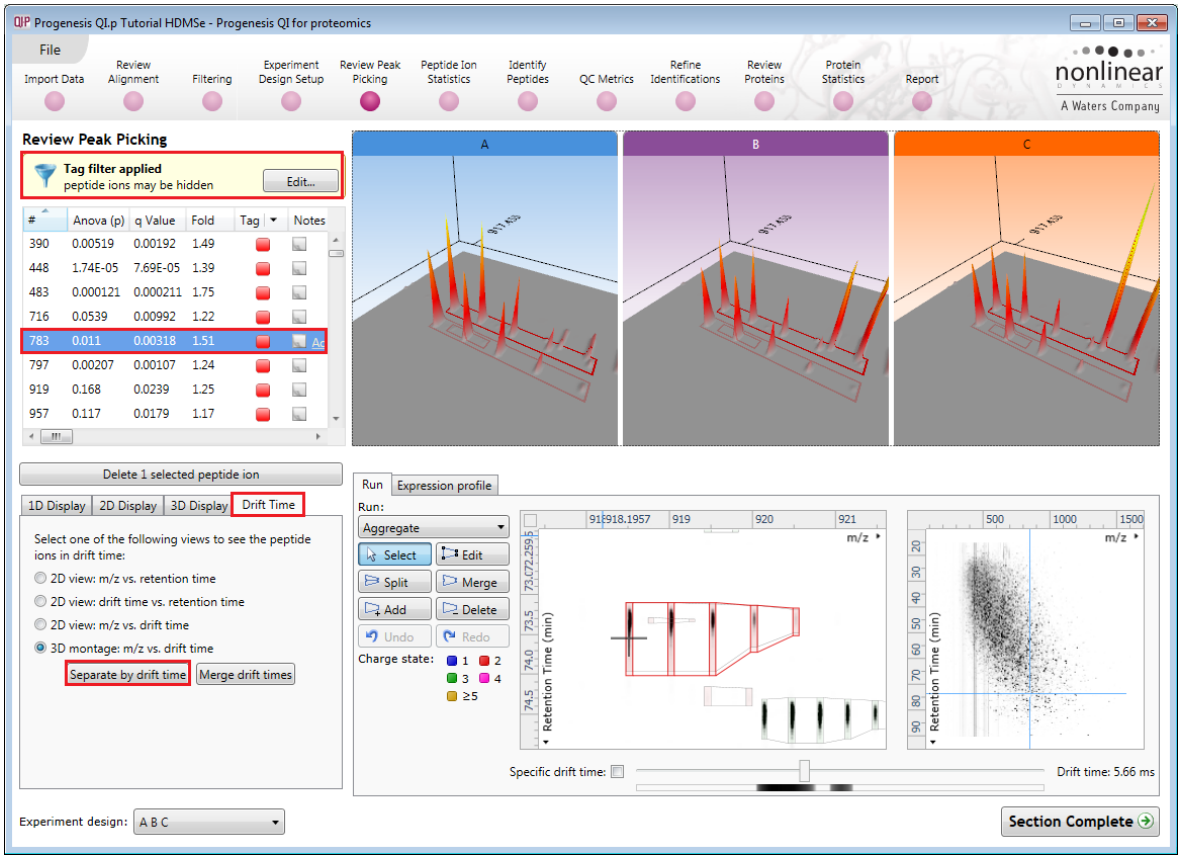
Click on **Create** on the filter panel above the table.

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.



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



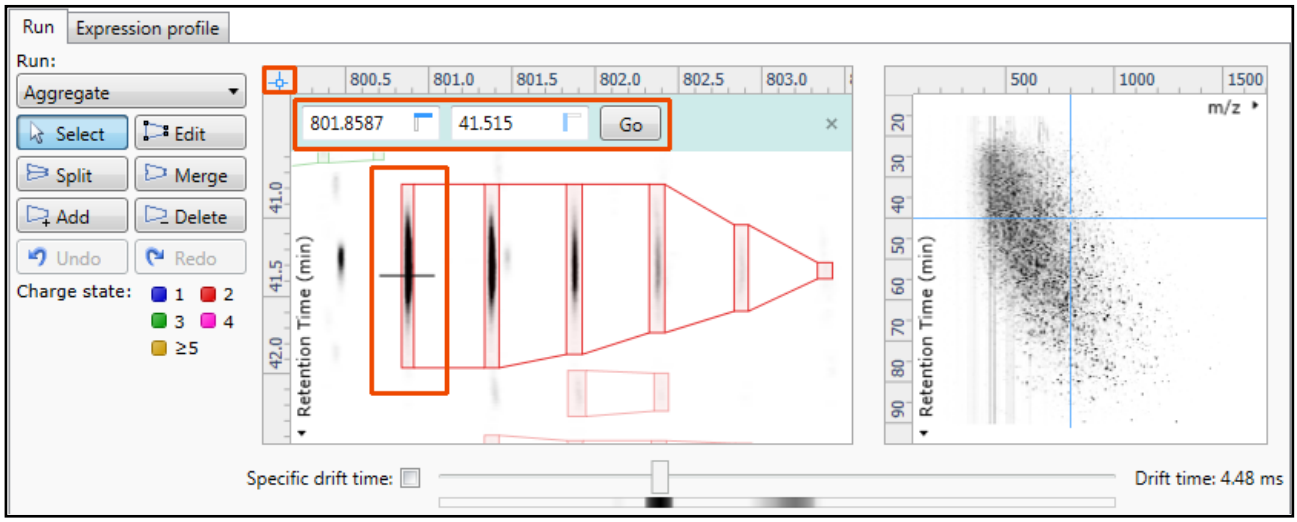
Note: with this **Tag filter applied** you can easily review the effect of Drift time separation for the peptide ions.

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

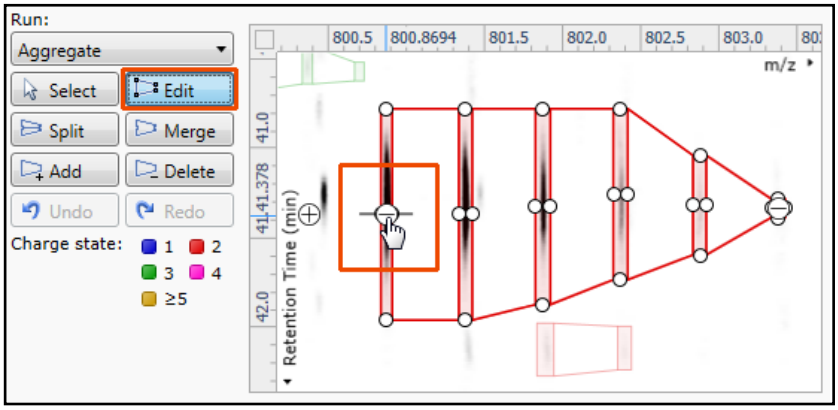
Editing of peptide ions in the View Results stage

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.

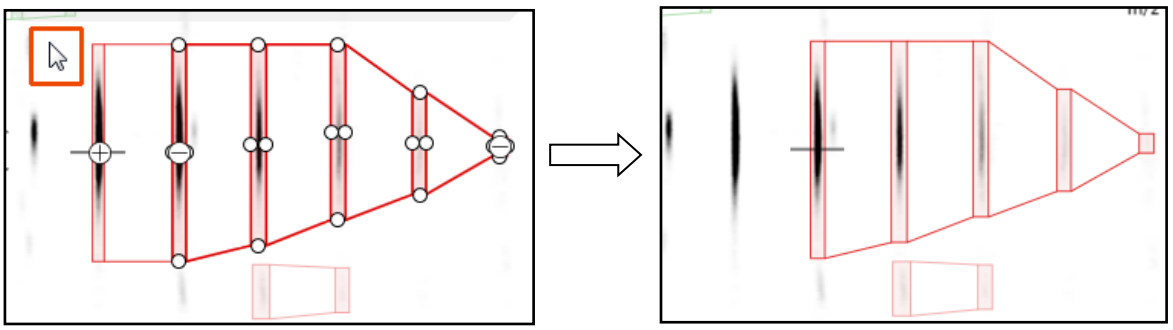
1. Locate the peptide ion at approx 801.86 m/z and 41.5 min using the **Go To Location** tool (top left of zoomed ion map), right click and zoom out and click the cross hairs on the monoisotopic peak to set the zoom.



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.

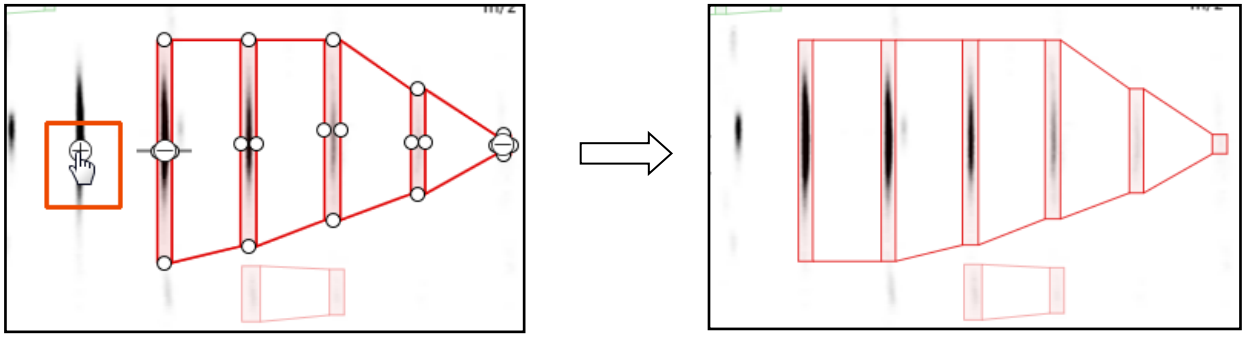


3. Click on the 'minus' handle over the monoisotopic peak to remove it.



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.

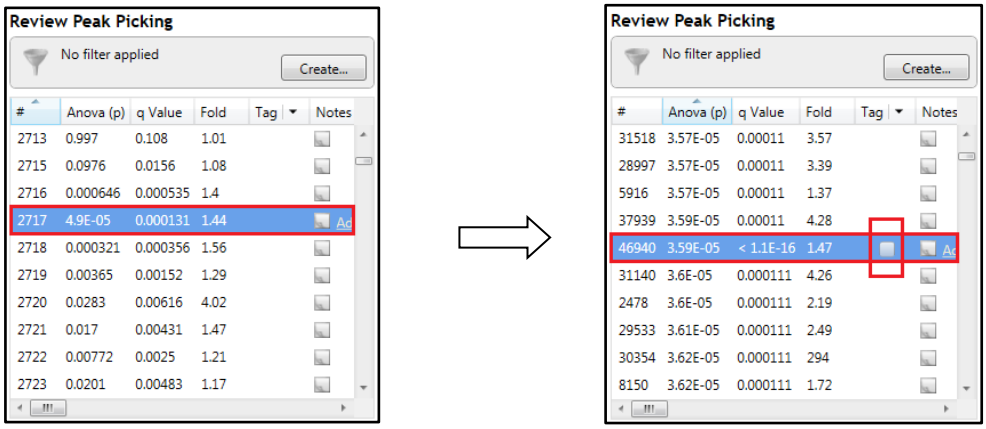
6. Click on the 'plus' handle on the peak to add it.



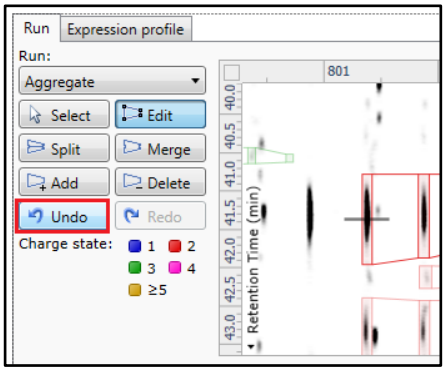
7. Then click outside the peptide ion to update the view.

8. **Note**: If you are not satisfied with the editing use the **Undo** button and retry.

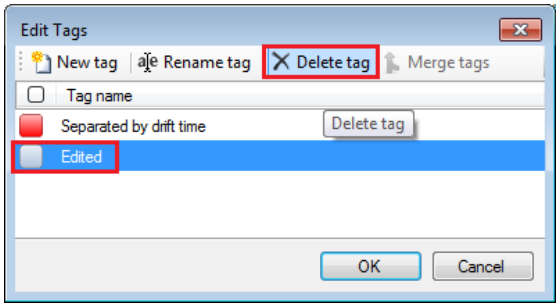
9. **Note**: that a tag is automatically added to the edited peptide ion in the table and the peptide ions id. number is changed to the next available one at the end of the list.



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



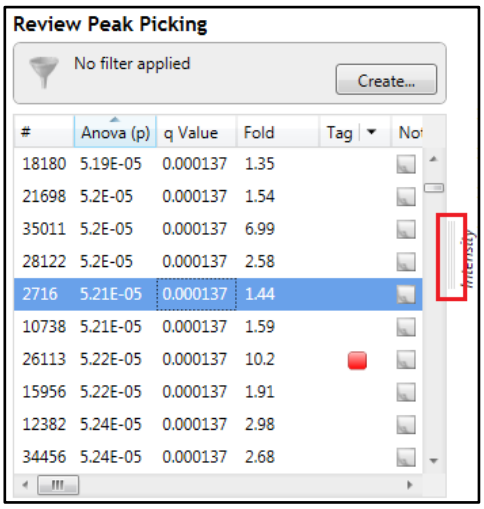
Note: if you have been exploring the editing of peptide ions then a quick way of reversing the edit is to repeatedly press **Undo**.



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

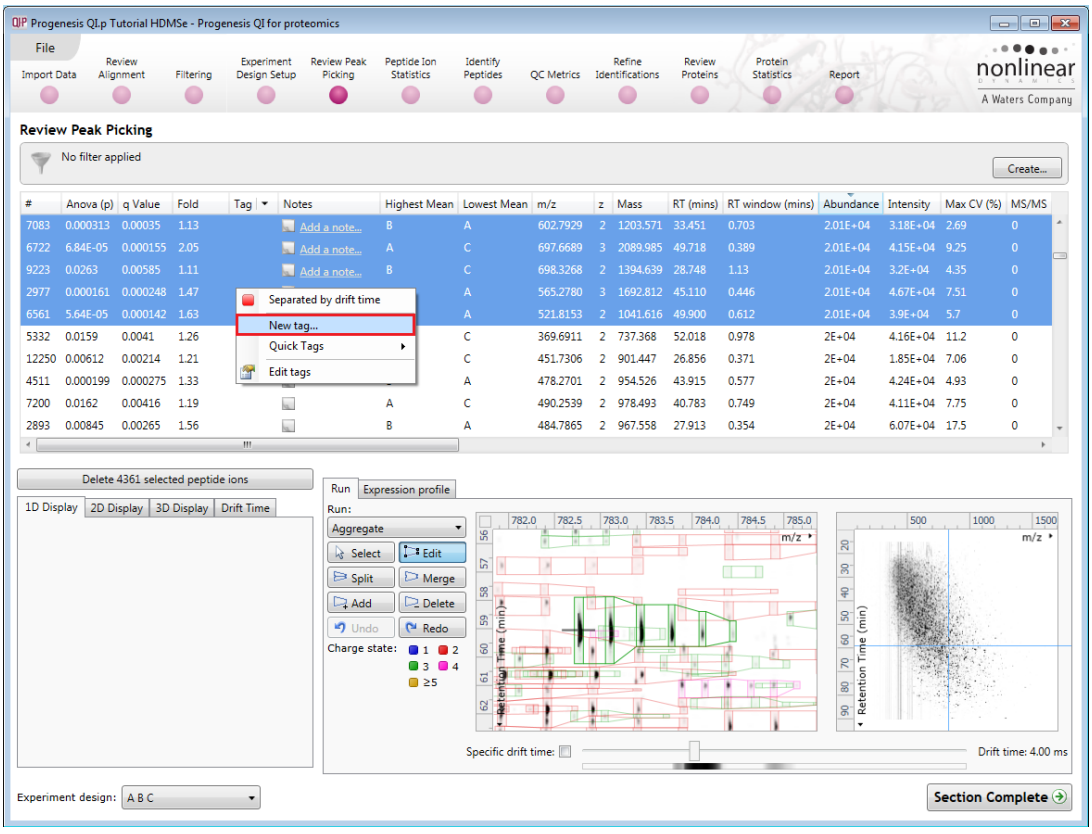
There are a number of ways to 'refine' your 'Ranked List' of analysed peptide ions before examining them with the Statistical tools in **Peptide Ion Statistics**. These make use of simple 'Selection' and 'Tagging' tools that can be applied to the various groupings created in Stage 6 (page 29). An example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



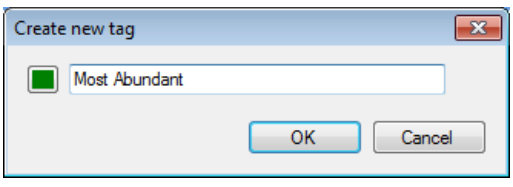
First expand the 'Peptide ions' table to show all the details by double clicking on the 'Splitter Control' to the right of the Review Peptide ions table.

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

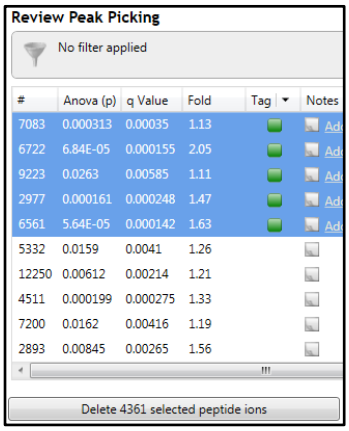
Right click on the highlighted peptide ions and select 'New Tag '.Give the Tag a name. i.e. **'Most Abundant'**.



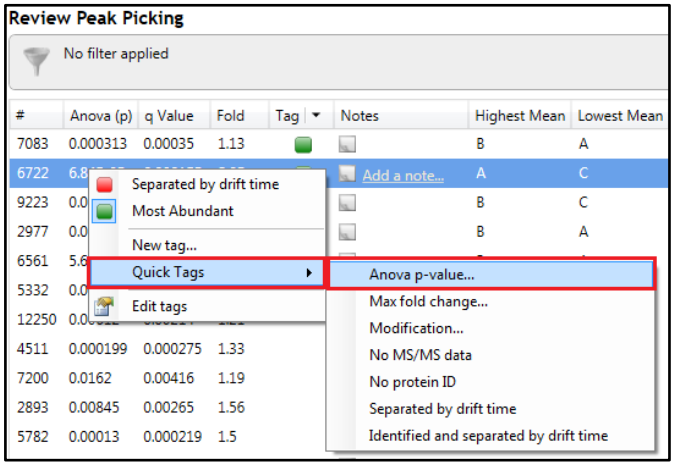
Note: there is already a red tag present that was assigned to those peptide ions that are **Separated by drift time,** which you created in the previous section.

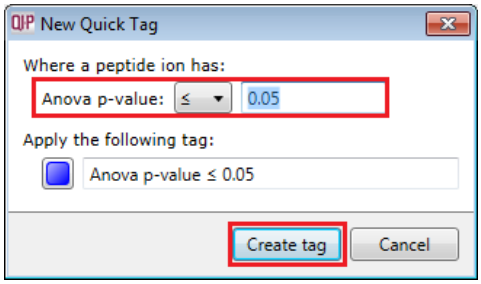


On clicking **OK** the Tag is added to the peptide ions highlighted in the table (signified by a coloured square, green in this example).

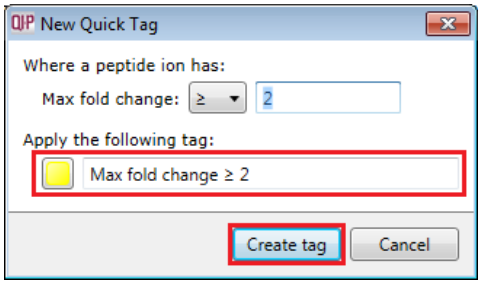


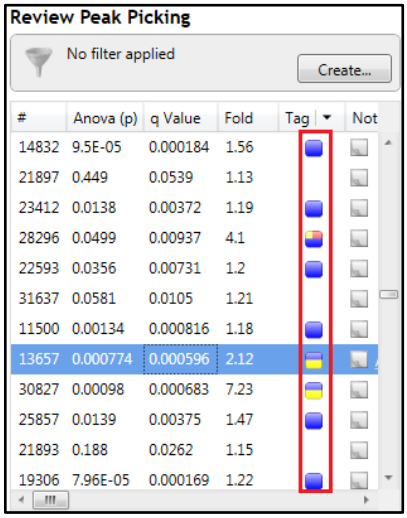
Now right click on any peptide ion in the table and select **Quick Tags** this will offer you a number of standard tag options. Select **Anova p-value....** Then set the threshold as required and adjust default name as required and click **Create Tag**.





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 ≥ 2**



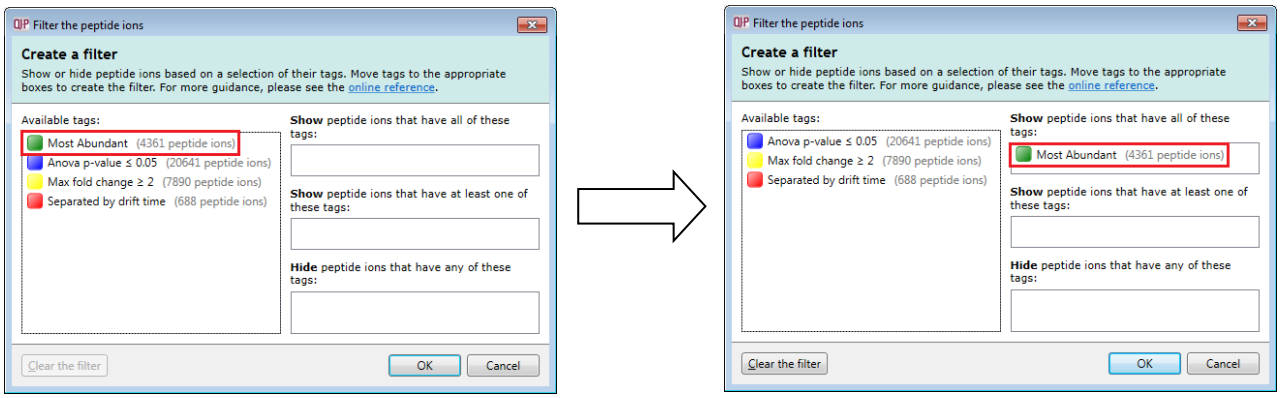


The table now displays peptide ions with multiple tags.

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

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.

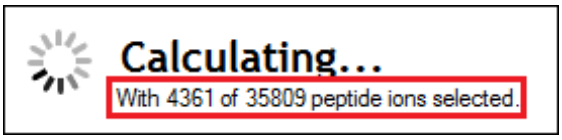


To move to the next stage in the workflow, Peptide Ion Statistics, click **Section Complete.**

Stage 8: Peptide Ion Statistics on Selected Peptide ions

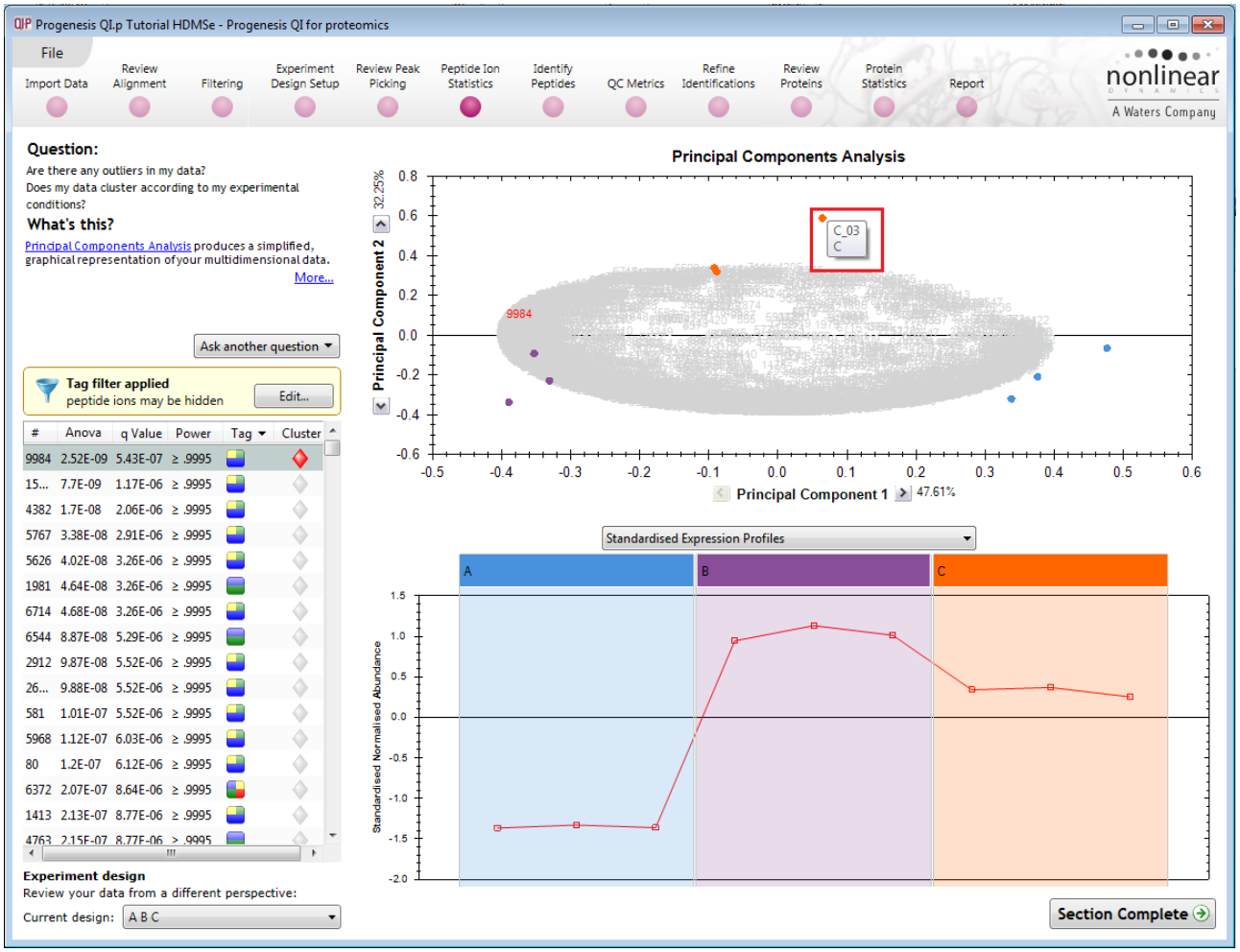
The user guide now describes the functionality of the Multivariate Statistics.

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.

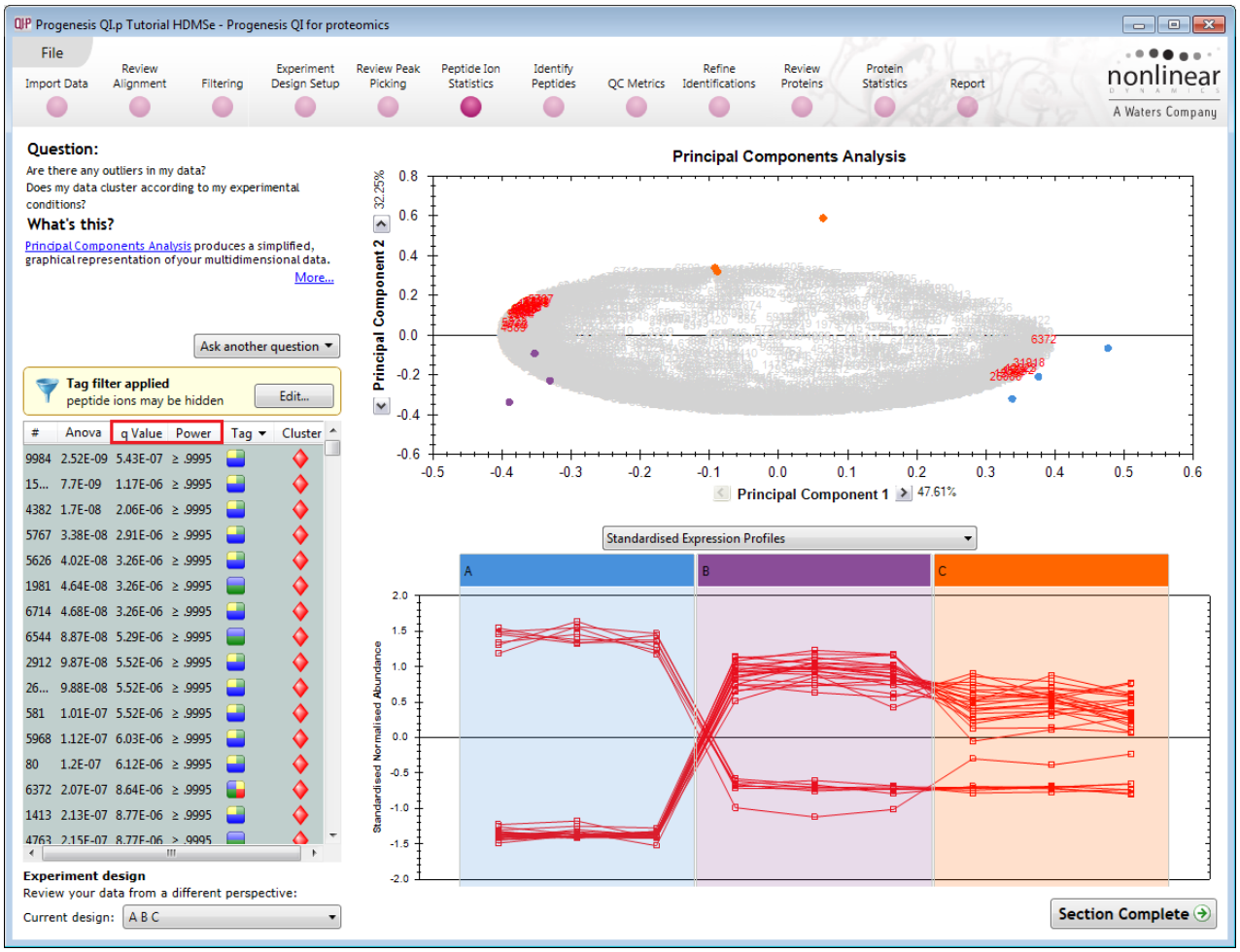
Principal Component Analysis (PCA)

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.

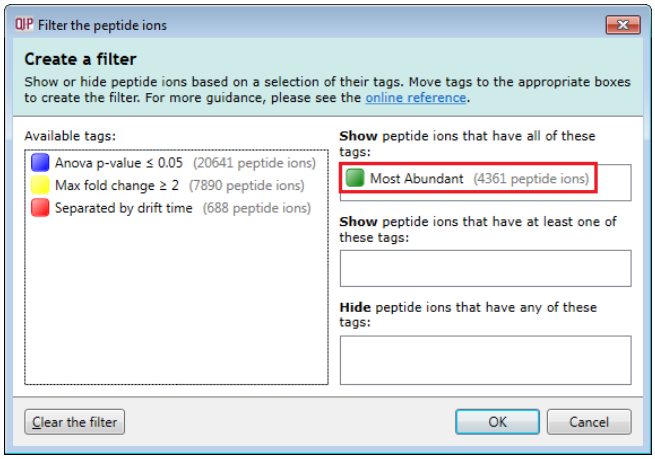
Power: can be defined as the probability of finding a real difference if it exists. 80% or 0.8 is considered an acceptable value for power. The Power Analysis is performed independently for each peptide ion, using the expression variance, sample size and difference between the means.

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 91)

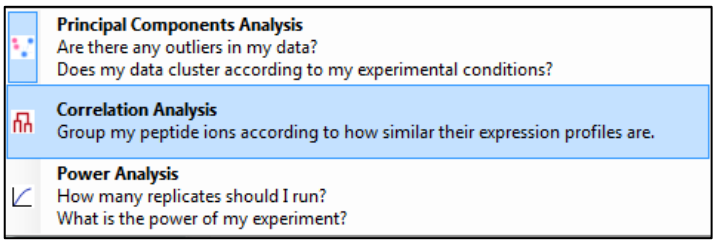
Correlation Analysis

With the tag filter still set to display only the top 4361 **Most Abundant** peptide ions, we are going to explore the Correlation Analysis of these peptide ions.

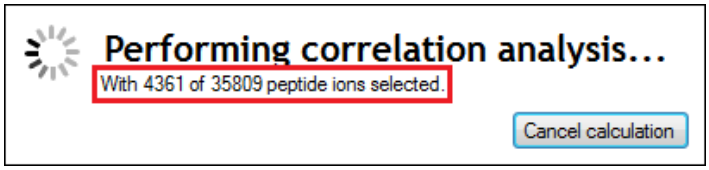


To set up the **Correlation Analysis** using this filtered data set click on **Ask another question** (above the table)

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'

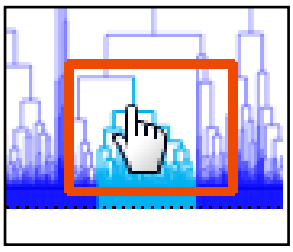
The question is answered by:

'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

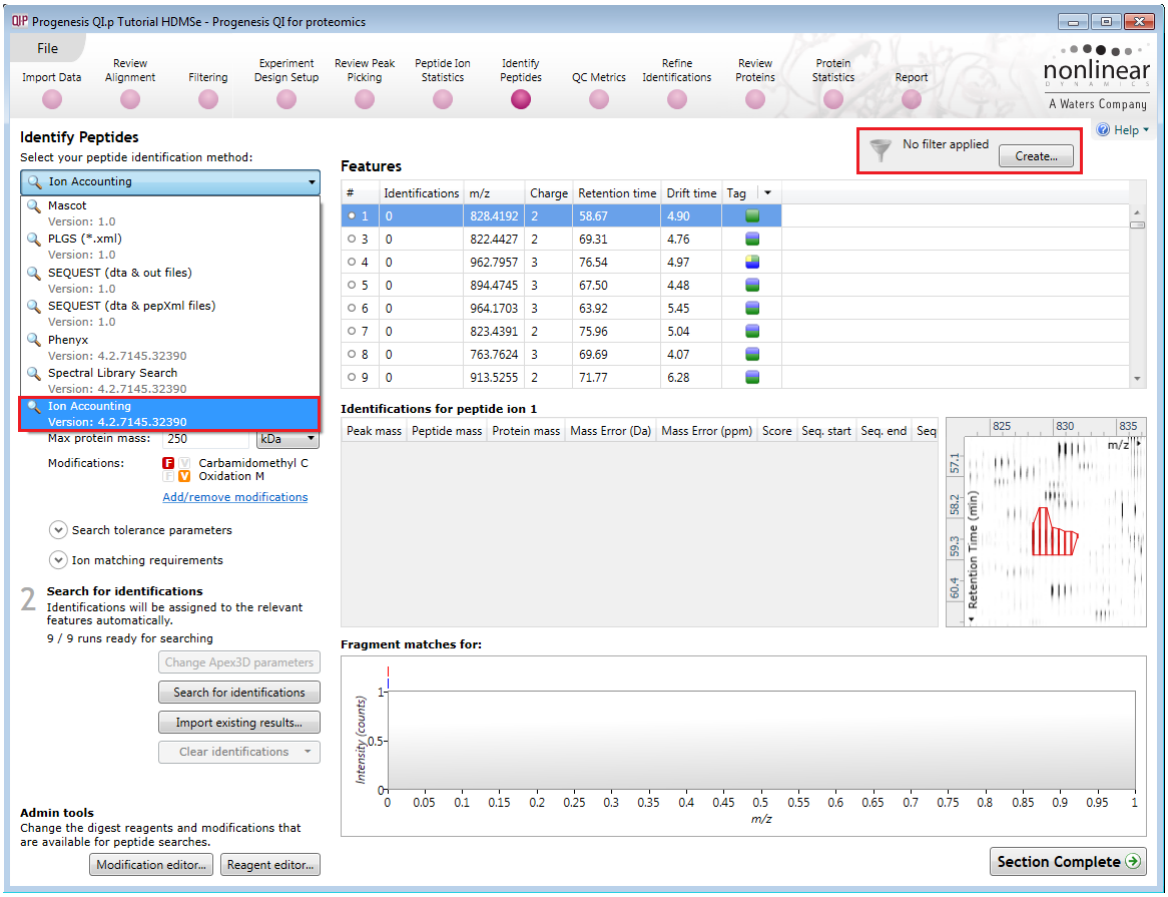
Tip: when reviewing the tags (see above) if you are not applying a new filter then use the **Cancel** button to return to the main view, this prevents unnecessary recalculation of your data.

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.



For this example we are using the direct method **Ion 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**.**

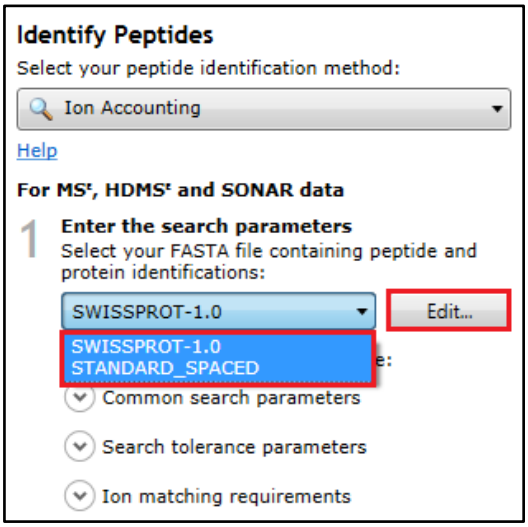
Entering Search Parameters

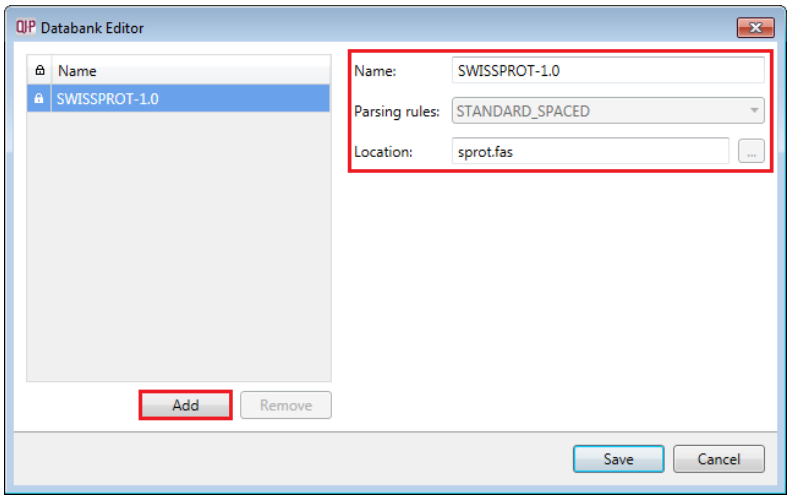
Firstly you need to select the FASTA file containing peptide and protein identifications.

SWISSPROT-1 is provided with the installation of the software.

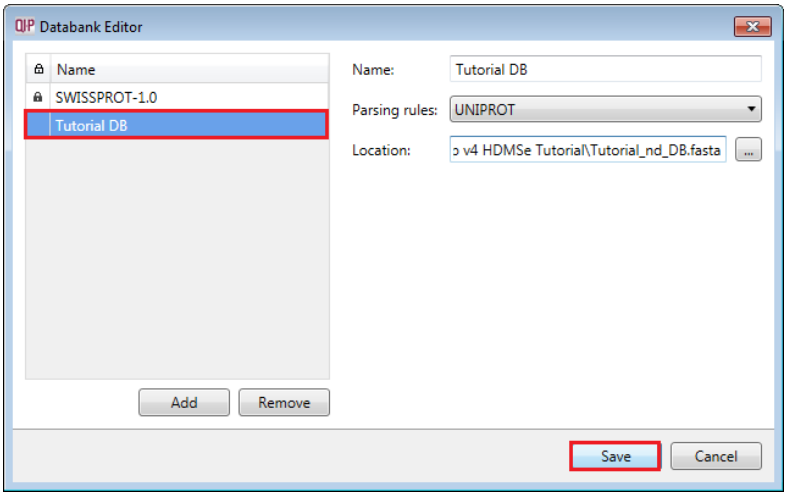
To add new Databanks in the form of FASTA files click on **Edit…** to open the Databank editor

Note: the SWISSPROT-1.0 is locked



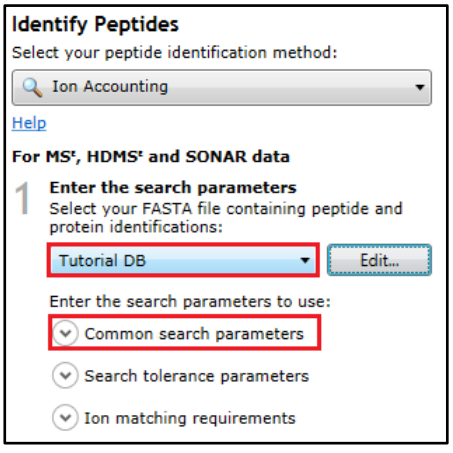


For a new Databank you need to give it name, select the parsing rules and specify the location of the FASTA file, see the example below.



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**

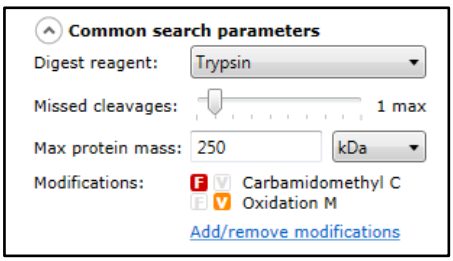
The default settings are displayed:

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...**

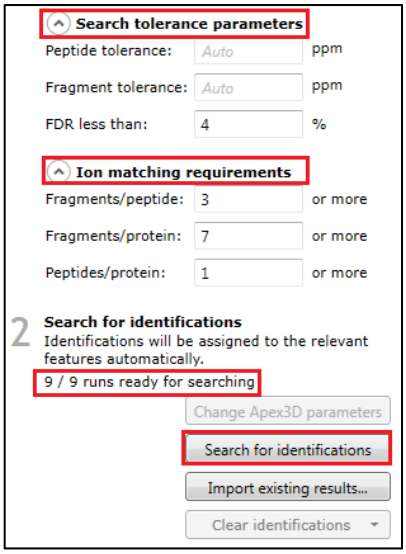
Missed cleavages: is set as 1.

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…**



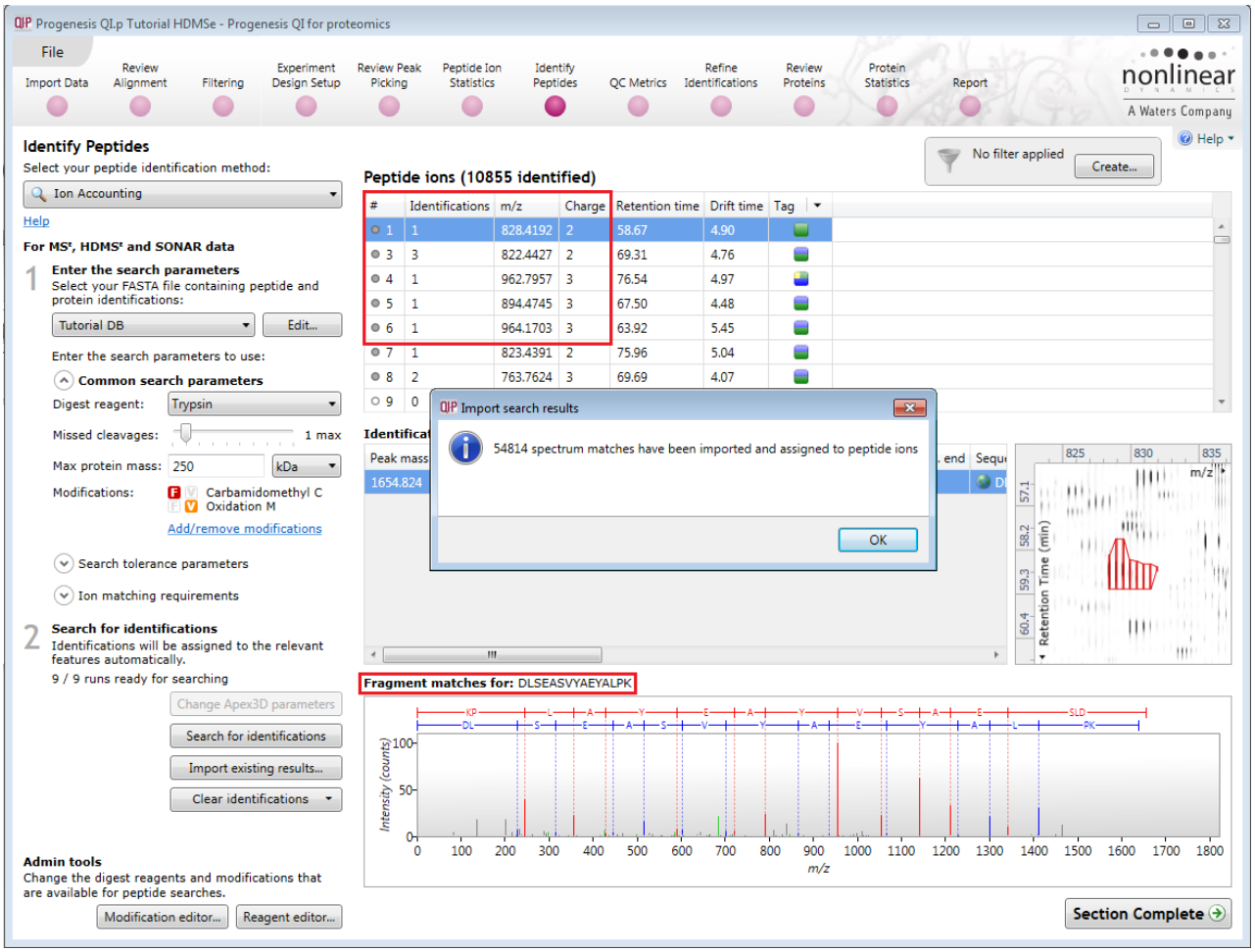
Having selected the Databank then set the Search tolerance parameters and ion matching parameters as shown to the right.



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.



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.

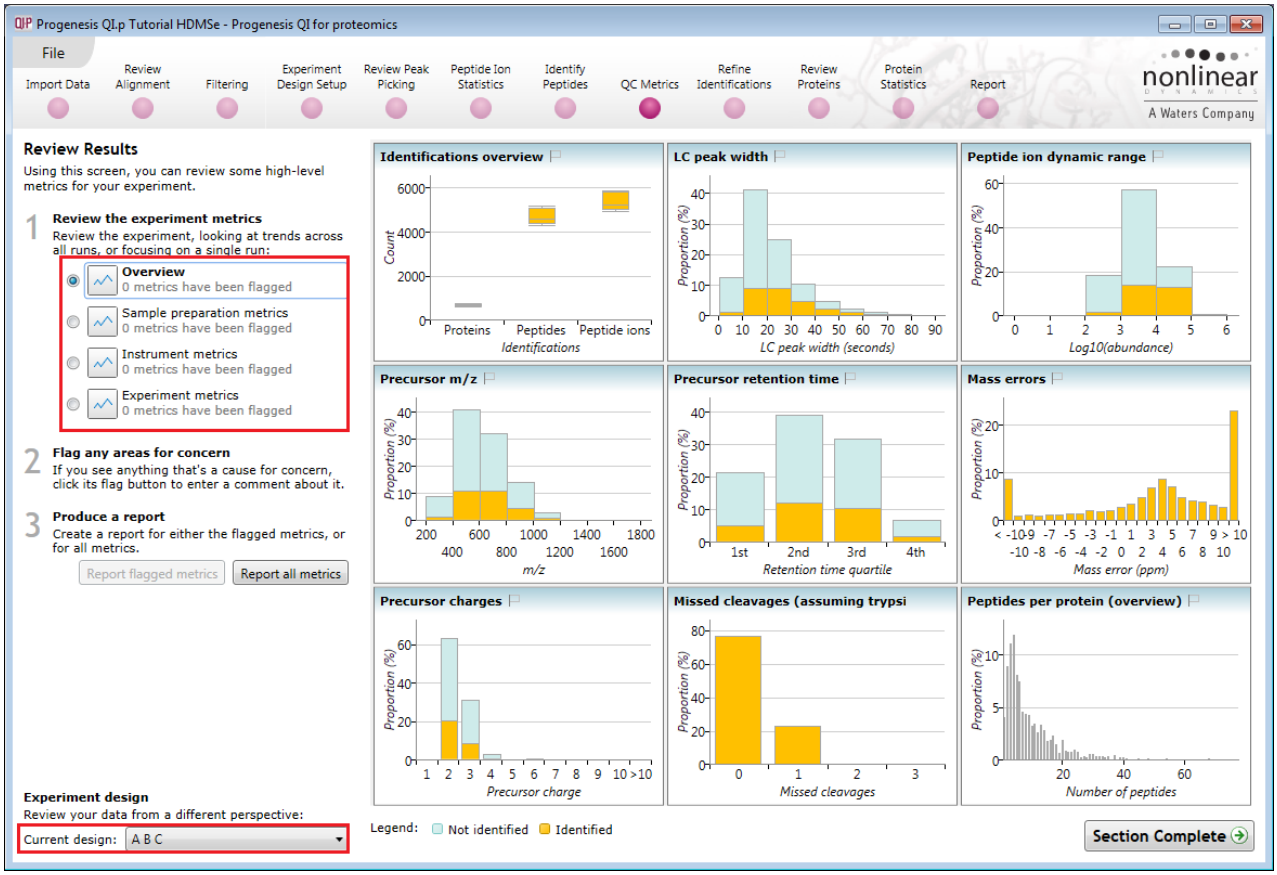
Note: if you want to perform the search with a new set of parameters then first select **Clear all identifications**

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

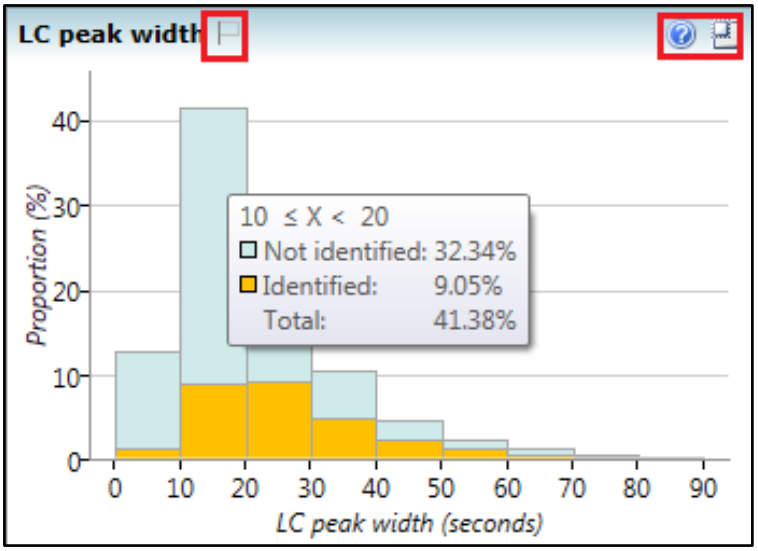
More detailed information on the QC metrics is available on the FAQ pages.

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.

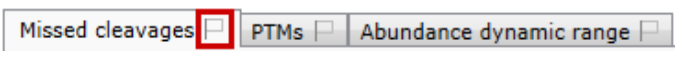
Note: QC measurements are designed to flag up potential issues for investigation, hence there must be an assignable cause to the variation observed to render QC practical.

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.

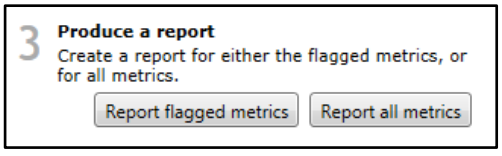
You can also tag metrics of interest or concern with a comment for your records, which is saved with the experiment. To do this, click the empty flag icon in the tab header for the given metric (or in the tile header on the overview screen):



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:



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

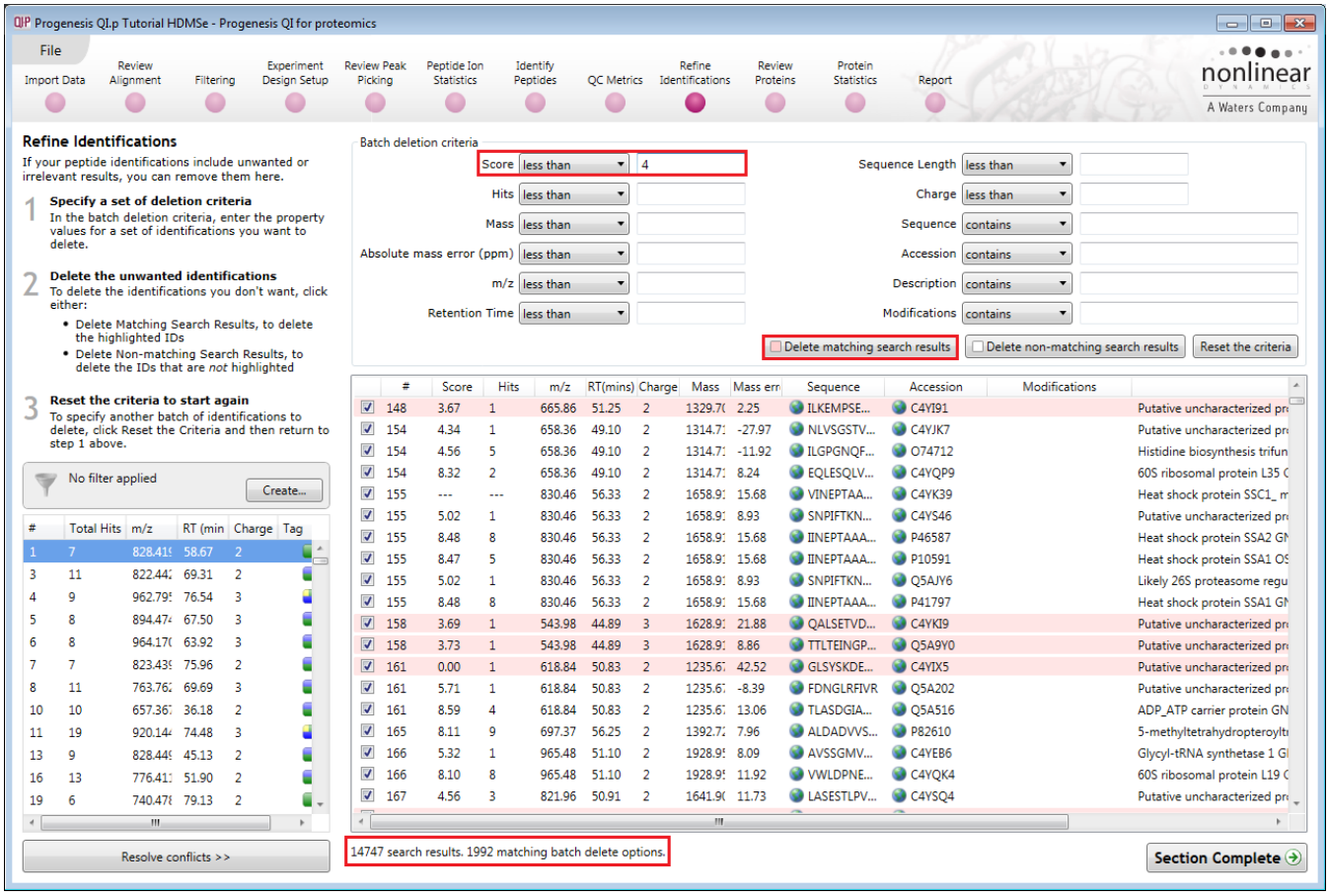
In this example we are going to apply a number of filters to 'refine' the quality of the Databank search.

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'

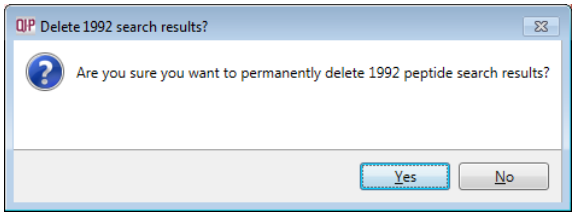
To perform these filters, on the Batch detection options panel, set the Score to less than 4, then **Delete matching search results**.



Note: the search results matching the filter criteria turn pink and the total is displayed at the bottom of the table (in this example: 1992 matching out of 14747)

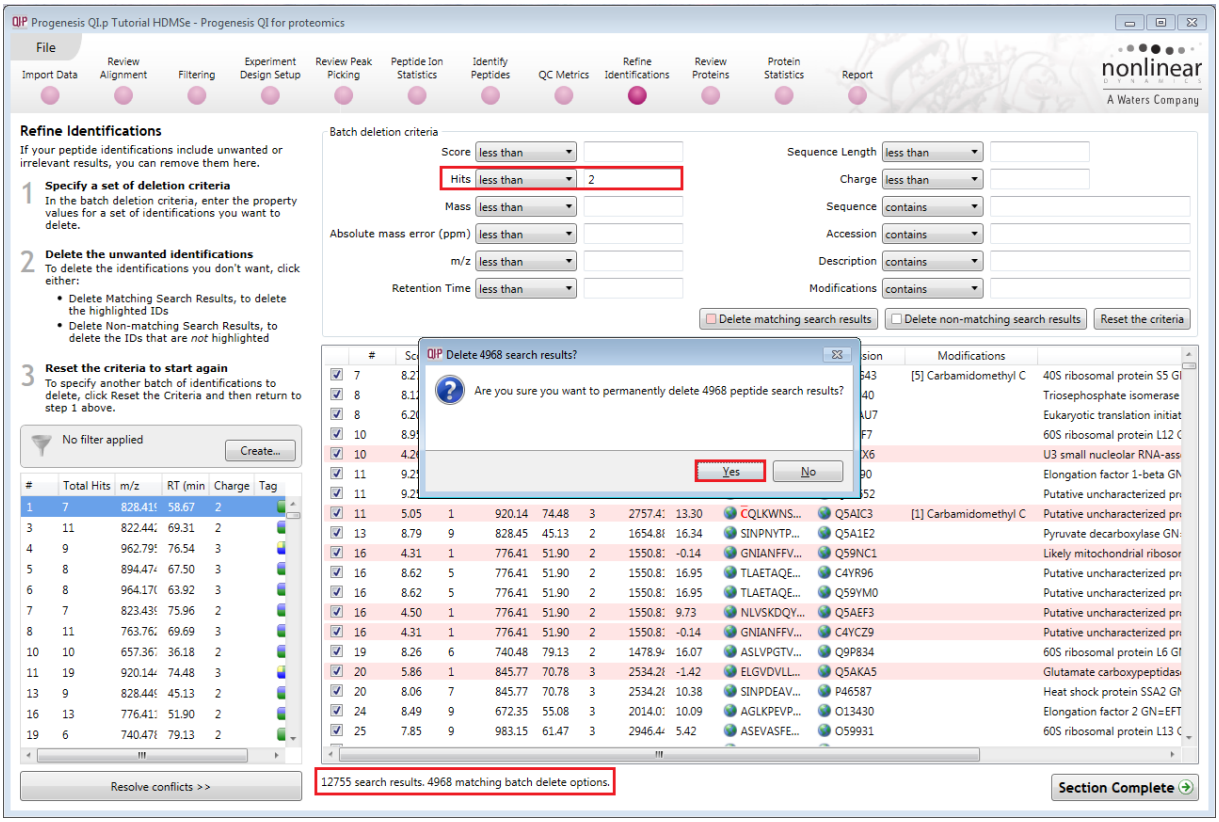
Note: a dialog warns you of what you are about to delete

Click **Yes**.

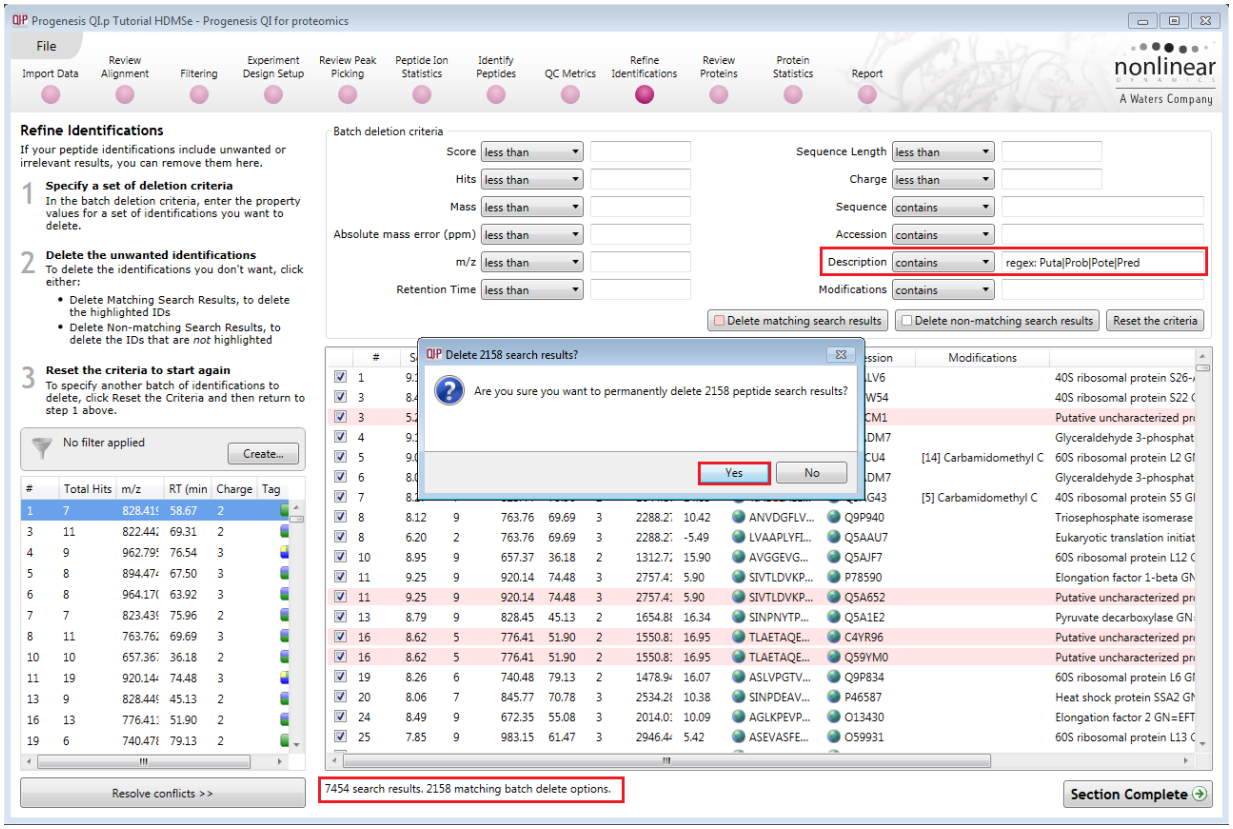


Now click **Reset the criteria** to clear the previous threshold then apply the next filter 'Hits: less than 2'.

Tip: always click **Reset the Criteria** after each deletion.



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.



Having applied all the filters there will be **5296** search results remaining

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

Resolving Conflicts

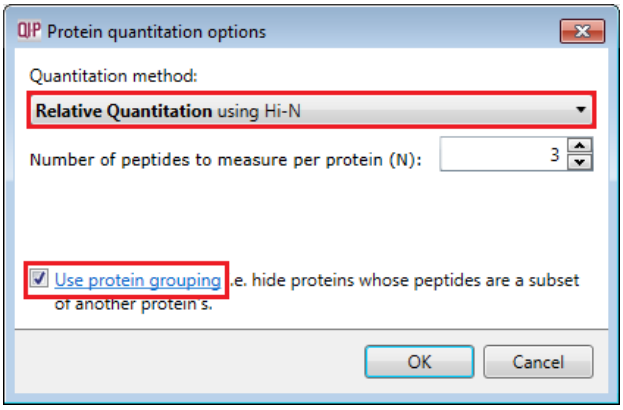
This stage allows you to examine the behaviour of the identified peptides and choose to resolve any conflicts for the various peptide assignments at the protein level.

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 92)

For this guide: we will NOT resolve the conflicts.



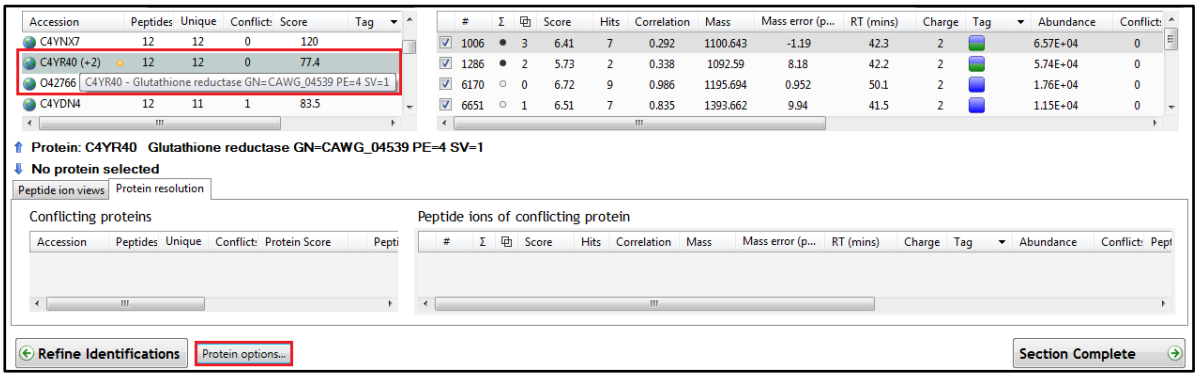
The following pages describe the handling of Protein grouping and Protein Quantitation. Options for these are available at both Resolve Conflicts and Review Proteins

Note: the default settings are to **Use Protein Grouping** and **Relative Quantitation using Hi-N**

Protein Grouping

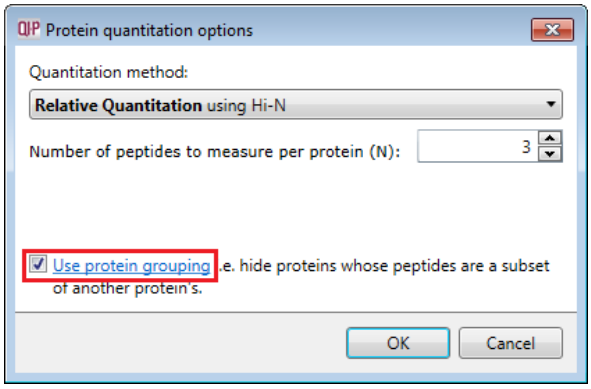
Where proteins are identified containing the same peptides then they are effectively indistinguishable aside from score. Also where one protein contains only peptides that represent a subset of another protein's peptides, the protein with fewer peptides can be subsumed into that with the greater number.

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.

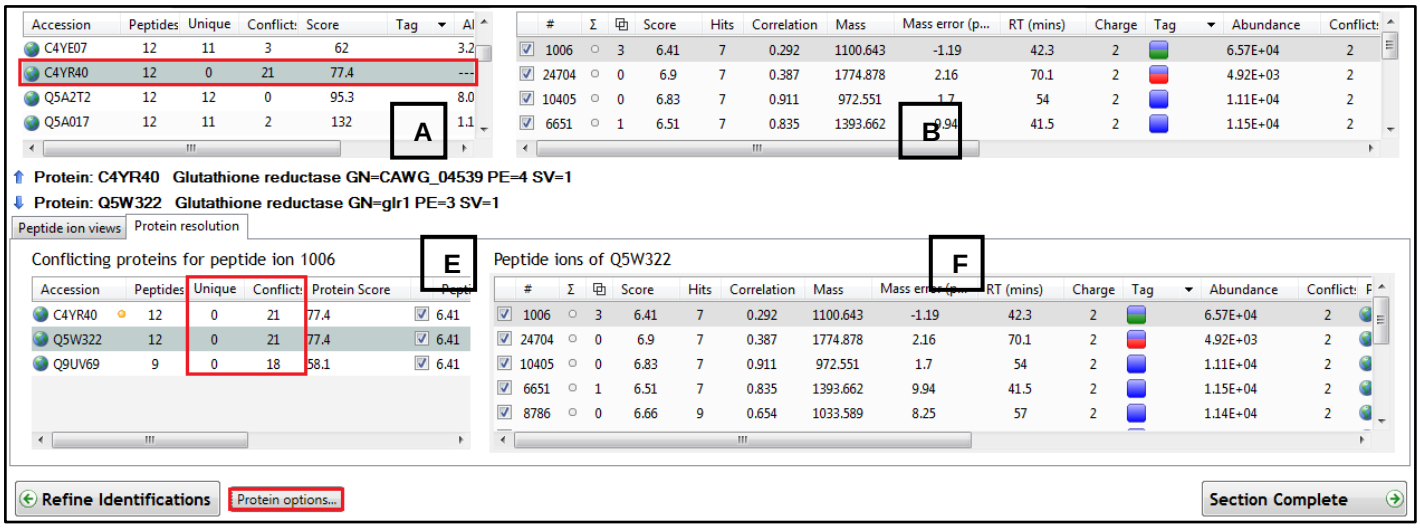


Although Conflict resolution has not been performed this protein has no conflicting peptides.

Protein grouping is switched on by default. To switch it off click on **Protein options**… (bottom left) and untick the option.



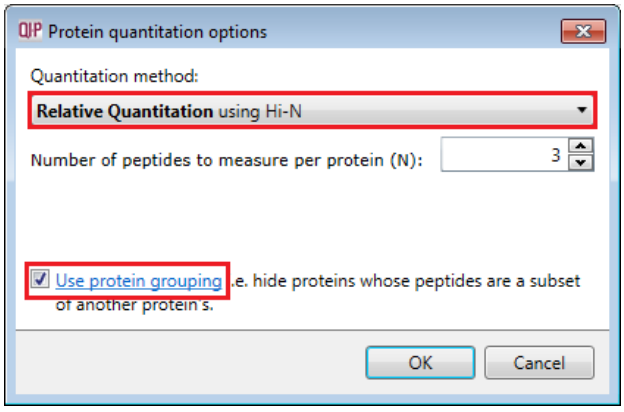
As grouping is switched off the grouped proteins appear with conflicts to the other group members.



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.

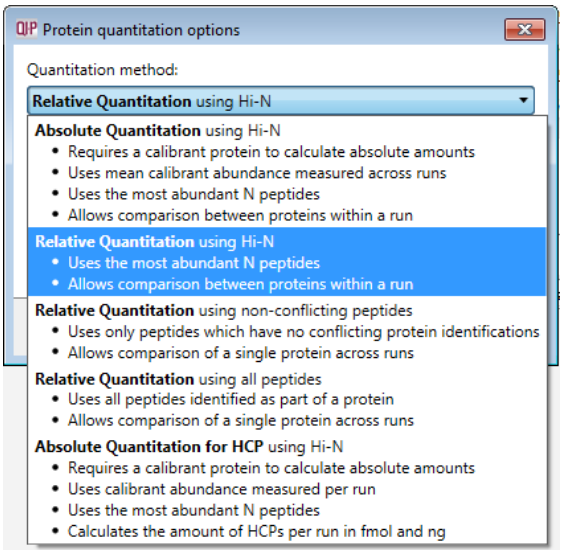
The next section in this guide describes the use of alternatives to the default method shown to the right.



Note: If you are using the default method, you can go directly to the **Review Protein** stage page 61 using the workflow.

Protein Quantitation options

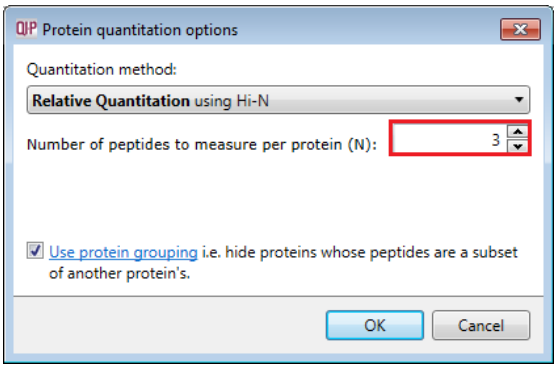
There are 5 options with which to control how the Protein Quantification is performed by Progenesis QI for proteomics.



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.

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.

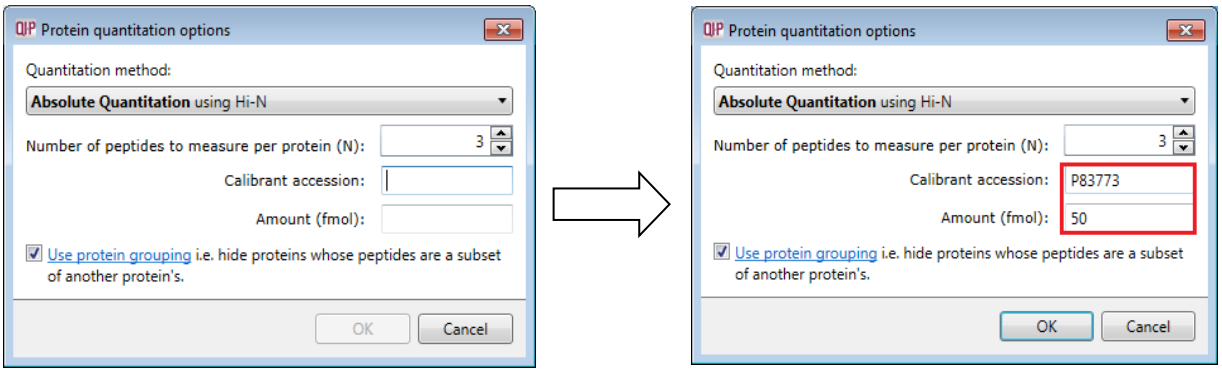


For each protein, the N most abundant peptides (N being set according to the user selection) have their **abundances averaged** to provide a reading for the protein signal.

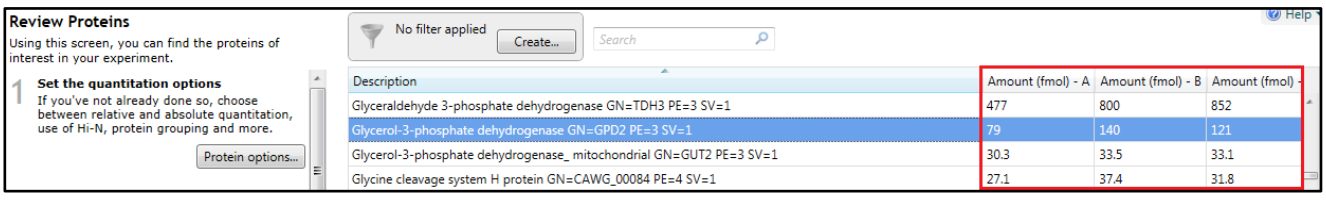
The ranking of peptide abundance is based on the integrated value across all the runs, allowed by the accurate alignment and lack of missing values. This gives added confidence in the peptide selection, taking all runs into account to make the ranking robust.

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.



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.



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.

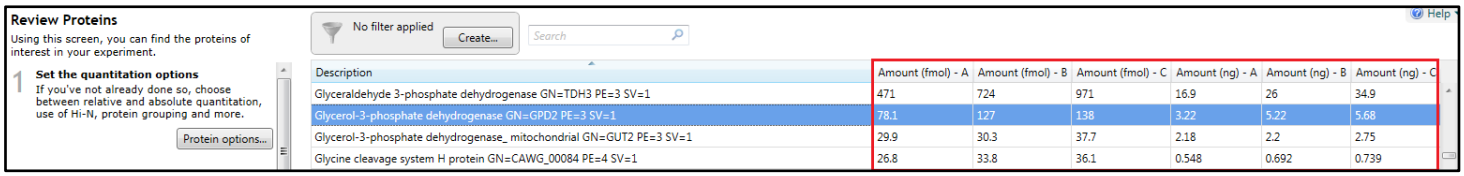
Note: When there are **peptide conflicts** (a peptide is shared between two proteins, for example) it is important to assign the signal correctly for absolute quantitation. To do this, Progenesis QI for proteomics carries out a two-step process. Firstly, Hi-N is carried out only on the N most abundant **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.

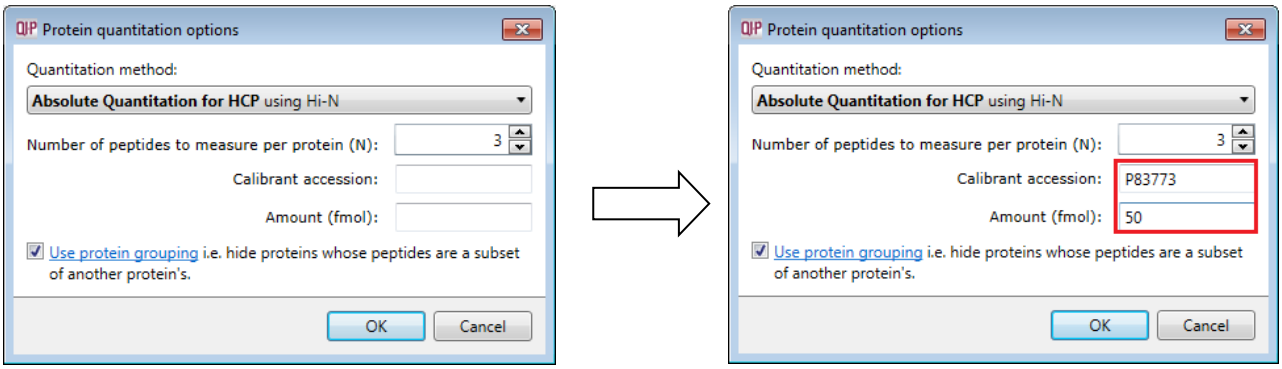
To generate values for **Absolute Quantitation for HCP** (Host Cell Proteins) select the 5th option on the drop down at the beginning of this section.

Note: there are 2 differences between this HCP-specific option and the Absolute Quantitation option:

- The mass of protein present is also reported in ng in addition to the amount in fmol
- The calculation of protein amounts and masses present is based on measurements of the calibrant protein present in the same run, and not a pooled measurement over all the runs. It does still assume a specified amount of a calibrant in every run. However, it does not pool information across the runs to derive its relationship between the calibrant and observed abundance. The amount of a contaminant is instead calculated directly by relation to the observed amount of the calibrant in the same run.



As for **Absolute Quantitation using Hi-N,** enter the accession number and amount for the calibrant.



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.

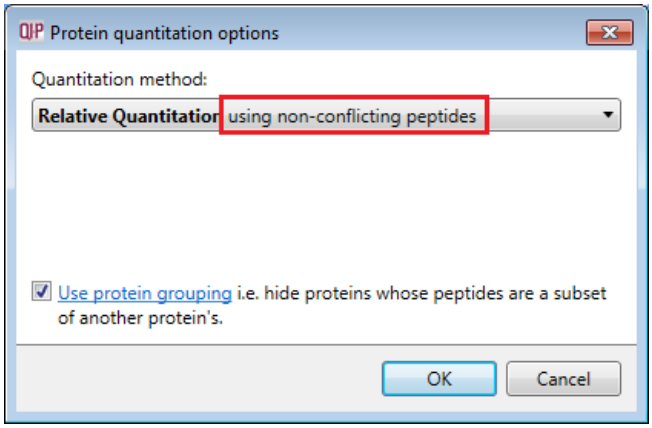
How the values for the calibrant behave across runs comparing Absolute Quant with Absolute Quant for HCP using the same calibrant (Acetyl-CoA hydrolase)

Description	Amount (fmol) - A	Amount (fmol) - B	Amount (fmol) - C
Acetolactate synthase small subunit_ mitochondrial GN=CAWG_03647 PE=4 SV=1	23.1	26	30.7
Acetyl-CoA hydrolase GN=ACH1 PE=1 SV=2	50.6	55.3	44
Acetyl-coenzyme A synthetase 1 GN=ACS1 PE=3 SV=1	6.66	8.89	7.95

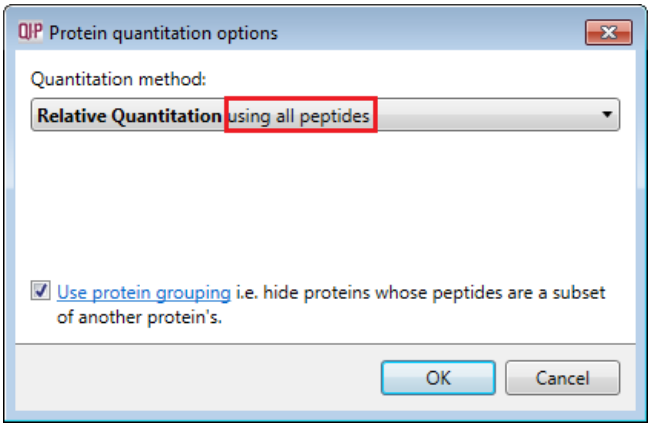
Description	Amount (fmol) - A	Amount (fmol) - B	Amount (fmol) - C	Amount (ng) - A	Amount (ng) - B	Amount (ng) - C
Acetolactate synthase small subunit_ mitochondrial GN=CAWG_03647 PE=4 SV=1	22.9	23.5	35	0.764	0.785	1.17
Acetyl-CoA hydrolase GN=ACH1 PE=1 SV=2	50	50	50	2.91	2.91	2.91
Acetyl-coenzyme A synthetase 1 GN=ACS1 PE=3 SV=1	6.59	8.03	9.03	0.496	0.605	0.68

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



The relative Quantitation can also be performed using all peptides.



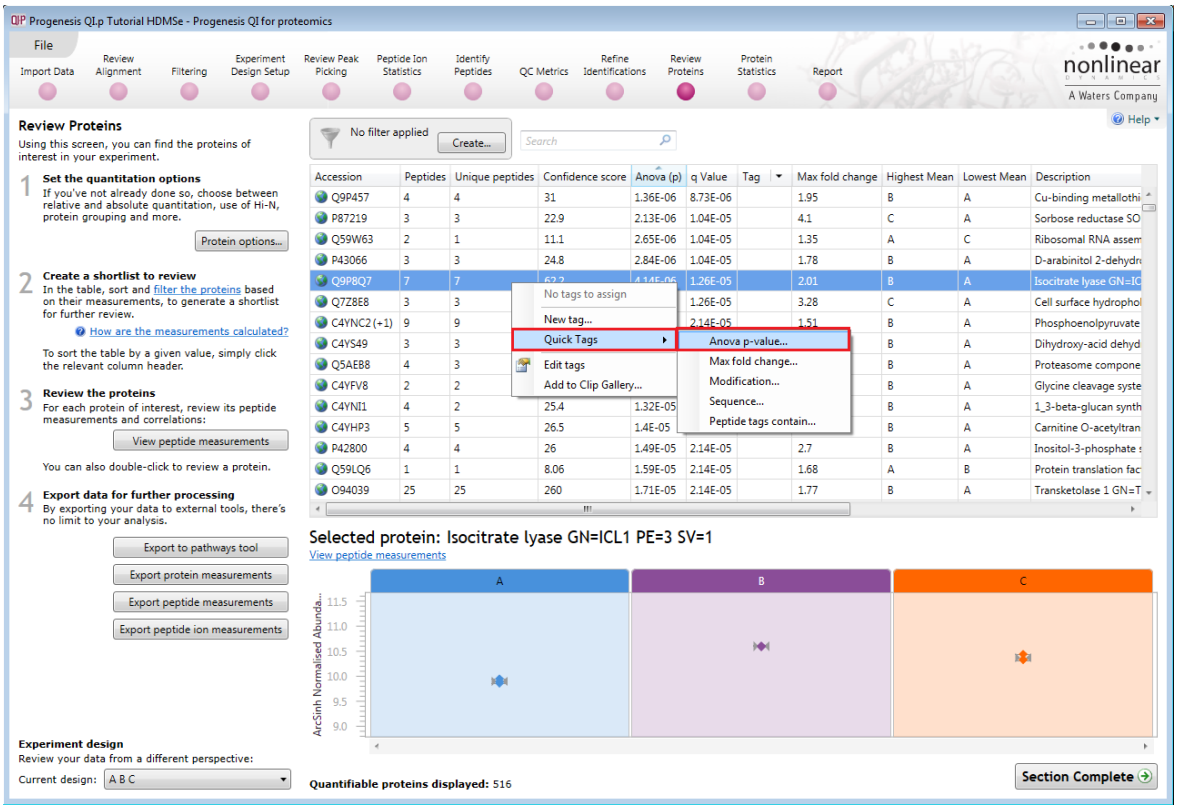
Note: if you have performed conflict resolution then there will be no difference between these methods.

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.

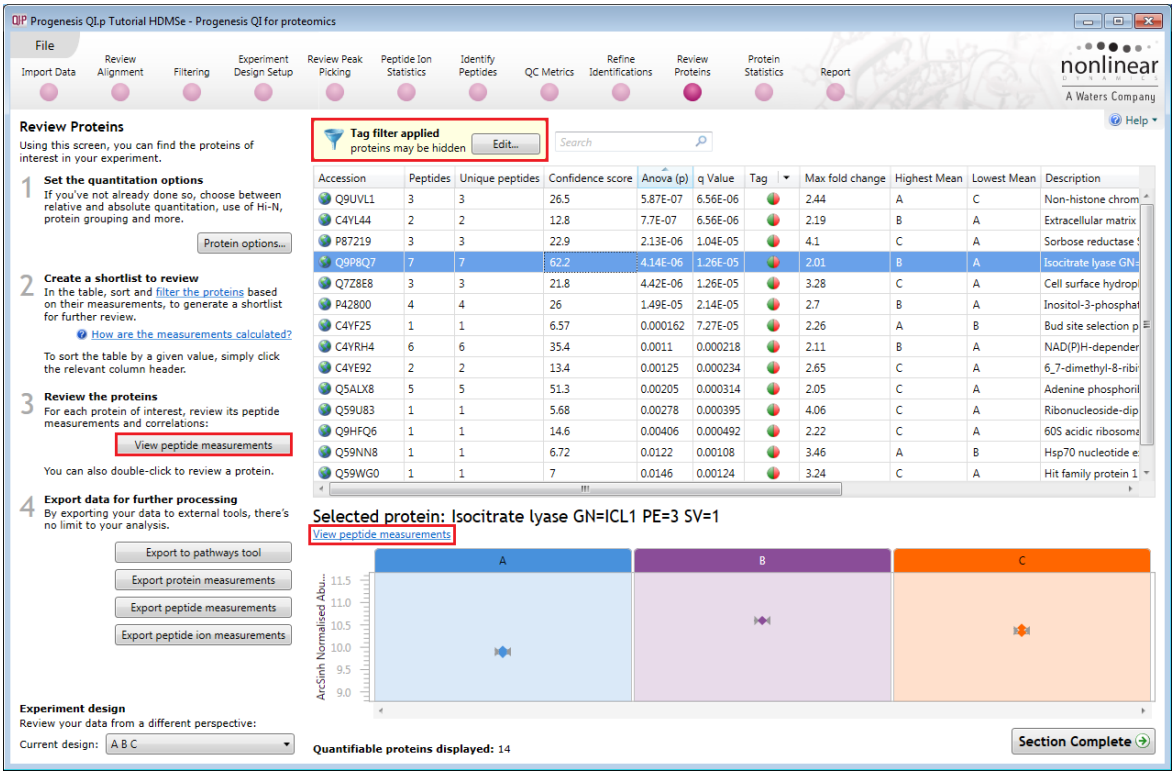
Stage 12: Review Proteins

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 ≤ 0.05 and Max Fold change ≥ 2.



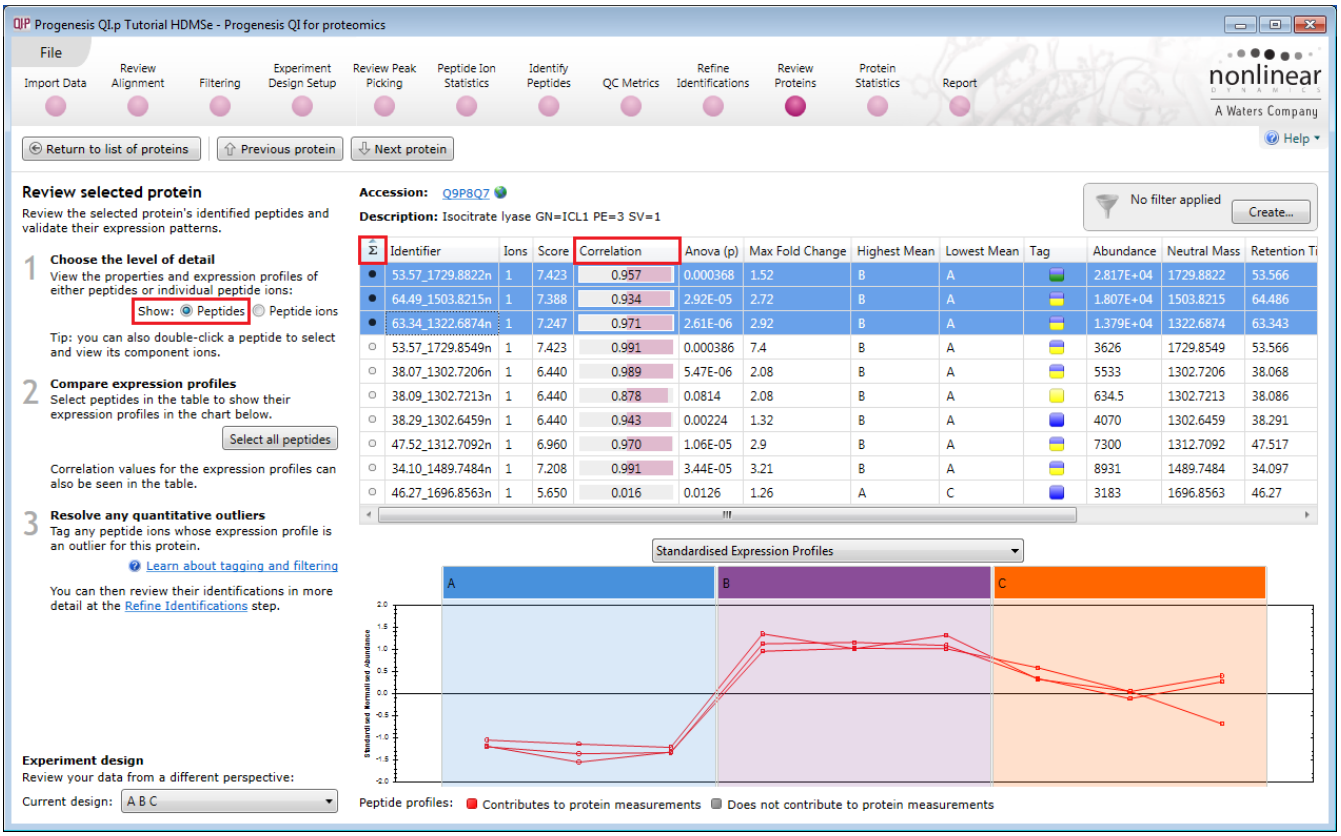
As an example, let us explore Isocitrate Lyase.

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



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

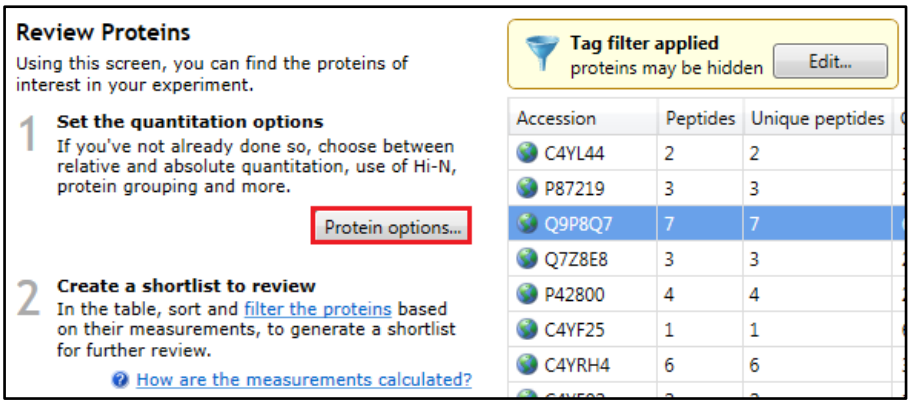
To view the corresponding peptide measurements for the current protein either double click on the protein in the table or use the **View peptide measurements** beside table and ensure that Show **Peptides** is selected.



The solid icon in **Σ** column indicates that the peptide contributes to protein measurements.

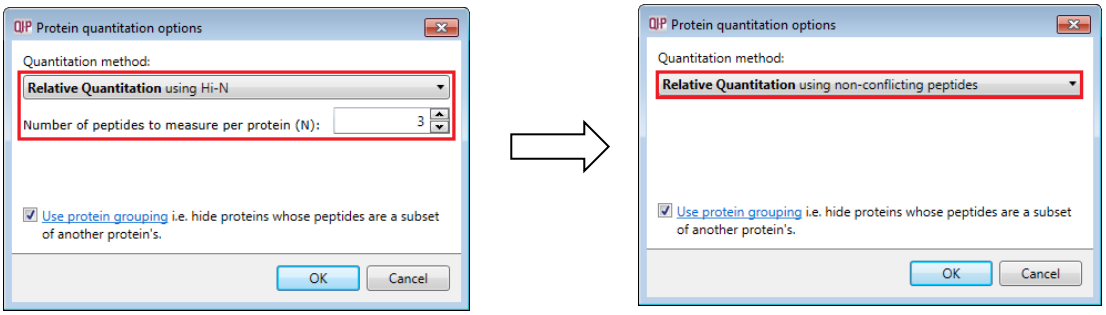
Note: a correlation score (between -1 and 1) for each peptide is available to facilitate the validation of peptide expression.

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

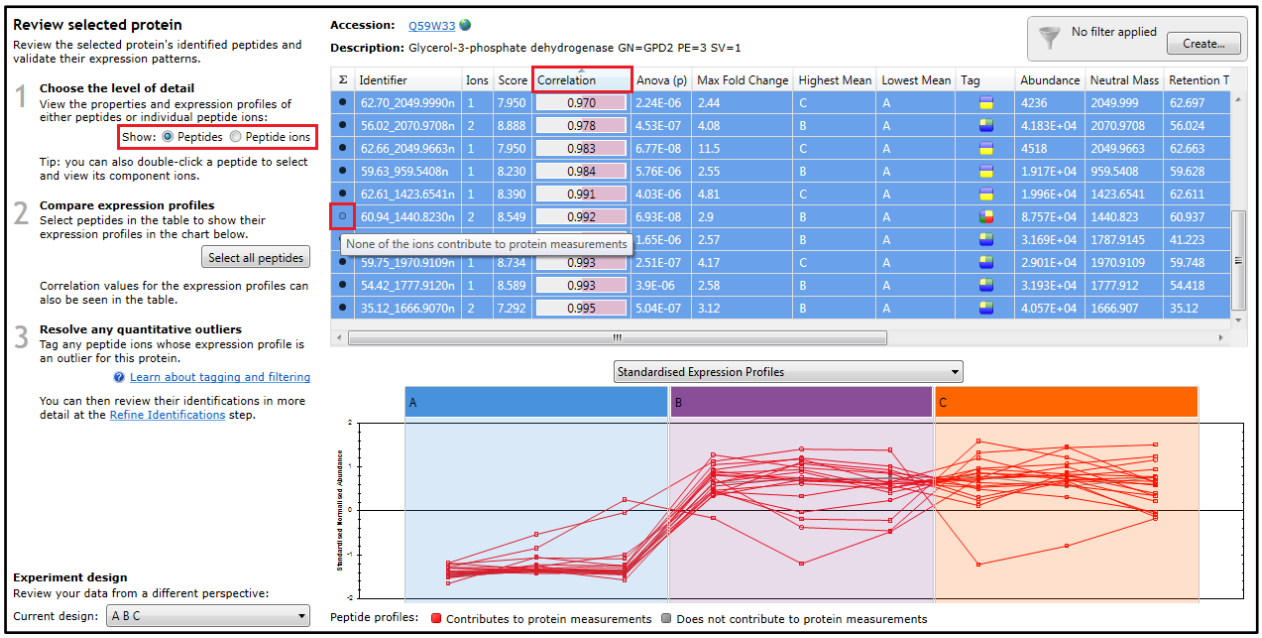


Note: the default quantitation method is based on the Relative Quantitation using Hi-N, in this case n=3 so the top 3 most abundant peptides are used to determine the relative abundance for each protein. Where there are multiple charge states the combined abundance of the charge states is used.

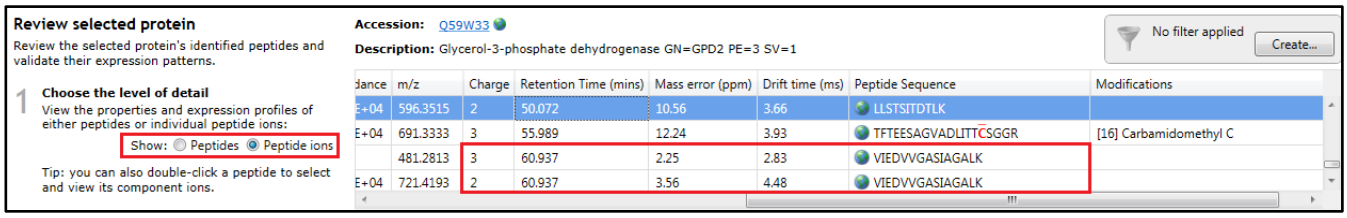
Changing to **Relative Quantitation using non conflicting** peptides will utilise all non conflicting peptides in the abundance calculation. (as shown below)



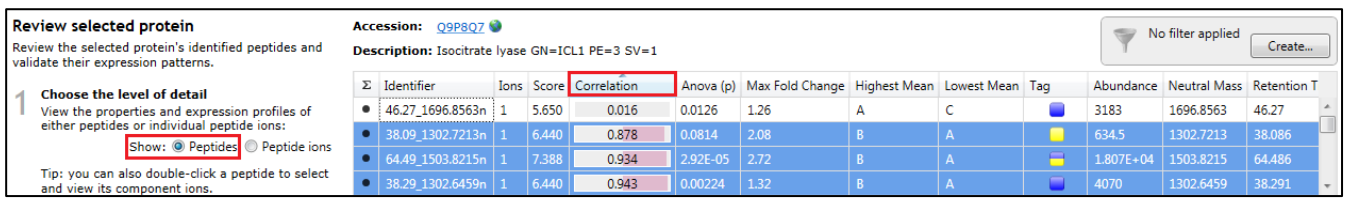
Note: doing this will update the peptide table to show open symbols against conflicting peptides which will not contribute to the quantitation of the protein.



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

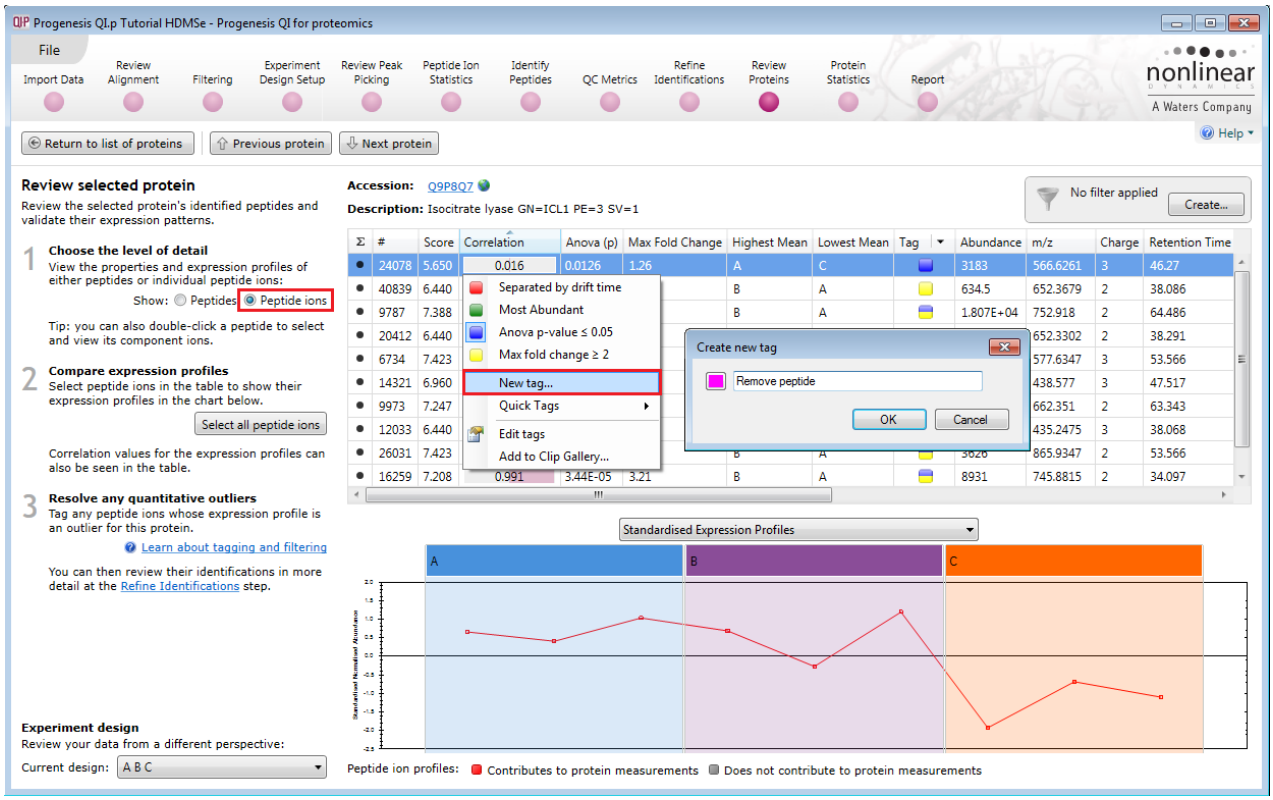


Returning to Isocitrate Lyase and the **Peptides** view of the table

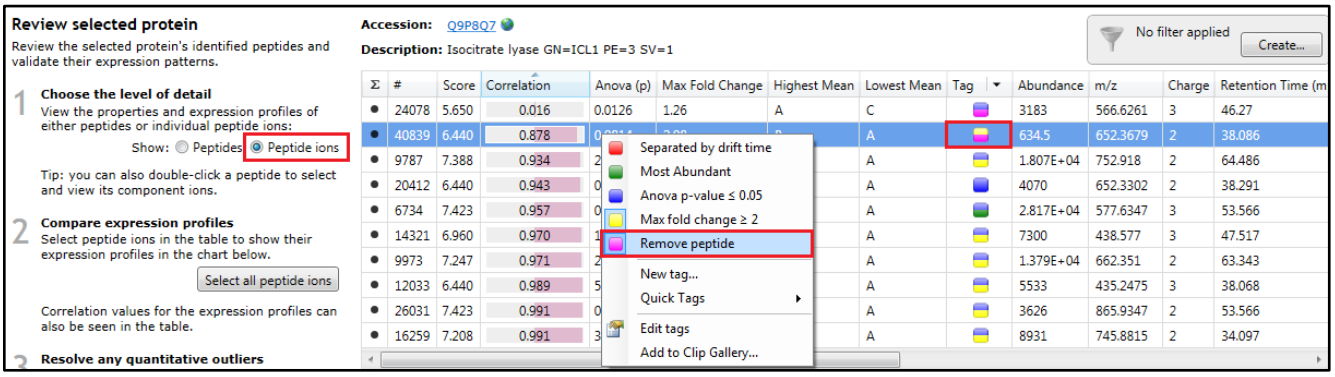


If you order the Peptide table using the **Correlation** column then hold down the Ctrl key and then click on the first peptide (with the lowest correlation) you can remove them from the expression profiles.

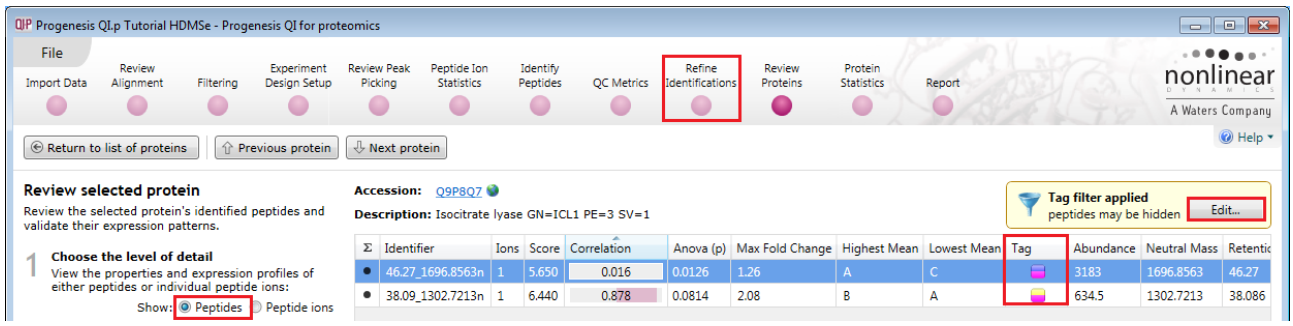
As an example: the removal of a poorly correlated peptide from a protein requires you to first create a tag (at the peptide ion level for the corresponding peptide ions of the peptide you wish to remove (see below).



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.

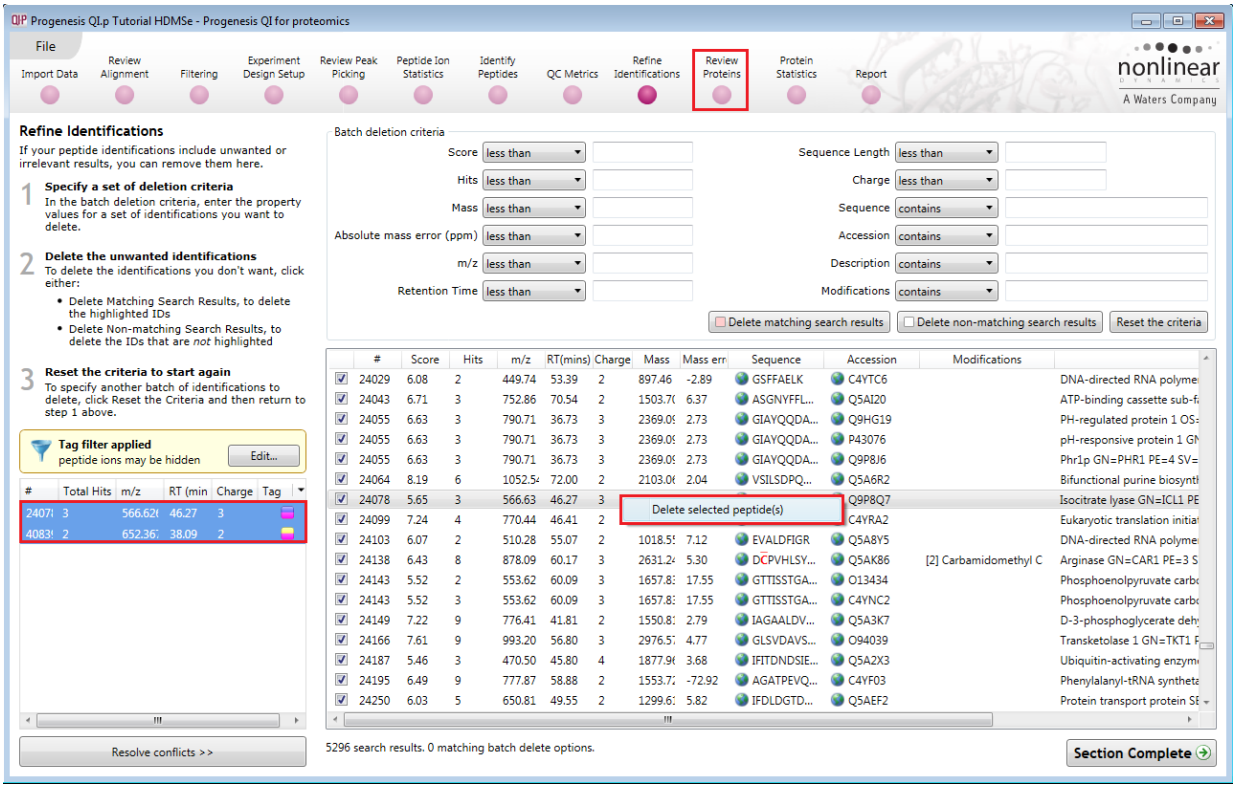


Now at the Peptides level, set up a Tag Filter to display only the poorly correlated peptide ions in this example 2 (with the **Remove peptide** tag).



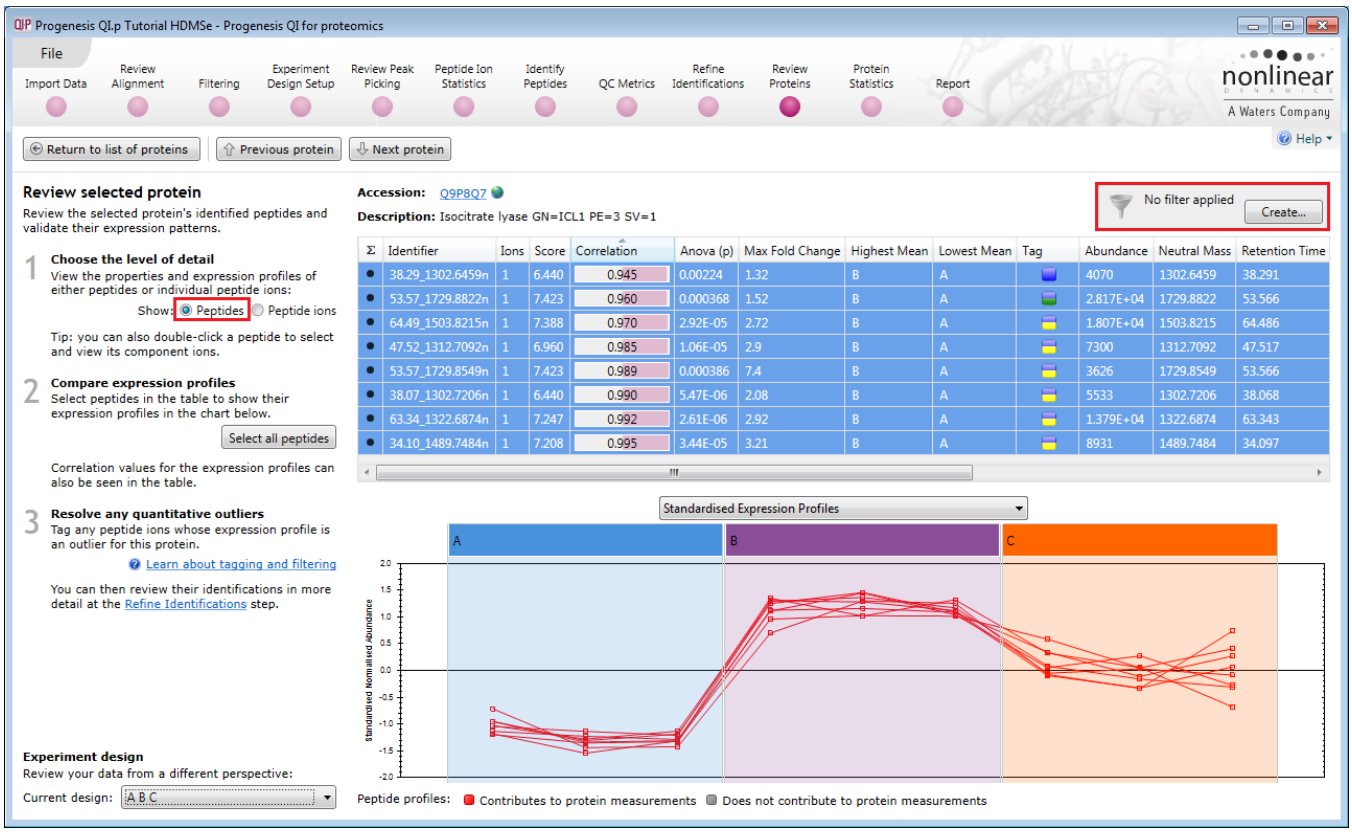
Then click on **Refine Identifications** on the workflow.

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)**.



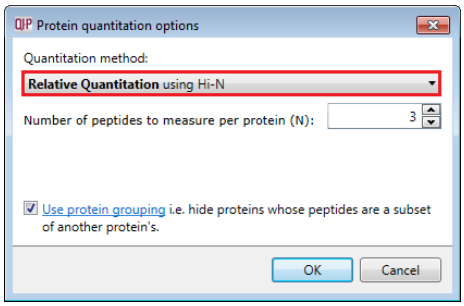
Now return to **Review Proteins,** double click on Isocitrate Lyase (Q9P8Q7) and go to the **Peptides** view. Clear the filter, to view the remaining peptides, and then click **Select all peptides** for Isocitrate Lyase.

Note: the poorly correlated peptides are no longer present.

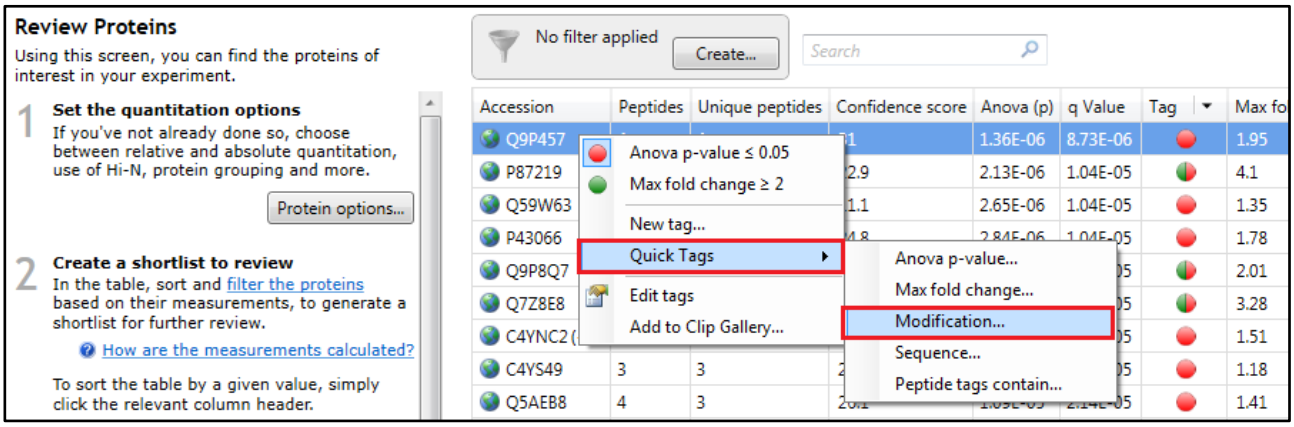


Now return to the protein level by clicking on Return to list of proteins (top left).

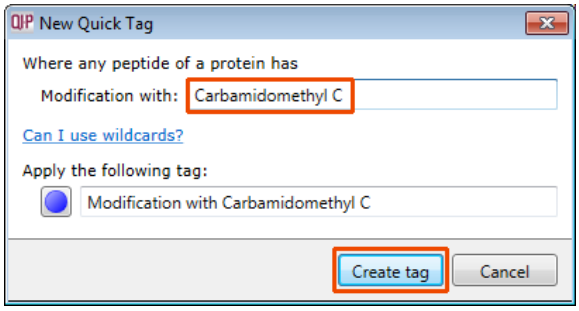
Before creating tags for proteins containing modified peptides, reset the Quantitation method to **Relative Quantitation using Hi-N** using the **Protein options…**

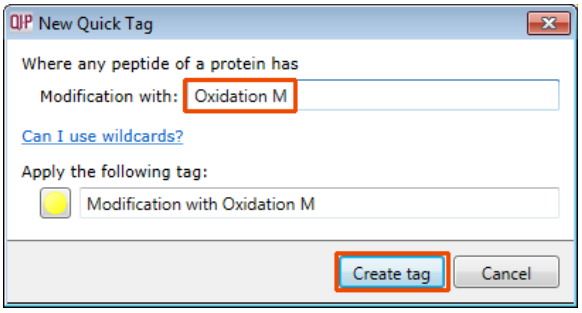


Now right click on the Protein table and select **Modification** from the **Quick tags**.

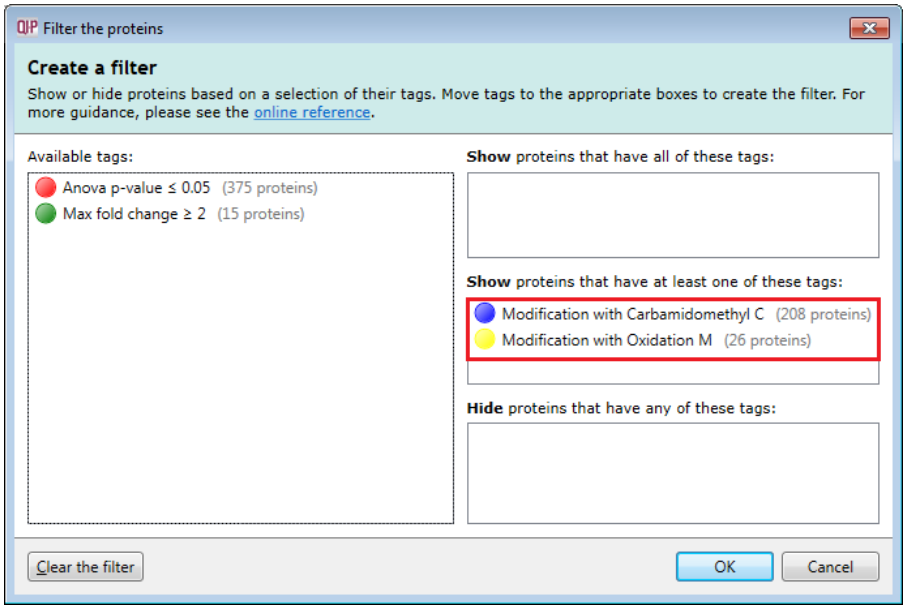


To find those proteins containing peptides with Carbamidomethylated cysteine and Oxidated methionine residues create Quick tags for each modification as shown below.

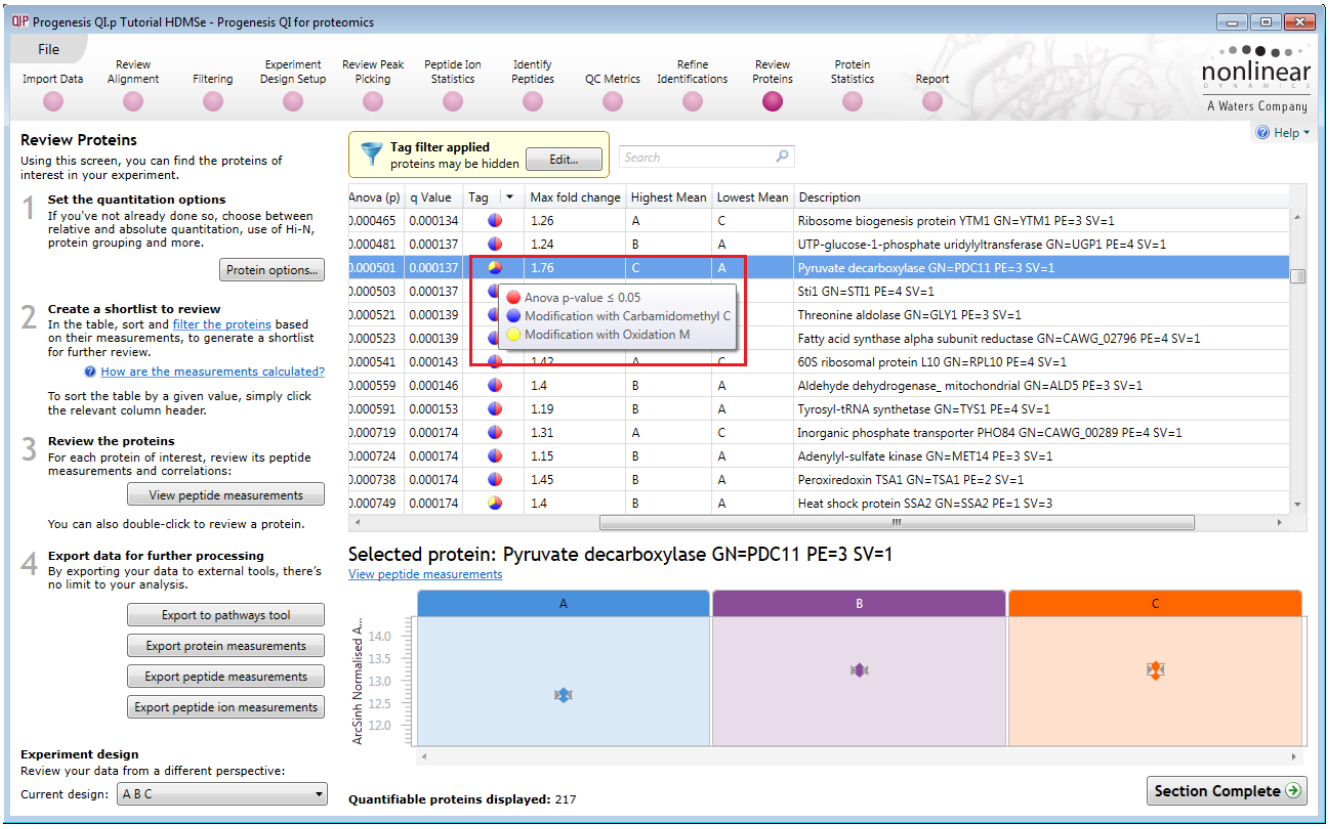




To reduce the table to displaying only these proteins with modified peptides (on cysteines and or methionines) use a tag filter to focus on these proteins by placing the appropriate tags in the **Show proteins that have at least one of these tags:**



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

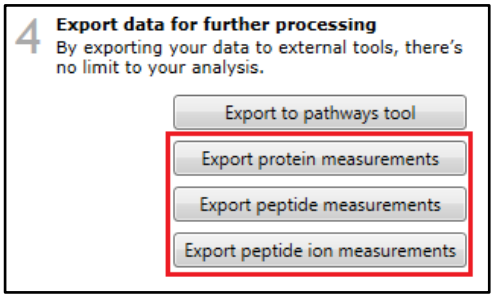


Note: hold the cursor over the tags for a description of the protein's current tags.

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

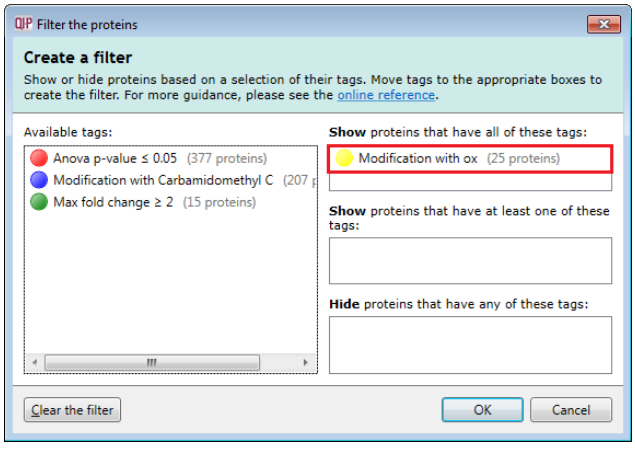
Stage 13: Exporting Protein Data

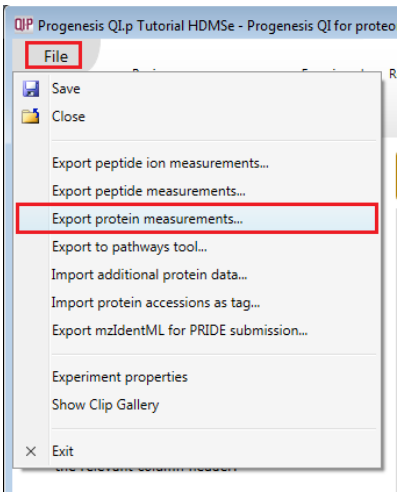
Data can be exported in a csv file format. You can either export the **protein, peptide or peptide ion measurements** using the options in the File Menu or use the buttons under Step 4 both available at the **Review Proteins** stage**.**



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

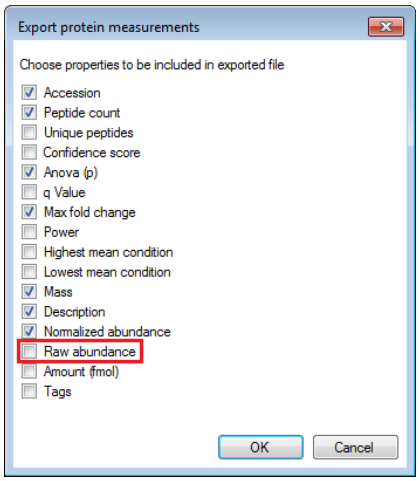
First set the tag filter as shown below. Then select **Export Protein Measurements**.

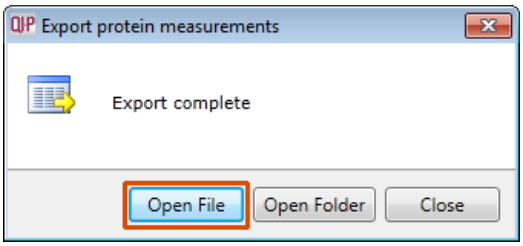




The Export Protein Measurements dialog opens. Select the required fields and click OK. **Save** the file and then open the exported data file using the dialog that opens.

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



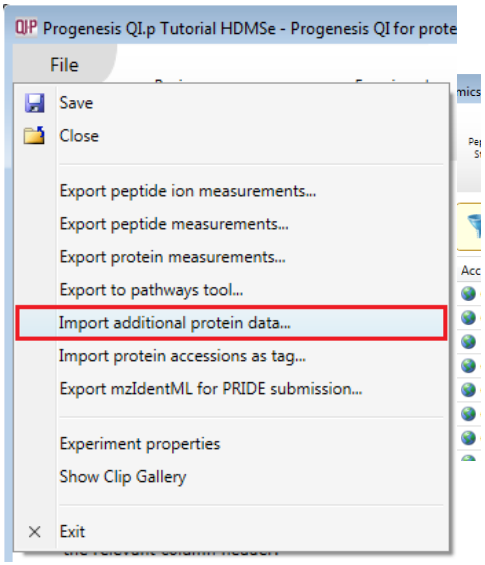


Excel will open displaying the exported protein measurements

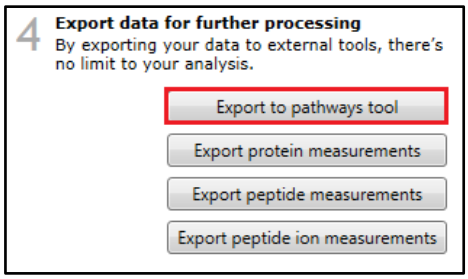
						Normalized abundance								
						A			B			C		
Accession	Peptide count	Anova (p)	Max fold change	Mass	Description	A_01	A_02	A_03	B_01	B_02	B_03	C_01	C_02	C_03
Q59WG3	19	0.000286	1.393265562	91276.19	Cell division control protein 48 GN=CDC48 PE=3 SV=1	6.04165	6.104069	6.382303	7.810237	8.469195	8.124259	9.235389	8.411273	8.167793
Q5A1E2	19	0.000501	1.757985787	62783.45	Pyruvate decarboxylase GN=PDC11 PE=3 SV=1	30.47532	34.81616	35.18093	61.06671	57.59173	54.2401	64.75432	61.17964	50.6951
P46587	34	0.00075	1.395119219	70241.81	Heat shock protein SSA2 GN=SSA2 PE=1 SV=3	26.34676	27.96146	28.2539	36.62435	40.75456	37.80508	33.07268	33.9658	30.17681
Q9Y725	5	0.002606	1.242929018	39469.74	Mannose-1-phosphate guanyltransferase 1 OS=Candid	5.923814	6.143487	6.088348	5.100299	4.794784	4.712066	5.066129	5.133183	4.493118
Q59Z65	5	0.003284	1.239495422	31564.35	Proteasome component PRE2 GN=PRE2 PE=3 SV=1	2.14134	2.00107	1.959733	2.456694	2.641729	2.465155	2.293807	2.248603	2.465606
Q5ADM7	20	0.004259	1.785445695	35947.04	Glyceraldehyde 3-phosphate dehydrogenase GN=TDH	28.27132	37.47768	35.05756	66.21397	52.08768	50.68558	65.07962	63.1759	51.72912
Q59LW3	14	0.007171	1.929316718	187412.7	DNA-directed RNA polymerase GN=RPA190 PE=3 SV=1	4.219746	3.771511	3.794856	6.538796	4.287708	4.245464	8.246794	7.903809	6.588543
C4YTC6	10	0.00903	1.22209187	132058.3	DNA-directed RNA polymerase I 135 kDa polypeptide (	9.059609	10.47197	9.842404	10.69975	11.48251	11.08544	9.237917	9.320345	8.663667
Q59N00	15	0.009063	1.182776974	27285.6	40S ribosomal protein S3 GN=RPS3 PE=4 SV=1	30.95634	32.97579	31.92331	30.09772	32.01223	30.41159	27.94132	28.06409	25.03729
C4YSV1	6	0.020115	1.197574763	15842.55	60S ribosomal protein L25 GN=CAWG_05170 PE=4 SV=1	19.09519	21.06827	20.28896	21.3714	20.04349	20.17272	17.93076	18.02852	15.46766
C4YQM1	4	0.022335	1.124601067	15995.81	40S ribosomal protein S15 GN=CAWG_02780 PE=4 SV=1	7.402242	7.74925	7.425526	7.501278	7.528884	7.452533	6.944223	6.957132	6.174225
P30575	18	0.026576	1.713187284	47231.58	Enolase 1 GN=ENO1 PE=1 SV=1	21.86692	29.96505	28.59235	51.95777	35.8221	35.52757	50.63594	49.372	37.77399
C4YHD6	7	0.031059	1.083204829	76870.01	NADPH-cytochrome P450 reductase GN=CAWG_03482	4.676369	5.02495	4.740551	5.020557	4.936881	4.881926	4.561702	4.682273	4.455524
C4YTC4	11	0.035844	1.174491751	91821.43	Glutaminyl-tRNA synthetase GN=CAWG_05411 PE=4 SV	9.448774	10.68459	9.762518	9.3306	8.400595	8.364039	8.782928	8.725951	7.945433
C4YRA2	9	0.041867	1.150593676	59119.25	Eukaryotic translation initiation factor 2 gamma subun	4.030048	4.073439	4.109665	5.053444	4.451311	4.54762	4.49272	4.689095	4.194535
P41797	27	0.056709	1.244772849	70495.2	Heat shock protein SSA1 GN=SSA1 PE=1 SV=2	15.05905	11.85971	11.42823	15.36704	16.33804	16.0282	13.4975	13.78449	12.17764
Q5A516	12	0.059216	1.179345683	32926.06	ADP_ATP carrier protein GN=PET9 PE=3 SV=1	30.36237	34.1933	32.87809	34.50778	35.19443	33.92876	30.93623	30.89512	26.04023
Q5ABS1	7	0.06238	1.069201884	14423.79	Ubiquinol-cytochrome c reductase complex 14 kDa prc	7.27818	7.431786	7.889224	8.234258	7.998214	7.930625	7.727017	7.876603	7.862713
Q5A900	9	0.078281	1.248516734	26987.34	40S ribosomal protein S2 GN=RPS21 PE=3 SV=1	15.07801	16.72155	15.13405	14.1835	12.10492	11.30308	15.55814	15.47995	12.76609
Q59M82	19	0.08783	1.124167619	190630.7	Clathrin heavy chain GN=CHC1 PE=4 SV=1	9.715146	10.26357	10.03633	10.34802	10.91702	9.966933	9.749938	9.565339	8.467036
C4YR46	38	0.099847	1.121176792	117623.1	Elongation factor 3 GN=CAWG_04545 PE=4 SV=1	23.97338	26.44841	25.42621	27.59592	29.83916	27.60393	26.99834	26.37865	23.40255
Q5ACQ0	11	0.137185	1.118347026	77833.41	Long-chain-fatty-acid-CoA ligase 4 GN=FAA4 PE=4 SV=	9.58484	10.05479	9.555411	9.596871	8.595828	8.515559	8.989106	9.185083	7.931336
P16017	19	0.234967	1.194311094	50468.03	Elongation factor 1-alpha GN=TEF1 PE=1 SV=1	68.59949	67.33289	65.09681	70.54589	49.78468	48.19559	69.49089	73.01285	58.76892
Q59ZX4	14	0.314524	1.114106362	39269.36	60S ribosomal protein L4-B GN=RPL4B PE=4 SV=1	37.76197	40.11539	38.34233	39.72134	36.06694	34.58901	37.5628	36.55015	30.20356

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.



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.

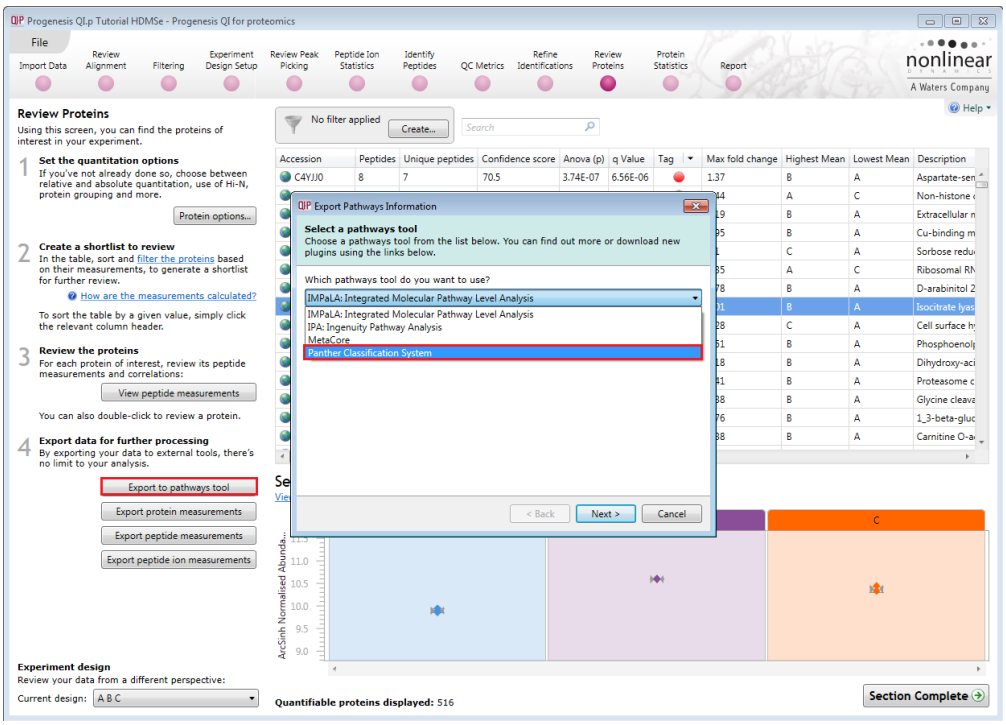
MetaCore: MetaCore is an integrated software suite for functional analysis of experimental data. MetaCore is based on a curated database of human protein-protein, protein-DNA interactions, transcription factors, signaling and metabolic pathways, disease and toxicity, and the effects of bioactive molecules.

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 100)

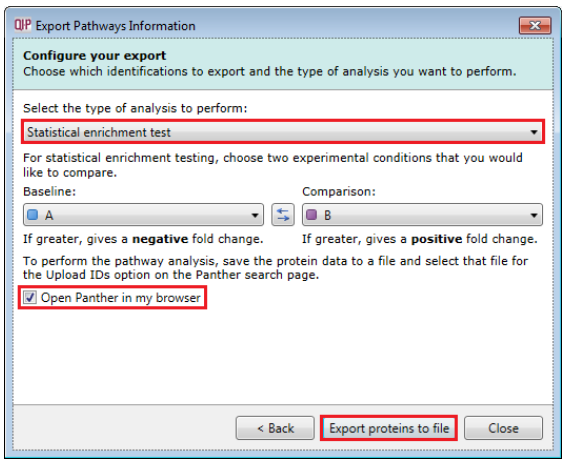
As an example of exporting to a Pathways tool first use the tag filtering to 'focus' on the set to export. Then click **Export to pathways tool**, select **Panther** and then the test to be performed.

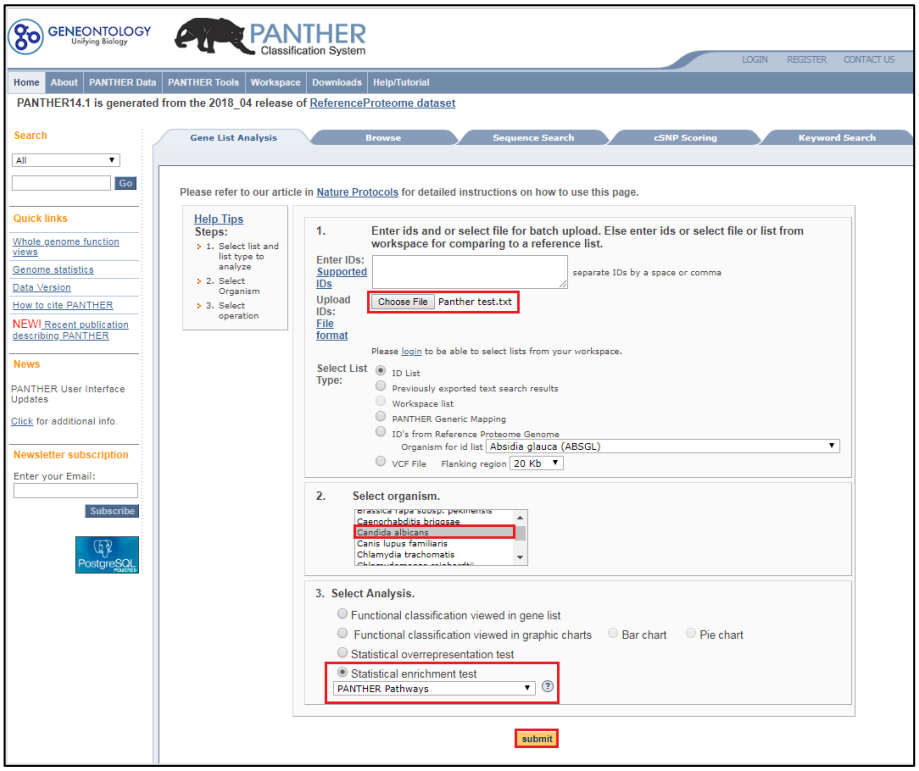


Select either **Statistical over-representation test** or **Statistical enrichment test.**

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

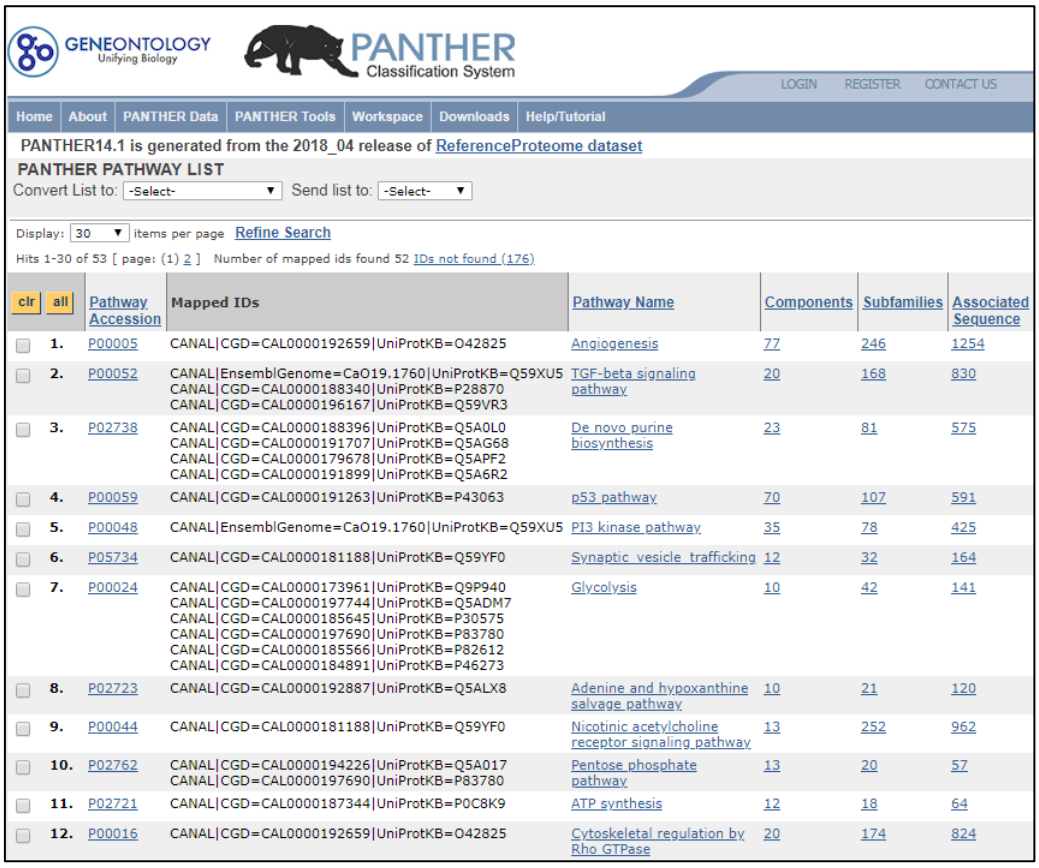
When Panther opens locate the file, select the appropriate organism (Candida albicans) and tick Statistical enrichment test.





Click **submit**.

Analysis results list is returned:



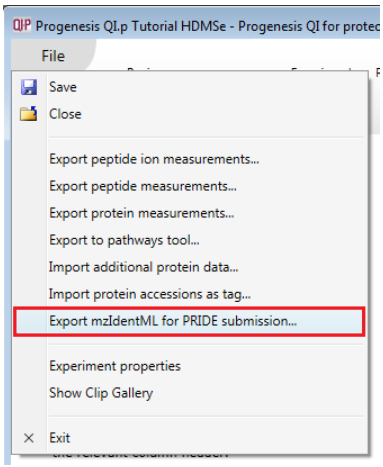
Click to explore returned analysis.

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

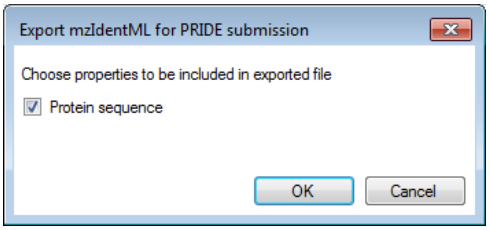
If you are submitting your raw data and results to PRIDE using the **mzIdentML** Complete submission process (described in section 2A on the PRIDE site) then it is now possible to export the identification results as mzIdentML which can be checked in **Pride Inspector tool** as part of the submission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

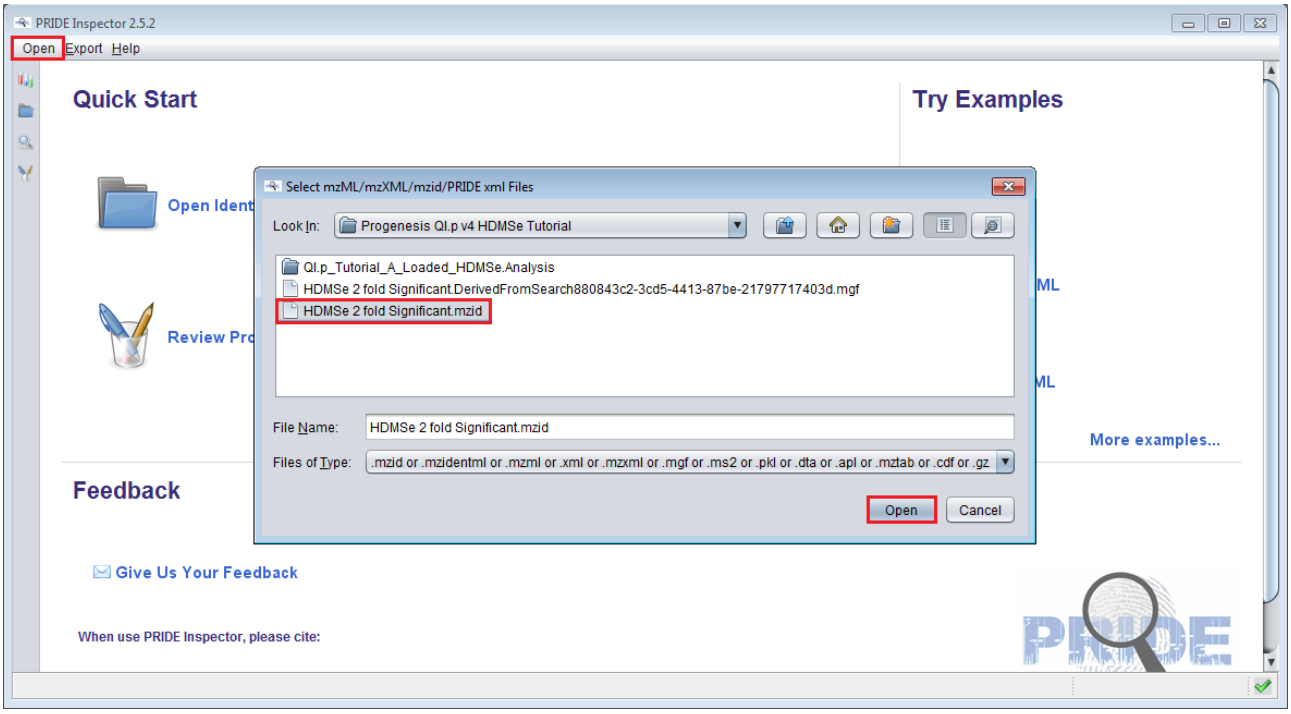
Export Identifications is carried out at the Review Proteins stage of the workflow. Click on the **File** menu and select **Export mzIdentML for PRIDE submission….**

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.

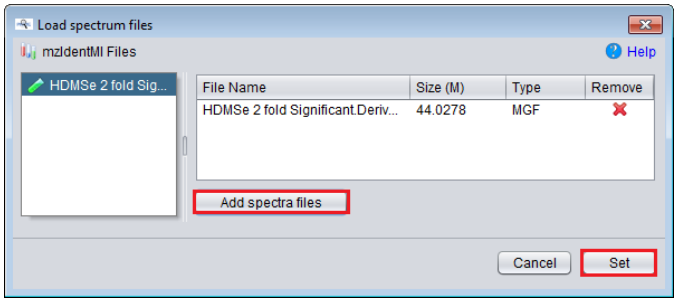
Open PRIDE Inspector and locate and select the mzid file to import.



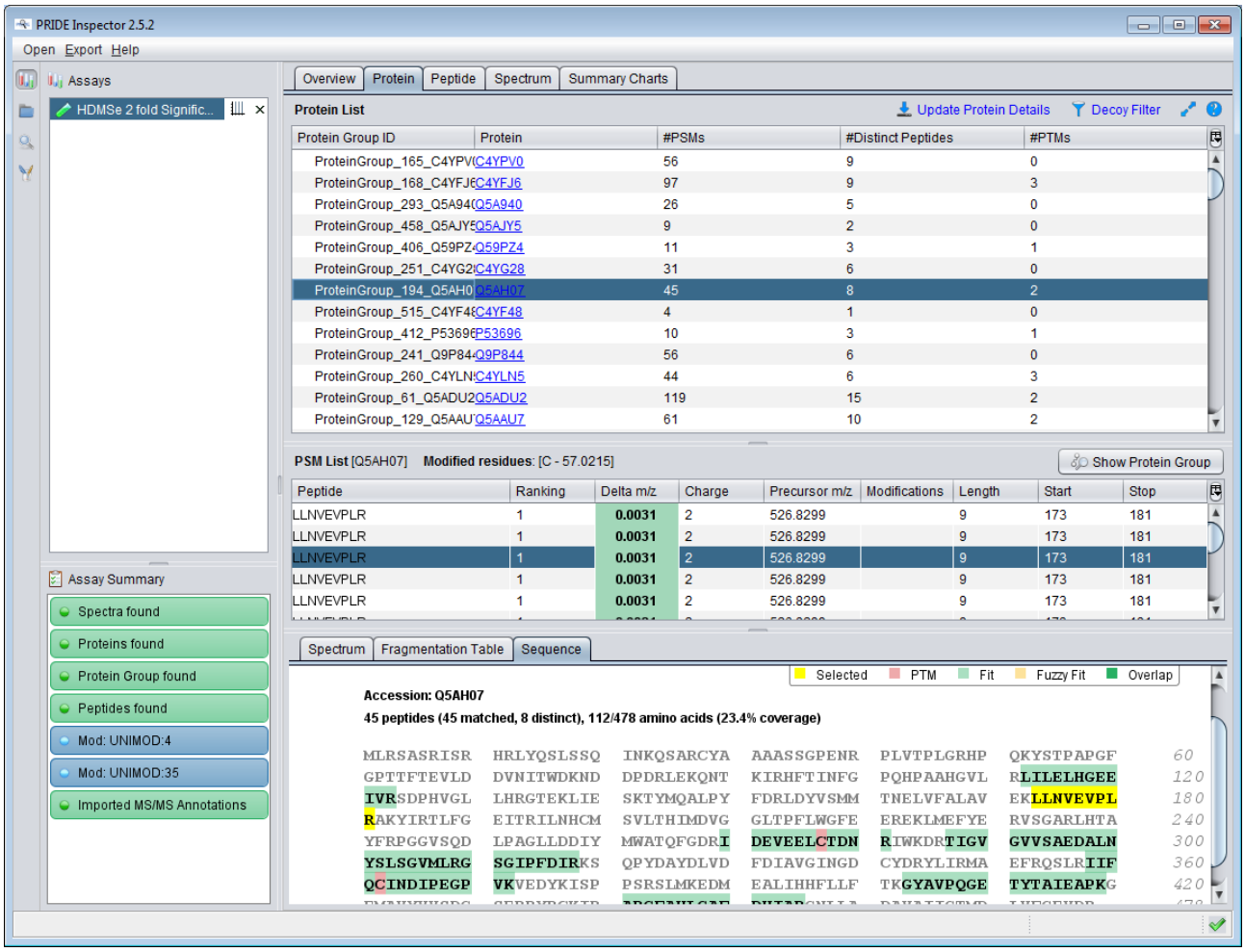
Click open.

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

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



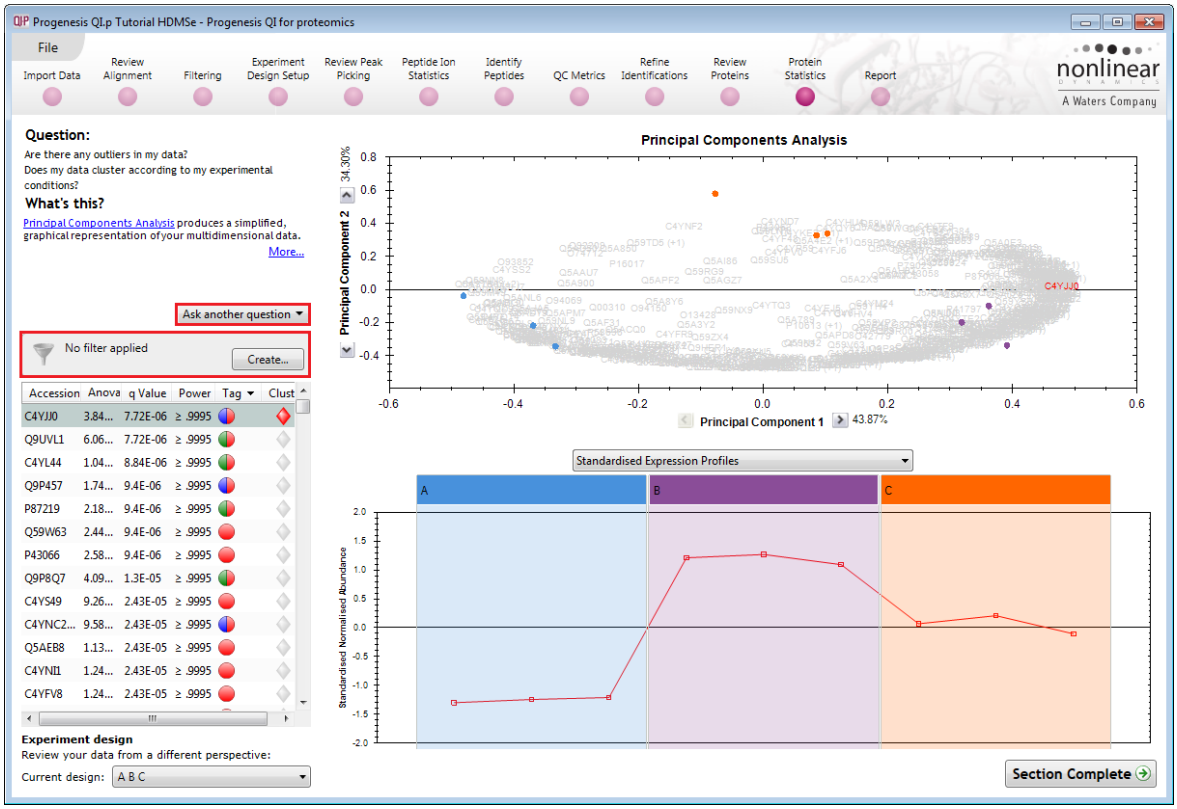
PRIDE Inspector will open allowing you to check the data.



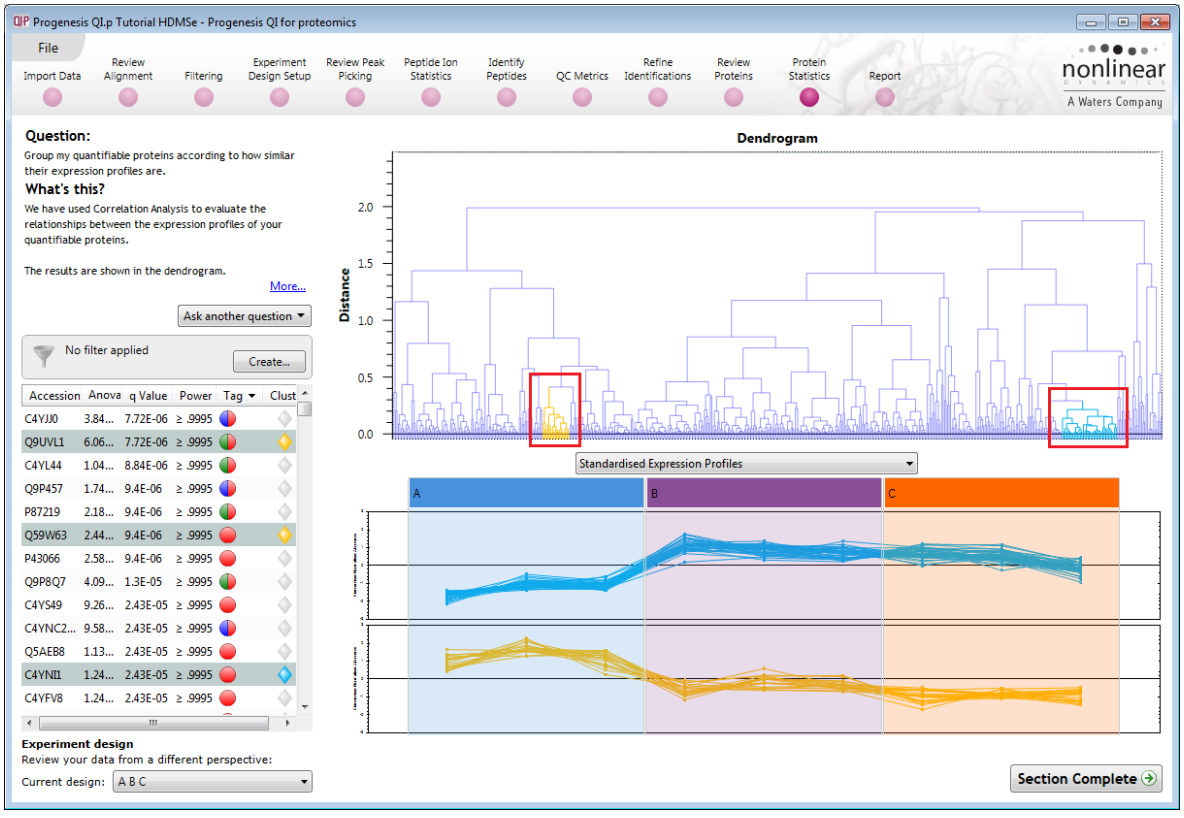
Once checked you can submit the results data to PRIDE, along with your raw data, as part of your submission process, following the guide lines provided on the PRIDE site.

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.

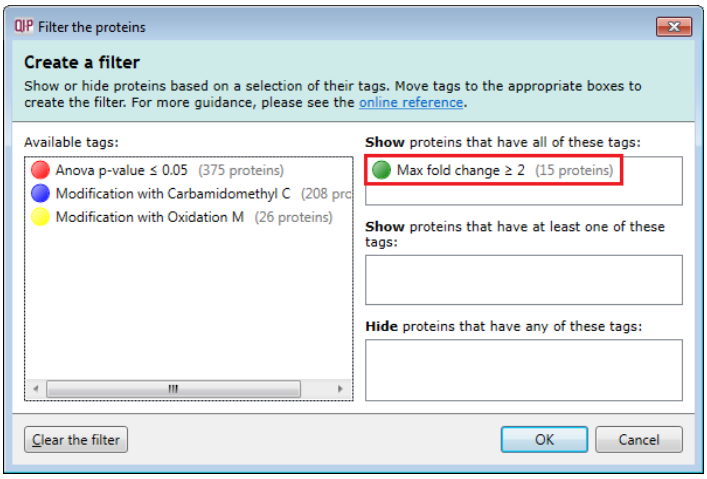


Now move to the **Report** section to report on Proteins and /or peptides.

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.**

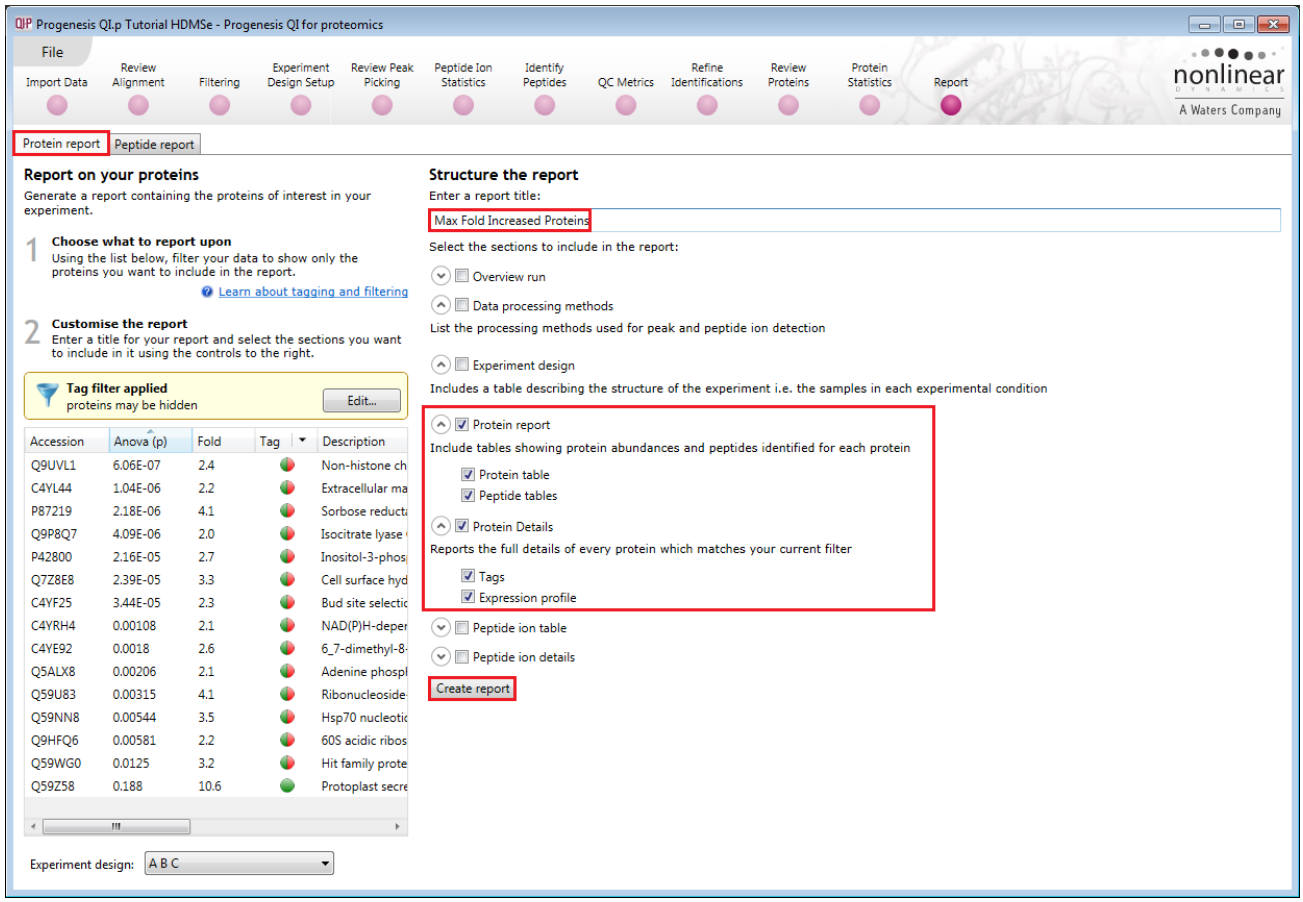
Note: this facility is used to generate Html reports on a limited selection of Proteins in your data. Creating a report on all the data in your experiment can take a long time



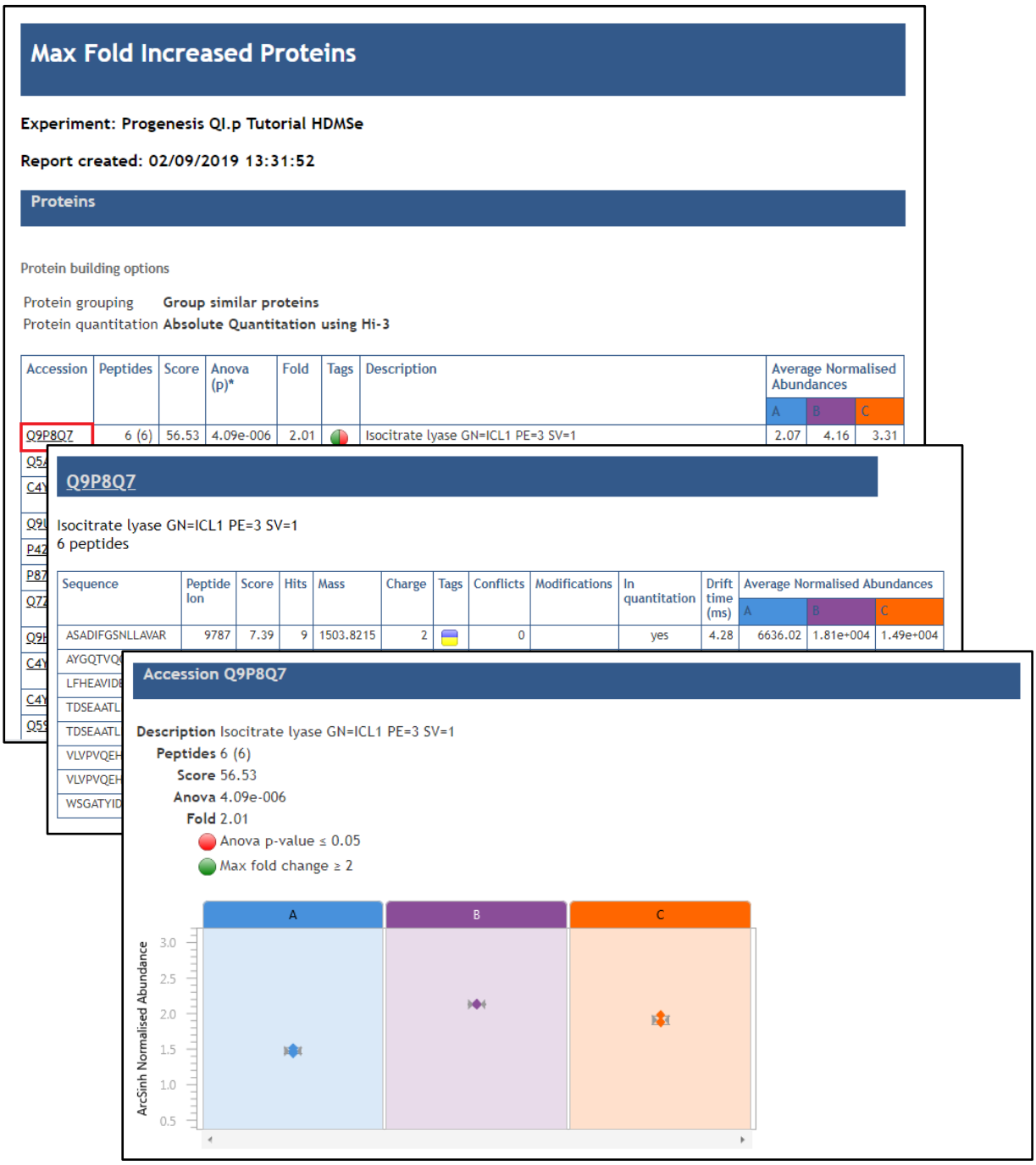
As an example we will create a report for **only** the proteins showing a Max Fold change of greater than 2.

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.



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



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.

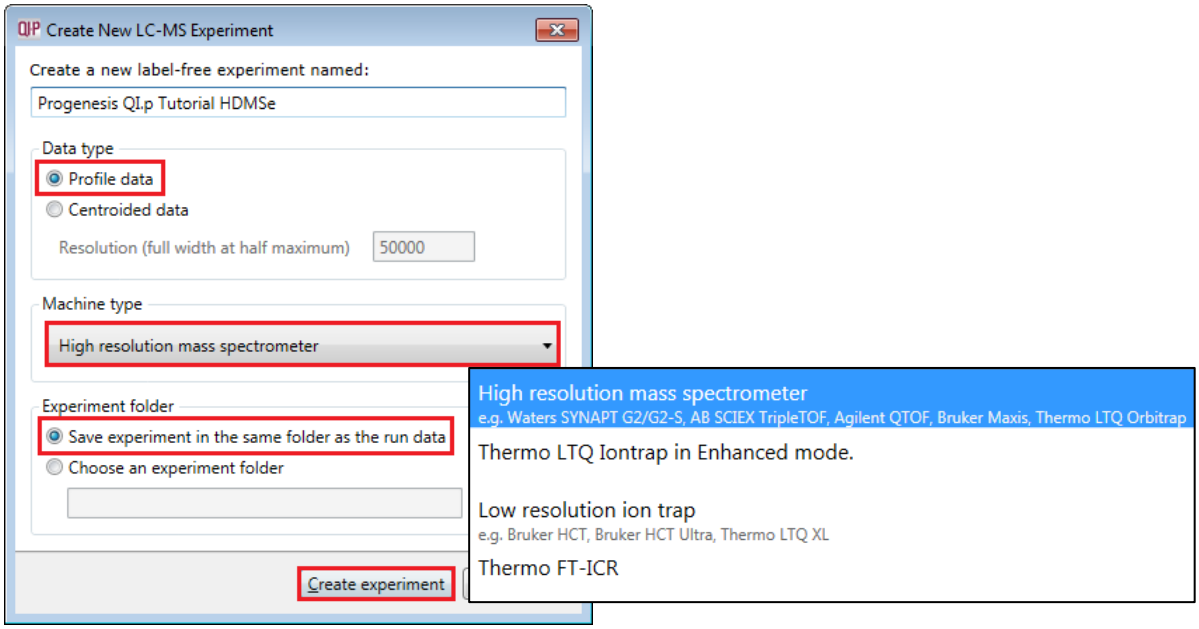
Note: there are separate panels for reporting on Proteins and Peptides.

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'.

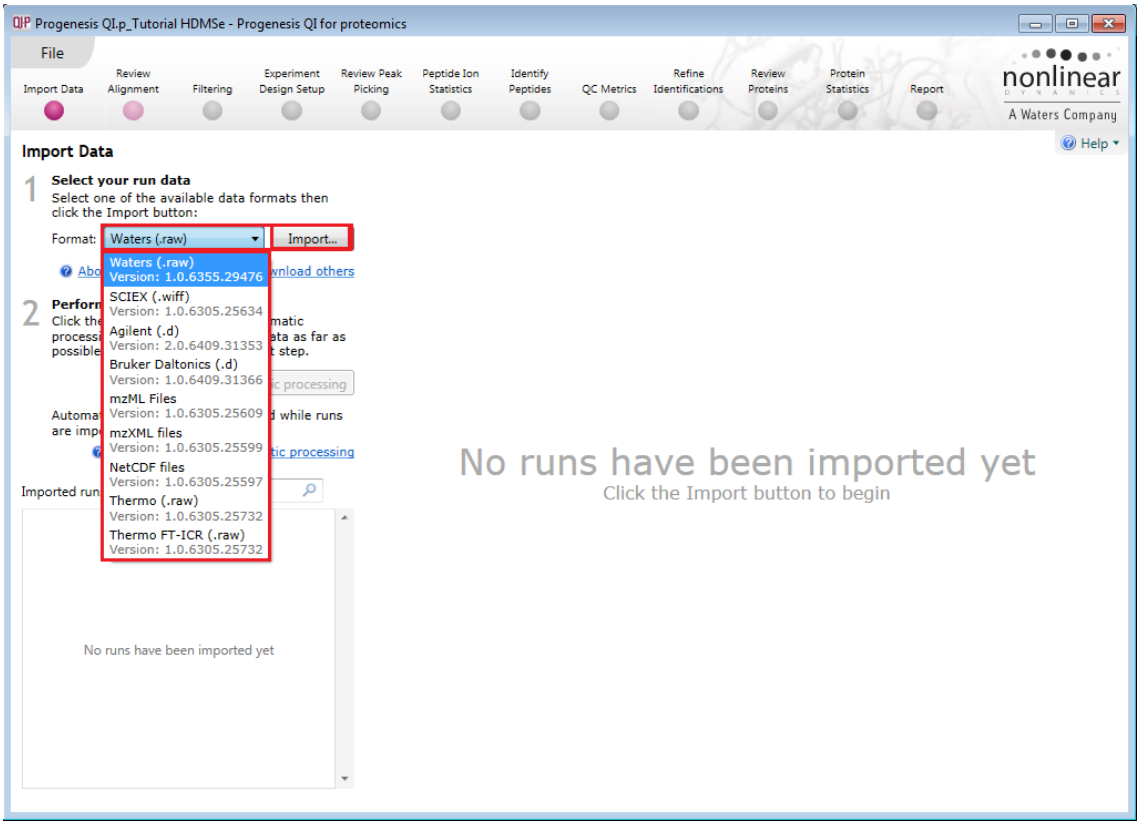
Note: if you have converted or captured the data as centroided then select Centroided data and enter the Resolution for the MS machine used.



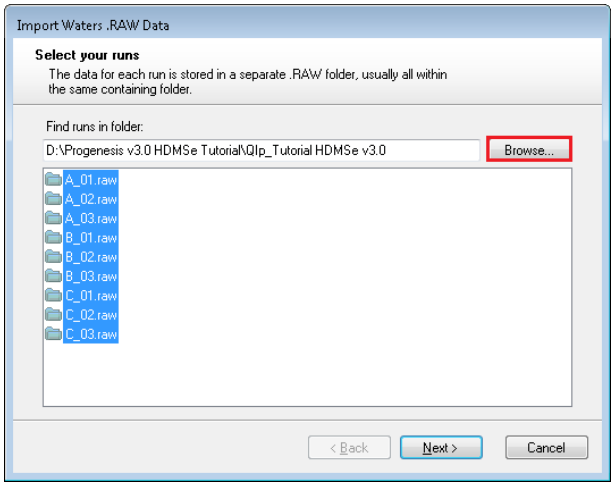
Click **Create experiment** to open the LC-MS Data Import stage of the workflow.

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

Then locate your data files using **Import...**

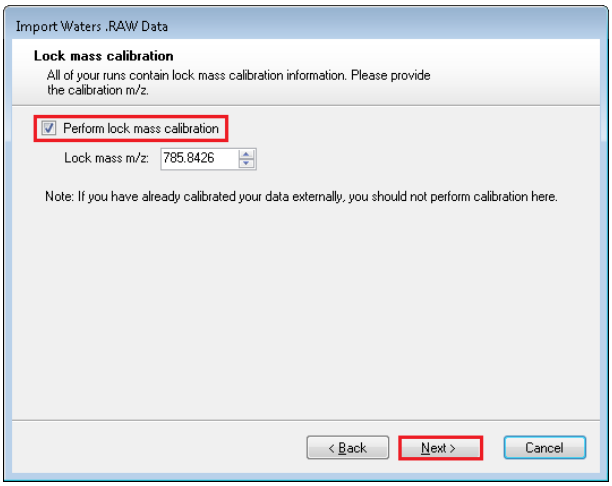


Locate and select all the .RAW folders (A_01 to C_03).



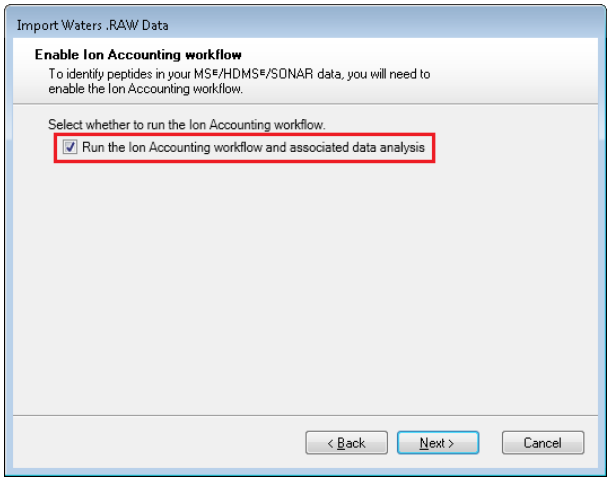
On importing, the lock mass calibration is read and presented on this dialog

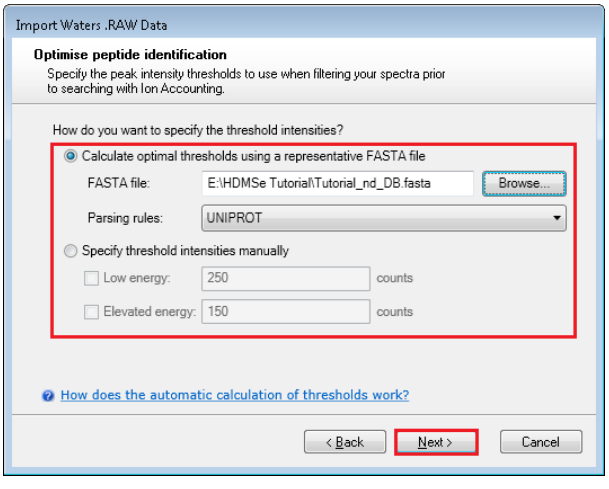
You can, if required, alter the lock mass calibration at this step.



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.





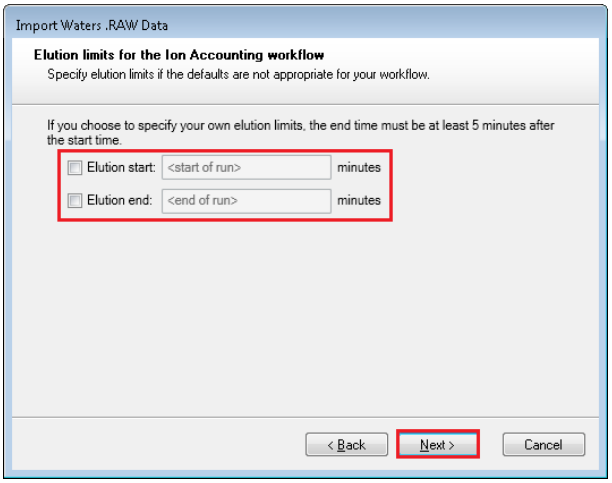
If you choose to determine the thresholds automatically then Progenesis determines appropriate thresholds by sampling each run and finding the thresholds that yield the most protein identifications in the sample area. For each run, Progenesis performs the following steps:

- 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.

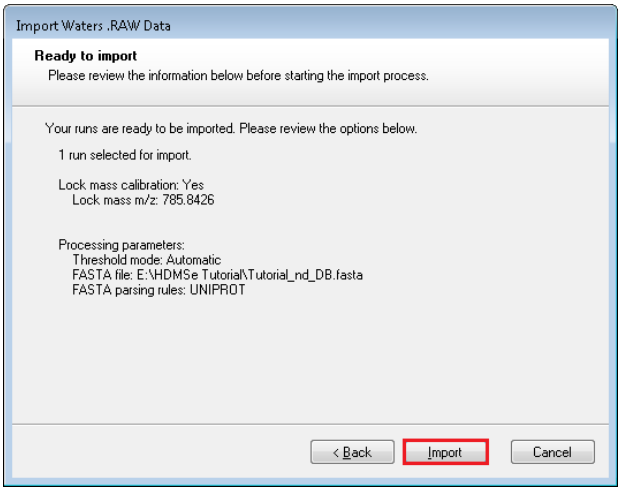
- Finally, it selects the thresholds that resulted in the largest number of protein identifications and applies those to the whole run. If more than one set of thresholds results in similar numbers of identifications (within 10% of the maximum), it will choose the highest thresholds as a way of optimising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

Note: for HDMSe the default settings are 150 and 30 and for MSe the default 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.



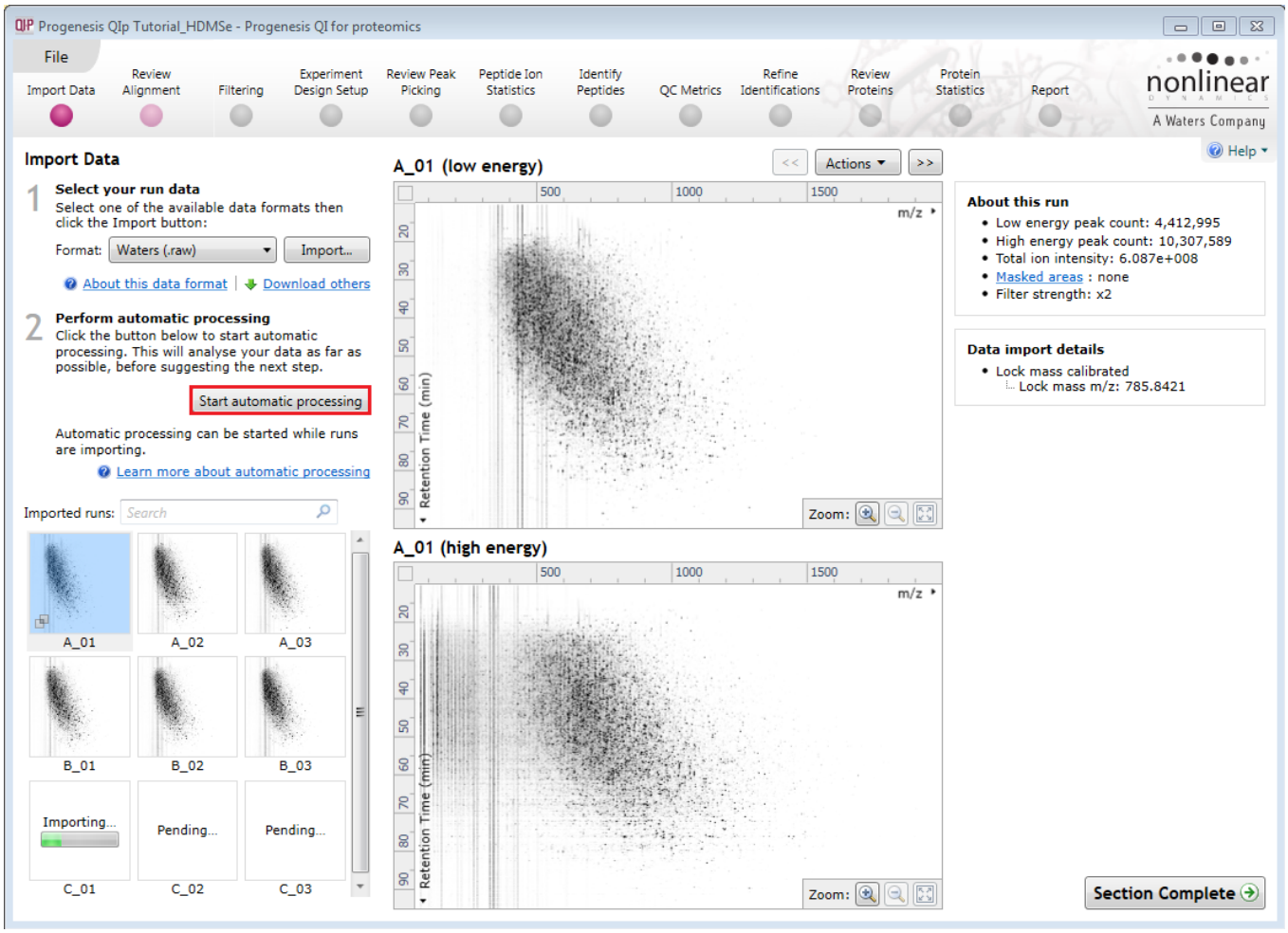
A summary of the loading parameters is provided before you click Import



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.

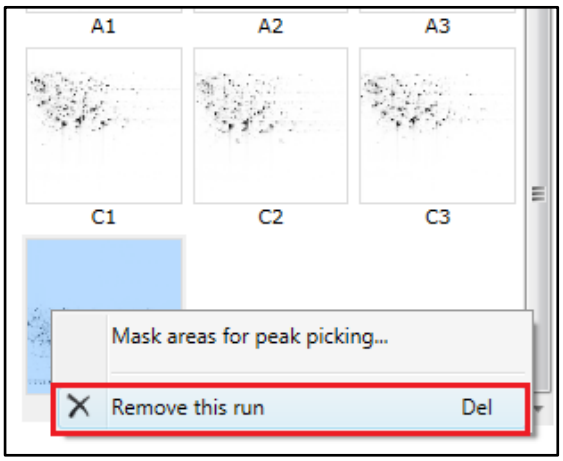


For details of setting up the steps in the automatic processing wizard return to Stage 2A page 7.

Review Chromatography

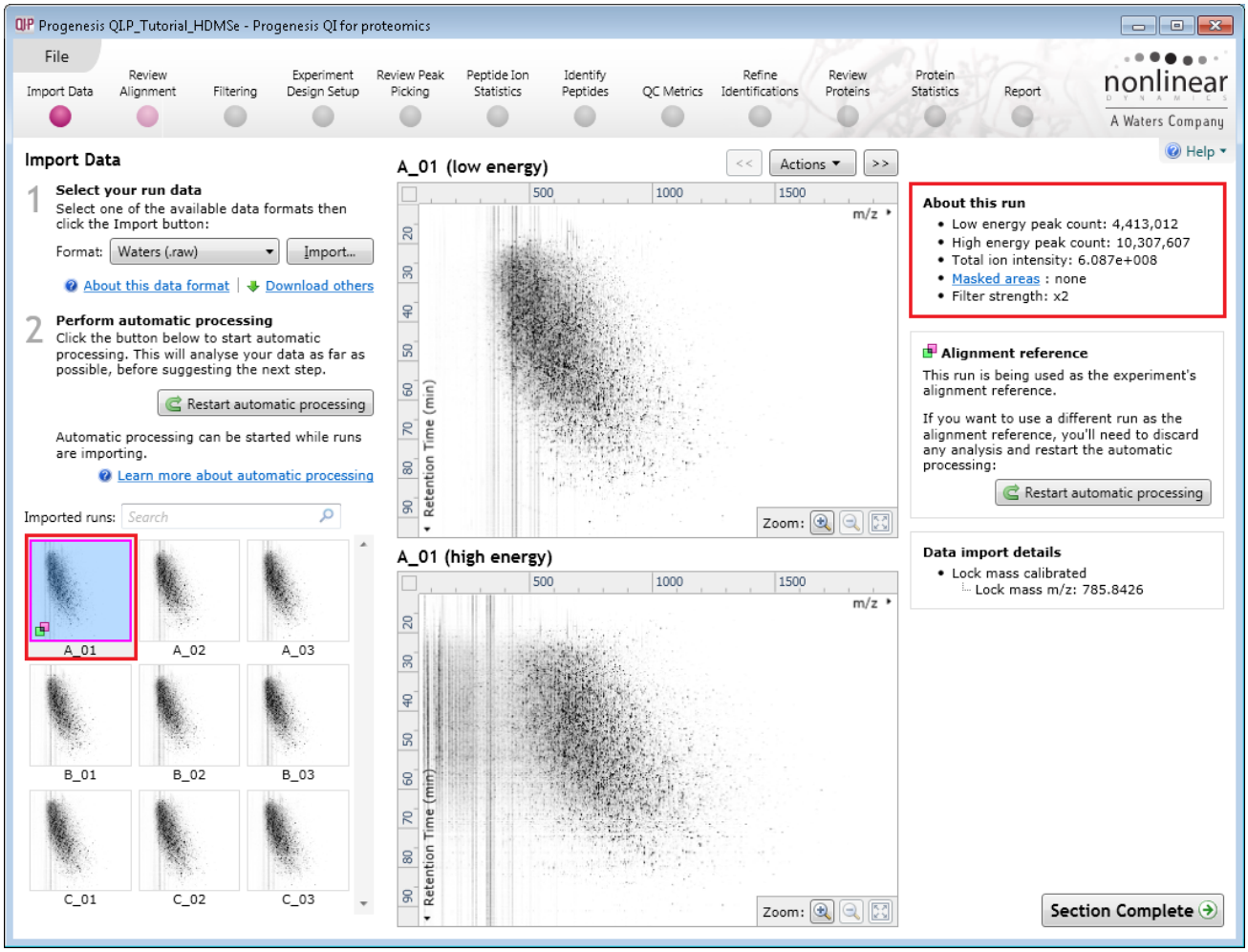
Each data file appears as a 2D representation of the run. If you created a **profile** experiment, at this stage you will be warned if any of the data files have been 'centroided' during the data acquisition and conversion process, as files must be of one format or the other.

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.

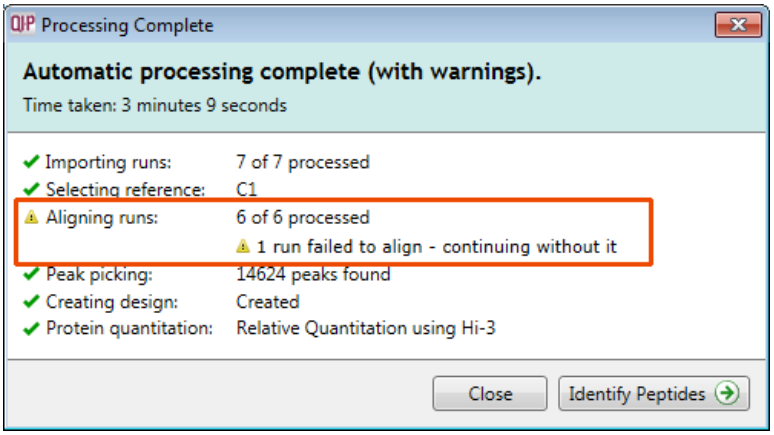
Note: you will be offered the automatic alignment if you have not performed it automatically already.

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

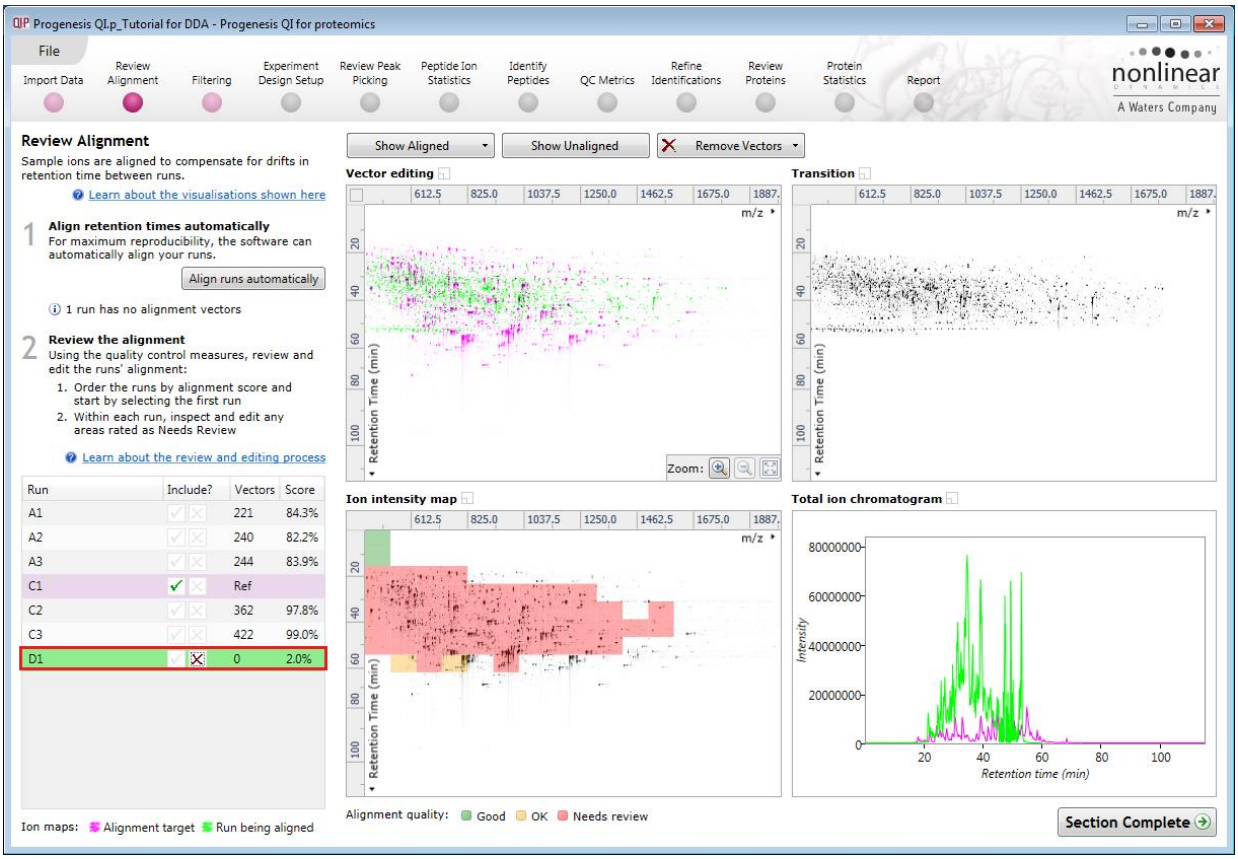
Appendix 2: Stage 1 Processing failures

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.



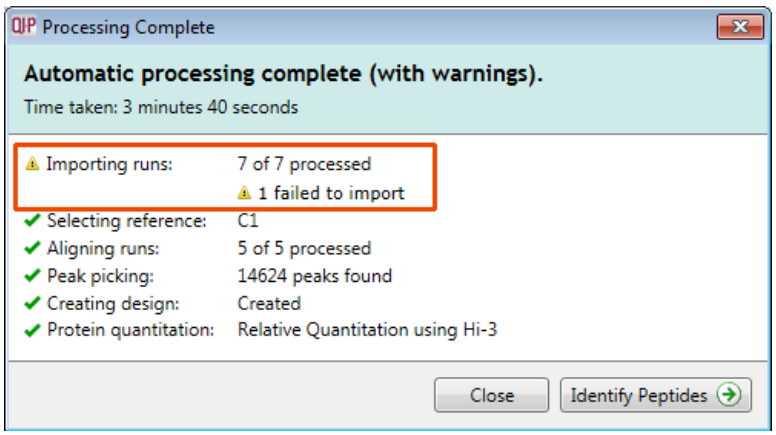
Note: in this example the run that failed to align will not contribute to the peak picking and will be excluded at the alignment stage (a cross appears in the include column).



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.

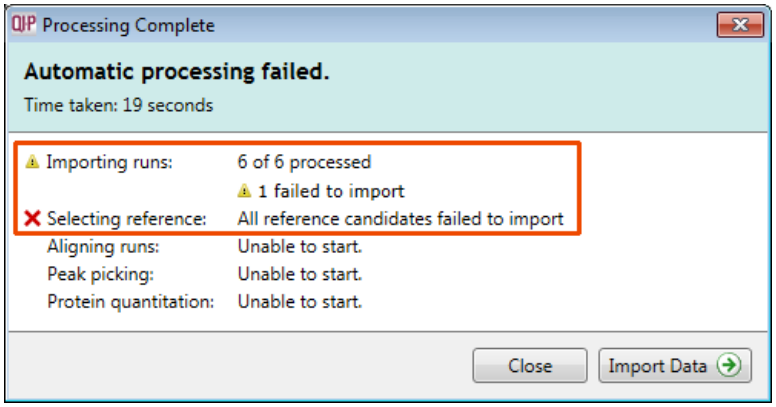
If some runs in a data set fail to import (but not all), the automatic processing will continue informing you that one or more runs have failed to import.



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).



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.

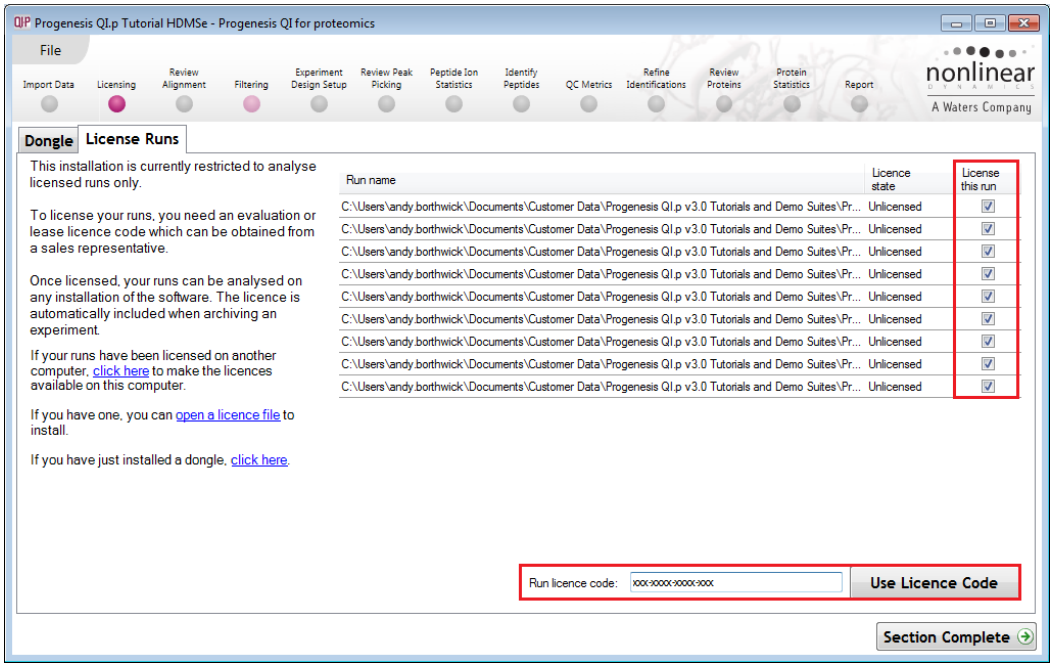
To use this page to License your Runs you must first either obtain an **'Evaluation' Licence Code from a Sales Person or purchase a licence code directly**.

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

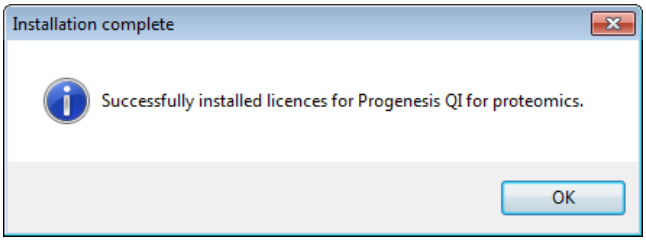
The runs in your experiment will be listed as shown below.

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.



A message confirming successful installation of your licences will appear.

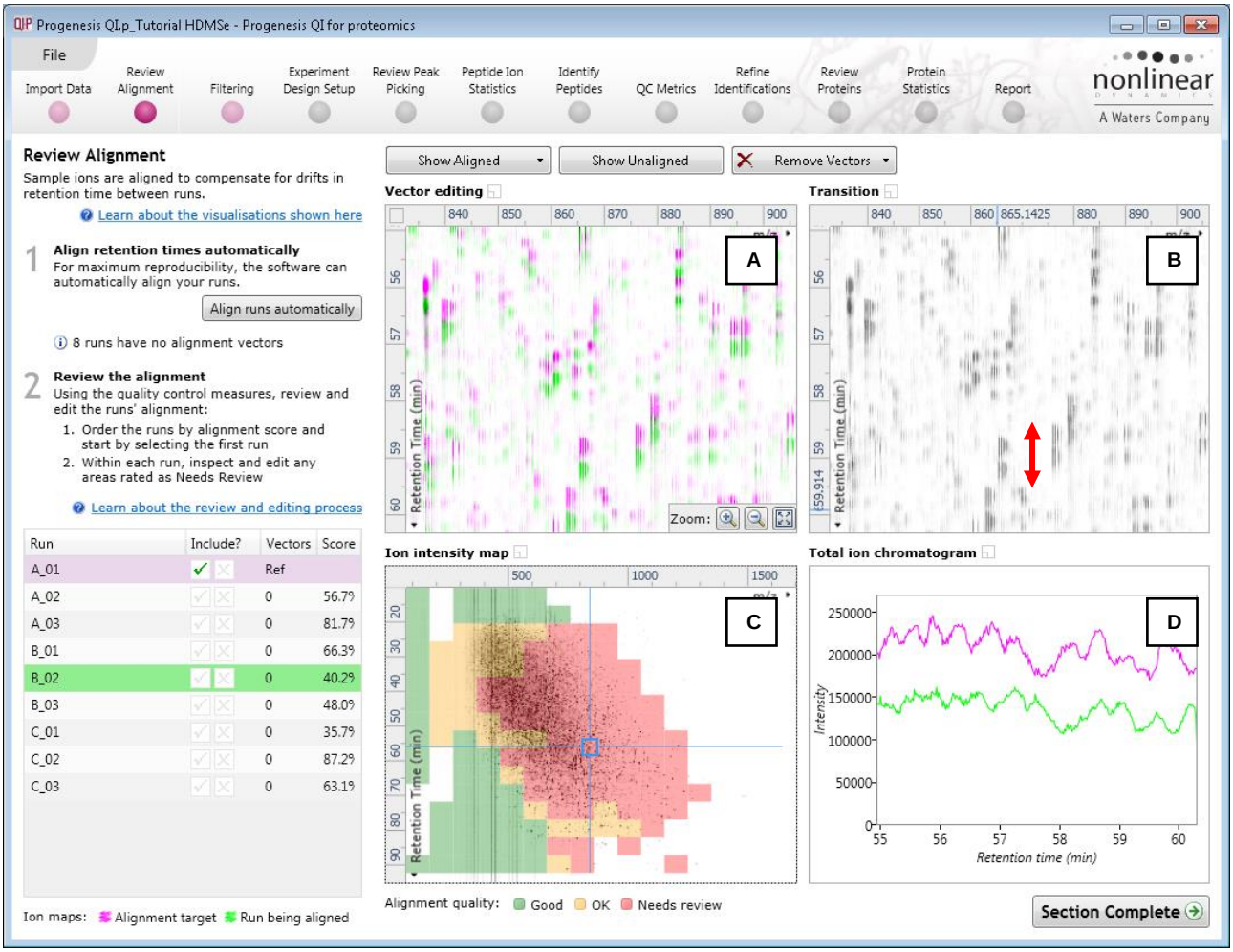


Click **OK**, the view will update and Alignment, the next stage in the workflow, will open with the licensed files.

Appendix 4: Manual assistance of Alignment

Approach to alignment

To place manual alignment vectors on a run (B_02 in this example):



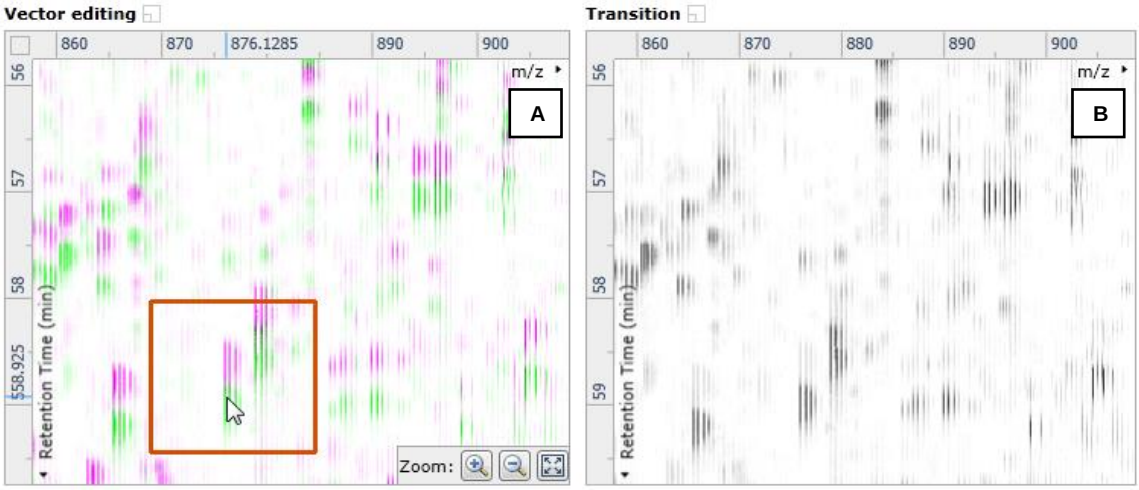
1. Click on Run B_02 in the **Runs** panel, this will be highlighted in green and the reference run (A_01) will be highlighted in magenta.
2. You will need to place approximately 5 - 10 **alignment vectors** evenly distributed from top to bottom of the whole run (RT range).
3. First drag out an area 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.

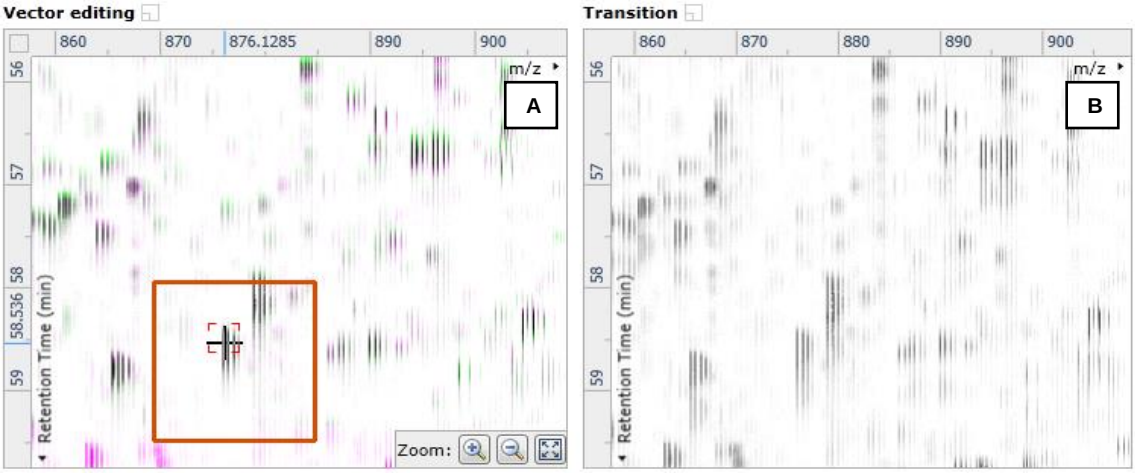
Note: the Ion Intensity Map gives you a colour metric, visually scoring the current alignment and an overall score is placed next to the Vectors column in the table. With each additional vector added this score will update to reflect the 'changing' overall quality of the alignment. The colour coding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

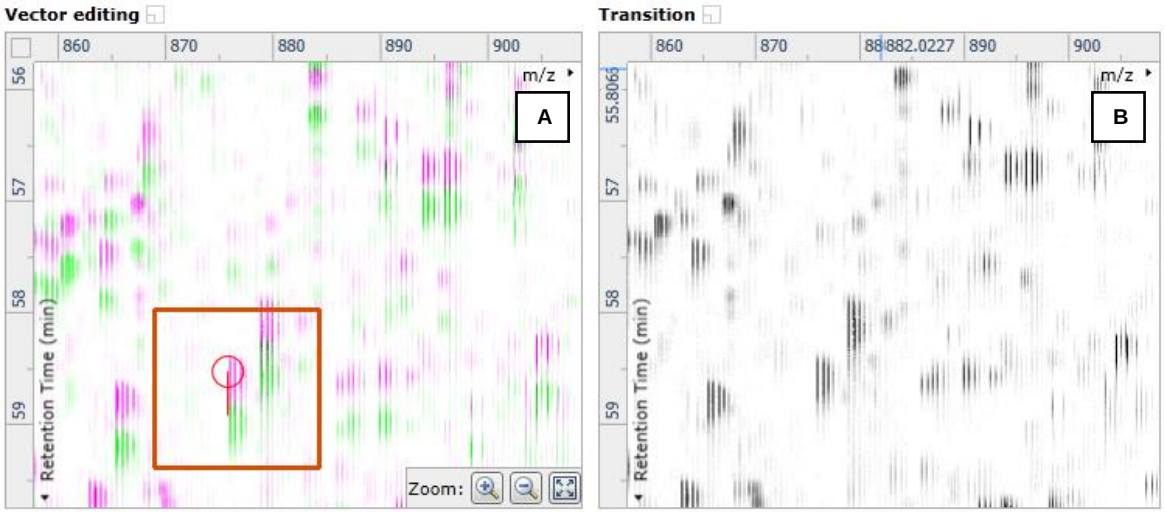


5. As you are holding down the left mouse button (depending on the severity of the misalignment), the alignment vector will automatically find the correct lock. If not, drag the green peptide ion over the corresponding magenta peptide ion of the reference run. The red box will appear as shown below indicating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



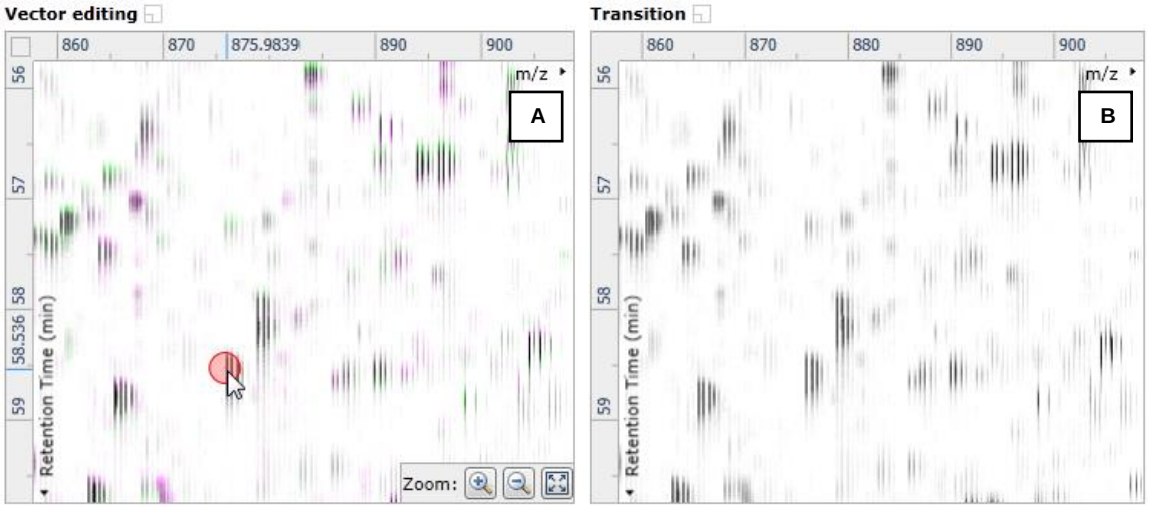
***Tip*:** while holding down the mouse button hold down the **Alt** key. This will allow smooth movement of the cursor as the **Alt** key allows you to override the 'automatic alignment' performed as you depress the mouse button.

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.

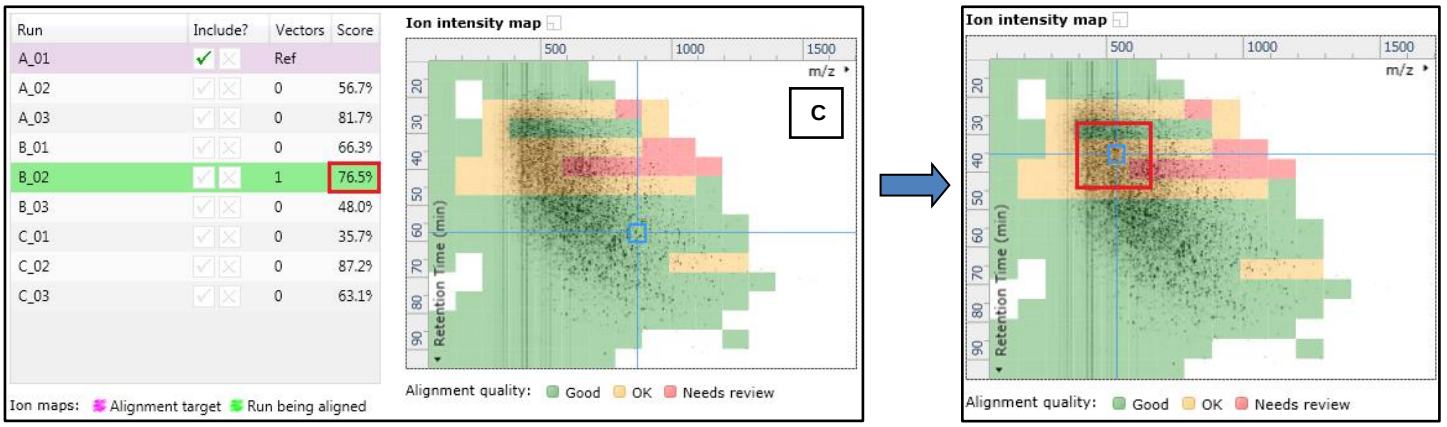


Note: an incorrectly placed vector is removed by right clicking on it in the **Vector Editing** window and selecting delete vector.

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.

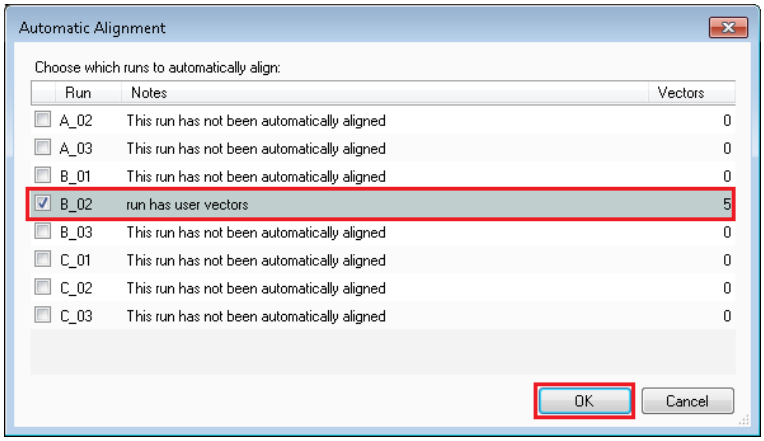


9. Adding an additional vector will improve the alignment further as shown below.

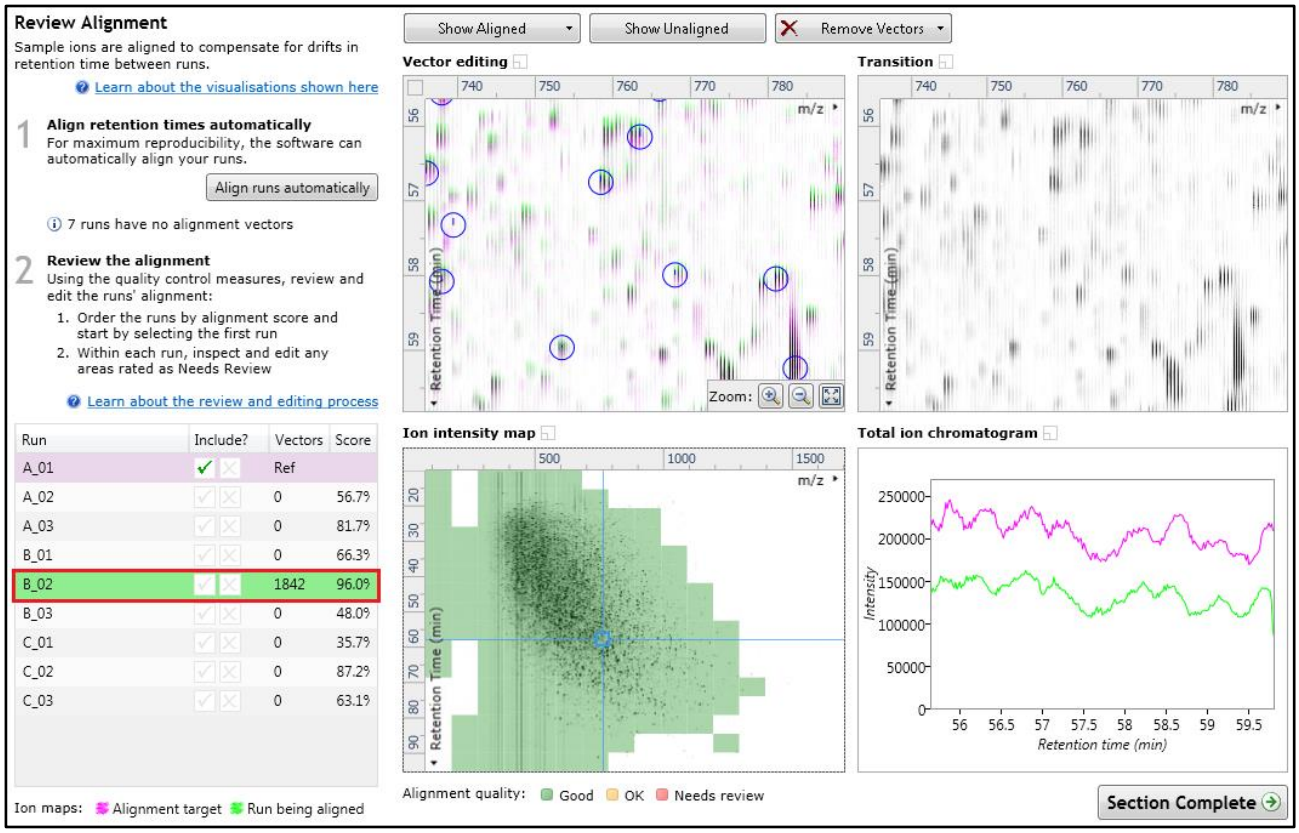
10. The shift in the Retention Time (RT) is as a result of incorrect running of the chromatography. In many of these cases if the Automatic Alignment fails to generate a good alignment then removing all the alignment vectors for this run and placing a single manual vector to act as a ‘seed’ for the Automatic 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.

12. 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.



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.

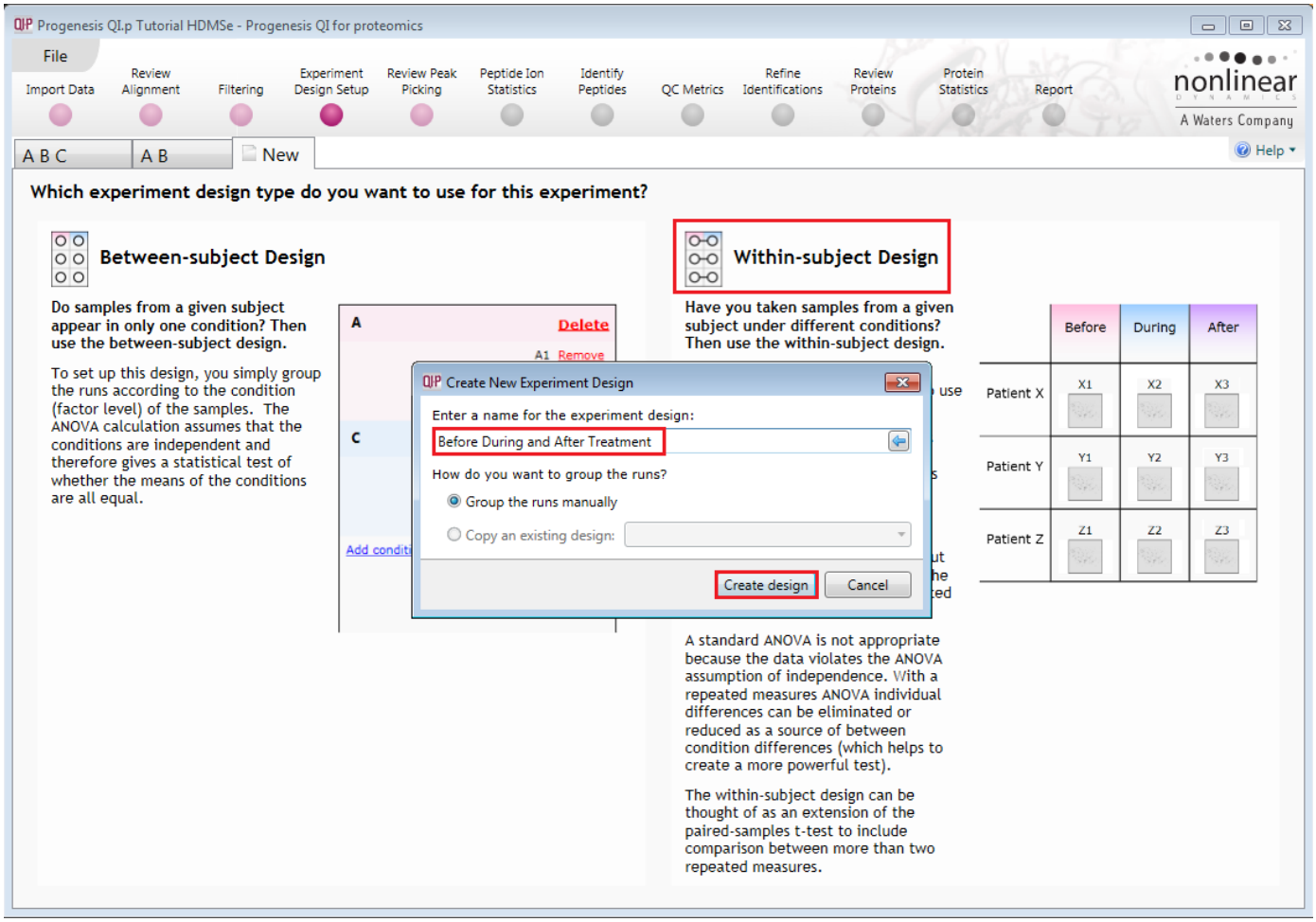
Also the 'ease' of addition of vectors is dependent on the actual differences between the LC-MS runs being aligned.

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

Appendix 5: Within-subject Design

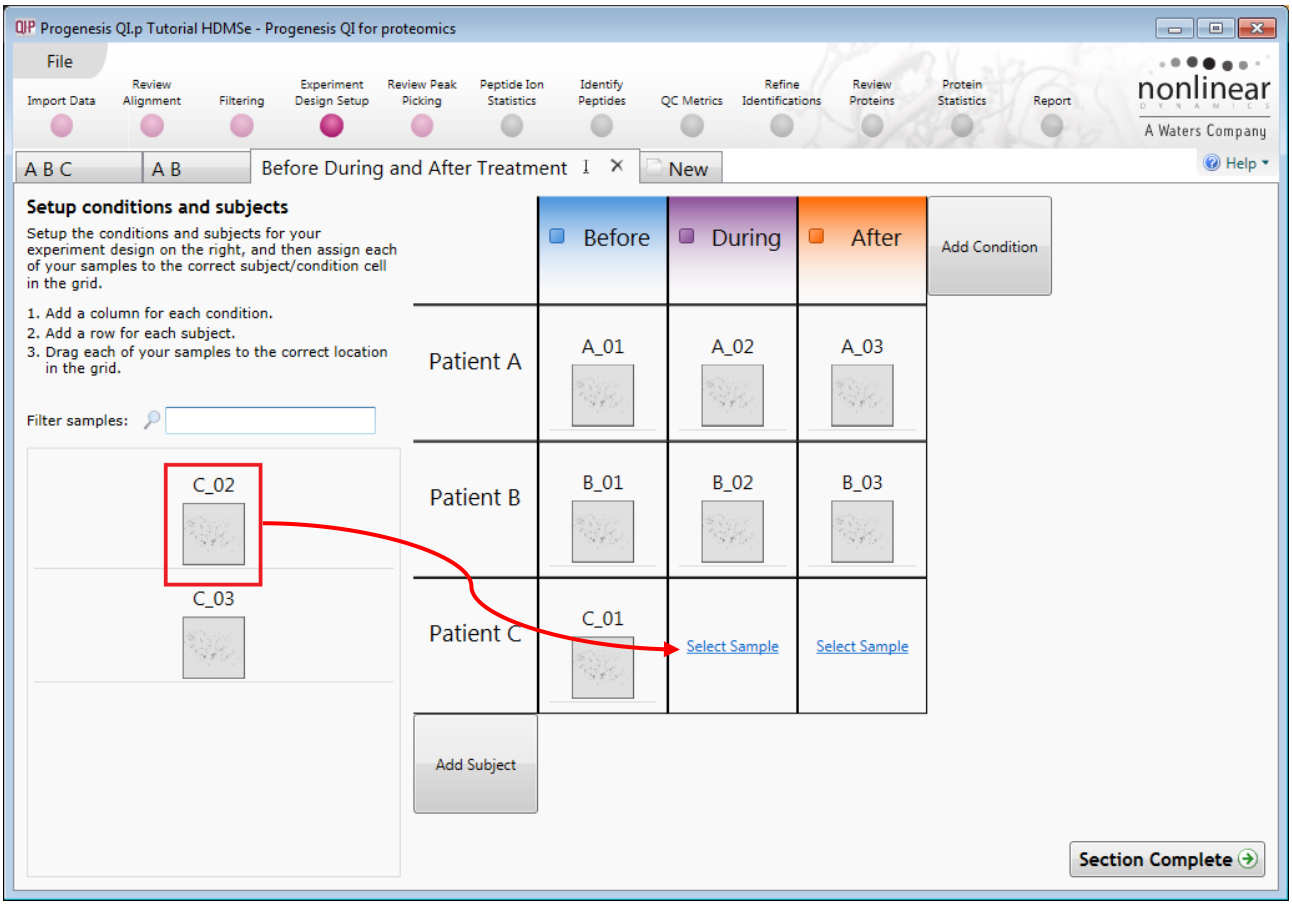
To create a **Within-subject Design** for your data set select this option on the **Experiment Design Setup** page and enter the name of the design.

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



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.



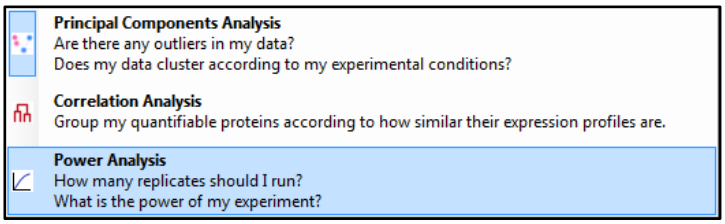
You can create additional Experimental Designs using the New tab

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.



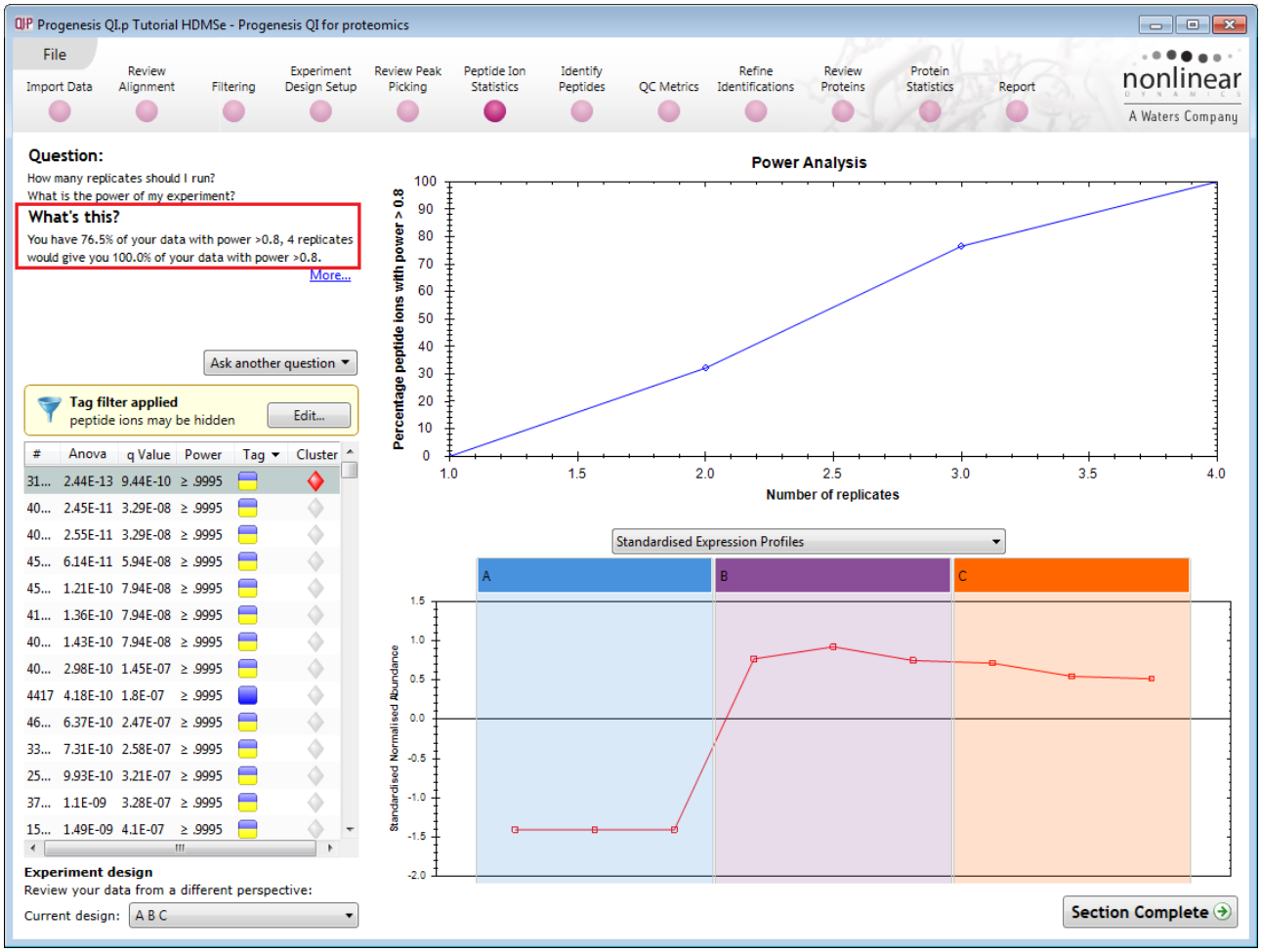
Select the option

'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 (20641),** as an example, view the power analysis.



This is displayed graphically showing that 76.5% of the 20641 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%.

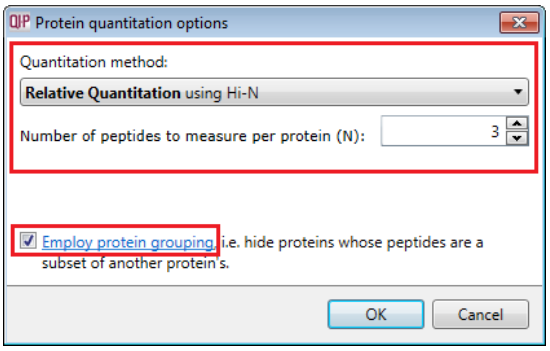
Appendix 7: Resolve Conflicts

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



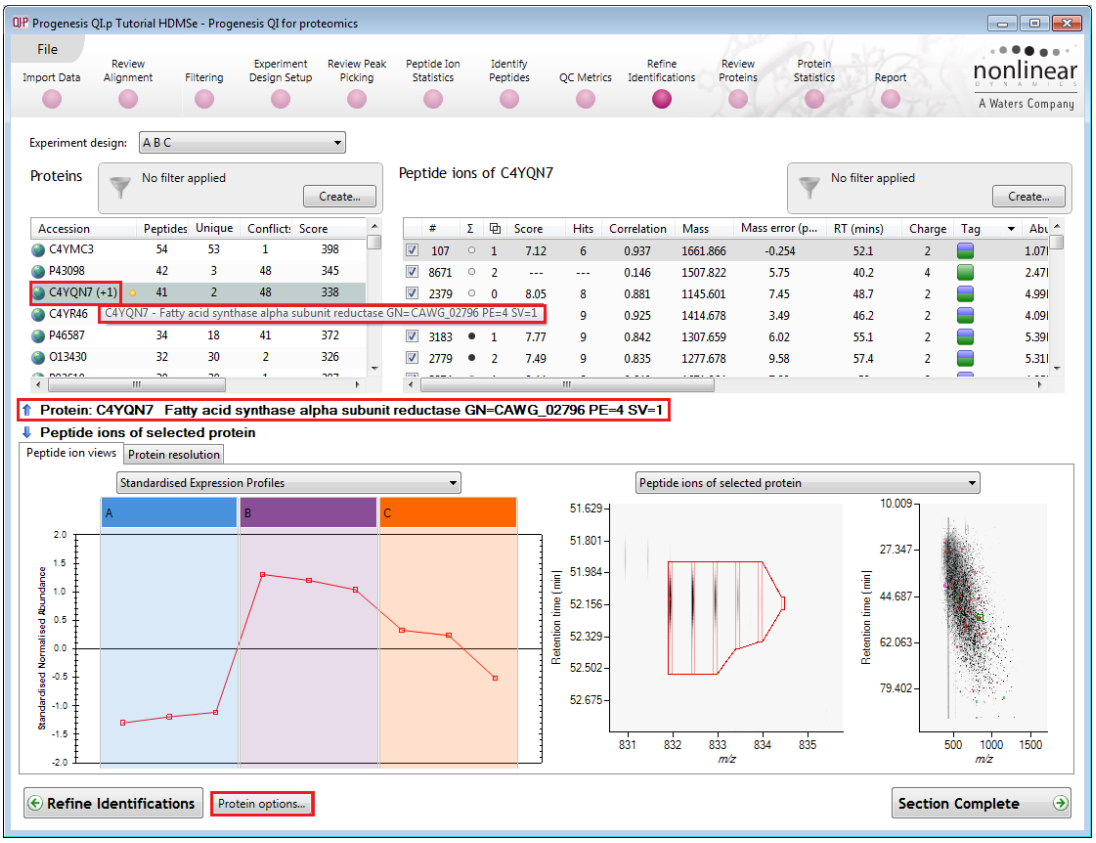
This means that if you choose **not** to resolve the conflicts then proteins, to be considered for quantitation, require at least one unique peptide (see Unique column).

For this guide: we will not resolve the conflicts but click on Section Complete to go to Review Proteins (page 61).

(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.

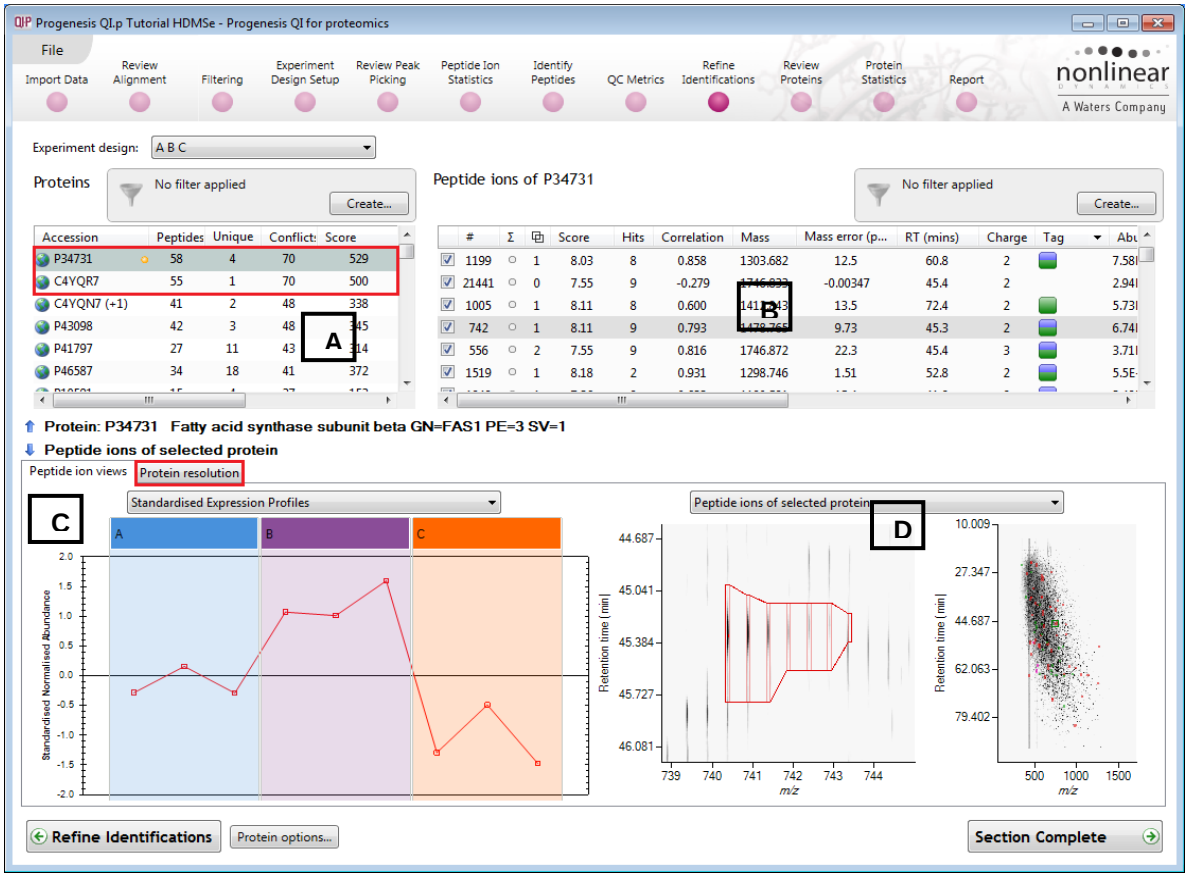


The number of the **Peptides** used for quantitation is displayed in the **Unique** column.

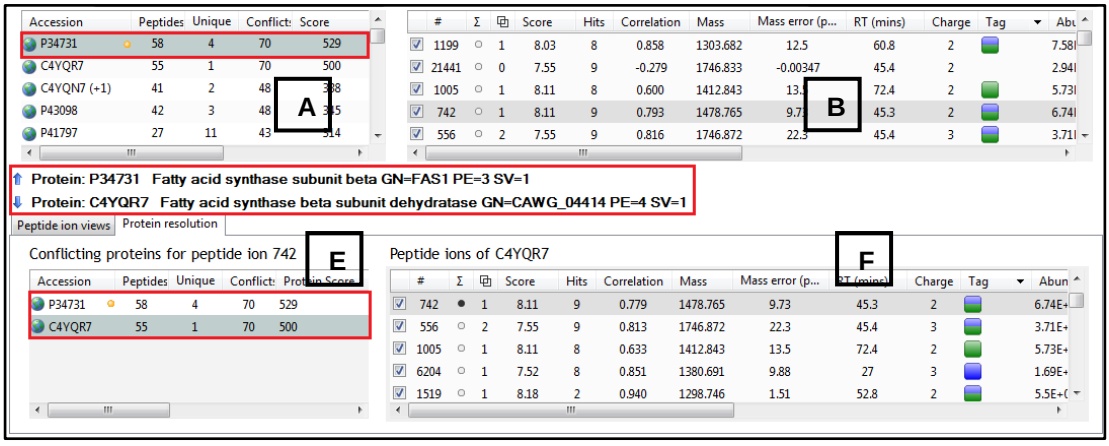
The **Resolve Conflicts** stage provides a number of interrelated graphical and tabular views to assist you in the validation of the peptides that have been assigned to proteins and also to review the relevance of the data returned from the search.

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 70 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

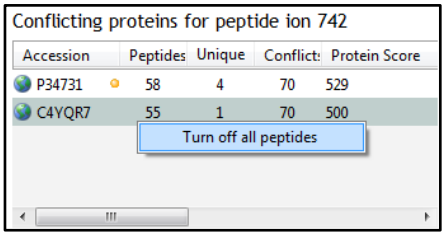
Panel D shows the details for the selected peptide.

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

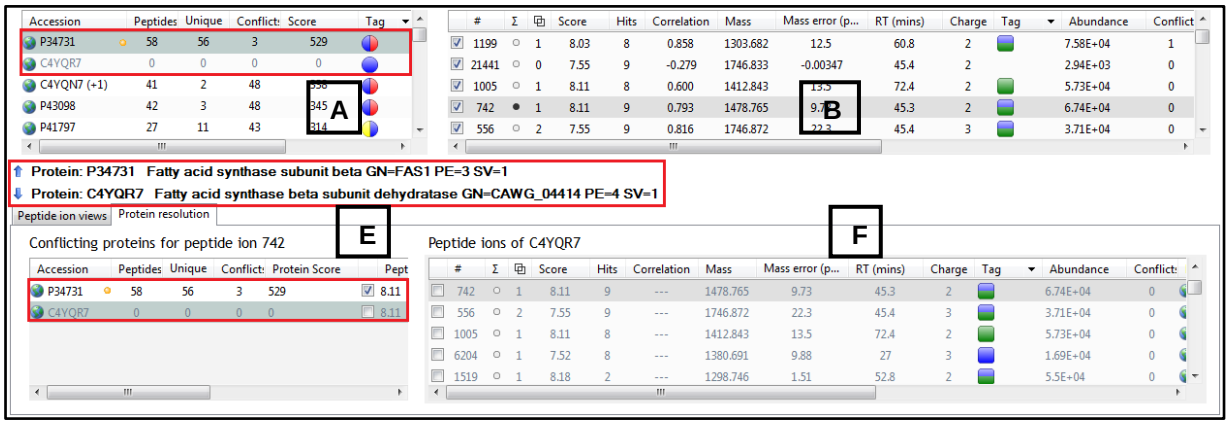
The lower left panel E displays the Conflicting proteins for the peptide ion highlighted in panel B this includes the current protein in panel A as indicated by the orange ball to the right of the accession.

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.

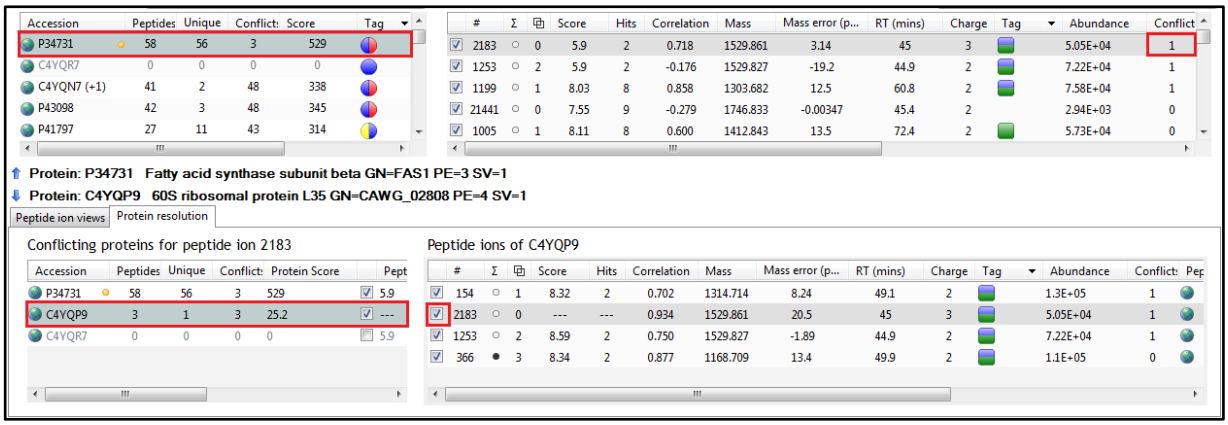
One way to do this is to right click on the lower scoring protein in panel E which only has one unique peptide and turn off all its peptides



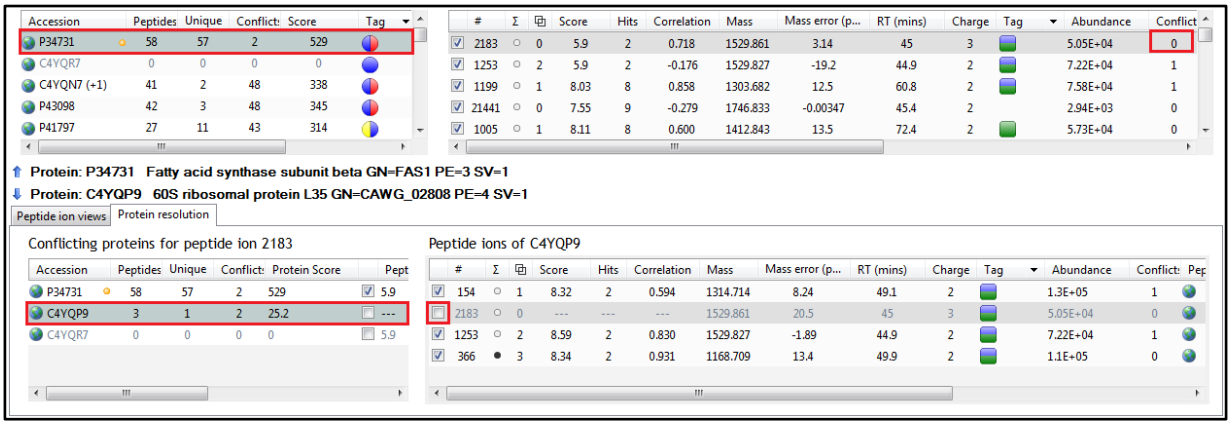
All the peptides are now switched off in panel B and all the entries for the lower scoring protein are set to zero. The higher scoring protein now has 56 non-conflicting peptides and only 4 remaining conflicts



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.

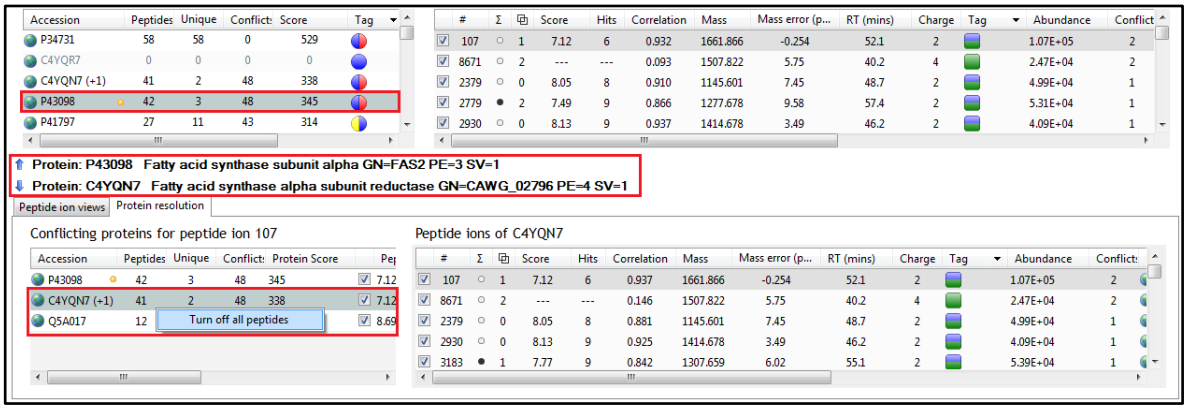


Favouring the protein with the higher score, but this time resolve the conflict by switching off (or un-assigning) the peptide in panel F for the protein with the lower score. By doing this the other 3 panels update to show the change in conflicts.

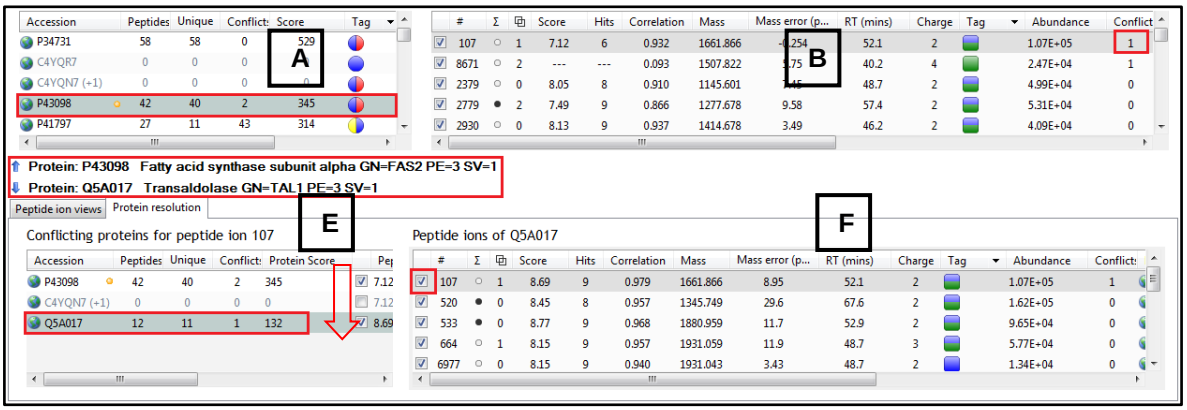


Repeat this process until there are no conflicts remaining for the current protein in Panel A.

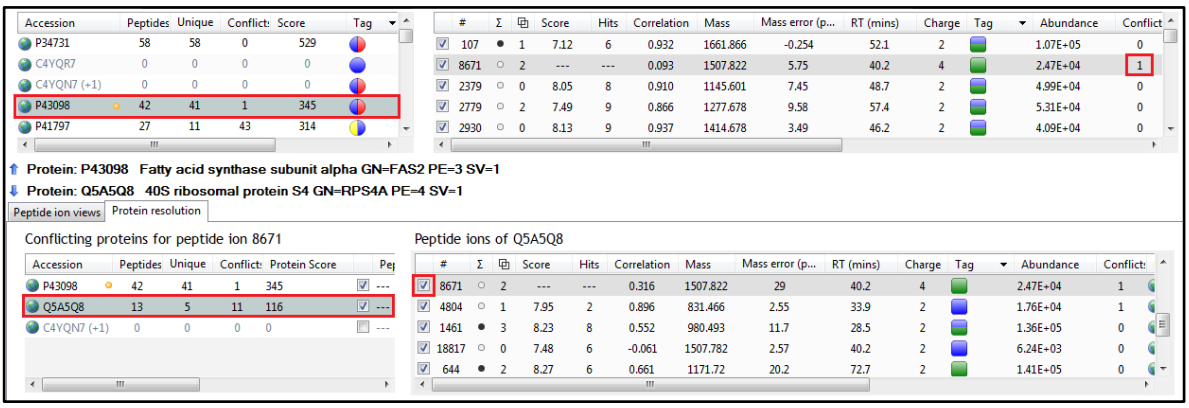
Now repeat using a similar approach for the next protein in Panel A, here the situation is similar.



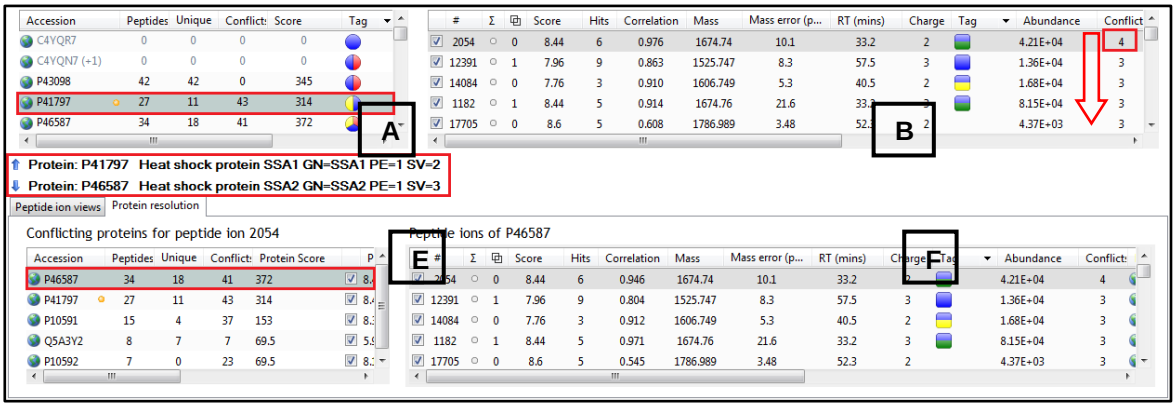
Resolution of conflicts for this protein



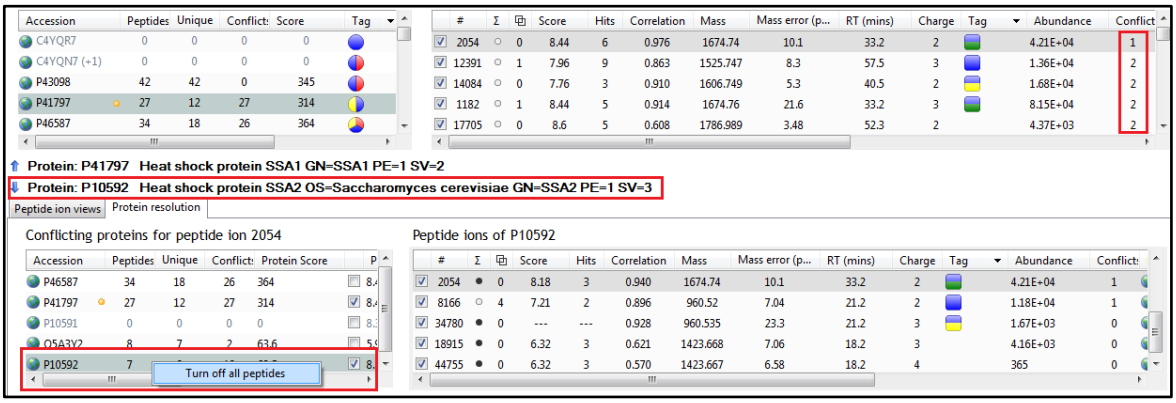
Finally move to the remaining conflict in panel B, and favour the higher scoring protein



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

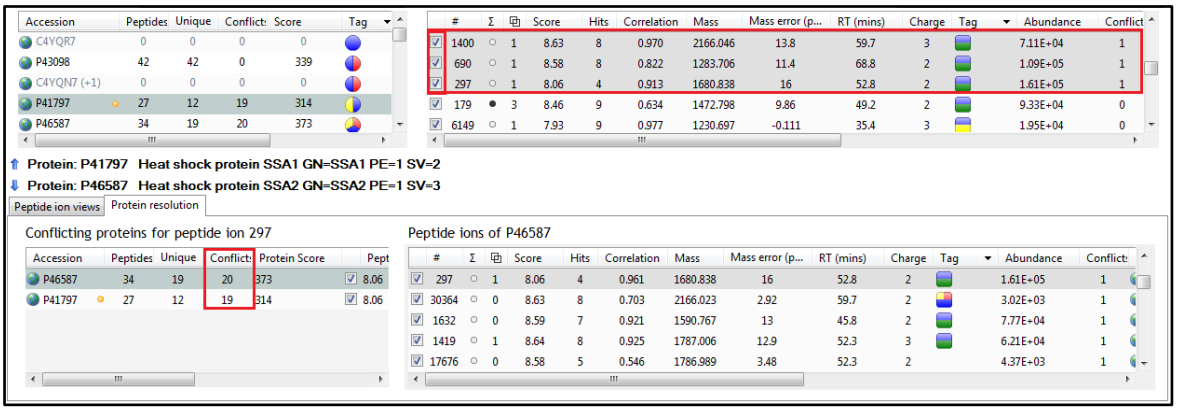


In this case the first peptide for protein (P41797) has 4 conflicting proteins in panel E. Resolve the conflict in favour of the protein with the higher score (P46587) by unticking the peptide ion in panel B then move on to the next conflicting peptide ion in Panel B (which has 3 conflicts) and resolve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

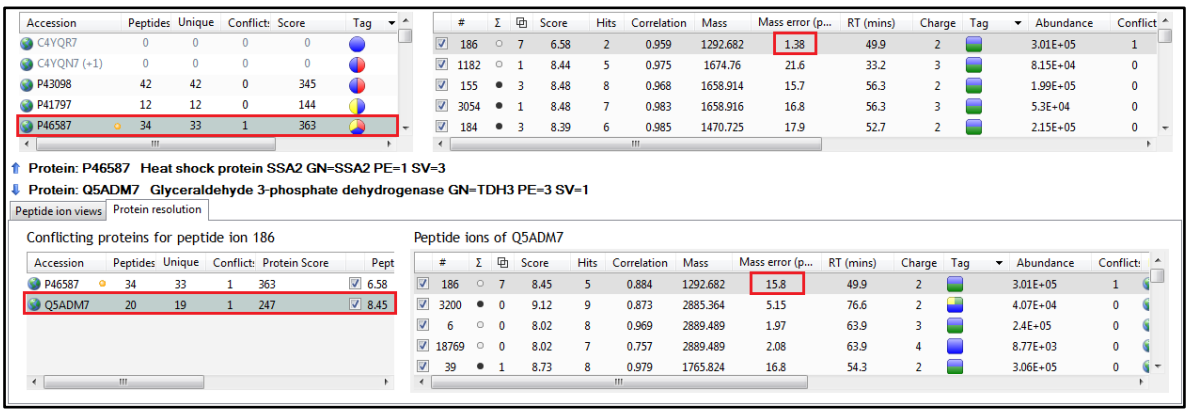


Alternatively you can review the other conflicting proteins. In this case P10591 and P10592 are from a similarly named protein but a different species so you can right click and turn off all the peptides.

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.

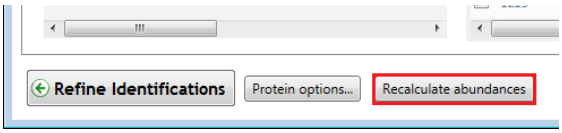


The display will update to show one remaining conflict for P46587. Click on this protein in panel A.



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

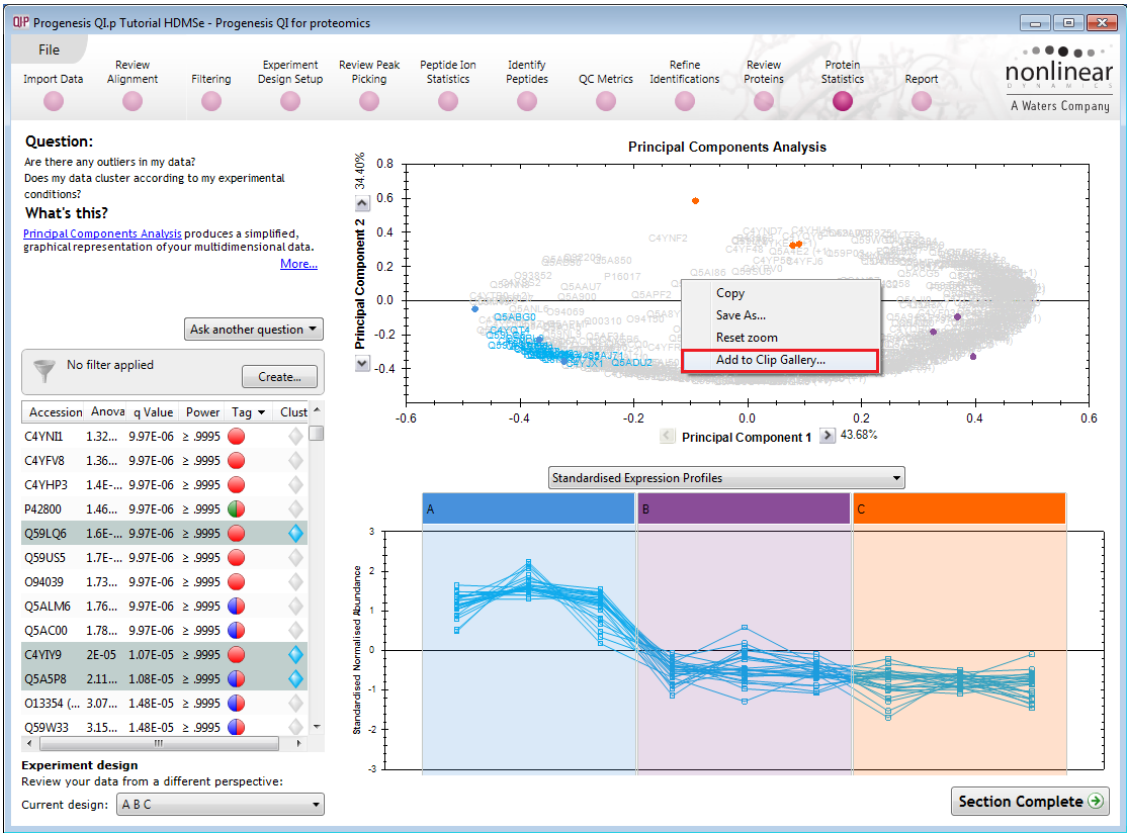


Appendix 8: Using Clip Gallery to Save and Export Pictures and Data

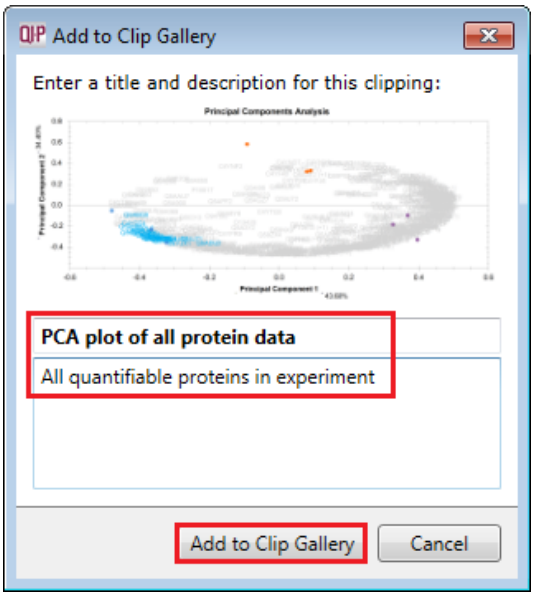
At every stage of the Progenesis QI for proteomics workflow the views and data tables can be added to the Clip Gallery.

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…**

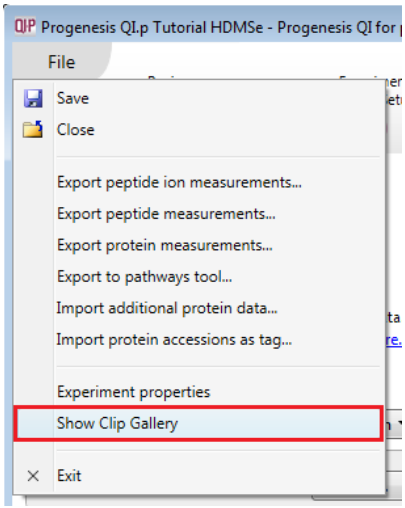


This will open a dialog displaying what is to be saved and allows you to alter the title and provides a description of the image for later reference.

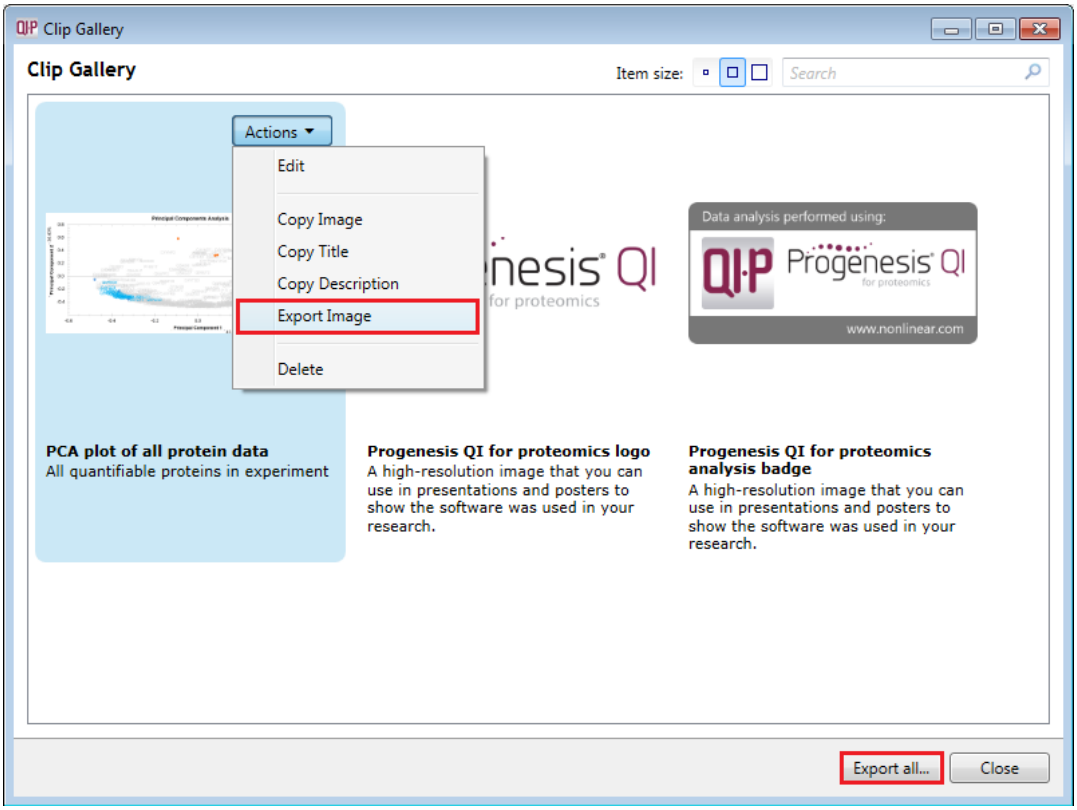


Enter details as required and click **Add to Clip Gallery**

To view, edit and/or export from the clip gallery the gallery can be accessed from the **File** menu.



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



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

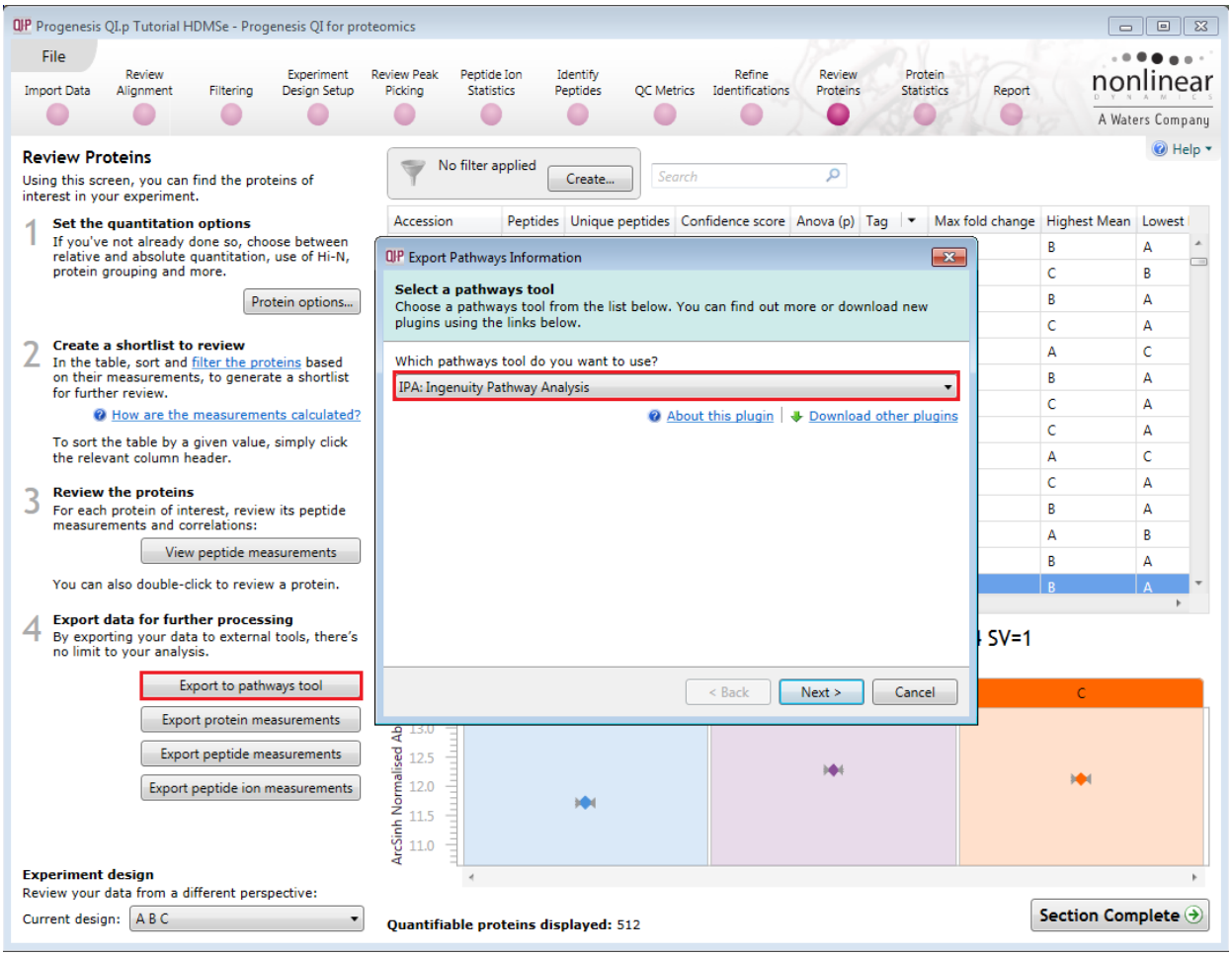
IPA (Ingenuity Pathway Analysis): is a commercial web-based software application for the analysis, integration and interpretation of data derived from any experiments that generate gene and chemical lists with expression data, such as metabolomics and proteomics.

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.

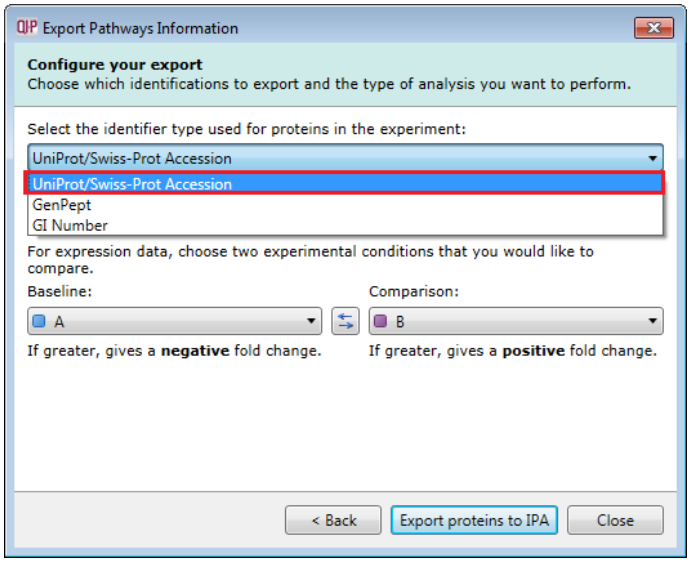
Note: the use of IPA requires a licensed copy, for licensing details please visit the IPA website.

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**.



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

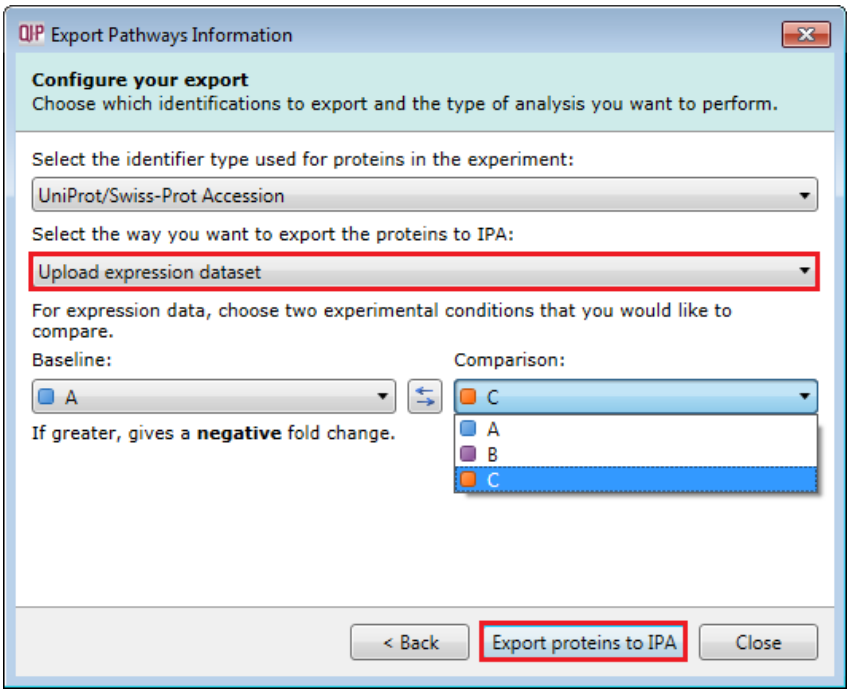


Note: for proteins with different identifier types, filter the protein list to contain only compounds with single identifier type at a time, then perform the export operation and merge the resulting lists in IPA.

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

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.

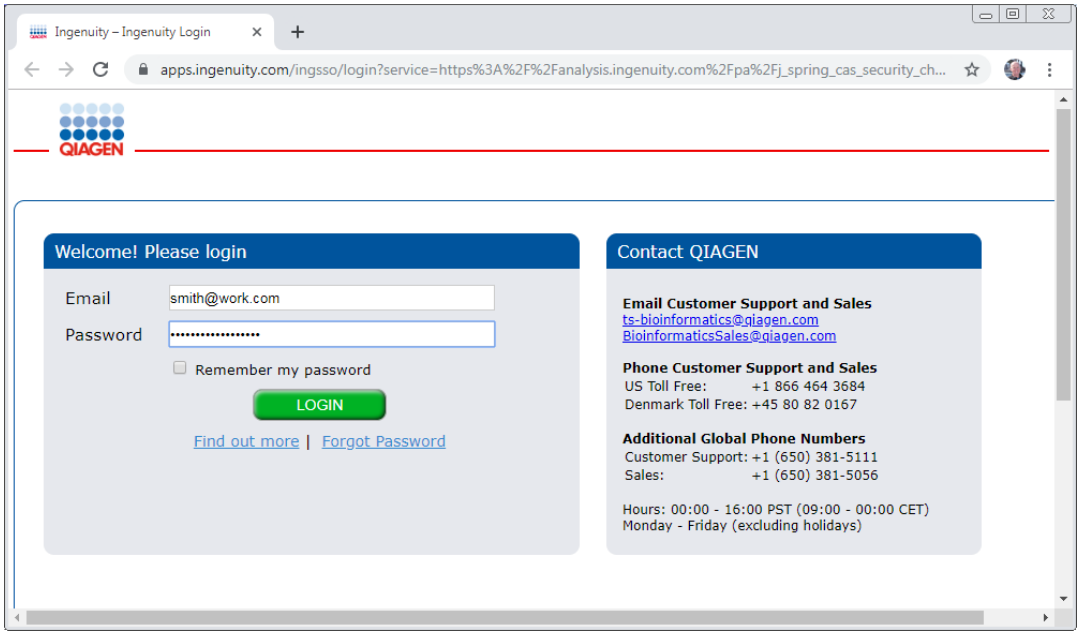
If you require to perform enrichment analysis or other types of analyses that use protein expression data, select the option to **Upload expression dataset** to IPA.



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.

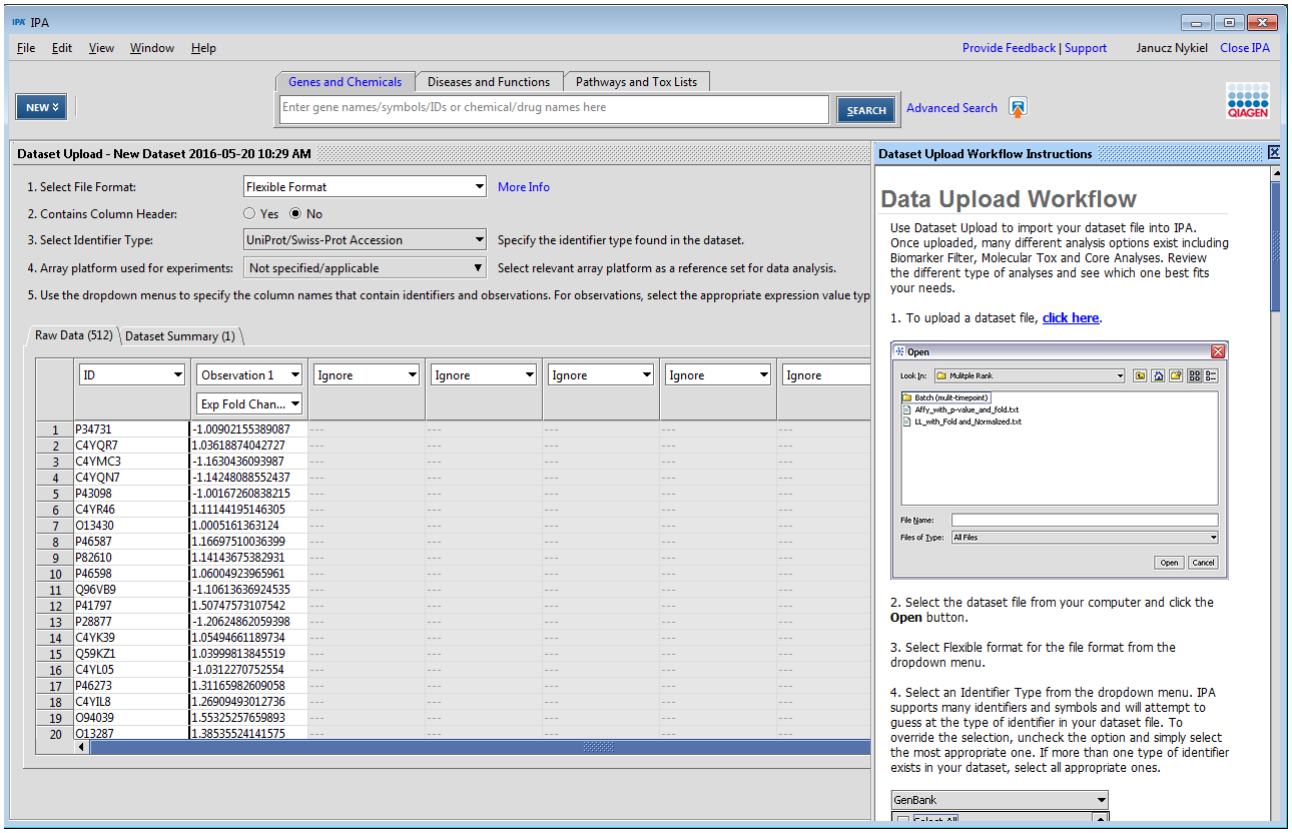
The operation exports data from a single observation at a time - select the two experimental conditions (taken from the current **experiment design as set in Progenesis QI for proteomics** you wish to compare as that observation).

Click **Export compounds to IPA**.



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.



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.

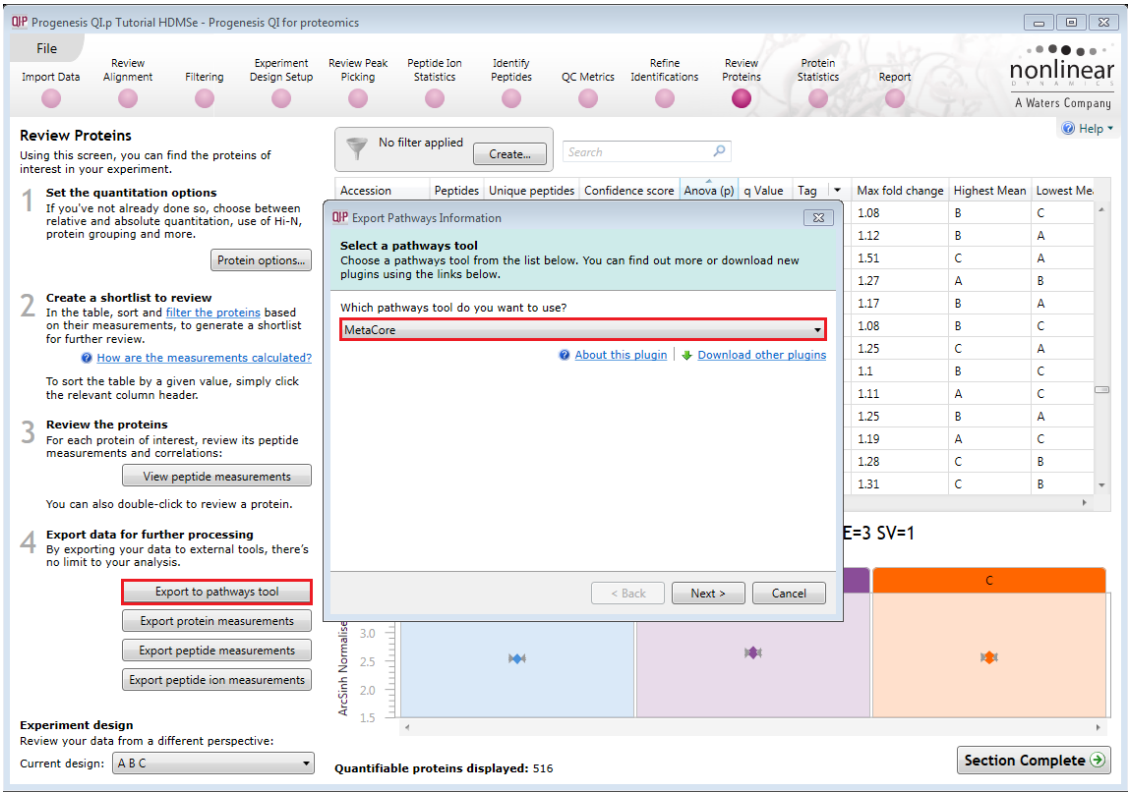
Appendix 10: Exporting protein identities to Metacore

Metacore: 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.

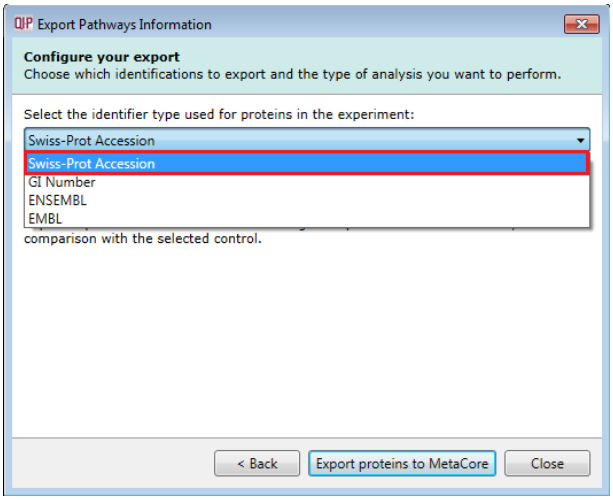
Before exporting data to MetaCore, make sure that you can launch MetaCore using the default browser on your system. The plug-in relies on the browser to upload the Progenesis-exported data to MetaCore.

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 MetaCore

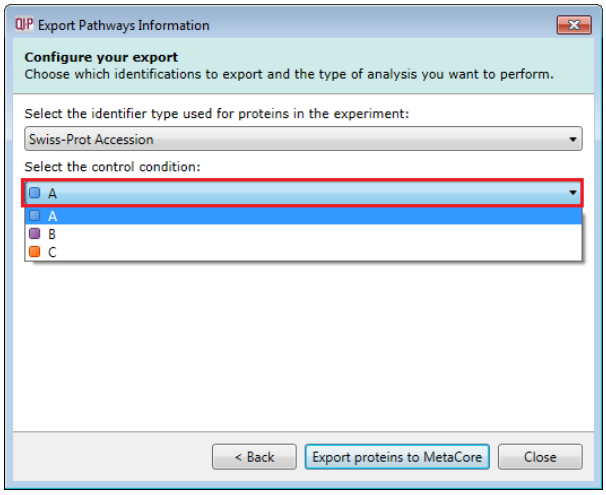


Then click **Next** to Configure your export page .



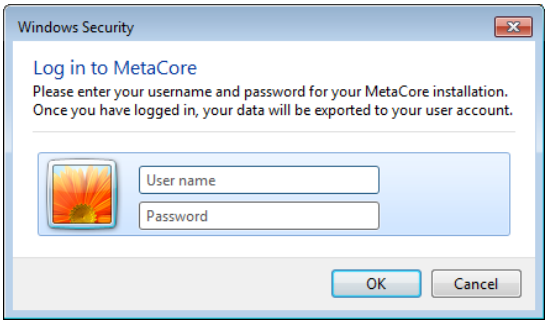
Select the appropriate identifier type used for proteins in the experiment.

If you have multiple conditions in your current experiment design you can select which condition should be used as the control



Finally click **Export proteins to MetaCore**.

If you have not already done so, you will be prompted to enter your MetaCore *User name* and *Password*; once you have done this, your data will be exported to your MetaCore account, and the MetaCore user interface will open in your default web-browser.



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

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

Appendix 11: 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: Lenovo

Processor: Intel® Xeon® CPU E5-1630 v3 @ 3.70GHz 12core K40c GPU card

RAM: 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^E Search		13min 15s	
	(re-opening at Identify Peptides)		10s	
Total Analysis Time:	Excluding Background Apex Processing		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 $LCMS^E$