Progenesis LC-MS Fractionation User Guide

Analysis workflow guidelines

for version 4.1

Contents

Introduction

This user guide takes you through the processes involved in the analysis of a fractionated label free LC-MS experiment. Where the method of fractionation can be gel based on electrophoresis: 1D, 2D, Off gel etc or chromatography using an additional LC step prior to the LC-MS.

In this example, to demonstrate the processes at each stage, an experiment using samples that have been fractionated using ion exchange chromatography is described. As each fraction constitutes the full application of the label free workflow as described in the main LC-MS user guide a shortened version of these analysis steps are described here.

It starts with LC-MS data file loading then Alignment, followed by Analysis that creates a list of interesting features (peptides) which can be explored within Progenesis Stats using multivariate statistical methods, then onto Protein identity.

How to use this document

This document is designed to be used as a guide to the processes involved in the analysis of fractionated samples. Currently a full data set is not provided as this would result in a considerable download. The initial section of the document is concerned with an abbreviated description of the main experimental workflow as applied to a single fraction (a more comprehensive description of the main analysis workflow is available in the main User guide). The second section describes the process of recombining these individual fraction experiments into a 'Multi-fraction' experiment.

How can I analyse my own runs using LC-MS?

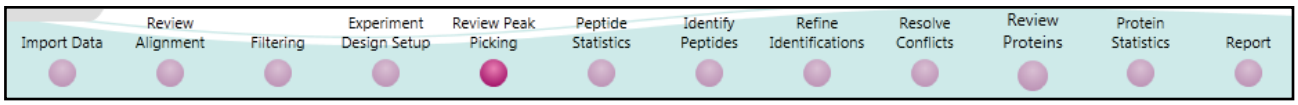
You can freely explore the quality of your LC-MS data using Data Import and then licence your own LC-MS runs using this evaluation copy of Progenesis LC-MS. Instructions on how to do this are included in a section at the end of the user guide document. Alternatively if you would like to arrange a demonstration in your own laboratory contact support@nonlinear.com and we will help you.

LC-MS Data used in this user guide

NLD would like to thank Dr Robert Parker and Prof Haroun Shah at the Health Protection Agency, London, UK for providing the example data used in this user guide as well as invaluable discussion on the handling of the data.

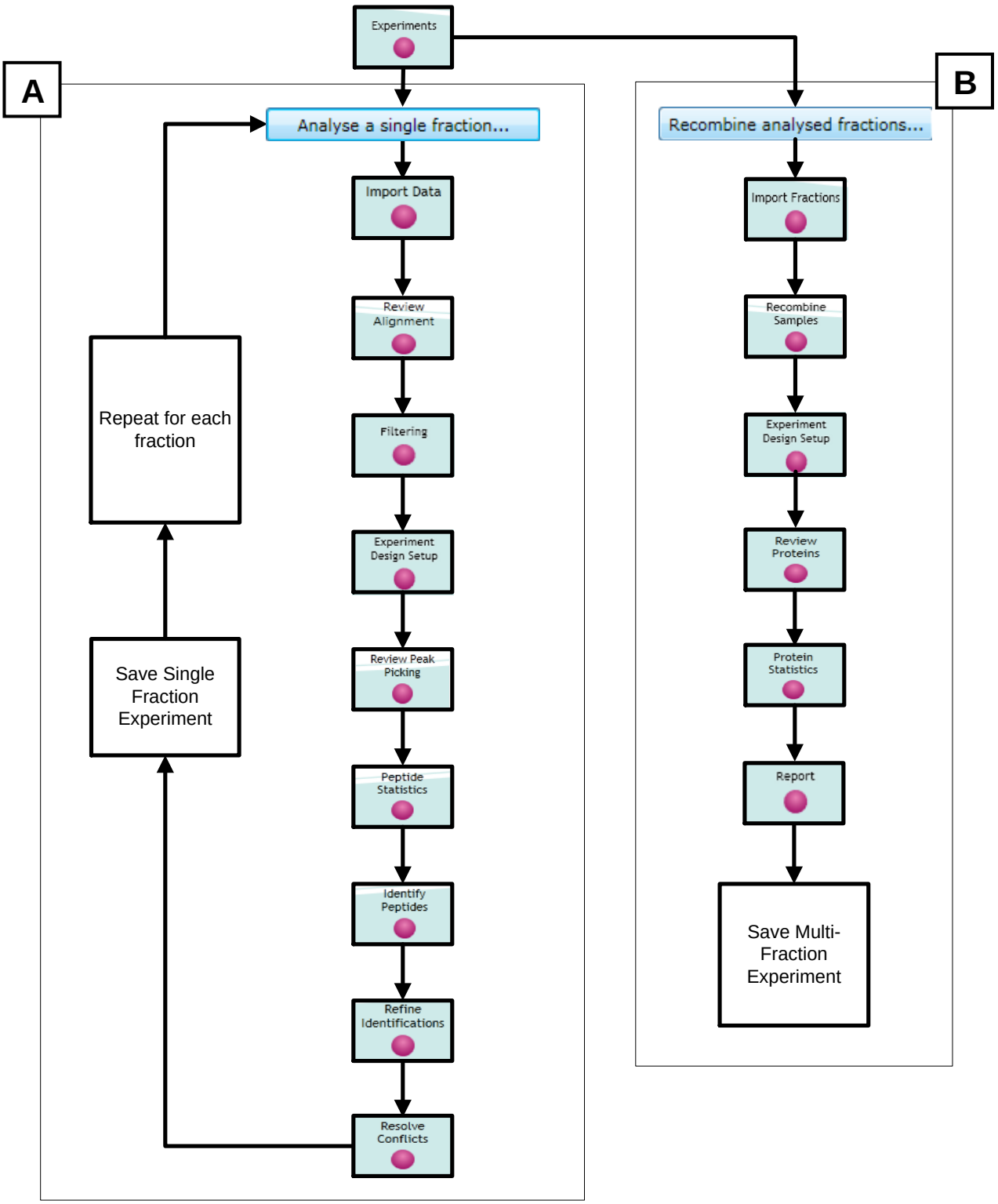
Workflow approach to LC-MS run analysis

Progenesis LC-MS 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 analysis of fractionated samples.



Overview of a typical Fractionation Analysis workflow

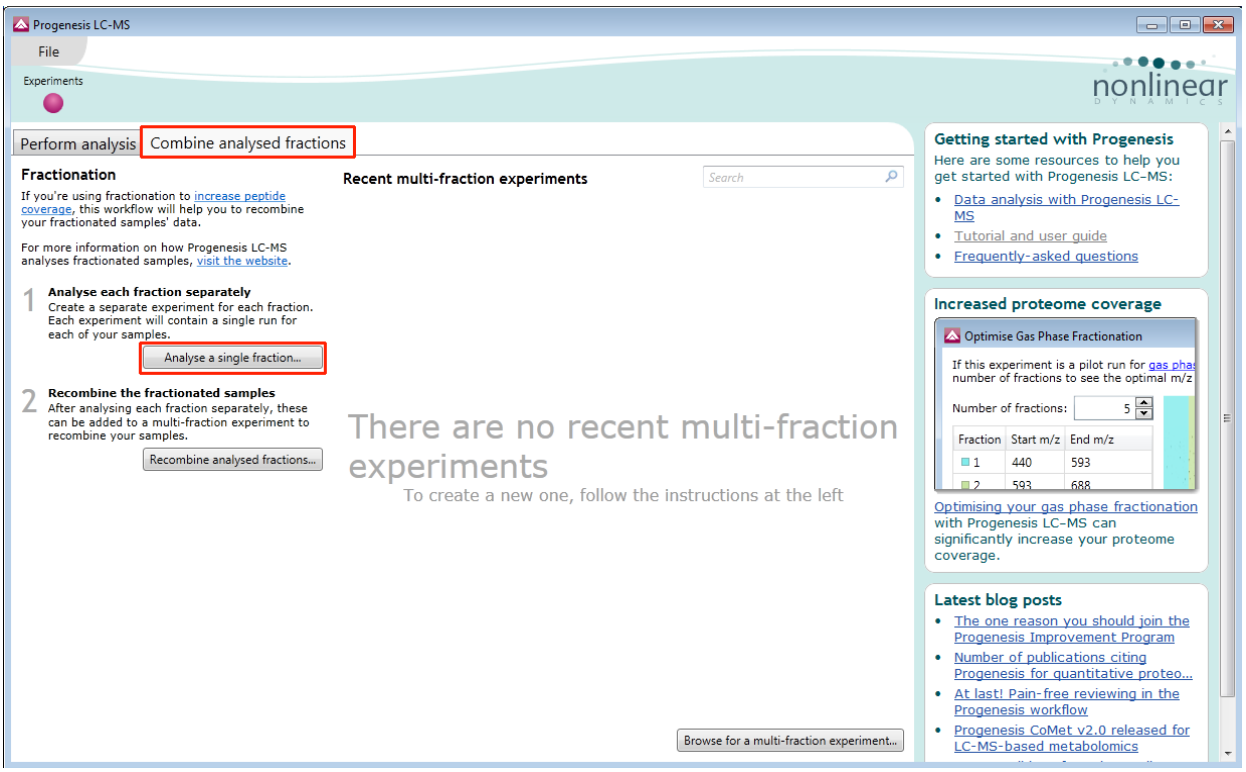
The workflow outlines the steps performed in the analysis of a typical fractionated experiment. In this example there were 6 fractions hence the main analysis workflow was performed 6 times. To analyse the data, select the **Combine analysed fractions** tab then (A) Analyse a single fraction then (B) Recombine the analysed fractions into a multi-fraction experiment.



Analysing a single fraction

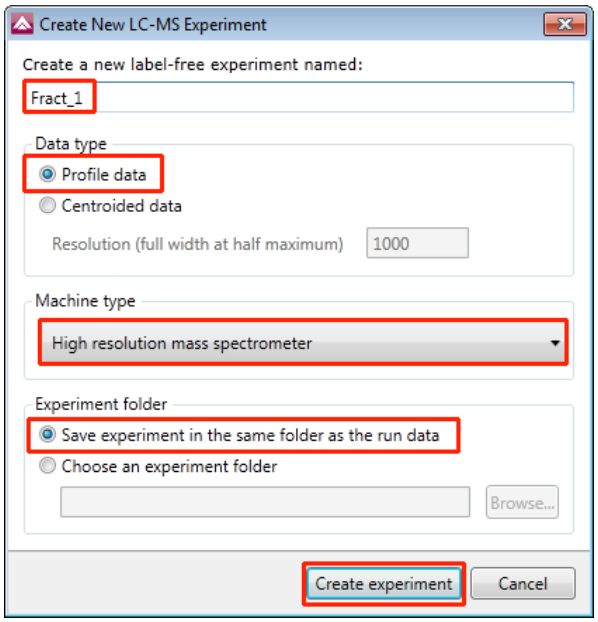
Open Progenesis LC-MS and click on the **Combine analysed fractions** tab to start the processing of Fractionated Samples.

To start the analysis of the LC-MS runs for a fraction, click on **Analyse a single fraction...**



This opens the 'Create New Experiment' dialog.

Name the fraction to be analysed (**Fract_1**) then adjust the Data and Machine types accordingly and set the Experiment folder as required .

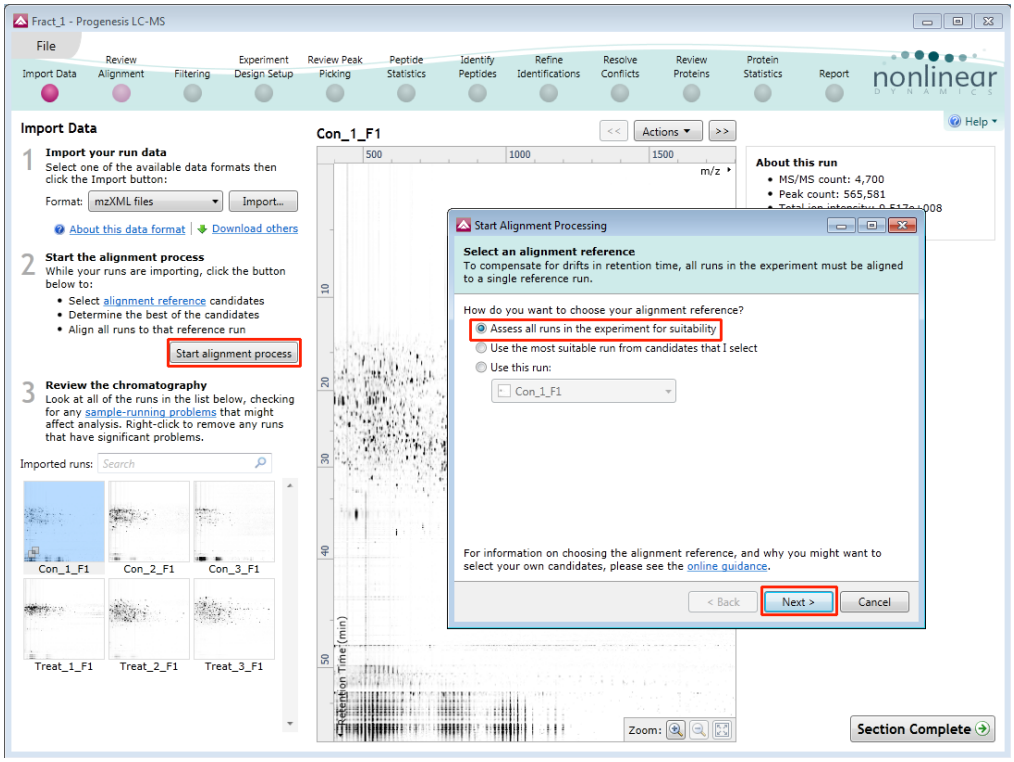


Note: current settings shown are the defaults

Click **Create experiment**

Stage 1: Data import and QC review of LC-MS data set for a fraction

Use the Import facility to select your runs for Fraction 1 (Fract_1). As your LC-MS runs start to load click **Start alignment process** and select how you want to choose the alignment reference.



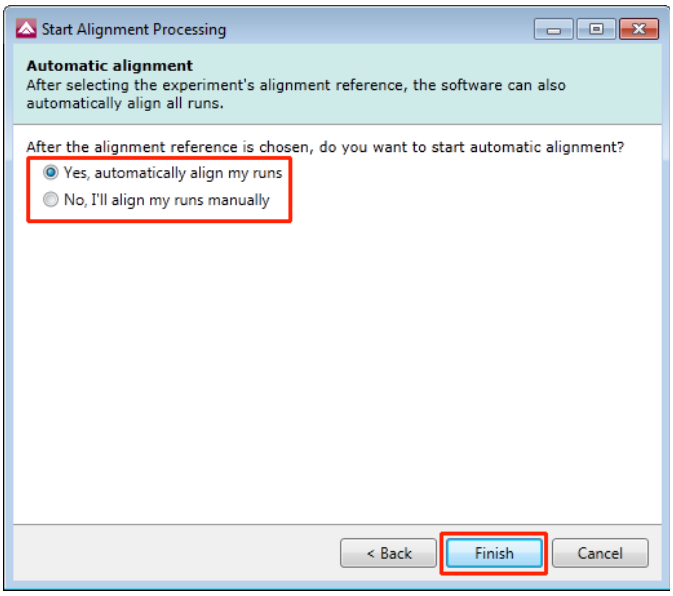
In this example we will select the first option **Assess all runs in the experiment for suitability.** This allows the software to manage the choice automatically, see Appendix 1 in the main User Guide for more details on the selection of the alignment reference.

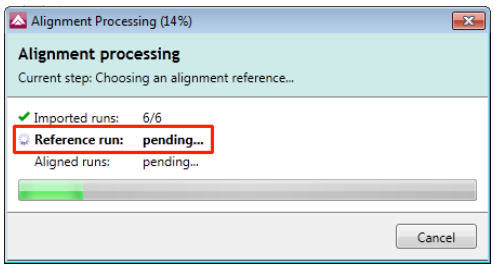
You will now be asked if you want to Align your runs automatically or manually.

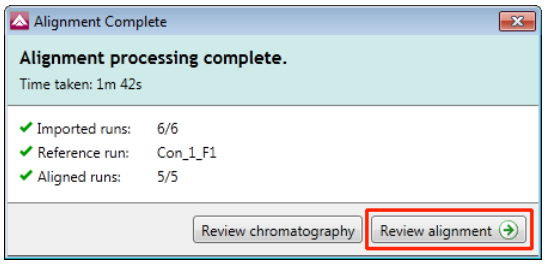
Select **Yes, automatically**…. and click finish.

The Alignment process starts with the automatic selection of Con_1_F1 as the alignment reference.

Then the alignment of the other 5 runs to Con_1_F1.







Now move to the next stage in the workflow by clicking **Review Alignment.**

Stage 2: Reviewing quality of alignment vectors

At this stage Progenesis LC-MS Alignment opens displaying the alignment of the runs to the Reference run (Con_1_F1).

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 chosen 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.

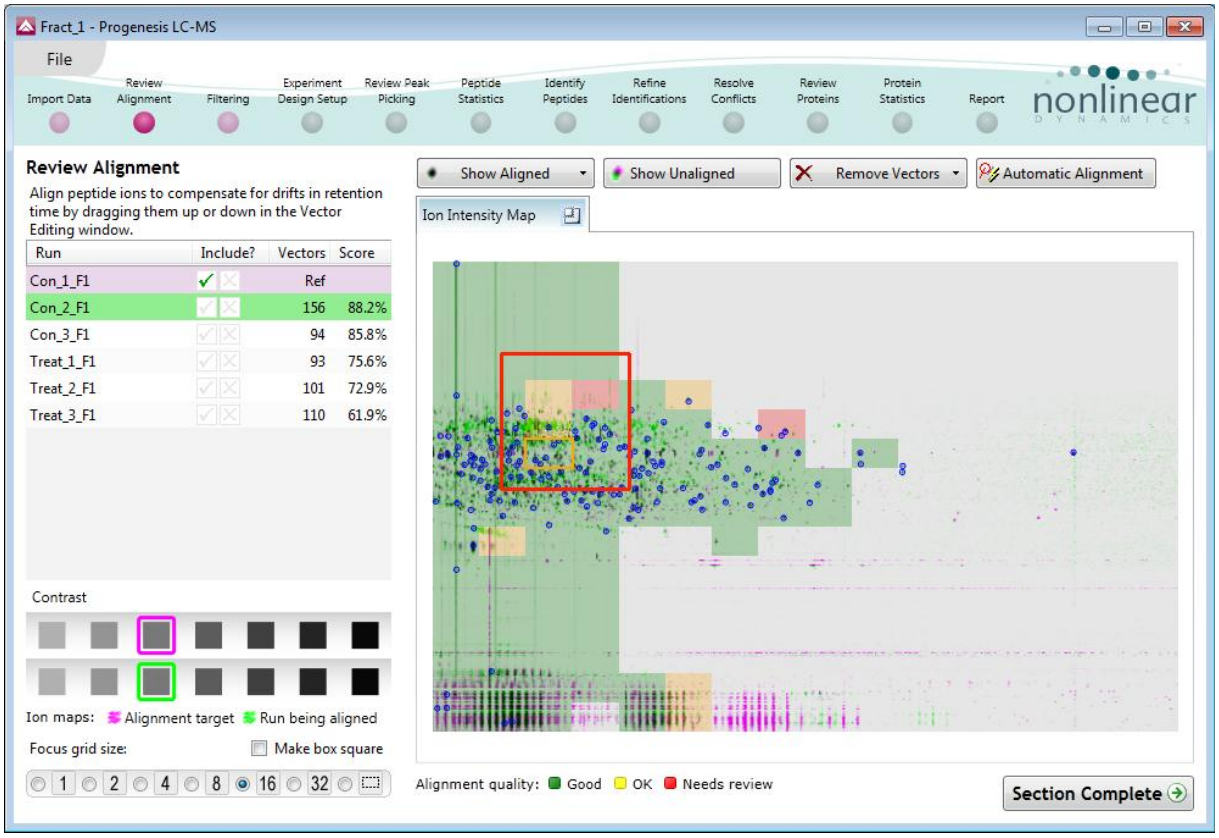
Transition (Window B): uses an **alpha blend** to animate between the current and reference runs. Before the runs are aligned, the features appear to move up and down. Once correctly aligned, they will appear to pulse. During the process of adding vectors, this view will change to show a zoomed view of the area being aligned to help accurate placement of manual vectors.

Whole Run (Window C): shows the **focus** for the other windows. When you click on the view the orange 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 using the controls in the bottom left of the screen or 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 (**note**: this can be switched off using the options in the View menu) which focuses your review of the alignment process.

Total Ion Chromatograms (Window D): shows the current **total ion** chromatogram (green) overlaid on the Reference chromatogram (magenta). As the features 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 **Whole Run** view (Window C).

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.

If the alignment has worked well then in Windows A and C the grid lines (option under **View** menu) should show minimal distortion, Window B (Transition) will show features pulsing slightly but not moving up and down.

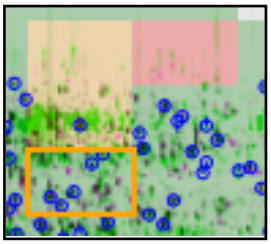


Note: you can use the icon to the right of the panel name to expand or contract each view

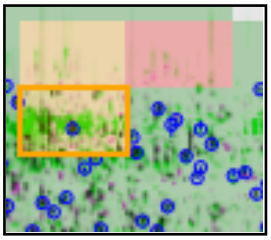
Reviewing Quality of Alignment

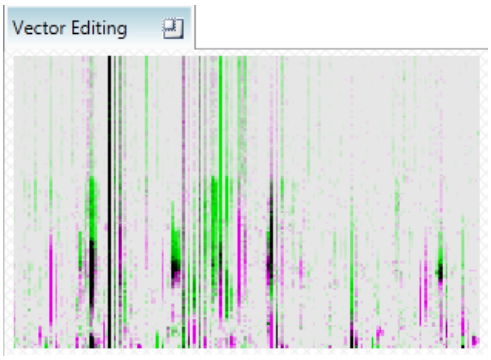
At this point the quality metric, overlaid on the Ion Intensity Map as coloured squares, acts as a guide drawing your attention to areas of the alignment. These range from Good (Green) through OK (Yellow) to Needs review (Red). When reviewing individual squares set the grid size to 16, (and untick the Make box square option) using the **'Focus grid size'** control at the bottom left of the window. 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.

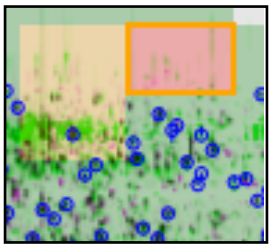


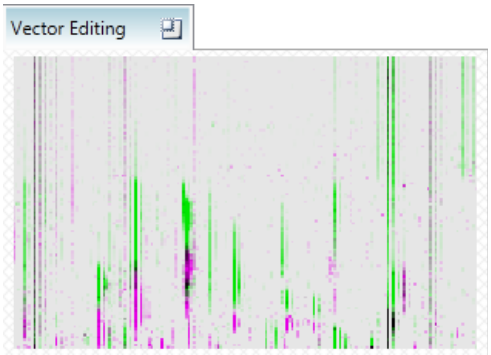
For a 'yellow' square some of the data appears overlapped (black) indicating OK alignment. When viewed in the Transition view some of the data appears to pulse.





For a 'red' square little 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 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 alignment.

Note: a marked red area combined with a low score clearly indicates a 'miss alignment' and may require some manual intervention (see Appendix 1, page 33).

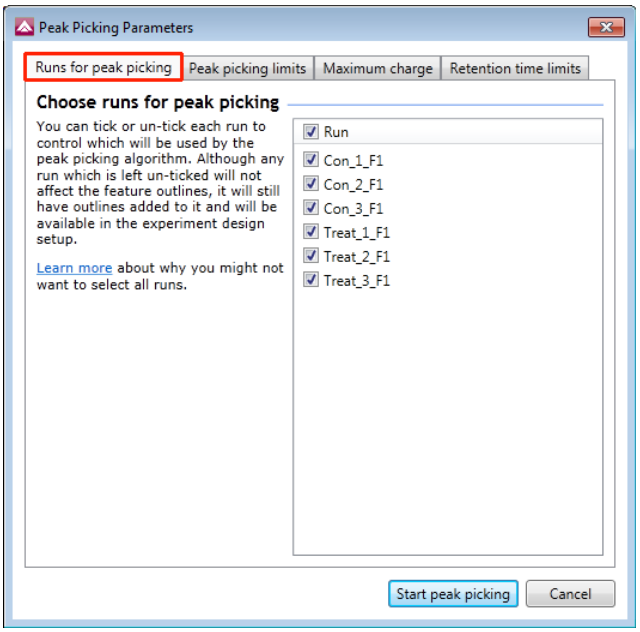
In each square, you can, if required edit the vectors to improve the run alignment (for more detailed information on performing the alignment of your runs refer **Appendix 1** (page 33) and also to the main LC-MS analysis User guide.

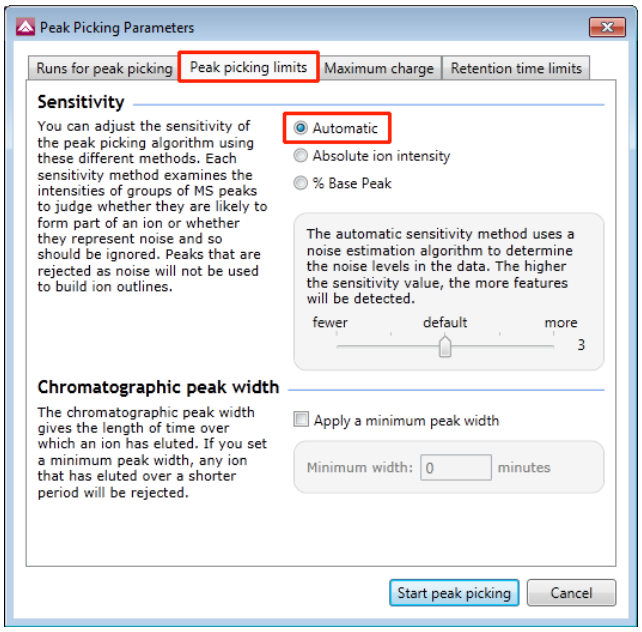
Stage 3: Filtering

Now that you have reviewed your aligned Runs, you are ready to analyse them. Move to the **Filtering** stage, by either clicking on **Section Complete** (bottom right) or on Filtering on the workflow.

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 feature 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 feature detection pattern. This may be important when one or more of the runs appear noisy due to non-optimal chromatography or sample handling.

Note: feature 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 is controlled by adjusting settings under the **Peak picking limits** tab.

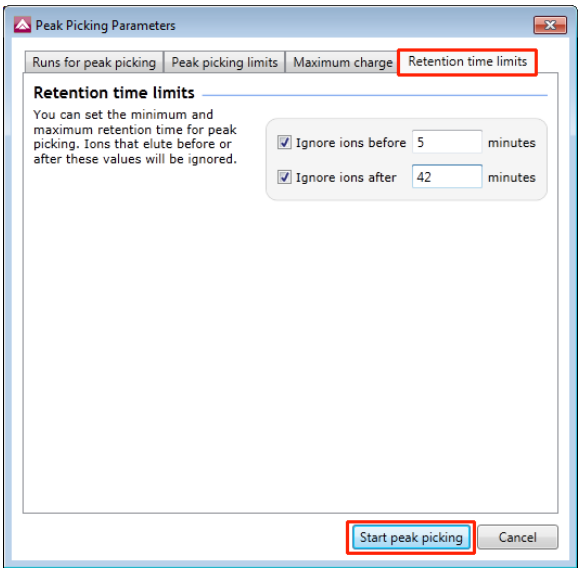
For this example the default settings for the **Automatic** method were used.

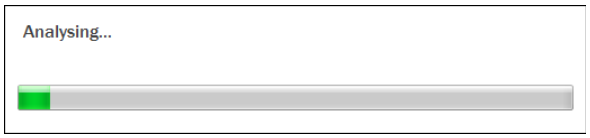
The third tab allows you to set the **maximum charge** of the ions that will be detected. The default setting is a charge state of 20.

In the fourth tab set the Retention time limits to before 5 and after 42 minutes.

Press **Start peak picking** to start the detection process.

During the few minutes that the automatic analysis requires, a progress bar will appear telling you that it is Analysing.

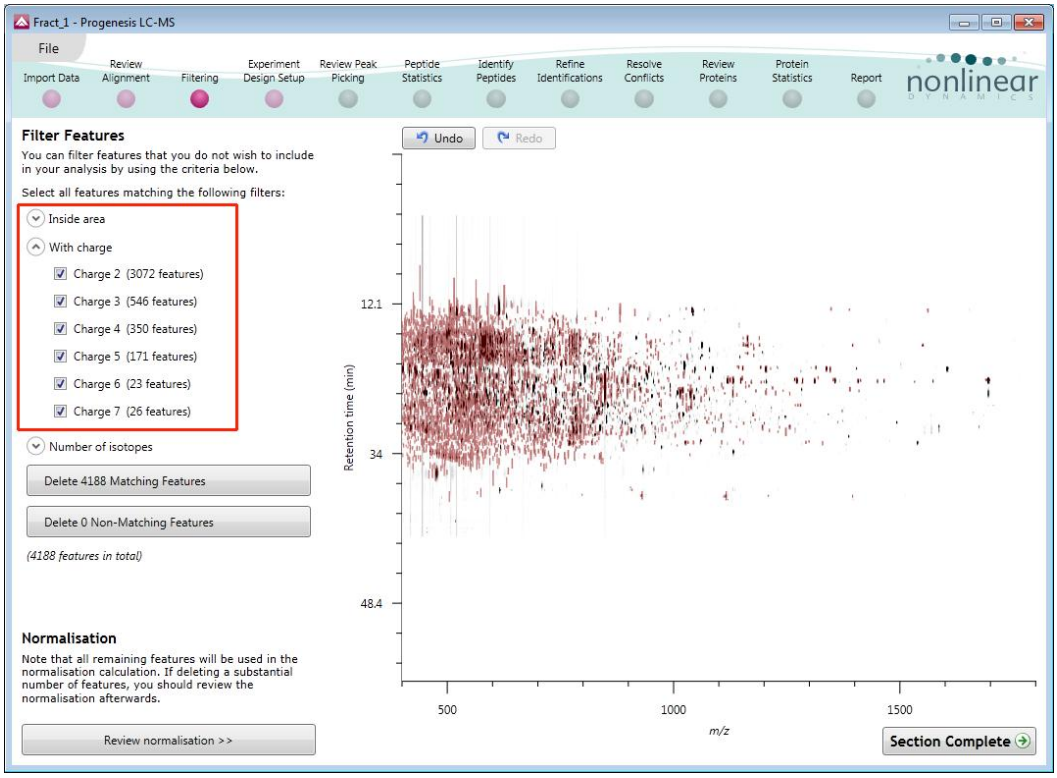




More details on the management of sensitivity are available in the How to do on **Adjusting the Sensitivity of Feature detection.**

On completion of analysis the Filtering stage will open displaying the number of features.

If required you can remove features based on position, charge state, number of isotopes or combinations of these feature properties.



The example here shows the removal of features with a charge state of **less than 2 and greater than 7**.

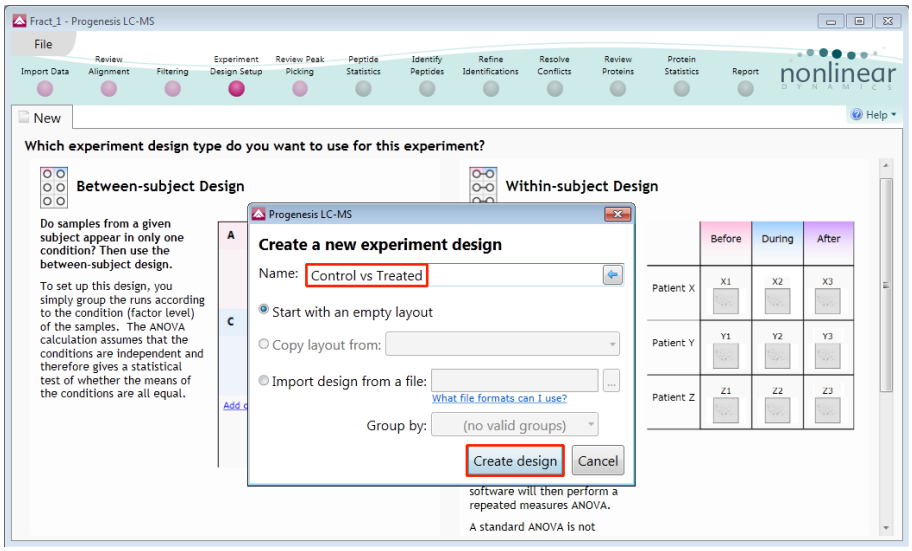
Having removed features the Normalisation will recalculate as you move to the next section.

Stage 4: 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: where samples from any given subject appear in only one condition.



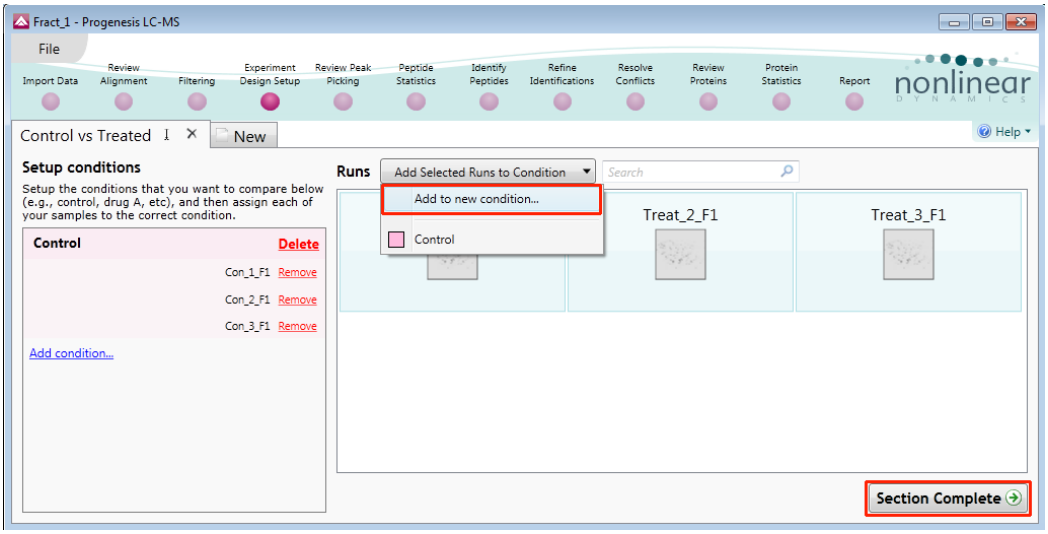
Within-subject design: where samples have been taken from a given subject under different conditions

(Additional information on how to apply the Within-subject Design is in **Appendix 2** page 38)

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.

Select **Between-subject** and give design an appropriate name.

Highlight the runs, to add them on to a new condition by clicking on **Add Selected Runs to Condition**

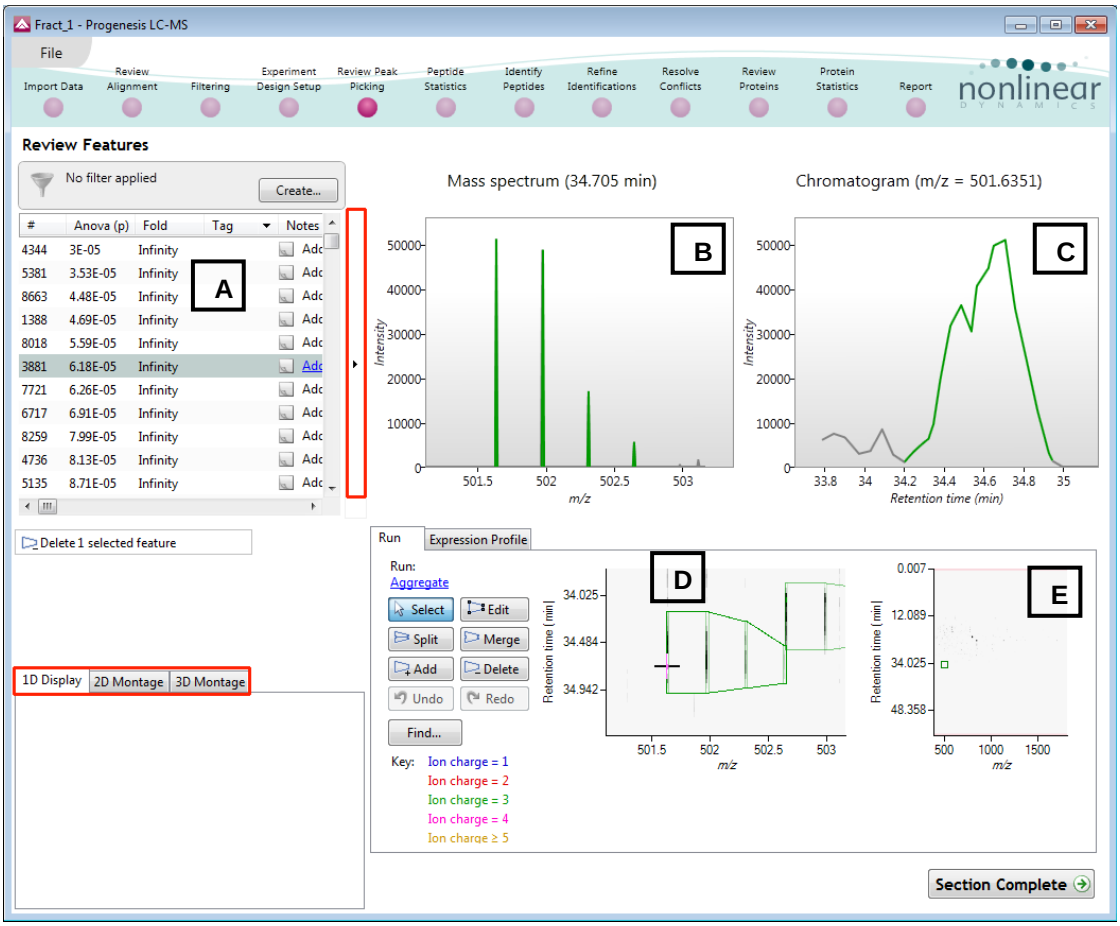


Click **Section Complete** to move to Review Peak Picking.

Stage 5: Validation, review and editing of results

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

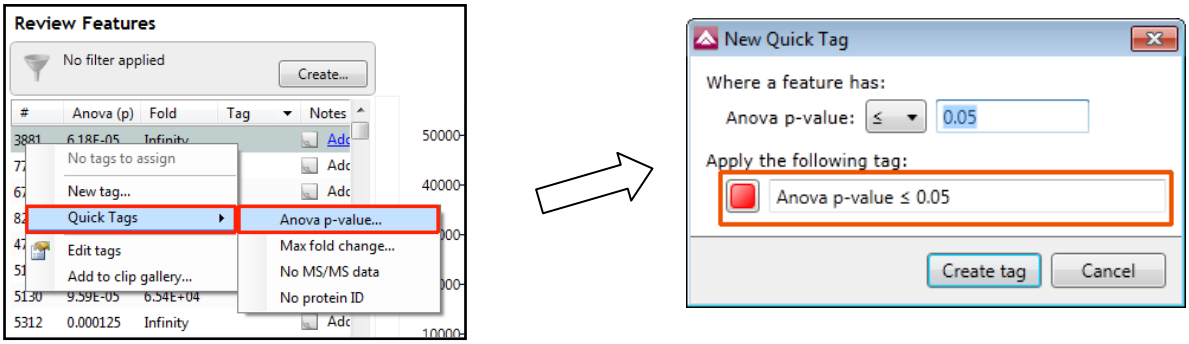
The review stage has 4 display modes: 1D, 2D, 3D and Feature Details controlled by the tabs on the bottom left of the display and the expander bar to the right of the table. Each display has multiple views to allow comparative exploration of the detected features on the aligned LC-MS runs.



Details on how to use the various views and table are described in detail in the Main analysis workflow user guide.

For the purposes of this example we require to identify all those features that demonstrate a significant Anova value (p≤0.05) between the 2 conditions being studied. We will create a Tag identifying just those features.

Right click on a feature in the table and select **Quick Tags** then **Anova p-value**.. set the required threshold and either accept the tag name or overtype it.

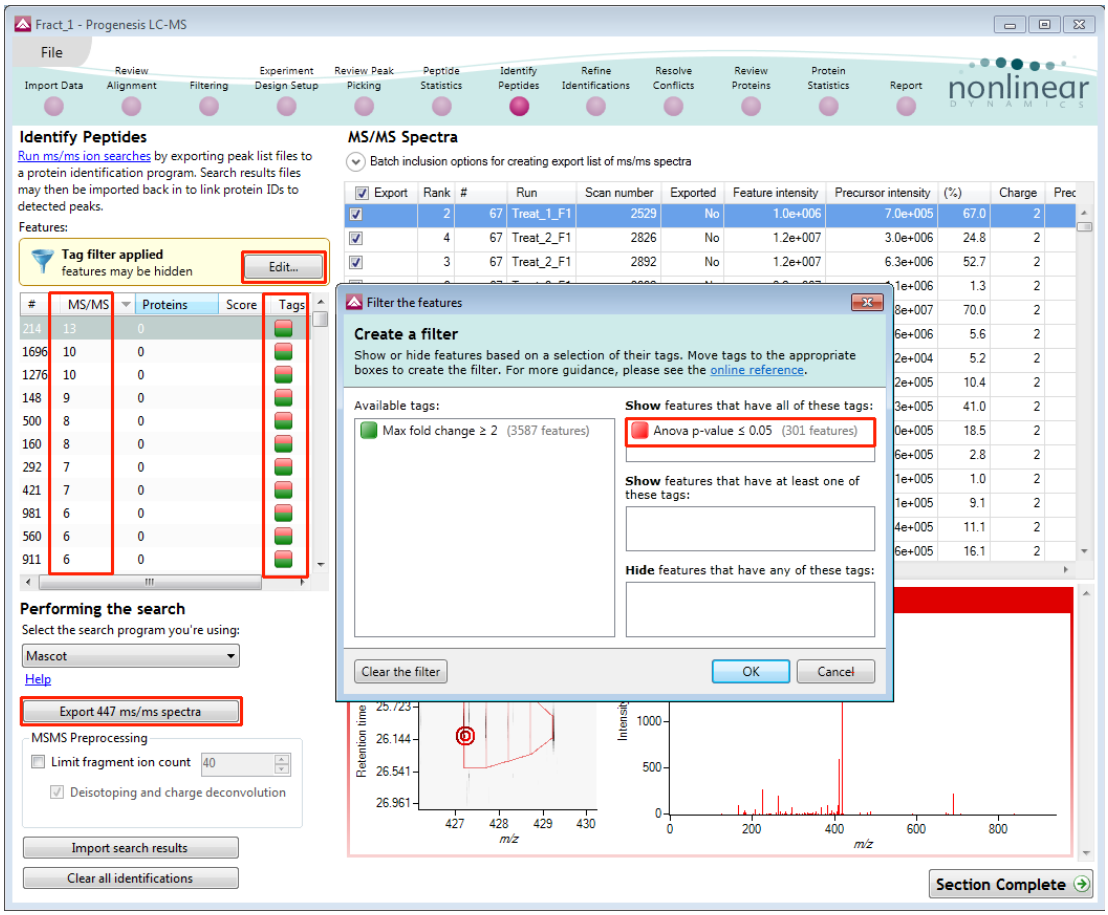


Now move to the **Identify Peptides** stage in the workflow using the icon on the workflow**.**

Stage 6: Identify Peptides

Progenesis LC-MS does not perform peptide identifications itself. Instead it supports identifications by allowing you to export a set of 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 LC-MS, using a number of different file types, and matched to your detected features.

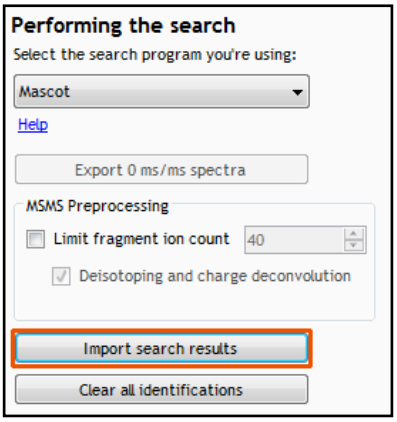
Determining protein identification is dependant on the availability of MS/MS data for the LC-MS runs. This data may be available but limited if the LC-MS was performed in a data dependant MS/MS detection mode due to under sampling. Under these conditions MS/MS data acquisition is dependant on thresholds and parameters set prior to the acquisition of the LC-MS run.



For this example we are using LC-MS runs containing MS/MS data where the data was acquired in a data dependant mode.

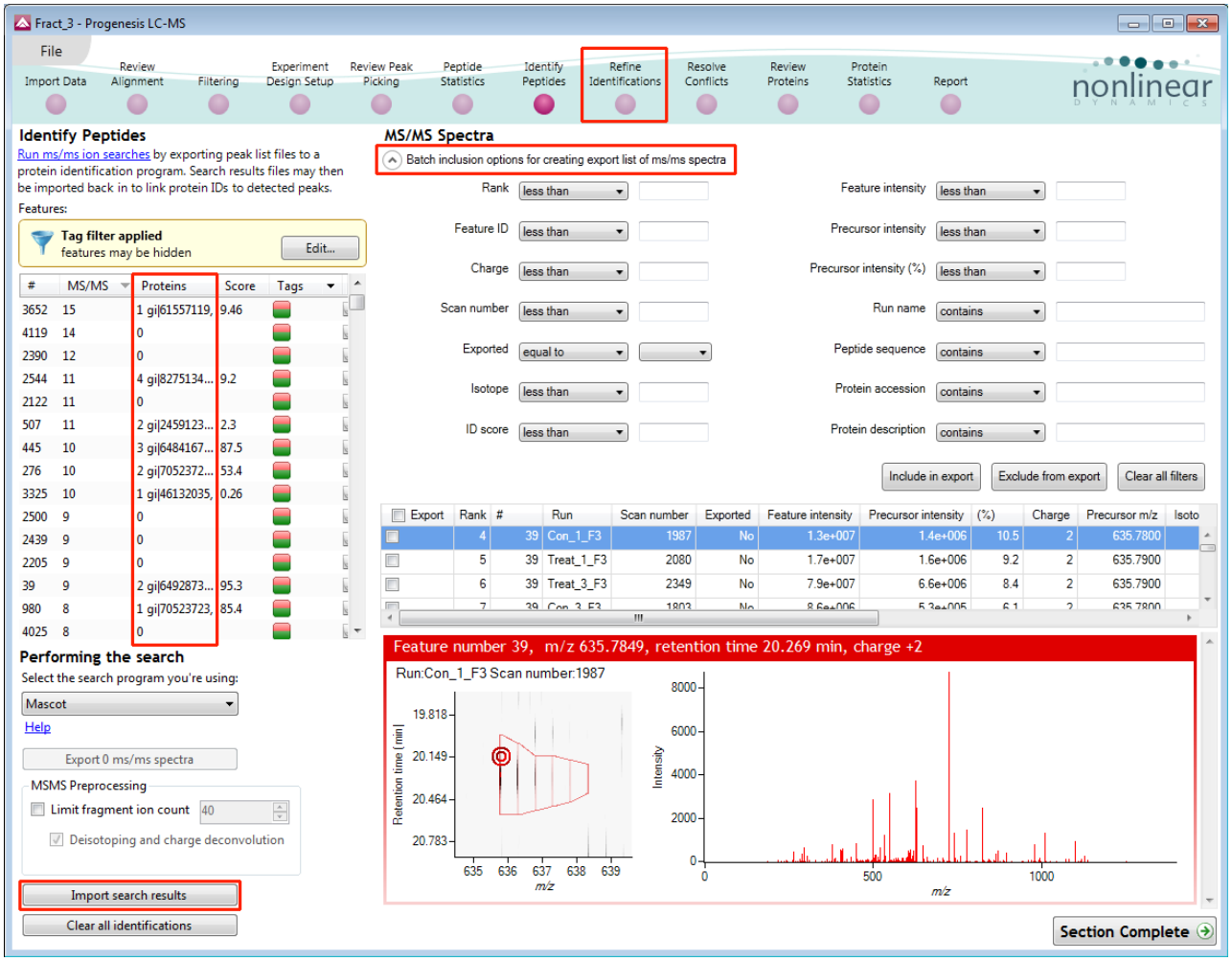
The Peptide Search page shows the number MS/MS that have been matched to each feature in the Feature list (see above). MS/MS scans are matched to a feature if their precursor *m/z* and aligned retention time fall within the area of one of the isotopes of the feature. The MS/MS scans which are matched to the displayed features are shown in the MS/MS spectra list on the right.

The first step is to decide which MS/MS scans you wish to export to be identified. By default this is all the available spectra for the Features displayed in the Features list. Using the tag created in the previous section you can filter the table to only those showing a significant change (Anova p≤0.05) between the conditions. This number of spectra to be exported is visible on the Export button.



Performing an MS/MS Ion Search

1. Select appropriate search engine i.e. Mascot
2. Click ‘Export current query set’ to save search as file
3. Perform search on appropriate search engine and save results file
4. Click ‘Import search results’, locate results file and open
5. On importing the Search results the Features table updates to reflect the identified proteins and the relevant score for each searched feature.



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

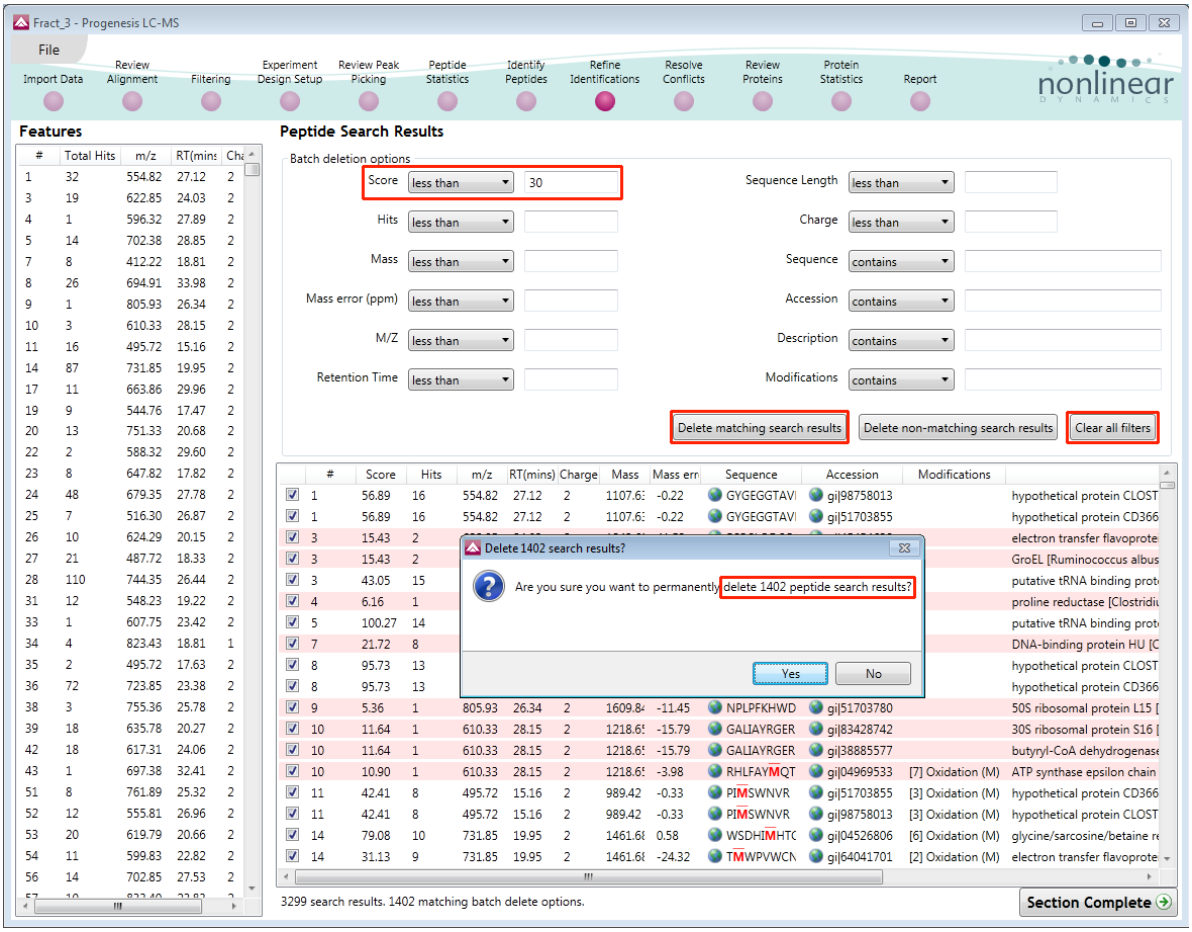
Stage 7: Refine Identifications

In this example the organism under study is *Clostridium difficile*

As an **example** 'Acceptance Criteria' on which to base the sequential filtering of the Peptide results, the following thresholds will be applied:

- Remove identifications with a Score less than 30
- Remove identifications where less than 2 hits were returned
- Remove all identifications where the Protein Description **Contains** 'hypothetical'
- Remove all identifications where the Protein Description **Doesn't contain** 'Clostridium difficile'

On the Batch detection options panel, set the Score to less than 30, then **Delete matching search results**.



Note: the search results matching the filter criteria turn pink and the number of search results being deleted is displayed

Now **Clear all filters** and then apply the next filters as described above.

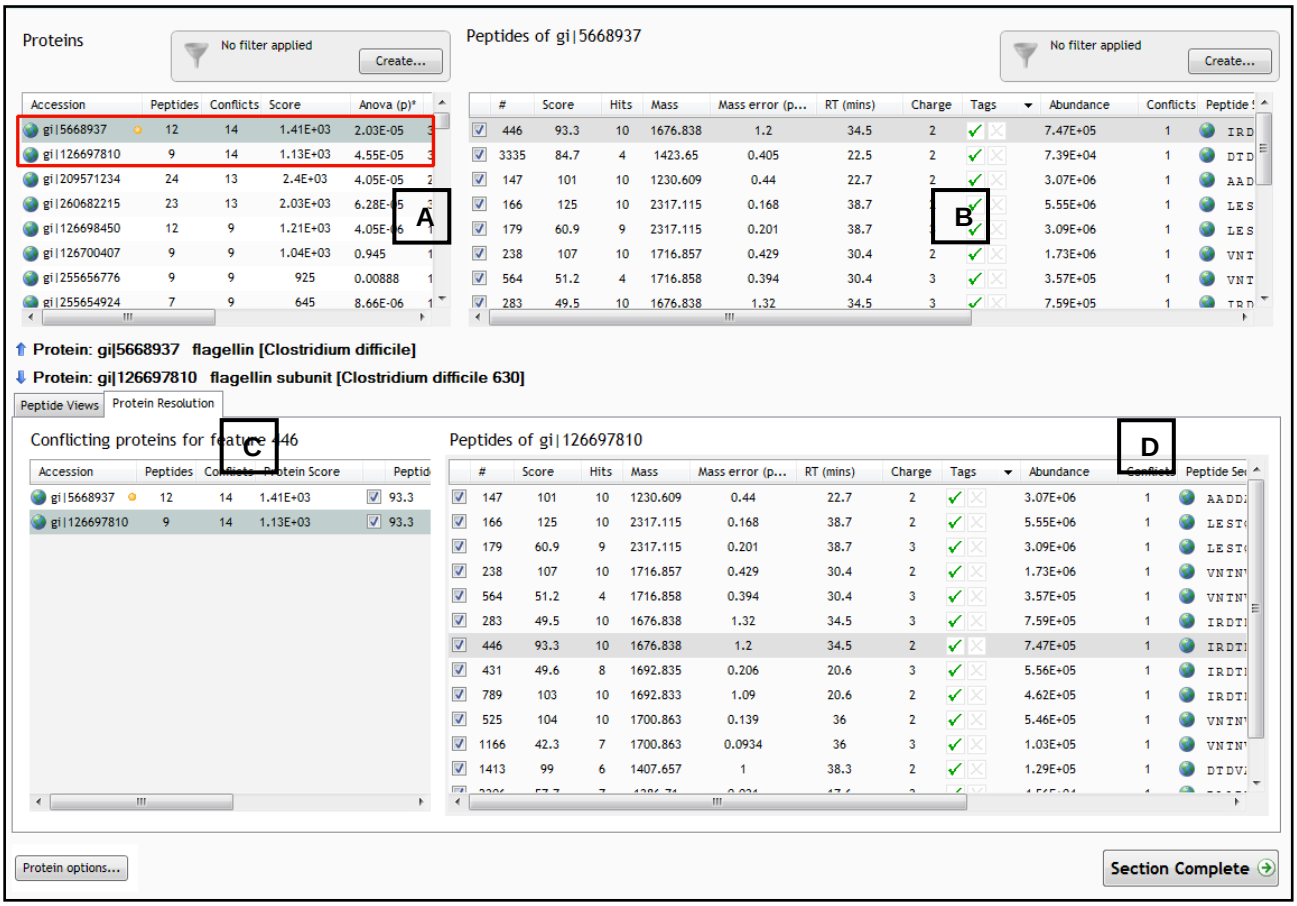
To validate the Peptide search results at the protein level select the next stage in the workflow by clicking on **Protein View**.

Stage 8: Resolve Conflicts

The Resolve Conflicts view 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.

Open the Resolve Conflicts and 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 section may vary slightly.

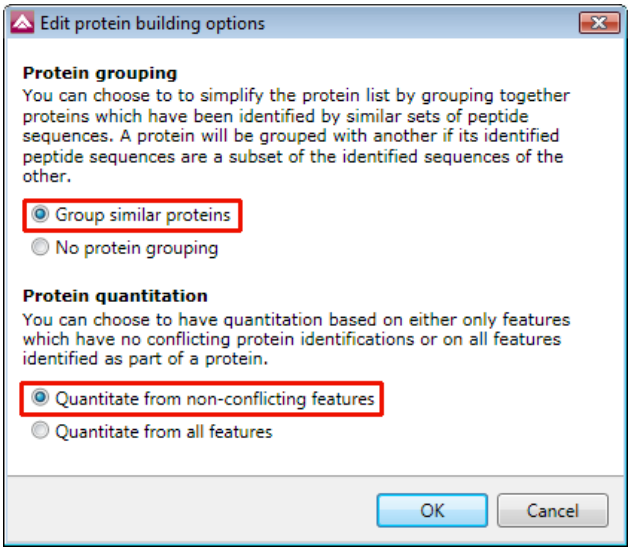


In the above example the conflict would be resolved in favour of the protein with 12 peptides as the flagellin subunit does not contain any unique peptides as compared to flagellin.

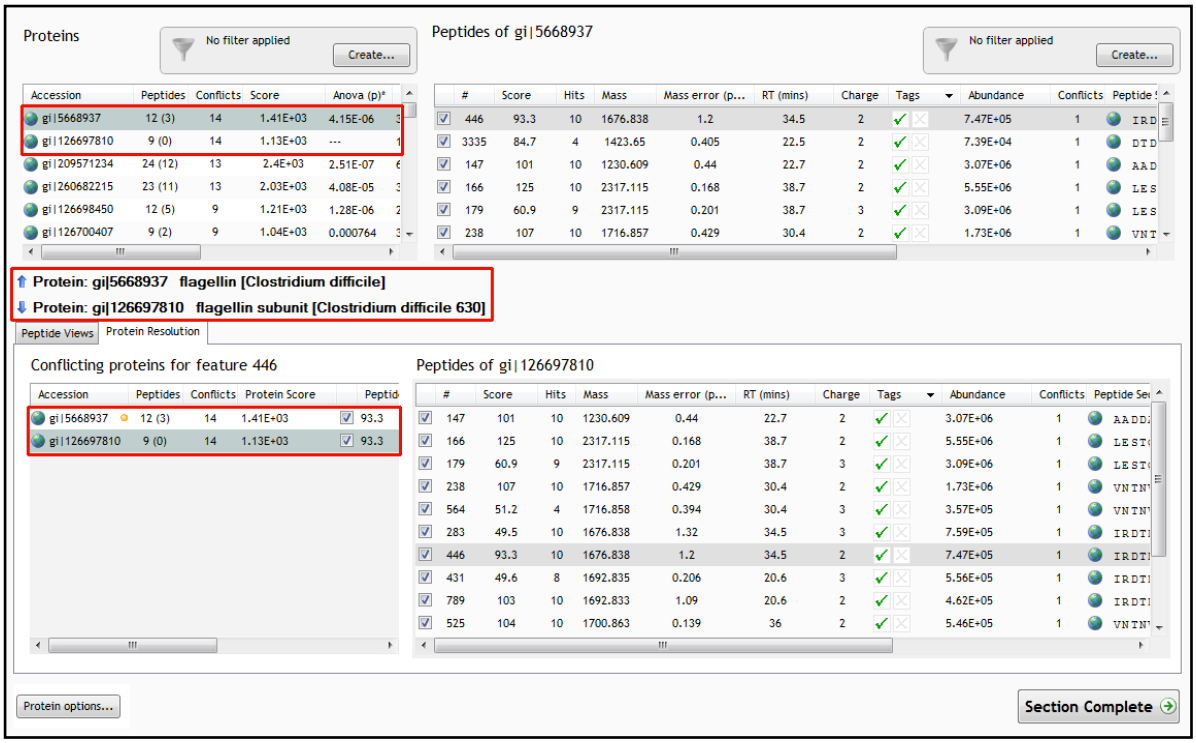
Note: the number of conflicts you have to resolve will depend on the scope and stringency of the filters you apply at the **Peptide Search** stage,

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

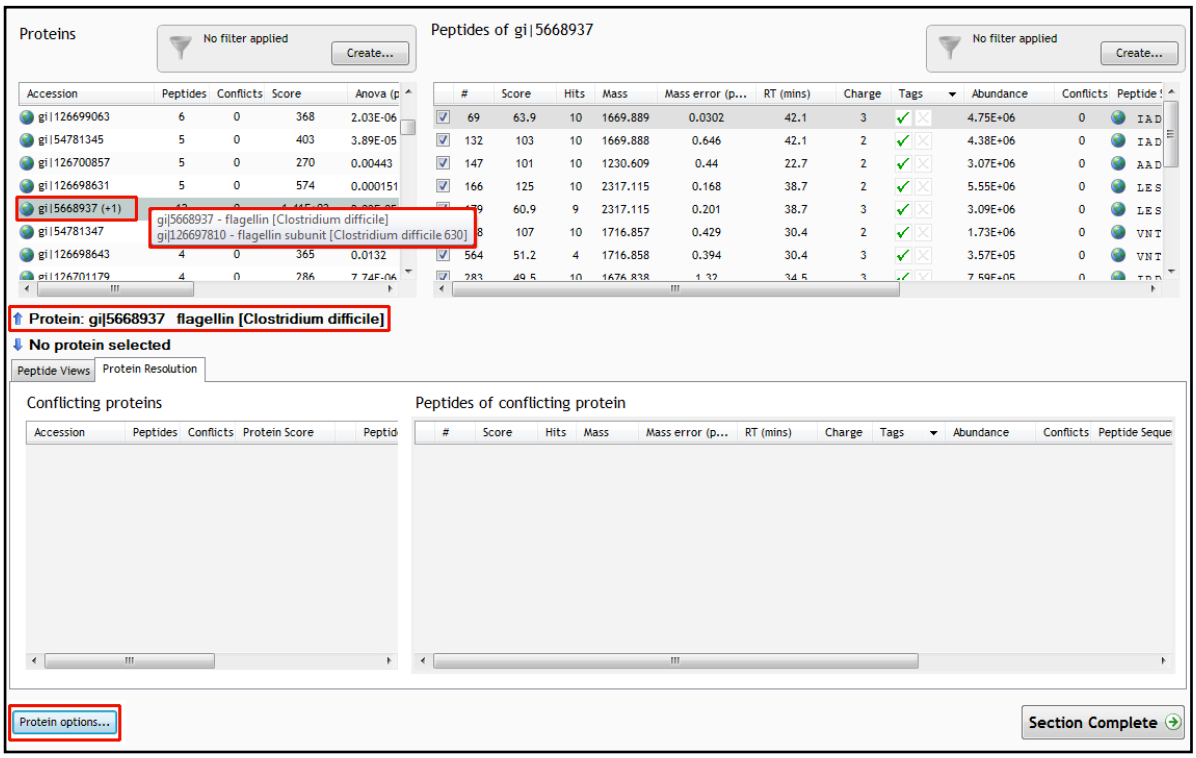
Having performed the conflict resolution with **Group similar proteins** and **Quantitate from non-conflicting features** now switch off the protein grouping.



As grouping is switched off the grouped proteins appear with conflicts to the other group members and the number of unique peptides that are used for quantitation appear in brackets after the peptide number.



With protein grouping switched on protein groups and the additional members are indicated by a bracketed number located after the Accession number. Taking **flagellin** as an example, when the cursor is held over the accession number the group members appear in a tool tip.

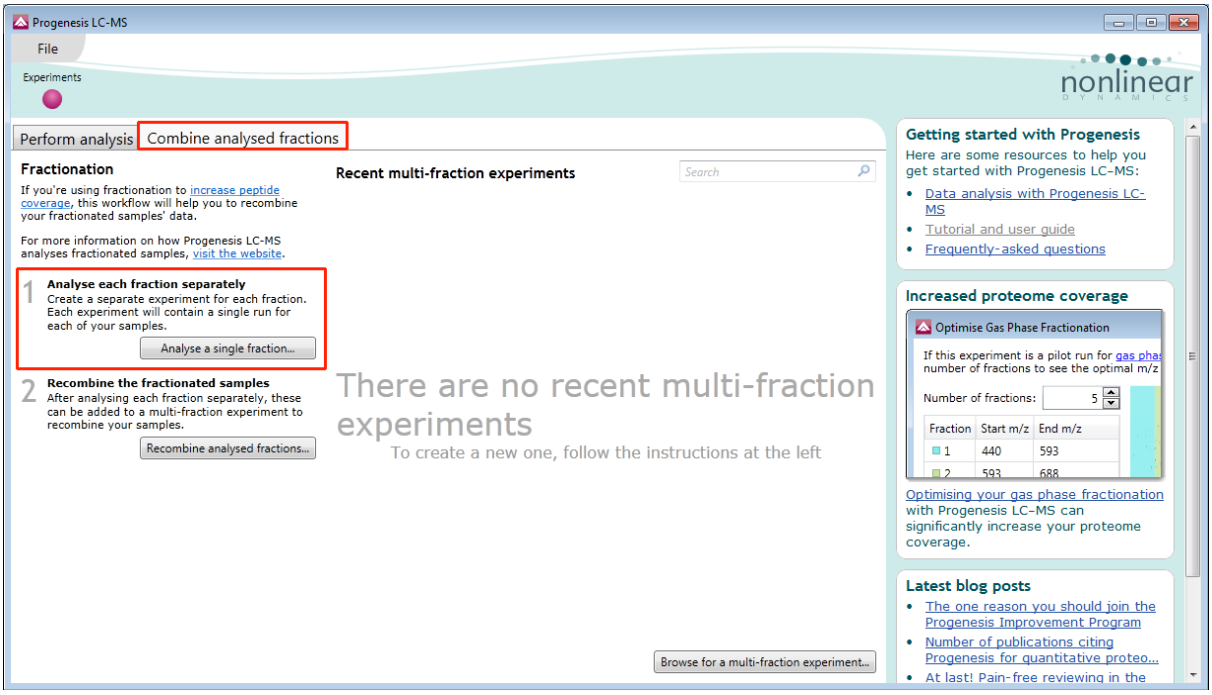


Note: the flagellin subunit has **no unique** peptides (brackets after the peptides field in the Proteins table as shown above) as they are all present in flagellin protein hence the reason for grouping. As a result all the conflicts are internal to the group.

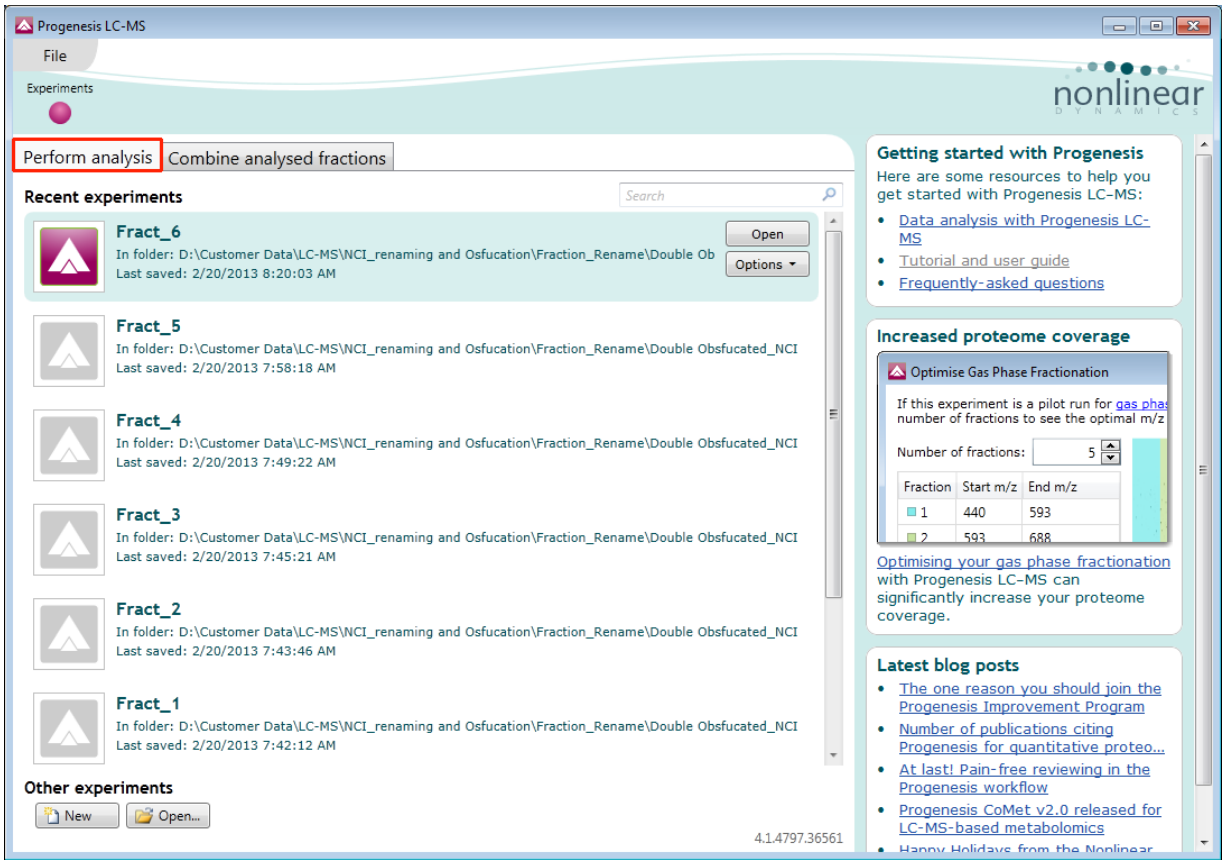
Before recombining the fractions make sure all the single fraction experiments are complete.

Completion of Fractionation Workflow Step 1

The first stage in the analysis of a Fractionated experiment is completed when you have analysed all the single-fraction experiments, in this example there are 6 fractions, therefore 6 experiments. For each experiment this includes the identification of proteins and resolution of any peptide conflicts as described in the previous sections.



The six experiments will appear in the Perform Analysis tab and can be accessed individually.

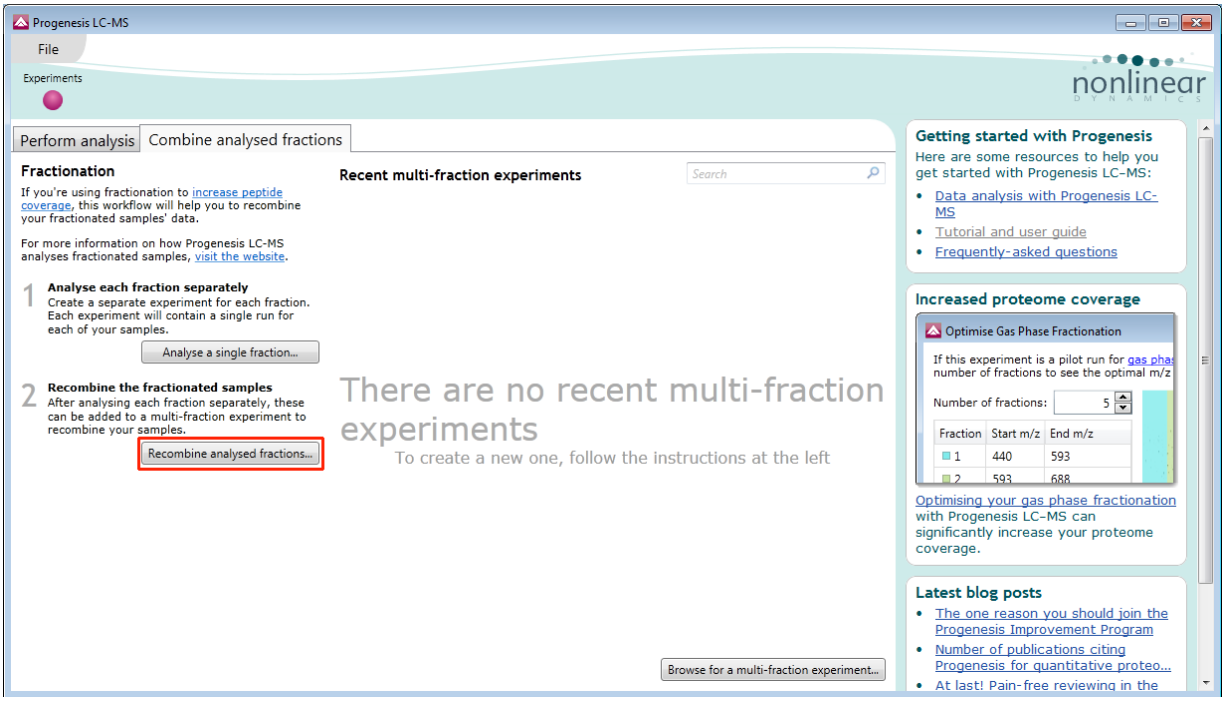


The next section describes the workflow involved in the 'Combining' of these single fraction experiments to generate a 'multi-fraction' experiment.

Fractionation Workflow Step 2

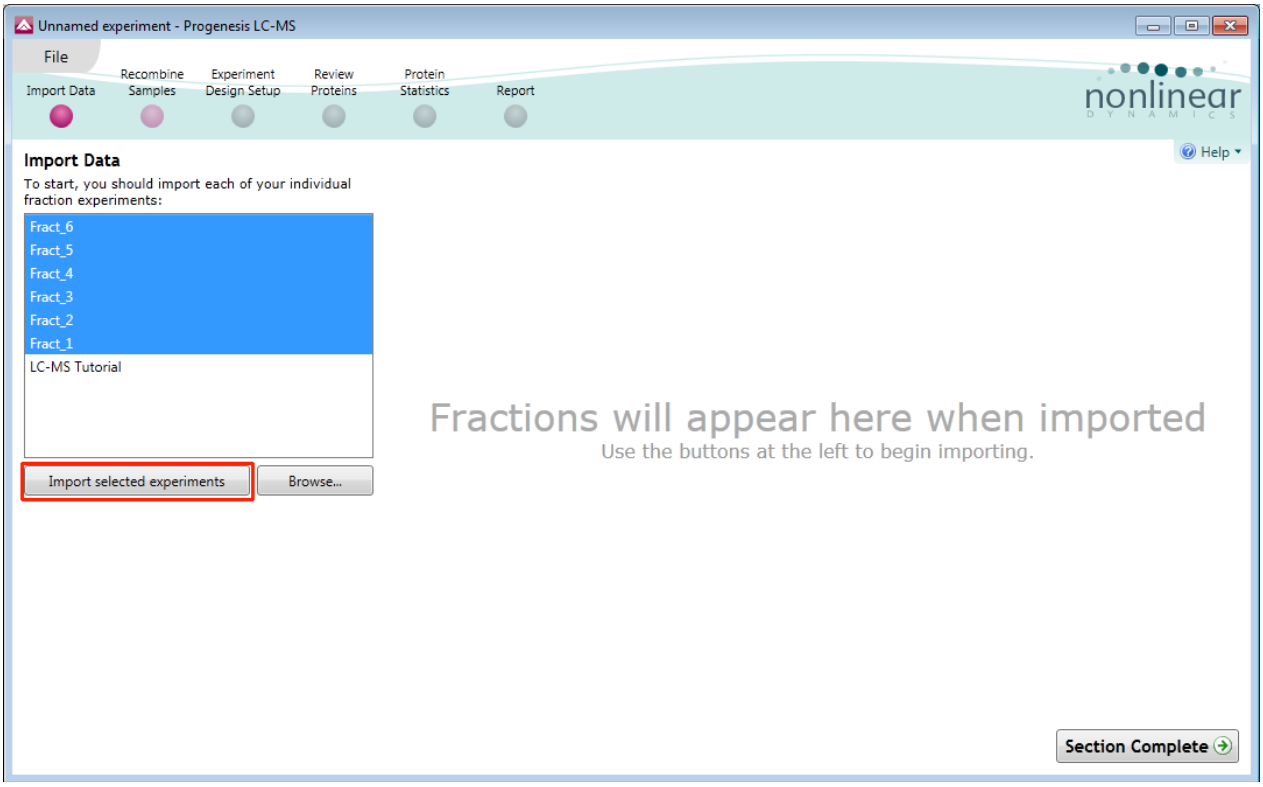
The combining of the single fraction experiments is performed in the second stage of the Fractionation workflow.

Select **Recombine analysed fractions...**



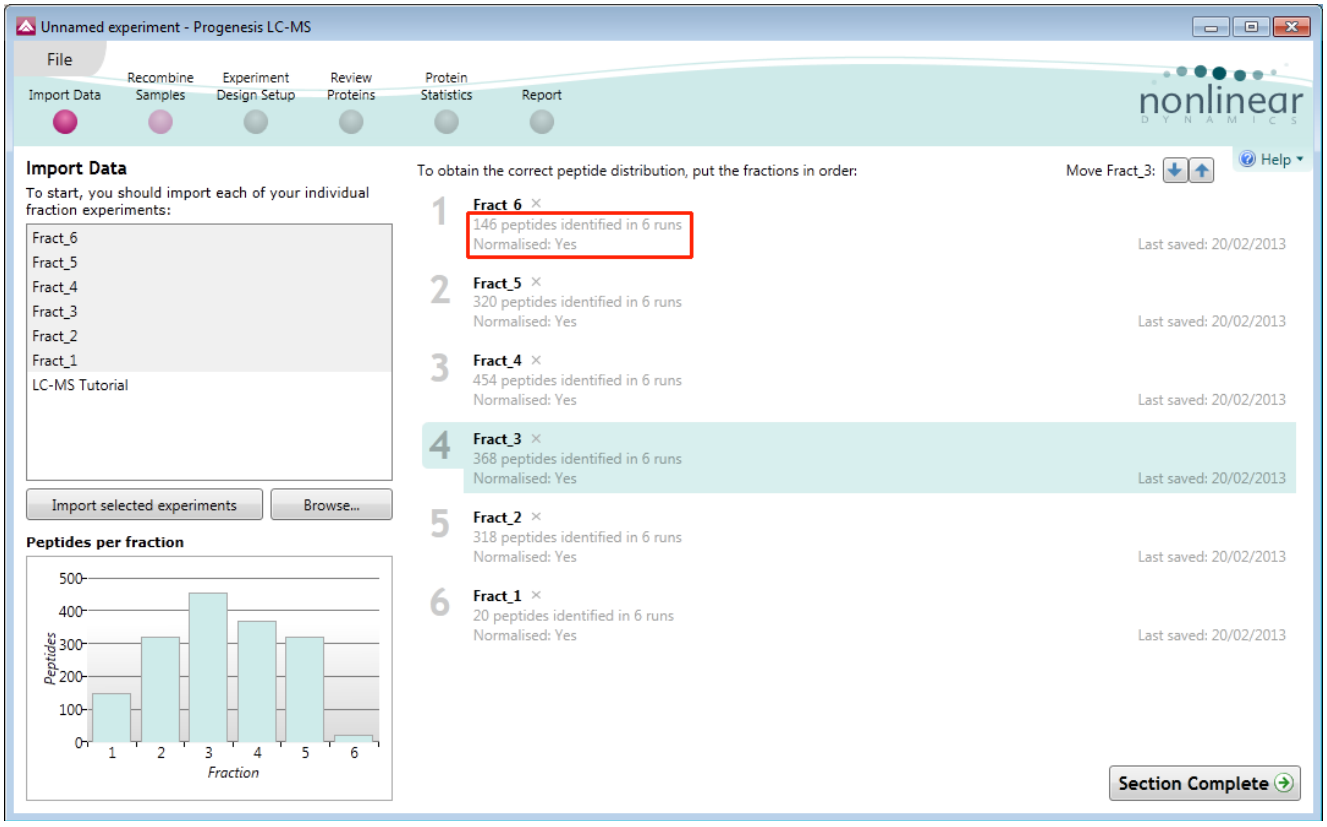
Stage 1 Import Fractions

The Import Fractions stage of the work opens, select the experiments that correspond to the (6) fractions.

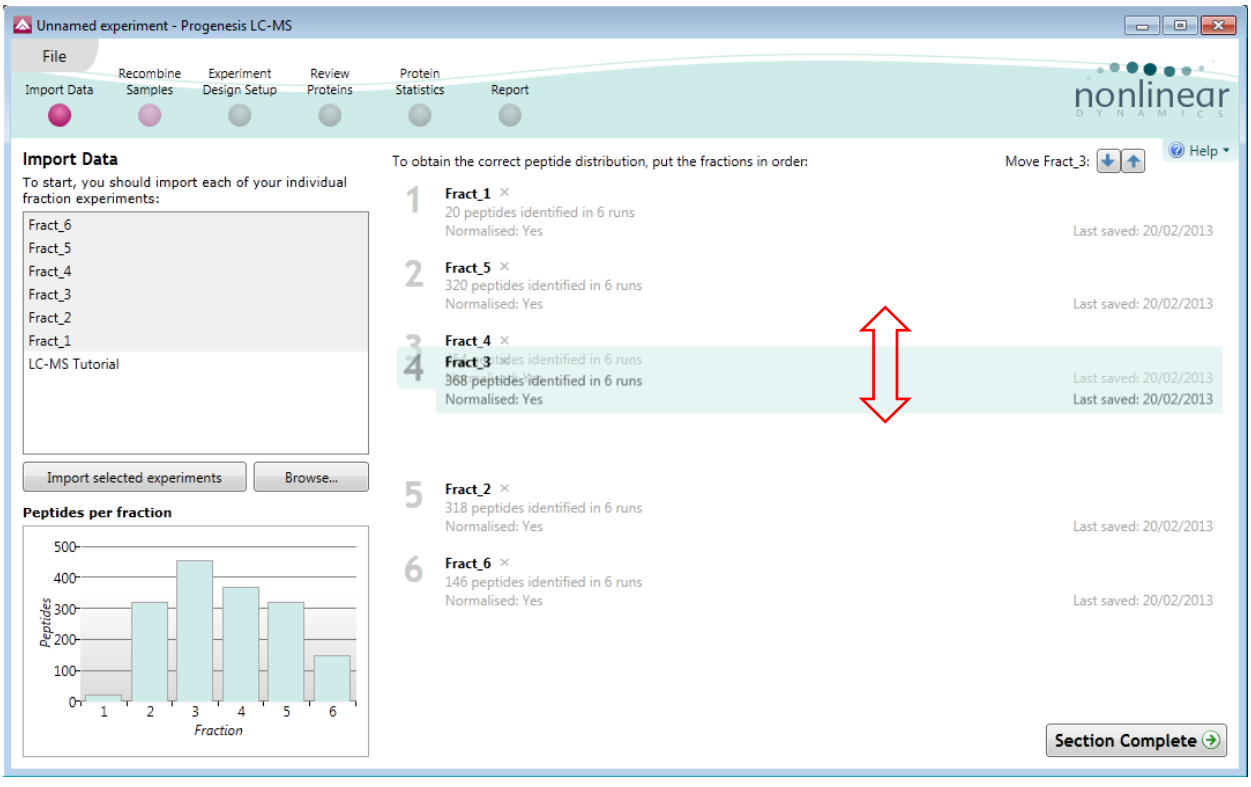


Click **Import selected experiments**

Details for each single fraction experiments appear in the panel showing numbers of identified peptides.



You can adjust the order of the single fraction experiments to reflect the order of the fractions by dragging the single fraction experiments to the correct position.



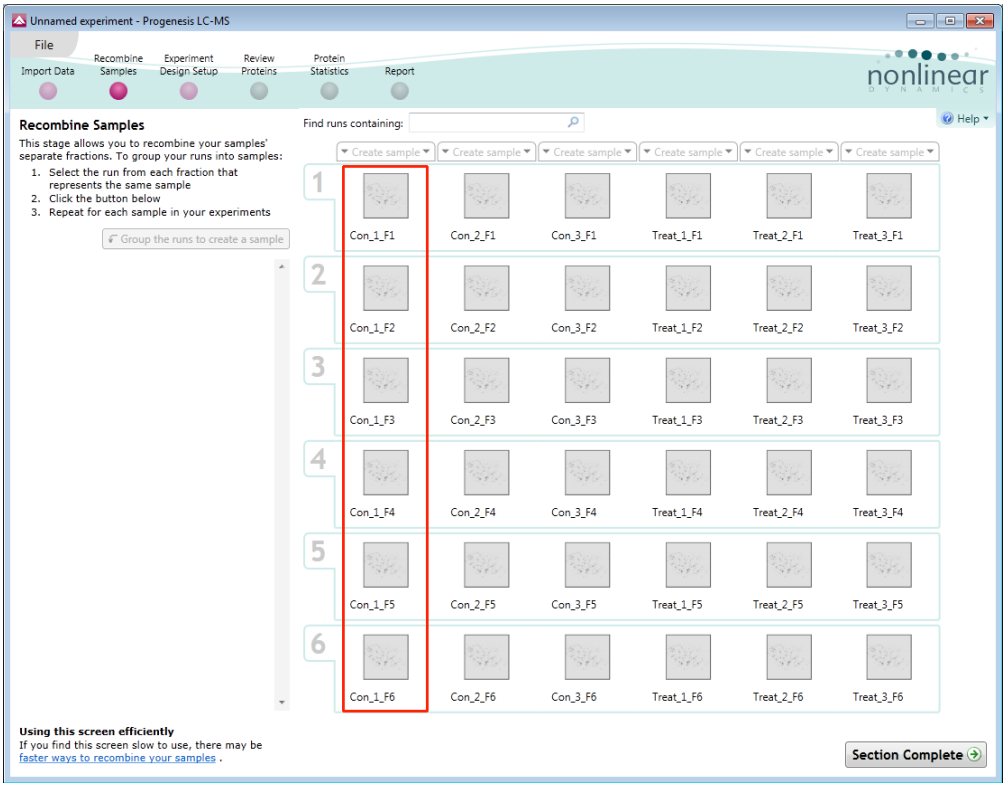
Note: the graph of 'peptides per fraction' updates to reflect the new order.

To move to the **Recombine Samples** stage click **Section Complete**.

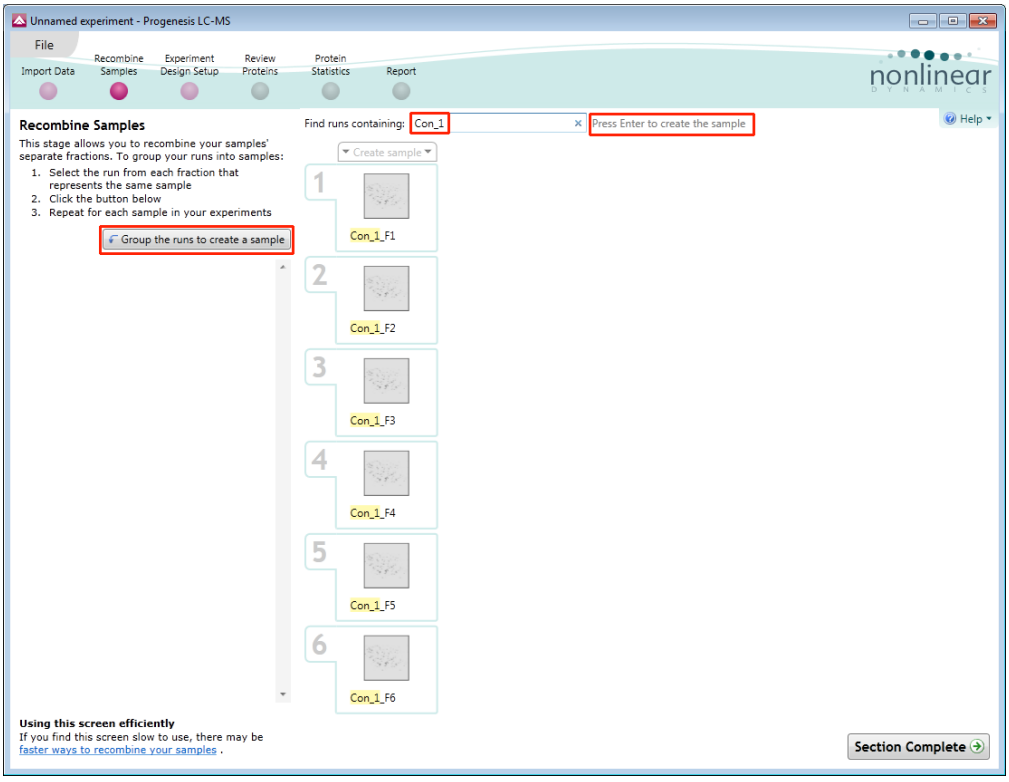
Stage 2 Recombine Samples

At this stage you will recombine the samples by selecting the runs that correspond to each sample from the single fraction experiments.

Note: how efficiently you use this page will depend on how methodically you have named the various sample runs. For this example Samples are Con_1, 2 and 3 and Treat_1, 2 and 3

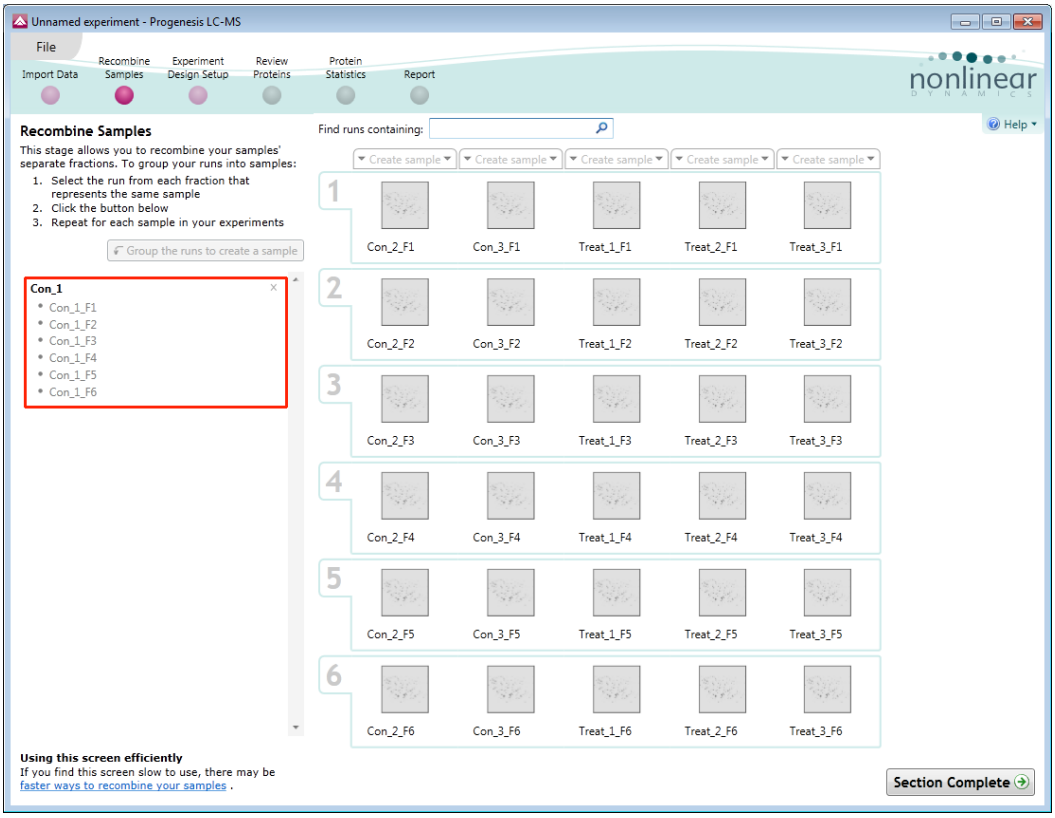


Typing **Con_1** in the **Find runs containing** search box will locate the runs corresponding to sample Con_1.

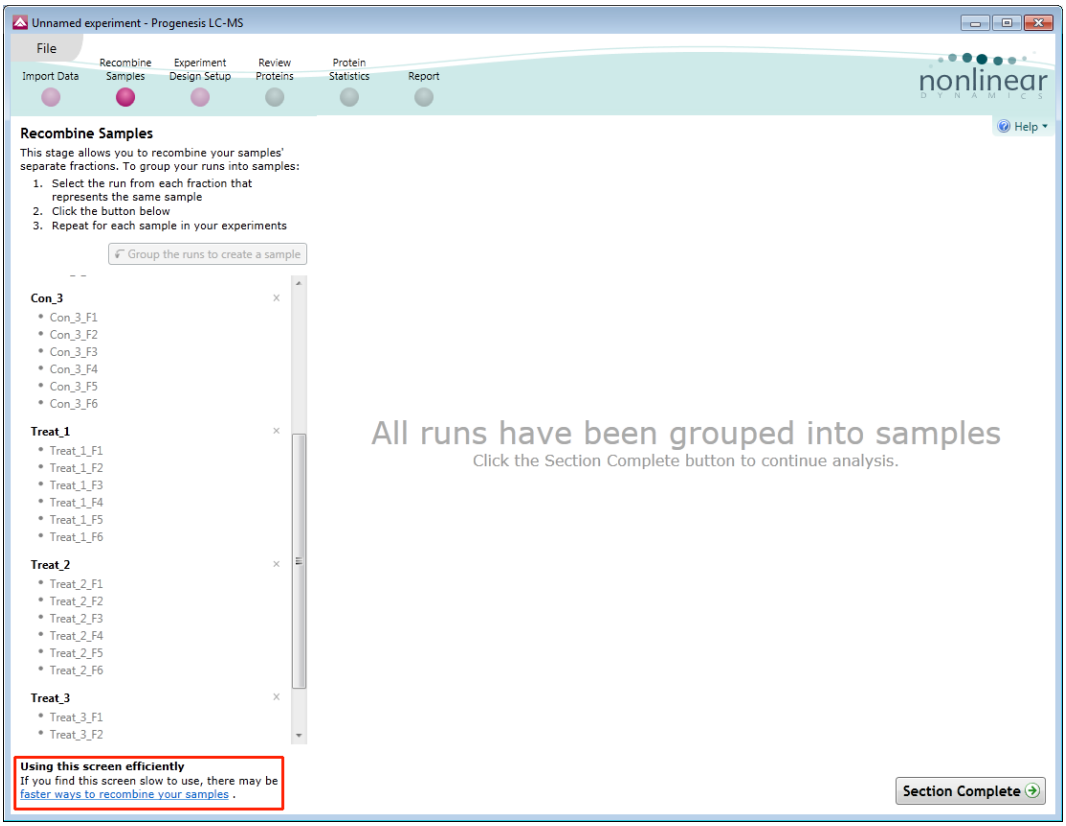


Click '**Enter**' or **Group the runs to create a sample** in the left hand panel

Note: using Enter will set the sample name as **Con_1**, overtype to rename as required.



Repeat for the remaining samples.



Note: as mentioned before other ways of Recombining the samples can be applied, depending on the naming conventions used; use the link, bottom left, to see the alternatives.

Having completed the recombination of the samples, click **Section Complete**.

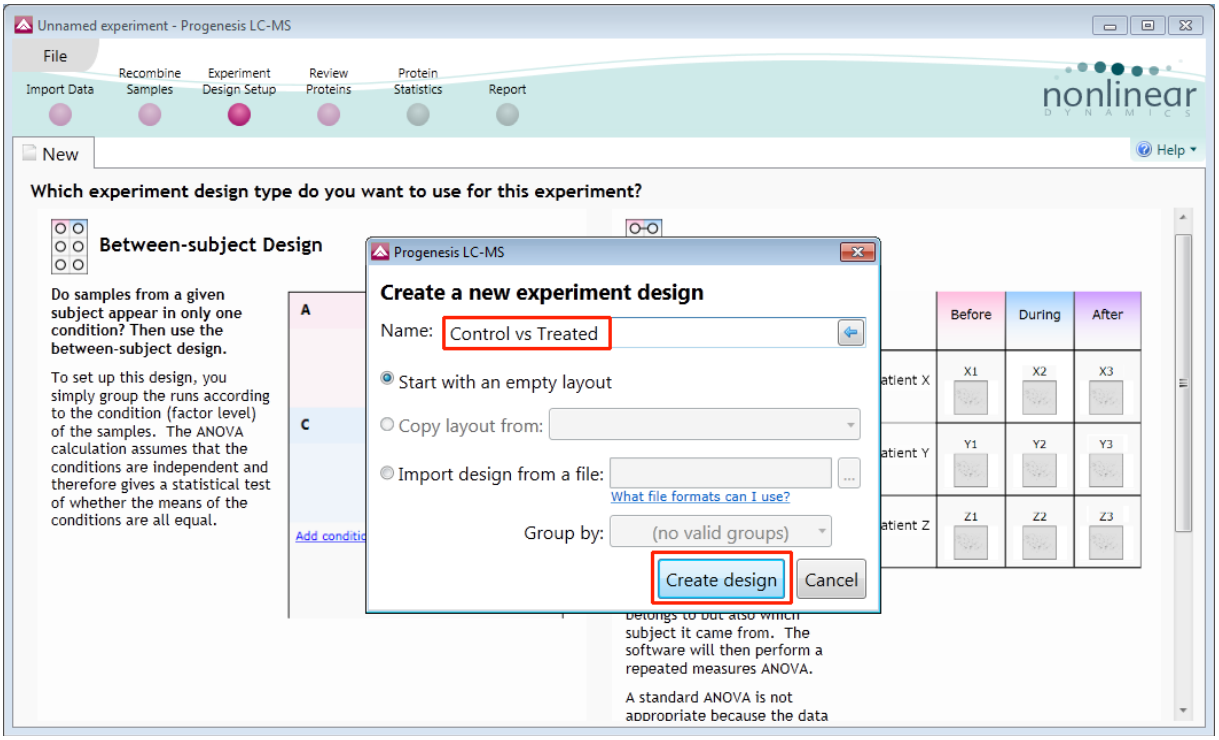
Note: at this point the data is re-normalised to account for the fractionation of the samples.

Stage 3 Experiment Design Setup

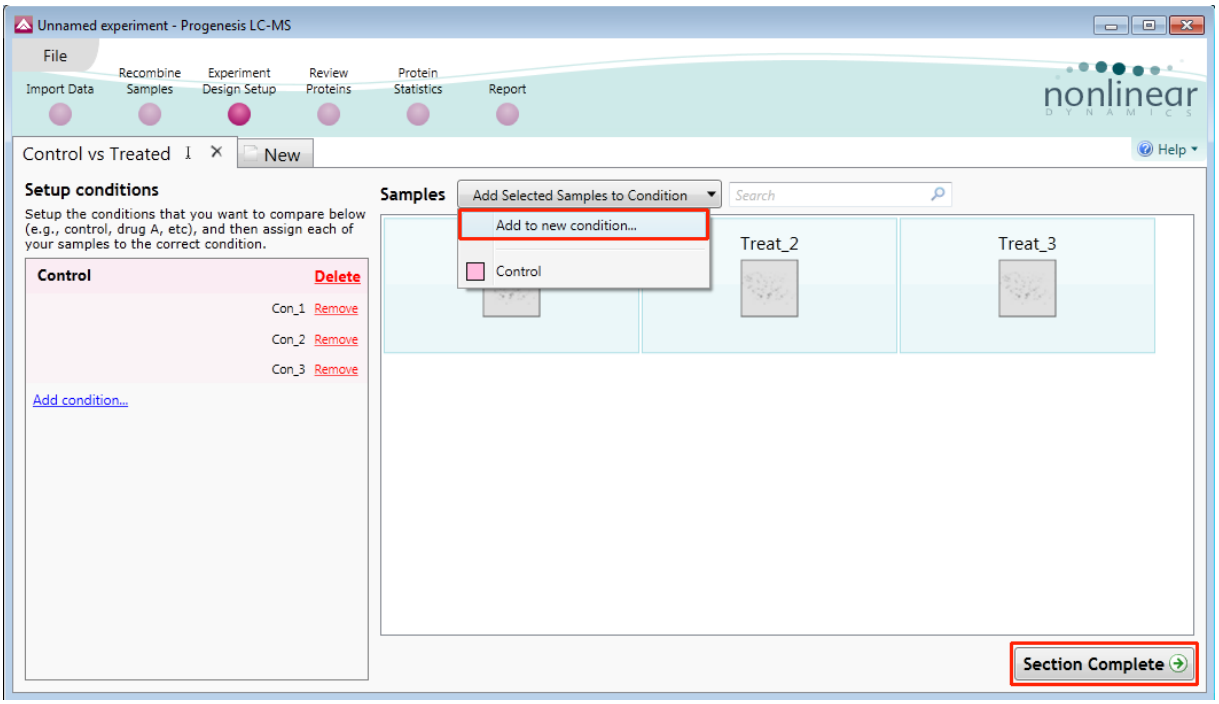
Having recombined your samples you can now define the experimental designs most appropriate for your original experiment.

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

Select **Between-subject** and give design an appropriate name.



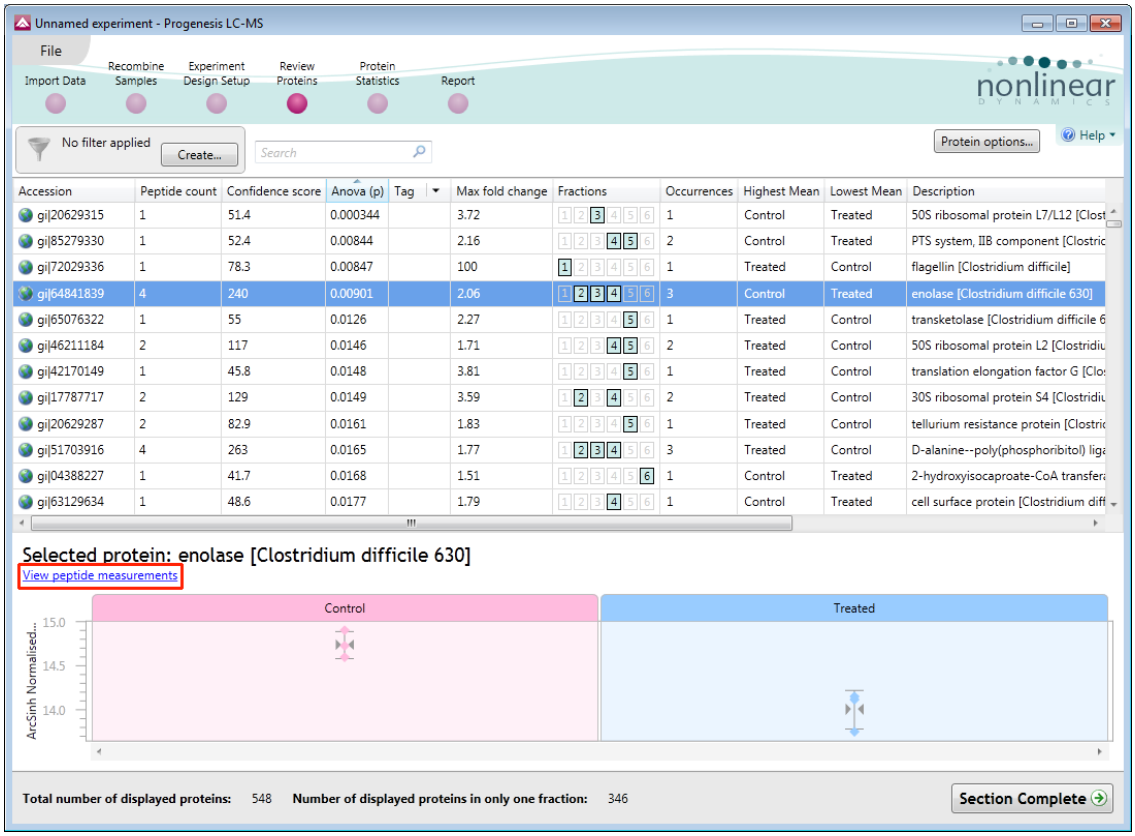
Highlight the samples, to add them on to a new condition click on **Add Selected Samples to Condition**



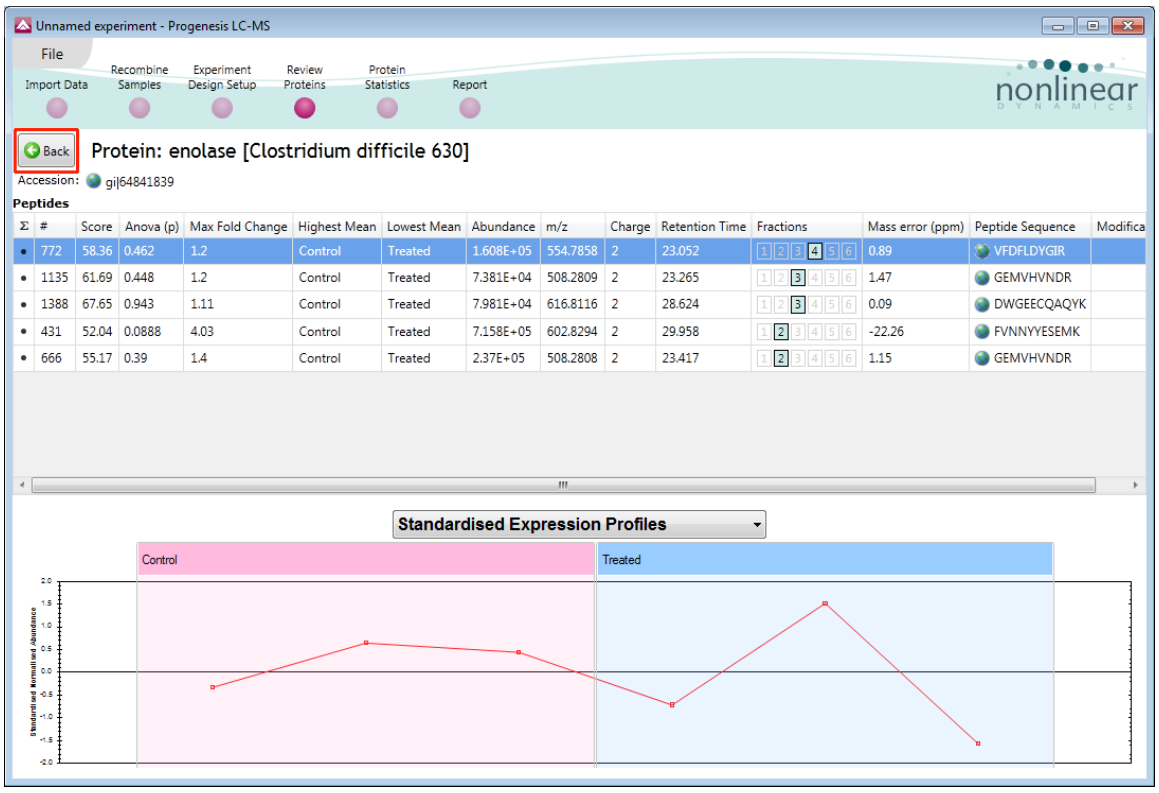
Click **Section Complete** to move to Review Proteins.

Stage 4 Review Proteins

The recombined data can now be viewed at the level of the Proteins.



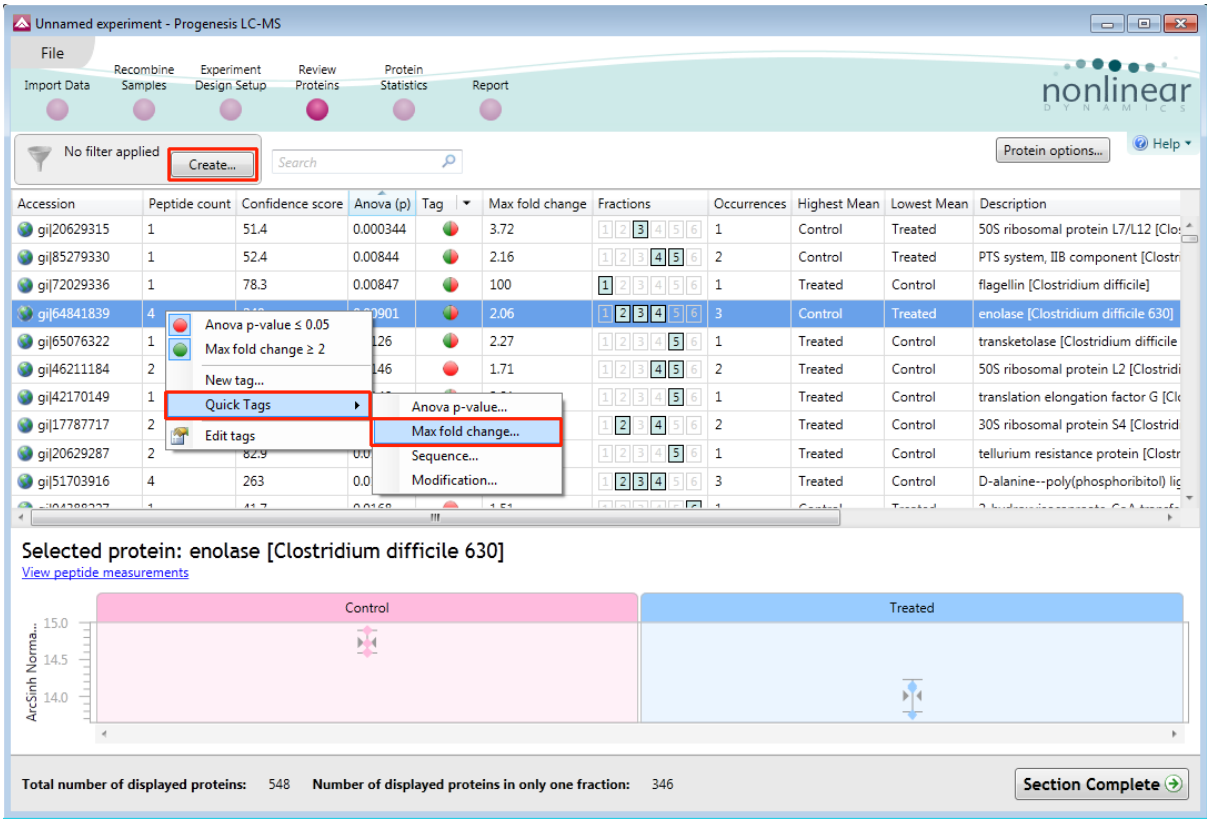
And at the peptide level when you click **View peptide measurements**



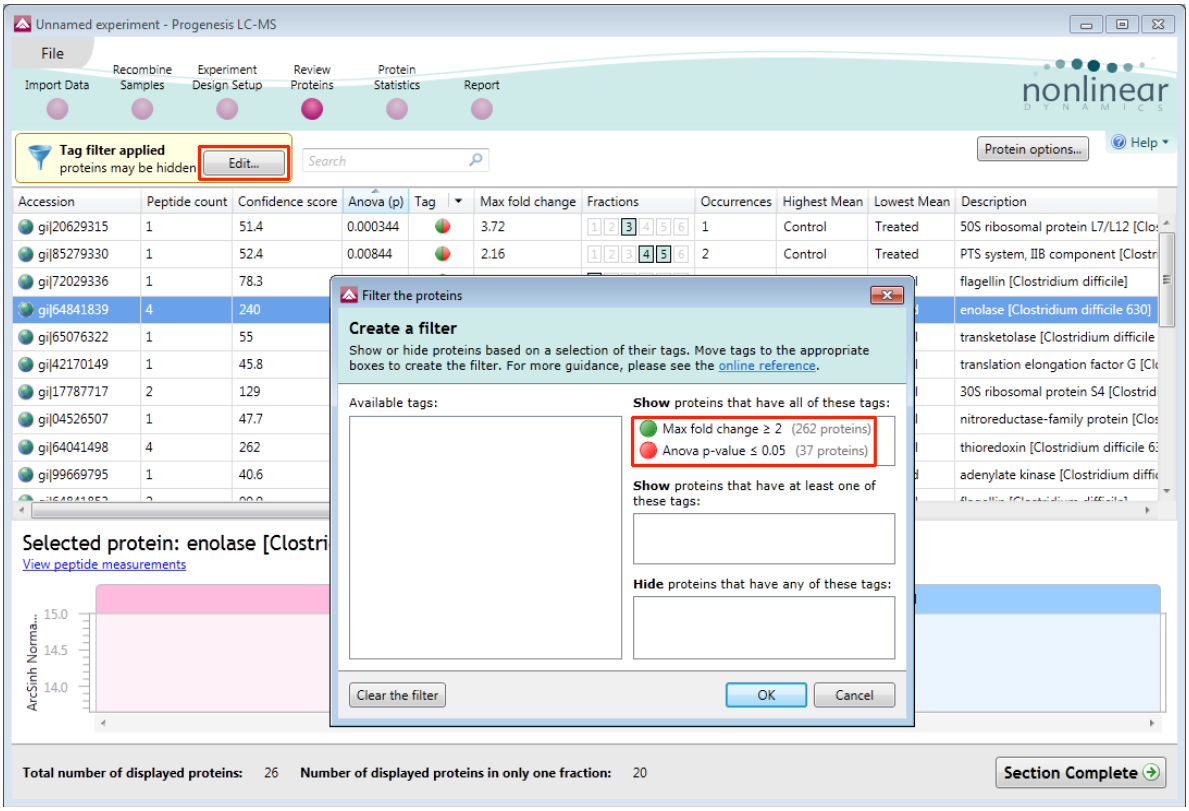
Click **Back** to return to the **Protein View**

Using the Protein Tags you can generate a list of proteins based on similar properties and thresholds.

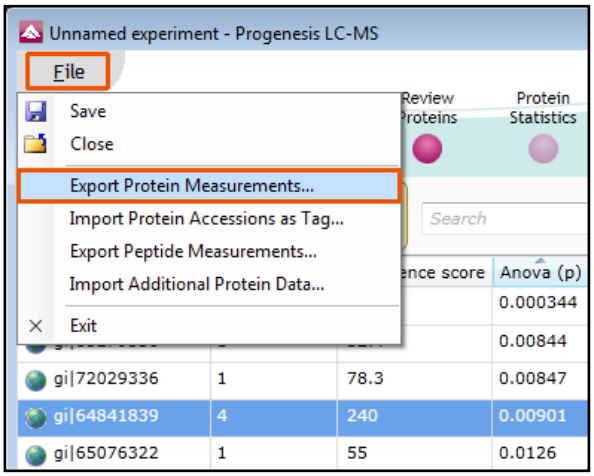
Right click on a protein in the table and use the Quick Tags to generate tags for proteins with **Anova p-value≤0.05** and a **Max fold change≥2**.

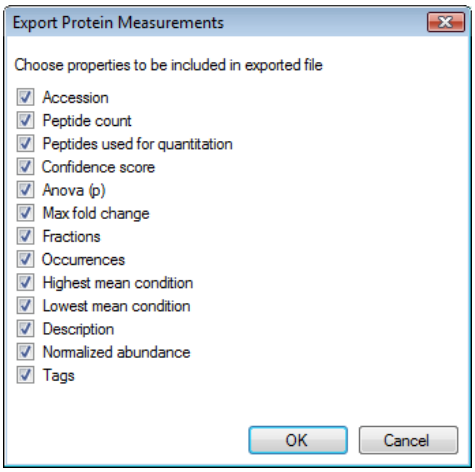


Using the 'Tag' filters the list is reduced to the relevant proteins. Details of these proteins can be exported by selecting **Export Protein Measurements** from the **File** menu.



Details of these proteins can be exported by selecting **Export Protein Measurements** from the **File** menu.





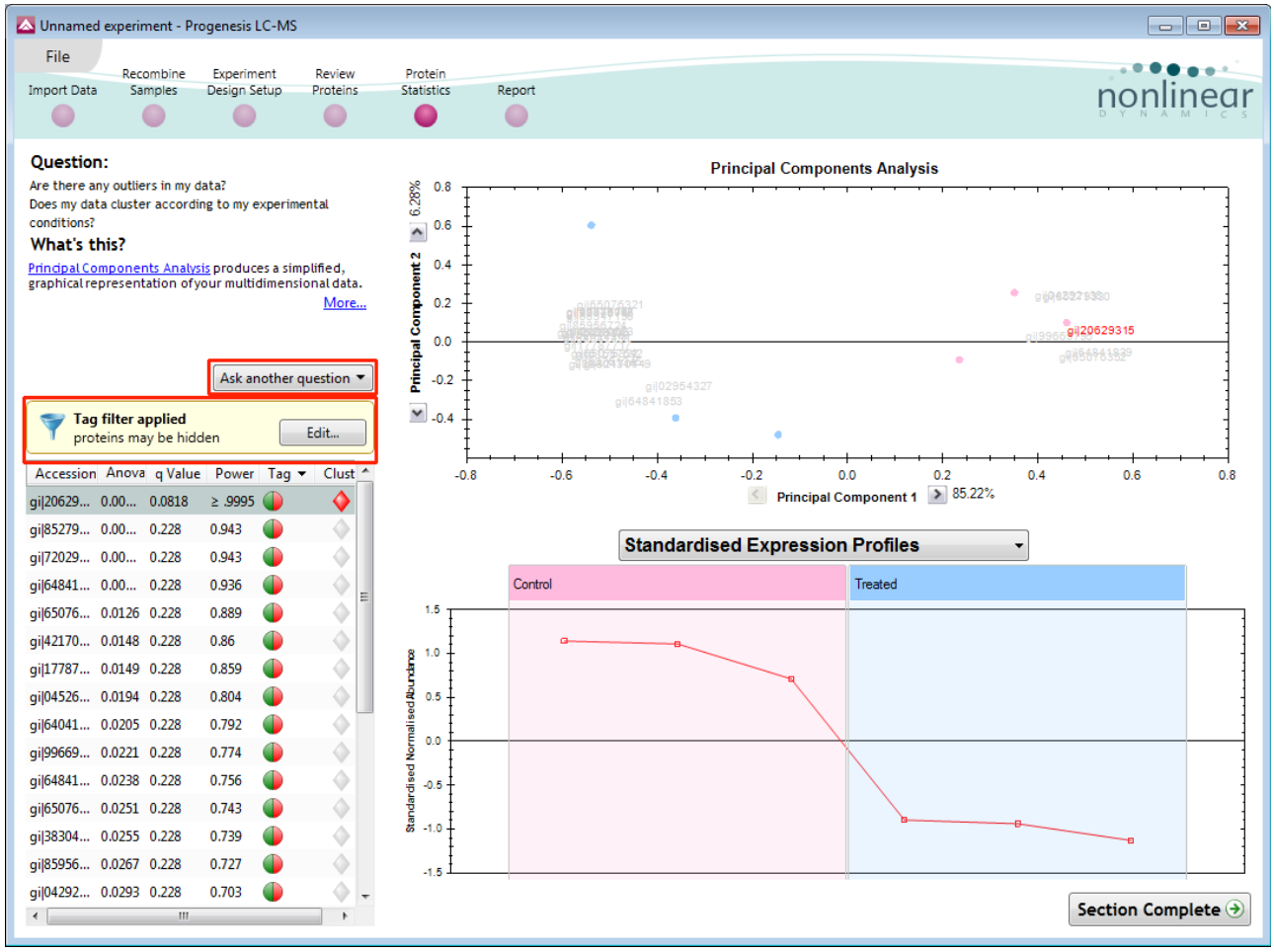
A dialog allows you to control the details of the output file.

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

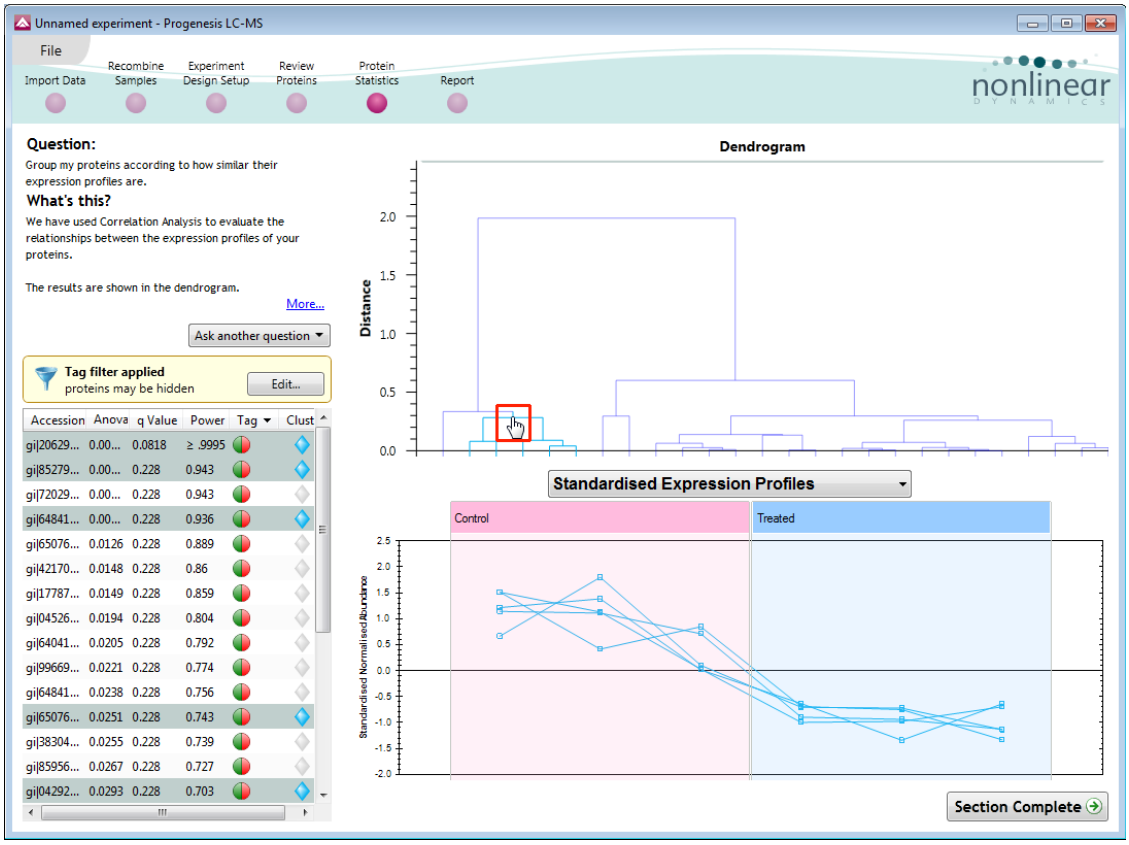
Finally your recombined data can be saved as a **Multi-fraction experiment**.

Stage 5: 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.

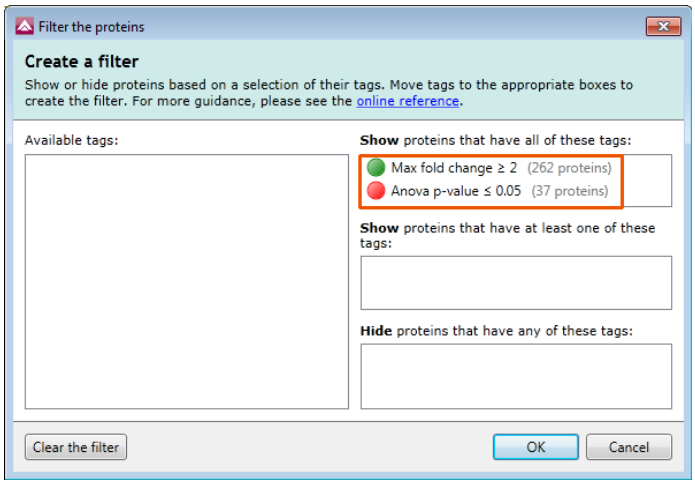


Now move to the **Report** section to report on Proteins and /or peptides.

Stage 6: Reporting

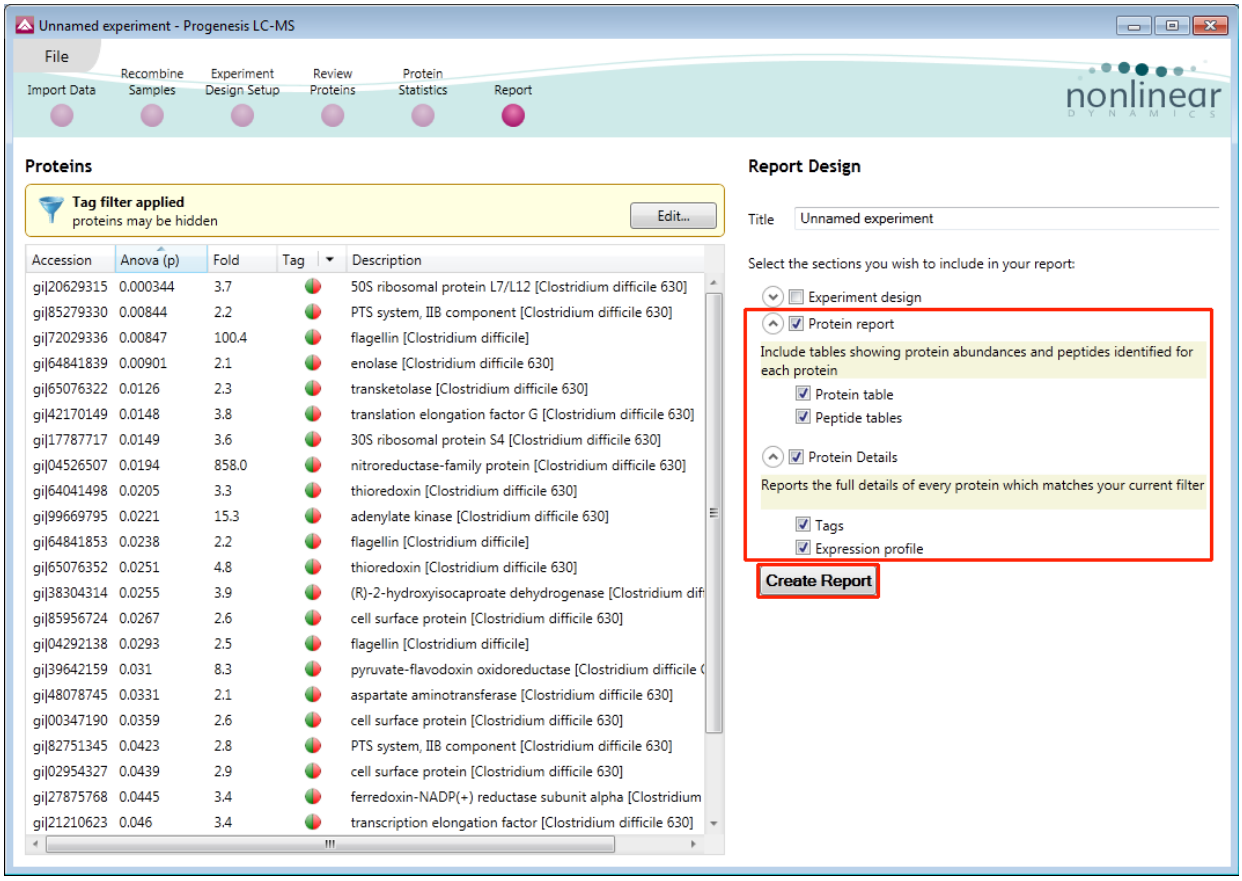
The **Report Design** stage allows you to select what views you want to include in a report based on the list of **currently selected proteins.**

As an example we will create a report for **only** the proteins with **Anova p-value≤0.05** and a **Max fold change≥2.**

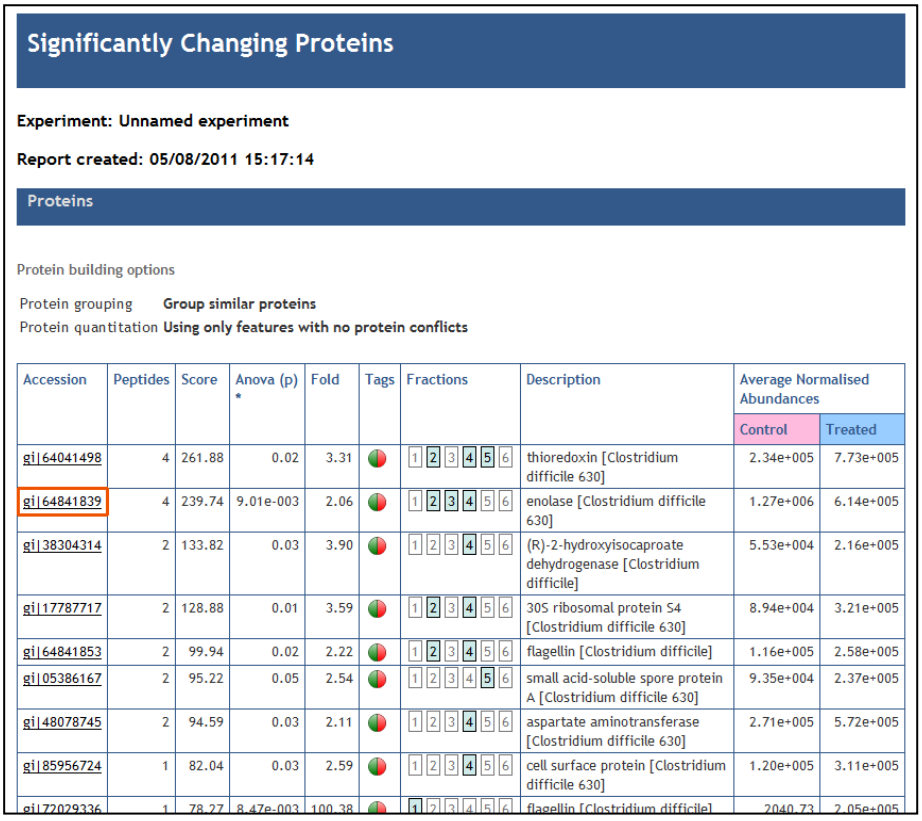


1. First reduce the proteins to report on by selecting the tags: **Anova p-value≤0.05** and a **Max fold change≥2.**
2. Expand the various Report Design options (by default they are all selected)
3. Un-tick as shown below

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.



Significantly Changing Proteins

Experiment: Unnamed experiment

Report created: 05/08/2011 15:17:14

Proteins

Protein building options

Protein grouping **Group similar proteins**
Protein quantitation **Using only features with no protein conflicts**

Accession	Peptides	Score	Anova (p) *	Fold	Tags	Fractions	Description	Average Normalised Abundances	
								Control	Treated
gi|64041498	4	261.88	0.02	3.31		1 2 3 4 5 6	thioredoxin [Clostridium difficile 630]	2.34e+005	7.73e+005
gi|64841839	4	239.74	9.01e-003	2.06		1 2 3 4 5 6	enolase [Clostridium difficile 630]	1.27e+006	6.14e+005
gi|38304314	2	133.82	0.03	3.90		1 2 3 4 5 6	(R)-2-hydroxyisocaproate dehydrogenase [Clostridium difficile]	5.53e+004	2.16e+005
gi|17787717	2	128.88	0.01	3.59		1 2 3 4 5 6	30S ribosomal protein S4 [Clostridium difficile 630]	8.94e+004	3.21e+005
gi|64841853	2	99.94	0.02	2.22		1 2 3 4 5 6	flagellin [Clostridium difficile]	1.16e+005	2.58e+005
gi|05386167	2	95.22	0.05	2.54		1 2 3 4 5 6	small acid-soluble spore protein A [Clostridium difficile 630]	9.35e+004	2.37e+005
gi|48078745	2	94.59	0.03	2.11		1 2 3 4 5 6	aspartate aminotransferase [Clostridium difficile 630]	2.71e+005	5.72e+005
gi|85956724	1	82.04	0.03	2.59		1 2 3 4 5 6	cell surface protein [Clostridium difficile 630]	1.20e+005	3.11e+005
gi|72029336	1	78.27	8.47e-003	100.38		1 2 3 4 5 6	flagellin [Clostridium difficile]	2040.73	2.05e+005

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

gi|64841839

enolase [Clostridium difficile 630]
4 peptides

Sequence	Feature	Score	Hits	Mass	Charge	Fraction	Modifications	In quantitation	Average Normalised Abundances	
									Control	Treated
DWGEECQAQYK	1388	67.65	2	1231.6086	2	1 2 **3** 4 5 6		yes	7.98e+004	7.18e+004
FVNNYYESEMK	431	52.04	1	1203.6443	2	1 **2** 3 4 5 6		yes	7.16e+005	1.78e+005
GEMVHVNDR	666	55.17	5	1014.5470	2	1 **2** 3 4 5 6		yes	2.37e+005	1.70e+005
GEMVHVNDR	1135	61.69	2	1014.5473	2	1 2 **3** 4 5 6		yes	7.38e+004	6.17e+004
VFDFLDYGIR	772	58.36	1	1107.5570	2	1 2 3 **4** 5 6		yes	1.61e+005	1.33e+005

gi|38304314

(R)-2-hydroxyisocaproate dehydrogenase [Clostridium difficile]
2 peptides

Sequence	Feature	Score	Hits	Mass	Charge	Fraction	Modifications	In quantitation	Average Normalised Abundances	
									Control	Treated
AFSCMSEGGLSCK	1747	78.62	7	1279.6782	2	1 2 3 **4** 5 6		yes	4.74e+004	1.65e+005
FCPWWCHYTNHER	4756	55.20	1	1425.6537	2	1 2 3 **4** 5 6		yes	7844.75	5.06e+004

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.

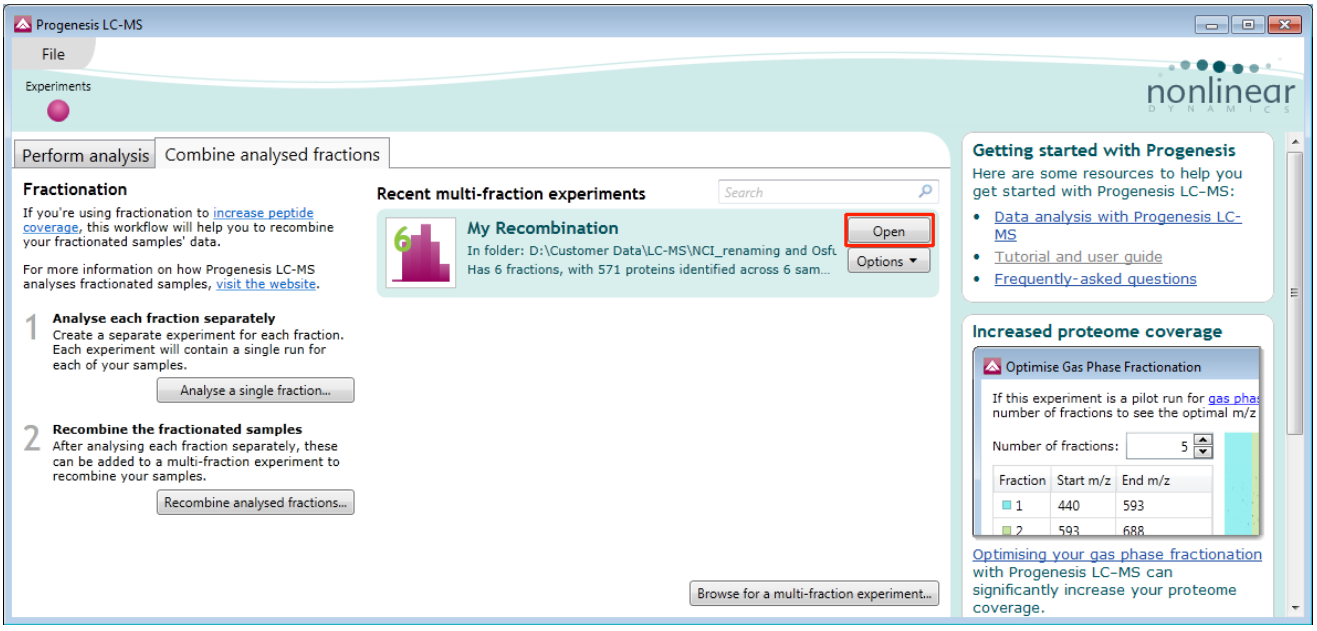
Note: there are separate panels for reporting on Proteins and Peptides

Finally your recombined data can be saved as a **Multi-fraction experiment**.

Stage 7: Saving a Multi-Fraction experiment

When you opened the Fractionation workflow and started working with the recombination of your 'Single Fraction Experiments' the workflow recognises the current experiment as '**Unnamed**' this status will change as you close and/or save the experiment.

On saving the new multi-fraction experiment appears on the **Combine analysed fractions** page



Details of the new experiments location (folder), number of fractions, samples and total identified proteins.

Note: you can reopen the experiment by either double clicking on it or using **open.**

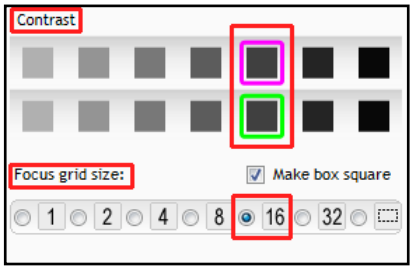
This completes a guided tour through using Progenesis LC-MS to analyse fractionated data.

Appendix 1: Manual assistance of Alignment

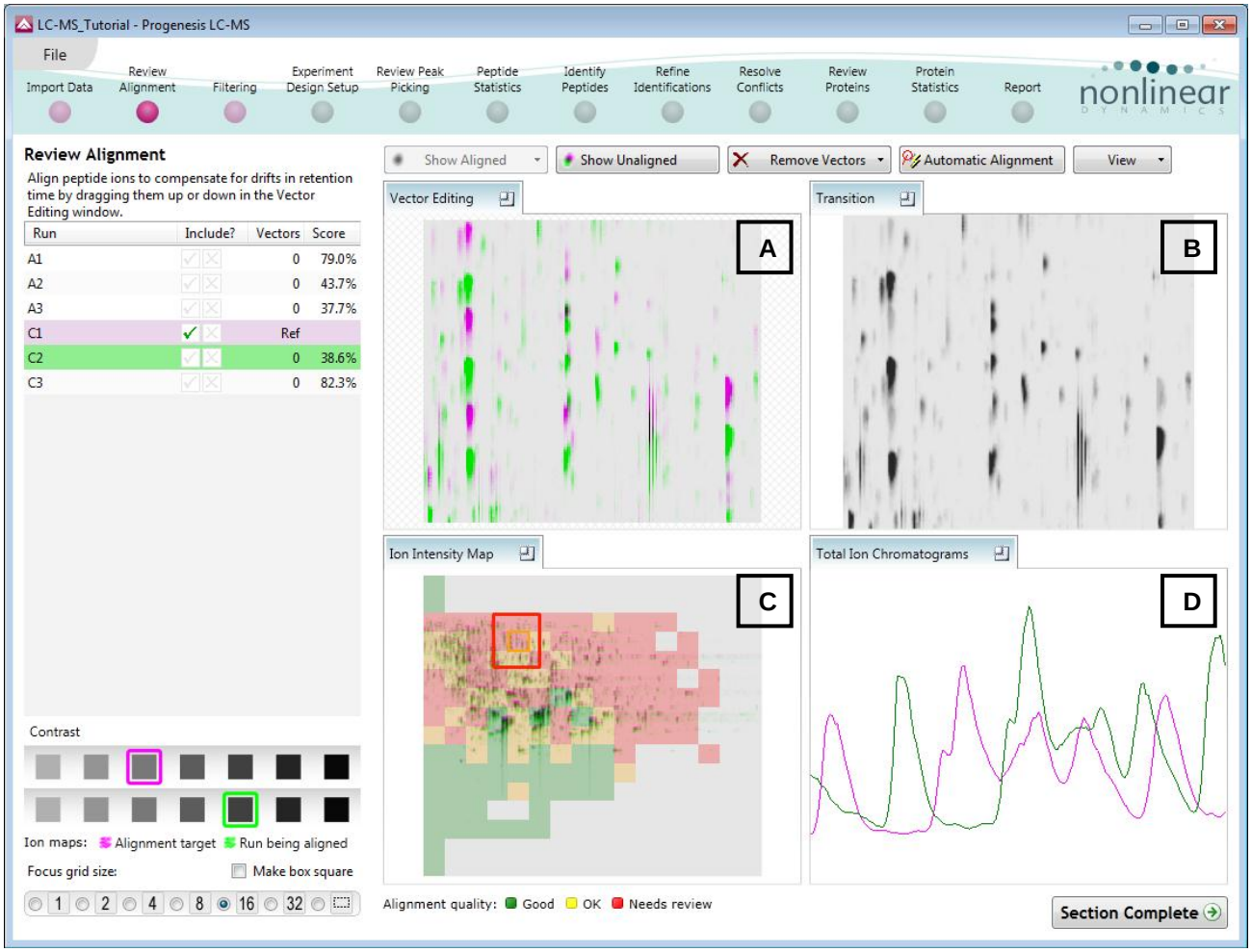
Approach to alignment

To place manual alignment vectors on a run (A2 in this example):

1. Click on Run A2 in the **Runs** panel, this will be highlighted in green and the reference run (A1) will be highlighted in magenta.
2. You will need approximately 5 - 10 **alignment vectors** evenly distributed from top to bottom of the whole run.
3. First ensure that the size of the focus area is set to **8 or 16** in the Focus grid size on the bottom left of the screen.



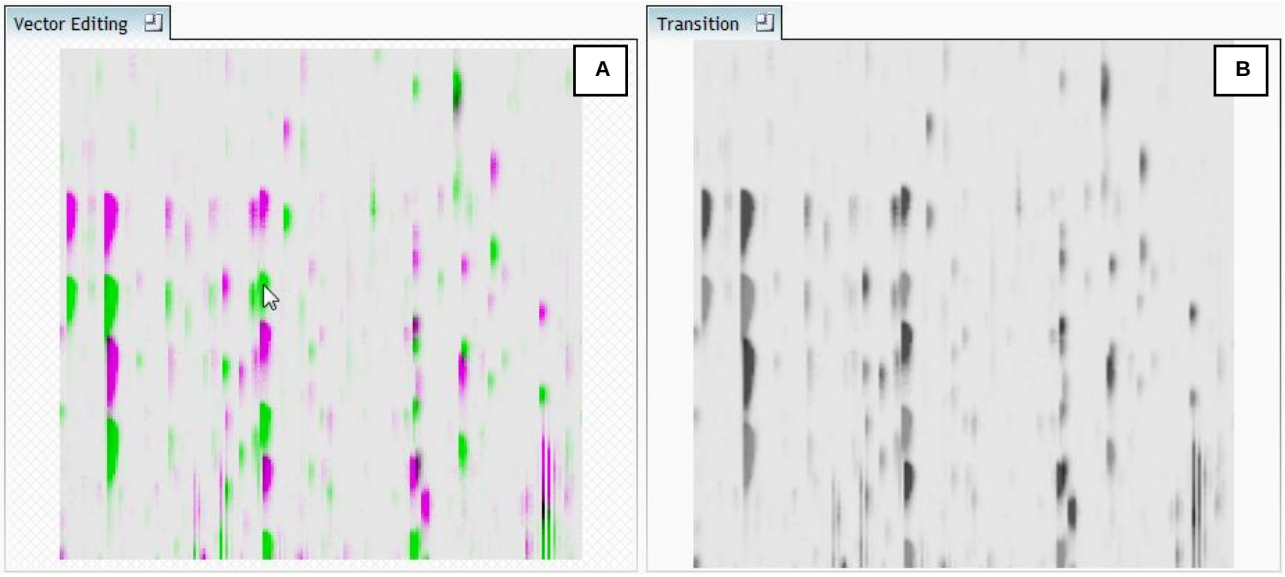
Click on an area (see below) in the **Whole Run** view (C) to refocus all the windows. Adjust Contrast as required.



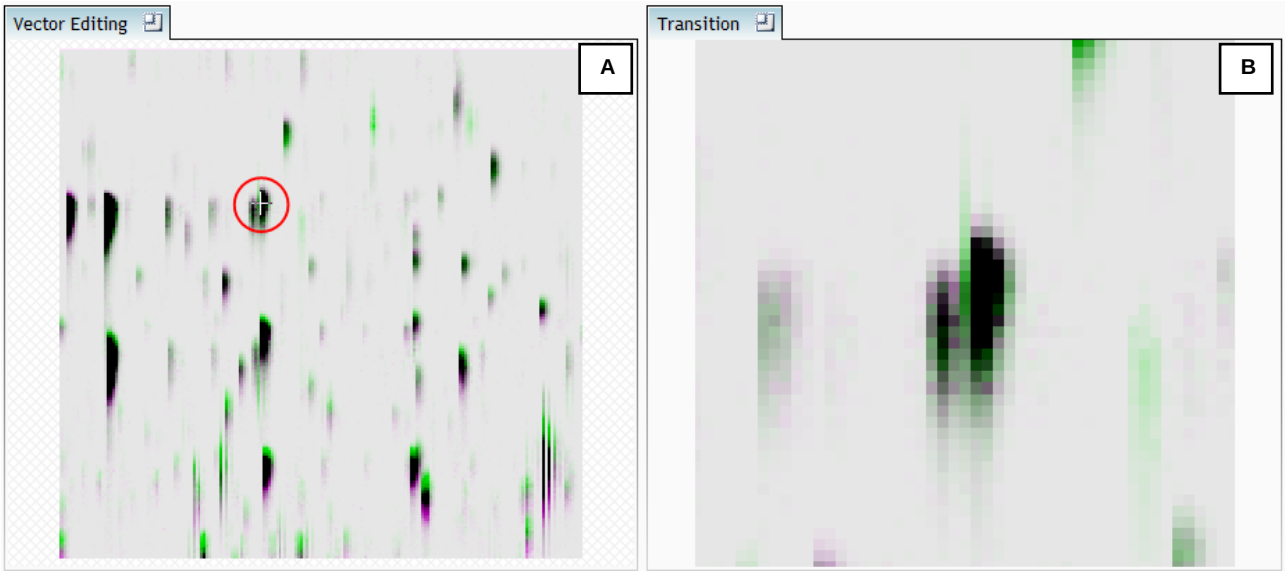
Note: the features moving back and forwards between the 2 runs in the **Transition** view indicating the misalignment of the two LC-MS runs

Note: The **Total Ion Chromatogram** view also reflects the misalignment of the 2 runs for the current Retention Time range (vertical dimension of the current Focus grid in the **Whole Run** view.

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

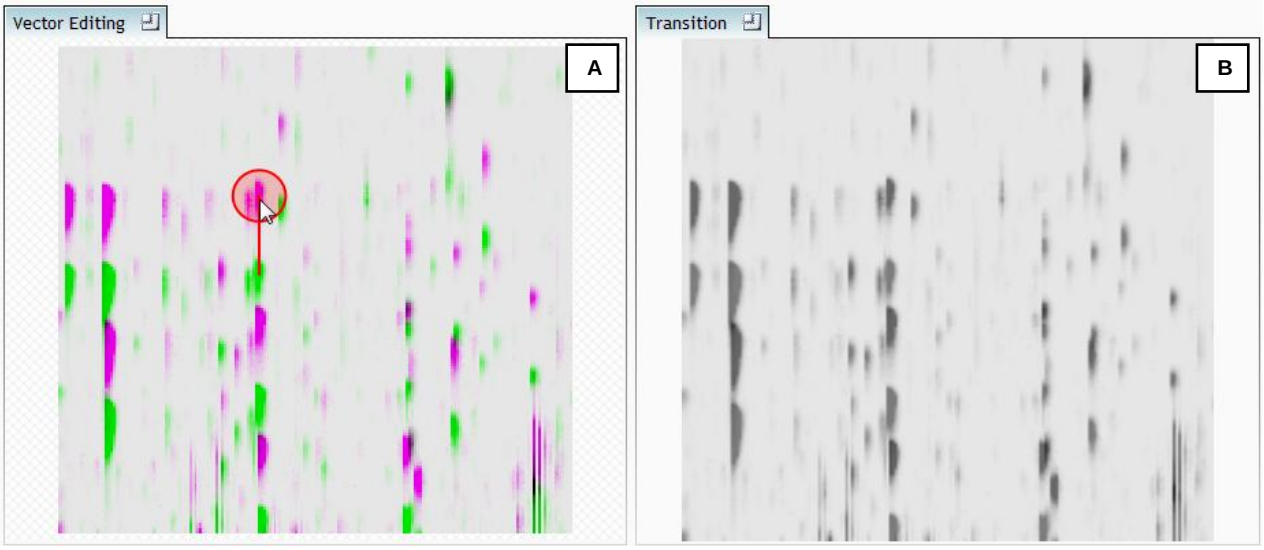


5. As you are holding down the left mouse button drag the green feature over the corresponding magenta feature of the reference run. The red circle will appear as shown below indicating that a positional lock has been found for the overlapping features.



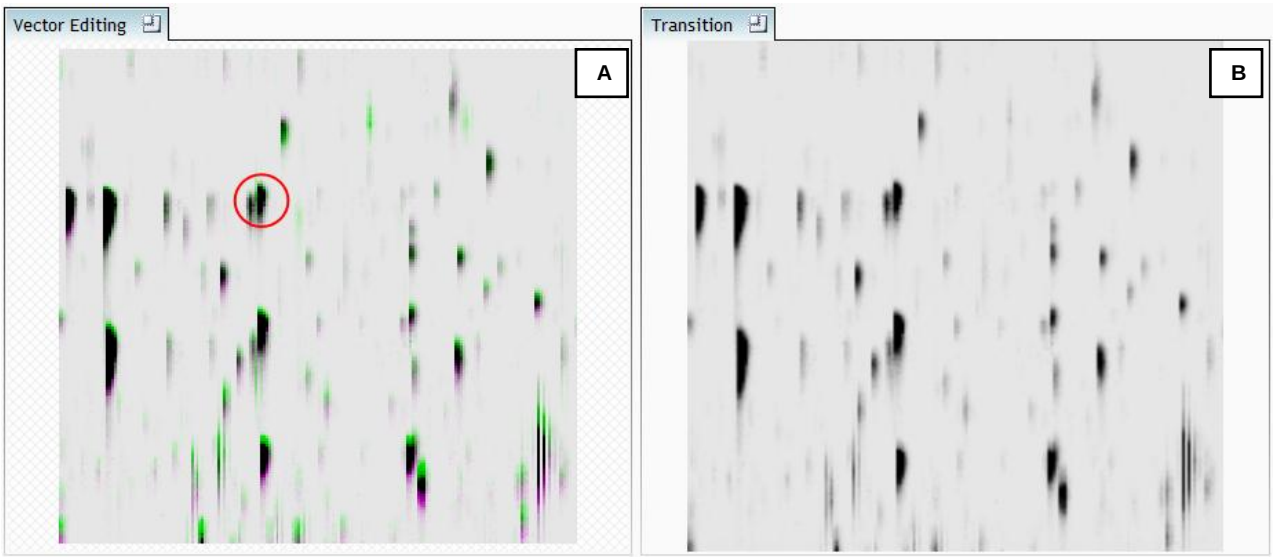
Note: as you hold down the mouse button, window B zooms in to help with the alignment.

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

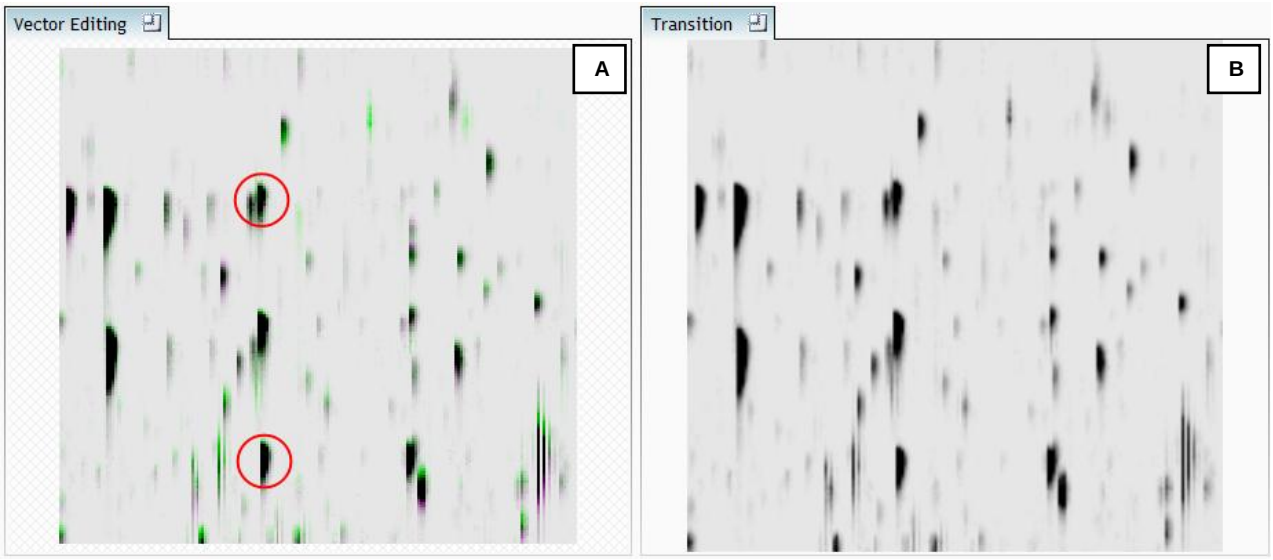


Note: an incorrectly placed vector is removed by right clicking on it in the **Vector Editing** window

7. Now click **Show Aligned** on the top tool bar to see the effect of adding a single vector.



8. Additing an additional vector will improve the alignment further. **Note** this time as you click to add the vector it 'jumps' automatically to the correct position using the information from the existing alignment vector.



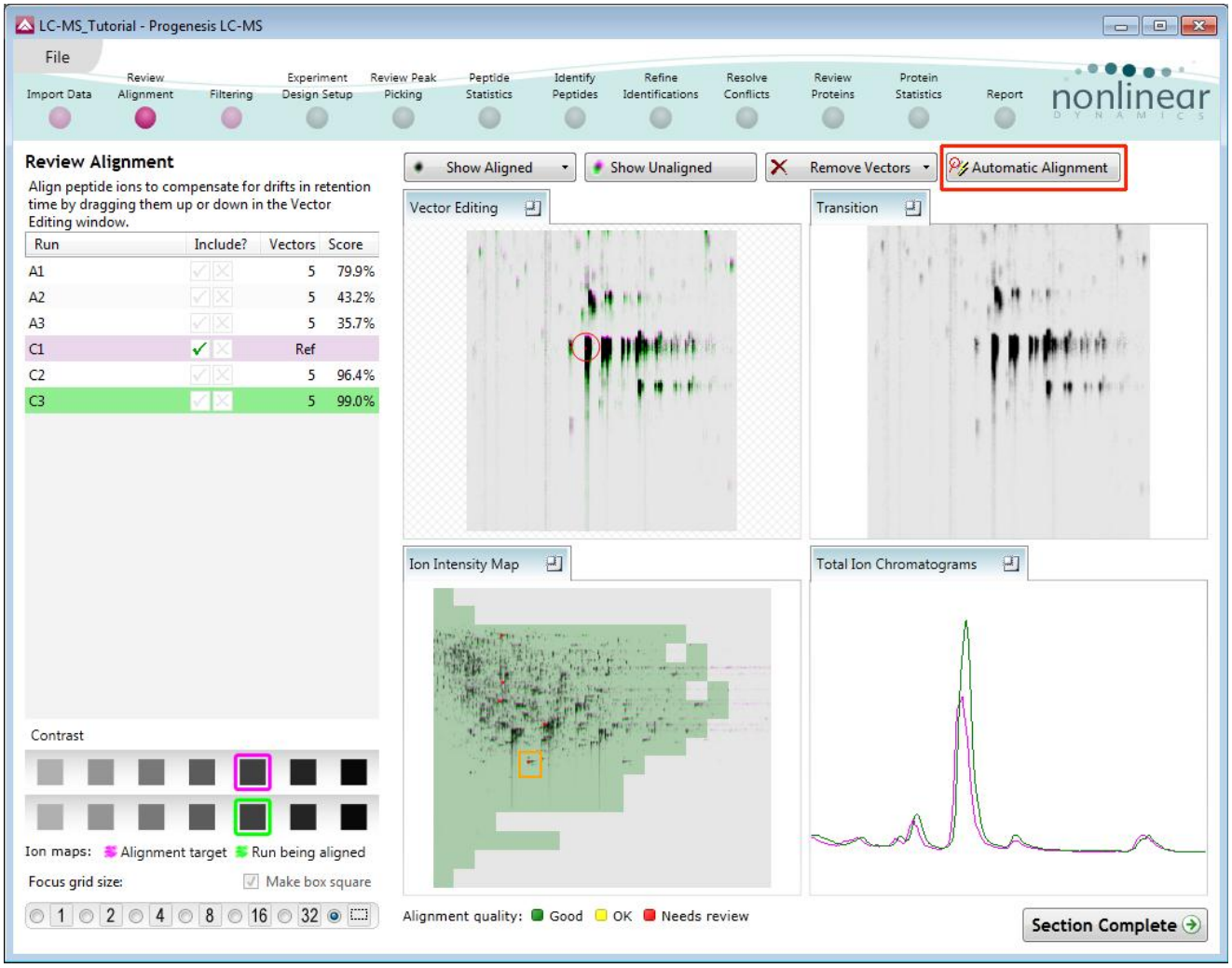
9. Repeat this process moving the focus from top to bottom on the **Whole Run** view

Note: the number of vectors you add is recorded in the **Runs** table also with each vector addition the Score and alignment quality updates. This can help guide the number of manual vectors you need to add before applying the automatic alignment.

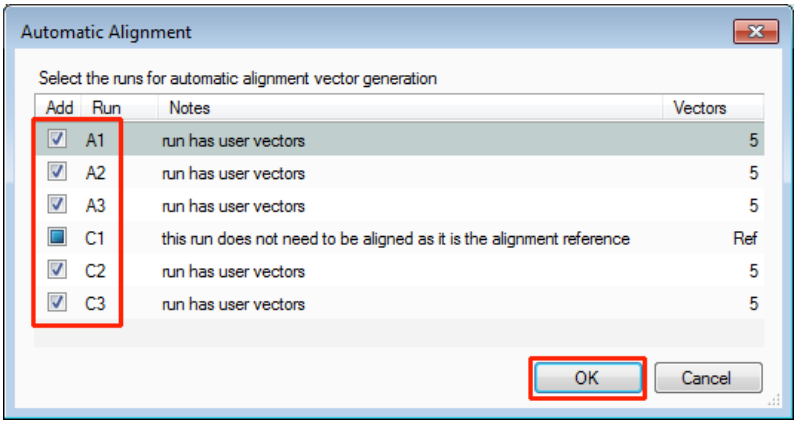
10. Repeat this process for all the runs to be aligned.

The number of manual vectors that you add at this stage is dependant on the misalignment between the current run and the Reference run. In many cases only using the Automatic vector wizard will achieve the alignment.

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



11. Then select Automatic Alignment to bring up the Automatic Alignment dialog and click **OK**. The automatic alignment process will begin, using the manual vectors you have added to aid in automatic vector placement.



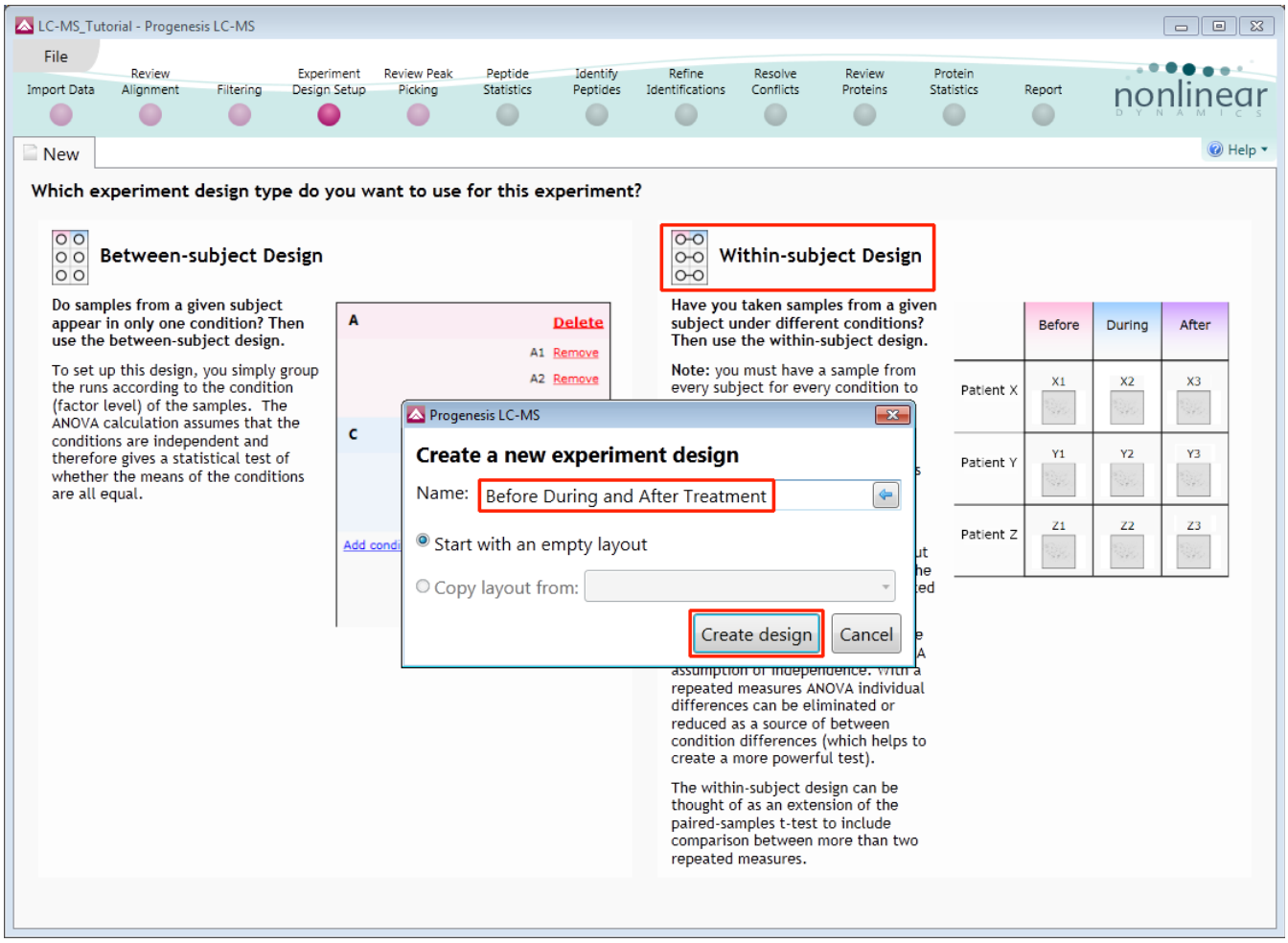
Note: the tick boxes next to the 'Run name control' which control whether vectors will be generated for each run.

To review the vectors, automatic and manual return to page 8

Appendix 2: Within-subject Design

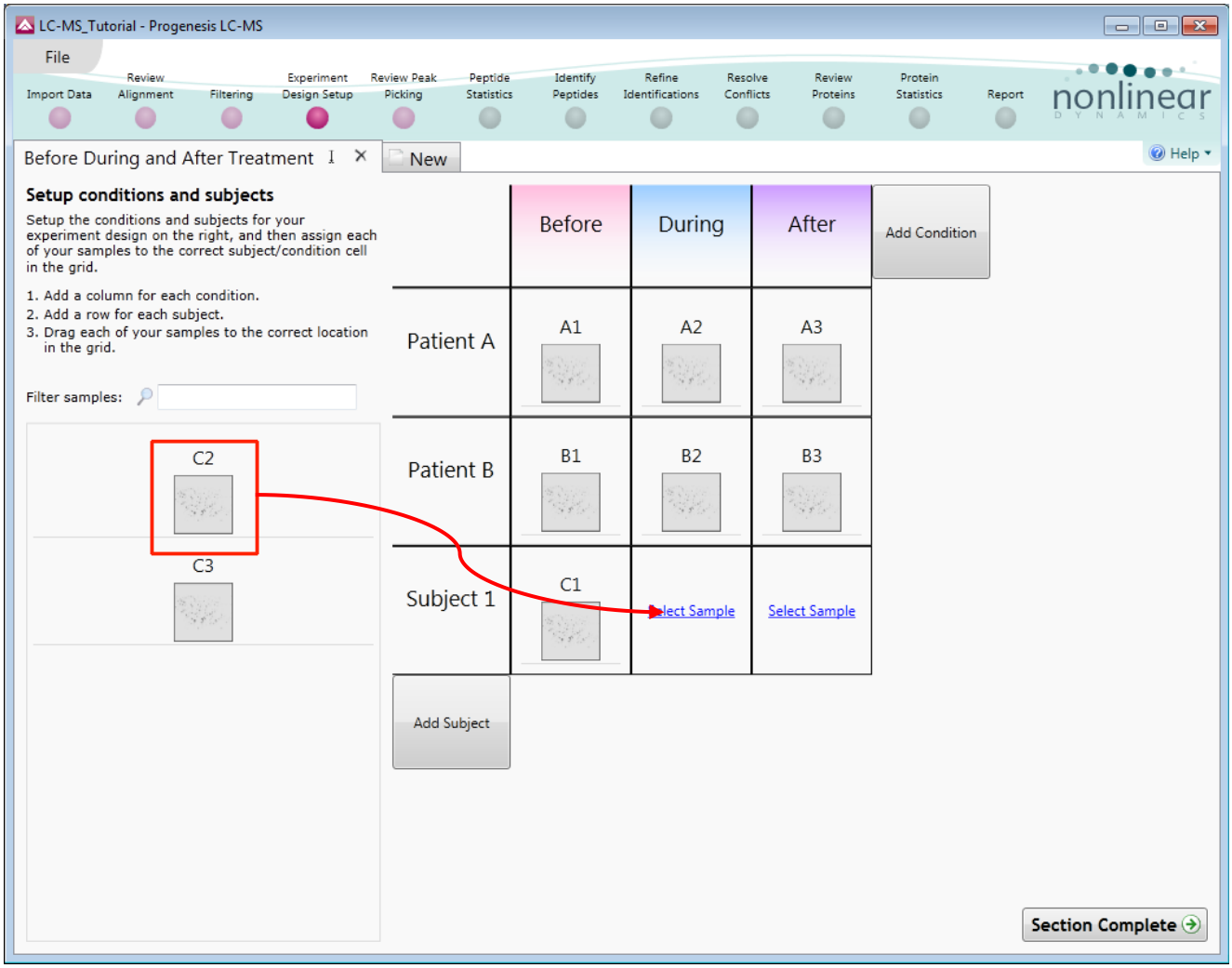
To create a **Within-subject Design** for your data set select this option on the **Experiment Design Setup** page and enter the name of the design.

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 all the following stages in the LC-MS workflow.