

Progenesis LC-MS Fractionation User Guide

Analysis workflow guidelines

for version 4.1



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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 <u>support@nonlinear.com</u> 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

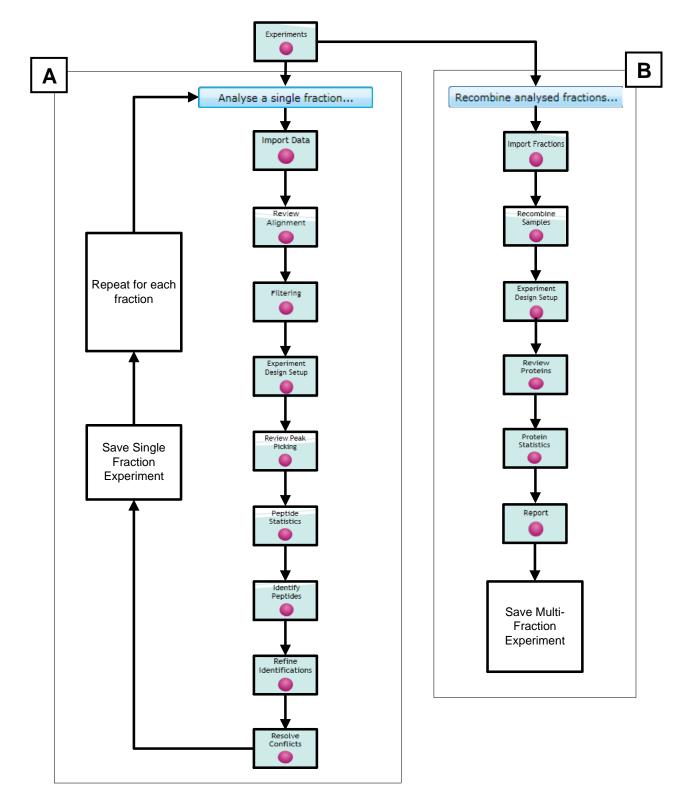
Progenesis LC-MS adopts an intuitive **Workflow** approach to performing comparative LC-MS data analysis. The following user guide describes the various stages of this workflow (see below) focusing mainly on the analysis of fractionated samples.

Import Data	Review Alignment	Filtering	Experiment Design Setup	Review Peak Picking	Peptide Statistics	Identify Peptides	Refine Identifications	Resolve Conflicts	Review Proteins	Protein Statistics	Report
				•							

Stage	Description	Page
Import Data	LC-MS Import Data: Selection and review of data files for single fraction analysis.	7
Review Alignment	Review Alignment: automatic and manual run alignment	8
Filtering	Filtering : defining filters for features based on Retention Time, m/z , Charge and Number of Isotopes.	11
Experiment Design Setup	Experiment Design Setup: defining one or more group set ups for analysed aligned runs	12
Review Peak Picking	Review Peak Picking : review and validate results, edit peak detection, tag groups of features and select features for further analysis	13
Identify Peptides	Identify Peptides : managing export of MS/MS spectra to, and import of peptide ids from Peptide Search engines	14
Refine Identifications	Refine Identifications: manage peptide ids and filters	16
Resolve Conflicts	Resolve Conflicts : validation and resolution of peptide id conflicts for data entered from Database Search engines	17
Import Fractions	Import fractions: import multiple analysed fractionated experiments	21
Recombine Samples	Recombine samples: regenerate samples from fractions	23
Experiment Design Setup	Experiment design Setup: define original experimental design	25
Review Proteins	Review Proteins: review protein and peptide expression and identity	26
Protein Statistics	Protein Statistics: perform multivariate statistics on Proteins	28
Report	Report: generate reports on proteins of interest	29

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...**

Progenesis LC-MS		
File Experiments		nonlinear
Perform analysis Combine analysed fraction Fractionation If you're using fractionation to increase peptide coverage, this workflow will help you to recombine your fractionated samples data. For more information on how Progenesis LC-MS analyses fractionated samples, visit the website.	8 Recent multi-fraction experiments Search	Getting started with Progenesis Here are some resources to help you get started with Progenesis LC-MS: • Data analysis with Progenesis LC-MS: • MS • Tutorial and user guide • Frequently-asked questions
 Analyse each fraction separately Create a separate experiment for each fraction. Each experiment will contain a single run for each of your samples. Analyse a single fraction Recombine the fractionated samples After analysing each fraction separately, these can be added to a multi-fraction experiment to recombine your samples. Recombine analysed fractions 	There are no recent multi-fracti experiments To create a new one, follow the instructions at the left	Increased proteome coverage Optimise Gas Phase Fractionation If this experiment is a pilot run for gas phase Increased proteome coverage. Mumber of fractions to see the optimal m/z Number of fractions: <u>5 Fraction Start m/z End m/z <u>1 440 593 <u>0 mining your gas phase fractionation with Progenesis LC-MS can significantly increase your proteome coverage. </u></u></u>
	Browse for a multi-fraction exper	Latest blog posts The one reason you should join the Progenesis Improvement Program Number of publications citing Progenesis for quantitative proteo At last! Pain-free reviewing in the Progenesis workflow Progenesis CoMet v2.0 released for LC-MS-based metabolomics

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 .

Create New LC-MS Experiment	×
Create a new label-free experiment named:	
Fract_1	
Data type Profile data Centroided data	
Resolution (full width at half maximum) 1000	
Machine type	5
High resolution mass spectrometer	•
Experiment folder	
Save experiment in the same folder as the run data	
Choose an experiment folder	_
Browse	

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.

File	Review Alignment	Filtering	Experiment Design Setup	Review Peak Picking	Peptide Statistics	Identify Peptides	Refine Identifications	Resolve Conflicts	Review Proteins	Protein Statistics	Report	non	linea
port Dat	a			Con_1_F	1			<< Ac	ctions 🔻 📄 >>				🕜 Helj
Select o click the	your run data ne of the availat Import button: mzXML files	ole data fo •	Import		00		1000		1500 m/z •	Peal	MS count: 4 c count: 56	5,581	008
@ Abo	out this data form	nat 🛛 🖊 🖸	ownload others	2 -			lignment Proces	-					
	e alignment p our runs are imp		ck the button	10		To comp	in alignment r ensate for drifts le reference rur	in retention	time, all runs ir	the experim	ent must b	e aligned	
Det Alig Review Look at for any affect ar that hav	ect alignment re ermine the best n all runs to tha the chromatog all of the runs in sample-running nalysis. Right-cli e significant pro	of the car t reference Start alig graphy the list b problems ck to remo	ndidates er run elow, checking that might ove any runs	20	ч йн хотор (тр.)	O Use	ess all runs in th	e experiment	Inment reference for suitability andidates that I s				
ported run	s: Search		Q	8		11							
	957	starys. 1769											
Con_1_F1	Con_2_F	1 Co	on_3_F1	40					nment reference ee the <u>online gu</u>		u might wa	int to	
17 942	-		ŧć.	(min)					< Bac	k Nex	t >	Cancel	
	1	1 Tre	eat 3 F1	50 Time (n									

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.

Start Alignment Processing

You will now be asked if you want to Align your runs	After selecting the experiment's alignment reference, the software can also automatically align all runs.
automatically or manually.	After the alignment reference is chosen, do you want to start automatic alignment?
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.	< Back Finish Cancel
Alignment Processing (14%)	🔼 Alignment Complete
Alignment processing Current step: Choosing an alignment reference	Alignment processing complete. Time taken: 1m 42s
Imported runs: 6/6 Reference run: pending Aligned runs: pending Cancel	 ✓ Imported runs: 6/6 ✓ Reference run: Con_1_F1 ✓ Aligned runs: 5/5 Review chromatography Review alignment →
Cancel	Keview chromatography

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.

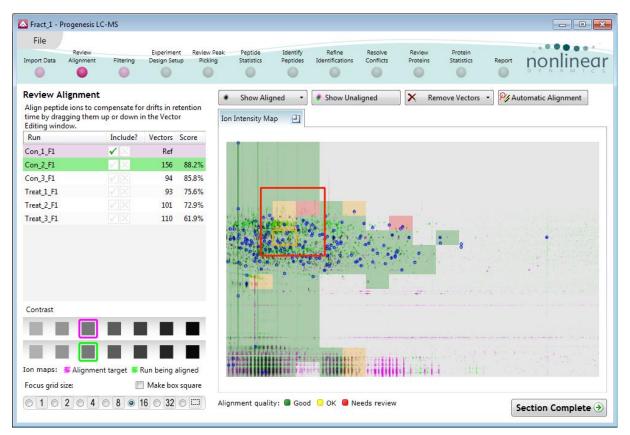
Transition (Window B): uses an **alpha blend** to animate between the current and reference runs. Before the runs are aligned, the features appear to move up and down. Once correctly aligned, they will appear to pulse. During the process of adding vectors, this view will change to show a zoomed view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

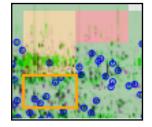


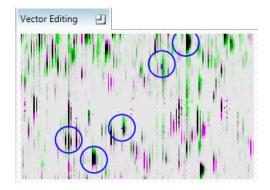
Note: you can use the icon to the right of the panel name to expand or contract each view

Reviewing Quality of Alignment

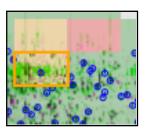
At this point the quality metric, overlaid on the Ion Intensity Map as coloured squares, acts as a guide drawing your attention to areas of the alignment. These range from Good (Green) through OK (Yellow) to Needs review (Red). When reviewing individual squares set the grid size to 16, (and untick the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

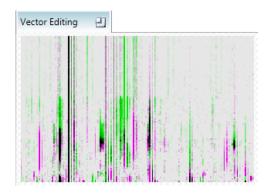
For a 'green' square the majority of the data appears overlapped (black) indicating good alignment. When viewed in the Transition view the data appears to pulse.



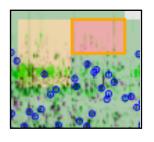


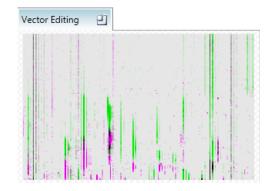
For a 'yellow' square some of the data appears overlapped (black) indicating OK alignment. When viewed in the Transition view some of the data appears to pulse.





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.

🔼 Peak Picking Parameters	(×	🔼 Peak Picking Parameters	—
Runs for peak picking Peak picking lim	its Maximum charge Retention time limits		Runs for peak picking Peak picking li	mits Maximum charge Retention time limits
Choose runs for peak picking You can tick or un-tick each run to control which will be used by the peak picking algorithm. Although any	✓ Run ✓ Con 1 F1		Sensitivity You can adjust the sensitivity of the peak picking algorithm using these different methods. Each	Automatic Absolute ion intensity
run which is left un-ticked will not affect the feature outlines, it will still have outlines added to it and will be	 Con_2_F1 ✓ Con_2_F1 ✓ Con_3_F1 		sensitivity method examines the intensities of groups of MS peaks to judge whether they are likely to	© % Base Peak
available in the experiment design setup. Learn more about why you might not want to select all runs.	✓ Treat_1_F1 ✓ Treat_2_F1 ✓ Treat_2_F1 ✓ Treat 3 F1		form part of an ion or whether they represent noise and so should be ignored. Peaks that are rejected as noise will not be used	The automatic sensitivity method uses a noise estimation algorithm to determine the noise levels in the data. The higher the sensitivity value, the more features
want to select all runs.			to build ion outlines.	fewer default more 3
			Chromatographic peak width	
			The chromatographic peak width gives the length of time over which an ion has eluted. If you set	Apply a minimum peak width
			a minimum peak width, any ion that has eluted over a shorter period will be rejected.	Minimum width: 0 minutes
	Start peak picking Cancel			Start peak picking Cancel

Tip: It may be appropriate only to pick peaks that are present in a limited number of your runs. In which case un-tick the runs that you do NOT want to contribute to the 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.

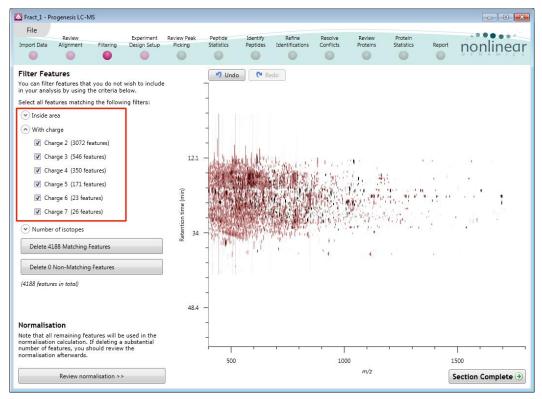
The sensitivity of the detection is controlled by adjusting settings under the **Peak picking limits** tab.

For this example the default settings for the Automatic method were used.	Peak Picking Parameters Runs for peak picking Peak picking limits Maximum charge Retention time lim							
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.	You o maxii pickir	ention time li can set the minin imum retention t ing. Ions that elu r these values wi	mum and time for peak ite before or	✓ Ignore ions before ✓ Ignore ions after	_	minutes minutes		
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.								
Analysing				Start pe	ak picking	Cancel		

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.

Kract_1 - Progenesis LC-MS												
File Import Data Alignment Filtering	Experime Design Se		Peptide Statistics	Identify Peptides	Refine Identifications	Resolve Conflicts	Review Proteins	Protein Statistics	Rep	m nç	onlin	ear
New											(🕖 Help 🔻
Which experiment design typ	e do y	ou want to u	ise for thi	s experin	nent?							
Between-subject De	esign				0-0 0-0 Wit	thin-subj	ect Des	ign				*
Do samples from a given	\square	🔼 Progenesis LC	-MS				•					
subject appear in only one condition? Then use the	A	Create a n	ew expe	eriment	design				Before	During	After	
between-subject design. To set up this design, you simply group the runs according		Name: Cor					-	Patient X	X1	X2	X3	
to the condition (factor level) of the samples. The ANOVA calculation assumes that the	c	Start with Copy layo		layout			Ŧ	Patient Y	Υ1	Y2	Y3	
conditions are independent and therefore gives a statistical test of whether the means of the conditions are all equal.		Import de							Z1	Z2	23	
the conditions are all equal.	Add o		Grou	ıp by:	t file formats ca (no valid g		¥	Patient Z		98) 		
					Create d	esign C	ancel					
	l					ill then perf neasures AN						
					A standard	ANOVA is no	ot					*

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

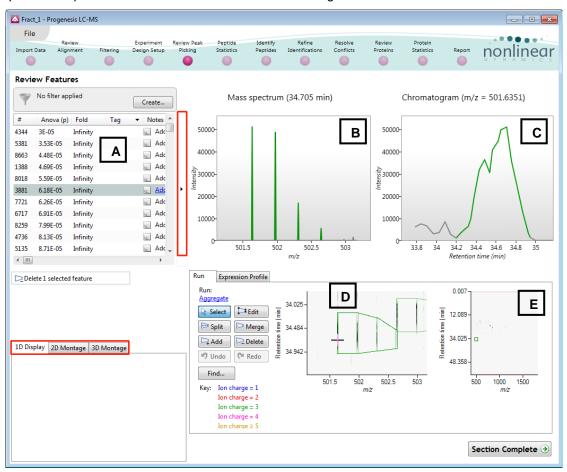
SFract_1 - Prog	genesis LC-N	IS											
File Import Data	Review Alignment	Filtering	Experiment Design Setup	Review Peak Picking	Peptide Statistics	Identify Peptides	Refine Identifications	Resolve Conflicts	Review Proteins	Protein Statistics	Report	nonli	near
Control vs T	Freated I	×	New										🕜 Help 🔻
Setup cond	itions			Runs	Add Select	ted Runs to (Condition 🔻	Search		Q			
Setup the cond (e.g., control, your samples t	drug A, etc)	, and then	assign each o		Add to	new conditi	on	Trea	at_2_F1		T	reat_3_F1	
Control			Dele	te	Contro	bl		15	2000			and the second	
		(Ion_1_F1 <u>Remo</u>	<u>/e</u>	1	1.11							
		c	Ion_2_F1 Remo	<u>/e</u>									
		c	Ion_3_F1 <u>Remo</u>	<u>/e</u>									
Add condition	<u>1</u>												
												Section Com	olete 🏵

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.

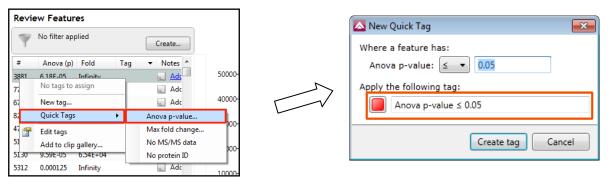
The review stage has 4 display modes: 1D, 2D, 3D and Feature Details controlled by the tabs on the bottom left of the display and the expander bar to the right of the table. Each display has multiple views to allow comparative exploration of the detected features on the aligned LC-MS runs.



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 ($p \le 0.05$) 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.

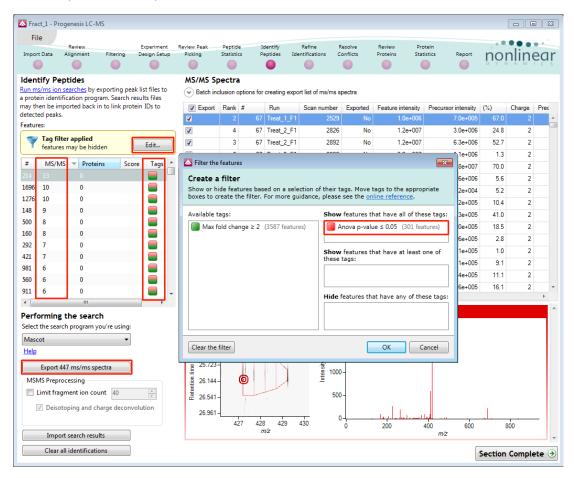


Now move to the Identify Peptides stage in the workflow using the icon on the workflow.

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 dependent 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 dependent MS/MS detection mode due to under sampling. Under these conditions MS/MS data acquisition is dependent 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 dependent 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

 $p \le 0.05$) 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.

Performing the search Select the search program you're using:
Mascot 👻
Help
Export 0 ms/ms spectra
MSMS Preprocessing
Limit fragment ion count 40
$\ensuremath{\overline{\mathbb{J}}}$ Deisotoping and charge deconvolution
Import search results
Clear all identifications

🔼 Fra	ct_3 - Progen	esis LC-MS												
Fi							E E							
Impo		eview onment Filt	tering	Experiment Design Setup	Review Peak Picking	C Peptide Statistics	Identify Peptides	Refine Identifications	Resolve Conflicts	Review Proteins	Protein Statistics	Report	-	onlinger
		•		0		•					•		D	nonlinear
Iden	tify Peptic	les			MS/	MS Spectra								
		<u>ches</u> by exportir n program. Sear			. E	Batch inclusion optic	ons for creating (export list of ms	/ms spectra					
be imp	orted back in	to link protein				Rank	less than	•			Feature intensit	less than	•	
Featur						Feature ID	(I				Precursor intensit	N (11		
7	Tag filter a	pplied y be hidden		Edit.		reature ID	less than	•			Frecursor intensi	V less than	•	
<u> </u>	reatures ma	y be hidden	_			Charge	less than	•		P	recursor intensity (%) less than	•	
#	MS/MS	Proteins	Score	Tags 🔹	· •		(
3652	15	1 gi 61557119	, 9.46			Scan number	less than	•			Run nam	e contains	•	
4119	14	0			8						5 1			
2390	12	0			8	Exported	equal to	•	•		Peptide sequenc	contains	•	
2544	11	4 gi 8275134	. 9.2		2	Isotope	less than	•			Protein accessio	n contains	•	
2122	11	0			2		1000 11011					Containe		
507	11	2 gi 2459123			8	ID score	less than	•			Protein descriptio	n contains	•	
445	10	3 gi 6484167			8									
276	10	2 gi 7052372			3						Includ	e in export	Exclude from exp	Clear all filters
3325	10	1 gi 46132035	, 0.26		8		-							
2500	9	0				Export Rank #	Run 39 Con_1_1	Scan nur			-		Charge 10.5 2	Precursor m/z Isoto 635,7800
2439	9	0		_		5	39 Treat_1					1.6e+006	9.2 2	635,7900
2205	9	0	05.0	-		6	39 Treat_3					6.6e+006	8.4 2	635.7900
39 980	9	2 gi 6492873 1 gi 70523723		-		7	39 Con 3	-				5 20105	61 2	635.7800
4025	8	0	, 85.4		× *									•
	orming the			_		ature number	39, m/z 6	35. 78 49, re	etention tir	ne 20.269	min, charge +2	2		*
	-	e searcn rogram you're u	isina:		Ru	un:Con_1_F3 Sca	an number:19	87	1			1		
Maso		ogram joure a	-						8000 -					
Help			•		_	19.818-								
					jii j			>	6000 -					
	Export 0 m	s/ms spectra			time	20.149-		Intensity	4000-					
MS	VS Preproces	sing			lici	20.464 -		Ē	1000					
	-	nt ion count 40		A V	Retention time (min				2000 -					
l	Deisotopir	ng and charge d	leconvolu	ution		20.783-			0	العاد .	القيسا للوجيا والبايي	السليل الم	لىارا	<u> </u>
	Import se	arch results				635 63	6 637 638 <i>m/z</i>	639	0		500	m/z	1000	-
	Clear all id	entifications											Sec	tion Complete 🏵

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**.

	Fract_	3 - Proge	nesis LC-N	/IS															
	File																		
Im	port [lata A	Review lignment	Filter	rina		operime sign Se		Review Pea Picking		istics	Identify Peptide		Refine tifications	Resolve Conflict		Protein Statistics	Report	
		/010 /				00		lup		510			.5 10011						nonlinear
Fe	atur	res 🛛					Pep	tide	Search	Results									
	#	Total Hit	m/z	RT(min	s Cha	A	Ba	tch del	etion optio	ons									
1		32	554.82	27.12	2				Score	e less ti	an	▼ 30	1			Sequence	Length less tha	n T	
3		19	622.85	24.03	2												5 (iess and		
4		1	596.32	27.89	2				Hit	less th	an	•					Charge less tha	n 🔻	
5		14	702.38	28.85	2														
7		8	412.22	18.81	2				Mas	less ti	ian	•				Se	quence contain	s •	
8		26	694.91	33.98	2														
9		1	805.93	26.34	2			Mass e	error (ppm	less ti	an	•				Ac	cession contain	s •	
10)	3	610.33	28.15	2				M/2			•				Dec	ription contain	•	
11		16	495.72	15.16	2				191/2	less ti	ian	•				Desi	contain	5 •	
14		87	731.85	19.95	2			Rete	ntion Time	less ti	an	•				Modifi	cations contain	· ·	
17		11	663.86	29.96	2					1055 0							contain		
19		9	544.76	17.47	2														
20		13	751.33	20.68	2										Dele	te matching search	Delet	e non-matching sear	ch results Clear all filters
22		2	588.32	29.60	2												1		
23		8	647.82	17.82	2			#	Score		m/z		ns) Charg		Mass err	Sequence	Accession	Modifications	A ===
24		48	679.35	27.78	2			1	56.89	16	554.82			1107.63		GYGEGGTAVI	gi 98758013		hypothetical protein CLOST
25		7	516.30	26.87	2			1	56.89	16	554.82	27.12	2	1107.63	-0.22	GYGEGGTAV	gi 51703855		hypothetical protein CD366
26		10	624.29	20.15	2			3	15.43	2	🔼 De	elete 1402	2 search r	esults?				83	electron transfer flavoprote
27		21	487.72	18.33	2				15.43	2									GroEL [Ruminococcus albus
28		110 12	744.35 548.23	26.44 19.22	2		V	3	43.05	15	?	Are y	ou sure y	ou want to	o permane	ntly delete 1402 pe	eptide search resu	lts?	putative tRNA binding prot
31		12	548.23 607.75	23.42	2				6.16	1 14									proline reductase [Clostridit
34		4	823.43	18.81	1		_	7	100.27	8									putative tRNA binding prote
35		2	495.72	17.63	2			8		13									DNA-binding protein HU [C
36		2 72	723.85	23.38	2		V	8 8	95.73 95.73	13						Yes	No		hypothetical protein CLOST hypothetical protein CD366
38		3	755.36	25.78	2			9	5.36	15	805.93	26.34	2	1609.84	-11.45	NPLPFKHWD	🔮 gi 51703780		50S ribosomal protein L15 [
39		18	635.78	20.27	2			10	11.64	1	610.33		-		-15.79	GALIAYRGER	 gij51703780 gij83428742 		30S ribosomal protein S16 [
42		18	617.31	24.06	2			10	11.64	1	610.33				-15.79	GALIAYRGER	gi 38885577		butyryl-CoA dehydrogenase
43		1	697.38	32.41	2			10	10.90	1	610.33			1218.65		RHLFAYMQT	gi[00005577]	[7] Oxidation (M)	ATP synthase epsilon chain
51		8	761.89	25.32	2			11	42.41	8	495.72			989.42	-0.33		gi[51703855		hypothetical protein CD366
52		12	555.81	26.96	2			11	42.41	8	495.72		-	989.42	-0.33		gij91765055 gij98758013	[3] Oxidation (M)	hypothetical protein CLOST
53		20	619.79	20.66	2			14	79.08	10	731.85			1461.68			gij90750015	[6] Oxidation (M)	glycine/sarcosine/betaine re
54		11	599.83	22.82	2			14	31.13	9	731.85				-24.32	TMWPVWCN	gij64041701		electron transfer flavoprote +
56		14	702.85	27.53	2		4			-								(()))
57		10	012 40	22.02	2	*	3290	earch	h results. 1	402 mate	hing batch	h delete	ontions						Section Complete 🌖
4					•		52.95	, scarci				. acrese	options						Section complete

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.

Proteins	Vo No 1	filter applied	Create	Pe	eptide	es of gi	56689	37					No filter ap	plied	Create
Accession Pe	ptides Confli	cts Score	Anova (p)*		#	Score	Hit	s Mass	Mass error (p	. RT (mins)	Charge	e Tags	 Abundance 	Conf	licts Peptide
🌛 gi 5668937 🛛 🧿	12 14	1.41E+03	2.03E-05 3		446	6 93.	3 10	1676.838	1.2	34.5	2	√ ×	7.47E+05	1	🌖 IRD
🌛 gi 126697810	9 14	1.13E+03	4.55E-05 3		333	5 84.	7 4	1423.65	0.405	22.5	2	✓ ×	7.39E+04	1	S DTE
🌒 gi 209571234	24 13	2.4E+03	4.05E-05 2		147	7 101	1 10	1230.609	0.44	22.7	2	√ ×	3.07E+06	1	🔇 AAD
谢 gi 260682215	23 13	2.03E+03	6.28E-05		166	6 125	5 10	2317.115	0.168	38.7	1	B	5.55E+06	1	🔇 LES
谢 gi 126698450	12 9	1.21E+03	4.05E-06		179	9 60.	99	2317.115	0.201	38.7	-	⊳ ∕ ×	3.09E+06	1	🔇 LES
谢 gi 126700407	9 9	1.04E+03	0.945 1		238	8 107	7 10	1716.857	0.429	30.4	2	 ✓ × 	1.73E+06	1	🔇 VNI
🔮 gi 255656776	9 9	925	0.00888 1		564	4 51.	2 4	1716.858	0.394	30.4	3	\checkmark ×	3.57E+05	1	🔇 VNI
a) gi 255654924 ∢ III	7 9	645	8.66E-06 1			3 49.	5 10	1676.838	1.32	34.5	3	V X	7.59E+05	1	C TRT
Protein: gi 12669 eptide Views Protein R Conflicting protein	esolution		Clostridium d			0] ofgi 12	266978	10						D	
	esolution		Clostridium d				266978	10						D	
eptide Views Protein R Conflicting protein Accession Pep	esolution ns for feat otides Co nflic	Ce 46	Peptid	Pept	tides #	of gi 12 Score	Hits	Mass 1		RT (mins)	-	-	✓ Abundance	D	Peptide Sec
eptide Views Protein R Conflicting protein Accession Pep gi 15668937 9 1	esolution ns for feat bides Confident 2 14	te 46 te Protein Score 1.41E+03	Peptid	Pept	tides # 147	of gi 12 Score 101	Hits 10	Mass /	0.44	22.7	2	< ✓ ×	3.07E+06	Conflict	S AADD
eptide Views Protein R Conflicting protein Accession Pep Sgi 15668937 9 1	esolution ns for feat otides Co nflic	Ce 46	Peptid	Pept	tides # 147 166	of gi 12 Score 101 125	Hits 10 10	Mass / 1230.609 2317.115	0.44	22.7 38.7	2 2	< ≺ ×	3.07E+06 5.55E+06	LConflict 1 1	 AADDI LEST(
eptide Views Protein R Conflicting protein Accession Pep gi 15668937 9 1	esolution ns for feat bides Coldie 2 14	46 ++++++++++++++++++++++++++++++++++++	Peptid	Pept	tides # 147 166 179	of gi 12 Score 101 125 60.9	Hits 10 10 9	Mass / 1230.609 2317.115 2317.115	0.44 0.168 0.201	22.7 38.7 38.7	2 2 3		3.07E+06 5.55E+06 3.09E+06	Conflict 1 1 1	 AADDI LEST(LEST(
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eptide Views Protein R Conflicting protein Accession Pep gi 15668937 9 1	esolution ns for feat bides Coldie 2 14	46 ++++++++++++++++++++++++++++++++++++	Peptid	Pept	tides # 147 166 179 238 564	of gi 12 Score 101 125 60.9 107 51.2	Hits 10 10 9 10 4	Mass // 1230.609 2317.115 2317.115 1716.857 1716.858	0.44 0.168 0.201 0.429 0.394	22.7 38.7 38.7 30.4 30.4	2 2 3 2 3		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05	1 1 1 1 1 1	 AADD; LEST(LEST(VNTN' VNTN'
eptide Views Protein R Conflicting protein Accession Pep gi 15668937 9 1	esolution ns for feat bides Coldie 2 14	46 ++++++++++++++++++++++++++++++++++++	Peptid	Pep1	tides # 147 166 179 238 564 283	of gi 1; <u>score</u> 101 125 60.9 107 51.2 49.5	Hits 10 10 9 10 4 10	Mass J 1230.609 2317.115 2317.115 1716.857 1716.858 1676.838	0.44 0.168 0.201 0.429 0.394 1.32	22.7 38.7 38.7 30.4 30.4 30.4 34.5	2 2 3 2 3 3 3		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05	Conflict 1 1 1 1	 AADD; LEST; LEST; VNTN; VNTN; IRDT;
Protein R Conflicting protein Accession Pep gi 15668937 • 1	esolution ns for feat bides Confident 2 14	46 ++++++++++++++++++++++++++++++++++++	Peptid	Pep1	tides (# 147 166 179 238 564 283 446	of gi 12 Score 101 125 60.9 107 51.2 49.5 93.3	Hits 10 9 10 4 10 10	Mass Mass 1230.609 2317.115 2317.115 1716.857 1716.858 1676.838 1676.838 1676.838	0.44 0.168 0.201 0.429 0.394 1.32 1.2	22.7 38.7 30.4 30.4 34.5 34.5	2 2 3 2 3 3 3 2		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05	Conflict 1 1 1 1 1 1 1 1	 AADD; LEST; LEST; VNTN; VNTN; IRDT; IRDT;
eptide Views Protein R Conflicting protein Accession Pep gi 15668937 9 1	esolution ns for feat bides Confident 2 14	46 ++++++++++++++++++++++++++++++++++++	Peptid	Pep1	tides (# 147 166 179 238 564 283 446 431	of gi 12 Score 101 125 60.9 107 51.2 49.5 93.3 49.6	Hits 10 9 10 4 10 10 8	Mass // 1230.609 2317.115 2317.115 1716.857 1716.858 1676.838 1676.838 1692.835	0.44 0.168 0.201 0.429 0.394 1.32 1.2 0.206	22.7 38.7 30.4 30.4 34.5 34.5 20.6	2 2 3 2 3 3 3 2 3 3 2 3		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05 5.56E+05	Conflict 1 1 1 1 1 1 1 1 1	 AADD: LEST: LEST: VNTN' VNTN' IRDTI IRDTI IRDTI
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eptide Views Protein R Conflicting protein Accession Pep gi 15668937 9 1	esolution ns for feat bides Confident 2 14	46 ++++++++++++++++++++++++++++++++++++	Peptid	Pept	tides / # 147 166 179 238 564 283 446 431 789	of gi 12 Score 101 125 60.9 107 51.2 49.5 93.3 49.6 103	Hits 10 10 9 10 4 10 10 10 8 10	Mass / 1230.609 2317.115 2317.115 1716.857 1716.858 1676.838 1676.838 1692.835 1692.833	0.44 0.168 0.201 0.429 0.394 1.32 1.2 0.206 1.09	22.7 38.7 30.4 30.4 34.5 34.5 20.6 20.6	2 2 3 2 3 3 2 3 2 3 2 2 2		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05 5.56E+05 4.62E+05	Conflict 1 1 1 1 1 1 1 1 1 1 1 1 1	 AADD: LEST: LEST: VNTN' VNTN' IRDTI IRDTI IRDTI
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Protein R Conflicting protein Accession Pep gi 15668937 • 1	esolution ns for feat bides Confident 2 14	46 ++++++++++++++++++++++++++++++++++++	Peptid	Pept V V V V V	tides 4 # 147 166 179 238 564 283 446 431 789 525 1166	of gi 12 Score 101 125 60.9 107 51.2 49.5 93.3 49.6 103 104 42.3	Hits 10 9 10 4 10 10 8 10 10 7	Mass / 1230.609 / 2317.115 / 2317.115 / 1716.857 / 1676.838 / 1676.838 / 1692.833 / 1692.833 / 1692.833 / 1700.863 / 1700.863 /	0.44 0.168 0.201 0.429 0.394 1.32 1.2 0.206 1.09 0.139 0.0934	22.7 38.7 30.4 30.4 34.5 20.6 20.6 36 36	2 2 3 2 3 3 2 3 2 2 3 2 2 3		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05 5.56E+05 4.62E+05 5.46E+05 1.03E+05	Conflict 1 1 1 1 1 1 1 1 1 1 1 1 1	 AADD: LEST: LEST: VNTN' VNTN' IRDTI IRDTI IRDTI IRDTI IRDTI VNTN' VNTN'

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.

🔼 Edit protein building options 🛛 💽
Protein grouping You can choose to to simplify the protein list by grouping together proteins which have been identified by similar sets of peptide sequences. A protein will be grouped with another if its identified peptide sequences are a subset of the identified sequences of the other.
 Group similar proteins No protein grouping
Protein quantitation You can choose to have quantitation based on either only features which have no conflicting protein identifications or on all features identified as part of a protein.
 Quantitate from non-conflicting features Quantitate from all features
OK Cancel

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.

roteins	Y	No filte	r applied	Create		Pep	lides	of gi 566	0937						No filter ap	plied		Create
Accession	Peptides	Conflicts	Score	Anova (p)*	*		#	Score	Hits Mas	ss Mass e	ror (p	RT (mins)	Charge	Tags	 Abundance 	Confl	icts	Peptide !
) gi 5668937	12 (3)	14	1.41E+03	4.15E-06	3	V	446	93.3	10 167	6.838	.2	34.5	2	V X	7.47E+05	1	6) IRD
gi 126697810	9 (0)	14	1.13E+03		1	V	3335	84.7	4 143	23.65 0.	405	22.5	2	VX	7.39E+04	1	6	🕽 ртр
gi 209571234	24 (12)	13	2.4E+03	2.51E-07	6	1	147	101	10 123	0.609 0	.44	22.7	2	 X 	3.07E+06	1	6	aad
gi 260682215	23 (11)	13	2.03E+03	4.08E-05	3	1	166	125	10 231	7.115 0.	168	38.7	2	√ ×	5.55E+06	1	6	LES
gi 126698450	12 (5)	9	1.21E+03	1.28E-06	2	1	179	60.9	9 231	7.115 0.	201	38.7	3	√ ×	3.09E+06	1	6	LES
gi 126700407	9 (2)	9	1.04E+03	0.000764	3 +	1	238	107	10 171	6.857 0.	429	30.4	2	VX	1.73E+06	1	6	🕽 vnt
III					P.	•				III								Þ
Protein: gi 126	ein Resolutio	n		Clostridiur				gi 12669	7810									
Protein: gi 126	ein Resolutio	feature		Clostridiur	F		des of	gi 12669 ore Hit		Mass error	(p R	(T (mins)	Charge	Tags	 Abundance 	Conflicts	; Рер	tide Ser
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Protein: gi 126 ptide Views Prote Conflicting prot Accession gil5668937 •	ein Resolution D teins for Peptides C	n feature Conflicts 14	e 446 Protein Score	Pept	F id [Peptio # ▼ 1. ▼ 1.	des of Sc 47	ore Hit	Mass 1230.6	09 0.44			2			Conflicts 1 1	3	
Protein: gi 126 ptide Views Prote Conflicting prot Accession gil5668937 •	ein Resolution Oteins for Peptides (C 12 (3)	n feature Conflicts 14	e 446 Protein Score 1.41E+03	Pept	id [Peptio # √ 1. √ 1.	des of Sc 47 66 79	ore Hit 101 10 125 10 60.9 9	Mass Mass 1230.60 2317.1 2317.1	09 0.44 15 0.168 15 0.201		22.7 38.7 38.7	2 .		3.07E+06 5.55E+06 3.09E+06	Conflicts 1 1 1	3 3	AADD
Protein: gi 126 ptide Views Prote Conflicting prot Accession	ein Resolution Oteins for Peptides (C 12 (3)	n feature Conflicts 14	e 446 Protein Score 1.41E+03	Pept	id [Peptie # ▼ 1. ▼ 1. ▼ 1. ▼ 1. ▼ 2.	des of 5c 47 66 79 38	ore Hit 101 10 125 10 60.9 9 107 10	Mass Mass 1230.66 2317.1 2317.1 0 1716.8	09 0.44 15 0.168 15 0.201 57 0.429		22.7 38.7 38.7 30.4	2 2 3 2		3.07E+06 5.55E+06 3.09E+06 1.73E+06	Conflicts 1 1 1 1	3 3 3	AADDI LEST(
Protein: gi 126 ptide Views Prote Conflicting prot Accession gi15668937 •	ein Resolution Oteins for Peptides (C 12 (3)	n feature Conflicts 14	e 446 Protein Score 1.41E+03	Pept	id [Peptie # ▼ 1- ▼ 10 ▼ 11 ▼ 22	des of Sc 47 66 79 38 64	ore Hitt 101 10 125 10 60.9 9 107 10 51.2 4	 Mass 1230.6i 2317.1¹ 2317.1¹ 1716.8i 1716.8i 	09 0.44 15 0.168 15 0.201 57 0.429 58 0.394		22.7 38.7 38.7 30.4 30.4	2 2 3 2 3 3		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05	Conflicts 1 1 1 1 1 1	3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3	AADD; LEST(LEST(VNTN VNTN
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Protein: gi 126 ptide Views Prote Conflicting prot Accession gi15668937 •	ein Resolution Oteins for Peptides (C 12 (3)	n feature Conflicts 14	e 446 Protein Score 1.41E+03	Pept	id. [[[[Peptie # 1- 1- 1- 1- 1- 1- 1- 1- 1- 1-	des of 5c 47 66 79 38 64 83 46	J Hit 101 10 125 10 60.9 9 107 10 51.2 4 49.5 10 93.3 10	Mass 1230.6 2317.1 2317.1 1716.8 1716.8 1676.8 0 1676.8	09 0.44 15 0.168 15 0.201 57 0.429 58 0.394 38 1.32 38 1.2		22.7 38.7 38.7 30.4 30.4 34.5 34.5	2 2 3 2 3 3 3 2 2		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05	Conflicts 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1		AADD; LEST(LEST(VNTN' VNTN' IRDT; IRDT;
Conflicting pro	ein Resolution Oteins for Peptides (C 12 (3)	n feature Conflicts 14	e 446 Protein Score 1.41E+03	Pept	id (Peptie # 1. 1. 1. 1. 1. 1. 1. 1. 1. 1.	des of 5c 47 56 54 83 83 46 31	ore Hit 101 10 125 10 60.9 9 107 10 51.2 4 49.5 10 93.3 10 49.6 8	Mass 1230.60 2317.11 2317.11 1716.82 1716.83 1676.83 1676.83 1692.83	09 0.44 15 0.168 15 0.201 57 0.425 58 0.394 38 1.32 38 1.2 35 0.206		22.7 38.7 38.7 30.4 30.4 34.5 34.5 20.6	2 1 2 1 3 2 3 1 3 1 3 1 3 1 3 1 3 1 3 1 3 1 3 1 3 1		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05 5.56E+05	Conflicts 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1		AADD; LEST(LEST(VNTN ¹ IRDT1 IRDT1 IRDT1
Protein: gi 126 ptide Views Prote Conflicting prot Accession	ein Resolution Oteins for Peptides (C 12 (3)	n feature Conflicts 14	e 446 Protein Score 1.41E+03	Pept	id [[[[[[[[[# # 1 <	des of 5c 47 66 79 38 64 83 83 46 31 89	ore Hitt 101 10 125 10 60.9 9 107 10 51.2 4 49.5 10 93.3 10 49.6 8 103 10	Image: second	09 0.44 15 0.168 15 0.201 57 0.429 58 0.394 38 1.32 38 1.2 35 0.206 33 1.09		22.7 38.7 30.4 30.4 34.5 34.5 20.6 20.6	2 2 3 2 3 3 3 3 2 3 2 3 2		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05 5.56E+05 4.62E+05	Conflicts 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1		AADD; LEST(UNTN IRDTI IRDTI IRDTI IRDTI
Protein: gi 122 ptide Views Prot. Conflicting prod Accession gi 1566937 0 gi 126697810	ein Resolution Oteins for Peptides (C 12 (3)	n feature Conflicts 14	e 446 Protein Score 1.41E+03	 Pept ✓ 93.3 ✓ 93.3 	F id [[[[[[[[[[[[[[[[Peptic # # 1 1 1 1 1 1 2 1 1 2 1 2 2 2 5 7 2 2 4 4 4 7 7	des of 5c 47 56 54 83 83 46 31	ore Hit 101 10 125 10 60.9 9 107 10 51.2 4 49.5 10 93.3 10 49.6 8	Image: second	09 0.44 15 0.168 15 0.201 57 0.429 58 0.394 38 1.32 38 1.2 35 0.206 33 1.09		22.7 38.7 38.7 30.4 30.4 34.5 34.5 20.6	2 2 3 2 3 3 3 2 3 2 3 2		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05 7.59E+05 7.47E+05 5.56E+05	Conflicts 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1		AADD; LEST(LEST(VNTN' IRDTI IRDTI IRDTI

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.

	V N	lo filter app	olied	Create	Рер	tides	of gi 56	668937						Vo f	filter applied		Create
					_												
Accession		Conflicts		Anova (p ^		#	Score	Hits	Mass	Mass error (p		Charge	Tags				Peptide 9
gi 126699063	6	0	368	2.03E-06		69	63.9	10	1669.889	0.0302	42.1	3	 ✓ × 	4.758	E+06) (🜒 IAD
gi 54781345	5	0	403	3.89E-05	1	132	103	10	1669.888	0.646	42.1	2	 ✓ × 	4.38	E+06) (🗿 IAI
gi 126700857	5	0	270	0.00443	V	147	101	10	1230.609	0.44	22.7	2	\checkmark \times	3.076	E+06) (🗿 AAD
gi 126698631	5	0	574	0.000151	V	166	125	10	2317.115	0.168	38.7	2	\checkmark \times	5.558	E+06) (LES
gi 5668937 (+1)	ail5668937	7 - flagellir	Clostridiur	n difficile1		- 79	60.9	9	2317.115	0.201	38.7	3	\checkmark \times	3.098	E+06) (LES
gi 54781347				Clostridium dif	ficile 63	30] <mark>8</mark>	107	10	1716.857	0.429	30.4	2	 ✓ × 	1.738	E+06) (🗿 VNI
gi 126698643	4	0	365	0.0132	V	564	51.2	4	1716.858	0.394	30.4	3	 ✓ × 	3.576	E+05) (s vn
ei1126701179	4	0	286	7 74F-06	3	283	49 5	10	1676 838	1 37	34.5	3	1	7 598	F+05	n (а тр
Conflicting prote	eins				Peptio	des of	f conflict	t <mark>ing</mark> pr	otein								
	eins Peptides Conf	flicts Prot	ein Score	Peptid	Peptio #					ass error (p R	RT (mins)	Charge T	ags	✓ Abundar	nce Conflic	ts Pe	otide Sec
		flicts Prot	ein Score		-					ass error (p R	(T (mins)	Charge T.	ags i	▼ Abundar	nce Conflic	ts Pej	otide Seç
Conflicting proto	Peptides Conf	Ricts Prot	ein Score	Peptid	-					ass error (p R	RT (mins)	Charge T	ags	✓ Abundar	nce Conflic	ts Per	otide Seq

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.

Not the second s		
File Experiments		nonlinear
Perform analysis Combine analysed fraction Fractionation If you're using fractionation to increase peptide coverage, this workflow will help you to recombine your fractionated samples' data. For more information on how Progenesis LC-MS analyses fractionated samples, visit the website.	Recent multi-fraction experiments Search	Getting started with Progenesis Here are some resources to help you get started with Progenesis LC-MS: Data analysis with Progenesis LC-MS: Tutorial and user guide Frequently-asked questions
 Analyse each fraction separately Create a separate experiment for each fraction. Each experiment will contain a single run for each of your samples. Analyse a single fraction Recombine the fractionated samples After analysing each fraction separately, these can be added to a multi-fraction experiment to recombine your samples. Recombine analysed fractions 	There are no recent multi-fract experiments To create a new one, follow the instructions at the left	Fraction Start m/z End m/z
	Browse for a multi-fraction exp	The one reason you should join the Progenesis Improvement Program Number of publications citing Progenesis for quantitative protec At last! Pain-free reviewing in the

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

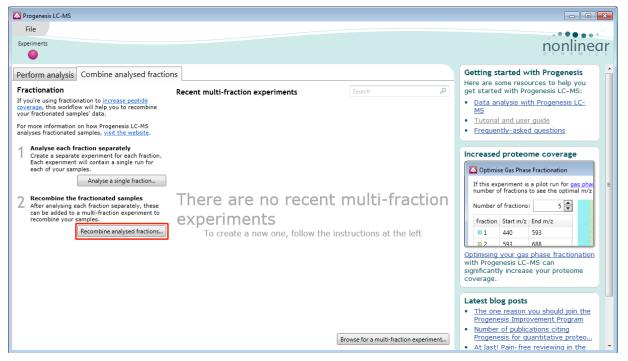
A Progenesis LC-MS	
File	
Experiments	nonlinear
Perform analysis Combine analysed fractions	Getting started with Progenesis Here are some resources to help you
Recent experiments Search P	get started with Progenesis LC-MS:
Fract_6 In folder: D:\Customer Data\LC-MS\NCI_renaming and Osfucation\Fraction_Rename\Double Ob Last saved: 2/20/2013 8:20:03 AM	Data analysis with Progenesis LC- MS <u>Tutorial and user guide</u> <u>Frequently-asked questions</u>
Fract_5 In folder: D:\Customer Data\LC-MS\NCI_renaming and Osfucation\Fraction_Rename\Double Obsfucated_NCI Last saved: 2/20/2013 7:58:18 AM	Increased proteome coverage
Fract_4 In folder: D:\Customer Data\LC-MS\NCI_renaming and Osfucation\Fraction_Rename\Double Obsfucated_NCI Last saved: 2/20/2013 7:49:22 AM	If this experiment is a pilot run for <u>gas phar</u> number of fractions to see the optimal m/z Number of fractions: 5 T
Fract_3 In folder: D:\Customer Data\LC-MS\NCI_renaming and Osfucation\Fraction_Rename\Double Obsfucated_NCI Last saved: 2/20/2013 7:45:21 AM	1 440 593 2 593 688 Optimising your gas phase fractionation with Progenesis LC–MS can
Fract_2 In folder: D:\Customer Data\LC-MS\NCI_renaming and Osfucation\Fraction_Rename\Double Obsfucated_NCI Last saved: 2/20/2013 7:43:46 AM	significantly increase your proteome coverage.
Fract_1 In folder: D:\Customer Data\LC-MS\NCI_renaming and Osfucation\Fraction_Rename\Double Obsfucated_NCI Last saved: 2/20/2013 7:42:12 AM Other experiments	The one reason you should join the Progenesis Improvement Program Number of publications citing Progenesis for quantitative proteo At last! Pain-free reviewing in the Progenesis workflow
New Open 4.1.4797.36561	Progenesis CoMet v2.0 released for <u>LC-MS-based metabolomics</u> Happy Holidays from the Nonlinear

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

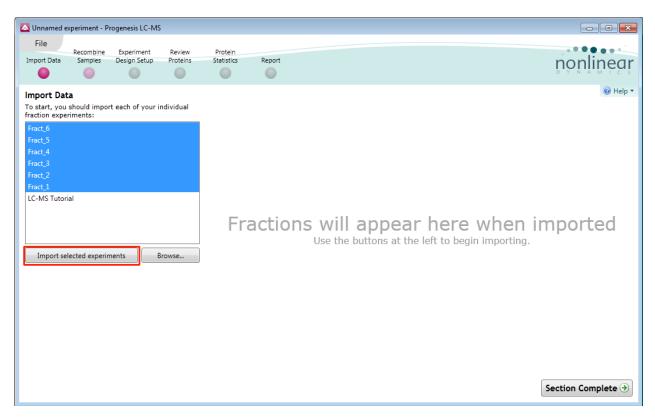
The combining of the single fraction experiments is performed in the second stage of the Fractionation workflow.

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.

🔼 Unnamed experiment - Progenesis LC-MS		
File Recombine Experiment Review Proteins	Protein Statistics Report	nonlinear
Import Data To start, you should import each of your individual fraction experiments: Fract_6 Fract_5 Fract_4 Fract_3 Fract_2 Fract_1 LC-MS Tutorial	To obtain the correct peptide distribution, put the fractions in order: 1 Fract 6 × 1 46 peptides identified in 6 runs Normalised: Yes 2 Fract 5 × 3 20 peptides identified in 6 runs Normalised: Yes 3 Fract 4 × 454 peptides identified in 6 runs Normalised: Yes 4 Fract 3 × 368 peptides identified in 6 runs	Move Fract_3: Last saved: 20/02/2013 Last saved: 20/02/2013 Last saved: 20/02/2013
Import selected experiments Browse Peptides per fraction 500- 400- 400- 500- 400- 1 2 3 4 5 6 Fraction	Normalised: Yes 5 Fract 2 × 318 peptides identified in 6 runs Normalised: Yes 6 Fract 1 × 20 peptides identified in 6 runs Normalised: Yes	Last saved: 20/02/2013 Last saved: 20/02/2013 Last saved: 20/02/2013

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.

Unnamed experiment - Progenesis LC-MS		
File Recombine Experiment Review Import Data Samples Design Setup Proteins	Protein Statistics Report	nonlinear
Import Data To start, you should import each of your individual fraction experiments: Fract_6 Fract_7 Fract_4 Fract_3 Fract_2 Fract_1 LC-MS Tutorial	To obtain the correct peptide distribution, put the fractions in order: 1 Fract_1 × 20 peptides identified in 6 runs Normalised: Yes 2 Fract_5 × 320 peptides identified in 6 runs Normalised: Yes 388 peptides/identified in 6 runs Normalised: Yes	Move Fract_3: Image: Image: Action and Actio
Import selected experiments Browse Peptides per fraction 500- 400- 500- 500- 400- 500-	 Fract_2 × 318 peptides identified in 6 runs Normalised: Yes Fract_6 × 146 peptides identified in 6 runs Normalised: Yes 	Last saved: 20/02/2013 Last saved: 20/02/2013 Section Complete 🕑

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

🔼 Unnamed e	xperiment - P	rogenesis LC-MS	5								
File			Review	Protei							
Import Data	Recombine Samples	Experiment Design Setup	Proteins	Statisti						nonli	noar
					Ó						
Recombine	e Samples			Find rur	ns containing:		٩				🕜 Help 🔻
		ecombine your s up your runs int		ſ	▼ Create sample ▼	Create sample	Create sample	▼ Create sample ▼	Create sample	▼ Create sample ▼	-J
	the run from ents the same	each fraction th	at	1							
2. Click th	ne button belo		eriments	4	386.) 1	384 A.	1996	2396	1996	2310	
		the runs to crea			Con_1_F1	Con_2_F1	Con_3_F1	Treat_1_F1	Treat_2_F1	Treat_3_F1	
			^	2	846).			Sec.		Sec.	
					Con_1_F2	Con_2_F2	Con_3_F2	Treat_1_F2	Treat_2_F2	Treat_3_F2	
				3							
					Con_1_F3	Con_2_F3	Con_3_F3	Treat_1_F3	Treat_2_F3	Treat_3_F3	
				4					E.		
					Con_1_F4	Con_2_F4	Con_3_F4	Treat_1_F4	Treat_2_F4	Treat_3_F4	
				5	SPS.						
					Con_1_F5	Con_2_F5	Con_3_F5	Treat_1_F5	Treat_2_F5	Treat_3_F5	
				6		SP.					
			-		Con_1_F6	Con_2_F6	Con_3_F6	Treat_1_F6	Treat_2_F6	Treat_3_F6	
If you find th		w to use, there i	may be							Section Com	-
faster ways t	o recombine	your samples .								Section Com	piete 🤿

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

Unnamed experiment - Progenesis LC-MS		_ 0 ×
File Recombine Experiment Design Setup Proteins	Protein Statistics Report	nonlinear
<text><text><list-item><list-item></list-item></list-item></text></text>	Find runs containing: Con_1 × Press Enter to create the sample Create sample Con_1 F1 Con_1 F2 Con_1 F3 Con_1 F3 Con_1 F3 Con_1 F5 Con_1 F5 Con_1 F5	€ Help Υ
Using this screen efficiently If you find this screen slow to use, there may be faster ways to recombine your samples .		Section Complete)

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.

Unnamed experiment - Progenesis LC-MS							- • •
File Recombine Experiment Review Proteins	Protein Statistic						nonlinear
Recombine Samples	Find runs	s containing:		P			🔞 Help 🔻
This stage allows you to recombine your samples' separate fractions. To group your runs into samples:		• Create sample •	▼ Create sample ▼	▼ Create sample ▼	🔻 Create sample 🔻	🕶 Create sample 💌	
 Select the run from each fraction that represents the same sample Click the button below Repeat for each sample in your experiments 	1	S.			S.C.		
		Con_2_F1	Con_3_F1	Treat_1_F1	Treat_2_F1	Treat_3_F1	
Con_1 × * * Con_1_F1 * Con_1_F2	2					N.	
• Con_1_F3 • Con 1 F4		Con_2_F2	Con_3_F2	Treat_1_F2	Treat_2_F2	Treat_3_F2	
• Con_1_F5 • Con_1_F6	3						
		Con_2_F3	Con_3_F3	Treat_1_F3	Treat_2_F3	Treat_3_F3	
	4						
		Con_2_F4	Con_3_F4	Treat_1_F4	Treat_2_F4	Treat_3_F4	
	5						
		Con_2_F5	Con_3_F5	Treat_1_F5	Treat_2_F5	Treat_3_F5	
	6	Sp.	S. S				
		Con_2_F6	Con_3_F6	Treat_1_F6	Treat_2_F6	Treat_3_F6	
Using this screen efficiently If you find this screen slow to use, there may be faster ways to recombine your samples .							Section Complete)

Repeat for the remaining samples.

File						
	Recombine	Experiment	Review	Protein		
mport Data	Samples	Design Setup	Proteins	Statistics	Report	nonlinear
						D Y N A M I C S
Recombine	Samples					@ Help
		combine your s				
		up your runs int each fraction th				
represe 2. Click the	nts the same e button belo	sample w				
Repeat	for each sam	ple in your exp	eriments			
	🖌 Group	the runs to crea	te a sample			
Con 3			×			
• Con 3 F	L					
• Con_3_F						
 Con_3_F. 	3					
 Con_3_F 	1					
 Con_3_F 						
 Con_3_F 	5					
Treat_1			×	Δ	ll ru	ns have been grouped into samples
• Treat_1_	1					
• Treat_1_	2					Click the Section Complete button to continue analysis.
 Treat_1_ 	-3					
 Treat_1_ 						
 Treat_1_ 						
 Treat_1_ 	-6					
Treat_2			× =			
 Treat_2_ 	1					
• Treat_2_	2					
 Treat_2_ 						
 Treat_2_ 						
• Treat_2_						
 Treat_2_ 	-6					
Treat_3			×			
 Treat_3_ 	1					
 Treat_3_ 	2		-			
Jsing this so		ently v to use, there i	nav be			
		v to use, there i your samples .	nay be			Section Complete 🤿

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.

Vinnamed experiment - Progenesis LC-M	5									• 🔀
File Recombine Experiment Import Data Samples Design Setup	Review Proteins	Protein Statistics	Report					ņç	pnlin	ear
New									(🕜 Help 🔻
Which experiment design type	e do you v	want to use	for this exp	perime	nt?					
00 00 Between-subject De	sign	A Progenesis	LC-MS			×				
Do samples from a given subject appear in only one condition? Then use the between-subject design.	A		new expe ontrol vs Tre		-	-	Before	During	After	
To set up this design, you simply group the runs according to the condition (factor level)	c	Start wit Copy lay	th an empty	layout		atient X	X1	X2	X3	E
of the samples. The ANOVA calculation assumes that the conditions are independent and therefore gives a statistical test			design from		/hat file formats can I use?	atient Y	Y1	Y2	Y3	
of whether the means of the conditions are all equal.	Add condition		Grou	up by:	(no valid groups)	atient Z	Z1	Z2	Z3	
					Create design Cance	el .				
					subject it came from. The software will then perform a repeated measures ANOVA.					
					A standard ANOVA is not appropriate because the data					-

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

🔼 Unnamed experiment - Progenesis LC-MS				
File				
Recombine Experiment Review Import Data Samples Design Setup Proteins	Protein Statistics	Report		poplinear
		•		D Y N A M I C S
Control vs Treated I × 🕒 New				🔞 Help 🔻
Setup conditions	Samples	Add Selected Samples to Condition	▼ Search	Q
Setup the conditions that you want to compare below (e.g., control, drug A, etc), and then assign each of your samples to the correct condition.		Add to new condition	Treat_2	Treat_3
Control Delete		Control		
Con_1 Remove			a starter	13952
Con_2 Remove				
Con_3 Remove				
Add condition				
				Section Complete 🏵

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.

File Import Data		ign Setup Protein		tics R	eport					nonlinea
Wo filter	applied Crea	ste		Q						Protein options 🕜 Help
ccession	Peptide co	unt Confidence sco	re Anova (p)	Tag 💌	Max fold change	Fractions	Occurrences	Highest Mean	Lowest Mean	Description
🔰 gi 20629315	1	51.4	0.000344		3.72	123456	1	Control	Treated	50S ribosomal protein L7/L12 [Clost
🔰 gi 85279330	1	52.4	0.00844		2.16	123456	2	Control	Treated	PTS system, IIB component [Clostric
🔰 gi 72029336	1	78.3	0.00847		100	123456	1	Treated	Control	flagellin [Clostridium difficile]
🕽 gi 64841839	4	240	0.00901		2.06	123456	3	Control	Treated	enolase [Clostridium difficile 630]
🔰 gi 65076322	1	55	0.0126		2.27	123456	1	Treated	Control	transketolase [Clostridium difficile 6
🄰 gi 46211184	2	117	0.0146		1.71	123456	2	Treated	Control	50S ribosomal protein L2 [Clostridiu
🕽 gi 42170149	1	45.8	0.0148		3.81	123456	1	Treated	Control	translation elongation factor G [Clo
) gi 17787717	2	129	0.0149		3.59	123456	2	Treated	Control	30S ribosomal protein S4 [Clostridiu
) gi 20629287	2	82.9	0.0161		1.83	123456	1	Treated	Control	tellurium resistance protein [Clostri
🔰 gi 51703916	4	263	0.0165		1.77	123456	3	Treated	Control	D-alaninepoly(phosphoribitol) liga
🔰 gi 04388227	1	41.7	0.0168		1.51	123456	1	Control	Treated	2-hydroxyisocaproate-CoA transfer
🔰 gi 63129634	1	48.6	0.0177		1.79	123456	1	Control	Treated	cell surface protein [Clostridium diff
Selected p		olase [Clostri	dium dif	ficile 6	30]					•
15.0			Control						Treated	
ArcSinh Normalised.			¥							

And at the peptide level when you click View peptide measurements

	Jnnam	ied expe	eriment - Pro	ogenesis LC-MS									_	×
	File port Da		Samples		roteins Sta		port						nonlin	ear
Acc	Back ession		otein: e 164841839	nolase [Clost	ridium dif	ficile 630	l							
Σ	#	Score	Anova (p)	Max Fold Change	Highest Mean	Lowest Mean	Abundance	m/z	Charge	Retention Time	Fractions	Mass error (ppm)	Peptide Sequence	Modifie
•	772	58.36	0.462	1.2	Control	Treated	1.608E+05	554.7858	2	23.052	123456	0.89	😵 VFDFLDYGIR	
•	1135	61.69	0.448	1.2	Control	Treated	7.381E+04	508.2809	2	23.265	123456	1.47	GEMVHVNDR	
•	1388	67.65	0.943	1.11	Control	Treated	7.981E+04	616.8116	2	28.624	1 2 3 4 5 6	0.09	DWGEECQAQYK	
•	431	52.04	0.0888	4.03	Control	Treated	7.158E+05	602.8294	2	29.958	123456	-22.26	FVNNYYESEMK	
	666	55.17	0.39	1.4	Control	Treated	2.37E+05	508.2808	2	23.417	123456	1.15	GEMVHVNDR	
(Standard	lised Exp	ression	Profile	S]
			Control						Treated					
tundardi sed Normali sed Abundance	20 1.5 1.0 0.5 0.0 0.5 -1.0 -1.5			B	B									

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**.

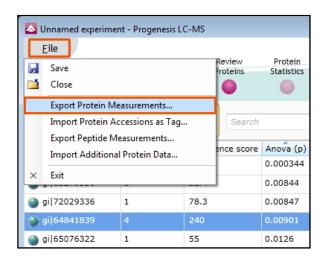
	combine amples	Experim Design S		Prote Statist	tics R	leport					nonlinea
Wo filter ap		Create	Search		Q						Protein options 🕡 Help
ccession	Peptide	e count	Confidence score	Anova (p)	Tag 💌	Max fold change	Fractions	Occurrences	Highest Mean	Lowest Mean	Description
) gi 20629315	1		51.4	0.000344	۹	3.72	123456	1	Control	Treated	50S ribosomal protein L7/L12 [Clo
gi 85279330 gi	1		52.4	0.00844	۹	2.16	123456	2	Control	Treated	PTS system, IIB component [Clostr
gi 72029336 gi	1		78.3	0.00847	۹	100	123456	1	Treated	Control	flagellin [Clostridium difficile]
) gi 64841839	4841839 4 <mark>● Anova p-value ≤ 0.05</mark> 2901 ● 2.06						123456	3	Control	Treated	enolase [Clostridium difficile 630]
🕽 gi 65076322	1	Max fold change ≥ 2		126	۲	2.27	123456	1	Treated	Control	transketolase [Clostridium difficile
gi 46211184	2	Newt	New tag		-	1.71	123456	2	Treated	Control	50S ribosomal protein L2 [Clostrid
gi 42170149	1		Quick Tags		Anova p-value		123456	1	Treated	Control	translation elongation factor G [Cl
gi 17787717 gi	2	Edit ta	05	_	Max fold ch		1 2 3 4 5 6	2	Treated	Control	30S ribosomal protein S4 [Clostrid
) gi 20629287	2		82.9	0.0	Sequence		123456	1	Treated	Control	tellurium resistance protein [Clost
) gi 51703916	4		263	0.0	Modificatio	n	123456	3	Treated	Control	D-alaninepoly(phosphoribitol) lie
-::04200227	4		A1 7	0.0169		4 54		4	C	T	a Ludan da anna a Aanaa
/iew peptide meas			se [Clostrid	ium dif	ficile 6	30]				Treated	
14.5				¥						▶ ▼	
otal number of (: 548 Numb			eins in only one fra	action: 346				Section Complete

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.

🔼 Unnamed exp	eriment - Progene	sis LC-MS										2
File												
Import Data			Review Proteins	Protei Statist		Report					nonlin	ear
	•			0								
Tag filter proteins r	applied nay be hidden	Edit	Search	'n		<u>م</u>					Protein options) Help 🔻
Accession	Peptide cour	nt Confidenc	e score	Anova (p)	Tag 🛛 🔻	Max fold change	Fractions	Occurrences	Highest Mean	Lowest Mean	Description	
🎯 gi 20629315	1	51.4		0.000344	۹	3.72	123456	1	Control	Treated	50S ribosomal protein L7/L1	2 [Clo: *
🌒 gi 85279330	1	52.4		0.00844	۹	2.16	123456	2	Control	Treated	PTS system, IIB component [Clostri
🎯 gi 72029336	1	78.3		📐 Filter the	a proteinc	•			1	×	flagellin [Clostridium difficile] E
🔇 gi 64841839		240									enolase [Clostridium difficile	630]
🔇 gi 65076322	1	55		Create a		ins based on a sele	ction of their tags	Move tags to	the appropriate		transketolase [Clostridium d	ifficile
🎯 gi 42170149	1	45.8				filter. For more gu					translation elongation factor	G [Clo
🎯 gi 17787717	2	129	-	Available	tags:		Show pro	teins that hav	/e all of these t	ags:	30S ribosomal protein S4 [Cl	lostrid
🎯 gi 04526507	1	47.7			3		· · ·		2 (262 proteins		nitroreductase-family protei	n [Clos
🔇 gi 64041498	4	262						-	05 (37 proteins)		thioredoxin [Clostridium diff	icile 6:
🎯 gi 99669795	1	40.6					Show pro	teins that hav	/e at least one	of d	adenylate kinase [Clostridiur	n diffic
A -::CA0410ED	2	00.0					these tags				Description (Processing States)	, ×
Selected p View peptide m 15.0 14.5 14.0	protein: eno easurements	lase [Clo	ostri	Clear the	filter		Hide prot	eins that have	e any of these t			•
Total number o	of displayed prote	eins: 26	Numbe	er of displa	yed prote	ins in only one frac	tion: 20				Section Comple	te Э

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

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Export Protein Measurer	nents		
Choose properties to be in	cluded in exporte	ed file	
Accession			
Peptide count			
Peptides used for qua	ntitation		
Confidence score			
📝 Anova (p)			
Max fold change			
Fractions			
Occurrences			
Highest mean condition	n		
Lowest mean condition	1		
Description			
Normalized abundance	е		
Tags			
	_		
		ОК	Cancel

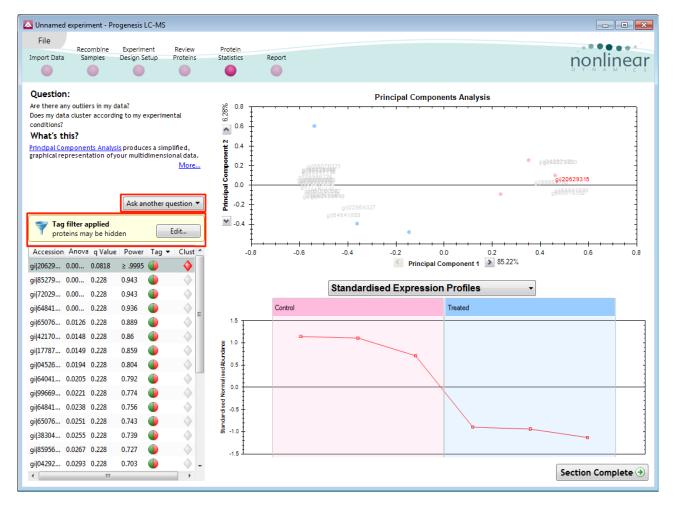
A dialog allows you to control the details of the output file.

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

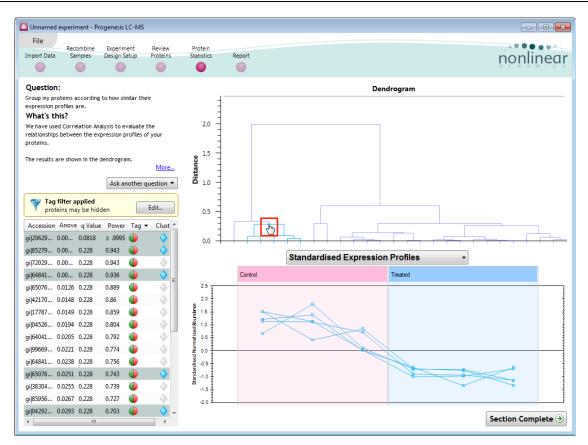
Finally your recombined data can be saved as a Multi-fraction experiment.

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.**

Filter the proteins	
Create a filter Show or hide proteins based on a selection of the create the filter. For more guidance, please see t	
Available tags:	Show proteins that have all of these tags:
	Max fold change ≥ 2 (262 proteins)
	Anova p-value ≤ 0.05 (37 proteins)
	Show proteins that have at least one of these tags: Hide proteins that have any of these tags:
Clear the filter	OK Cancel

- 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

🔼 Unnamed experiment - P	rogenesis LC-MS		
File Recombine Import Data Samples		eview Protein teins Statistics Report	nonlinear
Proteins Tag filter applied proteins may be hid Accession Anova (p) gi 20629315 0.000344 gi 85279330 0.00844 gi 72029336 0.00847 gi 64841839 0.00901 gi 65076322 0.0126 gi 42170149 0.0148 gi 17787717 0.0149 gi 04526507 0.0194 gi 6969795 0.0221	dden Fold Tag 3.7 2.2 100.4 2.1 2.3 3.8 3.6 858.0 3.3 15.3	Edit Description S0S ribosomal protein L7/L12 [Clostridium difficile 630] PTS system, IIB component [Clostridium difficile 630] flagellin [Clostridium difficile 630] transketolase [Clostridium difficile 630] translation elongation factor G [Clostridium difficile 630] a0S ribosomal protein S4 [Clostridium difficile 630] nitroreductase-family protein [Clostridium difficile 630] trioredoxin [Clostridium difficile 630] adenylate kinase [Clostridium difficile 630]	Report Design Title Unnamed experiment Select the sections you wish to include in your report: Experiment design Protein report Include tables showing protein abundances and peptides identified for each protein Protein table Protein table Protein Details Reports the full details of every protein which matches your current filter Include tables in the full details of every protein which matches your current filter
gij64841853 0.0238 gij65076352 0.0251 gij38304314 0.0255 gij85956724 0.0267 gij04292138 0.0293 gij39642159 0.031 gij48078745 0.0331 gij00347190 0.0359 gij82751345 0.0423 gij02954327 0.0439 gij27875768 0.0445 gij21210623 0.046 e	2.2 4.8 3.9 2.6 2.5 8.3 2.1 2.6 2.8 2.9 3.4 3.4	flagellin [Clostridium difficile] thioredoxin [Clostridium difficile 630] (R)-2-hydroxyisocaproate dehydrogenase [Clostridium difficile 630] flagellin [Clostridium difficile] pyruvate-flavodoxin oxidoreductase [Clostridium difficile 630] cell surface protein [Clostridium difficile 630] ferredoxin-NADP(+) reductase subunit alpha [Clostridium transcription elongation factor [Clostridium difficile 630]	✓ Tags ✓ Expression profile Create Report Output

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

Signific	antly	Cha	nging F	Prote	ins								
Experiment	periment: Unnamed experiment												
Report crea	port created: 05/08/2011 15:17:14												
Proteins													
Protein buildi Protein grou Protein quan	ping G	roup sim	nilar protein / features v		protei	n conflicts							
Accession	Peptides	Score	Anova (p) *	Fold	Tags	Fractions	Description	Average Nor Abundances					
								Control	Treated				
<u>gi 64041498</u>	4	261.88	0.02	3.31		123456	thioredoxin [Clostridium difficile 630]	2.34e+005	7.73e+005				
<u>gi 64841839</u>	4	239.74	9.01e-003	2.06		123456	enolase [Clostridium difficile 630]	1.27e+006	6.14e+005				
gi 38304314	2	133.82	0.03	3.90		123456	(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	305 ribosomal protein 54 [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		123456	small acid-soluble spore protein A [Clostridium difficile 630]	9.35e+004	2.37e+005				
<u>gi 48078745</u>	2	94.59	0.03	2.11		123456	aspartate aminotransferase [Clostridium difficile 630]	2.71e+005	5.72e+005				
gi 85956724	1	82.04	0.03	2.59		123456	cell surface protein [Clostridium difficile 630]	1.20e+005	3.11e+005				
gi172029336	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

Sequence	Feature	Score	Hits	Mass	Charge	Fr	actio	on			Modifications	In quantitation	Average Normalised Abundances	
													Control	Treated
DWGEECQAQYK	1388	67.65	2	1231.6086	2	1	2	3 4	4 5	6		yes	7.98e+004	7.18e+004
FVNNYYESEMK	431	52.04	1	1203.6443	2	1	2	3 4	4 5	6		yes	7.16e+005	1.78e+005
GEMVHVNDR	666	55.17	5	1014.5470	2	1	2	3 4	4 5	6		yes	2.37e+005	1.70e+005
GEMVHVNDR	1135	61.69	2	1014.5473	2	1	2	3 4	4 5	6		yes	7.38e+004	6.17e+004
	772	58.36	1	1107.5570	2	1	2	3 4	4 5	6		yes	1.61e+005	1.33e+005
vFDFLDYGIR gi 3830431 R)-2-hydroxyiso peptides Sequence	4	e deh	ydro			ım d		icile	e]	6	Modificatio		Average N	lormalised
gi 3830431 R)-2-hydroxyiso peptides	<u>4</u> ocaproat	e deh	ydro	genase [Clo	ostridiu	ım d	liffi	icile	e]	6	Modificatio	ns In	Average N	lormalised
gi 3830431 R)-2-hydroxyiso peptides	4 ocaproat	e deh	ydrog ore H	genase [Clo	ostridiu Char	ım d	liffi Fra	icile	e] on	6		ns In	Average N Abundance	lormalised ces Treated

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

File		
(periments		nonlinea
erform analysis Combine analysed fractio	าร	Getting started with Progenesis Here are some resources to help you
ractionation	Recent multi-fraction experiments Search	p get started with Progenesis LC-MS:
you're using fractionation to increase peptide verage, this workflow will help you to recombine our fractionated samples' data.	My Recombination	Pen Data analysis with Progenesis LC- MS
or more information on how Progenesis LC-MS alyses fractionated samples, visit the website.	Has 6 fractions, with 571 proteins identified across 6 sam	• <u>Tutorial and user guide</u> • <u>Frequently-asked questions</u>
Analyse each fraction separately Create a separate experiment for each fraction. Each experiment will contain a single run for each of your samples.		Increased proteome coverage
Analyse a single fraction		If this experiment is a pilot run for <u>gas phas</u> number of fractions to see the optimal m/z
Recombine the fractionated samples After analysing each fraction separately, these can be added to a multi-fraction experiment to		Number of fractions: 5
recombine your samples.		Fraction Start m/z End m/z
Recombine analysed fractions		1 440 593
		Optimising your gas phase fractionation with Progenesis LC–MS can

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**.

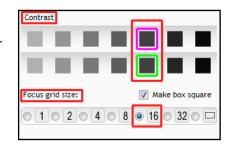
This completes a guided tour through using Progenesis LC-MS to analyse fractionated data.

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.



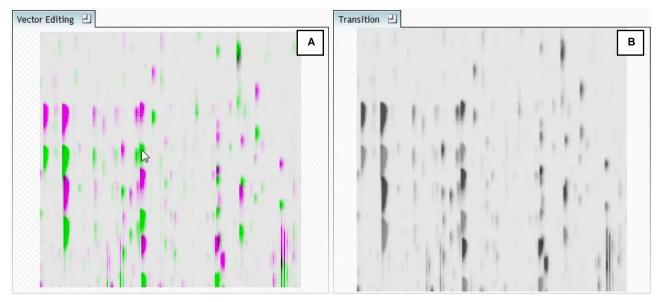
Click on an area (see below) in the **Whole Run** view (C) to refocus all the windows. Adjust Contrast as required.



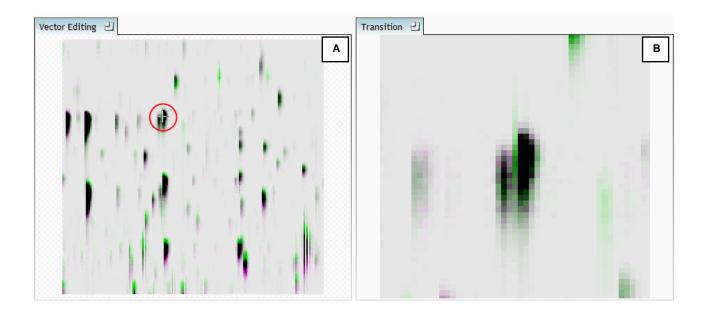
Note: the features moving back and forwards between the 2 runs in the **Transition** view indicating the misalignment of the two LC-MS runs

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.



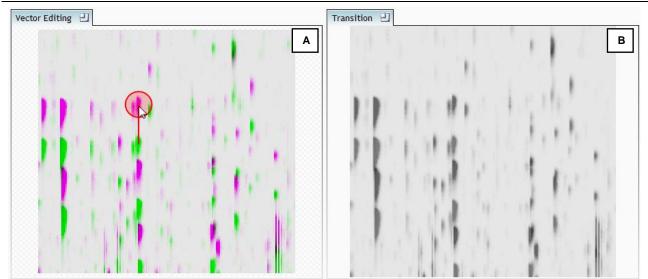
5. As you are holding down the left mouse button drag the green feature over the corresponding magenta feature of the reference run. The red circle will appear as shown below indicating that a positional lock has been found for the overlapping features.



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

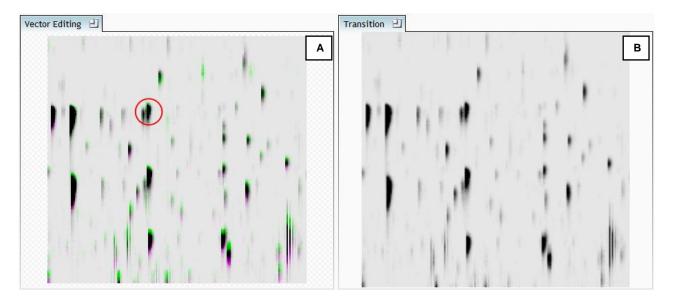
6. On releasing the left mouse button the view will 'bounce' back and a red vector, starting in the green feature and finishing in the magenta feature will appear.

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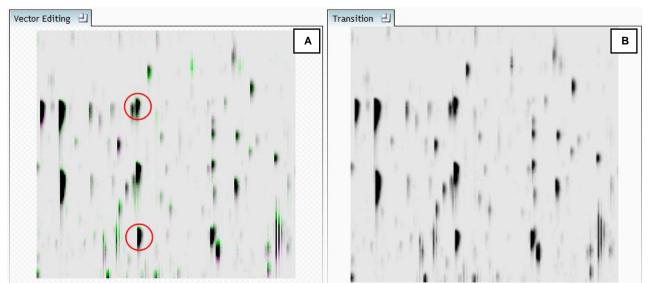


Note: an incorrectly placed vector is removed by right clicking on it in the Vector Editing window

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

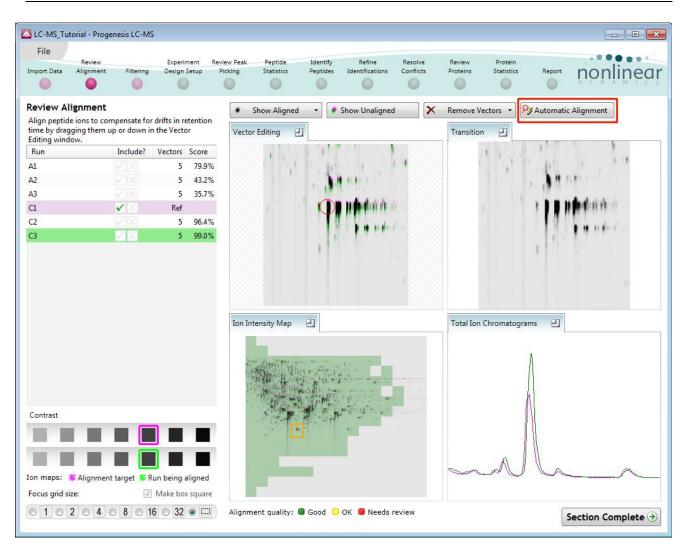


Note: the number of vectors you add is recorded in the **Runs** table also with each vector addition the Score and alignment quality updates. This can help guide the number of manual vectors you need to add before applying the automatic alignment.

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.

		lignment	- ×
	Run		Vectors
V	A1	run has user vectors	5
V	A2	run has user vectors	5
V	A3	run has user vectors	5
	C1	this run does not need to be aligned as it is the alignment reference	Ref
1	C2	run has user vectors	5
v	C3	run has user vectors	5
		ОК	Cancel

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

Review Exper		Picking	Peptide Statistics	Identify Peptides	Refine Identifications	Resolve Conflicts	Review Proteins	Protein Statistics	Report	noi	nlinear
New Which experiment design type do y Between-subject Design Do samples from a given subject	/ou wan	t to use '	for this ex	(periment	0-0 0-0 0-0		ject Design les from a give]			Ø Help ▼
appear in only one condition? Then use the between-subject design. To set up this design, you simply group the runs according to the condition	A			Delete Remove Remove	subject u Then use Note: you	nder differe the within- must have a	nt conditions? subject design		Before X1	During X2	After X3
(factor level) of the samples. The ANOVA calculation assumes that the conditions are independent and therefore gives a statistical test of whether the means of the conditions are all equal.	C Add cone	Creat Name:		ouring and	every subject for every condition to ent design After Treatment			Patient X Patient Y Patient Z	atient X Y1	Y2 Z2	Y3 Z3
		© Cop	y layout fro	om:	assumption repeated difference reduced a condition create a r The within thought o paired-sar	measures AN es can be eli s a source o differences nore powerf n-subject de f as an exter mples t-test	Cancel e Cancel e NoVA individual foto and a foto and a	e			

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.

LC-MS_Tutorial - Progenesis LC-MS						
File						
Review Experiment Re Import Data Alignment Filtering Design Setup	eview Peak Peptide Picking Statistics	Identify Peptides	Refine Reso Identifications Conf	olve Review flicts Proteins	Protein Statistics R	
	•	0	0			
Before During and After Treatment \perp X	New					🔞 Help 🔻
Setup conditions and subjects						
Setup the conditions and subjects for your experiment design on the right, and then assign each of your samples to the correct subject/condition cell in the grid.		Before	During	After	Add Condition	
 Add a column for each condition. Add a row for each subject. 					1	
Drag each of your samples to the correct location in the grid.	Patient A	A1	A2	A3		
Filter samples: 🔎						
C2		B1	B2	B3		
1967	Patient B	3870-10-1				
		and the second				
C3					1	
	Subject 1	C1	lect Sample	Select Sample		
3,7,3,-						
					-	
	Add Subject					
						Section Complete

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.