

# Progenesis LC-MS Fractionation User Guide

Analysis workflow guidelines

for version 3.0



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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 userguide 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 Userguide). 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.

	Reference Run			Experiment						
Data Import	Selection	Alignment	Filtering	Design Setup	View Results	Progenesis Stats	Peptide Search	Peptide Filter	Protein View	Report

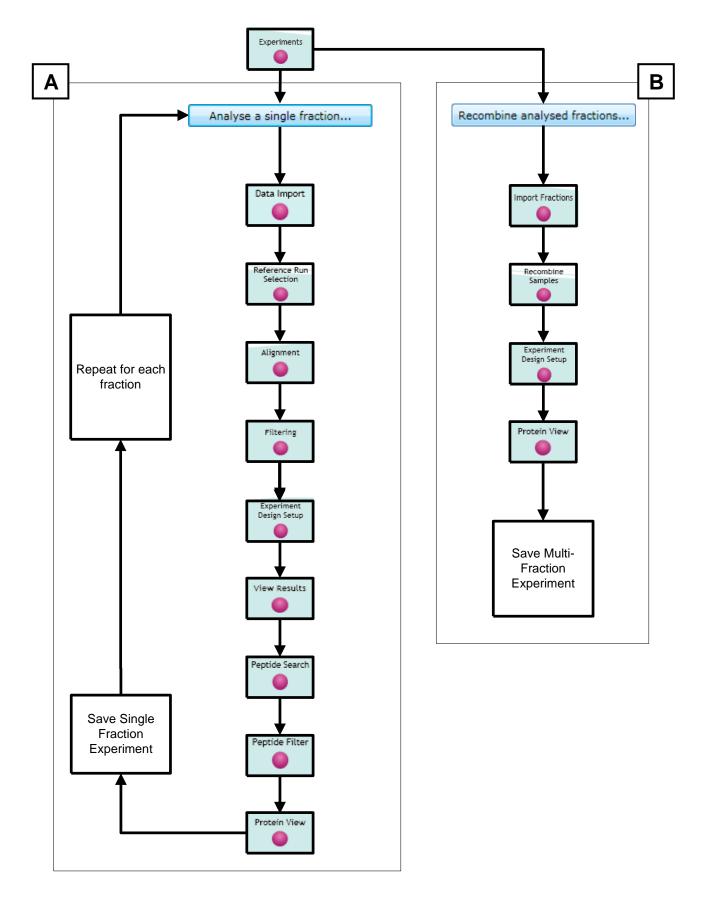
Stage	Description	Page
Data Import	<b>LC-MS Data Import:</b> Selection and review of data files for single fraction analysis.	7
Reference Run Selection	Reference Run Selection: Select run to align to.	7
Alignment	Alignment: automatic and manual run alignment	8
Filtering	<b>Filtering</b> : defining filters for features based on Retention Time, m/z , Charge and Number of Isotopes.	10
Experiment Design Setup	<b>Experiment Design Setup</b> : defining one or more group set ups for analysed aligned runs	11
View Results	<b>View Results</b> : review and validate results, edit feature detection, tag groups of features and select features for further analysis	12
Peptide Search	<b>Peptide Search</b> : managing export of MS/MS spectra to, and import of peptide ids from Peptide Search engines	13
Peptide Filter	Peptide Filter: manage peptide ids and filters	14
Protein View	<b>Protein View</b> : validation and resolution of peptide id conflicts for data entered from Database Search engines	15
Import Fractions	Import fractions: import multiple analysed fractionated experiments	19
Recombine Samples	Recombine samples: regenerate samples from fractions	21
Experiment Design Setup	Experiment design Setup: define original experimental design	23
Protein View	Protein View: review protein and pentide identity	24

Protein View: review protein and peptide identity

24

# **Overview of a typical Fractionation Analysis workflow**

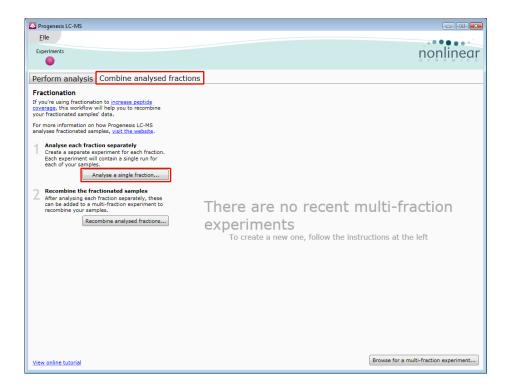
The workflow outlines the steps performed in the analysis of a typical fractionated experiment. In this example there were 6 fractions hence the main analysis workflow was performed 6 times. To analyse the data, select the **Combine analysed fractions** tab then (A) Analyse a single fraction then (B) 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 (**Fraction\_1**) then adjust the Data and Machine types accordingly and set the Experiment folder as required .

🔼 Create New Experiment 📃	٢.
Create a new label-free experiment named:	
Fraction_1	
Data type	
Profile data	
Centroided data	
Resolution (full width at half maximum) 5000	
Machine type	
High resolution mass spectrometer 🔹	
Experiment folder	
Save experiment in the same folder as the run data	
Choose an experiment folder	
Browse	
	5
Create experiment Cancel	

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

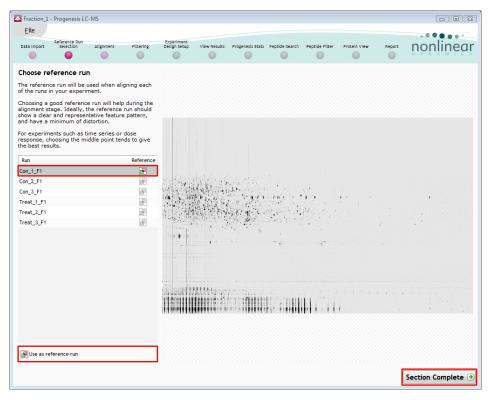
All LC-MS runs for the first Fraction are loaded into a new LC-MS experiment.

Kenter Stratter - Progenesis LC-MS		
Eile		
Reference Run Data Import Selection Alignment	Experiment Filtering Design Setup View Results Progenesis Stats Peptide Search Peptide Filter Protein View	Report nonlinear
• • •		
Import Data	Data processing methods:	
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About this data format	Peak processing method: Profile data	
Include?	No problems found	<u>^</u>
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Con_1_F1 V X		E .
Con_2_F1		
Con_3_F1		
Treat_1_F1 Pending		
Treat_2_F1 Pending Treat_3_F1 Pending	a Maria da Arra	
		-
	No problems found	
	The data file was imported with no problems.	
	The data appears to be in the correct format to be analysed by this software.	
✓ Include run in analysis ★ Don't include run in analysis		
B Exclude areas from selected run		Section Complete $ i$

Once all the files have been imported move to the next stage in the workflow by clicking Section Complete.

### Stage 2: Reference Run selection

This stage in the analysis workflow allows you to review and select the most appropriate Reference LC-MS run to align all the other runs to.

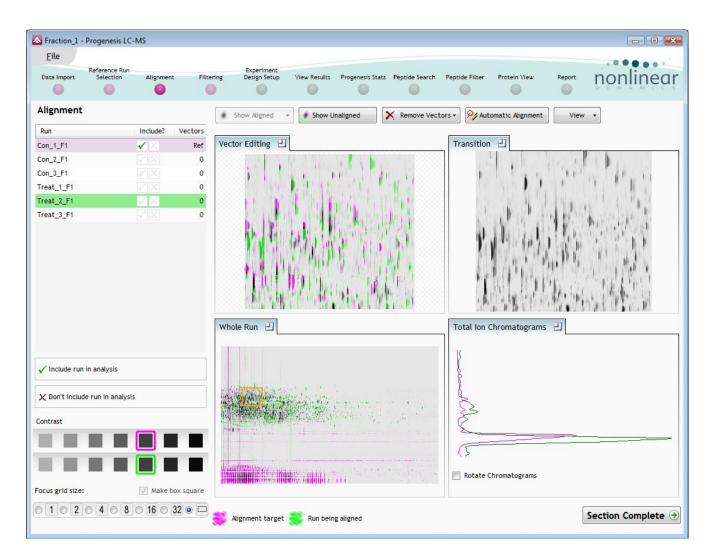


To select a Reference run either click on the run in the list and then click **Use as reference run** or double click on the run in the list.

Now move to the next stage in the workflow by clicking Section Complete.

# Stage 3: Alignment

At this stage Progenesis LC-MS Alignment opens displaying your data.



#### Generation of alignment vectors

The alignment of LC-MS runs is required in the LC (retention time) direction, this is key to correcting for the variable elution of peptides during the chromatographic separation.

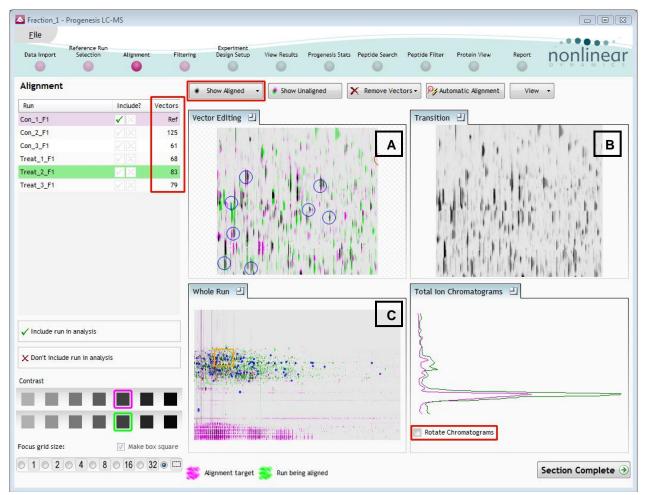
The alignment vectors are generated automatically for all the LC-MS runs by using the 'Automatic vector wizard' accessed by clicking on **Automatic Alignment** on the top tool bar, and making sure all the runs are selected before clicking OK.

ide Search	Peptide Filter	Protein V
s 🕶 🎯 AL	utomatic Alignmen	t Vi
	Transition	n 🕘

### **Reviewing generation of alignment vectors**

After applying **Automatic alignment** the number of vectors 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 should show minimal distortion, Window B (Transition) will show features pulsing slightly but not moving up and down.



At this point, you should check the automatically placed (blue) vectors. This will be easier with a larger grid size. Make sure the grid size is set to 4 using the **'Focus grid size'** control at the bottom left of the window.

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 27) and also to the main LC-MS analysis Userguide.

# Stage 4: 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.



#### **Detection Parameters**

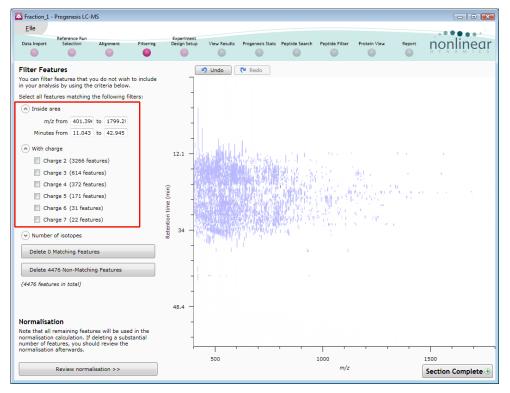
The Detection Parameters dialog, showing all the runs in the current experiment and a tick against each run, opens. This is the default setting, where the feature detection algorithm uses information from all of the runs to contribute to the pattern of feature outlines.

Detection Parameters		Detection Parameters	
Runs for detection Detection limits		Runs for detection Detection limits	
Choose runs for detection		Sensitivity	
control which will be used by the feature detection algorithm. Although any run which is left un-ticked will not affect the detected feature outlines, it will still have outlines added to it and will be available in the experiment design setup.	<pre>   Con_1_F1   Con_2_F1   Con_3_F1   Treat_1_F1   Treat_2_F1   Treat_3_F1 </pre>	You can adjust the sensitivity of the detection algorithm using these different methods. Each sensitivity method examines the intensities of groups of MS peaks to judge if they are likely to form part of a peptide ion or whether they represent noise and so should be ignored. Peaks which are rejected as noise will not be used to build peptide ion outlines.	Automatic     Absolute ion intensity     % Base Peak  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 will be detected. fewer default more     3
		Minimum retention time wind	iow
		The retention time window is the period over which a peptide has eluted. If you set a retention time window limit, any peptide which has eluted over a shorter period will be rejected.	Apply a retention time window limit      RT window limit:     0
	Detect Cancel		Detect Cancel

For the runs in this example the default settings for the Automatic method were applied.

Press Detect to start the detection process.

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 shows the removal of features with a charge state of **less than 2 and greater than 7** and outside a defined retention time window

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

### Stage 5: 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 32)

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.

Fraction_1 - Progenesis LC-MS											• 🛛
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Which experiment design type	e do you	u want to use fo	r this exp	eriment?							
<b>Between-subject</b> De	-			0-0 0-0 0-0	Within-su	ıbject Desi	gn				•
Do samples from a given subject appear in only one condition? Then use the between-subject design.	A	Create a new	experim	nent desig	n	<b>•</b> ו		Before	During	After	
To set up this design, you simply group the runs according to the condition (factor level)	c	Name: Control		-		Patient X	X1	X2	X3		
of the samples. The ANOVA calculation assumes that the conditions are independent and therefore gives a statistical test		○ Copy layout fr	rom:			- -	Patient Y	Y1	¥2	Y3	
of whether the means of the conditions are all equal.	Add con-				ate design	Cancel	Patient Z	Z1	Z2	Z3	
				the so condit belon	ftware not on tion (factor le gs to but also tt it came from	lý which vel) each run which m. The					

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

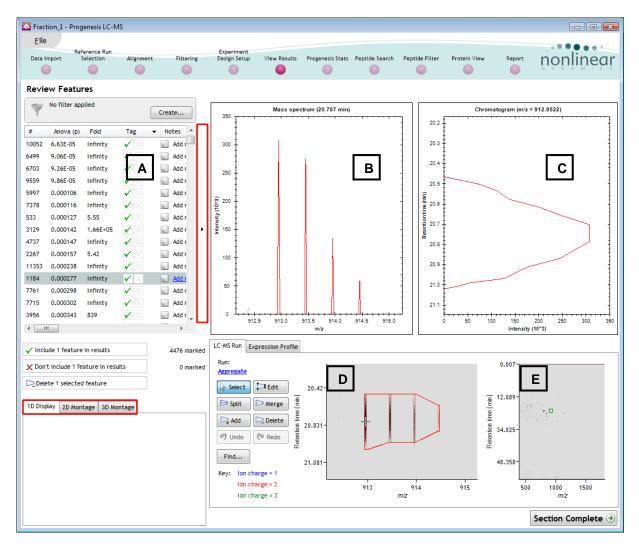
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Setup conditions			Runs Add	Selected Runs	to Condition	•				
Setup the conditions the (e.g., control, drug A, e your samples to the co	at you want to co etc), and then ass rrect condition.	mpare below ign each of	A	dd to new con	dition	Trea	t_2_F1		Treat	_3_F1
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	Con_2_	F1 <u>Remove</u>								
	Con_3_	F1 <u>Remove</u>								
Add condition										
									Se	ection Complete 🧿

Click Section Complete to move to View Results.

## Stage 6: 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.

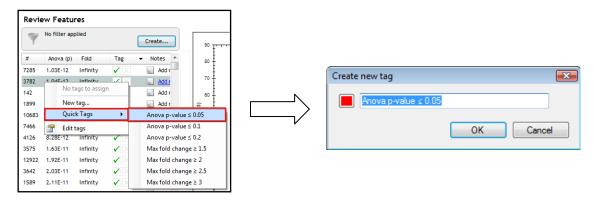
The review stage has 4 display modes: 1D, 2D, 3D and Feature Details controlled by the tabs on the bottom left of the display and the expander bar to the right of the table. Each display has multiple views to allow comparative exploration of the detected features on the aligned LC-MS runs.



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

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 =<0.05 and accept the name



Now move to the **Peptide Search** stage in the workflow using the icon on the workflow.

## Stage 7: Peptide Search

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.

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Data	Import	Reference Run Selection	Alignm	ent Filter	ing	Experiment Design Setup	Vi	iew Resul	ts Prog	enesis Stat	Peptide Sear	ch Peptide Filt	er Protein View	Repo	ort I	nonlin	oar
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3849	17	0	0								📕 Anova p	-value ≤ 0.05 (7	'61 features)				
Data Import       Selection       Alignment       Filtering       Design Setup       View Results       Progenesis Stats       Peptide Search       Report       Difference         Peptide Search Bun ms/ms ion searches by exporting peak list files of the import debuk in to link protein fibs to detected peaks.       Image: Search results files may then import debuk in to link protein fibs to detected peaks.       Image: Search results files may then import debuk in to link protein fibs to detected peaks.       Image: Search results files may then import debuk in to link protein fibs to detected peaks.       Image: Search results files may then import debuk in to link protein fibs to detected peaks.       Image: Search results files may then import debuk in to link protein fibs to detected peaks.       Image: Search results files may then import debuk in to link protein fibs to detected peaks.       Image: Search results files may then import debuk in to link protein fibs to detected peaks.       Image: Search results files may then import debuk in to link protein fibs to detected peaks.       Image: Search results files may then import debuk in to link protein fibs to detected peaks.       Image: Search results files may then import debuk in to link protein fibs to detected peaks.       Image: Search results files may then import debuk in the files may then import deb																	
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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

Performing the search Select the search program you're using:
Mascot 👻
Help
Export 0 ms/ms spectra
Import search results
MSMS Preprocessing
Limit fragment ion count
$\overrightarrow{\mathbb{V}}$ Deisotoping and charge deconvolution

On importing the Search results the Features table updates to reflect the identified proteins and the relevant score for each searched feature.

Fraction_3 - P	rogenesis LC-MS																-	
Eile																		
	Reference Run					Experime												•••
Data Import	Selection	Alignn	nent	Filte	ering	Design Set	tup V	/iew l	Results Prop	enesis Stats	Peptide Search	Peptide Filte	r Protein View		port	r	າonl	inec
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eatures:									greater triair	•			,	less tildi	•			
Tag filter features	applied may be hidden			Edit	•		Feature	e ID	less than	•		I	Precursor intensity	less than	•			
# MS/MS	<ul> <li>Proteins</li> </ul>	Score	Tags	- I	Not 🔺		Cha	rge	less than	•		Prec	cursor intensity (%)	less than	•			
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4572 24	6 gi 706104	5.35	$\checkmark$ ×		1		Expor	ted	equal to	•	•		Peptide sequence	contains	•			
3849 17	3 gi 454546	13	$\checkmark$ ×		4													
2358 15	0	D	$\checkmark$ $\times$		1		Isoto	ope	less than	•			Protein accession	contains	•			
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276 10	4 gi 705237	53.4	$\checkmark$ $\times$		1	<b>V</b>	3	39	Con_3_F	3 1867	No	8.6e+006	9.9e+005	11.4	2	635.7900	1	95.3
145 10	5 gi 648416	87.5	$\checkmark$ $\times$		4	<b>V</b>	4	39	Con_1_F	3 1987	No	1.3e+007	1.4e+006	10.5	2	635.7800	1	68.2
325 10	4 gi 177878	1.57	$\checkmark$ $\times$		1	<b>V</b>	5	39	Treat_1_	3 2080	No	1.7e+007	1.6e+006	9.2	2	635.7900	1	87.2
9 9	2 gi 649287	95.3	$\checkmark$ $\times$		4	<b>√</b>	6	39	Treat_3_	3 2349	No	7.9e+007	6.6e+006	8.4	2	635.7900	1	91.5
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MSMS Preproce	ssing					20.783	<sup>3</sup> -L					r de	. I <b>I</b> . I . I . I . I . I . I . I . I . I .		1.0	1		
Limit fragm	ent ion count 10	00	×				635	636		639	0-		500	CID ALKALAN K	- Abdala -	1000		
Deisoto	ping and charge de	convoluti	on						m/z					m/z				

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

### **Stage 8: Peptide Filter**

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.

Fraction_3 - Pro																		- 0
	Reference	Run				Exp	eriment											
Data Import	Selectio		Alignment	Filte	ering		gn Setup	View Re	sults	Progenesi	s Stats	Peptide Sea	rch Peptide F	ilter	Protein View	Report		nonline
													•					
eatures				Pept	ide Se	arch l	Results											
# Total Hits	m/z	RT(mins	Charge 🔺	Batch	n deletio	n option	s											
3011 1	489.21	13.44	2		1	Score	less than	•	30				c	harge	less than	•		
3006 3	540.89	47.97	2		L		tess than							-	topo chan			
2967 1	404.23	47.15	2			Hits	less than	•					Seg	uence	contains	•		
2931 1	452.83	48.03	2				tess than								contains			
2928 1	843.44	34.67 44.87	4			Mass	less than	•					Aco	ession	contains	•		
2910 1 2888 2	617.37 761.89	44.87	1				tess than								contains			
2000 2	481.73	12.43	2	Ma	ass error	(ppm)	less than	•			_		Descr	ription	contains	•		
2871 4	754.30	13.10	2				isso main	•										
2843 4	805.42	33.57	2			M/Z	less than	•					Modifi	cation	contains	•		
2842 2	584.91	47.92	2				Cos undit	•							contains			
2824 1	1084.10	53.23	1	F	Retentior	Time	less than	•										
2812 1	821.40	33.28	2				tess triair						_					
2794 1	834.87	36.62	2	Se	quence l	ength	less than	•					6	Delete i	matching search res	ults Delete r	non-matching search re	sults Clear all filter
2781 1	889.39	13.44	1				tess tildir											
2764 2	435.56	32.69	3															
2680 4	1075.00	52.64	1		#	Score	Hits	m/z		s) Charge	Mass	Mass erro	Sequence	-	Accession	Modifications		
2652 2	785.89	45.30 33.34	2		13011	17.28	1	489.21	13.44	2	976.40	2.21	GQSEDFPVK	-	gi 61791817			Clostridium difficile Q
2628 1 2599 1	597.81 497.19	33.34 15.08	2	<b>V</b> 1	3006	5.89	1	540.89	47.97	2	1079.76	164.11	TQSMHKMHC			] Oxidation (M)	cell surface protein	Clostridium difficile 63
2599 1	918.96	33.16	2	V 1	3006	5.89	1	540.89	47.97	2	1079.76	164.11	🕥 тозмнкйно	ск 🌍	gi 58272948 [7	Oxidation (M)	putative di-trans,pol	y-cis-decaprenylcistrar
2560 4	496.86	48.09	2	<b>V</b> 1	3006	1.59	1	Delete 54	63 searc	h results?	?					- 23	oligopeptide ABC tra	nsporter, substrate-bir
2511 1	538.82	32.86	2	V 1	2967	1.10	1										RecName: Full=Endo	glucanase D; AltName:
2503 1	839.89	40.62	2	<b>V</b> 1	2931	1.18	1	(?)	Are yo	u sure yo	u want	o permaner	ntly delete 5463	peptid	le search results		nitroreductase-famil	protein [Clostridium
2497 3	795.39	34.91	2	V 1	2928	1.70	1	-									hypothetical protein	CD2964 [Clostridium d
2494 3	631.37	49.86	1		2910	8.04	1											n L27 [Clostridium diff
2467 1	682.85	45.12	2		2888	5.21	1						_	_				CLOSTASPAR 00821 [C
2465 2	685.84	36.54	2		2888	52.02	1							Yes	No			ANASTE_02219 [Anaer
425 1	474.85	48.03	2					101.75	10.15	-		04.07	CTTEROT	0				
2404 2	542.25	13.70	2		2874	10.02	1	481.73	12.43	2	961.44	36.97	GTTFEGITK	-	gi 04439756			CD3667 [Clostridium d
2364 2	457.53	13.84	3		2874	10.02	1	481.73	12.43	2	961.44	36.97	GTTFEGITK	-	gi 51703556			lium phytofermentans
2328 1 2313 1	415.70 558.23	13.38 16.10	2		2874	10.02	1	481.73	12.43	2	961.44	36.97	GTTFEGITK	-	gi 70610755			trate-binding protein (
277 4	658.23 658.36	16.10 34.61	3	<b>V</b> 1	2871	8.77	1	754.30	13.10	2	1506.58	113.34	FHEYHVVEG	AE 🌍	gi 04388269		hypothetical protein	CLOBOL_01554 [Clostr
263 2	694.91	50.21	2	<b>V</b> 1	2871	12.10	1	754.30	13.10	2	1506.58	141.88	WEEIHQCKSI	INI 🌍	gi 70610538		ATP synthase F1 sect	or subunit beta [Lacto
2251 1	579.35	34.99	3	<b>V</b> 1	2871	8.77	1	754.30	13.10	2	1506.58	113.34	FHEYHVVEG	AE 🌍	gi 94534676		protein of unknown f	unction DUF342 [Clost
2237 3	466.72	12.60	2	V 1	2871	12.10	1	754.30	13.10	2	1506.58	141.88	WEEIHQCKSI	INI 🌍	gi 17787522		rubrerythrin [Clostric	fium difficile 630]
2187 1	741.41	34.38	2		2843	2.79	1	805.42	33.57	2	1608.83	29.66	YASALIPDQY	'FI 🙆	gi 70610763			a subunit (Subdoligran
2180 2	808.74	51.94	1		2843	2.79	1	805.42	33.57	2		29.66	S YASALIPDQY	-	gi 70610762			n S3 (Clostridium diffic
2166 5	716.40	34.33	2	<				5051.12		-								) and a second second
2072 1	479,72	15.53	2	7400.0				g batch del	-									

**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 in the previous page.

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

### **Stage 9: Protein View**

The Protein View combines the quantitative LC-MS data with the qualitative MS/MS results at the protein level, highlighting proteins of interest between experimental groups. This stage allows you to examine the behaviour of the identified peptides and resolve any conflicts for the various peptide assignments at the protein level.

The Protein 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 Protein View 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	P	No f	filter applied	Create		Pept	tides	of gi 5	66893	7					No filter ap	oplied	Create
Accession	Peptide	s Conflic	cts Score	Anova (p)*	A		#	Score	Hits	Mass	Mass error (p	. RT (mins)	Charg	e Tags	✓ Abundance	Confli	cts Peptide !
🎯 gi   5668937 🛛 😐	12	14	1.41E+03	2.03E-05 3		<b>V</b>	446	93.3	10	1676.838	1.2	34.5	2	$\checkmark$ ×	7.47E+05	1	🔮 IRD
🚳 gi 126697810	9	14	1.13E+03	4.55E-05 3		V	3335	84.7	4	1423.65	0.405	22.5	2	$\checkmark$ ×	7.39E+04	1	🔇 ртр
🕥 gi 209571234	24	13	2.4E+03	4.05E-05 2		$\checkmark$	147	101	10	1230.609	0.44	22.7	2	- 🗸 🛛	3.07E+06	1	🕥 AAD
🔮 gi 260682215	23	13	2.03E+03	6.28E-05		V	166	125	10	2317.115	0.168	38.7	1	B	5.55E+06	1	🕥 LES
🔮 gi 126698450	12	9	1.21E+03	4.05E-16		V	179	60.9	9	2317.115	0.201	38.7		Pv×	3.09E+06	1	🕥 LES
🗿 gi 126700407	9	9	1.04E+03	0.945 1		1	238	107	10	1716.857	0.429	30.4	2	<ul> <li>✓ ×</li> </ul>	1.73E+06	1	S VNT
🎯 gi   255656776	9	9	925	0.00888 1		V	564	51.2	4	1716.858	0.394	30.4	3	$\checkmark$ ×	3.57E+05	1	🔇 VNT
3 gi   255654924	7	9	645	8.66E-06 1	Ŧ		283	49.5	10	1676.838	1.32	34.5	3	<b>7</b> X	7.59E+05	1	S TRD
Peptide Views Prote	teins fo	or feat	<b>C</b> e <sup>46</sup>		Pe			gi 126								D	
	Peptides		te Protein Score	Peptid		#						RT (mins)	Charge	5	<ul> <li>Abundance</li> </ul>	Conflicts	Peptide Set ^
🔮 gi   5668937 🧕	12	14	1.41E+03	93.3	<b>V</b>			101		1230.609	0.44	22.7	2	✓×	3.07E+06	1	S AADDI
🎯 gi 126697810	9	14	1.13E+03	✓ 93.3	V			125		2317.115	0.168	38.7		<ul> <li>✓ X</li> <li>✓ X</li> </ul>	5.55E+06	1	S LEST
					✓ ✓	17		60.9 107		2317.115 1716.857	0.201	38.7 30.4	3 2	✓× ✓×	3.09E+06 1.73E+06	1	S LEST
					v V	 56		51.2		1716.858	0.429	30.4	2	<ul> <li>✓ ×</li> <li>✓ ×</li> <li>✓ ×</li> </ul>	3.57E+05	1	S VNTN
					<ul> <li>✓</li> </ul>	28		49.5		1676.838	1.32	34.5	3	VA.	7.59E+05	1	VNTN
					v V			49.5 93.3		1676.838	1.2	34.5	2	<ul> <li>✓ ×</li> <li>✓ ×</li> </ul>	7.59E+05	1	S IRDTI
					▼ ▼			49.6		1692.835	0.206	20.6	3	v A VX	5.56E+05	1	S IRDII
					V			103		1692.833	1.09	20.6	2	× ∧ √X	4.62E+05	1	S IRDII
					V			104		1700.863	0.139	36		• ∡⊠	5.46E+05	1	S VNTN
					1			42.3		1700.863	0.0934	36	3	· ⊡ √X	1.03E+05	1	S VNTN
					V			99		1407.657	1	38.3	2	· ∩ √X	1.29E+05	1	S DTDV
					110				-	1007 74	0.024		-		4.545.04		· · · · · ·
۰ II				+	•		-	_			III						•
Protein options																Section	Complete

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.

Kan a second sec	×
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.	
<ul> <li>Group similar proteins</li> <li>No protein grouping</li> </ul>	
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.	
<ul> <li>Quantitate from non-conflicting features</li> <li>Quantitate from all features</li> </ul>	
OK Cance	:

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.

	Y	No filt	er applied	Create	•	тср	tides of	gi120009	57					No filter ap	plied	Create
Accession	Peptides	Conflict	Score	Anova (p)*	•		# Sc	ore Hi	ts Mass	Mass error (p	RT (mins)	Charge	Tags	<ul> <li>Abundance</li> </ul>	Confl	cts Peptid
🔰 gi   5668937	12 (3)	14	1.41E+03	4.15E-06	3		446	93.3 1	0 1676.838	1.2	34.5	2	✓ ×	7.47E+05	1	🕥 IR
gi 126697810	9 (0)	14	1.13E+03		1	<b>V</b>	3335	84.7	4 1423.65	0.405	22.5	2	VX	7.39E+04	1	S DT
🔰 gi   209571234	24 (12)	13	2.4E+03	2.51E-07	é	V	147	101 1	0 1230.609	0.44	22.7	2	<ul> <li>X</li> </ul>	3.07E+06	1	🕥 AA
gi 260682215	23 (11)	13	2.03E+03	4.08E-05	3	<b>V</b>	166	125 1	0 2317.115	0.168	38.7	2	<ul> <li>X</li> </ul>	5.55E+06	1	🌖 LE
🔰 gi   126698450	12 (5)	9	1.21E+03	1.28E-06	2	1	179	60.9	9 2317.115	0.201	38.7	3	VX	3.09E+06	1	🌖 LE
👂 gi   126700407	9 (2)	9	1.04E+03	0.000764	3 +	1	238	107 1	0 1716.857	0.429	30.4	2	VX	1.73E+06	1	S vn
(					Þ	•				III						
Protein: gi 120	6697810 ein Resolutio	flagel	in subunit [					1266978	310							
Protein: gi 120	6697810 ein Resolutio Dteins for	flagel n featur	in subunit [		P			1266978 Hits		Mass error (p F	₹T (mins)	Charge 1	Tags	<ul> <li>Abundance</li> </ul>	Conflicts	Peptide Se
Conflicting pro	6697810 ein Resolutio Dteins for Peptides (	flagel n featur	in subunit [ e 446	Clostridiun	F	eptio	les of gi Score	Hits		Mass error (p F 0.44	RT (mins) 22.7	-	Tags	Abundance     3.07E+06	Conflicts	Peptide Se
Protein: gi 120 ptide Views Prot Conflicting prot Accession gi 15668937	6697810 ein Resolutio Dteins for Peptides (	flagel n featur Conflicts	e 446 Protein Score	Clostridiun	F id	Peptic # ₹ 14 ₹ 16	des of gi Score 47 10	Hits 10	Mass I			2	-		Conflicts 1 1	
Protein: gi 120 ptide Views Prot Conflicting prot Accession gi 15668937	6697810 ein Resolutio oteins for Peptides ( 12 (3)	flagel n featur Conflicts	e 446 Protein Score 1.41E+03	Pepti	F id	Peptio # ▼ 14	des of gi Score 47 10 56 12	Hits 10 10	Mass /	0.44	22.7	2 2 3		3.07E+06	Conflicts 1 1 1	S AADD
Protein: gi 120 ptide Views Prot Conflicting prot Accession gi 15668937	6697810 ein Resolutio oteins for Peptides ( 12 (3)	flagel n featur Conflicts	e 446 Protein Score 1.41E+03	Pepti	id I	Peptic # 7 14 7 16 7 17 7 23	des of gi Score 47 10 56 12 79 60.	Hits 10 10 9 9	Mass 1230.609 2317.115	0.44	22.7 38.7	2 2 3		3.07E+06 5.55E+06	Conflicts 1 1 1 1	<ul> <li>AADD</li> <li>LEST</li> </ul>
Protein: gi 120 ptide Views Prot Conflicting prot Accession gi 15668937	6697810 ein Resolutio oteins for Peptides ( 12 (3)	flagel n featur Conflicts	e 446 Protein Score 1.41E+03	Pepti	id C	Peptic # 7 14 7 16 7 17 7 23 7 56	des of gi Score 47 10 56 12 79 60. 38 10	Hits 10 10 9 9 10	Mass / 1230.609 2317.115 2317.115	0.44 0.168 0.201	22.7 38.7 38.7	2 v 2 v 3 v 2		3.07E+06 5.55E+06 3.09E+06	Conflicts 1 1 1 1 1 1	<ul> <li>AADD</li> <li>LEST</li> <li>LEST</li> </ul>
Protein: gi 120 ptide Views Prot Conflicting prot Accession gi 15668937	6697810 ein Resolutio oteins for Peptides ( 12 (3)	flagel n featur Conflicts	e 446 Protein Score 1.41E+03	Pepti	id I I I I I I I I I I I I I I I I I I I	Peptic # 7 14 7 16 7 17 7 23 7 56 7 28	des of gi Score 47 10' 56 12! 79 60. 38 10' 54 51. 33 49.	Hits 10 10 9 9 7 10 2 4 5 10	Mass // 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 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	Conflicts 1 1 1 1 1 1 1	<ul> <li>AADD</li> <li>LEST</li> <li>LEST</li> <li>VNTN</li> </ul>
Protein: gi 120 ptide Views Prot Conflicting prot Accession gi 15668937	6697810 ein Resolutio oteins for Peptides ( 12 (3)	flagel n featur Conflicts	e 446 Protein Score 1.41E+03	Pepti	id C C	Peptic # 2 14 2 16 2 17 2 23 2 56 2 24 2 44	des of gi Score 47 10' 56 12! 79 60. 38 10' 54 51. 33 49.	Hits 10 10 9 9 7 10 2 4 5 10	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 34.5 34.5	2 2 3 2 3 3 3 2 2 3 2		3.07E+06 5.55E+06 3.09E+06 1.73E+06 3.57E+05	Conflicts 1 1 1 1 1 1 1 1 1	<ul> <li>AADD</li> <li>LEST</li> <li>LEST</li> <li>VNTN</li> <li>VNTN</li> </ul>
Protein: gi 120 eptide Views Prot Conflicting prot Accession	6697810 ein Resolutio oteins for Peptides ( 12 (3)	flagel n featur Conflicts	e 446 Protein Score 1.41E+03	Pepti	id I I I I I I I I I I I I I I I I I I I	Peptic # V 14 V 16 V 17 V 22 V 24 V 24 V 44 V 44	des of gi Score 47 10 56 122 79 60. 38 100 54 51. 33 49. 46 93. 31 49.	Hits 10 10 9 9 10 2 4 5 10 8 10 5 8	Mass         J           1230.609         2317.115           2317.115         1716.857           1716.857         1676.838           1676.838         1676.838           1692.835         1692.835	0.44 0.168 0.201 0.429 0.394 1.32 1.2 0.206	22.7 38.7 38.7 30.4 30.4 34.5 34.5 20.6	2 2 3 2 3 3 2 3 3 2 3 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	Conflicts 1 1 1 1 1 1 1 1 1 1 1	<ul> <li>AADD</li> <li>LEST</li> <li>LEST</li> <li>VNTN</li> <li>VNTN</li> <li>IRDT</li> </ul>
Protein: gi 120 ptide Views Prot Conflicting prot Accession gi 15668937	6697810 ein Resolutio oteins for Peptides ( 12 (3)	flagel n featur Conflicts	e 446 Protein Score 1.41E+03	Pepti		Peptic # 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2	des of gi Score 47 10 56 122 79 60. 38 10 54 51. 33 49. 46 93. 31 49. 39 10	Hits 10 10 9 9 10 2 4 5 10 3 10 5 8 10 5 8 10	Mass         J           1230.609         2317.115           2317.115         1716.857           1716.858         1676.838           1676.838         1692.835           1692.833         1692.833	0.44 0.168 0.201 0.429 0.394 1.32 1.2 0.206 1.09	22.7 38.7 38.7 30.4 30.4 34.5 34.5 20.6 20.6	2 2 3 2 3 2 3 3 2 2 3 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	Conflicts 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	<ul> <li>AADD</li> <li>LEST</li> <li>LEST</li> <li>VNTN</li> <li>VNTN</li> <li>IRDT</li> <li>IRDT</li> </ul>
Protein: gil12 ptide Views Prot Conflicting prot Accession ≩ gil5668937 ● ≩ gil126697810	6697810 ein Resolutio oteins for Peptides ( 12 (3)	flagel n featur Conflicts	e 446 Protein Score 1.41E+03	Clostridiun		Peptic # V 14 V 16 V 17 V 22 V 24 V 24 V 44 V 44	des of gi Score 47 10 56 122 79 60. 38 10 54 51. 33 49. 46 93. 31 49. 39 10	Hits 10 10 9 9 10 2 4 5 10 3 10 5 8 10 5 8 10	Mass         J           1230.609         2317.115           2317.115         1716.857           1716.857         1676.838           1676.838         1676.838           1692.835         1692.835	0.44 0.168 0.201 0.429 0.394 1.32 1.2 0.206	22.7 38.7 38.7 30.4 30.4 34.5 34.5 20.6	2 2 3 2 3 3 2 3 3 2 3 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	Conflicts 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	<ul> <li>AADD</li> <li>LEST</li> <li>LEST</li> <li>VNTN</li> <li>VNTN</li> <li>IRDT</li> <li>IRDT</li> <li>IRDT</li> </ul>

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.

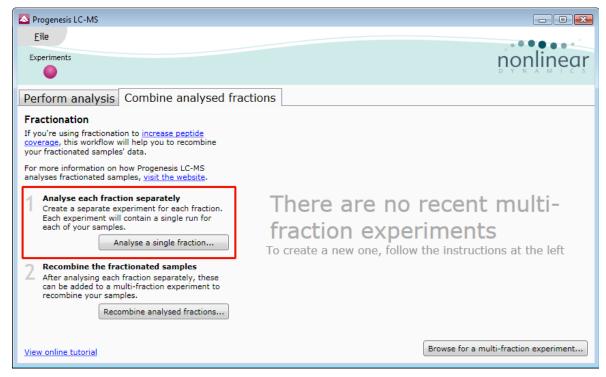
	- N	o filter app	lied		Рер	tides	of gi 56	568937					ĺ		No filter appli	ied	
	1			Create									l	1			Create
Accession	Peptides	Conflicts	Score	Anova (p 🔺		#	Score	Hits	Mass	Mass error (p	RT (mins)	Charge	Tags	- /	Abundance	Conflict	s Peptide
🕽 gi   126699063	6	0	368	2.03E-06		69	63.9	10	1669.889	0.0302	42.1	3	$\checkmark$ ×	4	.75E+06	0	🕥 IAI
gi 54781345	5	0	403	3.89E-05	V	132	103	10	1669.888	0.646	42.1	2	$\checkmark$ ×	4	.38E+06	0	IAI
gi 126700857	5	0	270	0.00443	$\checkmark$	147	101	10	1230.609	0.44	22.7	2	$\checkmark$ ×	3	.07E+06	0	🔇 AAI
gi 126698631	5	0	574	0.000151	$\checkmark$	166	125	10	2317.115	0.168	38.7	2	$\checkmark$ ×	5	.55E+06	0	🔇 LES
gi 5668937 (+1)	wij5669027		Clostridiur	o con on		• 79	60.9	9	2317.115	0.201	38.7	3	$\checkmark$ ×	3	.09E+06	0	🔇 LE:
gi 54781347				Clostridium dif	ficile 63	30] 8	107	10	1716.857	0.429	30.4	2	$\checkmark$ ×	1.	.73E+06	0	S VN:
gi 126698643	4	0	365	0.0132	V	564	51.2	4	1716.858	0.394	30.4	3	<b>√</b> ×	3	.57E+05	0	S VN:
gi1126701179	4	0	286	7 74F-06	7	783	49 5	10	1676 838	1 37	34 5	3	ZV	7	59F+05	0	🙆 тв
					Peptie	des of	f conflict	ting pr	otein								
eptide Views Protein Conflicting prote Accession Pe		licts Prote	ein Score	Peptid	Peptic #			ting pr		ass error (p R	T (mins)	Charge T	ags	✓ Abur	ndance	Conflicts F	Peptide Se
Conflicting prote	ins	licts Prote	ein Score		<u> </u>			5.		ass error (p R	T (mins)	Charge T	ags	✓ Abur	ndance	Conflicts F	Peptide Seq
Conflicting prote	ins	licts Prote	ein Score	Peptid	<u> </u>			5.		ass error (p R	T (mins)	Charge T	ags	✓ Abur	Idance	Conflicts F	Peptide Sec

**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.

Progenesis LC-MS Eile Experiments	nonlinear
Perform analysis Combine analysed fractions Recent experiments	Online content
<ul> <li>Date: Yesterday</li> <li>Fraction_4</li> <li>Fraction_5</li> <li>Fraction_6</li> <li>Fraction_3</li> <li>Fraction_2</li> <li>Fraction_1</li> <li>LC-MS Tutorial</li> <li>Date: Thursday</li> <li>My New data set</li> </ul>	Open •   • Frequently asked questions
Other experiments           Image: New         Image: Browse           View online tutorial         Image: State Stat	3.0.3958.22939 check for updates

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

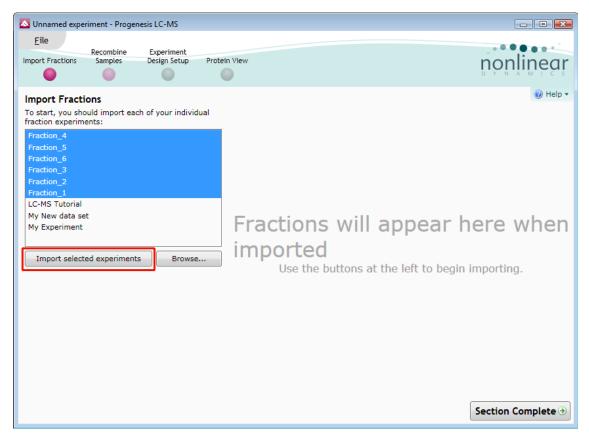
The combining of the single fraction experiments is performed in the second stage of the Fractionation workflow.

#### Select Recombine analysed fractions...

Service Progenesis LC-MS	
<u>F</u> ile	
Experiments	nonlinear
Perform analysis Combine analysed fracti	ons
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.         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	There are no recent multi- fraction experiments To create a new one, follow the instructions at the left
2 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 <u>View online tutorial</u>	Browse for a multi-fraction experiment

## **Stage 1 Import Fractions**

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



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

Unnamed experiment - Progenesis LC-MS			
Eile Recombine Experiment Import Fractions Samples Design Setup Pr	otein View		nonlinear
Import Fractions	To obt	ain the correct peptide distribution, put the fractions in order:	🔸 🛧 🔞 Help 👻
To start, you should import each of your individual fraction experiments: Fraction_4 Fraction_5 Fraction_6 Fraction_3 Fraction_2 Fraction_1 LC-MS Tutorial My New data set My Experiment	1 2 3 4	Fraction 4 × 454 peptides identified in 6 runs Normalised: Yes Fraction_5 × 320 peptides identified in 6 runs Normalised: Yes Fraction_6 × 146 peptides identified in 6 runs Normalised: Yes	Last saved: 02/11/2010 Last saved: 02/11/2010 Last saved: 02/11/2010
Import selected experiments Browse Peptides per fraction	5	Fraction_2 × 318 peptides identified in 6 runs Normalised: Yes	Last saved: 02/11/2010
500- 400- 99300- 200- 100- 0-	6	Fraction_1 × 20 peptides identified in 6 runs Normalised: Yes	Last saved: 02/11/2010
1 2 3 4 5 6 Fraction			Section Complete →

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			
Eile Import Fractions Samples Design Setup Protein	View		nonlinear
Import Fractions To start, you should import each of your individual fraction experiments: Fraction_4 Fraction_5 Fraction_6 Fraction_2 Fraction_1 LC-MS Tutorial My New data set My Experiment Import selected experiments Browse Peptides per fraction 500- 400- 400- 100-	1 2 3 6 4 5	Fraction_2 × 318 peptides identified in 6 runs Normalised: Yes Fraction_4 × Science the identified in 6 runs Fraction_3 × 368 peptides identified in 6 runs Normalised: Yes La Fraction_5 × 320 peptides identified in 6 runs Normalised: Yes La	3: () () Help () st saved: 02/11/2010 st saved: 02/11/2010 st saved: 02/11/2010 st saved: 02/11/2010 st saved: 02/11/2010
0+ 1 2 3 4 5 6 Fraction		S	ection 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 experiment - Progenesis LC-MS								- • •
Eile Import Fractions Samples Design Setup Prote	in View						non	linear
Recombine Samples	Find ru	ns containing: 🔎						🕜 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 🔻	▼ Create sample ▼	
<ol> <li>Select the run from each fraction that represents the same sample</li> <li>Click the button below</li> <li>Repeat for each sample in your experiments</li> </ol>	1			S.C.				
✓ Group the runs to create a sample		Con_1_F1	Con_2_F1	Con_3_F1	Treat_1_F1	Treat_2_F1	Treat_3_F1	
í	2							
		Con_1_F2	Con_2_F2	Con_3_F2	Treat_1_F2	Treat_2_F2	Treat_3_F2	
	3		$\mathbb{Q}(\mathbb{R})$					
		Con_1_F3	Con_2_F3	Con_3_F3	Treat_1_F3	Treat_2_F3	Treat_3_F3	
	4		$\mathbb{S}[\mathbb{R}]$					
		Con_1_F4	Con_2_F4	Con_3_F4	Treat_1_F4	Treat_2_F4	Treat_3_F4	
	5							
		Con_1_F5	Con_2_F5	Con_3_F5	Treat_1_F5	Treat_2_F5	Treat_3_F5	
	6							
		Con_1_F6	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 🤊

Typing **Con\_1** in the **Find runs containing** search box will locate the runs corresponding to sample Con\_1.

Unnamed experiment - Progenesis LC-MS		- • •
Eile Recombine Experiment Import Fractions Samples Design Setup Prot	sin View	nonlinear
Recombine Samples This stape allows you to recombine your samples' separate fractions. To group your runs into samples: . Select the run from each fraction that groups and the sample in your experiments . Click the button below . Click the button below . Click the button below . Group the runs to create a sample of group the runs to create a sample . Coupt the runs to create a sample . Click the button below . Cli	Find runs containing:     Con_1     Con_1_F1     Con_1_F2     Con_1_F3     Con_1_F3     Con_1_F3     Con_1_F5     Con_1_F6	i Help ∙
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							- • •
Eile Recombine Experiment Import Fractions Samples Design Setup Prot	ein View						nonlinear
Recombine Samples	Find ru	ıns containing: 🎾					🕜 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 ▼	
<ol> <li>Select the run from each fraction that represents the same sample</li> <li>Click the button below</li> <li>Repeat for each sample in your experiments</li> </ol>	1						
✓ Group the runs to create a sample		Con_2_F1	Con_3_F1	Treat_1_F1	Treat_2_F1	Treat_3_F1	
Con_1  × * * Con_1_F1 * Con_1_F2	2		Sec.				
• 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	M.				SPL.	
		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	S.C.					
-		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 ④

Repeat for the remaining samples.

Unnamed experiment - Progenesis LC-MS		
<u>F</u> ile		
Recombine Experime Import Fractions Samples Design Set		
import Practions Samples Design Sec	up Protein view	nonlinear
Recombine Samples		🕖 Help 🔻
This stage allows you to recombine your sa	amples'	
separate fractions. To group your runs into		
<ol> <li>Select the run from each fraction tha represents the same sample</li> </ol>	Jt.	
2. Click the button below		
<ol><li>Repeat for each sample in your expe</li></ol>	riments	
Group the runs to create	e a sample	
Con 3	× *	
• Con_3_F1		
<ul> <li>Con_3_F2</li> </ul>		
<ul> <li>Con_3_F3</li> <li>Con_3_F4</li> </ul>		
<ul> <li>Con_3_F4</li> <li>Con_3_F5</li> </ul>		
• Con_3_F6		
Treat_1	×	All runs have been grouped into comples
Treat_1_F1		All runs have been grouped into samples
<ul> <li>Treat_1_F2</li> </ul>		Click the Section Complete button to continue analysis.
<ul> <li>Treat_1_F3</li> <li>Treat 1 F4</li> </ul>		
Treat_1_F5		
• Treat_1_F6		
Treat 2	×	
• Treat 2 F1	E	
Treat_2_F2		
• Treat_2_F3		
<ul> <li>Treat_2_F4</li> <li>Treat_2_F5</li> </ul>		
• Treat_2_F6		
Treat 3	×	
* Treat 3 F1		
Treat_3_F2		
• Treat_3_F3	Ŧ	
Using this screen efficiently		
If you find this screen slow to use, there m	nay be	
faster ways to recombine your samples.		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.

Unnamed experiment - Progenesis LC-MS								• X
Eile Import Fractions Samples Design Setu		ein View				ņ	onlin	near
New								🕜 Help 🔻
Which experiment design type of	do you v	vant to use for this experime	nt?					
Between-subject Desi	gn		0-0 0-0 0-0 0-0	n				
Do samples from a given subject appear in only one condition? Then use the between-subject design.	A	Progenesis LC-MS	Have you taken samples from a		Before	During	After	E
To set up this design, you simply group the runs according to the condition (factor level) of the		Create a new experimen Name: Control vs Treated	nt design	i dicitante i	x1	X2	X3	
samples. The ANOVA calculation assumes that the conditions are independent and therefore gives a statistical test of whether the	c	<ul> <li>Start with an empty layout</li> <li>Copy layout from:</li> </ul>	ż	S Patient Y	Y1	¥2	¥3	
means of the conditions are all equal.	Add cond		Create design Cancel	Patient 2	Z1	Z2	Z3	
			(factor level) each run belongs to but also which subject it came from. The software will then perform a repeated measures ANOVA.				•	- -

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

Vunnamed experiment - Progenesis LC-MS	
Eile Import Fractions Samples Design Setup Protein View	near
Control vs Treated I × New	🕜 Help 🔻
Setup conditions         Samples         Add Selected Samples to Condition         P           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 Con_1 Remove Con_2 Remove Con_3 Remove	
Add condition  Add condition  Section Comple	ata 👁

Click Section Complete to move to Protein View

# **Stage 4 Protein View**

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

<u>F</u> ile									
port Fractions	Recombine Samples	Experiment Design Setup	Protein View						nonlinea
		0							D Y N A M I C
No filter a	crea	te P							Protein options
cession	Peptide count	Confidence score	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 [Clostridium difficile e
gi 85279330	1	52.4	0.00844	2.16	123456	2	Control	Treated	PTS system, IIB component [Clostridium difficile 63
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 630]
gi 46211184	2	117	0.0146	1.71	123456	2	Treated	Control	50S ribosomal protein L2 [Clostridium difficile 630]
gi 42170149	1	45.8	0.0148	3.81	123456	1	Treated	Control	translation elongation factor G [Clostridium difficile
gi 17787717	2	129	0.0149	3.59	123456	2	Treated	Control	30S ribosomal protein S4 [Clostridium difficile 630]
gi 20629287	2	82.9	0.0161	1.83	123456	1	Treated	Control	tellurium resistance protein [Clostridium difficile 63
gi 51703916	4	263	0.0165	1.77	123456	3	Treated	Control	D-alaninepoly(phosphoribitol) ligase subunit 2 (D
gi 04388227	1	41.7	0.0168	1.51	123456	1	Control	Treated	2-hydroxyisocaproate-CoA transferase [Clostridium
gi 63129634	1	48.6	0.0177	1.79	123456	1	Control	Treated	cell surface protein [Clostridium difficile QCD-63q4
gi 04526507	1	47.7	0.0194	858	123456	2	Treated	Control	nitroreductase-family protein [Clostridium difficile 6
elected pi		olase [Clostri		"" le 630]					
15.0 -			Control						Treated
15.0			Þ.						
14.0									) 4

And at the peptide level when you click view peptide measurements

	le													
E	le	F	lecombine	Experiment										
mpor	t Fracti		Samples		Protein View								nor	nlinea
													D Y N	A M I C
<b>3</b> E	lack	Prote	ein: eno	lase [Clostric	dium diffic	ile 630]								
nes	sion: (		841839	-		-								
	les	gile.	0.1000											
	#	Score	Anova (p)	Max Fold Change	Highest Mean	Lowest Mean	Abundance	m/z	Charge	Retention Time	Fractions	Mass error (ppm)	Peptide Sequence	Modification
Y	772	58.36	0.462	1.2	Control	Treated	1.608E+05	554.7858	2	23.052	123456	0.89	S VFDFLDYGIR	
2	1135	61.69	0.448	1.2	Control	Treated	7.381E+04	508.2809	2	23.265	123456	1.47	GEMVHVNDR	
2	1388	67.65	0.943	1.11	Control	Treated	7.981E+04	616.8116	2	28.624	123456	0.09	DWGEECQAQYK	
2	431	52.04	0.0888	4.03	Control	Treated	7.158E+05	602.8294	2	29.958	123456	22.26	FVNNYYESEMK	
V	666	55.17	0.39	1.4	Control	Treated	2.37E+05	508.2808	2	23.417	123456	1.15	GEMVHVNDR	
						Stand	ardised E		n Profi	les	•			
			Control			Stand	ardised E		n Profi		•			
2	Ŧ		Control			Stand	ardised E				•			
1.	-		Control			Stand	ardised E				•			
Abundance			Control			Stand	lardised E				•			
1. 1. 1.			Control			Stand	lardised E				•			
11 and Sprindance	5		Control			Stand	lardised E				•			
1.1 0.0 0.0 0.0	5		Control			Stand	ardised E				•			
1.1 1.1 0.0 0.0 0.0 0.0 0.0 0.0 0.0	5		Control			Stand	lardised E				•			

Click back to return to the Protein View

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

i.e. the list can be reduced to show only those with a p value less than 0.05 and containing 2 or more peptides

Eile	Recombine Ex	operiment								
			rotein View							nonlinear
<b>Tag filter ap</b> proteins may		dit 🔎								Protein options
ccession	Peptide count	Confidence s	core Anova (p)	Tag 👻	Max fold change	Fractions	Occurrences	Highest Mean	Lowest Mean	Description
gi 64841839	4	240	0.00901		2.06	123456	3	Control	Treated	enolase [Clostridium difficile 630]
gi 46211184	2	117	0.0146		1.71	1 2 3 4 5 6	2	Treated	Control	50S ribosomal protein L2 [Clostridium difficile
gi 17787717	2	129	0.0149		3.59	123456	2	Treated	Control	30S ribosomal protein S4 [Clostridium difficile
gi 20629287	2	82.9	0.0161		1.83	123456	1	Treated	Control	tellurium resistance protein [Clostridium diffici
gi 51703916	4	263	0.0165		1.77	123456	3	Treated	Control	D-alaninepoly(phosphoribitol) ligase subunit
gi 64041498	4	262	0.0205		3.31	123456	3	Treated	Control	thioredoxin [Clostridium difficile 630]
gi 64841853	2	99.9	0.0238		2.22	123456	2	Treated	Control	flagellin [Clostridium difficile]
gi 38304314	2	134	0.0255		3.9	123456	1	Treated	Control	(R)-2-hydroxyisocaproate dehydrogenase [Clo
gi 48078745	2	94.6	0.0331		2.11	123456	1	Treated	Control	aspartate aminotransferase [Clostridium diffici
gi 17787672	2	92.4	0.0383		1.56	1 2 3 4 5 6	2	Treated	Control	pyruvate-flavodoxin oxidoreductase [Clostridiu
a qi 23157299	3	160	0.0422		1.66	123456	1	Control	Treated	Cwp66 [Clostridium difficile]
Selected pro		lin [Clostri	idium diffic	ile]						
: 13.5 -			Control						Trea	ated
Pucsing 13.5			<u>→</u>							

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	
<u>F</u> ile			
🛃 Save		ent tup Pro	tein View
Close			•
Export Prot	ein Measurements		
Export Pept	ide Measurement	5	
× Exit		fidence sco	re Anova (p)
🌒 gi 17787717	2	129	0.0149
🌒 gi 20629287	2	82.9	0.0161
🌒 gi 51703916	4	263	0.0165
🌍 gi 64041498	4	262	0.0205
👔 gi 64841853	2	99.9	0.0238

Export Protein Measurements		×
Choose properties to be included	in exported file	
Accession     Peptide count     Peptides used for quantitation     Confidence score     Anova (p)     Max fold change     Fractions     Occurrences		
<ul> <li>Highest mean condition</li> <li>Lowest mean condition</li> </ul>		
<ul> <li>Description</li> <li>Normalized abundance</li> </ul>		
Tags		
	OK Ca	ncel

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

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

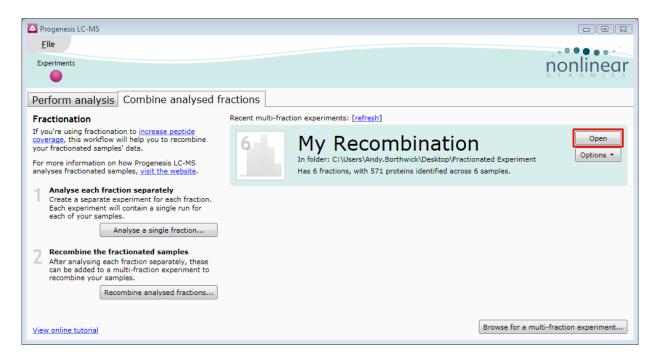
### **Stage 5 Saving a Multi-Fraction experiment**

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.

<b></b>	Jnnamed <mark>experimer</mark>	nt - Progenesis L	C-MS	
	<u>F</u> ile			
	Save		ent itup	Protein View
	Close			
	Export Protein Me Export Peptide Me			۶
×	Exit		fidenc	e score Anov
	gi 17787717	2	129	0.01

\land Save				×
Save in:	Fractionated Experiment	•	3 🌶 📂 🛄 🗸	
Save in: Recent Places Desktop Andy Borthwick	Fractionated Experiment Name Fract_1.Analysis Fract_2.Analysis Fract_3.Analysis Fract_3.Analysis Fract_5.Analysis Fract_6.Analysis	Date modified 02/11/2010 09:26 02/11/2010 09:50 02/11/2010 09:52 02/11/2010 10:01 02/11/2010 09:59 02/11/2010 09:53	③       ♪       ▷          Type       File Folder       File Folder         File Folder       File Folder       File Folder         File Folder       File Folder       File Folder	Size
Computer	File name: My Recombine     Save as type: Multi-fraction	nation Experiment (*.Progene	▼ sisMultiFrac ▼	Save Cancel

On saving the new mult-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**.

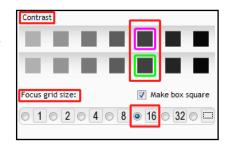
This completes a guided tour through using Progenesis LC-MS to analyse fractionated data.

# Appendix 1: Manual assistance of Alignment

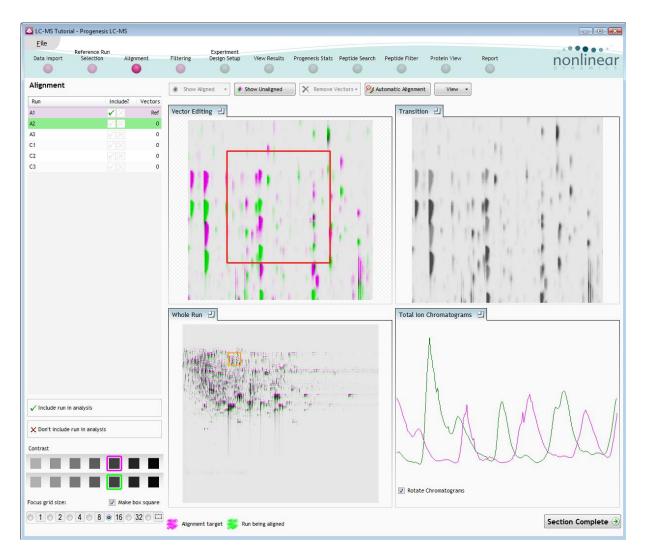
### Approach to alignment

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.



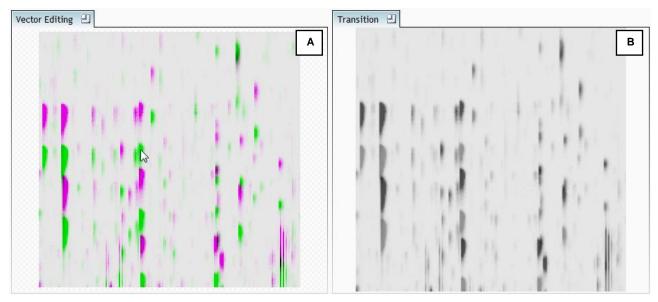
Click on an area (see below) in the **Whole Run** window (C) to refocus all the windows. Adjust Contrast as required



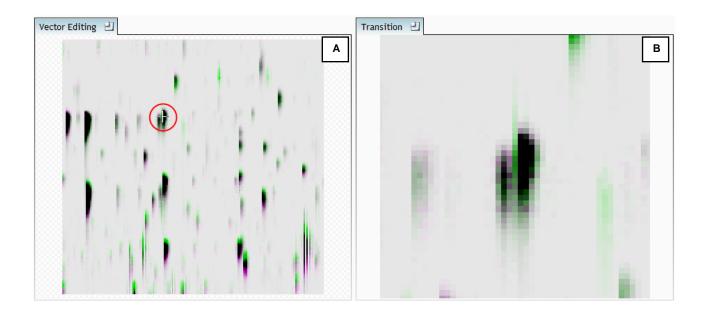
**Note**: the features moving back and forwards between the 2 runs in the **Transition** view indicating the misalignment of the two LC-MS runs

**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.



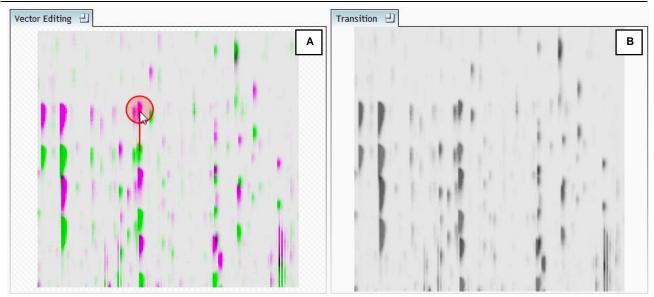
5. As you are holding down the left mouse button drag the green feature over the corresponding magenta feature of the reference run. The red circle will appear as shown below indicating that a positional lock has been found for the overlapping features.



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

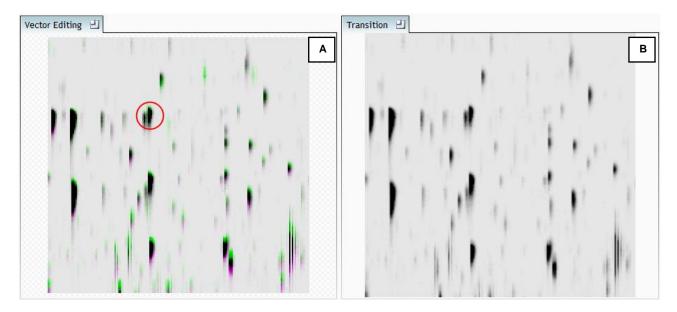
6. On releasing the left mouse button the view will 'bounce' back and a red vector, starting in the green feature and finishing in the magenta feature will appear.

#### Progenesis LC-MS Fractionation User Guide

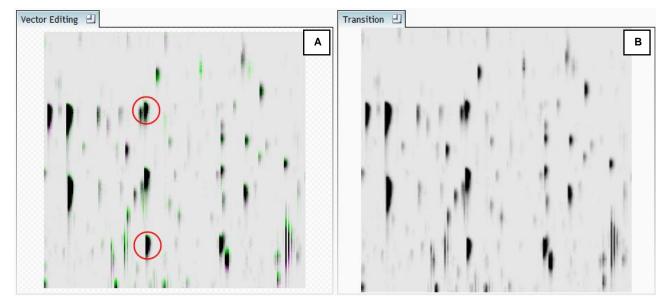


Note: an incorrectly placed vector is removed by right clicking on it in the Vector Editing window

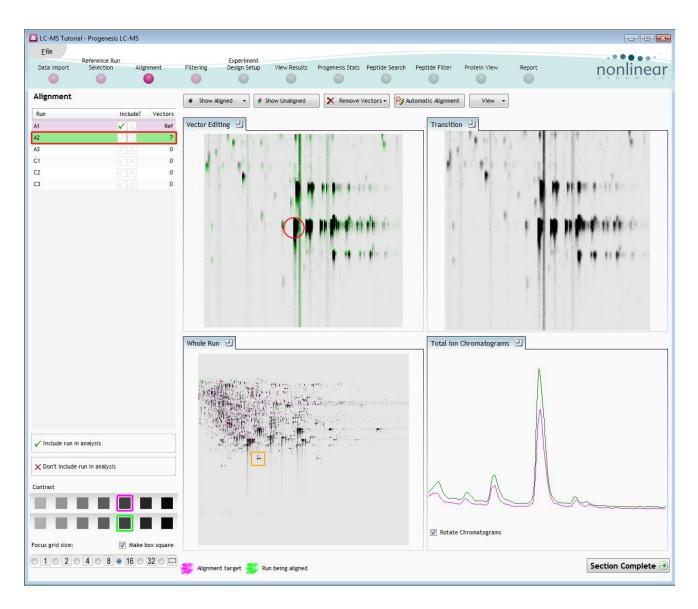
7. Now click **Show Aligned** on the top tool bar to see the effect of adding a single vector.



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.



#### Repeat this process moving the focus from top to bottom on the Whole Run view

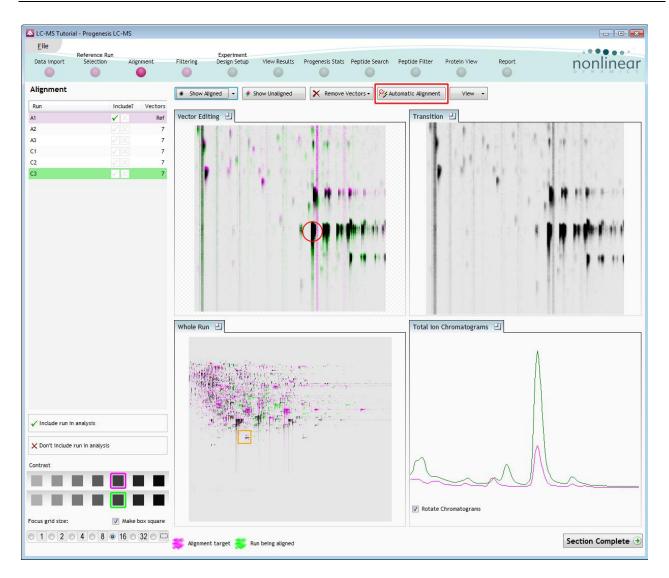


Note: the number of vectors you add is recorded in the Runs table

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.

Automatic Al	gnment	×
Select the ru	ns for automatic alignment vector generation	
Add Run	Notes	Vectors
🔳 A1	this run does not need to be aligned as it is the alignment reference	Ref
🔽 A2	run has user vectors	7
🔽 A3	run has user vectors	7
🔽 C1	run has user vectors	7
🔽 C2	run has user vectors	7
🔽 C3	run has user vectors	7
	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 9

### **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 X,Y and Z) who have been individually sampled: Before(1), During (2) and After (3) treatment

e	Reference Image	Mask Of			Experiment								
ige QC	Selection	Disinterest	Alignment	Filtering	Design Setup	View Results	Progenesis Stats	Spot Picking	Report			ņọ	nlin
ew nich exp	eriment desi	ign type do	you want t	o use for t	his experimer	nt?							
0 0 0 0 Be	tween-subj	ect Design				ſ	0-0 0-0 Within-	subject Des	sign				
	es from a given condition? The esign.			A	gel1	Delete Remove	Have you taken s subject under di use the within-s	fferent condit	ions? Then		Drug A	Drug B	Drug C
mages ac evel) of t issumes t	this design, you cording to the o he samples. Th hat the conditio	ondition (fact e ANOVA calc ons are indepe	or ulation ndent Drug					ave a sample f condition to u	se a within-	Person 1	gel1	gel5	gel9
nd there vhether t qual.	fore gives a stat he means of the	istical test of conditions ar	e all	Creat Name:	e a new exp		<b>_</b>		ns type of ht where t each time software	Person 2	gel2	gel6	gel10
			Dru	Star	t with an empt y layout from:	y layout		Ŧ	evel) each ubject it en perform a	Person 3	gel3	gel7	gel11
							Create desig ANOVA individual eliminated or red condition differe	differences ca luced as a source	ce of between	Person 4	gel4	gel8	gel12
							more powerful te The within-subje as an extension o to include compa repeated measur	est). ct design can b f the paired-sa rison between	e thought of mples t-test				

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.

My_Timecourse - Progenesis LC-MS					
<u>F</u> ile					
Reference Run E Data Import Selection Alignment Filtering De	xperiment sign Setup View Results	Progenesis Stats Pepti	ide Search Peptide Filte	r Protein View	
Patients Progress I × New					
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
<ol> <li>Add a column for each condition.</li> <li>Add a row for each subject.</li> <li>Drag each of your samples to the correct location in the grid.</li> </ol>	Patient X	X1	X2	X3	
Samples 🔎			1400	15400	
Z2	Patient Y	Y1	Y2	Y3	
Z3	Patient Z	Z1	Stect Sample	<u>Select Sample</u>	
	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