

Progenesis QI for proteomics HCP Spectral Library User Guide

Analysis workflow guidelines for MS^E data

THE SCIENCE OF WHAT'S POSSIBLE.

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Introduction

This user guide takes you through how to construct a Spectral Library from the analysis of a set of 12 LC-MS runs with 4 groups (3 replicate runs per group) using the unique Progenesis QI for Proteomics workflow. It describes the initial analysis of the data followed by the creation and use of a spectral library to identify proteins in 5 Batches of additional samples. More detailed descriptions of each step in the analysis workflow is described in the DDA and HDMSe User guides.

To allow ease of use the tutorial is designed to start with the restoration of Archived experiments where the data files have already been loaded. The document covers all the stages in the workflow, initially focusing on the Automatic Processing of the data then the use of the individual analysis stages. More details of the samples and the proteins present in them are available on page 4.

If you are using your own data files please refer to Appendix 1 (page 38) then start at page 6.

Note: the images used in Appendices 1 and 4 refer to the HDMSe User guide data set.

How to use this document

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

If you experience any problems or require assistance, please contact us at support@nonlinear.com

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

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

LC-MS Data used in this user guide

For the purposes of this data set the MS^E parameters were set to 250:150 the default settings as defined in Appendix 1 (page 38). This was to done to reduce the time taken to demo the data analysis.

Tutorial Data

The samples were originally generated to examine the use of Spectral Libraries to monitor the presence of Host Cell Proteins (HCPs) in batches of the NIST mAb product. The idea being to simulate contamination of the mAb preparation with Chinese Hamster Ovary (CHO) cell proteins and therefore to monitor Host Cell Proteins with high accuracy using mass spectrometry.

Label free LC-MS was performed using a 30min gradient on a CSH C18 (M-class ACQUITY UPLC) attached to a Xevo-G2XS

There are 2 data sets: HCP_Spectral Library_**Created** and HCP_mAb_**Batches** containing the samples as indicated below, run in triplicate.

CHO proteins ppm	Create	Batches
50		Batch_5
100	CreateA	
500		Batch_4
1000	CreateB	
5000		Batch_3
10000	CreateC	
25000		Batch_2
50000		Batch_1
100000	CreateD	

The idea of the data sets is to allow you to explore the processes involved in making spectral libraries and then their use in the identification and quanification of Host Cell Proteins using different Batches where the HCPs have been simulated by introducing different levels of CHO proteins.

The raw data has already been loaded into Progenesis QI for proteomics 4.2 in the form of 2 experiments and licenced archives of these experiments have been created:

HCP_Spectral Library_Created.ProgenesisQIPArchive

HCP mAb_Batches.ProgenesisQIPArchive.

Note: these will open at the Identify Peptides screen when restored in Progenesis QI for proteomics

A fasta file, **NIST AB_chaperone_CHO.fasta**, is provided for identification of the proteins in the HCP_Spectral Library Created experiment. The FASTA contains sequences for CHO cell proteins, the NIST mAb product and the internal spiked E.coli Chaperone protein ClpB which was added to all the samples (200 fmol/sample).

All samples contain 41 pmol of NIST mAb product protein.

Note: there are also 2 experiment design setup (Create.spl and mAb_Batches.spl) files and 6 example Spectral Libraries .msp available.

The files described above are all available in the 2 Spectral Library Tutorial.zip files downloaded from http://www.nonlinear.com/progenesis/qi-for-proteomics/v4.2/user-guide/.

The following sections explain how to restore and process the data in Progenesis QI for proteomics v4.2.

Restoring the Tutorial

Open Progenesis QI for proteomics and download the Compressed files (.zip),

Library_creation_data_set.zip and Library_search_data_set.zip from the 'User guide and tutorial' link shown below, placing them in a **new folder** on your desktop (i.e. Spectral Library Tutorial). Before restoring the tutorial in the software **you must** first right click on the (.zip) files and extract them to the same folder.

There are 2 archive files, 2 Grouping files, a fasta file and example Library files.

Now restore the 2 archive files (HCP_Spectral Library_Created and HCP_mAb_Batches). To do this, locate the 'name'.Progenesis QIP Archive file using the **Open** button and press Open.

QIP Progenesis QI for proteomics	
File	1
Experiments	nonlinear
	A Waters Company
Perform analysis Combine analysed fractions	New to Progenesis QI for proteomics?
Recent experiments Search	Here are some resources to help you get started with Progenesis QI for proteomics:
Image: Constraint and the second s	The Progenesis OI for proteomics workflow User quide and tutorial data Frequently-asked questions
Organize 🔻 New folder	
MASCOT Mini NZ MILINI TmAb Atribut List NILINI_TmAb Atribut List NILINI_TmAb Sept 2019 OLD DRIE recover Progenesis vz Expressionist QIP_4.2 Testing SHORTCUTS Sysinternals v < mm ,	Quickly go to an ion map location Want to quickly validate your sample running by zooming to a known ion?
File <u>n</u> ame: Experiments and Archives (*,Prt v Qpen v Cancel	corner of the ion maps.
Other experiments	42.7207.22925 • Identification scoring in Progenesis

This opens the "Import Experiment from Archive" dialog.

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

UP Import Experiment from Archive Import experiment from archive After importing the experiment from this archive, any changes to the experiment will be saved to the location below, not back to the archive.					
 Replace an existin Experiment to replace Create a new experiment 	ace:				
Experiment name:	HCP_Spectral Library_Created				
Save to folder:	E:\Customer Data\Progenesis QIp_4.2 Tutorials and Browse				
	Import Cancel				

Then click Import.

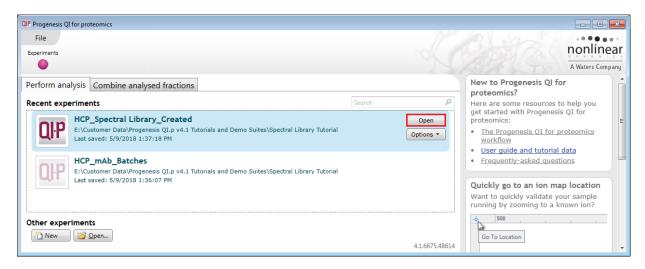
After the import is complete select **Close** from the **File menu** and then repeat the process for the other archive (Batches), restoring them into the same folder.

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

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

Stage 1: Perform automatic processing of the Create Library samples

Having opened the Progenesis archive files the experiments will now be displayed when you open Progenesis QI for proteomics. Select the HCP_Spectral Library_Created experiment and click **Open**.



Note: having restored the **HCP_Spectral Library_Created** experiment, go to Stage 9 (page 26), where you can proceed with the creation of the various spectral libraries from the analysed experiment.

Alternatively, you can use the first 7 stages of this guide to perform the Automatic processing of the Created data set and then go through the processes involved in the selection and review of Peptide ions to add to the Spectral libraries. To do this you can generate a third experiment using the same HCP_Spectral Library_Created archive then remove the analysis by clicking **Restart automatic processing** as described below.

First you must create an additional folder called **My Analysis** in the same folder you restored the original archives, to do this right click on the HCP_Spectral Library_Created experiment and select **Open file**

QIP Progenesis QI for proteomics		
File Experiments		nonlinear A Waters Company
Perform analysis Combine analysed fractions Recent experiments HCP_Spectral Library_Created C:\Users\@ Open Last save		New to Progenesis QJ for proteomics? Here are some resources to help you get started with Progenesis QI for proteomics: DI for proteomics
HCP_m Copy to archive C:Users Delete Last save Open file location	Organize ▼ @ Open Include in library ▼ 4+ Organize ▼ @ Open Include in library ▼ Share with ★ Favorites Mame Mame ▲ Desktop ↓ HCP_Spectral Library ▲ Downloads ↓ HCP_mAb_batches, patches, patc	ith - Burn >> BIF - O ion map location idate your sample g to a known ion?
<u></u>	Desktop Greate.spl Grea	Create snl
ation , right click and create a alysis.	New folder called My	Desktop Libraries Documents Music Pictures v
en copy the 3 files, as shown, c orial folder to the My Analys		3 items

Now select **Open** on the Progenesis Experiments page and Navigate to the **My Analysis** folder.

Select the HCP_Spectral Library_Created.Progenesis QIPArchive and click Open

Progenesis QI for proteomics		
File		nanlina
periments		nonline
•		A Waters Comp
erform analysis Combine analysed fractions		New to Progenesis QI for proteomics?
ecent experiments Search	Q	Here are some resources to help you
HCP_Spectral Library_Created	Open	get started with Progenesis QI for proteomics:
C:\Users\ukabor\Desktop\Spectral Library Tutorial	Options -	The Progenesis OI for proteomics
Last saved: 10/23/2019 8:48:10 AM	Options *	workflow
HCP_mAb QP Open Experiment		User guide and tutorial data Frequently-asked guestions
		• <u>Frequency asked questions</u>
Last saved: Search My Analysis - 49 Search My Analysis	Q	Quickly go to an ion map location
Organize 🔻 New folder 🛛 🕮 👻		Want to quickly validate your sample
MASCOT ^ Name		running by zooming to a known ion?
Mini NZ MII INI TmAb Atribut List HCP_Spectral Library_Created.ProgenesisQIPArchive		
Nilini Trastuzumab Feb Dea		Go To Location
NILINI_TmAb Sept 2019		and the second states of the second states
LD DRIE recover		20
Progenesis vs Expressionist QIP 4.2 Testing		
SHORTCUTS		Jump to a specific m/z and RT using the
🕒 Spectral Library Tutorial		Go To Location tool in the top-left
🕌 Sysinternals 👻 🤟 🎹	E.	corner of the ion maps.
File name: HCP_Spectral Library_Created.Prot - Experiments and Archives (*.Prc 👻	Latest blog posts
Qpen 🚽 Can	cel	Are you missing out on how
		Progenesis QI can help your research?
There experiments		Why are reviews important?
invew ig Open	4.2.7207.22925	<u>Identification scoring in Progenesis</u> OI

The following dialog will open asking you to Replace an existing experiment, do not do this.

QP Import Experiment from Archive						
Import experiment from archive After importing the experiment from this archive, any changes to the experiment will be saved to the location below, not back to the archive.						
Replace an existing	g experiment					
Experiment to repla	ce: HCP_Spectral Library_Created					
- 🔘 Create a new expe	riment					
Experiment name:	HCP_Spectral Library.Created (2)					
Save to folder:	C:\Users\ukabor\Desktop\HCP_Spectral Library_Cn Browse					
	Import					

Select Create a new experiment and amend the experiment name to HCP_Spectral Library_My Creation

OPP Import Experiment from Archive					
Import experiment from archive After importing the experiment from this archive, any changes to the experiment will be saved to the location below, not back to the archive.					
© Replace an existing experiment Experiment to replace: HCP_Spectral Library_Created					
Create a new experiment Experiment name: HCP_Spectral Library_My Creation Save to folder: ibor\Desktop Spectral Library Tutorial\My Analysis Browse					
Import					

Then make sure that Save to folder is set to My Analysis using the Browse facility.

Then click Import

The newly created **HCP_Spectral Library_My Creation** experiment will open at the **Identify Peptides** stage on the workflow.

Click on **Import Data** and then click **Restart automatic processing.** This will bring up a warning dialog.

Select Discard all analysis and continue this will remove all existing analysis of this data set.

	P_Spectral Lil	brary_My Creatio	n - Progenesis Ql	for proteor	mics									
Fi Impo	F	Review gnment Filt	Experin ering Design S		ew Peak icking	Peptide Ion Statistics	Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report		nonlinear A Waters Company
Imp	ort Data										<			
· ·	Select your	www.data		Cr	reateA_0	1 (low en			1050			Actions •		
	Select one of	f the available d	ata formats ther	. F.	-		525		1050		1575	·	m/z •	About this run
	click the Imp					1.1		1.1.1.1.1.1.1						 Low energy peak count: 2,473,597 High energy peak count: 2,833,840
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	processing. 1		your data as fai	as g	ſ		I from editing				×		1.1.1.1.1.1.1	 Lock mass calibrated Lock mass m/z: 556.2766
			utomatic process	ing 8	(min)	Discord	- Lanalusia f	or all runs?						EGCK 11855 11/2, 550.2700
					e									
	are importing		started while ru	ns e				rocessing will ri ient analysis to	eset ALL analysis be repeated.	requiring alig	nment, peak			
	🕜 Lea	irn more about	automatic proces	ising ທ	Retention				ysis state, you sl	and a sub-line a				
Impo	rted runs: Se	earch	Q		Ret		starting analys		ysis state, you si	jouid archive t	this experiment	m: 🔍	0.2	
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Cr	eateC_01	CreateC_02	CreateC_03	Ŧ	∝ •						Z	loom: 🔍	9.2	Section Complete 🏵

The Auto processing wizard opens and a series of dialogs allow you to select how you want the runs to be analysed in Progenesis.

Click Cancel and the Close from the File menu (Top left of the Screen)

QIP Progenesis (QI for proteomics		
File Experiments			nonlinear A Waters Company
Perform an Recent exp	alysis Combine analysed fractions	Search P	New to Progenesis QI for proteomics? Here are some resources to help you
QŀP	HCP_Spectral Library_My Creation E:\Customer Data\Progenesis QIp_4.2 Tutorials and Demo Suites\Spectral Library Tutorial\My Analysis Last saved: 10/2/2019 12:15:16 PM	Open Options -	get started with Progenesis QI for proteomics: • <u>The Progenesis OI for proteomics</u> <u>workflow</u> • User guide and tutorial data
QŀP	HCP_Spectral Library_Created E:\Customer Data\Progenesis QIp_4.2 Tutorials and Demo Suites\Spectral Library Tutorial Last saved: 10/2/2019 8:16:23 AM		Frequently-asked questions Quickly go to an ion map location
QŀP	HCP_mAb_batches E:\Customer Data\Progenesis QIp_4.2 Tutorials and Demo Suites\Spectral Library Tutorial Last saved: 10/2/2019 8:12:43 AM	-	Want to quickly validate your sample running by zooming to a known ion?
Other expe	rriments	4.2.7171.46980	8 (1) (1) (1) (1) (1) (1) (1) (1)

Note: the appearance of the new experiment in the list

The following pages describe how to proceed with the full analysis of this data.

Click Open and move to the next stage.

Stage 2: Automatic Processing of your data

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

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

2	Perform automatic Click the button below processing. This will a possible, before sugg	v to start automatic analyse your data as far as
		Start automatic processing
	Automatic processing are importing.	can be started while runs
	② Learn more	about automatic processing

For the processing of the 12 files in this experiment make the following selections:

Step 1: Select the third option, **Use this run**. This tells QIp to set the Alignment reference for to your chosen image. (In this example CreateD_01)

OP Start automatic processing
Select an alignment reference To compensate for drifts in retention time, all runs in the experiment must be aligned to a single reference run.
How do you want to choose your alignment reference?
Assess all runs in the experiment for suitability
Use the most suitable run from candidates that I select
Use this run:
CreateD_01 •
For information on choosing the alignment reference, and why you might want to select your own candidates, please see the <u>online guidance</u> .
< Back Next > Cancel

Click Next. The option to perform Automatic alignment will be selected by default.

QP Start automatic processing
Automatic alignment After selecting the experiment's alignment reference, the software can also automatically align all runs.
After the alignment reference is chosen, do you want to start automatic alignment? Yes, automatically align my runs
< Back Next > Cancel

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

Step 2: For the purposes of this User guide we will use the default settings for peak picking which you can review by clicking on Set parameters.

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

Click Next.

Step 3: To apply the experiment design in advance Click **Set up an experiment design** then locate the appropriate file (**Create.spl** available in the folder you extracted the .zip file) using Browse.

Note: if you do not enter a name for the experiment design it will adopt the name of the first column in the Create.spl, in this case Conditions, this can be changed as required in the main workflow.

QIP Start automatic processing								
Experiment design Experiment designs allow you to group and compare your samples according to their experimental conditions.								
By defining an experiment design in advance, statistical measures such as ANOVA can be calculated automatically.								
Enter a name for the experiment design:								
Conditions								
Load the criteria for grouping runs from this file:								
storials and Demo Suites\Progenesis QIp_v4.2 HCP Tutorial\Create.spl								
Group runs by: Condition 🔹								
What file formats are supported? A Back Next > Cancel								

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

Select Conditions and then click Next.

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

Step 4: To automatically process the identification of peptides using there MS^E fragmentation data in the tutorial data click on **Set parameters**. The default Databank is for Swissprot-1.0 (which is a locked example).

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

QP Start automatic processing	OP Enter search parameters	
Identify peptides Get identifications for the peptides in your samples using their MS ^t fragmentation data.	Enter the search parameters Select your FASTA file containing peptide and protein identifications: SWISSPROT-1.0	Edit
After peak picking has completed, do you wish to automatically identify peptides? Use MS ^t data from my runs to identify peptides <u>Set parameters</u>	Enter the search parameters to use: Common search parameters Digest reagent: Trypsin Missed cleavages: Max protein mass: 250 Modifications: Search tolerance parameters Search tolerance parameters So Ion matching requirements Admin tools Change the digest reagents and modifications that are available for peptide seas Modification editor Rease	1 max •
< Back Next > Cancel		arameters

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

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

QIP	D	atabank Editor		×
ť	9	Name	Name:	NIST AB_Chaperone_CHO
é	a	SWISSPROT-1.0	Parsing rules:	
		NIST AB_Chaperone_CHO	Location:	and Tutorial\NIST AB_Chaperone_CHO.fas
			Location.	and Tutorial/NIST AB_Chaperone_CHO.fas
		Add Remove		
				Save Cancel

The new Data bank will appear in the left panel now click **Save** to return to the Search parameters. If your databank is not already displayed then select it from the drop down list.

Check the Common search parameters

The default settings are displayed:

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

Missed cleavages: is set as 1.

Maximum protein mass: is set at 250kDa

Modifications: are set Carbamidomethyl C (Fixed) and Oxidation M (Variable). More modifications are available from the list and additional ones can be added to the list using the Modification editor... In this example Deamidation on N and Q have been added

Search Tolera (FDR) has been

Ion matching r Fragments/pep Peptides/protei

Click Save par

QIP Start automati Protein Quant If you've include can calculate th Ouantitation me Relative Quant Number of pept

Use protein g protein's.

	FUR less than: 1 %					
nce parameters: The false discovery rate n set to 1% requirements: are set at tide: 3, Fragments/protein: 7 and n: 1 by default	Ton matching requirements Fragments/peptide: Fragments/protein: Peptides/protein: Change the digest reagents and modifications that are available for peptide searches. Modification editor Reagent editor					
ameters and then Next.	Save parameters					
tation (d known amount of a calibrant protein in each of your samples, you can cu cu can cu c	t automatic processing					
des to measure per protein (N):	elative Quantitation using Hi-N beolute Quantitation using Hi-N Requires a calibrant protein to calculate absolute amounts Uses mean calibrant abundance measured across runs Uses the most abundant N peptides Allows comparison between proteins within a run elative Quantitation using Hi-N Uses the most abundant N peptides Uses the most abundant N peptides					
rouping i.e. hide proteins whose peptides are a subset of another < Back Finish Cancel	Allows comparison between proteins within a run tive Quantitation using non-conflicting peptides Uses only peptides which have no conflicting protein identifications Allows comparison of a single protein across runs tive Quantitation using all peptides Uses all peptides identified as part of a protein Allows comparison of a single protein across runs Jute Quantitation for HCP using Hi-N Requires a calibrant protein to calculate absolute amounts Uses calibrant abundance measured per run Uses the most abundant N peptides Calculates the amount of HCP user run imfol and ng					

OP Enter search parameters

NIST AB_Chaperone_CHO

Digest reagent:

Aissed cleavages

Max protein mass:

Peptide tolerance:

Fragment tolerance:

Modifications:

Enter the search parameters Select your FASTA file containing peptide

Trypsin

250

Carbamidometh
Carbamidation N
C Deamidation Q
C Oxidation Q

Add/remove modifications

Carbamidomethyl C

Enter the search parameters to use: Common search parameters

Search tolerance parameters

- - -

Edit...

-

1 ma:

kDa

ppm

The Protein Quantitation dialog opens displaying the default method, Relative Quantitation using Hi-N which uses up to 3 peptides per protein to compute the relative amount of each 'identified' protein. Use the drop down to reveal the alternative methods for protein quantitation

Select the Default option and click Finish.

The process starts with the selection of an alignment reference and completes with Protein quantitation.

QIP Automatic Processing (30%)	
Automatic processing Current step: Aligning 'CreateA_01'	Automatic processing complete.
✓ Importing runs: 12 of 12 processed ✓ Selecting reference: CreateD 01	Time taken: 16 minutes 20 seconds
Aligning runs: 1 of 11 processed Peak picking: Pending Creating design: Pending Identifying peptides: Pending Protein quantitation: Pending Creating design: Creating Creating design: Creating Creating design: Creating Creating design: Creating	 ✓ Importing runs: 12 of 12 processed ✓ Selecting reference: CreateD_01 ✓ Aligning runs: 11 of 11 processed ✓ Peak picking: 53615 peptide ions found ✓ Creating design: Created ✓ Identifying peptides: 6999 search hits ✓ Protein quantitation: Relative Quantitation using Hi-3
	<u>⊆lose</u> QC Metrics ⊙

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

Note: if processing fails to complete successfully there are a number of suggested strategies you can use to proceed with your analysis. These are out lined in Appendix 2 (page 43).

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

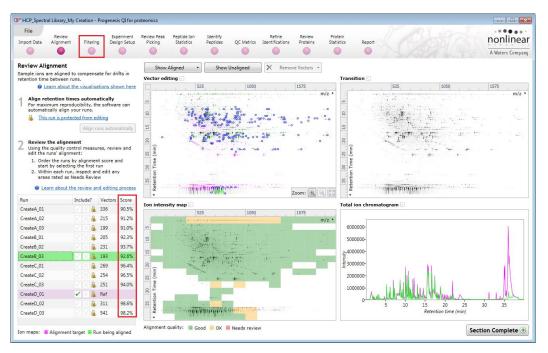
HCP_Spectral Library_My Creation - Progenesis QI for p	oteomics	
File Review Experiment Import Data Alignment Filtering Design Setup	Review Pesk Peptide Ion Identify Refine Review Protein Picking Statistics Peptides QC Metrics Identifications Proteins Statistics Report	nonlinea
	0 0 0 0 0 0 0 0	A Waters Compar
mport Data	CreateD 01 (low energy)	• >>
Select your run data Select one of the available data formats then click the Import button:	525 1050 1575	m/z * About this run • Low energy peak count: 4,274,249
Format: Waters (<i>raw</i>) Maters (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i>raw</i>) Mater (<i></i>	8	Low energy peak count: 4,2/4,249 High energy peak count: 5,110,277 Total ion intensity: 1.179e+009 Masked areas : none
Perform automatic processing Click the button below to start automatic processing. This will analyse your data as far as possible, before suggesting the next step.	2 2 0 0 P Processing Complete	Alignment reference This run is being used as the experiment's alignment reference.
Automatic processing can be started while runs are importing.	Automatic processing complete.	If you want to use a different run as the alignment reference, you'll need to discard any analysis and restart the automatic processing:
Learn more about automatic processing ported runs: Search P		C Restart automatic processing
1	Creating design: Created Created Creating design: Created Created Creating design: Created	Lock mass calibrated Lock mass m/z: 556.2766
CreateA_01 CreateA_02 CreateA_03	CreateD_01 (high ene	
		m/z *
CreateB_01 CreateB_02 CreateB_03	[1] The second secon	
	a the second	<u></u>
CreateC_01 CreateC_02 CreateC_03		
	an Time and Time	
CreateD_01 CreateD_02 CreateD_03	Zoom: @	Section Complete 🟵

When Processing completes click on Close and then on Review Alignment in the Workflow

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

Note: additional details on alignment are available in Appendix 4 (page 46).

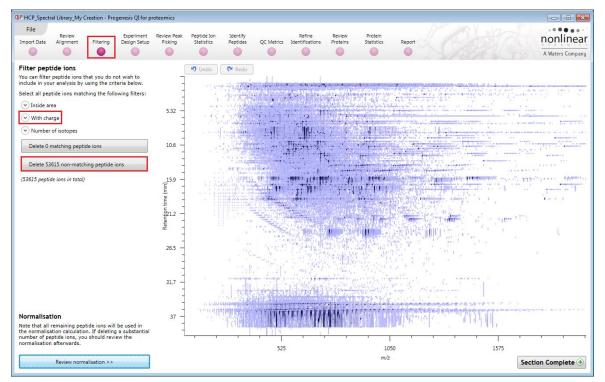
For this data set, the quality of the alignment (as indicated by the high scores) and detection following automatic processing does not require to be re-performed.



Click on Filtering on the workflow to move to the next section.

Stage 3A: Filtering

At Filtering you can review the total number of peptide ions detected (53,615) and choose to keep all or those which meet certain filtering criteria.

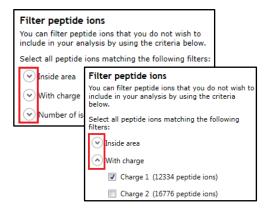


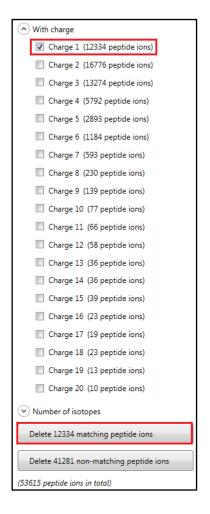
For this particular data set we will reduce the peptide ions to having a 'charge state' of 2 and above by filtering out the Charge state 1 peptide ions.

Select **With charge** and tick the Peptide ions you wish to remove from the analysis.

Following deletion of the peptide ions that do not meet the criteria this leaves 41,281 peptide ions with a range of charge states 2 to 20.

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





Now move on to Normalisation by clicking on 'Review normalisation', bottom left of the Filter page.

Stage 3B: Normalisation

At this stage in the workflow you can review and change the normalisation method; by default it will be set to normalise to all proteins.

To change it click on the Normalisation Method tab and select **Normalise to a set of housekeeping proteins** from the drop down. Enter 'Light chain' in the proteins you want to normalise against. Select all of the peptide ions for this protein by selecting all the rows in the table and ticking the **Use** box.

File mport Data A	Review lignment	Experi Filtering Design			Peptide Ion Statistics		dentify eptides Q		efine ifications	Review Proteins	Protein Statistics	Report				nonline
		•			_		•	•				•	10	1A	18	A Waters Compa
leview norm	alisatior	ı	Norm	alisation	n Graphs N	orma	lisation Metho	od								
lormalisation is ifferent sample		o allow comparisons a	Norm	alise to	a set of hous	ekeep	ping proteins	•								
		ant number of peptide												4	💚 No filter aj	oplied Create
se the factor by	which the	ental conditions, we ca e sample as a whole v		k the ho	usekeeping p	orotei	ins you want t	o normalise agair	nst Light	Chain	×				1	Create
o normalise bac			Use	#^	m/z	z	Mass	Retention time	Tag 🔹	Accession	Protein Descrip	tion				
ote: for each si vithin its robust	ample, onl estimation	ly the peptide ions falli n limits (see graphs) a	ng 🔽	1	711.8746	4	2843.4693	15.55		867100	Light Chain					
sed to calculate	the norm	alisation factor. Furthe	r 🛛 🔽		937.4697	2	1872.9249	16.69		867100	Light Chain					
Normalisation					946.9597	2	1891.9048	10.76		867100	Light Chain					
CreateC_02	reference:				948.8270	3	2843.4592	15.57		867100	Light Chain					
_				6	631.6429	3	1891.9069	10.75		867100	Light Chain					
ormalisation fa	_				701.0759	4	2800.2744	10.03		867100	Light Chain					
Run	Factor	Log(factor)					1872.9268			867100	Light Chain					
CreateA_01	1.00	-0.001			561.0633		2800.2802				Light Chain					
CreateA_02	1.02	0.0072			934.4296						Light Chain					
CreateA_03	1.01	0.0037			904.5116	2	1807.0087	17.44		867100	Light Chain					
CreateB_01	0.97	-0.012			924.9028		1847.7909	9.46		867100	Light Chain					
CreateB_02	0.93	-0.032			848.7167		2543.1283	15.80		867100	Light Chain					
CreateB_03	0.94	-0.025					2543.1355	15.80		867100	Light Chain					
CreateC_01	1.05	0.021					1185.6517	11.04			Light Chain					
CreateC_02	1.00	0			938.4740		1874.9334			867100	Light Chain					
CreateC_03	1.04	0.015			661.3477	2	1320.6808	9.10		867100	Light Chain					
CreateD_01	0.84	-0.076			476.7766		951.5386	8.65		867100	Light Chain					
CreateD_02	0.82	-0.084			557.8091	4	2227.2073	15.66		867100	Light Chain					
CreateD_03	0.88	-0.057			712.6654		2134.9745	5.23		867100	Light Chain					
					603.6716		1807.9930	17.85		867100	Light Chain					
					559.9427		1676.8063	11.30		867100	Light Chain					
				42	1068,4944		2134.9742	5.23		867100	Light Chain					
				43	899.4560		1796.8975	18.87		867100	Light Chain					

Note: the Normalisation factors will update, reflecting the use of the selected protein for normalisation.

Note: As the same amount of mAb protein (41 pmol) is present in each sample the normalisation was performed against the peptide ions for this protein.

Now move on to Experiment design setup by clicking on the icon on the workflow.

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.

Currently the Between subject experiment design has been set during the Automatic processing of the data, with 4 conditions: CreateA to D (CHO proteins spiked @ 100 ppm to 100000ppm).

File Review Alignment	Filtering	Experiment Design Setup	Review I Pickir		Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report		nonline
	•			-	•	•			•	02		A Waters Compa
onditions I × 🛛	New											@ Hel
etup conditions			Runs	Add Selected F	uns to Condition	Search		Q				
etup the conditions that y e.g., control, drug A, etc), our samples to the correc	and then as	ompare below sign each of										
CreateA		Delete										
	Create	A_01 <u>Remove</u>										
	Create	A_02 <u>Remove</u>										
	Create	A_03 <u>Remove</u>										
CreateB		Delete										
	Create	B_01 <u>Remove</u>										
	Create	B_02 Remove										
	Create	B_03 <u>Remove</u>										
CreateC		Delete										
		C_01 Remove										
		C_02 Remove										
	Create	C_03 Remove										
CreateD		Delete										
		D_01 <u>Remove</u>										
		D_02 <u>Remove</u>										
and a second before	Create	D_03 Remove										
Add condition												
											ſ	Section Complete

Note: you can use the New tab to create another Experiment design as required.

Details on the use of the next 2 steps in the Workflow: **Review Peak Picking** and **Peptide Ion Statistics** are available in the main HDMSe User guide.

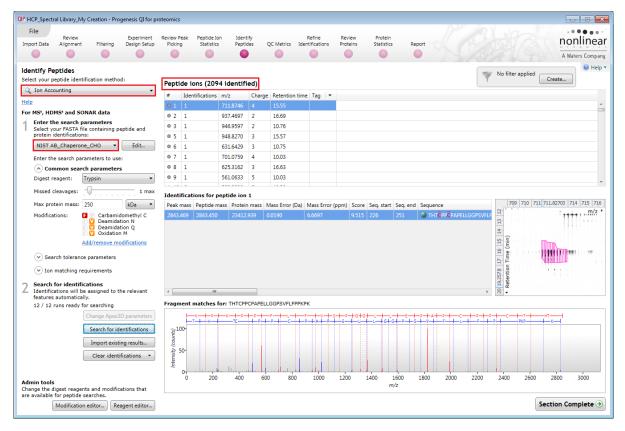
For the purposes of this Tutorial we will move directly to the **Identify Peptides** stage by clicking on the workflow.

File										1		
mport Data	Review Alignment	Filtering	Experiment Design Setup	Review Peak Picking	Peptide Ion Statistics	Identify Peptides	OC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report	nonlinea

Stage 5: Identify peptides

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

The data described in this tutorial was acquired on a Waters Xevo G2XS it is MS^E and therefore the peptide identification method used was **Ion accounting** as shown below.



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

Note: Following the automatic processing, described in Stage 2 (page 9) of this guide, the Identify Peptides page currently displays the full list of the detected peptide ions in your experiment.

Note: At the Filtering stage you will have removed the charge state 1 peptide ions, so you should re-perform the search using the parameters shown on the right.

There will be 2094 identified.

The left hand panel will display the Fasta File used (NIST AB_Chaperone_CHO) in the search and the parameters and settings used to control the search.

For MS¹, HDMS¹ and SONAR data

1	Enter the search parameters Select your FASTA file containing peptide and protein identifications:							
	NIST AB_Chaperon	e_CHO 🔻	Edit					
	Enter the search par	ameters to use:						
	Common searce	ch parameters						
	Digest reagent:	Trypsin	•					
	Missed cleavages:		1 max					
	Max protein mass:	250	kDa 🔻					
	Modifications: Carbamidomethyl C Carbamidation N Comparison Q Comparison M Add/remove modifications							
	 Search toleran 	ce parameters	E .					
	Peptide tolerance:	Auto	ppm					
	Fragment tolerance:	Auto	ppm					
	FDR less than:	1	%					
	 Ion matching 	requirements						
	Fragments/peptide:	3	or more					
	Fragments/protein:	7	or more					
	Peptides/protein:	1	or more					

Stage 6: Refine Identifications

Before attempting to create a Spectral library from this data one should first refine the quality of the search results by filtering out peptide identifications base on the score, number of hits and absolute mass error.

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

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

- Remove identifications with a Score less than 5
- Hits less than 2
- Absolute mass error (ppm) greater than 10

To perform these filters, on the Batch detection options panel, set the Score to less than 5, then **Delete matching search results**.

File nport Data	Review Alignment	Filtering	Experiment Design Setup	Review Pick	ng	Peptide Ion Statistics		dentify eptides	QC Metri	cs Ide	Refine ntifications	Revie Protei		Protein Statistics	Report						A Waters C	M T
	entifications			Bat	ch dele	tion criteria				_		_								_		
	de identification sults, you can re					L.	Score	less than	•	5		_			Sequence Length	less th	an	•				
	a set of delet						Hits	less than	•						Charge	less th	an	•				
values f	oatch deletion cr for a set of ident						Mass	less than	•						Sequence	contai	ns	•				
delete.				Abs	olute n	nass error (ppm) (less than	•						Accession	contai	ns	•				
	the unwanted						m/z	less than	•						Description	contai	ns	•				
 To delet either: 	te the identificat	tions you do	n't want, click			Detertion			•									•				
• Del	lete Matching Se highlighted IDs	earch Result	s, to delete			Retention	nine (less than	•						Modifications	conta	115	•				_
• Del	lete Non-matchi	ng Search R	esults, to											🔲 Delete	matching search res	ults	Delete	e non-m	atching search re	esults	Reset the cri	iteri
del	ete the IDs that	are not higi	hlighted		#	Score	Hits	m/z	RT(mins	Charge	Marc	Mass err	Sec	quence	Accession			Modific	rations			_
	the criteria to				115	10.13	12	905.68	12.17	4	3618.69			NALQS	Accession			Wound	auons		Light Chain	
	ify another batc click Reset the (116	0.00	1	836.42	16.63	2	1670.82		_	VLDSDG	867100						Light Chain	
step 1 a	above.				117	10.13	12	1207.24	12.17	3	3618.7(-0.67	🕥 VD	NALQS	867100						Light Chain	
🔛 No fi	ilter applied]		118	8.41	6	419.76	6.09	2	837.51	14.07	🌒 Alf	PAPIEK	867100						Light Chain	
Υ		l	Create		120	8.59	4	418.23	7.19	2	834.44	15.57	🌒 DTI	LMISR	867100						Light Chain	
Tata	l Hits m/z	RT (min Chi	Ten Ten		128			1422.74	15.57	2	2843.41	-1.11	🔘 LG(GTIDD <mark>C</mark>	G3HDR3	[8] Car	bamidon	nethyl C			T-complex p	orot
3		15.55 4	arge rag	1	128	9.45	5	1422.74		2	2843.47		-	TCPPCP	867100	[4] Car	bamidon	nethyl C	[7] Carbamidome	ethyl C	Light Chain	
2		16.69 2			130	9.19	5	811.40	5.86	2	1620.79		-	YMHWY	867100						Light Chain	
11	946.95				131	8.98	6	558.05	16.26	4	2228.18		_	SVLTVL	867100		amidatio			_	Light Chain	
8		15.57 3		V	132 145	8.62	8	581.81	12.13	2	1161.61		-	VSLTCL	867100				arbamidomethyl	C	Light Chain	
10	631.642					9.44 0.00	12	609.68 912.03	9.00 15.57	2	3043.38 1822.04		-	WQQGN	867100 867100	[11] Ca	rbamido	metnyi G			Light Chain Light Chain	
11	701.075				159	4.76	1	807.40	6.91	2	1612.75		-	AIFTIHL	G3H3J9	101.01	rbamido	methyl (Kinesin-like	Dre
6	625.31(160	9.01	3	406.21	5.86	4	1620.8(-	YMHWY	867100	[10] 00	irbarnido	meanyre	-		Light Chain	pre
		10.03 5			175	9.07	12	743.41	15.66	3	2227.2(-	SVLTVL	867100						Light Chain	
2 11	934.429			_	189			501.59	13.00	3	1501.75		_	FVESDE	G3I216						Triosephosp	hat
3 10	904.511				189	8.96	4	501.59	13.00	3	1501.75	1.69	S DS	TYSLSST	867100						Light Chain	
11	924.902				198	5.91	1	981.87	14.53	5	4904.30	5.36	🔇 IVG	GYT <mark>C</mark> A	P00761	[7] Car	bamidon	nethyl C	[25] Carbamidon	nethyl	Trypsin OS=	Su
5 12	848.71(15.80 3			198	6.17	1	981.87	14.53	5	4904.3(5.36	🌒 IVG	GGYT <mark>C</mark> A	P00761	[7] Car	bamidon	nethyl C	[15] Deamidatior	n Q [25	Trypsin OS=	Su
7 1	751.889	13.01 2		1	199	6.17	1	818.39	14.53	6	4904.30	4.85	🔘 IVG	GYT <mark>C</mark> A	P00761	[7] Car	bamidon	nethyl C	[15] Deamidation	n Q [25	Trypsin OS=	Sus
12	1272.57	15.80 2			199	5.91	1	818.39	14.53	6	4904.30		-	GGYT <mark>C</mark> A	P00761				[25] Carbamidon	nethyl	Trypsin OS=	Sus
0 1	593.833	11.04 2			206	9.59	6	1273.06		2	2544.11		_	YPSDIA	867100		amidatio				Light Chain	
2 1	938.474	6.92 2			206	9.34	4	1273.06		2	2544.11		-	YPSDIA	867100	[16] De	amidatio	on Q			Light Chain	
7 0	661 34	910 2	Ŧ		220	8.51	7	429.73	2.16	2	857.44	1.64	S HY	NPSLK	867100						Light Chain	

Note: the search results matching the filter criteria turn pink and the total is displayed at the bottom of the table (in this example: 462 matching out of 2867)

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



Then click **Reset the criteria** and enter the next criteria and repeat the process

Having applied the 3 filters there will be **1320** search results remaining

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

Stage 7 Select Peptide ions for Spectral Library at Resolve Conflicts

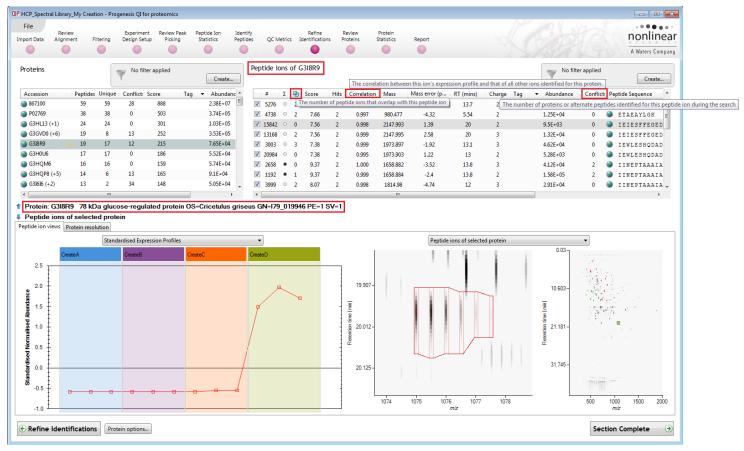
This stage allows you to examine the behaviour of the identified peptides and choose to resolve any conflicts for the various peptide assignments at the protein level.

In addition to indicating the number of conflicting peptide identifications other tools have been introduced at this stage in the workflow that will facilitate the review and selection of peptide ions (Spectra) to be entered into the spectral library.

This allows you to choose spectra on the basis of:

- a) Unique peptide ions, no alternative conflicting peptide identifications
- b) No overlapping peptide ions
- c) Correlation of the peptide ions expression profile with other peptide ions identified for the same protein

The Resolve Conflicts stage can be accessed at the bottom left of the Refine Identifications stage.



Note: If you decide to resolve all the conflicts before applying the selection strategy described below then refer to Appendix 6 (page 53)

For this tutorial, Conflict resolution was NOT performed.

The current protein, G3I8R9 (left hand table), has a total number of 19 peptides, 17 of which are uniquely assigned to this protein, the remaining 2 have a total of 12 conflicting protein assignments.

A possible strategy for the selection of peptide ions for the current protein is to use the tools in the peptide ion table to order and select only those peptide ions that have:

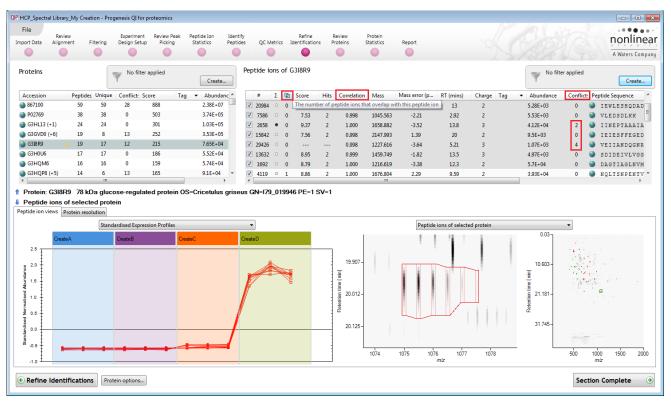
- a) No overlapping peptide ions
- b) No conflicts
- c) Good correlation (i.e >0.925) of the peptide ion's expression with all the other ions identified for this protein

To achieve this you will make use of tagging the 'candidate' ions to be added to the library.

In this example data set we will create 2 spectral libraries:

- A) HCP library_All which will contain all the selected peptide ions for all the Host Cell proteins
- B) NIST_Product Library which contains all the selected peptide ions for the mAb product

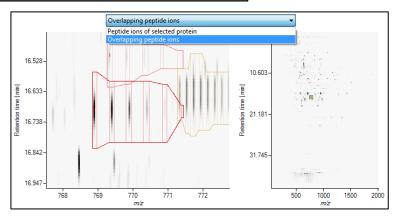
Starting with the HCP library_All we will continue with the G3I8R9 protein, as described above. First order the peptide ion table on Overlapping peptide ions and highlight all of the zero entries.



Note: the good correlation of the expression of the highlighted peptide ions as shown in the bottom left graph as well as the **Correlation** scores highlighted in the peptide ion table.

сp		5113		318R9									W No filter	applied		Create
	#	Σ	囤	Score	Hits	Correlation	Mass	Mass error (p	RT (mins)	Charge	Tag	•	Abundance	Conflict:	Pe	ptide Sequence
V	5276	0	2	8.19	2	0.999	1396.78	-1.22	13.7	2			2.28E+04	0	3	ELEEIVQPIIS
V	3999	0	2	8.07	2	0.998	1814.98	-4.74	12	3			2.91E+04	0	3	IINEPTAAAIA
7	36326	0	2			0.997	1315.63	0.323	11.2	3			1.02E+03	0	3	NELESYAYSLK
V	13168	0	2	7.56	2	0.999	2147.995	2.58	20	3			1.32E+04	0	3	IEIESFFEGED
V	1938	٠	2	9.09	2	0.999	1535.792	1.09	16.7	2			8.93E+04	0	3	TFAPEEISAMV
7	4738	0	2	7.66	2	0.997	980.477	-4.32	5.54	2			1.25E+04	0	3	ETAEAYLGK
V	2724	0	2	8.29	2	0.999	1524.87	-4.03	11.3	3			4.14E+04	0	3	KELEEIVQPII
7	2726	0	2	8.95	2	1.000	1459.751	-0.819	13.5	2			4.46E+04	0	3	SDIDEIVLVGG .

Note: if you highlight a peptide ion with overlapping peptide ions, these can be displayed in the bottom right panel by selecting 'Overlapping peptide ions' from the drop down.

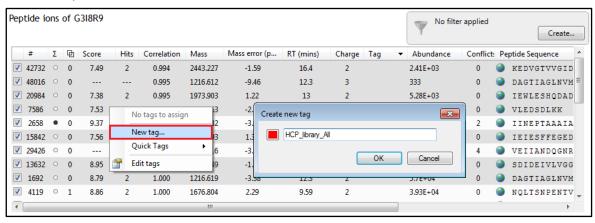


Returning to the highlighted peptide ions for protein with no overlapping peptide ions, now holding down the **Ctrl** key deselect the 2 ions showing 2 and 4 conflicts.

ер	tide io	ons	of C	318R9								Wo filter	applied	Create
	#	Σ	몓	Score	Hits	Correlation	Mass	Mass error (p	RT (mins)	Charge	Tag 🔻	Abundance	Conflict:	Peptide Sequence
V	42732	0	0	7.49	2	0.994	2443.227	-1.59	16.4	2		2.41E+03	0	KEDVGTVVGID
V	48016	0	0			0.995	1216.612	-9.46	12.3	3		333	0	🕥 DAGTIAGLNVM
V	20984	0	0	7.38	2	0.995	1973.903	1.22	13	2		5.28E+03	0	🔮 IEWLESHQDAD
V	7586	0	0	7.53	2	0.998	1045.563	-2.21	2.92	2		5.53E+03	0	🔮 VLEDSDLKK
1	2658	٠	0	9.37	2	1.000	1658.882	-3.52	13.8	3		4.12E+04	2	🚳 IINEPTAAAIA
V	15842	0	0	7.56	2	0.998	2147.993	1.39	20	2		9.5E+03	0	IEIESFFEGED
1	29426	0	0			0.998	1227.616	-3.64	5.21	3		1.07E+03	4	🔇 VEIIANDQGNR
V	13632	0	0	8.95	2	0.999	1459.749	-1.82	13.5	3		4.97E+03	0	SDIDEIVLVGG
V	1692	0	0	8.79	2	1.000	1216.619	-3.38	12.3	2		5.7E+04	0	🕥 DAGTIAGLNVM
1	4119	0	1	8.86	2	1.000	1676.804	2.29	9.59	2		3.93E+04	0	🌒 NQLTSNPENTV
•														•

This leaves 7 remaining peptide ions, with no conflicts and no overlapping peptide ions all showing good correlation of their expression profiles.

Now create a new tag for these peptide ions by right clicking on the highlighted ions, select **New tag** and call it HCP_library_All



To keep track of which proteins you have reviewed, create a protein tag (HCP_library) for the left hand table.

Accession	Peptides	Unique	Conflict:	Score	Tag	 Abundanc ^ 		#	Σ	Φ	Score	Hits	Correlation	Mass	Mass error (p	RT (mins)	Charge	Tag	 Abundance 	Conflic	t: Peptide Sequence
867100	59	59	28	888		2.38E+07 E		42732		0	7.49	2	0.994	2443.227	-1.59	16.4	2		2.41E+03	0	KEDVGTVVGID
P02769	38	38	0	503		3.74E+05		48016		0			0.995	1216.612	-9.46	12.3	3		333	0	DAGTIAGLNVM ^E
🚳 G3HL13 (+1)	24	24	0	301		1.03E+05		20984		0	7.38	2	0.995	1973.903	1.22	13	2		5.28E+03	0	IEWLESHQDAD
3 G3GVD0 (+6)	19	8	13	252		3.53E+05		7586		0	7.53	2	0.998	1045.563	-2.21	2.92	2		5.53E+03	0	VLEDSDLKK
🎯 G3I8R9 🗾 🧕	19	17	12	215	0	7.65E+04		2658	٠	0	9.37	2	1.000	1658.882	-3.52	13.8	3		4.12E+04	2	IINEPTAAAIA
🎯 G3H0U6 📃 🥌	HCP_lib	rary	0	186		5.52E+04		15842		0	7.56	2	0.998	2147.993	1.39	20	2		9.5E+03	0	IEIESFFEGED
🚳 G3HQM6	New tag	j	0	159		5.74E+04		29426	0	0			0.998	1227.616	-3.64	5.21	3		1.07E+03	4	VEIIANDQGNR
G3HQP8 (+5)	Quick T	ags 🕨	13	165		9.1E+04		13632		0	8.95	2	0.999	1459.749	-1.82	13.5	3		4.97E+03	0	SDIDEIVLVGG
🎯 G31616 (+2) 👩	Edit tag	5	34	148		5.05E+04	V	1692		0	8.79	2	1.000	1216.619	-3.38	12.3	2		5.7E+04	0	DAGTIAGLNVM
🎯 G3IAQ0 (+5) 💻	15	- 15	0	139		4.22E+04		4119	0	1	8.86	2	1.000	1676.804	2.29	9.59	2		3.93E+04	0	NQLTSNPENTV _
-		III				•	•							III							

Now work down the list of proteins (ordered on Peptides) in the Left hand table, highlight in turn all the 'non overlapping' peptide ions (in the peptide ion table). Then while holding down the **Ctrl** key (to retain the selection) click on the peptide ions with conflicts to un-highlight them. Finally **right** click on the remaining highlighted peptide ions and select the **HCP_library_All** tag.

Accession	Peptides	Unique	Conflict:	Score	Tag	✓ Abundanc ▲	4	ŧ	Σ	Ð	Score	Hits	Correlation	n Mass	Mass error (p	RT (mins)	Charge	Tag	▼ Abundance	Confli	ct: Peptide Sequence
867100	59	59	28	888		2.38E+07	V 8	3561		0	8.94	2	0.979	1926.789	-1.29	6.47	2		1.68E+04	0	CCAADDKEACF
P02769	o 38	38	0	503		3.74E+05	V 8	8900		0			0 999	1141 702	-4.76	9.46	3		4.28E+03	0	KQTALVELLK
🎯 G3HL13 (+1)	24	24	0	301		1.03E+05	V 1	560		0	8.94	2	📒 НСР_	library_All	-1.36	4.84	2		7.63E+04	0	¥ICDNQDTISS
G3GVD0 (+6)	19	8	13	252		3.53E+05	V 1	3269		0	8.94	2	New	ag	-3.73	4.84	3		3.88E+03	0	¥ICDNQDTISS
G3I8R9	19	17	12	215	•	7.65E+04	V 1	3677	0	1	8.69	2	Quick	: Tags	 -2.17 	10.5	2		1.59E+04	0	RPCFSALTPDE
G3H0U6	17	17	0	186		5.52E+04	V 1	198	0	1	9.29	2	🔗 Edit t	200	-0.9	8.16	2		1.04E+05	0	HLVDEPQNLIK
🚳 G3HQM6	16	16	0	159		5.74E+04	V 1	202	0	1	8.82	2	0.995	/88.462	-2.76	5.77	2		4.7E+04	0	LVTDLTK
G3HQP8 (+5)	14	6	13	165		9.1E+04 🕌	V 1	.062	0	1	8.95	2	0.999	1013.615	2.44	13	2		9.23E+04	0	QTALVELLK
•						•	•														F.

Repeat this process for all the HCP proteins with un-conflicted peptide ions.

Now highlight the NIST mAb protein, in this case the one with the greatest number of peptides, right click and tag it (NIST_Ab_Product). Then in the Peptide lons table order on overlapping peptide ions and highlight all those with no overlaps. Un-highlight those with Conflicts. Then right click on the remaining highlighted peptide ions and create the tag **NIST_product library**.

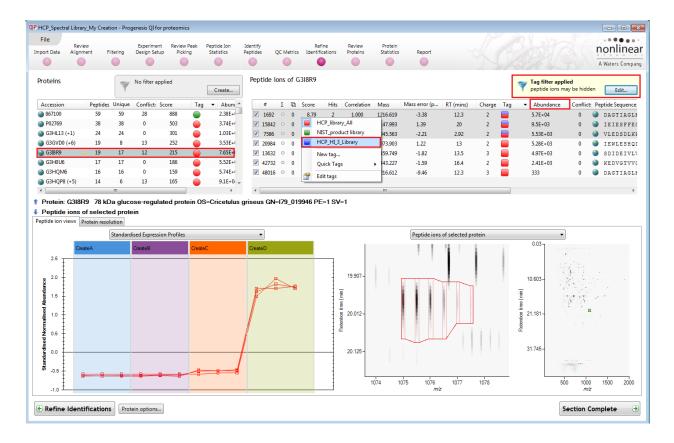
Proteins			Y	No filter	applied		Create	Pepti	de io	ons of	f 867 [.]	100							W No filter app	olied	Create
Accession		Peptides	Unique	Conflict:	Score	Tag	✓ Abunda ^		#	Σ	e Sc	ore Hit	s Correlation	Mass	Mass error (p	RT (mins)	Charge	Tag	 Abundance 	Confli	t: Peptide Sequence ^
3 867100	۰	59	59	28	888		2.38E+0	V	1406	0	0	3.53 12	0.415	868.347	-3.2	2	2		1.28E+04	0	SFNRGEC
P02769		38	38	0	503		3.74E+0!	V	1352	0			0.152	1074.914	-2.89	6.91	4		3.91E+04	0	🕥 VYAČEVTH
🚳 G3HL13 (+1)		24	24	0	301		1.03E+0!		7847	0	o 📙	HCP_lik	7-	87.887	-0.68	19.9	2		7.45E+03	0	ALEWLADI
🎯 G3GVD0 (+6)		19	8	13	252		3.53E+0!		618	0	o 🕒	NIST_p	oduct library	16.966	-3.25	4.66	3		1.54E+05	0	🎯 VGYMHWYQ
G318R9		19	17	12	215		7.65E+04	V	19	0	D	New ta	j	43.135	4.47	15.8	2		2.71E+06	0	GFYPSDIA
G3H0U6		17	17	0	186		5.52E+04	V	42	0	D	Quick T	ags	34.974	5.99	5.23	2		1.97E+06	0	VDNALQSG
🚳 G3HQM6		16	16	0	159		5.74E+04		7288	0	0	P Edit tag	s	07.973	-5.6	17.8	4	_	3.02E+03	1	VVSVLTVL
G3HQP8 (+5)		14	6	13	165	- 	9.1E+04 👻	V 3	4655	0	0		0.871	1807.971	-7.02	18.2	4		235	0	🔇 VVSVLTVL 🗸
•							•	٠ -					III								F.

Note: at this stage, in addition to the tagged groups described above you **can** also create a very conservative library by only tagging the 3 most abundant peptide ions, assigned to different sequences that have no overlapping peptides, no conflicts and good correlation of their expression profiles.

To do this first apply a Filter so that only the Peptide ions that are tagged **HCP_library_All** are shown in the Peptide lons table

Create a filter Show or hide peptide ions based on a selection create the filter. For more guidance, please see	of their tags. Move tags to the appropriate boxes to the <u>online reference</u> .
Available tags:	Show peptide ions that have all of these tags:
 HCP_HI_3_Library (119 peptide ions) NIST_product library (55 peptide ions) 	HCP_library_All (242 peptide ions) Show peptide ions that have at least one of thes tags:
	Hide peptide ions that have any of these tags:

For example returning to the G3I8R9 protein, order on Abundance and highlight the 3 most abundant peptide ions with different sequences



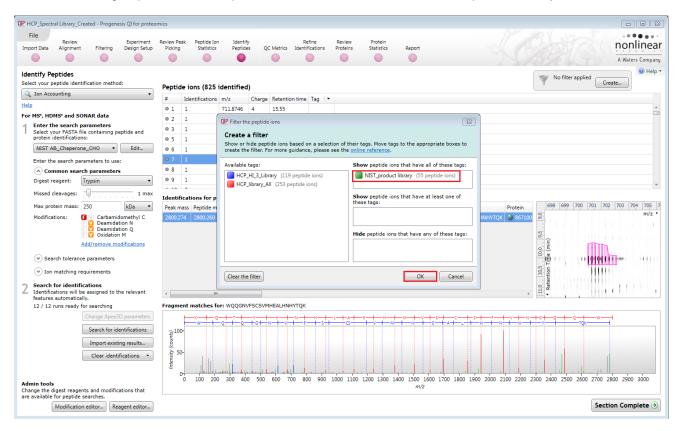
Now, before actually creating the Spectral Libraries from the tagged groups of peptide ions you can review the Quality of fragmentation for each 'candidate' spectra being entered into the library and add additional tags to indicate quality of fragmentation. This then allows to easily control the creation and addition of spectra to a library through application of tag filters.

Stage 8 Review fragmentation quality of candidate spectra

Once you have tagged your candidate spectra at the Resolve Conflicts stage you can now return to the Identify Peptides stage and review the quality of fragmentation for each peptide ion, before adding it to the spectral library.

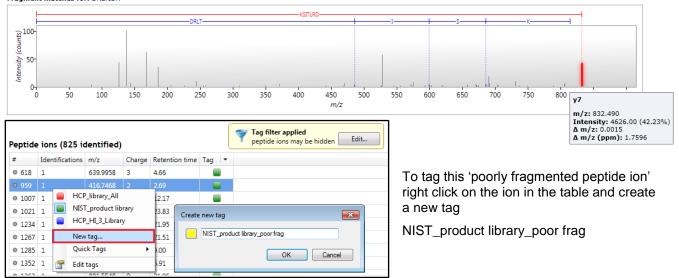
Note: the following section describes one 'possible strategy' on how to decide which spectra to add to the spectral library on the basis of the number and quality of fragment matches. It outlines the processes involved **but** it should be clear that it is by no means definitive as there will always be an element of subjectivity, quality is not an 'exact metric'.

First select the group of candidate spectra to review i.e. start with the NIST_product library



Before going through the filtered list of tagged peptide ions for the NIST Ab product you should first create a new tag to attach to all the spectra that are judged poor in terms of fragmentation or no matches.

For example: for the fragmentation of DRLTISK, shown below, there is only a single match for the y (in this case y 7 representing the un-fragmented precursor) and only 4 b ions matched.



Fragment matches for: DRLTISK

Return to the top of the filtered list of Peptide ions, then step through the list tagging the 'poorly fragmented ions where there is a low number of Fragment matches, poor coverage of the peptide ion sequence or no matched fragments (due to inherited identification from another charge state).

Tag filter applied tide ions may be hidden Edit... Peptide ions (825 identified) Identifications m/z Charge Retention time Tag 0 2 937.4697 2 16.69 1 Ξ • 12 • 13 904.5116 2 17.44 1 1272.5750 2 • 19 15.80 1 • 42 1 1068.4944 2 5.23 43 1 899.4560 2 18.87 • 59 1 541.2741 2 3.24 87 839.4070 11.29 1 2 Identificatio tide ion 12 93(930.8519 935 940 Protein Products BY Matches m/z VFSCSVM 0 10.3 10.2 10.1 10.0 9.9 9.6729. Products The number of matched fragment ime to C Ш Fragment matches for: WOOGNVFSCSVMHEALHNHYTOK sitv 50 3000 200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400 2600 2800 m/z

Example of good fragmentation matches, leave untagged.

Example of poor fragmentation matches, right click on the peptide ion in the table and select the NIST_product library_poor frag

Peptide	ions (825 id	entified)							Tag filter peptide id	r applied ons may be h	idden 🗌	Edit	Ø Help ▼
#	Identifications	m/z	Charge	Retention time	Tag 💌								
1406	1	435.1808	2	2.00									
● 1440	1	565.2901	2	1.99									
• 1524	1	1052.7869	4	19.25									
● 1776	1	705.0713	4	10.02									
• 1976	1	802.5004	10	21.93									=
• 2693	1	925.9694	4	HCP_libra									
© 2741	1	1401.1638	2	HCP_librar NIST_prod									
3234	1	387.8804	3	HCP_HI_3									
0.0000	4	2121444	-		uct library_po	oor frag							•
Identifica	ations for pept				accupiany_pe	sormag				924	925 9	26 927	
	_		Protein	New tag	_					924 8	925 9	26 927	928 92
/KPTQTLTL	TCTFSGFSLSTAG	MSVGWIR	8671	Quick Tag	5	•				_			1111-1
				🚰 Edit tags						21.0			
										21.2 (min)		10000	
										me (r			1.1.1
										- F	Ц		
										ntior			
										21.6 21 Retention			
4									•	- •			
Fragment	t matches for:	ESGPALVKP	TQTLTLT	CTFSGFSLSTAG	MSVGWIR								
F	RIW	-+G+v+s-	-MG-	A+T+S+L+			SFGS	FTCTLTLTQTPKVLAPG	SE				
					ESGPALVK	(PTQTLTLTCTFSGFSLS	AGMSVGWIR					— í	
Intensity (counts) -05	I												
Inte													
0	200 40	0 600	800	1000 1200	1400 160		00 2200 m/z	2400 2600	2800 30	00 3200	3400	3600 3800	4000

Here there is not only limited Fragment matches for y ions but also only the matching of the precursor ion.

Finally where you have initially tagged peptide ions, where the identity has been inherited from another charge state, this will show no Fragment matches. At this point, the option would be not to have tagged it in the first place, when you were creating the original tagged list as described in the previous section.

Peptide	ions (825 ide	entified)					Tag filter applied peptide ions may be hidden Edit
#	Identifications	m/z	Charge	Retent	tion time	Tag 💌	
1007	1	1810.3587	2	12.17			
● 1021	1	1242.3072	3	23.83			
● 1234	1	1002.8708	8	21.95			
1267	1	1119.7197	6	21.51			
• 1285	1	1015.4662	3	9.00	н нс	CP_library_All	
● 1352	1	469.7359	4	6.91	_	IST_product library	
● 1363	1	891.5548	9	21.95		CP_HI_3_Library	
© 1371	1	1603.9956	5	21.94	_	IST_product library_poor frag	
0 1400	4	435 1000	2	2.00	_	ew tag	
Identifica	ations for pept					uick Tags	4045
_			Protein	Pro			1015 1020
GNVFS C SV	MHEALHNHYTQ	К	§) 86710	0 0	🚰 Edi	lit tags	
*							z/m
						ched fragments fo dentification is inherited from anoth	

Once you have completed the review process of the NIST_product library then repeat the process for the remaining 2 candidate libraries (HCP_library_All and as required HCP_Hi_3_Library) creating additional tags for the corresponding poor fragmentation (i.e. HCP_library_poor frag).

Having done this you will have 6 tagged groups of peptide ions as shown below.

QIP Filter the peptide ions	×
Create a filter Show or hide peptide ions based on a selection of the the filter. For more guidance, please see the <u>online re</u>	
Available tags:	Show peptide ions that have all of these tags:
 NIST_product library (55 peptide ions) NIST_product library_poor frag (34 peptide ions) HCP_HI_3_Library (119 peptide ions) HCP_library_All (253 peptide ions) HCP_Library_poor frag (189 peptide ions) HCP_HI_3_library_poor frag (83 peptide ions) 	Show peptide ions that have at least one of these tags:
<u>Clear the filter</u>	OK Cancel

Note: the actual numbers will vary depending on how the spectra were reviewed.

Now move forward to the next section to see how these tags can be used to create Spectral Libraries.

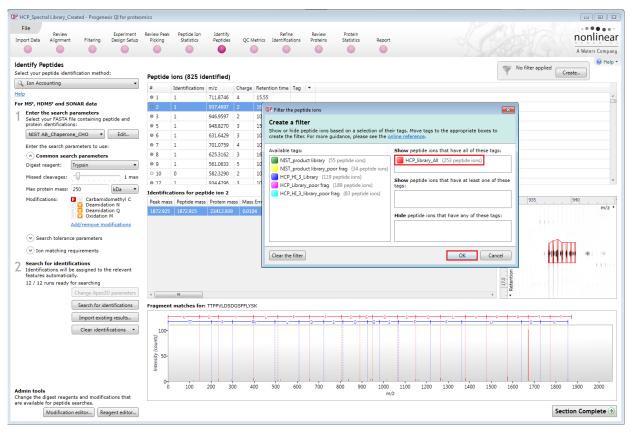
Stage 9: Creating a Spectral Library

Having tagged and reviewed the 'candidate' lists of peptide ions in the previous section you can now create the actual libraries by first filtering to the required list of peptide ions and then exporting them to a Spectral library.

Note: if you have come straight to this point in the workflow and you have already unzipped the **Library_search_data_set.zip** into same folder then examples of the spectral libraries will already exist as described below

As an example, create two Spectral Libraries firstly a Spectral Library called **HCP_library_All_raw** which is based on all peptide ions tagged **HCP_library_All** and a second library **HCP_library_All_reviewed** is the same as the first library minus the spectra judged as having poor fragmentation.

Click on **Create** and drag the **HCP-library_All** tag on to the Show panel and click OK. This will reduce the table to showing only those peptide ions which have this tag.



To create the library from the tagged filter group, select **Export spectral library...** from the file menu

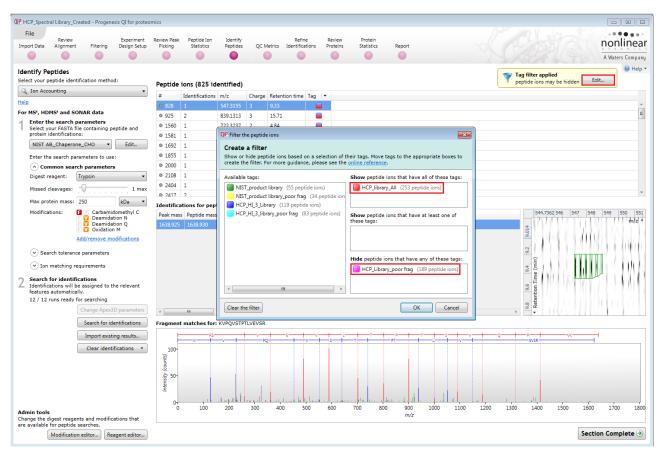
Then save the new spectral library as HCP_library_All_raw.msp

File Save	Review Peak Picking	Peptide Ion Statistics	Identify Peptides	QC M	Refir letrics Identifica		eviev oteir
Close) (
Export peptide ion data							
Export deconvoluted peptide ion data	Peptide	ions (825 id	entified)				
Import peptide ion numbers as tag	#	Identifications	m/z	Charge	Retention time	Tag 💌	
Export all identifications	• 828	1	547.3155	3	9.33		
Export spectral library	• 893	2	768.8687	2	6.79		
Export Peptide3D spectra to MGF	© 925	2	839.1313	3	15.71	-	
		3	566.7668	2	6.27	-	
Export inclusion list	• 1414	5				_	
	• 1414 • 1533	1	675.3708	2	13.13	-	
	h	-	675.3708 722.3237	2 2	13.13 4.84	-	
Export inclusion list	© 1533	1		-		-	

Now create the HCP_library_All_raw_reviewed spectral library.

First modify the applied tag filter to hide the spectra that have been judged as poorly fragmented.

Click on **Edit** to open the existing Tag filter. Then drag the tag HCP_Library_poor frag on to the **Hide** panel while leaving the existing tag in the **Show** panel



This will further reduce the number of tagged peptide ions in the table.

As before to create the library from the tagged filter group, select **Export spectral library...** from the file menu

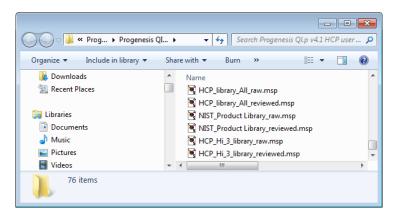
Then name and save the library, **HCP_library_All_reviewed**.msp

Tip: if saving the library to the same folder as the example library files, use a different name if you wish to retain both libraries.

Now clear the filter by clicking on **Edit** followed by **Clear the filter**, then repeat the process for the **NIST_product library** and the **HCP Hi_3_Library**.

Once you have done this you will have 6 .msp files that you can use for searching the Batch samples described in the next section.

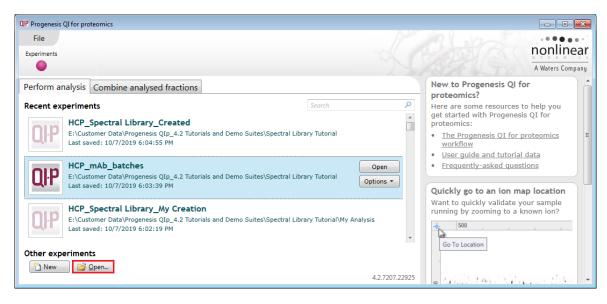
1	File								
-	Save	Review Pea	k Peptide Ion Statistics	Identify Peptides	QC N	Refir Identifica			levie rotei
1	Close		•						0
	Export peptide ion data								
	Export deconvoluted peptide ion data	Peptide	e ions (825 id	entified)					
	Import peptide ion numbers as tag	#	Identifications	m/z	Charge	Retention time	Tag	-	
	Export all identifications	• 828	1	547.3155	3	9.33			
	Export spectral library	© 893	2	768.8687	2	6.79			
	Export Peptide3D spectra to MGF	© 925	2	839.1313	3	15.71			
	Export inclusion list	0 1533	1	675.3708	2	13.13			
		0 1581	1	456.7962	2	8.87			
	Experiment properties	© 1855	1	751.8094	2	6.27			
	Show Clip Gallery	© 2000	1	552.6267	3	12.28			
			5	454.2643	2	9.67			



Note: Examples of these Spectral Libraries are provided with the Tutorial data that you downloaded to perform this tutorial.

Stage 10: Autoprocessing of the Batch Samples

As an example of using the spectal library you have just created use it to identify and measure the Host Cell Proteins (HCP) present in the **HCP_mAb_Batches** of NIST Ab product. To do this use the second tutorial archive that you restored at the beginning of this tutorial.



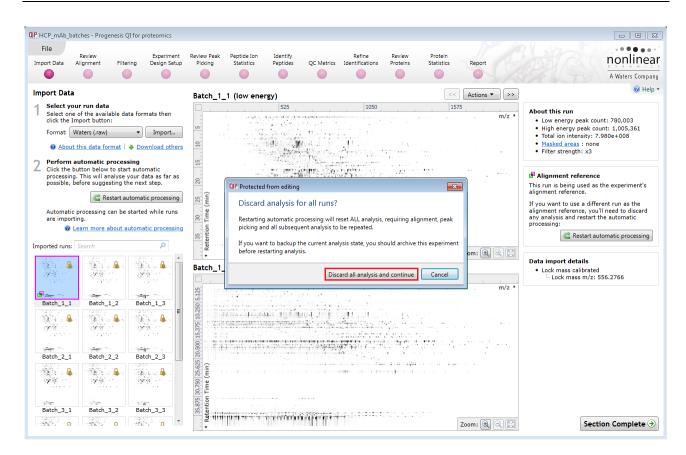
When you open the restored **HCP_mAb_Batches** experiment it will be already at the Identify Peptides stage but no identification has been performed.

QP HCP_mAb_Batches - Progenesis QI for proteomics								
File	Review Peak Peptide Picking Statisti		QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report	nonlinear A Waters Company
Identify Peptides Select your peptide identification method:	Features							Whelp *
-	# Identification	m/z Ci	harge Retentio	n time 🛛 Tag 🛛 💌				
Help For MS', HDMS' and SONAR data	• 1 0	711.8729 4						A ====================================
Enter the search parameters	0 2 0	937.4669 2						
Select your FASTA file containing peptide and protein identifications:	030	946.9574 2						
SWISSPROT-1.0 Edit	04 0	948.8245 3						
	050 060	603.3439 1 701.0740 4						
Enter the search parameters to use:	070	631.6414 3						
Common search parameters Digest reagent: Trypsin	08 0	625.3142 3	16.63					
Missed cleavages: Max protein mass: 250 kDa • Modifications: Search tolerance parameters Modifications will be assigned to the relevant features automatically. 15 / 15 runs ready for searching	Identifications for	peptide ion 1	ass Mass Error	(Da) Mass Error (ppm) Score	Seq. start See	a, end Sequence Prote	in Product
Change Aper3D parameters Search for identifications Import existing results Clear identifications * Admin tools Change the digest reagents and modifications that are available for peptide searches.							1 selecte	ment ions
Modification editor Reagent editor								Section Complete $ i $

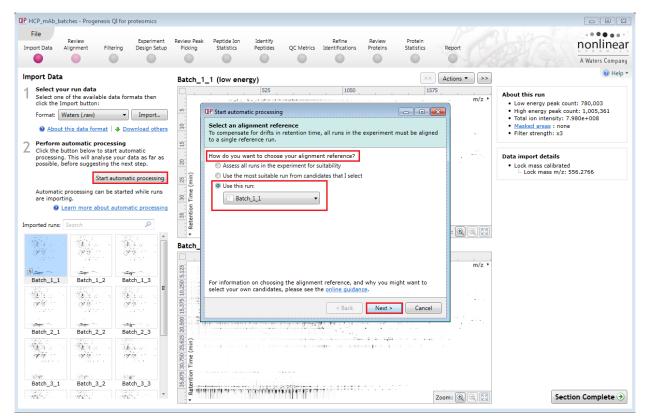
Note: the data has been autoanalysed and an Experiment Design applied as part of this automatic analysis.

To perform Identification using the Spectral Libraries that you made in the previous section go to Stage 11 (page 32)

The following pages describe the automatic analysis that has been performed on this data which you can repeat by first discarding the automatic analysis by clicking **Restart automatic processing**.



On clicking **Discard all analysis and continue** the first Automatic processing dialog will open asking how you want to choose the alignment reference to be determined



The list below describes what options were applied at each stage to automatically analyse the Batches data set.

Setting up the steps for the auto processing:

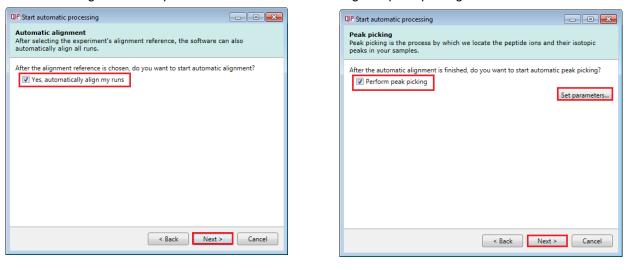
The Alignment Reference was set as 'Batch 1_1' (as shown above).

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Progenesis QI for Proteomics Spectral Library User Guide

Automatic alignment was performed and the default settings for peak picking.



The default Peak picking limits are set as Automatic (as shown below)

QP Peak Picking Parameters	QP Start automatic processing
Runs for peak picking Peak picking limits Maximum charge Retention time limits Sensitivity	Experiment design Experiment designs allow you to group and compare your samples according to their experimental conditions.
the peak picking algorithm using these different methods. Each sinestivity method examines the intensities of groups of KB speaks to judge whether they are likely to form part of an ion or whether they represent noise and so should be ignored. Peaks that are rejected as noise will not be used to build ion outlines. The sensitivity value, the more peptide ions will be detected. Set the set of the value of the detected.	By defining an experiment design in advance, statistical measures such as ANOVA can be calculated automatically. Set up an experiment design Enter a name for the experiment design: Batches Load the criteria for grouping runs from this file: styProgenesis QLp v4.1 HCP user guide and Tutoria the Batches.spl Browse
Chromatographic peak width	Group runs by: Condition
The chromatographic peak width gives the length of time over which an ion has eluted. If you set a minimum peak width, any ion that has eluted over a shorter period will be rejected.	
	What file formats are supported?
OK Cancel	< Back Next > Cancel

For Experiment design setup: use file mAb_Batches.spl (available in same folder that the **Library_search_data_set.zip** was extracted.

QP Start automatic processing	OP Start automatic processing
Identify peptides Get identifications for the peptides in your samples using their MS ⁺ fragmentation data.	Protein Quantitation If you've included a known amount of a calibrant protein in each of your samples, you can calculate the absolute amounts of each identified protein.
After peak picking has completed, do you wish to automatically identify peptides?	Quantitation method:
Use MS ^E data from my runs to identify peptides	Relative Quantitation using Hi-N
Set parameters	Number of peptides to measure per protein (N):
 Without peptide identifications, only a limited set of experimental QC metrics will 	Use protein grouping i.e. hide proteins whose peptides are a subset of another
be available.	protein's.
< Back Next > Cancel	< Back Finish Cancel

Do not perform identification by deselecting Use MS^E data...... untick this option

Use default settings for Quantitation (Relative Quantitation using Hi-N) and Use protein grouping

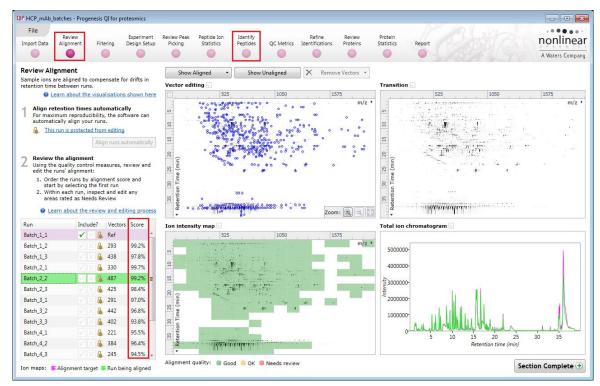
Click Finish to start the analysis.

The automatic processing completes in approx. 5min (depending on your machine spec).

	Review ignment Filter	Experiment ing Design Setup	Review Peak Picking	Peptide Ion Identif Statistics Peptide		Review Protein Proteins Statistics	Report	nonlinea
	•							A Waters Compan
nport Data			Batch 1	1 (low energy)		<	Actions ->>	🔞 Help
Select your	run data			_1 (1011 chergy)	1050	1575	5	
Select one o click the Imp	f the available dat	ta formats then		a series and the second				About this run Low energy peak count: 780.003
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Click the but	tomatic process ton below to star This will analyse y		20 15	QP Processing Complet			.	P Alignment reference
	ore suggesting th		-	Automatic proces	· · ·			This run is being used as the experiment's alignment reference.
			35 30 25 Retention Time (min)	Time taken: 4 minutes	21 seconds		_	If you want to use a different run as the alignment reference, you'll need to discard
are importin		started while runs	Time	 Importing runs: 	15 of 15 processed			any analysis and restart the automatic processing:
0 <u>Lea</u>	arn more about au	utomatic processing	tion	 Selecting reference: Aligning runs: 	Batch_1_1 14 of 14 processed			Restart automatic processing
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्रिके से स्थिति संसर्वे		2). 73	Batch_1				_	 Lock mass calibrated
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Batch_3_1	Batch_3_2	Batch_3_3	8 a					
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Having repeated the analysis you can either **Close** it, where it will remain at the Import Data stage and you can step through the Alignment and other stages or you can open it directly at the **Identify Peptides** stage.

Here we will close and then click on Review Alignment on the workflow at the top of the screen.



Note: the high quality of retention time alignment of the runs as indicated by the high % Score. Now click on Identify Peptides and go to the next section.

Stage 11: Identifying and measuring HCP proteins using a spectral library

As an example of using the spectral libraries you have just created use it to identify and measure the Host Cell Proteins (HCP) present in the **HCP_mAb_Batches** of NIST Ab product. To do this use the second tutorial archive that you restored at the beginning of this tutorial.

If you have just reanalysed this you will be currently at the Identify Peptide stage in the workflow.

Select Spectral Library Search from the peptide identification methods

GIP HCP_mAb_batches - Progenesis QI for proteomics						- • •
File						
Review Experiment Import Data Alignment Filtering Design Setup	Review Peak Peptide Io Picking Statistics		Refine QC Metrics Identifications	Review Protein Proteins Statistics	Report	nonlinear
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Identify Peptides						🕜 Help 🔻
Select your peptide identification method:	Features				No filter applied	reate
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Version: 1.0			15.56			
Q PLGS (*.xml) Version: 1.0	0 2 0	937.4693 2	16.68			
SEQUEST (dta & out files)	030	946.9596 2	10.75			
Version: 1.0	04 0	948.8268 3	15.57			
SEQUEST (dta & pepXml files) Version: 1.0	050	701.0757 4	10.02			
Q Phenyx	° 6 0	631.6430 3	10.75			
Version: 4.2.7207.22925	070	603.3453 1	17.43			
Spectral Library Search Version: 4.2.7207.22925	080	625.3157 3	16.63			Ŧ
Q Ion Accounting	Identifications for p	eptide ion 1				
Version: 4.2.7207.22925	Peak mass Peptide n	nass Protein mass	Mass Error (Da) Mass Error	(ppm) Score Seq. start Se	peno bequence rioteni r	712 713 714 715 716
Max protein mass: 250 kDa 🔻					51 1	·m/z ·
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Add/remove modifications					U U U U U U U U U U U U U U U U U U U	N. C.
Search tolerance parameters					17 16 15 n Time (min)	
 Ion matching requirements 					8 13 11 Retention	
7 Search for identifications					Ret Ret	
Identifications will be assigned to the relevant features automatically.						
15 / 15 runs ready for searching						
Change Apex3D parameters						
Search for identifications						
Import existing results			No ider	tification	selected	
Clear identifications		Se	elect an identificatio	n above to view its	matched fragment ions	
Admin tools						
Change the digest reagents and modifications that are available for peptide searches.						
Modification editor Reagent editor					Sect	tion Complete 🏵
Mouncation Editor						

As no searches have been performed there will be no libraries to select. Click **Browse** and select the **NIST_Product Library_raw.msp** from the folder you restored the Tutorial data.

OP HCP_n	nAb_batches	- Progen	esis QI for p	proteomics											
File Import D		nent	Filtering	Experiment Design Setup	Review Peak Picking	Peptide Ion Statistics	Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report			nonlinear
-					•								1811	C T	A Waters Company
	y Peptides				Identificat	tions:					V	Show only spe	tral library ID:	S Peptide ion: #	1 😢 Help 🔹
	our peptide i		tion metho	d:	# Pr	otein	Sequence				Score	Fragment mat	thes Peak ma	709 710 7	11 712 713 714 715 716
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Help					OP Open								×	4	
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O 346	966.4609	3	15.55					Sea	ren tor iden	uncation	is using th	e paner ac	the left		
0 347		1	1.39												
0 348		1	8.61												
0 349	760.5861	1	36.71												

Now check the tolerances as shown below

The NIST_Product Library_raw will appear as the current spectral library

_	batches - Proge	nesis QI for p	proteomics										_ • •
File Import Data	Review Alignment	Filtering	Experiment Design Setup	Review Peak Picking	Peptide Ion Statistics	Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protein Statistics	Report		A Waters Company
Identify Pe	eptides		_	Identifica	tions:	_				V	Show only spectral I	ibrary IDs Peptide ion: #1	(2) 11-1-
Spectra Help 1 Enter se Select th NIST_F	Precursor tolera Fragment tolera	ters ry to search raw create a sp ince: 10 ince: 10	• Browse ppm • ppm •	# Pr	otein	Sequence				Score	Fragment matches	Peak ma	L 712 713 714 715 71 m/z
_	tention time wi gments per pep		or more		S	Searching						▲ Rete	
	Share hits acro	ss charge s	tates 🕜 <u>Why?</u>							Cano	el		
📕 Identific	for identificat ations will be as automatically. Search for ider	ssigned to t	he relevant										

The Spectral library search parameters are described below

Precursor tolerance

Specifies the mass error below which a precursor is considered a match to a spectral library entry.

Fragment tolerance

Specifies the m/z error below which a fragment is considered a match to a spectral library entry's fragments.

Retention time within

Specifies the retention time error, below which a precursor is considered a match to a spectral library entry. If this is not selected, or a spectral library entry has no retention time information, it has no effect.

Fragments per peptide

The minimum number of fragment matches required for a match to be accepted. If not selected, all matches are accepted, even those with no fragment matches. If a spectral library entry has fewer fragments than the given value, all of its fragments must match. If a spectral library entry has no fragments, matches to that entry will be accepted regardless of this value.

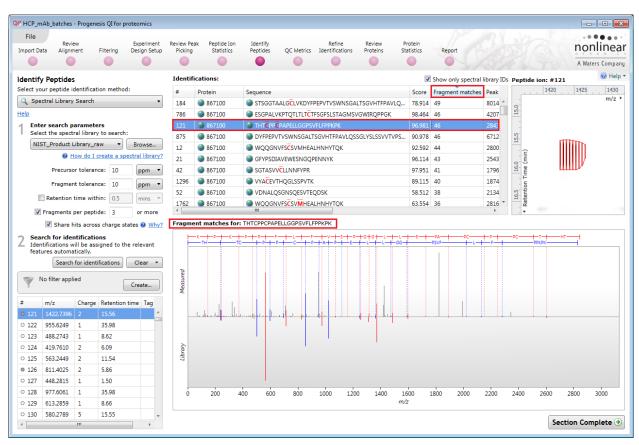
Share hits across charge states

If selected, charge state deconvolution of identifications will be performed. This means that if only some charge states of a given peptide were identified by the spectral library search, the other charge states will inherit the same identifications.

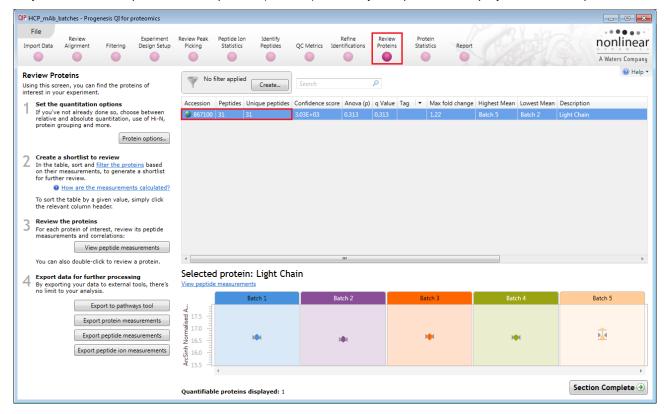
Tick the tolerances as shown above

Start the search by clicking Search for identifications

When the search completes order the table on Fragment matches, and select an example in the list. The 'mirror' plot will now display the fragment matches for the current peptide sequence showing the Measured and Library b and y ions.



Now click on **Review proteins** on the workflow to examine the quantified proteins. As the Product library only contained the spectra for one protein (867100) then only one protein is displayed with 31 Peptides.

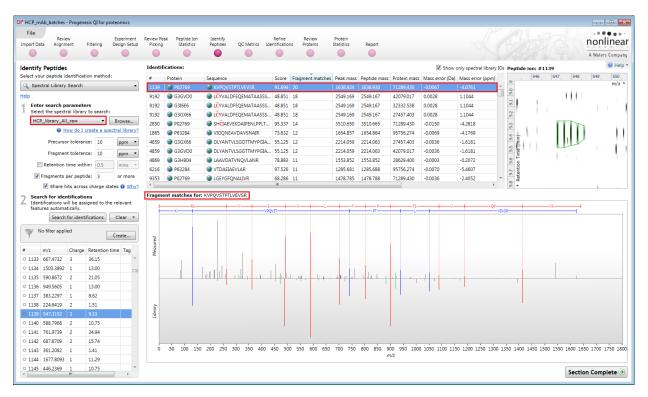


The protein quantitation (options) are currently set to Relative Quantitation based on the 3 most abundant peptides (Hi-N).

Note: having searched using the NIST_ product Library_raw you can set the normalisation to use only the peptide ions for this protein, as the same amount of this protein is present in all the batches, refer to page 15 for details on how to change the normalisation).

Now use the HCP_library_All_raw.msp to search the batches by first browsing for the new library then

Click Search for identifications



Change the method for Protein Quantitation to **Absolute Quantitation for HCP** using Hi-N, by clicking on **Protein options** and use the peptides identified as belonging to the Chaperone Protein ClpB from E.coli, that was spiked into each sample at 200fmol, as the Calibrant (Accession P63284).

HCP_mAb_batches - Progenesis QI for proteomics												
File Review Experiment nport Data Alignment Filtering Design Setup	Review Peak Peptide Picking Statist		entify ptides	QC Metrics	Refine Identifications		Protein Statistics	Report				nonlinea A Waters Compa
						•	•	-		1 201 101	CALL?	
eview Proteins sing this screen, you can find the proteins of terest in your experiment.	No filter app	lied Cre	eate	Search	ş	þ						🕑 Help
Set the quantitation options	es Confidence score	Anova (p)) q Value	Tag 🔹	Max fold change	Highest Mear	Lowest Mear	Description		*		
If you've not already done so, choose between relative and absolute guantitation, use of Hi-N,	90.4	< 1.1E-16	< 1.1E-10	5	Infinity	Batch 1	Batch 3	ATP syntham	e subunit beta OS=	Cricetulus griseus GN=I79_013474	4 PE=3 SV=1	
protein grouping and more.	66.1	1.53E-07	3.05E-07		8.23E+06	Batch 1	Batch 4	Calreticulin	OS=Cricetulus grise	us GN=H671_3g9525 PE=3 SV=1		
Protein options	171	0.00106	0.00128		1.74	Batch 5	Batch 3	Chaperone	protein ClpB OS=Es	herichia coli (strain K12) OX=833	33 GN=clp8 PE=1 SV=1	
	301	1.09E-05	1.64E-05		2.08	Batch 1	Batch 4	Elongation	factor 1-alpha 1 OS=	Cricetulus griseus GN=179_00993	35 PE=4 SV=1	
Create a shortlist to review	119	2.22E-16	1.07E-15		Infinity	Batch 1	Batch 4	Heat shock	cognate 71 kDa pro	tein OS=Cricetulus griseus GN=17	9_021800 PE=3 SV=1	
 In the table, sort and <u>filter the proteins</u> based on their measurements, to generate a shortlist 	68.2	9.66E-07	1.665/						tognate 71 kDa pro	tein OS=Cricetulus griseus GN=I7	9 022974 PE=3 SV=1	
for further review.	95.5	< 1.1E-16	< 1.1	P Protein qui	antitation options			— ×	vdrogenase A chai	n OS=Cricetulus griseus GN=I79_I	009741 PE=4 SV=1	
How are the measurements calculated?	119	< 1.1E-16	< 1.1	Quantitation	method:					GN=I79 022400 PE=4 SV=1		
To sort the table by a given value, simply click the relevant column header.	94,3	9.11E-05	0.000	Absolute Q	uantitation for H	CP using Hi-N		•	lyl cis-trans isomera	ase OS=Cricetulus griseus GN=179	001023 PE=3 SV=1	
	14.6	1.82E-13	4.856					3 🗣		griseus GN=179 002954 PE=4 SV:	-	
For each protein of interest, review its peptide	16.5	3E-07	5.54E	Number of p	eptides to measu	re per protein		5 💌		DS=Cricetulus griseus GN=I79 00		
measurements and correlations:	136	2.93E-05			c	alibrant access	ion: P6			DS=Cricatulus arisaus GN=179 00		
View peptide measurements	81.8	1.06E-06			orant protein can'		w will P63284	4				
You can also double-click to review a protein.	834	1.45E-08		measure	ments be calcula	ted?	G3IHP		IpB OS=Escherichia (coli (strain K12) OX 80 PE=3 SV=	-	
	99.2		0.005			Amount (fn			lpha 1 OS=Cricetulu	s griseus GN=I79		
Export data for further processing By exporting your data to external tools, there's	55	< 1.1E-16		Use prote	in grouping i.e. hi	de proteins who				us griseus GN=179_008223 PE=3 S	51/-1	
no limit to your analysis.	55	< 1.1E-10	1.1	of anothe	r protein's.				chain OS=Cricetuit	us griseus GIV=1/9_008223 PE=3 3	5V=1	
Export to pathways tool						_						
	Selected prot		aper				ОК	Cancel	DX=83333 GN	l=clpB PE=1 SV=1		
Export protein measurements	View peptide measur	ements							1			
Export peptide measurements	a 13.096667		Batch 1			Batch 2		Bat	ch 3	Batch 4	В	latch 5
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There are 24 quantifiable proteins identified, across the batches that you are testing

PHCP_mAb_batches - Progenesis QI for proteomics File Review Experiment I Import Data Augment Filtering Design Setup		estide Ion Identify Refine Refine Potoens	Protein Statistics Report										nonlinear A Waters Company
Review Proteins Using this screen, you can find the proteins of interest in your experiment.	W No filte	er applied Create											🥹 Help
Set the quantitation options	Lowest Mean	Description		Amount (fmol) - Batch 1	Amount (fmol) - Batch 2	Amount (fmol) - Batch 3	Amount (fmol) - Batch 4	Amount (fmol) - Batch 5	Amount (ng) - Batch 1	Amount (ng) - Batch 2	Amount (ng) - Batch	3 Amount (ng) - Batch	4 Amount (ng) - Batch
If you've not already done so, choose between relative and absolute quantitation, use of Hi-N.	Batch 4	Protein disulfide-isomerase A3 OS=Cricetulus griseus GN=I79_0037	65 PE=4 SV=1	53.4	24.9	0.048	0	0	2.69	1.26	0.00242	0	0 ^
protein grouping and more.	Batch 5	Trypsin OS=Sus scrofa PE=1 SV=1		55.5	53.5	47.6	44	34.3	1.39	1.34	1.19	1.1	0.857
Protein options	Batch 5	Protein disulfide-isomerase A6 OS=Cricetulus griseus GN=I79_0076	16 PE=4 SV=1	63.8	29.4	0.495	0.314	0.161	1.83	0.841	0.0142	0.009	0.00461
	Batch 4	Heat shock cognate 71 kDa protein OS=Cricetulus griseus GN=I79_	21800 PE=3 SV=1	65	29.4	0.101	0	0	1.48	0.668	0.00231	0	0
Create a shortlist to review In the table, sort and filter the proteins based	Batch 5	78 kDa glucose-regulated protein OS=Cricetulus griseus GN=179_01	9946 PE=1 SV=1	92.6	43.5	2.66	0.584	0.0863	6.72	3.16	0.193	0.0424	0.00626
on their measurements, to generate a shortlist	Batch 5	Pyruvate kinase OS=Cricetulus griseus GN=179_004880 PE=3 SV=1											0
for further review. Bow are the measurements calculated?	Batch 5	Tubulin beta-3 chain OS=Cricetulus griseus GN=I79_000194 PE=3 S	/=1	140	67.8	2.56	0.511	0.165	12.2	5.88	0.222	0.0443	0.0144
To sort the table by a given value, simply click	Batch 4	Peroxiredoxin-1 OS=Cricetulus griseus GN=I79_002954 PE=4 SV=1		148	71.9	1.57	0	0	3.33	1.62	0.0353	0	0
To sort the table by a given value, simply click the relevant column header.	Batch 5	60 kDa heat shock protein_ mitochondrial OS=Cricetulus griseus GN	=I79_011398 PE=3 SV=1	148	66.8	1.15	0.0187	0	8.41	3.79	0.0652	0.00105	0
Review the proteins	Batch 5	Peptidyl-prolyl cis-trans isomerase OS=Cricetulus griseus GN=I79_0	01023 PE=3 SV=1	153	82.3	12.1	6.16	4.03	2.58	1.39	0.204	0.104	0.0682
For each protein of interest, review its peptide	Batch 4	Calreticulin OS=Cricetulus griseus GN=H671_3g9525 PE=3 SV=1		155	68.3	0.0509	1.95E-05	0.000105	7.52	3.31	0.00246	9.44E-07	5.06E-06
measurements and correlations:		Chaperone protein ClpB OS=Escherichia coli (strain K12) OX=83333	GN=clp8 PE=1 SV=1	200	200	200	200	200	19.2	19.2	19.2	19.2	19.2
View peptide measurements	Batch 5	Heat shock cognate 71 kDa protein OS=Cricetulus griseus GN=I79	122974 PE=3 SV=1	233	111	3.92	0.0546	0	5.38	2.57	0.0908	0.00126	0
You can also double-click to review a protein.	Batch 4	Actin_ cytoplasmic 1 OS=Cricetulus griseus GN=I79_001666 PE=3 S	/=1	279	132	4.77	0	0	11.8	5.57	0.201	0	0
Export data for further processing	Batch 5	Serum albumin BSA		473	232	14.8	1	0.178	33.7	16.5	1.05	0.0713	0.0127
By exporting your data to external tools, there's no limit to your analysis.	Batch 5	Elongation factor 1-alpha 1 OS=Cricetulus griseus GN=I79_009935	E=4 SV=1	706	520	496	354	298	39.2	28.9	27.6	19.7	16.5
Export to pathways tool Export protein measurements	- Selected p	protein: Pyruvate kinase OS=Cricetulus grise	us GN=179_004880 PE=3 SV=	:1				.11					
Export peptide measurements		Batch 1	Batch 2			Batch 3			Batch 4			Batch 5	
Export peptide ion measurements	Sinh Normalised Abundance	yêk	141			⊉ r			1 4 1			bot	
[₹ -1 -	proteins displayed: 24										s	ection Complete

Note: having used Absolute Quantification for HCP using Hi-N, the spiked protein (Calibrant (Accession P63284) will show the same Amount (either fmol or ng) across all Batches under test.

Now order the data at the **Review Proteins** stage, starting with Batch 1. The first protein in the list shows decreasing presence as you go from Batch 1 to 2 to essentially undetected in Batch 3.

OP HCP_mAb_batches - Progenesis QI for proteomics File Review Experim	ent Review Peak Peptide Ion Identify Ret		tein							J.	31.47	nonlinear
Import Data Alignment Filtering Design S	etup Picking Statistics Peptides QC Metrics Identifi		istics Report									A Waters Company
Review Proteins Using this screen, you can find the proteins of interest in your experiment.	Vo filter applied Create	٩										😧 Help 🕶
Set the quantitation options	Description		Amount (fmol) - Ba	tch 1 Amount (fmol)	Batch 2 Amount (fmol) - Batch	3 Amount (fmoi) - Ba	tch 4 Amount (fmol) - Batch	5 Amount (ng) - Batc	h 1 Amount (ng) - Batch	2 Amount (ng) - Batcl	Amount (ng) - Ba	tch 4 Amount (ng) - Batch 5
If you've not already done so, choose between relative and absolute	L-lactate dehydrogenase A chain OS=Cricetulus griseus GN=I79_00974	1 PE=4 SV=1	19.4	9.17	0	0	0	0.24	0.113	0	0	0 *
	Annexin OS=Cricetulus griseus GN=I79_022682 PE=3 SV=1		25.6	10.5	0	0	0	0.699	0.287	0	0	0
	Tubulin beta chain OS=Cricetulus griseus GN=I79_008223 PE=3 SV=1		30.7	13.6	0	0	0	1.54	0.68	0	0	0
Protein options	Alpha-enolase OS=Cricetulus griseus GN=I79_019072 PE=4 SV=1		35	37.8	40.3	40.2	33.7	0.618	0.668	0.713	0.712	0.597
Create a shortlist to review	ATP synthase subunit beta OS=Cricetulus griseus GN=I79_013474 PE=	SV=1	35.6	16.8	0	0	0	2.01	0.951	0	0	0
	ATP synthase subunit alpha_ mitochondrial OS=Cricetulus griseus GN=	179_007391 PE=4 SV=1	37.6	16.9	0.584	0.453	0.0795	0.264	0.119	0.0041	0.00318	0.000557
generate a shortlist for further review.	Nucleolin OS=Cricetulus griseus GN=179_022400 PE=4 SV=1		39.5	17.7	0	0	0	2.07	0.93	0	0	0
	14-3-3 protein eta OS=Cricetulus griseus GN=I79_011108 PE=3 SV=1		45.7	22.4	0.394	0	0	1.25	0.613	0.0108	0	0
calculated?	Protein disulfide-isomerase A3 OS=Cricetulus griseus GN=I79_003765	PE=4 SV=1	53.4	24.9	0.048	0	0	2.69	1.26	0.00242	0	0
To sort the table by a given value, simply click the relevant column header.	Trypsin OS=Sus scrofa PE=1 SV=1		55.5	53.5	47.6	44	34.3	1.39	1.34	1.19	1.1	0.857
Review the proteins	Protein disulfide-isomerase A6 OS=Cricetulus griseus GN=I79_007616	PE=4 SV=1	63.8	29.4	0.495	0.314	0.161	1.83	0.841	0.0142	0.009	0.00461
3 For each protein of interest, review its	Heat shock cognate 71 kDa protein OS=Cricetulus griseus GN=I79_021	800 PE=3 SV=1	65	29.4	0.101	0	0	1.48	0.668	0.00231	0	0
	78 kDa glucose-regulated protein OS=Cricetulus griseus GN=179_0199	46 PE=1 SV=1	92.6	43.5	2.66	0.584	0.0863	6.72	3.16	0.193	0.0424	0.00626
View peptide measurements	Pyruvate kinase OS=Cricetulus griseus GN=I79_004880 PE=3 SV=1		109	47.8	0.214	0.0003	0	5.67	2.49	0.0111	1.56E-05	0
	Tubulin beta-3 chain OS=Cricetulus griseus GN=179_000194 PE=3 SV=	1	140	67.8	2.56	0.511	0.166	12.2	5.88	0.222	0.0443	0.0144
protein.	Peraxiredoxin-1 OS=Cricetulus griseus GN=I79_002954 PE=4 SV=1		148	71.9	1.57	0	0	3.33	1.62	0.0353	0	• 0
	Selected protein: L-lactate dehydrogenase A View peptide measurements	chain OS=Cricetuli	us griseus GN=179_009	9741 PE=4 SV=	1							×
Export protein measurements	Batch 1		Batch 2		Bat	h 3		Batch 4			Batch	5
Export peptide measurements	oorepui 4 ₩											
Export peptide ion measurements	Articlet Monadiane Atom		*		ж	м) 0 1			ы	
	* [,							÷
	Quantifiable proteins displayed: 24											Section Complete 🤿

As you order on Batch 3, 5 of the proteins originally present in Batch 2 are no longer measurable in Batch 3, this rises to 13 in Batch 5.

Protein measurements are exported as .csv the content being dependant on the choices made on the dialog shown to the right.

You can export these measurements to Excel by clicking on **Export Protein Measurements**

Alternatively, right click on the table and select **Add to clip gallery...** (refer to Appendix 7 (page 59) for more details)

Export protein measurements	×
Choose properties to be included in exported file	
Accession	
Peptide count	
Unique peptides	
Confidence score	
📝 Anova (p)	
📝 q Value	
Max fold change	
Power	
Highest mean condition	
Lowest mean condition	
Mass	
Description	
Normalized abundance	
Raw abundance	
Amount (fmol)	
Amount (ng)	
Tags	
OK Cano	cel

You can clear the current searches and repeat the searches with the other libraries provided or try the ones you have created.

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

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

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

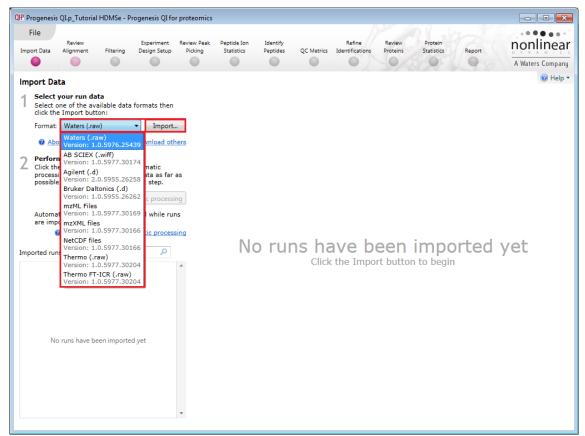
Note: if you have converted or captured the data as centroided then select Centroided data and enter the Resolution for the MS machine used.

QIP Create New LC-MS Experiment	
Create a new label-free experiment named:	
Progenesis QI.P_Tutorial_HDMSe	
Data type Ordile data Centroided data Resolution (full width at half maximum) 50000	
Machine type High resolution mass spectrometer	•
Experiment folder	High resolution mass spectrometer e.g. Waters SYNAPT G2/G2-S, AB SCIEX TripleTOF, Agilent QTOF, Bruker Maxis, Thermo LTQ Orbitrap
 Save experiment in the same folder as the run data Choose an experiment folder 	Thermo LTQ Iontrap in Enhanced mode.
Brow	Low resolution ion trap e.g. Bruker HCT, Bruker HCT Ultra, Thermo LTQ XL
Create experiment	Thermo FT-ICR

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

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

Then locate your data files using Import...



Locate and select all the .RAW folders (A_01 to C_03).	Import Waters .RAW Data Select your runs The data for each run is stored in a separate .RAW folder, usually all within the same containing folder. Find runs in folder: D:\Progenesis v3.0 HDMSe Tutorial\QIp_Tutorial HDMSe v3.0 A_01 raw A_02 raw B_01 raw B_02 raw B_03 raw C_02 raw D_33 raw
	< <u>B</u> ack Next > Cancel
	Import Waters .RAW Data
	All of your runs contain lock mass calibration information. Please provide the calibration m/z.
	Perform lock mass calibration Lock mass m/z: 785.8426
	Note: If you have already calibrated your data externally, you should not perform calibration here.
On importing, the lock mass calibration is read and presented on this dialog	
You can, if required, alter the lock mass calibration at this step.	
	< Back Next > Cancel

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

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

Enable Ion Accounting workflow	Optimise peptide identification						
To identify peptides in your MS#/HDMS#/SONAR data, you will need to enable the Ion Accounting workflow.	Specify the peak intensity thresholds to use when filtering your spectra prior to searching with Ion Accounting.						
Select whether to run the Ion Accounting workflow.	How do you want to spec	ify the threshold inter	isities?				
Run the Ion Accounting workflow and associated data analysis	 Calculate optimal the 	iresholds using a repr	resentative FASTA file				
	FASTA file:	E:\HDMSe Tutoria	al\Tutorial_nd_DB.fasta Browse				
	Parsing rules:	UNIPROT					
	Specify threshold in	tensities manually					
	Low energy:	250	counts				
	Elevated energy	y: 150	counts				
	How does the autom	atic calculation of t	thresholds work?				
			< <u>B</u> ack <u>N</u> ext> Car				

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

- It finds the 5-minute retention time window that contains the highest total intensity. ٠
- It extracts the ions within this window and performs multiple Ion Accounting searches, each one • using a different set of threshold values.

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

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

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

	it		
you choose to spe e start time.	city your own elution lir	nits, the end time must be	e at least 5 minutes
Elution start:	<start of="" run=""></start>	minutes	
Elution end:	<end of="" run=""></end>	minutes	

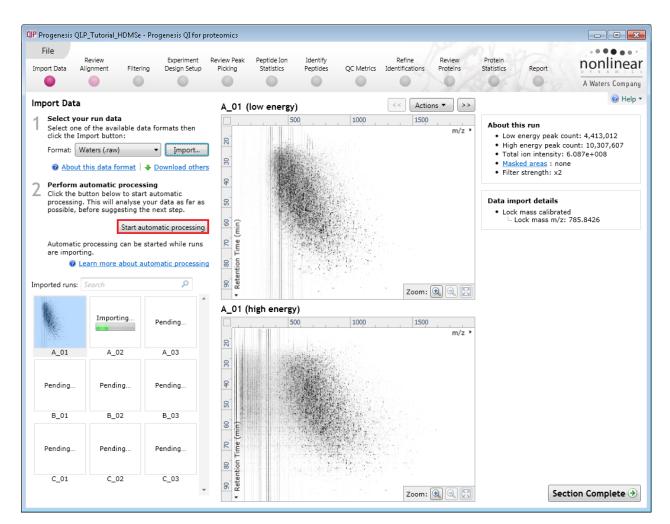
	Waters ,RAW Data
	dy to import sase review the information below before starting the import process.
Yo	ur runs are ready to be imported. Please review the options below.
9	Fruns selected for import.
L	.ock mass calibration: Yes Lock mass m/z: 785.8426
٩	dS≊ parameters: Low energy threshold: 150 Elevated energy threshold: 30
	< Back Import Cancel

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

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

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

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



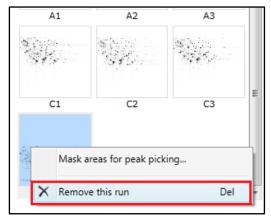
For details of setting up the steps in the automatic processing wizard return to Stage 2A page 9.

Review Chromatography

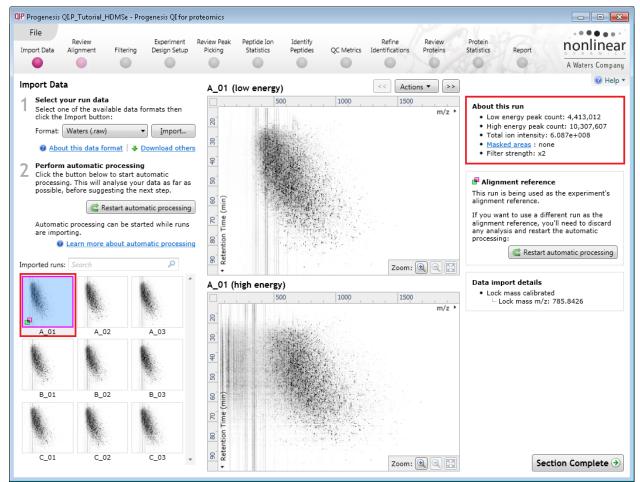
Each data file appears as a 2D representation of the run. If you created a **profile** experiment, at this stage you will be warned if any of the data files have been 'centroided' during the data acquisition and conversion process, as files must be of one format or the other.

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

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



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



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

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

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

Now move to the next stage in the workflow (page 9 in this user guide) by clicking Section Complete.

Appendix 2: Stage 2 Processing failures

If a stage fails to complete successfully or only partially completes, the automatic processing dialog will warn you of the problem. This may or may not allow the automatic processing to complete.

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

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

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



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

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

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

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

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

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

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

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

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

Appendix 3: Licensing runs

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



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

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

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

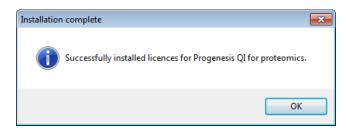
The runs in your experiment will be listed as shown below.

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

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

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

A message confirming successful installation of your licences will appear.

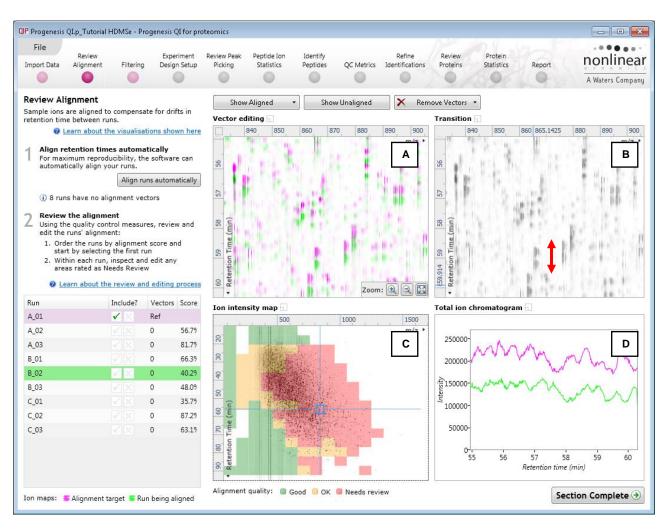


Click OK, the view will update and Alignment, the next stage in the workflow, will open with the licensed files.

Appendix 4: Manual assistance of Alignment

Approach to alignment

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

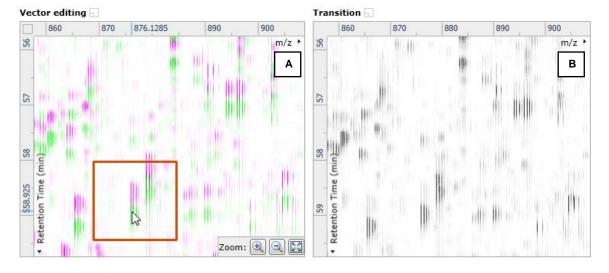


- 1. Click on Run B_02 in the **Runs** panel, this will be highlighted in green and the reference run (A_01) will be highlighted in magenta.
- 2. You will need to place approximately 5 10 **alignment vectors** evenly distributed from top to bottom of the whole run (RT range).
- 3. First drag out an area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

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

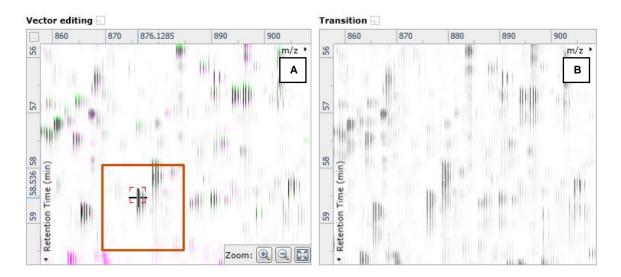
Note: the lon Intensity Map gives you a colour metric, visually scoring the current alignment and an overall score is placed next to the Vectors column in the table. With each additional vector added this score will update to reflect the 'changing' overall quality of the alignment. The colour coding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

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



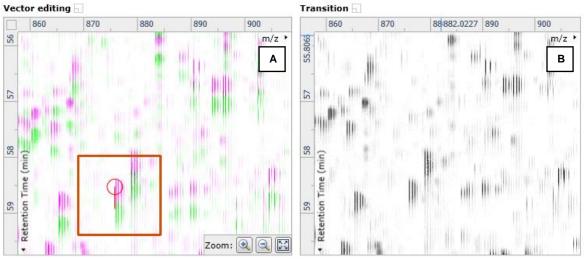
4. Click and hold on a green peptide ion in Window A as shown below.

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



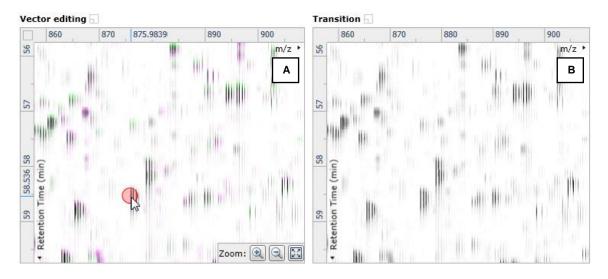
Tip: while holding down the mouse button hold down the **Alt** key. This will allow smooth movement of the cursor as the **Alt** key allows you to override the 'automatic alignment' performed as you depress the mouse button.

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

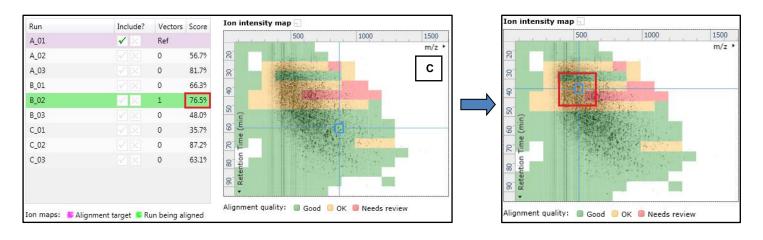


Note: an incorrectly placed vector is removed by right clicking on it in the **Vector Editing** window and selecting delete vector.

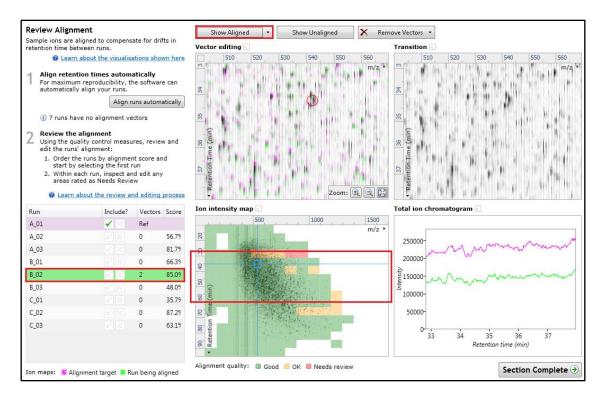
7. Now click Show Aligned on the top tool bar to see the effect of adding a single vector.



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



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



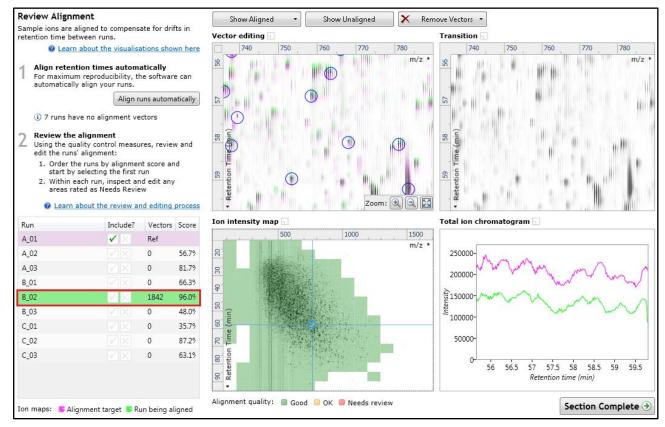
- 10. The shift in the Retention Time (RT) is as a result of incorrect running of the chromatography. In many of these cases if the Automatic Alignment fails to generate a good alignment then removing all the alignment vectors for this run and placing a single manual vector to act as a 'seed' for the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



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

A	itomatic Ali	gnment	— ×
	Choose whic	h runs to automatically align:	
	Run	Notes	Vectors
	🗖 A_02	This run has not been automatically aligned	0
	🗖 A_03	This run has not been automatically aligned	0
	B_01	This run has not been automatically aligned	0
	✓ B_02	run has user vectors	5
17	B_03	This run has not been automatically aligned	0
	C_01	This run has not been automatically aligned	0
	C_02	This run has not been automatically aligned	0
	C_03	This run has not been automatically aligned	0
			OK Cancel

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



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

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

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

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

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

Appendix 5: Within-subject Design

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

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

File nport Data	Review Alignment	Filtering	Experiment Design Setup	Review Peak Picking	Peptide Ion Statistics	Identify Peptides	QC Metrics	Refine Identifications	Review Proteins	Protei Statisti		port	0	onlin Waters Cor
B C Vhich ex	A B	esign typ		ant to use	e for this e	periment?								۲
Do samp	etween-su les from a giv n only one co between-sub	ubject De	esign nen A			Delete	0-0 Have y subjec	Within-sub rou taken samp t under differe ise the within-	ples from a g	iven is?		Before	During	After
To set up the runs (factor le	this design, according to evel) of the sa alculation ass	you simply the condition amples. The	group on e	Ente	eate New Experi r a name for th	ne experiment o	lesign:		×	use	Patient X	X1	X2	X3
therefore	ns are indeper e gives a stati the means of qual.	istical test o		How		After Treatment o group the run s manually				s	Patient Y	Y1	Y2	Y3
			Add	conditi	Copy an existi	ng design:	C	reate design	• Cancel	ut he :ed	Patient Z	Z1	Z2	Z3
							becaus assump repeat differe reduce conditi create The wi though paired- compai	Jard ANOVA is 1 e the data viol tion of indepe- ed measures Al nces can be eli d as a source o on differences a more powerf thin-subject de t of as an exte -samples t-test rison between d measures.	ates the ANO ndence. With NOVA individu iminated or f between (which helps ful test). esign can be nsion of the t to include	te VA a Jal				

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

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

	eomics iew Peak Peptide Ic licking Statistics		Refin QC Metrics Identifica		Protein Statistics Report	nonlinear
A B C A B Before During an	After Treatm	ent I × 🖻	New			A Waters Company 🕡 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. Drag each of your samples to the correct location in the grid. Filter samples: P	Patient A	A_01	A_02	A_03		
C_02	Patient B	B_01	B_02	B_03	-	
C_03	Patient C	C_01	Select Sample	Select Sample		
	Add Subject				1	
					S	ection Complete 🏵

You can create additional Experimental Designs using the New tab

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

Appendix 6: Resolve Conflicts

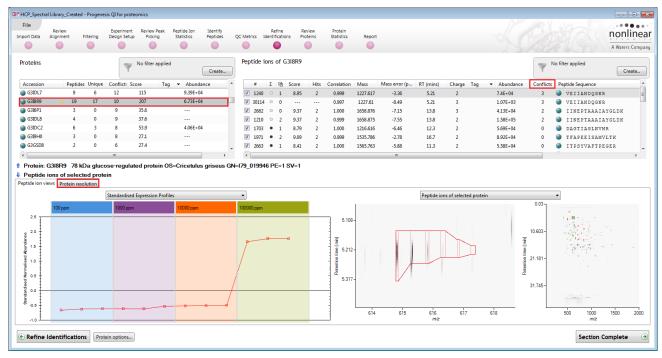
This stage allows you to examine the behaviour of the identified peptides and choose to resolve any conflicts for the various peptide assignments at the protein level.

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

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

Currently no Conflict resolution was performed on the HCP_Spectral Library_Created data set.

Example of performing conflict resolution on G3I8R9 (left hand table), which has a total number of 19 peptides, 17 of which are uniquely assigned to this protein, the remaining 2 have a total of 10 conflicting protein assignments.



On the peptides table order on conflicts and select the first peptide (which has 3 conflicts) and click on the Protein resolution tab to show the conflicting proteins

The Conflicting proteins are ordered on the basis of Protein Score in the bottom left table

Accession	Peptides	Unique	Conflict:	Score	Tag 👻	Abundanc	e	^	#	Σ	色 Sci	ore	Hits Correlat	on Mass	Mass error (p	RT (mins)	Charge Ta	g 🝷 Abundance	e Co	onflicts Pe	ptide Sequence	
G3H5Q0 (+2)	12	1	12	123		9.46E+04			/ 124	0 0	1 8	8.95	2 0.999	1227.61	-3.36	5.21	2	7.4E+04		3 🕥	VEIIANDQGNR	
G3IDL7	9	6	12	115		9.39E+04			7 3011	14 0	0		0.997	1227.61	-8.49	5.21	3	1.07E+03		3 🌒	VEIIANDQGNR	
👌 G318R9 🛛 💿	19	17	10	207		6.73E+04		S	/ 268	2 0	0 9	9.37	2 1.000	1658.876	-7.15	13.8	3	4.13E+04		2 🌒	IINEPTAAAIAYGLDK	
G3IDL8	4	0	9	37.6				3	/ 121	0 0	2 9	9.37	2 0.999	1658.87	-7.55	13.8	2	1.58E+05		2 🌒	IINEPTAAAIAYGLDK	
G3I6P1	3	0	9	35.6				5	170	в •	1 8	8.79	2 1.000	1216.610	-6.46	12.3	2	5.69E+04		0 🌒	DAGTIAGLNVMR	
G319H0	3	0	8	27.1				* R	/ 197	1 0	2 (9.09	2 0.999	1535 78/	-2 7R	167	2	8 97F+04		<u>n</u> 🚳	TEAPEETSAMULTE	
	Z8 Heat	shock-	related a	/0 kDa pro	tein 20	S=Cricetuli	is grisei	us GN=	1/9_(0536	2 PE=3	SV=1										
Protein: G3H42																						
Protein: G3H42 atide ion views Pi Conflicting prof	rotein resolu	ution	ion 124	0				Pepti	de ior	ns of (G3H4Z8	3										
tide ion views P	rotein resolu teins for	ution peptide		0 rotein Score	Pep	tide Score	Tag	Peptie	de ior		G3H4Z8 Score		Correlation	Mass N	lass error (p R1	T (mins) C	Charge Tag	 Abundance 	Conflict	t: Peptide S	equence	
tide ion views P	rotein resolu teins for Peptides Ur	ution peptide		rotein Score	Pep ▼ 8.95		Tag	Peptie	Σ					Mass N 1227.617	lass error (p R] -3.36	T (mins) C 5.21	Charge Tag 2	 Abundance 7.4E+04 	Conflict 3		equence LANDQGNR	
tide ion views Prof onflicting prof Accession F G31BR9 0	rotein resolu teins for Peptides Ur	ution peptide nique C	onflict: Pr	rotein Score 7			Tag		Σ 240	· •	Score	Hits							Conflict 3 3	🕥 VEI:		
tide ion views Profilecting prof Accession P G3IBR9 O G3H4Z8 (+4)	rotein resolu teins for Peptides Ur 19	ution peptide nique C	onflict: Pr 10 20	rotein Score 7 7	₹ 8.95		Tag	# 11 30	Σ 240 114	· •	Score 8.95	Hits 2	0.986	1227.617	-3.36	5.21	2	7.4E+04	3	VEIVEI	IANDQGNR	
tide ion views Pr onflicting prot Accession F G3IBR9 • G3IBR9 • G3IH4Z8 (+4) G3IDL7	rotein resolu teins for Peptides Ur 19	ution peptide nique C	onflict: Pr 10 20 21 11	rotein Score 7 7 5	✓ 8.95✓ 8.95		Tag	# ♥ 11 ♥ 30 ♥ 20	240 Ω	E 🔁 1 0 0	Score 8.95	Hits 2	0.986 0.982	1227.617 1227.61	-3.36 -8.49	5.21 5.21	2	7.4E+04 1.07E+03	3 3	 VEI VEI VEI IINI 	IANDQGNR IANDQGNR	
tide ion views Pr conflicting prot Accession F G318R9 • G318428 (+4) G31DL7	rotein resolu teins for Peptides Ur 19	ution peptide nique C	onflict: Pr 10 20 21 11 12 11	rotein Score 7 7 5	 ✓ 8.95 ✓ 8.95 ✓ 8.95 		Tag	# 11 30 20 20	Σ 240 1114 582	E E 1 0 0 0 2	Score 8.95 9.37	Hits 2	0.986 0.982 0.988	1227.617 1227.61 1658.876	-3.36 -8.49 -7.15	5.21 5.21 13.8	2 3 3	7.4E+04 1.07E+03 4.13E+04	3 3 2	 VEI: VEI: VEI: IINI TTP: 	IANDQGNR IANDQGNR EPTAAAIAYGLDK	
tide ion views Pr conflicting prot Accession F G318R9 • G318428 (+4) G31DL7	rotein resolu teins for Peptides Ur 19	ution peptide nique C	onflict: Pr 10 20 21 11 12 11	rotein Score 7 7 5	 ✓ 8.95 ✓ 8.95 ✓ 8.95 		Tag	 # ✓ 11 ✓ ✓	240 Ω 1114 Ω 582 Φ 932 Ω	E E 1 0 0 2 2 2	Score 8.95 9.37 9.06	Hits 2	0.986 0.982 0.988 0.987	1227.617 1227.61 1658.876 1486.684	-3.36 -8.49 -7.15 -6.61	5.21 5.21 13.8 9.81	2 3 3 2	7.4E+04 1.07E+03 4.13E+04 4.25E+04	3 3 2	 VEI: VEI: VEI: IINI TTP: 	IANDQGNR IANDQGNR EPTAAAIAYGLDK SYVAFTDTER EPTAAAIAYGLDK	
tide ion views P Conflicting prof Accession F	rotein resolu teins for Peptides Ur 19	ution peptide nique C	onflict: Pr 10 20 21 11 12 11	rotein Score 7 7 5	 ✓ 8.95 ✓ 8.95 ✓ 8.95 		Tag	 # ✓ 12 ✓ 30 ✓ 20 ✓ 22 ✓ 12 	240 Ω 1114 Ω 582 Φ 932 Ω 210 Φ 1860 Ω	E E 1 0 0 2 2 2	Score 8.95 9.37 9.06 9.37	Hits 2	0.986 0.982 0.988 0.987 0.988	1227.617 1227.61 1658.876 1486.684 1658.875	-3.36 -8.49 -7.15 -6.61 -7.55	5.21 5.21 13.8 9.81 13.8	2 3 3 2 2	7.4E+04 1.07E+03 4.13E+04 4.25E+04 1.58E+05	3 3 2 2 2	VEI VEI VEI IINI TTP: IINI	IANDQGNR IANDQGNR EPTAAAIAYGLDK SYVAFTDTER EPTAAAIAYGLDK	
tide ion views Pr conflicting prot Accession F G318R9 • G318428 (+4) G31DL7	rotein resolu teins for Peptides Ur 19	ution peptide nique C	onflict: Pr 10 20 21 11 12 11	rotein Score 7 7 5	 ✓ 8.95 ✓ 8.95 ✓ 8.95 		Tag	# ✓	Σ 240 1114 582 932 210 860 564	 1 0 0 2 2 0 	Score 8.95 9.37 9.06 9.37 8.03	Hits 2 2 2 2 2 2 2	0.986 0.982 0.988 0.987 0.988 0.988	1227.617 1227.61 1658.876 1486.684 1658.875 803.432	-3.36 -8.49 -7.15 -6.61 -7.55 -8.28	5.21 5.21 13.8 9.81 13.8 3.13	2 3 3 2 2 2	7.4E+04 1.07E+03 4.13E+04 4.25E+04 1.58E+05 2.13E+03	3 2 2 2 2 2	VEI VEI VEI IINI TTP: IINI	LANDQGNR LANDQGNR SPTAAAIAYGLDK SYVAFTDTER SPTAAAIAYGLDK INDK DFF <mark>N</mark> GK	

Untick the peptide on the bottom right table to favour the current protein based on the highest protein score Then move down the Conflicting proteins table to the next protein. Here also untick the peptide in the bottom right table.

Accession	Peptide	s Unique	Conflict:	Score	Tag 🔻 Abundan	ce	^	#	Σ	ф	Score	Hits Correlat	on Mass	Mass error (p	RT (mins)	Charge	Tag 🔻 Abundan	ce Ci	onflicts Peptide Sequence	
G3H5Q0 (+2)	12	1	12	123	9.46E+04			V 1	240	1	8.95	2 0.999	1227.61	7 -3.36	5.21	2	7.4E+04	Г	2 🔮 VEIIANDQGNR	
G3IDL7	9	6	11	115	9.39E+04			V 30	0114 0	0		0.997	1227.61	-8.49	5.21	3	1.07E+03		3 🔮 VEIIANDQGNR	
) G318R9	o 19	17	9	207	6.73E+04			2	682	0	9.37	2 1.000	1658.87	5 -7.15	13.8	3	4.13E+04		2 🔇 IINEPTAAAIAYGLDK	
G3IDL8	4	0	9	37.6				V 1	210	2	9.37	2 0.999	1658.87	5 -7.55	13.8	2	1.58E+05		2 🔮 IINEPTAAAIAYGLDK	
G316P1	3	0	9	35.6				V 1	703	• 1	8.79	2 1.000	1216.61	5 -6.46	12.3	2	5.69E+04		0 💿 DAGTIAGLNVMR	
G319H0	3	0	8	27.1			-	V 1	971 (, ,	9.09	2 0.999	1535.78	5 -2.78	167	2	8 92F+04		0 🚳 ΤΕΣΡΕΕΤΟΣΜΟΤ.ΤΚ	
ptide ion views	Protein res	olution			tein OS-Cricetulus	s griseus														
ptide ion views	Protein res	olution			tein OS-Cricetulus	s griseus														
ptide ion views F Conflicting pro	Protein res oteins fo	olution r peptic	le ion 124	0			Pep	tide i	ons of	G3IDL	.7									_
ptide ion views F Conflicting pro Accession	Protein reso Dteins fo Peptides	olution r peptic Unique	le ion 124 Conflict: P	0 rotein Score	Peptide Score	s griseus Tag	Pep	tide i	ons of Σ@	G3IDL Score	.7 e Hits					Charge Tag	✓ Abundance		t: Peptide Sequence	
otide ion views F Conflicting pro Accession G318R9 •	Protein res oteins fo	olution r peptic	le ion 124 Conflict: P 9 20	0 rotein Score 7	Peptide Score		Pep'	tide i # 1240	ons of Σ@	G3IDL Scort 8.9	.7 e Hits 5 2	0.999	1227.617	-3.36	5.21	Charge Tag 2	7.4E+04	Conflic 2	VEIIANDQGNR	
Accession G318R9 G314Z8 (+4)	Protein reso Dteins fo Peptides	olution r peptic Unique	le ion 124 Conflict: P	0 rotein Score 7	Peptide Score		Pep'	tide i # 1240 1210	ons of Σ@	G3IDL Score	.7 e Hits 5 2									
Accession G318R9 G314ZB (+4)	Protein rese Dteins fo Peptides 19	olution r peptic Unique	le ion 124 Conflict: P 9 20	0 rotein Score 7 8	Peptide Score		Pep'	tide i # 1240 1210 2932	ons of Σ @ ○ 1 ● 2 ○ 2	G3IDL Scort 8.9	.7 e Hits 5 2 7 2	0.999	1227.617	-3.36	5.21	2	7.4E+04	2	VEIIANDQGNR	
tide ion views F Conflicting pro Accession G318R9 G314Z8 (+4) G31DL7	Protein rese Dteins fo Peptides 19	olution r peptic Unique 17 2	le ion 124 Conflict: P 9 20 18 10	0 rotein Score 7 8 5	Peptide Score		Pep'	tide i # 1240 1210 2932	ons of Σ @ ○ 1 ● 2 ○ 2	G3IDL Scort 8.9 9.3	.7 Hits 5 2 7 2 6 2	0.999	1227.617 1658.875	-3.36 -7.55	5.21 13.8	2 2	7.4E+04 1.58E+05	2	 VEIIANDQGNR IINEPTAAAIAYGLDK 	
tide ion views F Conflicting pro Accession G318R9 G314Z8 (+4) G31DL7	Protein rese Dteins fo Peptides 19	r peptic Unique 17 2 6	le ion 124 Conflict: P 9 20 18 10 11 11	0 rotein Score 7 8 5	Peptide Score		Pep V V	tide i # 1240 1210 2932	ons of Σ @ ○ 1 ● 2 ○ 2	G3IDL Score 8.9 9.3 9.0	.7 Hits 5 2 7 2 6 2 	0.999 0.999 1.000	1227.617 1658.875 1486.684	-3.36 -7.55 -6.61	5.21 13.8 9.81	2 2 2	7.4E+04 1.58E+05 4.25E+04	2 2 2	 VEIIANDOGNR IINEPTAAAIAYGLDK TTPSYVAFTDTER 	
tide ion views F Conflicting pro Accession G318R9 G314Z8 (+4) G31DL7	Protein rese Dteins fo Peptides 19	r peptic Unique 17 2 6	le ion 124 Conflict: P 9 20 18 10 11 11	0 rotein Score 7 8 5	Peptide Score		Pep'	tide i # 1240 1210 2932 42255	ons of Σ 俚 ○ 1 ○ 2 ○ 2 ● 0	G3IDL Score 8.9 9.3 9.0	.7 Hits 5 2 7 2 6 2 5 2	0.999 0.999 1.000 0.990	1227.617 1658.875 1486.684 1198.658	-3.36 -7.55 -6.61 -7.91	5.21 13.8 9.81 13.8	2 2 2 3	7.4E+04 1.58E+05 4.25E+04 344	2 2 2 0	 VEIIANDOGNR IINEPTAAAIAYGLDK TTPSYVAFTDTER DAGTIAGLNVLR 	
Accession G318R9 G318R9 G314Z8 (+4) G31DL7	Protein rese Dteins fo Peptides 19	r peptic Unique 17 2 6	le ion 124 Conflict: P 9 20 18 10 11 11	0 rotein Score 7 8 5	Peptide Score		Pep'	tide i # 1240 1210 2932 42255 2585	ons of Σ @ ○ 1 ○ 2 ○ 2 ○ 0 ○ 1	G3IDL Score 9.3 9.0 8.6	.7 e Hits 5 2 7 2 6 2 - 5 2 7 2	0.999 0.999 1.000 0.990 0.999	1227.617 1658.875 1486.684 1198.658 992.511	-3.36 -7.55 -6.61 -7.91 -6.46	5.21 13.8 9.81 13.8 7.82	2 2 2 3 2	7.4E+04 1.58E+05 4.25E+04 344 2.77E+04	2 2 2 0 0	 VEIIANDOGNR IINEPTAAAIAYGLDK TTPSYVAFTDTER DAGTIAGLNVLR EIAEAYLGK 	
tide ion views F Conflicting pro Accession	Protein rese Dteins fo Peptides 19	r peptic Unique 17 2 6	le ion 124 Conflict: P 9 20 18 10 11 11	0 rotein Score 7 8 5	Peptide Score		Pep V V V	tide i # 1240 1210 2932 42255 2585 1265	ons of Σ @ ○ 1 • 2 • 0 ○ 1 • 0 ○ 1 • 3	G3IDL Score 9.3 9.0 8.6 9.3	7 Hits 5 2 7 2 6 2 5 2 7 2 4 2	0.999 0.999 1.000 0.990 0.999 1.000	1227.617 1658.875 1486.684 1198.658 992.511 1198.662	-3.36 -7.55 -6.61 -7.91 -6.46 -3.85	5.21 13.8 9.81 13.8 7.82 13.8	2 2 2 3 2	7.4E+04 1.58E+05 4.25E+04 344 2.77E+04 9.37E+04	2 2 0 0 0	 VEIIANDQGNR IINEPTAAAIAYGLDK TIFSYVAFTDTER DAGTIAGLNVLR EIAEXIGK DAGTIAGLNVLR 	

Note: as you untick each Peptide ion for the conflicting protein the number of conflicts is reduced in the top 2 tables

Repeat this process for all the conflicting proteins for this peptide ion

	Peptides	Unique	Conflict	Score	Tag 🔻 Abundar	nce	^	4	• •	ΣΦ	Score	Hit	ts Correlati	n Mass	Mass error (p	RT (mins)	Charge Ta	g 🔹 Abundanc	e Cor	nflicts Peptide Sequence
G3H5Q0 (+2)	12	1	12	123	9.46E+04			V 1	240	• 1	8.95	2	0.999	1227.61	7 -3.36	5.21	2	7.4E+04		0 🔮 VEIIANDQGNR
G3IDL7	9	6	9	106	9.39E+04			V 3	0114	0			- 0.997	1227.61	-8.49	5.21	3	1.07E+03		3 🔮 VEIIANDQGNR
🗿 G318R9 🛛 🥥	19	18	7	207	6.73E+04			V 2	682	0	9.37	2	1.000	1658.87	5 -7.15	13.8	3	4.13E+04		2 🕥 IINEPTAAAIAYGLDK
G3IDL8	4	0	9	37.6				V 1	210	0 2	9.37	2	0.999	1658.87	-7.55	13.8	2	1.58E+05		2 🕥 IINEPTAAAIAYGLDK
G3I6P1	3	0	9	35.6				V 1	703	• 1	8.79	2	1.000	1216.61	5 -6.46	12.3	2	5.69E+04		0 🕥 DAGTIAGLNVMR
G319H0	3	0	8	27.1			*	V 1	971	• 2	9.09	2	0 999	1535 78		167	2	8 92F+04		0 a TRADEFISAMULTE
			III			+		< _							III					
Conflicting pro						-	÷		ions o							.			0.00	a
Accession F	Peptides U	nique	Conflict:	Protein Score	Peptide Score	Tag	_	#		₿ Sc	ore H	lits C	Correlation	Mass N	lass error (p R	T (mins)	Charge Tag	 Abundance 	Conflict	Peptide Sequence
🎯 G318R9 🛛 🍳	19	18	7 2	107	8.95		V	30114	0 (D			0.999	1227.61	-8.49	5.21	3	1.07E+03	3	VEIIANDQGNR
🕥 G3H4Z8 (+4)	10	2	18 1	.08	8.95		\mathbf{v}	2932	0 2	2 !	9.06	2	1.000	1486.684	-6.61	9.81	2	4.25E+04	2	TTPSYVAFTDTER
G3IDL7	9	6	9 1	06	8.95		\checkmark	12860	0 (0 1	8.03	2	0.997	803.432	-8.28	3.13	2	2.13E+03	2	ITITNDK
G3HZE5	4	0	11 3	1.5	8.95		V	7832	0) :	7.21	2	0.999	2773.307	-4.64	15.5	3	2.84E+04	2	QTQTFTTYSDNQPGVLIQVYEGE
							\mathbf{v}	12769	0 () (7.21	2	0.999	2773.323	1.26	15.5	2	1.92E+04	2	QTQTFTTYSDNQPGVLIQVYEGE
							m	1240	0 1		8.95	2		1227.617	-3.36	5.21	2	7.4E+04	0	VEIIANDQGNR

Now move to the second Peptide ion (with 3 conflicts) on the top right table

Then move through the Conflicting proteins on the bottom left table unticking the peptide ions on the right hand tables as appropriate

Accession	Peptide	s Unique	Confl	ict: Score	Tag 🔻 🖊	Abundance		^	#	Σ	中 Se	core	Hits Correlati	on Mass	Mass error (p	RT (mins)	Charge Tag	 Abundance 	e Cont	flicts Peptide Sequence	
G3H5Q0 (+2)	12	1	12	123	9.	9.46E+04			124	• 0	1	8.95	2 0.999	1227.617	-3.36	5.21	2	7.4E+04	() 🔇 VEIIANDQGNR	
G3IDL7	9	6	9	106	9	9.39E+04			V 301	14 O	0		0.997	1227.61	-8.49	5.21	3	1.07E+03		3 🔮 VEIIANDQGNR	
G318R9 <	o 19	18	7	207	6.	5.73E+04			268	2 O	0	9.37	2 1.000	1658.876	-7.15	13.8	3	4.13E+04		2 🥥 IINEPTAAAIAYGLDK	
G3IDL8	4	0	9	37.6			-		12	0 0	2	9.37	2 0.999	1658.875	-7.55	13.8	2	1.58E+05	2	2 🔮 IINEPTAAAIAYGLDK	
G316P1	3	0	9	35.6					17 0	3 •	1	8.79	2 1.000	1216.616	-6.46	12.3	2	5.69E+04	() 💿 DAGTIAGLNVMR	
G319H0	3	0	8	27.1				*	10	•	,	9.09	2 0.999	1535 786	-2 78	167	>	8 92F+04) 🚳 ΤΕΣΡΕΕΤΟΣΜΟΙ.ΤΚ	
tide ion views P	Protein resi	olution			tem 2 03-0	Circetuius	s griser				G3H4Z	8									
Protein: G3H4 tide ion views P onflicting pro	Protein resi	olution			tem 2 03-0	CITCERING	s griser					8									
ide ion views P onflicting pro Accession	Protein res Dteins fo	olution r peptic	le ion 3		Peptide		Tag	Pept	ide io	ns of E ⊕	G3H4Z	8 Hits	Correlation				harge Tag	 Abundance 	Conflict:	Peptide Sequence	
de ion views P onflicting pro	Protein res Dteins fo	olution r peptic	le ion 3	0114				Pept	ide io	ns of E ⊕	G3H4Z		Correlation 0.980	Mass M 1227.61	lass error (p R -8.49	T (mins) C 5.21	harge Tag 3	Abundance 1.07E+03	Conflict: 3	Peptide Sequence	
ide ion views P onflicting pro accession a G318R9 o	Protein resi Dteins for Peptides	olution r peptic Unique	le ion 3	0114 Protein Score	Peptide ✓ ✓			Pept	ide io # 1	ns of E ⊕	G3H4Z Score	Hits	0.980								
ide ion views P onflicting pro Accession G3IBR9 O G3IH4Z8 (+4)	Protein reso Dteins fo Peptides 19	olution r peptic Unique	le ion 3 Conflict 7	0114 Protein Score 207	Peptide			Pept	ide io # 1 0114 2682	nsof E⊞ 0	G3H4Z Score 	Hits 2	0.980	1227.61	-8.49	5.21	3	1.07E+03	3 (VEIIANDQGNR	
ide ion views P onflicting pro Accession 6318R9 0 6314428 (+4) 631DL7	Protein reso Dteins fo Peptides 19	olution r peptic Unique	le ion 3 Conflict 7	0114 Protein Score 207 108	Peptide ✓ ✓			Pept	ide io #	ns of E 12 0 0	G3H4Z Score 9.37	Hits 2	0.980 0.986 0.985	1227.61 1658.876	-8.49 -7.15	5.21 13.8	3	1.07E+03 4.13E+04	3 (VEIIANDQGNR IINEPTAAAIAYGLDK	
ide ion views P onflicting pro Accession 6318R9 0 6314428 (+4) 631DL7	Protein reso Dteins fo Peptides 19	olution r peptic Unique	le ion 3 Conflict 7 18 9	0114 Protein Score 207 108 106	Peptide ✓ ✓ ✓			Pept	ide io #	ns of E 12 0 0 2	G3H4Z Score 9.37 9.06	Hits 2 2 2	0.980 0.986 0.985	1227.61 1658.876 1486.684	-8.49 -7.15 -6.61	5.21 13.8 9.81	3 3 2	1.07E+03 4.13E+04 4.25E+04	3 2 2	VEIIANDQGNR IINEFTAAAIAYGLDK TTPSYVAFTDTER	
de ion views P onflicting pro accession 4 G3IBR9 0 G3IH4Z8 (+4) G3IDL7	Protein reso Dteins fo Peptides 19	olution r peptic Unique	le ion 3 Conflict 7 18 9	0114 Protein Score 207 108 106	Peptide ✓ ✓ ✓			Pept	ide io #	ns of E 1 (1) 0 0 0 2 2 2	G3H4Z Score 9.37 9.06 9.37	Hits 2 2 2 2 2	0.980 0.986 0.985 0.986	1227.61 1658.876 1486.684 1658.875	-8.49 -7.15 -6.61 -7.55	5.21 13.8 9.81 13.8	3 3 2 2	1.07E+03 4.13E+04 4.25E+04 1.58E+05	3 (2 (2 (2 (VEIIANDQGNR IINEPTAAAIAYGLDK TTPSYVAFTDTER IINEPTAAAIAYGLDK	
de ion views P onflicting pro accession 4 G3IBR9 0 G3IH4Z8 (+4) G3IDL7	Protein reso Dteins fo Peptides 19	olution r peptic Unique	le ion 3 Conflict 7 18 9	0114 Protein Score 207 108 106	Peptide ✓ ✓ ✓			Pept	ide io # 20114 2682 2932 1210 2860 5205	ns of E 中 0 2 2 0	G3H4Z Score 9.37 9.06 9.37 8.03	Hits 2 2 2 2 2 2 2 2 2	0.980 0.986 0.985 0.986 0.986 0.986	1227.61 1658.876 1486.684 1658.875 803.432	-8.49 -7.15 -6.61 -7.55 -8.28	5.21 13.8 9.81 13.8 3.13	3 3 2 2 2	1.07E+03 4.13E+04 4.25E+04 1.58E+05 2.13E+03	3 2 2 2 2 2 2 2	VEIIANDOGNR IINEPTAAAIAYGLDK TIPSYVAFIDTER IINEPTAAAIAYGLDK IIINDK	
ide ion views P onflicting pro	Protein reso Dteins fo Peptides 19	olution r peptic Unique	le ion 3 Conflict 7 18 9	0114 Protein Score 207 108 106	Peptide ✓ ✓ ✓			Pept	ide io # 20114 2082 22932 1210 2860 5205 5664	ns of E 12 0 0 2 0 2 0 1	G3H4Z Score 9.37 9.06 9.37 8.03 8.19	Hits 2 2 2 2 2 2 2 2 2	0.980 0.986 0.985 0.986 0.986 0.986 0.987 0.987	1227.61 1658.876 1486.684 1658.875 803.432 772.403	-8.49 -7.15 -6.61 -7.55 -8.28 -6.2	5.21 13.8 9.81 13.8 3.13 4.96	3 3 2 2 2	1.07E+03 4.13E+04 4.25E+04 1.58E+05 2.13E+03 5.18E+03	3 2 2 2 2 2 2 2	VEIIANDOGNR IINEPTAAAIAYGLDK TIFPSYVAFIDTER IINEPTAAAIAYGLDK IIIINDK DNNLLGK	

The tables will update to reflect the resolved conflicts.

Accession		s Unique	Conflict:			dance	^		#		D Sco		Hits Correlat		Mass error (p		Charge T	-	ce C	onflicts	s Peptide Sequence	_
G3H5Q0 (+2)	12	1	12	123	9.468	04		V	1240	•	1 8	8.95	2 0.999	1227.61	7 -3.36	5.21	2	7.4E+04		0	VEIIANDQGNR	
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🌏 G318R9	o 19	18	4	207	6.738	04		V	2682	0	0 9	9.37	2 1.000	1658.87	5 -7.15	13.8	3	4.13E+04		2	IINEPTAAAIAYGLDK	
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Repeat this process until there are no remaining conflicts on the top 2 tables for the current protein G3I8R9.

	leview		Experiment	Review Peak	Peptide Ion	Identify			Refine	Revie									3721	nonlin																																										
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G319H0 Protein: G318 Protein: G318 tide ion views conflicting pr Accession G318R9 G314Z8 (+4)	3 IBR9 78 IDL7 He Protein r proteins 1 Peptide 9 19 I) 8	0 kDa gluc at shock esolution for peptid Unique 19 2	8 m cognate 71 e ion 1210 Conflict: Prot 0 207 11 89.2	27.1 ed protein (kDa protein tin Score	DS-Cricetulu: OS-Cricetul Peptide Score 9.37 9.37	us griseus	F Image: Constraint of the second secon	1971 019946 0218 e ions (Σ 0 0 0 0 0 0 0 0 0 0 0 0 0		a no a no SV-1 3 3 SV-1 a SV b SV a SV b S a S b S b S b S b S c S c S c S c S c S c S c S c S c S c S	2 0.99 Correlation 0.991 0.999 1.000	Mass Mass 1658.875 1227.61 1198.658 992.511 1198.662	Mass error (p) -7.55 -3.36 -7.15 -8.49 -7.91 -6.46 -3.85	16.7 (mins) (13.8 5.21 13.8 5.21 13.8 7.82 13.8	2 Charge Tag 2 2 3 3 3 3 2	 ▲ Abundance 1.58E+05 7.4E+04 4.13E+04 1.07E+03 344 2.77E+04 9.37E+04 	0 0 0 0	0 1 1 1 1 1 1 1 1 1 1 1 1 1	de Sequence INE PTAALAYGLDK EITANOGONR HIMEFTAALAYGLDK EITANDOGNR AGTIAGLMVLR AGTIAGLMVLR																																											

To Resolve the conflicts for the whole data set work through this process with all of the proteins in the top left hand table, that display conflicts, until there are no remaining conflicts.

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

< III		۴.	•
• Refine Identifications	Protein options	Recalculate a	bundances

Protein Quantitation options

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

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

If you have selected one of the other options during the setup of the Auto Processing of your data (Stage 2) then this option will be applied.

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

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

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

QP Protein quantitation options
Quantitation method:
Relative Quantitation using Hi-N
Absolute Quantitation using Hi-N • Requires a calibrant protein to calculate absolute amounts • Uses mean calibrant abundance measured across runs • Uses the most abundant N peptides • Allows comparison between proteins within a run
Relative Quantitation using Hi-N Uses the most abundant N peptides Allows comparison between proteins within a run
Relative Quantitation using non-conflicting peptides • Uses only peptides which have no conflicting protein identifications • Allows comparison of a single protein across runs
Relative Quantitation using all peptides • Uses all peptides identified as part of a protein • Allows comparison of a single protein across runs Absolute Quantitation for HCP using Hi-N
 Requires a calibrant protein to calculate absolute amounts Uses calibrant abundance measured per run Uses the most abundant N peptides Calculates the amount of HCPs per run in fmol and ng
QIP Protein quantitation options
Quantitation method:
Relative Quantitation using Hi-N
Number of peptides to measure per protein (N): 3
Use protein grouping i.e. hide proteins whose peptides are a subset of another protein's.
OK Cancel

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

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

QP Protein quantitation options	QP Protein quantitation options
Quantitation method:	Quantitation method:
Absolute Quantitation using Hi-N	Absolute Quantitation using Hi-N
Number of peptides to measure per protein (N):	Number of peptides to measure per protein (N):
Calibrant accession:	Calibrant accession: P63284
Amount (fmol):	Amount (fmol): 200
Use protein grouping i.e. hide proteins whose peptides are a subset of another protein's.	Use protein grouping i.e. hide proteins whose peptides are a subset of another protein's.
OK Cancel	OK Cancel

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

Review Proteins Using this screen, you can find the proteins of interest in your experiment.	Vo filter applied Create					🔞 Hi
1 Set the quantitation options	Description	Amount (fmol) - Batch 1	Amount (fmol) - Batch 2	Amount (fmol) - Batch 3	Amount (fmol) - Batch 4	Amount (fmol) - Batch 5
If you've not already done so, choose between relative and absolute quantitation,	L-lactate dehydrogenase A chain OS=Cricetulus griseus GN=I79_009741 PE=4 SV=1	19.6	8.62	0	0	0
use of Hi-N, protein grouping and more.	Nucleolin OS=Cricetulus griseus GN=I79_022400 PE=4 SV=1	40.1	16.8	0	0	0
Protein options	Peptidyl-prolyl cis-trans isomerase OS=Cricetulus griseus GN=I79_001023 PE=3 SV=1	155	77.1	8.95	5.71	4.83
	Peroxiredoxin-1 OS=Cricetulus griseus GN=I79_002954 PE=4 SV=1	150	67.7	1.2	0	0

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

account to make the ranking robust. These Hi-N methods allow the relative and absolute comparison of proteins within the same run.

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

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

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

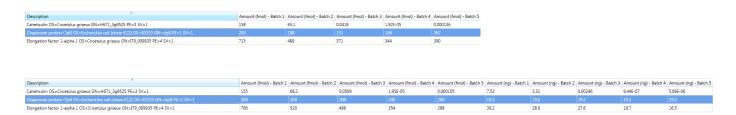
Review Proteins Using this screen, you can find the proteins of interest in your experiment.	Vo filer applied Create										😢 Hi
 Set the quantitation options 	Description	Amount (fmol) - Batch 1	Amount (fmol) - Batch 2	Amount (fmol) - Batch 3	Amount (fmol) - Batch 4	Amount (fmol) - Batch 5	Amount (ng) - Batch 1	Amount (ng) - Batch 2	Amount (ng) - Batch 3	Amount (ng) - Batch 4	Amount (ng) - Batch 5
If you've not already done so, choose between relative and absolute guantitation,	L-lactate dehydrogenase A chain OS=Cricetulus griseus GN=I79_009741 PE=4 SV=1	19.4	9.17	0	0	0	0.24	0.113	0	0	0
use of Hi-N, protein grouping and more.	Nucleolin OS=Cricetulus griseus GN=179_022400 PE=4 SV=1	39.5	17.7	0	0	0	2.07	0.93	0	0	0
Protein options	Peptidyl-prolyl cis-trans isomerase OS=Cricetulus griseus GN=I79_001023 PE=3 SV=1	153	82.3	12.1	6.16	4.03	2.58	1.39	0.204	0.104	0.0682
	Peroxiredoxin-1 OS=Cricetulus griseus GN=I79_002954 PE=4 SV=1	148	71.9	1.57	0	0	3.33	1.62	0.0353	0	0

As for Absolute Quantitation enter the accession number and amount for the calibrant.

QP Protein quantitation options	QIP Protein quantitation options
Quantitation method:	Quantitation method:
Absolute Quantitation for HCP using Hi-N	Absolute Quantitation for HCP using Hi-N
Number of peptides to measure per protein (N):	Number of peptides to measure per protein (N):
Calibrant accession:	Calibrant accession: P63284
Amount (fmol):	Amount (fmol): 200
Use protein grouping i.e. hide proteins whose peptides are a subset of another protein's.	Use protein grouping i.e. hide proteins whose peptides are a subset of another protein's.
OK Cancel	OK Cancel

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

How the values for the calibrant behave across runs comparing Absolute Quant and Absolute Quant for HCP using the same calibrant



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

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

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

Using non conflicting peptides

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

The relative Quantitation can also be performed using all peptides.

QP Protein quantitation o	ptions		×
Quantitation method:			
Relative Quantitation	using all peptides		•
Use protein grouping i.e. hide proteins whose peptides are a subset of another protein's.			
		OK Canc	el

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

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

Appendix 7: Using Clip Gallery to Save and Export Pictures and Data

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

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

As an example of using the Clip Gallery, at the **Review Proteins** stage right click on the Protein Table and select **Add to clip gallery...**

QIP HCP_mAb_Batches - Progenesis QI for proteomics				
File Review Experiment R Import Data Alignment Filtering Design Setup	eview Peak Peptide Ion Identify Refine Review Protein Picking Statistics Peptides QC Metrics Identifications Proteins Statistics Report			A Waters Company
Review Proteins Using this screen, you can find the proteins of interest in your experiment. Search P				
Set the quantitation options	n Description	Amount (fmol) - Batch 1 Ar	mount (fmol) - Batch 2 Amount (fmol) - Batch 3	Amount (fmol) -
If you've not already done so, choose between relative and absolute quantitation, use of Hi-N,	Eukaryotic translation initiation factor 5A OS=Cricetulus grinters CAL TO 015001 DF 2 CV 1	5.93 2.3	39 0	0 🔺
protein grouping and more.	Protein S100 OS=Cricetulus griseus GN=I79_008074 PE=3 No tags to assign	17.3 7.4	82 0	0
Protein options	10 kDa heat shock protein_ mitochondrial OS=Cricetulus g New tag	24.3 8.9	92 0.00632	0.00581
	Heat shock protein HSP 90-beta OS=Cricetulus griseus GN Quick Tags	28.2 12	2.7 0	0
7 Create a shortlist to review	Annexin OS=Cricetulus griseus GN=179_022682 PE=3 SV= 🚰 Edit tags	40 19	9.2 0.0771	0.0143
In the table, sort and <u>filter the proteins</u> based on their measurements, to generate a shortlist	Protein disulfide-isomerase A3 OS=Cricetulus griseus GN= Add to Clip Gallery	40.1 19	9.9 0.341	0.0347
for further review.	Proliferating cell nuclear antigen OS=Cricetulus griseus GN=I79_005005 PE=3 SV=1	45.9 22	2.7 5.17	0.869
	How are the measurements calculated? L-lactate dehydrogenase OS=Cricetulus griseus GN=I79_017480 PE=3 SV=1		4.2 0.48	0
To sort the table by a given value, simply click the relevant column header. Glutathione S-transferase P OS=Cricetulus griseus GN=I79_018157 PE=3 SV=1		50.3 24	4.2 0.16	0.106

G3HXX5	•	Unique peptides	54.9
G3HC31	-	-	46.5
G3HYJ8	_	1	82.6
G3HC84	1	1	98.3
G3IG05	3	3	243
G3H0U6	3	3	186
G3H412	2	2	162
G3I255	4	4	379
G3I3Y6	2	2	114
G3HCL2	1	1	75.5
G3HRN0	1	1	95.3
Batches Protein Table_Abs Quant Table containing Absolute Quantitation measurements for Batches of NIST mAb			

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

Enter details as required and click Add to clip gallery

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

Click Show Clip Gallery

QIP B	OP Batches - Progenesis QI for proteomics				
	File				
	Save				
	Close				
	Export peptide ion measurements				
	Export peptide measurements				
	Export protein measurements				
	Export to pathways tool				
	Import additional protein data				
	Import protein accessions as tag				
	Export mzIdentML for PRIDE submission				
	Experiment properties				
	Show Clip Gallery				
×	Exit				

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

QIP Clip Gallery		
Clip Gallery	Item size	e 🗉 🔲 Search 🔎
G3HC31 1 1 G3HVJ8 1 1 G3HC84 1 1 G3IG05 3 3 G3H0U6 3 3 G3H412 2 2 G3I255 4 4	Edit Copy Table Copy Title Copy Description Export Table	Data analysis performed using: Progenesis QI for proteomics www.nonlinear.com
G31376 2 2 G3HCL2 1 1 Batches Protein Table_Abs Quant Table containing Absolute Quantitation measurements for Batches of NIST mAb	Progenesis QI for proteomics logo A high-resolution image that you can use in presentations and posters to show the software was used in your research.	Progenesis QI for proteomics analysis badge A high-resolution image that you can use in presentations and posters to show the software was used in your research.
		Export all Close

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

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

References

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