Agilent G3835AA MassHunter Mass Profiler Professional Software

Application Guide

What is Agilent Mass Profiler Professional?

Agilent Mass Profiler Professional (MPP) software is a powerful chemometrics platform designed to exploit the high information content of mass spectra (MS) data and can be used in any MS-based differential analysis to determine relationships among two or more sample groups and variables. MPP provides advanced statistical analysis and visualization tools for GC/MS, LC/MS, CE/MS, and ICP-MS data analysis. MPP also integrates smoothly with Agilent MassHunter Workstation, Spectrum Mill, and ChemStation software and is the only platform that provides integrated identification/ annotation of compounds and integrated pathway analysis for metabolomic and proteomic studies. The system also enables Automated Sample Class Prediction that revolutionizes mass spectrometer-based qualitative analysis of unknown samples in many applications. MPP is ideally suited for applications characterized by complex sample matrices such as metabolomics, proteomics, natural products, food, beverages, flavors, fragrances, and environmental analyses.

Agilent Technologies

Where is MPP used in your experiment?

MPP is used to import, organize, and analyze the data you acquired. Your unbiased differential analysis experiment may include the following steps with MPP beginning at step four: (1) prepare for your experiment, (2) acquire your data, (3) find the spectral features, (4) import and organize your data, (5) create your initial analysis, (6) identify the features, (7) save your project, and (8) perform advanced analysis operations. Figure 1 shows the Agilent software tools used in your experiment.

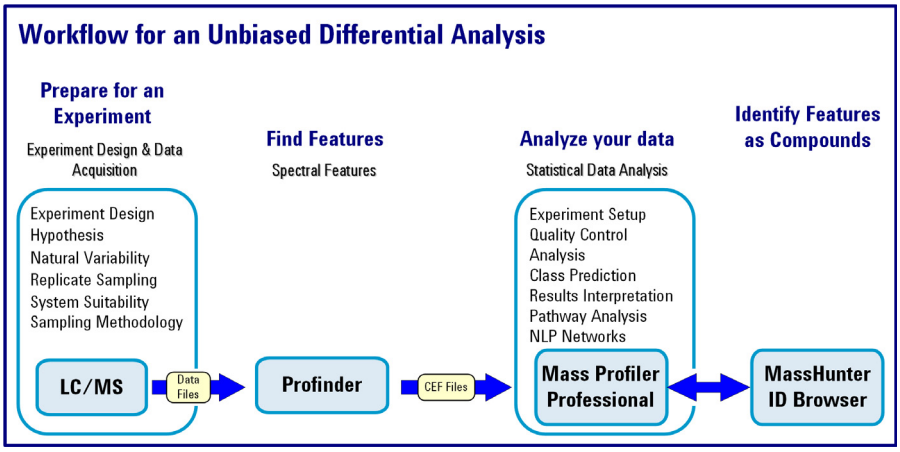


Figure 1 Agilent software that may be used in an unbiased differential analysis for LC/MS data in conjunction with Mass Profiler Professional.

How do I use MPP to analyze my data?

MPP helps you analyze your data through the use of sequential dialog boxes and wizards as shown in Figure 2 on page 3.

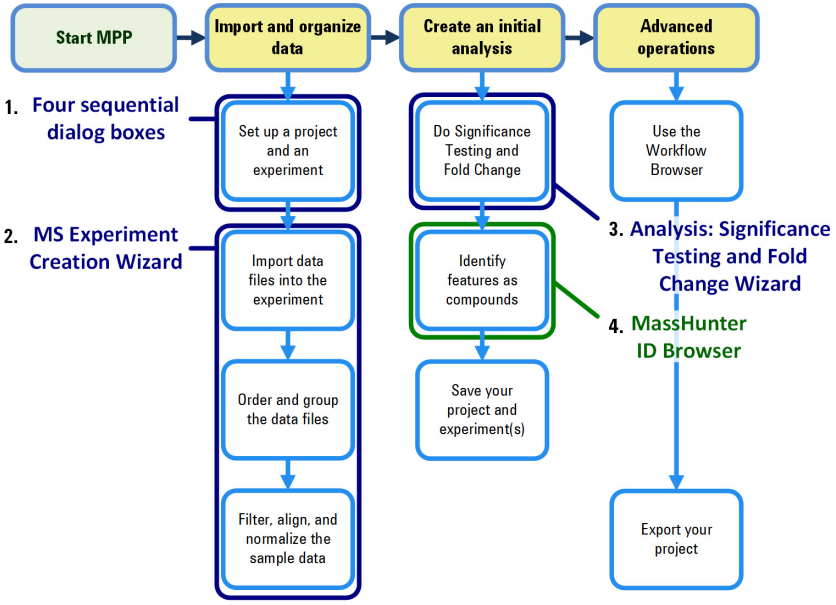


Figure 2 Overview of the four main sets of sequential dialog boxes and wizards that help you use MPP.

Where do I get more information?

The Agilent workflow guides and overviews, online Help, and user guides provide you with additional detail, techniques, and explanations to perform advanced analysis operations.

- *Agilent MassHunter Mass Profiler Professional User Manual*. You can find a PDF copy in the MPP installation folder **C:\Program Files\Agilent\MassHunter\Workstation\Mass Profiler Professional\docs\manual\MS**.
- *Agilent Metabolomics Workflow - Discovery Workflow Guide* (p/n 5990-7067EN, Revision B)
- *Agilent Metabolomics Workflow - Discovery Workflow Overview* (p/n 5990-7068EN, Revision B)
- *Integrated Biology with Agilent Mass Profiler Professional - Workflow Guide* (p/n 5991-1909EN)

- *Integrated Biology with Agilent Mass Profiler Professional - Workflow Guide Overview* (p/n 5991-1910EN)
- *Class Prediction with Agilent Mass Profiler Professional - Workflow Guide* (p/n 5991-1911EN)
- *Class Prediction with Agilent Mass Profiler Professional - Workflow Guide Overview* (p/n 5991-1912EN)
- MassHunter Profinder *Quick Start Guide* and online Help

1. Prepare for your Experiment

An experiment consists of the analysis of a set of replicate samples collected over a range of well defined parameters, treatments, and/or exposures known as independent variables, including parameter controls representing minimal or normal perturbations (control samples). The results of changes observed in the samples is designed to provide an answer to your hypothesis. The hypothesis may be proved or disproved by analyzing the correlation of the independent variables on the resulting expression of a large number of dependent variables - the features (compounds) that are measured in your samples. The results must be significant beyond natural variability.

After you obtain your samples, acquire your data, and find the features in your sample data, MPP takes you through data extraction, processing, and statistical analysis so that you can prove or disprove your hypothesis.

Elements to consider in planning your experiment

The hypothesis

The hypothesis is the question that is answered by your analysis. For example, the question may be a statement that proposes a possible correlation, or cause and effect, between a set of independent variables and the resulting features in your data.

Natural variability

It is important to understand how any one sample in your data represents the population as a whole. Because of natural variability and the uncertainties associated with both the measurement and the population, no assurance exists that any single sample from a population represents the mean of the population. Thus, increasing the sample size greatly improves the accuracy of the sample set in describing the characteristics of the population.

Replicate sampling

Sampling the entire population is not typically feasible because of constraints imposed by time, resources, and finances. On the other hand, fewer samples increase the probability of making a false positive or false negative correlation.

System suitability

System suitability involves collecting data to provide you with a means to evaluate and compensate for drift and instrumental variations to assure quality results. Techniques employed by your Agilent MassHunter software include (1) retention time alignment, (2) intensity normalization, (3) chromatographic deconvolution, and (4) baselining to produce the highest quality results. The best results are achieved by maintaining your instrument and using good chromatography.

Sampling methodology

Improved data quality comes from matching the sampling methodology to the experimental design so that replicate data is collected to span the parameter values for each parameter. A larger number of samples appropriate to the population under study results in a better answer to your hypothesis. An understanding of the methodologies used in sampling and using more than one method of sample collection have a positive impact on the significance of your results.

Where to find more information to help you prepare for your experiment

Step-by-step detail of the process for preparing for your experiment and performing an unbiased differential analysis is presented in the *Agilent Metabolomics Workflow - Discovery Workflow Guide* (p/n 5990-7067EN, Revision B).

2. Find the Features in your Data

Before you analyze your data with MPP, the features (compounds or molecular features) in your data must be extracted. For Agilent data, you can use MassHunter Mass Profiler, MassHunter Profinder, or the MassHunter Qualitative Analysis to find the features in your sample data. MPP imports and analyzes features that are saved in CEF files created by MassHunter software and features that are saved in CSV files created by non-Agilent software.

MassHunter Profinder

MassHunter Profinder software is a stand-alone program, optimized for batch feature extraction from TOF and Q-TOF based profiling data files. Feature extraction, combined with chromatographic alignment across multiple data files, is a critical step in the peak finding and data reduction workflow that minimizes the appearance of both false positive and false negative features by "binning" the features in the chromatographic time domain.

MassHunter Profinder batch feature extraction offers three different feature extraction workflows and provides you with an intuitive user interface to inspect and review each feature across the files associated with your data set. With Profinder you can review and compare extracted ion chromatograms and mass spectral data associated with each feature.

Refer to the *MassHunter Profinder Software - Quick Start Guide* (p/n 53835-90014, Revision B) and online Help for additional details.

Non-Agilent Data

You can use Mass Profiler Professional to process non-Agilent data. Once imported into Mass Profiler Professional, you can do statistical analysis and visualizations on non-Agilent data in the same way that you analyze Agilent data, except *you are not able to do*:

- Spectral visualization
- Compound identification (using ID Browser)

- Create a recursion list (for further mining of the data once interesting features are identified)
- Create an MS/MS inclusion list

1 Use your non-Agilent data acquisition and analysis program to extract the features in your sample data.

2 Export your non-Agilent data to a spreadsheet file in comma-separated, tab-separated, or Excel (.xls and .xlsx) format. Make sure that these required columns exist in the spreadsheet in this order:

- RT
- Mass
- Compound Name
- Formula
- CAS ID
- One or more Signal columns with non-specific column headers and only numeric values in each column

Please note that:

- Mass Profiler Professional recognizes only the named required columns (**RT**, **Mass**, **Compound Name**, **Formula**, and **CAS ID**), plus these optional columns: **KEGG ID**, **ChEBI ID**, **HMP ID**, **Lipid ID**, **NCBI gi ID**, and **Swiss-Prot ID**.
- Mass Profiler Professional identifies all other columns that contain numeric values as signal columns. However, you can clear any column that MPP incorrectly identifies as a signal column.
- Only the protein entities with a valid **UniprotKB Accession** values specified in the **Swiss-Prot ID** column are considered for mapping in pathway analysis. **UniprotKB Entry name** is not considered for mapping.
- A generic file (see Figure 3) can contain one or more samples and many such files can be used to create an experiment. Given a set of generic files, if the same sample name occurs multiple times in the same file or across files, Mass Profiler Professional uses only the first instance (in alphanumeric file name order) of the sample.

- Mass Profiler Professional supports Identified, Unidentified, and Combined (Identified + Unidentified) types of experiment creation for Generic data. Select one of these three experiment types during Experiment Description. The first entry that appears in any one of the **Compound Name**, **CASID**, **Swiss Prot ID**, and **Formula** columns (in that order) is used to name the entity. If no entry appears in any of these four columns, the compound is considered to be unidentified.

Mass	RT	Compound Name	Formula	CAS ID	KEGG ID	Ch
410.0033	0.298625					
352.0715	0.3075	Griseofulvin		126-07-8		
693.9807	0.32					
695.9493	0.32025					
433.9571	0.318625					
611.9776	0.321375					
529.9748	0.321375					
791.9571	0.321875					
541.0607	0.419	cyclic adenosine diphosphate ribose		119340-53-3		
427.0293	0.45175	Zidovudine diphosphate		106060-89-3		

Figure 3 Example spreadsheet to import non-Agilent data. Click this image to open the spreadsheet file for use as a template.

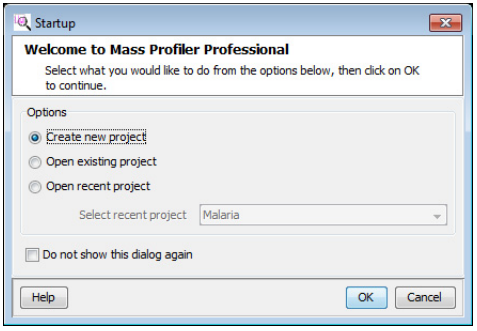
3. Import and Organize your Data

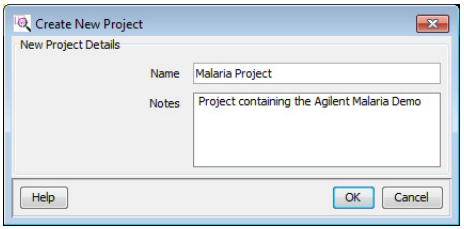
Create a new project and experiment for your data

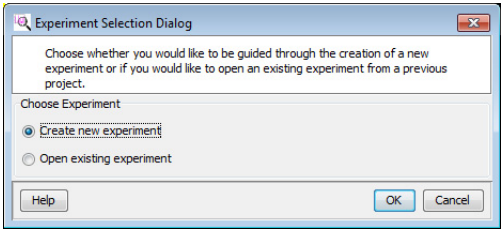
You are guided through four sequential dialog boxes to create a new project and experiment to receive your data:

1. **Startup:** Select the option to create a new project.
2. **Create New Project:** Type descriptive information about your project.
3. **Experiment Selection:** Select the option to create a new experiment as part of your project.
4. **New Experiment:** Set up the information to store with your experiment and to guide the analysis process.

Follow the steps below to setup your new project. The Agilent *Malaria Demo* data set is used as an example in each step. You are encouraged to substitute the demo information and data files with your own data.

Steps	Detailed Instructions	Comments
1 Start Mass Profiler Professional.	**a** Click the **Mass Profiler Professional** icon on your desktop.	• When MPP starts, if you choose, you are immediately guided through four sequential dialog boxes to create a new project and experiment.
2 Create a new project from the **Startup** dialog box. 	**a** Click **Create new project**. **b** Click **OK**.	• **Create new project** provides you with the option to create a new experiment or import an experiment from an existing project into the new project. • After closing an open project, you may create a new project from the Menu bar; click **Project > New Project,** or from the Toolbar; click the **New project** button .

Steps	Detailed Instructions	Comments
3 In the **Create New Project** dialog box, enter your project information. 	**a** Type `Malaria Project` or your project information in **Name**. **b** Type descriptive information in **Notes**. **c** Click **OK**.	• The project name and notes may be viewed and edited at any time using the **Project Inspector** by clicking **Project > Inspect Project** from the menu bar.

4 In the **Experiment Selection Dialog** dialog box, create a new experiment.
 
	a Click **Create new experiment**.
	b Click **OK**.
		• You can also create a new experiment in your project from the:
		  • Menu bar: Click **Project > New Experiment.**
		  • Toolbar: Click the **New experiment** button  .
		• **Open existing experiment** opens a project and the experiment(s) that are stored in the project. You may also click the **Add experiment** button  to add an existing experiment to your project.

5 In the **New Experiment** dialog box, enter and select information that guides your experiment creation.	**a** Type a descriptive name for the experiment in **Experiment name**. **b** Select **Mass Profiler Professional** for **Analysis type**. **c** Select **Unidentified** or **Combined (Identified + Unidentified)** for the **Experiment type**. **d** Select **Analysis: Significance Testing and Fold Change** for **Workflow type**. **e** Type descriptive information in **Experiment notes**. **f** Click **OK**.	• Regardless of your personal expertise, it is recommended to select the **Analysis: Significance Testing and Fold Change** for the **Workflow type** to provide you with quality control to your analysis that improves your results. • At the conclusion of the **Analysis: Significance Testing and Fold Change** workflow, you may save your project and customize your entire analysis using the operations available in the Workflow Browser.

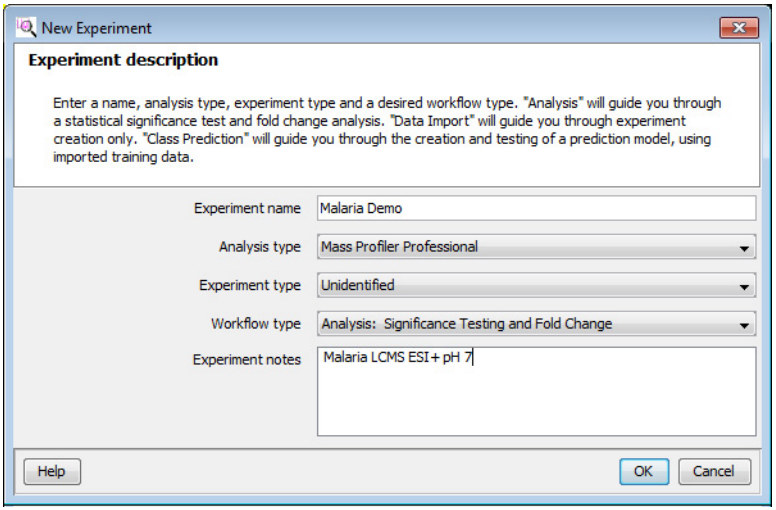
Steps	Detailed Instructions	Comments
		• Table 1 on page 13 and Table 2 on page 13 show the selection and entry options available to you for the **New Experiment** dialog box • **Experiment type** (see also Table 2 on page 13) determines how Mass Profiler Professional manages the data: ◦ Select **Combined (Identified + Unidentified)** when you are unsure if the data has been identified in full or in part, or when MassHunter Qualitative Analysis has been previously used to identify some of the compound features. ◦ Select **Identified** or **Proteomics (Identified)** when the compounds have been identified by compound, formula, and/or CAS number. ◦ Select **Unidentified** when the compounds have only been identified by their molecular features of neutral mass and retention time. • If you selected **Analysis: Significance Testing and Fold Change** or **Data Import Wizard** for the **Workflow type** in the **New Experiment** dialog box, you immediately begin the data import process.

Table 1 Table of selections and entries for the New Experiment dialog box

Dialog Box Option	Your Choices	Comments
Experiment name	<none>	Describe this experiment
Analysis type	Mass Profiler Professional <other choices depending on Order IDs>	"Mass Profiler Professional" must be selected.
Experiment type	Combined (Identified + Unidentified) Identified Proteomics (Identified) Unidentified	<see Table 2>
Workflow type	Analysis: Significance Testing and Fold Change Class Prediction: Build and Test Model Data Import Wizard	
Experiment notes		Enter other experimental notes.

Table 2 Table of data sources and file extensions based on Experiment Type

Experiment Type	Data Source	File Types	Comments
Combined	MH Qual/Profinder	.CEF	Find by Molecular Feature Extractor (MFE) and Find by Formula (FbF)
	MH Qual (GC Scan)	.CEF	Find by Chromatographic Deconvolution and Library Search
	MH ICP-MS	.CEF	Identified by ICP-MS software
	AMDIS	.ELU	Targets and components discovered by AMDIS
	Generic	.XLS .XLSX .CSV .TXT	Combination of entries identified by and not identified by Compound (column C), Formula (column D), CASID (column E)

Table 2 Table of data sources and file extensions based on Experiment Type (continued)

Experiment Type	Data Source	File Types	Comments
Identified	MH Quant	.CEF	Compounds identified by MassHunter Quantitative Analysis
	MH Qual/Profinder	.CEF	Find by Formula (FbF)
	MH Qual (GC Scan)	.CEF	Identified by Unit Mass Library
	MH ICP-MS	.CSV	Identified by ICP-MS software
	ChemStation	.FIN	Identified by ChemStation Quantification or Screener processes
	AMDIS	.FIN	Compound identified by an AMDIS target library
	Chenomx	.CSV	Compound identified using Chenomx NMR Suite
	Generic	XLS .XLSX .CSV .TXT	Entries identified by Compound (column C), Formula (column D), CASID (column E)
Proteomics	Spectrum Mill	.APR	Compounds identified by Spectrum Mill and saved as an Agilent Proteomics Results file
Unidentified	MH Qual/Profinder	.CEF	Find by Molecular Feature Extractor (MFE)
	MH Qual (GC Scan)	.CEF	Find by Chromatographic Deconvolution
	ICP-MS	.CEF	Identified by ICP-MS software
	AMDIS	.ELU	Components identified by AMDIS that are not identified by an AMDIS target library
	Generic	XLS .XLSX .CSV .TXT	Entries not identified by Compound (column C), Formula (column D), CASID (column E)

Import and organize your data

After you set up your project and create an experiment the **MS Experiment Creation Wizard** (Figure 4) immediately guides you through the necessary steps to organize your experiment, import your data, define your experiment variables, and prepare your data for analysis; data preparation includes grouping, filtering, alignment, normalization, and baselining.

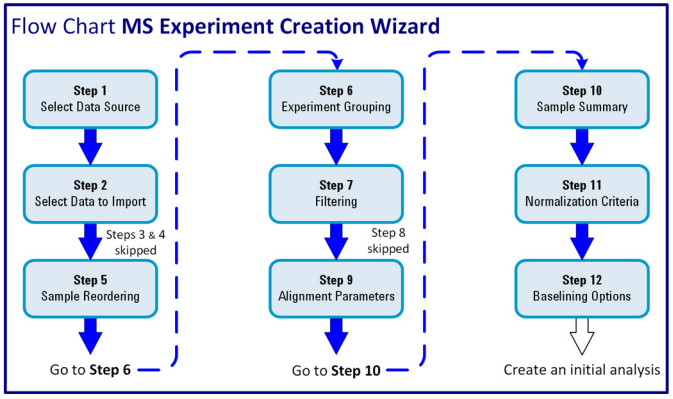
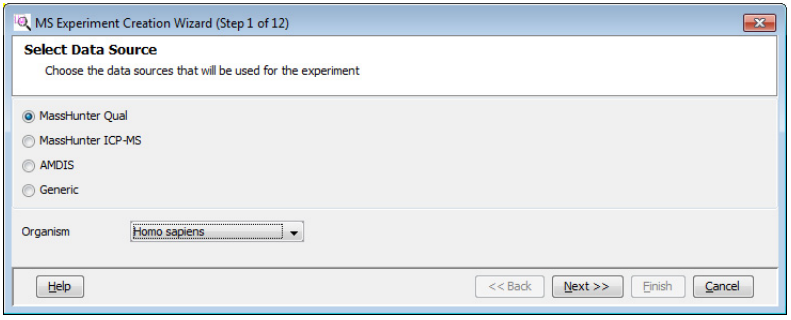
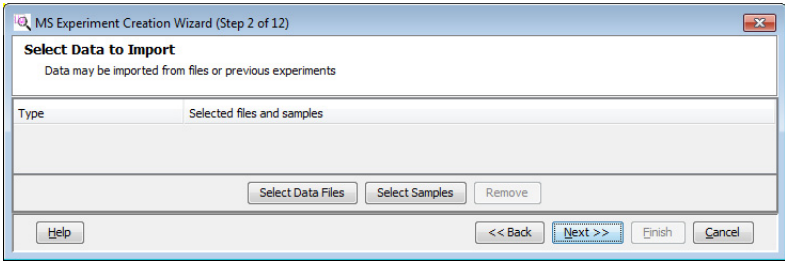
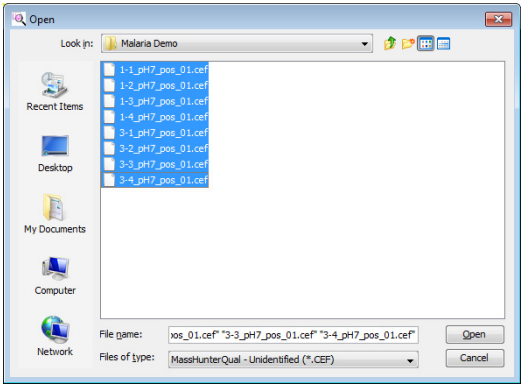


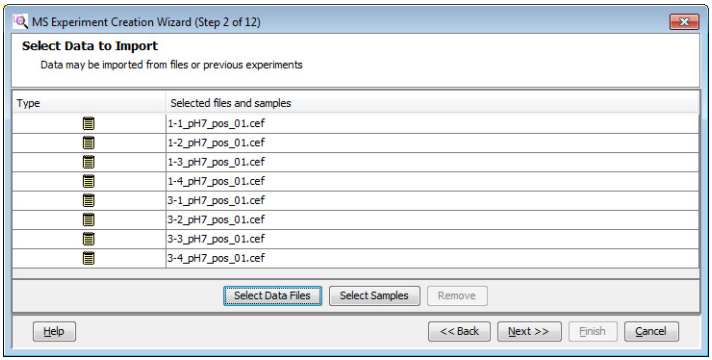
Figure 4 Steps in the **MS Experiment Creation Wizard**

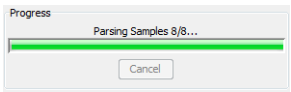
Steps	Detailed Instructions	Comments
1 Select the data source that generated the molecular features for your experiment in the **MS Experiment Creation Wizard (Step 1 of 12)**.	**a** Click **MassHunter Qual** and select **Homo sapiens** for the **Organism** if you are using the *Malaria Demo* data set. **b** Click **Next**.	• If you are using your own data set, click the source of your sample files, and select the **Organism** of the sample files or select **None**. • Selecting an **Organism** is most important when you use the Pathway Analysis features of MPP. • If you are importing a non-Agilent data file, click **Generic**.

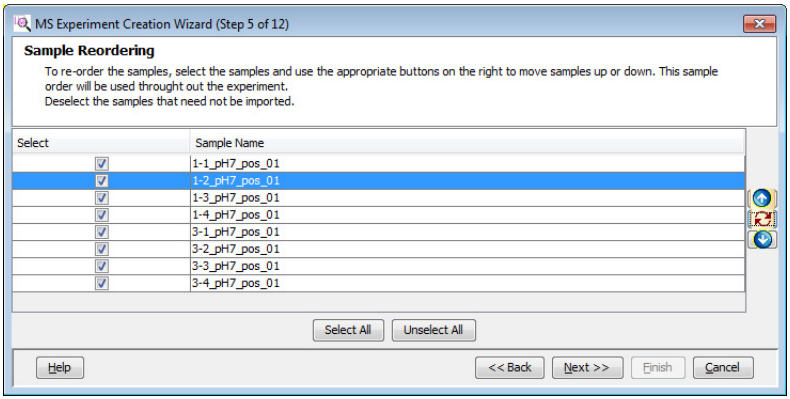


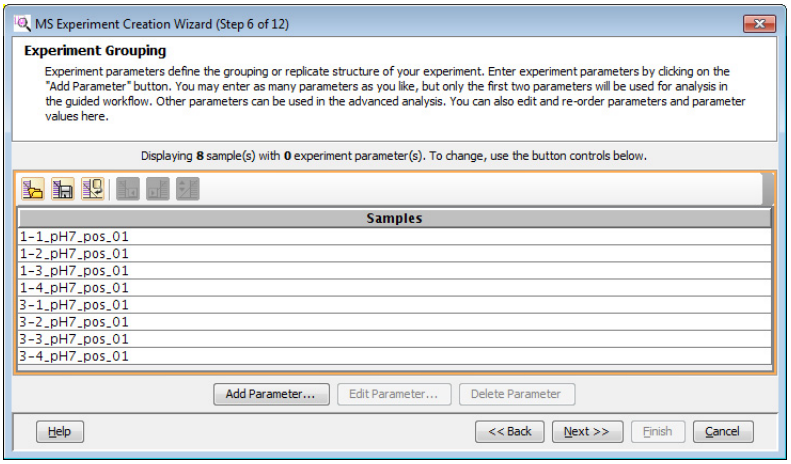
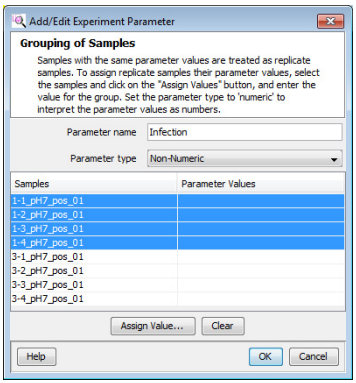
Steps	Detailed Instructions	Comments
2 Select the molecular feature sample files to import in the **MS Experiment Creation Wizard (Step 2 of 12)**.	**a** Click **Select Data Files**.	• The file type you need to select depends on the data source you selected in the **MS Experiment Creation Wizard (Step 1 of 12).** ◦ See Table 2 on page 13 for a comprehensive list of data sources you can select from based on your experiment type.
		• To control your progress through the wizard dialog boxes: ◦ Click Next >> to go to the next step. ◦ Click << Back to return to prior steps and make modifications to your settings and previous entries. ◦ Click **Cancel** to end the **MS Experiment Creation Wizard** without saving.
	b Select your samples in the **Open** dialog box. If necessary, browse to **C:\Program Files\Agilent\MassHunter\Workstation\Mass Profiler Professional\samples\Malaria Demo** for the Malaria Demo. **c** Click the sample molecular feature **data files** to import into the experiment. The example Malaria data files are: ◦ 1-1_pH7_pos_01.cef ◦ 1-2_pH7_pos_01.cef ◦ 1-3_pH7_pos_01.cef ◦ 1-4_pH7_pos_01.cef ◦ 3-1_pH7_pos_01.cef ◦ 3-2_pH7_pos_01.cef ◦ 3-3_pH7_pos_01.cef ◦ 3-4_pH7_pos_01.cef	• You can select a continuous range of files with a click on the first file and press **Shift** and click on the last file that includes the range of files you want to select. • You may select discontinuous, individual by pressing **Ctrl** and clicking on additional files.
		
	d Click **Open** to load the selected files.	

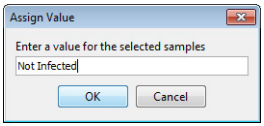
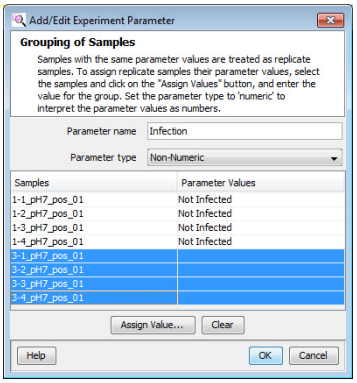
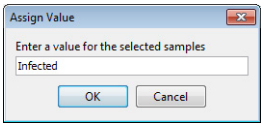
Steps	Detailed Instructions	Comments
	e Click **Next**.	• Replicate samples are from the collection of multiple identical samples from a population. When replicate samples are evaluated a result is obtained that more closely approximates the true value of the population. • You can review and make changes to your selection during the next step before finalizing the experiment creation.
		• A progress indicator is shown while your files are imported into MPP.
3 Review and order the sample files based on the independent variables in your experiment in the **MS Experiment Creation Wizard (Step 5 of 12)**.	**a** Click one or more samples that you want to reorder. **b** Click the **Up** or **Down** button to reorder the selected sample(s). **c** Repeat the reordering actions as often as necessary to obtain your order. **d** Mark the sample names that you want to import into your experiment. **e** Click **Next**.	• ***Note:*** This step is the only opportunity to reorder your samples. After completing the data import, create a new project or experiment and repeat this process to reorder your samples. • You may select a continuous range of files with a click on a first file and a Shift-click on a last file that includes the range of files you want to select. • Click the **Restore** button at any time to return the sample order to your starting point when this step was begun.

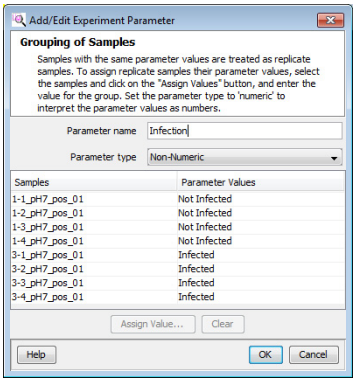
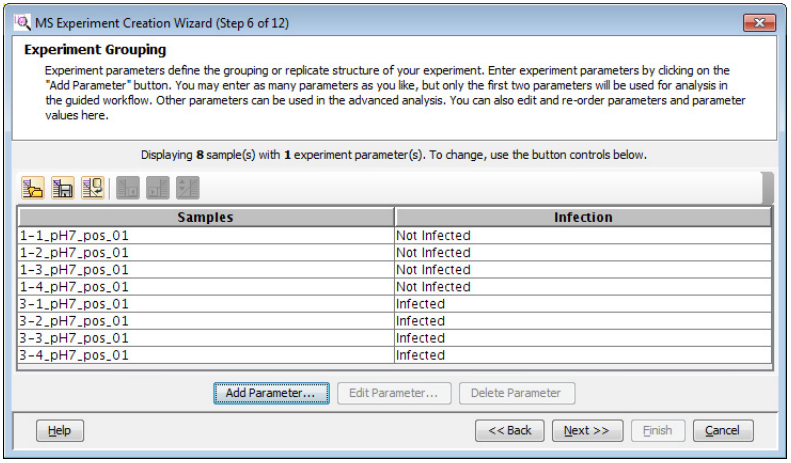




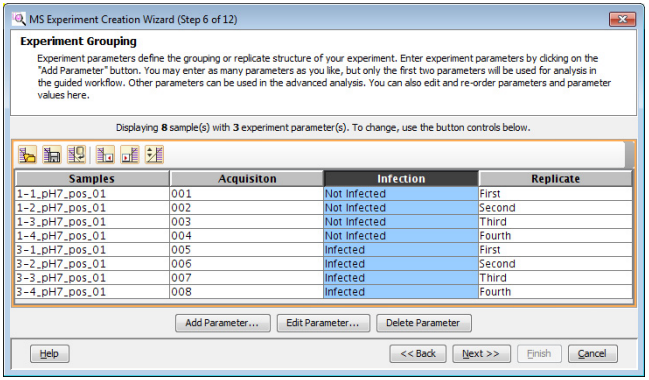


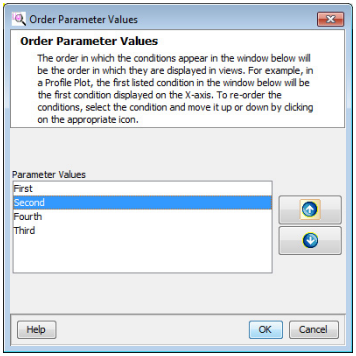
Steps	Detailed Instructions	Comments
4 Define the sample grouping with respect to the independent variables and the replicate structure of your experiment in the **MS Experiment Creation Wizard (Step 6 of 12)**.	**a** Click **Add Parameter**.	• ***Note:*** Grouping at this time is optional. You may add grouping or change your grouping during the **Analysis: Significance Testing and Fold Change Wizard** or at any time thereafter.
		• An independent variable is an essential element, constituent, attribute, or quality in a data set that is deliberately controlled in your experiment. An independent variable is referred to as a parameter and is assigned a parameter name. • The attribute values within an independent variable are referred to as parameter values. Samples with the same parameter value and the same parameter name are treated as replicates.
	**b** Type a name for your **Parameter name** in the **Add/Edit Experiment Parameter** dialog box. Type `Infection` for the *Malaria Demo*. **c** Click your replicate **Samples** that share the same first parameter value in your data. For example: • 1-1_pH7_pos_01 • 1-2_pH7_pos_01 • 1-3_pH7_pos_01 • 1-4_pH7_pos_01 **d** Select the **Parameter type** for your grouping. **Non-Numeric** is selected for the *Malaria Demo*. **e** Click **Assign Value**.	• Parameter Type options: ◦ Select **Non-Numeric** if the grouping is not a quantitative value. ◦ Select **Numeric** if the grouping value is quantitative or a value that reflects a degree of proportionality among the samples with respect to an independent variable. A numeric parameter type allows some data plots to be scaled by the parameter values.

Steps	Detailed Instructions	Comments
	**f** Type the value for your first grouping in the **Assign Value** dialog box. For the *Malaria Demo* type `Not Infected`. **g** Click **OK**.	• In this example the samples are assigned parameter values representing the Infection parameter.
	**h** Click your replicate **Samples** that share the same second parameter value in your data. For example: • 3-1_pH7_pos_01 • 3-2_pH7_pos_01 • 3-3_pH7_pos_01 • 3-4_pH7_pos_01 **i** Click **Assign Value**.	• The highlighted samples are assigned the value typed in the **Assign Value** dialog box.
	**j** Type the value for your second grouping in the **Assign Value** dialog box. For the Malaria data type `Infected`. **k** Click **OK**. **l** Repeat the value assignment steps with your own data until you have assigned a parameter name, type, and value to all of your samples. **m** Review your entries and grouping assignment accuracy in the **Add/Edit Experiment Parameter** dialog box. **n** Repeat the value assignments for individual or multiple samples as necessary to make corrections or changes. **o** Click **OK** when the grouping for this parameter name is complete.	

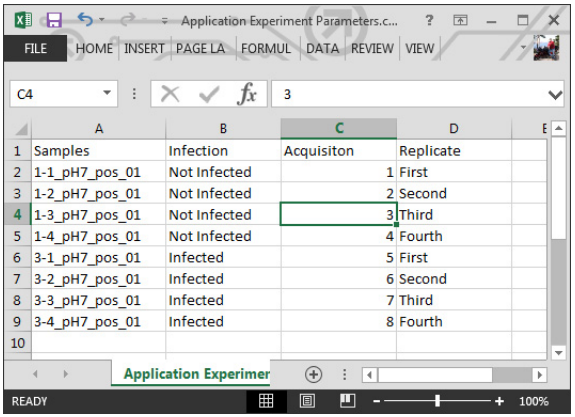
Steps	Detailed Instructions	Comments
	**p** Repeat **Add Parameter** if your data has more than one independent variable. • Click **Add Parameter**. • Repeat the steps above until you have assigned a parameter name, type, and value to all of your data. **q** Review step 5 *OPTIONAL: Re-order your parameter values* and step 6 *OPTIONAL: Saving and importing experiment grouping information in a spreadsheet.* These steps provide advanced instructions to manage your parameters and parameter name assignments using the wizard toolbar and a spreadsheet application. **r** Click **Next** when you have completed your experiment grouping.	• You may change the value of any sample, or group of samples; highlight the sample and click **Assign Value** or **Clear**. • ***Note:*** You may add grouping or change your grouping during the **Analysis: Significance Testing and Fold Change Wizard** and at any time thereafter.
		

Steps	Detailed Instructions	Comments
5 OPTIONAL: Re-order your parameter values.	**a** Click any one value under the parameter column to select the whole parameter column. **b** Re-order the parameter column, click the **Left** or **Right** button.	• When you have more than one parameter associated with your samples, each parameter and its values is displayed in a separate column in the **MS Experiment Creation Wizard (Step 6 of 12)** dialog box. • When the parameter column is selected the column is highlighted.
	c Re-order the parameter values by selecting a parameter column, then click the **Re-order parameter values** button. **d** Click one or more values that you want to reorder. **e** Click the **Up** or **Down** button to reorder the selected value(s). **f** Click **OK** when the order for this parameter is complete.	
6 OPTIONAL: Saving and importing experiment grouping information in a spreadsheet.	**a** Save the experiment parameters and parameter values to a .tsv. Click the **Save experiment parameters to file** button. **b** Load your experiment parameter grouping values from a .tsv file, instead of using the MPP user interface. Click the **Load experiment parameters from file** button.	• An example experiment grouping file that is in the *Malaria Demo* directory named "MALARIA EXPERIMENT PARAMETERS (to be loaded from file).tsv" • The .tsv file is organized using tab separated values (tsv) that may be created, edited, and viewed using Microsoft Excel or Notepad.

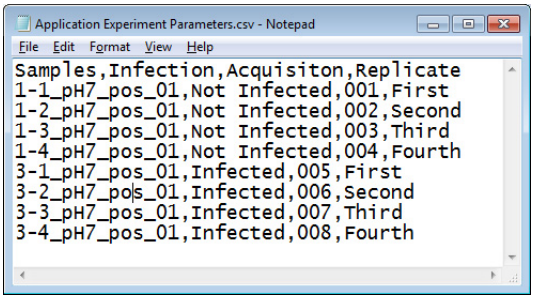




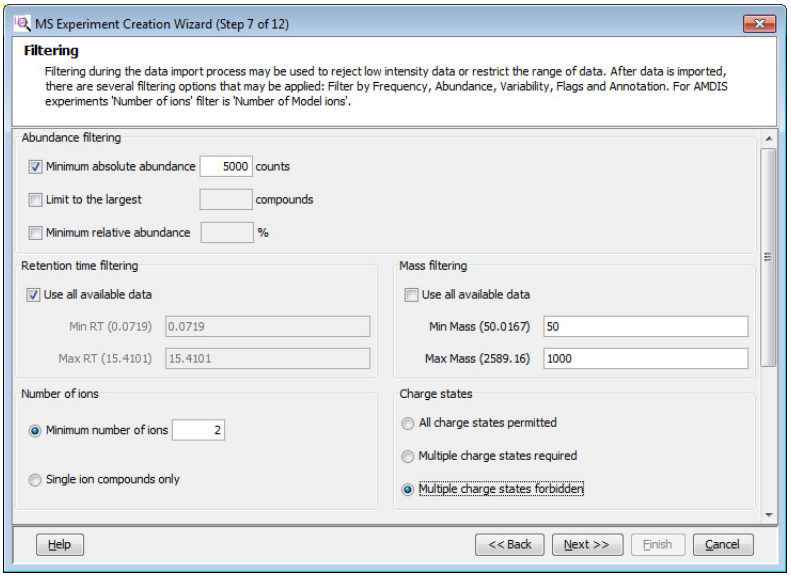
Steps	Detailed Instructions	Comments
	c Load your experiment parameter grouping values from a sample file, if applicable, by clicking the **Import parameters from samples** button.	• Creating and editing experiment parameter groupings may be more convenient for you using Microsoft Excel. Save your file as a .tsv file.



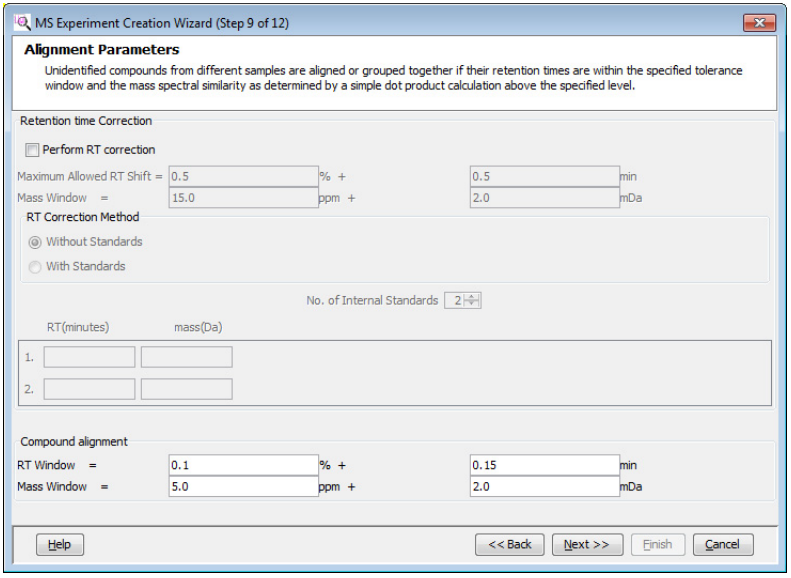
	A	B	C	D
1	Samples	Infection	Acquisiton	Replicate
2	1-1_pH7_pos_01	Not Infected	1	First
3	1-2_pH7_pos_01	Not Infected	2	Second
4	1-3_pH7_pos_01	Not Infected	3	Third
5	1-4_pH7_pos_01	Not Infected	4	Fourth
6	3-1_pH7_pos_01	Infected	5	First
7	3-2_pH7_pos_01	Infected	6	Second
8	3-3_pH7_pos_01	Infected	7	Third
9	3-4_pH7_pos_01	Infected	8	Fourth
10				



Steps	Detailed Instructions	Comments
7 Filter the molecular features by abundance, mass range, number of ions per feature, and charge state in the **MS Experiment Creation Wizard (Step 7 of 12)**.	**a** Mark the **Minimum absolute abundance** check box under Abundance filtering. **b** Type a value of `5000` **counts**. **c** Clear the **Limit to the largest** and **Minimum relative abundance** check boxes.	• The filtering parameters dialog box is unique for each experiment type. More information may be found in the online Help. • **MassHunter Qual** as the selected data source, used in this example, presents the most active fields. • Filtering during the data import process may be used to reject low-intensity data or restrict the range of data. • In a Find by Molecular Feature (MFE) generated data file the term abundance actually refers to the feature volume. • In a Find by Formula (FbF) generated data file the term abundance actually refers to the feature chromatographic area.
	d Mark the **Use all available data** check box under Retention time filtering. **e** Clear the **Use all available data** check box and type `50.00` for the **Min Mass** and `1000` for the **Max Mass** under Mass filtering. **f** Click the **Minimum number of ions** button and type `2` under Number of ions. **g** Click **Multiple charge states forbidden** under Charge states. **h** Click **Next**.	• Filtering by maximum mass may improve your statistical analysis by rejecting masses that are not significant to the experiment. This is especially relevant to metabolomic samples. • The filter parameters may be cleared to preserve the prior filtering that was used to generate the feature data file. • Filtering works with both GC/MS and LC/MS data.

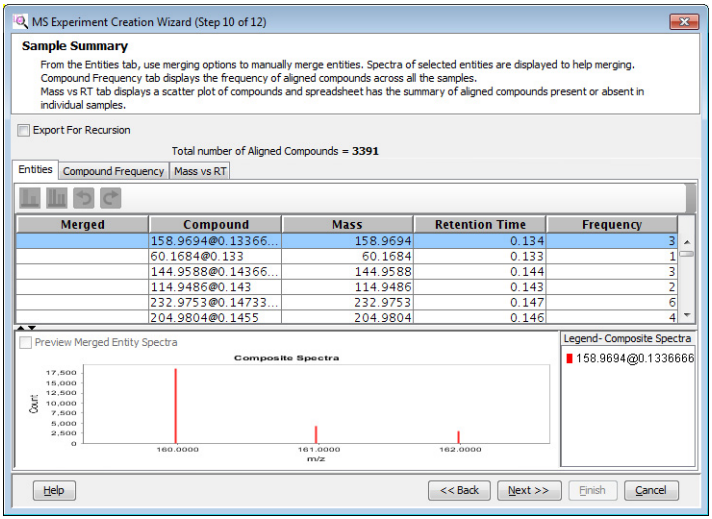


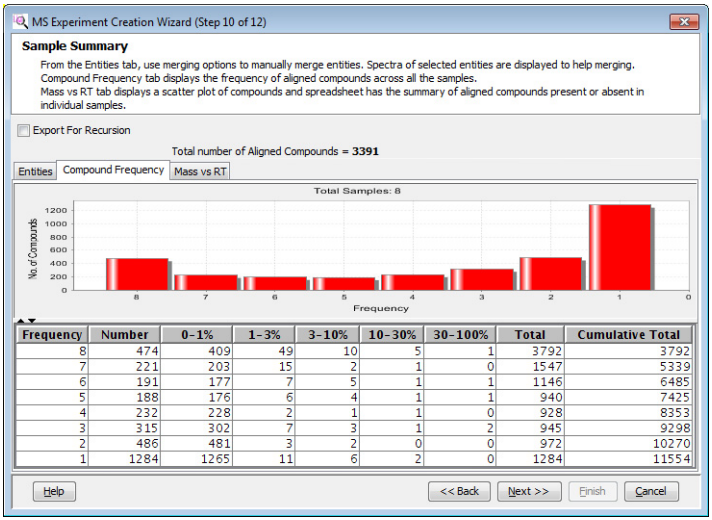
Steps	Detailed Instructions	Comments
8 Align the features across the samples based on tolerances established by retention time and mass in the **MS Experiment Creation Wizard (Step 9 of 12)**.	**a** Clear the **Perform RT correction** check box. **b** Type `0.1` **%** and `0.15` **min** for **RT Window**. A smaller value reduces compound grouping and leads to a larger list of unique compounds. **c** Type `5.0` **ppm** and `2.0` **mDa** for Mass Window. It is not recommended to set the mass window less than 2.0 mDa for higher masses. **d** Click **Next**.	• This step is omitted when the experiment type is "identified." • GC/MS data alignment includes retention time difference and mass spectral match factor. • A large retention time shift may be used to compensate for less than ideal chromatography.

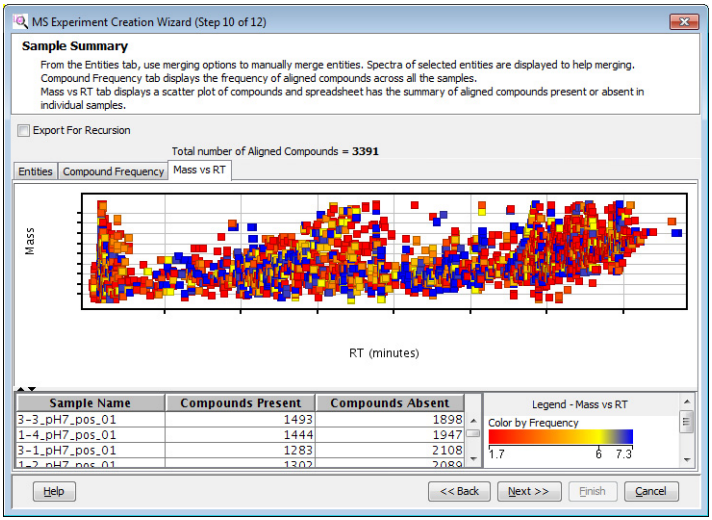


- If retention time correction is used, it is recommended to use at least two widely spaced standards, and to use standards that are present in every sample. The correction is based on a piecewise linear fit.
- Unidentified compounds from different samples are aligned or grouped together if (1) their retention times are within the specified tolerance window and (2) the mass spectral similarity are above the specified level.
- Retention alignment rewrites the retention times in the data file.
- If your CEF files are from Profinder the compound alignment performed by Profinder can be exported with the CEF files and imported into MPP. With these VCEF files the compound alignment settings can be set as follows: **RT Window** is 0% and 0.0001 min and **Mass Window** is 0.0001 ppm and 0 mDa.

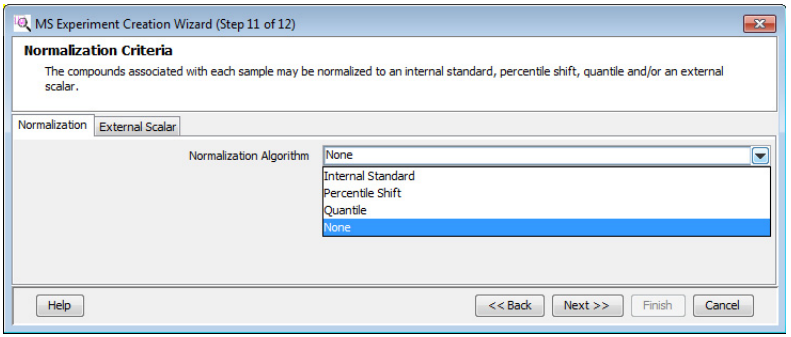
Steps	Detailed Instructions	Comments
9 Review the compounds present and absent in each sample in the **MS Experiment Creation Wizard (Step 10 of 12)**.	**a** Click the **Compound Frequency** tab. **b** Clear the **Export for Recursion** check box. **c** Click **Next**.	• This step shows a summary of the compounds present and absent in each of the samples based on the experiment parameters, including the application of the filter and alignment parameters. • The **Entities** table allows you to merge two or more compounds into a single merged compound. This tab is only visible for Experiment types: **Combined (Identified + Unidentified)** and **Unidentified**. • The **Compound Frequency** chart and table report the number of *common* entities that appear in your samples (i.e., there are 474 entities that appear in all 8 samples and 1,284 entities that appear in only 1 sample - "one-hit wonders"). The percent columns show you abundance distribution of the *identical* entities normalized to the most abundant *common* entity. • If most of the "one-hit wonders" have a low relative abundance your sample data alignment is likely good. If the "one-hit wonders" have a high relative abundance (i.e., in the 30-100% column) then you may need to improve your sample data alignment.

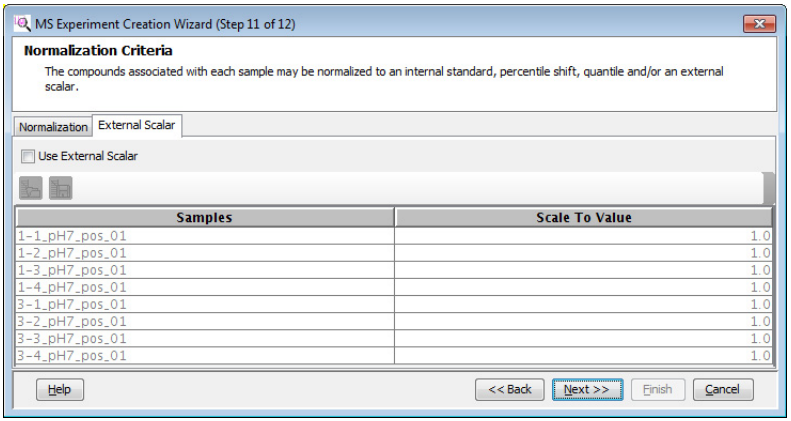




Steps	Detailed Instructions	Comments
		• In the **Mass vs. RT** table, replicate samples are expected to have a similar number of compounds present and absent. • Use the **Back** and **Next** feature to independently assess the effects of your retention time alignment versus compound alignment. • It is not recommended to export the compounds for recursion at this step in your experiment. Better results are obtained after the data has been filtered for significance.
10 Select whether to normalize the data to reduce the variability caused by sample preparation and instrument response in the **MS Experiment Creation Wizard (Step 11 of 12).**	**a** Select **None** for the **Normalization Algorithm**. **b** Clear the **Use External Scalar** check box. **c** Click **Next**.	• You may use normalization and external scalar techniques to reduce the variability in your data that was caused by sample preparation and instrument response.

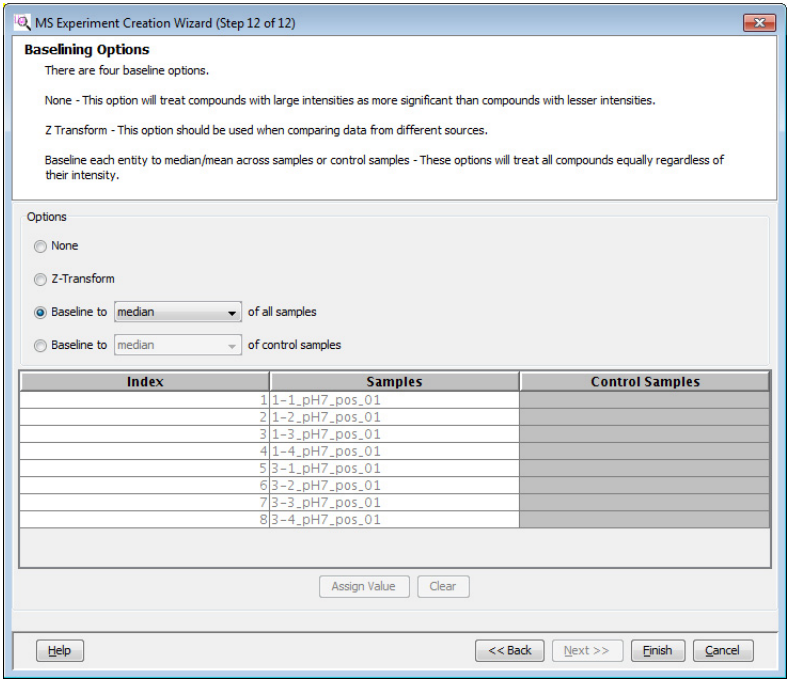
Steps	Detailed Instructions	Comments





Steps	Detailed Instructions	Comments

11 Compare the features in each sample to the response of each feature across multiple samples, or the control samples, in the **MS Experiment Creation Wizard (Step 12 of 12).**	**a** Click the **Baseline to ____ of all samples** button.
	b Select **median** for the Baseline to ____of all samples.
	c Click the **Finish** button << Back.
		• There are four baselining options:
		  • **None:** Recommended if only a few features in the samples exist.
		  • **Z-Transform:** Recommended if the data sets are from different sources or if the data sets are very dense, i.e., with data where very few instances of compounds are absent from any sample, such as a quantitation data set from recursion.
		  • **Baseline to ____ of all samples:** The abundance for each compound is normalized to its selected statistical abundance across all of the samples. This has the effect of reducing the weight of very large and very small compound features on later statistical analyses.
		  • **Baseline to ____ of control samples:** The abundance for each compound is normalized to its selected statistical abundance across just the samples selected as the control samples. This has the effect of weighting the compound features to a known value that is considered to be normal in the population while reducing the effect of large and small compound features.
		• If you selected **Analysis: Significance Testing and Fold Change** for the **Workflow type** in the **New Experiment** dialog box you immediately begin your analysis.



4. Create your Initial Analysis

The **Analysis: Significance Testing and Fold Change Wizard** (Figure 5) improves the quality of your results and helps you create an initial differential expression from your data. The steps are predetermined and based on the experiment type, experiment grouping, and conditions you entered when creating your project and setting up your experiment. Some steps may be automatically skipped for your experiment.

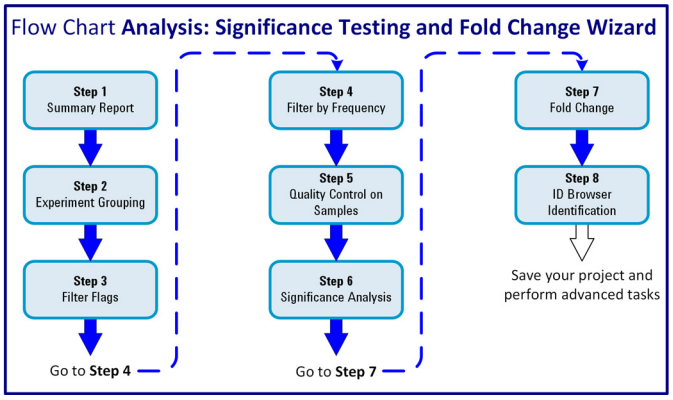
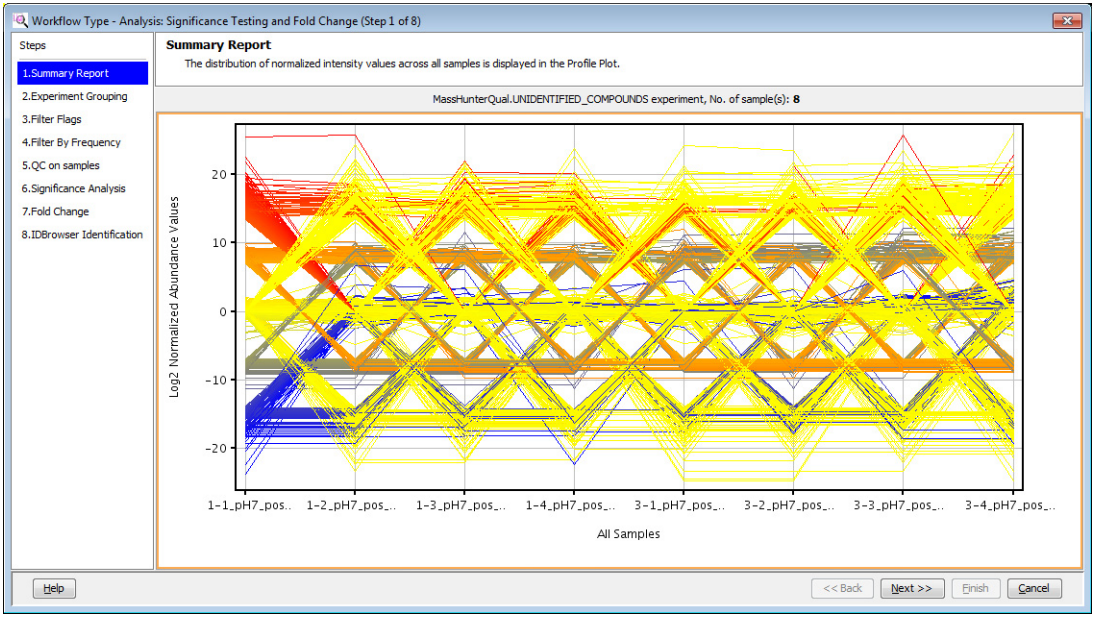
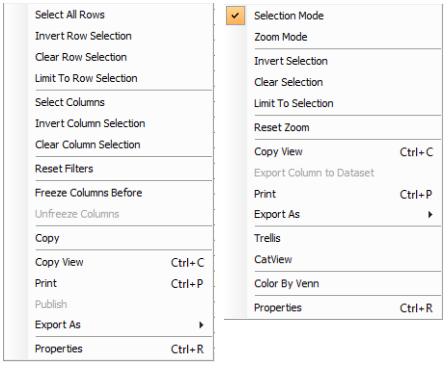


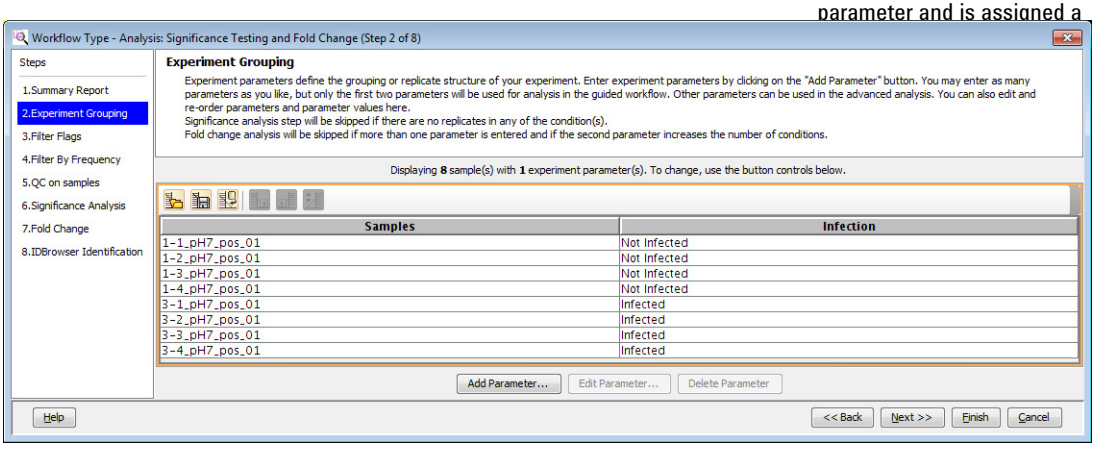
Figure 5 Steps in the **Analysis: Significance Testing and Fold Change Wizard**

Steps	Detailed Instructions	Comments
1 Review the summary of your new experiment. **Summary Report (Step 1 of 8)**.	**a** Review the Summary Report. **b** Click and right-click features on the plot, or spreadsheet, to review the data, change the plot view, export selected data, or export the plot to a file. **c** Click **Next**.	• Familiarize yourself with the tools available to you in the summary report view. • The Summary Report is displayed as a spreadsheet view when you have more than 30 samples.

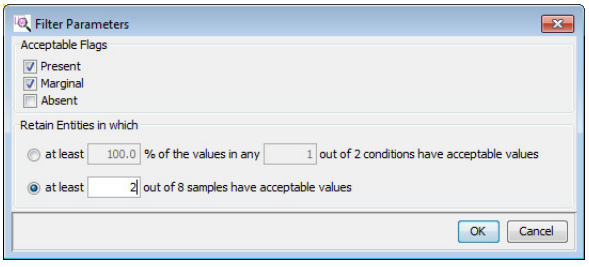




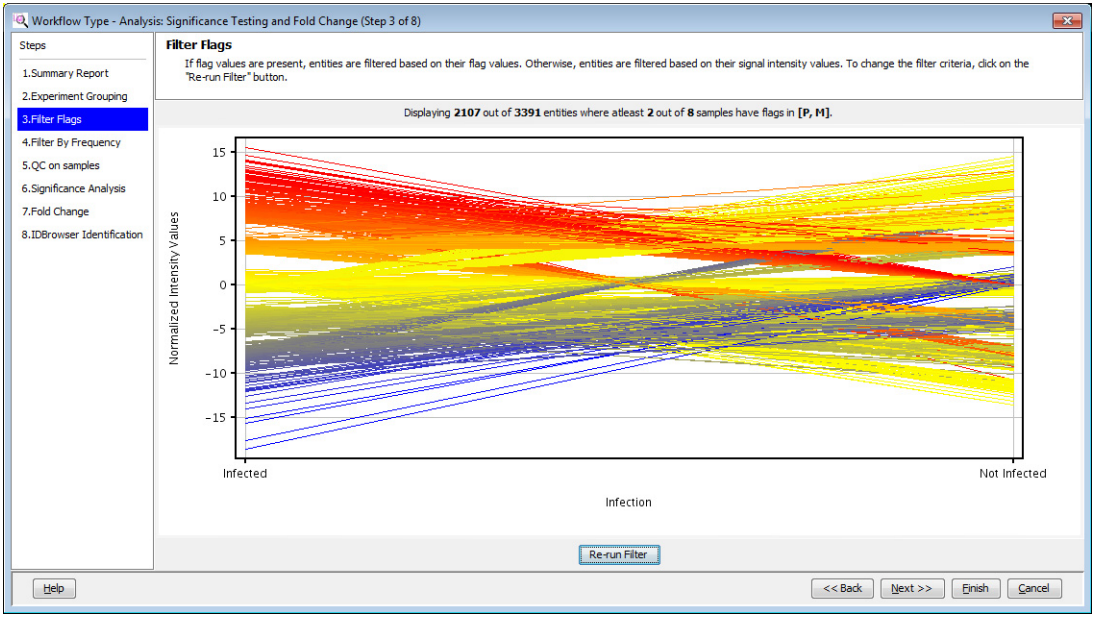
Steps	Detailed Instructions	Comments
2 Define or adjust the sample grouping with respect to the independent variables and the replicate structure of your experiment. **Experiment Grouping (Step 2 of 8).**	**a** Click **Add Parameter** to define or adjust your experiment grouping. **b** Follow the steps in "Define the sample grouping with respect to the independent variables and the replicate structure of your experiment in the MS Experiment Creation Wizard (Step 6 of 12)." on page 18. **c** Click **Next** when you have completed your experiment grouping.	• ***Note:*** In order to proceed to the next step at least one parameter with two parameter values must be assigned. • An independent variable is an essential element, constituent, attribute, or quality in a data set that is deliberately controlled in an experiment. An independent variable is referred to as a parameter and is assigned a



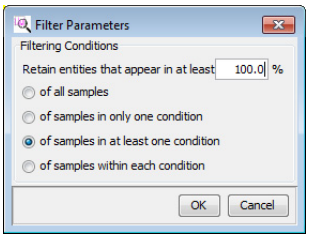
3 Filter entities from your samples based on the quality of their presence in specified samples and conditions. **Filter Flags (Step 3 of 8).**	**a** Review the summary plot. **b** Click **Re-run Filter** to enter parameters into the **Filter Parameters** dialog box. **c** Mark the **Present** and **Marginal** check boxes.	• A flag is a term used to denote the quality of an entity within a sample. A flag indicates if the entity was detected in each sample as follows: **Present** means the entity was detected, **Absent** means the entity was not detected, and **Marginal** means the signal for the entity was saturated. • This filter removes irreproducible entities from further consideration by your analysis.

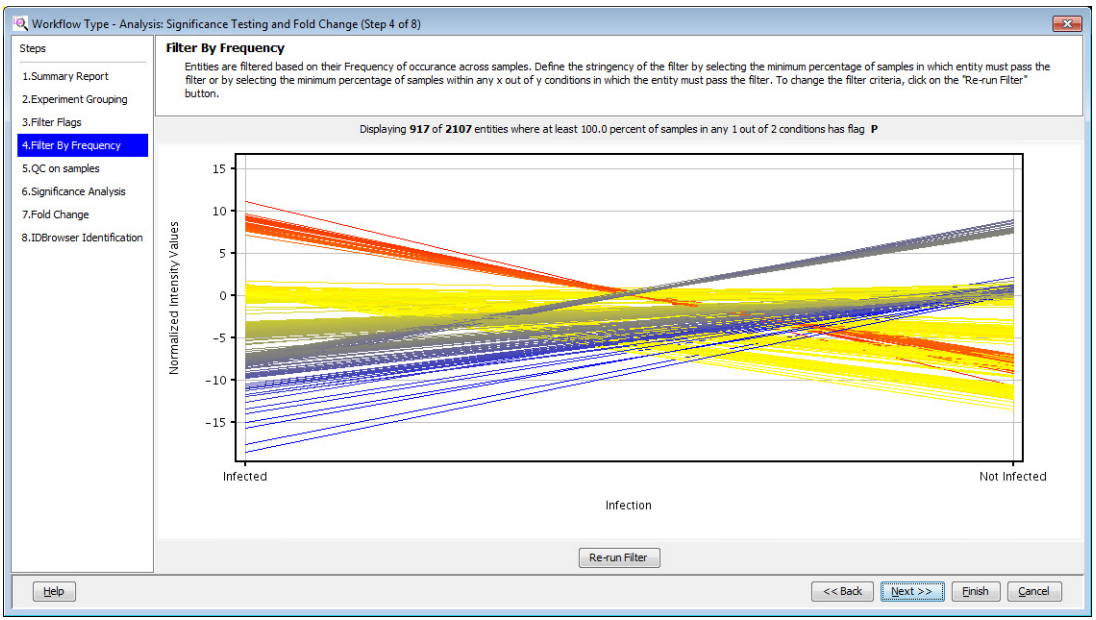


Steps	Detailed Instructions	Comments
	d Clear the **Absent** check box. This flag is useful when you want to identify missing entities in the sample data. **e** Click **at least ___ out of X samples have acceptable values**. The "X" is replaced in your display with the total number of samples in your data set. **f** Type 2 in the entry box. By setting this parameter to a value of two or more, "one-hit wonders" are filtered. **g** Click **OK**. **h** Review the profile plot. You are encouraged to repeat the **Re-run Filter** until you obtain the best results for your experiment. **i** Click **Next**.	• The number of entities displayed above the profile plot is expected to decrease as you progress through the workflow. • A "one-hit wonder" is an entity that appears in only one sample, is absent from the replicate samples, and does not provide any utility for statistical analysis.

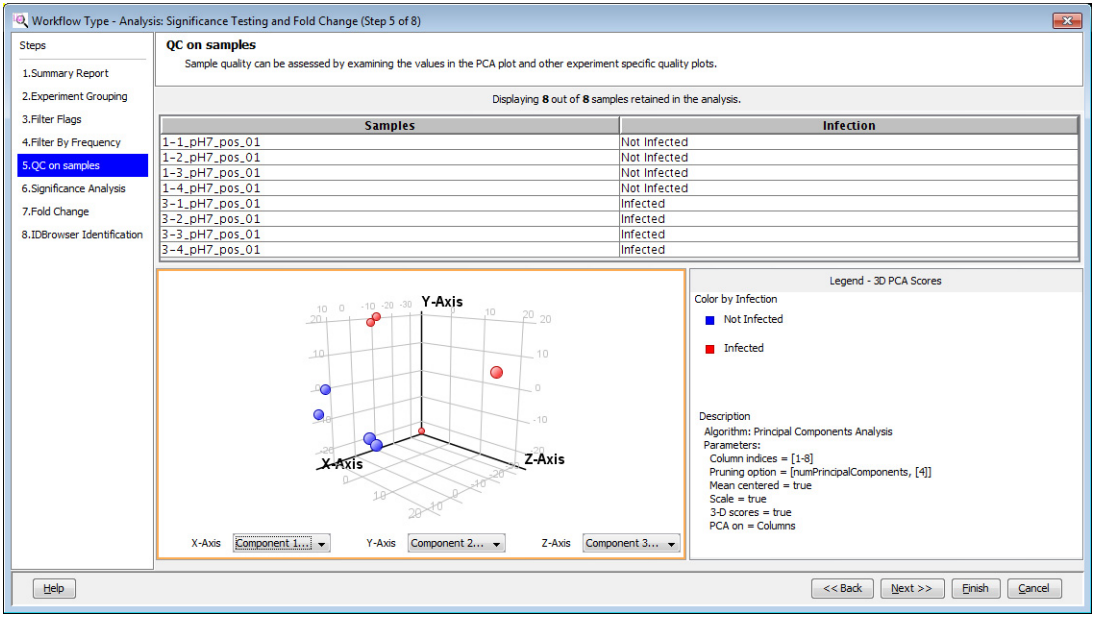


Steps	Detailed Instructions	Comments
4 Filter the remaining entities in your samples based on their frequency of occurrence among the samples and conditions. **Filter by Frequency (Step 4 of 8).**	**a** Review the summary plot. **b** Click **Re-run Filter** to enter parameters into the **Filter Parameters** dialog box. **c** Type `100` in the **Retain entities that appear in at least**. **d** Click **of samples in at least one condition**. **e** Click **OK**. **f** Review the profile plot. You are encouraged to repeat the **Re-run Filter** until you obtain the best results for your experiment. **g** Click **Next**.	• Set the minimum % and the applicable condition of samples that an entity must be present to pass the filter: (1) of all samples (conditions are not evaluated), (2) of samples in only one condition (one and only one condition) (3) of samples in at least one condition (one or more conditions), and (4) of samples within each condition (all conditions). • For experiments that contain five or fewer replicates, 100% of all samples is recommended. For experiments with a larger number of replicates, the filter frequency percentage may be lowered. A larger % removes more entities.

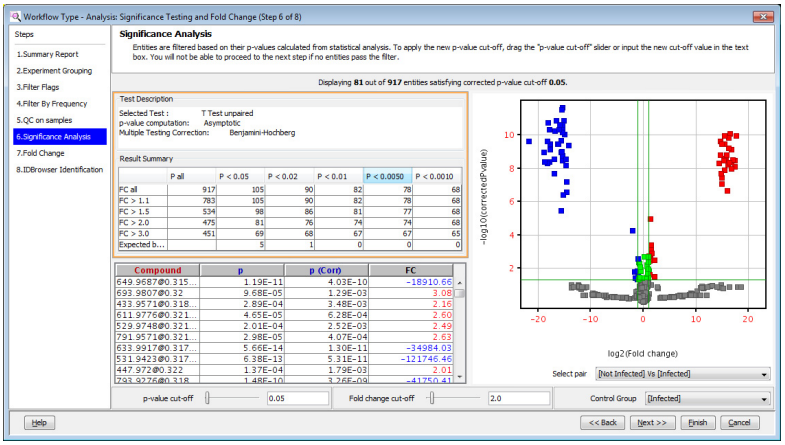


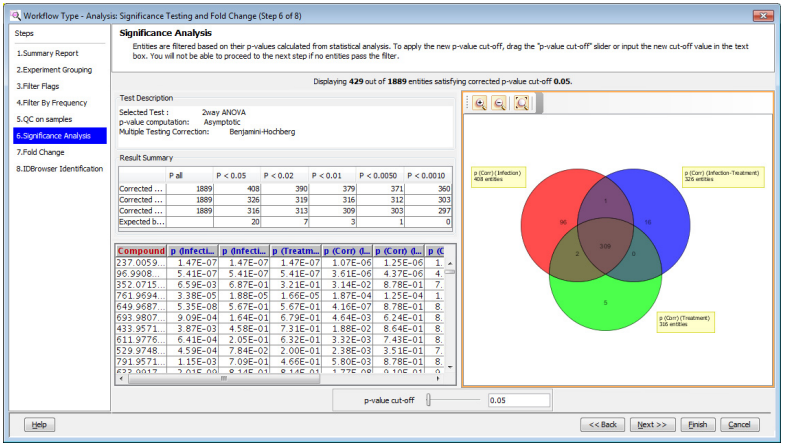


Steps	Detailed Instructions	Comments
5 Assess the sample quality of your experiment. **QC on samples (Step 5 of 8).**	**a** Review the summary plot. **b** *Highly recommended:* Click **Back** to make adjustments to prior steps in the workflow to improve the results. **c** Click **Next**.	• QC on samples provides you with the first view of the data using a Principle Component Analysis (PCA). PCA allows you to assess the data by viewing a 3D scatter plot of the calculated principle components. • You want your samples to form discrete groups in the 3D PCA Scores view based on their parameter assignments.

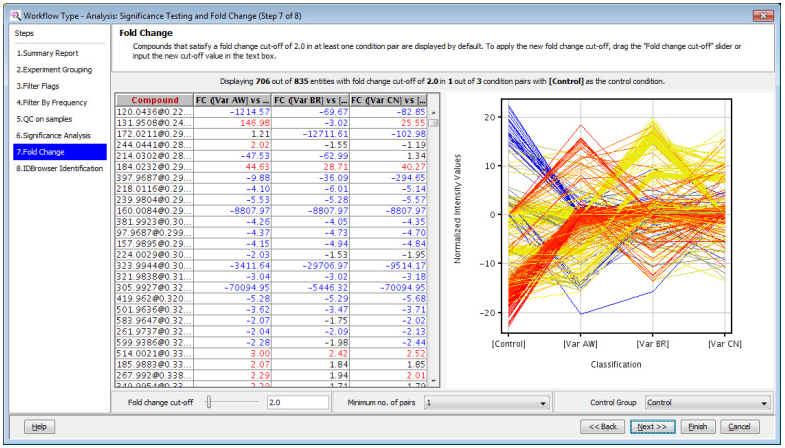


Steps	Detailed Instructions	Comments
6 Assess the differential significance of your samples. **Significance Analysis (Step 6 of 8).**	**a** Review the summary plot. **b** *Highly recommended:* Click **Back** to make adjustments to prior steps in the workflow to improve the results. **c** Customize the window panes. **d** Move the **p-value cut-off** slider(s) or type a value to change the **p-value cut-off** value(s). A larger p-value passes a larger number of entities.	• The statistical analysis is either a T-test or an Analysis of Variance (ANOVA) based on the samples and experiment grouping. • The last row of data in the Result Summary spreadsheet shows the number of entities that would be expected to meet the significance analysis by random chance based on the p-value specified in each column heading. If the number of entities expected by chance is much smaller than those based on the corrected p-value, your entities show significance among the parameter values. • The display of a diagram (Venn Diagram, Fold Change, Spreadsheet, or other plot) depends on your samples and experiment grouping for the analysis. For the Malaria Demo samples the Significance Analysis is a T-Test, the upper figure to the left.

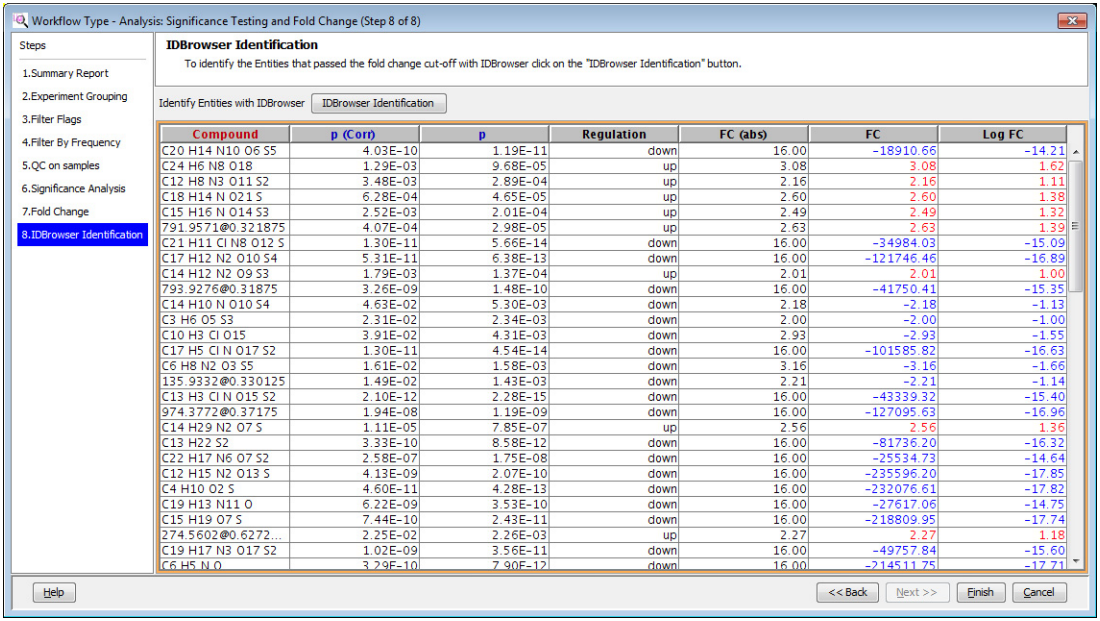




Steps	Detailed Instructions	Comments
7 Filter the remaining entities in your samples based on their relative abundance ratios among the samples and conditions. **Fold Change (Step 7 of 8).**	**a** Review the summary plot. **b** Move the **Fold change cut-off** slider or type a value to change the **Fold change cut-off**. The default value is 2.0. A larger cut-off value passes a smaller number of entities through to the final results. **c** Select a value for the **Minimum number of pairs** of conditions that must have entities with a fold change greater than the cut-off. The default value is 1. **d** Click **Next**.	• The Fold Change workflow step may be automatically skipped depending on your experiment setup (it is skipped using the *Malaria Demo*). If your experiment has a parameter that contains at least three parameter values, the Fold Change step is available. • Fold change is a signed value that describes how much an entity changes from its initial to its final value. For example, when an entity changes from a value of 60 to a value of 15, the fold change is -4. The quantity experienced a four-fold decrease. Fold change is the ratio of the final value to the initial value. • Fold change analysis is used to identify entities with abundance ratios, or, for example, differences between a treatment and a control, that are in excess of specified cut-off or threshold value. Fold change is calculated between the conditions where Condition 1 and another condition, Condition 2, are treated as a single group.

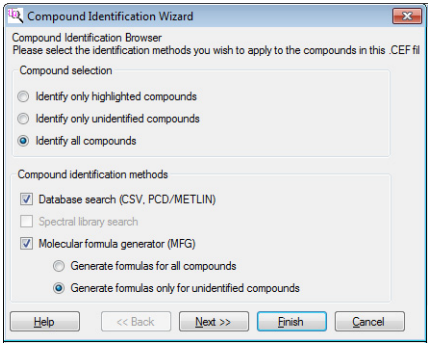


Steps	Detailed Instructions	Comments
8 Export the significant entities in your experiment for identification. **ID Browser Identification (Step 8 of 8).**	**a** Review the summary plot. **b** *Highly recommended:* Click **Back** to make adjustments to prior steps in the workflow to improve the results. **c** Click **IDBrowser Identification** to export your entity list to Agilent MassHunter ID Browser. ID Browser is started and automatically prompts you to set up your identification method parameters.	• Processing your entities with ID Browser performs the following automatically: save the selected entity list into a CEF file format, open Agilent MassHunter ID Browser, and import the saved CEF file for identification. • Once identification is completed, ID Browser returns an identified CEF file. This CEF file is imported into the MPP experiment and annotations are automatically updated.

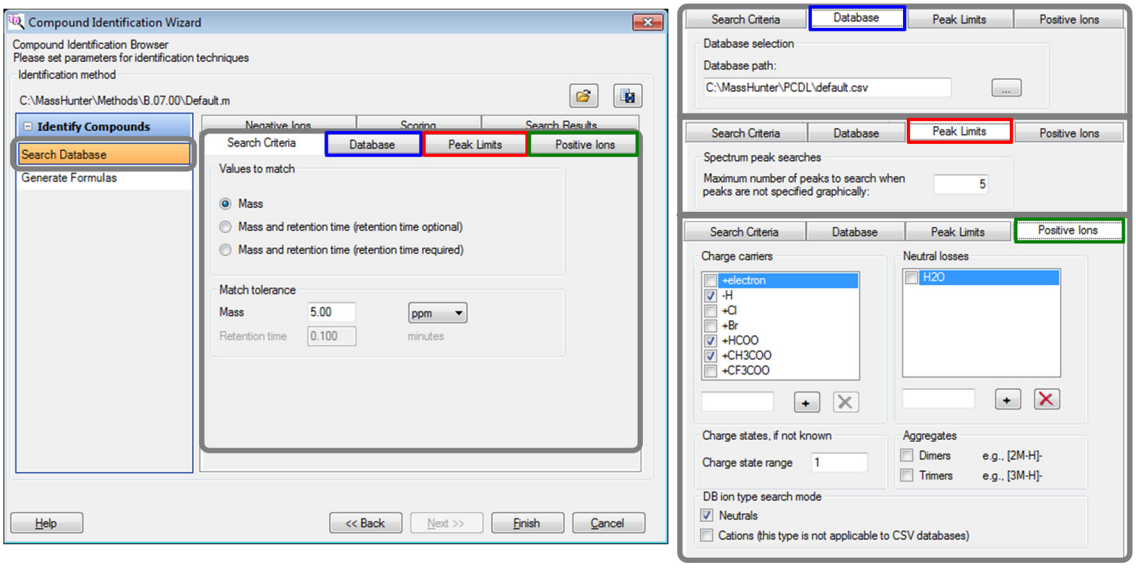


Compound	p (Corr)	p	Regulation	FC (abs)	FC	Log FC
C20 H14 N10 O6 S5	4.03E-10	1.19E-11	down	16.00	-18910.66	-14.21
C24 H6 N8 O18	1.29E-03	9.68E-05	up	3.08	3.08	1.62
C12 H8 N3 O11 S2	3.48E-03	2.89E-04	up	2.16	2.16	1.11
C18 H14 N O21 S	6.28E-04	4.65E-05	up	2.60	2.60	1.38
C15 H16 N O14 S3	2.52E-03	2.01E-04	up	2.49	2.49	1.32
791.9571@0.321875	4.07E-04	2.98E-05	up	2.63	2.63	1.39
C21 H11 Cl N8 O12 S	1.30E-11	5.66E-14	down	16.00	-34984.03	-15.09
C17 H12 N2 O10 S4	5.31E-11	6.38E-13	down	16.00	-121746.46	-16.89
C14 H12 N2 O9 S3	1.79E-03	1.37E-04	up	2.01	2.01	1.00
793.9276@0.31875	3.26E-09	1.48E-10	down	16.00	-41750.41	-15.35
C14 H10 N O10 S4	4.63E-02	5.30E-03	down	2.18	-2.18	-1.13
C3 H6 O5 S3	2.31E-02	2.34E-03	down	2.00	-2.00	-1.00
C10 H3 Cl O15	3.91E-02	4.31E-03	down	2.93	-2.93	-1.55
C17 H5 Cl N O17 S2	1.30E-11	4.54E-14	down	16.00	-101585.82	-16.63
C6 H8 N2 O3 S5	1.61E-02	1.58E-03	down	3.16	-3.16	-1.66
135.9332@0.330125	1.49E-02	1.43E-03	down	2.21	-2.21	-1.14
C13 H3 Cl N O15 S2	2.10E-12	2.28E-15	down	16.00	-43339.32	-15.40
974.3772@0.37175	1.94E-08	1.19E-09	down	16.00	-127095.63	-16.96
C14 H29 N2 O7 S	1.11E-05	7.85E-07	up	2.56	2.56	1.36
C13 H22 S2	3.33E-10	8.58E-12	down	16.00	-81736.20	-16.32
C22 H17 N6 O7 S2	2.58E-07	1.75E-08	down	16.00	-25534.73	-14.64
C12 H15 N2 O13 S	4.13E-09	2.07E-10	down	16.00	-235596.20	-17.85
C4 H10 O2 S	4.60E-11	4.28E-13	down	16.00	-232076.61	-17.82
C19 H13 N11 O	6.22E-09	3.53E-10	down	16.00	-27617.06	-14.75
C15 H19 O7 S	7.44E-10	2.43E-11	down	16.00	-218809.95	-17.74
274.5602@0.6272...	2.25E-02	2.26E-03	up	2.27	2.27	1.18
C19 H17 N3 O17 S2	1.02E-09	3.56E-11	down	16.00	-49757.84	-15.60
C6 H5 N O	3.29E-10	7.90E-12	down	16.00	-214511.75	-17.71

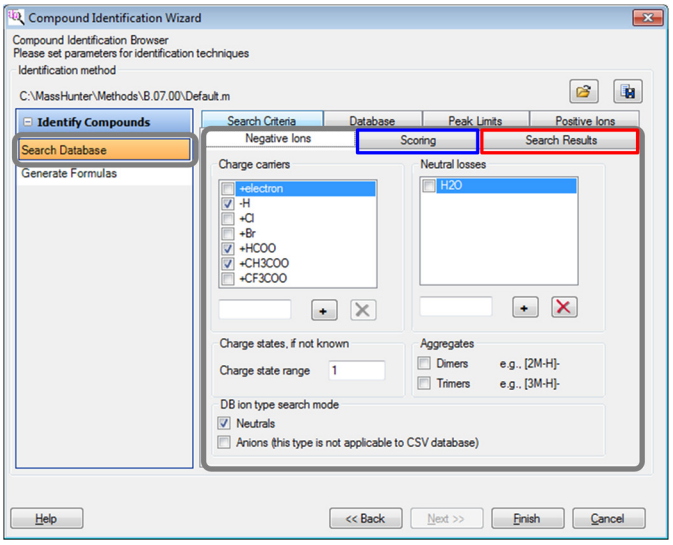
Steps	Detailed Instructions	Comments
	d Select the compounds to identify and mark the identification method for your experiment in the **Compound Identification Wizard** dialog box. **e** Click **Next**.	

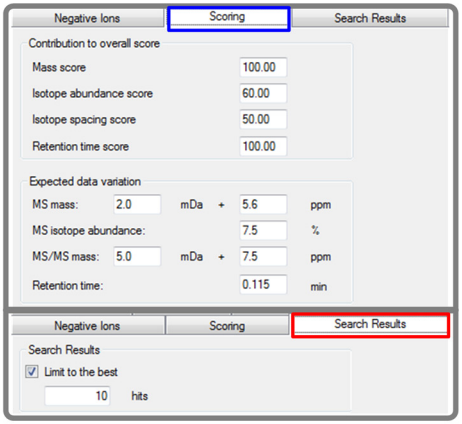


	f Setup the parameters and values for your database search.	

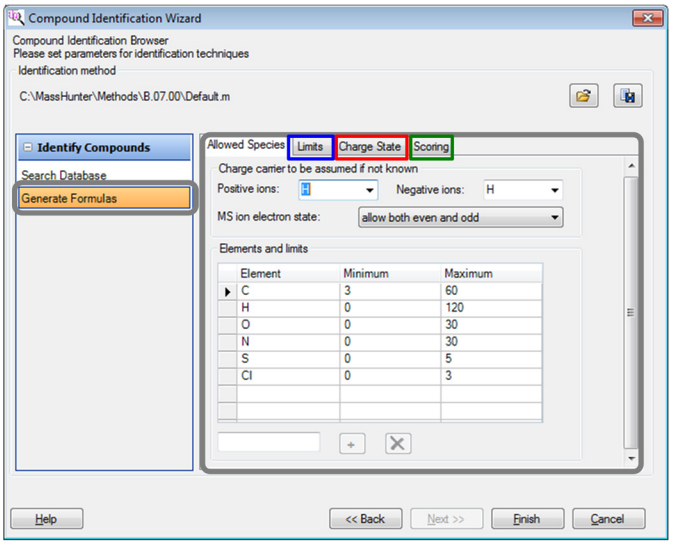


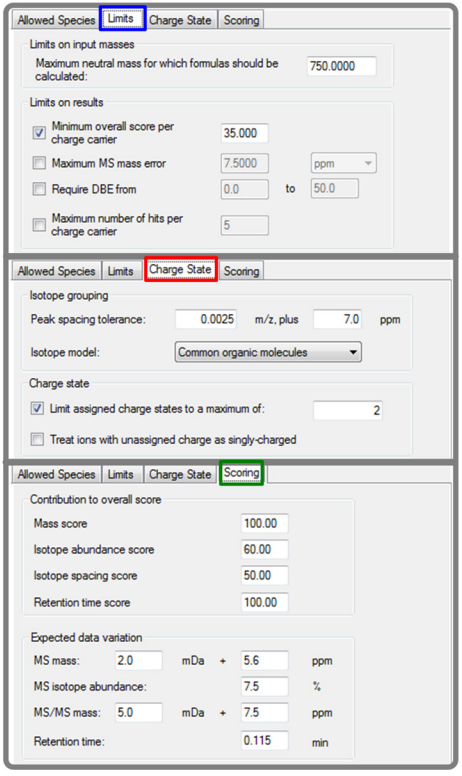
Steps	Detailed Instructions	Comments



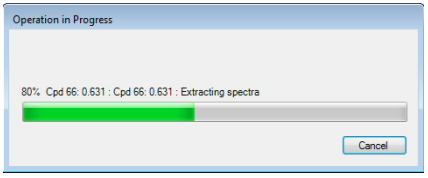


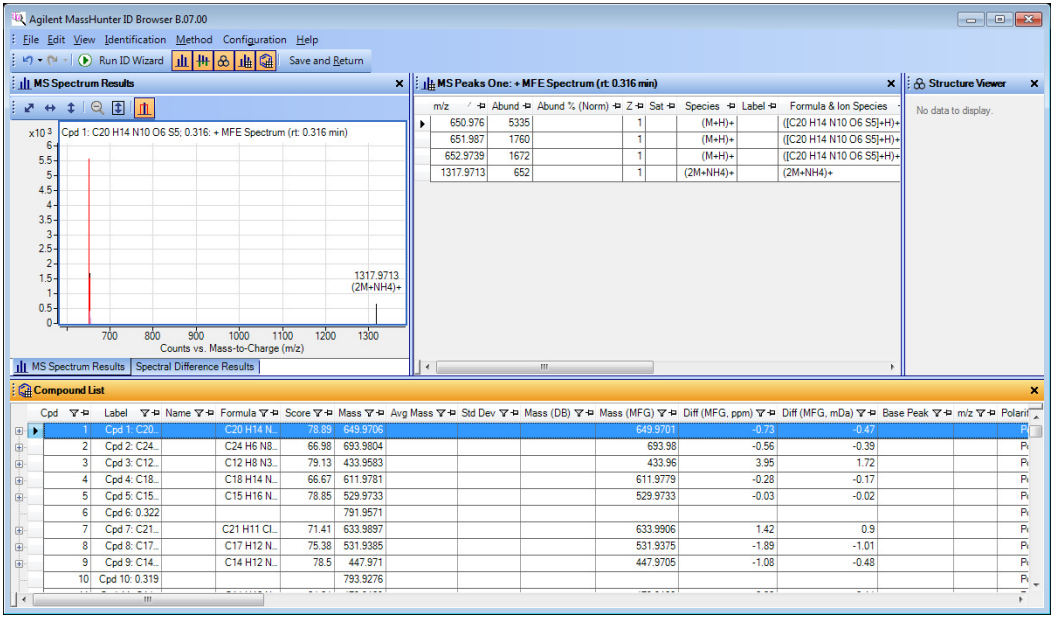
Steps	Detailed Instructions	Comments



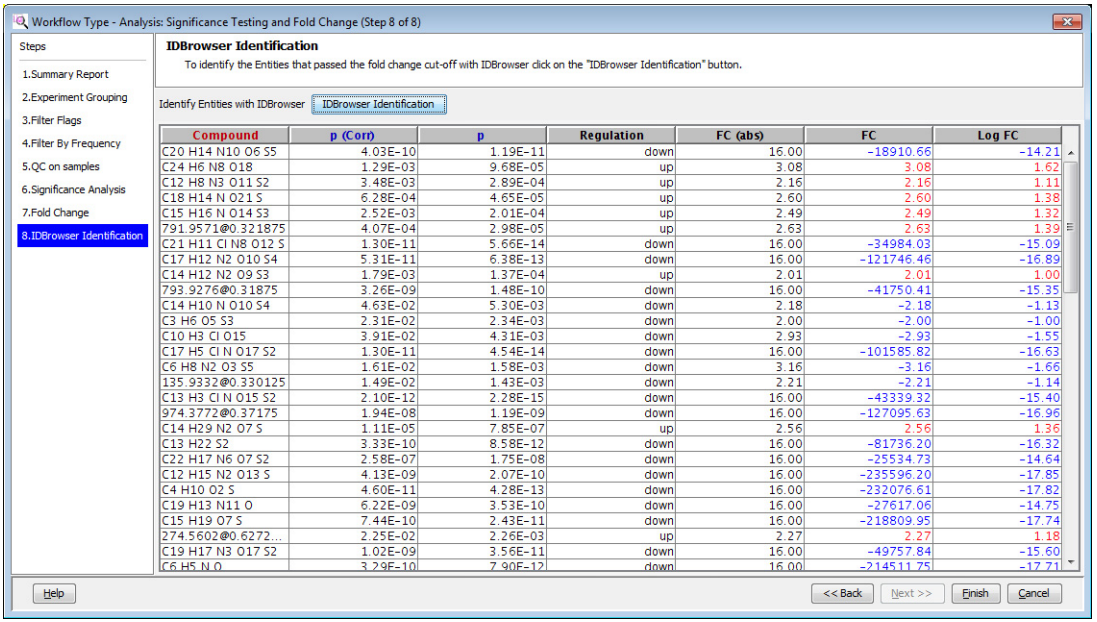


g Click **Finish** when you have the method set up for your experiment. ID Browser automatically begins identifying your entities and shows a progress bar.



Steps	Detailed Instructions	Comments
	h Review and make adjustments to the entity identifications as necessary using the ID Browser interface. **i** Click **Save and Return** Save and Return to export your entity list back to your experiment in MPP. You are automatically returned to the MPP user interface.	
	 		
	k Review your identified entity list in the ID Browser Identification results. The molecular formula now replaces the mass and retention time for identified entities in the compound column. **l** Click **Finish** when you have completed the ID Browser Identification.	• The **Analysis: Significance Testing and Fold Change** workflow is now complete and you are immediately returned to the main MPP interface.

Steps	Detailed Instructions	Comments



Compound	p (Corr)	p	Regulation	FC (abs)	FC	Log FC
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C12 H8 N3 O11 S2	3.48E-03	2.89E-04	up	2.16	2.16	1.11
C18 H14 N O21 S	6.28E-04	4.65E-05	up	2.60	2.60	1.38
C15 H16 N O14 S3	2.52E-03	2.01E-04	up	2.49	2.49	1.32
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C21 H11 Cl N8 O12 S	1.30E-11	5.66E-14	down	16.00	-34984.03	-15.09
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C14 H12 N2 O9 S3	1.79E-03	1.37E-04	up	2.01	2.01	1.00
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C3 H6 O5 S3	2.31E-02	2.34E-03	down	2.00	-2.00	-1.00
C10 H3 Cl O15	3.91E-02	4.31E-03	down	2.93	-2.93	-1.55
C17 H5 Cl N O17 S2	1.30E-11	4.54E-14	down	16.00	-101585.82	-16.63
C6 H8 N2 O3 S5	1.61E-02	1.58E-03	down	3.16	-3.16	-1.66
135.9332@0.330125	1.49E-02	1.43E-03	down	2.21	-2.21	-1.14
C13 H3 Cl N O15 S2	2.10E-12	2.28E-15	down	16.00	-43339.32	-15.40
974.3772@0.37175	1.94E-08	1.19E-09	down	16.00	-127095.63	-16.96
C14 H29 N2 O7 S	1.11E-05	7.85E-07	up	2.56	2.56	1.36
C13 H22 S2	3.33E-10	8.58E-12	down	16.00	-81736.20	-16.32
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C15 H19 O7 S	7.44E-10	2.43E-11	down	16.00	-218809.95	-17.74
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C19 H17 N3 O17 S2	1.02E-09	3.56E-11	down	16.00	-49757.84	-15.60
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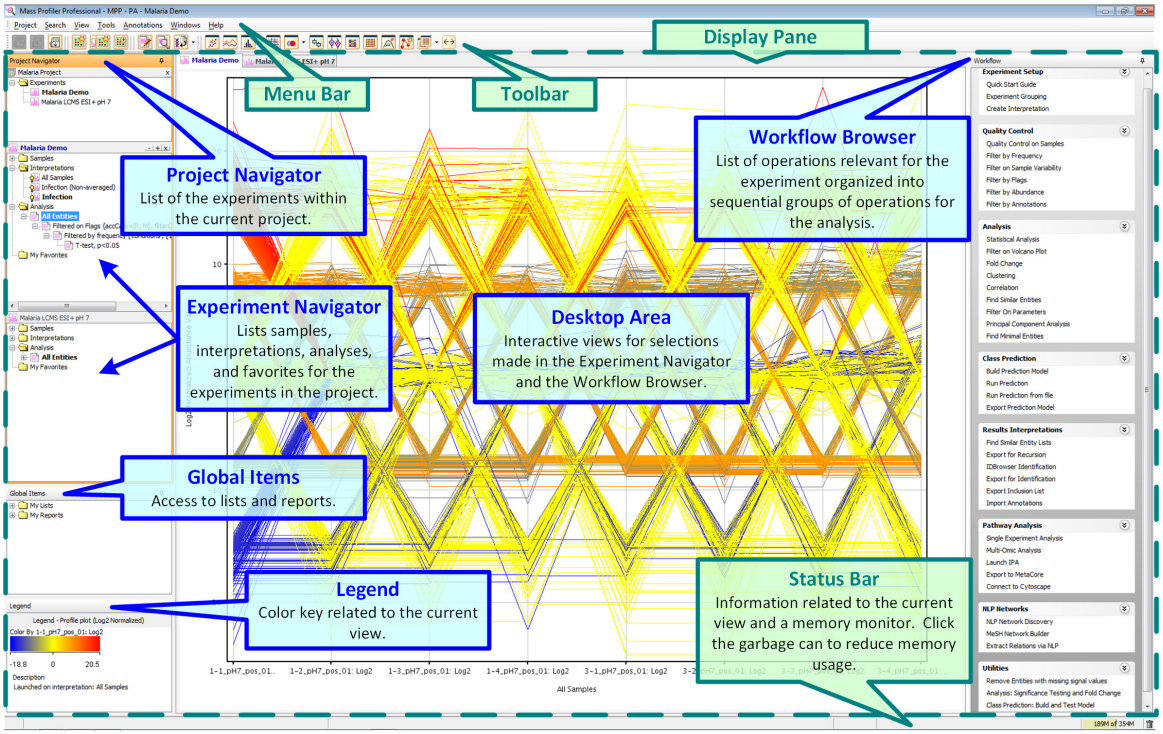
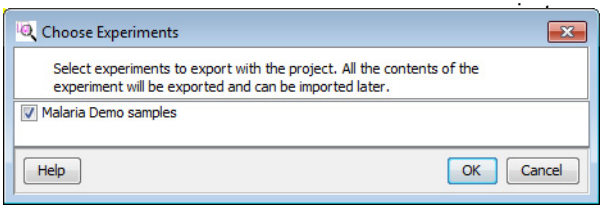
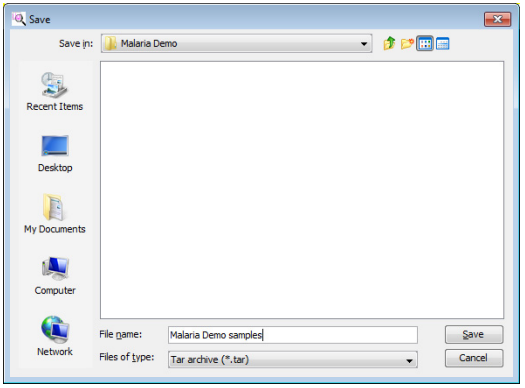
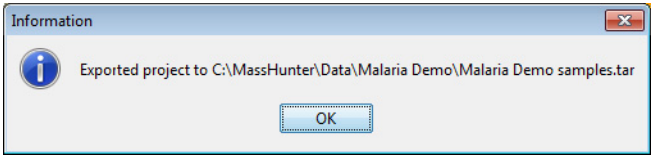
Steps	Detailed Instructions	Comments



5. Save your project

Save your current analysis as a TAR file for archiving, restoration of any future analysis to the current results, sharing the data with a collaborator, or sharing the data with Agilent customer support.

Steps	Detailed Instructions	Comments
1 Export your project to a TAR file.	**a** Click **Project > Export Project**. **b** Mark the check box next to the experiment you wish to save **c** Click **OK**.  **d** Select or create the file folder. **e** Type the File name. **f** Click **Save**.  **g** Click **OK**. 	• You have completed creating your project and analyzing an experiment. It is recommended to archive your progress by exporting

6. Perform Advanced Operations

The operations available in the Workflow Browser provide the tools necessary for analyzing features from your mass spectrometry data depending upon the need and aim of the analysis, the experiment design, and the focus of the study. This helps you create different interpretations to carry out the analysis based on the different filtering, normalization, and standard statistical methods.

7. Acknowledgment and Citations

BioCyc Pathway/Genome Databases

Includes BioCyc Pathway/Genome databases from the Bioinformatics Research Group at SRI International®, used under license.

http://www.biocyc.org/

Citation based on use of BioCyc databases or the Pathway Tools software

If you use BioCyc databases or the Pathway Tools software in your research, cite relevant publications as described on the BioCyc website:

http://biocyc.org/publications.shtml

For example, users who publish research results in scientific journals based on use of data from the EcoCyc Pathway/Genome database should cite:

Keseler et al., *Nucleic Acids Research* **39**:D583-90, 2011.

Users who publish research results in scientific journals based on use of data from most other BioCyc Pathway/Genome databases should cite:

Caspi et al., *Nucleic Acids Research* **40**:D742-53, 2012.

KEGG Database

Includes KEGG (Kyoto Encyclopedia of Genes and Genomes) databases developed by Kanehisa Laboratories.

http://www.genome.jp/kegg/

Citation based on use of KEGG Database

If you use the KEGG database in your research, cite relevant publications as described on the KEGG website:

http://www.genome.jp/kegg/kegg1.html

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www.agilent.com

In this book

The *Agilent G3835AA MassHunter Mass Profiler Professional Software - Application Guide* presents additional detail of the software interface and helps you use MPP with your data.

This guide applies to MassHunter Mass Profiler Professional Software 14.0 and higher until superseded.

Revision A, November 2015

G3835-90028

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