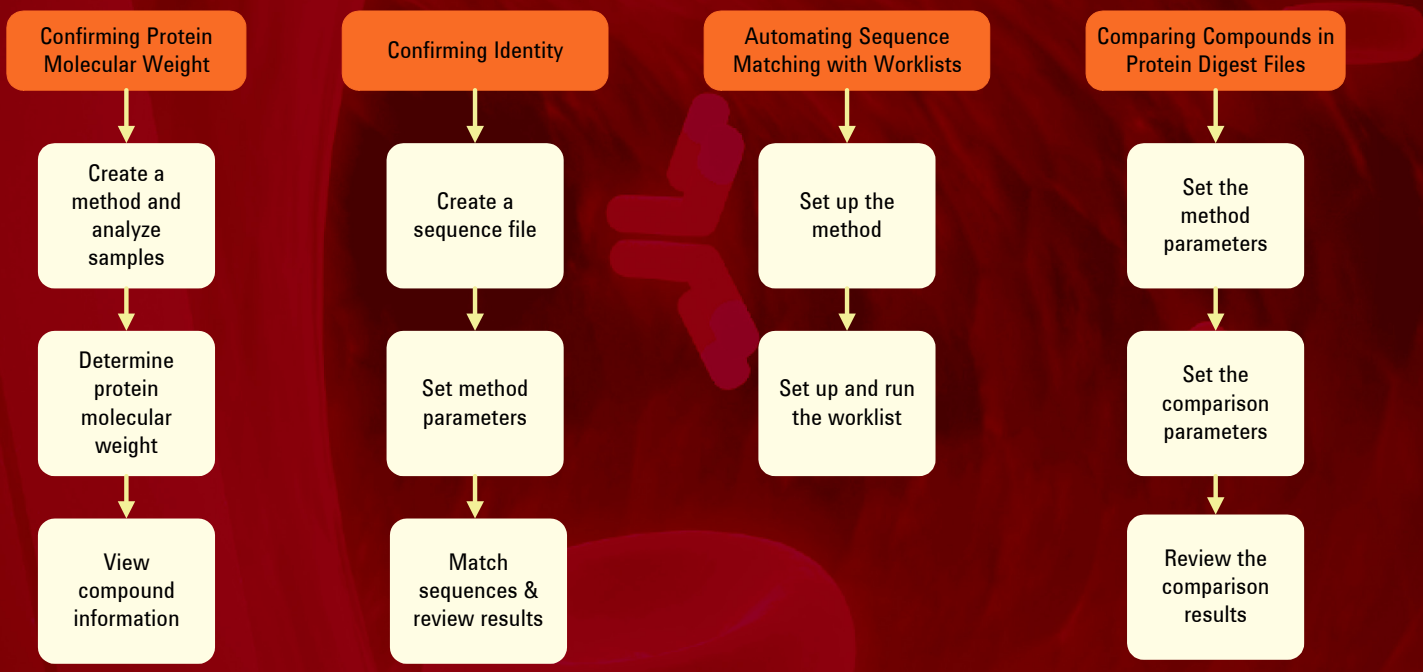
Agilent MassHunter BioPharma

Workflow Overview



For more information:

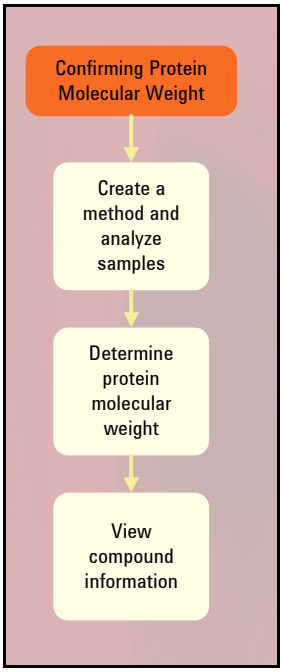
See the *MassHunter BioPharma Workflow Guide* (p/n 5990-7064EN)

Agilent Technologies

Confirming Protein Molecular Weight

Here are the high-level steps and products required to confirm protein molecular weight.

- Agilent 6200 Series Accurate-Mass TOF LC/MS or Agilent 6500 Series Accurate-Mass Q-TOF LC/MS instrument
- MassHunter Data Acquisition B.06.01 software
- MassHunter Qualitative Analysis B.07.00 software
- MassHunter BioConfirm B.07.00 program



1. Create an acquisition method and analyze samples.

a In the MassHunter LC/MS Data Acquisition program, set LC method set points.

b Set MS method set points.

c Save the method with a new name.

d Place the samples in the ALS or well-plate sampler.

e Set up to run your samples in one of the following ways:
- Use the **Sample** tab for a single sample.
- Use the **Worklist** pane for multiple samples.

f Start the run in one of the following ways:
- Click **Sample > Run** for a single sample.
- Click **Worklist > Run** for multiple samples.

2. Determine the protein molecular weight.

a Open the data file of interest in the MassHunter Qualitative Analysis software.

The TIC is automatically displayed in the Chromatogram Results window.

b To integrate and extract peak spectra, click **Actions > Integrate and Extract Peak Spectra**.

c Set **Deconvolution mass range** and **mass step** in the Deconvolute (MS): Protein section of the Method Editor.

d Click to select the extracted MS peak of interest, then click the run button on the Method Editor toolbar to deconvolute the spectrum. The results appear in the Deconvolution Results window.

e To view Peak information:
- Click the spectrum in the Deconvolution Results window to select it.
- Right-click the spectrum and select **View MS Spectrum Peak List 1** from the shortcut menu.
- Click twice on the Height column heading to sort results by spectral peak height.

3. View compound information.

a To view the compound list, click the button on the main toolbar. See Figure 1.

Compound List

Automatically Show Columns

Show/Hide	Cpd	File	Name	Start	End	Mass	Fit Score	Min
☑	1	myoglobin.d	Compound 1	3.209	3.724	16951.505	10	
☑	2	myoglobin.d	Compound 2	3.209	3.724	16934.6536	9	
☑	3	myoglobin.d	Compound 3	3.209	3.724	16973.3253	8	
☑	4	myoglobin.d	Compound 4	3.209	3.724	16994.5588	9	
☑	5	myoglobin.d	Compound 5	3.209	3.724	17006.64	9	
☑	6	myoglobin.d	Compound 6	3.209	3.724	16894.8039	8	
☑	7	myoglobin.d	Compound 7	3.209	3.724	17049.8039	9	
☑	8	myoglobin.d	Compound 8	3.209	3.724	16781.0765	8	
☑	9	myoglobin.d	Compound 9	3.209	3.724	16763.5782	9	
☑	10	myoglobin.d	Compound 10	3.209	3.724	16748.1799	8	

Figure 1 Left half of Compound List window for myoglobin.d

b Click to select the mass of interest.

c Click to select the ion set spectrum of interest in the MS Spectrum Results window.

d Click the button on the main toolbar to view the charge states found for the protein along with their ppm error. This information appears in the MS Peak List 2 window, as shown in Figure 2.

MS Peaks Two: + Scan

Peak	Cpd Mass	Calc Mass	Diff (ppm)	Diff (Da)	m/z	m/z (Calc)	Diff (m/z)	Z	Abund	Abund %	Fo
1	16951.51	16951.65	-8.29	0.14	628.8457	628.8405	-0.0052	27	1473.48	0.77	
2	16951.51	16951.34	9.98	-0.17	652.9814	652.988	0.0065	26	8753.23	4.55	
3	16951.51	16951.56	-3.52	0.06	679.0696	679.0672	-0.0024	25	28119.4	14.61	
4	16951.51	16951.48	1.51	-0.03	707.3186	707.3197	0.0011	24	61537.37	31.97	
5	16951.51	16951.5	0.46	-0.01	738.0286	738.029	0.0003	23	103180.77	53.6	
6	16951.51	16951.54	-1.95	0.03	771.5315	771.53	-0.0015	22	143109.5	74.34	
7	16951.51	16951.57	-3.67	0.06	808.2245	808.2215	-0.003	21	175718.11	91.28	
8	16951.51	16951.55	-2.77	0.05	848.5846	848.5823	-0.0024	20	192514.36	100	
9	16951.51	16951.55	-2.57	0.04	893.1938	893.1915	-0.0023	19	188235.27	97.78	
10	16951.51	16951.49	0.78	-0.01	942.7565	942.7573	0.0007	18	164246.91	85.32	
11	16951.51	16951.46	2.94	-0.05	998.1514	998.1544	0.0029	17	122562.37	63.66	
12	16951.51	16951.45	3.08	-0.05	1060.4728	1060.4761	0.0033	16	88387.08	45.91	
13	16951.51	16951.45	3.35	-0.06	1131.1035	1131.1073	0.0038	15	69181.33	35.94	
14	16951.51	16951.42	5.01	-0.08	1211.8227	1211.8288	0.0061	14	55564.74	28.86	
15	16951.51	16951.43	4.4	-0.07	1304.9632	1304.9689	0.0057	13	37056.82	19.25	
16	16951.51	16951.42	5.29	-0.09	1413.6249	1413.6324	0.0075	12	22214.49	11.54	
17	16951.51	16951.51	-0.16	0	1542.0532	1542.0529	-0.0003	11	16660.98	8.65	
18	16951.51	16951.51	-0.14	0	1696.1577	1696.1575	-0.0002	10	8065.46	4.19	
19	16951.51	16951.65	-8.35	0.14	1884.5233	1884.5076	-0.0157	9	1449.6	0.75	
20	16951.51	16951.59	-4.88	0.08	2119.9555	2119.9451	-0.0103	8	246.62	0.13	

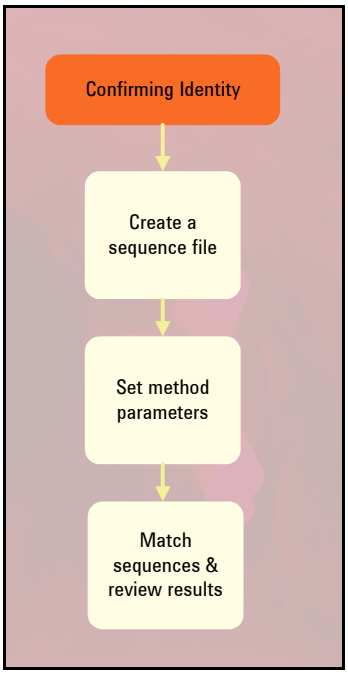
Figure 2 MS Peaks Two window for myoglobin.d

e Select **Compound Report** from the **File > Print** menu to print a compound report.

Confirming Identity

Here are the high-level steps and products needed to create and match sequences.

- MassHunter Qualitative Analysis B.07.00 software
- MassHunter BioConfirm B.07.00 program



1. Create a sequence file.

a Click **BioConfirm Workflow > Define and Match Sequences** in the Method Explorer window to display the Define and Match Sequences section of Method Editor.

b Click **New Sequence** on the Sequence menu.

c In the Sequence Editor window, type a name and select the appropriate **Sequence Type** for the new sequence (Protein, Protein Digest, Synthetic peptide, or Oligonucleotide)

d To enter the amino acid sequence, type in individual amino acids one at a time between the N-term and C-term symbols. Use the single-character (letter) amino acids abbreviations, as shown in the Amino acid list on the left side of the Sequence Editor window.

e Add links and modifications, if they are present in the sequence.

f Save the sequence.

A saved sequence file can be imported for use in other methods or referenced from worklists.

2. Set method parameters.

a Open the appropriate method. The following methods are provided with BioConfirm:

For Proteins:

- **BioConfirmIntactProtein-Default** (for Maximum Entropy or LMFE Deconvolution)
- **BioConfirmIntactProteinHighMass-Default** (for protein >25 kDa and LMFE Deconvolution)

For Protein Digests:

- **BioConfirmProteinDigest-Default** (with MFE Deconvolution)

For Synthetic Peptides:

- **BioConfirmSynthetic Peptide-Default** (with MFE Deconvolution)

For Oligonucleotides:

- **BioConfirmOligonucleotideSmall-Default** (for Oligonucleotides <7 kDa and MFE Deconvolution)
- **BioConfirmOligonucleotideLarge-Default** (for Oligonucleotides >7 kDa and LMFE or MaxEnt Deconvolution)

---

b Open the data file of interest. The following example data files are provided on the BioConfirm setup disk:

- For Proteins, use **myoglobin.d**
- For Protein Digests, use **enolase-Chip-final.d**
- For Synthetic Peptides, use **SynPep3.d**
- For Oligonucleotides, use **DNA-2ug-r001.d**

The TIC is automatically displayed in the Chromatogram Results window.

c Find compounds.

- Click **BioConfirm Workflow > Find by Molecular Feature** in the Method Explorer window to display and review the parameters in the Find Compounds by Molecular Feature section of Method Editor.
- To start the compound search, click the process button on the Method Editor toolbar.
- Review the results in the Compound List window.

d To select the appropriate layout for your data, click **Load Layout** on the **Configuration > Window Layouts** menu, and select one of the following layouts:

For Proteins:

- **BioConfirm-IntactProtein-MaximumEntropy-Default** (for Maximum Entropy Deconvolution)
- **BioConfirmIntactProtein-LMFE** (for LMFE Deconvolution)

For Protein Digests:
- **BioConfirm-ProteinDigest** (with MFE Deconvolution)

For Synthetic Peptides:
- **BioConfirm-Synthetic Peptide** (with MFE Deconvolution)

For Oligonucleotides:
- **BioConfirm-Oligonucleotide** (for MFE, LMFE, or MaxEnt Deconvolution)

e To import the sequence file:
- Click **BioConfirm Workflow > Define and Match Sequences** in the Method Explorer window.
- Click the **Sequences** tab.
- Click the **Import** button.

f Select the sequence file of interest, then click **Open**. The following sequence files are provided on the BioConfirm setup disk:
- For Proteins, use **myoglobin.psq**.
- For Protein Digests, use **EnolaseDigest.psq**.
- For Synthetic Peptides, use **SynPep3.psq**.
- For Oligonucleotides, use **21mer_oligo.psq**.

g Assign digest reagents and view the digest list.
(For Protein Digests only)

h To select matching rules, right-click in the Sequence Editor window and select **Edit Matching Rules** from the shortcut menu to open the Rules dialog box. Use **Ctrl+click** to select multiple rules from the list, then click **OK**.

For Proteins:
- Intact protein
- Predicted Modifications

For Protein Digests:
- Complete Digest
- Incomplete Digest
- Predicted Modifications

For Synthetic Peptides:
- Intact Peptide
- Extra Amino Acid
- Missing Amino Acid
- Fmoc blocking groups

For Oligonucleotides:
- Intact oligonucleotide
- Oligonucleotide truncation

3. Match sequences and review the results.

i To select the match source:
- Click the **Source** tab in the Define and Match Sequences section of the Method Editor window.
- Click **Qualitative method**.
- Mark the **Sequences** check box.

---

a To start the match sequence, click  on the Method Editor toolbar. The results appear in the Compound List window.

b To review search results, click the  button on the main toolbar to display the Compound Identification Results window.

The results for the first compound that is highlighted in the Data Navigator (that is marked to show) are displayed in the Compound List window.

c View results for other compounds by clicking on them in the Data Navigator or Compound List or Compound Identification Results windows.

d To see other information for compounds in the list, right-click in the table, then click **Add/Remove Columns** from the shortcut menu.

e Evaluate/confirm the match quality:
- If the value in the **Score (Bio, MS)** column is good and the value in the **Score (Bio, MS/MS)** column is high, then it probably is a true match.
- If the value in the **Score (Bio, MS)** column is very good, but value in the **Score (Bio, MS/MS)** column is low, then it may be a false positive.

f To identify the location of predicted modification for proteins or protein digests, look for the entry in the **Pred Mods** column that has the highest value in the Score (Bio, MS/MS) column.

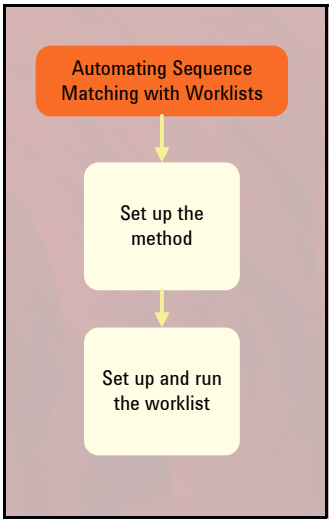
g You can also view sequence match results in the Compound List Window, which is a nested table of results.

h Save the method for later use.

Automating Sequence Matching with Worklists

Here are the high-level steps and products required to set up a worklist to automatically confirm the presence of a selected protein sequence in previously acquired samples.

- MassHunter Data Acquisition B.06.01 software
- MassHunter Qualitative Analysis B.07.00 software
- MassHunter BioConfirm B.07.00 program



1. Set up the method in Qualitative Analysis.

a Open the method of interest, such as one of the following default methods:

For Proteins:

- **BioConfirmIntactProtein-Default** (for Maximum Entropy or LMFE Deconvolution)
- **BioConfirmIntactProteinHighMass-Default** (for protein >25 kDa and LMFE Deconvolution)

For Protein Digests:

- **BioConfirmProteinDigest-Default** (with MFE Deconvolution)

For Synthetic Peptides:

- **BioConfirmSynthetic Peptide-Default** (with MFE Deconvolution)

For Oligonucleotides:

- **BioConfirmOligonucleotideSmall-Default** (for Oligonucleotides <7 kDa and MFE Deconvolution)
- **BioConfirmOligonucleotideLarge-Default** (for Oligonucleotides >7 kDa and LMFE or MaxEnt Deconvolution)

---

b Select Worklist as the match source.

- Click the **Source** tab on the **Define and Match Sequences** in Method Editor.

- Click **Worklist.** This selection causes the software to get the sequence from the worklist rather than the method.

c Click **Worklist Automation > Worklist Actions** in the Method Explorer window.

d Select **Find Compounds by Molecular Feature** and set the following worklist automation options:
- **Extract Defined Chromatograms**
- **Integrate and Deconvolute**
- **Match Sequences**
- **Generate Compound Report**

e Save the modified method with a new name.

---

2. Set up and run a worklist in Data Acquisition.

a Open the MassHunter Workstation Data Acquisition software window, if it is not already open.

b Display the Worklist pane.

c Click **Worklist > Add Sample** to add a new sample row to the Worklist table.

d To specify the sequence file in the worklist:
- Click **Worklist > Add Column**.
- On the Add Column dialog box, select **Protein** as the **ColumnType**.
- Enter an appropriate description in the **Column name**, such as **Sequence**.
- Enter the sequence file of interest as the **Value**, such as iii_**myoglob.psq**.
- Click **OK**.

e Specify the appropriate method in the worklist. Use the method you modified and saved in the previous section.

f Specify the appropriate data file in the worklist. The following example data files are provided on the BioConfirm setup disk:
- For Proteins, use **myoglobin.d**
- For Protein Digests, use **enolase-Chip-final.d**
- For Synthetic Peptides, use **SynPep3.d**
- For Oligonucleotides, use **DNA-2ug-r001.d**

g On the Worklist Run Parameters dialog box:
- Select **DA Only** as the **Part of method to run**.

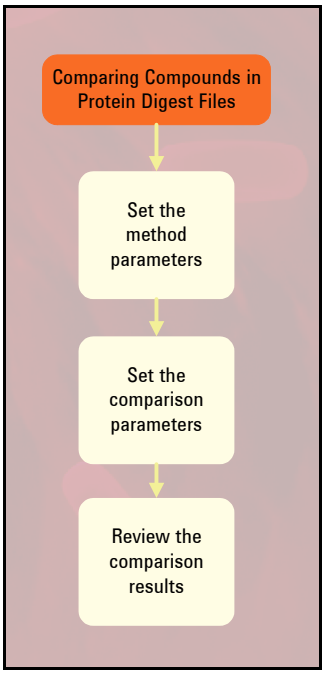
h Click **Worklist > Run** to start the worklist.

i Review the printed Compound reports.

Comparing Compounds in Protein Digest Files

These are the high-level steps and products required to compare compounds in two or more protein digest data files.

- MassHunter Qualitative Analysis B.07.00 software
- MassHunter BioConfirm B.07.00 program



Tip: You can find and identify compounds in either of the following ways:

- Manually as described in "Confirming Identity" on page 4, or
- By clicking **Wizards > Compare Protein Digest Files: Find Results, Identify and Compare** to have the wizard guide you through the process.

1. Set up the method.

a Open the data files to compare.

b View the method parameters that were used to find and identify compounds in the following sections of the Method Editor:

- BioConfirm Workflow > **Find by Molecular Feature**
- BioConfirm Workflow > **Define and Match Sequences**

2. Set the comparison parameters.

a To start the compare protein digest files wizard, click **Wizards > Compare Protein Digest Files: Compare Existing Results**.

b On the Select Reference file and Sample file(s) page, select the data files to compare. Once you select the **Reference file**, the remaining files in the list are automatically added to the list of **Sample files**.

c Click **Next** to display the next page of the wizard.

d On the Alignment Information page, set the compound correlation parameters.

e When finished setting the parameters, click **Finish**.

3. Review the comparison results.

a Review the results displayed in the Compound Compare List window in the MassHunter Comparative Analysis program. Note that compound information is presented in three sections:

- Section 1 (purple shading): correlated compound information
- Section 2 (red shading): reference file information
- Section 3 (blue shading): sample file information

An example of the Comparative Analysis program window is shown in Figure 3. It shows comparison results for the following example protein digest data files, which are provided on the BioConfirm setup disk:

- **enolase-chip-final.d** (Reference file)
- **enolase-oxidized-chip-final.d** (Sample file)

b Review comparison results in the following other windows, which are updated automatically when you select different compounds in the Compound Compare List window:

- Chromatogram Compare Results window
- MS Spectrum Compare Results window
- MS/MS Spectrum Compare Results window
- Sequence Compare Coverage Map window
- Reference MS Spectrum Peak List window
- Sample MS Spectrum Peak List window

If the window of interest is not currently visible, select it from the **View** menu.

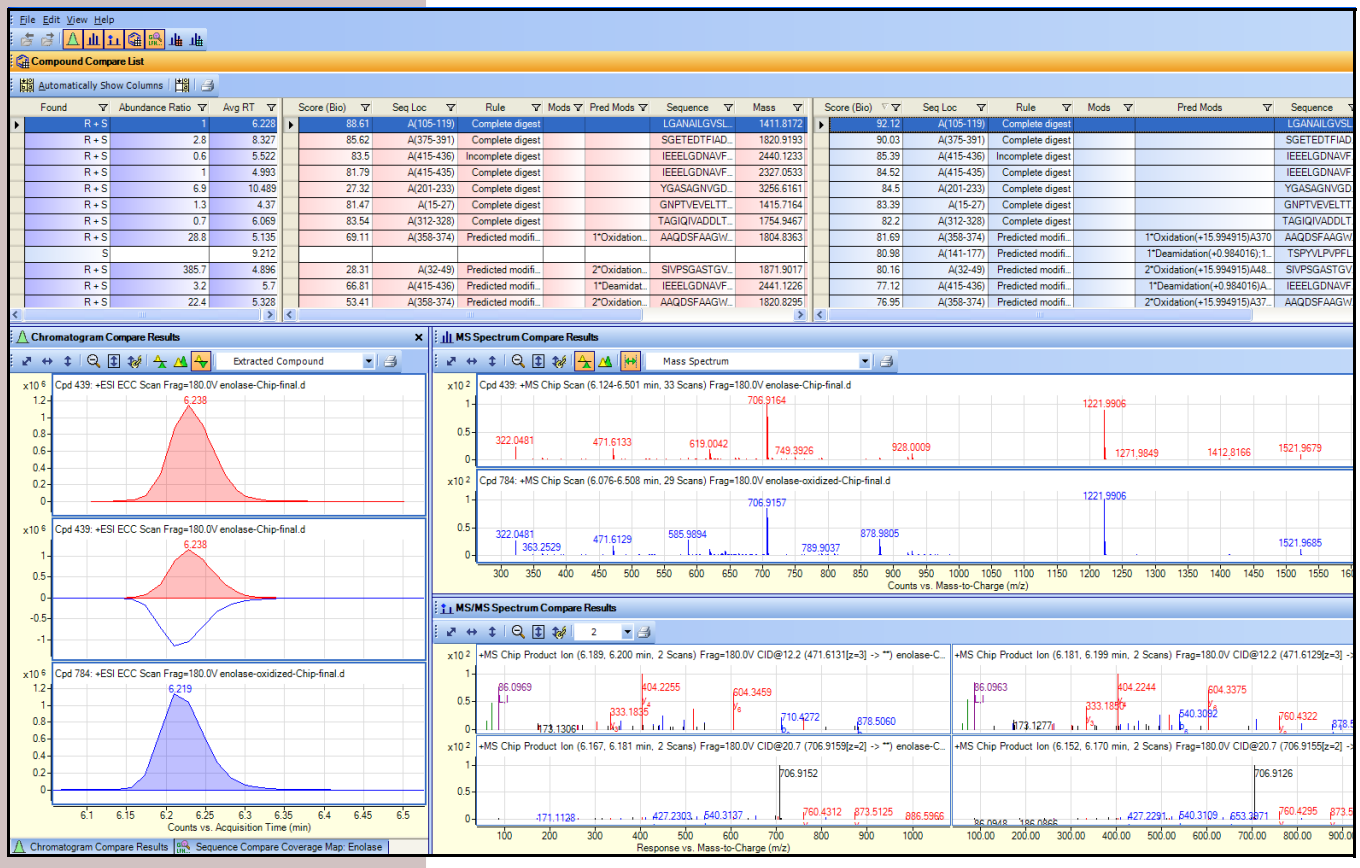


Figure 3 MassHunter Comparative Analysis Program Window

www.agilent.com

Printed in USA, January 2016

5990-7065EN

Agilent Technologies