Agilent

MassHunter BioConfirm Software

Introduction Workbook

Notices

Document Identification

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Software Revision

This Guide is valid for the 14.0 revision or higher of Agilent MassHunter BioConfirm until superseded.

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CAUTION

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A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

Contents

1 Introduction

About this Training Workbook

This workbook provides instructions for MassHunter BioConfirm.

For additional information on the software and detailed instructions not covered in this workbook, refer to the Online Help.

Use the following exercises to experience how to utilize MassHunter BioConfirm. Example data is provided with the installation of the software to introduce these steps.

This workbook is your introductory guide for the set-up and execution of basic procedures with MassHunter Explorer BioConfirm This workbook is divided into chapters, each building upon the last, so we recommend that each chapter is completed in succession. During each chapter, lessons are guided by video support.

By completing this learning event, you will have an introductory level of experience in the use of BioConfirm

How to use this Workbook

This learning experience introduces basic concepts in a learning-by-doing, guided manner. Each chapter uses step-by-step instructions.

Exercises to be completed are marked like this:

Exercise Name

Exercise Instructions

Task steps look like this:

1. Tasks or items needed to complete tasks look like this.

If you are expected to enter any information or if something is important, it is set in italicized type like this:

Type *Blank One* in the field.

If you are expected to press a key on the keyboard or button on the software screen, the key is displayed in bold like this:

Press **Enter**.

What this Workbook covers

In this learning experience, the goal is to get up and running using the software as quickly as possible. After completing this learning MassHunter BioConfirm event with your Agilent consultant, you will have an introductory level of experience in the use of MassHunter BioConfirm 14.0 .

This learning experience introduces basic concepts in a learning-by-doing, guided manner. Each chapter uses-step by-step instructions and is supported by online videos to view and review the material as needed. Scan the code to the right or use this link to access the videos). If you have a question or get stuck, find your local sales and support contact by visiting the following page using the link below.

- https://www.agilent.com/en/contact-us/page

For technical support, visit the following page:

- https://www.agilent.com/en/support

The following exercises are designed to support the execution of theBioConfirm workflow(s) along with video support. The videos are intended to provide visual support for working with the software. We recommend reviewing the video demonstrations first, then attempting the workflow processes, using each exercise as a guide. If at any time you have questions or run into an event that is not in alignment with this workbook, please reach out to your Agilent Consultant.

Video Support

This Introduction Workbook has supplemental video support available. Each chapter uses step-by-step instructions supported by online videos to view and review the material as needed. Scan the code below or use the link to access the videos.

https://aglt.co/BioConfirmIntro

Agilent Community

Agilent Community

To get answers to your questions, join over 10,000 users in the Agilent Community. Review curated support materials organized by platform technology. Ask questions to industry colleagues and collaborators. Get notifications on latest videos, documents, tools, and webinars relevant to your work.

https://community.agilent.com/

Requirements

To complete the chapters in this training workbook, you must have installed the software to the most recent release recommended. The software is found on the installation media provided in the install kit, along with specially prepared data sets to load onto the software system before the start of this learning exercise.

Please refer to the Online Help or Installation Guide for further information. As always, feel free to contact Agilent Support for additional assistance.

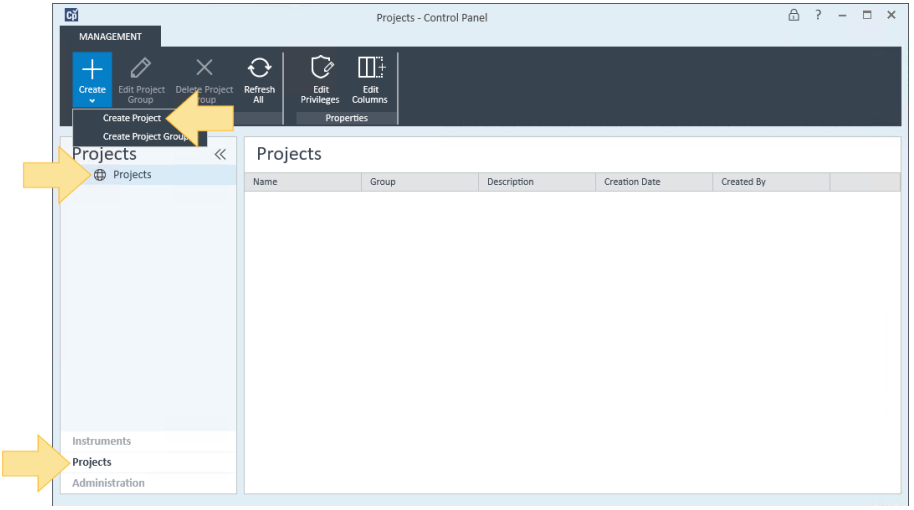
2 Setup

Projects in Agilent Control Panel organize your data files, sequences, and workflow settings in one place. Creating a project is the first step before running any BioConfirm workflow. This ensures consistency and makes it easy to save and reopen your work later. In this section, you will set up a project using either MassHunter or OpenLab Control Panel for use with all thetraining exercises In this Introduction Workbook.

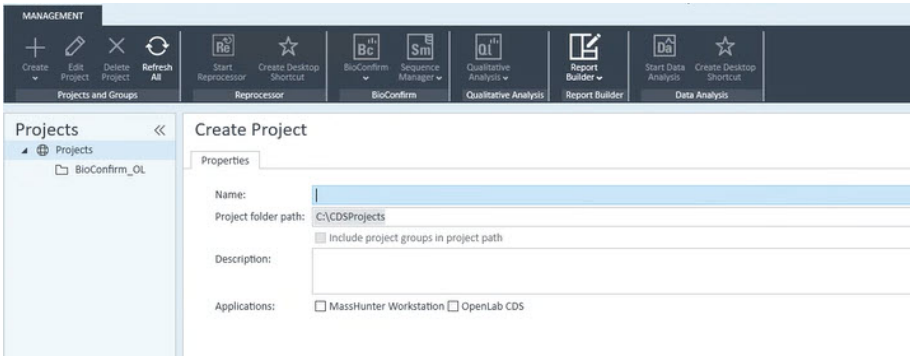
MassHunter workstation is a local, instrument-PC-based setup for running and processing data. OpenLab CDS is a centralized, login-based system that manages instruments, users, and data under controlled workflows.

Document which system used to acquire data:

1. Click **Projects** and select Projects or any project group.
2. Click **Create > Create Project**.



3. In the **Name** text box, type a name for the project. In this example, type *Training*.
4. Select an **Application**.

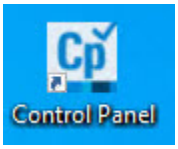


5. Click **OK**.

3 User Interface and General Navigation

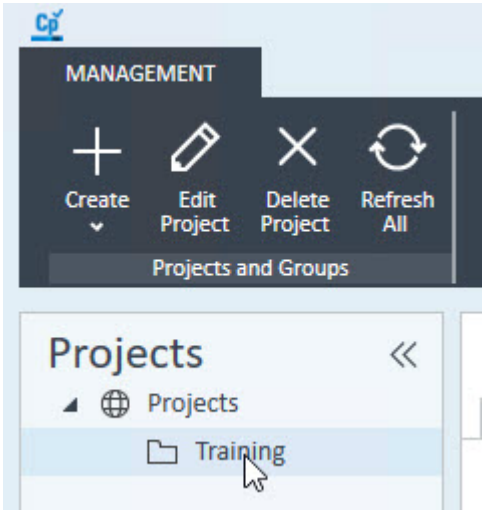
Launching MassHunter BioConfirm

1. Double-click on the Control Panel icon to launch the software.

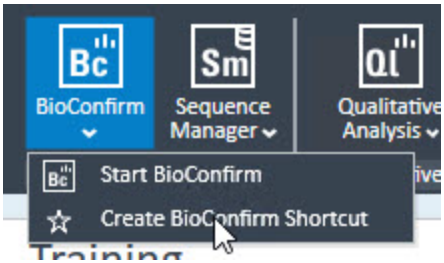


   The software splash screen displays the software initialization, then the Control Panel user interface loads.

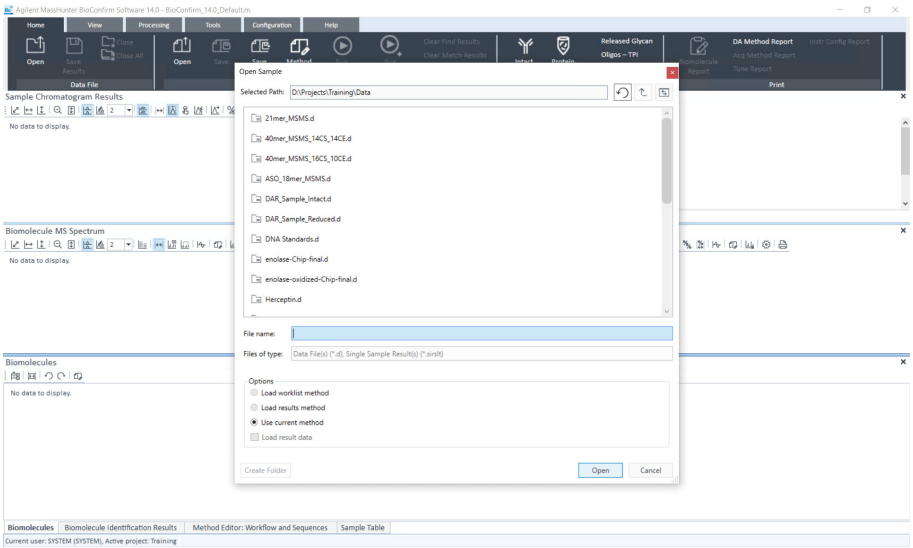
2. Click **Projects** and select the **Training** project.



3. On the Ribbon, in the BioConfirm section of the MANAGEMENT tab, click the BioConfirm icon and select **Start BioConfirm Shortcut.**



4. The Main Window loads and the Open Sample dialog box displays.

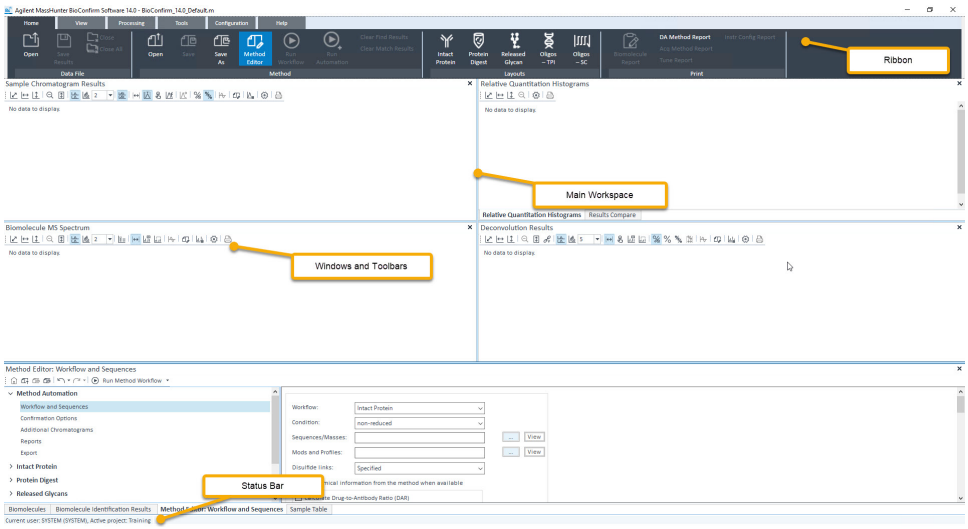


User Interface

NOTE

This section provides a high-level overview of the user interface. For complete details, including advanced options and step-by-step instructions, refer to the Online Help. Access the Online Help by selecting **Help > Contents** from the Ribbon or pressing **F1**.

The BioConfirm workspace contains three functional areas:



1. Main Window
2. Ribbon
3. Windows and toolbars

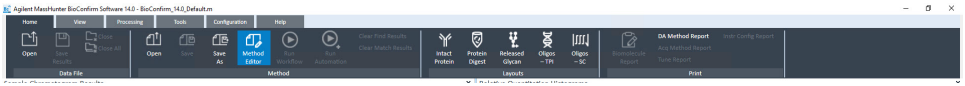
Main Window

The BioConfirm main window is organized with the following components:

- **Ribbon Toolbar** at the top: Provides quick access to workflows (Intact Protein, Protein Digest, Sequence Confirmation), methods, and reporting tools.
- **Main Workspace** in the center: Shows active views such as chromatograms, deconvoluted spectra, histograms, and sample tables. Includes panes for Sample Table, Method Editor, and Results Visualization. These panels can be resized, floated, or docked for a customized layout.
- **Status Bar** at the bottom: Displays processing status, messages, and progress indicators.

Ribbon

The Ribbon provides access to actions such as opening or closing data files, opening and saving methods as well as saving results and printing reports.



Windows and Toolbars

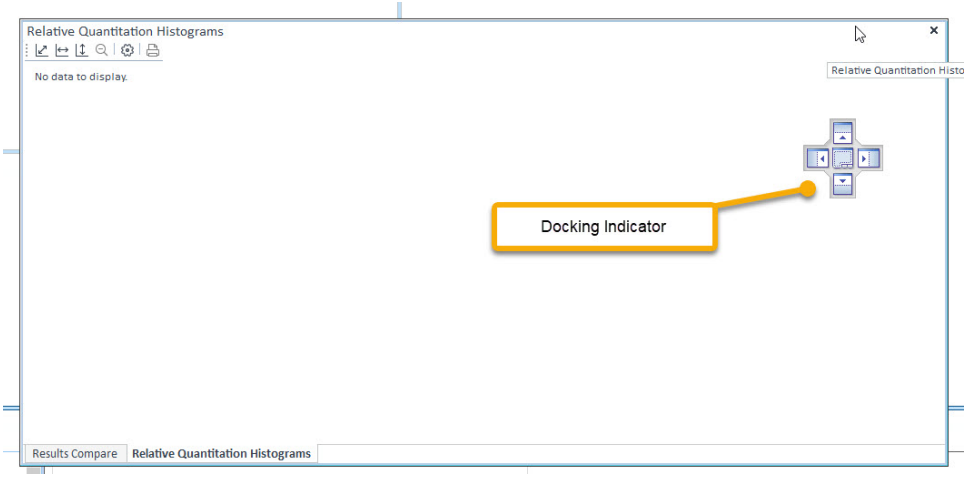
The Main Workspace displays windows with toolbars and tabs used for visualizing and interpreting results.

Some examples include:

- **Sample Chromatogram Panel**: Shows chromatographic data for selected samples, with tools for zooming, overlaying, and exporting views.
- **Deconvolution Results Panel**: Displays deconvoluted spectra with peak annotations and comparison tools.
- **Relative Quantitation Histogram Panel**: Presents glycoform or peptide-level relative abundance comparisons across samples.
- **Sample Table Panel**: Lists all samples with confirmation status and key metrics, supporting sorting and filtering.
- **Sequence Coverage and Fragment Ladder Panels**: Highlight sequence confirmation details for digests or oligonucleotides.

Change the position or tab the windows

Layouts can be rearranged so that any window is positioned above, to the right, below or to the left of any other visible window, or at one of the edges of the main program window. It is also possible to place a window on top of another window, so that the windows are tabbed. The window whose tab is selected is visible, and only the tabs of the other windows are visible.

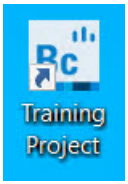


4 Workflows

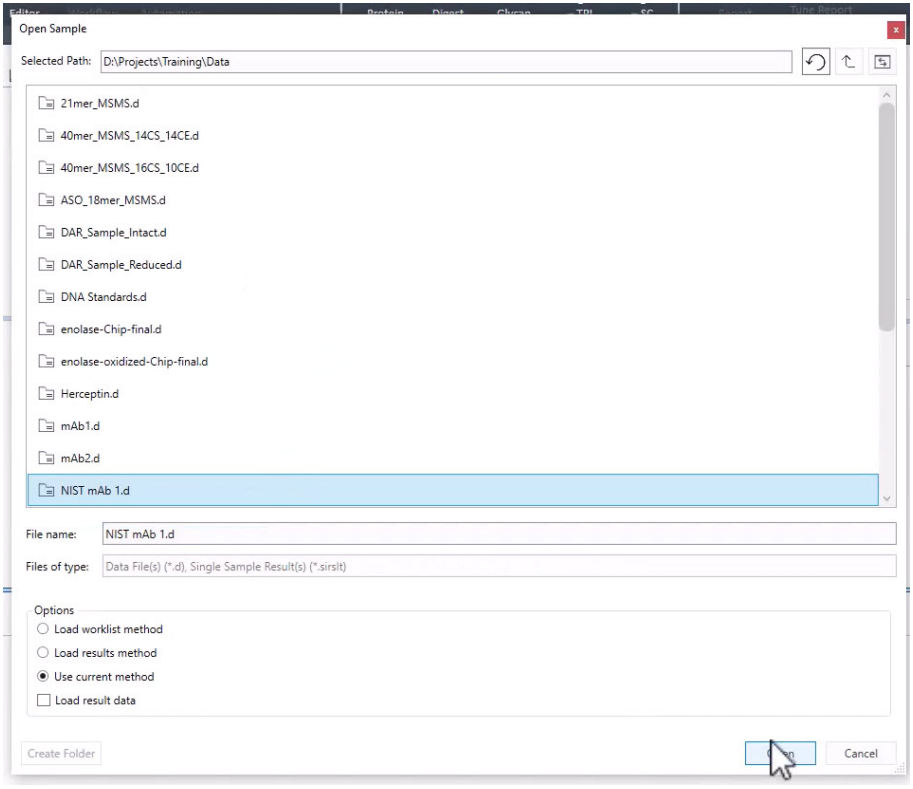
Intact Protein Workflow

The Intact Protein workflow in MassHunter BioConfirm is designed to analyze whole proteins without prior digestion, providing accurate molecular weight determination and confirmation of protein identity. This workflow uses high-resolution MS data to deconvolute complex charge states into a single neutral mass, enabling detection of post-translational modifications, glycoforms, and other variants. It is ideal for verifying protein integrity, assessing heterogeneity, and supporting biopharmaceutical characterization.

1. Using the Training Project shortcut, double-click to open MassHunter BioConfirm.

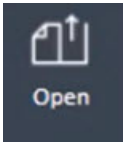


2. In the Open Sample window, select **NIST mAb 1.d** and click **OK**.

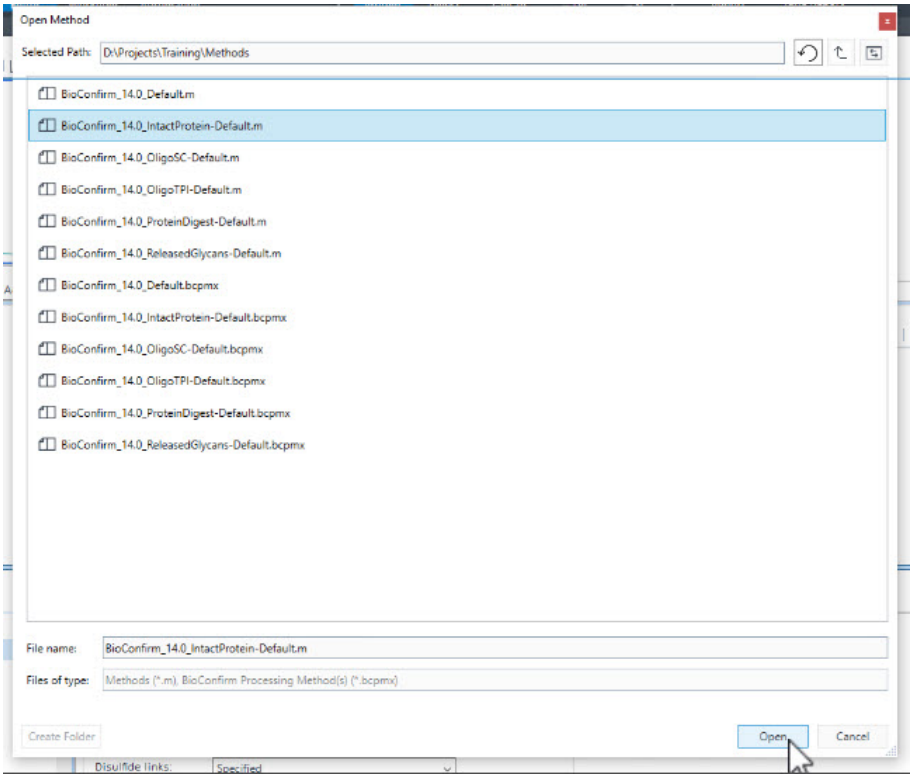


3. In the Home tab Layout section, click **Intact Protein** to select.

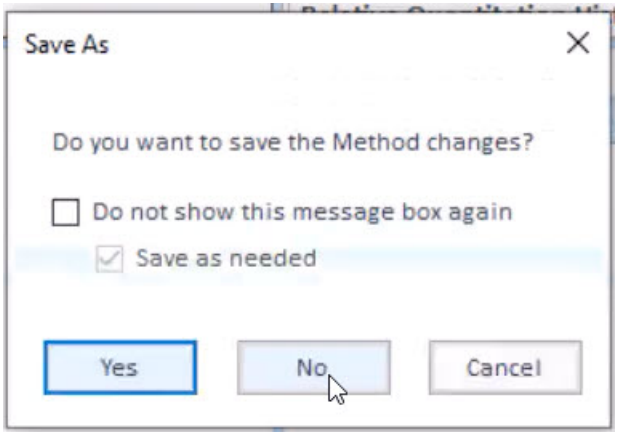
4. In the Method section, click **Open**.



5. In the Open Method dialog box, select **BioConfirm_14.0_IntactProtein-Default.m**, then click **Open**.

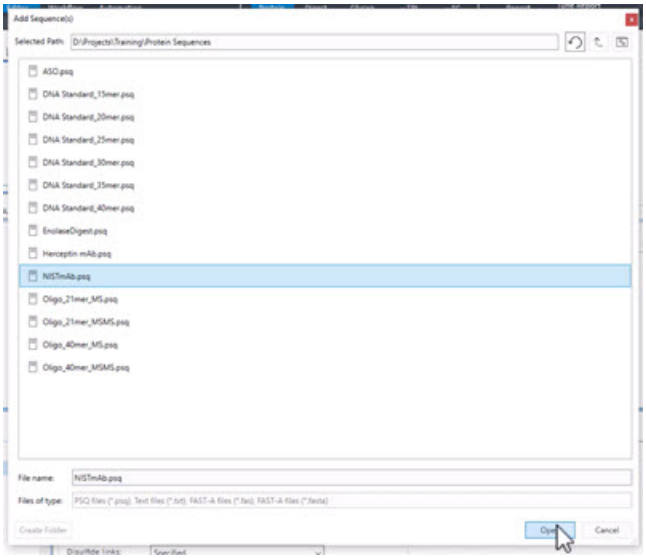


6. In the Save As dialog box, click **No**.

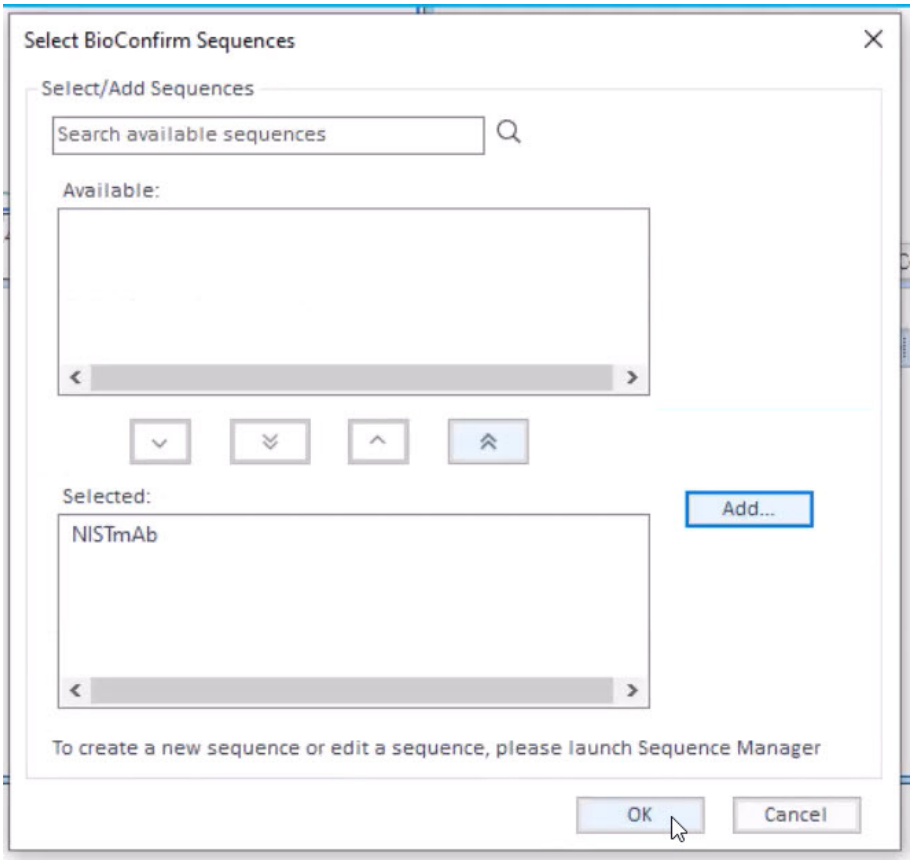


7. In the Method Editor: Workflow and Sequences window, Workflow and Sequences section, ensure that **Intact Protein** for Workflow and **non-reduced** for Condition is selected.

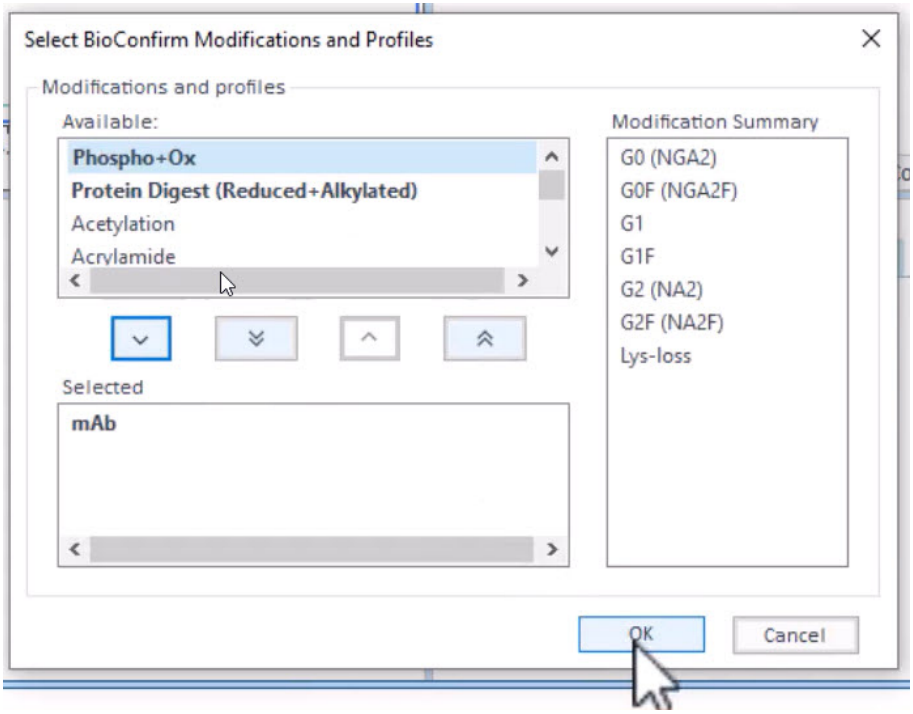
8. To select a sequence, click ....
9. In the Select BioConfirm Sequence dialog box, click **Add.**
10. In the Add Sequence(s) dialog box, select **NISTmAb.psq**, then click **Open**.



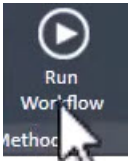
11. The selected sequence is listed in the Selected: field of the Select BioConfirm Sequences. Click **OK**.



12. Click ... to make a selection for **Mods and Profiles**.
13. In the Select BioConfirm Modifications and Profiles dialog box, select **mAb** and click ⌄, then click **OK**.



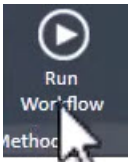
14. Once selections are complete, click **Run Workflow** on the Ribbon or **Run Method Workflow** in the Method Editor window to run the workflow.



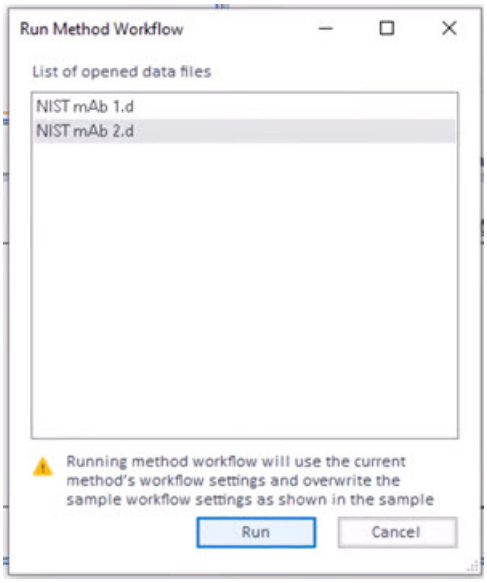
Add a data file to Intact Protein workflow

1. In the Data File section of the Ribbon, click **Open**.
2. In the Open Sample window, select **NIST mAb 12.d** and click **OK**.

3. Once selections are complete, click **Run Workflow** on the Ribbon or **Run Method Workflow** in the Method Editor window to open the Run Method Workflow dialog box.



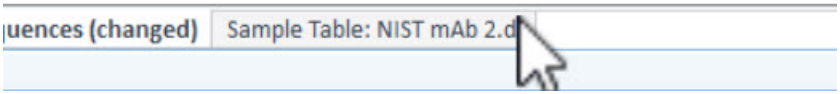
4. In the Run Method Workflow dialog box, select **NIST mAb 2.d**, then click **Run**.



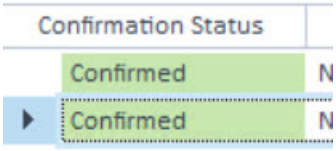
The second data file processes and loads into the Main Window.

Confirm if the sample is present

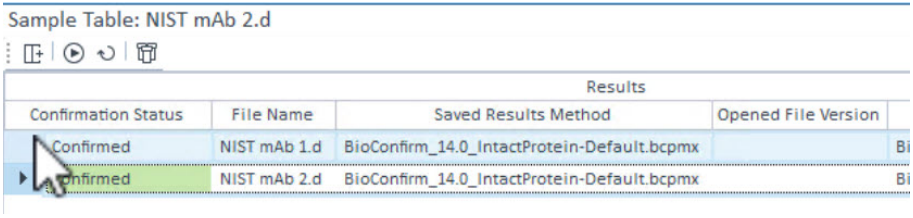
1. Click the **Sample Table** tab in the Method Editor Window.



2. Review the table for the Confirmation Status column.

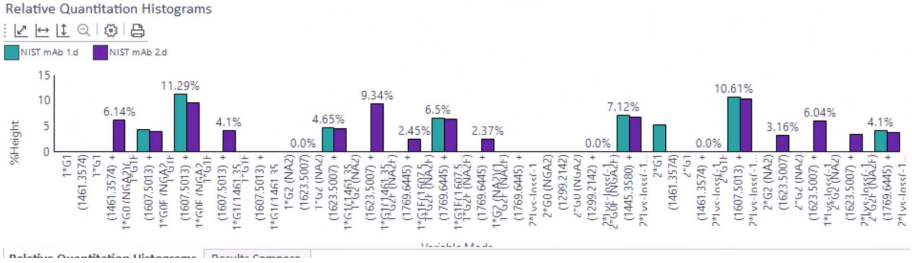


3. Press CTRL and click to highlight both samples in the Sample Table.



Review Relative Quantitation Histograms

1. Review the Relative Quantitation Histograms window.



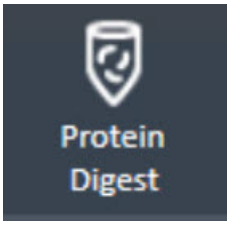
The histogram displays glycoform relative abundances of the samples.

2. By selecting multiple samples in the Sample Table, the histogram shows multiple color sets for comparison.

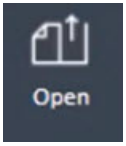
Protein Digest Workflow

The Protein Digest workflow in BioConfirm enables detailed peptide-level characterization of proteins following enzymatic digestion. Using high-resolution MS and MS/MS data, the workflow identifies peptides generated by proteolysis, calculates sequence coverage, and confirms protein identity based on matched fragment ions. It also supports detection of post-translational modifications, missed cleavages, and other sequence-based variants. This workflow is ideal for verifying primary amino-acid sequence, assessing digestion efficiency, and supporting in-depth characterization of biotherapeutic proteins.

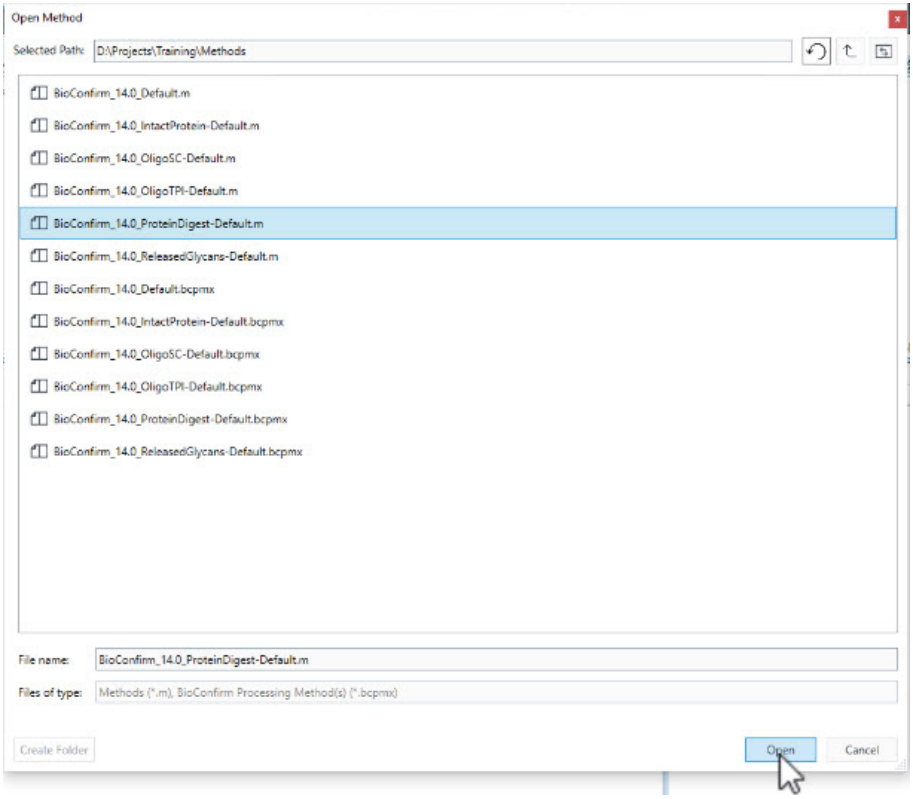
1. In the Home tab Layout section, click **Protein Digest** to select the layout.



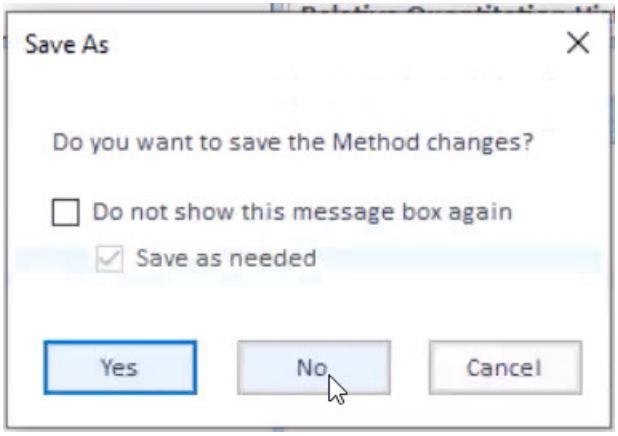
2. In the Method section, click **Open**.



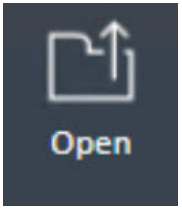
3. In the Open Method dialog box, select **BioConfirm_14.0_ProteinDigest-Default.m**, then click **Open**.



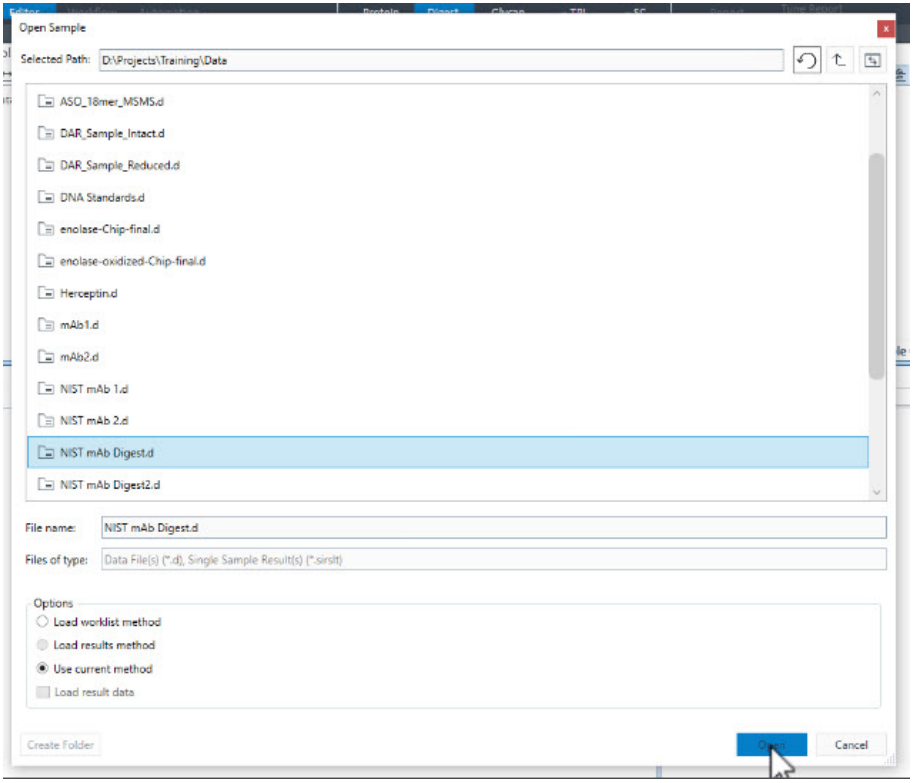
4. In the Save As dialog box, click **No**.



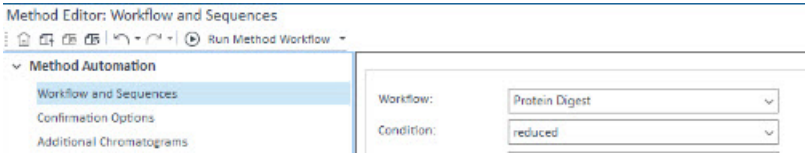
5. In the Data file section of the Home tab, click **Open**.



6. In the Open Sample Window, select **NIST mAb Digest.d** and click **Open**.

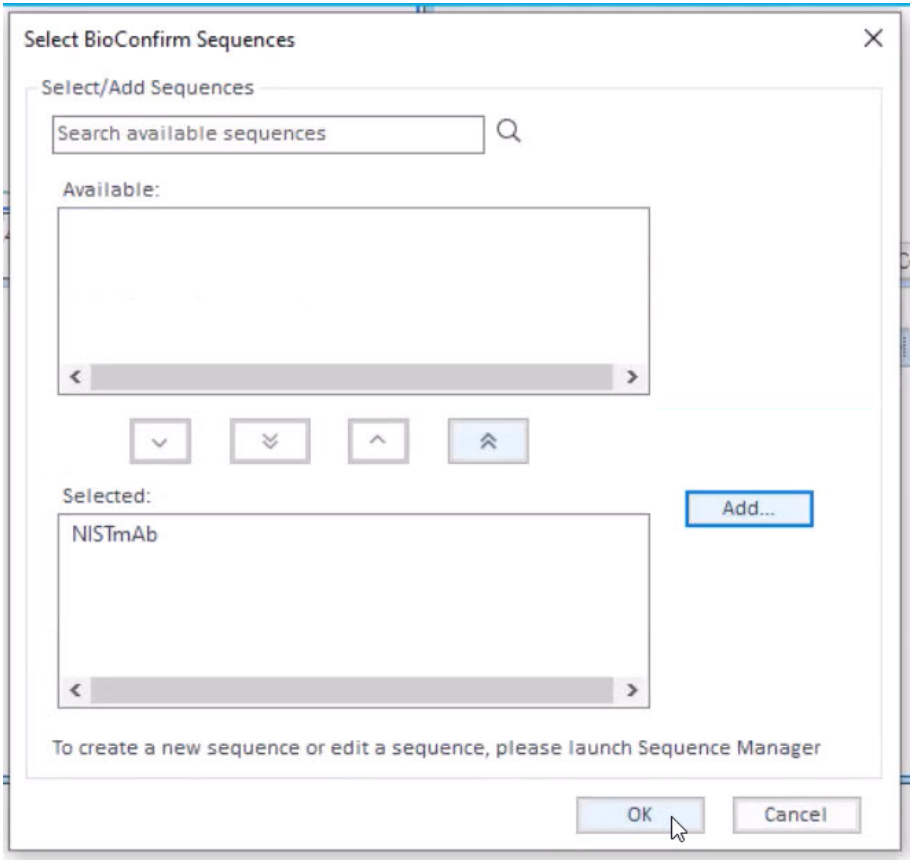


7. In the Method Editor: Workflow and Sequences window, Workflow and Sequences section, ensure that **Protein Digest** for Workflow and **reduced** for Condition is selected.

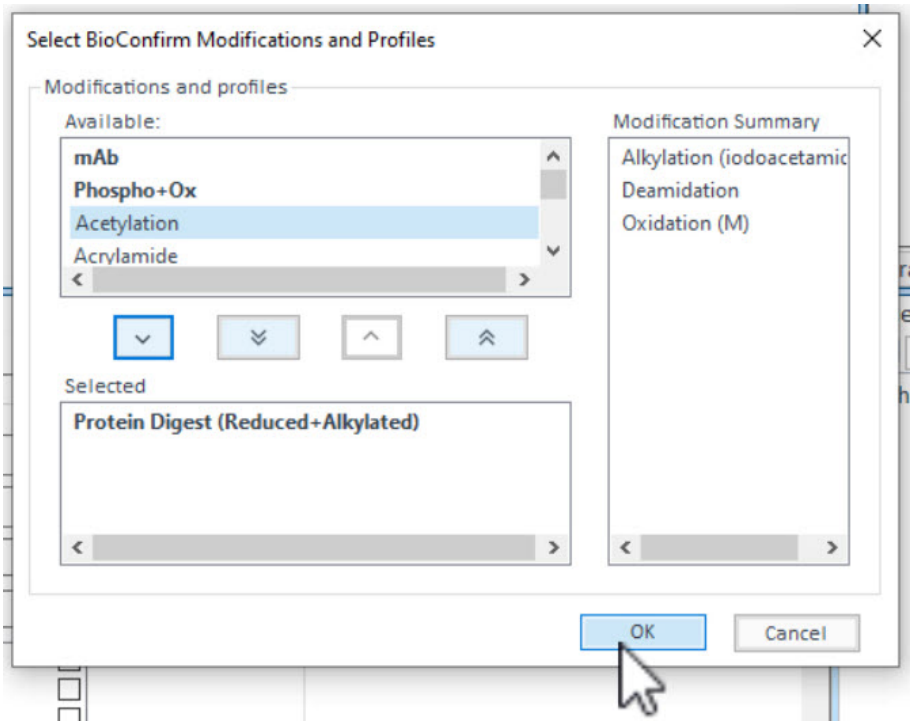


8. To select a sequence, click ... .
9. In the Available field of the Select BioConfirm Sequences window, select NISTmAb and click the ⌄ .

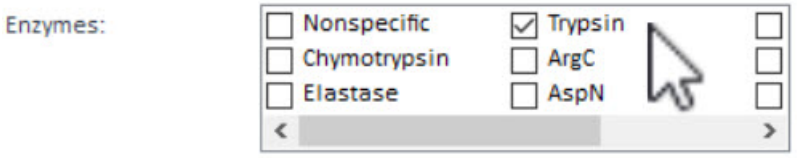
10. The selected sequence is listed in the Selected: field of the Select BioConfirm Sequences. Click **OK**.



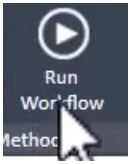
11. Click ... to make a selection for **Mods and Profiles**.
12. In the Select BioConfirm Modifications and Profiles dialog box, select **Protein Digest (Reduced + Alkylated)**, click ⌄, then click **OK**.



13. Confirm the enzyme **Trypsin** is selected.

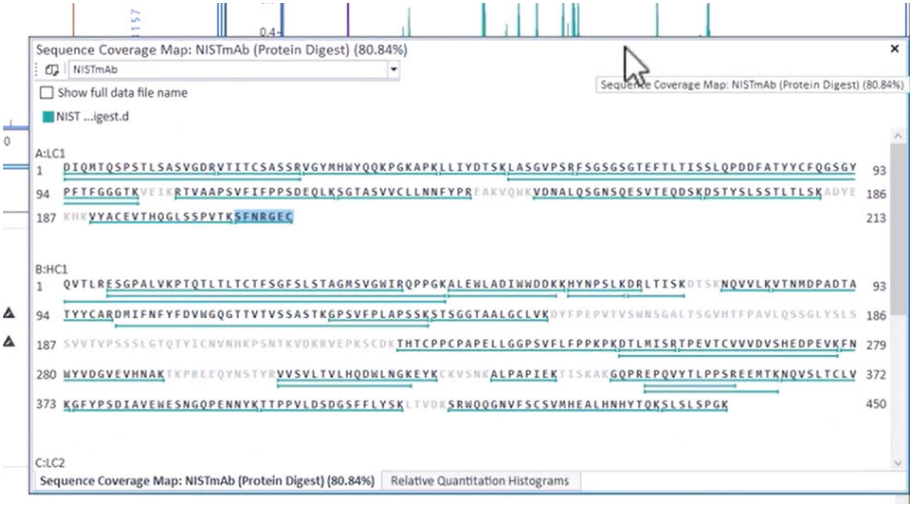


14. Once selections are complete, click **Run Workflow** on the Ribbon or **Run Method Workflow** in the Method Editor window to run the workflow.

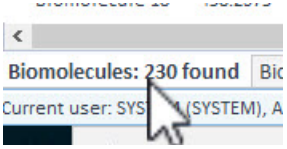


Reviewing Sequence Coverage and Adjusting the Coverage Window

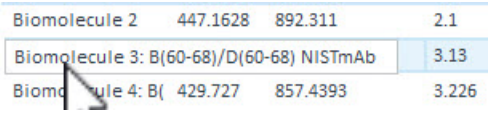
1. Double-click the **Sequence Coverage Map** to float the window.



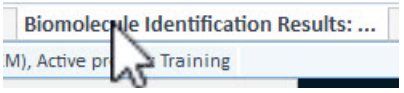
2. Review the percentage shown, which indicates how much of the theoretical peptide sequence was identified.
3. Double-click the **Sequence Coverage Map** window to redock it to the Main Window.
4. Click the **Biomolecules tab**.



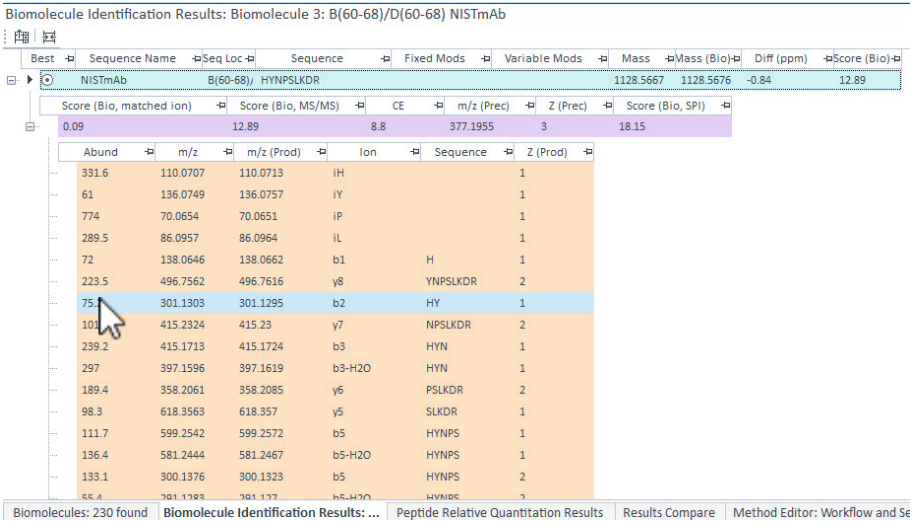
5. Select a matched biomolecule, in this example, **Biomolecule 3**.



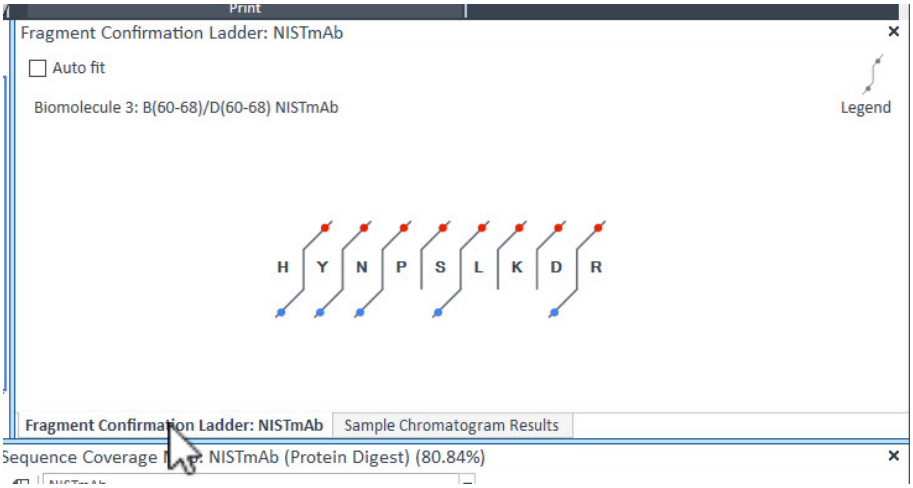
6. Click the **Biomolecule Identification Results** tab.



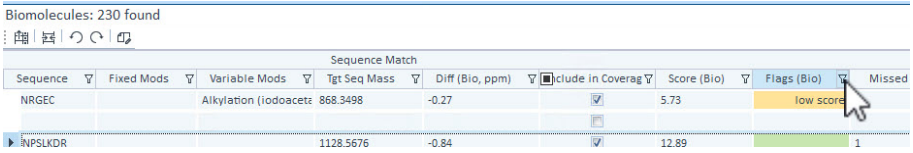
7. Review identified fragment in the **Biomolecule Identification Results** table.



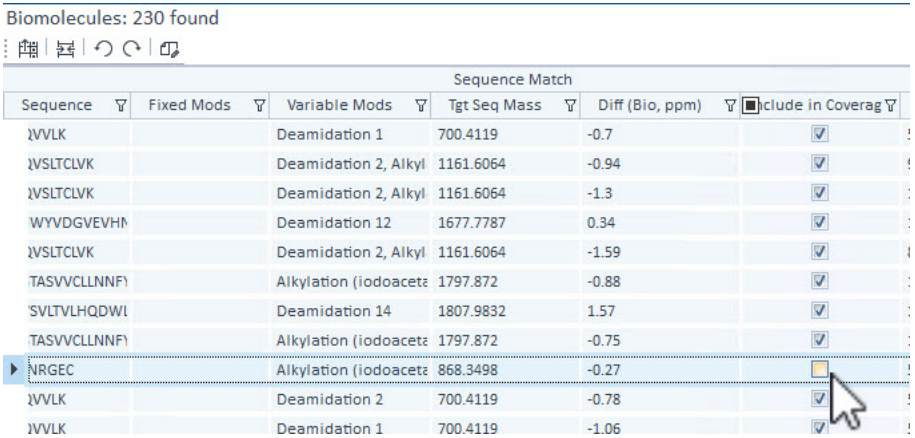
8. Review the visual map of those fragments along the peptide sequence in the **Fragment Ladder**.



9. In the Biomolecules table, sort by the **Flags** column to screen for marginal identifications.

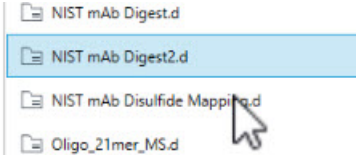


10. For any peptides that are incorrect after reviewing their fragments, uncheck **Include in Coverage**. The coverage percentage updates accordingly.

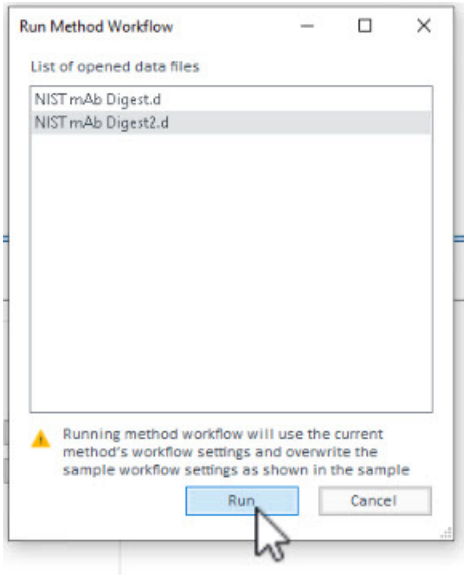


Cumulative Sequence Coverage Across Files

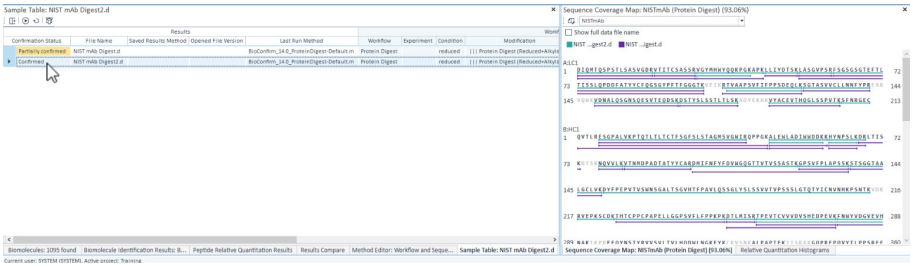
1. From the Home tab, click **Open** to load another data file.
2. In the Open Sample window, select **NIST mAb Digest 2.d**.



3. Click **Open**.
4. Click **Run Method Workflow** again.
5. In the Run Method Workflow window, select **NISTmAb Digest2.d** and click **Run**.



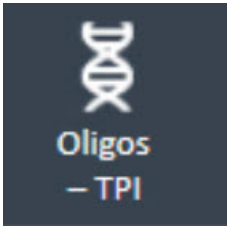
6. Review the Sequence Coverage Map and confirm that peptides from both files are combined. The sequence coverage percentage updates.



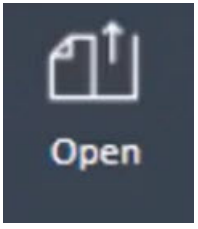
Oligo Target Plus Impurities Workflow

The Oligo Target Pluse Impurities workflow in BioConfirm is designed to confirm the presence of a known oligonucleotide sequence and evaluate synthesis-related impurities. Using high-resolution mass spectrometry data, the workflow matches observed masses to the intended target and a predefined set of impurity candidates, such as truncated sequences and common adducts. It provides relative quantitation of the target and impurities to assess sample purity and applies acceptance criteria to determine confirmation status. This workflow is well suited for verifying oligonucleotide identity, monitoring synthesis quality, and supporting routine purity assessment.

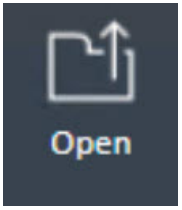
1. In the Home tab Layout section, select the **Oligos - TPI** layout.



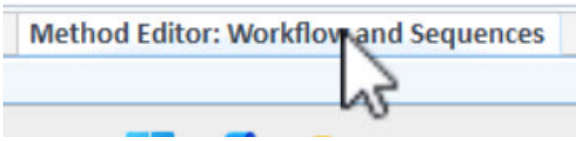
2. In the Method section, click **Open Method**.



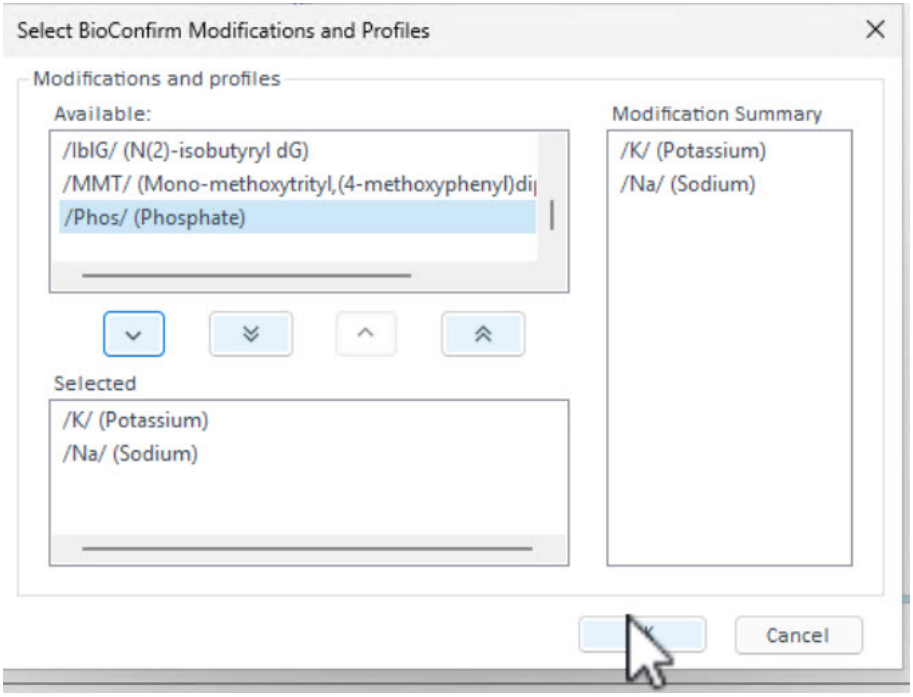
3. In the Open Method dialog box, select **BioConfirm_14.0_OligoTPI-Default.m** method file and click **Open**.
4. Click **Open Data File**.



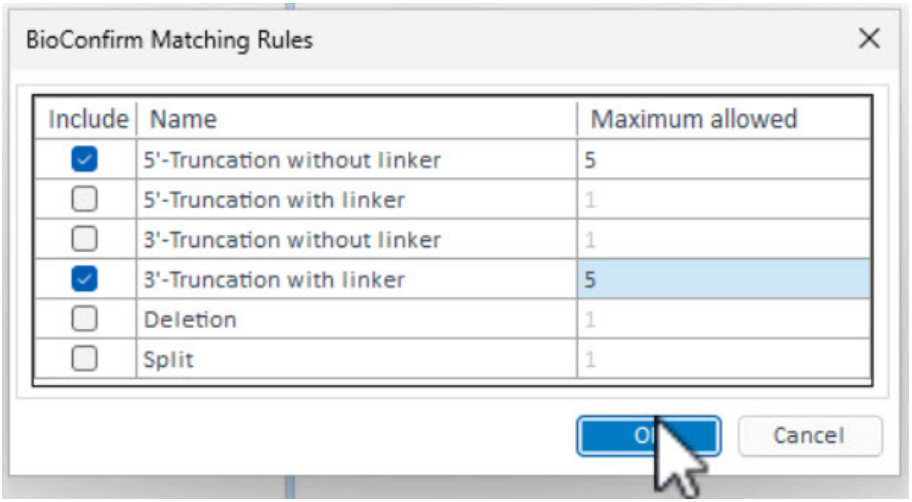
5. In the Open Sample dialog box, select **Oligo_40mer_MS_1**, then click **Open**.
6. Open the **Method Editor: Workflow and Sequences** window.



7. In the Workflow and Sequences - Sequences/Masses section, click ....
8. In the Select BioConfirm Sequences window, click **Add**.
9. Select **Oligo_40mer_MS** and click **Open**.
10. Click ... to make a selection for **Mods and Profiles**.
11. In the Select BioConfirm Modifications and Profiles dialog-box, select the Potassium and Sodium adducts, then click the ⌄ to move them to the Selected window and click **OK**.



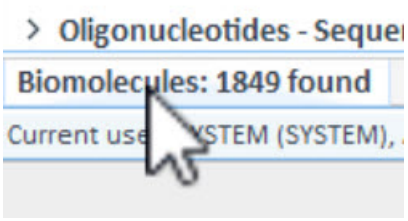
12. Click ... to make a selection for **Matching Rules**.
13. Select **5' without linker** and **3' truncation with linker** options.
14. Set the maximum allowed truncations to **5** for both the 5' and 3' ends.



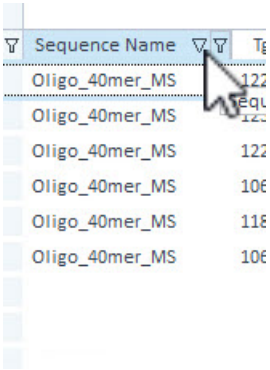
15. Click **Run Workflow**.

Assess Sample Purity

1. Navigate to the **Biomolecules** table.

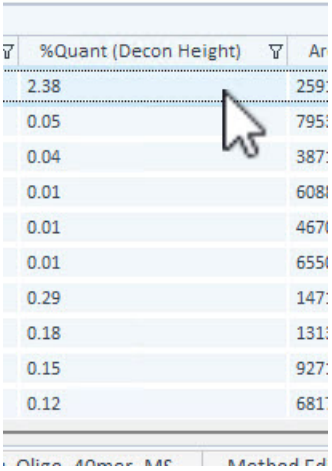


2. Scroll to the **Sequence Name** column and sort by clicking on the column header.



The top biomolecule is typically the target oligonucleotide, identified by having no variable modifications listed and the highest relative abundance. Review additional data as needed.

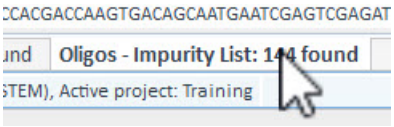
3. Scroll to the **%Quant (Decon Height)** column in the Biomolecules table.



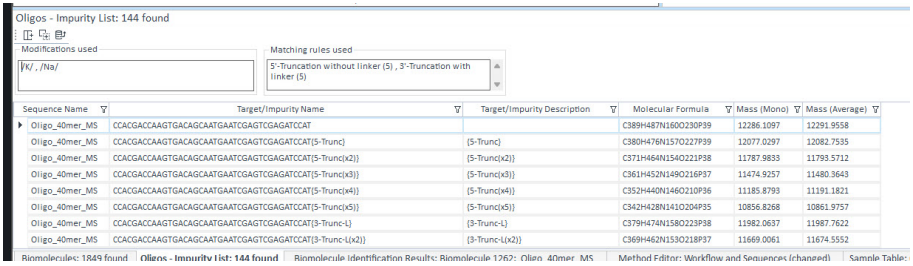
4. Review the relative abundance values. Confirm whether the target molecule accounts for the majority of the signal.

Review Impurity Candidates and Confirmation Status

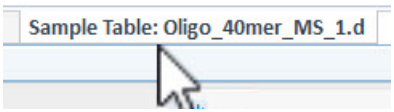
1. Select the **Oligos Impurity List** tab.



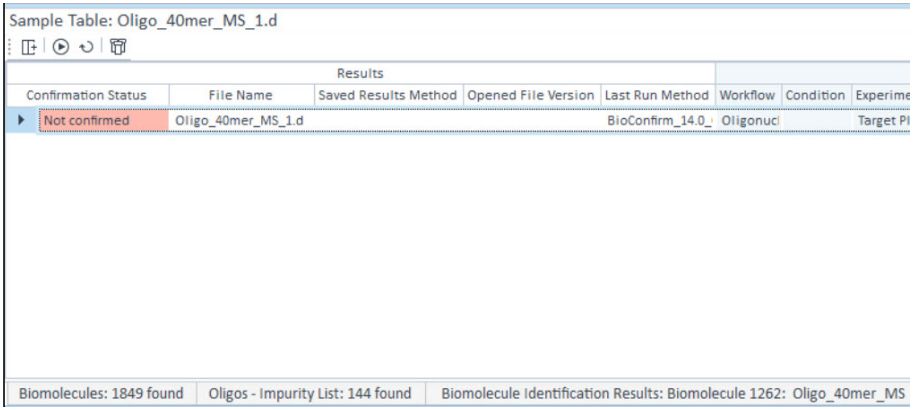
2. Review the table of impurity candidates. This is a theoretical list generated from Matching rules and Modification settings.



3. Navigate to the **Sample Table** tab and locate the Confirmation Status column.



4. Review the status. The confirmation result is based on the relative purity of the target molecule. If target purity is below predefined acceptance criteria, the sample is marked Not Confirmed. In this example, the target accounts for approximately 20%, resulting in a "Not confirmed" status.

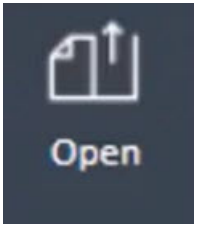

Sample Table: Oligo_40mer_MS_1.d
Results
Confirmation Status
File Name
Saved Results Method
Opened File Version
Last Run Method
Workflow
Condition
Experime
Not confirmed
Oligo_40mer_MS_1.d
BioConfirm_14.0_
Oligonucl
Target Pl
Biomolecules: 1849 found
Oligos - Impurity List: 144 found
Biomolecule Identification Results: Biomolecule 1262: Oligo_40mer_MS

Oligo Sequence Confirmation Workflow

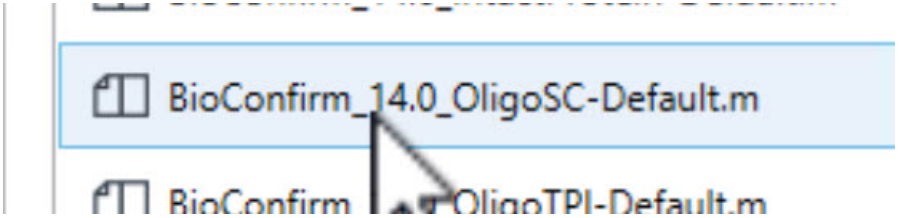
The Oligo Sequence Confirmation workflow in BioConfirm is used to verify the base-by-base sequence of a known oligonucleotide using fragment-based MS/MS data. Rather than assessing purity or synthesis-related impurities, this workflow confirms that each nucleotide is present and correctly ordered by mapping observed fragment ions to the expected sequence. It supports evaluation of individual and cumulative sequence coverage across multiple data files, providing high confidence in oligonucleotide sequence identity. This workflow is especially suited for sequence verification, method development confirmation, and investigations requiring detailed structural validation of oligonucleotides.

1. In the Home tab Layout section, select the **Oligos SC layout**.

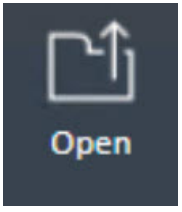
2. In the Method section, click **Open Method**.



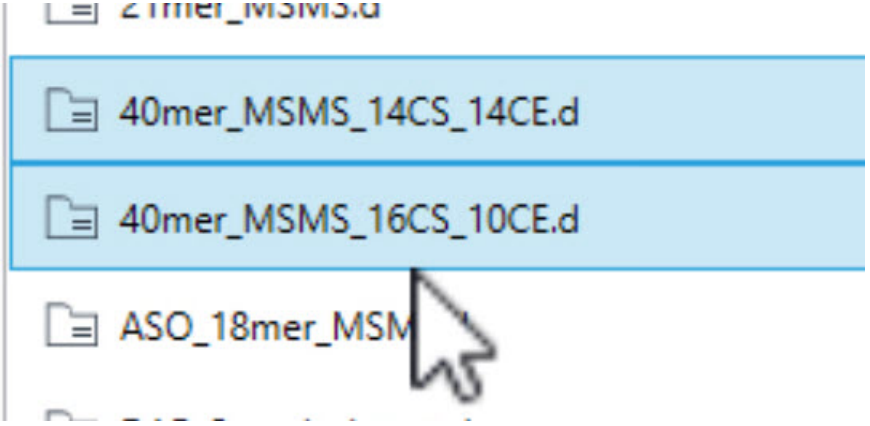
3. In the Open Method dialog box, select **BioConfirm 14.0 SC Default.m** method file and click **Open**.



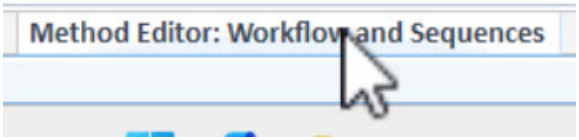
4. Click **Open Data File**.



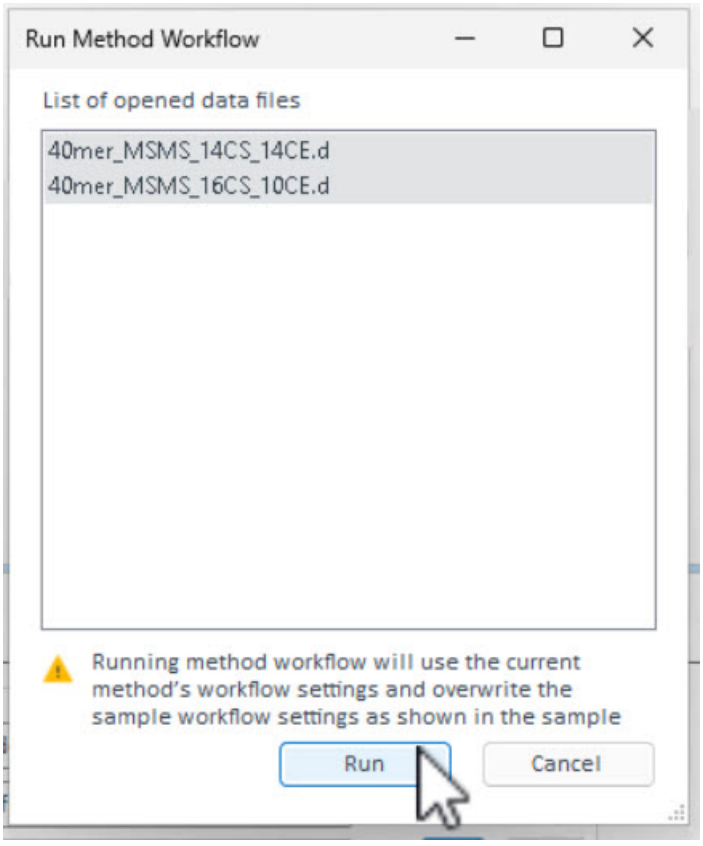
5. In the Open Sample dialog box, select **40mer_MSMS_14CS.d** and **40mer_MS_MS_16CS.d**, then click **Open**.



6. Open the **Method Editor: Workflow and Sequences** window.

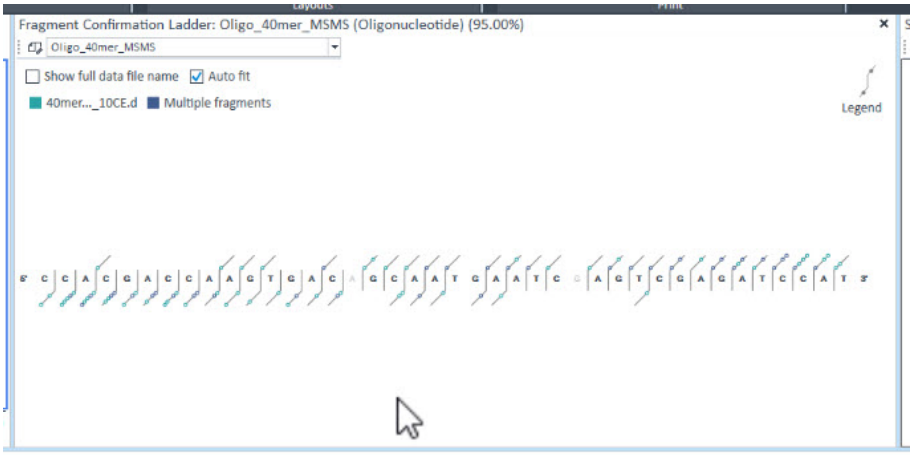


7. In the Workflow and Sequences - Sequences/Masses section, click ....
8. In the Select BioConfirm Sequences window, click **Add**
9. Select **Oligo_40mer_MSMS** , click and click Open.
10. Click **Run Workflow**.
11. In the Run Method Workflow window, select both files, then click **Run**.



Review Sequence Coverage

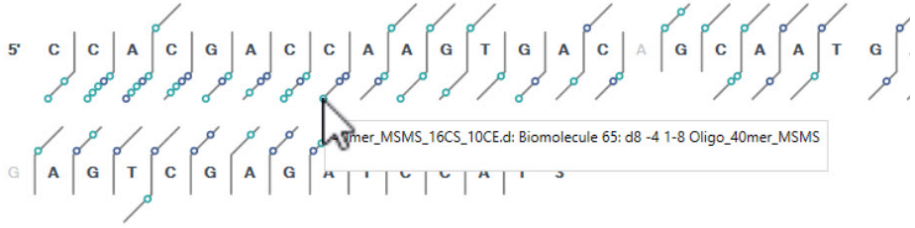
1. Navigate to the **Fragment Confirmation Ladder**.



2. Uncheck **Auto Fit** to expand the field of view.

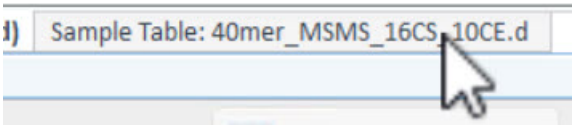
3. Review the Sequence Coverage percentage displayed at the top of the ladder.

4. Hover over a **circle** to view contributing biomolecules. Each circle represents one or more confirmed fragments at a specific sequence position.

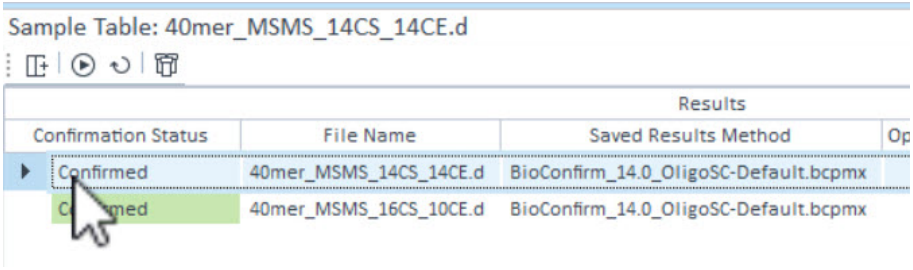


Exclude Incorrect Fragment Assignments and Validate Cumulative Sequence Coverage

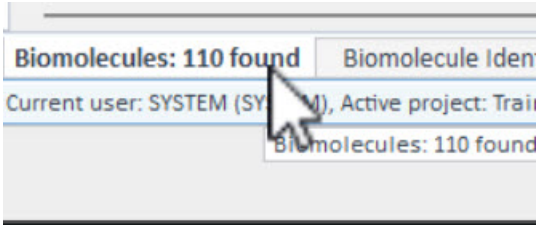
1. Navigate to the **Sample Table** tab.



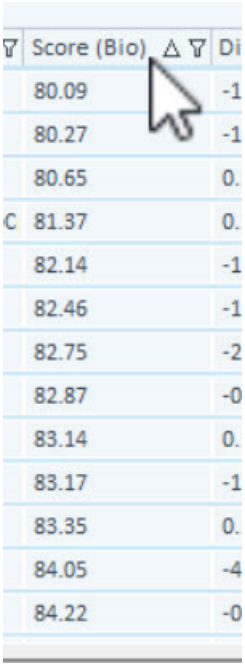
2. Select the **40mer_MSMS_14CS_14CE.d** file.



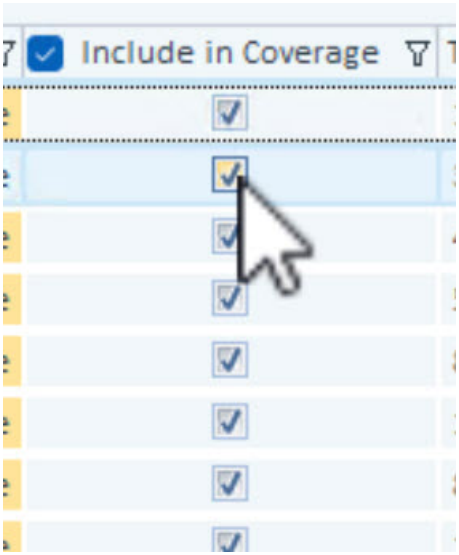
3. Navigate to the **Biomolecules** table.



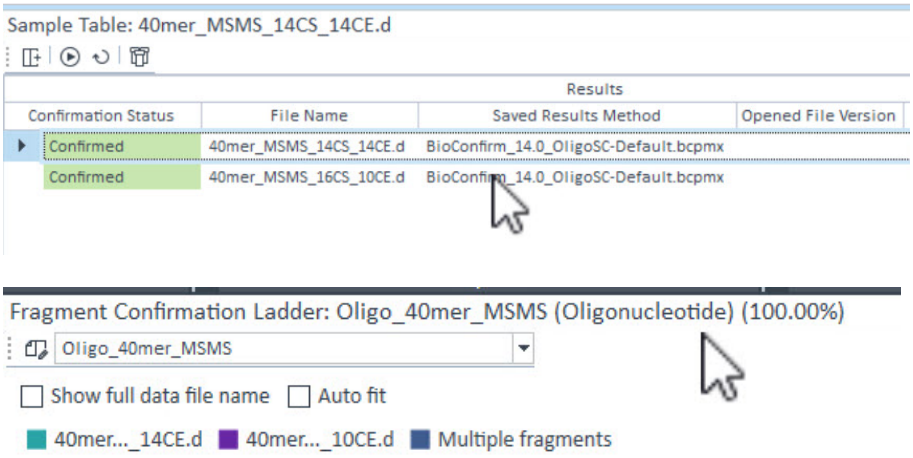
4. Sort the table by the **Score (Bio)** column.



5. If a fragment appears incorrectly identified, uncheck **Include in Coverage** for that biomolecule. The sequence coverage percentage updates automatically. In this example, deselect **Biomolecule 24**.



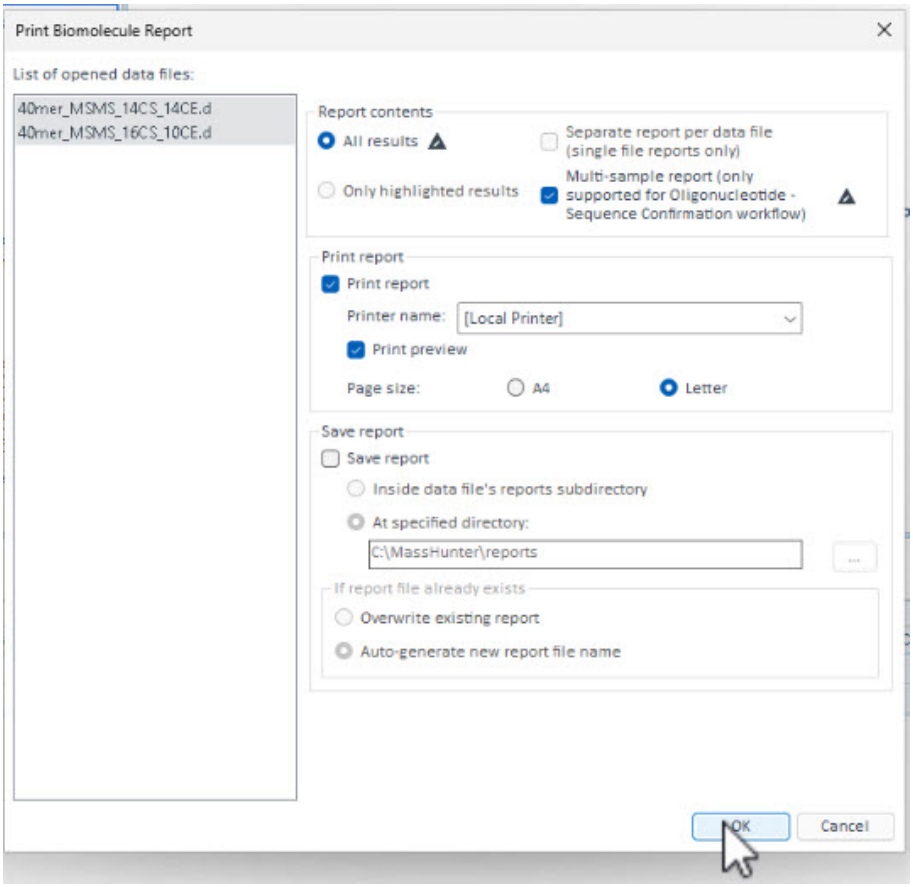
6. To demonstrate cumulative sequence coverage, navigate back to the **Sample Table**, and select both data files. The Sequence Confirmation Ladder updates. Confirm that fragment coverage is combined across files.



Generate Sequence Confirmation Reports

1. In the **Home tab > Print section**, select **Biomolecule Report**.

2. In the Print Biomolecule Report dialog box, select **All Results**.
3. Click to enable **Multi-sample report**.
4. Click **OK** to generate the report.



The report summarizes individual and cumulative sequence coverage across all selected files using the same target sequence.

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