

# An Open-Source Tool for the Creation of Multiplexed, Targeted Proteomic Instrument Methods Utilizing Isobaric Labels

Christopher M. Rose<sup>1,2</sup>, Brian K. Erickson<sup>2</sup>, Steven P. Gygi<sup>2</sup>, Donald S. Kirkpatrick<sup>1</sup>

<sup>1</sup>Genentech, South San Francisco, CA, <sup>2</sup>Harvard Medical School, Boston, MA

Genentech  
A Member of the Roche Group

## Introduction

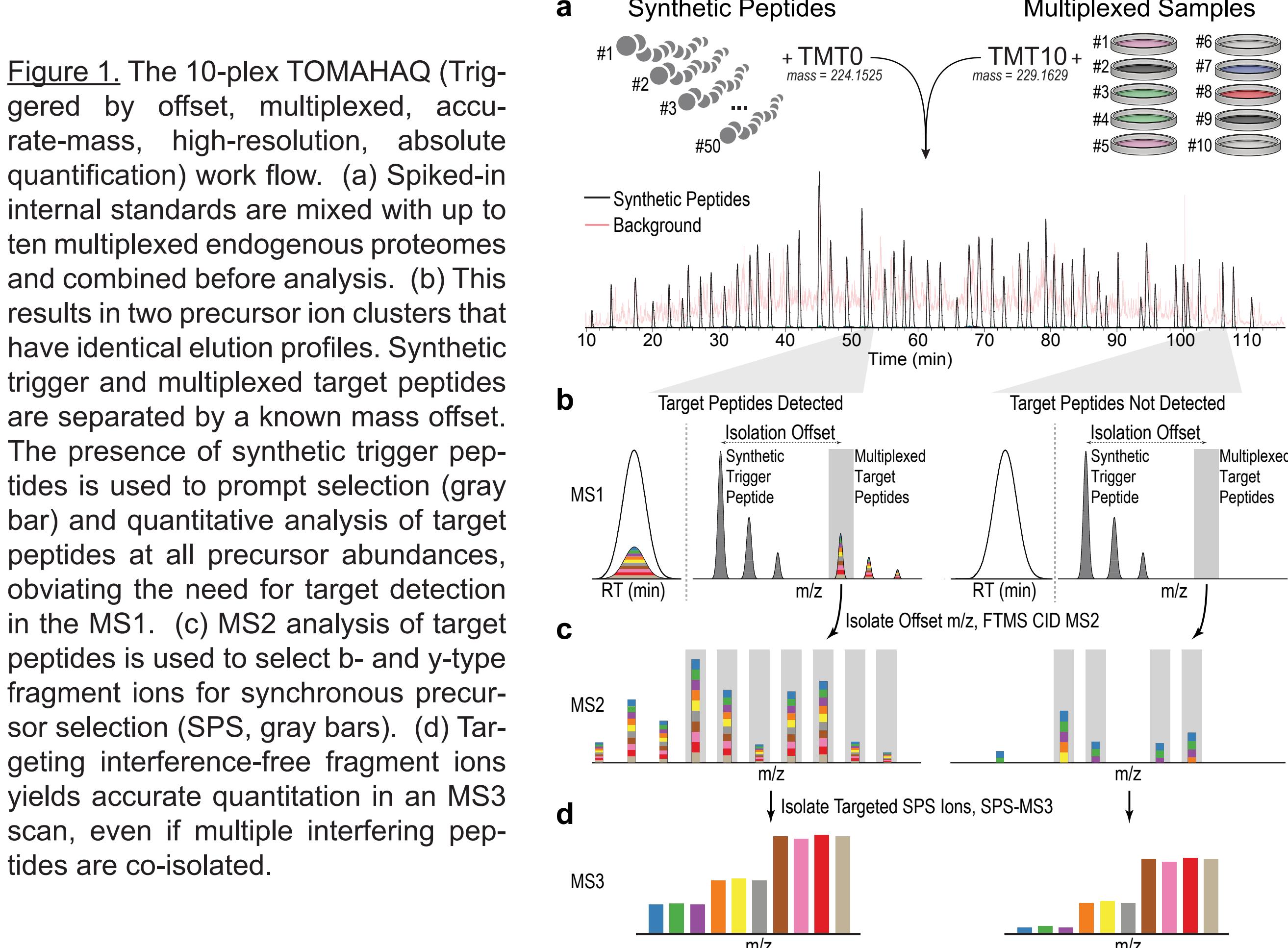
Traditionally, the combination of peptide and sample multiplexing in targeted proteomics has been used to analyze enriched subproteomes (e.g., pY IP). This is due to large amounts of co-isolated precursor interference when targeting low abundance peptides. Recently, a new method TOMAHAQ – Triggered by Offset, Multiplexed, Accurate mass, High resolution, and Absolute Quantitation – was introduced to overcome these challenges. TOMAHAQ utilizes synthetic trigger peptides labeled with chemical tags identical in structure, but different in mass, to the tag used for sample multiplexing. Synthetic peptides are spiked into the multiplexed samples and used to prompt quantitative analysis of multiplexed samples.

Due to the number of scan events and filters necessary for optimal TOMAHAQ performance construction of instrument methods can be time consuming, specifically when building a method to quantify more than 10 peptides. To expedite method construction we have created a Windows application called TomahaqCompanion that utilizes a ".csv" input of peptide sequences along with a template method to create TOMAHAQ instrument ".meth" files.

TomahaqCompanion requires peptide sequences, but to enable flexibility users can specify the preferred peptide charge, trigger peptide m/z values used to prompt target peptide analysis, and/or target peptide m/z values used for targeted SPS-MS3 analysis. Users can select the modifications used to differentiate the trigger and target peptides (e.g., TMT0 and TMT10). Once peptides are imported and modifications are selected TomahaqCompanion modifies the template method to create a TOMAHAQ method for Orbitrap Fusion/Lumos instruments.

TomahaqCompanion enables visualization of TOMAHAQ data collected on Thermo Fusion or Lumos instruments. Data visualization includes trigger and target peptide extracted ion chromatograms, target peptide MS/MS spectra, target peptide SPS-MS3 spectra, as well as quantitative information for all SPS-MS3 scans. The data can then be exported and analyzed utilizing offline tools (e.g., R).

## TOMAHAQ - Sample multiplexing & targeted proteomics



## TOMAHAQ + TomahaqCompanion sample analysis work flow

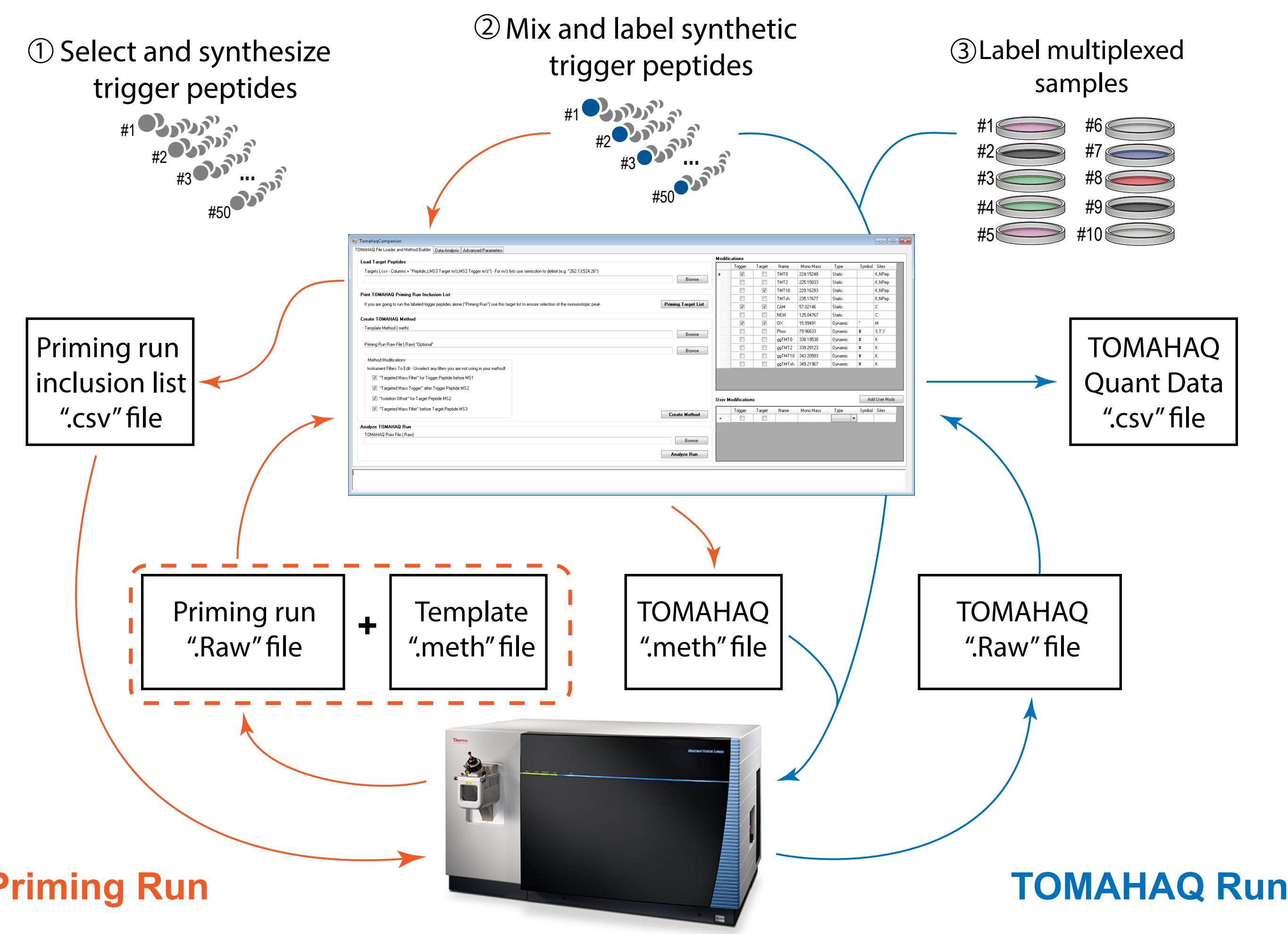


Figure 2 illustrates the TOMAHAQ sample analysis work flow utilizing TomahaqCompanion. There are two stages of analysis. 1) Priming run. This is an analysis of the peptides alone that is required when developing an assay. A target list for the priming run is generated by TomahaqCompanion and the results of the priming run can be viewed by TomahaqCompanion. The priming run can be combined with a template TOMAHAQ method to produce a TOMAHAQ method file. 2) TOMAHAQ run. After generating the TOMAHAQ method you can mix the synthetic peptides with your sample of interest and analyze the experimental sample. The final data can be viewed, curated, and exported using TomahaqCompanion.

## TomahaqCompanion GUI and input formats

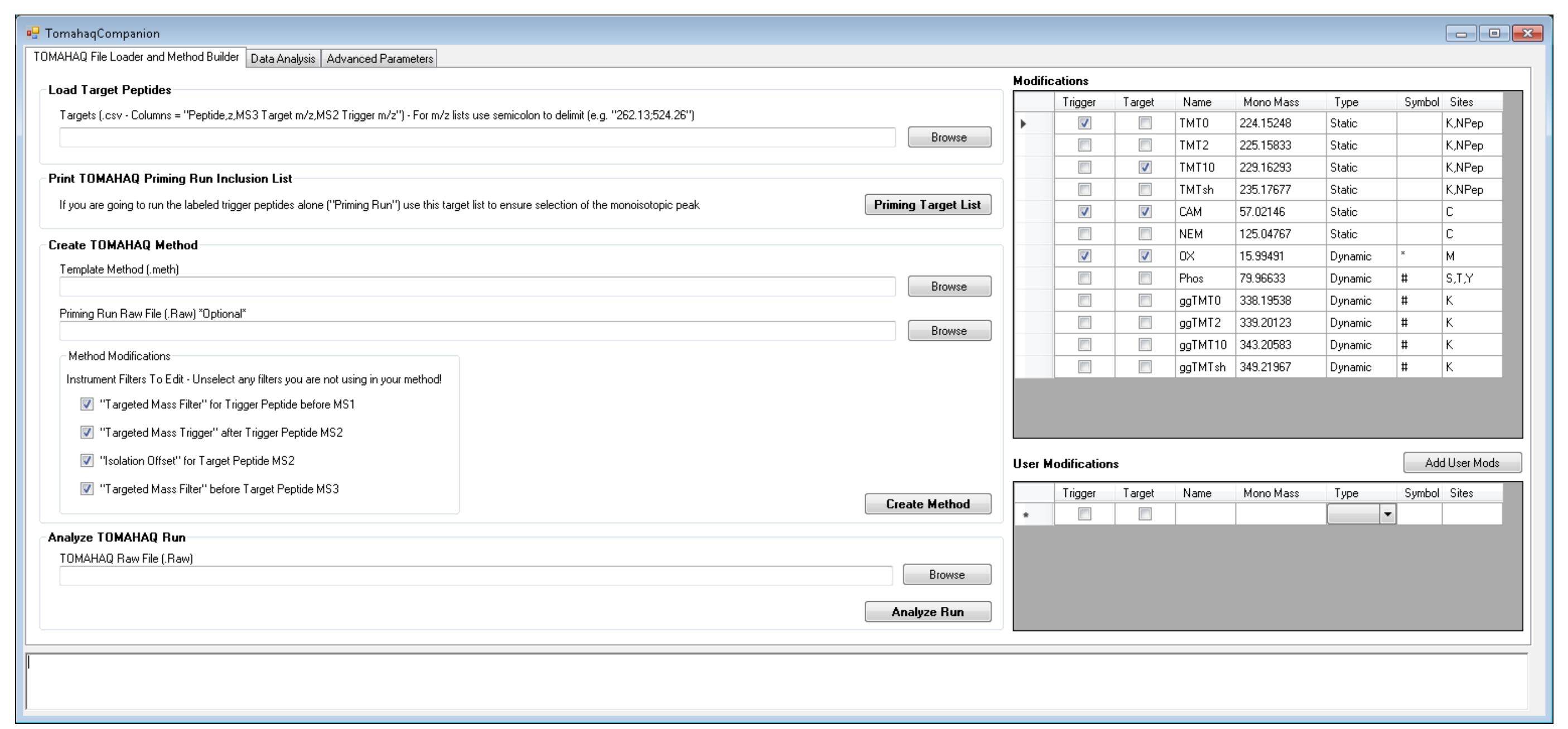


Figure 3. TomahaqCompanion input formats. TomahaqCompanion was designed to work with a varying complexity of input formats. a) The simplest input is a ".csv" file with a list of peptide sequences in a single column labeled "Peptide". If a charge state is not provided TomahaqCompanion will consider a range of charge states for each peptide. This includes automatic creation of TOMAHAQ methods and analysis of TOMAHAQ method if a priming run is provided. The best charge state is determined by the trigger peptide intensity. b) The user can provide both the sequence and charge state to ensure that a particular charge state is used when creating a TOMAHAQ method or analyzing a TOMAHAQ experiment. c) TomahaqCompanion will automatically populate both trigger and target ions for the trigger MS2 and SPS-MS3, respectively.

c. Sequence, charge, and target MS3 m/z

Peptide	z	MS3 Target m/z
STPVNPVISQK	3	557.3451,626.8585
LAAQFIPK	2	485.3285,760.4555

d. Sequence, charge, target MS3, and trigger MS2 m/z

Peptide	z	MS3 Target m/z	MS2 Trigger m/z
STPVNPVISQK	3	557.3451,626.8585	554.8399,624.3533
LAAQFIPK	2	485.3285,760.4555	480.3180,755.4540

## Peptide modifications within TomahaqCompanion

Figure 4. TOMAHAQ can utilize various combinations of isobaric labels and heavy labeled synthetic peptides to produce the mass shift that separates trigger and target peptides. TomahaqCompanion utilizes a graphical user interface to allow users to select the modifications that exist on trigger or target peptides. a) For example, if super heavy TMT is used as the trigger label then TMT10 will be selected only for the target peptides. All other modifications will be selected for both peptides as they can exist on both species. Modifications can be set to either Static or Dynamic and the sites of modification must be provided. For Dynamic modifications, the symbol in the sequence must match the sequence in the GUI.

b) If heavy amino acids are used to induce the mass shift they may be added through the GUI. In this case Heavy lysine (+8 Da) and arginine (+10 Da) were used. Heavy amino acids are treated as dynamic modifications and must include the mass of the isobaric label if they can be labeled. For example, the heavy lysine has an additional 229 Da to account for TMT10. The inclusion of the TMT10 mass is due to the modification of the lysine being replaced by the user modification. Dynamic modification symbols must be unique or the modification will fail to the last modification on the GUI list. Multiple sites of modification are denoted by multiple identifiers separated by a comma. All modifications must have a unique name, but can have the same exact masses.

## Generating a priming run target list

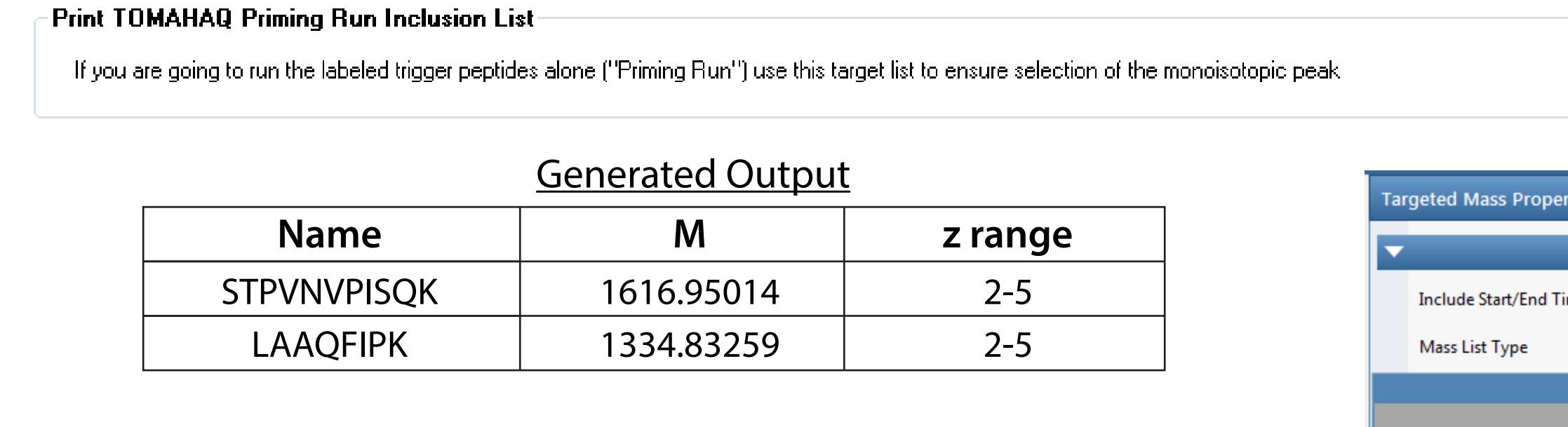


Figure 5. An analysis of the trigger peptides alone, also called a "priming run" helps to determine the ions that will be used for fragment ion triggering and targeted SPS-MS3. When performing a priming run it is important to select the monoisotopic peak of the isotopic cluster. TomahaqCompanion will use the input peptide list to produce a ".csv" file that contains the peptide, its neutral mass, and the charge range to interrogate. This ".csv" can be uploaded into the instrument method when performing the priming run. To ensure that peptides are selected the "Ignore charge state requirement for unassigned ions" should be selected.

## Peptide QC and selection of representative spectra

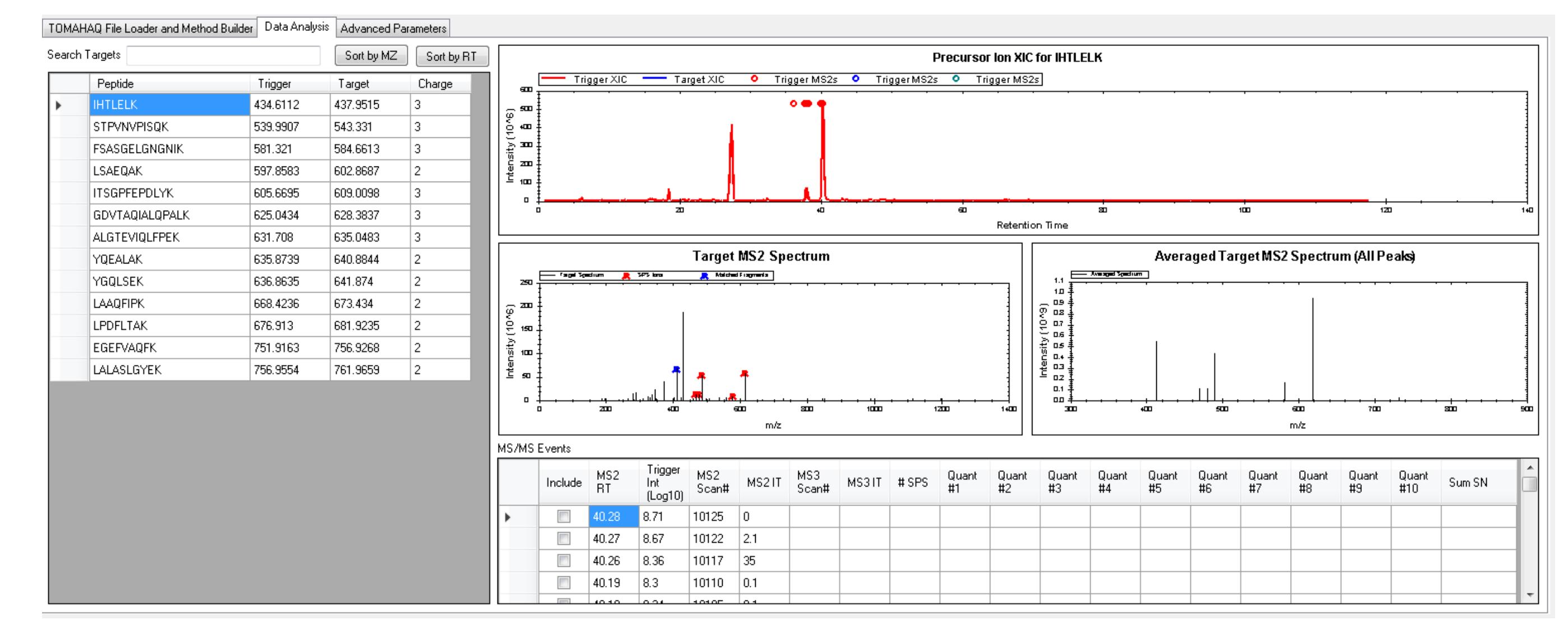


Figure 6. Trigger peptide quality control. Once synthetic trigger peptides have been run on the mass spectrometer, they can be analyzed using TomahaqCompanion. If the target peptide list (.csv) and the raw data (.Raw) files are provided the user can press the 'Create Method' button which will analyze the raw data to produce extracted ion chromatograms of each peptide. Additionally, each MS2 will be presented along with matching b- or y- ions that are denoted with either a red or blue star. The blue star indicates that the b- or y-ion matched but was not added to the SPS-MS3 target list. An red star indicates that the ion will be added to the SPS-MS3 target list. An averaged spectrum displays the matching ions and their relative intensity across all MS2 spectra.

## Creation of a TOMAHAQ template method

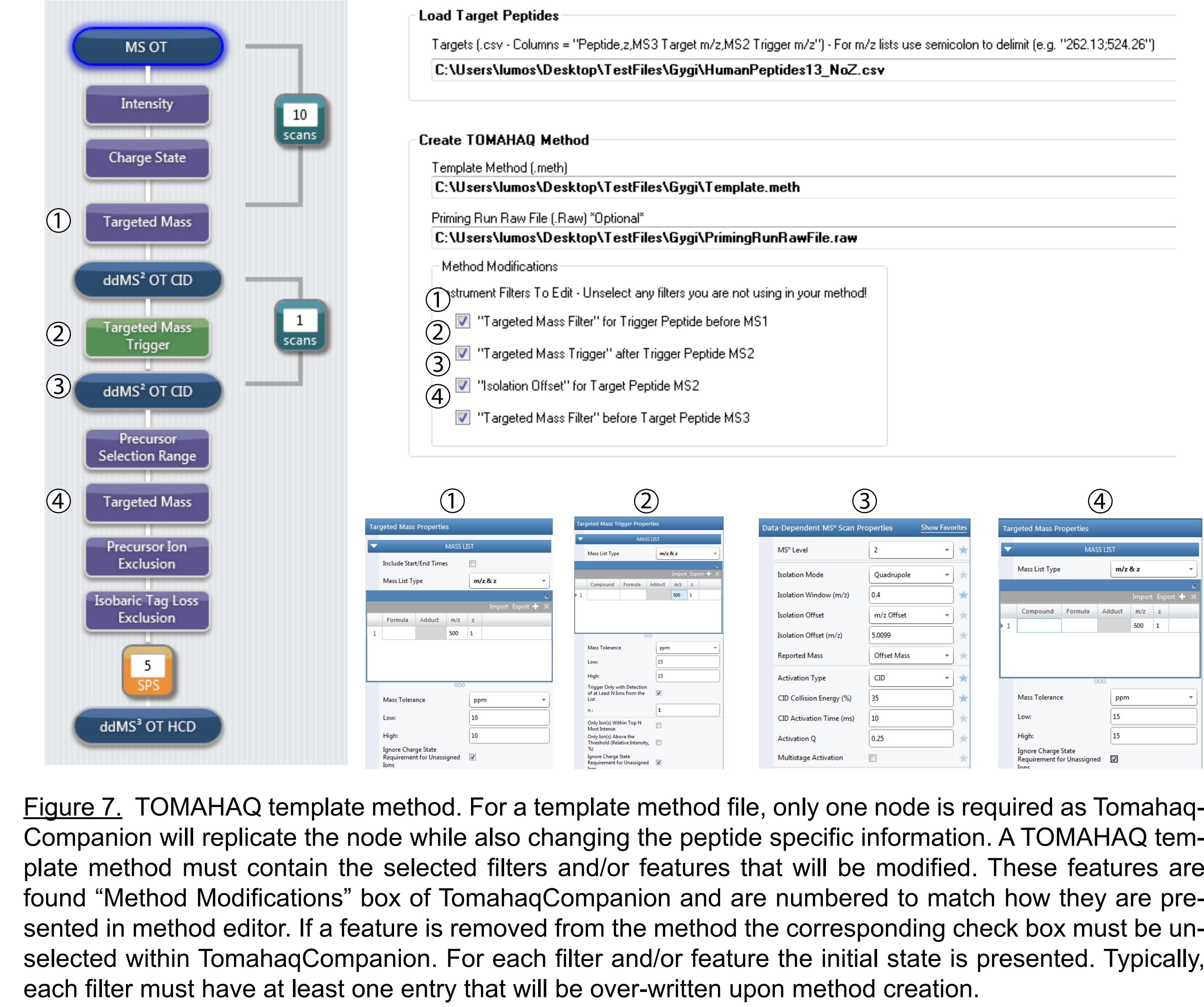


Figure 7. TOMAHAQ template method. For a template method file, only one node is required as TomahaqCompanion will replicate the node while also changing the peptide specific information. A TOMAHAQ template method must contain the selected filters and/or features that will be modified. These features are found "Method Modifications" box of TomahaqCompanion and are numbered to match how they are presented in method editor. If a feature is removed from the method the corresponding check box must be unselected within TomahaqCompanion. For each filter and/or feature the initial state is presented. Typically, each filter must have at least one entry that will be over-written upon method creation.

## Automatic creation of TOMAHAQ methods

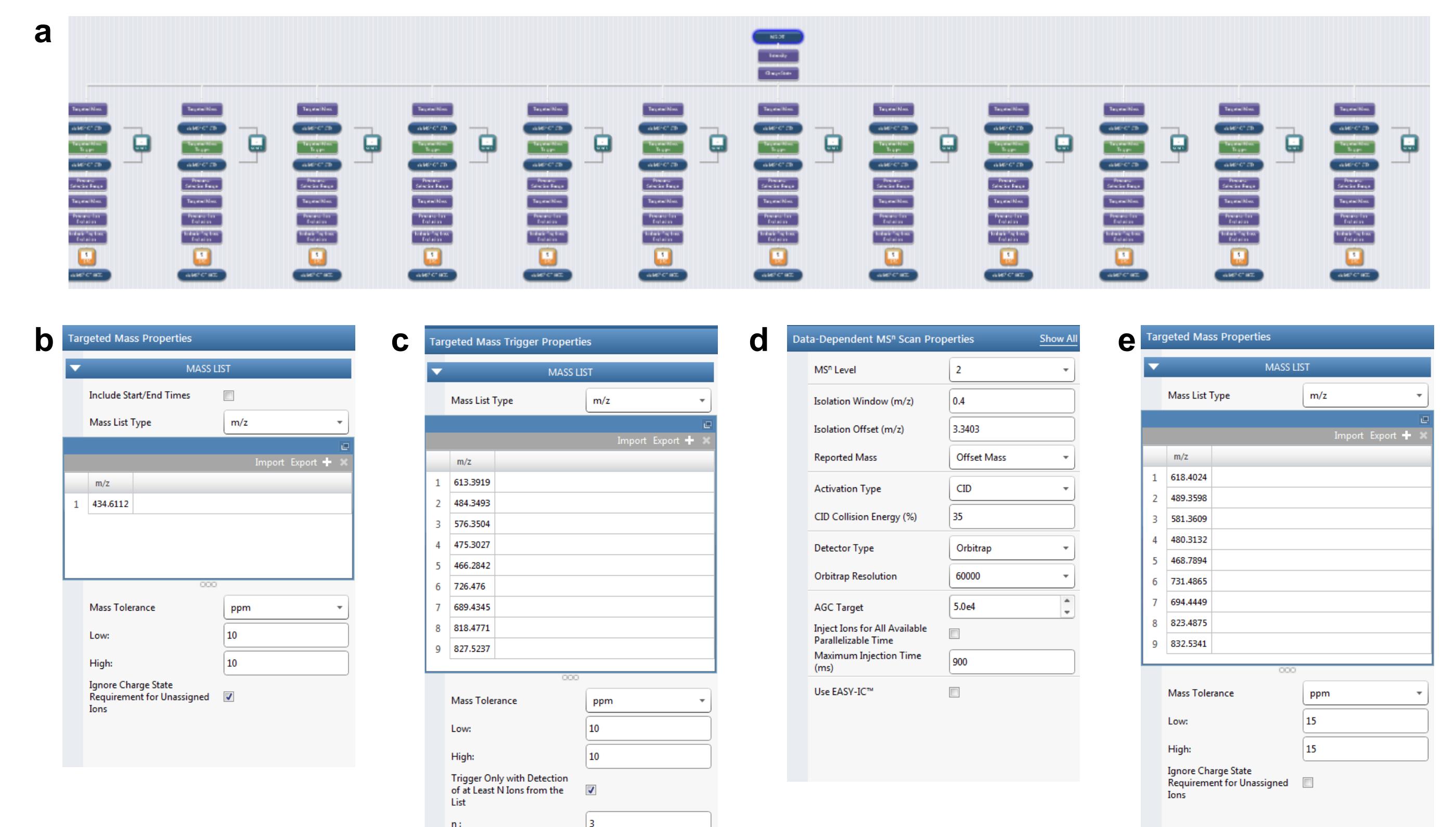


Figure 8. Automatic creation of TOMAHAQ methods. To create a method a target peptide list and a template method are required (Fig. 7). This will create an instrument method that includes theoretical fragments for each of the peptides that will be targeted. If a priming run is provided when creating a method, TomahaqCompanion will use the priming run for the trigger peptides for a range of charge states, match the fragment ions present in the FTMS2, and calculate the mass of the target peptides and fragment ions based on the modifications from the user input. If no charge state is provided TomahaqCompanion will choose the best charge state based on the intensity of the trigger peptide. a) The scan tree of TOMAHAQ method. Each node was automatically generated specifically for a single peptide. Peptide specific information is placed in the parameters selected within the TomahaqCompanion GUI (Fig. 7). b-e) The scan filters and definitions for the panels displayed in Fig 7. The m/z values and isolation offset have been replaced with values that correspond to either the trigger or target peptide. TomahaqCompanion has been used to create methods for over 100 peptides; however, opening methods larger than 75 peptides may not be possible.

## Interactive analysis of TOMAHAQ data

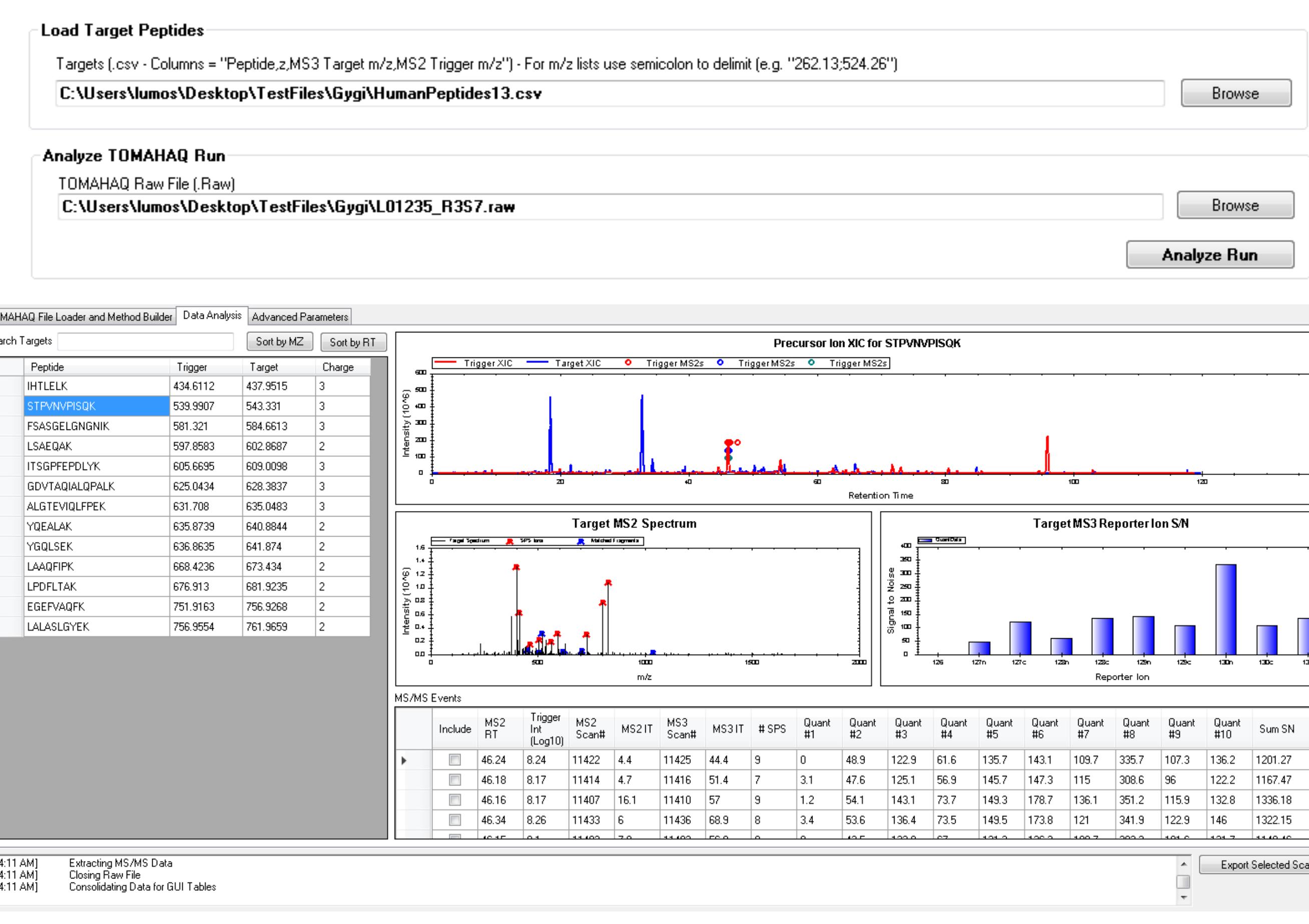


Figure 9. Data analysis with TomahaqCompanion. The user provides the target peptide list (.csv) and a ".Raw" file of a TOMAHAQ run and TomahaqCompanion will search the run for MS2 scans of the trigger peptide. Next, any subsequent MS2 or SPS-MS3 scans on the target peptide will be linked to the trigger peptide event. TomahaqCompanion will extract the SPS ions that were used for the targeted scan and report the signal to noise (SN) values. The data are displayed in the GUI where users can zoom in on an area of interest in the chromatogram and view the quantitation associated with each MS3 scan along with the target peptide MS2 that proceeded the MS3 scan (see Fig. 10).

## Data curation, export, and analysis

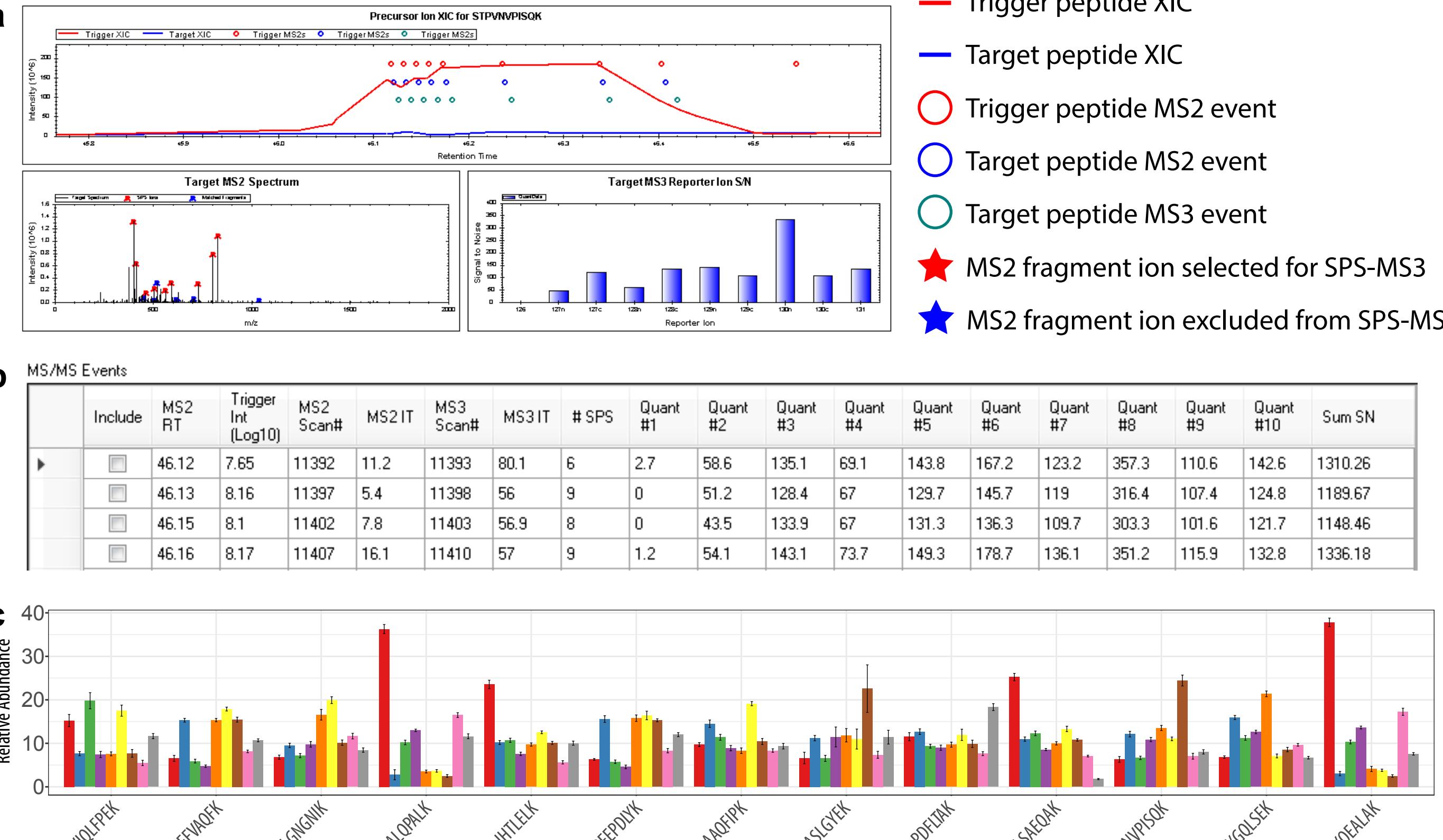


Figure 10. Data curation, export, and analysis within TomahaqCompanion. a) TomahaqCompanion will display the trigger peptide (red line) and target peptide (blue line) extracted ion chromatograms to allow users to determine when the peptide elutes. Additionally, MS/MS scans are denoted with open circles indicating when a trigger peptide MS2 (red), target peptide MS2 (blue), or target peptide SPS-MS3 (teal) scan was acquired. Clicking on a circle will display the most closely associated target MS2 spectrum and the associated SPS-MS3 quantitation (blue bars). The target MS2 spectrum denotes matched fragment ions (blue) and unmatched fragment ions used for SPS-MS3 (red). This allows users to assess the potential sources of interference. b) Data for each target MS2 and SPS-MS3 scan are displayed in an interactive table. Clicking on a given row will display the data within the viewer. While data for all scans is saved as a ".csv" file upon analysis, scans can be individually selected and exported allowing users to only use the scans that are within the correct retention time range or do not demonstrate a great level of interference. c) Exported data can then be analyzed in external data analysis software, such as R.

## Conclusions and future directions

- TomahaqCompanion is an open-source tool central to the development and analysis of TOMAHAQ methods.
- TomahaqCompanion generates an inclusion list for priming runs, enables analysis of priming runs to validate the presence of labeled trigger peptides and ensure they are of equal intensity, creates TOMAHAQ instrument methods that can be used for data collection.
- Using a graphical user interface TOMAHAQ data can be analyzed, curated, and exported for further analysis.
- In the future, TomahaqCompanion outputs will be used with the instrument API such that TOMAHAQ experiments can be performed without the use of an instrument method.

## Acknowledgments

We would like to thank Derek Bailey from ThermoFisher Scientific and Robert Everley from Harvard Medical School for helpful discussions regarding the creation and features of the work presented here.

## References

Erickson BE<sup>1</sup>, Rose CM<sup>1</sup>, Braun CR<sup>1</sup>, Erickson AR<sup>1</sup>, Knott J<sup>1</sup>, McAlister GC<sup>1</sup>, Wuhr M<sup>1</sup>, Paulo JA<sup>1</sup>, Everley RA<sup>1</sup>, Gygi SP<sup>1</sup>. "A Strategy to Combine Sample Multiplexing with Targeted Proteomics Assays for High-Throughput Protein Signature Characterization." Molecular Cell, 2017, 65(2):361-370.