TECHNICAL MANUAL

6 × 5 LC-MS/MS Peptide Reference Mix

Instructions for Use of Products
V7491 and V7495

Revised 5/18
TM425

6 × 5 LC-MS/MS Peptide Reference Mix

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

Data generated by scientific instruments and decisions based on that data depend on optimal instrument performance (1,2). Clinical assays rely on mass spectrometric (MS) data for accurate results so that correct health-related results are gained and appropriate results-based decisions are made (3,4). However, there are no generally agreed upon tools nor performance standards (system suitability tests) for mass spectrometry. Furthermore, while several software tools exist that serve to assist with the analysis of instrument performance, a dedicated reagent-software package has yet to be created (5–7).

For optimal liquid chromatography (LC) performance, parameters like retention time, peak width and peak height are typically reported. Commonly monitored MS parameters include mass accuracy, mass resolution, signal-to-noise, sensitivity, limit of detection (LOD), limit of quantitation (LOQ) and dynamic range. There is a specific need for a reliable reagent to aid in method development and optimization, and for monitoring instrument performance.

Toward that end, we have developed the 6 × 5 LC-MS/MS Peptide Reference Mix[a]. The product is a mixture of 30 peptides: 6 sets of 5 isotopologues of the same peptide sequence. The isotopologues (Figure 1) differ only by the number of stable, heavy-labeled amino acids incorporated into the sequence (see Section 9.A., Table 5 for a list of the peptides). The labeled amino acids consist of uniform ^{13}C and ^{15}N atoms. Each of the isotopologues is indistinguishable chemically and chromatographically. However, since they differ in mass, they are clearly resolved by mass spectrometry.

In our formulation, the isotopologues of each peptide are present in a series of tenfold differences in concentration or molar abundance. If 1pmol of the mixture is loaded onto an LC column, the next lighter isotopologue would be 100fmol, the next 10fmol, the second lightest 1fmol, and the lightest 100amol. This range allows assessment of the instrument's dynamic range and sensitivity from a single run. The sensitivity measurement is contingent on loading enough peptide so that all of the isotopologues can be detected.

Several other design characteristics are worth noting. Peptides with a wide range of hydrophobicities were chosen to enable reporting of LC column performance. The most hydrophilic peptide gives users a tool to optimize the capture of hydrophilic peptides that might be difficult to capture otherwise, but that are too precious to use for method development. In addition, the peptides were chosen for maximal stability. Amino acids prone to artificial post-translational modification (i.e., methionine, asparagine, etc.) were excluded from the sequences. None of the peptides have internal lysines or arginines, and will therefore not be affected by trypsin or lys-C. Finally, there is a mass separation between the isotopologues of at least 4 Daltons so that none of the mass peaks will overlap.

The 6 × 5 LC-MS/MS Peptide Reference Mix is intended for use in method development and optimization, and for routine performance monitoring. Figure 2 provides a workflow for the use of the mixture. To assist in data processing, we provide a complementary software tool, the 6 × 5 LC-MS/MS Peptide Reference Mix Analysis Software (PReMiS™ Software). Data files from various vendors (Thermo or AB SCIEX) or .mzML files (which can be derived from most of the other major vendors) can be directly read into the software tool. The software is available at:
www.promega.com/resources/tools/6x5-premis-software-download/
PReMiS™ Software produces a tabular report of calculated instrument parameters, graphical analysis of linearity curves (Figure 3) as well as reporting the history of user-selected parameters such as LC retention time, peak height and mass accuracy. If the laboratory has a collection of instruments, there is also an option to compare parameters across instruments. Finally, there is an option to view the eXtracted Ion Chromatograms (XIC) of all 30 peptides. Table 1 lists some of the parameters reported by the software.

If you are using Thermo instruments, you may need to install the MS filereader software, available at:
www.promega.com/resources/tools/premis-software-data-formats/

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Table 1. LC-MS/MS Instrument Parameter and Feature Details.

Parameter/Feature	Category	Calculated by Software	Details
Retention Time	LC	Yes	Time required for peptide being separated to elute from the chromatography column.
Peak Width	LC	Yes	Peak width is an indication of column efficiency.
Base Peak Height	LC	Yes	This measure of the signal response can be correlated to the ionization efficiency of the peptide.
Graphical XIC Analysis	LC	Yes	A graphical XIC (eXtracted Ion Chromatogram) is displayed for all 30 ions for visual confirmation of detectability of all masses. This is especially important for analysis of the peptide mixture spiked into a complex background.
Lowest Detectable Quantity	MS	Yes	Lowest abundance detected is reported for each peptide sequence.
Linear Fit Analysis (Linear Dynamic Range)[1]	MS	Yes	The software does not directly calculate the full dynamic range of the instrument. However, up to 5 logs of dynamic range can be determined directly from the Linear Fit Analysis.
LOD and LOQ[2]	MS	No	The AAA- (amino acid analysis) qualified peptides allow the mix to confirm instrument LOD and LOQ, the basis for the amount loaded, which then can be used to confirm consistency of detection limits.
Slope and R^2 of Sensitivity Curve: Graphical Analysis	MS	Yes	A plot of the log of peptide amount in moles versus log of peak height is reported to provide information on LOD and LOQ.
Mass Accuracy	MS	Yes	—
Performance Over Time	Both	Yes	Parameters like LC retention time, peak height and mass accuracy are graphically displayed based on date and time.
Multi-Instrument Comparisons	Both	Yes	—

[1]A dynamic range of up to 5 logs is confirmed. For instruments with ranges above 5, multiple runs should be considered.
[2]The LOD and LOQ need to be determined by titration of the peptide mix. Once the limits are established, the reagent can be used to confirm linearity and detection of the lowest abundance isotopologue in a single injection.

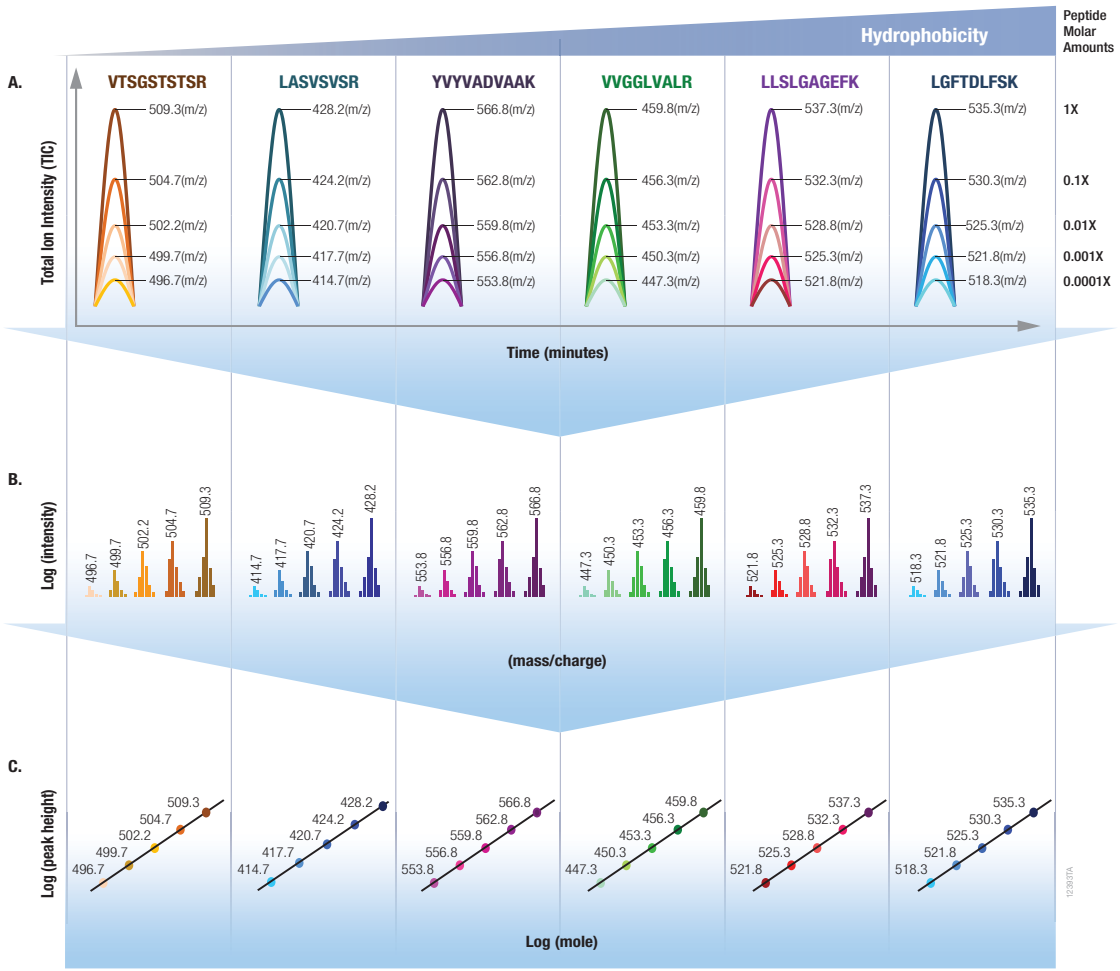


Figure 1. A mixture of 6 peptide sets is actually 30 peptides in total (6 × 5). Each of the peptide sets is a mixture of five isotopologues, distinguished only by mass through the incorporation of stable, heavy-labeled amino acids. **Panel A.** The isotopologues are chemically and chromatographically identical and therefore appear as 6 peaks. **Panel B.** Each of the isotopologues is mixed with a tenfold molar increase (lightest to heaviest). **Panel C.** A plot of the log of peak height versus the log (molar amount) on column has a slope = 1. This analysis enables confirmation of detection limits (LOD and LOQ), and can also be used to confirm linearity up to 5 logs of dynamic range.

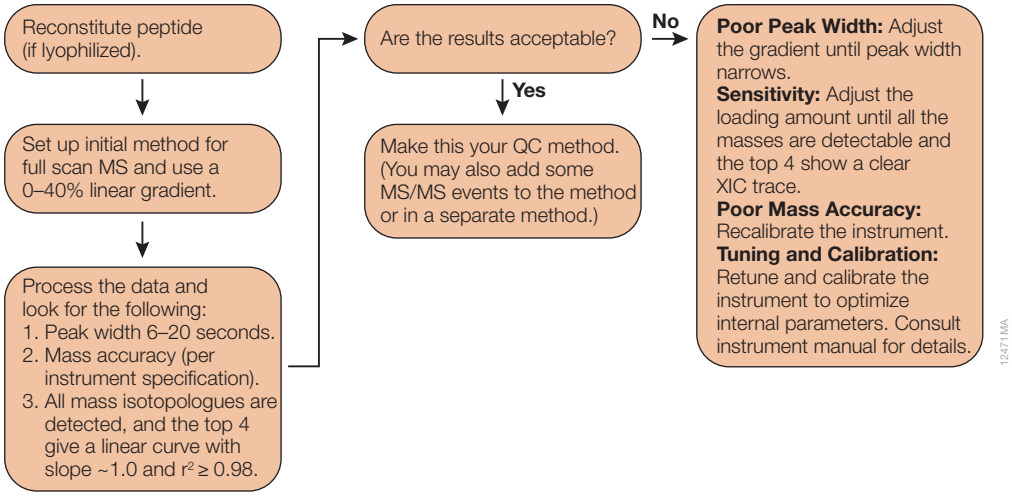


Figure 2. Suggested workflow for the 6 × 5 LC-MS/MS Peptide Reference Mix system.

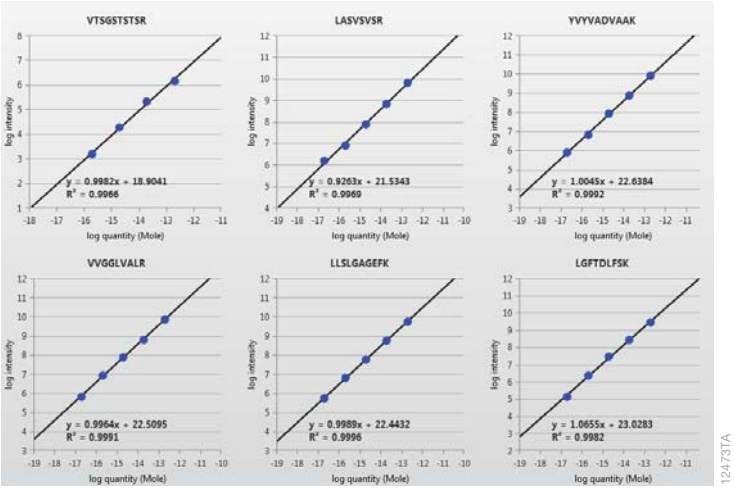


Figure 3. Linearity analysis of the peptide mixture is used to measure and confirm instrument sensitivity as well as dynamic range. Note: Not all isotopologues need to be detected in order for the software to perform correctly. In this example, the first peptide (due to its extremely hydrophilic nature) failed to capture and therefore to detect the lowest mass isotopologue.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
6 × 5 LC-MS/MS Peptide Reference Mix	**50µl***	**V7491**

For Research Use.
*Supplied as a liquid (25pmol of the heaviest peptide in 50µl). This system contains sufficient reagents for 50 injections at 500fmol/injection. Includes:

- 50µl 6 × 5 LC-MS/MS Peptide Reference Mix
- 6 aliquot tubes

PRODUCT	SIZE	CAT.#
6 × 5 LC-MS/MS Peptide Reference Mix	**200pmol****	**V7495**

For Research Use.
**Supplied lyophilized. This system contains sufficient reagents for 40 injections at 5pmol (of the heaviest peptide)/injection. Includes:

- 200pmol 6 × 5 LC-MS/MS Peptide Reference Mix
- 6 aliquot tubes

Storage Conditions: Store the 6 × 5 LC-MS/MS Peptide Reference Mix at –30°C to –10°C. Cat.# V7491 is supplied as a liquid. Divide it into aliquots after thawing, using the supplied tubes. Cat.# V7495 is supplied lyophilized. Upon reconstitution, store aliquots for up to 3 months at –20°C.

3. Before You Begin

3.A. Select the Loading Amount

The peptide mixture is intended for both nanoflow (75–150µm; I.D.) and microflow (1–4.6mm) columns (see Section 9.A., Table 4). For nanoflow injections, we recommend loading 0.5–1pmol (heaviest peptide), and for microflow loading, at least 1–5pmol (heaviest peptide). Please note that loading 500fmol of the heaviest peptide will give 50amol (attomole) of the lightest peptide. If you are concerned that your instrument will not detect 50amol, consider loading more peptide initially and titrate until you see a clear chromatographic peak corresponding to the lightest mass in the set. (Detection of all 5 isotopologues is not required.) The PReMiS™ Software includes a "View XIC" feature that allows for the visual inspection of the co-elution of all 5 isotopologues. This feature is a quick way to establish that mass chromatograms are produced for all of the peptides.

3.B. Buffer Selection

The first eluting peptide (VTSGSTSTSR) is extremely hydrophilic. If your goal is to capture this peptide, Buffer A should not contain more than 2% acetonitrile. If you are less concerned with capturing this first peptide (VTSGSTSTSR) and require a small amount of organic solvent in your initial mobile phase, then we recommend 2–5% acetonitrile/94.9–98.9%water/0.1% formic acid.

3.C. LC Gradient

We recommend running the mixture as a neat sample onto a capillary or microbore C_{18} reverse-phase chromatography column. (If you wish to use the mixture as an internal standard, see Section 3.G., Spiking into Complex Mixtures.) For best results, start with a 1-hour method of 0–40% Buffer B, with at least 5 minutes held, initially at 0% (particularly if you want to capture the most hydrophilic peptide). Note that, if you are using a guard column, it might be difficult to see hydrophilic peptides. If you are concerned about the capture of hydrophilic peptides, we recommend direct on-column loading. Capture of the hydrophilic peptide will depend on loading times, which may have to be optimized depending on the system. Figure 4 shows a representative chromatogram.

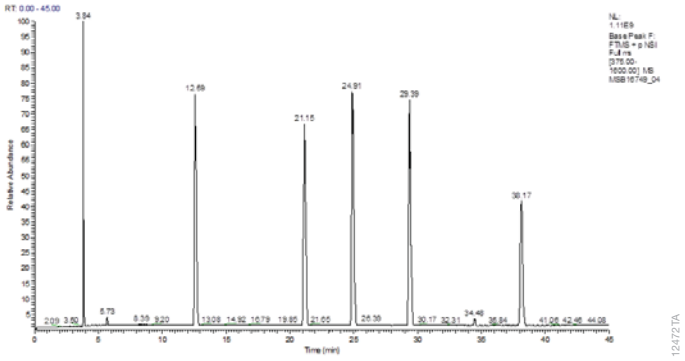


Figure 4. LC-MS analysis of the peptide mixture.

3.D. MS Method

For best results, you should create an MS method that enables maximal data sampling on the chromatographic timescale. For this reason, we recommend that, in addition to your standard Top N (N=3–15) method, you run a single-stage (i.e., full-scan) method alone. We have found this method to give the most sensitive analysis of chromatographic performance and measurement of sensitivity. The majority of the peptides will show up in the doubly charged form, so you can select an appropriate mass range around these masses. A 375–1,600 mass range, in full-scan mode, will encompass detection of all masses in the mixture. Keep in mind that your peptide mixture injection method should be a reflection of the actual method that you routinely use to collect data.

3.E. Preparing the Sample for Injection

For nanoflow columns we recommend injecting at least 500fmol of the heaviest isotopologue of each peptide on column in a volume of 5µl, so that you can detect all 5 of the isotopologues in each set. For the liquid format, this will require a 1:5 dilution (from 500fmol/µl to 100fmol/µl). For microflow columns, we recommend resuspending the lyophilized peptide mix in 200µl of 99.9% water/0.1% formic or trifluoroacetic acid (1pmol/µl final concentration of the heaviest isotopologue of each peptide) and injecting 5µl. If necessary, aliquot the stocks using the provided Axygen® tubes (Corning Cat.# SCT-150-SS-Y-S or similar; sterile tubes are not required). Store the peptides at –20°C or below after reconstitution in LC Buffer A.

3.F. Determining Sensitivity and Dynamic Range

We suggest analyzing a wide range of peptide loadings (100amol to 10pmol based on the heaviest isotopologue of each peptide per set) as neat samples. Analysis of the peak heights of the most abundant peptides can provide a true analysis of the limit of detection (LOD), the limit of quantitation (LOQ) and the true dynamic range of the instrument. The best mass spectrometers are capable of 3.5–5 orders of dynamic range. It is therefore imperative to find the loading amount that captures the instrument dynamic range and establishes the true sensitivity range and limits of the instrument. In conjunction with the software, mass spec instrument operators have a unique opportunity to confirm that their instrument is operating at optimal sensitivity limits on a consistent basis.

3.G. Spiking into Complex Mixtures

The peptide mixture can be spiked into complex mixtures. All of the peptides in the set contain one or more heavy-labeled amino acid, which overcomes the challenge of isobaric interference. We suggest spiking in enough peptide so that all of the isotopologues can be easily detected. For nanoflow experiments, spiking in 0.5–1pmol (with a background of 0.5µg complex mixture or less) initially, and then diluting the spike per your experimental requirements. The PReMiS™ Software algorithm has been optimized to allow for the correct peptide identification in complex mixtures. The **View XIC Anlaysis** feature in the PReMiS™ Software is provided to confirm optimal peptide loading and correct identification.

4. Protocol

1. Reconstitute the peptides (if necessary) as follows (additional information is available in Section 3.E.):
   a. The reagent in **liquid** form (25pmol in 50µl; Cat.# V7491) is supplied in 5% acetonitrile/0.1% trifluoroacetic acid (TFA)/94.9% water. This format is ready to inject. We suggest diluting 1:5 with 1% acetonitrile/0.1% TFA/98.9% water, to a final concentration of 100fmol/µl and injecting 5µl.
   b. For **lyophilized** reagent (200pmol; Cat.# V7495), reconstitute in 50–200µl of 2% acetonitrile/0.1% TFA/98.9% water (1–4pmol/µl).
2. Dilute with HPLC Buffer A to a final concentration of at least 100fmol/µl (nanoflow LC) or 1pmol/µl (microflow LC; flow rates above 10µl per minute).
3. Inject the samples using your standard procedure and based on a method developed using the suggestions above (Section 3.D).
4. When the results are available, process the data using the available (by download) 6 × 5 LC-MS/MS Peptide Reference Mix Analysis Software Tool (PReMiS™ Software).

5. The PReMiS™ Software Analysis Tool

5.A. Introduction

The PReMiS™ Software analyzes mass spectrometric data collected from the 6× 5 LC-MS/MS Peptide Reference Mix run either neat or spiked into complex backgrounds. It calculates values for parameters that report on LC-MS/MS instrument performance. Data collected from Thermo (.raw files) or AB SCIEX instruments (.wiff and associated files) can be analyzed directly. Other data formats must first be converted to .mzML formats prior to analysis.

5.B. Hardware Requirements

A Windows® PC is required to run PReMiS™ Software.

- Dual-Core x86 based processor, 4GB Memory, 100GB hard drive
- Video 1024 × 768 pixels
- Microsoft Windows® XP® SP3, Vista® 32/64, Windows® 7 32/64, or Windows® 8 32/64

5.C. Installation

The PReMiS™ Software package is available as a free download. It is available at:
www.promega.com/resources/tools/6x5-premis-software-download/
Double-click on the installer (filename) and follow the instructions on the screen.

5.D. Other Required Software

If you are using Thermo instruments, you may need to install the MS filereader software version 2.2 and above, available at:
www.promega.com/resources/tools/premis-software-data-formats/

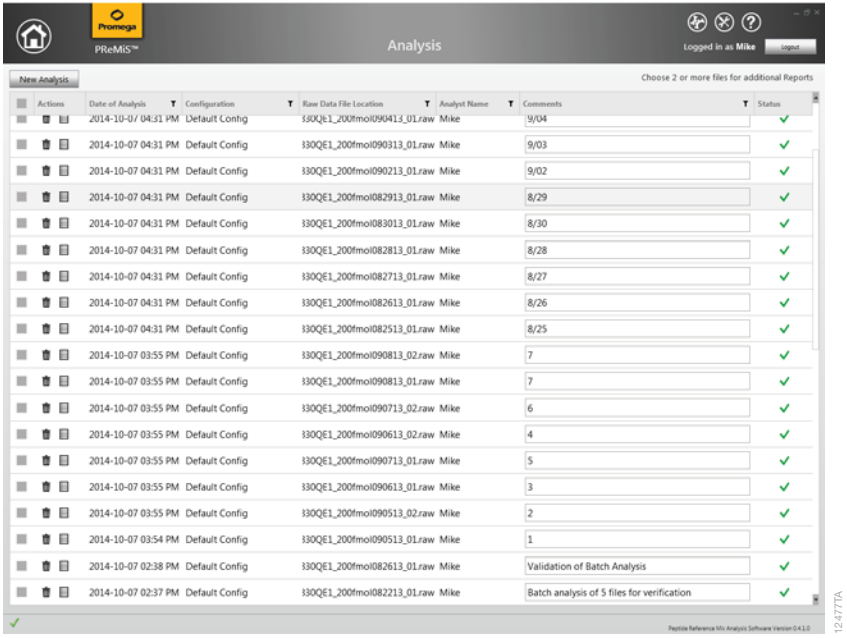
6. Instructions for Using PReMiS™ Software

6.A. Creation of a Login

After installation of the software, you can log in (although this is not required). By creating a login you can track who performs analyses with PReMiS™ Software.

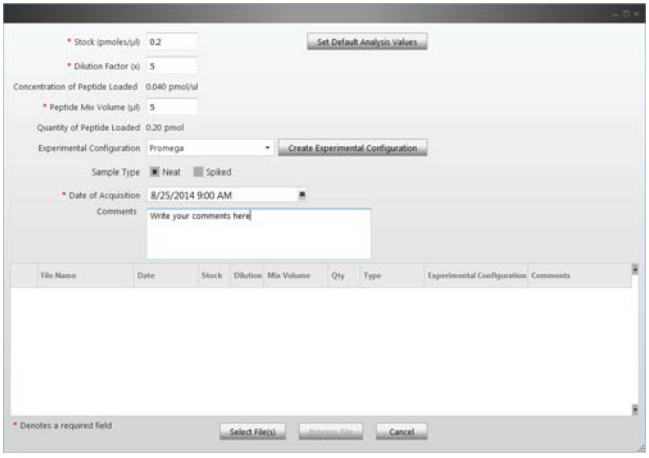
6.B. Analysis of Raw Data and Creation of Reports

1. To analyze a data file, click the gray **New Analysis** button as displayed here.



6.B. Analysis of Raw Data and Creation of Reports (continued)

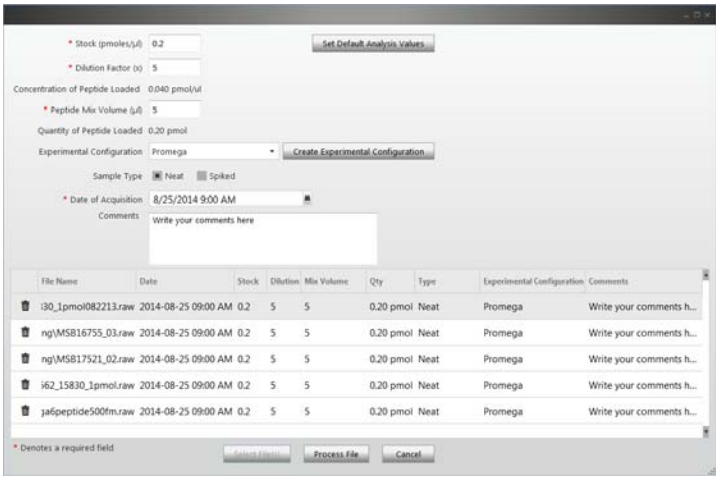
2. The following screen will appear.



3. Enter the concentration stock in pmol/µl (of the most abundant isotopologue), the Dilution Factor and the Peptide Mix Volume loaded on column. For example, the liquid format is supplied at a concentration of 0.5pmol per µl (of the heaviest isotopologue). It can be diluted fivefold (10µl diluted into 40µl of 0.1% formic acid in water) resulting in a loaded peptide concentration of 100fmol per µl. If 0.5pmol of the most abundant isotopologue of each peptide is loaded (on column), then the other isotopologues would be present at 50, 5, 0.5, and 0.05fmol. The quantity of peptide loaded on column will then automatically be calculated, based on input values.

4. Specify the **Sample Type**. If you are injecting the peptide mixture alone, click on the **Neat** button. If the sample has been spiked into a complex background, perhaps as a loading control or for continuous monitoring of instrument performance, then you will want to click the **Spiked** button. Please note that if the **Spiked** button is clicked incorrectly, it will not affect the analysis.

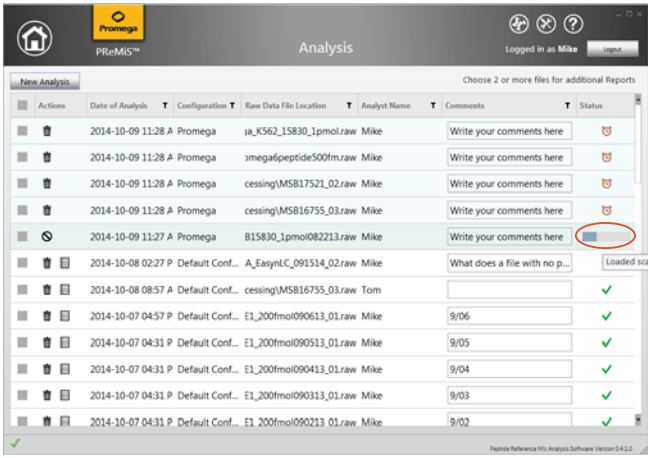
5. Enter the date and the time as shown in the figure below (Date of Acquisition). We suggest adding the date and time of analysis to the comments section so that this detail will show up in the main screen and will make recalling the date and time possible when processing files for the **Instrument History Analysis**. (The date and time entered here will be the x-axis value in the Instrument History Analysis.)



6. Select **File(s) feature.** The software can process either a single file, or multiple files (batch processing) in a single process. If multiple files are selected and **Open** is selected, you will need to process those files before additional files are processed. Once a single process is started, another **New Analysis** can be carried out. Note that if multiple files are to be processed at one time, care must be taken to edit the appropriate values so as to generate accurate calculations and ensure that the correct date and time of acquisition are properly documented. These values cannot be changed once the files are processed. If an erroneous value is entered, the result file will have to be deleted and the raw data file processed again. Comments, however, can be added after the files are processed.

6.B. Analysis of Raw Data and Creation of Reports (continued)

7. Select **Load and Process Mass Spec Data** file.

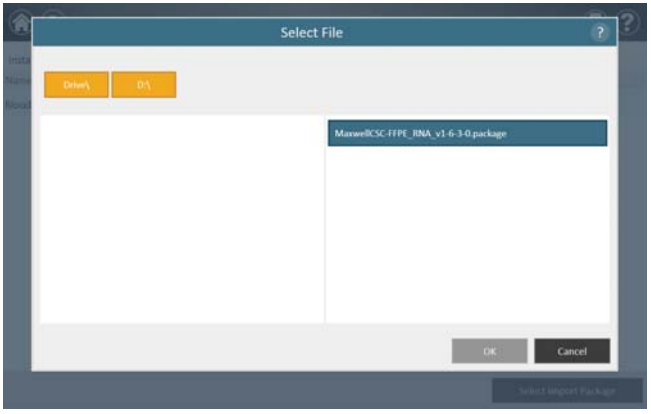


8. The software will begin to process the Mass Spec raw data file, as shown below.

9. As the MS scan files are loaded, you will see movement of the progress bar (circled in red in the image above). If you hold the mouse over the progress bar, you can determine how many scans of the raw data (out of the total number of scans) are read into the software.

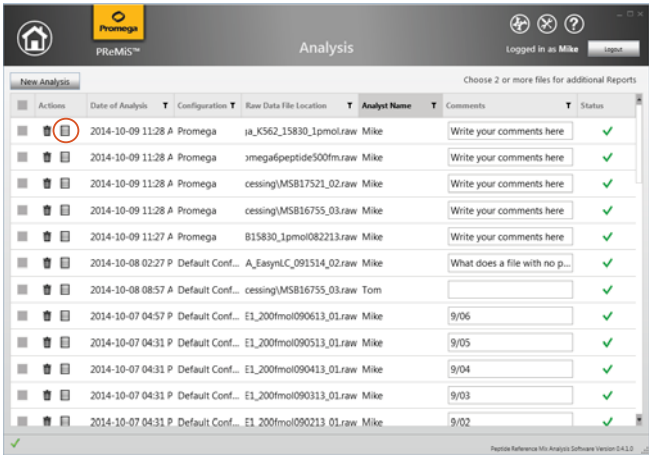
6.B. Analysis of Raw Data and Creation of Reports (continued)

10. When the analysis is complete a **green check mark** will be visible next to the file name on the analysis screen.

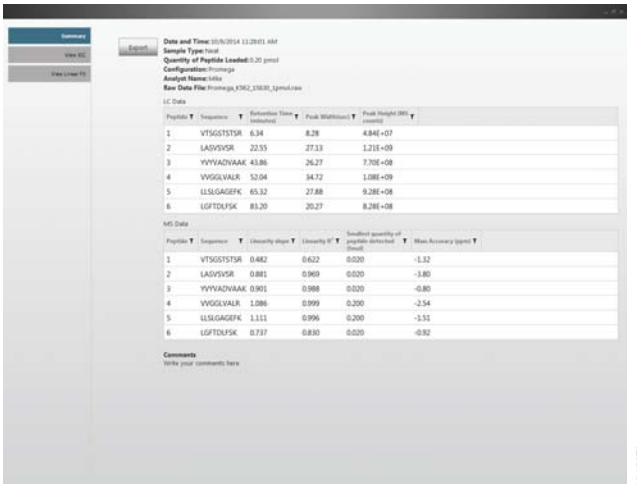


11. Select the data file when it finishes processing by clicking any of the fields in the row. The default file location can be set on the Administration page (see Section 7.H). The amount of processing time will depend on the size of the raw file.

12. To view results, click on the report icon, circled in red in the figure below.



13. The following screen will appear.



14. This is a summary report of the calculated instrument parameters determined from the data from the LC-MS/MS analysis. The data can be exported as a .csv file using the gray export button.

15. The following parameters are reported:

    a. **Retention Time:** The software will determine the retention time for each of the peptides (in both a neat and complex mixture). The retention time is a measure of the column's ability to separate a given peptide. Retention times may vary due to various factors including composition and polarity of mobile phases, LC gradient conditions, and stationary phases. It is therefore very important to optimize conditions for the separation of the peptide mixture, before establishing the QC method. Drifts in retention time (over the course of days, weeks or months) may indicate that cleaning or column replacement may be required.

    b. **Peak Width:** The peak width (in seconds at 50% half-maximum) can be a metric of the column efficiency. The more narrow the peak width, the better the column separation. An increase in peak width (from one run to the next) could mean that the column is in need of regeneration or possibly replacement. In addition, if the column shows an abrupt change in peak width, there may be some type of malfunction in the HPLC hardware.

    c. **Peak Height:** The peak height is directly related to the Total Ion Current (TIC) from the Mass spectrometer. Changes in the peak height over the course of time can be an indication of the instrument's decrease in sensitivity. These types of changes may indicate the need for maintenance, calibration or possibly service of the instrument.

6.B. Analysis of Raw Data and Creation of Reports (continued)

d. **Slope of Linear Analysis:** A plot of the $\log_{10}$(molar amount) of peptide on the LC column versus $\log_{10}$(Peak Height) should be linear (depending on the linear dynamic range (LDR)) with a slope = 1.0. For example, if your instrument has an LDR of up to 5, then the 5 points that appear on the plot should have an R^2 value of >0.95 (and ideally 0.99). If you find that all 5 points (especially the lowest mass) do not give this value, export the data (.csv format) and use a data-graphing program (i.e., Microsoft Excel®) and analyze the 4 "heaviest" masses to ensure an R^2 value of 0.99. Repeat this process until the fit is 0.99 or better. From experience we have found that points that deviate from linearity tend to be outside of the Limit of Quantitation for that particular peptide on the instrument in question. If you find that you are unable to obtain a result of 0.99 or better, then you may be loading an amount that is outside of the linear range of the instrument (either too much or too little; refer to Section 6.F for suggestions on establishing the linearity of the instrument) or your instrument is in need of calibration or maintenance.

e. **R^2 Value of Linear Analysis:** As described in the previous section, a value of 0.95 or better should be considered acceptable with 0.99 being ideal.

f. **Lowest Detected Quantity:** We suggest that you load at least 500fmol for LC systems with flow rates less than 10µl per minute, and 5pmol for systems with flow rates above 10µl per minute. By loading 500fmol of peptide on column (heaviest peptide per set), and detecting all 5 of the masses with linearities above 0.95, the lowest detectable quantity will be 0.05fmol or 50amol. This metric is particularly useful for tracking the ability of the instrument to maintain a consistent level of sensitivity.

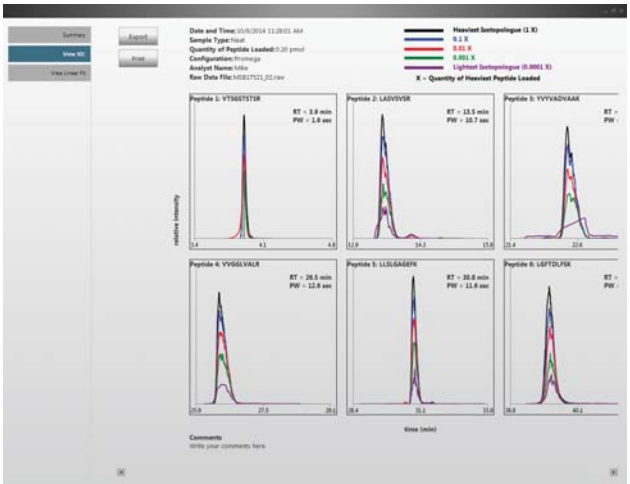
g. **Mass Accuracy:** The mass accuracy of the instrument will depend on various factors, particularly instrument types. High-resolution instruments like Q-TOF or FTICR (i.e., Orbitrap/Q-Exactive) tend to have mass accuracies ranging from several hundred parts per billion (ppb) to no more than 15ppm (parts per million). The typical specification for this type of instrument is 3–6ppm. The software will give a report on the instrument mass accuracy, then provide results that may indicate the need for recalibration. We recommend that your initial QC method refinement and implementation be done immediately after calibration of your instrument.

Note: PReMiS™ Software is not compatible with low mass accuracy instruments such as ion traps and triple quadrupole mass spectrometers.

6.C. View XIC

To view the eXtracted Ion Chromatograms (XIC) of all of the 30 peptides (mass specific), click on the **View XIC** button, which first appears below the **Summary** button.

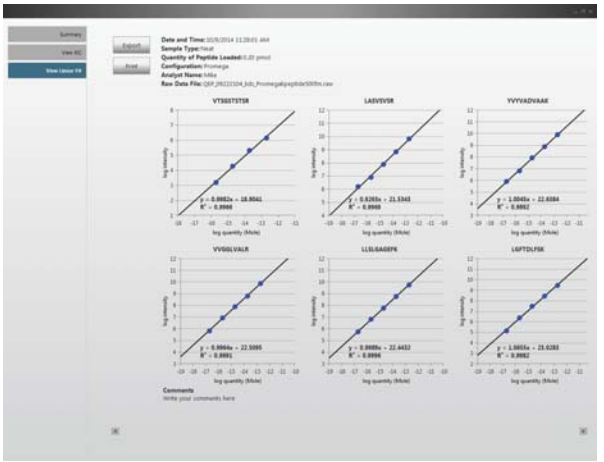
1. The following graphical feature will appear.



2. This feature is a convenient way to check that all of the peptide's isotopologues are co-eluting as expected and that their detection is valid (and not an artifact). Detection of all 5 isotopologues is a good indication that your MS instrument is able to detect and resolve all 5 peptides and that they are being identified correctly. While this may not seem like a major challenge for peptides run as neat samples, the problem is compounded when these peptides are spiked into complex mixures. The XIC analysis is a very quick indication that the correct peptides have been identified. (The linear analysis plot will confirm the LDR.) The y-axis values are scaled to allow for visualization of all five isotopologues simultaneously. Absolute intensities can be obtained by exporting data from the Linear Fit analysis section (Section 6.D.).

6.D. Linear Fit

Click on the **View Linear Fit** button to view the linear analysis that pertains to all 6 peptides in the set. A graph of log signal intensity versus log isotopologue quantity is displayed for all 6 peptides. In the figure below, 4 masses of the peptide VTSGSTSTSR are detected. The other 5 peptide sets show detection of all 5 isotopologues (masses). The lowest peptide amount is 50amol (0.05fmol or 5×10^{-17} moles), based on an initial load of 0.5pmol. The slope in these types of analyses should be very close to 1.0 and the R^2 should be greater than 0.95.



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1. The sensitivity and linearity of the instrument should be established during method development using the 6 × 5 LC-MS/MS Peptide Reference Mix.
2. Once this is confirmed, this analysis can be used to confirm the sensitivity and dynamic range of the instrument on a consistent basis.
3. The linear analysis data can be exported by clicking on the **Export** button (.csv format) and further analyzed. The intensity data is exported in log format. If you wish to plot the data manually, you can plot the data in columns (x axis: Log Quantity; y axis: Log Intensity). If you wish to generate the actual Peak Intensity, you can make this conversion in Microsoft Excel® or on an equivalent software tool. To do this, enter the following formula into the cell:

   = 10^(cell location (i.e., G19)) based on the Log Intensity

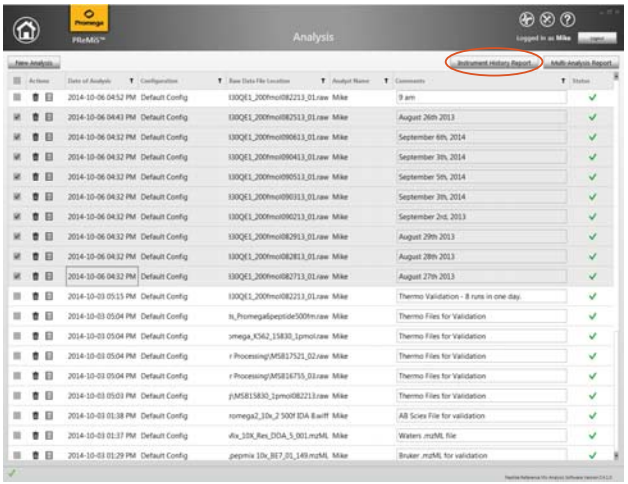
   This will give you the peak height. If you wish to determine the molar amounts, use this equation for the Log Quantity.
4. Because the lowest detected quantity loaded (i.e., lowest isotopologue) will be at attomole levels (for loads in the femtomole or picomole amount of heaviest isotopologue), the MS detector will be challenged to detect these specific peptides. It is therefore expected that the fluctuation in the MS signal for these peptide species will show greater fluctuation in signal (than their heavier counterparts). It is therefore important to evaluate the precision of this signal by running a series of replicates as you establish the initial QC method. In doing so, you will also have data for all of the other isotopologues in the set.

6.E. Instrument History Report

PReMiS™ Software has a convenient feature that facilitates the tracking of the following parameters: Retention Time, Peak Height, Peak Width, Mass Accuracy, Linear Fit, lowest quantity detected and slope of the linear fit.

1. To use this feature, select 2 or more files. To select a continuous series of files, click on the uppermost file in the series. It should appear gray. Then holding the shift bar down, select the lowest file in the series. Following this, all of the files should appear to be selected (the check boxes should be checked).

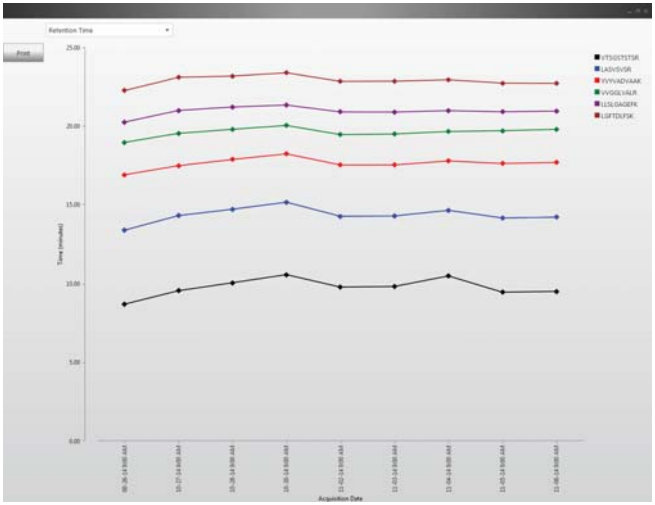
2. If you want to select a non-continuous set of files, simply click on the check mark on the left side of the page. Select the files that you wish to analyze.



3. Click **Instrument History Report** circled in red above.

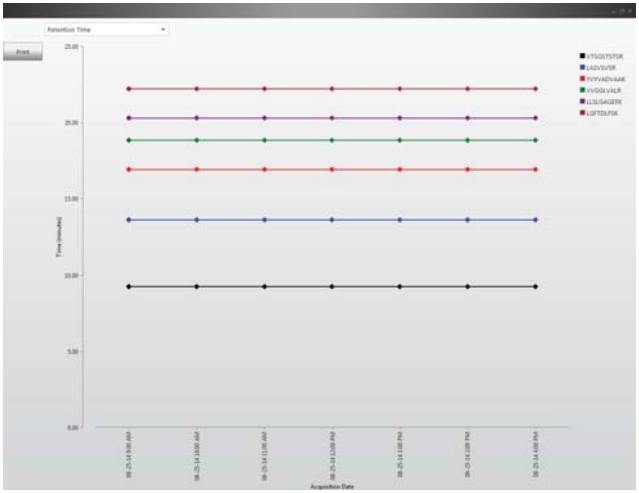
6.E. Instrument History Report (continued)

4. The following screen will be produced, with the default value of retention time. If you want to look at one sample per day, the following type of graph will be displayed.



12512TA

5. If you want to monitor a series of datafiles for samples that were recorded every hour, the following type of figure will be displayed. **Note:** It is critical to add the correct date and time before processing the sample as these values will be the x-axis values for the Instrument History Analysis.



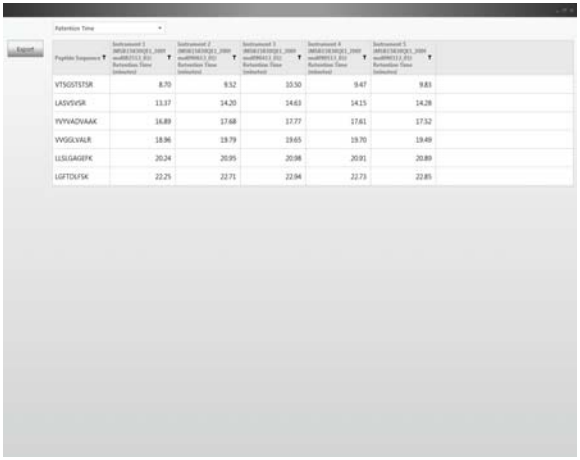
12513TA

6. To plot other parameters select the drop-down arrow located on the top left of the screen.

6.F. Multi-Analysis Report

Different instruments and analyses can be compared in the Multi-instrument Report.

1. To use this feature, select the files to be compared and click the **Multi-Analysis Report** button.
2. The following type of analysis table will be displayed.



3. The data may be exported with the **Export** button.
4. You can change the parameters to be displayed by clicking on the drop-down menu located on the top left (currently Retention Time is displayed).

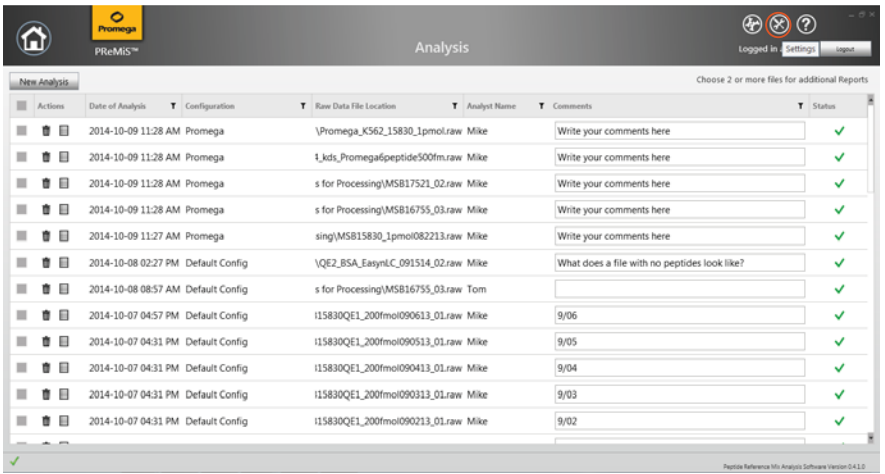
7. Instrument Configuration Settings (Optional)

7.A. Creating an Instrument Configuration

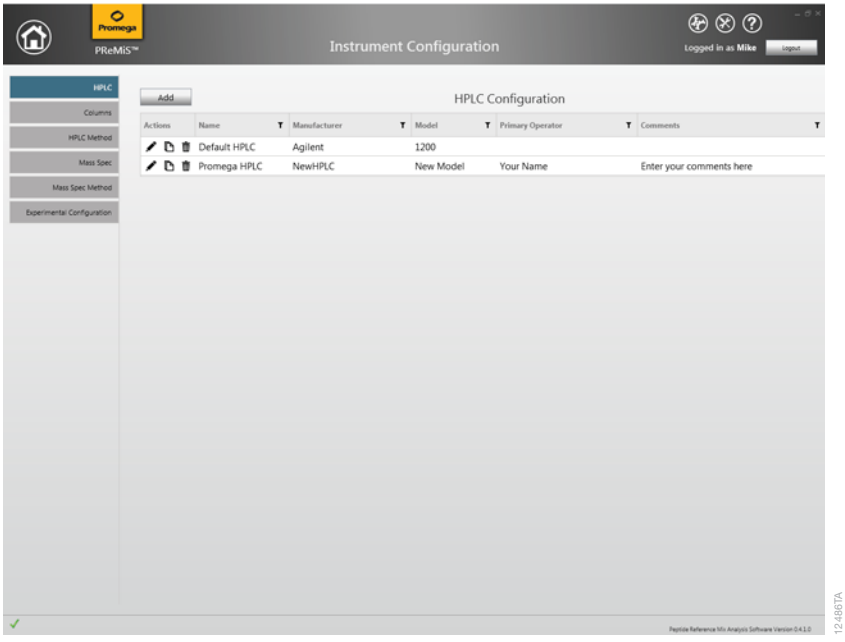
The instrument configuration allows you to record information about the conditions used to collect data with the 6 × 5 LC-MS/MS Peptide Reference Mix. **The instrument configuration is for reference only and does not affect the analysis of data**. This feature provides a quick and easy way to refer back to the method details in order to preserve method consistency.

7.A. Creating an Instrument Configuration (continued)

To add a new configuration, click on the tools icon (red circle) at the top of the analysis page, upper right of the screen below, above the logout button.



The following screen will appear. Click on the blue HPLC button on the top left.



7.B. HPLC

By clicking on the blue HPLC icons on the left side of the screen (previous figure), you will see a default HPLC setup.

1. Click on the **Add** button and the following screen will appear (in this case the screen has been filled with sample information).

2. To add additional **Manufacturers** and **Models**, click on the **Edit List** and add the name of your Manufacturer. Repeat the process for the Model. For example:

3. Click **Add** to add a new Manufacturer (and go to the Model field in the previous screen to enter the Model or Brand).

7.C. Columns

After setting up the HPLC portion, select the **Columns** tab. The tab should appear blue.

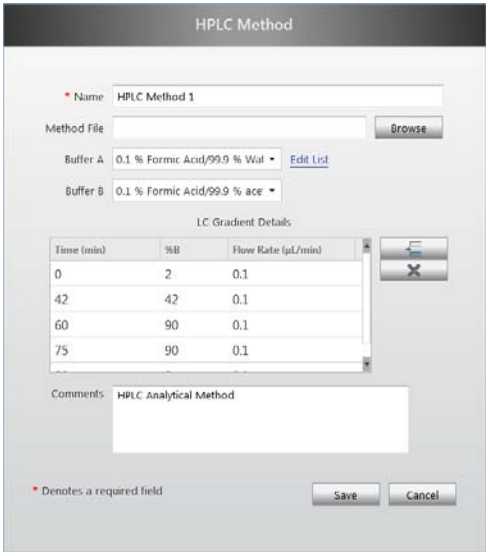
1. Select the **Add** button. The following screen will appear:

2. The example above lists a typical setup for an analytical column. If your system contains a guard column, repeat this process but select the guard button, and fill in the appropriate information for the guard column being used. Be sure to select **Save** to preserve your settings.

7.D. HPLC Method

This step will allow users to capture the HPLC method used for instrument performance monitoring.

1. To add an HPLC method, click the **HPLC Method Tab** and then click **Add**. The following screen will appear.



2. Fill the fields based on your specific method. To add buffers specific to your setup, simply click the Edit list feature and enter the name of your new buffer.

3. If, in the future, you need to change the HPLC Method, click on the pencil icon (circled in red below) to make any of the necessary edits to the method.

7.E. Mass Spectrometer

As in the previous steps, after clicking the add button, fill in the fields as they pertain to your specific MS instrument. If your instrument and/or manufacturer are not in the drop-down list, you can add them to the list by clicking on the edit list feature. To edit any details of this section, select the **pencil** icon to make any necessary changes. Once this section is filled out, it should not be changed.

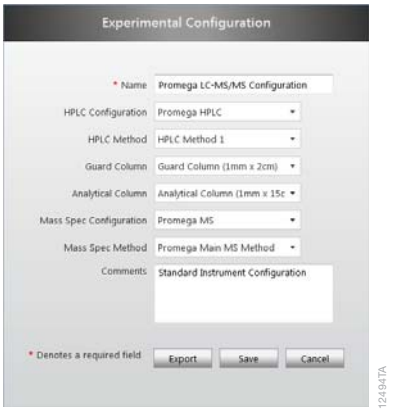
7.F. Mass Spectrometric Method

Fill out this section as per the previous sections. Because this is a very generic overview, any additional instrument-specific details should be captured in the Comments section, as shown in the figure below.

7.G. Experimental Configuration

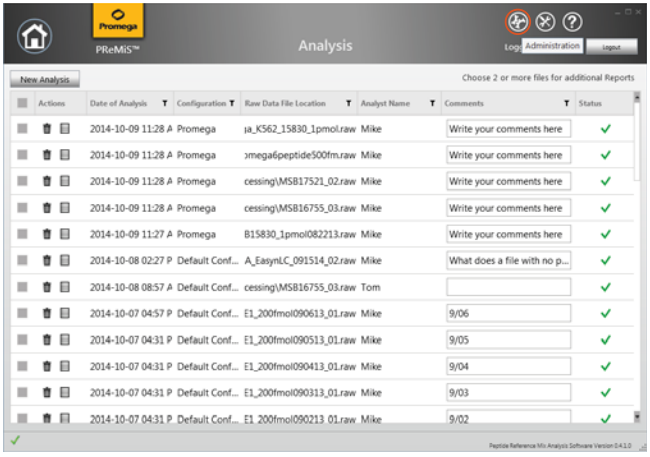
The experimental configuration for a given set of HPLC and MS methods summarizes all of the details that were documented in the previous sections.

1. Edits can be made by clicking the pencil icon on the main **Experimental Configuration** screen.
2. To scroll between different sections (HPLC, HPLC method, columns, etc.), click the gray button. The gray button will change to blue.



7.H. Administration

1. To set default file locations, click on the gears icon (circled in red below).



2. Specify the location of the raw data files (**Default Mass Spec Data File** location) and also the saved files (**Default Save File** location). This is where the analysis file results will be found once the analysis is complete.

7.H. Administration (continued)

3. Once complete, proceed to setting up your instrument configuration, or to analyzing data.

8. Converting to .mzML Format

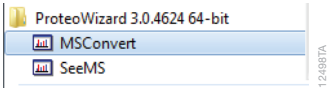
The PReMiS™ Software supports .raw (Thermo), .wiff (AB SciEx) and .mzML (a HUPO Proteomics Standards Initiative developed generic format) file formats. If your data comes from a Thermo or AB Sciex instrument, the software will read it in its native format, but for other instruments it will need to be converted to .mzML. Although Thermo and SCIEX data could be converted to .mzML data, such conversion is not recommended as analysis will be slower and the conversion process can alter the data slightly.

A free software tool to prepare .mzML data files is MSConvert, an open source software tool that is part of the ProteoWizard proteomics data analysis package: http://proteowizard.sourceforge.net/downloads.shtml **or** http://proteowizard.sourceforge.net/ downloads.shtml (also found at: **www.promega.com/resources/tools/premis-software-data-formats/**)

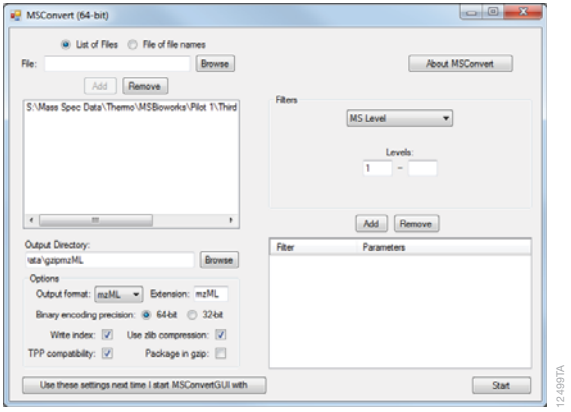
If you need assistance with installation of ProteoWizard, please contact Promega Technical Support.

To convert your files to .mzML data, run MSConvert as follows:

1. Start MSConvert from the Start menu: Start>All Programs>ProteoWizard>MSConvert



2. The following screen will appear:



8. Converting to .mzML Format (continued)

3. Set the options as follows:
   a. Output format: mzML
   b. Extension: mzML
   c. Binary Precision: 64-bit
   d. Write index: checked
   e. TPP compatibility: checked
   f. Use zlib compression: checked
   g. Package in gzip: unchecked
4. Set your Output directory to a convenient location, such as the default data file directory set in your PReMiS™ Software.
5. Browse to and Add the file(s) you wish to convert and click **Start**.
6. The amount of time it takes for conversion to finish depends on the file size.

9. Appendix

9.A. Informational Tables

Table 2. Suggested MS Method Settings: Thermo Orbitrap/Exactive™ Family.

Parameter	Suggested Value
Mass Range	375–1,600
Resolution	12,500–120,000
AGC Target	1–3 × 10^6 (Full MS)
Microscans	1
Ion Transfer Time	10–120ms
Scan Mode	Profile
Scan Mode	Full Scan
Number of MS/MS Experiments	0**; 5

**We recommend that you run at least one of your experimental replicates in full scan mode only to maximize the number of points across the chromatographic peak.

Table 3. Representative SIM Scan Method.

Mass (m/z)	Polarity	Start (minutes)*	End (minutes)*
409.74073	Positive	7	16.
414.74456	Positive	7	16
417.75146	Positive	7	16
420.75836	Positive	7	16
424.26696	Positive	7	16
428.27406	Positive	7	16
442.29038	Positive	22	27
447.29421	Positive	22	27
450.30111	Positive	22	27
453.30801	Positive	22	27
456.31496	Positive	22	27
459.82351	Positive	22	27
491.74418	Positive	0	7
496.74801	Positive	0	7
499.75496	Positive	0	7
502.26016	Positive	0	7
504.76541	Positive	0	7
509.27416	Positive	0	7
514.27713	Positive	33	57
517.79823	Positive	27	33
518.28396	Positive	33	57
521.79251	Positive	33	57
521.80506	Positive	27	33
525.30111	Positive	33	57
525.31361	Positive	27	33
528.82221	Positive	27	33
530.31471	Positive	33	57
532.33081	Positive	27	33
535.32836	Positive	33	57
537.34441	Positive	27	33
549.79568	Positive	16	22
553.80251	Positive	16	22

Mass (m/z)	Polarity	Start (minutes)*	End (minutes)*
556.80941	Positive	16	22
559.81631	Positive	16	22
562.82321	Positive	16	22
566.83031	Positive	16	22

*Note that start and end times will depend on the chromatographic method.

Table 4. Suggested Loading Amounts.

Loading Amount	Column Diameter
500fmol	75µm
5pmol	1mm
20pmol	2.1mm
100pmol	4.6mm

Table 5. Masses of Various Peptides. Underlined letters indicate peptides labeled with stable, heavy isotopes.

Number	Set	Peptide	Mass	$[M + 2H]^{2+}$	Relative Loading Abundance
1	1	VTSGSTSTSR	1016.533	509.2739	1.0000
2	1	VTSGSTSTSR	1007.515	504.7651	0.1000
3	1	VTSGSTSTSR	1002.505	502.2599	0.0100
4	1	VTSGSTSTSR	997.494	499.7547	0.0010
5	1	VTSGSTSTSR	991.480	496.7478	0.0001
6	2	LASVSVSR	854.532	428.2738	1.0000
7	2	LASVSVSR	846.518	424.2667	0.1000
8	2	LASVSVSR	839.501	420.7581	0.0100
9	2	LASVSVSR	833.487	417.7512	0.0010
10	2	LASVSVSR	827.473	414.7443	0.0001
11	3	YVYVADVAAK	1131.645	566.8300	1.0000
12	3	YVYVADVAAK	1123.631	562.8229	0.1000
13	3	YVYVADVAAK	1117.617	559.8160	0.0100
14	3	YVYVADVAAK	1111.603	556.8091	0.0010
15	3	YVYVADVAAK	1105.603	553.8022	0.0001
16	4	VVGGLVALR	917.631	459.8232	1.0000
17	4	VVGGLVALR	910.614	456.3147	0.1000
18	4	VVGGLVALR	904.600	453.3078	0.0100
19	4	VVGGLVALR	898.587	450.3009	0.0010
20	4	VVGGLVALR	892.573	447.2940	0.0001
21	5	LLSLGAGEFK	1072.673	537.3441	1.0000
22	5	LLSLGAGEFK	1062.646	532.3305	0.1000
23	5	LLSLGAGEFK	1055.629	528.8219	0.0100
24	5	LLSLGAGEFK	1048.612	525.3134	0.0010
25	5	LLSLGAGEFK	1041.594	521.8048	0.0001
26	6	LGFTDLFSK	1068.641	535.3281	1.0000
27	6	LGFTDLFSK	1058.614	530.3145	0.1000
28	6	LGFTDLFSK	1048.587	525.3008	0.0100
29	6	LGFTDLFSK	1041.569	521.7923	0.0010
30	6	LGFTDLFSK	1034.552	518.2837	0.0001

9.B. General Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and comments
No peptide signal is detected.	Check for leaks in the plumbing or air bubbles in the injector.
	Increase the amount of peptide loaded by severalfold and carry out analysis until peaks are detected (see Section 3.F, Determining Sensitivity and Dynamic Range). If adding five- to tenfold more peptide fails to result in peptide detection, instrument may need service.
	If peptides cannot be detected in a complex mixture, run them alone (i.e., neat).
Set 1 peptides (VTS) not detected.	This is probably due to the presence of organic material in the mobile phase. If you are using a guard column, it may not be possible to detect this peptide. If you need to detect this peptide, consider using a direct on-column injection.
Retention times are shifting.	Your column may need cleaning or changing.
Peaks are too broad.	Sharpen the LC gradient or optimize plumbing connections. This is especially important for the NanoLC.
Peaks are tailing.	Check your system for leaks or optimize the packing material. May also want to sharpen the LC gradient (see above, Peaks are too broad).
Peaks heights are decreasing over time.	There is a bubble in the injector syringe or LC plumbing.
	Your instrument is losing sensitivity and needs maintenance (cleaning) or recalibration.
Masses don't match theorhetical results or are off by more than 25ppm.	Recalibrate.
Software does not detect peptides.	Check that the instrument is calibrated properly. If the mass is off by more than 25ppm, the software will not detect peptides. A value of zero will be reported if the software cannot find the peptides.
Less than 5 peptides are detected or lowest mass XIC is not well-resolved or chromatographic in appearance.	This is not an issue and may be seen with peptides in complex samples. If you want to see all five isotopologues increase the amount of peptide loaded.

9.C. PReMiS™ Software Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and comments
Peptide peaks = 0.000	Peptide mass is not detected. Check to ensure that sample was stored properly and the correct vial was selected. Make sure there are no leaks in your LC injection system.
	Load a larger quantity of peptide onto the analytical column.
Unable to process *.raw files	Analysis of *.raw files requires MS File Reader version 2.2 or above to be installed.
*.raw file error message: A critical error occurred during execution	Install MS File Reader and retry analysis.
Error message: "The analyzer could not be started because the analyzer was incompatible with this system. The vendor driver may be incompatible or incorrectly detected."	If this issue persists please contact Technical Services at: 1-800-356-9526 or **techserv@promega.com**
For *.raw file analysis, Analysis Status = 	Analysis of *.raw files requires MS File Reader to be installed.
Tool tip: "The analysis failed to initialize." Please contact Promega Technical Services at: 1-800-356-9526 or **techserv@promega.com**	Install MS File Reader and retry analysis.
	Analysis may have been interrupted by user logout, user switch or shutdown.
	Delete the failed analysis and attempt new analysis.
	If issue persists, please contact Promega Technical Services: 1-800-356-9526 or: **techserv@promega.com**
For any file type Analysis Status = 	Analysis may have been interrupted by user logout, user switch or shutdown.
Tool tip = "Data could not be analyzed, please attempt new analysis."	Delete the failed analysis and attempt new analysis.
	Data file may be corrupted. Run sample on LC/MS generating a new data file. If this issue persists please contact Technical Services at: 1-800-356-9526 or: **techserv@promega.com**
	If the file being processed is stored in a network location, copy the file to your local workstation and reprocess.

9.C. PReMiS™ Software Troubleshooting (continued)

Symptoms	Causes and comments
Closing software with analysis running message: "There is currently an analysis processing in the background. This analysis will continue to completion in the background unless the current windows user logs out, switches users or shuts down."	Exiting software and user log out, switching user or shut down will result in Analysis Status =  Delete the failed analysis and attempt new analysis.

9.D. References

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2. Percy, A.J. *et al.* (2013) Standardized protocols for quality control of MRM-based plasma proteomic workflows. *J. Prot. Res.* **12**, 222–33.
3. Hoofnagle, A.N. *et al.* (2008) Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. *Clin. Chem.* **54**, 1796–1804.
4. Boja, E. *et al.* (2011) Evolution of clinical proteomics and its role in medicine. *J. Prot. Res.* **10**, 66–84.
5. Pichler, P. *et al.* (2012). SIMPATIQCO: A server-based software suite which facilitates monitoring the time course of LC-MS performance metrics on Orbitrap instruments. *J. Prot. Res.* **11**, 5540–7.
6. Ma, Z.-Q. *et al.* (2012) QuaMeter: Multivendor performance metrics for LC-MS/MS proteomics instrumentation. *Anal. Chem.* **84**, 5845–50.
7. Bereman, M.S. *et al.* (2014) Implementation of statistical process control for proteomic experiments via LC MS/MS. *J. Am. Soc. Mass. Spectrom.* **25**, 581–7.

9.E. Related Products

Mass Spec-Compatible Yeast and Human Protein Extracts

Product	Size	Cat.#
MS Compatible Yeast Protein Extract, Digest	100µg	V7461
MS Compatible Human Protein Extract, Digest	100µg	V6951
MS Compatible Yeast Protein Extract, Intact	1mg	V7341
MS Compatible Human Protein Extract, Intact	1mg	V6941

Enzymes, Proteases and Surfactants

Product	Size	Cat.#
IdeS Protease	5,000 units	V7511
Trypsin Gold, Mass Spectrometry Grade	100µg	V5280
rLys-C, Mass Spec Grade	15µg	V1671
Asp-N, Sequencing Grade	2µg	V1621
Arg-C, Sequencing Grade	10µg	V1881
Glu-C, Sequencing Grade	50µg (5 × 10µg)	V1651
Chymotrypsin, Sequencing Grade	25µg	V1061
	100µg	V1062
Endoproteinase Lys-C, Sequencing Grade	5µg	V1071
Elastase	5mg	V1891
Thermolysin	25mg	V4001
PNGase F	500 units	V4831

9.F. Summary of Changes

The following changes were made to the 5/2018 revision of TM425:

Table 5 was updated to add underlines to the letters that represent peptides labeled with heavy isotopes.

[a] Patent Pending.

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Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.