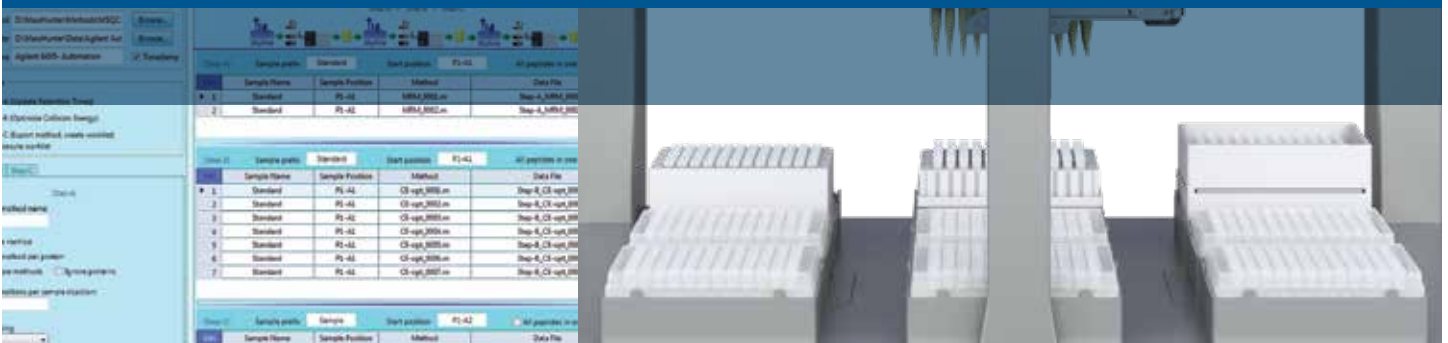
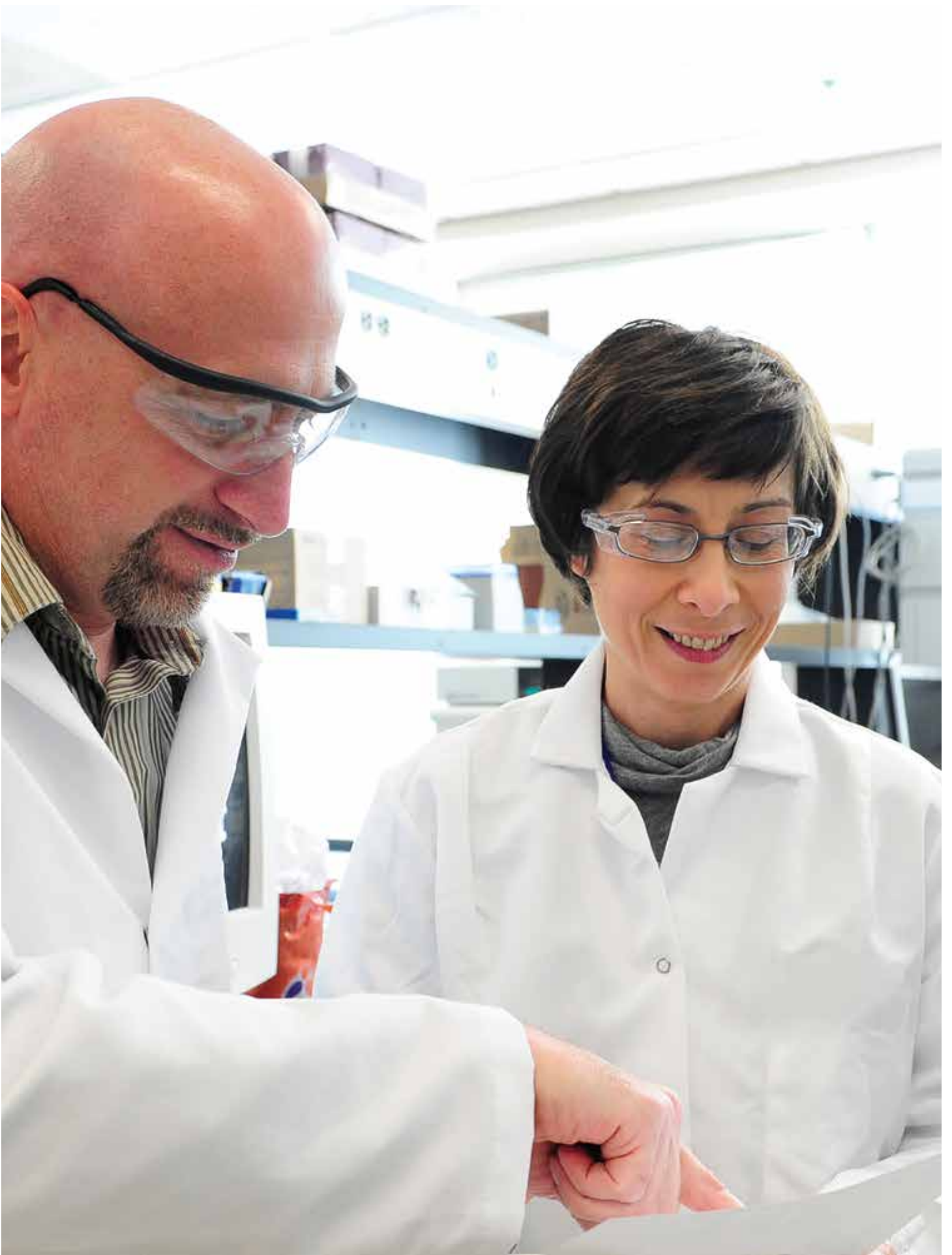


Guide to Peptide Quantitation

Agilent clinical research





Peptide Quantitation for the Clinical Research Laboratory

Peptide quantitation is rapidly growing in clinical research as scientists are translating peptide biomarkers identified in basic research to clinical research and utility. This quantitation approach measures unique peptides from the proteins that are expected to be present in clinical research samples based on prior knowledge. That is, from literature, genomic and proteomic studies, or combined proteogenomic analysis.

As you develop peptide quantitation methods for your clinical research, we can offer expert advice at every step: sample preparation, liquid chromatography (columns, consumables, and analytical methodologies for separations), peptide mass spectrometry using sensitive triple quadrupole instrumentation, and data analysis using reliable software.

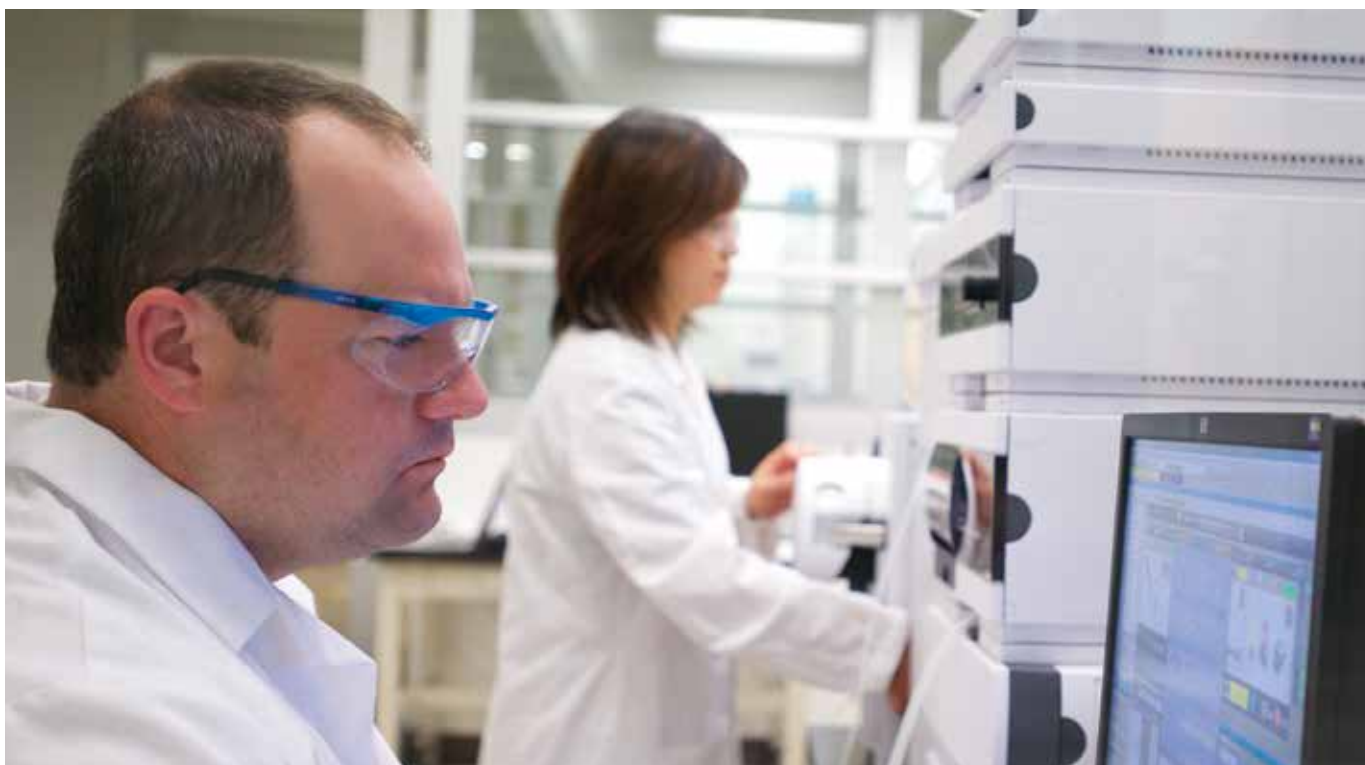
Agilent is ideally qualified and happy to be the company that can assist you in your peptide quantitation journey.



The Agilent solution for peptide quantitation: Agilent AssayMAP Bravo liquid handling system, Agilent 1290 Infinity II liquid chromatography system, Agilent 6495 Triple Quadrupole LC/MS system and Agilent AdvanceBio Peptide Mapping columns.

Small Versus Large Molecule Analysis

Analyzing small molecules, and analyzing larger molecules such as peptides, are fundamentally different undertakings. Sample preparation for traditional small molecules involves a variety of techniques such as extraction and protein precipitation, depending on the analyte of interest. In contrast, peptide analysis follows a relatively standard protocol. Proteins in solution are first denatured, then reduced and alkylated before enzymatic digestion. While the same type of liquid chromatography and similar mobile phases are used to analyze peptides, the gradients are much shallower and the run times can be longer due to sample complexity.



When developing a method for analyzing peptides via mass spectrometry, you will need to consider that a single peptide may exist in multiple charge states and that there will be many product ions, some of which will have higher m/z than the

precursor ion. Also keep in mind, that the most abundant product ion may not be the best choice for the analysis due to interferences. The best MRM transition will provide both selectivity and analytical sensitivity. As with traditional small

molecule analysis, acquiring the data is only the beginning. Data analysis requires sophisticated, but straightforward, software that can extract the most information from your experimental results.

Automated Peptide Sample Preparation for Mass Spectrometric Analysis

Reproducibility in sample preparation has always been critical in clinical research. Preparing peptide samples involves many timed steps and reagent additions that can impact analytical reproducibility. Automating this process reduces variability and provides error-free operation with sample tracking. The Agilent AssayMAP Bravo platform efficiently prepares protein and peptide samples for LC/MS analysis. This platform offers a simplified user interface with laboratory-tested peptide and protein sample preparation protocols to meet your clinical research needs.

Automated peptide sample prep with Agilent AssayMAP Bravo



Preparing samples for peptide quantitation requires a series of steps, some of which are optional. Within the software interface of the AssayMAP Bravo platform, the Peptide Sample Prep Workflow Navigator provides a simplified interface for choosing and using the optimal peptide sample

preparation workflow. Each workflow includes an easy-to-follow protocol, which can be modified if desired, and a reagent calculator with a workflow guide. The In-Solution Digestion Workflow for denaturation, reduction, and alkylation of disulfide bonds, and enzymatic digestion, is

always performed. The Peptide Cleanup Workflow is an optional step that desalts the samples prior to LC/MS analysis. Agilent has developed AssayMAP Bravo applications for each of these workflows, so your clinical research lab can create combinations that meet the specific requirements of your automated sample preparation.



The Agilent AssayMAP Bravo liquid handling system.

Chromatographic Separation

After the samples have been prepared and are ready for analysis, the next step is developing an efficient chromatographic method for the target peptides. The Agilent 1290 Infinity II liquid chromatography system gives you the ability to perform demanding separations with exceptional reliability and robustness. The UHPLC capability of the Agilent 1290 Infinity II LC system offers a variety of flow rates and column choices so that you can optimize your chromatographic methods for target peptides in complex matrices such as biofluids.



“The flexibility of being able to run the triple quadrupole at a higher flow rate allows us to get robustness and reproducibility. By using the 1290 Infinity II LC system’s higher flow rate, we’ve been able to determine that the precision and the reproducibility of retention times is so certain that we can almost integrate the peak areas without visually inspecting the peaks. Clearly, as we move forward to a clinical test, that’s going to become increasingly important for the vast majority of the data to be extracted automatically. I think that the tools, the reproducibility, and the software around the Agilent instrumentation allows us to do that.”

– **Stephen R. Pennington**, Professor of Proteomics and Senior Fellow at the Conway Institute of Biomolecular and Biomedical Research, University College Dublin. Founder, CEO, and CSO of Atturos Ltd.

The chromatographic columns used for peptide separations typically have larger pores to accommodate the larger molecules and include chromatographic media that minimizes non-specific binding of analytes for improved recovery. Agilent AdvanceBio Peptide Mapping columns deliver consistent, exceptional performance for separating and characterizing peptides.

This column is superficially porous and has a 120 Å pore size, which is optimal for analyzing the peptides produced by enzymatic digestion of proteins. The 2.7 µm particle size enables higher flow rates and better resolution of the target peptides in a complex

mixture. Improved chromatographic resolution, when used in conjunction with mass spectrometry, will help reduce interferences, and sharper chromatographic peaks will improve sensitivity, leading to easier data processing and increased result confidence. In addition, AdvanceBio Peptide Mapping columns are specially tested with a challenging peptide mix to ensure reliable peptide chromatographic separations and column-to-column reproducibility. Since many peptide quantitation analyses are multiplexed using retention time windows, stable retention times are key for robust analytical methods and thus high-throughput environments.

Alternatively, Agilent AdvanceBio Peptide Plus columns are a great choice when using formic acid. In addition to being a 120 Å superficially porous particle column, it is also a charged surface C18 hybrid column. These characteristics provide excellent peak shape when using formic acid and alternative selectivity, which enables better separation of closely eluting post-translational modifications such as deamidated species.

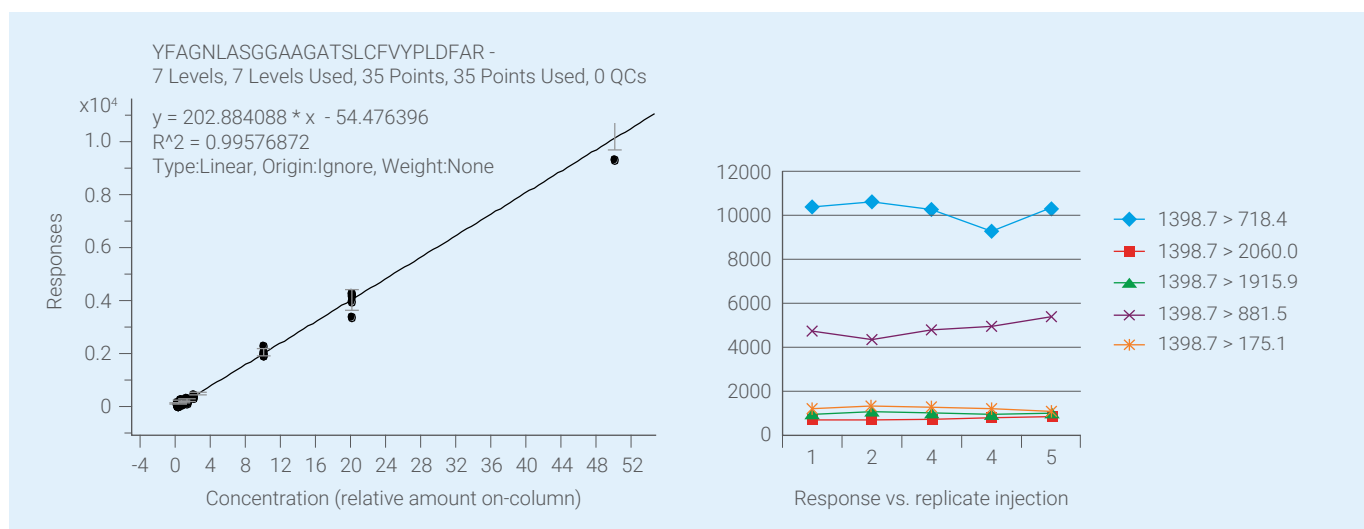
Both columns provide near UHPLC results with greater robustness leading to less downtime associated with switching and conditioning columns for mass spectrometry.



The Agilent 1290 Infinity II liquid chromatography system and Agilent AdvanceBio Peptide Mapping columns.

Mass Spectrometric Detection

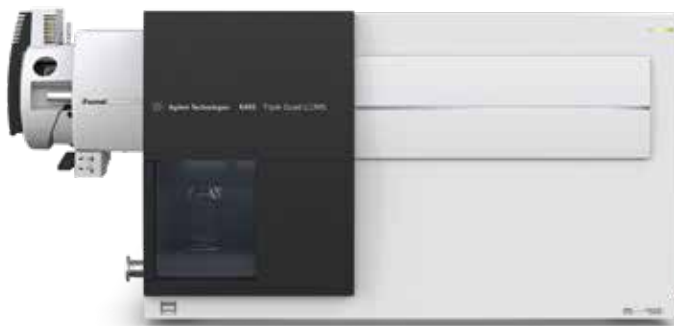
For peptide quantitation, sensitivity and a wide dynamic range can be critical because the protein concentrations in biofluids may span many orders of magnitude. The Agilent 6495 Triple Quadrupole LC/MS system incorporates iFunnel technology. When combined with the Agilent Jet Stream ion source, iFunnel technology dramatically increases ion sampling, which results in lower detection limits. The 6495 is the proven choice for quantitative applications requiring superior analytical sensitivity, reliability, and overall system robustness. When maintenance is needed, the newly incorporated gate valve enables higher uptime and increased productivity by allowing you to replace the capillary without having to vent the instrument.



The extended mass range (up to m/z 3000) of the 6495 allows you to select a wider range of precursor and product ions, thus broadening your choice of

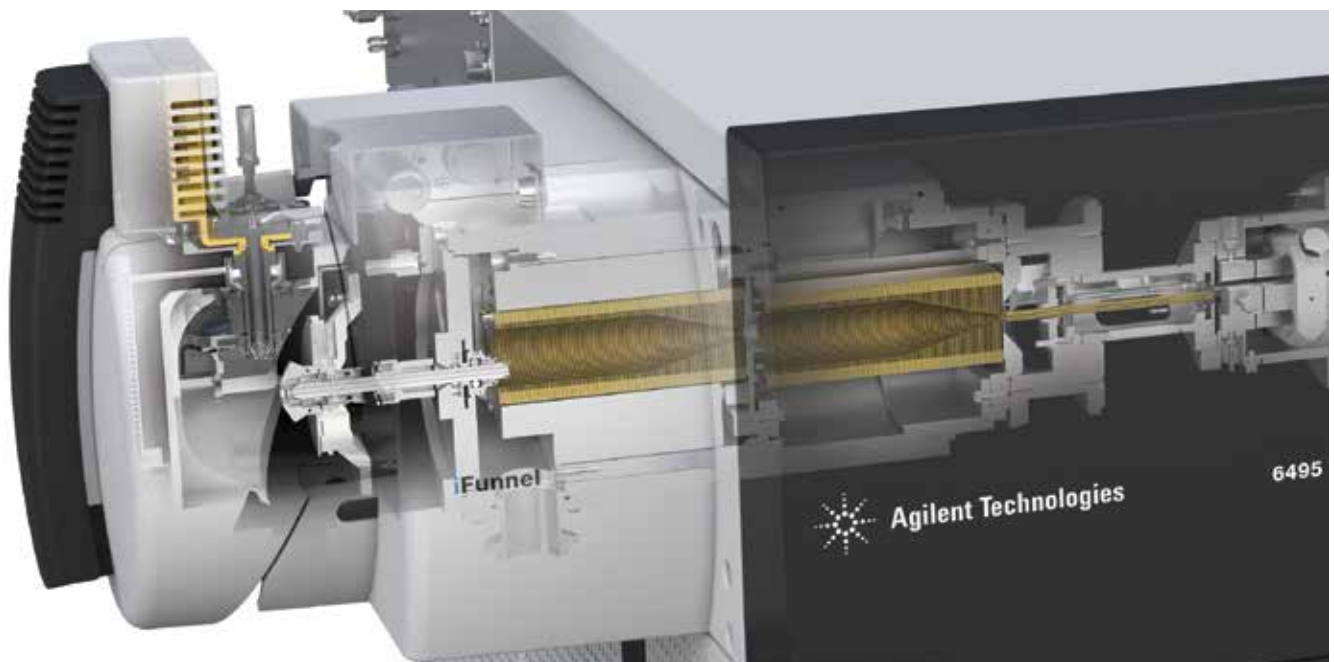
peptides and MRM transitions when optimizing for protein quantitation. The example above shows quantitation for a peptide where two of the selected

product ions are higher than m/z 1900. Such high m/z product ions provide high specificity for confirmation of the targeted peptide.



The Agilent 6495 Triple Quadrupole LC/MS system incorporates iFunnel technology combined with the Agilent Jet Stream ion source.

Agilent's unique Jet Stream ion source provides an interface that is three to five times more sensitive than standard electrospray ionization, and is not concentration dependent. This enables your lab to use standard 2.1 mm id columns and higher LC flow rates without the loss of sensitivity typically associated with such an approach. For peptide quantitation, clinical researchers that are not sample-limited can achieve near-nanoflow HPLC analytical sensitivity using conventional-flow HPLC with the Jet Stream source. As a result, your LC/MS analysis of complex proteomics samples will be fast, robust, and reproducible.



Agilent Jet Stream provides a 3 to 5X increase in signal for peptides compared to electrospray.

MassHunter Software

Agilent MassHunter Workstation software includes an acquisition module for instrument control as well as both quantitative and qualitative data analysis. Data processing can be automated with MassHunter Quantitative Analysis tools to rapidly provide peptide quantitation results. These tools enable you to customize the results criteria that you use and will highlight the results that warrant further review.

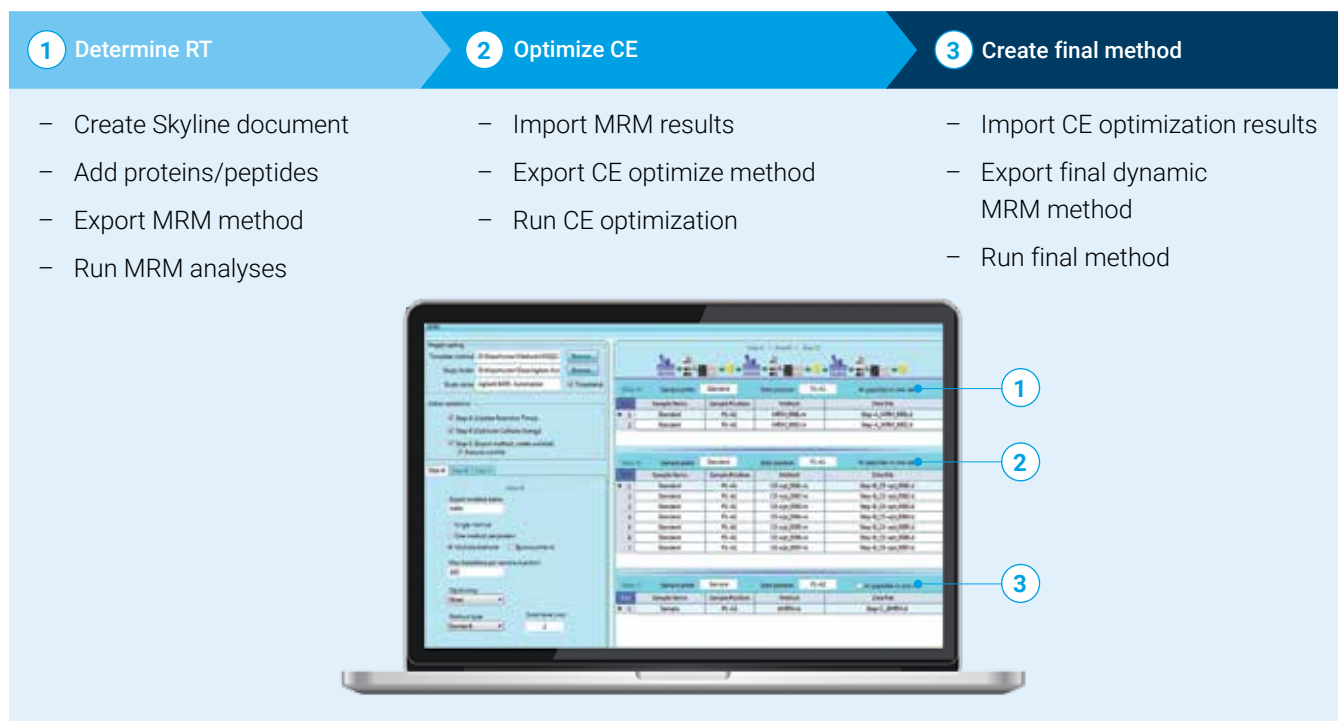


MassHunter Workstation software can also integrate directly with your Laboratory Information System (LIS) so you can import sample lists for analysis, and ultimately export your results.

Skyline Software

Agilent MassHunter software provides tight integration with Skyline software. Designed for proteomics researchers, Skyline software (from the MacCoss Group at the University of Washington) supports MRM, dynamic MRM (dMRM), and triggered MRM (tMRM) modes in MassHunter so you can acquire the desired quantifier and qualifier signals in increasingly complex samples.

Automated MRM in three easy steps using the Agilent Skyline Automation tool



Agilent's unique Skyline automation tool makes creating new methods much simpler by automating important method development and optimization processes. Skyline and MassHunter work together to provide an easy solution: just put your sample in a vial, set up the parameters, and let the software do the rest of the

optimization. Come back a few hours later and your method is ready to run samples.

When changing columns or methods, adjusting retention times in complex methods can be time-consuming. The retention time calibration tool in MassHunter acquisition software enables you to easily adjust methods

to address changes in retention time. Skyline also includes iRT capabilities to store and predict peptide retention times. In short, Agilent MassHunter and Skyline software make your peptide quantitation analyses faster, easier, and more productive.

Agilent offers solutions for all aspects of the peptide quantitation workflow, including sample preparation, LC/MS analysis, and data processing. Agilent is proud to be your company of choice in peptide quantitation for clinical research.

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