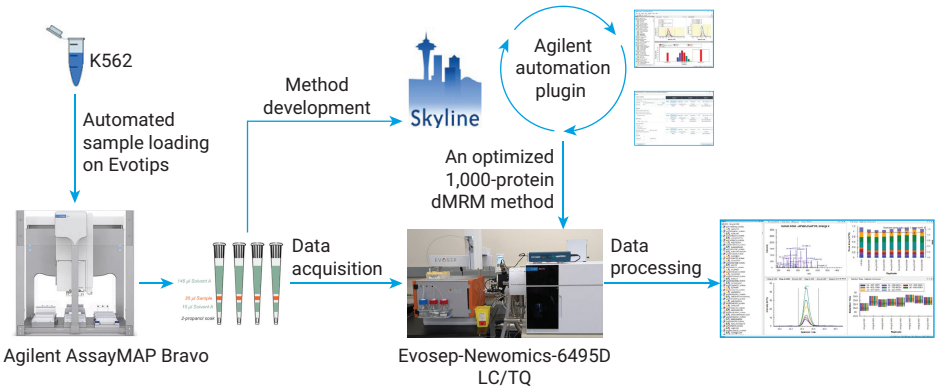
Application Note

Proteomics

Large-Panel Targeted Proteomics with Single-Cell-Equivalent Sensitivity Using Nanoflow 6495D LC/TQ



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Abstract

Quantitative proteomics analysis of small input samples, such as single cells, requires exceptionally sensitive and precise instrumentation. This study showcases the performance of an enhanced nanoflow liquid chromatography triple quadrupole (LC/TQ) system, integrating the Evosep One LC, Newomics UniESI ion source, and Agilent 6495D triple quadrupole LC/MS, for large-panel targeted proteomics in trace amounts of human cell lysate samples. Multiple hardware and software enhancements were implemented to accelerate method development and expand analytical capabilities for nanoflow LC/MS workflows. The optimized platform delivers high specificity, sensitivity, and throughput, enabling robust quantitation from sample loads equivalent to those used in single-cell applications. These results highlight the system's potential for advancing proteomic studies where input material is extremely limited.

Introduction

Understanding biological processes, particularly those with significant cellular heterogeneity such as those involved in cancer research, may require the ability to analyze protein and peptides in extremely low quantities—down to the scale of individual cells. Sensitive, precise, and accurate peptide quantification at this scale is essential for revealing subtle yet critical variations in protein expression and post-translational modifications, which can drive diverse cellular behaviors but are often masked in bulk sample analyses. Proteome research, particularly in single-cell proteomics, has traditionally relied on liquid chromatography mass spectrometry (LC/MS) using high-resolution accurate mass spectrometry (HRAMS) platforms, which, while powerful, are often prohibitively expensive and less accessible to some research laboratories.

In this application note, we demonstrate the potential of a state-of-the-art triple quadrupole LC/MS system for researchers to push the boundaries of protein biomarker discovery in this emerging frontier. A comprehensive targeted quantification assay for 1,000 proteins in human K562 cell lysate was developed using an enhanced nanoflow LC/TQ platform. The system integrates the Evosep One LC, Newomics UniESI ion source, and Agilent 6495D triple quadrupole mass spectrometer. The results show excellent quantification performance, enabling reliable protein quantification at an extremely low amount, approximating single-cell level detection.

Key aspects of the analytical instrument and workflow

1. Agilent MassHunter acquisition software was modified to support up to 10,000 transitions in a single dynamic MRM (dMRM) method on the 6495D LC/TQ, which significantly accelerates the method development process and expands proteome coverage for targeted protein analysis.
2. The Newomics UniESI ion source was upgraded and fully integrated with the Agilent LC/MS system to improve usability and nanospray ESI robustness.
3. The MRM-based targeted method development workflow employs human cell protein extract digest without synthetic peptide standards, utilizing Skyline software integrated with the Agilent automation tool, offering a rapid and cost-effective solution for comprehensive proteome analysis.

Experimental

Instrumentation

- Evosep One LC system
- Newomics UniESI ion source for Agilent MS (IS-A01U)
- Agilent 6495 triple quadrupole LC/MS system (G6495D)
- Agilent AssayMAP Bravo platform (G5571AA)

Materials

- MS-compatible human protein extract digest (Promega Corporation)
- Protein LoBind Tube 0.5 mL (Eppendorf)
- Evotip (Evosep, Inc.)
- Aurora Elite XT C18 UHPLC column (15 cm × 75 µm id, 1.7 µm, IonOpticks)

Sample preparation

For method development, 50 ng of the purchased protein extract digest of the K562 human cell line was automatically loaded onto Evotips in batch using the AssayMap Bravo platform. For calibration curve analysis, the cell protein extract digest was serially diluted two-fold across twelve levels in protein LoBind Eppendorf tubes and then loaded onto Evotips, ranging from 64 ng to 31.25 pg on-column for LC/MS analysis.

Method development

Skyline software (v24.1.0.199) from the MacCoss lab at the University of Washington was used for MRM-based targeted proteome method development and data analysis.[1] An upgraded Agilent MassHunter Workstation for triple quadrupole LC/MS data acquisition (pre-release beta version) was used for LC/MS data acquisition.

A large-panel dMRM method covering 1,000 human proteins was developed using Skyline software integrated with the Agilent automation tool. The method development process comprised the following key steps (Figure 1).

1. Create a Skyline document with custom peptide and transition settings, using the Uniprot human reference proteome as the background. Enforce peptide uniqueness by genes, select peptides matching library and filter criteria, rank by picked intensity, and limit to one peptide per protein. Use the human K562 peptide library, apply carbamidomethyl (C) as a fixed modification, and select eight product ions per precursor (six minimum). The human K562 peptide library was built based on proteome identification data acquired previously on the Agilent 6550 LC/Q-TOF.[2] For other biological samples, users can either

generate in-house peptide MS/MS spectral libraries or download species- or tissue-specific libraries from public databases such as PeptideAtlas, the NIST peptide spectral library, or ProteomeTools.[3–6]

2. A list of 1,000 target protein names was imported into the Skyline, which automatically generated corresponding peptides and transitions. Using the Agilent automation tool plugin, multiple standard MRM methods were programmatically created and applied for LC/MS data acquisition on the nanoflow Agilent 6495D LC/TQ system with K562 cell lysate. This workflow is defined as Step-A (Update Retention Time) within the Skyline Automation project interface.
3. To establish a retention time (RT) predictor, 33 high-abundance peptides were selected based on their confident detection and even distribution across the LC gradient. Their RTs were used to build a custom predictor on the operating system, which was then saved and applied in the Skyline document for targeted method development. Users may also choose to build a custom RT predictor prior to initial data acquisition in Step-A. This enables the creation of dMRM methods to determine peptide RTs, thereby reducing the number of required sample injections.
4. Import all the acquired MRM data into Skyline and refine peak picking for all target peptides. At this step, it is recommended to review all target peptides to ensure correct peak selection.
5. Apply multi-parameter filters—dotp > 0.75, peak found ratio > 0.75, shape correlation ≥ 0.9, retention time deviation from prediction ≤ 6 minutes—to retain confidently detected peptides. These criteria enhance detection accuracy and can be adjusted based on the acquisition method and dataset.
6. (Optional) Optimize collision energy (CE) for peptide transitions following Step-B in the Skyline automation tool interface. CE optimization using complex biological samples may result in suboptimal settings for some peptides due to peak picking errors, requiring manual curation. Since the default Agilent QQQ CE equation in Skyline already provides near-optimal performance, it is recommended to skip CE optimization during initial method development to help streamline the process.
7. Export the finalized assay as a single dMRM method containing 7,942 transitions total for 1,000 peptides, each uniquely mapping to one of the 1,000 target protein groups.

For a subset of proteins, the initially selected peptides exhibited suboptimal peak performance, which was re-optimized through alternative peptide selection to improve assay performance in large-scale panels. RT windows were also refined in the MassHunter acquisition software to account for RT drift across injections, resulting in 89% of target peptides being assigned RT windows greater than 2 minutes.

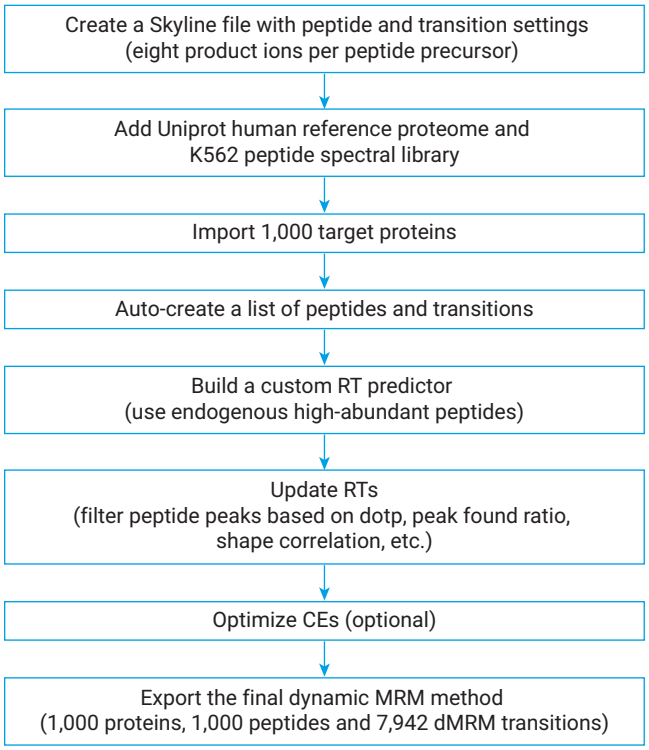

Figure 1. Method development workflow using Skyline software with Agilent automation tool, human cell protein extract digest, and peptide spectral library without any synthetic peptides.

LC/MS analysis

All samples were analyzed on an IonOpticks Aurora Elite XT C18 UHPLC column, operated with the Whisper Zoom 20 SPD LC method. Data acquisition was performed using MassHunter software (pre-release beta version) on a 6495D LC/TQ system (Table 1). The analytical column was heated at 50 °C. The samples for calibration curve analysis were analyzed in triplicates from the lowest to the highest levels.

Table 1. LC/MS parameters.

Parameter	Value
Evosep One LC System	
Column	IonOpticks Aurora Elite XT column (15 cm × 75 µm id, 1.7 µm C18)
Column Temperature	50 °C
LC Method	Whisper Zoom 20 SPD
LC Flow Rate	200 nL/min throughout the primary LC gradient
Agilent 6495D Triple Quadrupole Mass Spectrometer	
Ion Source	Newomics UniESI source platform with IonOpticks column stage
Polarity	Positive
Gas Temperature	200 °C
Drying Gas	11 L/min
Capillary Voltage	1,600 V (the final optimized capillary voltage may vary case-by-case)
MS1/MS2 Resolution	Wide/Unit
Autotune Mode	Large molecule mode
Total dMRM Transitions	7,942
Maximum Concurrent MRM	500
Minimum Dwell Time	0.64 ms
Cycle Time	1,400 ms
iFunnel Mode	Standard

Results and discussion

Method development for large-panel targeted proteomics

To evaluate the system capability of the enhanced nanoflow 6495D LC/TQ, a large-panel dynamic MRM method was developed using the endogenous human K562 cell protein extract digest and a pre-existing human K562 peptide spectrum library.[2] The final target proteome method contained 7,942 dMRM transitions corresponding to 1,000 peptides, each uniquely mapping to one of the 1,000 target protein groups. For each peptide precursor, up to eight product ions were monitored. Figure 2 illustrates the distribution of concurrent transitions in the finalized large panel dMRM method, as supported by the updated MassHunter acquisition software. Peptides monitored in this method exist across a broad dynamic range of MS signal intensities, spanning over four orders of magnitude (Figure 3 and Table 1).

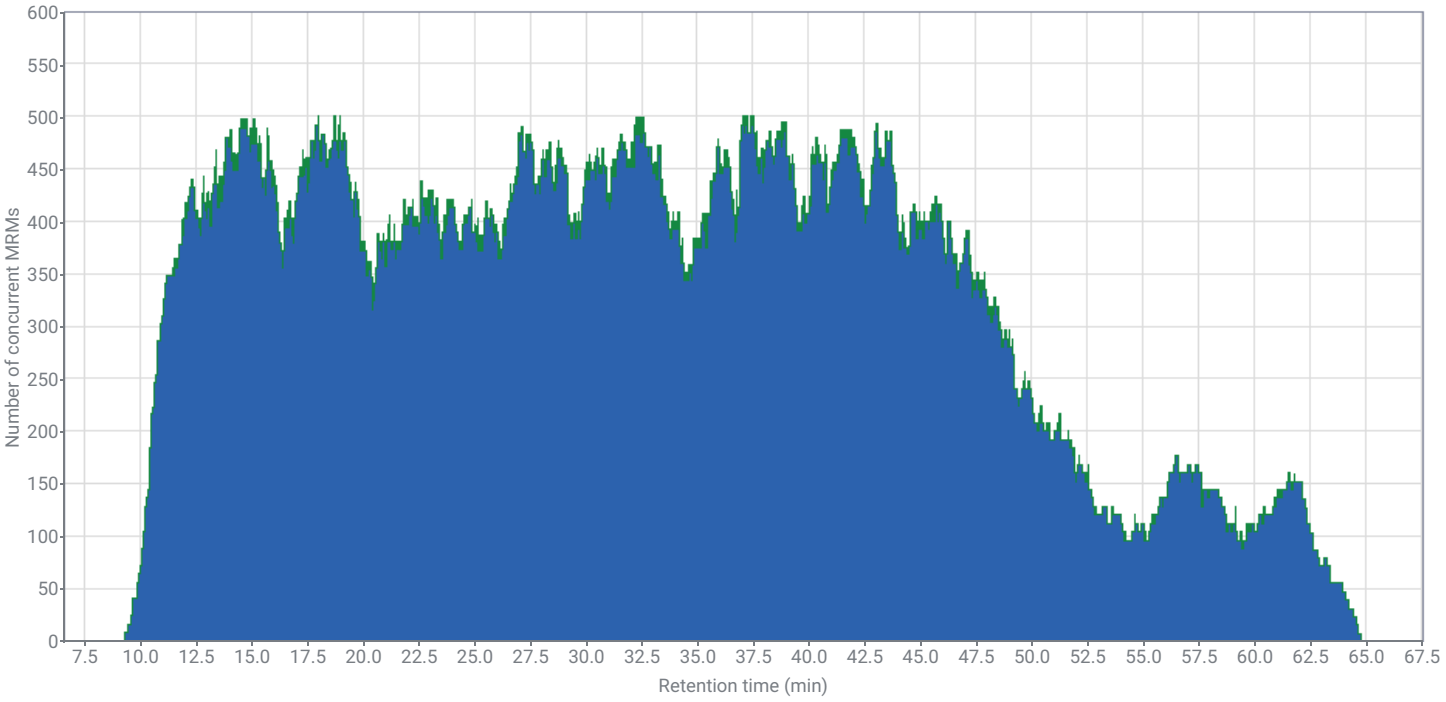

Figure 2. Distribution of concurrent MRMs in the final large-panel dMRM method.

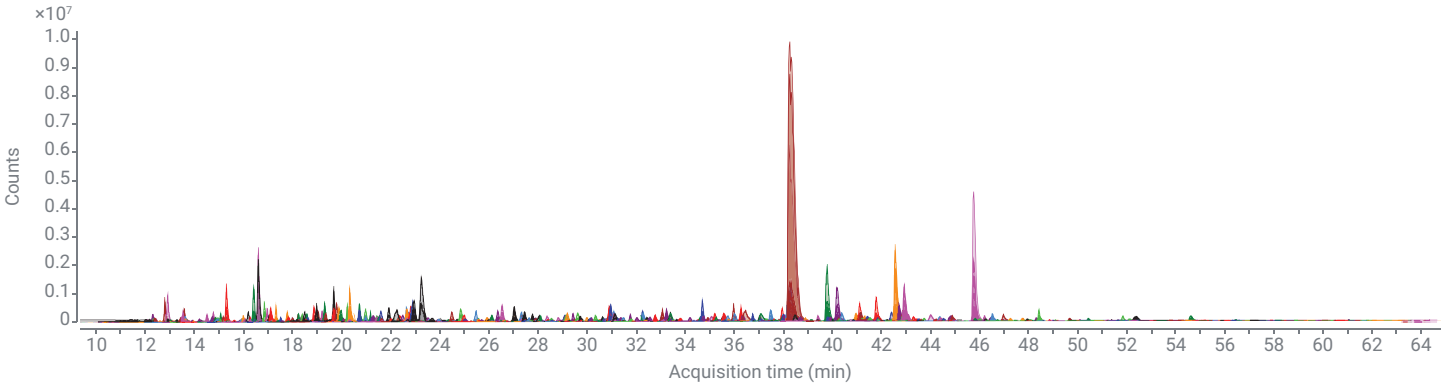

Figure 3. Total MRM chromatograms of 64 ng K562 cell digest analyzed by the 1,000-target proteome assay.

High-confidence detection

To assess the identification specificity and accuracy of the MRM-based workflow, the same LC system, column, and ion source were connected to a high-resolution accurate mass LC/Q-TOF for proteomic discovery using data-dependent acquisition (DDA). Among the identified peptides, 456 overlapped with the 1,000-target assay. Retention times from both workflows were compared (Figure 4), showing 98.5% consistency, confirming high detection confidence by the MRM-based workflow. Incorporating more product ions per peptide significantly improved accuracy compared to a previous study using only four product ions (68% accuracy).[7] These results demonstrate that the unit-mass Agilent 6495D LC/TQ can achieve high-confidence identification by targeting a greater number of product ions per precursor.

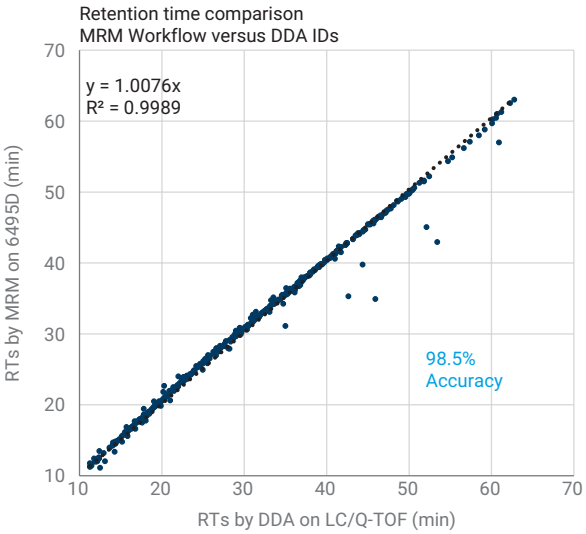

Figure 4. Peptide retention times show strong correlation between MRM (Agilent 6495D LC/TQ) and DDA (LC/Q-TOF) workflows, confirming the high detection accuracy of the MRM approach.

Method reproducibility

Excellent quantification performance was achieved using the 1,000-target proteome assay on the nanoflow 6495D LC/TQ system. The overall peak area coefficient of variation (CV) for all target peptides displayed excellent reproducibility across the full LC gradient, resulting in a median CV of 7.5% across all target peptides and 96.2% of target peptides exhibiting CVs below 20%, highlighting the high precision and reproducibility of this large panel dMRM method (Figure 5).

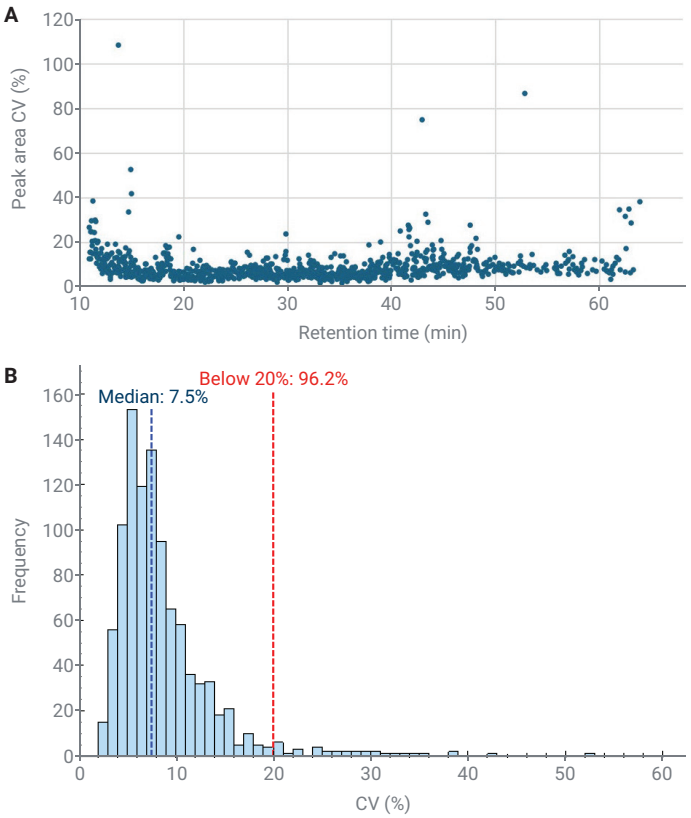

Figure 5. Distribution of peptide peak area CV with 64 ng cell protein extract digest on-column (n = 8). (A) Plot of peptide peak area CV against LC retention time. (B) Histogram of peptide peak area CV distribution.

Protein quantification approximating single-cell sensitivity

Precise and accurate protein quantification at single-cell levels is crucial for revealing subtle yet biologically significant variations in protein expression and post-translational modifications. The total protein mass of a single cell varies considerably depending on cell type and physiological state. For instance, the estimated protein content of a single lymphocyte ranges from approximately 20 to 200 pg, depending on its activation status.[8] In contrast, a human HeLa cell contains around 200 pg of proteins, while a human oocyte holds approximately 100 ng per cell.[9,10] To assess system capability for quantifying proteins at levels approximating single-cell detection, a sample loading calibration curve was generated using 31.25 pg to 64 ng of K562 cell protein extract digest on-column, analyzed in triplicate with the 1,000-target proteome method (Figures 6–8). The results showed outstanding quantification performance:

- Excellent linearity for the twelve levels tested, with $R^2 > 0.99$ for 57.8% of target proteins and $0.90 < R^2 \leq 0.99$ for 25.6% of target proteins (Figure 6A).
- Low-level quantification sensitivity was demonstrated, with 10% of target proteins (100 out of 1,000) quantifiable at 31.25 pg on-column. Quantifiable coverage increased to 33.6% and 47.8% with 250 pg and 500 pg loadings, respectively (Figure 6B).
- Outstanding quantification performance of two representative peptides—histone peptide LLLPGELAK and Poly(rC)-binding protein 1 peptide LVVPATQCGSLIGK—demonstrated excellent quantification sensitivity, linearity, precision, and accuracy at all tested levels (Figures 7 and 8).
- High-quality MRM chromatograms and consistent product ion ratios were obtained for both peptides, even at ultra-low sample loads (Figures 7B–C and 8B–C).
- The MS signal intensities of these two representative peptides differed by approximately 30-fold, highlighting the broad dynamic range and proteome coverage potentially achievable with this nanoflow 6495D LC/TQ system for quantitative analysis (Figures 7B and 8B).

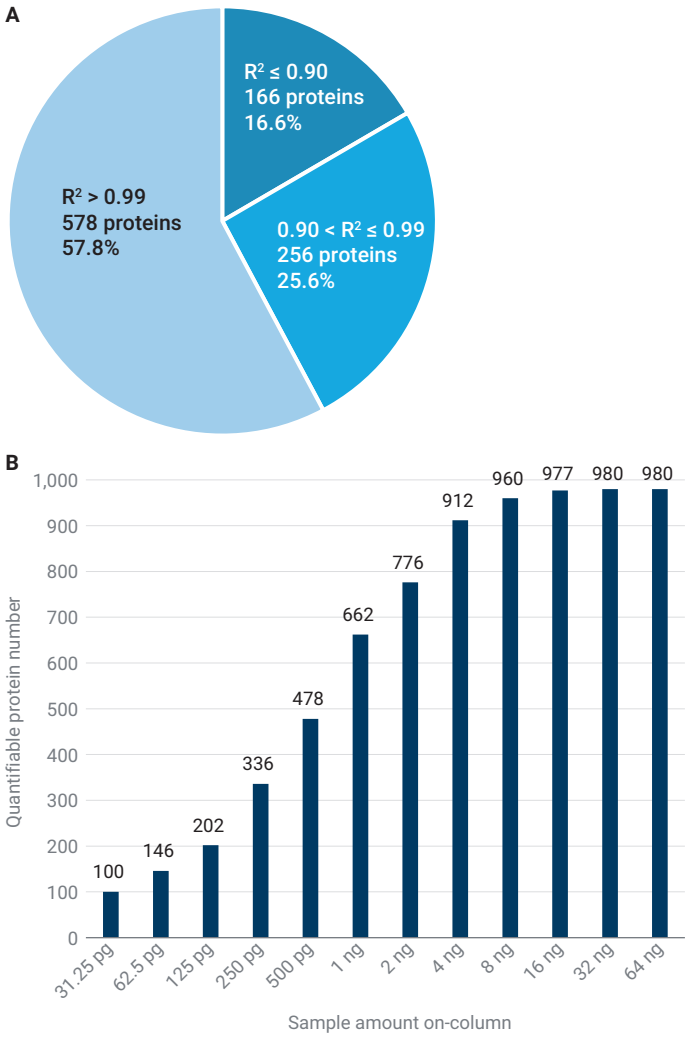

Figure 6. Overview of quantification performance for all the target proteins. (A) R^2 of linear curve fitting for all the 1,000 proteins with sample loading from 31.25 pg to 64 ng on-column. (B) Quantifiable protein counts at each sample loading level.

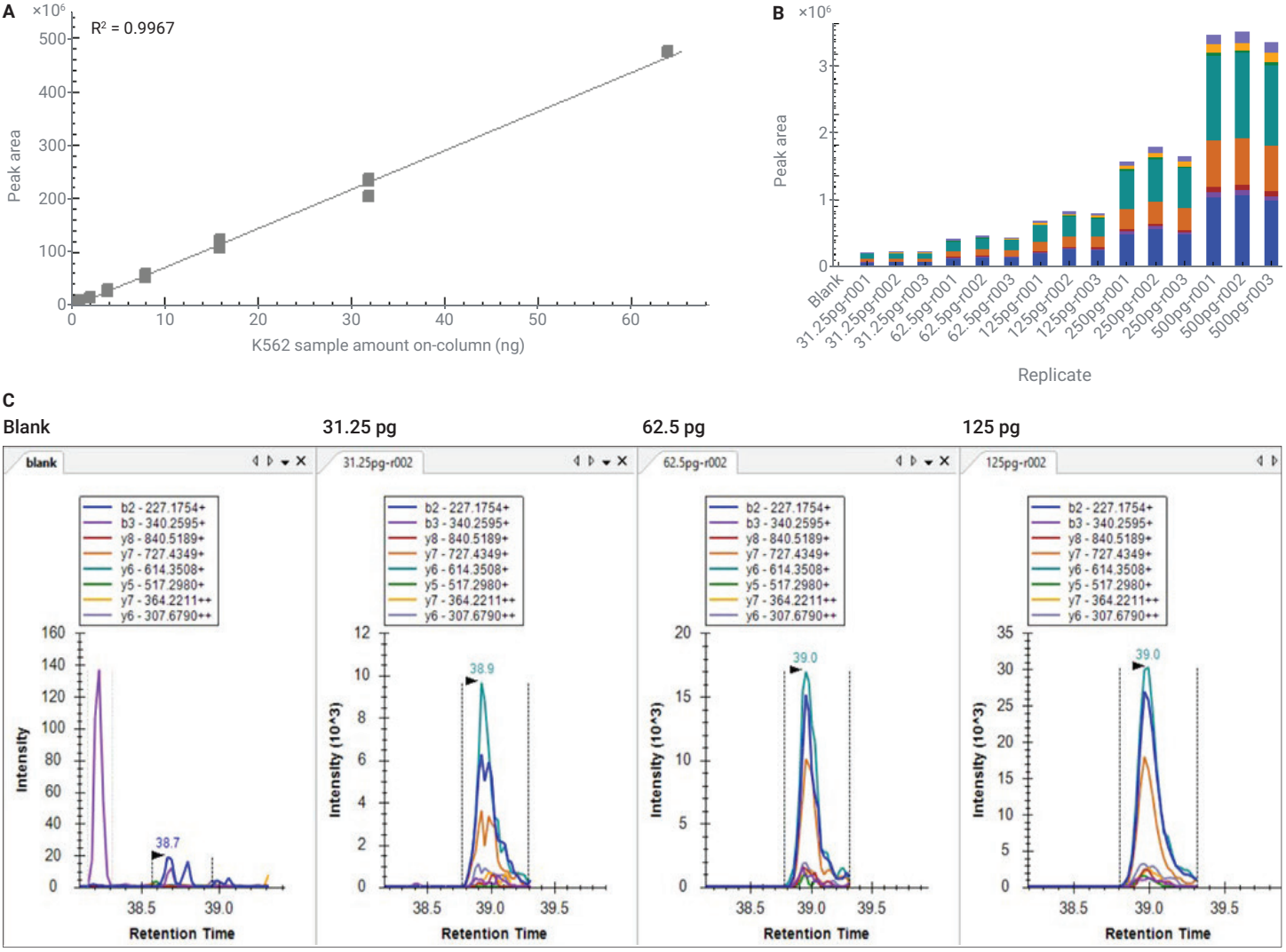

Figure 7. Quantification performance of histone peptide LLLPGELAK. (A) Sample loading linear calibration curve (n = 3). (B) Triplicate peptide peak areas for all target transitions at blank and low levels. (C) Peptide MRM chromatograms at blank and low levels.

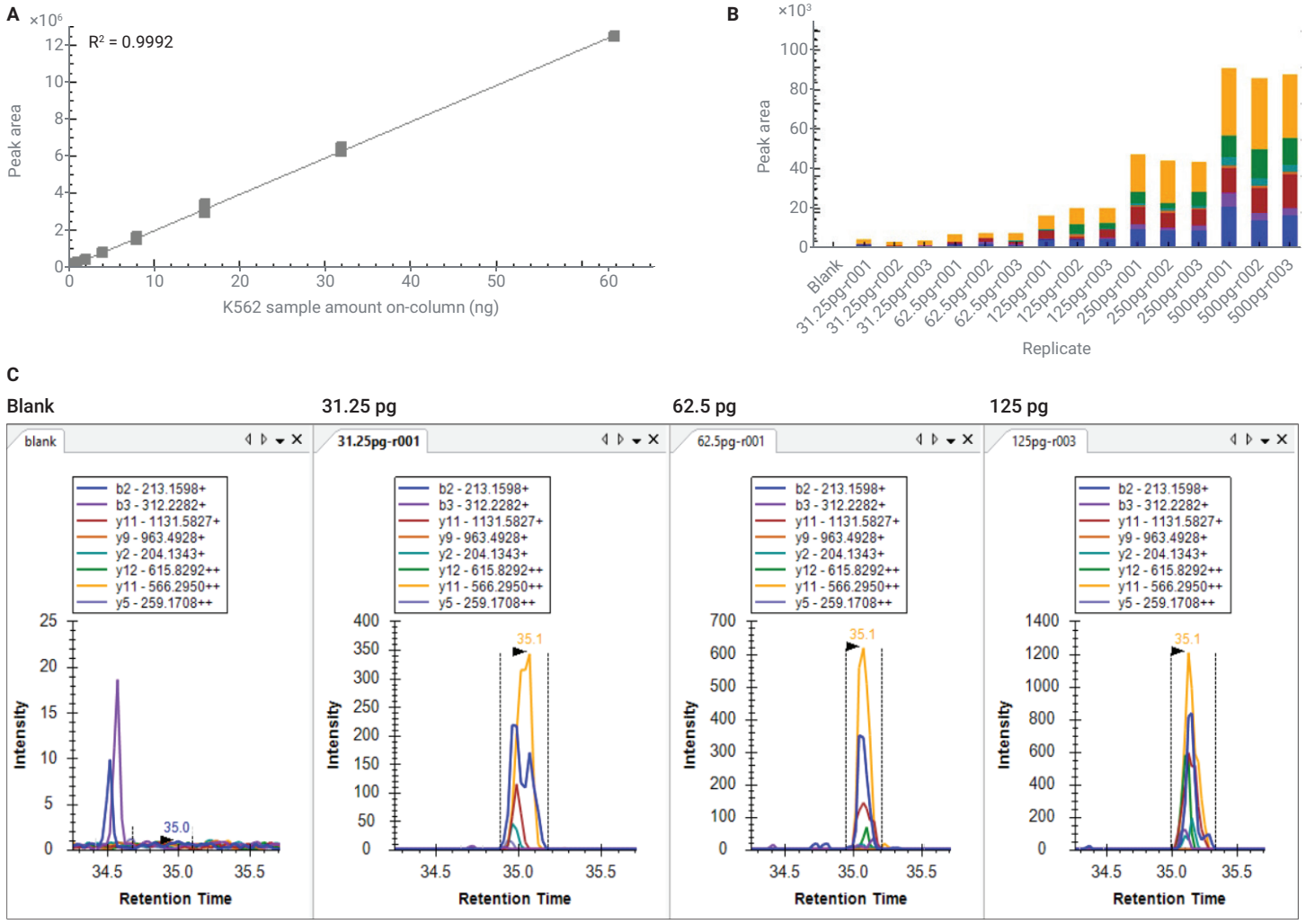

Figure 8. Quantification performance of Poly(rC)-binding protein 1 peptide LVVPATQCGSLIGK. (A) Sample loading linear calibration curve (n = 3). (B) Triplicate peptide peak areas for all target transitions at blank and low levels. (C) Peptide MRM chromatograms at blank and low levels.

Conclusion

This study showcases a powerful advancement in targeted proteomics: a fully integrated nanoflow LC/TQ system combining Evosep One LC, Newomics ion source and the Agilent 6495D LC/TQ. Designed for comprehensive and high-sensitivity applications, this solution enables large-scale targeted protein quantification at levels approximating single-cell detection.

Key highlights include:

- **Cost-effective method development:** Leveraging Skyline software and Agilent 6495D LC/TQ, the dMRM workflow eliminates the need for stable isotope-labeled (SIL) peptides, significantly reducing assay development costs without compromising performance.
- **Excellent sensitivity and precision:** The system delivers quantification with high precision and accuracy, even at ultra-low sample inputs, making it ideal for limited or precious biological samples.
- **Scalable for large panels:** Capable of quantifying 1,000 proteins in a single LC/MS run, this system is optimized for large panel studies, biomarker analysis, and translational research.

In summary, this enhanced workflow empowers researchers to push the boundaries of targeted proteomics, offering a practical, scalable, and highly sensitive solution for biomarker discovery.

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