

# ABSOLUTE QUANTIFICATION OF FULLY CUSTOMIZABLE PROTEIN PANELS USING **TRUESIGNATURE** TARGETED MASS SPECTROMETRY

**Targeted mass spectrometry (MS) has become a powerful tool for precise protein quantification without the need for antibody development, making it ideal when affinity reagents are unavailable or with poor specificity.**

By using stable isotope-labeled standards (SIS) as spike-in references, this approach ensures unmatched specificity. Recent advances, such as integrating Field Asymmetric Ion Mobility Spectrometry (FAIMS) with Parallel Reaction Monitoring (PRM), have significantly enhanced sensitivity, enabling reliable detection of low-abundance proteins with greater confidence.<sup>1</sup> Furthermore, the matrix-agnostic and species-independent nature of mass spectrometry makes it applicable across a wide range of biological samples, including peripheral blood mononuclear cells (PBMCs)<sup>2</sup>, crucial for pharmacodynamic (PD) assessments in clinical trials, and formalin-fixed, paraffin-embedded (FFPE) tissues.<sup>3</sup>

Biognosys' TrueSignature targeted proteomics platform offers a robust CRO service for precise protein and proteoform quantification across diverse biological matrices and species. It enables drug developers to monitor pharmacodynamic (PD) markers or patient response with high precision and accuracy, even in complex clinical matrices, and offers several key advantages:

**Unparalleled specificity:** Proteotypic peptides – peptides that uniquely represent one target protein in a given species – ensure precise quantification of proteins with minimal background interference.

**Customization and multiplexing:** Fully tailored panel design means assays can address specific biological questions such as post-translational modifications, isoforms, cleavage products and mutations. Additionally, assays can be multiplexed to enable the simultaneous detection of up to 50 targets per panel for enhanced throughput.

**Flexibility across matrices and species:** By eliminating the need for antibody generation, the TrueSignature platform enables rapid assay development and reduces overall development timelines. This flexibility means assays can be adapted to suit different biological matrices and species, enabling their applicability in preclinical studies.

**Quantitative robustness:** Using SIS peptides as synthetic spike-in references not only ensures the selectivity of the assay but also improves quantification by compensating for factors such as ion suppression or matrix effects.<sup>4</sup> This enhances the precision of measurements and guarantees consistent performance across longitudinal studies.

This application note outlines the TrueSignature workflow (Figure 1), demonstrated by a case study quantifying a 9-protein panel applied in a human PBMC matrix.

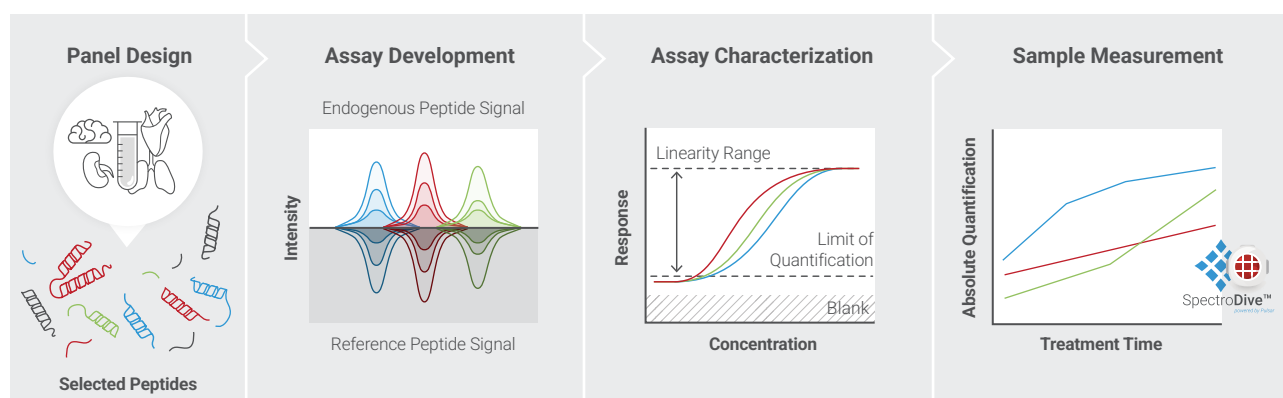


Figure 1. TrueSignature targeted proteomics workflow

# CUSTOMIZED PROTEIN PANEL QUANTIFICATION IN HUMAN PBMCS

This case study highlights TrueSignature’s customizable assay development, characterization and application for clinical sample analysis.

## Panel design

The panel design process involves selecting and evaluating proteotypic peptides for the proteins of interest. Candidate peptides that meet predefined selection criteria are identified from in-house data repositories and discovery proteomics datasets acquired from the customer project. For specific proteoforms, such as mutations, peptide sequences can also be predicted in silico.

In this study, peptides were selected for 9 target proteins (Table 1). Figure 2 illustrates this process using CD44 as an example. Multiple candidate peptides were initially detected in a discovery proteomics experiment.

Following experimental evaluation in a relevant biological matrix, the final peptide (red) was selected for assay development.

## Assay development

SIS reference peptides for all 9 proteins were synthesized and underwent quality control, alongside systematic optimization of assay parameters, leading to the final assembly of a FAIMS-PRM panel. SIS peptides mitigate sample-specific ionization effects – such as ion suppression and interferences that impact quantitation – and ensure reproducibility across different laboratories and time points.<sup>4</sup>

## Assay characterization

Nine SIS peptides were spiked into a human PBMC background matrix to generate an eight-point calibration curve (seven concentration levels and a

Figure 2. Selected proteotypic peptide for human CD44 from extracellular domain

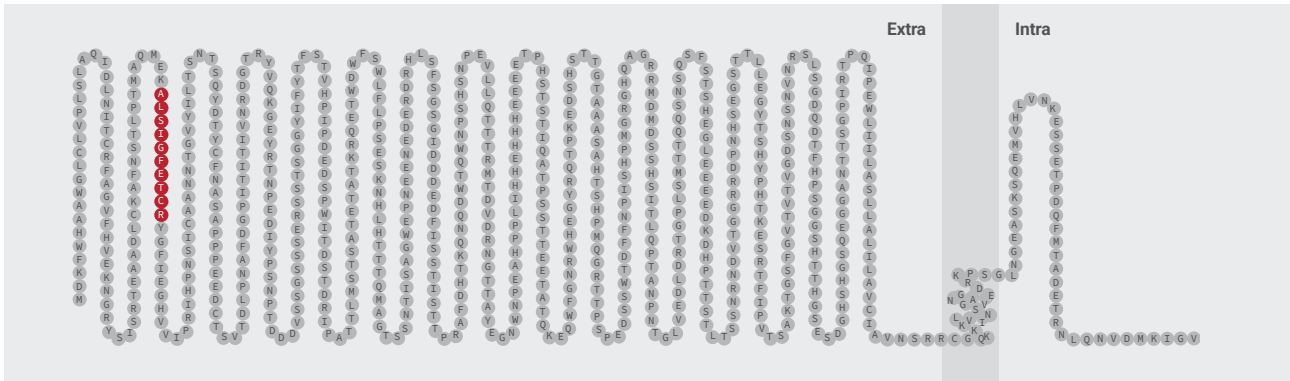


Figure 3. Calibration curve and LLOQ determination for CD44 based on proteotypic peptide

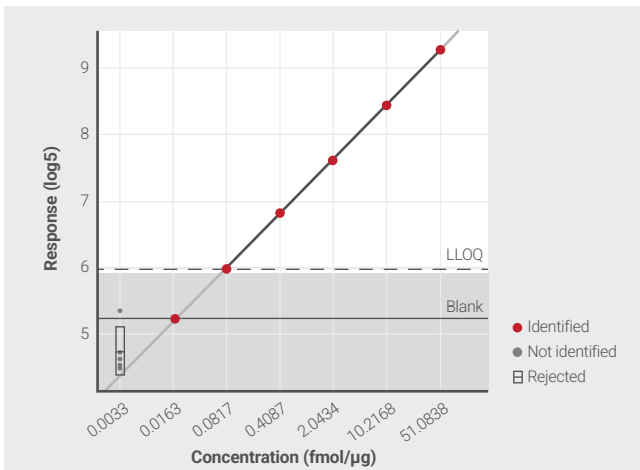
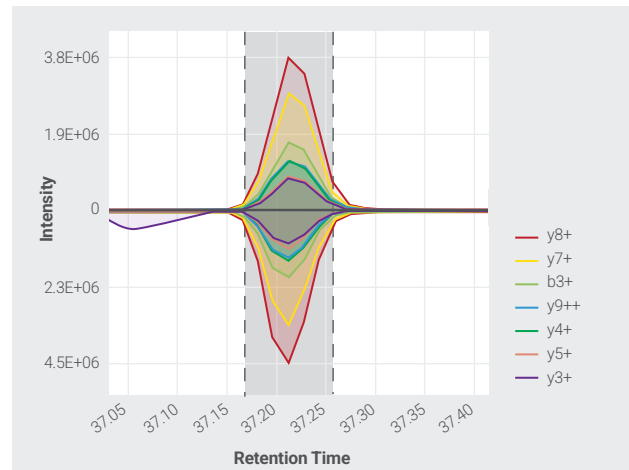


Figure 4. Absolute quantification of CD44 in human PBMC. Upper panel: endogenous peptide, lower panel: spike-in SIS reference



blank) for each analyte. This allowed for the evaluation of linearity across a broad concentration range, ensuring quantification of analytes in both low and high concentration samples. The calibration curve was used to assess the relationship between peptide concentration and signal intensity, ensuring reliable quantification.

The lower limit of quantification (LLOQ) for each analyte was defined as the lowest spike-in concentration that could be quantified with a coefficient of variation (CV) < 25% and a signal intensity at least 2× greater than the blank (Figure 3). Establishing this calibration curve and LLOQ enables precise, multipoint quantification of analytes across a wide dynamic range.

For clinical applications, we recommend a comprehensive, fit-for-purpose assay qualification process to ensure required performance criteria are met. This typically includes evaluation of assay sensitivity, LLOQ, linear range, carry-over, selectivity, intra- and inter-batch precision and stability. The assay can be performed under GCP guidelines, ensuring highest quality data for regulatory submissions.

### Sample measurement

The assay panel successfully quantified 9 target proteins in human PBMC samples. Figure 4 illustrates endogenous (light, L) and reference (heavy, H) analyte detection. Absolute quantification was determined using the heavy-to-light (H/L) ratio, based on the intensity-to-quantity relationship established in the multi-point calibration curve (Figure 5).

Data processing and quantitative analysis were performed using SpectroDive™, an advanced and versatile software platform for targeted proteomics data analysis. SpectroDive features automatic false discovery rate estimation, powerful peak picking algorithm, intuitive data visualization, and flexible reporting, providing streamlined and robust data processing of hundreds of samples.



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Figure 5. Box plots showing absolute quantification of target proteins (fmol/μg) in 5 sample replicates

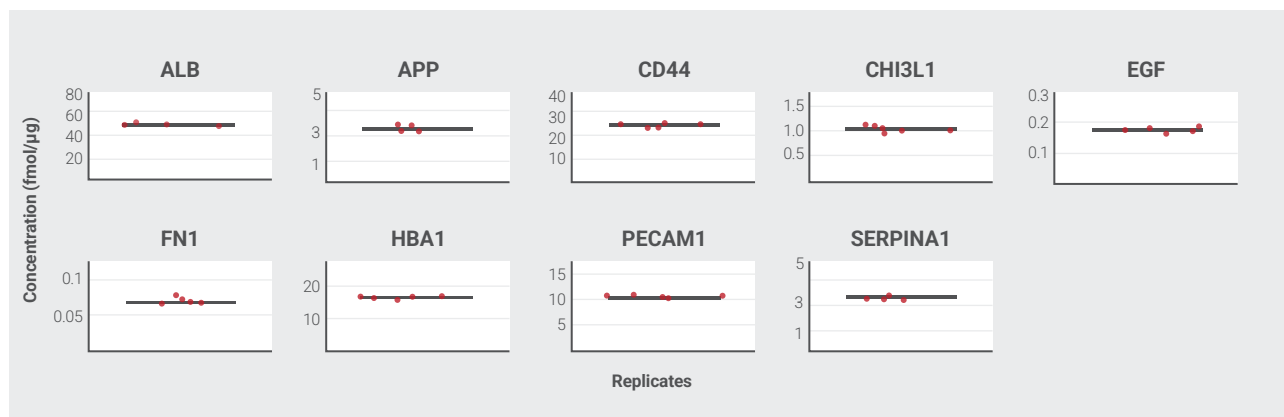


Table 1. Summary of quantification

Gene ID	UniProt ID	Protein Name	Peptide Used for Quantification (Charge State)	LLOQ (fmol/μg)	Average CV (%)
ALB	P02768	Albumin	FQNALLVR (2+)	0.0084	1.5
CD44	P16070	CD44 antigen	YGFIEGHVVIPR (2+)	0.0470	1.7
HBA1	P69905	Hemoglobin subunit alpha	VGAHAGEYGAEALER (2+)	0.0045	1.4
PECAM1	P16284	Platelet endothelial cell adhesion molecule	STESYFIPEVR (2+)	0.0410	1.2
APP	P05067	Amyloid-beta precursor protein	THPHFVIPYR (2+)	0.7300	3.8
SERPINA1	P01009	Alpha-1-antitrypsin	GKWERPFEVK (3+)	0.0084	2.1
CHI3L1	P36222	Chitinase-3-like protein 1	GNQWVGYYDDQESVK (2+)	0.0140	3.5
EGF	P01133	Pro-epidermal growth factor	ALLETSEK (2+)	0.0600	5.9
FN1	P02751	Fibronectin	SYTITGLQPGTDYK (2+)	0.2000	5.3

# KEY TAKEAWAYS

**Biognosys' TrueSignature targeted proteomics platform offers a robust CRO service for precise protein quantification across diverse biological matrices and species, offering several key advantages:**

## Unmatched specificity

Reference peptides enable simultaneous monitoring of endogenous targets and internal standards, ensuring assay specificity across diverse matrices.

## Proteotypic peptide selection

Eliminates the need for antibodies, allowing precise monitoring of protein variants such as mutations, truncations and isoforms.

## Multiplexing flexibility

Fully customizable assay panels can accommodate up to 50 targets of interest.

## Application in clinical settings

Automated sample preparation and Good Clinical Practice (GCP)-compliant workflows ensure suitability for clinical studies.

## References

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