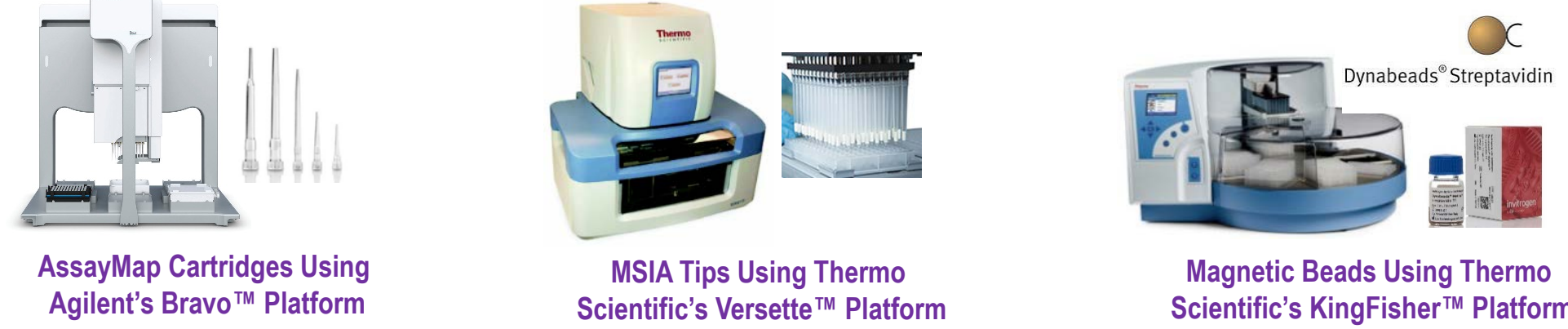


Integration of Automated Bead- and Cartridge-Based Immunocapture Workflows on a Generic Tecan Platform for LC-MS Bioanalysis of Protein Therapeutics and Biomarkers

Naiyu Zheng, Huidong Gu, Kristin Taylor, Yan J. Zhang, Jianing Zeng
Analytical and Bioanalytical Operations, Bristol-Myers Squibb Company, Princeton, NJ 08543

PURPOSE

- Recently, LC-MS technology has been increasingly used for the bioanalysis of protein therapeutics and biomarkers to complement ligand-binding assays (LBA) in drug discovery and development.
- Regardless of the direct mass spectrometric detection of intact proteins or indirect measurement of their tryptic surrogate peptides, immunocapture (IC) has become the sample preparation technique of choice to achieve the best sample cleanup for ultrasensitive detection of protein analytes by LC-MS.
- Current IC workflows include manual procedure (e.g. using magnetic beads), automated bead-based automated IC (e.g. Thermo Fisher Scientific's KingFisher™ platform) and cartridge-based automated IC (e.g. AssayMap Cartridges with Agilent Bravo, MSIA tips on Thermo Scientific's Versette platform, or PhyTip® columns on TECAN platform).



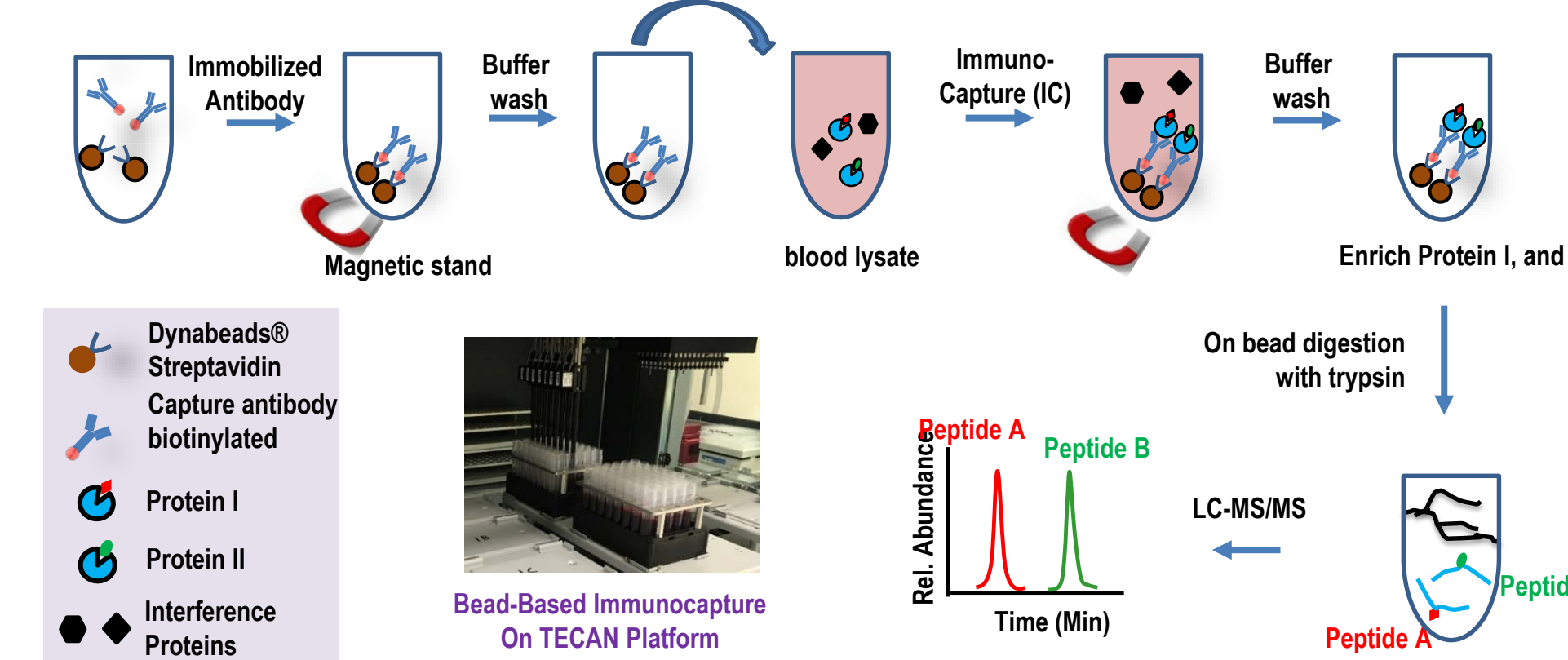
Integration of Immunocapture (IC) Workflow on a Generic Tecan Freedom EVO® Platform

- Many commercially available robotic liquid handlers (RLH) specialized for IC are highly vendor-specific. These IC workflows usually require significant capital investment for new RLH and the use of costly cartridges (tips) from the same vendor. In addition, they generally can do only one IC workflow: either bead-based or cartridge-based IC.
- The purpose of this study is to develop an integrated IC strategy for both bead- and cartridge-based IC on the existing Tecan Freedom EVO® platforms for LC-MS bioanalysis of protein therapeutics and biomarkers for diverse applications without additional capital investment.



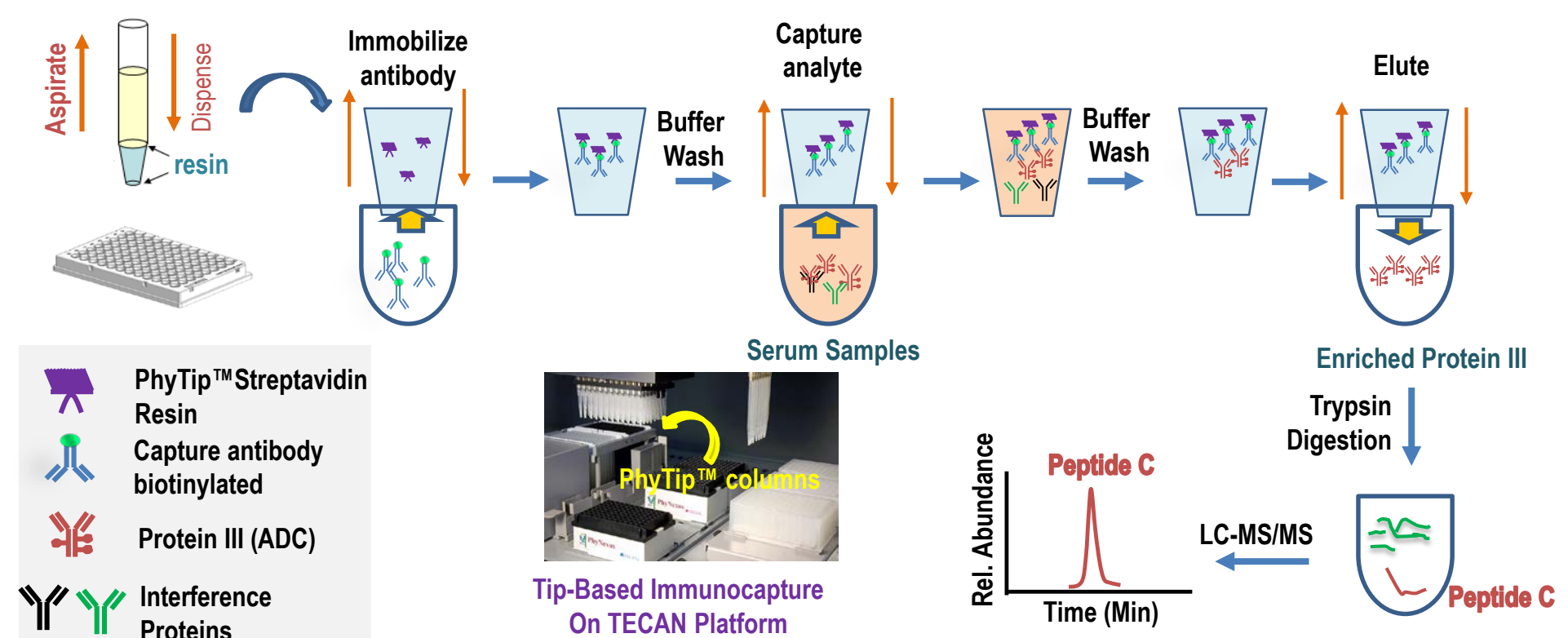
ASSAY 1: Bead-Based Immunocapture (IC) Used for the Quantitation of Two Modified Protein Biomarkers, Protein I and Protein II, in Monkey Blood Lysate

- To improve LC-MS/MS assay sensitivity, IC of the analytes from 3 mL blood lysate was required.
- Due to large sample volume, IC in 96-well format is impossible. Automated sample enrichment using bead-based IC in 4-mL sample tubes on Tecan is desirable.



ASSAY 2: Tip-Based IC Used for the Quantitation of An Antibody Drug Conjugate (ADC), Protein III, in Rat Serum by LC-MS/MS

- IC-LC-MS/MS for the bioanalysis of Protein III (ADC) in rat plasma was previously developed and validated using an Agilent Bravo™ platform. Evaluation of new IC workflow using PhyTip® columns on an existing Tecan has several advantages, such as cost saving in capital investment of new IC platform, less expensive with the tips, more flexibility and capability with Tecan than using standalone IC platforms.



METHODS

Assay 1: Bead-Based IC: LC-MS Bioanalysis of Two Modified Protein Biomarkers, Protein I and Protein II that Derived from an Endogenous Protein (MW: 90 KDa) in Monkey Blood Lysate.

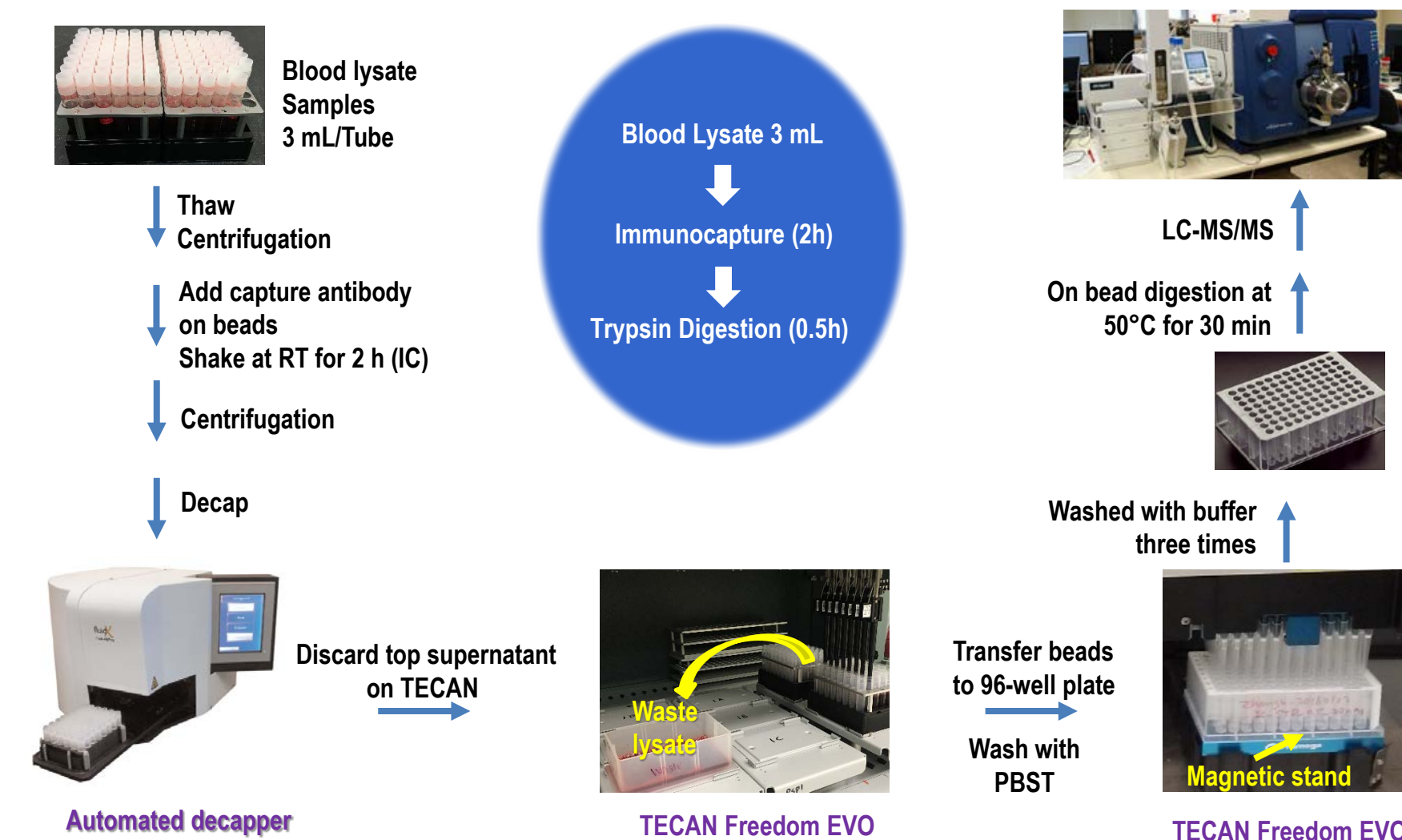
- A Fluidx® XSD-48 automated capping/decapping system was used for automated capping and decapping during IC and subsequent washes.
- Dynabeads® MyOne™ streptavidin T1 beads with the use of a biotinylated antibody were evaluated.
- IC of protein analytes was achieved by incubation of 3 mL of monkey blood lysate with the immobilized biotinylated antibody on magnetic beads in 4-mL automation friendly Greiner Bio-One tubes (Cat No: 127261).
- After IC of the protein analytes by shaking at room temperature for 2 h, followed by centrifugation, the unwanted biological matrix were transferred to the waste, and the remaining beads in the tubes were transferred into a 96-well plate on top of a MagnaBot 96 magnetic separation stand. After two PBST washes, followed by one wash with 25 mM NH₄OAc with 0.05% Tween-2, the samples were used for on-bead digestion.
- Quantitation of Proteins I and II was achieved by LC-MS/MS analysis of two representative surrogate peptides. For each processed sample, a 50 µL volume of the digest was injected into a Waters CORTECS™ UPLC C18+ column (2.1 x 100 mm, 1.6 µm) and analyzed on a Triple Quad 5500 system using MRM detection for Peptide A, Peptide B and SIL-Peptide A (IS).

Assay 2: Cartridge-Based IC: LC-MS Bioanalysis of an Antibody Drug Conjugate (ADC), Protein III in Rat Serum

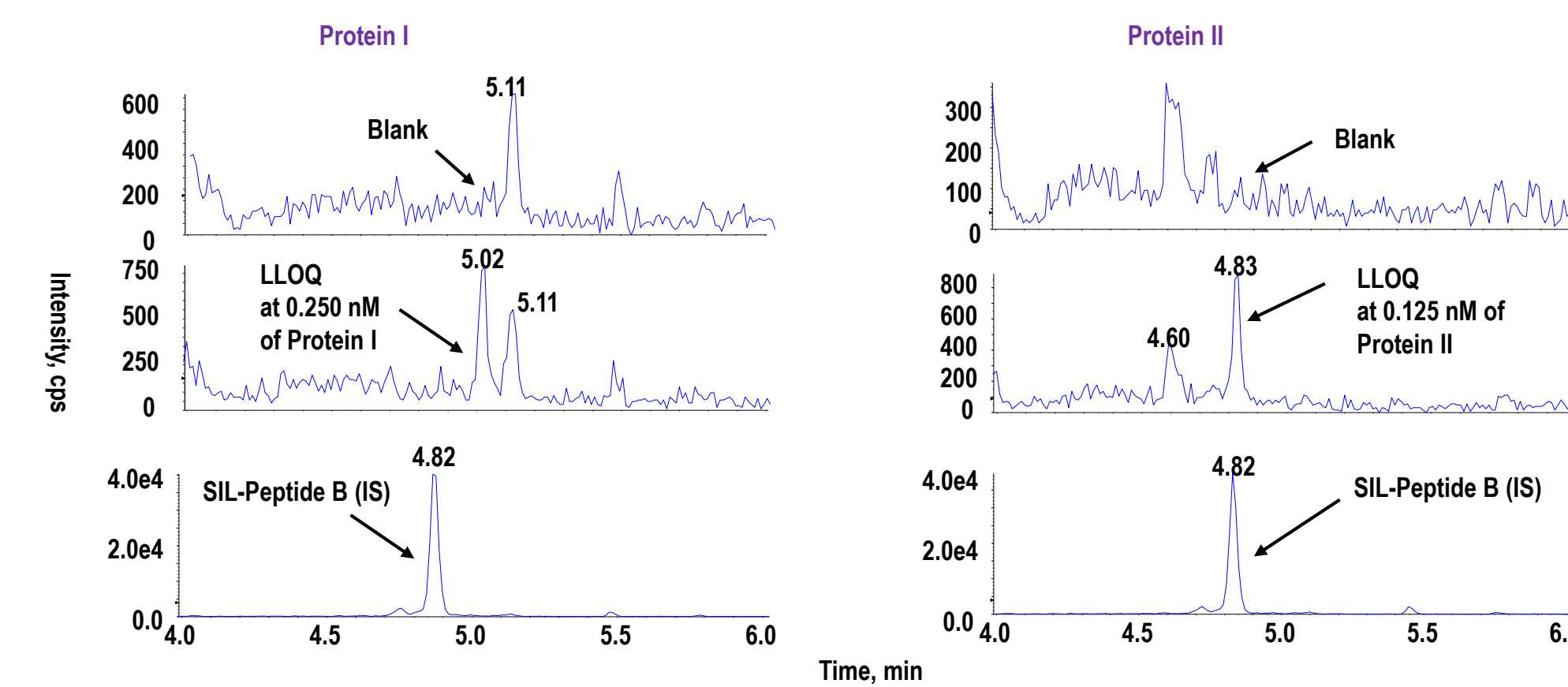
- PhyTip® streptavidin columns (PhyNexUS, Inc) (20 µL resin bed size on 200-µL Tecan tips) with the use of another biotinylated antibody were evaluated.
- Cartridge-based IC was evaluated for LC-MS bioanalysis of an antibody drug conjugate (ADC), Protein III in rat serum (15 µL serum + 145 µL PBS).
- The IC was performed by aspiration and dispensing of the serum samples through PhyTip® columns for 16 cycles (4.2 µL/sec, 10 s delay), followed by a wash prior to acid elution and trypsin digestion.
- Quantitation of Protein III was achieved by LC-MS/MS analysis of a surrogate peptide. For each processed sample, a 40 µL volume of the digest was injected into a Waters Acquity™ UPLC HSS T3 column (2.1 x 50 mm, 1.8 µm) and analyzed on a Triple Quad 6500 system with MRM detection for Peptide C and the IS (SIL-Peptide C), respectively.

RESULTS

Assay 1: Automation of Bead-Based IC Workflow on Tecan Platform

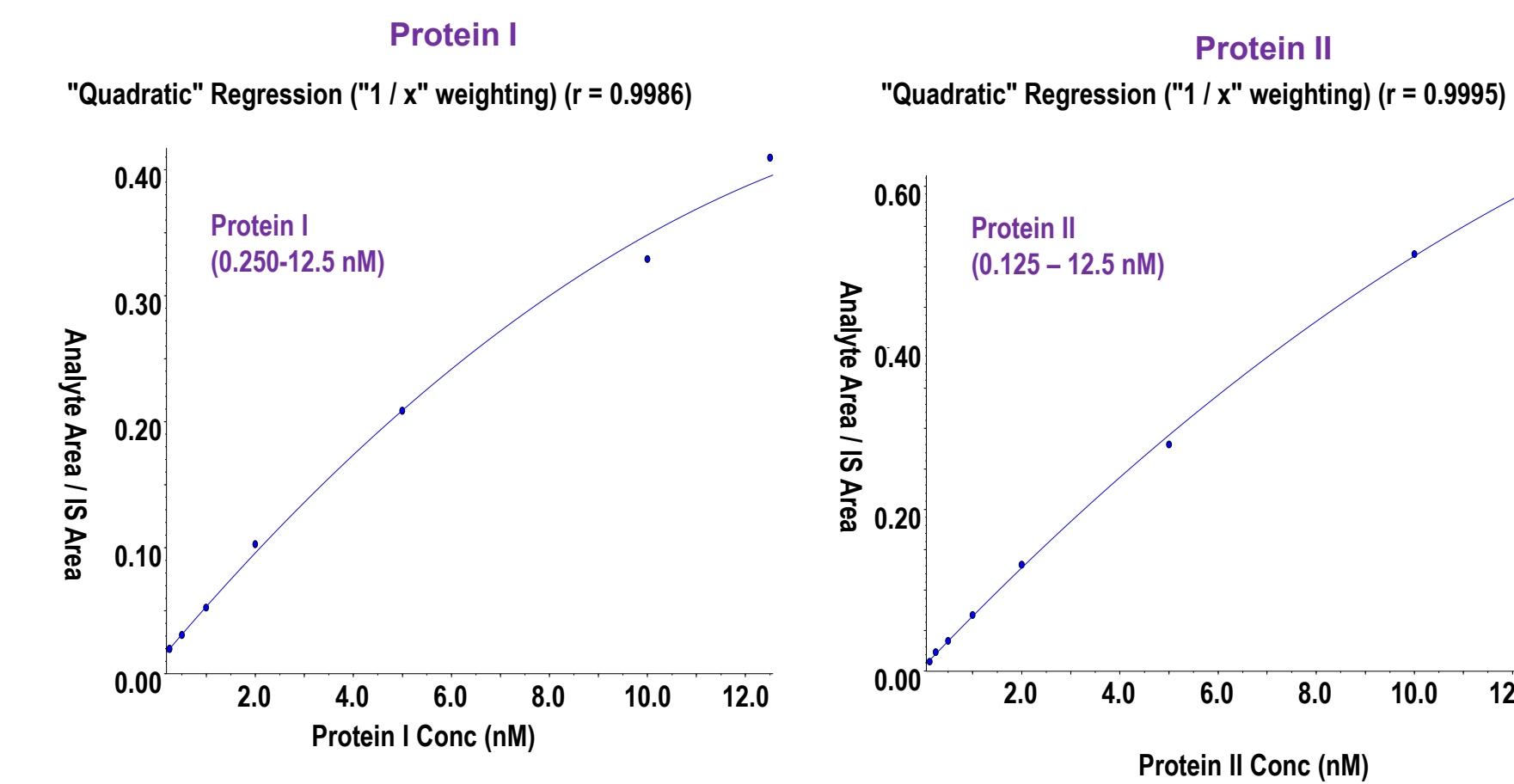


Assay 1: Representative Chromatograms for the Modified Protein Biomarker, Protein I and Protein II



RESULTS (Continued)

Assay 1: LLOQ of 0.125 nM and 0.250 nM in monkey blood lysate were achieved for Protein I and Protein II

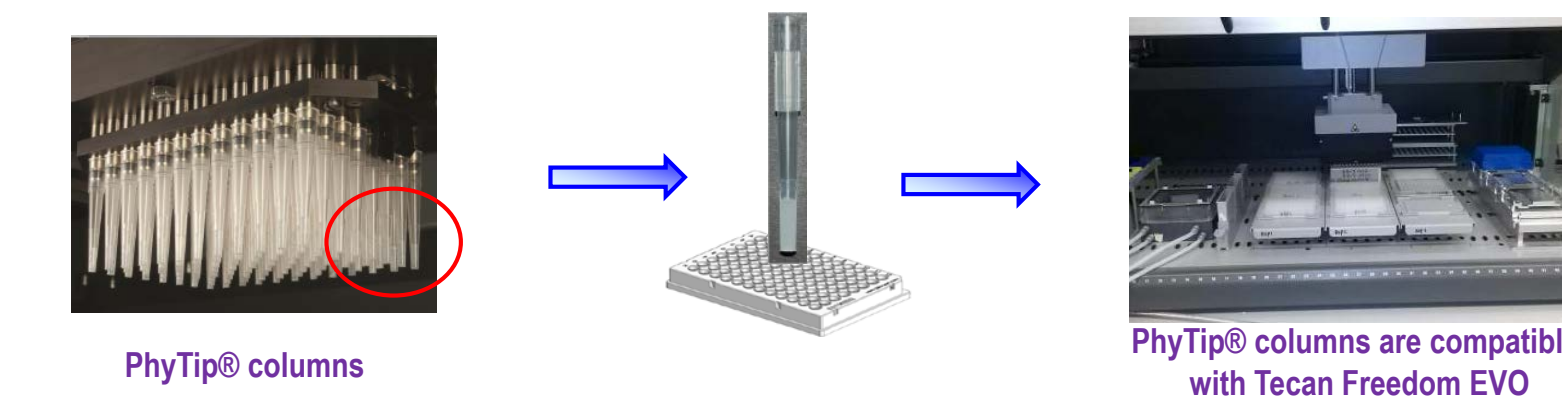


Assay 1: In Addition to Cost-Effectiveness, Bead-based Immunocapture Using a Tecan Allows the Sensitivity Improvement from Being Able to Process More Sample Volume in Each Sample With High Throughput

- One of the approaches to improve LC-MS/MS assay sensitivity for protein quantitation is to enrich protein analytes from a large sample volume using IC. However, when the volume is larger than the capacity of the 96-well format, it could be very challenging.
- By using a Tecan Freedom EVO platform with a Fluidx® XSD-48 automated capping/decapping system, sample preparation throughput was increased from 32 samples per batch performed manually to 96 samples per batch when performed on Tecan.

Platforms	TECAN Freedom EVO	Manual
Sample Racks	2 Fluidx® XSD-48 Racks	4 DynaMag™-5 Magnet Racks
Sample Size Per Process	96 Samples	32 Samples
Time for 96 Samples	1 day (< 4 hours)	3 days (3 separate batches are required)
Productivity Improvement	3-fold	N/A
Comment	Existing TECAN platform; Capable to handle all liquid transfer as well.	N/A

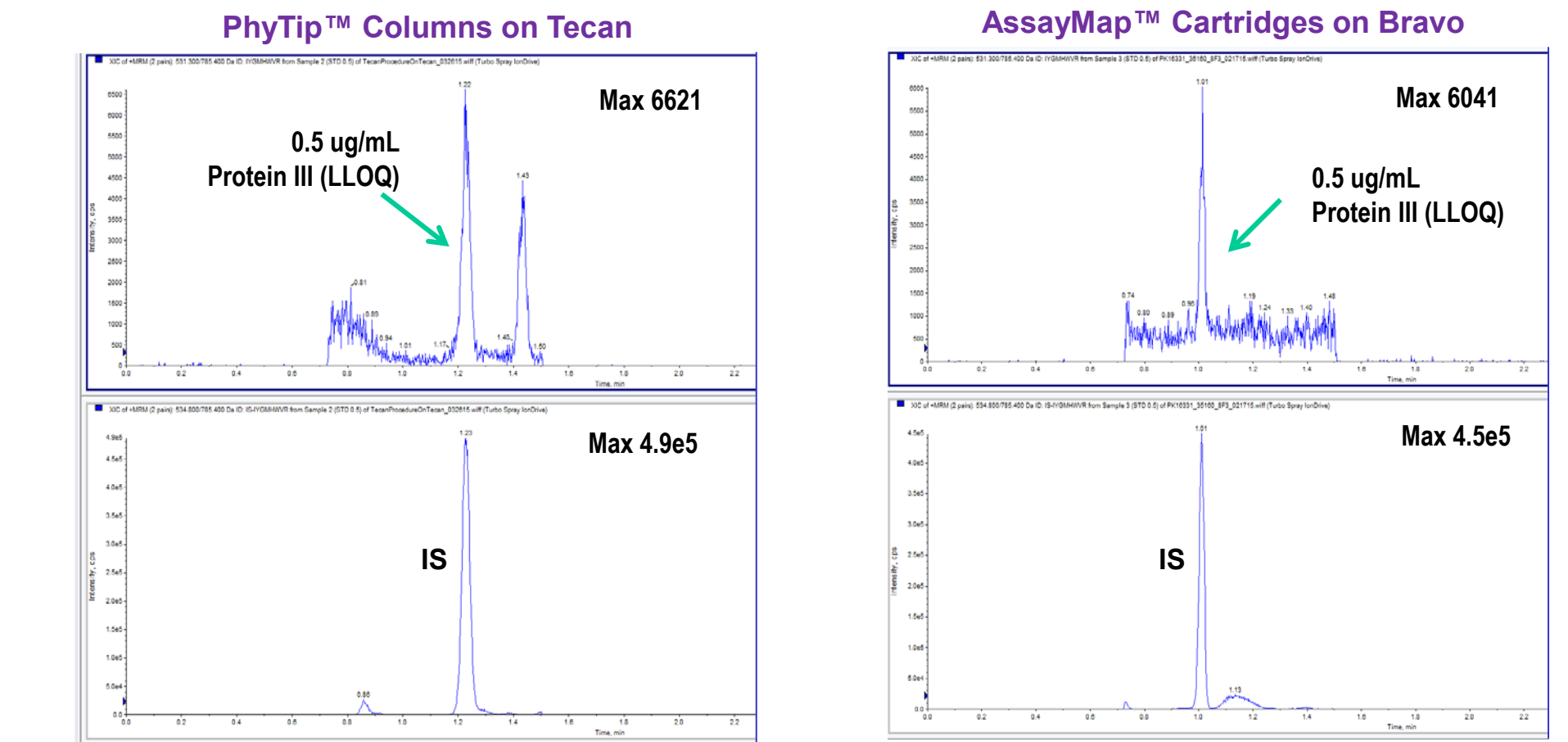
Assay 2: Automated Cartridge-Based Immunocapture (IC) Workflow on Tecan Platform



Step 1	Load PhyTip™ columns:	200-µL PhyTip™ columns packed with 20-µL of Streptavidin resin
Step 2	Equilibration:	200 µL PBS (pH 7.4, 10 mM), 4 cycles.
Step 3	Load antibody:	100 µL of 100 µg/mL, 8 cycles.
Step 4	Wash:	200 µL PBS (pH 7.4, 10 mM), 2 cycles.
Step 5	Capture from Serum:	160 µL (15 µL serum + 145 µL PBS), 16 cycles.
Step 6	Wash:	200 µL PBS (pH 7.4, 10 mM), 2 cycles.
Step 7	Elute:	40 µL 30% ACN with 0.1% formic acid, 4 cycles.
Step 8	Neutralize:	10 µL of 1 M NH ₄ HCO ₃ → Reduction/alkylation → Trypsin Digestion.

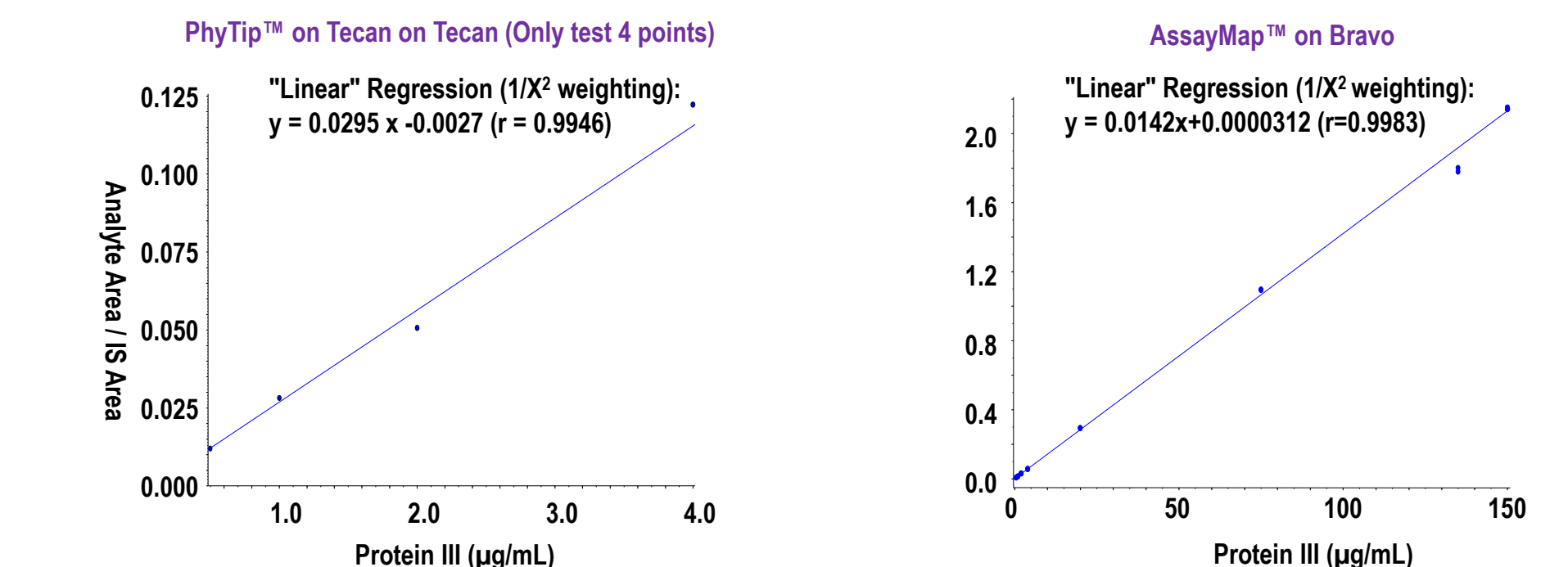
RESULTS (Continued)

Assay 2: Comparable LC-MS Responses at LLOQ Concentration Level Achieved from Immunocapture Using PhyTip™ Columns vs. AssayMap™ Cartridges



Assay 2: Comparison of Standard Curves for Protein III Obtained from Immunocapture Using Two Platforms (PhyTip™ Column on Tecan vs AssayMap™ Cartridges on Bravo)

- Preliminary results indicated that the standard curve generated from the 4 lowest concentration levels using PhyTip™ columns were comparable with those obtained from the previously validated method using AssayMap™ cartridges.



Assay 2: Immunocapture Using PhyTip™ on Tecan Freedom EVO Is More Cost-Effective than Agilent's Bravo When New Capital Investment is not Required

Platforms	TECAN Freedom EVO	Agilent's Bravo
Tips or Beads	PhyTip™ Column	AssayMap™ Tip
Typical Volume Size for Tips	200 µL	50 µL
Typical Packed Resin Bed on Tips	20 µL	5 µL
Maximal capacity for antibody loading	138 µg IgG	100 µg IgG
Time for One Plate	45 min	> 1 h
Capital Investment	Existing platforms	\$100K
Comment	PhyTip™ columns are also compatible with Tecan, Hamilton and other platforms; Capable to handle other liquid transfer as well.	May need other liquid handlers for other liquid handling, such as sample dilution.

CONCLUSIONS

- New immunocapture (IC) strategy has been successfully developed by integrating and automating both bead- and cartridge-based IC on existing and generic Tecan Freedom EVO® platforms for LC-MS bioanalysis of protein therapeutics and biomarkers.
- This strategy allows the IC enrichment of protein analytes from diverse biological sample sizes and applications with high flexibility and high throughput without additional capital investment.

ACKNOWLEDGEMENTS

We would like to thank Dr. Lee Hoang from PhyNexus Inc., Ms. Adrienne Chow and Mr. Bob Clark from Tecan US for their help in Tecan setup for cartridge-based immunocapture using PhyTip™ columns.