Alturas Analytics, Inc.

The LC-MS Experts

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Highly Efficient Bioanalysis of Proteins by Immunocapture Microtips and MFLC-MS/MS

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Senior Principal Scientist
Alturas Analytics, Inc.

Bioanalytical Challenges

- **Selectivity**
  - mg/mL concentrations of proteins in plasma
  - Thousands of possible peptides after digestion
  - Identical peptide fragment to target
  - Matrix effects from co-eluting peaks
    - Ion suppression/enhancement

- **Sensitivity**
  - Peptide is a small % of total protein
  - Low recovery from multistep processes

Research Goals

- Develop Accurate/precise/selective large molecule methods
- Methods can be validated per BMV Guidance
- Methods can be automated

Gains in Selectivity and Sensitivity

- High resolution mass spectrometer
- Chromatographic improvements
  - Microflow LC-MS – optimized source
  - More resolution
  - Different stationary phase or orthogonal chromatography
- **Off-line sample preparation**

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MFLC Coupled to Sciex 6500+ MS/MS

MFLC-MS/MS Advantages

- Higher ionization efficiency at lower flowrates
- Higher overall analyte response
- Decreased solvent consumption
- Less waste production
- Cleaner instrument

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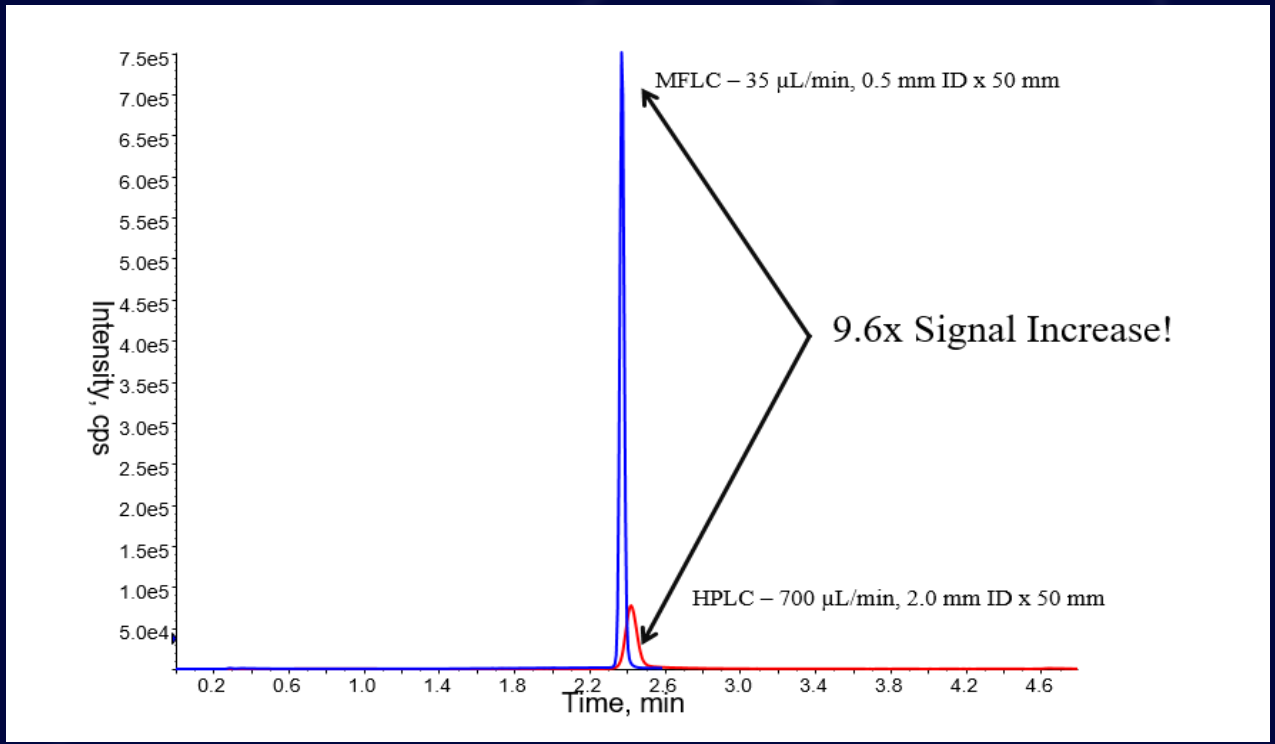
MFLC Signal Enhancement

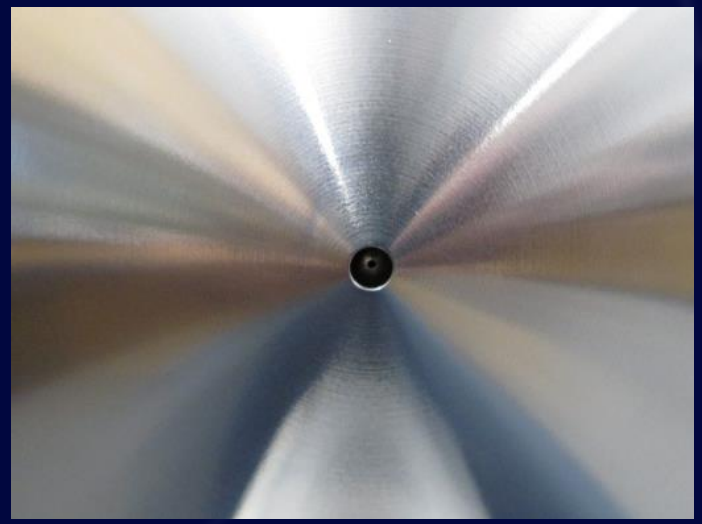
MFLC-MS/MS and HPLC-MS/MS chromatograms from the analysis of a 2.0 µL injection of Methotrexate extracted from human plasma



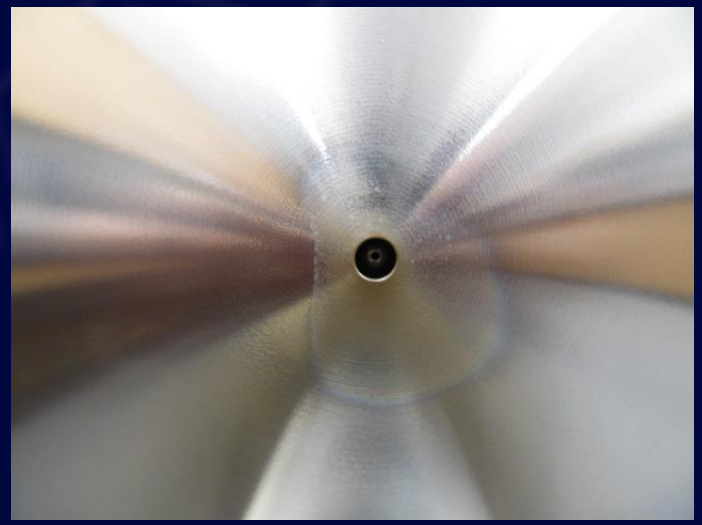
Cleaner Instrument

SCIEX 5500 interface plate after approximately 400 injections

MFLC System



HPLC System



Off-line Sample Preparation Techniques

- Pellet Digestion
- Magnetic bead/resin capture
- Membrane-based affinity purification
- MSIA

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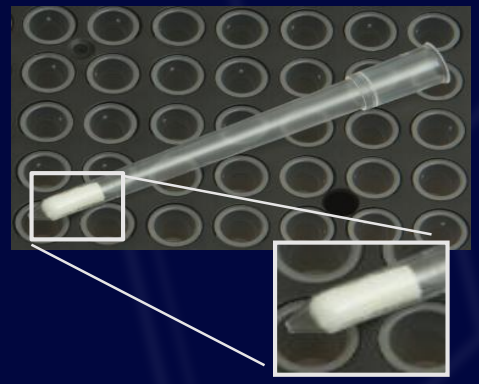
What is MSIA (Mass Spec Immuno Assay)?



- Monolithic microcolumn bonded with affinity ligand
- 96 well format for easy automation
- Compatible with Novus I, Tecan EVO and Versette
- Available in Streptavidin, Insulin, Protein A, G, A/G and Custom (direct attachment of an Ab, protein or peptide)

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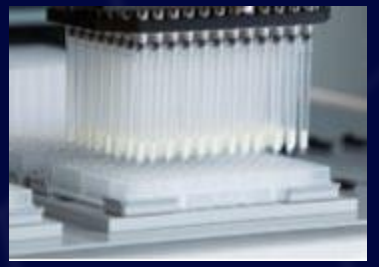
Magnetic Beads

MSIA



Criteria	Magnetic Beads	MSIA
Automation	Difficult	Simple
Selective	Good	Great
Elution Volume	~100 µL	30 µL
Capacity	Unlimited	Limited
Versatility	Flexible to any automation	Limited
Speed	Time/Labor Intensive	≥2X Faster

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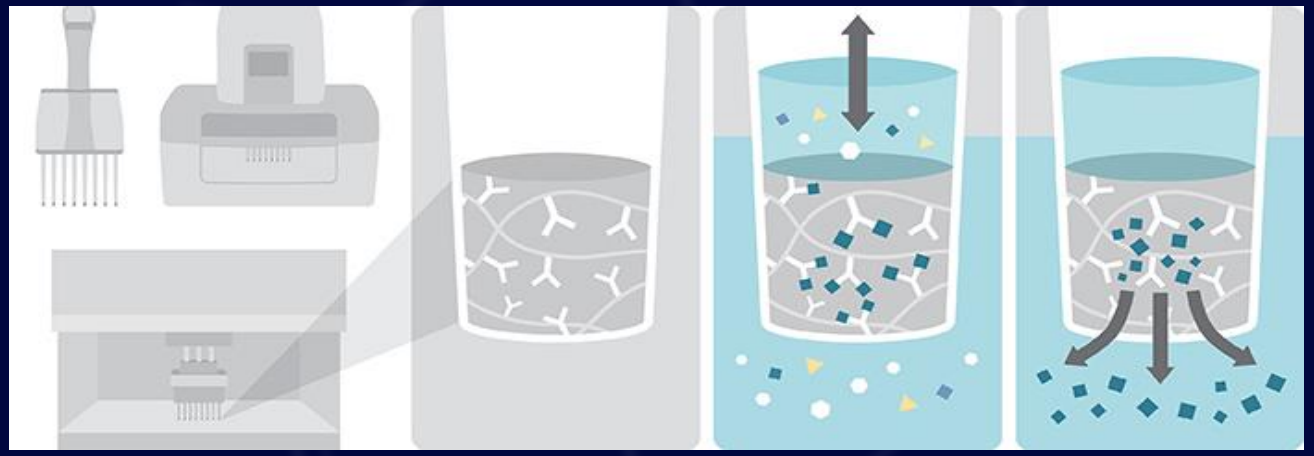
Preparation Procedure MSIA™

- Aliquot plasma
- IS
- Rinses
- *Biotinylated Molecule
- Elution Solvent



- *Capture Biotinylated Molecule
- Rinse

- Capture Target
- Rinse

- Elute Water 2% FA

Denature, Reduce, Digest Eluent

* If necessary

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Example #1: MSIA Humira

Humira LC-MS/MS Challenges

- **Selectivity**
  - Fully Human
  - NCBI BLAST Results
    - Light Chain: 97% amino acid sequence match
    - Heavy Chain: 94% amino acid sequence match
  - SKYLINE Peptide Predictions
    - Zero selective peptides predicted from trypsin digest
- **Sensitivity**
  - How low is good enough?

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Heavy Chain Skyline Predictions

Parameters: 8-20 amino acids

Sequence	Q1	Q3
GPSVFPLAPSSK	593.8	1129.6
GPSVFPLAPSSK	593.8	846.5
GPSVFPLAPSSK	593.8	699.4
GPSVFPLAPSSK	593.8	602.4
NSLYLQMNSLR	669.8	1024.5
SLSLSPGK	394.7	701.4
SLSLSPGK	394.7	588.3
VVSVLTVLHQDWLNGK	904.5	1209.6
VVSVLTVLHQDWLNGK	904.5	1110.6
EPQVYTLPPSR	643.8	1157.6
EPQVYTLPPSR	643.8	932.5
EPQVYTLPPSR	643.8	833.5
EPQVYTLPPSR	643.8	670.4
EEQYNSTYR	595.3	931.4
EEQYNSTYR	595.3	803.4
EEQYNSTYR	595.3	640.3
FNWYVDGVEVHNAK	893.4	1067.5
FNWYVDGVEVHNAK	893.4	968.5
EVQLVESGGGLVQPGR	812.9	840.5
EVQLVESGGGLVQPGR	812.9	1056.5
EVQLVESGGGLVQPGR	812.9	927.5
ALPAPIEK	419.8	654.4

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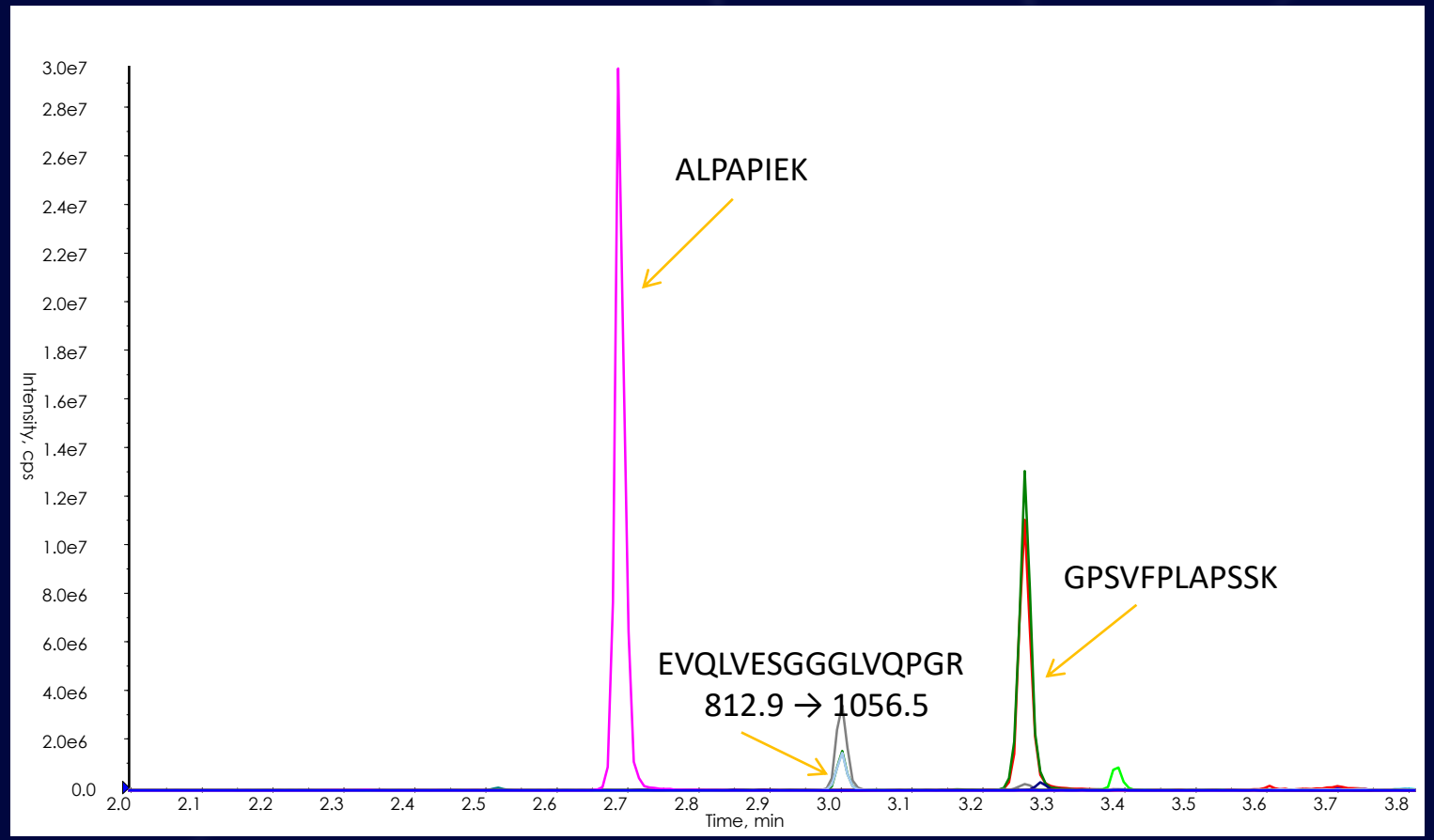
Humira in Buffer

Skyline MRM Predictions



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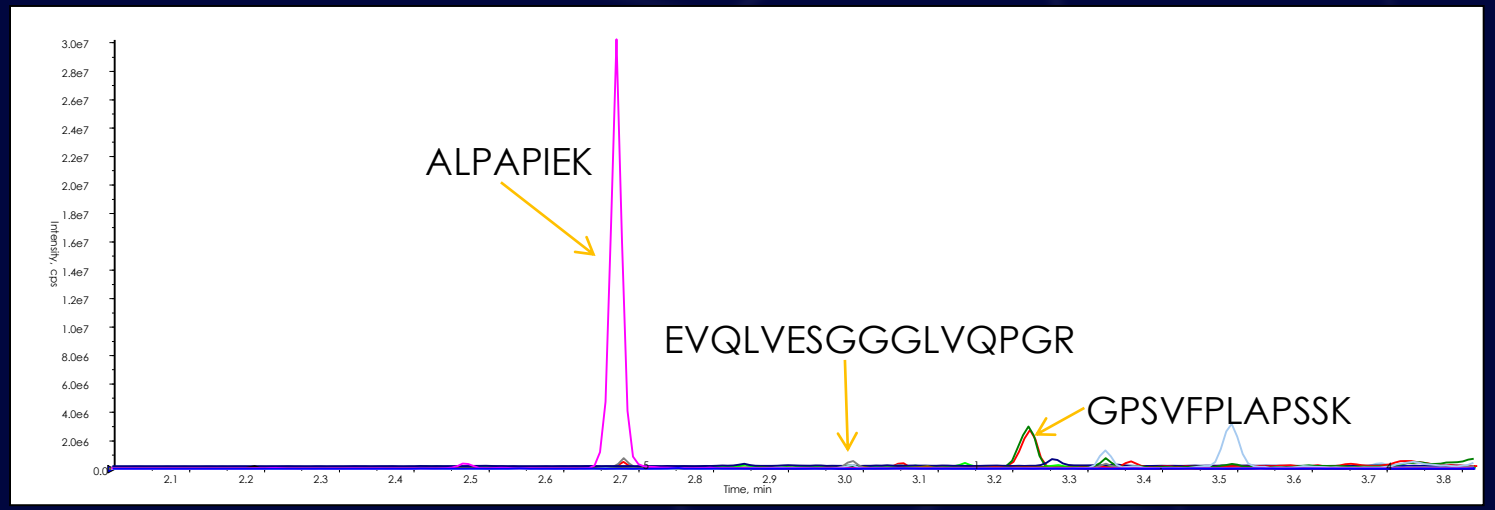
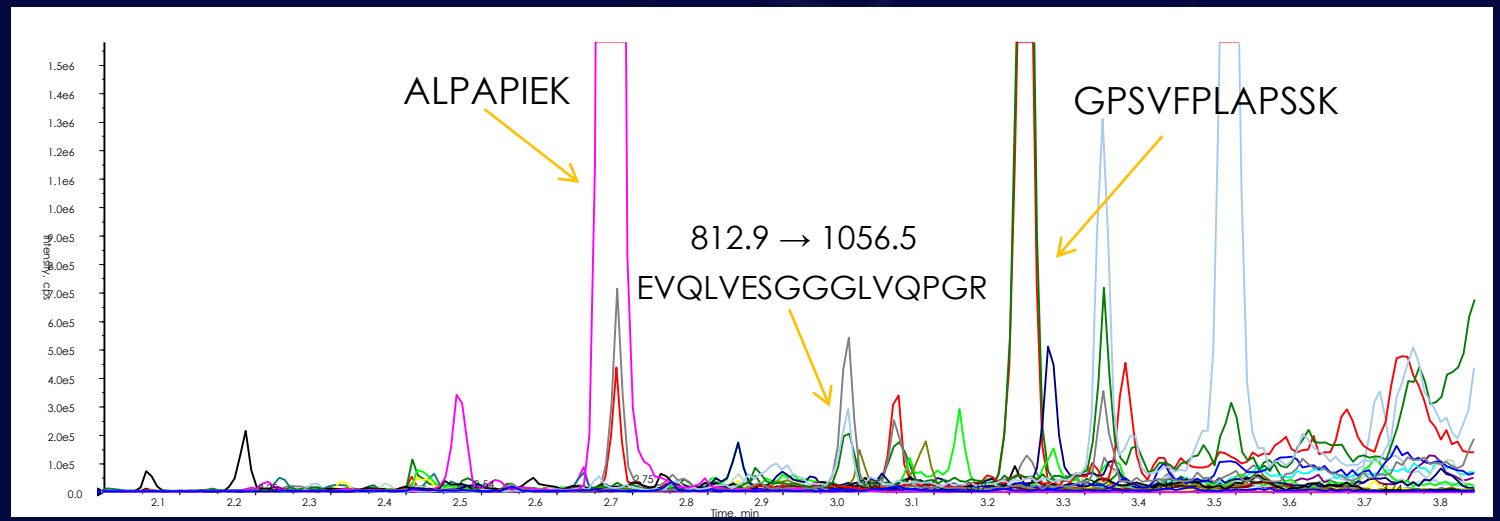
Pellet Digestion of Human Plasma

Skyline MRM Predictions





Materials

- SigmaMAb Adalimumab – Sigma
- Internal Standard: SILu™MAb Adalimumab Stable-Isotope Labeled Monoclonal Antibody [$^{13}C_6$, $^{15}N_4$]-Arginine and [$^{13}C_6$, $^{15}N_2$]-Lysine - Sigma
- 300 μL Mass Spectrometric Immunoassay (MSIA) Streptavidin D.A.R.T.S -Thermo Scientific
- Biotinylated Human TNFα Protein - ACROBiosystems
- Water, formic acid, PBS Buffer, TCEP and Trypsin (Promega)

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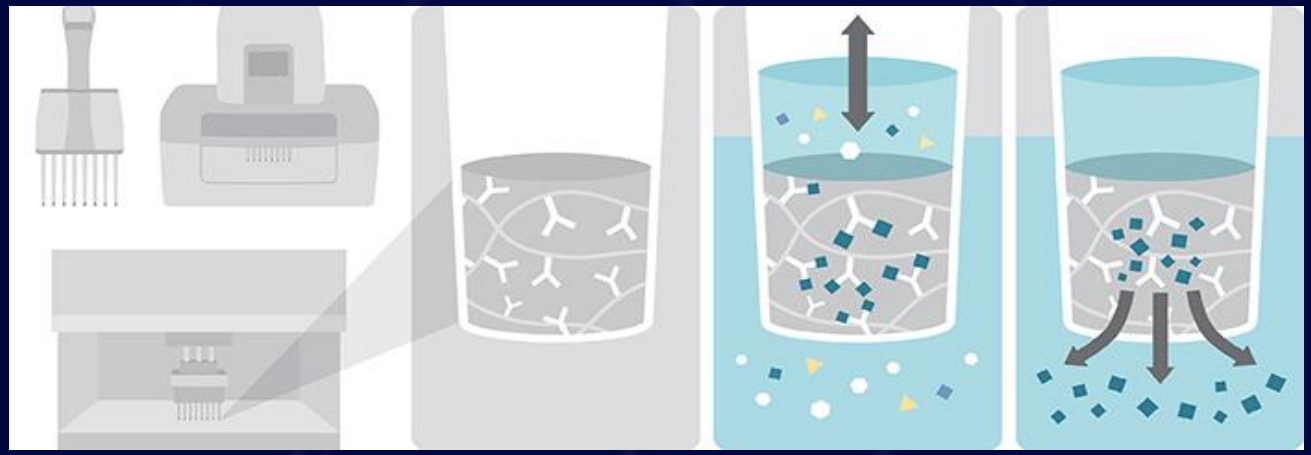
Preparation Procedure MSIA™

- Aliquot plasma
- IS
- Rinses
- Biotin-TNFa
- Elution Solvent



- Capture Biotin-TNFα
- Rinse

- Capture Humira
- Rinse

- Elute Water 2% FA

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Methods

Sample Preparation MSIA Eluent

- Adjust pH 2M Tris buffer
- Denature/Reduce with Heat (80°C) & 0.1 M TCEP 15 minutes
- Add Ammonium Bicarbonate/Calcium Chloride
- Digest with 10 µL of trypsin (0.8 µg/mL)
- Incubate at 50°C for 1 hour
- Stop digestion with 40% formic acid solution

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Methods

MFLC-MS/MS

- Quantitation peptide: EVQLVESGGGLVQPGR (2X Charge)
  - Humira: 812.9 → 1056.5
  - Internal Standard: 818.101→1066.6
- Waters Acquity M-Class Binary LC Systems
- Gradient using acetonitrile and water with 0.1% formic acid
- Flow rate: 10 µL/min
- Column: Halo Biphenyl (50 X 0.3 mm, 3 µm)
- Column temperature: 50°C
- Sciex 6500+ operating in MRM mode
- ESI
- Positive ion mode
- Optiflow Source (1-50 µL/min probe)

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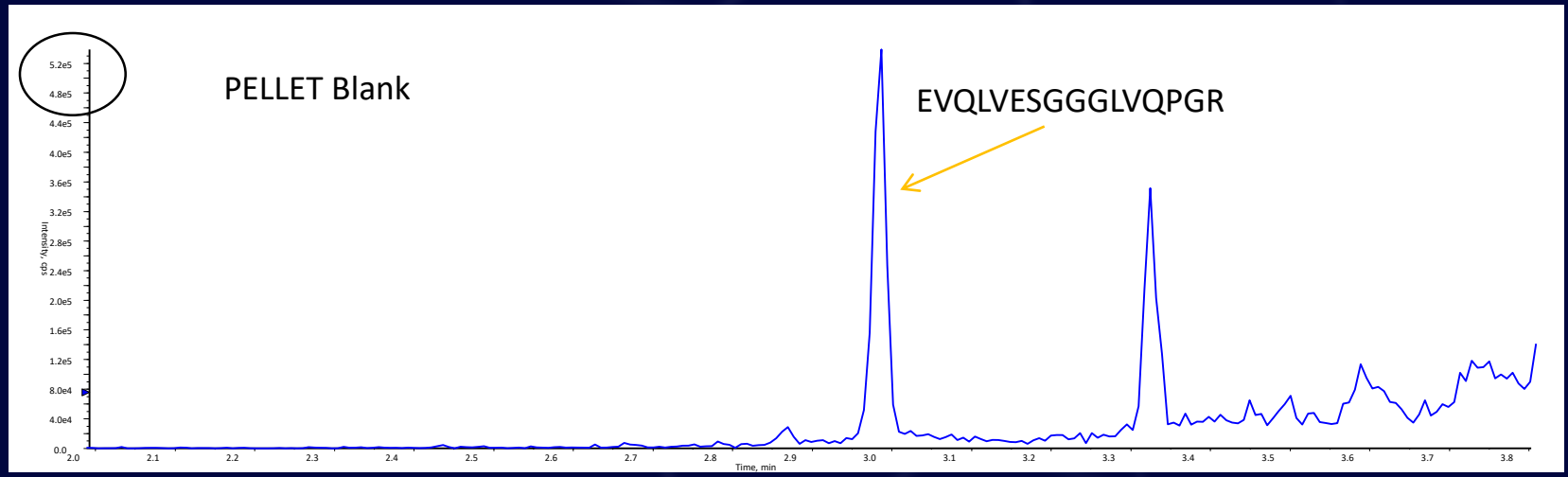
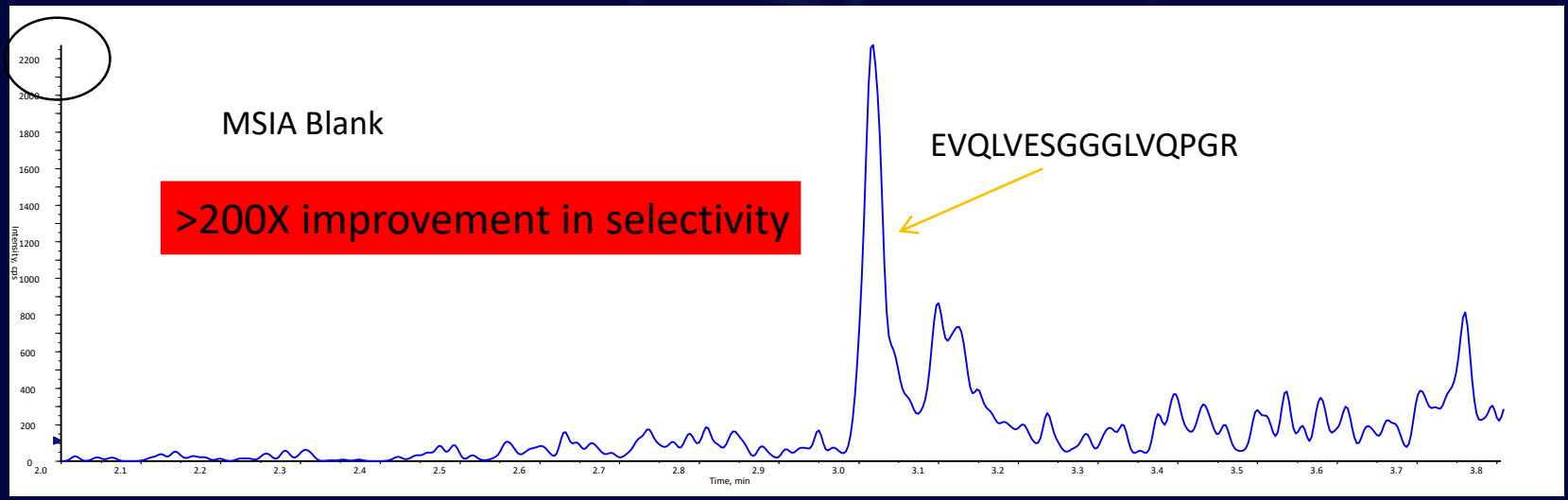
Pellet Extraction vs MSIA (Blank Plasma)





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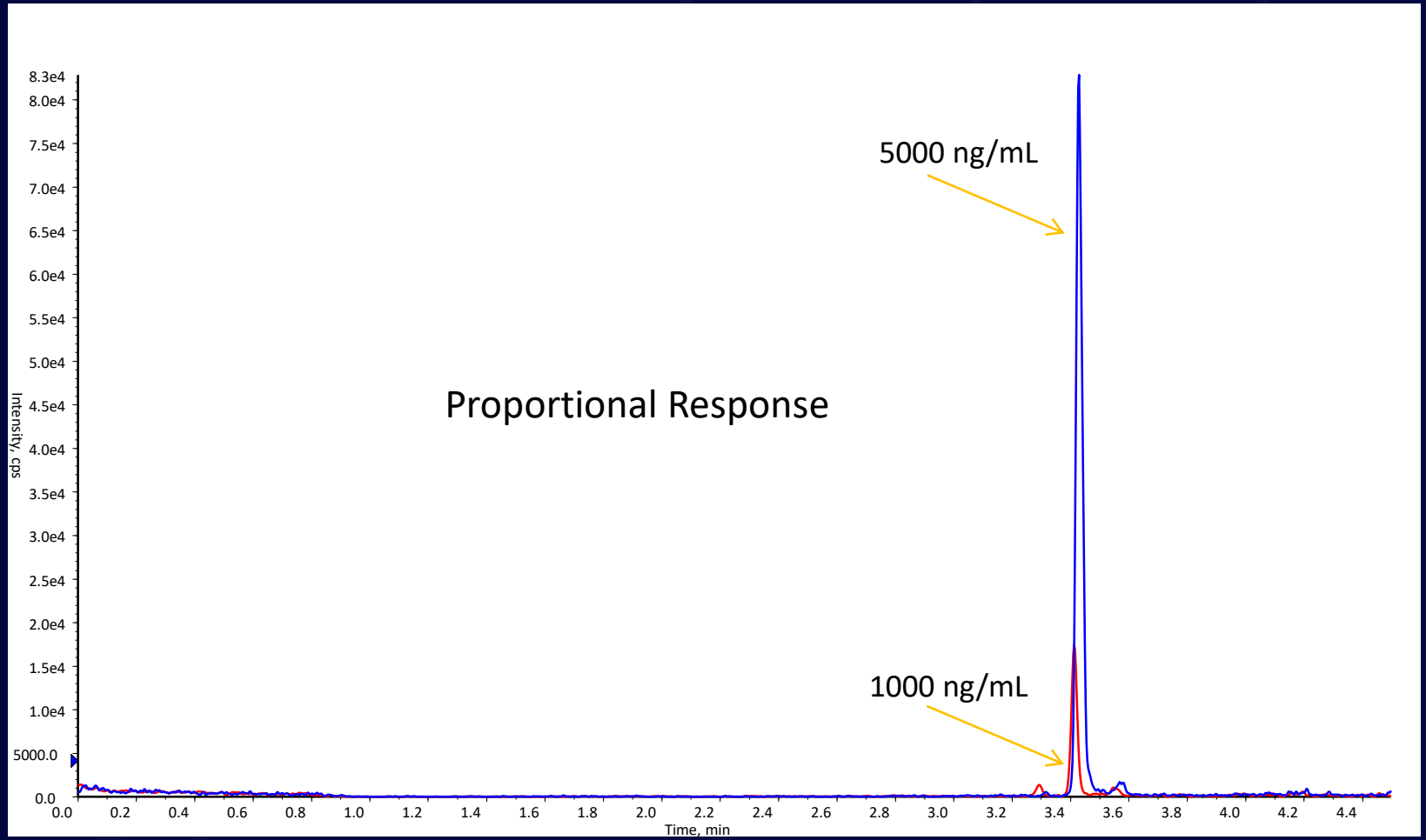
1000 and 5,000 ng/mL Humira Extracted from Human Plasma



Analytical Results

- 1000-20,000 ng/mL dynamic range
- A/P 90% ± 5%
- Blanks <15% of LLOQ Response

Example #1 Conclusions

- Accurate/precise/selective method developed to analyze Humira using MSIA and MFLC-MS/MS
- Sample Preparation ~2X Faster than bead method
- Method can be validated to support clinical studies

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Example #2:
MSIA Antibody Drug Conjugate

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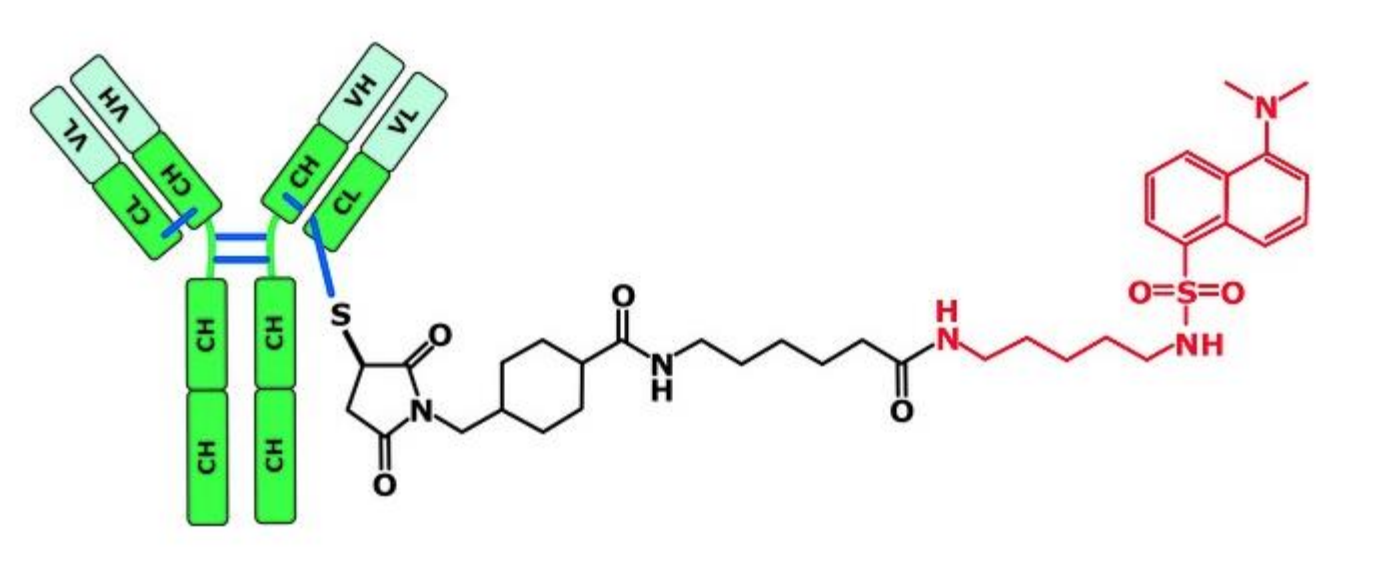
SigmaMAb ADC Conjugate Mimic



- Recombinant Monoclonal IgG1 Human Antibody
- Linked to dansyl-fluorophores
- Extracted from Rat Plasma

Materials

- SigmaMAb ADC Conjugate Mimic – Sigma
- Internal Standard: SILu™MAb Infliximab- Sigma
- 300 μL Mass Spectrometric Immunoassay (MSIA) Streptavidin D.A.R.T.S -Thermo Scientific
- Biotinylated anti-igG-Fc - Thermo

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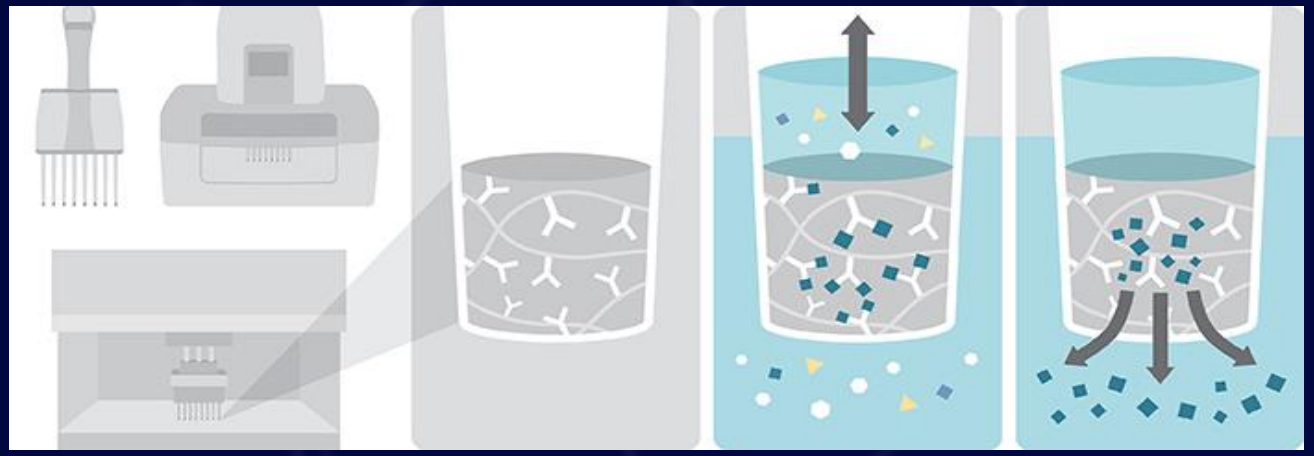
Preparation Procedure MSIA™



- Aliquot 25 µL plasma
- IS
- Rinses
- Biotin-anti-igG
- Elution Solvent

- Capture Biotin-anti-igG
- Rinse

- Capture ADC
- Rinse

- Elute Water 2% FA

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Methods

Sample Preparation MSIA Eluent

- Adjust pH 2M Tris buffer (14 µL)
- Denature/Reduce with Heat (80°C) & 0.1 M TCEP 15 minutes (5 µL)
- Add Ammonium Bicarbonate (10 µL)
- Add Calcium Chloride (8 µL)
- Digest with 10 µL of trypsin (80 mg/mL)
- Incubate at 50°C for 1 hour
- Stop digestion with 40% formic acid solution (5 µL)

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Methods

MFLC-MS/MS

- Quantitation peptide
  - ADC: ALPAPIEK (2X Charge)
  - Internal Standard: YASESMSGIPSR
  - ADC: 420 → 327
  - Internal Standard: 648 → 845
- Waters Acquity M-Class Binary LC Systems
- Gradient using acetonitrile and water with 0.1% formic acid
- Flow rate: 50 µL/min
- Column: Kinetex Biphenyl (50 X 1.0 mm, 3 µm)
- Column temperature: 50°C
- Sciex 6500+ operating in MRM mode
- ESI Positive ion mode
- Optiflow Source (1-50 µL/min probe)

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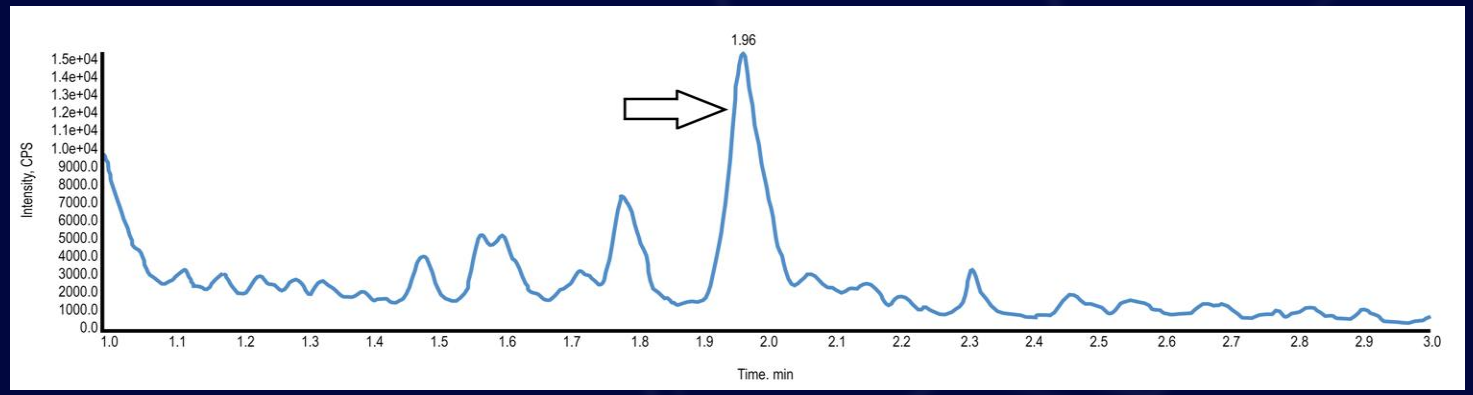
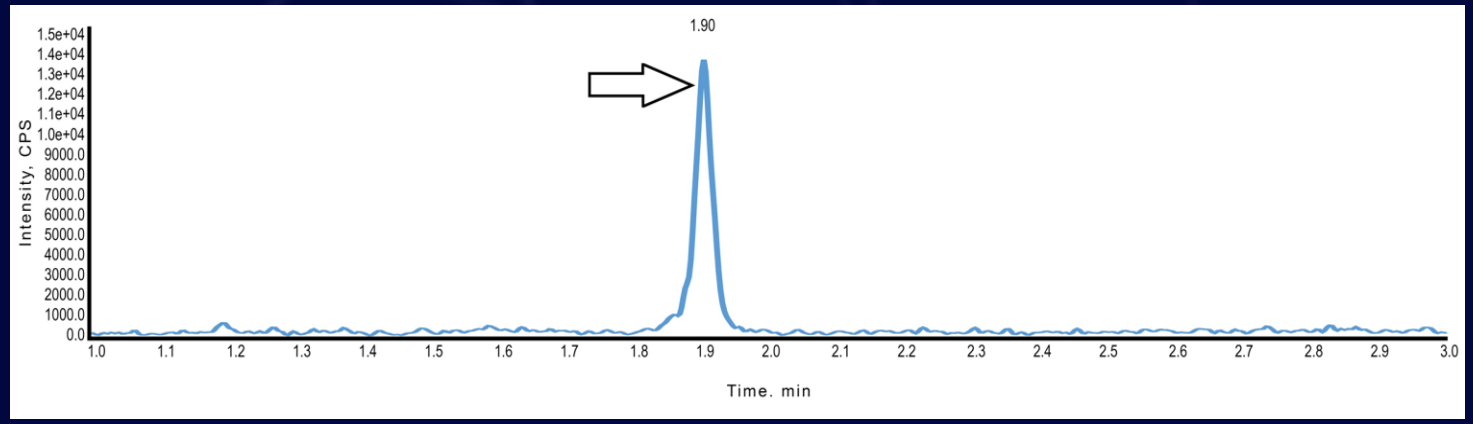
Pellet Extraction vs MSIA

SigmaMAb ADC Mimic (Digested to peptide) 100 ng/mL Extracted from Rat Plasma using Pellet Digestion



SigmaMAb ADC Mimic (Digested to peptide) 10.0 ng/mL Extracted from Rat Plasma using MSIA Procedure



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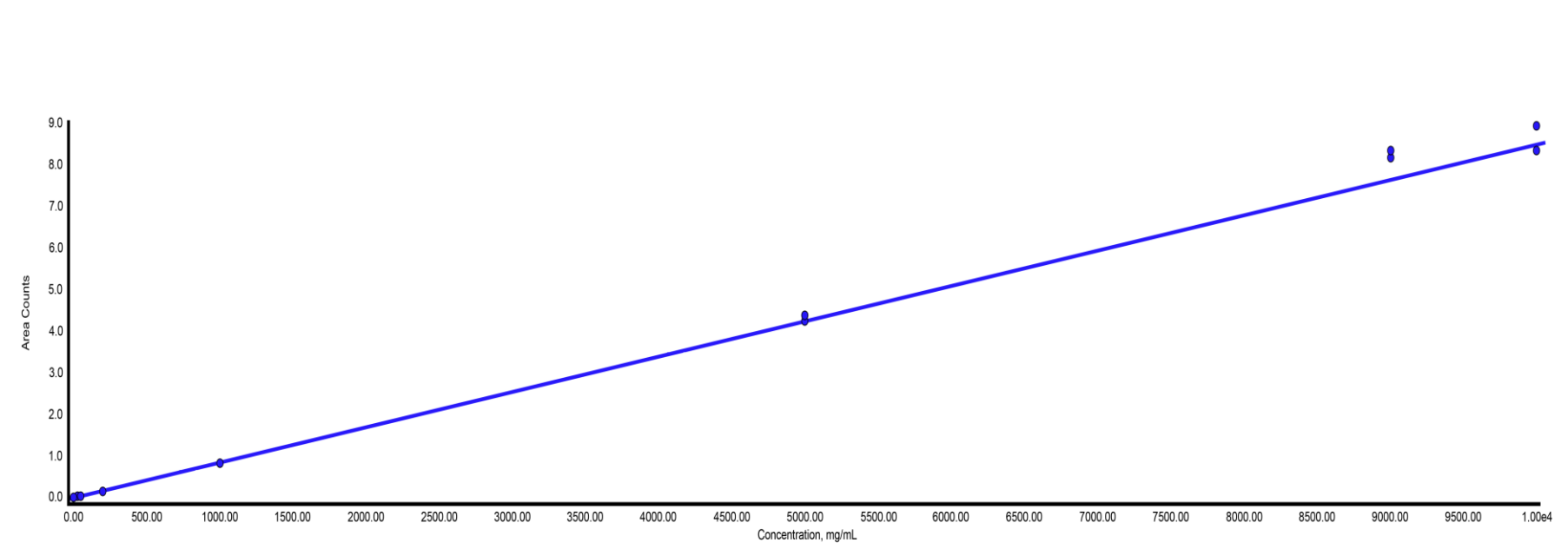
Linear Calibration Curve Generated from the Analysis of 16 Standard Curve Samples (8 Concentrations in Duplicate) Extracted from Rat Plasma
r=0.9959, 10.0-10,000 ng/mL



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Analytical Results

- 10.0-10,000 ng/mL dynamic range
- Linear response
- Method Accurate/Precise

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Emerging Technology

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TaKaRa Rapid Capturem Purification

- Rapid purification with minimal steps
- High-capacity, membrane-based affinity purification
- Compatible with any 96 plate automation
- Available in Streptavidin, Protein A and G
- High recovery of protein

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Example Method

Sample Preparation Capturem Protein A

- Dilute plasma with 500 µL Capturem Buffer
- Condition wells with 500 µL Capturem Buffer
- Add entire sample volume
- Wash with 500 µL Capturem Buffer
- Elute with 250 µL Elution buffer
- Add digestion component and Incubate
- Evaporate and reconstitute
- Inject onto LC-MS/MS

Conclusions

- MFLC couple with optiflow results in greater sensitivity for target peptides
- MSIA sample preparation provides clean, reproducible extracts using automation
- Capturem purification products offer rapid, simple and automatable preparation procedure

Acknowledgements

- Team Alturas
    - Sharon DeChenne
    - Jennifer Zimmer
    - Cody Hawkins

- Thermo-MSIA™
- TaKaRa
- Sciex
- Waters

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Questions?

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