Validation of an immunoprecipitation, digestion and immunoaffinity LC/LC/nanoLC-MS/MS assay for human β-nerve growth factor (NGF) and implementation in support of clinical trials

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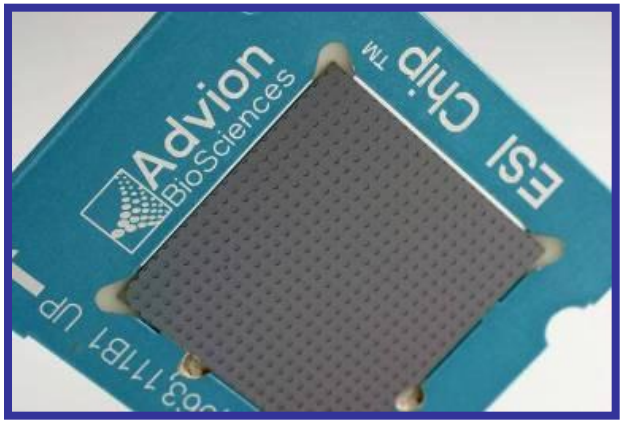
Macromolecule & Biomarker LC/MS

- Quantitative determination of biotherapeutics, antibodies, proteins, peptides, lipids, and small molecule biomarkers
  - GLP-biotherapeutic studies
  - Quantitative analysis for PD/PK/TK
  - Biomarker studies
  - Immunogenicity determination



- Advanced techniques
  - Hamilton Star automated liquid handling robotics
  - Immunoprecipitation using capture antibodies (magnetic bead assays)
  - Immunoaffinity columns using capture antibodies (made in-house)
  - Dionex Ultimate 3000 for multi-dimensional (up to 4-D) LC/NanoLC/MS
  - Advion TriVersa NanoMate® with ESI Chip™ Technology
  - Thermo TSQ Vantage triple quadrupole mass spectrometers

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Special Considerations for Protein Quantitation

- Incomplete characterization of the reference compound
  - particularly for protein biomarkers
- Should the internal standard be the isotopically-labeled protein?
  - Ideal, high expense
  - may be necessary to achieve small molecule 4/6/15 bioanalytical regulatory guidance
- Second best, isotopically-labeled peptide as internal standard that undergoes enzymatic cleavage on each end of the target peptide
  - more cost effective but more likely 4/6/20 due to differences in extraction and/or recovery yield between analyte and IS
- LCMS signature is often a proteolytic fragment of a target protein
- Assays are often complex (eg: immuno-enrichment →proteolysis → multi-dimensional LC → nano-electrospray ionization)

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LC/MS/MS Method Validation of Endogenous Compounds

- No formal regulatory guidance (EMA or FDA) exists for LC-MS/MS method validation for endogenous biochemicals (biomarkers)
- Anticipated that the next release of FDA BMV guidance will include endogenous compound assays
- Fit-for-Purpose (FFP) approach recommended by J.W. Lee paper*
- Many follow modified version of FDA BMV guidance

- Adopt Standard Operating Procedure (SOP) for Validation of Biomarkers by LC-MS, but allow for Fit for Purpose approach:
  - Use the SOP as a menu of experiments, with clear guidance on how each experiment is performed
  - Use a Validation Plan to select the experiments and define acceptance criteria consistent with the unique challenges of the specific biomarker and with the intended use of the data

*J. W. Lee et. al. Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement, Pharmaceutical Research, Volume 23, No. 2, February 2006, pp 312 – 328

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Assay Overview

- Human β– Nerve Growth Factor (β–NGF)
  - 13.5 kDa protein
  - Bound by Monoclonal Antibody Drug
- First immunoprecipitation method at Advion
- First assay to utilize nanoLC-MS
- First use of TriVersa NanoMate® with ESI Chip™ Technology
- Unique challenges to method development:
  - Three day sample prep
  - Addition of IS following immunoprecipitation
  - Monoclonal antibody competitively binds NGF
  - 3D LC separation
  - 35 hour run time (210 injections) with nanoflow LC-MS/MS

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Standard, QC and Sample Preparation

- All samples are prepared in an ice bath
- Calibration standards are prepared in a surrogate matrix consisting of 5% BSA in PBS
- Calibration standards are prepared in a surrogate matrix
- Quality Control samples are prepared 2x concentration in human serum and diluted with surrogate matrix to the target concentration
- 300µL serum sample is incubated overnight at 4°C with biotinylated anti-β-NGF polyclonal antibody
- The antibody captures total β-NGF
- Analytical QC concentrations corrected for endogenous level of β -NGF

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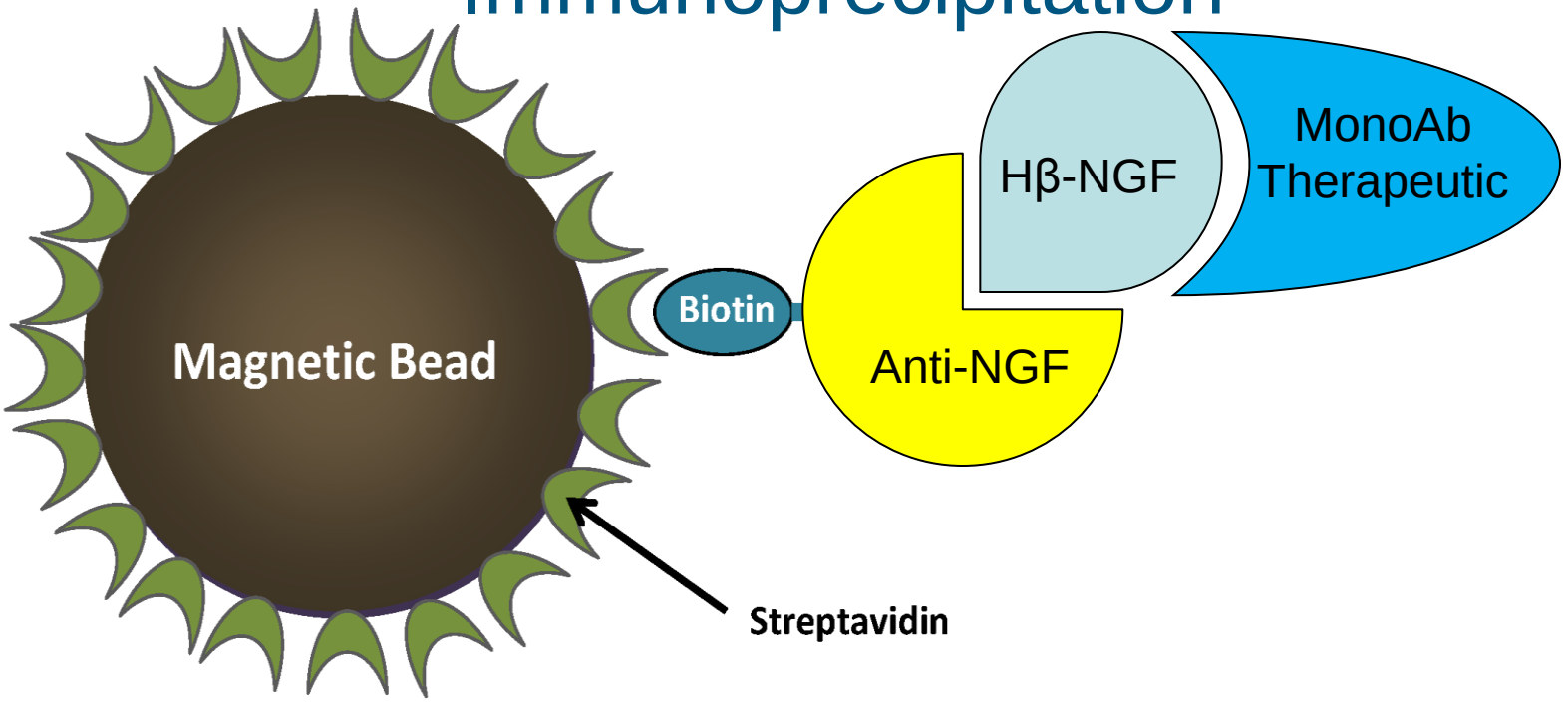
Immunoprecipitation



- Streptavidin-coated magnetic beads are combined with the samples the following morning using a Hamilton MicroLab STAR
- A 16 Step immunoprecipitation extraction procedure is used to capture the biotinylated anti-NGF/h β-NGF
- The beads are washed and eluted by lowering the pH.

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Reduction, Alkylation and Digestion

- Internal standard is added following elution from beads
- TCEP and IA followed by overnight tryptic digestion

β–NGF tryptic peptide for quantitation:
Ile-Asp-Thr-Ala-Cys-Val-Cys-Val-Leu-Ser-Arg

Internal Standard:
Ala-Trp-Arg-Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys-Val-Cys- (Val-$^{15}N_1{}^{13}C_5$)-

(Leu-$^{15}N_1{}^{13}C_6$)- Ser-Arg-Lys-Ala-Val-Arg-Arg-Ala

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LC/LC/NanoLC-MS/MS

- **Analysis by immunoaffinity capture and nanoflow LC-MS/MS**
- **3D LC separation using a Dionex Ultimate 3000 UHPLC**
  - Anti-peptide Column (Custom made at Advion)
  - 130 µL injection to anti-peptide column isolates β–NGF and IS
  - Trap and elute to nanoLC column (PepMap C18, 75 µm x 150 mm)
  - Nano LC flow rate, 600 nL/min
  - 3.5 minute retention time with 10 minute cycle time
- **Advion TriVersa NanoMate with ESI Chip**
  - 1.7kV spray voltage
- **Thermo Vantage Triple Quadrupole MS**
  - 20 ms SRM
  - m/z 647.3 > m/z 893.4 – β-NGF
  - m/z 653.9 > m/z 906.4 – β-NGF IS

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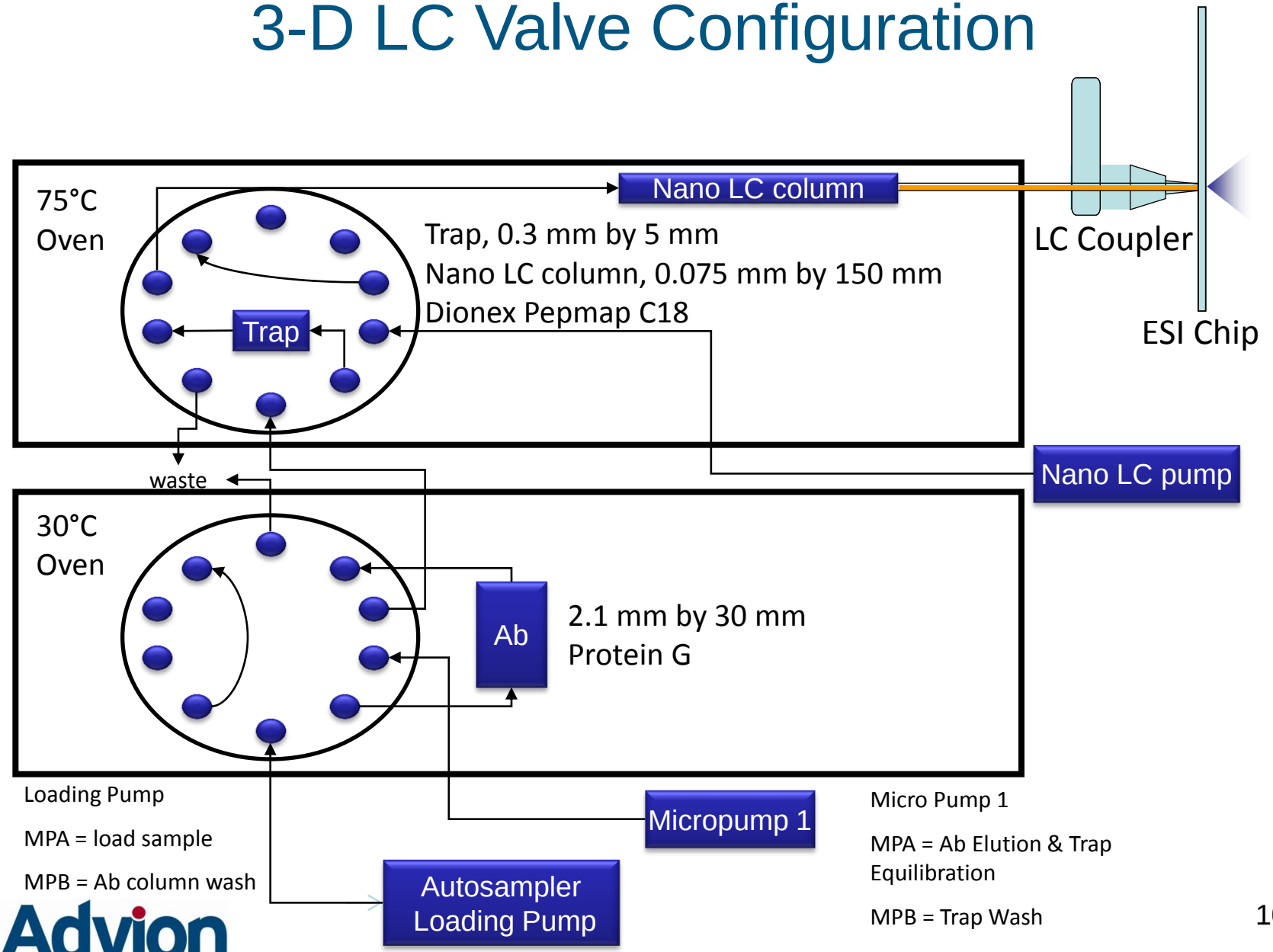
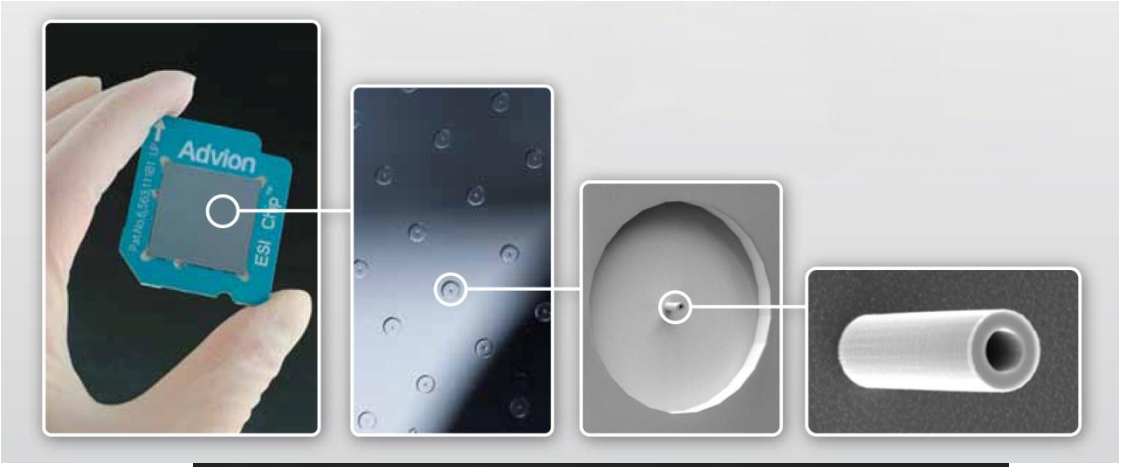
3-D LC Valve Configuration



TriVersa NanoMate® with ESI Chip™ Technology

- Robust
- Reproducible
- Spray Sensing Technology



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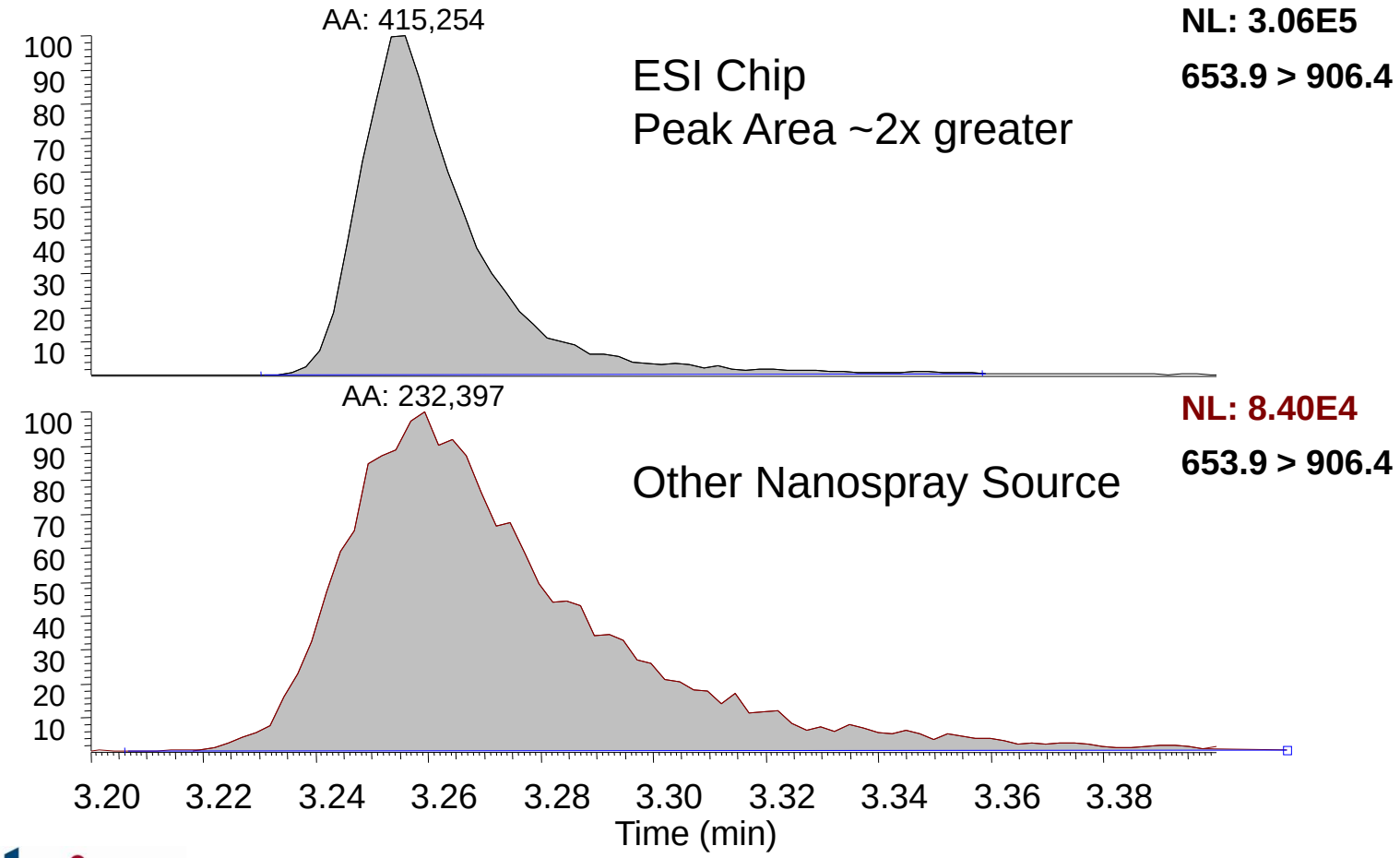
Peak Area Comparison



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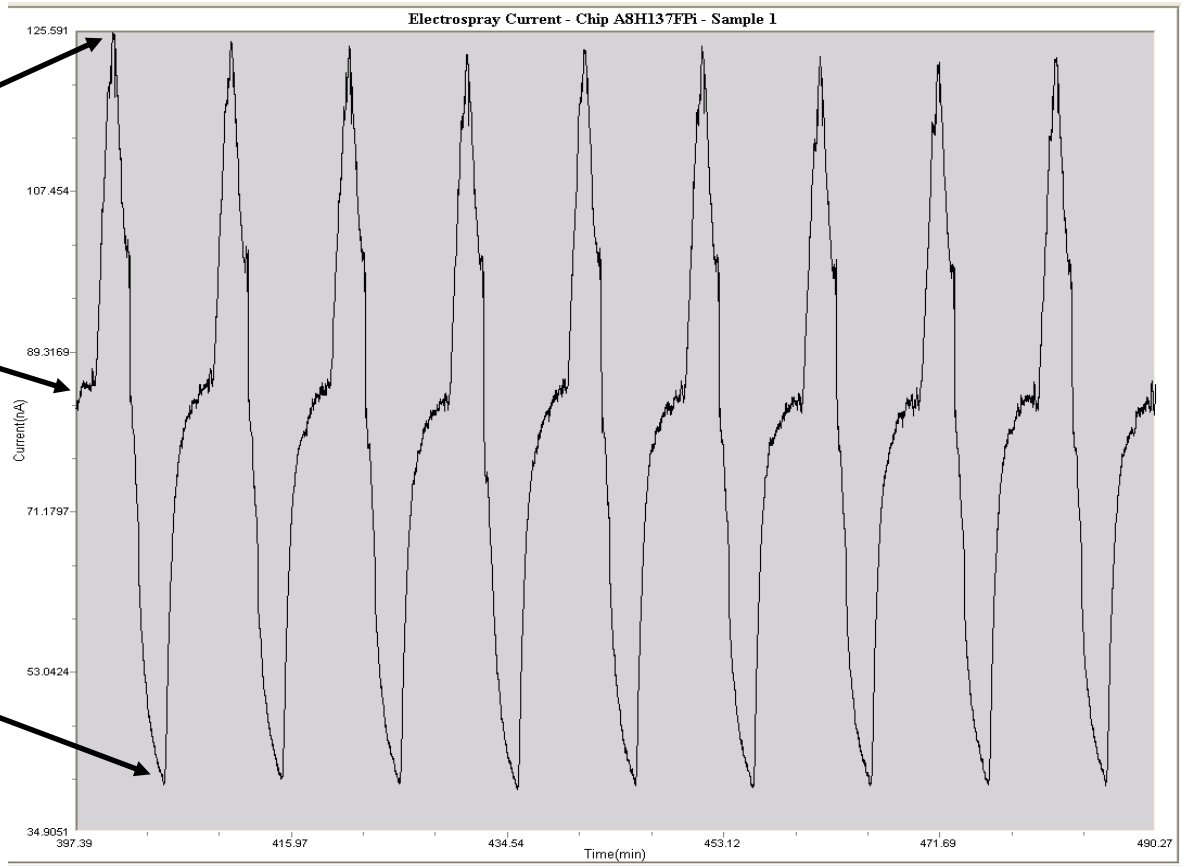
TriVersa Spray Sensing Technology

High aqueous, higher spray current ~125nA

Re-equilibration of analytical column 80% aqueous

Low aqueous, lower spray current ~40nA



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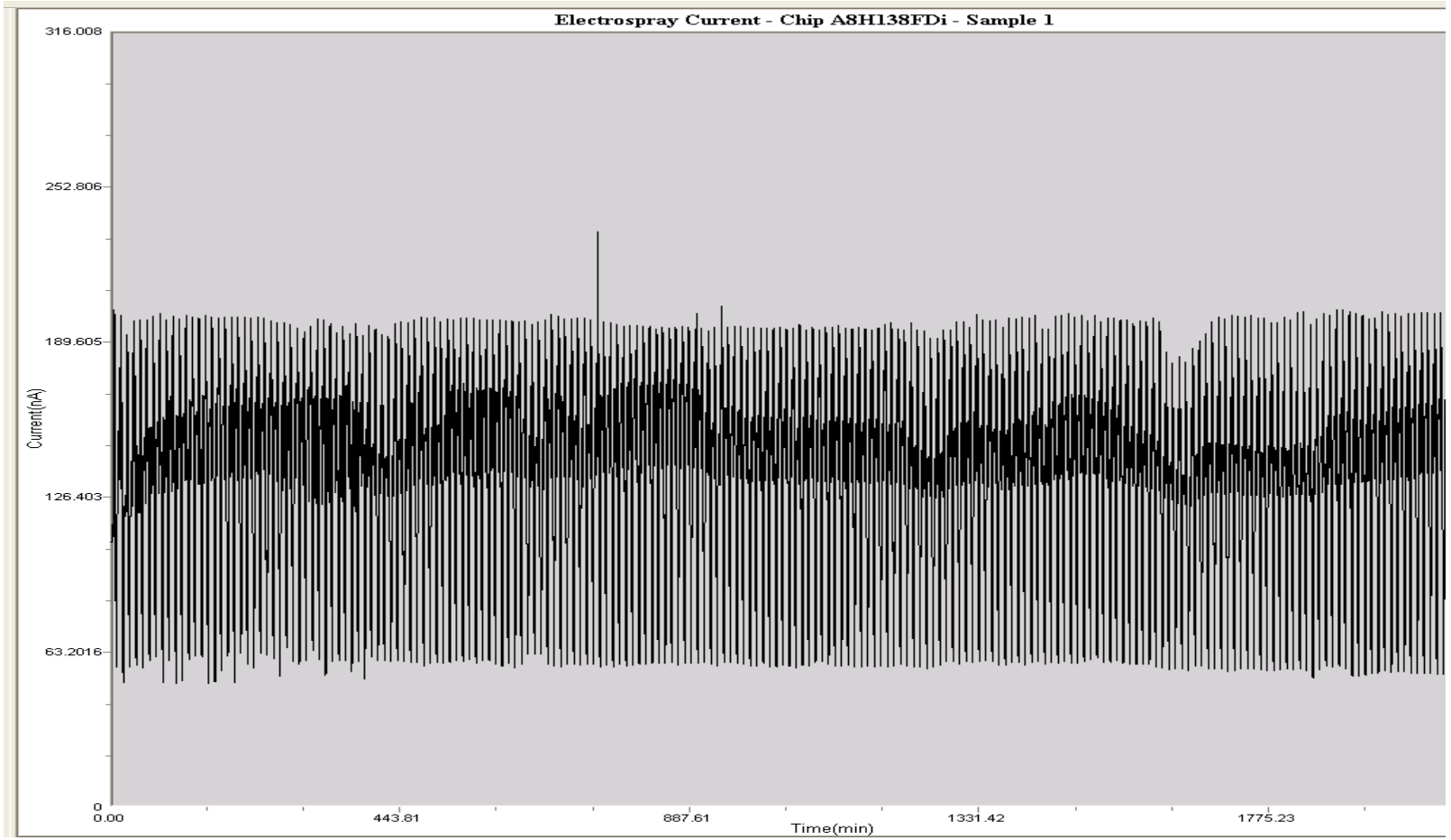
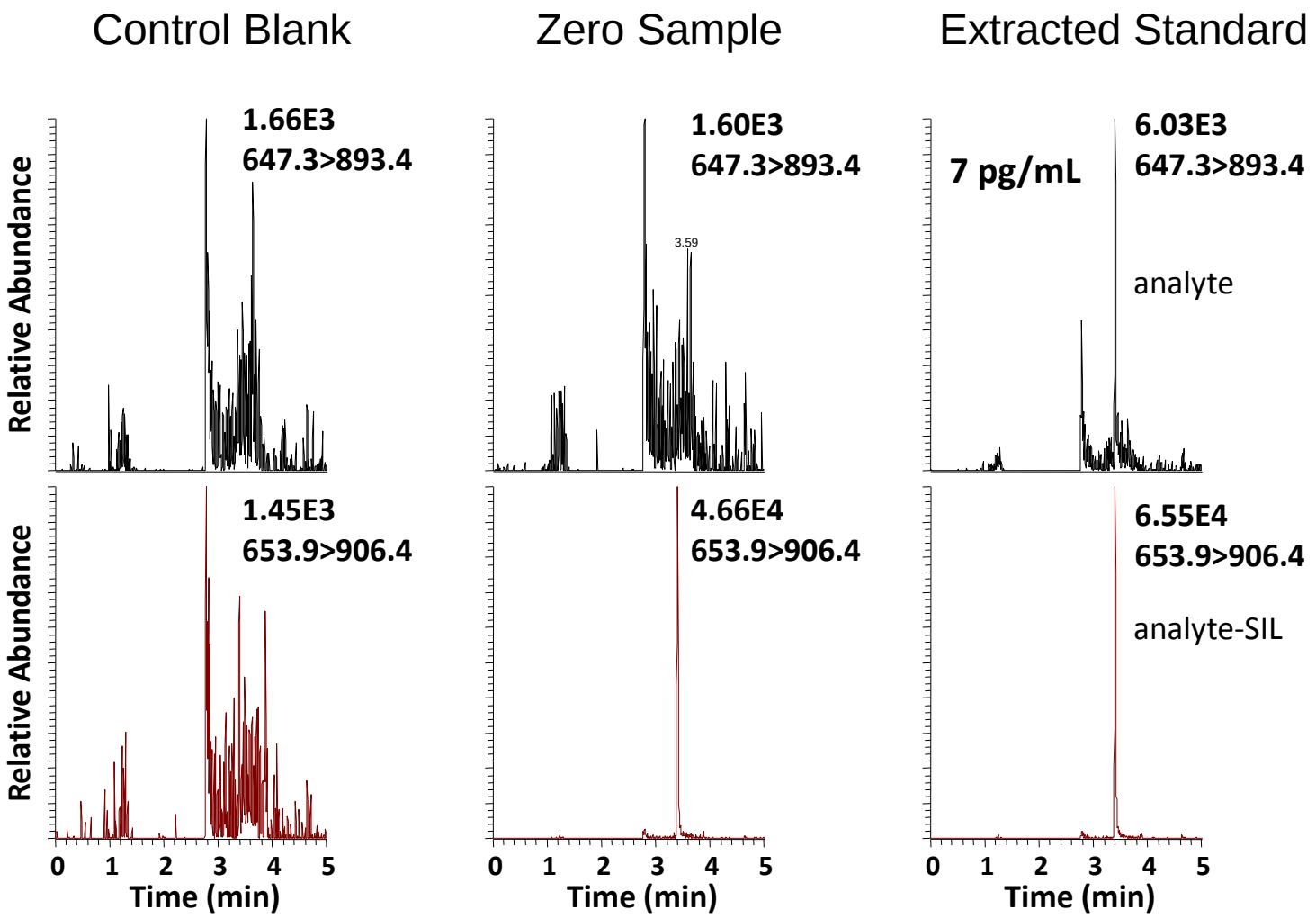
Spray Sensing over 35 hour run

Spray Current, nA



Time

Selectivity



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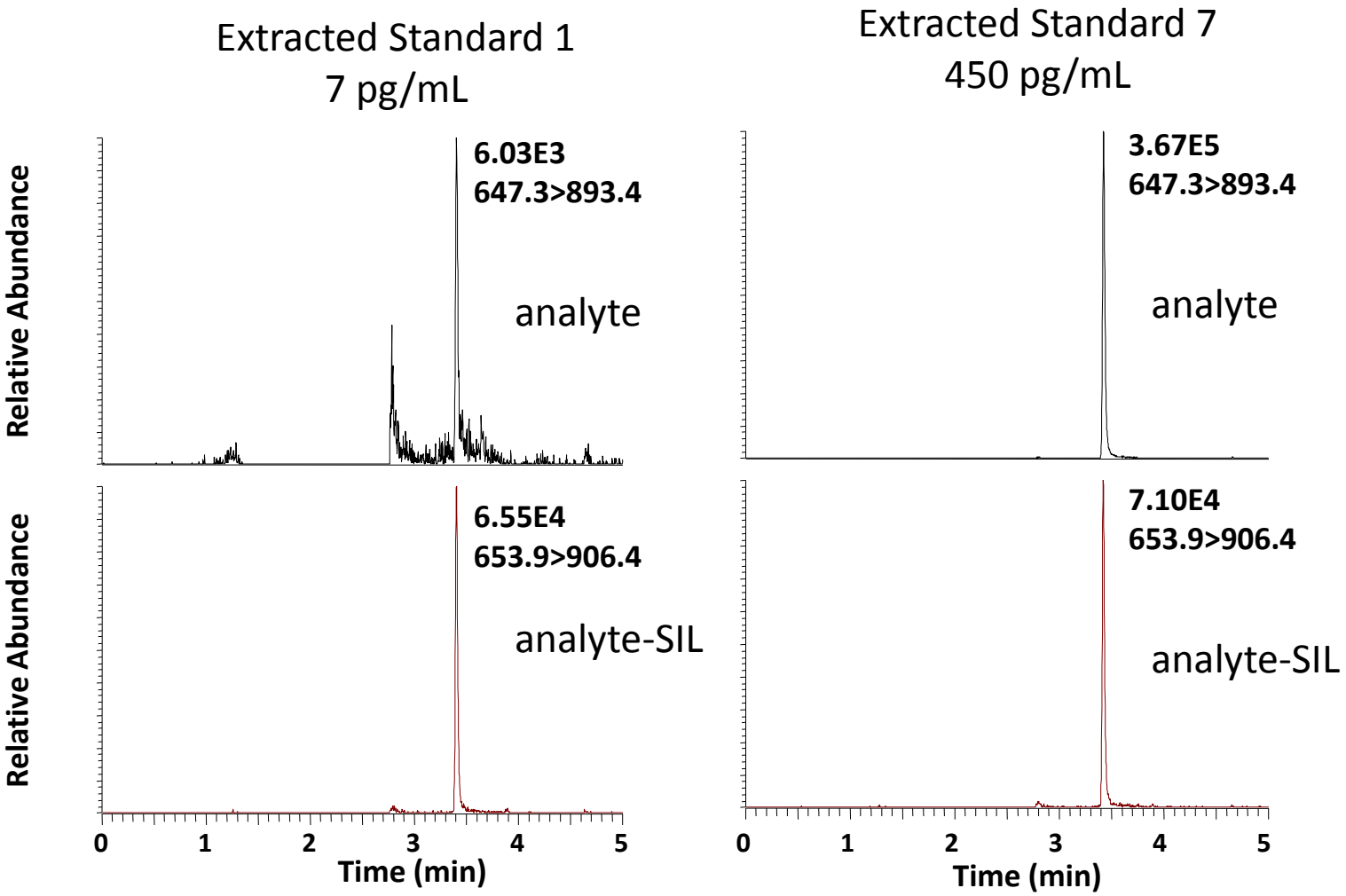
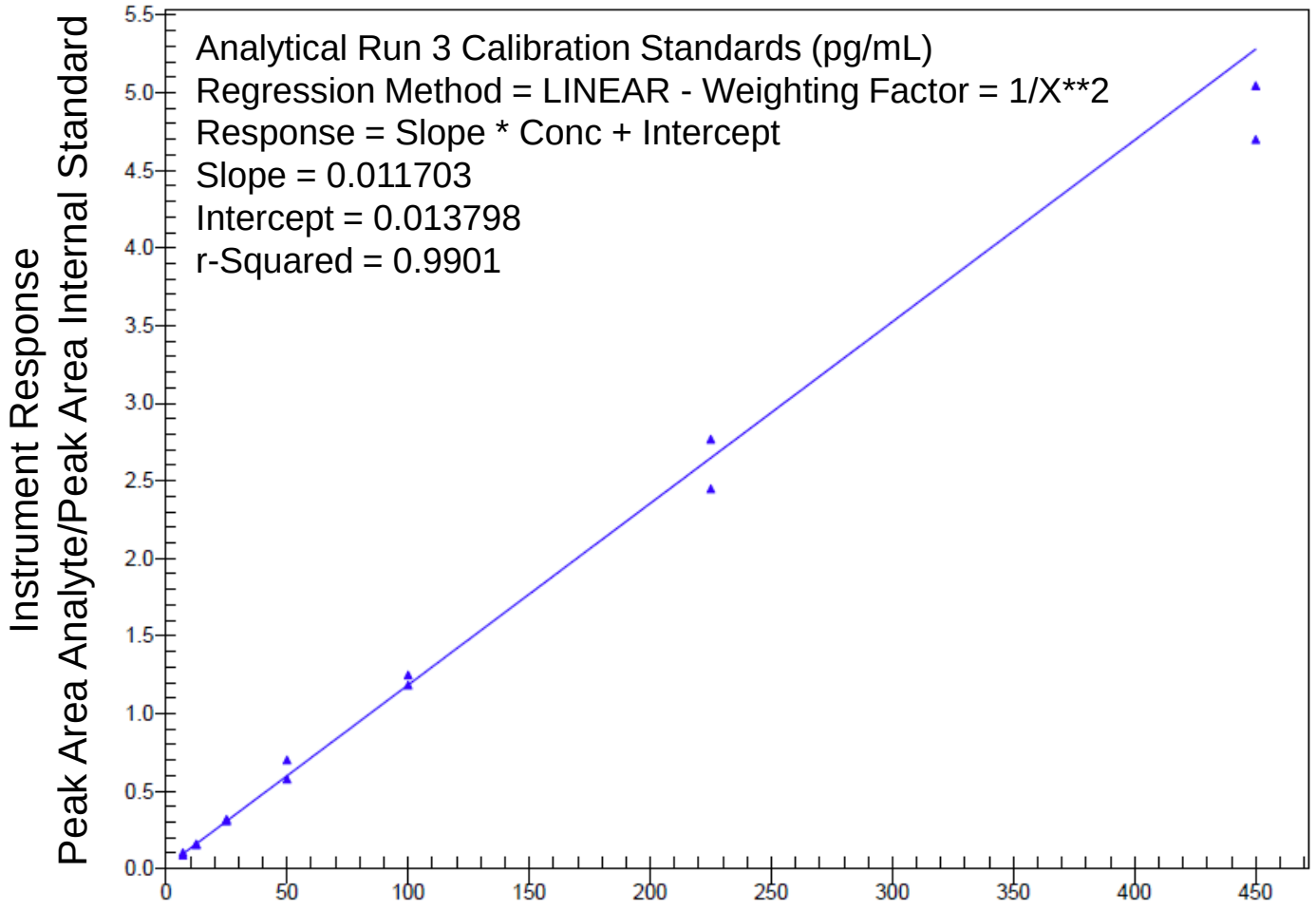
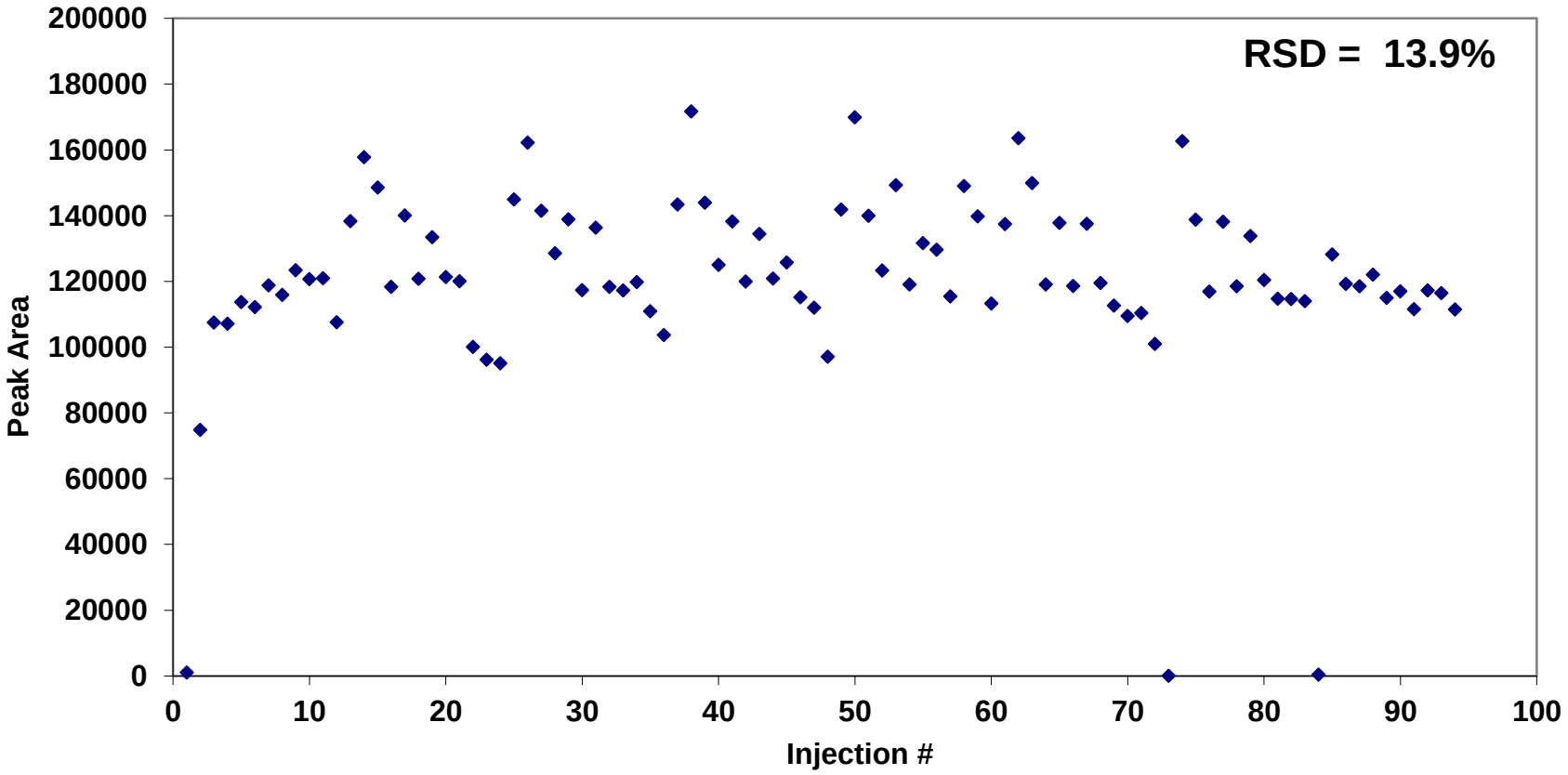
Sensitivity



Dynamic Range



Internal Standard Peak Area Trend – Accuracy & Precision Run 03



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Assay Performance

QC (pg/mL)	VS LLOQ 7.000	2X END 8.105	END 16.21	VS Low 18.11	VS Mid 208.1	VS High 333.1	VS ULOQ 450.0	100X Dil 8016
Mean	8.140	8.960	16.21	18.15	185.2	282.4	392.9	8184
%CV	12.1	21.2	16.6	12.6	7.0	8.3	9.7	9.4
%Theor.	**116.3**	**110.6**	**N/A**	**100.3**	**89.0**	**84.8**	**87.3**	**102.1**
n	18	17	18	18	18	18	17	17

- **Inter-run Precision and Accuracy (n=3):**
  - The inter-run accuracy ranged from -15.2% to 16.3% across all validation samples
  - The inter-run assay precision ranged from 7.0% to 21.2% across all validation samples
- **Stability:**
  - 64 hours of extract stability at 37°C prior to trypsin deactivation
  - 53 hours of reinjection stability at 4°C
  - 27 days of extract stability at -20°C
  - 4 freeze/thaw cycles at -20°C and -70°C
  - 179 days of long-term freezer storage stability at -20°C and -70°C
- **Robustness:**
  - Nanoelectrospray stability over 35 hour run time using a single nozzle
  - 2.0% Run failure rate

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Conclusions

- This presentation highlighted the performance and successful implementation of a validated bioanalytical assay for a protein biomarker.
- This immunoprecipitation, immunoaffinity LC/LC/nanoLC-MS/MS assay has proven to be robust and reproducibly sensitive.
- This assay supports clinical development programs of a therapeutic antibody, for which biomarker immunoassay development was unsuccessful.

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