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DEVELOPMENT AND VALIDATION OF A LC-MS/MS HYBRID ASSAY USING nSMOL FOR BEVACIZUMAB QUANTIFICATION IN HUMAN SERUM

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What is nSMOL?

...nano-Surface and Molecular-Orientation Limited Proteolysis

- *A unique approach for signature peptide assays (IgG antibody drugs)*
- *Developed by Shimadzu and comes as a kit with all reagents for analysis of up to 100 samples*

Signature Peptides from Tryptic Digestion

NRRPCHSHTKECESAWKNRPCHSHTKKPCHSHTKKNRKVWKIPPFFW

- Trypsin will cut the protein sequence mainly at Arginine and Lysine amino acids.
  - ✓ Choose a unique (signature) peptide for the matrix of interest.
  - ✓ Choose peptide with high sensitivity.
  - ✓ Primary signature peptide is used for quantification.
  - ✓ Secondary peptides may be useful for monitoring purposes.

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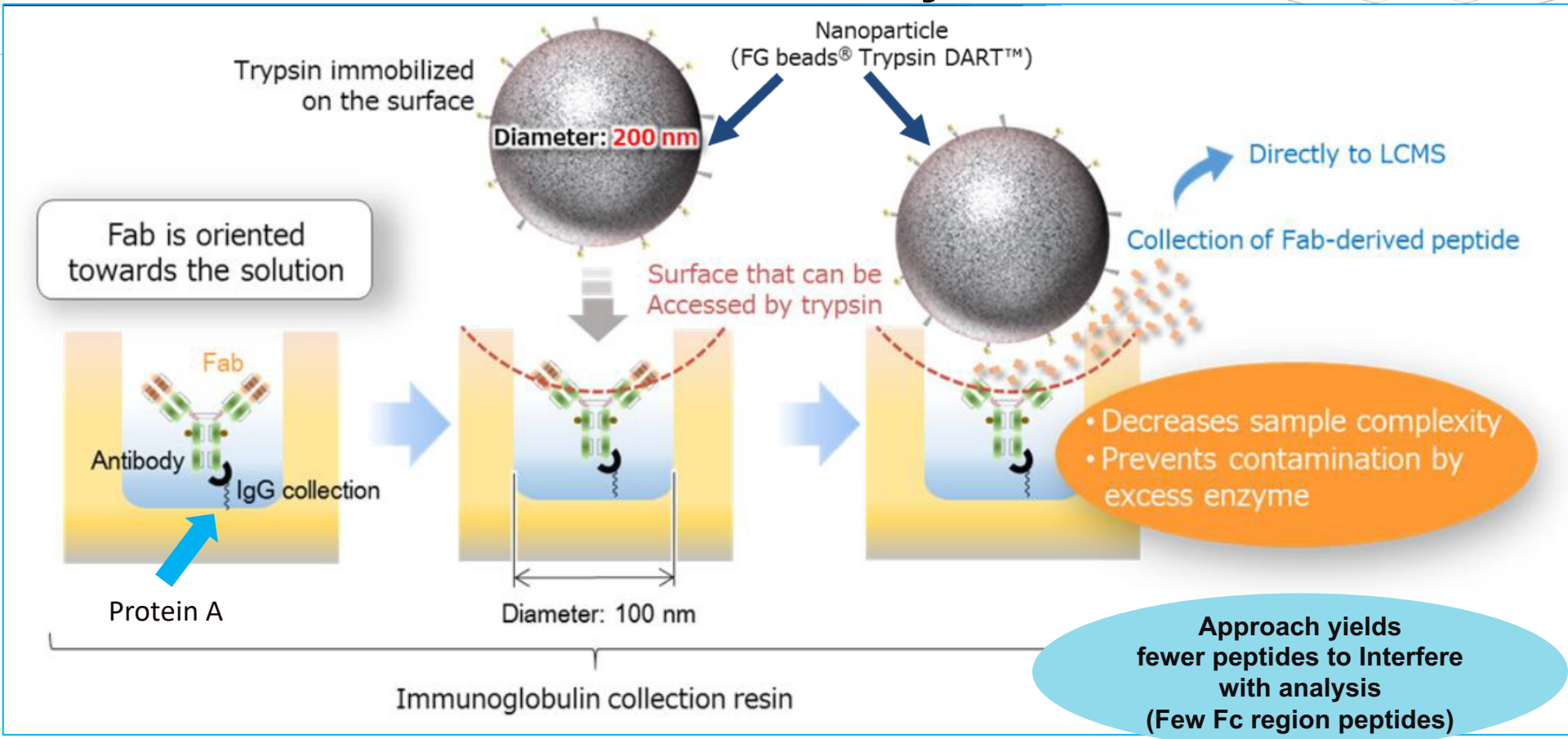
HOW nSMOL WORKS: Fab Selectivity



Approach yields fewer peptides to Interfere with analysis (Few Fc region peptides)

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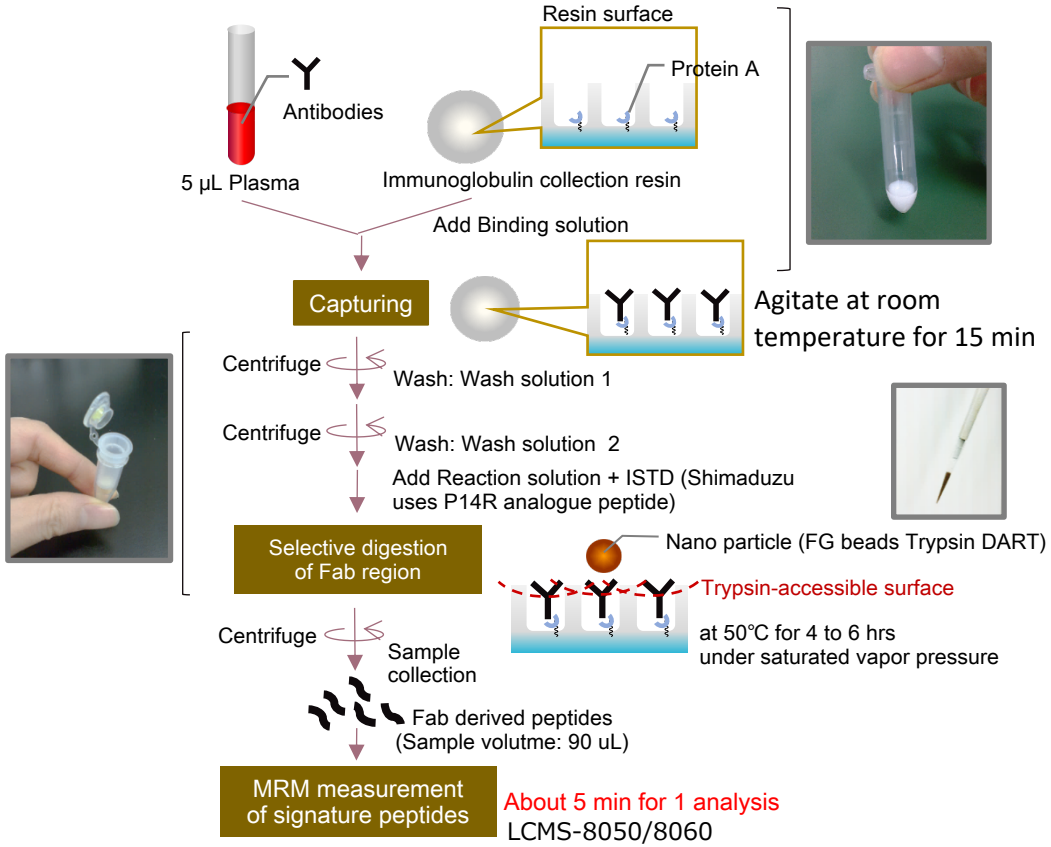
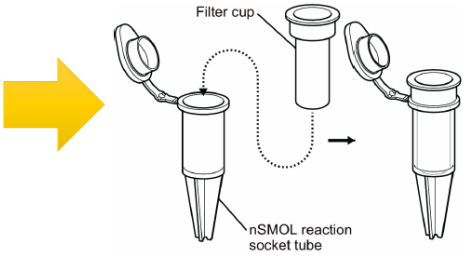
STANDARDIZED PROTOCOL: “nSMOL”



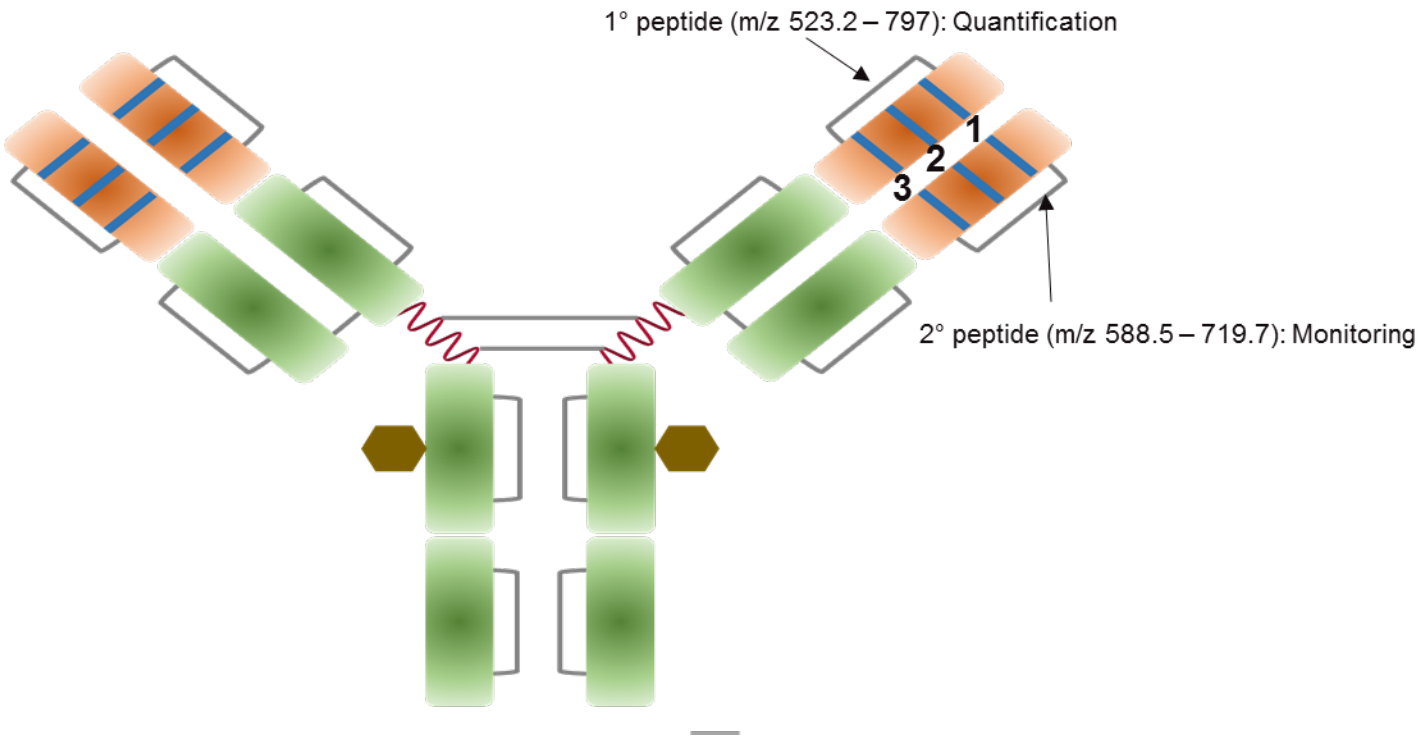
Simple Workflow

- The protocol eliminates the necessity of troublesome protein denaturing, alkylation and solid phase extraction after trypsin digestion, which results in highly repeatable data.
- After preparation, samples can be injected directly into the LCMS.



Trypsinization on a filter cup using nSMOL™ Reaction Socket Tube.

SITES OF PRIMARY AND SECONDARY PEPTIDES

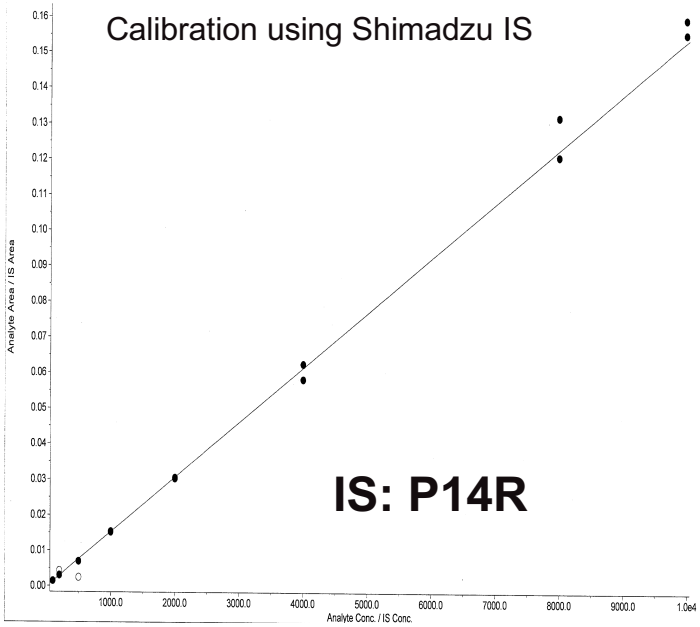


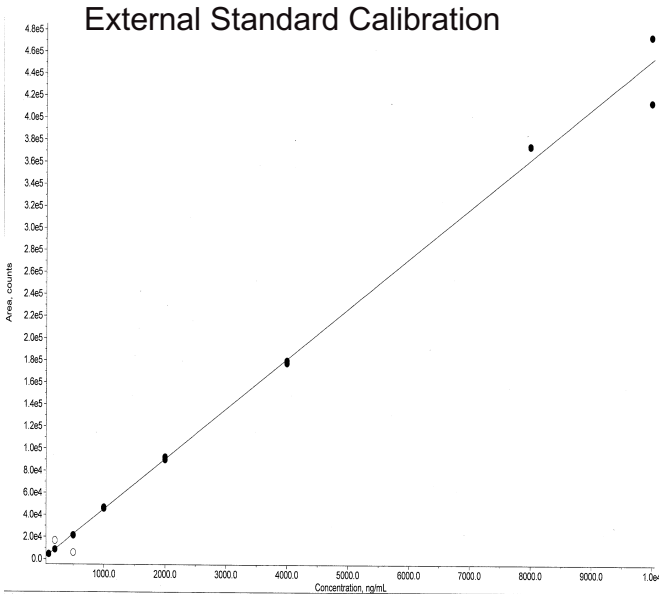
LCMS Procedures

- Intertek modified sample processing procedures to reflect Internal laboratory processes and adjusted chromatography…

Sciex	Shimadzu
API-6500 Mass Spectrometer	LCMS-8060 Mass Spectrometer
Shimadzu LC-20AD XR HPLC Pumps	Shimadzu LC-30AD HPLC Pumps or Mikros LC Pumps (micro-flow)
Leap PAL HTC-XT Autosampler	Shimadzu SIL-30AC Autosampler

Calibration Curve Performance (MV-1)





Bevacizumab Intra-Assay Accuracy and Precision (Using P14R Internal Standard)

Run #	QC Concentration (ng/mL)							
	100		300		3000		7500	
	Conc. (ng/mL)	%Dev (%)	Conc. (ng/mL)	%Dev (%)	Conc. (ng/mL)	%Dev (%)	Conc. (ng/mL)	%Dev (%)
34176_001	96.5	-3.50	266	-11.3	2450	-18.3	5190	-30.8
	95.4	-4.60	249	-17.0	2390	-20.3	4850	-35.3
	85.8	-14.2	275	-8.33	2430	-19.0	4990	-33.5
	109	9.00	247	-17.7	2600	-13.3	4900	-34.7
	100	0.00	246	-18.0	2520	-16.0	5210	-30.5
	103	3.00	261	-13.0	2810	-6.33	5040	-32.8
Mean	98.3		257		2530		5030	
SD	7.84		11.9		154		148	
%CV	7.98		4.62		6.10		2.94	
%Dev	-1.70		-14.3		-15.7		-32.9	

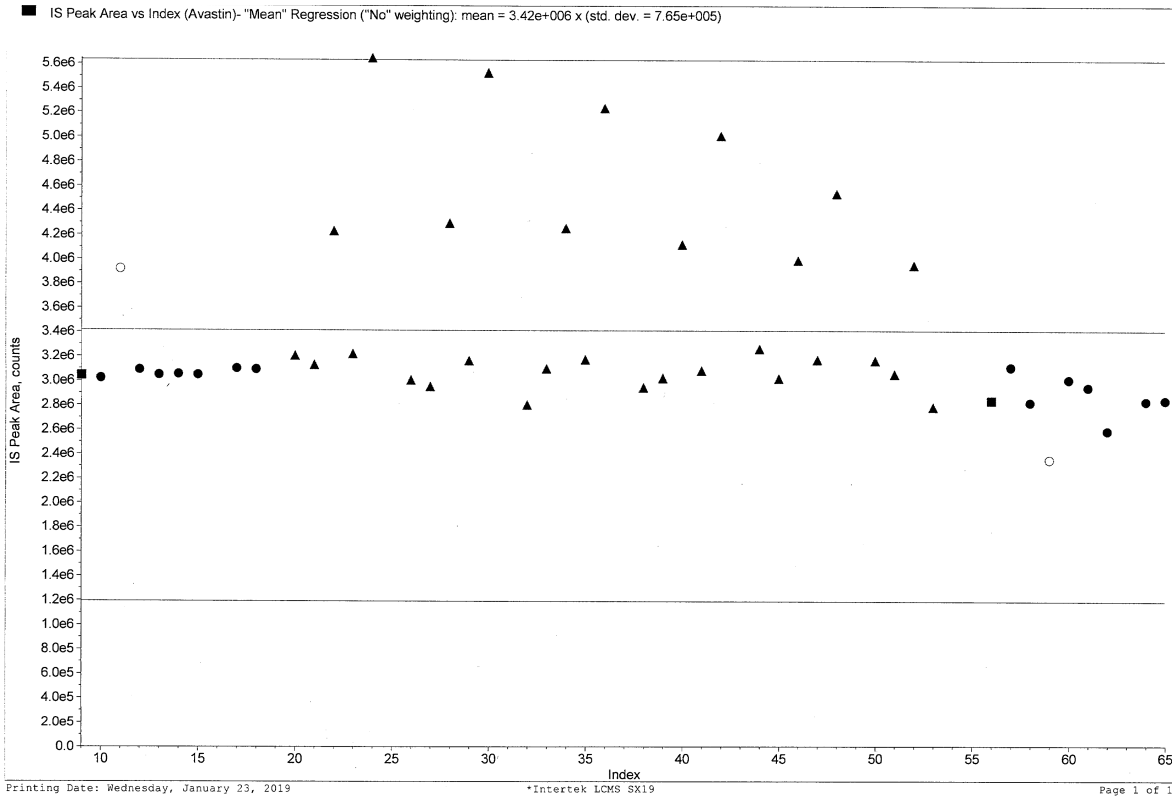
- Shimadzu published validation work did not see this using <u>different</u> chromatography.

Bevacizumab Intra-Assay Accuracy and Precision (External Standardization-No IS Correction)

Run #	QC Concentration (ng/mL)							
	100		300		3000		7500	
	Conc. (ng/mL)	%Dev (%)	Conc. (ng/mL)	%Dev (%)	Conc. (ng/mL)	%Dev (%)	Conc. (ng/mL)	%Dev (%)
34176_001	99.0	-1.00	285	-5.00	2670	-11.0	7430	-0.933
	92.6	-7.40	251	-16.3	2560	-14.7	7050	-6.00
	87.0	-13.0	259	-13.7	2620	-12.7	7200	-4.00
	109	9.00	244	-18.7	2720	-9.33	6840	-8.80
	99.4	-0.600	269	-10.3	2710	-9.67	7040	-6.13
	103	3.00	277	-7.67	2660	-11.3	6740	-10.1
Mean	98.3		264		2660		7050	
SD	7.73		15.7		59.6		248	
%CV	7.86		5.93		2.24		3.52	
%Dev	-1.70		-12.0		-11.3		-6.00	

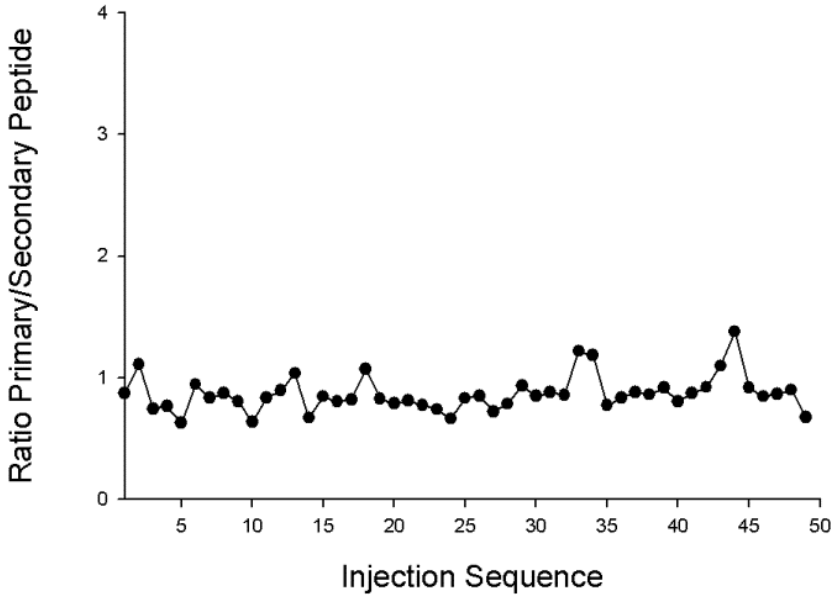
- External standard quantification data suggests the nSMOL capture step works well.

What's Happening?... Internal Standard Plot



- Freshly frozen QCs run against a freshly prepared calibration curve using the same control matrix.

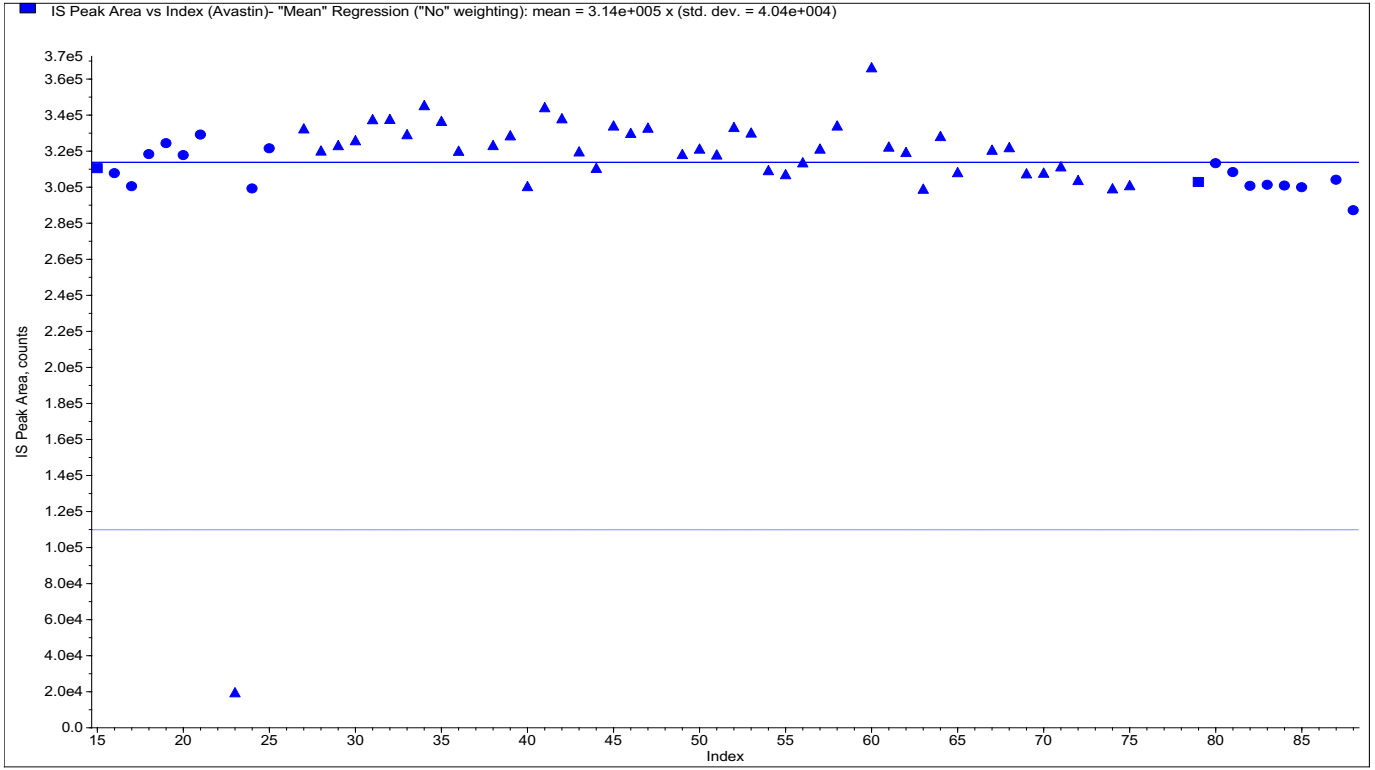
Evaluation of Secondary Peptide



UPDATED (96-WELL) METHOD …

- The internal standard issue had to be addressed to validate the method and a method with higher throughput was desired.
  - ✓ Change assay format to <u>96-well</u> processing (5→10 µL aliquot).
  - ✓ Update method for use of a stable labeled peptide internal standard.
  - ✓ Revised chromatography (stable label allowed for better selectivity)

Internal Standard Plot (Using Stable Label)



Updated Method: Validation Run 1
Intra-Accuracy and Precision (with IS Correction)

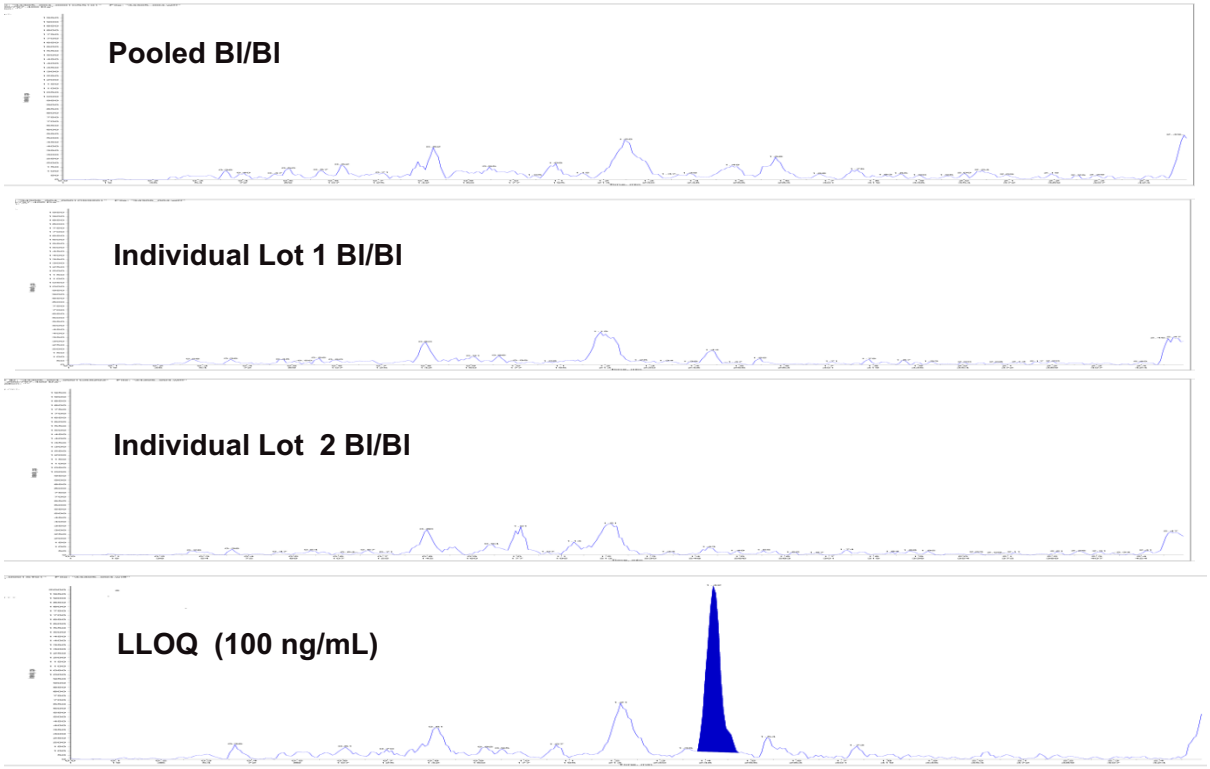
Run #	Quality Control Concentrations (ng/mL)							
	100		300		3000		7500	
	Amount Found (ng/mL)	Dev. (%)	Amount Found (ng/mL)	Dev. (%)	Amount Found (ng/mL)	Dev. (%)	Amount Found (ng/mL)	Dev. (%)
34305_001	97.1	-2.90	276	-8.00	2540	-15.3	7110	-5.20
	91.2	-8.80	264	-12.0	2910	-3.00	6960	-7.20
	88.1	-11.9	306	2.00	3030	1.00	8370	11.6
	105	5.00	276	-8.00	2910	-3.00	7660	2.13
	90.2	-9.80	238	-20.7	2860	-4.67	7850	4.67
	79.1	-20.9	292	-2.67	3110	3.67	8030	7.07
Mean	91.8		275		2890		7660	
sd	8.72		23.4		196		542	
%CV	9.50		8.50		6.78		7.07	
%Dev	-8.22		-8.22		-3.56		2.18	

Updated Method:
Intra- and Inter-Assay Accuracy and Precision

Run #		Quality Control Concentrations (ng/mL)			
		100	**300**	**3000**	**7500**
34305_001	Mean	91.8	275	2890	7660
	sd	8.72	23.4	196	542
	%CV	9.50	8.50	6.78	7.07
	%Dev	-8.22	-8.22	-3.56	2.18
34305_002	Mean	94.2	293	2860	6990
	sd	51.4	15.4	188	280
	%CV	54.5	5.26	6.56	4.01
	%Dev	-5.82	-2.20	-4.53	-6.85
34305_004	Mean	103	279	2780	7430
	sd	11.7	17.3	205	468
	%CV	11.4	6.20	7.39	6.30
	%Dev	2.98	-7.00	-7.33	-0.933
Between Batch	Mean	102	282	2850	7380
	sd	14.1	19.9	190	515
	%CV	13.8	7.05	6.65	6.98
	%Dev	2.17	-5.89	-5.04	-1.66

- Accuracy, precision and sensitivity were similar on API-6500 and LCMS-8060 instruments

Selectivity and Sensitivity



Matrix Effects (Individual Lot QCs)

Run #	Lot	Quality Control Concentrations (ng/mL)			
		300		7500	
		Conc. (ng/mL)	Dev. (%)	Conc. (ng/mL)	Dev. (%)
34305_004	1	340	13.3	8160	8.80
	2	393	31.0	8680	15.7
	3	351	17.0	8620	14.9
	4	225	-25.0	5540	-26.1
	5	326	8.67	7350	-2.00
	6	342	14.0	8110	8.13
Mean		330		7740	
sd		56.0		1180	
%CV		17.0		15.2	
%Dev		9.83		3.24	
(Lots ≤20%)		4/6		5/6	

Hyperlipemic and Hemolysis Effects (QCs)

Run #	Quality Control Concentrations (ng/mL)			
	300		7500	
	Conc. (ng/mL)	Dev. (%)	Conc. (ng/mL)	Dev. (%)
34305_004 **(Hyperlipemic Serum)**	373	24.3	8670	15.6
	342	14.0	8550	14.0
	346	15.3	8450	12.7
Mean	354		8560	
sd	16.9		110	
%CV	4.77		1.29	
%Dev	17.9		14.1	
34305_004 **(Hemolyzed Serum)**	301	0.333	7810	4.13
	252	-16.0	7610	1.47
	276	-8.00	7420	-1.07
Mean	276		7610	
sd	24.5		195	
%CV	8.87		2.56	
%Dev	-7.89		1.51	

%Recovery (Capture Step)

	Peak Area for Bevacizumab					
	300 ng/mL		3000 ng/mL		7500 ng/mL	
	QC	Post-Spike	QC	Post-Spike	QC	Post-Spike
Method 1*						
Mean	8293	4662	83133	45153	201169	108607
sd	405	388	4940	3187	7068	19193
%CV	4.89	8.33	5.94	7.06	3.51	17.7
%Recovery	178		184		185	
Method 2**						
Mean	6321	3876	62758	40668	172170	99508
sd	852	89	1164	406	6321	6405
%CV	13.5	2.30	1.85	1.00	3.67	6.44
%Recovery	163		154		173	
Method 3***						
%Recovery	23 ?					

- Processed matrix blanks spiked post capture step, but prior to digestion step

*Kit Digestion only

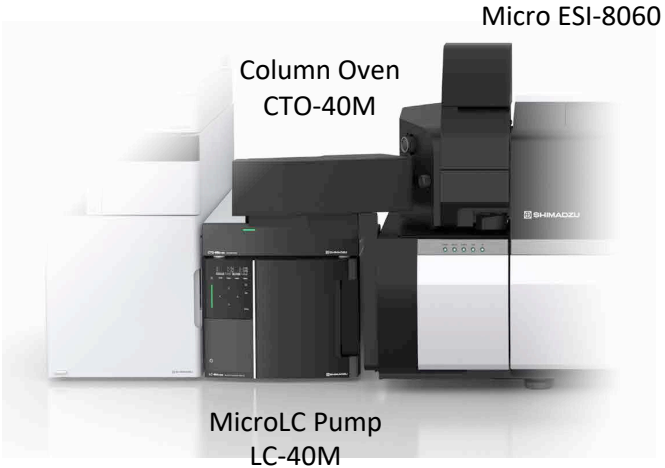
**Kit Digestion + added free trypsin

***Internal standard as reference for calculation (mid concentration, <u>not</u> corrected for Isotope effect)

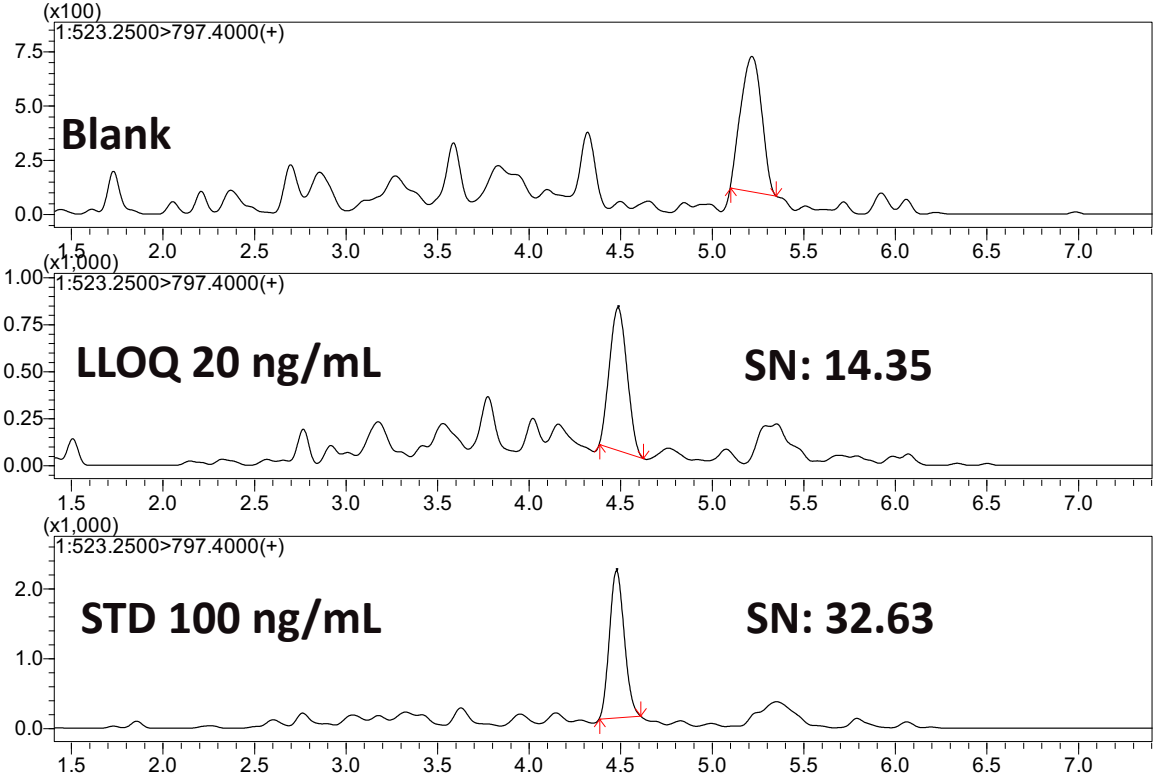
- %Recovery not clearly determined

Enhanced Sensitivity with micro-flow HPLC

- As sensitivity can be an issue for LCMS bioanalysis of proteins, micro-flow HPLC was evaluated.
  - ✓ Shimadzu LCMS-8060 MS coupled to the Micro-ESI interface
  - ✓ LLOQ evaluated: 20 ng/mL



Representative Chromatograms with micro-flow HPLC



- 20-25 ng/mL achievable with existing methodology
- Preliminary chromatography used: additional development will improve selectivity

nSMOL Considerations

- nSMOL sample processing procedure is confirmed to be acceptable:
  - ✓ Accuracy and Precision was good and consistent.
  - ✓ Consistent sensitivity across multiple kit lots.
  - ✓ Method can be modified to higher throughput, 96-well analysis.
  - ✓ Matrix Effects were minor, but acceptable.
  - ✓ Internal standard choice is critical (as with any LCMS method).
    - Winged internal standards to track digestion step will likely not work for nSMOL (based on recovery experiments).

Acknowledgements

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