Absolute Quantitation of Biotherapeutic Drug Product and its Endogenous Protein in Human Serum Using a Hybrid Immunoassay-LC/MS Approach

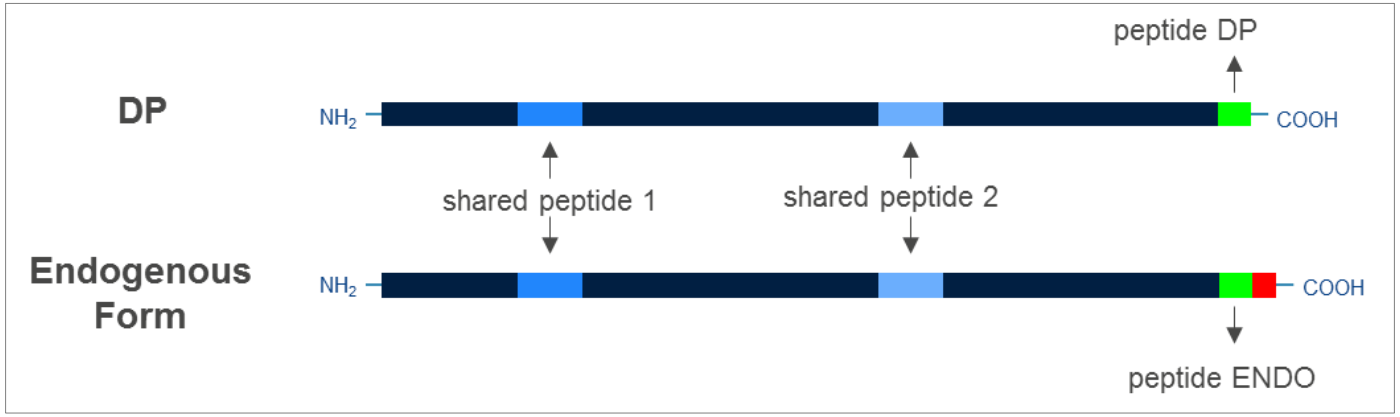
November 6, 2018

Rudolf Guilbaud

Caprion Biosciences

Session Description and Objectives

- An antibody-based assay was unable to differentiate a biotherapeutic protein from it's endogenous form.



- A mass spectrometry-based assay PK assay was thus developed, which provided several advantages:
  1) Selectivity to distinguish between the near-identical analytes,
  2) Multiplexing for measurement of both the biotherapeutic and the endogenous forms within a single assay,
  3) Sensitivity for reliable measurement of the two target proteins at the dosed and endogenous levels found in plasma study samples.
- The assay combines single-antibody capture of both target proteins with LC/MS analysis of peptides unique to each target. Through extensive troubleshooting at the sample work-up and LC/MS instrumentation levels, an optimized assay was developed and successfully qualified for an exploratory analysis of plasma from dosed subjects.

Contact Information

Rudolf Guilbaud

Director, Operations, Caprion Biosciences

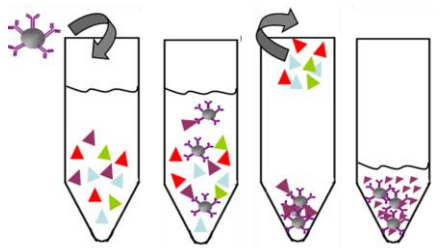
Montreal, Canada

rguilbaud@caprion.com

Hybrid Immunoassay-LC/MS Approach Overview

Single Ab pulldown of DP & ENDO proteins

Digestion to peptides



+heavy labelled peptide standards

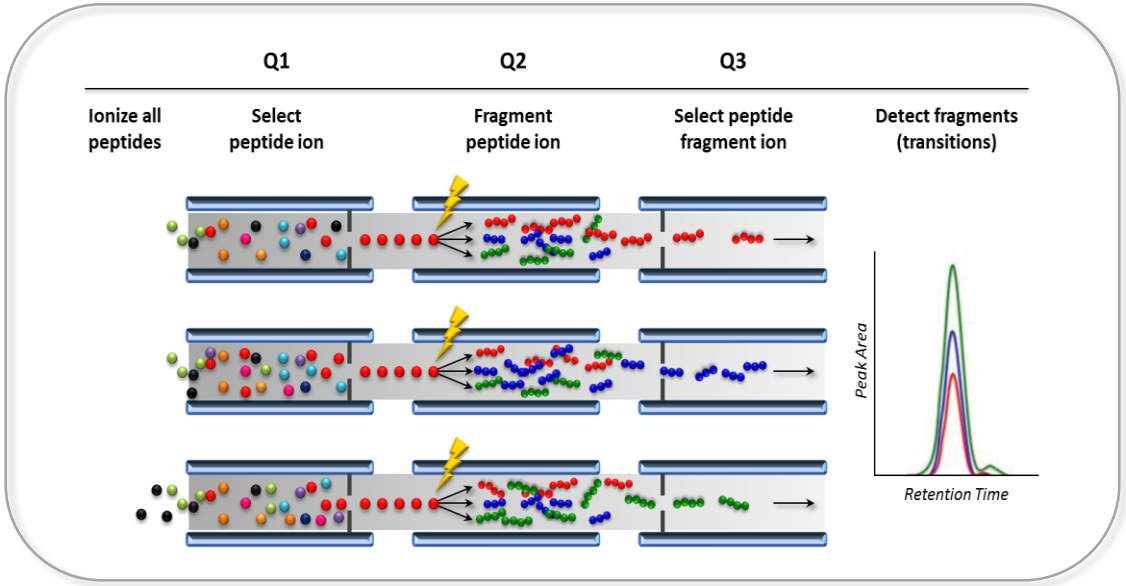
Shared peptide 1

Shared peptide 2

DP peptide

ENDO peptide

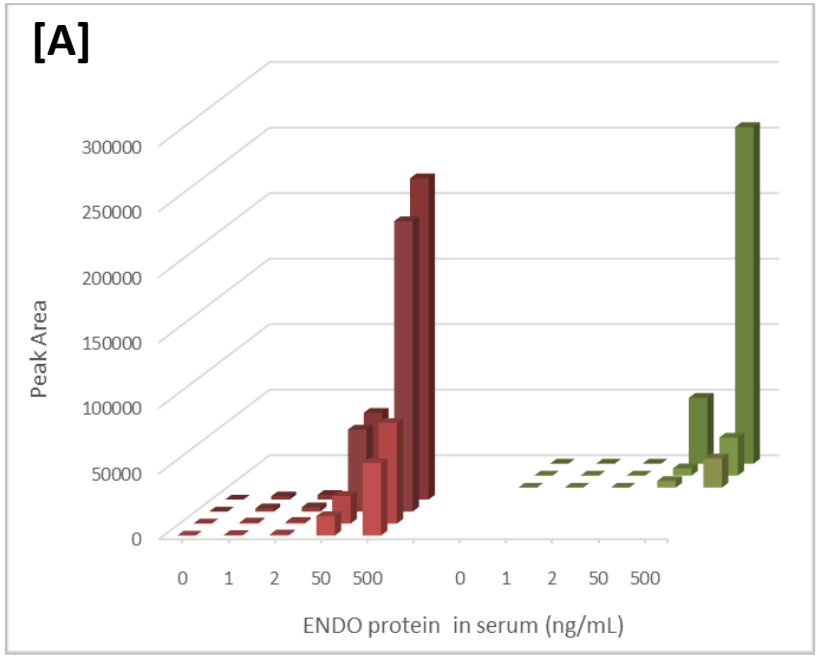
LC-MS Analysis of DP and ENDO specific peptides and shared peptides
(Shown: Targeted Multiple Reaction Monitoring LC-MRM/MS approach)

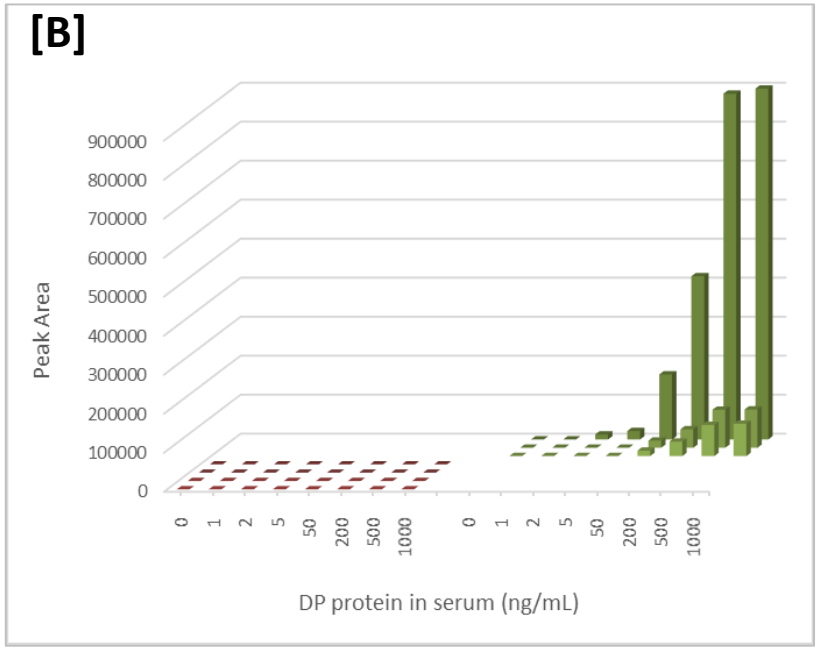


- Use of the DP and ENDO specific peptides presented various analytical challenges:
  (1) Digestion (2) Sensitivity (3) Linearity

(1) Digestion: Non-specific proteolysis and peptide conversion

- Serum spiked with protein ENDO [A] or DP [B] at a range of concentrations were analyzed for DP and ENDO peptide levels using initial assay conditions

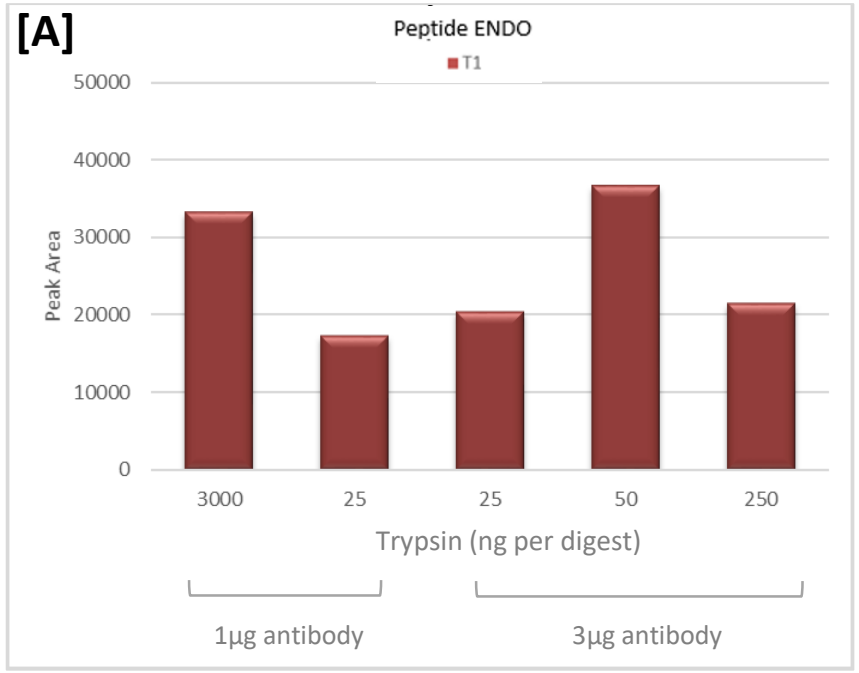


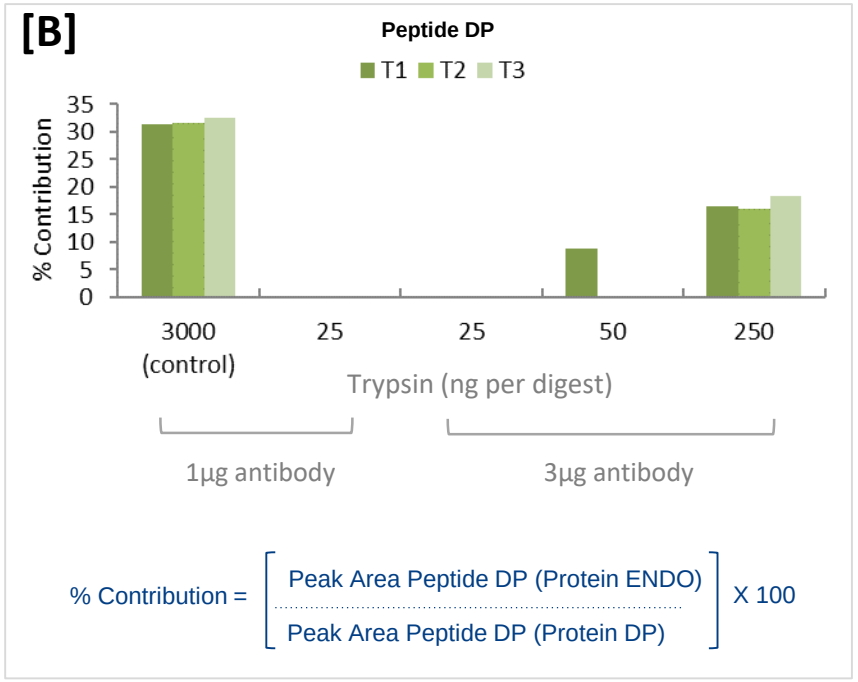


- Peptide DP was unexpectedly detected in the ENDO protein spiked sample, due to non-specific proteolysis and peptide conversion from ENDO to DP

(1) Digestion: Optimization and impact on peptide conversion

- Serum spiked with protein ENDO was tested using [A] lower trypsin amounts and more antibody for pulldown. [B] Impact of reduced trypsin on conversion.





- Reducing trypsin had a moderate, non-linear impact on signal intensity and also reduced conversion. Use of 3µg antibody vs 1µg for pulldown improved linearity in the upper range.

(1) Digestion: Testing other proteases to minimize conversion activity

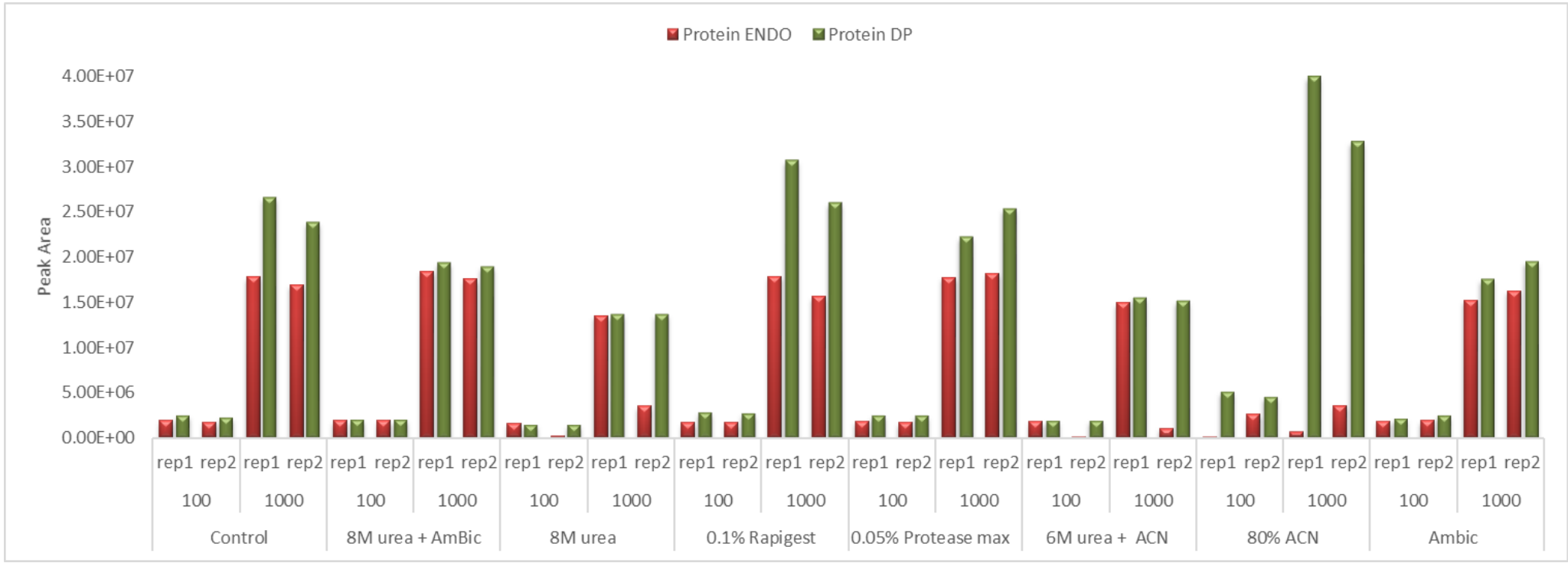
- Test samples spiked with protein ENDO or DP were digested with various enzymes. Results shown are for Lys-C enzyme.

		Protein spiked in serum (µg/mL)			
		Protein ENDO		Protein DP	
		200	20	200	20
Peak Area	Peptide ENDO	2.74E+08	3.00E+07	ND	ND
		1.97E+08	3.08E+07	ND	ND
		1.25E+08	1.74E+07	ND	ND
	Peptide DP	2.22E+06	3.93E+05	2.54E+08	2.74E+07
		1.61E+06	2.29E+05	2.19E+08	2.49E+07
		1.06E+06	1.57E+05	2.18E+08	2.19E+07

- Digestion using 500ng Lys-C produced peptide ENDO and peptide DP for analysis, with a conversion rate of ~1%.

(1) Digestion: Optimization of Lys-C digestion protocol

- Lys-C digestion with a range of denaturing conditions was tested to determine conditions for the best signal intensity and LOD for the target peptides



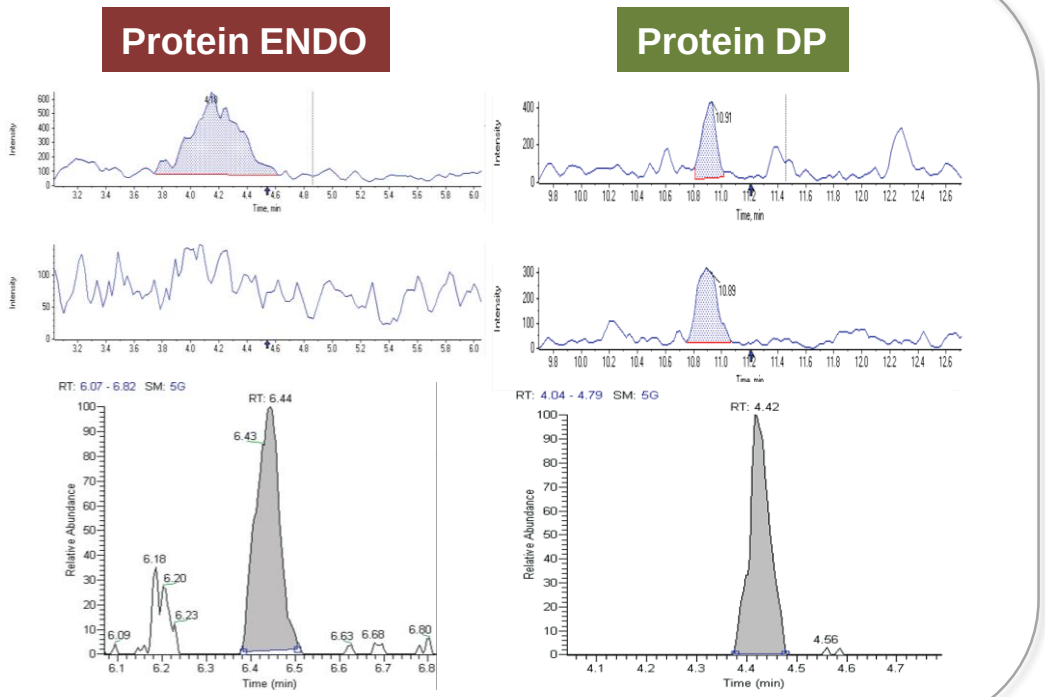
- Rapigest was selected over the control TFE-based protocol due to having the slightly better signal sensitivity, good reproducibility and signal intensity, and ease of use.

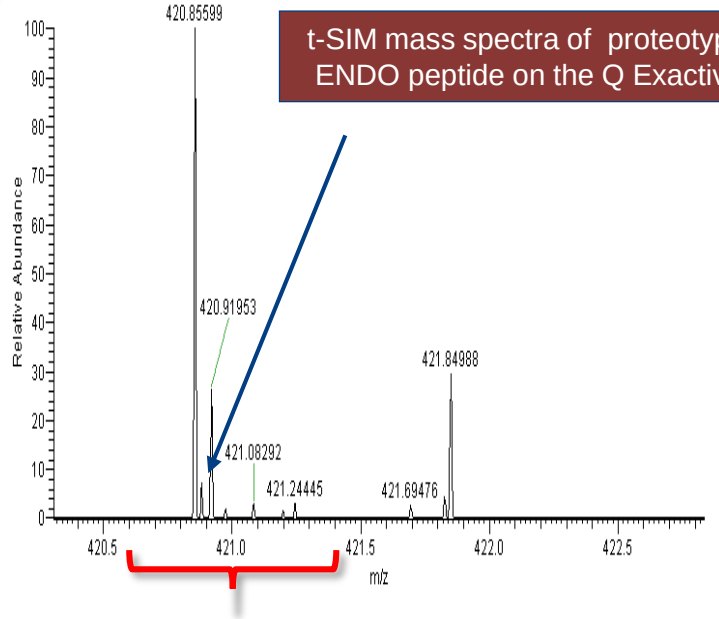
(2) Sensitivity: Optimization of peak shape and LC-MS conditions

- To meet the 1ng/mL target sensitivity, the MS analysis was shifted to a different MS system and acquisition method, and an ionic modifier was used prior to injection

QTRAP
(T1, T2 peaks with poor sensitivity and poor peak shape)

Q Exactive
(MS1 peak with +10% TCA and t-SIM mode)





Approximate width of the isolation window on the lower resolution QTRAP.

- Switching to the high-resolution Q Exactive instrument for quantitation on the MS1 peak avoided the poor signal issues observed with MRM, and use of 10% TCA improved the peak shape

(3) Linearity: Immunoaffinity pulldown optimization

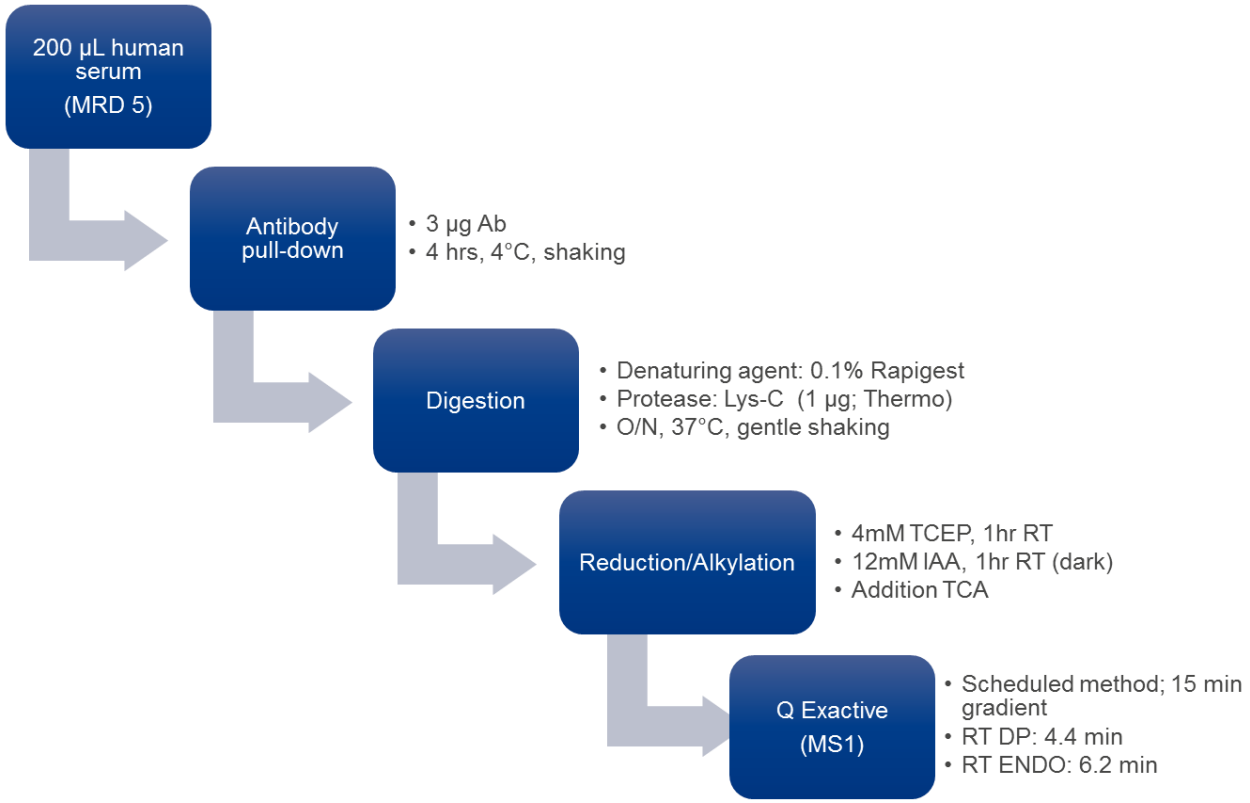
- Initial pulldown testing of spiked DP and ENDO proteins from serum used 1µg antibody. Results shown are for DP calibration and QC standards.

		DP Calibration Standards (ng/mL)										DP QC Samples (ng/mL)				
1 µg antibody	**Nominal**	**0.5**	**1**	**2**	**5**	**10**	**400**	**800**	**1000**	**1600**	**2000**	**0.8**	**1.6**	**4**	**500**	**1400**
	Calculated	0.54	1.04	1.99	5.99	10.54	192.4	396.22	399.46	481.61	416.63	0.94	1.25	4.33	235.25	425.69
		0.48	0.88	2.1	4.4	8.79	225.52	388.93	372.39	447.36	425.69	0.97	1.97	3.98	265.9	423.38
	Mean	0.51	0.96	2.04	5.2	9.66	208.96	392.58	385.92	464.49	421.16	0.95	1.61	4.16	250.58	424.53
	SD	8%	12%	4%	22%	13%	11%	1%	5%	5%	2%	2%	31%	6%	9%	0%
	%Difference	1%	-4%	2%	4%	-3%	-48%	-51%	-61%	-71%	-79%	19%	1%	4%	-50%	-70%

- Saturation was observed at the upper ranges, therefore pulldown conditions were optimized using different antibody amounts and incubation conditions.

Final Assay Conditions and Performance

[A]



[B]

			DP Calibration Standards (ng/mL)							
3 µg antibody	Nominal		1	2	10	50	200	800	1600	2000
	Run 1	Rep 1	1.1	2.2	10	46.9	220	881.2	1476	1912.3
		Rep 2	1	1.6	8.8	54.5	205.1	832.4	(R)	1962.4
	Run 2	Rep 1	1.2	2.1	10.5	56.7	196.2	811.2	(R)	(R)
		Rep 2	0.8	2	9.9	49.3	193.6	779.3	(R)	1776.3
	Run 3	Rep 1	(R)	2.1	9.7	48.4	201.1	840.6	1596.3	2024.2
		Rep 2	1	1.9	9.9	51.2	194.3	846.2	1551.7	1948.3
	Inter-day P&A	Mean	1	2	9.8	51.2	201.7	831.8	1541.3	1924.7
		CV	13%	10%	6%	7%	5%	4%	4%	5%
		% Bias	1%	-1%	-2%	2%	1%	4%	-4%	-4%

			DP QC Samples (ng/mL)				
3 µg antibody	Nominal		1	2	3	750	1500
	Run 1	Rep 1	1	2.1	3.3	790.2	1441.7
		Rep 2	1.3	2	2.9	808.7	1426.3
		Rep 3	1.3	1.9	3.3	809.7	1635.3
	Run 2	Rep 1	1.1	1.4	2.8	603.1	1166.4
		Rep 2	0.9	1.9	2.6	747.6	1251.1
		Rep 3	0.8	1.8	2.4	738.3	1361.2
	Run 3	Rep 1	1.2	2	2.8	708.2	1510.3
		Rep 2	1.1	2	3.2	778.7	1538
		Rep 3	0.9	1.8	3.1	765.6	N/A
	Inter-day P&A	Mean	1.1	1.9	2.9	750	1416.3
		CV	17%	11%	10%	9%	11%
		% Bias	7%	-6%	-3%	0%	-6%

Summary and Conclusions

- Lack of antibody selectivity for the DP vs ENDO forms meant that immunoassay could not be used
- A hybrid immunoassay-mass spectrometry based approach enabled independent measurement of the DP and ENDO forms in a single assay
- Systematic troubleshooting and optimization enabled the development of final assay conditions with sensitivity (LLOQ) equivalent to immunoassay (MesoScale®), despite the non-optimal characteristics of the peptides needed to obtain assay selectivity
- Successful assay qualification was performed for the intended uses:
  (1) Primary use: PK assay for DP measurement in serum at high concentrations
  (2) Secondary use: Measurement of ENDO in serum at lower concentrations, for academic interest

➢ **This mass spectrometry based approach is generally applicable when available antibodies are not sufficiently selective (e.g. for protein isoforms, phosphorylation or other PTMs), and use of immunoassay is not feasible or inappropriate**

Questions

Rudolf Guilbaud
Director, Operations, Caprion Biosciences
rguilbaud@caprion.com
caprion.com
LinkedIn | Twitter | Facebook

CAPRION
ACCELERATING PRECISION MEDICINE