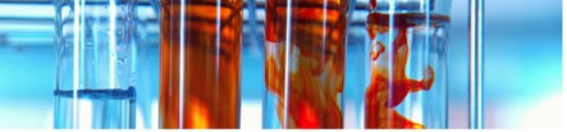
KCAS

ACTIVE BIOMARKERS a KCAS company

FlowMetric a KCAS company

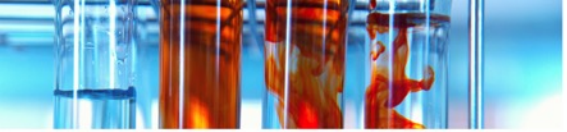
Using the Flexibility of Hybrid LC-MS/MS to Address Typical Challenges in Quantitation of Large Molecules

Dawn Dufield, Ph.D., Rathna Veeramachaneni, Ph.D.
John R. Perkins, Ph.D.



Agenda

- Introduction to large molecule bioanalysis
- The increasing role of hybrid LC-MS/MS in large molecule bioanalysis
- Case Studies
- Conclusions

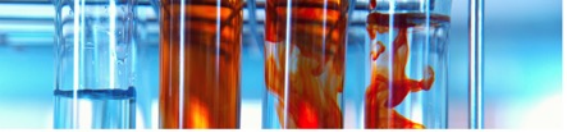


Introduction

- Instinct when thinking about bioanalysis of large molecules is towards ligand binding assays (LBA) .

- Typical sandwich assay requires two antibodies for successful analysis
  - Capture antibody to bind target analyte
  - Detection antibody with conjugated to allow detection

- Lack of available reagents often call for alternative approaches for large molecule bioanalysis
  - Opportunity for MS-based approaches



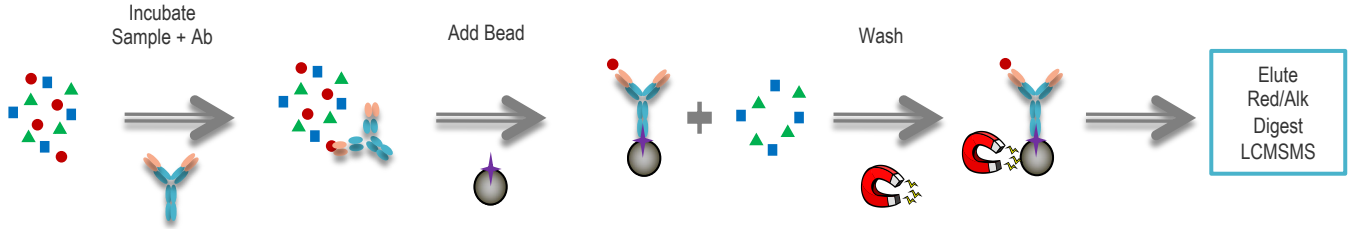
Principles of Hybrid LC-MS/MS

- Definition: Hybrid LC-MS/MS is a technique which combines an enrichment step (typically an antibody enrichment on beads or columns) with the selectivity and sensitivity provided by LC-MS/MS
- Target analytes have masses in the range of tens to hundreds of thousands of daltons
- Typical mass range of triple quadrupole mass spectrometer is 5-2000 Da (SCIEX API-6500) so surrogate peptide approach is necessary
- Following digestion, unique peptide is extracted and quantitated allowing PK modelling for large molecules
- The ability to optimize multiple steps plays into the flexibility of hybrid LC-MS/MS

Typical Hybrid LC-MS/MS Methodology

Capture

1.

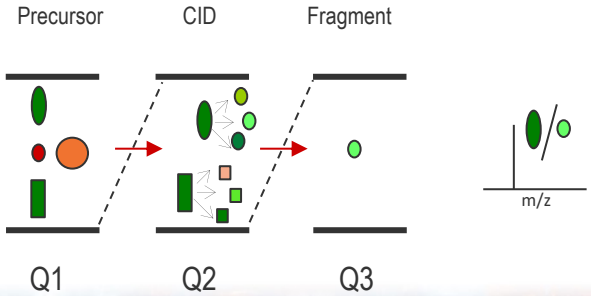


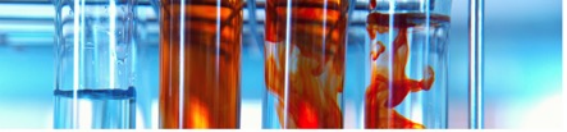
Detector

2.

- MS detector allows one to "dial" in selectivity
- Only need 1 Ab

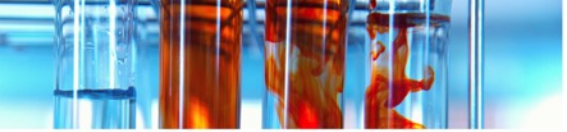
Multiple Reaction Monitoring





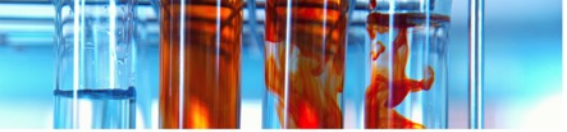
Typical Project Workflow for Hybrid LC-MS/MS

- In-silico modelling to predict potential target peptides following enzymatic digestion (trypsin, chymotrypsin, Lys-C etc)
- Identify peptides with chain length to work well with LC-MS/MS
- Digestion in solution & LC-MS/MS of putative candidates (screening for sensitivity, selectivity)
- Performance of key peptides in matrix
- Selection of primary peptide for further development & synthesis of stable-labeled extended peptide



Hybrid LC-MS Value to Large Molecule Bioanalysis

- Lack of availability of reagents does not prevent development of an LC-MS/MS assay
  - Feasible to develop LC-MS/MS assay with one or no good reagents
- Use selectivity from extraction, chromatography and MS/MS to avoid interferences
- Ready translation between species with some sequence changes. Less concern on effect on Ab binding
- Ability to work around and detect simple sequence changes (e.g. – 1 amino acid, phosphorylation)
- Flexibility in analysis – not tied to a single molecular entity
  - Ability to provide multiple data assessments at any time
  - Same protein but different parts of the molecule

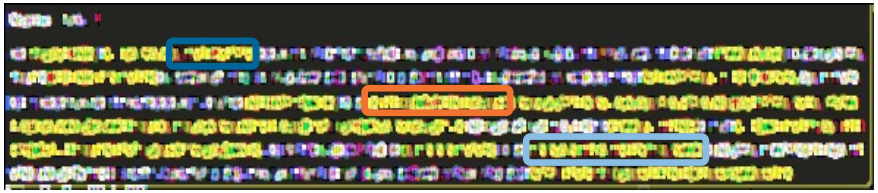


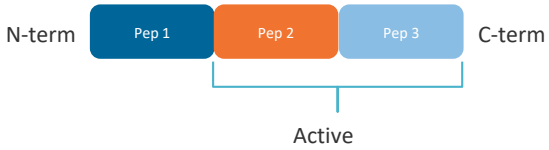
Case Studies

- Case Study #1 – Flexibility in Analyte Candidate Selection
- Case Study #2 – Bioanalysis of Biomarker Long & Short Forms
- Case Study #3 – Rapid Assay Development for ADC Stability Screening & PK Support
- Case Study #4 – Lack of Availability of Protein Reference Standard for Opthalmic Assay

Case Study #1 – Flexibility in Analyte Candidate Selection

- Original project scope – PK assay for biotherapeutic using LBA
- Observed issues with instability impacting assay development & quantitation
- Pivot to hybrid LC-MS/MS to troubleshoot instability

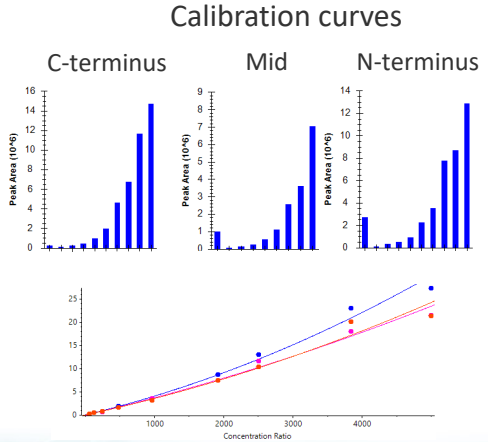


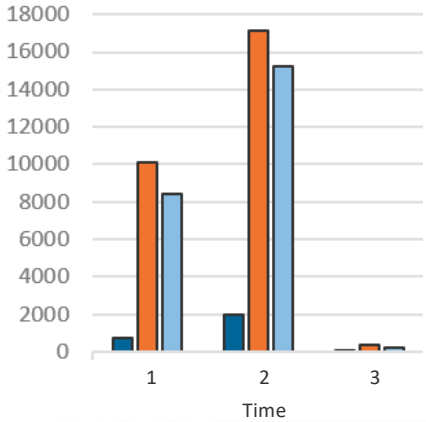


Multiplexed hybrid LC-MS/MS assay using selected peptides from different regions of protein

Case Study #1 – Flexibility in Analyte Candidate Selection

- Quantify several peptides
- Choose across entire protein (N-term, Mid, C-term)
- PK assay as well as stability information – correlated with activity/efficacy etc.

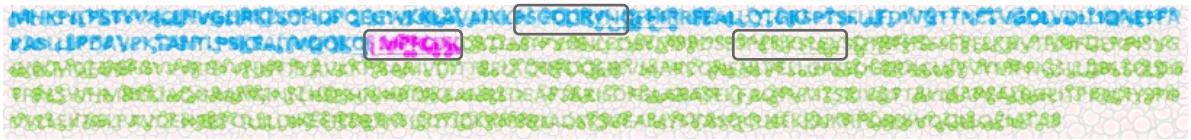




- N-terminal peptide rapidly lost compared to other protein regions
- Drug was still active so assumption - N-term not required for activity

Case Study #2 – Bioanalysis of Biomarker Long & Short Forms

- Goal: To develop an assay for short and long isomers of a protein biomarker



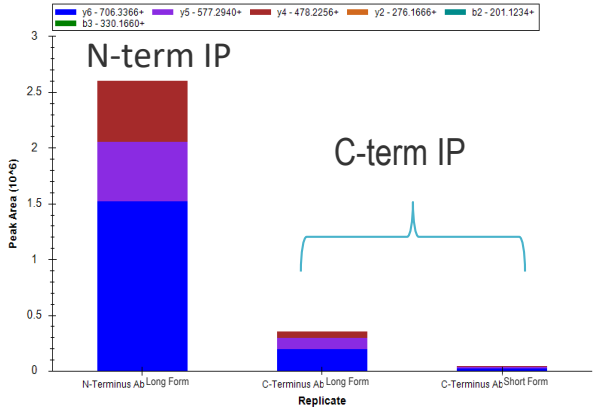
Cyan – specific to long form peptide

Magenta – specific to short form peptide

Green – Common/total peptide

- Challenges
  - Identify unique peptide for each isoform
  - Only 1 peptide can differentiate “short” form
  - Can we get a 2 plex assay – short and long forms?
- Initial Approach
  - 2 Plex assay
    - IP with C-term Ab – capture both short and long isoforms
- Findings
  - C-terminal could not be used for 2 plex assay
  - Insufficient sensitivity for long and short forms
- Pivot to alternative approaches
  - IP with N-term Antibody for long form
  - How to measure short form?
    - Incorporate total assay

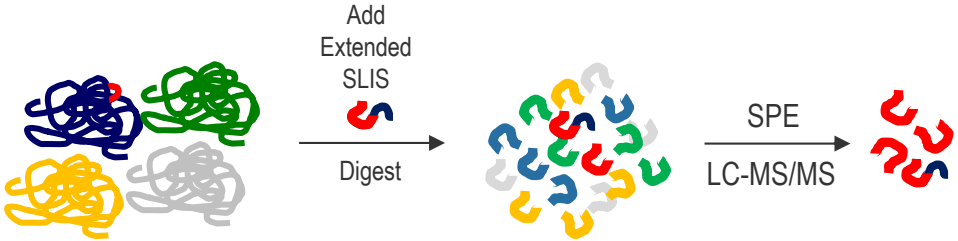
Case Study #2 – Bioanalysis of Biomarker Long & Short Forms



- Results
  - N-terminal IP yielded much better sensitivity for Long Peptide
  - No direct measurement possible for short peptide
  - Dual workflow

Total Measurement - Traditional LC-MS/MS

PBMCs → Direct Digest → SPE → LC-MS/MS 7500



Long Isoform - Hybrid LC-MS/MS

PBMCs → IP-Enrichment → STD/ISTD Digestion → LC-MS/MS 7500

Short = Total – Long

Case Study #2 – Bioanalysis of Biomarker Long & Short Forms

Total Assay – Traditional LC-MS/MS

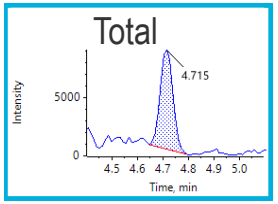
Standard Curves in 0.1% BSA in Deionized Water

Component Name	Actual Conc (ng/mL)	Num. Values	Mean	% CV	% Accuracy
Common - Total	5	2 of 2	5.2	1.9	104.6
Common - Total	10	2 of 2	9.2	1.4	91.7
Common - Total	20	2 of 2	19.5	6.5	97.7
Common - Total	40	2 of 2	39.6	4.6	98.9
Common - Total	80	2 of 2	81.3	7.3	101.7
Common - Total	160	2 of 2	165.2	1.8	103.2
Common - Total	320	2 of 2	308.6	10.5	96.5
Common - Total	640	2 of 2	692.2	9.0	108.2
Common - Total	1280	2 of 2	1265.3	4.2	98.9
Common - Total	2560	2 of 2	2526.8	2.4	98.7

Quality Controls in 0.1% BSA in Deionized Water

Name	Actual Conc (ng/mL)	Dilution Factor	Num. Values	Mean	% CV	% Accuracy
Common	20	1	3 of 3	17.0	10.4	85.2
Common	60	1	3 of 3	46.5	6.9	77.6
Common	500	1	3 of 3	381.7	3.6	76.4
Common	2000	1	3 of 3	1578.7	5.2	78.9
Common	10000	10	3 of 3	10239.3	2.0	102.4

Detection of Total and Long forms in isolated PBMC's from about 1 million cells.



Lysis with 400 µL TPER buffer
~11 ng/mL

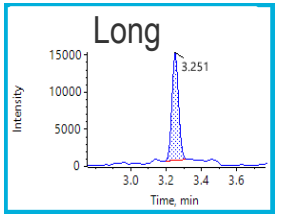
Long Isoform Assay – Hybrid LC-MS/MS

Standard Curve in 0.1% BSA in Deionized Water

Name	Actual Conc (ng/mL)	Num. Values	Mean	% CV	% Accuracy
Long	5	2 of 2	4.8	6.46	96.53
Long	10	2 of 2	10.8	7.03	107.76
Long	20	2 of 2	19.5	8.37	97.4
Long	40	2 of 2	41.1	0.21	102.72
Long	80	2 of 2	80.7	5.06	100.89
Long	160	2 of 2	154.7	5.69	96.71
Long	320	2 of 2	305.1	7.15	95.36
Long	640	2 of 2	654.6	7.05	102.28
Long	1280	2 of 2	1285.3	11.57	100.41
Long	2560	2 of 2	2559.2	3.13	99.97

Quality Controls in 0.1% BSA in Deionized Water

Name	Actual Conc (ng/mL)	Dilution Factor	Num. Values	Mean	% CV	% Accuracy
Long	5	1	3 of 3	5.1	4.8	102.3
Long	15	1	3 of 3	14.8	1.9	98.8
Long	500	1	3 of 3	491.2	6.6	98.2
Long	2000	1	3 of 3	2127.4	14.5	106.4
Long	10000	10	3 of 3	9972.7	6.1	99.7

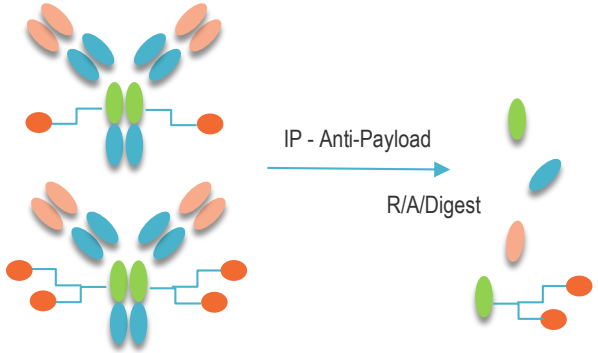


Lysis with 400 µL TPER buffer
~9 ng/mL

Case Study #3 – Rapid Assay Development for ADC Stability Screening & PK Support

- Goal: To develop a PK assay for multiple human ADC in preclinical species for *in-vitro* and *in-vivo* studies
  - Various DAR's with same payload (MMAE)
  - Step 1 – In-vitro stability to identify most stable ADCs
  - Step 2 – In-vivo PK for top 3 ADCs

- Experimental Approach –
  - MMAE Antibody immunoprecipitation followed by digestion and LCMSMS
  - +/- Reduction/Alkylation
  - Monitor Common Fc peptides (Several to choose from (Trp sites))
  - DAR insensitive – Will capture any DAR > 0
  - 20 different candidate ADC's screened

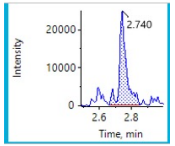


ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSD
GSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Case Study #3 – Rapid Assay Development for ADC Stability Screening & PK Support

- 1.5 to 2 Day MD – verify peptide sensitivity, assess accuracy & precision
- Typical range 10-5000 ng/mL (25 µL mouse plasma)
- Surrogate Matrix STD's (0.1% BSA) and Matrix QC's
- Several Fc peptides (FNW, VVSV, GPS, etc)

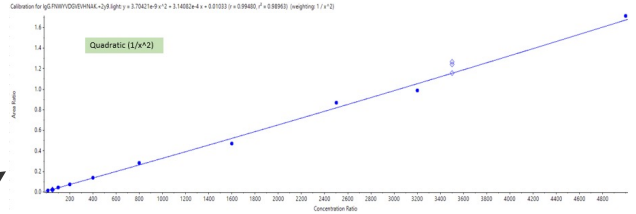
Standard 10ng/mL



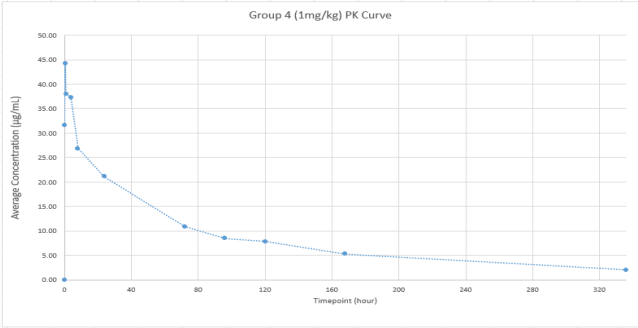
Component Name	Actual Concentration (ng/mL)	Num. Values	Mean (ng/mL)	Average Accuracy	Value #1 (ng/mL)
IgG.FNWYVDGVEVHNAK.+2y9.light	10	1 of 1	10.3	**102.8**	10.3
IgG.FNWYVDGVEVHNAK.+2y9.light	50	1 of 1	39.2	**78.4**	39.2
IgG.FNWYVDGVEVHNAK.+2y9.light	100	1 of 1	112	**112.5**	112
IgG.FNWYVDGVEVHNAK.+2y9.light	200	1 of 1	207	**103.4**	207
IgG.FNWYVDGVEVHNAK.+2y9.light	400	1 of 1	411	**102.9**	411
IgG.FNWYVDGVEVHNAK.+2y9.light	800	1 of 1	858	**107.2**	858
IgG.FNWYVDGVEVHNAK.+2y9.light	1600	1 of 1	1452	**90.7**	1452
IgG.FNWYVDGVEVHNAK.+2y9.light	2500	1 of 1	2648	**105.9**	2648
IgG.FNWYVDGVEVHNAK.+2y9.light	3200	1 of 1	3006	**93.9**	3006
IgG.FNWYVDGVEVHNAK.+2y9.light	5000	1 of 1	5113	**102.3**	5113

Component Name	Actual Concentration (ng/mL)	Num. Values	Mean (ng/mL)	Standard Deviation	Percent CV	Average Accuracy Across Replicates	Value #1 (ng/mL)	Value #2 (ng/mL)	Value #3 (ng/mL)
IgG.FNWYVDGVEVHNAK.+2y9.light	0	0 of 3	N/A	N/A	N/A	N/A	0	0	0
IgG.FNWYVDGVEVHNAK.+2y9.light	50	3 of 3	43.7	7.8	17.88	87.5	34.7	47.9	48.6
IgG.FNWYVDGVEVHNAK.+2y9.light	3500	3 of 3	3694.3	166.3	4.50	105.6	3507	3752	3824

Standard Curve



PK Curve

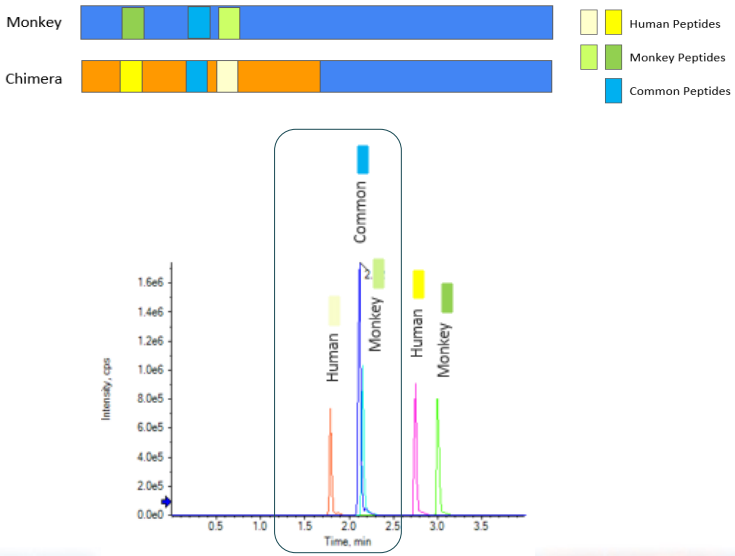


Case Study #4 – Lack of Availability of Protein Reference Standard

- Goal: Development of an assay to be able to differentiate endogenous monkey from chimeric forms of expressed protein following administration of gene therapy

- Approach/Strategy
  - IP with C-term Ab – capture monkey and chimera
  - Identify peptides for total target protein and expressed chimera
  - No protein STD – use Peptide STD's (long/flanked ISTD) and bridged to protein with cell lysates

Retina → IP-Enrichment → STD/ISTD Digestion → LC-MS/MS 7500



Case Study #4 – Lack of Availability of Protein Reference Standard

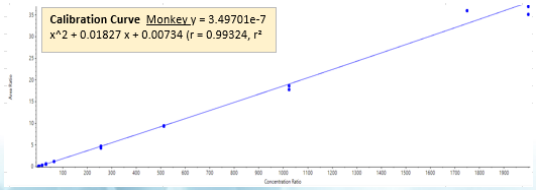
Typical Peptide Standard Curve (NHP)

Calibration Curve Statistics - Peptide Standatrds in Surrogate Matrix

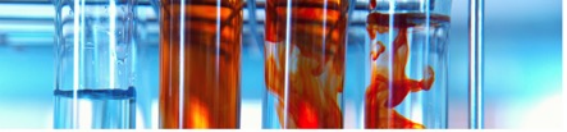
Component Name	Actual Conc (pM)	Replicate #1	Replicate #2	Replicates Used	Mean	St Dev	% CV	Average % Accuracy
Monkey	0.5	0.415	0.56	2 of 2	0.487	0.102	21.0	97.4
Monkey	1	~~1.62~~	~~1.837~~	0 of 2	N/A	N/A	N/A	N/A
Monkey	2	2.70	1.90	2 of 2	2.30	0.562	24.5	114.9
Monkey	4	3.48	3.75	2 of 2	3.62	0.19	5.3	90.4
Monkey	16	15.2	17.8	2 of 2	16.5	1.85	11.2	103.1
Monkey	32	28.4	32.5	2 of 2	30.4	2.958	9.7	95.1
Monkey	64	66.8	66.2	2 of 2	66.5	0.41	0.6	104.0
Monkey	256	235	257	2 of 2	246	15.2	6.2	96.1
Monkey	512	513	507	2 of 2	510	4.2	0.8	99.5
Monkey	1024	959	1000	2 of 2	980	29.52	3.0	95.7
Monkey	1750	1900	1899	2 of 2	1899	0.6	0.0	108.5
Monkey	2000	1951	1858	2 of 2	1905	65.3	3.4	95.2

Peptide Quality Controls in Surrogate Matrix

Component Name	Actual Conc (pM)	Replicate #1	Replicate #2	Replicate #3	Rep Used	Mean	St Dev	% CV	Average % Accuracy
Monkey	1	(Ctrl)	1.07	0.88	3 of 3	1.00	0.106	10.6	100.2
Monkey	3	~~3.93~~	3.05	3.40	2 of 3	3.23	0.25	7.8	107.6
Monkey	50	51.4	53.1	52.6	3 of 3	52.4	0.90	1.7	104.7
Monkey	1500	1388	1568	1542	3 of 3	1499	97.0	6.5	100.0

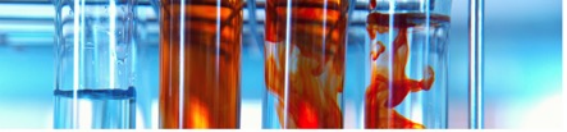


- Fit-for-purpose assay qualified to detect chimera and confirm presence of human, NHP or common peptides
- Use of SCIEX API-7500 to achieve the most sensitivity needed for the retinal punches.
- Ability to report approximate picomolar concentrations for protein (0.5 to 2000 pM).
- Matrix effects checked with various dilutions of an overexpressed lysate to test the protein recovery was consistent. Use as “QCs”



Conclusions

- Flexibility of hybrid LC-MS/MS key to successful assay development
  - Ability to measure multiple different peptides within an analyte
  - Optimization of capture reagents, extraction, digestion, downstream analysis
- Lack of availability of reagents does not prevent development of an LC-MS/MS assay
- Successful fit-for-purpose assays can be developed for proteins or biomarkers when no protein standards are available.
- Combination of hybrid LC-MS/MS with traditional extraction approaches can provide more complete picture
- Use of “generic” assays can leverage rapid assay development



Thank you for your attention