The Road Ahead - What's Next for Host Cell Protein Analytics?

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OUR MISSION

We empower accurate and economical impurity detection, supporting our customers at every stage of their journey toward delivering safe and effective biotherapeutics.

CYGNUS TEAM 2026

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Cygnus Expertise

Unmatched Product and Service Portfolio

- Generic HCP ELISA kits for **24 expression platforms**
- Custom HCP kits for late stage and marketed products
- Generic ELISA kits for purification leachates and cell culture media additives (Protein A, BSA, Insulin, etc)
- Custom services for orthogonal assay characterization

Decades of Experience

- Pioneer in HCP Analytics with **over 25 years** as the market leader
- Deep understanding of all areas of process-related impurity testing: purification development, assay qualification, orthogonal testing, regulatory expectations

Credibility

- Thought leaders within HCP Analytics market
- Known as **"Gold Standard"** globally
- Support our clients with best practices in HCP analytical development

Trusted by Industry and Regulators

- Established relationships with customers and regulators
- **Over 600 AAE/2D-PAGE and AAE-MS** coverage analysis projects
- **Over 200 process-specific HCP assay** supporting late stage and marketed biologics
- **Cygnus kits are filed in 27 of 27 approved CAR-T cell and Gene Therapy products**

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Introduction: A Trip Down Memory Lane

- Where we started: HCP as a process monitoring tool
- Methods used to monitor HCP clearance
- Discovery of immunogenicity caused by HCPs
  - 1D Western Blot
  - 2D Western Blot
  - HCP ELISA
  - SDS-PAGE
- Updated regulatory expectations

Clearance studies, which could include spiking experiments at the laboratory scale, to demonstrate the removal of cell substrate derived impurities such as nucleic acids and host cell proteins may sometimes be used to eliminate the need for establishing acceptance criteria for these impurities. Guidance for Industry Q6B: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products

Current Regulatory Guidelines

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Current Regulatory Guidelines

- ICH Q6B ” Test Procedure and Acceptance Criteria for Biotechnological/Biological Products “ - Recommendations on monitoring HCPs (1999)
- ICH S6(R1) – Guidance on the safety assessment of biologics, including the HCP related risks
- ICH Q2(R2) “Validation of Analytical Procedures” – Guidance on HCP analysis method validation (March 2024)
- ICH Q8(R2) – Highlights the importance of identifying and controlling CQAs, during pharmaceutical development
- ICH Q14 – introduces risk-based, science driven approach to develop and maintain analytical procedures to identify and quantify individual process impurities (March 2024)

- USP Chapters 1132, 1132-1
- European Pharmacopoeia 2.6.34

HCP Immunoassays and HCP Analysis by LC-MS

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HCP Analysis by ELISA

ELISA is a gold standard for process monitoring and product lot release

- Semi-quantitative measurement of a statistical sample of HCPs in a sample
- Excellent sensitivity and selectivity
- Does not require special expertise to run
- Effective in relatively high levels of product protein
- Easily transferable, reliable, robust

+

- Reports total HCP equivalents (not ng/mL)
- Does not identify individual HCPs
- No information regarding which HCPs are detected

-

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Opportunities in HCP analytics by LC-MS

Broadly applicable

- All expression systems

Not dependent on animal immune response and antibody reactivity to HCPs

- HCP signal in ELISA is proportional to HCP abundance, antibody coverage and affinity to HCPs
  - LC-MS signal is not

Individual HCP identity/quantification

- Partition total HCP signal via ELISA into individual HCPs

Rapidly evolving technology

- Sample prep chemistries, instruments, software improvements facilitated by AI, etc.

Challenges in HCP analytics by LC-MS

Variable elements affecting LC-MS

- Digestion, ionization, intensity, fragmentation, matrix interference

Units of measurement

- Mass spectrometry is measured in moles; ELISA is measured in mass
  - Conversion is required

Limited Dynamic Range of MS Instruments/Sensitivity and Accuracy

- Enrichment is popular but makes quantification more difficult
  - AAE, combinatorial libraries, fractionation, native digestion
- Accuracy is typically based on non-HCP standards

HCP Antibody Coverage Analysis by AAE-MS™

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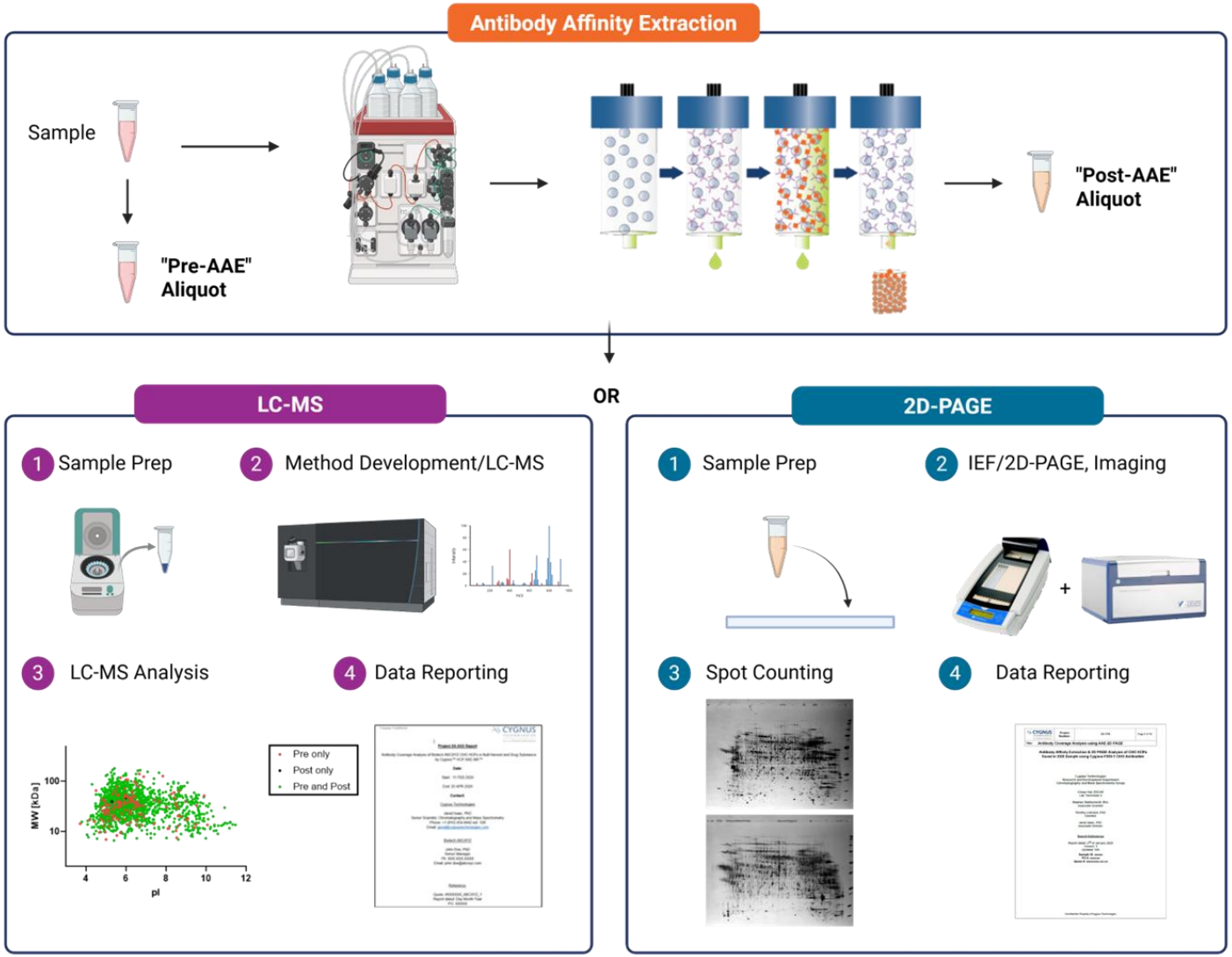
Antibody Affinity Extraction (AAE™)

- Overview of the technology
- Advantages over previous methods

Mass Spectrometry

- Identification of HCP in mock/null harvest for coverage
- Identification of HCP in product expressing harvest for coverage
- Identification of HCP in drug substance

Antibody Affinity Extraction (AAE™)



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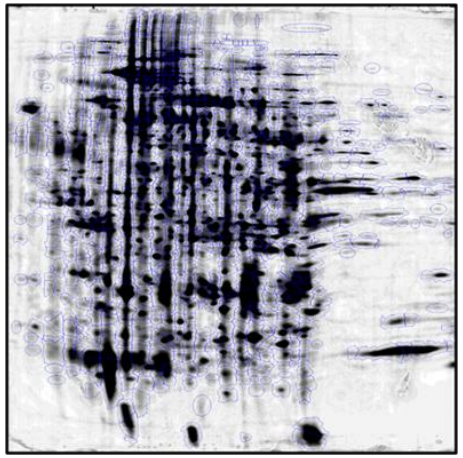
CHO HCP Ab Coverage Assessment

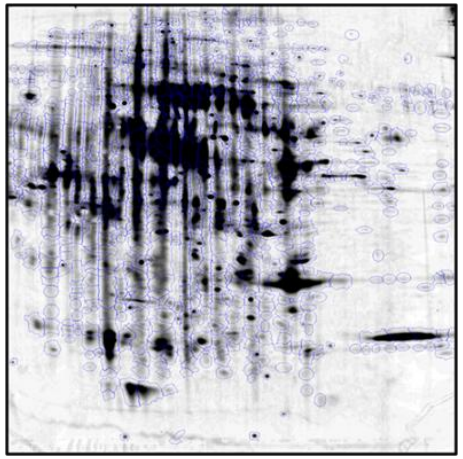
F550-1 Anti-CHO HCP pAb Coverage of CHO Harvest Sample

A

Silver Stain of "**Pre-AAE**" CHO Harvest Sample



Silver Stain of "**Post-AAE**" CHO Harvest Sample



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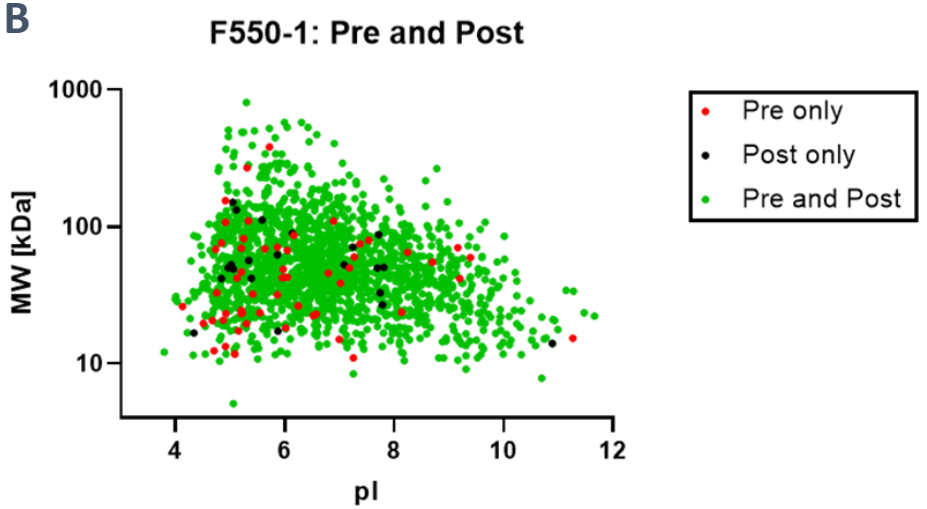

Detection Method	Pre-AAE	Post-AAE	% pAb Coverage
2D-PAGE	1176	1005	86
MS	1694	1639	97

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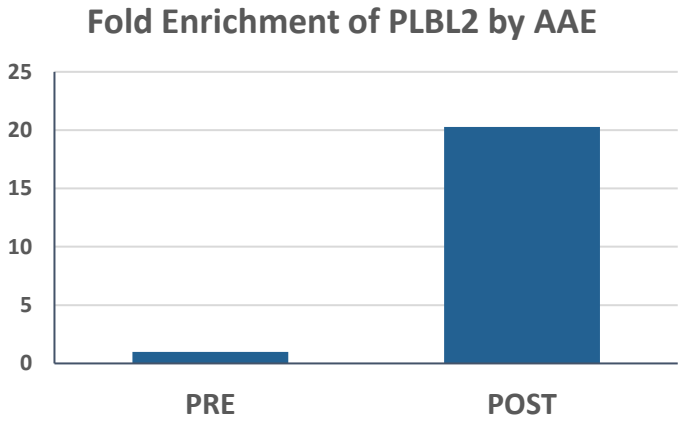
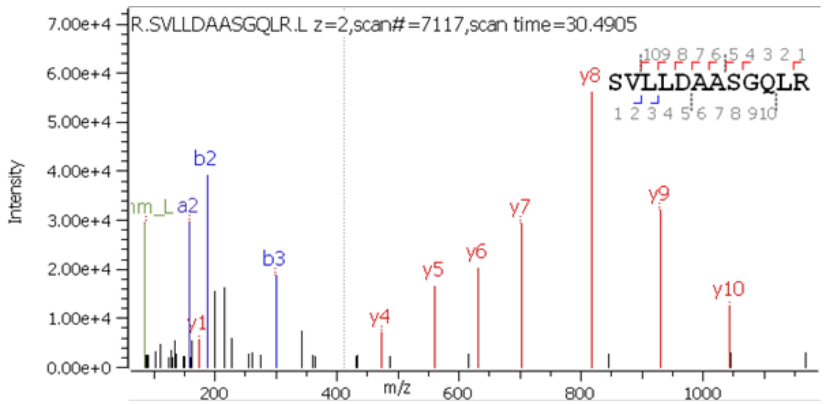
Individual CHOP Reactivity to Anti-CHO HCP pAB by AAE-MS™

Phospholipase B like 2 [PLBL2] is Immunoreactive with CHO 3G





#	Potential High Risk HCPs	PRE	F550	F550-1	pI	MW
1	78 kDa glucose regulated protein (BiP, HSPA5)	Y	Y	Y	5.07	72379.1
2	Actin (ACTB)	Y	Y	Y	5.29	41736.7
3	Aldose reductase related protein 2	Y	Y	Y	5.85	46698.3
4	Alpha enolase	Y	Y	Y	5.98	50011.7
5	Carboxypeptidase D	Y	Y	Y	5.3	48265.1
6	Cathepsin B (CTSB)	Y	Y	Y	5.73	35646.9
7	Cathepsin D	Y	Y	Y	6.54	44110.9
8	Cathepsin E	N	N	N	4.61	42726.4
9	Chondroitin sulfate proteoglycan 4	Y	Y	Y	5.4	252012.3
10	Clusterin	Y	Y	Y	5.58	51557.5
11	Cofilin 1	Y	Y	Y	8.22	18532.5
12	Elongation factor 1a1	Y	Y	Y	9.39	55106.0
13	Elongation factor 2	Y	Y	Y	6.41	95324.1
14	Flagellin	N	N	N	4.5	51295.0
15	Galectin 3 binding protein	Y	Y	Y	5.05	63802.2
16	Glutathione S transferase P	Y	Y	Y	7.64	23638.2
17	Glyceraldehyde 3 phosphate dehydrogenase	Y	Y	Y	8.49	35747.9
18	G-protein coupled receptor 56	Y	Y	Y	9.06	77370.5
19	Heat shock cognate 71 kda protein	Y	Y	Y	5.23	70804.9
20	Heat shock protein HSP 90	Y	Y	Y	4.94	83166.1
21	Lipoprotein Lipase	Y	Y	Y	7.94	52900.3
22	Lysosomal protective protein	Y	Y	Y	5.64	56110.7
23	Matrix Metalloproteinase 19	Y	Y	Y	7.71	58942.0
24	Metalloproteinase inhibitor 1	Y	Y	Y	8.84	22401.0
25	Monocyte chemoattractant protein 1 (C-C motif chemokine)	Y	Y	Y	9.32	15858.4
26	Nidogen-1	Y	Y	Y	4.72	83103.0
27	Peptidyl-prolyl cis-trans isomerase	Y	Y	Y	9.59	23634.4
28	Peroxiredoxin 1	Y	Y	Y	8.22	22262.6
29	Phosphoglycerate kinase 1	Y	Y	Y	8.02	44562.5
30	Phospholipase A2 (Group XV lysosomal)	Y	Y	Y	6.16	87100.0
31	Phospholipase B like 2	Y	Y	Y	5.63	61824.4
32	Procollagen C endopeptidase enhancer 1	Y	Y	Y	8.16	50446.5
33	Procollagen lysine 2 oxoglutarate 5 dioxygenase 1	Y	Y	Y	6.46	83550.2
34	Procollagen-lysine 5-dioxygenase (PLOD3)	Y	Y	Y	6.57	83327.9
35	Protein disulfide isomerase	Y	Y	Y	5.98	56796.4
36	Pyruvate kinase	Y	Y	Y	6.88	57893.8
37	Serine protease HTRA1	Y	Y	Y	6.62	34404.5
38	SPARC	Y	Y	Y	7.1	51081.6
39	Sulfated glycoprotein 1	N	N	N	5.31	65758.2
40	T-complex protein	Y	Y	Y	5.7	60338.6
41	Thioredoxin 1	Y	Y	Y	6.94	44611.3

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Problematic CHO Host Cell Proteins and Identification of Immunoreactive Proteins in DS samples

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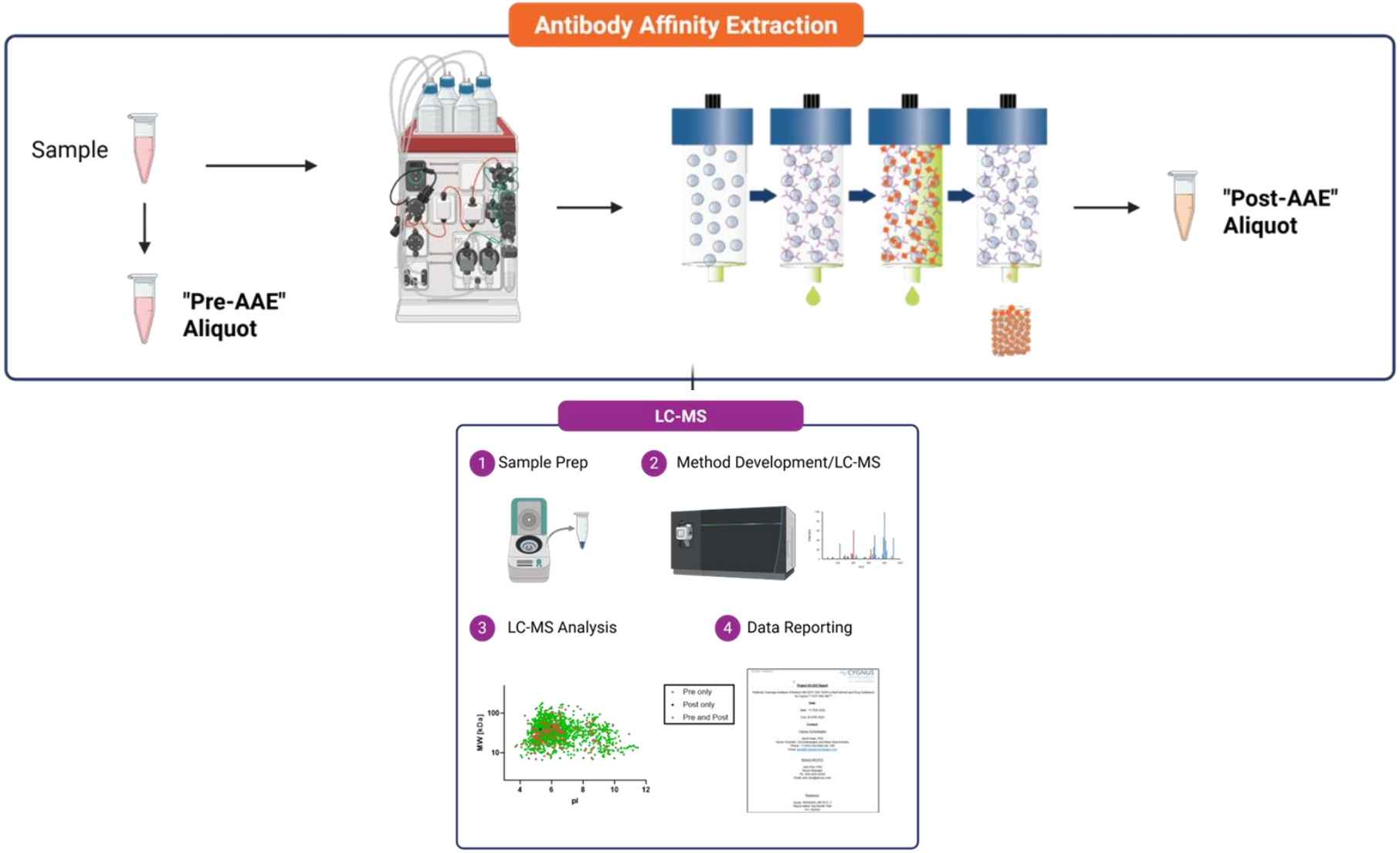
Antibody Affinity Extraction (AAE™)



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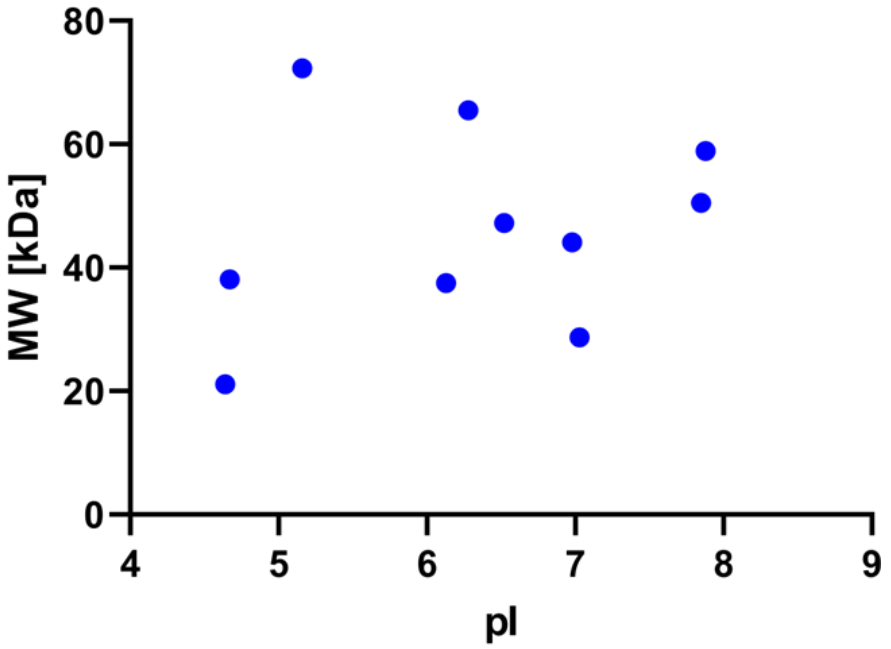
HCPs and their potential mechanisms



Jones, M. et al *Biotechnol. Bioeng*. 2021
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Levy, N. et al. *Biotechnol Bioeng.* 2014

Drug Degradation

Serine Protease HTRA1
Cathepsins
Matrix Metalloproteinase

Immunogenicity

Phospholipase B domain containing 2

PS20 PS80 Degradation

Lipoprotein lipase
Phospholipase A2 Group XV
Phospholipase A2 Group VII
Phospholipase A1

Drug Aggregation

Protein Disulfide Isomerase

Endoplasmic reticulum chaperone BiP
Heat shock proteins

Drug Binding

Clusterin

N-Glycan Degradation

Hexaminidase B (HEXB)

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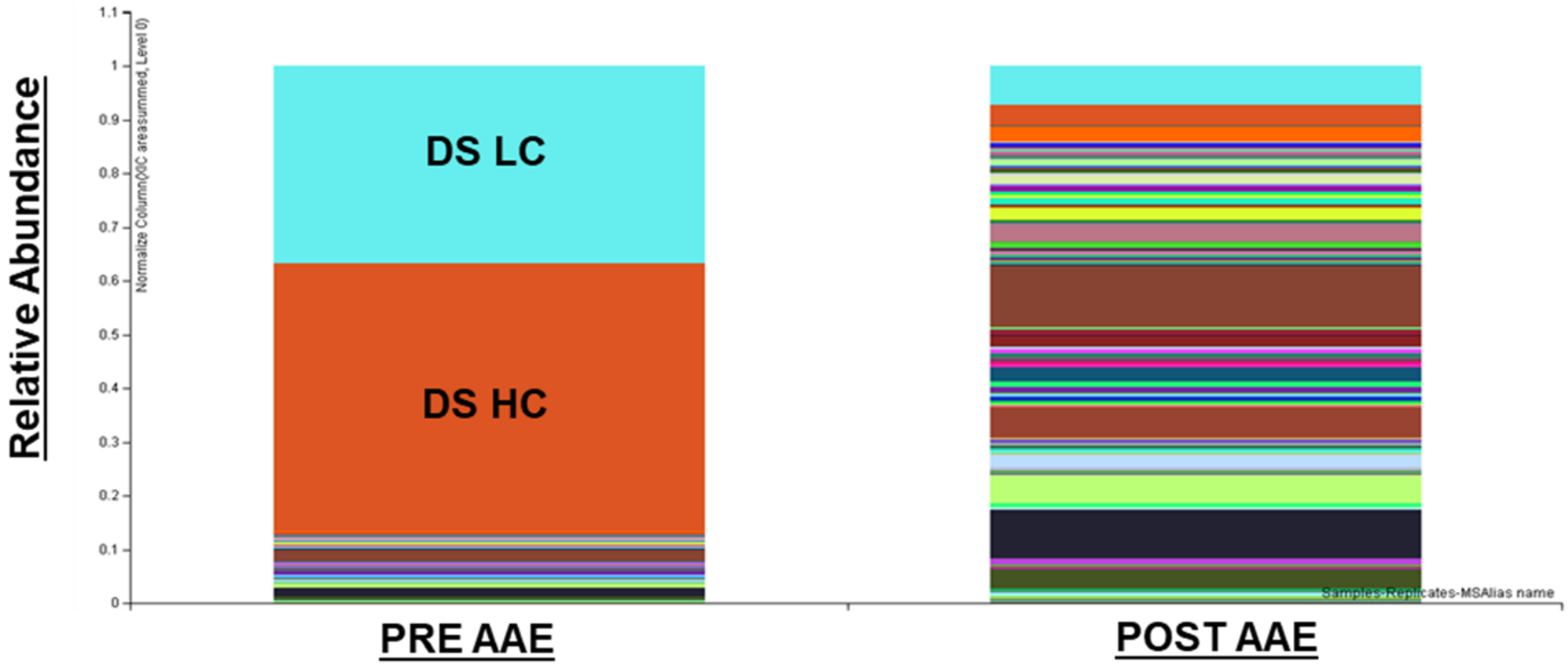
AAE™ Enriches HCP and Depletes DS



The relative abundance of DS and HCPs according to their extracted ion chromatograms were normalized to one and graphed in a stacked bar chart.

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Importance of LC-MS/MS-based HCP Profiling

Host Cell Protein Clinical Safety Risk Assessment—An Updated Industry Review (Biophorum Consortium Publication; *Biotechnology and Bioengineering, Jul 2025*)

*"It is recommended to perform HCP profiling by mass spectrometry **as early as possible** to guide process optimization at each stage of clinical development leading toward commercialization to reduce "high-risk" HCPs to levels that ensure patient safety."*

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USP Chapter 1132.1
“Residual Host Cell Protein Measurement in Biopharmaceuticals by Mass Spectrometry”

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USP Chapter 1132.1 - Establishes Common Industry Terminology and Methodology

- Acknowledges lack of standardized methods (a Wild-West type situation)
- Provides an overview of the capability of MS methods for HCP identification and quantitation as well as their limitations.
- Discusses best practices for instrument selection, sample preparation, LC separation, MS data acquisition, MS data analysis and reporting.
- Emphasizes that MS and HCP-ELISA are different methods, and thus MS results may differ from those obtained by ELISA.
- Makes the LC-MS analysis comparable between different labs
- Expands our HCP analysis toolbox

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Analytical Methods in USP Chapter 1132.1

	Product Protein	Spiked-in Proteins	Spiked-in Peptides
Sensitivity	Poor	Moderate	High
Quantitative Range	Unclear	2-4 orders of magnitude	1-2 orders of magnitude
Precision	High (CV < 20%)	High (CV < 20%)	High (CV < 20%)
Accuracy	Poor	Acceptable (Order of magnitude)	Great (within 2-fold)
Ease of use	Simple; no additional reagents	Moderate; no complex method development	Difficult; thorough method development required

Global Relative Quantification of HCP by inSPECt™ MS with the Cygnus Protein Standard (CPS)

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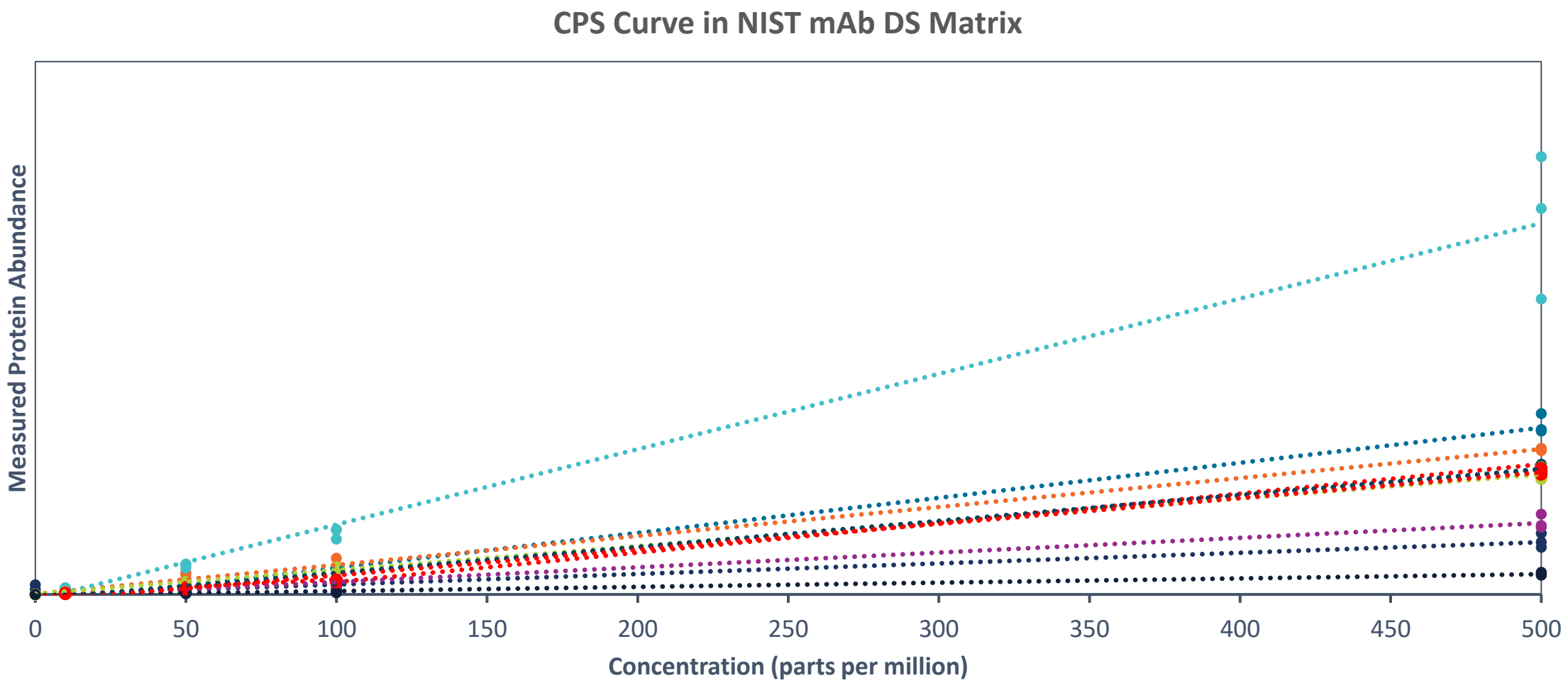
Global Relative Quantification Based Upon the Median Response Factor of CPS



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inSPECt™ MS Quantification of HCP in NIST mAb is Comparable to Prior Reports

HCP	Beaumal *et al.* 2023	inSPECt™ MS
Fructose-biphosphate aldolase A	189 ppm	240 ppm
Fructose-biphosphate aldolase C	84 ppm	83 ppm
Protein-disulfide isomerase A6	83 ppm	148 ppm
Glucose-6-phosphate isomerase	32 ppm	87 ppm

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inSPECt™ MS: Spike and Recovery for Accuracy and Precision

HCP	100 ppm		500 ppm		1000 ppm	
	%Error	%CV	%Error	%CV	%Error	%CV
Lysosomal phospholipase A and acyltransferase	26%	2%	32%	2%	34%	4%
Clusterin	6%	3%	5%	3%	11%	6%
Glutathione S-transferase P 1	23%	5%	27%	2%	31%	7%
Phospholipase B-like	34%	2%	24%	5%	26%	3%

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Absolute HCP Quantification by PRM-MS

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The Cygnus CHO 6xLipase™ MS Assay

CHO Lipases of concern:

- Known roles in excipient stability and immunogenicity
- Can be present in low abundance in downstream bioprocess samples
  - Low concentration ≠ low activity (i.e. sub ppm and ppb levels)

Phospholipase A2 Group XV lysosomal lipase (GXVP_A2/PLA2G15)	**Lysosomal Acid Lipase (LAL/LIPA)**
Lipoprotein Lipase (LPL)	**Phosphoinositide phospholipase C (PIPL_C)**
Phospholipase A1 (PL_A1/PLA1A)	**Phospholipase B like 2 (PLBL2)**

** 2 – 6 peptides per protein for method robustness*

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Parallel Reaction Monitoring (PRM)

Targeted proteomic analysis

- High-resolution mass spectrometry (unlike QQQ)

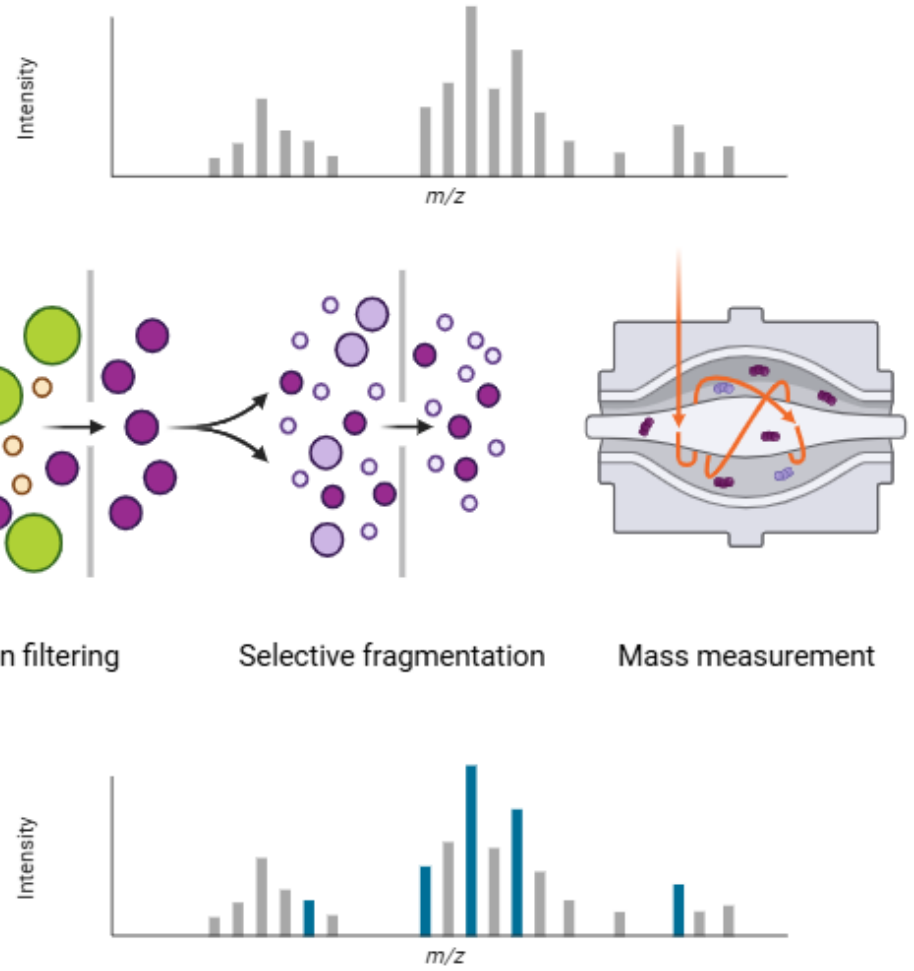
High specificity and sensitivity

- All fragment ions measured
- Wider dynamic range than MRM (Peterson et *al*. Molecular & Cellular Proteomics 2012)

Retention time scheduling

- Free up mass spec to analyze peptides only when they elute off the column

Same instrument as DDA/DIA-based antibody coverage analysis services



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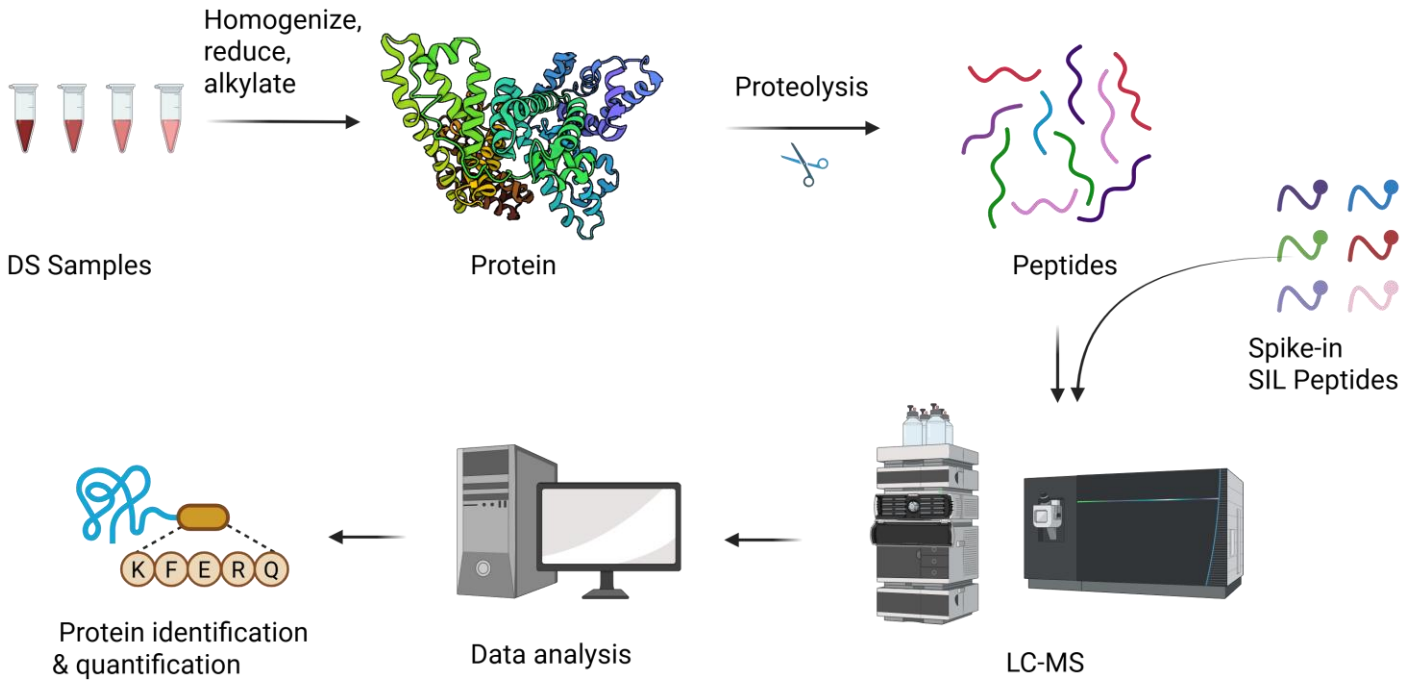
PRM Sample Preparation

General workflow:

- Minimal sample manipulation
- Spike-in of SIL peptides



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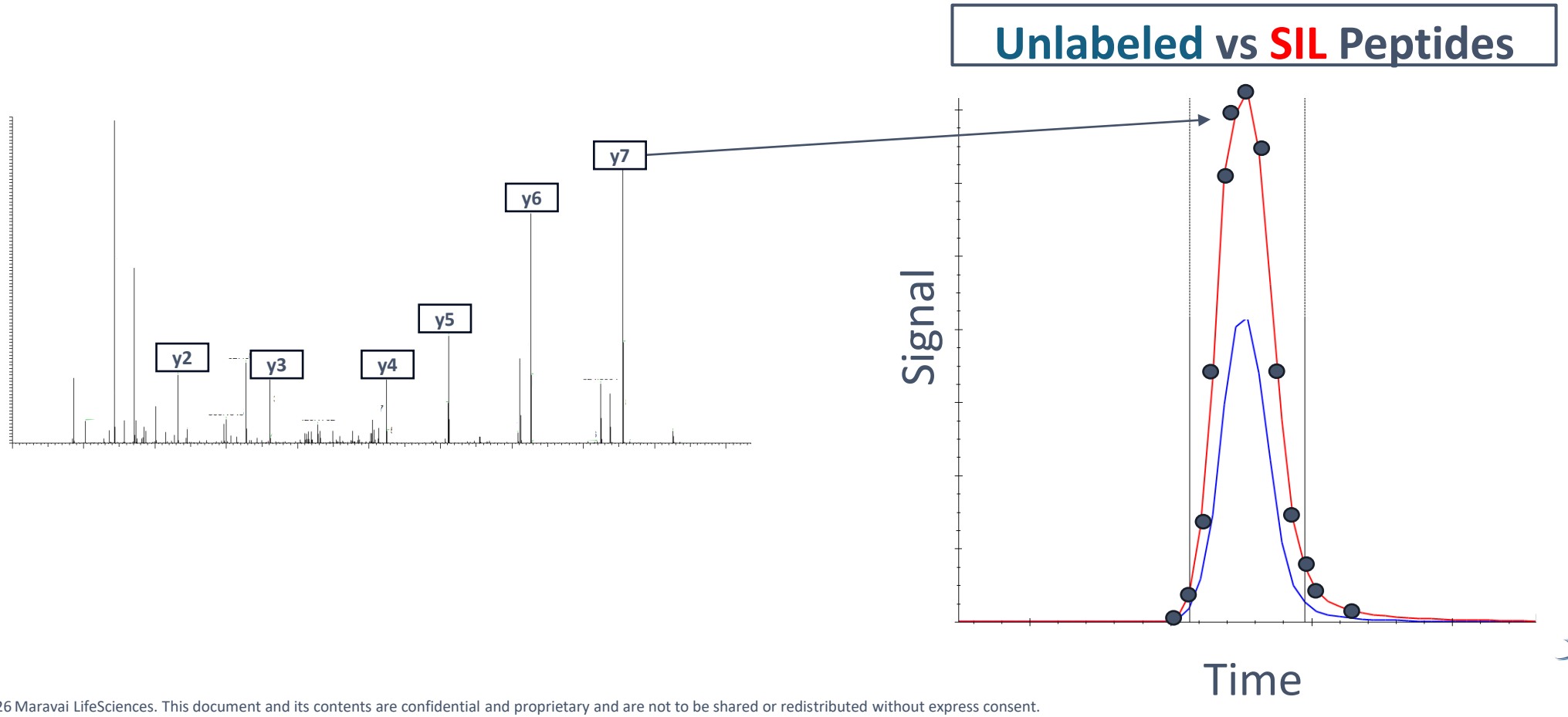
Peak Area Ratio for Absolute Quantification



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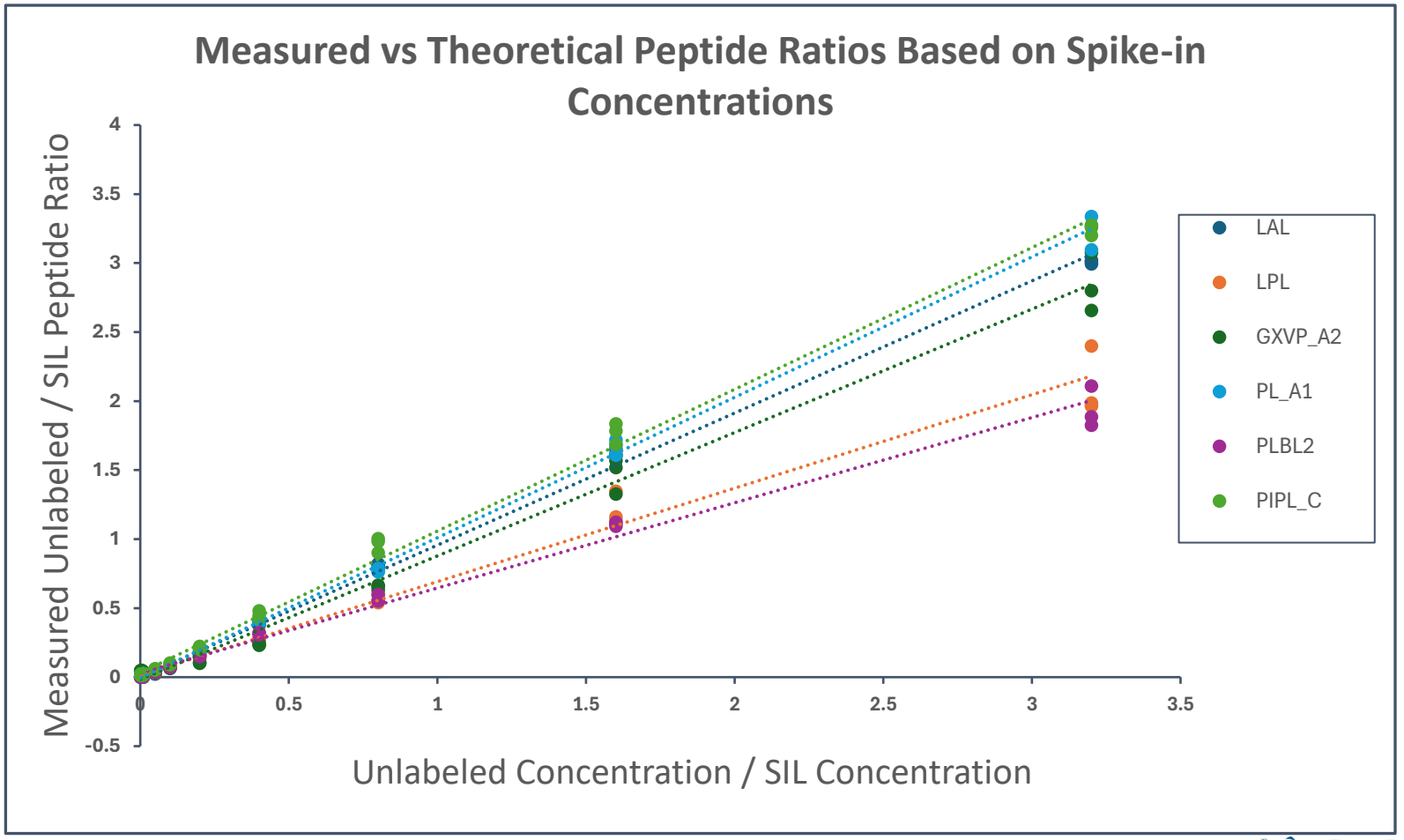
High linearity in PRM calibration curve

HCP	R^2
LAL	1.00
LPL	0.98
GXVP_A2	1.00
PL_A1	1.00
PLBL2	0.99
PIPL_C	1.00



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Acceptance Criteria: Accuracy and Precision

Accuracy

- Error:
  - (Observed – Expected) / Expected * 100
    - 35%

Precision

- %CV
  - Stdev(peak areas) / mean(peak areas) * 100
    - 25%

Linear range: LLOQ - ULOQ

- Must pass ^both criteria
  - E.g., Liposomal Acid Lipase (LAL_1)
    - LLOQ = Point 5
    - ULOQ = Point 10

Cal Curve Point	Theoretical Ratio	Observed Ratio	%CV	%Error
1	0.001	0.0	NA	100.0%
		0.0		
		0.0		
2	0.005	0.0	173.2%	94.7%
		0.0		
		0.0		
3	0.01	0.0	NA	100.0%
		0.0		
		0.0		
4	0.05	0.02	9.9%	50.3%
		0.02		
		0.03		
5	0.1	0.07	5.6%	30.2%
		0.07		
		0.07		
6	0.2	0.17	0.5%	17.0%
		0.17		
		0.17		
7	0.4	0.39	2.9%	2.4%
		0.40		
		0.38		
8	0.8	0.79	1.8%	1.1%
		0.81		
		0.82		
9	1.6	1.65	1.5%	1.4%
		1.61		
		1.60		
10	3.2	3.02	0.5%	6.0%
		2.99		
		3.01		

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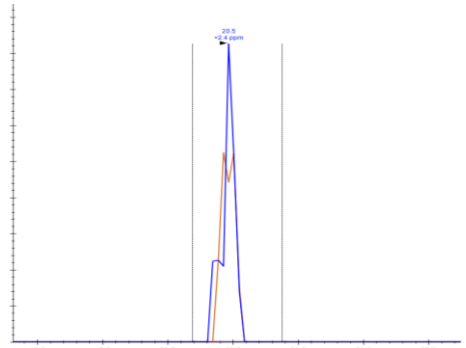
PRM Assay Analytical Limits

Protein	LOD (ppm)	Quantitative Range (ppm)
LAL	0.15	18.25 - 292.06
LPL	0.08	0.10 - 323.34
GXVP_A2	0.03	4.72 - 302.31
PL_A1	0.12	0.49 - 311.67
PLBL2	0.56	0.66 - 419.46
PIPL_C	0.09	13.91 - 890.07

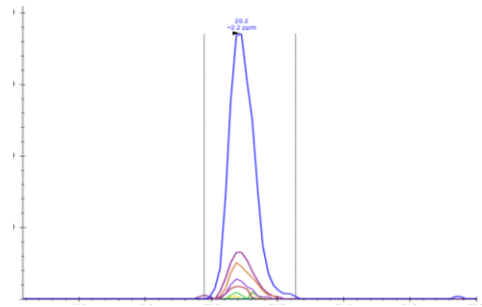
Example Peak: LPL

Blank Injection

Injection near LOD



Injection at LLOQ



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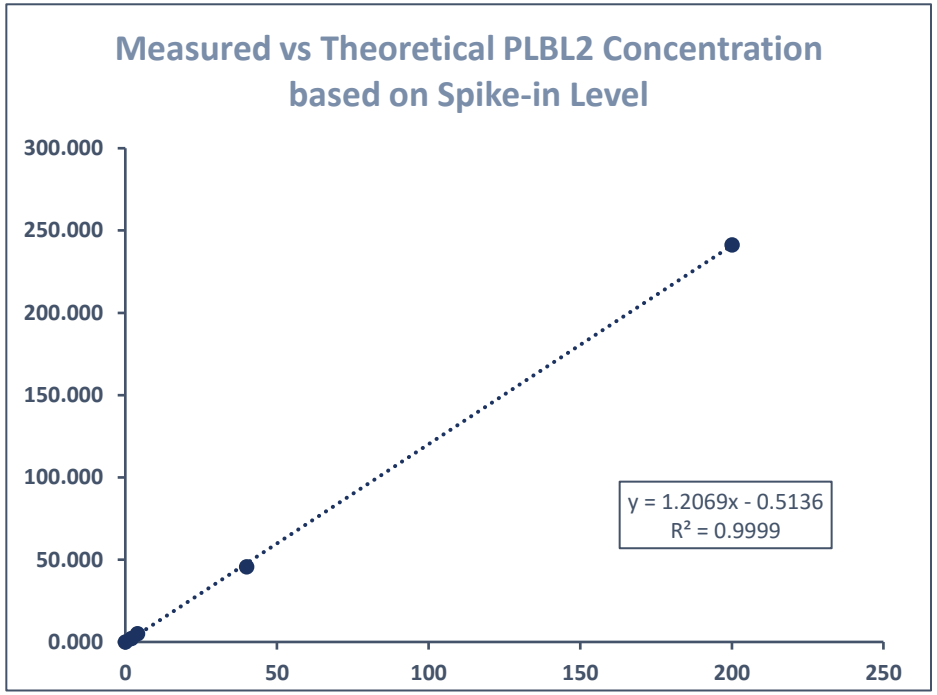
Spike and Recovery with Recombinant PLBL2 (USP)

HCP	Measured PLBL2 (ppm)	Expected PLBL2 (ppm)	%CV	%Error
PLBL2	0.09	0	80%	NA
	2.21	2	15%	10%
	5.22	4	11%	30%
	45.84	40	3%	15%
	241.34	200	15%	21%



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Company A: Two Drug Substances tested by CHO 6xLipase™ MS Assay

Drug Substance 1 (top)

- Fc-fusion protein
- Measurable PS20 degradation
    - Completely degraded in 3 months

Protein	Estimated concentration (ng / mL)	%CV
GXVP_A2	1.15	5.2%
LAL	2.76	9.6%
LPL	6.94	2.3%
PIPL_C	<LOD	NA
PL_A1	0.23	3.2%
PLBL2	<LLOQ	12.4%

Drug Substance 2 (bottom)

- mAb
- No measurable PS20 degradation

Protein	Estimated concentration (ng / mL)	%CV
GXVP_A2	<LOD	NA
LAL	<LOD	NA
LPL	<LOD	NA
PIPL_C	<LOD	NA
PL_A1	<LOD	NA
PLBL2	3.06	3.20%

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Quantitative Comparison: PRM vs Global Semi-quantitative Approach

PRM method tested against DIA-based label-free quantification approach

- DIA- relative to spiked-in protein standards
  - Top3 and Median Response Factor approach
- PRM- relative to spiked-in peptide standards
  - Same method, but analyzed on a longer column and unscheduled acquisition
    - Unscheduled acquisition leads to noisier quantification

Protein	Expected ppm	PRM %Error	PRM %CV	DIA %Error	DIA %CV
LAL	4.6	2%	15%	161%	7%
LAL	9.1	1%	13%	129%	31%
LAL	18.3	0%	8%	176%	26%
LPL	5.1	10%	22%	246%	17%
LPL	10.1	19%	28%	128%	34%
LPL	20.2	19%	32%	162%	35%
GXVP_A2	4.7	7%	5%	435%	12%
GXVP_A2	9.4	8%	0%	224%	16%
GXVP_A2	18.9	7%	4%	208%	7%
PL_A1	4.9	15%	5%	135%	13%
PL_A1	9.7	17%	17%	65%	29%
PL_A1	19.5	9%	3%	106%	26%
PLBL2	6.6	16%	54%	453%	15%
PLBL2	13.1	13%	19%	308%	50%
PLBL2	26.2	12%	11%	253%	36%
PIPL_C	13.9	17%	4%	26%	13%
PIPL_C	27.8	17%	32%	16%	32%
PIPL_C	55.6	24%	27%	22%	32%

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Conclusions

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Summary

- LC-MS is a powerful technique to identify and quantify HCPs in diverse bioprocess samples
- PRM combined with SIL peptides is the most accurate method for absolute quantification
- The CHO 6x Lipase™ MS Assay is highly accurate and precise within a range relevant for downstream bioprocess samples
- New panels and improved acquisition will continue to push the boundaries towards global HCP quantification

Cygnus Technologies Services - End-to-end, comprehensive MS solutions for HCP assessment in your bioprocess

Coverage Analysis by AAE-MS™

- More predictive of HCP antibody performance
- Identifies unique proteins, not spots
- The only reliable choice for coverage of HCPs in DS-containing harvest samples

Identification of immunoreactive HCPs in IPC and final DS

- AAE-MS identifies immunoreactive HCPs in the final DS
- Identifies HCPs thar persist through DSP and may contribute to loss of dilution linearity
- Enables targeted downstream process optimization

HCP inSPECt™ MS Quantification

- DIA-based HCP quantification relative to Cygnus optimized protein standard
- Resolves and quantifies thousands of HCPs with exceptional accuracy and precision

PRM-MS for absolute HCP quantification

- Absolute quantification of six CHO lipases using targeted proteomic approach and SIL peptide standards (CHO 6xLipase™ MS Assay)
- Additional assays in development

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Roadmap of Mass Spectrometry Trajectory

	Short-Term	Mid-Term	Long-Term Strategy
Process Development	• Generic/Platform HCP ELISA	• Generic/Platform HCP ELISA	• Generic/Platform HCP ELISA
Lot Release	• Custom/Generic HCP ELISA	• Custom/Generic HCP ELISA	• GMP MS Testing
Incorporate MS	– Coverage Analysis – Investigations – Guide Process Development – Relative Global Quant – Absolute Quant of Specific Proteins	– Lot Release testing when HCP ELISA not available – Coverage Analysis – Investigations – Guide Process Development – Relative Global Quant – Absolute Quant of Specific Proteins	– GMP Lot Release testing – Coverage Analysis – Investigations – Guide Process Development – Relative Global Quant – Absolute Quant of Specific Proteins

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Thank You

Driving your success at every stage

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