Analytics for Process-related Impurities in Viral Vector Manufacturing

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CASSS CGTP 2023

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part of **Maravai LifeSciences**

Experts in Impurity Analysis

1998-2007

Published article on methods for HCP analysis

Released 1st generic ELISA for CHO & *E. coli* HCP

Product line expansion for various expression systems

2008

Added additional orthogonal services such as 2D WB & 2D HPLC for identification of individual HCPs

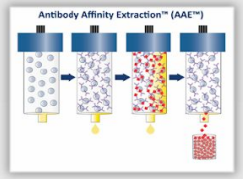
2012

Added host cell DNA extraction & detection kits

2014

Introduced Antibody Affinity Extraction (AAE™) propriety analysis to replace 2D WB

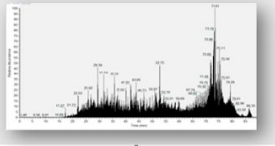
Launched Protein A Mix-N- Go™



2016

Published White Paper on integration of orthogonal method data from MS & AAE

Became part of Maravai Life Sciences



2018-2019

Added Mass Spec Services completing orthogonal offerings

EndonulceaseGTP® ELISA Kit launch

HEK293 HCP ELISA has been utilized for product lot release of multiple commercialized gene therapies

2020

Introduced novel MockV kits for viral clearance studies

Launched CHO HCP 3G Robotics Kit

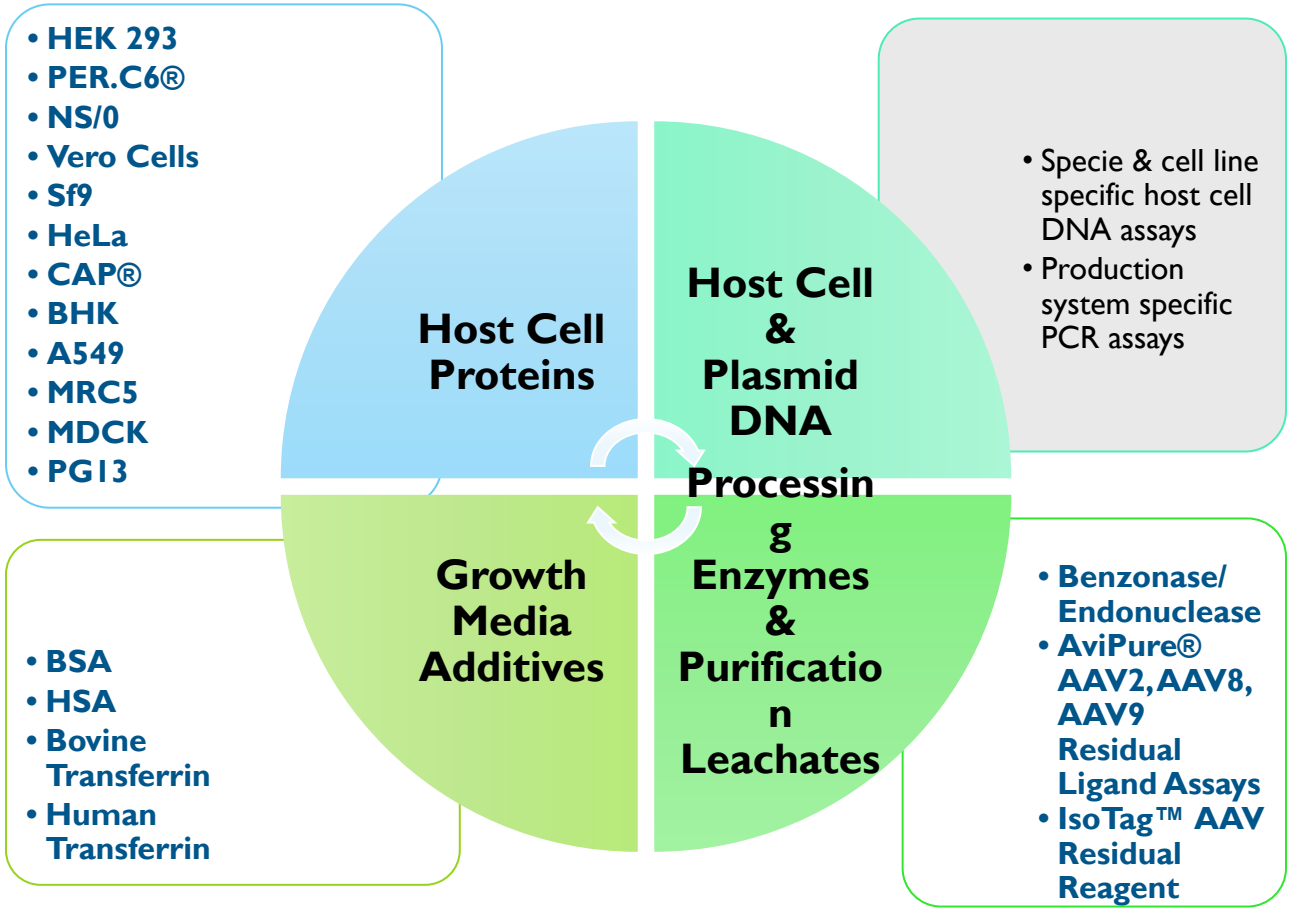
Partnerships with Gyros Protein Technologies and Protein Simple for assay automation

Driving your success at every stage.

Regulatory Environment

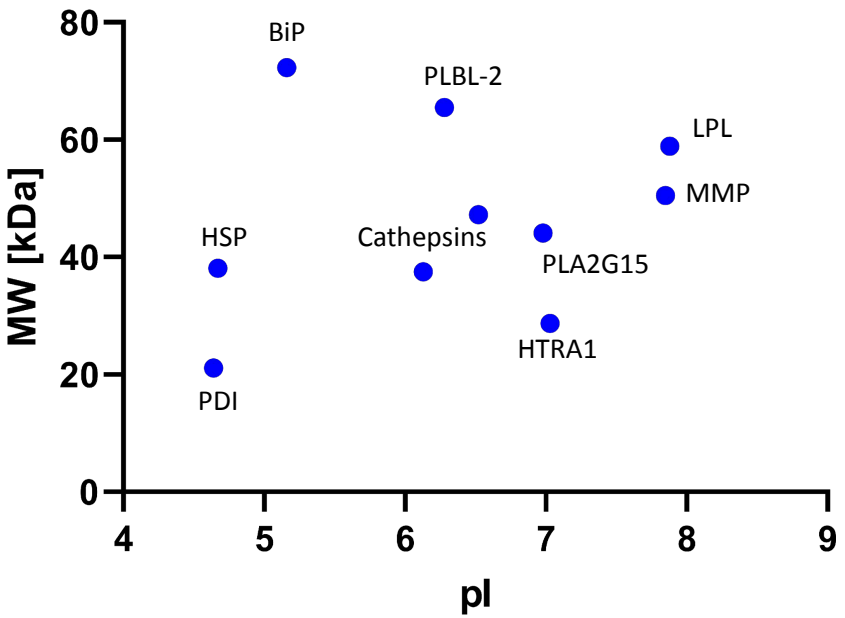
- What did we learn from mAb bioprocess?
  - HCP Impurities are a real safety concern [immunogenicity, DS stability]
  - Updated USP and European guideline are more harmonized for mAb and rProteins (<100 pm with a trend to <10 ppm for mAbs)
  - Regulators are fully aware of limitations of current methods and now reference AAE and Mass Spec as acceptable orthogonal methods
  - HCP assessment is not a “Check the box ” exercise
- FDA Guidance for Industry: CMC Information for Human Gene Therapy IND Applications require testing for process impurities such as HCP, HC + “production system” DNA, and other process-related impurities (BSA, Benzonase/Endonuclease, AAV affinity resins leachates)

Process-related Impurities



Developed by and available from Cygnus = blue font

Potentially Problematic HCPs



Drug Degradation

Serine Protease
HTRA1
Cathepsins
Matrix Metalloproteinase

Drug Aggregation

Protein Disulfide Isomerase

Endoplasmic reticulum chaperone BiP
Heat shock proteins

Lipases

Lysophospholipase 2
Lysphospholipase-like 1
Lysosomal acid lipase A

Immunogenicity and Loss of ELISA dilutional linearity

Phospholipase B domain containing 2

PS20 PS80 Degradation

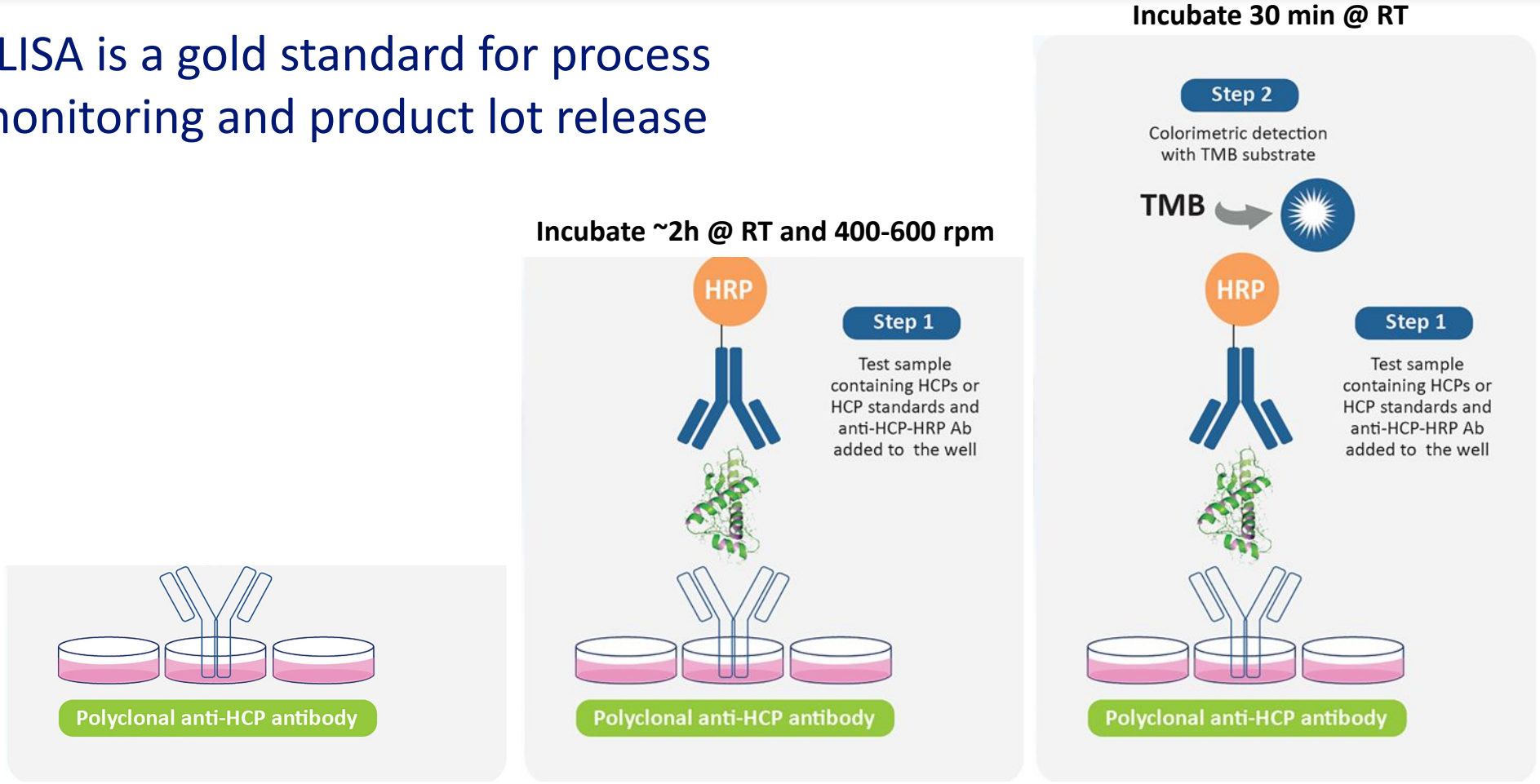
Lipoprotein lipase
Phospholipase A2 Group XV
Phospholipase A2 Group VII
Phospholipase A1

Jones, M. et al *Biotechnol. Bioeng*. 2021
Li, X. et al *bioRxiv*. 2020
Wang, F. et al Bioprocess Intl. 2018
Vanderlaan, M. et al *Biotechnol. Prog*. 2018
Durocher, V. et al. *J. of Biotech*. 2017
Valente, K. et al *Biotechnol. Prog.* 2015
Levy, N. et al. *Biotechnol Bioeng.* 2014

HCP Immunoassays

HCP Analysis by ELISA

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



Typical Cygnus HCP ELISA – Simultaneous Protocol

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)
  - Results subject to change with change in kit/reagents
  - Can take 12-months to generate new reagents

Assay Qualification

- Dilution Linearity
- Accuracy
- Precision
- Range: LOD/LLOQ/ULOQ
- Coverage by AAE™ /2D-PAGE
- Coverage & Characterization by AAE-MS™

•ICH Q11 "Development and Manufacture of Drug Substances (Chemical Entities Biotechnological/Biological Entities)", 2012
•ICH Q6B "Test Procedures and Acceptance Criteria for Biotechnological/Biological Products," 1999
•USP Chapter 1132 "Residual Host Cell Protein Measurement in Biopharmaceuticals," 2016
•USP Chapter 1132.1 "Residual Host Cell Protein Measurement in Biopharmaceuticals by Mass Spectrometry", 2023

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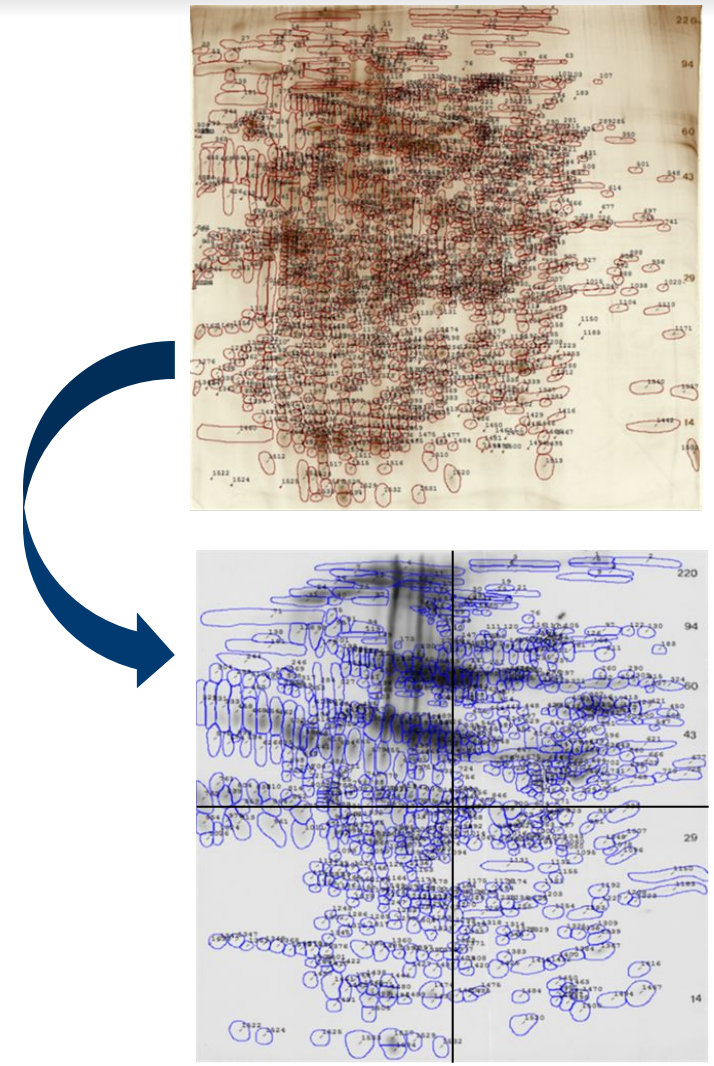
Methods for Determining Antibody Coverage

ELISA Antibody Coverage Analysis

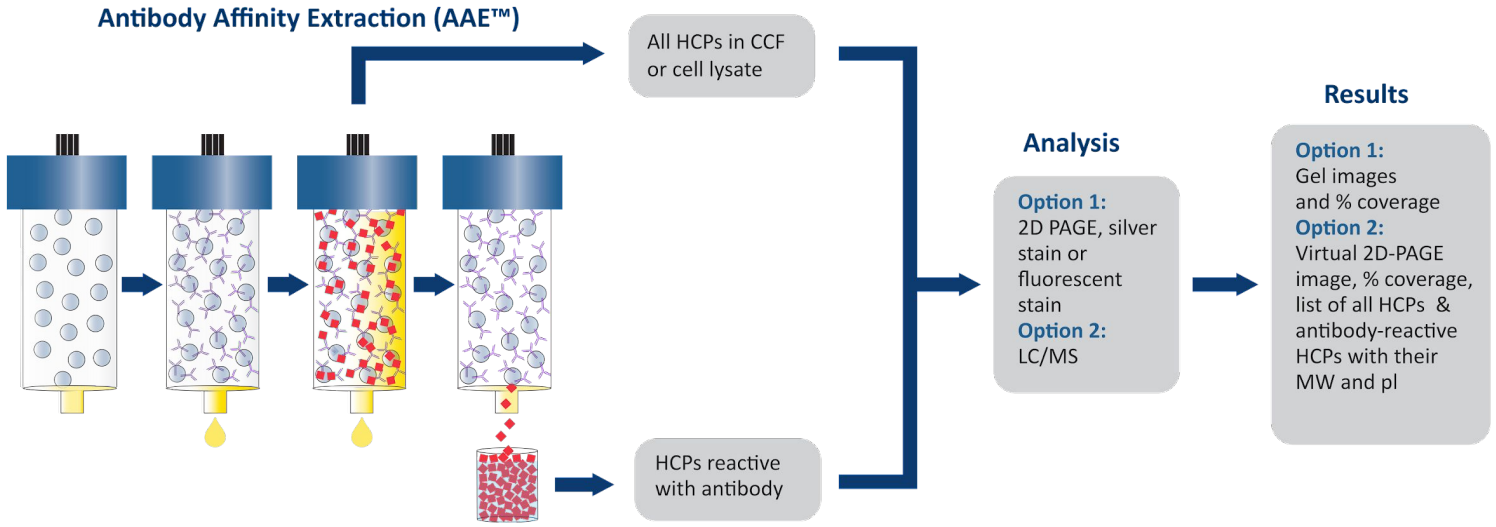
- Performed to demonstrate that the Ab used in the ELISA are broadly reactive to the HCPs in the expression platform
  - Conventional Method
    - 2D Western blot compared to Silver Stain
    - Specifications:
      - Reactive with the majority of spots found on the silver stain gel
      - Must have spots in each quadrant of the gel where spots are found on the silver stain
  - Antibody Affinity Extraction (AAE™) with Silver Stain or DIGE detection
  - AAE™ with LC-MS



Antibody Affinity Extraction (AAE™)

Antibody Affinity Extraction (AAE™)

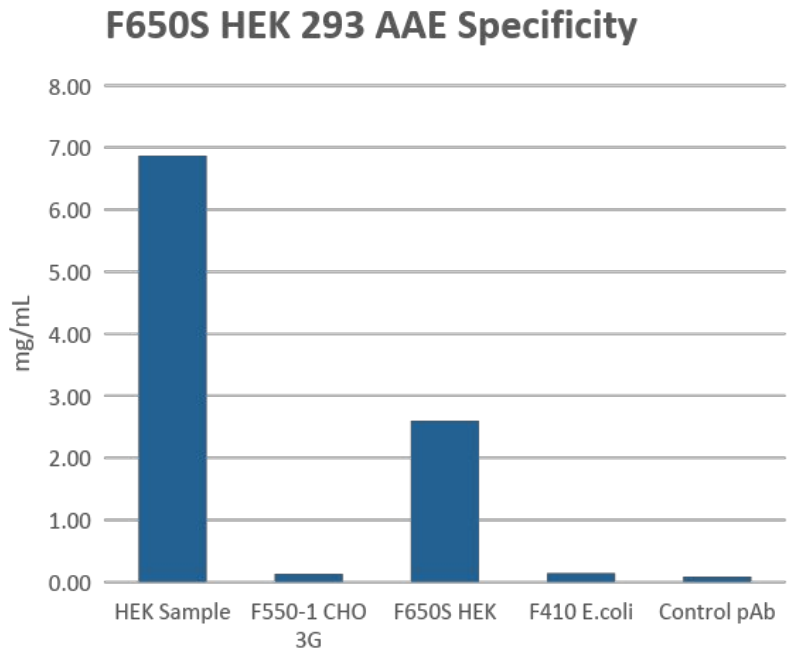
Harvest Material



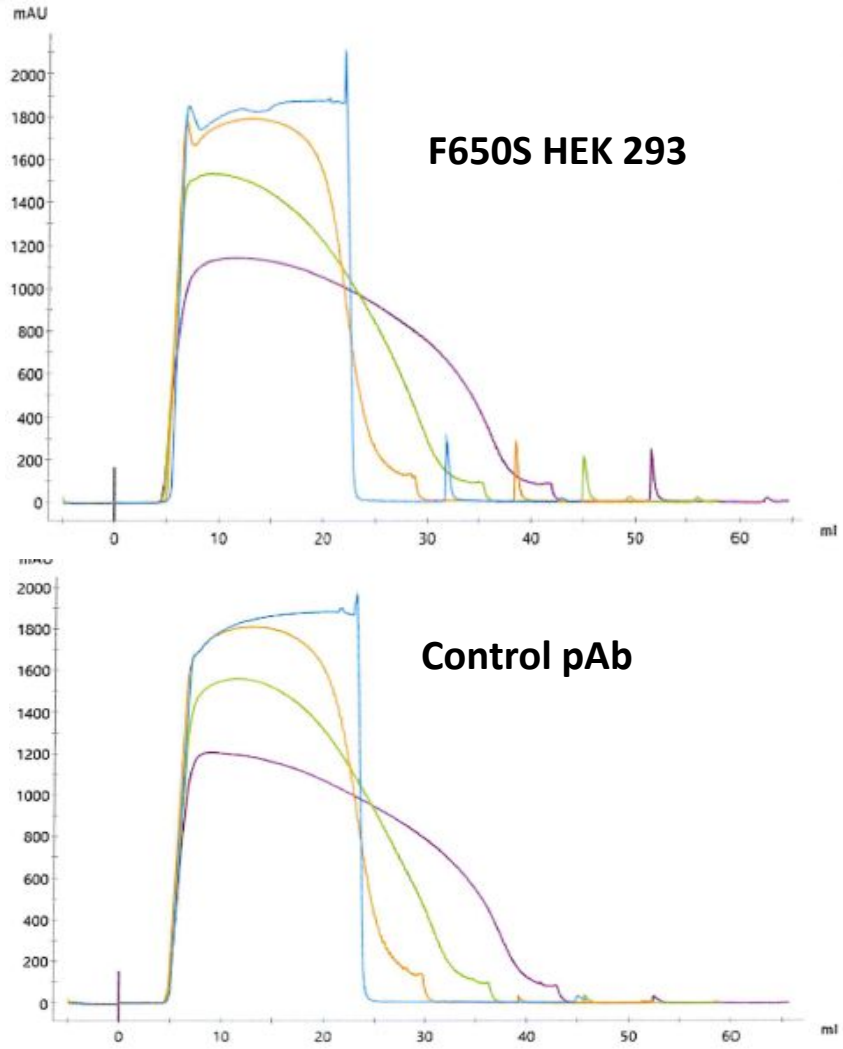
Method

- AAE columns with antibodies from HEK 293 [F650S], CHO [F550-1], E. coli [F410] HCP ELISA Kits
- Negative controls: AAE columns with non-immune goat/rabbit IgG
- Antibody Affinity Extraction was performed with HEK 293 HCP
- LC-MS Sample Preparation:
  - the Pre- and Post-AAE HCPs were precipitated, dissolved in 8M urea, reduced, alkylated, digested with trypsin, desalted, and concentrated
- HCP Identification by LC-MS:
  - the Pre- and Post-AAE samples were analyzed with the custom LC-MS method independently in triplicate and in a randomized sequence. Blank washing runs were implemented in between sample injections to minimize sample carryover.
- HCP Quantification by LC-MS:
  - Cygnus Protein Standard (CPS) was spiked into all quantification samples prior to LC-MS sample preparation. Quantification of HCPs in ppm were calculated relative to CPS at 1000 ppm**.** The ng/mL of HCPs were calculated by multiplying the mg/mL of the sample to the ppm value.

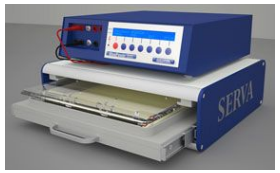
AAE™ Method Specificity Assessment –HEK 293 Harvest Sample

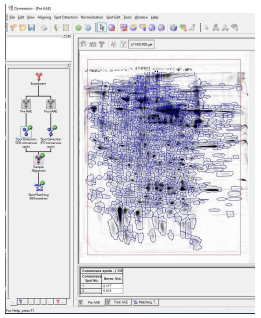


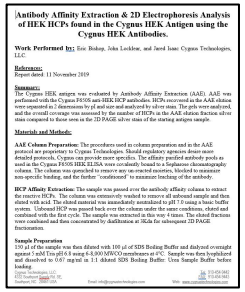
HEK 293 HCP sample was passed over the F550-1 3G CHO, F650S, F410 E. coli, and IgG control columns by FPLC. Immunoreactive HCPs were quantified by LC-MS



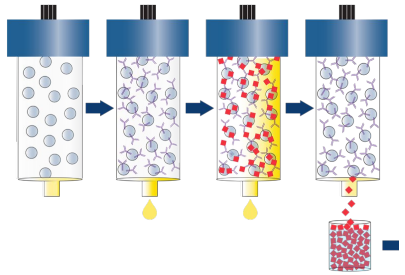
2D-PAGE Workflow in HCP Analytics



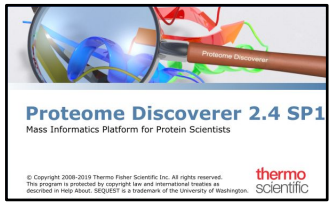


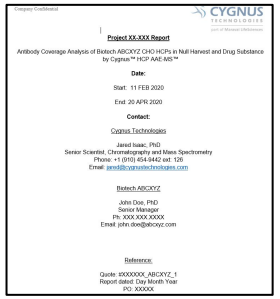


AAE → Sample Preparation → 2D-PAGE + Imaging → Spot Counting + Data Analysis → Data Reporting

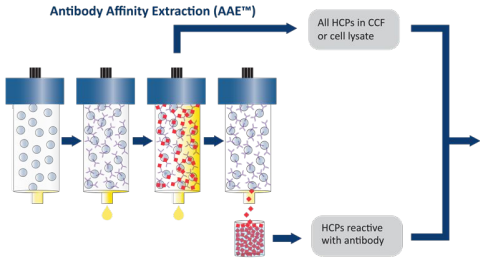


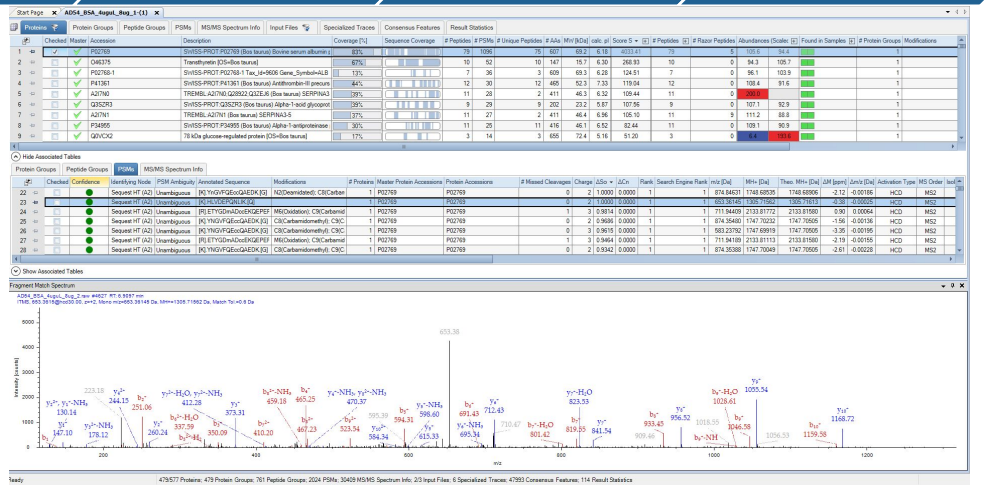
MS Workflow in HCP Analytics





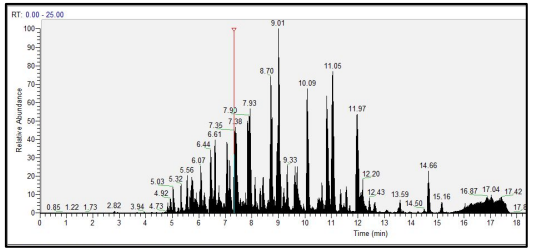
AAE → Sample Preparation → Method Development → LC-MS Analysis → Data Reporting

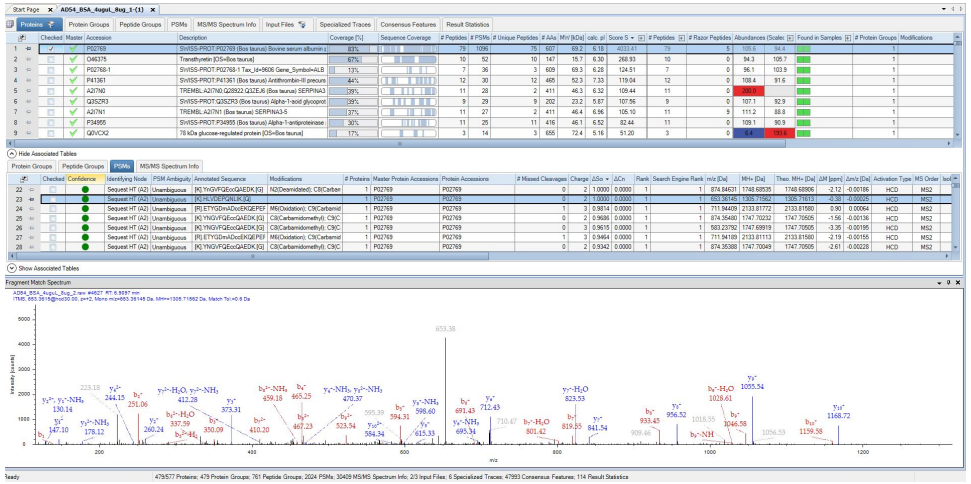




Curated Proteomic Databases

1. *Homo sapiens* (HEK293, HeLa)
2. *Cricetulus griseus* (CHO)
3. Vero
4. *Bos taurus*
5. *Mus musculus (*NS/O and PG13*)*
6. *E. coli*
7. Sf9
8. *S. cerevisiae*
9. *P. pastoris*
10. Goat
11. Rabbit
12. MRC5
13. *S. aureus*
14. *P. fluorescens*
15. *L. lactis*
16. Per.C6
17. BHK
18. MDCK



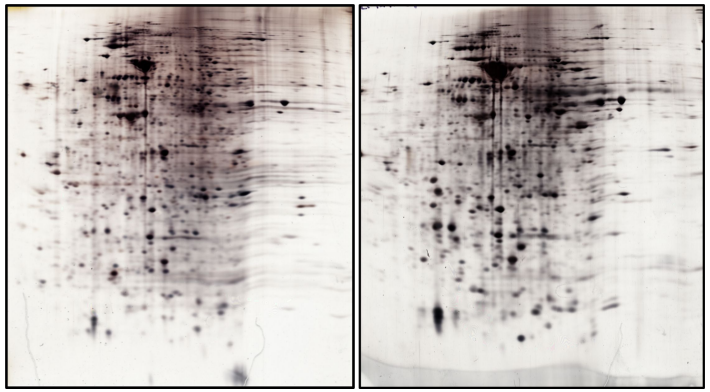


F650S Anti-HEK293 pAb Coverage of HEK 293 Harvest Sample

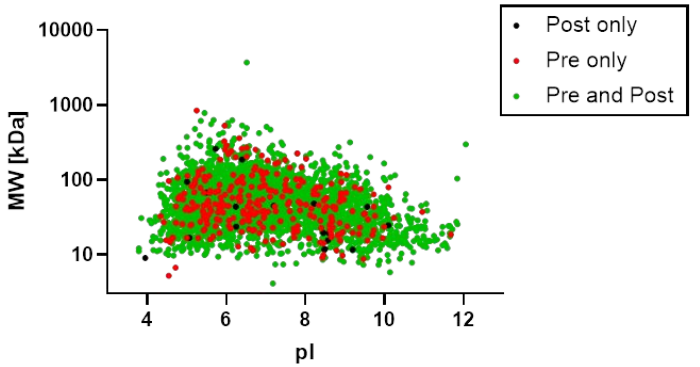
Silver Stain of "Pre-AAE" HEK 293 Antigen Sample

Silver Stain of "Post-AAE" HEK 293 Antigen Sample

A

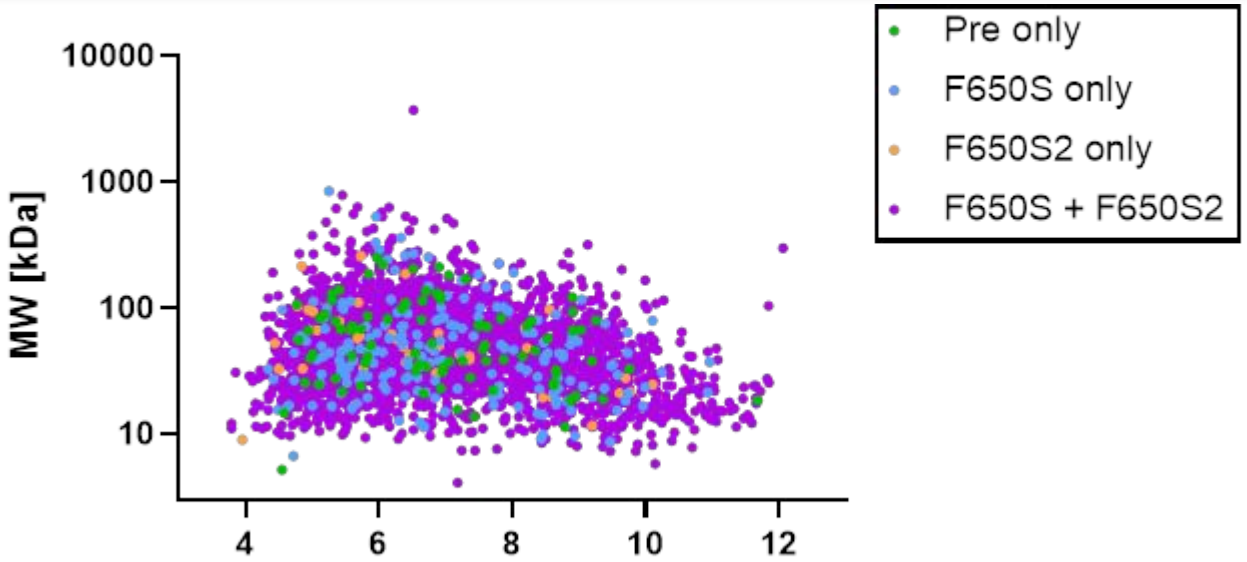


B



Detection Method	PRE-AAE (all HCPs)	POST-AAE (immunoreactive HCPs)	% pAb Coverage
2D-PAGE	1687	1604	95
MS	3075	2812	91

Virtual 2D Gels of Cygnus HEK 293 Master Antigen with F650S and F650S R1 HCP Antibodies: MW vs pI

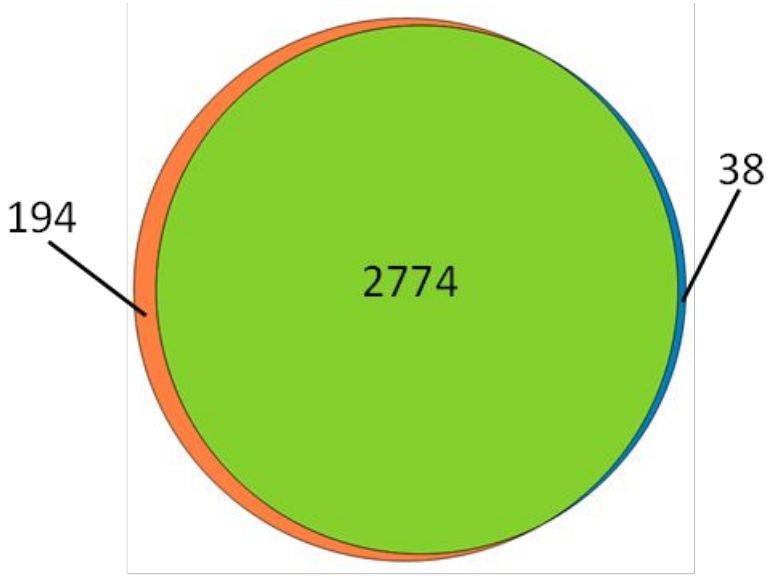


Sample		AAE (number of protein ids)				% Antibody Coverage	
Name	AAE	Total	Unique to each fraction	Total Unique	Matching	Lower Boundary	Upper Boundary
F650S	Pre	3075	119	3278	2765	90.54%	96.52%
	Post	2968	12				
F650S Resupply	Pre	3075	281	3093	2794	90.91%	91.45%
	Post	2812	18				

Determining Equivalency of Two HEK 293 HCP Antibodies

Comparison of F650S and F650S Resupply Protein Identifications

Antibody	Total Protein ID	Matching Proteins	Unique Proteins	% Similarity
F650S	2968	2774	194	92.3%
F650S Resupply	2812		38	



Quantitative Venn Diagram to assess the % similarity of F650S and F650S Resupply antibodies binding to the Cygnus HEK 293 antigen. Unique identifications of F650S Resupply (blue) and F650S (orange) are displayed along with proteins identified by both antibodies (green).

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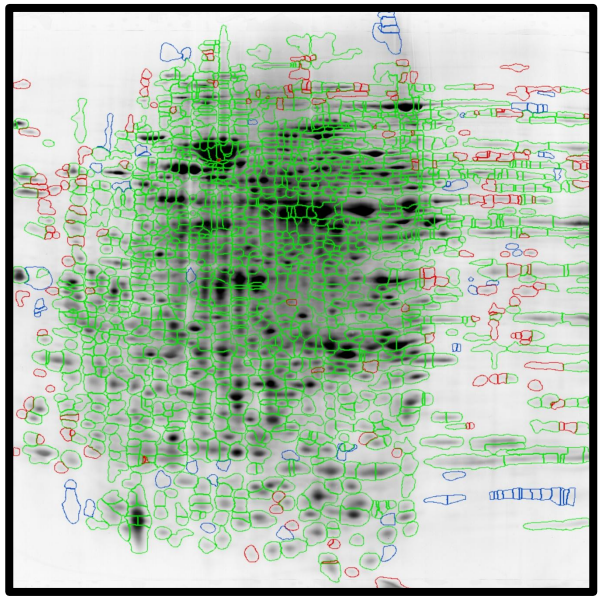
Individual HCP pAb Reactivity by MS

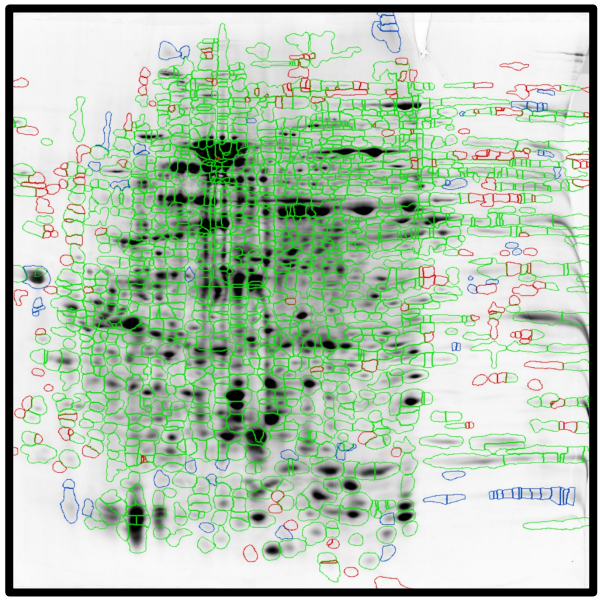
#	Potential High-Risk HCPs	PRE	F650S	F650S R	pI	MW
1	78 kDa glucose regulated protein (BiP)	Y	Y	Y	5.07	72332.96
2	Actin	Y	Y	Y	5.29	41736.73
3	Aldose reductase related protein 2 (Aldo-keto reductase)	Y	Y	Y	7.66	36019.6
4	Alpha enolase	Y	Y	Y	7.01	47169.0
5	Carboxypeptidase D	Y	Y	Y	5.68	152931.1
6	Cathepsin B	N	N	N	5.88	37821.6
7	Cathepsin D	Y	Y	Y	6.1	44552.2
8	Cathepsin E	N	N	N	4.69	42793.5
9	Chondroitin sulfate proteoglycan 4	N	N	N	5.27	250536.8
10	Clusterin	N	N	N	5.88	52494.6
11	Cofilin 1	Y	Y	Y	8.22	18502.5
12	Elongation factor 1a1	Y	Y	Y	9.1	50140.9
13	Elongation factor 2	Y	Y	Y	6.41	95338.1
14	Flagellin (Salmonella paratyphi A (strain ATCC 9150 / SARB42)	N	N	N	5.03	51950.2
15	Galectin 3 binding protein	Y	Y	Y	5.12	65331.0
16	Glutathione S transferase P	Y	Y	Y	5.43	23355.8
17	Glyceraldehyde 3 phosphate dehydrogenase	Y	Y	Y	8.57	36053.2
18	G-protein coupled receptor 56	N	N	N	8.79	57473.2
19	Heat shock cognate 71 kDa protein	Y	Y	Y	5.37	70898.1
20	Heat shock protein HSP 90	Y	Y	Y	4.94	84659.7
21	Lipoprotein Lipase	N	N	N	8.37	53162.5
22	Lysosomal protective protein	N	N	N	6.16	54466.1
23	**Matrix Metalloproteinase 19**	N	N	N	7.22	57357.0
24	Metalloproteinase inhibitor 1	N	N	N	8.46	23170.9
25	**Monocyte chemoattractant protein 1 (C-C motif chemokine 2)**	N	N	N	9.4	11025.0
26	**Nidogen-1**	N	N	N	5.12	136377.0
27	Peptidyl-prolyl cis-trans isomerase	Y	Y	Y	8.27	19257.2
28	Peroxiredoxin 1	Y	Y	Y	8.27	22110.4
29	Phosphoglycerate kinase 1	Y	Y	Y	8.3	44614.7
30	Phospholipase A2 (Group XV lysosomal)	Y	Y	Y	6.26	46657.8
31	Phospholipase B like 2	Y	Y	Y	6.34	65471.8
32	Procollagen C endopeptidase enhancer 1	N	N	N	7.41	47972.5
33	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1	Y	Y	Y	6.46	83550.19
34	Procollagen-lysine (Procollagen-lysine 5-dioxygenase)	Y	Y	Y	6.54	83504.2
35	Protein disulfide isomerase	Y	Y	Y	4.76	57116.37
36	Pyruvate kinase	Y	Y	Y	7.96	57936.9
37	**Serine protease HTRA1**	N	N	N	8.09	51287.0
38	**SPARC (Osteonectin)**	N	N	N	4.73	34632.2
39	Sulfated glycoprotein 1 (Prosaposin)	Y	Y	Y	5.06	58112.6
40	T-complex protein 1 subunit alpha	Y	Y	Y	5.8	60343.6
41	Thioredoxin 1	Y	Y	Y	4.82	11737.5

Custom HEK 293 ELISA with Customer Sample

PRE AAE



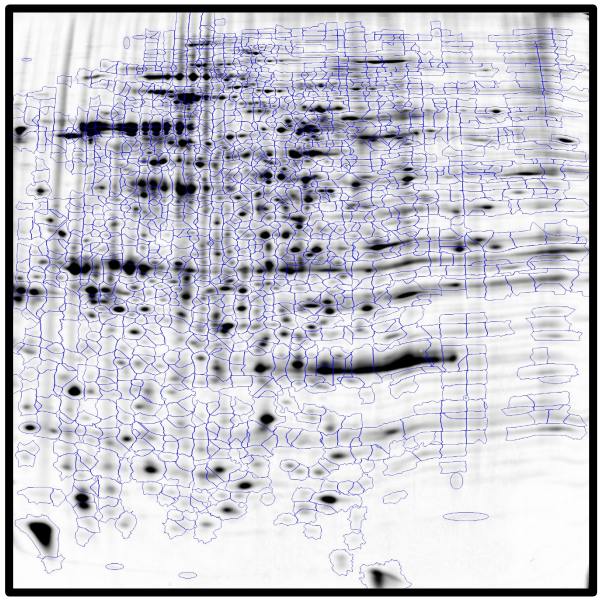
POST AAE



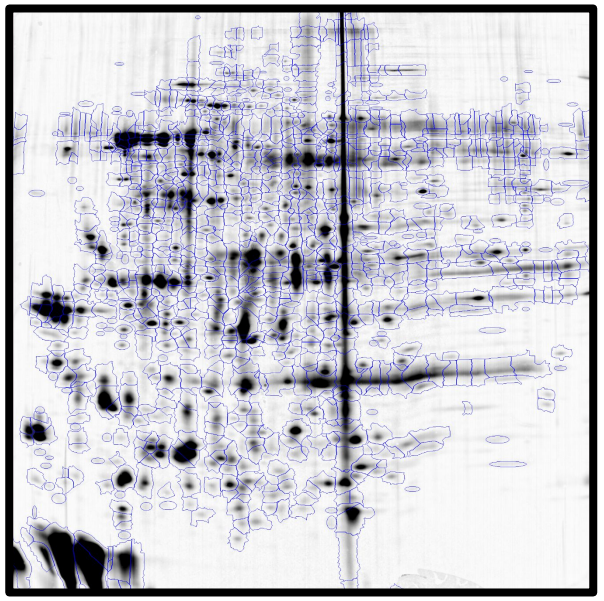
There were 1454 spots in the sample and 1356 spots in the AAE elution fraction. **Based on our assessment the coverage of the antibodies is 92%**.

Custom Sf9 ELISA with Customer Sample

PRE AAE



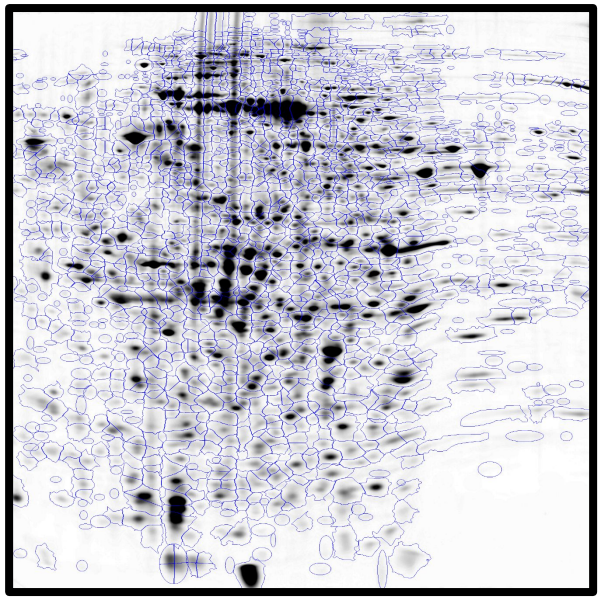
POST AAE



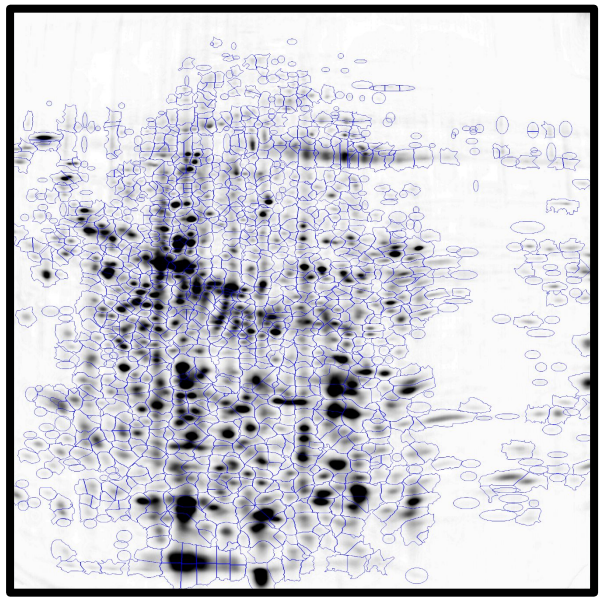
There were 1585 spots in the sample and 1214 spots in the AAE elution fraction. **Based on our assessment the coverage of the antibodies is between 64% - 78%**.

Platform F975 Vero AAE 2D-PAGE

PRE AAE



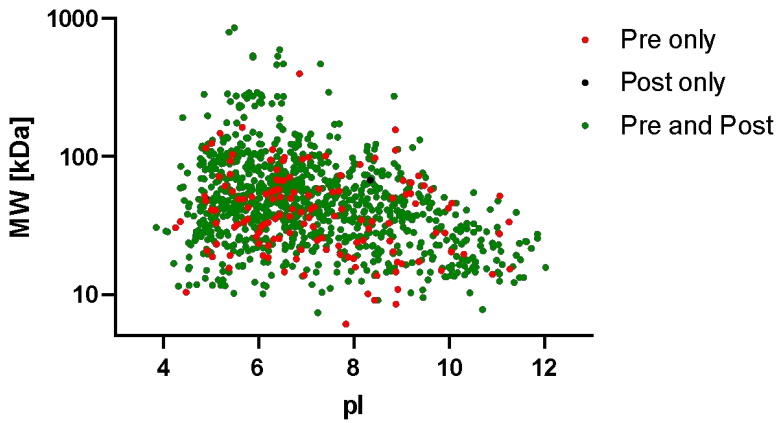
POST AAE



There were 1517 spots in the sample and 1193 spots in the AAE elution fraction. Based on our assessment the coverage of the antibodies is between 62% - 79%.

Platform F975 Vero AAE-MS with Customer Sample

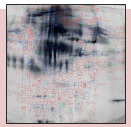
Vero F975 AAE-MS



	AAE		
	PRE	POST	% Antibody Coverage
no of HCPs	1129	993	88%

#	Potential High-Risk HCPs	Pre AAE	Post AAE	IEP	MW
1	Protein 1	N	N	5.07	72379.1
2	Protein 2	Y	Y	5.29	41736.7
3	Protein 3	Y	Y	5.85	46698.3
4	Protein 4	Y	Y	5.98	50011.7
5	Protein 5	N	N	5.3	48265.1
6	Protein 6	Y	Y	5.73	35646.9
7	Protein 7	Y	Y	6.54	44110.9
8	Protein 8	N	N	4.61	42726.4
9	Protein 9	N	N	5.4	252012
10	Protein 10	N	N	5.58	51557.5
11	Protein 11	Y	Y	8.22	18532.5
12	Protein 12	Y	Y	9.39	55106
13	Protein 13	Y	Y	6.41	95324.1
14	Protein 14	N	N	4.5	51295
15	Protein 15	N	N	5.05	63802.22
16	Protein 16	Y	Y	7.64	23638.2
17	Protein 17	Y	Y	8.49	35747.9
18	Protein 18	N	N	9.06	77370.5
19	Protein 19	Y	Y	5.23	70804.9
20	Protein 20	Y	Y	4.94	83166.1
21	Protein 21	Y	Y	7.94	52900.29
22	Protein 22	N	N	5.64	56110.7
23	Protein 23	N	N	7.71	58942
24	Protein 24	N	N	8.84	22401
25	Protein 25	N	N	9.32	15858.4
26	Protein 26	N	N	4.72	83103
27	Protein 27	Y	Y	9.59	23634.4
28	Protein 28	Y	Y	8.22	22262.6
29	Protein 29	Y	Y	8.02	44562.5
30	Protein 30	N	N	6.16	87100
31	Protein 31	Y	Y	5.63	61824.4
32	Protein 32	N	N	8.16	50446.5
33	Protein 33	Y	Y	6.46	83550.2
34	Protein 34	N	N	6.57	83327.9
35	Protein 35	Y	Y	5.98	56796.4
36	Protein 36	Y	Y	6.88	57893.8
37	Protein 37	Y	Y	6.62	34404.5
38	Protein 38	Y	Y	7.1	51081.6
39	Protein 39	N	N	5.31	65758.2
40	Protein 40	Y	Y	5.7	60338.6

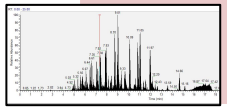
Coverage is Dependent on Method



2D WB = 50-60%

AAE-2D PAGE

Silver = 60-80%
Fluorescent = 70-80%
Cy3 and Cy5 = 70-85%

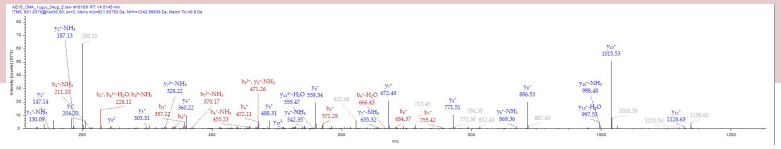


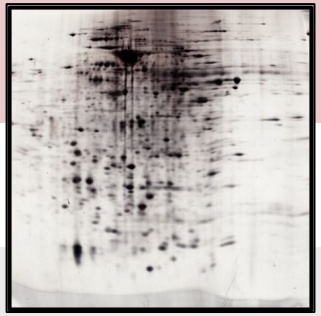
AAE-MS = 70-90%

How is ELISA pAb Antibody Coverage Determined?

Lower boundary = (Post AAE spots/Unique spots)
Upper boundary = (Post AAE spots/Pre AAE-spots)

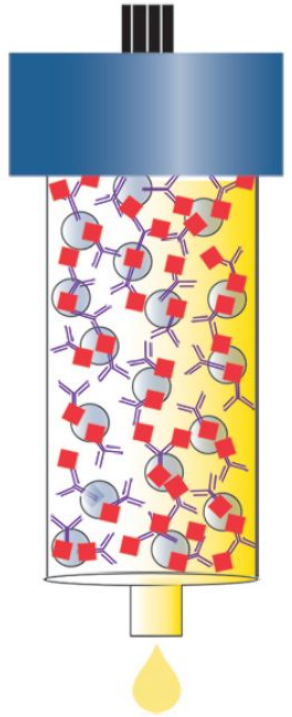
Spots and Ions





AAE in Completing the HCP Picture

- Antibody coverage analysis
  - 2D WB = 50%
  - AAE and 2D SDS PAGE= 65-75% (Silver Stain)
  - AAE and MS > 80-85%
- Coverage is dependent upon detection method
- AAE is more predictive of how the Ab will perform in ELISA
- Increased sensitivity and specificity as compared to 2D WB
- Sufficient sensitivity to evaluate individual HCPs that persist through purification process



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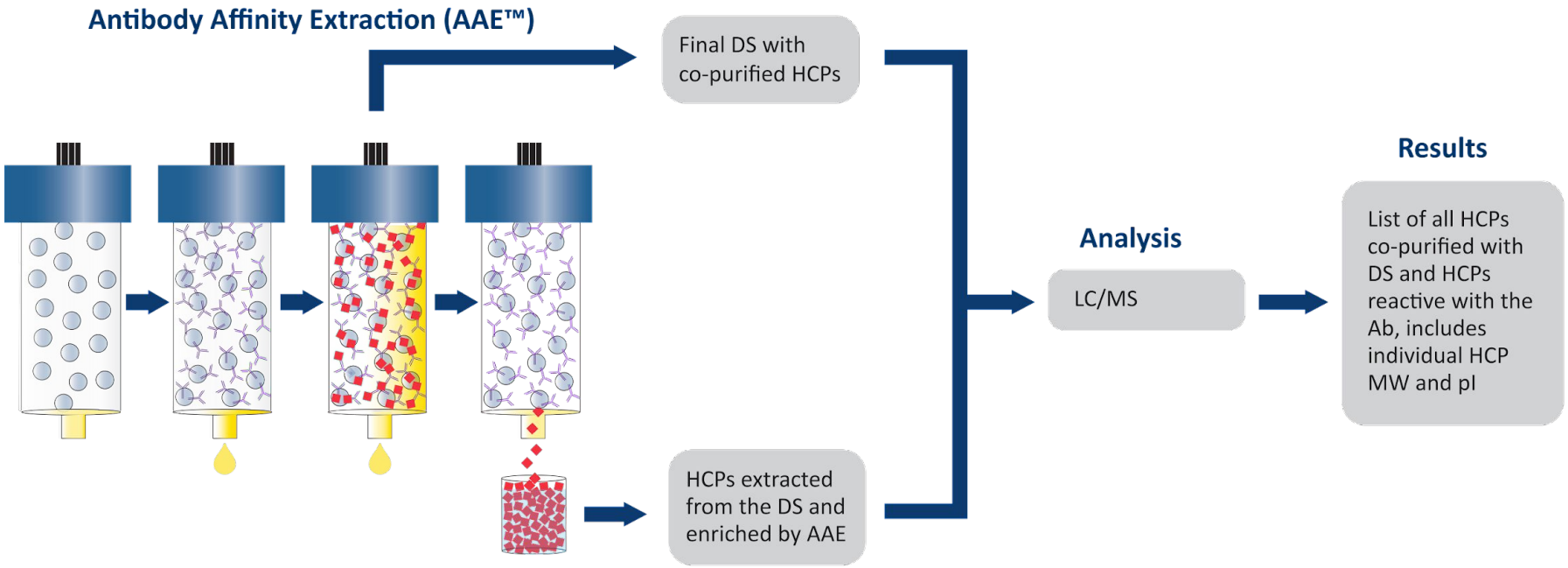
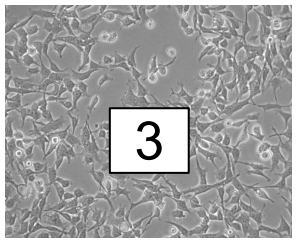
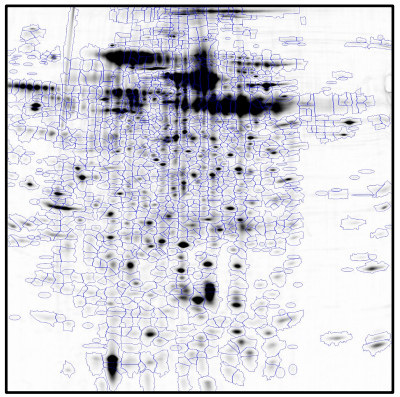
HCP Identification in DS and IP Samples by AAE-MS™

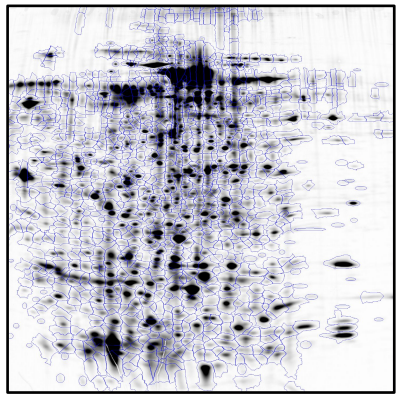
DS Material

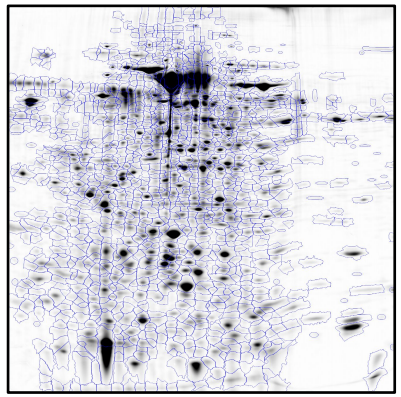

Antibody Affinity Extraction (AAE™)
Final DS with co-purified HCPs
HCPs extracted from the DS and enriched by AAE
Analysis
LC/MS
Results
List of all HCPs co-purified with DS and HCPs reactive with the Ab, includes individual HCP MW and pI

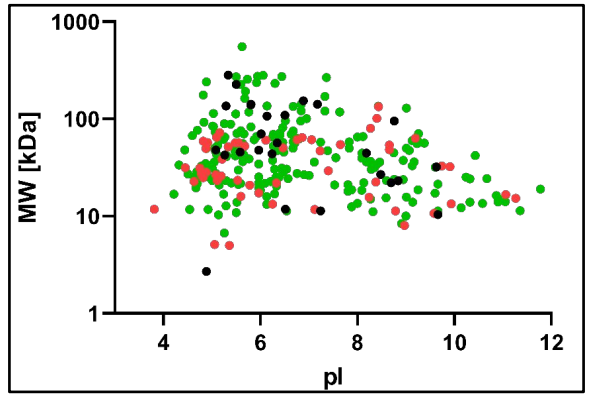
Gene Therapy HEK 293 F650S Coverage by AAE 2D-PAGE and MS











<u>69-93%</u>
1221 Post AAE spots

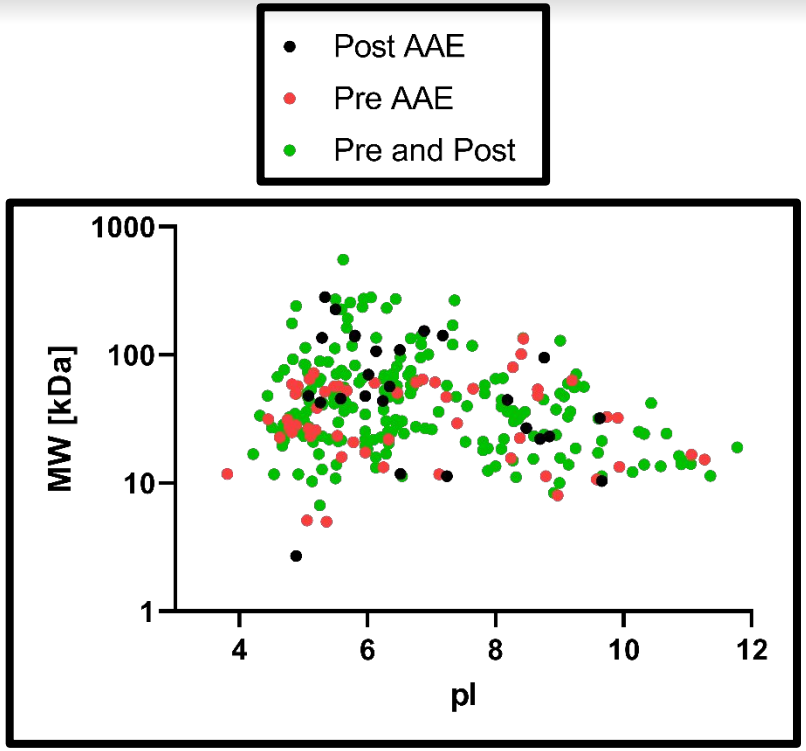
<u>66-91%</u>
1302 Post AAE spots

<u>66-93%</u>
1358 Post AAE spots

<u>80-87%</u>
218 Post AAE Proteins

MS is well suited for antibody coverage of DS

F650S Antibody Reactivity to HCPs in the Final DS



F650S: 87%

- 251 Pre AAE spots
- 218 Post AAE spots

Custom Services

HCP Antibody Coverage Analysis and Assay Qualification

- Comprehensive qualification of in-house assays or generic kits by orthogonal methods (**AAE**, **Mass Spec**)
- AAE method to demonstrate coverage to those individual HCPs that co-purify with drug substance & identify those HCPs by MS
- ELISA testing including dilution linearity and spike recovery analysis

Process-specific antibody and assay development:

- Proven antibody generation & purification
- Cygnus develops, qualify, & manufacture a robust kit that meets customer specific monitoring, release testing & regulatory needs

techsupport@cygnustechnologies.com

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- Eric Bishop
- Jared Isaac
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- Jacob Stubbs
- Stephen Stahlschmidt
- Johanna Barnhill

Thank You

Driving your success at every stage

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