Enhanced Analysis of Host Cell Proteins Using the Agilent 6545XT AdvanceBio LC/Q-TOF

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Introduction

Residual Host Cell Protein (HCP) are process impurities remained in a purified drug product

- HCPs can influence product stability and cause immune response in patients

- FDA requires that HCP contaminants in the final product are measured and reported

- ELISA is still a widely accepted method for HCP quantification
  - Strengths:
    - Very sensitive (ppb detection limits)
    - High level of reproducibility
    - High-throughput (plate format, automation)

  - Challenges:
    - Lack of specificity, no identification of individual HCPs
    - Lack of coverage for non-immunoreactive HCPs
    - Quantitation is based on a cohort of HCPs

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# LC/MS as a Solution for Host Cell Protein Analysis

Advantages:

- Identify individual protein including immunogenic HCPs

- Improve early purification process development

- Doesn't require protein specific antibodies

- High analytical sensitivity (low ppm)

- Provide both qualitative and quantitative information

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Challenge for LC/MS Analysis of HCPs

- Low abundant HCP peptides co-elute with very intense "product" mAb peptides
- Need broad dynamic range and better separation, and 2D-LC is often used
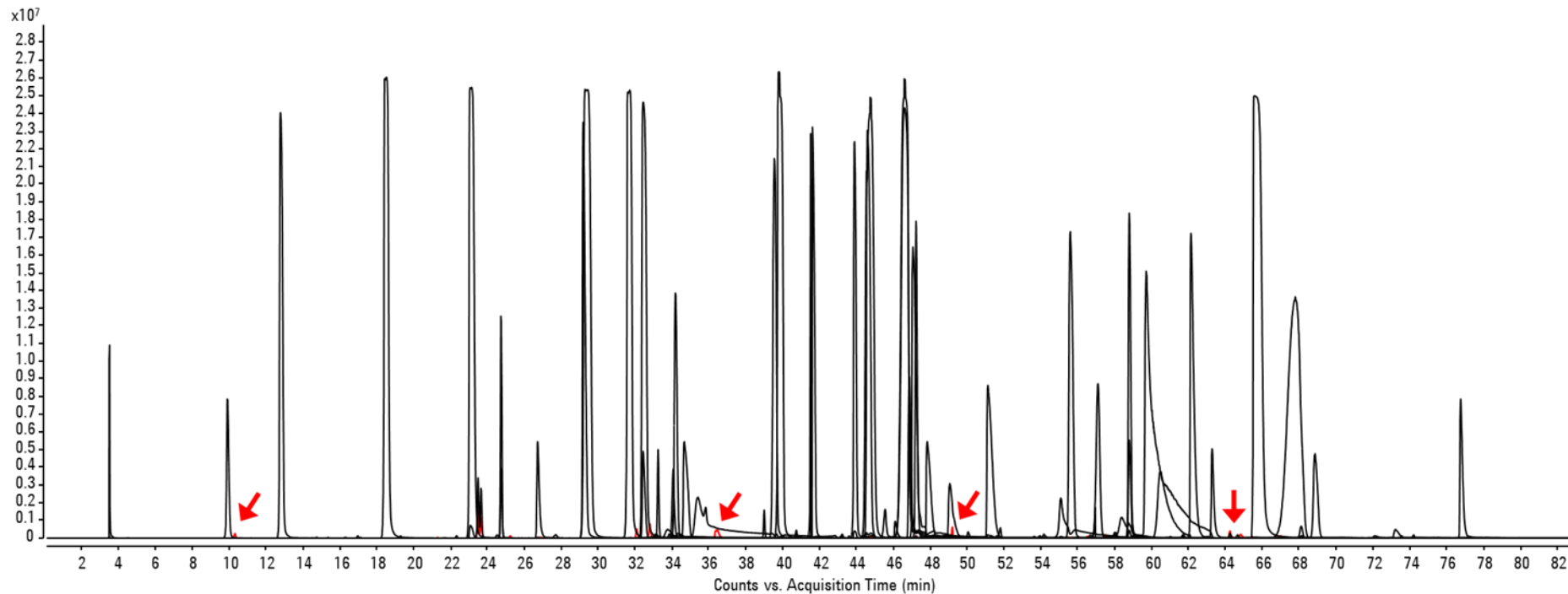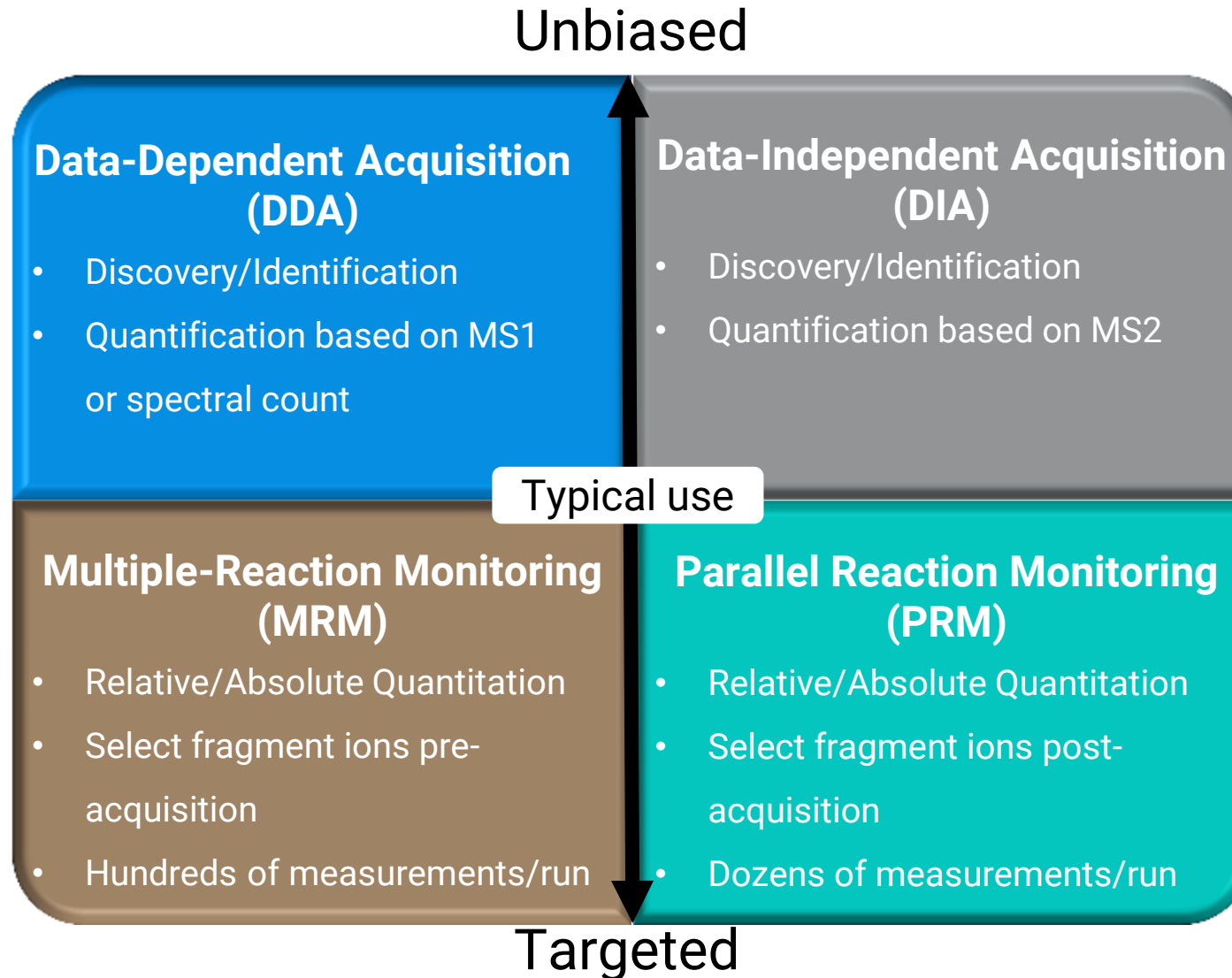
Host Cell Protein Analysis Workflow

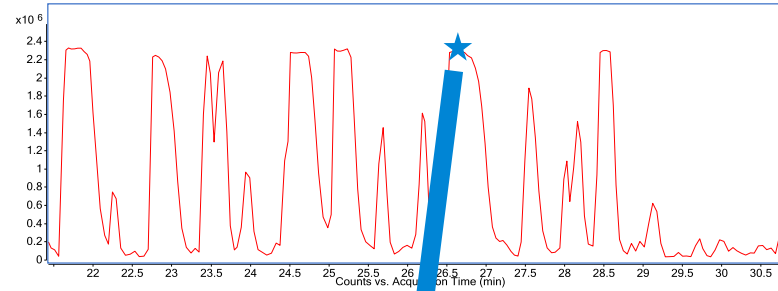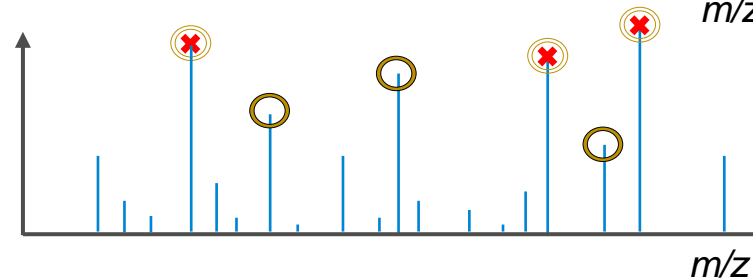| Protein Reduction, Alkylation and Digestion | 6545XT AdvanceBio LC/Q-TOF Using IterativeMS/MS | Match MS/MS Spectra to Database |

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Different MS/MS Scan Types

Unbiased

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Data-Dependent Acquisition (DDA)

- Discovery/Identification
- Quantification based on MS1 or spectral count

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Data-Independent Acquisition (DIA)

- Discovery/Identification
- Quantification based on MS2

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Multiple-Reaction Monitoring (MRM)

- Relative/Absolute Quantitation
- Select fragment ions pre-acquisition
- Hundreds of measurements/run

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Parallel Reaction Monitoring (PRM)

- Relative/Absolute Quantitation
- Select fragment ions post-acquisition
- Dozens of measurements/run

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Typical use

Targeted

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Automated IterativeMS/MS Acquisition

Precursors selected for MS/MS

Rolling excluded precursors

Experiment Design

- Spike-in UPS2 standards in purified CHO-cultured mAb to assess low-level HCP identification and quantification
  - Proteomics Dynamic Range Standard (UPS2) Commercial mix of 48 proteins at 6 concentrations, spanning 6 orders of magnitude, 8 proteins per concentration level
  - mAb without UPS2 spike was used as a negative control

- Sample preparation without off-line fractionation or desalting

- Standard-flow 60min 1D LC on an AdvanceBio Peptide Plus column (2.1x150 mm)

- Data-dependent acquisition (DDA)

- Data analysis
  - IterativeMS/MS vs. AutoMS/MS
  - HCP identification sensitivity
  - HCP label-free quantification precision
  - HCP quantification reproducibility

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IterativeMS/MS Decision Engine Improves Protein Identification
IterativeMS/MS with 5 injections vs. autoMS/MS with 5 injections

Protein name	Actual Protein Level (ppm)	Unique peptide #		Spectra #	
		Iterative MS/MS	Auto MS/MS	Iterative MS/MS	Auto MS/MS
mAb heavy chain	6.8E+05	589	491	5193	9738
mAb light chain	3.2E+05	261	208	2012	3925
Serum albumin	313.0	33	21	82	144
Carbonic anhydrase 2	137.3	12	9	34	53
Carbonic anhydrase 1	135.6	11	9	29	62
Leptin	76.2	6	3	14	26
Hemoglobin subunit beta	74.8	8	7	21	44
Hemoglobin subunit alpha	71.3	7	4	15	22
Ubiquitin	50	4	2	13	18
Small ubiquitin-related modifier 1	18.3	6	0	9	0
Peroxiredoxin 1	10.4	3	2	7	18
Peptidyl-prolyl cis-trans isomerase A	9.5	3	0	4	0
Myoglobin	8	0	3	0	11

Data analysis using Byonic® with 1%FDR:

- IterativeMS/MS -> more unique peptides
- IterativeMS/MS -> less redundant spectra

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Comparison of Identified Protein Levels
-- five experiments with different loading amount, injection numbers and acquisition modes

Protein name	Molecular weight (Da)	Actual Protein Level (ppm)	Unique peptide #				
			IterativeMS/MS (24µg x 5 inj.)	IterativeMS/MS (32µg x 3 inj.)	AutoMS/MS (24µg x 5 inj.)	AutoMS/MS (24µg x 1 inj.)	AutoMS/MS (32µg x 1 inj.)
Serum albumin	66,357	313.0	33	32	21	16	22
Carbonic anhydrase 2	29,115	137.3	12	9	9	5	7
Carbonic anhydrase 1	28,739	135.6	11	9	9	5	6
Leptin	16,158	76.2	6	4	3	2	3
Hemoglobin subunit beta	15,867	74.8	8	7	7	5	3
Hemoglobin subunit alpha	15,126	71.3	7	3	4	3	2
Ubiquitin	10,597	50.0	4	5	2	2	5
Complement C5/C5a anaphylatoxin	8,563	40.4	1	1	3	2	1
Catalase	59,625	28.1	0	3	8	3	0
Small ubiquitin-related modifier 1 (SUMO-1)	38,815	18.3	6	2	7	0	0
NAD(P)H dehydrogenase [quinone] 1	30,736	14.5	0	2	0	0	2
Peroxiredoxin 1	21,979	10.4	3	5	2	2	5
Peptidyl-prolyl cis-trans isomerase A	20,176	9.5	3	4	3	1	2
Myoglobin	17,053	8.0	1	1	3	0	0
Cytochrome b5	16,022	7.6	0	0	0	0	0
Pro-epidermal growth factor (EGF)/Epidermal growth factor	6,353	3.0	0	0	0	0	0
Histidyl-tRNA synthetase, cytoplasmic	58,233	2.7	6	4	8	2	0
Creatine kinase M-type	43,101	2.0	0	0	0	0	0
Ribosyldihydronicotinamide dehydrogenase	25,821	1.2	0	0	0	0	0

Data analysis using Byonic® followed by Byologic® software

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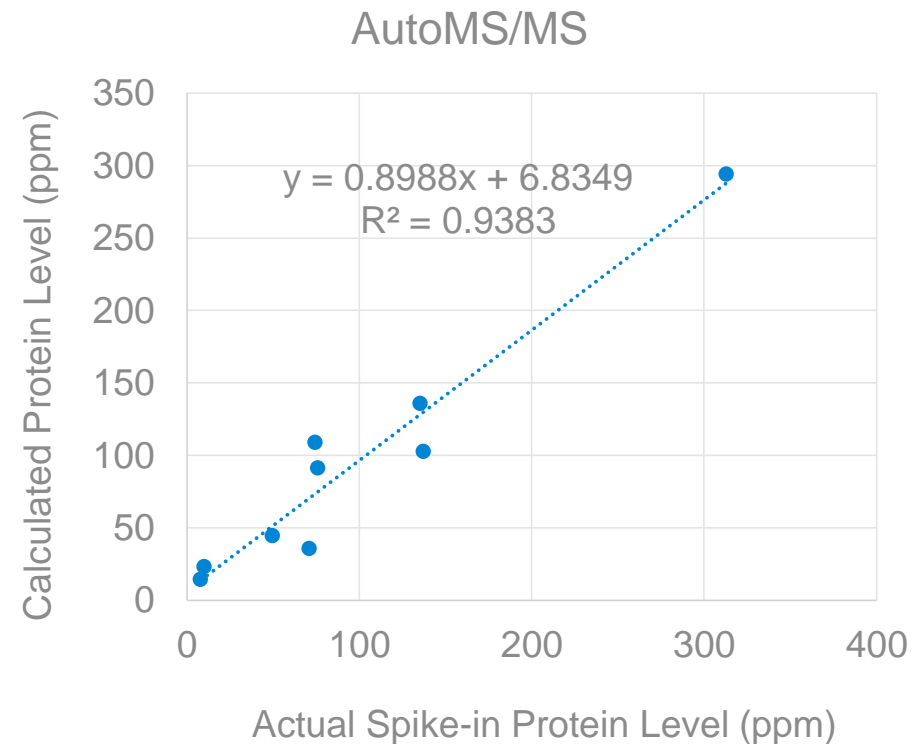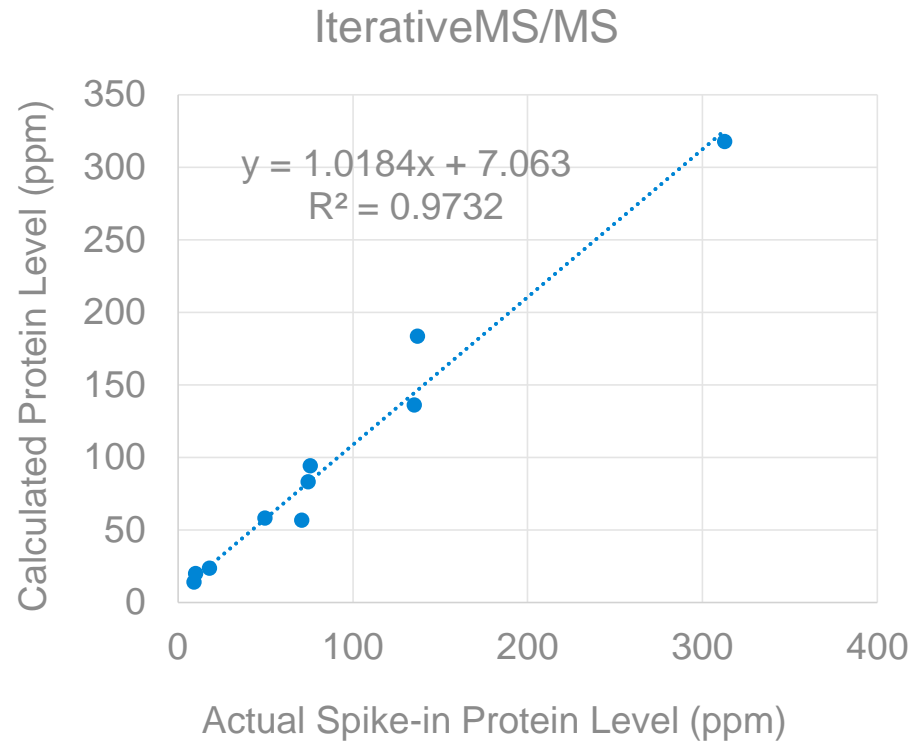
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- Triplicate injections with IterativeMS/MS allows identification of HCPs in single-digit ppm level
- Multiple unique peptides identified for HCP at 2.7 ppm

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Excellent Precision for HCP Label-free Quantification
HCP levels were calculated based on one internal reference protein

IterativeMS/MS

$y = 1.0184x + 7.063$
$R^2 = 0.9732$

Calculated Protein Level (ppm)

Actual Spike-in Protein Level (ppm)

AutoMS/MS

$y = 0.8988x + 6.8349$
$R^2 = 0.9383$

Calculated Protein Level (ppm)

Actual Spike-in Protein Level (ppm)

- UPS2 protein levels were calculated based on Carbonic Anhydrase 1, and then plotted with their actual spike-in levels:
  - Excellent linearity was achieved by using either IterativeMS/MS ($R^2 = 0.9732$) or AutoMS/MS ($R^2 = 0.9383$) dataset

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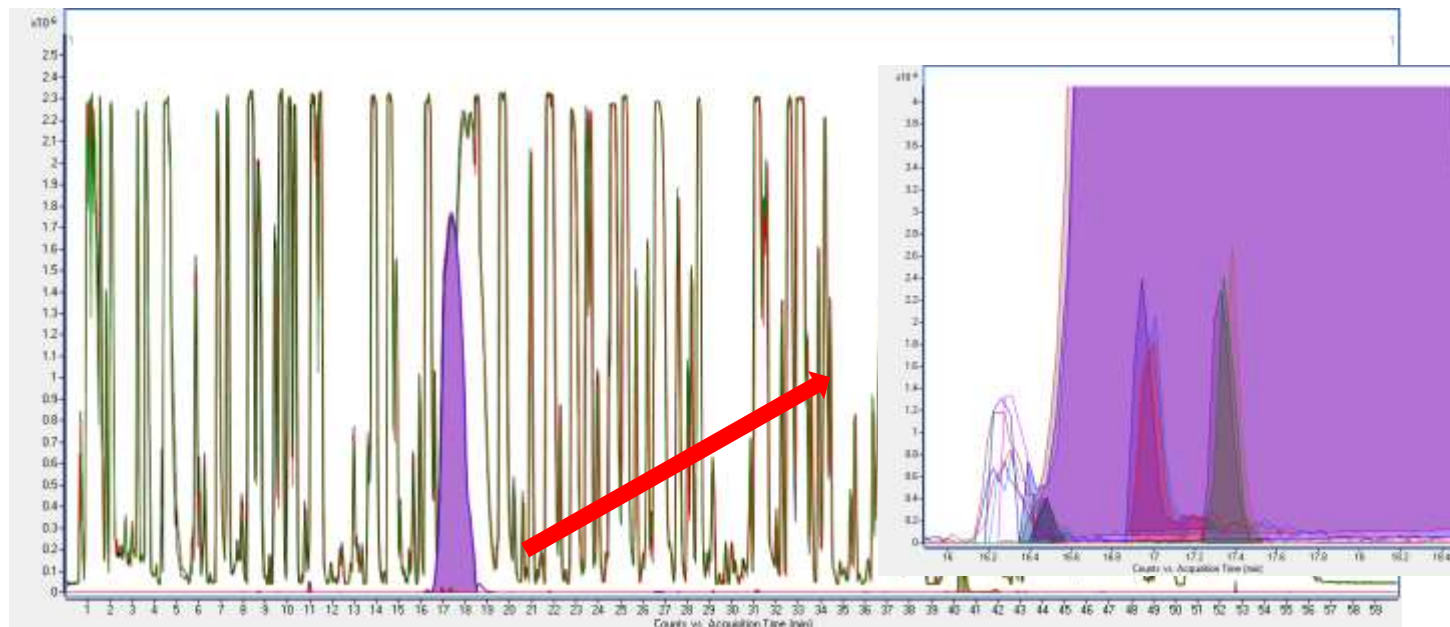
Broad Dynamic Range for Co-eluting Peptides
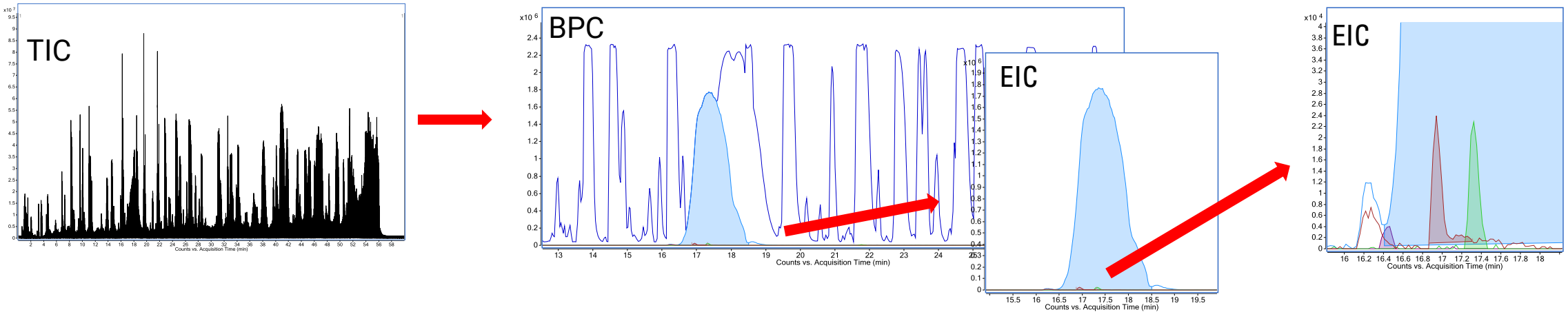
Peptide	Precursor ion (m/z)	Mass error (ppm)	Intensity	Actual Protein Level (ppm)	Protein name
ALELFR	374.7208	-1.1	6.76E+03	8	Myoglobin
SAVTALWGK	466.7659	4.8	1.36E+05	74.8	Hemoglobin subunit beta
TIAQDYGVLK	554.3049	-1.8	1.51E+05	10.4	Peroxiredoxin 1
EPQVYTLPPSR	643.844	1.0	1.38E+08	1.0E+06	mAb

- High loading capacity (32 µg on-column)
- Broad dynamic range for co-eluting peptides
  - peptide intensity > 4 orders
  - protein in weight > 5 orders

Excellent Chromatography Reproducibility
Overlay chromatograms of triplicate runs

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EPQVYTLPPSR	643.844	1.0	1.38E+08	1.2%	1.0E+06	mAb

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Summary

- 1D LC/MS solution: no off-line fractionation or desalting

- Simple data processing with DDA data

- Automated IterativeMS/MS improves protein identification coverage

- Identification of low-level (< 10ppm) HCPs

- Simultaneous identification and quantification

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Acknowledgements

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