

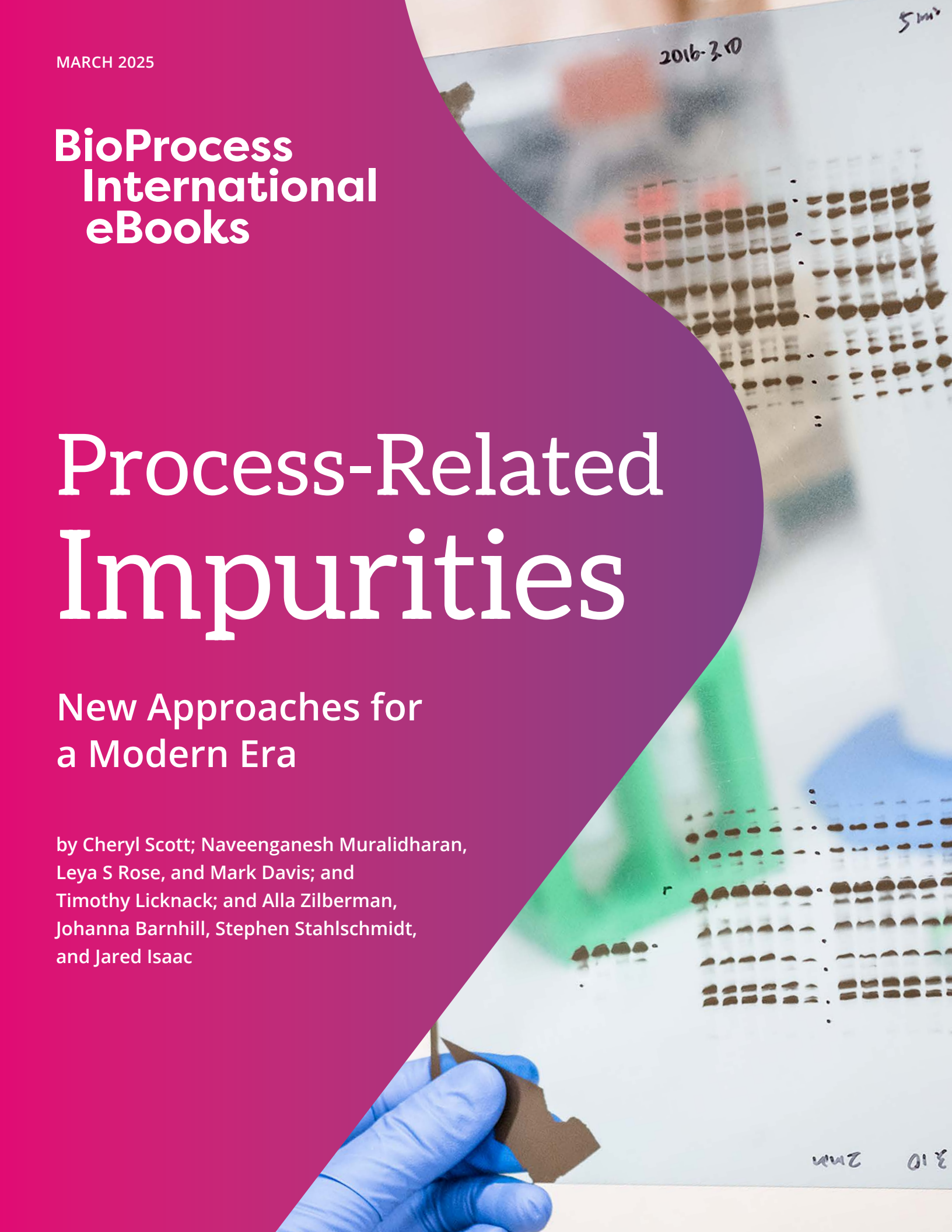
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# Process-Related Impurities

**New Approaches for  
a Modern Era**

by Cheryl Scott; Naveenganesh Muralidharan,  
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Johanna Barnhill, Stephen Stahlschmidt,  
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# Quantification of Six HCP Lipases from CHO-Derived Biotherapeutics

## Using Parallel Reaction Monitoring and Stable Isotope Labeled Peptides

Timothy Licknack, Alla Zilberman, Johanna Barnhill, Stephen Stahlschmidt, and Jared Isaac

**H**ost cell proteins (HCPs) copurify with biological drug substances (DSs) and pose risks for both patients and drug manufacturers. Although many HCPs are benign, some are immunogenic; others can diminish DS efficacy and/or stability. Although the total concentration of HCPs commonly is measured and reported to meet regulatory requirements, even trace levels of certain HCPs can influence drug product efficacy and stability. Total levels of HCPs typically are measured through an enzyme-linked immunosorbent assay (ELISA). Although that technique is highly sensitive and easy to use, it cannot discern the identity or complexity of the HCP profile present; such information can be determined using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) approaches.

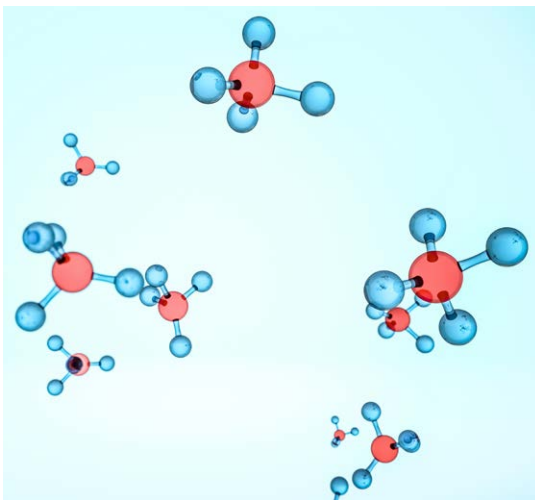
### LC-MS/MS FOR HCP ANALYSIS

LC-MS/MS is an analytical technique that can identify and quantify analytes with both high sensitivity and selectivity. HCP applications typically use bottom-up workflows, in which HCPs are digested with trypsin and their peptides are separated and measured by LC-MS/MS. When thousands of HCPs are of interest, the MS detection method is either a data-dependent (DDA) or data-independent (DIA) acquisition. Currently, neither approach provides accurate measurements of individual HCPs at actionable levels in DS samples.

Targeted proteomic workflows restrict analysis to a predefined list of peptides. During parallel reaction monitoring (PRM), target peptides are fragmented, and the entire fragmentation spectrum is measured at high resolutions to provide selective measurements of each fragment ion and peptide/protein. Stable isotope labeled (SIL) peptides are used for absolute quantification. SIL peptides behave identically with respect to chromatographic or fragmentation properties. That characteristic allows SIL peptides to be spiked at a known concentration, and their peak areas can be compared with those of endogenous peptides.

We conducted a study in which six Chinese hamster ovary (CHO) HCP lipases — lysosomal acid lipase (LAL), lipoprotein lipase (LPL),

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phospholipase A1 (PLA1), group XV lysosomal phospholipase A2 (GXVPA2), phospholipase B-like 2 (PLBL2), and phosphoinositide phospholipase C (PIPLC) – were selected and measured by PRM with SIL peptides. The quantitative assay was qualified using peptide calibration curves, spike and recovery with PLBL2 proteins, and a spike-in experiment with a CHO secretome standard. Our study demonstrates the utility of that assay to quantify six CHO lipases in DSs from bioprocesses.

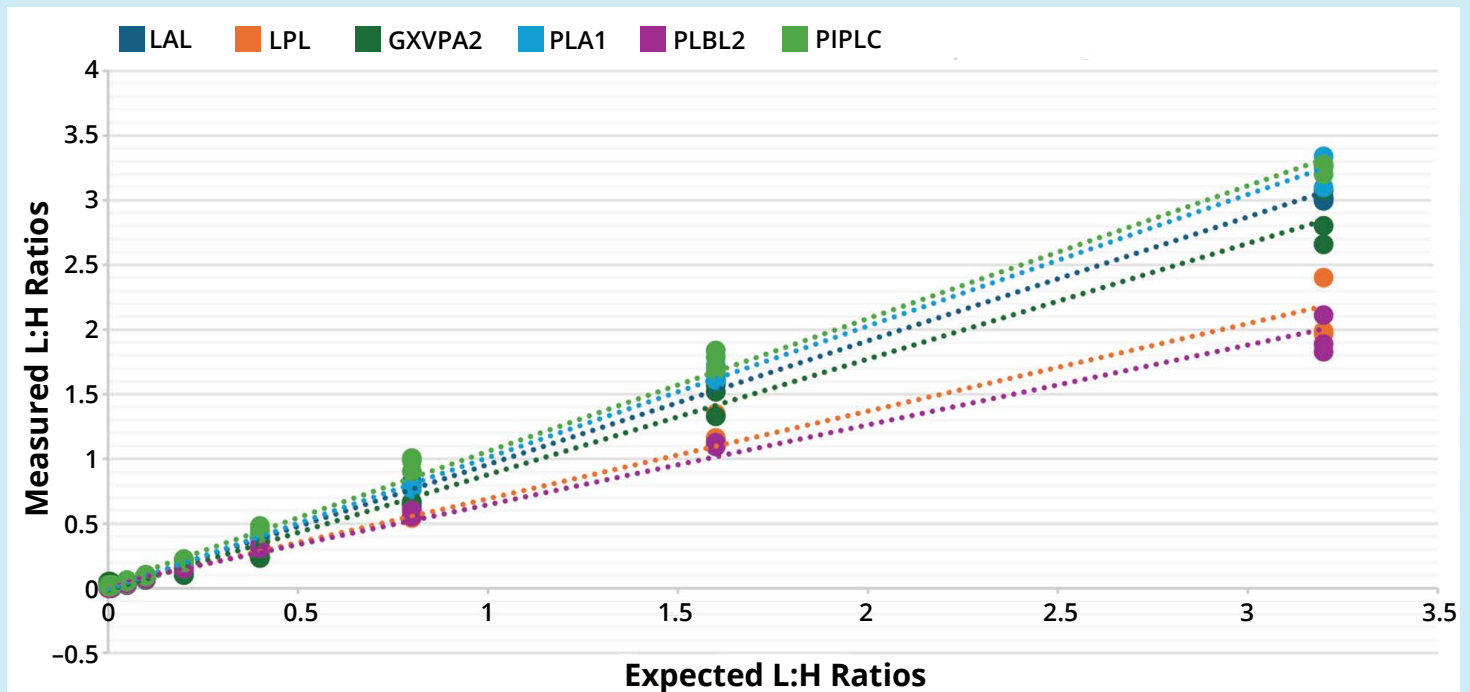
**Establishing PRM Assay Linear Range:** The six CHO lipases were selected for targeted proteomic analysis due to their possible role in DS stability. The lower (LLOQ) and upper (ULOQ) limits of quantification were estimated with a calibration curve including both SIL and unlabeled peptides to mimic endogenous

**Table 1:** Linear ranges of peptide calibration curves; peptide-level and protein-level metrics for two peptides per protein are displayed from calculations using 100 pM of stable isotope labeled (SIL) peptides. LLoQ = lower limit of quantitation, MW = molecular weight, ppm = parts per million, ULoQ = upper limit of quantitation

Protein	Peptide	LLOQ (pM)	ULOQ (pM)	MW (Da)	LLOQ (ng/mL)	ULOQ (ng/mL)	LLOQ (ppm)	ULOQ (ppm)
Lysosomal acid lipase (LAL)	LAL_1	10	320	45,635	0.46	14	9	292
	LAL_2	20	80		0.91	3	18	73
Lipoprotein lipase (LPL)	LPL_1	1	320	50,522	0.05	16	1	323
	LPL_2	10	320		0.51	16	10	323
Group XV lysosomal phospholipase A2 (GXVPA2)	GXVPA2_1*	5	320	47,236	0.24	15	4	302
	GXVPA2_2	5	320		0.24	15	4	302
Phospholipase A1 (PLA1)	PLA1_1*	5	320	48,698	0.24	15	4	311
	PLA1_2	0.5	320		0.02	15	0.49	311
Phospholipase B-like 2 (PLBL2)	PLBL2_1	1	320	65,541	0.07	21	1.3	419
	PLBL2_2	0.5	160		0.03	10	0.66	209
Phosphoinositide phospholipase C (PIPLC)	PIPLC_1	160	320	139,074	22.00	44	445	890
	PIPLC_2*	5	320		0.70	44	14	890

\* Thresholds did not apply across entire calibration range.

**Figure 1:** Peptide calibration curve; ratio of light to heavy peptides (L:H) is plotted for the quantifier peptide of each protein, with stable isotope labeled (SIL) peptide concentration fixed to 100 pM. Data were acquired using a Vanquish Neo ultra-high performance liquid-chromatography (UHPLC) system and an Orbitrap Eclipse Tribrid mass spectrometer operated in targeted MS2 mode (Thermo Fisher Scientific).



LAL = lysosomal acid lipase, LPL = lipoprotein lipase, PLA 1 = phospholipase A1, GXVPA2 = group XV lysosomal phospholipase A2, PLBL2 = phospholipase B-like 2, PIPLC = phosphoinositide phospholipase C

peptides; each was spiked into a background of control peptides (from NISTmAb reference material) to mimic a DS sample. The unlabeled peptide concentration varied between 0.1 and 320 pM, whereas the SIL-peptide concentration was at a fixed concentration of 100 pM. The ratio of light to heavy peptides (L:H) was calculated using the peak area of the same summed fragment ions corresponding to each one. Linearity was observed for all peptides except LAL\_2 (Figure 1 and Table 1). All displayed acceptable accuracy and precision and were chosen as surrogates of their respective proteins.

A targeted proteomic assay must be both precise and accurate for estimating protein abundance in diverse samples. *Precision* is defined as the closeness of agreement between a series of measurements obtained from multiple samplings, whereas *accuracy* is defined as how close those measurements are to their true values. The LLoQ is defined as the lowest point in the calibration curve, in which the coefficient of variation (%CV) is <25% and the nominal standard deviation (%nominal) is ±35% (Table 1). The ULoQ is the equivalent metric on the highest point in the curve. Those values define the assay's linear range.

The median peptide-level LLoQ for quantifier peptides was 5 pM, which resulted in protein-level LLOQs of 0.49–18.25 ppm, depending on the protein's molecular weight (MW) (Table 1). Except for LAL\_2 and PLBL2\_2, the highest tested point in the peptide calibration curve was typically the ULoQ (e.g., 320 pM), corresponding to protein-level ULOQs of 73.02–890.07 ppm.

**Determining Analytical Limits of Detection and Quantification:** Sensitivity is defined by the limit of detection (LoD) and limit of quantification (LoQ). In our study, matrix blanks were used when DS and SIL peptides were present, but not with unlabeled peptides. LoD and LoQ estimates were derived from those blank samples. All peptides had LoD values of ≤4.8 pM, and protein-level LoD values were between 0.00–0.22 ng/mL or 0.03–4.39 ppm (Table 2). Peptide-level LoQ values were 0.11–16 pM,

**Table 2:** Analytical limits of detection (LoD) and quantification (LoQ), calculated for two peptides per protein and reported in both peptide-level and protein-level concentrations from blanks, with 100 pM stable isotope labeled (SIL) peptide concentration; values were rounded to the third decimal place and are reported as 0.00 if they are <0.0049; MW = molecular weight, ppm = parts per million.

Peptide	LoD (pM)	LoQ (pM)	MW (Da)	LoD (ng/mL)	LoQ (ng/mL)	LoD (ppm)	LoQ (ppm)
LAL_1	0.16	0.54	45,635	0.01	0.02	0.15	0.49
LAL_2	4.8	16		0.22	0.72	4.39	14
LPL_1	0.30	1.0	50,522	0.02	0.05	0.31	1.0
LPL_2	0.08	0.25		0.00	0.01	0.08	0.26
GXVPA2_1	1.9	6	47,236	0.09	0.30	1.8	6.1
GXVPA2_2	0.04	0.12		0.00	0.01	0.03	0.11
PLA1_1	0.39	1.3	48,698	0.02	0.06	0.38	1.3
PLA1_2	0.13	0.42		0.01	0.02	0.12	0.41
PLBL2_1	0.66	2.2	65,541	0.04	0.14	0.86	2.8
PLBL2_2	0.41	1.3		0.03	0.09	0.53	1.76
PIPLC_1	0.03	0.11	139,074	0.00	0.02	0.09	0.31
PIPLC_2	1.0	3.4		0.14	0.47	2.9	9.5

GXVPA2 = group XV lysosomal phospholipase A2, LAL = lysosomal acid lipase, LPL = lipoprotein lipase, PLA1 = phospholipase A1, PIPLC = phosphoinositide phospholipase C, PLBL2 = phospholipase B-like 2

**Table 3:** Phospholipase B-like 2 (PLBL2) spike-in experiment with a NIST monoclonal antibody (mAb) drug substance (DS); precision and accuracy of spike and recovery experiment with 100 pM SIL peptide concentration. Different concentrations of recombinant PLBL2 were spiked into the NIST mAb; CV = coefficient of variation, NA = not applicable, ppm = parts per million.

Peptide	Expected ppm	%CV	%Error
PLBL2_1	0	37.0%	NA
	2	6.8%	34%
	4	1.0%	72%
	40	3.4%	65%
	200	6.7%	56%
PLBL2_2	0	86.8%	NA
	2	8.8%	10%
	4	3.6%	30%
	40	1.1%	15%
	200	8.9%	21%

and protein-level LoQ values were 0.01–0.72 ng/mL or 0.11–14 ppm.

### **Accurate Recovery of Recombinant PLBL2 After Sample Preparation and Acquisition by PRM:**

Although calibration curves are useful in assessing accuracy when unlabeled peptides are spiked in immediately before data acquisition, such curves do not account for variation introduced during sample preparation. To measure variation in a relevant context, recombinant PLBL2 (United States Pharmacopeia, USP) was spiked into NISTmAb solution at four different concentrations, and SIL peptides were spiked to a final concentration of 100 pM (Table 3). The PLBL2\_2 peptide was highly accurate (<30%), whereas PLBL2\_1 was less accurate (<72%; Table 3). The performance of PLBL2\_2 supports the accuracy of the PRM assay.

Two lipases were known to exhibit a linear response when CHO antigen is spiked into a mAb DS solution: The previous experiments were performed on samples with known analyte concentrations. A final experiment was performed in which a CHO secretome standard – containing all lipases at some unknown concentration – was diluted into a mAb DS in which LPL and PLBL2 were detected previously by deep proteomic profiling via Antibody Affinity Extraction analysis coupled with LC-MS/MS (AAE-MSTM) (1). The CHO standard was spiked into the mAb DS (Table 4). SIL peptides were spiked in at a final concentration of 100 pM. A linear response was observed for both LPL and PLBL2.

## **SUMMARY**

We determined LoD, LoQ, precision, accuracy, LLoQ, and ULoQ for a LC-MS/MS quantification assay for CHO lipases using SIL and PRM. Previous studies have accomplished similar results using either multiple reaction monitoring (MRM) or PRM (2–4). Comparison of peptide- and protein-level LLoQ values reported by MRM (5) with our PRM data shows excellent performance for LPL and PLBL2.

Six CHO lipases were quantified by this assay: LAL, LPL, GXVPA2, PLA1, PLBL2, and PIPLC. All figures of merit have been determined for this PRM assay with SIL peptides and were within generally accepted guidelines. Based on both precision and accuracy, the median peptide-level quantitative range was 5–320 pM, which resulted in a protein-level range from ≈0.5 to 890 ppm. Taken together, this PRM assay demonstrated applicability to CHO downstream DS bioprocess samples.

*Absolute quantification of six CHO HCP lipases by PRM with SIL peptides is now available as a service from Cygnus Technologies. Contact [techsupport@cygnutechnologies.com](mailto:techsupport@cygnutechnologies.com) to discuss your projects.*

**Table 4:** Chinese hamster ovary (CHO) antigen spike-in experiment with monoclonal antibody (mAb) drug substance (DS); lysosomal acid lipase (LPL), phospholipase B-like 2 (PLBL2), and phospholipase A1 (PLA1) abundance increased as CHO antigen spike level increased. LPL and PLBL2 responses were linear from 64–1024 ppm, although many points were below the qualified limit of detection (LoD) and/or lower limit of quantitation (LLoQ). All three lipases were detected and quantified at the 1024-ppm spike level.

CHO Spike (ppm)	LPL Mean Conc. (ppm)	PLBL2 Mean Conc. (ppm)	PLA1 Mean Conc. (ppm)
1024	7.2	2.2	0.81
256	1.5	0.62**	0.14**
64	0.38**	0.24*	0.06*
0	0.6*	0.08*	0.07*

\* Below reported LoD \*\* Below reported LLoQ

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