

LC-MRM Quantification of Hamster Phospholipase B-Like 2 Protein (PLBL2) in Therapeutic Monoclonal Antibodies at Part-per-Billion (ppb) Levels

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APPLICATION BENEFITS

- An ultra sensitive LC-MRM assay was developed for quantification of a ubiquitous low-level host cell protein (PLBL2) known to affect the stability of mAb drug substances.
- Compared with the traditional process-specific ELISA assays used for quantification of individual host cell proteins (HCPs), the LC-MRM assays achieve similar sensitivities while they are much more flexible, faster and cheaper to develop

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KEYWORDS

Phospholipase B-like 2 protein (PLBL2), host cell protein (HCP), monoclonal antibody (mAb), LC-MRM, ppb-level protein quantification, Xevo TQ-S MS

INTRODUCTION

Protein biopharmaceuticals are typically produced by recombinant DNA technology in well-selected cell lines. Even after extensive purification, low-level protein impurities (1–100 ppm) derived from the host cell line – HCPs, can still be present in the formulated drug substance (DS). The presence of HCPs in the biopharmaceutical product can have several detrimental effects, most concerning being the possibility to produce unwanted immunogenic effects in patients. In addition, HCPs can affect drug stability and efficacy or be responsible for drug degradation. For these reasons, regulatory agencies require that several HCP identification and quantification assays are performed prior to drug approval.

Phospholipase B-Like 2 (PLBL2) is a 65.5 kDa host-cell-protein (HCP) that co-purifies frequently in mAb preparations produced in Chinese hamster ovary (CHO) cells which was identified for the first time by mass spectrometry by a group of scientists from Roche/Genentech.¹ The same protein or similar PLBL2 isoforms were later identified in mAb preparations by other researchers from Janssen,² Eli Lilly and Company³ and Shire.⁴ The protein was shown to be responsible for enzymatic degradation of polysorbates 20 and 80, which are common adjuvants present in formulated DS.²⁻³ For this reason, PLBL2 affects DS (mAb) stability and needs to be removed from mAb preparations down to acceptable levels. The interactions between PLBL2 and mAbs were recently investigated to understand the factors responsible for the presence of PLBL2 following Protein A purifications.⁵

The acceptable levels tolerated for this HCP are in the sub ppm range.¹⁻⁴ While the typical assays used for HCP quantification, like the general CHO ELISA kits or the process-specific immunoassays (e.g. ELISAs) are very sensitive (<1 ppm), they cannot provide individual HCP measurements. It is possible to develop ELISA assays for each individual HCP, but in reality this approach is not feasible to be implemented for each HCP impurity due to time constraints (>6 months) and development costs (>\$500K). A process specific ELISA PLBL2 assay had to be developed in house recently¹ because the commercial ELISA PLBL2 kit failed to detect the protein.

Another alternative to the process-specific PLBL2 ELISA assays, is the LC/MRM assay described here which was developed in significantly less time (one week) and with considerably less resources compared to the PLBL2 ELISA assay. The LC-MRM assay presented here is able to quantify PLBL2 from mAb preparations down to concentrations of 0.00625 nM (or 25 attomoles or 1.6 pg of PLBL2 digest loaded on-column). For a total mAb digest amount of 10 µg loaded on-column, the LOQ of the PLBL2 assay developed here is ~160 ppb (part-per-billion). The ppb level quantification of PLBL2 was confirmed with four tryptic peptides using their corresponding MRM transitions. Peak area relative standard deviation (RSD) for replicate measurements (n=3) was better than 15% when using the corresponding ¹³C¹⁵N-isotopically labeled peptide internals standard. In addition, the assay had a wide dynamic range of at least three orders of magnitude – 1600. Assay robustness was demonstrated by performing 90 injections for a concentration near the LOQ level (0.025 nM PLBL2 digest or 100 attomoles protein digest loaded on-column) when the peak area RSDs were also better than 15% for all four peptides.

Clearly the LC-MRM assay described here opens the door for flexible, high-throughput, sub-ppm quantification assays of multiple HCPs across multiple preparations of biopharmaceuticals.

EXPERIMENTAL

Sample preparation

A highly purified mAb (NIST mAb candidate reference LRM 8670) produced in a murine suspension cell culture was acquired from the National Institute of Standards and Technology (NIST) at a concentration of 10 mg/mL. The NIST mAb was denatured with 0.05% *RapiGest* surfactant (60 °C, 15 min), reduced with 25 mM DTT (60 °C, 1h), alkylated with 10 mM IAM (RT, 30 min in the dark) and digested with porcine trypsin (Promega, Madison, WI, USA) overnight (16 h, 37 °C) using a 10:1 molar ratio of mAb : enzyme. After digestion, the *RapiGest* surfactant was decomposed by adding 5 µL of formic acid (FA, Sigma-Aldrich, St. Louis, MS, USA) and the digest was incubated for 30 min at 37 °C and centrifugated (15 min, 4,000 rpm) to separate the insoluble component of *RapiGest* by precipitation. The supernatant was then transferred to a TruView™ LCMS certified clear glass vial ([P/N 186005663CV](#)). LC/MS-grade organic solvents (acetonitrile-ACN, isopropanol-IPA and methanol-MeOH) were purchased from Thermo Fisher Scientific.

Since the hamster (CHO) PLBL2 protein was not commercially available, the rat PLBL2 protein, obtained from MyBioSource Inc. (San Diego, CA, USA), was used instead. The rat PLBL2 protein (0.2 mg with a concentration of 5 mg/mL) was digested with trypsin following the NIST mAb protocol described above.

All seven tryptic peptides used for PLBL2 quantification were obtained from New England Peptide (Gardner, MA, USA): four light (non-isotopically labeled) peptides (T10, T16, T34, and T39, see Figure 1 and Table I) and three heavy (¹³C¹⁵N-isotopically labeled) peptides (T10-HVY, T16-HVY, and T34-HVY, see Figure 1 and Table I as well). The light peptides were spiked in the NIST mAb digest at eight different concentrations: 0.00625; 0.0125; 0.025; 0.125; 0.25; 1.25; 2.5, and 10 nM (or fmoles/µL), while the heavy peptides were used as internal standards at a fixed concentration of 1 nM (or 1 fmole/µL). With an injection volume of 4 µL, the amounts of light peptides loaded on-column for the 8 concentrations mentioned above were: 25; 50; 100; 500; 1000; 5000; 10000 and 40000 attomoles, while the amount of NIST mAb digest was kept constant at 10 µg for every injection. The amount of each heavy peptide was also kept constant for every injection (4 fmoles on-column). Sample blanks, containing only the NIST mAb digest without the spiked PLBL2 peptides, were prepared as well.

MAAPMDR_SPGGR_AVR_ALR_LALALASLTEVLLNCPAGALPTQGPGR_R_R_QNLDPPVSR_VR_SVLLDAASGQLR_LVDGIHPYAVAWANLTNAIR_ETGWAYLDLGTNGSYNDSLQAYAAGVVEASVS EELIYMHMWMNTMVNYCGPFYEYGVYCEK_LK_SFLEINLEWMQR_EMELSQDSPYWHQVR_LTLLQ LK_GLEDSYEGR_LTFPTGR_FTIC_PLGFLLLQIAGDLEDLEQALNK_TSTK_LSLGSGSCSAIK_LL PGAR_DLLVAHNTWNSYQNMLR_IJK_K_YQLQFR_QGPQEAYPLIAGNNLVFSSYPGTIFSGDDFYI LGSGLVTLETTIGNK_NPALWK_YVQPOGCVLEWIR_NIVANR_LALDGATWADIFK_QFNSGTYYN QWMIVDYK_AFIPNGPSPGSR_VLTILEQIPGMVVVADK_TEDLYK_TTYWASYNIPFFEIVFNASGL QDLVAQYGDWFSYTK_NPR_AQIFQR_DQSLVEDMNSMVR_LIR_YNNFLHDPLSLCEACIPKPNA ENAISAR_SDLNPANGSYPPQALYQRPHGGIDVK_VTSFSLAK_R_MSMLAASGPTWDQLPPFQWS LSPFR_SMLHMGQPDWTFSPISVPWD

Figure 1. The sequence of CHO phospholipase B-Like 2 protein (PLBL2) indicating the four tryptic peptides (T10, T16, T34 and T39, highlighted in red) used for LC-MRM protein quantification.

Table 1. Optimized MRM/SRM transitions used by the LC-MRM assay for quantification of hamster PLBL2 in the present study. Two transitions were monitored for each peptide and the highlighted product ions were used for PLBL2 quantification.

Peptide ID	Peptide sequence	Retention time (min)	Peptide precursor/charge	Product ions (m/z)	Collision energy (eV)	Dwell time (ms)
T10	SVLLDAASGQLR	15.5	615.35 (+2)	702.39	28	72
				817.42	22	72
T10-HVY	SVLLDAASGQLR-HVY	15.5	620.35 (+2)	712.40	28	72
				827.42	22	72
T16	LTLLQLK	16.8	414.78 (+2)	614.42	14	72
				715.47	16	72
T16-HVY	LTLLQLK-HVY	16.8	418.79 (+2)	622.44	14	72
				723.49	16	72
T34	AFIPNGPSPGSR	12.3	600.31 (+2)	657.33	27	142
				868.43	19	142
T34-HVY	AFIPNGPSPGSR-HVY	12.3	605.32 (+2)	667.34	27	142
				878.43	19	142
T39	AQIFQR	8.8	381.72 (+2)	450.24	11	297
				563.33	13	297

LC conditions

LC system: ACQUITY UPLC I-Class
 Column: 1.0 x 150 mm, packed with 1.7 μm CSH C18 particles ([P/N 186005294](#))
 Column temp.: 60 °C
 Flow rate: 50 μL/min
 Mobile phases: Solvent A: 0.1% FA in DI water
 Solvent B: 0.1% FA in acetonitrile
 Gradient table:

Time (min)	Flow rate (μL/min)	Solvent A composition (%)	Solvent B composition (%)	Gradient curve
0	50	98	2	6
2	50	98	2	6
20	50	75	25	6
21	50	10	90	11
26	50	98	2	11
35	50	98	2	11

Injection volume: 4 μL, partial loop injection using a 10 μL injection loop
 Wash solvents: SNW (1:1:1 IPA:ACN:MeOH:H₂O with 0.2% FA) WNW (0.1% FA in H₂O)

MS conditions

MS system: Xevo TQ-S Tandem Quadrupole Mass Spectrometer
 Ionization mode: ESI (+)
 ESI probe: Low flow probe ([P/N 186007529](#))
 Capillary voltage: 3.0 kV
 Cone voltage: 35 V
 Desolvation temp.: 600 °C
 Desolvation flow: 1000 L/hr
 Nebulizer gas flow: 7 bar
 Cone gas flow: 150 L/hr
 Collision cell pressure: 3.83 e⁻³ Torr
 Data acquisition software: MassLynx 4.1 SCN 950
 Quantification software: TargetLynx

RESULTS AND DISCUSSION

Hamster phospholipase B-Like 2 (PLBL2) was recently identified as a low-level HCP contaminant in several high-purity mAb preparations produced in several CHO cell lines.¹⁻⁴ The full protein sequence, containing 585 AA, is displayed in Figure 1. Four tryptic peptides (highlighted in red) were selected for LC-MRM quantification based on high-resolution peptide mapping data obtained for the rat PLBL2 digest. Four stable proteotypic peptides, highlighted in Figure 1, were selected based on the strong response of their precursors in ESI-MS. The LC-MRM signals were individually optimized for each peptide transition in terms of collision energy and the MRM/SRM experimental conditions of the assay are summarized in Table I.

As shown in Figure 2, all four tryptic PLBL2 peptides monitored (T10, T16, T34, and T39) were detected with similar sensitivities at the lowest spiked concentration: the LOQ concentration was 0.00625 nM, corresponding to 25 attomoles of PLBL2 peptides loaded on-column. Also shown in the same figure are the LC-MRM chromatograms recorded for another concentration, near the LOQ level: 0.0125 nM or 100 attomoles PLBL2 peptides loaded on-column.

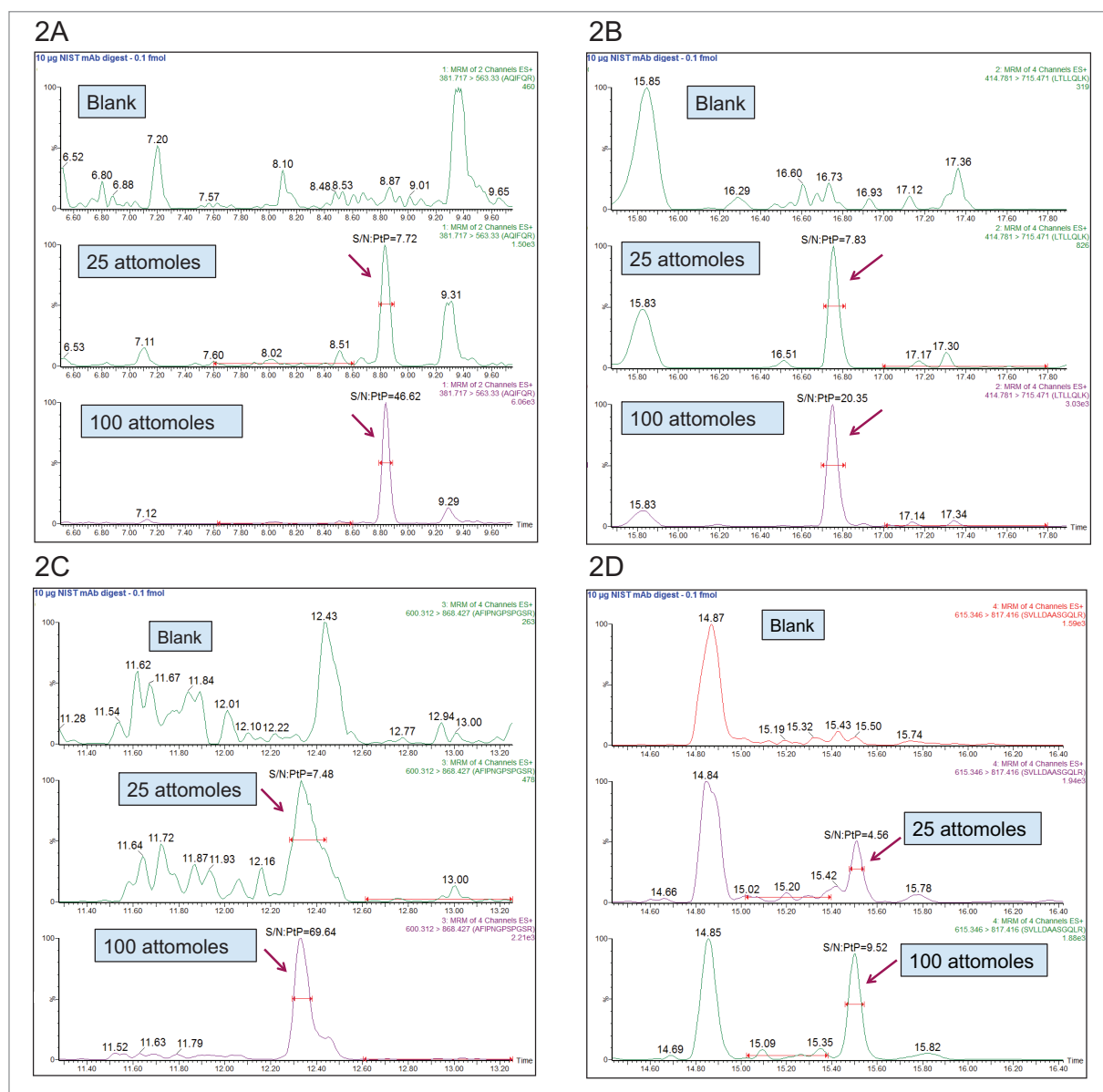


Figure 2. LC-MRM chromatograms recorded for T10 (panel A), T16 (panel B), T34 (panel C) and T39 (panel D) PLBL2 tryptic peptides spiked in the NIST mAb digest. Two chromatograms are shown for each peptide, one obtained at the LOQ of the assay (0.00625 nM concentration or 25 attomoles on-column) and the other at a higher concentration 4 times above the LOQ (0.0125 nM or 100 attomoles on-column). Each of the four panels includes the corresponding NIST mAb digest blanks (with no PLBL2 spiked peptides). The calculated single-to-noise (S/N) ratios are indicated as well.

The reproducibility of the assay is illustrated by the data presented in Table II which shows the results obtained for replicate injections (n=3) across all eight concentrations investigated. Individual TargetLynx calibration curves obtained for each peptide are presented in panels A-D of Figure 3.

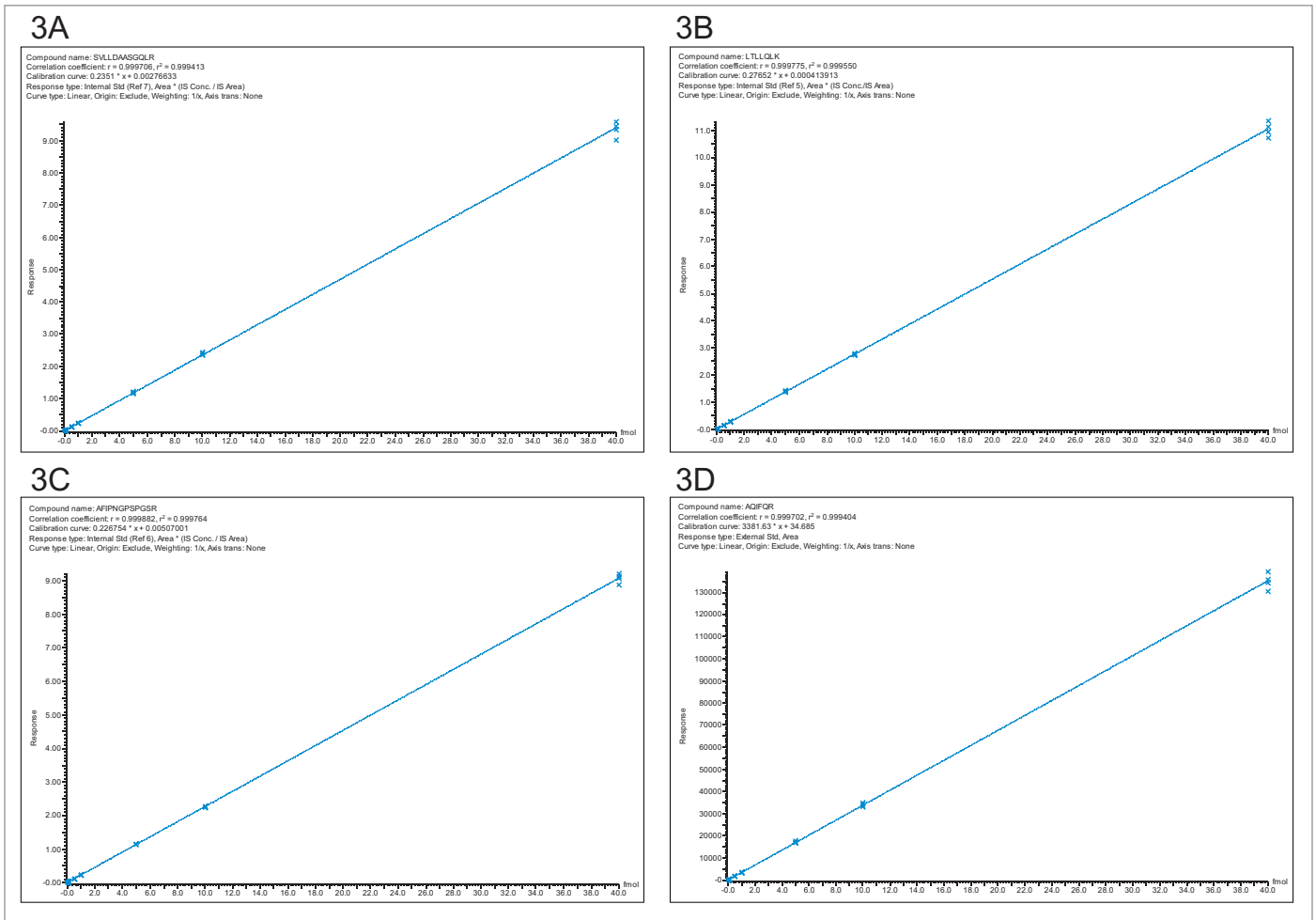


Figure 3. TargetLynx calibration curves for 4 PLBL2 tryptic peptides across 8 different concentrations (0.00625; 0.0125; 0.025; 0.125; 0.25; 1.25; 2.5, and 10 nM); (A) T10; (B) T16; (C) T34; (D) T39 peptides.

Table II. Reproducibility of the PLBL2 LC-MRM assay. Ratios of light peptide peak areas vs heavy ¹³C₁₅-labeled peptide IS peak areas for three PLBL2 tryptic peptides are displayed in panels (A) T10 peptide; (B) T16 peptide and (C) T34 peptide. Peak areas recorded for the T39 peptide across 3 replicate injections are shown in panel (D). The relative standard deviation (RSD) was better than 15% for all peptides over the entire concentration range investigated.

A.	Conc (nM)	Amount on-column (attomoles)	Peak area ratios of T10: SVLLDAASGQLR peptide				
			Rep01	Rep02	Rep03	Mean	RSD (%)
	0.00625	25	0.008	0.010	0.009	0.009	11.1
	0.0125	50	0.015	0.013	0.011	0.013	15.4
	0.025	100	0.021	0.025	0.028	0.025	14.2
	0.125	500	0.133	0.124	0.124	0.127	4.1
	0.25	1000	0.238	0.246	0.240	0.241	1.7
	1.25	5000	1.206	1.222	1.187	1.205	1.5
	2.5	10000	2.432	2.350	2.351	2.378	2.0
	10	40000	9.028	9.450	9.590	9.356	3.1

B.	Conc (nM)	Amount on-column (attomoles)	Peak area ratios of T16: LTLQLK peptide				
			Rep01	Rep02	Rep03	Mean	RSD (%)
	0.00625	25	0.006	0.007	0.007	0.007	8.7
	0.0125	50	0.014	0.014	0.013	0.014	4.2
	0.025	100	0.028	0.033	0.029	0.030	8.8
	0.125	500	0.143	0.146	0.143	0.144	1.2
	0.25	1000	0.285	0.297	0.273	0.285	4.2
	1.25	5000	1.423	1.366	1.367	1.385	2.4
	2.5	10000	2.744	2.799	2.734	2.759	1.3
	10	40000	10.941	10.721	11.355	11.006	2.9

C.	Conc (nM)	Amount on-column (attomoles)	Peak area ratios of T34: AFIPNGSPGSR peptide				
			Rep01	Rep02	Rep03	Mean	RSD (%)
	0.00625	25	0.008	0.006	0.004	0.006	33.3
	0.0125	50	0.010	0.012	0.013	0.012	13.1
	0.025	100	0.027	0.023	0.030	0.027	13.2
	0.125	500	0.123	0.125	0.119	0.122	2.5
	0.25	1000	0.227	0.242	0.229	0.233	3.5
	1.25	5000	1.156	1.153	1.157	1.155	0.2
	2.5	10000	2.267	2.243	2.286	2.265	1.0
	10	40000	9.206	9.130	8.879	9.072	1.9

D.	Conc (nM)	Amount on-column (attomoles)	Peak areas of T39: AQIFQR peptide				
			Rep01	Rep02	Rep03	Mean	RSD (%)
	0.00625	25	99	108	107	104	4.6
	0.0125	50	201	206	201	203	1.4
	0.025	100	392	381	336	370	8.0
	0.125	500	1849	1718	1741	1769	4.0
	0.25	1000	3624	3392	3404	3473	3.8
	1.25	5000	17634	16867	16840	17114	2.6
	2.5	10000	33054	33411	34366	33610	2.0
	10	40000	130354	134363	135693	133470	2.1

In addition, the robustness of the assay was tested with 90 injections performed near the LOQ level (100 attomoles on-column for each peptide) and the peak area trend plots are shown in Figure 4.

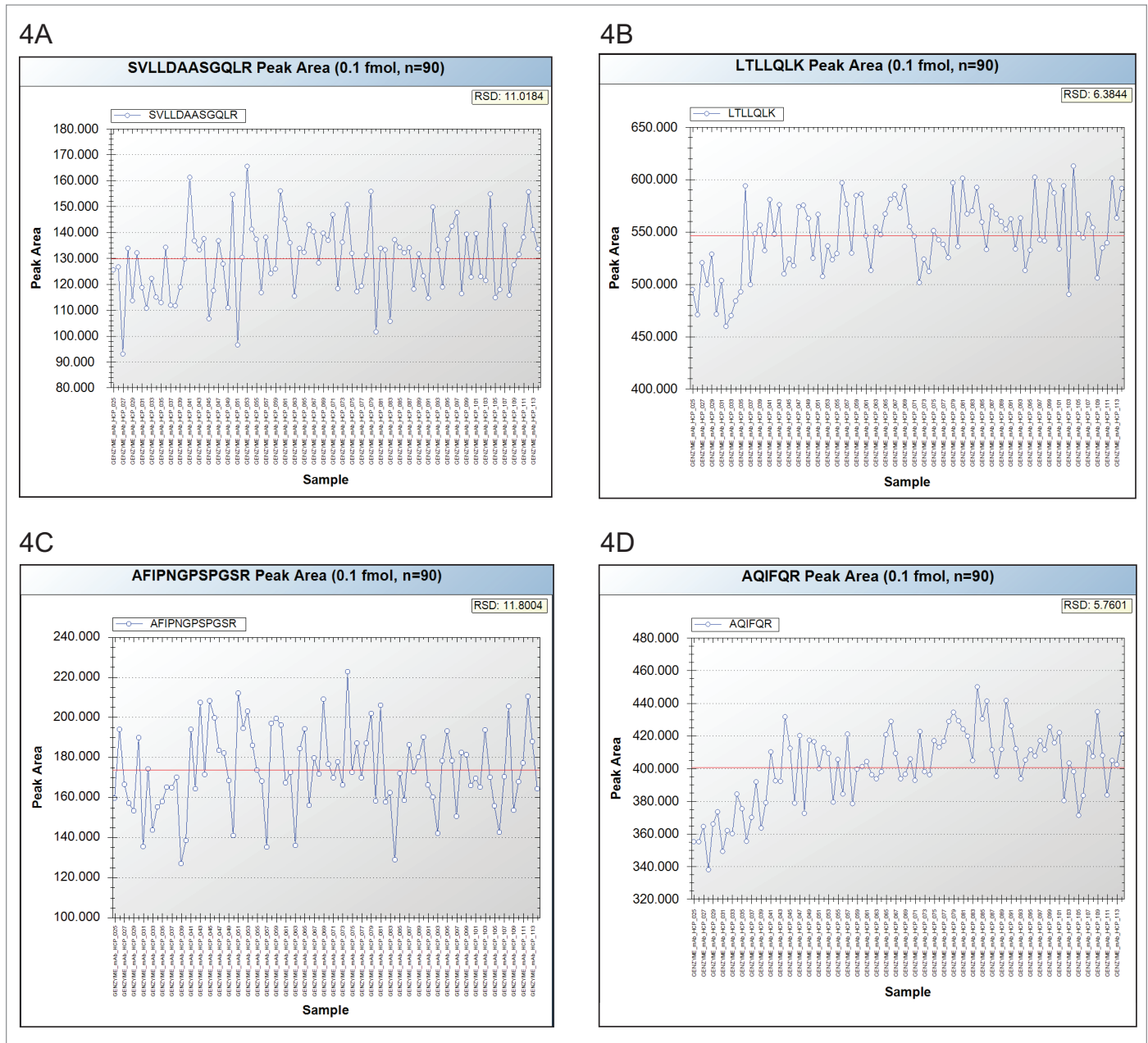


Figure 4. Peak area trend plots demonstrating the robustness of the LC-MRM assay: 90 injections were performed for one of the digest containing the PLBL2 peptides spiked near the LOQ of the assay (0.025 nM concentration or 100 attomoles PLBL2 peptides on-column). The four panels display the data obtained for (A) T10; (B) T16; (C) T34; (D) T39 peptides.

DISCUSSION

The exquisite sensitivity of the PLBL2 LC-MRM assay is highlighted by the chromatograms shown in Figure 2. All four peptides (T10, T16, T34, and T39), carefully selected to cover several critical protein domains, were detected at the lowest spiked concentration (0.00625 nM, 25 attomoles loaded on-column) with S/Ns in the range of 5–10. In addition, as indicated by the blank chromatograms recorded for the free (not spiked) NIST mAb digest, the assay was free of significant MRM/SRM interferences even in the presence of a significant amount (10 µg) of peptide matrix. The LOQ of the assay for three peptides (T10, T16, and T39) was the same as the LOD, indicating the ability of the LC-MRM assay to measure PLBL2 in mAb preparations down to ~160 ppb (part-per-billion). For the fourth peptide (T34, Figure 2C), the LOQ of the assay was 0.0125 nM (50 attomoles on-column), which corresponds to ~360 ppb. The reproducibility of the assay (shown in Table II), which was tested by replicate injections (n=3) across the entire concentration range investigated (0.00625–10 nM, 1600 dynamic range), was better than 15% at all concentrations at and above the LOQ.

It is worth mentioning that the original work that led to the mass spectrometric identification of CHO PLBL2 in mAb preparations was triggered by nonlinear dilution effects observed in the original total CHO protein (CHOP) ELISA assay.¹ This general CHOP assay was able to measure PLBL2 accurately at low concentrations (10 ng/mL or ~10 ppm), but at higher concentrations (300 ng PLBL2 per mg mAb or 300 ppm) it failed to provide reliable measurements. By contrast, the LC-MRM assay presented here has the appropriate dynamic range for PLBL2 measurements. At least three orders of magnitude are demonstrated by the four calibration curves displayed in Figure 3.

Finally, the assay robustness was evaluated by 90 consecutive injections performed at a concentration level near the LOQ (0.025 nM PLBL2 digest or 100 attomoles protein digest loaded on-column). The trend plots shown in Figure 4 indicate that the peak area RSDs were also better than 15% for all four peptides.

CONCLUSIONS

- An LC-MRM assay able to detect PLBL2 (hamster phospholipase B-like 2 protein) down to ~160 ppb in mAb preparations was successfully developed.
- The reproducibility and robustness of the assay was better than 15% at all concentrations above the LOQ (0.00625 nM or 25 attomoles PLBL2 peptides loaded on-column).
- Linearity of the assay was maintained over three orders of magnitude.
- Compared with the traditional ELISA assays used for quantification of individual HCPs, the LC-MRM assays are able to achieve similar sub-ppm sensitivities while being more flexible, faster, and cheaper to develop.

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