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Practice of Protein Biomarker Assay Development and Validation From Drug Discovery to Development

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Introduction

Protein biomarkers have been widely used in clinical diagnostics, disease prognosis, drug discovery and development (e.g., assessment of drug efficacy and toxicity). The challenges in quantifying protein biomarkers are the complexity of protein molecules, sensitivity, specificity of detecting a specific targeted protein in biological fluids/tissues, and the stability etc. LC/MS/MS offers an alternative tool if ELISA assay becomes limited due to cross reactivity or lack of available antibodies.

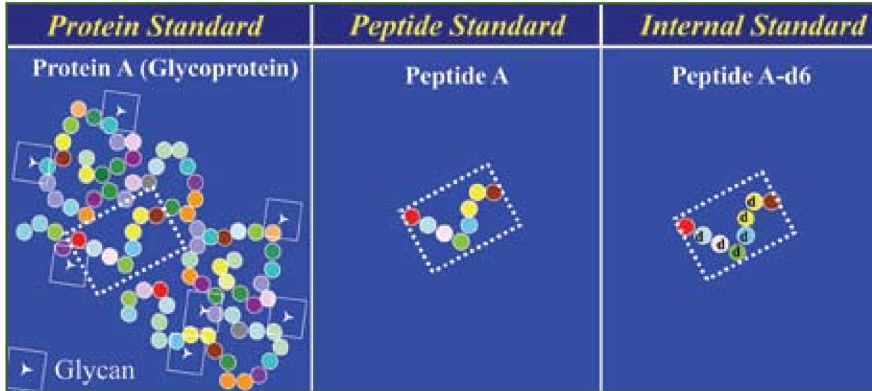
Protein standards and synthesized peptides (non-labeled and stable-labeled) are used for protein biomarker assay development and validation. To develop a robust LC/MS/MS assay for protein quantification, the choices of analytical references (standards and internal standards), the sources of digestion enzymes, the performance of calibration curves, the types of QC samples are required to be examined. The assay precision and accuracy, sensitivity, specificity, and stability are reported.

Materials and Chemicals

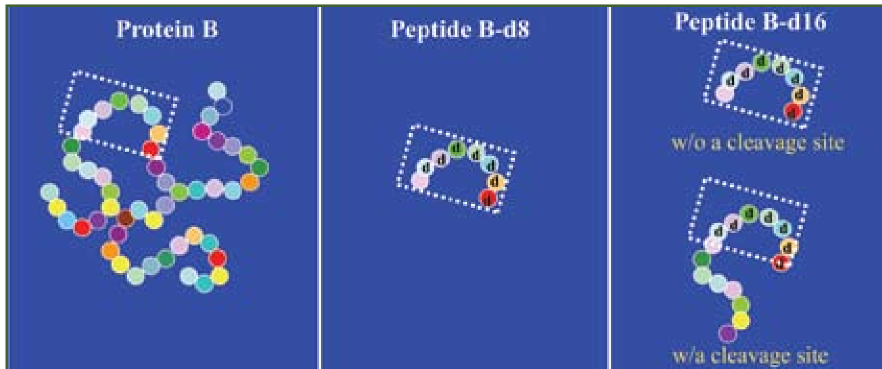
Chondrotinase ABC, Keratinase I & II, and CarboRelease kits were used to remove glycans from the glycoprotein. The reduction and alkylation reactions were performed using Dithiothreitol (DTT) and Iodoacetamide. For digestion, Chymotrypsin was used to cleave the proteins into peptide fragments. Two protein standards (Protein A and Protein B) were chosen for assay development and validation. Peptide A and Peptide B-d8 were synthesized to be used for the quantification of Protein A and Protein B. Peptide A-d6 and Peptides B-d16 (with and without a cleavage site) were chosen as internal standards. The quantifications of Protein A and Protein B were conducted in human synovial fluid and human serum, respectively.

Protein and Peptide Standards

Peptide A: a non-labeled epitope peptide cleaved from Protein A



Peptide B-d8: a stable-labeled epitope peptide cleaved from Protein B



Sample Preparation

PROTEIN A (GLYCOPROTEIN)

- A non-labeled synthetic Peptide A was used as a standard to make calibration curves in buffer (10 mM ammonium acetate, pH 7.5).
- QC samples were made by adding non-labeled Peptide A in human synovial fluid (200 ng/mL and 2000 ng/mL).
- For each sample, 10 µL of human synovial fluid were diluted with water (1:10).
- 100 µL of 1:10 diluted human synovial fluid and standards were processed.

PROTEIN A PROCESS

STDs/QCs/Study Samples

Deglycosylation

Denaturation (70°C)

Reduction (DTT)

Alkylation (Iodoacetamide)

Advanced Deglycosylation

Chymotrypsin Digestion

Protein Precipitation

PROTEIN B

- A stable-labeled synthetic Peptide B (Peptide B-d8) was used to prepare standard curves in human serum.
- QC samples were made by adding Peptide B-d8 in human serum (50, 500, and 1250 ng/mL).
- For each sample, 10 µL of human serum were diluted with water (1:10) two times.
- 100 µL of 1:100 diluted human serum were processed.

PROTEIN B PROCESS

STDs/QCs/Study Samples

Denaturation (100°C)

Reduction (DTT)

Alkylation (Iodoacetamide)

Chymotrypsin Digestion

Protein Precipitation

Methods

LC-TOF MS AND LC-TOF MS/MS

The identification of specific peptide fragments after the enzymatic digestion was achieved using LC-TOF MS and LC-TOF MS/MS.

HPLC SEPARATION

- Column – Phenomenex Syngergi Hydro RP (150 x 2.0 mm)
- Column Heater: ambient temperature
- Mobile Phase A: 95/5, H2O/ACN (0.1% formic acid)
- Mobile Phase B: 50/50 MeOH/ACN (0.1% formic acid)
- Injection Volume: 20 µL
- Gradient

Time (min)	%B	Flow Rate (mL/min)
0.1	0	0.15
2.0	0	0.15
62.0	50	0.15
80.0	90	0.15
82.0	90	0.15
82.5	0	0.15
90.0	0	0.15

MASS SPECTROMETRY

- QSTAR XL (Applied Biosystem/Sciex)
- TOF Q1 Full Scan: *m/z* 500 – 8000
- Product Ion Scans
- Declustering Potential: 60 V

LC/MS/MS

HPLC SEPARATION

- Column – VARIAN, Metasil C18 (50 x 2.0 mm)
- Column Heater: 40°C
- Mobile Phase A: 95/5, H_2O/ACN (0.1% formic acid)
- Mobile Phase B: 50/50 MeOH/ ACN (0.1% formic acid)
- Injection Volume: 10 µL
- Gradient

Case 1 Study

Time (min)	%B	Flow Rate (mL/min)
0.00	0	0.5
3.00	5	0.5
6.00	90	0.5
8.00	90	0.5
8.10	0	0.5
10.00	0	0.5

Case 2 Study

Time (min)	%B	Flow Rate (mL/min)
0.00	0	0.6
2.00	25	0.6
2.75	90	0.6
3.50	90	0.6
3.60	0	0.6
4.50	0	0.6

Methods continued

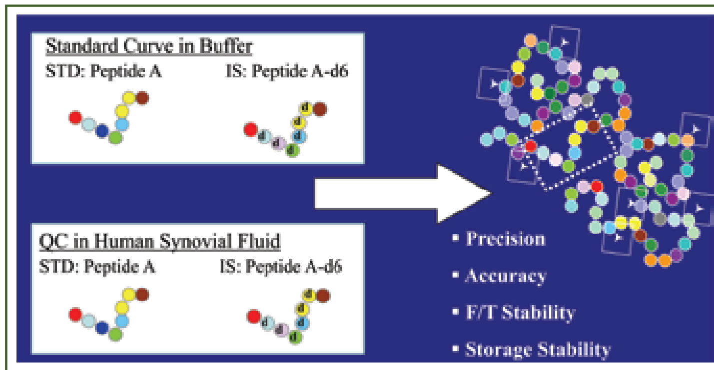
MASS SPECTROMETRY

- API 5000 (Applied Biosystem/Sciex)
- MRM: 40 to 50 multiple transitions
- Product Ion Scans: singly, doubly, and/or triply charged peptide ions
- Declustering Potential: 60 V
- Collision Energy: 10 – 70 eV

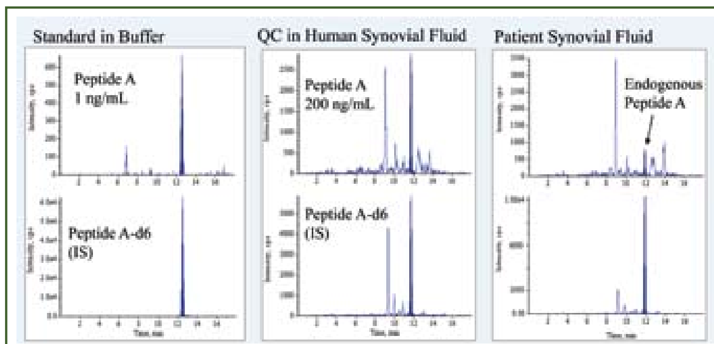
Results and Discussion

Case 1 Study

Measurement of Protein A (Glycoprotein) in Human Synovial Fluid



Representative Mass Chromatograms of Peptide A

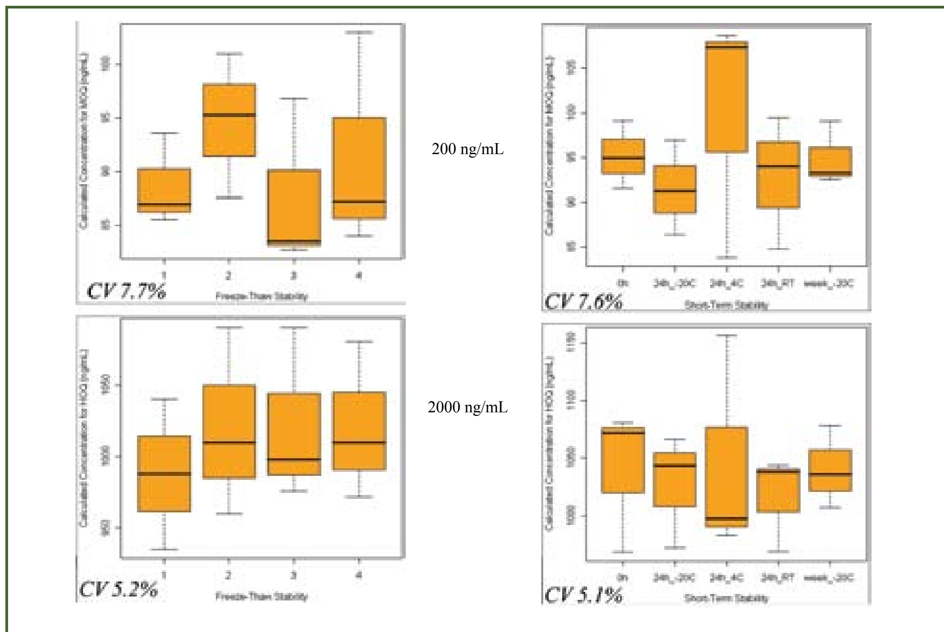


Results and Discussion continued

A Summary of Intra- and Inter-Assay Precision and Accuracy

	Expected Concentration (ng/mL)	Number Of Values Used	Mean	Standard Deviation	%CV	Accuracy
Run 1	200	3 of 3	88.65	4.33	4.89	44.32
	2000	3 of 3	985.96	50.68	5.14	49.30
Run 2	200	3 of 3	94.60	6.63	7.01	47.30
	2000	3 of 3	1019.50	67.14	6.59	50.98
Run 3	200	3 of 3	87.65	7.92	9.03	43.83
	2000	3 of 3	1022.15	62.02	6.07	51.11
Run 4	200	3 of 3	91.29	10.01	10.96	45.65
	2000	3 of 3	1022.48	55.79	5.46	51.12

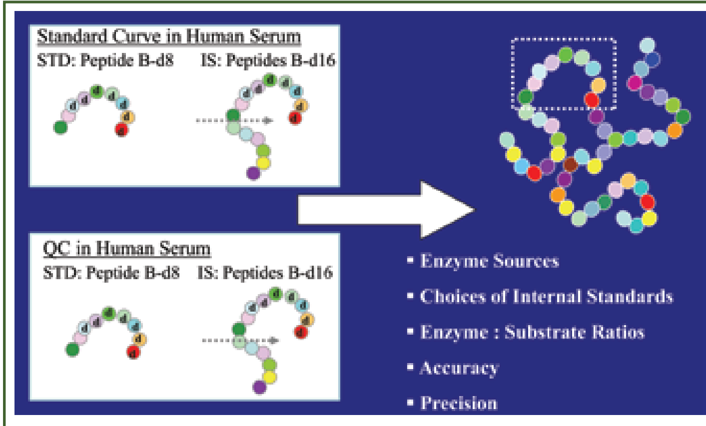
Freeze/Thaw Stability *Short-Term Storage Stability*



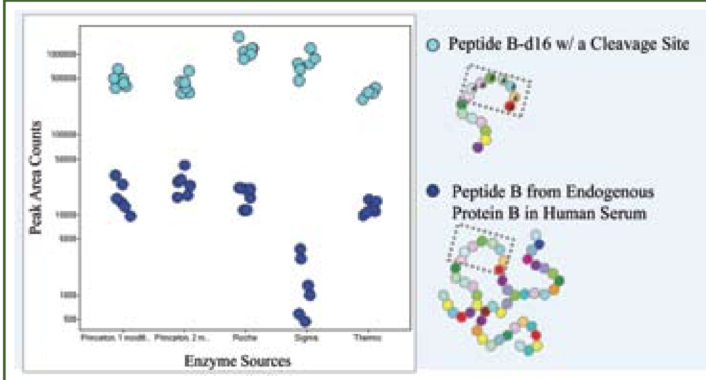
Results and Discussion continued

Case 2 Study

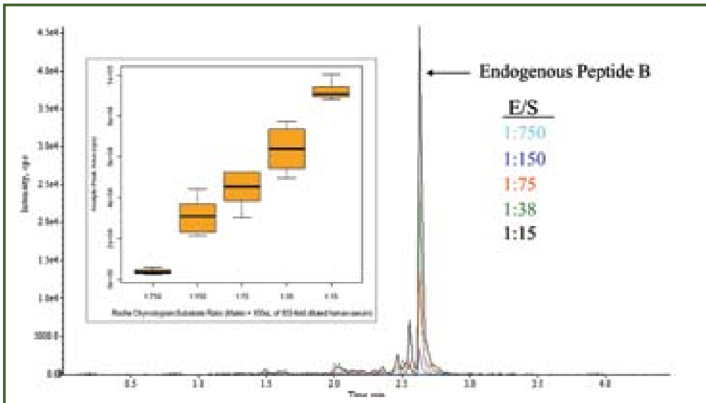
Measurement of Protein B in Human Serum



Evaluation of Enzyme Sources



Effect of Enzyme:Substrate Ratios on Protein B Digestion



Results and Discussion continued

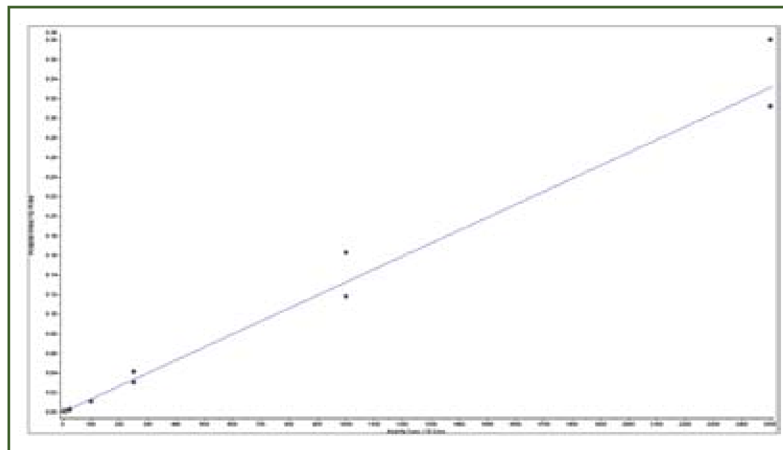
CHOICES OF INTERNAL STANDARDS

Internal Standard w/o a Cleavage Site

Expected Conc. (ng/mL)	Mean (ng/mL)	STDEV	%CV	Accuracy (%)	Data Point #1	Data Point #2
10	10.6	NA	NA	106	10.6	NR
25	23.0	NA	NA	92	NR	23.0
100	82.6	0.5	0.6	83	82.3	83.0
250	269.8	58.7	21.7	108	311.2	228.3
1000	1059.5	237.4	22.4	106	891.6	1227.4
2500	2613.4	361.1	13.8	105	2358.1	2868.8

NR: data dropped, not reported NA: not applicable

A Standard Calibration Curve Using an Internal Standard w/o a Cleavage Site



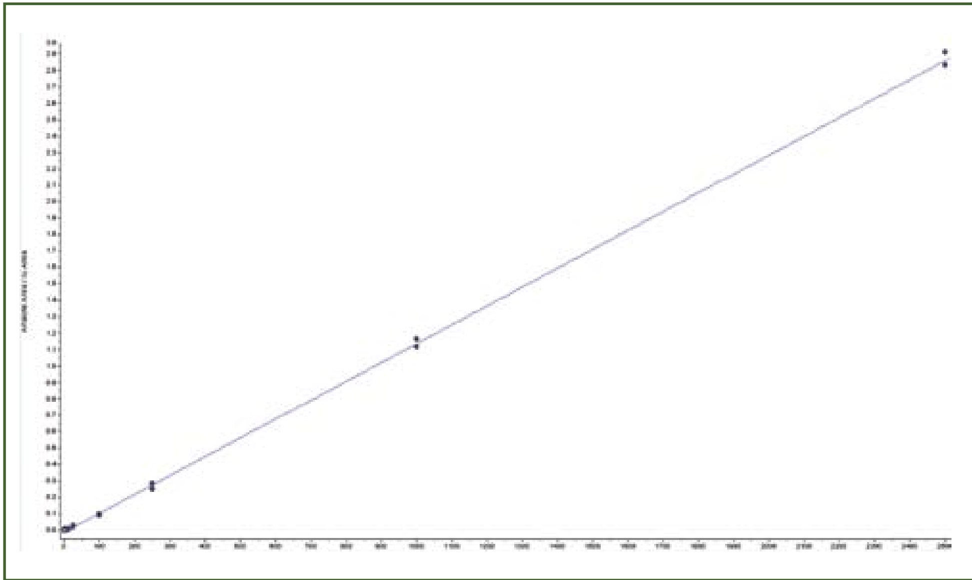
Internal Standard w/a Cleavage Site

Expected Conc. (ng/mL)	Mean (ng/mL)	STDEV	%CV	Accuracy (%)	Data Point #1	Data Point #2
10	11.2	0.3	2.8	112	11.5	11.0
25	25.2	4.1	16.3	101	28.1	22.3
100	84.6	4.3	5.0	85	87.6	81.6
250	267.2	NA	NA	107	267.2	NR
1000	938.0	33.0	3.5	94	914.7	961.4
2500	2567.3	381.5	14.9	103	2837.1	2297.6

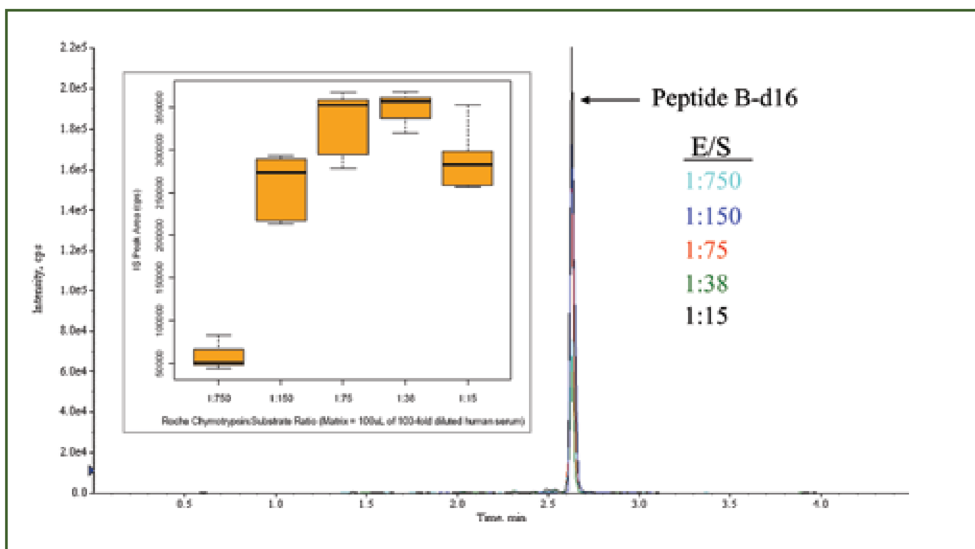
NR: data dropped, not reported NA: not applicable

Results and Discussion continued

A Standard Calibration Curve Using an Internal Standard w/a Cleavage Site

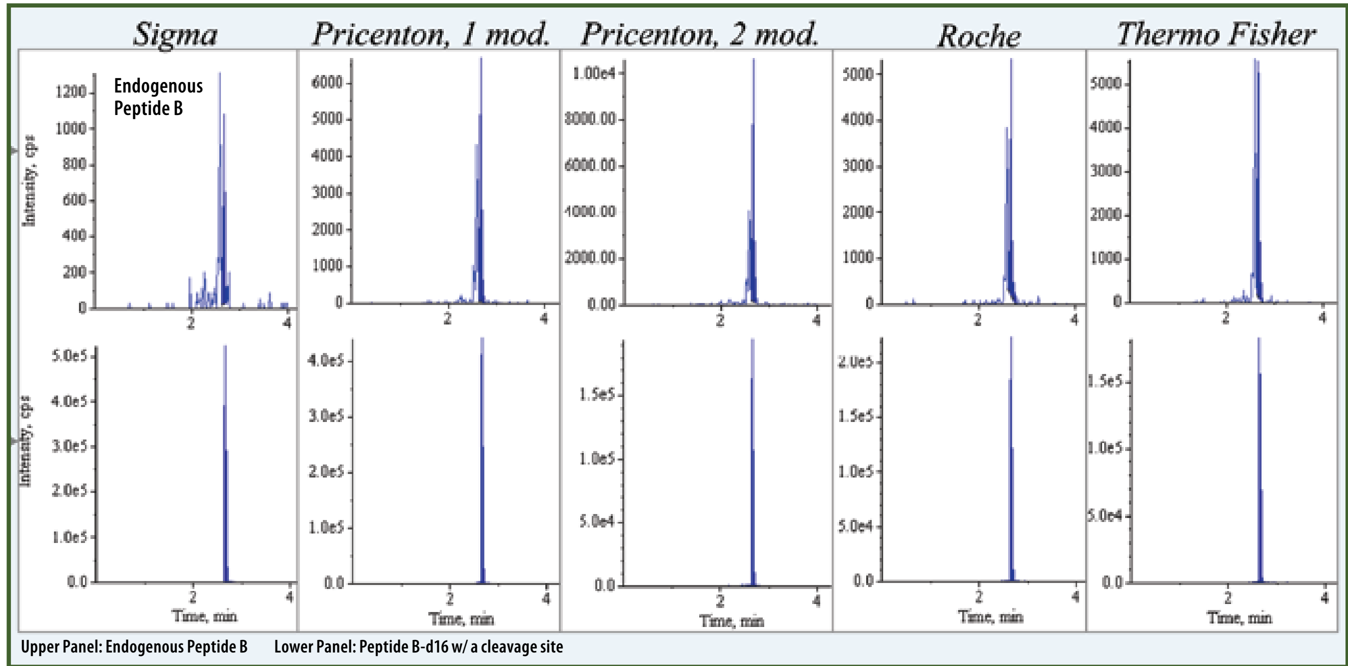


Effect of Enzyme:Substrate Ratios on Peptide B-d16 Digestion



Results and Discussion continued

Representative Mass Chromatograms of Peptide B and Peptide B-d16



Upper Panel: Endogenous Peptide B   Lower Panel: Peptide B-d16 w/ a cleavage site

ASSAY ACCURACY AND PRECISION

A Summary of QC Samples in Human Serum

Expected Conc. (ng/mL)	Mean (ng/mL)	STDEV	%CV	Accuracy (%)	Data Point #1	Data Point #2	Data Point #3
50	52.1	6.5	12.6	104	47.5	56.8	NR
500	492.7	51.5	10.4	99	538.7	502.2	437.0
1250	1134.1	66.7	5.9	91	NR	1086.9	1181.3

Results and Discussion

ASSAY ACCURACY AND PRECISION

The cleaved epitope peptide from a target protein served as a protein surrogate. To measure the endogenous epitope peptide, the standards were examined for assay performance. As seen in Case 1 and 2 studies, there was no impact on assay precision with non-labeled or stable-labeled peptide as a standard. Assay accuracy of ~50% was observed using non-labeled peptide standard curves in a surrogate matrix (e.g., buffer). The 50% error was due to the differences in matrix suppression, digestion efficiency, and recovery between surrogate and biological matrices.

SELECTION OF INTERNAL STANDARDS

Peptides B-d16 w/ and w/o a cleavage site were used as internal standards to monitor the processes of digestion, extraction, and sample analysis. There was no significant difference in assay performance in regards to the presence of a cleavage site on the internal standard.

DIGESTION PERFORMANCE

To validate digestion performance, the sources of an enzyme were tested. The protein digestion varied depending on the manufacturing of this enzyme. Compared to protein digestion, peptide digestion was less affected by the sources of the enzyme. The protein digestion could be improved using more enzymes by increasing the enzyme:substrate (E/S) ratios.

STABILITY

It was essential to establish stability of a targeted protein and the epitope peptide for assay validation during the freeze/thaw and storage processes. Once a robust assay was developed, the stability results could be obtained though the assay accuracy was off as demonstrated in Case 1 study.

Conclusions

A non-labeled peptide could be used as a standard if using a surrogate matrix to prepare standard calibration curves for protein quantification. Once the assay demonstrated to be reproducible, it would be valid to utilize it as a tool to support discovery stage of work, though the assay accuracy might be off.

To accurately measure the protein quantity in biological matrix, the performances of enzyme (sources and E/S ratios) and the assay (linearity, lower limit of quantitation, accuracy and precision) had to be validated. Additionally, the stability of the endogenous protein and the epitope peptide (as a standard) should be included for the assay validation.