Quantitation of a target protein in crude samples using targeted peptide quantification by Mass Spectrometry

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Abstract

Background: A target protein is used foremost as additives in feed by our client. The target protein product is not purified since the purification leads the loss of activity. The quantitation of target in a final crude product is a challenging task.

Methods: The concentration of target in crude samples is measured via targeted quantitation of tryptic peptide comparing with an isotope labeled spike peptide reference. The assay is carried out by using a Dionex Ultimate 3000 RSLCnano system coupled with a thermo Q-Exactive mass spectrometer.

Results: Proteomic mapping of pure and crude target samples identified 205**AXIXQXXXK**213 as the optimal targeted peptide for quantitation. Linear dynamic range of detection and quantitation for this peptide were 10 fmol to 1 pmol (R^2=0.9997) for synthesized isotope labeled peptide, and 50 fmol to 5 pmol ((R^2=0.9906) for target samples. The CV of measurement of target from different of runs of the same samples is less than 1% on a high performance nano-LC system coupled with a quadrupole-Orbitrap mass spectrometer (Thermo Q-Exactive). The CV derived from sample's preparation including protein denature, reduction, alkylation, trypsin digestion and C18-Zip-tip cleanup tryptic peptides, and LC-MS analysis is an average of 10%. The concentration and purity of target (60% to 70%) in crude samples which are determined by the targeted peptide quantitation measurement and BCA total protein assay is consistent with the activity assay.

Introduction

Targets are used foremost as additives in feed. The quantitation of target in a final crude product is a challenging task.

Mass spectrometry (MS)-based "shotgun" proteomics has emerged as a powerful and universal method for the global measurement of proteins. It can identify and quantify protein mixtures at the same time on a large scale. In short, after enzymatic digestion of the proteins, the generated peptides are analyzed by means of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in a data dependent mode. The peptide mixture is loaded directly onto a reversed-phase C18 column and the peptides are separated by hydrophobicity and charge. As the peptides elute from the column, they are ionized and separated by m/z in the first stage of tandem mass spectrometry (MS1). The selected ions undergo collision-induced dissociation or other process to induce fragmentation. The charged fragments are separated and measured in the second stage of tandem mass spectrometry (MS2). The "fingerprint" of each peptide's fragmentation mass spectrum is used to identify the protein from which they derive by searching against a sequence database with commercially available software such as SEQUEST. Therefore, this whole analysis is also

called LC-MS/MS. Based on the intensity and peak area of peptide ion chromatograph detected in MS1 the relative abundance of each identified peptide ion can be quantified. However, the complexity of the digested protein mixtures under investigation and the wide range of protein abundances limit the reproducibility and the sensitivity of this stochastic approach, which is critical if one aims at the systematic quantification of the proteins. Thus, alternative MS approaches have emerged for the systematic quantitative study of complex samples, the MS-based targeted proteomics. In this approach, only specific subsets of analytes (a few targeted peptides used as surrogates for the proteins of interest) are selectively measured in predefined m/z ranges and retention time windows, which overcomes the bias toward most abundant compounds commonly observed with shotgun proteomics. The measurements provided by targeted peptide quantitation are highly specific since several performance characteristics of the analyte including its mass, the masses of several of its fragment ions, chromatographic retention time, as well as how well these characteristics match to a heavy isotope labeled internal standard of the analyte, are measured. Addition of the heavy internal standard also allows for absolute abundance of the target analyte to be measured.

In this study we present a targeted quantitation of peptide for measuring the concentration of target in crude samples. We tested the reproducibility across sample replicates as well as between different operators. We then applied the developed assay to measure target levels in three lots of crude target product, and the results were correlated with activity assay.

Materials and Methods

Samples and Reagents

Samples listed below from Elanco Animal Health are stored at 4°C.

1. Purified D-target (lot#07102014, 120 mM NaCl, 0.1 mL)
2. Crude-1 sample (Native target in Sorbitol)
3. HC001 (in buffer containing Proxel at 1 mg/mL)
4. HC003 (in buffer containing Proxel at 1 mg/mL)
5. HC005 (in buffer containing Proxel at 1 mg/mL)

Urea (CAS 57-13-6, Lot 130397) was purchased from Fisher Scientific; Pierce Trypsin Protease, MS grade (Pro# 90057) was purchased from Pierce; DTT (Pro#1000748546, lot 109k01691) and Iodoacetamide (Pro#122270250 lot A0320373) were purchased from sigma; LC-MS/MS grade water, Methanol, and Acetonitrile were purchased from sigma.

Preparation of Protein trypsin digestion

The total protein content in pure target or crude target samples is determined by Pierce BCA Protein Assay Kit (Lot#PB195642, Prod# 23250). 2 to 5 µg of total protein from each sample were treated by 8 M urea for denature followed by DTT for reduction, then iodoacetamide for alkylation, and further diluted by 25 mM NH_4HCO_3 to 0.6 M urea before adding trypsin for digestion. The tryptic peptides mixture was cleaned using C18 Zip-Tip and reconstituted in 0.1% formic acid before nanospray LC/MS/MS analysis. For targeted quantitation of peptide the known amount spike isotope labeled peptide is added into the tryptic peptide mixture before the zip-tip cleanup step or before trypsin digestion.

Nanospray LC/MS/MS analysis and database search

The LC/MS/MS analysis was carried out using a Thermo Scientific Q-Exactive hybrid Quadrupole-Orbitrap Mass Spectrometer and a Thermo Dionex UltiMate 3000 RSLCnano System. Peptides from

trypsin digestion were loaded onto a peptide trap cartridge at a flow rate of 5 µL/min. The trapped peptides were eluted onto a reversed-phase 25 cm C18 PicoFrit column (New Objective, Woburn, MA) using a linear gradient of acetonitrile (3-36%) in 0.1% formic acid. The elution duration was 45 min or 60 min at a flow rate of 0.3 µL/min. Eluted peptides from the PicoFrit column were ionized and sprayed into the mass spectrometer, using a Nanospray Flex Ion Source ES071 (Thermo) under the following settings: spray voltage, 1.6 kV, Capillary temperature, 250°C.

The Q Exactive instrument was operated in the data dependent mode to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (m/z 300−2000) were acquired in the Orbitrap with 70,000 resolution (m/z 200) after accumulation of ions to a 1×10^6 target value based on predictive AGC from the previous full scan. Dynamic exclusion was set to 10 s. The 12 most intense multiply charged ions ($z \geq 2$) were sequentially isolated and fragmented in the octopole collision cell by higher-energy collisional dissociation (HCD) using normalized HCD collision energy 25% with an AGC target 1e5 and a maxima injection time of 100 ms at 17,500 resolution.

Raw data files were searched against the custom target sequence or 161-protein sequence database using the Proteome Discoverer 1.4 software (Thermo, San Jose, CA) based on the SEQUEST and percolator algorithms. The false positive discovery rates (FDR) is set on 1%.

Targeted peptide quantitation assay development

For development of the targeted peptide quantitation assay, pure target and crude target protein samples was digested with trypsin and the resultant peptides are analyzed as described above and illustrated in figure 1. Software programs Proteome Discoverer 1.4 and Xcalibur 2.1 (Thermo Scientific, San Jose, CA) were used to identify the optimal tryptic peptides for targeted quantitation analysis based on reproducible peak heights, retention times, chromatographic ion intensities, and distinctive/reproducible transition ion ratios. The peptide **AXIXQXXXK**, comprising residues 205–213 within the target protein's surface domain, was found to be unique to target by comparing this sequence to the entire proteins database provided by the client using the BLASTP function within the BLAST search engine (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Heavy **(AXIXQXXXK [^{13}C6, ^{15}N2])** versions of this peptide were synthesized to develop and perform the assay (4.9 pmol/µl in 5 % of Acetonitrile (Thermo Scientific, San Jose, CA). After calibration of the linear dynamic range of targeted peptide in pure and crude samples, and calibration of the linear dynamic range of isotope labeled heavy peptide the suitable amount of tryptic digestion of target samples and spike peptide for LC-MS/MS analysis are determined. The target samples (2.5 µg or 5 µg of total protein based on BCA assay) are processed for trypsin digestion. After overnight digestion a portion of the digestion is mixed with certain amount of isotope labeled peptide (spike peptide) for C18 zip-tip cleanup. Alternatively the spike peptide is added into the processed sample solution before trypsin digestion. After cleanup the peptides mixture with spike peptide is analyzed by LC-MS/MS.

Calibration of linear dynamic range of targeted peptide

A standard curve for the linear dynamic range of targeted peptide was developed by analysis of a serial dilution of tryptic peptide mixture of pure target sample or crude target samples. The peak area of targeted peptide AXIXQXXXK was measured for each dilution by proteome discoverer and Xcalibur software.

A standard curve for the linear dynamic range of isotope labeled heavy peptide was developed by analysis of a serial dilution of the peptide by LC/MS/MS. The peak area of heavy peptide **AXIXQXXXK [^{13}C6, ^{15}N2]** is measured for each dilution by Proteome discoverer and/or Xcalibur software.

Calculation of target concentration and statistical analysis

Since both of spike peptide and targeted light peptide from samples are co-eluted at the same time the ratio of the two peptide ion peak area based on MS1 can be determined. The MS1 peak area of spike peptide and the MS1 peak area light peptide from samples were quantified by Proteome discoverer and/or Xcalibur software. The concentration of target in crude samples is calculated based on the ratio of targeted peptide to spike peptide and the concentration of pure target sample as the reference. The calculation formula:

$$\text{Target (mg/mL)} = \text{DA} \times \text{PM} \times \text{S-ratio } \left(\frac{LightPA}{HeavyPA}\right) / \text{P-ratio } \left(\frac{LightPA}{HeavyPA}\right)$$

DA: dilution factor;
PM: target concentration of pure target reference (mg/mL);
S-ratio: ratio of light peptide versus heavy peptide (spike peptide) from analysis of sample;
P-ratio: ratio of light peptide versus heavy peptide (spike peptide) from analysis of reference pure target.
The MEAN, STDEV, CV and R^2value are calculated using Microsoft Excel.

Evaluation of Assay Precision

To demonstrate the precision of the assay, the same set of five samples from Elanco were analyzed independently by two different operators on different days.

Results

Targeted quantitation assay development

For development of the Targeted quantitation of target assay, the tryptic digests of pure target and crude target samples were analyzed using LC-MS/MS. As shown in **Figure 2** a dominant peak at 36.6 min (retention time) representing the peptide ion 205AXIXQXXXK213 was identified using a 60 min LC/MS/MS run time (or at 27 min when using a 45 min LC/MS/MS run time). This peptide provided the most reproducible peak height, retention time, chromatographic ion intensity, clean elution profile. Therefore, this peptide was selected for assay development.

To determine the assay's linear dynamic range of targeted peptide a standard curves was generated by serial dilution of tryptic digestion of pure or crude target samples (does not shown). As shown in **Figure 3A** the linear dynamic range is between 0.1 pmol and 5 pmol. When the amount of peptides mixture analyzed reaches to 15 pmol the detection of the peak area is not in the dynamic range (Figure 3B).

To calibrate the linear dynamic range of isotope labeled peptide a standard curves was generated by serial dilution of the synthesized heavy peptide AXIXQXXXK [13C6, 15N2]. As shown in **Figure 3C** the range is between 1 fmol and 250 fmol. The coefficient of variation (CV) derived

from LC/MS system also was determined by analysis of triplicate of the same sample (**Figure 3D&E)**. 98 fmol of heavy peptide was analyzed in triplicates. The results demonstrated that the CV is less than 2%.

Measurement of target content in crude sample by targeted quantitation assay

Next, to measure the target concentration in pure or crude target samples a known amount of a heavy peptide is added to the tryptic peptide mixtures. Analysis of the peptides mixture by LC-MS/MS allows to quantify the amount of analyte in the sample by comparing the ratio of peak area of targeted peptide from sample versus spike heavy peptide. Two analyses performed by two operators and the results are summarized in **Tables 1, 2 and 3.** The assay results demonstrated that the purity of the crude samples from three different lots is between 60% and 75% (**Table 4**), which is consistent with activity assay result (Elanco data, not shown here). This result suggests that the targeted peptide quantitation for measuring target in crude samples is a reliable and reproducible method.

The CV between different assays is about 10%, which is much larger than the CV derived from different LC/MS/MS runs. Although this CV value is comparable with other analytical methods it is still possible to reduce it by optimizing the assay method. The higher CV is probably due to the efficiency of sample processing during the steps of denature, trypsin digestion and C18 Zip-tip purification, especially the denature step. Since 8 M urea used for denaturing/unfolding protein was reduced to 0.6 M by dilution with 25 mM NH_4HCO_3 before addition of trypsin for digestion, which may cause the recovery of denatured target and decrease the digestion efficiency. It is known that the target is resistant to 1% SDS. It may be impossible to make complete denaturing/unfolding of target using either 8M Urea or/and 1% SDS. One way to resolve this problem is to develop isotope labeled target, which can be used as the internal control reference for quantitation and digestion efficiency control to replace synthesized isotope labeled spike peptide.

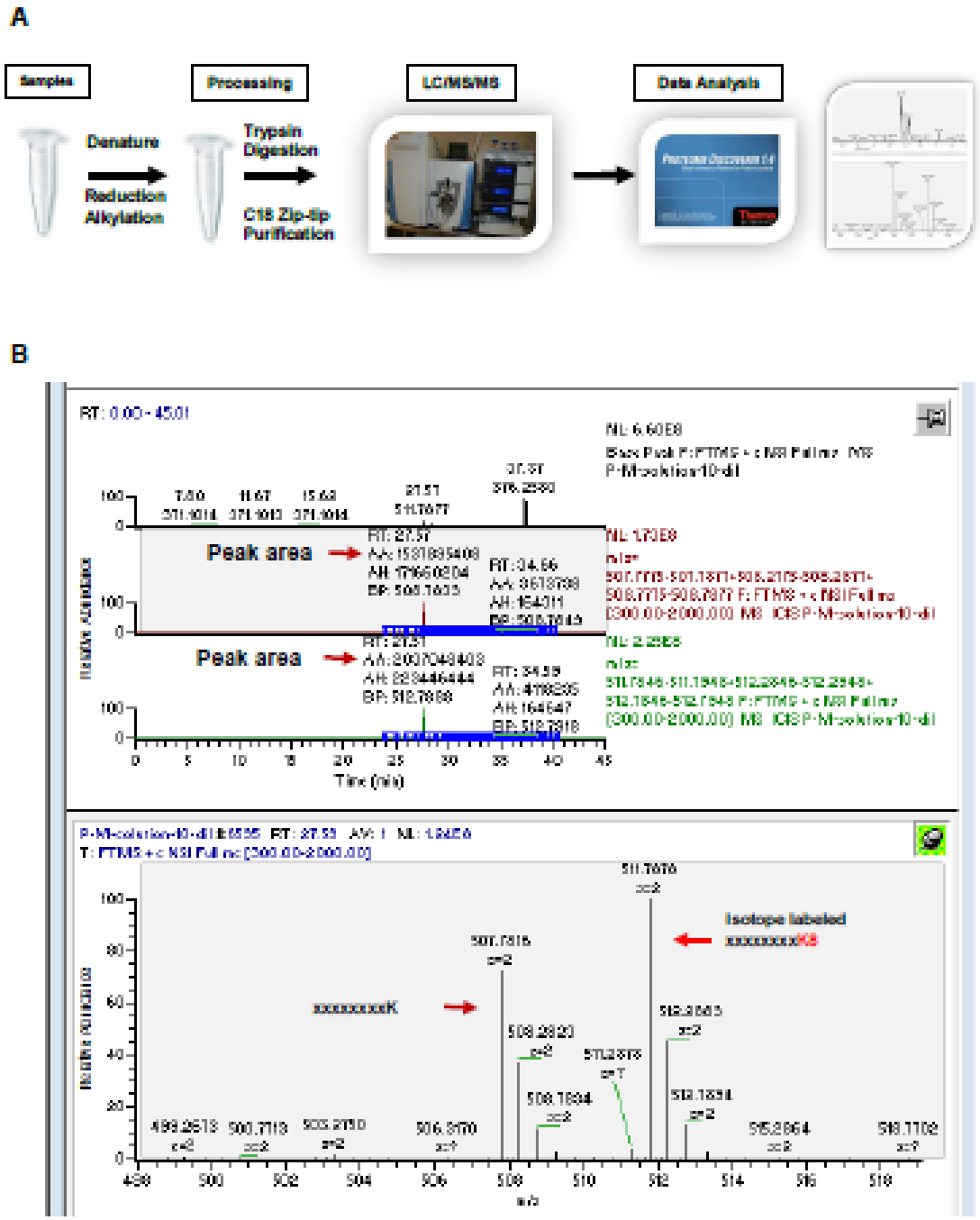


Figure 1: **Targeted quantitation of peptide workflow for quantification of target in crude samples.** Crude samples are processed for trypsin digestion to produce a tryptic peptide mixture. A known amount of heavy isotope labeled internal standard peptide is added to the mixture and the sample is analyzed using targeted peptide to measure the abundance of the endogenous peptide of interest.

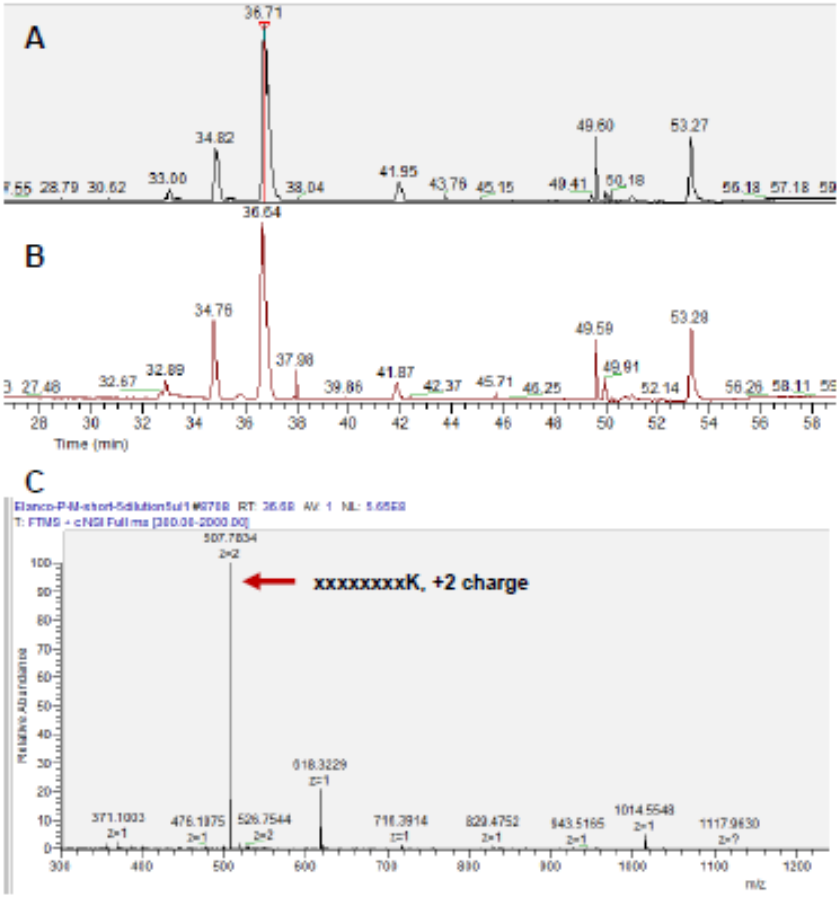
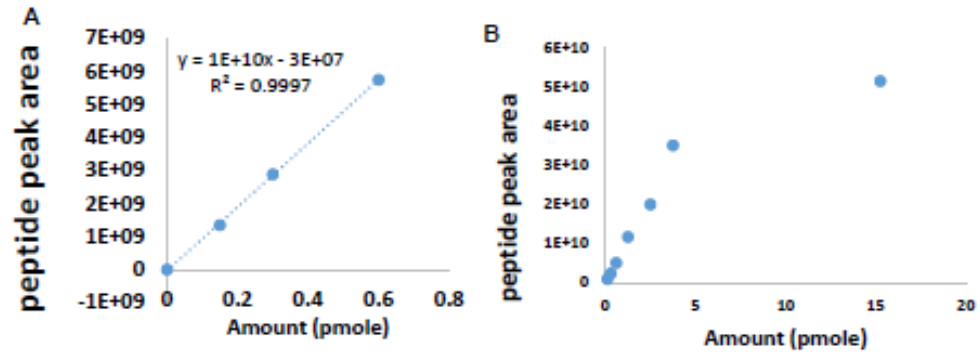
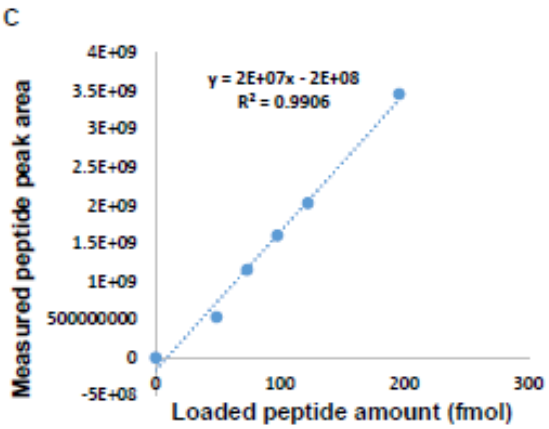


Figure 2: **Peptides mapping and identification of peptide for targeted quantitation.** The total peptides ion chromatograms for the pure target sample (A), for the crude target sample (B); (C) The ion chromatogram showing the m/z measurement for selected peptide AXIXQXXXK at 36.7 min (the retention time).





D

Sequence	xxxxxxxxK8
Amount	98 fmol
Modifications	C-Term(K8)
MH+ [Da]	1022.571031
run-1: Peak Area	2627468712
run-2: Peak Area	2606291598
run-3: Peak Area	2536781045
Average	2590180452
STD	47441964.78
CV	1.83%

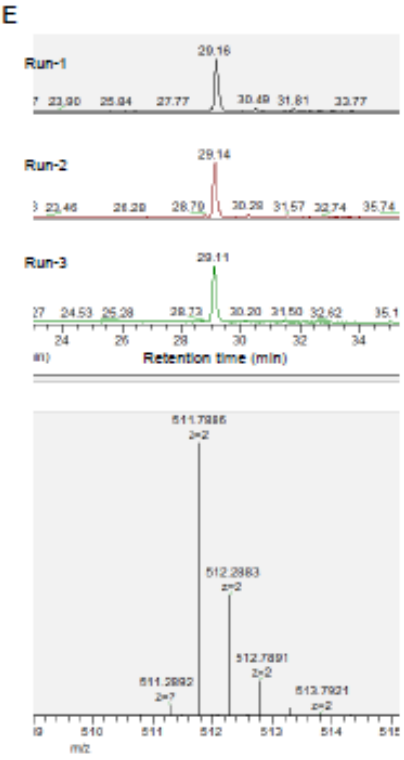


Figure 3: **Determination of linear dynamic range of targeted peptide for quantitation method development.** (A) and (B), the standard curve generated by analysis of tryptic peptides from pure target sample; (C), the standard curve generated by analysis of synthesized heavy peptide AXIXQXXXK [13C6, 15N2]; (D) and (E), Determination of CV based on triplicates analysis of same sample (98 fmol of the heavy peptide).

Table 1: **Results of Assay one performed by Operator A**

Sample	Measured peak area		Ratio (light/heavy)	Dilution factor	Result (mg/mL)
	xxxxxxxxK8 (heavy)	xxxxxxxK (light)			
Pure	2219217905	1681743033	0.76	160	8.07
Crude-1	2778705410	1661195485	0.60	54	2.15
HC001	2786864342	4509182323	1.62	190	20.46
HC003	3166775591	4416263593	1.39	190	17.64
HC005	4878006937	7327632936	1.50	190	19.00

Table 2: **Results of Assay two performed by Operator B**

Sample	Measured peak area		Ratio (light/heavy)	Dilution factor	Result (mg/mL)
	xxxxxxxxK8 (heavy)	xxxxxxK (light)			
Pure	1772605338	2145663773	1.21	2000	8.07
Crude-1	1165484282	2001714768	1.72	400	2.29
HC001	2032344525	7131200718	3.51	2000	23.39
HC003	2120253869	7167190447	3.38	2000	22.54
HC005	2060966804	6965581947	3.38	2000	22.53

Table 3: **Final results based on two assays**

Sample	Pure	Crude-1	HC001	HC003	HC005
Target (mg/mL)	8.07	2.22 ± 0.1	21.93 ± 2.07	20.09 ± 3.47	20.76 ± 2.50

Table 4: **Determination of purity of crude sample based on BCA assay and LC/MS quantitation**

Sample	BCA (mg/mL)	LC/MS/MS (mg/mL)	Purity
Pure	8.07	N/D	N/D
Crude-1	2.71	2.22	81.90%
HC001	29.39	21.93	74.61%
HC003	32.14	20.09	62.50%
HC005	29.99	20.76	69.24%

References

1. User Guide: Pierce BCA Protein Assay Kit - Reducing Agent Compatible (https://tools.thermofisher.com/content/sfs/manuals/MAN0011538_Pierce_BCAProtein_Asy_ReducAgntComp_UG.pdf)
2. Hellman U, Wernstedt C, Gonez J, Heldin CH. Improvement of an “In-Gel” digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. Anal Biochem 1995. 224(1):451–5. [PubMed: 7710111]
3. Biopharmaceutical Characterization Application Compendium (http://www.thermoscientific.de/content/dam/tfs/ATG/CMD/cmd-documents/sci-res/app/ms/lc-ms/Orbitraps/AN-biopharmaceutical-characterization-application-compendium-AI64242-EN.pdf)