

Enhanced Detection of Host-Cell Proteins in Biotherapeutic Preparations using Preparative Electrophoresis Followed by LC –Ion Mobility -MS

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OVERVIEW

- Investigate the use of an off-line preparative electrophoresis device to fractionate Host-cell proteins (HCPs) away from the biotherapeutic protein prior to LC-MS analysis.
- Fractionate both the intact and reduced monoclonal antibody to determine which separation enables enhanced detection of low- level HCPs.
- The SageELF separation device results in reproducible protein level fractionation across duplicate experiments.
- Sub-ppm level HCP detection was achieved, i.e., 58 HCPs were detected ≤ 1 ppm with 3 unique peptides/protein.

INTRODUCTION

- Host-cell proteins (HCPs) are proteins produced by the host cells during recombinant protein drug production. These HCPs contaminate the biotherapeutic drug protein at low levels (1-100 ppm) even after several steps of extensive purification [1].
- Since even low level HCPs can stimulate a immunogenic response, regulatory guidelines mandate that these HCPs must be identified and quantified to protect patient safety [2].
- Current HCP mass spectrometry (MS) characterization involves some level of pre-fractionation (i.e., electrophoresis, reversed-phase (RP) separation, 2D RP/RP LC, etc.) prior to analysis [1].
- Objective: investigate the use of an off-line preparative electrophoresis device (Figure 1) to fractionate the HCPs away from the biotherapeutic protein prior to LC-MS analysis.
- Goal: mitigate the matrix effects of the high concentration biotherapeutic protein to enable the enhanced detection and quantification of sub-ppm level HCPs.

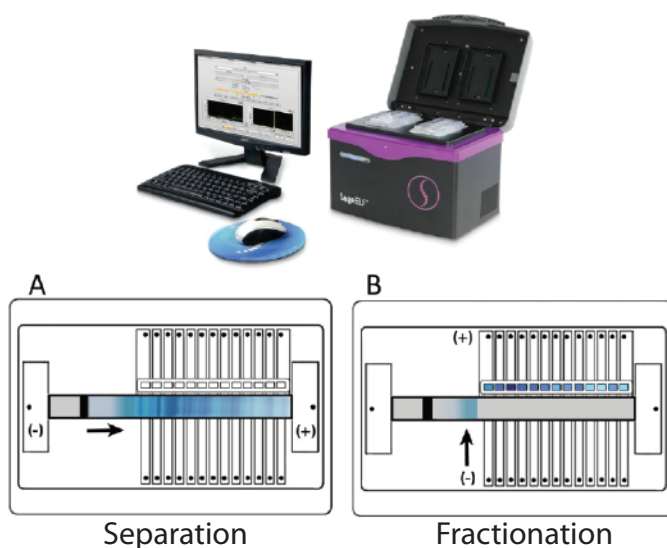


Figure 1. Image of the SageELF off-line preparative electrophoresis device. (A) SDS agarose separation cassette during protein level separation and (B) orthogonal separation into discrete in-solution protein fractions.

METHODS

Sample: SILu Lite Sigma mAb Universal Antibody Standard human (Sigma Aldrich, MSQC4) – expressed in CHO cells ($\geq 90\%$ pure)

SAGE ELF Conditions:

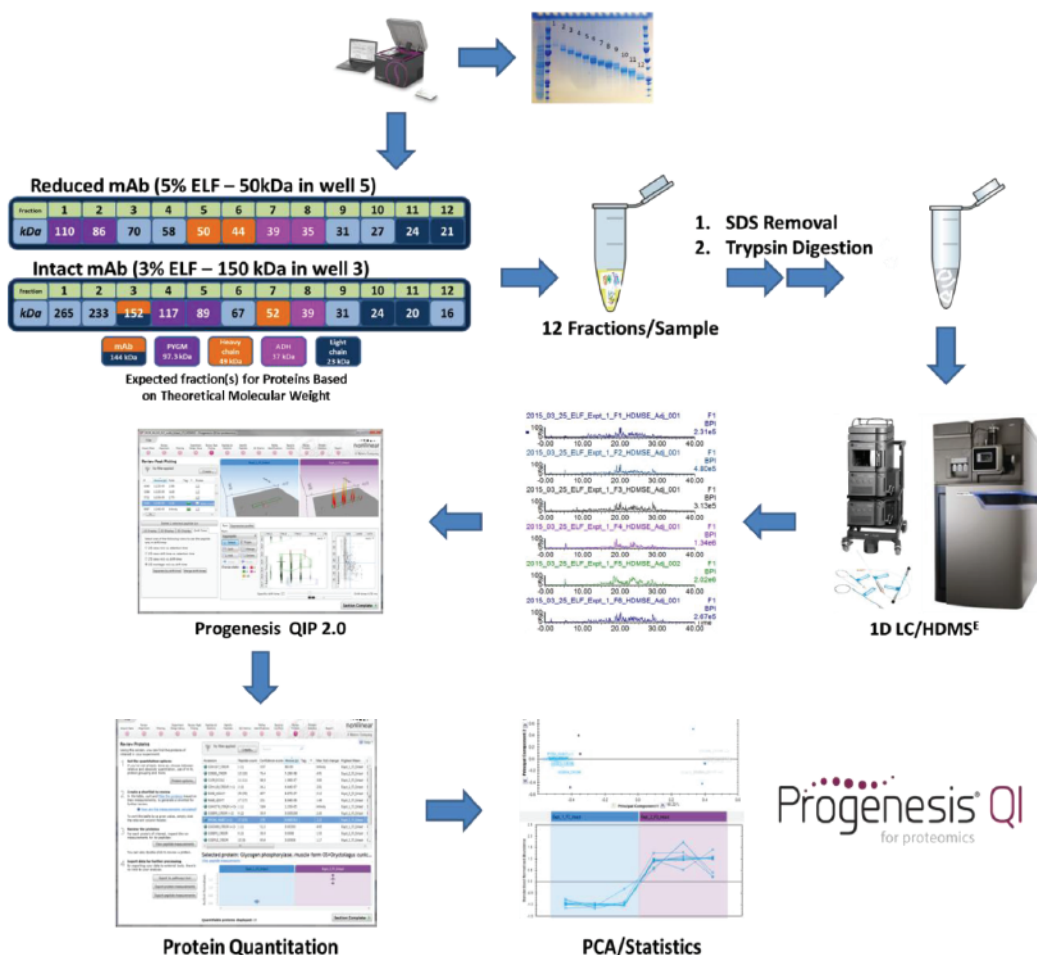
- Intact mAb: 3% Agarose separation gel (See Scheme 1)
- Reduced mAb: 5% Agarose separation gel
- Experiment 1: 100 μg mAb + 1000 ppm Phosphorylase B (PYGM_RABIT) + 100 ppm Yeast Alcohol Dehydrogenase (ADH1_YEAST)
- Experiment 2: 100 μg mAb + 100 ppm PYGM_RABIT + 10 ppm (ADH1_YEAST)

LC Conditions:

- Column: 1.8 μm HSS T3 C18 75 μm x 150 mm w/ Symmetry C18 trap column
- Gradient: 1.5% B/Column Volume (5-35% B)

MS Conditions:

- Mass spectrometer: SYNAPT G2-Si HDMS (Waters Corporation)
- Data Acquisition: ion mobility-assisted data independent acquisition (IMS-DIA) using a drift time specific collision energy profile [3].
- Database: Cricetulus griseus (Chinese hamster) reference proteome - Uniprot (UP000001075)
- Data Processing: Progenesis QIP 2.0 was used for protein identification and quantification – (Chaperone B from E. coli (CLPB_ECOLI) Hi3 standard was used for relative protein quantitation, 5 fmol/ μL each fraction).



Scheme 1. Experimental Design and Workflow for the Intact and Reduced Antibody experiments with the predicted fractions shown for each respective experiment. The subsequent sample preparation and LC-HDMSE analysis are illustrated for all twelve resulting SageELF fractions.

RESULTS

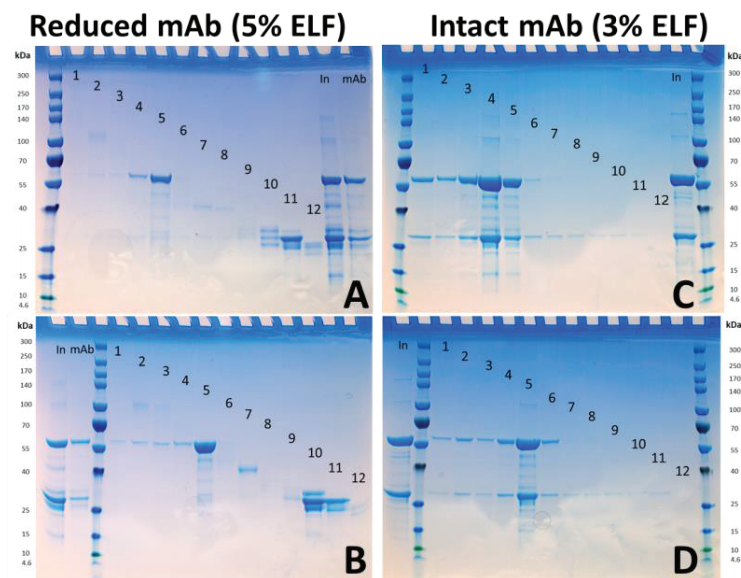


Figure 2. SDS-PAGE (4-20%) following fractionation of mAb protein using the SageELF. Reduced mAb experiment 1 (A) and 2 (B). Intact mAb experiment 1 (C) and 2 (D). The 12-fractions, input (In), and antibody (mAb) are indicated on the gel images.

Figure 3. Distribution of the mAb heavy and light chain expressed as the ppm concentration for each respective SageELF fraction 1-12. Reduced mAb experiment (A) heavy chain and (B) light chain. Intact mAb experiment (C) heavy chain and (D) light chain. The protein quantitation was performed using the top 5 ionizing peptides from Chaperone B ClpB (CLPB_ECOLI, 5 fmol/ μ L) and the normalization was performed in Progenesis QIP 2.0 using all peptide

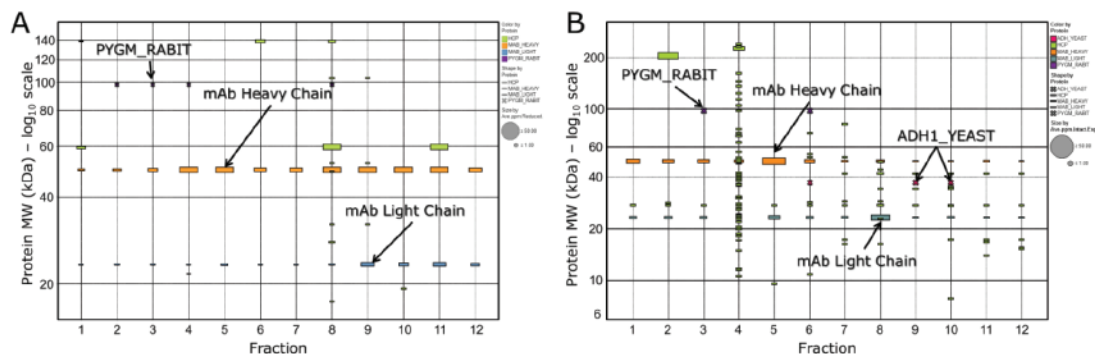
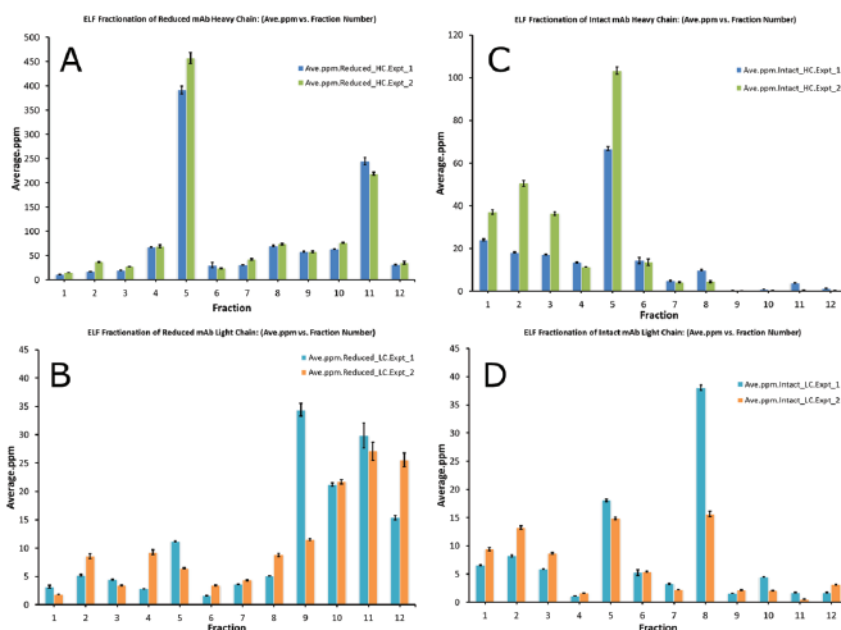


Figure 4. Distribution of protein molecular weight (kDa) vs. SageELF fraction number. (A) Reduced mAb experiment and (B) Intact mAb experiment. Filter: the proteins must have 1 unique peptide used for quantitation (2 peptides for Intact mAb) and the proteins shown are quantified $\leq 20\%$ CV. The mAb heavy / light chain and the spiked standards (PYGM_RABIT and ADH1_YEAST) are color coded and sized by ppm level.

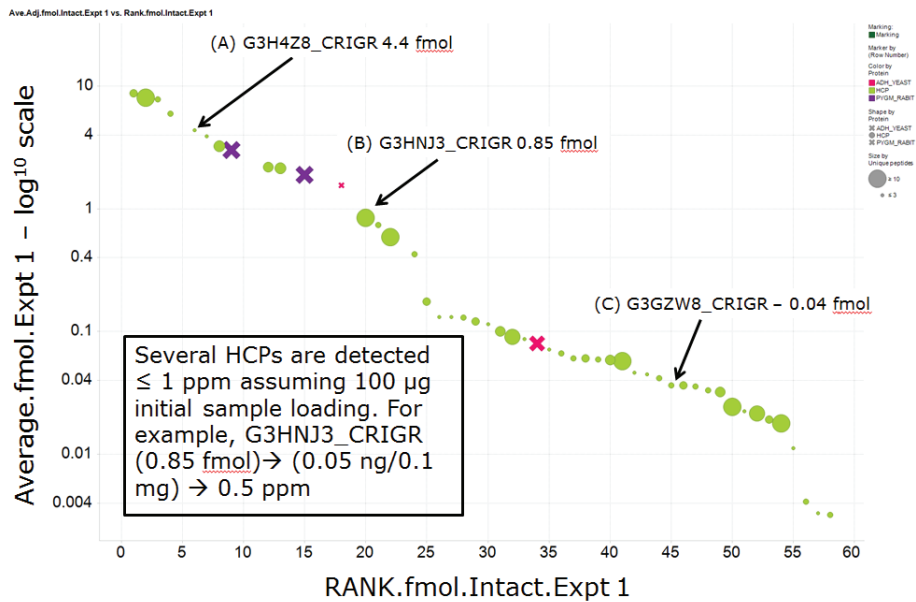


Figure 5. Dynamic range plot of average ppb (log10 scale) vs. RANK.ppm for the identified HCPs found within the mAb standard. The quantified proteins have been filtered by a minimum of 3 unique peptides/protein and the proteins are quantified $\leq 20\%$ CV. Three proteins are labeled as **(A)** G3H4Z8_CRIGR (Heat shock-related 70 kDa protein 2) – **4.4 fmol**, **(B)** G3HNJ3_CRIGR (Clusterin) – **0.85 fmol**, and **(C)** G3GZW8_CRIGR (Phosphoglycerate mutase 1) – **0.04 fmol**.

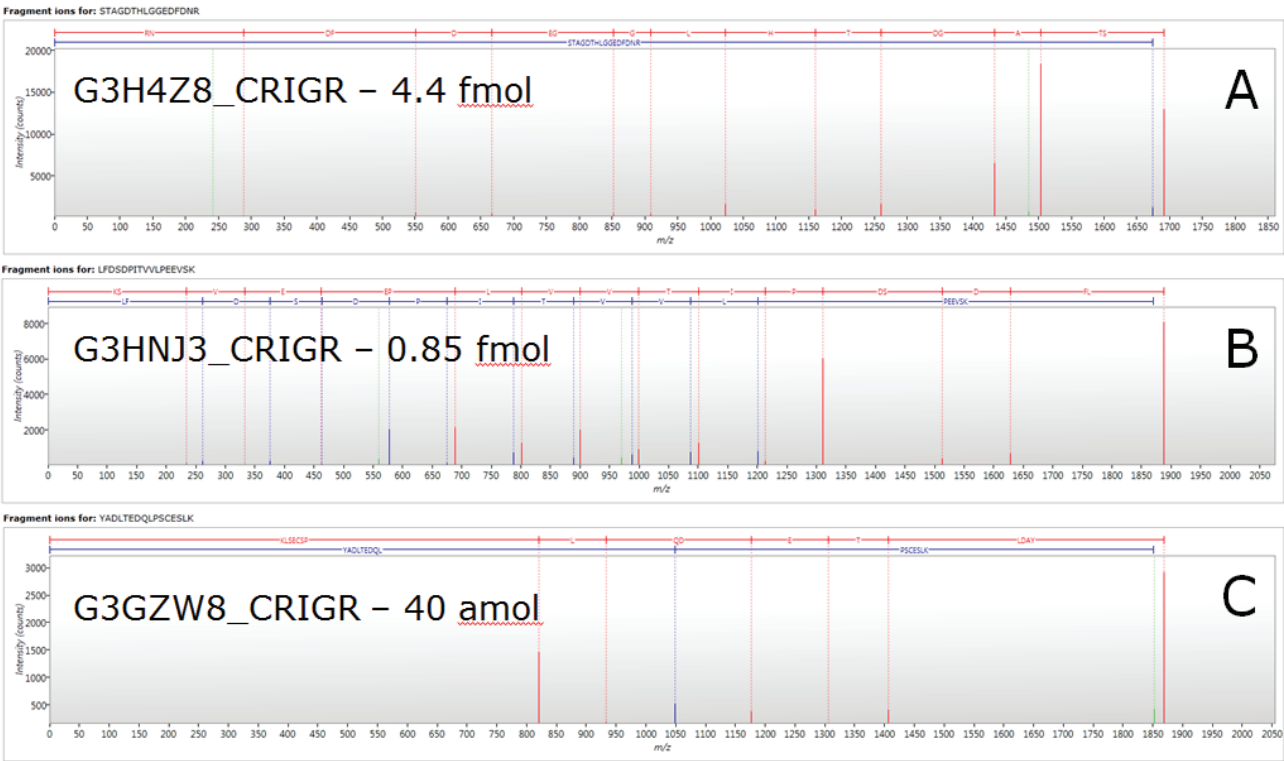


Figure 6. Representative elevated energy mass spectra for the proteins denoted on the dynamic range plot (Figure 4) at decreasing ppb HCP levels. **(A)** Heat shock-related 70 kDa protein 2 - STAGDTHLGGEDFDNR (m/z 564.5847) at 4.4 fmol, **(B)** Clusterin – LFDSDPITVVLPEEVSK (m/z 944.5041) at 0.85 fmol, and **(C)** Phosphoglycerate mutase 1 – YADLTEDQLPSCESLK at 0.04 fmol.

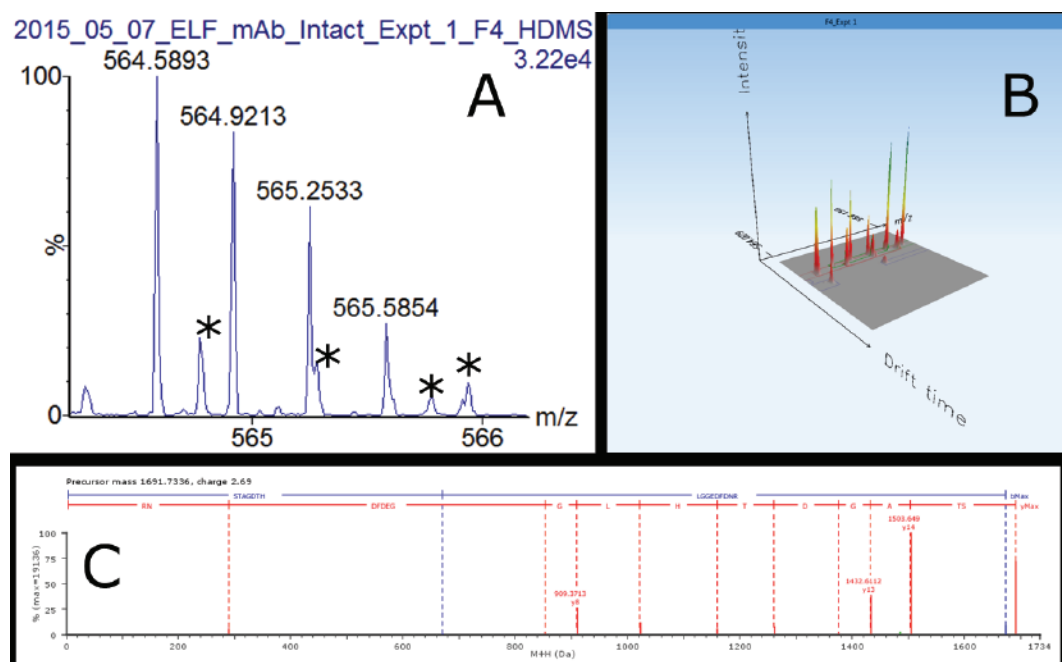


Figure 7. (A) Multiple precursor ions (denoted with asterisks) are observed in the mass spectrum at $R_t = 12.58$ min for $[M+3H]^3+$ ion at 584.58 m/z (B) Visualization of m/z vs. drift time (dt) for the ion mobility separation of the precursor ion with a drift time of 3.67 ms and (C) ion mobility resolved elevated energy mass spectrum for the tryptic peptide STAG-DTHLGGEDFDNR from Heat shock-related 70 kDa protein.

Accession	Protein MW (Da)	Peptide count	Unique peptides	Fraction	Confidence score	Description	Ave.Adj.fmol.Intact.Expt 1	Ave.Adj.fmol.Intact.Expt 2	Ave.ng.Intact.Expt 1	Ave.ng.Intact.Expt 2
G3I8R9_CRIGR	72550	20	18	4	188	78 kDa glucose-regulated protein OS=Cricetulus griseus GN=I79_012331 PE=3 SV=1	0.058	0.712	0.004	0.052
G3HNJ3_CRIGR	52385	16	16	6	145	Clusterin OS=Cricetulus griseus GN=I79_012331 PE=3 SV=1	0.847	0.692	0.044	0.036
G3HSL4_CRIGR	97669	15	15	4	115	Elongation factor 2 OS=Cricetulus griseus GN=I79_013848 PE=3 SV=1	0.024	0.568	0.002	0.055
G3IH63_CRIGR;G	226993	15	15	4	94	Myosin-9 OS=Cricetulus griseus GN=I79_023145 PE=4 SV=1	8.040	1.596	1.825	0.362
G3HG95_CRIGR	64099	14	14	4	119	Lamin-A/C OS=Cricetulus griseus GN=I79_009616 PE=3 SV=1	0.588	0.381	0.038	0.024
G3HN14_CRIGR	239982	13	13	4	85	Transitional endoplasmic reticulum ATPase OS=Cricetulus griseus GN=I79_012331 PE=3 SV=1	0.018	0.198	0.004	0.047
G3I4H6_CRIGR;G	39838	9	9	4	75	Fructose-bisphosphate aldolase OS=Cricetulus griseus GN=I79_012331 PE=3 SV=1	0.090	0.276	0.004	0.011
G3H3W4_CRIGR	113786	9	9	4	58	Insulin-degrading enzyme OS=Cricetulus griseus GN=I79_004916 PE=3 SV=1	0.022	0.215	0.002	0.024
G3IBF4_CRIGR	28832	7	7	8	44	Serine protease HTRA1 OS=Cricetulus griseus GN=I79_020974 PE=3 SV=1	2.153	3.312	0.062	0.095
G3ICD3_CRIGR	16266	7	7	8	41	Lactadherin OS=Cricetulus griseus GN=I79_021326 PE=4 SV=1	3.253	1.371	0.053	0.022
G3HKN1_CRIGR;	44331	6	6	8	45	Protein SET OS=Cricetulus griseus GN=I79_011260 PE=3 SV=1	2.192	1.595	0.097	0.071
G3HD42_CRIGR	42500	6	6	4	40	Serpin B5 OS=Cricetulus griseus GN=I79_008416 PE=3 SV=1	0.059	0.276	0.003	0.012
G3HC84_CRIGR	48150	5	5	4	49	Heat shock protein HSP 90-beta OS=Cricetulus griseus GN=I79_012331 PE=3 SV=1	0.175	0.521	0.008	0.025
G3HQX0_CRIGR	19848	5	5	4	31	40S ribosomal protein SA OS=Cricetulus griseus GN=I79_013216 PE=3 SV=1	0.060	0.098	0.001	0.002
G3IE79_CRIGR	30320	5	4	4	37	Arginase-1 OS=Cricetulus griseus GN=I79_022023 PE=3 SV=1	7.802	5.946	0.237	0.180

Table 1. Top 15 identified HCP impurities identified and quantified from the intact mAb experiments. The proteins within this table are the top 15 proteins with the most unique peptides used for quantitation. Note that a total of 66 HCP proteins are quantified from the intact mAb experiments with a minimum of 3 unique peptides/protein and quantified at $\leq 20\%$ CV.

- The ELF separation device results in reproducible protein level fractionation across duplicate experiments for the intact and reduced mAb protein separations.
- Based on our results, the SAGE ELF pre-fractionation of intact non-reduced protein samples has shown to identify/quantify more sub-ppm level HCPs than the reduced antibody separation method.
- The spiked control proteins PYGM_RABIT and ADH1_YEAST in the intact mAb experiment are successfully identified in ± 1 predicted ELF fraction at 100 and 10 ppm levels, respectively.
- Sub-ppm level HCP detection was achieved - 58 HCP proteins detected ≤ 10 fmol with 3 unique peptides/protein

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