To perform against custom databases. Final results were filtered using an E ProSightPC Intact protein spectra were Data Analysis protein A MATERIALS AND METHODS Sample Preparation A number of protein candidates expected to satisfy the following criteria: 1) having a MW range of 150D – 600K, 2) possessing mostly clear modification and added 3) having ESI charge state distributions covering a wide m/z range from 500-2,000, were identified and then re-processed E, polypeptide and puffed. All were screened for ionization efficiency, purity, MW, charge state distribution by LC/MS, and protein hits resulting in selection of the final candidates for the recombinant standard. Mixing ratios were adjusted such that all proteins could be detected simultaneously in a single LC/MS experiment. Quality and stability of the selected proteins were verified by SDS-PAGE, UV/MS, Insolution MS, and LC-MS/MS using a Thermofisher™ Orbitrap Fusion™ Lumos™ Tribrid™ or a Thermofisher™ Q Exactive™ Hybrid quadrupole-Orbitrap™ mass spectrometer in intact protein mode. High resolution CID, ETn/HD and HCD spectra were collected to confirm the sequences assigned to the final protein list. Data Analysis Protein candidates were deconvoluted with PeptideProphet (7) or ProSightPC (6) and using the sliding window deconvolution algorithm in Thermo Fisher Scientific™ Protein Deconvolution™ 4.0 software. The top down data were analyzed with Thermo Scientific™ ProSightPC™ v2.1 and Thermoscientific™ Proteome Discoverer™ 2.1 utilizing the ProSightPC™ rookie software packages. All searches were performed against custom databases. Final results were filtered using an E value cutoff of 1 x 10^-7 and search engine rank 1.

RESULTS To extend the ability of the protein standard mixture to the broader intact protein analysis community, the protein standard mixture should satisfy several additional requirements, such as demonstrating long term stability and the absence of animal origin proteins as per FDA regulations. As such, new non-animal, recombinant protein candidates were synthesized, affinity captured, and purified. Affinity tag removed and proteins were debiased using a de novo algorithm. All proteins were verified in Masslynx 5.0 software. The de novo searches were performed on those which met purity, MW, and m/z distribution requirements (Figure 3–7). The new pre-commercial mixture is presented here.

Figure 1. Workflow for recombinant protein mixture generation

Figure 2. Prototype of recombinant protein mixture standard

Figure 3. MS Analysis of selected candidates. Left panel shows the full scan spectrum of each candidate protein collected on a Thermo Scientific™ G Exactive™ Q Exactive™ UltiMate™ Q Exactive™ 4.0 software. In red, were deconvoluted with PeptideProphet (7) or ProSightPC (6) and using the sliding window deconvolution algorithm in Thermo Fisher Scientific™ Protein Deconvolution™ 4.0 software and the top down data were analyzed with Thermo Scientific™ ProSightPC™ v2.1 and Thermoscientific™ Proteome Discoverer™ 2.1 utilizing the ProSightPC™ rookie software packages. All searches were performed against custom databases. Final results were filtered using an E value cutoff of 1 x 10^-7 and search engine rank 1.

Figure 4. Final recombinant protein mixture infusion-MS spectra. Top panel – infusion spectra of new candidate mixture at 15K resolution and corresponding deconvolution results from Protein Deconvolution 4.0 software. All proteins typically detected, including larger proteins. Protein AG and Klenow. Bottom panel – infusion spectra of the optimized mixture using new recombinant protein candidates at 120K resolution and corresponding deconvolution results from Protein Deconvolution 4.0 software. IGF-LR3, ProTG, Protein G, and Carbonic Anhydrase are detected at this resolution setting.

Figure 5. UHPLC analysis of recombinant proteins. Thermo Scientific™ Dionex™ Ultimate™ 3000 RSL Column system, Column: Proteomix Thermo Scientific™ MANPex™ 250 mm × 3.0 mm Mobile Phase A: Water + 0.1% Formic acid or TFA, B: 100% ACN + 0.1% Formic acid or TFA, Flow Rate: 2 µL/min. Temperature 60°C. Sample 100 µg protein mix (see chromatogram). Yields: 0.1 µL. Detection: UV 214 nm

Figure 6. LC/MS/MS analysis of selected recombinant proteins. Left panel –tandem proteins were analyzed on an UltiMate 3000 RSLC system using a NanoPro PX™ frit = 150 µm inlet column. Mobile Phase A: Water + 0.1% Formic acid, B: 100% ACN + 0.1% Formic acid at 250 nL/min. Flow Rate: 200 nL/µmL. Temperature: 50°C. Sample: 500 µg protein mix (see chromatogram). Right panel – Samples were analyzed on a XEactive™ mass spectrometer in intact protein mode. MS/MS spectra were acquired using Top 5-20 DIA method settings of 15 at m/z 250 and 20 at m/z 250 at a resolution of 120K at m/z 250. Top panel shows deconvolution results from Protein Deconvolution 4.0 software and Bottom panel shows top-down results from ProSightPD 1.1 node in Proteome Discoverer 2.1 software.

Figure 7. Preliminary stability studies. Shown here is Protein G reconstituted in water and analyzed immediately, or after 7 days incubated at 37°C, corresponding to 1 year storage at 20°C.

CONCLUSIONS A high quality intact protein standard was developed that meets needs for HPLC, LC-MS, LC-MS/MS quality control, method development, and optimization. The completely recombinant nature of the sample makes it compatible for clinical research applications. The proteins cover a wide MW range, iso range, and demonstrate good chromatographic separation.

ACKNOWLEDGEMENTS We would like to acknowledge Jason Fink, Eugene Dano, Tina Argon, Xueke Sun for their help with early testing of, and feedback on, the R&D mixture. We would like to thank the Kelleher lab for testing, creating custom protein database and feedback on candidate proteins for the final mixture.

TRADEMARKS/LICENSING © 2017 Thermo Fisher Scientific Inc. All rights reserved. ProSightPC and ProSightPD are trademarks of Proteomixus, Inc. RePro is a trademark of Positive Probability Ltd. All other trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others.