Supporting Information

Improved Protein and PTM Characterization with a Practical Electron-Based Fragmentation on Q-TOF Instruments

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Supplemental Figure 1. The ExD-Collision Cell Combination replaces the standard collision cell in Agilent QTOF Instruments. The collision cell is shortened by 18%, which has little discernible impact on CID fragmentation.

Supplemental Figure 2. Increasing CID energy to 30 V with ECD. ECD fragments are still comparable to the majority of CID fragments, while far more low intensity peaks congest the baseline compared to Fig. 3 in the results section.
Supplemental Figure 3. ECD spectrum of the 6+ charge state of bovine ubiquitin obtained with an Agilent 6545 Q-TOF. The protein in 100 mM ammonium acetate was infused using the Agilent Jet Stream electrospray source. 7 V collision energy was applied. An enlargement of z_{66}^{3+} shows good signal-to-noise ratios for large fragments with relatively low abundance.
Supplemental Figure 4. Coverage of a Lys-C digest of the antibody Infliximab. Light and heavy chain. The N-glycosylated residue is indicated in red. Infliximab has one of two major glycans, G0F or G1F, bound at this site. Each of the ions was selected in a targeted LC-MS run and subjected to ECD. All ions were scored by MSGF+ based on the ECD spectrum, and those with sub-standard scores are not shown.
**Experimental Methods**

Substance P was dissolved at 0.01 mg/mL in 50% methanol, 50% water, 0.1% formic acid, and infused at 10 µL/min through the reference nebulizer of the Agilent Dual AJS source. The collision energy of 22 V for CID was calculated using Agilent’s recommended formula:

\[
3.1 \cdot \frac{(674 \text{ m/z})}{100} + 1 = 22 \text{ V}
\]

A stock solution of bovine insulin (Sigma) was prepared at 0.9 mg/mL in 5% acetic acid. This was diluted to 10 µM with 20% acetonitrile, 0.1% formic acid for infusion at 16.7 µL/min with the Agilent Jet Stream source.

Ubiquitin (bovine, Sigma) was dissolved in 100 mM ammonium acetate to give 10 µM, and infused above by a syringe pump the Agilent Dual AJS source.

**Automated Phosphopeptide enrichment and cleanup.** Two samples identified as “Phosphopeptide” and “Phosphopeptide-Yeast” were received from the HUPO organization [13]. Both samples were resuspended in 100 µL of 80% acetonitrile (ACN), 0.1% trifluoracetic acid (TFA) and contained some 94 phosphorylated peptides with sequences found in the human proteome. The concentrations of phosphopeptides were 3.3, 10, or 30 fmol/µL. The Agilent AssayMAP Phosphopeptide Enrichment v2.0 App was used for automated phosphopeptide enrichment using an Fe(III)-NTA cartridge and then desalt the enriched phosphopeptides with C18 cartridges. Each sample was dried at room temperature in a SpeedVac vacuum concentrator, resuspended in 25 µL of 10% ACN, 0.1% formic acid (FA), and sonicated for two minutes. The sample was further diluted with 25 µL of 0.1% FA in water to yield 50 µL of the final sample in 5% ACN with 0.1% FA.

**Nano-LC/MS Analysis of phosphopeptides.** The 1290 Infinity II LC system was converted to nanoflow LC by coupling with the Agilent Infinity UHPLC Nanodapter. This nanoflow LC was connected to the Agilent nanospray ESI source and coupled with the Agilent 6550 iFunnel Q-
TOF LC/MS equipped with an e-MSion ExD cell for peptide identification. A 75 μm × 25 cm C18 column was kept at 60 °C and used for peptide separation with a 90-minute gradient in a total 120-minute LC run time (see [14] for the detailed conditions). A targeted list of precursor ions that contains m/z, charge state, retention time, delta retention time, isolation width, and acquisition time was generated using the phosphopeptide list identified from the data-dependent acquisition (DDA) experiment. For ECD the collision energy was set to 0 eV, and the same precursor ions generated in the target list were fragmented four to five times in one run.

For identifying phosphorylation sites, the Byonic software package (Protein Metrics, Cupertino) was used to analyze ECD spectra in the first round. The Swiss-Prot human protein database was used with trypsin as the digestion enzyme and up to two missed cleavages were allowed. Carbamidomethylation was chosen as the fixed modification, while deamidation (N, Q) and phosphorylation of serine (S), threonine (T), and tyrosine (Y) were set as variable modifications. Further spectra analysis was done by using the program ExDProcess.

Peptide identification and phosphopeptide quantitation was performed using DDA together with Skyline [15, 16]. Phospho-site localization was analyzed using ECD and Byonic software.

**Antibody Digestion with Lys C.** Infliximab (European Pharmacopoeia Reference Standard) was denatured in 6 M guanidinium hydrochloride (Millipore ULTROL Grade) for 30 min at 37 °C before reduction with TCEP (tris(2-carboxyethyl)phosphine hydrochloride, Aldrich) for 60 min at 37 °C. Alkylation with iodoacetamide (SigmaUltra) was carried out for 30 minutes at room temperature, protected from light. The reduced and alkylated antibody was exchanged into 50 mM Tris buffer pH 8.0 (Invitrogen UltraPure) before digestion with Lys C (Promega rLys-C, Mass Spec Grade) at 37 °C for 2 hours, with an antibody-to-enzyme ratio of 1:40.

An Agilent PLRP-S column (2.1 x 50 mm, 5 μm particle size, 1000 Å pore size) at 40°C was used, with a 30-minute gradient at 0.3 ml/min of 10-40% acetonitrile with 0.1% FA. An initial MS1-
only run was used to identify possible peptide precursors and establish elution times. Most peptides were identified using Agilent BioConfirm software (version 10).

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**Data Collection and Processing of antibody Lys-C digests.** Spectra were acquired on an Agilent 6545XT AdvanceBio LC/Q-TOF equipped with an e-MSion ExD cell for ECD fragmentation and an Agilent 1290 Infinity II LC system. 20 µL of Ly-C digested antibody (500 pmol) was injected on an Agilent PLRP-S column (2.1 x 50 mm, 5 μm particle size, 1000 Å pore size) at 40°C. The peptides were separated with a 30 minute 10-40% gradient of buffer B (0.1 % formic acid in acetonitrile). An initial MS1-only run was used to identify possible peptide precursors and establish elution times. Most peptides were identified using Agilent BioConfirm software (version 10). A targeted MS2 method was created such that each peptide was subjected to ECD fragmentation across the apex of the peak.