



P003.0P – Complete protocol for the generation of capillary LC-MS/MS analysis used to generate an accurate mass and time (AMT) tag database and capillary LC-MS analysis to generate quantitative data.

**Proteomics sample preparation**

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Ammonium bicarbonate and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA), and sequencing-grade modified trypsin was purchased from Promega (Madison, WI). Bicinchoninic acid (BCA) assay reagents and standards were obtained from Pierce (Rockford, IL); and purified, deionized water, >18 M $\Omega$ , (Nanopure Infinity ultrapure water system, Barnstead, Dubuque, IA) was used to make all aqueous buffers. The protein concentrations of sample homogenates are determined by BCA protein assay and diluted to uniform volume in 50 mM ammonium bicarbonate, pH 7.8. Proteins are reduced with 10 mM dithiothreitol, followed by alkylation of free sulfhydryl groups with 40 mM iodoacetamide at 37°C in the dark; each reaction is performed for 1 h at 37°C with constant shaking at 550 rpm. Denatured and reduced samples are diluted 10-fold with 50 mM ammonium bicarbonate, pH 7.8, and CaCl<sub>2</sub> is added to a final concentration of 1 mM prior to enzymatic digestion. Sequencing-grade modified trypsin is activated by adding 20  $\mu$ L of 50 mM ammonium bicarbonate, pH 7.8, to 20  $\mu$ g lyophilized trypsin and incubating for 10 min at 37°C. Activated trypsin is added to the samples at 1:50 (w/w) trypsin-to-protein ratio, and samples are digested at 37°C for 3 h with constant shaking at 800 rpm; reactions are quenched by rapid freezing in liquid nitrogen. Digested samples are desalted using solid phase extraction columns (Discovery C18, Supelco, Bellefonte, PA), which are conditioned with 3 mL of methanol and rinsed with 2 mL of 0.1% trifluoroacetic acid (TFA). Digest-loaded columns are



washed with 4 mL of H<sub>2</sub>O/acetonitrile (95:5, v/v) containing 0.1% TFA, and peptides are eluted with 1 mL of acetonitrile/H<sub>2</sub>O (80:20, v/v) containing 0.1% TFA. Samples are concentrated to 100 µL *in vacuo* (Speed-Vac SC 250 Express, Thermo Savant, Holbrook, NY), and a BCA protein assay is performed to verify final peptide concentrations. Samples are stored at –80°C until strong cation exchange fractionation with liquid chromatography-tandem mass spectrometry (LC-MS/MS) or quantitative LC-MS analyses.

### **Strong cation exchange fractionation**

Strong cation exchange fractionation is performed on pooled samples using an Agilent 1100 HPLC System (Agilent, Palo Alto, CA) equipped with a quaternary pump, degasser, diode array detector, peltier-cooled autosampler, and fraction collector (set at 4°C). Peptides (350 µg per injection) are separated with a PolySulfoethyl A (PolyLC Inc., Columbia, MD) column (200 mm × 2.1 mm; 5 µm particles with 300-Å pores) with a 10 mm × 2.1 mm guard column packed with the same material at a flow rate of 0.2 mL/min. The solvents consist of (a) 10 mM ammonium formate, pH 3.0, and 25% acetonitrile, and (b) 500 mM ammonium formate [pH 6.8] and 25% acetonitrile. The following linear gradient is used: 100% of solvent (a) for 10 min; ramp to 50% solvent (b) in 40 min; ramp to 100% solvent (b) in the next 10 min; followed by a 10 min hold in 100% solvent (b). Routinely, 24 fractions are collected from minute 30 to minute 65 of the gradient; and they are subsequently dried *in vacuo* and stored at -80°C until LC-MS/MS analysis.

### **Reversed-phase capillary LC-MS/MS and LC-MS analyses**

Capillary LC-MS/MS analysis was used to generate an accurate mass and time (AMT) tag database for virus-infected lung homogenates (see below). For this, dried peptide fractions were reconstituted in 30 µL of 25 mM ammonium bicarbonate, pH 7.8, and analyzed using a 4-column custom-built capillary LC system coupled online to a linear ion trap mass spectrometer (LTQ; Thermo Scientific, San Jose, CA) by way of an in-house manufactured electrospray ionization interface. Electrospray emitters were custom made using 150 µm outer diameter (o.d.) x 20 µm inner diameter (i.d.) chemically



etched fused silica. Reversed-phase capillary columns were prepared by slurry packing 3- $\mu\text{m}$  Jupiter C18 bonded particles (Phenomenex, Torrance, CA) into a 75  $\mu\text{m}$  x 65 cm fused silica capillary (Polymicro Technologies, Phoenix, AZ) using 0.5 cm sol-gel plugs for particle retention. Mobile phases consisted of (a) 0.1% formic acid in water and (b) 0.1% formic acid in acetonitrile, and they were degassed on-line using a Degasys Model DG-2410 vacuum degasser (Dionex, Germany); the HPLC system was equilibrated at 10,000 psi with 100% mobile phase (a) for initial starting conditions. After loading 2.5  $\mu\text{g}$  of peptides onto the column, the mobile phase was held at 100% mobile phase (a) for 50 min. Exponential gradient elution was initiated 50 min after sample loading with a column flow rate of 300 nL/min, and the mobile phase was ramped from 0% to 55% mobile phase (b) over 100 min using a 2.5 mL stainless steel mixing chamber, followed by a rapid increase to ~100% (b) for 10 min to wash the column. To identify the eluting peptides, the LTQ was operated in a data-dependent MS/MS mode (400-2,000  $m/z$ ), in which a full MS scan was followed by ten MS/MS scans using a normalized collision energy of 35%. A dynamic exclusion window of 1 min was used to discriminate against previously analyzed ions. The temperature of the heated capillary and the electrospray ionization (ESI) voltage were 200°C and 2.2 kV, respectively.

Following sample homogenate/virus AMT tag database generation, capillary LC-MS analyses are performed on all virus infected and mock-infected samples to generate quantitative data. For this, dried peptide samples (see above) are reconstituted in 30  $\mu\text{L}$  of 25 mM ammonium bicarbonate, pH 7.8, and analyzed in triplicate and random order using identical chromatographic and electrospray conditions as for capillary LC-MS/MS analyses. The LC system is interfaced to an Exactive Orbitrap mass spectrometer (Thermo Scientific), and the temperature of the heated capillary and the ESI voltage are 250°C and 2.2 kV, respectively. Data are collected over the mass range 400-2,000  $m/z$ .



### **Development of the AMT tag database for virus-infected model systems**

A novel AMT database is generated for peptides the specific model system, using mock-infected and virus-infected. To generate the AMT tag database, aliquots of the virus infected or mock-infected samples are combined to make the appropriate sample pools. Each pool is subjected to strong cation exchange fractionation as described above, and each fraction is analyzed by capillary LC-MS/MS. The SEQUEST analysis software is used to match the MS/MS fragmentation spectra with sequences from the appropriate UniProt/Swiss-Prot protein database. When searching, SEQUEST used a dynamic mass modification on methionine residues corresponding to oxidation (15.9949 Da) and a static mass modification on cysteinyl residues to account for alkylation by iodoacetamide (57.0215 Da). Peptides passing the following filter criteria are stored as AMT tags in a Microsoft SQL Server database: 1) SEQUEST DelCn2 value (normalized Xcorr difference between the top scoring peptide and the second highest scoring peptide in each MS/MS spectrum)  $\geq 0.10$  and 2) SEQUEST correlation score (Xcorr)  $\geq 1.6$ , 2.4, and 3.2 for fully tryptic peptides with 1+, 2+, and 3+ charge states, respectively, and Xcorr  $\geq 4.3$ , and 4.7 for partially tryptic or non-tryptic protein terminal peptides with 2+, and 3+ charge states, respectively. Non-tryptic peptides are excluded, and a minimum peptide length of 6 amino acid residues is required. The elution times for these peptides were normalized to a range of 0 to 1 using a predictive peptide LC normalized elution time (NET) model and linear regression. A NET average and standard deviation are assigned to each identified peptide if the same peptide is observed in multiple analyses. Both calculated monoisotopic masses and observed NETs of identified peptides are included in the AMT tag database.

