Identification of signature peptide (SP) candidates

Candidates of signature peptides(SP) were discovered experimentally by analyzing tryptic digests of the HCV mAb using nano-LC-LTQ/Orbitrap MS. Following the reduction, alkylation and an on-pellet-digestion step (specified in the following sections), the peptide mixture was separated on an Eksigent two-dimensional nano-LC system (Eksigent Technologies, Dublin, CA, USA). Solvents used were water/0.1% formic acid (mobile phase A) and 85% acetonitrile/0.1% formic acid (mobile phase B). Samples containing 4µg of peptides were loaded onto a large-ID trap (300 µm ID × 5 mm, 3-µm C18) with 3% B at a flow rate of 10 µL/min, and the trap was washed for 3 min. A shallow, 90-min gradient was used to back-flush the trapped samples onto the nano-LC column (75 µm ID × 25 cm, packed with Pepmap 3-µm C18 material). The flow rate was 250 nL/min. High resolution MS analysis was performed on an LTQ/Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). MS/MS analysis was performed using a survey scan in FT mode (m/z 350–1500) with a resolution of 60,000 and an ion accumulation target value of $8 \times 10^6$, followed by fragmentation of the 7 most intensive peaks in the linear ion trap. Identification of peptides was carried out by BioWorks 3.3.1 embedded with SEQUEST (Thermo Fisher), searching against a FASTA database containing the forward and reversed sequences of target mAb. The precursor mass tolerance was 25 ppm and the mass tolerance for CID fragments was 1.0 mass unit. Cross correlation score (Xcorr) criteria were ≥4 for 4+ and higher charge states, ≥3 for 3+ ions, ≥2.2 for 2+ ions, and ≥1.7 for 1+ ions. The identified peptides were filtered to select the potential SP candidates for further consideration. The criteria include: i) the peptides containing labile residues such as methionine and miss-cleavages were discarded; and ii) only peptides unique to the target mAb and not found in the target species (rat) were selected for further consideration.

On-the-fly orthogonal array optimization of all SP candidates

SRM parameters of SP candidates were obtained in a high-throughput manner by an on-the-fly Orthogonal Array Optimization (OAO) method developed by our lab. HCV antibody was spiked into pooled blank rat plasma at a concentration of 10 µg/mL, and then the sample processed and digested following an efficient acetone precipitation/on-pellet digestion procedure (described in the rat plasma sample treatment section). The digested sample was analyzed on a micro-flow LC coupled to a TSQ Quantum triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The product ions, declustering (i.e. Tube Lens voltages), and collision energies were selected as the three main factors to be optimized as they can significantly affect the sensitivity achievable by the peptides. For each factor, five levels were evaluated. The experimental design was fitted in an L25 orthogonal array in order to provide a comprehensive evaluation of all factors. The five most abundant product ions obtained from the identification
experiments were used as the five levels for product ions; levels for declustering energies and collision energies were predefined based on a pilot analysis of more than 20 unique candidates. One set of 25 SRM trials were performed for each candidate. The range of collision energies was estimated based on the empirical values we established through pilot analyses of the unique tryptic peptides. The optimization was conducted by programming 25 independent SRM measurements with strategically varied parameters. The peak area, peak height and S/N ratio were extracted from each SRM channel and exported to a statistic analysis module, which automatically calculated the effect curves for each parameter. The TSQ was programmed to perform the 25 SRM trials dictated by the orthogonal array design within a 4-min chromatographic window that centered on the retention time of each candidate peptide. In the event that the elution windows of two or more candidates overlapped partially, an 8-min window that included all closely eluting candidates was used, and the SRM trials for these candidates were performed sequentially within each MS scan cycle. The dwell times in each trial ranged from 20-50 ms and were based on the precursor intensities obtained in the nano-LC/LTQ/Orbitrap experiment. For example, a dwell time of 20 ms was used for candidates having precursor intensities >1 \times 10^7 in the identification experiments, whereas a dwell time of 50 ms was used for those candidates having intensities <1 \times 10^7 (by Orbitrap).

The results were processed using the “fractional DOE” function of Minitab (State College, PA), which included analysis of variance and backward regression. Although effect curves for product ions and TL voltages were obtained directly from these analyses, the effect curves for collision energies were calculated individually for each product ion: for a given product ion, five SRM trials were performed with different levels of collision energy and TL voltage. In order to isolate the effects of collision energy on sensitivity from those exerted by the TL voltages, the S/N obtained from these trials was normalized individually against the effects of the corresponding TL voltages. Thus, the optimal collision energy for each candidate transition was obtained.

**Comprehensive evaluation of SP candidates for stability and sensitivity in plasma digest**

Before final selection of the SP, the candidate peptides were evaluated for both stability and sensitivity in blank rat plasma. Therefore, HCV mAb was spiked into a pooled rat plasma sample at 10 µg/mL each and digested as described in the following plasma sample treatment section, and then the sample was used for the stability assessments. Peptide stabilities were evaluated by the continued incubation under the conditions of (i) prolonged tryptic digestion (37 °C, pH 8.5) and (ii) LC/MS queue storage in the autosampler (4 °C, pH 2.8). For evaluation of stability under digestion conditions, the solution was sampled at 0, 2, 4, 6, 10, 14, and 24h after the completion of digestion. At each time point, a 20 µL sample was taken, acidified with formic acid witha final concentration of 1% (v/v), and then analyzed immediately by micro-LC/MS using the optimized conditions obtained in the OAO procedure. For the
assessment of candidate stability under autosampler storage conditions, the digestion mixture was
acidified immediately upon completion of the digestion, incubated in the refrigerated autosampler, and
analyzed after 0, 1, 2, 4, 8, 12, 24, and 48 h. Both stability assessments were conducted in triplicate. Any
candidate that degraded more than 20% within either evaluation period was eliminated. For each
remaining candidate, the S/N was evaluated under the optimal SRM conditions using pooled rat plasma
spiked with 1 µg/mL of the HCV antibody. The most sensitive and stable candidates from each of the
heavy and light chain, i.e. GPSVFPLAPSSK (GPS) and TVAAPSVFIFPPSDEQLK (TVA) were selected
as the final signature peptides. GPS belongs to the heavy chain of the antibody, while TVA is from the
light chain.

**Validation of the five calibration approaches**

The five calibration approaches were validated using QC samples prepared by spiking the corresponding
calibrators (peptides, extended-peptides and protein) into blank plasma samples, at three levels: 1.6, 10
and 80 µg/ml mAb protein in plasma (or equal molar concentrations of peptides or extended-peptides).
For peptide-level calibration, GPS peptide and TVA peptide were respectively spiked at 0.512, 3.2 and
25.6 ng/mL and 0.832, 5.2 and 41.6 ng/mL in the blank plasma digest; for extended-peptide-level
calibration, extended-GPS peptide and extended-TVA peptide were respectively spiked at 0.832, 5.2 and
41.6 ng/mL and 1.2, 7.52 and 60 ng/mL in the digestion buffer before digestion of the blank plasma. For
protein-level and the two hybrid calibration approaches, the full-length HCV mAb was spiked into blank
plasma at 1.6, 10 and 80 µg/ml before sample preparation. Spiked solutions were vortexed, incubated at
4 °C for overnight to allow equilibrium of binding, and then precipitated, digested and analyzed by
LC/SRM-MS. For each approach, each sample was analyzed in triplicate for both inter- and intra-day
analyses.