Full Automation of the SISCAPA® Workflow Using the Biomek NX®P Laboratory Automation Workstation

Morty Razavi¹, Michael Kowalski², Tara Jones-Roe², Christie L Hunter³, Leigh Anderon¹, Richard Yip¹, Matt Pope¹, Terry Pearson¹
¹SISCAPA Assay Technologies, Washington, DC, ²Beckman Coulter Life Sciences, Indianapolis, IN, ³SCIEX, Redwood City, CA

With the growing translational research application of mass spectrometry (MS), the need for automation of sample preparation upstream of MS analysis has become increasingly important. Specifically, the multi-step workflows involved in mass spectrometric approaches for measurement of proteins are often fastidious and labor intensive, requiring a level of expertise rarely present in translational research settings. To overcome this challenge, the SISCAPA (Stable Isotope Standards and Capture by Anti-Peptide Antibodies) technology for targeted protein quantitation has been automated.

SISCAPA is an immuno-mass spectrometric approach in which a high affinity antibody specific for a specific proteotypic peptide is used to enrich the target analyte from a complex protein mixture such as a human plasma digest and quantify it against a known amount of a stable isotope internal standard. This targeted workflow enables reproducible quantitation on much lower abundance proteins from complex samples with much higher throughput. The workflow utilizes an ‘addition-only’ protocol whereby liquid reagents are added to the samples throughout the procedure without any separation steps (e.g. centrifugation, long chromatographic separation etc.). Hence, the sample preparation procedure simplifies to a series of liquid handling steps, which can easily be programmed onto liquid handling robots. In fact, we recently demonstrated that by automating the SISCAPA workflow, proteins of various abundance and size can be measured with intra-run precisions of ~5%¹.

In this report, we present the first completely hands-free SISCAPA workflow implemented on a Biomek NX®P Workstation. The workflow starts with human plasma/serum/blood samples, analyte-specific SISCAPA antibodies, the corresponding stable isotope standards and the necessary reagents as input material. The automated protocol carries the samples through a) tryptic digestion, b) a SISCAPA enrichment of target analytes and c) the proprietary SISCAPA wash and elute procedures. The enriched peptides are eluted into an elution plate and analyzed using a QTRAP® 6500 system. The protocol is designed to be hands-free and high-throughput with maximum capacity of 10 plates (960 samples) per day per robot.

Summary
- In this report we present the first hands-free, fully automated version of the SISCAPA workflow implemented on a Beckman Coulter Biomek NX®P platform.
- The workflow was used to measure endogenous levels of five proteins whose abundance spans 8 orders of magnitude.
- Total workflow CVs ranged from 2.1% - 6.7% for different proteins.
- The platform is capable of processing two 96-well plates (192 samples) through denaturation, tryptic digestion and SISCAPA peptide enrichment in 4.5 hours, or a maximum of 960 samples per day.
Methods

Peptides, Antibodies and Plasma: Five proteotypic, surrogate tryptic peptides were chosen to represent human plasma proteins of varying size and abundance (Table 1). Stable isotope labeled versions of the target (endogenous) peptides were synthesized using solid-phase methods by New England Peptide (MA, USA). For the stable isotope standard (SIS) peptides, an isotope labeled C-terminal arginine or lysine was used to provide mass shifts of +10 amu and +8 amu, respectively.

The SISCAPA Workflow: The “addition only” digestion protocol has been described in detail\(^1\) and is summarized in Figure 1. Briefly, the samples were first denatured in a mixture of urea, Trizma and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) such that their respective concentrations were 9M, 0.2M and 0.03M during the reaction. After denaturation, the samples were alkylated using 0.06M iodoacetamide, diluted in 0.2M Trizma to a final urea concentration of 1M and digested using trypsin at a protein:enzyme ratio of 10:1. We chose a digestion time of 3 hours, as has been previously determined to yield maximum endogenous signal for all proteins under study. The SIS peptides were added before digestion. After digestion, the assay-specific antibodies coupled to protein-G magnetic beads were added to the samples to capture the endogenous analyte and the corresponding SIS peptide. The enriched peptide targets, now bound to the antibody, were then subjected to a series of wash steps followed by acid-elution from the antibody.

Experimental Design: The pooled human plasma was processed as 16 replicates. The eluates from eight of the replicates were pooled at the end of the process to evaluate the imprecision contributed by the mass spectrometric measurements. The remaining eight replicates were processed separately to evaluate the total workflow coefficient of variation (CV). The following sum of squares model was used to calculate the imprecision contributed by the upstream workflow implemented on the Biomek NX\(^\circ\) platform:

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CV_{\text{Biomek}}^2 = CV_{\text{Total Workflow}}^2 - CV_{\text{MS}}^2
\]

Table 1. List of Analytes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Approximate ‘Normal’ range (mg.ml)</th>
<th>MW (Da)</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>35,000,000 – 50,000,000</td>
<td>69,367</td>
<td>LVNEVTEFAK</td>
</tr>
<tr>
<td>Apolipoprotein</td>
<td>400,000 – 1,250,000</td>
<td>515,605</td>
<td>FPEVDVLTK</td>
</tr>
<tr>
<td>CystatinC</td>
<td>600 – 1,500</td>
<td>15,799</td>
<td>ALDFAVGEYNK</td>
</tr>
<tr>
<td>Soluble Transform Receptor</td>
<td>1,800 – 4,600</td>
<td>84,871</td>
<td>GFVEDHYVVGAQR</td>
</tr>
<tr>
<td>Mesothelin</td>
<td>10 - 80</td>
<td>68,986</td>
<td>LLGPHVEGLK</td>
</tr>
</tbody>
</table>

Pooled human plasma was obtained from BioReclamation, Inc. (NY, USA)
Mass Spectrometer Configuration: For each target peptide, the MS parameters (collision energies and declustering potentials) were optimized by injecting 10 µL of 100 fmol/µL solution of the peptide mixture into the LC-MS/MS system and analyzed using Skyline software (University of Washington, USA). The LC-MS/MS platform consisted of a nanoLC™ 425 system operating in microflow mode coupled to a QTRAP® 6500 System (SCIEX, USA). A 15 µL aliquot of each sample was loaded on a trap-elute configuration where a conserved flow gradient was developed at 3% for 2 minutes before switching the injection valve to separate the peptides on a 2.7µm HALO Peptide-ES C18 column (SCIEX, Part No. 5039576) with a flow rate of 10 µL/min. The target peptides were separated using a 10 minute analytical gradient with 0.1% formic acid / 5% DMSO in water as solvent A and 90% acetonitrile / 5% DMSO in 0.1% formic acid in water as solvent B. The peak area ratios were analyzed using MultiQuant™ software (SCIEX).

Results

The workflow presented here was designed with three key requirements in mind: a) to demonstrate liquid handling precision b) to be completely hands-free and c) to be high-throughput (capable of processing ~1000 samples per day).

The two key steps of the workflow that significantly impacted the precision were determined to be the SIS peptide addition step and the Clean Plate transfer step. The SIS peptide addition was optimized by manipulating the liquid handling parameters: a 5 µL leading air-gap was aspirated before aspirating the SIS mixture, the content was blown out in the liquid without a tip touch at a speed of 10 µL/s. The Clean Plate was introduced into the washing procedure to reduce the amount of non-specific background peptides thus improving the signal to noise ratio and the overall CV (Figure 3).

Figure 3. Reduction of Non-Specific Binding Through Efficient Washing Steps. The chromatogram for endogenous ApoB peptide (blue) and the corresponding SIS peptide (pink) with (A) and without (B) the Clean Plate in the protocol.
Typical chromatograms for the five endogenous targets are shown in Figure 4, illustrating the difference in abundance. The CV analysis for the experiment is presented in Table 2. The total workflow CV ranged from 2.1% to 6.7% for the different proteins. Consistent with previous reports, the imprecision seen with some assays (albumin and sTfR) was primarily contributed by the mass spectrometric measurements hence making it impossible to accurately estimate the very low imprecision due to the automated SISCAPA workflow itself. In cases where measuring the imprecision of upstream sample preparation was possible, the results ranged from 3.6% to 5.0% for different proteins, which includes the imprecision due to digestion variability and the liquid handling imprecision of the Biomek during the SISCAPA procedure.

While the automated method is flexible to accommodate the smaller sample number tested here, it is designed to fully process two 96-well plates (192 samples) in 4.5 hours. Performing five consecutive runs would enable the processing of 960 samples per instrument per day, thereby achieving our desired throughput.

**Table 2. CV Analysis.** Eight technical replicates were used to calculate the total workflow CV. Eight replicates were pooled at the end of the workflow and before MS analysis to calculate the MS CV. The Biomek CV was estimated using the sum of squares model.

<table>
<thead>
<tr>
<th></th>
<th>Alb</th>
<th>ApoB</th>
<th>CC</th>
<th>sTfR</th>
<th>Meso</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MS CV</strong></td>
<td>4.1%</td>
<td>3.3%</td>
<td>1.0%</td>
<td>3.2%</td>
<td>5.7%</td>
</tr>
<tr>
<td><strong>Biomek CV</strong></td>
<td>N/A</td>
<td>4.6%</td>
<td>5.0%</td>
<td>N/A</td>
<td>3.6%</td>
</tr>
<tr>
<td><strong>Total CV</strong></td>
<td>2.1%</td>
<td>5.6%</td>
<td>5.1%</td>
<td>2.7%</td>
<td>6.7%</td>
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</table>
Conclusions
The results indicate that the automated SISCAPA workflow implemented on the Biomek NX™ platform can be used for precise measurement of proteins of varying sizes and concentrations in plasma. Importantly, this workflow was designed to be completely hands-free and high-throughput and thus is suitable for widespread translational research applications.

References: