A fast and simple workflow for surrogate peptide bioanalysis: NISTmAb case study

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Application benefits
• Fast and simple workflow for the quantitative analysis of monoclonal antibodies with 96 samples manually processed in under four hours
• Highly reproducible extractions using immunocapture, tryptic digestion demonstrating single digit accuracy and precision across the analytical range
• Selective and sensitive analysis with LLOQ of 10 ng/mL from just 50 µL of sample.

Goal
To test a streamlined workflow for the quantitative bioanalysis of chimeric or humanized monoclonal antibodies from a non-human matrix such as rat plasma using affinity capture, tryptic digestion and LC-MS/MS analysis.

Introduction
This application note describes a fast and simple approach to sample preparation of biotherapeutics that reduces the workflow from a day or more to a few hours (Figure 1) and is simple to implement. This workflow maintained the necessary high levels of accuracy, precision (1–9% calculated concentration deviation and variability), and sensitivity with a linear range of 10 to 10,000 ng/mL, using only 50 µL of sample.
The ability to perform accurate and robust quantitative bioanalysis of therapeutic monoclonal antibodies (mAbs) is critical to successfully bring new drugs to market. Accurate data on pharmacokinetics must be available as early as possible in the development process as it contributes to the final success or failure of the compound. The initiation of early absorption, distribution, metabolism, and excretion (ADME) screening has dramatically decreased the proportion of compounds failing in clinical trials. The main aim of preclinical ADME is to eliminate weak drug candidates in the early stages of drug development; this allows resources to be focused on more promising potential drug candidates. Undesirable pharmacokinetic properties such as poor absorption or extensive first-pass metabolism are a major contributor to the failure of many drug candidates in early stages of drug development programs.

Early-stage studies, typically within the drug discovery or development phase, have adopted the use of generic methodologies, or assay starting points, to quickly and cost effectively develop analytical methods. Studies in these phases have the goal of generating blood concentration data as quickly as possible in order to make fast decisions whether to progress a drug candidate to the next phase, or to ‘fail quick, fail cheap’ for candidates that, for example, do not show the expected ADME performance.

It is difficult to justify extensive method development times as short studies and quick data turn-around is required to efficiently progress candidates. The use of enabling technologies to simplify the workflow and help to reduce failure rates are vital tools for the bioanalytical scientist.

Many pharmaceutical companies and clinical research organizations (CRO), with limited bio-molecule experience, are now being asked to perform accurate quantitative analyses of biotherapeutics in a variety of matrices across the drug’s lifecycle, from discovery through to clinical studies. While small molecule scientists are experts in techniques required for small molecule analysis, they often have little hands-on experience with quantifying large molecules.

One of the most common methods for protein quantitation is the surrogate peptide approach, particularly in mass spectrometry (MS)-based analyses. Proteins are digested, or broken down into smaller peptides, which can be easier to analyze and interpret than the intact proteins. Peptides are generally more amendable to triple quadrupole MS detection, which is still the tool of choice for fast and sensitive bioanalytical quantitation. This approach, however, can be time-consuming and complex due to the number of steps and reagents required for sample preparation.

After the protein has been digested (typically through the use of trypsin), one or more peptides with good selectivity and specificity are chosen as a surrogate measure for the protein and analyzed by liquid chromatography (LC)-MS or LC-MS/MS. However, direct digestion of plasma is often not advisable due to its lack of selectivity. The final extract will contain peptides derived from every protein in the sample, such as abundant immunoglobulins. This can have a dramatic effect on the level of sensitivity that can be achieved.

Immunoaffinity capture can be used prior to digestion to selectively enrich for the target protein. Addition of this step results in a far cleaner sample and improves the sensitivity and reproducibility of the assay as many similar matrix components are removed.
Highly reproducible and sensitive immunocapture and protein digestion methods can be achieved in a single well using Thermo Scientific™ SMART Digest™ ImmunoAffinity (IA) kits. These kits were designed to remove the challenges associated with the processing of biomolecule quantitation by providing a simple and easy capture-digestion workflow that takes only hours instead of overnight processing. Both capture purification and tryptic digestion can be performed on the same magnetic bead. The kits are available with Protein A, Protein G, or Streptavidin options, as well as magnetic or non-magnetic based beads.

Here we describe a generic approach with the goal of creating a set of conditions that can be used to quantitatively analyze fully human to chimeric mAbs in non-human matrices, such as rodent plasma. By loading an antibody onto the SMART Digest IA beads that target the human IgG Fc region, a generic affinity-based extraction can be performed to purify a range of proteins, from chimeric mAbs to human IgGs with a simple and fast workflow.

Effective LC gradient conditions were used to provide excellent retention time precision by combining the Thermo Scientific™ Vanquish™ Horizon UHPLC system with a Thermo Scientific™ Acclaim™ 120 C18 column. The Acclaim column features an ultrapure silica substrate with extremely low metal content to minimize tailing effects and deliver symmetrical peak shapes. The unique bonding chemistry yields excellent surface coverage to provide highly predictable separations that are unaffected by secondary interactions.

Designed with innovative technology, the Vanquish Horizon UHPLC system delivers a new standard in high-end UHPLC. This fully integrated and biocompatible system features high sample capacity for high-throughput workflows, industry-leading pumping performance, linearity, two-mode column thermostating, and active mobile phase preheating.

Detection of peptides was performed with the Thermo Scientific™ TSQ Quantiva™ triple quadrupole mass spectrometer, which uses active ion management to exceed even the most stringent analytical requirements with superb sensitivity, speed, and dynamic range.

Tryptic peptides from the mAb Fc region were purposefully chosen to be monitored for analysis because they are abundant in a range of chimeric, humanized, and fully human mAbs, but are not present in rat or mouse plasma. More specific peptides from the mAb variable region can also be monitored, along with the use of a more specific capture antibody once progressing to human matrices, to improve method specificity when required. The conditions demonstrated here were chosen to minimize the method development required to create a robust, quantitative assay for the analysis of chimeric to fully human mAbs in a non-human matrix.

**Experimental Consumables**
- Fisher Scientific™ Optima™ UHPLC-MS grade water (P/N 10154604)
- Fisher Scientific™ Optima™ UHPLC-MS grade methanol (P/N A458-1)
- Fisher Scientific™ Optima™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Fisher Scientific™ Analytical grade formic acid (P/N F/1900/PB08)
- Fisher Scientific™ Analytical grade ammonia (P/N A/3295/PB05)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Acclaim 120 C18 column, 50 × 2.1 mm, 2.2 µm (P/N 068989)
- SMART Digest ImmunoAffinity (IA) Protein G kit (P/N 60112-101)

**Sample preparation**

**Sample preparation system**

**Instrumentation:**
- Thermo Scientific™ Thermal Mixer (P/N 13687720)
- Thermo Scientific™ Virtuoso™ Vial Identification system (P/N 60180-VT100)

**Compounds:**
- RM 8671 - NISTmAb, Humanized IgG1κ Monoclonal Antibody

**Internal standards:**
- Stable-isotope-labeled human IgG containing $^{13}$C$_6$, $^{15}$N$_2$-labeled arginine and $^{13}$C$_6$, $^{15}$N$_2$-labeled lysine
Capture antibody: Anti-human IgG (Fc region)
Matrix: Rat Plasma (lithium heparin)
Affinity capture and digestion product: SMART Digest IA kit, Protein G magnetic (P/N 60112-104)

**Bead preparation**
SMART Digest IA kit Protein G magnetic beads were bulk prepared with anti-human IgG antibody as directed by the technical guide; for every 30 µL of SMART Digest Protein G resin, 5 µg of capture antibody was added and mixed for 30 minutes to allow binding to occur. Following three washes with phosphate buffer solution (PBS) to remove any unbound material, a solution of 0.01% glutaraldehyde was used as a cross linking agent. Finally, TRIS buffer solution was added to quench the reaction.

**Sample preparation**
Blank rat plasma was spiked with various concentrations of RM 8671 - NISTmAb, Humanized IgG1κ Monoclonal Antibody to produce a calibration curve from 10 to 10,000 ng/mL. Additional matrix was spiked at 50, 500, and 5000 ng/mL to act as QC samples. Blank matrix samples were also prepared.

50 µL of each concentration of calibration standard (n=2 for LLOQ and ULOQ), QC sample (n=6), and blank sample (n=4) were aliquoted into lo-bind centrifuge tubes.

200 µL of internal standard (stable isotope labeled human IgG containing $^{13}$C$_6$, $^{15}$N$_2$-labeled arginine and $^{13}$C$_6$, $^{15}$N$_2$-labeled lysine), prepared at 1 µg/mL in PBS, was added to each tube except for matrix blanks, where 200 µL of analyte-free PBS was added.

Finally, 30 µL of prepared SMART Digest IA kit, Protein G magnetic beads were added to each tube. Protein G variant of the kit was chosen in this case due to high affinity to the antibody strain. The variant is typically selected based on high affinity to the antibody and low affinity to the matrix species.

**Affinity capture and digestion**
The samples were mixed at 14,000 rpm at room temperature for approximately two hours. Each sample was washed by centrifuging the tube, removing the supernatant, and replacing volume to volume with SMART Digest IA wash buffer (included in the kit). This step was repeated four times. On the final wash step, 50 µL of liquid was left behind in the sample. 150 µL of SMART Digest buffer was added to each sample. All samples were mixed at 1400 rpm at 70 °C for 1 hour to facilitate tryptic digestion.

Finally, the samples were centrifuged, the supernatant removed, diluted 1:1 with 1% TFA (aq) in a lo-bind 96 well plate (included with SMART Digest kit), and placed into an autosampler set to 4 °C ready for analysis.

**Method optimization**
To find suitable affinity capture times, a time-course experiment was performed, varying the time of the capture step, and concluding after the monitored tryptic peptide (TTPVLDSDGFLYSK) no longer increased in signal (Figure 2). An affinity capture time of 120 minutes was chosen for this assay.

![Figure 2. Time course experiment for capture step.](image)

Following affinity capture, digestion time was optimized using a similar time course experiment, again monitoring tryptic peptide (TTPVLDSDGFLYSK) until the signal reached a plateau (Figure 3). The optimal digestion time of 60 minutes was chosen for this assay.
The Acclaim 120 C18 50 × 2.1 mm id, 2.2 µm analytical column was chosen for analysis due to sufficient pore size and retentive characteristics for peptide analysis. A simple water/acetonitrile (with acid modifier) gradient was employed as a starting point for separation as this was likely to separate many potential of target peptides. This gradient can be easily modified for a more targeted analysis if needed (Figure 4).

Peptides used for quantitation

Two tryptic peptides, VVSVLTVLHQDWLNGK and TPPVLDSDGSFFLYSK, from the Fc region of the analyte were chosen for analysis due to their abundance in a range of chimeric, humanized, and fully human mAbs (highlighted examples in Figure 5). Additionally, the highlighted peptides are not present in either rat or mouse plasma, making them suitable for a “semi-generic” mAb quantitation method. Where more selective methods are required, specific peptides from the variable region of the mAb can be monitored, along with the use of a more specific capture antibody. This is often required once progressing to human matrices.

For comparative purposes rituximab, RM 8671 NISTmAb, and human IgG were spiked separately into water and rat plasma, extracted and analyzed under the same conditions. Very similar responses and recoveries were observed upon analysis of each sample, demonstrating the potential for this generic methodology (Figure 6).

Stable isotope labeled human IgG containing $^{13}$C$_6$, $^{15}$N$_2$-labeled arginine and $^{13}$C$_6$, $^{15}$N$_2$-labeled lysine was chosen as the internal standard, which, in general behaved as an excellent extraction mimic, compensating for small inconsistencies as well. However, the standard did contain an approximate 1% impurity of unlabeled material (Figure 7), which could compromise the selectivity of the
The concentration of the internal standard was kept as low as possible, and the lower limit of the assay set appropriately so as negate the impact of the impurity.

![Figure 7. Example chromatogram of the internal standard. Stable isotope labeled human IgG containing $^{13}$C$_6$, $^{15}$N$_4$-labeled arginine and $^{13}$C$_6$, $^{15}$N$_2$-labeled lysine, injected at a high concentration to measure the level of impurity.](image)

**LC conditions**

**Instrumentation**

Vanquish Horizon UHPLC system consisting of the following:

- System Base Vanquish Horizon (P/N VH-S01-A)
- Binary Pump H (P/N VH-P10-A)
- Split Sampler HT (P/N VH-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (P/N 6732.0110)
- MS Connection Kit Vanquish (P/N 6720.0405)

**Separation conditions**

Mobile phase A: 0.1% formic acid in water
Mobile phase B: 0.1% formic acid in 90:10 acetonitrile/water (v/v)

**LC gradient conditions:**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>1.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>4.0</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>4.0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>4.5</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>4.5</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>5.5</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Flow rate: 0.5 mL/min
Column temperature: 50 °C, still air
Injection details: 20 µL
Injection wash solvent: 1:1 mix of mobile phase A and B

**MS conditions**

**Instrumentation**

TSQ Quantiva triple quadrupole mass spectrometer (IQLAAEAAAXFAOUMZZZ)

**MS settings**

MS settings are provided in Tables 1 and 2.

**Table 1. MS source parameters.**

<table>
<thead>
<tr>
<th>MS Source Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Thermo Scientific™ Ion Max ion source with HESI-II probe</td>
</tr>
<tr>
<td>Polarity</td>
<td>Positive ionization</td>
</tr>
<tr>
<td>Spray voltage (V)</td>
<td>3500</td>
</tr>
<tr>
<td>Vaporizer temperature (°C)</td>
<td>400</td>
</tr>
<tr>
<td>Sheath gas pressure (psi)</td>
<td>50</td>
</tr>
<tr>
<td>Aux gas pressure (Arb)</td>
<td>15</td>
</tr>
<tr>
<td>Ion transfer tube temperature (°C)</td>
<td>350</td>
</tr>
<tr>
<td>CID gas pressure (mTorr)</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Table 2. Compound transition details.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>TTPPVLDSDFSFLYSDK</th>
<th>IntStd</th>
<th>VVSVLTVLHGDWLNK</th>
<th>IntStd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor (m/z)</td>
<td>937.5</td>
<td>941.5</td>
<td>603.3</td>
<td>606.0</td>
</tr>
<tr>
<td>Products (m/z)</td>
<td>836.6</td>
<td>840.5</td>
<td>805.6</td>
<td>809.5</td>
</tr>
<tr>
<td>Collision energy</td>
<td>25</td>
<td>25</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>
Data processing
The Thermo Scientific™ Chromeleon™ 7.2 SR5 Chromatography Data System (CDS) was used for data acquisition and analysis.

Results and discussion
A generic method for the analysis of chimeric mAbs is described. The method consisted of immunoaffinity capture followed by rapid digestion in the same well using SMART Digest IA Protein G kits. The assay described would be suitable for the analysis of a wide range of mAbs, with little to no optimization, making it an effective method to quickly implement for analysis of target mAbs in a non-human matrix.

SMART Digest IA kits provide a streamlined approach to immunoaffinity capture and digestion. The incorporation of both capture antibody and digestion enzyme on the same bead limits the amount of manual transfers within a workflow and so minimizes processing errors. Heat-stable trypsin speeds up the digestion time and minimizes addition of reagents that may have an adverse effect on the assay.

While human IgG and rituximab were used to demonstrate the applicability of the method to alternative analytes, RM 8671 – NISTmAb, Humanized IgG1κ Monoclonal Antibody was used as a case study example for quantitative analysis. A method was created with a 1000-fold linear range from 10 to 10,000 ng/mL using only 50 µL of sample.

Table 3. Accuracy values showing linearity of the calibration curve and mean values of the low, mid, and high QC levels

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linearity Range (ng/mL)</th>
<th>Coefficient of determination</th>
<th>Mean Relative Error (%) at QCL (50 ng/mL) n=6</th>
<th>Mean Relative Error (%) at QCM (500 ng/mL) n=6</th>
<th>Mean Relative Error (%) at QCH (5000 ng/mL) n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTPPVLDSDGSFFLYSK</td>
<td>10–10,000</td>
<td>0.9985</td>
<td>-5.99%</td>
<td>-3.35%</td>
<td>1.14%</td>
</tr>
<tr>
<td>VWSVLTVLHQDWLNGK</td>
<td>10–10,000</td>
<td>0.9984</td>
<td>-2.70%</td>
<td>-5.56%</td>
<td>8.87%</td>
</tr>
</tbody>
</table>

Accuracy and precision data were excellent over the dynamic range with single digit mean accuracy values obtained with the QC samples, and a minimum correlation value of 0.99 for both peptides, with a linear 1/x fitting applied (Figure 8 and Table 3).
Table 4 shows the excellent precision obtained for six replicates of QC samples at low, mid, and high levels spanning the calibration range.

### Table 4. Precision values for the QC samples.

<table>
<thead>
<tr>
<th>Compound</th>
<th>QCL (50 ng/mL) n=6</th>
<th>QCM (500 ng/mL) n=6</th>
<th>QCH (5000 ng/mL) n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTPPVLDSDGSFLYSK</td>
<td>6%</td>
<td>3%</td>
<td>1%</td>
</tr>
<tr>
<td>VSVLTVLHQDWLNGK</td>
<td>3%</td>
<td>4%</td>
<td>9%</td>
</tr>
</tbody>
</table>

Recovery of the immunoaffinity capture step from water and from plasma were measured by comparing a standard spiked into water and digested, to a standard enriched from water and plasma, then digested. High and precise recovery from water was observed at 97% with precision of less than 1.5 RSD, and average recovery from plasma was 73% with 8.6% RSD. Matrix effects were calculated by comparing a standard spiked into an extracted rat plasma blank to the aforementioned digested standard (Tables 5 and 6).

### Table 5. Recovery levels from water and plasma.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average Peak Area (n=4)</th>
<th>% Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested standard</td>
<td>342883</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Capture from water</td>
<td>337436</td>
<td>97%</td>
<td>1.42%</td>
</tr>
<tr>
<td>Capture from plasma</td>
<td>233047</td>
<td>73%</td>
<td>8.60%</td>
</tr>
</tbody>
</table>

### Table 6. Matrix effects.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Signal Suppression (Matrix Effects)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTPPVLDSDGSFLYSK</td>
<td>6%</td>
</tr>
<tr>
<td>VSVLTVLHQDWLNGK</td>
<td>11%</td>
</tr>
</tbody>
</table>

Representative chromatograms for the lower limit of quantitation (LLOQ) (Figure 9) and a blank sample spiked with internal standard (Figure 10) show excellent signal-to-noise for the LLOQ and good selectivity for the internal standard. As discussed previously, assessment of the internal standard showed approximately 1% impurity and so the internal standard concentration and the LLOQ were selected to negate any selectivity from the impurity.
Conclusions

- Significantly faster sample preparation than traditional in solution digestion protocols, typically a few hours

- Significantly simplified sample preparation workflow compared to traditional in-solution digestion protocols due to fewer steps and reduction in reagents

- Accurate and precise results achieved (1–9% accuracy and precision values obtained)

- Lower limit of quantitation of 10 ng/mL obtained with only 50 µL of sample

- Possibility to transfer to other biotherapeutic mAbs, with little to no modification

References

2. SMART Digest ImmunoAffinity (IA) Kit User Manual, Version 1, Revision A
3. Thermo Scientific Application Note 21504: Fast Digestion Method Optimization
   https://tools.thermofisher.com/content/sfs/brochures/AN-21504-SP-SMART-Digest-Digestion-Endpoint-AN21504-EN.pdf
4. Thermo Scientific Acclaim 120 C18 columns
   https://www.thermofisher.com/order/catalog/product/059142

Find out more at
thermoscientific.com/BuiltForBiopharma
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thermoscientific.com/appslab