A Fast and Simple Workflow for Surrogate Peptide Bioanalysis: NIST mAb Case Study

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ABSTRACT

This poster describes a fast and simple approach to the sample preparation of biotherapeutics which reduces the workflow from a day or more to a few hours; and is simple to implement.
The use of immunoaffinity capture, coupled with heat stable, immobilized tryptic digestion, results in a clean extract containing specific surrogate peptides used for accurate quantitation.

Thermo Scientific™ SMART Digest™ ImmunoAffinity (IA) Protein G kit was used to isolate RM 8671 - NISTmAb, a humanized IgG1κ Monoclonal Antibody, from rat plasma before heat assisted tryptic digestion was performed. The capture antibody and digestion enzyme are both immobilized on the same bead and are active under different conditions. As a result, all sample preparation steps in this methodology are performed with minimal sample manipulation. This reduces the potential for manual handling errors and also simplifies the workflow.

The assay maintained high levels of accuracy, precision and sensitivity (1-9% calculated concentration deviation and variability) with a linear range of 10 to 10,000 ng/mL, using only 50 µL of plasma.

INTRODUCTION

One of the most common methods for protein quantitation is the surrogate peptide approach, particularly in mass spectrometry (MS)-based analyses. Proteins are digested into smaller peptides which can be easier to analyze and interpret than the intact proteins.

Peptides are generally more amenable 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 potentially 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 the sample. The addition of this step results in a significantly cleaner sample and improves the sensitivity and reproducibility of the assay where as many similar matrix components are removed.

Figure 1; Thermo Scientific™ SMART Digest™ ImmunoAffinity (IA) Protein G kit and optional accessories

MATERIALS AND METHODS

Consumables:

Thermo Scientific™ Acclaim™ 120 C18 50 x 2.1 mm, 2.2 µm column	(P/N 068989)
SMART Digest ImmunoAffinity Protein G	(P/N 60112-101)
Thermo Scientific™ Thermal Mixer	(P/N 13687720)

Compounds:
RM 8671 - NISTmAb, Humanized IgG1κ Monoclonal Antibody
Internal standards:
Stable isotope labelled Human IgG
Capture antibody:
Anti-human IgG (FC region)
Matrix:
Rat plasma (Lithium heparin)

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 labelled human IgG containing $^{13}C_6$, $^{15}N_4$-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. The Protein G variant of the kit was chosen in this case due to high affinity to the antibody type. 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 1400 rpm at room temperature for approximately 2 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. Optimal capture and digestion times were determined experimentally (figure 2 and 3).

Finally, the samples were centrifuged, the supernatant removed, and 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.

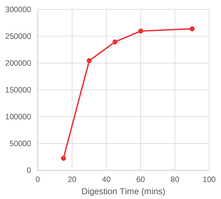

Figure 2; Time course experiment to determine the optimal digestion time by observing response of surrogate peptide TTPPVLDSDGSFFLYSK

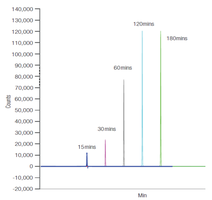

Figure 3; Time course experiment to determine the optimal capture time by observing response of surrogate peptide TTPPVLDSDGSFFLYSK

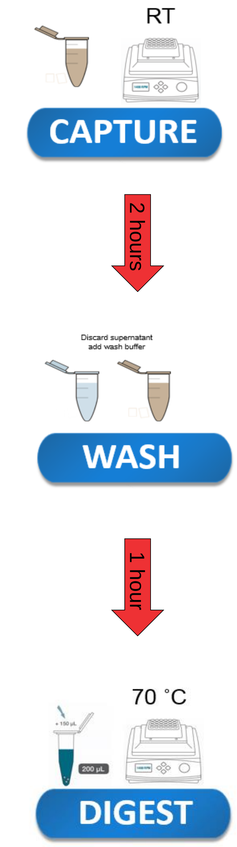

Figure 4; Three step process, all carried out on the same bead, for capture, wash and digestion of target mAb.

ANALYSIS

Assessment of methodology
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. The average recovery from plasma was 73% with 8.6% RSD.

Compound	Average Peak Area (n=4)	% Recovery	%RSD
Digested standard	342883	-	-
Capture from water	337436	97%	1.42%
Capture from plasma	233047	73%	8.60%

Table 1; Extraction recovery for surrogate peptides TTPPVLDSDGSFFLYSK from water and rat plasma

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 regression applied (figure 5). Precision values for low, mid and high QC are shown in table 2.

Compound	QCL (50 ng/mL) n=6	QCM (500 ng/mL) n=6	QCH (5000 ng/mL) n=6
TTPPVLDSDGSFFLYSK	6%	3%	1%
VVSVLTVLHQDWLNGK	3%	4%	9%

Table 2; Precision values for QC samples extracted n=6

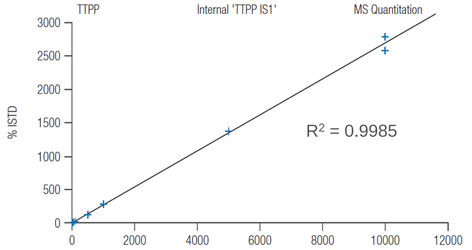

Figure 5; Calibration line extracted from rat plasma over the range of 10 to 10,000 ng/mL

The Thermo Scientific™ Chromeleon™ 7.2.6 Chromatography Data System (CDS) was used for data acquisition and control of the Thermo Scientific™ Vanquish™ Horizon UHPLC system. 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 target peptides. This gradient can be easily modified for a more targeted analysis if needed.

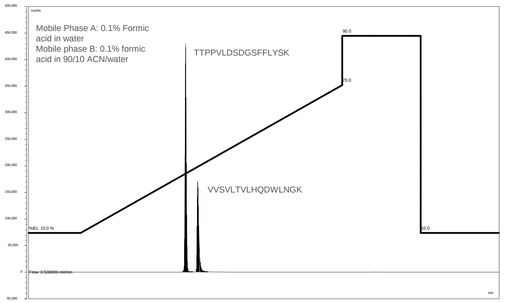

Figure 6; Liquid chromatography conditions used in the analysis on an Acclaim 120 C18 2.2 µm 50 x 2.1 mm id analytical column

Compound	TTPPVLDSDGSFFLYSK	IntStd	VVSVLTVLHQDWLNGK	IntStd
Precursor (*m/z*)	937.5	941.5	603.3	606.0
Products (*m/z*)	836.6	840.5	805.6	809.5
Collision energy	25	25	15	15

Table 3; Monitored transitions for surrogate peptides TTPPVLDSDGSFFLYSK and VVSVLTVLHQDWLNGK

CONCLUSIONS

- Significantly faster sample preparation than traditional in solution immune affinity and digestion protocols, typically a few hours
- Significantly simplified sample preparation workflow compared to traditional in-solution digestion protocols
- 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

FURTHER READING

- SMART Digest ImmunoAffinity (IA) Kit User Manual, Version 1, Revision A
- Thermo Scientific Application Note 21504: Fast Digestion Method Optimization
- Thermo Scientific Application Note AN21767-EN 0418S: A fast and simple workflow for surrogate peptide bioanalysis: NISTmAb case study

TRADEMARKS/LICENSING

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