APPLICATION NOTE 22107

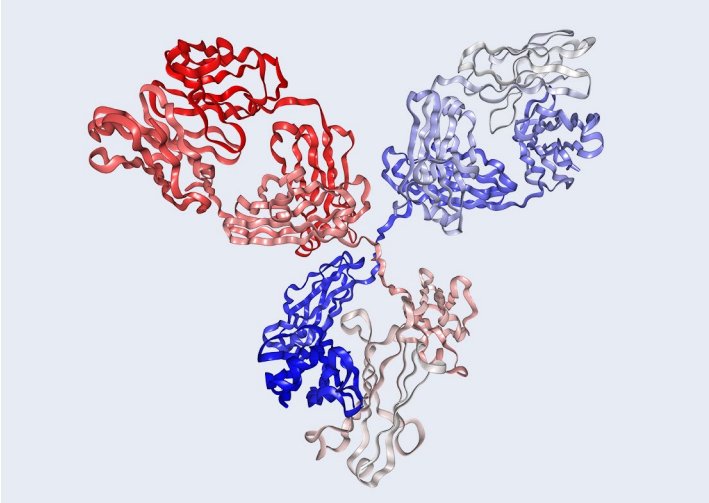
Reproducible IA-LC-MS/MS quantitation of human IgG from plasma with SMART Digest Fractionation Kits

Authors: Kevin Meyer, Perfinity Biosciences, Inc., West Lafayette, IN, USA

Matthew Franklin, Thermo Fisher Scientific, Runcorn, Cheshire, UK

Mike Oliver, Thermo Fisher Scientific, Runcorn, Cheshire, UK

Keywords: Immunoaffinity-capture, SMART Digest bulk fractionation, surrogate peptide quantitation, human IgG, animal matrix, digestion, Accucore C18 column



Goal

To demonstrate the reproducible immunoaffinity capture and tryptic digestion of human IgG using Thermo Scientific™ SMART Digest™ Bulk Fractionation kits to improve sensitivity and sample cleanliness.

Introduction

The global biologics market is one of the fastest-growing areas in the pharmaceutical industry, with total revenues exceeding $231 billion in 2017 alone.[1] While the production and use of these drugs continue to grow, better, more robust and reproducible ways of analyzing, monitoring, and measuring these structures must adapt and change to keep pace. In the past, biotherapeutics were quantified using immunoaffinity methods like ELISA (enzyme-linked immunoassay). However, these types of assays are susceptible to cross-reactivity and a notable lack of specificity.[2]

With the advent of bottom-up proteomics, whereby surrogate peptides of the biotherapeutic structure are produced using enzymatic digestion and analyzed, immunoaffinity capture methods are increasingly coupled to LC-MS/MS to improve assay specificity.[3] However, the sample preparation of these molecules, such as immunoaffinity capture and enzymatic digestion, can be complicated and susceptible to variation.

A popular option is to combine these two steps into one easy product, such as with the Thermo Scientific™ SMART Digest™ ImmunoAffinity (IA) kits. The products utilize a co-immobilized bead that is a seamless, easy to use option that can reduce a 24 hour sample capture-digestion into only an hour.[4] While these kits enable the most sensitive workflow, there is a need for a preparative biotinylation or linkage step before IA-capture.

The streptavidin, Protein A, or Protein G on the surface of these beads needs to be conjugated to the anti-antibody of your target immunoglobulin, which means that this step must be validated every time a new capture antibody is utilized.

SMART Digest Bulk Fractionation Kits utilize Protein G enrichment for improved selectivity over digest and inject workflows while removing the requirement to validate a capture antibody. These kits remove the challenges often associated with protein cleanup by providing a simple and easy capture workflow. Unlike other SMART Digest IA products, the SMART Digest Bulk Fractionation kits contain beads that only have the Protein A or Protein G immunoaffinity capture chemistry on them. This means that the proteolytic digestion of proteins occurs in a secondary step with the addition of soluble SMART Digest trypsin. One of the advantages of this is that all target immunoglobulins, such as IgG in this case, are captured, irrespective of species or class. As a result, the beads can be washed thoroughly to remove matrix interferences and other protein structures, such as albumin, without worrying about losing excess target molecule. The extra cleanliness of these samples equates to significant gains in sensitivity.

With the SMART Digest Bulk Fractionation kits, there is also no need to perform an anti-antibody linkage step to the Protein A or G capture protein. While the anti-antibody linker/biotinylation step itself is not entirely cumbersome, the removal of it means that revalidating your method every time a new capture antibody is utilized is no longer necessary. This makes these kits particularly amenable to generic workflows that can be applied to a wider range of biopharmaceutical processing.

In this workflow, specific “universal” surrogate peptides of the Fc region of human IgG antibodies, that are notably absent from animal proteomes, were selected for quantitation from murine plasma. Protein G was chosen as the immunocapture reagent due to its greater binding affinity for human IgG subclasses.[5] The inclusion of an immunoaffinity-enrichment step in a workflow allows for the capture and concentration of proteins that are typically found at low levels in complex biological matrices. For LC-MS analysis, these surrogate peptides were monitored with selected reaction monitoring (SRM).

Experimental

Consumables

Affinity and digestion

- SMART Digest Bulk Fractionation kit, Protein G, magnetic, with soluble trypsin (P/N 60115-104)

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Chemical™ Optima™ acetonitrile (ACN) (P/N A955-4)
- Fisher Chemical™ formic acid (FA) (P/N F/1900/PB08)
- Mouse plasma from a reputable supplier
- Human IgG from a reputable supplier
- Fisher BioReagents™ pH 7.4 tris buffered saline (TBS) (P/N BP2471500)

Sample handling

- Thermo Scientific™ Matrix™ SepraSeal™ (P/N 4463)
- Eppendorf™ Deepwell™ Plates 96 (P/N E951032905)
- Thermo Scientific™ Nunc™ EZFlip™ Conical Centrifuge Tubes, 15 mL (P/N 362694)
- Thermo Scientific™ Graduated Safelock Microcentrifuge Tubes, 1.5 mL (P/N 3457PK)
- Thermomixer from a reputable supplier

Preparation of calibration and quality control (QC) samples

Human IgG was spiked into mouse plasma at concentrations between 0.1 and 100 µg/mL. Quality control samples were prepared at 0.1, 1, 10, and 100 µg/mL in mouse plasma.

Capturing human IgG

Murine plasma samples (2.5 µL), spiked with human IgG at varying concentrations, were added to a 96-deep well plate. The samples were then diluted 40-fold into pH 7.4 tris buffered saline (TBS) and added to 30 µL of magnetic Protein G beads. Samples were incubated at room temperature and shaken at 1,400 rpm for 30 min. After incubation, samples were magnetically pulled down and 80 µL were decanted. The beads were then washed twice with the addition and removal of 400 µL of TBS.

Tryptic digestion

After the final wash, 150 µL of SMART Digest buffer and 5 µL of soluble SMART Digest were added to the samples. The samples were capped with SepraSeals then incubated for 90 min at 70 °C and 1,400 rpm in a deep well plate on the thermomixer (with lid on). After incubation, the samples were decanted into an equal volume of 1% trifluoroacetic acid (TFA). 50 mL of the final solution was injected onto the LC-MS for analysis.

Separation conditions

Instrumentation

Thermo Scientific™ UltiMate™ 3000 Rapid Separation Dual System equipped with:

- SRD-3600 Solvent Racks with Degasser (P/N 5035.9230)
- DGP-3600RS Rapid Separation Pump (P/N 5040.0066)
- WPS-3000TRS Rapid Separation Thermostatted Well Plate Autosampler (P/N 5841.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)

Column

Thermo Scientific™ Accucore™ C18 column, 2.1 mm × 50 mm, 2.6 µm (P/N 17126-052130)

LC settings

Parameter	Value
Mobile phase A	0.1% formic acid in 98% water and 2% acetonitrile
Mobile phase B	0.1% formic acid in 10% water and 90% acetonitrile
Flow rate	0.5 mL/min
Column temp.	50 °C
Injection details	50 µL
Gradient	See Table 1

Table 1. LC gradient conditions

Time (min)	% A	% B
0	90	10
1	90	10
5	30	70
5.1	10	90
6.5	10	90
6.6	90	10
8	90	10

MS conditions

MS Instrumentation

Thermo Scientific™ Velos Pro™ ion trap mass spectrometer

MS settings

Parameter	Value
Settings	HESI
Mode	Positive
Heater temp.	350°C
Sheath gas	60
Aux gas	20
Spray voltage	4 kV
Capillary temp.	375 °C
S-lens RF level	55%
MS fragment	(See Table 2) *m/z* 603.4, 937.7

Note: Flow is diverted to waste using the divert valve until 1.5 min into the gradient. Flow is sent to the source from 1.5 min to 3 min into the gradient, and then sent to waste again at 3 min into the gradient.

Table 2. MS fragments information

Q1 mass	Q3 mass	Act Q	Act time (ms)	CE	Peptide sequence
603.4	805.4	0.25	10	35	VVSVLTVLHQDWLNGK
937.7	836.5	0.25	10	35	TTPPVLDSDGSFFLYSK

Data processing

The LC/MS instrument was controlled by Thermo Scientific™ Xcalibur™ software.

Results and discussion

Calibration of human IgG with SMART Digest IA Protein G kit

The SMART Digest IS Protein G kit produced exceptional reproducibility and linearity across the dynamic range from 0.1 µg/mL to 100 µg/mL. Two sets of calibration standards were used to produce two linear calibrations curves for both the 603 and 937 *m/z* surrogate peptides, achieving coefficients of determination of over 0.999 for each (Figures 1 and 2).

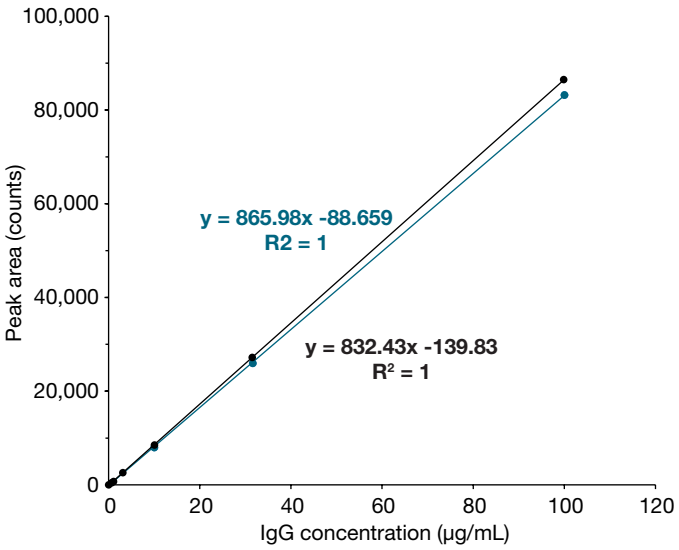


Figure 1. Calibration curve of the *m/z* 603 surrogate peptide fragment from the extraction of human IgG from mouse plasma using the SMART Digest IA Bulk Fractionation kit, Protein G, with soluble trypsin

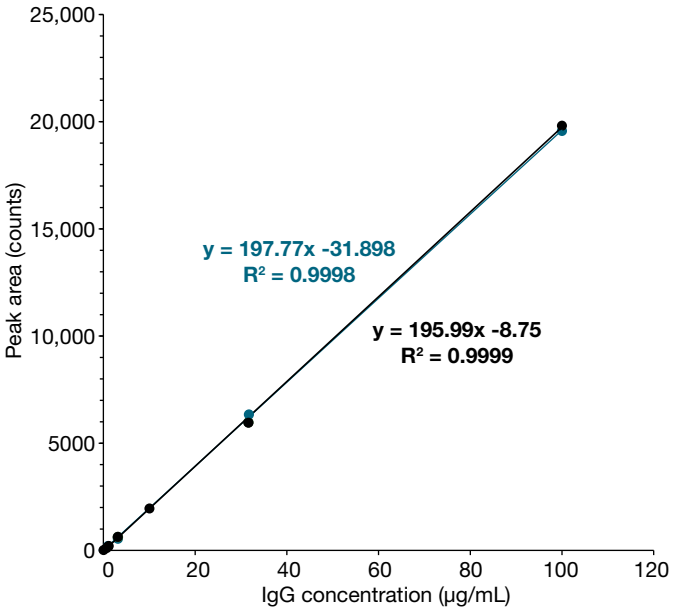


Figure 2. Calibration curve of the *m/z* 937 surrogate peptide fragment from the extraction of human IgG from mouse plasma using the SMART Digest IA Bulk Fractionation kit, Protein G, with soluble trypsin

The reproducibility of the method is also demonstrated with precision (CV%) of less than 6.5% when assessed using quality control standards for each surrogate peptide (Tables 3 and 4). This supports the use of SMART Digest Bulk Fractionation kits to confidently extract low concentrations of human IgG from murine plasma.

Table 3. Extraction of human IgG from mouse plasma using the SMART Digest IA Bulk Fractionation kit, Protein G, with soluble trypsin; low-, mid-, and high-quality control results for *m/z* 603 (n=6)

	Hu IgG concentration (µg/mL)		
Replicate	1	10	100
R1	828	8270	92083
R2	789	8419	90406
R3	793	8454	93881
R4	827	8714	95103
R5	786	8422	91019
R6	743	8883	88548
R7	769	9078	89658
R8	791	8661	90724
Average	791	8613	91428
St. Dev.	28	272	2174
CV (%)	3.5	3.2	2.4

Table 4. Extraction of human IgG from mouse plasma using the SMART Digest IA Bulk Fractionation kit, Protein G, with soluble trypsin; low-, mid-, and high-quality control results for *m/z* 937 (n=6)

	Hu IgG concentration (µg/mL)		
Replicate	1	10	100
R1	217	2045	22319
R2	221	2052	22545
R3	208	2175	22972
R4	233	2229	22542
R5	199	2159	21702
R6	193	2221	22439
R7	200	2284	23639
R8	202	2158	22815
Average	209	2165	22622
St. dev.	13	83	558
CV (%)	6.4	3.9	2.5

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Conclusion

The accurate and reproducible processing of human IgG antibodies for LC-MS analysis is achieved using SMART Digest Bulk Fractionation kits to enrich target concentrations, thoroughly clean and completely digest samples.

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AN22107-EN 1020S

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