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APPLICATION NOTE 21692

Rapid quantitation of therapeutic antibodies in animal plasma

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Keywords

Biopharmaceutical, bioanalysis, quantitation, peptide analysis, SRM, MRM, affinity proteomics, biotherapeutic, IgG, monoclonal antibody (mAb), human IgG, immunoaffinity, immunocapture, Protein A, Protein G, Streptavidin, SMART Digest™ Immunoaffinity (IA) Kits, UHPLC, Accucore™ C18, biotin, protein digestion, protein enzyme digestion, protein magnetic beads, sample preparation, 96 well plates

Application benefits

- Rapid sample preparation workflow - 15 step process reduced to five steps
- Simplified workflow - enrichment and digestion are carried out in one well
- Significant time reduction, digestion normally complete in under one hour
- Immunoaffinity capture improves extract cleanliness and increases LC/MS/MS method sensitivity more than ten-fold
- Improved quantitative LC/MS/MS analysis of low-level protein applicable to a wide range of mAb

Goal

To demonstrate the rapid quantitation of a low-level human IgG in animal plasma by LC/MS/MS using the Thermo Scientific™ SMART Digest™ Immunoaffinity (IA) kits (including magnetic and non-magnetic versions of the Streptavidin kit, Protein A kit, and Protein G kit), which combine the IA capture and digestion process into a single well. The assay is required to be both selective and accurate.

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Introduction

Workflows for the quantitative analysis of proteins from biological matrices are complex and time consuming. The most sensitive methods involve immunoaffinity (IA) enrichment and proteolytic digestion followed by liquid chromatography and mass spectrometric detection. These IA-LC/MS/MS approaches allow for the quantification of low abundance proteins in complex sample matrices.[1]

While sensitive, IA-LC/MS/MS methods are multistep workflows that typically span across multiple days. The digestion of proteins has been the biggest bottleneck and one of the largest sources of variation in sample preparation. Sources of variability arise from the multiple steps required to concentrate the compound of interest and remove matrix interferences, enzymatically digest proteins to surrogate peptides with better mass spectrometric properties, and modify the sample matrix or buffering system to achieve compatibility with LC separation and mass spectrometric detection systems.

The bottlenecks and variability associated with these workflows can be removed by using the SMART Digest IA Kit, which provides the following benefits:

- Total time for protein enrichment and digestion is reduced by a factor of five compared to a conventional tryptic digest[2]
- Improves kinetics and drives the reaction to completion, improving digestion reproducibility[2]
- Simplifies sample pretreatment (three times fewer steps than traditional tryptic digestion)
- Compatible with automation[3]

The SMART Digest IA Kit contains immunocapture reagents and temperature-activated thermally stable trypsin, which are co-immobilized onto beads (either magnetic or non-magnetic). During enrichment carried out at room temperature, the enzyme is inactive. Following enrichment, the enzyme is activated by elevating the temperature to 70°C, which also facilitates accelerated digestion under protein denaturing conditions. When denaturation and digestion are performed simultaneously on the same stationary phase used for enrichment, the need for elution, sample transfers, and pretreatment prior to digestion (e.g. denaturation, reduction, and alkylation) is eliminated. As such, the total time needed for the sample preparation workflow is decreased from around 24 hours to 3–4 hours, depending on the target analyte. This represents a time savings of over 80%.

This application demonstrates the use of each of the three types of SMART Digest IA kits (SMART digest IA Streptavidin, Protein A, and Protein G). Streptavidin is a biotin-binding protein. Biotin is a small molecule that can be conjugated to a protein without altering its biological activity. The affinity of streptavidin for biotin results in the strongest known non-covalent biological interaction. As such, capture reagents can be biotinylated, bound to streptavidin stationary phases, then readily applied to immunoaffinity methods.

Protein A and G specifically bind IgGs. As such, they are frequently used for antibody capture. Protein A and G bind IgG subtypes with varying affinities. Protein A is generally preferred for rabbit, pig, dog, and cat IgG. Protein G has better binding capacity for a broader range of mouse and human IgG subclasses (IgG1, IgG2, etc.).[4]

Depending on the type of immunocapture reagent (or bait) used, users can choose between these affinity reagents for customization of their immunoaffinity workflow. This application demonstrates the use of each of the three types of SMART digest IA kits (SMART digest IA Streptavidin, Protein A and Protein G). Human IgG was used as a surrogate for a humanized biotherapeutic and its concentration was determined in mouse plasma.

In the first example, anti-human IgG Fc was biotinylated and adhered to the SMART Digest IA Streptavidin Kit. When combined with the plasma samples, human IgG is captured by the anti-human IgG Fc while all other proteins are washed away. The enriched human IgG is then digested prior to analysis (refer to Figure 1 for a representation of the process). While biotinylated anti-human IgG was used as the capture reagent in this example, streptavidin can be used to bind a wide variety of capture reagents ranging from biotinylated proteins to small molecules. In the other two examples, the anti-human IgG Fc antibody was cross-linked to SMART Digest IA Protein A and Protein G kits and then used to enrich human IgG.

For LC/MS analysis, ‘universal’ surrogate peptides found in the Fc region of most human mAb candidates (which are absent from the proteomes of animals)

were monitored using selected reaction monitoring. These peptides are typically used to support preclinical development (mouse). The resulting workflows provide a general approach for the quantification of low abundance mAbs from animal plasma.

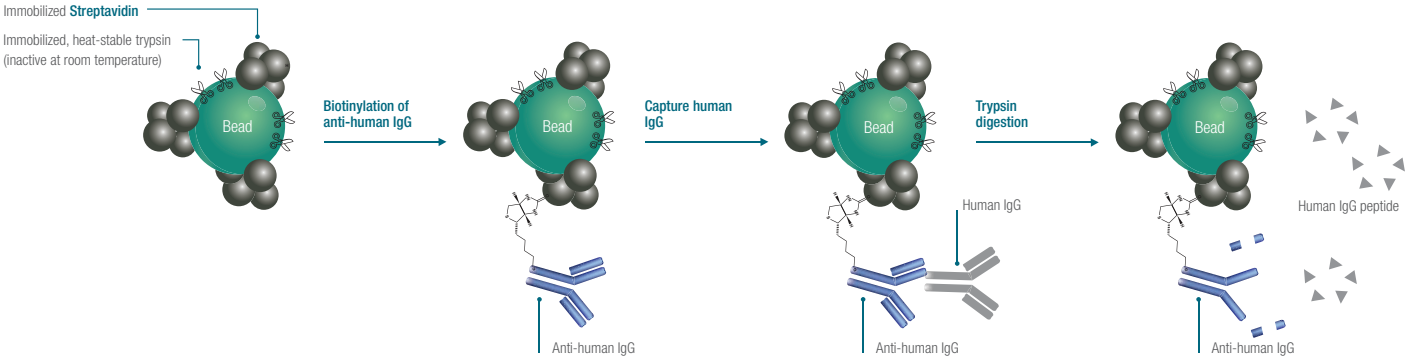

Figure 1. SMART Digest™ Streptavidin IA kit workflow.

Experimental

Affinity and Digestion

- SMART Digest IA kit, Streptavidin (Av) magnetic (P/N 60110-104), non-magnetic (P/N 60110-101)*
- SMART Digest IA kit, Protein A magnetic (P/N 60111-104), non-magnetic (P/N 60111-101)*
- SMART Digest IA kit, Protein G magnetic (P/N 60112-104), non-magnetic (P/N 60112-101)*

* Each SMART Digest IA kit includes beads, wash buffer, and digest buffer

Chemicals

- Deionized water, 18.2 MΩ•cm resistivity
- Fisher Scientific™ Optima™ acetonitrile (ACN) (A955-4)
- Fisher Scientific™ formic acid (FA) (F/1900/PB08)
- Thermo Scientific™ Pierce™ dimethylsulfoxide (DMSO), LC/MS Grade (85190)
- Thermo Scientific™ EZ-Link™ NHS-Biotin (20217)
- Mouse plasma from a reputable supplier
- Anti-human IgG from a reputable supplier
- Human IgG from a reputable supplier
- Glutaraldehyde from a reputable supplier
- pH 7.4 phosphate buffered saline (PBS) (BP24384)
- pH 7.4 tris buffered saline (TBS) (BP2471500)

Sample handling

- Thermo Scientific™ Sepraseal (4463)
- Fisher Scientific™ Deepwell™ Plates 96 (E951032905)
- Thermo Scientific™ Nunc™ EZFlip™ Conical Centrifuge Tubes 15 mL (362694)
- Thermo Scientific™ Graduated Safelock Microcentrifuge Tubes 1.5 mL (3457PK)
- Thermomixer from a reputable supplier

Preparation of calibration and quality control (QC) samples

For Experiment 1, human IgG was spiked into mouse plasma at concentrations between 5 ng/mL and 1.6 µg/mL. Quality control samples were prepared at 5, 50, and 500 ng/mL in mouse plasma. For Experiments 2 and 3, human IgG was spiked into mouse plasma at concentrations ranging from 6 ng/mL to 6 µg/mL.

Experiment 1 - Quantification of human IgG in animal plasma using SMART Digest IA Streptavidin beads

Preparing a biotinylated antibody/capture moiety

To prepare 0.5 mg of biotinylated anti-human mouse IgG, 2.5 µL of 5 mg/mL NHS-Biotin (in DMSO) was added to 1 mL of 0.5 mg/mL anti-human mouse IgG. Then, 30 µL of SMART Digest IA Streptavidin beads and 6 µL of biotinylated antibody were added to 36, 1.5 mL micro centrifuge tubes.

Capturing human IgG

Four hundred microliters of the murine plasma samples, spiked with human IgG at varying concentrations, was added to each of the bead containing tubes. Samples were incubated at room temperature and shaking at 1,400 rpm for 60 minutes. After incubation, the samples were washed 3 times with 600 µL of wash buffer.

Trypsin digestion

After the final wash, the sample volume was reduced to 50 µL by centrifuging the samples and decanting the supernatant, then followed by the addition of 150 µL of SMART Digest Buffer. The samples were capped then incubated for 60 minutes at 70°C and 1,400 rpm (see Results and discussion). The samples were acidified with 200 µL of 1% trifluoroacetic acid, decanted, and placed into a new 96-well plate for analysis.

Experiments 2 and 3 – Quantification of human IgG in animal plasma using SMART Digest IA Protein A beads (Experiment 2) and SMART Digest IA Protein G beads cross-linking anti human IgG (Experiment 3)

Preparation of the SMART Digest Immunoaffinity Protein A and G beads - cross-linking anti human IgG

The following components were added to a 15 mL centrifuge tube, which were 750 µL SMART Digest IA Protein A or G beads, 250 µL of 0.5 mg/mL mouse anti-human IgG antibody, and 500 µL of PBS. The beads were mixed at 1,400 rpm for 30 minutes at room temperature, centrifuged, decanted (1,300 µL of solution) and washed three times with PBS (add 1,500 µL of PBS, decant 1,500 µL PBS). After the final wash, 450 µL of PBS and 2,500 µL of 0.01% glutaraldehyde in PBS were added to the beads, then mixed at 1,400 rpm for 2.5 hours at room temperature. The cross-linking reaction was quenched by the addition of 250 µL of 0.5 M TBS and the beads mixed at 1,400 rpm for 10 minutes at room temperature. The beads were centrifuged and 2,750 µL of solution decanted. 30 µL of modified SMART Digest IA Protein A or G beads were added to 24, 1.5 mL micro centrifuge tubes.

Capturing human IgG

Five hundred microliters of the murine plasma samples, spiked with human IgG at varying concentrations, was added to each of the bead containing tubes. Samples were incubated at room temperature and shaking at 1,400 RPM for 60 minutes. After incubation, the samples were washed three times with 650 µL of wash buffer.

Trypsin digestion

As in Experiment 1, after the final wash, the sample volume was reduced to 50 µL by centrifuging the samples and decanting the supernatant, then followed by the addition of 150 µL of SMART Digest Buffer. The samples were capped, then incubated for 60 minutes at 70°C and 1,400 RPM (see Results and discussion). The samples were acidified with 200 µL of 1% trifluoroacetic acid, decanted, and placed into a new 96-well plate 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

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 Temperature	50°C
Injection Details	50 µL for Streptavidin IA, 25 µL for Protein A and G IA
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

Thermo Scientific™ Velos Pro™ ion trap mass spectrometer.

Instrumentation

MS 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 minutes into the gradient. Flow is sent to the source from 1.5 minutes to 3 minutes into the gradient, and then sent to waste again at 3 minutes into the gradient

Data processing

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

Table 2. MS fragment 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

Results and discussion

To quickly and efficiently quantify human IgG, optimization for immunocapture and digestion time were investigated with the SMART Digest IA Streptavidin kit.

Determination of digestion time

The optimal digestion time for human IgG was determined by dispensing 200 µL of 1 µg/mL human IgG, and 30 µL of SMART Digest IA Streptavidin beads (biotinylated anti-human mouse IgG), into each of eight wells. The samples were vortexed then placed into the heated block set to 70°C. Samples were removed at 15 minute intervals, centrifuged, and decanted, and the resulting peptides collected and analyzed by LC/MS. It was determined that the digestion of IgG reaches completion after 60 minutes (Figure 2).

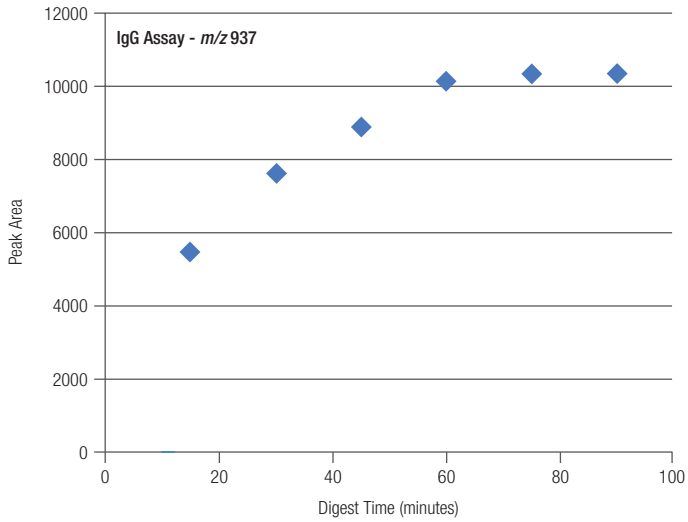

Figure 2(A). SMART Digest IA Streptavidin kit sample processing method optimization. Fragment peak area at different digest times.

Determination of immunocapture time

Optimization of capture time was determined by dispensing 400 µL of mouse plasma containing 1 µg/mL human IgG, and 30 µL of SMART Digest IA Streptavidin beads (biotinylated anti-human mouse IgG), into each of eight tubes. The samples were placed into a shaker at room temperature. Samples were removed at 15 minute intervals and rinsed four times. Following the addition of digest buffer, samples were digested at 70°C for 60 minutes. Samples were then centrifuged and decanted, and the resulting peptides collected and analyzed by LC/MS. It was determined that the capture of IgG reaches completion after 60 minutes.

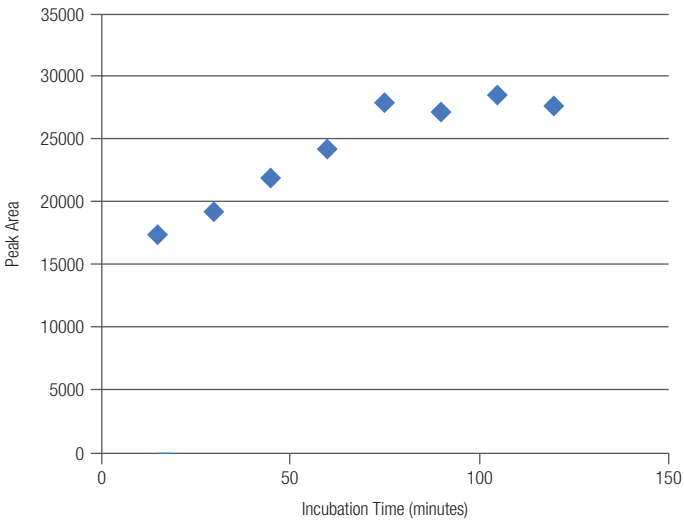

Figure 2(B). SMART Digest IA Streptavidin kit sample processing method optimization. Fragment peak area at different incubation (immunocapture) times.

Calibration and quantification for human IgG with SMART Digest IA Streptavidin kit

The SMART Digest IA Streptavidin kit provided excellent reproducibility and linearity across a wide dynamic range. Calibration and quality control samples were prepared as previously described in the materials and methods section. Calibration ranges were from 5 ng/mL to 1,580 ng/mL. Quality control samples were prepared at low, medium, and high ranges. Six-point calibration curves of IgG in plasma yielded linear fits for R^2 greater than 0.995 for both front and back calibration curves (Figure 3 shows both, front in blue and back in red). Quality control samples prepared at 5, 50, and 500 ng/mL were accurate and precise (Table 3). Variability was determined and CV values were obtained for IgG in mouse plasma ranging from 6% at the LLOQ to 2.3% at the ULOQ (n=6) even without internal standard.

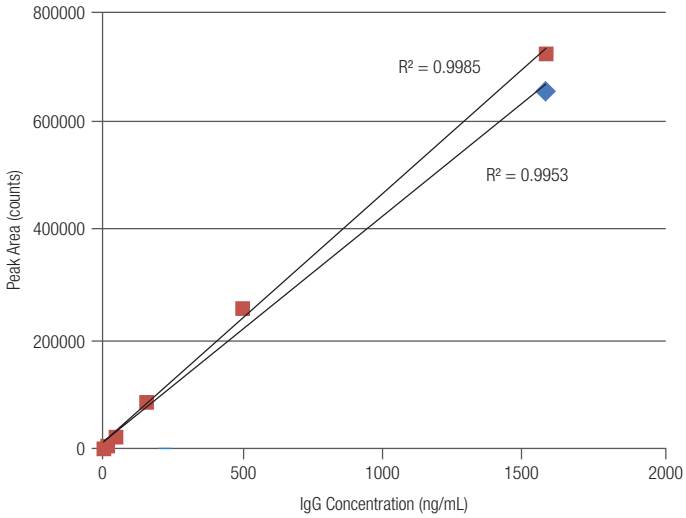

Figure 3. Extraction of human IgG from mouse plasma using SMART Digest IA Streptavidin kit, front (blue) and back (red) calibration curve.

Calibration of human IgG with SMART Digest IA Protein A/G kits

The SMART Digest IA Protein A and G kits also provided excellent reproducibility and linearity across a wide dynamic range from 6 ng/mL to 6000 ng/mL. For both kits, 6 ng/mL was readily and reproducibly detected. Linear fits of all six calibration curves with three lines in each kit were 0.99 or better (Figure 4).

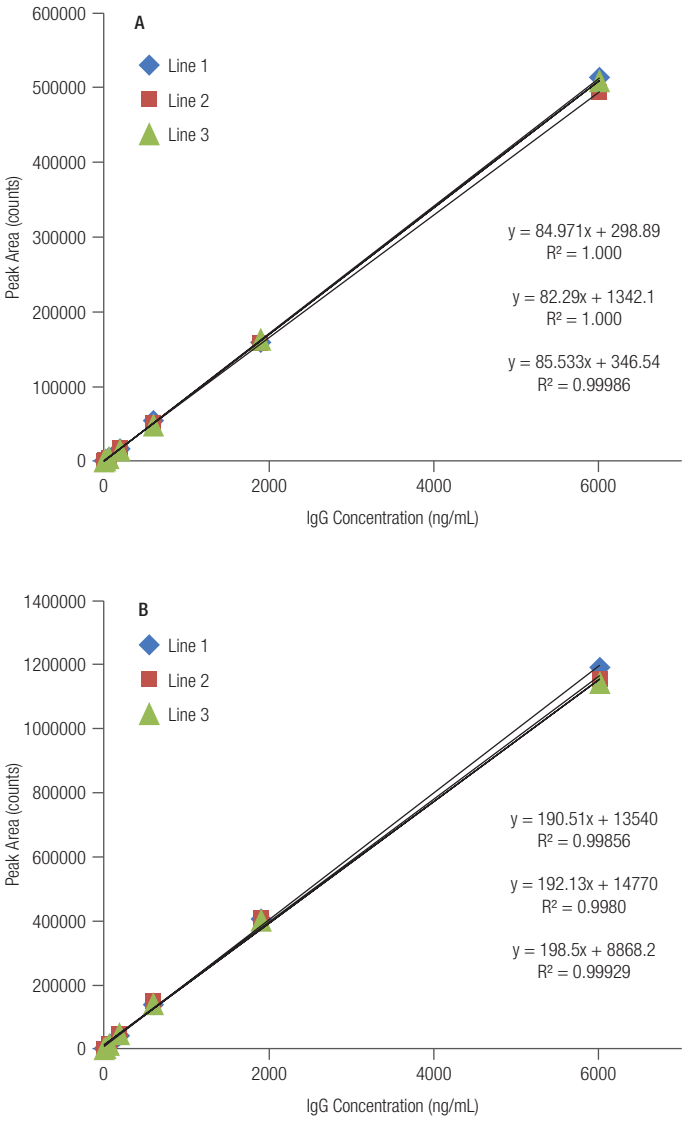

Figure 4. Calibration curve for extraction human IgG from mouse plasma (A: SMART Digest IA Protein A kit; B: SMART Digest IA Protein G kit).

Without cleanup, the targeted analysis of mAbs in complex biological matrices is generally limited to concentrations above 100 ng/mL.[5] Historical attempts to add IA enrichment have resulted in time-consuming, multi-step workflows and highly variable results. The SMART Digest IA kits streamline the process, reducing the sample preparation time to less than three hours with the detection limit reduced to <10 ng/mL. This workflow also combined the use of a general anti-Fc antibody and the monitoring of 'universal' surrogate peptides. This combination results in a single method that can be used for the measurement of a wide range of monoclonal antibodies, keeping method development time to a minimum. Additionally, three stationary phases (Streptavidin, Protein A, and Protein G) allow for the use of a variety of capture moieties as required.

The SMART Digest IA workflow has been repeated using magnetic beads (similar protocol, linearity ranged from 10 ng/mL to 10 µg/mL with $R^2 > 0.99$), 1 mL deep well plates, and SepraSeal caps. No significant changes in sensitivity or reproducibility were observed while the magnetic bead format allowed for further simplification of the wash steps. Furthermore, these results suggest that the process is readily amenable to automation using a number of existing instruments such as the Thermo Scientific™ KingFisher™ system as long as the heating unit is capable of heating the plate evenly.

Table 3. Extraction of human IgG from mouse plasma using SMART Digest IA Streptavidin kit, quality control results (N=6).

	IgG concentrations (ng/mL)		
Replicates	**5 (QCL)**	**50 (QCM)**	**500 (QCH)**
R1	4416	30074	284410
R2	4087	29392	272466
R3	4301	30199	268620
R4	4101	28610	270032
R5	4004	30550	267826
R6	3714	29413	270273
Average	4104	29706	272271
Std Dev	244	704	6156
CV (%)	6.0	2.4	2.3

Conclusions

- The rapid processing of antibodies using the SMART Digest IA kits allows development of faster methods compared to conventional protein enrichment and digestion by simplifying sample preparation and enabling a reproducible, sensitive, and fully integrated LC/MS/MS workflow.

- This application shows a rapid, simple, and sensitive method for the quantification of human IgG in mouse plasma using the SMART Digest IA kits with a total time of 3–4 hours that compares to a process that can take up to 24 hours.

- The method described provides a significant simplification for the affinity proteomics process within a single well providing increased sensitivity and reduced sample preparation time.

- This Application Note provides a general approach for quantifying low abundance mAbs with reproducible results across a broad dynamic range for all three types of SMART Digest (IA) kits (SMART digest IA Streptavidin, Protein A, and Protein G).

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