A fast and simple immuno-mass spectrometry method for preclinical bioanalysis for IgG1 mAb

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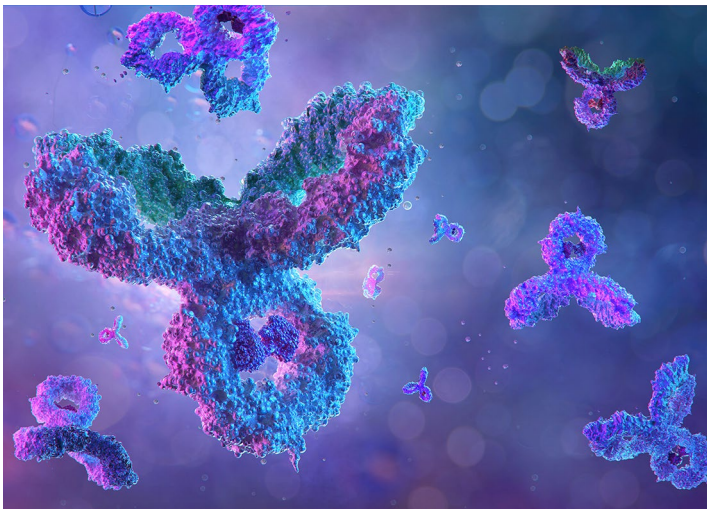

Benefits

- **Simple and fast:** The sample preparation step takes only 4–5 hours, and the whole process can be automated to enable high-throughput analysis.
- **Universal method:** This can be applied to all IgG1 type mAbs in all animal serum matrices at the preclinical phase without any modification.
- **Comprehensive:** Capable of both monitoring and quantifying mAb in vivo to obtain a comprehensive drug metabolism profile.
- **High sensitivity and selectivity:** Immuno-mass spectrometry combines the sensitivity of immunoassay and the selectivity of mass spectrometry. LLOQ as low as 20 ng/mL is achieved using only 50 µL of serum sample.

Introduction

The importance of bioanalysis in the initial discovery and preclinical/clinical development of biotherapeutics has been well recognized. Accurate, sensitive, selective, robust, and high throughput quantification is essential to obtain fundamental temporal data for pharmacokinetic (PK), pharmacodynamic (PD), and toxicokinetic (TK) analyses.[1] Ligand Binding Assay (LBA), such as ELISA, is considered the gold standard in bioanalysis of biotherapeutics due to its sensitivity and throughput.

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However, this approach suffers several major limitations due to some inherent issues:

- It relies on generation of high quality antibodies to achieve specificity, which may take a long time to develop.
- It provides only a limited linear dynamic range, which may in certain cases not be sufficient to meet the requirements of pharmacokinetics.
- It is sensitive to matrix interferences, a potential risk if the same method is transferred to different matrices.
- It has high susceptibility to induce anti-drug antibody (ADA) production during animal studies.

Since the advances in targeted proteomics technology, LC-MS strategies have emerged as a promising alternative for the bioanalysis of biotherapeutics. The most prominent method employed is the bottom-up approach in which the proteins are first digested, the selected transition pairs (parent/daughter ion) from the surrogate peptide is then monitored, either in selected reaction monitoring (SRM) mode on a triple quadrupole mass spectrometry or in parallel reaction monitoring (PRM) mode on a high-resolution mass spectrometry, for the quantitation of the biotherapeutics. Several digestion protocols, including in-solution digestion,[3,4] on-pellet digestion[5,6] and Filter Aided Sample Preparation (FASP)[7] have all been used for the bioanalysis of biotherapeutics. Unfortunately, quantitation based on these crude sample preparation techniques may sometimes lack sufficient sensitivity or selectivity.[8,9]

By combining LBA and mass spectrometry, this hybrid method provides unparalleled solution for selectivity and sensitivity. In fact, a considerable number of pharmaceutical and CRO companies have employed this type of immuno-mass spectrometry for the bioanalysis of biotherapeutics.[10,11] Capture method and signature peptides should be optimized according to different study phase when utilizing immuno-mass spectrometry method; usually different specific antibodies or ligands were required for target drug enrichment. Moreover, the traditional immuno-mass spectrometry method is labor intensive and time-consuming in daily operation. In this work, a simple and fast immuno-MS method was developed for universal bioanalysis of IgG1 type mAb drugs at preclinical phase using Thermo Scientific™ SMART Digest™ ImmunoAffinity (IA) Streptavidin Kit and Thermo Scientific™ CaptureSelect™ specific ligands. The SMART Digest IA kit enables immuno-capture at room temperature, followed by denaturation and enzymatic digestion simultaneously at 70 °C, thereby shortening the whole sample preparation time to 4 hours, as frequent sample transfer, denaturation, reduction/alkylation, and the subsequent solid phase extraction (SPE) step were avoided. CaptureSelect ligands bind specifically to the Fc region of the humanized mAb, enabling a universal immunocapture of humanized mAb from any non-human matrices. By using the general IgG1 kappa type heavy-isotope-labeled antibody as an internal standard and selecting multiple signature peptides in the constant region to monitor the intact antibody, a completely universal bioanalysis method in preclinical stage for all IgG1 type mAbs has been implemented. By monitoring the signature peptides from multiple domains, the structural integrity was investigated while quantifying the antibody drug in vivo. This method is suitable for all biopharmaceutical and CRO companies, saving precious time for most pharmaceutical participants.

Experimental

Reagents and consumables

- SMART Digest IA Kit, Av magnetic (P/N 60110-104)
- Thermo Scientific™ CaptureSelect™ Human IgG-Fc PK Biotin Conjugate (P/N 7103322500)
- SILu™Mab K1 - Stable Isotope Labeled Universal mAb Standard (Sigma-Aldrich, P/N MSQC6)

Equipment

- Thermo Scientific™ DynaMag™-2 Magnet (P/N 12321D)
- Thermo Scientific™ KingFisher™ Duo Prime system (P/N 5400110) or KingFisher Flex system (P/N 5400630)
- Thermo Scientific™ Thermal Mixer (P/N 13687722)
- Thermo Scientific™ HulaMixer™ Sample Mixer (P/N 15920D)

Preparation of calibration standards (STDs), quality control (QC) samples, and internal standard (IS) solutions

The target mAb (NIFDC IgG1) preparation was diluted using animal serum to the final concentration of 0.1 µg/µL. Each aliquot of 100 µL of this stock solution was stored at -80 °C until use. The calibrators were prepared from target mAb stock solution to the concentrations of 39.06, 78.13, 156.25, 312.5, 625, 1250, 2500, 5000, and 10,000 ng/mL through serial dilution with animal serum. Three levels of QC samples were home prepared to the final concentrations of 100, 800, and 6000 ng/mL by diluting target mAb stock solution with animal serum. Each aliquot of 300 µL QC sample was stored at -80 °C and thawed before daily use. Stable isotope labeled mAb standard was dissolved in 0.1% FA/H_2O to the concentration of 0.2 µg/µL following the instructions of the manufacturer, and then diluted to the concentration of 9 ng/µL in 1% BSA/PBS solution. This IS working solution was aliquoted and stored at -80 °C until use.

Sample preparation

- Ligand immobilization: CaptureSelect Human IgG-Fc PK Biotin Conjugate (hereinafter referred to as anti-Fc ligand) was diluted into 0.5% BSA/PBS solution at the ratio of 1:30 (v/v), then the SMART Digest IA magnetic beads (SDIA beads for short) were bulk prepared with the anti-Fc ligand solution above at the ratio of 1:1 (v/v) at room temperature for 40 minutes. The SDIA beads with capture reagent immobilized were resuspended to its original volume with 0.1% BSA/PBS after one gentle wash.
- Target mAb capture: Each aliquot of 30 µL immobilized anti-Fc beads were pipetted into 2.0 mL ep tubes or 96-well plates (30 µL is the unit volume of SDIA magnetic beads defined in the manual). 50 µL serum sample (or calibrator, QC sample), 10 µL SILuMAB K1 heavy-isotope-labeled antibody as an internal standard (final concentration of internal standard is 1.5 µg/mL), and 140 µL PBS were added into each well containing anti-Fc beads and then incubated at room temperature for 90 minutes by gentle shaking. Washing with 0.1%BSA/washing buffer was performed to remove non-specific binding proteins (washing buffer is provided in powder form in the SDIA kit).
- Target mAb digestion: 200 µL SMART Digest buffer was added after removing the supernatant (this buffer is provided in the SDIA kit in the form of a solution). Trypsin digestion was performed at 70 °C for 60 minutes with shaking at 1400 rpm.
- Preparation for injection: 190 µL of supernatant was transferred into a new tube and centrifugated for 2 min with high speed to remove any remaining magnetic beads (filter plates were the option for 96-well plates). 24 µL of 10% FA/50% ACN was added to 175 µL of supernatant to eliminate nonspecific absorption. Then 40–80 µL of sample was injected for LC-MS analysis.

LC method

Thermo Scientific™ Vanquish™ Binary Flex UHPLC system consisting of the following:

- System Base Vanquish Flex (P/N VF-S01-A)
- Binary Pump F (P/N VF-P10-A-01)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- MS Connection Kit Vanquish (P/N 6720.0405)
- Vanquish F Pumps 100 µL Mixer Set (P/N 6044.5100)
- Vanquish Split Sampler HT Sample Loop, 100 µL (P/N 6850.1913)

Separation gradient

- Solvent A: 0.1% FA/Water; Solvent B: 0.1% FA/ACN
- Column: Thermo Scientific™ Accucore™ 150 C18 2.6 µm, 2.1 mm × 50 mm (P/N 16126-052130)
- Column temperature: 50 °C, still air

Table 1. UHPLC gradient

Time (min)	Flow rate	Flow path	%A	%B
0.0	0.5 mL/min	To waste	96	4
1.4	0.5 mL/min	Main path	96	4
1.7	0.5 mL/min	Main path	96	4
6.5	0.5 mL/min	Main path	66	34
7.0	0.5 mL/min	Main path	10	90
8.9	0.5 mL/min	Main path	10	90
9.0	0.5mL/min	Main path	96	4
10.0	0.5mL/min	Main path	96	4

Mass spectrometry

- Thermo Scientific™ Orbitrap ID-X™ Tribrid™ Mass Spectrometer
- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer

Table 2. Mass spectrometry parameters

	Orbitrap ID-X settings	Q Exactive Plus settings
Source parameters		
Polarity	Positive	
Spray voltage	3700 V	
Sheath gas	50 Arb	
Aux gas	15 Arb	
Sweep gas	0 Arb	0 psi
Aux temp.	300 °C	
Capillary temp.	320 °C	
MS settings		
PRM scan		
S-lens RF	50	
Iso. window	1.4 Th	
MS2 resolution	30,000	17,500
Fragmentation	HCD	
AGC target	1e5	2e5
MicroScan	1	

Results and discussion

The immuno-mass spectrometry workflow based on SMART Digest IA

Figure 1 shows the total immuno-mass spectrometry workflow based on the SMART Digest IA kit. This kit provides simultaneous affinity capture and tryptic digestion of the target protein using its proprietary magnetic bead design, in which both the affinity linker, such as streptavidin (Av), and heat-activated trypsin are both immobilized on the same magnetic bead. The heat-activated trypsin was engineered such that it has mild activity at room temperature and will be activated when the temperature rises to 70 °C to digest the target mAb captured on beads. Only four steps were needed for sample preparation:

1. Immobilization of anti-Fc ligand at room temperature
2. Capture of target mAb from the matrix at room temperature
3. Removal of non-specific binding proteins through gentle washing
4. Elevating the temperature to 70 °C for SMART Digest trypsin digestion

No detergent or denaturant was needed as heat was used to denature the target protein; thus, the subsequent SPE step could be skipped. Compared with traditional immuno-mass spectrometry, this workflow avoided frequent sample transferring, protein denaturation, reduction/alkylation, and the subsequent solid-phase extraction step, shortening the original 2-day protocol to 4 hours.

Table 3. Signature peptides transition list – Orbitrap ID-X

Sequence		Target peptide (Light)				Internal standard (Heavy)		CE	Retention time (min)
		Precursor (*m/z*)	Quan ion (*m/z*)	Confirmation ion (*m/z*)	Ion Ratio	Precursor (*m/z*)	Fragment ion (*m/z*)		
Peptide 2	ALPAPIEK	419.756	486.292 ($y4^{+}$)	327.695 ($y6^{2+}$)	0.499	423.762	494.305 ($y4^{+}$)	29	4.04
Peptide 3	GPSVFPLAPSSK	593.828	699.405 ($y7^{+}$)	846.473 ($y8^{+}$)	0.710	597.834	707.417 ($y7^{+}$)	25	5.13
Peptide 5	DSTYSLSSTLTLSK	751.887	1036.589 ($y10^{+}$)	836.474 ($y8^{+}$)	0.841	755.891	1044.602 ($y10^{+}$)	25	5.23
Peptide 7	TTPPVLDSDGSFFLYSK	937.967	836.918 ($y15^{2+}$)	397.208 ($y3^{+}$)	0.144	941.973	840.924 ($y15^{2+}$)	31	6.05

Table 4. Signature peptides transition list – Q Exactive Plus

Sequence		Target peptide (Light)				Internal standard (Heavy)		CE	Retention time (min)
		Precursor (*m/z*)	Quan ion (*m/z*)	Confirmation ion (*m/z*)	Ion Ratio	Precursor (*m/z*)	Fragment ion (*m/z*)		
Peptide 2	ALPAPIEK	419.756	654.381 ($y6^{+}$)	486.292 ($y4^{+}$)	0.629	423.762	494.305 ($y4^{+}$)	16	4.06
Peptide 3	GPSVFPLAPSSK	593.828	699.405 ($y7^{+}$)	846.473 ($y8^{+}$)	0.702	597.834	707.417 ($y7^{+}$)	22	5.13
Peptide 5	DSTYSLSSTLTLSK	751.887	1036.589 ($y10^{+}$)	836.474 ($y8^{+}$)	0.913	755.891	1044.602 ($y10^{+}$)	28	5.23
Peptide 7	TTPPVLDSDGSFFLYSK	937.967	836.918 ($y15^{2+}$)	397.208 ($y3^{+}$)	0.150	941.973	840.924 ($y15^{2+}$)	44	6.05

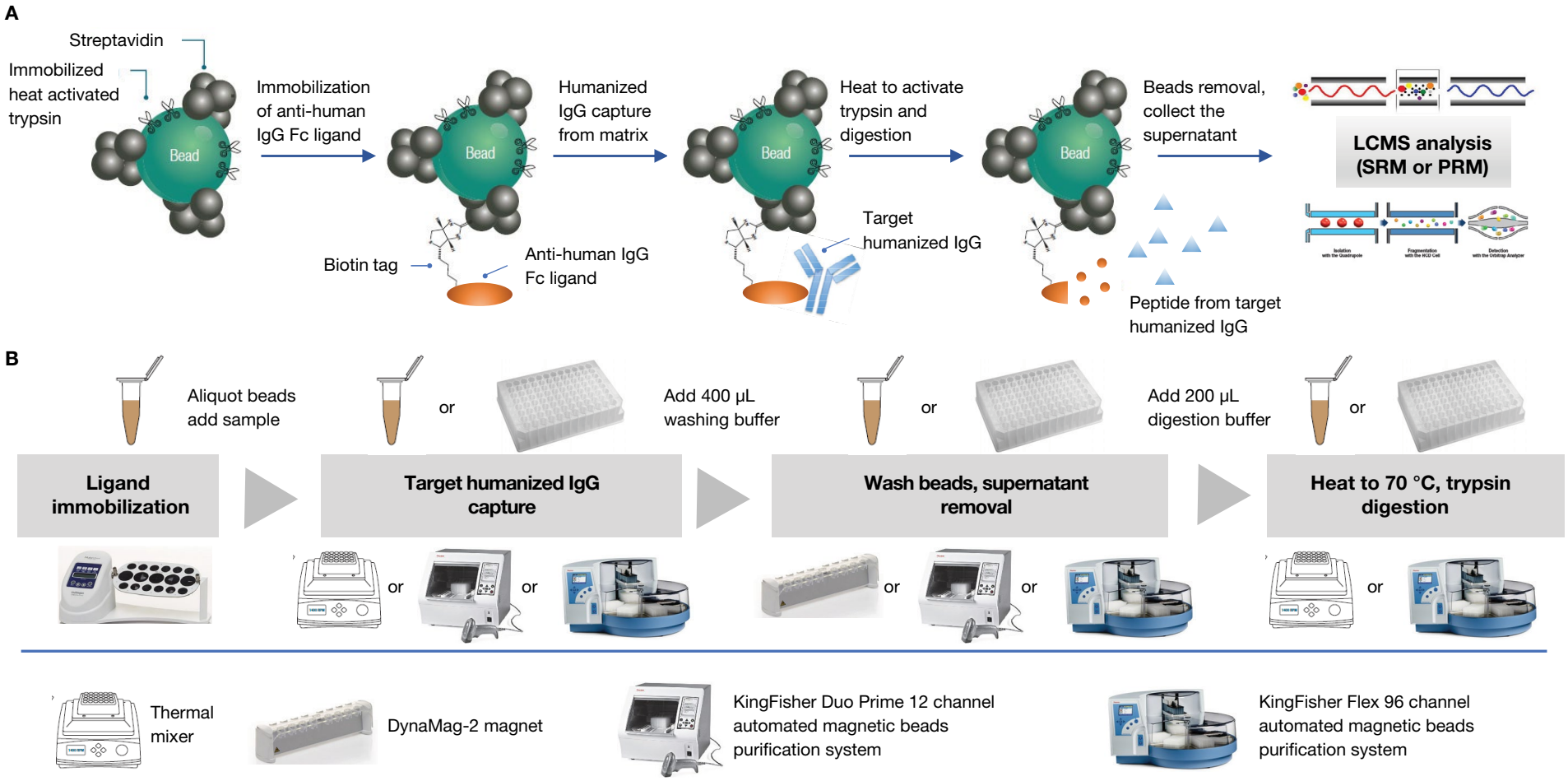


Figure 1. Principle and operation step of immuno-mass spectrometry method using SMART Digest IA streptavidin magnetic beads

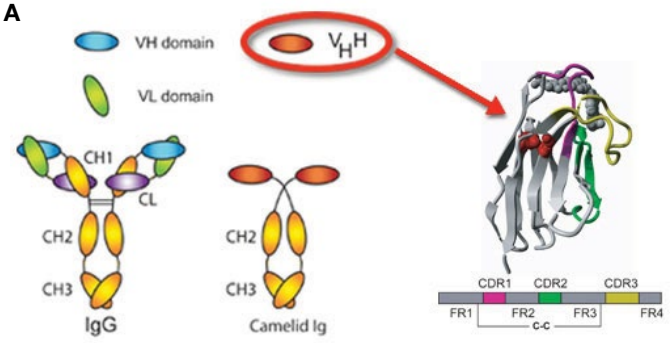
This workflow is compatible with KingFisher magnetic beads purification technology to achieve up to 12-channel or 96-channel automated sample preparation, further improving the analysis throughput and reproducibility.

To enrich target monoclonal antibodies from animal serum matrix, CaptureSelect Human IgG-Fc PK Biotin Conjugate (anti-Fc ligand for short) was chosen as the affinity ligand. This anti-Fc ligand was composed of the fragment belonging to the variable region of camelid IgG, with the size of 13 KDa. (Figure 2A). It recognizes the Fc region of all human IgG subtypes (Figure 2B) while showing no cross reactivity with the animal models we used in the preclinical research, such as mice, rats, dogs, and cynomolgus monkeys (Figure 2C). Compared with traditional protein A and anti-Fc antibody enrichment, this anti-Fc ligand showed obvious advantages:

- A unified sample preparation protocol was able to be established through using this ligand, which was not possible if taking protein A as the choice for affinity ligand due to different binding reactivity of protein A towards IgG in different species.
- The CaptureSelect ligand showed less interference for target mAb analysis due its much smaller molecular weight compared with antibody.

Moreover, biotin-streptavidin showed more stable interaction, which made the capture efficiency more reliable. To investigate the SDIA beads loading capacity of anti-Fc ligand 0.5 µg, 1 µg, 2 µg, 4 µg, and 8 µg of anti-Fc ligand were added to the unit volume (30 µL) of SDIA magnetic beads. Free anti-Fc ligand in the supernatant was digested and then monitored by mass spectrometry. 30 µL of SDIA beads offered the capacity of up to 4 µg of this anti-Fc ligand. 1 µg of anti-Fc ligand was immobilized on 30 µL of SDIA beads for the experiments below. Since 200 pg–3.5 mg of total protein could be digested by unit volume of SDIA beads according to the user manual, 1 µg of anti-Fc ligand immobilized on 10–15 µL of SDIA beads for each sample offered a more economic option.

A



B

Binding selectivity Human IgG-Fc PK Biotin

Antibody target	Isotype/subclass	Binding selectivity
Ab fragments	Human IgG Fc	✓
	Human IgG Fab	–
IgG subclasses	Human IgG1	✓
	Human IgG2	✓
	Human IgG3	✓
	Human IgG4	✓
Ab isotypes	Human IgGA	–
	Human IgGM	–

C

Antibody target	Isotype/subclass	Binding selectivity
Species	Chimpanzee	✓
	Cynomologus macaque and Rhesus macaque	–
	Rat, Mouse	–
	Sheep, Goat	–

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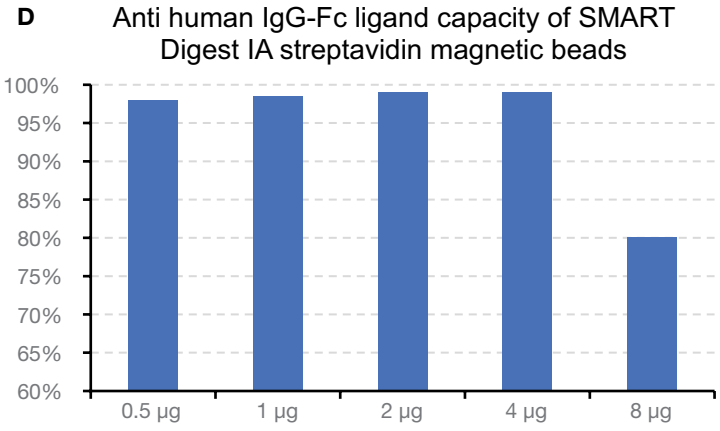

	Quantitative ion	Confirming ion
Peptide 1 of anti-Fc ligand	442.707 -> 696.328	442.707 -> 565.288
Peptide 2 of anti-Fc ligand	628.699 -> 688.373	628.699 -> 560.315

Figure 2. Information of CaptureSelect human IgG-Fc PK biotin conjugate. (A) Structure of human IgG, camelid IgG; (B) binding selectivity of human IgG-Fc biotin conjugate; (C) species reactivity of human IgG-Fc biotin conjugate; (D) anti-human IgG-Fc ligand capacity of SMART Digest IA streptavidin magnetic beads calculated by measuring free anti-Fc ligand in supernatant using PRM method.

Recovery of immuno-capture

The free target mAb in the supernatant was measured after immuno-capture using unit volume SDIA beads immobilized with 1 µg of anti-Fc ligand to investigate the anti-Fc beads' loading capacity. For the matrix, 50 µL of 1% BSA/PBS were used with target mAb concentration from 20 µg/mL to 160 µg/mL. The free mAb in the supernatant was digested using the on-pellet digestion protocol,[12] and the four signature peptides from different domains were monitored by PRM mode on an Orbitrap instrument (Figure 3A). The LOD of this on-pellet digestion mass spectrometry method was 0.2 µg/mL, enough for evaluating the free target in the supernatant (data not shown). Signature peptides in different domains digested from the free target mAb in the supernatant showed similar concentrations. The immuno-capture recovery was greater than 95% for 40 µg/mL or less target mAb from matrix (Figure 3B), fully meeting the needs of the ULOQ. Next, it was investigated whether a consistent immuno-capture recovery from different matrices was able to be achieved. Figure 3C shows that the similar immuno-capture recovery as in 1% BSA matrix for sera in all preclinical animal models could be expected, offering preliminary evidence for method universality.

On-bead digestion and signature peptides determination

The digestion efficiency was different if proteins were captured on bead compared to dissolved in solution and was domain dependent. This phenomenon might be caused by the structural change due to binding of anti-Fc ligand and steric hindrance effects on the magnetic beads (Figure 5C). Therefore, the appropriate signature peptides should be determinated based on the on-bead digestion after immuno-capture. The signature peptides should fulfil these criteria:

- Located in the constant region, and completely conserved in all IgG1 mAb drugs
- (Strong and stable PRM signal upon on-bead digestion after immuno-capture
- Similar recovery of on-bead digestion compared to in-solution digestion
- Cover more domains in the constant region
- No matrix interference towards the chosen transition in the matrix blank sample (which will be discussed later)

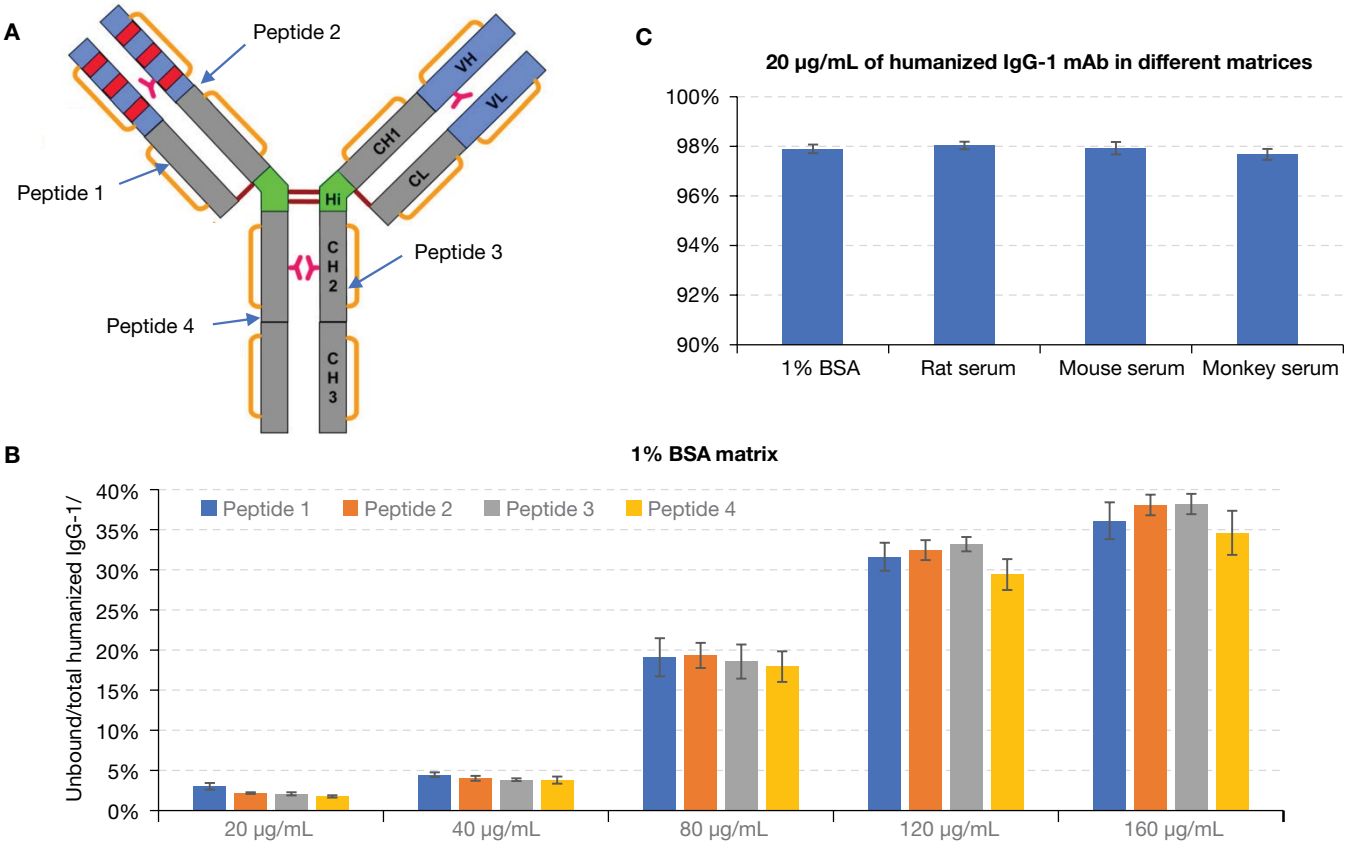

Figure 3. Tests of capacity and recovery of target mAb immuno-capture using SMART Digest IA beads immobilized with 1 µg of anti-Fc ligand. (A) Location of four signature peptides used for PRM assay in the mAb; (B) target mAb capacity test of these anti-Fc beads from 1% BSA matrix; (C) immuno-capture recovery test of this anti-Fc beads from different matrices.

Two peptides in the CL domain (peptides 1 and 5) were selected, one peptide in the CH1 domain (peptide 3), two peptides in the CH2 domain (peptides 4 and 8), two peptides in the CH3 domain (peptides 6 and 7), and one peptide (peptide 2) between the CH2 and the CH3 domain for the following evaluation (Figure 4A). These eight peptides were completely conserved among humanized IgG1 type mAbs commercially available (Figure 4B, 4C).

Before or after immuno-capture (50 µL of matrix diluted with 150 µL of PBS), 25 ng mAb drug was spiked into 50 µL of rat serum matrix followed by digestion at 70 °C. The resulting eight signature peptides were monitored with PRM. Signature peptides located in the CH1 domain and CH3 domain showed digestion recovery higher than 90% when compared with SMART Digest digestion performed in solution without immuno-capture (Figure 5A, 5B), indicating that the digestion efficiency is similar for target mAb captured on bead compared to presented in solution. Peptide 8 of the CH2 domain showed very strong MS signal for SMART Digest performed in solution; however, the efficiency of on-bead digestion was only about 30%. The CH2 domain is the epitope of human IgG recognized by CaptureSelect human IgG-Fc ligand (see CaptureSelect anti-Fc ligand data sheet). The binding of anti-Fc ligand with CH2 domain probably blocked the digestion site of trypsin, resulting in a low recovery rate (Figure 5C). The two signature peptides in the CL domain showed relatively low recovery of on-bead digestion, but peptide 5 was still chosen as a monitoring peptide to investigate the structure integrity of mAb *in vivo.* Finally, peptide 5 of the CL domain, peptide 3 of the CH1 domain, peptide 7 of the CH3 domain, and peptide 2 located between the CH2 and CH3 domain were taken into account. The sequence and transitions information are listed in Tables 3 and 4.

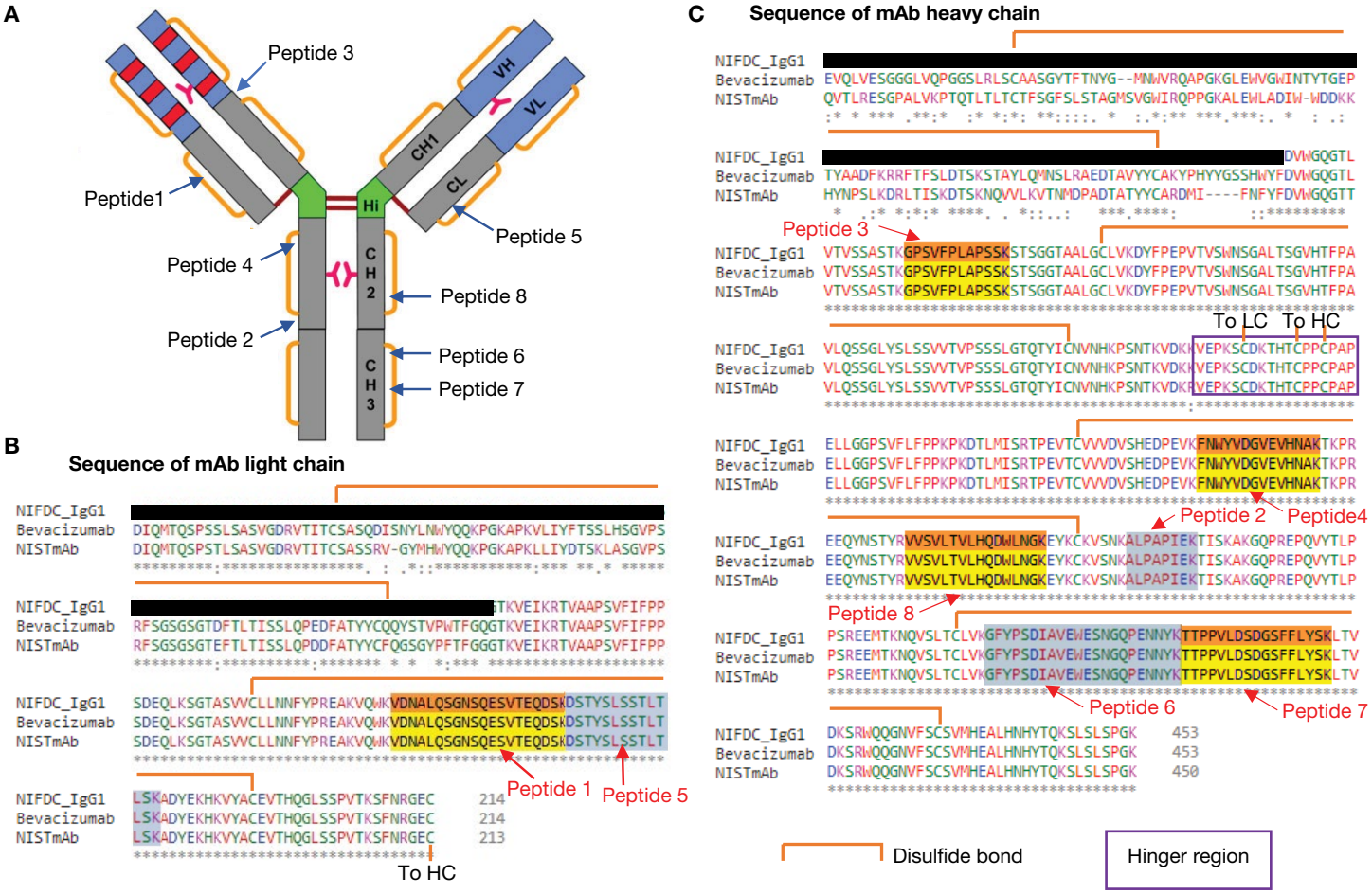

Figure 4. Multiple sequence alignment of humanized IgG1 type mAb and candidates of signature peptides

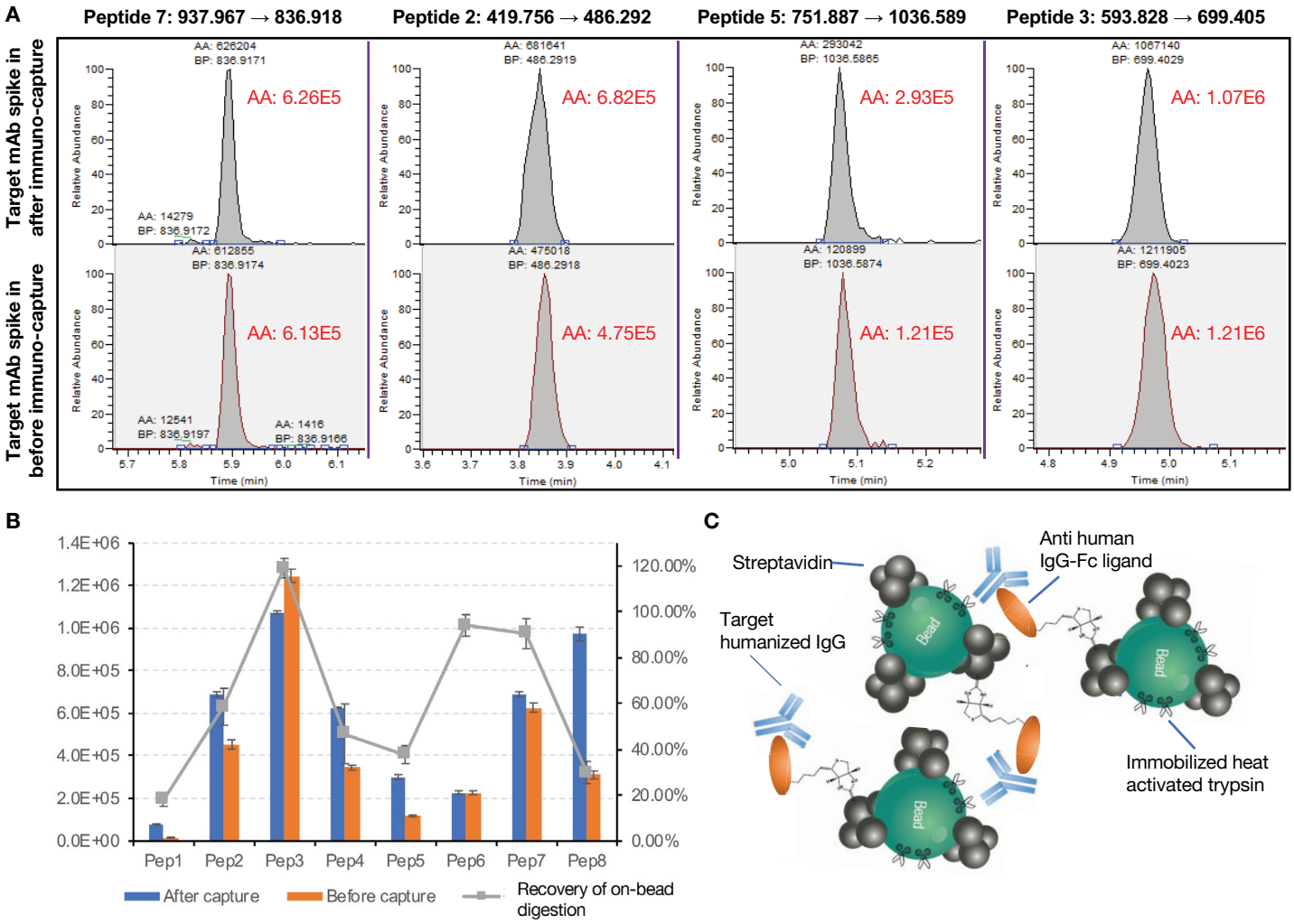

Figure 5. Digestion recovery of on-bead digestion after immuno-capture. (A and B) Target mAb was spiked into matrix before or after immuno-capture to mimic on-bead and in-solution digestion. (A) The XIC plot of transitions of four signature peptides; (B) the statistical results, in which on-bead digestion recovery was calculated by transition peak area via on-bead digestion divided by in-solution digestion. (C) Mechanism of on-bead digestion after immuno-capture.

Performance of isotope labeled IgG1 kappa universal internal standard

In previous targeted proteomics experiments, heavy-isotope-labeled peptides were usually spiked into enzymatically digested samples as internal standards to quantify the target protein. However, the heavy-isotope-labeled protein would be a better choice, as it would eliminate system variables across the entire workflow, including immuno-capture, digestion, and LCMS analysis. SILuMAB K1 heavy-isotope-labeled universal mAb standard was used as internal standard in the workflow. It shared the highly conserved constant region with human IgG1 kappa type immunoglobin, and all lysine and arginine were substituted with heavy-isotope-labeled ones (Figure 6A). The four signature peptides (peptide 2, 3, 5, 7) selected in the target mAb shared exactly the same sequence with that in the internal standard, and a mass difference of +8 Da could be observed between the light and heavy peptides after digestion, indicating the potential of SILuMAB K1 as a universal internal standard. Then, the only question remaining of using isotope-labeled protein as internal standard was investigated; that is, do the heavy-isotope-labeled amino acids in the sequence have enough isotopic purity to avoid interference to target a light peptide digested from the target mAb drug. 75 ng of SILuMAB K1 isotope-labeled antibody (mimicking the final working concentration) was spiked into 50 µL of 1% BSA/PBS matrix. After immuno-capture and digestion, PRM was used to monitor the heavy-labeled peptide and the corresponding light peptide to see whether signal from the light peptide channel could be observed in the case that no target mAb drug was spiked in. Figure 6B showed no response in the channel of the light peptide when the signal of heavy-labeled peptide is normal, indicating this heavy-isotope-labeled antibody as the perfect internal standard at the current concentration (1.5 µg/mL).

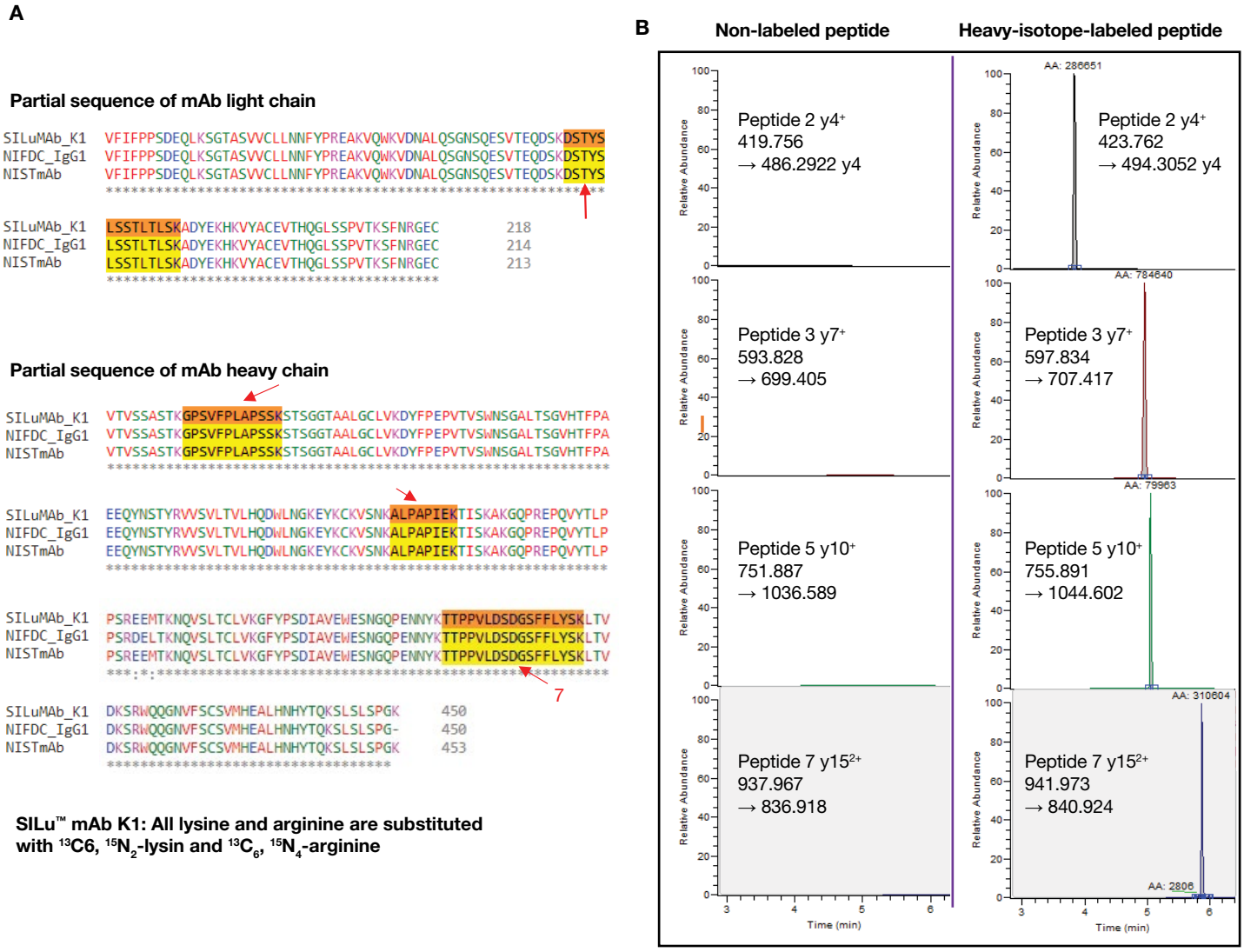

Figure 6. Internal standard information. (A) Sequence alignment of the internal standard protein (SILuMAB K1) and IgG1 type mAb (partial sequence displayed); the four selected IgG1 universal signature peptides were highlighted; (B) investigating interference generated from internal standard. The internal standard was spiked into the BSA matrix. PRM was performed to detect the four signature peptides and their corresponding non-labeled forms after immuno-capture and digestion.

Investigation of method specificity in different matrices

The method specificity of the universal method compatible for all IgG1 mAbs and all animal models in preclinical research was invetigated. Transition signals of four signature peptides observed in matrix blank sample were much lower than that in the LLOQ sample (less than 10% of the LLOQ response, data not shown). It is worth noting that strong matrix interference would be observed for signature peptide 3 and peptide 7 in cynomolgus monkey serum matrix if on-pellet digestion without immuno-capture was used as the sample preparation protocol (Figure 7A). However, the interference signal from the matrix was eliminated if immuno-capture was performed before digestion (Figure 7B), probably due to the inability of the interfering protein to bind with the anti-Fc ligand. Therefore, immuno-capture offered additional specificity together with mass spectrometry analysis in the workflow, making the method universal in the matrix of all preclinical animal models.

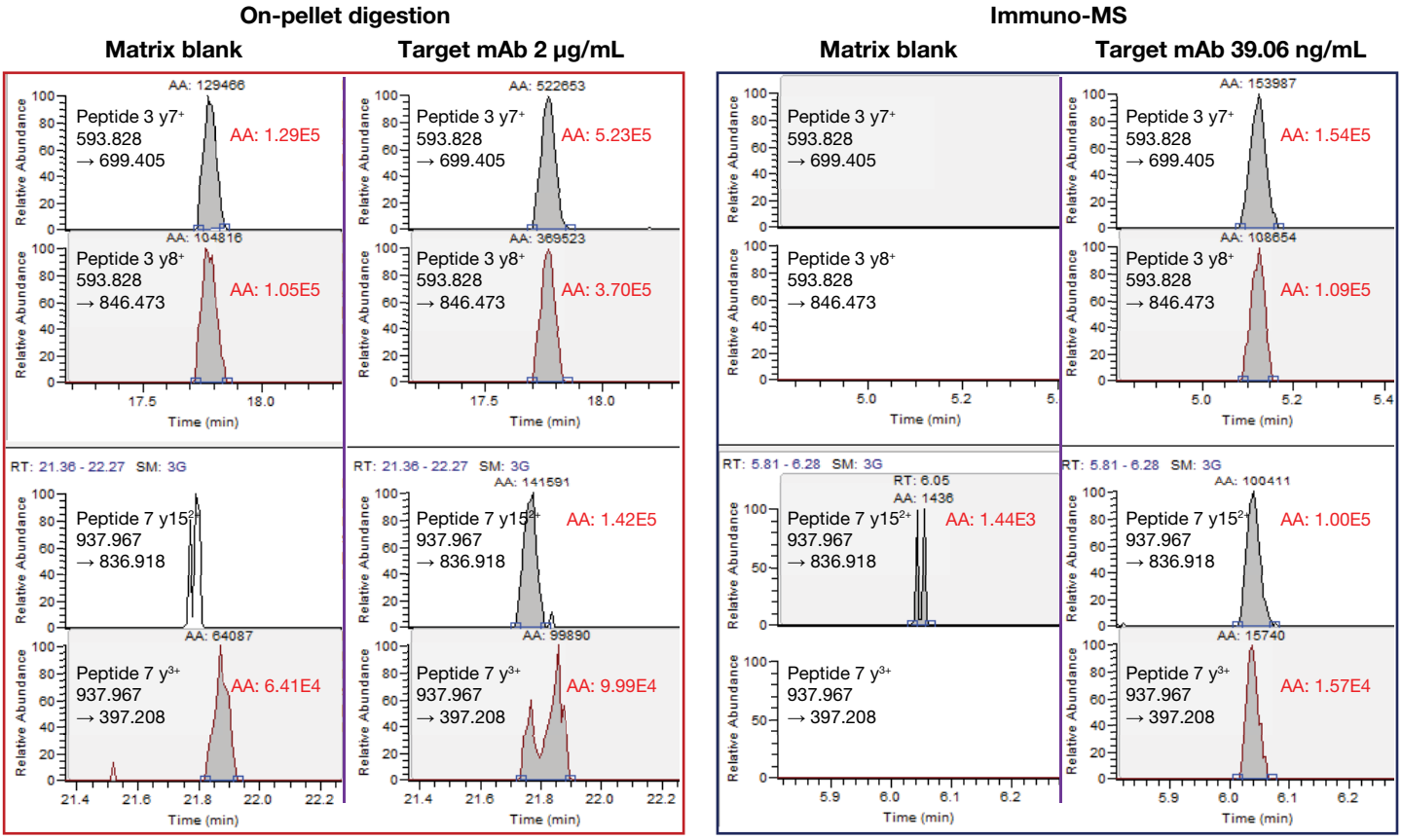

Figure 7. Investigation of method specificity in matrices. (A) Cynomolgus monkey serum matrix, spiked with 0 µg/mL or 2 µg/mL of target mAb. Samples were processed using on-pellet digestion protocol, and signature peptides were then monitored by PRM; (B) Cynomolgus monkey serum matrix, spiked with 0 ng/mL or 39.063 ng/mL of target mAb. Samples were processed using the SMART Digest IA kit based immuno-MS protocol; signature peptides were then monitored by PRM.

Quantification performance

Overall quantification performance in different matrices was evaluated. In different matrices, the four signature peptides all showed good linearity (Figure 8). Peptides 2, 3, and 7 had the linear range of 39.063 to 10,000 ng/mL, and peptide 5 had the range of 78.125 to 10,000 ng/mL (Table 5). The internal standard showed highly stable peak area response for all four signature peptides (RSDs within 5%), indicating the excellent reproducibility of the sample preparation step. Data from four consecutive days showed an LLOQ of 20 ng/mL for peptide 2 and peptide 3, 50 ng/mL for peptide 5, and 30 ng/mL for peptide 7. Quantification recovery at the LLOQ was within the range of 80% to 120% (Table 5). This linear range and LLOQ fully meet the current demands from pharmacokinetic and pharmacodynamic studies of mAbs. Since only 50 µL of serum sample was used in the current work, higher sensitivity could be expected through simply increasing the sample amount.

Table 5. Standard curve linear range, r^2, and LLOQ – Orbitrap ID-X

	Sequence	Linear range	r^2, linear fit, $1/x^2$ weight	LLOQ	IS CV%
Peptide 2	ALPAPIEK	20–10,000 ng/mL	0.996	20 ng/mL	4.50
Peptide 3	GPSVFPLAPSSK	20–10,000 ng/mL	0.993	20 ng/mL	3.34
Peptide 5	DSTYSLSSTLTLSK	50–10,000 ng/mL	0.994	50 ng/mL	4.51
Peptide 7	TTPPVLDSDGSFFLYSK	30–10,000 ng/mL	0.998	30 ng/mL	4.54

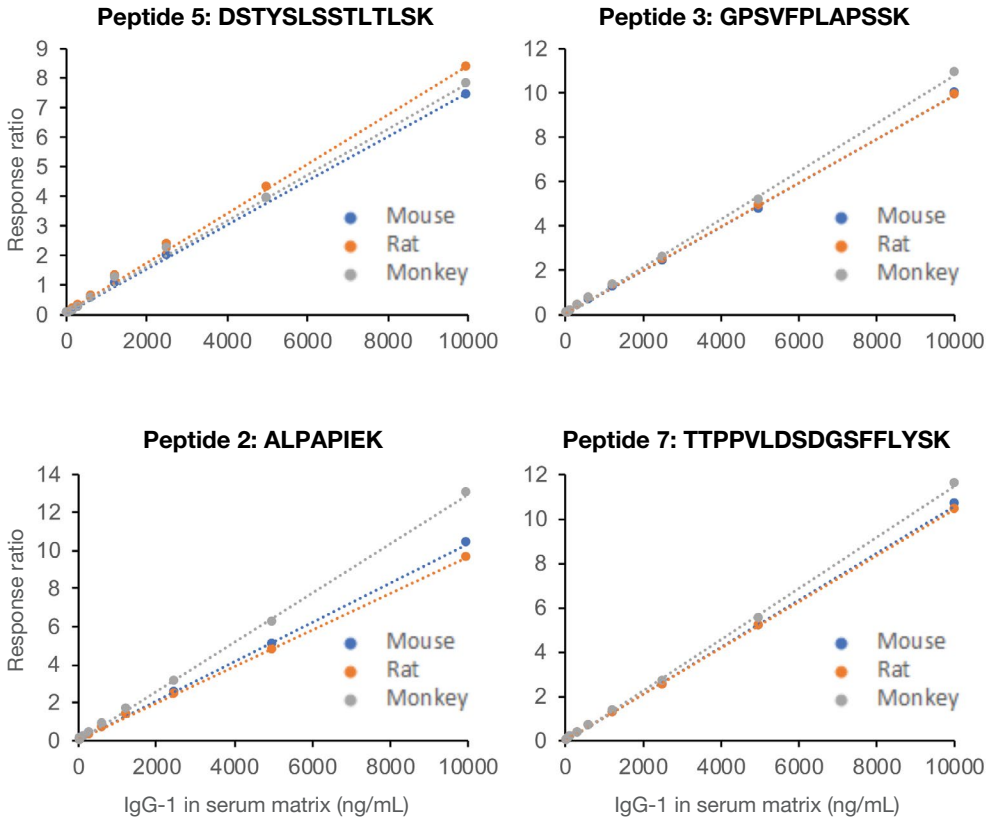

Figure 8. Linearity of four universal signature peptides of IgG1 in animal serum matrices. The range of the calibrator was 39.063–10,000 ng/mL.

Finally, quantification precision and accuracy were evaluated on three level QC samples (Table 6). The intra-assay standard deviations of the four peptides were within 10%. Except signature peptide 5 in the QCL sample, which had 10.4% of standard deviation, all peptides in every level of QC samples showed inter-assay CV within 10%, and the recoveries were all between 90% and 110%, demonstrating the excellent quantification performance. Peptide 3 was finally defined as the quantitative peptide to measure the concentration, and the remaining three peptides as monitoring peptides to investigate the structure integrity of the target mAb *in vivo.*

Table 6. Quantification precision and accuracy of the quality control samples

	QCL (100 ng/mL)			QCM (800 ng/mL)			QCH (6,000 ng/mL)		
	Intra CV% (n = 6)	Inter CV% (n = 4)	Accuracy	Intra CV% (n = 6)	Inter CV% (n = 4)	Accuracy	Intra CV% (n = 6)	Inter CV% (n = 4)	Accuracy
Peptide 2	2.26	2.42	102.1%	0.87	1.91	103.4%	2.08	2.09	100.5%
Peptide 3	2.87	3.53	102.8%	0.98	1.89	102.2%	0.87	1.64	99.9%
Peptide 5	6.54	10.40	98.5%	6.47	7.06	109.5%	4.96	8.66	109.3%
Peptide 7	4.28	6.08	107.6%	1.50	3.03	109.5%	3.70	5.38	101.5%

Table 7. Standard curve linear range and r^2 – Q Exactive Plus

	Sequence	Linear range	r^2, linear fit, $1/x^2$ weight
Peptide 2	ALPAPIEK	39.063–10,000 ng/mL	0.993
Peptide 3	GPSVFPLAPSSK	39.063–10,000 ng/mL	0.999
Peptide 5	DSTYSLSSTLTLSK	78.125–10,000 ng/mL	0.991
Peptide 7	TTPPVLDSDGSFFLYSK	39.063–10,000 ng/mL	0.998

This method was initially developed and validated on the Orbitrap ID-X platform; it was then transferred to the Q Exactive Plus platform to check the method versatility across instrument platforms. LC performance was kept completely the same as the identical mixer, sample loop, column, and tubing were used. Mass spectrometry parameters, especially the collision energy and ion ratio, should be updated since the Q Exactive series instrument adopted a different formula in the collision energy calculation compared to the Tribrid™ series instrument (Tables 2 and 4). The quantification performance on Q Exactive Plus platform is highly consistent with that on the Orbitrap ID-X platform, showing the convenience of method transfer among different instrument platforms.

Conclusion

An immuno-mass spectrometry-based universal method for IgG1 mAb bioanalysis in preclinical phase successfully established using the SMART Digest IA Av Magnetic Kit, CaptureSelect Human IgG-Fc PK Biotin Conjugate, and SILuMAB K1 - Stable Isotope Labeled Universal mAb Standard. This method enjoyed the benefit of fast simple workflow, high sensitivity, and method universality, and was also able to monitor the structural integrity while quantifying the mAb in vivo. It can be adopted for early stage of drug development for screening candidates with excellent kinetic feature to enter the next research phase with the principle of “fail quick, fail cheap”. It also works for the preclinical phase of drug development, providing data on pharmacokinetics and pharmacodynamics necessary for registration. This method is expected to save time during bioanalysis method development for pharmaceutical and CRO companies, and to provide more dimensional information for helping pharmaceutical scientists judge the probability of successful drug development with the candidate antibodies in the early stage.

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