

Comparison of alternative approaches to trypsin protein digestion for reproducible and efficient peptide mapping analysis of monoclonal antibodies

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Keywords

NIBRT, biopharmaceutical, biotherapeutic, CQA, monoclonal antibody (mAb), IgG, peptide mapping, sequence coverage, NISTmAb RM8671, Humira®, adalimumab, SMART Digest kit, Magnetic SMART Digest kit, KingFisher Duo Prime Purification System, Vanquish Flex Binary UHPLC System, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer

Application benefits

- A simple and rapid protein digestion approach to peptide mapping analysis has been identified using the Thermo Scientific™ SMART Digest™ kit with digestion taking only 45 min with subsequent reduction and alkylation taking a further 30 min each
- 100% sequence coverage was attained for all the studied digestion protocols and low levels of sample preparation-induced post-translational modifications (PTMs) were observed for the SMART Digest kit approach
- The proposed SMART Digest kit approach allows easily automated peptide mapping resulting in less sample handling, increased productivity, and improved reproducibility

Goal

To compare peptide mapping data acquired using the SMART Digest kit and the SMART Digest Trypsin kit, Magnetic Bulk Resin option, to those obtained by alternative digestion methods including an in-solution protocol and a rapid protein digestion method. The study focused on reproducibility, protein sequence coverage and identification of post-translational modifications (PTMs), including deamidation and oxidation.

Introduction

Due to their long half-life in humans and high specificity to target antigens, monoclonal antibodies (mAbs) are the leading biotherapeutics used for the treatment of multiple disease states, including cancer, inflammatory, autoimmune, cardiovascular, and infectious diseases.¹ Sales of €125 billion worldwide by the year 2020 are predicted at the current approval rate.² However, due to their complexity and the possibility of protein degradation, extensive characterization of mAbs must be carried out prior to clinical trials and for routine quality control assessments.³

Peptide mapping is a “gold standard” tool in biotherapeutic characterization, used to measure critical quality attributes (CQAs) of mAbs to ensure product quality, efficacy, and safety. Peptide mapping consists of the enzymatic or chemical treatment of a protein, resulting in peptide fragments that are then separated, detected, and interpreted. The protease trypsin is most frequently employed to digest therapeutic mAbs for peptide mapping analysis due to its specificity for cleavage at lysine and arginine residues. This results in the production of peptides with the preferred mass range for mass spectrometry detection.

Various approaches to peptide mapping have been developed, utilizing different protein denaturation procedures and digestion times. However, there can often be a lack of reproducibility and confidence in results with commonly used digestion protocols for peptide mapping. This leads to the incurring of excessive cost and reduced productivity due to the laborious sample handling procedures and long digestion times (up to several hours). Furthermore, modifications such as deamidation and oxidation may be induced by sample preparation methods, thereby distorting results.⁴ Hence, a simple, robust and reproducible method is essential for peptide mapping.

In this study, a traditional overnight trypsin digestion method and a recently applied rapid digestion protocol⁵ were compared to two SMART Digest kit options (the standard kit and the magnetic bulk resin option). These kits contain thermally stable, immobilized trypsin that enables high temperature protein denaturation without a requirement for addition of denaturants. The SMART Digest trypsin kit (magnetic bulk resin option) was used in conjunction with the Thermo Scientific™ KingFisher™ Duo Prime purification system, which allows easy automation

of magnetic bead based applications and is therefore suitable for high-throughput, reproducible sample analysis.⁶

Two monoclonal antibodies were used to assess the alternative digestion methods: adalimumab, the world's top selling biotherapeutics product² and the reference monoclonal antibody NISTmAb RM 8671 (NISTmAb), a standard commonly applied for evaluation of analytical method performance.⁷ All samples were analyzed on a high-resolution analytical platform consisting of a Thermo Scientific™ Vanquish™ Flex Binary UHPLC and a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer with BioPharma Option.

Four sample preparation methods for peptide mapping were investigated to evaluate the impact of sample preparation on reproducibility and PTMs detected. For each preparation method evaluated (reference experimental section), samples were prepared by two different analysts, with varying sample preparation experience, over three different days and analyzed by liquid chromatography-mass spectrometry (LC-MS) detection. Data analysis was performed using Thermo Scientific™ BioPharma Finder™ software. The analytical platform enabled excellent reproducibility of chromatographic data enabling 100% sequence coverage of mAbs.

Peptide reproducibility and PTMs induced by sample preparation were investigated, highlighting the benefits of using a heat stable immobilized trypsin for sample preparation compared to other trypsin formulations that require high pH buffers for protein denaturation. In addition, sample reproducibility was found to be further improved using trypsin immobilized on magnetic beads in combination with an automated handling system, e.g. KingFisher Duo Prime purification system.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ-cm resistivity
- Water, Fisher Chemical™ Optima™ LC/MS grade (P/N 10505904)
- Acetonitrile, Fisher Chemical™ Optima™ LC/MS grade (P/N 10001334)
- Water with 0.1% formic acid (v/v), Fisher Chemical™ Optima™ LC/MS grade (P/N 10188164)

- Acetonitrile with 0.1% formic acid (v/v), Fisher Chemical™ Optima™ LC/MS grade (P/N 10118464)
- Trifluoroacetic acid (TFA), Fisher Chemical™ (P/N 10294110)
- SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- SMART Digest Trypsin Kit (P/N 60109-102)
- Dithiothreitol (DTT), Fisher Bioreagents™ (P/N 10386833)
- Iodoacetic acid (IA), Acros Organics™ (P/N 10235940)
- MS Grade Trypsin Protease, Thermo Scientific™ Pierce™ (P/N 13464189)
- 8M Guanidine-HCl, Thermo Scientific™ Pierce™ (P/N 10167783)
- 1M Triethylammonium bicarbonate (TEAB), Thermo Scientific™ (P/N 90114)
- Tris-HCl, Fisher Chemical™ (P/N 10142400)
- Thermo Scientific™ KingFisher™ Deepwell, 96 well plate (P/N 95040450)
- Thermo Scientific™ KingFisher™ Duo 12-tip comb (P/N 97003500)
- Thermo Scientific™ Acclaim™ VANQUISH™ C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Equipment

- KingFisher Duo Prime Purification System (P/N 5400110)
- Vanquish Flex Binary Flex UHPLC system including:
 - Binary Pump F (P/N VF-P10-A)
 - Column Compartment H (P/N VH-C10-A)
 - Split Sampler FT (P/N VF-A10-A)
 - System Base Vanquish Horizon (P/N VH-S01-A)
- Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer with BioPharma Option (P/N IQLAAEGAAPFALGMBDK)

- Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer (P/N ND-2000)

Sample pre-treatment

Commercially available monoclonal antibody adalimumab drug product was supplied at a concentration of 50 mg/mL. NISTmAb reference material 8671, was supplied by National Standard of Standards and Technology (NIST), also at 10 mg/mL.

Sample preparation

Monoclonal antibody samples were prepared in triplicate by different analysts on different days.

In-solution digestion protocol using heat for denaturation (in solution, heat)

Samples were diluted to 2 mg/mL in water. Reduction of disulfide bonds was carried out by the addition of 10 mM DTT in 100 mM TEAB, pH 8.5, to the mAb samples with incubation at 70 °C for 75 minutes. Alkylation was carried out by the addition of 20 mM IA. The samples remained in darkness for 30 minutes, and 11 mM DTT was added to quench IA. Trypsin was added to mAb samples at a protein/trypsin ratio of 1:50, and tryptic digestion was carried out at 37 °C for 16 hours, followed by quenching with 10% TFA.

Alternative rapid digestion protocol (alternative rapid digest)

A peptide mapping sample preparation method described by Rogers et al.⁵ was used for this analysis. Samples were diluted to 2 mg/mL, denatured, and reduced using 10 mM DTT in 7.5 M guanidine, pH 8.3, for 30 minutes at ambient temperature. Following reduction, samples were alkylated in 20 mM IA and incubated for 20 minutes in darkness. The reaction was quenched with 11 mM DTT. Sample solutions were desalted using BioSpin™-P6 columns (Bio-Rad) and eluted in 100 mM Tris Buffer, pH 8.0. Protein concentration was measured with a NanoDrop 2000 Spectrophotometer. Trypsin was added to samples at a trypsin/protein ratio of 1:10 and incubated at 37 °C for 30 minutes, followed by reaction quenching with 10% TFA.

Protocol for sample preparation using a SMART Digest trypsin kit (SMART Digest)

Samples were diluted to 2 mg/mL in water. Then, 150 µL SMART Trypsin Buffer was added to SMART Digest vials, followed by 50 µL of sample (100 µg). MAb samples were incubated at 1400 rpm at 70 °C for 45 minutes. Following

tryptic digestion, samples were spun down for 2 minutes at 7000 rpm and supernatant placed in a fresh tube. Reduction of disulfide bonds was carried out with 10 mM DTT for 30 minutes at 57 °C. Samples were alkylated in 20 mM IA for 30 minutes in darkness. Quenching of IA and trypsin was carried out with 11 mM DTT and 10% TFA, respectively. (Note: use of IA and TFA is not required as part of the SMART Digest protocol, however, these were added to ensure that peptides induced by these reagents would be uniform across all sample preparations).

Protocol for sample preparation using a SMART Digest trypsin kit, magnetic bulk resin option (Magnetic SMART Digest)

Samples were diluted to 2 mg/mL in water. For each sample digest, sample and buffers were added to each lane of a KingFisher Deepwell 96 well plate as outlined in

Table 1. Bead “wash buffer” was prepared with by diluting SMART Digest buffer 1:4 (v/v) in water. Bead buffer was neat SMART Digest buffer. Digestion was performed using a KingFisher Duo Prime purification system with Thermo Scientific™ BindIt™ software (version 4.0), using the protocol outlined in Table 2. Samples were incubated for 45 minutes at 70 °C on medium mixing speed (to prevent sedimentation of beads), with post-digestion cooling carried out to 10 °C. Following digestion, disulfide bond reduction was performed with 10 mM DTT for 30 minutes at 57 °C and subsequently alkylated with 20 mM IA in darkness for 30 minutes. The reaction was quenched with 11 mM DTT followed by 10% TFA. (Note: use of IA and TFA is not required as part of the SMART Digest protocol, however, these were added to ensure that peptides induced by these reagents would be uniform across all sample preparations).

Table 1. KingFisher Duo Prime plate layout utilized for sample preparation. Reagents and associated volumes placed in each well are outlined.

Lane	Content	Volume Applied to Each Well (µL)
A	SMART Digest buffer	150
	Sample (2 mg/mL)	50
B	Tip Comb	
C	Empty	
D	Magnetic SMART Beads	15
	Bead Buffer (SMART Digest buffer)	100
E	Bead Wash Buffer (SMART Digest buffer 1:4 (v/v))	200
F	Waste Lane (Water)	250

Table 2. Protocol for automated peptide mapping using a KingFisher™ Duo Prime system

Step	Release Bead	Mixing	Collect Beads	Temperature	Lane
Collect Bead	–	10 s Bottom Mix	3 count, 1 s	–	D
Bead Wash	Yes	1 min Medium Mix	3 count, 1 s	–	E
Digest and Cool	Yes	45 min Medium Mix	3 count, 15 s	During digestion: 70 °C Post-digestion: 10 °C	A
Release Beads	Yes, Fast	–	–	–	F

LC conditions

Mobile phase A:	Water with 0.1% formic acid (v/v)
Mobile phase B:	Acetonitrile with 0.1% formic acid (v/v)
Flow rate:	0.3 mL/min
Column temperature:	25 °C (Still air mode)
Autosampler temperature:	5 °C
Injection volume:	10 µL
Injection wash solvent:	Methanol/water, 10:90
Needle wash:	Enabled pre-injection
Gradient:	See Table 3 for details

Table 3. Mobile phase gradient for UHPLC separation of peptides

Time (min)	Flow (mL/min)	% Mobile Phase B	Curve
0.000	0.300	2.0	5
45.000	0.300	40.0	5
46.000	0.300	80.0	5
50.000	0.300	80.0	5
50.500	0.300	2.0	5
65.000	0.300	2.0	5

Table 5. MS method parameters utilized for peptide mapping analysis

General	Setting	MS ² Parameters	Setting
Runtime	0 to 65 min	Resolution settings	17,500
Polarity	Positive	AGC target value	1.0 × 10 ⁵
Full MS parameters	Setting	Isolation width	2.0 m/z
Full MS mass range	200–2000 m/z	Signal threshold	1.0 × 10 ⁴
Resolution settings	70,000	Normalized collision energy (HCD)	28
AGC target value	3.0 × 10 ⁶	Top-N MS ²	5
Max injection time	100 ms	Max injection time	200 ms
Default charge state	2	Fixed first mass	–
SID	0 eV	Dynamic exclusion	7.0 s
Microscans	1	Loop count	5

Data processing

Acquisition software:	Thermo Scientific™ Xcalibur™ software 4.0
MS data analysis software:	Thermo Scientific™ BioPharma Finder 3.0

MS conditions

Detailed MS method parameters are shown in Tables 4 and 5.

Table 4. MS source and analyzer conditions

MS Source Parameters	Setting
Source	Thermo Scientific™ Ion Max source with HESI II probe
Sheath gas pressure	40 psi
Auxiliary gas flow	10 arbitrary units
Probe heater temperature	400 °C
Source voltage	3.8 kV
Capillary temperature	320 °C
S-lens RF voltage	50 V

MS data processing

Detailed parameter settings are shown in Table 6.

Table 6. BioPharma Finder software parameter settings for analysis of peptide mapping data

Component Detection	Setting
Absolute MS signal threshold	1.60×10^5 counts
Identification	Setting
Mass accuracy	5 ppm
Minimum confidence	0.8
Maximum number of modifications for a peptide	1
Unspecified modification	-58 to +162 Da
N-glycosylation	CHO
Protease specificity	High
Static Modifications	Setting
Side chain	Carboxymethylation
Variable Modifications	Setting
Side chain	Deamidation (NQ) Glycation (K) Oxidation (MW)

Results and discussion

Figure 1 shows chromatograms generated for six different sample preparations of NISTmAb using all four protein digestion methods, highlighting both the reproducibility of the sample preparation method and LC-MS platform.

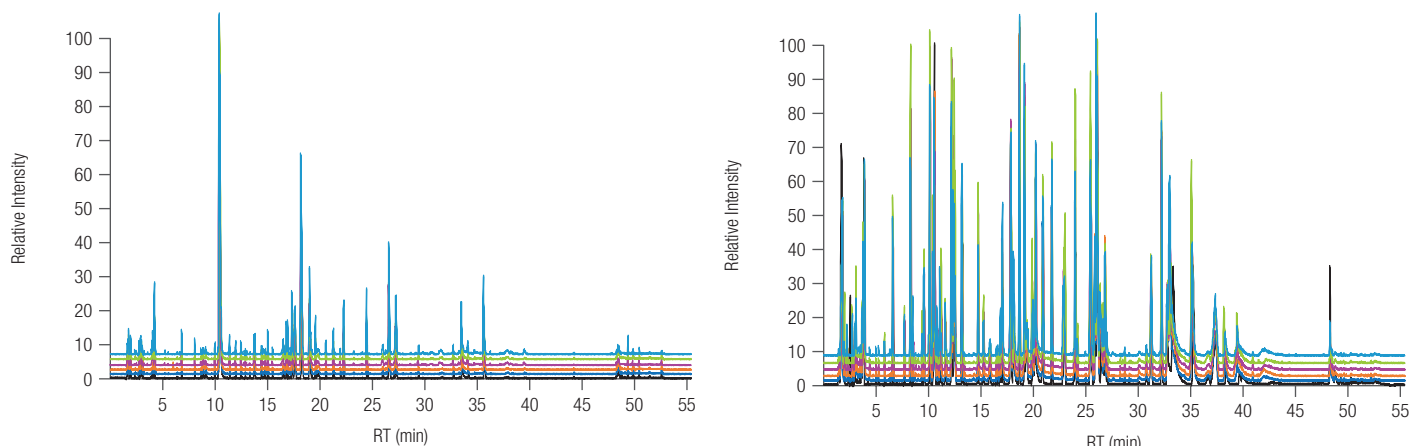


Figure 1A. Base Peak Chromatograms (BPC) overlay from six peptide mapping sample preparations of NISTmAb using in solution, heat digest (left) and alternative rapid digest (right). Samples were prepared by two individual operators on different days.

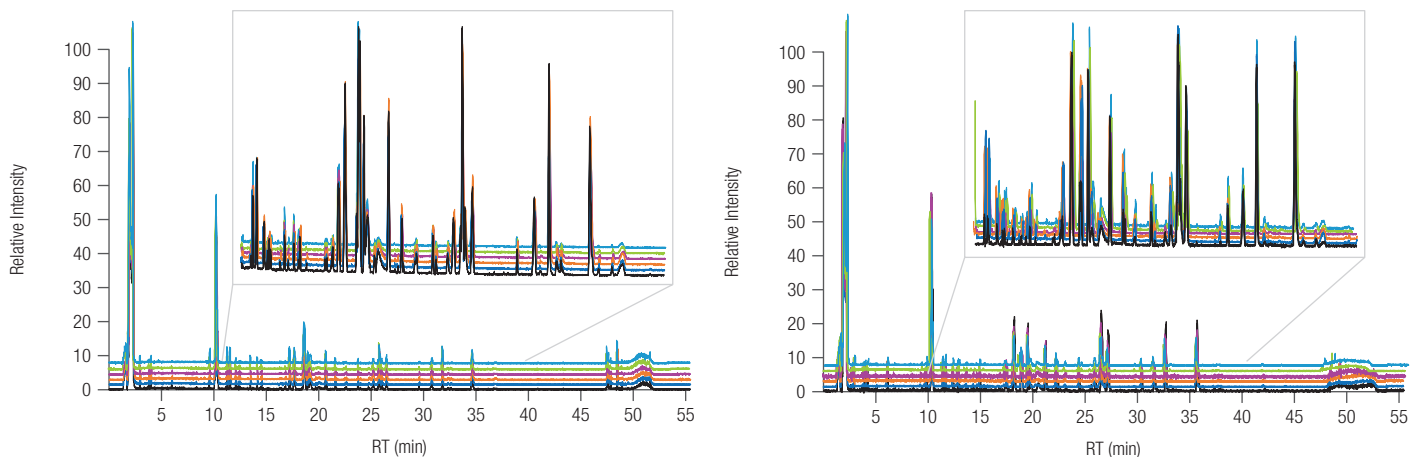


Figure 1B. Base Peak Chromatograms (BPC) overlay from six peptide mapping sample preparations of NISTmAb using SMART Digest (left) and Magnetic SMART Digest (right). Samples were prepared by two individual operators on different days.

Peptide mapping analysis provides relative quantification of post-translational and chemical modifications; therefore, very high sequence coverage for both mAb light and heavy chains is essential. For nearly all protein

digestion methods 100% coverage was achieved (Table 7). Both the rapid digestion protocol and SMART Digest protocol resulted in full sequence coverage using digestion times of < 45 minutes.

Table 7. Sequence coverage determined for adalimumab and NISTmAb following sample preparation with various digestion methods

Proteins	Number of MS Peaks	Sequence Coverage	Relative Abundance	Sample, n=6
NISTmAb RM8671 Light Chain	425	100%	16.01%	Magnetic SMART Digest, 45 minutes
	268	100%	22.77%	SMART Digest, 45 minutes
	493	100%	46.61%	Alternative rapid digest
	549	100%	26.52%	In-solution, heat
NISTmAb RM8671 Heavy Chain	1340	100%	83.94%	Magnetic SMART Digest, 45 minutes
	1045	100%	77.23%	SMART Digest, 45 minutes
	1002	100%	53.40%	Alternative rapid digest
	1652	100%	73.48%	In-solution, heat
Adalimumab Light Chain	753	100%	38.14%	Magnetic SMART Digest, 45 minutes
	627	100%	51.88%	SMART Digest, 45 minutes
	510	100%	23.30%	Alternative rapid digest
Adalimumab Heavy Chain	911	100%	49.40%	In-solution, heat
	1461	99.10%	61.86%	Magnetic SMART Digest, 45 minutes
	1409	99.10%	48.13%	SMART Digest, 45 minutes
	870	100%	45.48%	Alternative rapid digest
	1167	100%	50.60%	In-solution, heat

It has previously been reported that certain aspects of sample preparation may induce modifications, such as deamidation and oxidation of amino acid residues. Ren et al. reported that both the buffer pH and length of digestion time may artificially induce deamidation modifications.⁴ Similarly, oxidation may be induced by sample preparation or digestion. To determine the effect of the sample preparation methods evaluated on oxidation and deamidation modifications, the percentage relative abundance of each modified form of a peptide identified in each sample was determined using BioPharma Finder software. The average percent relative abundance of each deamidated and oxidized peptide from different sample preparations and replicate injections was calculated and is shown in Tables 8 and 9 for adalimumab and NISTmAb, respectively. For both mAbs, the percentage relative abundance of PTMs, including deamidations, oxidations, glycation, and N-glycosylation were examined. To demonstrate and compare the relative abundance, plots of oxidation and deamidation modifications are shown for all digestion methods for adalimumab and NISTmAb. For both oxidation and deamidation modifications, the alternative rapid digest method, the SMART Digest, and the magnetic SMART Digest kits demonstrated the lowest amount of modified peptide forms for the studied mAbs, except for adalimumab where SMART digestion showed similar levels of total deamidation than In-solution, heat digestion (44.30% and 44.32%, respectively). Adalimumab showed higher levels of modifications than NISTmAb for all studied digestion methods. In-solution, heat digestion showed the highest levels of oxidation and deamidation for adalimumab and NISTmAb.

SMART digest protocols were performed close to neutral pH (pH 7.2), while the in solution, heat method and alternative rapid digest method were carried out in basic conditions (pH 8.5 and pH 8.3, respectively). Studies have shown high buffer pH and long digestion times may lead to higher levels of deamidated peptides. As Figures 2 and 4 show, a combination of elevated buffer pH and digestion times over a number of hours lead to dramatically increased abundance of some deamidated peptide forms (N319/N318 and N329/N328 for adalimumab and NISTmAb, respectively).

The presence of certain deamidated peptides at high levels resulted in a significant increase in the relative total amount of deamidated species detected for the in-solution, heat method, in particular the presence of deamidated form of N318 (11.94%) in the in-solution, heat digests for NISTmAb and N319 (16.23%) in the in-solution, heat samples for adalimumab. Certain asparagine residues are more susceptible to sample preparation induced modifications. For example, N319 displayed low levels of deamidation for adalimumab when prepared using the SMART Digest kit (5.22%) and Magnetic SMART Digest kit (1.93%).

A therapeutic protein's higher order structure may have an effect on the proportion of artificially induced modifications observed following sample preparation with different protein digestion methods.

High levels of methionine oxidation were found following sample preparation using the in-solution, heat digestion method that were not observed following sample processing with the SMART Digest kit, Magnetic SMART Digest kit and the alternative rapid digestion protocol. For example, M256 was found to be 10.79% modified, contributing to the larger oxidation based relative abundance of this method for the adalimumab sample (Figure 3). The analysis of NISTmAb did not result in large percent relative oxidation modifications with only 3.69% of average modification for M431 when using the in-solution, heat digestion method. Similar profiles were observed for the percentage relative oxidized forms of M255 following sample preparation using the SMART Digest kit (1.52%), Magnetic SMART Digest kit (2.17%), and alternative rapid digestion method (1.54%).

Other commonly targeted modifications are lysine (K) glycations, which are listed in Tables 8 and 9. In total between 6 (SMART Digest method) to 26 (alternative rapid method) lysine glycations could be identified and relatively quantified for NISTmAb < 2.20% and for adalimumab sample < 0.90%.

One of the most noted PTMs for therapeutic mAbs is their varied N-linked glycan structures, which include galactosylation, fucosylation, mannosylation, and sialylation. High abundance of glycosylation of the heavy chain is also observed for the studied mAbs at the Fc part at position N301 for adalimumab or N300 for NISTmAb. The main glycans are complex biantennary oligosaccharides containing from 0 to 2 non-reducing galactoses with fucose attached to the reducing end of N-acetylglucosamine (A2G0F, A2G1F, A2G2F and A1G0F), afucosylated biantennary (A1G0, A2G0), and high mannose (M5, M6, and M7) structures (Tables 8 and 9).

The different approaches to peptide mapping sample preparation vary greatly in the number of preparation steps and amount of sample handling required. For example, the use of guanidine in the alternative rapid digest protocol results in the requirement for a desalting step in the sample preparation process, which causes a 30% loss in sample recovery. A lengthy and manual sample preparation protocol has the potential to lead to variations in peptide samples generated. To evaluate the reproducibility of sample preparation for each method, standard deviation (stdev) values were investigated for oxidation and deamidation modifications. For the data shown, low stdev values for modified peptides were observed for samples digested with the SMART Digest kits. The ease of automation for the digest method, and therefore the potential for high-throughput analysis of samples, make these methods ideal for a QC environment where precise and robust high-throughput analysis of samples is required.

Overall, the data shows that while 100% sequence coverage may be achieved using different methods, the method of denaturation and buffers used for sample preparation have a potentially large impact on the level of deamidation and oxidation modifications reported. Use of heat stable trypsin enzyme, high temperature protein denaturation and neutral pH reaction buffers may dramatically reduce sample preparation induced protein modifications when compared to in-solution, heat digestion method. The data shows a high level of reproducibility with SMART Digest kits and low levels of PTMs induced by sample preparation when compared to alternative methods evaluated.

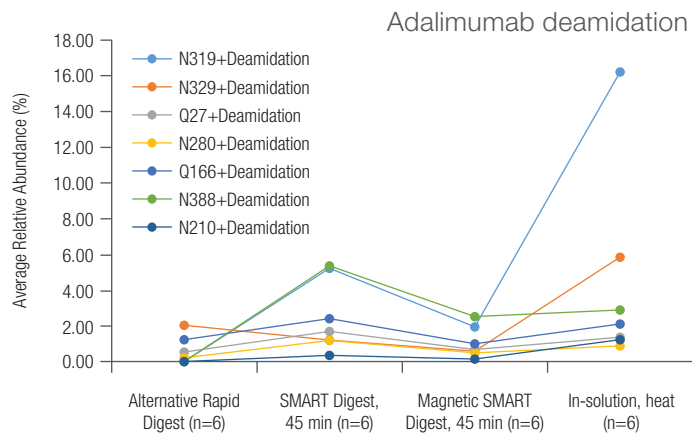


Figure 2. Average relative abundance of seven identified deamidation modifications for adalimumab with various digestion methods

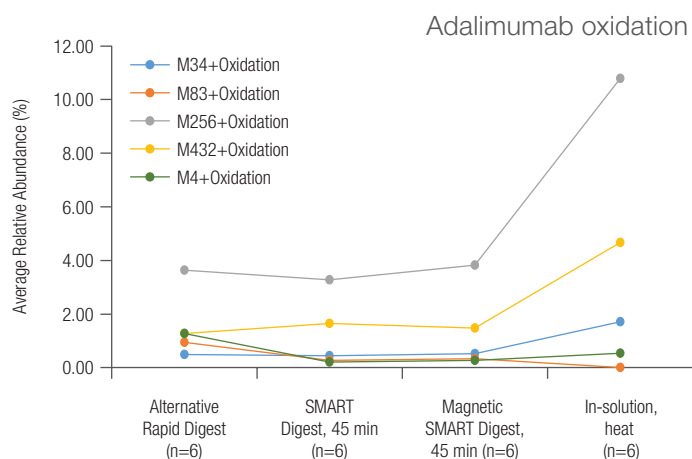


Figure 3. Average relative abundance of five identified oxidation modifications for adalimumab with various digestion methods

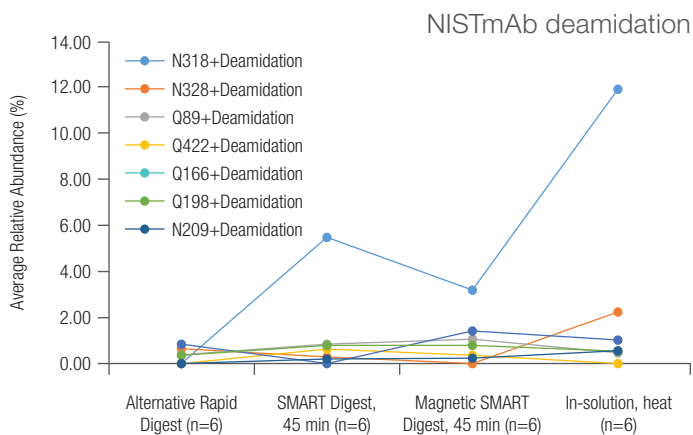


Figure 4. Average relative abundance of seven identified deamidation modifications for NISTmAb with various digestion methods

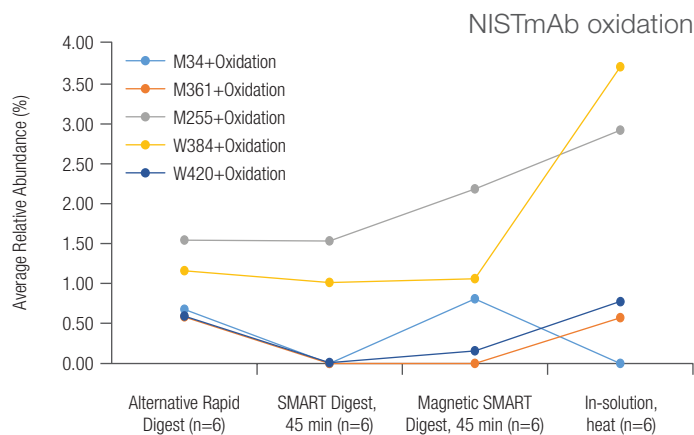


Figure 5. Average relative abundance of five identified oxidation modifications for NISTmAb with various digestion methods

Table 8A. Comparison of relative abundance (%) of post-translational modifications identified with the different digestion methods for adalimumab. A tilde (~) before the modification indicates the modification was found on the tryptic peptide but could not be localized on a specific amino acid with MS/MS spectra.

Modification	Alternative Rapid Digest (n=6)	SMART Digest, 45 min (n=6)	Magnetic SMART Digest, 45 min (n=6)	In-solution, heat (n=6)
~Q13+Deamidation	1.654	2.693	–	1.999
N74+Deamidation	–	–	–	0.805
N77+Deamidation	0.224	3.498	1.595	–
Q82+Deamidation	0.514	1.414	0.652	–
N84+Deamidation	0.259	1.359	0.577	–
Q113+Deamidation	0.567	–	–	–
N163+Deamidation	–	–	0.661	–
N290+Deamidation	0.218	1.174	0.496	0.873
N301+Deamidation	0.051	0.285	0.149	–
N319+Deamidation	–	5.221	1.933	6.229
N329+Deamidation	2.041	3.875	0.586	5.855
Q346+Deamidation	0.873	–	–	–
N365+Deamidation	0.127	1.880	0.910	0.170
Q366+Deamidation	0.423	1.268	0.571	0.905
~N388+Deamidation	–	5.367	2.523	2.892
~Q423+Deamidation	–	1.062	–	–
~N393+Deamidation	2.430	–	–	–
~Q3+Deamidation	1.016	1.224	1.046	–
~Q6+Deamidation	0.830	1.325	0.330	–
Q27+Deamidation	0.540	1.682	0.664	1.366
N31+Deamidation	0.131	–	0.571	–
~Q37+Deamidation	1.144	–	1.131	1.421
~Q38+Deamidation	–	1.476	–	–
Q55+Deamidation	0.787	1.263	0.327	0.726
Q100+Deamidation	0.689	1.941	0.541	1.195
~N137+Deamidation	0.388	2.088	0.588	1.510
Q147+Deamidation	0.661	1.049	0.387	0.801
~N158+Deamidation	–	1.777	–	1.741

Table 8B. Comparison of relative abundance (%) of post-translational modifications identified with the different digestion methods for adalimumab

Modification	Alternative Rapid Digest (n=6)	SMART Digest, 45 min (n=6)	Magnetic SMART Digest, 45 min (n=6)	In-solution, heat (n=6)
~Q155+Deamidation	–	–	0.436	1.431
~Q160+Deamidation	0.911	–	0.601	–
Q166+Deamidation	1.215	2.397	0.997	2.101
Q199+Deamidation	0.603	1.315	0.557	1.083
N210+Deamidation	–	0.338	0.148	1.220
M34+Oxidation	0.491	0.434	0.523	1.714
W53+Oxidation	0.084	–	–	–
W162+Oxidation	0.439	–	–	–
M256+Oxidation	3.636	3.278	3.828	10.798
~M432+Oxidation	1.268	1.654	1.477	4.670
M4+Oxidation	1.267	0.202	0.267	0.535
K76+Glycation	0.095	0.091	0.079	–
K137+Glycation	0.116	0.190	0.211	–
~K292+Glycation	0.135	0.214	0.224	–
K321+Glycation	0.046	0.031	–	–
~K330+Glycation	0.212	0.180	0.301	0.097
K338+Glycation	–	–	–	0.029
~K39+Glycation	0.364	–	–	–
K103+Glycation	0.194	0.265	0.177	0.217
K107+Glycation	0.041	–	–	–
K145+Glycation	0.050	–	–	–
K149+Glycation	0.209	–	0.361	0.252
K169+Glycation	0.127	–	–	–
~K183+Glycation	0.410	–	–	0.399
K188+Glycation	0.897	–	0.327	0.396
K190+Glycation	0.050	0.845	–	0.047
K207+Glycation	0.063	–	–	0.023
N301+A1G0	0.324	0.359	0.532	–
N301+A1G0F	3.238	6.531	11.534	4.942
N301+A1G1F	0.806	1.114	1.466	0.462
N301+A2G0	0.841	0.681	0.802	0.617
N301+A2G0F	66.339	67.081	61.945	75.617
N301+A2G0FB	0.223	–	0.338	–
N301+A2G1	0.028	–	0.098	–
N301+A2G1F	15.589	12.881	14.879	15.932
N301+A2G2F	1.003	0.796	0.988	1.071
N301+Gn	0.151	0.350	0.332	–
N301+M3	0.591	0.953	0.791	0.147
N301+M4	0.611	0.706	0.953	0.351
N301+M5	5.535	5.076	4.639	3.897
N301+M6	2.645	2.342	1.876	1.662
N301+M7	1.225	0.904	0.794	0.726
N301+M8	0.467	0.285	0.316	0.262
N301+Unglycosylated	1.631	3.606	3.471	0.425

Table 9A. Comparison of relative abundance (%) of post-translational modifications identified with the different digestion methods for NISTmAb RM8671. A tilde (-) before the modification indicates the modification was found on the tryptic peptide but could not be localized on a specific amino acid with MS/MS spectra

Modification	Alternative Rapid Digest (n=6)	SMART Digest, 45 min (n=6)	Magnetic SMART Digest, 45 min (n=6)	In-solution, heat (n=6)
Q1+Deamidation	0.041	–	–	0.041
N62+Deamidation	0.039	–	–	–
N78+Deamidation	0.116	0.584	0.690	–
Q79+Deamidation	0.380	0.845	1.059	0.477
~N279+Deamidation	–	–	–	0.469
N86+Deamidation	0.108	–	–	–
~N206+Deamidation	–	1.180	–	–
~N204+Deamidation	–	–	1.407	–
N289+Deamidation	0.180	1.056	0.869	0.591
N300+Deamidation	–	0.230	0.106	–
N318+Deamidation	–	5.502	3.206	11.942
N328+Deamidation	0.650	0.294	–	2.253
N364+Deamidation	0.040	1.849	0.440	0.288
Q365+Deamidation	0.304	0.853	1.694	0.324
~N392+Deamidation	3.002	–	4.429	2.736
~Q3+Deamidation	0.389	–	–	–
~Q6+Deamidation	0.508	–	–	–
~N387+Deamidation	–	4.579	–	–
~Q422+Deamidation	–	0.625	0.353	–
~Q36+Deamidation	0.690	1.553	1.440	–
~N136+Deamidation	0.273	1.027	0.932	0.618
Q146+Deamidation	0.284	0.724	0.603	0.310
~Q154+Deamidation	0.517	0.244	0.664	0.464
~Q159+Deamidation	0.537	–	–	–
~Q165+Deamidation	0.854	–	1.418	1.019
Q198+Deamidation	0.367	0.786	0.784	0.544
~N157+Deamidation	–	1.478	0.977	0.840
N209+Deamidation	–	0.195	0.241	0.555
~M34+Oxidation	0.674	–	0.803	–
M87+Oxidation	0.752	–	0.495	–
W161+Oxidation	0.245	–	–	–
M255+Oxidation	1.537	1.524	2.173	2.905
M361+Oxidation	0.583	–	–	0.568
~M431+Oxidation	1.157	1.007	1.055	3.691
M4+Oxidation	0.790	–	0.369	0.412
M32+Oxidation	0.589	0.013	0.160	0.774
K58+Glycation	0.601	–	0.430	–
K66+Glycation	0.369	–	–	–
K73+Glycation	1.036	0.964	0.998	0.796
K77+Glycation	0.281	–	–	–
K136+Glycation	0.309	–	0.360	–
K216+Glycation	0.336	–	–	–
K249+Glycation	0.590	–	–	–
~K291+Glycation	0.400	0.527	0.571	0.255
K320+Glycation	0.125	–	0.082	0.096
~K323+Glycation	0.167	–	2.198	–
K325+Glycation	0.703	–	0.716	0.317
K329+Glycation	0.360	0.429	0.422	0.256

Table 9B. Comparison of relative abundance (%) of post-translational modifications identified with the different digestion methods for NISTmAb RM8671

Modification	Alternative Rapid Digest (n=6)	SMART Digest, 45 min (n=6)	Magnetic SMART Digest, 45 min (n=6)	In-solution, heat (n=6)
K395+Glycation	0.080	–	–	–
K341+Glycation	–	–	–	0.036
K417+Glycation	0.048	–	–	–
K38+Glycation	0.124	–	0.207	–
~K41+Glycation	0.625	–	–	–
K44+Glycation	0.294	–	–	–
K52+Glycation	0.469	–	0.552	0.472
K106+Glycation	0.140	–	–	–
K144+Glycation	0.163	–	–	–
K148+Glycation	0.480	0.481	0.697	0.461
K168+Glycation	0.306	–	–	–
~K182+Glycation	0.498	–	–	0.724
K187+Glycation	0.855	–	0.554	0.910
K189+Glycation	0.158	2.060	–	0.131
K206+Glycation	0.104	0.024	–	0.085
K337+Glycation	–	–	0.101	0.089
K363+Glycation	–	–	0.123	–
~K208+Glycation	–	–	–	0.109
N300+A1G0	0.668	0.613	0.676	0.501
N300+A1G0F	3.549	6.003	12.143	3.349
N300+A1G0M4F	0.603	–	–	–
N300+A1G0M5F	1.332	–	–	0.879
N300+A1G1	0.098	–	0.066	0.047
N300+A1G1F	3.297	3.881	6.208	2.745
N300+A1G1M4F	1.512	0.985	1.400	1.084
N300+A1G1M5	0.231	–	–	–
N300+A1G1M5F	0.207	–	–	–
N300+A1S1M5	0.095	–	–	0.051
N300+A1Sg1	–	–	–	1.054
N300+A1Sg1F	1.049	0.968	1.006	0.797
N300+A2G0	0.118	0.097	0.168	–
N300+A2G0F	38.644	40.615	34.173	44.163
N300+A2G1	–	–	0.180	–
N300+A2G1F	36.308	34.641	33.831	38.902
N300+A2G2F	9.012	8.433	8.060	9.019
N300+A2G2M4F	1.836	–	–	–
N300+A2G2M5F	–	–	0.718	0.582
N300+A2Ga1G1F	1.637	1.670	2.052	1.739
N300+A2Ga2F	0.882	0.819	0.960	0.915
N300+A2S1G0	–	–	–	0.488
N300+A2S1G1	0.247	0.326	–	0.343
N300+A2Sg1G0F	0.304	–	0.383	0.161
N300+A2Sg1G1F	0.393	–	0.477	0.321
N300+A2Sg1Ga1F	0.563	0.430	–	0.300
N300+A3G0F	–	–	–	0.176
N300+A3G1F	0.558	–	–	0.422
N300+A3G2F	0.460	–	0.492	0.428
N300+A3G3F	0.257	–	0.294	–

Table 9C. Comparison of relative abundance (%) of post-translational modifications identified with the different digestion methods for NISTmAb RM8671

Modification	Alternative Rapid Digest (n=6)	SMART Digest, 45 min (n=6)	Magnetic SMART Digest, 45 min (n=6)	In-solution, heat (n=6)
N300+Gn	–	0.062	0.038	–
N300+M3	–	0.070	0.176	–
N300+M4	–	0.015	0.138	–
N300+M5	1.199	1.267	0.949	0.823
N300+M6	0.072	–	0.041	0.041
N300+Unglycosylated	0.708	1.610	1.043	0.498

Conclusions

- SMART Digest kits provide simple and rapid protein digestion for peptide mapping analysis in comparison with in solution digestion methods. The SMART Digest kit and Magnetic SMART Digest kit allow digestion in 45 min with little manual sample preparation.
- Analysis of monoclonal antibodies gives excellent quality data with high confidence in results. Excellent sequence coverage (100%) and low levels of sample preparation-induced post-translational modifications (PTMs) were observed with both versions of the SMART Digest kits (deamidation <5.5% and oxidation <3.8% for both adalimumab and NISTmAb).
- Easily automated peptide mapping resulting in less sample handling, increased productivity and improved reproducibility.

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