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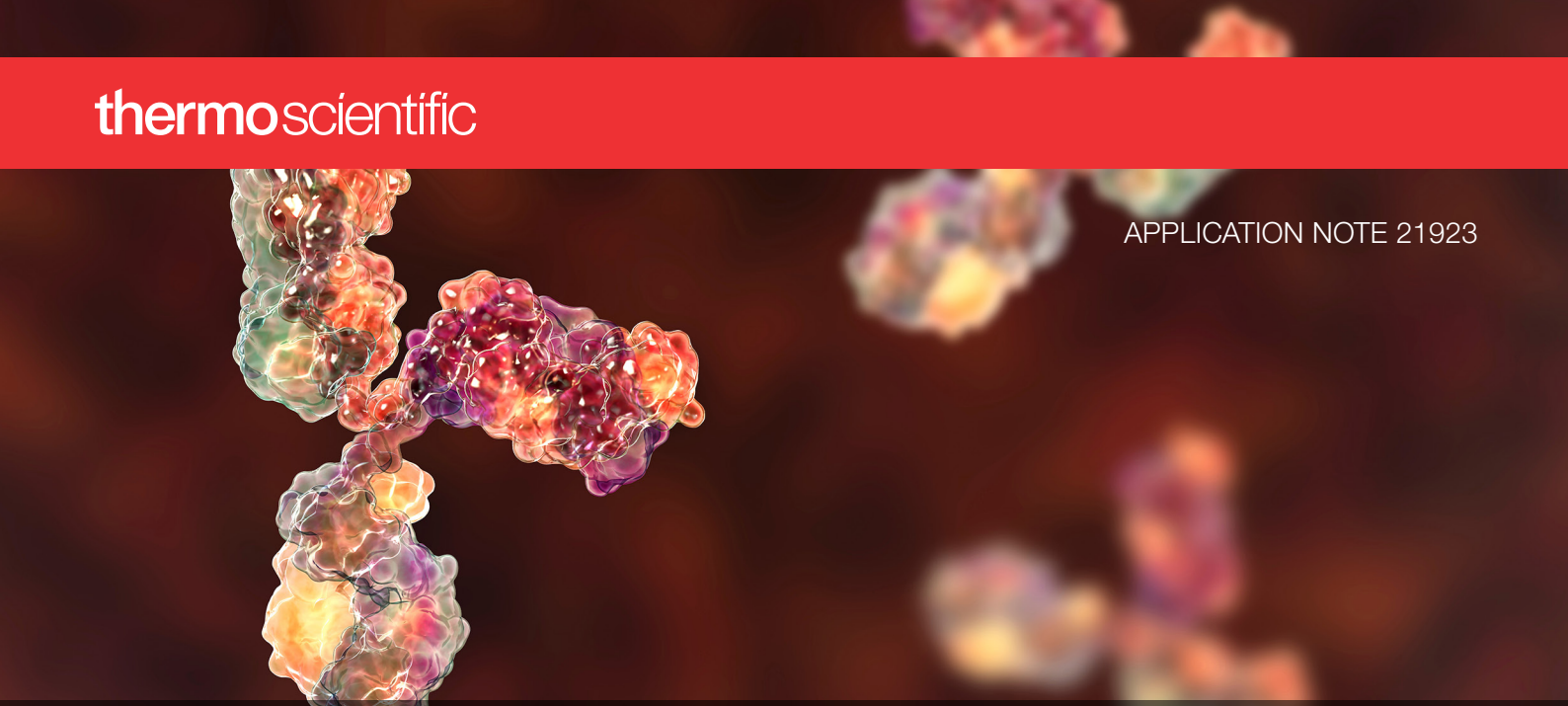

APPLICATION NOTE 21923

Automated biopharmaceutical protein digestion optimization for peptide mapping method development

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Keywords

Biotherapeutics, monoclonal antibodies (mAbs), IgG1, Yervoy® (ipilimumab), digest optimization, time-course, SMART Digest magnetic kit, KingFisher Duo Prime purification system, Vanquish UHPLC system, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer

Application benefits

Combining the Thermo Scientific™ SMART Digest™ magnetic kits with the Thermo Scientific™ KingFisher™ Duo Prime purification system provides an automated approach to protein digest method optimization.

- High quality, reproducible peptide mapping data eases the burden of method development, optimization, and validation.
- No denaturing reagents required—heat stable enzymes enable thermal denaturation.
- Immobilized trypsin in excess to sample—removes requirement to determine optimum enzyme to protease ratio.
- Precise termination of digestion—magnetic beads enable automated removal of immobilized trypsin by the KingFisher Duo Prime system.
- Downloadable Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) eWorkflow™ procedures and Thermo Scientific™ BindIt™ software time-course protocol to determine minimum optimal digest time.

Goal

A robust and reproducible, automated digestion time-course protocol to determine the optimal digest time for a biotherapeutic during development of a peptide mapping method.

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Introduction

Peptide mapping is a powerful analytical technique that when coupled with high resolution accurate mass (HRAM) mass spectrometry (MS) can be used to characterize the primary structure of a biotherapeutic. Alternatively, the use of optical detection, such as UV, allows peptides that either denote critical quality attributes (CQAs) or are specific to the biotherapeutic of interest to be monitored. Peptide monitoring performed in this way is viewed as the gold standard identity and quality test in QA/QC environments.

Bioanalytical scientists are challenged with developing reproducible and robust analytical methods that are easily transferable and easily implementable by non-experts. Traditional peptide and monitoring methods are notoriously difficult to validate due to the number of steps required to ensure a successful and reproducible protein digest. The aim, therefore, is to simplify this process and develop methods that are easily validated for in-process testing, lot/batch release, and stability testing within QC laboratories.

Biotherapeutics are often complex proteins comprised of multiple globular domains with disulfide bridges and electrostatic interactions. Denaturing agents, such as guanidine hydrochloride and urea, are often required to denature and unfold the proteins to enable high fidelity proteases, such a trypsin, to access cleavage sites and accomplish a complete digestion of the protein into its constituent peptides.[1]

During analytical method development, there are critical parameters that will impact the overall performance and reproducibility of digestion. As part of a peptide mapping method optimization, the length of the digest is one such parameter, as this will ultimately influence the overall reproducibility of the method.[2]

Here, an automated digestion time-course approach, using SMART Digest kits coupled with the KingFisher Duo Prime purification system is presented. The protocol facilitates determination of optimal incubation time to ensure complete digestion of a biotherapeutic, during peptide mapping method optimization.

The automated digestion time-course BindIt protocol can be downloaded from Thermo Scientific AppsLab Library and easily transfered, via USB connection, to the KingFisher Duo Prime system. Five-minute time intervals are built into the protein digestion stage of the protocol, where after each interval, the digest is paused as the SMART Digest magnetic beads, supporting the immobilized, heat-stable trypsin, are removed by the KingFisher Duo Prime system, allowing a sample aliquot to be taken at that timepoint.

Experimental

Recommended consumables

- Water, Optima™ LC/MS grade (Fisher Chemical) (P/N 10505904)
- Water with 0.1% formic acid (v/v), Optima™ LC/MS grade (Fisher Chemical) (P/N 10188164)
- Acetonitrile with 0.1% formic acid (v/v), Optima™ LC/MS grade (Fisher Chemical) (P/N 10118464)
- Thermo Scientific™ SMART Digest™ Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- Thermo Scientific™ KingFisher™ Deepwell, 96 well plate (P/N 95040450)
- Thermo Scientific™ KingFisher™ Duo 12-tip comb (P/N 97003500)
- Thermo Scientific™ MAbPac™ RP LC column, 4 µm, 2.1 × 50 mm (P/N 088648)
- Thermo Scientific™ Acclaim™ VANQUISH™ C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)

Equipment

- Thermo Scientific™ KingFisher™ Duo Prime purification system (P/N 5400110)
- Thermo Scientific™ Vanquish™ Horizon UHPLC System including:
  - System Base Vanquish Horizon/Flex (P/N VF-S01-A-02)
  - Binary Pump H (P/N VH-P10-A-02)
  - Column Compartment H (P/N VH-C10-A-02) with Active Pre-heater VH-C1 (P/N 6732.0110) and Post-column Cooler 1 µL VH-C1 (P/N 6732.0510)
  - Split Sampler HT (P/N VH-A10-A-02)
  - Diode Array Detector HL (P/N VH-D10-A) equipped with LightPipe™ Standard Flow Cell (P/N 6083.0100). UV detector was bypassed for LC-MS work.
- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer with BioPharma option (P/N 0726055)

Sample preparation

Commercially available ipilimumab (Yervoy®) monoclonal antibody (mAb) drug product, supplied at a concentration of 5 mg/mL, was diluted to a final concentration of 2 mg/mL (final volume 650 µL) with water (Optima, LC/MS grade).

Table 1. Monoclonal antibody used in the study

Drug	Concentration	Type
Ipilimumab	5 mg/mL	Recombinant Human IgG1κ mAb

To prepare the KingFisher Deepwell 96 plate, 150 µL SMART Digest buffer was aliquoted into each sample well in Lane A (A1–A12). To this, 50 µL ipilimumab (2 mg/mL) was added, resulting in a final sample concentration of 0.5 mg/mL. Additional lanes of the KingFisher Deepwell 96 plate were prepared as outlined in Table 2.

Table 2. KingFisher Deepwell 96 well plate layout

Lane	Content	Volume per well (µL)
A (Samples)	SMART Digest Buffer	150
	Sample (2 mg/mL)	50
B (Tips)	Tip Comb	N/A
C	Empty	N/A
D (Resin)	Magnetic SMART Beads	15
	SMART Digest Buffer	100
E (Bead Wash)	Bead Wash Buffer (SMART Digest Buffer diluted 1:4 (v/v) with water, Optima LC/MS grade)	200
F (Waste)	Water, Optima LC/MS grade	200

Digestion was performed using the Kingfisher Duo Prime purification system with BindIt software (version 4.0), using the time-course protocol outlined in Table 3, which is available for download via the Thermo Scientific™ AppsLab Library of Analytical Applications. Samples were incubated at 70 °C with medium mixing speed to prevent sedimentation of beads.

Detailed chromatographic conditions are listed in Tables 4 and 5 for residual intact mAb assessment and Tables 6 and 7 for peptide mapping assessment.

Table 3. Kingfisher Duo Automated digest time-course protocol with BindIt software

Step	Release Beads	Mixing	End of Step*	Temperature	Lane
Collect Beads	-	10 s (slow mix)	3 count, 1 s	-	D
Bead Wash	Yes	1 min (medium mix)	3 count, 1 s	-	E
Digest**	Yes	5 min (medium mix)	3 count, 5 s	70 °C	A
Pause***	-	-	-	70 °C	-
Leave (Tip comb)	-	-	-	-	F

*At the end of each step in the time-course protocol, the magnetic beads will be collected by the KingFisher magnetic rods three times with a collection time of either 1 or 5 s.
**The automated digest time-course protocol is set to perform 12 × 5 min digest steps at 70 °C. Refer to Figure 1.
***At each Pause, the KingFisher plate returns to the loading position, allowing collection of a timepoint sample.

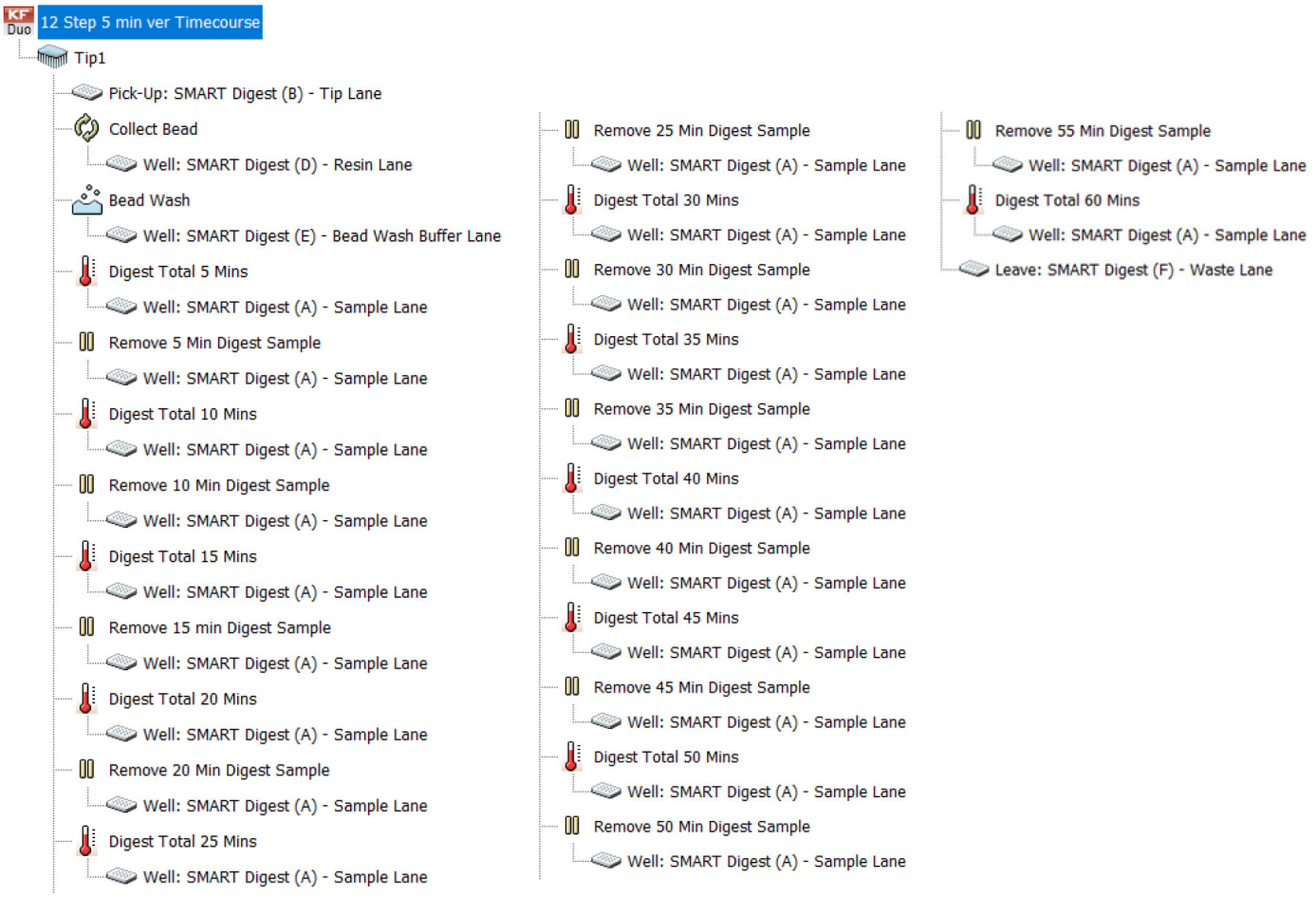

Figure 1. BindIt automated digest time-course protocol

Table 4. Chromatographic conditions for residual intact mAb assessment

Conditions	
Column	MAbPac RP LC column, 4 µm, 2.1 × 50 mm
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in acetonitrile
Flow Rate	0.3 mL/min
Column Compartment Temperature Settings	Column compartment: 80 °C Active pre-heater: 80 °C Post-column cooler: 40 °C
Injection Volume	8 µL
Detector Settings	Detection wavelength: 280 nm Data acquisition rate: 100 Hz Response time: 0.05 s
Gradient	See Table 5 for details

Table 5. Mobile phase gradient for residual intact mAb assessment

Time (min)	Flow mL/min)	% Mobile Phase B	Curve
0.0	0.30	5.0	5
1.0	0.30	5.0	5
11.0	0.30	80.0	5
12.0	0.30	80.0	5
12.1	0.30	5.0	5
15.0	0.30	5.0	5

Table 6. Chromatographic conditions for peptide mapping assessment

Conditions	
Column	Acclaim VANQUISH C18 column, 2.2 µm, 2.1 × 250 mm
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in acetonitrile
Flow Rate	0.3 mL/min
Column Compartment Temperature Settings	Column compartment: 60 °C Active pre-heater: 60 °C Post-column cooler: 40 °C
Injection Volume	5 µL
Gradient	See Table 7 for details

Table 7. Mobile phase gradient for peptide mapping assessment

Time (min)	Flow mL/min)	% Mobile Phase B	Curve
0.0	0.30	2.0	5
40.0	0.30	40.0	5
42.0	0.30	80.0	5
45.0	0.30	80.0	5
45.5	0.30	2.0	5
60.0	0.30	2.0	5

MS conditions

Detailed MS method parameters are listed in Tables 8 and 9.

Table 8. MS tune settings for peptide mapping assessment

MS Source Setting	Value
Source	Thermo Scientific™ Ion Max source with HESI II probe
Sheath Gas	35 arb
Aux Gas	10 arb
Sweep Gas	0 arb
Spray Voltage	3.5 kV
S-lens RF Level	50
Aux Gas Temp.	250 °C
Capillary Temp.	250 °C

Table 9. MS tune settings for peptide mapping assessment

MS Method Setting	Value
General	
Runtime	0 to 60 min
Polarity	Positive
Default Charge State	2
Inclusion	-
Exclusion	-
Tags	-
Full MS	
Resolution	70,000
AGC Target	$3e^6$
Maximum IT	100 ms
Scan Range	200–2000 *m/z*
dd-MS² / dd-SIM	
Resolution	17,000
AGC Target	$1e^5$
Maximum IT	200 ms
Loop Count	5
TopN	5
Isolation Window	2.0 *m/z*
Fixed First Mass	-
(N)CE/Stepped (N)CE	NCE: 30
dd Settings	
Minimum AGC Target	$2.00e^3$
Intensity Threshold	$1.0e^4$
Apex Trigger	-
Charge Exclusion	Unassigned
Peptide Match	Preferred
Exclude Isotopes	On
Dynamic Exclusion	10.0 s

Data processing

Chromeleon CDS software version 7.2.9 (Cat. No. CHROMELEON7) was used for LC-MS data acquisition and analysis. For data processing, Thermo Scientific™ BioPharma Finder™ software version 3.1 was applied. Detailed parameter settings are shown in Table 10.

Table 10. BioPharma Finder software version 3.1 settings for peptide mapping assessment

Database Parameters	
Protease	Trypsin (*C*-term KR)
Specificity	High
Fixed Modification	-
Variable Modifications	Deamidation (N) Deamidation (Q) Glycation (K) NH_3 loss (NQ) Oxidation (MW) Lys (*C*-term) Gln→Pyro-Glu (*N*-term) *N, O*-Glycans (CHO)
Component Detection Parameters	
Absolute MS Signal Threshold	1.02E+6
Typical Chromatographic Peak Width (min)	0.15
Maximum Chromatographic Peak Width (min)	2.16
MS Noise Level	8000.00
S/N Threshold	128.00
Relative MS Signal Threshold (% Base Peak)	1.00
Relative Analog Threshold (% of Highest Peak)	1.00
Width of Gaussian Filter (Represented as 1/n of Chromatographic Peak Width)	3
Minimum Valley to be Considered as Two Chromatographic Peaks (%)	80.0
Minimum MS Peak Width (Da)	1.20
Maximum MS Peak Width (Da)	4.20
Mass Tolerance (ppm for high-res or Da for low-res)	4.00
Maximum Retention Time Shift (min)	3.30
Maximum Mass (Da)	30,000
Mass Centroiding Cutoff (% from base)	15
Identification	
Search by Full MS Only	No
Use MS/MS	Use All MS/MS
Maximum Peptide Mass	12,000
Mass Accuracy (ppm)	5
Minimum Confidence	0.50
Maximum Number of Modifications for a Peptide	2
Unspecified Modification	No
N-Glycosylation	CHO
Search for Amino Acid Substitutions	None
Disulfide Search	
Perform Disulfide Search	Yes
Allow Free Cys	Yes
Maximum Number of Hits	2048
Maximum Number of Disulfide Bonds	3
Maximum Number of Identical Chains in the Molecule	2

Results and discussion

Taking a stepwise approach, we compared the results from the time-course protocol on the KingFisher Duo Prime system by analyzing each 5 min digest timepoint at both the intact protein and peptide level. From this, the optimal digest length for ipilimumab in non-reduced conditions was determined.

Residual intact mAb assessment

An assessment of digest efficiency was initially made at the intact protein level by analyzing each timepoint on a Vanquish Horizon UHPLC with UV detection following separation with a MAbPac RP column as per Table 4 and 5 in the Experimental section. The progress of the ipilimumab digestion is easily tracked from a 0 min control sample (no digestion) by monitoring the loss of the intact mAb peak, eluting at approximately 5.5 min (Figure 2).

The SMART Digest magnetic trypsin takes immediate effect, generating large peptide peaks even by the first 5 min timepoint where a reduction in the peak height of the intact mAb peak is evident. The intact mAb peak is not visible after the 45 min timepoint, implying that digestion of the intact mAb has gone to completion.

For a more comprehensive assessment, additional factors must be taken into consideration to confirm the completeness of digestion of ipilimumab within 45 min. The percentage of missed and non-specific cleavages, total intensity of identified peptides, level of trypsin autolysis, post-translational modifications (PTMs), and sequence coverage cannot be assessed by UV detection at the intact level. Therefore, samples were also analyzed at the peptide level.

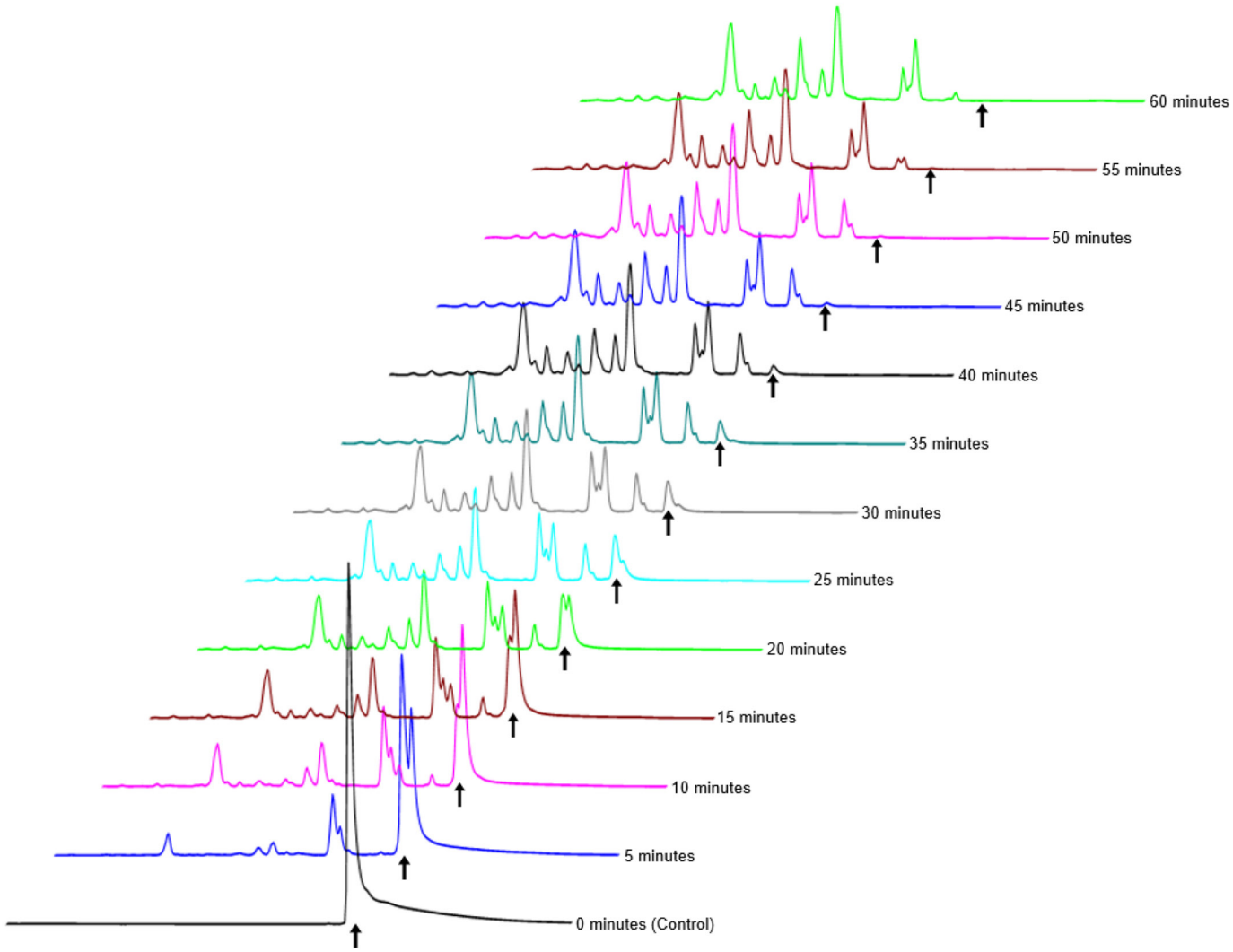

Figure 2. Residual intact mAb—digestion completion assessment. Overlay of UV chromatograms of the digestion timepoints for ipilimumab separated on a MAbPac RP column and detected at 280 nm. Arrows indicate intact mAb peaks.

Peptide mapping assessment

Post-20 min digestion, the intact assessment (Figure 2) provided confidence that the level of remaining intact mAb was acceptable for peptide mapping analysis. Therefore digest timepoints from 20 to 60 min were assessed by LC-MS/MS with a Q Exactive Orbitrap mass spectrometer, using the conditions defined in Tables 6–9 in the Experimental section, with peptide separations performed on an Acclaim VANQUISH C18 UHPLC column. Following acquisition, data were processed in BioPharma Finder software version 3.1 using the parameters stated in Table 10. It should be noted that since no reducing agent was added during digestion, a disulfide bond search was included in the search parameters.

Digestion efficiency at each timepoint was assessed by comparing the relative levels of missed cleavages, non-specific cleavages, and trypsin autolysis. Relative levels were calculated using the "Average MS Area" value generated for peptides with missed cleavages, non-specific cleavages (i.e. not at lysine (K) or arginine (R) residues), and for trypsin autolysis peptides; the amino acid sequence for trypsin was included in the search (UniProt accession number: P00761). The exporting results feature in BioPharma Finder software version 3.1 enabled the relative percentage of each peptide type to easily be determined.

Missed and non-specific cleavages

The 60 min timepoint had the lowest relative level of missed cleavages (Figure 3a), this is expected, as longer digestion times increase the access time for trypsin to the mAb. However, the relative percentage of non-specific cleavages (Figure 3b), increased with longer digestion times. Non-specific cleavage of peptides is best kept to a minimum, as additional unexpected peptides may unnecessarily complicate a peptide map, increasing the number of peaks and can result in method irreproducibility. The timepoint where the lowest amounts of observed non-specific cleavage peptides was 20 min, suggesting that longer digestion times under these conditions, can have a detrimental effect on trypsin specificity. A compromise should therefore be made between the relative amount of missed cleavages and non-specific cleavages. With the evidence collected from the time-course protocol, the optimal digestion time was determined to be 40 min.

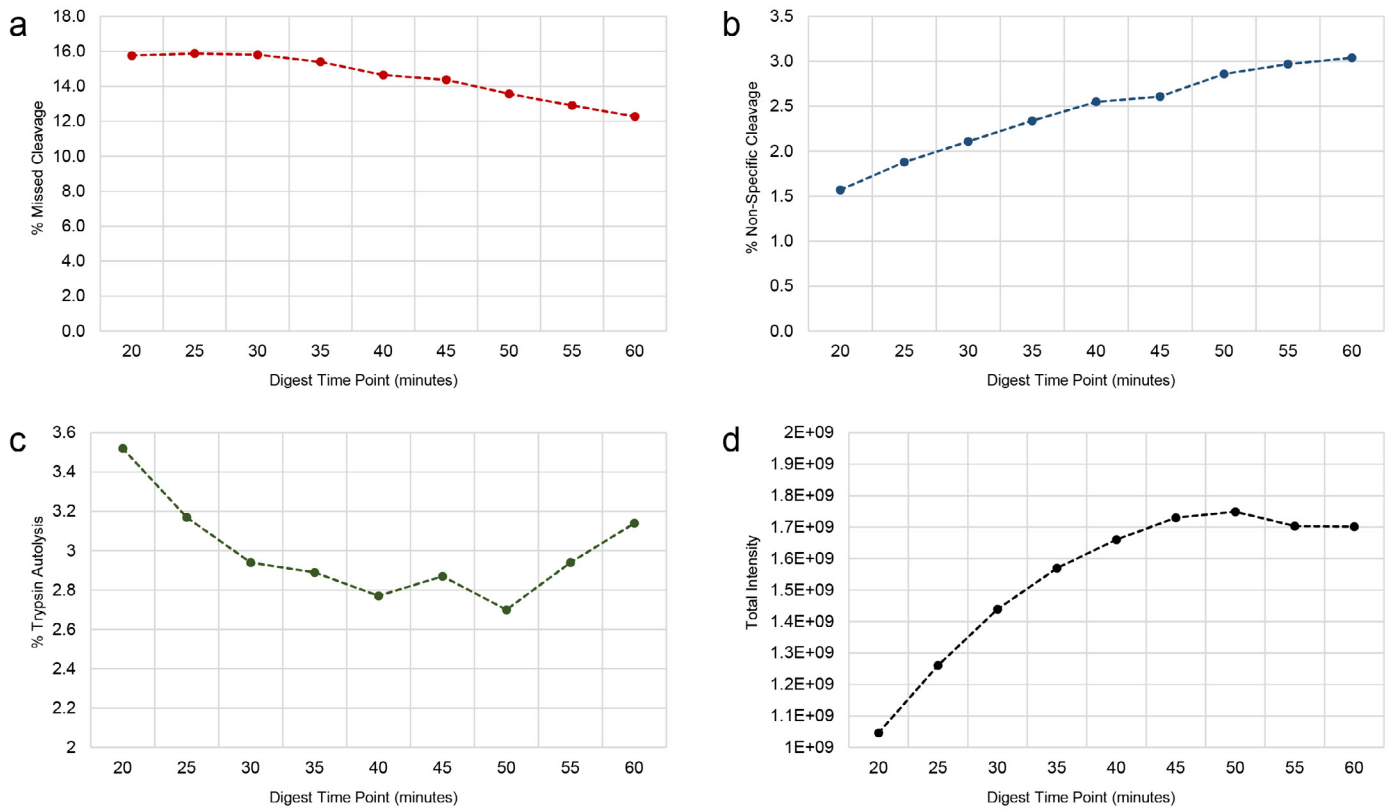

Figure 3. Relative digest efficency at digest timepoints from 20 to 60 min. Ipilimumab was digested with SMART Digest Magnetic Trypsin for different digest lengths on the KingFisher Duo Prime sytem, followed by LC-MS/MS analysis. Digest efficiency was evaluated by examining relative levels of (a) missed cleavage; (b) non-specific cleavage; (c) trypsin autolysis; and (d) total intensity of identified peptides.

Trypsin autolysis and total identified peptide intensity

Trypsin is a high-fidelity protease with high specificity for catalyzing the hydrolysis of amide bonds of K or R, including those in its own sequence, except when followed by a proline (P) residue. Trypsin autolysis has long been recognized as a potential source of artefacts in peptide mapping analysis.[3] For this reason the relative level of trypsin autolysis peptides should be considered when assessing digest efficiency. The 40 to 50 min timepoints had the lowest trypsin autolysis levels (Figure 3c). A plateau in total identified peptide intensity (Figure 3d) occurs after 40 min indicating that the mAb digestion is complete.

Post translational modifications (PTMs)

As part of biotherapeutic analytical characterization, peptide mapping can highlight PTMs–covalent and/or general enzymatic modification of proteins during or after protein biosynthesis. PTMs are important components in cell signaling and can occur on amino acid side chains or at the *C*- or *N*-termini of the protein. Here we assess some CQAs that are typically monitored within biopharmaceuticals. Changes in the PTM profile can be detrimental to the safety, efficacy, and stability of a biotherapeutic.

Some PTMs can also be induced by the digestion process itself such as asparagine (N) deamidation and methionine (M) oxidation, therefore, we assessed a range of common modifications at each digestion timepoint from 20 to 60 min to determine the effect of digestion length.

One example of a common mAb PTM is cyclized pyroglutamic acid (E). This reaction involves the cyclization of the *N*-terminal amine and subsequent loss of NH_3 (-17.027 Da). As this is a source of mAb heterogeneity, the biopharmaceutical industry must identify the presence and percentage of each variant. Digest length has no impact on the relative level of pyroE in the heavy chain of ipilimumab, as indicated by a relative modification above 97% (Figure 4a).

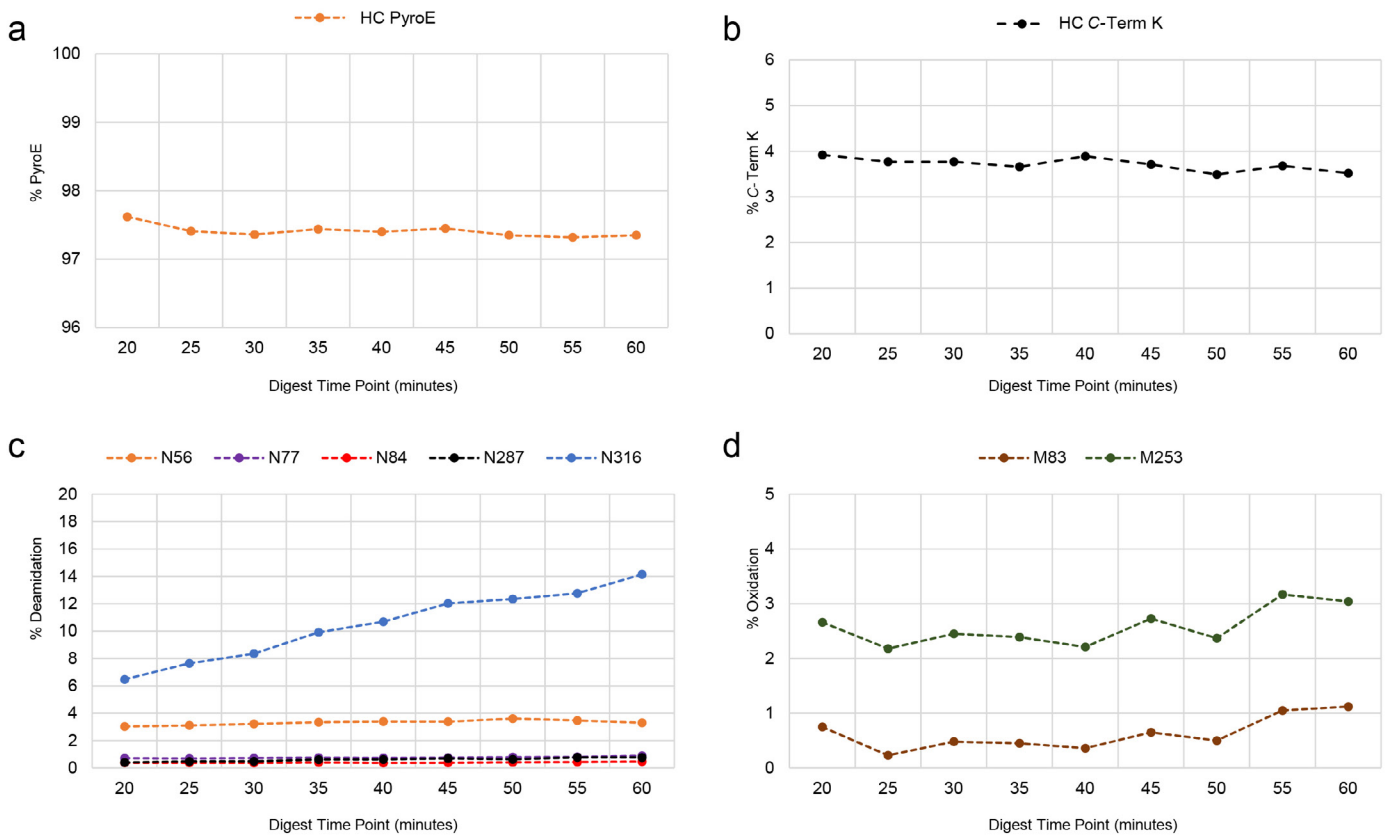

Figure 4. Relative levels of amino acid modification at digest timepoints from 20 to 60 min. Ipilimumab was digested with SMART Digest Magnetic Trypsin for different digest lengths on the KingFisher Duo Prime system, followed by LC-MS/MS analysis. Data were examined in BioPharma Finder software version 3.1 to compare the relative levels of (a) *N*-terminal pyroE formation (b) remaining *C*-terminal K (c) N deamidation, and (d) M oxidation at each digest timepoint.

Another common mAb PTM is *C*-terminal K truncation. Lysine residues at the *C*-termini of mAb heavy chains are often absent in the final drug product, which can reflect on manufacturing consistency and should therefore be monitored. The relative level of *C*-terminal K remains consistently below 4% throughout this time-course (Figure 4b), indicating that digest length also has no impact on this PTM.

Deamidation is a common structural modification in biotherapeutics, with varying effects on drug activity and stability. Deamidation occurs primarily on N residues (to a lesser extent glutamine, Q) and determining deamidation levels is a significant challenge within the biopharmaceutical industry.[4] During this time-course, digestion length had a minimal effect on the relative level of deamidation for residues N56, N77, N84, and N287 in the heavy chain of ipilimumab (Figure 4c). The relative modification level on residue N316 increased to >14% at the final digest timepoint of 60 min. The adjacent amino acid residue to N316 is glycine (G). The N+1 residue is particularly important since its size and charge influence the local flexibility of the peptide backbone; a G residue at N+1 has the greatest effect on the rate of deamidation, followed by histidine (H) and serine (S).[5,6] Therefore, to minimize the deamidation at N316, a compromise must be made in overall digestion length.

Methionine residues are susceptible to oxidative damage due to sulfur atoms acting as oxygen radicals.[7] Oxidation can alter the physiochemical characteristics of a mAb and have a negative effect on potency and immunogenicity.[4] M83 and M253 were identified as being susceptible to oxidation in the ipilimumab heavy chain. As with the deamidation at N316, there is a slight increase in the relative amount of oxidation up to the 60 min timepoint. Although relative levels remain below 3%, suggesting that digest length does not have a pronounced impact on M oxidation within ipilimumab.

Sequence coverage

The most common indicator of digestion efficiency is the percentage sequence coverage, i.e., the number of amino acids confirmed through peptides identified against a theoretical amino acid sequence. A high sequence coverage, as close to 100% as possible, for the light and heavy chains, improves confidence in protein identity.

The sequence coverage for each digest timepoint was determined using the parameters defined in Table 10 of the Experimental section. Sequence coverage can be exported from the Coverage tab in BioPharma Finder software version 3.1. Additional information relating to the number of MS peaks, MS peak area, and abundance can also be exported for further interrogation. The sequence coverage percentage at different minimum recovery thresholds (1–50%) was assessed. At a specific minimum recovery value, an identified peptide must have a recovery value higher than the threshold for it to be included in the coverage map. A good recovery is greater than 10%; a fair recovery, greater than 1%; and a poor recovery, less than 1%.

a

Light Chain % Minimum Recovery in BioPharma Finder

Digest Length (minutes)	1	5	10	15	20	25	30	35	40	45	50
20	98.60	98.60	98.60	94.90	90.70	89.30	89.30	79.50	76.30	76.30	76.30
25	98.60	98.60	98.60	96.70	90.70	90.70	90.70	85.60	76.30	76.30	76.30
30	98.60	98.60	98.60	98.60	90.70	90.70	90.70	88.80	76.30	76.30	76.30
35	98.60	98.60	98.60	98.60	90.70	90.70	90.70	85.60	76.30	76.30	76.30
40	98.60	98.60	98.60	98.60	93.00	90.70	90.20	90.20	85.60	76.30	76.30
45	98.60	98.60	98.60	96.70	90.70	90.70	90.20	88.80	85.60	76.30	76.30
50	98.60	98.60	98.60	96.70	90.70	90.70	90.20	88.80	85.60	85.60	76.30
60	98.60	98.60	98.60	96.70	92.60	90.70	88.80	88.80	85.60	85.60	76.30
55	98.60	98.60	98.60	96.70	95.30	89.30	88.80	88.80	88.80	85.60	85.60

Heavy Chain % Minimum Recovery in BioPharma Finder

Digest Length (minutes)	1	5	10	15	20	25	30	35	40	45	50
20	98.44	94.40	74.30	66.70	55.10	52.00	47.80	35.30	33.00	27.20	23.40
25	98.44	94.40	89.50	74.30	66.70	63.20	60.70	51.10	44.20	40.60	33.00
30	98.44	94.40	93.80	74.30	72.80	64.70	60.70	59.40	51.10	47.50	43.10
35	98.44	94.40	90.80	74.30	74.30	68.30	60.70	60.30	59.40	47.50	47.50
40	99.78	94.40	90.80	74.30	74.30	70.80	64.70	60.70	59.80	55.80	47.50
45	99.33	94.40	90.80	74.30	74.30	72.30	68.30	59.40	59.40	56.30	52.70
50	99.33	94.40	90.80	74.30	74.30	69.90	68.30	59.40	59.40	55.80	52.70
60	97.99	87.50	75.70	73.20	71.40	68.50	63.80	61.20	56.30	52.70	49.30
55	90.85	76.80	75.70	72.30	67.40	60.30	60.30	50.20	45.30	31.90	24.80

b

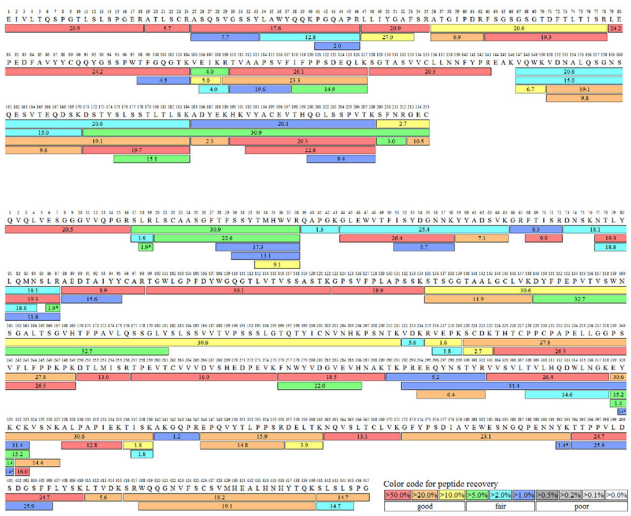

Figure 5. Ipilimumab sequence coverage assessment. Ipilimumab was digested with SMART Digest Magnetic Trypsin for different digest lengths on the KingFisher Duo Prime system, followed by LC-MS/MS analysis. Data were examined in BioPharma Finder software version 3.1 to determine the optimal digest length for % sequence coverage for the light and heavy chains. (a) Conditinal formating in an Microsoft® Excel® Workbook to determine the opitmal digest length using the % minimum recovery feature in BioPharma Finder software version 3.1 (b) Sequence coverage map (40 min digest timepoint) of ipilimumab light (top) and heavy (bottom) chains obtained using BioPharma Finder software version 3.1.

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The highest % sequence coverage, when the minimum recovery threshold is increased from 1% to 50%, was consistently at the 40 and 55 min digest lengths for the ipilimumab light chain and the 40, 45, and 50 min digest lengths for the heavy chain. An example of the sequence coverage generated in BioPharma Finder software version 3.1 for the 40 min timepoint can be observed in Figure 5b.

Therefore, the digest length for optimal sequence coverage of the light and heavy chains of ipilimumab, with all necessary parameters assessed, was determined to be 40 min.

Conclusions

- SMART digest magnetic kit, coupled with the KingFisher Duo Prime purification system, provide an automated approach to protein digestion.
- The developed time-course BindIt protocol facilitates rapid assessment of digest efficiency, especially when compared to a more traditional in-solution digest time-course experiment, significantly reducing method development times.
- Residual intact mAb can be assessed by LC-UV on a MAbPac RP column, providing an efficient means of determining digest efficiency in combination with the downloadable BindIt digest time-course protocol for the KingFisher system.
- A compromise in digestion length can minimize the impact of missed cleavages, non-specific cleavages, trypsin autolysis, and relative levels of PTMs, while maximizing the potential total peptide intensity and sequence coverage. BioPharma Finder software version 3.1 can determine the level of all relevant attributes and can aid in optimizing biotherapeutic digest length in peptide mapping method development.
- The regular sampling during this digest time-course enables the optimum digest length of 40 min for ipilimumab to be selected, taking into account minimizing undesirable modifications and methodological artefacts while maximizing efficiency of the SMART digest magnetic kit.

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