

## Monitoring Impurities Using a High Throughput Focused Gradient With the Alliance™ iS Bio System

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### Abstract

The Alliance iS Bio HPLC System provides an efficient HPLC platform for routine monitoring of quality indicating attributes in protein drug substances. Innovative products such as Waters IonHance™ difluoroacetic acid, when used in conjunction with the Titanium diffusion bonded mixer, and the integrated sample metering pump, provides improved UV-detection through reduced baseline noise, increased sensitivity, and reliable detection for improved peak integration. This unique configuration increases confidence in monitoring low-abundant impurities by retention time matching in UV-based assays, making it particularly well-suited for quality control environments. In this study, a shortened focused gradient targeting an oxidized peptide and its native species was developed via mass confirmation and transferred to the Alliance iS Bio HPLC System which provides high throughput and efficient monitoring.

### Benefits

- The Titanium diffusion bonded mixer reduces baseline noise for increased confidence in detecting low-abundant impurities via retention time matching
  - The sample metering pump and large volume needle loop of the Alliance iS Bio HPLC System is capable of
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precise injections up to 100  $\mu$ L, bypassing the need for external loops

The Alliance iS Bio HPLC System consistently monitors low-abundant impurities over extended analysis periods

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## Introduction

Monoclonal antibody (mAb) therapies have a distinct advantage in their capability to target specific cellular epitopes and have proven performance in disease diagnosis and treatment of infections and cancer. As a biologic, the complexity in the structure of mAb-based drug substances requires robust methods in the analysis and monitoring of impurities to ensure safety and compliance standards are met. Peptide mapping is frequently used as part of upstream development activity to identify undesired modifications to amino acid residue side chains such as deamidation, isomerization, N-terminal cyclization, and oxidation. Characteristically, these assays typically require lengthy analyses to increase the resolution of chromatographically resolved impurities and predominantly utilize mass spectrometry for analysis and identification. As part of the drug development process, these characterization methods are often transferred downstream to manufacturing labs for further optimization and routine monitoring of quality indicating attributes.

In manufacturing activities, these methods may be translated into a UV-based assay and optimized as a targeted assay with reduced run time to increase assay efficiency and robustness as part of routine monitoring. The purpose of this study is to demonstrate instrument and method considerations that can expedite development and transfer of targeted RPLC-UV assay from a RPLC-MS method for easier deployment in a QC lab environment. For this study, an enzymatically treated standard of NISTmAb RM 8671 was analyzed with the Alliance iS Bio HPLC System under Empower™ 3 CDS control as a representative sample and LC platform deployed in QC labs. A methionine containing peptide fragment was monitored for its oxidation degradation to evaluate assay reproducibility and robustness.

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## Experimental

MS grade water and acetonitrile were purchased from Honeywell. MS grade IonHance Difluoroacetic Acid was purchased from Waters Corporation (p/n: [186009201](#) <

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<https://www.waters.com/nextgen/global/shop/standards--reagents/186009201-ionhance-difluoroacetic-acid-1-vial.html> ). MS grade formic acid was purchased from Fisher Chemical. The mAb Tryptic Digestion Standard purchased from Waters Corporation (p/n: [186009126](https://www.waters.com/nextgen/global/shop/standards--reagents/186009126-mab-tryptic-digestion-standard.html) < <https://www.waters.com/nextgen/global/shop/standards--reagents/186009126-mab-tryptic-digestion-standard.html> ) was reconstituted using 0.1% formic acid in water at a concentration of 0.5 µg/µL. Oxidized T21 peptide was custom-made and dissolved in water.

LC system:	Alliance iS Bio HPLC System (QSM) with a 690 µL Titanium diffusion bonded mixer  Arc™ Premier System (BSM) with a 680 µL mixer with a 2489 TUV and an ACQUITY QDa™ Mass Detector
Detection:	TUV, λ=220 nm
Column:	XSelect™ Premier Peptide CSH™ C <sub>18</sub> Column, 130 Å, 2.5 µm, 4.6 x 100 mm (p/n: 186009908) (+eConnect™ 186009908RF)
Column temperature:	60 °C
Sample temperature:	10 °C
Injection volume:	30 µL
Flow rate:	0.90 mL/min
Mobile phase:	A: 0.1% DFA in water B: 0.1% DFA in acetonitrile
Chromatography software:	Alliance iS Bio HPLC System: Empower 3.8 Arc Premier System: Empower 3, FR4

## Gradient Table for Peptide Mapping

Time (min)	Flow rate (mL/min)	%A	%B	Curve
initial	0.90	99.0	1.0	initial
1.0	0.90	99.0	1.0	6
55.0	0.90	65.0	35.0	6
61.0	0.90	15.0	85.0	6
65.0	0.90	15.0	85.0	6
72.0	0.90	99.0	1.0	6
86.0	0.90	99.0	1.0	6

## Gradient Table for T21 Peptide Focused Gradient

Time (min)	Flow rate (mL/min)	%A	%B	Curve
initial	0.90	93.0	7.0	initial
2.0	0.90	93.0	7.0	6
8.5	0.90	88.5	11.5	6
10.5	0.90	35.0	65.0	6
13.0	0.90	35.0	65.0	6
15.0	0.90	93.0	7.0	6
20.0	0.90	93.0	7.0	6

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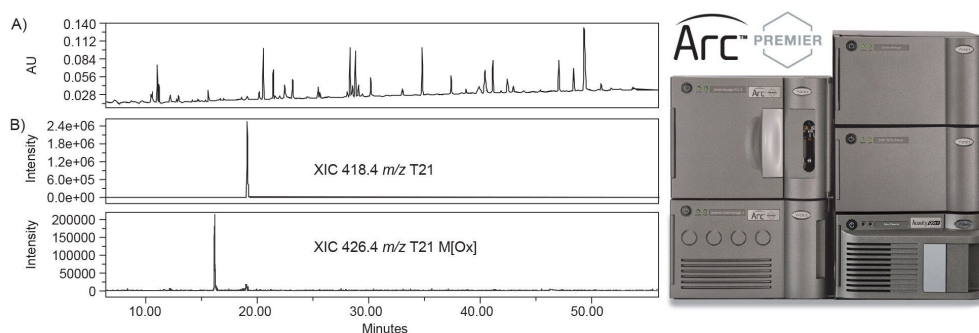
## Results and Discussion

Common mobile phase additives for peptide mapping methods include formic acid, difluoroacetic acid (DFA), and trifluoroacetic acid; each of them exhibits increasing ion pairing strength and ionization suppression. While upstream laboratories frequently use formic acid in mass spectrometry to increase assay sensitivity, downstream

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laboratories prefer using stronger ion-pairing agents such as trifluoroacetic acid to improve peak shape and reproducibility in optical based methods. To streamline method transfer from upstream to downstream environments, we chose DFA as a mobile phase additive that improves peak shape with moderate ionization suppression for method development purposes while also providing better baseline response for UV-detection to increase assay reproducibility.<sup>1</sup>

To demonstrate this, an 80-minute peptide mapping gradient typical of characterization and development workflows was run on an Arc Premier System configured with an ACQUITY QDa Mass Detector using mobile phase containing 0.1% DFA (v/v). As shown in Figure 1A, The Arc Premier System sufficiently separated the peptide fragments, aligning well with previous results in terms of peak profiles.<sup>2</sup> The lower ion-suppression of DFA allows for efficient method development for MS enabled workflows. An example of which is shown in Figure 1B for the T21 peptide fragment. The T21 peptide fragment of NISTmAb generated by the trypsin digestion includes the amino acid sequence of DTLMISR ( $[M+2H]^{+2} = 418.4 \text{ m/z}$ ,  $[M+H]^+ = 835.6 \text{ m/z}$ ). Methionine is subject to oxidation and produces the oxidized T21 fragment ( $[M+2H]^{+2} = 426.4 \text{ m/z}$ ,  $[M+H]^+ = 851.6 \text{ m/z}$ ). These two species were quickly identified eluting at 19 and 16 minutes, respectively using the extracted ion chromatograms (XIC) functionality within the Empower 3 CDS (Fig. 1B). The ability to use complimentary mass data to monitor peaks enables users to quickly develop targeted assays using focused gradients to monitor quality attributes.



*Figure 1. Arc Premier System supports simultaneous data collection of both UV and mass data via a 2489 TUV and an ACQUITY QDa Mass Detector. (A) UV absorption spectrum of NISTmAb trypsin digested peptides on the Arc Premier System (QSM). (B) Extracted ion chromatograph (XIC) mode of ACQUITY QDa Mass Detector provides confidence in peak identification via mass confirmation.*

An example of this is shown in Figure 2. Gradient conditions were quickly screened resulting in a 20-minute focused gradient that fully resolved the native peptide (Fig. 2A) and its oxidized form (Fig. 2B). This shortened gradient provides peaks with baseline resolution with high spectrum purity as shown in the top panel of Figure 2 where the mass analysis review window displays the spectrum of the leading peak area (peak start to 5% peak height), the apex, and the trailing peak area (5% peak height to the peak end). With peak identity and purity confirmed, the 20-minute targeted method was transferred to the Alliance iS Bio HPLC System.

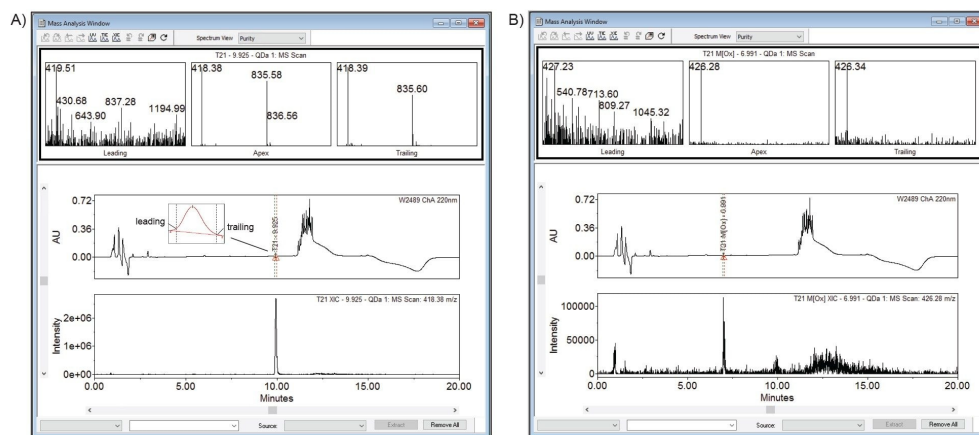


Figure 2. The mass analysis window of (A) T21 peptide and (B) oxidized T21 peptide. The spectrum window displayed the spectrum of the leading area (peak start to 5% peak height), the apex, and the trailing area (5% peak height to the peak end), which confirms the peak identity by mass.

Based on previous results, the oxidized impurity of interest is observed at relatively low abundance in the NISTmAb trypsin digested peptides when not exposed to air or oxidative stress.<sup>3</sup> In consideration of this, a Titanium diffusion bonded mixer was used in lieu of the standard 680  $\mu$ L Titanium beaded mixer to enhance solvent mixing to reduce baseline noise and increase accuracy in the detection and integration of low-abundant impurities.<sup>4-5</sup> An example of baseline performance of the Alliance iS Bio System using the diffusion bonded mixer is shown in Figure 3. The overlay of 49 consecutive injections of blank (Fig. 3A) exhibited notably minimal variance which translates to a highly stable baseline for focused gradients as shown in the overlay of 17 injections of the NISTmAb trypsin digested sample (Fig. 3B). This reliable baseline performance increases the confidence in detecting low-abundant impurities like the oxidized T21 peptide via retention time matching. An

example of this is presented in Figure 4, where a synthetic oxidized T21 standard was used as a system suitability standard to confirm the retention time and identity of the oxidized T21 peak in the NISTmAb trypsin digested sample.

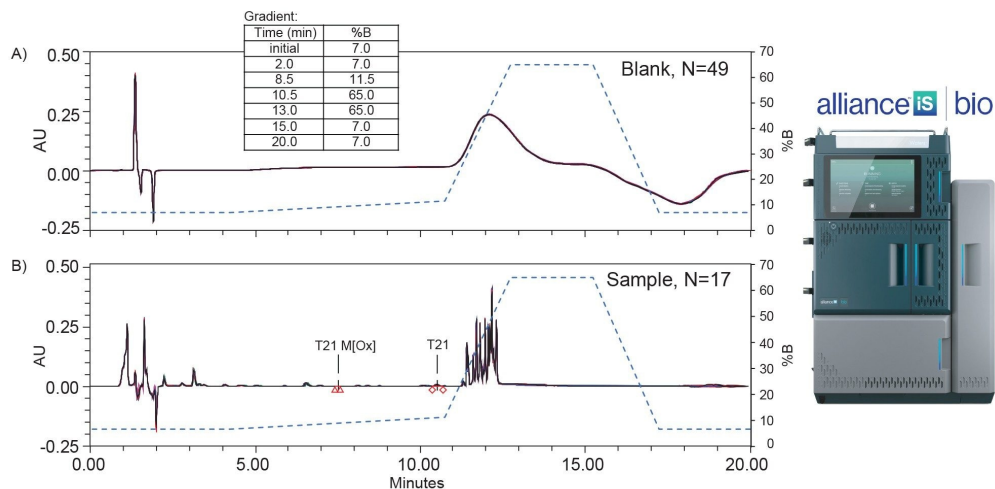


Figure 3. The Alliance iS Bio System featuring a Titanium diffusion bonded mixer consistently delivers a highly stable and reproducible baseline when using a focused gradient. The chromatogram overlay shows (A) 49 consecutive injections of sample diluent and (B) 17 injections of NISTmAb trypsin digested peptides analyzed in a period of 30 hours. The blue trace represents the real time mobile phase composition as in %B (0.1% DFA in acetonitrile) with an offset.

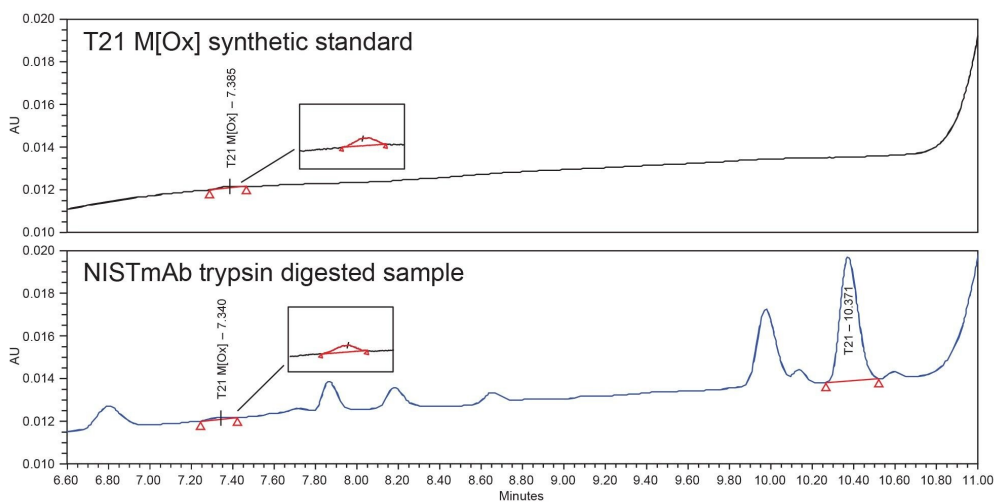


Figure 4. For the shortened 20-minute gradient, a synthetic standard of oxidized T21 peptide was used to identify this impurity contained in the NISTmAb trypsin digest sample via retention time matching.

To further enhance detection of the oxidized impurity, on-column mass load was scaled and evaluated for linearity. As part of the Alliance iS Bio HPLC System, the sample metering pump is capable of precise injections up to 100  $\mu\text{L}$ . This in combination with the large volume capacity of the needle loop eliminates the need of adding an extension loop which introduce additional dispersion volume and potential points of failure. As shown in Figure 5, using the calibration curve function within the Empower processing method to plot peak area, peptide T21 was observed to scale linearly when injecting 10 to 100  $\mu\text{L}$  (indicated as amount) of a sample prepared at a concentration of 0.5  $\mu\text{g}/\mu\text{L}$ .



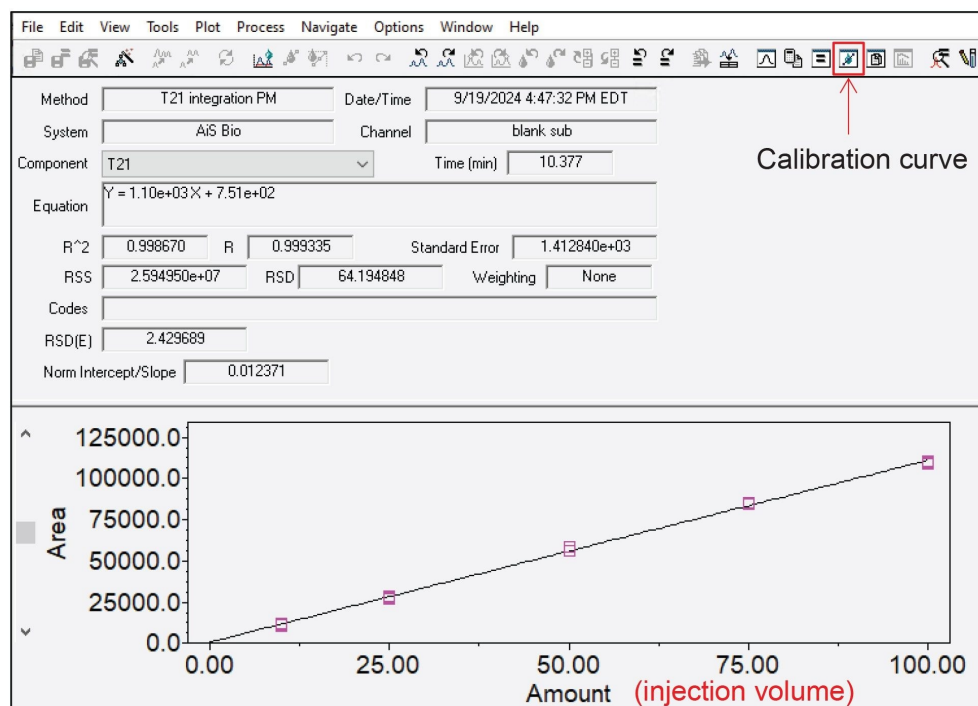
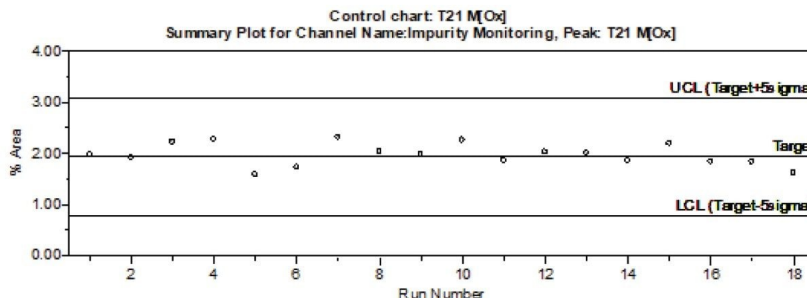


Figure 5. The T21 peptide area increases linearly with the injection volume of 10 to 100  $\mu\text{L}$ , which represents total peptide mass load of 5 to 50  $\mu\text{g}$ , as displayed in the calibration curve option in the Empower review window.

Using 60  $\mu\text{L}$  injection volumes, the high throughput 20-minute method was evaluated over an extended 30-hour period. A sample set consisting of 90 injections was performed with four injections of blank (sample diluent) followed by one injection of sample containing the NISTmAb trypsin digested sample for a total of 18 sample injections over 30-hours. As shown in Figure 6, using the summary plot functionality within the reporting features of Empower 3 CDS for visualization purposes, the relative amount of oxidized T21 was found to be stable over the 30-hour injection series (mean %Area  $\leq 2.0$ , % RSD  $\cong 12\%$ ). In this instance, the results show the oxidation was controlled and well within a Gaussian distribution represented as  $5\sigma$  for the upper and lower limit of the control chart using the Limits tab of the processing method. Collectively, these results demonstrate the Alliance iS Bio HPLC System is capable of delivering consistent and reliable UV-based results in an efficient manner for routine monitoring of quality indicating attributes in a QC lab setting.



T21 M[Ox] Summary Table

	Sample Name	RT	% Area
1	mAbtryps in-1	7.52	1.82
2	mAbtryps in-2	7.51	1.84
3	mAbtryps in-3	7.51	1.84
4	mAbtryps in-4	7.50	2.19
5	mAbtryps in-5	7.48	1.85
6	mAbtryps in-6	7.51	2.00
7	mAbtryps in-7	7.51	2.02
8	mAbtryps in-8	7.53	1.88
9	mAbtryps in-9	7.52	2.26
10	mAbtryps in-10	7.52	1.98
11	mAbtryps in-11	7.53	2.04
12	mAbtryps in-12	7.51	2.32
13	mAbtryps in-13	7.50	1.73
14	mAbtryps in-14	7.50	1.58
15	mAbtryps in-15	7.49	2.28
16	mAbtryps in-16	7.49	2.23
17	mAbtryps in-17	7.49	1.92
18	mAbtryps in-18	7.49	1.98
Mean		7.51	1.97
Std. Dev.		0.01	0.22
% RSD		0.18	11.18

T21 Summary Table

	Sample Name	RT	% Area
1	mAbtryps in-1	10.53	98.38
2	mAbtryps in-2	10.52	98.16
3	mAbtryps in-3	10.53	98.16
4	mAbtryps in-4	10.52	97.81
5	mAbtryps in-5	10.52	98.15
6	mAbtryps in-6	10.52	98.00
7	mAbtryps in-7	10.54	97.98
8	mAbtryps in-8	10.56	98.14
9	mAbtryps in-9	10.55	97.74
10	mAbtryps in-10	10.54	98.02
11	mAbtryps in-11	10.54	97.96
12	mAbtryps in-12	10.54	97.68
13	mAbtryps in-13	10.52	98.27
14	mAbtryps in-14	10.53	98.42
15	mAbtryps in-15	10.51	97.72
16	mAbtryps in-16	10.51	97.77
17	mAbtryps in-17	10.51	98.08
18	mAbtryps in-18	10.50	98.02
Mean		10.53	98.03
Std. Dev.		0.02	0.22
% RSD		0.15	0.23

Figure 6. The amount of oxidized T21 peptide was stable over time (30 hours) as shown in the summary plot generated via the Empower 3 CDS reporting feature.

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## Conclusion

Consideration of ion-pairing agents and various mixer designs as part of the method development process can be critical when monitoring low-abundant species. In this study, DFA emerged as a suitable mobile phase ion pairing agent which improved peak shape in UV-detection while also supporting upstream MS-based workflows, facilitating easier method transfer between labs. The Titanium diffusion bonded mixer, available as an optional mixer for the Alliance iS Bio HPLC System, provides reliable baseline performance and increases the confidence in detecting low-abundant impurities via retention time matching. Additionally, the sample metering pump of the Alliance iS Bio HPLC System in conjunction with the large volume needle loop, allows for precise injections up to 100 µL thus eliminating the need for extension loops and enabling direct method scaling, which saves both time and resources. In conclusion, the Alliance iS Bio HPLC System proves to be an ideal HPLC platform for QC labs that can deliver reliable and consistent results for UV-based monitoring assays. It efficiently monitors low-abundant impurities over extended analysis times, thereby reducing errors and operation costs in manufacturing environments.

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720008591, November 2024



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