Middle-Down Analyses of Unmodified and Stressed Monoclonal Antibodies Using an Orbitrap Fusion Lumos **Tribrid Mass Spectrometer**

ABSTRACT

Purpose: To characterize unmodified and stressed monoclonal antibodies using a middle-down approach

Methods: Samples were first digested with IdeS and MS1 and targeted MS2 data were acquired. Two different modes of fragmentation, electron-transfer dissociation (ETD) and ultraviolet photodissociation (UVPD), were used, and data were processed with Thermo Scientific[™] BioPharma Finder[™] software version 3.0.

Results: By combining different activation times and modes of fragmentation, very high sequence coverage was obtained for the NIST mAb. One targeted MS2 experiment was enough to pinpoint some sites of oxidation for a mAb after an oxidative stress. Specific insights on hotspots were also revealed.

INTRODUCTION

Peptide mapping is the gold standard for in-depth characterization of biotherapeutics, but the sample preparation, LC runtime, and data analysis can be time-consuming. Considering that subunit mass analysis is a common assay in the biopharmaceutical industry, getting sequence information using the same sample preparation and LC settings would be advantageous. Automated data processing has historically been one of the bottlenecks for middle-down experiments; however, dedicated software developed for biotherapeutics characterization is available today.

MATERIALS AND METHODS

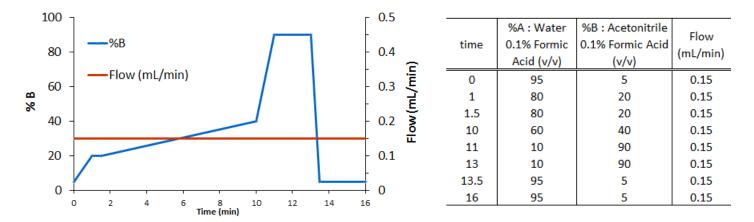
Sample Preparation

Trastuzumab and NIST mAbs were used as standard samples. The stressed samples were generated by incubating mAbs in 0.015% (v/v) of H_2O_2 for 5 or 24 hours. Stressed samples were then buffer exchanged in 0.1 M Tris HCI using a spin column. Finally, all samples were digested with IdeS protease and then reduced under denaturing conditions to generate three ~25 kDa subunits (scFc, LC, Fd).

Methods

For MS1 and MS2 experiments, data were acquired at 120K resolution. MS2 Targeted (wide isolation window or multiplexing) LC-MS experiments were performed using a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid[™] mass spectrometer with an ETD source and 213 nm UVPD source coupled with a Thermo Scientific[™] Vanguish[™] UHPLC system. A Thermo Scientific[™] MAbPac[™] 1 mm × 100 mm column was used for all experiments.

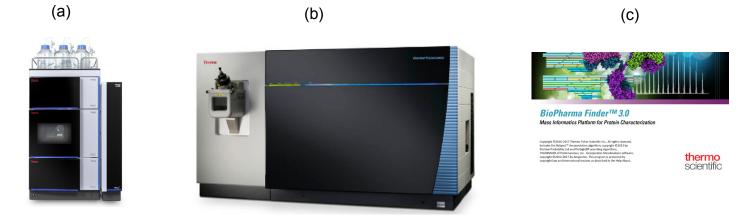
Figure 1. Gradient details



Data Analysis

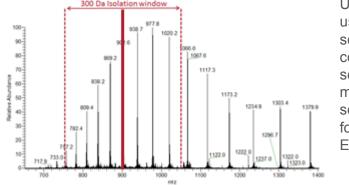
LC-MS raw files were processed with BioPharma Finder 3.0 software. For MS1 experiments, the sliding window coupled to the Xtract deconvolution algorithm was used. Time-defined MS/MS spectra were automatically deconvoluted using Xtract, and fragment ion maps were directly generated within the software. The signal-to-noise ratio (SNR) threshold for fragment peak picking was set to 7 and the mass tolerance of fragment ions that can be matched to theoretical fragments was set to 10 ppm. The different proteoforms with one or two oxidations were generated automatically inside BioPharma Finder software by defining the number of oxidation and the site of modification.

Figure 2. Data were collected using a Vanquish UHPLC system (a) coupled to an Orbitrap Fusion Lumos mass spectrometer (b) and processed with BioPharma Finder 3.0 software (c).



RESULTS

Figure 3. Illustration of ions that will be fragmented for the LC when a wide isolation window of 300 Da centered at 900 *m/z* is used.





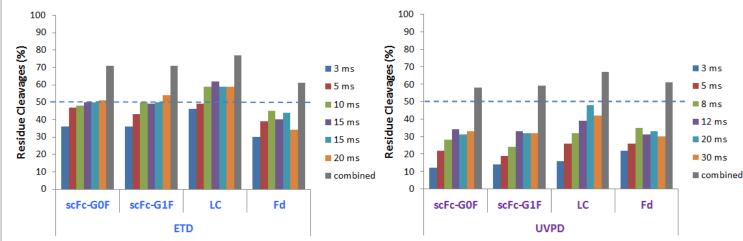


Figure 5. Comparison of the sequence coverage when all of the different reactions for ETD or/and UVPD are combined.

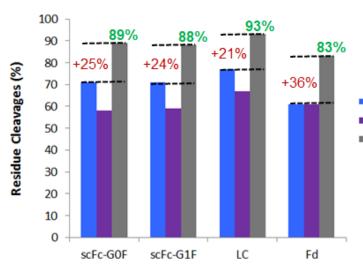
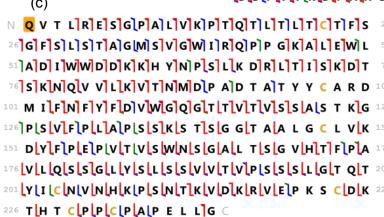


Figure 6. Fragment ion maps for scFc (a), LC (b), and Fd (c) using all reaction times for ETD and UVPD. Sequence coverages for scFc-G0F, LC, and Fd are 89%, 93%, and 83%, respectively.

¹**[T[Q[K[S[L[S[L S P G ⊂**



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A) NIST mAb: Acquisition with a wide isolation window and ETD and UVPD modes of fragmentations

A wide isolation window of 300 Da centered at 900 m/zwas used for all three subunits. For each subunit, ETD fragmentation will provide higher sequence coverage than UVPD (Figure 4). On average, the sequence coverages using ETD were respectively 47%, 56%, and 39% for the scFc, LC, and Fd. For UVPD, on average the sequence coverages were respectively 26%, 34%, and 29% for the scFc, LC, and Fd. For UVPD or ETD, by combining the matched fragment ions from different reaction times, the sequence coverage will increase. The sequence coverage for the combined reaction times ranged from 61 to 77 for ETD and from 58 to 67 for UVPD.

Figure 4. Sequence coverage for the NIST mAb subunits for different reaction times using ETD or UVPD.



ETD (all) UVPD (all) Combined

The highest sequence coverages, between 83% and 93%, are only observed when the ETD and UVPD runs are combined (Figure 5). For any sub-unit, adding the matched fragments ions from UVPD at different reaction times will increase the sequence coverage by at least 21%. UVPD is a complementary fragmentation technique to ETD.

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B) Oxidative stress of trastuzumab

1) MS1 experiment

Table 1. Results for duplicate raw files for each condition (unmodified or 0 h and after an oxidative stress of 5 h or 24 h)

								📕 76 D WLLIN GIKEYKCKV SLNLKLAL PAPILELKLTLI 100 76 D WLL NGKEYKCKV SLNLKLAL PAPILELKLTLI 100
	Modification	Monoisotopic Mass (mean)	Theoretical Mass (Da)	Matched Mass Error (ppm) (mean)	Relative Abundance	Number of Files Observed	Apex RT (mean)	101 SK AKGQ P RE PQ V YT L P PSRE EM TKN 125101 SK AKGQ P RE PQ V YT L P PSRE EM TKN 125126Q V S L T C L VK G FY P SD IAV E WESNGQ 150126Q V S L T C L VK G FY P SD IAV E WESNGQ 150151 P ENNYK T T P P V L DSDGS F FLYSKLIT 175
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LC		23428.614	23428.524	3.9	100.00	6	6.695	Figure 11. scFc of trastuzumab after 5 h of oxidative stress. Data searched with G0F on asparagine at
Fd		25367.619	25367.517	4.0	65.50	6	7.543	position 61 and one oxidation at position 16 (a) or 192 (b).
scFc	1xA2G0F	25220.550	25220.463	3.4	21.00	6	5.825	M16. Residue Cleavages: 28% M192. Residue Cleavages: 39%
scFc	1xA2G1F	25382.588	25382.516	2.8	17.33	6	5.807	(a) M16. Residue Cleavages: 28% g p s v]F]L]F p plk plk]D]T]L M I]S]R T Ple v]T C 25 (b) M192. Residue Cleavages: 39% G p s v]F]L]F p plk plk]D]T]L M I S]R]T Ple v T]C 25
scFc	1xA2G1F,1xOxidation (MW)	25398.589	25398.511	3.1	8.67	4	5.706	26 V V V D V S HJEJD P E VJKJFJNJWJY V D G VJE V HJNJ 50 26 V V VJD V S HJEJD PJEJVJKJFJNJWJY VJD G V E V HJNJ 50 51 AJK TJK PJRJEJEJQ YJMJS T YJR V V S V L T V L H Q 75 51 A K T K P RJEJEJQ YJMJS T YJR V V S V L T V L H Q 75
scFc	1xA2G0F,1xOxidation (MW)	25236.540	25236.458	3.2	6.28	6	5.722	76 D W L N G]K E Y K C K V S N K A L P A P I E K T I 100 76 D W L N G K E Y K C K V S N K A L P A P I E K T I 100
Fd	1xOxidation (MW)	25383.612	25383.512	3.9	6.17	4	7.411	101 S K A KIG Q P R E P Q V Y T L P PIS RIE E M T KIN 125 101 S K A KIG Q P R E P Q V Y T L P PIS RIE E M T KIN 125 126 Q V S L T C L V K G F Y P S D I A V E W EISIN G Q 150 126 Q V S L T C L V K G F Y P S D I A V E W E S N G Q 150
scFc	1xA2G1F,2xOxidation (MW)	25414.570	25414.506	2.5	2.30	2	5.616	151 P E N NLY K T T P P V L D S D G S FLFL Y S K LLT 175 151 P E N N Y K T T P P V L D S D G S F F L Y S K L T 175
scFc	1xA2G2F	25544.618	25544.569	1.9	1.75	6	5.811	- 176 V D K S R W Q Q G N V F S C S V M H E A L H N H Y 200 201 T Q K S L S L S P G C 201 T Q K S L S L S P G C
scFc	1xA2G0F,2xOxidation (MW)	25252.521	25252.453	2.7	1.62	4	5.68	Figure 12. scFc of trastuzumab after 24 h of oxidative stress. Data searched with G0F on asparagine at
scFc	1xA2G0	25074.471	25074.405	2.6	1.45	6	5.853	position 61 and one oxidation at position 16 (a) or 192 (b).
								(M16 Pasidus Clasvaras, 26% (M102 Pasidus Clasvaras, 20%



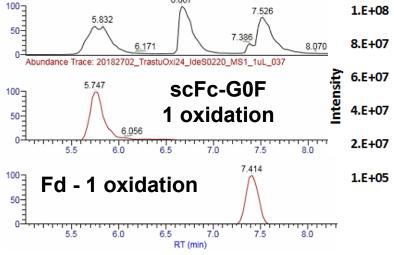
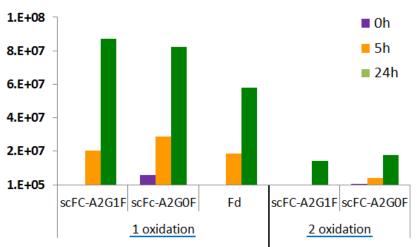
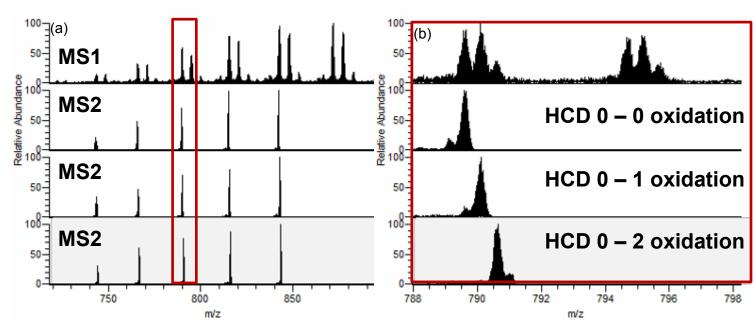


Figure 8. Intensity of oxidized subunits of trastuzumab unmodified or after 5 hours or 24 hours of an oxidative stress with H₂O₂



2) Visualization of the multiplexing capability to target specific level of oxidation (non modified, 1 oxidation or 2 oxidation).

Figure 9. Difference between an MS1 acquisition or acquisition by multiplexing five charge states (MSX5) for the different level of oxidation on scFc (a). Zoom in the mass range between 788 and 798 *m/z* (b).



All of the subunits of trastuzumab were identified and matched to the theoretical mass at less than 4 ppm (Table 1). Oxidized forms of scFc and Fd eluted before the non-modified subunit (Figure 7). As expected, the level of oxidation increased for longer times of incubation in H_2O_2 (Figure 8).

To test the isolation of the unmodified and oxidized subunits of trastuzumab for the multiplexing mode of acquisition, MS2 raw files were acquired using the HCD mode of fragmentation but with no collision energy. Different masses of trastuzumab scFc-G0F corresponding to the unmodified or with one or two oxidations were targeted (Figure 9a), and each subunit can be separated from each other (Figure 9b).

201 T Q K S L S L S P G C

3) MS2 experiments: ETD (8 ms activation time) on scFc-G0F by multiplexing five charge states

Figure 10. scFc of trastuzumab unmodified. Data searched with G0F on asparagine at position 61 and no oxidation (a) or one oxidation at position 16 (b) (modified peptides are highlighted in green). Residue Cleavages: 57% M16 Pasidua Class at 220/

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Figure 13. scFc of trastuzumab after 24 h of oxidative stress. Data searched with G0F on asparagine at position 61 and the first oxidation at position 16 (a-f) or 192 (g-l) and the second oxidation on different methionine or tryptophan.

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N 26 51 76 101 126 151 151 201	G V A] D S] Q P V T	0 1 0 1 0 1 0 1 0 1 0 1 0 1 0 1 0 1 0 1	SV D T K N N K S K S K S	F V G G T Y R L	L] S R] K Q C K W S	FI HI EI FI FI T Q L	P P E D E D C K K E V K K F P C G S P	K P] C P G P N G	P]K E]V N S K V Q V F Y V I V F C	D K S S P S	T Y N N S S C	C L N W R V K A L P D I D G S V	I V V L P A S M	S 1 V S P 2 S 1 S 1 S 1 F 1 H 1	R T O G V L A P R E E M F L E A	P V T E E V E	E V E V E K M I S N S K H N	T H H T K T K I G L H	1C1 1N1 Q 1I 1N Q T LY	50 75 100 125 150 175	26 51 76 101 126 151 176 201	G V A D S I Q P V T	P K K K K Z E L I D I Q	SV VD KK NAK SL NK S K S	V P G IG T LY L	L]F SH R]E KE QP CL KT WQ SL	P E Y R V T	P 1 Q 2 K 0 E 1 K 0 P 1 G 1 P 0	K P E Y N C K Q G F V G C	K V S V V Y L F	D K T S L T S L T S C S C	FN FN K L K L K L K C S C S	M V A P I G V	I S Y V L H P S H M	5 R 7 D 5 V 7 A 7 R 7 F 7 F 1 E	G L P L U L L A	PH VH TV EL YS LH	E V E V E K E K S K H N	T H] H [T] K] G[L] [H]	C 2 N 5 Q 7 I 10 N 12 Q 15 T 17 Y 20
N 26 51 76 101 126 151	G V A D S L Q P V	D 1 E 1 K 7 K 7 F	SV D T K L N A K S L N]F V P G LG T LY R	ILI S R] K Q C K	FI H]H E]H E Z F H L V T 2 Q (PP ED EQ K K R E V K K F P Q G	K P] Y] C P G P N	P]K E]V N S K V Q V F Y V I V F	D K T S Y S Y P D	T Y N N S S	C L N W R V K A L P D I D G S V	I Y V L P A [S	S 1 V 1 S 1 S	R T D G V L A P R E W F L L	P] V T I] E L Y	E V E V V I E K M T S K	7]T H H T T K I G K L	C N Q I N LQ LT	50 75 100 125 150 175	26 51 76 101 126 151	G V A D S C P V	P : V V K 2 K 2 E 1 D 1	SV VD SK NN SL NN SL NN S	IFI V P G LG T LY	L]F SH R]E KE QF CL KT WQ	P E E V R V	P D K E K C P G	K P P E Y N C K P Q G F Q F V N V	IK V S V V Y L	D] K T S L I Y Z D S	F N Y R N K F L S D S D	M V A P I G	I S Y V S I P S H	5 R 7 D 5 V 7 A 7 R 7 F	T G L P LE W	P 1 V 1 T V E 1 F 2 Y 2	EV EV Z K E K K S K S K	T H] H []] K] G[L]	C 2 N] 5
N 20 5: 70 10: 120	G 6 V 1 A 6 D 1 S 6 Q	P K W K	SVI VII TIP LN AP SI	V]E V E N G K C L 1	F I I I I I I I I I I I I I I I I I I I	F H] E E P L	PH E]I E](YH RH VL	2 K 2 P 2 Y 2 P 3 C 5 P 3 C 5 C 5 C 6 C 6 C	P E N K Q F	K]D ST V]S V]S V Y F	T T T T	N R K L D	I V V A P P I A] S] V] S , P] S , V	R 2 D 0 V 1 A 1 R I E 1	r p G V L T P I E E W	IEI E V LEI LM S L	V] VH LH K] TH N([]C H]N H Q []I K]N G Q	25 50 75 100 125 150		G V A] D S] Q	P K W K V	SV VD IK LN AK SL	V P G LG T	L]F SH R]E KE QF CI	' P E E Y R V	P D K E K	K P P]E Y]N C K P Q G F	K V S V V Y	D]: K]I T S[I Y P S	T]L F]N Y]R N[K T L S[D	1M 1W1 V 1A P I	I] S Y] V V] S L H P] S A V	5 R 7 D 5 V 2 A 5 R 7 E	G L P LE W	P]H VH TV I]H E[M E	E]V E V 7 L E]K 4 T 5 N]T] H] H]T] K] GL	C] 2 N] 5 Q 7 I 10 N 12 Q 15

176 V D K S RIW QIQIGIN VIF S C S VIMIH E A L HINIHIY 200 176 V D K S RIW QIQIGIN VIF S C S VIMIH E A L HINIHIY 200

201 T Q K S L S L S P G C

For a rapid identification of the oxidation sites, only one raw file per level of oxidation was acquired and the only mode of fragmentation used was ETD with an activation time of 8 ms. For the unmodified scFc, masses corresponding to only GOF as a modification were targeted. By multiplexing five charge states, a sequence coverage of 57% was observed (Figure 10a). The same deconvolution masses were searched against scFc-GOF with one oxidation at position 16, and no N terminal fragments were matched after the methionine at position 16 assuring that random matching or false positive is at a low level or not occurring.

For scFc-G0F with one modification, the highest sequence coverage for 5 h or 24 h was identified with the oxidation on the methionine at position 192 and the sequence coverage was 39% (Figures 11b and 12b). Numerous fragment ions also support an oxidation on methionine at position 16 (Figures 11a and 12a). These two sites of oxidation are unambiguous. The other sites of oxidation for scFc with one oxidation can not be pinpointed because every fragment ion with the oxidation at a different position could be explained with the oxidation at M16 or M192. For scFc-G0F with two oxidations, the oxidation on M16 was fixed and we did not observed N or C terminal fragments supporting the second oxidation until the oxidation was on methionine position 192 (Figures 12a to 12g). These results suggest that for scFc-G0F with two oxidations, M16 can only be oxidized when M192 is also oxidized. On the other hand, three fragment ions are also supporting the oxidation on M192 and M181 at the same time (Figure 13j).

4) MS2 experiments: ETD (8 ms activation time) on Fd by multiplexing 5 charge states Figure 14. Fd of unmodified trastuzumab

(no oxidation).

Residue Cleavages: 53%	For th
N E V Q]L V]E S G G G L V]Q P]G]G]S L R L]S]C A A]S 25	53%
26 G]F]N I K]D]T]Y I]H]W V]R]Q]A P G]K G L E]W V]A]R 50	oxida
51 IJY P TNNGYTTRY ADDSVK G RFT I SADTS 75	the o
76 K N]T]A]Y]L Q]M]N]S]L R]A]E D]T]A V Y Y C S R W G]100 101 G]D]G]F]Y]A M D Y W]G O G T]L V T V S]S A S T K]G 125	obse
126 P S V F PLLA PLS S K S T S G G T A A L G C L V K 150	
151LD YLF PLE P V T VLS WNSGLA L TLS G VHTFF P A 175	(Figu
176 V L Q S S G L Y S L S S V V T V P S S S L G T Q T 200	down
2011Y I CINIVINIHIK P SINITIKIVIDIKIKIVIE PIKIS CIDIK 225	
226 T H T C P P C P A P E L L G C	

Figure 15 Ed of trastuzumab after 24 h of oxidative stress. Data searched with one oxidation (in green) at

Figure 15. Ed of trastuzumab after 24 h of oxidative stress. Data searched with one oxidation (in green) at
different positions.
(a) W36. Residue Cleavages: 29% (d) W99. Residue Cleavages: 43%
N E V Q L V]E]S G G G L]V]Q P]G]G S L R L]S]C A A S 25 N E V Q L V]E]S G G G L]V]Q P]G]G S L R L]S]C A A S 25
26 GJFJN I KJDJTJY IJHJM V R Q A P G K G L E W V A R 50 26 GJFJN I KJDJTJY IJHJW V RJQJA P G K G L EJW V AJR 50
51 I Y P T N G Y T R Y A D S V K G R F T I S A D T S 75 51 I Y P T N G Y T R Y A D S V K G R F T I S A D T S 75
76 K N T A Y L Q M N S L R A E D T A V Y Y C S R W G 100 76 K N T A Y L Q M N S L R A E D T A V Y Y C S R 🖬 G 100
101 G D G F Y A MLDY W G Q G T L V T VLSISLA S TLKLG 125 101 G D G F Y A MLDY W G Q G T L V T VLSISLA S TLKLG 125
126 P S V F P LA P S SKIS T S G G TA A L G C L V K 150 126 P S V F P LA P S SKIS T S G G TA A L G C L V K 150
151 D Y]F PLE P V T VLS WLNLS GLA L T S G VLH TLF PLA 175 151 D Y]F PLE P V T VLS WLNLS GLA L T S G VLH TLF PLA 175
176 VLLQISISIGILIYISILISISIV VLTIV PISISISILIG T QLT 200 176 VLLQISISIGILIYISILISISIV VLTIV PISISISILIG T QLT 200
201LY I C N VINIHIK P SINITIKIVIDIKIKIVIE PIK S C D K 225 201LY I C N VINIHIK P SINITIKIVIDIKIKIVIE PIK S C D K 225
226 THTCPPCPAPELLGC (b) MOT Decidus Cleaverer 240/
(b) W87. Residue Cleavages: 31% (e) M107. Residue Cleavages: 44%
N E V Q L V]E]S G G G L]V]Q P]G]G S L R L]S]C A A S 25 N E V Q L V]E]S G G G L]V]Q P]G]G S L R L]S]C A A S 25
26 G]F]N I K]D]T]Y I]H]W V R]Q]A P G]K G L E]W V A R 50 26 G]F]N I K]D]T]Y I]H]W V R]Q]A P G]K G L E]W V A]R 50
51 I Y P T N G Y T R Y A D S V K G R F T I S A D T S 75 51 I Y P T N G Y T R Y A D S V K G R F T I S A D T S 75
76 K N T A Y L Q M N S L R A E D T A V Y Y C S R W G 100 76 K N]T]A]Y]L]Q]M[N]S]L R A]E D]T]A V Y Y C S]R W G 100 101 G D G F Y A M[D]Y W G Q G T L V T V[S]S]A S T[K]G 125 101 G D]G]F Y A M]D]Y W G Q G T L V T V[S]S]A S T[K]G 125
101 G D G F Y A MLDIY W G Q G T L V T VLSLSLA S TLKLG 125 101 G D G F Y A MLDIY W G Q G T L V T VLSLSLA S TLKLG 125 126 P S V F P LLA P S SLKLS T S G G TLA A L G C L V K 150 126 P S V F P LLA P S SLKLS T S G G TLA A L G C L V K 150
126 P S V F P LIA P S SIRIS T S G G TIA A L G C L V K 150 126 P S V F P LIA P S SIRIS T S G G TIA A L G C L V K 150 151 D YIF PLE P V T VIS WINIS GIA L T S G VIH TIF PLA 175 151 D YIF PLE P V T VIS WINIS GIA L T S G VIH TIF PLA 175
151 D ILF PLE P V T VLS WINIS GIAL T S G VIH TIF PLA 175 151 D YLF PLE P V T VLS WINIS GIAL T S G VIH TIF PLA 175 176 VLLQISISIGILIYISILISISIV VITIV PLSISISILIG T QLT 200 176 VLLQISISIGILIYISILISISIV VITIV PLSISISILIG T QLT 200
201LY I C N VINIHIK P SINITIKIVIDIKIKIVIE PIK S C D K 225 201LY I C N VINIHIK P SINITIKIVIDIKIKIVIE PIK S C D K 225
226 THTCPPCPAPELLGC (C) M83. Residue Cleavages: 40% N EVQLVIEISGGGLIVIQPIGIGSLRLISICAAS 25 N EVQLVIEISGGGLIVIQPIGIGSLRLISICAAS 25
26 G]F]N I K]D]T]Y I]H]W V R]Q]A P G]K G L E]W V A]R 50 26 G]F]N I K]D]T]Y I]H]W V R]Q]A P G]K G L E]W V A]R 50
51 IJY P TNGYT R YADJS VKG RJFT I SJADJTS 75 51 IJY P TNGYT R YADJS VKG RJFT I SJADJTS 75
76 K N]T]A]Y]L]Q]M N S L R A E D T A V Y Y C S R W G 100 76 K N]T]A]Y]L]Q]M[N]S]L R A]E D]T]A V Y Y C S]R W G 100
101 G D G F Y A M D Y W G Q G T L V T V S S A S T K G 125 101 G D G F Y A M D Y W G Q G T L V T V S S A S T K G 125
126 P S V F P L A P S S K S T S G G T A A L G C L V K 150 126 P S V F P L A P S S K S T S G G T A A L G C L V K 150
151 D Y]F PLE P V T VLS WINIS GIA L T S G VIH TIF PLA 175 151 D YJF PLE P V T VLS WINIS GIA L T S G VIH TIF PLA 175
176 VLLQISISIGILIYISILISISIV VITIV PISISISILIG T QIT 200 176 VLLQISISIGILIYISILISISIV VITIV PISISISILIG T QIT 200
201 Y I C N VINHIK P SINITIKIVIDIKIKIVIE PIK S C D K 225 201 Y I C N VINHIK P SINITIKIVIDIKIKIVIE PIK S C D K 225
226 THTCPPCPAPELLGC 226 THTCPPCPAPELLGC

CONCLUSIONS

- High sequence coverage of mAb subunits can be obtained by combining different modes of fragmentation (ETD and UVPD) and reaction times.
- Multiplexing different charge states representing different levels of oxidation of a stressed mAb can be a strategy to quickly identify sites of oxidation and can provide unique information.
- The identification of the oxidation site through a middle-down experiment can be unambiguous, but when subunits are oxidized at numerous sites, the analysis can be challenging.

TRADEMARKS/LICENSING

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or the unmodified Fd, a sequence coverage of 6 was observed (Figure 14). For Fd with one lation, N and C terminal fragments supporting oxidation on methionine at position 107 were erved and the sequence coverage was 44% ure 15e). All of the sites identified by middlein were also confirmed by peptide mapping.

