

### **ThermoFisher** SCIENTIFIC

### **Advanced Fragmentation Techniques for BioPharma Characterization**

Global BioPharma Summit

The world leader in serving science

### Different modes of fragmentation to answer different questions or for different assays



(Figure from Coon et al., Analytical Chemistry, 2009)



### MS Tools for Major Biopharma Characterization Workflows

### Thermo Scientific<sup>™</sup> Q Exactive<sup>™</sup>

**MS Family** 



For all the routine characterization workflows.

#### Mode of fragmentation: HCD

### Thermo Scientific<sup>™</sup> Orbitrap Fusion<sup>™</sup> Tribrid<sup>™</sup> MS Family



From routine to the most challenging tasks.

Mode of fragmentation: HCD CID ETD EThcD ETciD (UVPD - not commercial)



### Etanercept: O-glycosylation



### O-glycosylation of Etanercept

#### Etanercept (trade name Enbrel)



LPAQVAF**T**PYAPEPGSTC<sub>18</sub>RLREYYDQTAQMC<sub>31</sub>C<sub>32</sub>SKC<sub>35</sub>SPGQHAKVFC<sub>45</sub>TKTSDTVC<sub>53</sub>DS C<sub>56</sub>EDSTYTQLWNWVPEC<sub>71</sub>LSC<sub>74</sub>GSRC<sub>78</sub>SSDQVETQAC<sub>88</sub>TREQNRIC<sub>96</sub>TC<sub>98</sub>RPGWYC<sub>104</sub>ALS KQEGC<sub>112</sub>RLC<sub>115</sub>APLRKC<sub>121</sub>RPGFGVARPGTETSDVVC<sub>139</sub>KPC<sub>142</sub>APGTFS**N**TTSSTDIC<sub>157</sub>R PHQIC<sub>163</sub>NVVAIPG**N**ASMDAVC<sub>178</sub>TSTSPTR**S**MAPGAVHLPQPV**ST**RSQH**T**QP**T**PEP**ST**AP**ST S**FLLPMGP**S**PPAEGSTGDEPKS**C**<sub>240</sub>DKTH**TC**<sub>246</sub>PP**C**<sub>249</sub>PAPELLGGPSVFLFPPKPKDTLMIS RTPEVTC<sub>281</sub>VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**N**STYRVVSVLTVLHQDWLN GKEYKC<sub>341</sub>KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC<sub>387</sub>LVKGFYP SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC<sub>445</sub>SVMHEALHNH YTOKSLSLSPGK

3 N-glycan sites
 13 reported O-glycosylation sites

Sialic acids from the O-glycan were removed prior trypsin digestion

Peptide mapping was performed on the tryptic digest



### Results for peptide SMAPGAVHLPQPVSTR after processing HCD data

|   | HC             | D  |                           |                    |                             |                        |                  |                    |                                     |                    |                   |                 |  |                                  | <b>▼</b> ×         |
|---|----------------|--|---------------------------|--------------------|-----------------------------|------------------------|------------------|--------------------|-------------------------------------|--------------------|-------------------|-----------------|--|----------------------------------|--------------------|
| l |                | ŧ  | 7                         | No.                | Peptide Sequence            | Modification           |                  | Delta (ppm)        | RT (min)                            | M/Z                |                   | Charge<br>State | Mono Mass Exp.                                       | Mono Mass Theo. 🔺                | MS Area            |
|   | T <sub>x</sub> |  |                           | = - T <sub>x</sub> | <u>A</u> a • T <sub>2</sub> | Aa                     | • T <sub>x</sub> | = • T <sub>x</sub> |                                     | T <sub>26</sub> =  | ▼ 1/ <sub>x</sub> | $=$ $T_{x}$     | = • T <sub>x</sub>                                   | = • T <sub>x</sub>               | = • T <sub>x</sub> |
| L | ÷              | 1  | 1                         | 1339               | SMAPGAVHLPQPVSTR            | None                   |                  | -2.15              | 32,                                 | 41                 | 824.434           | 2               | 1646.8527  | 1646.8562                        | 14,750.08          |
|   | •              | 2  | $\checkmark$              | 855                | SMAPGAVHLPQPVSTR            | O_core                 |                  | -2.09              | 30.                                 | 41                 | 1007.501          | 2               | 2011.9843  | 2011.9885                        | 185,478.92         |
| L | •              | 3  | 1                         | 1076               | SMAPGAVHLPQPVSTR            | O_core                 |                  | -2.33              | 31.                                 | 59                 | 1007.500          | 2               | 2011.9838  | 2011.9885                        | 756,140.69         |
| Ļ | +              | 4  | $\checkmark$              | 599                | SMAPGAVHLPQPVSTR            | O_core ,O_core         |                  | -2.90              | 29.                                 | )8                 | 1190.066          | 2               | 2377.1138  | 2377.1207                        | 33,328,382.00      |
| L | ÷              | 5  | $\checkmark$              | 378                | SMAPGAVHLPQPVSTR            | O_core ,O_core ,O_core | 2                | -2.29              | 27.                                 | 75                 | 1372.633          | 2               | 2742.2466  | 2742.2529                        | 201,459.91         |
|   |                |  |                           |                    |                             |                        |                  |                    |                                     |                    |                   |                 |  |                                  |                    |
|   | 10             |  | 32.43                     |                    |                             |                        |                  |                    |                                     |                    |                   |                 | BioPharma Finder<br>Mass Informatics Platform for Pr | TM 2.0<br>Otein Characterization |                    |
|   | 5              | 0- <u>-</u>  |                           |                    |                             | S <sub>186</sub> MAPG  | iAV              | HLPQF              | 2VS <sub>199</sub>                  | Γ <sub>200</sub> Ϝ | <b>{</b>          |                 |  |                                  |                    |
|   | 10<br>5        | 0<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1 |                           |                    |                             |                        | 30.4             | 40                 |                                     | 31.57              |                   |                 | <u>S</u> MAPGA                                       | AVHLPQPV                         | <u>ST</u> R +      |
|   | 10<br>5        | 0<br>0<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1<br>1                                    | 29.07                     |                    |                             |                        |                  |                    | <u>S</u> MAPGAVHLPQPV <u>ST</u> R + |                    |                   |                 |  |                                  |                    |
|   | 10<br>5        |  | 27.75<br>SMAPGAVHLPQPVSTR |                    |                             |                        |                  |                    |                                     |                    |                   |                 |  |                                  |                    |
|   |                | <  | 27.5                      | 28.0               | 28.5 29.0                   | 29.5 30.0              | 1                | 30.5 31<br>T       | ime (min)                           | 1.5                | 32.0              | 32.5            | 33.0 33  | 3.5 34.0                         | 34.5 35.0          |



### CID and HCD spectra of peptide SMAPGAVHLPQPVSTR + 1 O-core1 ( 365.1322 Da)



Average Structural Resolution = 1.8 residues



Color Code for Ion Intensity >1.8e+004 >1.1e+004 >6.2e+003 >3.6e+003 >2.1e+003

Average Structural Resolution = 1.1 residues

#### $\overset{1}{S} + \overset{2}{\underline{M}} + \overset{3}{\underline{A}} + \overset{4}{\underline{P}} + \overset{5}{\underline{G}} + \overset{6}{\underline{A}} + \overset{7}{\underline{V}} + \overset{8}{\underline{H}} + \overset{9}{\underline{L}} + \overset{10}{\underline{P}} - \overset{11}{\underline{Q}} + \overset{12}{\underline{P}} + \overset{13}{\underline{V}} + \overset{14}{\underline{S}} - \overset{15}{\underline{T}} + \overset{16}{\underline{R}} + \overset{16}{\underline{V}} + \overset{16}{\underline{N}} + \overset{16}{\underline{V}} + \overset{16}{\underline{N}} + \overset{16}{\underline{V}} + \overset{16}{\underline{V}} + \overset{16}{\underline{N}} + \overset{16}{\underline{V}} + \overset{16}{\underline{V} + \overset{16}{\underline{V}} +$

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 S M A P G A VHL P Q P V S T R b12-H20[2+](767.4) y4(462.2) b4(752.4) y12(1261.7) y15[2+](781.4) y14(1429.8) y11-3H20(1150.6) y9(1034.6) y8(897.5) y7(784.4) y5(559.3) y3-3H20[2+] y1

>1.6e+004 >9.0e+003 >5.1e+003 >2.9e+003 >1.6e+00

Color Code for Ion Intensity

HCD spectrum provides good peptide backbone fragmentation.

Site of glycosylation cannot be identified with the HCD or CID spectra.



### Data acquisition method on a Fusion Lumos: ETD methods

Flexibility and ease of use to design acquisition method



DDA method used to collect data to identify the sites of O-glycosylation.



### Data acquisition method on a Fusion Lumos: ETD methods







### Data acquisition method on a Fusion Lumos: ETD methods

Other useful methods:

- \* HCD followed by ETD for every precursors.
- \* HCD followed by ETD when specific product ions are present in the HCD MS2 spectrum





### ETD spectrum of SMAPGAVHLPQPVSTR (No O-core1 tag)





### ETD MS/MS of O-glycan structural isomers: SMAPGAVHLPQPVSTR + 1 core1 tag.





### ETD spectrum of SMAPGAVHLPQPVSTR + 2 core1 tag.







### ETD spectrum of SMAPGAVHLPQPVSTR + 3 core1 tag.





### Results for peptide SMAPGAVHLPQPVSTR after processing ETD data

| E   | ETD 📃          |  | Peptide Sequence                | Modification           | Site               | Delta (ppm)          | Confidence Score   | RT (min)           | M/Z                | Charge<br>State Mono Mass Exp. |                    | Mono Mass Theo. 🖌 |  |
|-----|----------------|--|---------------------------------|------------------------|--------------------|----------------------|--------------------|--------------------|--------------------|--------------------------------|--------------------|-------------------|--|
|     | T <sub>x</sub> |  | <u>A</u> a sma ▼ ¥ <sub>x</sub> | <u>A</u> a 🔹 🛙         | , <u>A</u> a ▼ 17, | . = • T <sub>x</sub> | = • T <sub>x</sub> | = • T <sub>x</sub> | = - T <sub>x</sub> | $=$ $T_{x}$                    | = • T <sub>x</sub> | = - T             |  |
| ٠   | 1              |  | SMAPGAVHLPQPVSTR                | None                   |                    | -1.19                | 100.0%             | 32.35              | 549.959            | 3                              | 1646.8542          | 1646.856          |  |
| ÷   | 2              |  | SMAPGAVHLPQPVSTR                | O_core                 | ~T200              | -0.94                | 100.0%             | 30.35              | 672.003            | 3                              | 2011.9866          | 2011.988          |  |
| ٠   | 3              |  | SMAPGAVHLPQPVSTR                | O_core                 | ~S199              | -1.06                | 100.0%             | 31.54              | 672.003            | 3                              | 2011.9863          | 2011.988          |  |
| ٠   | 4              |  | SMAPGAVHLPQPVSTR                | O_core ,O_core         | S199,T200          | -1.97                | 100.0%             | 29.07              | 595.537            | 4                              | 2377.1160          | 2377.120          |  |
| ÷   | 5              |  | SMAPGAVHLPQPVSTR                | O_core ,O_core ,O_core | S186,S199,T200     | -2.11                | 100.0%             | 27.71              | 686.569            | 4                              | 2742.2471          | 2742.252          |  |
| ٠ [ |                |  |                                 |                        |                    |                      |                    |                    |                    |                                |                    | •                 |  |

All of the O-glycosylation sites of peptide SMAPGAVHLPQPVSTR were successfully identified



BioPharma Finder<sup>TM</sup> 2.0 Mass Informatics Platform for Protein Characterizatio





# Etanercept: leveraging ETD and MSn for disulfide bond mapping



### Disulfide bond mapping: Etanercept

## Etanercept (trade name Enbrel)



LPAQVAF**T**PYAPEPGSTC<sub>18</sub>RLREYYDQTAQMC<sub>31</sub>C<sub>32</sub>SKC<sub>35</sub>SPGQHAK VFC<sub>45</sub>TKTSDTVC<sub>53</sub>DSC<sub>56</sub>EDSTYTQLWNWVPEC<sub>71</sub>LSC<sub>74</sub>GSRC<sub>78</sub>SSD QVETQAC<sub>88</sub>TREQNRIC<sub>96</sub>TC<sub>98</sub>RPGWYC<sub>104</sub>ALSKQEGC<sub>112</sub>RLC<sub>115</sub>APL RKC<sub>121</sub>RPGFGVARPGTETSDVVC<sub>139</sub>KPC<sub>142</sub>APGTFS**N**TTSSTDIC<sub>157</sub> RPHQIC<sub>163</sub>NVVAIPG**N**ASMDAVC<sub>178</sub>TSTSPTR**S**MAPGAVHLPQPV**ST** RSQH**T**QP**T**PEP**ST**AP**ST**SFLLPMGP**S**PPAEGSTGDEPKSC<sub>240</sub>DKTH**T** C<sub>246</sub>PPC<sub>249</sub>PAPELLGGPSVFLFPPKPKDTLMISRTPEVTC<sub>281</sub>VVVDV SHEDPEVKFNWYVDGVEVHNAKTKPREEQY**N**STYRVVSVLTVLHQDW LNGKEYKC<sub>341</sub>KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEM TKNQVSLTC<sub>387</sub>LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSC<sub>445</sub>SVMHEALHNHYTQKSLSLSPGK



### Disulfide bond mapping: Etanercept

#### Etanercept (trade name Enbrel)



The N-terminal side of this sample is not as expected. The first 2 amino acids are missing.



### **Disulfide Bond Mapping: Etanercept**

#### Etanercept (trade name Enbrel)



LPAQVAFTPYAPEPGSTC<sub>18</sub>RLREYYDQTAQMC<sub>31</sub>C<sub>32</sub>SKC<sub>35</sub>SPGQHAFVFC<sub>45</sub>TKTSDTVC<sub>53</sub>DS C<sub>56</sub>EDSTYTQLWNWVPEC<sub>71</sub>LSC<sub>74</sub>GSRC<sub>78</sub>SSDQVETQAC<sub>88</sub>TREQNRIC<sub>96</sub>TC<sub>98</sub>RPGWYC<sub>104</sub>ALS KQEGC<sub>112</sub>RLC<sub>115</sub>APLRKC<sub>121</sub>RPGFGVARPGTETSDVVC<sub>139</sub>KPC<sub>142</sub>APGTFSNTTSSTDIC<sub>157</sub>R PHQIC<sub>163</sub>NVVAIPGNASMDAVC<sub>178</sub>TSTSPTRSMAPGAVHLPQPVSTRSQHTQPTPEPSTAPST SFLLPMGPSPPAEGSTGDEPKSC<sub>240</sub>DKTHTC<sub>246</sub>PPC<sub>249</sub>PAPELLGGPSVFLFPPKPKDTLMIS RTPEVTC<sub>281</sub>VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN GKEYKC<sub>341</sub>KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC<sub>387</sub>LVKGFYP SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC<sub>445</sub>SVMHEALHNH YTQKSLSLSPGK

The N-terminal side peptide contains one cysteine. The HCD search result of the non-reduced sample suggests that the N-terminal peptide is linked to two other peptides through disulfide bonds.



### Leveraging ETD and MSn for disulfide bond mapping

#### Workflow of the MS2 ETD – MS3 HCD for disulfide bond peptide identification





### Leveraging ETD and MSn for disulfide bond mapping

Workflow of the MS2 ETD – MS3 HCD for disulfide bond peptide identification





#### Peptide: AQVAFTPYAPEPGSTC<sub>18</sub>R-EYYDQTAQMC<sub>31</sub>C<sub>32</sub>SK-VFC<sub>45</sub>TK





#### MS3 HCD spectrum of AQVAFTPYAPEPGSTC<sub>18</sub>R.





#### Peptide: AQVAFTPYAPEPGSTC<sub>18</sub>R-EYYDQTAQMC<sub>31</sub>C<sub>32</sub>SK-VFC<sub>45</sub>TK

MS3 HCD spectrum of EYYDQTAQMC $_{31}C_{32}SK$  using a) the Orbitrap or b) the ion trap mass analyzers.





#### Peptide: **AQVAFTPYAPEPGSTC**<sub>18</sub>**R-EYYDQTAQMC**<sub>31</sub>**C**<sub>32</sub>**SK-VFC**<sub>45</sub>**TK**

MS3 HCD spectrum of VFC<sub>45</sub>TK.





### Antibody Drug Conjugates



### ADCs are heterogeneous mixture



Light Chain: 12 Lysines Heavy Chain: 32 Lysines



### Trastuzumab emtansine peptide mapping by SMART Digest<sup>™</sup>

Trastuzumab emtansine SMART Digest (75 min) - reduction (non optimized protocol)



**BioPharma Finder<sup>TM</sup> 2.0** Mass Informatics Platform for Protein Characterization



| Proteins         | Number of MS Peaks | MS Peak Area | Sequence Coverage | Abundance (mol) |
|------------------|--------------------|--------------|-------------------|-----------------|
| 1:LC_Trastu_Emta | 1029               | 25.2%        | 100.0%            | 38.62%          |
| 2:HC_trastu_Emta | 2479               | 62.8%        | 100.0%            | 61.38%          |
| Unidentified     | 25270              | 12.0%        |                   |                 |

#### BioPharma Finder Variable PTMs K: MCC-DM1 Glycan: CHO N, Q: Deamidation M, W: Oxidation



### *Trastuzumab emtansine*: conjugated peptides

#### Subset of identified conjugated peptides

|   | ŧ  | No.            | Peptide Sequence      | # K | -                | Modification                  | Site  | Deita (ppm)        | ID Type        | RT (min)           | M/Z                | Charge<br>State | Mono Mass Exp.     | Mono Mass Theo.    | MS Area            | Protein        |   |
|---|----|----------------|-----------------------|-----|------------------|-------------------------------|---|--------------------|----------------|--------------------|--------------------|-----------------|--------------------|--------------------|--------------------|----------------|---|
| ٦ | ж  | = (Custom) 🔹 🟹 | <u>A</u> a            |     | · T <sub>x</sub> | <u>A</u> a ( ▼ ¥ <sub>×</sub> | $\underline{A}a \bullet \underline{\nabla}_{\!_{\!$ | = • T <sub>x</sub> | <u>A</u> a 🔻 🟹 | = • T <sub>x</sub> | = • T <sub>x</sub> | $= T_{\rm x}$   | = • T <sub>x</sub> | = • T <sub>x</sub> | = • T <sub>x</sub> | <u>A</u> a 🔹 🟹 | × |
| ÷ | 1  | 21049          | ADYEKHK               |     |                  | DM1                           | K188  | 0.60               | MS2            | 50.16              | 616.272            | 3               | 1845.7949          | 1845.7938          | 20,170,934.00      | LC_Trastu_Emta | а |
| ÷ | 2  | 21419          | ADYEKHK               | 2   |                  | DM1                           | K188  | -0.13              | MS2            | 50.94              | 616.272            | 3               | 1845.7936          | 1845.7938          | 14,392,560.00      | LC_Trastu_Emta | а |
| ÷ | 3  | 22848          | AKGQPR                | 1   |                  | DM1                           | K343  | -0.09              | MS2            | 53.88              | 806.878            | 2               | 1611.7408          | 1611.7410          | 1,016,677.25       | HC_trastu_Emta | a |
| ÷ | 4  | 23430          | AKGQPR                |     |                  | DM1                           | K343  | -0.17              | MS2            | 54.75              | 806.878            | 2               | 1611.7407          | 1611.7410          | 790,552.56         | HC_trastu_Emta | a |
| ٠ | 5  | 26843          | ALPAPIEKTISK          |     |                  | DM1                           | K337  | 0.64               | MS2            | 64.62              | 742.381            | 3               | 2223.1206          | 2223.1192          | 462,441.38         | HC_trastu_Emta | a |
| ÷ | 6  | 26950          | ALPAPIEKTISK          | ۷   |                  | DM1                           | K337  | 0.42               | MS2            | 65.11              | 742.381            | 3               | 2223.1201          | 2223.1192          | 353,500.62         | HC_trastu_Emta | a |
| ÷ | 7  | 25889          | ASQDVNTAVAWYQQKPGK    |     |                  | DM1                           | K39   | 0.40               | MS2            | 60.66              | 983.794            | 3               | 2946.3564          | 2946.3553          | 99,419.80          | LC_Trastu_Emta | а |
| ÷ | 8  | 25971          | ASQDVNTAVAWYQQKPGK    | 2   |                  | DM1                           | K39   | 0.56               | MS2            | 61.18              | 983.794            | 3               | 2946.3569          | 2946.3553          | 78,092.43          | LC_Trastu_Emta | а |
| ÷ | 9  | 23503          | ASQDVNTAVAWYQQKPGKAPK | 2   |                  | DM1                           | ~K42  | 0.58               | MS2            | 54.93              | 1082.521           | 3               | 3242.5420          | 3242.5401          | 3,348,808.00       | LC_Trastu_Emta | а |
| ÷ | 10 | 23773          | ASQDVNTAVAWYQQKPGKAPK | 3   |                  | DM1                           | ~K42  | 0.20               | MS2            | 55.50              | 1082.521           | 3               | 3242,5408          | 3242.5401          | 2,925,363.50       | LC_Trastu_Emta | а |
| ÷ | 11 | 27147          | DSTYSLSSTLTLSKADYEK   | 2   |                  | DM1                           | K183  | 0.50               | MS2            | 66.91              | 1023.135           | 3               | 3064.3821          | 3064.3806          | 72,910.94          | LC_Trastu_Emta | а |
| ÷ | 12 | 27210          | DSTYSLSSTLTLSKADYEK   | 2   |                  | DM1                           | K183  | 0.10               | MS2            | 67.35              | 1023.136           | 3               | 3064.3809          | 3064.3806          | 42,332.44          | LC_Trastu_Emta | а |
| ÷ | 13 | 22964          | TKPR                  | 1   |                  | DM1                           | K293  | -1.18              | MS2            | 54.16              | 729.342            | 2               | 1456.6698          | 1456.6715          | 1,648,967.12       | HC_trastu_Emta | a |
| ÷ | 14 | 23556          | TKPR                  |     |                  | DM1                           | K293  | -0.01              | MS2            | 55.02              | 729.343            | 2               | 1456.6715          | 1456.6715          | 1,267,997.38       | HC_trastu_Emta | a |
|   |    |                |                       |     |                  |                               |   |                    |                |                    |                    |                 |                    |                    |                    |                |   |





### Identification of peptide ASQDVNTAVAWYQQKPGKAPK



### Identification of peptide ASQDVNTAVAWYQQKPGKAPK



#### $\dot{A} = \overline{s} + \overline{2} \overline{2} + \overline{2} \overline{2} + \overline{2} \overline{1} + \overline{1} + \overline{1} \overline{2} + \overline{2} \overline{1} + \overline{2} \overline{$



The HCD spectra of the non-conjugated peptide contains numerous intense y fragments ions. On the opposite, most of y ions for the conjugated peptide are at low intensity.

The HCD spectra of the conjugated peptide contains a characteristic neutral loss ion at m/z 547.2.

#### $\overset{1}{A} + \overset{2}{\underline{S}} + \overset{3}{\underline{O}} + \overset{4}{\underline{D}} + \overset{5}{\underline{N}} + \overset{4}{\underline{T}} + \overset{2}{\underline{A}} + \overset{10}{\underline{W}} + \overset{11}{\underline{W}} + \overset{11}{\underline{W}} + \overset{11}{\underline{U}} + \overset{11}{\underline{W}} + \overset{11}{\underline{W} + \overset{11}{\underline{W}} + \overset{11}{\underline{W}}$





### Trastuzumab emtansine site occupation: ETD spectrum





### Trastuzumab emtansine site occupation



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### *Trastuzumab emtansine*: conjugated peptides

#### Subset of identified conjugated peptides

|    | ŧ  | No.            | Peptide Sequence      | # K   | -                | Modification                  | Site           | Deita (ppm) | ID Type                            | RT (min)      | M/Z                | Charge<br>State | Mono Mass Exp.     | Mono Mass Theo.    | MS Area            | Protein    |                  |
|----|----|----------------|-----------------------|-------|------------------|-------------------------------|----------------|-------------|------------------------------------|---------------|--------------------|-----------------|--------------------|--------------------|--------------------|------------|------------------|
| ٣, | c  | = (Custom) 🔹 🏹 | <u>A</u> a            | ,     | · T <sub>x</sub> | <u>A</u> a ( ▼ ¥ <sub>×</sub> | <u>A</u> a ▼ 🏹 | = • T,      | <u>A</u> a <b>▼</b> 7 <sub>x</sub> | $r = r T_{x}$ | = • T <sub>x</sub> | $= T_{\rm x}$   | = • T <sub>x</sub> | = • T <sub>x</sub> | = • T <sub>x</sub> | <u>A</u> a | ▼ T <sub>x</sub> |
| ÷  | 1  | 21049          | ADYEKHK               |       |                  | DM1                           | K188           | 0.60        | MS2                                | 50.16         | 616.272            | 3               | 1845.7949          | 1845.7938          | 20,170,934.00      | LC_Trast   | tu_Emta          |
| ÷  | 2  | 21419          | ADYEKHK               | 2     |                  | DM1                           | K188           | -0.13       | MS2                                | 50.94         | 616.272            | 3               | 1845.7936          | 1845.7938          | 14,392,560.00      | LC_Trast   | tu_Emta          |
| ÷  | 3  | 22848          | AKGQPR                | 1     |                  | DM1                           | K343           | -0.09       | MS2                                | 53.88         | 806.878            | 2               | 1611.7408          | 1611.7410          | 1,016,677.25       | HC_tras    | tu_Emta          |
| ÷  | 4  | 23430          | AKGQPR                |       |                  | DM1                           | K343           | -0.17       | MS2                                | 54.75         | 806.878            | 2               | 1611.7407          | 1611.7410          | 790,552.56         | HC_tras    | tu_Emta          |
| +  | 5  | 26843          | ALPAPIEKTISK          | _ 2 _ |                  | DM1                           | K337           | 0.64        | MS2                                | 64.62         | 742.381            | 3               | 2223.1206          | 2223.1192          | 462,441.38         | HC_tras    | tu_Emta          |
| +  | 6  | 26950          | ALPAPIEKTISK          | ۷     |                  | DM1                           | K337           | 0.42        | MS2                                | 65.11         | 742.381            | 3               | 2223.1201          | 2223.1192          | 353,500.62         | HC_tras    | tu_Emta          |
| ÷  | 7  | 25889          | ASQDVNTAVAWYQQKPGK    | _ 2 _ |                  | DM1                           | K39            | 0.40        | MS2                                | 60.66         | 983.794            | 3               | 2946.3564          | 2946.3553          | 99,419.80          | LC_Trast   | tu_Emta          |
| •  | 8  | 25971          | ASQDVNTAVAWYQQKPGK    | ۷     |                  | DM1                           | K39            | 0.56        | MS2                                | 61.18         | 983.794            | 3               | 2946.3569          | 2946.3553          | 78,092.43          | LC_Trast   | tu_Emta          |
| ÷  | 9  | 23503          | ASQDVNTAVAWYQQKPGKAPK | 2     |                  | DM1                           | ~K42           | 0.58        | MS2                                | 54.93         | 1082.521           | 3               | 3242.5420          | 3242.5401          | 3,348,808.00       | LC_Trast   | tu_Emta          |
| +  | 10 | 23773          | ASQDVNTAVAWYQQKPGKAPK | 3     |                  | DM1                           | ~K42           | 0.20        | MS2                                | 55.50         | 1082.521           | 3               | 3242.5408          | 3242.5401          | 2,925,363.50       | LC_Trast   | tu_Emta          |
| ÷  | 11 | 27147          | DSTYSLSSTLTLSKADYEK   |       |                  | DM1                           | K183           | 0.50        | MS2                                | 66.91         | 1023.135           | 3               | 3064.3821          | 3064.3806          | 72,910.94          | LC_Trast   | tu_Emta          |
| •  | 12 | 27210          | DSTYSLSSTLTLSKADYEK   | 2     |                  | DM1                           | K183           | 0.10        | MS2                                | 67.35         | 1023.136           | 3               | 3064.3809          | 3064.3806          | 42,332.44          | LC_Trast   | tu_Emta          |
| •  | 13 | 22964          | TKPR                  | 1_    |                  | DM1                           | K293           | -1.18       | MS2                                | 54.16         | 729.342            | 2               | 1456.6698          | 1456.6715          | 1,648,967.12       | HC_tras    | tu_Emta          |
| •  | 14 | 23556          | TKPR                  | I     |                  | DM1                           | K293           | -0.01       | MS2                                | 55.02         | 729.343            | 2               | 1456.6715          | 1456.6715          | 1,267,997.38       | HC_tras    | tu_Emta          |



### Identification of peptide TKPR





Abundance

Relative

### Middle-down: ETD



Main parameters that can be controlled for ETD fragmentation on an Orbitrap<sup>™</sup> Fusion<sup>™</sup> mass spectrometer.

• Isolation window





- AGC target for precursor ions and reagent
- Reaction time
- Supplemental energy



### Top-Down sequencing: Trastuzumab

Light Chain of Trastuzumab





Due to the complexity of the spectra in top-down analysis, high resolution is required



### Effect of parent ion AGC and ETD reaction time on sequence coverage (Trastuzumab)







% residue cleavages for AGC target value of 3E5 or 1E6 at different ETD reaction times







#### % residue cleavages for AGC target value of 3E5 or 1E6 at different ETD reaction times







### Middle Down: Orbitrap Tribrid Fusion Lumos



Type:

Observed:

Theoretical:

Mass Diff. (Da):

Mass Diff. (ppm):

Monoisotopic

25,220.46

25,220.46

0.002

0.07

590.62

2e-53

Ŧ

High sequence coverage for the light chain, Fc and Fd were obtained from the combined ETD and EThcD experiments.



60 %

Monoisotopic

25,367.52

25,367.52

0.002

0.08

641.24

2.3e-57

18 %

Precursor Mass

Type:

<u>Scores</u>

P-Score:

PCS:

Observed:

Theoretical:

Mass Diff. (Da):

Mass Diff. (ppm):

% Fragments Explai...

Modification (E1)

No Modification

Custom

x

% Residue Cleavages: 60 %

Scores 101 SKAKJGQ PREPQVYTL PPSREEMTKN 125 PCS: 126 Q V S L T C LLVK GLFY P SDIIALVEWELSNGQ 150 P-Score: % Fragments Explain... 20 % 151 PENNYK T T P P VLLDSDGSFFLLYSKLLT 175 % Residue Cleavages: 67 % 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 Modification (G1) 201 T Q K S L S L S P G C No Modification Custom Matching Fragments (Count: 454) X

26 VVVDVDVSHEDPEVKFNWVVDGVEVHN 50

<sup>51</sup>AKTKPREEQYNSTYRVVSVLTVLHQ <sup>75</sup>

76 DWLNGKEYKCKVSNKALPAPILEKTI 100

### NIST mAb: middle down experiment using ETD and UVPD

#### ETD 10 ms + UVPD 12 ms

N DIQMTQSPSTLSASVGDRVTITCSA 24 <sup>26</sup>]S]S]R]V]G]Y]M]H]W]Y]Q]Q]K]P G]K]A]P]K]L]L]I]Y]D]T 5 51 S K L A S G V P S R F S G S G S G T E F T L T I S 75 76 SLLQPDDFAT YLYC F QG S GYP FT FGGGG 100 101 T KUVELIK RTVAAAPLS VLF ILFPPPSDEQ LLK 125 S GITIALS VIV CIL L NINE Y P REAKVQWK VD 150 151 NALLQIS GINISIQLE SIVITLE QUDISIK DIST Y SILLS 175 176 STLL TLLSKADYEKHKVYA CLEVTHQ GLL 200 201 S S P V T K S F N R G E C % Residue Cleavages: N G P S V F L F P P K P K D T L M I S R T P E V T C 25 0 <sup>51</sup>AKTKPREEQYNSTYRVVSVLTVLHQ <sup>75</sup> <sup>76</sup>]DWLNGKEYKCK)VSNKAL PLIEKTI 100 101 SLK ALKLGQP R ELP QLV Y TLLPLPLS R ELE MLT KN 125 L26 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 150 151 PENNYK TTPPVLLDSDGSFFLYSKLLT 175 ١ĭ 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 TLQLKLSLSPGC % Residue Cleavages: 67 % N OVTLRESGPALVKPTQTLTLTCTFS 25 26]G FSLSTAGMS V GW IROP P GKALJEW L 50 <sup>51</sup> AD IWWDDKKHYNPSLKDRLTISKDT <sup>75</sup> 8 76 SKNQVVLKVTNMDPADTATYYCARD 100 101 M I FNFY FD VWGQ GT TV TV SA ST KG 125 126 P S V]FÌP LÌAÌP S SÌKLS TÌSLG G T A A L G C L VLK 150 151 DYFFEPEVTVSWNSGALTSGVHTFPA 175 176 VLLQSSGLLYSLSSVVVTVPSSSLLGT QT 200 226 THTCLPLPCLPLALPELLG % Residue Cleavages: 61 9

#### One acquisition with UVPD 12 ms

N DIQMITQISIPSTLISASVGDRIVITITCISA 25 26]SJSRIVJGYIMHWIYIQIQIKIPGIKAIPIKILILIYDT 50 51 SKILASGIVIPSRIFISGISGSGTEIFITLTIJS 75 76]SLIQIPDDFIAITYLYLCFQLGSGIYIPFTFGLGG100 101 TKVEIKRTVAIAIPLSVFILFIPIPLSDEQLLK125 126 SGTLASVLVCLLLNNFYPREAKVQWKVD150 151 NALQLSGNSQELSLVLTEQDSKDSTLYLS175 176 STLTLLSKLALDLYLEKLHLKVLYLACLELVLTLHQGL 200 201LSLSLPLVLTLKSFNRGEC%Residue Cleavages: 40%

N GPSVF]L]F]PPK]P]K]D]TL]M]I]SR]TPE]V]T]C 25 26 VVVDVDVSHEDPEVKFNVVDGVEVHN 50 <sup>51</sup> A K T K P R E E Q Y N S T Y R V V S V L T V L H Q <sup>75</sup> 76]DWLNGKEYKCK]VSNKAL]PAPLIEKTI 100 101 SKAKGQPREPQVYTLPPSREEMTKN 125 126 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 150 151 PENNYKTTPPVLDSDGSFFLYSKLT 175 176 V D K S R WLQ Q G NLV FLS CLS V M HLELA L HLNLH Y 200 201 TQKSLSLSPGC % Residue Cleavages: 32 % N OVTLRESGPALVKPTQTLTLTCTFS 25 <sup>26</sup>]GF]S]L]S]TA]GM]SVG]WI]R]Q]PPGKAL]E]WL <sup>50</sup> <sup>51</sup> A D I W W D D K K H Y N P S L K D R L T I S K D T 76 SKNQVVLKVTNMDPADTATYYCARD 100 101 M I F N F Y F D V W G Q G T T V T V S S A S T K G 126 PSVFPLAPSSKSTSGGTAALGCLVK 150 151 DYFLPLEPVTVSWNSGALLTSGVHTFLPA 175 176 V LOS SG LYS L SSVVTVPS S S L G T Q T 200 201 Y I C N V N H K P S N T K V D K R V E P K S C D K 226 THTCLPPCLPALPELLG % Residue Cleavages: 26 %

N DIQMTQSPSTLSASVGDRVTITCSA 25 26 S S R VJGJYMHWJJQJQJK P GJKJA PJKJLJIJYDJT SISKLASGV PS RFSGSGS GT E F TLTIS 7 76 SLQ PDDFATYYCFQGSGYPFTFGGGG 100 101 T KVEIIK RT VA A PS VF IF P P SDEQ LK 125 10 126 S G T A S V V C L L N N F Y P R E A K V Q W K V D 150 151 NALLQ S GNSQES VITEQDSKDSTYSLS 175 176 STL TLSKADYEKHKVYACEVTHQGL 200 201 S P V T K S F N R G E C % Residue Cleavages: N G P S V F L F P P K P K D T L M I S R T P E V T C 25 % 26 V V VD V SHED PEVKFNWYVDGVEVHN 50 <sup>51</sup>AKTKPREEQYNSTYRVVSVLTVLHQ <sup>75</sup> O 3 76 DWLNGKEYKCKVSNKALPAPILEKTI 100 S 101 SKAKGQPREPQVYTLPPSREEMTKN 125 126 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 U 151 PLE NNYKTTPPVLDSDGSFFLLYSKLLT 175 LĽ 176 VDKSRWOOGNVFSCSVMHEALHNHY 200 201 TOKSLSLSPGC % Residue Cleavages: 52 % N Q V T L R E S G P A L V K P T Q T L T L T C T F S 25 26 GFSLSTAGMSVGWIRQPPGKALEWL 50 AD IWWDDKKHYNPSLKDRLTISKDT 0 2 76 SKNQVVLKVTNMDPADTATYYCARD 100 0 (0)  $\mathbf{c}$ 101 M I FNFY FD VWGQGTTV TV S SAST KG 125 N PSVFPLAPSSKSTSGGTAALGCLVK 150 151 DYFPEPVTVSWNSGALTSGVHTFPA 175 σ 176 VLQSSGLYSLSSVVTVPSSSLGTQT 200  $\mathbf{O}$ 226 THTCPPCPAPELLGC % Residue Cleavages: 43 %

One acquisition with ETD 10 ms



### NIST mAb: middle down experiment using ETD and UVPD

|          | One acquisition with E   | ETD 10   | ms  | One acquisition wi  | th UVPD 12 ms  | ETC   | 0 10 ms + UVPD 12 ms  |   |
|----------|--|--|---|---|--|---|---|---|
| LC (60%) | N DI QM TQS PSTLSASVGC<br>26 SSRVGYMHWYQQK PGKA<br>51SKLASGV PSRFSGSGSG<br>76SLQ PDDFATYYCFQGSG<br>101 TKVEIKRTVAAPSVFIL<br>126 SGTASVVCLLNNFYPRE<br>151NALLQSGNSQESVTEQDS<br>176STLTLSKADYEKHKVYA | D R V T I<br>A P K L L L<br>G T E F T<br>G Y P F T<br>F P P S D<br>E A K V Q<br>S K D S T<br>A C E V T | T C S A 25       N         I]Y]D]T 50       26]         L]T]I]S 75       51         F[G]G]G 100       76]         E[Q L[K 125       101         W]K V[D 150       126         Y[S]L[S 175       151         H[Q G L 200       176 | DIQMÌTQÌSÌPSTLÌSAS<br>SÌSRÌVÌGYÌMÌHWÌYÌQÌQÌKÌF<br>SKÌLASGÌVÌPSRÌFÌSGÌS<br>SLÌQÌPDDFÌAÌTYLYLCFQ<br>TKVEIKRTVAÌAÌPLSV<br>SGTLASVLVCLLLNNFY<br>NALQLSGNSQELSLVLTE<br>STLTLLSKLALDLYLEKLHLK | S V G D R V T I T C S A       25         P G K A P K L L L I Y D T       50         S G S G T E F T L T I S       75         Q G S G Y P F T F G G G 100         Y F I F P P S D E Q L K 125         Y P R E A K V Q W K V D 150         E Q D S K D S T Y S L S 175         X V Y A C E V T H Q G L 200 | N D I QMTQ<br>26]S]S]RV]G]Y<br>51]S]K]L]A]S]G<br>76]S[L]Q[P]D]D<br>101 T K[V]E]I]K<br>126 S G[T[A]S V<br>151[N]A[L]Q[S G]<br>176[S[T[L T[L]S] | S)P S T L]S A S V G D R]V]T]I T]C]S A       25         M]H]W]Y]Q]Q]K]P G]K]A]P]K]L]L]I]Y]D]T       50         V]P]S R]F]S]G]S]G]S G]T E]F]T]L]T]I]S       75         IF]A]T Y[Y[C F Q[G S G]Y]P F]T F[G]G]G       100         R]T]V]A]A]P[S V]F I[F]P]P[S]D[E]Q L[K       125         [V C[L L N]N[F Y P R]E[A[K[V]Q]W]K V[D       150         [N]S[Q[E[S[V T]E[Q]D[S[K]D[S]T[Y]S[L[S       175         [K]A[D]Y[E[K]H[K[V]Y[A C[E[V]T]H[Q G[L       200  |   |
|          | 201 S PVT KSFNR G E C % Resid  | lue Cleavag  | es: 60 %  | SLSLPLVLTLK S F N R G E C 9   | 6 Residue Cleavages: 40 %  | 201 <b>ͺϛͺϛͺϼͺνͺ</b> ϯͺκ  | SLFNRGEC % Residue Cleavages: 78 %  | % |
| (%)      | N G P S VJFJLJF P PJK PJKJDJTJLJM<br>26JV V VJD V SJHJEJD PJEJVJKJFJNJWJ<br>51JAJKJTJK PJRJEJEJQJY <mark>N</mark> S T Y RJV  | I]S]R]T P]   | EIVITIC 25 N  | GP S V FILIFIP P KIPIKIDI   | ages   | N GIP S VIFILI  | F]P P]K]P]K]D]T]L]M]I]S]R]T P]E]V]T]C 25<br>H]E]D]P]E]V]K]F]N]W]Y]V]D]G]V]E]V]H]N 50<br>E]E]Q]Y <mark>]N</mark> ]S T Y R]V V S V L T V L H Q 75   |   |
| 22       | <sup>76</sup> DWLNG <mark>KEYKCKVSNKA</mark>   |  | ETD 10 ms   | UVPD 12 ms  | ETD + UVPD   | % increase  | EYKCKÌVSNKALÌPLAPLILELKÌTLI 100   |   |
| С<br>С   | 101 SLK ALKLELO P K E P Q V Y TLL P<br>126 Q V S L T C L VLK GLFLY PLS D I<br>151 PLE NNYLK T T P P V L DLSDG  | LC   | 60  | 40  | 78   | 30.0%   | PRELPQLVYTLL PLPLSRELEMLTKLN125<br>LVLKGLFLYPLSDILAVEWLELSNLGLQ150<br>TTLPPVLLDLSDLGLSLFFLLYLSKLLT175   |   |
|          |  | Fc   | 52  | 32  | 67   | 28.8%   | χ[Q[G[N[V]F[S c[S V M[H[E[A L[H[N[H[Y 200          κ p]c            κ p]c           κ p]c           κ p]c           κ p]c           κ p]c           κ p]c           κ p]c           κ p]c           κ p]c           κ p]c           κ p]c           κ p]c           κ       γ           κ       γ       γ           κ       γ       γ       γ       γ       γ   |   |
|          | N QVTLR]E]S]GPALV]KP]T]Q]T   | Fd   | 43  | 26  | 61   | 41.9%   | SIG P A LIVIKIPITIQITILITILITICITIFIS 25  | _ |
| 3%)      | <sup>26</sup> G F]S L S T A G M S V G W I R]Q P <sup>51</sup> A]D I]W]W]D]D]K K H Y]N P S]L K D <sup>76</sup> ]S]K]N]Q]V V L]K]V]T]N M]D P A D T <sup>101</sup> M I F]N]F]Y F]D V]W]G O G T T]V T  | PGIKIAIL<br> R L TIS<br>ATYYC  | EWL 50 20<br>[K]D]T 75 51<br>A R D 100 76<br>T K]G 125 00 101   | <del>В FISILISIT А В МІЗ V ВІМ.</del><br>А D I W W D D KÌK H YÌNÌP S<br>S K N Q V V L K VÌT N M DÌI<br>M I F N F Y F D V W GÌQ G I  | S L K D R L T I S K D T 75<br>P A D T A T Y Y C A R D 100<br>F T V T V S S A S T K G 125   | 51 A]D I]W]W]D]<br>76]S]K]N]Q]V V<br>101 M I F]N]F]Y  | A]G M]S V G[W I]R]Q]P P G[K A L]E]W L       50         D]K]K H Y]N]P S]L K D]R]L]T I S]K]D]T       75         L]K]V]T]N M]D]P A D T A T Y Y C A R D       100         F]D V]W]G]Q G[T T]V T]V S[S[A S T K]G       125   |   |
| 4        | 126 P S V]F P L]A P S S]K[S T]S[G G T<br>151 D Y]F P[E P V T V[S W N[S[G[A L T   | A A L G C<br>S G V H T   | L V K 150<br>F P A 175  | P S V FÌP L AÌP S S K S T S<br>D Y FÌPLELP V T V S W N S C  | S G G T A A L G C L V K <sup>150</sup><br>S ALL T S G V H T FLP A <sup>175</sup>   | 126 P S V F P L<br>151 D Y F F F F F  | A) P S S)KLS TLSLG G T A A L G C L VLK $150$<br>V T VLS W NLSLGALL TLSLG V H T FLP A $175$  |   |
| й        | <sup>⊥</sup> /º LV LLQLSLS GLLYLSLLSLSLV VLTLV P<br>201 LY I C N V N H K P S N T K V D K R<br>226 T H T C P P C P A P E L L G C % R  | S LS LS LL LG<br>LV LE P K LS<br>esidue Cleav  | c [D [K 225]       201         ages:       43 %       226   | Y I C N V N H[K[P[S]N T K N<br>T H T C[P P C[P A[P E L L C  | V DLK R V ELP K S C D K 225<br>% Residue Cleavages: 26 %   | 226 T H T CLPP  | LL       LL <td< td=""><td>5</td></td<> | 5 |



### Hydrogen Deuterium Exchange



### HDX-MS workflow





### Moving from MS only to MS/MS HDX experiment using ETD

Under normal operation condition, minimal scrambling is observed on the ETD Spectrum



•Minimal deuterium scrambling ETD measurement with different instruments and source conditions (No deuterium is retained on Histidine due to fast exchange with solvent)



### Moving from MS only to MS/MS HDX experiment using ETD



#### **Pinpoint the Protein Ligand Binding Site with ETD**

Deuterium labeled protein and protein+ligand biding sample's C and Z fragments mass differences vs. sequence position. From C3 to C14 |4mass differences were from 0.03 to 0.2. Start from 1 C17, the  $\Delta$  mass increased to around 1 and stay at the same level for the rest of the C fragments. C14 4 was identified as significant change area.



Significant change was observed around  $Z_8$ - $Z_{10}$  is consistent with the C fragments plot. Combine the two plot results, the potential binding site was predicted.



### Summary

• ETD is a must have type of fragmentation for in-depth characterization of biotherapeutic proteins.

 High sequence coverage for middledown experiments allows quick characterization assays.

 Orbitrap Fusion Lumos offers multiple types of fragmentation and ease of use to tackle the most challenging task.





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