Middle-down Approach Using Proton Transfer Charge Reduction Enables Unambiguous Drug-Payload Localization in Cys-linked Antibody Drug Conjugates

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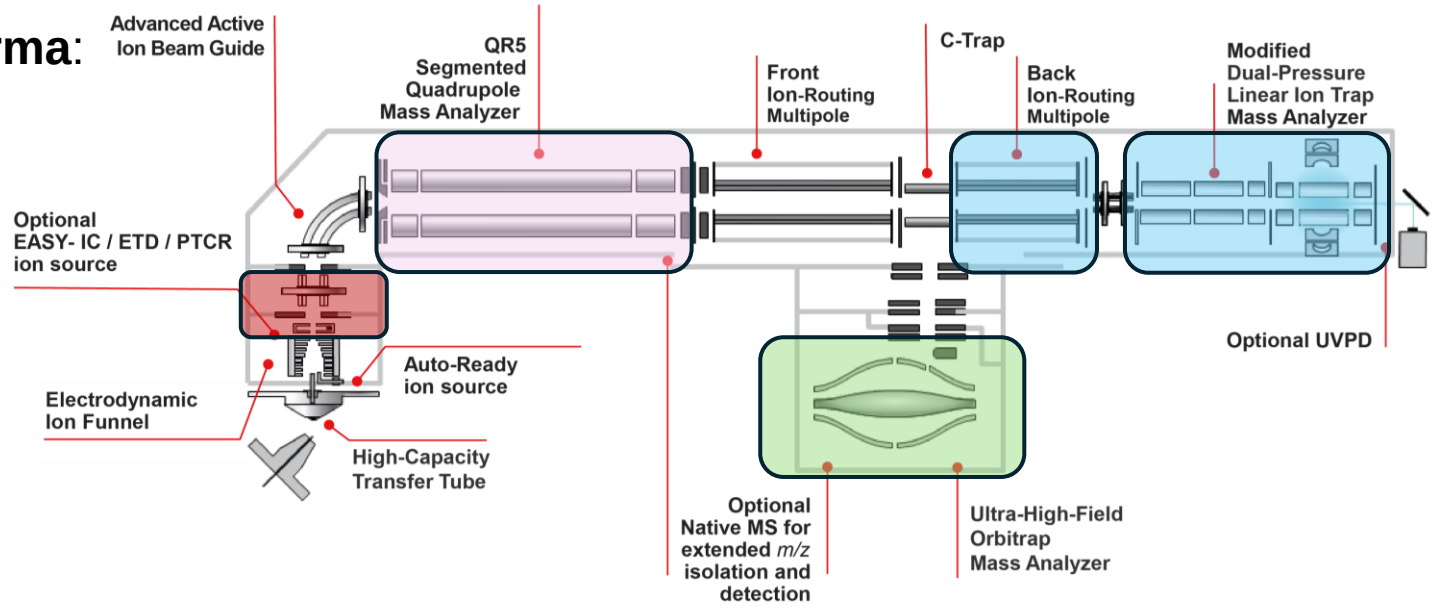
Outline

- Proton transfer charge reduction (PTCR) on Orbitrap Ascend tribrid
- Middle-down MS analysis of IgGs
- Middle-down MS analysis of antibody-drug conjugate
- Summary and future directions

Orbitrap Ascend Biopharma Edition

Relevant features of the **Orbitrap Ascend BioPharma**:



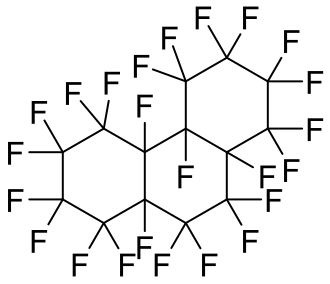
BioPharma Edition

Proton Transfer Reactions in the gas-phase

- Primary Ion/Ion Reactions for Analyte Cations

Proton Transfer

$(M + 5H)^{5+} + A^- \rightarrow (M + 4H)^{4+} + HA$



$C_{14}F_{24}$

m/z **623.9**

A^- : Perfluoroperhydrophenanthrene

Commercial implementation:
Proton Transfer Charge Reduction
(PTCR)

Ion/Ion Reactions in the Gas Phase: Proton Transfer Reactions Involving Multiply-Charged Proteins

James L. Stephenson, Jr., and Scott A. McLuckey*

Contribution from the Chemical and Analytical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6365

Received April 9, 1996[⊗]

Anal. Chem. **1996,** *68,* 4026−4032

Ion/Ion Proton Transfer Reactions for Protein Mixture Analysis

James L. Stephenson, Jr., and Scott A. McLuckey*

Chemical and Analytical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6365

ION/ION CHEMISTRY OF HIGH-MASS MULTIPLY CHARGED IONS

Scott A. McLuckey, James L. Stephenson, Jr.
Chemical and Analytical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6365

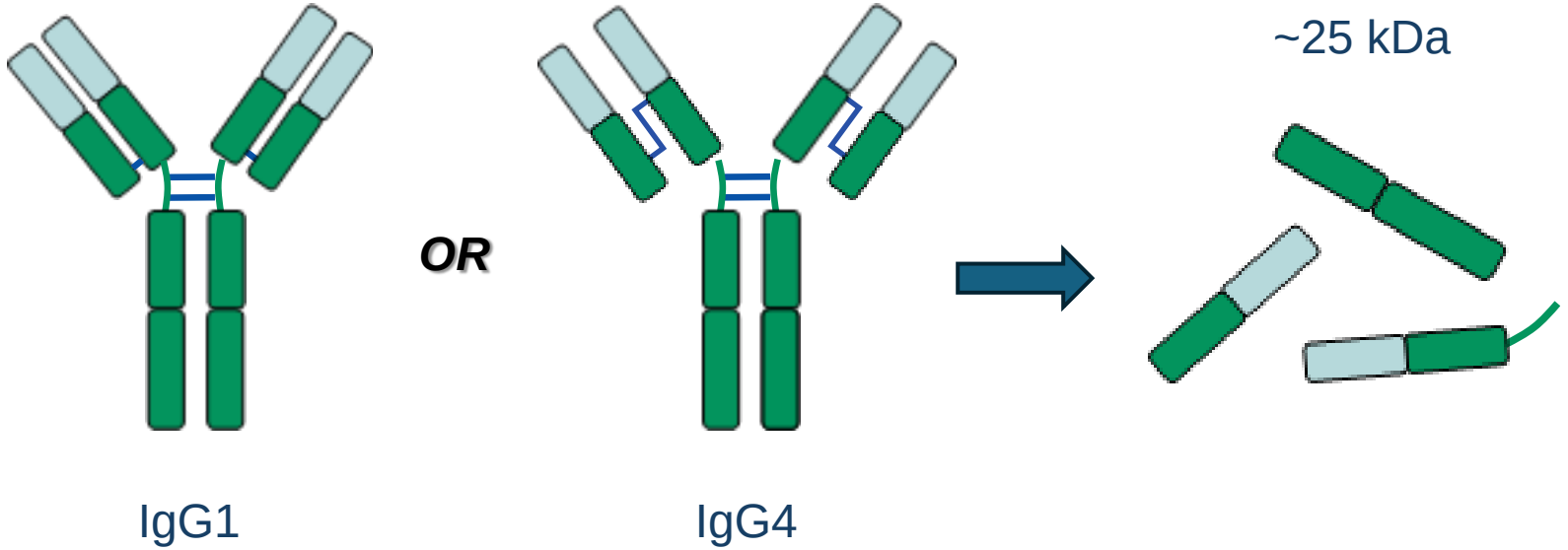
Received 1 October 1998; accepted 3 December 1998

Middle-down MS analysis of IgGs

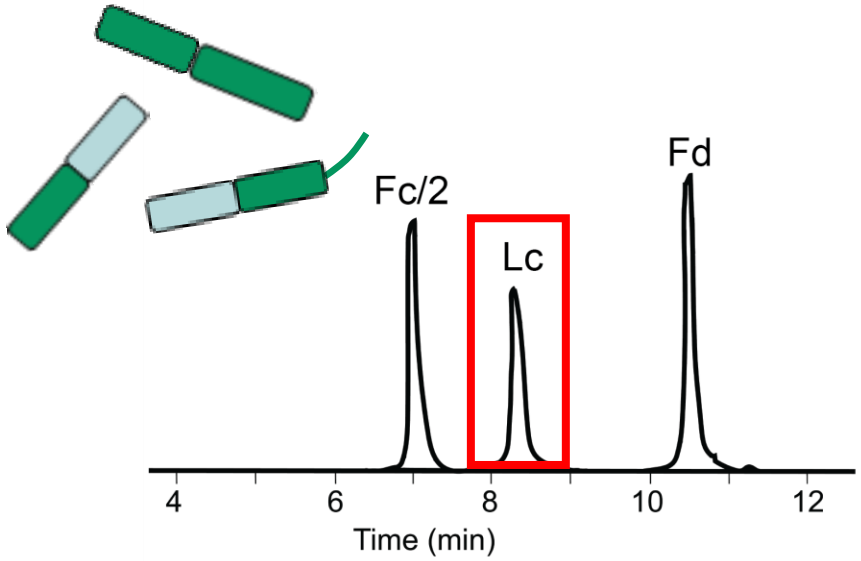
Middle-down MS for IgG characterization

Middle-down MS workflow:

- NIST (IgG1), SILu Lite (IgG1), SILu K4 (IgG4)
- IdeS digestion, reduction (TCEP) + denaturation (guanidinium HCl)
- RPLC separation + MS analysis
- Performed on an Orbitrap Eclipse Tribrid



PTCR in targeted IgG middle-down MS analysis

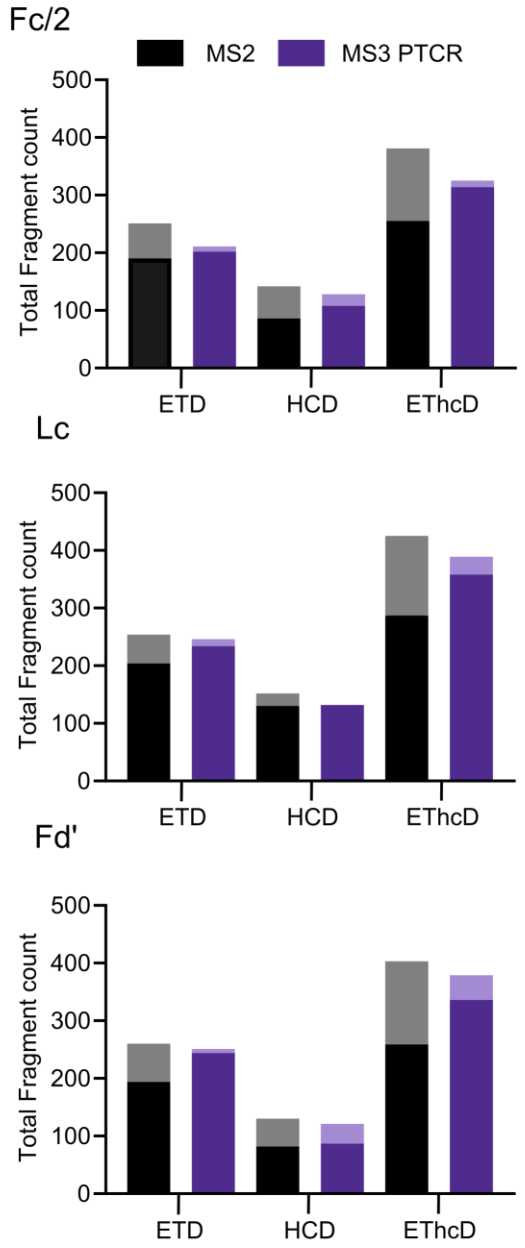


PTCR in targeted IgG middle-down MS analysis

PTCR improves sequencing metrics:

- Sequence coverage
- Number of unique fragments identified
- Number of complementary ion pairs
- Average size of matched fragments

Increased confidence in fragment assignment

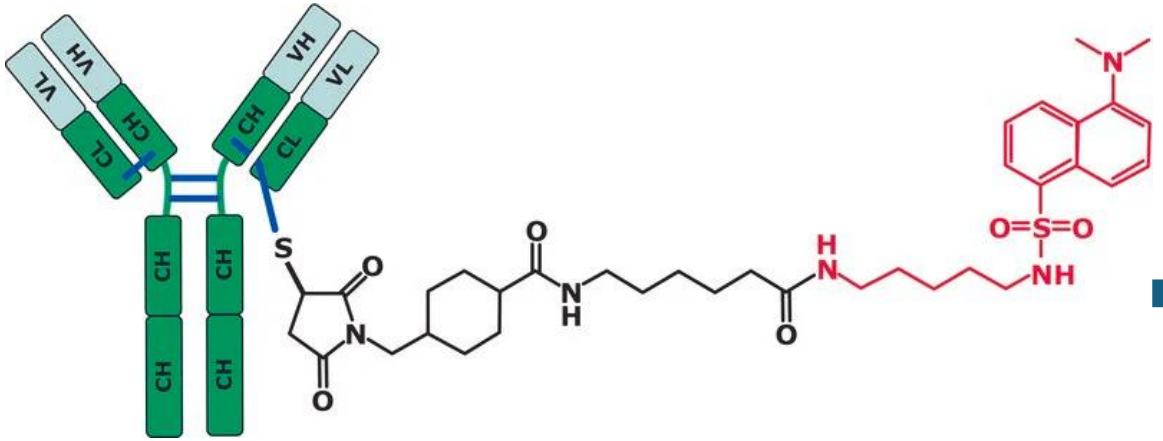


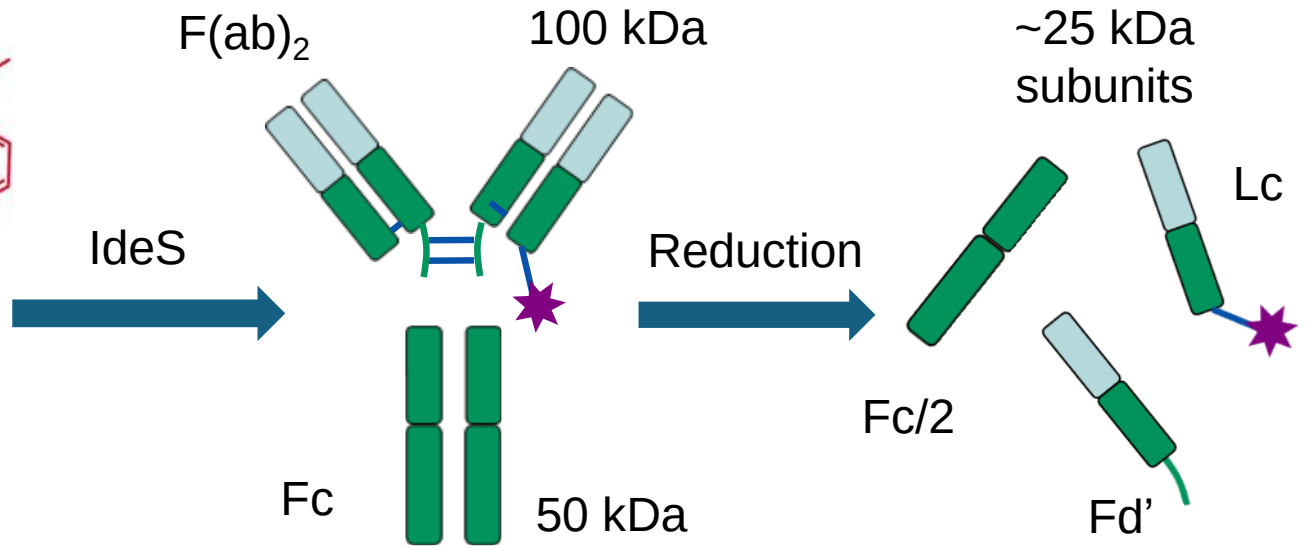

```
N  G P S V F  L F P  P K  P K D T L M I S R T P E V T C  25
26 V V V D V S H E D P E V K F N W Y V D G V E V H N  50
51 A K T K  P R E E Q Y N S T Y R V V S V L T V  L H Q  75
76 D W L N G K E Y K C K V S N K A L P A P I E K T I 100
101 S K A K G Q P R E P Q V Y T L P  P S R E E M T K N 125
126 Q 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 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
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
```

Combined Fc/2 – 95.7% sequence coverage!

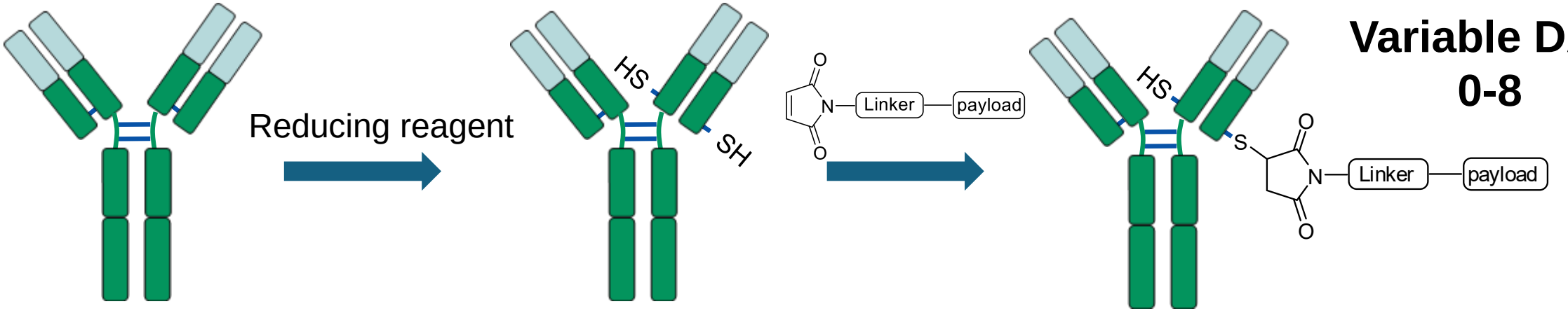
Middle-down MS analysis ADC subunits

Middle-down MS Workflow



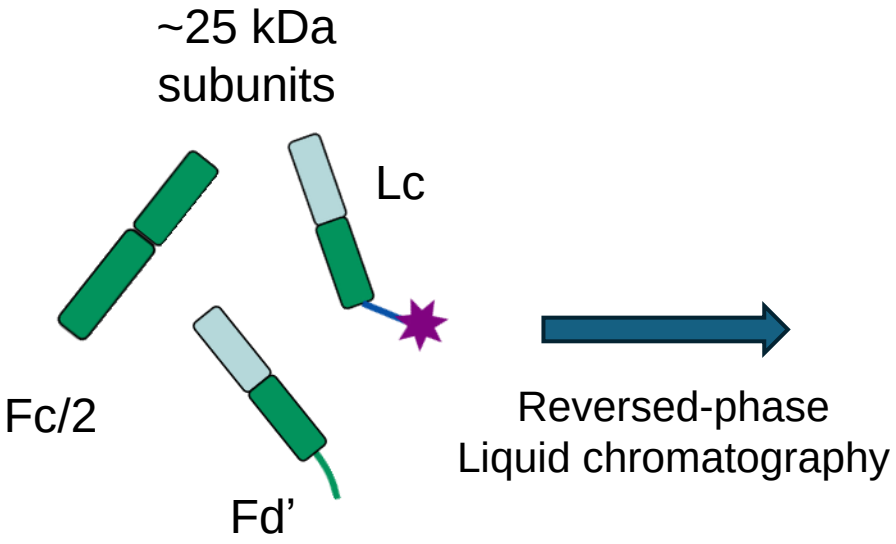


- SigmaMAb ADC mimic
- Cys-conjugation to dansyl-cadaverine-SMCC

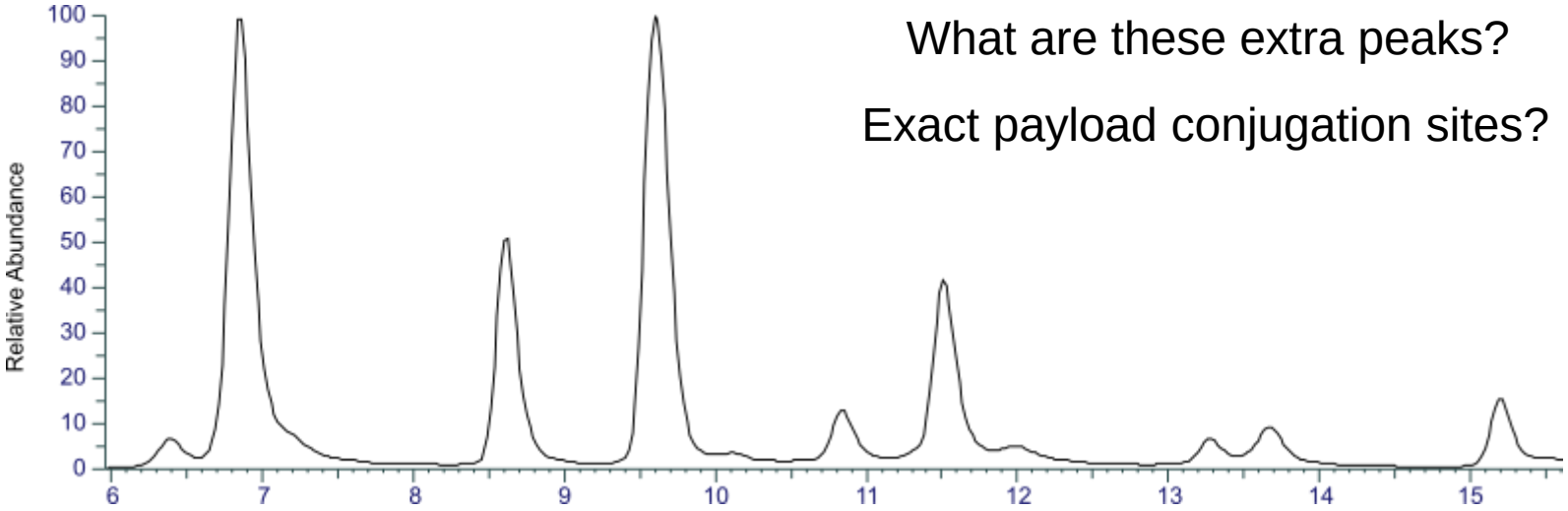


Variable DAR 0-8

Middle-down MS Workflow



Reversed-phase Liquid chromatography



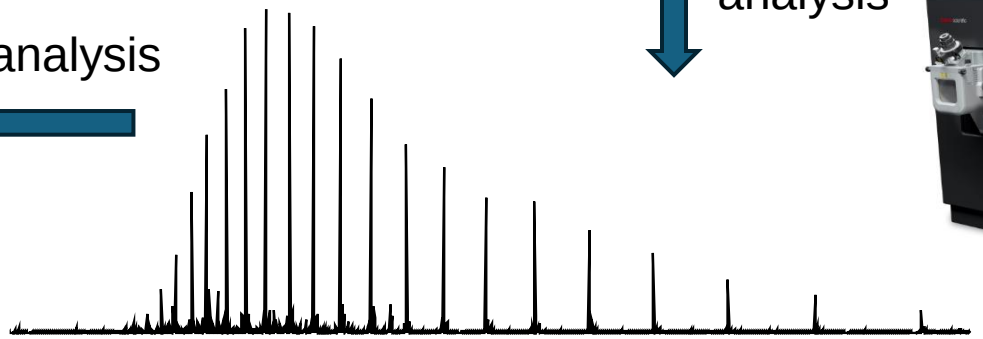
What are these extra peaks?

Exact payload conjugation sites?

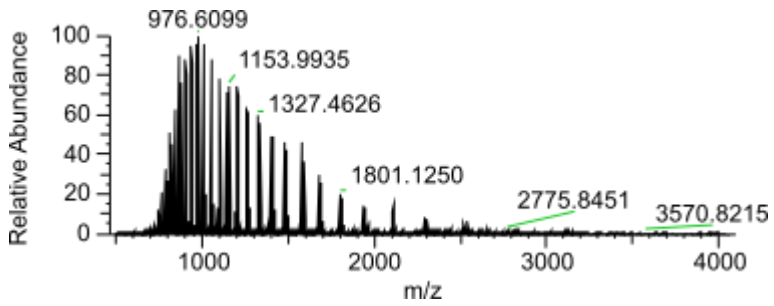
Manual data validation using TDValidator

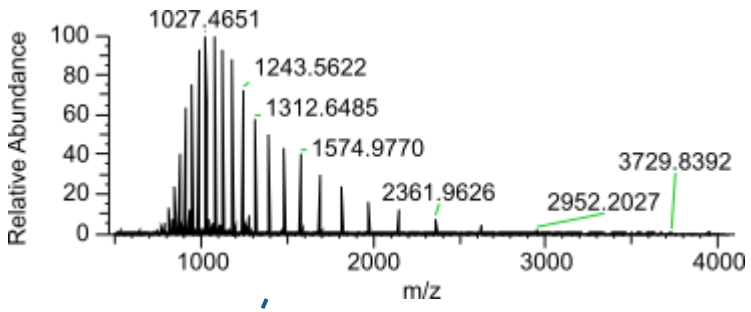
Mass spectrometry analysis

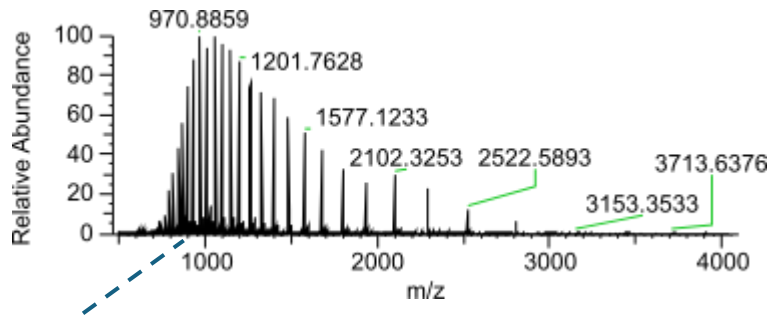
Data analysis



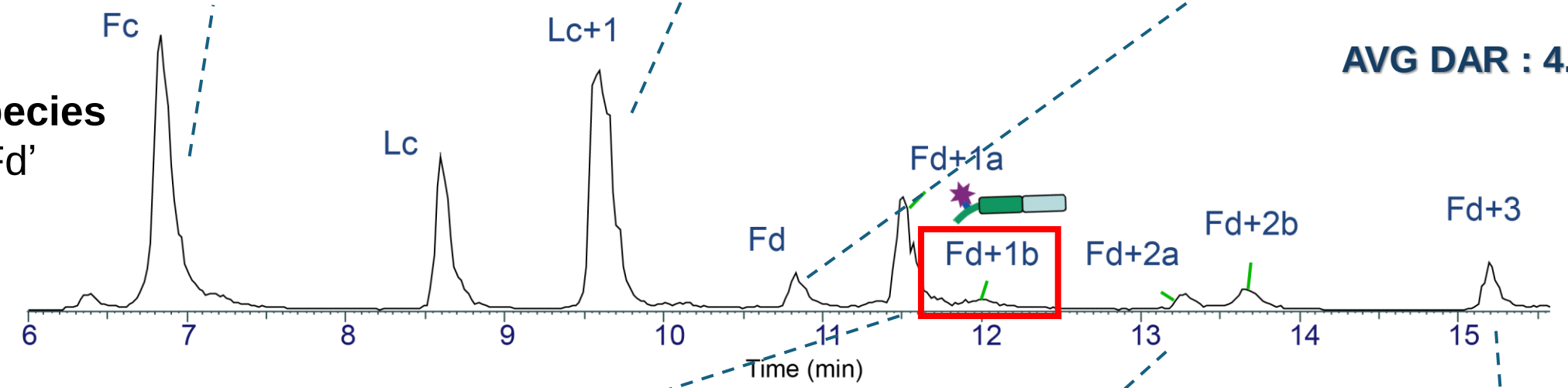
Middle-down MS: Payload localization



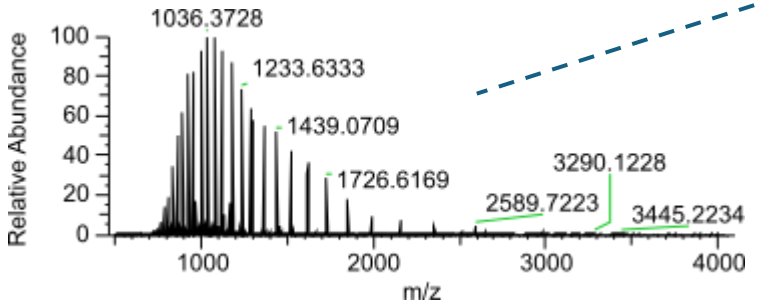


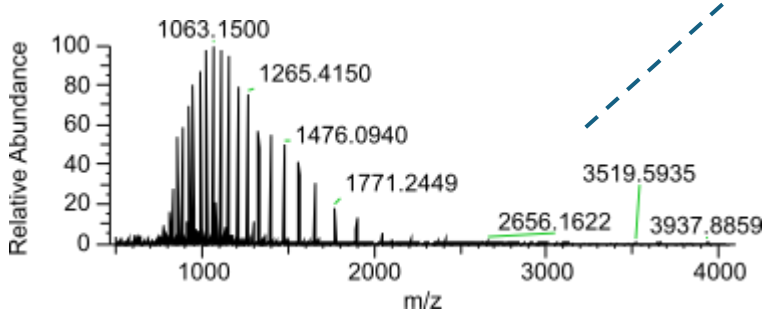


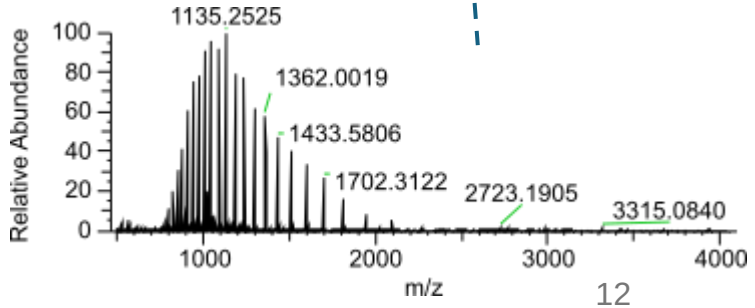
Isobaric species present at Fd' subunit



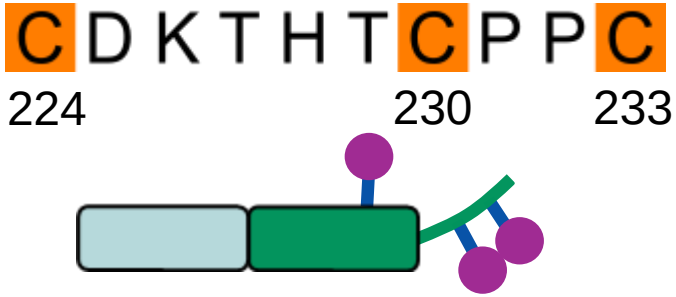
AVG DAR : 4.18



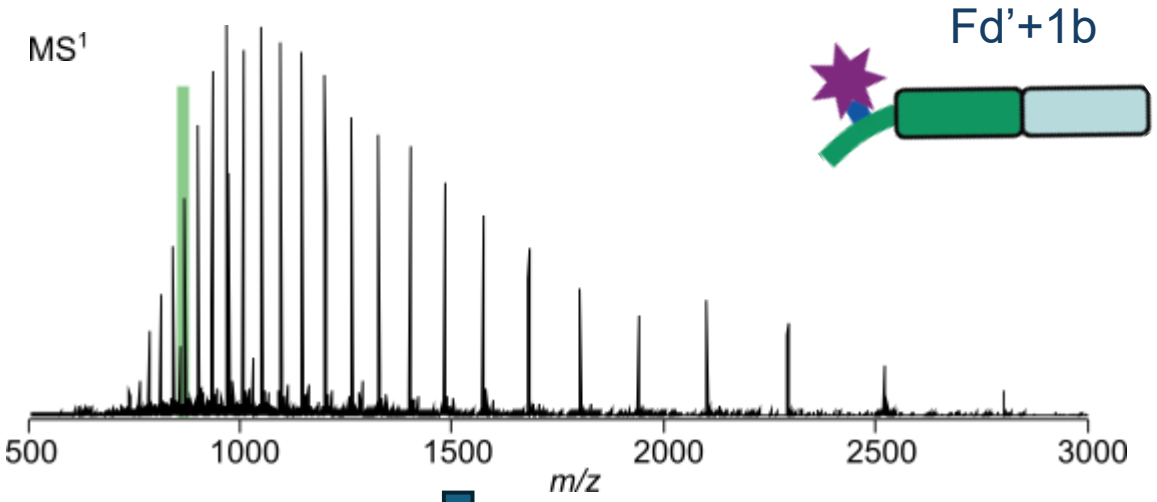




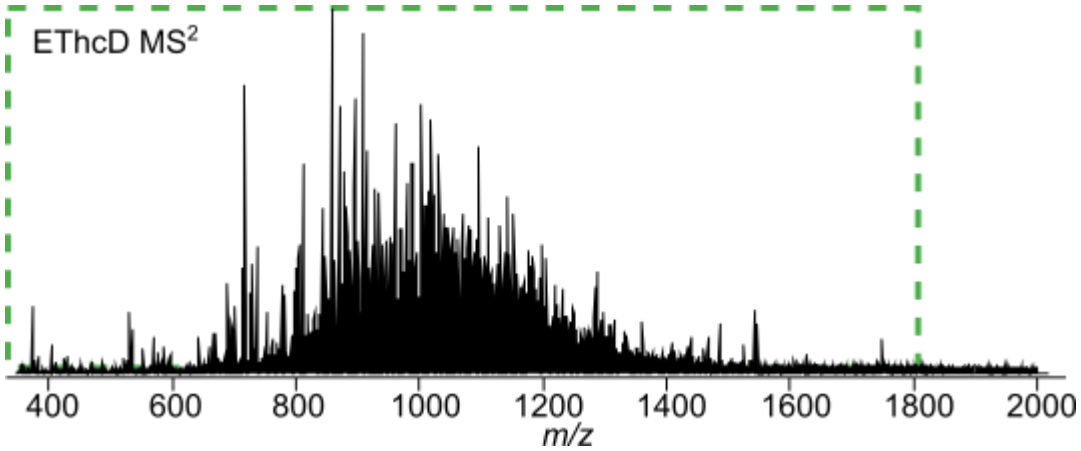
Middle-down MS: Payload localization



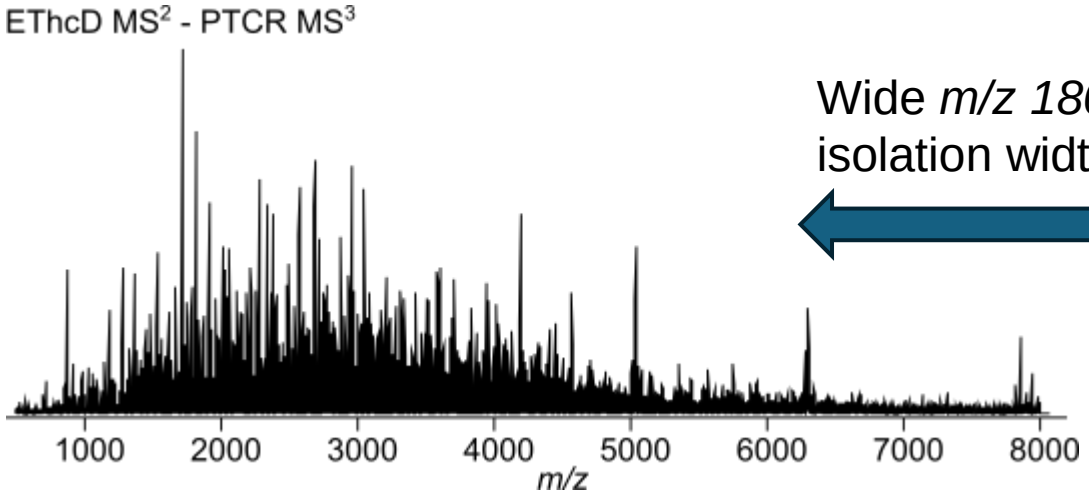
- 3 possible sites conjugation sites for Fd' subunit!
- Why is PTCR necessary?



Single precursor *m/z* 5 isolation width



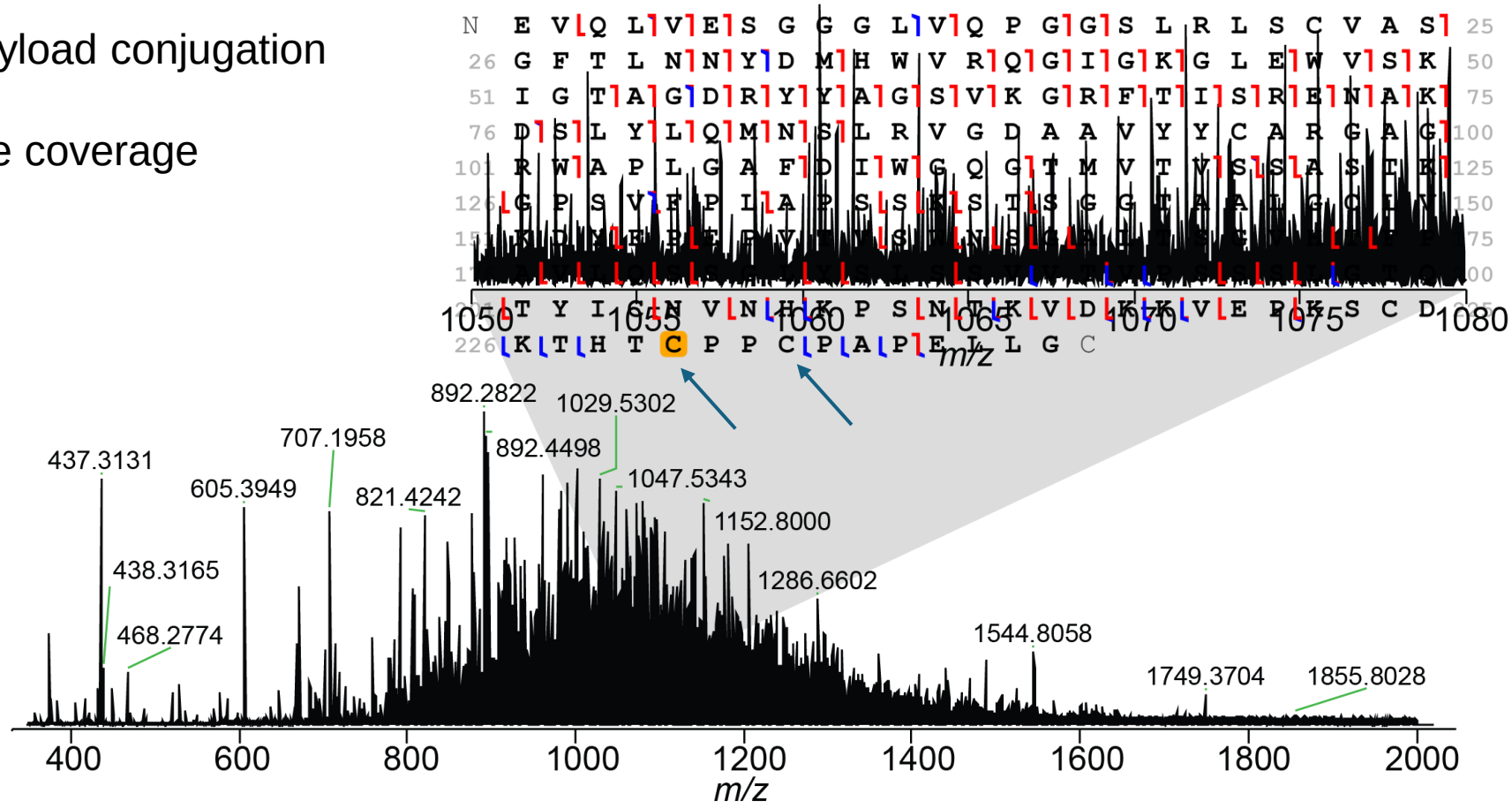
Wide *m/z 1800* isolation width



Middle-down MS² for ADC characterization: Fd+1b subunit

Traditional targeted MD MS²:

- Ambiguity in payload conjugation
- ~50% sequence coverage

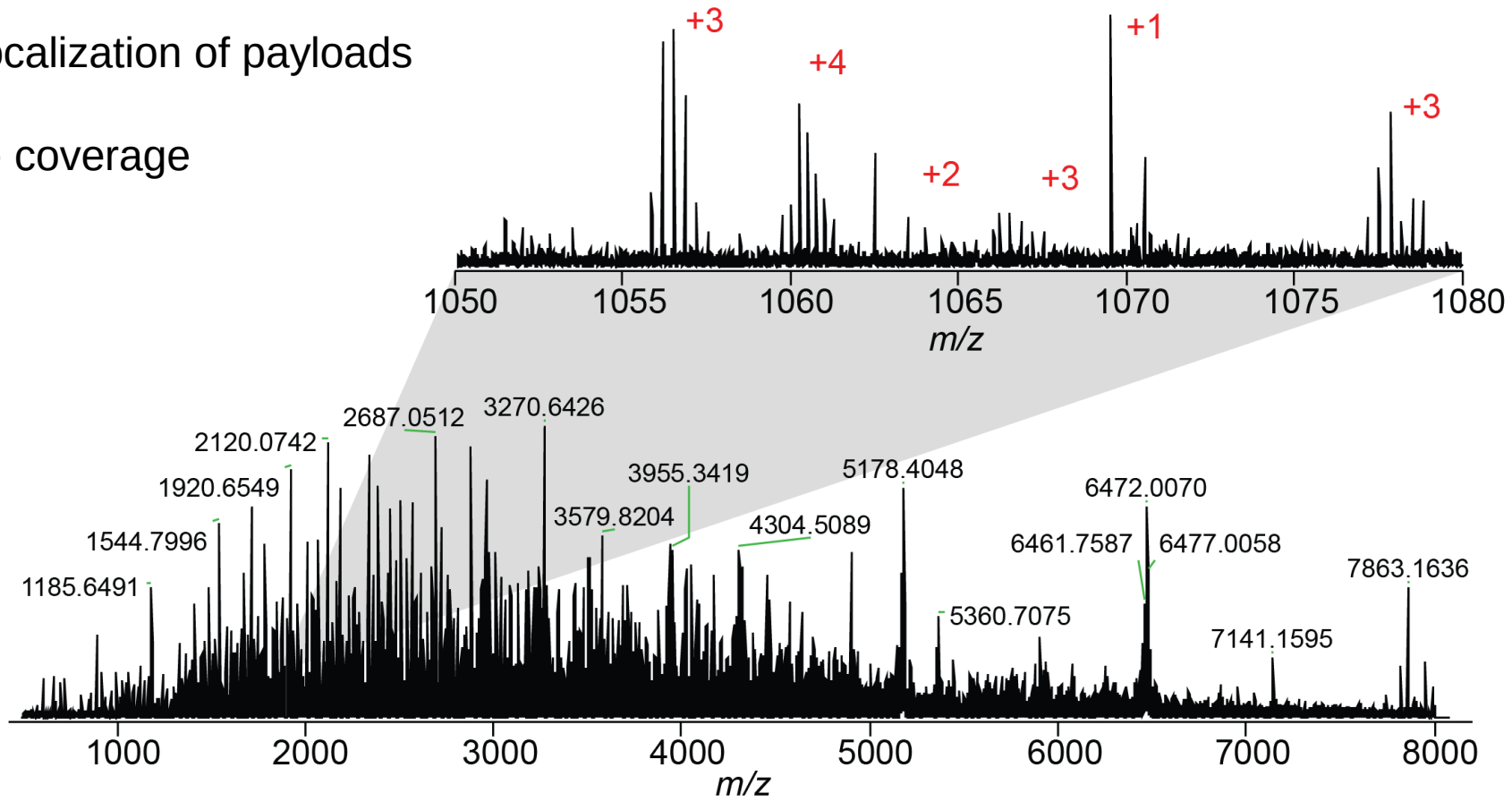


EThcD MS²

Middle-down MS^3 for ADC characterization: Fd+1b subunit

Targeted MD MS^2 – PTCR MS^3:

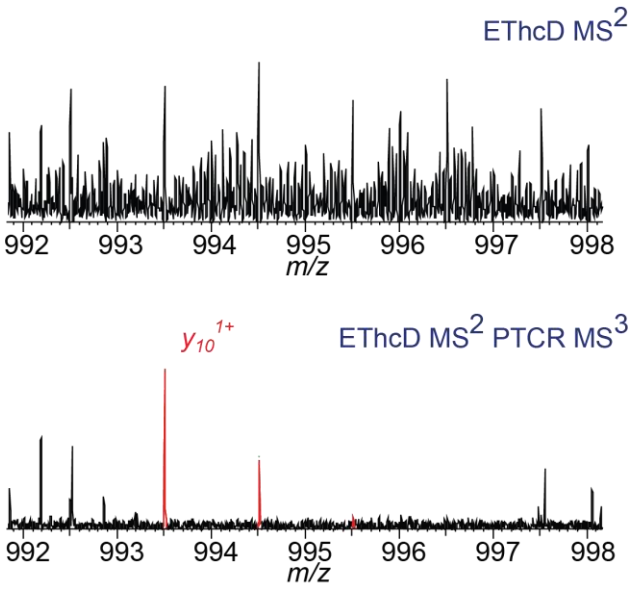
- Unambiguous localization of payloads
- >70% sequence coverage

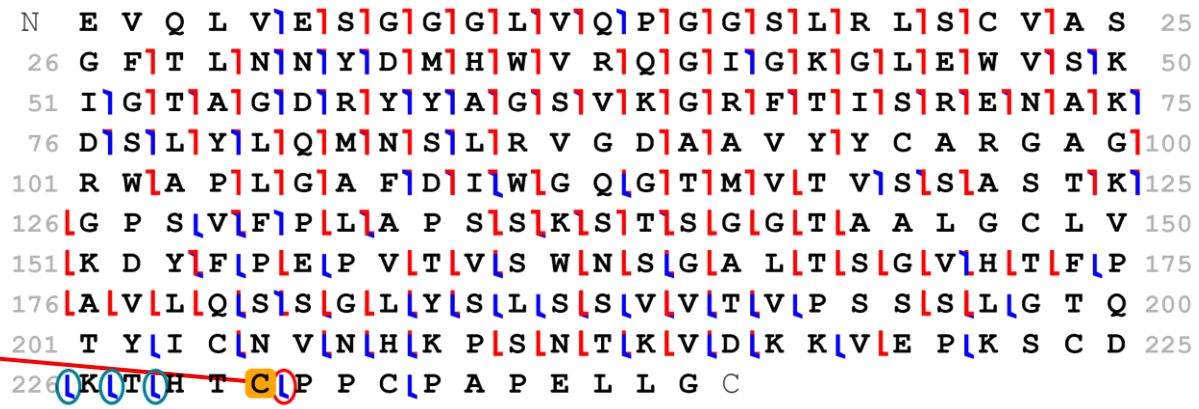


Lowly charged product ions

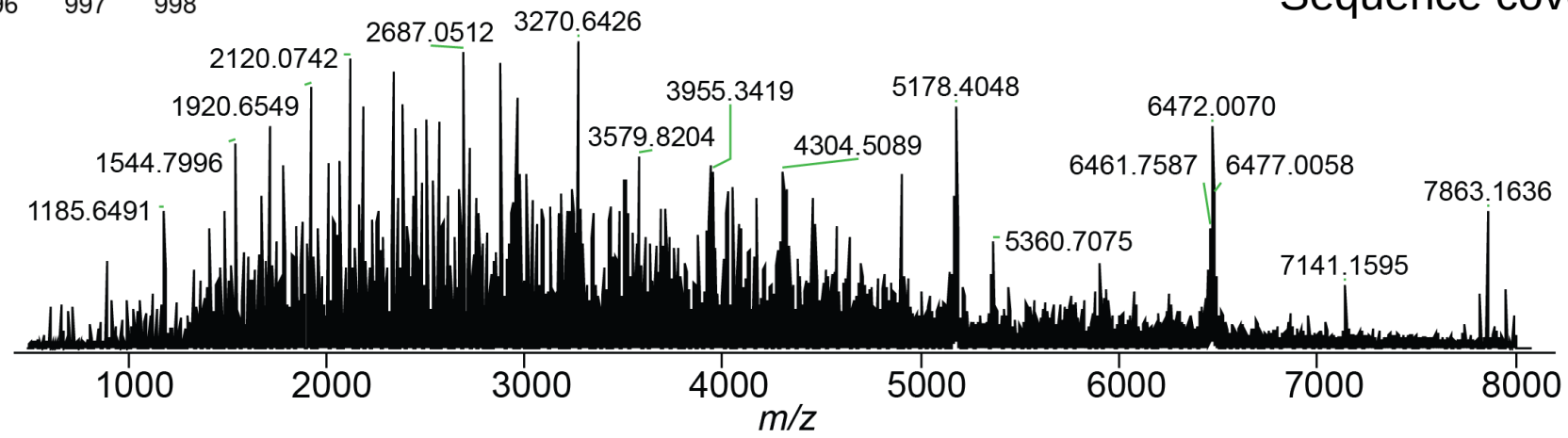
EThcD MS^2 – PTCR MS^3

Middle-down MS³ for ADC characterization: Fd+1b subunit





Sequence coverage: 70.7%



EThcD MS² – PTCR MS³

PTCR kinetics proportional to z^2

Multiple ion activations for enhanced sequencing

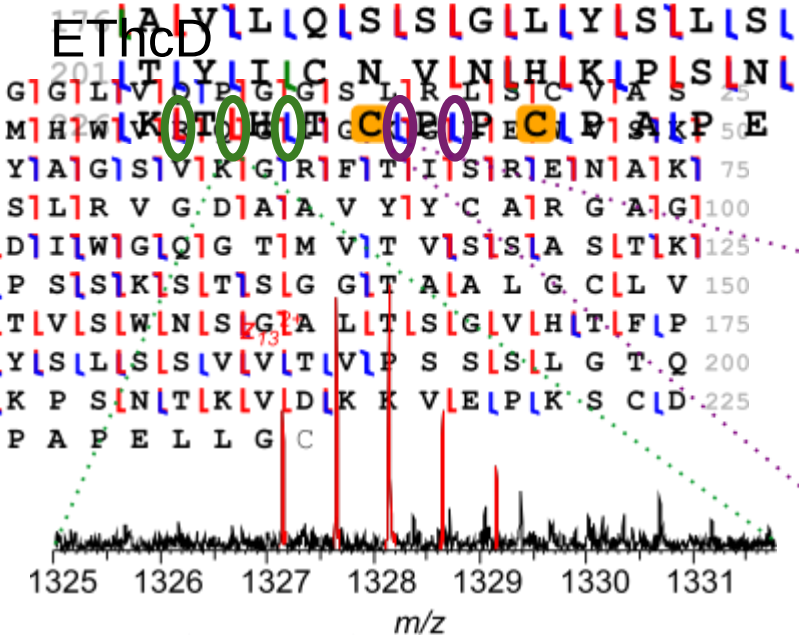
ETD

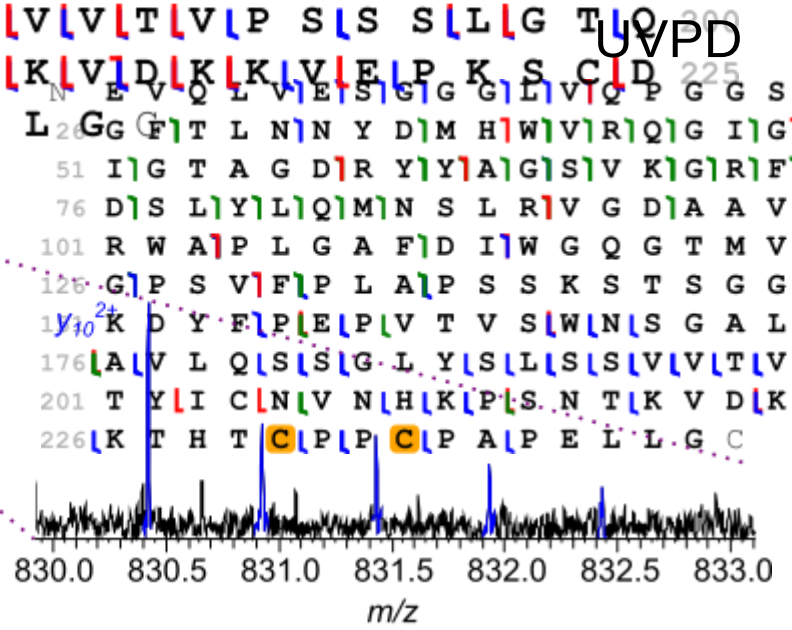
~85% sequence coverage!

HCD

EThcD

UVPD





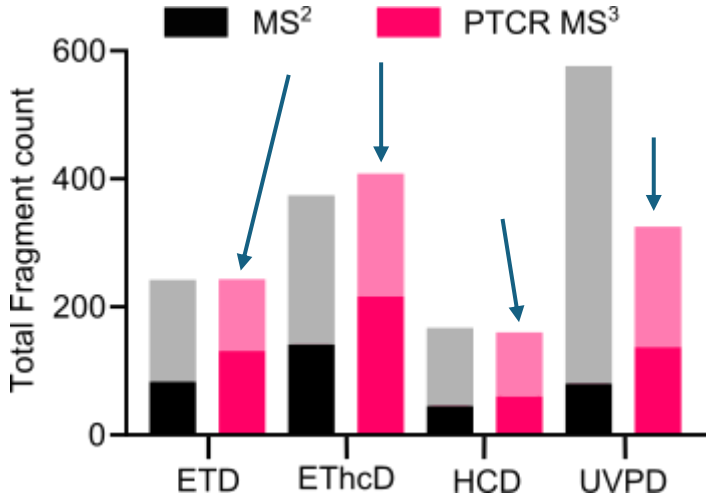
EThcD MS² – PTCR MS³

UVPD MS² – PTCR MS³

Summary: PTCR enables unambiguous localization

- Enhanced ADC characterization
  - Improved sequence coverage
  - Unambiguous payload localization

Subunit / Experiment		Fd + 1a		Fd + 1b		Fd + 2a		Fd + 2b	
		Sequence Coverage (%)	Localization	Sequence Coverage (%)	Localization	Sequence Coverage (%)	Localization	Sequence Coverage (%)	Localization
MS^2	HCD	23.9	C224 or C230	25.9	Ambiguous	25.9	Ambiguous	28	C230+C233
	ETD	40.6	C224	41.4	C230 or C233	41.4	C224+C230 or C224+C233	43.9	C230+C233
	UVPD	30.5	C224 or C230	29.3	C230 or C233	27.6	C224+C230 or C224+C233	30.1	C230+C233
	EThcD	50.6	C224	52.7	C230 or C233	53.6	C224+C230	55.6	C230+C233
PTCR - MS^3	HCD	27.6	Ambiguous	28	Ambiguous	30.5	Ambiguous	31.4	C230+C233
	ETD	60.3	C224	57.3	C230 or C233	56.9	C224+C230 or C224+C233	61.9	C230+C233
	UVPD	43.1	C224 or C230	45.2	C230 or C233	40.6	C224+C230 or C224+C233	41.1	C230+C233
	EThcD	71.1	C224	70.7	C230	72	C224+C233	71.1	C230+C233
	Combined	87.9		79.5		86.6		85.4	



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ThermoFisher SCIENTIFIC

PROTEINCEOUS

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Thank you!