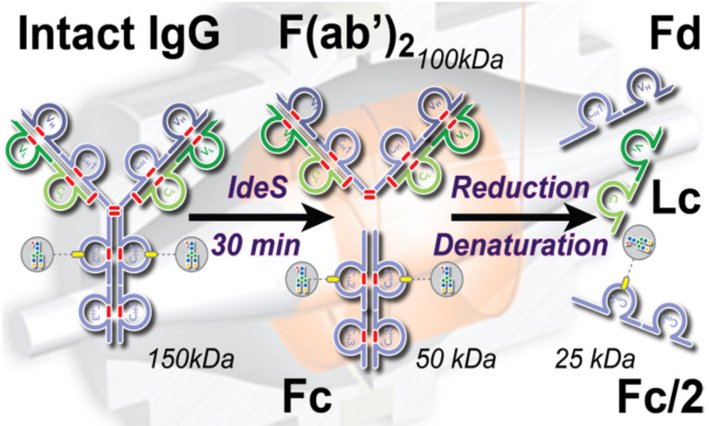
Mass Spectrometry Strategies for Rapid and Artifact-Free Antibody Structure Analysis

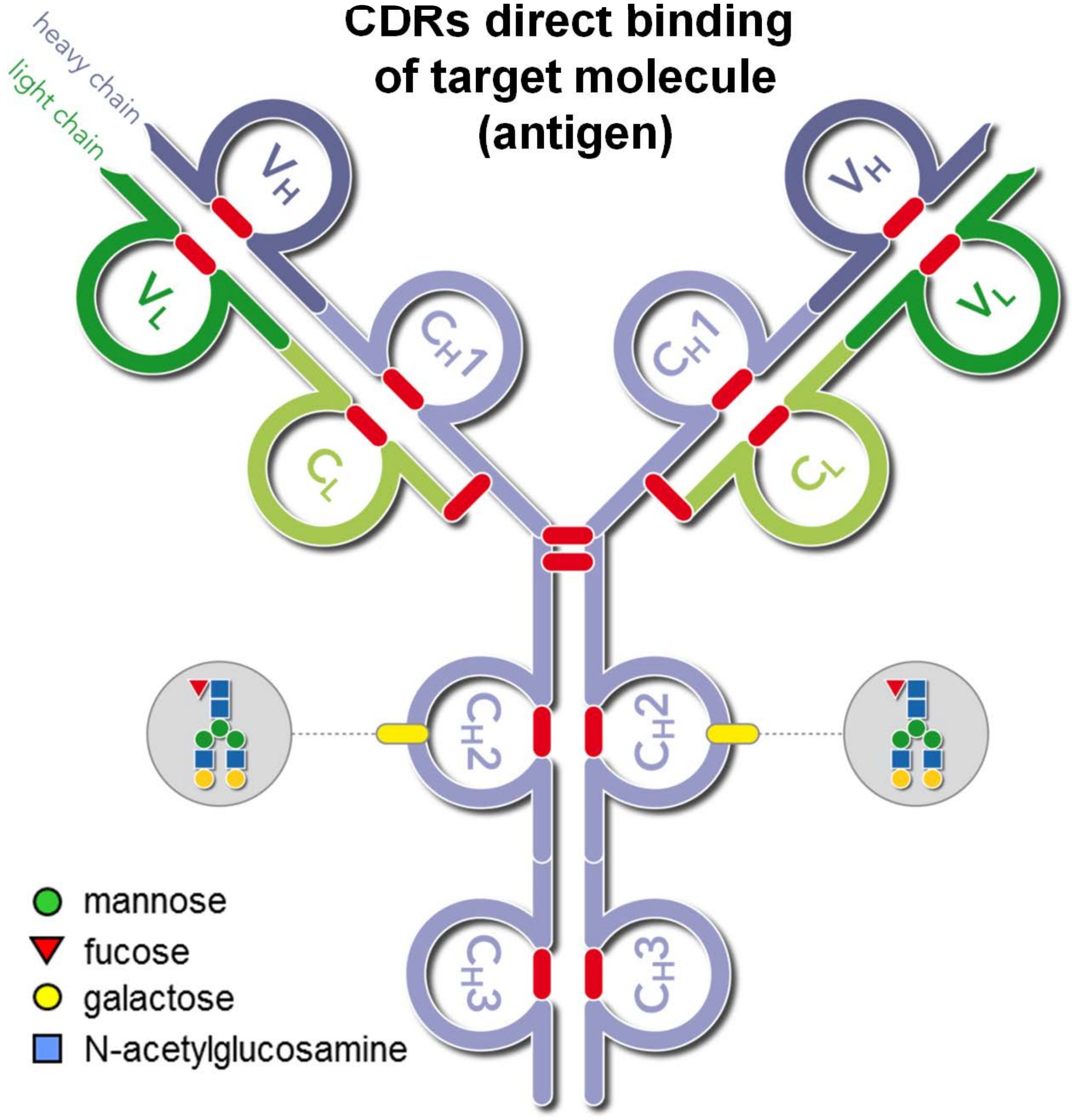
Yury O. Tsybin

Ecole Polytechnique Fédérale de Lausanne, Switzerland



20 June 2014, Baltimore, MD

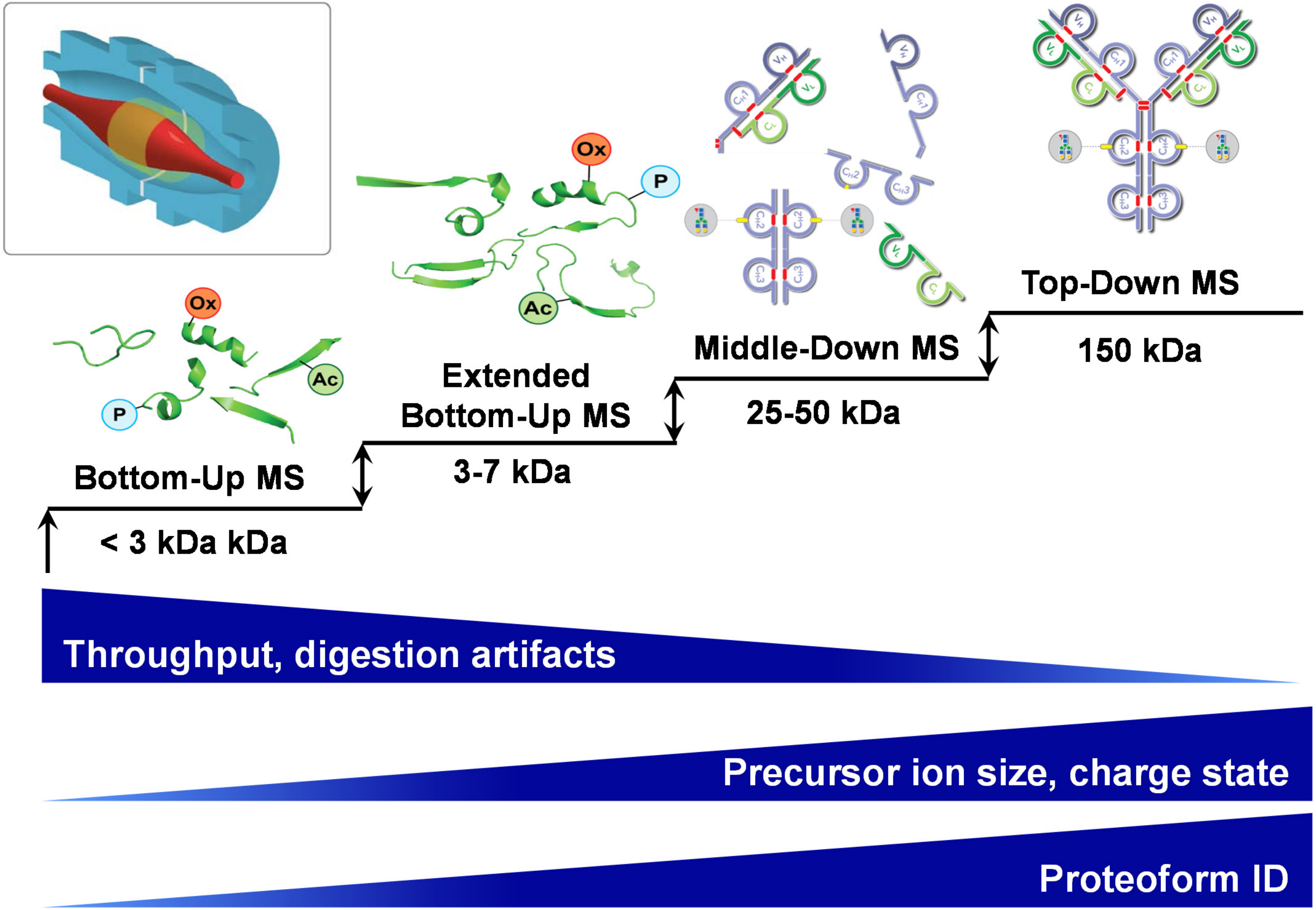
Antibodies Structural Questions



Schematics of immunoglobulin G1, IgG1
CDRs: Complementarity Determining Regions

- Sequence verification, **fast & artifact-free**
- **Modifications**: oxidation, deamidation, glycosylation...
- CDRs **connectivity**
- Light&heavy chains **pairing**
- **Mixtures**: LC-MS/MS
- *de novo* sequencing
- Disulfide bond scrambling
- Ligand, drug binding (ADC)
- Aggregation
- High order structure

MS/MS-Based Approaches For IgG Analysis



Bottom-Up MS

< 3 kDa kDa

Extended Bottom-Up MS

3-7 kDa

Middle-Down MS

25-50 kDa

Top-Down MS

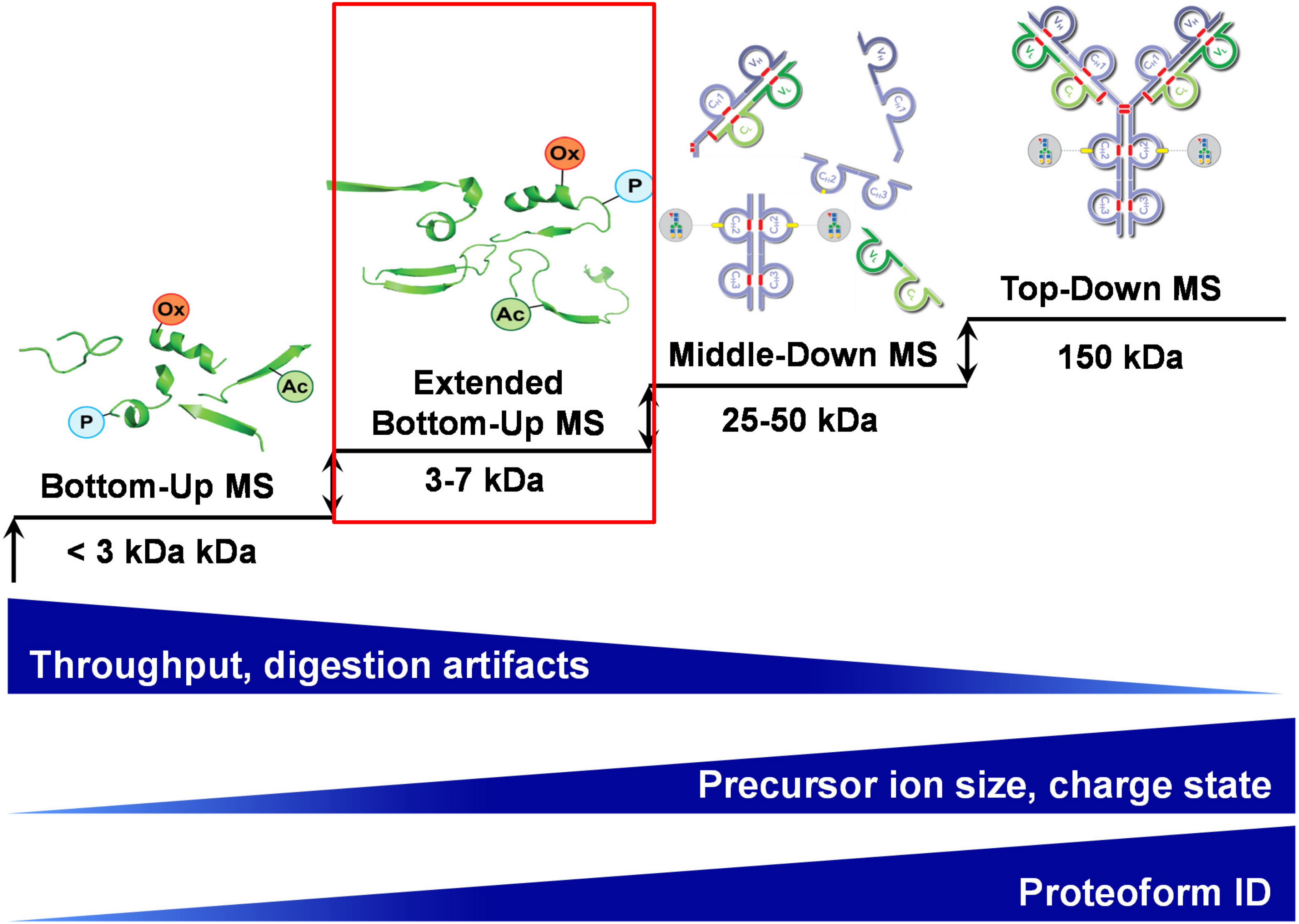
150 kDa

Throughput, digestion artifacts

Precursor ion size, charge state

Proteoform ID

MS/MS-Based Approaches For IgG Analysis



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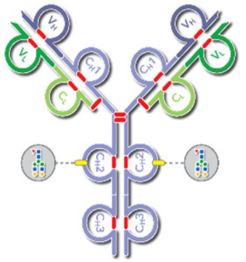
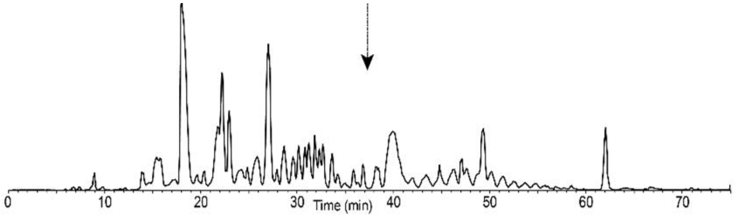
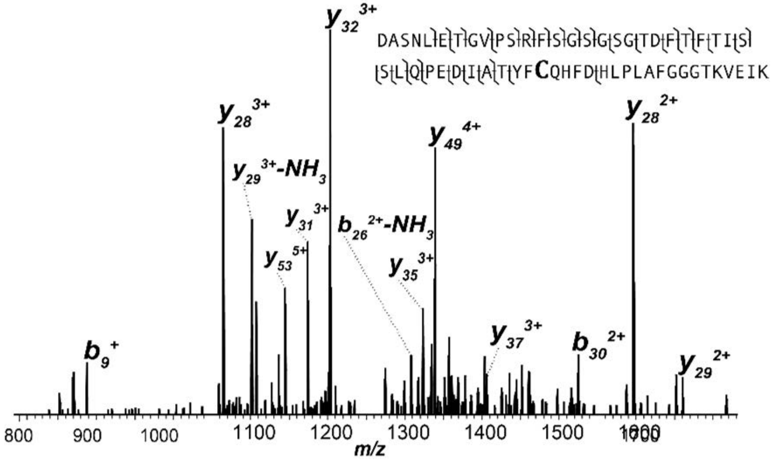
Extended bottom-up MS for IgG characterization



Sap 9 digestion
pH 5.5

3 – 7 kDa
EXTENDED Bottom-Up





Sap9 → aspartic protease from *Candida albicans,* active in a broad pH range, with cleavage preference for dibasic sites (e. g., RR, KK) or proximal dibasic sites in slightly acidic conditions.

IgG deamidation:

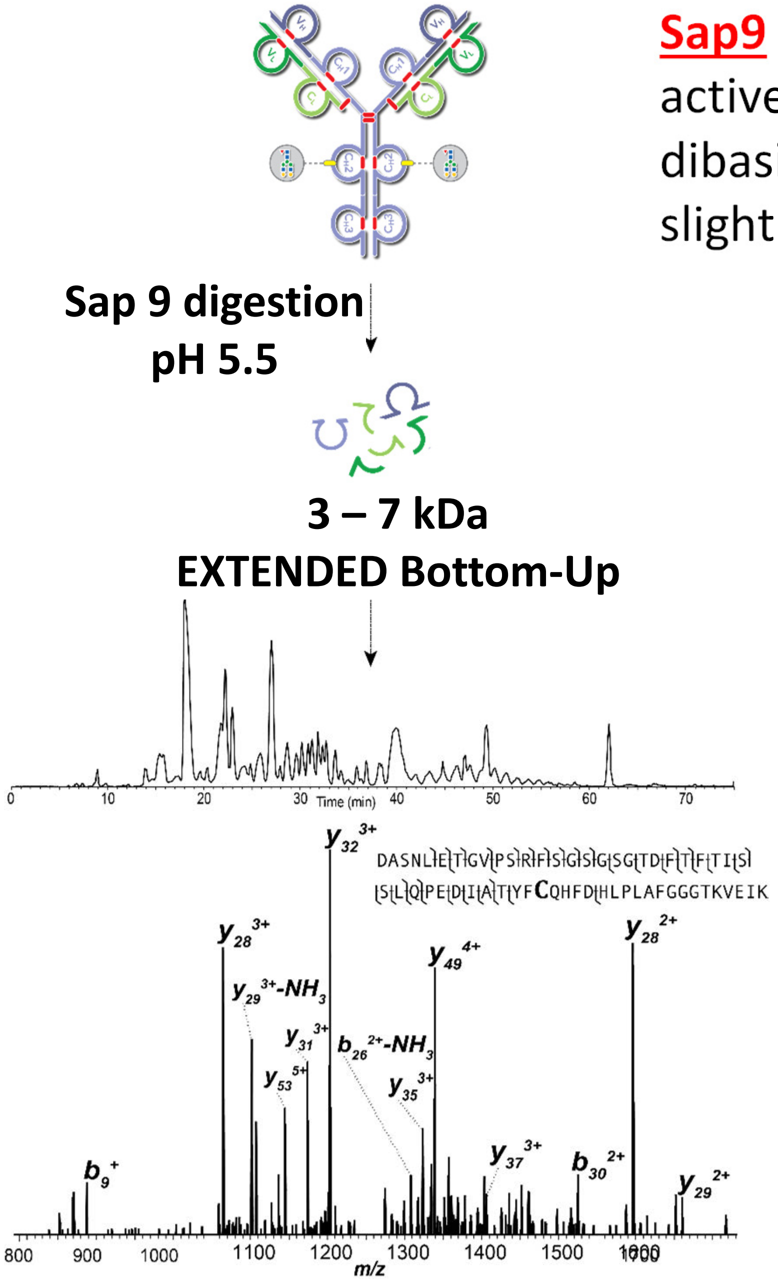
- Major quality attribute
- Alters antigen binding when occurs in CDRs
- Classic bottom-up: Basic digestion pH → artifactual deamidation
- Inefficient enzyme digestion at lower pH

Sap9 digestion:

- Fast: 1 hour
- Slightly acidic pH: 5.5 – 6.0: No artifactual deamidation

Extended bottom-up MS for IgG characterization

Sap9 → aspartic protease from *Candida albicans,* active in a broad pH range, with cleavage preference for dibasic sites (e. g., RR, KK) or proximal dibasic sites in slightly acidic conditions.

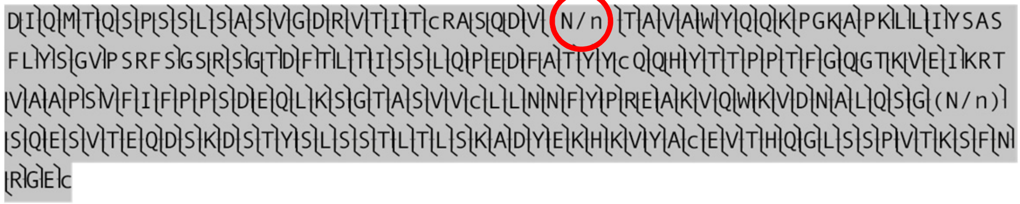


Sap 9 digestion
pH 5.5

3 – 7 kDa
EXTENDED Bottom-Up

Trastuzumab

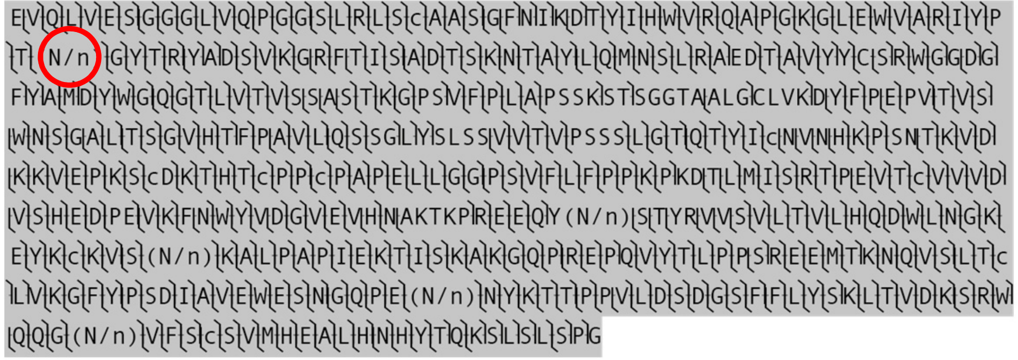
Light chain: Sequence coverage: **100 %**



DIQMTQSPSSLSASVGDRVTITcRASQDV(N/n)TAVAWYQQKPGKAPKLLIYSAS
FLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYcQQHYTTPPTFGQGTKVEIKRT
VAAPSVFIFPPSDEQLKSGTASVVcLLNNFYPREAKVQWKVDNALQSG(N/n)S
QESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYAcEVTHQGLSSPVTKSFN
RGEc

(Average: 3.5 kDa, 4.0+)

Heavy chain: Sequence coverage: **100 %**



EVQLVESGGGLVQPGGSLRLScAASGFNIKDTYIHWVRQAPGKGLEWVARIYP
T(N/n)GYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYcSRWGGDG
FYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGcLVKDYFPEPVTVS
WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIcNVNHKPSNTKVD
KKVEPKScDKTHTcPPcPAPELLGGPSVFLFPPKPKDTLMISRTPEVTcVVVD
VSHEDPEVKFNWYVDGVEVHNAKTKPREEQY(N/n)STYRVVSVLTVLHQDWLNGK
EYKcKVS(N/n)KALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTc
LVKGFYPSDIAVEWESNGQPE(N/n)NYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQG(N/n)VFScSVMHEALHNHYTQKSLSLSPG

(Average: 3.3 kDa, 4.2+)

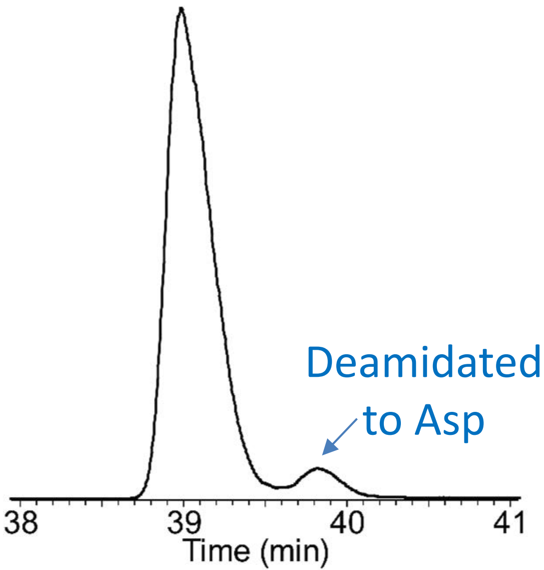
○ CDR deamidation hotspot

Harris et al., J Chrom B, 2001, 233-245

Deamidation assesment by **e**-bottom-up with Sap9

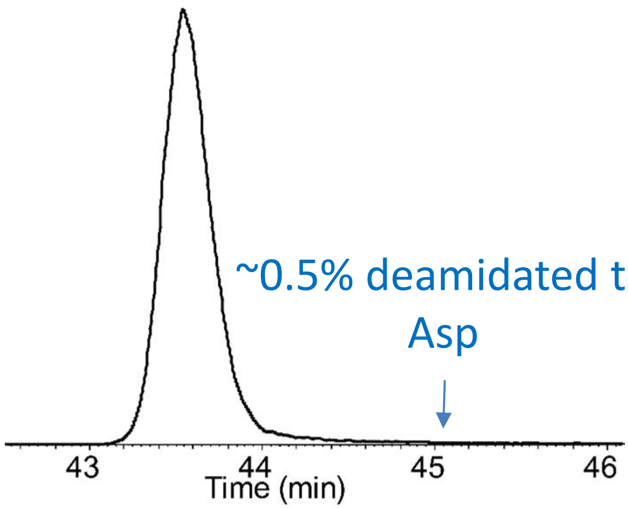
Trastuzumab

Sap 9 Lc peptide containing N_{30}



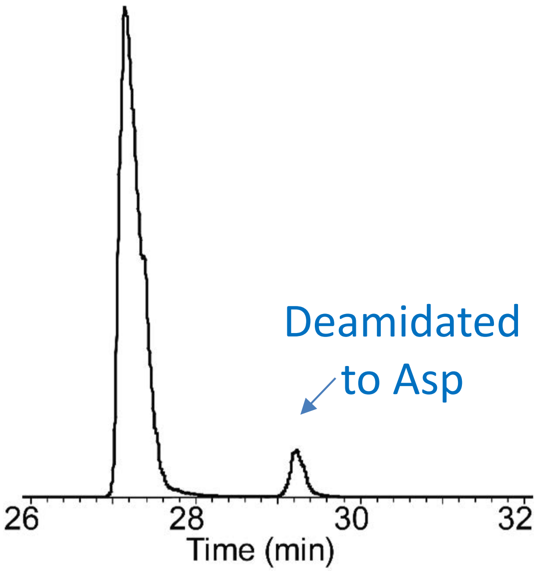
Deamidated to Asp

Sap 9 Hc peptide containing N_{55}



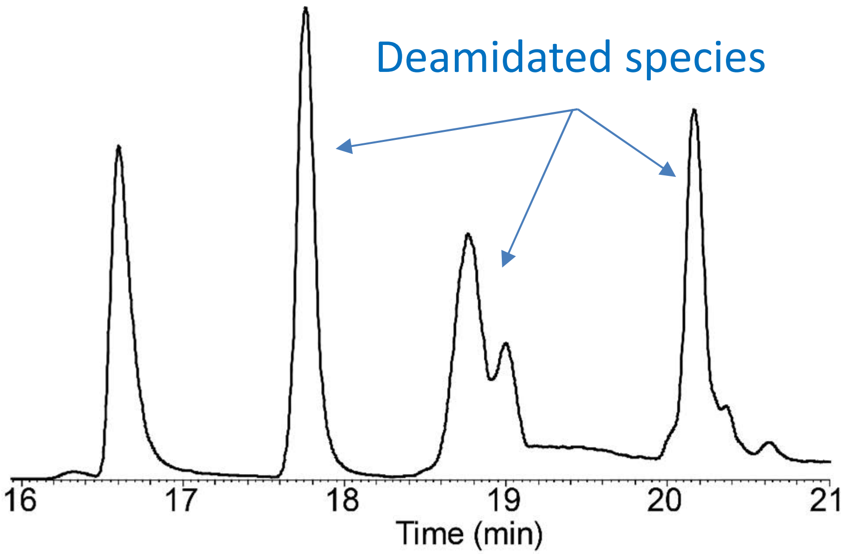
~0.5% deamidated to Asp

Tryptic Lc peptide containing N_{30}



Deamidated to Asp

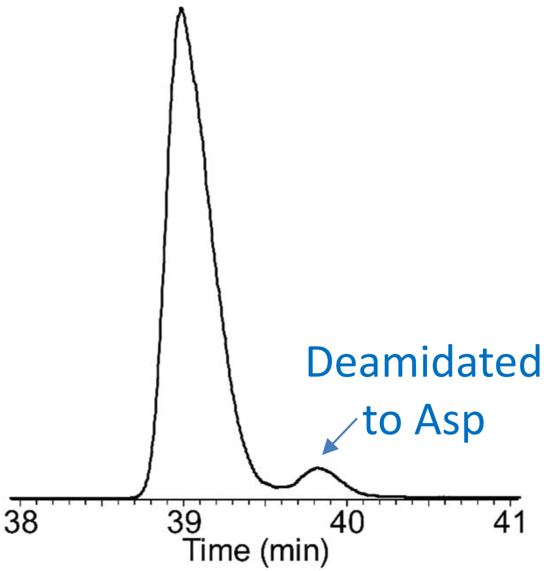
Tryptic Hc peptide containing N_{55}



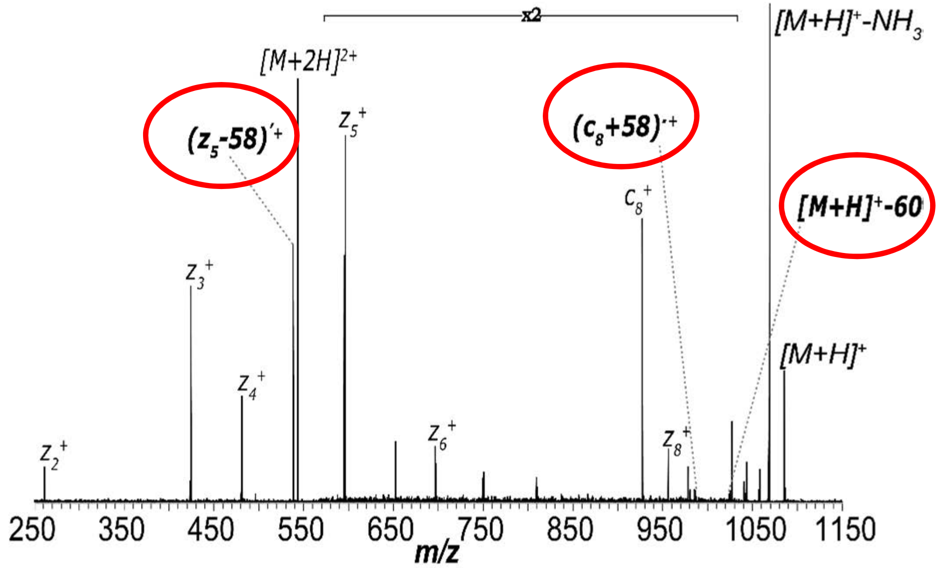
Deamidated species

Deamidation assesment by **e**-bottom-up with Sap9

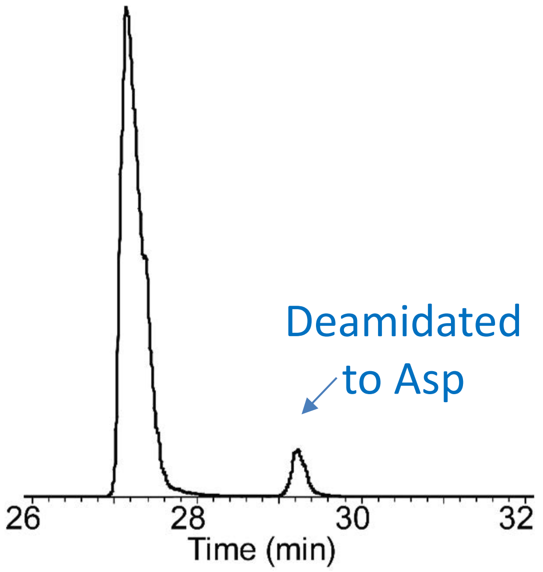
Sap 9 Lc peptide containing N_{30}



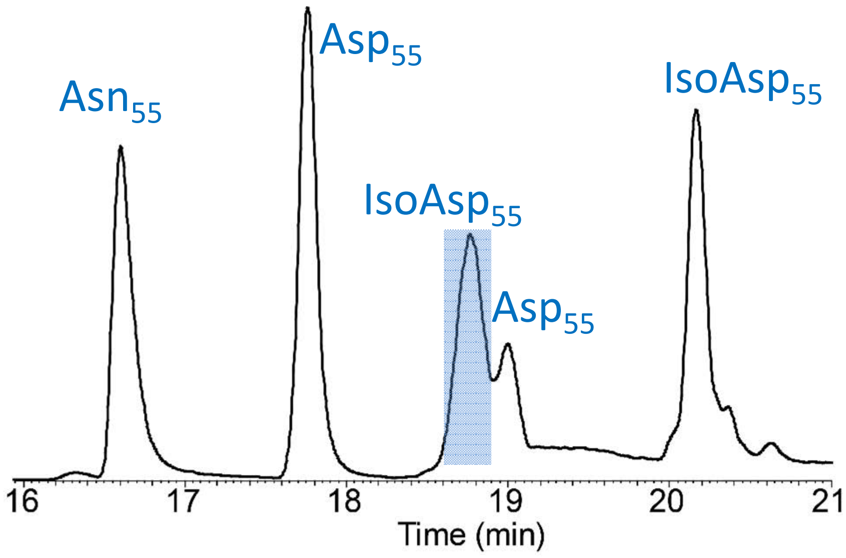
ETD spectrum with IsoAsp_{55} diagnostic ions



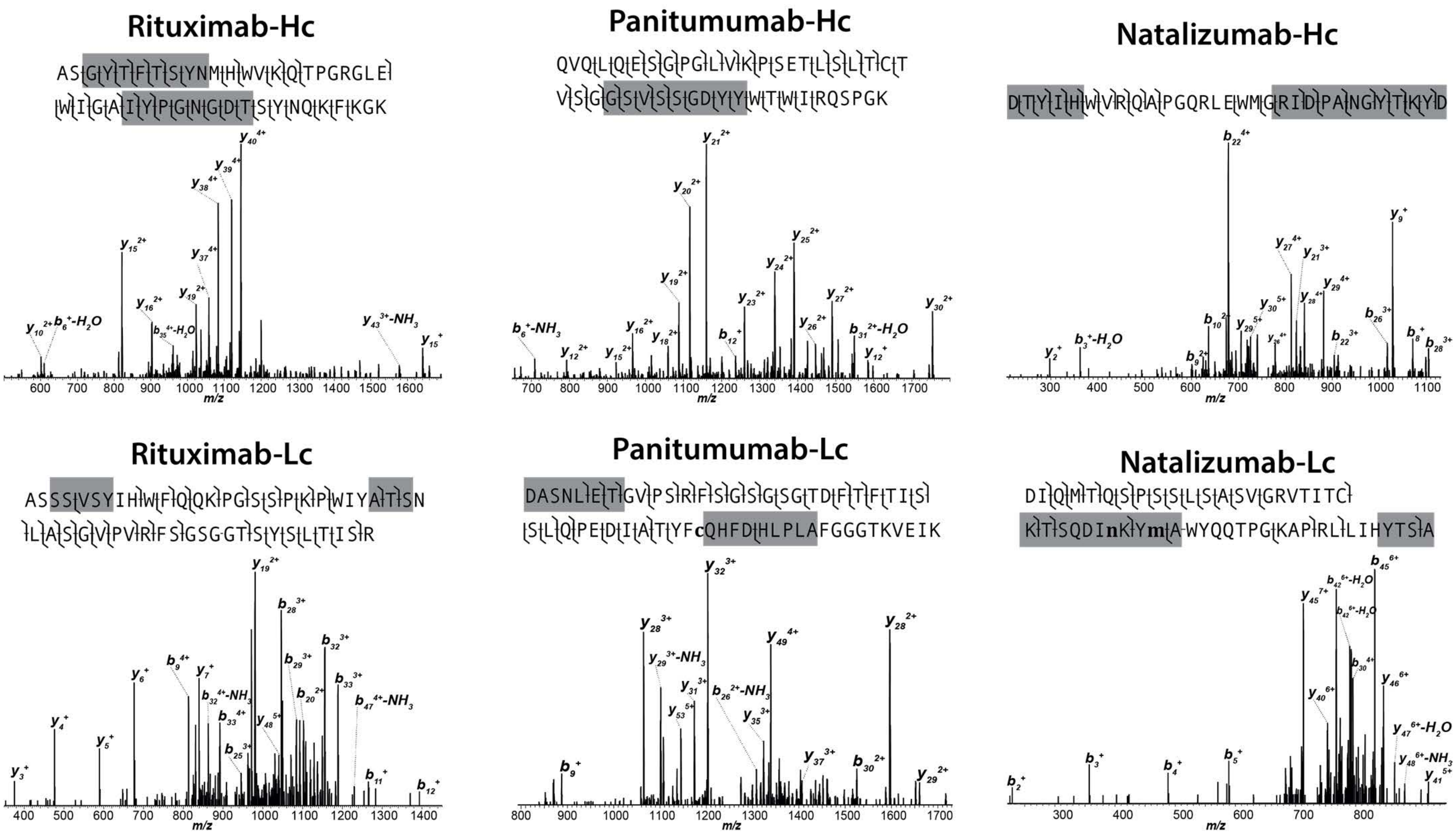
Tryptic Lc peptide containing N_{30}



Tryptic Hc peptide containing N_{55}

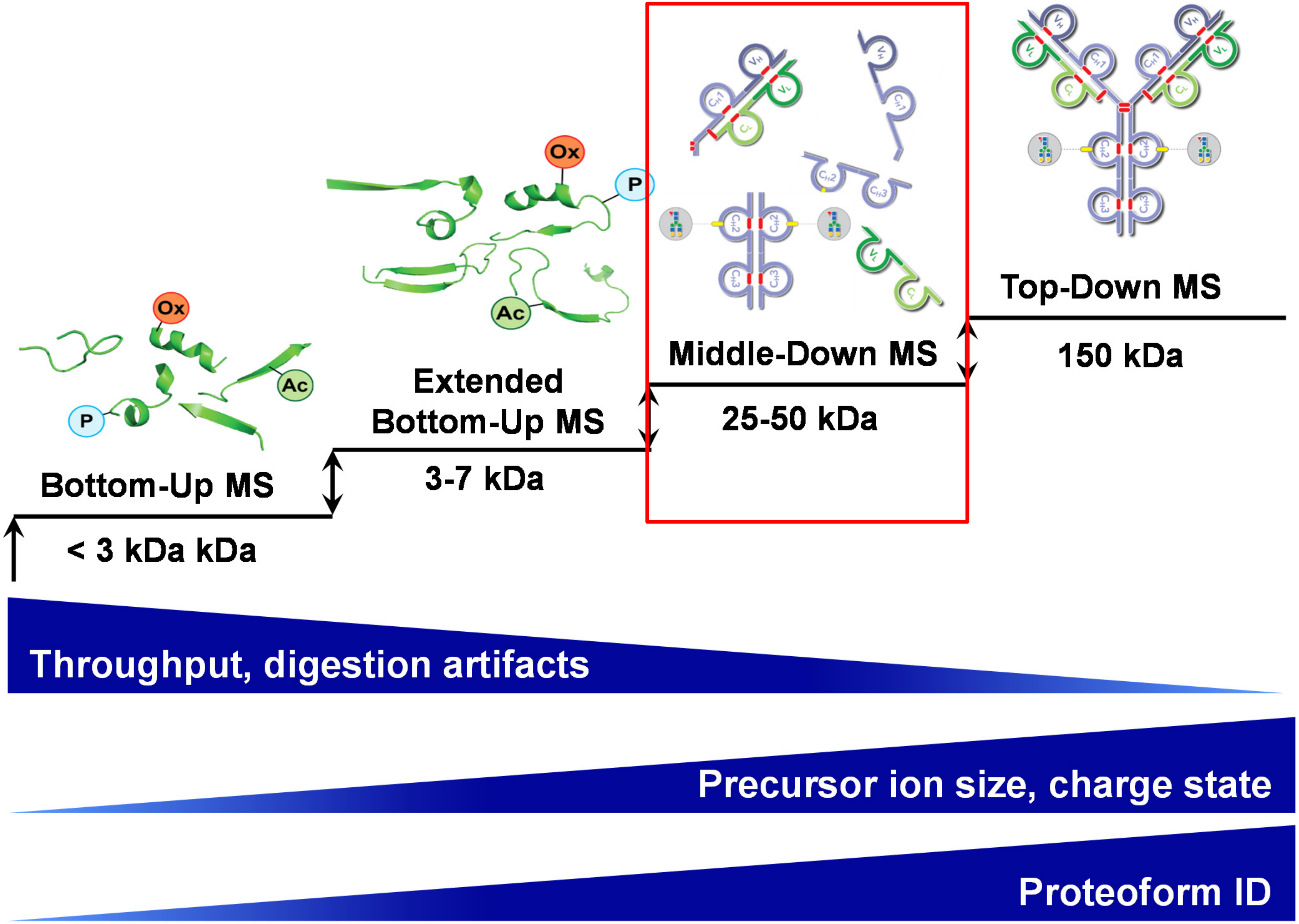


Six IgGs mixture by **e**-bottom-up with Sap9



Identification of proteotypic peptides with two CDRs;
Sequence coverage > 90 % in a mixture

MS/MS-Based Approaches For IgG Analysis



Bottom-Up MS

< 3 kDa kDa

Extended Bottom-Up MS

3-7 kDa

Middle-Down MS

25-50 kDa

Top-Down MS

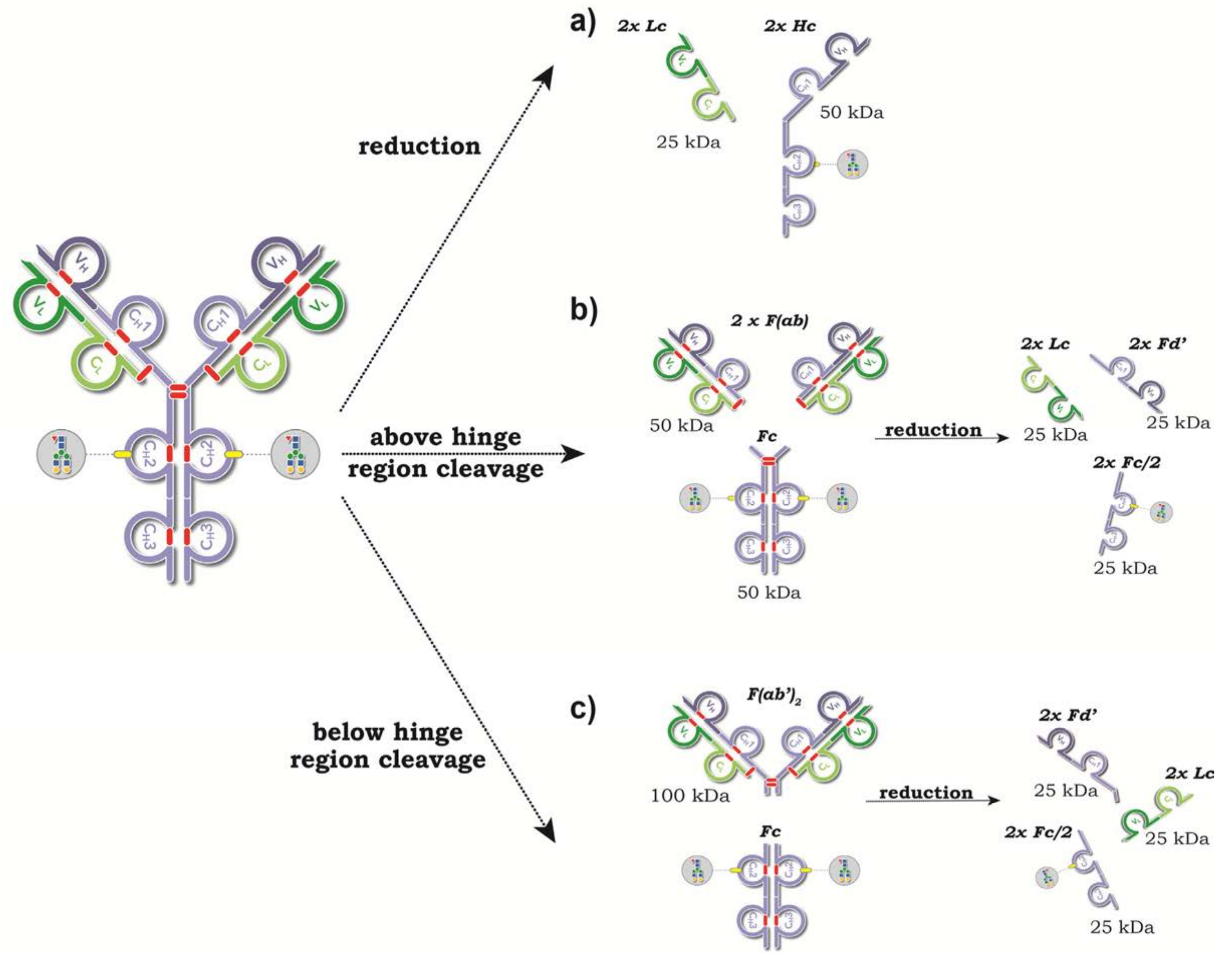
150 kDa

Throughput, digestion artifacts

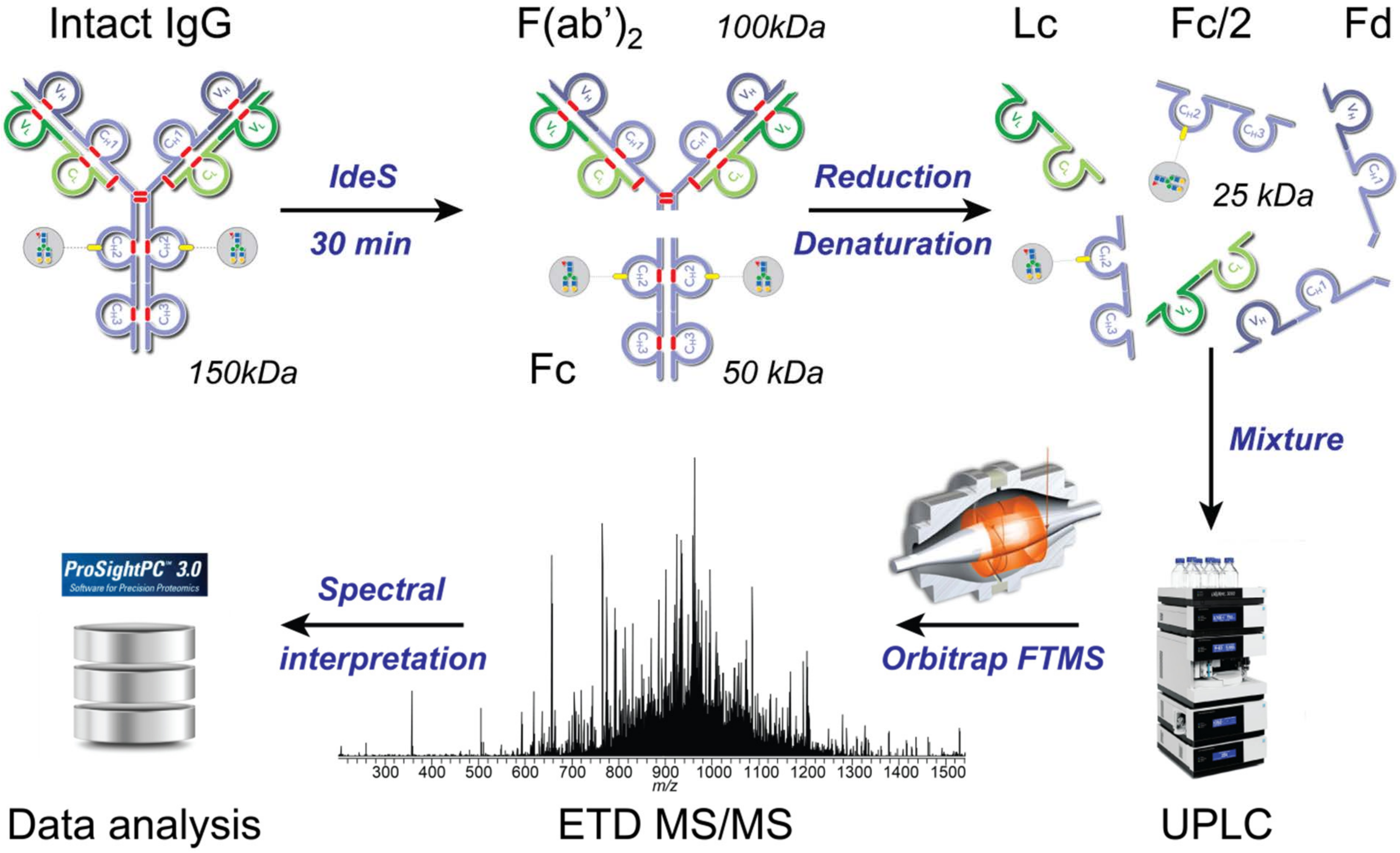
Precursor ion size, charge state

Proteoform ID

Top-Down View on IgG: 150, 100, 50, 25 kDa subunits



Middle-up and –down MS workflows: **Ides** proteolysis



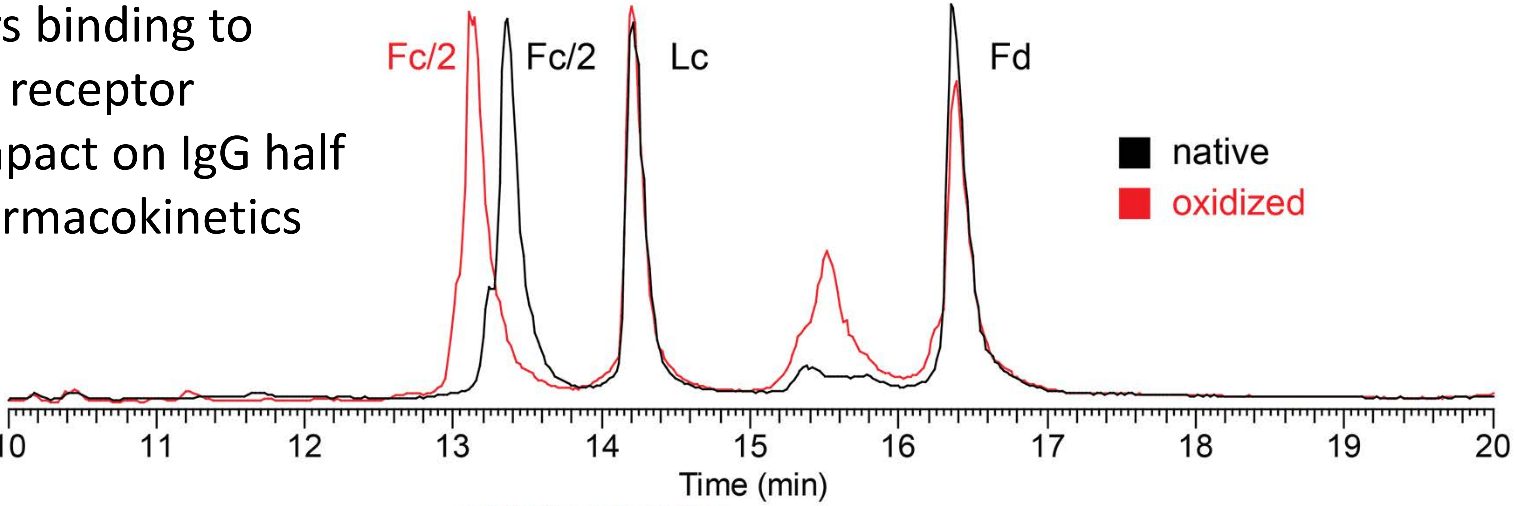
- Ides cleaves in formulation buffer: no artifacts
- Quick protocol, sample preparation in less than 1h
- CDRs 1, 2 & 3 sequenced
- Up to - 68% sequence coverage
- Glycosylation site identified
- Effective in IgG mixtures analysis

Fornelli et al., Anal. Chem. 2014, 3005-3012

Oxidation assesment with middle-down MS

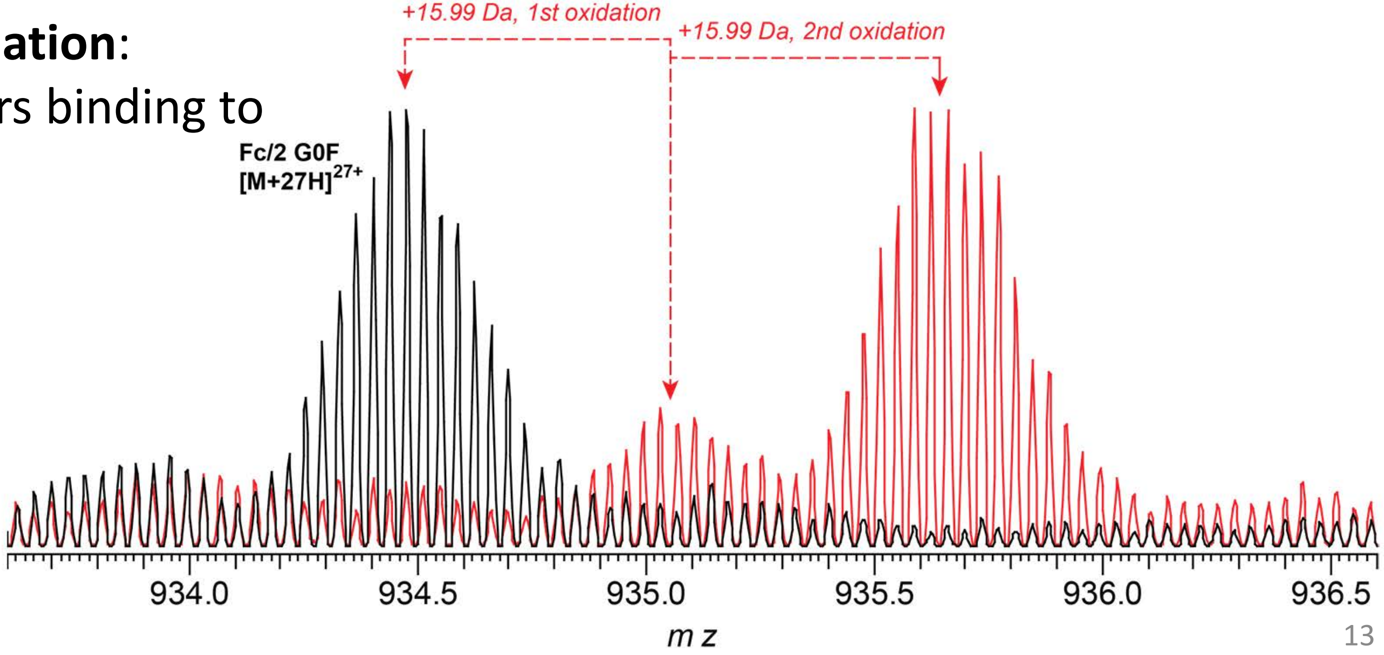
Methionine oxidation:

- In Fc it alters binding to neonatal Fc receptor
- Negative impact on IgG half life and pharmacokinetics



Tryptophane oxidation:

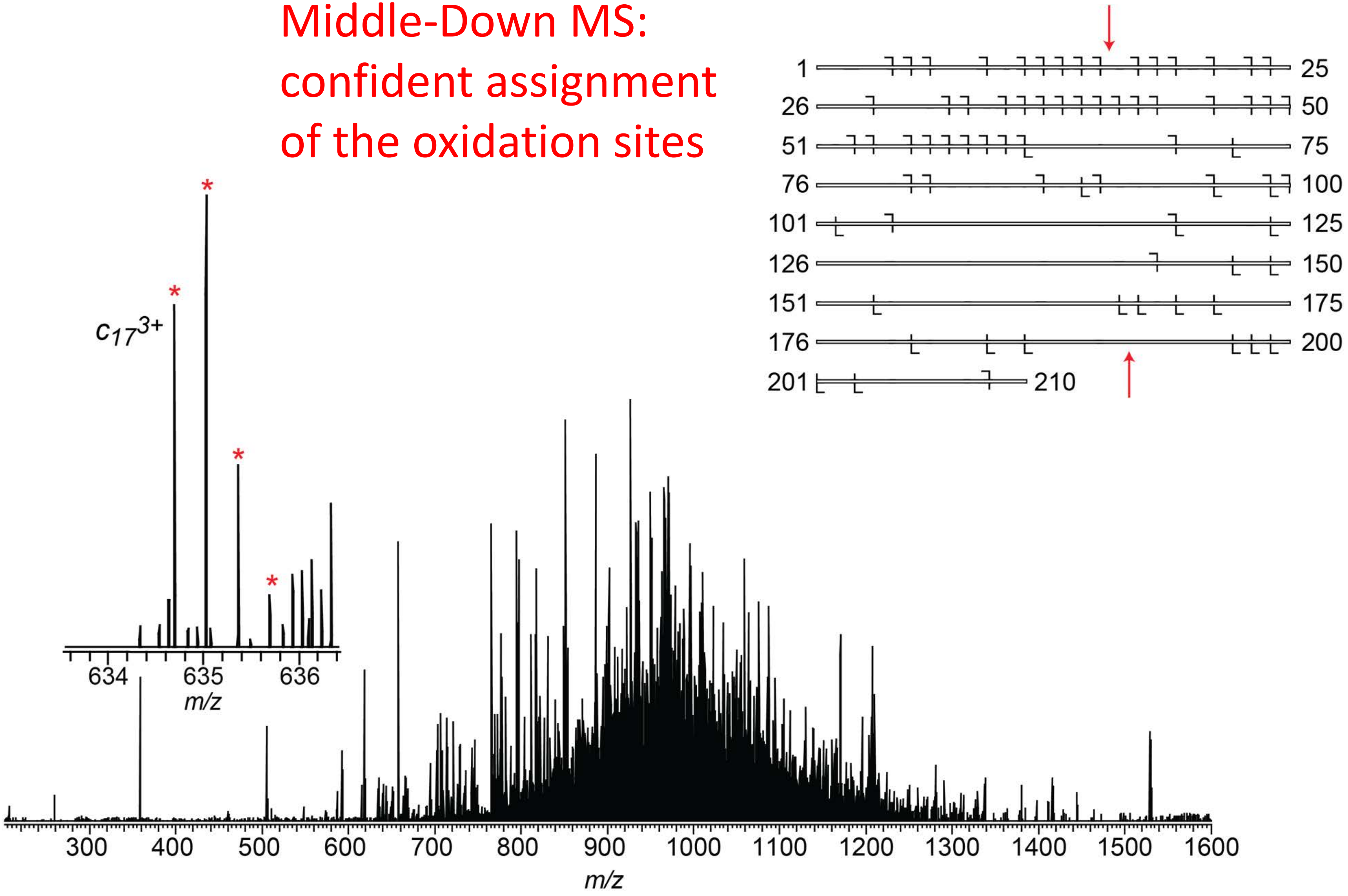
- If in CDR it alters binding to antigen



Fornelli et al., Anal. Chem. 2014, 3005-3012

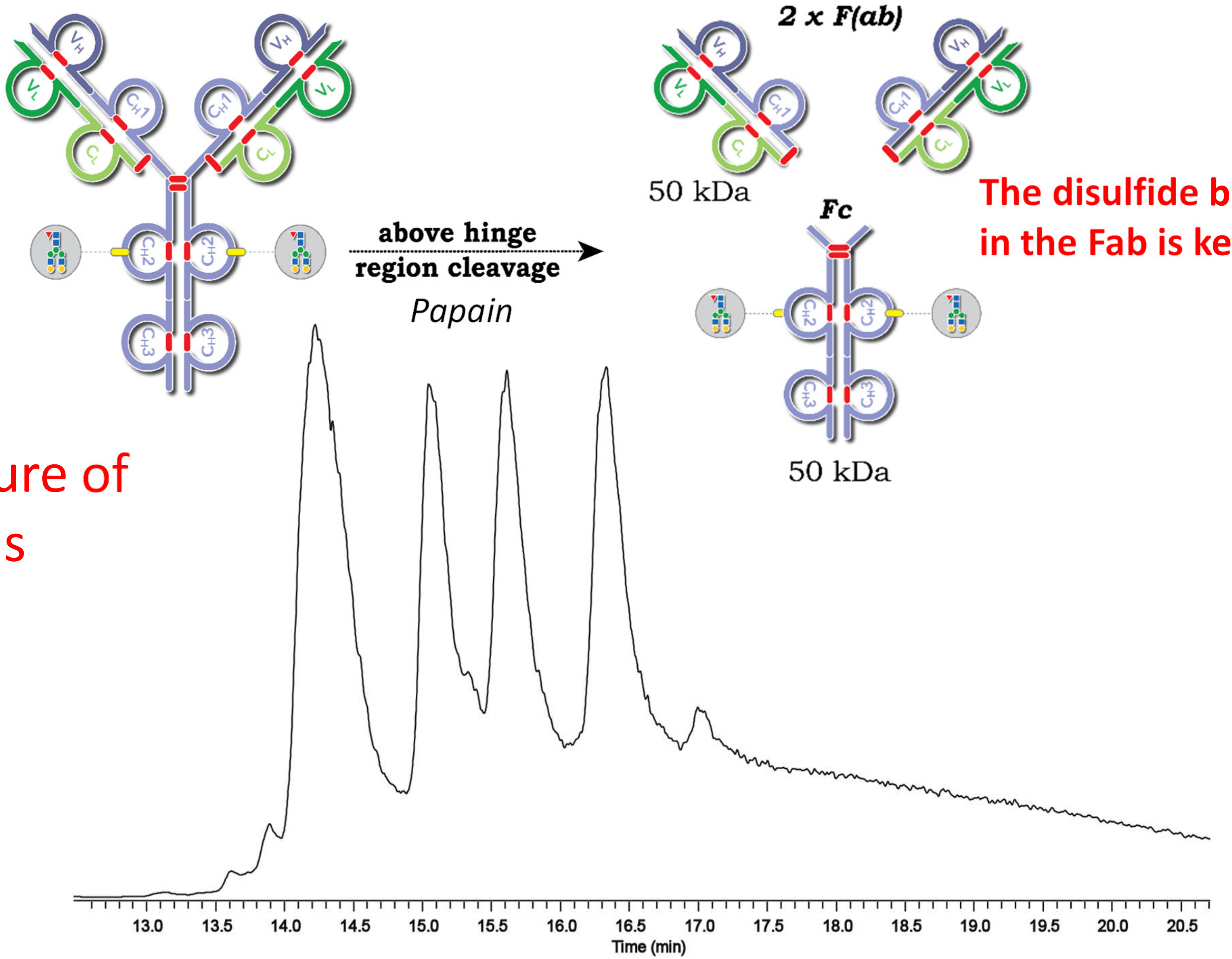
Oxidation assesment with middle-down (ETD) MS

Middle-Down MS:
confident assignment
of the oxidation sites



Fornelli et al., Anal. Chem. 2014, 3005-3012

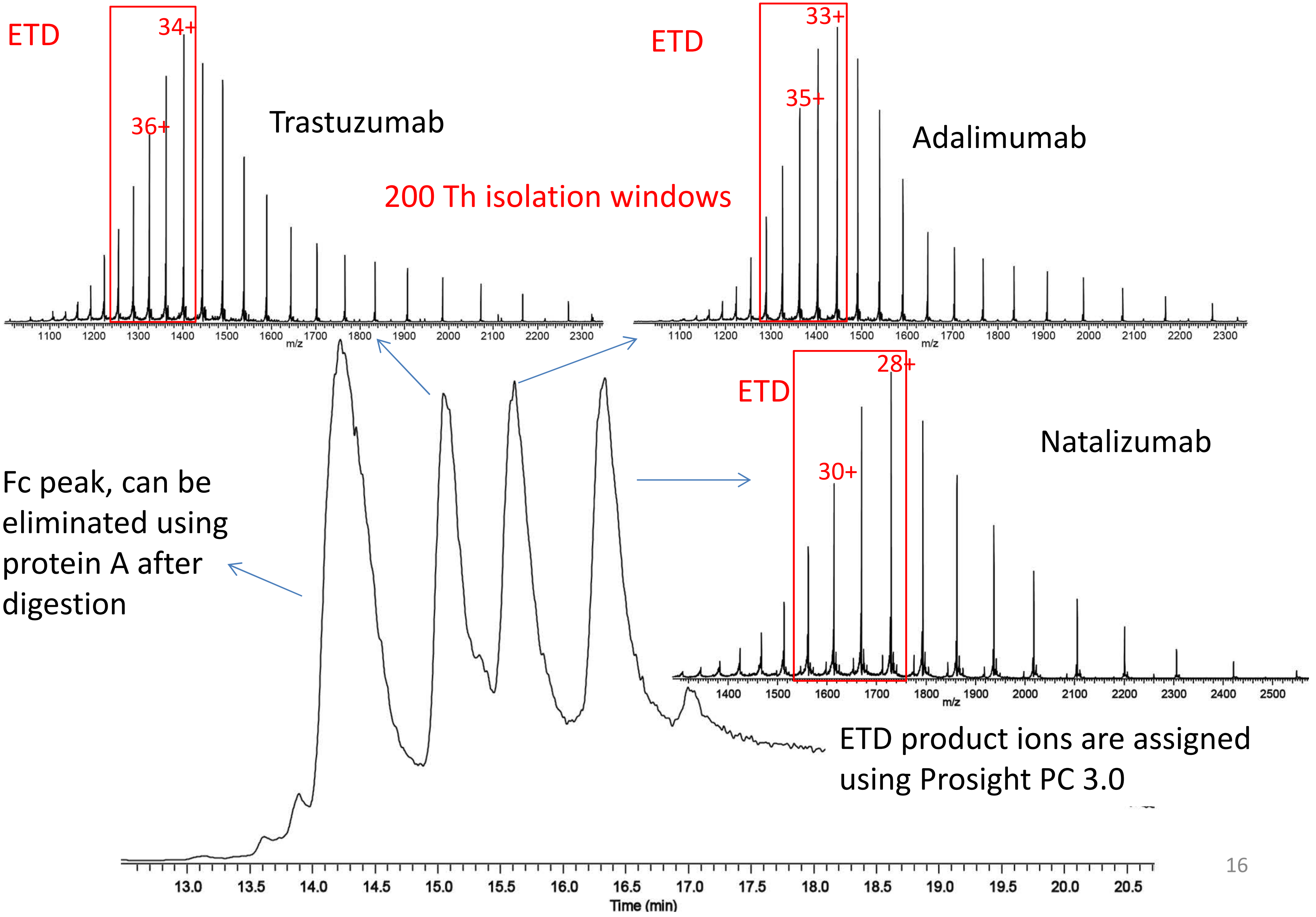
Fab (50 kDa) analysis with middle-down (ETD) MS



The disulfide bond in the Fab is kept

Mixture of 3 IgGs

Fab (50 kDa) analysis with middle-down (ETD) MS

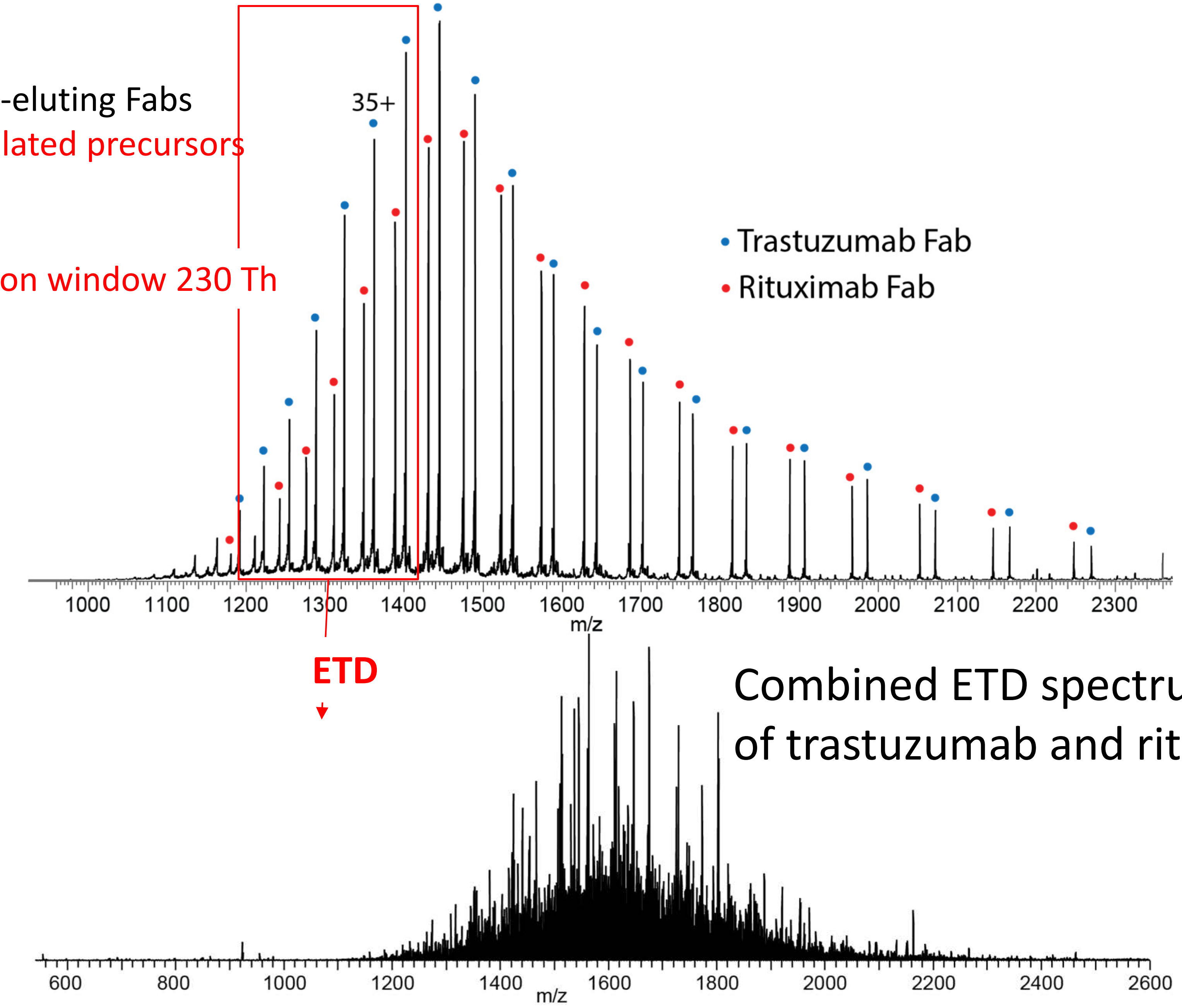


200 Th isolation windows

Fc peak, can be eliminated using protein A after digestion

ETD product ions are assigned using Prosight PC 3.0

Pairing information @ Fab level with middle-down MS



Co-eluting Fabs

Co-isolated precursors

Isolation window 230 Th

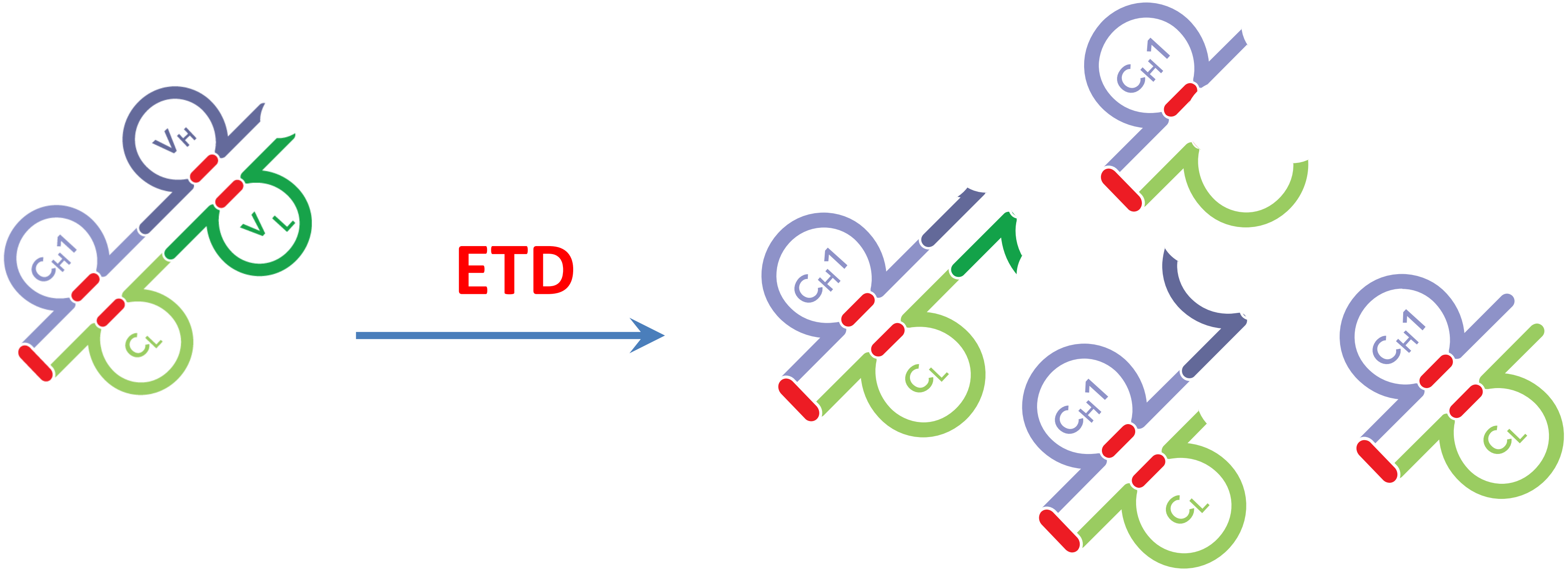
● Trastuzumab Fab

● Rituximab Fab

ETD

Combined ETD spectrum
of trastuzumab and rituximab

Assignment of ETD product ions

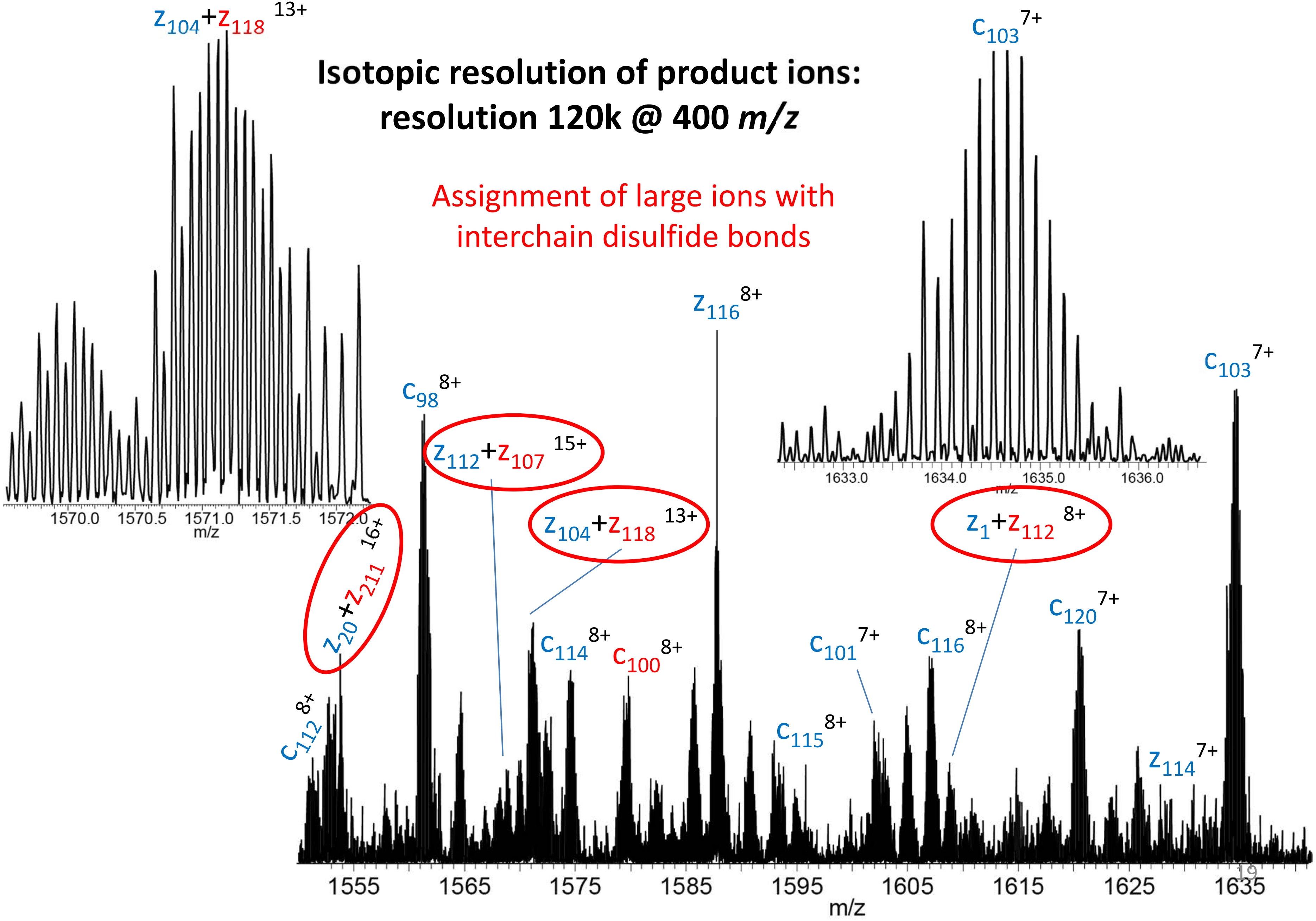


- S-S bonds may remain intact upon ETD MS/MS
- Current top-down search algorithms do not account for these ions
- **A dedicated ETD product ion analysis tool has been developed to assign these S-S-bond containing ions**

Pairing information @ Fab level with middle-down MS

Isotopic resolution of product ions: resolution 120k @ 400 *m/z*

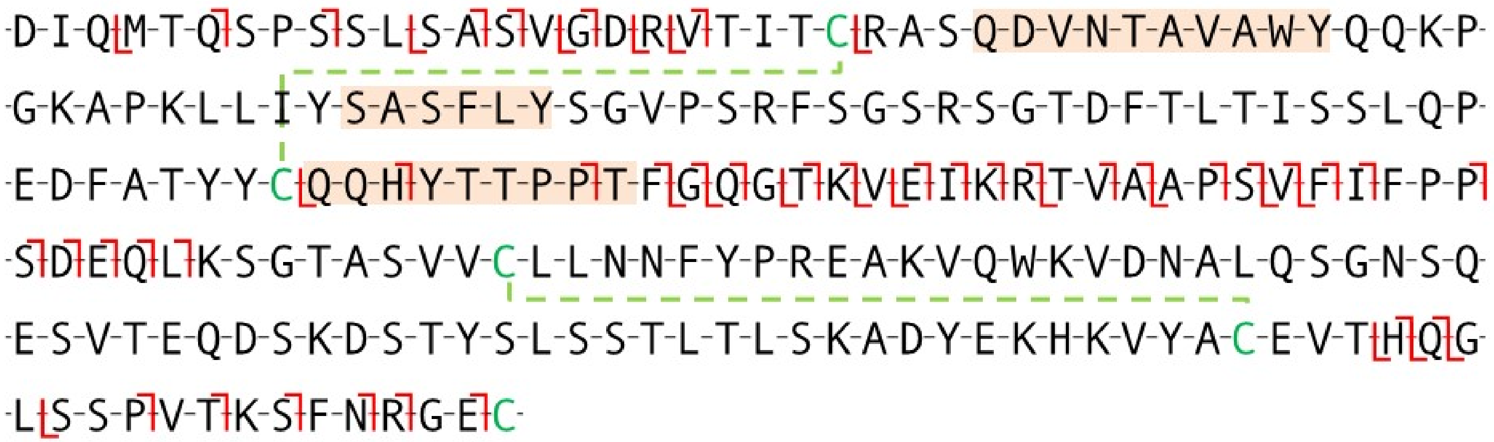
Assignment of large ions with interchain disulfide bonds

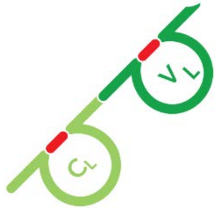


Pairing information @ Fab level with middle-down MS

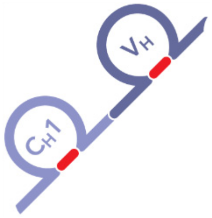
Trastuzumab, ETD product ions with the interchain S-S bond cleaved

Light chain: Sequence coverage 23.0 %





Heavy chain Fd: Sequence coverage 26.1 %



Pairing information @ Fab level with middle-down MS

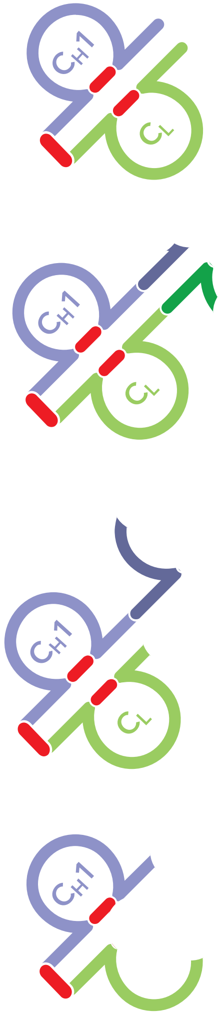
Trastuzumab, ETD product ions with the interchain S-S bond **NOT** cleaved

Light chain: Sequence coverage 16.4 %

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKP
GKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQP
EDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ
ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC

Heavy chain Fd: Sequence coverage 16.8 %

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQA
PGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAY
LQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSS
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS
WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQT
YICNVNHKPSNTKVDKKVEPKSCDKTH

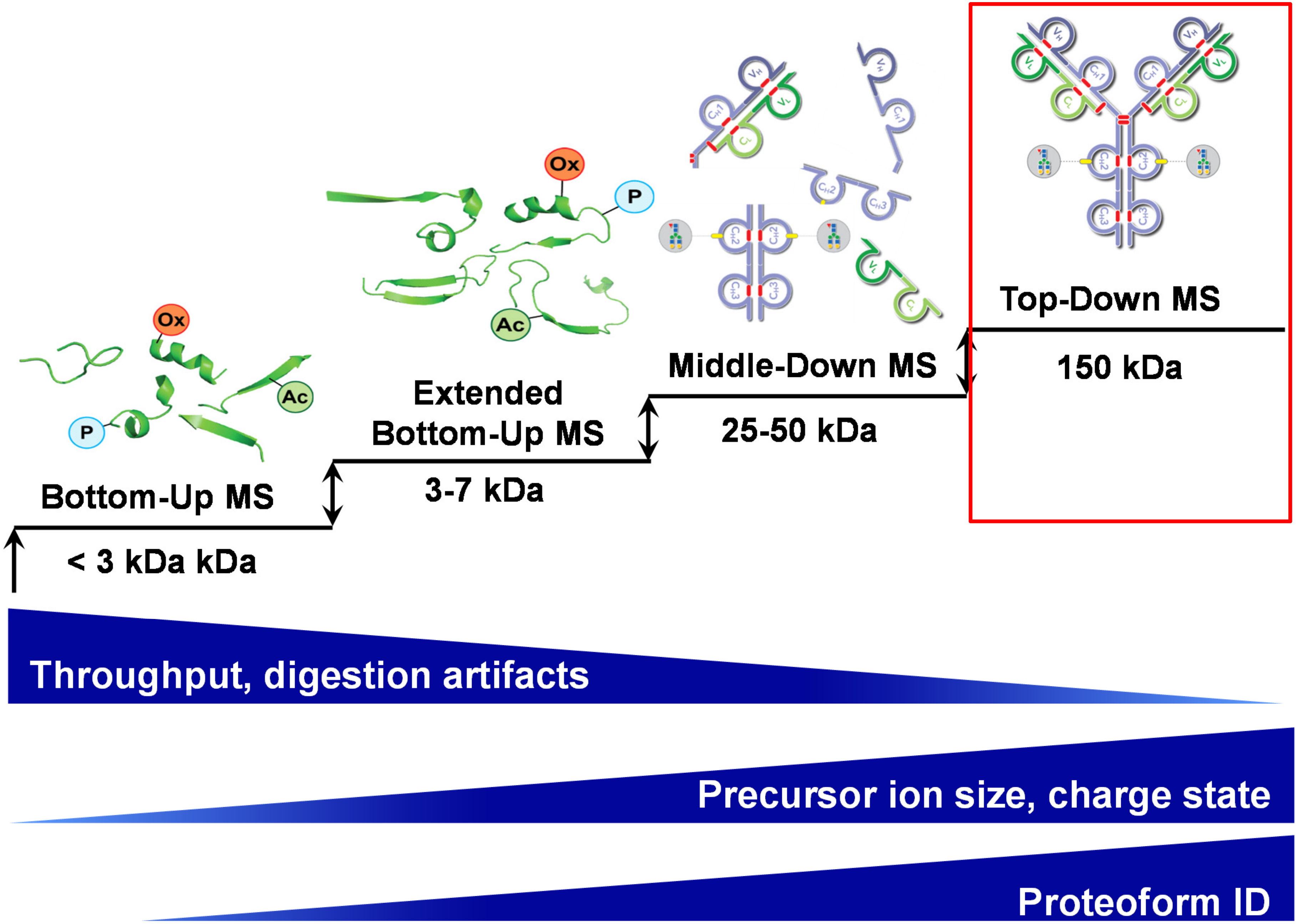


Intermediate Conclusions

- **Extended bottom-up MS for IgG analysis**:
  - Faster sample preparation than in bottom-up MS
  - More effective sequencing of single and mixture IgGs
  - **Deamidation** assesement without artifact introduction
  - Identification of heavy and light chains from sera
- **Middle-down (ETD) MS for IgG analysis**:
  - 25 kDa subunits (Ides):
    - Sequencing
    - PTMs characterization: **Oxidation** sites assesement
  - 50 kDa subunits (Fab, Papain):
    - Identification of Lc and Hc chain **pairs**
    - Assignment of disulfide-bridged ETD product ions

Challenge: High complexity serum mixtures of IgGs → Improving separation, fractionation (High IgG structural similarity)

MS/MS-Based Approaches For IgG Analysis



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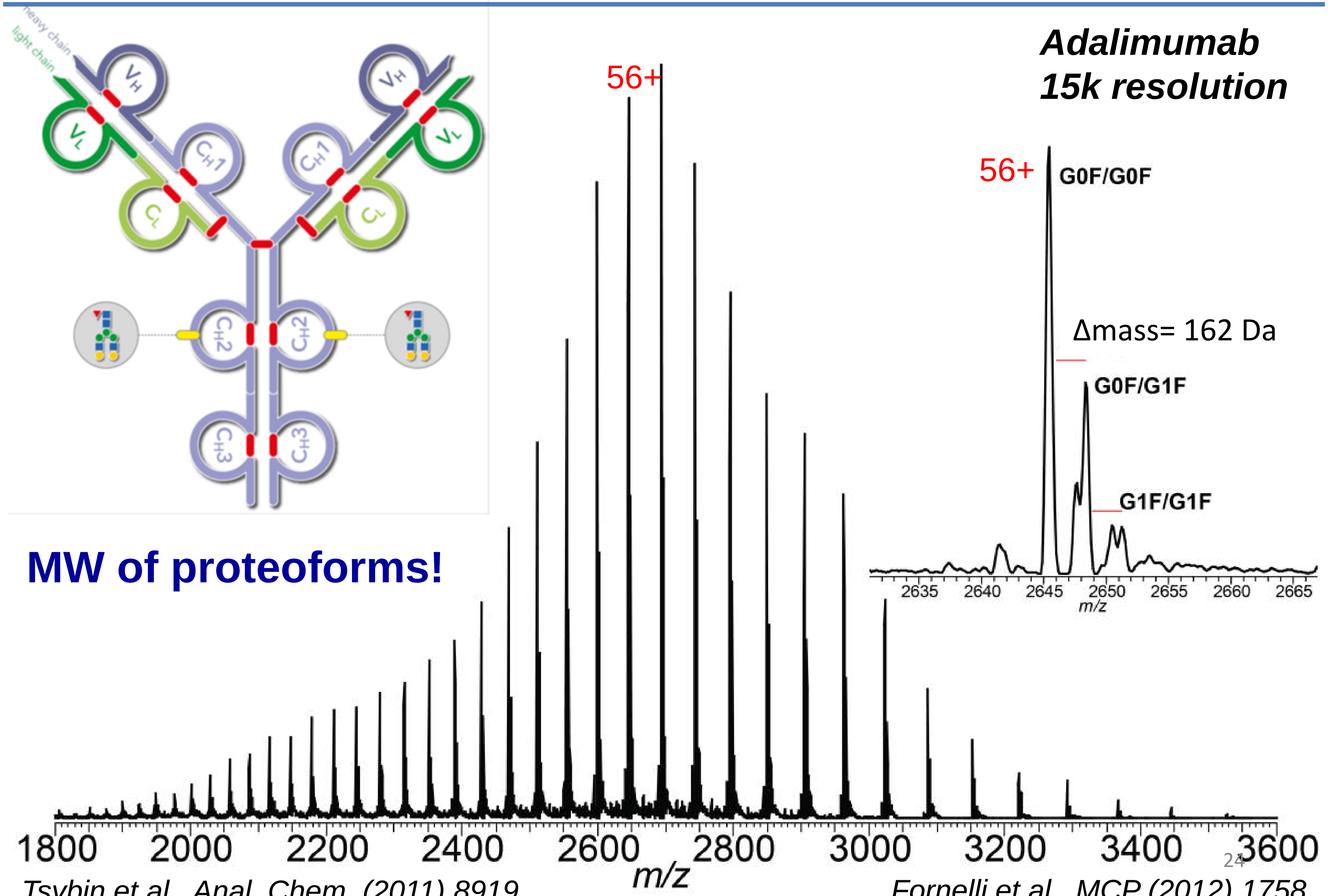
Proteoform ID

Intact Antibody Mass Analysis: Proteoforms



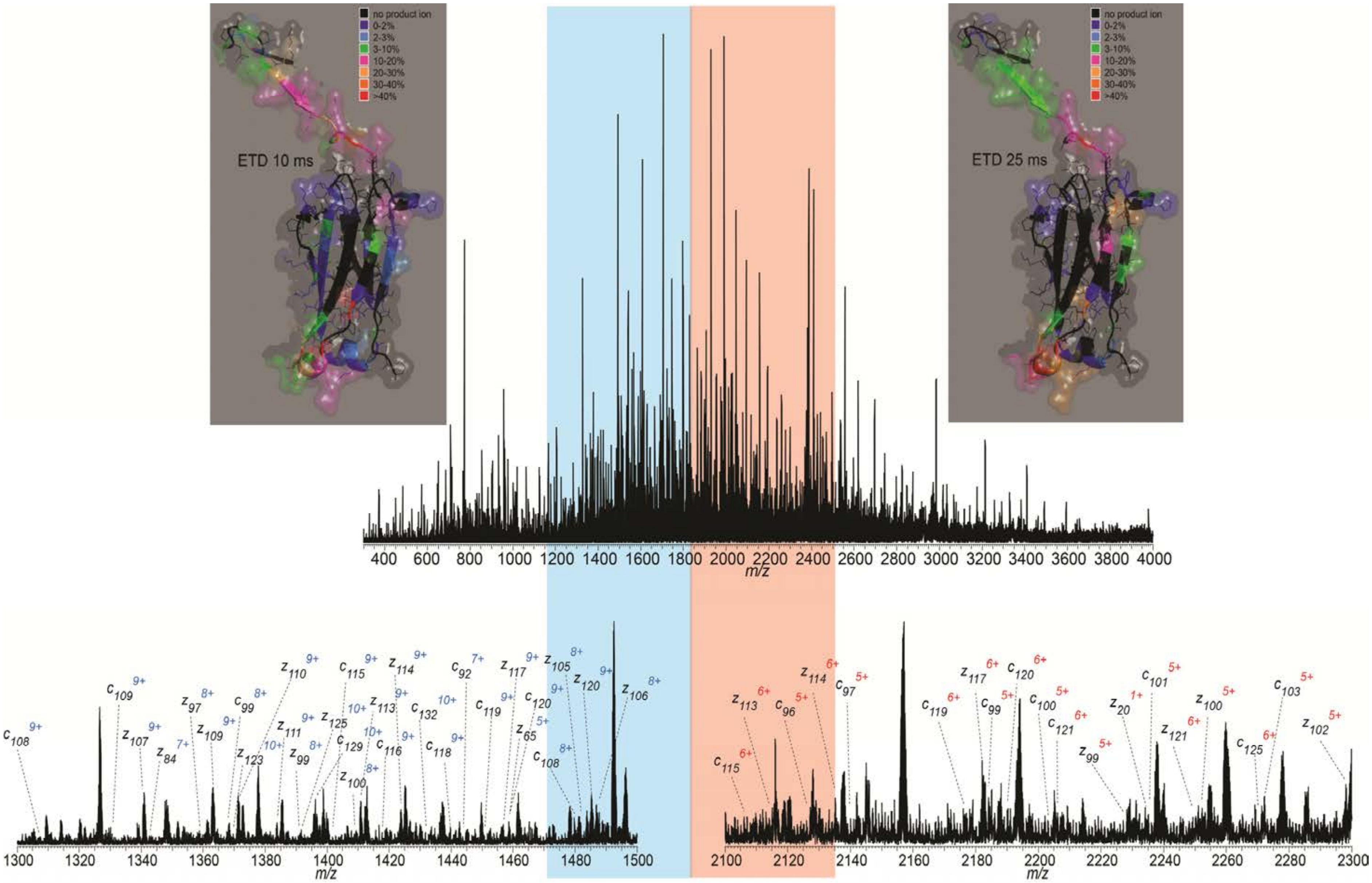
Adalimumab
15k resolution

MW of proteoforms!

Tsybin et al., Anal. Chem. (2011) 8919

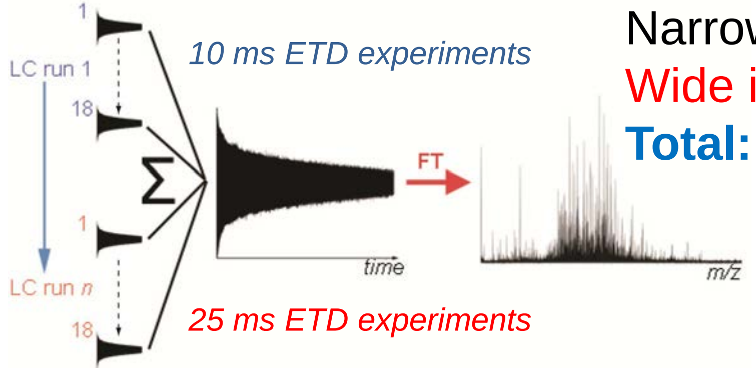
Fornelli et al., MCP (2012) 1758

Intact Antibody Structure Analysis: ETD MS/MS



Intact Antibody Structure Analysis: ETD MS/MS

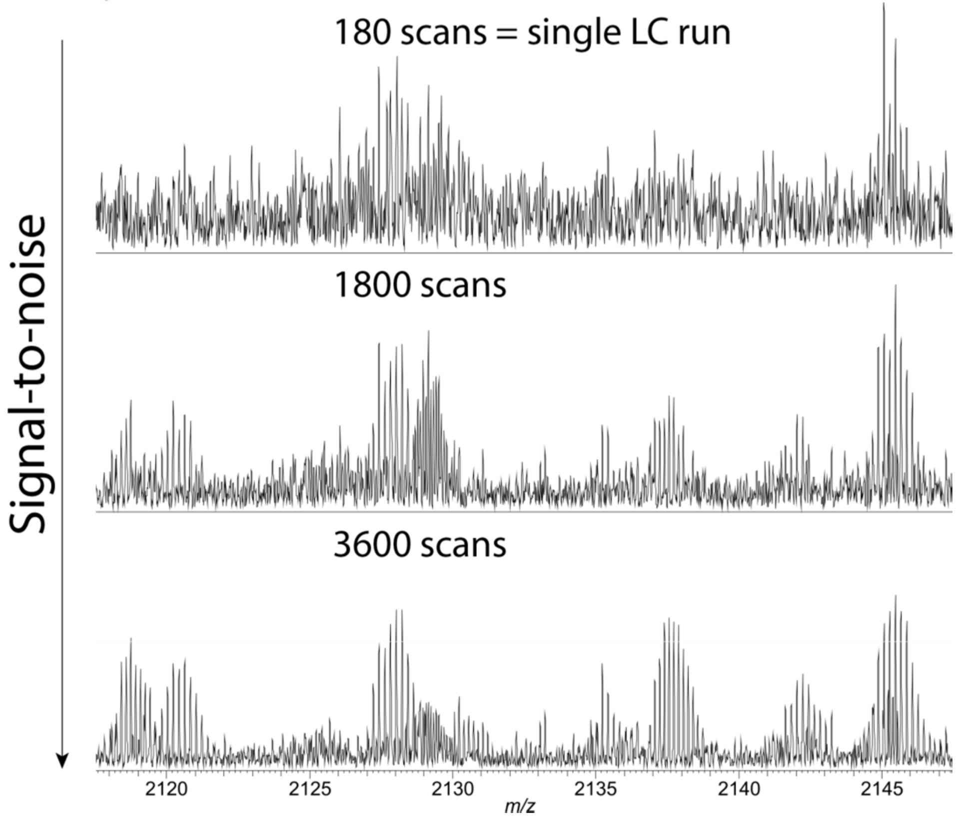
Averaging many MS scans (in time or in **spectral domain**) for improving SNR:

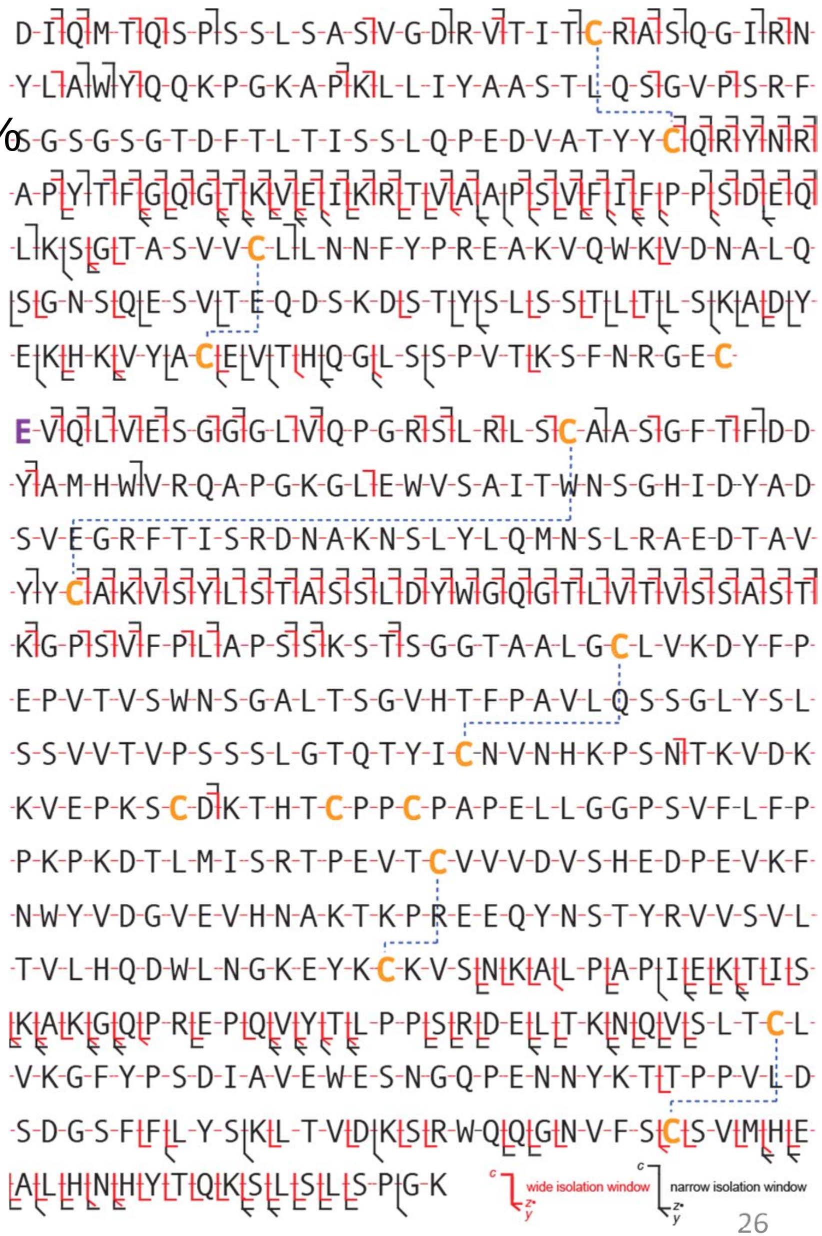


Narrow i.w.: 24.4%

Wide i.w.: 27.1%

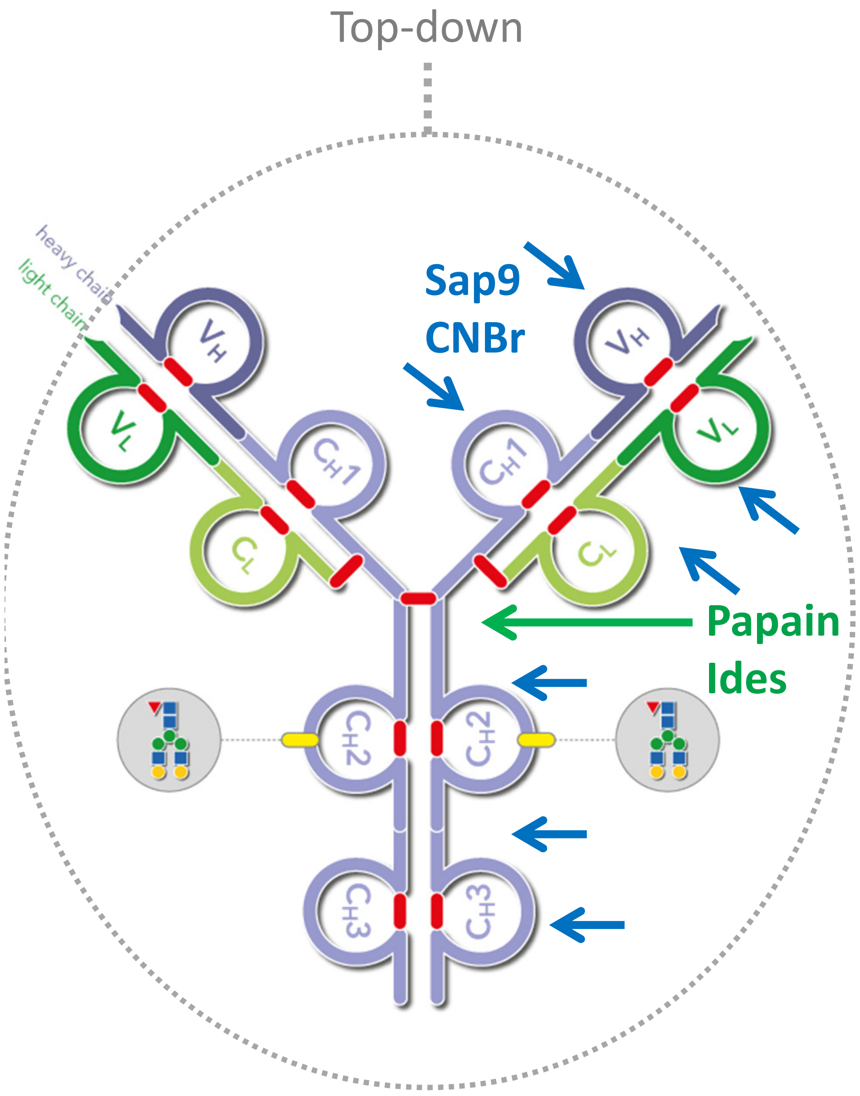
Total: 32.7%





Fornelli et al., MCP (2012) 1758

Summary: MS Approaches For IgG Analysis



	Pros	*Cons*
TDP: **ETD/HCD**	• No artifact induction • Proteoform ID	• ~30% sequence coverage • Only CDR3
MDP: **Papain**	• ~2x sequence coverage TDP • Large PTM ID • Chain pairing	• Long protocol • Non specific cleavages • Difficult small PTM ID (deam.)
MDP: **Ides**	• No artifact induction (fast) • Specific cleav.	• Difficult small PTM ID (deam.) • 70% seq. cov.
Extended BUP: Chemicals (CNBr, NTCB)	• No substrate specificity • Large (>3 kDa) peptides	• Stoichiometry is very important • Special handling conditions needed • Secondary reactions
Extended BUP: **Sap9**	• Works in acidic pH, fast (1 h) • 100% seq. cov. • Large peptides • Small PTM ID	• Not very specific, overlapping peptides • Longer digestion times lead to shorter peptides

TDP: Top-Down Proteomics
MDP: Middle-Down Proteomics
BUP: Bottom-Up Proteomics

Acknowledgements

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erc

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