A Novel, Rapid Chip-Based iCIEF-MS Analysis of Therapeutic mAb Charge Variants Under Forced Degradation Conditions and Comparison to Traditional Methods

Daniel Donnelly[1], Xiaoqing Hua[1], Jay Desai[1], Bhumit Patel[1], Wei Chen[2], Mariam ElNaggar[2], Maggie Ostrowski[2], Scott Mack[2], Erik Gentalen[2], Douglas Richardson[1]

[1]Analytical Research and Development, Merck & Co. Inc., Kenilworth, NJ[1]

[2]Intabio, Inc., Newark, CA

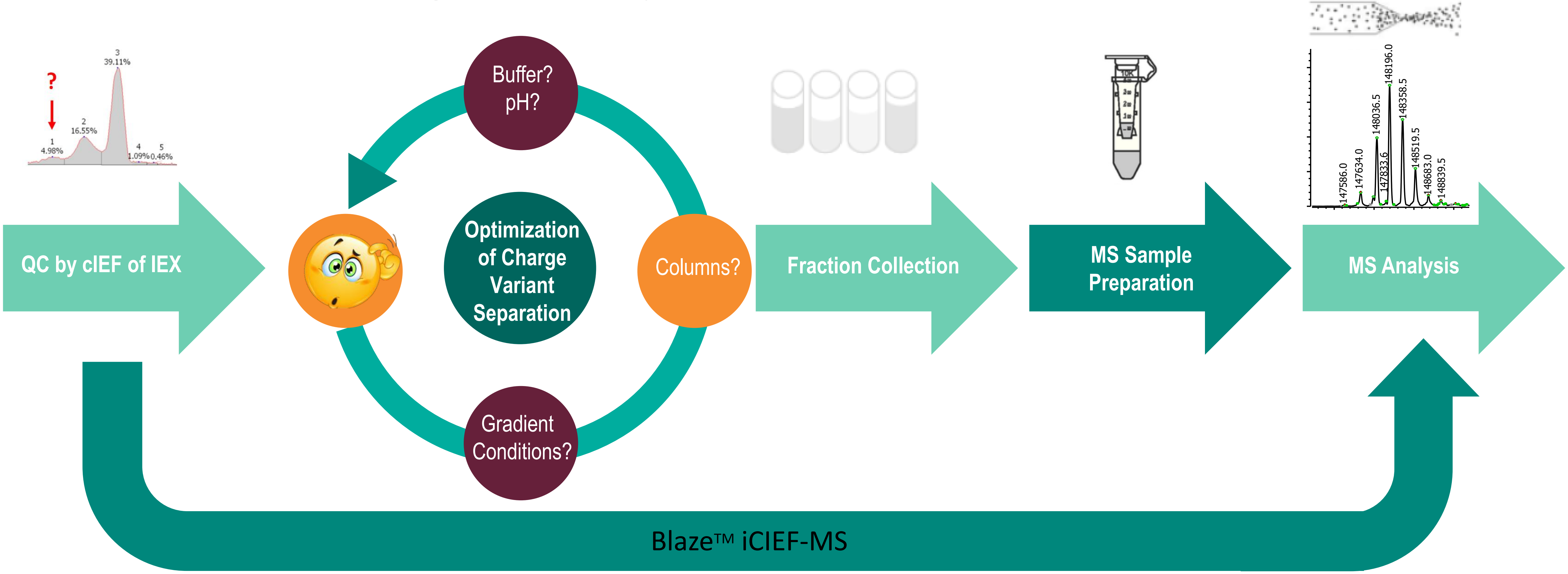
Background and Challenge Statement

- Charge heterogeneity analysis is essential for the successful development and production of therapeutic antibodies.
- Traditionally, charge variant analysis and peak identification are performed by two separate offline assays (cIEF/IEX and Mass Spectrometry).
- MS identification of a charge variants requires a tedious, multi-step process including fractionation, sample preparation, and off-line MS analysis. Additionally, IEX (which is the current preferred charge variant method at Merck) can suffer from extensive method development, column robustness, and long run times.

INTABIO

MERCK
INVENTING FOR LIFE

Online cIEF-MS Significantly Reduces Peak ID Efforts



QC by cIEF of IEX

Buffer? pH?

Optimization of Charge Variant Separation

Columns?

Gradient Conditions?

Fraction Collection

MS Sample Preparation

MS Analysis

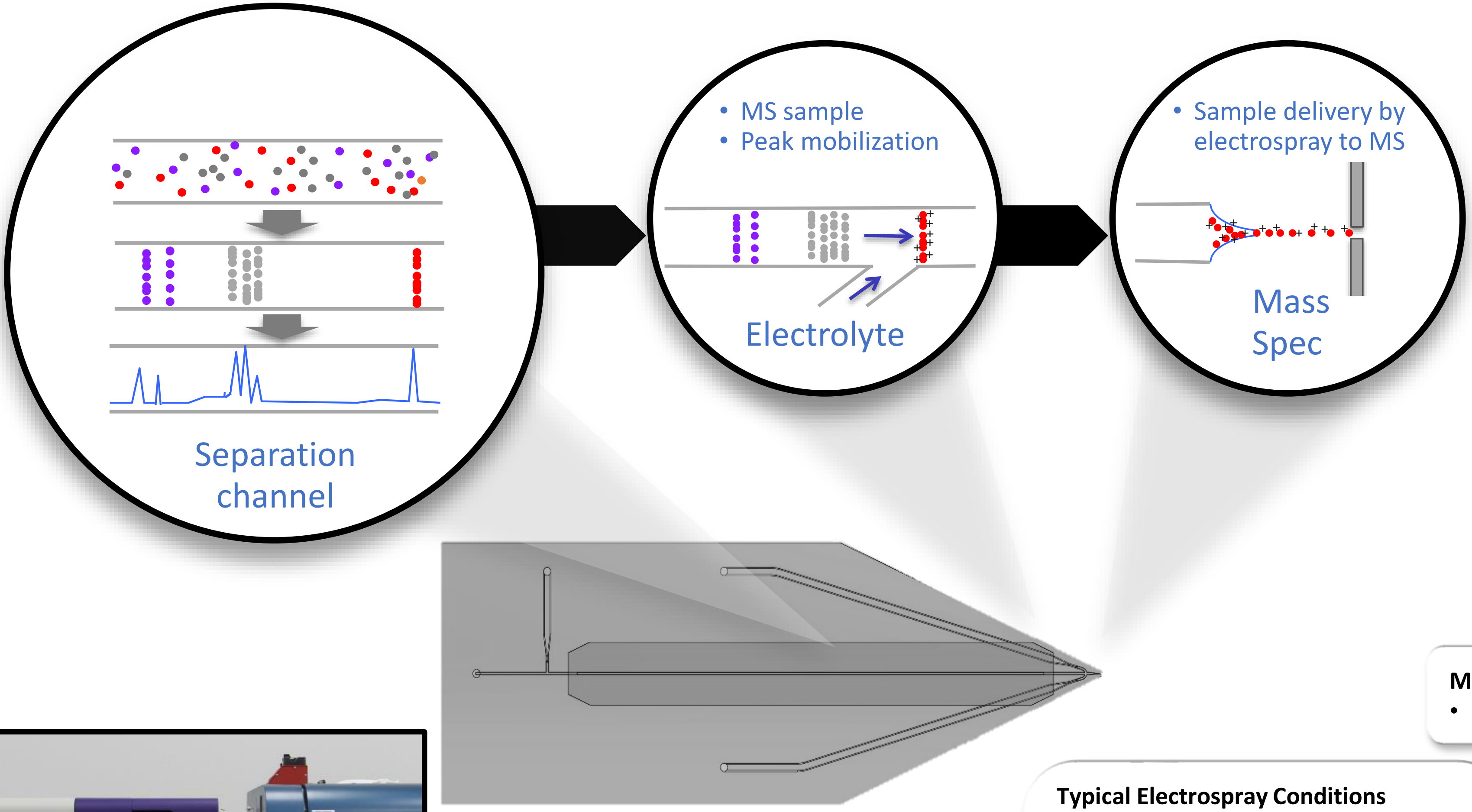
Blaze™ iCIEF-MS

- A cIEF-MS platform can turn a multistep (3-4 steps) workflow into a one step analysis
- Following CQA determination by more in-depth methods (RPM) monitoring levels of deamidation or oxidation for extended timepoint studies (e.g., stability, forced degradation) can be done more efficiently

INTABIO

MERCK
INVENTING FOR LIFE

Intabio proprietary microchip technology integrates key iCIEF-MS analytical functions



Separation channel

- MS sample
- Peak mobilization

Electrolyte

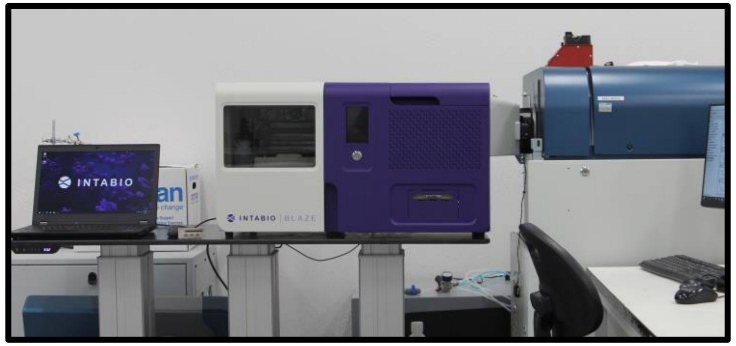
- Sample delivery by electrospray to MS

Mass Spec

Sample Consumption
- 50 µg mAb in 200 µL CIEF cocktail
- ~3 µL mAb cocktail per injection

Sensitivity
- >14x concentration in focused peaks increases signal intensity

Mobilization Conditions
- 1% Formic Acid, 1% Diethylamine



Blaze^{TM} iCIEF-MS System with SCIEX 6600+

Typical Electrospray Conditions
- 1% Formic Acid in 1:1 Acetonitrile/Water
- Tip voltage 4000 volts
- (+) mode
- *m/z* 2000-6000, 35000 Resolution
- 150 eV DP, 50 eV CE
- Capillary Temperature 150 °C
- SCIEX 6600+

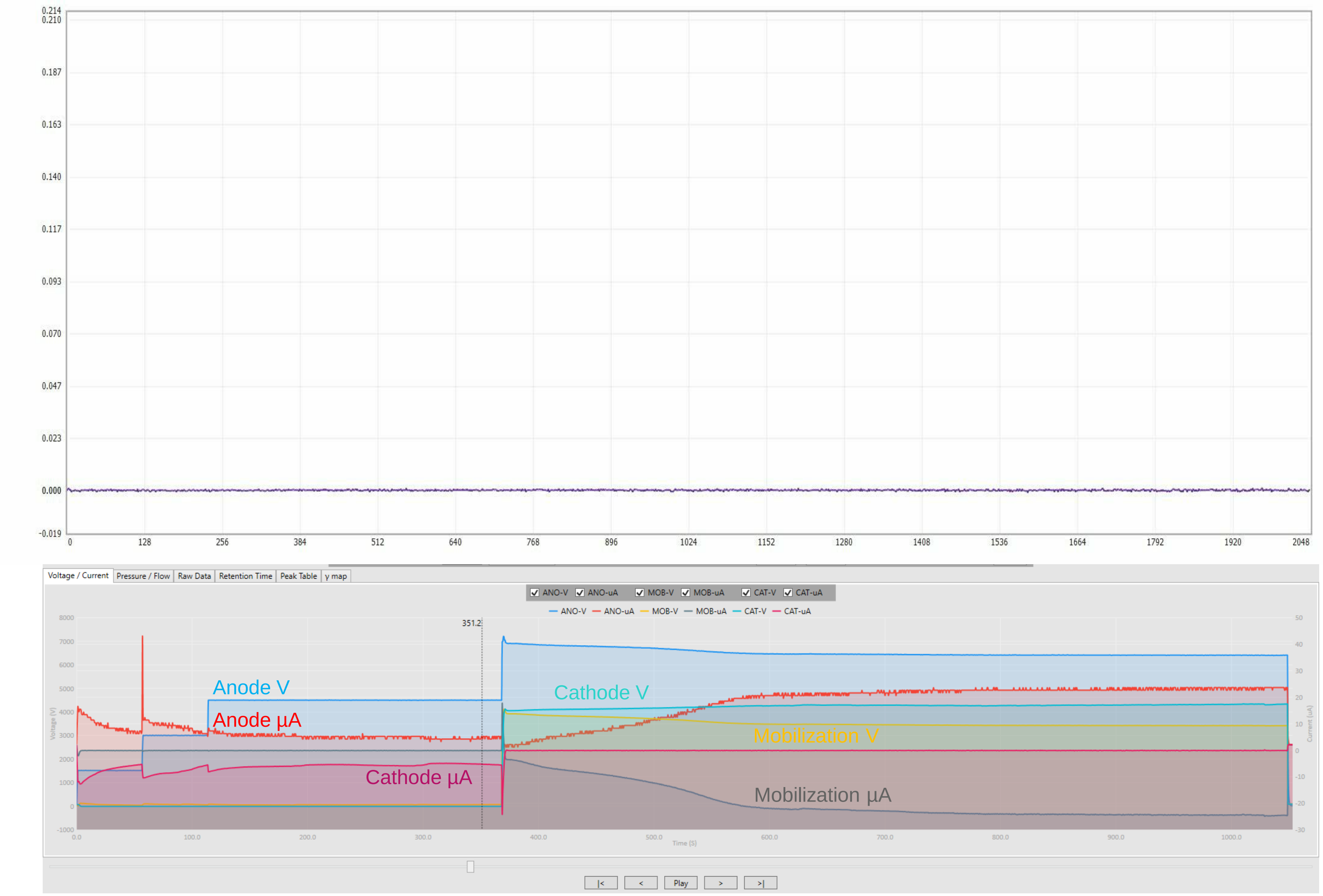
INTABIO

PROTEIN METRICS

MERCK
INVENTING FOR LIFE

Blaze real-time Imaged cIEF separation

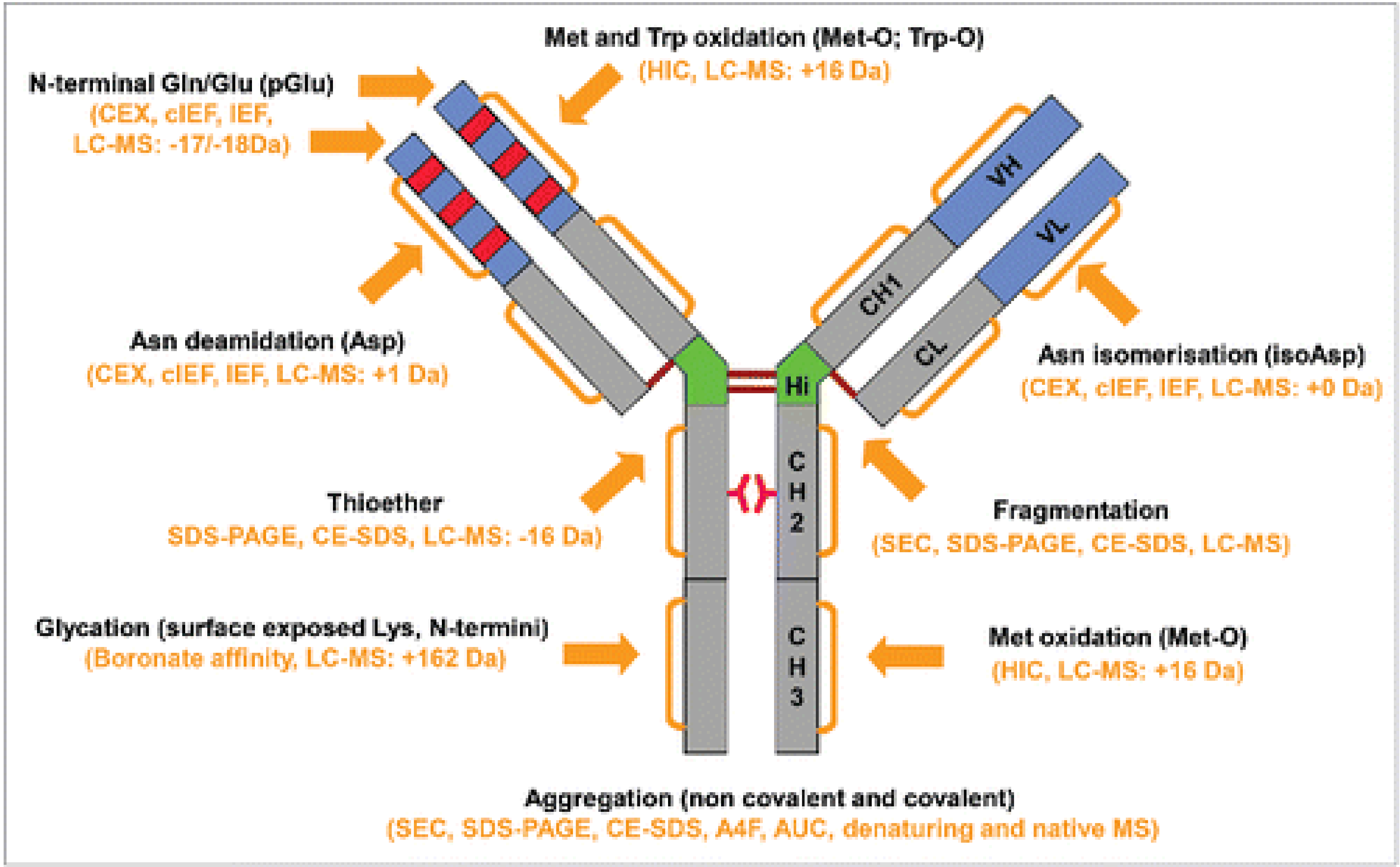
- Whole column, UV absorbance during focusing & mobilization
- Stepped voltage and current control during focusing enable real-time feedback
- Enables rapid cIEF method development & peak tracking during mobilization



INTABIO

MERCK
INVENTING FOR LIFE

Major degradation pathways of recombinant monoclonal antibodies and Traditional Assays to Monitor them[1]



Met and Trp oxidation (Met-O; Trp-O)
(HIC, LC-MS: +16 Da)

N-terminal Gln/Glu (pGlu)
(CEX, cIEF, IEF,
LC-MS: -17/-18Da)

Asn deamidation (Asp)
(CEX, cIEF, IEF, LC-MS: +1 Da)

Asn isomerisation (isoAsp)
(CEX, cIEF, IEF, LC-MS: +0 Da)

Thioether
SDS-PAGE, CE-SDS, LC-MS: -16 Da)

Fragmentation
(SEC, SDS-PAGE, CE-SDS, LC-MS)

Glycation (surface exposed Lys, N-termini)
(Boronate affinity, LC-MS: +162 Da)

Met oxidation (Met-O)
(HIC, LC-MS: +16 Da)

Aggregation (non covalent and covalent)
(SEC, SDS-PAGE, CE-SDS, A4F, AUC, denaturing and native MS)

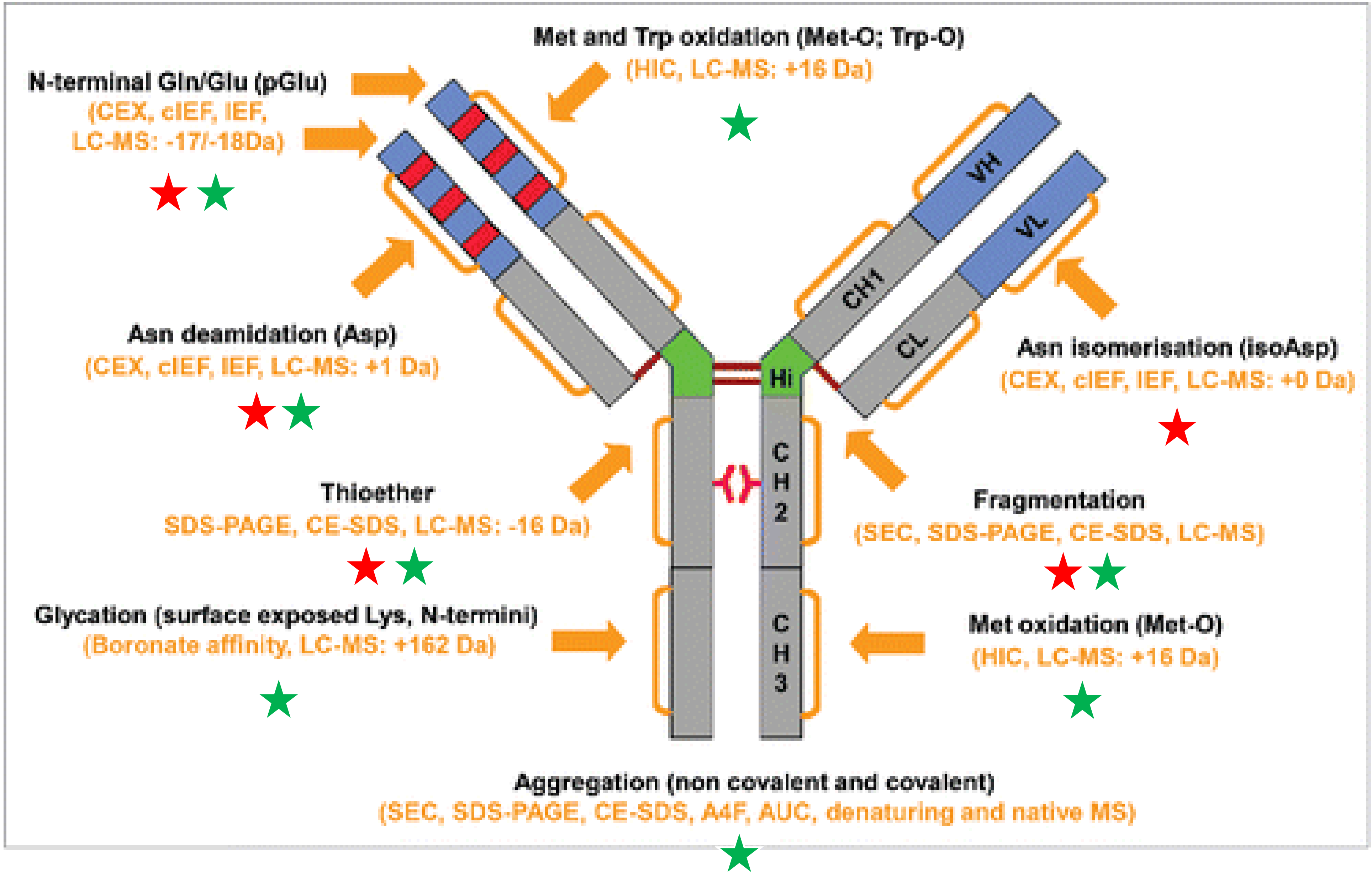
INTABIO

MERCK
INVENTING FOR LIFE

---

[1] Nowak et al (2017) Forced degradation of recombinant monoclonal antibodies: A practical guide, mAbs, 9:8, 1217-1230

Major degradation pathways of recombinant monoclonal antibodies and Traditional Assays to Monitor them[1]



N-terminal Gln/Glu (pGlu)
(CEX, cIEF, IEF, LC-MS: -17/-18Da)

Met and Trp oxidation (Met-O; Trp-O)
(HIC, LC-MS: +16 Da)

Asn deamidation (Asp)
(CEX, cIEF, IEF, LC-MS: +1 Da)

Asn isomerisation (isoAsp)
(CEX, cIEF, IEF, LC-MS: +0 Da)

Thioether
SDS-PAGE, CE-SDS, LC-MS: -16 Da)

Fragmentation
(SEC, SDS-PAGE, CE-SDS, LC-MS)

Glycation (surface exposed Lys, N-termini)
(Boronate affinity, LC-MS: +162 Da)

Met oxidation (Met-O)
(HIC, LC-MS: +16 Da)

Aggregation (non covalent and covalent)
(SEC, SDS-PAGE, CE-SDS, A4F, AUC, denaturing and native MS)

★ **LC/MS**

★ **cIEF**

Combining cIEF and MS analysis can successfully cover all degradation pathways of interest in a single shot.

INTABIO

MERCK
INVENTING FOR LIFE

---

[1] Nowak et al (2017) Forced degradation of recombinant monoclonal antibodies: A practical guide, mAbs, 9:8, 1217-1230

Outline of cIEF-MS Evaluation of Forced Degradation Study

1. Subject Merck mAb to traditional forced deg conditions
    - Heat
    - Light Exposure
    - High pH
    - Oxidizing Conditions

2. Analysis by traditional in-house cIEF Assay

3. Identification of major PTMs by in-house RPM assay

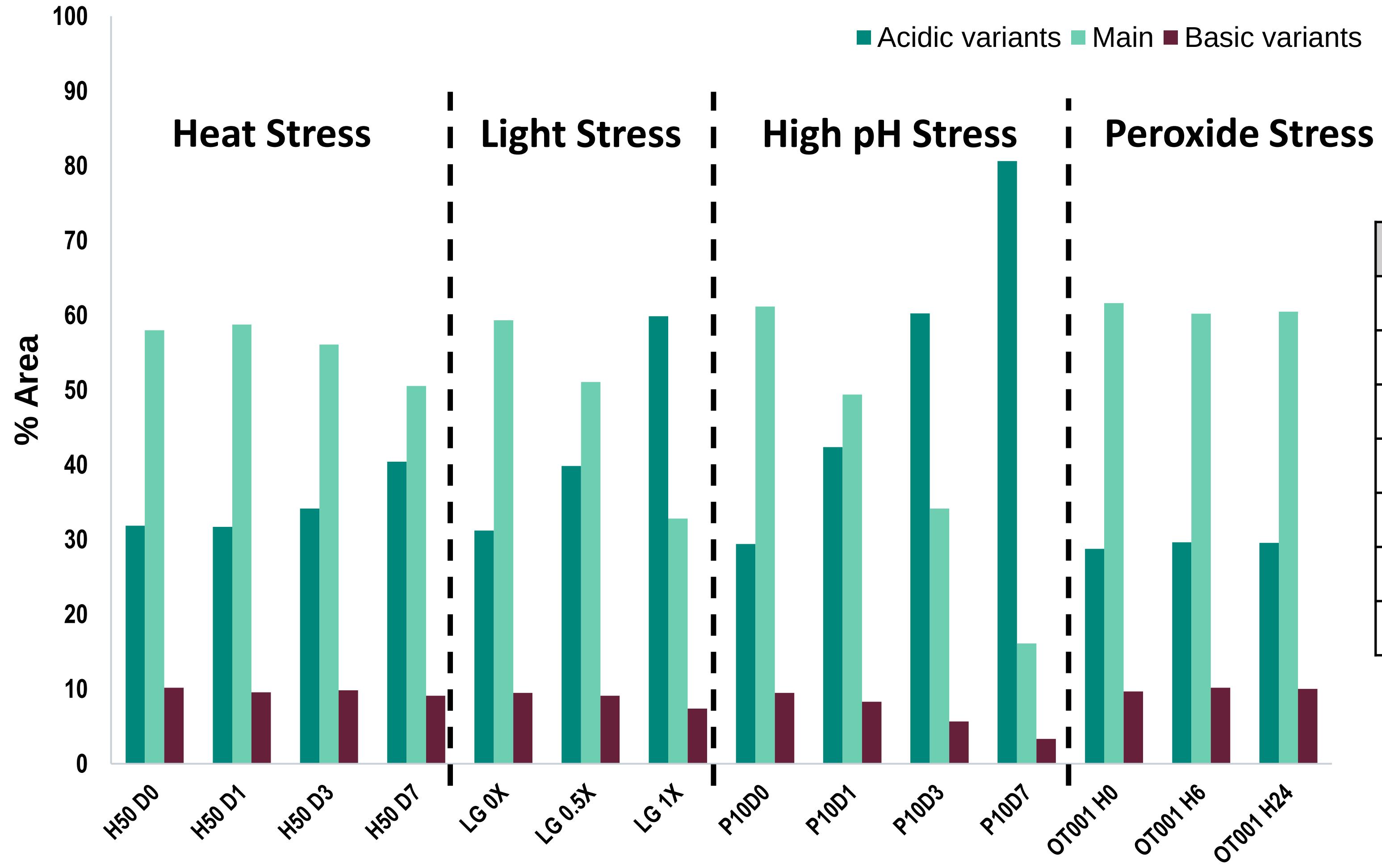
4. Analysis of samples by novel cIEF-MS platform

5. Correlation of Data between all assays

INTABIO

MERCK
INVENTING FOR LIFE

Traditional cIEF Analysis of Merck mAb using in-house methods following forced degradation conditions.



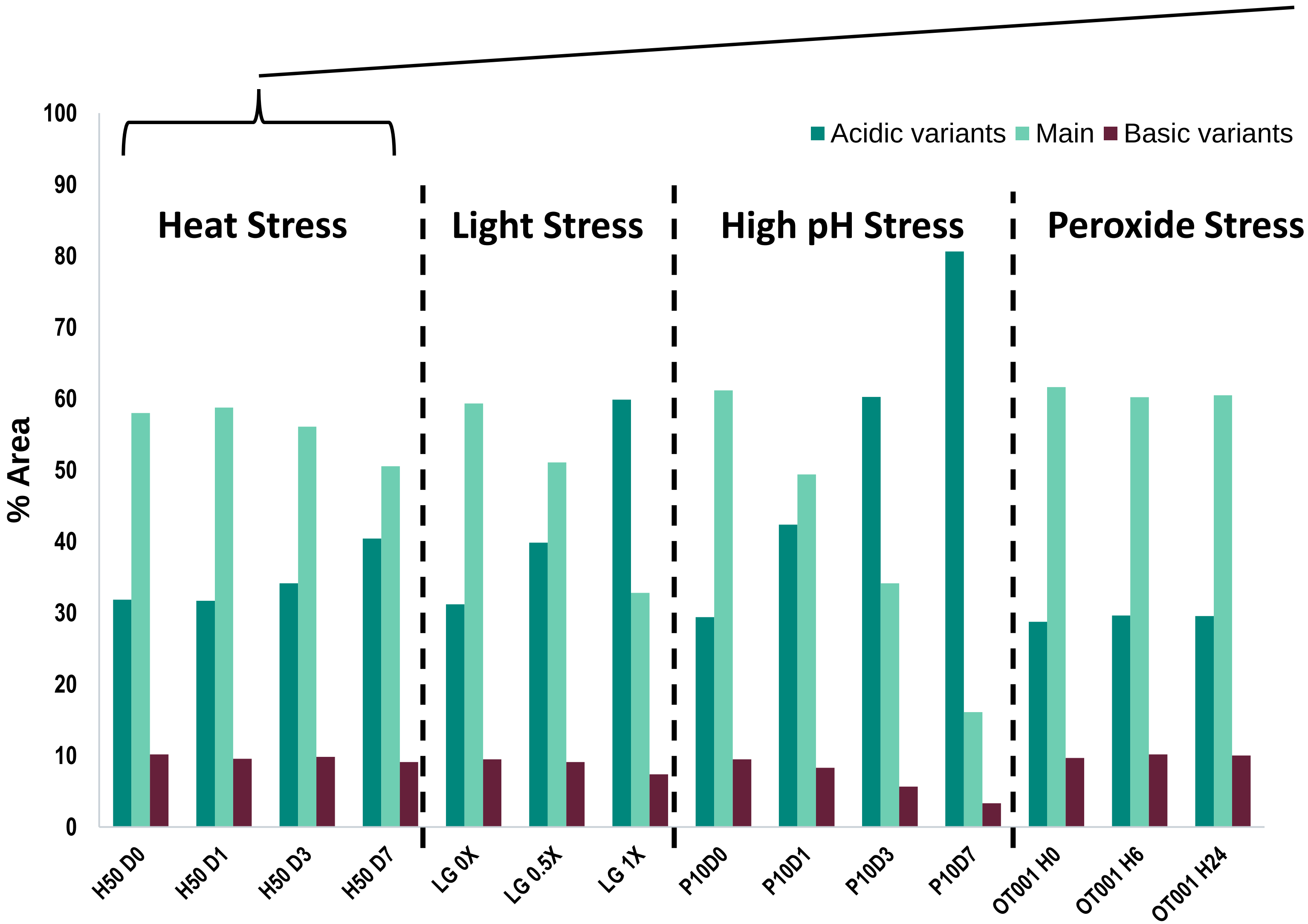
Common Acidic Modifications[1]
Sialic Acid
Deamidation
Trisulfide bonds
Thiosulfide Modification
Cysteinylation
Free thiols
Fragments

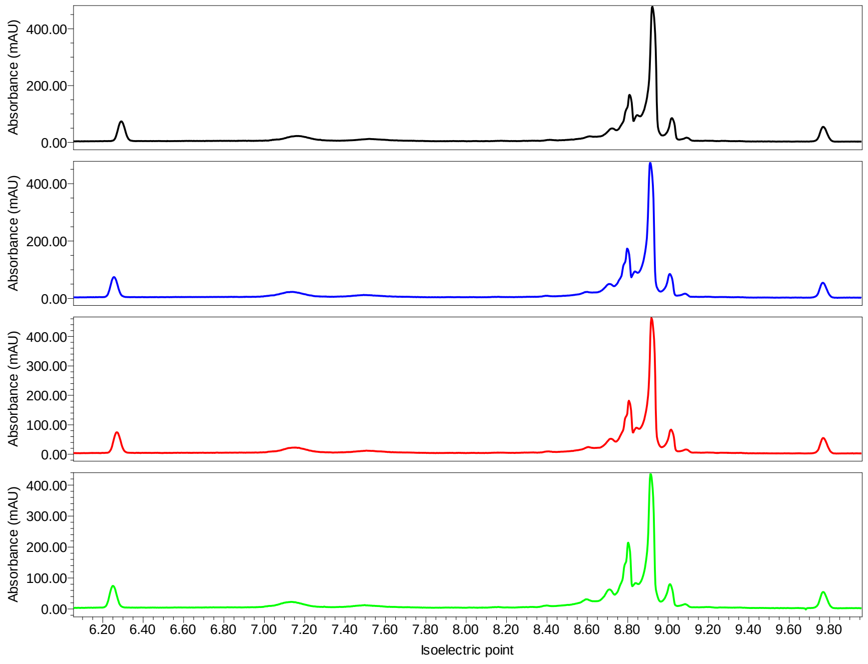
Common Basic Modifications[1]
C-terminal Lysine
N-terminal Glu
Isomerization of Asp
Met Oxidation
Amidation
Ser->Arg mutation
Fragments

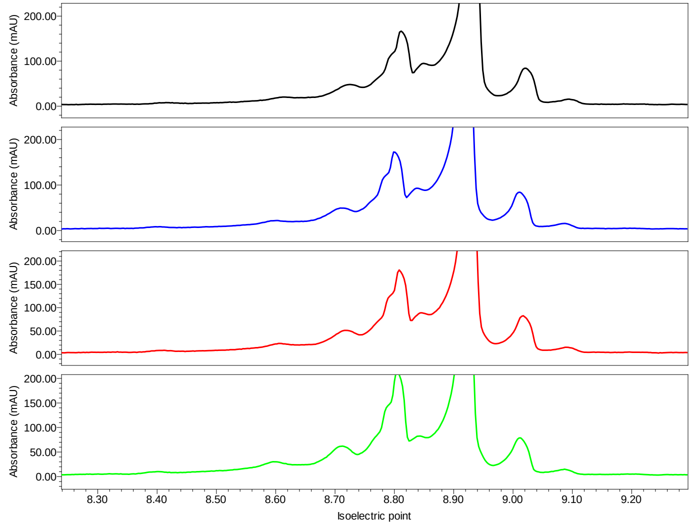
---

[1] Du, Yi et al. (2012) Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies." *mAbs* vol. 4,5, 578-85.

Traditional cIEF Analysis of on Maurice (Protein Simple) Instrument



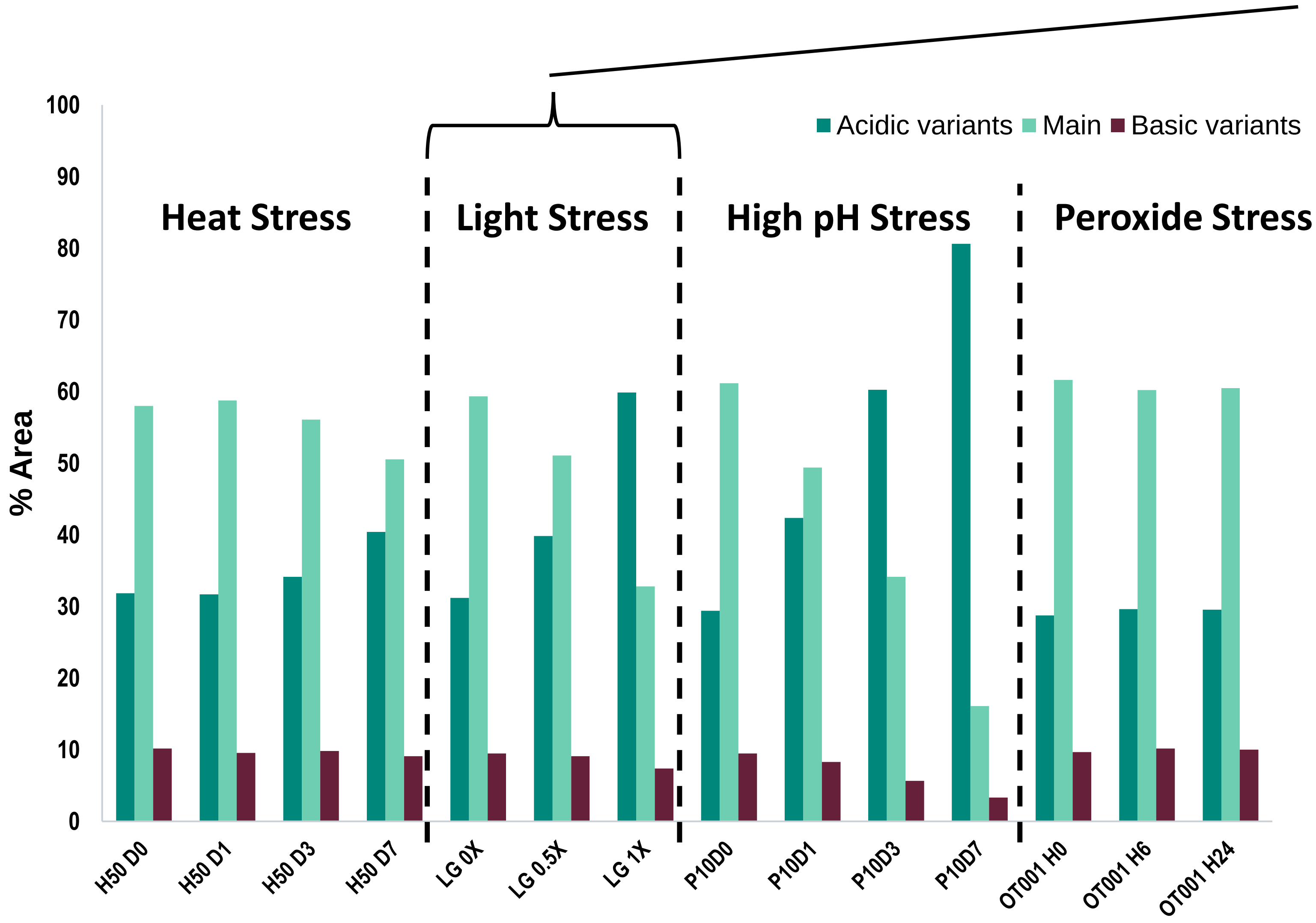


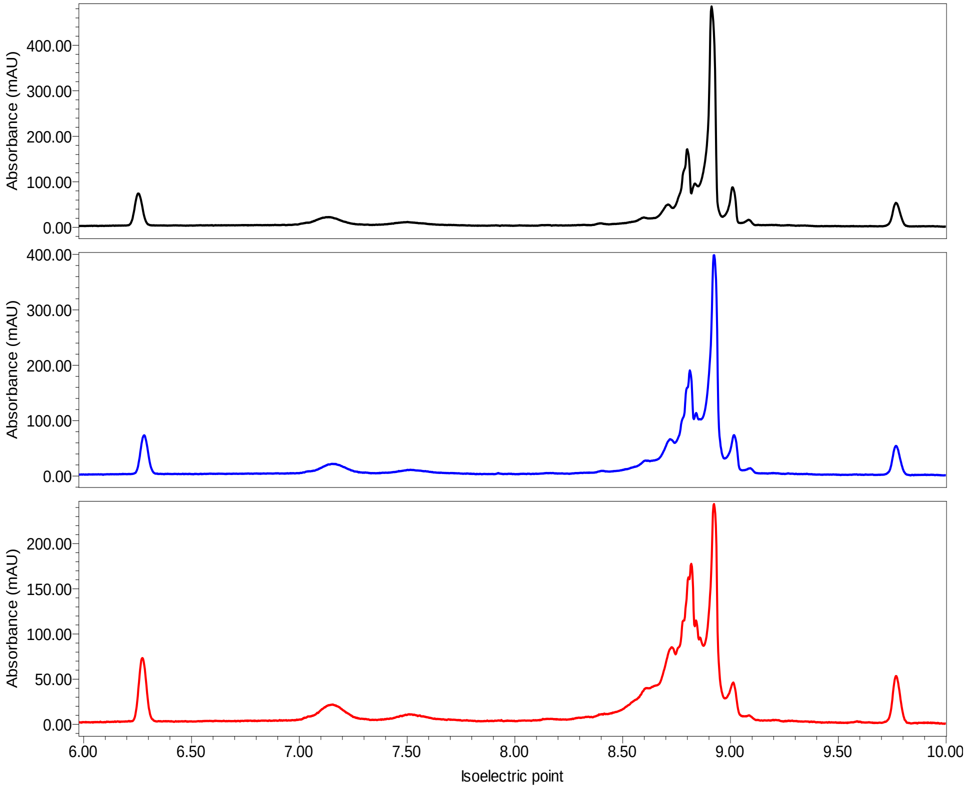


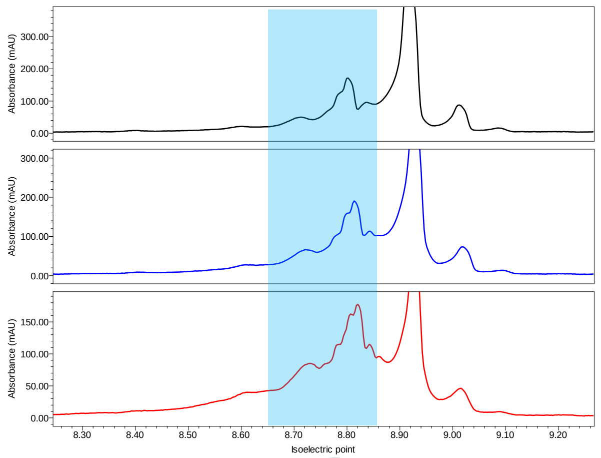
INTABIO

MERCK
INVENTING FOR LIFE

Traditional cIEF Analysis of on X instrument



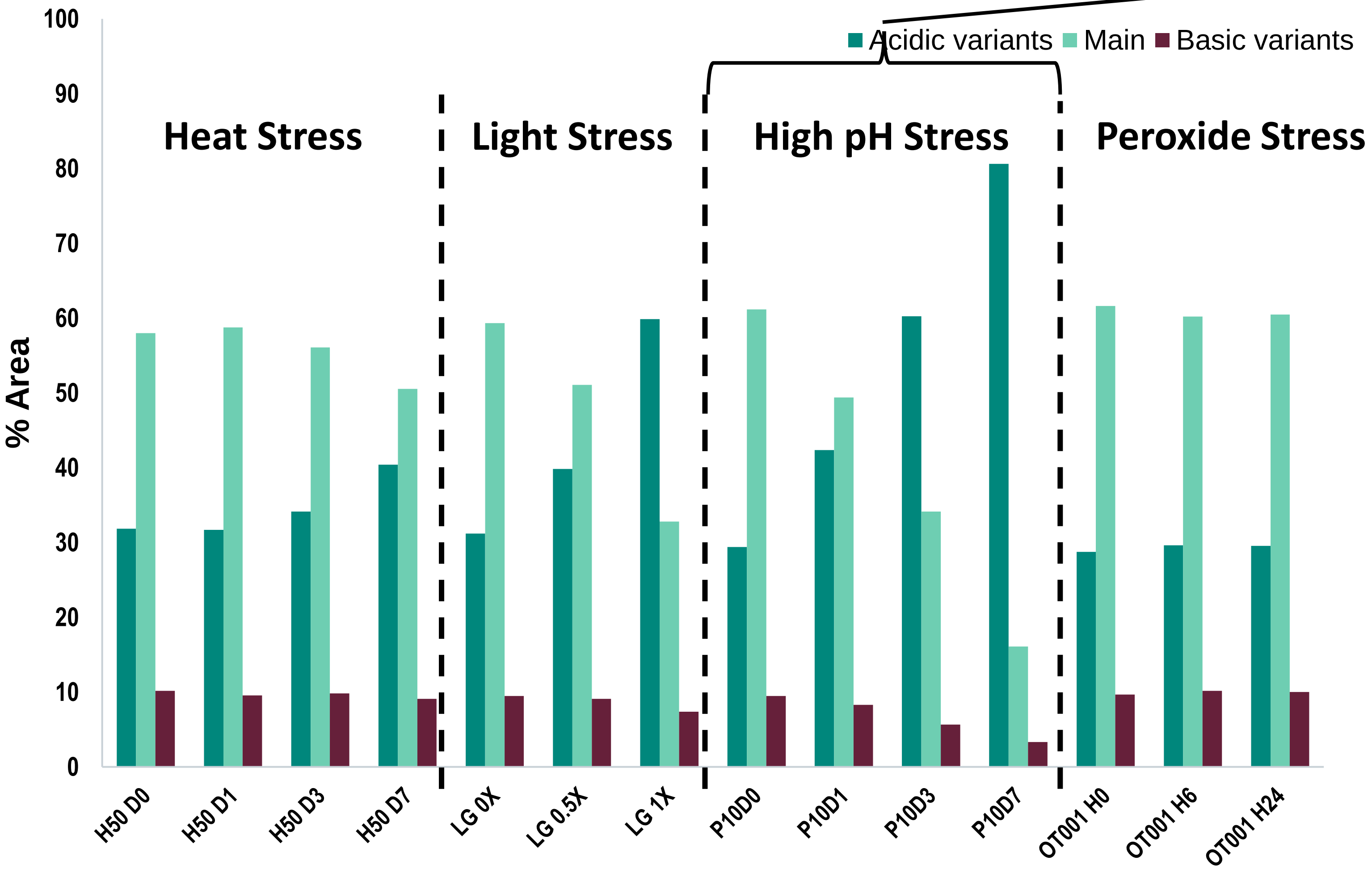


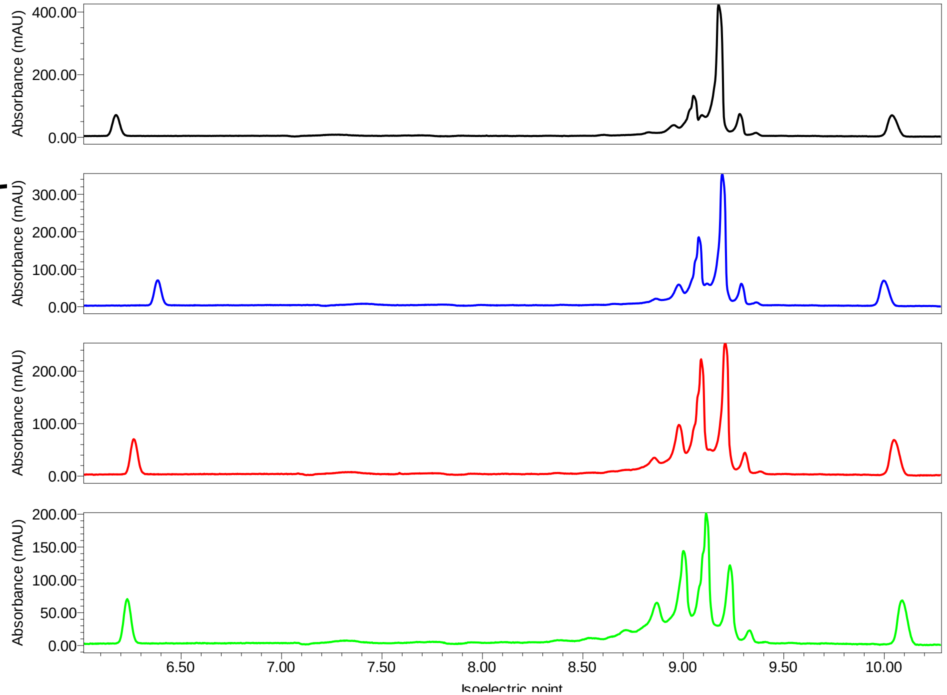


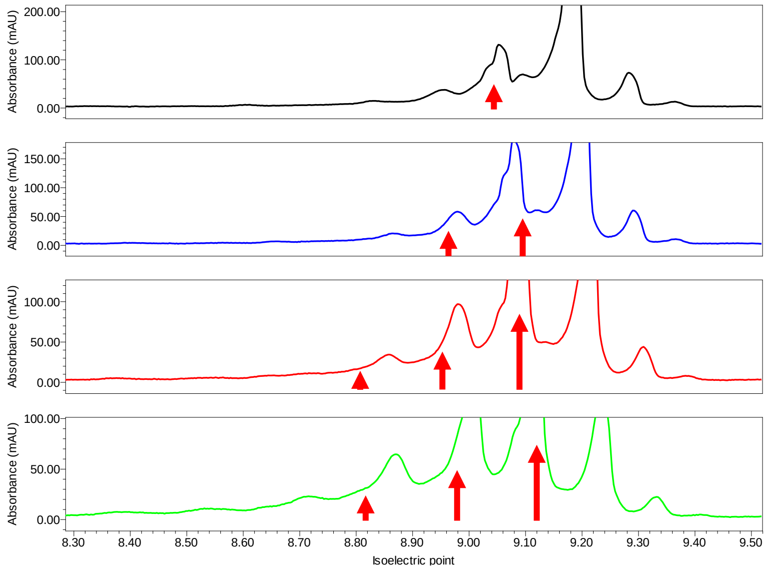
INTABIO

MERCK
INVENTING FOR LIFE

Traditional cIEF Analysis of on X instrument



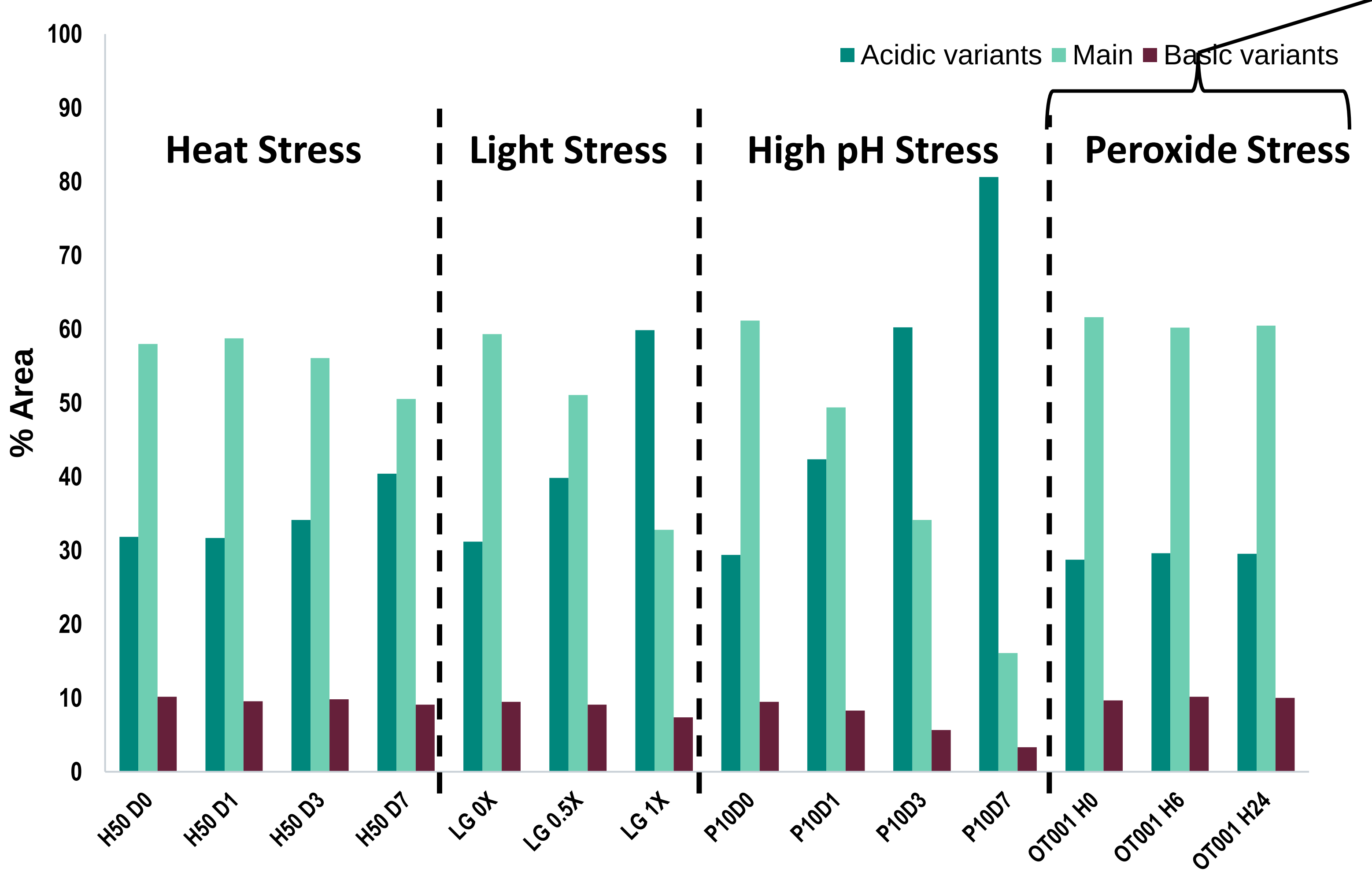


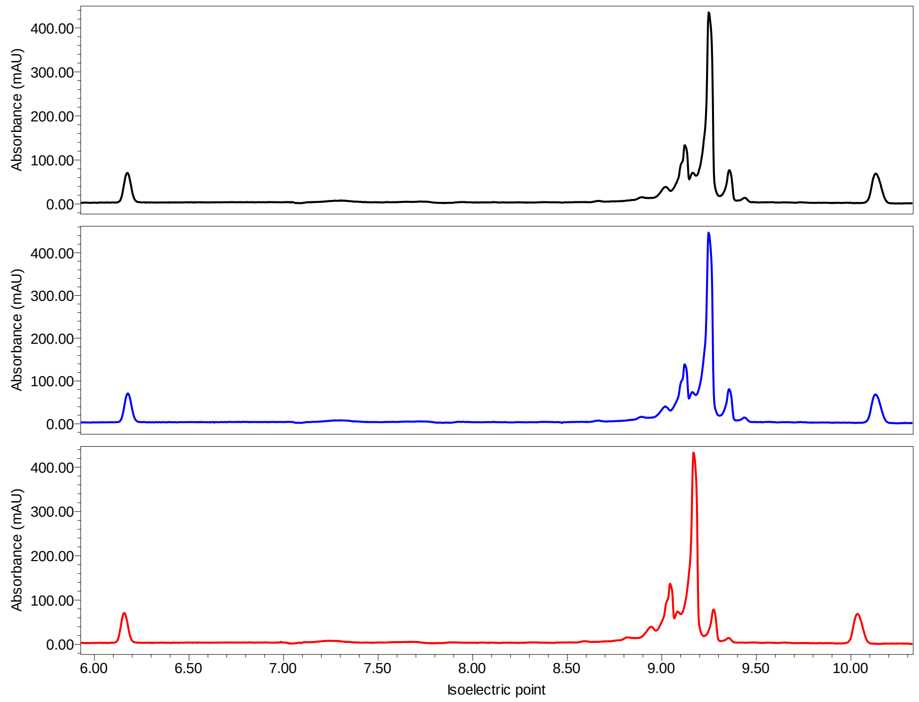


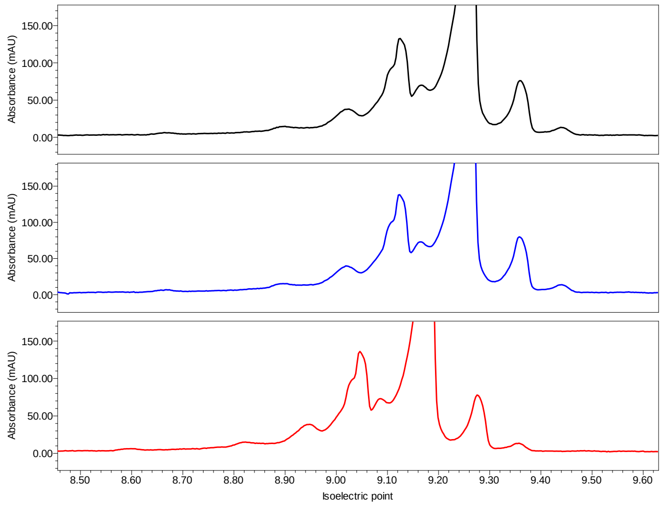
INTABIO

MERCK
INVENTING FOR LIFE

Traditional cIEF Analysis of on X instrument





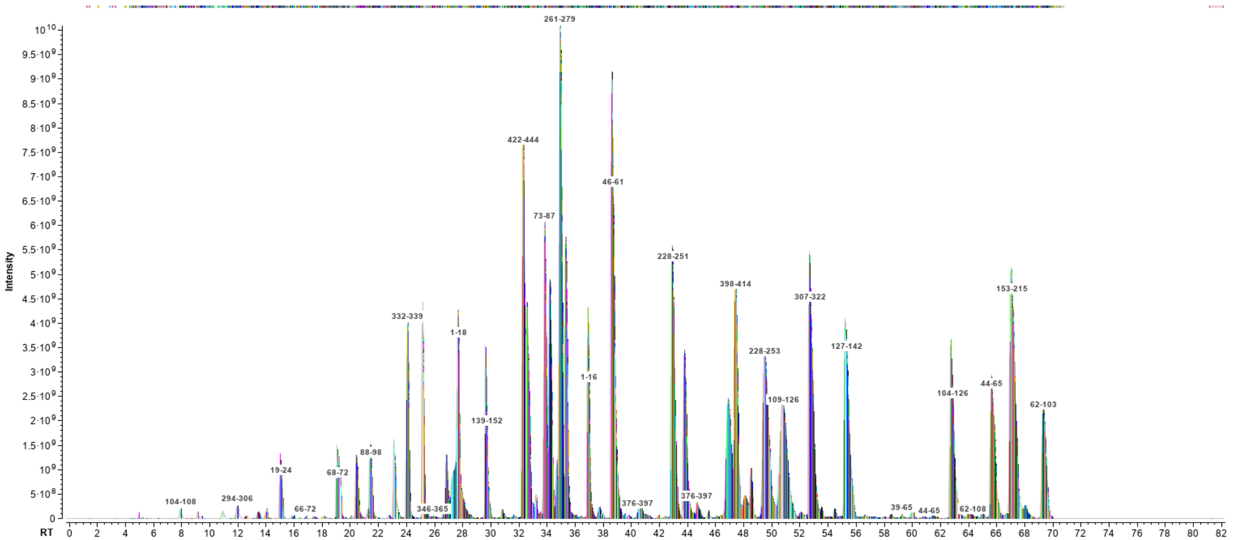


INTABIO

MERCK
INVENTING FOR LIFE

Major Modification Sites Identified by Reduced Peptide Mapping

	N terminal Pyroglutamate	Deamidation	Oxidation	Oxidation	C-terminal Lysine Retention
	N-terminus (Q)	Asparagine #1	Methionine #1	Methionine #2	C-terminus (K)
No Stress (411)	97.9	3.2	0.5	2.2	9.2
H50 –D0 (412)	97.9	3.4	0.7	2.0	8.3
H50 – D1 (413)	97.8	3.9	0.8	2.2	8.3
H50 – D3 (414)	97.4	3.6	0.9	2.4	8.6
H50 – D7 (415)	97.6	4.8	1.0	3.2	9.1
LG – 0X (417)	97.5	4.0	1.0	2.8	8.6
LG – 0.5X (418)	97.5	3.8	3.6	17.2	8.7
LG – 1X (419)	97.5	4.1	7.2	39.7	7.4
OT001 – H0 (420)	97.6	4.0	0.9	2.2	7.0
OT001 – H6 (421)	97.2	3.9	1.1	5.2	7.8
OT001 – H24 (422)	97.5	4.3	2.3	19.2	7.6
P10 – D0 (423)	97.6	4.4	1.0	3.6	7.7
P10 – D1 (424)	97.3	11.0	1.0	3.3	7.7
P10 – D3 (425)	97.5	23.7	1.0	3.9	7.4
P10 – D7 (426)	97.4	42.8	1.0	4.0	7.0



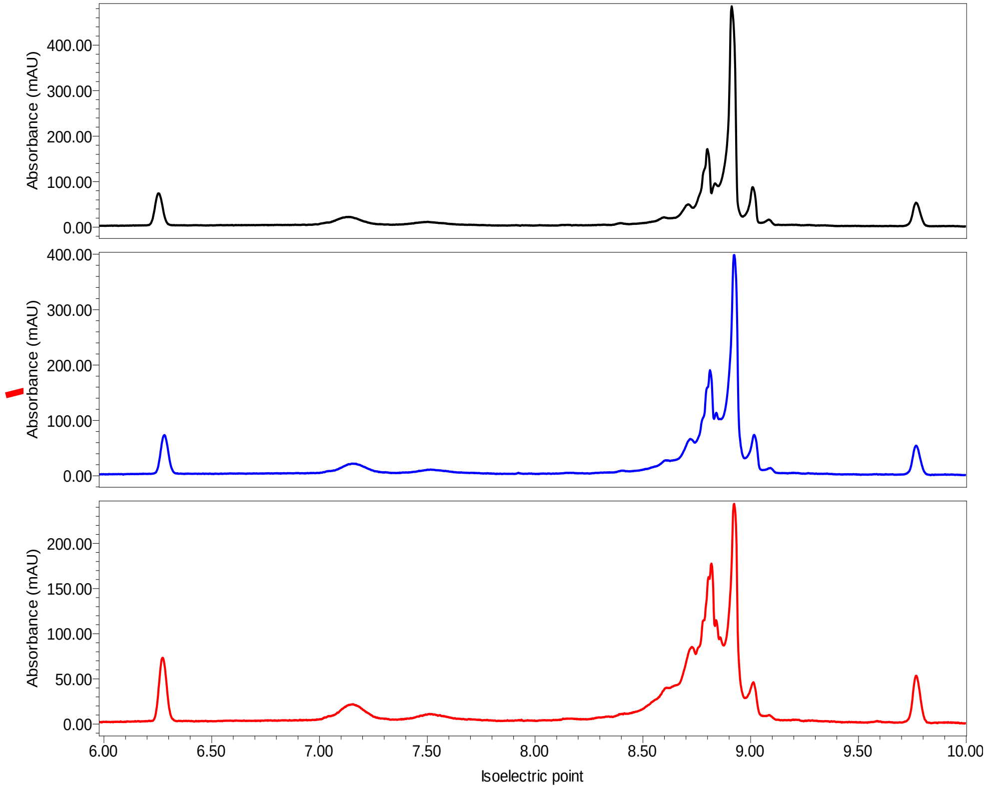
>95% sequence coverage

INTABIO

MERCK
INVENTING FOR LIFE

Major Modification Sites Identified by Reduced Peptide Mapping

	N terminal Pyroglutamate	Deamidation	Oxidation	Oxidation	C-terminal Lysine Retention
	N-terminus (Q)	Asparagine #1	Methionine #1	Methionine #2	C-terminus (K)
No Stress (411)	97.9	3.2	0.5	2.2	9.2
H50 –D0 (412)	97.9	3.4	0.7	2.0	8.3
H50 – D1 (413)	97.8	3.9	0.8	2.2	8.3
H50 – D3 (414)	97.4	3.6	0.9	2.4	8.6
H50 – D7 (415)	97.6	4.8	1.0	3.2	9.1
LG – 0X (417)	97.5	4.0	1.0	2.8	8.6
LG – 0.5X (418)	97.5	3.8	3.6	17.2	8.7
LG – 1X (419)	97.5	4.1	7.2	39.7	7.4
OT001 – H0 (420)	97.6	4.0	0.9	2.2	7.0
OT001 – H6 (421)	97.2	3.9	1.1	5.2	7.8
OT001 – H24 (422)	97.5	4.3	2.3	19.2	7.6
P10 – D0 (423)	97.6	4.4	1.0	3.6	7.7
P10 – D1 (424)	97.3	11.0	1.0	3.3	7.7
P10 – D3 (425)	97.5	23.7	1.0	3.9	7.4
P10 – D7 (426)	97.4	42.8	1.0	4.0	7.0

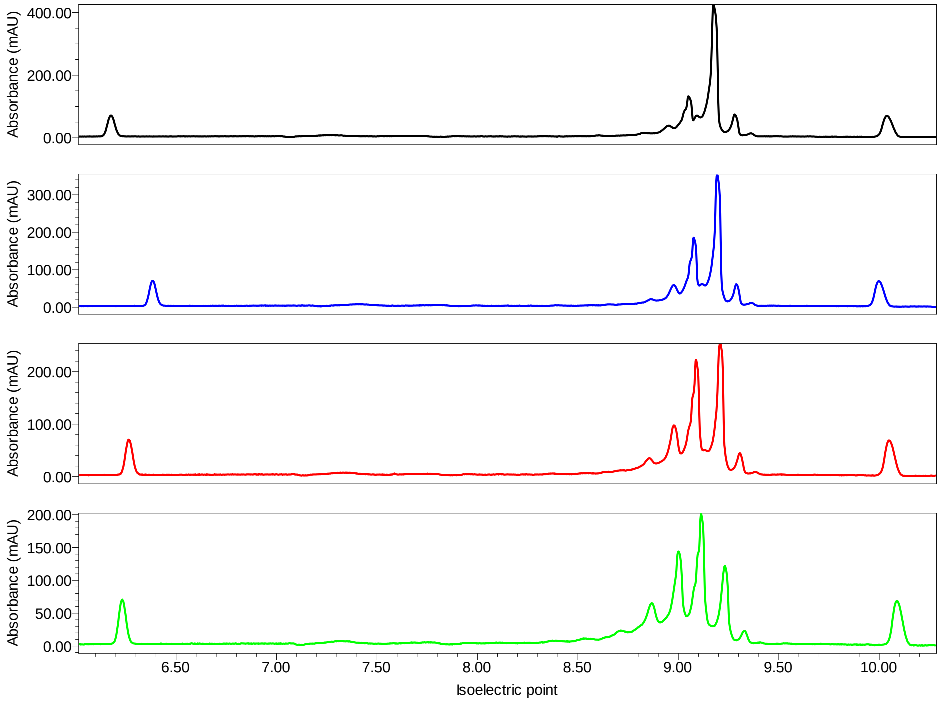


INTABIO

MERCK
INVENTING FOR LIFE

Major Modification Sites Identified by Reduced Peptide Mapping

	N terminal Pyroglutamate	Deamidation	Oxidation	Oxidation	C-terminal Lysine Retention
	N-terminus (Q)	Asparagine #1	Methionine #1	Methionine #2	C-terminus (K)
No Stress (411)	97.9	3.2	0.5	2.2	9.2
H50 –D0 (412)	97.9	3.4	0.7	2.0	8.3
H50 – D1 (413)	97.8	3.9	0.8	2.2	8.3
H50 – D3 (414)	97.4	3.6	0.9	2.4	8.6
H50 – D7 (415)	97.6	4.8	1.0	3.2	9.1
LG – 0X (417)	97.5	4.0	1.0	2.8	8.6
LG – 0.5X (418)	97.5	3.8	3.6	17.2	8.7
LG – 1X (419)	97.5	4.1	7.2	39.7	7.4
OT001 – H0 (420)	97.6	4.0	0.9	2.2	7.0
OT001 – H6 (421)	97.2	3.9	1.1	5.2	7.8
OT001 – H24 (422)	97.5	4.3	2.3	19.2	7.6
P10 – D0 (423)	97.6	4.4	1.0	3.6	7.7
P10 – D1 (424)	97.3	11.0	1.0	3.3	7.7
P10 – D3 (425)	97.5	23.7	1.0	3.9	7.4
P10 – D7 (426)	97.4	42.8	1.0	4.0	7.0

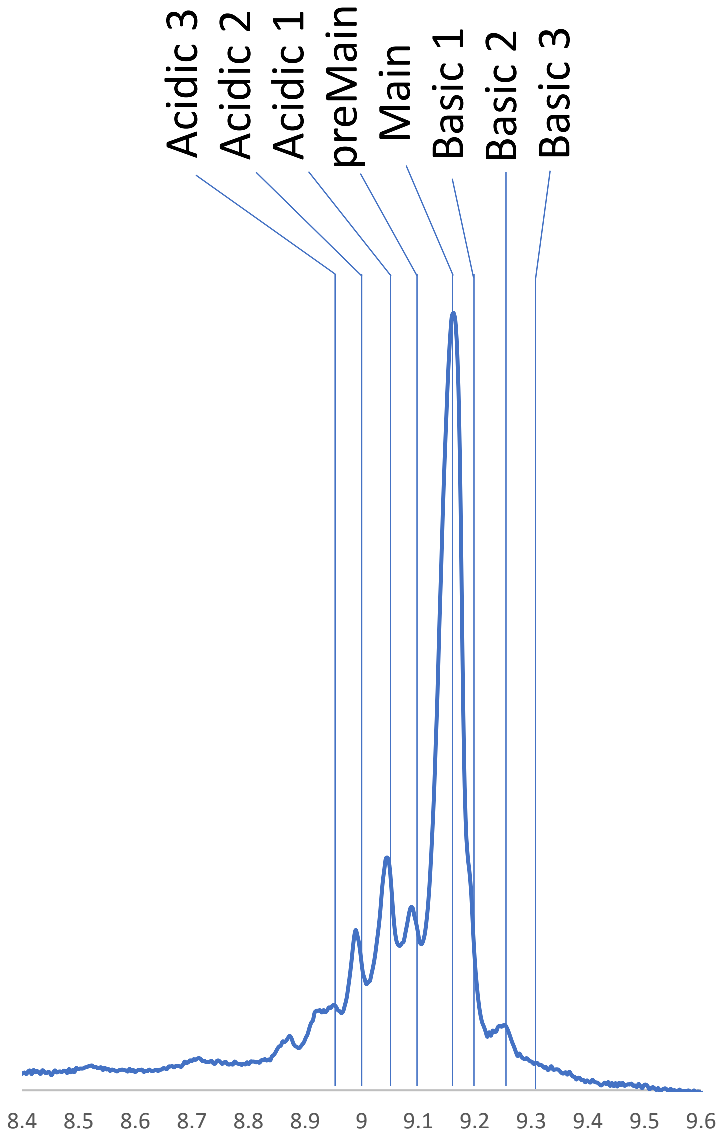


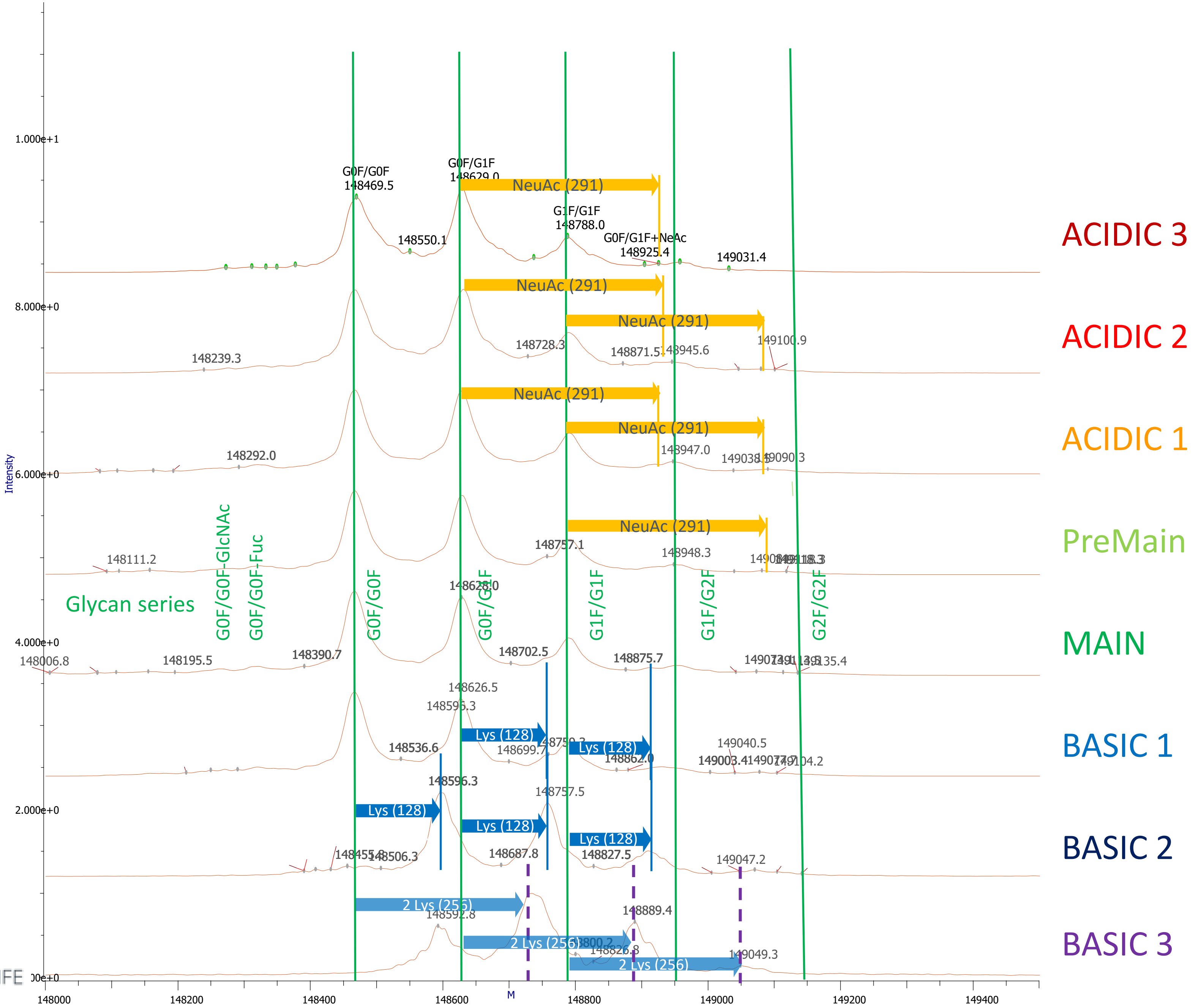
Change in acidic variant abundance as seen is cIEF analysis can most likely be attributed to deamidation at the PENNY peptide

INTABIO

MERCK
INVENTING FOR LIFE

Blaze iCIEF-MS Analysis of Charge Variants – Non-stressed Control



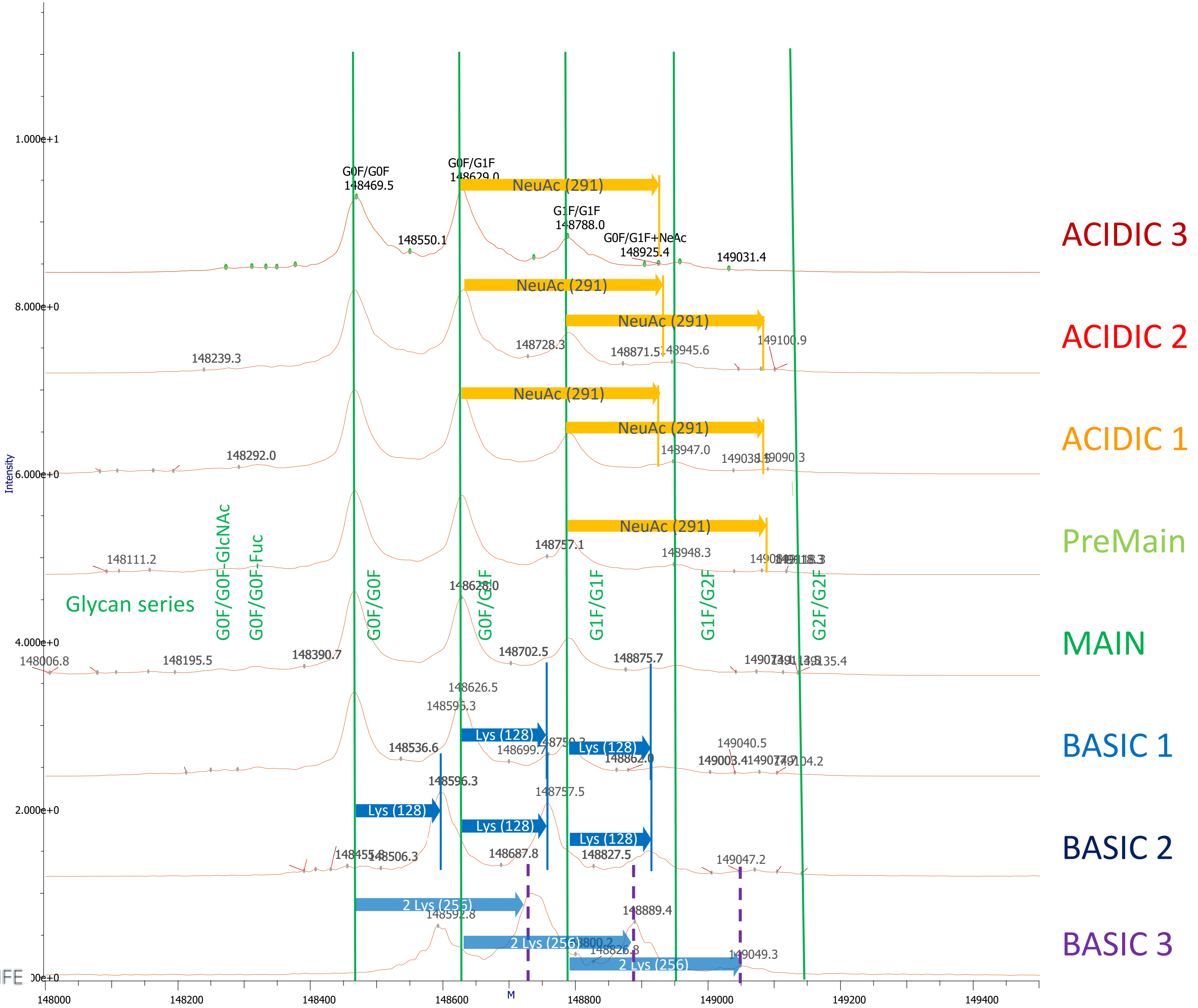


INTABIO

MERCK
INVENTING FOR LIFE

Blaze iCIEF-MS Analysis of Charge Variants – Non-stressed Control

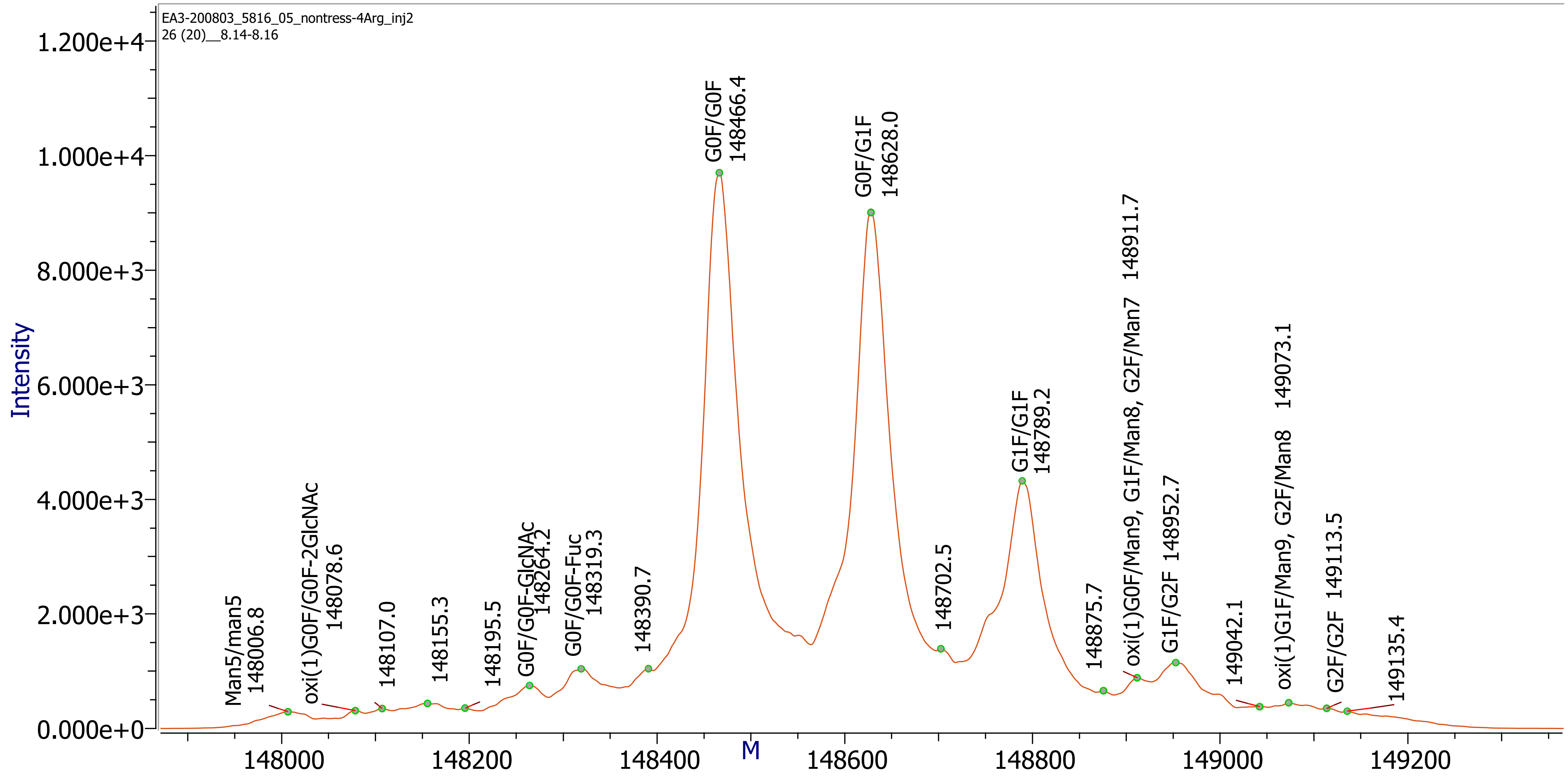
- Mass shifts of 162 Da denote the galactose series of G0F, G1F and G2F glycans
- Basic charge variants with a mass addition of 128 Da correspond to unprocessed lysine(s)
- Acidic charge variants correspond to sialic acid additions



INTABIO

MERCK
INVENTING FOR LIFE

Blaze iCIEF-MS Analysis of Non-stressed Control Main Peak Putative Modifications



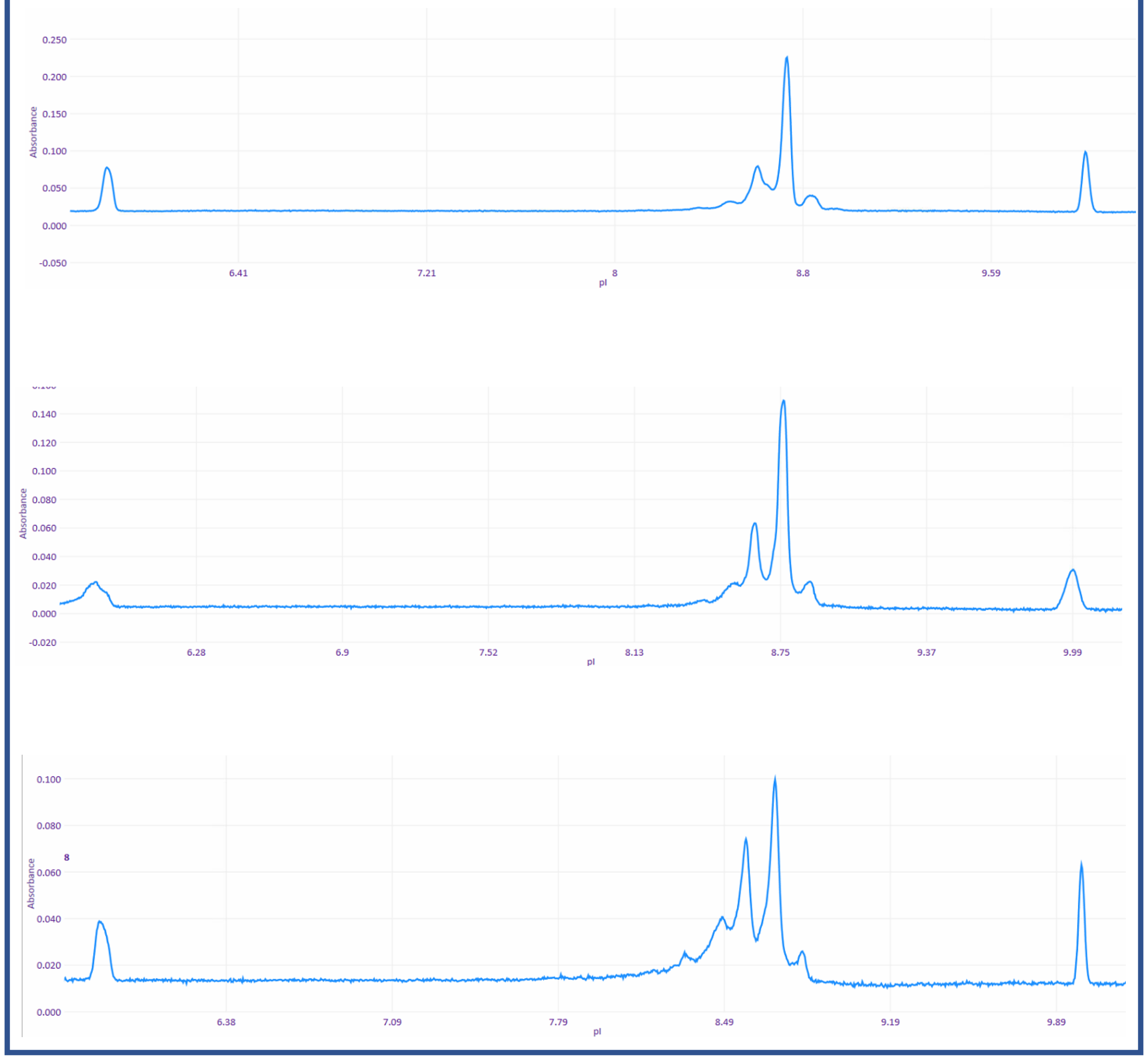
Blaze analysis of the 3 light stressed conditions shows comparable cIEF profiles to Merck cIEF analysis

Blaze cIEF-MS analysis

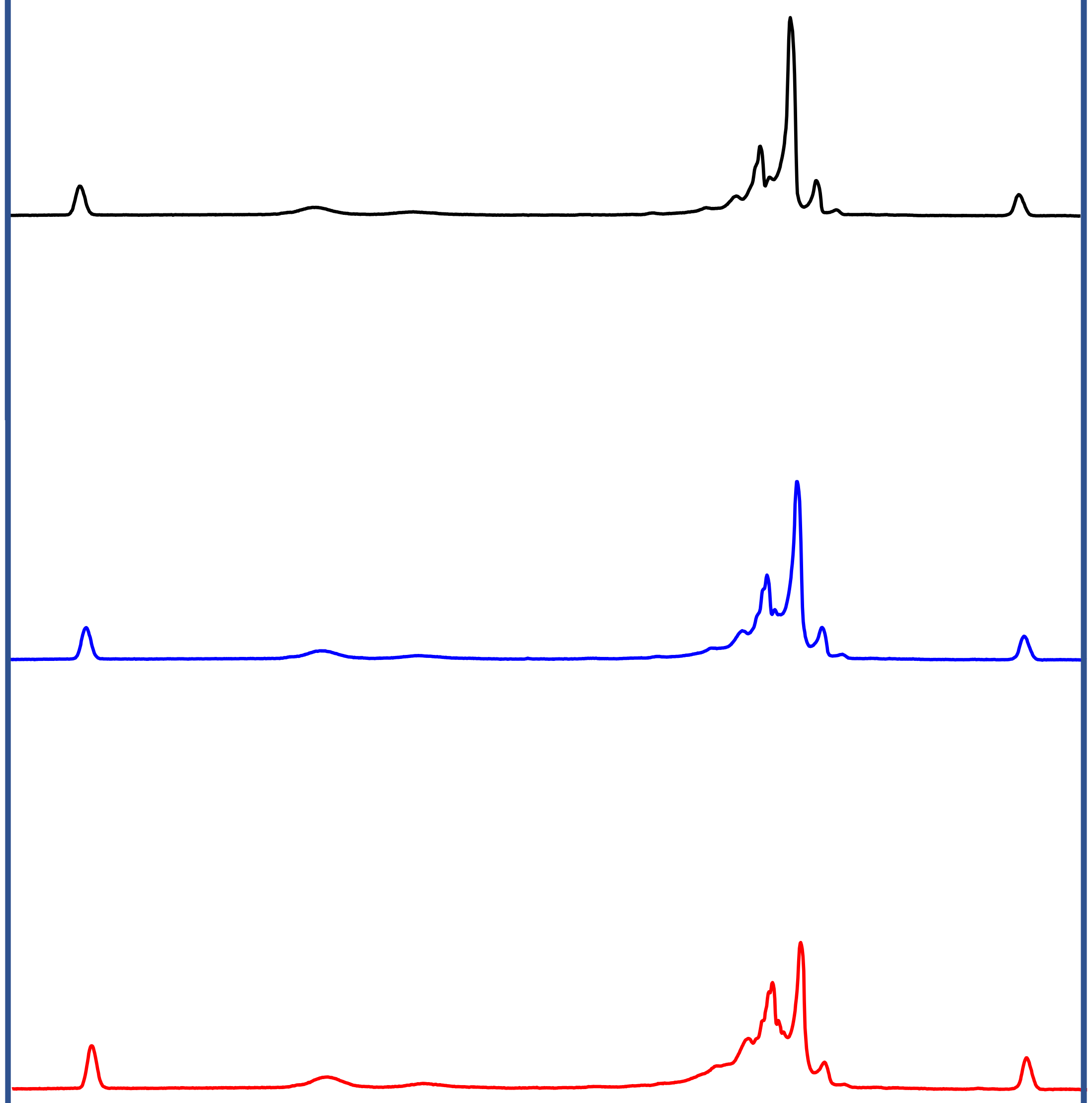
0X

.5X

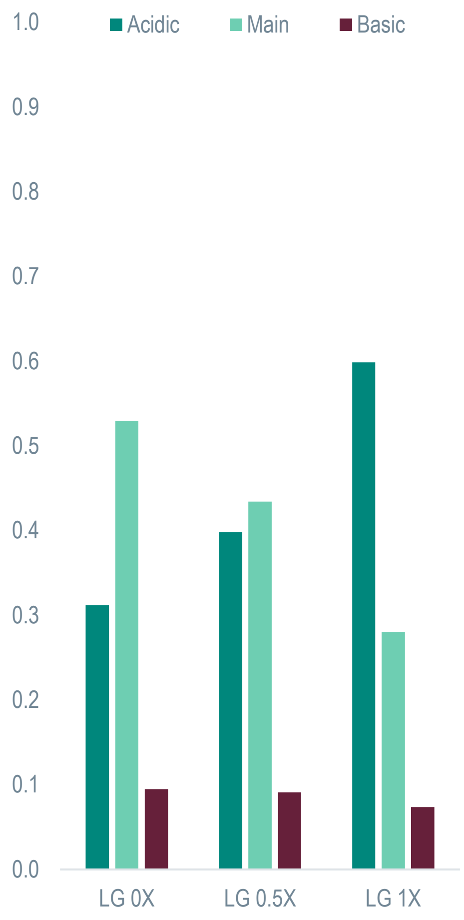
1X

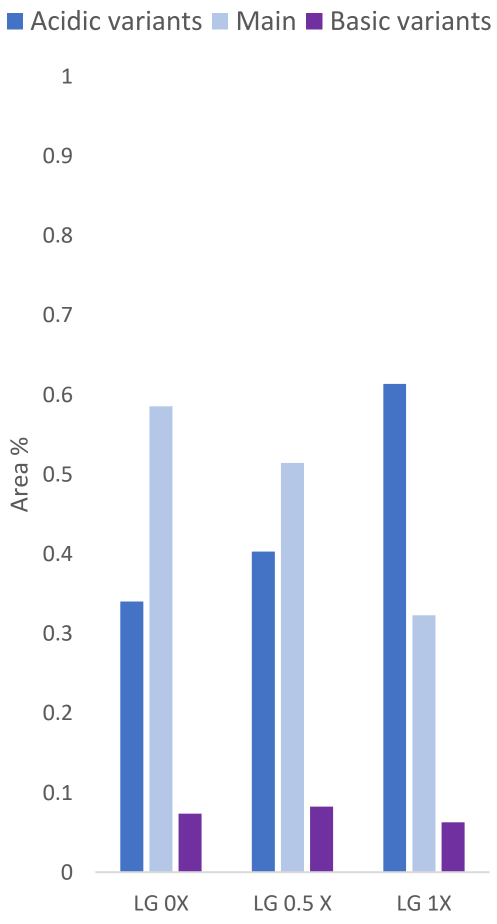


Merck in-house cIEF analysis



Comparable Quantitation of Blaze ciEF with Merck In-House Data

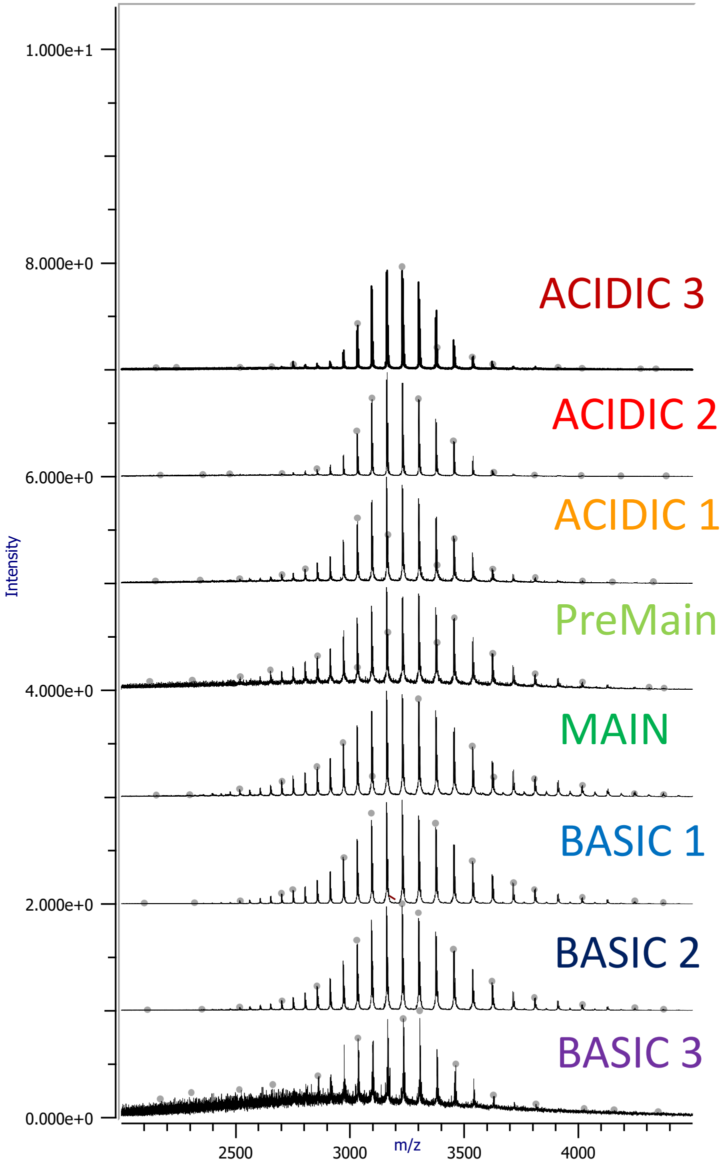


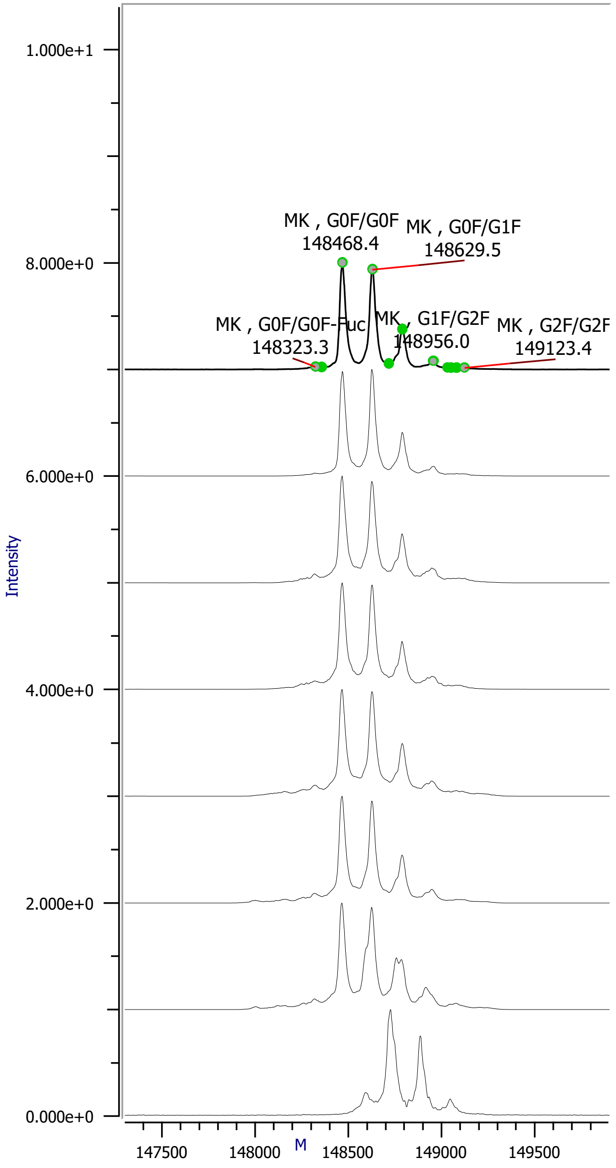


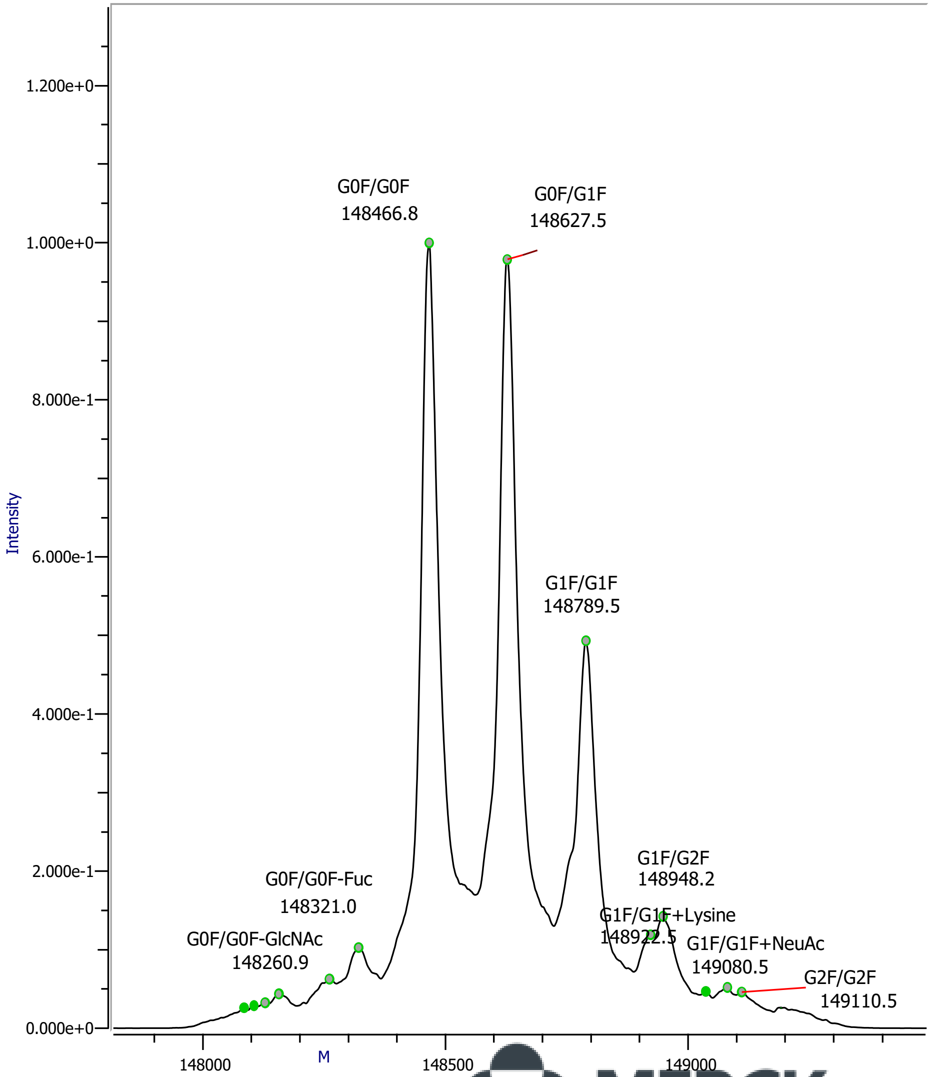
INTABIO

MERCK
INVENTING FOR LIFE

Blaze iCIEF-MS Analysis 0X Control


Raw mass spectra


Deconvoluted spectra

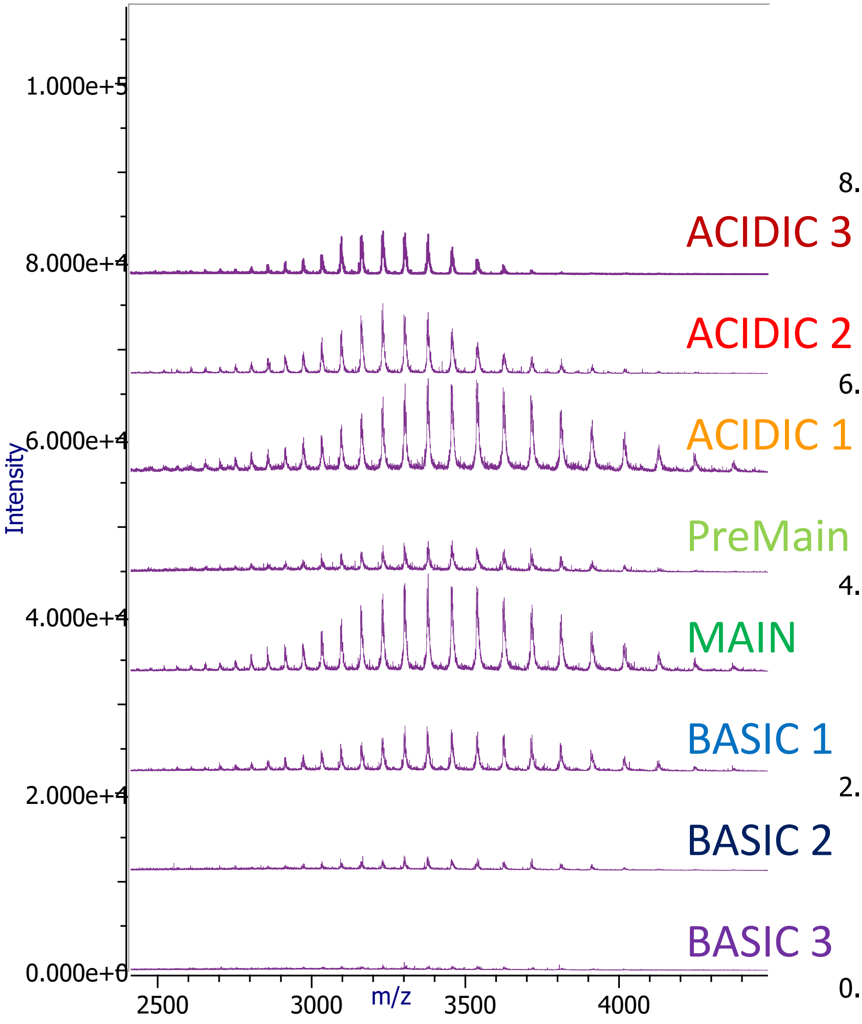

Main peak deconvoluted spectra

INTABIO

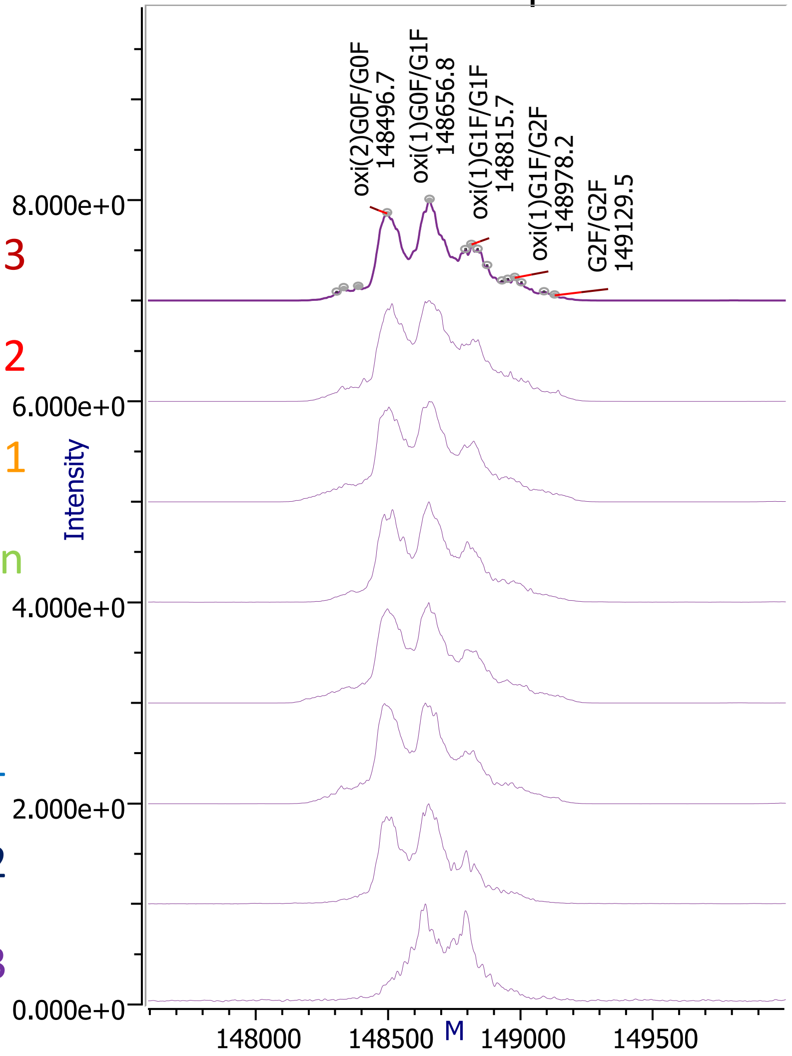
MERCK
INVENTING FOR LIFE

Blaze iCIEF-MS Analysis of 0.5X Light Stress Shows 1-2 Oxidation Sites

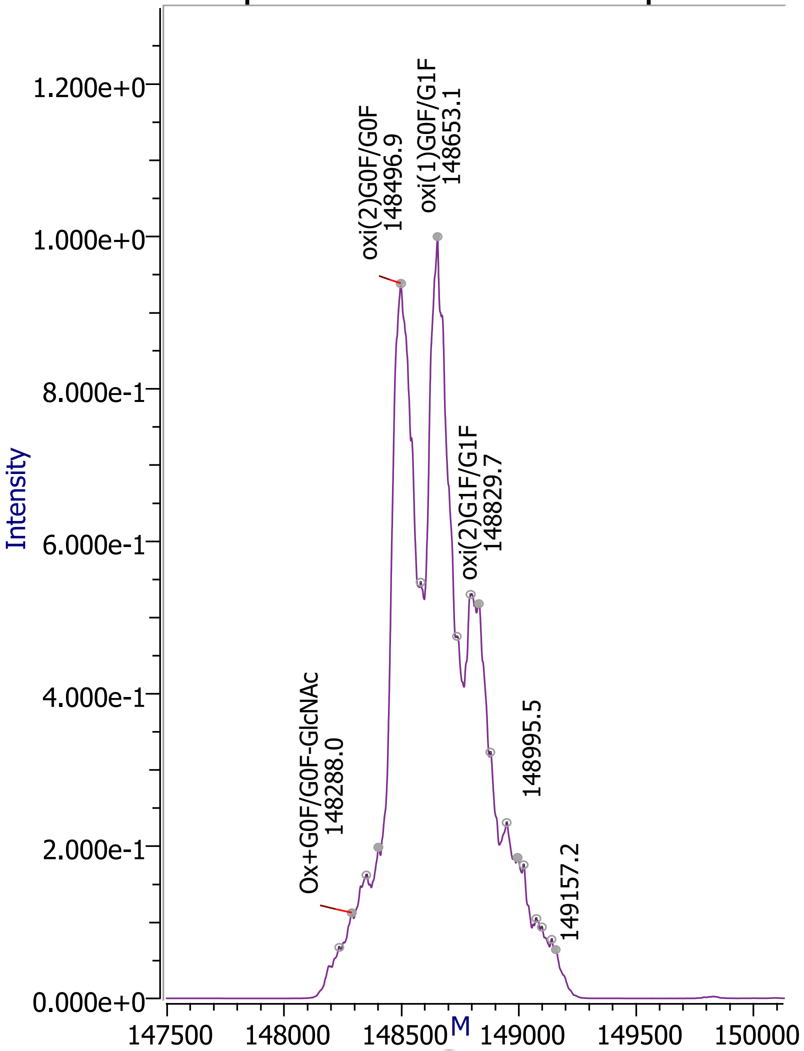
Raw mass spectra



Deconvoluted spectra



Main peak deconvoluted spectra

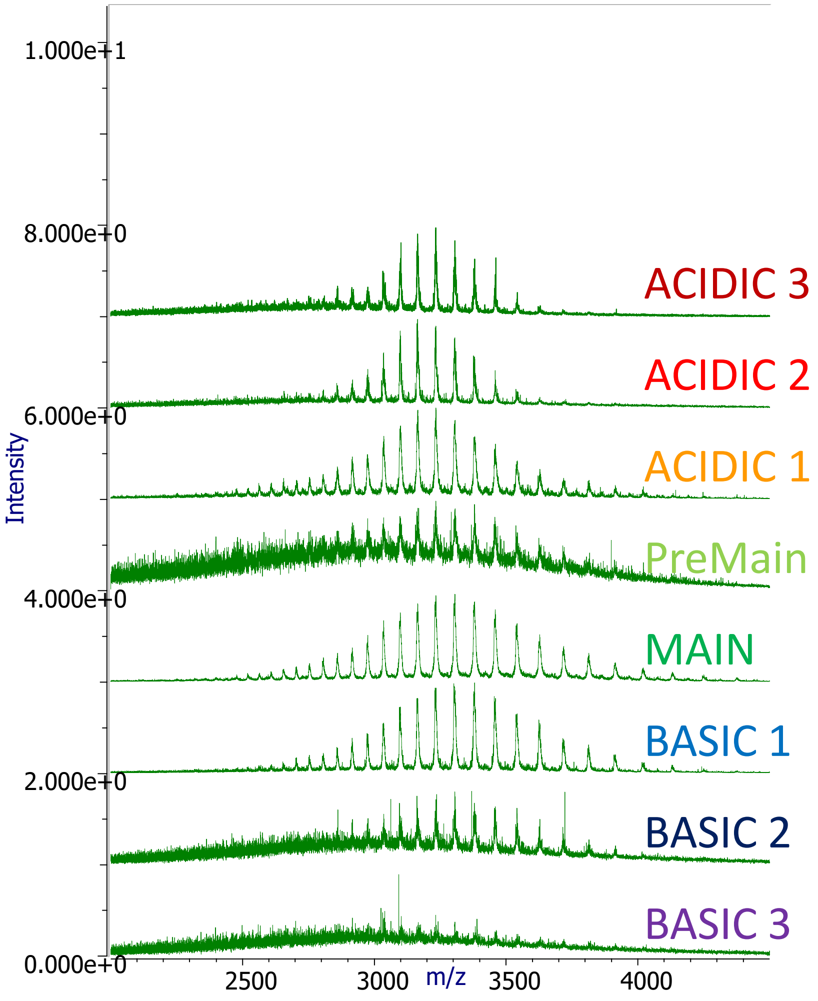


INTABIO

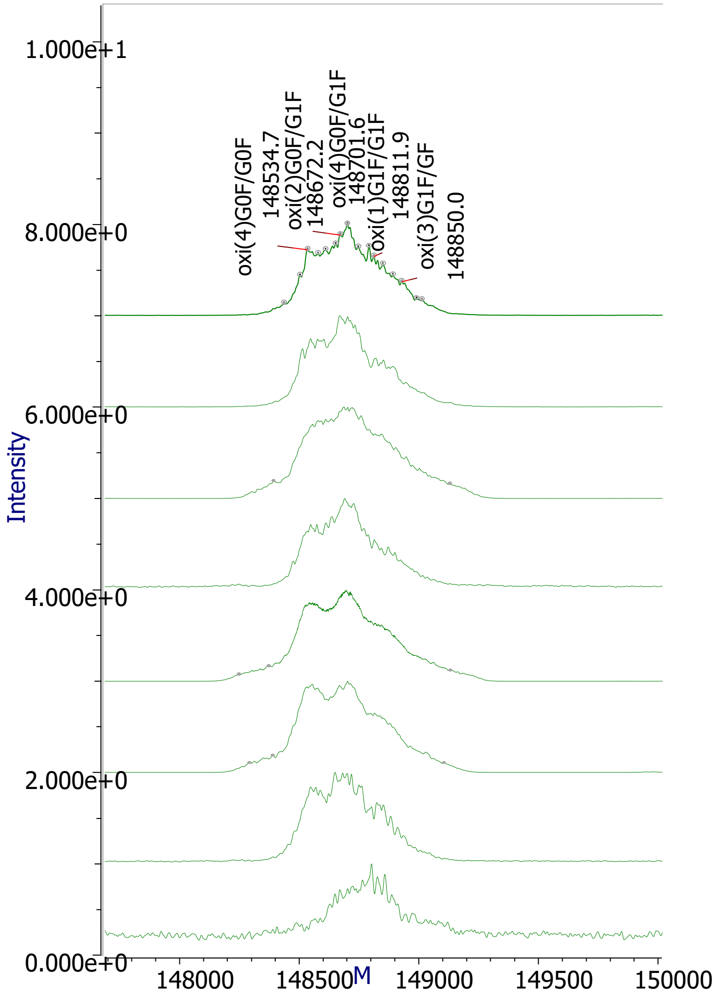
MERCK
INVENTING FOR LIFE

Blaze iCIEF-MS Analysis of 1X Light Stress Shows 3-4 Oxidation Sites

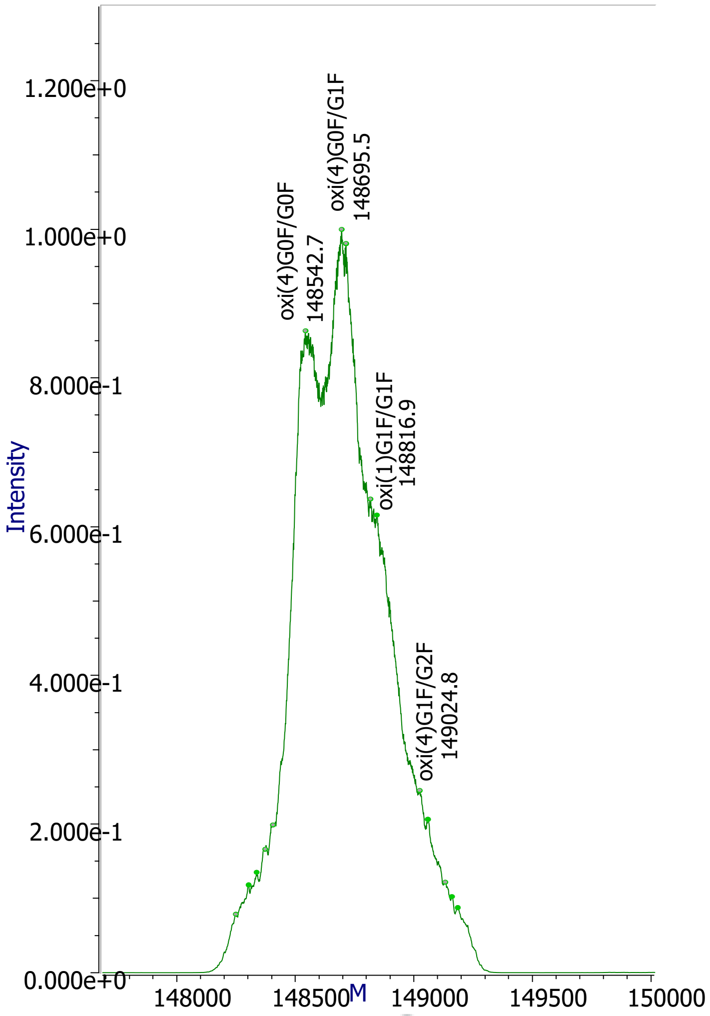
Raw mass spectra



Deconvoluted spectra



Main peak deconvoluted spectra



INTABIO

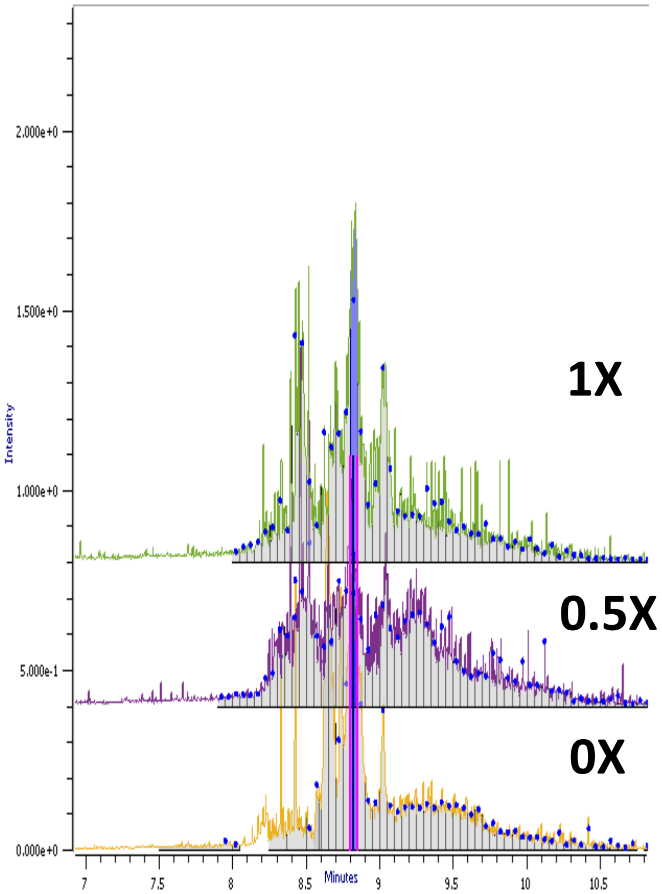
MERCK
INVENTING FOR LIFE

Comparison of Oxidation Levels by iCIEF-MS vs. Peptide Mapping

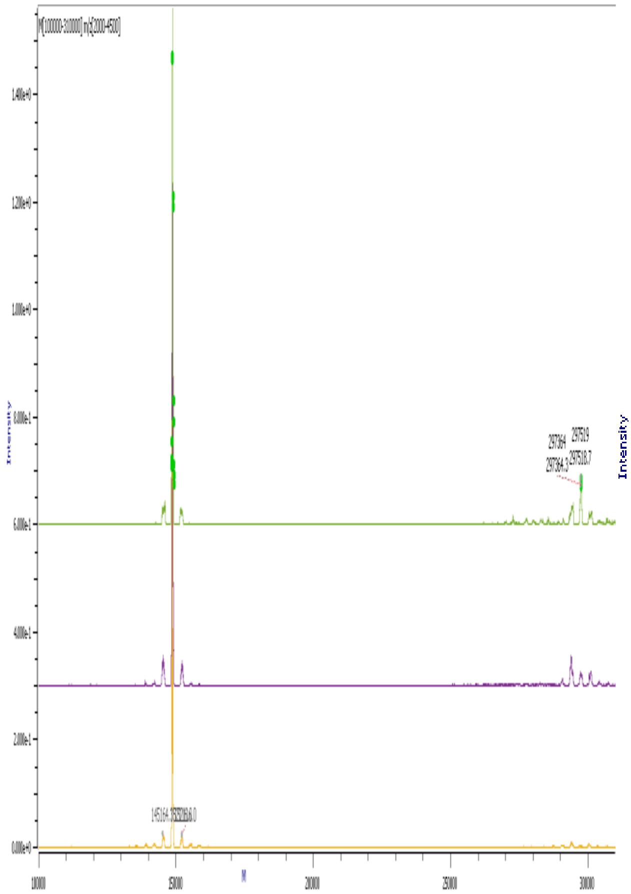
Light Stressed samples	% of oxidized protein measured with blaze CiEF-MS analysis	% of oxidized amino acid residue with blaze CiEF- MS analysis	Average % oxidized Methionine (Methionine #1 & Methionine #2) with RPM	% of oxidation of Methionine #1 (RPM)	% of oxidation of Methionine #2 (RPM)
0X	2.81%	2.53%	1.90%	1.00%	2.80%
0.5X	20.16%	13.59%	10.40%	3.60%	17.20%
1x	36.65%	25.56%	23.45%	7.20%	39.70%

Increase in Dimer Species is observed by iCIEF-MS in Light Stress Material and Verified with Size Exclusion Chromatography

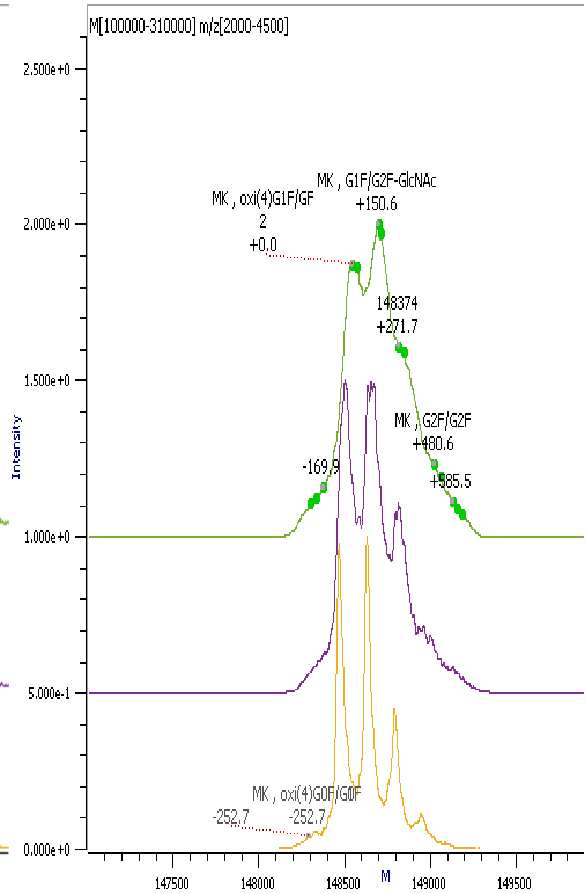
Base peak



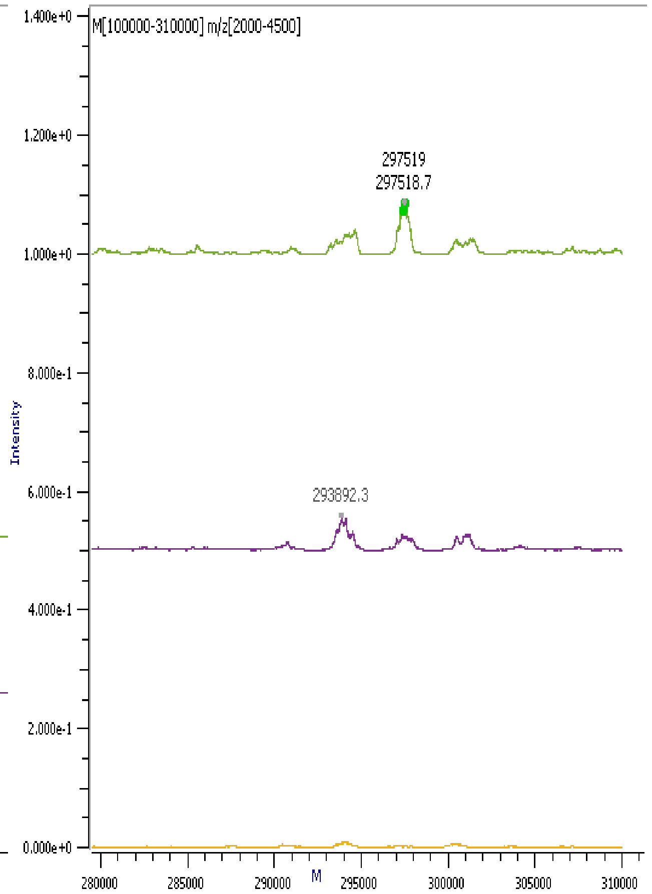
Normalized deconvoluted mass



Monomer



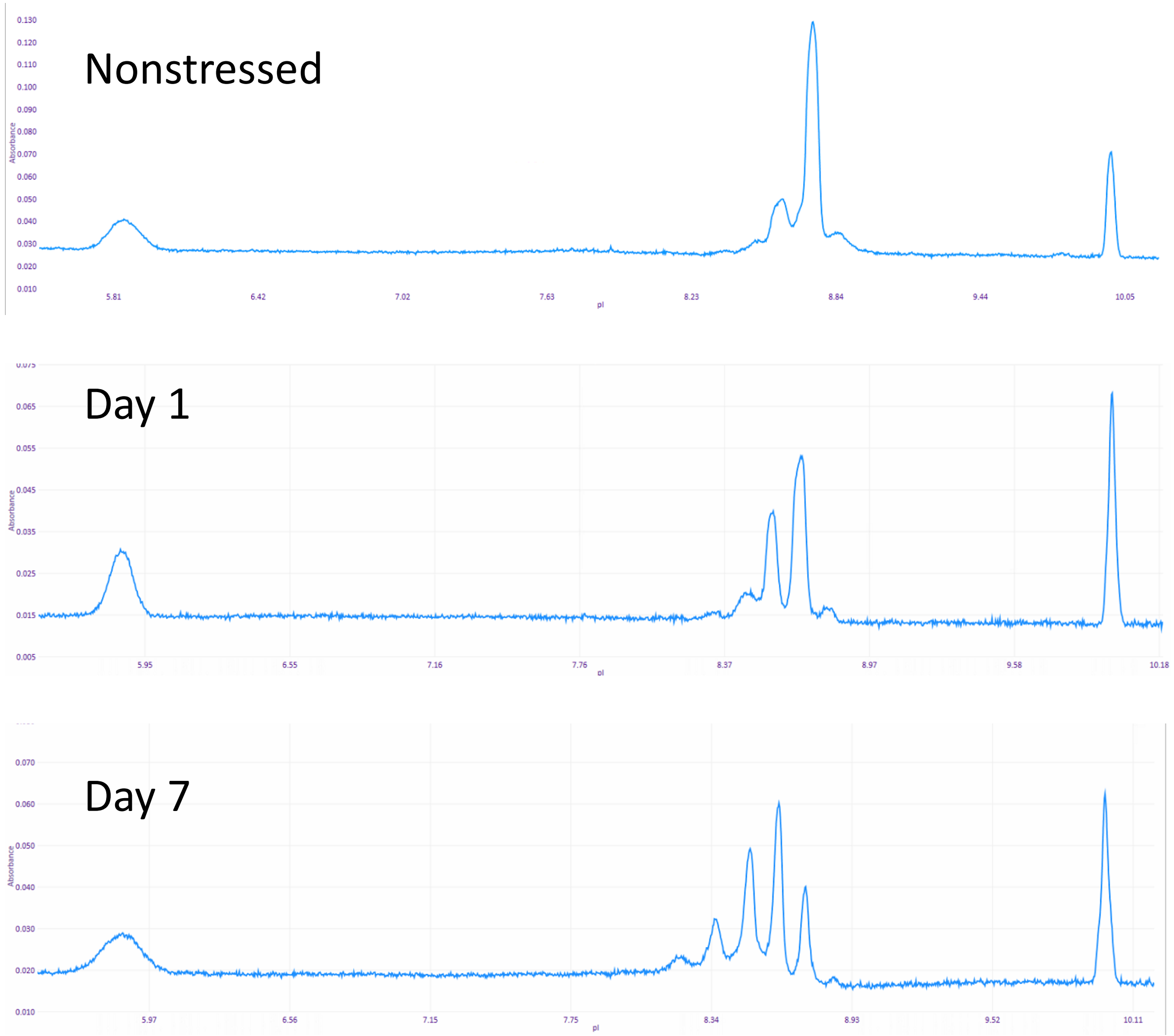
Dimer

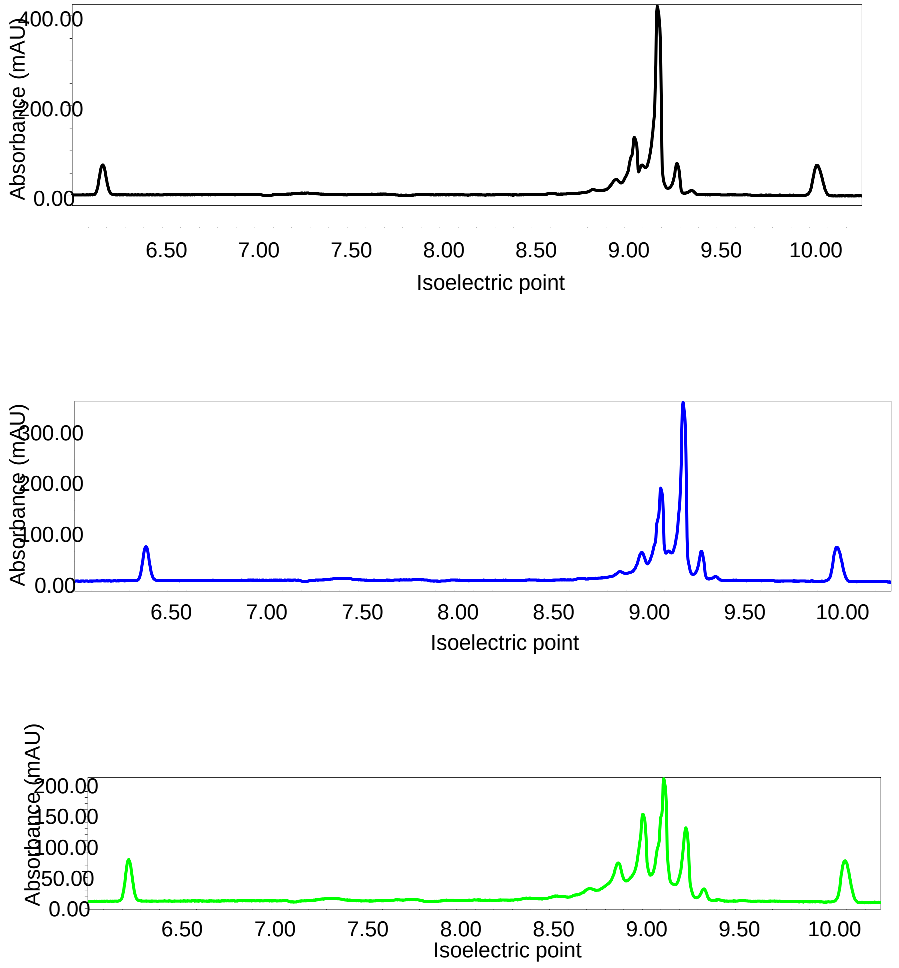


INTABIO

MERCK
INVENTING FOR LIFE

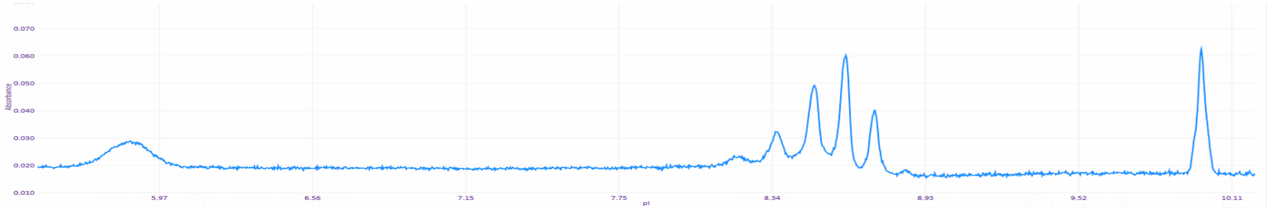
iCIEF Analysis of High pH Samples on Blaze vs. in-house iCIEF

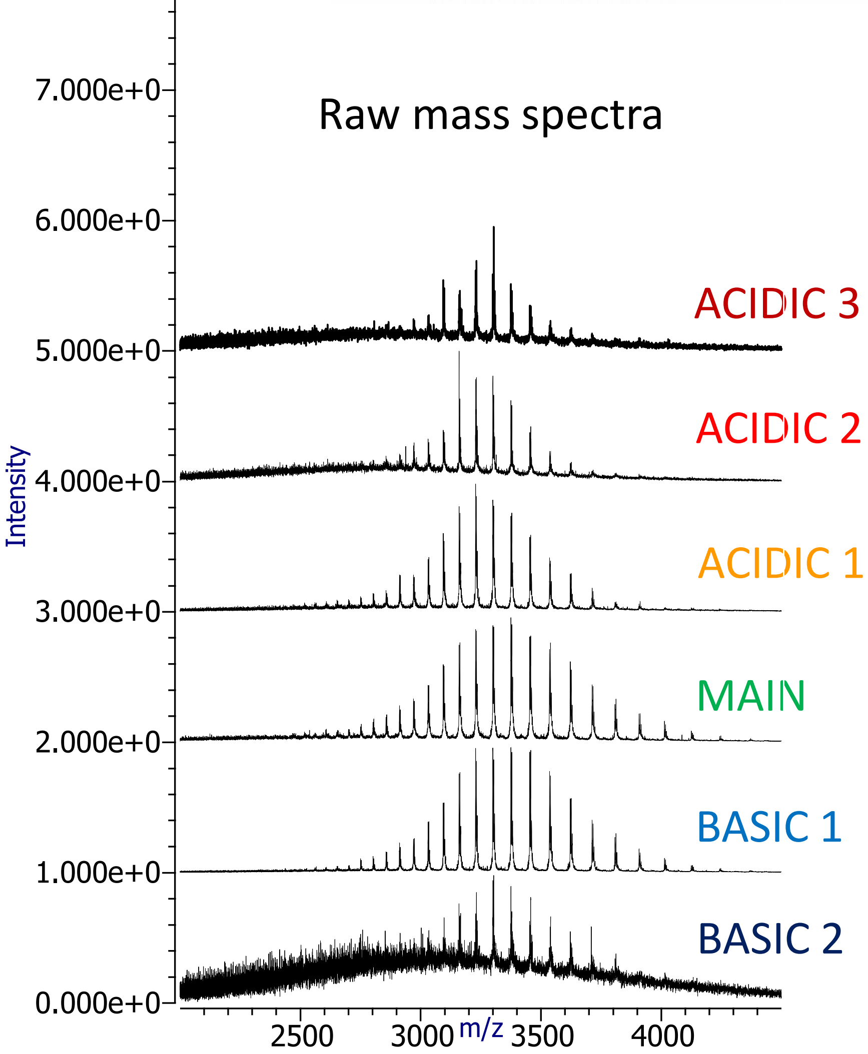

Blaze cIEF-MS analysis

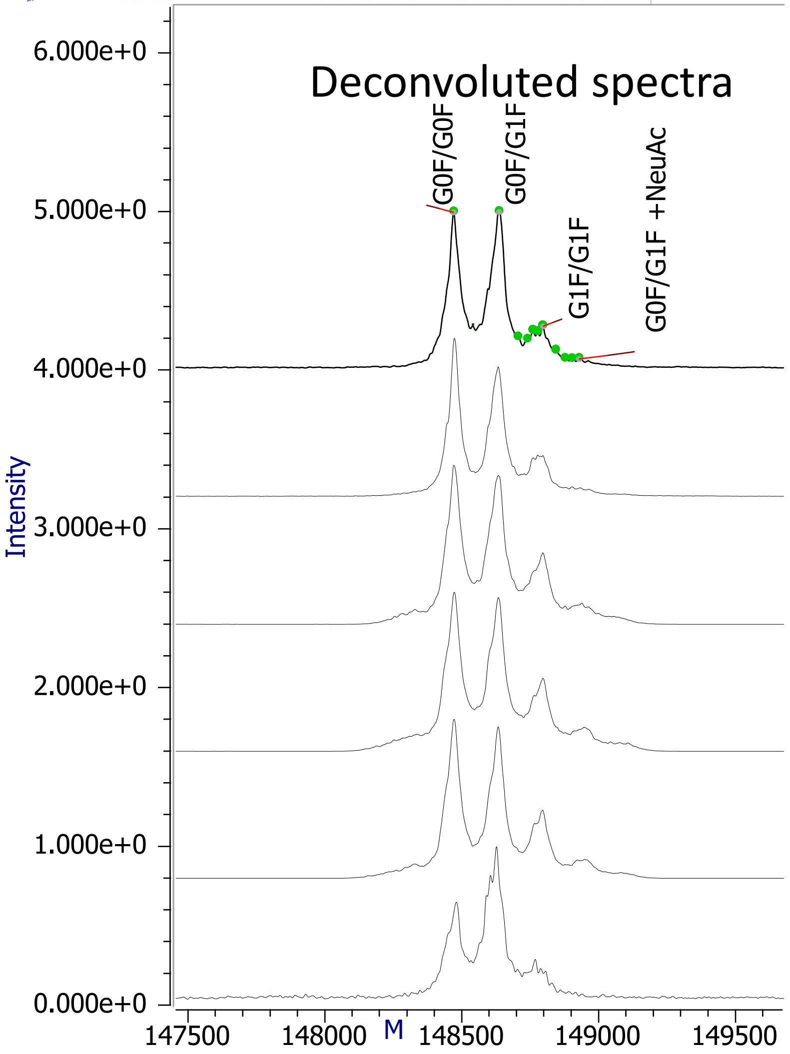

Merck in-house cIEF analysis

INTABIO MERCK INVENTING FOR LIFE

Blaze iCIEF-MS Analysis of all Charge Variants - High pH Day 7







Common Acidic Modifications
Sialic Acid
Deamidation
Trisulfide bonds
Thiosulfide Modification
Cysteinylation
Free thiols
Fragments

- Succinimide 2X higher in high pH day 7, compared to control.
- No additional shift in mass from acidic to main peak identified suggesting presence of deamidation (+1 Da, unresolved on TOF instrument), agreeing with RPM data.

INTABIO

MERCK
INVENTING FOR LIFE

Conclusions

1. Complete Blaze iCIEF-MS analysis in < 20 minutes

2. Rapid method develop and easy method transfer from legacy iCE instruments, <1 day

3. iCIEF electropherograms from Intabio Blaze analysis and in-house analysis are comparable throughout control and all stressed conditions.

4. Analysis of non-stressed material identifies expected modifications including glycosylation series (G0F/G0F, G0F/G1F, etc), unprocessed lysines, loss of GlcNAc, and sialic acid additions.

5. iCIEF-MS analysis of light-stressed samples identifies increasing oxidative states of mAb (1-4 oxidation sites). Total amount of oxidation observed generally matches RPM analysis. Increase in the presence of dimer is observed across all electrophoretic peaks of 1X light-stressed material.

6. RPM identified one main deamidation site in high-pH stress (Day 7). cIEF data shows an increase in acidic peaks but intact mass unable to resolve +1 Da mass shift. Analysis on higher resolution MS may better resolve this mass shift.

Acknowledgements

Merck Team:

Daniel Donnelly
Xiaoqing Hua
Jay Desai
Bhumit Patel
Doug Richardson

Intabio Team:

Wei Chen
Mariam ElNaggar
Erik Gentalen
Maggie Ostrowski
Kristine Bissell
Lena Wu
Wally Ausserer
Scott Mack

NIIMBL The National Institute for Innovation in Manufacturing Biopharmaceuticals

INTABIO

MERCK INVENTING FOR LIFE