Glycoanalysis of a Glycoengineered VHH-Radioligand Therapy Designed For Improved Biodistribution

David Bush
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RLT platform concept strategy
What is RLT?

By harnessing the **power of radioactive atoms** and applying it to advanced cancers through radioligand therapy, RLTs are able to deliver radiation to **target cells** anywhere in the body.

RADIOLIGAND THERAPY

1. **Radioisotope** emits therapeutic radiation that damages cells and may cause cell death.[1]

2. **Target ligand** binds selectively to specific markers on target cells.[2]

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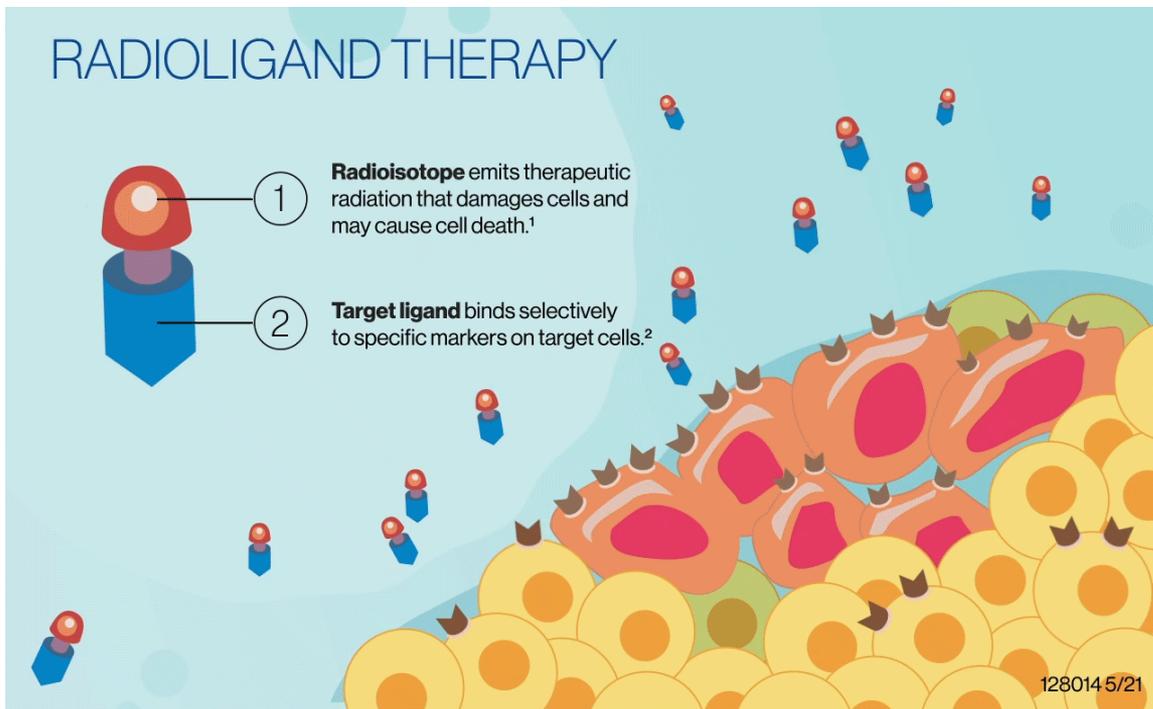
References: 1. Jadvar H. Targeted radionuclide therapy: an evolution toward precision cancer treatment. *AJR Am J Roentgenol.* 2017;209(2):277-288. **2.** Jurcic JG, Wong JYC, Knox SJ, et al. Targeted radionuclide therapy. In: Tepper JE, Foote RE, Michalski JM, eds. *Gunderson & Tepper's Clinical Radiation Oncology.* 5th ed. Elsevier, Inc; 2021;71(3):209-249.

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Exploration of a broad range of formats as RLT vectors

IgG	Fab	scFv	sdAb	Non-Ab scaffolds	Mini-proteins	Peptides, Peptido-mimetics	LMW
149 kDa	48 kDa	26 kDa	13 kDa	7- 20 kDa	3-4 kDa (< 40 aa)	< 2 kDa (< 20 aa)	0.5 kDa

LUTATHERA® (lutetium Lu 177 dotatate) injection, for intravenous use

PLUVICTO® lutetium Lu 177 vipivotide tetraxetan INJECTION FOR INTRAVENOUS USE

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Manufacturing process
RLT radioactive products structure

Radioligand

Cancer cell
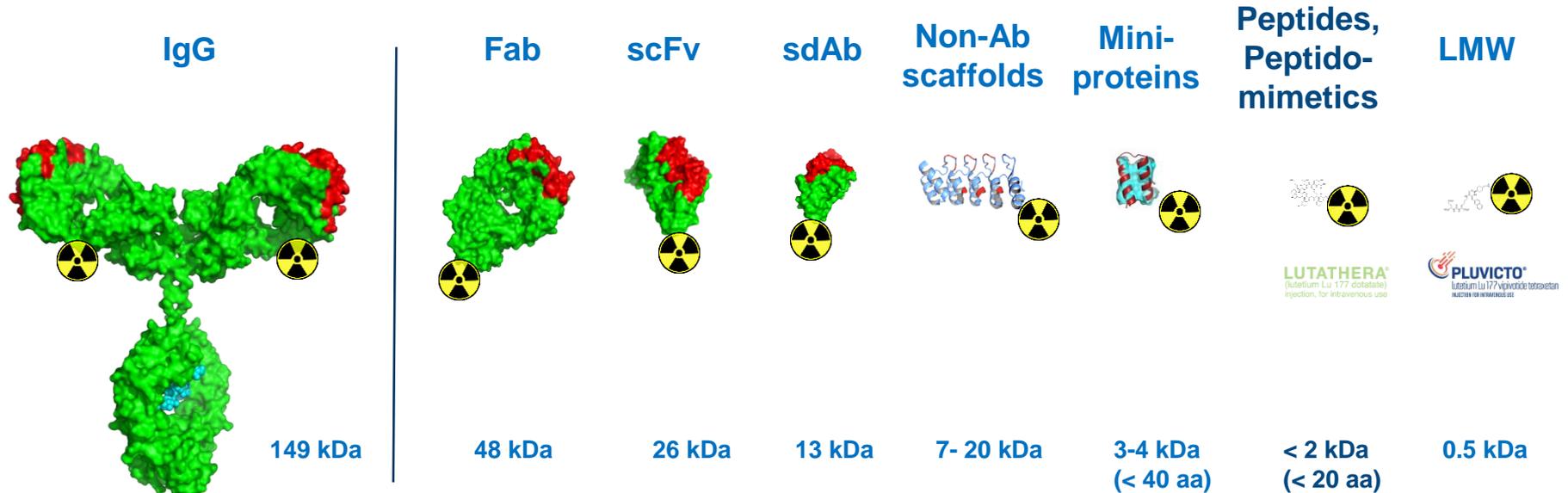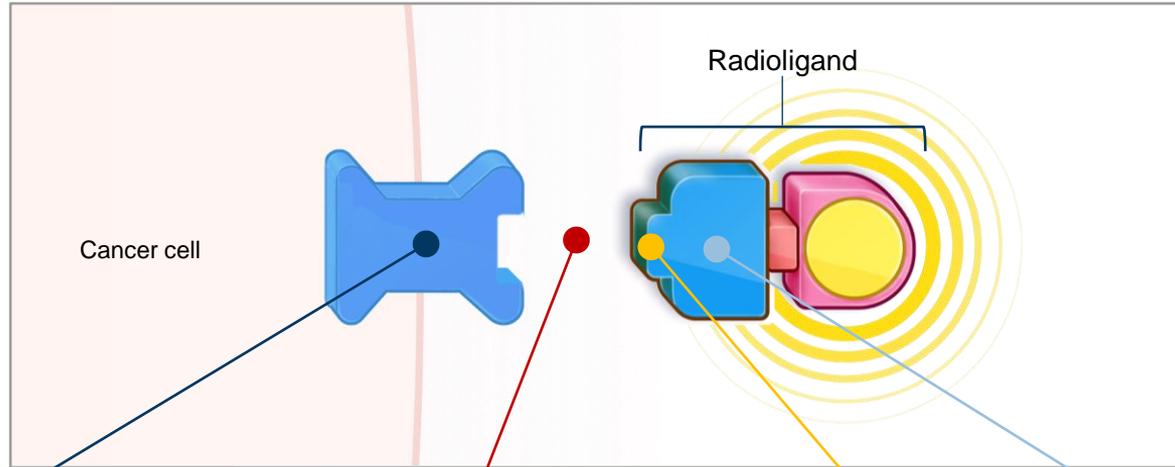
Target
Receptor preferentially expressed on tumor cells or in tumor microenvironment

Vector/Ligand
Small molecule, peptide or Ab fragment binding a target with high affinity

Chelator/Linker
Small molecular adaptor enabling attachment of isotope to the ligand

Isotope
Radioisotope releasing γ rays for imaging, or β⁻ or α particles for therapy

▪ Jurcic JG, Wong JYC, Knox SJ, et al. Targeted radionuclide therapy. In: Gunderson LL, Tepper JE, eds. *Gunderson & Tepper's Clinical Radiation Oncology*. 4th ed. Elsevier, Inc.; 2016:423-437.e19.

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Manufacturing process
RLT radioactive products structure
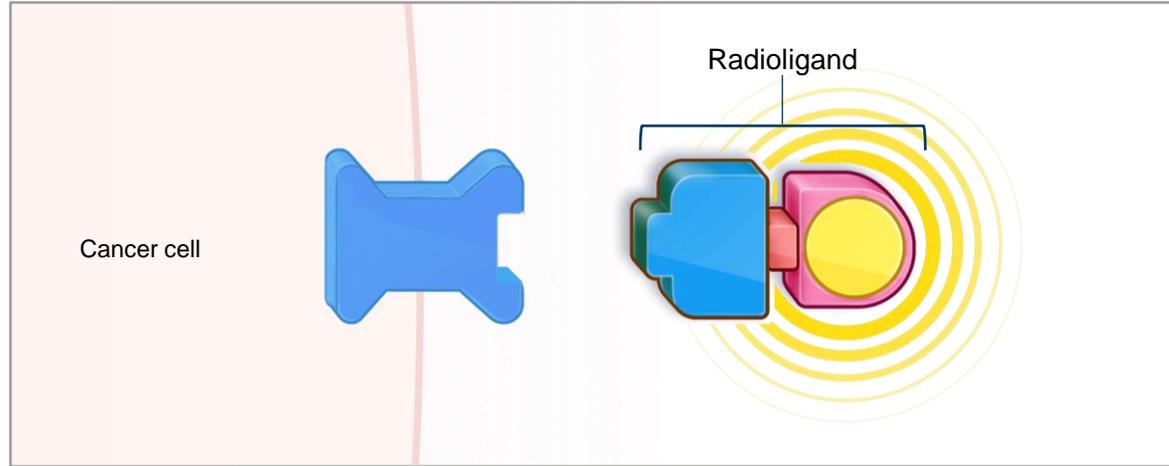
- **Assessment of a VHH-RLT:**
    - DOTA / linker is conjugated to the C-terminal side of VHH
    - N-glycan sequon was added to increase size / lower pI in an attempt to lower undesired off-target tissue accumulation
    - VHH expressed in a **HEK293**

- **Analytical request from production teams:**
    1. Is the produced VHH fully glycosylated?
    2. Does glycosylation affect the conjugation?
    3. What is the glycan profile and does it affect biodistribution?

Comparison of Human vs. CHO glycans
Rapifluor tag: EX 265/EM 425 nm

VHH expressed in HEK293
Fc-cytokine fusion expressed in CHO
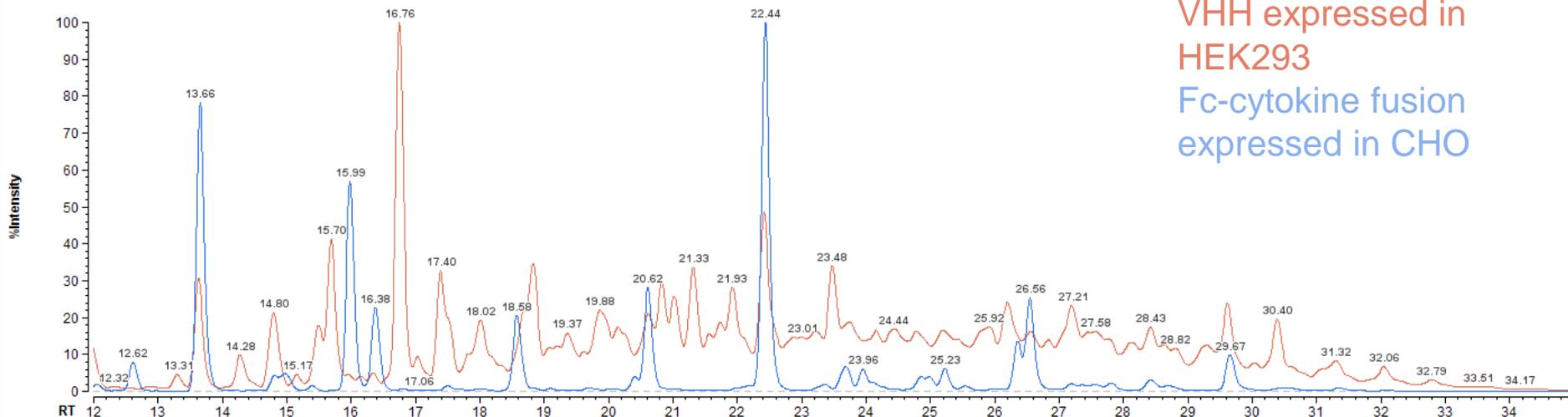
Comparison of Human vs. CHO glycans
Full LC/MS Ion Map
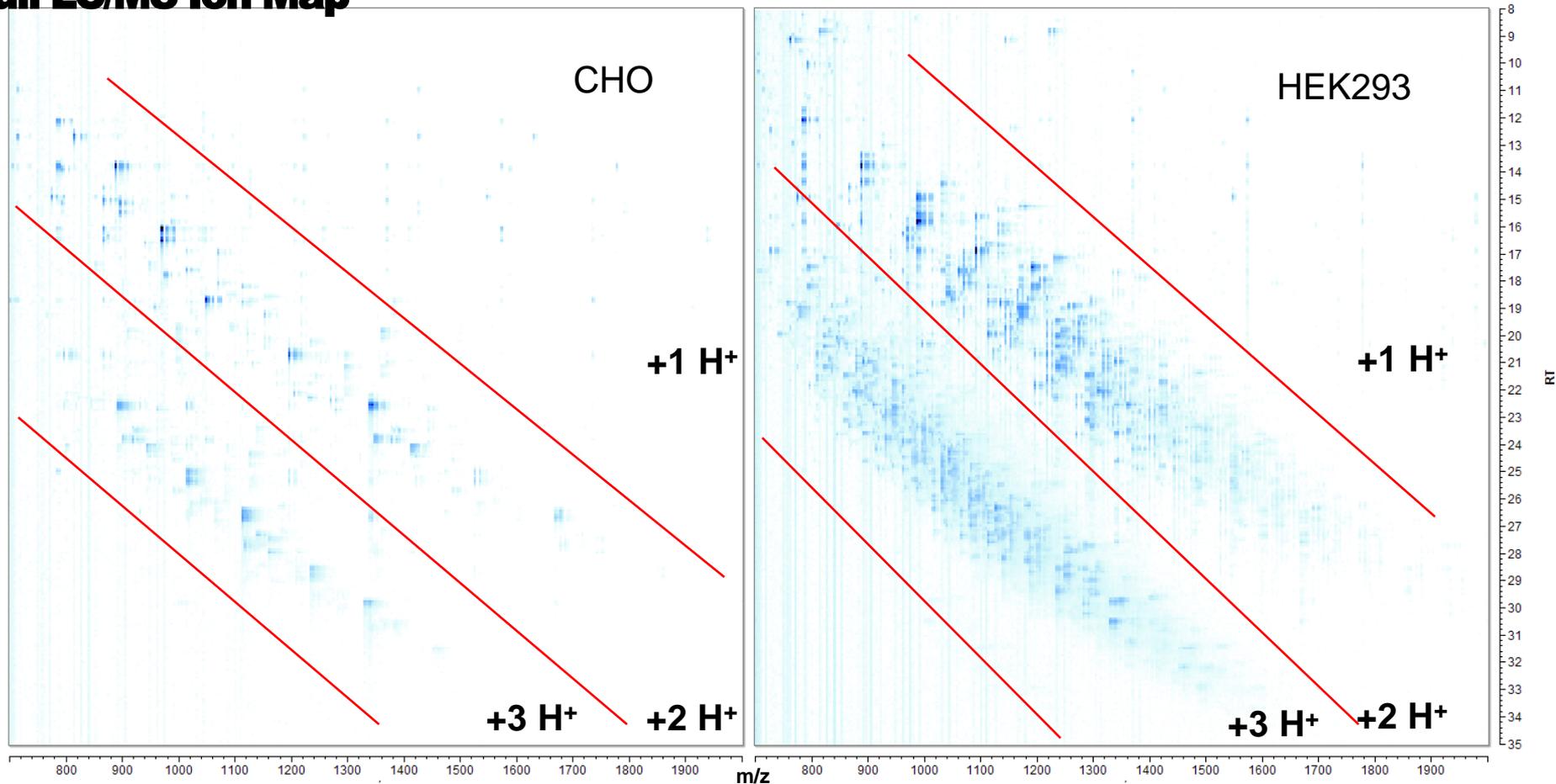
Peptide Mapping: Materials and Methods

- The tryptic digest of the unconjugated VHH gives a peptide with the N-glycan on the His-Tag (ionizes well)
  - Before Conjugation: ...XXNITXnXHHHHHHHH
  - After Conjugation: ...XXNITXnXXXX*

- Reduce with DTT, alkylate with iodoacetamide, digest with trypsin (1:40 enzyme:VHH).

- MS: Thermo QE-HFX

- LC: Thermo EASY-nLC 1200

- Data analysis: Genedata Expressionist v. 17.0; generic Thermo pepmap workflow
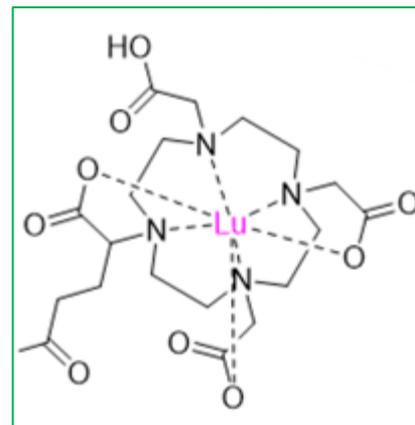
Released Glycan: Materials and Methods

- Followed Waters Glycoworks protocol (PNGase glycan release, RapiFluor labeling, SPE cleanup)

- MS: Waters Synapt-XS
  - Top-8 MS/MS, CE ramp 20-35 eV
  - In-line FLD

- LC: Waters I-class
  - ACQUITY UPLC Glycan BEH Amide, 130 Å, 1.7 µm, 2.1 x 150 mm

- Data analysis: Genedata Expressionist v. 17.0; generic Waters released glycan workflow
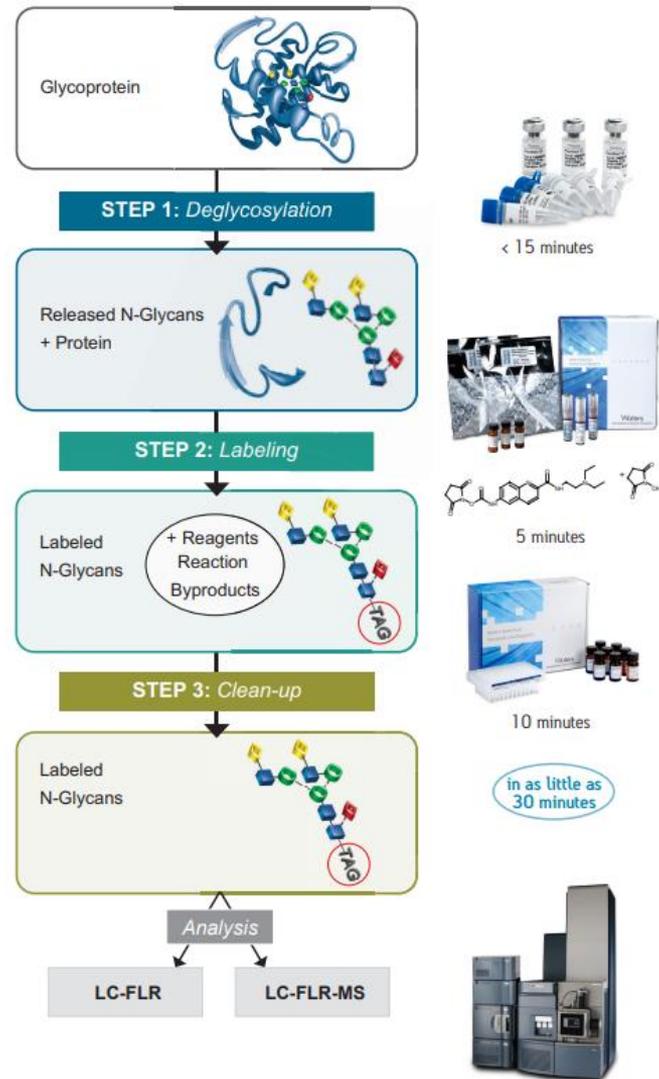
Data Analysis Strategy:

- Acetylated sialic acid and antenna sulfation are treated as variable glycan modifications)
- 'Humanize' our curated CHO database of N-glycans to include common human glycan moieties
  - NANA +=> NGNA (up to 2)
  - Gal +=> GalNAc
  - + Antennae Fucose
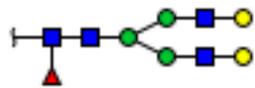
FA2G2
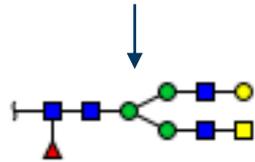
FA2G1GalNAc1
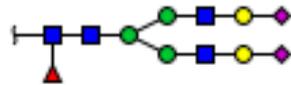
FA2G2S2

FA2F1G2S2

FA2G2S1Sg1

FA4G4S4

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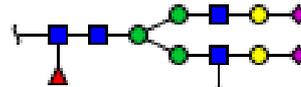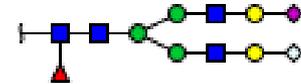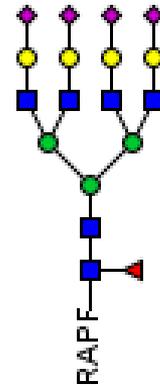
Glycopeptide Ion Map

Glycopeptide Ion Map
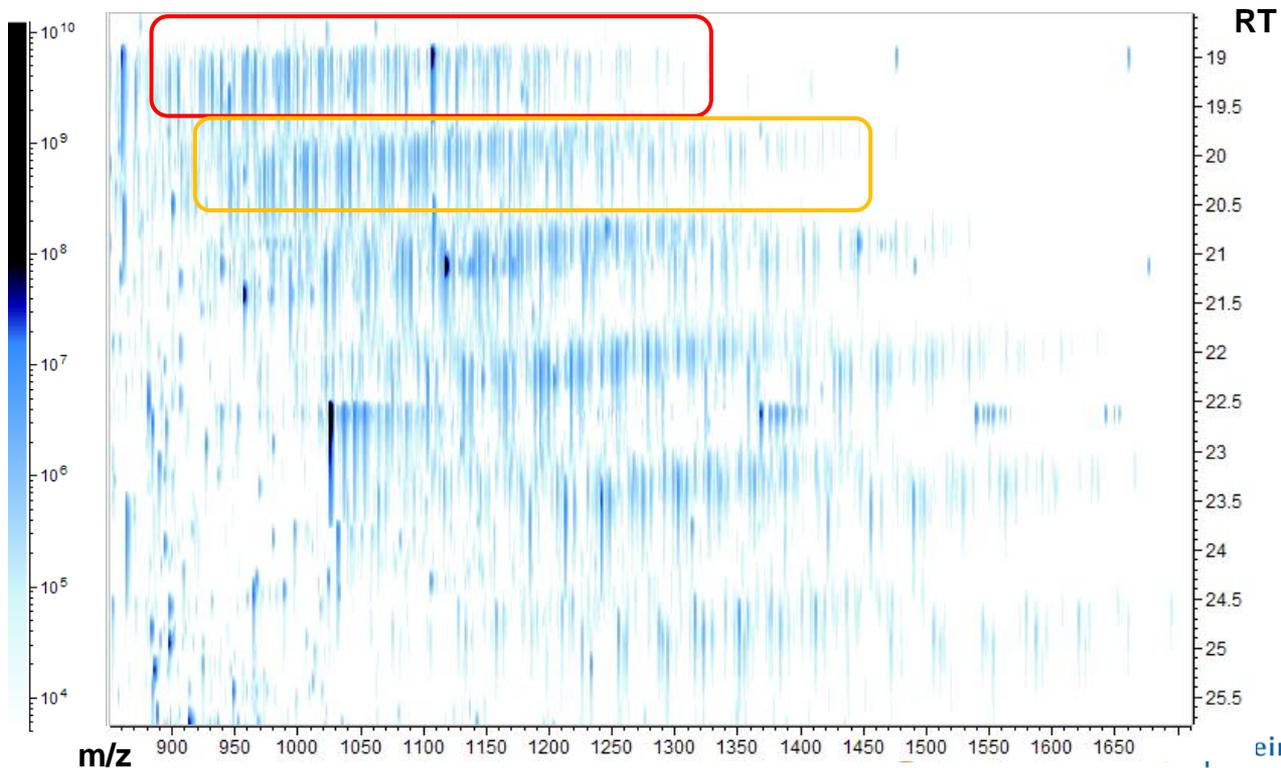
Acid moieties (NANA, NGNA, Sulfation):   0, 1, 2, 3, 4, 5

Glycopeptide Ion Map

Acid moieties (NANA, NGNA, Sulfation):   **0**, **1**, **2**, **3**, **4**, **5**
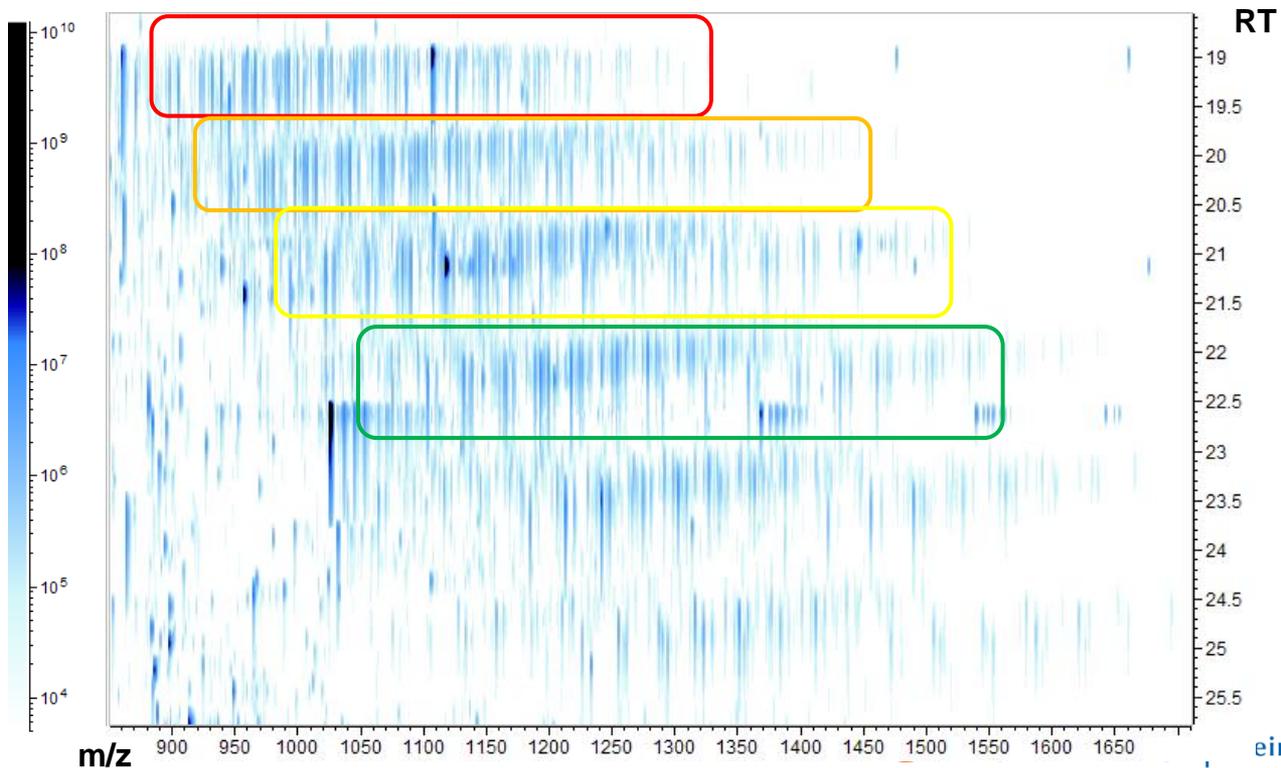
Glycopeptide Ion Map

Acid moieties (NANA, NGNA, Sulfation): **0, 1, 2, 3, 4, 5**
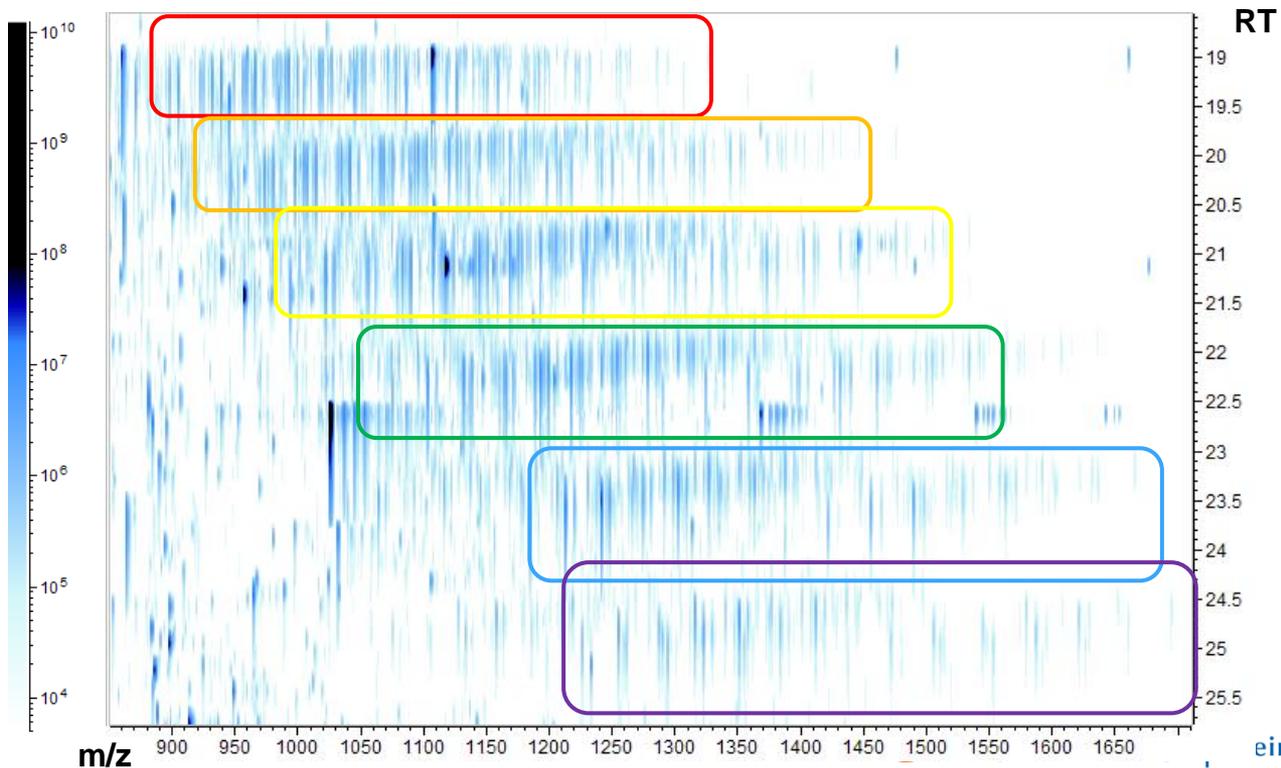
Glycopeptide Ion Map

Acid moieties (NANA, NGNA, Sulfation):   **0**, **1**, **2**, **3**, **4**, **5**
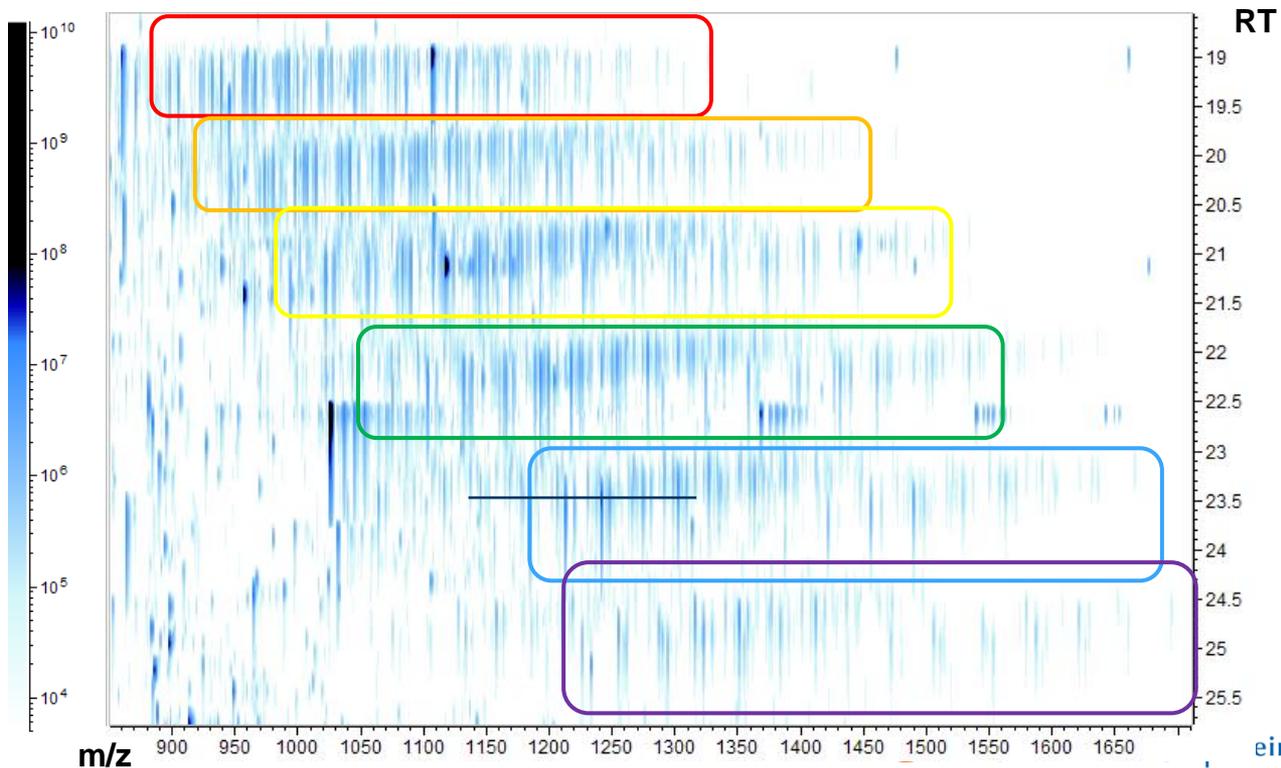
Mass Spectrum, 4 acidic-moiety glycan range
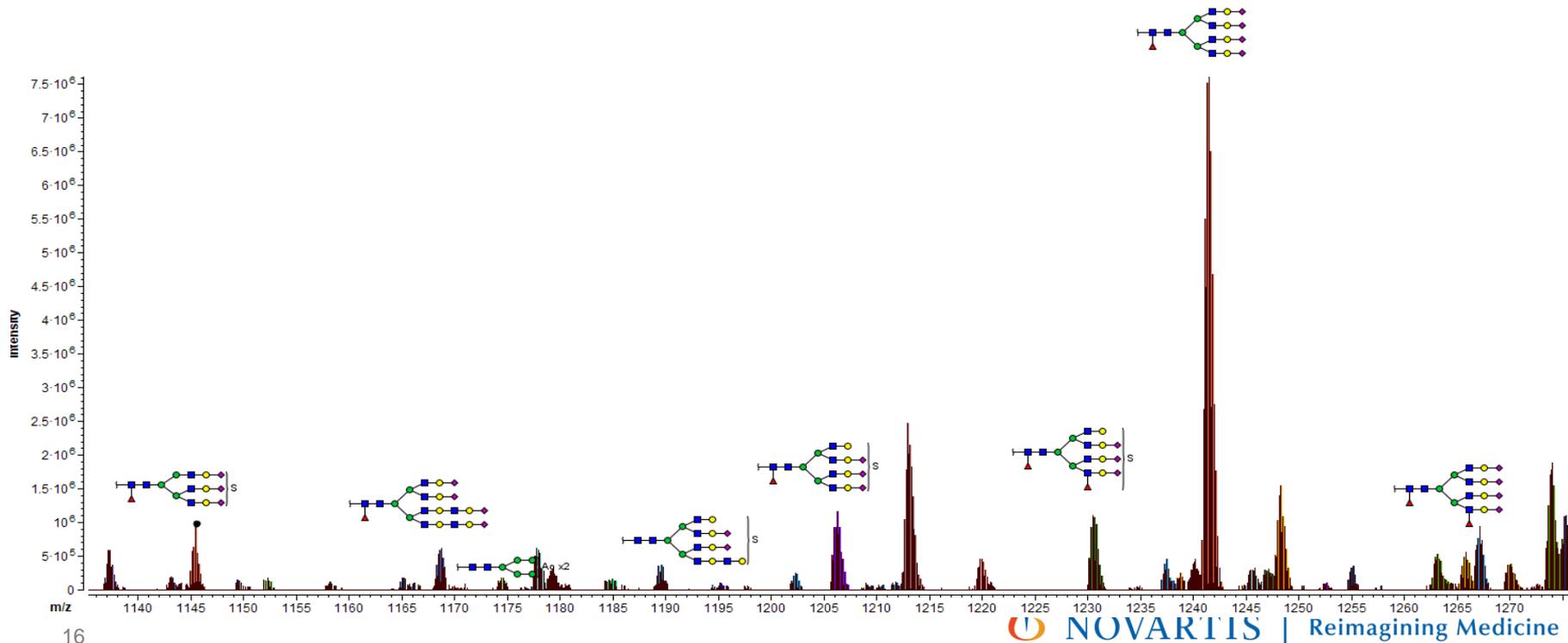
Lu:DOTA conjugated peptide does not ionize well...

Acid moieties (NANA, NGNA, Sulfation):   0, 1, 2, 3, 4, 5

Pre-conjugation (+His Tag)
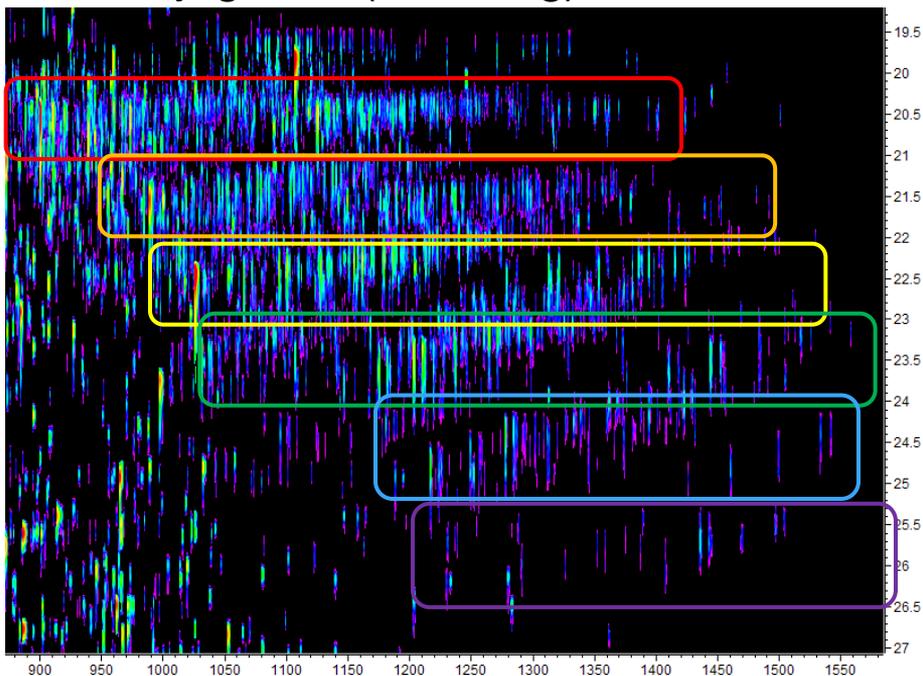
Post-conjugation (-His Tag, +Lu:DOTA)

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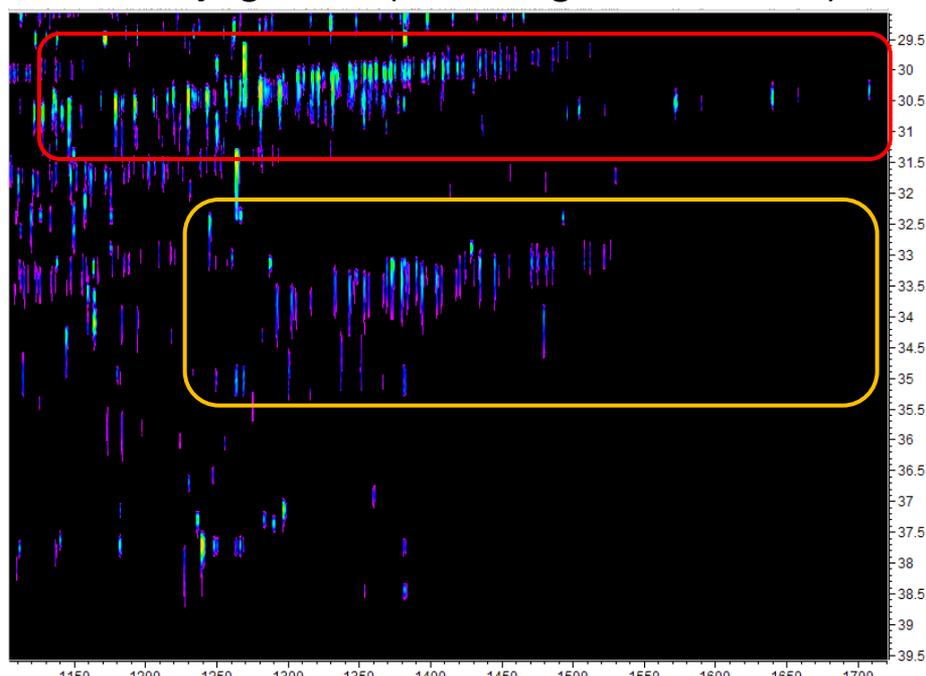
Released Glycan Profiles
Rapifluor tag: EX 265/EM 425 nm Day 1
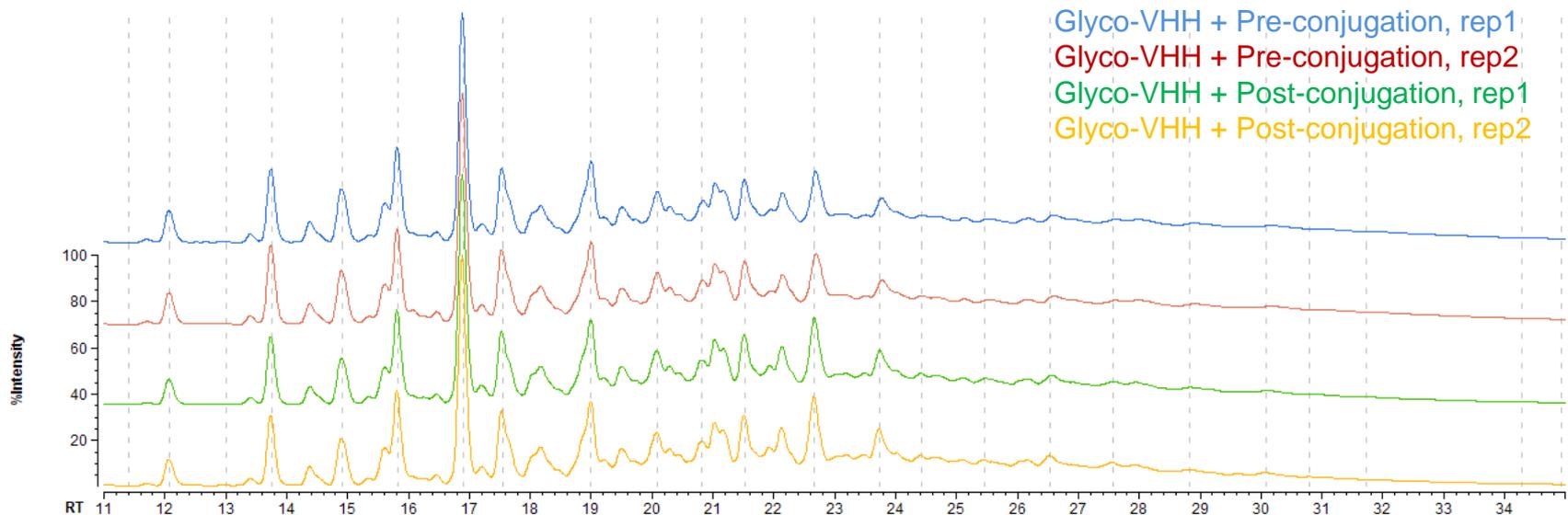
- Sample prep by Andrew robot

Released Glycan Profiles
Rapifluor tag: EX 265/EM 425 nm Day 2

- Manual Sample Prep.

Modified Bi-antennary Glycans
Modified: FA2G2S2
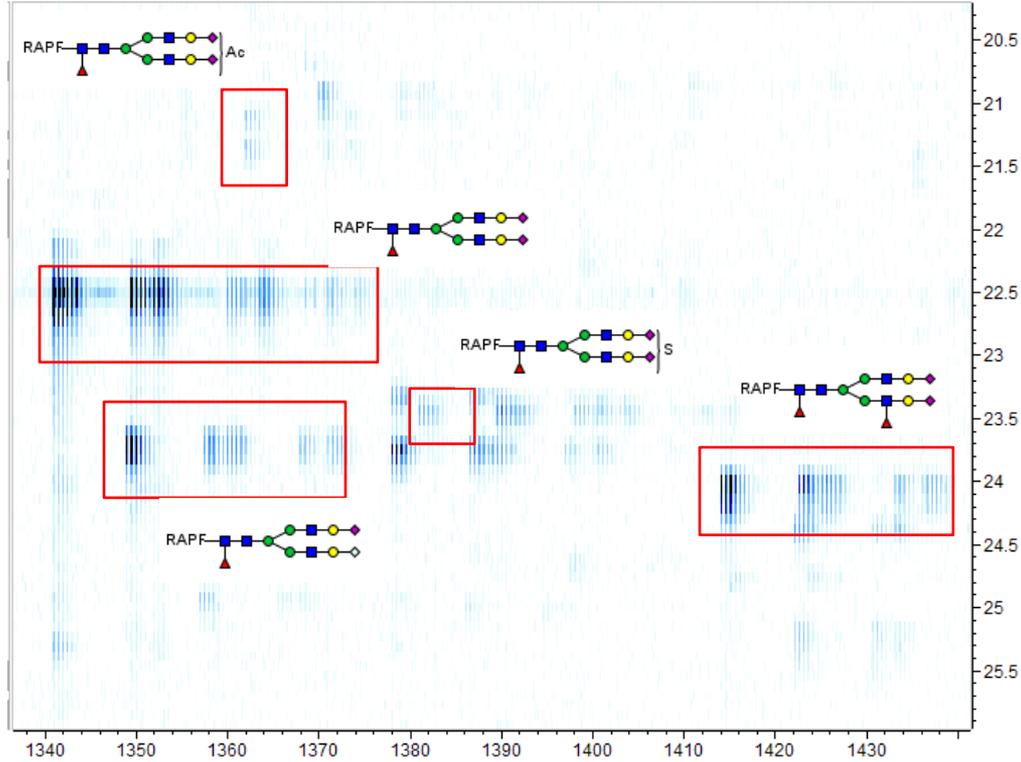
Fragmentation confirmation of NGNA
FA2G2S1Sg1 / FA2G2S2
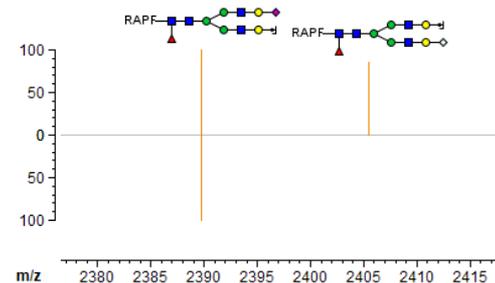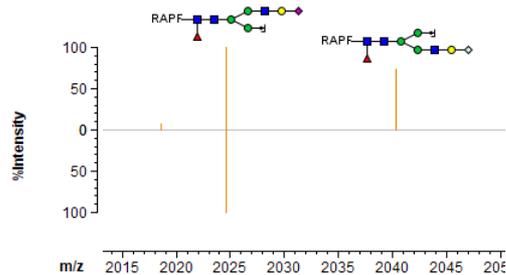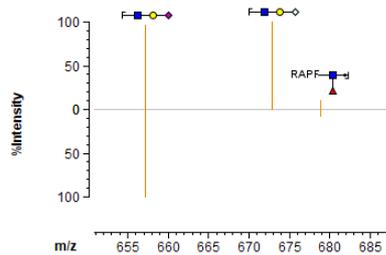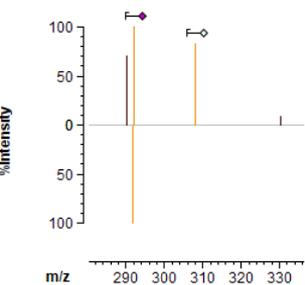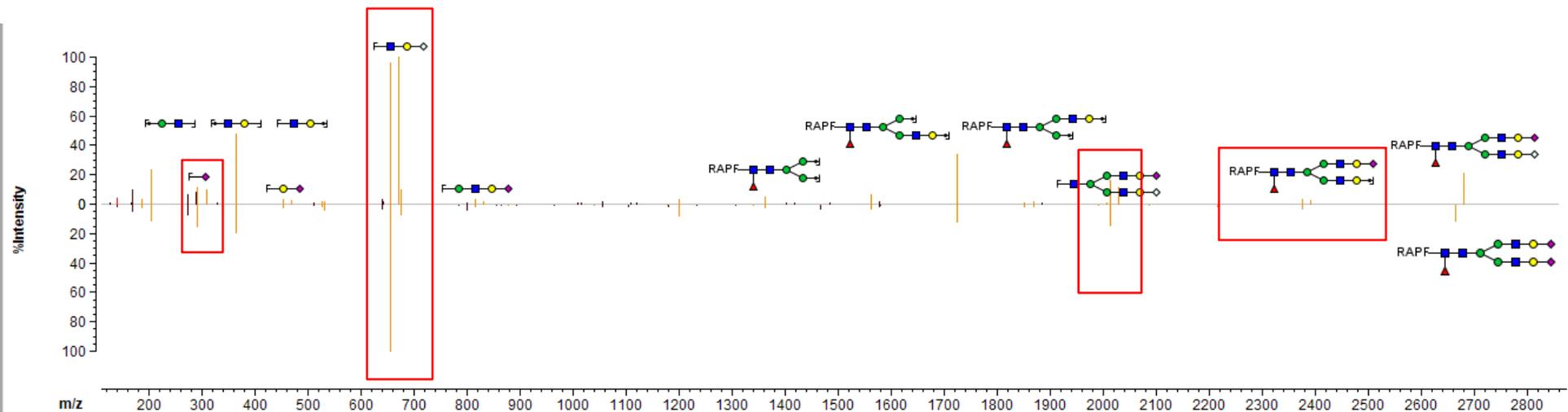
Fragmentation Confirmation of O-Ac-NANA

FA2G2S2_OAc1 / FA2G2S2
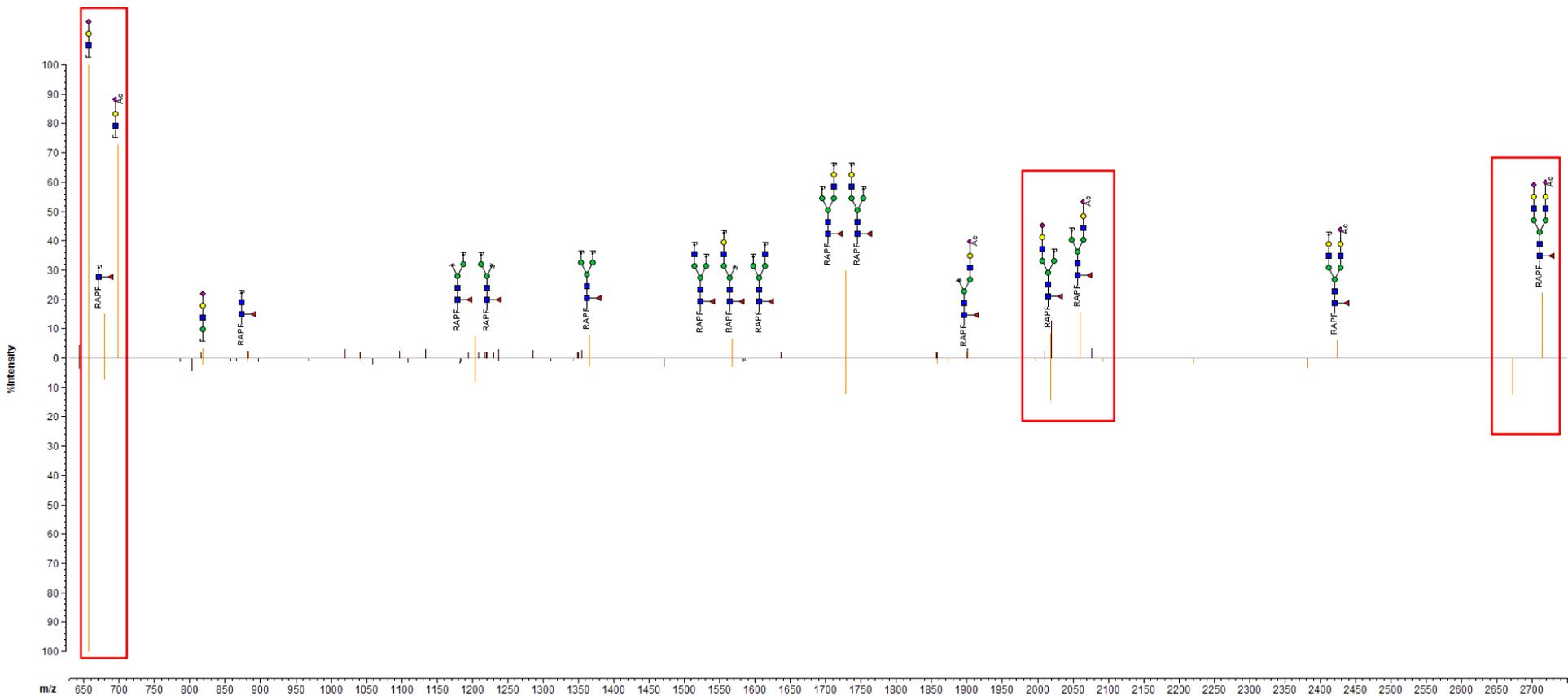
Fragmentation Confirmation of Antenna-Fuc

FA2F1G2S2/FA2G2S2
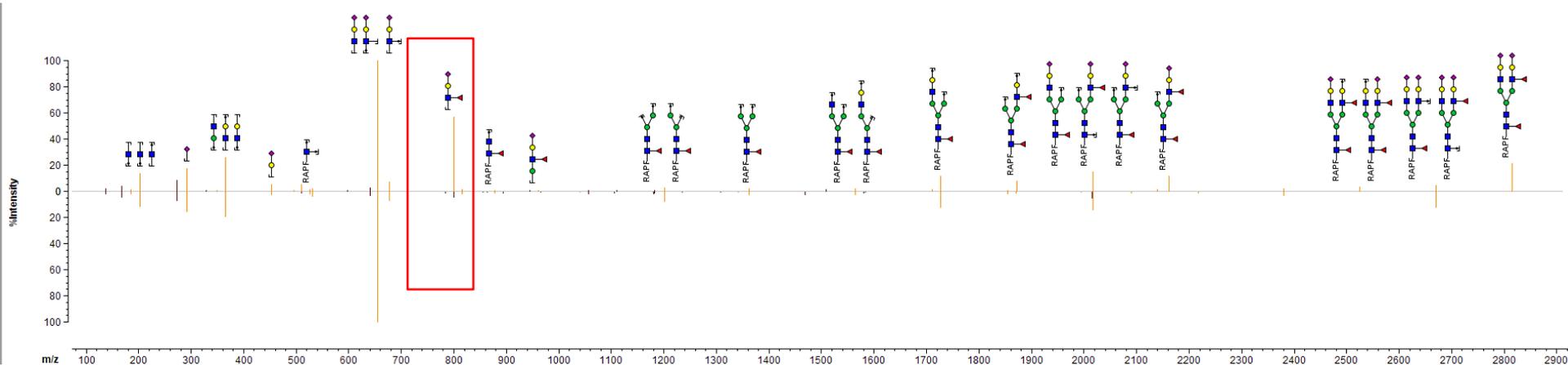
FA2GalNAc2Su1

Fragmentation Confirmation of Sulfated-Gal / GalNAc

FA2G2S2Sulf
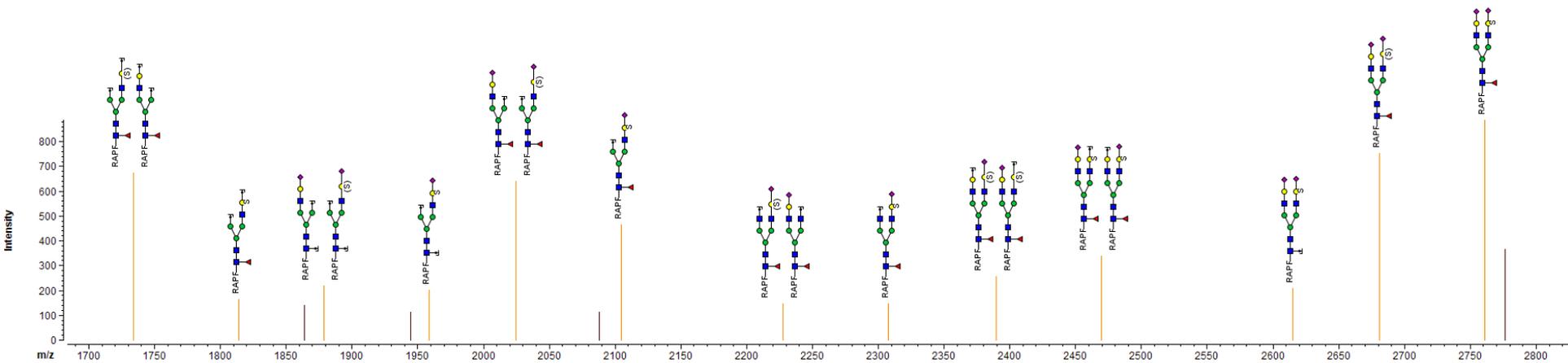
Distilling Information from Results

- Tables with 100's of glycan ID's and quantities do not facilitate decision making

- Mapping glycan attributes to annotations via a generic glycan name provides information that can be summarized and reported to non-experts...

Glycan	Core Fuc	Antenna (High Estimate)	Antena (low estimate)	Antenna Fuc	Sialic Acid	NANA	NGNA	GalNAc	...	Intensity
A3G3	0	3	2	0	0	0	0	0		2.3215
A3G3S1	0	3	2	0	1	1	0	0		3.6121
A3G3S2	0	3	2	0	2	2	0	0		2.8231
A3G3S3	0	3	3	0	3	3	0	0		1.5918
FA3G3	1	3	2	0	0	0	0	0		5.2351
FA3G3S1	1	3	2	0	1	1	0	0		6.2354
FA3G3S2	1	3	2	0	2	2	0	0		8.7354
FA3G3S3	1	3	3	0	3	3	0	0		8.9542
FA3G3Sg1	1	3	2	0	1	0	1	0		1.3852
FA3G3S1Sg1	1	3	2	0	2	1	1	0		1.2719
FA3F1G3S1	1	3	2	1	1	1	0	0		2.3245

(Next slide)

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Example Reports

Example 1: Nearly indistinguishable glycan profiles of the His-tagged VHH and the conjugated (Lu:DOTA ligated) samples

# of Anteanna	HIS Tag Rep1	HIS Tag Rep2	LuDOTA Rep1	LuDOTA_Rep2
0	1.0	1.0	0.8	0.9
1	1.3	1.4	1.2	1.4
2	58.4	59.0	57.0	56.6
3	31.4	31.3	33.1	33.0
4	8.0	7.3	7.9	8.2
# of Fucosylation				
0	3.3	3.4	3.3	3.4
1	79.0	79.1	78.7	78.6
2	17.4	17.2	17.7	17.7
3	0.3	0.2	0.3	0.3
Sulf	3.4	3.3	3.5	3.4
O-acetyl	0.5	0.5	0.5	0.5
Uncapped Gal/GalNAc, high estimate				
0	2.1	2.2	2.1	2.2
1	26.0	26.2	27.6	28.0
2	50.2	49.9	48.0	47.7
3	20.0	20.1	20.5	20.3
4	1.6	1.6	1.8	1.9
High-mannose / hybrid	0.5	0.5	0.5	0.5
# NANA/NGNA				
0	62.5	62.7	59.8	59.5
1	27.6	27.6	30.0	30.1
2	7.5	7.6	8.4	8.6
3	1.7	1.5	1.4	1.4
4	0.7	0.6	0.4	0.4

Example 2: Comparing Fc glycan-profiles from different glycoengineered cell lines

Protein ID	Production Conditions	High Mannose [%]	Terminating Gal [%]	Afucosylation [%]	Sialylation [%]
Prot1	A	1	30	4	0
Prot2	A	1	28	9	0
Prot3	A	0	37	1	0
Prot4	A	0	25	8	0
Prot5	A	2	24	6	0
Prot6	A	0	24	4	0
Prot2	B	1	22	100	0
Prot3	B	0	31	100	0
Prot4	B	0	25	100	0
Prot5	B	1	20	100	0
Prot6	B	0	19	100	0
Prot2	C	4	26	4	46
Prot4	C	0	9	0	78
Prot6	C	0	21	2	61

Conclusions

- Glycopeptide mapping and released glycan analysis of glycoengineered VHH's provided useful insights to support the production and conjugation teams
  - Peptide mapping was used primarily for determining aglycan levels
  - Released glycans showed that glycoengineering had no detectable effect on conjugation
  - Although human glycans are too complex for a 1D LC experiment to resolve chromatographically for fluorescence quantitation, differences in relative MS signal between samples can provide useful insights

- Impacts of glycosylation on VHH biodistribution
  - With glycosylation, the ratio of VHH at tumor vs undesirable locations increased
  - However, accumulation in the liver, while not a toxicity concern, was increased
  - This is likely due to glycans with exposed Gal and GalNAc binding to ASGPR on the surface of hepatocytes
  - Judicious selection of expression system and culture conditions could further improve biodistribution

- Misc. conclusions
  - Well curated human and CHO glycan databases have since shown to be useful investments
  - Mapping glycan attributes to quantified glycans is a quick way provide actionable summaries of glycan profiles

Acknowledgements

- Jenifer Kaplan
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- Francis Bitsch
- Patrick Schindler
- Hayretin Yumerefendi
- Daniela Miranda
- Rainer Kneuer

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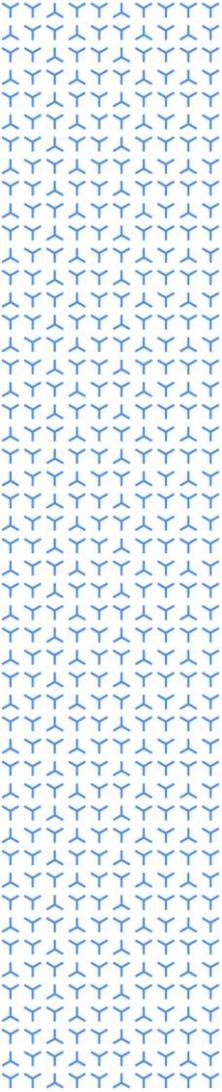
Backups

Glycan Structure: Key Differences Between CHO and Human

A2
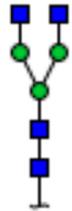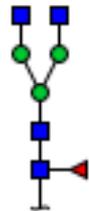
FA2
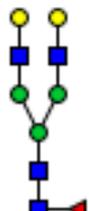
FA2G2

FA2G2S2

FA2F1G2S2

S
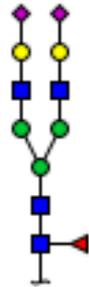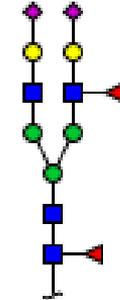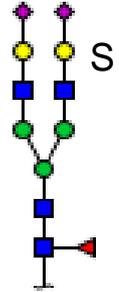
FA2G2S2Su1 (FA2G2S2_S1)
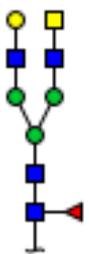
FA2G1GalNAc1
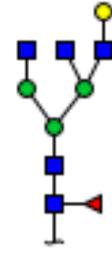
FA3G1
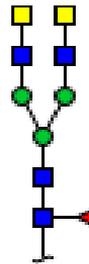
FA2GalNAc2

FA4

FA2G2S1Sg1

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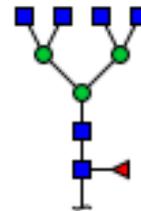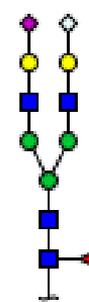
Comparison of Human vs. CHO glycans
Full LC/MS Ion Map

Comparison of Human vs. CHO glycans
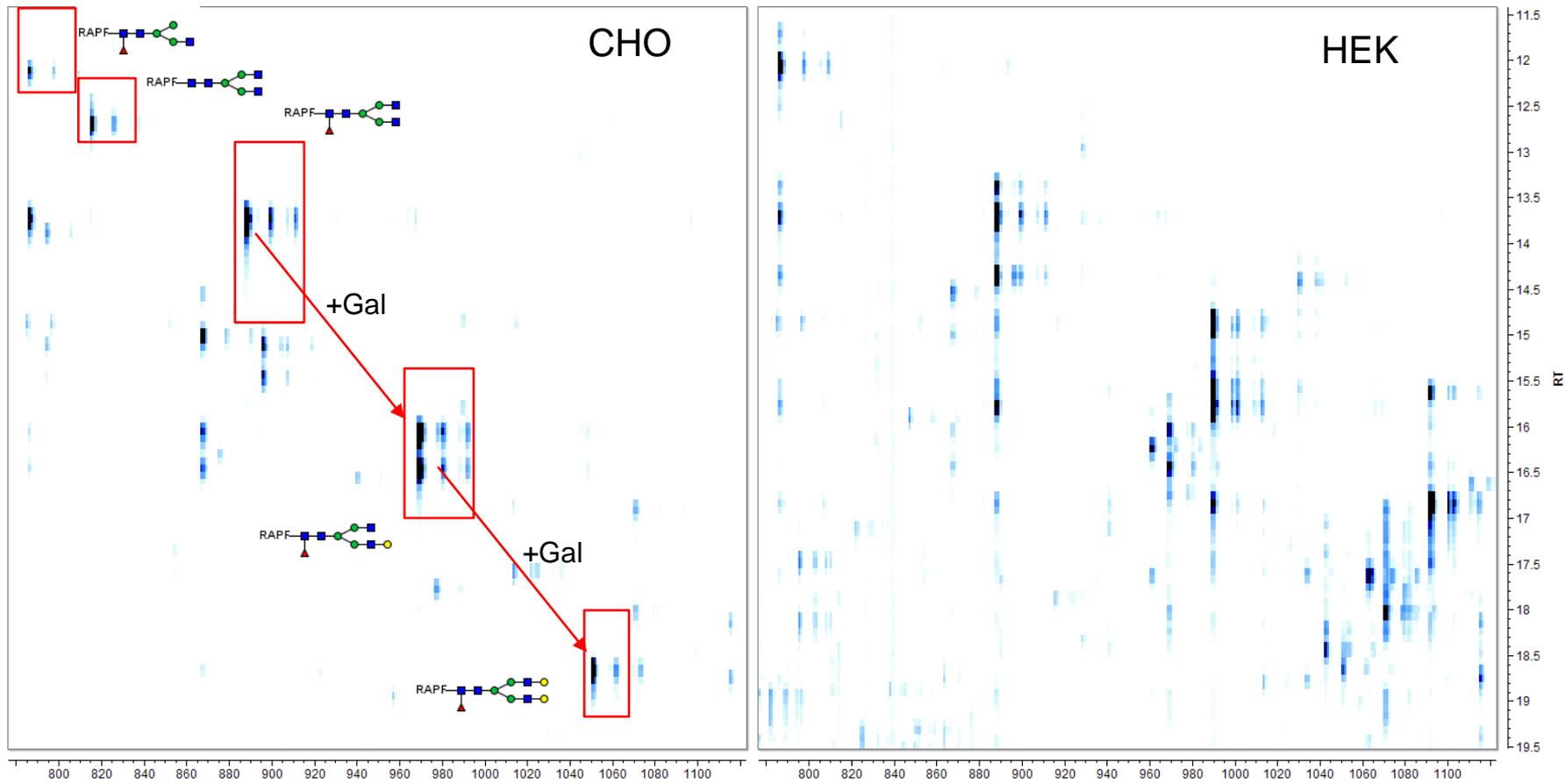
Non-sialylated Biantennary species

Comparison of Human vs. CHO glycans
Non-sialylated Biantennary species

Comparison of Human vs. CHO glycans
Non-sialylated Biantennary species

CHO

HEK

+GlcNAc

+Gal

+GlcNAc

+Gal

+GlcNAc

+Gal

+Gal
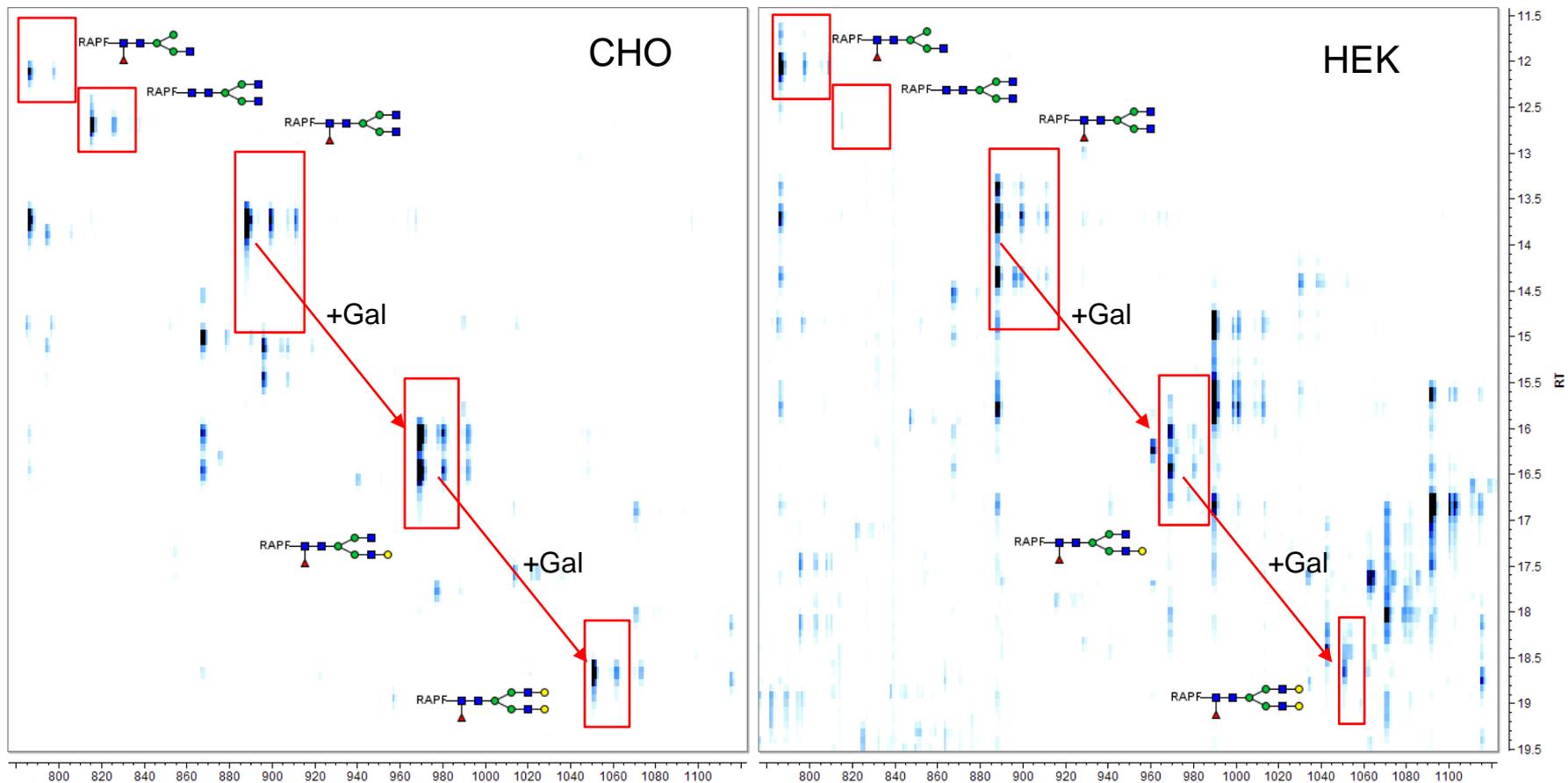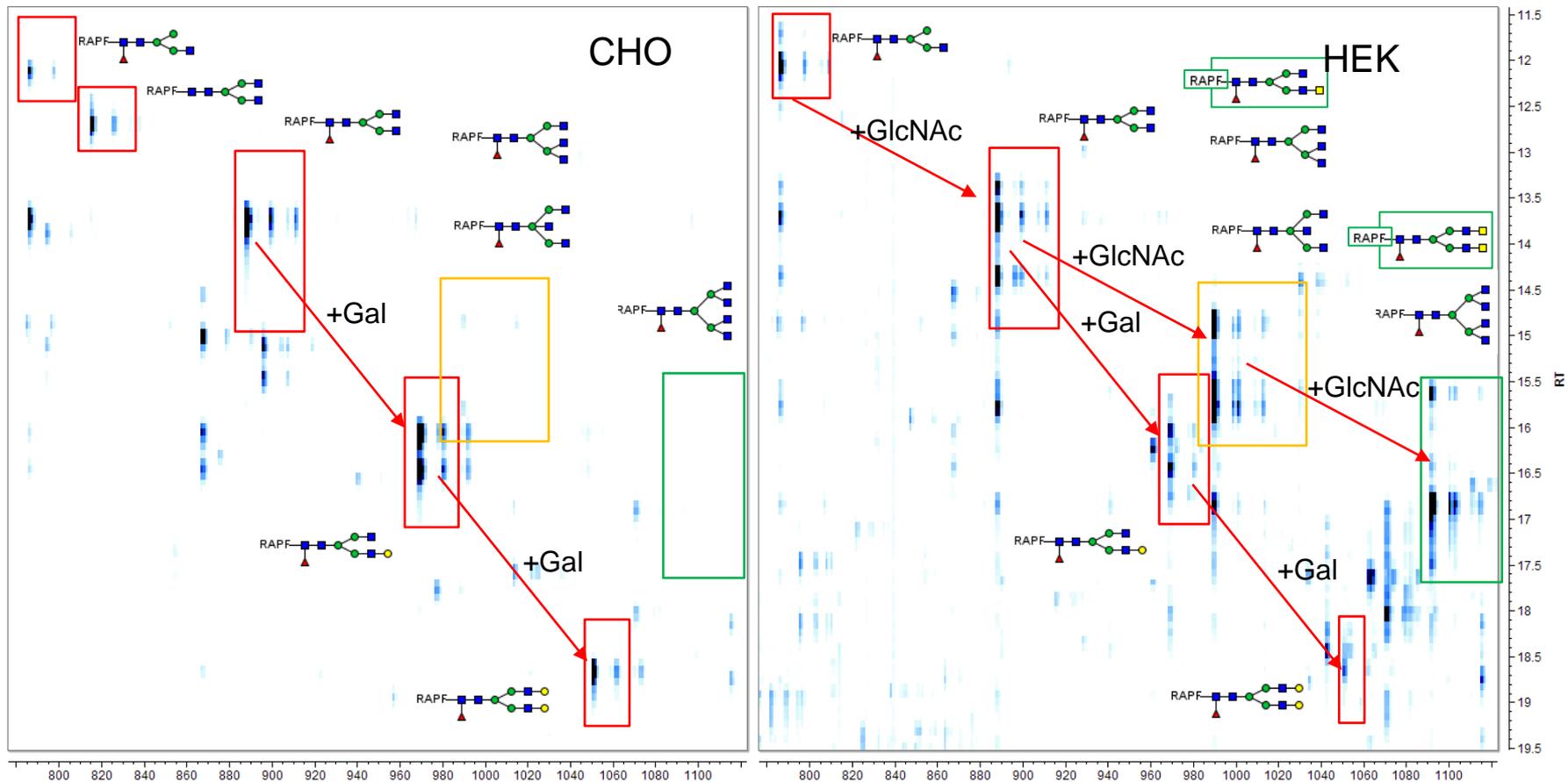
Comparison of Human vs. CHO glycans
Non-sialylated Biantennary species
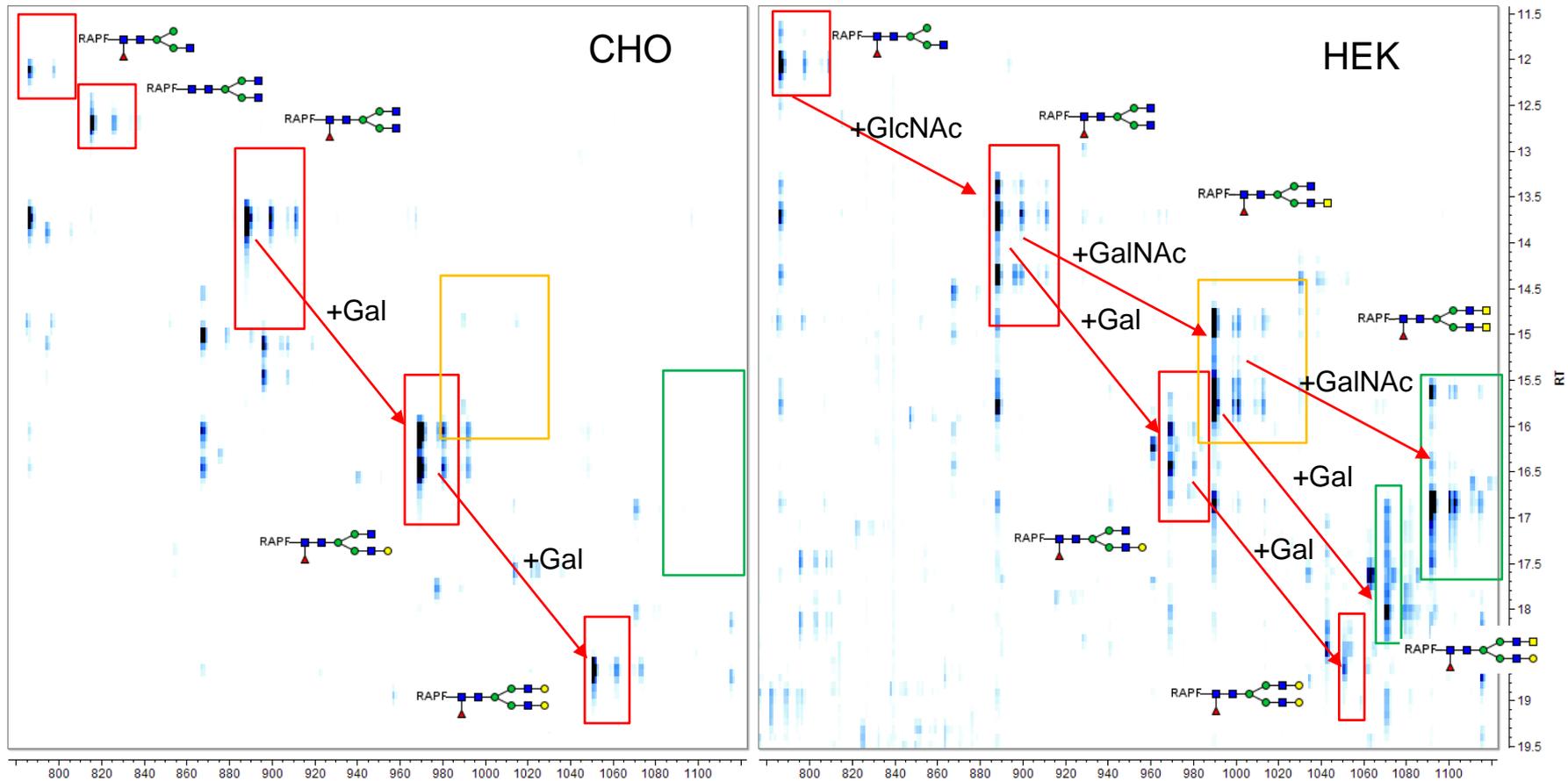
Glycan Structure Refresher:
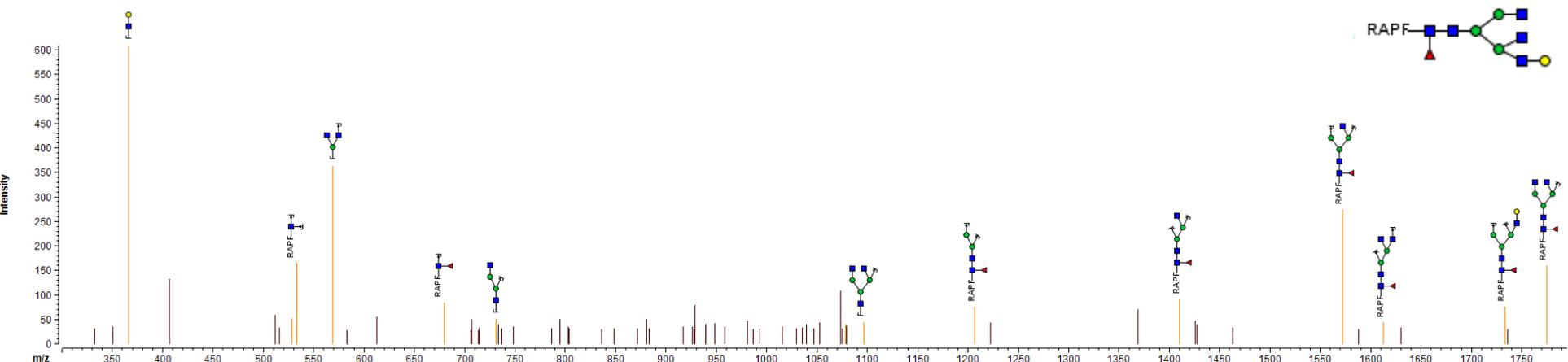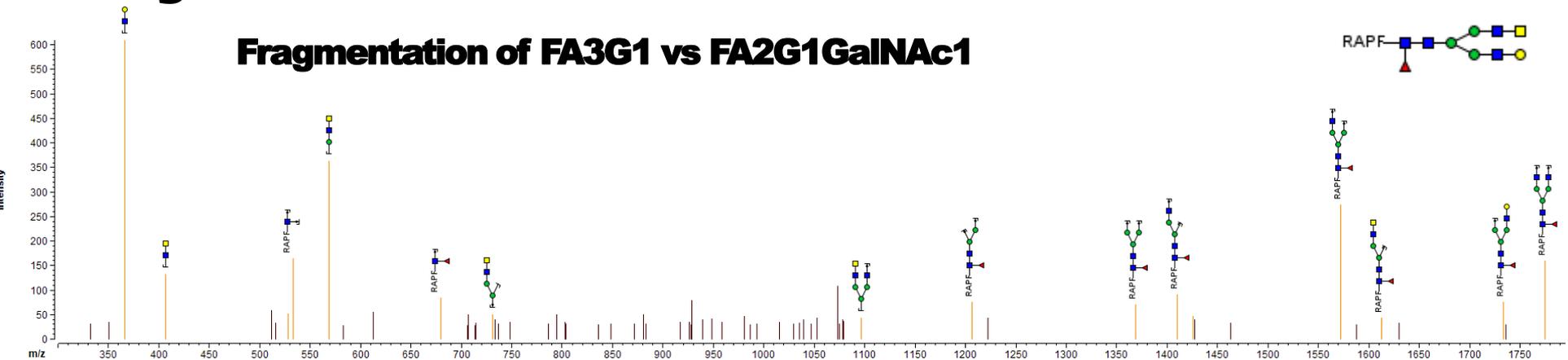
Fragmentation of FA3G1 vs FA2G1GalNAc1

Glycan Structure Refresher:

Fragmentation of FA3G1 vs FA2G1GalNAc1
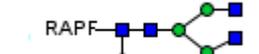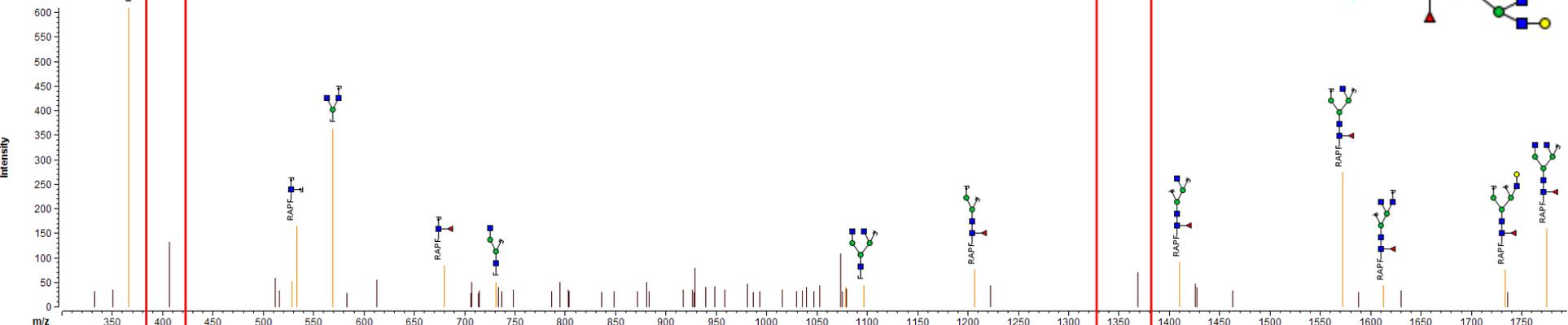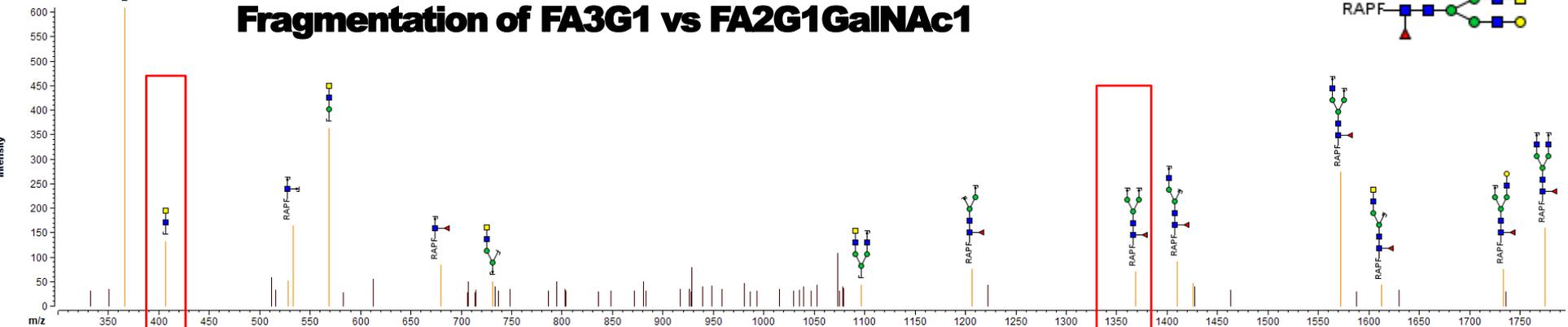
Glycan Structure Refresher:

Fragmentation of FA4 vs FA2GalNAc2
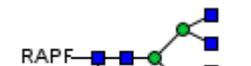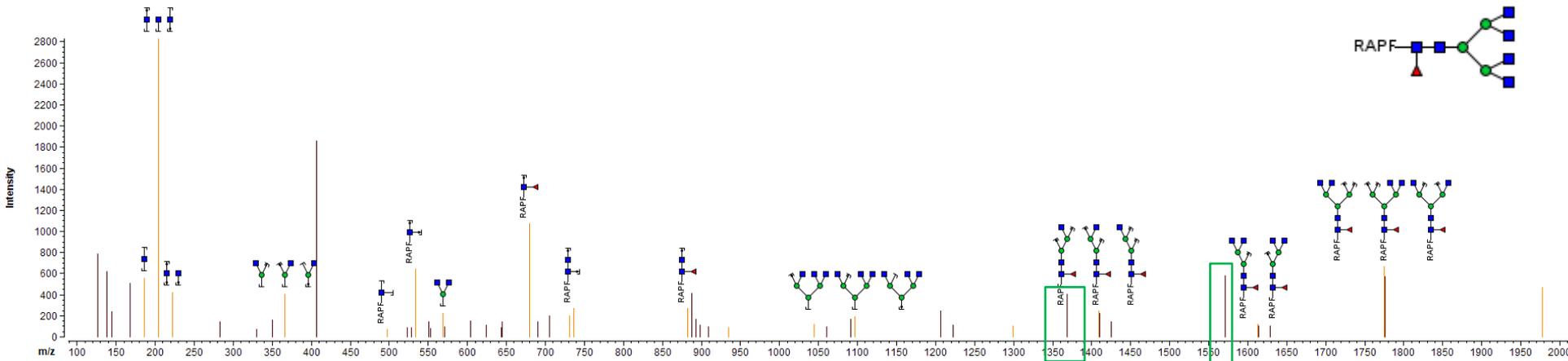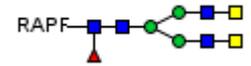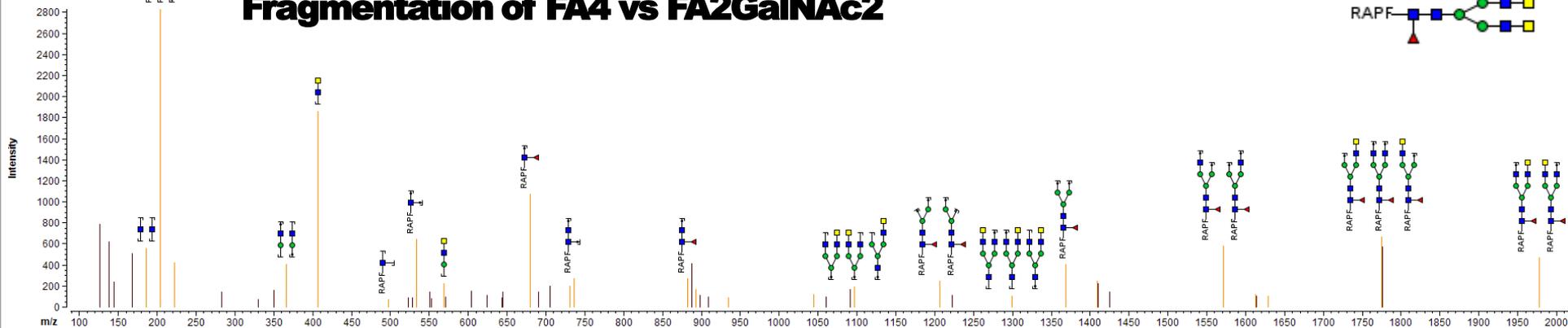
Glycan Structure Refresher:
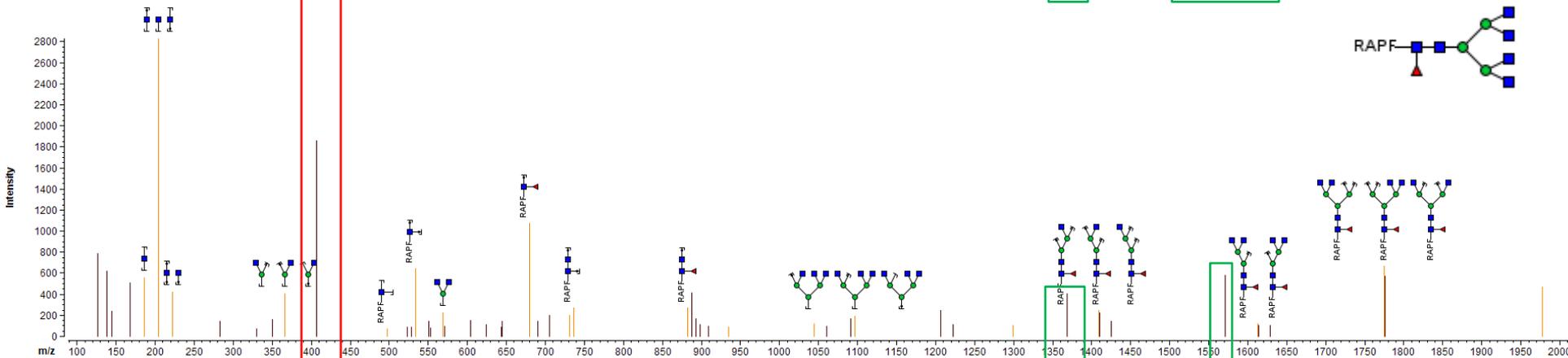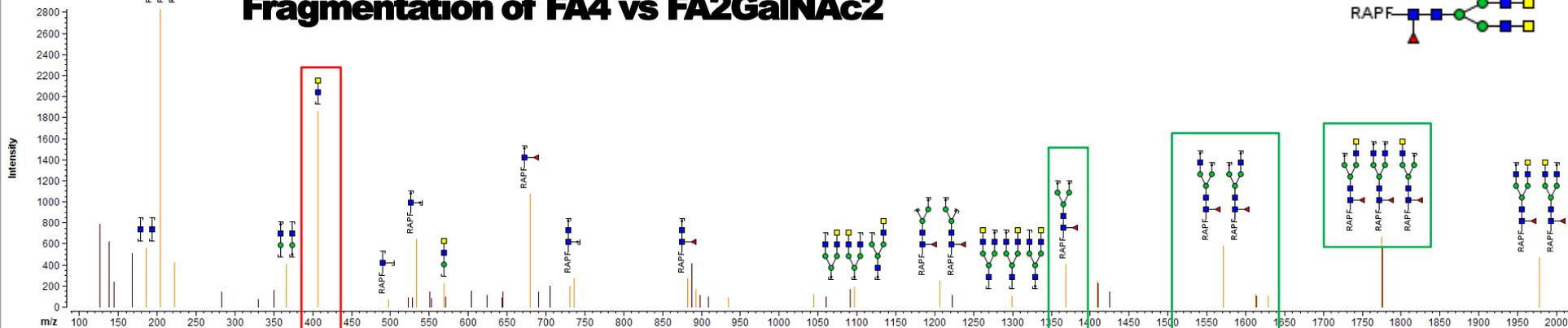
Fragmentation of FA4 vs FA2GalNAc2

Comparison of Human vs. CHO glycans
Sialylated Biantennary species
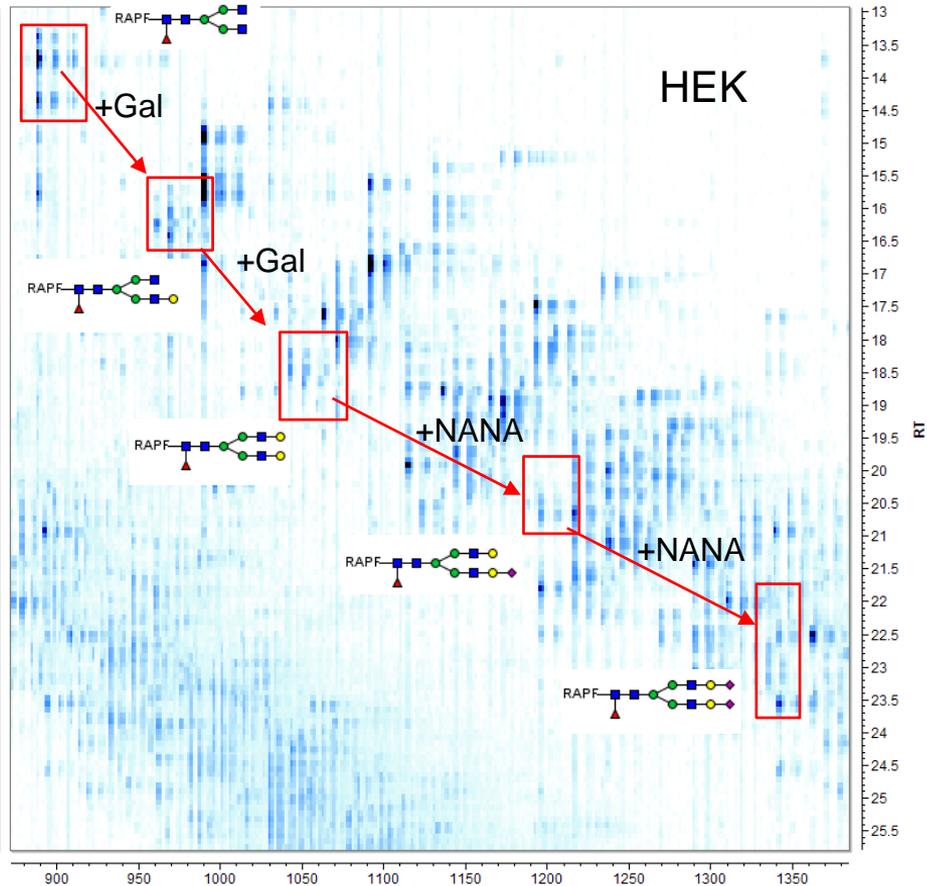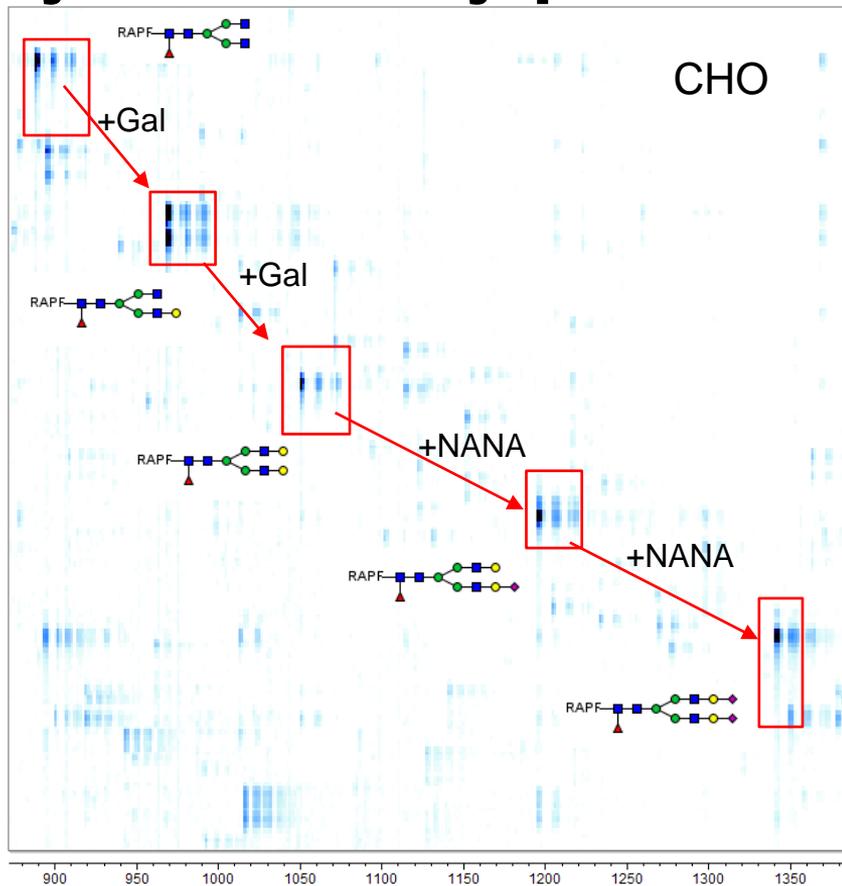
Comparison of Human vs. CHO glycans

Large sialylated species

Comparison of Human vs. CHO glycans
Large sialylated species

CHO

HEK

+NANA

+NANA

+NANA

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Glycan Structure Refresher

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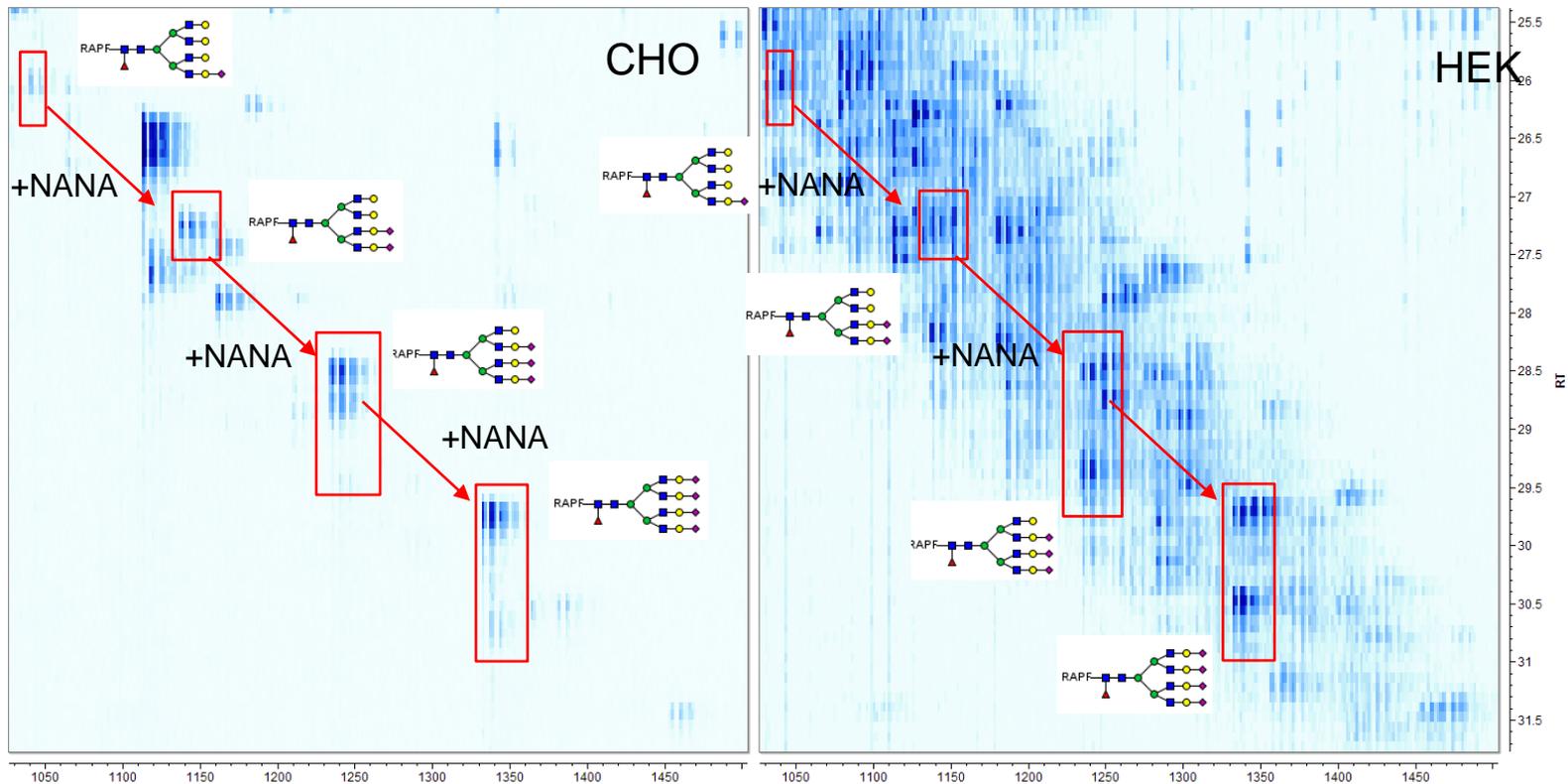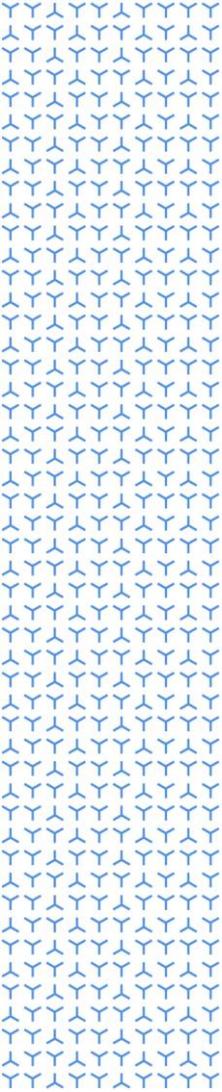
Glycan Structure Refresher

Class

High Mannose
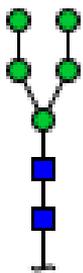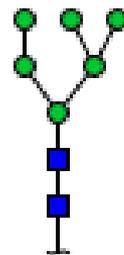
Man5   Man6

Complex

A2   FA2   FA2G2   FA2G2S2

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Glycan Structure Refresher: Isomers 1

FA2G1
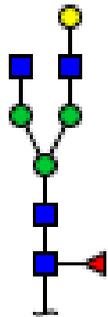
FA2G1
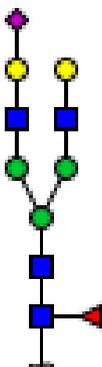
FA2G2S1
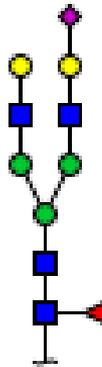
FA2G2S1
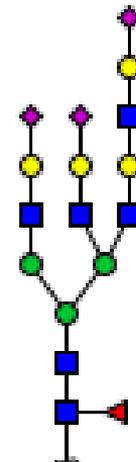
FA3G3Lac1S3
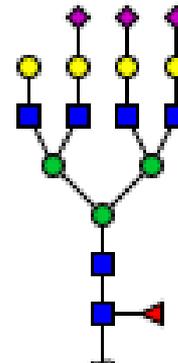
FA4G4S3

Glycan Structure Refresher: Isomers 2
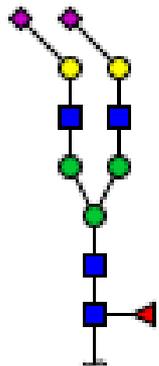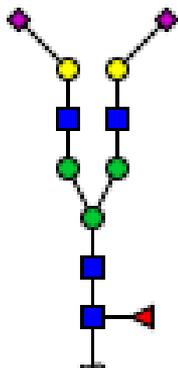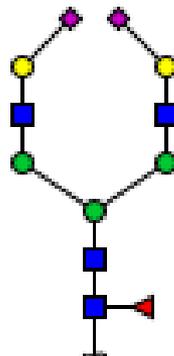
α[2→3], α[2→3]   α[2→3], α[2→6]   α[2→6], α[2→3]   α[2→6], α[2→6]
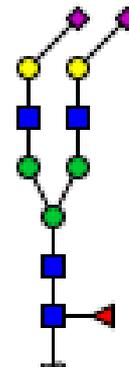
FA2G2S2

Glycan Structure Refresher: Isomers 2
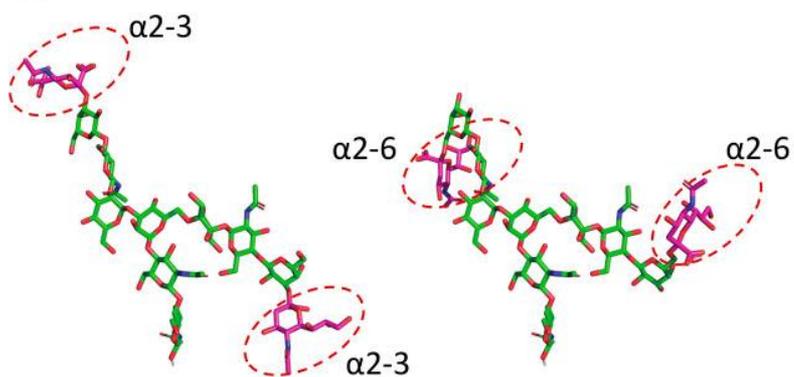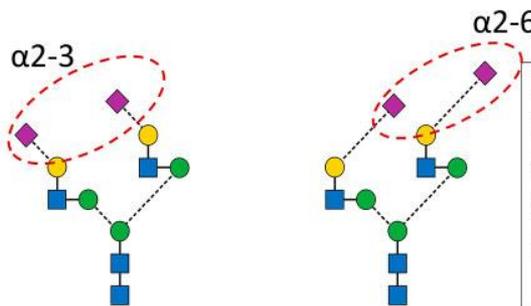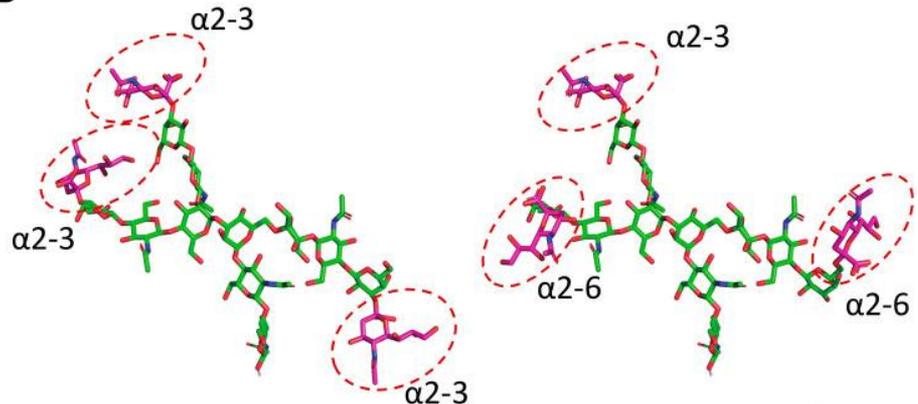
Dias FA, Ullao-Aguirre A 2021 Front. Endocrinol. 12,

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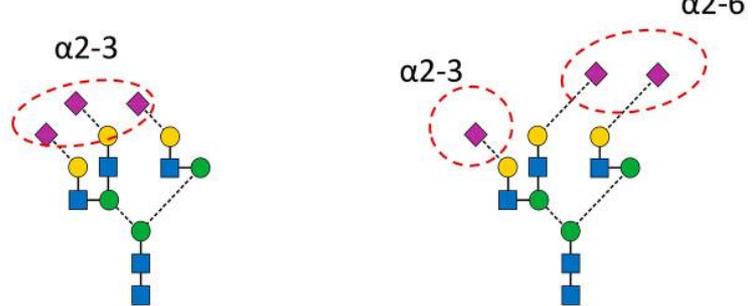
Glycan Structure Refresher: Isomers 3
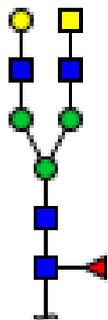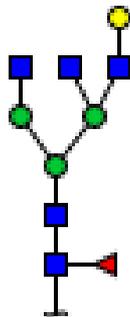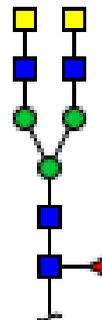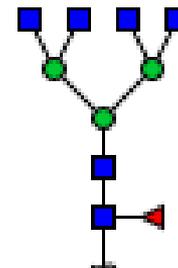
FA2G1GalNAc1   FA3G1   FA2GalNAc2   FA4

Harvey DJ et. Al. 2021 Anal. Bioanal. Chem. 413, 7229

Do KY, Do SI, Cummings RD 1997 Glycobiol. 7, 183

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Glycan Structure Refresher: Etc.

+42

+41

Ac

FA2G2S1Ac1

FA2G1GalNAc1S1

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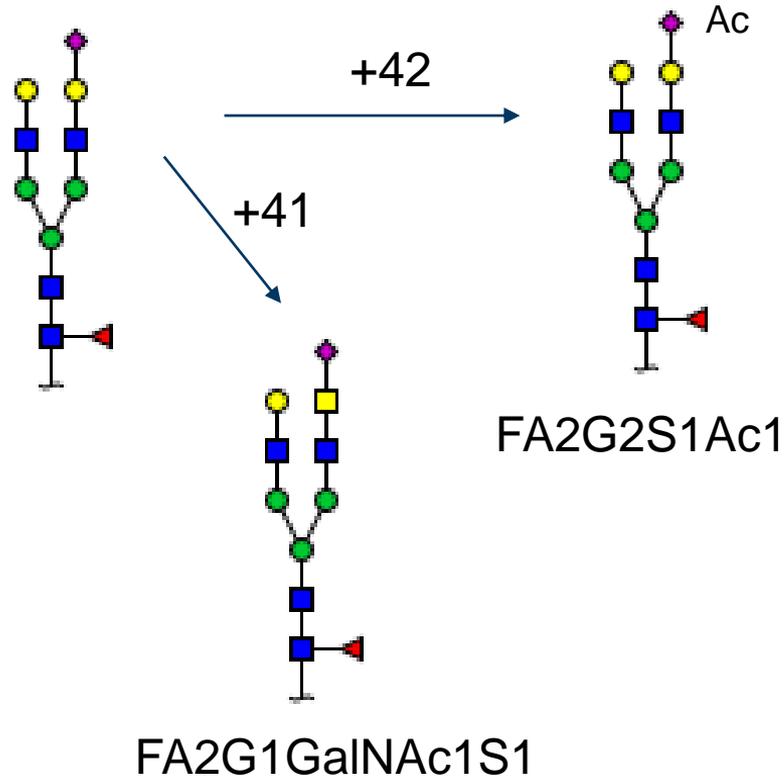
Glycan Structure Refresher: Etc.
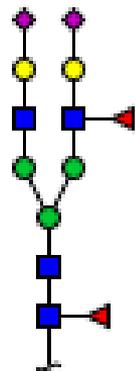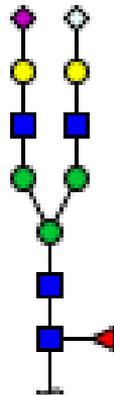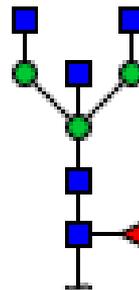
	FA2F1G2S2	FA2G2S1Sg1	FA2B	FA2G2S2Su1
Mass Shift	+146, -16	+16	+204, -41	+80
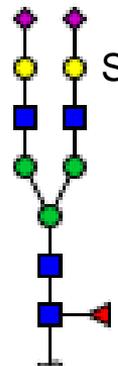
+162 Hex, +291 NeuAc

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