Variation of Site-Specific Glycosylation Profiles for Influenza Glycoproteins from Different Vaccines and Recombinant Sources

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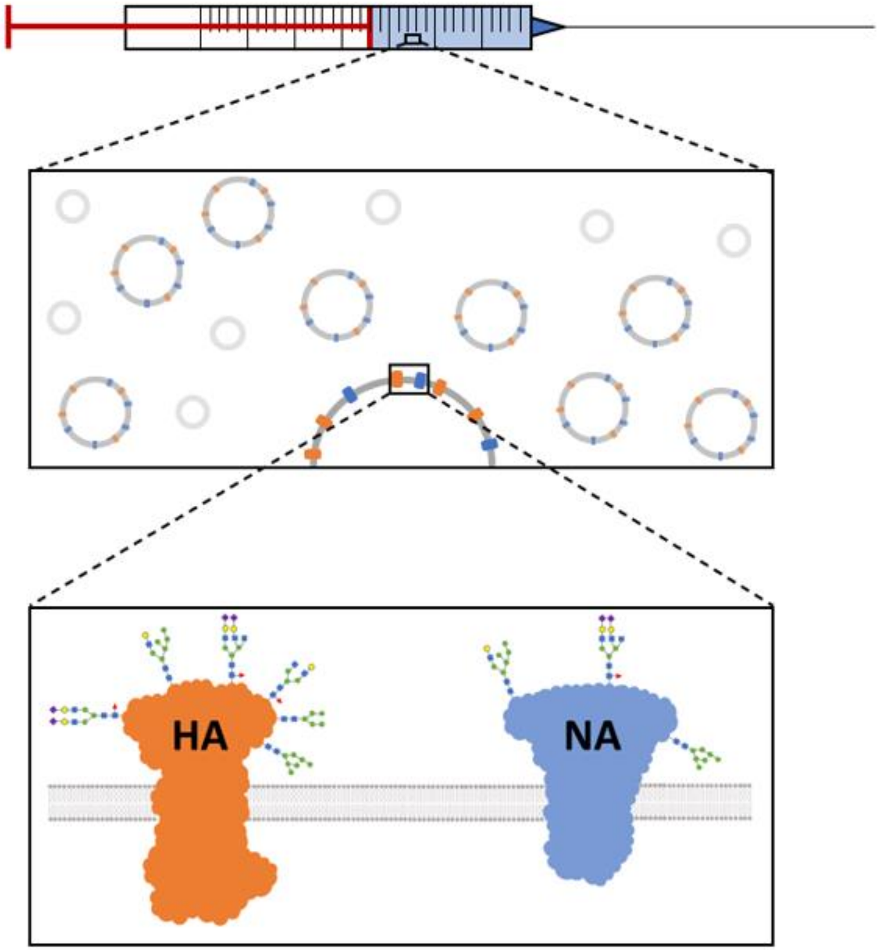
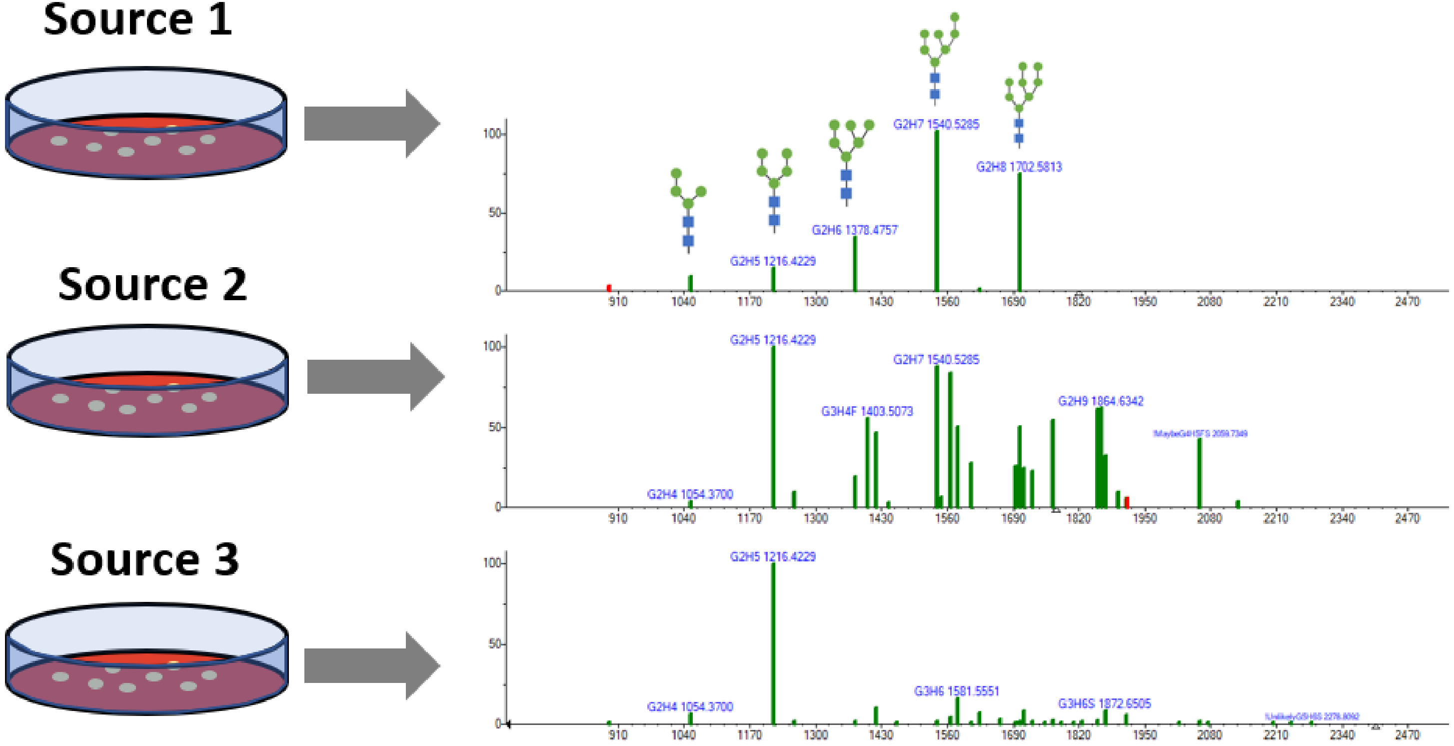
March 20, 2023

National Institute of Standards and Technology
U.S. Department of Commerce

Mass Spectrometry Data Center

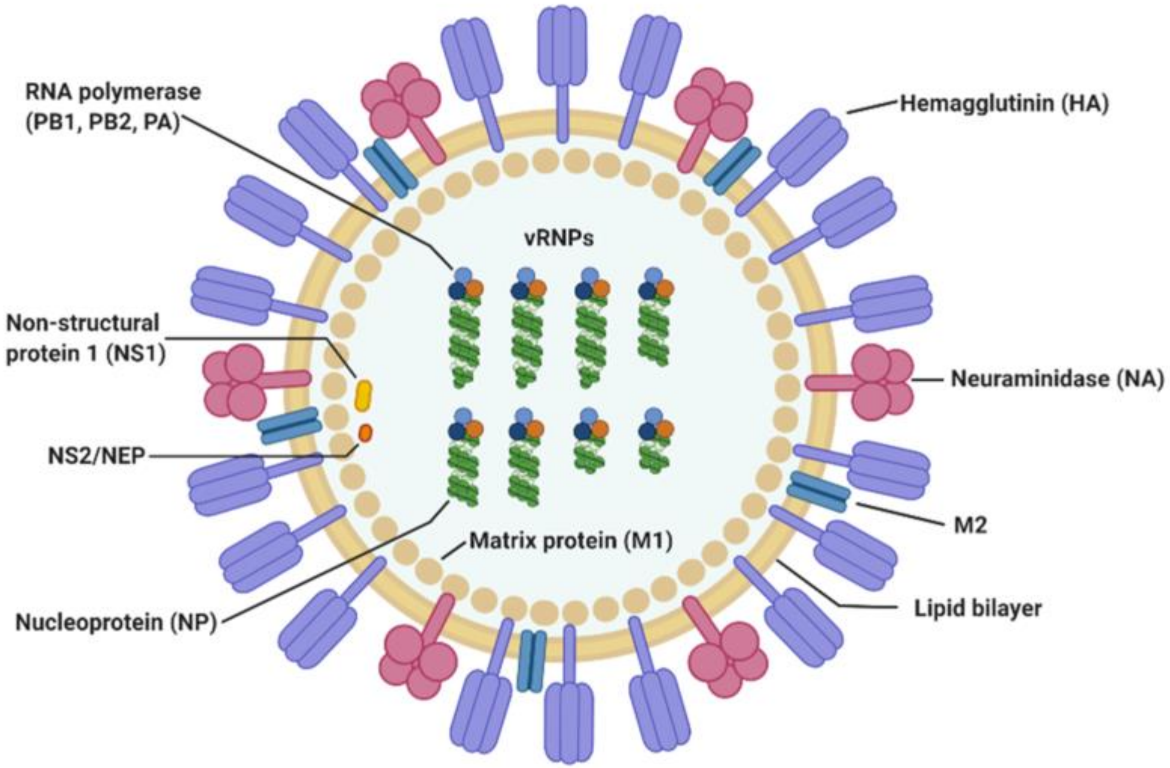
<u>G</u>lycopeptide <u>A</u>bundance <u>D</u>istribution <u>S</u>pectrum





Influenza Virus

- 10 proteins
- Hemagglutinin (HA) and neuraminidase (NA) transmembrane proteins.
- Diversity of strains arise through two mechanisms: point mutations in the viral genome or reassortment between two co-circulating strains.

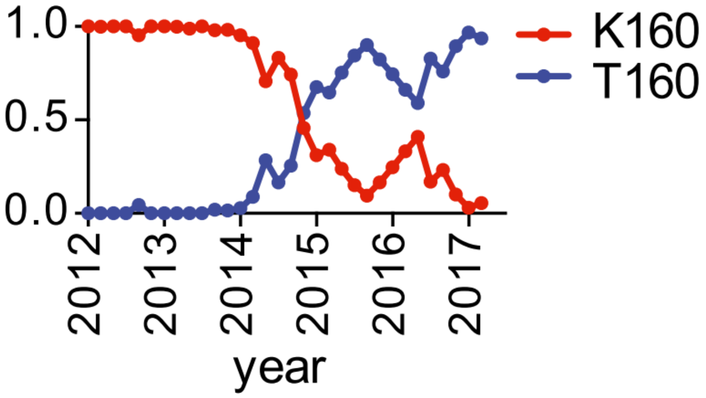


Jung et al., 2020

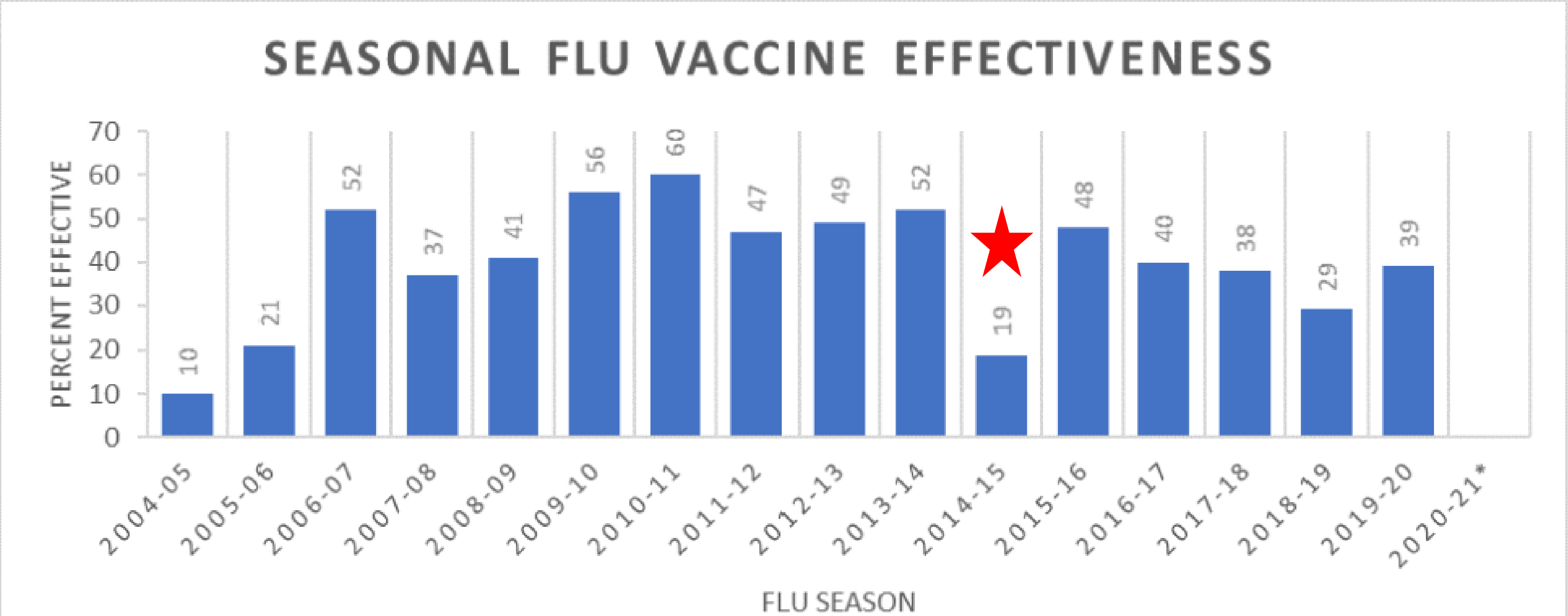
Why Glycosylation Matters For Vaccines

…THLNFKYPAL…
…THLNFTYPAL…

Sequon motif
NXT/S X≠P

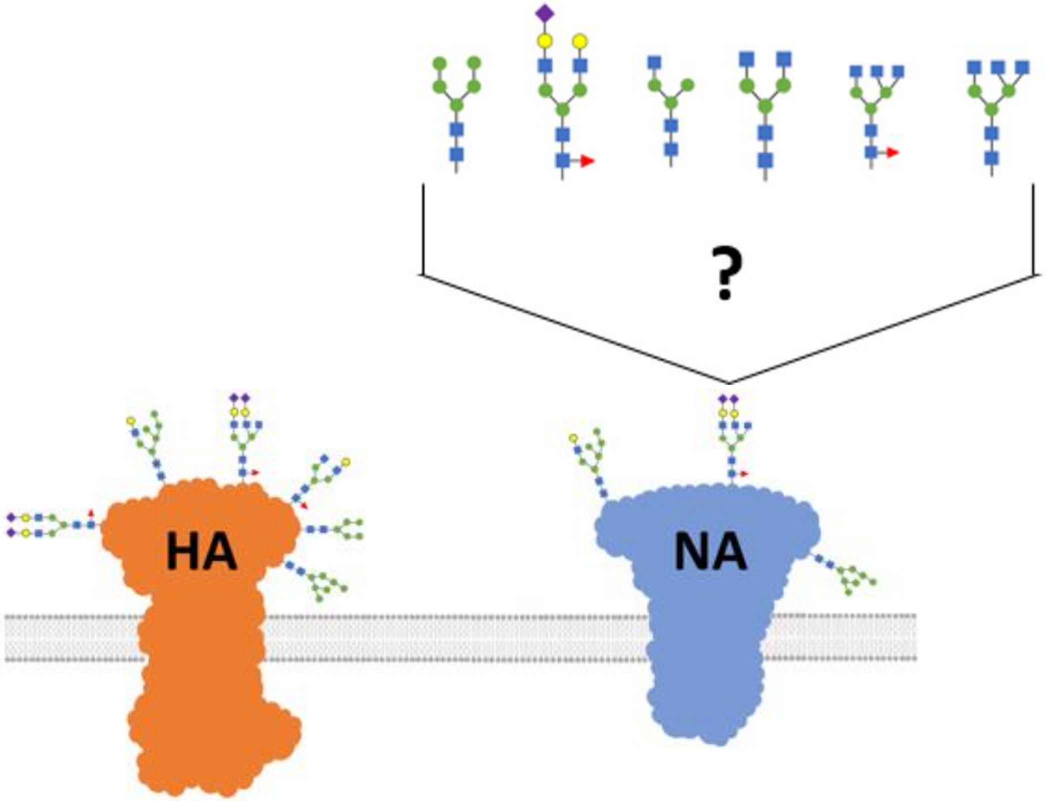


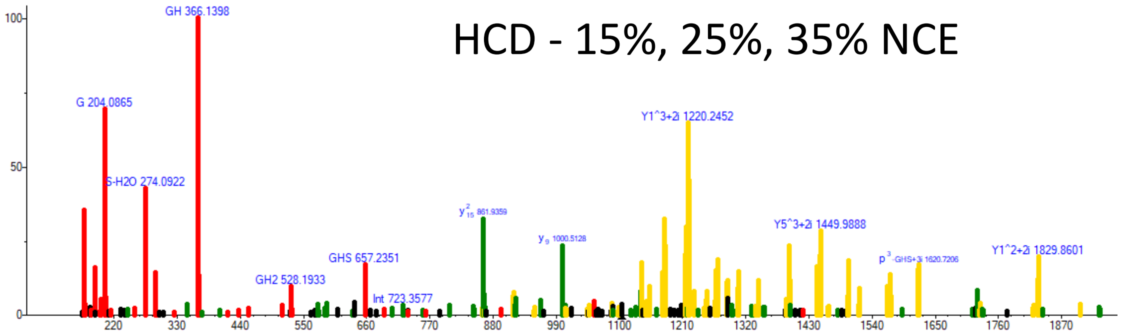
Zost et al., 2017



www.cdc.gov

Site-Specific Glycosylation



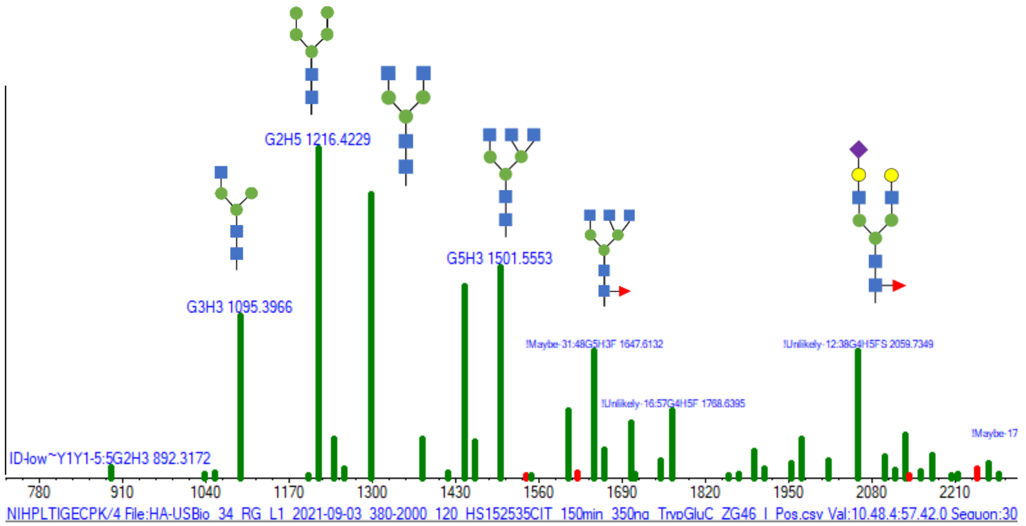


HCD - 15%, 25%, 35% NCE

Oxonium ions
Peptide backbone ions
Glycopeptide ions

Visualizing Glycan Distributions

1. Glycopeptide search using Byonic software
2. Tandem library creation and spectral validation via create_glycopeptide_lib.exe and MS_Piano.exe.
3. GADS creation using make-gads.exe



Glycopeptide Abundance Distribution Spectrum

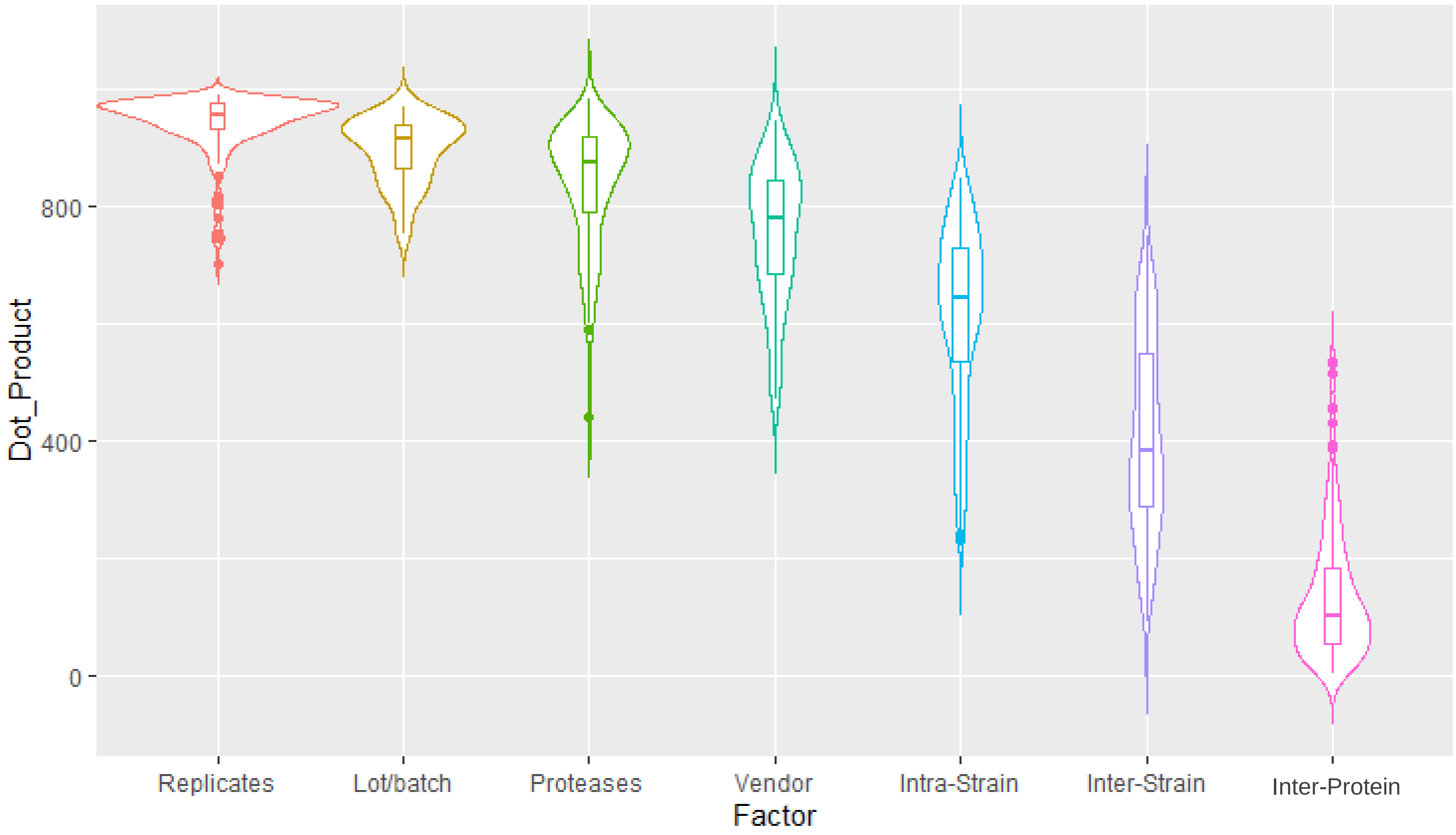
Recombinant Proteins

TABLE 1 Recombinant proteins analyzed for site-specific glycosylation

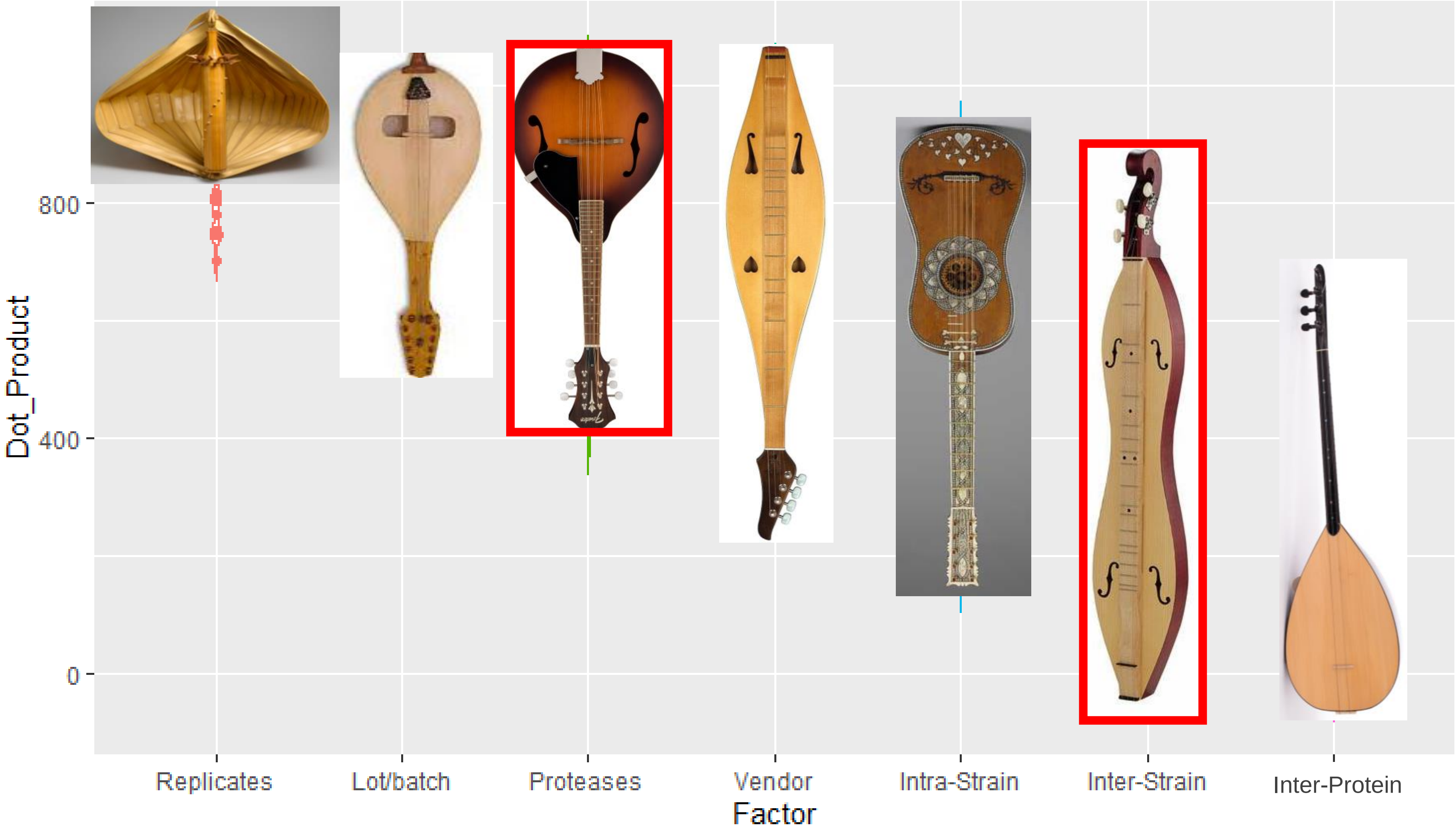
Abbreviation	Protein	Strain	Subtype	Vendors	Number of sequons	Protein mass* (kDa)
HA-CA09	**HA**	**A/California/04/2009**	**H1N1**	**Creative Biomart**	**8**	**63**
HA-NC99	**HA**	**A/New Caledonia/20/1999**	**H1N1**	**Sino Biological**	**10**	**63**
HA-JP57	**HA**	**A/Japan/305/1957**	**H2N2**	**Creative Biomart**	**8**	**63**
HA-HK14	**HA**	**A/Hong Kong/485197/2014**	**H3N2**	**Biovision**	**13**	**64**
HA-HK97	**HA**	**A/Hong Kong/483/1997**	**H5N1**	**BioVision, US Biological, Sino Biological**	**8**	**64**
NA-AZ08	**NA**	**A/Arizona/13/2008**	**H1N1**	**Sino Biological**	**9**	**52**
NA-TH04	**NA**	**A/Thailand/1(KAN-1)/2004**	**H5N1**	**BioVision, US Biological, Sino Biological**	**3**	**49**
NA-NL03	**NA**	**A/Netherlands/219/2003**	**H7N7**	**Creative Biomart**	**11**	**52**

*Unglycosylated

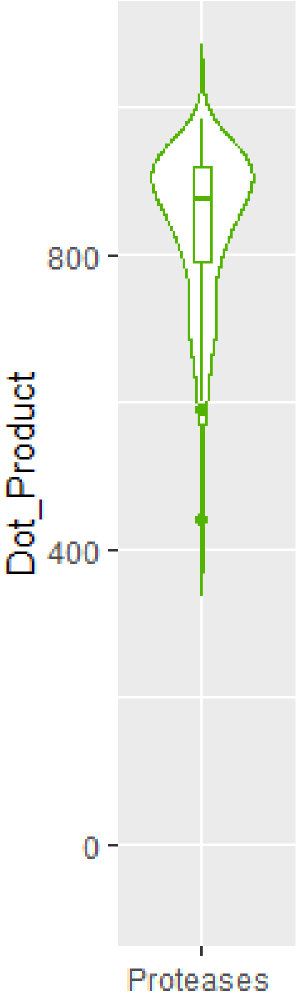
Review - Variation in glycosylation profile



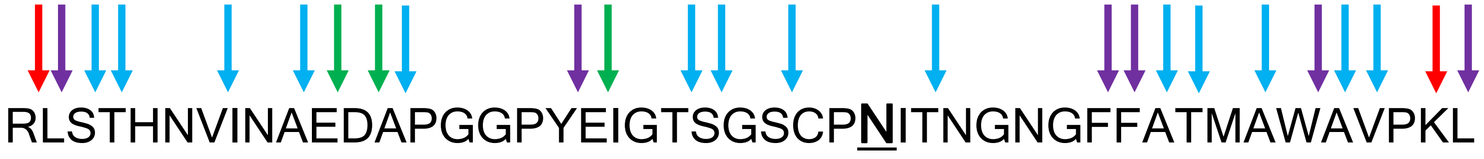
Review - Variation in glycosylation profile



Variation From Different Protease Digestions

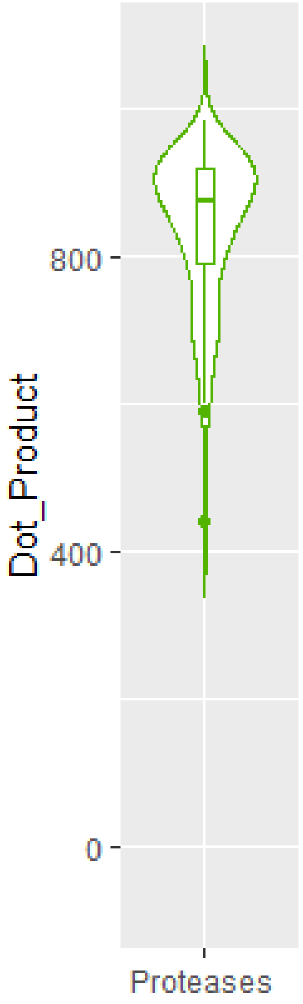


Protease Combination	Amino Acid Cleavage Sites
Trypsin + Lys-C	KR
Trypsin + Glu-C	KRED
Trypsin + Chymotrypsin	KRFWYL
Chymotrypsin + Glu-C	FWYLED
Chymotrypsin	FWYL
Alpha-lytic	TASV



RLSTHNVINAEDAPGGPYEIGTSGSCP<u>N</u>ITNGNGFFATMAWAVPKL

Variation From Different Protease Digestions



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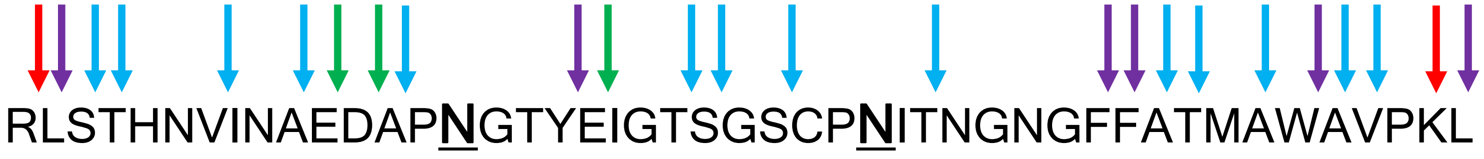
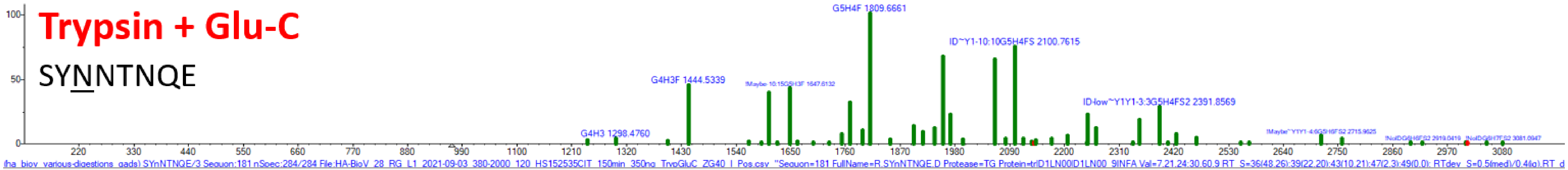
Trypsin + Lys-C	KR
Trypsin + Glu-C	KRED
Trypsin + Chymotrypsin	KRFWYL
Chymotrypsin + Glu-C	FWYLED
Chymotrypsin	FWYL
Alpha-lytic	TASV

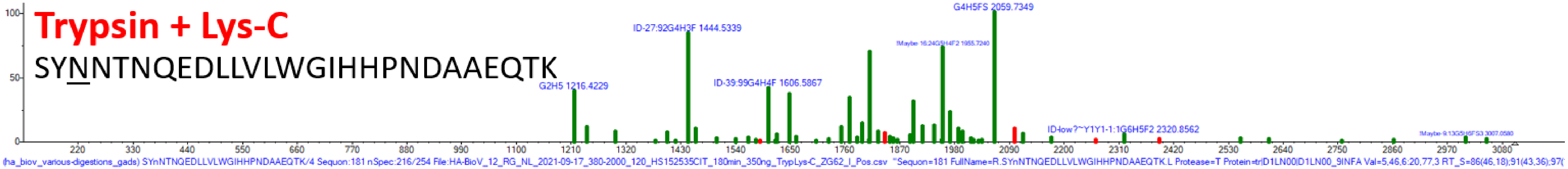


Variation From Different Protease Digestions



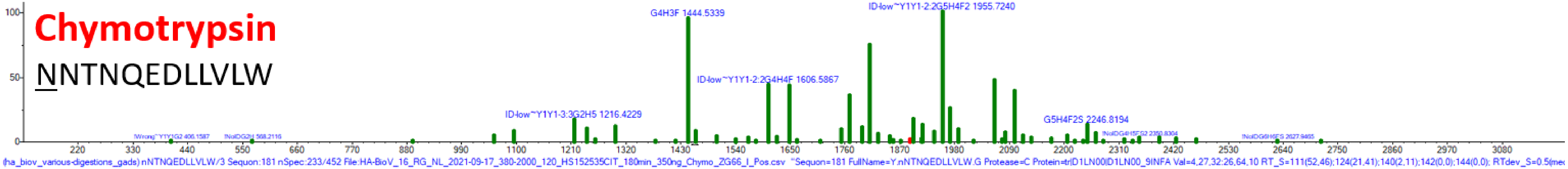
Trypsin + Glu-C

SYNNTNQE



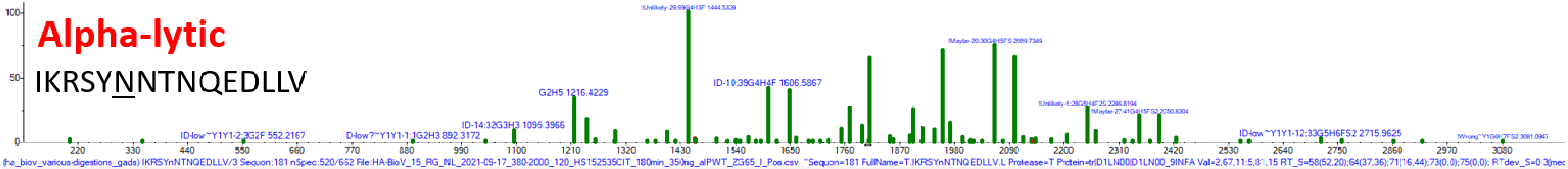
Trypsin + Lys-C

SYNNTNQEDLLVLWGIHHPNDAAEQTK



Chymotrypsin

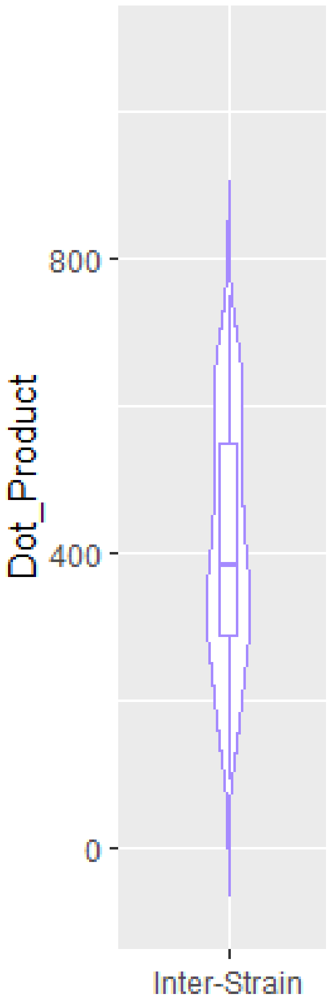
NNTNQEDLLVLW



Alpha-lytic

IKRSYNNTNQEDLLV

Variation From Different Strains



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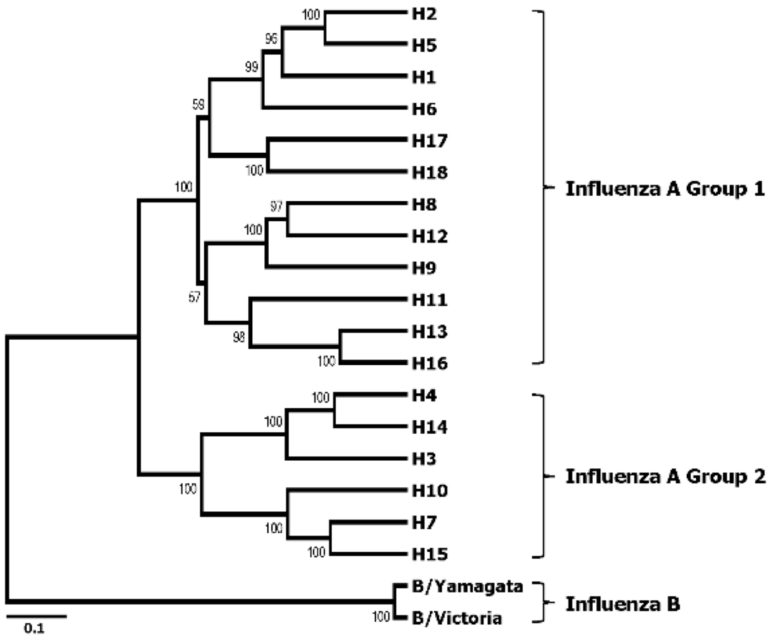
HA	**A/California/04/2009**	**H1N1**
HA	**A/New Caledonia/20/1999**	**H1N1**
HA	**A/Japan/305/1957**	**H2N2**
HA	**A/Hong Kong/485197/2014**	**H3N2**
HA	**A/Hong Kong/483/1997**	**H5N1**
NA	**A/Arizona/13/2008**	**H1N1**
NA	**A/Thailand/1(KAN-1)/2004**	**H5N1**
NA	**A/Netherlands/219/2003**	**H7N7**



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MKAILVVLLYTFA----------TANADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLL
-MEKIVLLLATVS----------LVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDIL
--MAIIYLILLFT----------AVRGDQICIGYHANNSTEKVDTILERNVTVTHAKDIL
    :: :   .                 :* *  :  .   *.*  : .: **    :

QNSSIGEICDSPH------QILDGENCTLIDALLGDPQCDGFQN-KKWDLFVERS-KAYS
EDSHNGKLCLLKGI-----APLQLGNCSVAGWILGNPECELLISKESWSYIVETPNPENG
EDKHNGKLCKLRGV-----APLHLGKCNIAGWILGNPECESLSTASSWSYIVETPSSDNG
ERTHNGKLCDLNGV-----KPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKASPAND
EKTHNGKLCKLNGI-----PPLELGDCSIAGWLLGNPECDRLLSVPEWSYIMEKENPRDG
     . :.                .*.  .  *. * *         . : *      .

NCYPYDVPDYASLRSLVATSGTLE---FNNE----SFNWTGVTQ-NGTSSACIRR-SSSS
TCYPGYFADYEELREQLSSVSSFERFEIFPK----ESSWPNHTV-TGVSASCSHN-GKSS
TCYPGDFIDYEELREQLSSVSSFERFEIFPK----TSSWPNHDSNKGVTAACPHA-GAKS
LCYPGNFNDYEELKHLLSRINHFEKIQIIPK-----SSWSNHDASSGVSSACPYL-GKSS
LCYPGSFNDYEELKHLLSSVKHFEKVKILPK-----DRWTQHTTT-GGSRACAVS-GNPS
 *:*    :  .::         *   :  .               * : :*    .  .

FFSRLNWLTHLNYTY-----PALNVTMPNNEQFDKLYIWGVHHPGTDKDQIFLYAQSSGR
FYRNLLWLTGKNGLY-----PNLSKSYVNNKEKEVLVLWGVHHPPNIGNQRALYHTENAY
FYKNLIWLVKKGNSY-----PKLSKSYINDKGKEVLVLWGIHHPSTSADQQSIYQNADTY
FFRNVVWLIKKNSTY-----PTIKRSYNNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTY
FFRNMVWLTKKGSDY-----PVAKGSYNNTSGEQMLIIWGVHHPNDETEQRTLYQNVGTY
*:  : *                .         : : :**.*          ::

IT-VSTKRSQQAVIPNIGSRPRI-----RDIPSRISIYWTIVKPGDILLINSTGNLIAPR
VS-VVSSHYSRRFTPEIAKRPKV-----RDQEGRINYYWTLLEPGDTIIFEANGNLIAPW
VF-VGSSRYSKKFKPEIAIRPKV-----RDQEGRMNYYWTLVEPGDKITFEATGNLVVPR
IS-VGTSTLNQRLVPEIATRPKV-----NGQSGRIEFFWTILKPNDAINFESNGNFIAPE
VS-VGTSTLNKRSTPEIATRPKV-----NGQGGRMEFSWTLLDMWDTINFESTGNLIAPE
     :.        :*.  *           .*:   : : .  .   :    ..: *
```

```
GYFKI-RSGKSSIMRSDAPIGKCKSECITPNGSIPNDKPF-QNVNRITYGACPRYVKHST
YAFALSRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPF-QNVHPVTIGECPKYVRSAK
YAFAMERNAGSGIIISDTPVHDCNTTCQTPKGAINTSLPF-QNIHPITIGKCPKYVKSTK
YAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGAINSSMPF-HNIHPLTIGECPKYVKSNR
YGFKISKRGSSGIMKTEGTLENCETKCQTPLGAINTTLPF-HNVHPLTIGECPKYVKSEK
  :       * ::  .              *.: .  *:  . :  : * ** :*:

LKLATGMRNVPEKQ----TRGIFGAIAGFIENGWEGMVDGWYGFRHQNSEGRGQAADLKS
LRMVTGLRNIPSIQ----SRGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKS
LRLATGLRNIPSIQ----SRGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADLKS
LVLATGLRNAPQRERRRKKRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADQES
LVLATGLRNVPQIE----SRGLFGAIAGFIEGGWQGMVDGWYGYHHSNDQGSGYAADKES
* :..* :  *  :     **:*******:*.** **: **:*:   . .* . *** :*

TQAAIDQINGKLNRLIGKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLV
TQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLV
TQNAIDEITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFLDIWTYNAELLV
TQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLV
TQKAFDGITNKVNSVIEKMNTQFEAVGKEFSNLERRLENLNKKMEDGFLDVWTYNAELLV
** *:: :. ::* :      ::  :   :..:. .: :*:: ::*   *  : : ** *

ALENQHTIDLTDSEMNKLFEKTKKQLRENAEDMGNGCFKIYHKCDNACIGSIRNGTYDHN
LLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYP
LLENERTLDYHDSNVKNLYEKVRSQLKNNAKEIGNGCFEFYHKCDNTCMESVKNGTYDYP
LMENERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVKNGTYDYP
LMENERTLDFHDSNVKNLYDKVRMQLRDNVKELGNGCFEFYHKCDDECMNSVKNGTYDYP
 :.*:  ::  *..:  *  * :  *  .. ::*****:  ***:: *:  :   *::

VYRDEALNNRFQIKGVELK--SGYKDWILWI-SFAISCFLLCVALLGFIMWACQKGNIRC
KYSEESKLNREKIDGVKLE--SMGVYQILAIYSTVASSLVLLVSLGAISFWMCSNGSLQC
KYSEEAKLNREEIDGVKLE--STRIYQILAIYSTVASSLVLVVSLGAISFWMCSNGSLQC
QYSEEARLNREEISGVKLE--SMGTYQILSLYSTVASSLALAIMVAGLSLWMCSNGSLQC
KYEEESKLNRNEIKGVKLS--SMGVYQILAIYATVAGSLSLAIMMAGISFWMCSNGSLQC
 :   :     :* ...*.  .     **   : . ..: : : :  : .:  ....: *
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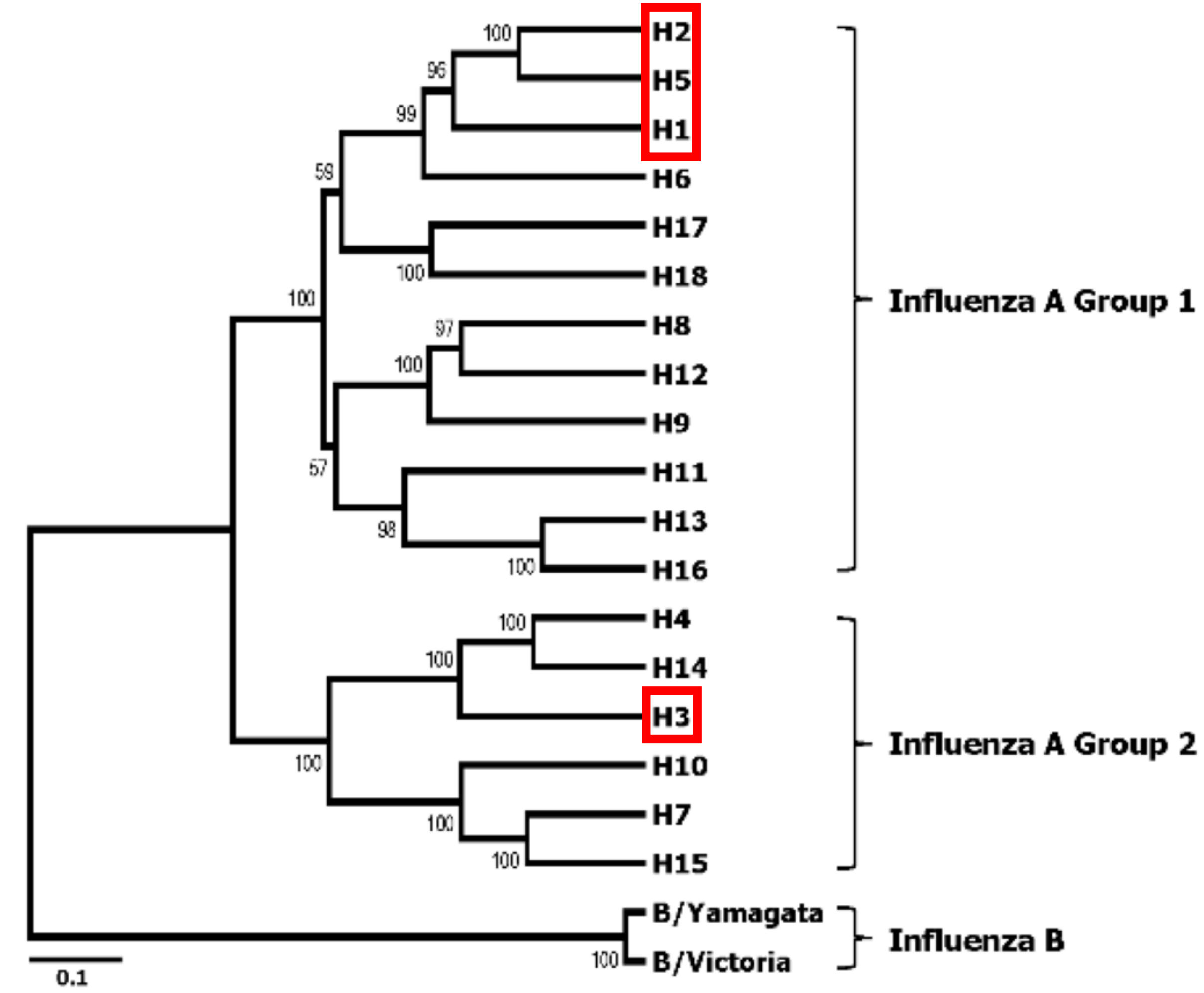
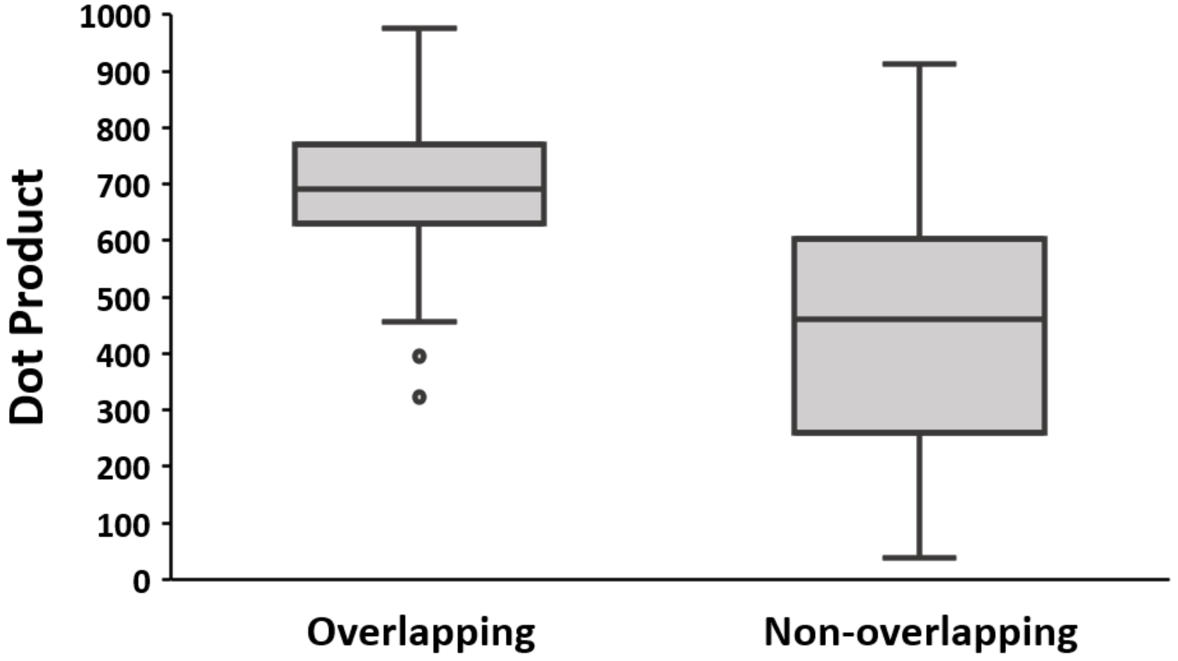
Conserved Regions Have Conserved Glycosylation Distribution



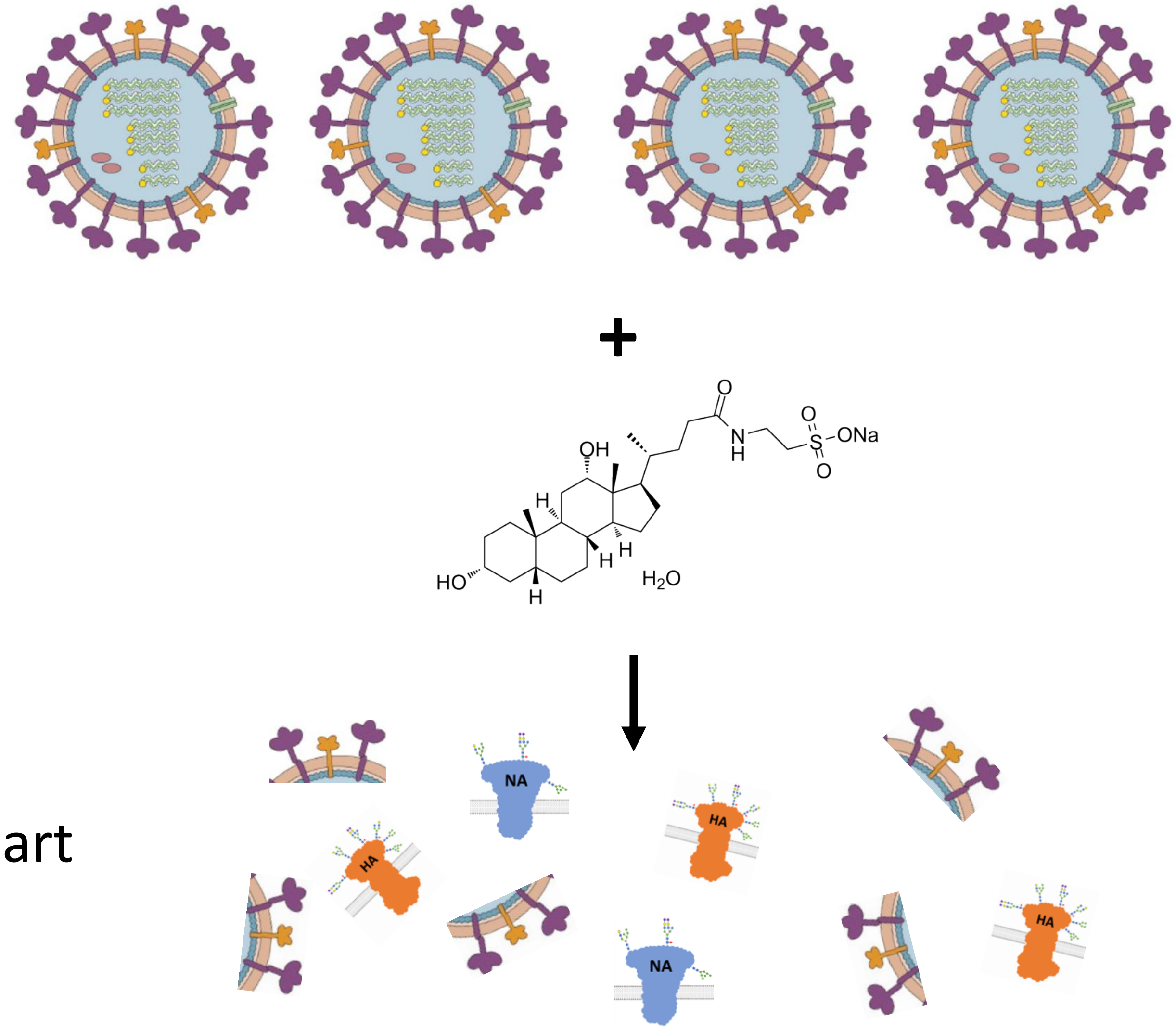


Jang et al., 2014

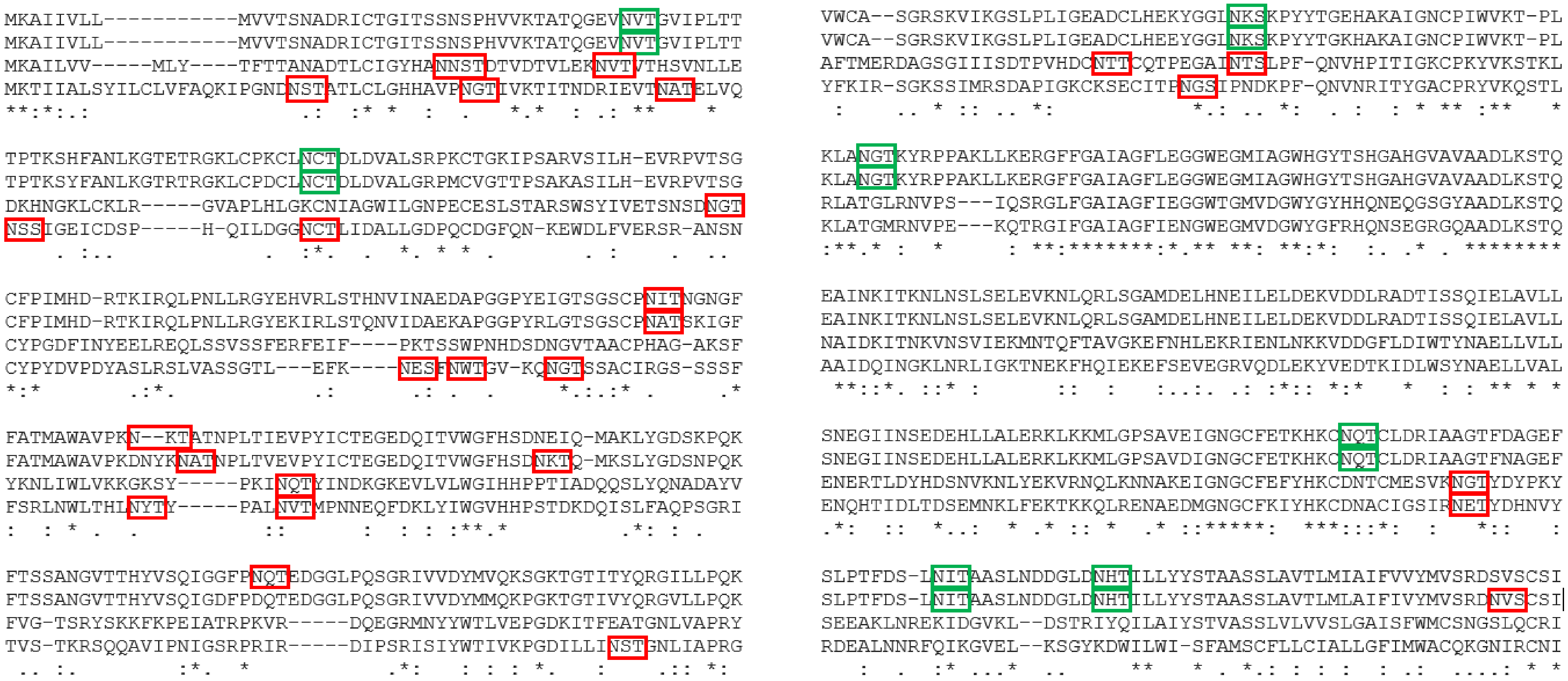
Influenza Vaccines

- Contents
    - Split virion
    - 60 mg HA per dose (15 mg per strain)
    - Thimerosal
    - Mercury
    - Sodium Chloride
    - Sodium Phosphate
    - Sodium Taurodeoxycholate
    - Ovalbumin
    - Sucrose………

- Afluria Quadrivalent
- 3 Monovalent vaccines from NIBSC
- 3 Monovalent vaccines from Creative Biomart



Quadrivalent Vaccine Challenges



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MKAIIVLL-----------MVVTSNADRICTGITSSNSPHVVKTATQGEVNVTGVIPLTT
MKAIIVLL-----------MVVTSNADRICTGITSSNSPHVVKTATQGEVNVTGVIPLTT
MKAILVV-----MLY----TFTTANADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLE
MKTIIALSYILCLVFAQKIPGNDNSTATLCLGHHAVPNGTIVKTITNDRIEVTNATELVQ
**:*:.:                 .:  :* *  :  .   *.*  : .: **    *

TPTKSHFANLKGTETRGKLCPKCLNCTDLDVALSRPKCTGKIPSARVSILH-EVRPVTSG
TPTKSYFANLKGTRTRGKLCPDCLNCTDLDVALGRPMCVGTTPSAKASILH-EVRPVTSG
DKHNGKLCKLR-----GVAPLHLGKCNIAGWILGNPECESLSTARSWSYIVETSNSDNGT
NSSIGEICDSP-----H-QILDGGNCTLIDALLGDPQCDGFQN-KEWDLFVERSR-ANSN
    . :..            .  :*.  .  *. * * .       . :    .  ..

CFPIMHD-RTKIRQLPNLLRGYEHVRLSTHNVINAEDAPGGPYEIGTSGSCPNITNGNGF
CFPIMHD-RTKIRQLPNLLRGYEKIRLSTQNVIDAEKAPGGPYRLGTSGSCPNATSKIGF
CYPGDFINYEELREQLSSVSSFERFEIF----PKTSSWPNHDSDNGVTAACPHAG-AKSF
CYPYDVPDYASLRSLVASSGTL---EFK----NESFNWTGV-KQNGTSSACIRGS-SSSF
*:*       .:*.           .:      .: .  .     *.:.:* .     .*

FATMAWAVPKN--KTATNPLTIEVPYICTEGEDQITVWGFHSDNEIQ-MAKLYGDSKPQK
FATMAWAVPKDNYKNATNPLTVEVPYICTEGEDQITVWGFHSDNKTQ-MKSLYGDSNPQK
YKNLIWLVKKGKSY-----PKINQTYINDKGKEVLVLWGIHHPPTIADQQSLYQNADAYV
FSRLNWLTHLNYTY-----PALNVTMPNNEQFDKLYIWGVHHPSTDKDQISLFAQPSGRI
:  : * .  .          ::      :  : : :**.*         .*: : .

FTSSANGVTTHYVSQIGGFPNQTEDGGLPQSGRIVVDYMVQKSGKTGTITYQRGILLPQK
FTSSANGVTTHYVSQIGDFPDQTEDGGLPQSGRIVVDYMMQKPGKTGTIVYQRGVLLPQK
FVG-TSRYSKKFKPEIATRPKVR-----DQEGRMNYYWTLVEPGDKITFEATGNLVAPRY
TVS-TKRSQQAVIPNIGSRPRIR-----DIPSRISIYWTIVKPGDILLINSTGNLIAPRG
 .. :.        :*.  *           .*:   : : : *.   :    .:: *:
```

```
VWCA--SGRSKVIKGSLPLIGEADCLHEKYGGLNKSKPYYTGEHAKAIGNCPIWVKT-PL
VWCA--SGRSKVIKGSLPLIGEADCLHEEYGGLNKSKPYYTGKHAKAIGNCPIWVKT-PL
AFTMERDAGSGIIISDTPVHDCNTTCQTPEGAINTSLPF-QNVHPITIGKCPKYVKSTKL
YFKIR-SGKSSIMRSDAPIGKCKSECITPNGSIPNDKPF-QNVNRITYGACPRYVKQSTL
 :    .. * :: .. *:           *.: .. *:  . :  : * ** :**   *

KLANGTKYRPPAKLLKERGFFGAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQ
KLANGTKYRPPAKLLKERGFFGAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQ
RLATGLRNVPS---IQSRGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADLKSTQ
KLATGMRNVPE---KQTRGIFGAIAGFIENGWEGMVDGWYGFRHQNSEGRGQAADLKSTQ
:**.* :  *     : **:*******:*.** **: **:*:  :. .* . ********

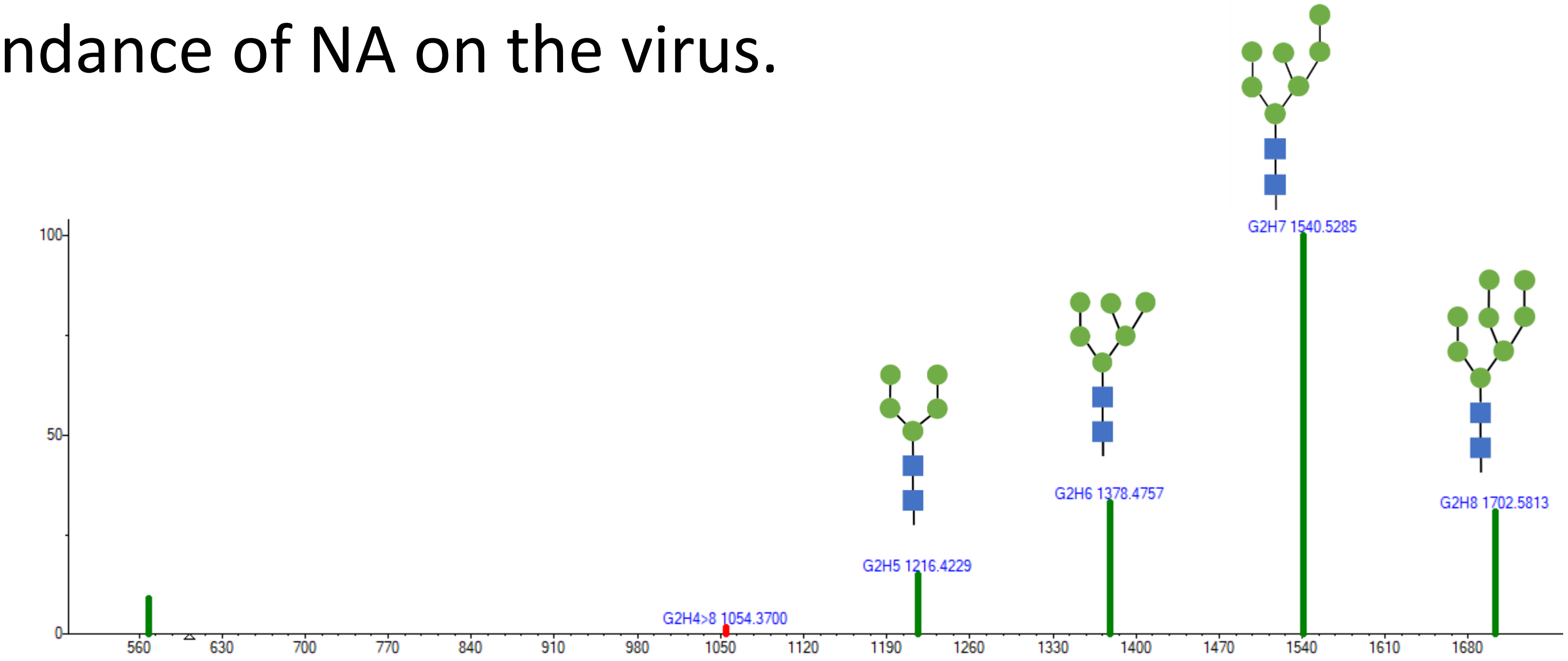
EAINKITKNLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTISSQIELAVLL
EAINKITKNLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTISSQIELAVLL
NAIDKITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFLDIWTYNAELLVLL
AAIDQINGKLNRLIGKTNEKFHQIEKEFSEVEGRVQDLEKYVEDTKIDLWSYNAELLVAL
 **::*. ::* :      ::  :   :..:. .: :*:: *:*   *  : : ** * *

SNEGIINSEDEHLLALERKLKKMLGPSAVEIGNGCFETKHKCNQTCLDRIAAGTFDAGEF
SNEGIINSEDEHLLALERKLKKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAGTFNAGEF
ENERTLDYHDSNVKNLYEKVRNQLKNNAKEIGNGCFEFYHKCDNTCMESVKNGTYDYPKY
ENQHTIDLTDSEMNKLFEKTKKQLRENAEDMGNGCFKIYHKCDNACIGSIRNETYDHNVY
.*:  ::  *..:  * .* :: *  .* ::*****:  ***:::*:  :   *::   :

SLPTFDS-LNITAASLNDDGLDNHTILLYYSTAASSLAVTLMIAIFVVYMVSRDSVSCSI
SLPTFDS-LNITAASLNDDGLDNHTILLYYSTAASSLAVTLMLAIFIVYMVSRDNVSCSI
SEEAKLNREKIDGVKL--DSTRIYQILAIYSTVASSLVLVVSLGAISFWMCSNGSLQCRI
RDEALNNRFQIKGVEL--KSGYKDWILWI-SFAMSCFLLCIALLGFIMWACQKGNIRCNI
   :  .  :* ...*  ..     **   * . *.: : : :  : .:  ....: * *
```

Afluria GADS

- 8→23 of the 40 sequons for the four hemagglutinin proteins identified. 27 are non-overlapping
- Three major GADS classes: high mannose, G4F complex, G5F complex
- No GADS were identified for NA proteins, probably due to a lower abundance of NA on the virus.



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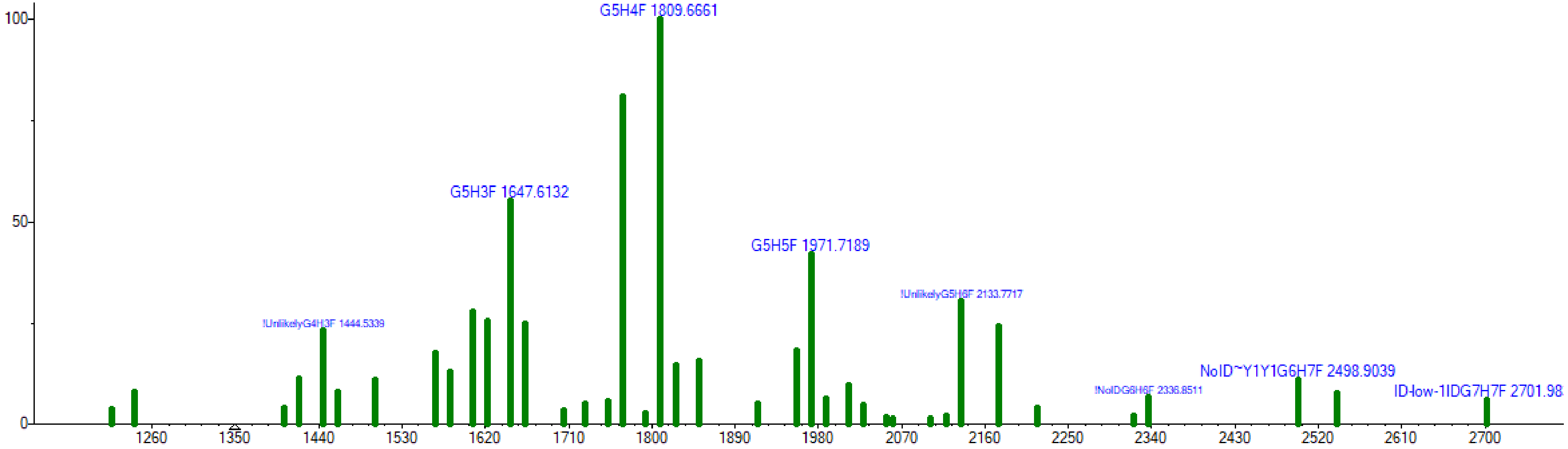
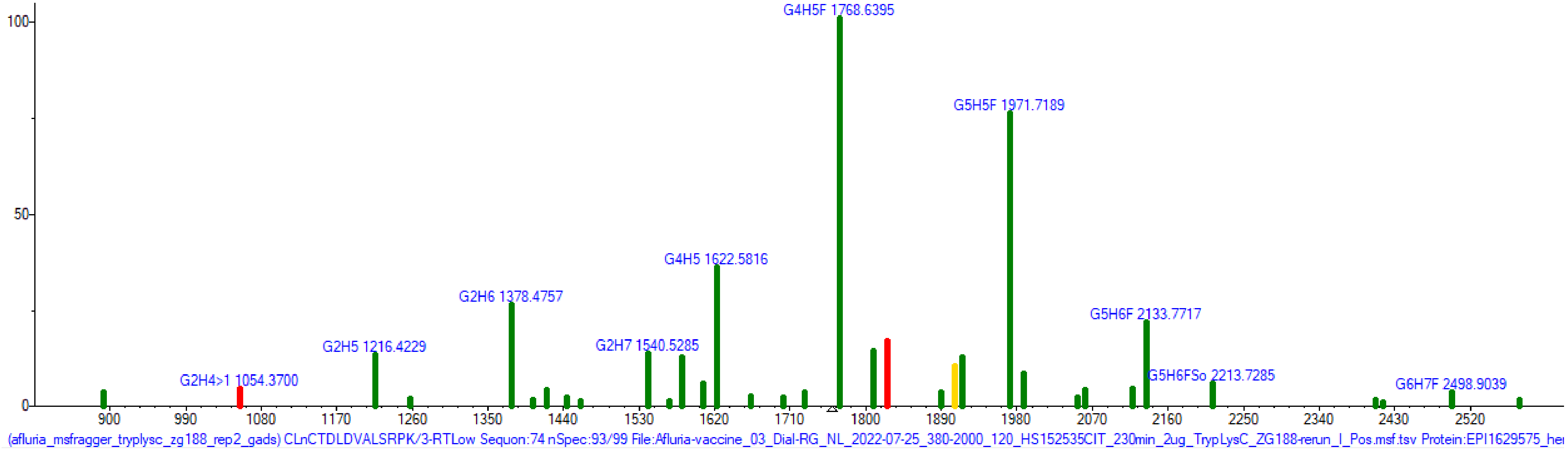


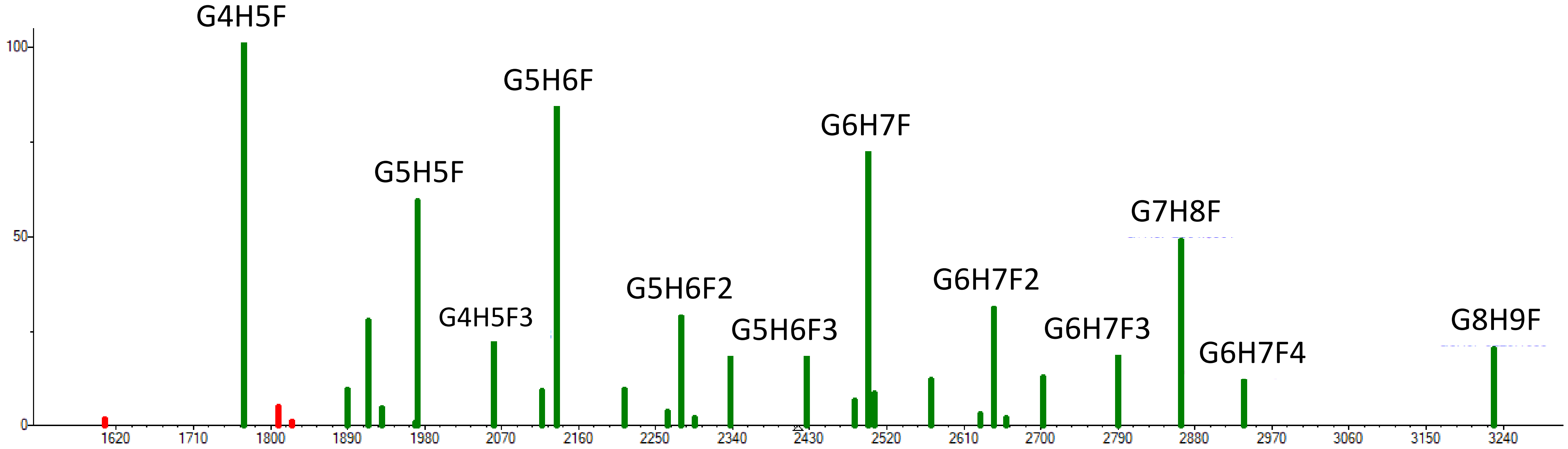
Afluria GADS

- 8→23 of the 40 sequons for the four hemagglutinin proteins identified. 27 are non-overlapping
- Three major GADS classes: high mannose, G4F complex, G5F complex
- No GADS were identified for NA proteins, probably due to a lower abundance of NA on the virus.



Monovalent vaccine GADS

- 90% sequons detected
- Three major GADS classes: high mannose, G4/G5, G4/G5 + high mannose
- Again, no major GADS identified for NA.

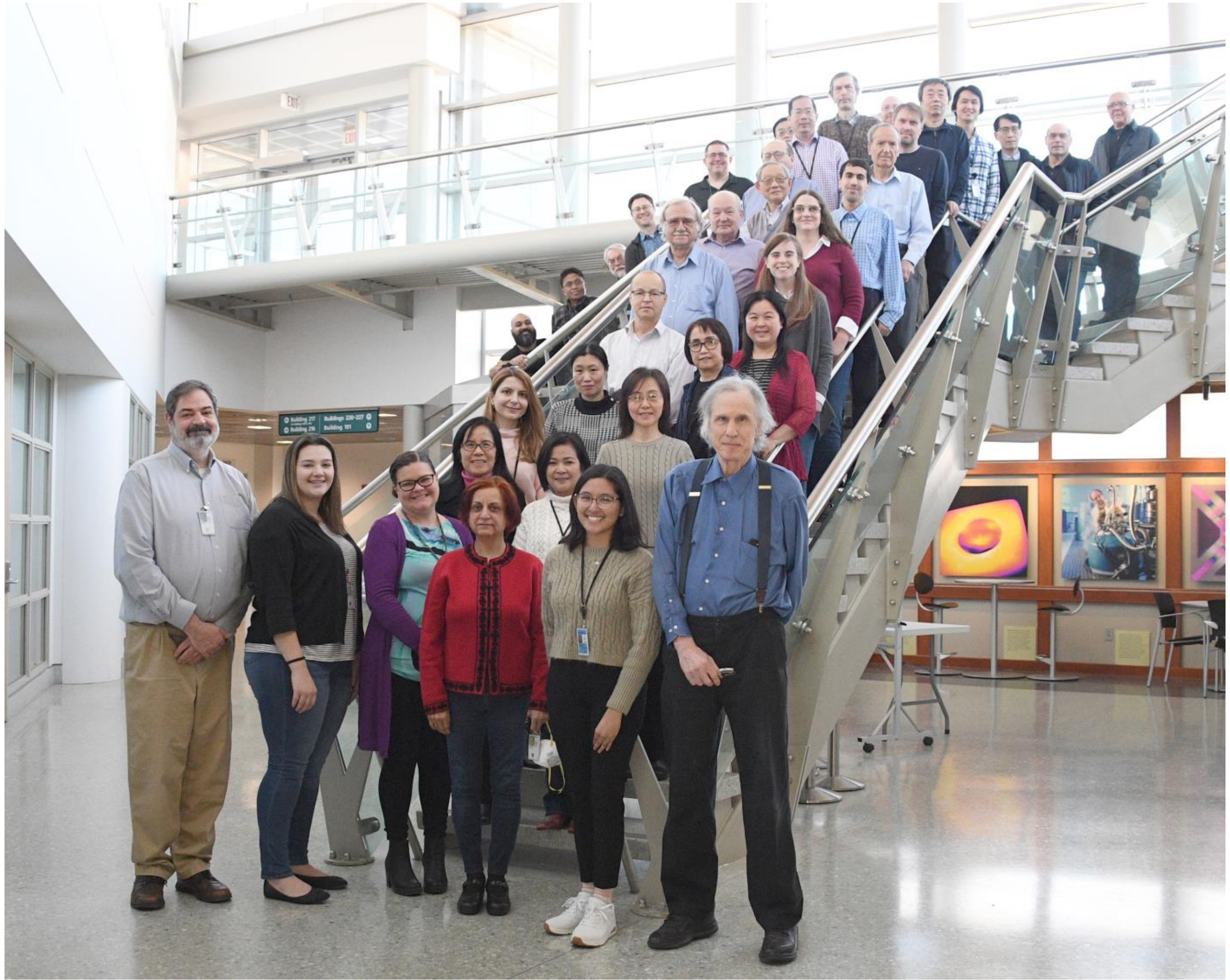


(zg271-276_filtered_gads) CLGHHAVPnGTLVKTITNDQIE/4 Sequon:38 nSpec:78/122 File:NIBSC-Vaccine(A-Philippines-2-1982)_13_Cyr_NL_2022-12-08_380-2000_120_HS152535CIT_230min_3ug_ChymoGluC_ZG274_I_Pos.msf.tsv Protein:ADJ41805.1_hemagglut

Conclusions

- Glycosylation profiles between replicates is most similar and between different proteins is least similar.
- Homologous sequence regions between different influenza strains have similar glycosylation distribution compared to non-conserved regions.
- Most glycans are high-mannose or mono-fucoylated complex in egg-based quadrivalent vaccines.
- Next steps:
  - Digest 2022-2023 vaccines and compare glycan distributions
  - Build reference MS2 and GADS libraries

Acknowledgments



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Questions?

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Supplemental Slides

Dot Product

Cosine of the angle between spectra represented as vectors

$$\frac{(\Sigma\, W_L W_U)^2}{\Sigma\, W_L^2 \; \Sigma\, W_U^2}$$

W_L = Weighted intensity of library

W_U = Weighted intensity of unknown

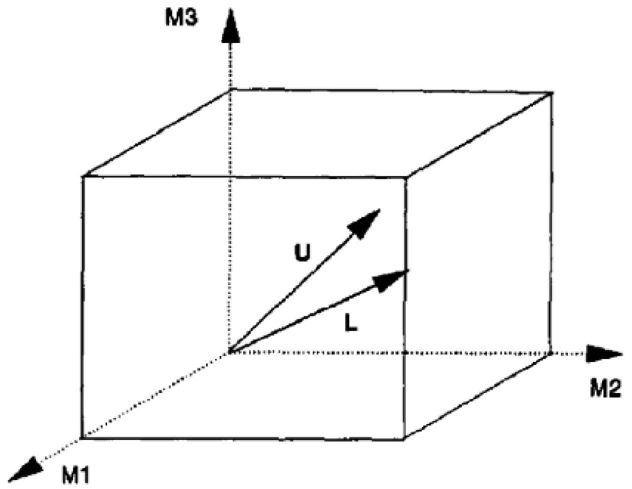


Figure 1. Vector representation of a hypothetical three-peak unknown (U) and library (L) mass spectrum in three-dimensional space (peaks have mass M1, M2, and M3).

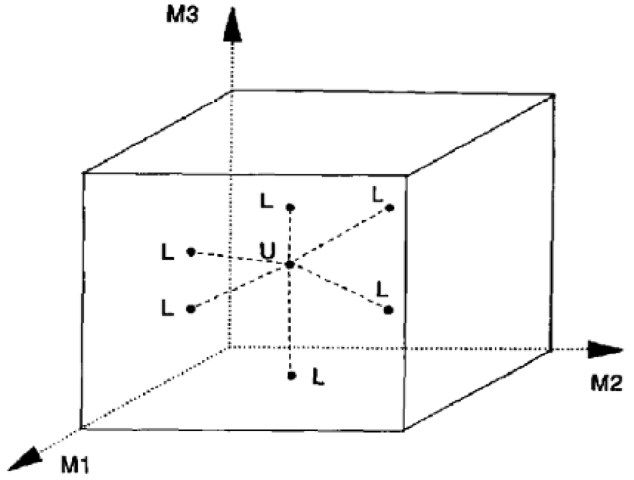
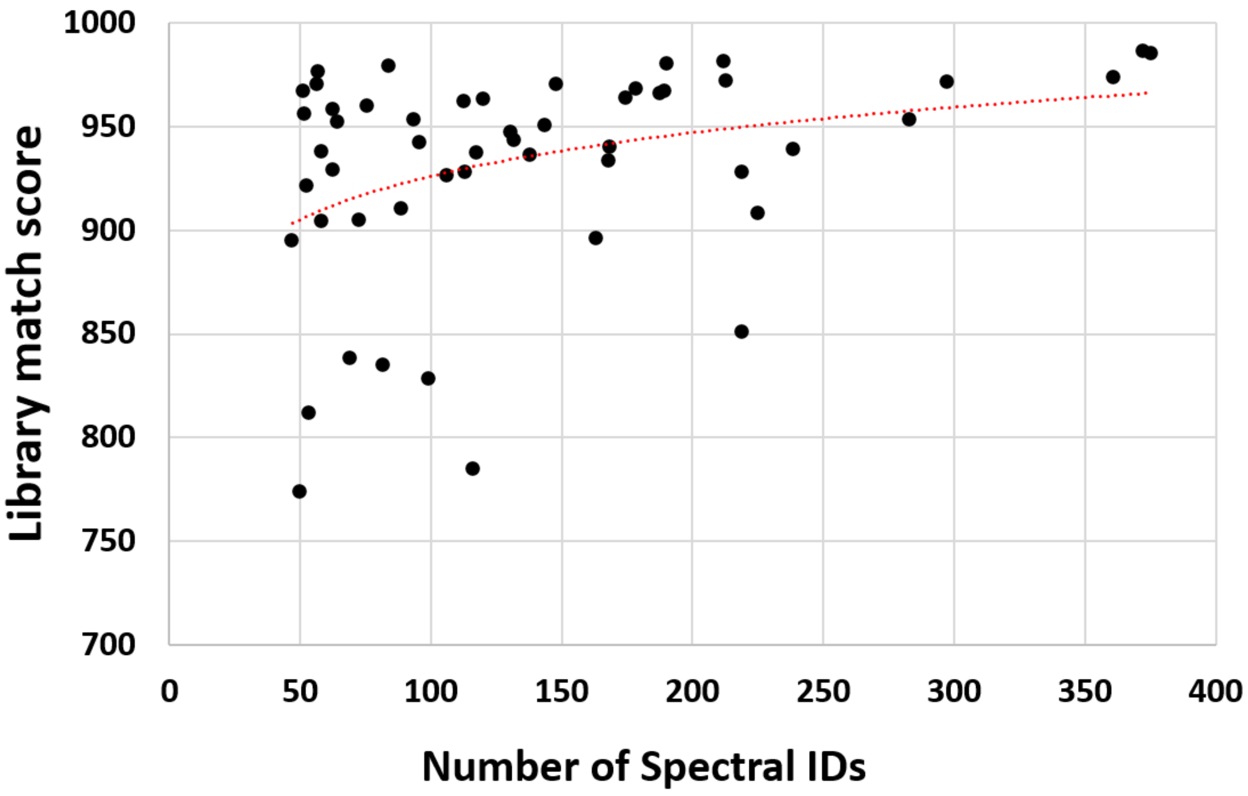


Figure 2. Point representation of library search results (L) for a hypothetical three-peak unknown (U) spectrum (masses M1, M2, and M3)

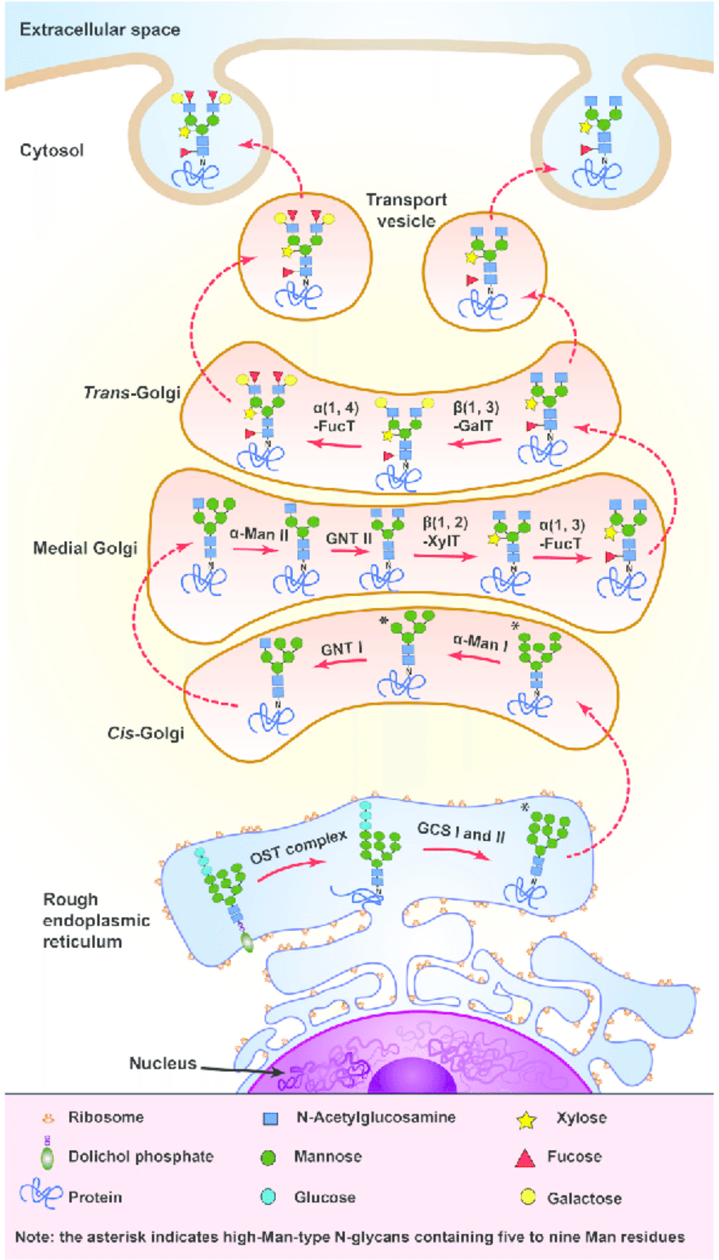
Supplemental Slides



Supplemental Slides

Glycobiology

- In viruses
  - Immune evasion
  - Host cell attachment
- In mammalian cells
  - Protein folding
  - Protein stabilization
  - Communication
  - Function

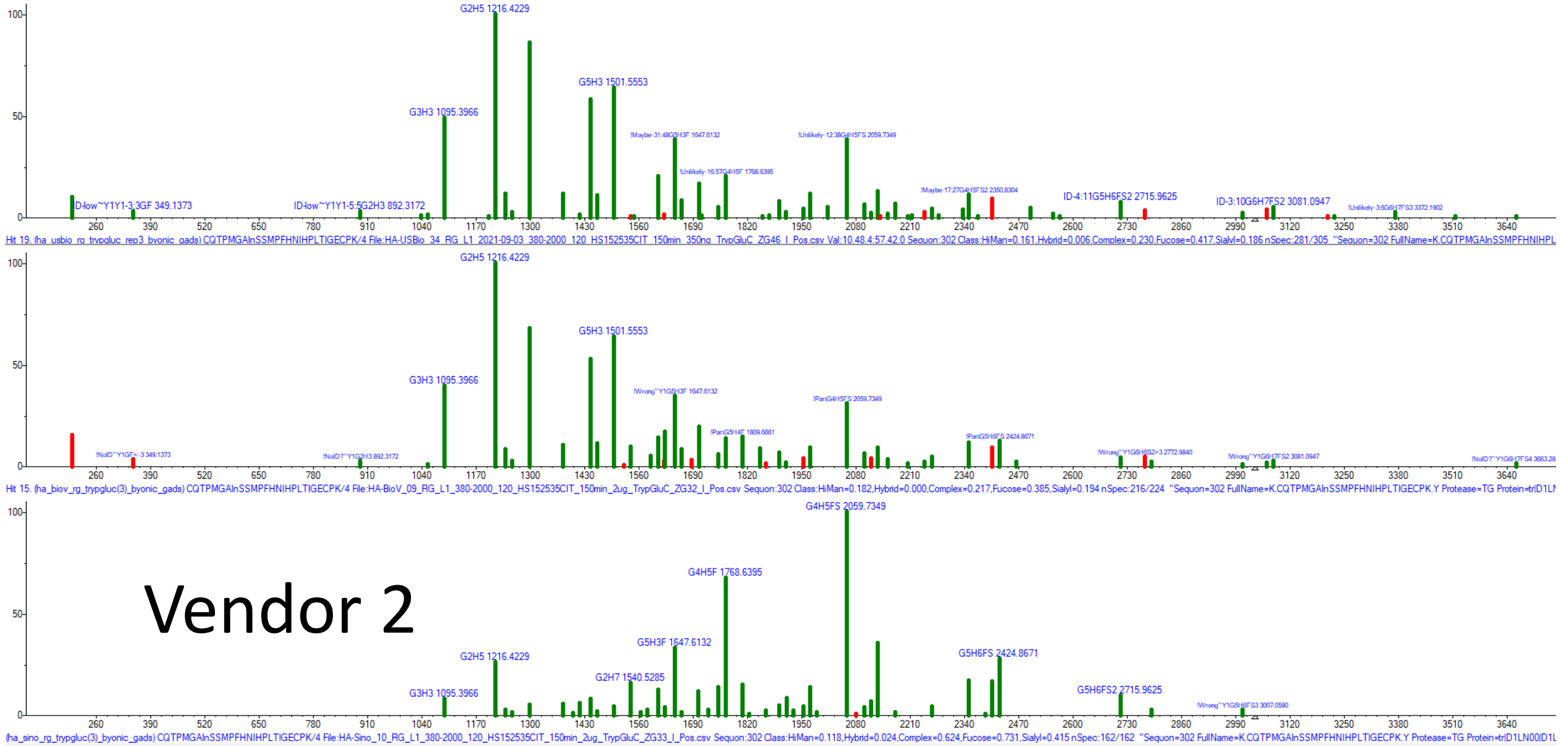


Note: the asterisk indicates high-Man-type N-glycans containing five to nine Man residues

Supplemental Slides

Vendor Comparison

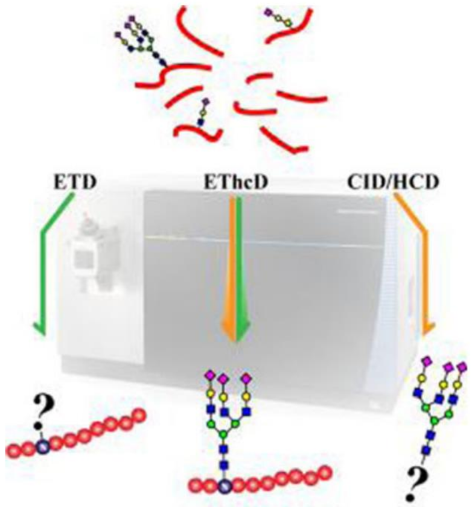
Glycan distribution was skewed toward higher mass (sialylated complex) glycans for vendor 2 and toward lower mass glycans (oligomannose and hybrid) for vendor 1 and vendor 3.

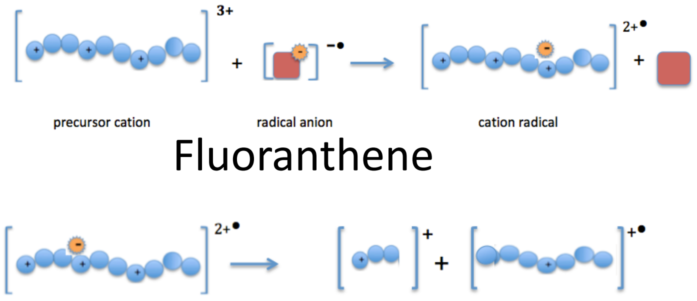


Vendor 2

Supplemental Slides

EThcD





Stepped HCD

Peptide bond
Glycosidic bond
N-glycosidic bond