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

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BMD Staff Seminar

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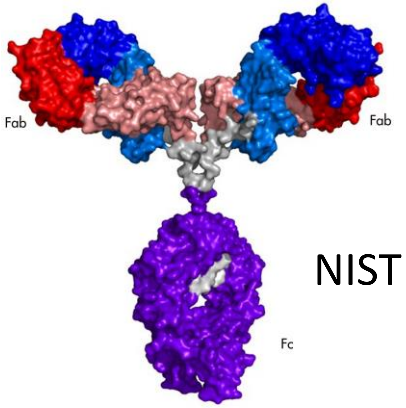
National Institute of
Standards and Technology
U.S. Department of Commerce

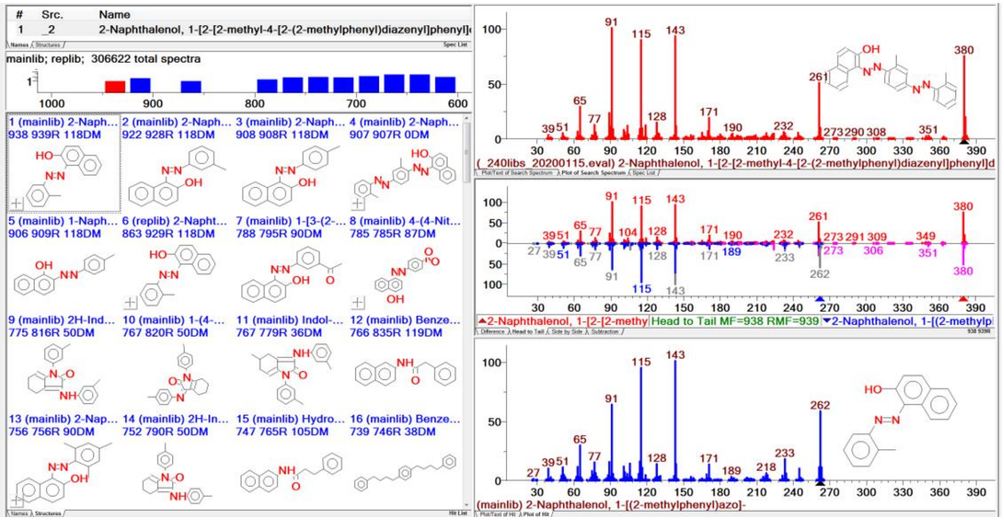
Mass Spectrometry
Data Center

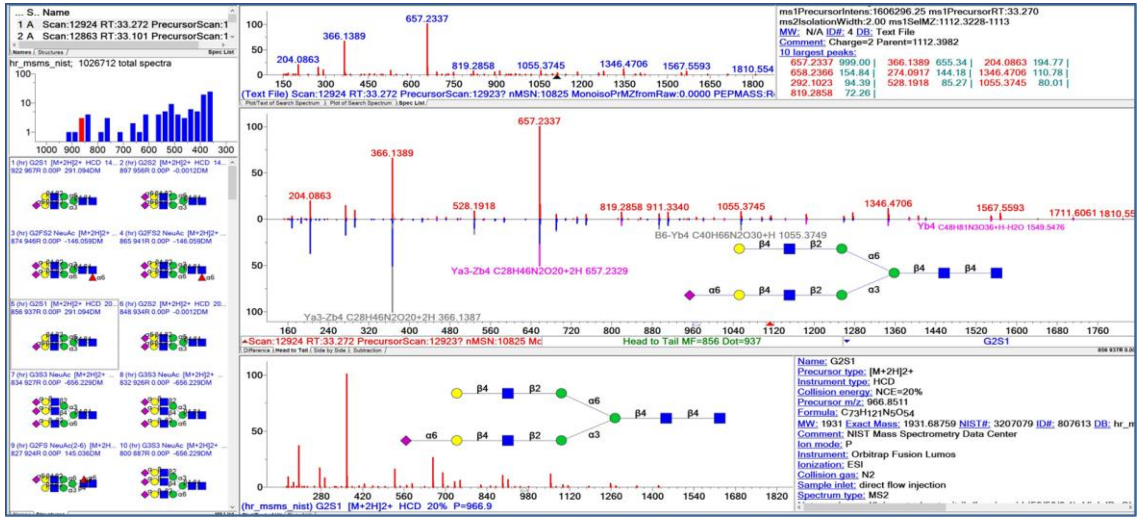
Why Glycopeptides at NIST?



NISTMab

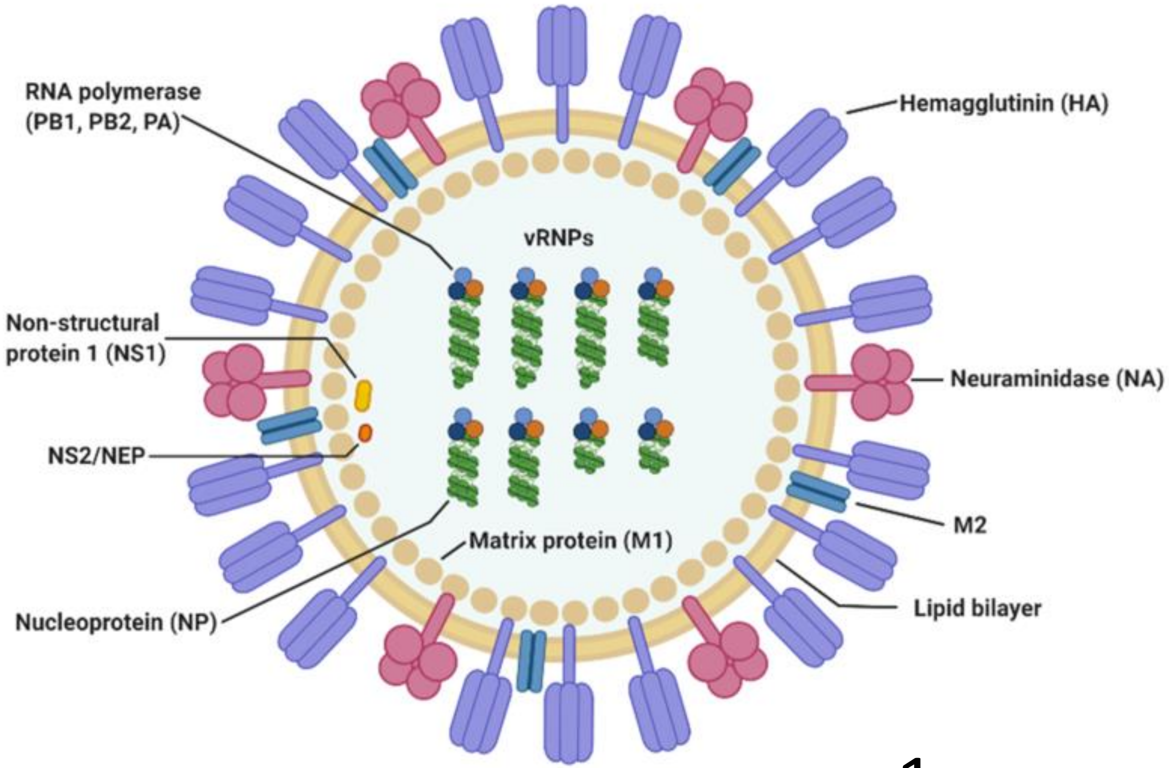
- Tandem libraries are growing.
- Glycopeptides prominent in biologics.
- Measurement of glycosylation is difficult and hard to reproduce.
- Variation in glycosylation pattern unknown. How reproducible?





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.

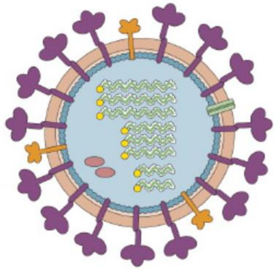


1

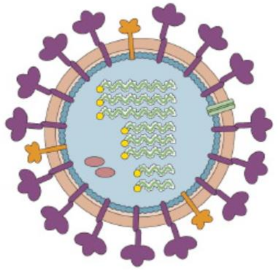
Influenza Vaccine

- Quadrivalent: 4 strains
  - 2 Influenza A strains
  - 2 Influenza B strains
- Embryonated chicken eggs
- Inactivated by rupturing membrane
- Adjuvants

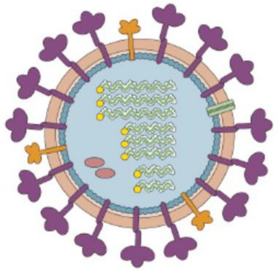
Influenza A (H3N2)



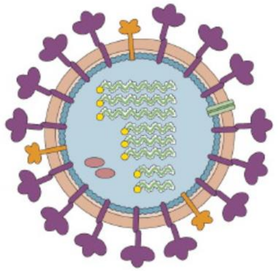
Influenza A (H1N1)



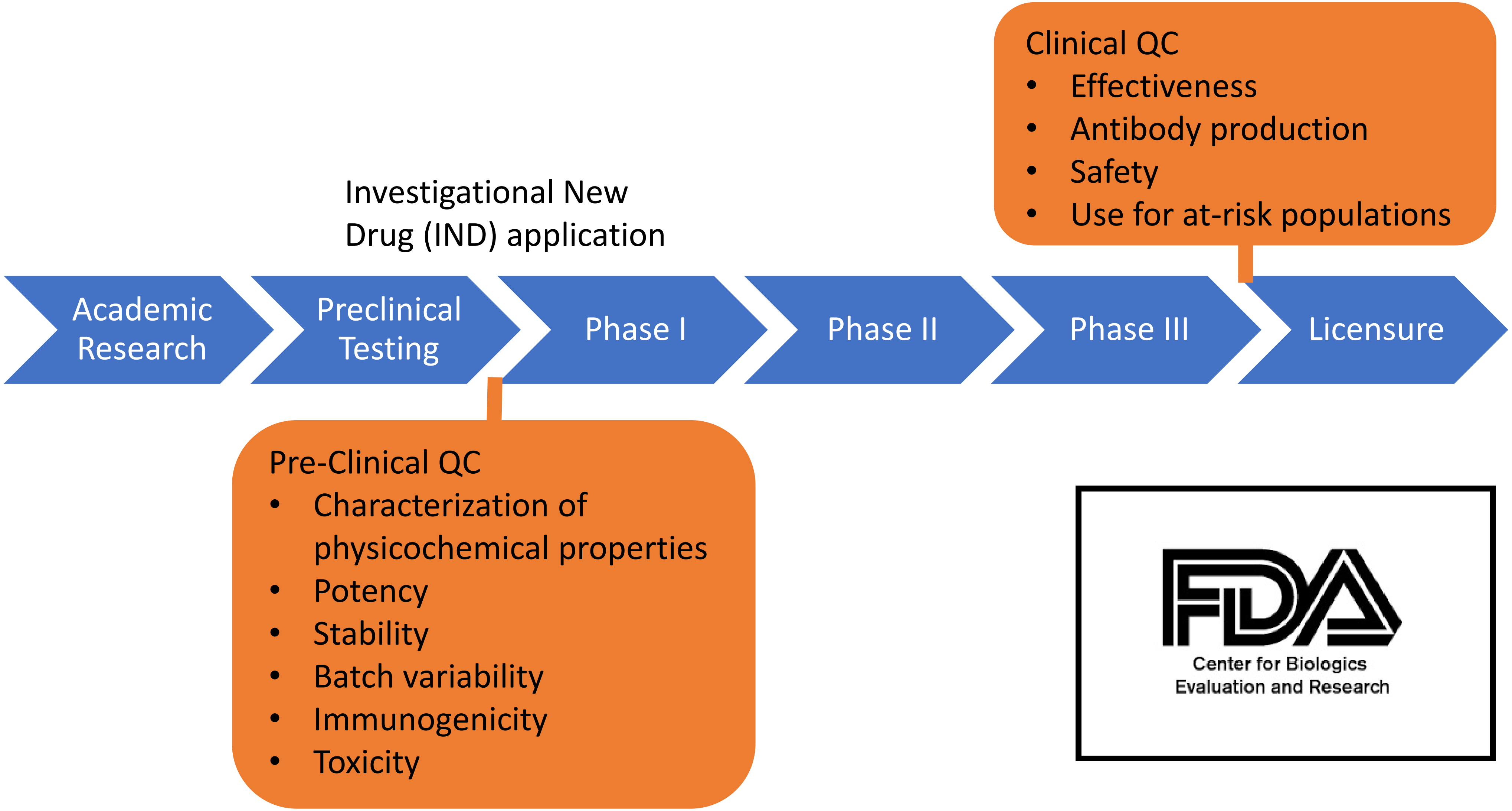
Influenza B (Victoria)



Influenza B (Yamagata)



Quality Control of Influenza Vaccines



Investigational New Drug (IND) application

Academic Research

Preclinical Testing

Phase I

Phase II

Phase III

Licensure

Clinical QC
- Effectiveness
- Antibody production
- Safety
- Use for at-risk populations

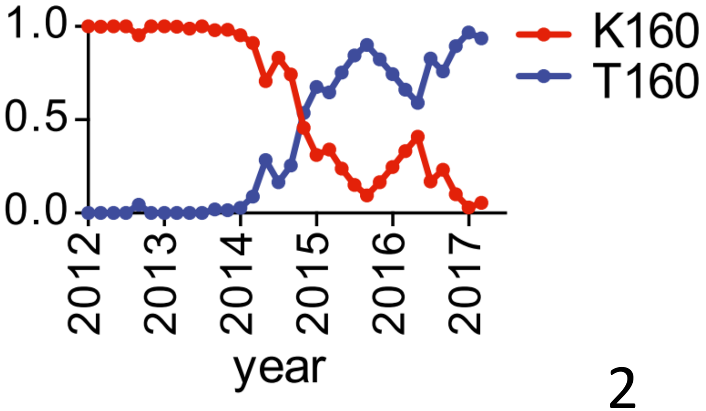
Pre-Clinical QC
- Characterization of physicochemical properties
- Potency
- Stability
- Batch variability
- Immunogenicity
- Toxicity

FDA
Center for Biologics
Evaluation and Research

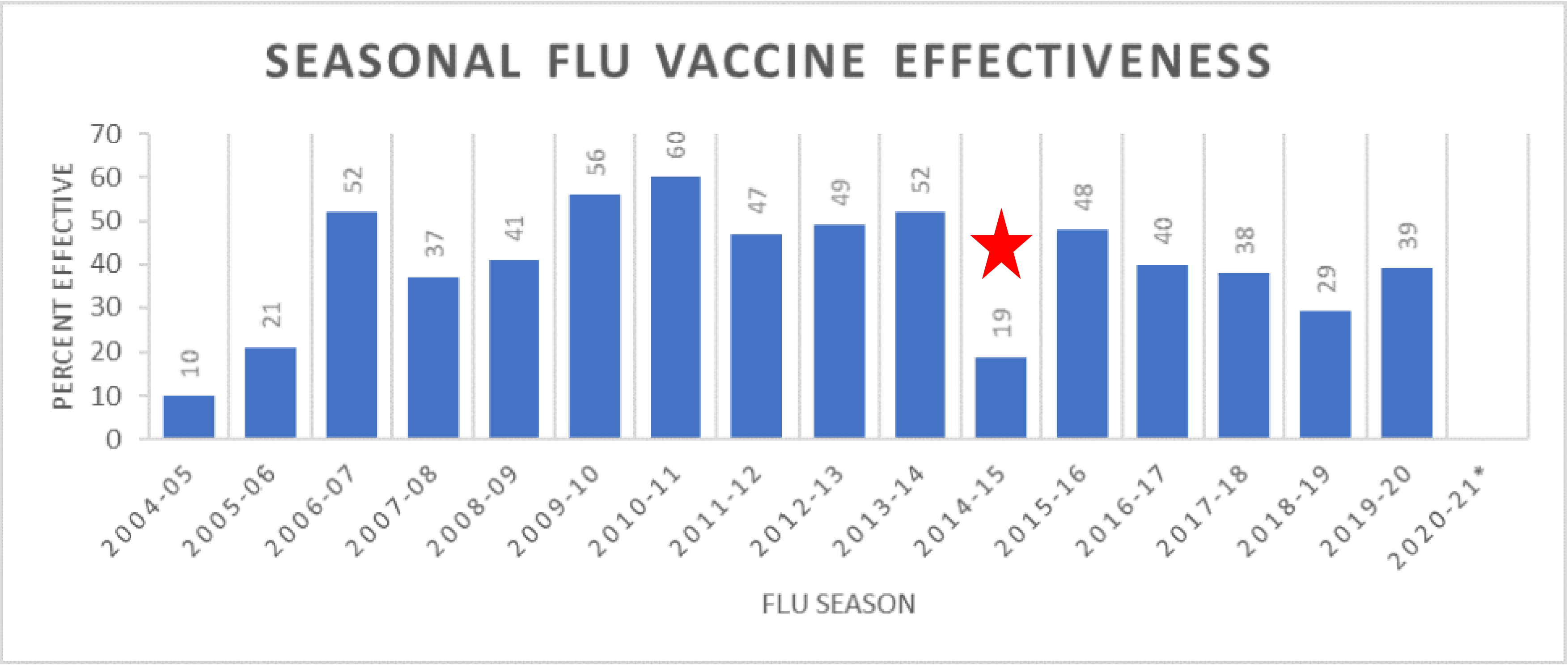
Why Glycosylation Matters For Vaccines

…THHLNFKYPAL…
…THLNFTYPAL…

Sequon motif
NXT/S X≠P

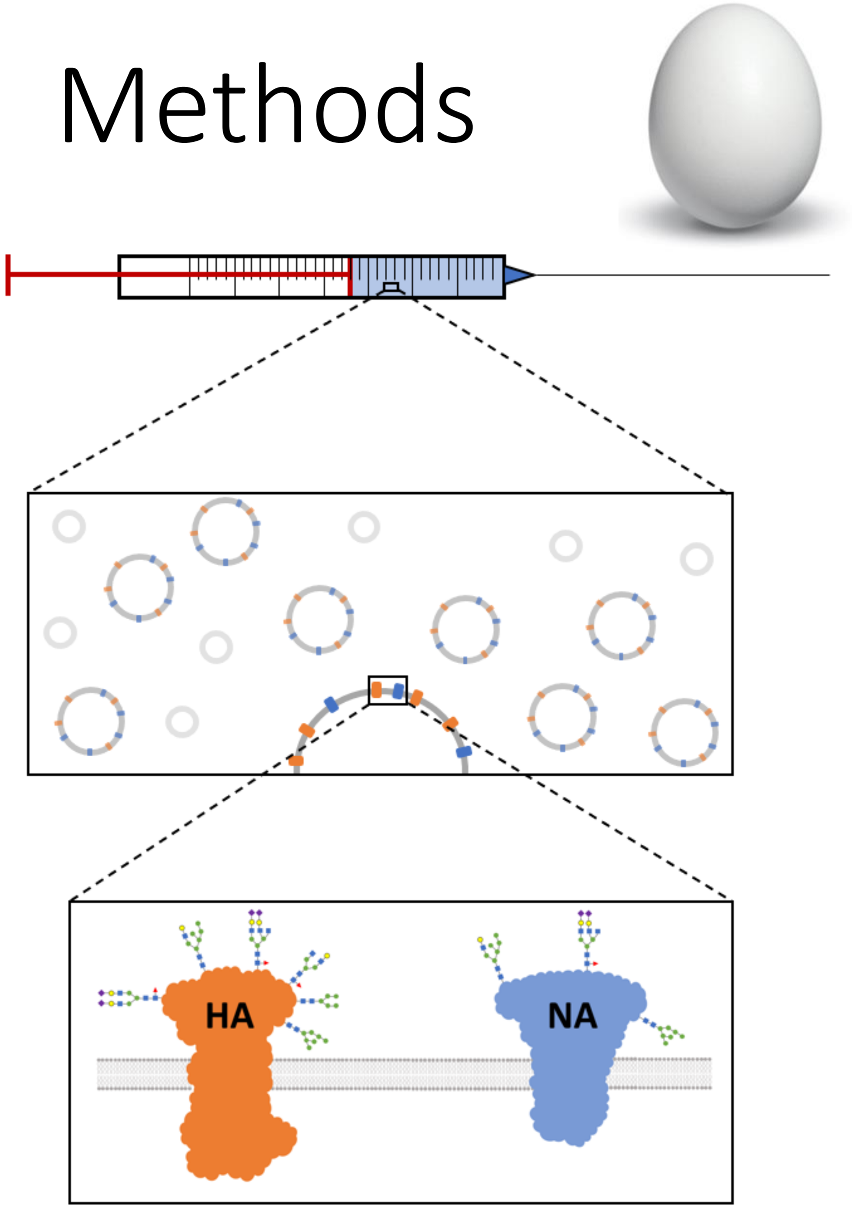


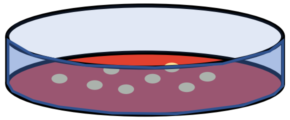
2



3

Methods

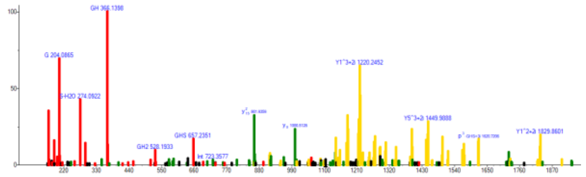




HEK293

Reduction
Alkylation
Digestion
Purification

HCD - 15%, 25%, 35% NCE

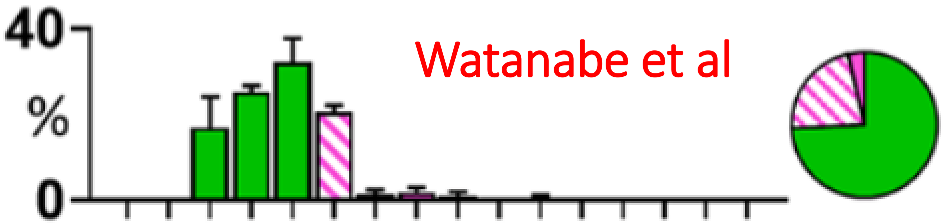


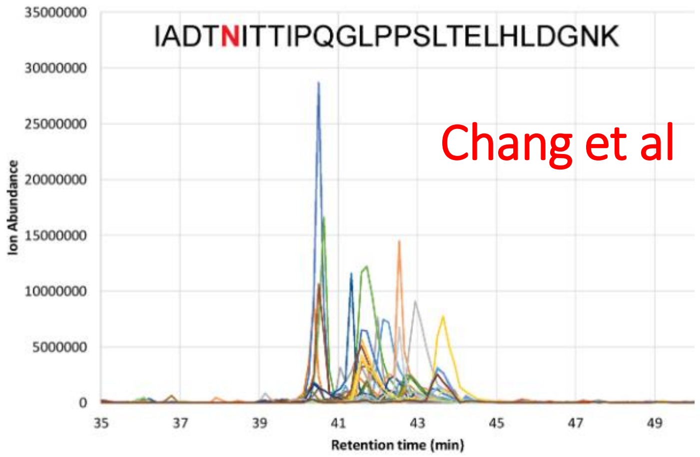
Oxonium ions
Peptide backbone ions
Glycopeptide ions

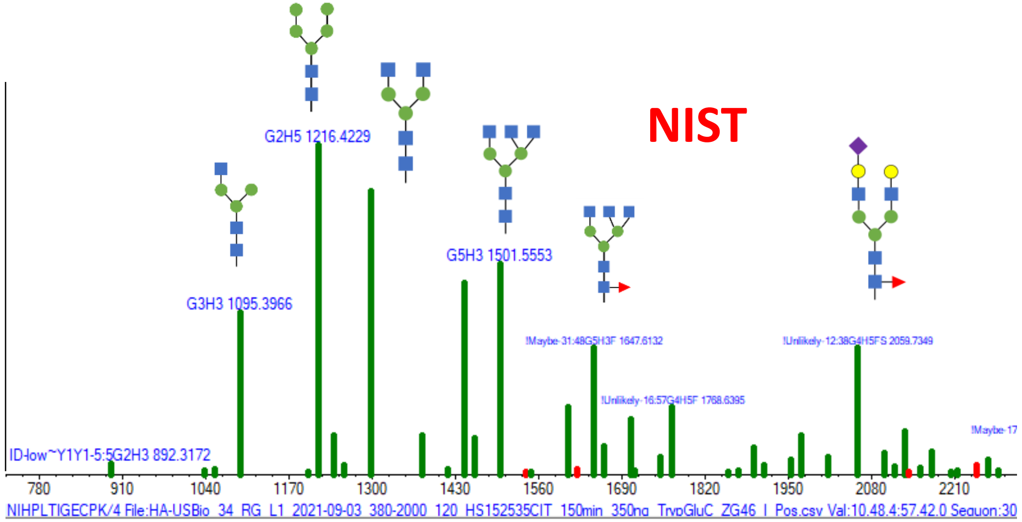
Methods – Data Processing

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

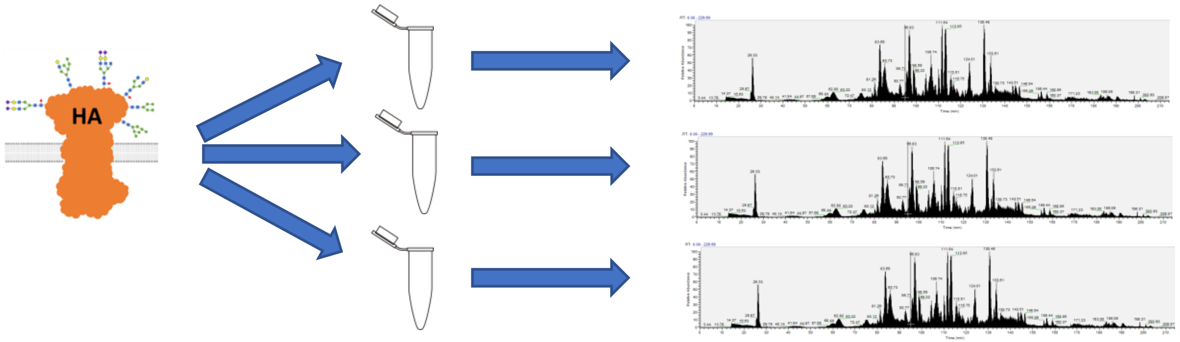

Watanabe et al


Chang et al


NIST

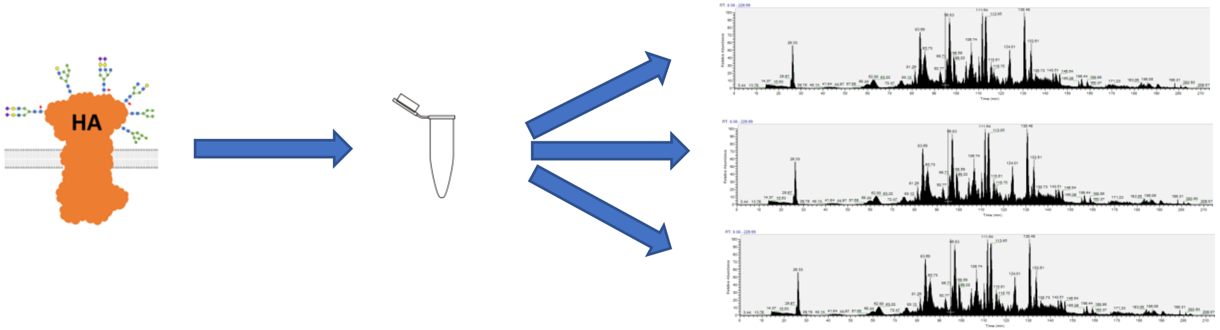
Variation in glycosylation profile - Replicate

Biological Replicate



Protein	Sequon	Average match score
Hemagglutinin	39	980 ± 2
	170	913 ± 60
	181	968 ± 16
	302	884 ± 92
	500	884 ± 27
Neuraminidase	68	973 ± 6
	126	945 ± 21
	215	959 ± 11
Total		939 ± 62

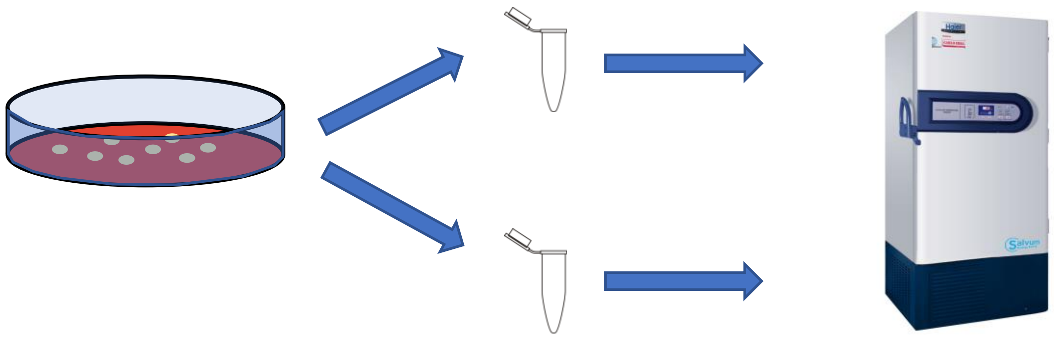
Technical Replicate



Protein	Sequon	Average match score
Hemagglutinin	39	963
	170	967
	181	939 ± 40
	302	946 ± 44
	500	926 ± 27
Neuraminidase	68	939 ± 14
	126	961 ± 24
	215	927 ± 68
Total		946 ± 38

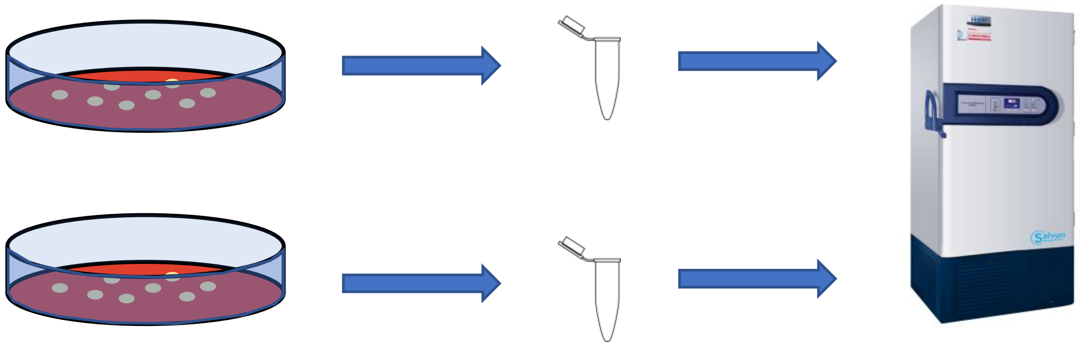
Variation in glycosylation profile - Lot

Batch Variation



Protein	Sequon	Average match score
Hemagglutinin	39	889 ± 75
	170	843 ± 38
	181	942 ± 19
	302	909 ± 45
	500	872 ± 69
Total		897 ± 57

Lot Variation



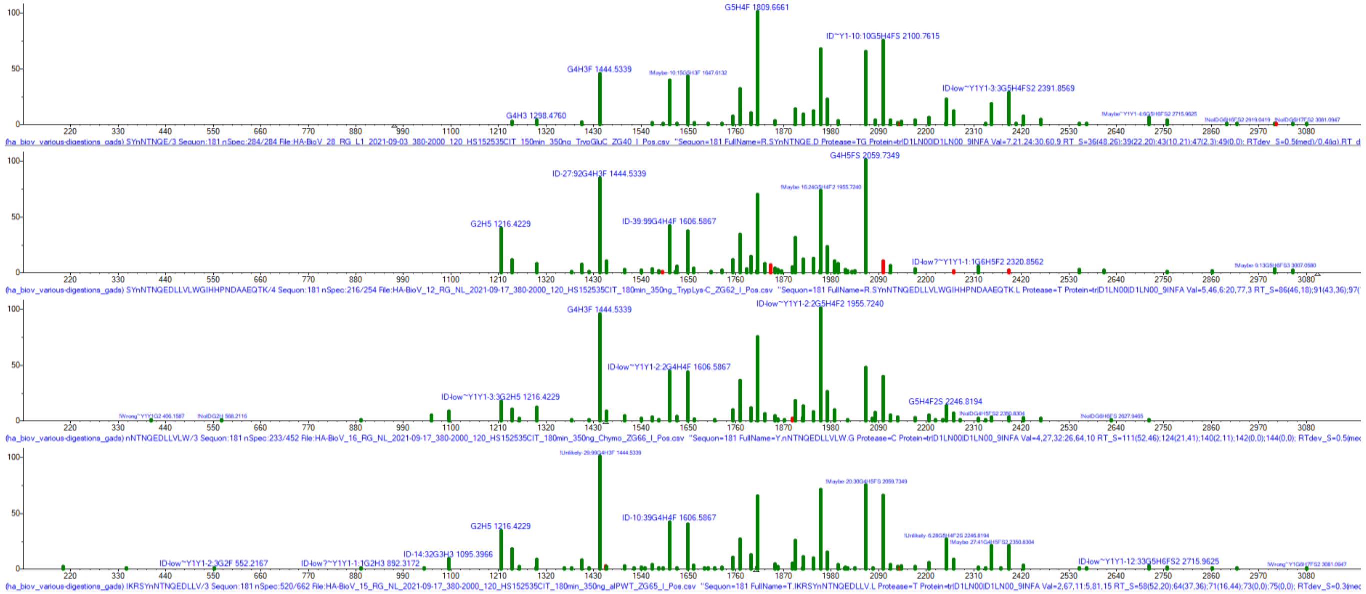
Protein	Sequon	Average match score
Neuraminidase	68	903 ± 39
	126	852 ± 72
	215	910 ± 48
Total		891 ± 57

Variation in glycosylation profile - Proteases

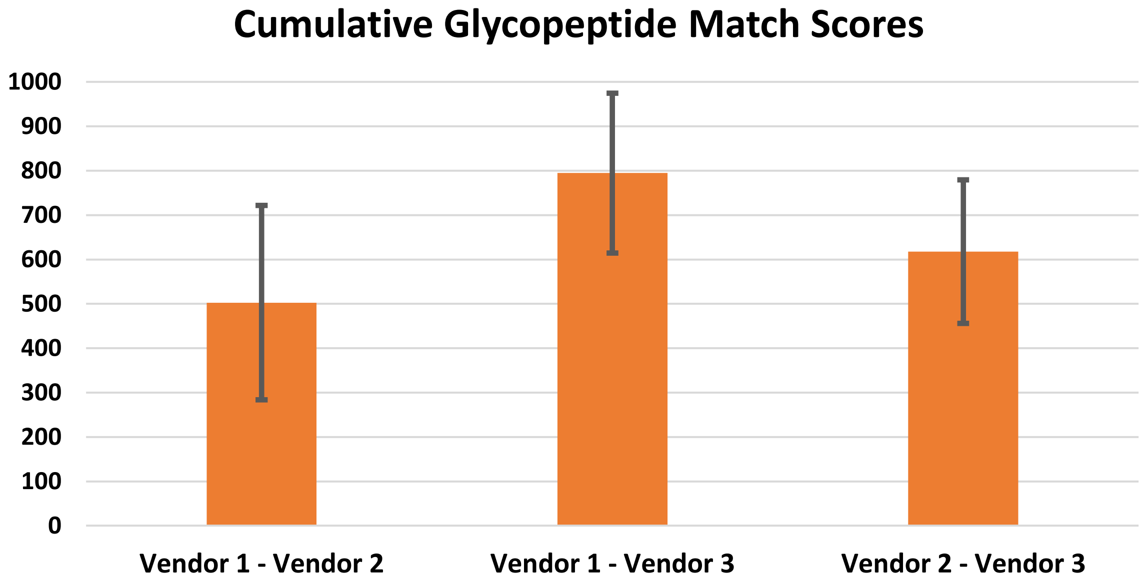
- Trypsin + Lys-C
- Trypsin + Glu-C
- Trypsin + Chymotrypsin
- Chymotrypsin + Glu-C
- Chymotrypsin
- Alphalytic protease

Average match: 819 ± 106

SYnNTNQE
SYnNTNQEDLLVLWGIHHPNDAAEQTK
nNTNQEDLLVLW
IKRSYnNTNQEDLLV



Variation in glycosylation profile - Vendor



Vendors

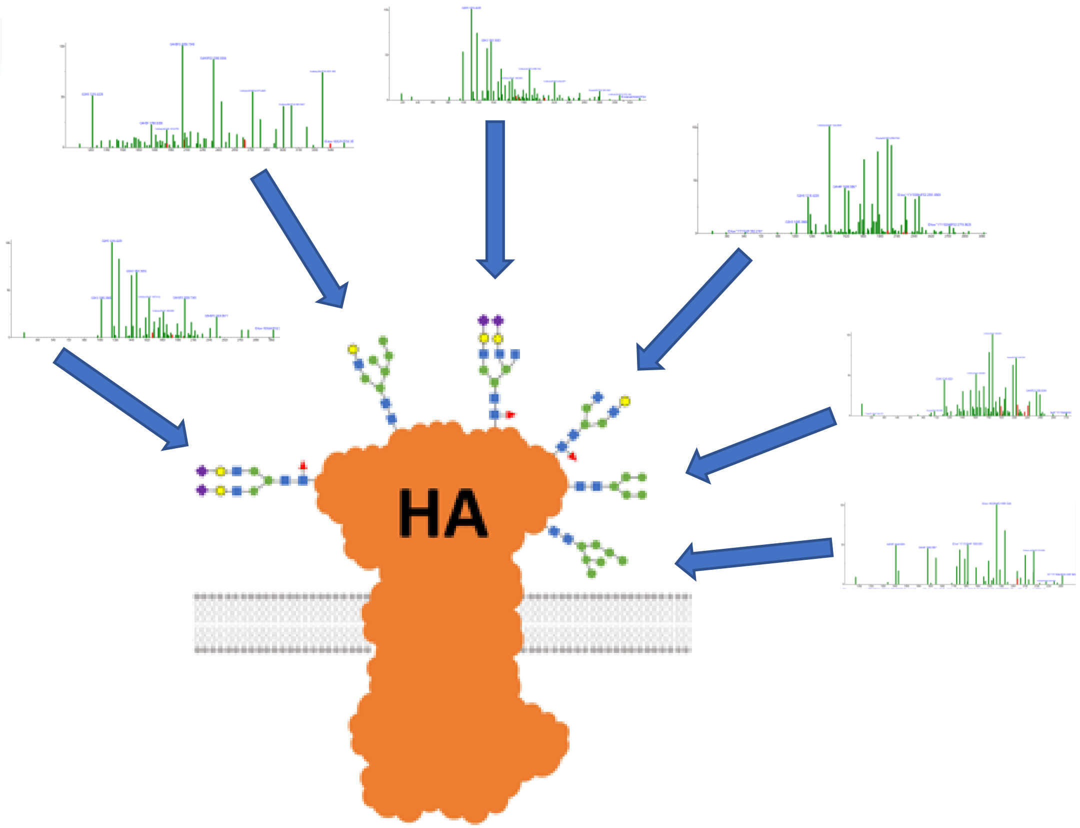
- BioVision
- Sino Biological
- US Biological

Strains

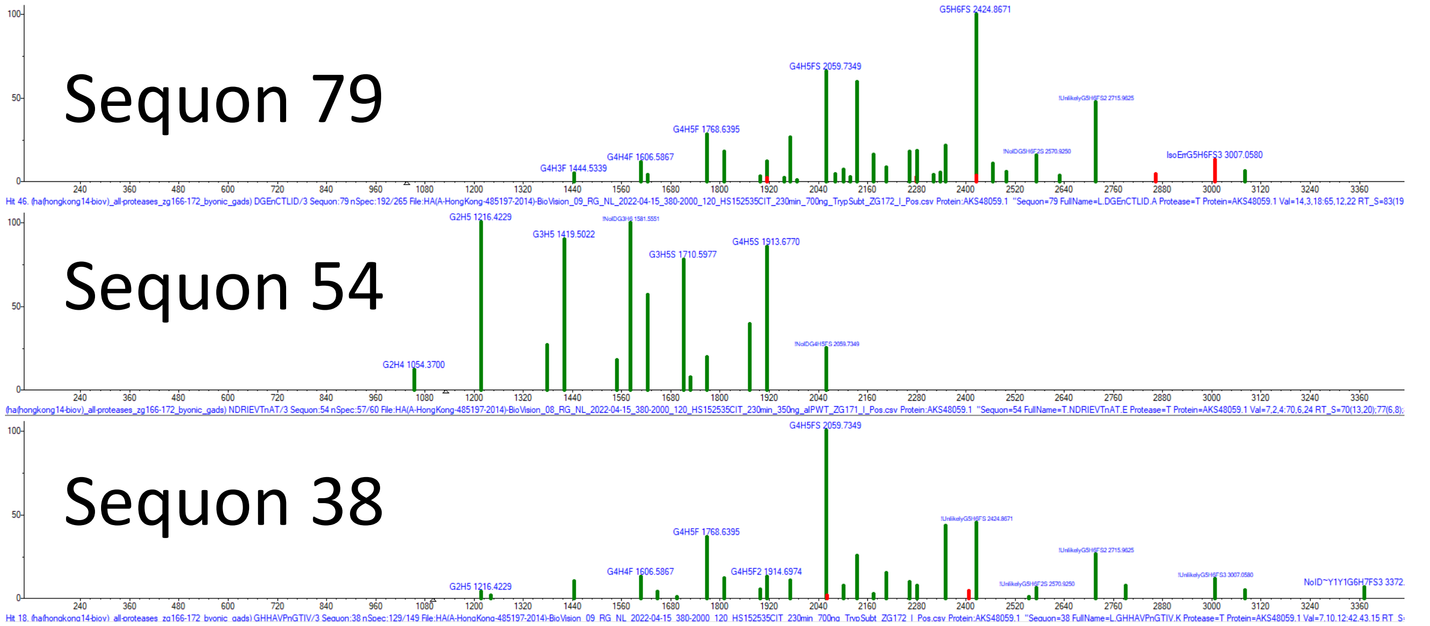
- HA (A/Hong Kong/483/1997) H5N1
- NA (A/Thailand/1(KAN-1)/2004) H5N1

Average match score: 638 ± 218

Variation in glycosylation profile – Same Strain



Average Match Score: 617 ± 151

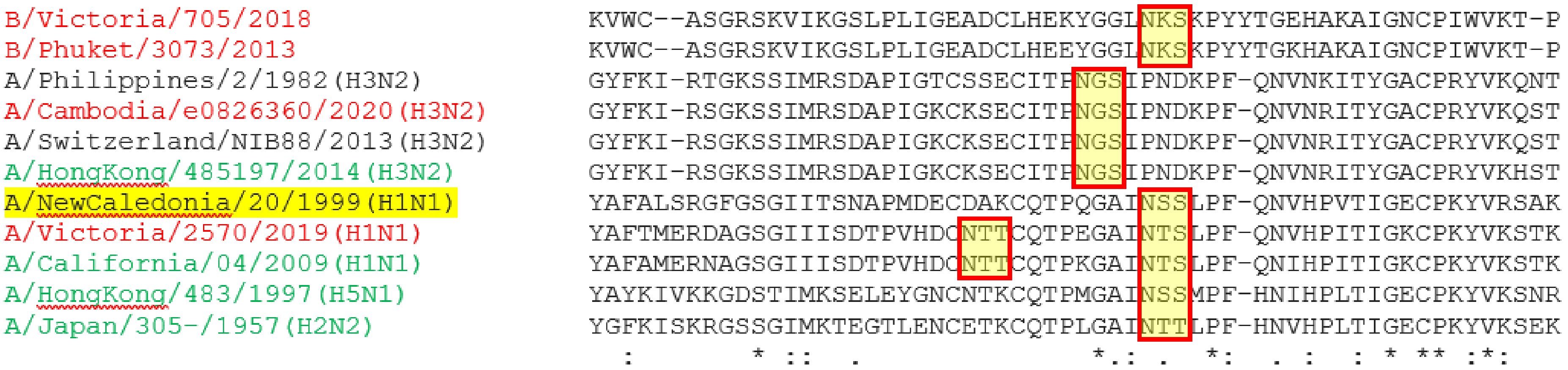


Variation in glycosylation profile – Different Strains

Protein	Strain	Subtype	Number of sequons	Protein mass* (kDa)
HA	A/California/04/2009	H1N1	8	63
NA	A/California/04/2009	H1N1	8	52
HA	A/Hong Kong/483/1997	H5N1	8	64
HA	A/Hong Kong/485197/2014	H3N2	13	64
HA	A/Japan/305/1957	H2N2	8	63
HA	A/New Caledonia/20/1999	H1N1	10	63
NA	A/Arizona/13/2008	H1N1	9	52
NA	A/Netherlands/219/2003	H7N7	11	52
NA	A/Thailand/1(KAN-1)/2004	H5N1	3	49

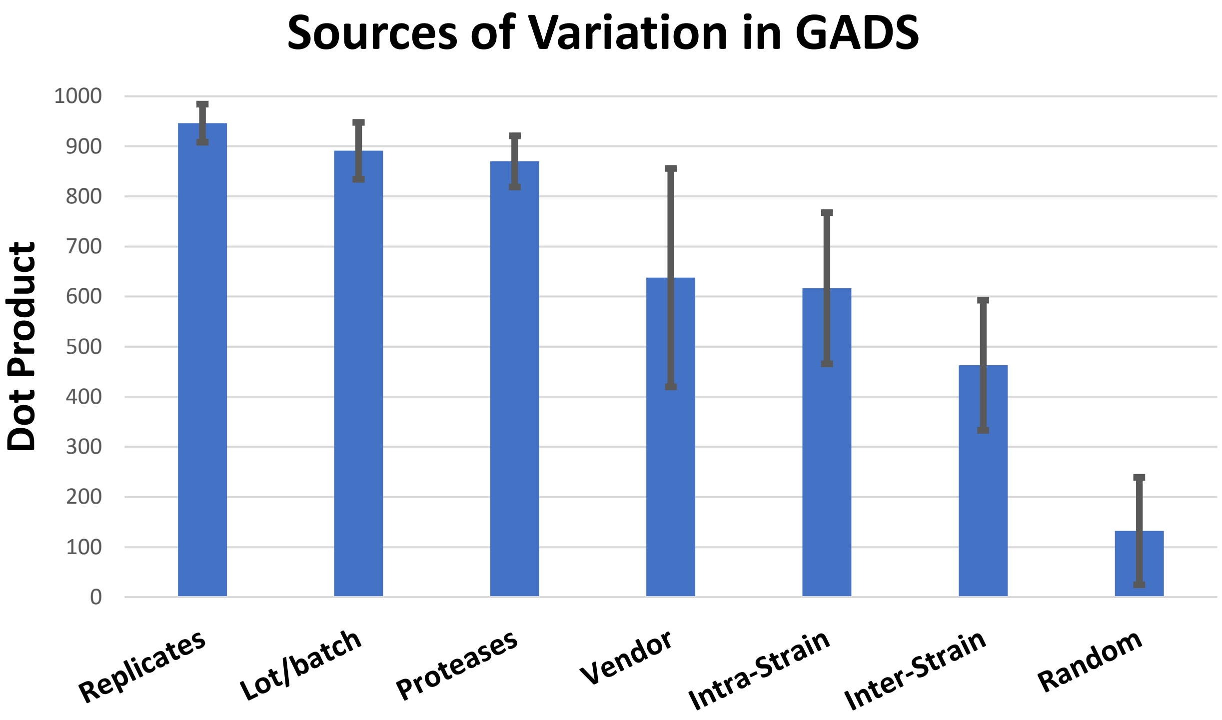
*Unglycosylated

Average Match Score:
463 ± 130



```
B/Victoria/705/2018              KVWC--ASGRSKVIKGSLPLIGEADCLHEKYGGLNKSKPYYTGEHAKAIGNCPIWVKT-P
B/Phuket/3073/2013               KVWC--ASGRSKVIKGSLPLIGEADCLHEEYGGLNKSKPYYTGKHAKAIGNCPIWVKT-P
A/Philippines/2/1982(H3N2)       GYFKI-RTGKSSIMRSDAPIGTCSSECITPNGSIPNDKPF-QNVNKITYGACPRYVKQNT
A/Cambodia/e0826360/2020(H3N2)   GYFKI-RSGKSSIMRSDAPIGKCKSECITPNGSIPNDKPF-QNVNRITYGACPRYVKQST
A/Switzerland/NIB88/2013(H3N2)   GYFKI-RSGKSSIMRSDAPIGKCKSECITPNGSIPNDKPF-QNVNRITYGACPRYVKQST
A/HongKong/485197/2014(H3N2)     GYFKI-RSGKSSIMRSDAPIGKCKSECITPNGSIPNDKPF-QNVNRITYGACPRYVKHST
A/NewCaledonia/20/1999(H1N1)     YAFALSRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPF-QNVHPVTIGECPKYVRSAK
A/Victoria/2570/2019(H1N1)       YAFTMERDAGSGIIISDTPVHDCNTTCQTPEGAINTSLPF-QNVHPITIGKCPKYVKSTK
A/California/04/2009(H1N1)       YAFAMERNAGSGIIISDTPVHDCNTTCQTPKGAINTSLPF-QNIHPITIGKCPKYVKSTK
A/HongKong/483/1997(H5N1)        YAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGAINSSMPF-HNIHPLTIGECPKYVKSNR
A/Japan/305-/1957(H2N2)          YGFKISKRGSSGIMKTEGTLENCETKCQTPLGAINTTLPF-HNVHPLTIGECPKYVKSEK
                                   :       * ::  .              *.: .  *:  . :  : * ** :*:
```

Summary of Variation



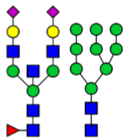
- Glycosylation of replicates is most similar and between strains is least similar.
- More deviation associated with lower match scores.
- “Random” is measured between different proteins from different vendors. (A1AG vs HA)

Isolation of Adjacent Sequons

- Site-specific glycosylation methods require a single sequon per peptide. Different proteases are used to maximize isolation.
- Influenza, HIV, Ebola, Herpesvirus, and MERS have adjacent sequons

NXT/S X≠P

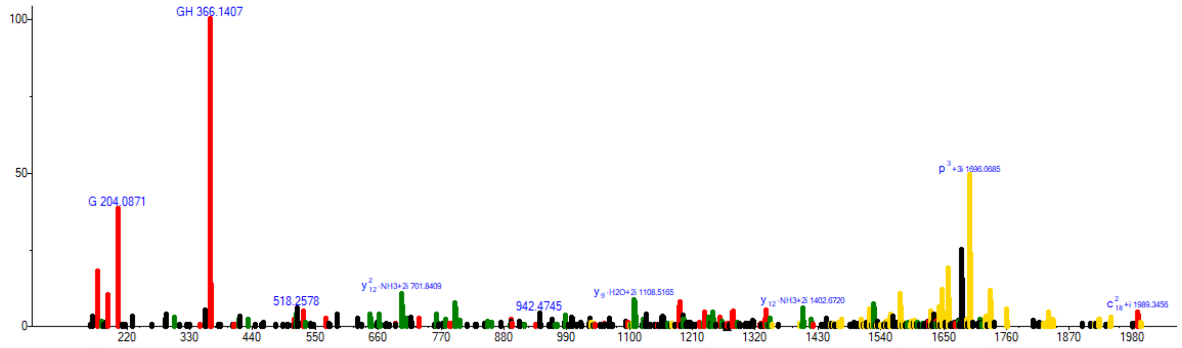
NNSS, NNTT, NNST, NNTS

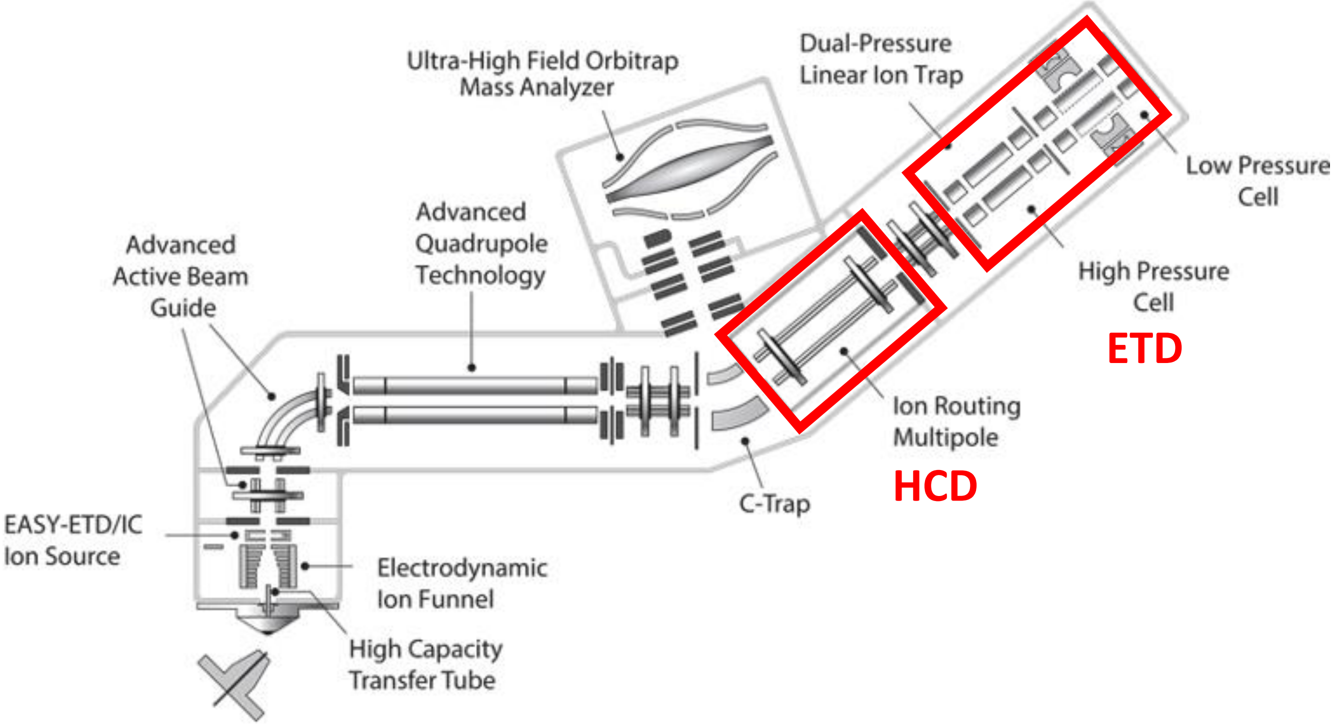


…PIDETEQGSYNNTSGEQMLIIWGVHHR…

Isolation of Adjacent Sequons - EThcD

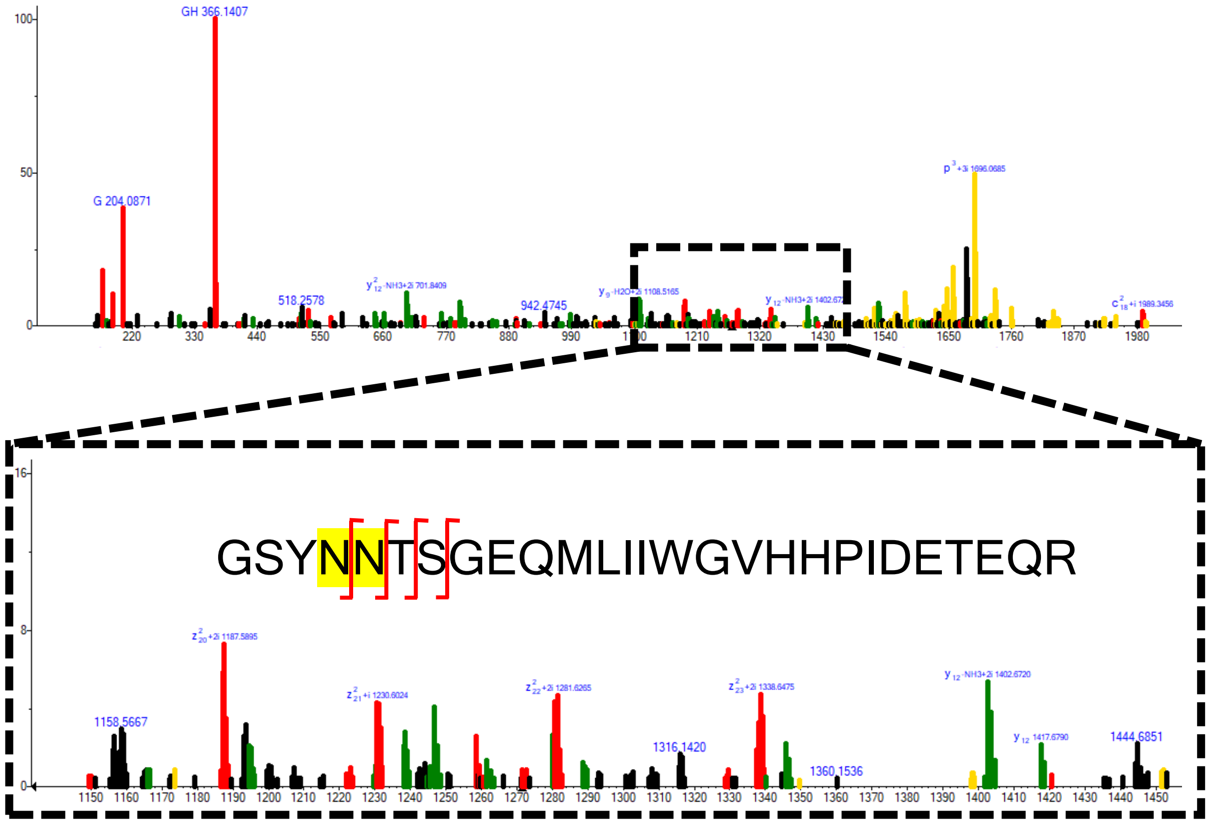
- ETD (Electron Transfer Dissociation)
- HCD (High-Energy Collisional Dissociation)

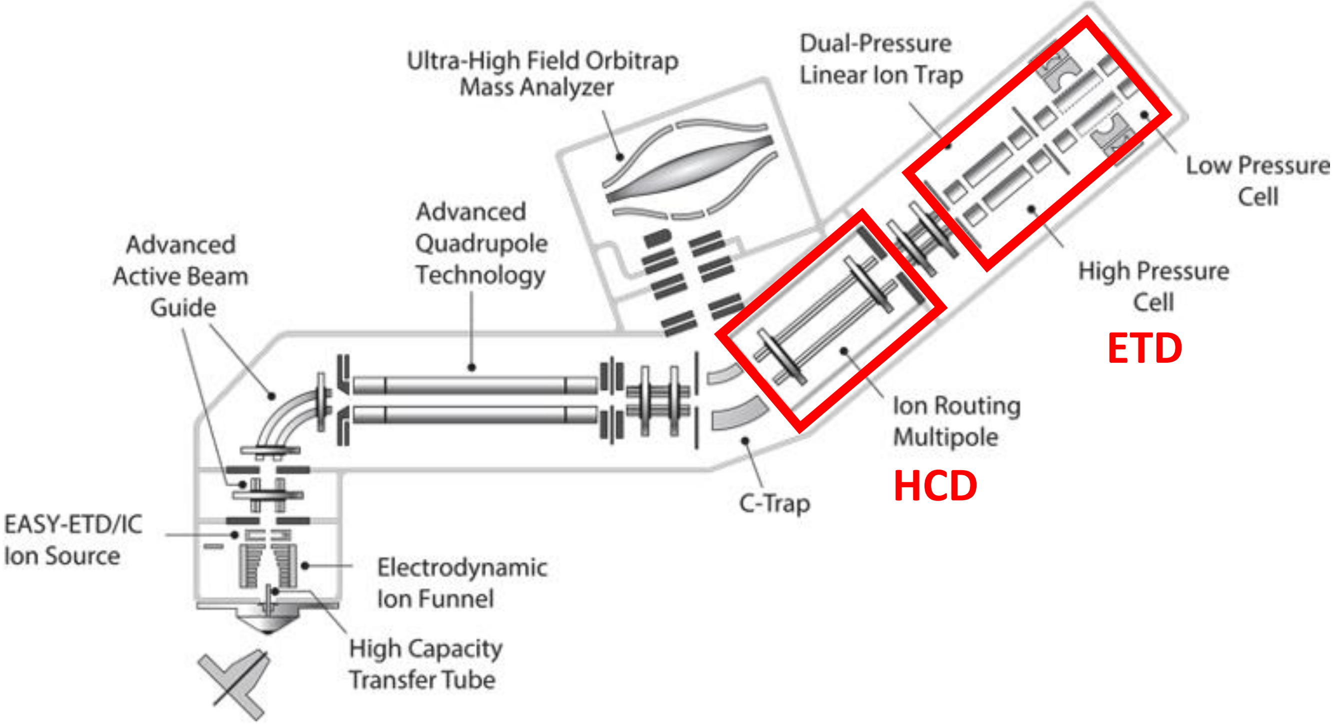




Isolation of Adjacent Sequons - EThcD

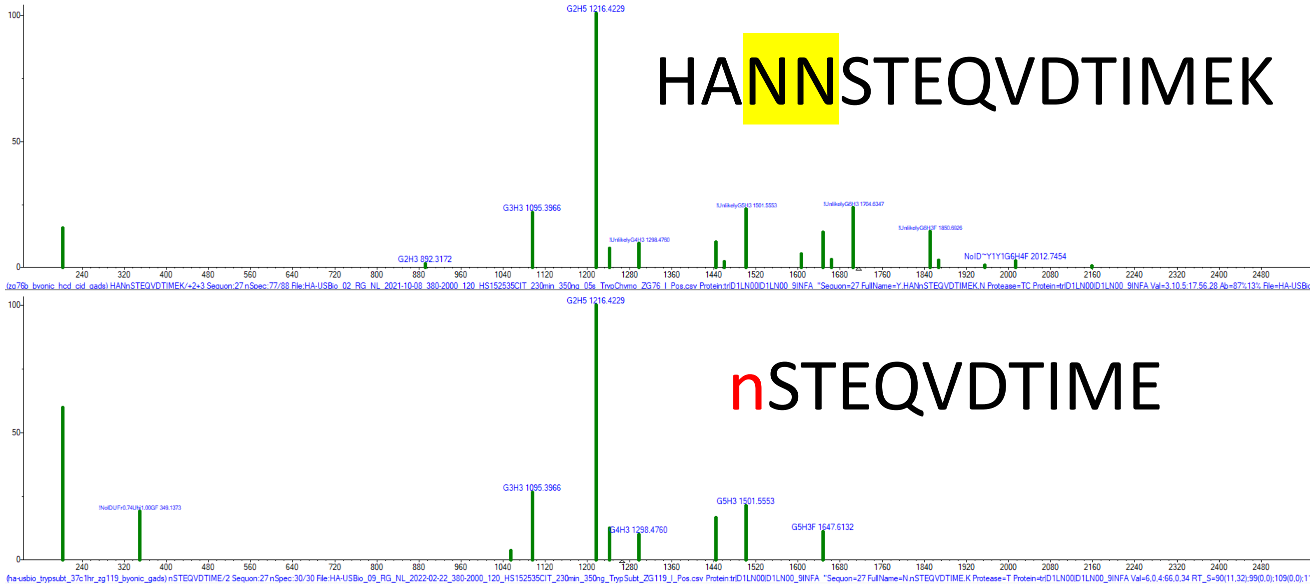
- ETD (Electron Transfer Dissociation)
- HCD (High-Energy Collisional Dissociation)





Isolation of Adjacent Sequons - Subtilisin

- *Bacillus subtilis* – extracellular serine endopeptidases



HANNSTEQVDTIMEK

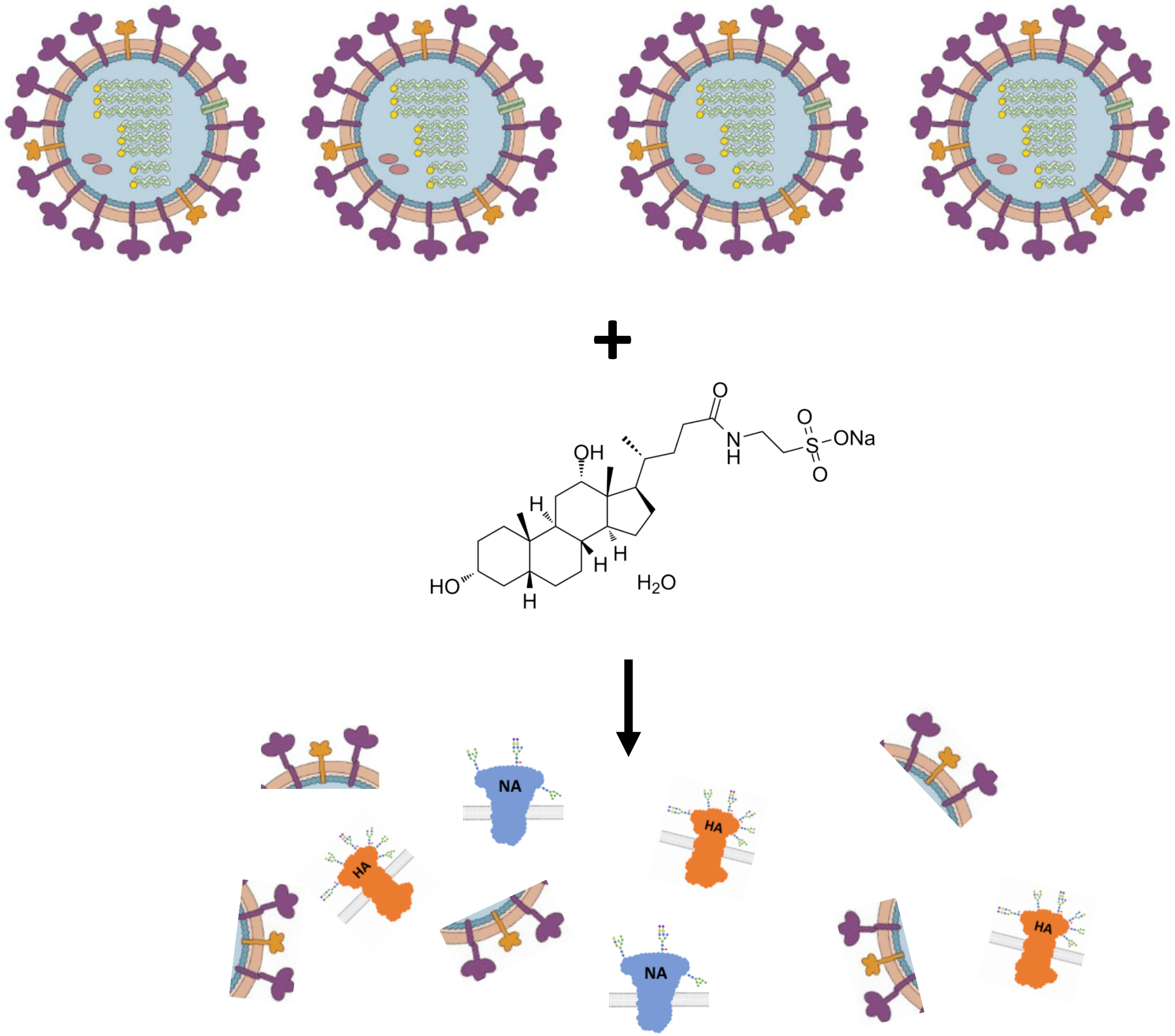
nSTEQVDTIME

DQICIGYHAn ???

Next Steps: Influenza Vaccines

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

- 67 sequons within all HA and NA proteins

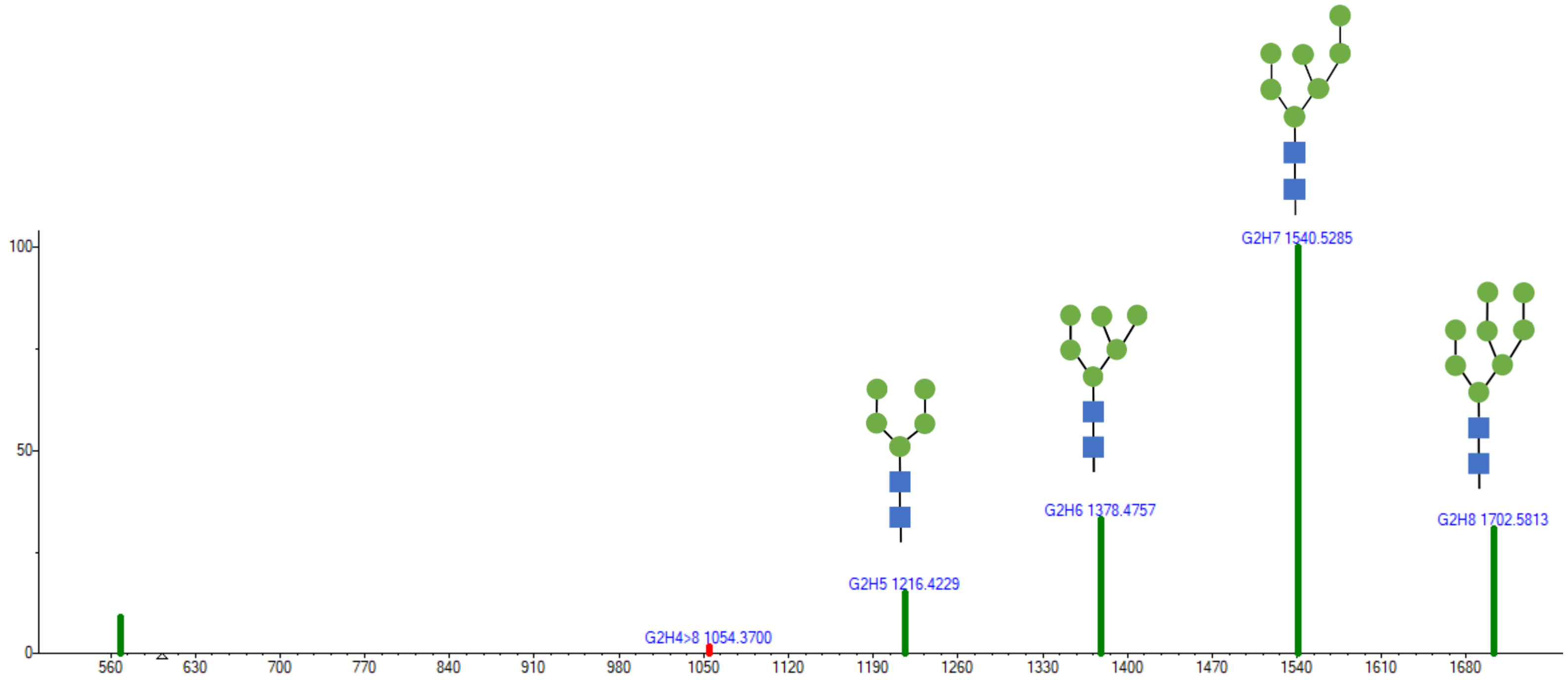


Influenza Vaccines – Challenges and Solutions

```
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 of the 40 sequons for the four hemagglutinin proteins identified.
- The GADS are majority high-mannose.
- No GADS were identified for NA proteins, probably due to a lower abundance of NA on the virus.



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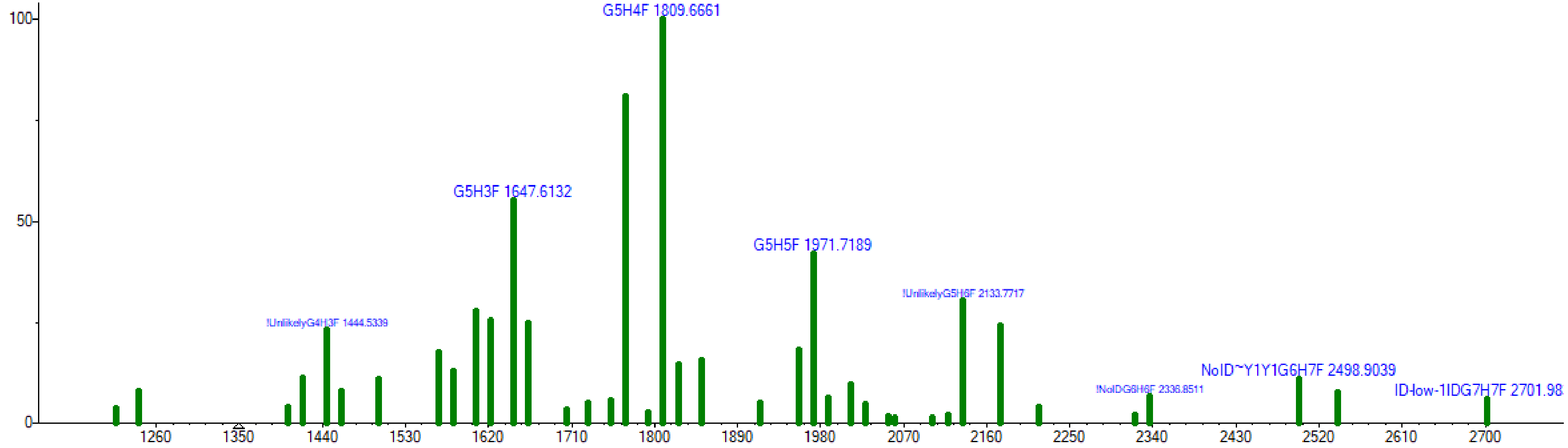
- The GADS are majority high-mannose.
- No GADS were identified for NA proteins, probably due to a lower abundance of NA on the virus.



Conclusions

- Glycosylation of replicates is most similar and between strains is least similar.
- Adjacent sequons may be isolated using EThcD fragmentation or non-specific cleavage using the protease subtilisin.
- Most glycans are high-mannose in egg-based quadrivalent vaccines.
- Next steps:
  - Optimize methods for influenza vaccine digestion.
  - Build reference MS2 and GADS libraries
  - Apply methods to other viral glycoproteins (HIV, Herpesvirus, Ebola…)

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Software

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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

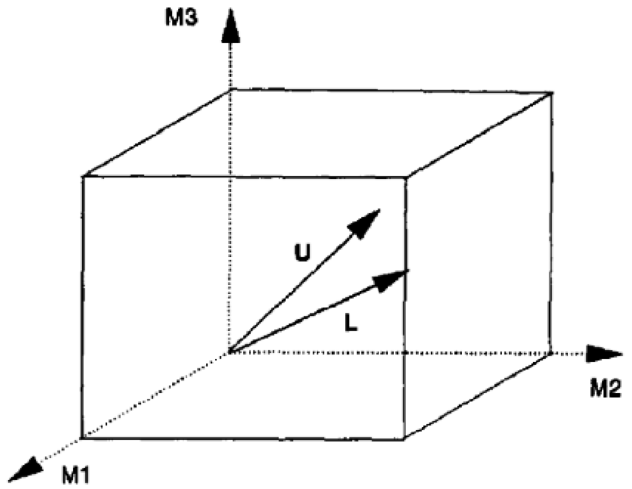


Figure 1. Vector representation of a hypothetical three-peak unknown (U) and library (L) mass spectrum in three-dimensional space (peaks have mass M1, M2, and M3).

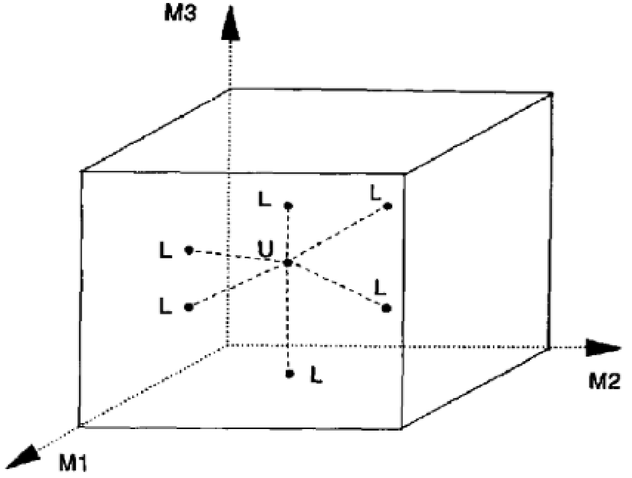
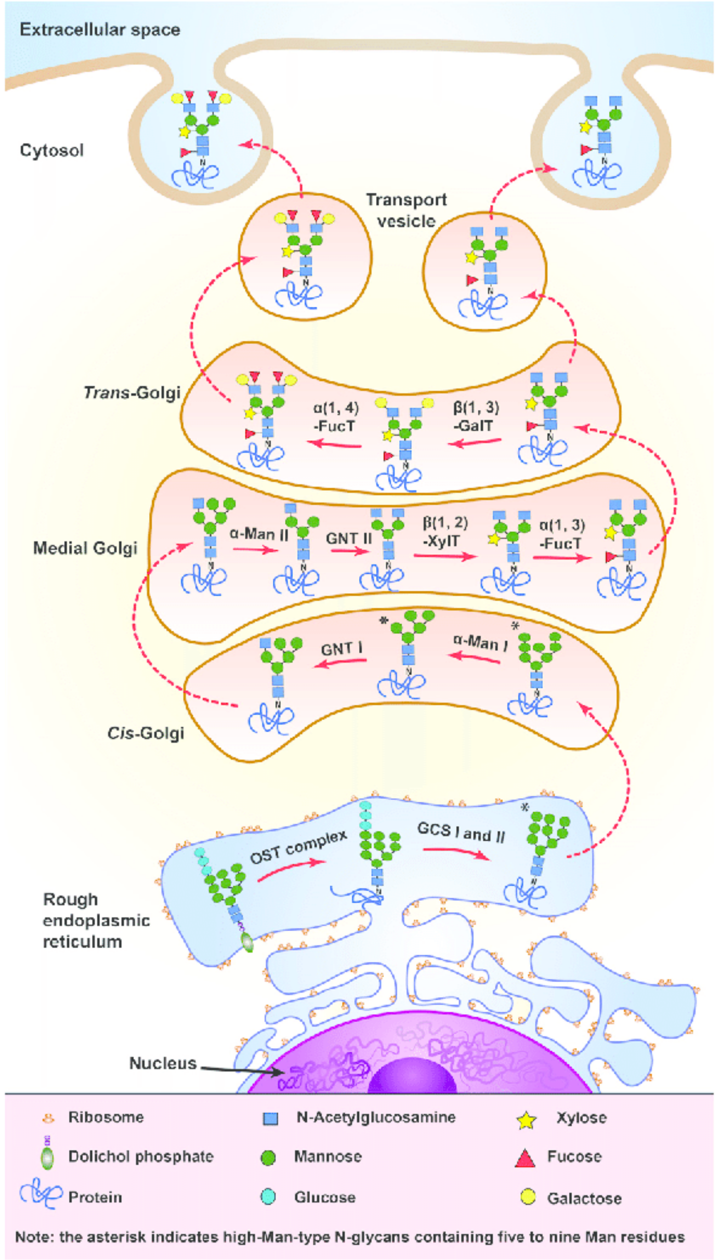


Figure 2. Point representation of library search results (L) for a hypothetical three-peak unknown (U) spectrum (masses M1, M2, and M3)

Supplemental Slides

Glycobiology

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

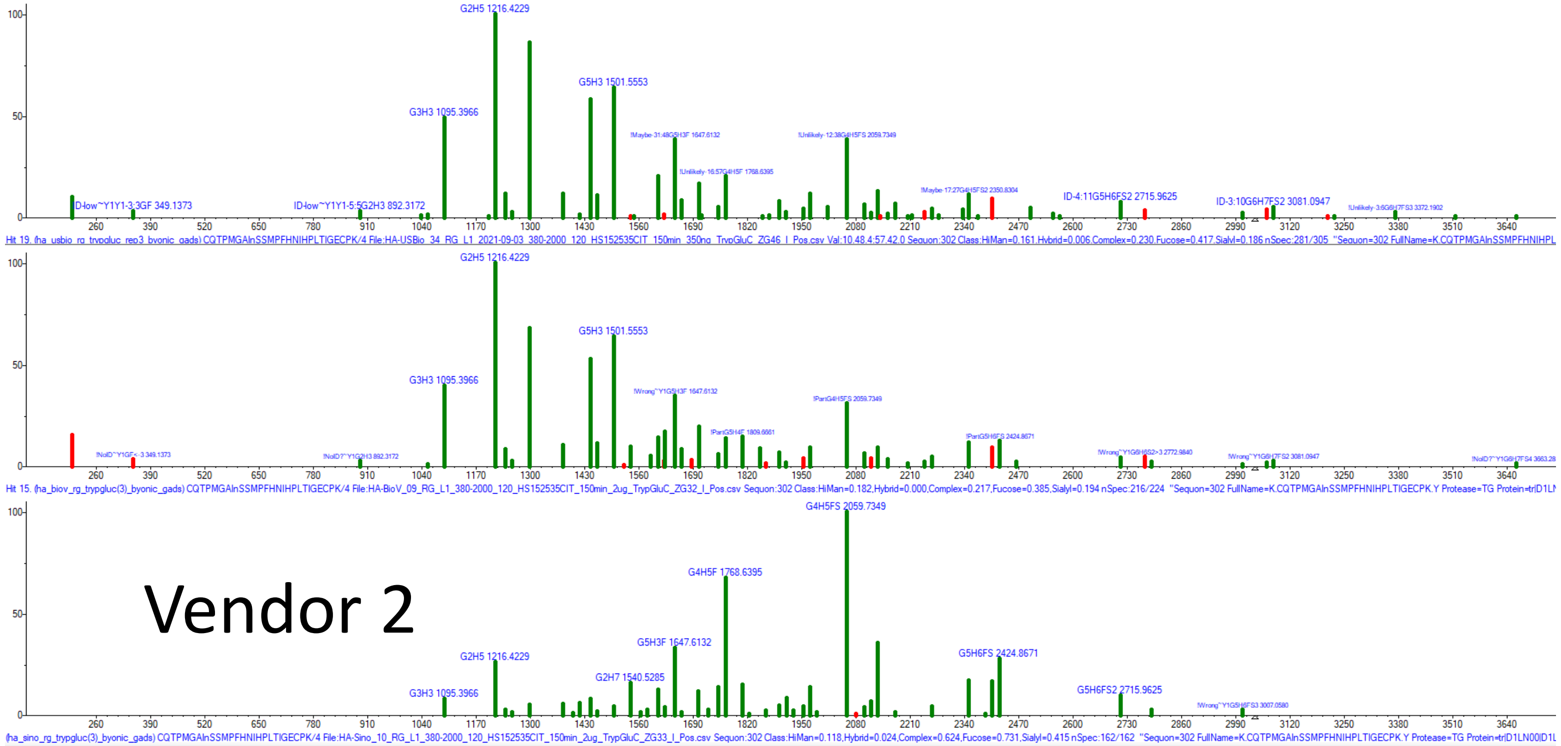


Note: the asterisk indicates high-Man-type N-glycans containing five to nine Man residues

Supplemental Slides

Vendor Comparison

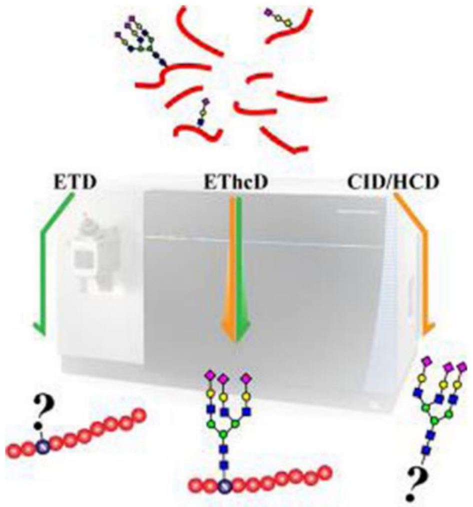
Glycan distribution was skewed toward higher mass (sialylated complex) glycans for vendor 2 and toward lower mass glycans (oligomannose and hybrid) for vendor 1 and vendor 3.

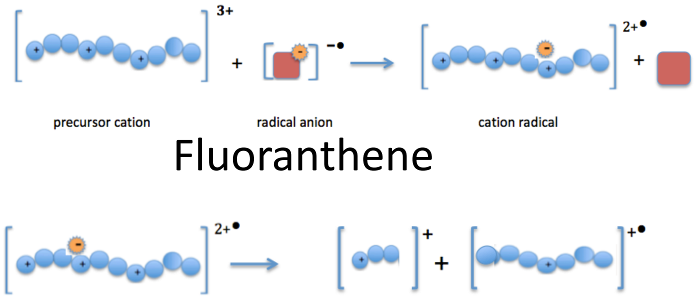


Vendor 2

Supplemental Slides

EThcD





Stepped HCD

Peptide bond
Glycosidic bond
N-glycosidic bond