國立臺灣大學生命科學院生化科學所

碩士論文

Institute of Biochemical and Sciences

College of Life Science

National Taiwan University

Master's Thesis

透過聚醣陣列探討A型流感血凝素與糖的結合專一性

Glycan Microarray Analysis of Influenza A Hemagglutinins Reveals Receptor Binding Specificities

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中華民國113年7月

July 2024

doi:10.6342/NTU202403230

致謝

碩士班的這兩年，短暫卻充實。我非常榮幸有機會來到翁啟惠老師實驗室學習，雖然翁老師事務繁忙，沒有辦法經常討論，但非常感謝老師總能在百忙之中撥空指導我，並提供了充足的實驗資源，我在大學時期並沒有太多的實驗經驗，對學術研究也只是一知半解，非常感謝翁老師提供我一個研究方向，感謝國翔、俊毅學長幫我完善題目並且提供了許多實驗技術的指導，特別感謝徐翠琳老師，徐老師如同我第二位指導教授，感謝徐老師願意撥空幫我釐清許多實驗上的問題，教導我如何對實驗結果進行正確判讀，以及老師願意借用自己實驗室的資源幫助我完成實驗。謝謝馬徹老師與林國儀老師願意擔任我的口試委員，並提供許多專業的建議，讓我的論文可以更完整。謝謝鍾興雅博士與揚諭學長花費大量時間與我一同校正 array printer，也感謝又雅在基因體對我的照顧。謝謝大家的幫助讓我能夠完成碩士班學業。

回顧了這兩年的碩班生涯，我學到了科學研究的基礎，提出問題，設計實驗，分析結果。許多實驗結果不如預期，但依舊要去好好分析，釐清問題，說不定也會有新的發現。希望我的研究能夠讓人類對流感病毒血凝素有更進一步的了解

我的碩士生涯要結束了，無論未來面臨什麼樣的挑戰，我都會以開放、勇敢和樂觀的心態去迎接。期許自己能夠保持這份積極的心態，繼續在人生的道路上精進和成長，希望能在未來的人生旅途中收穫更多的喜悅和成就感。

doi:10.6342/NTU202403230

中文摘要

流感病毒是一種致病性強的呼吸道病毒，主要分為 A、B、C 三種類型，其中 A 型流感病毒尤具高度變異性，易引發大規模流行。大多數人感染後僅出現輕微症狀，並通常能自行康復，但對於新生兒、老年人和慢性病患者，流感病毒可能引發嚴重併發症，如肺炎等，甚至導致死亡。在感染宿主細胞的過程中，流感病毒表面的血凝素（Hemagglutinin, HA）扮演關鍵角色。HA 蛋白與宿主細胞表面的糖結合，這一特性對病毒感染及疫苗研發至關重要。糖微陣列是一種高通量的生物分析技術，能夠在單一實驗中同時測量多種糖與蛋白質之間的相互作用。本研究利用實驗室自製的糖微陣列模擬宿主細胞表面的糖結構，研究不同糖結構對 HA 蛋白結合的影響，以探討流感病毒 HA 蛋白與不同糖類之間的結合專一性，從而為流感疫苗的設計提供更多資訊。我們調查了 14 種 H1N1、3 種 H5N1、2 種 B 型和 1 種 H3N2 的 HA 蛋白。結果顯示，部分 H1N1 HA 蛋白會與 Poly-LacNAc 且末端具有 α2,6 鍵結的 Neu5Ac 的糖結合。其中，H1N1 A/Beijing/262/1995 的 HA 蛋白能識別 α2,3 和 α2,6 鍵結的 Neu5Ac。而 H5N1 A/Vietnam/1194/2004（禽流感病毒）基於其結構特性，對末端具有 α2,3 聯結的糖具有強結合力。然而，其他株的 HA 蛋白在不同實驗條件下，在糖微陣列上未顯示任何結合。除了改變實驗條件，我們也透過紅血球凝集實驗檢查 HA 蛋白的活性，以及透過七種凝集素再次確認糖微陣列的可行性。

關鍵字: 流行性感冒病毒，血凝素，糖微陣列，凝集素、免疫

doi:10.6342/NTU202403230

Abstract

Influenza viruses, particularly type A, are highly pathogenic and prone to causing global pandemics. While most cases are mild, the virus can cause severe symptoms like pneumonia and even death, especially in infants, the elderly, and those with chronic illnesses. The hemagglutinin (HA) glycoprotein on the virus surface binds to glycans on host cells, playing a crucial role in infection and vaccine development. In this study, I used a glycan microarray with 182 different glycans to mimic host cell glycan structures and investigate HA protein binding. This helped explore the binding specificity between various influenza virus HA proteins and glycans, providing insights for vaccine design. I examined HA proteins from different strains and subtypes (14 H1N1, 3 H5N1, 2 influenza B, and 1 H3N2). Results showed that some H1N1 HA proteins bind to glycans with LacNAc repeats and α2,6-linked Neu5Ac, and H1N1 A/Beijing/262/1995 could recognize Neu5Ac with both α2,3 and α2,6 linkages. The H5N1 A/Vietnam/1194/2004 HA protein exhibited strong binding affinity to α2,3-linked glycans due to its structural characteristics. However, other HA proteins did not show any binding under various experimental conditions. In addition to modify the experimental conditions, we also examined the activity of the HA protein through HA assays, and confirmed the feasibility of the glycan microarray using seven different lectins.

Keywords: Influenza virus, Hemagglutinin, Glycan microarray, Lectin, Immunity

doi:10.6342/NTU202403230

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List of Abbreviations

HA	Hemagglutinin
NA	Neuraminidase
IAV	Influenza A Virus
IAB	Influenza B Virus
WHO	World Health Organization
CDC	Centers for Disease Control and Prevention
RNA	Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TRBCs	Lac Red Blood Cells
BSA	Bovine Serum Albumin
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline with Tween 20
LacNAc	N-acetyllactosamine
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
MAb	Monoclonal Antibody
PAb	Polyclonal Antibody
IgG	Immunoglobulin G
SNA	Sambucus nigra Agglutinin
LTL	Lotus Tetragonolobus Lectin
WGA	Wheat Germ Agglutinin
PHA-L	Phaseolus vulgaris Leucoagglutinin

doi:10.6342/NTU202403230

AOL	Aspergillus oryzae Lectin
RCA-I	Ricinus communis Agglutinin I
NCFG	National Center for Functional Glycomics
CRD	Carbohydrate Recognition Domain
NHS	N-Hydroxysuccinimide

doi:10.6342/NTU202403230

Introduction

The influenza virus is a highly infectious pathogen that can cause respiratory infections, fever, cough, rhinitis, headache, and muscle soreness. In severe cases, it can lead to complications such as pneumonia, particularly among the elderly, infants, and people with chronic diseases.[1, 2] Historically, the world has experienced four severe influenza pandemics, the 1918 Spanish flu caused by H1N1, the 1957 H2N2 Asian flu, the 1968 H3N2 Hong Kong flu, and the 2009 H1N1 swine flu. The 1918 Spanish flu was the most severe, causing an estimated 40-50 million deaths globally and infecting over one-third of the world's population.[3-5] Currently, the World Health Organization (WHO) primarily addresses influenza through designing suitable vaccine antigens based on the virus's variations and gene sequences.[6, 7] However, prediction is not entirely accurate, as seen in the 2019-2020 season, where WHO predicted the influenza virus strain to be 2A2B but the actual prevalent strain was the B-type Yamagata strain, resulting in a vaccine efficacy of only 30%.[8, 9] Therefore, efforts are focused on developing universal vaccines that can flexibly respond to the influenza virus's mutations, aiming to achieve true prevention of influenza.

Influenza viruses are members of the Orthomyxoviridae family, which is characterized by the presence of multiple segmented, negative-sense, single-stranded RNA segments encapsulated by a

doi:10.6342/NTU202403230

lipid envelope.[10, 11] The Orthomyxoviridae family includes four main types of influenza viruses: A, B, C, and D. These viruses are zoonotic pathogens, capable of transmitting between humans and various animal species, including pigs, horses, birds, and cattle. Only influenza A and B viruses are primarily responsible for causing pandemics in humans.[12] Influenza A virus (IAV) and influenza B virus (IBV) each have eight RNA segments that encode 11 or 12 proteins, which form the basic structure of the virus.[13] Upon entry into the host cell, the RNA-dependent RNA polymerase complex encoded by the PA, PB1, and PB2 segments transcribes the negative-sense RNA template into a positive-sense RNA template. The M segment encodes the matrix protein M1 and the ion channel M2 located on the viral membrane, which facilitates acidification of the virus within endosomes. The nucleoprotein (NP) segment is responsible for encoding the nucleoprotein, which plays a crucial role in packaging and protecting the viral RNA. Additionally, the NS segment can encode non-structural protein NS1, which counteracts the host's antiviral response, and nuclear export protein NS2 (NEP), which is essential for viral particle assembly and release.[4, 14-16] Most importantly, the HA and NA segments encode the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), respectively, which play critical roles in controlling virus entry and exit from host cells.[17]

Upon infecting host cells, the influenza virus initially binds to host cell receptors composed of sialic acids linked by either α2,3 or α2,6 glycosidic bonds.[18-20] This binding event triggers the virus's

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endocytosis, whereby the virus is internalized via the endocytic pathway. Subsequently, the viral envelope fuses with the endosomal membrane in a pH-dependent manner, releasing the viral genome into the cytoplasm. The viral genome is then transported to the nucleus, where replication and transcription into viral mRNA occur. The mRNA is translated into viral proteins, which are subsequently assembled into new viral particles.[21-24]

Hemagglutinin is a trimeric glycoprotein crucial for the infection and replication of influenza viruses. Serving as the primary surface antigen, HA induces an immune response in the host. Antibodies recognize and bind to HA, thereby preventing the virus from attaching to host cells and inhibiting infection. For example, studies have shown that antibodies targeting specific regions of HA can effectively neutralize viral particles, highlighting HA's importance as a vaccine target.[24, 25] Designing vaccines that focus on HA can therefore elicit protective immune responses tailored to combat circulating influenza strains. Unlike protein-protein interactions, the binding of HA to cell-surface receptors is mediated by N-acetylneuraminic acid (Neu5Ac) residues, and studies on various influenza virus subtypes and recombinant HAs have observed a correlation between sialoside binding preferences and viral species: human HA prefers α2,6 linkage to galactose, avian HA prefers α2,3 linkage to galactose, and swine HA can recognize both α2,6 and α2,3 linkages to galactose or glucose.[20, 26] However, the HA protein exhibits high variability, allowing influenza viruses to evade

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the immune surveillance induced by vaccines. This is why influenza vaccines need to be frequently updated to match new viral strains.[27, 28] By monitoring circulating influenza viruses, scientists can determine which HA subtypes should be included in new vaccines to provide effective protection. However, as mentioned earlier, this approach is not entirely accurate.[8, 9] To provide more durable protection against different strains and subtypes of the influenza virus, scientists are developing universal vaccines, and understanding the receptor binding specificity of HA proteins to glycans is crucial for designing more effective vaccines.[29-31] This exploration provides valuable information and guidance for vaccine research.

Glycan microarrays have emerged as powerful tools for analyzing HA-glycan interactions. These high-throughput platforms allow for the simultaneous screening of multiple glycan structures, providing detailed insights into the binding preferences of different HA proteins.[32] It has significant applications in the study of glycan-mediated diseases such as infectious diseases, inflammation, and cancer.[33] In this study, we use the self-made glycan microarray which is composed of 182 different glycan structures, including both natural and artificial glycans such as mannose, N-glycan, fucose, sialic acid, sulfate, GlcNAc, Gal, LacNAc, and GalNAc. The array is fabricated using NHS-coated slides, with glycans featuring a C_5H_2N group fixed onto the slides using an array printer.[34] The study specifically focuses on the interactions between HA proteins and glycans with terminal sialic acid in

doi:10.6342/NTU202403230

N-linked glycans, as well as those containing LacNAc units. Previous research has shown that certain viral strains of HA proteins exhibit strong binding to LacNAc units, while N-linked glycans are known to be present on cell surfaces.[35-37]

Over all, we utilize a self-made glycan microarray comprising 182 different glycan structures to investigate the interactions between HA proteins from various strains and subtypes (14 H1N1, 3 H5N1, 2 influenza B, and 1 H3N2) and their glycan receptors. By comparing the binding specificities among these different strains and subtypes, we aim to contribute to a deeper understanding of HA-glycan interactions and provide valuable insights for future vaccine design strategies.

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1. Material and Method

1.1 Materials

HA proteins, anti-HA antibodies (anti-H1N1 HA, Rabbit PAb; anti-H5N1 HA, Mouse MAb; anti-H3N2 HA, Mouse MAb; B type anti-HA, Rabbit Mab) were purchased from Sino Biological. Anti-mouse IgG fluor 647 and anti-rabbit IgG fluor 647 were obtained from laboratory. Alexa Fluor® 647 anti-His Tag antibody was purchased from BioLegend. SDS-PAGE tools were acquired from Thermo Scientific. Easy Stain for polyacrylamide gel staining was obtained from ECL. TRBCs stock was sourced from Innovative Research. Lectins were purchased from Sigma, Vector Laboratories, BioLegend, and EY Laboratories. NHS-coated glass slides were obtained from SCHOTT (Nexterion H). Glycans printed on the array slides were obtained from laboratory.

1.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The HA proteins, were subjected to NuPAGE™ gel (4 to 12%, Bis-Tris) and run electrophoresis in vertical gel electrophoresis apparatus with NuPAGE™ MOPS SDS Running Buffer (Thermo Scientific) at 120V for 50 minutes. The gels were then processed for protein staining with EASY Blue-Plus (ECL).

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1.3 Hemagglutination assay (HA assay)

The HA assay was performed using TRBCs, (Innovative^{TM}) diluted to a concentration of 0.5% (vol/vol) in PBS buffer. HA proteins were serially diluted twofold from 100 μg /mL in a total volume of 25 μl in V-shaped 96-well microtiter plates. Next, 25 μl of 0.5% turkey erythrocyte solution were added. The plates were tapped gently 10 times, and the hemagglutination was read after 60 minutes of incubation at room temperature.

1.4 Glycan microarray production

Glycan microarrays were prepared by printing (AD3200; BioDot) the glycans with pentylamine tail (C_5NH_2) prepared in the laboratories to the NHS-coated glass slide by robotic pin (Metal Industries Research & Development Centre) at room temperature with 80% humidity. The slides were spotted with glycan solutions at 100 μM with two duplicates in each chamber, storage in desiccator. The results were analyzed with GenePix Pro 6.0 (Molecular Device) to located and quantify the fluorescence intensity of all spots on the slides.

1.5 Glycan Microarray Binding of Lectin

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Lectins SNA, LTL, WGA, PHA-L, AOL, MAL-I, RCA-I, and Galectin-4, which contain either biotin tags or AlexaFluor-488, were prepared in TSM binding buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% Tween 20, 1% BSA) at concentrations ranging from 0.5 to 10 μg/ml. The array slides were blocked for 1 hour with SuperBlock™ Blocking Buffer, then washed three times with TSM wash buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% Tween 20). Samples were loaded onto the array slides and incubated for 1 hour at room temperature. For arrays containing biotin-tagged lectins, Streptavidin-AlexaFluor-488 (5 μg/mL in TSM binding buffer) was added to the array chambers and incubated for 1 hour at room temperature. The slides were then dipped five times in TSM wash buffer and three times in ddH_2O. The results were analyzed with GenePix Pro 6.0 (Molecular Devices) to locate and quantify the fluorescence intensity of all spots on the slides.

1.6 Glycan Microarray Binding of HA protein

1.6.1 Mouse Anti-His Ab Sequential

Block the total array slides with SuperBlock™ Blocking Buffer for 1 hour at room temperature. Wash the slide with PBST three times. The HA protein (5 μg/ml) in PBST (PBS with 0.05% Tween-20) with 3% BSA was first placed onto the array slide and incubated for 1 hour. Subsequently, Alexa Fluor® 647 mouse anti-His Tag antibody (5 μg/ml) was added and incubated for one hour. Dip the

doi:10.6342/NTU202403230

slides in PBST five times and ddH_2O three times. The results were analyzed with GenePix Pro 6.0 (Molecular Device). The median of fluorescence intensity of each spot was taken to calculate the median value of binding activities toward each sugar (duplicates for each glycan). The medians from at least three independent experiments were averaged for the figures.

1.6.2 Mouse Anti-His Ab Precomplexation

HA-antibody precomplexes were prepared by mixing HA proteins and Alexa Fluor® 647 mouse anti-His Tag antibody in a molar ratio of 2:1 in PBST (PBS with 0.05% Tween-20) with 3% BSA, incubate overnight at 4°C. Block the total array slides with SuperBlock™ Blocking Buffer for 1 hour at room temperature. Wash the slide with PBST three times and load the mixtures into the array chambers, incubate for 8 hours. Dip the slides in PBST five times and ddH_2O three times. The results were analyzed with GenePix Pro 6.0 (Molecular Device). The median of fluorescence intensity of each spot was taken to calculate the median value of binding activities toward each sugar (duplicates for each glycan). The medians from at least three independent experiments were averaged for the figures.

1.6.3 Mouse Anti-His Ab Precomplexation with Additional Fluorescent Secondary Ab.

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Base on the method of mouse anti-His Ab precomplexation, the additional anti mouse Fluro 647 were added. The molar ratio of HA protein: mouse anti-His Ab: anti mouse Fluro 647 = 4:2:1. Load the mixture into the array chambers incubate for 8 hours. Slides were subsequently washed by successive rinses in PBST, and deionized water, then immediately subjected to imaging.

1.6.4 Anti HA Ab sequential

Strain-specific antibodies were used: anti-H1N1 HA antibody for H1N1, anti-H5N1 HA antibody for H5N1, anti-B type HA antibody for B type, and anti-H3N2 HA antibody for H3N2. Block the total array slides with SuperBlock™ Blocking Buffer for 1 hour at room temperature. Wash the slide with PBST three times. The HA protein (5 μg/ml) in PBST (PBS with 0.05% Tween-20) with 3% BSA was first placed onto the array slide and incubated for 1 hour. Subsequently, Anti-HA antibodies (5 μg/ml) were added and incubated for one hour., Then, add secondary anti-mouse or anti-rabbit IgG fluor 647 antibodies (0.75 μg/ml), incubate for one hour. Dip the slides in PBST five times and ddH_2O three times. The results were analyzed with GenePix Pro 6.0 (Molecular Device) to located and quantify the fluorescence intensity of all spots on the slides.

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2. Results

Part I Characterization of HA protein

I.1 SDS PAGE of HA protein

The HA protein is composed of two subunits, HA1 and HA2, with molecular masses of approximately 50 kDa and 20 kDa, respectively. These subunits are cleaved from their precursor, HA0, which has a molecular mass ranging from 63 to 75 kDa, depending on the strain. In SDS-PAGE gels, the uncleaved HA0 typically appears at around 70 kDa. The results indicate that, except for part of HA proteins from A/Vietnam/1194/2004 were cleavage into HA1 and HA2 (Figure 1).

I.2 Hemagglutination assay (HA assay)

HA protein has the ability to agglutinate red blood cells, and HA assay is widely used to detect and quantify the presence of influenza viruses. In this study, we aim to assess the hemagglutination activity of commercial HA proteins from different influenza virus strains and subtypes using TRBCs. TRBCs are commonly used in hemagglutination assays due to their sensitivity in detecting a wide range of influenza viruses. The key to this experiment is observing the behavior of TRBCs in each well. In the absence of HA protein or if the HA proteins lack activity, the TRBCs will precipitate at

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the bottom of the well, forming a small red spot. The results show that not all commercial HA proteins can agglutinate TRBCs. The HA proteins from H5N1 A/Vietnam/1194/2004, H1N1 A/Beijing/262/1995, and H1N1 A/Hawaii/70/2019 can cause TRBCs to agglutinate even at very low HA protein concentrations (Figure 2), indicating strong activity. Meanwhile, H3N2 A/Darwin/9/2021 HA proteins exhibit intermediate activity. In contrast, HA proteins from H1N1 A/California/07/2009, A/Brisbane/59/2009, and A/Beijing/22808/2009 can cause TRBCs agglutination only at very high HA protein concentrations.

Part II　Part II HA proteins binding patterns on glycan microarray

II.1 Key glycans in HA protein studies

This study employed a glycan microarray containing 182 glycans, with a focus on N-glycans and poly-LacNAc structures. N-glycans are commonly found on the cell surface, featuring terminal α2,3 or α2,6 sialic acid residues. Poly-LacNAc chains, composed of repeating disaccharide units of N-acetyllactosamine (LacNAc), are also recognized by some strain of HA proteins. These glycans are abundant on the cell surface, making them crucial for HA protein binding. We selected and organized these glycans into figure 3

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II.2 HA binding patterns on glycan microarray

This study primarily employed two experimental methods: sequential and precomplexation. In the sequential method, the HA protein is first incubated on the array, followed by the addition of anti-His antibody. In the precomplexation method, the HA protein is precomplexed with the anti-His antibody before being incubated on the array. The sequential method more closely mimics the natural interaction between the HA protein and the glycans on the host cell surface. Previous research has shown that the precomplexation method can enhance binding signals. Therefore, this study will primarily discuss the data obtained from the sequential method, using the precomplexation method to provide additional support. A portion of H1N1 HA proteins were able to bind to glycans No. 29 and 30, with the strongest binding to the terminal α2,6 poly-LacNAc on glycan 30. (Figure 4 and 5) The results for H1N1 A/California/07/2009 (2009 Pandemic) and H1N1 A/Brisbane/59/2009 (2009 Seasonal) were consistent with previous studies.[36, 38] Additionally, A/Vietnam/1194/2004 (H5N1), an avian flu strain, was able to bind to any glycans with terminal α2,3 sialic acid (Figure 4 and 6), which also aligned with prior research.[38] H H1N1 A/Beijing/262/1995 recognized glycans with both terminal α2,3 and α2,6 sialic acids (Figure 7), indicating its potential for cross-species transmission. Within the α2,6-linked sialic acids, the H1N1 A/Beijing/262/1995 HA exhibited a preference for poly-LacNAc structures, with binding signals increasing as the number of LacNAc units increased. For

doi:10.6342/NTU202403230

α2,3-linked sialic acids, this HA showed a preference for tri- and tetra-antennary N-glycans. The remaining HA proteins from H1N1, H5N1, H3N2, and B type viruses did not exhibit binding specificity to any glycans on the microarray. (Appendix A and B)

Several of the HA proteins used in my experiments had been previously published, and our results matched those reports. However, this outcome did not meet our expectations, as HA proteins are theoretically capable of recognizing α2,3 or α2,6 sialic acid, but this pattern was not observed on the array. Therefore, we attempted to modify the experimental conditions, meanwhile, re-evaluate the HA proteins and self-made glycan microarray. The lack of binding specificity observed in my study suggests that the HA proteins may require other experimental conditions or additional factors to exhibit their full receptor binding pattern on the glycan microarray platform.

II.3 Comparison of anti-His Ab and anti-HA Ab on glycan microarray

According to the results, only two strains of HA protein demonstrated binding specificity, while the remaining proteins bound exclusively to glycans No.29 and 30, with some HA proteins showing no binding to any glycans. This contrasts with our expectations of binding to terminal sialic acid N-glycans or poly-LacNAc. To further investigate this phenomenon, I modified the experimental approach by substituting the antibody with anti-HA antibody, aiming to induce binding specificity of

doi:10.6342/NTU202403230

HA protein on glycan microarrays. The results indicated that the group using anti-HA antibody showed enhanced binding signals, but did not alter the glycans to which it binds (Figure 8).

II.4 Additional fluorescent secondary antibody detection

Regardless of whether a sequential or pre-complexation approach was used, or anti-HA antibody was substituted for detection, no binding specificity of the HA protein was observed. In the anti-His Ab and HA protein pre-complexation experiment, we additionally add anti-mouse Fluor 647 to enhance the signal. This method led to an amplification of the detection signal (Figure 9). However, the glycan-binding specificity of the HA protein did not change.

II.5 Comparison of HA Protein Monomer and Trimer Binding on Glycan Microarray

The HA protein used in our previous experiments was in its monomeric form, with each monomer possessing a single glycan-binding site. However, HA naturally exists as a trimer, which has three binding sites. It is plausible that the trimeric HA, through multivalent interactions, binds more strongly to glycans and exhibits distinct binding specificities. Therefore, I conducted a comparative analysis of the monomeric and trimeric forms of HA on an array. The experimental results demonstrated that the binding patterns of HA monomer and trimer on the glycan microarray were nearly identical (Figure 10). This suggests that, despite the potential for multivalent interactions

doi:10.6342/NTU202403230

in the trimeric form, the overall binding specificity and affinity of HA towards glycans remain consistent between its monomeric and trimeric forms.

Part III Lectin binding specificity on glycan microarray

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III.1 Lectin binding specificity on glycan microarray

To ensure the quality and reliability of glycan microarray data, quality control measures are essential. In this study, we conducted a quality control by examining 182 different glycan on the microarray using seven different lectins: WGA, PHA-I, AOL, LTL, Galectin-4, and RCA-I

Sambucus nigra Agglutinin (SNA, SNA-I)

SNA, commonly abbreviated as SNA, is a lectin isolated from elderberry bark and is one of the most widely used tools for detecting α2,6-linked sialic acids. Previous studies have indicated that α2,6-sialylated lactosamine (LacNAc) is the primary binding determinant for SNA. These studies have also revealed that SNA can recognize a variety of α2,6-sialic acid structures, including N-glycolylneuraminic acid (Neu5Gc) and KDN, in addition to the typical Neu5Ac. However, our experimental results show some discrepancies with previous research.[39, 40] We found that while SNA does selectively recognize α2,6-sialic acids, its tolerance for different α2,6-sialic acid structures is

doi:10.6342/NTU202403230

relatively low. Specifically, SNA only binds to α2,6-sialylated type II LacNAc structures and does not recognize other types of α2,6-sialylated structures (Appendice D). This finding contrasts with earlier reports suggesting that SNA can recognize a broader range of α2,6-sialic acid structures.

Lotus Tetragonolobus Lectin (LTL)

The binding pattern of LTL is not well-defined. Initially, it was thought to be a fucose-binding lectin, but no clear binding rule has been established. Recent studies have identified α1,3-fucose as the main recognition motif for LTL, although not all glycans containing this structure can be recognized.[40, 41] Our results align with these recent findings, showing that LTL does not exhibit a clear binding pattern (Appendice E). However, glycans containing the α1,3-fucose structure, such as Lewis x and Lewis y, were enriched in LTL binding assays

AOL (Aspergillus oryzae Lectin)

AOL is identified as an α-linked fucose-binding lectin Notably, AOL cannot bind blood group A and B antigens.[40] Our glycan microarray results indicate that AOL exhibits strong binding affinity for certain glycans, with signal saturation even at low concentrations, demonstrating AOL's strong fucose-binding capability (Appendice E). AOL shows high affinity for various fucosylated structures, including β-linked fucose (No. 76), but the sample size for β-linked fucose is limited, still need further

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research. Additionally, AOL does not bind to blood group A and B antigens (No. 144, 145). AOL also interacts with some non-fucosylated oligosaccharides or fails to bind certain fucosylated oligosaccharides, the patterns of these interactions remain unclear.

Wheat germ agglutinin (WGA)

WGA, derived from wheat germ (Triticum aestivum or Triticum vulgare), is one of the most extensively studied and widely utilized lectins. Although primarily classified as a lectin that binds N-acetylglucosamine (GlcNAc), WGA actually exhibits remarkably broad binding specificity. Previous studies have demonstrated that WGA can interact with a diverse array of terminal N-acetyl-containing glycans, including GlcNAc α-, GalNAc α-, GalNAcβ-, and Neu5Ac.[40, 42] My results are consistent with the prior findings, confirming the broad binding specificity of WGA (Appendice F).

Ricinus communis Agglutinin (RCA-I)

RCA-I exhibits a strong preference for terminal type II LacNAc (Galβ1-4GlcNAc) as its main binding determinant. It tolerates substitution at the 6-position of the terminal galactose but not at the 3-position.[40] My experimental results confirm this binding specificity observed in the literature. The data show that RCA-I preferentially binds to type II LacNAc structures, with a small subset of terminal structures containing α2,6-linked sialic acid (Neu5Ac) also being recognized (Appendice G).

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Interestingly, α2,3-sialylation of LacNAc blocked recognition by RCA-I, while α2,6-sialylation had little effect on RCA-I binding.

Phaseolus vulgaris-Leukoagglutinating (PHA-L)

Previous studies have observed that PHA-L predominantly binds to β1,6-branched N-glycans and can tolerate bisecting GlcNAc, core fucose, and α2,3 sialic acid, but its binding is inhibited by α2,6 sialic acid.[40] Our experimental results confirm and extend these findings, demonstrating that PHA-L exhibits broad binding specificity on the glycan microarray (Appendice H). Most N-glycans are recognized by PHA-L, and α2,6 sialic acid indeed inhibits binding, while core fucose and α2,3 sialic acid is tolerated. Additionally, our data suggest that PHA-L can also bind to certain linear glycans and may recognize GlcNAc.

Galectin-4

Galectin-4 is composed of two carbohydrate recognition domains (CRDs), located at the N-terminus and C-terminus, each with distinct binding specificities. Overall, Galectin-4 preferentially binds to glycans containing the core structures Galβ1-3GlcNAc or Galβ1-3GalNAc, and shows higher affinity for 3'-O-sulfated and 3'-sialylated disaccharides. Blood group antigens (ABH antigens) are considered to be the ligands with the highest affinity for Galectin-4.[43] In our study, we observed that

doi:10.6342/NTU202403230

Galectin-4 exhibits a broad range of glycan binding, which is consistent with previous findings (Appendice I). Our results demonstrate that Galectin-4 can bind to α2,3- and α2,6-linked sialic acids, as well as terminal galactose.

III.2 Array quality control with lectins

Based on the results, approximately 85% of the glycans were recognized by various lectins, confirming their presence on the array slides. Although some glycans with unique structures lacked suitable lectin matches, this did not compromise the array quality control functionality in this study. Importantly, nearly all lectins exhibited their expected binding specificity on the array, and all key glycans were successfully recognized by different lectins (Figure 11), demonstrating the effectiveness of the array quality control in this research.

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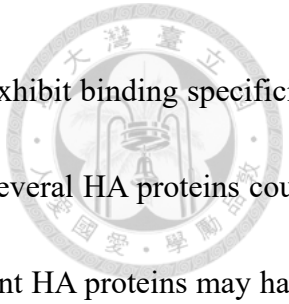
3. Discussion

Our glycan microarray analysis of various influenza hemagglutinin (HA) proteins revealed distinct binding patterns across different strains and subtypes (Table 1). Notably, 46% of H1N1 HA proteins demonstrated binding affinity to poly-LacNAc with terminal α2,6-linked Neu5Ac. This finding aligns with previous studies that have shown human influenza viruses preferentially bind to α2,6-linked sialic acids, which are predominant in the upper respiratory tract of humans. HA protein from H1N1 A/Beijing/262/1995, exhibits a preference for binding to glycans with α2,3-linked Neu5Ac, tri- or tetra-antennary N-glycans, and α2,6-linked poly-LacNAc structures. This dual recognition capability suggests a potential for cross-species transmission. Such strains with broader binding specificity may pose a higher risk for zoonotic transmission and could be important targets for surveillance and vaccine development efforts. The H5N1 A/Vietnam/1194/2004 HA protein showed strong binding affinity to α2,3-linked glycans, which is consistent with the typical preference of avian influenza viruses for α2,3-linked sialic acids. In this research, the H5N1 A/Vietnam/1194/2004 strain was primarily used as a positive control. The binding pattern of avian flu HA proteins has been repeatedly documented in literature. By detecting the known binding affinity of the H5N1 A/Vietnam/1194/2004 HA protein, we can confirm the reliability and accuracy of our experimental approach, thereby ensuring the credibility of our findings for other HA proteins tested in this study.

doi:10.6342/NTU202403230



However, it is notable that 46% of HA proteins in our study did not exhibit binding specificity under various experimental conditions. The lack of binding observed for several HA proteins could be due to several factors. Firstly, the quality and integrity of the recombinant HA proteins may have been compromised, potentially affecting their binding capabilities. Secondly, limitations in the glycan microarray itself, such as suboptimal glycan density or presentation, could have hindered the detection of binding interactions. Thirdly, the experimental methodology, detection methods, may not have been optimal for capturing the full range of HA-glycan interactions.

About the experimental methodology, we first use anti-His Ab with sequential and precomplex method, but some HA proteins did not demonstrate any binding on the glycan microarray. This unexpected result led us to modify our experimental approach in several ways. We first substituted the anti-His antibody with specific anti-HA antibodies. This change helped us to exclude the possibility that the anti-His antibody was unsuitable for use in this experiment. The results indicated that while the use of anti-HA antibodies did enhance binding signals, it did not alter the overall glycan binding specificity of the HA proteins. Additionally, we tested the addition of a fluorescent secondary antibody to amplify the detection signal. While the method leads to signal enhancement, they did not fundamentally change the glycan-binding specificity of the HA proteins. Considering the multivalent interactions involved in the binding of HA proteins to glycans, we also compared the differences

doi:10.6342/NTU202403230

between monomeric and trimeric forms of HA protein on glycan microarray. However, the results indicated that both forms exhibited the same binding patterns.

The hemagglutination (HA) assay results revealed that most of the commercial HA proteins used in our study were unable to cause agglutination of TRBCs. This suggests that the majority of these HA proteins lacked hemagglutination activity. Interestingly, we observed that the presence or absence of hemagglutination activity did not directly correlate with the ability of HA proteins to bind glycans on our microarray. For example, HA proteins from H1N1 A/Victoria/67/2022 and H1N1 A/Wisconsin/67/2022 exhibited hemagglutination activity but were still able to bind to glycans on the microarray. Conversely, some HA proteins that showed no hemagglutination activity still demonstrated binding on the glycan microarray. Based on the above results, the correlation between HA activity and its binding on the glycan array is not clear, this might cause by some reasons. An experiment conducted by Nature revealed that varying glycan densities affected the dissociation constants in the interaction with Siglec-8, indicating optimal binding at specific glycan densities.[44] The glycan density on glycan arrays may differ from that on the surface of red blood cells, leading to this phenomenon. Also, the composition of glycans can vary significantly between glycan arrays and natural cell surfaces.[45] Glycan arrays present only one type of glycan at each spot, whereas the surface of red blood cells has a more diverse array of glycans. This variability may also contribute to the differences in binding observed between glycan arrays and red blood cells.

doi:10.6342/NTU202403230

Lastly, HA assays typically use whole influenza viruses rather than HA protein alone, which may involve multivalent interactions.[46] The large number of HA trimers on the viral surface might provide multivalency, enhancing the strength of binding interactions.

This study marks the first comprehensive evaluation of our laboratory's glycan microarray using multiple lectins. The results of this quality control process were highly encouraging, demonstrating the reliability of our array. Approximately 85% of the glycans on our array were successfully recognized by different lectins, confirming their presence and accessibility on the array surface. The binding patterns observed for the various lectins were clear, and mostly consistent with previously reported specificities. For example, SNA showed the expected preference for α2,6-linked sialic acids, while AOL recognized fucosylated structures. The consistency of our results with established lectin binding patterns validates the quality and reliability of our glycan microarray. Moreover, the fact that our array includes about 70% of glycan structures that do not overlap with those in similar published experiments provides new data on binding specificities. The lectin binding specificities referenced in this study were primarily derived from a analysis conducted by the National Center for Functional Glycomics (NCFG). The NCFG's glycan array includes 562 glycan structures, providing a broad spectrum for lectin binding analysis. Despite our array containing only 182 glycan structures, 70% of glycans not overlapping with those in the NCFG array, offers new binding information and highlights

doi:10.6342/NTU202403230

the unique contribution of our glycan microarray in expanding the current knowledge base in glycobiology.

The glycan microarray format may not fully replicate the multivalent interactions that occur in vivo. This limitation could explain why some HA proteins did not exhibit expected binding patterns on the microarray even we already characterized the HA protein, and confirm the array reliability. Future studies could explore alternative methods, such as using techniques that allow for multivalent interactions, to better understand the binding specificities of HA proteins.

In conclusion, this work advances our understanding of influenza virus HA protein binding specificity on glycan microarray, and provides a foundation for future studies aimed at developing more effective strategies to combat influenza infections. As influenza viruses continue to evolve, such detailed characterizations of HA-glycan interactions will remain crucial for staying ahead of potential pandemic threats.

doi:10.6342/NTU202403230

4. Reference

(1) *Key Facts About Influenza (Flu)*. U.S. Centers for Disease Control and Prevention, https://www.cdc.gov/flu/about/keyfacts.htm (accessed 2024 2024 June 28).

(2) *Clinical Signs & Symptoms of Influenza*. U.S. Department of Health & Human Services, 2021. https://www.cdc.gov/flu/professionals/acip/clinical.htm#print (accessed June 27, 2024.

(3) Short, K. R.; Kedzierska, K.; van de Sandt, C. E. Back to the Future: Lessons Learned From the 1918 Influenza Pandemic. *Front Cell Infect Microbiol* **2018**, *8*, 343. DOI: 10.3389/fcimb.2018.00343 From NLM Medline.

(4) Krammer, F.; Smith, G. J. D.; Fouchier, R. A. M.; Peiris, M.; Kedzierska, K.; Doherty, P. C.; Palese, P.; Shaw, M. L.; Treanor, J.; Webster, R. G.; et al. Influenza. *Nat Rev Dis Primers* **2018**, *4* (1), 3. DOI: 10.1038/s41572-018-0002-y From NLM Medline.

(5) Palese, P. Influenza: old and new threats. *Nat Med* **2004**, *10* (12 Suppl), S82-87. DOI: 10.1038/nm1141 From NLM Medline.

(6) Perdue, M. L.; Nguyen, T. The WHO research agenda for influenza: two years later. *Bull World Health Organ* **2012**, *90* (4), 246. DOI: 10.2471/BLT.11.090175 From NLM Medline.

(7) *WHO Preferred Product Characteristics for Next-Generation Influenza Vaccines*. World Health Organization, 2017. https://iris.who.int/bitstream/handle/10665/258767/9789241512466-eng.pdf?sequence=1 (accessed.

(8) *Influenza season 2019–2020: early situation assessment*. World Health Organization, 2019. https://www.ecdc.europa.eu/sites/default/files/documents/influenza-situation-assessment-18-December-2019.pdf (accessed 2024 May, 13).

(9) *US Flu VE Data for 2019-2020*. U.S. Centers for Disease Control and Prevention, December 22, 2020. https://www.cdc.gov/flu/vaccines-work/2019-2020.html (accessed 2024 May 13).

(10) Noda, T. Native morphology of influenza virions. *Front Microbiol* **2011**, *2*, 269. DOI:

doi:10.6342/NTU202403230

10.3389/fmicb.2011.00269 From NLM PubMed-not-MEDLINE.

(11) Suzanne Clancy, P. D. Genetics of the Influenza Virus. *Nature Education 1(1):83* **2008**.

(12) S, B. *Orthomyxoviruses*. University of Texas Medical Branch at Galveston, 1996. https://www.ncbi.nlm.nih.gov/books/NBK8611/ (accessed 2023 August 18).

(13) Dadonaite, B.; Gilbertson, B.; Knight, M. L.; Trifkovic, S.; Rockman, S.; Laederach, A.; Brown, L. E.; Fodor, E.; Bauer, D. L. V. The structure of the influenza A virus genome. *Nature Microbiology* **2019**, *4* (11), 1781-1789. DOI: 10.1038/s41564-019-0513-7.

(14) Hao, W.; Wang, L.; Li, S. Roles of the Non-Structural Proteins of Influenza A Virus. *Pathogens* **2020**, *9* (10). DOI: 10.3390/pathogens9100812 From NLM PubMed-not-MEDLINE.

(15) Peiris., J. S. M. Avian influenza viruses in humans. *Revue scientifique et technique (International Office of Epizootics)* **2009**, 28 (21), pp.161-173.

(16) Bouvier, N. M., & Palese, P. . The biology of influenza viruses. *Vaccine* **2008**. DOI: 10.1016/j.vaccine.2008.07.039.

(17) Shi, W.; Lei, F.; Zhu, C.; Sievers, F.; Higgins, D. G. A complete analysis of HA and NA genes of influenza A viruses. *PLoS One* **2010**, *5* (12), e14454. DOI: 10.1371/journal.pone.0014454 From NLM Medline.

(18) Zhao, C.; Pu, J. Influence of Host Sialic Acid Receptors Structure on the Host Specificity of Influenza Viruses. *Viruses* **2022**, *14* (10). DOI: 10.3390/v14102141 From NLM Medline.

(19) Dedola, S.; Ahmadipour, S.; de Andrade, P.; Baker, A. N.; Boshra, A. N.; Chessa, S.; Gibson, M. I.; Hernando, P. J.; Ivanova, I. M.; Lloyd, J. E.; et al. Sialic acids in infection and their potential use in detection and protection against pathogens. *RSC Chem Biol* **2024**, *5* (3), 167-188. DOI: 10.1039/d3cb00155e From NLM PubMed-not-MEDLINE.

(20) Ayora-Talavera, G. Sialic acid receptors: focus on their role in influenza infection. *Journal of Receptor, Ligand and Channel Research* **2018**, *Volume 10*, 1-11. DOI: 10.2147/jrlcr.S140624.

(21) Aganovic, A. pH-dependent endocytosis mechanisms for influenza A and SARS-coronavirus.

doi:10.6342/NTU202403230

Front Microbiol **2023**, *14*, 1190463. DOI: 10.3389/fmicb.2023.1190463 From NLM PubMed-not-MEDLINE.

(22) AKIHIKO YOSHIMURA, K. K., 1 KAZUNORI KAWASAKI,1 SHOHEI; YAMASHINA, T. M. Infectious Cell Entry Mechanism of Influenza Virus. *JOURNAL OF VIROLOGY* **1981**, p. 284-293. DOI: 10.1128/jvi.43.1.284-293.1982.

(23) Lakadamyali, M.; Rust, M. J.; Zhuang, X. Endocytosis of influenza viruses. *Microbes Infect* **2004**, *6* (10), 929-936. DOI: 10.1016/j.micinf.2004.05.002 From NLM Medline.

(24) Mair, C. M.; Ludwig, K.; Herrmann, A.; Sieben, C. Receptor binding and pH stability - how influenza A virus hemagglutinin affects host-specific virus infection. *Biochim Biophys Acta* **2014**, *1838* (4), 1153-1168. DOI: 10.1016/j.bbamem.2013.10.004 From NLM Medline.

(25) Brandenburg, B.; Koudstaal, W.; Goudsmit, J.; Klaren, V.; Tang, C.; Bujny, M. V.; Korse, H. J.; Kwaks, T.; Otterstrom, J. J.; Juraszek, J.; et al. Mechanisms of hemagglutinin targeted influenza virus neutralization. *PLoS One* **2013**, *8* (12), e80034. DOI: 10.1371/journal.pone.0080034 From NLM Medline.

(26) Matrosovich, M.; Herrler, G.; Klenk, H. D. Sialic Acid Receptors of Viruses. *Top Curr Chem* **2015**, *367*, 1-28. DOI: 10.1007/128_2013_466 From NLM Medline.

(27) Gupta, D.; Mohan, S. Influenza vaccine: a review on current scenario and future prospects. *J Genet Eng Biotechnol* **2023**, *21* (1), 154. DOI: 10.1186/s43141-023-00581-y From NLM PubMed-not-MEDLINE.

(28) Lazniewski, M.; Dawson, W. K.; Szczepinska, T.; Plewczynski, D. The structural variability of the influenza A hemagglutinin receptor-binding site. *Brief Funct Genomics* **2018**, *17* (6), 415-427. DOI: 10.1093/bfgp/elx042 From NLM Medline.

(29) Boyoglu-Barnum, S.; Hutchinson, G. B.; Boyington, J. C.; Moin, S. M.; Gillespie, R. A.; Tsybovsky, Y.; Stephens, T.; Vaile, J. R.; Lederhofer, J.; Corbett, K. S.; et al. Glycan repositioning of influenza hemagglutinin stem facilitates the elicitation of protective cross-group

doi:10.6342/NTU202403230

antibody responses. *Nat Commun* **2020**, *11* (1), 791. DOI: 10.1038/s41467-020-14579-4 From NLM Medline.

(30) Fei, Y.; Sun, Y. S.; Li, Y.; Yu, H.; Lau, K.; Landry, J. P.; Luo, Z.; Baumgarth, N.; Chen, X.; Zhu, X. Characterization of Receptor Binding Profiles of Influenza A Viruses Using An Ellipsometry-Based Label-Free Glycan Microarray Assay Platform. *Biomolecules* **2015**, *5* (3), 1480-1498. DOI: 10.3390/biom5031480 From NLM Medline.

(31) Xu, R.; McBride, R.; Nycholat, C. M.; Paulson, J. C.; Wilson, I. A. Structural characterization of the hemagglutinin receptor specificity from the 2009 H1N1 influenza pandemic. *J Virol* **2012**, *86* (2), 982-990. DOI: 10.1128/JVI.06322-11 From NLM Medline.

(32) Liang, C. H.; Wu, C. Y. Glycan array: a powerful tool for glycomics studies. *Expert Rev Proteomics* **2009**, *6* (6), 631-645. DOI: 10.1586/epr.09.82 From NLM Medline.

(33) Liang, C. H.; Wang, S. K.; Lin, C. W.; Wang, C. C.; Wong, C. H.; Wu, C. Y. Effects of neighboring glycans on antibody-carbohydrate interaction. *Angew Chem Int Ed Engl* **2011**, *50* (7), 1608-1612. DOI: 10.1002/anie.201003482 From NLM Medline.

(34) Ruprecht, C.; Geissner, A.; Seeberger, P. H.; Pfrengle, F. Practical considerations for printing high-density glycan microarrays to study weak carbohydrate-protein interactions. *Carbohydr Res* **2019**, *481*, 31-35. DOI: 10.1016/j.carres.2019.06.006 From NLM Medline.

(35) Stevens, J.; Blixt, O.; Glaser, L.; Taubenberger, J. K.; Palese, P.; Paulson, J. C.; Wilson, I. A. Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. *J Mol Biol* **2006**, *355* (5), 1143-1155. DOI: 10.1016/j.jmb.2005.11.002 From NLM Medline.

(36) Chen, J. R.; Yu, Y. H.; Tseng, Y. C.; Chiang, W. L.; Chiang, M. F.; Ko, Y. A.; Chiu, Y. K.; Ma, H. H.; Wu, C. Y.; Jan, J. T.; et al. Vaccination of monoglycosylated hemagglutinin induces cross-strain protection against influenza virus infections. *Proc Natl Acad Sci U S A* **2014**, *111* (7), 2476-2481. DOI: 10.1073/pnas.1323954111 From NLM Medline.

doi:10.6342/NTU202403230

(37) Canales, A.; Sastre, J.; Orduna, J. M.; Spruit, C. M.; Perez-Castells, J.; Dominguez, G.; Bouwman, K. M.; van der Woude, R.; Canada, F. J.; Nycholat, C. M.; et al. Revealing the Specificity of Human H1 Influenza A Viruses to Complex N-Glycans. *JACS Au* **2023**, *3* (3), 868-878. DOI: 10.1021/jacsau.2c00664 From NLM PubMed-not-MEDLINE.

(38) Hsin-Yu Liao, C.-H. H., Shih-Chi Wang, Chi-Hui Liang,; Hsin-Yung Yen, C.-Y. S., Chien-Hung Chen, Jia-Tsrong Jan,; Chien-Tai Ren, C.-H. C., Ting-Jen R. Cheng, Chung-Yi Wu, and; Wong, C.-H. Differential Receptor Binding Affinities of Influenza Hemagglutinins on Glycan Arrays. *Journal of the American Chemical Society* **2010**.

(39) Xie, Y.; Sheng, Y.; Li, Q.; Ju, S.; Reyes, J.; Lebrilla, C. B. Determination of the glycoprotein specificity of lectins on cell membranes through oxidative proteomics. *Chem Sci* **2020**, *11* (35), 9501-9512. DOI: 10.1039/d0sc04199h From NLM PubMed-not-MEDLINE.

(40) Bojar, D.; Meche, L.; Meng, G.; Eng, W.; Smith, D. F.; Cummings, R. D.; Mahal, L. K. A Useful Guide to Lectin Binding: Machine-Learning Directed Annotation of 57 Unique Lectin Specificities. *ACS Chem Biol* **2022**, *17* (11), 2993-3012. DOI: 10.1021/acschembio.1c00689 From NLM Medline.

(41) Gao, Z.; Chen, S.; Du, J.; Wu, Z.; Ge, W.; Gao, S.; Zhou, Z.; Yang, X.; Xing, Y.; Shi, M.; et al. Quantitative analysis of fucosylated glycoproteins by immobilized lectin-affinity fluorescent labeling. *RSC Adv* **2023**, *13* (10), 6676-6687. DOI: 10.1039/d3ra00072a From NLM PubMed-not-MEDLINE.

(42) David Schwefel, C. M., Johannes G. Beck, Sonja Seeberger, Kay Diederichs, Heiko M. Moller, Wolfram Welte, and Valentin Wittmann. Structural Basis of Multivalent Binding to Wheat GermAgglutinin. *Journal of the American Chemical Society* **2010**.

(43) Slamova, K.; Cerveny, J.; Meszaros, Z.; Friede, T.; Vrbata, D.; Kren, V.; Bojarova, P. Oligosaccharide Ligands of Galectin-4 and Its Subunits: Multivalency Scores Highly. *Molecules* **2023**, *28* (10). DOI: 10.3390/molecules28104039 From NLM Medline.

doi:10.6342/NTU202403230

(44) Cao, H.; Antonopoulos, A.; Henderson, S.; Wassall, H.; Brewin, J.; Masson, A.; Shepherd, J.; Konieczny, G.; Patel, B.; Williams, M. L.; et al. Red blood cell mannoses as phagocytic ligands mediating both sickle cell anaemia and malaria resistance. *Nat Commun* **2021**, *12* (1), 1792. DOI: 10.1038/s41467-021-21814-z From NLM Medline.

(45) Broszeit, F.; van Beek, R. J.; Unione, L.; Bestebroer, T. M.; Chapla, D.; Yang, J. Y.; Moremen, K. W.; Herfst, S.; Fouchier, R. A. M.; de Vries, R. P.; et al. Glycan remodeled erythrocytes facilitate antigenic characterization of recent A/H3N2 influenza viruses. *Nat Commun* **2021**, *12* (1), 5449. DOI: 10.1038/s41467-021-25713-1 From NLM Medline.

(46) Nemanichvili, N.; Tomris, I.; Turner, H. L.; McBride, R.; Grant, O. C.; van der Woude, R.; Aldosari, M. H.; Pieters, R. J.; Woods, R. J.; Paulson, J. C.; et al. Fluorescent Trimeric Hemagglutinins Reveal Multivalent Receptor Binding Properties. *J Mol Biol* **2019**, *431* (4), 842-856. DOI: 10.1016/j.jmb.2018.12.014 From NLM Medline.

doi:10.6342/NTU202403230

5. Figures

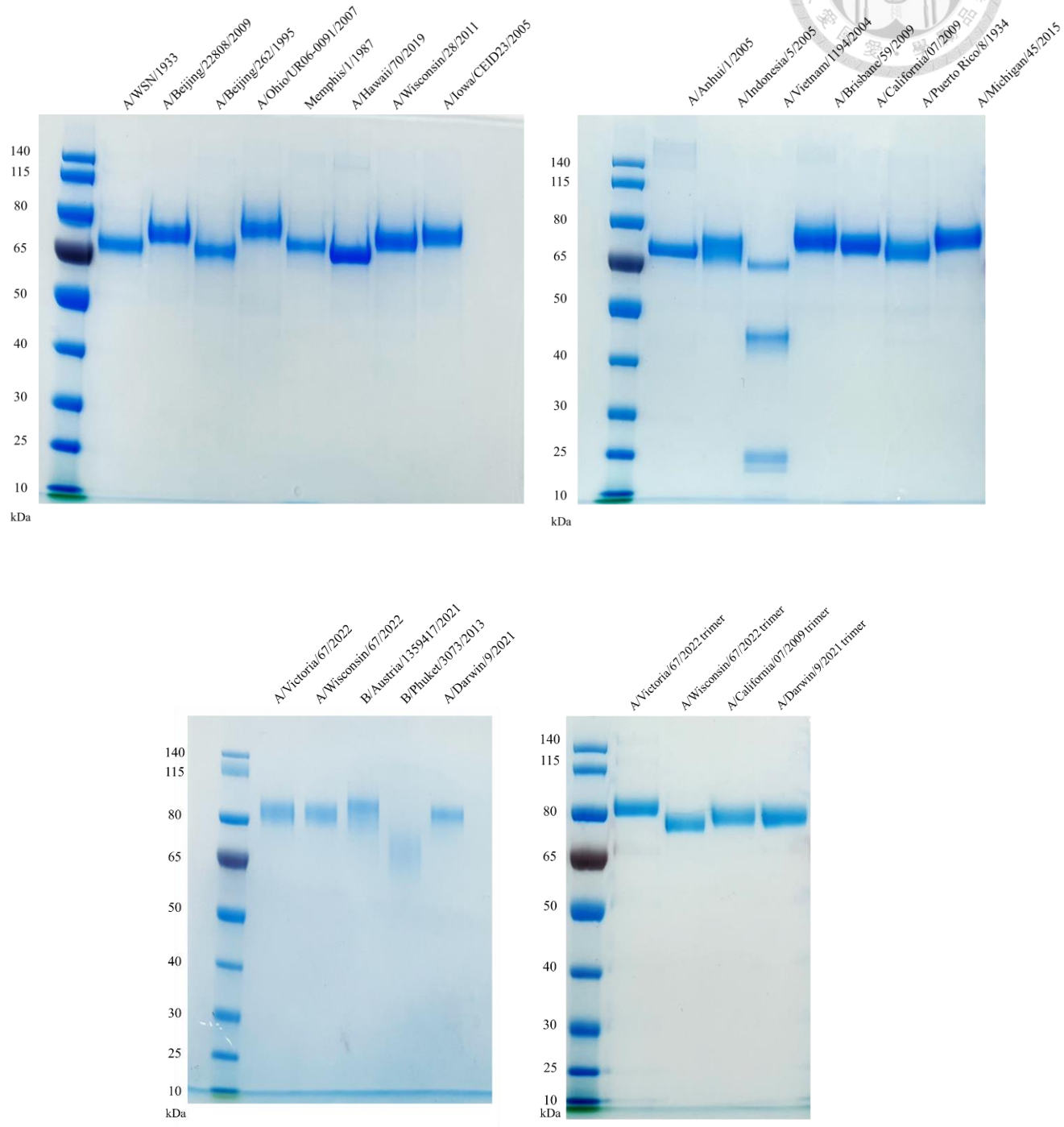


Figure 1. SDS-PAGE result of HA proteins.

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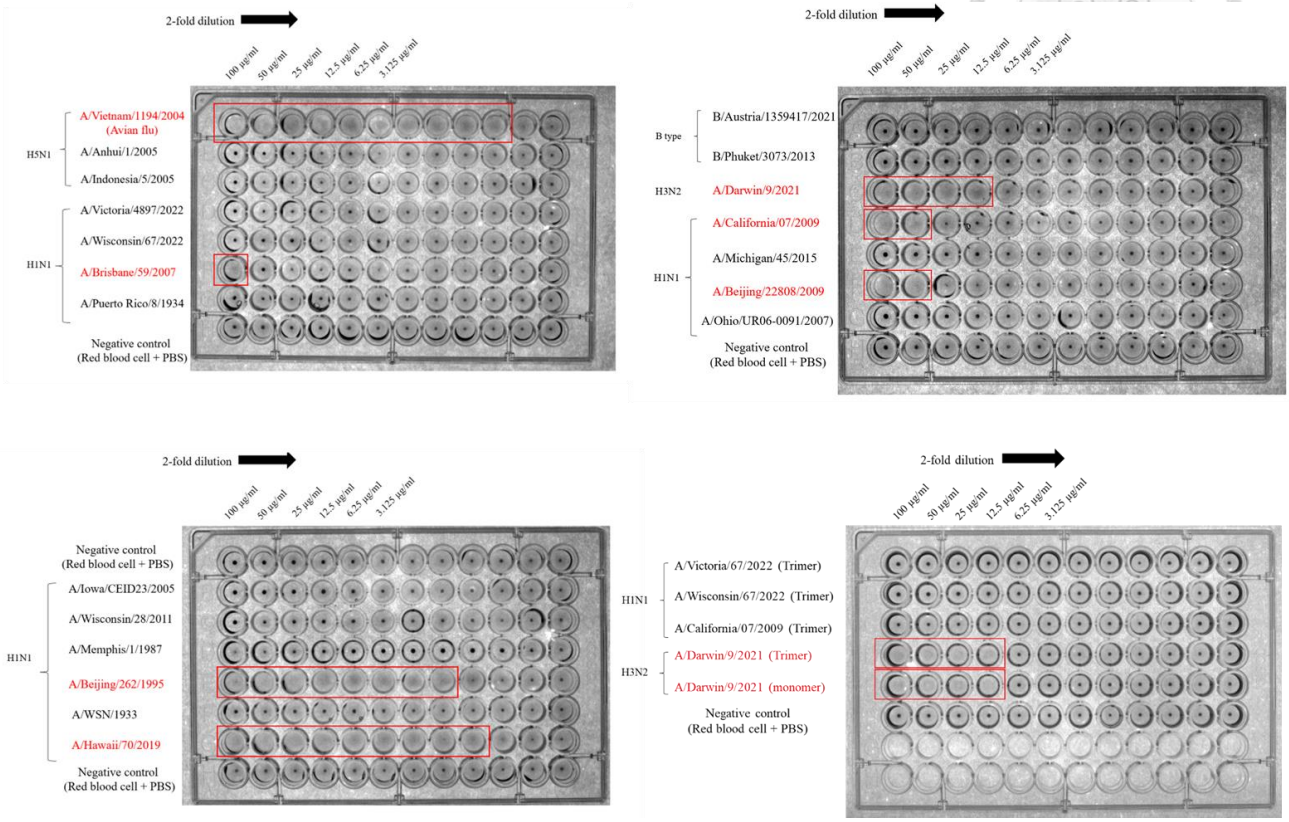

Figure 2. HA assay result.

doi:10.6342/NTU202403230

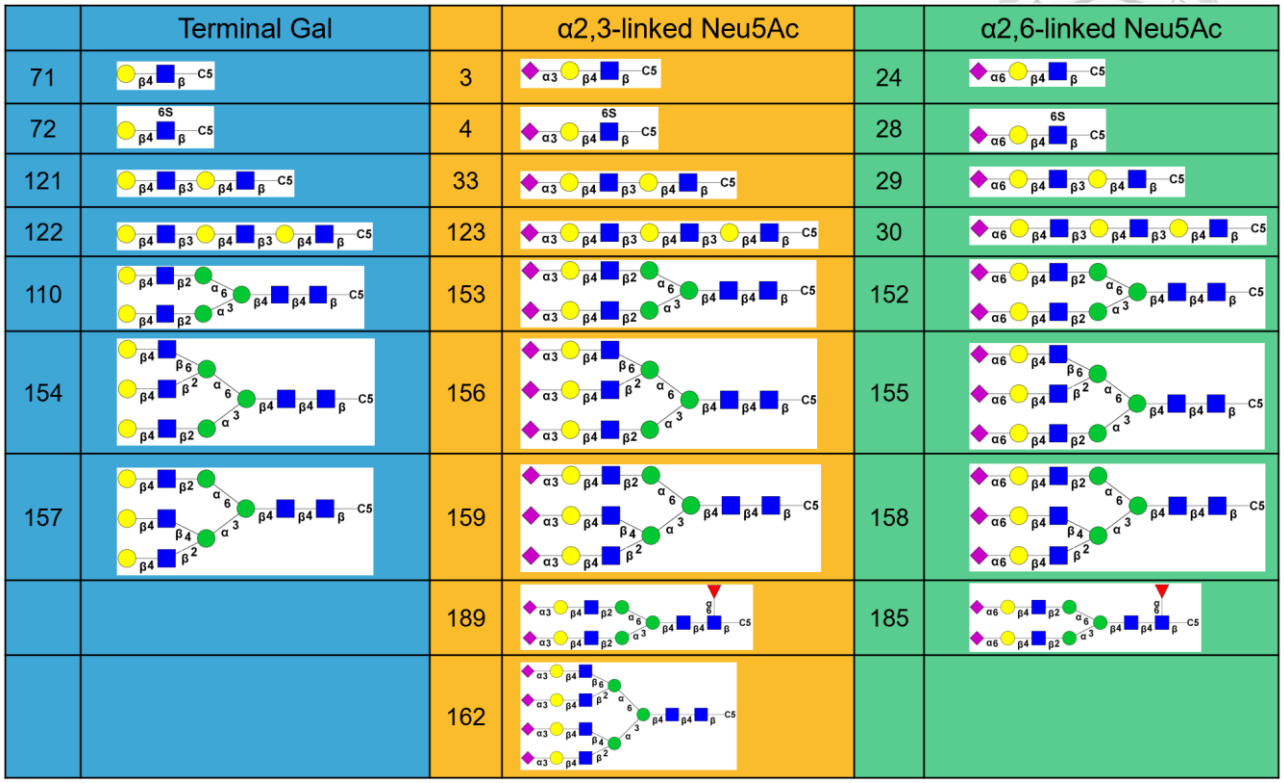

Figure 3. Key glycans in HA protein studies.

doi:10.6342/NTU202403230

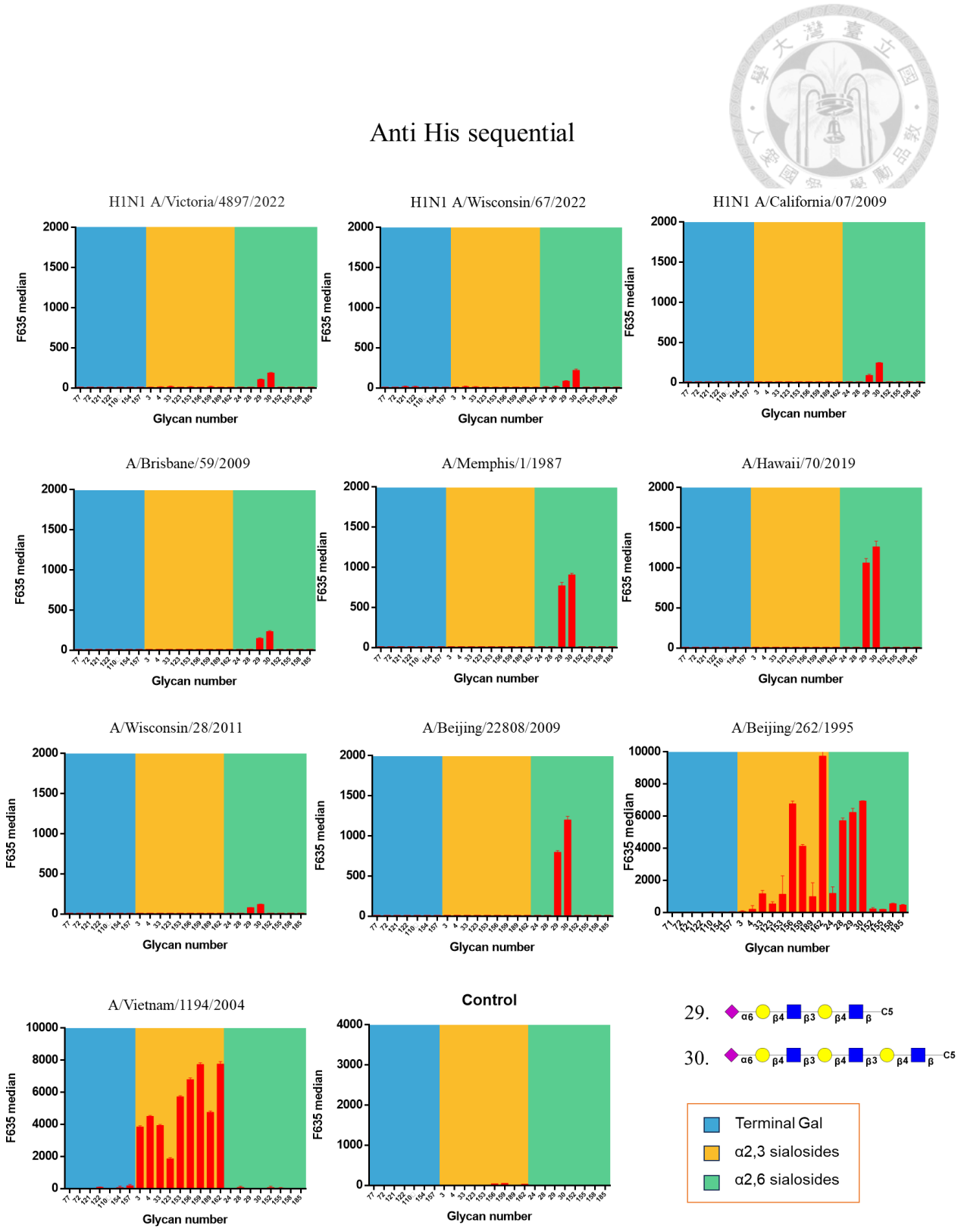

Figure 4. Sequential glycan microarray analysis of HA proteins.Results obtained from glycan microarray experiments using sequential methods. In the control group, only Alexa Fluor® 647 anti-His Tag Antibody was added.

doi:10.6342/NTU202403230

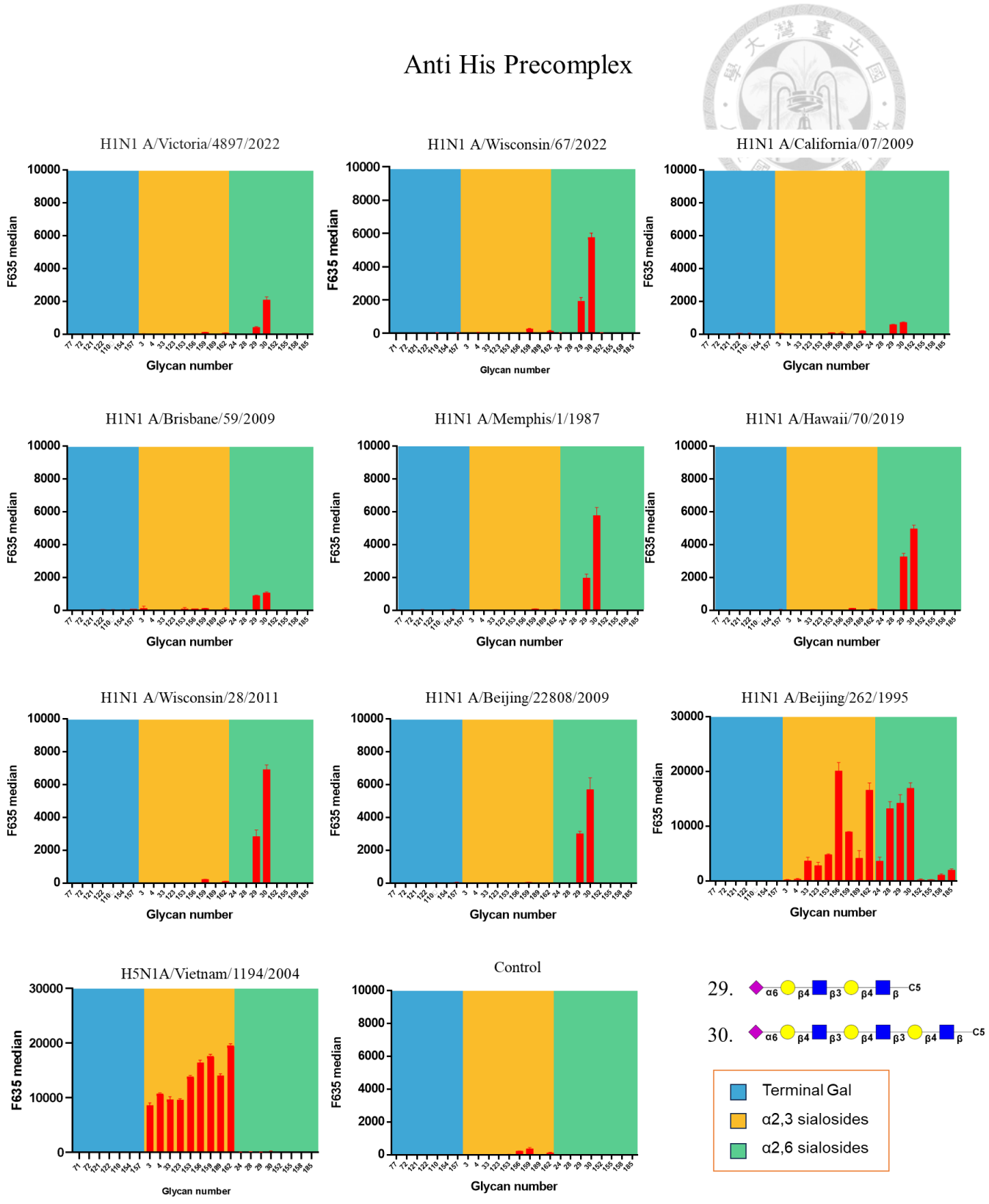

Figure 5. Precomplexation glycan microarray analysis of HA protein.

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Results obtained from glycan microarray experiments using precomplexed HA protein and anti-His Tag Antibody methods

doi:10.6342/NTU202403230

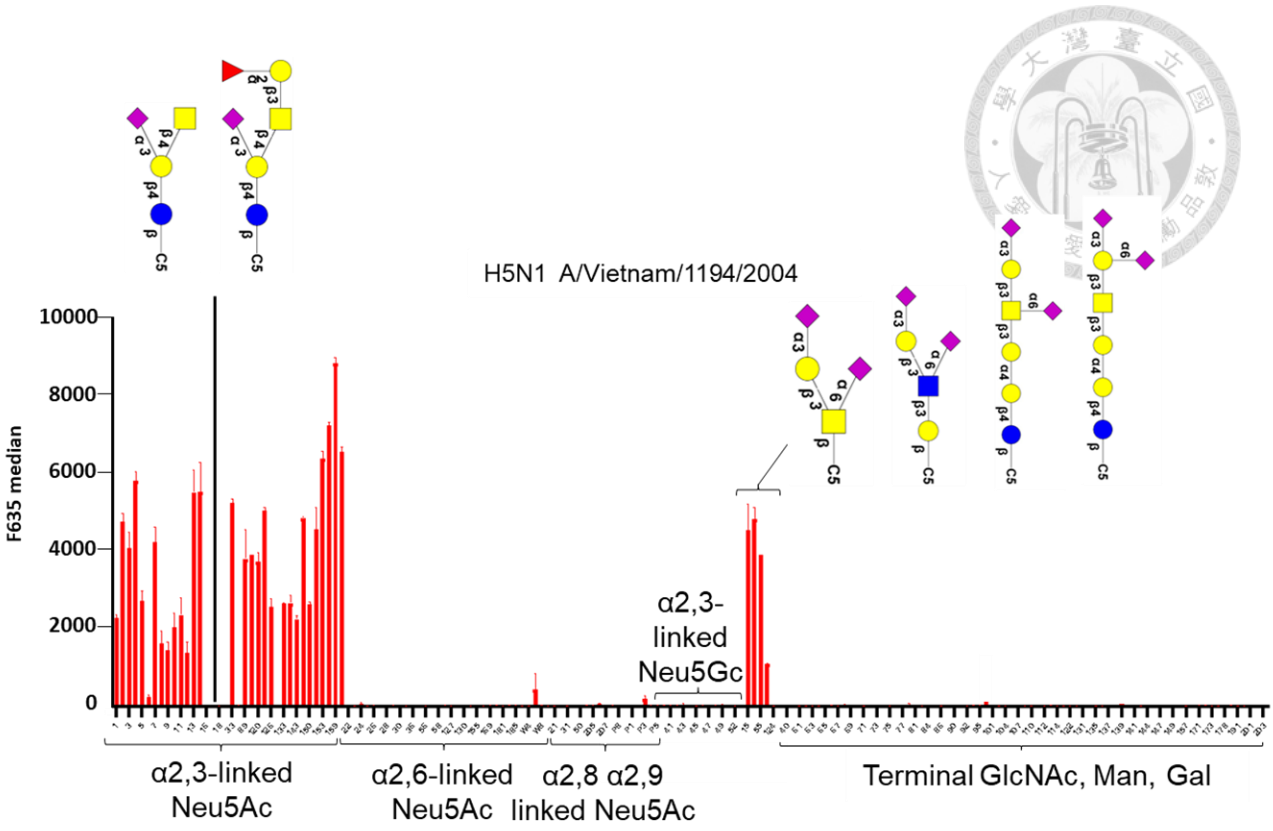


Figure 6. Full analysis of H5N1 A/Vietnam/1194/2004 on glycan microarray.

doi:10.6342/NTU202403230

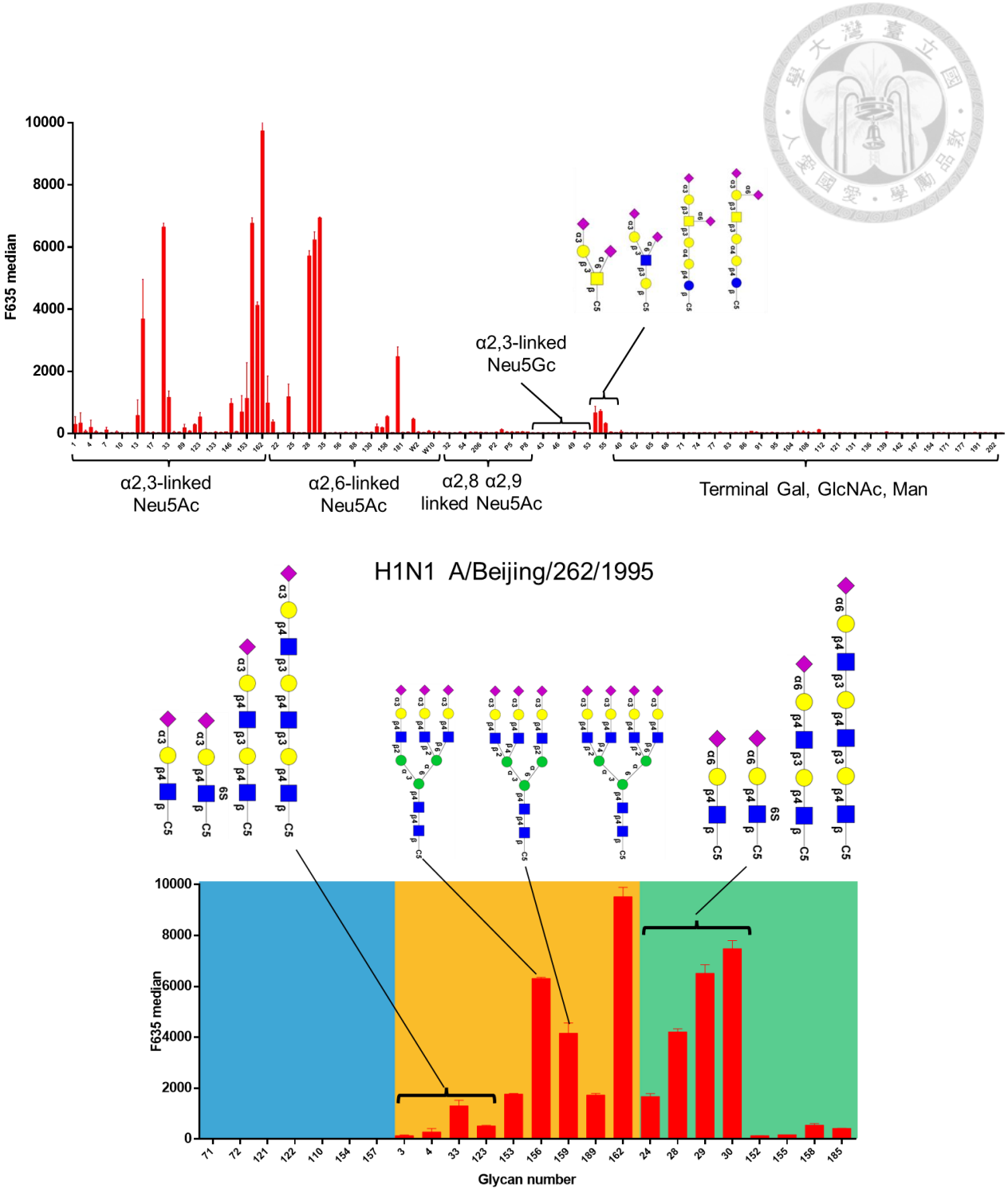


Figure 7. Full analysis of H1N1 A/Beijing/262/1995 on glycan microarray.

doi:10.6342/NTU202403230

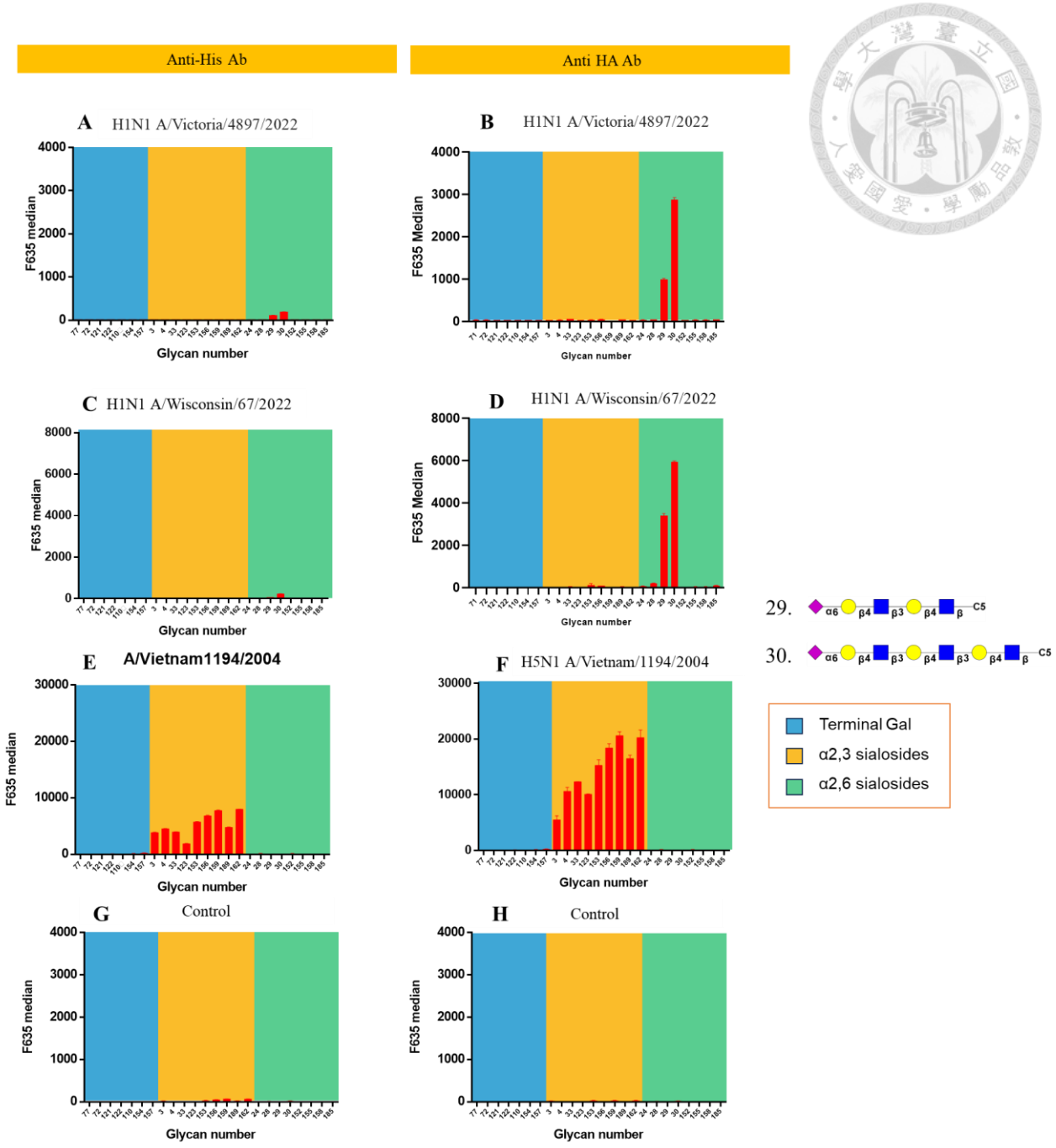


Figure 8. Sequential glycan microarray analysis of HA protein with anti-His and anti HA antibody.

doi:10.6342/NTU202403230

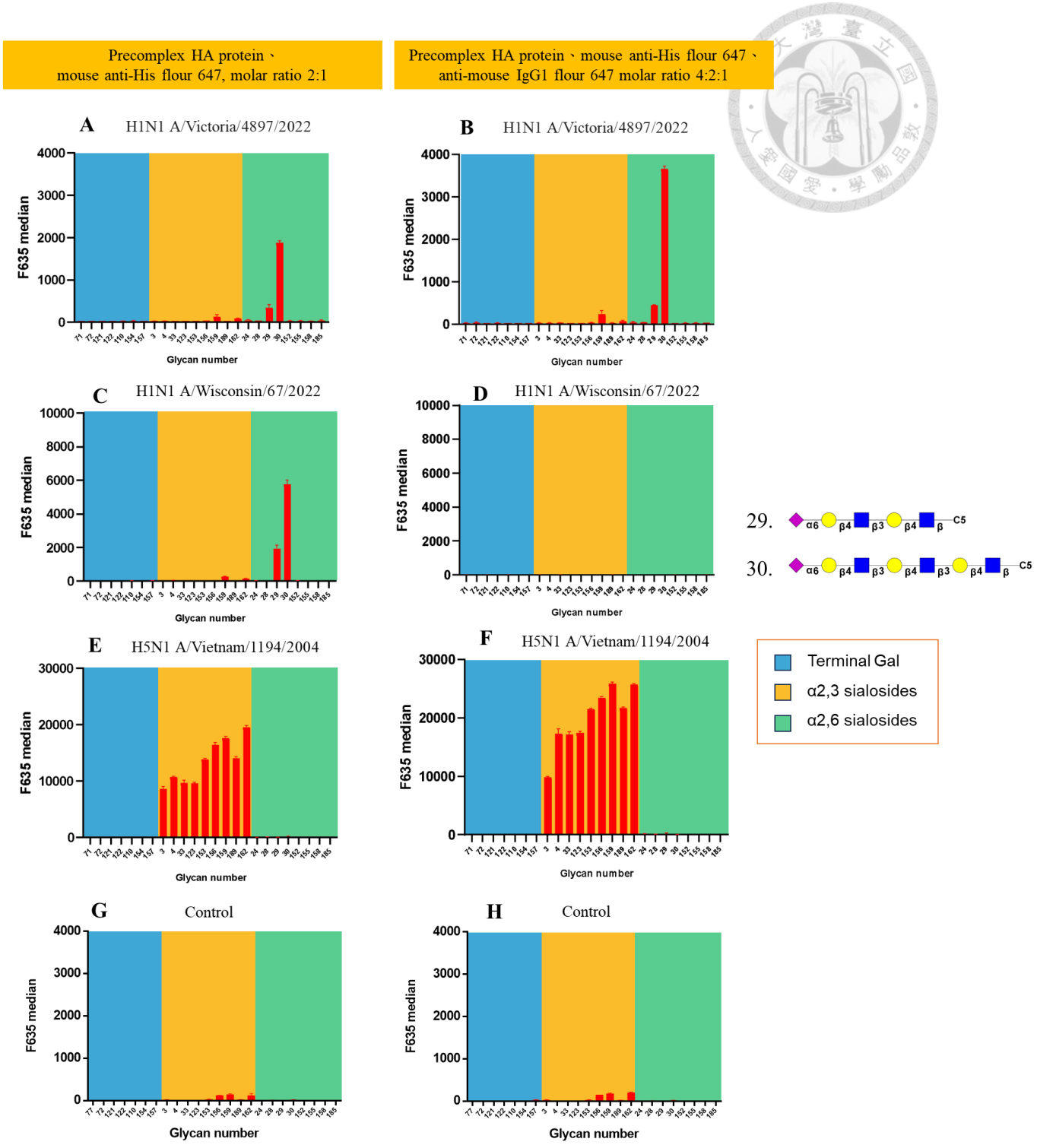


Figure 9. Precomplexation glycan microarray analysis of HA protein with additional fluorescent secondary antibody.

doi:10.6342/NTU202403230

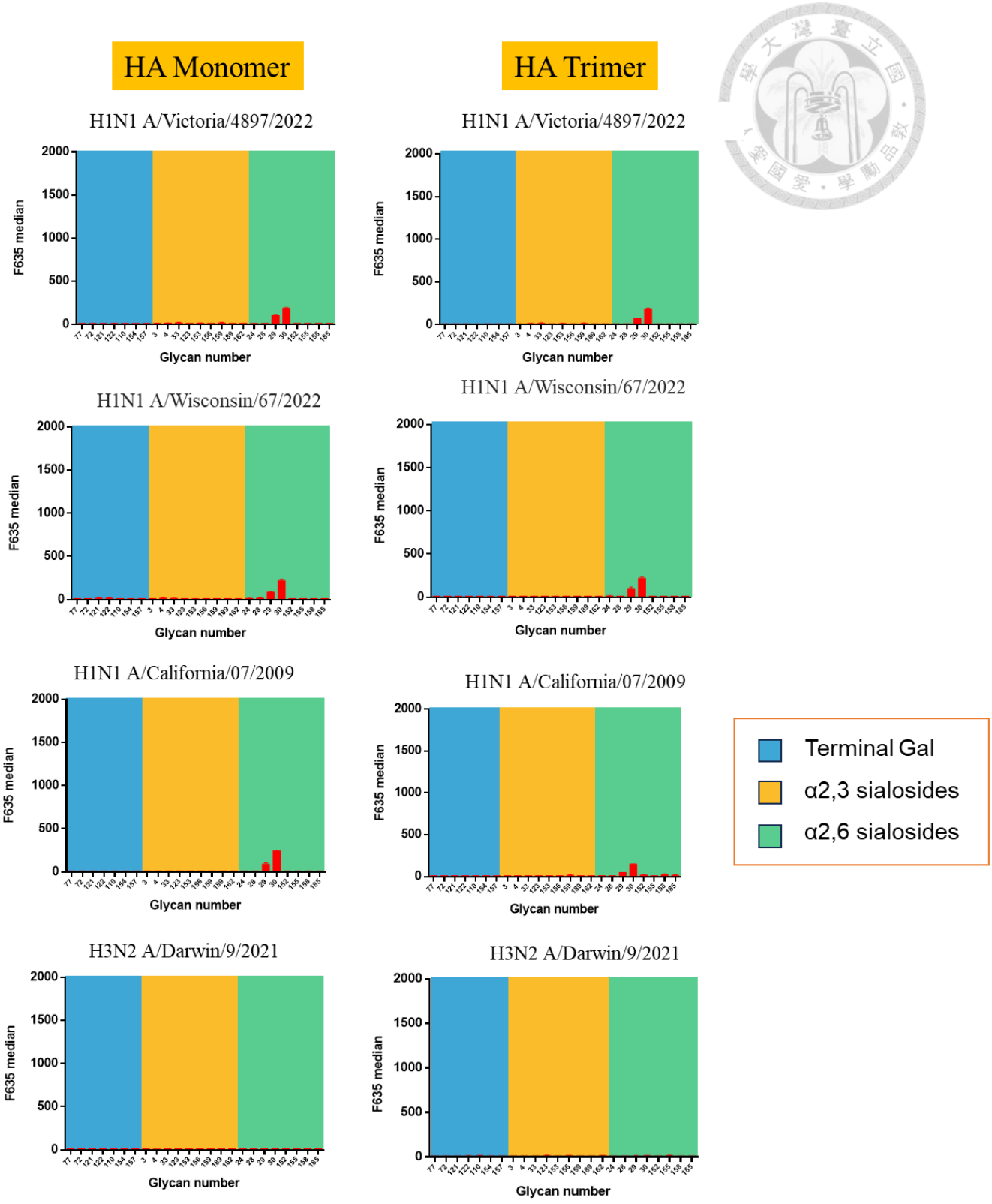


Figure 10. Sequential glycan microarray analysis of HA monomer and trimer.

doi:10.6342/NTU202403230

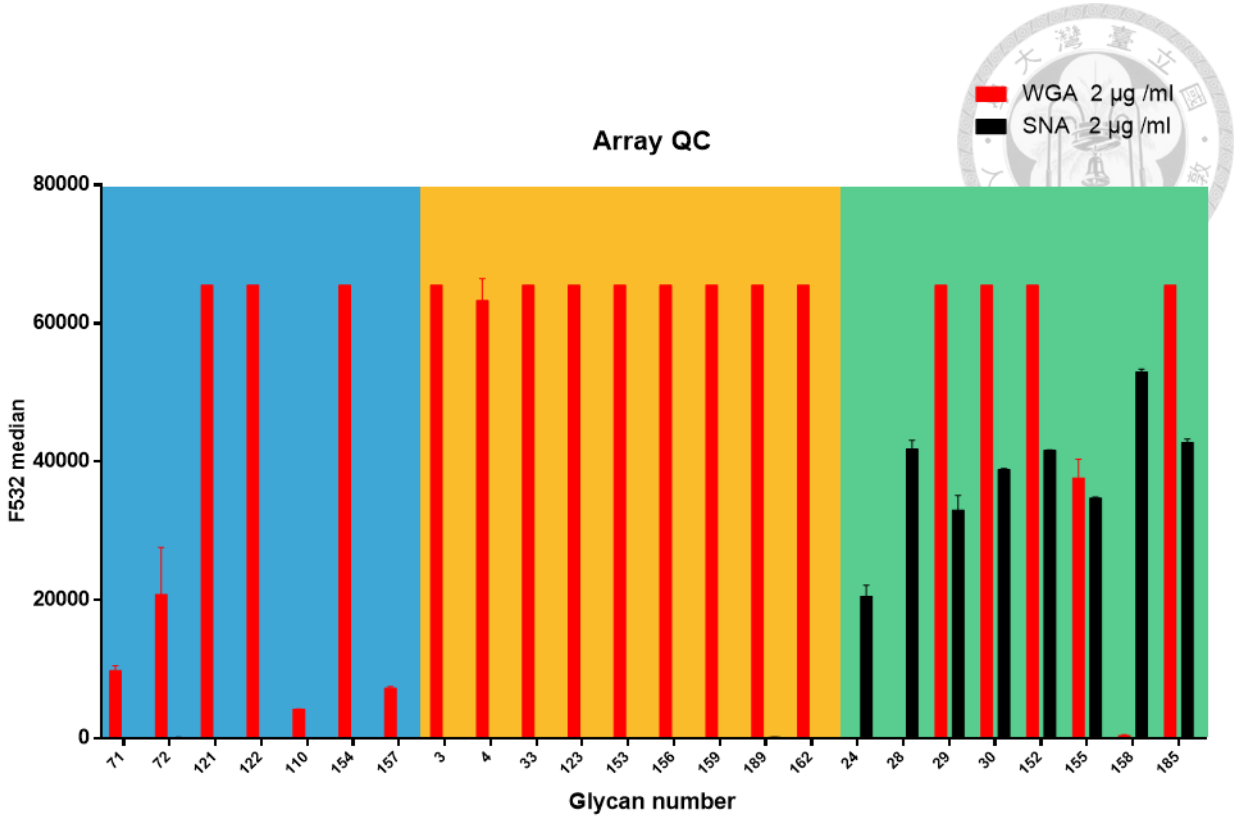


Figure 11. Array quality control with WGA and SNA.

doi:10.6342/NTU202403230

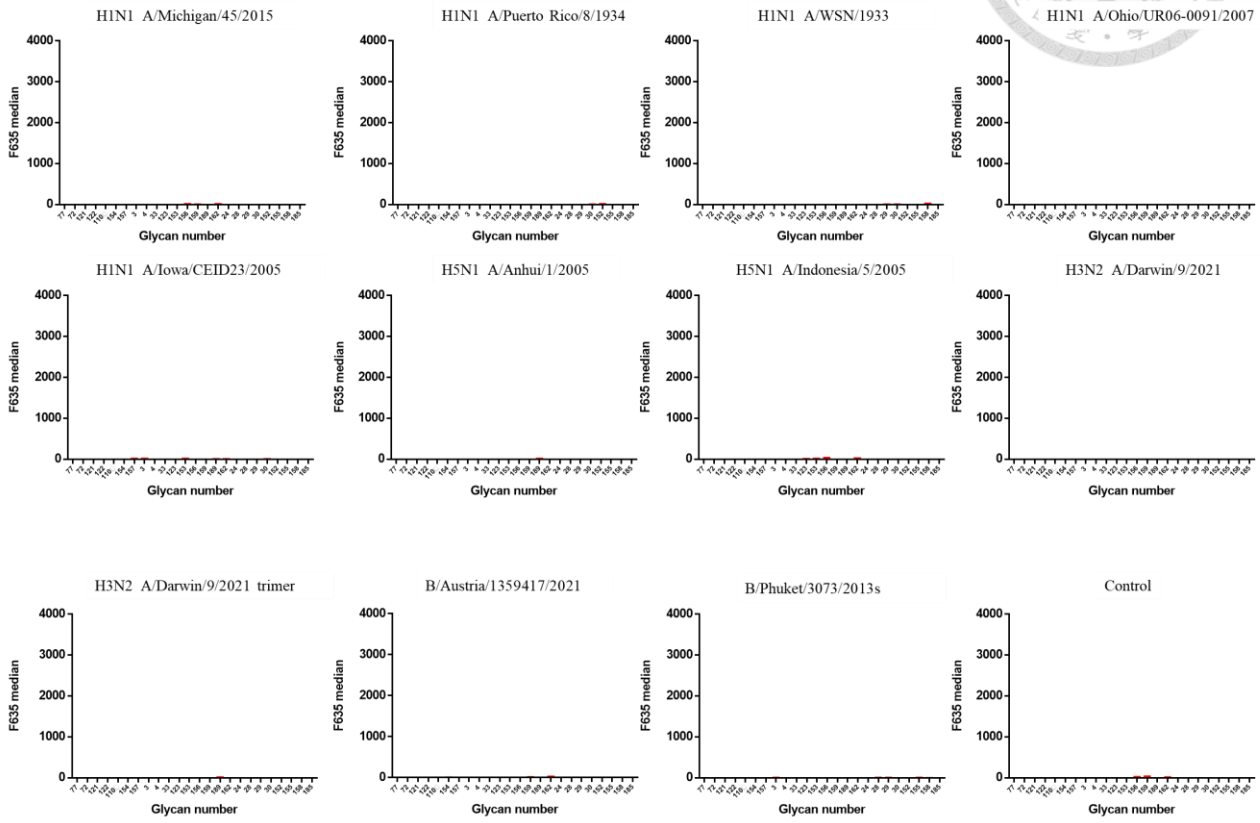
6. Table

HA Strain/Subtype	Hemagglutination Activity	Binding pattern	HA Strain/Subtype	Hemagglutination Activity	Binding pattern	HA Strain/Subtype	Hemagglutination Activity	Binding pattern
H1N1 A/Michigan/45/2015	X	No Binding	H1N1 A/California/07/2009	Low	Poly-LacNAc with terminal α2,6-linked Neu5Ac (No.29 and 30)	H1N1 A/Beijing/262/1995	High	α2,3-linked α2,6-linked Neu5Ac
H1N1 A/Puerto Rico/8/1934	X		H1N1 A/California/07/2009 (Trimer)	X				
H1N1 A/WSN/1933	X		H1N1 A/Victoria/67/2022	X		H5N1 A/Vietnam/1194/2004	High	α2,3-linked Neu5Ac
H1N1 A/Ohio/UR06-0091/2007	X		H1N1 A/Victoria/67/2022 (Trimer)	X				
H1N1 A/Iowa/CEID23/2005	X		H1N1 A/Wisconsin/67/2022	X				
H5N1 A/Anhui/1/2005	X		H1N1 A/Wisconsin/67/2022 (Trimer)	X				
H5N1 A/Indonesia/5/2005	X		H1N1 A/Brisbane/59/2009	Low				
H3N2 A/Darwin/9/2021	Medium		H1N1 A/Beijing/22808/2009	Low				
H3N2 A/Darwin/9/2021 (Trimer)	Medium		H1N1 A/Memphis/1/1987	X				
B/Austria/1359417/2021	X		H1N1 A/Hawaii/70/2019	High				
B/Phuket/3073/2013	X		H1N1 A/Wisconsin/28/2011	X				

Table 1. Summary of HA Protein Binding Specificity

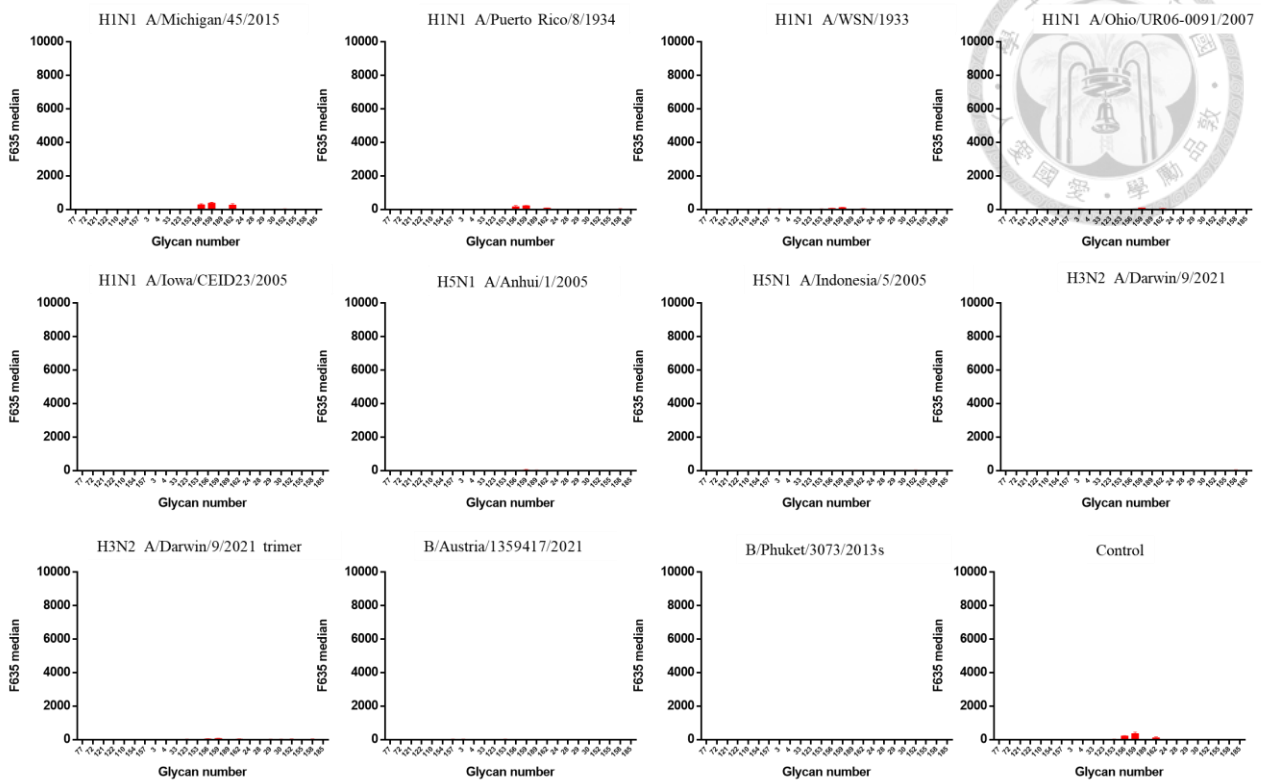
doi:10.6342/NTU202403230

7. Appendices



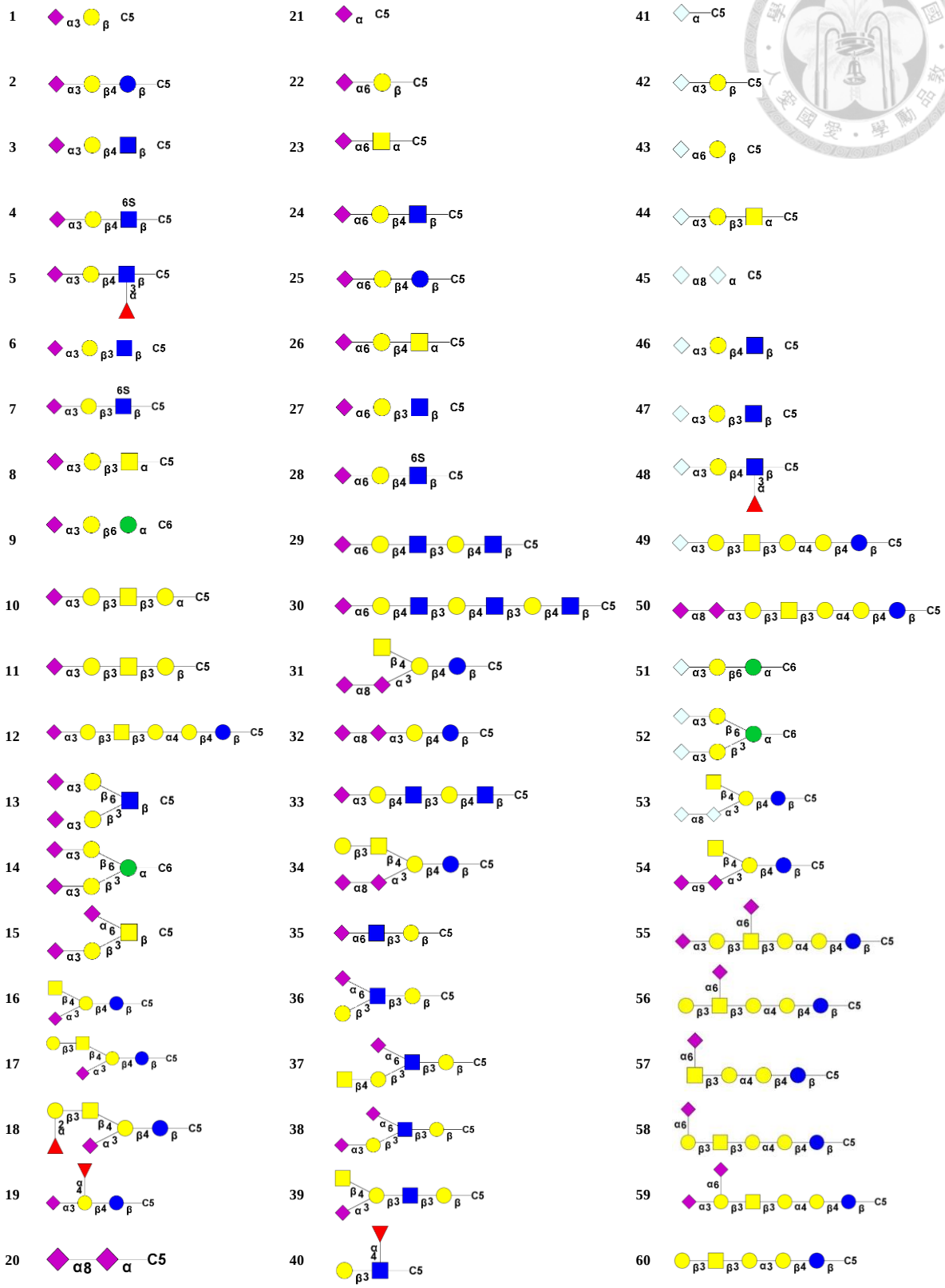
Appendix A. HA protein sequential results on glycan microarray with no binding specificity.

doi:10.6342/NTU202403230



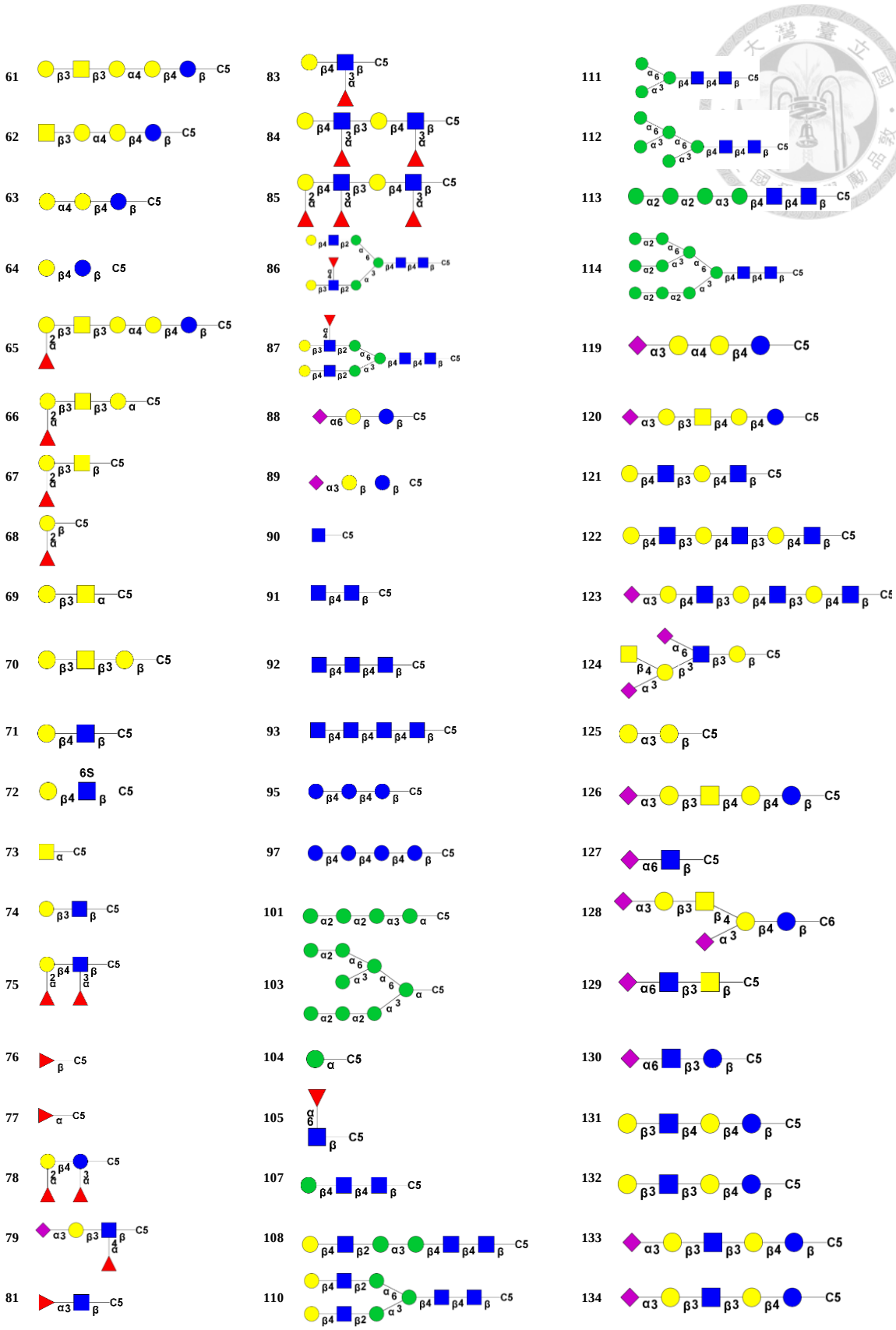
Appendix B. HA protein precomplexation results on glycan microarray with no binding specificity.

doi:10.6342/NTU202403230



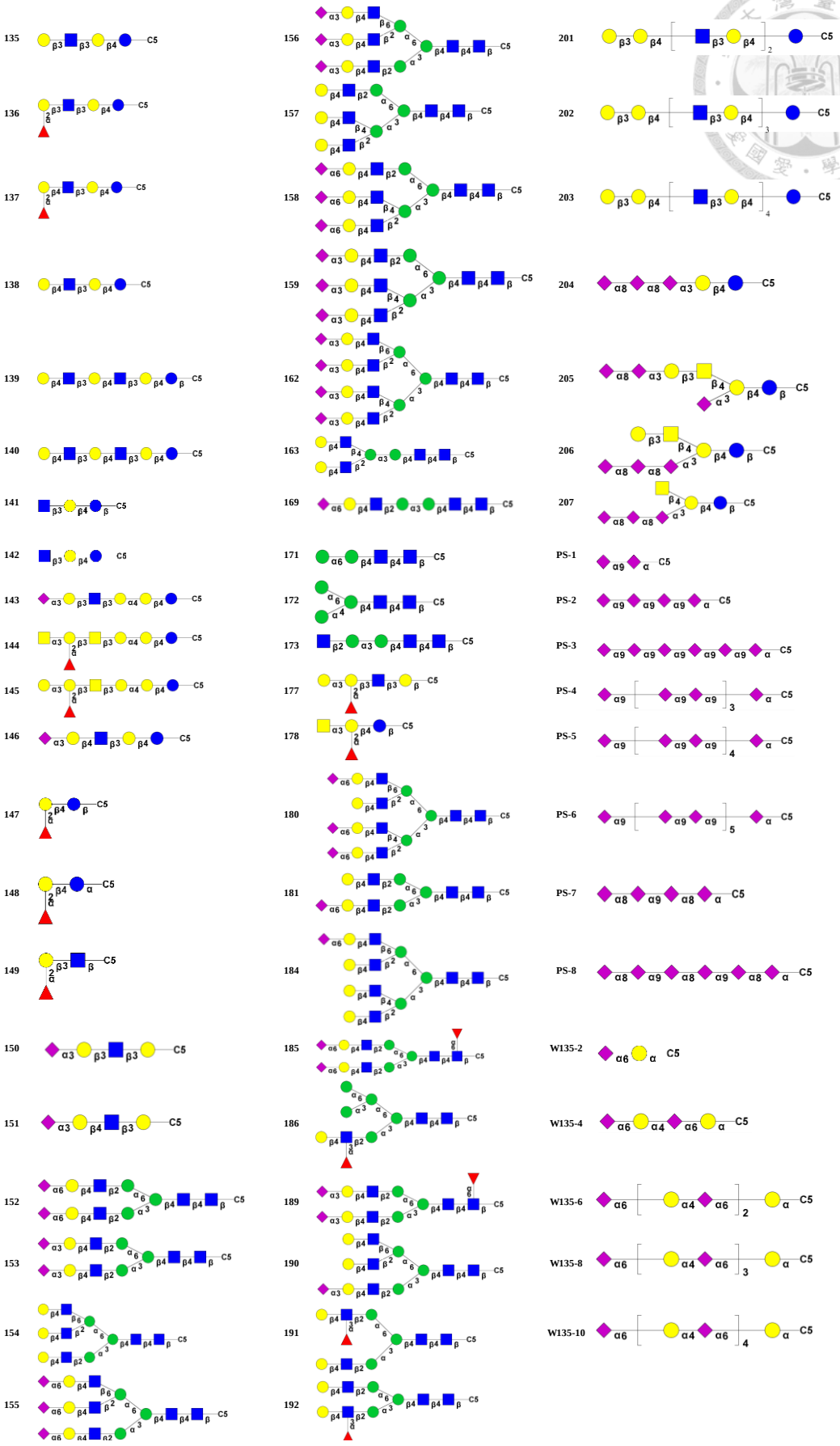
Appendix C continued

doi:10.6342/NTU202403230



Appendice C continued

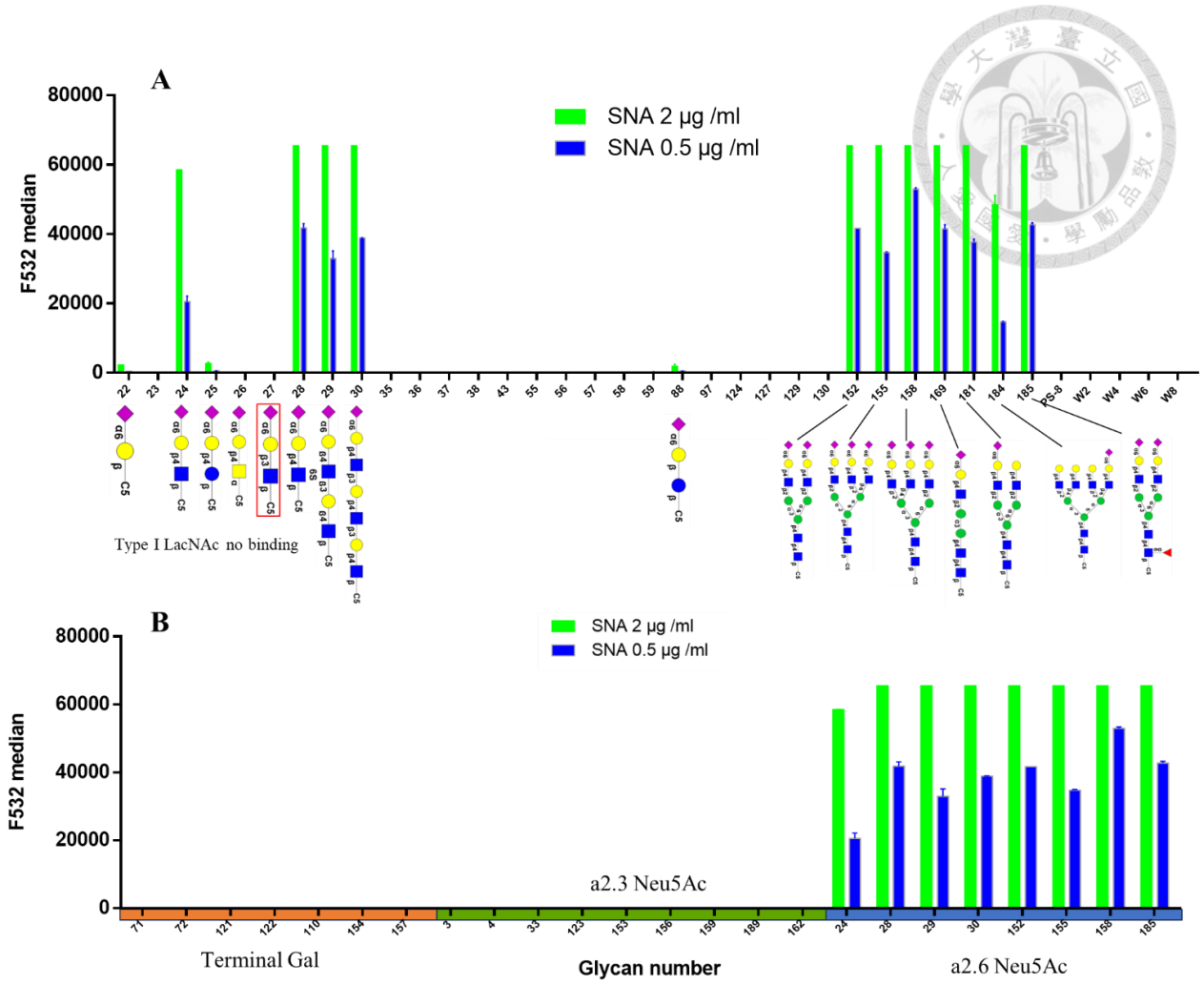
doi:10.6342/NTU202403230



doi:10.6342/NTU202403230

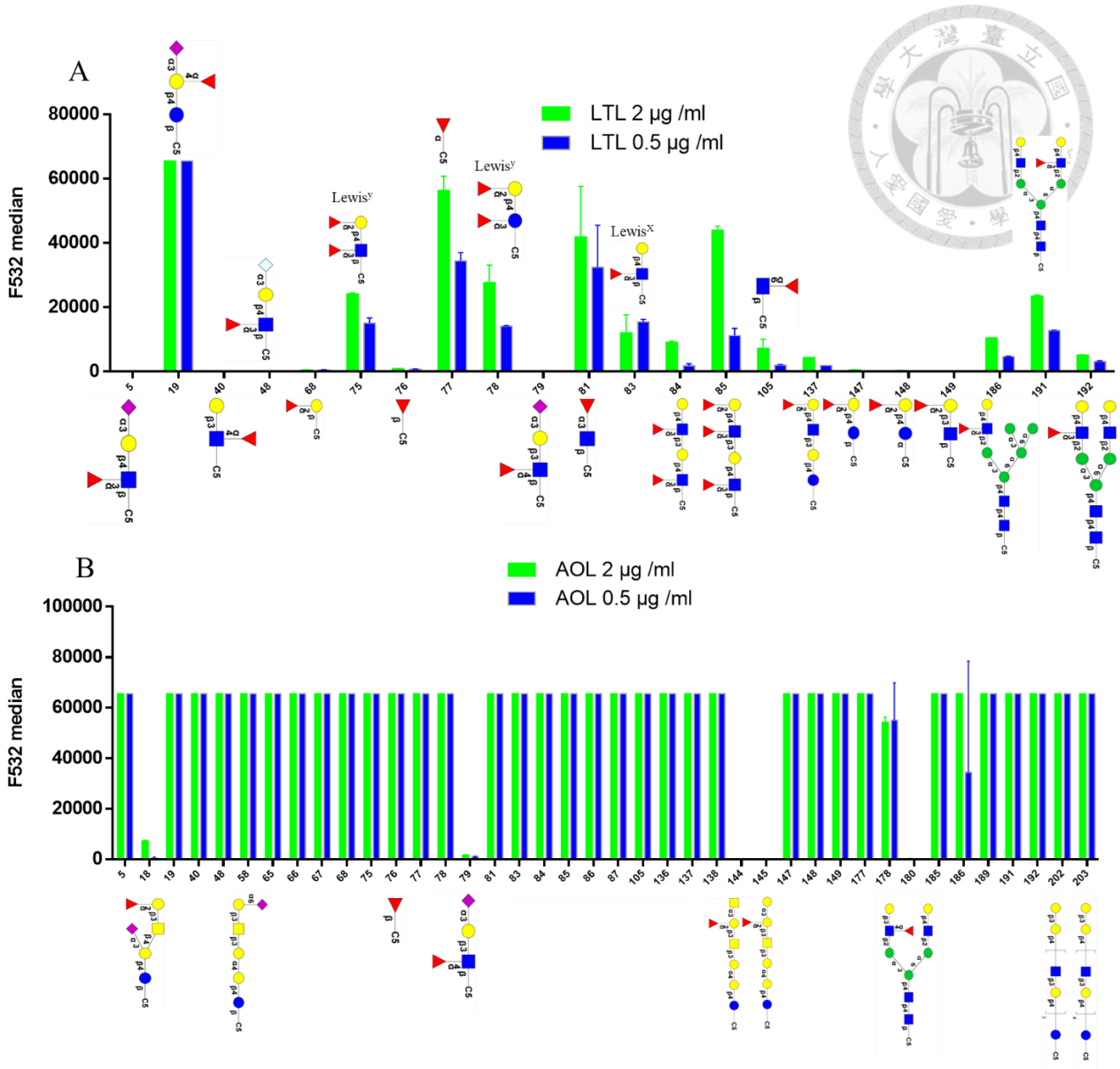
Appendice C. Complete set of glycans used in the glycan microarray for this study

doi:10.6342/NTU202403230



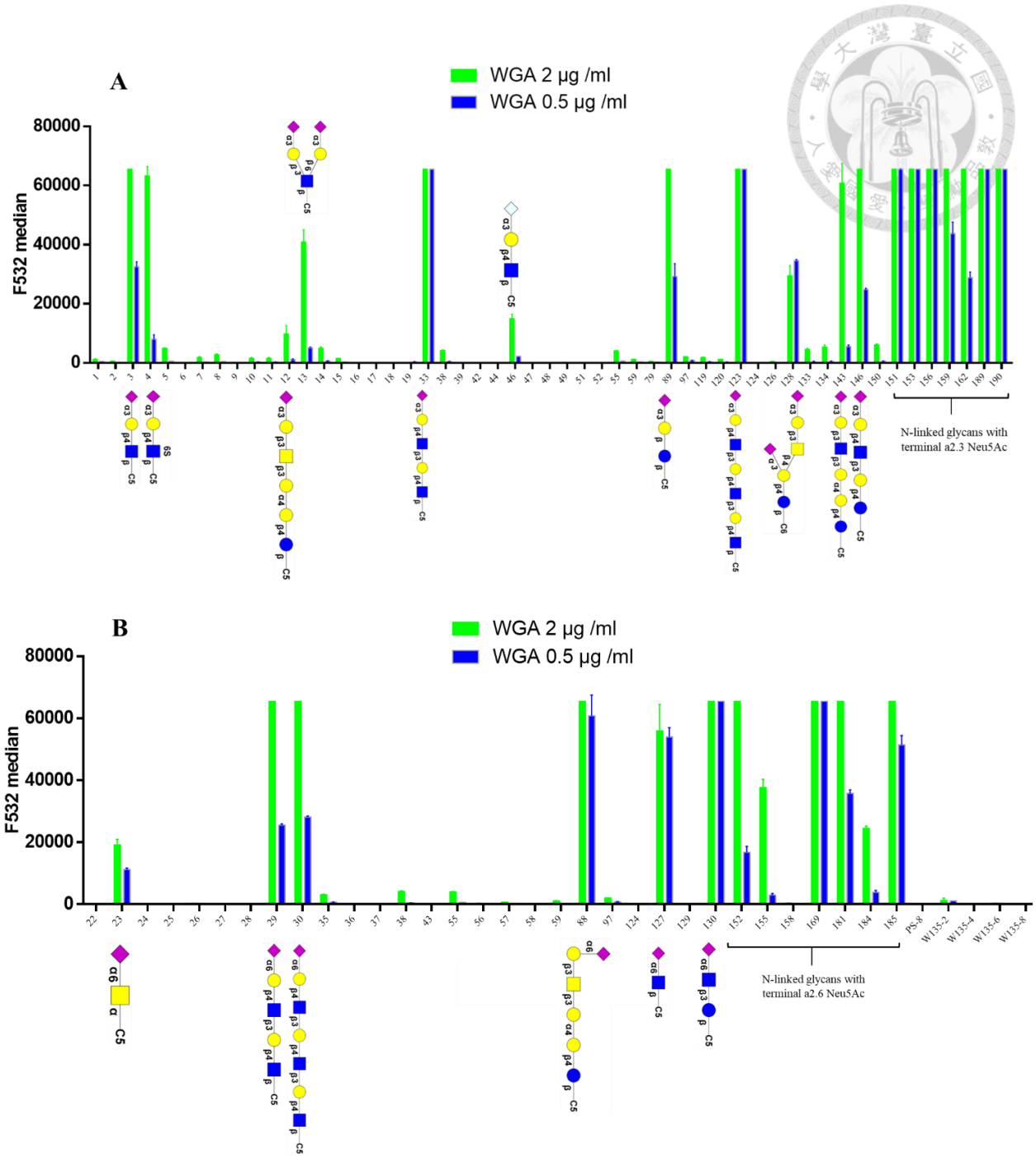
Appendice D. Glycan microarray analysis of SNA. (A) Binding specificity of SNA to α2,6-sialylated glycans identified by glycan microarray (B) Binding specificity of SNA to key glycans in HA protein study.

doi:10.6342/NTU202403230



Appendice E. Glycan microarray analysis of LTL and AOL. (A) Binding specificity of LTL to fucosylated glycans identified by glycan microarray (B) Binding specificity of SNA to key glycans in HA protein study.

doi:10.6342/NTU202403230



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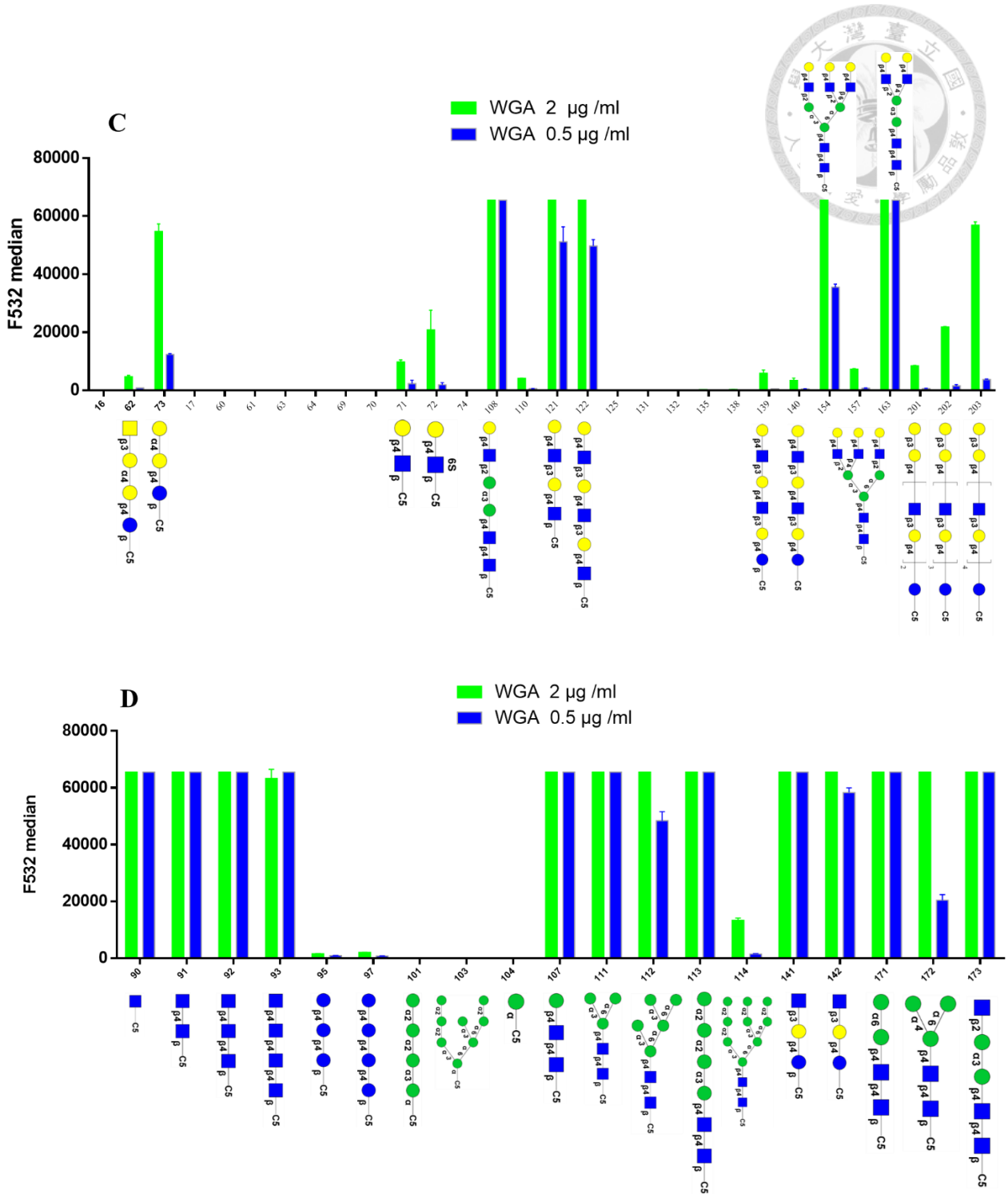
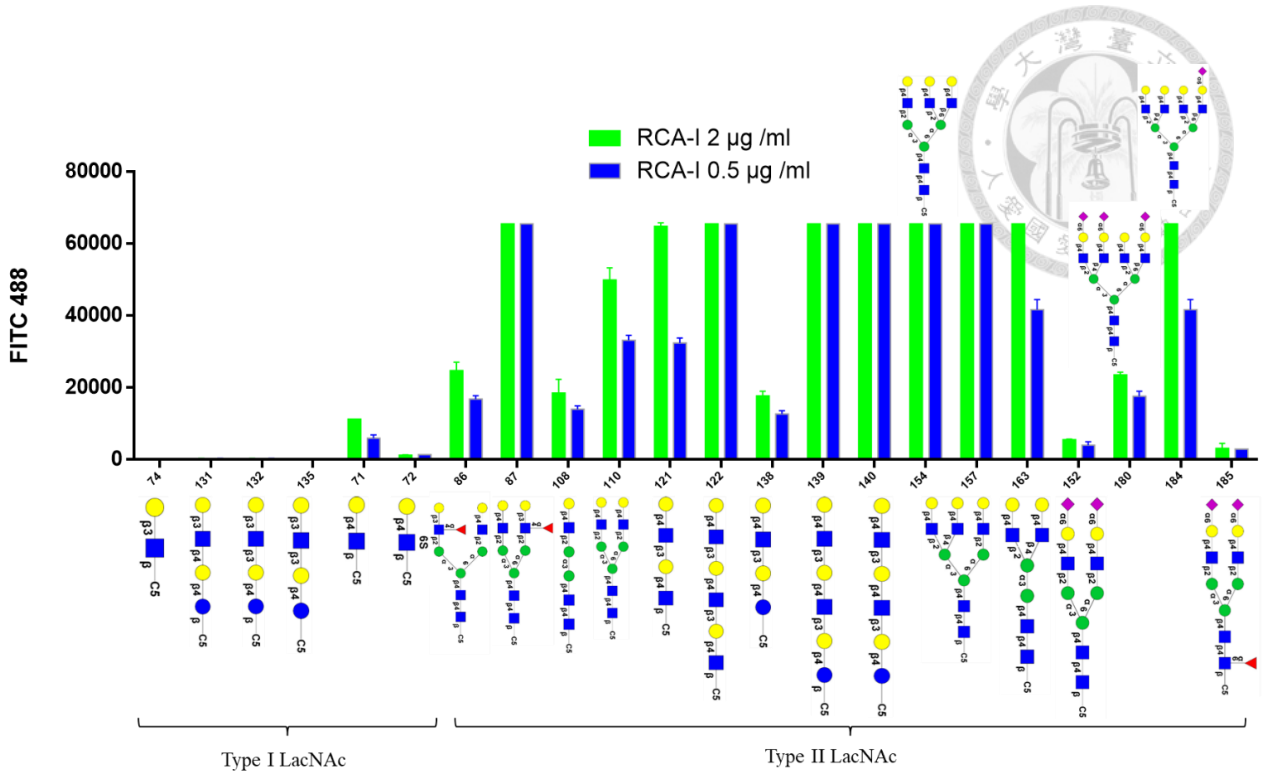


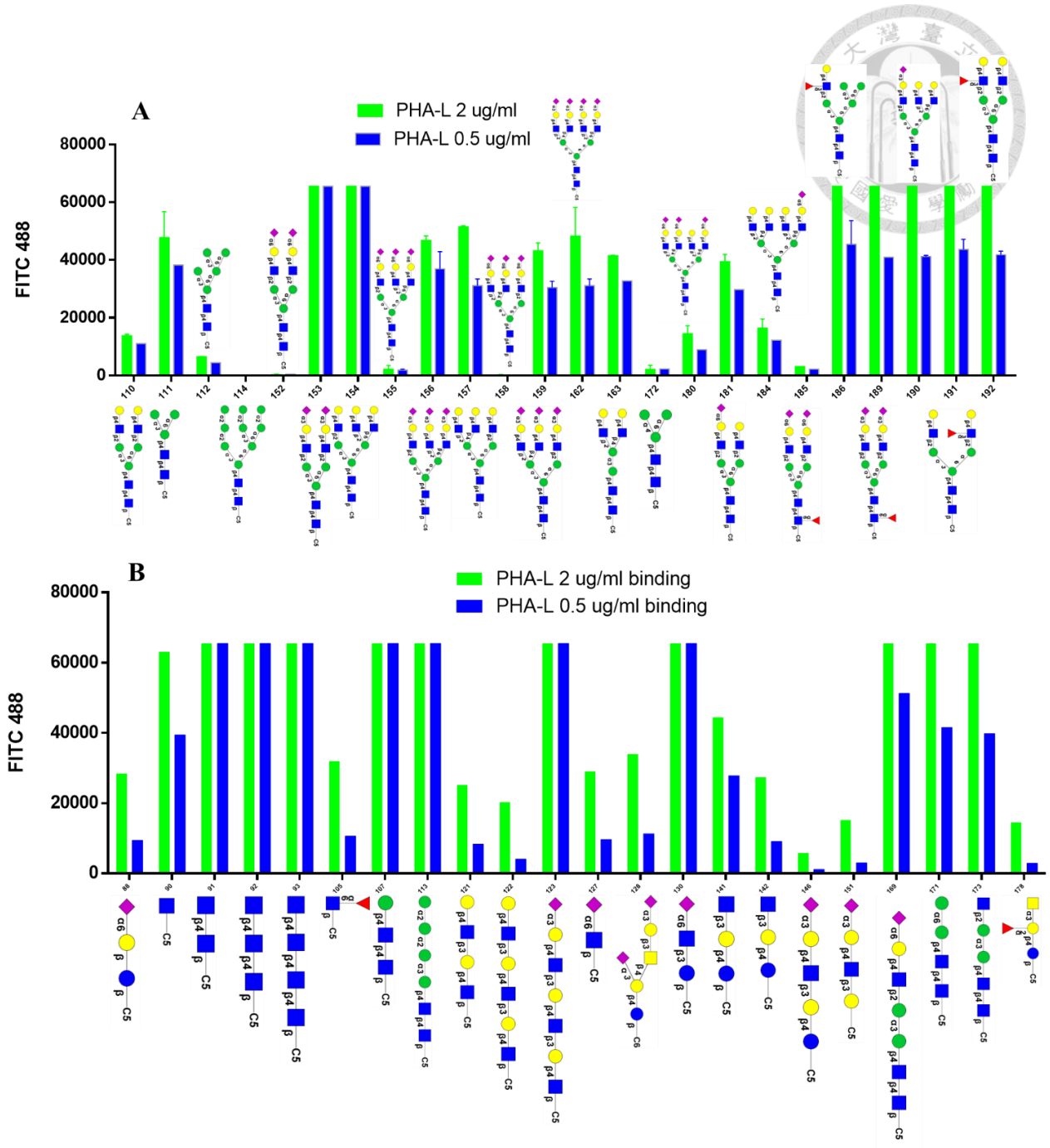
Appendice F. Glycan microarray analysis of WGA. (A) and (B) Binding specificity of WGA to terminal a2,3 and a2,6 sialylated glycans(C) Binding specificity of WGA to terminal Gal glycans (D) Binding specificity of WGA to terminal GlcNAc and mannose glycans.

doi:10.6342/NTU202403230



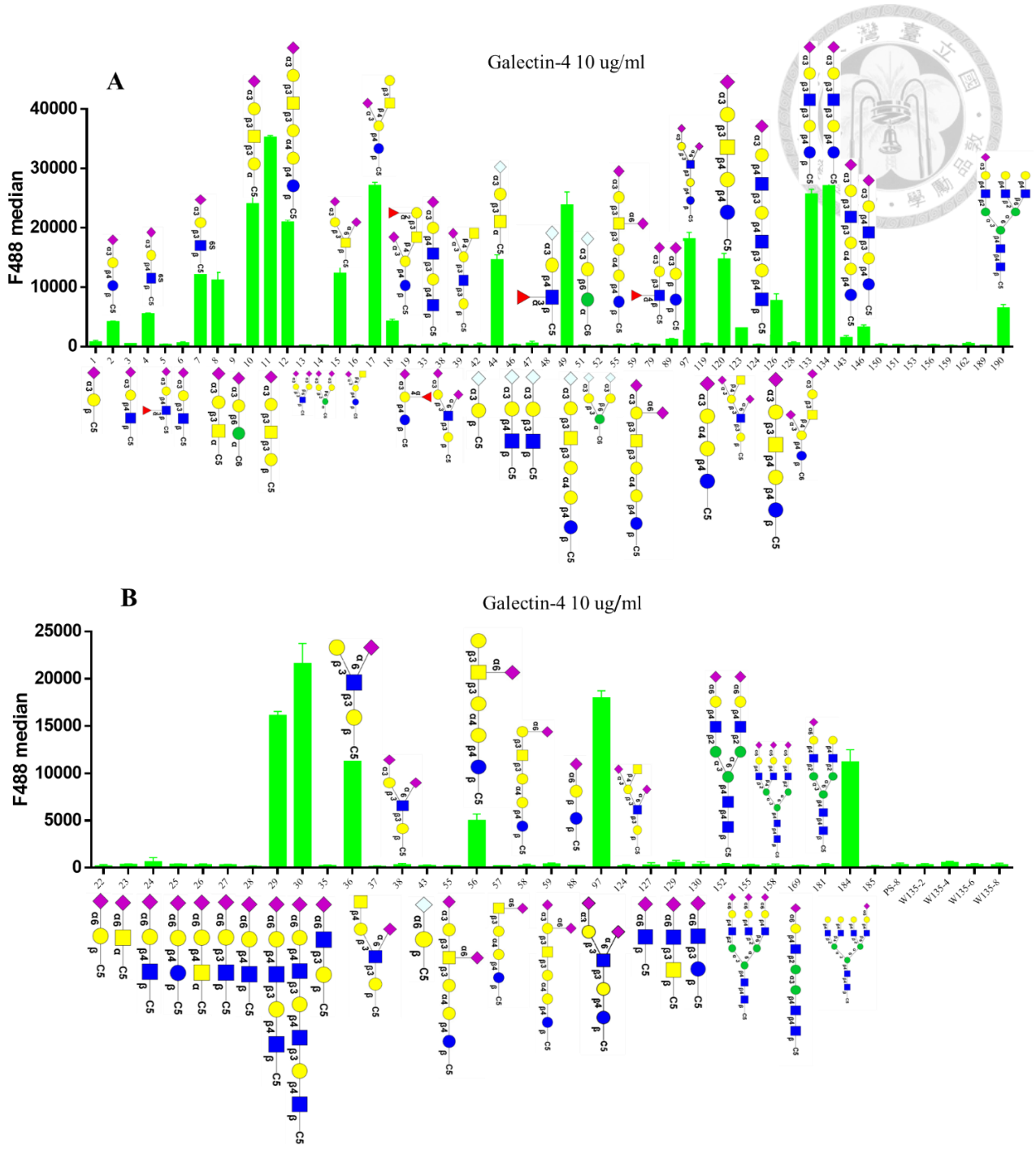
Appendice G. Glycan microarray analysis of RCA-I. Binding specificity of RCA-I to glycans with terminal LacNAc.

doi:10.6342/NTU202403230



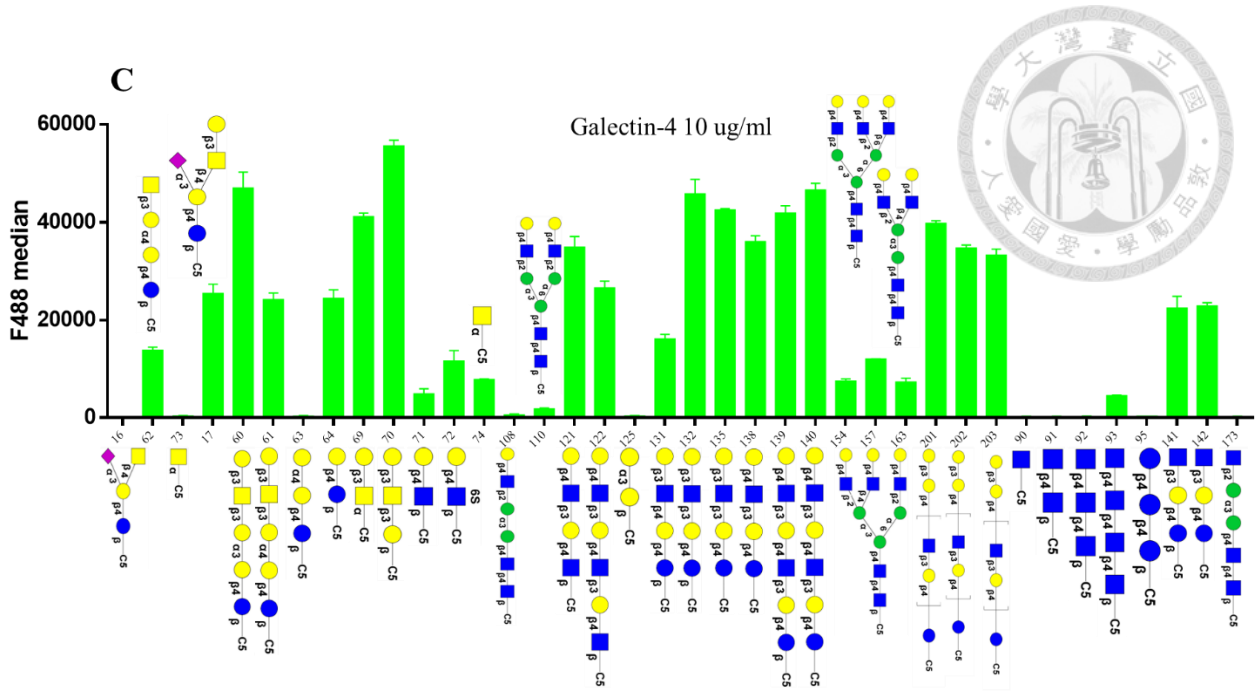
Appendice H. Glycan microarray analysis of PHA-L. (A) Binding specificity of PHA-L to N-linked glycans. (B) Other bindings of PHA-L on glycan microarray.

doi:10.6342/NTU202403230



Appendice I. continued.

doi:10.6342/NTU202403230

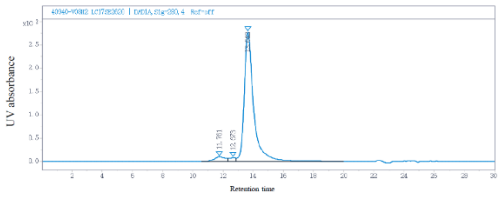


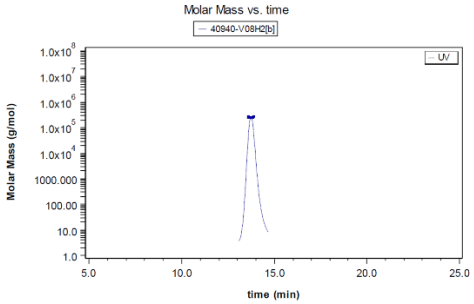
Appendice I. Glycan microarray analysis of Galectin-4. (A) and (B) Binding specificity of Galectin-4 to terminal a2,3 and a2,6 sialylated glycans(C) Binding specificity of Galectin-4 to terminal Gal and GlcNAc glycans.

doi:10.6342/NTU202403230

Influenza A H1N1 (A/Wisconsin/67/2022) Hemagglutinin / HA Trimer Protein

SEC-MALS





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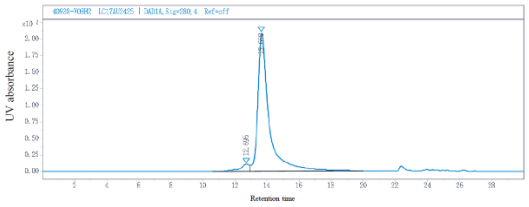
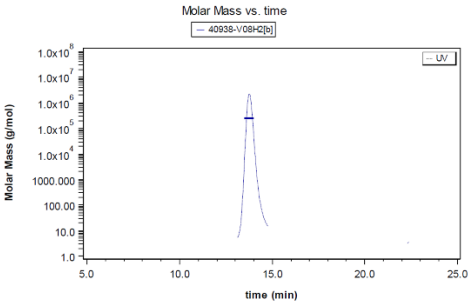
1	11.761	503.73	3.952	9.798	0.98196	1.753	DAD1A
2	12.673	244.177	1.916	7.888	1.91314	0.559	DAD1A
3	13.642	11997.586	94.132	277.716	0.59871	7.135	DAD1A

Influenza A H1N1 (A/Victoria/4897/2022) Hemagglutinin / HA Trimer Protein

SEC-MALS

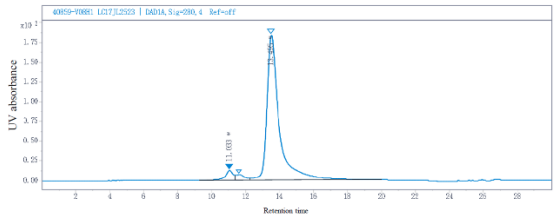


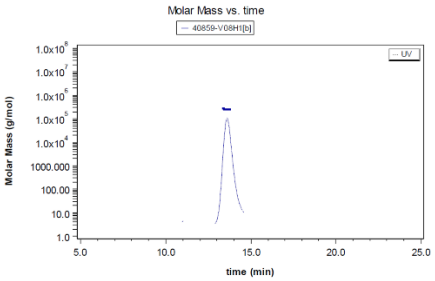


#	RT (min)	(mAU·s)	Peak area %	Peak height (mAU)	Symmetry	Peak width (min)	Signal
1	12.695	497.675	4.704	10.922	2.38443	2.351	DAD1A
2	13.658	10081.516	95.296	208.566	0.48498	7.053	DAD1A

Influenza A H3N2 (A/Darwin/9/2021) Hemagglutinin / HA trimer Protein (His Tag)

SEC-MALS





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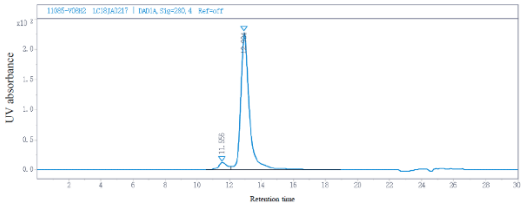
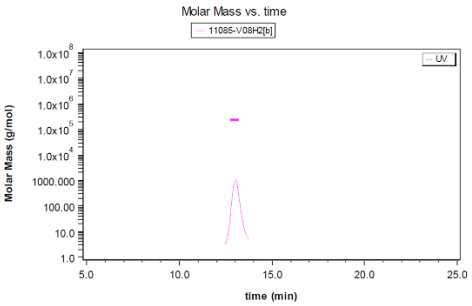
2	11.592	269.654	2.681	7.397	0.43646	0.88	DAD1A
3	13.495	9358.082	93.03	184.243	0.51308	7.752	DAD1A

Influenza A H1N1 (A/California/07/2009) Hemagglutinin / HA trimer Protein (His Tag)

SEC-MALS





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2	12.924	8604.567	94.567	228.651	0.64275	6.817	DAD1A

Appendice J. SEC-MAL data for HA trimer. Acessed from Sino Biological.

doi:10.6342/NTU202403230