Engineered Platforms for Molecular Characterization of Human ABO Blood Type Antigens

Vaishnavi Dhawan

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Reading Committee:

Daniel M. Ratner, Chair

Miklos Guttman

Jill M. Johnsen

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Abstract

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Vaishnavi Dhawan

Chair of the Supervisory Committee:
Associate Professor Dr Daniel M. Ratner
Bioengineering

The most prevalent typing procedures are based on the overly simplified characterization of erythrocyte membrane antigens into the commonly known ABO blood group system. Numerous subgroups of ABO have been described that reflect natural heterogeneity within the system. These variations cannot be accurately detected by current typing methods that rely on agglutination techniques. There is a need to deepen our understanding and characterize the natural variations of these antigens with implications in transfusion and transplant medicine. This collaborative project focuses on characterizing the ABO antigens on a genetic, phenotypic and structural level. To facilitate this process, the carbohydrate antigens are isolated via enzymatic methods and their reactivity is studied through biochemical qualitative and quantitative assays.

Released glycans will be structurally analyzed using LC-MS and derivatized on silicon photonics platform for the development of more accurate blood typing diagnostics.

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Chapter 1. INTRODUCTION

1.1 BRIEF INTRODUCTION TO ABO BLOOD GROUP SYSTEM & TRANSFUSION MEDICINE

Every 2 seconds someone in the United States needs blood, making blood transfusion one of the most frequently performed clinical procedures[1]. There are nearly 21 million blood components transfused every year during supportive care for cardiovascular surgery, transplant surgery, massive trauma, solid and hematological malignancy complications etc[1,2]. Prior to transfusion, blood typing is performed to categorize the donor and the recipient into the commonly known ABO blood group system. There are two ways to define an individual's blood type. The forward type is determined by the presence of carbohydrate antigens on the erythrocyte or red blood cell (RBC) membranes, while the reverse type is defined by the presence of antibodies in the plasma specific for foreign antigens. Interaction between transfused RBCs that are ABO incompatible and the recipient's immune system is one of the most serious complications of blood transfusion. Circulating antibodies in plasma recognize donor RBCs as foreign and initiate a hemolytic transfusion reaction (HTR) which causes the donor RBCs to lyse due to immune processes[3]. Acute HTRs (AHTRs) can occur within minutes of transfusions and in the most severe forms can even lead to organ failure and death[4]. Even though, the prevalence of AHTRs is established to be low, it is widely known to be underrepresented in reporting[3]. Patients receiving ABO-incompatible plasma transfusion can also be at risk for HTRs[5,6].

1.2 HISTORY OF ABO BLOOD GROUP SYSTEM

The ABO blood type system was first discovered by Karl Landsteiner at the beginning of the 20th century. One of his key observations was that serum from an individual did not agglutinate

with their own red cells but did agglutinate when mixed with red cells from certain other individuals. This simple yet profound observation introduced the concept of compatibility testing and led to the discovery of human blood type groups A, B and O. Based on this discovery, the first successful blood transfusion was performed by Dr. Reuben Ottenberg in 1907[7]. He was the first to suggest that group O RBCs could be transfused to recipients regardless of their blood types during medical emergencies, a practice that was widely adopted during World War II[8]. Transfusing RBCs was generally considered safe and resulted in relatively fewer medical complications, however, there were follow-up studies showing exceptions to this pattern for other blood components. High titers of anti-A and anti-B were observed in type O plasma, which resulted in complications upon plasma and platelet transfusions in non-type O recipients[9,10]. These clinical observations suggest the limitations of oversimplifying the ABO system for clinical practice and outline the medical implications for doing so.

To meet the growing need for blood transfusion with the onset of WWII, long-term anticoagulants, blood banking systems and blood collection containers were developed. These advancements contributed to the field of transfusion medicine and pushed for the structural investigation of the ABO blood group system[7].

In 1911, von Dungern found two types of blood group A erythrocytes: A_1 and A_2[11]. Additional rare subgroups with weaker serological reactivity within the ABO blood group system have since been identified and genetically characterized. These ABO subgroups continue to be an active area of research[12].

1.3 History of ABO Genetics

Even before researchers possessed the tools to determine the composition of the ABO blood group antigens, they observed A and B antigens to be inherited co-dominantly over the H

antigen in 1910[13] In 1924, Bernstein and colleagues proposed the "three allele model" which introduced A, B and O as three major alleles at the ABO locus, which was later extended to include the four alleles A_1, A_2, B and O[14,15]. In blood type O individuals, the O allele encodes for an inactive transferase enzyme which produces an H-antigen precursor, while the A and B alleles encode for an active glucosyltransferase producing A and B antigens on the erythrocyte membrane, respectively. These antigens were initially referred to as ABO(H) "substances" until much later when their structural composition was determined. In 1932, Sasaki and Schiff identified a "secretor" gene which was considered responsible for "secretor" individuals possessing ABH substances in their saliva and other bodily fluids, in addition to the surface of their RBCs. Contrastingly, the expression of ABH antigens was limited to RBC membranes in "non-secretor" individuals[16,17].

In the absence of methods that allowed for straight-forward isolation of ABH substances on erythrocytes membranes, human secretions were heavily used as sources of ABH-rich substances to study their structural composition [18]. Human semen., ovarian cyst fluids, vaginal fluids, saliva, gastric juice were all investigated and ovarian cyst fluid was found to be the most potent and accessible source of the water-soluble ABH substances[18,19].

1.4 Approaches for Structural Determination

The pioneering work in determining the structural composition of ABH substances was conducted by the Kabat Lab and the Watkins and Morgan group in late 1950-1960s[20]. Kabat's lab utilized fractionation procedures involving precipitation with ethanol from 90% phenol which capitalized on the high content of carbohydrate present in the secretion samples[21]. Secretions from A, B and O blood group individuals were shown to contain the same four sugars (L-fucose, D-galactose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine) and a peptide moiety rich in

serine, threonine and proline [16,22]. The first hint of specificity between the blood type antigens was observed from hemagglutination inhibition experiments performed with simple sugars as controls. These experiments showed that N-acetylgalactosamine (GalNAc) was a dominant sugar in blood type A, galactose (Gal) in blood type B, and fucose (Fuc) in blood type O[23-25]. In addition, fucose α1-2 linked to a galactose was identified as the complete H-antigen serving as the precursor structure, as expressed in type O individuals[24,26,27]. The A and B glucosyltransferase modified this precursor by adding α1-3 linked n-acetylgalactosamine (GalNAc) or Gal linked to the core Gal sugar, respectively. These di- and tri-saccharide motifs on the terminal end of the H, A and B antigens, respectively were termed as the immunodominant sugars[26]. Partial acid hydrolysis and alkaline degradation ("peeling" and "non-peeling") techniques were utilized to further study the structural base of the immunodominant sugars and the carriers anchoring them to the erythrocyte membranes[28,29].

Chemical approaches of delipidating erythrocyte membranes, performing chloroform-methanol followed by or prior to n-butanol extraction emerged as one of the common techniques for attempting the direct study of ABH substances on erythrocyte membranes. Extraction based methods were considered initially useful in isolating glycoprotein and glycolipid membrane components speculated to carry the ABH substances.

1.5 Distribution of ABH Antigens on Glycoprotein and Glycolipid Carriers

In the 1960-80s, studies presented conflicting data on whether the carbohydrate antigenic structures were carried predominantly on glycolipids or glycoproteins. While some studies claimed that ABH antigens were only present on glycolipids and not glycoproteins, others supported a contrasting hypothesis[30-32]. Most studies, however, provide data suggesting the presence of ABH

antigens on both glycolipids and glycoproteins. Yamakawa and Ida found ABH antigens on the erythrocyte membranes to be linked to glycosphingolipids (GSLs) [33,34]. These glycolipids were described as polar and water-soluble structures which caused them to bind to other polar glycoproteins in the membrane[35-37]. These sphingolipids, also termed as polyglycosylceramides are now known to carry 20-40 sugar residues per mole and speculated to be a potent source of ABH reactivity[38,39]. This finding potentially explains that even a relatively small volume of polar protein-binding GSLs could contaminate a glycoprotein fraction, otherwise selectively isolated from the erythrocyte membrane[40,41].

Finne *et al* first demonstrated the presence of ABH antigens on glycoprotein Band 3 and 4.5 on erythrocyte membranes[42]. Using radio-iodinated anti-A and anti-B lectins, they also illustrated two main types of glycoproteins, including the alkali-stable type polyglycosylpeptides (Bands 3 and 4.5) and alkali-labile type which were previously characterized as sialoglycoprotein antigens. Later, it was reported that 65-75% of the ABH antigens are expressed as alkali-stable glycoproteins, 5-15% alkali-labile glycopeptides, 10-15% polyglycosylceramides and the remaining 5% are simple GSLs[43]. The numbers of ABH glycoproteins are now thought to be about 80% of the total erythrocyte membrane ABH determinants[44]. Alkali-stable glycoproteins are further classified into N-linked and O-linked glycoproteins. N-linked glycoproteins are formed as a result of glycosylation occurring at asparagine side chains by linking N-acetylglucosamine to the amino group, while O-linked glycoproteins are created by linking N-acetylgalactosamine to the oxygen group at the threonine or serine side chains[45]. The glycolipids carrying the ABH antigens were also eventually categorized into lacto-, globo-, and ganglio-series[46,47].

1.6 ABH Core Structures and Types

With advancements in analytical chemistry tools like mass spectrometry and NMR spectroscopy combined with the introduction of monoclonal antibodies specific for the ABO blood group antigens (MoAbs), it became possible to distinguish between structural variants of these antigens[48]. These tools offered a closer look at the variations between the antigen epitopes carried on membrane lipids and proteins. At this point, the ABH carbohydrate antigens present on glycolipids and glycoproteins were understood to be identical in structure. However, there was unexplained variation observed between the two ABH reactive components. The sources of variation were hypothesized to be the inner structure the antigens are attached to and the repetitive structural units branching from the core structures [26,48,49]. These inner structures were defined by Clausen *et al* to be an extension from the glycosylation site towards the lipid and protein carriers and split into two components[48]. The peripheral structure is the Gal-GlcNAc or the Gal-GalNAc disaccharide structure, whereas the inner core structure is the R-group extending from GlcNac or GalNAc as shown in Table 1. These disaccharide cores serve as the precursor for various blood-group related glycosyltransferases. The peripheral cores are further divided into four main types numbered from 1 to 4 (Table 1) which are not only distributed between glycolipids and glycoproteins present in erythrocyte membranes but also among tissue lining and secretions. Within blood group A alone, heterogeneous expression of peripheral core structures was observed. For instance, Type 1 chain structures with A antigens are present on erythrocytes in minor quantities and their expression is dependent on secretor and Lewis blood group [50,51]. In contrast, Type 2 chain A structures carry a bulk of A antigens on erythrocytes with branches and unbranched molecular heterogeneity[52-54]. Type 3 chain A structures are composed of repetitive antigenic units

which immunologically resemble the A determinant O-linked to polypeptides (via serine or threonine)[54-56].

Table 1: Peripheral core structures and their principal tissue distribution (modified from Clausen and Hakomori, Storry and Olsson[48,49]).

Peripheral Core Type	**Structure**	**Distribution**
Type 1	Galβ1→3GlcNAcβ1 → R	Endodermal, secretions, plasma
Type 2	Galβ1→4GlcNAcβ1 → R	Ecto- and mesodermal (example: erythrocytes)
Type 3	Galβ1→3GalNAcα1 → R	O-linked mucin-type, repetitive A
Type 4	Galβ1→3GalNAcβ 1 → R	Glycolipids in kidneys (and erythrocytes)

1.7 ABO SUBGROUPS: A_1 VS. A_2

Understanding these structures provided a more comprehensive vocabulary to categorize the differences between the ABO blood type subgroups. Type A is the most common blood group with $A_1$and A2 being the most clinically significant subgroups, accounting for almost 99% of the type A individually[57,58]. The variation observed within the same blood group was a cue towards greater complexity than was originally discovered and characterized. Similar to the initial debate between the distribution of ABH antigens on glycoproteins versus glycolipids, there was a discourse around the nature of the phenotypic differences between the $A_1$and A_2 antigens. Some researchers observed solely quantitative differences while others pointed out qualitative differences as well, a discrepancy possibly attributable to the method chosen to study these antigens. Eventually, a general consensus of both quantitative and qualitative differences was

reached. It was found that there are approximately five times more A antigens present on A_1 erythrocytes than on A_2 erythrocytes[57]. This quantitative difference translates to a "stronger" A signal observed from A_1 individuals than from A_2 individuals on clinical agglutination tests[59-63]. As for the qualitative differences, it was attributed to the A_2 transferase being 10 times less efficient than the A_1 transferase and having a different pH optimum and pI for post-translational activity[63,64]. As a result, there are more precursor H-antigen structures that are not fully converted to A-antigen present on A_2 erythrocyte membranes than on A_1 erythrocytes [65]. Most of the primary investigations on differences in core structures between A_1 and A_2 erythrocytes were conducted on isolated glycolipid fractions. Clausen *et al* used an IgG monoclonal antibody (TH-1) which was shown to bind specifically to type 3 structures found on A_1 glycolipids and not glycoproteins[54]. To explain this phenomenon, they hypothesized that A_2 transferase is far less efficient at converting type 3 (repetitive) or type 4 (Globo) H chain structures to type 3 or type 4 A chain structures[54]. Even if the enzyme begins adding A structures at the base of the chain, due to the inefficiency of the enzyme the most terminal structure still remains the unconverted precursor H antigen structure masking the A antigen underneath. From a canonical perspective, this phenomenon increases the likelihood of A_2 antigen being recognized as an H antigen by clinical grade reagents. As a result, blood type A_2 individuals may incorrectly be typed as type O, which can lead to serious clinical implications[58].

Yamamanto and Hakomori later found some evidence of type 1, 2, 3 and 4 chain A structures on A_1 erythrocytes but only type 1 and 2 on the A2 erythrocytes[15]. To validate these phenotypic differences in A_1 and A_2, Svensson *et al* repeated these studies using purified glycolipids from donor erythrocytes, specific monoclonal antibodies and thin layer chromatography (TLC) immunostaining[66]. They found Type 1, 2 and 3 to be present on both A_1

and A_2 erythrocytes with Type 3 being the most abundantly present. Type 4 chains were detected mostly on A_1 erythrocytes and were reported to be missing in A_2 antigens. Based on these observations, they concluded Type 4 to be the major structural difference between A_1 and A_2 erythrocytes. However, it should be noted that these type 4 chain glycolipid structures are very minor contributors to the total glycolipid makeup and even exclude the complex polyglycosylceramides that are expressed on the erythrocyte membrane. Thus, these differences may be true only for a small proportion of the glycoconjugates found on A_1 and A_2 erythrocytes [67]. While these glycolipid-oriented studies have clarified structural variations in the lipid carrier structures for ABH antigens, they don't address whether similar structural variations between A_1 and A_2 erythrocytes are also expected to be present in glycoprotein carriers too. To address this discrepancy, Gehrie *et al* focused on identifying the potential qualitative and quantitative differences in the glycoproteins carrying the A_1 and A_2 blood group subtype antigens[67]. While they found smaller than expected quantitative differences between the number of antigens expressed, they found evidence to support more significant qualitative difference. They performed western blots probed with monoclonal Anti-A antibody and DBA lectin which recognizes A_1 antigen. Instead of finding weaker, fainter bands from the isolated glycoprotein fraction of A_2 as compared to A_1, they observed a complete absence of some of general protein bands, which would be expected if the differences were limited to quantity of antigen. They hypothesized that the weaker bands could be taken as evidence of slow A_2 transferase being unable to add A determinants as efficiently as A_1 transferase[67].

1.8 CLINICAL RELEVANCE

It is critical to understand and characterize the subgroups within the ABO system because they have significant impacts on allogenic ABO compatibility with implications in transfusion medicine, transplant, and pregnancy[49]. Due to the presence of H-antigen precursors on their erythrocyte membranes and anti-A antibodies in their plasma, A_2 donors and recipients are likely to get incorrectly forward and reversed-typed as blood type O[58]. Due to ABO incompatibility, transfusion of A_2 blood to an O recipient can cause fatal AHTRs in the recipient[3,4]. Factors like secretor status of the donor and recipient, transfusion volume, variation in antibody titer levels in recipient, heterogeneity within the ABH antigen phenotype of the donor can also be some of the key contributors to HTRs for patients receiving blood transfusions[9,68]. ABO incompatibility can play a role in the hemolytic disease of the unborn (HDN) when naturally occurring anti-A and anti-B antibodies in a type O mother cross the placenta to hemolyze fetal RBCs of A, B or AB blood type[69].

ABO incompatibility has also been shown to have serious clinical consequences for solid organ transplantation patients, increase hospitalization and decrease survival in some cases[70-73]. In addition, the association between ABH antigens and other diseases including cancer, cardiovascular diseases etc. indicate the relevance of the antigen phenotype even beyond blood transfusion and organ transplantation[74-76].

Clinical laboratories are often challenged with the task of explaining unexpected ABO antibodies, investigate adverse reactions caused by suspected ABO subgroup incompatibility, or classify ABO at a higher resolution beyond the blood types prior to transfusion and transplantation.

1.9 Need for Deeper Understanding

Despite being studied since the beginning of 20th century, the complexities of the ABO blood group system and attendant clinical implications have yet to be definitively elucidated. Even though ABO glycobiology, including the genetic and biochemical pathways, immunodominant sugars and their sub-types[49] is mainly understood, there are nuances in the antigenic structures, which are not yet fully elaborated. Individuals carrying "weak" phenotypes of the A antigen (A_2, A_x subgroups) often also carry anti-A antibodies in their plasma which is an important deviation from the enzymatic pathways and the expected clinical outcomes described in the literature. Moreover, the qualitative differences between A_1 and A_2 antigen phenotypes is not conclusively determined with regards to its distribution on glycoproteins[60,61,67,77].

It should be noted that historically most studies have used plant-based lectins and monoclonal antibodies to identify these subtle differences, however, this approach is based on circular logic. Antibodies were developed with the isolated human antigens as the target, without first understanding the structural composition of these antigens[78,79]. Thus, these antibodies only recognize a narrow range of structures which they were developed to identify, thereby limiting the discovery of nuanced structures that may be masked by terminal epitopes[48,80]. The natural variation and heterogeneity of the ABO blood group has become evident through the presence of sub-types, core and peripheral structures, branching, etc. and this complexity does not get adequately recognized by the very reagents that are developed for that purpose. Most notably, the ABO-binding antibodies and lectins do not accurately represent the natural human antibodies circulating in plasma which have been shown to not have a human target [81]. There is persistent ambiguity about the true interactions between heterogeneous ABH antigens and the natural antibodies, a mystery which cannot be fully solved using commercial reagents.

1.10 Introduction to Thesis Work

The structural differences of ABO blood types may be subtle but their effect on antigenicity is significant. There is a need to study them with more sophisticated tools to further elaborate the structures and fill the gaps in our understanding. Advancements in mass spectrometry techniques can help to investigate the nuances in the ABO carbohydrate core structures[82-88]. In addition, shotgun-glycomics technology provides a multiplex platform that supports a rigorous investigation into how these characterized structures are recognized by natural human antibodies in plasma[86-88].

The overarching goal of the ABO project, that this thesis is a part of, is to examine the structural basis of ABH antigens on a genetic, phenotypic, structural level and apply this knowledge to develop a new ABO blood group sensing platform. The insights into ABH structural determinants through this study will be valuable in explaining the basis of specific ABO subtypes that underlie discrepancies observed in clinical practice and impact outcomes in transfusion and transplant practice.

This thesis project sought to engineer and optimize methods that facilitate this structural characterization process. Chapter one provides the historical background on ABO blood group system with a brief discussion of its clinical relevance. Chapter two presents an overview of the methodology, including detailed protocols employed for sample processing and validation. Chapter three discusses the approaches attempted to isolate the glycoprotein and glycolipid fractions and includes discussion of preliminary methods. Chapter four concludes the thesis work and is oriented towards the foreseeable next steps to meet the long-term aims of the project.

Chapter 2. SAMPLE PREPARATION

2.1 Overview of Sample Pipeline

An overview of the project and the three main stages are illustrated in Figure 1 shown below. The black arrows signify a stage that has been completed and a dotted, grey arrow suggests future work. The first stage (I) was focused on preparing erythrocyte ghosts from donated blood and performing qualitative and quantitative tests to validate ABO reactivity using clinical grade reagents. In the second stage (II), the ghost membranes are processed to isolate the glycolipid and glycoprotein components and two different approaches were explored for this purpose: chemical and enzymatic. The latter approach was found to be more viable to effectively isolate the glycans from proteins and lipids. In the final stage (III), the free glycans will be derivatized with a bifunctional fluorescent linker for enhancing structural analysis on liquid chromatography mass spectrometry (LC-MS) and the development of more accurate blood typing diagnostics via silicon photonics platform.

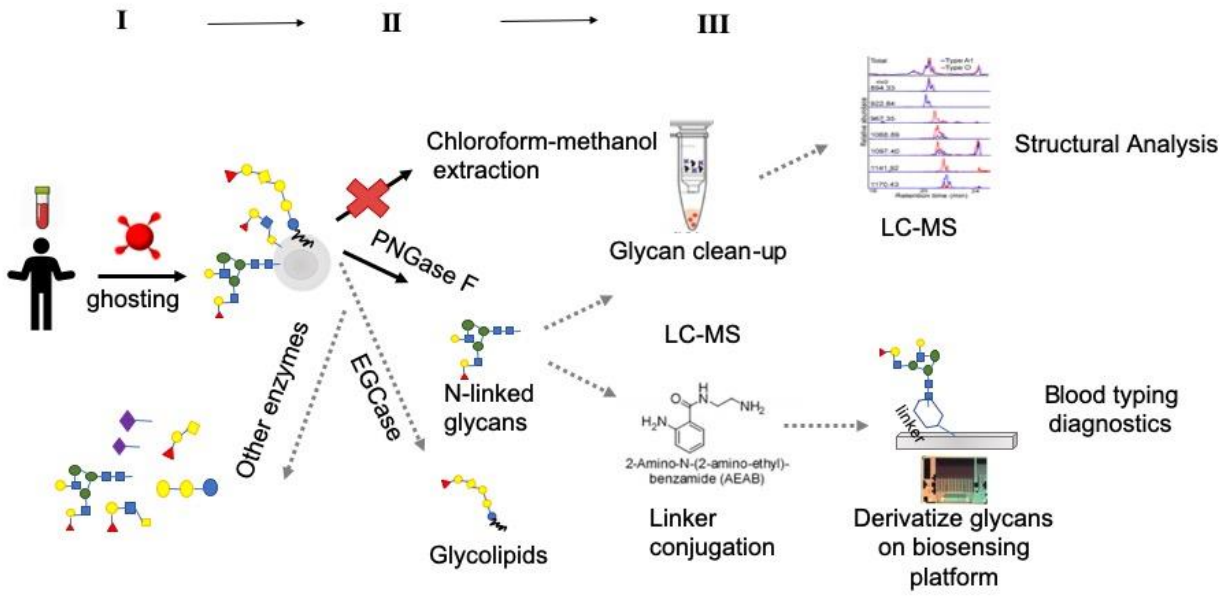


Figure 2: Schematic showing an overview of the project, divided into three main stages. The first stage entails the preparation of erythrocyte ghost membranes; the second stage is the isolation of glycolipid and glycoprotein membrane fractions to release free glycans; third stage is oriented towards derivatizing the free glycans obtained in stage two for structural analysis using mass spectrometry and to develop blood typing diagnostics based on the silicon photonics biosensing platform. The black arrows represent the stages that have been complete (stage I) and the grey, dotted arrows suggest future work (stage II and III).

2.2 Selection of Human Donors

Researchers have historically pooled human blood from various individuals of the same blood type to study the structure of ABO antigens. However, once the blood samples are pooled by their major ABO group, subtle variations within the group is concealed and retrieving genetic information from the phenotypic observations is challenging. To address this limitation, eligible human donors with A_1 and O phenotype were identified and selected as a canonical reference for the project. Johnsen Lab at Bloodworks Northwest Research Institute sequenced blood samples from these individuals to identify their genotype. Instead of pooling, the blood samples were

individually processed but identically treated. In doing so, we are prepared to study the underlying nuances of the blood type system, including sub-groups, diversity in core structures and their associated antigenicity.

Health Insurance Portability and Accountability Act (HIPPA) and local Institutional Review Board (IRB) guidelines about protecting the human donor privacy were strictly observed. Any identifiers were removed before the blood samples were obtained, thus blinding all researchers in the project, including Johnsen Lab personnel. Each individual has a unique ID by which the samples derived from their blood is referred to in this document: 1178 is type A_1 individual, 2207 is type O individual. Two additional donor samples were acquired for the purpose of optimizing the enzymatic digestion and clean-up protocols: 4390 is type A individual, 6197 is type O individual. Both of these individuals were not sequenced. A licensed phlebotomist drew blood from consenting donors which was processed according to detailed protocols described in the section 2.3.1 below.

2.3 METHODS

2.3.1 *Ghost Preparation*

Whole blood from individual donors is passed through leukoreduction filters to remove white blood cells and plasma, isolating the red blood cells. These erythrocytes undergo hemolysis in hypotonic solution, as described by Dodge *et al* to produce pearly-white erythrocyte ghosts devoid of any hemoglobin with intact membranes[89]. These ghost samples are used as a starting material for all the assays described in the following sections.

Table 2: Detailed erythrocyte ghosting protocol based on Dodge et al[89].

Materials	Pediatric leukoreduction filter(s) [Haemonetics] RBC ghost buffer: 5mM phosphate, pH 8.0 RBC resuspension buffer: PBS, pH 7.4 250mL polypropylene conical bottles or 300mL polystyrene centrifuge bottles 15mL polypropylene conical tubes
Recipe	20x phosphate buffer, 100mL • Solution A: 0.2M sodium phosphate, dibasic, dihydrate (Na2HPO4•2H2O FW = 178.05) • Solution B: 0.2M sodium phosphate, monobasic, monohydrate (NaH2PO4•H2O FW = 138.01) Combine 43.5mL of Solution A with 6.5mL of Solution B. Add H2O to 100mL.
Leukoreduction	1. Draw 30cc of whole blood into syringe prefilled with 4.3cc ACD anticoagulant 2. Dispense entire volume of syringe through leukoreduction filter and into collection bag via gravity flow 3. Dispense additional 10mL of 1x PBS through leukoreduction filter and into collection bag via gravity flow
RBC Ghosting	1. Distribute contents of leukoreduction collection bag evenly between three centrifuge bottles, each containing 200mL ice-cold RBC ghost buffer 2. Invert several times and incubate on ice for 5 minutes 3. Spin at 9000 x G x 20 minutes at 4°C 4. Discard supernatant 5. Resuspend each pellet in 10mL ice-cold PBS by gentle pipetting 6. Spin at 9000 x G x 20 minutes at 4°C 7. Discard supernatant 8. Resuspend each pellet in 200mL ice-cold RBC ghost buffer by gentle pipetting followed by inverting several times 9. Incubate on ice for 5 minutes 10. Spin at 9000 x G x 20 minutes at 4°C 11. Reserve supernatant and set aside. 12. Resuspend each pellet in 30mL ice-cold PBS 13. Spin at 9,000 x G x 20m at 4°C 14. Discard supernatant 15. Repeat steps 12-14 until pellet is yellow-white 16. Resuspend final pellets in residual liquid and transfer to 15mL conical

Reserved supernatant	1. Spin at 9000 x G x 60 minutes at 4°C 2. Remove supernatant and set aside. 3. Resuspend pellets in residual liquid and split evenly between 2-3 polypropylene round-bottom tubes 4. Add 25mL ghost buffer and resuspend 5. Spin at 9000 x G x 20 minutes at 4°C 6. Remove supernatant and set aside with previous 7. Resuspend pellets in 30mL PBS 8. Spin at 9000 x G x 20 minutes 9. Remove supernatant and set aside 17. If pellets appear clean, resuspend in residual liquid and combine with previous pellets in 15mL conical
Final sample processing	1. Combine ghosts well in single 15mL conical 2. If volume is <6mL, bring total volume to 6mL with PBS 3. If volume is >6mL, spin at max speed for 20 minutes and remove excess to bring total volume down to 6mL 4. Store excess (if any) in screw-cap vials. 5. Mix ghosts thoroughly and split equally between 6 screw-cap vials. 6. Label each tube with contents, donor ID, and date collected/processed

2.3.2 *BCA Assay*

Total protein concentration in the erythrocyte ghosts is determined using bicinchoninic acid (BCA) assay which is based on the colorimetric detection and quantification of protein in a sample. The macromolecular structure of protein, the number of peptide bonds and the presence of cysteine, cystine, tryptophan and tyrosine are responsible for reducing copper ion, resulting in a purple-colored product detected at 562 nm[90,91]. The working range of this assay is 20-2000 μg/mL and bovine serum albumin (BSA) is commonly used as a reference protein sample to create a linear standard curve. The unknown concentration of the protein is determined through extrapolation based on this linear curve. Pierce BCA Protein Assay Kit is used for performing the BCA assay and the protocol from the catalogues in included below, along with modifications. Due to limited ghost volume, 98-well microplate version was utilized for logistical purpose.

It is critical to perform the BCA assay prior to the downstream processes, in order to normalize the protein concentration between type A_1 and type O ghost samples and avoid artifacts on SDS-PAGE gels and blots. Since the erythrocyte membrane contain significant amounts of lipid which is known to interfere in the formation of the final complex, sodium dodecyl sulfate (SDS) is used to solubilize the lipids and added to the standards as well[92,93].

Table 3: Detailed BCA assay protocol adapted and modified from the Pierce BCA Protein Assay Kit (Thermo Scientific™) protocol[90,91].

Materials	Kit Contents: BCA Reagent A, 1000mL (in Product No. 23225) or 500mL (in Product No. 23227), containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide BCA Reagent B, 25mL, containing 4% cupric sulfate Albumin Standard Ampules, 2mg/mL, 10 × 1mL ampules, containing bovine serum albumin (BSA) at 2mg/mL in 0.9% saline and 0.05% sodium azide
Preparation of BCA Working Reagent (WR)	1. Use the following formula to determine the total volume of WR required: (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required. **Note**: 2.0mL of the WR is required for each sample in the test-tube procedure, while only 200 µl of WR reagent is required for each sample in the microplate procedure 2. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). For the above example, combine 50mL of Reagent A with 1mL of Reagent B. Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).
Sample Preparation	1. **Preparation of BSA standards** – use the standard BSA set. a. For a wider range of concentrations, dilute 0.125 mg/ml 1:2 to 0.0625 mg/ml followed by another 1:2 serial dilution to make 0.03125 mg/ml. Make dilutions in dH2O. b. For each BSA standard sample:

	i. 20 µl BSA standard + 5 µL of 10% SDS stock + 0 µl dH2O = 25 µl total ii. If doing triplicates (highly recommended), make a master mix in a microtube and aliquot into wells for triplicates (25ul per well). Follow the following recipe for making excess (multiplied by 3.5): 70 µl BSA standard + 17.5 µl 10% SDS stock + 0 µl dH2O = 87.5 µl total c. For blank sample i. 20µl dH2O + 5 µl of 10% SDS stock = 25 µl total ii. If doing triplicates, 70 µl dH2O + 17.5 µl of 10% SDS stock = 87.5 µl total 2. **Preparing unknown sample** a. For each ghost sample, follow the following recipe (25µl per well): i. For 1:1 ghost, 7.5 µl ghost + 5µl of 10% SDS stock + 12.5 µl dH2O = 25 µL total ii. For 1:2 ghost, 3.75 µl ghost + 5 µL of 10% SDS stock + 16.25 µL dH2O = 25 µL total b. If doing triplicates (highly recommended), make a master mix in a microtube and aliquot into wells for triplicates (25µl per well). Follow the following recipe for making excess (multiplied by 3.5): i. For 1:1 ghost, 26.25 µl ghost + 17.5µl of 10% SDS stock + 43.75 µl dH2O = 87.5 µl total ii. For 1:2 ghost, 13.125 µl ghost + 17.5 µl of 10% SDS stock + 56.87 µl dH2O = 87.5 µl total
Method (Sample to WR ratio = 1:8	1. Pipette 25µL of each standard or unknown sample replicate into a microplate well (working range = 20-2000µg/mL) (e.g., Thermo Scientific™ Pierce™ 96-Well Plates, Product No. 15041). b. For BSA samples, use 20µL of standard and add 5 µl of 5% SDS c. For ghost samples, use 7.5µl of ghost volume and the remaining volume is **Note**: If sample size is limited, 10µL of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125-2000µg/mL.

	2. Add 200µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds. 3. Cover plate and incubate at 37°C for 30 minutes. 4. Cool plate to RT. Measure the absorbance at or near 562nm on a plate reader. **Notes**: • Wavelengths from 540-590nm have been used successfully with this method. • Because plate readers use a shorter light path length than cuvette spectrophotometers, the Microplate Procedure requires a greater sample to WR ratio to obtain the same sensitivity as the standard Test Tube Procedure. If higher 562nm measurements are desired, increase the incubation time to 2 hours. • Increasing the incubation time or ratio of sample volume to WR increases the net 562nm measurement for each well and lowers both the minimum detection level of the reagent and the working range of the assay. As long as all standards and unknowns are treated identically, such modifications may be useful 5. Subtract the average 562nm absorbance measurement of the Blank standard replicates from the 562nm measurements of all other individual standard and unknown sample replicates. 6. Prepare a standard curve by plotting the average Blank-corrected 562nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample. **Note:** If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points

2.3.3 *SDS-PAGE*

Optimized protocols for SDS-PAGE were based on the method developed by Fairbanks *et al* to resolve and visualize erythrocyte membranes[91].

Table 4: Detailed SDS-PAGE protocol for evaluating the protein distribution on erythrocyte ghost membranes.

Materials	NuPAGE 4-12% Bis Tris 1.0mm 12 well gel NuPAGE LDS Sample Buffer 1x NuPAGE Antioxidant BenchMark protein ladder (for SYPRO stain) SeeBlue Pre-stained protein standard (for fluorescence imaging on western blots) Protein Precision Plus WesternC (for chemiluminescence imaging on western and dot blots) NuPAGE MES SDS Running Buffer 1x Fixing solution (50% ethanol, 7% acetic acid) De-stain solution (10% ethanol, 7% acetic acid) SYPRO Ruby stain 1M DTT
Sample Preparation	1. To load 10 µl of sample per well, prepare 20µl total sample volume to account for pipetting errors. Ghost volume is set to approximately 0.5 µL per lane, so adjust accordingly. Scale up for multiple lanes. d. Recipe: 5µl of 4x LDS buffer, 2 µL of 1M DTT, 0.5-1µl of protein (depending on ghost normalization), 11-12 µl of MQ water e. Make blanks by diluting LDS buffer to 1x, as needed. 2. Heat samples at 90C for 10 minutes. 3. For ladder prep, follow instructions on the product manual.
Running the gel	1. Open the sealed gel bag over the sink. Remove the tape strip at the bottom of the gel. Place the gel into the gel box setup. 2. Either use the bolt assembly (which is faster) or the classic sureLock tank. For the bolt setup the gel wells should face out towards you, while for the sureLock tank the gel wells face into the center reservoir. 3. Make up 1X running buffer. MOPS is better for resolving larger MW proteins (30-100kDa), while MES is better for smaller proteins (15-40kDa). 4. Remove the comb from the top of the gel. Pour the 1X running buffer into the main tank so it is completely full. Fill the outside tank so that it is about half full. 5. Load 10 µl of the samples into each gel with a gel loading pipette tip. If you have any empty/unused lanes, then load 10 µl of 1X LDS buffer (otherwise the gel will run distorted).

	6. Add 0.25mL of the antioxidant reagent to the front of the gel (for the bolt setup), or the center reservoir for the sureLock setup. 7. Close the gel box and run at 150V for 45 minutes. The dye front should move down the gel and should reach the bottom of the tank once the gel is done.
Fixing and Staining	1. Remove the gel from the running tank. Use the metal tool to pry the gel cassette open. Be careful not to rip the gel while doing this 2. Place the gel into a staining container, with 30-50mL of fix solution (50% ethanol, 7% acetic acid). Place on the gel rocker for 30 minutes to fix the gel. 3. For SyproRuby staining: Pour off the fix solution (into the gel waste container in the fume hood) and fix again for 30 minutes with another 30-50 mL of fix solution. 4. Pour off the fix solution and add 30-50mLs of SyproRuby stain. Cover the gel box with aluminum foil (the syproRuby stain is somewhat light sensitive). The gel will need to stain for at least an hour, but ideally leave it overnight. 5. Pour the stain back into the bottle (it's reusable) and add 30-50mLs of de-stain solution (10% ethanol, 7% acetic acid), and leave on the rocker for 15-30 minutes. After this the gel should be ready to image in the gel box.

2.3.4 *Western and Dot Blots*

Two types of blots were used to evaluate the distribution of reactivity across different proteins in the erythrocyte ghost samples: 1) Western blots to detect ABH reactivity present on glycoproteins transferred onto the PVDF membrane 2) dot bots to detect ABH reactivity present on both glycoproteins and glycolipids directly blotted on the nitrocellulose membrane. The protocol for both blots were based on established literature and further optimized in-house[94]. Clinical-grade Immunocor anti-A antibody and *Ulex europaeus* agglutinin I (UEA) lectin were used to identify A and H antigen, respectively. Donkey anti-mouse IgG conjugated to horseradish peroxidase (HRP) and streptavidin linked HRP were used as secondary antibodies with attached chemiluminescent tags.

Table 5: Detailed protocol for western and dot blots.

Materials	PVDF for western blots Nitrocellulose for dot blots NuPAGE transfer buffer
Semi-dry transfer to PVDF membrane	1. Soak PVDF in methanol for 1 min, then MQ H2O for 1 min. Leave in water until use. 2. Soak blotting pads in 1x transfer buffer 3. Stack the components in the following order into the electro-blotter apparatus. The bottom electrode is anode and fixed into place. The cathode electrode is adjustable within the lid of the apparatus. a. Bottom electrode: single piece of saturated Whatman grade filter paper b. PVDF membrane c. SDS-PAGE gel d. Top: single piece of saturated Whatman grade filter paper e. Check for bubbles in between the layers. Soak up any excess liquid on the bottom plate 4. Calculate power input by using 0.8mA/sq. cm of gel for larger gel transfers. Mini-gels (10 x 11cm) may be transferred at up to 100mA (5-25V) total current. Remember, smaller proteins (<20kd) require less time than medium size (<80kd) or larger proteins a. For two blots, transfer at 250 mA for 1 hour.
Blotting directly on nitrocellulose membrane	1. Prepare sample dilutions in PBS in one or more 96 well plate(s) (see figure 1) a. Sample dilutions will depend on the concentration of ABO antigen in your sample source. For example, buffy coat > whole blood > plasma > saliva b. Need enough for four blots (AntiA, UEA-1, WGA, DBA), 4 L per blot, make 40 mL total. i. For first row of 0.6 µg/l samples, load 30 mL PBS and 10 mL of sample (to dilute sample 0.25x for first spot). ii. For all other rows/wells, load 30 mL PBS iii. Serially dilute, taking 10µl from the first well, mixing in the next, and then repeating for each sample set. Be careful only serially dilute four wells at a time. Do not mix samples in the top half of the plate with the bottom half of the plate.

	2. Cut a piece of 0.45µm nitrocellulose to a little bit larger than the size of the plastic frame. Label the upper right-hand corner "A1" and set on a piece of extra thick blot paper 3. Place the plastic frame on top of the nitrocellulose and mark the corners (in case it moves during blotting) 4. Repeat steps 2-3 for as many replicates as desired. Note that a single frame can accommodate 2x96 spots 5. Using a multichannel pipette, aspirate 5µl of the dilutions from row A of dilution plate 6. Lower the tips onto the nitrocellulose using the frame as a guide, gently touching the surface of the membrane, and dispense the samples a. Applying too much pressure risks puncturing the membrane b. Applying not enough pressure risks droplets landing unpredictably c. It's really not that serious though, this is hard to do wrong 7. Repeat steps 5-6 for all wells and replicates a. If the whole plate is needed, then replicate all over again, or spot replicates one row at a time b. Change pipette tips if the sample changes (moving through multiple dilutions of a single sample with the same tip is fine) going through the rows. 8. Set plastic frame aside and allow the blots to air dry overnight. Blots can be stored in a clean, dry, air-tight container for many months before probing for ABO glycans.
Blocking & Probing for both	1. Block membrane in 2% BSA and 2 % Tween-20 in 1x TBS 15 minutes on rocker 2. Primary antibody in TBS for 1 hour on rocker a. For A antigen, dilute Immucor anti-A antisera at 1:50 b. For H antigen, dilute UEA lectin at 1:500 c. DBA: dilute 1:875-900 d. Glycophorin: dilute 1:2400-4000 (in process of optimization) e. WGA: dilute 2400-2700 3. Wash 3x 5 minutes each with TBST. Be generous with the wash buffer a. USE 20x TBST and dilute down to 1X 4. Secondary antibody and 1 mL Precision Protein StrepTactin-HRP Conjugate (4C) in TBS for 1 hour on rocker a. For A antigen, dilute donkey anti-mouse/HRP at 1:5000 b. For H antigen, DBA, WGA and Glycophorin, dilute streptavidin/HRP at 1:8000

	5. Wash membranes (TBST) as performed in step 3. Be generous with the wash buffer During the washes, prepare ECL reagent by combining equal parts of peroxide and luminol substrate. Millipore ECL is stable at room temperature for several hours and can be kept at 4°C after preparation overnight before use. Each blot takes about 5-6 mL of ECL to develop 6. Move to Imaging room Bring: Tweezers, paper towels, pipettes, blots, extra TBST, ECL Reagent, 50 ml Conical tubes, containers for blots, and gloves. 7. Develop blots with ECL (wash blot with ECL and drip off excess before photographing immediately.) a. Do not use saran wrap underneath blot (causes wrinkles). b. Check image export format. c. Clean machine after use.

2.4 BATCH VALIDATION

Three total batches were acquired from the two A_1 and O blood type donors. The first batch of ghosts was used to perform chloroform-extraction, while the second and third batch was reserved for enzymatic digestion processes. Validation studies were conducted to ensure that overall protein concentration and reactivity (to anti-A and UEA lectin) were comparable between batch two and three. BCA, SDS-PAGE gels, dot blots were performed before repeating any processes with the third batch. Figure 2 shows the BCA results comparing the protein concentration between batch two and three of type A_1 and type O. Figure 3 shows the results from comparing the protein concentration between type A and O from donors 4390 and 6197, respectively. These donor samples will be used for optimizing the protocol for downstream analysis. Table 6 summarizes the final calculated concentrations of based on extrapolation from the BSA standard curve. Batch two of type A (1178) and type O (2207) was normalized to approximately 1.95 mg/mL prior based on an earlier BCA (results not shown) and is similar in protein concentration across the two types. Batch three of 1178 (2.43 mg/mL) and 2207

(2.81mg/mL) are marginally different but are overall more concentrated than the previous batch. The samples type O (6197) (2.02 mg/mL) is approximately 1.23 times more concentrated in the protein content than type A (4390) (1.64mg/mL) sample, which would require normalization before performing any SDS-PAGE or western blot analyses. Figure 4 shows the dot blots comparing the ABH reactivity probed against anti-A antibodies (4A and 4B) and UEA lectin (4C and 4D), for detecting the A antigen and the H antigen, respectively. The ABO reactivity between batch two and three of type A (1178) in Figure 4 A and B is comparable with a positive signal observed in response to anti-A and a negative signal in response to UEA lectin. A similar pattern is observed between batch two and three of type O (2207) for Figure C and D.

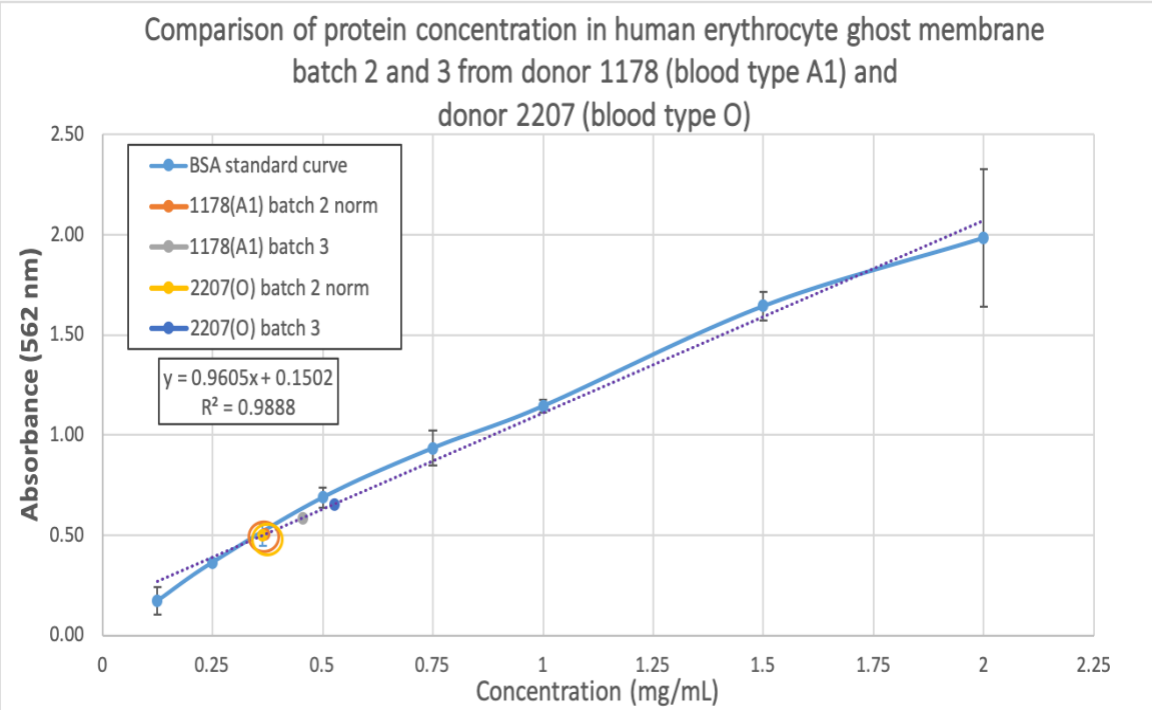


Figure 2: Comparison of protein concentration in erythrocyte ghost samples between batch two and three from type A_1 (donor 1178) and type O (donor 2207). Bovine serum albumin (BSA) was used as a reference linear curve.

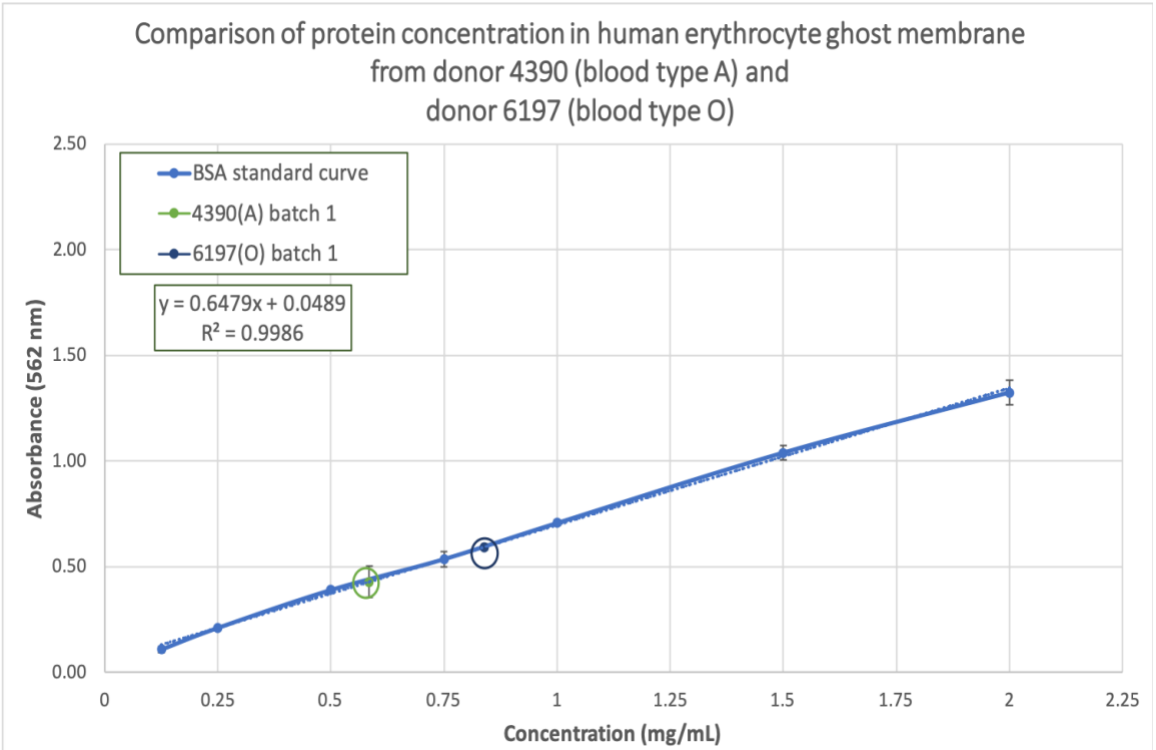


Figure 3: Comparison of protein concentration in erythrocyte ghost samples from type A (donor 4390) and type O (donor 6197). Bovine serum albumin (BSA) was used as a reference linear curve.

Table 6: Summarized protein concentration from blood type A and O across all donors, extrapolated from the BSA linear curve.

	Blood Type A		Blood Type O	
Protein Concentration (mg/mL)	4390	1178	6390	2207
Batch 1	1.64		2.02	
Batch 2		1.97		1.94
Batch 3		2.43		2.81

A and B were probed with Anti-A primary antibody with a donkey anti-mouse IgG /HRP secondary antibody. C and D were probed with biotinylated UEA lectin and streptavidin/HRP

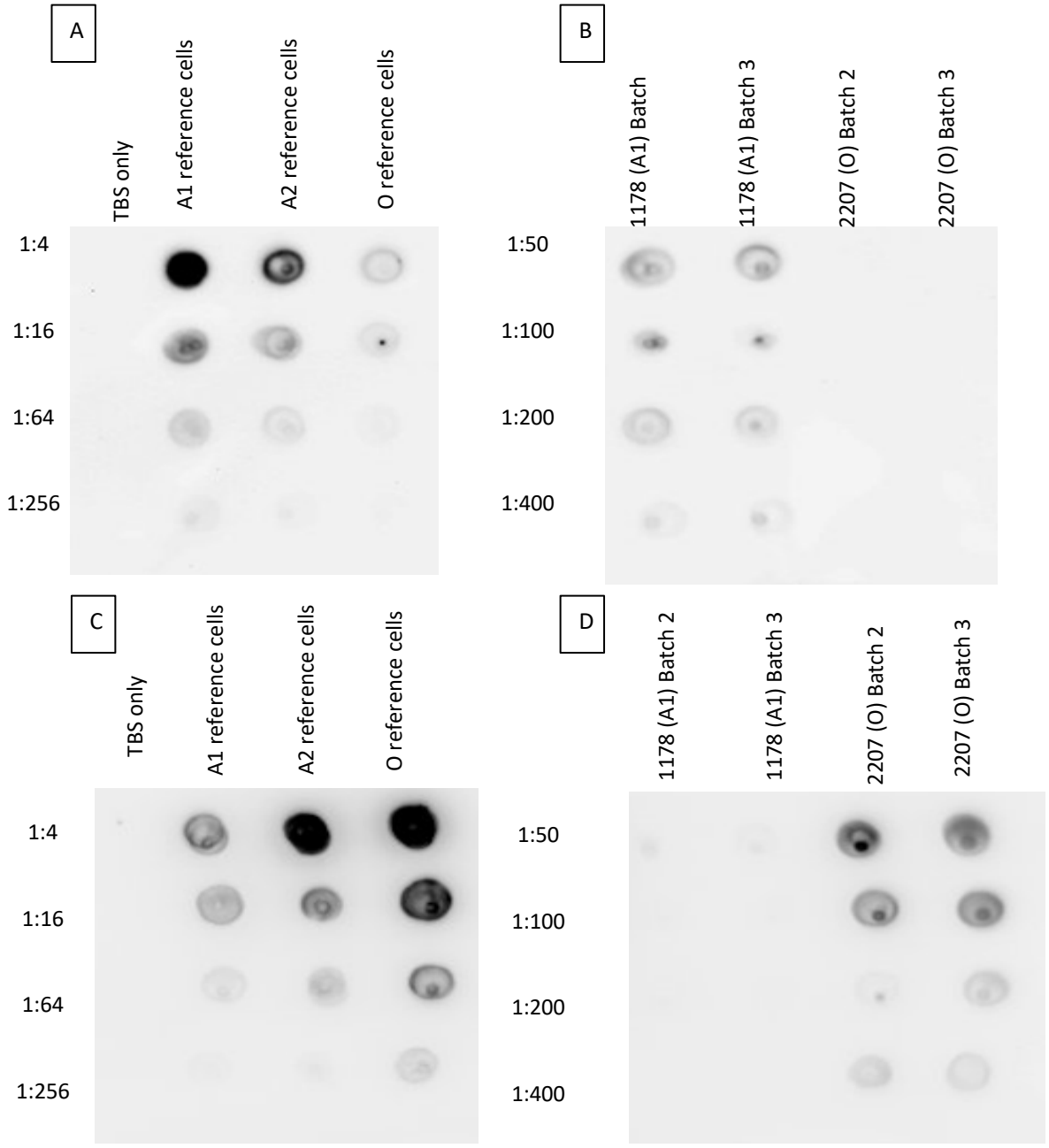


Figure 4: Dot blot results showing ABO reactivity between batch 2 and 3 of type A_1 and O donor from the validation studies. A_1, A_2 and O erythrocytes are used as reference cells to compare reactivity to standard anti-A antibody and UEA lectin. The columns represent different samples and the rows signify the serial dilutions conducted to cover a wider range of reactivity for the same sample. A and B represent the reactivity of both type A_1 and O to anti-A, and C and D show the reactivity of type A_1 and O to the UEA lectin.

Chapter 3. ISOLATION OF ABO BLOOD GROUP GLYCOLIPIDS AND GLYCOPROTEINS

3.1 INTRODUCTION

3.1.1 *Chemical Extraction Methods*

Complex biological samples such as human erythrocytes have to be processed in order to isolate the membrane glycoproteins and glycolipids carrying the ABH antigens. The ghosting procedure as described in the previous chapter removes the cytosolic components including hemoglobin, while still preserving the membrane glycoproteins and glycolipids[89]. For the structural study of glycans using analytical tools like mass spectrometry, these glycoconjugates must be strategically targeted to release the attached glycans[95]. Historically, a combination of different approaches using organic detergents, chaotropes and organic acids were employed to selectively extract the glycoproteins and glycolipids from the membrane [40,96-101]. While these approaches allowed for fractionation of glycoproteins and the assessment of the ABO reactivity from the attached glycans, the possible contamination with glycolipids cast doubt on accuracy of the results. The contaminating glycolipids are speculated to be glycosphingolipids (GSL), specifically polyglycosylceramides which have been shown to carry ABH antigens as well. They are described as polar and water-soluble causing them to tenaciously bind to other polar glycoproteins[34-37,102]. This phenomenon could contribute to the ABO reactivity observed in the glycoprotein fractions and interfere with the interpretation. Efforts to remove the contamination include addition of reagents like chaotropes and organic solvents, that require clean-up and pH adjustments which are often associated with sample loss[96].

Established chemical methods for de-glycosylation, such as hydrazinolysis for N-linked glycoproteins and β-elimination with mild-alkali for O-linked glycoproteins are also not entirely suitable for the purposes of studying ABH glycans[103-107]. These methods can be harsh, degrade the glycan and the protein, and lead to incomplete removal of the attached glycan. In addition, they have low specificity and can remove certain non-carbohydrate substituents as well[106-108].

3.1.2 *Enzymatic Digestion Methods*

In contrast to chemical methods, enzymatic cleavage via endoglycosidases provides a controlled de-glycosylation method with minimal degradation[106,107,109-111]. Peptide-N-Glycosidase F (PNGase F) is an amidase that cleaves between the innermost N-acetyl-glucosamine (GlcNAc) and asparagine (N) residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins[109]. PNGase F is known to be the most widely utilized enzymatic method to release an intact glycan, suitable for further analysis[112,113]. Endoglycoceramidase (EGCase I) is considered the enzyme equivalent for catalyzing the hydrolysis of the β-glycosidic linkage between the glycan and ceramides in glycosphingolipids, releasing intact glycans for analysis[114].

However, to date there has yet to be an equivalent broadly specific enzyme for cleaving the O-linked sugars on glycoproteins. Monosaccharides on O-linked glycans must be sequentially hydrolyzed by a series of exoglycosidases until only the core structure remains[115,116]. The other alternative is to employ harsh alkaline β-elimination techniques which degrades the protein backbone limiting the ability to do protein analysis via SDS-PAGE gel and blots[117,118] .

Commercial enzymes kits that target the cleavage of the terminal oligosaccharide epitopes have also emerged. One example is of Neuraminidase which catalyzes the hydrolysis of linear and branched non-reducing terminal sialic acid residues from glycoproteins[119,120]. Similarly, α-N-Acetylgalactosaminidase catalyzes the hydrolysis of terminal, non-reducing α- N-

acetylgalactosamine residues from oligosaccharides and N-glycans attached to proteins. These enzymes provide a tool-kit for targeting the ABH glycans of interest and facilitate the molecular characterization process[120].

3.2 Our Approach

This chapter covers the experimental design and preliminary results for isolating the glycoprotein and glycolipid fractions from human erythrocyte ghost membranes. Two main approaches were explored to fractionate the membrane: chloroform-methanol extraction and enzymatic cleavage of N-linked glycoproteins. The first approach was adapted from Hamaguchi *et al* with the detailed protocol included in section 3.3.1[121]. We employed established chemical techniques such as thin-layer chromatography (TLC) (Table 8) and phenol sulfuric acid assay (Table 9) to qualitatively and quantitatively assess the glycans in the fractionated membranes. Preliminary results and analysis in section 3.4.1 discuss the limitations of this method, including the possibility of glycolipid contamination in the fractionated layers, and the justification for discontinuing the chemical approach.

Cleaving the glycoproteins and glycolipids using enzymes was found to be the more viable approach for effectively isolating the ABH glycans. Part of the thesis work was focused on studying the effects of PNGase F cleavage of N-linked glycoproteins, which are speculated to express a significant distribution of ABH glycans[122]. It was hypothesized that after PNGase F cleavage, there would be an observable reduction in ABO reactivity to clinical reagents, such as anti-A antibody and UEA lectin. **This chapter will focus on the effect of PNGase F on N-linked glycoproteins carrying ABH antigens and the associated differences (if any) observed between type A_1 and type O erythrocytes.** Both type A_1 and O ghost samples will be tracked

pre- and post-enzyme treatment through SDS-PAGE gels and dot blots with standard ABO reagents to ensure that the enzymatic and chemical procedures are optimal for glycan removal. These measures are necessary for downstream glycan clean-up procedures and derivatization for structural analysis.

3.3 METHODS

3.3.1 *Chloroform-Methanol Extraction*

The protocol is adapted from Hamaguchi *et al* and optimized for the volume of erythrocyte ghosts we obtained from the whole blood samples[121]. Additional steps were performed after the extraction to solubilize the precipitate layer, as described in Table 1.

Table 7: Detailed protocol based on literature and further modified for the project [121].

Materials	15 ml Glass Conical tubes (Corning Part no. 05538127) LC/MS grade Methanol stock Chloroform Eppendorf tubes Pasteur pipettes
Procedure	1. To sample of 1ml starting volume add 4ml Methanol**.** Vortex well. 2. Add 1 ml Chloroform**.** Vortex well. 3. Add 3ml ddH20. Sample should appear cloudy. Vortex well. 4. Spin for 8 minutes at 10,000 x g. 5. Pipette off and save the top 3 ml of aqueous layer (top layer in the tube), careful to avoid removing any of the protein film/cloud/layer. This layer is denoted as aqueous layer A **(**in this project) which represents the bulk of the aqueous fraction Place this 3ml volume in a 15 ml conical tube. The protein precipitate exists between the aqueous (top) and organic (bottom) layers and may be visible as a thin/cloudy film. Freeze the 3ml aqueous fraction at -80^{0}C. Aspirate the remaining aqueous fraction, which is closer to the proteinaceous interface, referred to as aqueous layer B **(**in this project), without disturbing the interphase underneath. Save both fractions in the Eppendorf tube and freeze at -80^{0}C. 6. Add 8ml of Methanol. Vortex well.

	7. Spin for 8 minutes at 10,000 x g. NOTE: If pellet does not form, transfer in 1ml aliquots into 12-15 Eppendorf tubes and spin for 8 minutes at 10,000 x g. 8. Pipette and as much methanol as possible from the tube without disturbing the pellet. This fraction is referred to as methanol wash A or organic layer A (in this project)**.** 9. Save methanol in 15 ml conical tube and store at -80C. Rinse pellet by adding 10ml of methanol and invert 10x (pellet may or may not resuspend in methanol). 10. Spin for 8 minutes at 10,000 x g. Remove and save methanol used for rinse without disturbing pellet, label as 'rinse methanol' and store at -80^{0}C. This fraction is referred to the methanol wash B or organic layer B (in this project). Store pellet at -80^{0}C.
Solublization	Samples have just been through the Chloroform/Methanol Extraction procedure. 1. Spin samples at 9000 rpm or 9410 xg at 4^{0}C for 8 minutes but set the acceleration to 9 and the deceleration to 1, so the pellet will have more pressure on it and does not fall apart. 2. Using a glass Pasteur pipette remove as much of the organic layer as possible and place in the cone shaped glass tubes. Can pool same sample layers together. Let sit till pellet forms, because you are likely to suck out some of the free-floating protein. May take 1-2 hours. 3. At this point the pellet barely has any chloroform/methanol in it, but to make sure, let dry in hood for 10-15 minutes. 4. Add 2mL of 0.5% SDS and you can use a p1000 pipette to try to get as much off the walls as you can. Try doing this step a few times and centrifuging at about 4000-5000RPM at a deceleration of 5. 5. At this point a decision basically has to be made when this procedure seems to be have done enough and you are ready for bath sonication. 6. Take to bath sonicate and pool samples from same type together. Do not throw away tubes. 7. Re-do 2mL SDS step and try to get remaining off walls, bath sonicate and pool together. 8. Take the protein if any from the organic layer, decant organic layer, save for later use for glycolipid cleavage with EGCase and analysis. 9. Perform the SDS and bath sonication steps on this as well and pool with step 5 samples.

3.3.2 *Thin Layer Chromatography (TLC)*

TLC was used to qualitatively determine the presence of glycolipids in the fractionated organic and aqueous layer after the extraction was performed. The protocol was adapted from the established method described in literature[123]. GM1 and cholesterol were considered suitable controls since they resemble erythrocyte membrane glycolipids. Mobile phase was optimized by testing various ratios of $CaCl_2$, chloroform and methanol and the optimal ratio is included in the protocol. Both ceric ammonium molybdate and orcinol sulfuric acid stains were used to visualize the separation. Figure 5 shows the TLC results.

Table 8: Detailed TLC protocol based on methodology published in literature[124].

Materials	Samples TLC stains (CAM, orcinol) TLC solvent system (Chloroform: methanol: $CaCl_2$ (60:40:9 v/v/v) TLC stationary phase: silica gel glass plates with fluorescence Other materials: glass capillary tubes, solvent chamber, tongs, heat gun, ruler, pencil
Procedure	1. Score silica gel plates evenly into two plates per each sample (testing two different stains). Mark the plates with sample name, number of spots and stain type. Mark the baseline with a pencil about 2-3 cm from the bottom of the plate. Position the spots at least 0.5-1.0 cm from the edge of the plate. There should be a maximum of 5-6 separate evenly spaced positions on a single plate per samples 2. Using a capillary tube spot the sample on the plate while using an air source from the fume hood. Serially increase the number of spots on the plate. Allow sample to dry between spotting. 3. Prepare the solvent chamber by pouring fresh solvent. Insert a filter and wait 5 minutes to equilibrate the atmosphere in the chamber. For different day use, pour out used solvent, rinse with water and air dry before pouring fresh solvent. 4. Carefully immerse the spotted plate into the solvent container using tongs. Solvent level should initially be below the baseline on the plate. Make sure the plate is resting evenly against the wall and that the solvent rises evenly. Close the chamber and wait for 10-15 minutes until the solvent has travelled ¾ of the height. Mark with pencil. This the distance

	travelled by the solvent (R_x). Allow developed TLC plate to dry in the fume hood for 1-5 minutes. 5. Before staining, observe under UV lamp and mark any spots. 6. Heat the plate from the glass side (non-absorbent side) until plate is uncomfortably warm. 7. Prepare a glass container with the stain and immerse the hot developed TLC plate into the stain. Remove plate carefully and dab on a paper towel to remove excess stain. 8. Heat the glass side at highest setting using the hot gun until stains start to appear. Usually takes 1-3 minutes. 9. Mark the spots and measure the distance travelled by the solute. This distance is R_y. 10. Calculate R_f = (distance traveled by solute distance travelled by solvent) = (R_y/R_x).

3.3.3 *Phenol Sulfuric Acid Assay*

An established quantitative chemical assay based on the colorimetric detection of glycans and glycolipids was optimized for this project[123]. Due to the relatively small volumes of organic fractions available after chloroform-methanol extraction, the assay was modified to a 98-microwell plate assay for sample conservation. Parameters such as temperature, incubation time, reagents and their volume were tested, and the optimized version is included in the detailed protocol below.

Table 9: Detailed protocol for phenol sulfuric acid assay based on methods described by Dubois et al[125].

Materials	5% Phenol solution (1.5 µL phenol + 28.5 µL dH_20) Standard curve sample: 30 µL * 6 (2x 3 wells) = 180 µl/ dilution sample Unknown sample: 30 µl * 2x = 60 µl/ dilution sample Concentrated sulfuric acid (H_2SO_4) Standard curve sample: 150 µl *6 (2x 3 wells) = 900 µl/dilution sample Unknown sample: 150 µl * 2x = 300 µl/ dilution sample Standard curve solution 50 µl * 6 (2x 3 wells) = 300 µl/ dilution sample Unknown sample

	50 µl * 2x = 100 µl/ dilution sample
Sample Preparation	1. Standard curve samples**:** Make a stock 1mg/ml solution in the same buffer as the unknown sample. a. If using lactose or GM1 (50% glycan mass), perform serial dilutions (refer to lab notebook notes) to make the following concentrations for glycan standard curve: 4 µg/ml, 40 µg/ml, 100 µg/ml, 200 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml. Transfer 300 µl into reaction microtubes. 2. Unknown samples: Perform 1:10 and 1:2 dilutions using same buffer. Transfer 100 µl into reaction microtubes.
Microplate Assay	1. Rapidly inject the sulfuric acid to standard curve and unknown sample tubes- *in the fume hood.* 2. Shake for 30 minutes in the thermocycler at 650rpm - *Cover the lid with aluminum foil and shut the tube lid tight.* 3. Rapidly add 5 % phenol to standard curve and unknown samples. Meanwhile, set the desired temperature on the thermocycler to be 95^0C. *Avoid inhaling fumes.* 4. Load samples back in to the thermocycler and wait until the temperature reaches 95^0C, and when it does set a timer for 5 minutes. 5. After the 5 minutes, allow the thermocycler to cool down for 10 minutes until the temperature drops to below ~85^0 C. 6. Remove samples and place on counter top for additional cooling step for 10 minutes. Meanwhile, design the 96-well plate layout. 7. Vortex samples. Load 300 µl of standard curve and unknown samples into the 96-well plate. 8. Measure absorbance at 490 nm in the plate reader.
Disposal	Dump ice into sink, carefully immerse the plate into the bath, add sodium bicarbonate until excess is visible, flush down the drain using copious amounts of water. Discard the plate with lids in trash.

3.3.4 *Enzymatic Cleavage*

The protocol for PNGase F cleavage was based on the methodology described by Jensen *et al* and was subsequently modified for the ghost sample volumes[126]. Recombinant PNGase F was expressed and purified in-house by the Guttman Lab and its efficacy was previously validated using bovine fetuin as the substrate. Parameters such as incubation time with enzyme (12 hours versus 24 hours) and volume scale-up were tested (50 µL ghost volume versus 300 µL ghost

volume). The latter parameter was tested to produce a higher amount of free-glycans from the increased ghost volume for glycan-clean up prior to LC-MS. Prior heating of the substrate was also tested. The protocol versions are included in Table 10 and the results are shown in Figures 9 through 14.

Table 10: Detailed protocols for PNGase F digestion including two variations testing different parameters. The third version is the current protocol for the project.

<table>
<tr><td>Materials</td><td>Erythrocyte ghosts
1M DTT
5% SDS stock
10% NP-40 stock
1X PBS
PNGase F (produced in-house)</td></tr>
<tr><td>Version 1: Testing incubation time (12 hours vs. 24 hours)</td><td>1. To 50 μL ghosts, add
1. 1.5 μL 1M DTT
2. 15 μL 5%SDS
3. 52.2 μL PBS
2. Boil at 95 ^{0}C for 30 mins
3. Add 11.25 μL NP-40 to all samples
4. Add 10 μL PNGase
1. add PBS to mock samples
5. Incubated in heat block at 37 C for total 24 hrs
1. 10 μL PNGase/ PBS spike at 12 hrs. Take an aliquot for testing.
2. Both mock and digested samples incubated
6. Post-incubation ghost concentration (based on batch 2 BCA results of type A_1 & O, summarized in Table 6)
1. Mock: 27% ghost volume of total recipe volume
2. Digested: 29% ghost volume of total recipe volume
3. 0.5 μL ghost loaded per lane on SDS-PAGE gels from sample master-mix. Follow protocol in Table 4 for sample prep.</td></tr>
<tr><td>Version 2: Volume-scale up (total incubation time 24 hours with</td><td>Follow the above digestion protocol with the following volumes:
<table>
<tr><td>Volumes (μL)</td><td>Volume Controlled-Digest</td><td>Mini PNGase-Mock</td><td>Scaled PNGase-Digest</td></tr>
</table></td></tr>
</table>

an enzyme spike at 12 hours)	Ghost	50	50	300
	1M DTT	1	1	6
	5% SDS	10	10	60
	NP-40	11.25	11.25	67.5
	PNGase t = 0	10	10 (1x PBS)	60
	PNGase t = 12	6	6 (1x PBS)	36
	Total volume (μL)	88.25	88.25	529.5
<u>Version 3: Optimized Current protocol</u> (total incubation time 24 hours with an enzyme spike at 12 hours; total ghost volume 50μL)	1. To 50μl of ghost, add 1.5 μL 1M DTT, 15 μL 5% SDS, 52.2 μL 1x PBS 2. Boil at 95^0 C for 30 minutes. 3. Add 11.25 μl of 10% NP-40 for a final 1% in solution. 4. Add 10 μL PNGase F enzyme. 5. Add 10 μL of 1x PBS to PNGase mock control samples. 6. Incubate the sample at 37^0C in the shaker. 7. Spike 10 μL of PNGase F or 10 μL of 1x PBS at 12 hours. 8. Stop the incubation at 24hours. Aliquot samples for SDS-PAGE and blots. Store the remaining samples in -80^0C.			

3.4 RESULTS AND DISCUSSIONS

3.4.1 *Chloroform-Methanol Extraction*

Three visually distinct fractions were collected from the methanol-chloroform extraction process: aqueous, organic and precipitate. We expected the precipitate to contain hydrophobic membrane proteins, organic fraction to contain water-insoluble lipids and the aqueous fractions to contain water-soluble membrane components, possibly a combination of both glycosylated lipids and proteins. The aqueous and organic fractions were tested on TLC and dot blots, while the precipitate was solubilized to be resolved and visualized using SDS-PAGE.

Figure 5 shows aqueous and organic fractions spotted on the silica gel from type A_1 and O extracted sample. The distance travelled by the solvent and sample were measured to and divided to calculate the retention factor (R_f) for each spot using the equation:

$$R_f = \frac{distance\ travelled\ by\ sample}{distance\ travelled\ by\ solvent}$$

The R_f values are summarized in Table 11. The same solvent system travelled farther in the plate spotted with type O samples than in the plate with type A_1 samples. Due to this difference, the R_f values were higher for the plate with type O samples than the plate with A_1 samples, as shown in the table. The higher R_f values indicate that the compound is more non-polar, such as cholesterol, whereas GM1 is relatively polar due to the presence of sugar chains. The aqueous A fractions in both type A_1 and O show spots with high R_f suggesting the presence of residual non-polar impurities from extraction, but no detectable glycolipid spots. An absence of detectable glycolipid fractions was observed in Aqueous B samples for both type A_1 and O samples. In contrast, the organic sample in both type A_1 and O samples show four main spots with different R_f values indicating the presence of heterogenous glycolipid fractions.

Figure 6 shows the dot blot performed with pure ghost, aqueous and organic fractions probed with anti-A and UEA lectin. The aqueous fraction shows a positive signal in the type A_1 (1178) sample to anti-A, and in the type O (2207) sample to UEA lectin. The organic fractions show a weaker signal for both type A_1 and type O to anti-A and UEA lectin respectively, as compared to the signal observed in the pure ghost columns. However, it cannot be conclusively determined whether these antigens are carried on glycolipids or glycoproteins and to what extent. The absence of glycolipid-stained spots on the silica plate in Figure 5, does not completely rule out the presence of glycolipids in the aqueous fraction. It is possible that the aqueous fractions

contain a mix of water-soluble, polar glycolipids and glycoproteins which can interfere with the detection mechanism on the TLC.

Figure 7 shows the residual white precipitate collected from the extraction procedure. The pellet was solubilized as per the steps delineated in Table 7 and was loaded on SDS-PAGE gels. Figure 7 shows the resulting gel stained with SYPRO Ruby stain showing the ladder and the solubilized precipitate samples in lane two. In an effort to solubilize the pellet, exhaustive sonication had to be performed until the precipitate appeared to have dissolved into solution. However, the gel shows accumulated sample at the top of the wells suggesting the presence of insoluble precipitate. The smears and the lack of defined protein bands further suggests the degradation of the protein in the sample, most likely due to the sonication. Therefore, the insoluble precipitate fractions expected to contain the hydrophobic proteins carrying the ABH glycans could not be adequately resolved on SDS-PAGE gels.

The results shown in Figure 6 demonstrate promising ABO activity in the aqueous and organic fractions. We speculated a combined presence of polar, heavily glycosylated proteins and lipids contributing to the observed reactivity but could not extrapolate the exact source and distribution of ABH glycans. The process of further extracting and isolating the glycans was foreseeably challenging. To extract lipid from protein or vice versa, addition of reagents would be required such as chaotropes, organic solvents, detergents which necessitates further clean-up or pH adjustments before they can be analyzed on mass spectrometry instruments[96]. This clean-up process is also associated with sample dilution and loss which is undesirable given the small volumes of extracted ghost membranes[96]. Our preliminary experiments did show the presence of ABH antigens in the precipitate fraction (not shown), likely due to glycoproteins but exhaustive sonication is required to solubilize the pellet, which deteriorates the sample. Due to ambiguity in

determining the source of the ABH glycans and the limitations posed by the methods, we decided to discontinue this method and move forward with our second approach.

3.4.2 *Ghost samples Pre-PNGase enzyme treatment*

After enzymatic digestion was performed on erythrocyte ghost samples, the glycoproteins were separated and visualized on SDS-PAGE gels as described in section 2.3.3 Table 4. Figure 8 shows in-house PNGase F enzyme resolved on SDS-PAGE gels and visualized with SYPRO Ruby protein gel stain. Lane 6 shows the PNGase F prepared to load into the gel without any prior incubation, whereas lane 7 shows the PNGase F bands observed after performing a 24-hour incubation. The main PNGase F bands appear around the 60-70kDa mark while the smaller protein impurities are observed in the lower 20-50kDA range. These impurities are also observed in lanes loaded with digested ghost samples; however, they can be accounted for when using the unadulterated ghost lanes as a reference. In addition, the enzyme incubated for 24-hours at 37^0C degrades into two additional bands appearing around 50kDa and 40kDa in lane 7. These bands appear in subsequent SDS-PAGE gels performed post-incubation.

3.4.3 *Ghost Samples Post-PNGase enzyme treatment*

The ghost samples for both type A_1 and O were enzymatically cleaved by PNGase F based on the detailed protocols described in Table 10. The duration of incubation with enzyme and the scale-up of the ghost-volume were the tested parameters. Version three of the protocols describes the **current protocol** that will be used for future enzymatic digestions. Figure 9 and 10 show the ghost samples pre- and post-treatment with PNGase F on the SDS-PAGE gel and in-gel western, respectively. 0.5 μL of ghost of loaded in each lane for all the conditions across type A_1 and type O samples. The protocol for SDS-PAGE is described in section 2.3.3 Table 10 and the in-gel

western protocol is briefly described in Figure 10. The SDS-PAGE in Figure C shows the protein banding in A_1 and O ghost samples before and after the cleavage process. A PNGase F-mock ghost sample was prepared to serve as a control for monitoring any possible effects of the digestion conditions, such as addition of reagents and boiling in the absence of the enzyme. Both type A_1 and type O pure ghost samples show Band 3 and 4.5 which have been identified as N-linked glycoproteins carrying the ABH antigens[42]. Most notably, Band 3 in the ghost sample shifts down from the 95-100kDA range to the 80-90kDA range in the PNGase F digested ghost sample. This shift is likely a characteristic of the de-glycosylation of N-linked sugars, as the protein reduces in its molecular weight after the glycan is released. The analysis of other protein bands which are known to be representative of erythrocyte membrane proteins (such as Glycophorin, membrane transporters etc.) is not included in this document but will be performed in future iterations.

Between the 12-hour PNGase-digest (lane 4) and the 24-hour PNGase-digest condition (lane 6), the major observation is the presence of more defined bands in the 24-hours digest which is likely from the enzyme spike that was performed at the 12-hour mark. Since the 12-hour sample (lane 4) originated from an aliquot taken prior to the enzyme spike, the additional bands are absent which is expected. No other significant differences can be observed between the two samples.

While the same volume of ghost is loaded in each well across all conditions (pure ghost, PNGase-mock and PNGase digest), the ghost samples were not normalized based on the protein concentration prior to this experiment. Type O ghost samples contained a higher protein concentration as later reported by the BSA assay, potentially due to higher hematocrit in type O individuals as compared to their A_1 counterpart. Subsequent SDS-PAGE assays were performed with type A_1 and O ghost samples, normalized to approximately 1.94 mg/mL of protein concentration.

In-gel western technique was briefly employed prior to adopting the western blot procedure described in section 2.3.4, in an effort to address the under- and over-transfer of the proteins in an earlier version of the western blot protocol. Figure 10 shows the in-gel western result at the end of electrophoresis after the gel was fixed and probed with anti-A antibody and fluorescent goat anti-mouse IgG secondary antibody. Smears across the lanes containing the type A_1 samples suggest the possibility of specific interaction with anti-A and is similar to what we have observed in prior western in-gel experiments for these conditions (data not shown). However, reactivity observed in the defined protein bands for type O samples suggest the likelihood of non-specificity as well. Due to the contradicting reactivity observed, the in-gel western technique was discontinued, and the protocol detailed in Table 5 in section 2.3.4 was adopted. The western blot in Figure 12 and 13 are based on the updated western blot protocol described in Table 5.

Figure 11 shows the SDS-PAGE gel performed to the qualitatively evaluate any observable difference between the volume-controlled PNGase-digest lane and the scaled PNGase-digest lane. 0.5 μL of ghost volume was loaded for each lane. The aliquots for the volume-controlled PNGase-digest was taken from a recipe with 50 μL ghost volume, whereas the scaled PNGase-digest was taken from a recipe with 300 μL ghost volume. A similar shift of Band 3 and 4.5 is observed in lanes 3 and 4 for type A_1 and 8 and 9 for type O, showcasing reproducibility in deglycosylating the glycoproteins using PNGase F.

To further confirm the cleavage of ABH carbohydrate antigens, the gel was transferred to a PVDF blot following the semi-dry transfer process described in section 2.3.4. The resulting blot shown in Figure 12 with transferred membrane glycoproteins was probed with the clinical Immunocor anti-A antibody and UEA lectin to detect the A antigen and the H antigen, respectively.

Strong reactivity to anti-A antibody was observed in the lanes with type A_1 pure ghost and PNGase-mock lanes and relatively weak reactivity was observed in other lanes, possibly due to non-specific interactions. Similarly, strong reactivity to UEA lectin in Figure 13 was observed in lanes with type O pure ghost and PNGase-mock lanes and low reactivity in other lanes as well. The reduction in anti-A signal between the type A PNGase-mock lane and the PNGase-digest lane is significant and could be attributed to the cleavage of ABH glycans. An identical pattern was observed in the UEA lectin probing condition. The visible reduction in the signal observed between PNGase-mock and PNGase-digest condition suggests that a majority of the ABH antigens could be present on N-linked glycans which were cleaved in the digestion process. However, this observation needs to be confirmed by repeating the enzymatic digestion and performing a wider range of qualitative assays, not limited to western blots probed with canonical reagents. In addition, proper controls such as PNGase F only lane should be introduced to capture any specific interactions between UEA lectin and PNGase F enzyme.

Figure 14 A) and B) shows dot blots performed to evaluate the ABO activity expressed in the glycolipid and glycoprotein fractions of digested ghost membrane. The dot blots are based on the same principle as western blots, with the exception that the samples are manually spotted on the nitrocellulose directly instead of transferring from gels. In Figure 14 A), there is reduction in the signal captured between the rows containing pure ghost and the PNGase-digest spots, for anti-A antibody across type A_1 and type O. This reduction in signal is captured across a range of protein concentration in the ghost samples as indicated by the BCA results (Figure 2). The first spot has approximately 2.4 μg protein, second spot has 0.6 μg protein and finally 0.2 μg protein in the third spot. The remaining signal in each row can be attributed to the ABH glycans present on O-linked glycoproteins and ABO glycolipids. However, this pattern is not observed in the UEA lectin blot

where the post-PNGase samples maintain their reactivity to UEA lectin for both type A_1 and O. We anticipate cross-reactivity between the UEA lectin and the ghost samples due to the specificity of UEA lectin to bind to α-linked fucose which is present in the precursor structures of all ABO blood type antigens. The possible enhancement of signal after PNGase F cleavage could be due to specific interactions between UEA lectin and PNGase F enzyme. Additional controls and technical replicates of sample lanes would need to be included in the future iterations of dot blot experiments to conclusively determine the source of the reactivity observed.

Based on the observed reduction of signal in the post-PNGase F digested sample in the anti-A blot for both type A_1 and O samples when probed with canonical anti-A and UEA lectin, we believe that the deglycosylation process was successful. In addition, we speculate that the residual signal observed in the post-PNGase F condition for western and dot blots (Figure 12, 13, and 14) is due to the remaining ABH antigens present on O-linked glycoproteins and glycolipids carrying ABH antigens. If our interpretation is accurate then our results support the findings reported in literature that a majority of ABH antigens are carried by N-linked glycoproteins, and only a minority are present on the remaining O-linked glycoproteins and glycolipids[122].

However, due to the absence of certain controls, protein-normalized ghost volumes, and technical replicates, some of the observations could be artifacts of the assay. It is also possible that the canonical reagents we used to establish ABO reactivity in our western and dot blots is contributing to biased identification of blood group phenotypes. To account for any bias, it is critical to repeat the enzymatic digestion process with additional enzyme conditions and different probing reagents in the future iterations.

3.4.4 *Solublization*

One of the persistent challenges in this project has been the effective solublization of the ghost samples. We observed a tendency of the ghost membrane to precipitate at various stages during both chloroform-methanol extraction and the enzymatic digestion process. The inherent membrane hydrophobicity and the tenacious nature of the membrane glycolipids to bind to both polar and non-polar glycoprotein components could contribute to the observed insolubility[96,102].

The precipitation observed after the chloroform-methanol extraction process was expected since the extraction process fractionates the membrane based on solubility. However, even after multiple attempts at solubilizing in SDS, heating and sonicating, the insolubility persisted, and the aggregation seemed irreversible.

The ghost membranes were initially solubilized in SDS and DTT prior to starting the incubation for enzymatic digestion, but after 24 hours precipitation was still observed in the sample. We speculate that effective de-glycosylation by the PNGase F enzyme could lead to instability in the residual protein which may cause it to form aggregates in solution. Moreover, charged glycans are thought to impact the overall stability of glycoproteins since they can alter their isoelectric point (pI) which may explain the mechanism behind the precipitation[127]. Part of future work is targeted towards overcoming the challenge of insolubility and is discussed in Chapter 4.

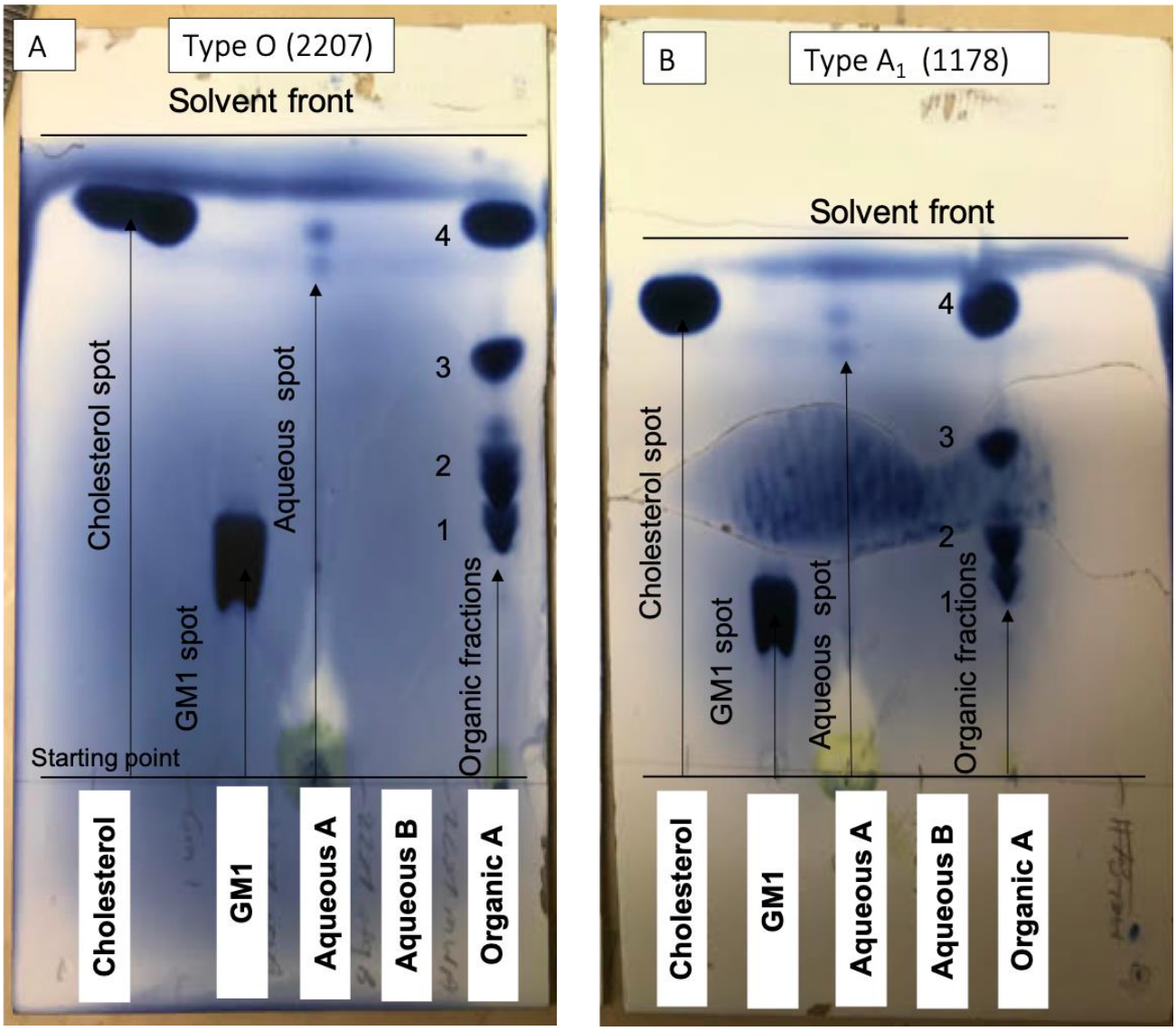


Figure 5: TLC silica gel plate showing the different fractions from the methanol chloroform extraction visualized with CAM stain migrating towards the solvent front. Left to right: cholesterol, GM1, aqueous A (bulk fraction), aqueous B (interphase fraction), organic fraction A for both type O (2207) and A_1 (1178). A) contains the sample fractions from 2207 and B) contains fractions from 1178. Respective R_f values were calculated using the solvent front migrated distance. Final calculated R_f are shown in the Table 11.

Table 11: Summary of calculated Rf values for each fraction obtained from methanol-chloroform extraction process. The Rf values represent the distance travelled by the solute relative to the solvent front.

Samples	**Type O R_f**	**Type A_1 R_f**
Cholesterol	0.95	0.85
GM1	0.44	0.33
Aqueous A	0.88	0.76
Aqueous B	0.98	0.83
Organic fraction 1	0.40	0.36
Organic fraction 2	0.52	0.42
Organic fraction 3	0.71	0.60
Organic fraction 4	0.98	0.83

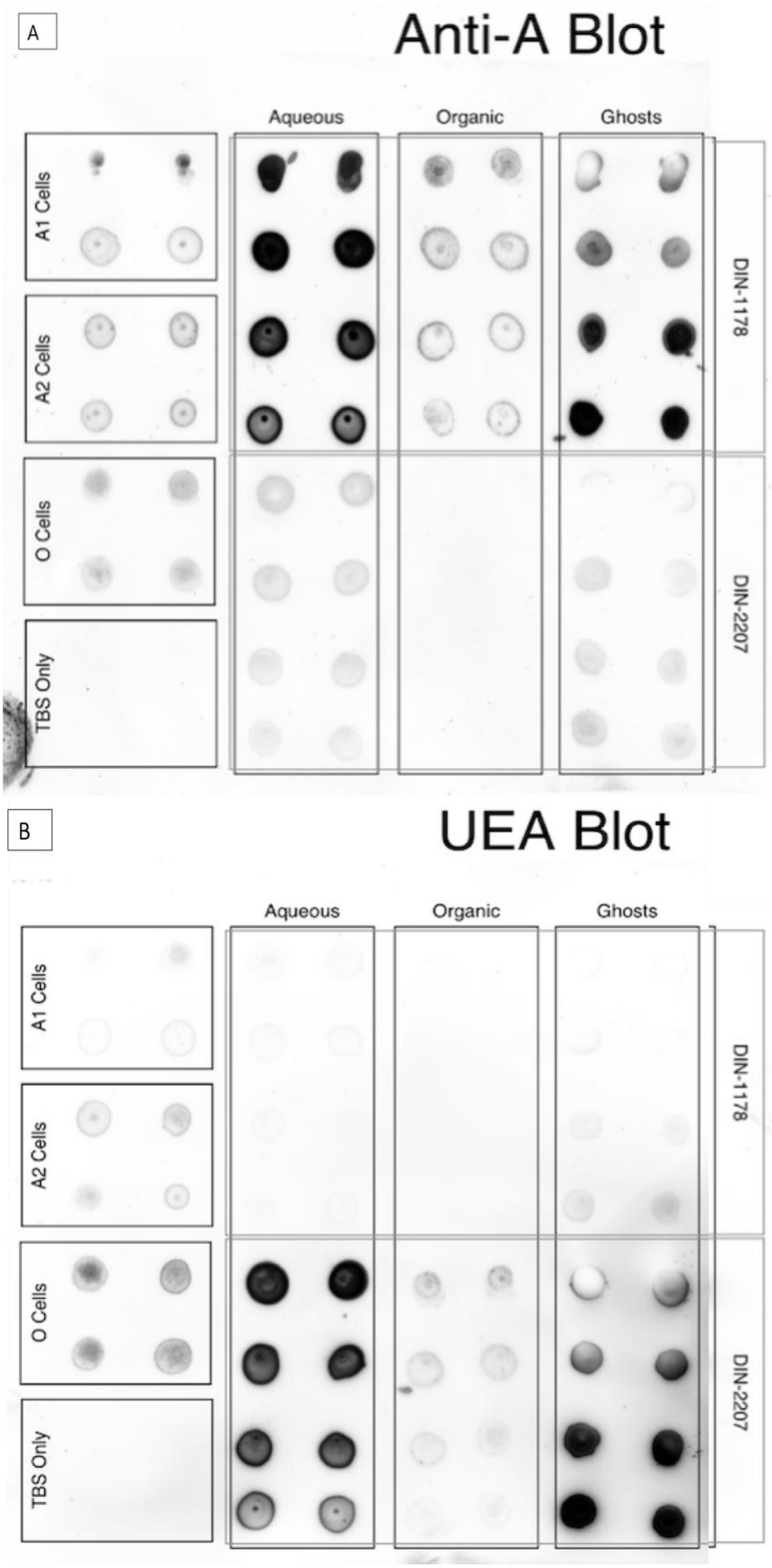
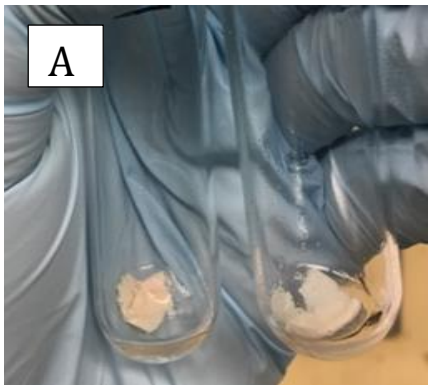


Figure 6: Dot blots performed on aqueous and organic fractions components from methanol-chloroform extraction for type A_1 (1178) and O (2207). The methodology used for dot blots is described in Table 5. A_1, A_2, and O erythrocytes are used as reference cells. A) shows ABO reactivity to anti-A and B) shows reactivity to UEA lectin in both type A_1(1178) and O (2207) samples.



B

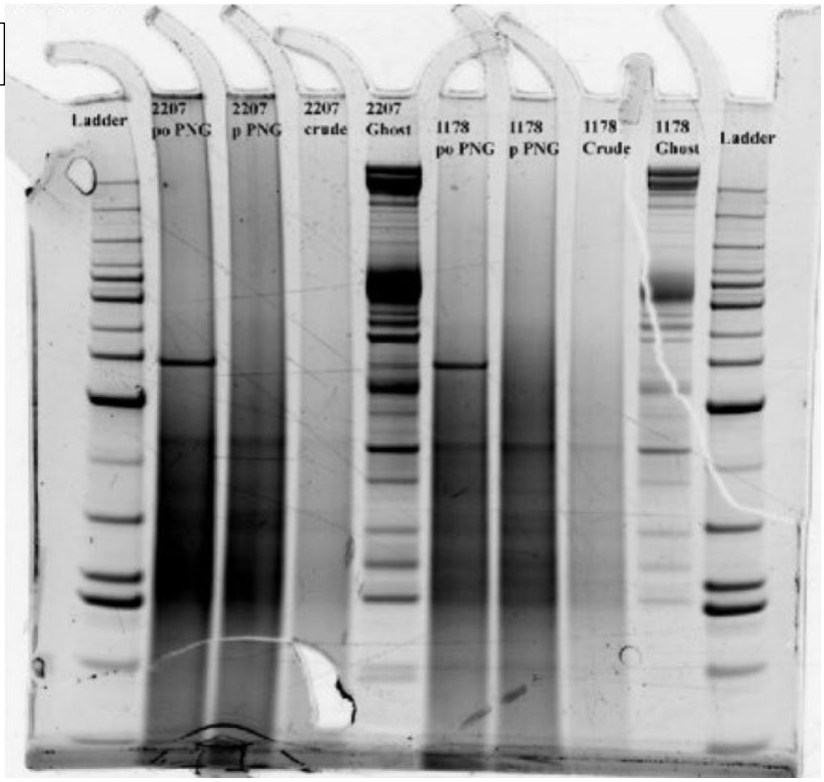
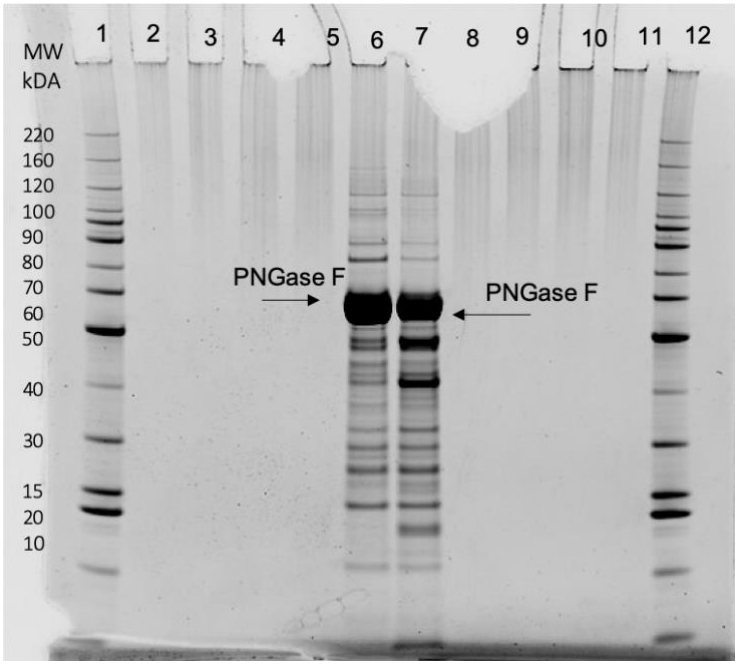


Figure 7: A) Left tube shows 2207 (type O) crude precipitate and right tube shows 1178 (type A_1) obtained from methanol-chloroform extraction. B) Precipitate fraction, crude, pre- and post-digestion visualized on SDS-PAGE stained with SYPRO Ruby stain. Left to right: Benchmark ladder, 2207(type O) post-PNGaseF, 2207 pre-PNGaseF, 2207 crude precipitate, 2207 erythrocyte ghost, 1178 (type A_1) post-PNGaseF, 1178 pre-PNGaseF, 1178 crude precipitate, 1178 erythrocyte ghost, Benchmark ladder.



1 Benchmark Pre-stained Protein Ladder
2 Blank
3 Blank
4 Blank
5 Blank
6 PNGase F
7 PNGase F post-24hr incubation
8 Blank
9 Blank
10 Blank
11 Blank
12 Benchmark Pre-stained Protein Ladder

Figure 8: SDS-PAGE showing in-house recombinant PNGaseF enzyme stained with SYPRO ruby stain.

1 Benchmark Pre-Stained Protein Ladder
2 Type A_1 erythrocyte ghost
3 Type A_1 mock (12hr)
4 Type A_1 PNGaseF (12hr)
5 Type A_1 mock (24hr)
6 Type A_1 PNGaseF (24hr)
7 Type O erythrocyte ghost
8 Type O mock (12hr)
9 Type O PNGaseF (12hr)
10 Type O PNGaseF (24hr)

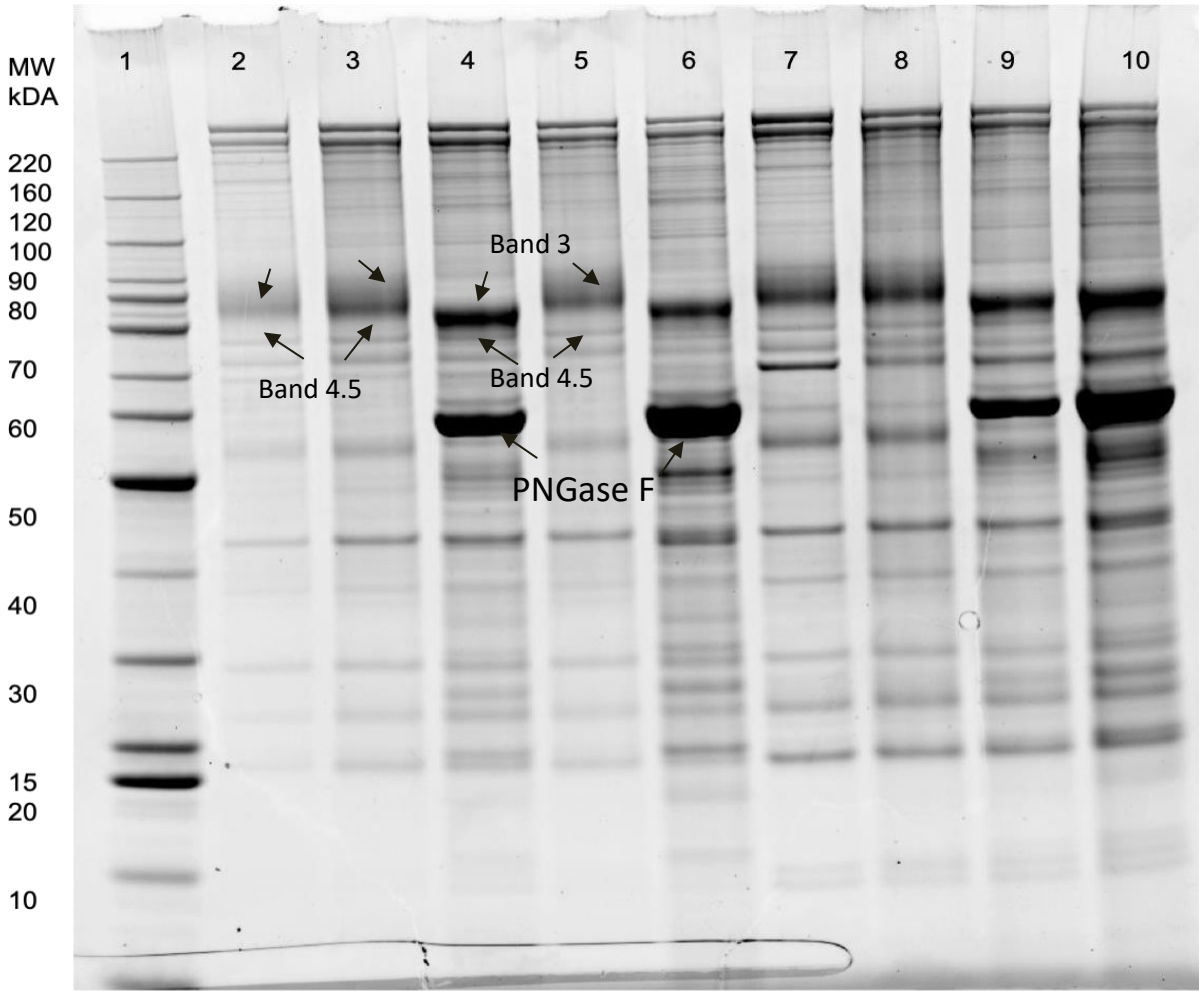


Figure 9: SDS-PAGE gel stained with SYPRO Ruby Protein stain to visualize pre- and post-PNGase F treatment, including a mock sample. Incubation times (12 vs. 24 hrs.) for digestion were tested.

1 SeeBlue Plus2 Pre-stained Protein Ladder
2 Type A_1 erythrocyte ghost
3 Type A_1 mock (12hr)
4 Type A_1 PNGaseF (12hr)
5 Type A_1 mock (24hr)
6 Type A_1 PNGaseF (24hr)
7 Type O erythrocyte ghost
8 Type O mock (12hr)
9 Type O PNGaseF (12hr)
10 Type O PNGaseF (24hr)

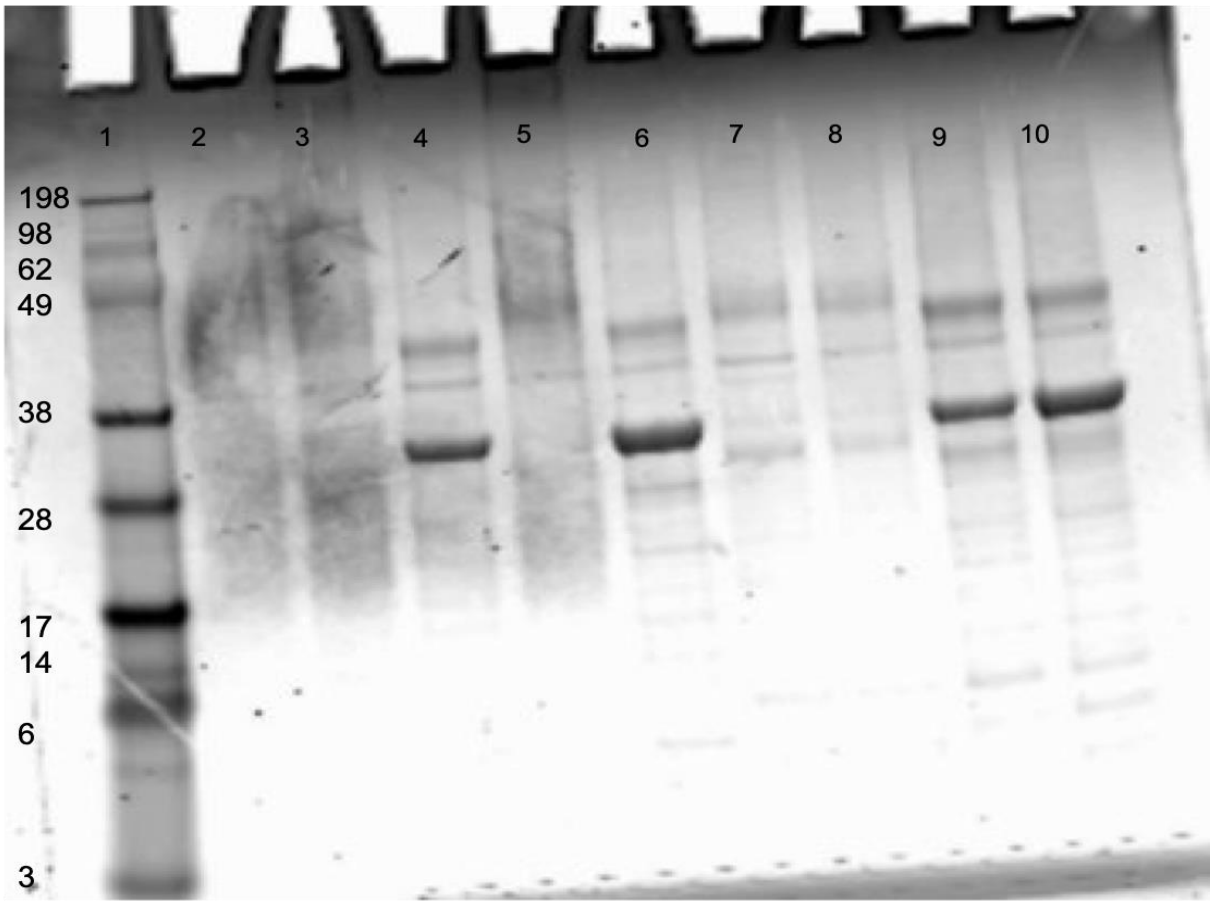


Figure 10: In-gel western procedure performed by fixing the gel in 7% acetic acid in ethanol after electrophoresis to test the ABO reactivity by probing with Anti-A (1:5) followed by fluorescent goat anti-mouse IgG secondary antibody (1:5000).

1 Benchmark Pre-Stained Protein Ladder
2 Type A_1 erythrocyte ghost
3 Type A_1 scaled PNGase-digest
4 Type A_1 volume-controlled PNGase-digest
5 Type A_1 mini PNGase-mock
6 Blank
7 Type O erythrocyte ghost
8 Type O scaled PNGase-digest
9 Type O volume-controlled PNGase-digest
10 Type O mini PNGase-mock
11 Blank
12 Benchmark Pre-Stained Protein Ladder

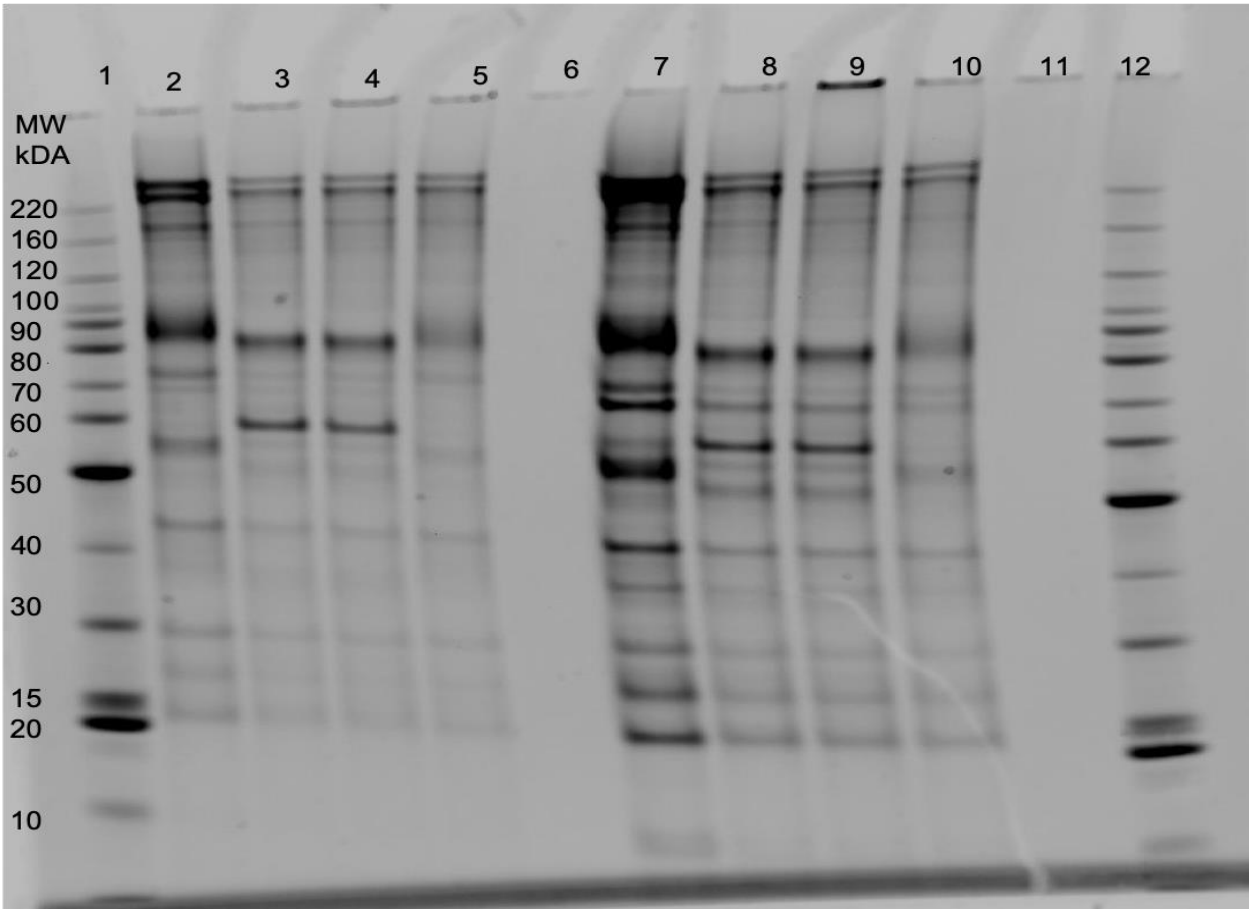


Figure 11: SDS-PAGE visualizing pre- and post-PNGase F treatment on erythrocyte ghost stained with SYPRO Ruby Protein stain for both type A_1 and O. Two volumetrically different recipes were tested: first recipe with a starting 50uL ghost volume (mini PNGaseF-digest) and second recipe with a starting 300 ul ghost volume (scaled PNGase-digest). A mock sample was included for the mini PNGase digest with a 50ul starting volume.

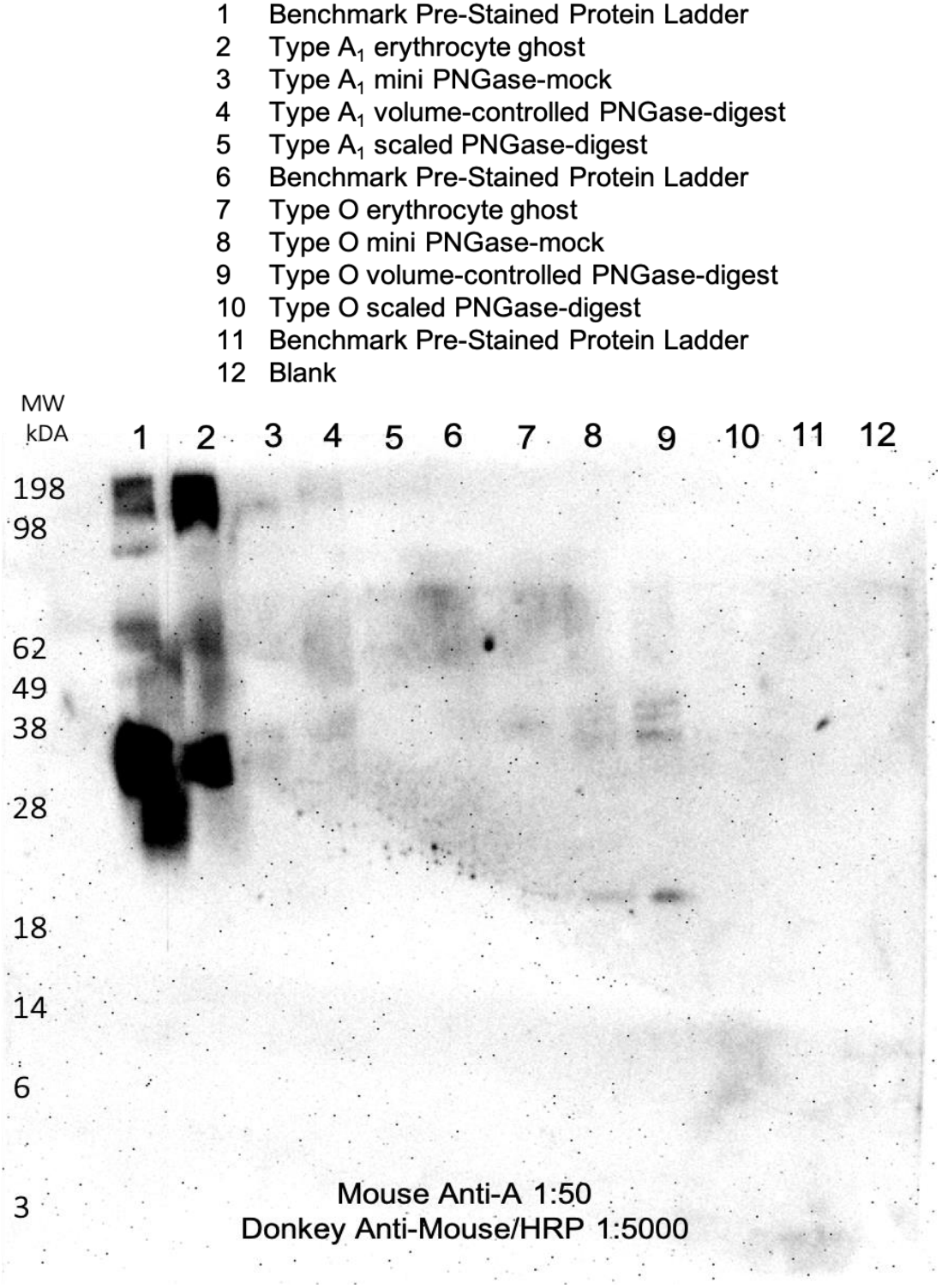


Figure 12: Western blot performed on the erythrocyte ghost samples pre- and post-PNGase F treatment for both Type A_1 and O samples, after transfer onto the PVDF membrane. Blots were probed with a mouse Anti-A antibody and a donkey anti-mouse/HRP IgG secondary antibody. Blots were developed with Pierce enhanced chemiluminescence (ECL) reagent western blotting substrate.

1 Benchmark Pre-Stained Protein Ladder
2 Type A_1 erythrocyte ghost
3 Type A_1 mini PNGase-mock
4 Type A_1 volume-controlled PNGase-digest
5 Type A_1 scaled PNGase-digest
6 Benchmark Pre-Stained Protein Ladder
7 Type O erythrocyte ghost
8 Type O mini PNGase-mock
9 Type O volume-controlled PNGase-digest
10 Type O scaled PNGase-digest
11 Benchmark Pre-Stained Protein Ladder
12 Blank

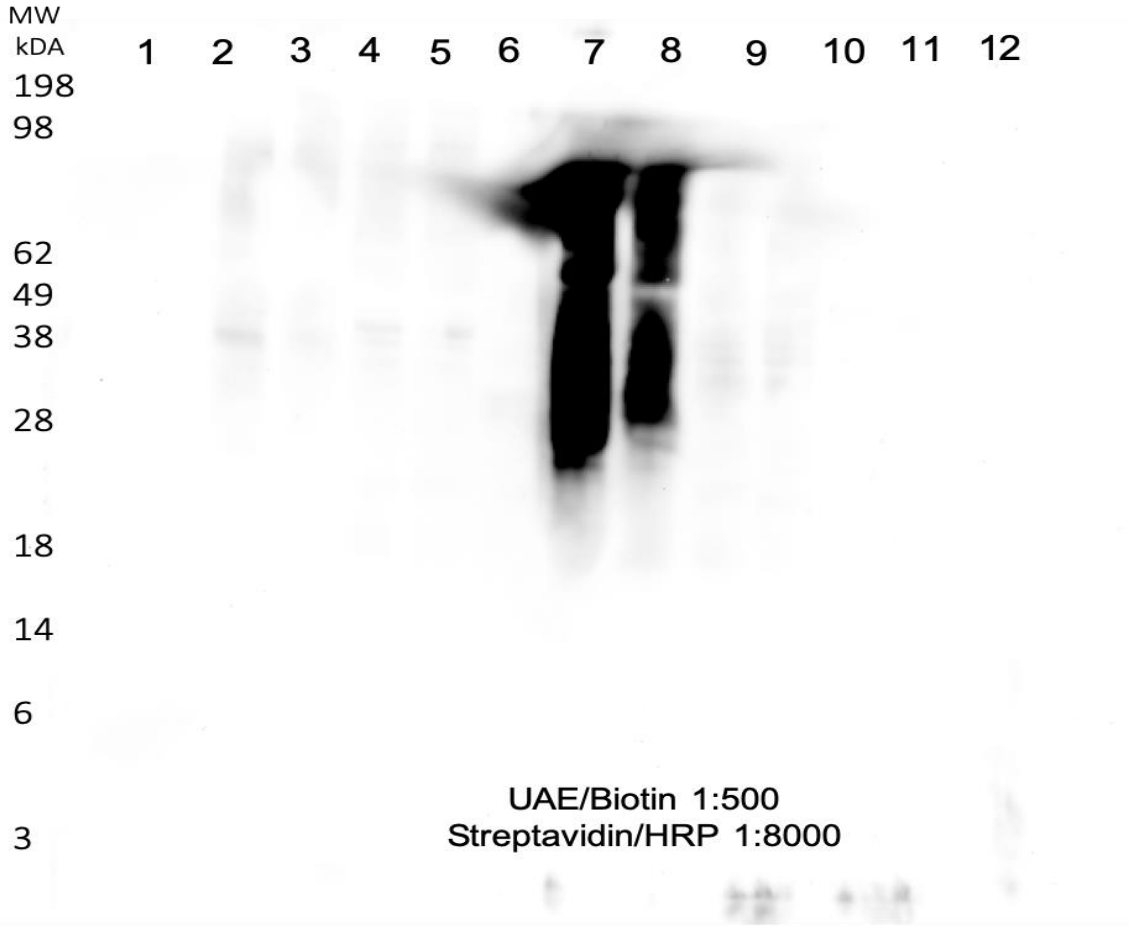
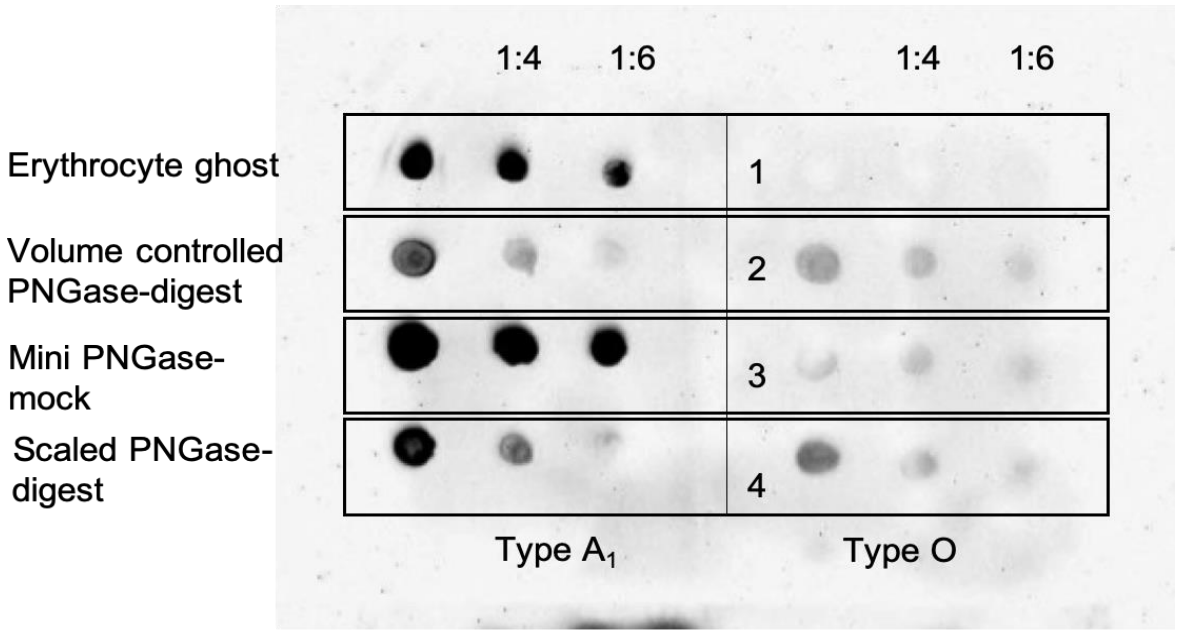
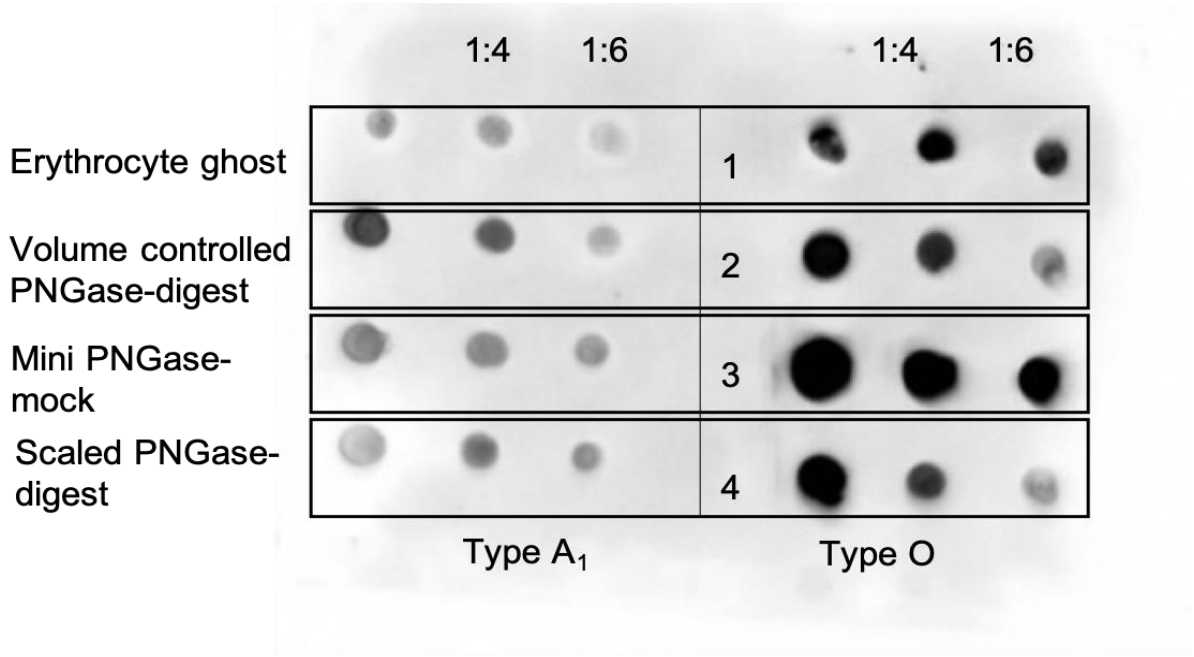


Figure 13: Western blot performed on the erythrocyte ghost samples pre- and post-PNGase F treatment for both Type A_1 and O samples, after transfer onto the PVDF membrane. Blots were probed with a biotinylated UEA lectin and a streptavidin-conjugated HRP secondary reagent. Blots were developed with Pierce enhanced chemiluminescence (ECL) reagent western blotting substrate.



Mouse Anti-A 1:50
Donkey Anti-Mouse/HRP 1:5000



UAE/Biotin 1:500
Streptavidin/HRP 1:8000

Figure 14: Dot blots performed on erythrocyte ghost samples pre- and post-PNGase F treatment for both Type A_1 and O samples. ABO reactivity to anti-A and UEA lectin was tested in A) and B), respectively. Blots were developed with Pierce enhanced chemiluminescence (ECL) reagent western blotting substrate.

Chapter 4. CONCLUSIONS AND FUTURE WORK

4.1 SUMMARY OF WORK

The ABO blood group system is inherently complex with structural nuances that are not fully understood. The overall collaborative project is aimed at elucidating the structural variances through the use of advanced mass spectrometry tools. The focus of this thesis was to develop and optimize the methodology for isolating the ABO(H) carbohydrate antigens from whole blood. Two individuals with blood type A_1 and O who consented to donate blood repeatedly were chosen and genotyped. Donated whole blood samples were processed to obtain erythrocyte ghost membranes and analyzed separately without pooling by their ABO blood group. Initial qualitative and quantitative analysis such as SDS-PAGE gels, dot blots, and BCA assays were performed on the ghost samples to validate the protein concentration and ABO reactivity between batches of ghosts obtained from different blood draws. Extraction of membrane glycolipids and glycoproteins from erythrocyte ghosts was performed using a chemical and an enzymatic approach. The latter approach was considered viable for isolating glycan components and performing further analysis. PNGase F was used to cleave N-linked glycoproteins on both type A_1 and O erythrocyte ghost samples. Preliminary SDS-PAGE and dot blot results showed a significant reduction in ABO reactivity for both type A_1 and O ghost samples after PNGase F digestion, suggesting the efficacy of the enzyme in cleaving N-linked glycans. Based on these early results, we hypothesize that a majority of ABH antigens are N-linked glycans. Further investigation is needed to support this hypothesis.

4.2 FUTURE WORK

The next step is to repeat enzymatic digestion on the ghost samples with additional enzyme conditions. EGCase I will be used to cleave glycans from the glycolipids, neuraminidase to cleave sialic acids from glycoproteins, and α-N-acetylgalactosamidase to cleave terminal non-reducing α-N-acetylgalactosamine (GalNAc) residues from N-glycans on glycoproteins. EGCase I and PNGase F digestion will be sequentially performed on the same ghost aliquot to ensure removal of glycans from both glycolipids and N-linked glycoproteins. We would expect to see a complete reduction of ABO reactivity to clinical reagents (anti-A, UEA lectin) on dot blot assays after the enzyme digestion. Since α-N-acetylgalactosamidase cleaves the immunodominant GalNAc sugar from type A glycoproteins, exposing the precursor structure, we would expect a weaker signal from anti-A antibody (recognizes the A antigen) and a stronger signal from UEA lectin (recognizes the H precursor antigen) on western and dot blots. *Dolichos biflorus* agglutinin (DBA) lectin which has specificity for α-linked GalNAc will be used a positive control to establish type A reactivity. Additionally, anti-glycophorin A antibody will be used to recognize the major sialoglycoproteins expressed on erythrocyte membranes, serving as a positive control for presence of membrane glycoproteins[128]. Strategic enzymatic cleavage will allow us to eliminate non-specific signal and determine the structural source of antigenicity. Additional controls (enzyme only samples), technical replicates, protein normalized ghost volume will be implemented for future iterations of biochemical analysis. Lastly, the insolubility observed in ghost samples post-digestion will be addressed by diluting the ghost samples prior to incubating with enzyme, adding a higher concentration of SDS detergent, and heating the ghost samples after the final incubation step.

After enzymatic cleavage, free-glycans will be conjugated to a bifunctional fluorescent linker 2-amino-N-(2-amino-ethyl-benzamide (AEAB) linker via reductive amination. The fluorescent tag allows for a facile fractionation on a number of different stationary phases as the analytes can be tracked by a fluorimeter by the Guttman Lab. The labeled glycans will then be analyzed by LC-MS using hydrophilic interactions chromatography (HILIC), which is capable of resolving subtle differences in glycan structures [129]. The second amine on the tag will allow for covalent coupling of the derivatized glycans to activate silicon oxide chip surface for downstream assay development[130]. We will continue to track all samples pre- and post-treatment through dot blots with standard ABO reagents for both glycolipids and glycoproteins to ensure that the enzymatic and chemical procedures are optimal for quantitative glycan removal. With our improved structural understanding, we hope to develop blood typing diagnostic tools using the biosensing platform to positively impact the field of transfusion medicine.

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