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# Specificity and utility of SubB2M, a new *N*-glycolylneuraminic acid lectin

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ABSTRACT

The B subunit of the subtilase cytotoxin (SubB) recognises *N*-glycolylneuraminic acid (Neu5Gc) containing glycans, the most prominent form of aberrant glycosylation in human cancers. We have previously engineered SubB by construction of a $SubB_{\Delta S106/\Delta T107}$ mutant (SubB2M) for greater specificity and enhanced recognition of Neu5Gc containing glycans. In this study, we further explore the utility of SubB2M as a Neu5Gc lectin by showing its improved specificity and recognition for Neu5Gc containing glycans over the wild-type SubB protein and an anti-Neu5Gc IgY antibody in a *N*-acetylneuraminic acid (Neu5Ac)/Neu5Gc glycan array and by surface plasmon resonance. Far-western blot analysis showed that SubB2M preferentially binds to bovine serum glycoproteins over human serum glycoproteins. SubB2M was also able to detect Neu5Gc containing bovine glycoproteins spiked into normal human serum with greater sensitivity than the wild-type SubB and the anti-Neu5Gc IgY antibody. These results suggest that SubB2M will be a useful tool for the testing of serum and other bodily fluids for cancer diagnosis and prognosis.



## 1. Introduction

The Shiga toxigenic *Escherichia coli* (STEC) Subtilase cytotoxin (SubAB) targets α2-3-linked *N*-glycolylneuraminic acid (Neu5Gc) via its pentameric B-subunit SubB [1,2]. Neu5Gc terminating glycans are not expressed at significant levels on healthy human tissues [3–6] as humans produce an inactive cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH) enzyme [7]. However, Neu5Gc containing glycans are the most prominent form of aberrant glycosylation in human cancers and can be explained by dietary intake of red meat and dairy products leading to the absorption of Neu5Gc [8].

In recent work we engineered the SubB protein to increase specificity and selectivity for Neu5Gc containing glycans by limiting Neu5Ac recognition and by broadening the types of Neu5Gc linkages recognised [9]. Of the six mutant SubB proteins produced, the $SubB_{\Delta S106/\Delta T107}$ mutant (SubB2M) fulfilled the aim of less Neu5Ac recognition and broadened Neu5Gc linkage recognition [9], including binding to Neu5Gcα2-6 containing glycans, possible cancer antigens [10]. The improved SubB2M lectin offers a potential new tool for the testing of serum and other bodily fluids from individuals with or suspected of having cancer. To further confirm the suitability of SubB2M as a tool for Neu5Gc detection in both laboratory and diagnostic settings we tested the protein with a new Neu5Ac/Neu5Gc paired structure glycan array and used the lectin in immunoassays, including, far-western blots and in Surface Plasmon Resonance (SPR) to detect Neu5Gc containing glycoproteins.

## 2. Materials and methods

### 2.1. Expression and purification of wild-type SubB and SubB2M

The wild-type SubB and SubB2M recombinant proteins were expressed and purified as previously described [9].

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### 2.2. Glycan array analysis of wild-type SubB and SubB2M

Neu5Ac/Neu5Gc glycan array slides were purchased from Z-Biotech (Aurora, Colorado, USA). The arrays were performed as per the manufacturer's instructions with the following modifications. For each subarray 2 μg of SubB proteins were pre-complexed with anti-His tag antibody (Cell signalling) and Alexa555 secondary and tertiary antibodies (rabbit anti-mouse; goat anti-rabbit; Thermo Scientific) at a ratio of 2:1:0.5:0.25 in a final volume of 100 μL. This 100 μl antibody protein complex was added to the silicon gene frame (Z-Biotech) without a coverslip. Washing was performed as previously described [11] and scanned using an Innoscan 1100AL scanner and analysed using Mapix analysis software as described in Day et al. 2017 [12].

### 2.3. Surface plasmon resonance of SubB with Neu5Ac/Neu5Gc glycan pairs

Surface plasmon resonance (SPR) was run using the Biacore T200 system (GE) as described previously [9]. Briefly, SubB, SubB2M and anti-Neu5Gc IgY (Biolegend) were immobilized onto flow cell 2–4 of a series S sensor chip CM5 (GE) using the NHS capture kit and flow cell 1 was run as a blank immobilization. Neu5Ac/Neu5Gc pairs were purchased from Chemily Glycoscience (Atlanta, GA). Glycans were used across a five-fold dilution series at a maximum concentration of 20 μM. Analysis was run using single cycle analysis and double reference subtraction on the Biacore T200 evaluation software.

### 2.4. Far-western blot analysis

#### 2.4.1. Glycoproteins

Each glycoprotein was diluted to 5 mg/ml and treated with 20 U of α2-3,6,8,9 neuraminidase (sialidase) A (New England BioLabs) in a final volume of 50 μl of 1 × GlycoBuffer 1 (New England BioLabs). 10 μg of human and bovine α1-acid glycoproteins (AGP), fetuin and asialofetuin (Sigma-Aldrich) and native bovine MUC-1 (Creative Bio-labs) (either with or without neuraminidase treatment) were combined with 1X NuPAGE sample buffer and 5% (v/v) β-mercaptoethanol, heated at 99 °C for 10mins and loaded into an SDS-polyacrylamide gel (NuPage 4–12% Bis-Tris gel, Invitrogen). The separated proteins were then transferred to nitrocellulose membrane for far-western blot analysis. The membranes were blocked with 1% fish gelatin (Sigma-Aldrich) in phosphate buffered saline (PBS) for 1 h and then washed once with PBS/0.05% Tween-20 (PBST). Then 1 μg/ml of wild-type SubB or SubB2M or chicken anti-Neu5Gc IgY antibody (1:10 000) (BioLegend) was used to detect Neu5Gc on glycoproteins. All membranes were washed in 1 × PBST three times. Blots were probed with monoclonal anti-polyhistidine-Alkaline Phosphatase mouse antibody (1:10 000) (Sigma-Aldrich) to detect binding of SubB and SubB2M or with rabbit anti-chicken IgY antibody (1:10 000) (Sigma-Aldrich) to detect binding of the anti-Neu5Gc antibody, both diluted in PBS. The membranes were washed with PBST three times, then developed with NBT/BCIP solution (Sigma-Aldrich) for 10 min.

#### 2.4.2. Sera from human and bovine

Serum samples were purchased from commercial suppliers: human (Sigma-Aldrich) and bovine (Sigma-Aldrich). Serum samples were diluted to 5 mg/ml and treated with 20 U of α2-3,6,8,9 neuraminidase (sialidase) A in a final volume of 50 μl of 1 × GlycoBuffer 1. Samples were then incubated at 37 °C for 2 h, as previously described [13]. 10 μg of total serum protein (either with or without neuraminidase treatment) was combined with 1X NuPAGE sample buffer and 5% (v/v) β-mercaptoethanol, heated at 99 °C for 10mins and loaded into an SDS-polyacrylamide gel. The separated serum proteins were then transferred to nitrocellulose membrane for far-western analysis. Membranes were probed with SubB, SubB2M and anti-Neu5Gc antibody and detected as described above for glycoproteins. Membranes were also probed with 1 μg/ml of *Sambucus nigra* agglutinin (SNA)-1-alkaline phosphatase conjugate (EY Laboratories) in PBS to detect sialic acids on glycoconjugates as an additional positive control.

### 2.5. Surface plasmon resonance of SubB proteins with Neu5Gc spiked human serum

Surface plasmon resonance (SPR) was run using the Biacore S200 system (GE) as described previously [14]. Briefly, SubB, SubB2M and anti-Neu5Gc IgY were immobilized onto flow cell 2–4 of a series S sensor chip CM5 (GE) using the NHS capture kit and flow cell 1 was run as a blank immobilization. Bovine α1-Acid glycoprotein and MUC1 were spiked into 1% normal human serum (NHS; Sigma-Aldrich) starting at 1μM. The protein was diluted 1:2 in 1% NHS across 5–6 dilutions and 1% NHS was used as the zero concentration control. Analysis was run using multi-cycle analysis and double reference subtraction on the Biacore S200 evaluation software.

## 3. Results

### 3.1. Glycan array and SPR analysis of wild-type SubB and SubB2M

To further assess the specificity of SubB2M for Neu5Gc and underlying glycan linkages, a Neu5Ac/Gc array from Z-Biotech was employed. The Z-Biotech array features 40 Neu5Ac/Gc pairs and one with both Neu5Ac and Neu5Gc on the one glycan, counted as Neu5Gc for analysis, with both linear and branched structures (Fig. S1). Binding to Neu5Gc structures was preferred by the wild-type SubB, but there were four out of 40 Neu5Ac glycans that were bound with greater than 5000 relative fluorescent units (RFU) above background and 14 out of 41 Neu5Gc structures that had binding below 5000 RFU (Fig. 1A and B). The SubB2M protein, however, showed no binding to Neu5Ac glycans with greater than 5000 RFU above background and only five out of 41 Neu5Gc structures had binding below 5000 RFU. Only eight of 40 Neu5Ac glycans showed any binding above background for SubB2M (Fig. 1A and C). It was also noted that SubB2M was better able to recognise fucosylated Neu5Gc containing glycans, sialyl-Lewis A and X, with improved binding to structures 3, 6, 9, 14, 17, 20, 23, 29 and 35 (Fig. 1B and C, Fig. S1). These are all structures containing Neu5Gc only present on the same terminal end as the sub-terminal fucosylation.

SPR analysis was performed on three sets of Neu5Ac/Gc pairs; α2-3Neu5Ac/Gc Lacto-N-neotetraose (Ac/Gc-SLNnT), Ac/Gc sialyl-Lewis X (Ac/Gc-SLeX) and Ac/Gc monosialylated ganglioside 1 (Ac/Gc-$G_{M1}$) (Table 1; Fig. S2). SubB2M and the anti-Neu5Gc IgY did not bind to any of the Neu5Ac structures, while wild-type SubB recognised the Ac-SLNnT with a $K_D$ of 2.68 μM (Table 1). Wild-type SubB bound Gc-SLeX with a similar $K_D$ (2.06 μM; Table 1) to the recognition of Ac-SLNnT and the addition of the branched fucose to the Gc-SLNnT (Gc-SLeX) reduced the $K_D$ by 300-fold. SubB2M bound Gc-SLeX with a $K_D$ (94.85nM), 21.7-fold better than the wild-type SubB but still 31-fold reduced affinity compared to the non-fucosylated Gc-SLNnT (Table 1). The anti-Neu5Gc IgY did not bind to Gc-SLeX at a maximum concentration of 20 μM (Table 1). The Gc-$G_{M1}$ was bound by all three proteins with 20–100 fold less affinity than the Gc-SLNnT demonstrating that all three of these proteins prefers Neu5Gc presented as the terminal sugar (exo) rather than on a internal branch (endo).

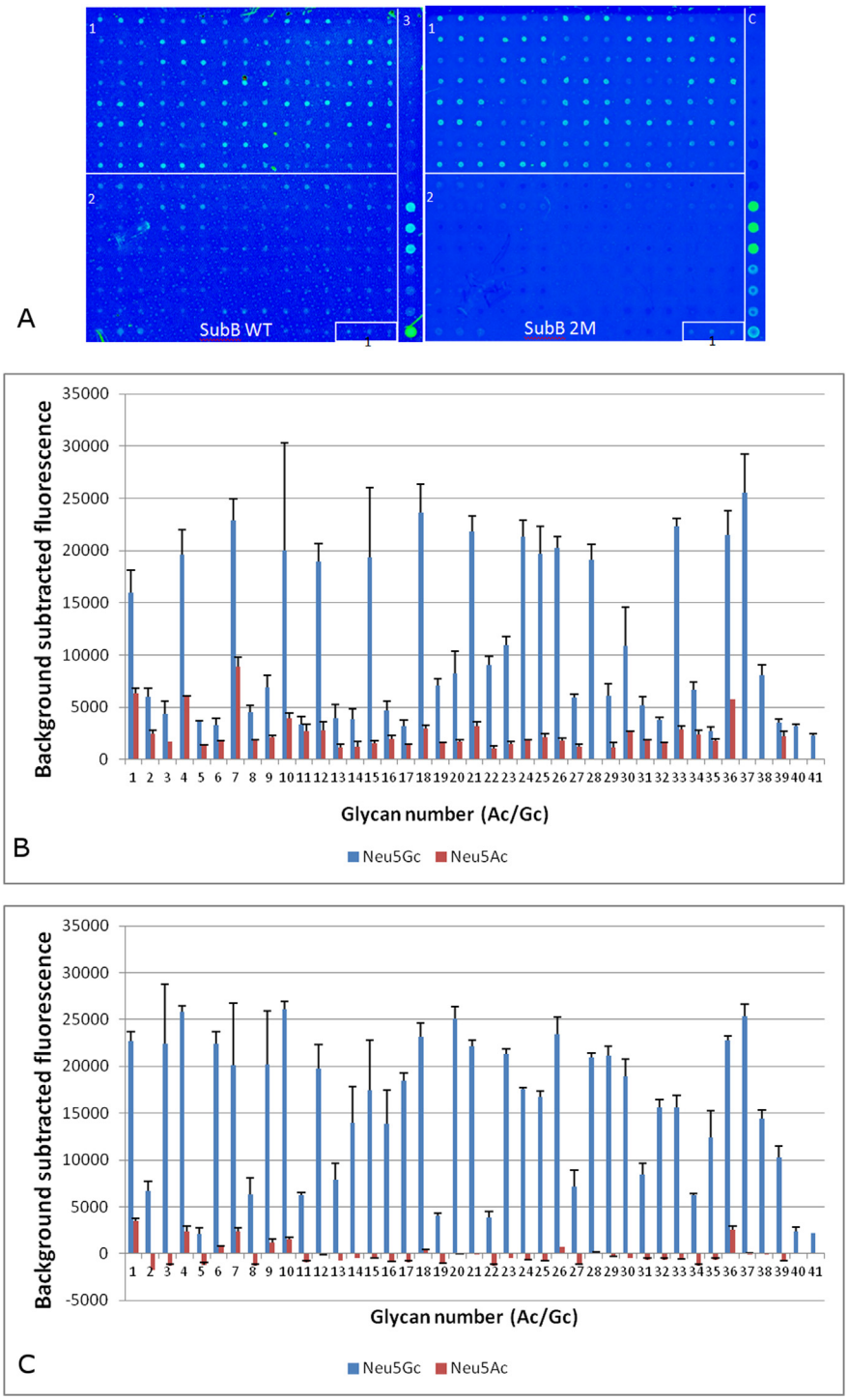
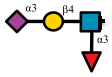
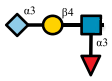
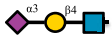
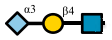



**Fig. 1. SubB WT and 2M analysis with Z-biotech Neu5Ac/Gc arrays. (A.)** Glycan array images. Region 1 (Top and 3 spots at bottom right of each subarray) = Neu5Gc Glycans. Region 2 (Bottom of each subarray) = Neu5Ac glycans. Region 3 (Right side of each subarray) = Control spots. (**B.**) Glycan array relative fluorescent units (RFU, above background fluorescence) of wild-type SubB protein and (**C.**) SubB2M protein. Neu5Gc glycans are shown in blue, Neu5Ac glycans are shown in red. For full list of glycans on the array see Fig. S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### 3.2. Far-western blot analysis of SubB and SubB2M

To confirm the specificity of SubB2M for Neu5Gc containing glycoproteins, a set of purified glycoproteins from human and bovine sources were analysed by far-western blotting. Bovine sources were selected as previous publications have demonstrated that bovine serum glycoproteins present a mixture of Neu5Ac and Neu5Gc sialylglycoconjugates [9,13], which are likely to be representative of human cancer antigens [3]. In our previous study, the human AGP was found to contain less than 1% Neu5Gc, while the bovine AGP contained a roughly 50% mixture of Neu5Gc and Neu5Ac, as determined by mass spectrometric analysis [9]. Fetuin, asialofetuin and purified MUC1 from bovine sources were also included in this analysis. While the high molecular weight of

**Table 1**
SPR analysis of Neu5Ac/Neu5Gc pairs.

| Structure | Terminal | SubB WT | SubB2M | NeuGc-IgY |
|---|---|---|---|---|
| Ac-SLeX |  | NCDI | NCDI | NCDI |
| Gc-SLeX |  | 2.058 μM ± 0.74 | 94.85 nM ± 6.6 | NCDI |
| Ac-SLNnT |  | 2.684 μM ± 1.4 | NCDI | NCDI |
| Gc-SLNnT |  | 6.78 nM ± 4.7 | 3.07 nM ± 0.58 | 164.4 nM ± 110 |
| Ac-$G_{M1}$ |  | NCDI | NCDI | NCDI |
| Gc-$G_{M1}$ |  | 606.2 nM ± 104 | 178.6 nM ± 24.3 | 3.482 μM ± 1.03 |

NCDI: No concentration dependent interaction with glycan up to 20 μM in concentration.
Gal GlcNAc GalNAc Neu5Gc Neu5Ac

bovine MUC1 (>400kDa) prevented this glycoprotein from efficiently resolving in SDS-PAGE, some smaller molecular weight glycoforms were resolved.

SubB2M had a clear preference for glycoproteins from the bovine sources compared to human and showed greater binding than wild-type SubB (Fig. 2). This binding was sialic acid dependent as binding was abolished upon treatment of the glycoproteins with an α2-3,6,8,9 neuraminidase. A minimal amount of binding was observed to the human AGP by SubB2M. This was likely due to the small amount of Neu5Gc present on this glycoprotein that was able to be detected by SubB2M only. There was also a small amount of reactivity with the asialofetuin, which was lost upon treatment with a broad range neuraminidase suggesting that there was incomplete desialylation of this protein (Fig. 2).

Positive controls performed with the anti-Neu5Gc IgY antibody verified the presence of Neu5Gc on the analysed glycoproteins, with binding of this antibody only observed to glycoproteins from the bovine sources (Fig. 2). The lectin SNA-1, which binds preferentially to α2-6 linked sialic acids, and to a lesser degree, α2-3 linked sialic acids, was also used to verify the presence of sialic acids on glycoproteins and the loss of sialic acid following neuraminidase treatment (Fig. 2). This lectin had weaker binding to the human and bovine AGP suggesting that the predominant sialic acid linkage on these glycoproteins is α2-3 linked Neu5Ac/Gc.

To further verify the specificity of SubB2M, this mutant protein was used to probe normal human serum and bovine serum, also by far-western blot analysis. As humans cannot produce Neu5Gc due to the lack of an active CMAH enzyme but can acquire Neu5Gc from dietary sources, normal human sera should have lower amounts of Neu5Gc glycoconjugates compared to bovine serum [7]. Both the wild-type SubB and SubB2M showed a preference for binding to glycoproteins from the bovine serum, with less binding observed for the human serum glycoproteins (Fig. 2). The binding to the human serum is likely due to the small amounts of Neu5Gc glycoconjugates present in this sample. Again the SubB2M showed stronger reactivity with the bovine serum glycoproteins compared to the wild-type version indicating a higher affinity for Neu5Gc containing structures. The binding by SubB and SubB2M was confirmed to be Neu5Gc dependent by treatment of serum samples with neuraminidase. This resulted in loss of binding by both SubB proteins (Fig. 2).

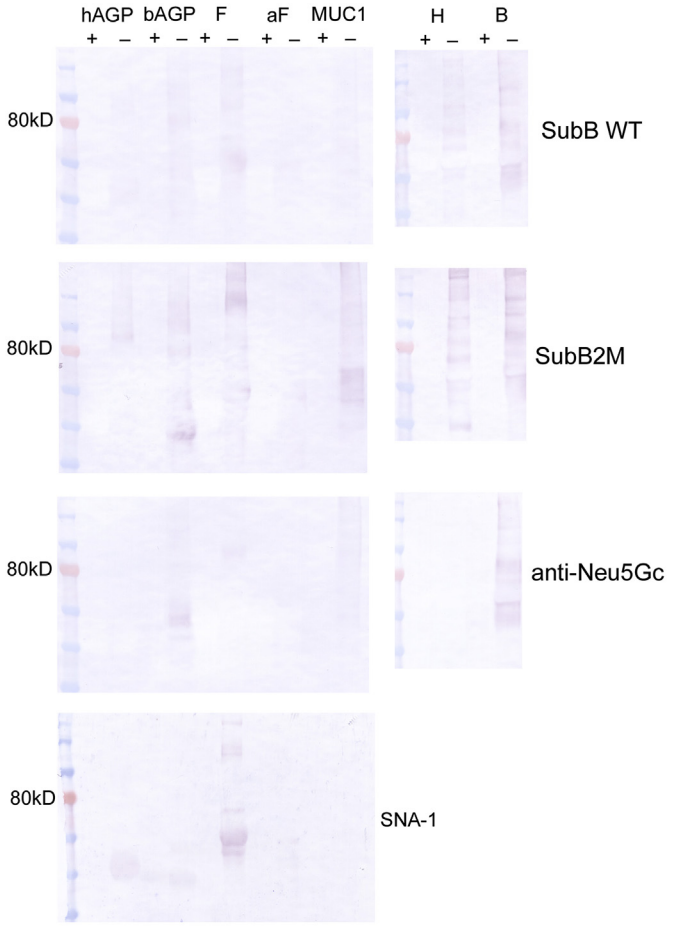



**Fig. 2. Far-western blot analyses of purified and serum glycoproteins from human and bovine sources**. Purified serum glycoproteins (hAGP = human α1-acid glycoprotein, bAGP = bovine α1-acid glycoprotein, F = bovine fetuin, aF = bovine asialofetuin, MUC1 = bovine MUC1) as well as normal human serum (H) and bovine serum (B) were treated with (+) or without (–) α2-3,6,8,9 neuraminidase to remove all sialic acids from glycoproteins. Membranes were probed with wild-type SubB, SubB2M, anti-Neu5Gc IgY antibody or SNA-1. Complete far-western blot images including controls and Coomassie stained gels can be found in Supplementary Material (Fig. S3 and Fig. S4).

The chicken anti-Neu5Gc IgY antibody only showed reactivity with bovine serum without neuraminidase treatment confirming the presence of Neu5Gc glycoconjugates in this sample. SubB2M showed greater reactivity to Neu5Gc-containing glycoproteins compared to the antibody (Fig. 2).

### 3.3. SPR analysis of Neu5Gc-glycoproteins spiked into human serum

To determine if the SubB2M protein is capable of detecting Neu5Gc containing glycoproteins in human serum, bovine AGP and native bovine MUC1 were spiked into normal human serum. The bovine AGP used has been previously examined using glycoproteomics [9] and was found to only have one Neu5Gc glycosylation on the protein, so initially the bovine AGP was used at a maximum concentration of 1μM, equivalent to 1μM of Neu5Gc containing glycan. The wild-type SubB interacted strongly with the NHS control and so interactions with the bovine AGP when present were less than the background (Fig. 3A). SubB2M was able to detect down to 62.5nM of bovine AGP above background (Fig. 3B). No binding was observed at any of these concentrations of bovine AGP tested using the anti-Neu5Gc antibody (Fig. 3C). Using these data as a guide, native bovine MUC1 was initially analysed at a maximum concentration of 200nM down to 1.5625nM across a 2-fold dilution series. This was recalibrated based on the results with SubB and SubB2M analysed and was started at 1nM (Fig. 3D and E), while the 200nM data was used for the anti-Neu5Gc IgY (Fig. 3F). SubB2M showed the same response to 0.125nM of bovine MUC1 above NHS background as wild-type SubB showed to 1nM and anti-Neu5Gc IgY to 200nM.

## 4. Discussion

Detection of Neu5Gc in human serum and other bodily fluids could be utilised as a diagnostic and prognostic marker in a range of human cancers including breast, ovarian, prostate, colon and lung cancer [5,6]. The SubB2M mutant was previously shown to have improved recognition of Neu5Gc compared to both wild-type SubB and the anti-Neu5Gc IgY antibody produced by Sialix (now supplied by Biolegend) but this was limited to a small subset of

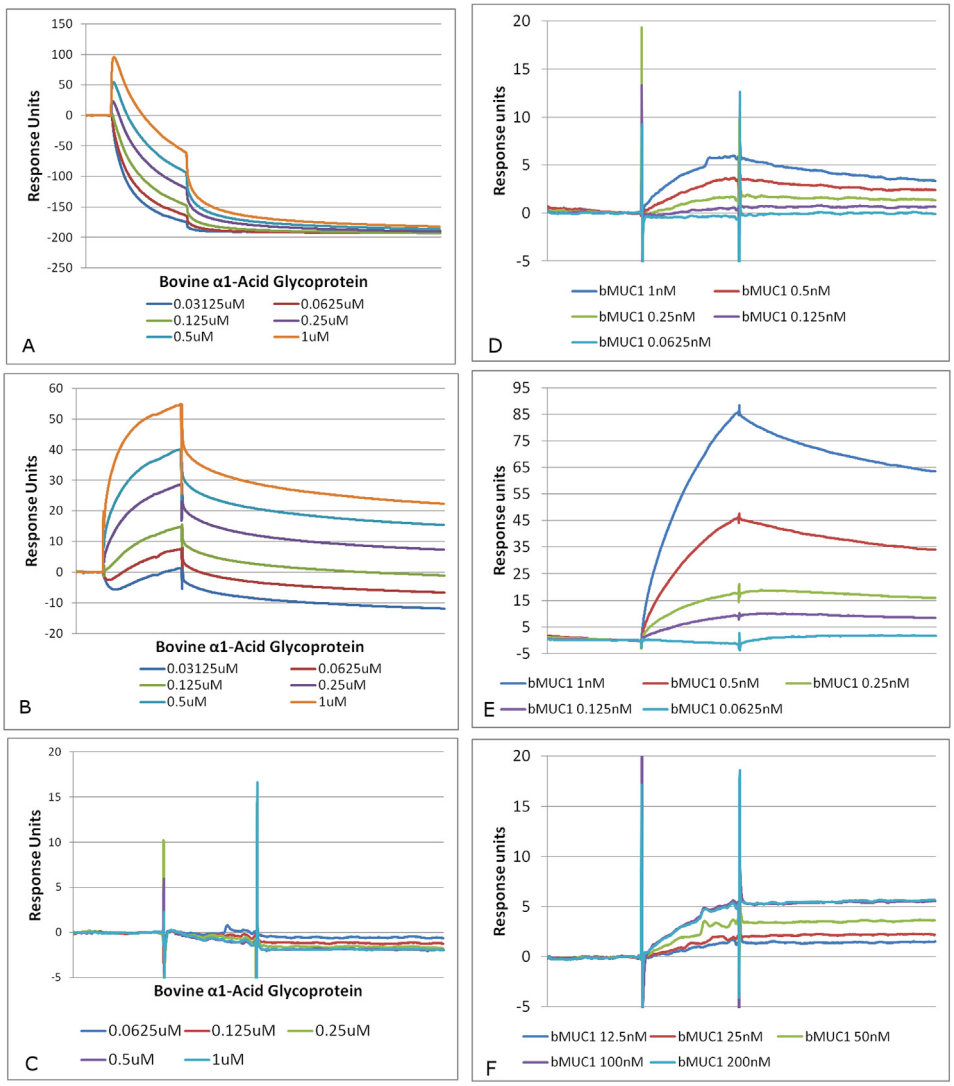



**Fig. 3. SPR analysis of SubB WT, 2M and anti-Neu5Gc IgY with spiked normal human serum (NHS).** NHS spiked with bovine α1-acid glycoprotein (AGP) with (**A.**) wild-type SubB protein, (**B.**) SubB2M protein and (**C.**) anti-Neu5Gc IgY. NHS spiked with bovine MUC1 (bMUC1) with (**D.**) wild-type SubB protein, (**E.**) SubB2M protein and (**F.**) anti-Neu5Gc IgY. Multiple cycle analysis of the binding of spiked serum above NHS serum background. Each line represents one concentration of protein in 1% NHS. Interactions are represented by binding values above zero.

Neu5Ac/Gc glycan pairs on a glycan array and in SPR [9]. In this study we used a comprehensive Neu5Ac/Gc glycan array to examine the full range of SubB2M binding. These studies demonstrated that SubB2M recognises Neu5Gc glycans exclusively, with only very low signals for a small number of Neu5Ac containing glycans. Compared to the SubB wild-type protein this represents a 4–5 fold improvement in selectivity and specificity. Importantly, SubB2M had improved recognition of the fucosylated Neu5Gc containing glycans, sialyl-Lewis A and X. These glycans have great potential to be cancer biomarkers [10]. The Neu5Ac/Gc array analysis demonstrated that the wild-type SubB protein did not recognise Neu5Gc very well when a sub-terminal fucose was present (Glycans 6, 9, 14, 17, 20, 23, 29 and 35; Fig. 1B and C, Fig. S1). The deficiency was overcome in the SubB2M protein and was not previously noted in our prior studies [1,9]. The SPR analysis also demonstrated that the anti-Neu5Gc IgY was not able to recognise sialyl-Lewis X at concentrations below 20 μM (Table 1; Fig. S2) indicating that SubB2M is also an improvement over the anti-Neu5Gc antibody when it comes to recognising Neu5Gc Lewis antigens.

Purified glycoproteins and serum glycoproteins from bovine sources were used in this study to represent glycoproteins enriched in Neu5Gc, and therefore to mimic the aberrant Neu5Gc glycosylation potentially present in human cancer serum samples. Far-western blot analysis clearly demonstrated that SubB2M had a preference for binding to glycoproteins from bovine sources, with stronger reactivity compared to the wild-type version, further demonstrating the utility of SubB2M as a cancer diagnostic tool and as a Neu5Gc specific lectin.

SPR was then used to determine if the SubB2M protein could detect elevated levels of Neu5Gc containing glycoproteins within normal human serum. The SubB2M protein was found to be able to detect 62.5nM bovine AGP spiked into NHS. This is equivalent to 62.5nM of Neu5Gc glycan due to there only being one Neu5Gc glycan per protein [9] or 6.25μM of Neu5Gc in 100% serum. The wild-type SubB and the anti-Neu5Gc IgY were unable to detect any amount of bovine AGP above the serum control at 1μM (100μM 100% serum equivalent) suggesting that the SubB2M protein is a significant improvement for a potential diagnostic over the wild-type protein and the IgY antibody.

A known human cancer serum biomarker is the heavily glycosylated mucin, MUC1 [15–17]. Levels of MUC1 are elevated in many kinds of epithelial tumors and tumor associated MUC1 has shortened glycosylation with extensive sialylation [18,19]. The presence of Neu5Gc has been confirmed on this human tumor antigen [20]. We used bovine MUC1, which is sialylated with both Neu5Gc and Neu5Ac, to mimic human tumor associated MUC1. The presence of Neu5Gc on bovine MUC1 was confirmed in our far-western blot analysis. When bovine MUC1 was spiked into NHS a much better signal to noise ratio was obtained for all three proteins; SubB2M, wild-type SubB and the anti-Neu5Gc IgY. SubB2M showed the best response to bovine MUC1 and detection at concentrations of <1nM.

SubB2M was previously found to have improved specificity and selectivity compared to the anti-Neu5Gc IgY antibody and the wild-type SubB protein [9]. Here we have provided further evidence that SubB2M offers a significant improvement over both wild-type SubB and the anti-Neu5Gc antibody with increased binding to differently linked Neu5Gc, and Neu5Gc on a wide range of structures, including fucosylated Lewis antigens, and on various serum glycoproteins. These outcomes suggest that SubB2M will be a useful tool for the testing of serum and other bodily fluids for the diagnosis and determining the prognosis of individuals with cancer or suspected of having cancer.

## Competing financial interests

The following authors (AWP, JCP, CJD and MPJ) declare that they inventors on a patent on matter contained in this manuscript.

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## Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.04.151.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.04.151.

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