

RESEARCH ARTICLE

Multiplex profiling of glycoproteins using a novel bead-based lectin array

Hong Wang^{1,2,3*}, Hong Li^{1,2*}, Wei Zhang^{1,2}, Liming Wei², Hongxiu Yu² and Pengyuan Yang^{1,2**}

¹ Department of Chemistry, Fudan University, Shanghai, China

² Institutes of Biomedical Sciences, Fudan University, Shanghai, China

³ Shanghai Institute for food and drug control, Shanghai, China

Lectin array is becoming important in profiling targeted glycan/glycoprotein, but weak interaction between lectin and glycan causes low sensitivity of the approach. This study aims to develop a bead-based lectin array for improving the sensitivity of glycosylation profiling. Lectins are chemically coupled to fluorescent dye coated microbeads, and glycan-lectin recognition is carried out three dimensionally. The performance of this platform was evaluated, and the LOD of lectin *Ricinus communis* agglutinin 120 (RCA120) was 50 pg/mL (1 pM) of asialofetuin, providing the bead-based lectin microarray with the highest sensitivity among the reported lectin microarrays. Furthermore, multiplexed assay was performed, which allowed the simultaneous detection of multiple carbohydrate epitopes in a single reaction vessel. The glycosylation patterns of hepatocellular carcinoma associated immunoglobulin G were analyzed, and increased (α -1,6) core fucosylation and (α -2,6) sialylation patterns were observed, which may provide significant clinical evidence for disease diagnosis.

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1 Introduction

Glycosylation is a crucial and complex PTM in eukaryotic proteins. Glycans are involved in cell differentiation, development, tumorigenesis, metastasis, and immunity [1]. Glycan structural alterations are a universal hallmark of tumors. Human serum glycoproteins, such as α -fetoprotein-L3, α 1-acid glycoprotein, haptoglobin (Hp), and IgG, are widely

used disease-specific biomarkers. Increased (α -2,6) sialylation of Hp measured by *Sambucus nigra* lectin (SNA) was detected in hepatocellular carcinoma (HCC) patients [2]. The alterations in the glycan structure of IgG were described in patients with ovarian, lung, gastric, and prostate cancers [3].

Despite the significant role, studies on glycosylation have been hindered by its structural heterogeneity and diversity due to its nontemplate-driven biosynthesis. Glycans with carbohydrate components have variable glycoside linkages and abundant isomers. Lectin microarray is a comprehensive platform for glycoproteomics in high-throughput investigations. Lectins selectively recognize specific oligosaccharide epitopes that provide crucial sugar/linkage information, and can discriminate structural isomers, such as (α -2,6)/(α -2,3)-linked sialic acid. Therefore, lectin microarray has been considered

Correspondence: Dr. Hongxiu Yu, Institutes of Biomedical Sciences, Fudan University, 138 Yi Xueyuan Road, Shanghai, 200032, China

E-mail: hongxiuyu@fudan.edu.cn

Fax: +86 21 5423-7961

Abbreviations: AAL, *Aleuria aurantia* lectin; ASF, asialofetuin; FET, fetuin; HCC, hepatocellular carcinoma; Hp, haptoglobin; mLAM, mouse laminin; PHA-E, *Phaseolus vulgaris* Erythroagglutinin; PNA, peanut agglutinin; PSA, *Pisum sativum* agglutinin; RCA120, *Ricinus communis* agglutinin 120; RNB, ribonuclease B; SNA, *Sambucus nigra* lectin; streptavidin-PE, streptavidin-R-phycoerythrin; WGA, wheat germ agglutinin

*These authors contributed equally to this work.

**Additional corresponding author: Professor Pengyuan Yang, E-mail: pyyang@fudan.edu.cn

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a key tool in profiling glycoproteins [4], cell surfaces [5], bacteria [6], and clinical biomarkers [7,8].

However, lectins and glycans have weak interactions ($K_d = 10^{-7}$ – 10^{-3} M) [9] that contribute to the low sensitivity of lectin microarray. Kuno et al. applied an evanescent-field fluorescence that detected lectin–glycan interactions under equilibrium conditions to increase the sensitivity of lectin microarray [9]. In antibody-overlay assay, the sensitivity was enhanced using a HRP-mediated tyramide signal amplification method [10]. Moreover, a ratiometric two-color approach provided a rapid and sensitive duplex assay [11]. Leipold et al. reported a multiplexed glycoprofiling method based on the attachment of metal-chelating polymers to lectins, which are more amenable to high-throughput analysis [12]. The construction of a novel fluorescent lectin array using a 3D hydrogel matrix has been reported [13].

Bio-Plex (Bio-Rad) suspension array system is a novel platform for immunoassay using microbeads coated with discrete amounts of two fluorescent dyes (red and infrared) instead of a flat surface for antibody immobilization. Up to 100 different types of commercially available beads, each showing a unique fluorescent signal and performing a separate immunoreaction, can be simultaneously detected by a flow cytometer. Simultaneously, the selectively captured proteins are measured by another fluorescent signal from the streptavidin-R-phycoerythrin (streptavidin-PE). Compared with the planar array, the bead-based array includes the 3D reaction and multiplexed assay, making it a sensitive and rapid immunoassay method [14]. The assay is performed in a 96-well plate, enabling high-throughput screening with improved reproducibility [15]. Bio-Plex assay also determines lectin specificities by coupling carbohydrates or glycopeptides on the microspheres, and reveals lectin and carbohydrate interactions [16,17]. However, the application to the bead-based lectin microarray has not yet been reported.

Bead-based lectin arrays have been used to isolate the glycoproteins in biological samples, which were then digested into peptides followed by LC-MS/MS identification [18,19]. In the present study, a novel bead-based lectin array system was developed. Lectins were conjugated to different microbead populations, and incubated with biotin-labeled glycoproteins in a suspended liquid condition. A double-laser fluorescence system simultaneously identified the immobilized lectin through the bead signal and lectin–glycan interactions through the PE signal. The previous strategy of combining bead lectin arrays with MS is suited to glycoprotein identification, while the present system enables direct, rapid, high-sensitive glycan pattern profiling. This platform has a lower LOD, 50 pg/mL (1 pM) of asialofetuin (ASF) using lectin *Ricinus communis* agglutinin 120 (RCA120), which is twice more sensitive than previously reported lectin microarrays [4]. A mixture of different bead types, each immobilized with a specific lectin, was incubated with a sample for multiplexed assay, providing greater information from one experiment. Furthermore, the quantitative analysis of HCC-associated IgG glycosylation alterations was performed. Com-

pared with healthy controls, increased core (α -1,6) fucosylation and (α -2,6) sialylation in IgG from HCC patients were observed, suggesting the bead-based lectin array is potentially powerful for clinical investigations.

2 Materials and methods

2.1 Coupling of magnetic beads

Lectins (Vector or EY) were coupled with activated, carboxylated magnetic beads (Bio-Rad) subsets #26, #45, and #64, using an amine coupling kit (Bio-Rad). For a $1 \times$ scale activation reaction, 100 μ L of the magnetic beads (1.25×10^6 beads) were activated with 500 μ g of EDC (Sigma-Aldrich) and 500 μ g of sulfo-NHS (Sigma-Aldrich) for 20 min at room temperature in the dark. The beads were then washed and resuspended in PBS. For the coupling reaction, 15 μ L of activated beads were incubated with varying lectin concentrations at 4°C overnight. Afterward, nonimmobilized lectins were washed out, and the magnetic beads were blocked with “Stabil Guard Choice” protein-free blocking solution (SurModics) at room temperature for 3 h. The coupled magnetic beads were then washed and resuspended in 45 μ L of storage buffer.

2.2 Direct array

ASF, fetuin (FET), HRP, ribonuclease B (RNB), BSA, mouse laminin (mLam), and IgG (all from Sigma-Aldrich) were labeled with biotin using a biotin labeling kit (Dojindo). The NH_2 -reactive biotin was added to the protein solution on a filter membrane, and incubated at 37°C for 10 min. Excess biotin was removed using a filtration tube. The labeled proteins were diluted to 58.5 μ L of incubation buffer (PBS containing 0.5% Tween-20), and incubated with 1.5 μ L lectin-coated beads (6000 beads) at 4°C overnight. For the multiplexed assay, two or three bead subsets (6000 beads per subset) were incubated with labeled proteins in 60 μ L of incubation buffer. Lactose or unlabeled glycoprotein was also added to the incubation buffer in the inhibition experiments. The beads were then washed three times with 100 μ L of wash buffer (PBS containing 0.1% Tween-20), and incubated with 50 μ L of 1 μ g/mL streptavidin-PE (Invitrogen) at room temperature for 40 min in incubation buffer. Finally, the beads were washed and resuspended in 60 μ L of wash buffer.

2.3 Antibody-overlay array

The biotin labeling of antihuman Hp antibody (Dako) was performed as described in Section 2.2. Antibody-overlay assay was performed as previously described [20]. Human serum Hp (Sigma-Aldrich) was diluted to 97.5 μ L of incubation buffer (PBS containing 1% Triton X-100), and incubated with 2.5 μ L of lectin-conjugated beads (10 000 beads) at 4°C overnight. For the multiplexed assay, two bead subsets (10 000

beads each) were incubated with Hp in 100 μ L of incubation buffer. Human polyclonal IgG (20 μ g) was added and incubated 30 min to mask the residual binding sites. Afterward, the beads were washed three times and incubated with 100 μ L of 0.3 μ g/mL biotinylated antihuman Hp antibody solution in incubation buffer at room temperature for 1 h. The beads were then washed and incubated with 100 μ L of 1 μ g/mL streptavidin-PE in incubation buffer at room temperature for 30 min. Finally, the beads were washed and resuspended in 100 μ L of incubation buffer.

2.4 Signal detection and data analysis

The streptavidin-PE-reporter signal was measured using the Bio-Plex system. A classification laser (635 nm) excited the fluorophore coated on the beads, classifying the set of beads. A reporter laser (532 nm) excited the fluorophore of streptavidin-PE, and the emitted fluorescence signal was recorded and converted to mean fluorescence intensity. The high-sensitivity range was used by selecting the “run at high RP1 target.” The signals were gated with DD gates at 5000 to 25 000 to exclude damaged or cross-linked beads. Seventy-five beads per bead set were measured. Two or three bead sets were selected for the multiplexed assay.

The mean background from the array was subtracted and divided by the signal intensity prior to evaluation. The mean value and SD of the net intensity value were determined for three replicates per sample. CVs were calculated to assess the reproducibility. LOD is the minimum concentration at which net signal/SD > 3, S/N > 1.5 were reached [8,21]. The statistical significances of the changes were evaluated with *t*-test, and a change associated with a *p*-value lower than 0.05 was considered to be significant.

2.5 Glycan cleaving by PNGase F and O-glycanase

ASF was divided into two identical aliquots, and digested with PNGase F (New England Biolabs) and O-glycanase (ProZyme), respectively (Supporting Information S-1). Deglycosylated ASF was collected and labeled with biotin using a biotin labeling kit.

2.6 Purification and validation of IgG from healthy and HCC sera

Pooled serum samples from three HCC patients and three healthy individuals at the Zhongshan Hospital (Shanghai, China) were stored at -80°C before processing. IgG from the pooled sera were purified using the Melon Gel IgG Spin Purification Kit (ThermoFisher). The purification support was transferred to the spin column, and washed with purification buffer. Afterward, 100 μ L of diluted serum was added and incubated for 5 min at room temperature. IgG was finally collected via centrifugation. Purified IgG concen-

trations were determined by Bradford assay. Approximately 20 μ g of purified IgG from healthy and HCC sera was labeled with biotin and analyzed using the bead-based lectin array in a direct array.

For lectin blotting validation, about 20 ng of unlabeled purified IgG was reacted with biotinylated SNA or *Pisum sativum* agglutinin (PSA; Vector). After incubated with Extravidin-HRP (Vector), bands were visualized using an ECL plus detection system. The detailed protocols for setting up the assay are in the Supporting Information (S-2).

3 Results and discussion

3.1 Bead-based lectin array approach

Seventeen lectins with a range of identified specificities, which played significant roles in the recognition of glyco-alteration, were selected (Supporting Information Table 1). In the bead-based lectin array, lectins immobilized on microspheres were spread three dimensionally, making them more accessible to the targets than in the traditional 2D microarray surface [22]. Figure 1 describes the key steps of the approach, which can be divided into single and multiplexed assays. The former was performed using a single type of lectin-modified beads, whereas the latter was performed using multiple types of lectin-modified beads. The multiplexed assay, which allows the simultaneous multiple glycan detection in a single workflow, can greatly expedite glycoprofiling and reduce time, cost, and labor.

The binding reaction can be further categorized into direct and indirect (antibody-overlay) profiling. In direct profiling, the lectin–glycan reaction was visualized by the interaction between streptavidin-PE and the biotinylated glycoprotein. In indirect profiling, a biotinylated antibody was used to detect its corresponding glycoprotein, and the reaction was visualized by the interaction between streptavidin-PE and biotinylated antibody, which can determine the glycan structures of target glycoprotein. Two solid-state lasers detected and recorded the fluorescence signal of the microsphere. The classification laser excited the fluorescent dye, whereas the reporter laser excited the streptavidin-PE.

3.2 Method validation of the single assay

A series of experiments were performed to optimize and validate the novel platform. The coupling reaction for lectin bead preparation was optimized for latter optimum binding between the bound lectin and its complementary glycoprotein. Supporting Information Fig. 1 and Table 2 show the different optimum lectin amounts evaluated by the mean fluorescence intensity trends of the glycoproteins and the corresponding negative controls (BSA and blank). To determine if the binding signals observed resulted from direct interactions between the lectins and glycans, we tested the specificity using the simple sugar inhibitors, lactose. Binding

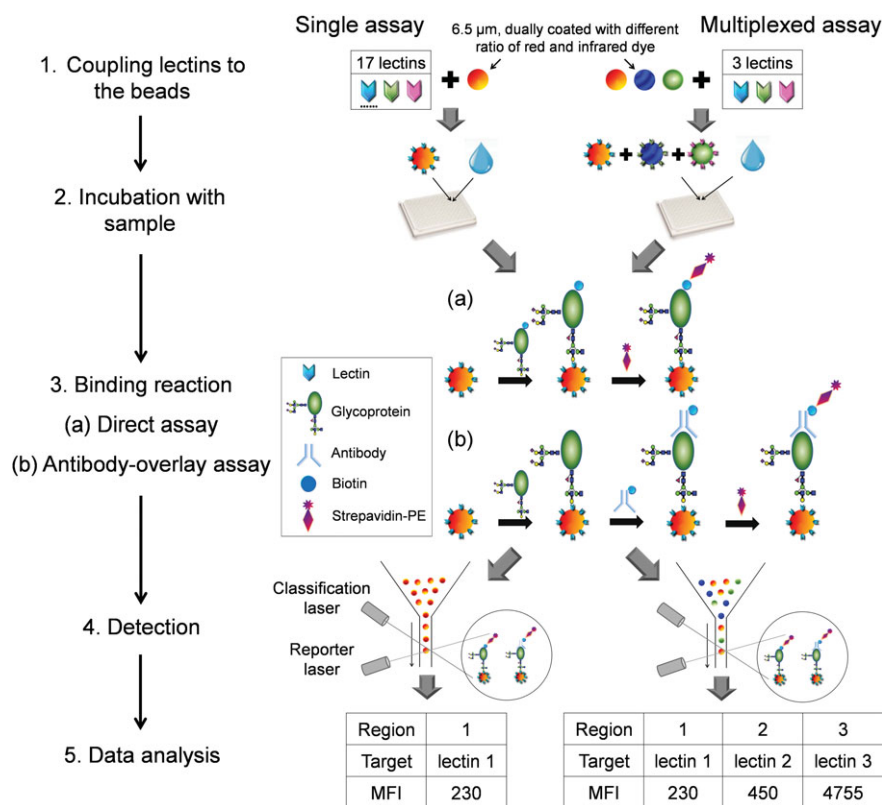


Figure 1. Diagram of the bead-based lectin array.

of bio-ASF to RCA120 was specifically inhibited in a competitor concentration-dependent manner (Supporting Information Fig. 2). The unlabeled ASF was also used as the competitor and the RCA120 binding to bio-ASF was inhibited competitively (Supporting Information S-3). These results strongly suggest that the observed signals on the lectin array are due to specific lectin affinity to the target glycans. Afterward, the sensitivity and linearity for both of the direct assay and antibody-overlay assay were evaluated.

3.2.1 Detection limit and response linearity of direct assay

The dose-dependent analysis was carried out to evaluate the LODs and linearities. Five model lectins were selected for the analysis: WGA, *Aleuria aurantia* lectin (AAL), Con A, peanut agglutinin (PNA), and RCA120. Four model glycoproteins (ASF, FET, HRP, and RNB) that can be specifically detected by the corresponding lectins were used. BSA and one glycoprotein with the same mole amounts were designed as two negative controls to obtain the specific signals from the target glycans. BSA and RNB were used as negative controls for WGA, AAL, PNA, and RCA120, whereas BSA and ASF were applied as negative controls for Con A. The mean background was subtracted from the signal intensity.

The bead-based lectin array achieved high sensitivity and linearities in direct assay (Fig. 2). The LOD of ASF determined

by RCA120 particularly reached 50 pg/mL, that is, 3 pg or approximately 1 pM (average MW of ASF, 48 kDa) with a CV of 14.7%. This result is possibly the highest sensitivity among reported lectin microarrays to date (Fig. 2A). Linear response of ASF for RCA120 was at a concentration range of 50 to 3333 pg/mL with correlation coefficient (R^2) of 0.994 (Fig. 2B). Similarly, the other lectins also showed low LODs and good linearities. In particular, AAL exhibited three orders of magnitude of linear response, with an average CV of 12.3% ($n = 3$; Supporting Information Fig. 3B).

Bio-Plex suspension array achieves biological affinity reactions on the microspheres in 3D format, and the fluorescent intensities of the microspheres are subsequently identified and detected individually, providing high sensitivity and specificity for immunoassay [14]. Previous report also proved that binding affinity between lectins and carbohydrates can be increased in 3D condition [23]. The present study took advantages of Bio-Plex system to conjugate lectins onto the microspheres optimally, thus, achieving higher sensitivity for glycosylation assay.

3.2.2 Detection limit and response linearity of antibody-overlay assay

In the antibody-overlay assay, bound glycoprotein was detected using the specific antibody. Compared with direct assay, antibody-assisted methods can be efficiently applied for

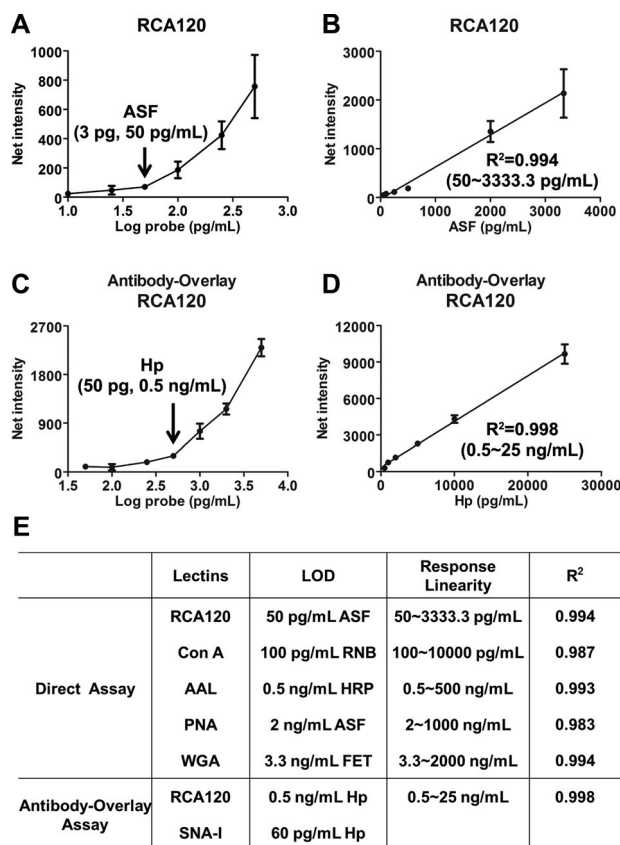


Figure 2. (A) LOD and (B) linearity of response of direct assay with RCA120 ($n = 3$). (C) LOD and (D) linearity of response of antibody-overlay assay for Hp profiling with RCA120 ($n = 3$). Error bars represent SD of triplicate determinants. (E) Summary of LOD and linearity of response for the five lectins. Signals were corrected for the mean background.

the target glycoprotein due to the specific signal observed in the antibody detection. Moreover, glycoprotein sample need not be labeled; thus, high-throughput analysis for clinical samples is feasible.

Hp is one of the acute-phase proteins that exhibit changes in both protein and glycosylation levels as a defense response to inflammation and infection, and was used here as the model glycoprotein probe with transferrin as negative control. Hp and transferrin have similar carbohydrate structures, but can be distinguished by the Hp antibody. In addition, excess amount of nonlabeled polyclonal IgG was applied to efficiently block the unbound sites of the lectins before the antibody detection.

Dose-dependent assays were performed to examine the LOD and linearity of this method. The LOD of Hp (average MW, 45 kDa) for RCA120 was at 0.5 ng/mL, 50 pg, approximately 11 pM (CV = 15.7%), with the linear response at the concentration range of 0.5 to 25 ng/mL ($R^2 = 0.998$, average CV = 10.9%; Fig. 2C and D). Meanwhile, the LOD of Hp for SNA-I was at 60 pg/mL, 6 pg, approximately 1.3 pM, with a CV

of 5.1% (Supporting Information Fig. 3C). Results for SNA-I and RCA120 lectins are shown in Fig. 2E. Compared with the LOD at the nanogram level in a previous report [10], the method increased the sensitivity of antibody-overlay lectin microarray, allowing the detection of target glycoprotein at low pictogram level with good linearity and high reproducibility.

To compare the sensitivities of bead-based direct assay and antibody-overlay assay, dose-dependent assays were performed (Supporting Information Fig. S4). The LOD of lectin RCA120 was 0.5 ng/mL of human Hp in both array formats. The sensitivities of two array formats were basically the same at a concentration range of 0.5 to 5 ng/mL.

3.3 Method validation of multiplexed assay

Multiplexing technology that allows simultaneous detection of multiple glycans can greatly improve throughput and performance of glycan profiling. A mixture of RCA120 and WGA beads, which can recognize the glycosylation of ASF and FET, respectively, was used to study the specific recognition in multiplexed assay (Fig. 3A). Both glycoproteins have the same amino acid sequence. However, ASF has β -galactosyl residues without sialic acid and can be recognized by RCA120, whereas FET has sialic acid and is specifically detected by WGA. Results showed that the ASF signal (500 ng/mL) was significantly increased for RCA120 compared with FET (500 ng/mL), but the ASF signal for WGA decreased due to the absence of sialic acid. Meanwhile, strong signals identified by RCA120 and WGA were observed for the mixtures of ASF and FET (500 ng/mL each), respectively. The remarkable signal changes suggest that the different glycan epitopes can be specifically distinguished by RCA120 and WGA. Moreover, RCA120 and WGA demonstrated specific signals in a dose-dependent manner for ASF and FET (Fig. 3B).

A mixture of RCA120 and PNA beads, incubated with BSA, RNB, and ASF individually, was also utilized to test the interaction specificities (Supporting Information Fig. 5A). In the dose-response assays, 10 ng/mL ASF can be detected simultaneously by RCA120 and PNA (Supporting Information Fig. 5B). The above results illustrate that multiplexed platform is capable of high-sensitive analysis, reporting specific reactions between various lectins and corresponding glycans in a single reaction vessel.

Three kinds of microbeads that correspondingly conjugated with RCA120, PNA, and Con A were analyzed after incubation with 12 ng/mL ASF using multiplexed assay. Several crucial evidences were observed in Fig. 3C. First, compared with BSA and HRP, remarkably enhanced signals for RCA120 and PNA were observed in the interactions with ASF, whereas both HRP and ASF were recognized by Con A due to the presence of mannose. Second, the recognition between lectin and carbohydrate exhibited similar behavior in single and multiplexed assays. The normalized signals for lectins in Fig. 3C were also statistically analyzed and p values were higher than 0.05 (Supporting Information Fig. 6A). In the

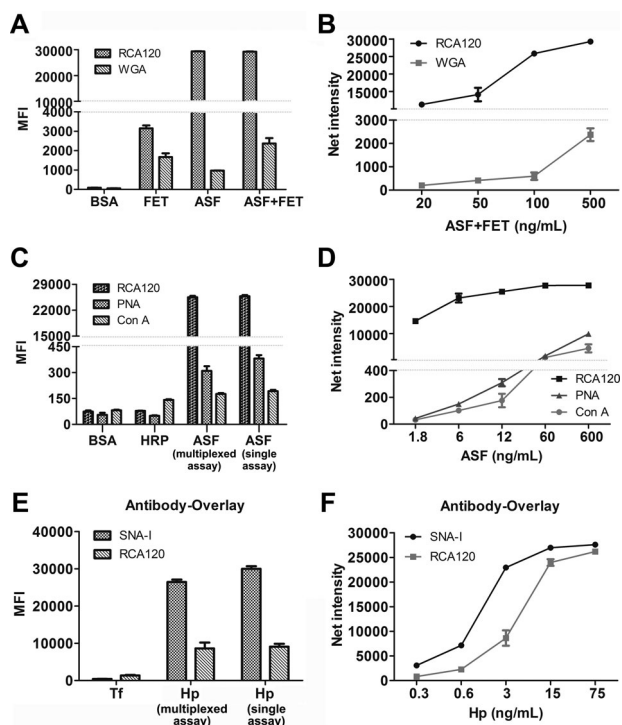


Figure 3. Multiplexed assay of bead-based lectin array ($n = 3$). (A) Multiplexed assay of RCA120 and WGA to test the specificities. ASF, FET (500 ng/mL each), and the same mole of BSA were used. (B) Dose-dependent net intensity of ASF and FET (20 to 500 ng/mL each) for RCA120 and WGA. (C) Multiplexed assay of RCA120, PNA, and Con A to test the specificities and compare the signals between single and multiplexed assay. ASF (12 ng/mL) and the same mole of HRP and BSA were used. (D) Dose-dependent net intensity of ASF (1.8 to 600 ng/mL) for Con A, RCA120, and PNA. (E) Multiplexed antibody-overlay lectin array of RCA120 and SNA-I. Hp (3 ng/mL) and the same mole of transferrin were used. (F) Dose-dependent net intensity of Hp (0.3 to 75 ng/mL) for RCA120 and SNA-I. Error bars represent SD of triplicate determinants.

dose-dependent assay, 6 ng/mL of ASF was sufficient for the measurement (Fig. 3D).

Multiplexed assay was further applied in the antibody-overlay profiling. Two kinds of microbeads coupling with RCA120 and SNA-I were utilized for Hp binding (Fig. 3E). Results showed that RCA120 and SNA-I can specifically reveal the glycosylation character of Hp. Moreover, the single and multiplexed assays did not show any distinct difference in binding signal with 3 ng/mL Hp. The normalized signals for lectins in Fig. 3E were statistically analyzed and p values were higher than 0.05 (Supporting Information Fig. 6B). The dose-dependent assay revealed that 0.6 ng/mL Hp can be effectively analyzed in multiplexed assay using RCA120 and SNA-I (Fig. 3F).

However, several commercially available lectins are already glycosylated and can potentially cross-react with each other in multiplexed lectin-based array [12]. In Bio-Plex system, only dispersed beads can be identified, thereby preventing the

interference of cross-reaction. Therefore, the application of multiplexed array will sensitively and specifically yield abundant information in a single reaction and avoid the side effect among lectins.

3.4 Glycosylation profiling test of 17 selected lectins

HRP (50 ng/mL), mLam (33 ng/mL), and ASF (50 ng/mL) were selected as representative glycoproteins to test the platform with 17 lectins. The sugar-binding specificities of these lectins can be classified into seven types, including α -/ β -galactose (Gal) binder, α -/ β -N-acetylglucosamine (GalNAc) binder, fucose (Fuc) binder, sialic acid (Sia) binder, lactose/N-acetylglucose (Lac/LacNAc) binder, mannose (Man) binder, and chitobiose (Chi) binder. Signal data of each glycoprotein were normalized as the signal intensity value (subtracted the background from BSA) relative to the maximum intensity, and shown as a horizontal bar graph. The glycosylation features of these model glycoproteins were described previously [24–26]. Figure 4A displays the glycosylation profiling results of HRP, mLam, and ASF. The signal patterns varied substantially, and the developed lectin array confirmed the diverse glycosylation features of the three well-known glycoproteins.

HRP is a plant glycoprotein that contains chitobiose core with xylose, fucose, and high mannose-type antennae [24]. In the detection of HRP, the AAL signal was significantly higher than the signal of the other Fuc-binders, including PSA, *Lens culinaris* agglutinin, and *Ulex europaeus* agglutinin-I (UEA-I), perhaps because HRP carries (a-1, 3) core fucosylation. The representative mLam glycoprotein extracted from Engelbreth–Holm–Swarm murine sarcoma was determined to possess bi- and/or tri-antennary complex-type N-glycans with partial core (α -1,6) fucose [25]. The signal patterns of mLam were more extensive than those of HRP. The specific signal on (α -2,3) sialic acid binder MAL-II displayed high intensity, thereby confirming the presence of (α -2,3) sialic acid in mLam. The presence of (GlcNAc)_n and α -mannose, recognized by WGA and Con A, respectively, was also evident in mLam.

ASF has both O-glycans (mainly T or Tn antigen) and bi- or tri-antennary complex type N-glycans without sialic acid. Unique signal on the Gal(β -1,3)GalNAc α -Thr/Ser (T) and GalNAc α -Thr/Ser (Tn) binder Jacalin in ASF confirmed the presence of T or Tn antigen, which was also described previously [9]. The high signal detected by RCA120 confirmed that ASF has β -galactosyl residues without sialic acid. Biantennary with bisecting GlcNAc binding with *Phaseolus vulgaris* Erythroagglutinin (PHA-E) was also exhibited in ASF, which was consistent with a previous report [26]. Glycosidases (PNGase F and O-glycanase) were used to confirm the existence of N-glycans and O-glycans to further verify the carbohydrate-lectin binding specificity (Fig. 4B). After the digestion of PNGase F, specific signals for RCA120 and PHA-E considerably decreased (Fig. 4C). Similarly, a significantly

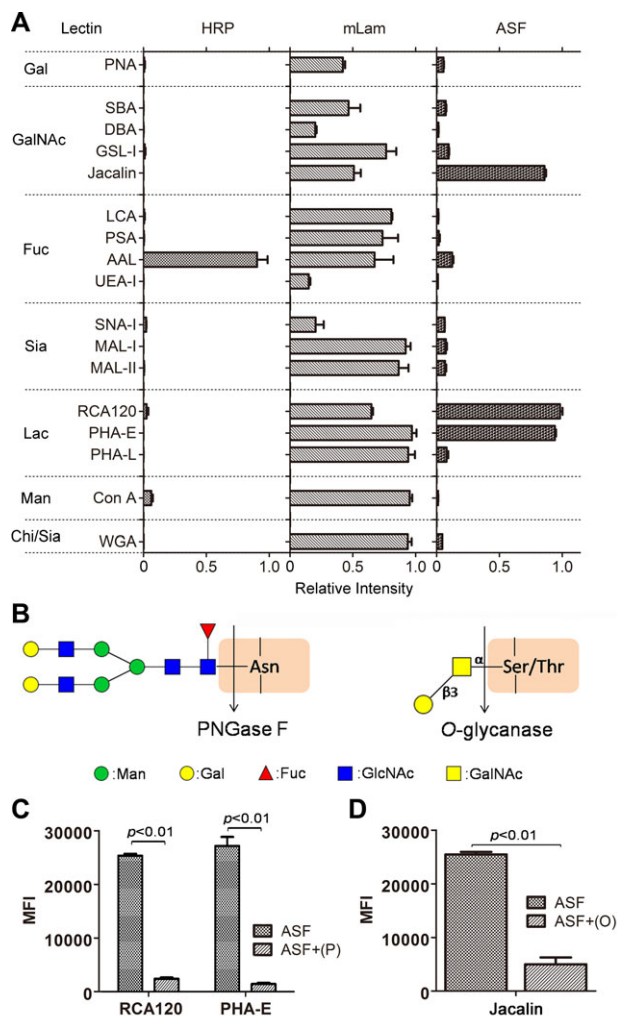


Figure 4. Glycan profiling with 17-lectin bead-based lectin array ($n = 3$). (A) Seventeen lectins with different binding specificities were incubated with three glycoproteins (50 ng/mL of HRP, 33 ng/mL of mLam, and 50 ng/mL of ASF) that contain different types of glycans. (B) The specificities and cleavage sites of PNGase F and O-glycanase. (C) Biotin-ASF (50 ng/mL) intact or digested by PNGase F was probed on the array. (D) Biotin-ASF (50 ng/mL) intact or digested by O-glycanase was probed on the array. Error bars represent SD of triplicate determinants.

reduced signal for Jacalin was detected after O-glycanase digestion (Fig. 4D). These results showed that the observed signals were strictly based on the specificities between lectins and glycans.

3.5 Differential glycosylation profiling of HCC-Associated IgG

Human IgG was selected as an example to validate the bead-based lectin array for profiling and relative quantification of glycan structures in clinical samples. IgG from healthy and

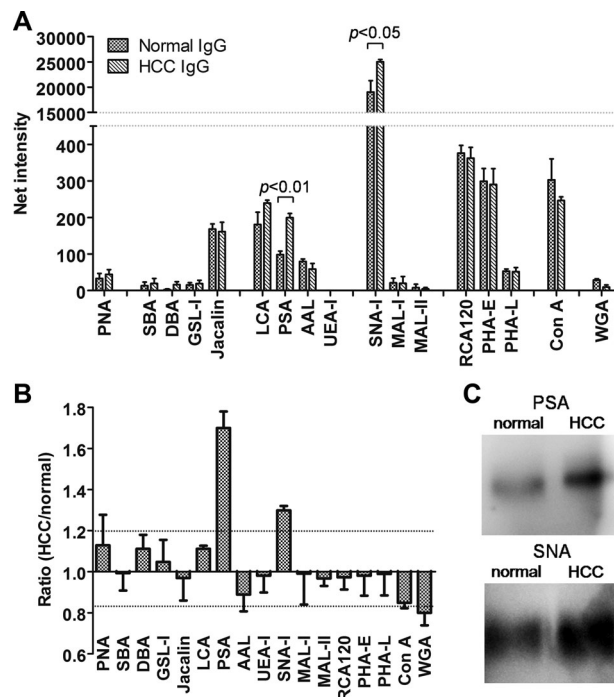


Figure 5. Analysis of HCC associated IgG glycosylation ($n = 3$). (A) Glycan profiles of IgG from healthy and HCC sera. (B) Relative quantification of fluorescence intensity from lectin–glycan interaction in healthy and HCC sera. Error bars represent SD of triplicate determinants. (C) Lectin (PSA and SNA-I) blottings to verify glycan change of IgG.

HCC sera were purified and subjected to the array with equal amounts (16.67 ng/mL).

The detectable signals of 17 lectins provided the following information: (i) (α -1,6) core fucosylation as confirmed by PSA, *Lens culinaris* agglutinin, and AAL; (ii) heavy (α -2,6) sialylation associated with strong signal on SNA-I; (iii) terminal Lac/LacNAc structures and bisecting GlcNAc associated with RCA120 and PHA-E; (iv) tetra-antennary N-glycans recognized by PHA-L; and (v) α -linked mannose structures associated with Con A (Fig. 5A). All data were consistent with previous reports [27–29].

Figure 5B depicts the ratios of specific IgG glycosylation signals of HCC samples to healthy controls. A change of more than 20% between the samples ($\text{HCC/normal} \leq 0.83$ or ≥ 1.20) was distinguishable, which served as a criterion to estimate the down- or upregulation of the glycan levels. In the three replicate analyses, the CVs of all the HCC/normal ratios ranged from 1.3 to 15.1%. The (α -2,6) sialylation was upregulated in IgG from HCC sera, with the HCC/normal ratio as 1.30 ± 0.02 ($p = 0.0004$), as verified by the increased signal on SNA-I. Furthermore, a considerable increment in (α -1,6) core fucosylation was detected through the increased binding to PSA in HCC sera, with the HCC/normal ratio as 1.70 ± 0.08 ($p = 0.0391$). Lectin blotting was performed to validate these changes in the glycan structures of IgG (Fig. 5C).

Results were consistent with those of the bead-based lectin array, further demonstrating the reliability of this approach.

Sialylation serves important intrinsic functions, including microbe binding, immune response regulation, and progression of malignancies. IgG is essential in immune system and acquires anti-inflammatory properties upon Fc sialylation. A close relationship may exist between IgG sialylation and HCC because inflammation affects almost every aspect of cancer progress [30]. An increased level of core fucosylation in N-glycans represents one of the cancer-related glycosylation alterations and is associated with the metastatic potential of hepatoma cells [31]. The presence or absence of core fucose is the most important feature with respect to antibody-mediated cellular cytotoxicity of IgG. The above results indicate that alterations in the glycan structure of IgG may become alternatives or complements to the clinical diagnosis.

4 Concluding remarks

This study demonstrated that the developed bead-based lectin array can be carried out in a rapid, sensitive, reproducible manner, and potentially fulfill qualitative and quantitative analysis of clinical samples. Compared with previously reported lectin microarrays, the novel platform has the following advantages: (i) this system allows for 3D reaction of lectins with specific carbohydrates and improves sensitivity, which is an important factor in clinical applications. It is thereby a promising approach for the analysis of lectins and glycans with relatively weak interactions; (ii) This method provides a multiplexed assay approach, which needs substantially fewer samples and reagents, and has higher throughput than the traditional lectin microarray; (iii) The approach was applied in differential analysis of IgG from healthy and HCC sera. The upregulations of (α -2,6) sialylation and (α -1,6) core fucosylation in HCC suggest that this method is effective for profiling glycosylation changes for diagnostic purposes. The substantial merits of multiplexed assay make it an extremely attractive platform and it is very likely to be a unique trend of bead-based lectin arrays.

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Supporting Information

S-1. Glycan Cleaving by PNGase F and O-glycanase

Deglycosylation with PNGase F was carried out using the manufacturer's (New England BioLabs, NEB) protocol. ASF (100 µg) was dissolved in water (90 µL) and denatured with denaturing buffer (10 µL, 5% SDS, 0.4M DTT) at 95°C for 10 min. After the addition of 20 µL of 10% NP-40, 20 µL of 10×G7 reaction Buffer (500 mM sodium phosphate) and 60µL of H₂O, 0.4 µL of PNGase F (500 units /µL) was added and the reaction mix was incubated for 36h at 37 °C.

The 100 µg ASF was dissolved in water (100 µL) and denatured at 95 °C for 10 min. The sample was then dried through vacuum centrifugation and redissolved in 50 mM sodium phosphate buffer (pH 5.0) for O-glycanase (ProZyme) digestion. About 2 µL of O-glycanase (≥1.25 units/mL) was then added to the sample, after which the sample was incubated at 37 °C overnight for digestion.

The glycans released were removed by 10 kDa MWCO centrifugal filter (Millipore) for about 2 h at 4 °C.

S-2. Supplementary Methods

- (1) Protect the beads from light as much as possible during the procedure.
- (2) It is important for the sensitivity and reproducibility of the assay that beads are completely resuspended as single monodisperse particles prior to use and remain in suspension during reaction.
- (3) Ensure there are sufficient lectin coupled beads (6,000 beads per subset in this study) in reaction vessel during incubation with the glycoprotein.
- (4) Saturated lectin binding should be avoided for quantification analysis.
- (5) In the antibody-overlay assays, select an antibody with high specificity. The concentration of biotinylated antibody needs to be optimized.
- (6) According to the instruction of Bio-plex system, daily calibration is recommended prior to reading the assay and validation needs to be performed periodically.

Table S1. Lectins used in this study.

Numb.	Abbr.	Origin	MW (kDa)	Specificity	Source
1	PNA	<i>Arachis hypogaea</i>	110	Gal(β -1, 3)GalNAc α -Thr/Ser (T)	Vector Lab
2	SBA	<i>Glycine max</i>	120	Terminal GalNAc (especially GalNAc α 1-3Gal)	Vector Lab
3	DBA	<i>Dolichos biflorus</i>	120	GalNAc α -Thr/Ser (Tn) and GalNAc α 1-3GalNAc	Vector Lab
4	GSL-I	<i>Griffonia simplicifolia</i>	115	α -GalNAc, GalNAc α -Thr/Ser (Tn) and α -Gal	Vector Lab
5	Jacalin	<i>Artocarpus integrifolia</i>	50	Gal(β -1, 3)GalNAc α -Thr/Ser (T) and GalNAc α -Thr/Ser (Tn)	Vector Lab
6	LCA	<i>Lens culinaris</i>	49	Fuc(α -1, 6)GlcNAc and α -Man, α -Glc	Vector Lab
7	PSA	<i>Pisum sativum</i>	46	Fuc(α -1, 6)GlcNAc and α -Man	Vector Lab
8	AAL	<i>Aleuria aurantia</i>	72	Terminal α Fuc and $\pm$ Sia-Le ^x	Vector Lab
9	UEA-I	<i>Ulex europaeus</i>	63	Fuc(α -1, 2)LacNAc	Vector Lab
10	SNA-I	<i>Sambucus nigra</i>	150	Neu5Ac(α -2, 6)Gal(β -1, 4)GlcNAc, Neu5Ac(α -2, 6)Gal(β -1, 4)Glc	EY Lab
11	MAL-I	<i>Maackia amurensis</i>	75	Gal(β -1, 4)GlcNAc, Neu5A(α -2, 3)Gal	Vector Lab
12	MAL-II	<i>Maackia amurensis</i>	140	Neu5Ac α 2-3	Vector Lab
13	RCA120	<i>Ricinus communis</i>	120	Lac/LacNAc	Vector Lab
14	PHA-E	<i>Phaseolus vulgaris</i>	125	NA2 and bisecting GlcNAc	Vector Lab
15	PHA-L	<i>Phaseolus vulgaris</i>	125	Tri and Tetra-antennary complex oligosaccharides	Vector Lab
16	Con A	<i>Canavalia ensiformis</i>	104	α -Man (inhibited by presence of bisecting GlcNAc)	Vector Lab
17	WGA	<i>Triticum unlgaris</i>	36	(GlcNAc) _n and multivalent Sia	Vector Lab

The sugar binding specificities and molecular weight of lectins refer to the product information from EY or Vector Laboratories, Inc. and Ref. 5, 9.

Table S2. The optimum amounts per coupling reaction with five lectins (WGA, AAL, Con A, PNA, RCA120).

Lectin	MW (kDa)	optimal lectin amount (μg)
WGA	36	21
AAL	72	20
Con A	104	2
PNA	110	5
RCA 120	120	10

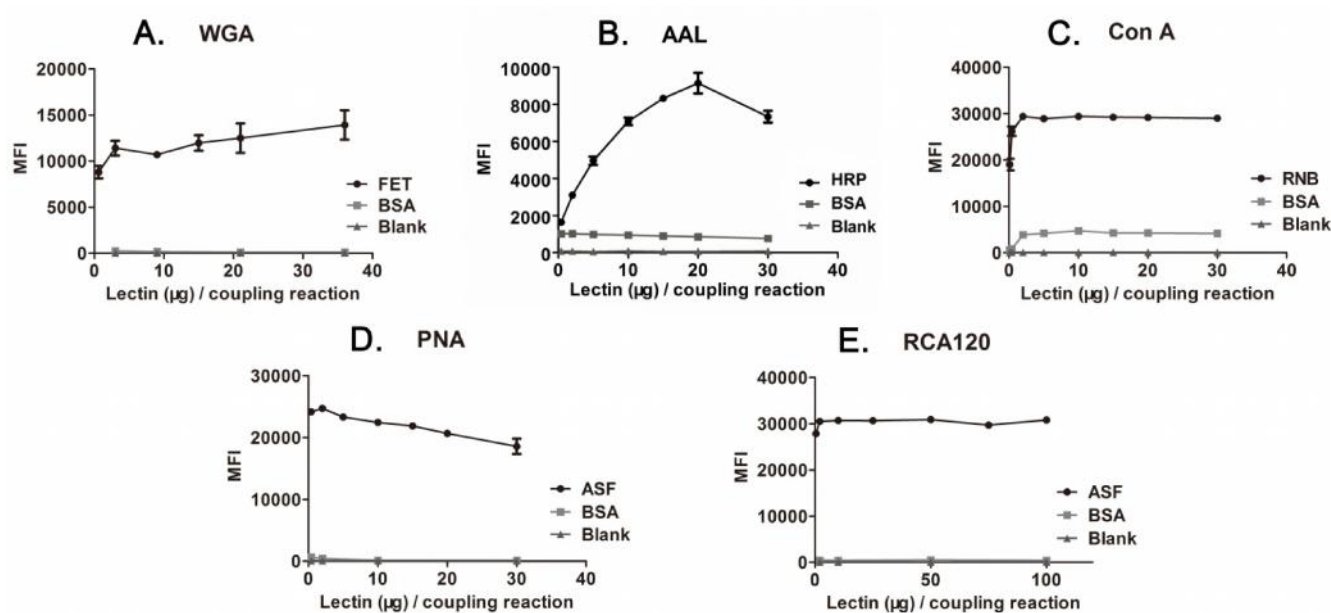


Figure S1. Signal curve made to obtain the optimum lectin amount per coupling reaction for (A) WGA, (B) AAL, (C) Con A, (D) PNA and (E) RCA120. The x-axis represents the various amounts of lectin coupled with 15 μL (approximately 1.875×10^5) beads. The y-axis represents the resulting fluorescence signal. The value of each spot on the y-axis presents the signal yield of each assay. The two gray curves represent negative controls. Error bars represent SD of triplicate determinants.

S-3. Inhibition Experiments with Competitors

When the RCA120-coated beads were incubated with bio-ASF (50 ng/mL), 250, 25, 2.5, 0.25, 0.025, 0.0025mM or 0mM of lactose was added to the incubation buffer. Binding of bio-ASF to RCA120 was specifically inhibited in a competitor concentration-dependent manner (Figure S2).

The unlabeled ASF was also used as the competitor in the inhibition experiments. 0.36, 0.036, 0.0036mM or 0mM of unlabeled ASF was added to the incubation buffer and the RCA120 binding to bio-ASF(50 ng/mL) was found to be inhibited competitively. The signals of bio-ASF in these inhibition experiments were similar to those of the negative control (BSA).

These results strongly suggest that the observed signals on the lectin array are due to specific lectin affinity to the target glycans.

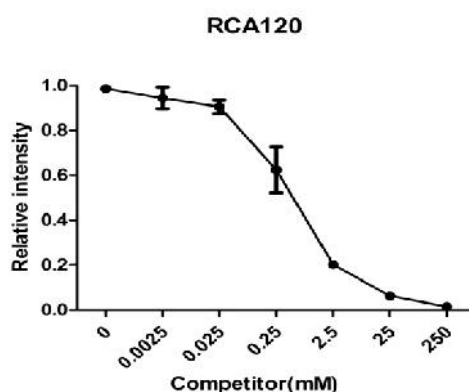


Figure S2. Competition assay of RCA120-ASF interaction with lactose. Error bars represent SD of triplicate determinants.

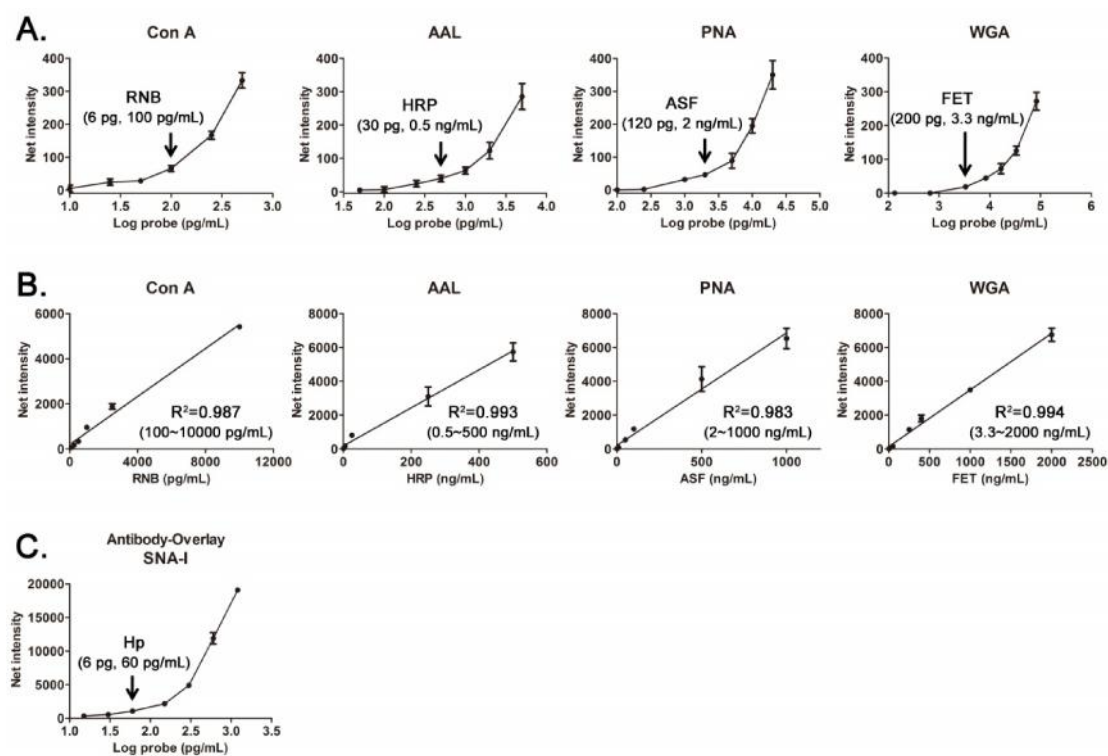


Figure S3. (A) LOD and (B) linearity of response of direct assay with Con A, AAL, PNA and WGA. (C) LOD of Hp through antibody-overlay profiling with SNA-I. Error bars represent SD of triplicate determinants.

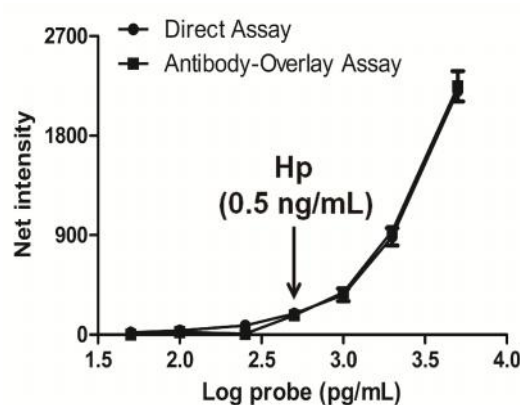


Figure S4. The sensitivities of bead-based direct assay and antibody-overlay assay were compared and found to be basically the same. The limit of detection of lectin RCA120 was 0.5 ng/mL of human Hp in both array formats. Error bars represent SD of triplicate determinants.

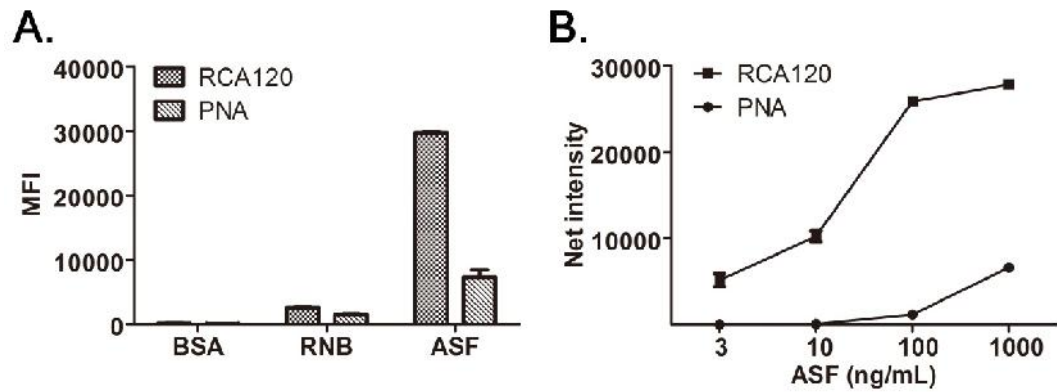


Figure S5. (A) Multiplexed assay of RCA120 and PNA to test the specific interaction. ASF (1 $\mu\text{g/mL}$), RNB and BSA with same mole were used. (B) Dose-dependent net intensity of ASF (3 ng/mL to 1000 ng/mL) for RCA120 and PNA. Error bars represent SD of triplicate determinants.

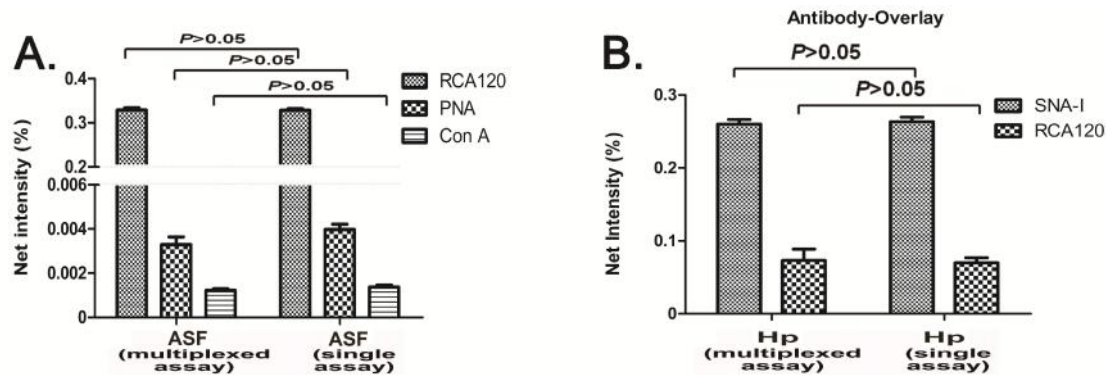


Figure S6. (A) The normalized signals for RCA120, PNA, and Con A in single and multiplexed direct assay were statistically analyzed. 12 ng/mL of ASF was used. (B) The normalized signals for RCA120 and SNA-I in single and multiplexed antibody-overlay lectin array were statistically analyzed. 3 ng/mL of Hp was used. Error bars represent SD of triplicate determinants.