absence of potential interaction of HRP with WFA was confirmed (Supporting Information Fig. S7), the reactivity of each mAb clone against WFA-binding rhM2BP was further qualified in the HRP-assisted sandwich ELISA using a microtiter plate (Fig. 2A and Supporting Information Methods). Two clones (Nos. 11 and 28) were selected as the most feasible detection antibody along with a conventional accelerated stability test (Fig. 2B). In the following spiking experiment with rhM2BP (see Supporting Information Methods), we finally concluded that clone No. 28 was the best mAb with resistance against inactivation of the antigen–antibody reaction by serum components (Fig. 2C).

We prepared our diagnostic agent for the direct measurement of serum WFA^{+}-hM2BP with this antibody. To examine for variations, we performed triplicate measurements for rhM2BP on different days and different production lots. The intraday variations in measurements indicated high reproducibility of the assay (CV = 1.3, 0.7 and 2.3%) using the fully automatic immunoanalyzer HISCL-2000i (Sysmex Co., Hyogo, Japan), whereas the overall difference among the lots was not minimized (CV = 10.4%). Therefore, we reduced the interday and lot variations by indexing the measured values with the value of an exact amount of rhM2BP as a calibrator in the following procedure.

1. The mean +2.5 SD value of the measured values of 800 samples arbitrarily selected from the data set of sera from 1000 healthy volunteers (HV) that we recently reported [8] was assigned as a COI of 1.0 (Fig. 3).
2. An rhM2BP solution was prepared at a concentration to yield this COI value. This solution was designated as the master calibration solution for future production.
3. This calibrator used as a positive control and the buffer as a negative control was measured at least three times for each run of the HISCL-2000i.
4. The measured values (WFA^+-hM2BP) were indexed with the obtained values using the following equation:

   $COI = ([WFA^+\text{-hM2BP}]_{sample} - [WFA^+\text{-hM2BP}]_{NC}) / ([WFA^+\text{-hM2BP}]_{PC} - [WFA^+\text{-hM2BP}]_{NC})$

   $[WFA^+\text{-hM2BP}]_{sample}$, WFA^+-hM2BP count of serum sample (PC, positive control; NC, negative control).

As a result, the mean was 377710, the SD 272230, and the mean +2.5 SD value 1058285. Reliability was then validated using the remaining samples, where the number of samples having COI >1 was 10 out of 200 (5.0% of the total).

Subsequently, we evaluated the reconstructed system with the data set from our previous study [8]. A total of 232 samples from 3 different sites had been measured without dilution (direct measurement) and after 10-fold dilution, of which 117 HV samples were measured on different days with the kits of different production lots and 114 CH samples on the same day with the same production lot (1 CH sample was excluded as the direct measurement exceeded the LOQ). In the resulting 2D plots, both slopes were approximated at 0.96 with high correlation coefficients (R^2 = 0.98 and 0.99). For clinical use, the COI would help in understanding disease severity. The significance of the COI is currently under validation using over 10,000 samples from 15 different sites. The validation will not only support reliability of the glycodiagnostic agent, but also provide advantageous information on basic science, enabling the focused glycoproteomics with well-defined target cases and controls to clarify the structure of the disease-related glyco-alterations on M2BP. Further experiments for glycoproteomics and molecular pathology of M2BP will elucidate the mechanism of M2BP with such glyco-alterations secreted into serum.

In conclusion, we have reconstructed our sandwich assay system as a robust diagnostic agent thanks to the development of a robust calibrator. This was

achieved using the evidence-based technology of lectin-assisted glycan profiling focusing on cell-to-cell and protein-to-protein glyco-alterations. Our development pipeline is highly efficient and applicable to a wide variety of glycodiagnostic agents and might accelerate the incorporation of glycan-based technologies into the clinical arena.

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Figure 1. Efficient construction of WFA-positive rhM2BP as a calibrator for a quantitative lectin–antibody sandwich immunoassay. Cell glycomes of culture supernatants of CHO and HEK293 cells cultivated in RPMI or DMEM were analyzed using a 45-lectin microarray (A). The obtained signal intensity was normalized against the means of signals on the 45 lectins. The asterisk indicates the signal intensity of WFA. (B) Comparative analysis of glycan profiles among four different rhM2BP production lots. Respective glycan profiles of two purification samples per production lot were averaged and are represented as a bar graph with error bars. The reactivity to WFA is displayed separately in the *left* panel of (C). The interlot variability on the WFA reactivity was further estimated by standardizing the signal on WFA using SSA in the *right* panel of (C).

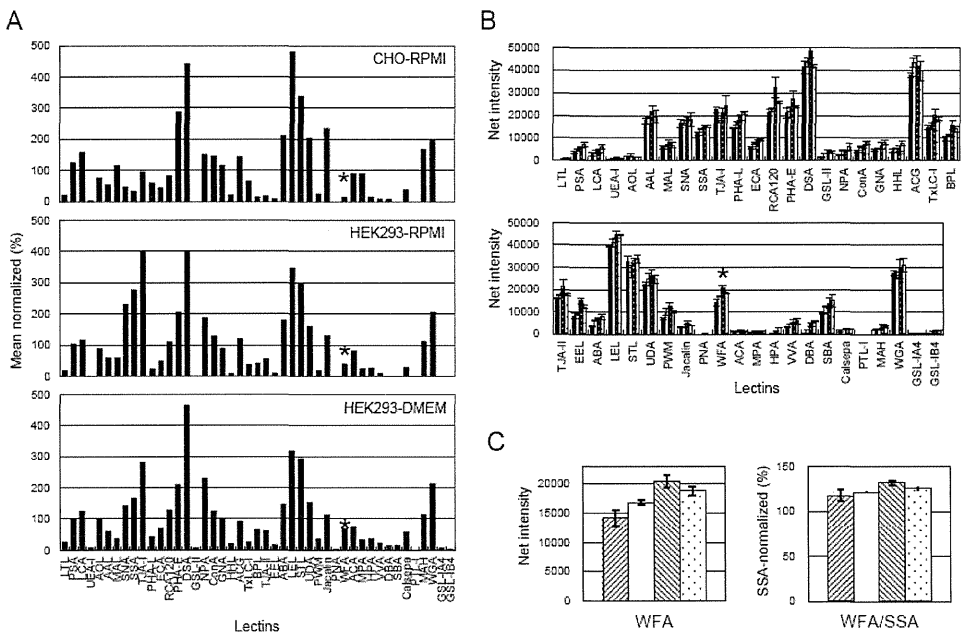
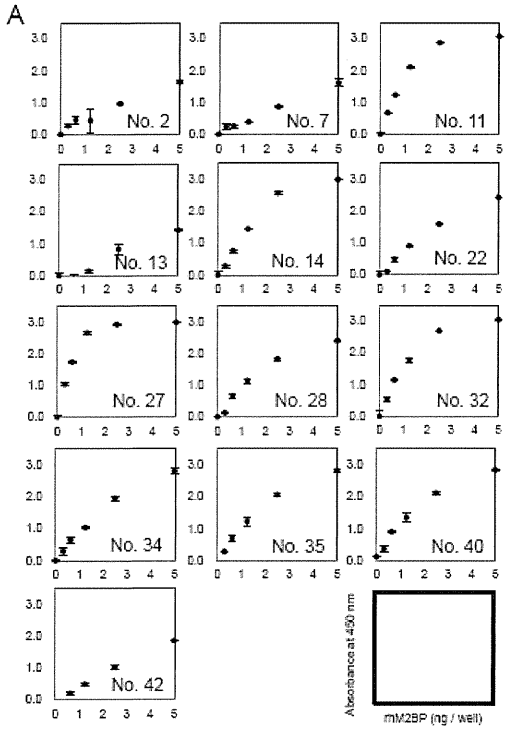


Figure 2. Sequential screening of the most reliable anti-hM2BP mAb using a lectin microarray with twofold serial dilution in a WFA–antibody sandwich ELISA (A), accelerated stability test (B) and spiking experiment (C). In the spiking experiment, appropriate amounts of rhM2BP were dissolved in the reaction buffer in the presence (open squares) or absence (closed squares) of serum samples where endogenous hM2BP had been depleted by immunoprecipitation.

A



B

Clone No.	IgG subclass	Stability (%)			
		Serum A		Serum B	
		Day 7	Day 14	Day 7	Day 14
2	IgG1	83	75	84	77
7	IgG1	83	73	85	76
11	IgG2a	94	92	100	87
13	IgG1	92	91	89	84
14	IgG1	87	73	90	77
22	IgG1	95	86	92	82
27	IgG2a	86	72	82	74
28	IgG1	93	90	95	85
32	IgG1	85	77	84	73
34	IgG1	79	75	79	72
35	IgG1	77	73	80	70
40	IgG1	93	77	96	83
42	IgG1	81	72	82	74

C

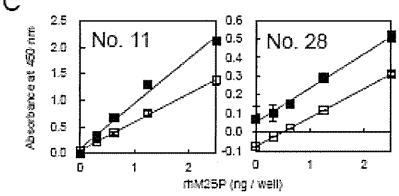


Figure 3. Cutoff index (COI) values for standardization of WFA^+-hM2BP counts showing the distribution of WFA^+-hM2BP counts of 800 samples from healthy volunteers.