ELECTRONIC SUPPLEMENTARY INFORMATION

Layer by layer assembly of biotinylated protein networks for signal amplification

Yu W. Chu,[a] Bo Y. Wang,[a] Huei-Shian Lin,[a] Tai-Yen Lin,[a] Yuan-Jen Hung,[a] David A. Engebretson,[b] Wonjae Lee*[c] and James R. Carey*[a]

[a] Department of Applied Chemistry, National University of Kaohsiung, 700 Kaohsiung University Road, Kaohsiung 811, Taiwan

[b] Department of Chemistry, Oklahoma City University, Oklahoma City, OK, United States

[c] College of Pharmacy, Chosun University, Gwangju 501-759, Republic of Korea

* To whom correspondence should be addressed: wlee@chosun.ac.kr and jcarey@nuk.edu.tw

TABLE OF CONTENTS

I. Materials

There are 7 types of phosphate buffered saline PBS (referred to as PBS-1,2,3….7) used in this work and are described sequentially below. PBS-1 consists of 140 mM NaCl, 10 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4 at pH 7.4. PBS-1 was prepared by diluting 10× PBS-1. 10× PBS-1 consists of 1.40 M NaCl (product number 3624-01, J. T. Baker, Phillipsburg, NJ), 0.10 M KCl (product number 1630-5150, Showa Chemical, Tokyo, Japan), 0.08 M Na_2HPO_4 $2H_2O$ (product number 30412, Sigma-Aldrich, St. Louis, MO), 0.02 M KH_2PO_4 (product number 30407, Sigma-Aldrich, St. Louis, MO) dissolved with DI water. PBS-2 consists of 100 mM sodium phosphate, 150 mM NaCl at pH 7.2. PBS-2 was prepared by dissolving PBS powder (product number 28372, Pierce, Rockford, IL) with DI water. PBS-3 was made from PBS-2 by adjusting the pH to 7.5. PBS-4 consists of 10 mM sodium phosphate at pH 7.4. PBS-4 was prepared by diluting 0.10 M sodium phosphate which was prepared by the ionic strength method as follows. 0.10 M NaH_2PO_4 (product number S0751, Sigma-Aldrich, St. Louis, MO) was added to 0.10 M Na_2HPO_4 $2H_2O$ (product number 30412, Sigma-Aldrich, St. Louis, MO) until the pH reached 7.4. Tween 20 (product number P5927, Sigma-Aldrich, St. Louis, MO) was used to prepare PBS-5 and PBS-6. PBS-5 was prepared by adding 1% BSA and 0.1% Tween 20 in PBS-1. PBS-6 was prepared by adding 0.05% Tween 20 in PBS-1. PBS-7 consists of 2.7 mM KCl, 1.5 mM KH_2PO_4, 137.0 mM NaCl, 7.7 mM Na_2HPO_4 at pH 7.9. PBS-7 was prepared by diluting commercial 10× PBS solution (product number UR-PBS001, UniRegion Bio-Tech, Taiwan) 10 fold with DI water.

PBS-1 buffer was used to prepare PBS-5 and PBS-6 unless otherwise stated. PBS-2 was used for the HABA assays, biotinylation reactions and the Bradford protein assays. Both PBS-3 and PBS-4 were used in the dilution of the ABC reagents. PBS-3 was used in Pierce's ABC kit and PBS-4 was used in Vector's ABC kit. PBS-5 was used to dilute reagents. PBS-6 was used as the wash buffer for washing steps. PBS-7 was also be used to prepare PBS-5 and PBS-6.

Biotinylated proteins were synthesized by reaction of EZ-Link NHS-PEG_4-biotin (product number 21329, Pierce, Rockford, IL) with BSA (product number A2153, Sigma-Aldrich, St. Louis, MO). Protein concentrations were measured by performing the Bradford protein assay (product number 500-0006, Bio-Rad, Hercules, CA) using purified BSA standards (product number 23209, Pierce, Rockford, IL). UV-vis spectra were collected using a Thermo Fisher Scientific Inc. BioMate^{TM} 3S. The MALDI-TOF-MS spectra were obtained using the Bruker microTOF-Q. The estimation of the mole-to-mole ratio of biotin to protein was achieved by performing the HABA assay kit (product number 28005, Pierce, Rockford, IL). Buffer changes, purification, and concentration of the protein samples were performed using Amicon Ultra centrifuge filters (MWCO 10 kDa, product number UFC501024, Millipore, Billerica, MA). Streptavidin was purchased from Jackson ImmunoResearch (product number 016-000-013), West Grove, PA or Pierce (product number 21135), Rockford, IL. Vectastain Elite ABC Staining Kit (product number PK-6100, Vector, Burlingame, CA) and Ultra-Sensitive ABC Peroxidase Staining Kit (product number 32050, Pierce, Rockford, IL) were used as the avidin-biotin-peroxidase complex (ABC) method in comparison experiments with the streptavidin biotinylated protein network (SBPN) method. Selected streptavidin and ABC staining kit for experiments were purchased from Pierce unless otherwise stated.

All the ABC, SBPN, and directly linked amplification (DLA) experiments were performed using a Maxisorp nunc-immuno module microtiter plate (product number 469949, NUNC, Roskilde, Denmark) and the IL-7 Eli-Pair Kit (product number 851.680.020, Gen-Probe, Besancon Cedex, France) which contained capture antibody, antigen, detection antibody (biotinylated antibody), streptavidin-HRP (S-HRP), and ready-to-use TMB. The results were recorded using an Epson Perfection V33 scanner and analyzed using ImageJ (NIH, Bethesda, MD)[1] after color development was complete.

II. Synthesis and characterization of BSA-PEG$_4$-biotin

Synthesis of BSA-PEG$_4$-biotin

2 mg (1 vial) of NHS-PEG$_4$-biotin was reconstituted using 170 µL DI water after the vial was equilibrated to rt. 1 mL of BSA (2.0 mg/mL in PBS-2) was mixed with the prepared NHS-PEG$_4$-biotin. The reaction mixture was placed at 4 ˚C for 2 d to maximize the labeling of biotin on BSA. The unreacted NHS-PEG$_4$-biotin was removed by centrifugation (MWCO 10 kDa). The solvent of NHS-PEG$_4$-biotin was changed to DI water using centrifugation (MWCO 10 kDa) before measuring the molecular weight by MALDI-TOF-MS. The molecular weight shifts from 68 kDa to a peak centered at 80 kDa as measured by MALDI-TOF-MS spectrum (Figures S1 and S2) indicating that the synthesis of BSA-PEG$_4$-biotin was realized. The molecular weight shift of BSA is caused by coupling with NHS-PEG$_4$-biotin molecules. Accordingly, we used 80 kDa as the molecular weight of BSA-PEG$_4$-biotin.

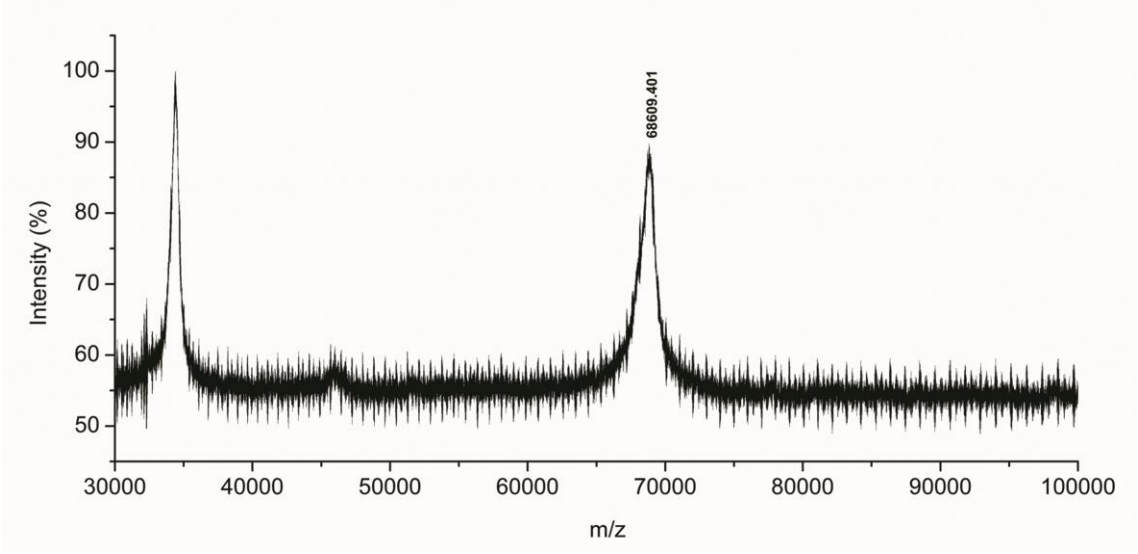


Figure S1. MALDI-TOF-MS spectrum of BSA. The peak at approximately 68 kDa is BSA. This mass corresponds to the data supplied with the reagent.

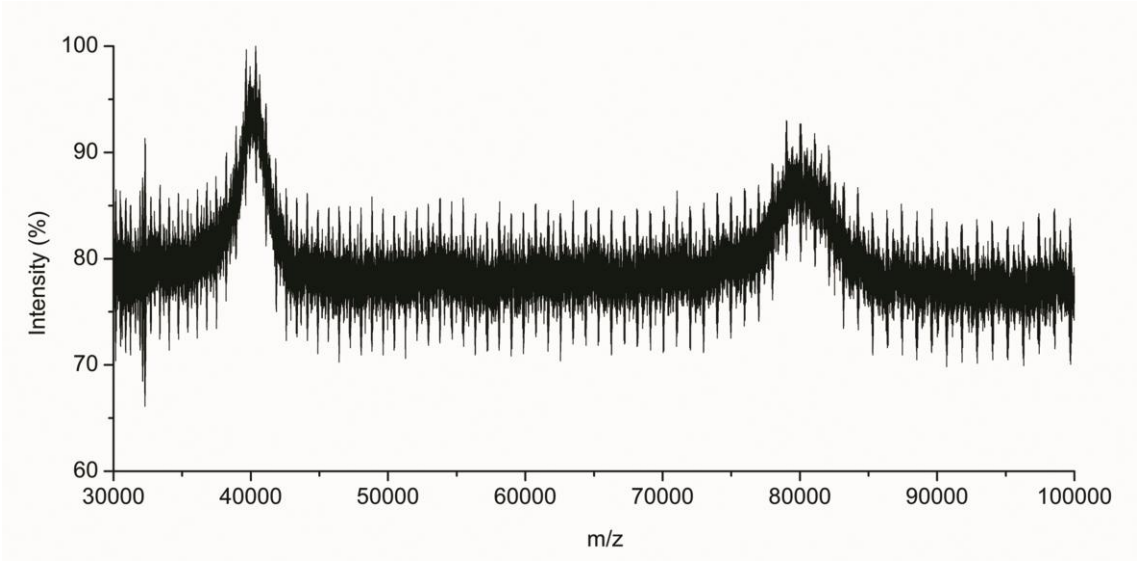


Figure S2. MALDI-TOF-MS spectrum of BSA-PEG$_4$-biotin. The molecular weight shift caused by the addition of NHS-PEG$_4$-biotin molecules on BSA. The molecular weight used in calculations for BSA-PEG$_4$-biotin is 80 kDa.

Determination of the concentration of BSA-PEG$_4$-biotin

The concentration of BSA-PEG$_4$-biotin was measured using the Bradford protein assay. The Bradford assay was performed following the product protocol sheet using purified BSA solution (2.0 mg/mL) as the standard reagent. The BSA standard (2.0 mg/mL) was diluted with DI water producing concentrations varying from 0.20 to 0.90 mg/mL. BSA-PEG$_4$-biotin was diluted 4 fold with PBS-2 before performing the Bradford assay. The calibration curve of the Bradford assay was generated (at 595 nm) after mixing the dye solutions with protein samples for 10 min at rt (Figure S3). The absorbance at 595 nm of diluted BSA-PEG$_4$-biotin was 0.33 resulting in the concentration for the diluted BSA-PEG$_4$-biotin to be 0.27 mg/mL. Hence, the concentration of the original BSA-PEG$_4$-biotin is 1.1 mg/mL.

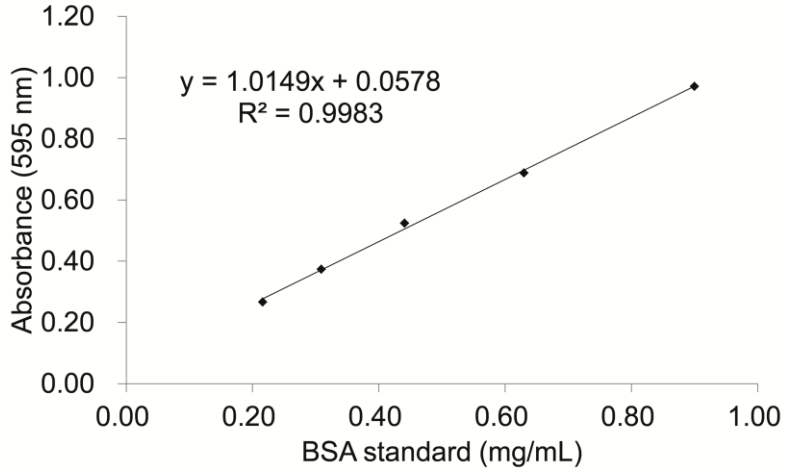


Figure S3. The calibration curve of the Bradford assay. The purified BSA standard solution was diluted producing concentrations varying from 0.20 to 1.00 mg/mL, at an interval of 0.20 mg/mL. The UV-vis absorbance at 595 nm was measured after BSA standards were mixed with dye solutions at rt for 10 min.

Biotin quantitative measurement of BSA-PEG$_4$-biotin

The biotin to BSA mol ratio of BSA-PEG$_4$-biotin was estimated using the HABA assay. All measurements and dilutions were carried out at rt. The HABA assay was performed by following the product protocol sheet provided with the reagent. BSA-PEG$_4$-biotin was diluted 4 fold with PBS-2 before performing the HABA assay. The HABA\avidin premixed reagent contained in the HABA assay kit was reconstituted using 100 µL DI water and mixed together with 800 µL PBS-2. The UV-vis absorbance at 500 nm of the HABA\avidin solution was measured. The absorbance at 500 nm was measured again after the addition of 100 µL BSA-PEG$_4$-biotin to the HABA\avidin solution. The biotin to protein mol ratio was calculated following the equations in the product sheet and are shown below:

$$\text{Calculation \# 1} = \text{mmol protein per mL} = \frac{\text{protein (mg/mL)}}{\text{molecular weight of protein (mg/mmol)}} \quad (1)$$

$$\text{Calculation \# 2} = \Delta A_{500} = (0.90 \times A_{500}\ \text{HABA\avidin}) - (A_{500}\ \text{HABA\avidin\protein}) \quad (2)$$

$$\text{Calculation \# 3} = \frac{\text{mmol biotin}}{\text{mL reaction mixture}} = \frac{\Delta A_{500}}{34{,}000 \times \text{light path length (cm)}} = \frac{\text{calculation \# 2}}{34{,}000 \times 1} \quad (3)$$

$$\text{Calculation \# 4} = \frac{\text{mmol biotin in protein}}{\text{mmol of protein}} = \frac{(\text{mmol of biotin in reaction mixture per mL}) \times 10}{\text{mmol of protein per mL}} = \frac{\text{calculation \# 3} \times 10}{\text{calculation \# 1}} \quad (4)$$

where the sample protein concentration is taken as 0.27 mg/mL from the Bradford assay, molecular weight of sample protein is 80 kDa measured by MALDI-MS, ΔA_{500} is the absorbance (500 nm) difference between HABA\avidin and HABA\avidin\sample. The calculation gives a mol ratio of PEG$_4$-biotin to BSA of 16:1 (Table S1) for BSA-PEG$_4$-biotin.

Table S1. The HABA assay results for BSA-PEG$_4$-biotin[a]

B-BSA MW[b]	B-BSA Con.[c]	A_{500}[d] (HABA\avidin)	A_{500} (HABA\avidin\B-BSA)	Calc.[e] # 1	Calc. # 2	Calc. # 3	Calc. # 4
80,000 g/mol	0.27 mg/mL	0.98	0.69	3.4×10^{-6}	0.19	5.6×10^{-6}	16

[a]The molecular weight of BSA-PEG$_4$-biotin (B-BSA) was measured by MALDI-MS. The concentration of B-BSA was measured using the Bradford assay. The calculations were performed following the equations in the product sheet described above. [b]Molecular weight of B-BSA. [c]Concentration of B-BSA. [d]UV-vis absorbance at 500 nm. [e]Calculation results.

III. Supporting experiments

General procedure for data workup

Each experimental condition was performed in three separate microtiter plate wells at the same time and the results were imaged using a desktop scanner. The color values (average of red, green, and blue values), tabulated in Tables S2, S4, S6, and S8, were estimated using ImageJ software. Using this software, the color values were selected based on a digital circular zone that was the size of the well. The smaller the color values measured, the darker the spots and thus the stronger the signal. The desk scanner suffers an inherent error caused by shadows during the scanning process. In order to minimize the scanning error, we measured the baseline color values from a BSA blocked well that contained biotinylated antibody and TMB. The results of each experiment were subtracted from the baseline color values to obtain relative color values. The relative color values are tabulated in Tables S3, S5, S7, and S9. The relative color values represent the change of color values; the larger the relative color values the darker the spots. For each experiment, the average of relative color values were calculated, called data(avg), and were plotted with error bars using one standard deviation (Figures S5, S11, S16, and 3 of manuscript). In addition, for each experiment, three background wells were performed. The average of the relative color values for the background experiments were calculated and are called background(avg). Finally, the background(avg) was subtracted from the data(avg) to give the normalized relative color values for each experiment. The normalized relative color values for each experiment are plotted in Figures S6, S12, S14, and S17.

Comparison of the various cycles of the SBPN method (0-10 cycles)

In order to determine the number of cycles for the SBPN method that provides the largest amplification, we compared several SBPN cycles that were performed on a biotinylated antibody coated microtiter plate. First, we compared 0, 1, 3, 5, 7, and 10 SBPN cycles. The SBPN method with various cycles were performed side by side and in triplicate using 3 different technicians. The 0 SBPN cycle is the same as the directly linked amplification (DLA) method described in the manuscript. The experiments were performed as follows. Streptavidin and BSA-PEG_4-biotin were diluted with PBS-5. The SBPN method was initiated by the addition of 100 µL/well streptavidin (7.5 µg/mL in PBS-5). The solution was emptied out immediately after the addition and the plates were washed 3 times with PBS-6 (400 µL/well). The addition of 100 µL/well BSA-PEG_4-biotin (1.5 µg/mL in PBS-5) was introduced and the first SBPN cycle was completed after the plates were washed 3 times with PBS-6 (400 µL/well). The various SBPN cycles were performed by following the same steps described above. S-HRP was introduced by addition of 100 µL/well S-HRP (1.5×10^{-4} mg/mL in PBS-5) after each specific SBPN cycle was completed. The plates were incubated on a 160 rpm shaker at 30 °C for 30 min. The plates were washed 3 times with PBS-6 (400 µL/well), followed by the addition of 100 µL/well ready-to-use TMB. The plates were placed in the dark at rt for 15 min and images of each experiment were recorded using a desktop scanner.

The image results from three different technicians indicate that the colors become darker when the number of SBPN cycles is increased (Figure S4). The color values (average of red, green, and blue values) were estimated using ImageJ software and are tabulated in Table S2. Using this software, the color values were selected based on a digital circular zone that was the size of the well. The data shown in Tables S2 and S3 and Figures S5 and S6 were generated as described in general procedure section above. The bar chart (Figure S5) is plotted from the data shown in Table S3. The bar becomes larger when the number of SBPN cycles increases. Although the background becomes larger when the number of SBPN cycles is increased, the signal is much greater than the background. Figure S6 was generated from the data in Table S3 following the background subtraction method described in general procedure section. The increasing signal tendency is confirmed when the background of each specific cycle is subtracted to generate a normalized bar graph (Figure S6).

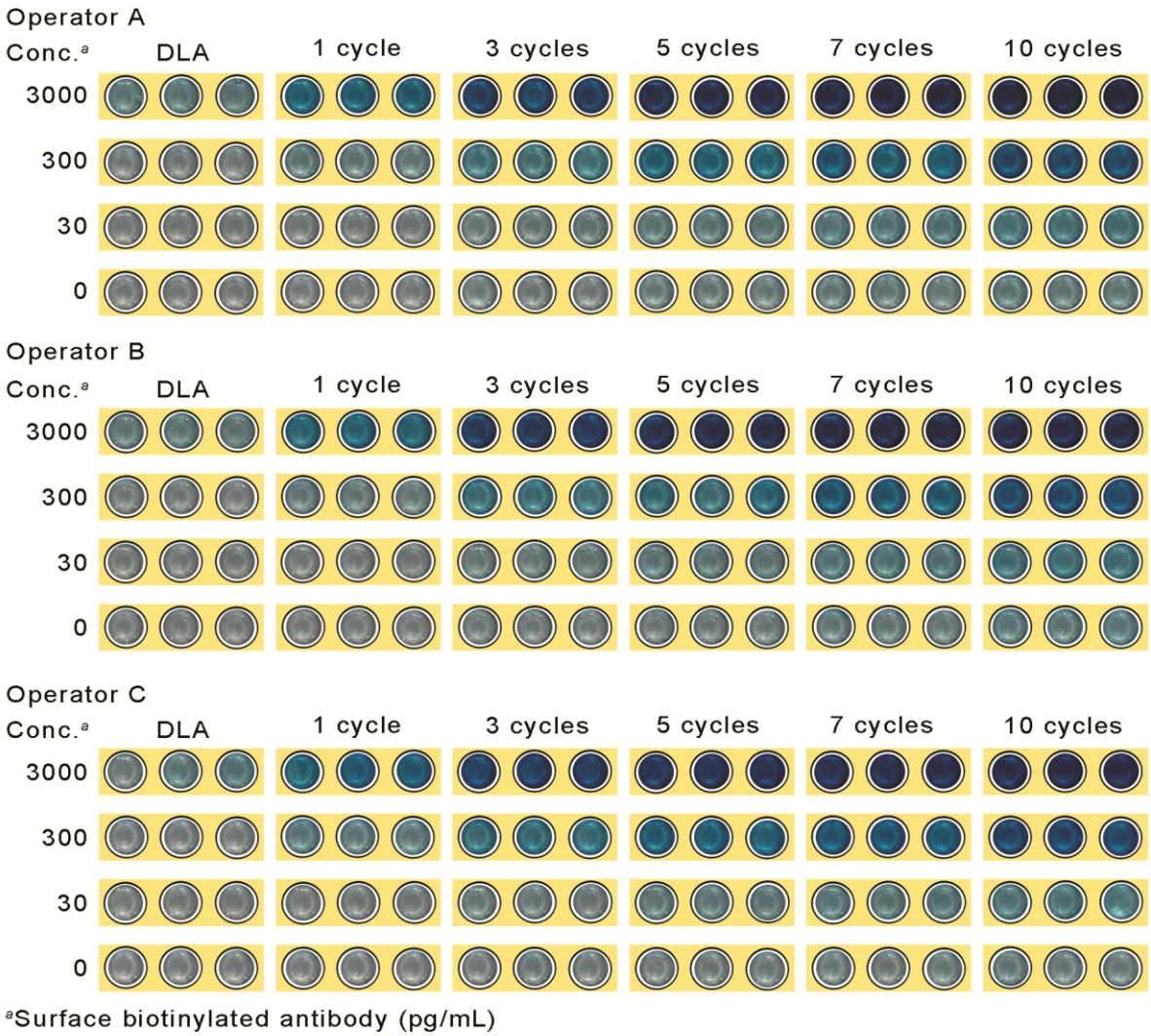


Figure S4. The microtiter well images generated by the DLA and SBPN (1-10 cycles) methods performed on a biotinylated antibody coated surface.

Table S2. Raw color values of the DLA and SBPN (1-10 cycles) methods performed on a biotinylated antibody coated surface[a]

		Operator A						Operator B						Operator C					
Conc.[b]	Baseline[c]	DLA	1 cycle	3 cycles	5 cycles	7 cycles	10 cycles	DLA	1 cycle	3 cycles	5 cycles	7 cycles	10 cycles	DLA	1 cycle	3 cycles	5 cycles	7 cycles	10 cycles
3000	126.193	112.418	74.581	50.066	39.818	34.248	30.391	112.167	77.745	48.051	40.402	35.048	36.359	123.187	77.779	52.104	44.141	40.746	37.282
	125.174	109.898	71.151	52.712	37.313	31.271	28.355	111.344	74.628	46.574	37.204	32.723	34.614	115.864	67.507	48.740	41.391	38.604	34.622
	122.754	106.391	70.181	47.816	36.451	31.318	27.466	107.835	72.889	46.829	35.980	32.311	32.717	107.541	66.872	48.247	39.446	37.520	34.700
300	129.091	126.456	114.676	100.458	71.966	62.865	53.098	123.551	115.104	93.186	84.776	63.688	56.418	128.457	115.735	86.752	70.331	65.264	54.663
	128.531	122.072	117.008	98.119	75.684	66.802	56.569	123.722	116.628	94.286	90.703	64.711	58.642	128.059	116.236	85.921	70.570	63.323	56.422
	129.345	125.440	118.920	98.307	78.747	67.840	56.543	125.475	118.978	97.670	79.293	69.033	59.305	129.437	117.360	88.976	72.714	68.226	58.166
30	126.755	124.982	124.125	119.616	112.121	107.849	99.061	124.216	124.308	116.986	109.446	105.762	95.796	129.472	127.091	121.988	114.797	111.582	105.177
	125.848	124.612	122.068	117.852	111.958	106.115	97.171	124.442	122.834	116.352	108.977	103.467	93.162	129.011	127.410	120.914	113.905	110.588	103.046
	122.892	121.012	121.463	114.195	108.720	102.783	94.521	121.061	119.991	112.513	106.264	101.155	93.953	125.294	124.819	118.964	112.263	107.242	111.335
0	129.836	127.845	129.350	128.615	123.821	118.969	114.874	126.099	126.599	122.510	120.983	117.012	114.655	135.126	134.005	127.513	125.964	126.210	119.708
	129.233	127.536	125.906	123.684	120.490	117.568	115.780	123.028	124.678	123.159	119.024	116.707	116.133	131.380	130.804	123.096	124.914	123.752	123.476
	129.811	127.283	127.714	125.696	123.156	119.460	116.460	124.977	127.480	124.885	122.654	118.448	114.930	131.149	132.781	126.924	125.818	124.622	123.218

[a]The color values (average of red, green, and blue values) were estimated using ImageJ software. Using this software, the color values were selected based on a digital circular zone that was the size of the well. The smaller color values indicate that the spots were darker. [b]Surface biotinylated antibody (pg/mL). [c]Baseline color values were measured from a BSA blocked well that contained biotinylated antibody and TMB.

Table S3. Relative color values generated by the DLA and SBPN (1-10 cycles) methods performed on a biotinylated antibody coated surface[a]

	Operator A						Operator B						Operator C					
Conc.[b]	DLA	1 cycle	3 cycles	5 cycles	7 cycles	10 cycles	DLA	1 cycle	3 cycles	5 cycles	7 cycles	10 cycles	DLA	1 cycle	3 cycles	5 cycles	7 cycles	10 cycles
3000	13.775	51.612	76.127	86.375	91.945	95.802	14.026	48.448	78.142	85.791	91.145	89.834	3.006	48.414	74.089	82.052	85.447	88.911
	15.276	54.023	72.462	87.861	93.903	96.819	13.830	50.546	78.600	87.970	92.451	90.560	9.310	57.667	76.434	83.783	86.570	90.552
	16.363	52.573	74.938	86.303	91.436	95.288	14.919	49.865	75.925	86.774	90.443	90.037	15.213	55.882	74.507	83.308	85.234	88.054
300	2.635	14.415	28.633	57.125	66.226	75.993	5.540	13.987	35.905	44.315	65.403	72.673	0.634	13.356	42.339	58.760	63.827	74.428
	6.459	11.523	30.412	52.847	61.729	71.962	4.809	11.903	34.245	37.828	63.820	69.889	0.472	12.295	42.610	57.961	65.208	72.109
	3.905	10.425	31.038	50.598	61.505	72.802	3.870	10.367	31.675	50.052	60.312	70.040	-0.092	11.985	40.369	56.631	61.119	71.179
30	1.773	2.630	7.139	14.634	18.906	27.694	2.539	2.447	9.769	17.309	20.993	30.959	-2.717	-0.336	4.767	11.958	15.173	21.578
	1.236	3.780	7.996	13.890	19.733	28.677	1.406	3.014	9.496	16.871	22.381	32.686	-3.163	-1.562	4.934	11.943	15.260	22.802
	1.880	1.429	8.697	14.172	20.109	28.371	1.831	2.901	10.379	16.628	21.737	28.939	-2.402	-1.927	3.928	10.629	15.650	11.557
0	1.991	0.486	1.221	6.015	10.867	14.962	3.737	3.237	7.326	8.853	12.824	15.181	-5.290	-4.169	2.323	3.872	3.626	10.128
	1.697	3.327	5.549	8.743	11.665	13.453	6.205	4.555	6.074	10.209	12.526	13.100	-2.147	-1.571	6.137	4.319	5.481	5.757
	2.528	2.097	4.115	6.655	10.351	13.351	4.834	2.331	4.926	7.157	11.363	14.881	-1.338	-2.970	2.887	3.993	5.189	6.593

[a]Relative color values were generated by subtracting the color values of each specific SBPN cycle in Table S2 from baseline color values. [b]Surface biotinylated antibody (pg/mL).

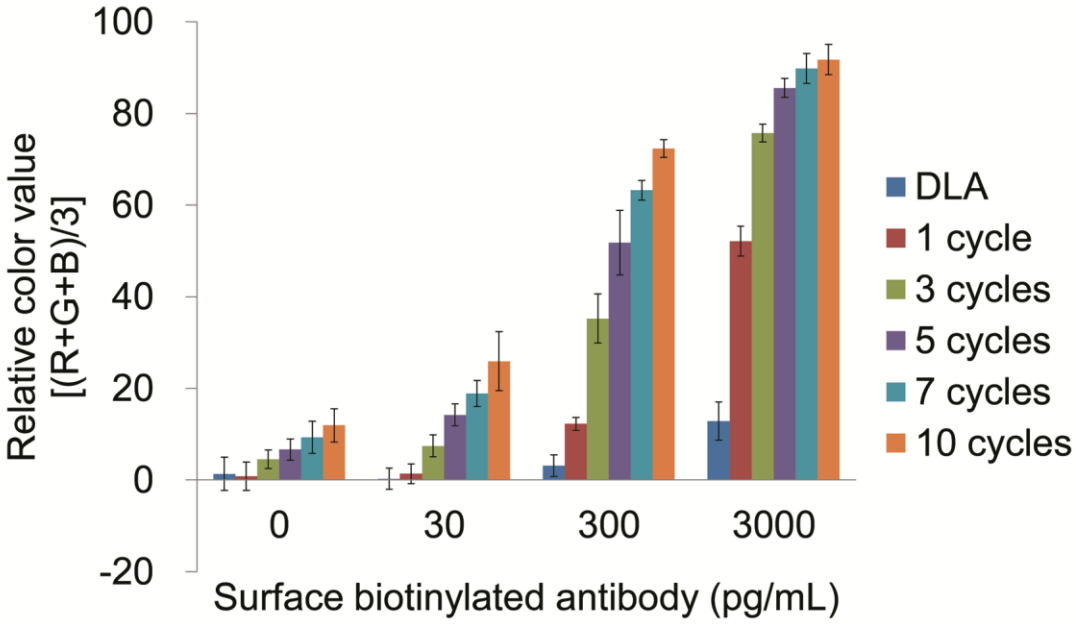


Figure S5. Comparison of the DLA and SBPN (1-10 cycles) methods. Various concentrations of biotinylated antibodies are plotted versus the average of relative color values. Error bars indicate ±1 standard deviation. The plot was generated from the data shown in Table S3. The relative color values become larger when the number of cycles is increased.

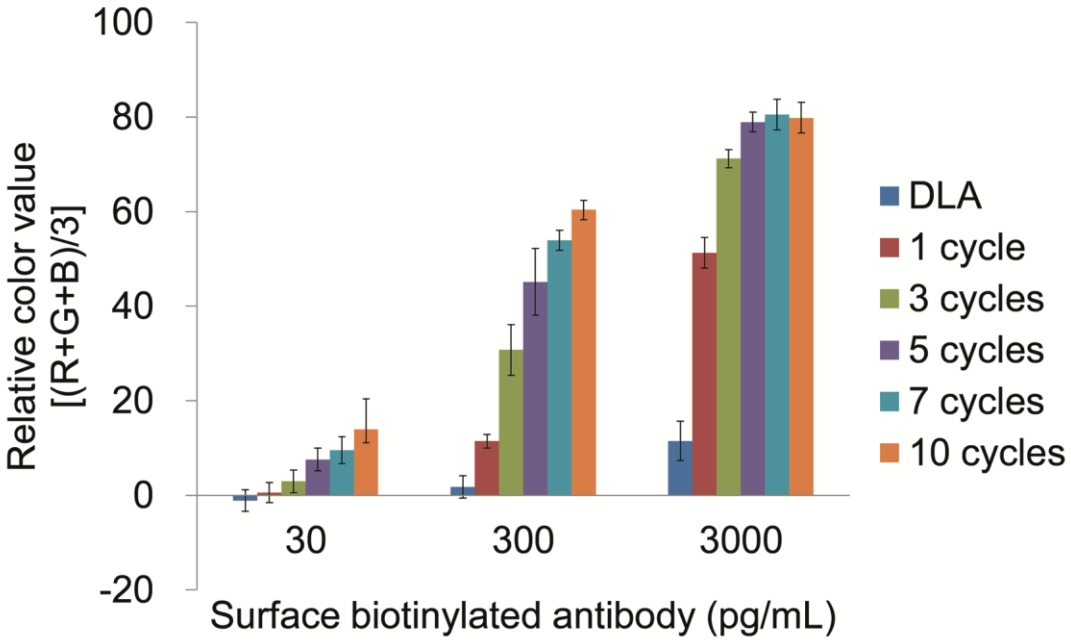


Figure S6. Comparison of the DLA and SBPN (1-10 cycles) methods using normalized data. Normalization was achieved by subtracting the average background signal from the average relative color values of each experiment (average of 9 total data points).

Comparison of the various cycles of the SBPN (5-30 cycles) method

In order to determine the optimal number of cycles for the SBPN method, we increased the number of SBPN cycles to 30 cycles. Studies were run in triplicate, and were performed using three technicians for each specific SBPN cycle. Each operator performed the DLA method as a positive control to ensure that the biotinylated antibody was coated on the microtiter plates and as an internal comparison to the SBPN method. All the reagents and methods were identical to the 1-10 SBPN cycles described above. All three experimental results agree that the signal increases after performing the SBPN method (Figures S7-S9). The images of the positive controls for the 15 and 20 cycles experimental set (Figure S7) is lighter than other two positive controls (Figures S8 and S9). This indicates that the amount of biotinylated antibody used for the experiment performed by operator A is less than the other two experiments. Thus, the performance of 15 and 20 cycles for this experiment was lower. All images are assembled in Figure S10 for a clear comparison. As mentioned above, the performance of 10 SBPN cycles at 3000 pg/mL surface biotinylated antibody is darker than 15 or 20 cycles.

The color values (average of red, green, and blue values) were estimated using ImageJ software. Using this software, the color values were selected based on a digital circular zone that was the size of the well and tabulated in Table S4. Data workup methods for Tables S4 and S5 and Figures S11 and S12 are described in general procedure section above. Figure S11 was generated from the data in Table S5. Since the relative color values from 20-30 cycles using 300 to 3000 pg/mL biotinylated antibody does not continue to increase (Figure S11), using the color value method it was determined that the signal is saturated at around 100. We will not discuss the data at 3000 pg/mL due to the saturation of the signals at 3000 pg/mL. It is obvious that the background dramatically increases from 20 to 25 cycles (Figure S11). Figure S12 was generated from the data in Table S5 following the background subtraction method described in general procedure section. When the background of each SBPN cycle at 30 and 300 pg/mL is subtracted, the increase of signal is terminated once 20 SBPN cycles is reached (Figure S12). The decrease in signal is mainly due from the sudden increase in background after 20 SBPN cycles. Taken together, the data clearly indicate that the optimal number of SBPN cycles is 20.

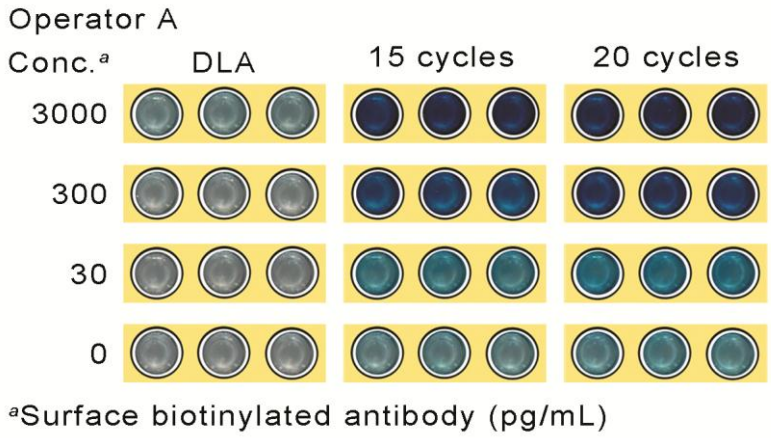


Figure S7. The microtiter well images generated from the DLA and SBPN (15 and 20 cycles) methods performed on a biotinylated antibody coated surface.

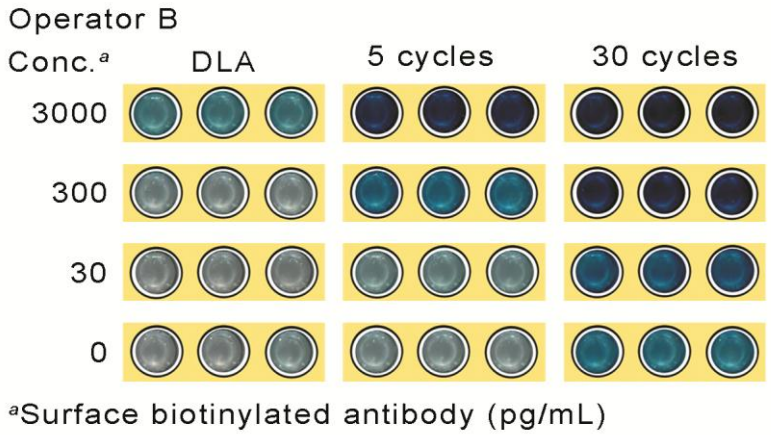


Figure S8. The microtiter well images generated from the DLA and SBPN (5 and 30 cycles) methods performed on a biotinylated antibody coated surface.

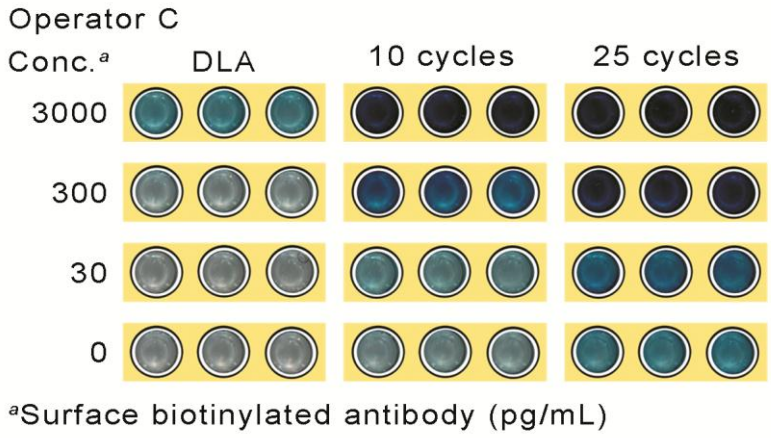


Figure S9. The microtiter well images generated from the DLA and SBPN (10 and 25 cycles) methods performed on a biotinylated antibody coated surface.

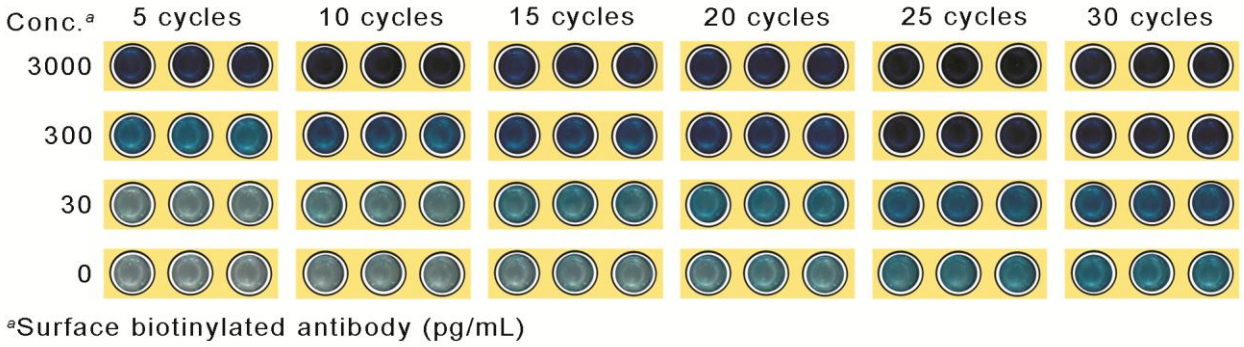


Figure S10. Comparison of the various SBPN cycles from 5 to 30 cycles performed on a biotinylated antibody coated surface.

Table S4. Raw color values of the DLA and SBPN (5-30 cycles) methods performed on a biotinylated antibody coated surface[a]

Conc.[b]	Baseline[c]	DLA (operator A)	DLA (operator B)	DLA (operator C)	5 cycles	10 cycles	15 cycles	20 cycles	25 cycles	30 cycles
3000	123.355	116.796	96.069	91.568	33.361	21.873	34.869	32.540	18.059	26.673
	121.629	113.771	92.157	88.074	31.447	19.469	30.948	29.389	14.650	21.396
	118.292	111.555	91.516	87.865	31.695	18.679	30.735	30.211	14.236	22.905
300	125.346	125.990	124.286	123.008	67.127	50.329	47.277	41.098	22.211	28.109
	125.705	125.247	125.537	122.328	69.466	52.938	48.162	41.056	23.846	27.451
	127.182	126.662	126.988	125.420	71.566	58.955	50.926	43.573	23.181	28.938
30	122.498	125.132	122.981	123.438	116.825	100.483	87.081	76.706	59.200	58.359
	121.864	123.641	123.794	123.288	116.075	100.305	84.793	74.554	56.967	56.489
	117.649	120.815	121.242	119.603	114.200	98.516	85.329	73.675	56.849	54.859
0	125.358	127.103	128.645	126.768	126.540	115.835	112.492	106.667	84.179	74.478
	126.244	125.812	126.290	125.014	127.156	111.836	107.044	101.294	82.722	79.243
	126.876	127.062	121.937	127.321	126.757	114.781	111.092	104.997	78.655	79.456

[a]The color values (average of red, green, and blue values) were estimated using ImageJ software. Using this software, the color values were selected based on a digital circular zone that was the size of the well. The smaller color values indicate that the spots were darker. [b]Surface biotinylated antibody (pg/mL). [c]Baseline color values were measured from a BSA blocked well that contained biotinylated antibody and TMB.

Table S5. Relative color values generated by the DLA and SBPN (5-30 cycles) methods performed on a biotinylated antibody coated surface[a]

Conc.b	DLA (operator A)	DLA (operator B)	DLA (operator C)	5 cycles	10 cycles	15 cycles	20 cycles	25 cycles	30 cycles
3000	6.559	27.286	31.787	89.994	101.482	88.486	90.815	105.296	96.682
	7.858	29.472	33.555	90.182	102.160	90.681	92.240	106.979	100.233
	6.737	26.776	30.427	86.597	99.613	87.557	88.081	104.056	95.387
300	-0.644	1.060	2.338	58.219	75.017	78.069	84.248	103.135	97.237
	0.458	0.168	3.377	56.239	72.767	77.543	84.649	101.859	98.254
	0.520	0.194	1.762	55.616	68.227	76.256	83.609	104.001	98.244
30	-2.634	-0.483	-0.940	5.673	22.015	35.417	45.792	63.298	64.139
	-1.777	-1.930	-1.424	5.789	21.559	37.071	47.310	64.897	65.375
	-3.166	-3.593	-1.954	3.449	19.133	32.320	43.974	60.800	62.790
0	-1.745	-3.287	-1.410	-1.182	9.523	12.866	18.691	41.179	50.880
	0.432	-0.046	1.230	-0.912	14.408	19.200	24.950	43.522	47.001
	-0.186	4.939	-0.445	0.119	12.095	15.784	21.879	48.221	47.420

[a]The relative color values were generated by subtracting the color values of each specific SBPN cycle in Table S4 from baseline color values. The larger relative color values indicates that the spots were darker. [b]Surface biotinylated antibody (pg/mL).

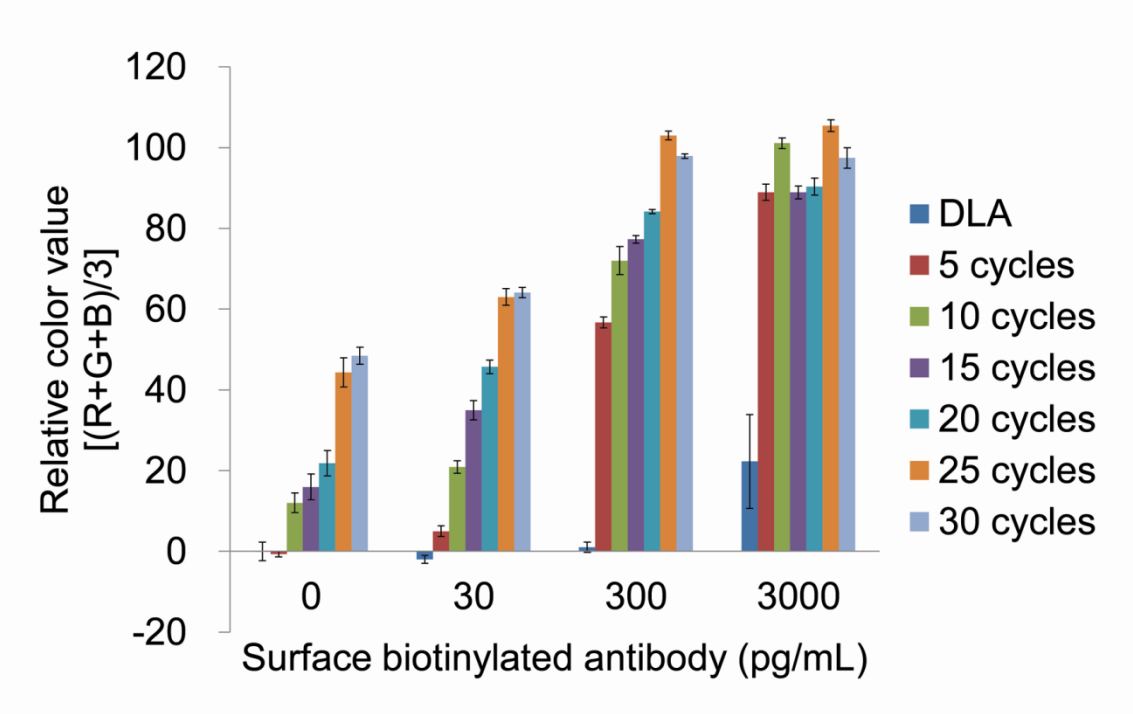


Figure S11. Comparison of the DLA and SBPN (5-30 cycles) methods performed on a biotinylated antibody coated surface. Various concentrations of biotinylated antibodies are plotted versus the average of relative color values. Error bars indicate ±1 standard deviation. The plot was generated from the data shown in Table S5. The relative color values become larger as the number of cycles is increased. The background increases dramatically when the number of SBPN cycles reaches 25.

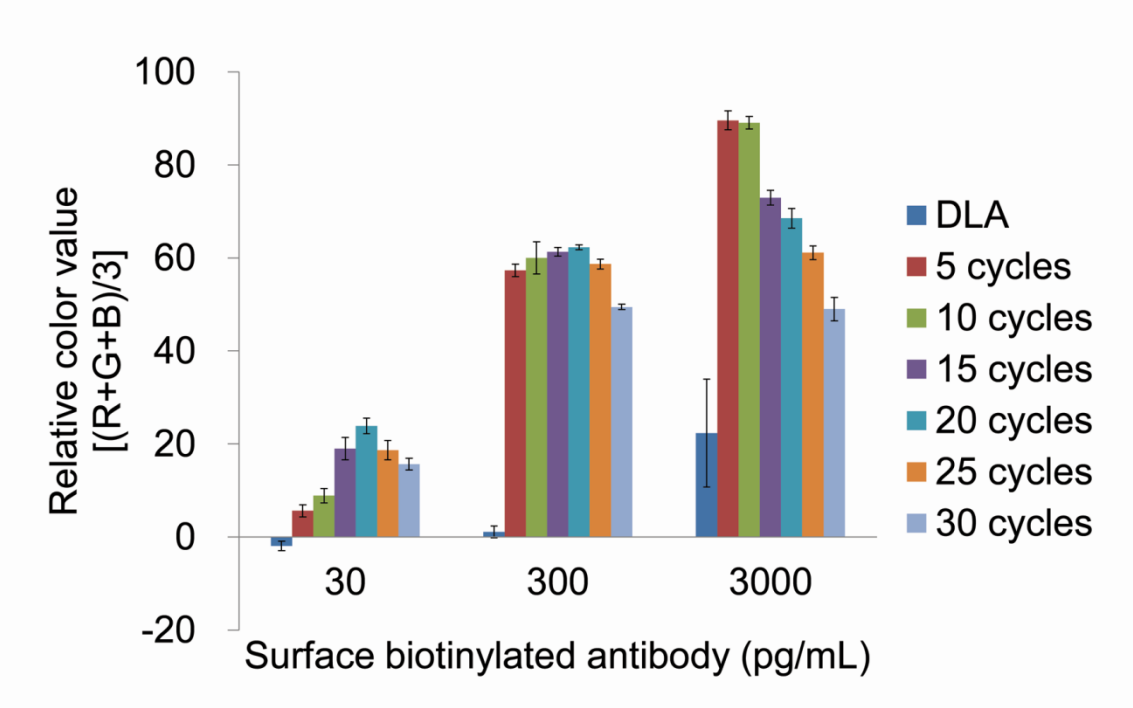
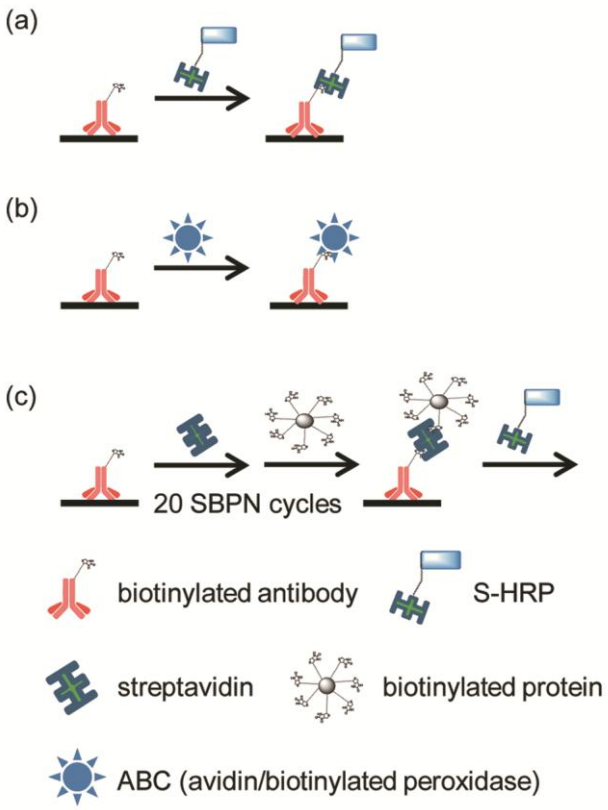


Figure S12. Comparison of the DLA and SBPN (5-30 cycles) methods performed on a biotinylated antibody coated surface using normalized data. Normalization was achieved by subtracting the average background signal from the average relative color values of each experiment.

Comparison of the ABC, DLA, and SBPN (20 cycles) methods performed on a biotinylated antibody coated surface.

Scheme S1 graphically displays the procedural differences of the ABC, DLA, and SBPN (20 cycles) amplification methods. The microtiter plates were coated with various concentrations of biotinylated antibodies by the addition of 100 µL/well of biotinylated antibody at various concentrations (0 to 3000 pg/mL in PBS-1). Controls were generated by the addition of 100 µL/well PBS-1 to wells without antibody. After coating specific wells with antibody, the plates were incubated at 4 °C overnight. After incubation, the plates were washed twice with PBS-6 (400 µL/well) to remove unbound biotinylated antibodies. The plates were then blocked at 30 °C by the addition of 250 µL/well blocking solution (PBS-1 containing 5% BSA) and incubated on a 160 rpm shaker for 2 h. After being emptied by dumping the solution and tapping the plates upside down on an absorbent paper, the plates were placed on the bench at rt for 24 h.

Scheme S1. Experimental procedure for the ABC, DLA, and SBPN methods.[a]



[a]Methods: (a) DLA method: S-HRP is directly added to the biotinylated antibody coated surface. (b) ABC method: The avidin-biotin-peroxidase complex (ABC) was prepared 30 min before direct addition to the biotinylated antibody coated surface. (c) SBPN method: Streptavidins and biotinylated proteins were added in a layer by layer fashion to complete 20 SBPN cycles followed by using S-HRP as the SGM.

When performing the SBPN method, streptavidin and BSA-PEG_4-biotin were diluted to 7.5 µg/mL and 1.5 µg/mL with PBS-5, respectively. The SBPN method was initiated by the addition of 100 µL/well streptavidin (7.5 µg/mL in PBS-5). The solution was emptied out immediately after the addition and the plates were washed 3 times with PBS-6 (400 µL/well). The addition of 100 µL/well BSA-PEG_4-biotin (1.5 µg/mL in PBS-5), followed by washing 3 times with PBS-6 (400 µL/well) completes the first SBPN cycle. 20 SBPN cycles were performed by following the same steps described above. S-HRP was introduced by the addition of 100 µL/well S-HRP (1.5×10^{-4} mg/mL in PBS-5) after the 20 SBPN cycles were completed. The plates were incubated on a 160 rpm shaker at 30 °C for 30 min. The plates were washed 3 times with PBS-6 (400 µL/well), followed by the addition of 100 µL/well ready-to-use TMB. The plates were placed in the dark at rt for 15 min and images of the microtiter wells were recorded using a desktop scanner.

The ABC method (Pierce, Rockford, IL) was performed according to the product protocol sheet. Briefly, 1 drop of avidin and 1 drop of biotinylated HRP were mixed together in 5 mL of PBS-3. The mixture was incubated at rt for 30 min. The biotinylated antibody coated plates were washed 3 times with PBS-6 (400 µL/well) before the addition of 100 µL/well of the ABC complex. The plates were incubated at 30 °C for 30 min while shaking at 160 rpm. 100 µL/well of ready-to-use TMB was added to the wells after washing each well 3 times with PBS-6 (400 µL/well). The plates were placed in the dark at rt for 15 min and scanned as above.

For the DLA method, the biotinylated antibody coated plates were washed 3 times with PBS-6 (400 µL/well) before the addition of 100 µL/well S-HRP (1.5×10^{-4} mg/mL in PBS-5). The plates were incubated at 160 rpm at 30 °C for 30 min. 100 µL/well of ready-to-use TMB was added to the wells after washing each well 3 times with PBS-6 (400 µL/well). The plates were placed in the dark at rt for 15 min and imaged as above.

Figure 2 of the manuscript presents the data performed by one of the three technicians. Here we provide the other two experimental results (Figure S13) and the data contained in Figure 3 of the manuscript (Tables S6 and S7). The images (Figure S13) indicate the same conclusion as Figure 2 of the manuscript, which clearly indicates that the SBPN method has the strongest signal amplification ability among the three methods. The color values (average of red, green, and blue values) were estimated using ImageJ software. Using this software, the color values were selected based on a digital circular zone that was the size of the well and tabulated in Table S6. Data in Tables S6 and S7 and Figures S14 and 3 of manuscript were generated as described in the general procedure section above. Figure 3 of manuscript was generated from the data in Table S7.

It is obvious that the signal of the SBPN method (20 cycles) is the largest of the ABC, DLA, and the SBPN methods at all concentrations of biotinylated antibody (Figures S14 and 3 of manuscript). Comparison of the ABC, DLA, and SBPN (20 cycles) methods at 30 pg/mL of biotinylated antibody are discussed below. For each set of 9 data points (3 experiments times 3 operators), the average relative color value was calculated and this number is called data(avg). In addition, for each experiment, three background wells were performed. The average of the relative color values for the background experiments were calculated called background(avg). Finally, the background(avg) was subtracted from the data(avg) to give the normalized relative color values for the three methods. These values for the ABC, DLA and SBPN methods are 0.701, 0.226, and 25.395, respectively and shown in Figure S14. The data shown in Figure S14 was calculated from Table S7. The SBPN method demonstrates a 36 and 112 fold larger signal amplification ability than the ABC, and DLA methods, respectively.

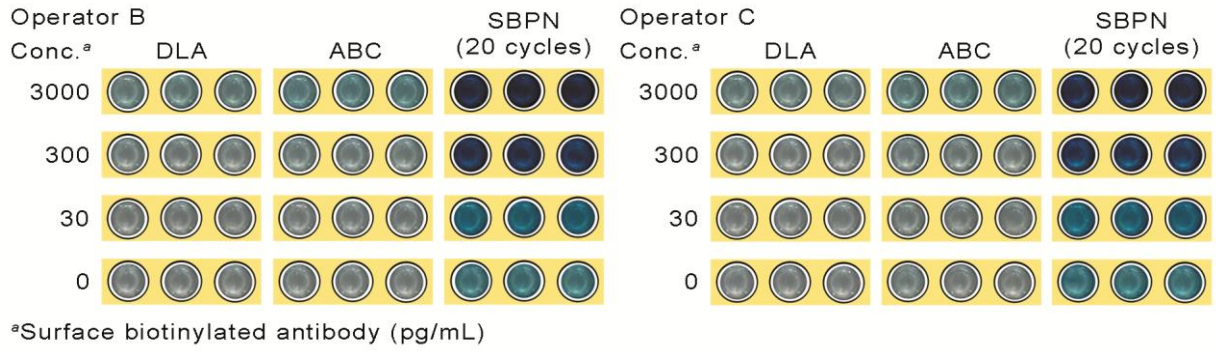


Figure S13. Comparison among the ABC, DLA, and SBPN (20 cycles) methods performed on a biotinylated antibody coated surface. These experiments were performed by three different technicians. The SBPN method produces the strongest signal among three methods. A detailed discussion is provided in the manuscript.

Table S6. Raw color values generated by the ABC, DLA, and SBPN (20 cycles) methods performed on a biotinylated antibody coated surface[a]

		Operator A			Operator B			Operator C		
Conc.[b]	Baseline[c]	SBPN	ABC	DLA	SBPN	ABC	DLA	SBPN	ABC	DLA
3000	124.311	41.473	111.696	118.170	28.896	109.395	118.565	31.817	108.760	116.716
	123.831	36.714	110.034	117.605	26.127	106.907	116.266	28.461	108.181	117.270
	120.609	35.872	107.872	115.709	25.567	106.533	113.928	29.687	104.672	115.866
300	130.545	48.638	123.629	129.664	35.772	126.858	127.469	40.576	123.298	128.668
	126.752	49.918	124.752	126.303	36.987	125.983	125.935	40.841	123.546	126.742
	129.210	53.006	126.163	128.649	39.296	126.052	128.050	41.293	125.871	126.155
30	123.531	83.300	124.068	124.421	67.954	125.743	125.185	67.624	121.703	127.865
	124.153	79.954	123.872	123.489	66.347	124.997	123.555	65.678	121.569	128.023
	120.982	81.285	120.303	121.274	64.608	123.345	121.242	62.751	119.549	125.165
0	128.607	112.942	130.120	128.159	94.998	130.285	129.151	92.838	124.916	131.848
	125.791	112.682	126.256	126.416	93.546	128.437	126.937	92.033	125.284	130.200
	127.662	116.843	128.979	128.316	98.351	128.595	129.862	94.006	128.768	131.545

[a]The color values (average of red, green, and blue values) were estimated using ImageJ software. Using this software, the color values were selected based on a digital circular zone that was the size of the well. The smaller color values indicate that the spots were darker. [b]Surface biotinylated antibody (pg/mL). [c]Baseline color values were measured from a BSA blocked well that contained biotinylated antibody and TMB.

Table S7. Relative color values generated by the ABC, DLA, and SBPN (20 cycles) methods performed on a biotinylated antibody coated surface.[a]

	Operator A			Operator B			Operator C		
Conc.[b]	SBPN	ABC	DLA	SBPN	ABC	DLA	SBPN	ABC	DLA
3000	82.838	12.615	6.141	95.415	14.916	5.746	92.494	15.551	7.595
	87.117	13.797	6.226	97.704	16.924	7.565	95.370	15.650	6.561
	84.737	12.737	4.900	95.042	14.076	6.681	90.922	15.937	4.743
300	81.907	6.916	0.881	94.773	3.687	3.076	89.969	7.247	1.877
	76.834	2.000	0.449	89.765	0.769	0.817	85.911	3.206	0.010
	76.204	3.047	0.561	89.914	3.158	1.160	87.917	3.339	3.055
30	40.231	-0.537	-0.890	55.577	-2.212	-1.654	55.907	1.828	-4.334
	44.199	0.281	0.664	57.806	-0.844	0.598	58.475	2.584	-3.870
	39.697	0.679	-0.292	56.374	-2.363	-0.260	58.231	1.433	-4.183
0	15.665	-1.513	0.448	33.609	-1.678	-0.544	35.769	3.691	-3.241
	13.109	-0.465	-0.625	32.245	-2.646	-1.146	33.758	0.507	-4.409
	10.819	-1.317	-0.654	29.311	-0.933	-2.200	33.656	-1.106	-3.883

[a]The relative color values were generated by subtracting the color values of each specific SBPN cycle in Table S6 from baseline color values. The larger relative color values indicates that the spots were darker. [b]Surface biotinylated antibody (pg/mL).

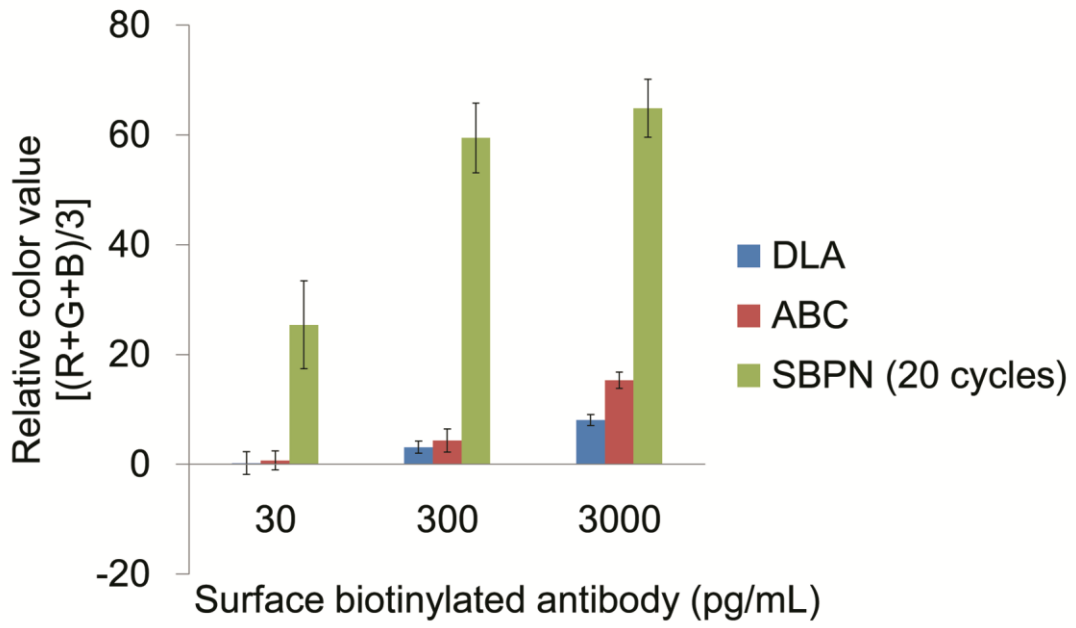


Figure S14. Comparison of the ABC, DLA, and SBPN (20 cycles) methods performed on a biotinylated antibody coated surface using normalized data. Normalization was achieved by subtracting the average background signal from the average relative color values of each experiment (average of 9 total data points).

Comparison of the ABC, DLA, and SBPN (5 cycles) methods performed on a biotinylated antibody coated surface.

In order to verify that the SBPN amplification is not reagent source dependent, we purchased our reagents from different sources and made comparisons. The following reagent changes were made for experiments described in this section. PBS-7 (product number UR-PBS001, UniRegion Bio-Tech, Taiwan) was used instead of PBS-1 (homemade). PBS-5 and PBS-6 were prepared from PBS-7 instead of using PBS-1. Streptavidin was purchased from Jackson ImmunoResearch (product number 016-000-013) instead of Pierce (product number 21135), and the ABC staining kit was purchased from Vector (product number PK-6100) instead of Peirce (product number 32050). Detailed reagent information is described in Materials section. For these comparison tests, 5 SBPN cycles were performed in order to reduce the reagents cost and time. The operation time of 5 SBPN cycles is approximately the same time that is needed to perform the ABC method. Each experimental condition was performed side by side in three separate wells at the same time and all experiments were performed by 4 different people on 4 different days.

Microtiter plates were coated with 100 μL/well of biotinylated antibody at various concentrations (0 to 3000 pg/mL in PBS-7). Controls were generated by the addition of 100 μL/well PBS-7 to wells without antibody. After coating, the plates were incubated at 4 °C overnight. Plates were washed twice with PBS-6 (400 μL/well) to remove unbound biotinylated antibodies. Plates were blocked at 30 °C by the addition of 250 μL/well blocking solution (PBS-7 containing 5% BSA) and incubated on a 160 rpm shaker for 2 h. After being emptied by dumping the solution and tapping the plate upside down on an absorbent paper, the plates were placed on the bench at rt for 24 h.

When performing the SBPN method, streptavidin and BSA-PEG_4-biotin were diluted with PBS-5. The SBPN method was initiated by the addition of 100 μL/well streptavidin (7.5 μg/mL in PBS-5). The solution was emptied out immediately after the addition and the plates were washed 3 times with PBS-6 (400 μL/well). 100 μL/well BSA-PEG_4-biotin (1.5 μg/mL in PBS-5) was introduced and the first SBPN cycle was completed after the plates were washed 3 times with PBS-6 (400 μL/well). 5 SBPN cycles were performed by following the same steps described above. S-HRP was introduced by addition of 100 μL/well S-HRP (1.5×10^{-4} mg/mL in PBS-5) after 5 SBPN cycles were completed. The plates were incubated on a 160 rpm shaker at 30 °C for 30 min. The plates were washed 3 times with PBS-6 (400 μL/well), followed by the addition of 100 μL/well ready-to-use TMB. The plates were placed in the dark at rt for 15 min and images of the experiments were recorded using a desktop scanner.

The ABC method was performed according to the protocol sheet supplied with the ABC kit. Briefly, 1 drop of avidin and 1 drop biotinylated HRP were added together in 2.5 mL PBS-4 containing 0.05% Tween 20. The mixture was incubated at rt for 30 min. The biotinylated antibody coated plates were washed 3 times with PBS-6 (400 μL/well) before the addition of 100 μL/well of each ABC reagents. The plates were incubated at 30 °C for 30 min while shaking at 160 rpm. 100 μL/well of ready-to-use TMB was added to the wells after washing each well 3 times with PBS-6 (400 μL/well). The plates were placed in the dark at rt for 15 min and imaged as above.

For the DLA method, the biotinylated antibody coated plates were washed 3 times with PBS-6 (400 μL/well) before the addition of 100 μL/well S-HRP (1.5×10^{-4} mg/mL in PBS-5). The plates were incubated at 160 rpm at 30 °C for 30 min. 100 μL/well of ready-to-use TMB was added to the wells after washing each well 3 times with PBS-6 (400 μL/well). The plates were placed in the dark at rt for 15 min and imaged as above.

The experimental images indicate that the SBPN method outperformed all other methods even though only 5 cycles of the SBPN method were performed (Figure S15). The color values (average of red, green, and blue values), generated by 5 SBPN cycles, were estimated using ImageJ software and are tabulated in Table S8. Using this software, the color values were selected based on a digital circular zone that was the size of the well. Data shown in Tables S8 and S9 and Figures S16 and S17 were generated as described in the general procedure section above. Figure S16 was generated from the data in Table S9. Figure S17 was generated from the data in Table S9 following the background subtraction method described in the general procedure section. Even though the SBPN method was reduced to 5 cycles, it still produces the largest signals (Figures S16 and S17). Figure S17 clearly indicates that the SBPN method provides the strongest amplification at the same concentration of surface biotinylated antibody.

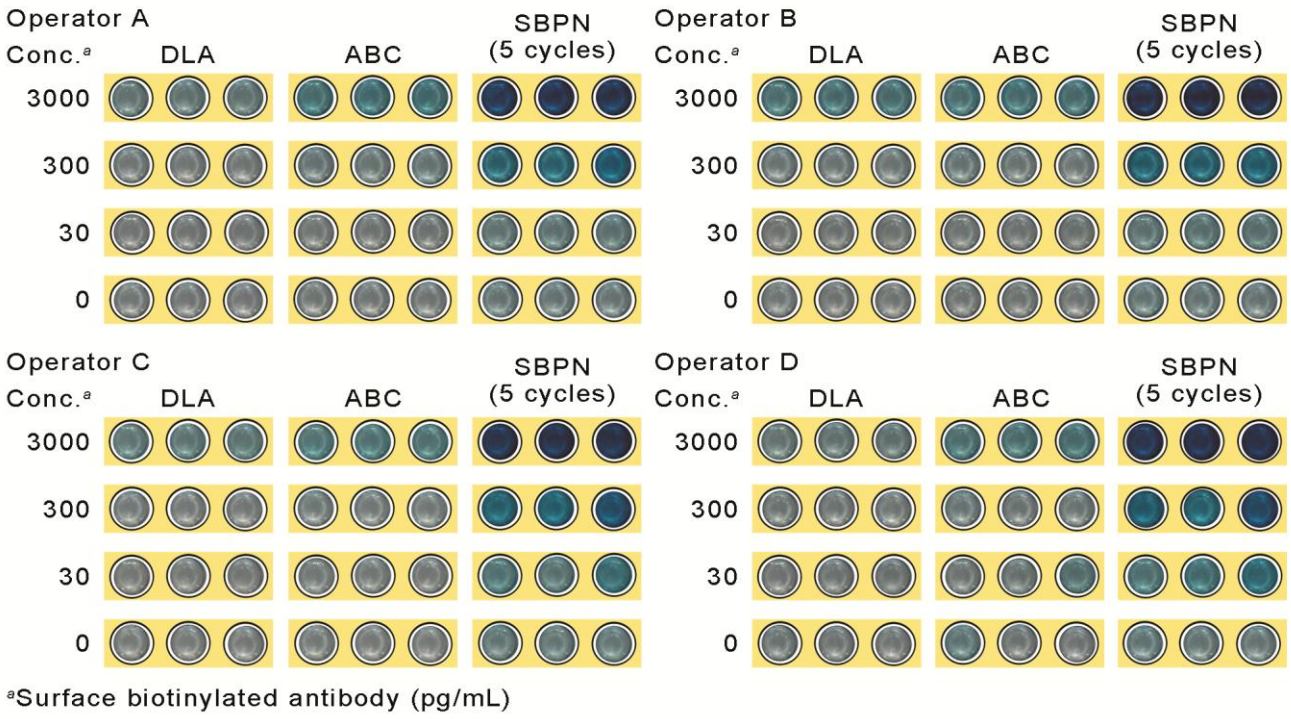


Figure S15. Comparison of the ABC, DLA, and SBPN (5 cycles) methods performed on a biotinylated antibody coated surface. These experiments were performed by four different technicians. The SBPN method produces the strongest signal of the three methods.

Table S8. Raw color values generated by the ABC, DLA, and SBPN (5 cycles) methods performed on a biotinylated antibody coated surface[a]

		Operator A			Operator B			Operator C			Operator D		
Conc.[b]	Baseline[c]	SBPN	DLA	ABC	SBPN	DLA	ABC	SBPN	DLA	ABC	SBPN	DLA	ABC
3000	127.158	46.286	117.945	98.972	37.611	103.188	101.531	40.660	119.690	110.177	41.379	118.903	103.303
	126.082	42.709	118.046	97.519	34.182	100.686	98.618	38.513	117.390	108.347	37.951	116.162	102.972
	124.186	41.962	116.343	95.803	34.152	99.437	98.197	38.089	116.095	107.892	36.454	113.837	98.787
300	129.680	82.219	129.921	123.013	74.798	125.996	122.538	75.259	132.998	132.456	68.523	124.715	124.034
	128.275	83.913	128.689	124.084	75.413	124.356	124.566	74.931	132.602	132.694	79.542	126.322	123.520
	129.581	66.760	129.128	122.171	74.070	125.897	126.044	58.793	132.680	133.366	56.179	128.157	124.797
30	127.418	116.491	127.105	126.371	112.273	123.183	122.653	106.585	130.416	130.832	105.528	124.969	122.177
	126.183	115.457	126.759	125.991	109.998	121.451	121.009	111.041	129.527	131.397	98.109	122.851	120.072
	123.718	112.758	124.982	124.343	108.311	119.714	118.242	89.476	127.943	129.957	83.699	118.954	105.375
0	130.014	122.235	131.181	130.724	119.005	127.381	124.515	118.650	133.443	135.541	123.054	123.902	116.863
	128.320	124.122	129.812	129.413	118.427	125.915	124.188	121.288	132.307	135.546	122.239	125.171	124.607
	129.353	123.458	130.435	129.705	121.128	127.612	125.290	120.838	133.316	136.024	124.176	127.528	127.385

[a]The color values (average of red, green, and blue values) were estimated using ImageJ software. Using this software, the color values were selected based on a digital circular zone that was the size of the well. The smaller color values indicate that the spots were darker. [b]Surface biotinylated antibody (pg/mL). [c]Baseline color values were measured from a BSA blocked well that contained biotinylated antibody and TMB.

Table S9. Relative color values generated by the ABC, DLA, and SBPN (5 cycles) methods[a]

	Operator A			Operator B			Operator C			Operator D		
Conc.[b]	SBPN	DLA	ABC	SBPN	DLA	ABC	SBPN	DLA	ABC	SBPN	DLA	ABC
3000	80.872	9.213	28.186	89.547	23.970	25.627	86.498	7.468	16.981	85.779	8.255	23.855
	83.373	8.036	28.563	91.900	25.396	27.464	87.569	8.692	17.735	88.131	9.920	23.110
	82.224	7.843	28.383	90.034	24.749	25.989	86.097	8.091	16.294	87.732	10.349	25.399
300	47.461	-0.241	6.667	54.882	3.684	7.142	54.421	-3.318	-2.776	61.157	4.965	5.646
	44.362	-0.414	4.191	52.862	3.919	3.709	53.344	-4.327	-4.419	48.733	1.953	4.755
	62.821	0.453	7.410	55.511	3.684	3.537	70.788	-3.099	-3.785	73.402	1.424	4.784
30	10.927	0.313	1.047	15.145	4.235	4.765	20.833	-2.998	-3.414	21.890	2.449	5.241
	10.726	-0.576	0.192	16.185	4.732	5.174	15.142	-3.344	-5.214	28.074	3.332	6.111
	10.960	-1.264	-0.625	15.407	4.004	5.476	34.242	-4.225	-6.239	40.019	4.764	18.343
0	7.779	-1.167	-0.710	11.009	2.633	5.499	11.364	-3.429	-5.527	6.960	6.112	13.151
	4.198	-1.492	-1.093	9.893	2.405	4.132	7.032	-3.987	-7.226	6.081	3.149	3.713
	5.895	-1.082	-0.352	8.225	1.741	4.063	8.515	-3.963	-6.671	5.177	1.825	1.968

[a]The relative color values were generated by subtracting the color values of each specific SBPN cycle in Table S8 from baseline color values. The larger relative color values indicates that the spots were darker. [b]Surface biotinylated antibody (pg/mL).

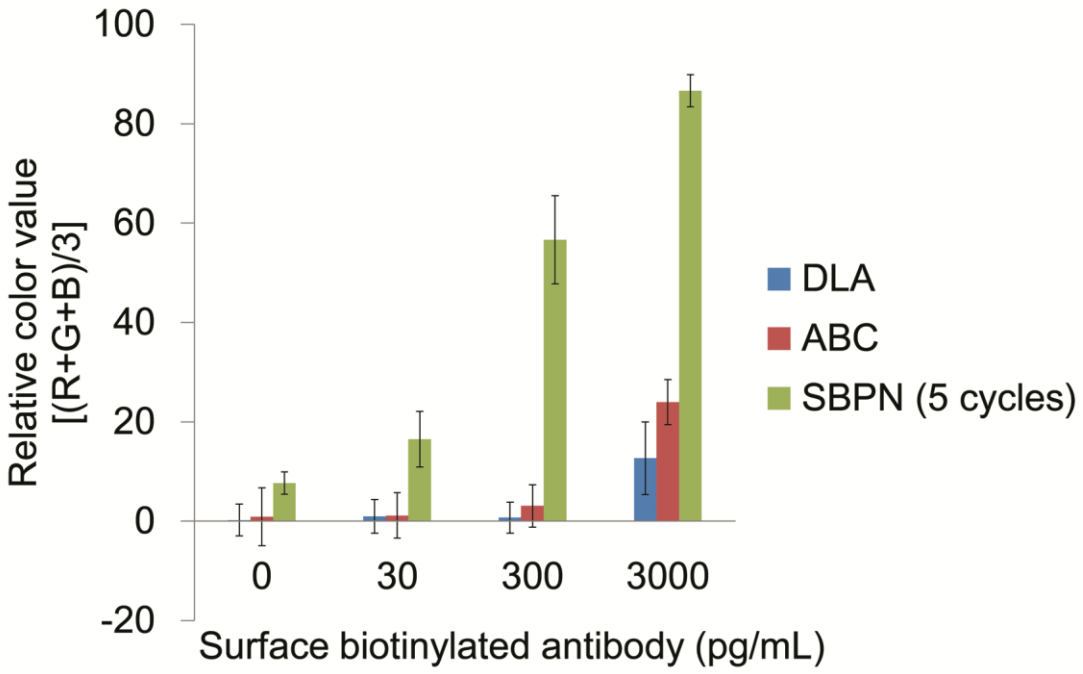


Figure S16. Comparison of the ABC, DLA, and SBPN (5 cycles) methods performed on a biotinylated antibody coated surface. Various concentrations of biotinylated antibodies are plotted versus the average of relative color values. Error bars indicate ±1 standard deviation. The plot was generated from the data shown in Table S9.

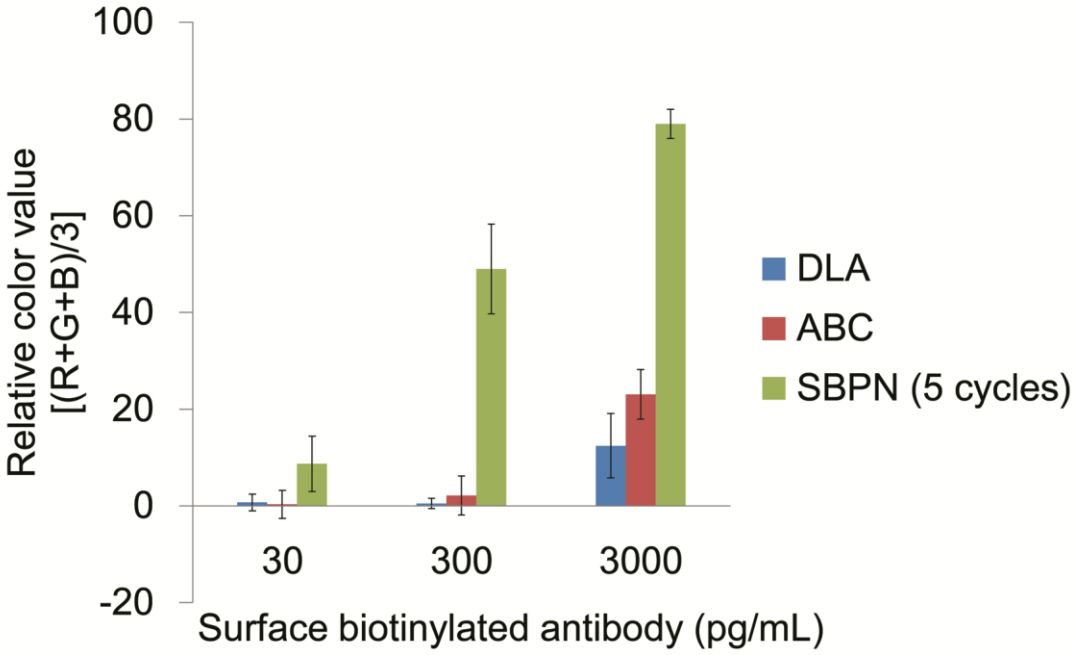


Figure S17. Comparison of the ABC, DLA, and SBPN (5 cycles) methods performed on a biotinylated antibody coated surface using normalized data. Normalization was achieved by subtracting the average background signal from the average relative color values of each experiment (average of 12 total data points).

IV. References

(1) ImageJ, version 1.43; software for image analysis; National Institutes of Health: Bethesda, MD 2006