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APPLICATION BRIEF #008

Spreeta™ Competitive Assay: Determination of Biotin Concentration

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Competitive Assay: Determination of Biotin Concentration

ABSTRACT

A competitive assay format is demonstrated for the determination of biotin using Spreeta technology. In this assay biotinylated bovine serum albumin is physisorbed directly to the gold surface. Solutions containing a fixed concentration of antibody and variable concentrations of free biotin are premixed and analyzed.

INTRODUCTION

The magnitude of the response obtained using SPR technology is proportional to the molecular weight of the molecule binding to the surface. Very low molecular weight molecules are best detected by competitive, inhibition or displacement assay formats. These three assay formats are similar in principle and only a competitive assay for biotin is described here. Simple physisorption of biotinylated-bovine serum albumin (BSA) was employed in place of other more sophisticated chemistries. It is possible to immobilize in this way because we are not concerned with surface orientation or denaturation. Orientation is not problematic due to the presence of many biotin residues per BSA molecule and, in contrast to other ligands such as antibodies, harsh regeneration solutions may be used to regenerate the surface since this conjugate is robust and possesses no innate biological binding activity.

Peripheral technologies are required to complete an application but are not intended to indicate any preference, or limitation, in the technologies that may be adapted to build a test instrument based on Spreeta. See **Application Note 12** for more details on the automated system employed here.

Materials and Methods

Goat anti-Biotin polyclonal antibodies and a BSA-Biotin conjugate were obtained from Pierce. All other reagents were from Sigma-Aldrich. HEPES buffered saline (HBS) buffer was used as constant running buffer. HBS containing 1 mg/ml BSA (HBS-BSA) was used as diluent when preparing biotin standards.

Surface Preparation

The exposed gold surface of a Spreeta sensor was plasma cleaned by sequential exposure to oxygen plasma and then hydrogen plasma for 1min each. This step is not essential and may be omitted. A swab with an ethanol-saturated kimwipe will clean the gold surface adequately. Care should be taken to prevent contact with the plastic at the face of the sensor during swabbing. 10 μl of BSA-biotin (100 μg/ml in PBS buffer) was allowed to dry onto the gold surface at room temperature. The surface was rinsed with ultra pure water and the sensor was docked with a flow cell. The sensor was allowed to warm for 5 min in the LED-on state and then initialized using a 70% glycerol in water solution.

10 mg of biotin was dissolved in 1 ml of DMSO and a 10 μg/ml stock solution was prepared by dilution in HBS-BSA buffer. 11 serial doubling dilutions were prepared giving a final concentration of 2500 ng/ml to 2.44 ng/ml. Each sample contained a final anti-biotin antibody concentration of 5 μg/ml. The BSA concentration was 1 mg/ml. 5 μg/ml goat IgG in HBS buffer was used as a control

Sample Analysis

The running buffer was maintained at 50 μl/min throughout the assay. Each sample was analyzed by injecting 150 μl at 50 μl/min. The surface was regenerated by injecting 30 mM NaOH and 30 mM HCl, sequentially. Sample was directly injected into the flow cell using a 2-position switch valve. A robotic arm holding the injection needle and syringe pump delivered the sample. Valve position A maintained continuous running buffer through the flow cell and valve position B enabled sample to be infused via a syringe pump. The system was automated using visual basic custom software to control the robotic arm, syringe pump and SPREETA software. Data was stored as a text data file using dynamic data exchange and processed using Microsoft Excel. The baseline drift associated with temperature fluctuations was found to be very low when flow injection analysis was employed, and hence, thermostat control was not used. A thermostat was used to keep the aluminum sample rack at 4° to minimize evaporation and denaturation of sample. The samples were analyzed in random order to ensure that a systemic bias was not introduced.

Results and Discussion

Control Studies

The average baseline drift was 3.5 ± 1.3 μRIU/min. The average baseline noise level, before sample injection, was 1.22 ± 0.1 μRIU. Baseline jumps associated with pump noise and air bubbles were minimal. However, regular spikes of 20-30 μRIU in magnitude were observed due to valve/pump related effects. It was found that the goat anti-biotin polyclonal antibody contained a population of antibodies that recognized epitopes composed of biotin and the biotin-linker region. This was concluded since 20 μg/ml of biotin failed to completely inhibit the binding response of the antibody to a biotin-BSA coated surface. In addition, a similar effect was observed when an ovalbumin-biotin conjugate coated surface was employed.

We concluded that the additional specific binding response could not be associated with epitopes composed of biotin-linker-protein regions. In addition, the presence of BSA, or ovalbumin, in the sample buffer did not remove this binding. Finally, non-specific goat IgG (5μg/ml in HBS) gave a negligible non-specific binding response of < 10 μRIU. The sample diluent (i.e. HBS-BSA) did not give a measurable binding response (i.e. < 2 μRIU). The maximum antibody binding response (R_{max}) in the absence of biotin was approximately 2000 RU. Hence it may be expected that a 10 fold reduction in anti-biotin concentration (i.e.0.5 μg/ml) may lower the expected R_{max} but without significantly effecting the assay performance. Obviously, minimization of baseline noise is important if expensive reagents are to be conserved.

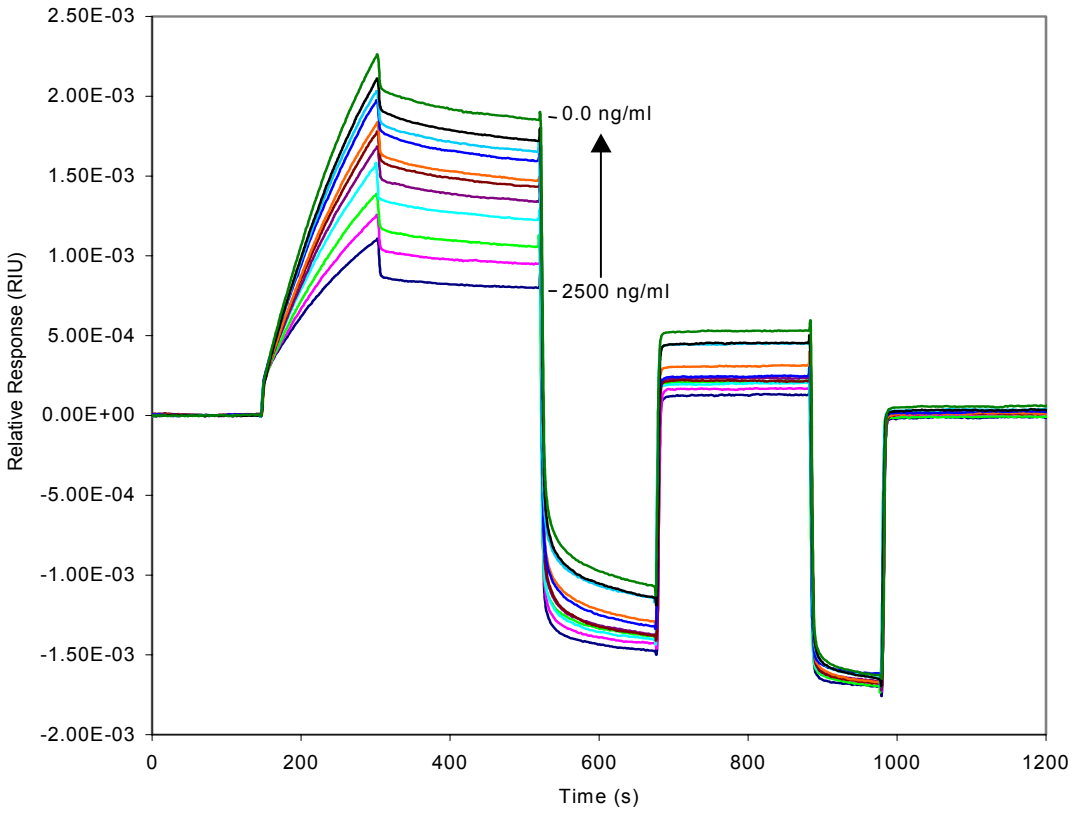

Figure 1. Overlaid sensorgrams for binding of anti-biotin to the BSA-biotin-coated surface. Each sample contains 2500.0, 1250.0, 625.0, 312.5, 156.2, 78.1, 39.0, 19.5, 9.7, 4.9, 2.4 and 0.0 ng/ml of biotin, respectively. The antibody binding response is inversely proportional to the biotin concentration.

A set of overlaid sensorgrams that are representational of the total set is shown in Figure 1 to show the quality of the data recorded. The sensorgrams show low drift and no artifacts associated with air-bubbles and backpressure effects. The sensorgrams have been plotted from raw data that has not been filtered in any way. The automation software simply records the biosensor response (refractive index units (RIU)) relative to a baseline that is set immediately before sample injection. Also, Figure 1 shows that regeneration of the surface after each sample cycle was effective. Some deterioration in surface binding capacity was observed during the course of the assay. Nevertheless, the acceptable %CV values indicate that physorption of the BSA-biotin conjugate produces a surface coating that is relatively stable, at least in the short-term.

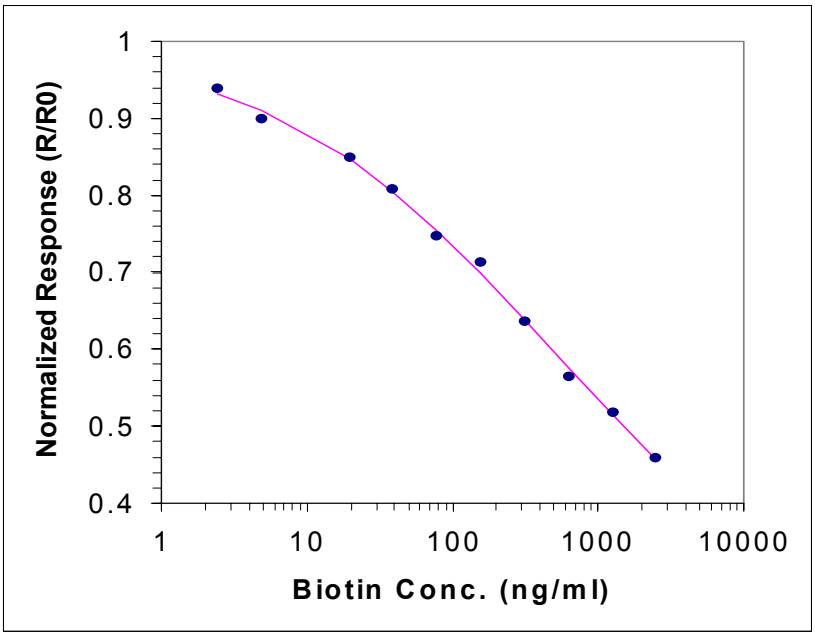

Figure 2. Calibration curve for the determination of biotin. Each sample was analyzed in triplicate. Each binding response (R) is normalized against the response obtained without biotin (R_0).

Note: The data obtained for a biotin concentration of 9.7 ng/ml was not recorded properly due to a software problem.

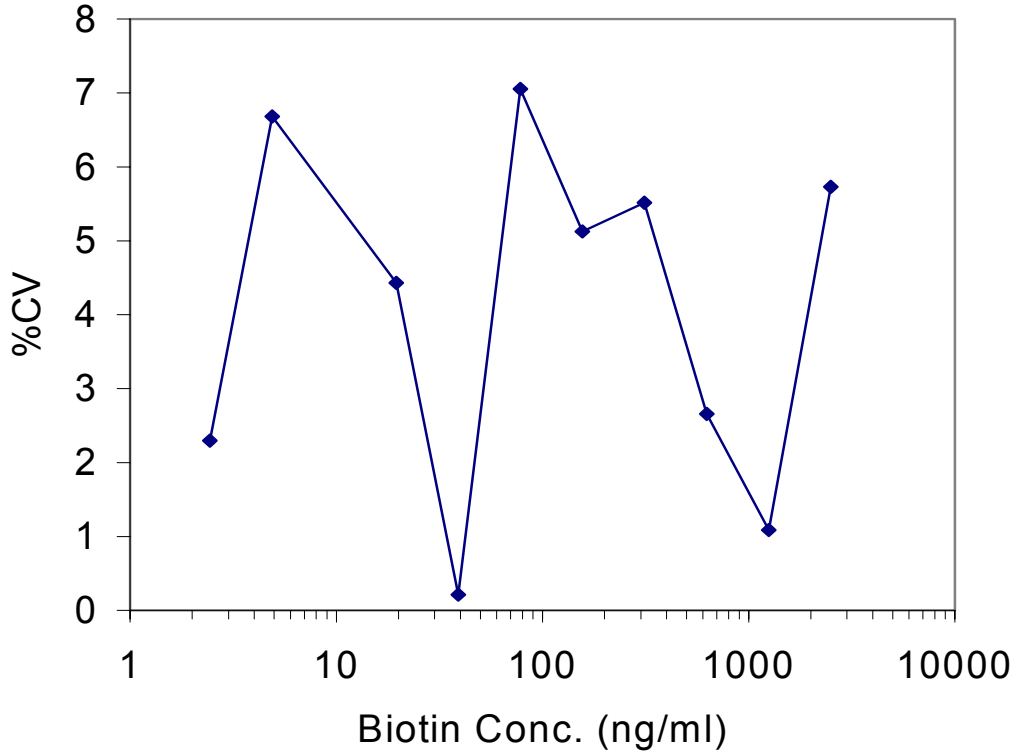

Figure 3. Plot of coefficient of variation for each biotin concentration.

A calibration curve for the immunoassay is shown in Figure 2. The binding response is obtained as the difference in response immediately before and after the sample injection and is an average over a 5 s time window. The measuring range extends from 2.4 ng/ml to 2500 ng/ml. The coefficients of variation for the assay are shown for each sample in Figure 3 (n=3). Samples prepared by spiking known concentrations of biotin into complex sample matrices and real samples were not prepared as the purpose of this application is to demonstrate the effectiveness of Spreeta and not of this particular assay.

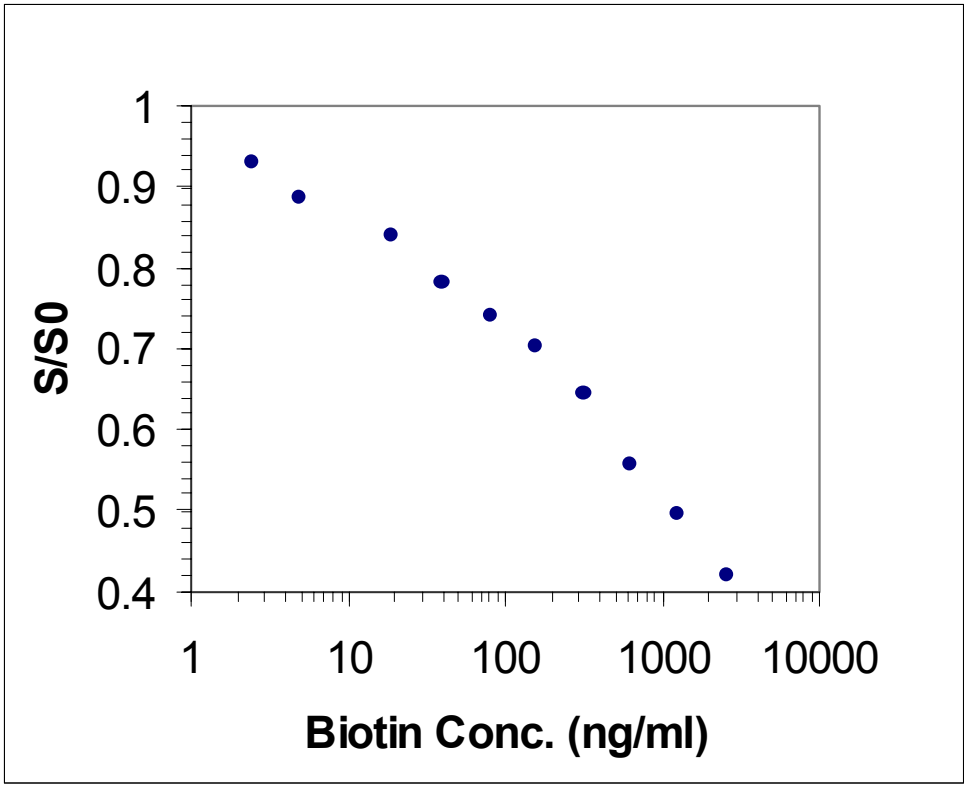

Figure 4. Calibration curve for the determination of biotin. The normalized response (S/So) was calculated by obtaining the slopes (i.e.RIU/s) of the sensorgram set shown in Figure 1. These slope values were divided by the slope obtained when biotin was not present giving the above data.

A second calibration curve for the immunoassay is shown in Figure 4. This was prepared for the same data set used in Figure 1 but the antibody-binding rate was employed rather than the change in response before and after sample injection. This method avoids the need to establish a reliable well behaved baseline before injection and also requires less than 30 seconds of binding data. A comparison with the previous calibration curve shows that they are almost identical. Hence, both methods are equivalent for this application.

Conclusion

The data presented demonstrates that high quality quantitative data can be obtained using the Spreeta biosensing device. The %CV values are acceptable for most assay applications despite the use of crude non-customized peripheral technologies. The baseline drift was very low without using temperature

control. This may be attributed to a stable room temperature and the use of flow injection analysis rather than cuvette based analysis. A loss in binding capacity of about 10% was observable after 40 binding regeneration cycles (data not shown). The use of more reliable coating chemistries will greatly enhance the assay by lowering %CV values.

APPENDIX

Raw Data

Conc (ng/ml)	N1	N2	N3	Mean	SE	SE/Ro	SD	%CV		R/R0
2500	932	870	832.4	878.1	29	0.015147875	50.3	5.728276962		0.458667
1250	1003.1	981.7	989.6	991.5	6.2	0.003238511	10.8	1.089258699		0.517901
625	1053.3	1083.5	1110.9	1082.5	16.6	0.008670852	28.8	2.660508083		0.565434
312.5	1290.3	1207.7	1157.2	1218.4	38.7	0.020214577	67.2	5.515430072		0.63642
156.25	1401.6	1408.73	1284.1	1364.8	40	0.02089362	70	5.128956624		0.71289
78.12	1463.3	1509.7	1316.4	1429.8	58	0.030295749	100.9	7.056931039		0.746842
39.06	1545.5	1550.2	1295.1	1547.8	2.38	0.0012431 7	3.32	0.214497997		0.808479
19.53	1651.5	1543.4	1680.1	1625	41.6	0.021729365	72	4.430769231		0.848803
4.88	1825.6	1740.4	1597.8	1721.2	66	0.034474473	115	6.68138508		0.899052
2.44	1830.72	1813.1	1752	1798.6	23.8	0.012431704	41.3	2.296230401		0.939482
R0	1922.1	1874.3	1947	1914.46	21	0.010969151	37	1.932659862		1