



APPLICATION BRIEF #006

# **Spreeta™** Affinity Constant Estimation

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# Affinity Constant Estimation

## ABSTRACT

**This note outlines solution phase affinity analysis of the interaction of an anti-dinitrophenyl monoclonal antibody with dinitrophenyl. The affinity constant is calculated by fitting an equilibrium model to a plot of free antibody concentration as a function of the DNP concentration in equilibrium mixtures.**

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## INTRODUCTION

In addition to providing a platform for concentration determination Spreeta allows the kinetic and affinity constants for an interaction to be determined. The affinity constant of any molecular interaction can be estimated by a number of approaches. Here we estimate the solution phase affinity constant for the interaction of a monoclonal antibody with dinitrophenyl (DNP). In this method a series of reaction mixtures containing a fixed concentration of antibody and variable concentrations of DNP are allowed to equilibrate. After equilibration the concentration of free antibody in each mixture is measured. The affinity constant is then calculated by fitting an equilibrium model to a plot of free antibody concentration as a function of the DNP concentration. In this method it is important to minimize disturbing the equilibrated mixture, and hence, short sample contact times are employed. Such analysis may be performed using enzyme-linked immunosorbent assay techniques but re-equilibration of the mixture is problematic during the long incubation period required. In contrast flow injection analysis and direct detection ensures that the concentration of free and complexed interactants remains constant thus improving the accuracy of the method.

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## EXPERIMENTAL SETUP

*As outlined in Application Note 12*

### Materials and Methods

All reagents were from Sigma-Aldrich.

DNP-BSA immobilization: 10  $\mu$ L of a BSA-DNP solution (1 mg/ml in PBS) was spread evenly over the gold surface of Spreeta and allowed to dry in air. The surface was then rinsed with 30 mM HCl followed by water. The sensor was docked with the fluidics, initialized, normalized and equilibrated.

### Assay

A 10 mg/ml stock solution of DNP was prepared in DMSO. From this solution a 10  $\mu$ g/ml working dilution was prepared. A series serial doubling dilutions were prepared. 0.5 mls of each DNP dilution was added to separate tubes containing a fixed concentration of anti-DNP monoclonal antibody. The final concentration range in the mixtures were from 4.9 to 2000 ng/ml DNP while the final antibody concentration was 3.5  $\mu$ g/ml. PBS containing 2 mg/ml BSA was used as diluent. The mixtures were allowed to reach equilibrium by incubating at room temperature for 4 hours. In order to estimate the free antibody concentration in the equilibrium and series of antibody standards from 78 to 3500 ng/ml were prepared in PBS-BSA diluent.

### Sample Analysis

The equilibrium mixtures and the antibody standards were analyzed as follows.

The running buffer (PBS, pH 7.4) was maintained at 50  $\mu$ L/min throughout the assay. Each sample was analyzed by injecting 150  $\mu$ L at 50  $\mu$ L/min. The surface was regenerated by injecting 30 mM NaOH and 30 mM HCl, sequentially. 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. The automation software simply records the biosensor response (refractive index units (RIU)) relative to a baseline that is set immediately before sample injection.

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## RESULTS AND DISCUSSION

Very little deterioration in surface binding capacity was observed during the course of the assay. This solution phase affinity analysis in essence is competitive assay format where the free antibody concentration is also determined. Figure 1 below is a calibration curve for the determination of DNP. This calibration curve is produced by mixing a fixed concentration of antibody with different DNP concentrations. Antibodies that have bound to the DSP in the sample cannot bind to my biosensor surface (coated with DNP-BSA) since the binding sites are already filled. However, free antibodies that have not bound DNP do bind to the surface. Hence, the binding response is inversely proportional to the DNP concentration in the sample.

The free antibody concentration in each sample was determined by back calculation from a calibration curve constructed with standard antibody concentrations in the absence of DNP. Figure 2 is the antibody calibration curve. The affinity constant is obtained by plotting the DNP concentration against the free antibody concentration. This plot describes concentrations of drug and antibody in each sample at equilibrium the affinity constant is estimated by fitting a solution phase equilibrium model. This model accounts for the bivalency of the antibody and allows for the probability of monovalent or bivalent occupation.

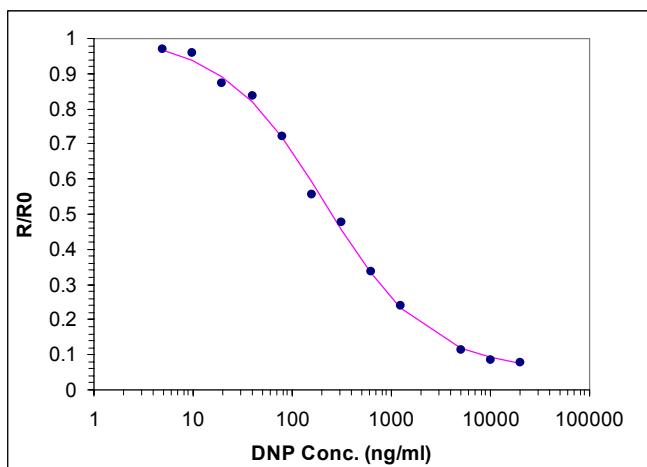


Fig.1. Curve showing the response for equilibrium mixtures containing a fixed conc. of anti-DNP antibody and a variable conc. of DNP.

$$y = B_{\max} * (1 - (x^n / (K^n + x^n))) + Y_2$$

Where:

Bmax	Bmax (SE)	K	K (SE)	Y2	Y2 (SE)	n
9.94e-1	2.38e-2	1.60e-6	1.18e-7	6.46e-3	1.62e-2	7.39e-1

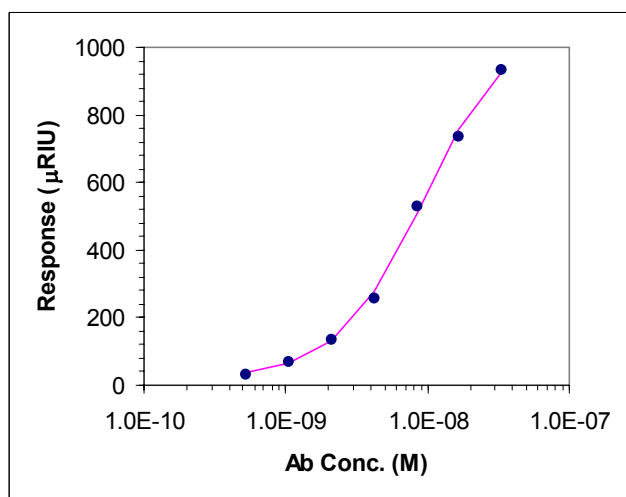


Fig.2. Calibration curve for the determination of the free antibody concentration contained in the equilibrium mixtures.

$$y = B_{\max} * (1 - (x^n / (K^n + x^n))) + Y_2$$

Where:

Bmax	Bmax (SE)	K	K (SE)	Y2	Y2 (SE)	n
-1.06e+3	8.96e+1	9.27e-9	1.07e-9	1.08e+3	7.36e+1	1.41e+0

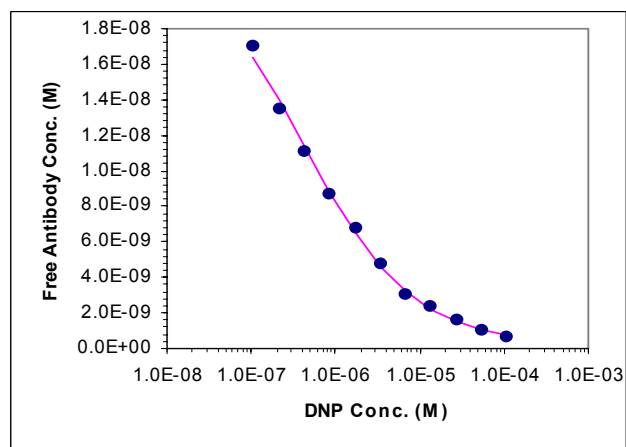


Fig.3. Affinity plot. A plot of the DNP concentration (A) against the free antibody concentration (B) contained in each equilibrium sample,

Where:

$$B(A) = \frac{B}{2} - \left[ \frac{B + A + Kd}{2} - \sqrt{\frac{(B + A + Kd)^2}{4} - AB} \right]$$

$$Kd = 4.0 \times 10^{-7} \text{ M}$$