

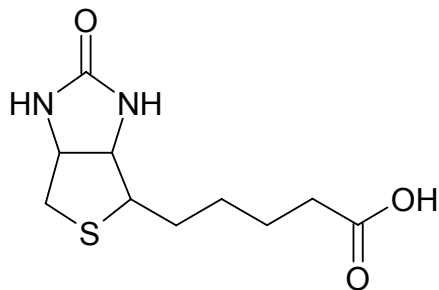
## Student Handout

### Spectrophotometric and Fluorimetric Streptavidin-Biotin Binding Assays

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#### Introduction:

Biotin (Vitamin H) is an essential cofactor for the transfer of carboxyl groups from bicarbonate to metabolic intermediates.



Biotin

A well-studied example of this would be the first step of gluconeogenesis whereby pyruvate is carboxylated by pyruvate carboxylase to form oxaloacetate. In this reaction, the biotin is bound via an amide linkage to a lysine residue on the enzyme. The nitrogen on the left side of the molecule in the urea-like moiety, as drawn above, is where the carboxyl group reversibly binds.

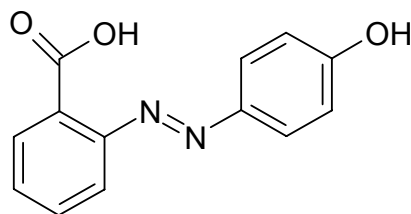
In addition to acting in a catalytic role, biotin-binding proteins such as avidin (from egg whites, among other animal tissues) and streptavidin (SA) (produced by the soil bacterium *Streptomyces Avidinii*, shown below) are thought to act as antimicrobial agents that sequester biotin from would-be invaders.



Streptavidin

The strong, specific binding of biotin by SA ( $K_D \approx 10^{-15}$  M) and avidin has been the ideal model system for protein-ligand binding studies, as well as serving as the 'lock and key' in a great many techniques and assays in the biotechnology arena<sup>1,2</sup>.

The traditional assay for biotin binding capacity utilizes 2-(4-Hydroxyphenylazo)benzoic acid (HABA), which is a weakly binding biotin analog.

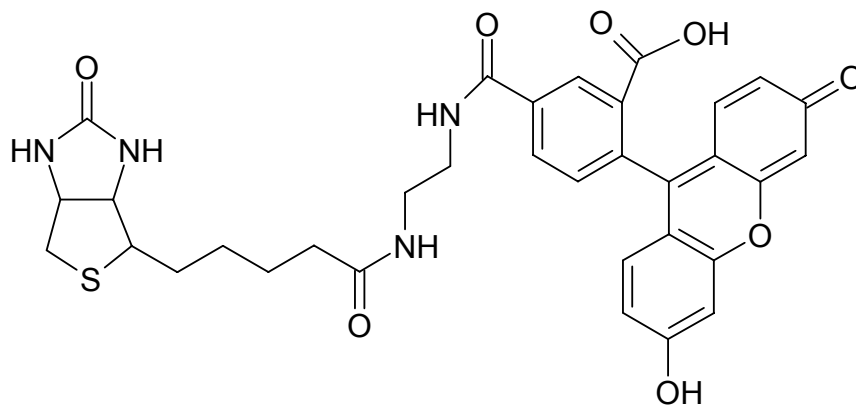


HABA

In that assay HABA is bound to avidin or SA, which alters its strong visible absorption. Upon the addition of biotin, the weaker binding HABA is displaced<sup>3</sup>. This is still often cited as the assay used by suppliers of the avidin proteins<sup>4</sup>. It is also frequently used in college teaching laboratories to illustrate quantitation of a protein-ligand binding interaction. The premise of this technique is that the extinction coefficient at 500 nm of free HABA ( $600 \text{ M}^{-1}\text{cm}^{-1}$ ) differs greatly from enzyme-bound HABA ( $34500 \text{ M}^{-1}\text{cm}^{-1}$ ). Thus, if one saturates avidin or SA with HABA, the majority of the absorbance at 500 nm is due to bound HABA. Binding of biotin to the protein will displace some or all of the HABA, resulting in a measurably lower absorbance.

Because of the wide applicability of this protein-ligand system, a variety of other techniques have been developed for the measurement of the binding interaction. Lin and Kirsch showed that the quenching of intrinsic tryptophan fluorescence ( $\lambda_{em}=350 \text{ nm}$ ) in avidin could be used to quantitate the binding of biotin<sup>5</sup>. A variety of modified avidin proteins<sup>6</sup>, as well as biotin analogs<sup>7</sup> have also been developed, including: radio-labeling of biotin or avidin<sup>8,9</sup> and functionalization of SA or biotin with fluorescent tags<sup>10-14</sup>. The assays which use these approaches have a range of sensitivities from  $\sim 3 \text{ pM}$  to  $1 \text{ }\mu\text{M}$ <sup>15</sup>.

Recently, Kada et al. synthesized biotin-4-fluorescein (B4F) and demonstrated its application to highly sensitive quantification of available SA units in solutions, including crude biofluids, where background absorbance would limit the use of HABA and related assays<sup>15,16</sup>. The primary principle of this assay is that B4F fluorescence is highly quenched ( $\geq 80\%$ ) upon binding to SA (polarization effects also accompany binding)<sup>15</sup>.



Biotin-4-fluorescein

Many of the available assays apply to limited ranges of protein-ligand concentration, are time consuming and/or use significant quantities of reagents. The B4F assay of Kada et al. is an exception to this general rule, but requires a reasonably sensitive fluorescence spectrometer to examine the lowest concentration ranges.

As it turns out a significant contributor to the observed fluorescence quenching of B4F upon binding to SA has recently been shown to be due to the large decrease in the extinction coefficient of B4F at 493 nm<sup>17</sup>. A new spectrophotometric assay for biotin binding capacity of SA was developed based on that principle. Using this technique, one may observe the stoichiometric binding via absorbance at 493 nm using either SA or B4F as the titrant. The sensitivity of the assay is at the lower end of similar fluorimetric and photometric assays. Though the sensitivity is not substantially better than comparable techniques, this assay offers additional flexibility in working range and instrumentation, since the same stock solutions may be used for this new photometric assay or the fluorescence assay for which this ligand was first developed.

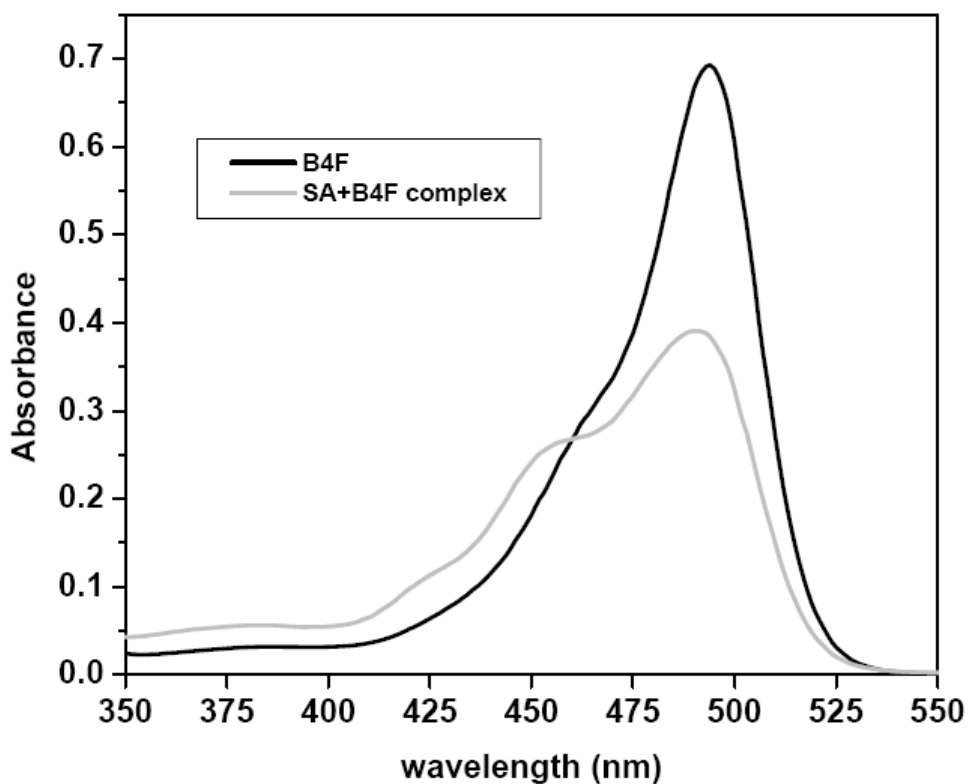


Figure 1: Visible absorption spectra of free and SA-bound biotin-4-fluorescein.

In this experiment we will examine the changes in the molar extinction coefficient of B4F that occur upon binding to SA. The use of B4F as a probe of SA activity is especially useful since the same reagent solutions may be used to carry out measurements using fluorescence or visible absorption spectroscopy.

## Materials & Methods:

**Reagents** The solvent is 10 mM triethanolamine (TEA) at pH=7.3 prepared using Type I, 18 M $\Omega$  water. The streptavidin stock solution has a nominal concentration of 140  $\mu$ M (~7 mg/mL). The actual SA concentration will be determined using  $A_{280}$  with  $\epsilon_{280}^{1\text{mg/ml}}=3.2\text{ cm}^{-1}\text{ }^{18}$ . The nominal stock D-biotin concentration is 1.8 mM, and the nominal stock biotin-4-fluorescein (B4F) is 33  $\mu$ M.

**Instruments & Accessories** Spectrophotometry will be performed using a Varian Cary 50 and ThermoSpectronic Genesys 20 uv-vis spectrophotometers. The Cary 50 is equipped with a thermostated Peltier sample holder that should be set to 25°C. Measurements will be made using quartz semi-micro cuvettes.

Fluorescence spectroscopy is carried out using a Photon Technology International (PTI, Lawrenceville, NJ) QuantaMaster Dual-Emission Spectrofluorimeter with a single, thermostatted and stirred sample cell will be used for all fluorescence analyses. Constant temperature will be maintained using a Lauda RE106 recirculating water bath (Lauda, Lauda-Königshofen, Germany). Excitation illumination at 493 nm is provided by a 75 W output power Xenon arc lamp. Bandpasses should be 2 nm and 8 nm for excitation and emission, respectively. When not collecting data, the excitation shutter should be closed to avoid photobleaching. When monitoring the fluorescence of B4F ( $\lambda_{\text{ex}}=493\text{ nm}$ ,  $\lambda_{\text{em}}=520\text{ nm}$ ) The PMT voltages should be 810 V with a gain of  $10^{-3}$ .

### Week 1

#### All groups

##### **Determination of SA concentration**

Use a quartz semi-micro cuvette in a Cary50 spectrophotometer to accurately measure the concentration of the SA. Dilute out the SA stock solution into TEA buffer to achieve an absorbance value somewhere in the range of 0.1 to 0.8. You can determine this from the nominal concentration of the SA stock and the molar extinction coefficient cited above. From that, calculate the millimolar concentration of protein, given the SA molecular weight of 52 kDa<sup>4</sup>.

##### **Spectrophotometric Titrations**

For all of these measurements, use a Genesys 20 spectrophotometer. Initially you will perform a “crude” titration of SA into B4F. You will start with a B4F concentration approximately 0.6 to 0.8  $\mu$ M (final concentration in cuvette), and titrate small aliquots of a diluted SA solution to determine the “break point.” The wavelength to be observed is 493 nm. You will need to determine the SA dilution and titration aliquots necessary to do this. The goal is to find the break point within about 6-8 aliquots.

The two groups done fastest will continue on to the next part, while the other four groups will pick up in the next session.

## Group 1

This group will standardize the B4F concentration by titration of SA with B4F via fluorescence titration. This begins with 2.00 mL of 30-80 nM SA in 0.01 M TEA in a quartz fluorescence cuvette. For B4F fluorescence, use 493 nm for excitation and 520 nm. Check to see that the photomultiplier tube (PMT) voltages are 810 V with a gain of  $10^{-3}$ . Collect data in time-based emission mode averaging over 4 seconds (1 second per point). Titrate with small (~2-3  $\mu\text{L}$ ) aliquots of the stock B4F solution. 10-12 aliquots should be sufficient. Don't forget to close the excitation shutter when not collecting data to avoid photobleaching of the B4F. Equilibrate solutions in the instrument (magnetic stirring on) for a minimum of 2 minutes after each addition of ligand. Do each titration in duplicate.

Correct the observed fluorescence for the increasing concentration of B4F (see below). Since each SA binds four B4F molecules, you will normalize the break point of the titration to a  $[\text{B4F}]/[\text{SA}]$  ratio of 4.00.

When bound to SA the fluorescence of B4F is found to be about 80% quenched. Therefore, the fluorescence intensity will increase slowly until there is excess unbound B4F. At that point the fluorescence will rapidly increase. Since you are increasing the concentration of B4F with each aliquot added, you will want to correct the observed fluorescence for this. Starting with the second addition of B4F you will want to multiply the observed fluorescence by a dilution correction factor:

$$\frac{V_{\text{after first addition}}}{V_{\text{total}}} = \frac{2002 \mu\text{L}}{(2000 + V_{\text{total B4F added}}) \mu\text{L}}$$

Plot corrected  $A_{493}$  vs.  $[\text{B4F}]:[\text{SA}]$ . Using the linear regression data for the two line segments determine the crossing point and average results.

In week 2 this group will perform a more detailed Spec20 titration of SA into B4F. This involves roughly 10-12 points, with at least three points before and after the breakpoint, performed in triplicate.

## Group 2

This group will use the Cary50 to titrate SA into B4F (a "reverse" titration) in fine detail (~10-12 points). For this group, assays begin with 1.00 mL of 0.35-1.0  $\mu\text{M}$  B4F. For the titration, measure absorbance at 493 nm and titrate with small aliquots of dilute SA. You should collect 10-12 points for each titration. There should be at least three points before and after the breakpoint at saturation. Upon each addition mix the solution by micropipette and equilibrate 2 min. Do this in duplicate.

Correct the absorbance to remove the contribution from the decreasing concentration of B4F. Starting with the first addition of SA, multiply the absorbance by the following dilution correction factor:

$$\frac{V_{\text{total}}}{V_{\text{initial B4F}}} = \frac{(1000 + V_{\text{total SA added}}) \mu\text{L}}{1000 \mu\text{L}}$$

Plot corrected  $A_{493}$  vs.  $[\text{SA}]:[\text{B4F}]$ . Using the linear regression data for the two line segments determine the crossing point and average results.

In week 2 this group will perform their Genesys 20 titration of in finer detail, 10-12 points, with at least three points before and after the breakpoint, performed in triplicate.

## Week 2

**Group 3:** Group 3 will use the Cary50 to perform “forward” titrations (B4F as the titrant). For this group, assays begin with 1 mL of 50-100 nM SA in a quartz semi-micro cuvette. For this titration, set the wavelength to 493 nm and titrate with small aliquots of 31  $\mu$ M B4F. You should collect 10-12 points for each titration, with at least three points before and after the breakpoint. Upon each addition the solution will be mixed by micropipette and allowed to equilibrate 2 min. Do this in triplicate.

Correct the absorbance to remove the contribution from the increasing concentration of B4F. Starting with the second addition of B4F, multiply the absorbance by the following dilution correction factor:

$$\frac{V_{\text{after first B4F addition}}}{V_{\text{total}}} = \frac{(1000 + V_{\text{first B4F aliquot}}) \mu\text{L}}{(1000 + V_{\text{total B4F added}}) \mu\text{L}}$$

Plot corrected  $A_{493}$  vs. [B4F]:[SA]. Using the linear regression data for the two line segments determine the crossing point and average results.

**Group 4:** Group 4 will use the Cary50 and perform “reverse” titrations (SA as the titrant). For this group, assays begin with 1.00 mL of 0.35-1.0  $\mu$ M B4F in a quartz cuvette. For the titration, set the absorbance at 493 nm and titrate with 10-12  $\mu$ L aliquots of dilute SA. You should collect 10-12 points for each titration, with at least three points before and after the breakpoint. Upon each addition mix the solution by micropipette and equilibrate 2 min. Do this in triplicate.

Correct the absorbance to remove the contribution from the decreasing concentration of B4F. Starting with the first addition of SA, multiply the absorbance by the following dilution correction factor:

$$\frac{V_{\text{total}}}{V_{\text{initial B4F}}} = \frac{(1000 + V_{\text{total SA added}}) \mu\text{L}}{1000 \mu\text{L}}$$

Plot corrected  $A_{493}$  vs. [SA]:[B4F]. Using the linear regression data for the two line segments determine the crossing point and average results.

**Group 5:** This group will begin with the fluorimetric B4F into SA titration as stated above (see Group 1 instructions). If results are consistent with the data from Group 1, go on to the next step. If the data are not consistent, do another repetition to see if that titration was flawed or if there is some systematic problem.

Once finished this group will then titrate biotin into SA. You will use the intrinsic fluorescence of SA tryptophan residues at 350 nm as the probe. This titration begins with 2.0 mL of 30-80 nM SA in 0.01 M TEA. To examine the intrinsic fluorescence of SA tryptophans, use 280 nm for excitation and 350 nm for emission. Dial the photomultiplier tube (PMT) voltages to 930 V with a gain of  $10^0$ . Collect data in time-based emission mode averaging over 4 seconds (1 second per point). When not collecting data, close the excitation shutter to avoid photobleaching of the SA. Titrate with 2-5  $\mu$ L aliquots of 100 times diluted biotin solution. Ten to twelve aliquots should be sufficient to obtain a good breakpoint, with at least three points before and after the breakpoint. Equilibrate solutions with magnetic

stirring for a minimum of 2 minutes after each addition of ligand. Do each titration in duplicate.

Correct the fluorescence to account for the decreasing concentration of SA as biotin is added. Starting with the first addition of biotin, multiply the absorbance by the following dilution correction factor:

$$\frac{V_{\text{total}}}{V_{\text{initial B4F}}} = \frac{(2000 + V_{\text{total biotin added}}) \mu\text{L}}{2000 \mu\text{L}}$$

Plot corrected fluorescence vs. [biotin]:[SA]. Using the linear regression data for the two line segments determine the crossing point and average results.

**Group 6:** This group will titrate SA into B4F using the fluorimeter (a reverse titration). This begins with 2.00 mL of 0.5-1.3  $\mu\text{M}$  B4F in 0.01 M TEA in a quartz fluorescence cuvette. For B4F excitation, use 493 nm for excitation and 520 nm for emission. Check to see that the photomultiplier tube (PMT) voltages are 810 V with a gain of  $10^{-3}$  for collection of B4F emission. Collect data in time-based emission mode averaging over 4 seconds (1 second per point). When not collecting data, close the excitation shutter to avoid photobleaching of the B4F. Titrate with small ( $\sim 2\text{-}3 \mu\text{L}$ ) aliquots of 50  $\mu\text{M}$  SA solution. 10-12 aliquots should be sufficient, with at least three points before and after the breakpoint. Equilibrate solutions with magnetic stirring for a minimum of 2 minutes after each addition of ligand. Perform each titration in triplicate.

Correct the fluorescence to account for the decreasing concentration of B4F as SA is added. Starting with the first addition of SA, multiply the absorbance by the following dilution correction factor:

$$\frac{V_{\text{total}}}{V_{\text{initial B4F}}} = \frac{(2000 + V_{\text{total SA added}}) \mu\text{L}}{2000 \mu\text{L}}$$

Plot corrected fluorescence vs. [SA]:[B4F]. Using the linear regression data for the two line segments determine the crossing point and average results.

Once complete, all groups will plot and share their data and come prepared next week to discuss the class findings.



## References:

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Figures: These figures are included for illustration purposes of what kind of data you may observe for some parts of this experiment.

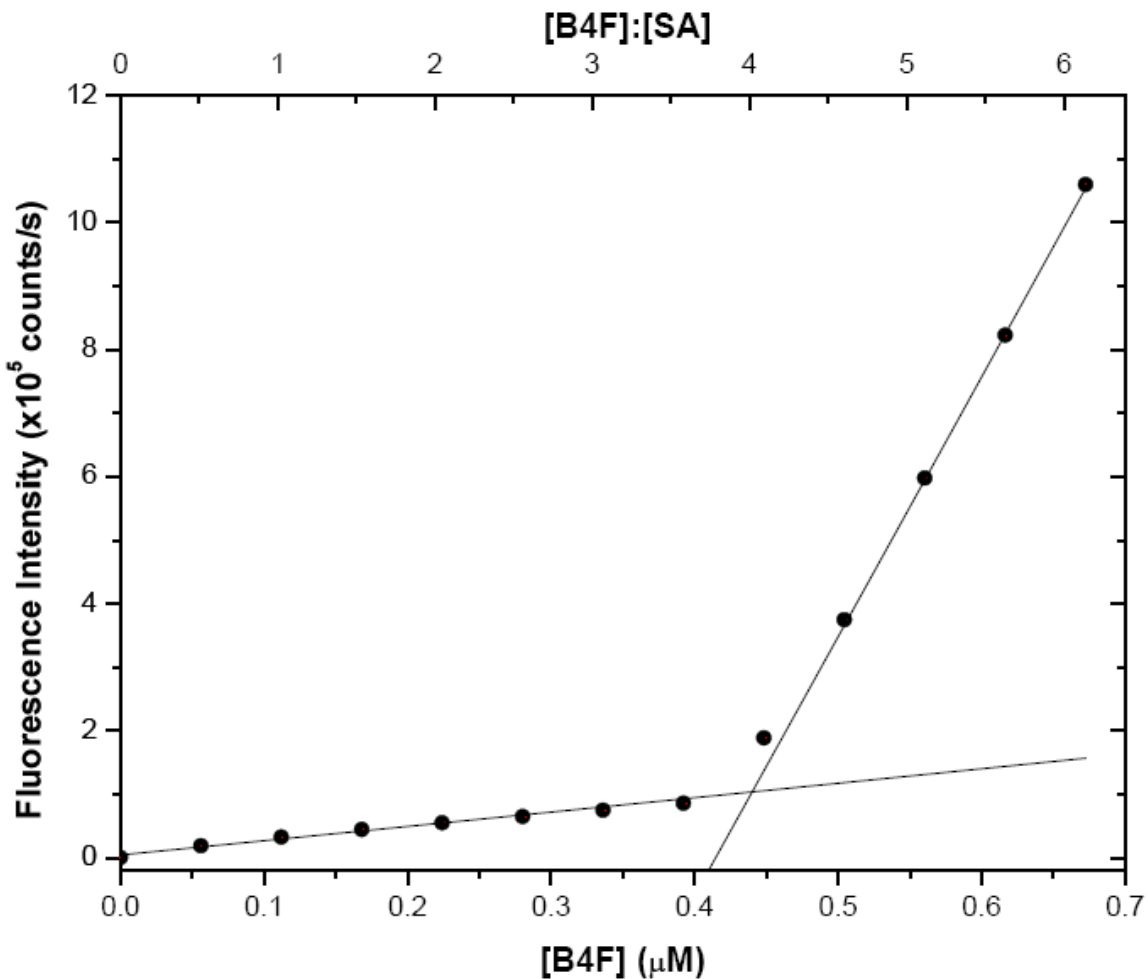


Figure 2: Fluorescence emission at 520 nm ( $\lambda_{\text{ex}}=493$  nm) upon titration of biotin-4fluorescein into 0.1  $\mu\text{M}$  streptavidin. Used to standardize the concentration of the B4F solution via the fluorescence assay of Kada et al.<sup>15,16</sup>.

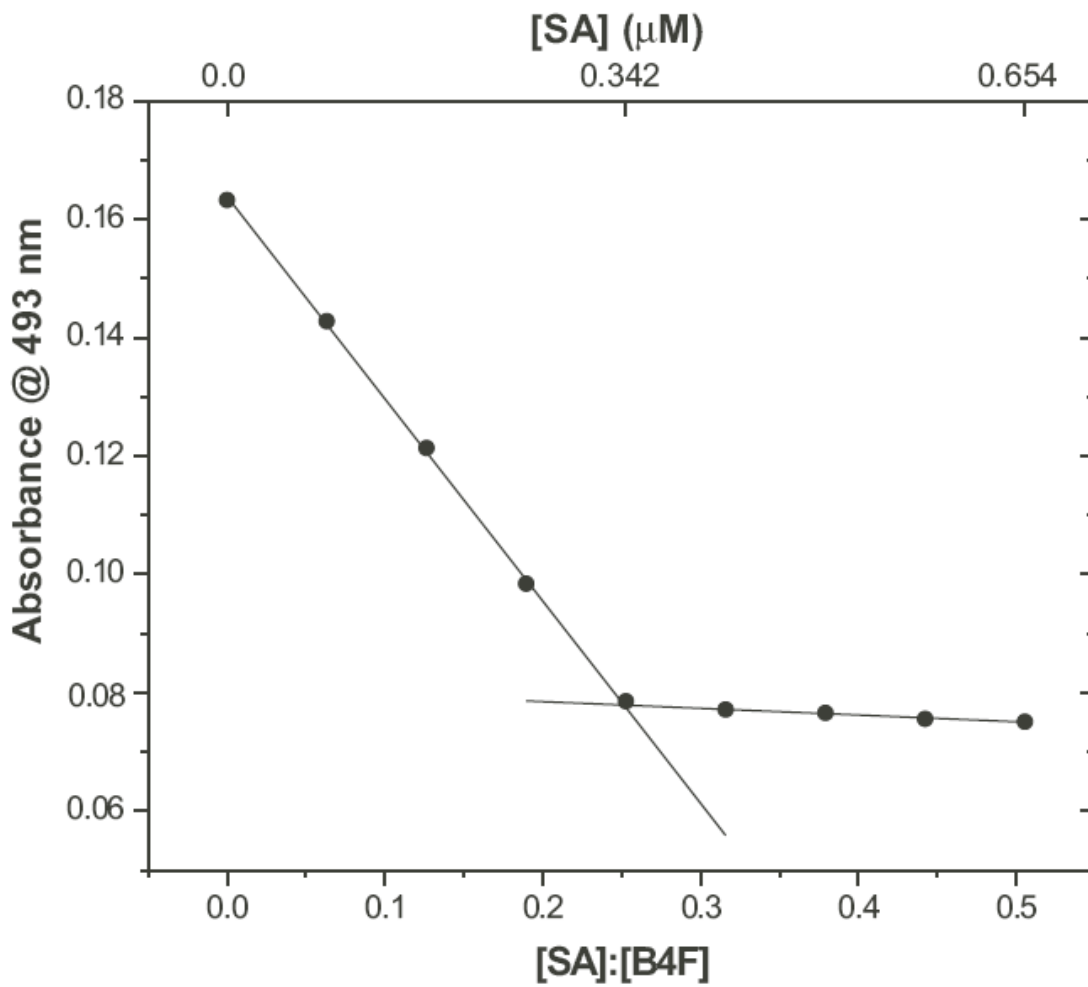


Figure 3: Absorbance ( $A_{493}$ ) titration of  $0.7 \mu\text{M}$  biotin-4-fluorescein with streptavidin ( $12 \mu\text{L}$  aliquots of  $0.4 \mu\text{M}$ ).

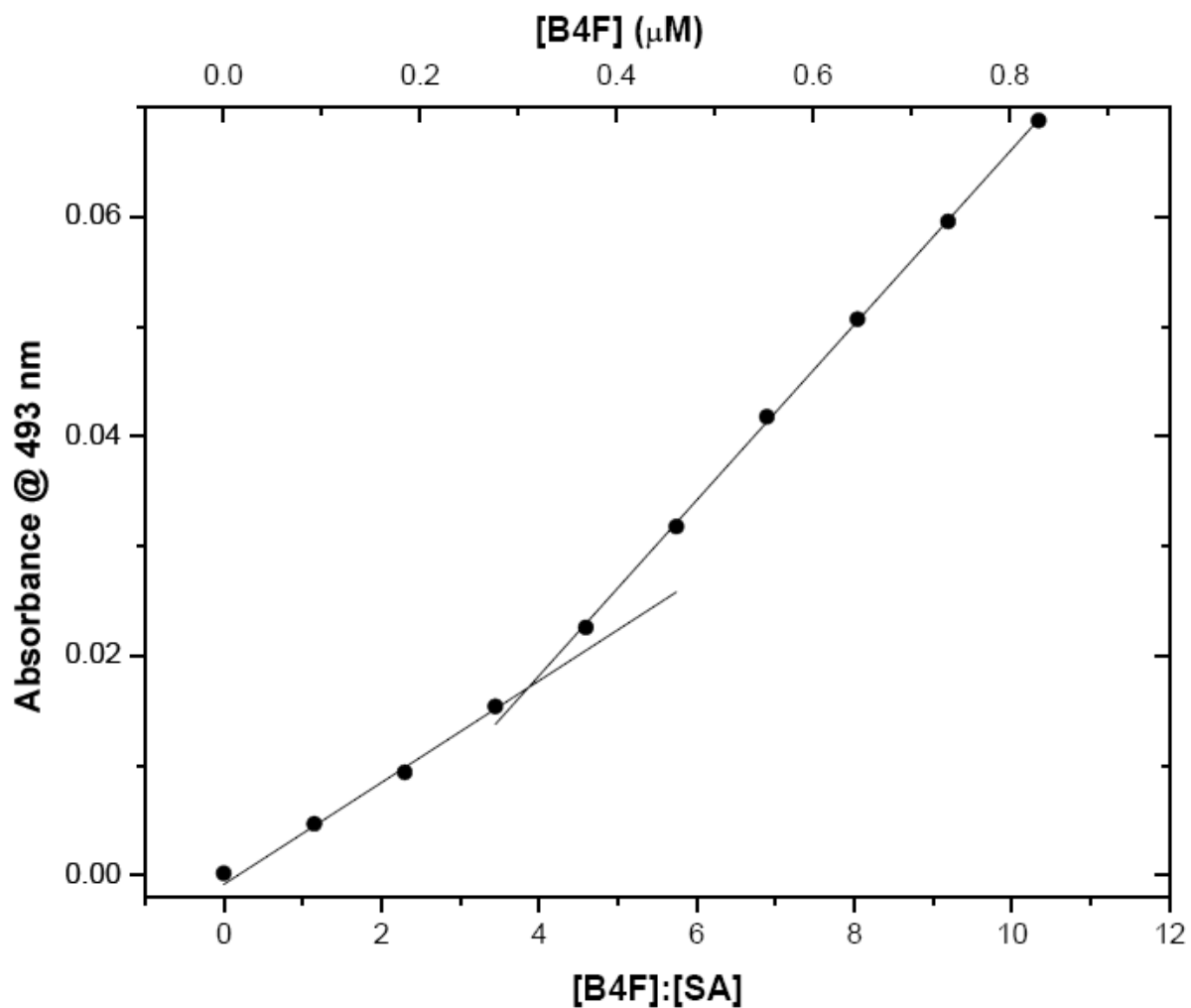


Figure 4: Absorbance ( $A_{493}$ ) titration of 93 nM streptavidin with biotin-4-fluorescein (3  $\mu$ L aliquots of 31  $\mu$ M).

**Instructor notes:**

- The two groups done fastest will continue on to the next part, while the other four groups will pick up in the next session. (p 5) Explain why...Throughput on fluorimeter...
- Our experiments were performed with thermostated sample holders at 25°C, however these assays are not very dependent on temperature. We have not found any significant alteration in the fluorescence titrations of biotin into SA at lower temperatures (to 10°C) or up to 35°C. Spectrophotometric results for titration of SA into B4F were not altered when performed at 30°C.
- Upon each addition the solution will be mixed by micropipette and allowed to equilibrate 2 min. (p 7 &...) Explain how – P200 pipet set to ~100 μL, fill at bottom, empty at top of solution.