Supplemental File 1

Assessment of streptavidin bead binding capacity to improve quality of streptavidin-based enrichment studies

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Table of Contents for Supporting Information

Other supplementary materials for this manuscript include the following:

Supplemental File 2. Skyline library file to enable extraction of streptavidin peptides

Supplementary Information Text

Methods

Cell Surface Capture (CSC) Technology

Ten million B cells (RPMI 1788) were taken through the CSC-Technology workflow as previously described in detail, with the exception that glycopeptide capture and bead washing was performed using an epMotion5073 (Eppendorf).[1–4] Cells were washed with PBS and oxidized by treatment with 1 mM sodium meta-periodate (Pierce, Rockford, IL) in PBS pH 7.6 for 15 min at 4°C followed by 4 mg/ml biocytin hydrazide (Biotium, Hayward, CA) in PBS pH 6.5 for 1 hour at 4°C. Cells were then collected and lysed in 600 µL of 2x Invitrosol (40% v/v; Thermo Fisher Scientific, Waltham, MA), 20% acetonitrile in 100 mM ammonium bicarbonate. Samples were sonicated (VialTweeter; Hielscher Ultrasonics, Teltow, Germany) by three ten-second pulses, set on ice for one minute, and then sonicated by three ten-second pulses. Samples were brought to 5mM TCEP and reduced for 30 min at 37°C on a Thermomixer at 1200 rpm. Samples were brought to 10 mM IAA and alkylated for 30 min at 37°C on a Thermomixer at 1200 rpm in the dark. The samples were incubated at a 1:40 peptide to trypsin/lysC (Promega. Madison, WI) ratio and digested at 37°C overnight on a Thermomixer at 1200 rpm. Peptides were quantified using Pierce™ Quantitative Fluorescent Peptide Assay and divided so that the streptavidin bead-based capture step of each CSC experiment within a comparison began with the same pool of 1000 µg total peptide. Peptides were incubated with 100 µl streptavidin beads for 1 hour at 25°C. Beads were sequentially washed with 2% SDS in ultrapure water, 80 mM sodium phosphate, 2 M NaCl, 0.2% Tween®20, 100 mM sodium carbonate, and 50 mM ammonium bicarbonate. Beads were resuspended in 50 mM ammonium bicarbonate and 20 units PNGaseF (Promega, Madison, WI) and incubated at 37°C on a Thermomixer at 1200 rpm. Samples were cleaned using SP2 to remove detergents and concentrate peptides.[5]

MS1 Peak Area Assessment of Streptavidin Peptides

RAW and searched MS data for CSC were imported into Skyline-daily (20.1.1.83).[6] Peptide inclusion criteria were (1) fully tryptic, (2) no missed cleavages, (3) length 6-30, (4) exclude N-terminal amino acids, and (5) no methionine residues.

RPMI 1788 CSC Data Acquisition and Searching

CSC data were acquired and searched as part of this study using parameters in Tables S1 and S2. Raw files are available on MASSIVE (massive.ucsd.edu; MSV000086213).

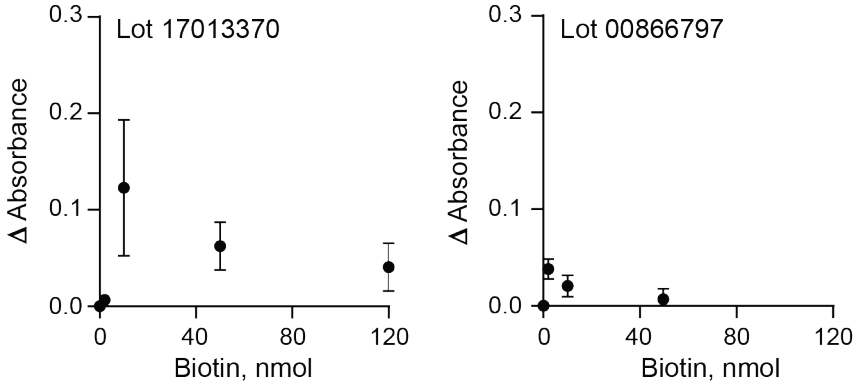
Table S1. Mass spectrometry acquisition settings

	Orbitrap Fusion Lumos: Cell Surface Capture	**Orbitrap Exploris 480: Cell Surface Capture**
Injection Mode	uL PickUp	User Defined Method
Sample Loop	14 µL	18 µL
Stationary Phase	Michrom Bioresources Magic C18AQ 200 Å, 3 µm, 10 cm	PepMap RSLC C18 100 Å, 3 µm, 15 cm
LC Solvent A	100% H_2O, 0.1% formic acid	100% H_2O, 0.1% formic acid
LC Solvent B	80% MeCN, 0.1% formic acid	80% MeCN, 0.1% formic acid
LC Gradient	5-5% B in 5 min 5-30% B in 32 min 30-40% B in 5 min 40-98% B in 2 min	5-5% B in 3 min 5-30% B in 52 min 30-55% B in 10 min 55-95% B in 1 min
LC Flow Rate	300 nL/min	300 nL/min
Mass Spectrometer	Thermo Orbitrap Fusion Lumos	Thermo Orbitrap Exploris 480
Method Type	Data dependent MS2, cycle time 3s	Data dependent MS2, cycle time 3s
Spray Voltage	2.0 kV	2.0 kV
MS^1 Detector	Orbitrap	Orbitrap
MS^1 scan range	300-1600 m/z	375-1800 m/z
MS^1 resolution	120,000 @ 200 *m/z*	120,000 @ 200 *m/z*
MS^1 AGC Target	400000	300 %
MS^1 Maximum IT	50 ms	50 ms
MS^2 Detector	Orbitrap	Orbitrap
MS^2 resolution	30,000 @ 200 m/z	30,000 @ 200 m/z
Isolation Window	1.6 m/z	1.6 m/z
MS^2 AGC Target	50,000	Custom
MS^2 Maximum IT	54 ms	Auto
Activation Type / Collision Energy	HCD 30%	HCD 30%
Intensity Threshold	Maximum intensity: 1e20, minimum intensity: 5e4	5e4
Dynamic Exclusion	60 s	15 s

Table S2. Peptide search and post-search validation parameters

Sample	**Cell Surface Capture (Hi-Hi)**
Platform	ProteomeDiscoverer 2.4
Search Algorithm	SequestHT MSFragger
Validation	Percolator Peptide Validator Protein FDR Validator
Database	SwissProt; Human; Trypsin, PNGase F, Streptavidin, Pierce PRTC, created 12/05/2019
Enzyme (semi/full)	Trypsin (semi)
Missed Cleavages	2
Precursor mass tolerance	20 ppm
Fragment mass tolerance	0.05 Da
Static Modifications	Carbamidomethyl (C)
Dynamic Modifications	Oxidation (M), Acetylation (N-term) Deamidation (N)
Target FDR (Strict):	0.01
Target FDR (Relaxed):	0.05
Validation basis	q-Value

Supplemental Figure 1. Biotin titration curves for Cytiva Sera-Mag Streptavidin-coated magnetic beads (lot 17013370) and Invitrogen Dynabeads MyOne Streptavidin C1 (lot 00866797). Changes in absorbance (Δ absorbance) are shown as means with SD error bars (n=3). Given the high variability in measurements for these two beads, these data are not considered usable titration curves but are provided for completeness.



General Considerations for Assessing Streptavidin Bead Fit-for-Purpose

1 Purpose

This general consideration for assessing streptavidin bead fit-for-purpose document is the result of our experiences regarding the challenges associated when evaluating the most apt streptavidin bead product for a given application. In an effort to promote repeatability among studies, we provide general guidelines to consider when using streptavidin bead products and advocate for the recording and reporting of key parameters of streptavidin beads to facilitate consistent and reproducible results within as well as among laboratories.

2 Outline

General Considerations: Details and strategies to be considered regarding physical bead characteristics, bead handling characteristics and performance, and manual manipulation versus automation.

Streptavidin Bead Key Parameter Record: Template for recording and reporting key parameter of streptavidin beads and their binding capacity that allows for conistent and reproducible results.

3 Physical Bead Characteristics

3.1 Type of Bead

There are a variety of commercially available products featuring streptavidin immobilized to beads. The form of streptavidin, the type of substrate it is bound to, and the size of the substrate differ among products. The protein may be avidin, streptavidin, and NeutrAvidin™, and substrates include magnetic, paramagnetic, and superparamagnetic particles, sepharose, acrylamide, and agarose beads. The type of bead and the format it is confined to can determine whether it is suitable for automation or requires manual manipulation. In general, liquid handling stations can accommodate magnetic beads relatively easily, whereas sepharose, acrylamide, and agarose beads might need to be confided to filter tips[7], columns, and/or well plates (*e.g.* auto Cell Surface Capture[7], Capturem technology by Takara) to allow automation.

3.2 Physical Properties

3.2.1 Size

Beads come in various sizes (1µm – 80µm), which should be considered when deciding on the format of the experiment (*i.e.* manual manipulation versus automation) as well as the scale of the method. When attempting to implement automated liquid handling devices or reduce the scale of the reaction volumes and tube sizes, bead volume may be a limiting factor due to size constraints of the beads.

3.2.2 Binding capacity

There are no universal reporting standards for describing the binding capacity of the beads.

Binding capacities are commonly reported as a range or “greater than” a specified value instead of a discrete measurement that informs the binding capacity of a specific product lot. Binding capacities have been reported in the following units:

- Free biotin (nmol/mL, pmol/mg)
- Biotinylated bovine serum albumin (reported as mg biotinylated BSA per mL resin)
- Biotinylated peptide (nmol/mL, pmol/mg)
- Biotinylated oligos (pmol/mL, pmol/mg)
- Biotinylated antibody (mg/mL, µg/mg)
- Human biotinylated IgG (reported as µg or mg human IgG per mg or mL beads)

Binding capacities reported using identical units cannot necessarily be directly compared among vendors because the concentration of beads within a transport solution as well as the size and substrate of the beads varies among products. Product descriptions frequently do not specify the method used to determine binding capacity. Therefore, we highly recommend users to test binding capacity of beads and report key parameters listed in section ***6 Streptavidin Bead Key Parameter Record***.

3.2.3 Shelf-life

Expiration date for products can be obtained from product container or datasheet. We recommend users refrain from using expired beads as streptavidin binding capacity can diminish over time and degradation products can potentially introduce interferences into the mass spectrometer. Vendors may not list an expiration date and it is up to the user to decide how long they want to continue using a bead product. We recommend users to routinely test the binding capacity of beads to ensure bead performance meets the expected standard.

4 Bead Handling Characteristics and Performance

4.1 Binding, Wash, and Elution Buffer

The behavior of beads is directly related to the type buffer that they are resuspended in. Common characteristics to observe during resuspension and pelleting:

- Clumping versus dispersion of beads
- Resistance to pelleting
- Pellet smearing during aspiration
- Adherence of beads to side of tube
- Resuspension behavior in binding buffer or elution buffer

In general, it is advantageous for beads to resupend easily in buffers and pellet tightly when centrifuged or placed on a magnetic rack.

For magnetic beads, it is recommended to use Neodymium magnetic rack of strength ≥ N38, or an alternative magnet of similar strength, as this greatly enhances ‘tightness’ of the pellet to avoid bead loss during aspiration of the supernatant.

4.1.1 Binding and wash buffer

The background buffer recommended for most streptavidin bead assays is either a Tris-HCl or sodium-phosphate-based buffer of neutral pH. Often additives such as salts (*e.g.* NaCl), detergents (*e.g.* SDS, Tween®20), and chelating agents (*e.g.* EDTA) are added to faciliate

the binding of biomolecules of interest and prevent binding of non-specific biomolecules. However, users should be aware that some bead products inherently bind a lot of non-specific binders despite the use of salts and detergent.

4.1.2 Elution buffer

Biomolecules can be eluted by various stratgies:

- On-bead digestion: trypsin, PNGaseF
- On-bead elution: 8M guanidine-HCl, glycine

Users should ensure elution buffers are compatible with on-bead digestion or elution strategy as well as bead product. For example, for optimal performance of on-bead PNGase F digestion, it is recommended to resupended PNGase F in 50 mM ammonium bicarbonate. PNGase F is incomptabile with detergents such as SDS and Tween®20.

4.2 Bead Performance

4.2.1 Specificity

Depending on the type of experiment, specificty can either be determined by the use of proper control experiments or by a direct readout that allows users to determine the percentage of specific versus non-specific binders. Although salts and detergents can often help prevent the binding of non-specific binders, users need to take into consideration that some beads inherently bind a lot of non-specific binders despite the use of such additives.

4.2.2 Aggregation behavior

Beads show enhanced resuspension and pelleting behavior when background buffers include detergents and salts. However, additives can often interfere with downstream analysis and users should test compatibility.

5 Manual Manipulation versus Automation

Manual manipulation can be achieved with centrifugation or vacuum filtration of filter tubes or plates. There are numerous formats available for automation, including tips packed with non-magnetic beads or freely dispersing magnetic beads. Depending on the needs of the user, each of these applications will have unique constraints related to the maximum volume of the bead product and wash and elution solutions to be used. There will also be limitations to the volume that can be aspirated. For example, implementing an automated liquid handling workstation for magnetic beads will likely require the user to optimize settings so that during washing steps, as much volume as possible can be aspirated but bead disturbance is minimized to avoid bead loss. Alternatively, workstations with vacuum capabilites can be considered.

6 Streptavidin Bead Key Parameter Record

To promote repeatability among studies, we advoate for the recording and reporting of key parameters of streptavidin beads to facilitate conistent and reproducible results within as well as among laboratories.

6.1 Protocol Details – Blank

This table includes information required to assess binding capacity of streptavidin bead products.

Streptavidin binding assay:			
Protocol details			
Name of beads:			
Type of bead:			
Size:			
Catalog # of beads:			
Lot # of beads:			
Volume of beads:			
Wash buffer:			
Binding buffer:			
Elution buffer:			
Biotin substrate:			
Amount of biotin substrate:			
If AVIDIY assay is used			
Amount of HABA:			
Reagent details			
Name	Catalog #	Vendor	Lot

6.2 Protocol Details - Example

Streptavidin binding assay: AVIDITY Assay			
Protocol details			
Name of beads:	Cytiva Sera-Mag SpeedBeads Neutravidin-Coated Magnetic Beads		
Type of bead:	Superparamagnetic		
Size:	1 µm		
Catalog # of beads:	78152104011150		
Lot # of beads:	17015347		
Volume of beads:	100 µL		
Wash buffer:	Same as binding buffer		
Binding buffer:	20 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, pH 7.5		
Elution buffer:	N/A		
Biotin substrate:	D-Biotin		
Amount of biotin substrate:	100 nmol		
If AVIDIY assay is used			
Amount of HABA:	25 nmol		
Reagent details			
Name	Catalog #	Vendor	Lot
D-Biotin	2031	Sigma	VF304235
HABA	28010	Thermo Fisher	3458120

Standard operating procedure: AVIDITY assay

1. REAGENT AND MATERIALS LIST

Item	Vendor	Catalog #
1 M Tris-HCl pH 7.5*	Quality Biologicals, Inc.	351-006-101
5 M NaCl*	Sigma	S6546
500 mM EDTA*	Sigma	324504
4-hydroxybenzene-2-carboxylic acid (HABA)	Thermo Scientific	28010
1N NaOH*	Sigma	S2567
D-biotin	Sigma	2031
15-mL conical tube	Various	
10 uL pipette tips and pipette	Various	
200 µL gel loading pipette tips and pipette	Various	
200 uL pipette tips	Various	
1000 uL pipette tips and pipette	Various	
Magnetic rack – recommended to use magnet strength ≥ N38	Various	
Mini centrifuge	Various	
Vortexer	Various	
1.5 mL microfuge tubes (low binding)	Various	
**Can be purchased from various vendors.*		
Solutions		
Binding buffer: 20mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5		
HABA solution: 10 mM HABA in purified water (18 MΩ)		
D-Biotin solution: 4 mM D-biotin in binding buffer		

2. IMPORTANT NOTES

- All steps are performed at room temperature, unless otherwise specified.
- All solutions are made fresh, unless otherwise specified.
- Follow the instructions specific to each bead type for all procedural steps.

- Per specific bead type, the HABA and d-biotin titration curve only need to be generated once to establish the proper amount of HABA and d-biotin to be used for the AVIDITY assay. It is recommended to repeat the HABA and d-biotin titration curve if a specific lot to be tested shows unusual behavior compared to previous lots tested.
- This protocol uses 100 µL of total bead volume. This volume can be adjusted to the needs of the user.
- The absorbance is measured in a 96-well microplate. If a larger volume is needed to perform experiments, user can scale up the volume and measure absorbance in a cuvette.
- It is recommended to perform each experiment in triplicate.
- The protocol described here is for magnetic beads. If non-magnetic beads are used, replace the bead collection steps (pelleting beads by magnet) with a centrifugation step that uses speed and duration appropriate to collect the beads being used.

3. PROTOCOL

3.1 Categorize Streptavidin Beads

Streptavidin beads were categorized according to: 1) the rate at which they form a tight pellet when placed near a magnet, 2) whether cell pellets smear when aspirating supernatant, and 3) how easily bead pellets can be resuspended in binding and/or elution buffer as this is a critical parameter that affects the experimental workflow (Table 1). If the streptavidin beads to be tested are not listed here, first determine how the streptavidin beads behave in binding buffer and pellet when placed on the magnetic rack.

Table 1 is a guide to assist users in selecting the protocol to be used to perform the AVIDITY assay. If a different binding buffer is more appropriate for the bead to be tested, then users should 1) test that the buffer is compatible with the AVIDITY assay and 2) update the protocol to include the buffer and also accommodate the amount of time it takes for the beads to pellet when placed on a magnetic rack as this may vary among beads and in different binding buffers. We have tested the following components and found them to be incompatible with the AVIDITY assay: SDS, Tween®20, 2M NaCl.

Table 1. Categorization of streptavidin bead products used in this study based on their behavior in binding buffer.

	Group A	Group B	Group C
Streptavidin beads	• GenScript Streptavidin Magbeads • ReSyn MagReSyn Streptavidin MAX beads	• Cytiva Sera-Mag SpeedBeads Neutravidin-Coated Magnetic Beads	• New England Biolabs Streptavidin Magnetic Beads • Invitrogen Dynabeads MyOne Streptavidin C1 Cytiva Sera-Mag Streptavidin-Coated Magnetic Beads
Pellet behavior on magnetic rack	Pellet fast	Slow, allow beads to settle for 30 seconds when placed on a magnetic rack	Slow, allow beads to settle for 30 seconds when placed on a magnetic rack
Pellet behavior during aspiration	Do not smear	Some smearing when supernatant is aspirated	More smearing when supernatant is aspirated
Resuspension behavior	Resuspend immediately	Triturate 3-5 times to fully resuspend pellet	Triturate 5-10 times to fully resuspend pellet

3.2 Prepare Solutions

3.2.1 Prepare 10 mM HABA solution.

1. Dissolve 24.2 mg of HABA in 9.8 mL purified water (18 MΩ) in a 15 mL conical tube.
2. Add 200 µL 1N NaOH.
3. Place tube in end-over-end rotator until HABA is dissolved completely.

   Note: This might take up to 20-30 minutes.

4. HABA solution may be stored at 4°C for up to one week.

   The calculation for preparation of this solution is shown here:

$$24.2\ mg\left(\frac{g}{1000mg}\right)\left(\frac{mol}{242.23\ g}\right)\left(\frac{1000\ mmol}{mol}\right)\left(\frac{1}{9.9\ mL}\right)\left(\frac{1000\ mL}{1\ L}\right) = 10\frac{mmol}{L} = 10\ mM$$

3.2.2 Prepare d-biotin solution.

1. Dissolve 10 mg of d-Biotin in 10 mL of **20mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5** in a 15 mL conical tube.
2. Vortex tube until d-biotin is dissolved.

   The calculation for preparation of this solution is shown here:

$$\frac{1mg}{mL}\left(\frac{g}{1000mg}\right)\left(\frac{mol}{244.31\ g}\right)\left(\frac{1000\ mmol}{mol}\right)\left(\frac{1000\ mL}{L}\right) = 4\frac{mmol}{L} = 4mM$$

3.3 Prepare Titration Curves for Group A Beads

3.3.1 Prepare HABA titration curve for Group A beads

1. Add 100µL of beads (*i.e.* beads in storage solution or bead slurry) to microfuge tube.

   Note: Follow vendor specific protocol on how to resuspend beads.

2. Quick spin microfuge tube in mini centrifuge.
3. Place microfuge tube on magnetic rack.
4. Aspirate storage solution using a 200-µL gel loading pipette tip.
5. Equilibrate beads by adding 250 µL of binding buffer.
6. Place microfuge tube on magnetic rack.
7. Aspirate supernatant using a 200-µL gel loading pipette tip.
8. Repeat steps 8-11 twice for a total of 3 washes.
9. Take microfuge tube off magnetic rack.
10. Add 200 µL binding buffer.
11. Place microfuge tube on magnetic rack.
12. Collect 200 µL of supernatant and add to one well of a 96-well plate using a 200-µL gel loading pipette tip, be careful not to collect any beads.

    *Note: This measurement is the blank, which will later be subtracted from all absorbance readings. See example calculations below in section **3.6 Calculations**.*

13. Read absorbance at 350 nm.
14. Return supernatant to microfuge tube containing beads.

    Note: This is the same tube that supernatant was collected from.

15. Add 10 mM HABA solution according to table below one addition at a time. Triturate 3 times. If liquid is found on the side of the tube, quick spin microfuge tube in mini centrifuge.

    Note: This is the same tube that supernatant was collected from. The successive addition of HABA solution to a single aliquot of beads allows the assay to be performed with a minimal amount of material (e.g. 100 µL bead slurry). While it is possible to perform this titration using separate aliquots of beads distributed into multiple different tubes, this would consume a considerable amount of product (700 µL bead slurry per single experiment and 2100 µL bead slurry if experiment is performed in triplicates).

16. Place microfuge tube on magnetic rack.
17. For addition 1, add 200.5 µL of supernatant to 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.
18. Read absorbance at 350 nm.
19. Return supernatant to microfuge tube containing beads.
20. Repeat steps 13-17 until all 7 additions are completed and their absorbance have been measured.

Number of additions	**Blank**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
HABA, nmol	0	5	10	25	50	70	85	100
HABA stock, µL	0	0.5	0.5	1.5	2.5	2	1.5	1.5
Total volume added to plate	200	200.5	201	202.5	205	207	208.5	210

21. Use the absorbance measurements and nmol of HABA to generate a linear curve. Then, determine the amount of HABA that leads to an absorbance of 0.9 to 1.5 to generate the biotin titration curve.

3.3.2 Prepare d-biotin titration curve for Group A beads

1. Add 100µL of beads (*i.e.* bead in storage solution or bead slurry) to microfuge tube.

   Note: Follow vendor specific protocol on how to resuspend beads.

2. Quick spin microfuge tube in mini centrifuge.
3. Place microfuge tube on magnetic rack.
4. Aspirate storage solution using a 200-µL gel loading pipette tip.
5. Equilibrate beads by adding 250 µL of binding buffer.
6. Place microfuge tube on magnetic rack.
7. Aspirate supernatant using a 200-µL gel loading pipette tip.
8. Repeat steps 8-11 twice for a total of 3 washes.

9. Take microfuge tube off magnetic rack.
10. Add 200 µL binding buffer.
11. Place microfuge tube on magnetic rack.
12. Collect 200 µL of supernatant and add to 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.

    *Note: This measurement is the blank, which will later be subtracted from all absorbance readings. See example calculations below in section **3.6 Calculations**.*

13. Read absorbance at 350 nm.
14. Return supernatant to microfuge tube containing beads.

    Note: This is the same tube that supernatant was collected from.

15. Add 2.5 µL of 10 mM HABA solution (25 nmol).

    Note: The amount of HABA added is determined from the HABA titration curve.

16. Place microfuge tube on magnetic rack.
17. Collect 202.5 µL of supernatant and add to 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.

    *Note: This measurement is the control, which will later be subtracted from the experimental absorbance measurements to obtain Δ absorbance. See example calculations below in section **3.6 Calculations**.*

18. Read absorbance at 350 nm.
19. Return supernatant to microfuge tube containing beads.

    Note: This is the same tube that supernatant was collected from.

20. Add 4 mM d-biotin solution 1 according to table below one addition at a time. Triturate 3 times. If liquid is found on the side of the tube, quick spin microfuge tube in mini centrifuge.

    Note: All addition will be added to the same microfuge tube containing beads. The successive addition of d-biotin solution to a single aliquot of beads allows the assay to be performed with a minimal amount of material (e.g. 100 µL bead slurry). While it is possible to perform this titration using separate aliquots of beads distributed into multiple different tubes, this would consume a considerable amount of product (700 µL bead slurry per single experiment and 2100 µL bead slurry if experiment is performed in triplicates).

21. Place microfuge tube on magnetic rack.
22. For addition 1, add 205 µL of supernatant to 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.
23. Read absorbance at 350 nm.
24. Return supernatant to microfuge tube containing beads.
22. Repeat steps 13-17 until all 7 additions are completed and their absorbance have been measured.

Number of additions	Blank	1	2	3	4	5	6	7
D-biotin, nmol	0	10	20	40	60	80	100	120
D-biotin stock, µL	0	2.5	2.5	5	5	5	5	5
Total volume added to plate	200	205	207.5	212.5	217.5	222.5	227.5	237.5

25. Use the absorbance measurements of the control and experimental group to calculate the change in absorbance (Δ absorbance). *See example calculations below in section **3.6 Calculations**.* Plot the change in absorbance versus the amount of d-biotin added to generate a hyperbolic curve. Then, determine the amount of d-biotin that leads to maximum displacement of HABA.

3.4 Prepare Titration Curves for Group B and C Beads

3.4.1 Prepare HABA titration curve for Group B and C beads

1. Add 100µL of beads (*i.e.* bead in storage solution or bead slurry) to microfuge tube.

   Note: Follow vendor specific protocol on how to resuspend beads.

2. Quick spin microfuge tube in mini centrifuge.
3. Place microfuge tube on magnetic rack.
4. Allow beads to settle for 30 seconds.
5. Aspirate storage solution using a 200-µL gel loading pipette tip.
6. Equilibrate beads by adding 250 µL binding buffer.
7. Place microfuge tube on magnetic rack.
8. Allow beads to settle for 30 seconds.
9. Aspirate storage solution using a 200-µL gel loading pipette tip.
10. Repeat steps 8-11 twice for a total of 3 washes.
11. Take microfuge tube off magnetic rack.
12. Add 200 µL binding buffer.
13. Place microfuge tube on magnetic rack.
14. Allow beads to settle for 30 seconds.
15. Collect 200 µL of supernatant and add to 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.

    *Note: This measurement is the blank, which will later be subtracted from all absorbance readings. See example calculations below in section **3.6 Calculations**.*

16. Read absorbance at 350 nm.
17. Return supernatant to microfuge tube containing beads.

    Note: This is the same tube that supernatant was collected from.

18. Add 10 mM HABA solution according to table below one addition at a time. Triturate 3 times. If liquid is found on the side of the tube, quick spin microfuge tube in mini centrifuge.

Note: All addition will be added to the same microfuge tube containing beads. The successive addition of HABA solution to a single aliquot of beads allows the assay to be performed with a minimal amount of material (e.g. 100 µL bead slurry). While it is possible to perform this titration using separate aliquots of beads distributed into multiple different tubes, this would consume a considerable amount of product (700 µL bead slurry per single experiment and 2100 µL bead slurry if experiment is performed in triplicates).

19. Place microfuge tube on magnetic rack.
20. Allow beads to settle for 30 seconds.
21. For addition 1, add 200.5 µL of supernatant to 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.
22. Read absorbance at 350 nm.
23. Return supernatant to microfuge tube containing beads.
24. Repeat steps 13-17 until all 7 additions are completed and their absorbance have been measured.

Number of additions	**Blank**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
HABA, nmol	0	5	10	25	50	70	85	100
HABA stock, µL	0	0.5	0.5	1.5	2.5	2	1.5	1.5
Total volume added to plate	200	200.5	201	202.5	205	207	208.5	210

23. Use the absorbance measurements and nmol of HABA to generate a linear curve. Then, determine the amount of HABA that causes an absorbance of 0.9 to 1.5 to generate the biotin titration curve.

3.4.2 Prepare d-biotin titration curve for Group B and C beads

1. Add 100µL of beads (*i.e.* bead in storage solution or bead slurry) to microfuge tube.

   Note: Follow vendor specific protocol on how to resuspend beads.

2. Quick spin microfuge tube in mini centrifuge.
3. Place microfuge tube on magnetic rack.
4. Allow beads to settle for 30 seconds.
5. Aspirate storage solution using a 200-µL gel loading pipette tip.
6. Equilibrate beads by adding 250 µL binding buffer.
7. Place microfuge tube on magnetic rack.
8. Allow beads to settle for 30 seconds.
9. Aspirate storage solution using a 200-µL gel loading pipette tip.
10. Repeat steps 8-11 twice for a total of 3 washes.
11. Take microfuge tube off magnetic rack.
12. Add 200 µL binding buffer.
13. Place microfuge tube on magnetic rack.
14. Add 200 µL of supernatant 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.

*Note: This measurement is the blank, which will later be subtracted from all absorbance readings. See example calculations below in section **3.6 Calculations**.*

15. Read absorbance at 350 nm.
16. Return supernatant to microfuge tube containing beads.

    Note: This is the same tube that supernatant was collected from.

17. Add 2.5 µL of 10 mM HABA solution (25 nmol).

    Note: The amount of HABA added is determined from the HABA titration curve.

18. Place microfuge tube on magnetic rack.
19. Add 202.5 µL of supernatant 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.

    *Note: This measurement is the control, which will later be subtracted from the experimental absorbance measurements. See example calculations below in section **3.6 Calculations**.*

20. Read absorbance at 350 nm.
21. Return supernatant to microfuge tube containing beads.

    Note: This is the same tube that supernatant was collected from.

22. Add 4 mM d-biotin solution 2 according to table below one addition at a time. Triturate 3 times. If liquid is found on the side of the tube, quick spin microfuge tube in mini centrifuge.

    Note: All addition will be added to the same microfuge tube containing beads. The successive addition of d-biotin solution to a single aliquot of beads allows the assay to be performed with a minimal amount of material (e.g. 100 µL bead slurry). While it is possible to perform this titration using separate aliquots of beads distributed into multiple different tubes, this would consume a considerable amount of product (400 µL bead slurry per single experiment and 1200 µL bead slurry if experiment is performed in triplicates).

23. Place microfuge tube on magnetic rack.
24. For addition 1, add 203 µL of supernatant 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.
25. Read absorbance at 350 nm.
26. Return supernatant to microfuge tube containing beads.
27. Repeat steps 13-17 until all 4 additions are completed and their absorbance have been measured.

Number of additions	**Blank**	**1**	**2**	**3**	**4**
D-biotin, nmol	0	2	10	50	120
D-biotin stock, µL	0	0.5	2	10	17.5
Total volume added to plate	0	203	205	215	323.5

28. Use the absorbance measurements of the control and experimental group to calculate the change in absorbance (Δ absorbance). *See example calculation below in section **3.6 Calculations***. Plot the change in absorbance versus the amount of d-biotin added to generate a hyperbolic curve. Then, determine the amount of d-biotin that leads to maximum displacement of HABA.

3.5 Determine Binding Capacity

3.5.1 Determine binding capacity of Group A streptavidin beads

1. Add 100µL of beads (*i.e.* bead in storage solution or bead slurry) to microfuge tube.

   *Note: Follow vendor specific protocol on how to resuspend beads. The volume of total beads will later be used to calculate binding capacity. See section **3.6 Calculations**.*

2. Quick spin microfuge tube in mini centrifuge.
3. Place microfuge tube on magnetic rack
4. Aspirate storage solution using a 200-µL gel loading pipette tip.
5. Equilibrate beads by adding 250 µL of binding buffer.
6. Place microfuge tube on magnetic rack.
7. Aspirate supernatant using a 200-µL gel loading pipette tip.
8. Repeat steps 8-11 twice for a total of 3 washes.
9. Take microfuge tube off magnetic rack.
10. Add 200 µL binding buffer
11. Place microfuge tube on magnetic rack.
12. Collect 200 µL of supernatant and add to 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.

    Note: This measurement is the blank, which will later be subtracted from all absorbance measurements. See example calculations below in section **3.6 Calculations**.

13. Read absorbance at 350 nm.
14. Return supernatant to microfuge tube containing beads.

    Note: This is the same tube that supernatant was collected from.

15. Add 2.5 µL of 10 mM HABA solution (25 nmol).

    *Note: The amount of HABA added is determined from the HABA titration curve determined in section **3.3.1**.*

16. Place microfuge tube on magnetic rack.
17. Collect 202.5 µL of supernatant and add to 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.

    Note: This measurement is the control, which will later be subtracted from the experimental absorbance measurements. See example calculations below in section **3.6 Calculations**.

18. Read absorbance at 350 nm.

19. Return supernatant to microfuge tube containing beads.

    Note: This is the same tube that supernatant was collected from.

20. Add 25 µL 4 mM d-biotin solution 1. Triturate 3 times and incubate for 5 minutes. If liquid is found on the side of the tube, quick spin microfuge tube in mini centrifuge.

    Note: The amount of d-biotin added is determined from the d-biotin titration curve determined in section 3.3.2.

21. Place microfuge tube on magnetic rack.
22. Add 227.5 µL of supernatant to 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.

    *Note: The volume of supernatant will later be used to calculate binding capacity and is dependent on the amount of HABA and d-biotin added. See section **3.6 Calculations**.*

23. Read absorbance at 350 nm.
24. Follow section **3.6 Calculations** below to use the absorbance measurement to calculate binding capacity of streptavidin beads.

    Note: If it is not possible to establish a biotin titration curve for Group C beads, then use amount of biotin that was established for Group A or Group B beads.

3.5.2 Determine binding capacity of Group B and C streptavidin beads

1. Add 100µL of beads (*i.e.* bead in storage solution or bead slurry) to microfuge tube.

   *Note: Follow vendor specific protocol on how to resuspend beads. The volume of total beads will later be used to calculate binding capacity. See section **3.6 Calculations**.*

2. Quick spin microfuge tube in mini centrifuge.
3. Place microfuge tube on magnetic.
4. Allow beads to settle for 30 seconds.
5. Aspirate storage solution using a 200-µL gel loading pipette tip.
6. Equilibrate beads by adding binding buffer.
7. Quick spin microfuge tube in mini centrifuge.
8. Place microfuge tube on magnetic.
9. Allow beads to settle for 30 seconds.
10. Repeat steps 8-11 twice for a total of 3 washes.
11. Take microfuge tube off magnetic rack.
25. Add 200 µL binding buffer.
26. Place microfuge tube on magnetic rack.
27. Allow beads to settle for 30 seconds.
28. Collect 200 µL of supernatant and add to 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.

    Note: This measurement is the blank, which will later be subtracted from all absorbance measurements. See example calculations below in section **3.6 Calculations**.

29. Read absorbance at 350 nm.
30. Return supernatant to microfuge tube containing beads.

    Note: This is the same tube that supernatant was collected from.

31. Add 2.5 µL of 10 mM HABA solution (25 nmol).

    *Note: The amount of HABA added is determined from the HABA titration curve determined in section **3.4.1**.*

12. Read absorbance at 350 nm.
13. Return supernatant to microfuge tube containing beads.

    Note: This is the same tube that supernatant was collected from.

14. Add 25 µL 4 mM d-biotin solution 1. Triturate 3 times and incubate for 5 minutes. If liquid is found on the side of the tube, quick spin microfuge tube in mini centrifuge.

    *Note: The amount of d-biotin added is determined from the d-biotin titration curve determined in section **3.4.2**.*

15. Place microfuge tube on magnetic rack.
16. Add 275.5 µL of supernatant to 96-well plate using a 200-µL gel loading pipette tip, careful not to collect any beads.

    *Note: The volume of supernatant will later be used to calculate binding capacity and is dependent on the amount of HABA and d-biotin added. See section **3.6 Calculations**.*

17. Read absorbance at 350 nm.
18. Follow section **3.6 Calculations** below to use the absorbance measurement to calculate binding capacity of streptavidin beads.

3.6 Calculations

Blank subtraction

$$A_{Experiment}\ or\ A_{Control} = A_{Sample} - A_{Blank}$$

$$A_{Experiment} = 1.2937 - 0.0605 = 1.2332$$

$$A_{Control} = 1.0544 - 0.0605 = 0.9939$$

Δ Absorbance

$$\Delta Absorbance = A_{Experiment} - A_{Control}$$

$$\Delta Absorbance = 1.2332 - 0.9939 = 0.2393$$

Concentration of D-Biotin

Beer-Lambert law:

$$A = k \times b \times C$$

Then, solve Beer-Lambert law for concentration.

$$C = \frac{A}{k \times b}$$

$$k_{HABA} = 20{,}500\ M^{-1}cm^{-1}$$

$$b = 0.5\ cm$$

$$C = \frac{A}{k \times b} = \frac{mol \times cm}{L \times cm} = \frac{mol}{L}$$

$$C = \frac{A_E - A_C}{20{,}500 \times 0.5} = \frac{A_E - A_C}{10{,}250} = \frac{mol}{L}$$

$$C = \frac{1.2332 - 0.9939}{20{,}500 \times 0.5} = \frac{0.2393}{10{,}250} = 0.00002335\frac{mol}{L}$$

Binding Capacity

Total bead volume

$$n = \frac{Molecule\ Concentration\ (Biotin) \times Volume\ of\ Supernatant \times 10^6}{Volume\ of\ total\ beads}$$

$$\left(\frac{L}{1000\ mL}\right)\left(\frac{1000mmol}{mol}\right)\left(\frac{1000\mu mol}{mmol}\right)\left(\frac{1000nmol}{\mu mol}\right) = \frac{nmol}{mL}$$

$$n = \frac{0.00002335 \times 0.2175 \times 10^6}{0.1} = 50.8\ \frac{nmol}{mL}$$

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