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Analysis of FLAG Proteins on the Octet® BLI Platform Using SAX or SAX2 Biosensors

Technical Note

Scope
This technical note walks the reader through the process of designing and optimizing a kinetics characterization assay for FLAG-tagged recombinant proteins on the Octet® Bio-Layer Interferometry (BLI) platform including the optimal biotinylation of anti-FLAG antibodies for immobilization onto the high precision streptavidin biosensor surfaces.

Abstract

Biotinylated anti-Flag antibody can be immobilized onto high-precision Octet® Streptavidin Biosensors (SAX or SAX2) and can be used with Octet® BLI platforms to enable kinetics characterization and quantitation of FLAG-tagged recombinant proteins. Sigma's Anti-FLAG M2 antibody is suggested as the ligand for creating a custom FLAG-protein binding surface on Octet® SAX or SAX2 Biosensors as it provides high affinity and specificity to FLAG-tagged proteins as well as baseline stability suitable for kinetic assays. The FLAG-tagged proteins can be captured onto the customized biosensor surface in either crude or purified form.

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Introduction

The DYKDDDDK polypeptide tag, commonly known as the FLAG® tag, is fused to recombinant proteins to facilitate their detection and purification. Octet® High Precision Streptavidin (SAX or SAX2) Biosensors are designed for customization by direct immobilization of biotin-labeled proteins for both kinetic characterization and quantitation measurements on Octet® BLI systems. By immobilizing a biotinylated anti-FLAG antibody onto SAX or SAX2 Biosensors, FLAG-tagged proteins can be easily captured from crude or purified samples for detection, quantitation, or affinity measurement to an interacting protein.

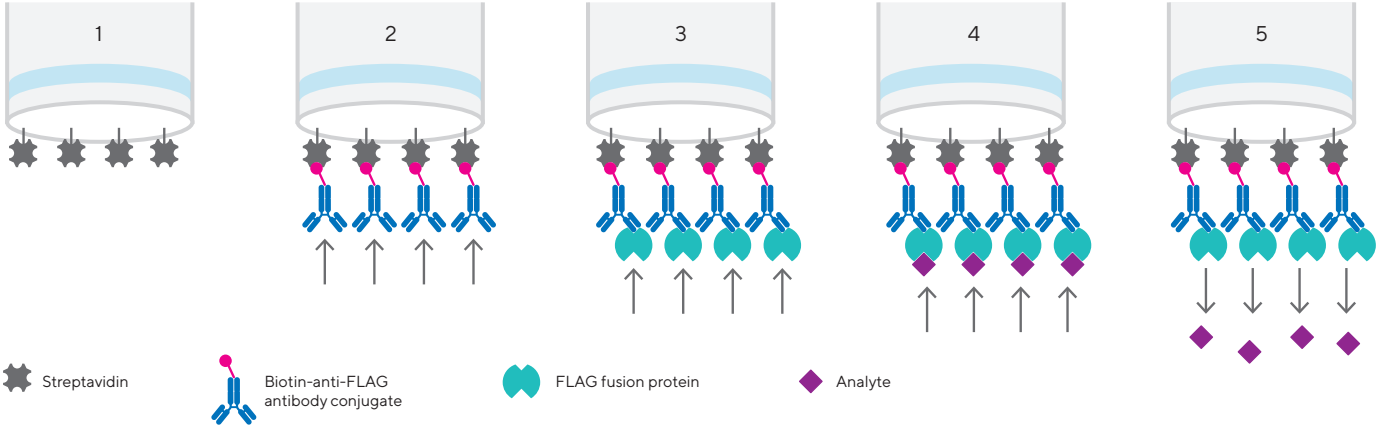
BLI Biosensors are fiber optic tips coated with specific ligand chemistries that enable detection, quantification, and kinetic analysis of a biomolecular target. The binding of the targeted molecule alters the interference pattern of light reflected from the biosensor tip to a detector, allowing molecular association and dissociation events to be measured in real time with the Octet® BLI platforms. Higher target concentrations result in both faster binding rates and larger signal amplitudes. Concentration of the target molecules in a sample can be determined by comparing either kinetic (binding rate) or equilibrium (signal amplitude) data to a standard calibration curve constructed from identical samples of known concentrations. In addition, detailed kinetic analysis can be performed in real time to determine affinity (K_{on}, K_{off}, K_D) of the target molecule to an analyte binding partner, for example an antibody-antigen interaction or receptor-ligand interaction. The biosensor tip is well suited for capture and analysis directly from complex mixtures as an alternative to chemical protocols such as EDC/NHS.

The Sigma Anti-FLAG M2 antibody is the suggested ligand for creating a custom FLAG-protein binding surface on SAX or SAX2 Biosensors, as it provides high affinity and specificity to FLAG-tagged proteins as well as baseline stability suitable for kinetic assays. The M2 antibody can easily be biotinylated following the procedure described in this document, then immobilized on SAX or SAX2 Biosensors prior to capture of FLAG-tagged protein for kinetic analysis. Figure 1 gives an overview of the FLAG-protein kinetic assay format to measure affinity of an interaction between FLAG-protein (ligand) and interacting its binding partner (analyte). First, biotinylated Anti-FLAG M2 conjugate is immobilized on SAX or SAX2 Biosensors to generate a FLAG-protein binding surface. Next, FLAG-protein is captured from either purified or crude samples. Then association and dissociation steps are performed with the analyte protein that interacts with the FLAG-protein. Octet® Analysis Studio Software is then used to process and align the data, followed by curve fitting and calculation of kinetic and affinity constants.

The following procedure has been optimized to create a FLAG-protein binding surface with a maximum response and the most reproducible results for kinetic assays.

Figure 1

Example Workflow for the Kinetic Characterization of an Interaction Between a FLAG-tagged Protein and Target Analyte.



Note. Step 1: Equilibration of SAX or SAX2 Biosensors in 1X Kinetics Buffer. Step 2: Immobilization of biotin-anti-FLAG antibody conjugate. Step 3: Loading of FLAG protein. Step 4: Association of interacting analyte. Step 5: Dissociation of analyte.

Biotinylation of Anti-FLAG M2 Antibody

Materials Required

- Mouse anti-Flag M2 antibody (Sigma part no. F1804)
- EZ-Link™ NHS-LC-LC-Biotin (Life Technologies part no. 21343)
- Dimethylformamide (DMF)
- BSA
- 10% (v/v) solution of Tween-20
- Slide-A-Lyzer™ Dialysis Cassette, MWCO 10,000 (Life Technologies)
- PBS buffer, pH 7.4

Procedure

1. Dilute antibody stock from Sigma 1:1 with PBS buffer (this step dilutes the glycerol in the stock buffer). A minimum of 200 µg of antibody should be used for the biotinylation reaction.
2. Prepare a 1 mg/mL solution of biotin-LC-LC-NHS by adding 1 mL DMSF to 1 mg reagent in a sterile glass or polypropylene tube. Vortex until all solids are dissolved.
3. Calculate the amount of biotin-LC-LC-NHS stock to add to diluted antibody for a 1:1 molar coupling ratio. Use the following formulas:

   150,000 = molecular weight of antibody

   1 = 1 antibody to 1 biotin (molar coupling ratio)

   567.7= molecular weight of biotin-LC-LC-NHS

   A. $\frac{\text{___mg of antibody x 1 x 567.7}}{150{,}000}$ = ___mg of biotin-LC-LC-NHS needed for reaction

   B. $\frac{\text{___mg of biotin-LC-LC-NHS needed}}{\text{1 mg/mL}}$ = ___mL of biotin-LC-LC-NHS in DMF needed for reaction

   The amount calculated in step 3B is the actual volume to be added to the antibody solution from step 1.
4. Add biotin-LC-LC-NHS to the antibody solution from step 1. Immediately vortex the solution. Incubate for 1 hour at room temperature.
5. Remove unincorporated biotin from reaction by dialysis (see next section).

Removal of Free Biotin

Important: The Slide-A-Lyzer cassette holding volume selected should be about twice the volume of the antibody sample to be dialyzed. The sample volume will expand during dialysis due to glycerol present in the original M2 antibody stock.

1. Following the manufacturer's procedure, wet the Slide-A-Lyzer Dialysis Cassette in PBS for at least 1 minute just prior to use.
2. Once the 1 hour incubation time for biotinylation (step 4, Procedure) is complete, inject the antibody sample into the dialysis cassette with a syringe. Remove excess air from the cassette with the syringe.
3. Dialyze for 3 hours against 1 liter PBS buffer or a minimum of 100X the sample volume.
4. Perform 4 buffer changes with a minimum of 3 hours incubation time in each buffer.
5. After dialysis is complete, remove the conjugated antibody solution from the cassette with a syringe. Determine the concentration of biotin-anti-FLAG antibody conjugate by UV-Vis method. The yield should be about 90%.
6. Add BSA and Tween-20 to the conjugated antibody solution for a final concentration of:
   - 0.1% BSA
   - 0.002% Tween 20

   Vortex gently until the additives are thoroughly dissolved.

Storage of Biotin-anti-FLAG Antibody Conjugate

The biotin anti-FLAG antibody conjugate should be stored at 4°C for up to two weeks. Do not freeze.

Kinetic Analysis of FLAG Proteins on SAX or SAX2 Biosensors

Materials Required

- Octet® BLI system with Octet® BLI Discovery and Analysis Studio Software
- High Precision Streptavidin (SAX or SAX2) Biosensors: (Sartorius part no. 18-5117 [tray]; 18-5118 [pack]; 18-5119 [case])
- Biotin-anti-FLAG antibody conjugate (see prior procedure)
- For all Octet® BLI systems: 96-well, black, flat-bottom, polypropylene microplate (Greiner Bio-One part no. 655209)
- Optional for Octet® RH16 and RH96 BLI systems:
  - 384-tilted well, black, flat-bottom, polypropylene microplate (Sartorius part no. 18-5080 [pack]; 18-5076 [case])
  - 384-well, black, flat-bottom, polypropylene microplate (Greiner Bio-One part no. 781209)

- **FLAG-containing protein for immobilization.** The FLAG-protein can be present in either buffer or a crude/complex matrix such as cell culture supernatant.

- **Analyte protein samples that interact with FLAG-protein.** For full kinetic characterization and determination of affinity constants (K_D), the analyte protein must be purified and of a known concentration. For screening purposes (target binding or off-rate), the interacting analyte can be from a crude sample of unknown concentration.

- **Assay buffer.** The SAX or SAX2 Biosensor is compatible with a wide range of buffers, although 1X Kinetics Buffer is recommended for kinetic assays. Dilute 10X Kinetics Buffer (Sartorius part no. 18-5032) 10-fold with PBS, pH 7.4.

Tips for optimal performance

- Equilibrate reagents and samples to room temperature prior to use. For frozen samples, thaw and mix thoroughly prior to use.
- Hydrate the SAX or SAX2 Biosensors for at least 10 minutes in 1X Kinetics Buffer before running the assay.
- Use a reference sample in the association step to correct for baseline downward drift. A reference sample is a buffer-only control with no analyte present in the association step.
- The baseline and dissociation steps should be performed in the same microplate well for every assay. This enables use of the inter-step correction feature to align the association and dissociation steps when processing data.
- Ensure that the Octet® BLI system is turned on and the lamp is warmed to room temperature for at least 60 minutes prior to starting the assay.
- Set the assay temperature to 30°C.

Assay procedure

Figure 2 shows an example microplate layout and assay design for a FLAG kinetic characterization assay. For all steps, use a 200 µL sample volume for 96-well plates, and 80 µL for 384-well plates.

Before the assay — Pre-hydration: Hydrate SAX or SAX2 Biosensors in 1X Kinetics buffer in a 96-well plate for a minimum of 10 minutes.

Assay Step 1 — Online equilibration of hydrated SAX or SAX2 Biosensors in 1X Kinetics Buffer (custom): Add 1X Kinetics Buffer to Column A of the sample plate according to the map in Figure 2.

Assay Step 2 — Immobilization of biotin-Anti-FLAG antibody conjugate (custom): Dilute the biotinylated anti-FLAG M2 antibody in 1X Kinetics Buffer to a concentration of 50 µg/mL. Add to the sample plate according to the map in Figure 2. See Figure 5 for example data from the immobilization step.

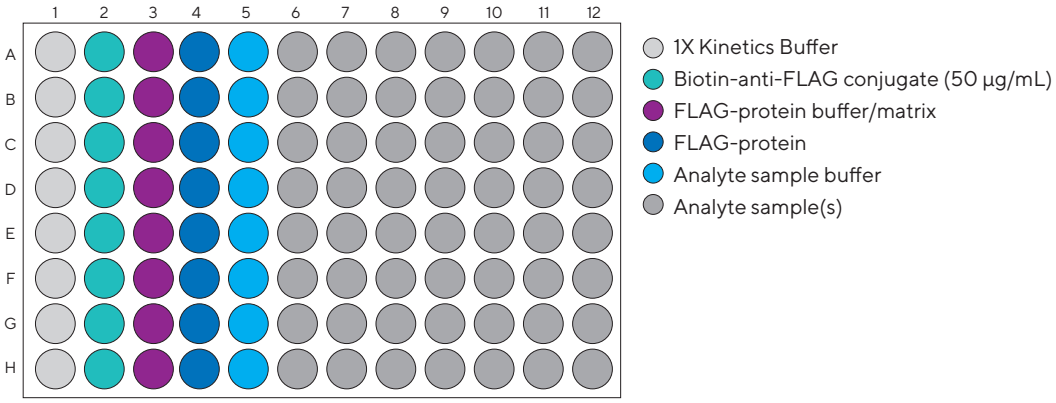
Assay Step 3 — Post-immobilization baseline (custom): After immobilization, the biotinylated ligand should produce a stable binding surface in the immobilization buffer (1X Kinetics Buffer). Check that the downward drift in this step is minimal (i.e. baseline is flat) to ensure immobilized conjugate is properly bound to the streptavidin surface.

Assay Step 4 — Equilibration in FLAG-protein buffer (custom): This step is necessary only if the FLAG-protein is diluted in a buffer other than 1X Kinetics Buffer. Add buffer or matrix to the sample plate that matches the FLAG-protein samples to be captured by the anti-FLAG antibody. For example, if capturing FLAG-protein from cell culture samples, perform baseline in the same cell culture medium minus the FLAG-protein. The baseline step should be run for a run for a sufficient time so that the biosensors are equilibrated in a new buffer matrix and any change in baseline drift has stabilized.

Figure 2

Sample Plate Map (A) and Assay Steps with Associated Parameters (B) for a FLAG-protein Kinetic Assay.

A.



B.

Step #	Column #	Description	Step Type	Time (Sec)	Flow (RPM)
Step 1	1	Equilibration in 1X Kinetics Buffer	Custom	60	1000
Step 2	2	Immobilization of biotin-anti-FLAG antibody conjugate	Custom	1200	1000
Step 3	1	Baseline in 1X Kinetics Buffer	Custom	120	1000
Step 4	3	Baseline in FLAG-protein buffer/matrix	Custom	120	1000
Step 5	4	Loading of FLAG-protein	Loading	120–600	0–1000
Step 6	5	Baseline in analyte sample buffer	Baseline	180–600	1000
Step 7	6–12	Association of analyte	Association	300–600	1000
Step 8	5	Dissociation of analyte	Dissociation	300–1800	1000

Note. This experimental design assumes anti-FLAG conjugate, FLAG-protein, and analyte samples are in different matrices. The assay can be repeated on new biosensors with analyte sample column offset (Assay Step 7) to analyze several analyte samples against a single FLAG-protein.

Assay Step 5 — Capture of FLAG-protein ligand (loading): Dilute FLAG-protein (ligand) to the appropriate concentration in 1X Kinetics Buffer or corresponding sample matrix, and add to the sample plate. The matrix or buffer used should match the one used for the baseline in Assay Step 4. The concentration of FLAG-protein to use will depend on its affinity for the anti-FLAG M2 antibody and its affinity to the associating analyte, as well as the size of both the ligand and analyte. For the best kinetic data, a loading optimization experiment should be performed to determine the optimal loading level. As a rule, load enough ligand so that the high concentration of analyte has adequate association signal at equilibrium (generally 4–5 nm). Loading more ligand than is needed can cause artifacts such as non-specific binding or mass transport limitation. For more details on optimization of ligand loading, refer to application note, *Biomolecular Binding Kinetic Assays on the Octet® Platform.*

Assay Step 6 — Baseline step in analyte sample buffer (baseline): Add 1X Kinetics Buffer or alternative buffer matching the analyte samples being analyzed to the sample plate according to Figure 2. It is important to match the baseline buffer matrix to the analyte samples. The baseline step should be run for a long enough time so that the biosensors are equilibrated in a new buffer matrix and any change in baseline drift has stabilized. We recommend 300–600 seconds of baseline if a new buffer matrix is used in this step. If the buffer is identical to the FLAG-protein buffer, a baseline step of 180 seconds should be adequate.

Assay Step 7 — Association to interacting analyte (association): If detailed kinetic characterization is being performed, the analyte protein must be purified and of known concentration. For a Global Fitting, it is recommended to run a titration series of at least four

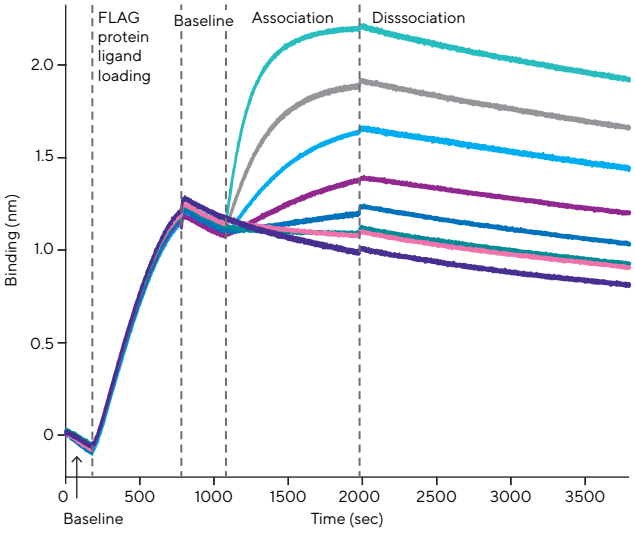
concentrations of the analyte protein. The highest concentration should be approximately 10 times the expected K_D. For example, concentrations of 90 nM, 30 nM, 10 nM, and 3 nM are recommended for an analyte with low-nanomolar affinity towards an immobilized ligand. For screening assays or qualitative interaction analysis, a single concentration of the interacting protein can be sufficient to assess binding. Analyte samples should be diluted in the same buffer used for the baseline and dissociation steps. Include a reference sample consisting of an assay buffer blank with no analyte present in this step to enable subtraction of background assay downward drift.

Assay Step 8 — Dissociation of interacting analyte (dissociation): The dissociation step is performed in the same buffer wells used for the baseline step. Using the same wells for baseline and dissociation enables the Octet® Analysis Studio Software's inter-step correction feature to be used in for more accurate curve fitting.

In the Octet® BLI Discovery Software, set up and run a kinetics assay according to the table in Figure 2B. Assay step times may vary according to the specific proteins being used. However, immobilization of the biotin-anti-FLAG conjugate should be performed exactly as recommended here for best results. Figure 3 shows example data starting from the FLAG-protein ligand loading step.

Figure 3

Example Raw Data Traces from a FLAG-protein Kinetic Experiment, Starting with the Pre-FLAG-protein Step Baseline.



Note. TRAIL-FLAG (23 kDa) was loaded followed by baseline in 10X Kinetics Buffer. A titration of mouse anti-TRAIL analyte in 10X Kinetics Buffer was used in the association step (5 concentrations) plus a reference sample containing 10X Kinetics Buffer only (orange trace) for correction of downward drift. Immobilization of biotin-anti-FLAG M2 conjugate was performed in a different experiment (Figure 5).

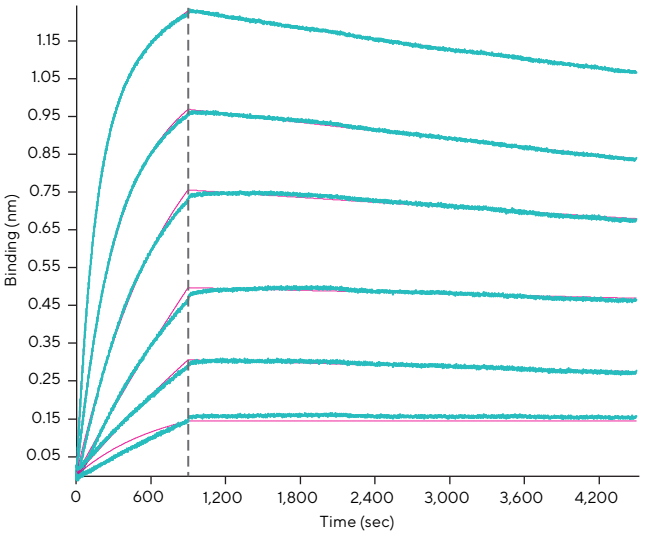
Process and Analyze Data

1. Load the data into the Octet® Analysis Studio Software.
2. Process the data by specifying methods for reference subtraction, y-axis alignment, inter-step correction and Savitzky-Golay filtering.
3. Analyze the data by specifying steps for analysis, fitting method (local or global), and window of interest.
4. To export the analyzed data, click "Save Report" to generate a Microsoft Excel® report.

Figure 4 and Table 1 show analyzed versions of the raw data shown in Figure 3. For more details on processing and analysis parameters or data exporting, please refer to the Octet® Software User Guide.

Figure 4

Kinetic Analysis of the Interaction Between Ligand TRAIL-FLAG (23 kDa) and an Analyte Mouse Anti-TRAIL (150 kDa).



Note. Assay steps included: 5 minutes of equilibration, 10 minutes of TRAIL-FLAG loading (2.5 µg/mL), 5 minutes of baseline stabilization, 15 minutes of ligand:analyte association, and 60 minutes of ligand:analyte dissociation. Analyte concentrations were 0.78, 3.13, 12.5, and 50 nM. 10X Kinetics Buffer was used as the matrix throughout and the assay temperature was 30°C.

Table 1

Kinetic Results for the Interaction Between Ligand TRAIL-FLAG (23 kDa) and an Analyte Mouse Anti-TRAIL (150 kDa) Using Anti-FLAG Biosensors.

k_a	k_d	K_D
1.20E+05 1/Ms	2.56E-05 1/s	2.13E-10 M

Batch Immobilization and Preservation of Custom Anti-FLAG Biosensors

An alternative approach to running the assay as described prior is to perform a batch immobilization of anti-FLAG M2 antibody conjugate on SAX or SAX2 Biosensors ahead of time, followed by a preservation step that allows the biosensors to be used in a separate experiment at a later time. The preserved biosensors can be stored long-term and subsequently re-hydrated for use in kinetic assays starting with immobilization of the FLAG-protein ligand. Using pre-coated, preserved biosensors can save a great deal of time when running FLAG-protein assays, allow room to analyze more samples in a single experiment, and also eliminate the need to perform repeated M2 antibody conjugations.

Materials Required

- Octet® BLI system with Octet® BLI Discovery and Analysis Studio Software
- High Precision Streptavidin (SAX or SAX2) Biosensors: (Sartorius part no. 18-5117 [tray]; 18-5118 [pack]; 18-5119 [case])
- Biotin-anti-FLAG antibody conjugate (see prior procedure)
- 1X Kinetics Buffer
- Sucrose
- For all Octet® BLI systems: 96-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. 655209)

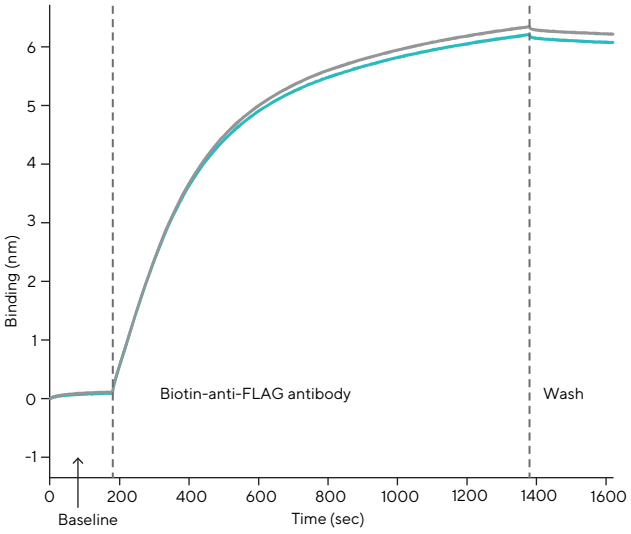
1. Prepare the biotin-LC-LC-anti-FLAG M2 antibody conjugate as described in "Biotinylation of Anti-FLAG M2 Antibody" on page 3.
2. Determine the number of biosensors to be prepared in the batch. If only part of a biosensor tray is being processed, carefully transfer the SAX or SAX2 Biosensors to an empty biosensor tray without touching the active tips of the biosensors. Store unused biosensors separately.
3. Hydrate the SAX or SAX2 Biosensors for at least 10 minutes in 1X Kinetics Buffer.
4. Prepare a sufficient volume of a 50 µg/mL solution of biotin-anti-FLAG conjugate in 1X Kinetics Buffer (200 µL/well – each well can be used to coat up to 5 biosensors).
5. Prepare a 96-well sample plate with the biotin-anti-FLAG conjugate solution, filling the appropriate number and location of wells.

Figure 5
Assay steps (A) and Raw Data Traces (B) for Immobilization of Biotin-anti-FLAG M2 Antibody Conjugate onto SAX or SAX2 Biosensors (Four Biosensors).

A.

Step	Column	Well Contents	Time	Flow	Step Type
Step 1	1	Buffer	120	1000	Baseline
Step 2	2	Biotin-anti-FLAG conjugate (50 µg/mL)	1200	1000	Loading
Step 3	3	Wash 1	30	1000	Custom
Step 4	4	Wash 2	30	1000	Custom

B.



6. Place the hydrated biosensors and sample plate in the Octet® BLI system. Set up and run a kinetics assay in Octet® BLI Discovery Software as described in Figure 5A.

   Important: Be sure the software is set to re-rack the biosensors.

   Program the assay to repeat as needed to coat all biosensor columns. To save reagent, each well containing biotin-anti-FLAG conjugate can be dipped into up to 5 times (Step 2) with 5 different biosensors to coat multiple times. Ensure that the signal in the Loading step approaches saturation, *i.e.* the slope of the raw data trace curves and flattens out. See Figure 5 for example immobilization data.
7. Prepare a 20% (w/v) solution of sucrose in distilled, deionized water. Sterile filter through a 0.2 µm filter. This solution is used to preserve the biosensors once the ligand is immobilized.

8. Add 200 µL per well of sucrose solution to a new 96-well sample plate, filling the wells corresponding to the locations of the biosensors being coated.
9. Once all desired biosensors have been coated with biotin-anti-FLAG conjugate, remove the biosensors in their tray from the Octet® BLI system. Place the biosensor tray into a bottom tray holder containing the sample plate with 20% sucrose solution. Incubate at room temperature without shaking for 2 minutes.
10. Remove the biosensors from the sucrose solution and allow them to dry completely. This can be done by letting them sit at room temperature for 10 minutes or in an incubator at 37°C for 3 minutes.
11. Store the preserved biosensors sealed in their original foil pouch with desiccant packet at room temperature for up to 6 months. Re-hydrate biosensors for at least 10 minutes in FLAG-protein buffer immediately before use.

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TN-4043 Rev C