SARTORIUS

Octet® HIS1K Biosensors for Label-free Analysis of His-tagged Proteins

Technical Note

Scope
Learn how Octet® HIS1K Biosensors along with the Octet® Bio-Layer Interferometry (BLI) platform provide a rapid and label-free method for analyzing His-tagged proteins.

Abstract

Octet® HIS1K Biosensors contain a penta-his antibody and are designed to provide a rapid and label-free method for both quantitation and kinetics characterization of His-tagged proteins in complex solutions such as cell lysates. The biosensors are not affected by chelating agents and can be used for the direct capture of His-tagged proteins. The biosensor's dynamic range for quantitation varies depending on sample matrix but will typically be in the range of 0.25–500 µg/mL. The biosensors can be regenerated for re-use in kinetics studies, however, this depends on the His-tagged protein ligand and should be optimized accordingly.

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Overview

The polyhistidine-tag (His-tag) is a common peptide tag added to recombinant proteins during cloning. Many tools have been developed that enable this tag to be used for detection and purification of tagged proteins. The Octet® HIS1K Biosensor from Sartorius comes pre-immobilized with Qiagen's highly specific Penta-His antibody. Combined with the Octet® Bio-Layer Interferometry (BLI) label-free analysis platform, the HIS1K Biosensors provide a rapid and label-free method for analyzing His-tagged proteins. His-tagged proteins can be quantified directly based on rate of binding to the HIS1K Biosensor tip. The high level of specificity provided by the Penta-His antibody enables quantification to be performed in complex solutions such as crude cell lysates or cell culture supernatants. Because the Penta-His antibody is not susceptible to chelating agents often used to elute His-tagged proteins from purification columns, HIS1K Biosensors can also be used for simple quantitation of metal affinity column eluates. The HIS1K Biosensor is also qualified for direct capture of His-tagged proteins for subsequent kinetic analyses due to its high affinity for the polyhistidine tag.

Principle

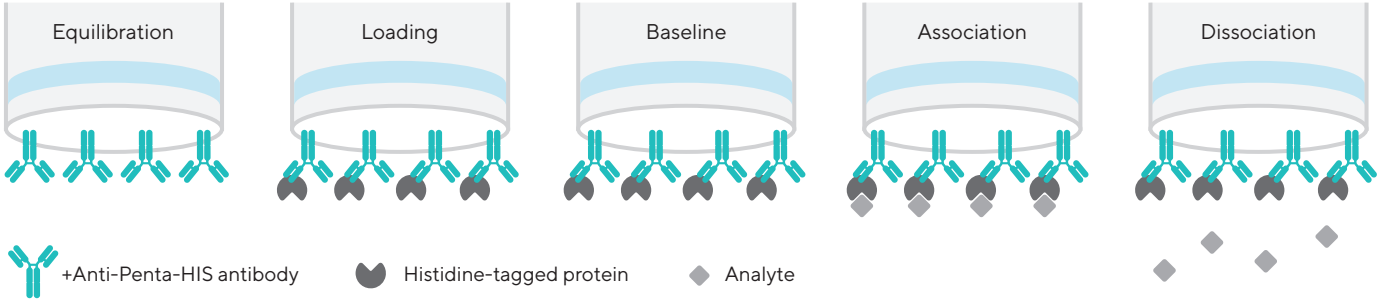
HIS1K Biosensors are disposable fiber optic tips for use with Sartorius' Octet® BLI systems. The biosensor tip surface is factory-coated with the monoclonal Penta-His antibody from Qiagen, enabling specific, high affinity capture and detection of His-tagged proteins. When the biosensor is dipped into a sample, the binding of protein molecules to the tip alters the interference pattern of light reflected from the biosensor surface to a detector, allowing molecular association and dissociation events to be monitored in real time with Octet® BLI systems. Since the rate of binding is proportional to concentration, His-tagged protein samples can be readily quantified against a standard curve. Kinetic analysis can also be performed with HIS1K Biosensors to determine affinity of a His-tagged protein to interacting biomolecular analytes (Figure 1). The surface enables capture and analysis directly from complex mixtures and can be used as an alternative to chemical protocols such as EDC/NHS and biotinylation.

Materials Required

- Octet® BLI system with Octet® BLI Discovery and Analysis Studio Software
- Octet® HIS1K Biosensors (Sartorius part no. 18-5120 [tray]; 18-5121 [pack]; 18-5122 [case])
- For all Octet® instruments: 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)
- Quantitation assays only:
  - **Purified His-tagged protein to be used as a calibration standard.** This calibration standard protein should be identical to the protein in the sample being quantified. Best results are obtained when all buffers/matrices are matched for all samples and standards.
  - **Octet® Sample Diluent (Sartorius part no. 18-1104) for dilution of all samples.** If undiluted crude samples will be quantitated, a matching blank matrix is required.

Figure 1

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



Note. The assay consists of 5 assay steps. Step 1: equilibration, Step 2: loading (capture) of His-tagged protein, Step 3: baseline, Step 4: analyte association, Step 5: analyte dissociation.

- Kinetic assays only:
  - **His-tagged protein for immobilization.** The target protein can be captured from either purified sample in buffer or unpurified sample in a complex mixture such as culture supernatant.
  - **Analyte proteins that interact with target protein.** The analyte proteins can be dissolved in buffer matrix or a complex mixture such as culture supernatant.
  - **Octet® Kinetics Buffer 10X (10X KB) (Sartorius part no. 18-1105).** The HIS1K Biosensor is compatible with a wide range of buffers, although 10X Kinetics Buffer is recommended.

Tips for Optimal Performance

- Fully equilibrate reagents, standards and samples to room temperature prior to preparation. For frozen samples, thaw and mix thoroughly prior to use.
- 10 minutes of biosensor hydration is required prior to an assay. Hydrating biosensors in a buffer consistent with the buffer used throughout the assay is recommended.
- Ensure that the Octet® BLI system is turned on and the lamp is warmed to room temperature for at least 40 minutes prior to starting the assay.
- Set the sample plate temperature in Octet® BLI Discovery Software by selecting **File > Experiment > Set plate** temperature and entering the desired temperature. Sartorius recommends running assays at 30°C. Using other temperatures may require modifying the assay times discussed in this protocol.
- Quantitation assays only:
  - His-tagged proteins will possess different binding kinetics due to amino acid sequence variations and differing steric exposure of the polyhistidine tag. Since binding rate is dependent on protein affinity and size, the calibration standard protein should be identical to the protein present in the test sample for best results.
  - Assay sensitivity and dynamic range will be highly protein-dependent, but typically range from 0.1 µg/mL to 500 µg/mL when measured at 1000 rpm.
  - Match the matrix of the samples, standards, references, and hydration solution as closely as possible.
  - Perform a dilution study and a dynamic range study as outlined in the Assay Optimization section.
  - Use a blank (buffer-only) negative control with a matching matrix for reference subtraction. This is especially important for accuracy in low-concentration samples.
- Kinetic assays only:
  - The inter-step correction processing feature in Octet® Analysis Studio Software corrects for misalignment between the association and dissociation steps. For the most effective inter-step correction, the baseline and the dissociation steps of an assay cycle should be performed in the same microplate well.
  - Use of a reference biosensor to correct for assay drift is always recommended. A reference biosensor should be loaded with the His-containing protein and run with a buffer-only blank for the association and dissociation steps.
  - The HIS1K Biosensor contains elements of the protein streptavidin. When using biotinylated analytes or matrices that contain biotinylated molecules, we recommend that open biotin binding pockets be blocked by incubating biosensors in 1 µg/mL of biocytin for 15 minutes.

Quantitation Assays

Assay Optimization

The following optimization steps are recommended each time a new matrix or new His-tagged protein is analyzed.

1. Determine the minimal dilution factor required to achieve the targeted assay performance (applicable only when the target protein is in a complex matrix such as cell lysate).
2. Perform a spike/recovery study to determine the assay dynamic range.
3. Establish data analysis parameters in Octet® Software.
4. Apply finalized protocol and data analysis parameters in routine assays.

Dilution Factor Determination for Sample Matrix

Components in complex matrices such as cell culture media can potentially interfere with assay performance. Diluting the sample matrix using Sartorius' Sample Diluent is an effective means of minimizing matrix effects. Dilution factor guidelines for various sample types are described in Table 1. However, before running a quantitation assay it should be empirically determined whether dilution of samples is needed.

Table 1

Recommended Minimum Dilution for Common Sample Types.

Sample Type	Minimum Recommended Dilution in Sample Diluent
Purified proteins	Dilute into assay range
Samples from column eluents	Dilute into assay range
Serum-free cell culture supernatants media	Neat or two-fold
Serum containing cell culture supernatants	Neat
Bacterial cell pellet lysates	Ten-fold

Note. In all cases, the matrix for the diluted samples, the standards and the biosensor hydration solution should be matched as closely as possible.

1. Prepare 1 mL each of sample matrix (without target protein) diluted both two-fold and ten-fold in Sample Diluent.
2. Add target His-tagged protein to each of the matrix dilutions, and also to neat matrix and to Sample Diluent as a control. The final concentration of target protein in each or the four samples should be in the middle of the desired quantitation range.
3. Transfer each sample to a 96-well or 384-well sample plate in duplicate (eight wells total).
4. Hydrate biosensors in the sample matrix that matches each sample type (e.g., biosensors to be used in wells with ten-fold diluted matrix should be hydrated in ten-fold diluted matrix). Place the sample plate and the hydrated biosensors into the Octet® BLI system. Recommended sample plate warm-up in the instrument and biosensor hydration time is 10 minutes. The delay timer can be used to automatically start the assay after 600 seconds.
5. Set up a Basic Quantitation assay according to the Octet® Software User Guide. Use the provided assay parameter file for the HIS1K Biosensor or use the **Modify** button in the Plate Definition tab to choose the appropriate Anti-Penta-HIS parameter file (parameter file availability will depend on software version number).
   a. **Anti-Penta-HIS Quantitation Assay:** Loads parameters for a 120-second assay at 1000 rpm, typically suited for a 0.25–500 µg/mL dynamic range (range will vary according to protein and sample matrix).
6. Run the experiment.
7. Data will be displayed in real time during the assay. Data and method files will be saved automatically.
8. Load data into Octet® Analysis Studio or BLI Discovery Software.
9. Visually inspect the real-time binding traces and determine the dilution required to:
   a. Minimize non-specific binding of matrix components.
   b. Show equivalent binding in the matrix spiked sample and the Sample Diluent control.
10. Use this dilution factor for routine assays.

Recovery and Precision Assay to Determine Quantitation Range

1. Prepare a series of His-tagged protein standards in the appropriate matrix diluent using the dilution factor determined in the Dilution Factor Determination for Matrix experiment. The dilution series should span the entire range of the assay, generally 0.1–500 µg/mL for assays run at 1000 rpm. The minimum volume needed in each well will vary with the plate used:
   - 200 µL/well in a 96-well microplate (all Octet® BLI systems)
   - 80 µL/well in a 384-well microplate (Octet® RH16 and RH96 BLI systems)
   - 40 µL/well in a 384-well tilted-bottom microplate (Octet® RH16 and RH96 BLI systems)
   - 4 µL in drop holder in the Octet® N1 instrument
2. Using the same matrix diluent as in Step 1, prepare two His-tagged protein samples of known concentration for measurement. The concentration of these samples should be within the range of the standard curve being generated, preferably one at the low end and one at the high end.
3. Transfer triplicates of the prepared standards and the samples to a sample plate. It is recommended to organize samples in columns, from A–H. Fill at least one well with blank diluted matrix for reference subtraction during data analysis. An example plate map is shown in Figure 2.

4. Hydrate biosensors for 10 minutes in matching matrix diluent. Place the sample plate and the hydrated biosensors in the Octet® BLI system. The recommended sample plate warm-up in the instrument and biosensor hydration time is 10 minutes. The delay timer can be used to automatically start the assay after 600 seconds.
5. Set up a Basic Quantitation assay using the assay parameters that were used in the Dilution Factor Determination for Matrix experiment.
6. Run the experiment. Data will be displayed in real time during the assay. Data files, method files and assay pictures will be saved automatically.
7. Load the data into Octet® Analysis Studio or BLI Discovery Software.
8. If blank matrix was included as a reference, use the reference subtraction option to correct the data as appropriate.
9. Calculate the binding rate. The results table will populate with calculated concentrations and data statistics.
10. Define assay dynamic range by selecting acceptable % CV values for the lower and upper concentration limits in the standard curve.
11. Exclude data points for the standard curve that lie outside the defined dynamic range.
12. Iteratively adjust the following processing parameters and re-calculate the binding rate:
    a. Adjust the read time window if necessary (typically 120 seconds).
    b. Adjust the zero concentration threshold if necessary (recommended 0.0001).
    c. Adjust the low concentration threshold to 0.001 (recommended).
    d. Select the appropriate equation to fit the standard curve.
13. Evaluate the calculated concentration value of the unknowns by defining acceptable values of % recovery (accuracy) and % CV (precision).

Running the Assay To Quantify His-tagged Protein(s) of Interest

1. Prepare samples, calibration standards and hydration solutions according to the conditions determined in optimization steps above.
2. Pipette standards, calibrators and samples into a black polypropylene microplate (see Figure 3 for a sample plate layout).
3. Pipette biosensor hydration solution into the wells of a 96-well black flat bottom microplate corresponding to the number and position of the biosensors to be used.

Figure 2
Example Plate Layout for a Spike-Recovery Assay.

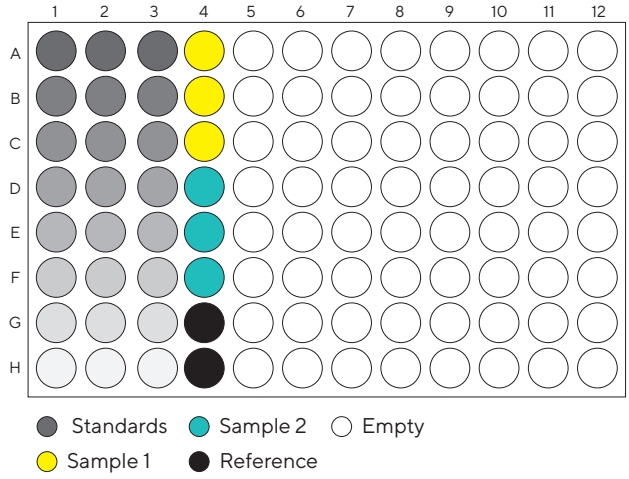
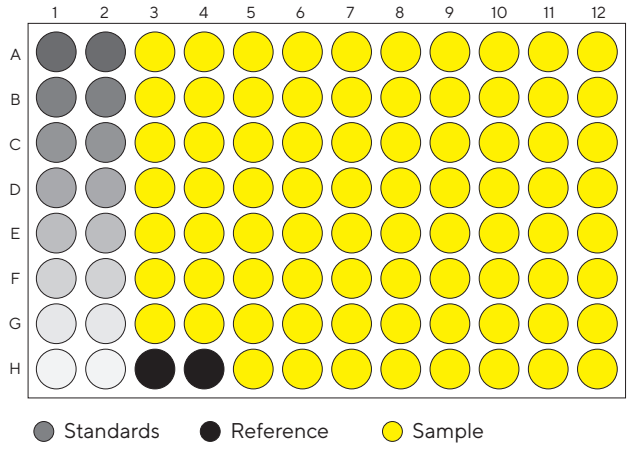


Figure 3
Example Plate Layout for a Routine Quantitation Assay Run in a 96-Well Microplate.



4. Place the biosensor tray with the hydration plate in the Octet® BLI system. Place the sample plate in the system. Warm the sample plate in the system and hydrate the biosensors for 10 minutes prior to starting the experiment. The delay timer can be used to automatically start the assay after 10 minutes (600 seconds).
5. Set up a Basic Quantitation using the parameters described previously in the optimization experiments above. For details on how to set up an assay see the Octet® Software User Guide.
6. Run the assay.
7. Load data into Octet® Analysis Studio or BLI Discovery Software. Analyze as in previous optimization steps to determine concentration of samples and data statistics.
8. To export the analyzed data, use the **Save Report** button to generate a Microsoft® Excel® report.

Representative Data

Figure 4 shows detection of His-PAI1 (50 kDa) or His-Protein A (43 kDa) using HIS1K Biosensors on the Octet® RH16 BLI system. A) His-PAI1 dose response. B) HIS-ProA dose response. C) and D) represent the resulting calibration curves from A and B respectively. Sample diluent was used as a matrix for all samples. See Table 3 for the statistical analysis of data from Figure 4.

Kinetic Analysis

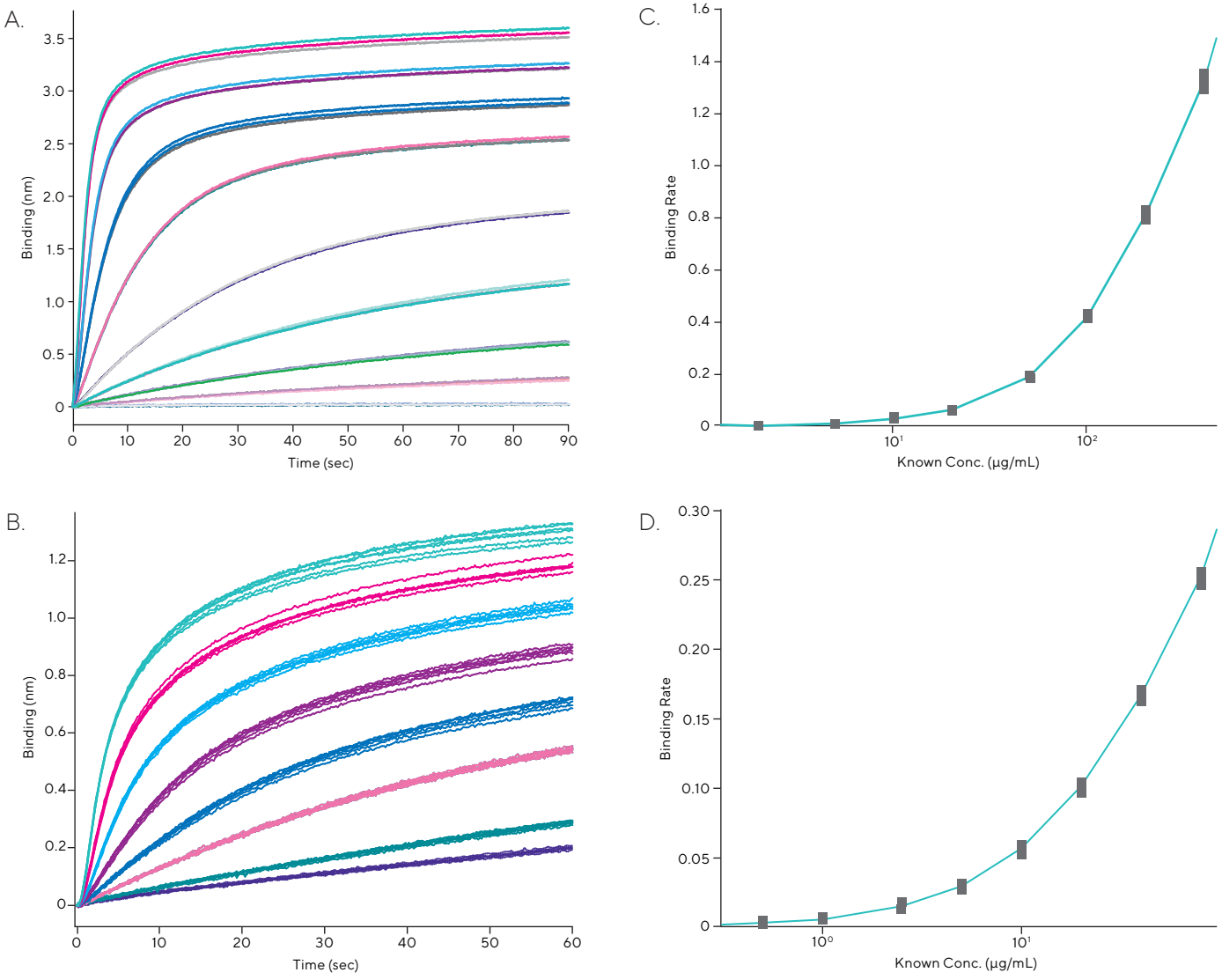
Assay Protocol

Overview

- Prepare assay solutions.
- Prepare the sample plate.
- Equilibrate both the hydrated biosensor assembly and the assay plate for 10 minutes on the Octet® BLI system.
- Run the assay.
- Process and analyze the data.
- Save the results.

Figure 4

Detection of His-PAI1 and His-Protein A Standards Using HIS1K Biosensors on the Octet® RH16 BLI System.



Note. A) Raw data, His-PAI1 dose response (n=3), B) Raw data, His-ProA dose response (n=6). C) and D) represent the corresponding calibration curves from A and B. Sample diluent was used as matrix for all samples, assay run at 1000 rpm.

Table 2

Concentration Analysis of His-PAI1 Standards.

Known Conc. (µg/mL) n=3	Average Binding Rate	Average Calculated Concentration	% Recovery	%CV
400	1.3267	400.0	100.0%	1.8%
200	0.8136	200.6	100.3%	2.6%
100	0.4211	100.1	100.1%	2.1%
50	0.1911	50.0	99.9%	3.3%
20	0.0635	20.0	99.8%	3.1%
10	0.0287	10.0	100.0%	2.3%
5	0.0117	5.0	99.9%	3.5%
2	0.0029	2.0	98.8%	14.1%

Note. Average calculated concentration and % CV of triplicates of His-PAI1 calibration standards for the data from Figure 4. Results may vary with individual His-tagged analytes and assay matrices.

Table 3

Concentration Analysis of His-Protein A Standards.

Known Conc. (µg/mL) n=6	Average Binding Rate	Average Calculated Concentration	% Recovery	%CV
80	0.2525	80.0	100.0%	1.8%
40	0.1672	40.2	100.4%	2.6%
20	0.1017	20.1	100.3%	2.1%
10	0.0570	10.0	100.3%	3.3%
5	0.0297	5.0	100.0%	3.1%
2.5	0.0154	2.5	100.0%	2.3%
1	0.0059	1.0	99.5%	3.5%
0.5	0.0035	0.5	102.4%	14.1%

Note. Average calculated concentration and % CV of triplicates of His-Protein A calibration standards for the data from Figure 4. Results may vary with individual His-tagged analytes and assay matrices.

Prepare the Samples

- Equilibrate reagents and samples to room temperature prior to preparation and mix thoroughly.
- **His-tagged protein ligand.** The ligand is the protein that will be captured on the biosensor tip surface. His-tagged ligands are typically immobilized (loaded) at a concentration between 1–25 µg/mL. The ligand solution can be recovered from the well after the assay and re-used, if desired. If the ligand is captured from a cell culture supernatant, dilution of the supernatant two-fold or greater with Octet® Kinetics Buffer 10X (10X KB) can potentially increase data quality. If dilution results in a low total concentration of the ligand, the biosensors can be incubated in the diluted supernatant overnight at 4°C to maximize loading. For tips on optimizing overnight loading, see the Technical Note, Batch Immobilization of a Biotinylated Ligand onto Streptavidin Biosensors.
- **Interacting protein (analyte).** During rigorous kinetic analysis, it is recommended to run a dilution 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 would be recommended for an analyte with low-nanomolar affinity towards an immobilized ligand. 200 µL/well, 80 µL/well and 40 µL/well of analyte solution are required for 96-well, 384-well, and 384-well tilted-well plates, respectively. The solution can be recovered from the well after the assay and re-used, if desired. For screening assays or qualitative interaction analysis, a single concentration of the interacting protein can be sufficient to characterize the binding based on off-rate.

Running the Assay

1. Ensure the Octet® BLI system and computer are turned on. It is essential that the instrument lamp warms up for at least 40 minutes before running an experiment.
2. Pipette 200 µL/well of biosensor hydration solution into wells of a 96-well, black, flat bottom microplate corresponding to the number and the positions of biosensors to be used.
3. Insert the hydration plate into the biosensor tray. Align the biosensor rack over the hydration plate and lower the biosensors into the wells, taking care not to scrape or touch the bottom of the biosensors.
4. Transfer 200 µL of each assay reagent into the appropriate wells of a black polypropylene microplate. Figure 5 shows an example plate map for a typical kinetic assay. Place the assay plate on the sample plate stage with well A1 toward the back right corner.
5. Place the biosensor hydration assembly in the Octet® BLI system on the biosensor plate stage.
6. Ensure that both the biosensor tray and sample plate are securely in place.
7. Equilibrate the plates in the instrument for 10 minutes prior to starting the experiment. The delay timer can be used to automatically start the assay after 10 minutes (600 seconds).
8. Set up a kinetic assay. For details, see the Octet® Software User Guide. Table 4 shows an example kinetic assay consisting of equilibration, ligand loading, baseline, association, and dissociation steps. Figure 5 shows an example plate map for a typical kinetic assay.
9. Run the assay.

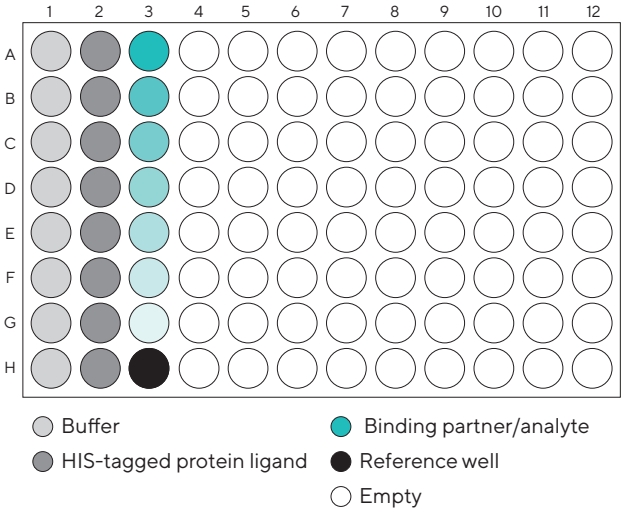
Process and Analyze the Data

1. Load 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, use the **Save Report** button to generate a Microsoft® Excel® report.

Note: For details on processing and analysis parameters and data exporting, refer to the Octet® Software User Guide.

Figure 5

Sample Plate Map - Kinetic Assay.



Note. Example plate map for a kinetic assay that includes equilibration, ligand loading, baseline, association, and dissociation steps. An assay step list using the sample plate is described in Table 1. The same buffer wells (column 1) should be used for the baseline and dissociation steps. Always include a reference well containing buffer only in the analyte step to subtract assay drift.

Table 4

Example Assay Steps and Associated Parameters.

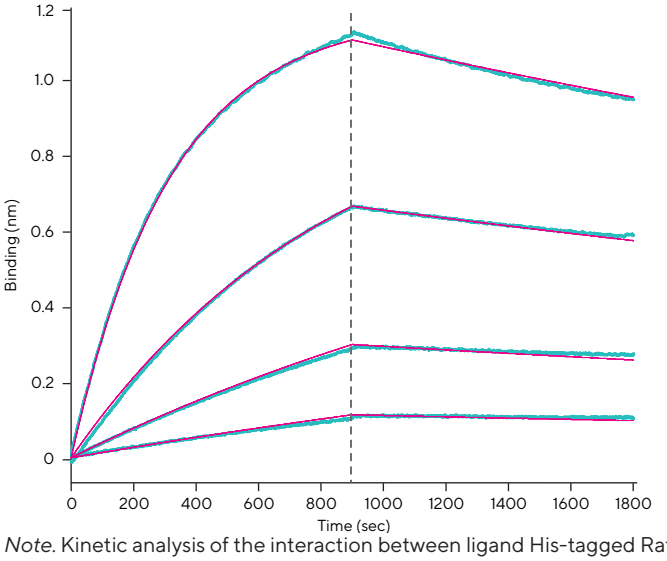
Step	Step Name	Time (s)	Shake Speed	Step Type
1	Equilibration	180–600	1000	Custom
2	Loading	300–600	1000	Custom
3	Baseline	180–600	1000	Baseline
4	Association	600–1800	1000	Association
5	Dissociation	600–3600	1000	Dissociation

Representative Data

Figure 6 shows kinetic analysis of the interaction between ligand His-tagged Rat C-Reactive Protein (24 kDa) and analyte Mouse Anti-Rat C-Reactive Protein (150 kDa). HIS1K Biosensors were hydrated for 10 minutes in 10X Kinetics Buffer prior to analysis. Assays steps included: 5 minutes of equilibration, 10 minutes of ligand loading (10 µg/mL), 5 minutes of baseline stabilization, 900 seconds of ligand : analyte association and 900 seconds of ligand : analyte dissociation. Analyte concentrations were 0, 10, 30, 90, and 270 nM. The kinetic results are reported in Table 5.

Figure 6

Kinetic Analysis of Mouse-Anti-Rat C-Reactive Protein Binding to His-tagged Rat C-Reactive Protein.



Note. Kinetic analysis of the interaction between ligand His-tagged Rat C-Reactive Protein (24 kDa) and analyte Mouse Anti-Rat C-Reactive Protein (150 kDa). 10X Kinetics Buffer was used as the matrix throughout and the assay temperature was 30°C. Data were processed and curve fit using a 1:1 binding model (curve fit lines in pink). The kinetic results are reported in Table 5.

Table 5

Binding Parameters for Mouse Anti-Rat C-Reactive Protein Interacting wth His-Tagged Rat C-Reactive Protein.

K_D (M)	k_{on}(1/Ms)	k_{dis}(1/s)
1.79E-09	9.25E+04	1.71E-04

Note. Kinetic results for the interaction between ligand His-tagged Rat C-Reactive Protein (24 kDa) and analyte Mouse Anti-Rat C-Reactive Protein (150 kDa) using HIS1K Biosensors.

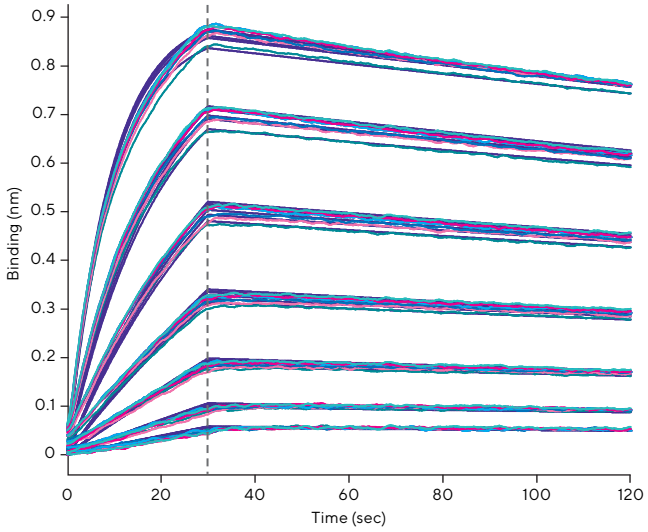
Regeneration of Biosensors

HIS1K Biosensors can be regenerated in kinetic assays. Regeneration provides a cost-effective solution for generating replicate data for ligand-analyte pairs, or for analyzing multiple analyte. The His-tagged capture protein and remaining bound analyte can be removed from the biosensors after the dissociation step by repeatedly dipping into a solution of 10 mM glycine, pH 1.5 for 5 seconds, followed by dip in assay buffer for 5 seconds to neutralize. This regeneration procedure should be repeated 3–4 times in sequence to fully remove bound complex. After regeneration, the biosensor can be reloaded with His-tagged ligand for a new interaction analysis. For best results, run the regeneration protocol before loading the first ligand as a biosensor pre-conditioning step. A small loss in binding capacity may occur after each regeneration cycle.

Regeneration results will depend highly on the His-tagged protein ligand. For each new ligand, regeneration feasibility and conditions should be tested, and the number of possible regeneration cycles determined experimentally. When association and dissociation steps are aligned for multiple assay cycles run on the same set of biosensors, data traces for each assay should overlap with no decrease in signal detected as the number of regeneration cycles increases. Figure 7 shows aligned association-dissociation data for an FcγRI- IgG1 kinetic assay over six total assay regeneration cycles. The data traces overlap closely, with low variability between calculated binding and affinity constants from cycle to cycle (Table 6).

Figure 7

Regeneration of HIS1K Biosensors.



Note. A kinetic assay was performed using His-tagged FcγRI as ligand and human IgG1 as analyte. A total of six assay cycles were performed on the same set of biosensors using regeneration with 10 mM glycine, pH 1.5. When association and dissociation steps are aligned for all six cycles, data traces overlap with very little signal decrease as cycles progress. Data was processed using 1:1 binding model (curve fit lines in pink). Table 6 shows average calculated binding and affinity constants and %CVs for each assay cycle. %CVs are very low, indicating successful regeneration.

Table 6

Regeneration Data Results.

	Average	%CV
K_D	4.1E-08	1.7%
k_{on}	3.0E+04	2.3%
k_{off}	1.3E-03	1.9%

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