



Ni-NTA biosensor kinetic assays

Overview

A polyhistidine tag (also known as hexa histidine-tag, 6xHIS-tag, or by the trademarked name HIS-tag) is commonly fused to recombinant proteins to facilitate their detection and purification. The polyhistidine sequence exhibits strong binding to nickel (Ni^{2+}). The Ni-NTA biosensor is pre-immobilized with novel nickel-charged tris-nitrilotriacetic (Tris-NTA) groups for quick and easy capture of HIS-tagged molecules. In conjunction with Biolayer Interferometry instruments such as the Octet® or BLItz® systems, the Ni-NTA biosensor provides a rapid and label-free method for HIS-tagged protein quantitation and kinetic analysis. For more information on quantitation analyses using the Ni-NTA biosensor, please see Technical Note 32, Ni-NTA Biosensor Quantitation Assays.

Principle

QIAGEN's Tris-NTA is charged with nickel (Ni^{2+}) and pre-immobilized onto the biosensor and will bind specifically to a HIS-tag attached to recombinant proteins. The Ni^{2+} captures HIS-tagged analytes for both quantitation and kinetic applications. The binding of the target analyte to the immobilized HIS fusion proteins alters the interference pattern of light reflected from the biosensor surface, allowing molecular association and dis-

sociation events to be monitored in real time using the Octet or BLItz® instrument platforms. The surface is well suited for capture and analysis directly from complex mixtures as an alternative to chemical protocols such as EDC/NHS and biotinylation.

Ni-NTA biosensors can be regenerated up to 10 times via a standard low-pH protocol in as little as two minutes for various applications such as acquisition of replicate data (same ligand/analyte pair) and bucket-based screening applications. Regeneration dissociates the HIS-tagged protein from the biosensor surface, allowing additional analyses. Once the biosensor is regenerated, it must be recharged with Ni^{2+} prior to loading the next HIS-containing proteins. For the highest quality affinity and kinetic results, using a new Ni-NTA biosensor for each unique capture ligand is recommended.

Materials required

- Octet instrument with Octet software
- Ni-NTA biosensors (ForteBio part no. 18-5101 [tray]; 18-5102 [pack]; 18-5103 [case])
- For all Octet instruments: 96-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. 655209)

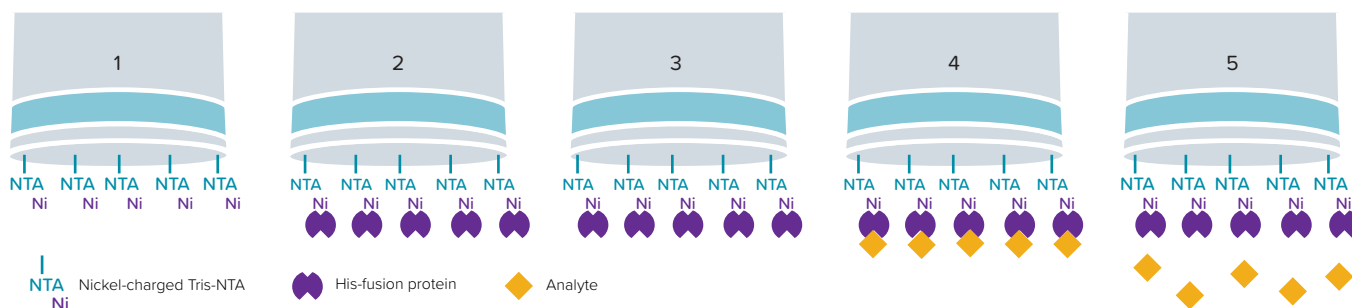


Figure 1: Example workflow for kinetic characterization of the interaction between a His-fusion protein and a target analyte. The assay consists of 5 assay steps. Step 1: equilibration, Step 2: loading (capture) of His-fusion Protein, Step 3: baseline, Step 4: association, Step 5: dissociation.

- Optional for Octet RED384 and Octet QK384 instruments:
 - 384-tilted well, black, flat bottom, polypropylene microplate (ForteBio part no. 18-5080 [pack]; 18-5076 [case])
 - 384-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. 781209)
- HIS-containing protein for immobilization. HIS-containing protein can be present in either buffer or a complex mixture such as culture supernatant.
- Analyte proteins that interact with HIS-fusion protein. The analyte proteins can be present in buffer matrix or a complex mixture such as culture supernatant.
- Kinetics Buffer. The Ni-NTA biosensor is compatible with a wide range of buffers although 1X Kinetics Buffer is recommended (dilute 10X Kinetics Buffer, ForteBio part no. 18-1042, 1:10 with PBS, pH 7.4). Best results are obtained when samples are free of chelating agents (e.g., EDTA, imidazole, etc.) and all matrices are matched as closely as possible.
- Optional:
 - Regeneration buffer (10 mM glycine, pH 1.7): required for surface regeneration. Further evaluation of the most appropriate pH and optimization may be required.
 - Re-charging reagent (10 mM NiCl₂ in H₂O): required after surface regeneration.
 - Cross-linking reagent (0.1 M EDC+0.025M NHS in H₂O): used to stabilize the captured protein. Further evaluation and optimization may be required.
 - Quenching reagent (1 M ethanolamine, pH 8.5): used after the cross-linking step.

Tips for optimal performance

- Some HIS-tagged ligands may dissociate from Ni-NTA surface, resulting in negative drift in 1X Kinetics buffer. To improve baseline and surface stability, the captured HIS-containing ligand can be stabilized by the chemical cross-linker EDC/NHS (typically 0.1M EDC + 0.025 M NHS, 1 min). Depending on the HIS-containing protein being used, the EDC/NHS concentration may require optimization. Subsequent quenching by exposing to 1M ethanolamine, pH 8.5 for 1 minute is required to de-activate the remaining NHS.
- The inter-step correction software processing feature 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.
- Typically, the biosensor surface can be regenerated by cycling it between 10 mM glycine, pH 1.7 (5 seconds) and 1X Kinetics Buffer (5 seconds) 3 times, then re-charging with 10 mM NiCl₂ (1 minute). Depending on the HIS-containing protein being used, the regeneration buffer formulation (buffering element and pH) may require optimization.

- Use of a reference biosensor to correct for drift is recommended. A reference biosensor should be loaded with the HIS-containing protein and run with a buffer blank for the association and dissociation steps.
- Equilibrate reagents and samples to room temperature prior to preparation. For frozen samples, thaw and mix thoroughly prior to use.
- Biosensors must be hydrated for 10 minutes before running an assay. Hydrating the biosensors in a buffer consistent with the buffer used throughout the assay is recommended.
- Turn on the Octet instrument at least 40 minutes before starting the assay, allowing the lamp to warm up.
- Set the sample plate temperature in the Octet software by selecting File > Experiment > Set plate temperature and entering the desired temperature. ForteBio recommends assaying at 30°C. Using other temperatures may require modifying the assay times discussed in this protocol.

Assay protocol

OVERVIEW

- 1 Prepare the Octet instrument and assay solutions.
- 2 Prepare the sample plate and biosensor hydration assembly; equilibrate both 10 minutes on the Octet instrument.
- 3 Run the assay.
- 4 Process and analyze the data.
- 5 Save the results.

PREPARE THE OCTET INSTRUMENT AND ASSAY SOLUTIONS

Note: Equilibrate reagents and samples to room temperature prior to preparation and mix thoroughly

- 1 Ensure that the Octet instrument and computer are turned on. It is essential that the instrument lamp warms up for at least 40 minutes before running an experiment. .
- 2 **HIS-containing ligand:** The ligand is the protein that will be immobilized on the biosensor tip surface. HIS-containing ligands are typically immobilized at a concentration between 5–25 µg/mL. If the concentration of the HIS-containing ligand is below 5 µg/mL, a longer loading time may be required (60 minutes loading at 1 µg/mL is equivalent to 10 minutes loading at 10 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 ten-fold or greater with 1X Kinetics Buffer 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 of the HIS-containing ligand. For

tips on optimizing overnight loading, see ForteBio Technical Note 10, *Batch Immobilization of a Biotinylated Ligand onto Streptavidin Biosensors*.

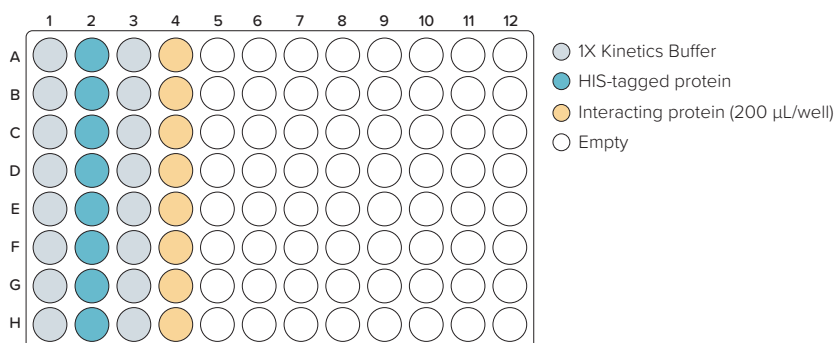
- Interacting protein (analyte):** During rigorous kinetic analysis, it is recommended to run a dilution series of at least four concentrations of the analyte protein. The highest concentration should be approximately 10 times the expected K_D . For example, concentrations of 90 nM, 30 nM, 10 nM and 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.
- Regeneration solution:** The captured HIS-containing ligand and the analyte can be removed from the biosensors by exposing them to 10 mM glycine pH 1.7 followed by a neutralization buffer (typically 1X Kinetics Buffer). 200 μL /well, 80 μL /well and 40 μL /well of regeneration and neutralization solutions are required for 96-well, 384-well, and 384-well tilted-well plates, respectively. After regeneration, the

biosensor can be re-charged with 10 mM NiCl_2 for 1 minute and then re-loaded with HIS-containing protein for a new interaction analysis. A small loss in binding capacity after a regeneration cycle is expected. Regeneration provides a cost-effective method of generating replicate data for ligand-analyte pairs. For the highest quality kinetic results, using a new biosensor to capture each unique ligand is recommended.

HYDRATE THE BIOSENSORS AND PREPARE THE SAMPLE PLATE

- 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.
- 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.
- Transfer 200 μL of each assay reagent into the appropriate wells of a black polypropylene microplate (refer to figures 2–4 for sample plate maps, and Tables 1–3 for corresponding assay steps). Place the assay plate on the sample plate stage with well A1 toward the back right corner.

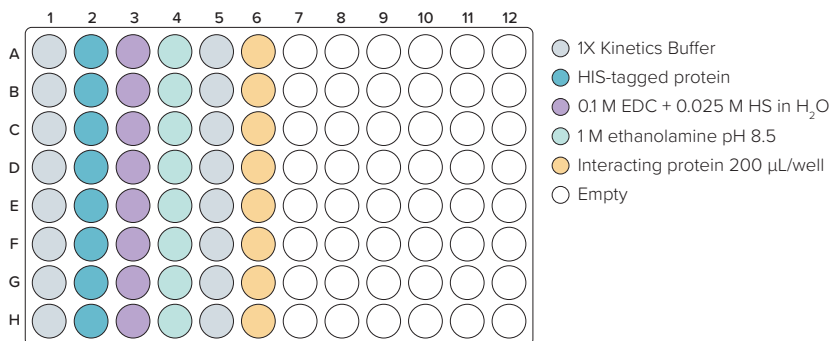
Figure 2: Example plate map for a kinetic assay that includes equilibration, ligand loading, baseline, association, and dissociation steps. Table 1 holds an assay step list for this sample plate. Column 3 is used for both the baseline and dissociation steps to make optimal use of the inter-step correction processing feature.



Step	Step name	Time (s)	Shake speed	Step type	Column
1	Baseline 1	180–300	1000	Baseline	1
2	HIS-fusion Protein	300–600	1000	Loading	2
3	Baseline 2	60	1000	Baseline	3
4	Association	600–1800	1000	Association	4
5	Dissociation	600–1800	1000	Dissociation	3

Table 1: Example assay steps and associated parameters. The same wells are used for the baseline and dissociation steps to make optimal use of the inter-step correction processing feature.

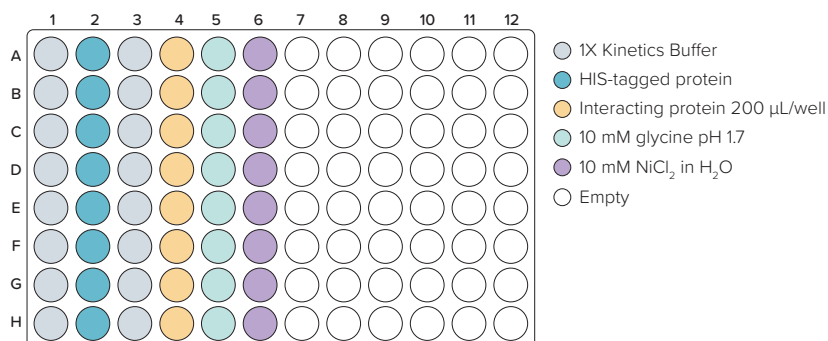
Figure 3: Example plate map for a kinetic assay that includes equilibration, ligand loading, EDC/NHS cross-linking, ethanolamine quenching, baseline, association, and dissociation steps. Table 2 holds an assay step list for this sample plate. Column 5 is used for both the baseline and dissociation steps to make optimal use of the inter-step correction processing feature.



Step	Step name	Time (s)	Shake speed	Step type	Column
1	Baseline 1	180–300	1000	Baseline	1
2	HIS-fusion Protein	300–600	1000	Loading	2
3	EDC + NHS	60	1000	Activation	3
4	Ethanolamine	60	1000	Quenching	4
5	Baseline 2	60	1000	Baseline	5
6	Association	600–1800	1000	Association	6
7	Dissociation	600–1800	1000	Dissociation	5

Table 2: Example assay steps and associated parameters. The same wells are used for the baseline and dissociation steps to make optimal use of the inter-step correction processing feature.

Figure 4: Plate map for a kinetic assay that includes equilibration, ligand loading, baseline association, dissociation, a regeneration cycle and a NiCl₂ re-charging step. Table 3 holds an assay step list for this sample plate. Column 3 is used for both the baseline and dissociation steps for optimal use of the inter-step correction processing feature.



Step	Step name	Time (s)	Shake speed	Step type	Column
1	Baseline 1	180–300	1000	Baseline	1
2	HIS-fusion Protein	300–600	1000	Loading	2
3	Baseline 2	60	1000	Baseline	3
4	Association	600–1800	1000	Association	4
5	Dissociation	600–1800	1000	Dissociation	3
6	Regeneration (10 mM glycine pH 1.7)	5	1000	Custom	5
7	Neutralization	5	1000	Custom	3
8	Regeneration (10 mM glycine pH 1.7)	5	1000	Custom	5
9	Neutralization	5	1000	Custom	3
10	Regeneration (10 mM glycine pH 1.7)	5	1000	Custom	5
11	Neutralization	5	1000	Custom	3
12	Re-charging (10 mM NiCl ₂ in H ₂ O)	60	1000	Custom	6

Table 3: Example a assay steps and associated parameters. After completing step 12, steps 1-12 would be repeated. The same wells are used for the baseline and dissociation steps to make optimal use of the inter-step correction processing feature.

- Place the biosensor hydration assembly in the Octet instrument on the left stage. Ensure that both the biosensor tray and sample plate are securely in place.
- Ensure that the Octet instrument and computer are turned on. It is essential that the instrument lamp warms up for at least 40 minutes before running an experiment.
- 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).

RUNNING THE ASSAY

- Set up a kinetic assay. For details, see the Octet Data Acquisition User Guide. Table 1 shows an example kinetic assay consisting of equilibration, ligand loading, baseline, association, and dissociation. Figure 2 shows an example plate map for a typical kinetic assay. Table 2 and Figure 3 show an example plate map and stepwise procedure for a kinetic assay with cross-linking of the HIS-tagged protein after loading. Table 3 and Figure 4 show an example plate map and stepwise procedure for a kinetic assay with sensor regeneration and recharging.
- Run the assay.

PROCESS AND ANALYZE THE DATA

- Load data into the Octet Data Analysis software.
- Process the data by specifying methods for reference subtraction, y-axis alignment, inter-step correction and Savitzky-Golay filtering. For details on each processing parameter, refer to the Octet Data Analysis User Guide.
- Analyze the data by specifying steps for analysis, fitting method (local or global) and window of interest. For details on each analysis parameter, refer to the Octet Data Analysis User Guide.
- To export the analyzed data, use the Save Report button to generate a Microsoft Excel report. For details on data exporting, refer to the Octet Data Analysis User Guide.

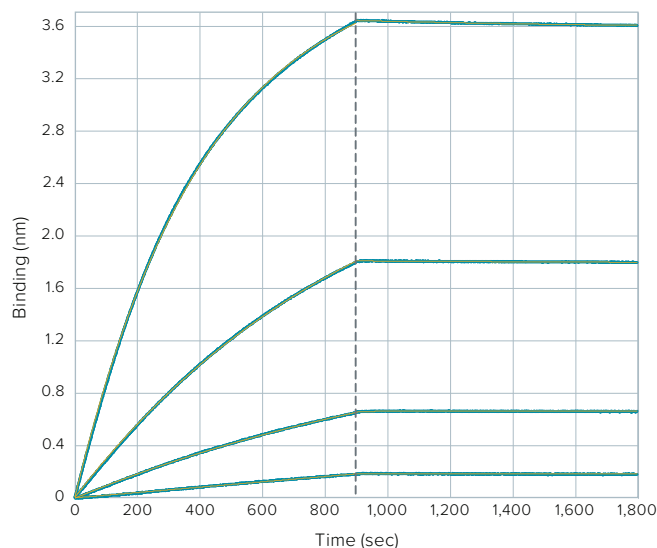


Figure 5: Kinetic analysis of the interaction between HIS-endostatin and its interaction partner anti-endostatin. 1X Kinetics Buffer was used as the matrix throughout and the assay temperature was 30°C. Data were processed and curve fit using a 1:1 binding model. The kinetic results are reported in Table 4.

k_{on}	k_{off}	K_D
8.1×10^4 1/M s	1.8×10^{-5} 1/s	0.22 nM

Table 4: Kinetic results for the interaction between HIS-endostatin and anti-endostatin using Ni-NTA biosensors.

Representative data

Figure 5 shows kinetic analysis of the interaction between HIS-endostatin and its interaction partner anti-endostatin. Ni-NTA biosensors were hydrated for 10 minutes in 1X Kinetics Buffer prior to analysis. Assay steps included: 5 minutes of equilibration, 5 minutes of HIS-endostatin loading (20 µg/mL), 5 minutes of baseline stabilization, 15 minutes of HIS-endostatin: anti-endostatin association and 15 minutes of HIS-endostatin: anti-endostatin dissociation. Analyte concentrations were 1.11, 3.33, 10, and 30 nM. 1X 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. The kinetic results are reported in Table 4.