Ni-NTA biosensor quantitation 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 (Ni2+). 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 kinetic analyses using the Ni-NTA biosensor, please see Technical Note 31, Ni-NTA Biosensor Kinetic 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 (refer to the QIAGEN QIAexpress Handbook for more information). This binding is monitored in real time using an Octet or BLItz system and can be compared to binding a known calibrator to determine concentration. With appropriate dilution, it is possible to measure analytes in complex matrix conditions (see Table 1). The concentration is calculated based on the binding kinetics of the HIS-tagged protein, therefore best results are achieved when the protein used for calibration and the unknown samples are identical. The use of Ni-NTA also requires that the samples and buffers be free from EDTA, imidazole, or other similar chelating agents.

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)
- 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)
- Standard protein containing a HIS-tag, to be used as a calibration standard. For best results this calibration standard protein should be identical to the protein in the sample
- Sample Diluent (ForteBio part no. 18-1000) for dilution of all samples

Tips for optimal performance

- HIS-tagged analytes possess different binding kinetics due to amino acid sequence variations and differing steric environments. Since quantitation performance is typically based on binding kinetics, for best results the calibration standard protein should be identical to the HIS-tagged protein in the unknown sample.
- Typical assay sensitivity ranges from 0.5–1000 µg/mL for assays run at 1000 rpm with a 2-minute read time. If a higher-sensitivity assay is needed, the assay time can be extended to 5 minutes.
- 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 negative control in a matching matrix for reference subtraction. This is especially important when optimizing accuracy and detecting low-concentration analytes.
- Fully equilibrate all reagents, calibrators and samples to room temperature prior to sample preparation. Thaw frozen samples completely and mix thoroughly prior to use.
- Hydrate the biosensors for a minimum of 10 minutes prior to use.
- 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 Experiment > Set Plate Temperature.... and entering the desired temperature. ForteBio recommends 30°C for accurate quantitation. In Octet Software version 6.4 and later, set the default startup temperature by selecting File > Options and entering the desired temperature under Startup.

Assay optimization

The following optimizations are recommended each time a new matrix or a new HIS-tagged protein is analyzed.

OVERVIEW

1 Determine the minimal dilution factor required to achieve the targeted assay performance.

2 Perform a spike/recovery study to determine assay dynamic range.

3 Determine data analysis parameters.

4 Apply finalized protocol and data analysis parameters in routine assay.

DETERMINING THE MATRIX DILUTION FACTOR

Differences between matrices can potentially influence assay performance. Diluting the sample matrix in ForteBio's Sample Diluent is a convenient and generally effective way to minimize matrix effects. Therefore, it is recommended to determine the minimum dilution factor using Sample Diluent that achieves the desired assay performance.

1 Prepare 2 mL of sample matrix diluted both two-fold and ten-fold in Sample Diluent. General guidelines for dilutions are given in Table 1.

2 Prepare a spiked sample of the HIS-tagged protein to be quantified in: Sample Diluent, neat matrix, two-fold diluted matrix and ten-fold diluted matrix by mixing the minimum volume of HIS-tagged protein and 0.5 mL of each matrix (four samples total). The final concentration of the HIS-tagged protein 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 in the Octet instrument. Recommended sample plate warm-up and sensor hydration time in the instrument 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 instructions in the Octet Data Acquisition User Guide. Modify Assay Settings to use Standard Assay and set the time to be 120s and shake speed to be 1000rpm.

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 Data Analysis software.

9 Visually inspect the real-time binding traces and determine the dilution required to:

   a Minimize non-specific binding due to the matrix

   b Show equivalent HIS analyte binding in the matrix-spiked sample and the Sample Diluent control.

10 Use this dilution factor for routine assays.

Sample type	Minimum recommended dilution in sample diluent
Purified proteins	Dilute into assay range
Samples from column eluents	Dilute into assay range (must be free from EDTA, imidazole, or similar chelating agents)
Serum-free cell culture supernatants	Ten-fold
Serum-containing cell culture supernatants	Ten-fold
Bacterial cell pellet lysed by sonication	Twenty-fold
Bacterial cell pellet lysed by B-PER	One hundred-fold

Table 1: Minimum recommended dilution for common sample types. In all cases the matrix for the diluted samples, the standards and the biosensor hydration solution should be matched as closely as possible.

SPIKE RECOVERY ASSAY

To determine the dynamic range and data analysis parameters suitable for a specific HIS analytes, establish a standard curve and spike recovery as described below.

1 Prepare a series of HIS standards in matrix using the dilution factor determined above. The typical range of the series spans 0.5–1000 µg/mL for the Standard Range Assay at 1000 rpm. A minimum volume of 200 µL/well in a 96-well microplate, 80 µL/well in a 384-well microplate or 40 µL/well in a 384-well tilted bottom microplate is required. Recommended concentrations for the standard curve are described in Figure 2.

2 Using the same sample matrix as in Step 1, prepare 700 µL of two unknown samples. The concentration of these samples should be within the assay dynamic range.

3 Transfer triplicates of the standards and the unknowns 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 1.

4 Hydrate biosensors in Sample Diluent or diluted matrix that matches the blank diluted matrix. Place the sample plate and the hydrated biosensors in the Octet instrument. The recommended sample plate warm-up in the instrument and sensor 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 either the Standard or High Sensitivity Assay parameters that were used in the matrix dilution assay above. Availability of the pre-loaded Ni-NTA assay parameters will be software version dependent.

6 Run the experiment. Data will be displayed in real time during the assay. Data files, method files and assay pictures (.jpgs) will be saved automatically.

7 Load the data into Octet Data Analysis 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.

10 Define a dynamic range by selecting acceptable % CV values for the lower and upper concentration limits. 10% is routinely used as a threshold, but may vary depending on the requirements of each assay.

11 Exclude data points for the standard curve that lie outside the defined dynamic range.

12 Individually adjust the following processing parameters and re-calculate the binding rate:

   a Adjust the zero concentration threshold if necessary (recommended 0.0001).

   b Adjust the read time window if necessary (typically 120 seconds).

   c Adjust the low concentration threshold to 0.003 (recommended).

   d Select the appropriate standard curve equation.

13 Evaluate the calculated concentration value of the unknowns by defining acceptable values of % recovery (accuracy) and % CV (precision). Frequently used threshold values are ± 15% recovery and 10% CV, but threshold values may vary depending on the requirements of each assay. See Figure 2 and Table 2 for representative data at 1000 rpm.

Assay protocol

1 Prepare samples, calibration standards and hydration solutions according to the information contained in Table 1. The minimum volume needed in each well varies with the plate used:

- 200 µL/well in a 96-well microplate (all Octet instruments)
- 80 µL/well in a 384-well microplate (Octet 384 instruments)
- 40 µL/well in a 384-well, tilted bottom microplate (Octet 384 instruments only).

2 Pipette standards, controls and samples into a black polypropylene microplate (see Figure 1 for a sample plate layout).

3 Pipette biosensor hydration solution into wells of a 96-well black flat bottom microplate corresponding to the number and positions of the biosensors to be used.

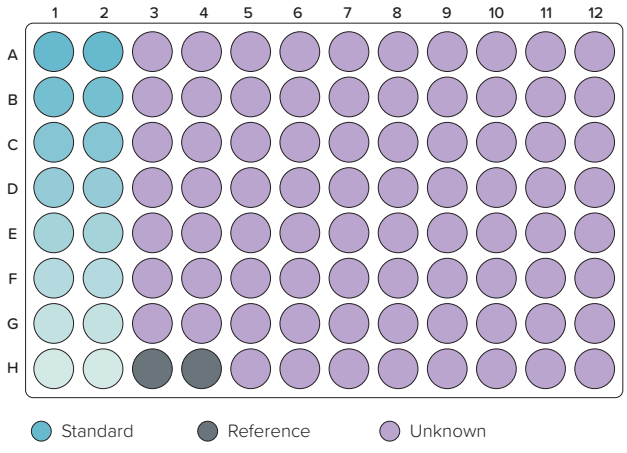

Figure 1: Example plate layout for a routine assay run in a 96-well microplate.

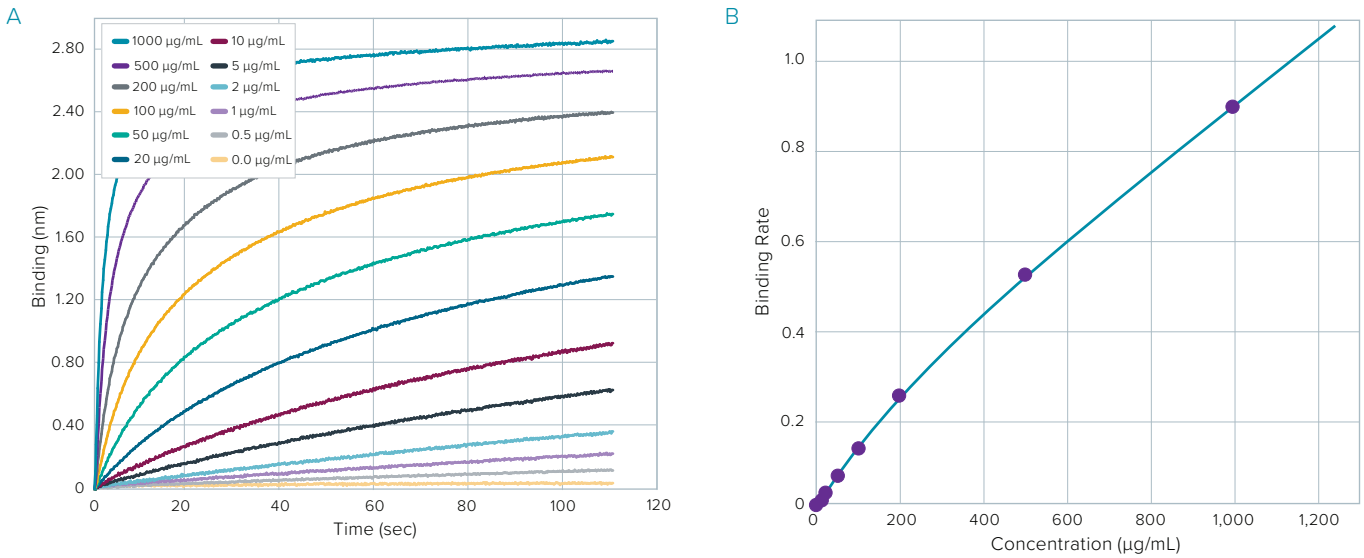

Figure 2: Quantitation of a concentration series of a HIS-Protein A standard using Ni-NTA biosensors on an Octet RED384 system with assay parameters for a standard dynamic range. A) Concentration measurement at 1000 rpm and 2 minutes read time. B) Calibration curve calculated from measuring the standard concentration series. Sample Diluent was used as the matrix for all samples.

4 Place the biosensor tray with the hydration plate in the Octet instrument. Place the sample plate in the Octet instrument. Warm the sample plate in the instrument 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 assay. An example plate map is shown in Figure 1. For details on how to set up an assay see the Octet Data Acquisition User Guide. The dynamic range of the assay can be tuned by changing the shake speed and the read time.

6 Run the assay.

7 Load data into Octet Data Analysis 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.

10 Define a dynamic range by selecting acceptable %CV values for the lower and upper concentration limits. 10% is routinely used as a threshold, but may vary depending on the requirements of each assay.

11 Exclude standard curve data points 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 concentrations threshold to 0.003 (recommended).
- d Select the appropriate standard curve equation.

13 To export the analyzed data, use the Save Report button to generate a Microsoft Excel report.

Representative data

Figure 2 shows detection of a HIS-Protein A standard using Ni-NTA biosensors on an Octet RED384 system with assay parameters for a standard dynamic range. A) Assay run at 1000 rpm and 2-minute read time. B) Calibration curve derived from A). Sample Diluent was used as a matrix for all samples. See Table 2 for the statistical analysis of the Figure 2 data.

	Standard range 1000 rpm 2 min. read time	
Expected concentration (µg/mL)	Avg. conc. µg/mL (N = 3)	% CV (N = 3)
1000	1000.00	0.2%
500	500.33	1.0%
200	200.17	0.7%
100	100.07	1.6%
50	50.00	0.5%
25	20.03	0.8%
10	9.97	2.0%
5	5.02	5.2%
2.5	1.99	3.7%
1	1.00	3.2%
0.5	0.50	8.2%

Table 2: Accuracy and precision for standard curve data. Average calculated concentration and %CV of triplicate HIS-Protein A calibration standards, derived from the data in Figure 2. Results may vary with individual HIS analytes and assay matrices.

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