



Anti-HIS (HIS2) Biosensor Quantitation Assays

OVERVIEW

The polyhistidine-tag (HIS-tag) is a common peptide tag fused 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 Anti-HIS (HIS2) Biosensor provides a rapid, label-free method for quantitation of HIS-tagged proteins on Octet® and BLItz® systems. This biosensor comes pre-immobilized with the next-generation high-affinity, high-specificity anti-HIS antibody from MBS (Maine Biotechnology Services), and is ready to use for detection and quantitation of HIS-tagged proteins.

PRINCIPLE

Anti-HIS biosensors are fiber optic devices coated with a monoclonal anti-HIS antibody for the quantitation of HIS fusion protein analytes. The binding of the molecules to the biosensor alters the interference pattern of light reflected from the surface, allowing binding events to be monitored in real time using either the Octet or BLItz platforms. Higher analyte concentrations result in both faster binding rates and larger signal amplitudes. Unknown concentrations are determined by comparing either binding rate data to a standard curve constructed from identical samples of known concentrations.

MATERIALS REQUIRED

- Octet instrument with Octet software 7.1 or higher
- Anti-HIS biosensors (Pall ForteBio part no. 18-5114 [tray]; 18-5115 [pack]; 18-5116 [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-well, black, tilted-bottom polypropylene microplate (Pall ForteBio part no. 18-5080 [pack]; 18-5076 [case])
 - 384-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. 781209)
- Purified HIS-tagged protein 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 (Pall ForteBio part no. 18-5028) for dilution of all samples. If undiluted crude samples will be quantitated, a matching blank matrix is required.

TIPS FOR OPTIMAL PERFORMANCE

- HIS-tagged proteins will possess different binding kinetics due to amino acid sequence variations and differing steric environments. Since quantitation performance is typically based on binding kinetics, the calibration standard protein should be identical to the protein present in the unknown sample for best results.
- Typical assay sensitivity ranges from 0.1–500 µg/mL (varies with HIS protein) for assays run at 1000 rpm with a 2-minute read time.
- 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 samples.

- 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 biosensors for a minimum of 10 minutes prior to use.
- Ensure the Octet instrument is turned on and the lamp is warmed for at least 40 minutes prior to starting the assay.
- Set the sample plate temperature in Octet Data Acquisition software by selecting Experiment > Set Plate Temperature....and enter the desired temperature. Pall ForteBio recommends 30°C for accurate quantitation. Set the default startup temperature (software version 6.4 and later) by selecting File > Options and enter the desired temperature under "Startup".

ASSAY OPTIMIZATION

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

- Determine the minimal dilution factor required to achieve the targeted assay performance.
- Perform a spike/recovery study to determine assay dynamic range.
- Determine data analysis parameters.
- Apply finalized protocol and data analysis parameters in routine assays.

Dilution Factor Determination for Matrix

Differences between matrices can potentially influence assay performance. Diluting the sample matrix using Pall ForteBio's Sample Diluent is a convenient and often effective means of minimizing matrix effects. It is therefore 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 described in Table 1.
- 2 Prepare a spiked sample of the HIS-tagged protein to be quantitated in: Sample Diluent, neat matrix, two-fold diluted matrix

and ten-fold diluted matrix by mixing the minimum volume of HIS analyte 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 hydrated biosensors into the Octet instrument. 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 Data Acquisition Software User Guide. Use the provided assay parameter file for the Anti-HIS Biosensor or use the Modify button in the Plate Definition tab to choose the appropriate Anti-HIS parameter file (parameter file availability will depend on software version number).
- Anti-HIS Quantitation Assay:** Loads parameters for a 120-second assay at 1000 rpm, typically suited for a 0.1-500 µg/mL dynamic range (varies with HIS analyte 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 Data Analysis software.
 - 9 Visually inspect the real-time binding traces and determine the dilution required to:
 - a Minimize non-specific binding from 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
Serum-free cell culture supernatant media	Neat or two-fold
Serum-containing cell culture supernatants	Neat
Bacterial cell pellet lysates	Ten-fold

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

Spike Recovery Assay

- 1 Prepare a series of HIS-tagged protein standards in matrix using the dilution factor determined in the Dilution Factor Determination for Matrix experiment. The typical range of the series spans 0.1–500 µg/mL at 1000 rpm. Recommended concentrations for the standard curve are shown in Table 2. 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)
- 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 the matrix that matches the blank diluted matrix. Place the sample plate and hydrated biosensors in the Octet instrument. 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. Availability of the pre-loaded HIS 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 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 Iteratively adjust the following processing parameters and recalculate the binding rate:
 - a Adjust the read time window if necessary (typically 120 seconds).

- b Adjust the zero concentration threshold if necessary (0.0001 recommended).
 - c Adjust the low concentration threshold to 0.001 (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). ± 15% recovery and 10% CV are frequently used threshold values but may vary depending on the requirements of each assay. See Representative Data section for example data.

ASSAY PROTOCOL

- 1 Prepare samples, calibration standards and hydration solutions according to the information 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, calibrators and samples into a black polypropylene microplate (see Figure 1 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.
- 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 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).

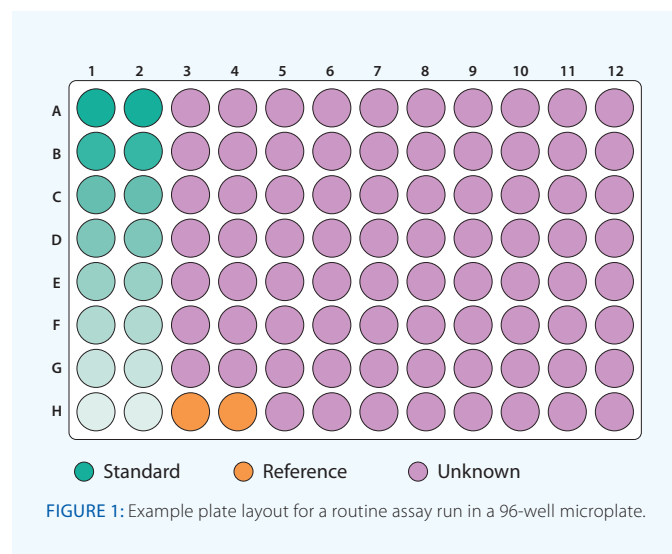


FIGURE 1: Example plate layout for a routine assay run in a 96-well microplate.

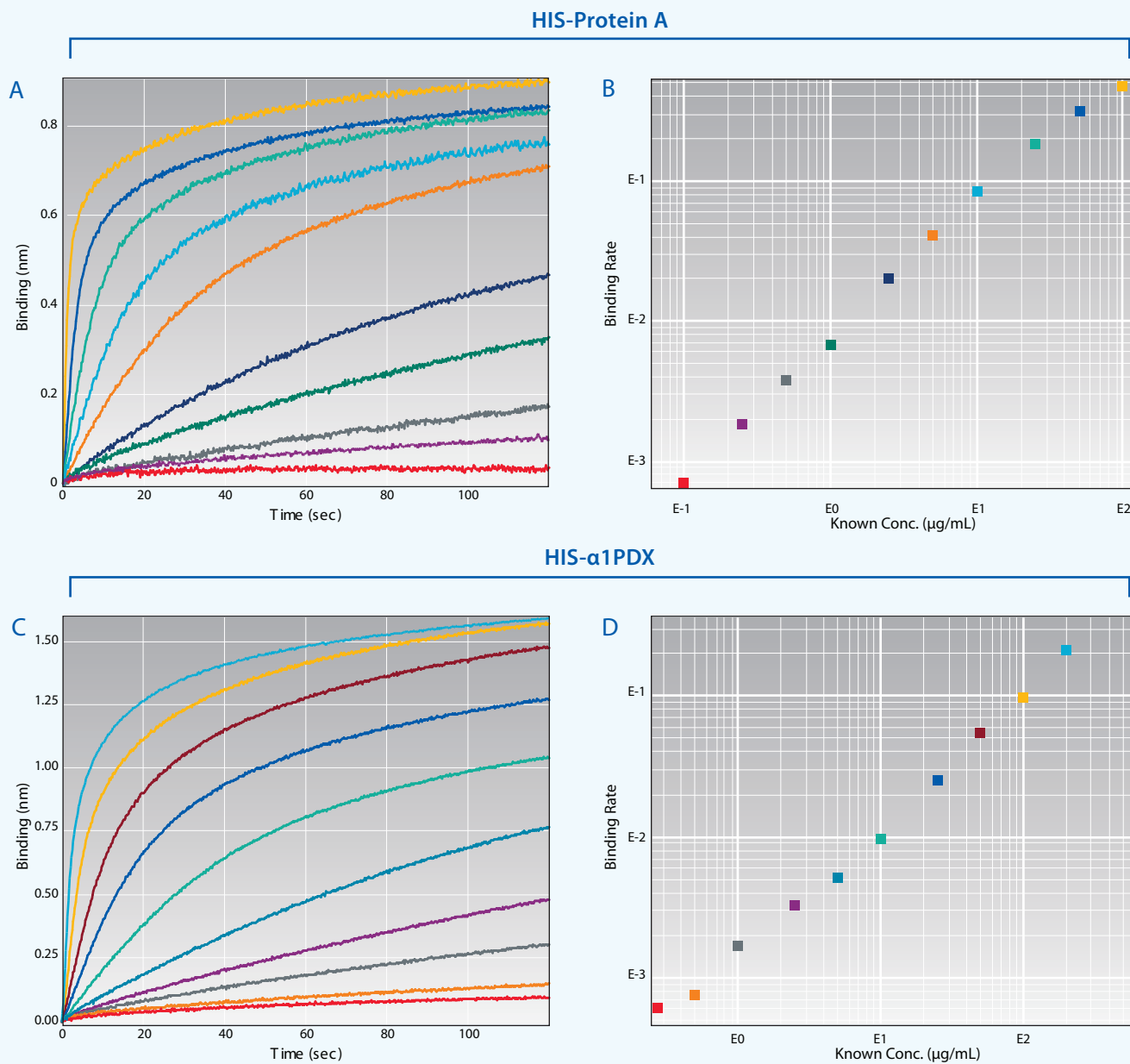


FIGURE 2: Detection of a HIS-Protein A (top) or HIS-α1PDX (bottom) standard using Anti-HIS biosensors on the Octet RED384 system with assay parameters (1000 rpm, 2 minutes) for a standard dynamic range. A) HIS-Protein A dose response. B) HIS-Protein A calibration curve. C) HIS-α1PDX dose response. D) HIS-α1PDX calibration curve. Sample diluent was used as a matrix for all samples.

- 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 Software User Guide.
- 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 data points for the standard curve that lie outside the defined dynamic range.
- 12 Iteratively adjust the following processing parameters and recalculate the binding rate:
 - a Adjust the read time window if necessary (typically 120 seconds).

Expected Conc. ($\mu\text{g/mL}$)	HIS-Protein A		HIS- α 1PDX	
	Avg. Conc. ($\mu\text{g/mL}$) N=3	% CV N=3	Avg. Conc. ($\mu\text{g/mL}$) N=3	% CV N=3
500			500.0	5.0%
200			201.6	4.1%
100			100.2	6.2%
50	50.4	4.9%	50.0	6.4%
25	25.2	7.3%	25.0	1.5%
10	10.0	1.9%	10.0	2.2%
5	5.00	2.7%	4.94	7.6%
2.5	2.50	2.1%	2.51	3.6%
1	0.99	4.7%	0.99	9.8%
0.5	0.50	3.6%		
0.25	0.25	2.8%		
0.1	0.10	4.2%		

TABLE 2: Average calculated concentration and % CV for triplicates of HIS-Protein A and HIS- α 1PDX calibration standards for the data from Figure 2. Results may vary with individual HIS analytes and assay matrices.

- b Adjust the zero concentration threshold if necessary (0.0001 recommended).
 - c Adjust the low concentration threshold to 0.001 (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 or HIS- α 1PDX standard using Anti-HIS biosensors on the Octet RED384 system with assay parameters of 1000 rpm, 2 minutes for a standard dynamic range. A) HIS-Protein A dose response. B) HIS- α 1PDX 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 2 for the statistical analysis of data from Figure 2.