SARTORIUS

Octet® VHH Biosensors

For Quantitation and Kinetic Characterization of VHH Antibodies

Technical Note

Scope: This Technical Note describes kinetic and quantitation assay workflows that use the Octet® VHH Biosensors for characterization of VHH antibodies.

Keywords or phrases: Octet®, Bio-Layer Interferometry, BLI, Kinetic analysis, Quantitation, VHHs, Nanobodies, Antibody capture, Biosensor regeneration.

Abstract

First discovered in the early 1990s, VHH antibodies, or nanobodies, have emerged as one of the most versatile and powerful tools for diagnostics, therapeutics, and many other research applications in biotechnology due to their small size, high stability, strong binding properties, and easy and economical modification and production.[1-3] To expedite the development and optimization of VHH antibodies, it is essential to have a rapid and robust method for their quantitation and kinetic characterization. In this context, the Octet® VHH Biosensors offer a reliable and cost-effective tool for label-free kinetic and quantitation analysis of VHHs in both crude and purified cell culture samples.

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Introduction

The Octet® VHH Biosensors exhibit high specificity towards VHH antibodies derived from camelids (including llama, alpaca, and camel), as well as humanized VHH antibodies (Figure 1A). These biosensors do not interact with Fab or Fc fragments from human, rabbit, or mouse, or with IgGs from mouse and rabbit. However, they do show limited binding to human and goat IgGs (Figure 1B). For VHH characterization, these biosensors offer high binding capacity and sensitivity, and a wide quantification dynamic range. In addition, these biosensors can be cost-effectively regenerated up to 10 times for both kinetics and quantitation assays, while maintaining consistent and precise measurements. Thus, Octet® VHH Biosensors are extremely useful for a wide range of high-throughput applications, including lead identification and optimization, cell line development, process development, and QC in both crude and purified protein samples. This Technical Note describes kinetic and quantitation assay workflows that utilize the Octet® VHH Biosensors to characterize VHHs and provides guidelines for assay optimization using these biosensors.

Kinetic Assay Workflow

The Octet® VHH Biosensors are pre-immobilized with an anti-VHH antibody cocktail, which enables the capture of camelid and humanized VHHs directly from crude or purified samples. These biosensors provide high binding capacity for alpaca, llama, camel, and humanized VHHs, making them suitable for sample analysis at low concentrations and for the characterization of a wide range of proteins.

An example assay workflow utilizing the Octet® VHH Biosensors to characterize the interaction between an analyte and a VHH antibody is outlined in Figure 2. For best results, this kinetic assay should be optimized for a) assay buffer, b) ligand loading concentrations, and c) analyte concentrations. It is equally important to precondition the Octet® VHH Biosensor surface with a preconditioning buffer before the ligand loading step. This ensures enhanced baseline stability and creates a more uniform and robust sensor surface for VHH capture and analyte binding.

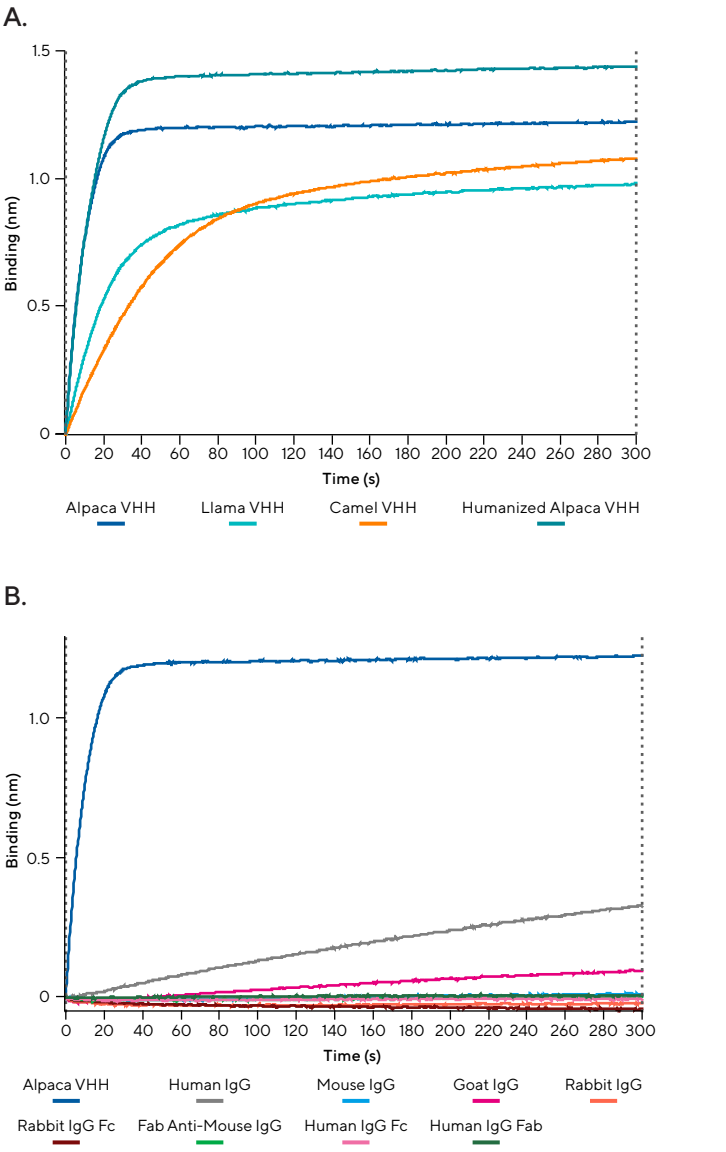


Figure 1. Specificity of the Octet® VHH Biosensors. (A) Strong binding to alpaca, llama, camel, and humanized VHHs. (B) No binding to Fab or Fc fragments from human, rabbit, or mouse, or IgGs from mouse and rabbit. All samples were tested at a concentration of 5 µg/mL.

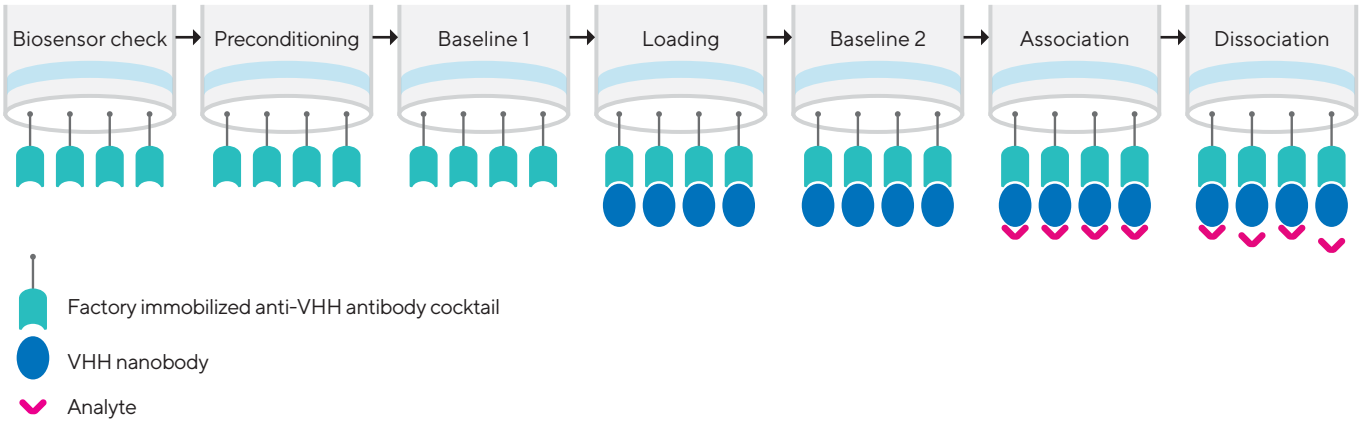
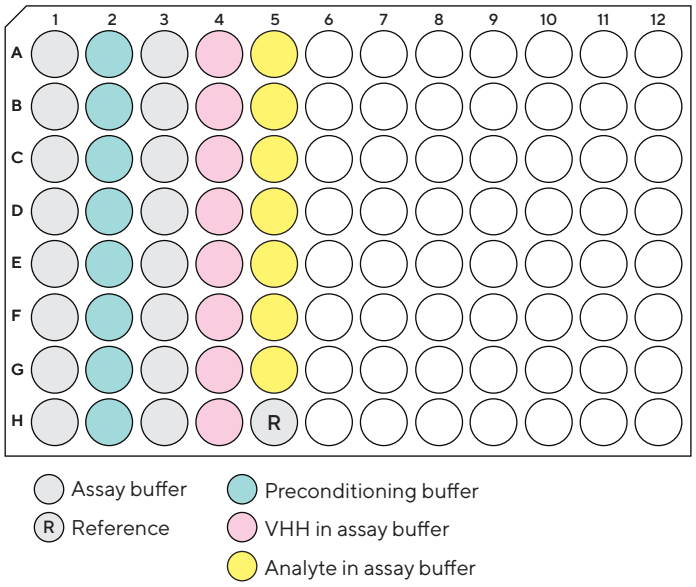


Figure 2. The Octet® VHH Biosensor kinetic assay workflow typically includes the following 7 steps: (1) Biosensor check. (2) Preconditioning for enhanced baseline stability. (3) Baseline 1 for assay drift check before ligand loading. (4) Loading (capture) of VHH antibody. (5) Baseline 2 for ligand stability check and assay drift check before analyte binding. (6) Association kinetics. (7) Dissociation kinetics.

Materials Required

- Octet® BLI instrument with Octet® BLI Discovery and Analysis Studio Software
- Octet® VHH Biosensors, Sartorius Part No. 18-5178 (Tray), 18-5179 (Pack), 18-5180 (Case)
- For all Octet® BLI instruments: 96-Well, Black, Flat Bottom Microplate, Sartorius Part No. 18-5172 (Pack), 18-5173 (Case)
- Optional for Octet® R8e, RH16 and RH96 instruments:
  - Octet® 384-Well, Black, Tilted Bottom Polypropylene Microplate, Sartorius Part No. 18-5166 (Pack), 18-5167 (Case)
  - 384-Well, Black, Flat Bottom, Polypropylene Microplate, Greiner Bio-One Part No. 781209
- **Assay buffer.** Octet® 1X Kinetics Buffer (1X KB) with 0.1% BSA is recommended. This buffer can be prepared by diluting the Octet® 10X Kinetics Buffer (Sartorius Part No. 18-1105) 10-fold with 1X PBS, pH 7.4 and adding 0.1% w/v BSA. Other buffers may also be used, provided they do not negatively affect the stability and activity of the immobilized VHH. For guidance on selecting the optimal assay buffer, please refer to the Best Practices for Kinetic Assays section.
- **Preconditioning buffer.** The optimal preconditioning buffer is the regeneration buffer for the model pair. If the regeneration buffer is not yet determined, then 10 mM glycine pH 1.7 can be used instead.
- **VHH antibody for immobilization.** The VHH antibody stock solution can be in a buffer solution or a complex mixture such as cell culture supernatant. Use the assay buffer to dilute and prepare the VHH antibody sample(s).
- **Analyte protein that interacts with the VHH antibody.** The analyte stock solution can also be in a buffer solution or a complex mixture such as cell culture supernatant. Use the assay buffer to dilute and prepare the analyte samples.
- For details on setting up a kinetic assay in the Octet® software, please refer to the Octet® BLI Discovery Software User Guide.[4] Figure 3 shows an example microplate layout and assay design for a kinetic characterization assay using the Octet® VHH Biosensors. For all steps, use a 200 µL sample volume for 96-well plates, 80–120 µL for standard 384-well flat-bottom plates, and 40–80 µL for tilted 384-well plates.

A.



B.

Step	Column	Description	Step Type	Time (Sec)	Shaking Speed (rpm)
Step 1	1	Biosensor Check in Assay Buffer	Custom/ Baseline	60	1000
Step 2	2	Preconditioning	Custom/ Regen	5	1000
Step 3	1	Preconditioning	Custom/ Neutralization	10	1000
Step 4	2	Preconditioning	Custom/ Regen	5	1000
Step 5	1	Preconditioning	Custom/ Neutralization	10	1000
Step 6	2	Preconditioning	Custom/ Regen	5	1000
Step 7	1	Preconditioning	Custom/ Neutralization	10	1000
Step 8	3	"Baseline 1" in Assay Buffer	Baseline	120	1000
Step 9	4	Loading of VHH in Assay Buffer	Loading	180–300	1000
Step 10	3	"Baseline 2" in Assay Buffer	Baseline	120	1000
Step 11	5	Association of Analyte in Assay Buffer	Association	180–300*	1000
Step 12	3	Dissociation of Analyte in Assay Buffer	Dissociation	180–300*	1000

Figure 3. (A) Sample plate map. (B) Assay steps with associated parameters for the VHH kinetic assay. *__NOTE:__ Association and dissociation time will be sample-dependent and should be empirically determined.

Kinetic Assay Procedure

Before the Assay

Warm up all reagents and samples to room temperature before biosensor hydration and assay setup. Pre-hydrate the VHH biosensors in 200 µL of assay buffer per well. Pre-hydration is performed in a 96-well, black, flat-bottom plate for a minimum of 10 minutes. Setup the assay procedures according to the plate map and assay steps shown in Figure 3, or a custom procedure.

Preconditioning of Pre-hydrated Biosensors

Add the regeneration buffer or 10 mM glycine pH 1.7 for the VHH into column 2 of the sample plate. Preconditioning is carried out after a quick biosensor check and typically involves three cycles of regeneration for 5 sec and neutralization for 5–10 sec.

Baseline-1

Add assay buffer to column 3 of the sample plate. This baseline step should be run for a long enough time (at least 120 seconds) to allow any observed baseline drift to stabilize.

Loading of VHH Antibody

Dilute the VHH antibody to the appropriate concentration in the assay buffer and add to column 4 of the sample plate. The concentration of ligand to use will depend on its affinity for the associating analyte, as well as the size of both the ligand and analyte. The typical immobilization concentration is 0.2–0.8 µg/mL for 300 sec and should be optimized for each interaction being studied.

For the best kinetic data and most accurate affinity constants, a loading optimization experiment should be performed to determine the optimal ligand loading concentration and time. When performing the loading step, slow loading for longer time ensures more uniform ligand immobilization. Ideally, the binding curve in the loading step will show a gradual increase in signal and should not be allowed to reach saturation. Use the lowest amounts of ligand loading, as well as analytes to minimize steric hindrance around the immobilized VHHs and ensure more effective binding.

Baseline-2

This baseline step is performed in the same buffer well(s) used for baseline-1 and should also be run for a long enough time (at least 120 seconds) to allow for the observation of diminished signal drift from residual ligand dissociation. See the Best Practices for Kinetic Assays section for additional information.

Association to Interacting Analyte
If detailed kinetic characterization is being performed, the analyte protein must be purified and of known concentration. It is recommended to run a titration series of at least five concentrations of the analyte protein and perform a global fitting of all concentrations to determine k_a, k_{dis} and K_D values. The highest analyte concentration should typically be 10 times greater than the expected K_D. For screening assays or qualitative interaction analysis, a single concentration of the interacting protein can be sufficient to characterize the binding. Analyte samples must be diluted in the same buffer used for the baseline and dissociation steps. Include a reference sample, consisting of assay buffer blank with no analyte present in this step, to enable subtraction of background baseline-2 drift. The association time will depend on the analyte sample and can range from about 30 to 300 seconds or longer.

Dissociation of Interacting Analyte
The dissociation step is performed in the same buffer well(s) used for the baseline steps 8 and 10. Using the same wells for the Baseline-2 and Dissociation steps enable the inter-step correction feature to be used in data analysis for more accurate curve fitting.

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

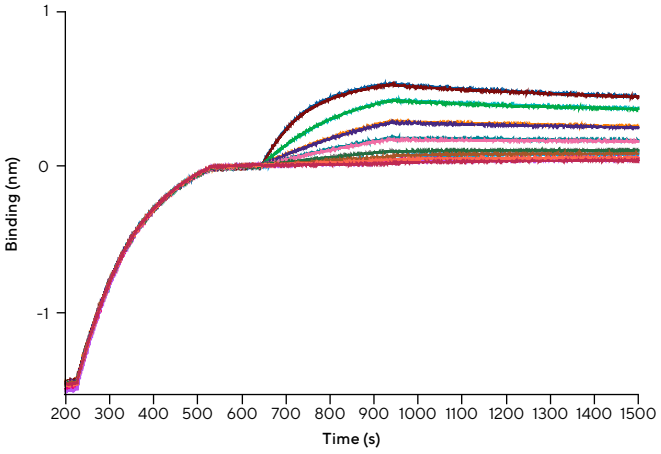
Best Practices for Kinetic Assays

- When using a capture-based biosensor such as the Octet® VHH Biosensor, some background level of dissociation of the captured VHH ligand from the biosensor will occur. This background dissociation (or baseline-2 drift in Figure 2), will be sample dependent, but can be mitigated by lowering the ligand concentration and using an optimized assay buffer.
- Because VHHs are small and sensitive to steric effects, both the ligand and analyte concentrations should be scouted in different buffers to determine the optimal assay conditions.
- Ligand loading optimization should take into account the ligand molecule's stability in different buffer conditions and should therefore include both optimal loading concentration and ligand stability in different buffer systems. For example, different assay buffers such as 1X KB + 0.1% BSA and 1X KB or other buffers of interest can be evaluated for their effects on the baseline-2 signals, while testing 3–4 ligand loading concentrations. The optimal ligand concentration and assay buffer will give baseline-2 signals in the range of ±0.03 nm over a 2-minute baseline run.
- Loading more ligand than what is needed may lead to uneven loading and cause artifacts such as non-specific binding, heterogeneity, or mass transport limitation. If not enough ligand is immobilized, however, the signal in the analyte association step may be too low to detect. For more details on optimization of ligand loading for kinetic assays, refer to the Application Note "Biomolecular Binding Kinetics Assays on the Octet® BLI Platform".[5]
- Any remaining baseline-2 drift can be corrected by subtracting from the reference sample in the association step. A reference sample is a biosensor that is dipped into the ligand solution during the loading step and is then dipped into buffer without analyte in the association step.
- The Baseline-2 and Dissociation steps should always be performed in the same well for each biosensor. This is critical, as it enables the inter-step correction feature to align the association and dissociation steps when processing data.
- For enhanced data quality, precondition the Octet® VHH Biosensors before ligand loading.

Representative Data

Figure 4 shows the Octet® VHH Biosensors loaded with anti-GFP VHH (Proteintech, Cat No. gt-250) at 0.4 µg/mL, followed by kinetic analysis of GFP (Proteintech, Cat No. Ag33633). The result of the kinetic analysis of this assay is summarized in Table 1.

A.



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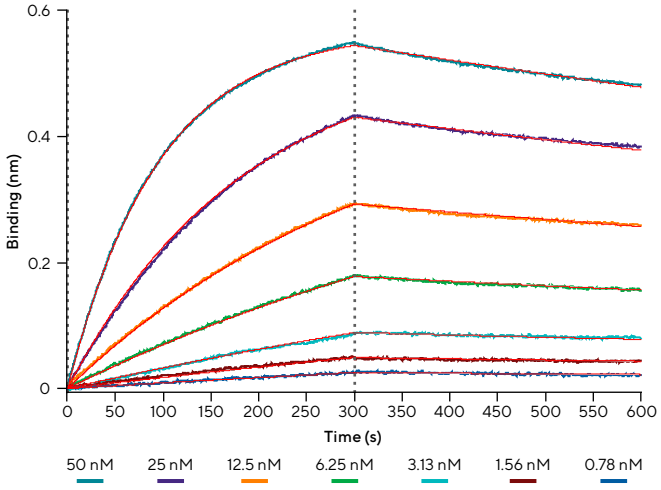

Figure 4. Binding kinetics of anti-GFP VHH and a GFP analyte on the Octet® R8 instrument. (A) The raw data for a full assay involving loading of anti-GFP VHH at 0.4 µg/mL, followed by kinetic analysis of the GFP analyte (MW 30.38 kDa). Octet® 1X KB + 0.1% BSA was used as the assay buffer, and 10 mM glycine pH 1.7 was used for preconditioning. (B) The association and dissociation curves after data processing (including reference subtraction using the 0 nM trace and fitting using the 1:1 binding model).

K_D (M)	k_a (1/Ms)	k_{dis} (1/s)	Full R^2
2.15E-09	2.01E-05	4.32E-04	0.9998

Table 1. Kinetic results (1:1 binding model, global fit) for the interaction between anti-GFP VHH and GFP using the Octet® VHH Biosensors for the data shown in Figure 4.

Quantitation Assay Workflow

The Octet® VHH Biosensors can be used to quantitate both crude and purified samples with concentrations in the range of 0.04–100 µg/mL depending on the assay conditions. For VHH samples with concentration range of 0.3–100 µg/mL, it is recommended to use a shaking speed of 400 rpm with a 1-minute assay time. However, if the VHH concentration is in the range of 0.04–1.25 µg/mL, it is recommended to use a 96-well plate as the sample plate and a shaking speed of 1000 rpm for 2 minutes for improved sensitivity

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Materials Required

- Octet® BLI instrument with Octet® BLI Discovery and Analysis Studio Software
- Octet® VHH Biosensors, Sartorius Part No. 18-5178 (Tray), 18-5179 (Pack), 18-5180 (Case)
- For all Octet® BLI instruments: 96-Well, Black, Flat Bottom Microplate, Sartorius Part No. 18-5172 (Pack), 18-5173 (Case)
- Optional for Octet® R8e, RH16 and RH96 instruments:
  - Octet® 384-Well, Black, Tilted Bottom Polypropylene Microplate, Sartorius Part No. 18-5166 (Pack), 18-5167 (Case)
  - 384-Well, Black, Flat Bottom, Polypropylene Microplate, Greiner Bio-One Part No. 781209
- Purified standard VHH antibody (that is of the same molecule as the unknown samples) to be used as a calibration standard.
- Octet® Sample Diluent (Sartorius Part No. 18-1104) for dilution of all samples. If undiluted crude samples are to be quantified, a blank buffer (that is free of the molecules of interest) with the same matrix is required.

Dilution Factor Determination for Sample Matrix Effect Mitigation

Components in complex matrices such as cell culture media can potentially interfere with assay performance. Diluting the sample matrix using the Octet® Sample Diluent is an effective means of minimizing matrix effects. Dilution factor guidelines for various sample types are described in Table 2. However, before running a quantitation assay it should be empirically determined whether dilution of samples is needed.

Sample Type	Minimum Recommended Dilution in Sample Diluent Buffer
VHH in Octet® Sample Diluent	Neat
VHH in CHO-SFM	1:3
VHH in DMEM	1:4
VHH in DMEM + 10% FBS	1:2
VHH in PBS	1:2
VHH in 2x YT	1:2

Table 2. Recommended minimum 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.

Assay Precision and Accuracy

Quantitation dynamic range may vary as a function of the sample matrix. To determine the quantitation range in any matrix, a precision and accuracy study should be carried out as follows:

1. Prepare a series of protein standards in the appropriate matrix diluent using the dilution factor determined in the Dilution Factor Determination for Sample Matrix experiment. The dilution series should span the entire range of the assay based upon the experimental goal, such as 0.3–100 µg/mL for assays run at 400 rpm.
2. Using the same matrix diluent as in Step 1, prepare two protein samples of known concentration for recovery 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. These will be defined as unknown samples in the assay for calculating recovery.
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 5.
4. Hydrate biosensors for 10 minutes in matching matrix diluent.
5. Set up a Basic Quantitation assay using the same assay parameters that were used in the Dilution Factor Determination for Sample Matrix experiment. Define sample Replicate Groups to calculate replicate averages and %CVs.
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 software.
8. If a 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, if necessary.
12. Select the appropriate equation to fit the standard curve. Start the fitting with a 4- or 5-parameter logistic model, although other models may be used with suitable validation. Use a 5-parameter logistic equation for better recovery results if there are lower concentration data points.
13. Evaluate the accuracy and precision of the assay using the calculated concentration value of the unknowns to determine %recovery and %CV.

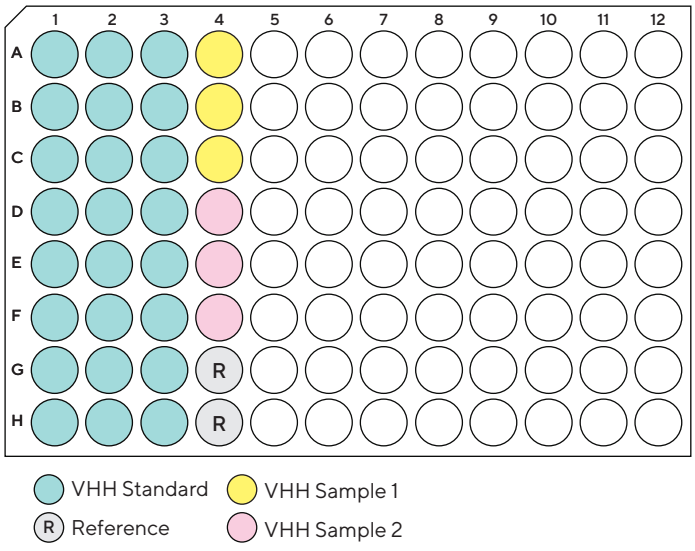

Figure 5. Example plate layout for a Spike Recovery Assay.

Running the Assay to Quantify Proteins of Interest

1. Prepare samples, calibration standards, and hydration solution according to the conditions determined in optimization steps in the prior sections.
2. Set up a Basic Quantitation assay using the parameters described previously in the optimization experiments. See Figure 6 for example assay set up.
3. Run the assay.
4. Load data into Octet® Analysis Studio software. Analyze as in previous optimization steps to determine concentration of samples and data statistics.
5. To export the analyzed data, click Save Report and select desired format, Excel or PDF.

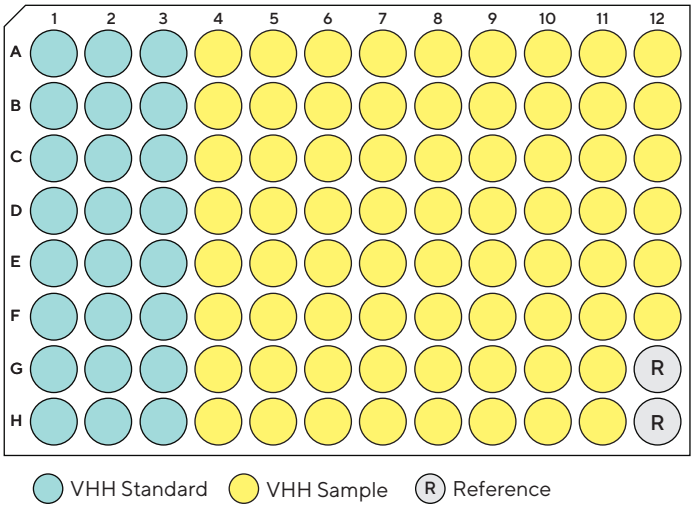

Figure 6. Example plate layout for a routine quantitation assay run in a 96-well microplate.

Best Practices for Quantitation Assays

The following optimization steps are recommended for the quantitation assays:

- The calibration standard should be identical to the molecule present in the unknown sample for best results.
- Concentrations of the calibration standards should bracket the expected range of concentrations in the unknown (test) samples.
- If expected test samples concentrations are >100 µg/mL, determine the minimal dilution factor required to achieve the targeted assay performance.
- Match the matrix of the test samples, standards, references, and hydration solution as closely as possible.
- Use a blank (zero-concentration) sample negative control in a matching matrix for background signal subtraction. This is especially important when optimizing accuracy and detecting low-concentration analytes.
- Perform a spike/recovery study to determine the assay dynamic range.

Representative Data

Figures 7 and 8 show the detection of VHH using the Octet® VHH Biosensors on the Octet® RH96 system. Two standard curves with two different assay settings were run to demonstrate the quantitation dynamic range of 0.04–100 µg/mL.

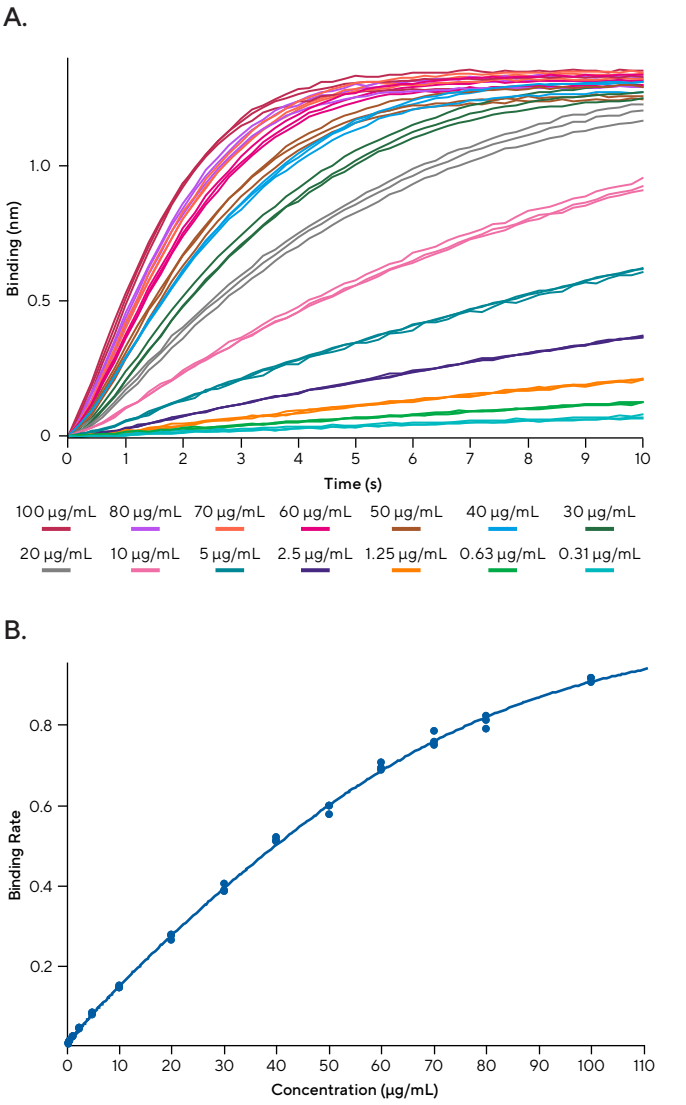

Figure 7. Quantitation of VHH using the Octet® VHH Biosensors. (A) VHH dose response for concentrations within the dynamic range of 0.3–100 µg/mL with three replicates on the Octet® RH96 instrument with assay parameters: 400 rpm shake speed and 1 min assay time, and 10 sec read time for data processing. (B) VHH standard calibration curve generated from three replicate samples and calculated using 5PL (Weighted Y) fitting model. The Octet® Sample Diluent Buffer was used as a matrix for all samples.

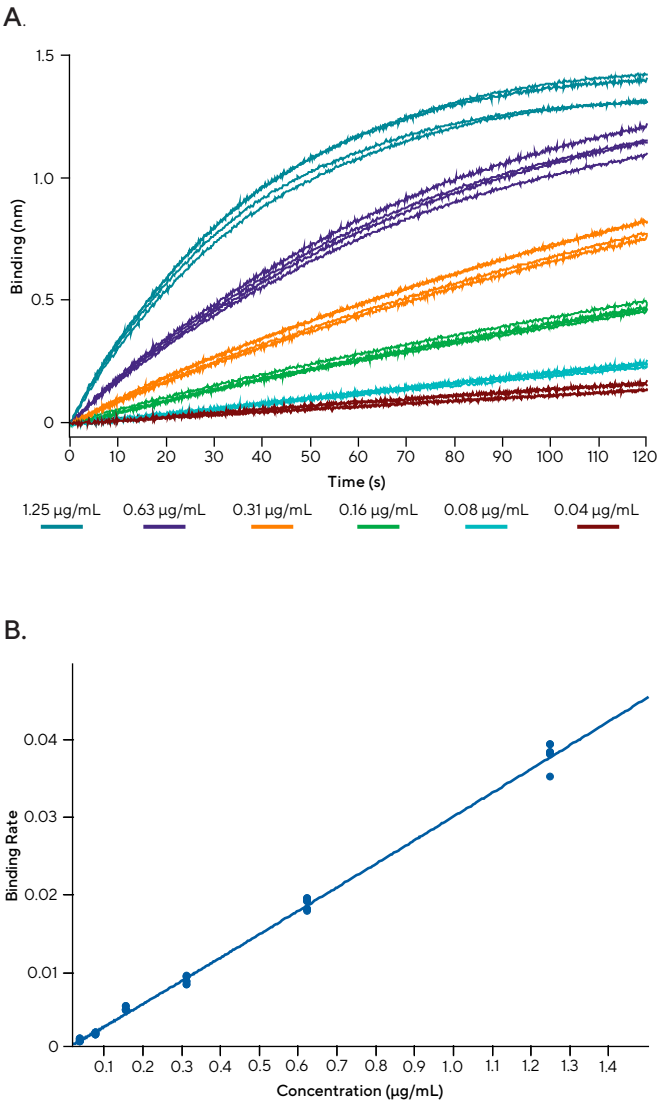

Figure 8. Quantitation of VHH using the Octet® VHH Biosensors. (A) VHH dose response for concentrations within the dynamic range of 0.04–1.25 µg/mL with three replicates on the Octet® RH96 instrument with assay parameters: 1000 rpm shake speed, 2-min assay time. (B) VHH standard calibration curve generated from three replicate samples and calculated using 5PL (Unweighted) fitting model. The Octet® Sample Diluent Buffer was used as a matrix for all samples.

Table 3 and 4 show the calculated concentrations, %CV among the replicates, and the %recovery for the concentrations. In order to calculate accurate data for a dynamic range, one more level of concentration is recommended to be added to the highest and lowest ends of the dynamic range. For example, to acquire accurate data for 100 µg/mL and 0.2 µg/mL, it is recommended to incorporate 200 µg/mL and 0.1 µg/mL at both ends of the dilution and standard curve.

Known Conc. (µg/mL)	Calculated Conc. (µg/mL)	%CV (n=3)	%Recovery
100	101.44	2%	101%
80	77.92	4%	97%
70	70.53	4%	101%
60	61.17	2%	102%
50	48.99	3%	98%
40	41.13	1%	103%
30	29.88	3%	100%
20	19.68	3%	98%
10	9.84	2%	98%
5	5.03	3%	101%
2.5	2.62	4%	105%
1.25	1.26	3%	101%
0.63	0.63	3%	101%
0.31	0.31	2%	97%

Table 3. Calculated concentrations, %CV and %Recovery for a VHH (0.3–100 µg/mL) quantitation assay with triplicates.

Known Conc. (µg/mL)	Calculated Conc. (µg/mL)	%CV (n=3)	%Recovery
1.25	1.25	5%	98%
0.63	0.63	3%	99%
0.31	0.31	4%	100%
0.16	0.18	6%	115%
0.08	0.07	5%	88%
0.04	0.04	14%	91%

Table 4. Calculated concentrations, %CV and %Recovery for a VHH (0.04–1.25 µg/mL) quantitation assay with triplicates.

Regeneration of Octet® VHH Biosensors

The Octet® VHH Biosensors can be cost-effectively regenerated and reused up to 10 times in both kinetic and quantitation assays for generating replicate data for ligand-analyte pairs, or for analyzing large numbers of samples in sequence. The regeneration is performed by dipping the biosensors into a solution of glycine for 5 seconds, followed by a dip in the assay buffer for 5–10 seconds. These regeneration steps should be repeated three times in sequence to fully remove bound VHH or the interaction complex. After regeneration, the biosensor can be immobilized with VHH for a new analysis. For best results, the concentration and pH of the glycine should be optimized for each assay since the biosensor regeneration performance is VHH sample-dependent. It is also recommended to precondition biosensors by running the regeneration protocol prior to loading the ligand for the first time.

Regeneration results will depend on the captured molecule and a small loss in binding capacity may occur after each regeneration cycle. The exact number of possible regenerations should be determined experimentally and will depend on assay precision requirements. For examples of kinetic and quantitation assays with 6 regeneration cycles, please refer to Figures 9 and 10, and Tables 5 and 6.

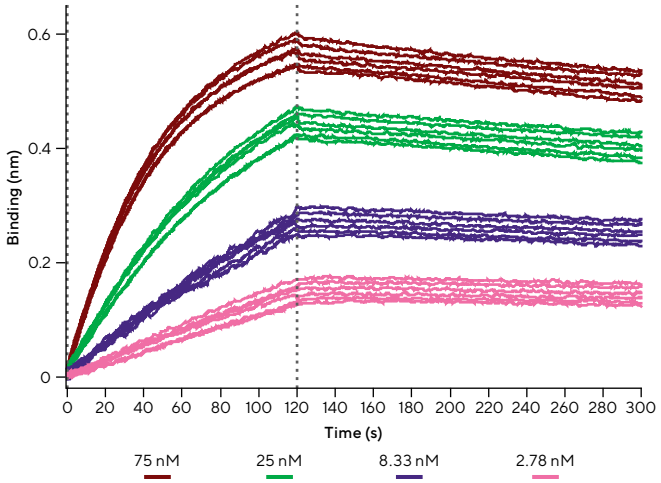

Figure 9. Overlay of association-dissociation curves for anti-GFPVHH-GFP kinetic assay after 6 regeneration cycles. The data traces overlap closely, with low variability between kinetic parameters from cycle to cycle for 6 regenerations.

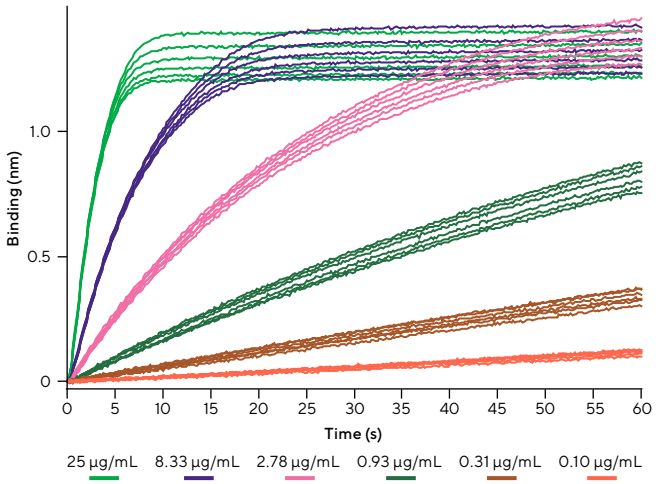

Figure 10. Overlay of binding curves for anti-GFP VHH quantitation assay for concentration range of 0.10–25 µg/mL after 6 regeneration cycles.

Cycle	K_D (M)	k_a (1/Ms)	k_{dis} (1/s)
Regen 1	2.78E-09	2.30E+05	6.40E-04
Regen 2	2.67E-09	2.16E+05	5.75E-04
Regen 3	2.53E-09	2.13E+05	5.39E-04
Regen 4	2.45E-09	2.24E+05	5.48E-04
Regen 5	2.57E-09	2.21E+05	5.68E-04
Regen 6	2.50E-09	2.18E+05	5.43E-04
Mean (6 regenerations)	2.58E-09	2.20E+05	5.69E-04
%CV (6 regenerations)	5%	3%	7%

Table 5. K_D, k_a, k_{dis} values and the corresponding %CVs for anti-GFP VHH-GFP binding assay over 6 cycles of regeneration using 50 mM glycine pH 1.9.

Known Well Concentration (µg/mL)	Average Calculated Concentration (6 Regenerations)	%CV (6 Regenerations)
25	25.03	2%
8.33	8.40	1%
2.78	3.07	6%
0.93	1.00	11%
0.31	0.33	11%
0.10	0.11	10%

Table 6. Calculated concentrations and %CV for 6 cycles of regeneration for anti-GFP VHH quantitation assay.

Regeneration Tips

- Regeneration buffer should be scouted to determine the optimal conditions for effective and safe removal of the protein ligand.
- It is recommended to precondition the biosensors before the first assay cycle for most consistent results when incorporating regeneration.
- It is important to ensure that the regeneration of biosensors for quantitation applications is complete. This is because the quantitation results are significantly dependent on the surface capacity of the biosensor. For example, a loss of 20% capacity over multiple regeneration cycles could affect precision of quantitation by 10–20%.

References

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