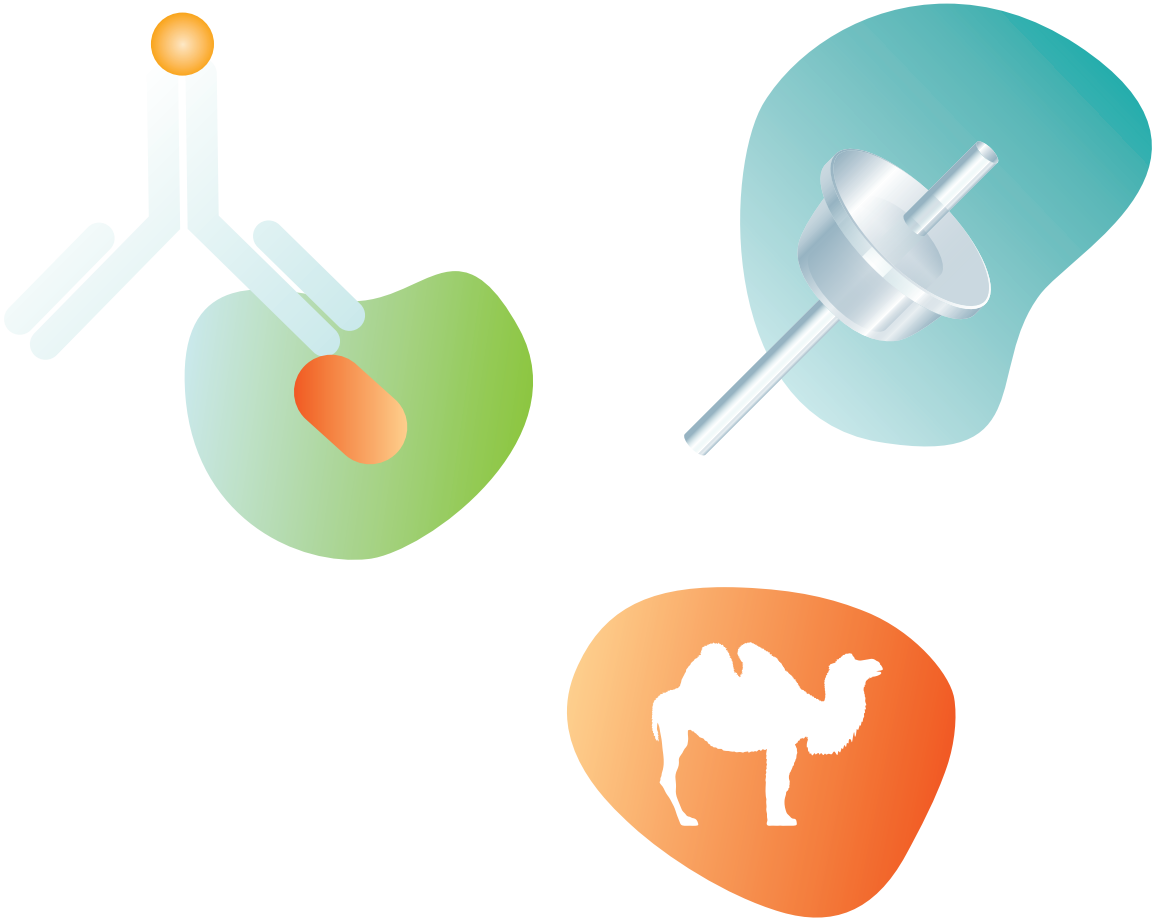
gator

Application Note



Gator® Anti-VHH Biosensors for Quantitation and Off-Rate Screening of Camelid VHH

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1. Introduction

The Gator® Anti-VHH biosensors represent a cutting-edge technology in the realm of label-free binding, specifically designed for the quantitation and off-rate screening of the variable domain of the heavy chain of the antibody (VHH), or the variable domain of the heavy chain antibody, derived from various camelid species.

This application note describes in detail how this biosensor and biolayer interferometry can be effectively used in biotherapeutics development and VHH research.

1.1 Overview of Camelid Nanobodies (VHH)

Camelid nanobodies also known as VHH or single-domain antibodies are a unique class of antibody fragments derived from the immune systems of camelids, which include camels, llamas, and alpacas. These nanobodies are gaining attention in biotechnology and medicine due to their distinctive structural and functional properties. Characterized by their small size, typically around 15 kDa, camelid nanobodies are significantly smaller than conventional antibodies. They consist of a single monomeric variable domain, known as VHH or nanobody, responsible for antigen recognition. This compact structure allows them to access and bind to hidden or recessed epitopes on target proteins that may be challenging for larger antibodies to reach.

VHHs are known for their stability, retaining their structural integrity under harsh conditions such as extreme pH, high temperatures, and exposure to proteolytic enzymes. This property makes them suitable for various applications, including diagnostics and therapeutic interventions.

Camelid VHHs exhibit high specificity and affinity for their target antigens reducing the likelihood of off-target effects and enhancing therapeutic efficacy. Nanobodies can be efficiently produced in microbial systems, such as bacteria or yeast, due to their smaller size and simplified structure. This makes their production cost-effective and scalable. The compact size of nanobodies facilitates better tissue penetration, enabling them to reach and bind to targets within solid tissues more effectively than larger antibodies.

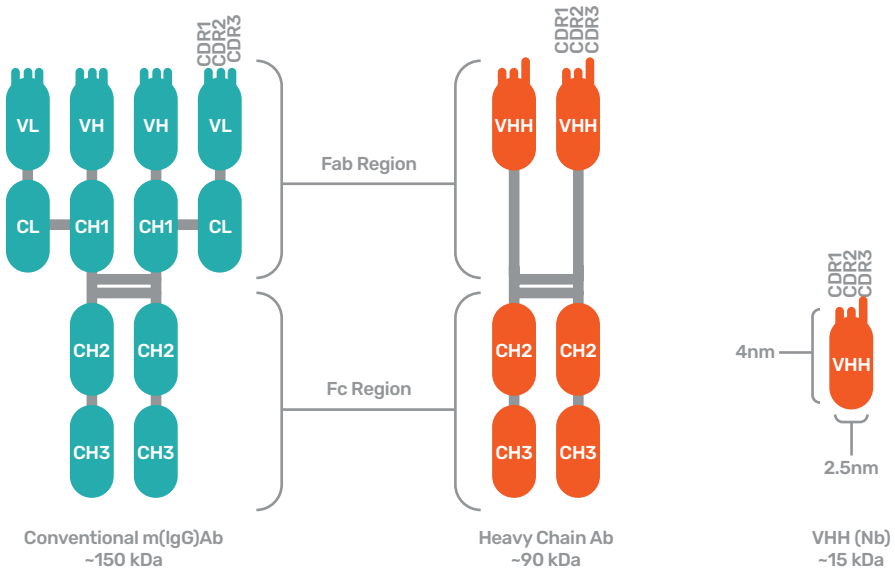

Figure 1: Illustration of the structural comparison among IgG, VHH-Fc, and VHH.

	VHH (Single-Domain Antibodies; nanobodies)	IgG (Immunoglobulin G)
Structure	Single, compact domain	Tetrameric structure with two identical heavy and two identical light chains
Size	Smaller size (~15 kDa)	Larger size (~150 kDa)
Production	Easily produced in microbial systems	Typically produced in mammalian cell cultures
Expression System	E. coli, yeast, plants, or mammalian cells	Mammalian cells (CHO cells, HEK cells) or hybridomas
Immunogenicity	Lower immunogenicity due to humanized or camelid origin	Higher immunogenicity, potential for immune response
Applications	Diagnostic assays, imaging, therapy	Therapy, diagnostics, research, in vivo imaging

Table 1: Comparison and Contrast of IgG and VHH

1.2 Significance of Gator® Anti-VHH Biosensors

Gator® Anti-VHH Biosensors are specifically engineered for the purpose of quantifying VHH nanobodies originating from camelid species. These biosensors employ the Biolayer Interferometry (BLI) technique to detect binding events in real time (as illustrated in figure 2).

Higher concentrations of VHH lead to both faster binding rates and larger signal amplitudes on the biosensor. This correlation between concentration and binding behavior is crucial for accurate quantitation. The determination of unknown VHH concentrations involves comparing the binding rate of the unknown to a standard curve constructed using samples with known concentrations, providing a reference for accurately quantifying VHH levels in unknown samples. In summary, Gator® Anti-VHH Biosensors leverage optical analytical technology, anti-VHH antibodies, and Biolayer Interferometry for real-time monitoring and quantification of VHH, offering a powerful tool for applications in research.

Anti-VHH are one-of-a-kind biosensors which offers quick and simple method for nanobody detection and off-rate ranking in a precise manner.

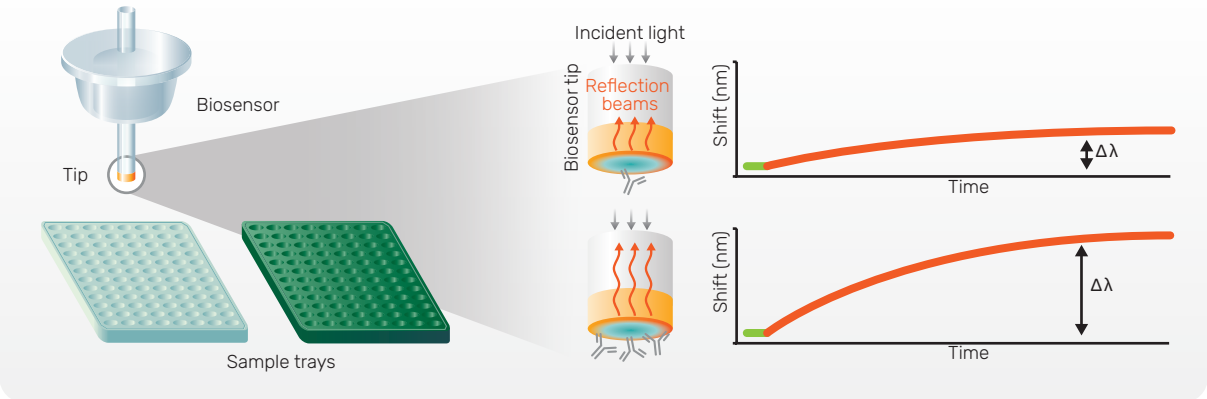

Figure 2: BLI Principle

2. Product Information: Key Features of Gator® Anti-VHH Probes

2.1 Materials and Methods

Materials Required	Storage Conditions
- Anti-VHH Probe, PN: 160032 - Quantitation (Q) buffer, PN: 120010 - Kinetics (K) buffer, PN: 120011 - Regen buffer (No Salt), PN: 120063 - Gator Max Plate, PN: 130062 - Gator BLI 96-Flat Plate, PN: 130260 - Precision Pipettes, User Supplied - Sterile Pipette Tips, User Supplied	**- Store the tray in its foil packaging pouch** **- Probes are stable at RT for 1 year** **- Buffers should be stored at 4° C.**

Table 2: Key Features of Anti-VHH Probes from Gator

3. Assay Protocol

3.1 Sample Preparation Protocol

Minimum Volume
Use 200 µL per well for a 96-well microplate (applicable to all Gator® BLI systems). Use 80 µL per well for a 384-well microplate (applicable to all Gator® BLI systems, except the Prime and Pilot). Use 40 µL per well for a 384-well, tilted-bottom microplate (applicable to all Gator® BLI system, except the Prime & Pilot).

3.2 Plate Preparation Protocol

Pipette standards, and samples into a black polypropylene microplate, adhering to the specified volume guidelines.

3.3 Biosensor Hydration

Pipette 250 µL biosensor hydration solution (same buffer as sample buffer) into the wells of a Max Plate (Gator Max Plate, PN: 130062). Match the number and position of the biosensors intended for use. The anti-VHH biosensors should be positioned on the Max Plate properly.

3.4 Instrument Setup

- Open the Assay set-up in the software and select the “Q assay” or “K assay” option.
- Place the Max Plate with the anti-VHH biosensors in the plate position in the Gator® BLI system.
- Place the sample plate in the Gator® BLI system.
- Warm the sample plate in the instrument.
- Hydrate biosensors for a minimum of 10 minutes at 1000 rpm before starting the experiment.

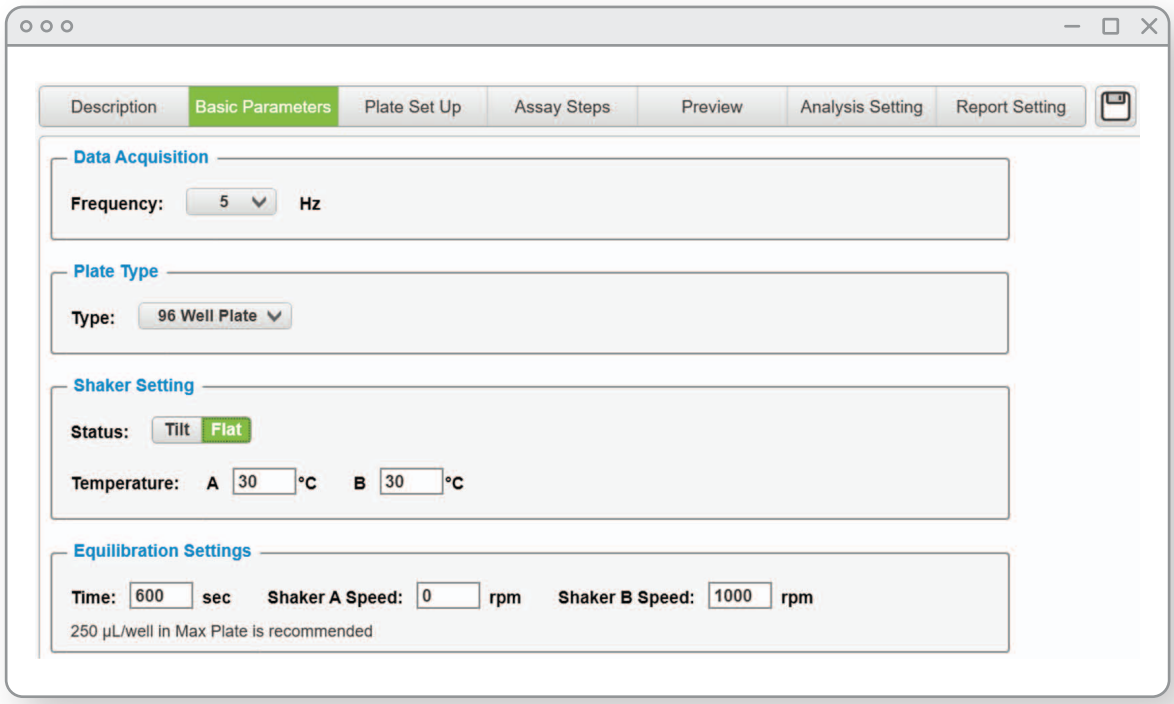

Figure 3: Establishing Fundamental Parameters for Instrument Setup

3.5 Assay Configuration

Below is an example of Quantitation Assay (Q Assay) configuration

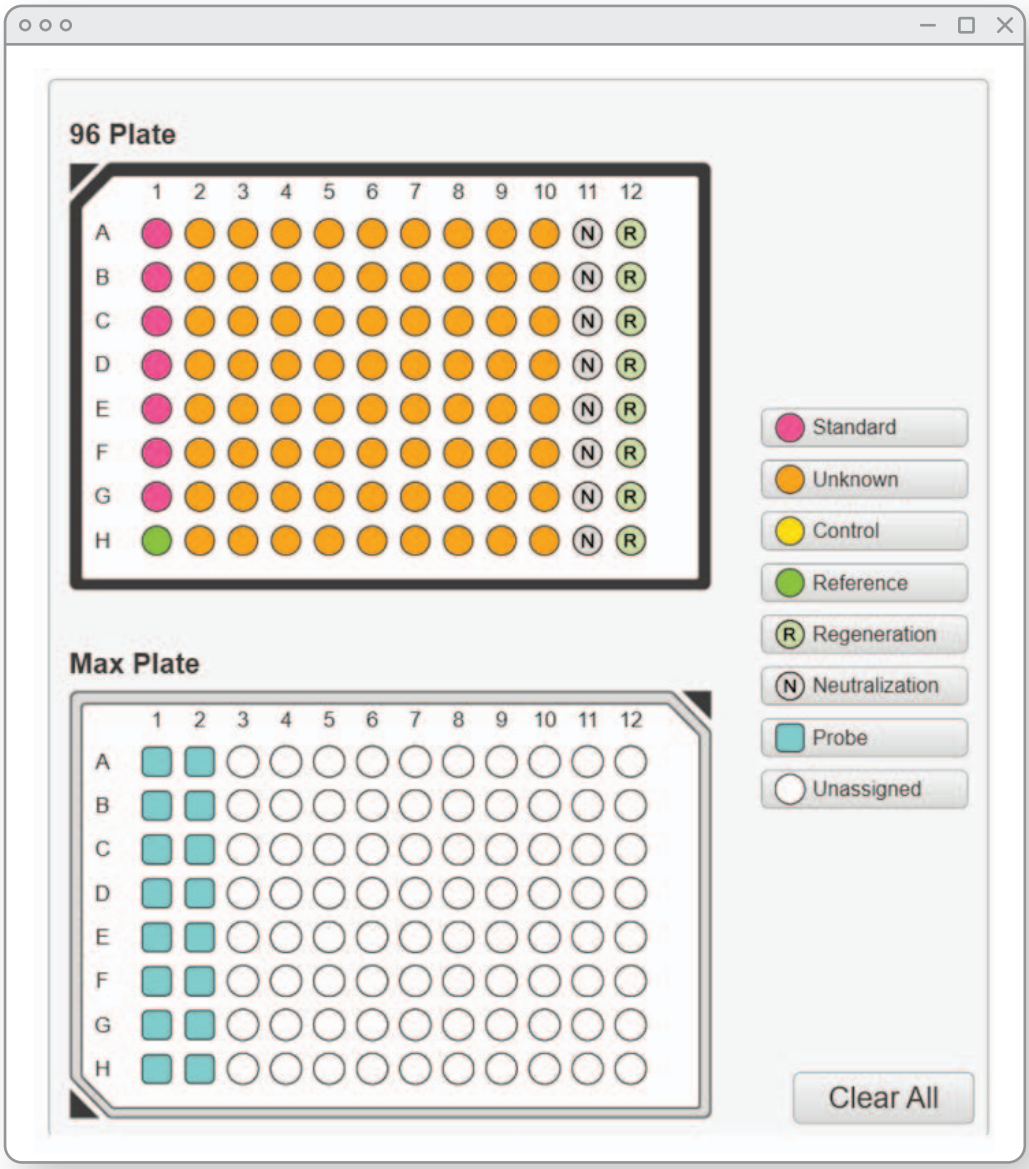

Figure 4: Example of a Q Assay set-up using the Gator software.

- Establish a Basic Q assay based on the experimental requirements.
- Use Figure 4 as a reference for an example plate map.

3.6 Assay Dynamic Range

Concentration Range	RPM (Revolutions per Minute)	Read Time (Seconds)
0.15 µg/mL - 10 µg/mL	200	120
0.03 µg/mL - 3 µg/mL	1000	300

Table 3: Dynamic Range of anti-VHH biosensors with assay parameters to obtain best data

3.7 Data Analysis

- Open the Results & Analysis section in GatorOne software to view the data.
- Reference Subtraction: If a blank matrix was included as a reference, use the reference subtraction option to correct the data appropriately.
- Binding Fitting: In this step it is recommended to select "initial optimal slope" for the binding rate determination. For the best fitting, set the "Optimal" setting to 2 seconds. Adjust the low concentration threshold to 0.001, as recommended.
- Select the appropriate Standard Curve Equation for optimal fitting.

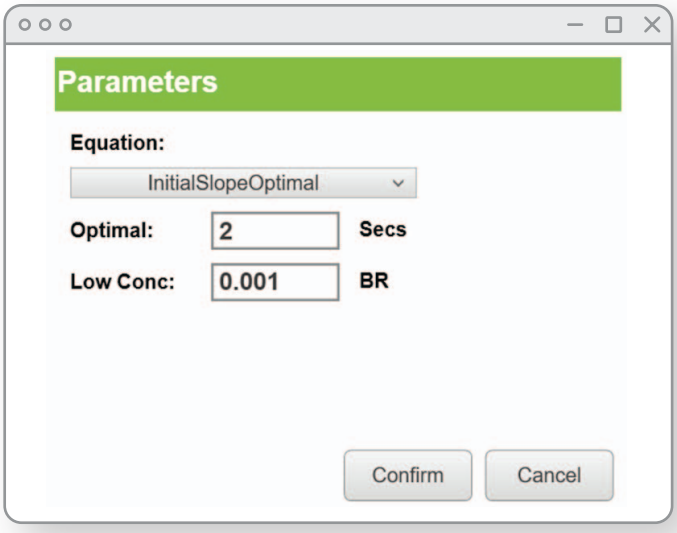

Figure 5: Snapshot of binding rate optimal fitting from the Gator software.

- Export analyzed data.
- Utilize the Save Report button within the software to generate a Microsoft Excel report containing the analyzed data.

4. Representative Results

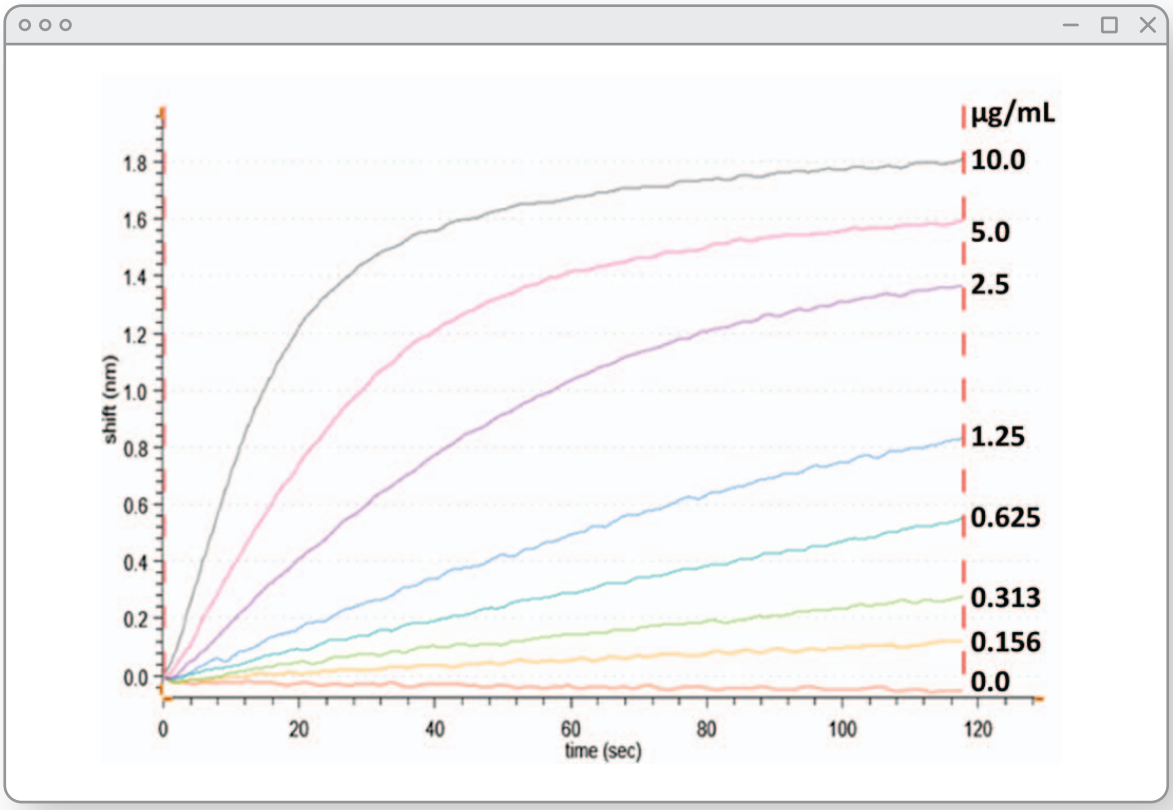

Figure 6: Binding curves were generated for Anti-RBD VHH with Anti-VHH probes in Q buffer, covering a concentration range from 0.16 µg/mL to 10 µg/mL, at a rotational speed of 200 rpm for 120 seconds

Concentration (µg/mL)	Binding Rate	Binding Rate %CV (n=4)	Calculated Conc. (µg/mL)	Calculated Conc. %CV (n=4)
10	0.112	0.85%	9.993	0.13%
5	0.0551	1.85%	4.995	2.59%
2.5	0.0272	2.38%	2.505	1.04%
1.25	0.0119	0.98%	1.268	1.41%
0.625	0.00438	1.59%	0.572	1.63%
0.313	0.00253	4.12%	0.365	1.95%
0.156	0.00115	1.80%	0.160	5.00%

Table 4: Accuracy and precision of VHH quantitation using the standard protocol. Concentrations were calculated using the initial binding rate. A total of 4 biosensors each concentration was measured and the CV% is shown in the table.

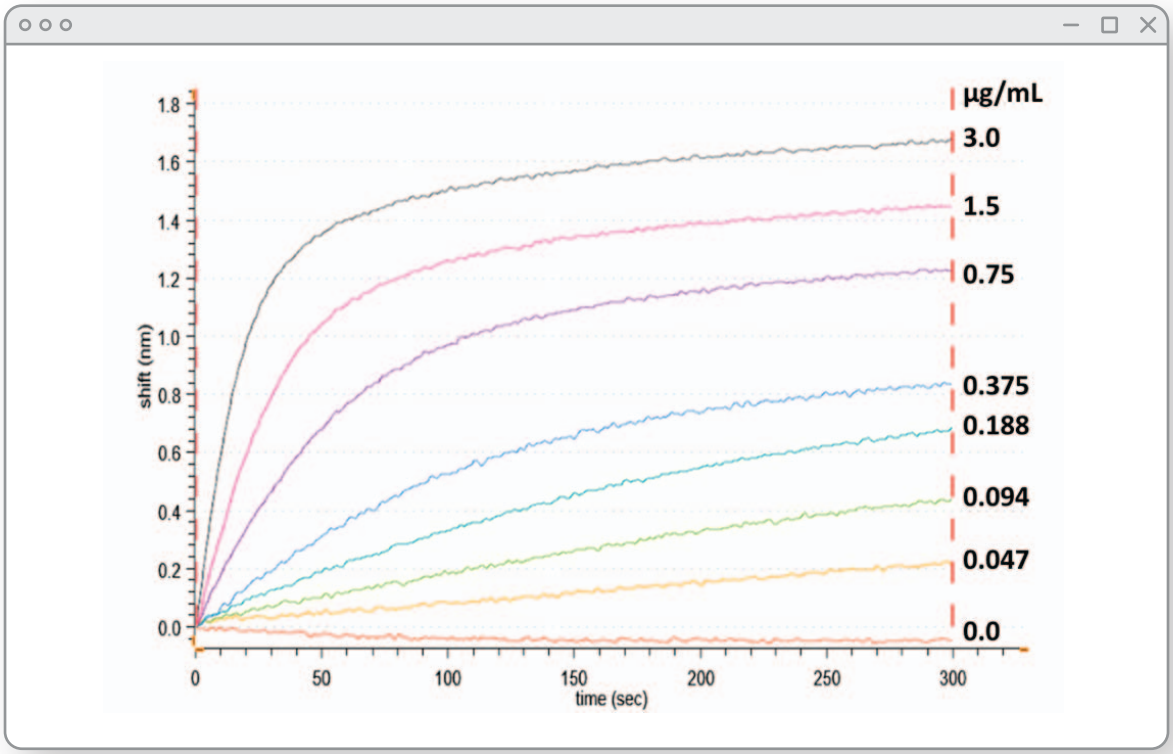

Figure 7: Binding curves generated for Anti-RBD VHH with Anti-VHH probes in Q buffer, from 0.05 µg/mL to 3 µg/mL, at 1000 rpm for 300 seconds.

Concentration (µg/mL)	Binding Rate	Binding Rate %CV (n=4)	Calculated Conc. (µg/mL)	Calculated Conc. %CV (n=4)
3	0.0804	1.03%	3.035	1.11%
1.5	0.0378	2.61%	1.463	3.22%
0.75	0.0189	3.79%	0.763	3.13%
0.375	0.0085	1.68%	0.404	2.92%
0.188	0.00206	3.09%	0.174	4.48%
0.094	0.0014	9.63%	0.098	9.20%
0.047	0.000786	12.11%	0.050	11.22%

Table 5: Accuracy and precision of VHH quantitation using the 1000 rpm protocol. Concentrations calculated using the initial binding rate.

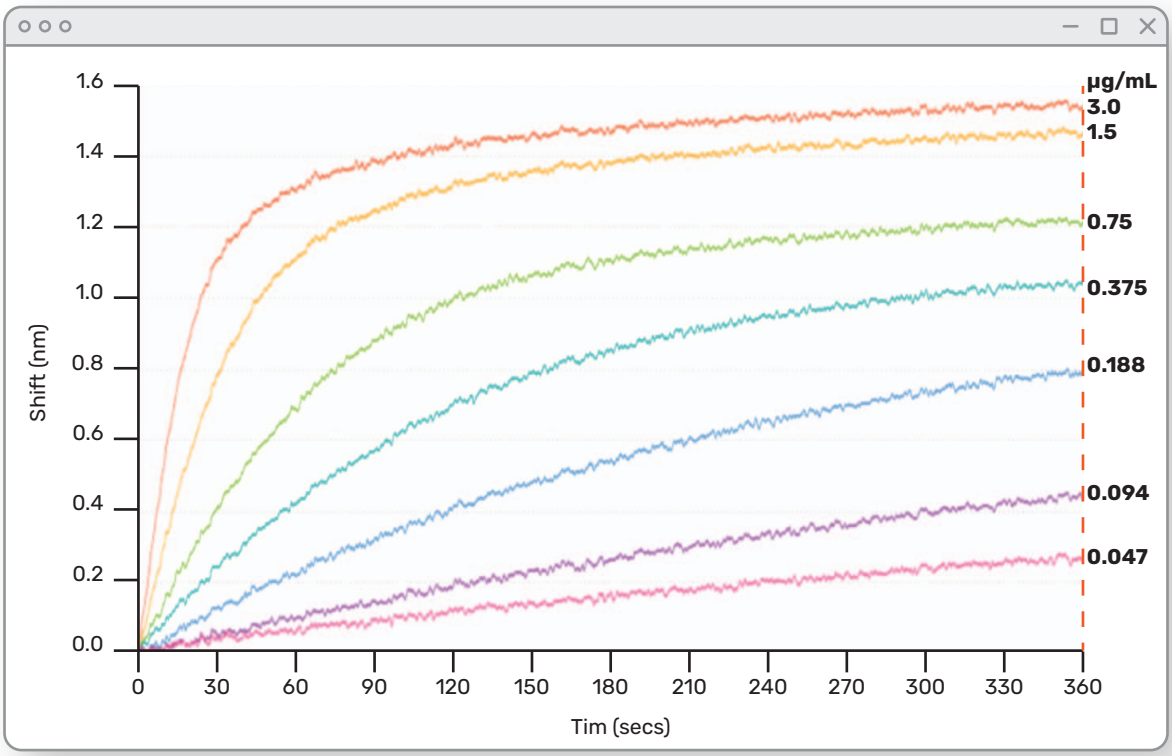

Figure 8: Binding curves for 0.05 to 3 µg/mL VHH in spent DMEM media diluted 100 x in Q buffer after reference subtraction. Comparable results were seen with spent 2x YT, spent CHO media and spent Expi293F media.

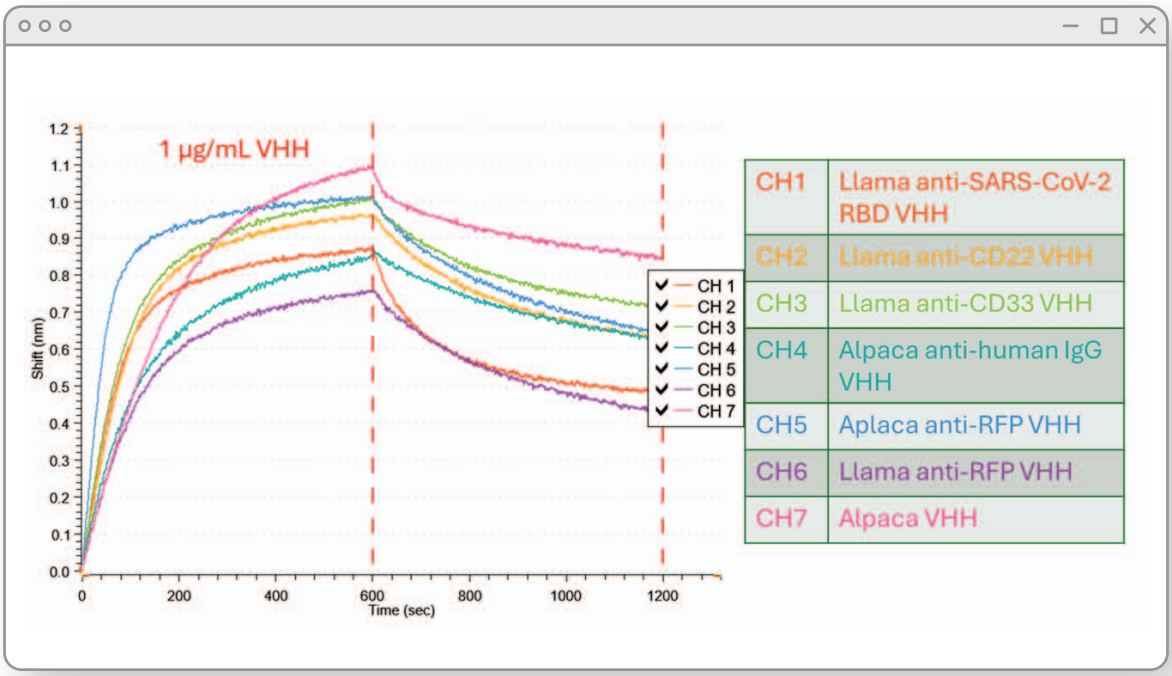

Figure 9: Illustration of binding of different camelid species to Anti-VHH Biosensor

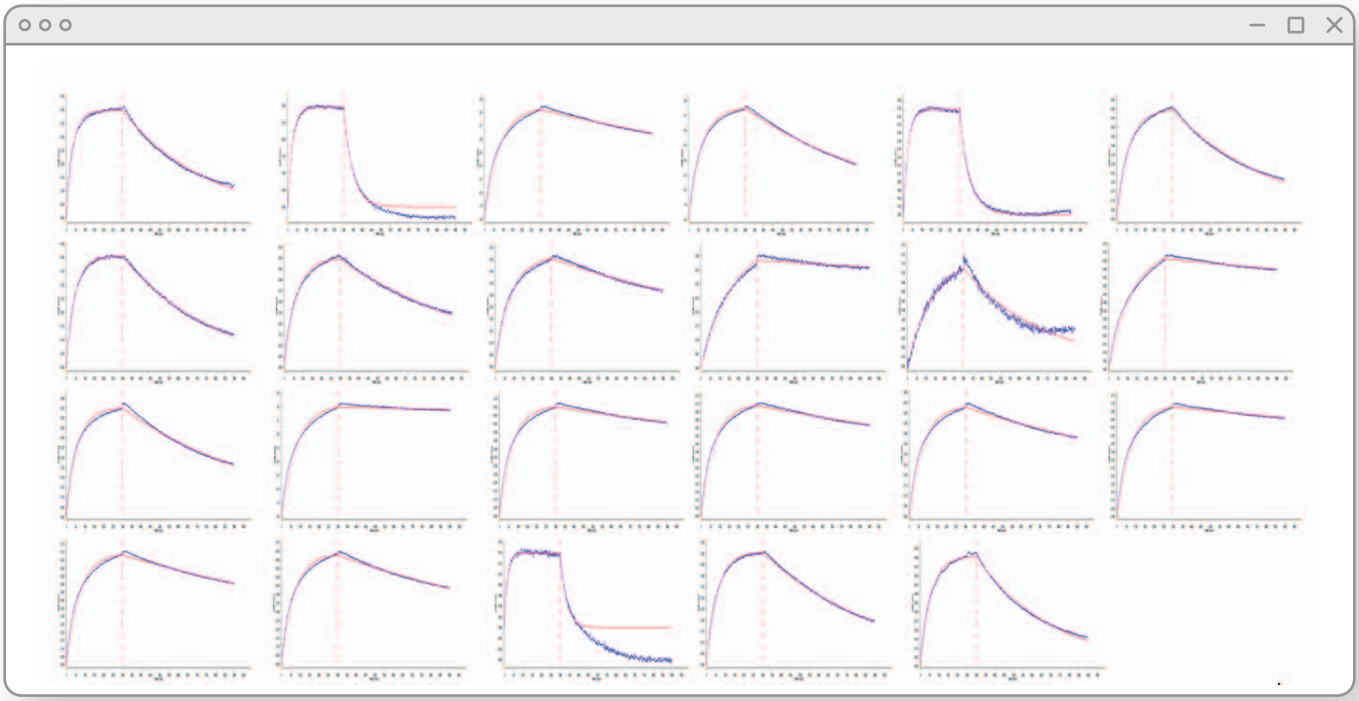

Figure 10: Illustrates the binding curves representing the association and dissociation of CD45 on 23 distinct VHHs captured on the anti-VHH biosensor surface.

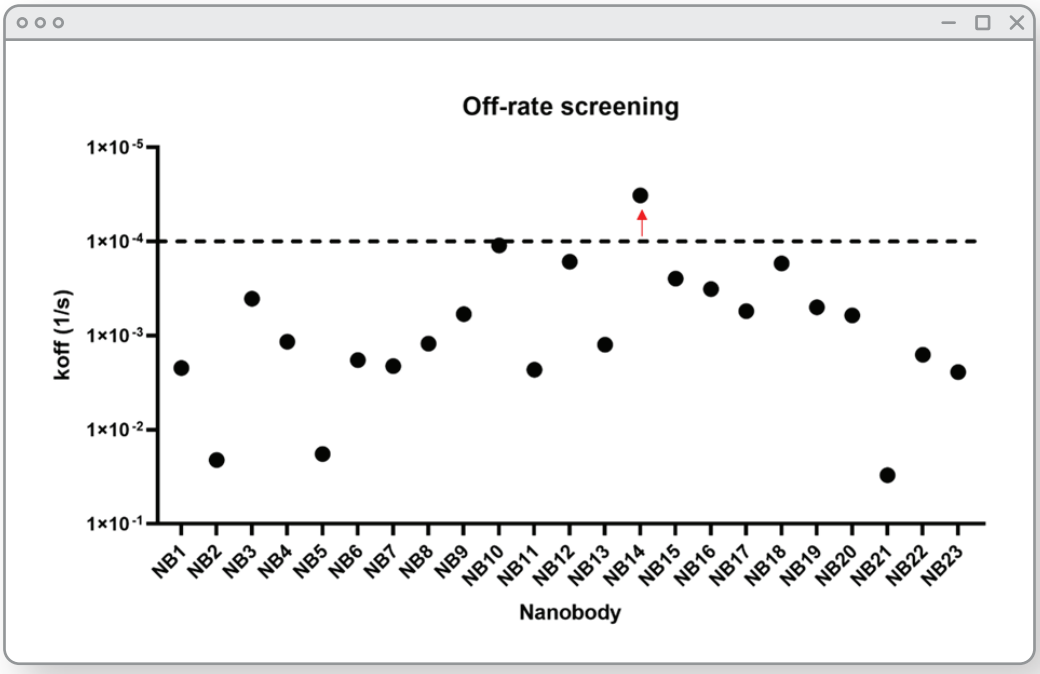

Figure 11: Illustrates the-off rate of CD45 antigen on 23 distinct VHHs captured on anti-VHH biosensor surface.

5 Strategies for Effective Assay Optimization

5.1 Quantitation Assay

Adjusting Assay Time for Low Sample Concentrations:

If the sample concentration is very low-specifically, less than 1 µg/mL- consider prolonging the Q assay time. Longer assay times provide increased sensitivity, allowing for better detection and quantification of low-concentration samples. Evaluate the impact of extended assay times on signal-to-noise ratio and assay precision, ensuring that the extended time enhances the assay's ability to detect and quantify low concentrations without compromising data quality.

Regeneration before assay:

Regenerate the probe before each assay to maintain run-to-run consistency. Failure to do so may result in a lower signal during the first run compared to subsequent runs.

Mitigating Lag Phase Effects in High-Concentration Samples:

Pre-data analysis truncation of initial 2 seconds.

5.2 Off-rate Ranking Assay

Sometimes the binding between the anti-VHH antibody and VHH is not characterized by high affinity, resulting in VHH frequently disassociating from the probe, for this reason the anti-VHH biosensors are not recommended for kinetic characterization. However, if the probe is used for off-rate screening and kinetic characterization, it is advisable to extend the baseline duration to more than 10 minutes after loading VHH to ensure thorough evaluation of the interaction dynamics.

- If an upward baseline drift is observed, consider incorporating one or two additional consecutive baseline steps after the initial baseline. This adjustment aids in stabilizing the probe surface.

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5.3 Common Issues and Solutions

Issue	Questions/Issues	Suggested Solutions
Q assay	Does the VHH probe bind to all types of nanobodies?	The VHH probe shows binding activity to all types of camelids nanobodies, llama, alpaca etc
	Samples show low binding	For quantitation, use the long protocol with 1000rpm
	Lag phase with high concentration of samples	In the analysis, remove the first 2 seconds of the assay prior to curve fitting
	What fitting model should be used for Q assay analysis?	InitialSlopeOptimal fitting equation
	Observing non-specific binding with the reference/media	Pre-wet the biosensors with the reference buffer for 15 minutes
	Cross-reactivity with other molecules	Anti-VHH probes only show mild cross-reactivity to hIgG1, IgG3 and IgG4
	Sensor regeneration before assay	Anti-VHH probes need to be normalized by regenerating before assay
	Sensor regeneration issues	Develop efficient regeneration protocols to maintain assay sensitivity over multiple cycles. We claim 10 regenerations with purified VHH samples.
	Using undiluted media	The Anti- VHH probes shows strong NSB to any undiluted media, it is suggested to dilute the media in PBST, Q or K buffer
	What type of buffer is ideal for the experiment?	Any buffer that has surfactant or surfactant with blocking agent together is needed for the experiments. The VHH tends to adhere to the plate wells if the buffer is not optimal.

Issue	Questions/Issues	Suggested Solutions
K assay	Can I use the Anti-VHH probes for K assays?	Yes
	After loading, VHH dissociation in the following step is strong. What should I do?	For all kinetic experiments, we recommend the wash step after loading the VHH should be a minimum of 10 minutes or optimized to your specific VHH
	I do not see any binding to my analyte/antigen after loading the VHH?	VHH is a 15 kDa small fragment, sometimes the antigen binding epitopes are hidden once immobilized. We would recommend reversing the assay format and immobilizing the antigen using appropriate biosensor.
	I see a lot of non-specific binding with the analyte binding to the Anti-VHH probes without loading	Please use a buffer which has surfactant and BSA, such as Q buffer. Also, pre-wet for a longer duration.
	I am using PBS as the running buffer, and I see VHH binding signal going down across regenerations	The 96 or 384 well plates need surfactant and BSA to block the surface. If you do not have these reagents present, the VHH will slowly start adhering to the plate surfaces.
	VHH have different off-rates after loading. Why is this?	The binding affinities for VHH varies to the anti-VHH antibodies on the probe.

Key Features	Details
Application	Quantitation/Kinetics (off-rate ranking)
Sample Types	Purified samples / Diluted Crude samples
Sensitivity for Q	0.05 µg/mL
Dynamic Range for Q	0.05-10 µg/mL (3.33-666.67 nM)
Precision	Tray to tray and within tray CV < 10%
Regeneration	At least 10 times
Regeneration Buffer	Regeneration Buffer (No Salt); pH 1.7 (PN: 120063)
Ease of Use	Ready to use Biosensors
Cross Reactivity	No cross-reactivity with mouse, rat, rabbit, goat IgG and human IgG2

Table 6: Key Features of Gator® Anti-VHH Probes

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