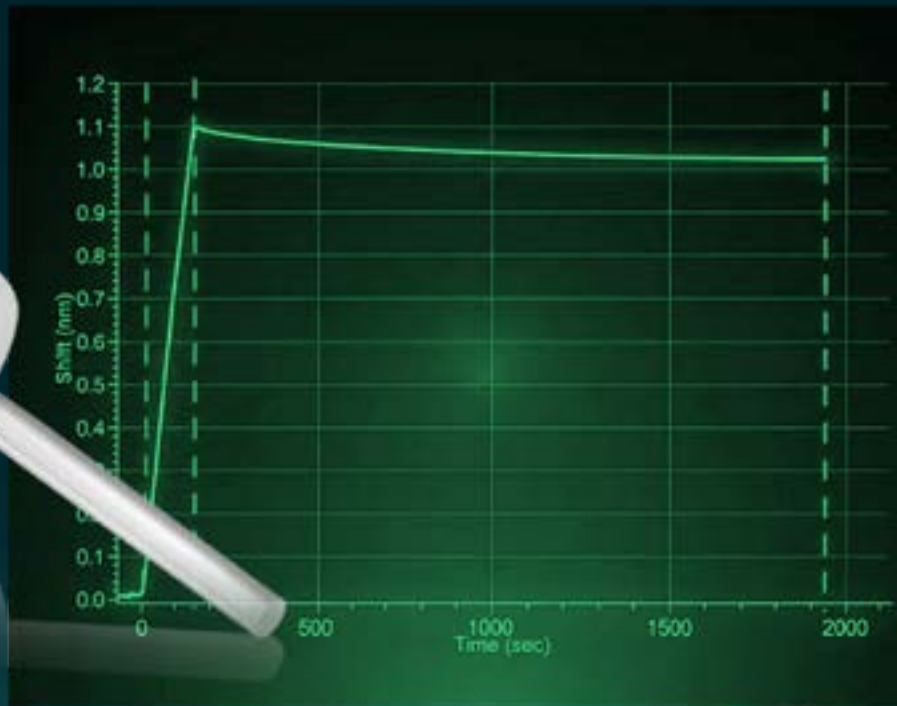




# HIS XT Probe: Advanced His-Tag Capture for Kinetics, Quantitation, and Epitope Binning



# Abstract

The Gator® Anti-His (HIS) XT Probe is a first-of-its-kind, next-generation label-free biosensor for rapid quantitation and kinetics of His-tagged proteins. It features an AI-designed anti-His NovoBody™ derived from Monod Bio's de novo protein design platform to enable specific poly-His capture with far greater capture half-life. The HIS XT Probe also features an advanced optical design that provides enhanced sensitivity to achieve high-magnitude signals even with smaller analytes. Combination of this NovoBody and Gator Bio's innovative sensor design provides high capture efficiency, improved baseline stability, superior resolution for kinetic assays and minimal consumption of valuable reagents. We demonstrate the sensor's utility in automated workflows, including repeat quantitation and epitope binning, highlighting its ability to maintain performance over multiple regeneration cycles.

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## Section 1: Introduction to HIS XT Probe

The polyhistidine tag (His-tag) is one of the most widely used peptide tags in recombinant protein expression, enabling efficient purification, detection, and characterization of target proteins throughout research and development. Label-free bioanalytical techniques such as Bio-Layer Interferometry (BLI) have expanded the utility of His-tags by allowing direct, real-time quantitation and kinetic analysis of tagged proteins. Conventional anti-His capture chemistries, including antibody-based and metal chelation approaches, have demonstrated utility but can be limited by susceptibility to chelating agents, nonspecific interactions, or batch-to-batch variability.

The Gator® HIS XT probe addresses these limitations through an AI-designed anti-His NovoBody™ capture ligand engineered specifically for bio-layer interferometry platforms. This application note demonstrates how HIS XT's industry-leading affinity and low drift characteristics enable reliable kinetics measurements from picomolar to micromolar affinities, a range previously inaccessible with traditional anti-His probes. The probe's enhanced binding kinetics reduce sample consumption by 5-fold, translating to approximately \$768 in reagent savings per 96-well tray when working with typical cytokine proteins. Additionally, HIS XT provides a 2.5-fold expanded dynamic capture range of 0.2-1000 µg/mL, offering greater flexibility for quantitation and epitope binning workflows.

Key performance improvements include:

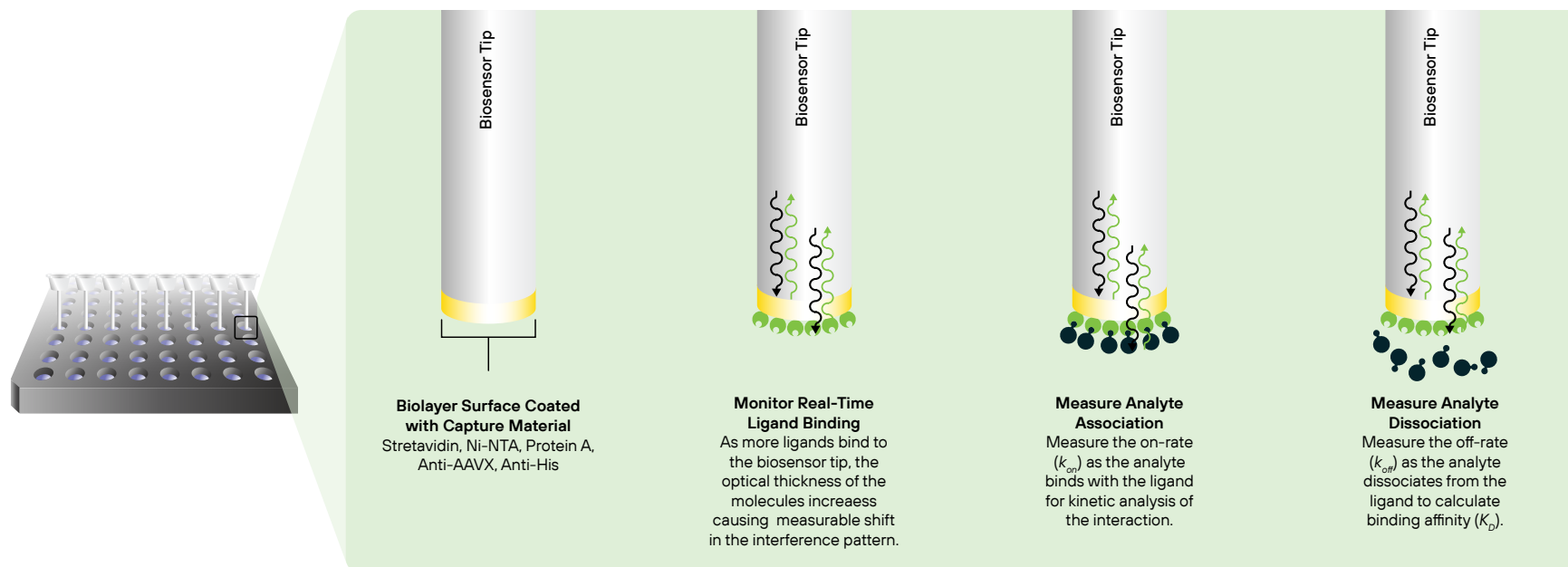
- Superior kinetics characterization with  $\text{Chi}^2 < 1.0$  across four logs of affinity
- Minimal signal drift enabling accurate measurement of picomolar affinity interactions
- 3-5x higher ligand loading capacity for enhanced resolution of small proteins and weak interactions
- 10x faster capture times reducing overall assay duration by 30-50%

The following sections detail experimental protocols and performance data demonstrating HIS XT's capabilities across representative therapeutic protein targets.

## Section 2: Basics of BLI-based detection of His-tagged reagents

Bio-Layer Interferometry (BLI) is a label-free, real-time method for measuring biomolecular interactions by detecting changes in optical thickness at the surface of a disposable probe tip. White light reflects from internal layers in the sensor. When biomolecules bind to the sensor surface, the bilayer grows, causing a measurable shift in the interference pattern reported as response versus time. BLI enables both quantitation (how much target is present) and kinetic characterization (how fast captured ligands bind their targets and how stable the complex is) without requiring fluorescent or enzymatic labels.

His-tagged proteins are well suited to BLI because the polyhistidine tag provides a convenient handle for direct capture onto probes. The performance of His-tag capture depends critically on the surface chemistry, which determines capture robustness, matrix tolerance, and regeneration behavior. Two primary approaches exist: antibody-based anti-His probes that immobilize proteins through epitope recognition, and metal chelation-based chemistries (such as Ni-NTA) that coordinate with histidine residues. Section 3 compares these approaches and details how the HIS XT probe's NovoBody chemistry improves upon traditional methods.



**Figure 1.** Real-time biolayer interferometry workflow showing biosensor tips with pre-coated capture surfaces used to monitor ligand binding, measure analyte association ( $k_{on}$ ), and measure analyte dissociation ( $k_{off}$ ) to determine binding affinity ( $K_D$ ).

## Section 3: Selection Guide - HIS XT vs Anti-His vs Ni-NTA

Gator offers three His-tag capture chemistries, each optimized for different experimental requirements. Selection depends on sample matrix complexity, assay type, buffer compatibility, and analyte characteristics.

### **Traditional Anti-His (PN: 160009)**

Traditional Gator Anti-His probes use a mouse polyclonal antibody to bind His-tagged proteins from purified or crude samples. The moderate capture density makes these probes suitable for kinetics applications and reduces avidity artifacts with large or multimeric analytes. However, the relatively high off-rate of traditional anti-His antibodies limits their utility for interactions requiring long association or dissociation phases, and the reduced signal intensity may not provide sufficient resolution for small molecule analytes or weak binding interactions.

### **Ni-NTA (PN: 160016)**

Ni-NTA probes capture His-tagged proteins through metal chelation, offering fast loading and standardized regeneration workflows. The high capture density can provide strong signals but increases the risk of avidity effects, requiring careful optimization of loading conditions for 1:1 kinetics. Ni-NTA is incompatible with reducing agents (e.g., BME, DTT above 10 mM) and metal chelators (e.g., EDTA up to 1 mM). Additionally, Ni-NTA exhibits promiscuous binding to proteins lacking polyhistidine tags, making it unsuitable for crude sample analysis. Ni-NTA probes are regenerated using Gator Ni-NTA Regen Buffer (PN: 120052) and Ni-NTA Neutral Buffer (PN: 120053), available together as the Ni-NTA Kit (PN: 350002).

### **Anti-His (HIS) XT (PN: 160050)**

HIS XT probes feature an AI-engineered NovoBody capture ligand that combines antibody-like specificity with superior binding kinetics. The dramatically reduced off-rate minimizes signal drift during extended association and dissociation phases, enabling reliable characterization of picomolar affinity interactions. Gator's proprietary XT coating technology delivers 3–5x higher ligand loading capacity, providing enhanced signal resolution for small analytes and weak interactions while maintaining flexibility to reduce loading density when working with large or multimeric proteins to minimize avidity. HIS XT probes tolerate crude samples and complex matrices while maintaining low nonspecific binding. Regenerate using Gator Regen Buffer pH 2.0 (no salt) (PN: 120008) as a starting point; optimization may be required depending on the His-tag position and protein characteristics. Do not use low-pH, high-salt regeneration buffers.

## Selection guide for biosensors that bind his-tagged proteins

**Table 1:** Recommended biosensors for specific samples or assay characteristics.

Sample / Assay Characteristics	Anti-His (PN: 160009)	Ni-NTA (PN: 160016)	Anti-His (HIS) XT (PN: 160050)
Crude samples or complex matrices	✓		✓
Buffers with reducing agents or chelators	✓		✓
Picomolar affinity measurements			✓
Small analytes (< 15kDa) or weak interactions			✓
Analysis of Fc receptor binding		✓	✓
Sample-limited applications			✓
Standardized, high-throughput workflows in clean buffers		✓	✓

**Table 2:** Feature comparison between Gator Bio Anti-His, Ni-NTA, and Anti-His (HIS) XT probes.

Feature	Anti-His (PN: 160009)	Ni-NTA (PN: 160016)	Anti-His (HIS) XT (PN: 160050)
Capture Surface	Monoclonal Antibody	Ni-NTA Coordination	AI-Designed NovoBody™
Applications	Q / K / EP	Q / K / EP	Q / K / EP
Quantitation Dynamic Range	0.25 – 500 µg/ml	0.1 -1000 µg/ml	0.2 – 1000 µg/ml
Sample Requirements	High	Moderate	Low
Compatible Sample Matrix	Purified proteins E coli lysate CHO Lysate Expi293 Lysate	Purified proteins	Purified proteins E coli lysate CHO Lysate Expi293 Lysate
Regeneration / Reuse	5-10 regenerations	10+ regenerations	10+ regenerations
Key Strengths	Specific His-tag recognition; compatible with many buffers	Simple, widely used capture chemistry; broad dynamic range	Higher signal response per binding event, excellent ligand affinity, low ligand use, optimized to reduce avidity

## Section 4: Standard HIS XT assay methods

### Kinetics Assay

HIS XT probes are designed for kinetic characterization of protein–protein interactions, including antibody and antibody fragment binding as well as protein–peptide interactions. The NovoBody-based surface provides high specificity and rapid on-rates, reducing the amount of His-tagged ligand required for capture. In most cases, 20–40 nM of His-tagged protein (1–2 µg/mL for a 50 kDa protein) is sufficient to load ligand onto the probe surface. For analytes smaller than 15 kDa or weak binding interactions, increase loading concentration to 50–100 nM or extend loading time to 60 seconds to achieve adequate signal resolution.

Kinetics are measured by capturing His-tagged ligand, then exposing the probe to a concentration series of analyte. Association and dissociation curves are fit to an appropriate binding model (typically 1:1 interaction) to determine  $k_{on}$ ,  $k_{off}$ , and  $K_D$  values.

#### Standard Kinetics Protocol:

1. Baseline: 180–600 s in assay buffer at 1000 rpm (Establishes stable baseline before loading)
2. Loading: 10–30 s in His-tagged ligand (20–40 nM) at 1000 rpm (Target 0.5–1.0 nm signal for most applications)
3. Baseline: 30–60 s in assay buffer at 1000 rpm (Allows signal stabilization and washes away unbound ligand)
4. Association: 60–600 s in analyte solution at 1000 rpm (Duration depends on binding kinetics; extend for slow on-rates)
5. Dissociation: 60–600 s in assay buffer at 1000 rpm (Duration depends on complex stability; extend for tight binders ( $K_D < 1$  nM))
6. Regeneration: 5 s in Regen Buffer pH 2.0 (no salt) (PN: 120008) at 1000 rpm (Returns sensor to baseline for next cycle)
7. Neutralization: 5 s in assay buffer at 1000 rpm (Prepares sensor for next sample)

Repeat steps 2–7 for each analyte concentration (typically 5–7 concentrations spanning 10-fold below to 10-fold above the expected  $K_D$ ).

#### Buffer Recommendations:

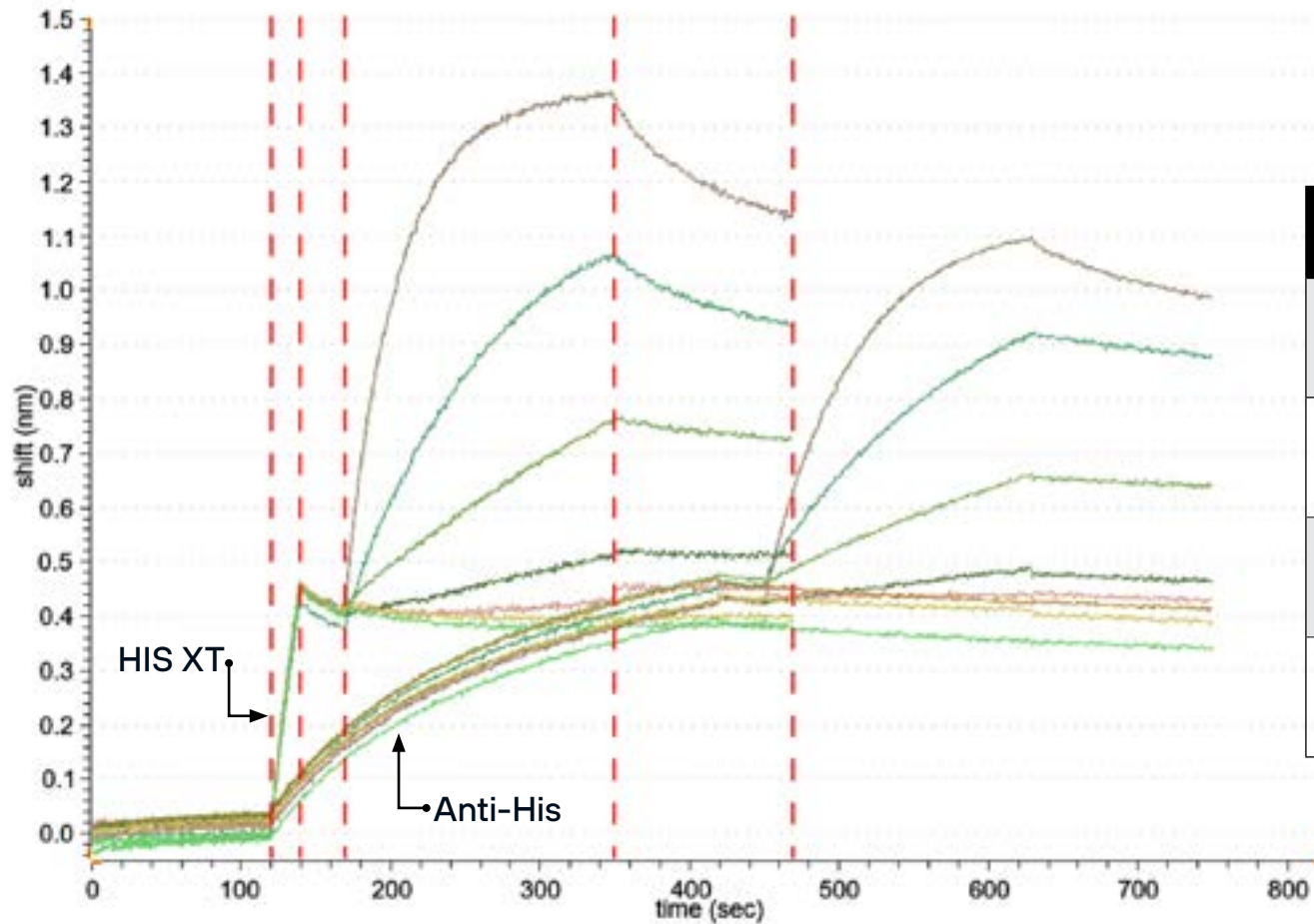
- Standard assay buffer: Q Buffer (PN: 120010) or K Buffer (PN: 120072)
- Maintain consistent buffer composition throughout all steps
- HIS XT tolerates complex matrices including cell culture supernatants and lysates

#### Optimization Notes:

- Adjust loading time/concentration to achieve 0.5–1.0 nm signal for most analytes
- For very tight binders ( $K_D < 100$  pM), extend dissociation to 1200–1800 s
- For fast kinetics ( $k_{off} > 0.01$  s<sup>-1</sup>), reduce association time to capture binding curve shape
- Target  $\text{Chi}^2 < 1.0$  for high-confidence fits

### HIS XT biosensors enable rapid capture of His-tagged PD-1

This kinetics assay compares human PD-1-His loading on conventional anti-His probes versus HIS XT biosensors, using the time to reach a 0.45 nm signal as the benchmark. HIS XT achieves equivalent loading in only 7% of the time required for anti-His probes, illustrating a substantial acceleration in assay setup and throughput for His-tagged protein studies.



	HIS XT	Anti-His
Loading Height	0.45 NM	0.45 nM
Loading Time	19 sec	300 sec
Analyte Height	1 nM	0.8 nM
$K_D$	10 nM	10 nM

**Figure 2.** Example kinetics assay demonstrating faster loading with HIS XT. Human PD-1-his was captured by both anti-His and HIS XT probes, and the time needed to capture up to a loading signal of 0.45 nm was measured. Loading on HIS XT zprobe occurred in just 7% time compared to traditional anti-His probe, enabling faster workflows.

## Quantitation Assay

HIS XT probes support both one-step and two-step quantitation workflows. In one-step quantitation, His-tagged analyte in unknown samples binds directly to the HIS XT surface and is quantified against a standard curve. In two-step quantitation, a His-tagged capture protein is first loaded onto the probe, followed by measurement of analyte binding to the captured protein. Both methods convert probe response (nm shift) to concentration using matrix-matched standard curves.

### One-Step Quantitation Protocol:

1. Baseline: 60-120 s in assay buffer at 400 rpm
2. Quantitation: 30-120 s in sample or standard at 400 rpm (Measure endpoint response or initial binding rate)
3. Regeneration: 5 s in Regen Buffer pH 2.0 (no salt) (PN: 120008) at 1000 rpm
4. Neutralization: 5 s in assay buffer at 1000 rpm

Repeat steps 2-4 for all samples and standards.

### Two-Step Quantitation Protocol:

1. Baseline: 60-120 s in assay buffer at 400 rpm
2. Loading: 10-30 s in His-tagged capture protein at 1000 rpm
3. Baseline: 30 s in assay buffer at 400 rpm
4. Quantitation: 30-60 s in sample or standard at 400 rpm
5. Regeneration: 5 s in Regen Buffer pH 2.0 (no salt) (PN: 120008) at 1000 rpm
6. Neutralization: 5 s in assay buffer at 1000 rpm

Repeat steps 2-6 for all samples and standards.

### Standard Curve Preparation:

Prepare a serial dilution series of purified His-tagged protein (one-step) or target analyte (two-step) in the same matrix as your samples. For example:

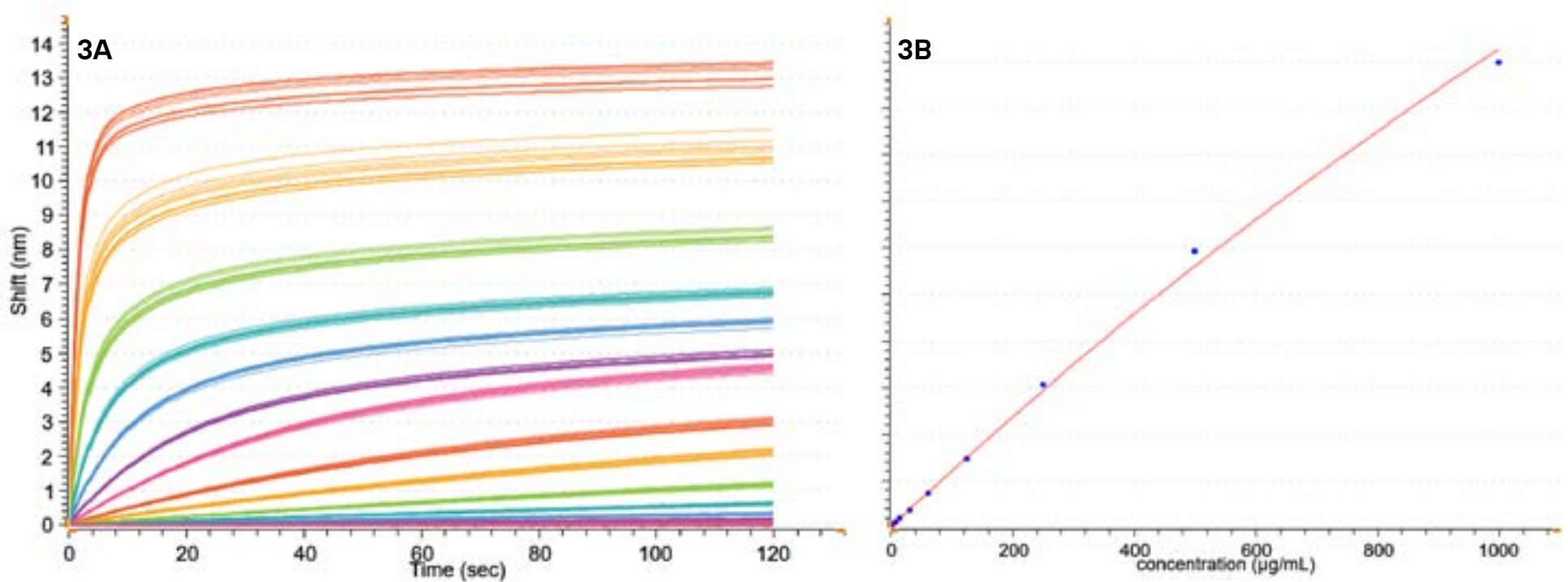
- His-tagged HSA: 1000 µg/mL → 0.12 µg/mL (2-fold serial dilution, 14 points)
- Adjust concentration range based on expected sample concentrations

### Data Analysis:

- Endpoint method: Measure response at end of quantitation step; plot response vs. concentration
- Rate-based method: Calculate initial binding rate (first 10-20 s); plot rate vs. concentration
- Use rate-based method for samples with high background or when endpoint response approaches sensor saturation
- Regeneration: HIS XT probes support >10 regeneration cycles with >90% binding capacity maintained using Regen Buffer pH 2.0 (No Salt) (PN: 120008).

### High-throughput analysis of His-tagged HSA across a broad dynamic range

These figures illustrate a kinetic binding workflow that tracks real-time association signals for multiple His-tagged HSA concentrations and converts the initial response rates into a calibration curve spanning more than three orders of magnitude, enabling precise determination of unknown sample levels.



**Figure 3. HIS XT quantitation assay.** (3A) His-HSA standards were prepared as a 2-fold dilution series from 1000 µg/mL to 0.12 µg/mL and measured using the Gator HIS XT probe with a 120 s acquisition at 400 rpm. Probes were regenerated and reused for 10 cycles between measurements. (3B) Standard curve generated for His-HSA quantitation from 0.12 µg/mL to 1000 µg/mL using rate-based method.

## Best Practices for Quantitation and Kinetics Assays:

### Control Loading Density

Avoid saturating the probe surface with ligand. Lower loading levels reduce mass-transport limitations and prevent rebinding artifacts during dissociation. Target 0.5-1.0 nm for most applications.

### Optimize for Analyte Size

- Large or multivalent analytes (>100 kDa, IgG, multimers): Reduce loading concentration/time, decrease temperature to 25°C, and use 400 rpm to minimize avidity effects
- Small analytes (<15 kDa, peptides, fragments): Increase loading concentration to 50-100 nM or extend loading time to improve signal resolution

### Match Sample Matrix

Prepare standards in the same buffer as your samples (e.g., culture media, lysate) to account for refractive index differences and reduce nonspecific binding artifacts. Equilibrate sensors in sample matrix before starting the assay.

### Implement Reference Subtraction

Include reference sensors (no ligand loaded) and buffer-only wells to subtract background signal and instrument drift from specific binding measurements.

### Validate Regeneration

Verify that regeneration fully removes bound material while maintaining capture performance across cycles. Test regeneration by running a reference standard after each cycle. Do not use low-pH, high-salt buffers with HIS XT probes.

**Table 3:** Recommended dilution conditions for loading from various sample matrices.

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 supernatants/media	Neat or 2-fold
Serum-containing cell culture supernatants	Neat
Bacterial cell pellet lysates	10-fold

## Section 5: Kinetics assay performance of HIS XT probe

We evaluated 20 different His-tagged proteins ranging from 15 to 150 kDa, sourced from 6 manufacturers and featuring both N-terminal and C-terminal His-tag orientation, to assess loading capacity and baseline stability on HIS XT compared to traditional anti-His probes. The protein panel included cytokines, receptors, enzymes, and antibody fragments to represent typical therapeutic development targets (complete list in Appendix 1).



**Figure 3.** Ligand binding performance of Gator HIS XT, Gator Anti-His, and legacy BLI anti-His probes. **3A:** Loading shift using 1  $\mu$ g/mL of protein for 2 minutes. Values reported are final response. **3B:** After loading experiment, loaded probes were placed in baseline buffer for 30 minutes. Total reduction in signal is reported as a percentage for ease of comparison.

## Enhanced Loading Capacity

Figure 3A demonstrates that HIS XT delivered an average of 4-fold higher loading signal compared to a commercially available anti-His probe and 3-fold higher signal than Gator's traditional anti-His probe when loading proteins at 1 µg/mL for 2 minutes. This improvement stems from Gator's proprietary XT optical coating technology, which generates more signal per bound protein molecule rather than simply increasing binding site density. Enhanced sensitivity enables detection of smaller analytes and allows researchers to deliberately reduce loading density when working with large or multivalent proteins to minimize avidity artifacts while maintaining adequate signal resolution.

Notably, hHER2/ErbB2 did not bind detectably to the legacy BLI Anti-His biosensor but loaded successfully on both Gator probes, suggesting broader compatibility of antibody-based capture chemistries across diverse His-tagged constructs.

## Superior Baseline Stability

Figure 3B compares ligand retention across the three platforms by measuring signal remaining 30 minutes after His-tagged protein capture. The commercially available legacy anti-His probe retained only 41% of the original signal on average, representing 59% signal loss due to ligand dissociation. Gator's traditional anti-His probe showed improved retention at 69% (31% loss), while HIS XT maintained 75% of bound protein after 30 minutes (25% loss).

HIS XT reduces signal loss by 58% compared to the alternate platform (from 59% loss to 25% loss), cutting drift by more than half. This improved baseline stability directly translates to more reliable kinetics measurements, particularly for interactions requiring long association or dissociation phases, and enables confident characterization of high-affinity (picomolar KD) interactions where ligand dissociation would otherwise compromise data quality.

## Performance Across Diverse Proteins

HIS XT demonstrated superior or equivalent performance for 17 of 20 proteins tested. Three proteins showed marginally better loading on the alternate platform: Interleukin-33 (N-terminal tag), hEGFR (C-terminal tag), and hCD30/TNFRSF8 (C-terminal tag). These cases represent < 15% of the panel and likely reflect protein-specific orientation effects.

## Practical Implications

The combination of higher loading capacity and improved baseline stability enables HIS XT users to:

- Measure picomolar affinities reliably ( $K_D < 100$  pM) without ligand dissociation artifacts
- Extend dissociation phases beyond 30 minutes for accurate off-rate determination
- Reduce His-tagged protein consumption by 5-fold while maintaining signal quality
- Characterize smaller analytes (< 15 kDa) with sufficient resolution for confident fitting

## Section 6: Summary and Conclusions

The Gator® Anti-His (HIS) XT Probe represents a significant advancement in His-tag capture technology for bio-layer interferometry, combining AI-designed NovoBody capture chemistry with proprietary XT optical coating to deliver superior performance across kinetics, quantitation, and epitope binning applications.

### Key Performance Advantages



#### 4-fold Higher Signal Resolution

HIS XT's advanced optical design delivers 4-fold higher response per captured protein molecule compared to competitor other commercially available anti-His probes and 3-fold higher than traditional Gator Anti-His probes. This enhanced sensitivity enables reliable characterization of small analytes (< 15 kDa), weak interactions, and low-abundance targets that would be challenging or impossible to measure with conventional capture surfaces.



#### 5-fold Reduction in Sample Consumption

The high-affinity NovoBody capture ligand achieves equivalent loading levels at 5-fold lower His-tagged protein concentrations (1 µg/mL vs 5 µg/mL). For a 96-well tray, this translates to approximately \$768 in reagent savings when working with typical cytokine proteins, making HIS XT particularly valuable for sample-limited applications and high-throughput screening.



#### Superior Baseline Stability for Accurate Kinetics

HIS XT retains 75% of bound ligand after 30 minutes, compared to 69% for traditional Gator Anti-His and only 41% for the legacy platform. This 58% reduction in signal drift enables confident measurement of picomolar affinities and accurate determination of slow off-rates in extended dissociation experiments, expanding the accessible affinity range from nanomolar to sub-picomolar KD values.



#### 10-fold Faster Loading for Increased Throughput

High on-rate kinetics enable typical loading in 30-60 seconds compared to 10-15 minutes for traditional anti-His probes. Combined with robust regeneration (>10 cycles with >90% capacity retention), HIS XT significantly reduces total assay time and increases daily sample throughput for screening and characterization workflows.



#### Optimized Avidity Control

Higher signal per molecule allows researchers to deliberately reduce loading density when working with large or multivalent analytes (IgG, fusion proteins, multimers), minimizing avidity artifacts while maintaining sufficient resolution for accurate kinetics measurements.

## When to Choose HIS XT

HIS XT is the optimal choice for:

- Picomolar affinity characterization ( $K_D < 100$  pM)
- Small analyte (<15 kDa) and weak affinity binder detection
- Sample-limited applications requiring minimal protein consumption
- Complex matrices (culture media, lysates)
- Extended kinetics experiments (>30 min dissociation)
- High-throughput screening workflows

For applications requiring compatibility with reducing agents >10 mM or metal chelators, or when working with simple purified samples in high-throughput standardized workflows, refer to Section 3 for guidance on selecting between HIS XT, traditional Anti-His, and Ni-NTA capture chemistries.

## Getting Started

Anti-His (HIS) XT Probes (PN: 160050) are provided in trays of 96 probes and are compatible with all Gator BLI instruments. Regenerate using Regen Buffer pH 2.0 (no salt) (PN: 120008). For assay development support, protocol optimization, or technical questions, contact Gator Technical Support or visit [www.gatorbio.com](http://www.gatorbio.com).

## References:

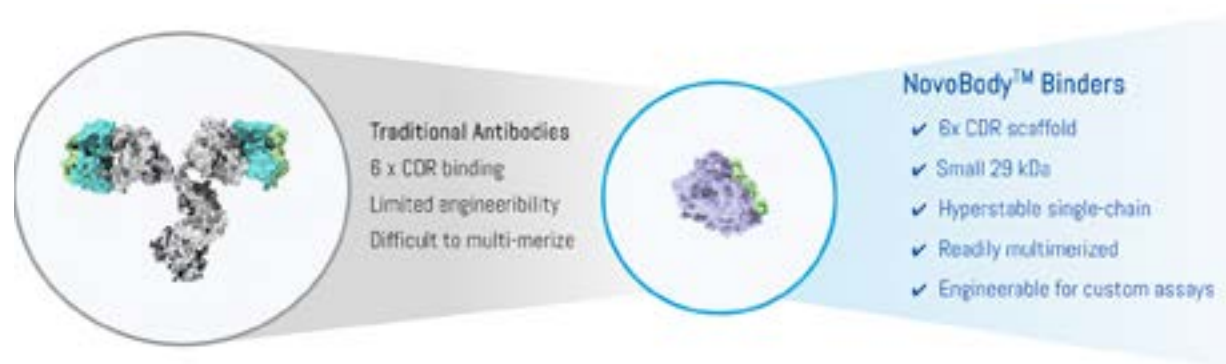
1. <https://monod.bio/novobody-2/>
2. <https://pmc.ncbi.nlm.nih.gov/articles/PMC7115108/>

## Appendix 1. Development of a fit-for-purpose ligand for BLI-based his-tagged reagent analysis

Monod Bio has developed the NovoBody™ platform, which leverages AI-enabled computational protein design to generate novel binding molecules based on the binding interfaces of existing antibodies or antibody fragments. The process eliminates suboptimal properties of the original molecules while enhancing key functionalities without requiring extensive discovery campaigns. The resulting NovoBody™ binding molecules are small (~30kDa), single-chain, highly stable, and easy to manufacture in both bacterial and mammalian cells, with customizable properties and formats.

The Gator® HIS XT probe leverages the NovoBody™ Platform to deliver a purpose-built capture molecule that outperforms traditional antibody-based capture molecules. By utilizing AI-guided de novo design, Monod Bio transformed a low-affinity starting point ( $\sim K_D$  approx 2,320 nM) into a best-in-class binder, achieving a 10,000x affinity improvement after optimization. The NovoBody™ binding molecule also eliminates the Fc region of the parental antibody, successfully removing Fc-mediated non-specific binding and ensuring cleaner baselines in complex matrices. In addition, because the NovoBody™ binding molecule is approximately 40% the size of an antibody, it localizes target analytes closer to the sensor surface, resulting in improved sensitivity.

Engineered for maximum versatility, the NovoBody™ binding molecule provides universal capture of N-terminal, C-terminal, and internal 6x or 8x His-tags, ensuring reliable performance regardless of the tag's position. Beyond this broad recognition, the evolutionary design process simultaneously enhanced the binder's physical properties, resulting in a hyperstable scaffold optimized to withstand harsh chemical environments and repeated regeneration cycles without loss of sensitivity. Furthermore, the platform prioritizes manufacturing efficiency by enabling high-yield soluble expression in *E. coli*, which ensures significantly lower production costs and superior lot-to-lot consistency compared to traditional mammalian cell-produced antibodies.



**Figure A1. NovoBody™ Platform:** AI-enabled computational protein design generates novel binding molecules based on the binding interfaces of existing antibodies or antibody fragments.

## Appendix 2: Ligands tested on Gator His XT, Gator Anti-His, and an alternative commercially available Anti-His probe

No	His-tagged Proteins Tested	Location of the Tag	Size (kDa)
1	Human PD-L2, His	C-terminus	62.8
2	Human EGF R, His	C-terminus	15.2
3	Human Angiopoietin-like 4, His	C-terminus	23.4
4	Human Latent Activin A / INHBA, His	C-terminus	70.2
5	SARS-CoV-2 Spike RBD-His	C-terminus	29.3
6	Human TROP-2, His	C-terminus	70.5
7	Human Serglycin/SRGN, His	N-terminus	27.9
8	Human Her2 / ErbB2 Protein, His Tag	N-terminus	45.9
9	TNF- $\alpha$ , His, Human	C-terminus	17.4
10	CD20, His, Human	C-terminus	26.54
11	EGF R, His, Human	C-terminus	29.4
12	Interleukin-33 Human Recombinant, His Tag	N-terminus	18.3
13	Human CD30/TNFRSF8 His-tag	C-terminus	16.8
14	Human CD45, His tag	N-terminus	95~115
15	Human PD-L1 / B7-H1 (19-134) Protein, His Tag	N-terminus	15.2
16	Human IL-2 R gamma / CD132 Protein, His Tag	C-terminus	22.49
17	Human PD-1 Protein (ECD, His Tag)	C-terminus	41
18	CD155 (D29-L348), His (Dimer)	C-terminus	150
19	CD155 (aa 21 - 343), His	C-terminus	92
20	Nectin 4, His	C-terminus	35.9

## Appendix 3: Application Examples Demonstrating HIS XT Versatility

The following examples demonstrate HIS XT probe performance across diverse interaction types and affinity ranges. All data were collected using standard protocols described in Sections 4 and 5.

### Protein-Protein Interactions

HIS XT probes enable reliable kinetic characterization of protein-protein interactions through specific capture of His-tagged ligands. The enhanced baseline stability and high signal resolution support accurate measurements across a wide affinity range.

Assay Conditions	Sensogram	Binding Constant
Biosensor: HIS XT  Ligand: Unknown protein with His-tag  Analyte: Binding partner (0-100 nM)		$K_D = 3.1 \text{ nM}$  Typical nanomolar-affinity protein-protein interaction demonstrating 1:1 binding kinetics
Biosensor: HIS XT  Ligand: Thrombopoietin-R His-tag  Analyte: Thrombopoietin Protein		$K_D = 2.9 \text{ nM}$  Cytokine-receptor interaction with tight nanomolar affinity
Biosensor: HIS XT  Ligand: Human PD-1-His  Analyte: Human PDL1		$K_D = 50 \text{ nM}$  Checkpoint protein interaction in the moderate nanomolar range

**Figure A2. Representative protein-protein binding interactions.** All protein-protein interactions show clean binding kinetics with minimal baseline drift, enabling confident 1:1 model fitting. The stable baseline during dissociation phases is critical for accurate  $k_{\text{off}}$  determination.

## Protein-Antibody and Fragment Interactions

HIS XT's low ligand dissociation rate and high sensitivity make it particularly well-suited for antibody characterization, including measurement of high-affinity interactions in the picomolar range that are challenging for traditional anti-His probes.

Assay Conditions	Sensogram	Binding Constant
Biosensor: HIS XT  Ligand: PD-1-His  Analyte: Anti-PD1 antibody		$K_D = 95 \text{ pM}$  High-affinity therapeutic antibody demonstrating picomolar binding. The minimal baseline drift during the extended dissociation phase enables accurate measurement of the slow off-rate.
Biosensor: HIS XT  Ligand: Human EGFR  Analyte: Anti-EGFR Fab		$K_D = 825 \text{ pM}$  Antibody fragment characterization in the picomolar range. The sensorgram complexity may reflect Fab-specific binding characteristics or sample matrix effects.
Biosensor: HIS XT  Ligand: Human Troponin-His  Analyte: Anti-human TNI antibody		$K_D = 2.9 \text{ nM}$  Cardiac biomarker antibody showing nanomolar affinity with clean 1:1 kinetics

**Figure A3. Antibody and fragment binding to His-tagged antigens.** These picomolar affinity measurements (95 pM and 825 pM) demonstrate HIS XT's ability to reliably characterize tight binding interactions. Traditional anti-His probes would show significant ligand loss during the extended dissociation phases required for these measurements, compromising data quality. The anti-EGFR Fab data shows some baseline complexity that may require additional optimization of assay conditions or buffer composition.

## Epitope Binning

Epitope binning classifies antibodies based on whether they recognize the same or different regions (epitopes) on a target antigen. HIS XT’s high loading capacity, stable baseline, and regeneration robustness make it ideal for tandem epitope binning workflows.

### Methodology

In tandem binning, His-tagged antigen is captured on HIS XT probes, followed by sequential exposure to two antibodies:

1. First antibody binds to the captured antigen
2. Second antibody is introduced without removing the first
3. Binding behavior determines epitope classification:
  - a. Competitive (same bin): Second antibody shows reduced or no binding because the first antibody blocks its epitope
  - b. Non-competitive (different bins): Both antibodies bind simultaneously to distinct epitopes



**Figure A4.** High-throughput epitope binning of anti-TnI antibody panel.

In the example provided in Figure A4, Human TnI-His antigen (10 µg/mL) was captured on HIS XT probes and exposed to all pairwise combinations of 16 anti-TnI antibodies with known epitope diversity. The complete 16 × 16 binning matrix (256 antibody pair combinations) was performed using a single row of regenerated probes in 217 minutes on the Gator Plus system in 384-well plate format.

## Results Interpretation

- Red cells: Competitive binding indicating antibodies recognize the same or overlapping epitopes (same bin)
- Green cells: Non-competitive binding indicating antibodies recognize distinct epitopes (different bins)

The heatmap correctly identified known epitope groups across all 16 antibodies. For example:

- Antibodies Fapon 043, 17F3, and MF4CC form one epitope bin (all show red/competitive binding with each other)
- Antibodies 3C7, M18CC, 801, and 14G5 form a different epitope bin
- Several antibodies (P-45-10, 267, 625) show unique epitope specificity (green with most others)

## Key Performance Features

- Stable baseline: Minimal drift during sequential antibody binding steps enabled clear discrimination between competitive and non-competitive pairs
- Robust regeneration: Single sensor row successfully regenerated >250 times without loss of binding capacity
- High throughput: Complete characterization of 256 antibody pairs in under 4 hours
- Accurate classification: Results matched known epitope diversity of the antibody panel

## Summary of Demonstrated Capabilities

These examples highlight HIS XT's versatility across:

- Affinity range: Reliable measurements from 95 pM to 50 nM (>500-fold dynamic range)
- Interaction types: Protein-protein, antibody-antigen, antibody fragments, checkpoint proteins, cytokine-receptor pairs
- Baseline stability: Minimal drift enables extended dissociation phases for accurate picomolar  $K_D$  determination
- Complex workflows: Tandem epitope binning with >250 regeneration cycles per sensor
- Data quality: Clean 1:1 binding kinetics with excellent curve fitting across diverse molecular interactions

For detailed protocols, assay optimization guidance, and regeneration conditions, refer to Sections 4 and 5.

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