

APPLICATION NOTE

Optimizing BLI Assays: Effective Strategies for Reducing Non-Specific Binding

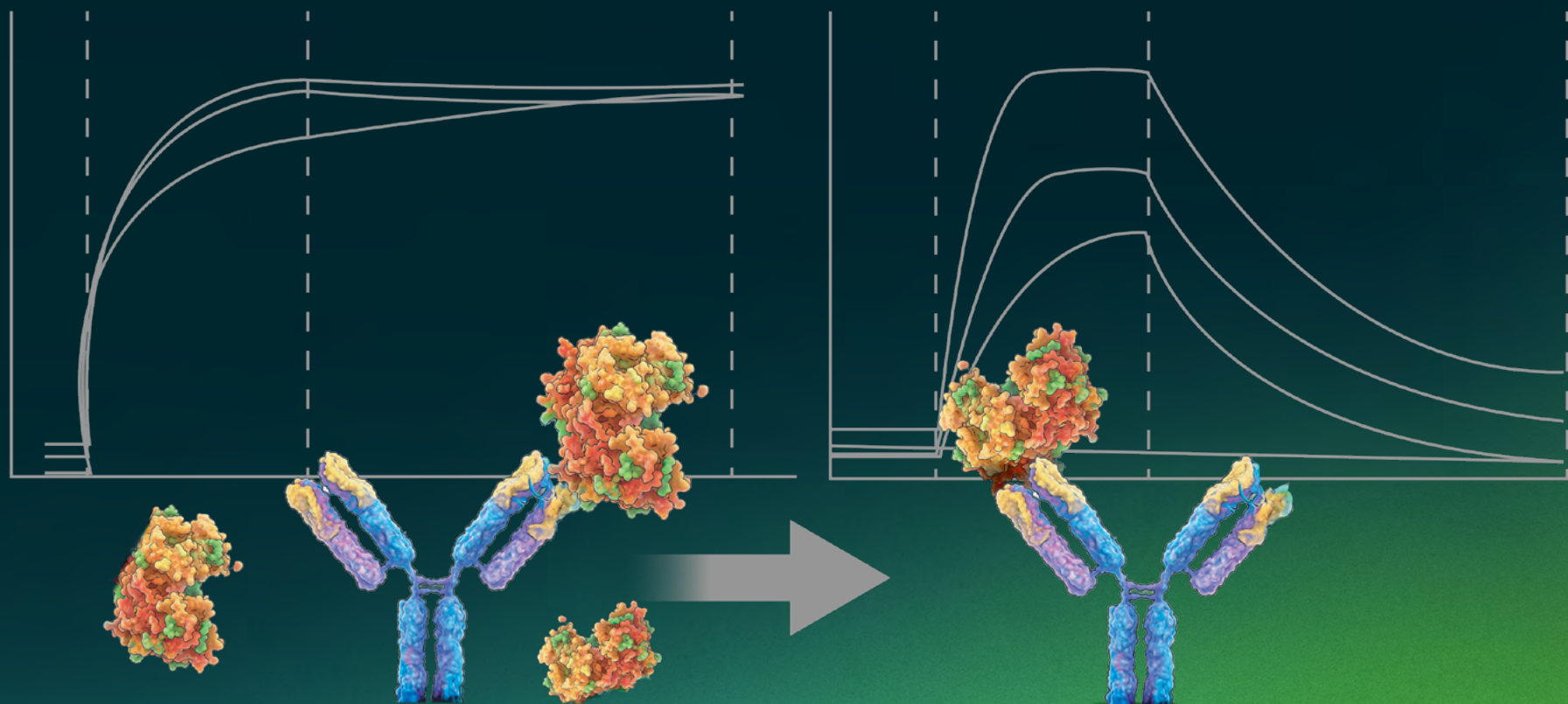


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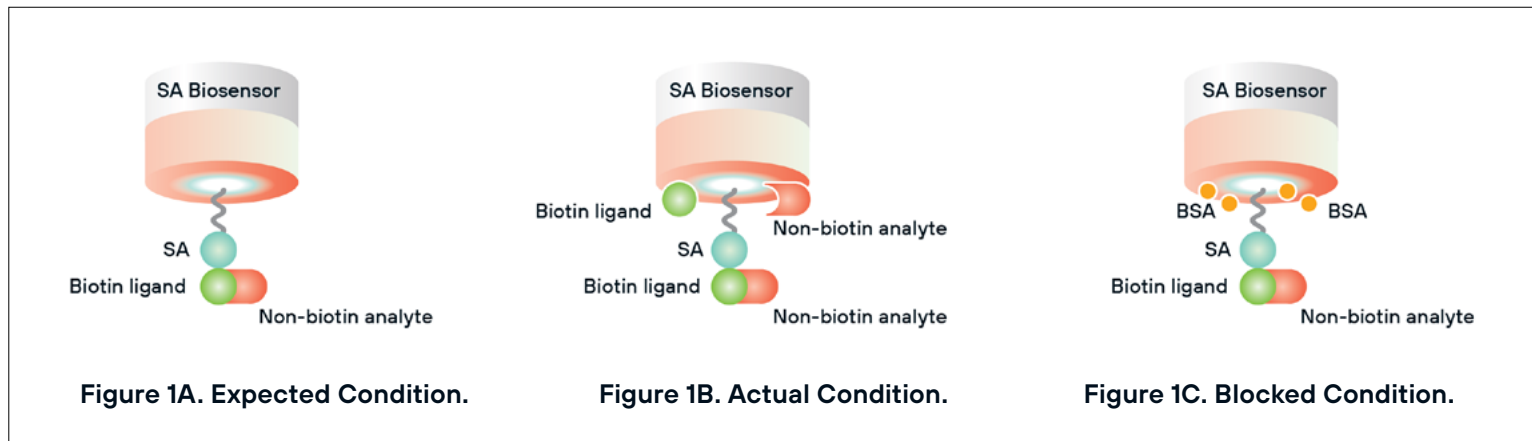
Part 1: Abstract

Label-free detection technologies do not inherently distinguish between wanted signal and unwanted signal as any binding event on the surface of the sensor changes the optical density and becomes detected. This effect, known as nonspecific binding (NSB), is a common challenge in biolayer interferometry (BLI) and other biochemical assays that compromise accurate kinetic and quantitative measurements through signal caused by unanticipated components of the sample buffer. NSB occurs when molecules in a sample including proteins, nucleic acids, lipids, or other biomolecules that bind unintentionally to unblocked regions of the biosensor surface rather than to the immobilized ligand. Incomplete surface blocking can promote hydrophobic and/or electrostatic interactions between sample components and the sensor, leading to elevated baselines, false association signals, and poor reproducibility. These effects are particularly pronounced when working with partially purified proteins or complex samples such as cell culture supernatants and crude lysates. NSB can occur across all types of BLI biosensors. This application note focuses on examples of NSB observed with Streptavidin (SA) biosensors and offers effective solutions to minimize NSB across a broad range of sample types, including purified biomolecules and complex matrices such as mammalian cell culture and E. coli crude lysates. By reducing off-target interactions, proper blocking methods improve assay accuracy, sensitivity, and overall data quality.

Part 2: Introduction

BLI enables real-time, label-free analysis of biomolecular interactions, but assay performance can be adversely affected by NSB [1]. Similar to ELISA assays, where unblocked microplate surfaces can bind biomolecules and generate background signals, unblocked regions of a BLI biosensor can also contribute to NSB.

Many BLI users assume that BLI biosensors are inherently specific and will interact only with the intended ligand or analyte. Consequently, a simple buffer (e.g., PBS) without the inclusion of a blocking agent is used for biosensor pretreatment and sample dilution, as illustrated in Figure 1A. In practice, BLI biosensors are made of many layers, starting from glass to polysaccharide to streptavidin to capture ligands. Each of these various layers can contain exposed surfaces that are susceptible to nonspecific interactions. Biomolecules present in sample buffers or crude matrices can bind to these exposed surfaces, resulting in significant NSB (Figure 1B). In BLI, NSB presents as an unusually high %CV in quantitation assays, an overestimation of the final result, a loss of signal across replicates or, in rare cases, a reduction of total binding signal. The goal of this application note is to provide tools to assess the total NSB present in samples and give clear guidelines for common troubleshooting approaches. Proper blocking (providing a molecule that binds to these spaces and ensure it's always present in experimental solutions) can significantly improve data accuracy, reproducibility, and reliability when working with purified or crude biological samples.



Part 3: Common NSB blocking agents

To mitigate NSB, we strongly recommend pre-blocking biosensors in conditions similar to the samples, and/or diluting protein samples in buffers containing one or more suitable blocking agents [2]. With effective blocking, NSB can be reduced to the point that true binding signals can be measured accurately and reliably (Figure 1C). Examples of successful blocking can be found in later sections.

When performing BLI assays, blocking agents can take a variety of forms. The most common non-specific blocking agents are:

1. Bovine Serum Albumin (BSA) – typically used at 0.02% to 5% of solvent
2. Detergents like Tween 20 – suitable up to 3% (Triton and SDS are not recommended)
3. Sucrose – typically 0.1-0.6 M
4. Imidazole – typically 5-20 mM (very useful for NSB related to anti-His or Ni-NTA biosensors)
5. Higher salt (increased ionic strength can limit charge-based interactions)

Additional NSB blocking agents have been shown to work for specific circumstances. For more information about potential blocking agents specific to your workflow, contact our support team.

In addition to these non-specific binding agents, specific blocking also plays a role in limiting signal from certain analytes. Biocytin (a soluble biotin analogue used as a specific blocking agent) and other biotin blocking agents can help to prevent interactions with Streptavidin. Blocking with biocytin after ligand loading can help to prevent NSB of analyte molecules that have hydrophobic motifs that interact with the hydrophobic pocket of the biotin binding site, or for analytes that are biotinylated. Biocytin can also be used in place of ligand loading to identify the extent of non-specific interactions that are occurring between the sensor surface and the molecules of interest by preventing all interactions with the streptavidin binding site. Figure 2A shows the extent of non-specific binding that is caused by a biotinylated antibody – showing that a significant percentage (almost 20%) of all binding signal on the streptavidin biosensor is caused by protein interacting with non-streptavidin surfaces. This is further demonstrated in Figure 2B, wherein non-biotinylated PD-1 is able to bind non-specifically to the sensor in the unloaded biosensor conditions. Note that blocking with biocytin does not block PD-1 NSB – this is caused by interaction with the sensor surface and not the streptavidin itself. For reference, while the experiments in this paper used biocytin at 10 µg/mL without ligand loading, a typical protocol can also incorporate a 60 second blocking step (at the same concentration) after ligand loading to prevent a biotinylated analyte from interacting with Streptavidin.

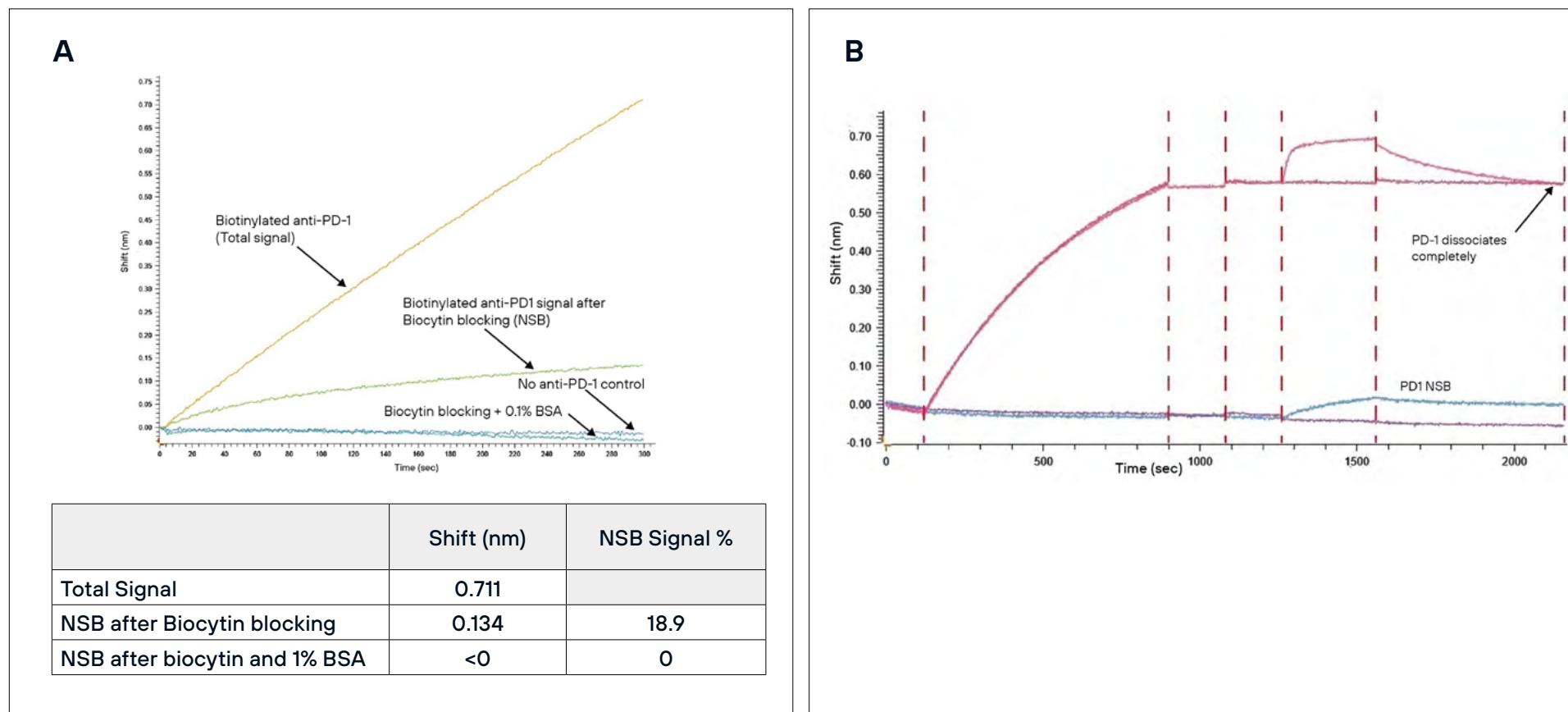


Figure 2 (A): Effect of biocytin blocking on biotinylated anti-PD-1 on SA biosensor. Note that approximately 18% of the total binding signal of biotinylated anti-PD-1 to the sensor was caused from interactions not associated with the Streptavidin-Biotin interaction. (B) Biotinylated anti-PD-1 (purple) or control (blue) are loaded onto SA biosensors and allowed to interact with either PD-1 or control well. PD-1 is not biotinylated – all binding signal here is non-specific interactions with the sensor surface. However, only the loaded biosensors dissociate to baseline after PD-1 interaction because anti-PD-1 provides adequate blocking of the biosensor, preventing PD-1 NSB.

Part 4: Non-specific signal from refractive index changes or through dissociation of blocking agents

While this application note focuses on non-specific interaction between ligand or analyte molecules and the biosensor surface, one common source of non-specific signal is caused by transitioning biosensors between wells containing solutions with different refractive indexes. NSB from protein interacting with the sensor surface can coexist with non-specific signal from buffer changes, making it difficult to troubleshoot. The most common causes of these interactions include:

- Glycerol (remember, lyophilized proteins can still have glycerol in the powder)
- Detergents (e.g. Tween20)
- Changes in pH
- Sugars (e.g. Sucrose)
- Imidazole (particularly for anti-his and Ni-NTA biosensors) – 5-20 mM
- DMSO, ethanolamine, imidazole, or other solvents

Some of these reagents can also be used as NSB blocking reagents. When used for blocking, these reagents can also be a cause of non-specific signal. Transferring from a baseline step in pure PBS to a step with glycerol will look like a binding curve (as glycerol slowly infiltrates the sensor surface). Conversely, when using a 1% BSA solution during a blocking step, transitioning to a step without that BSA can cause a downward trend (Figure 3) that can obscure binding events with other proteins.

To prevent blocking reagents from affecting the signal, ensure that all steps before and after the analyte binding step (second baseline and dissociation) contain the same buffer composition, and that they've had adequate time to come to equilibrium. If additives present the sample matrix is creating a non-specific signal, it is often easiest to perform a buffer exchange of the sample as serial dilution of the sample will also lead to serial dilution of matrix.

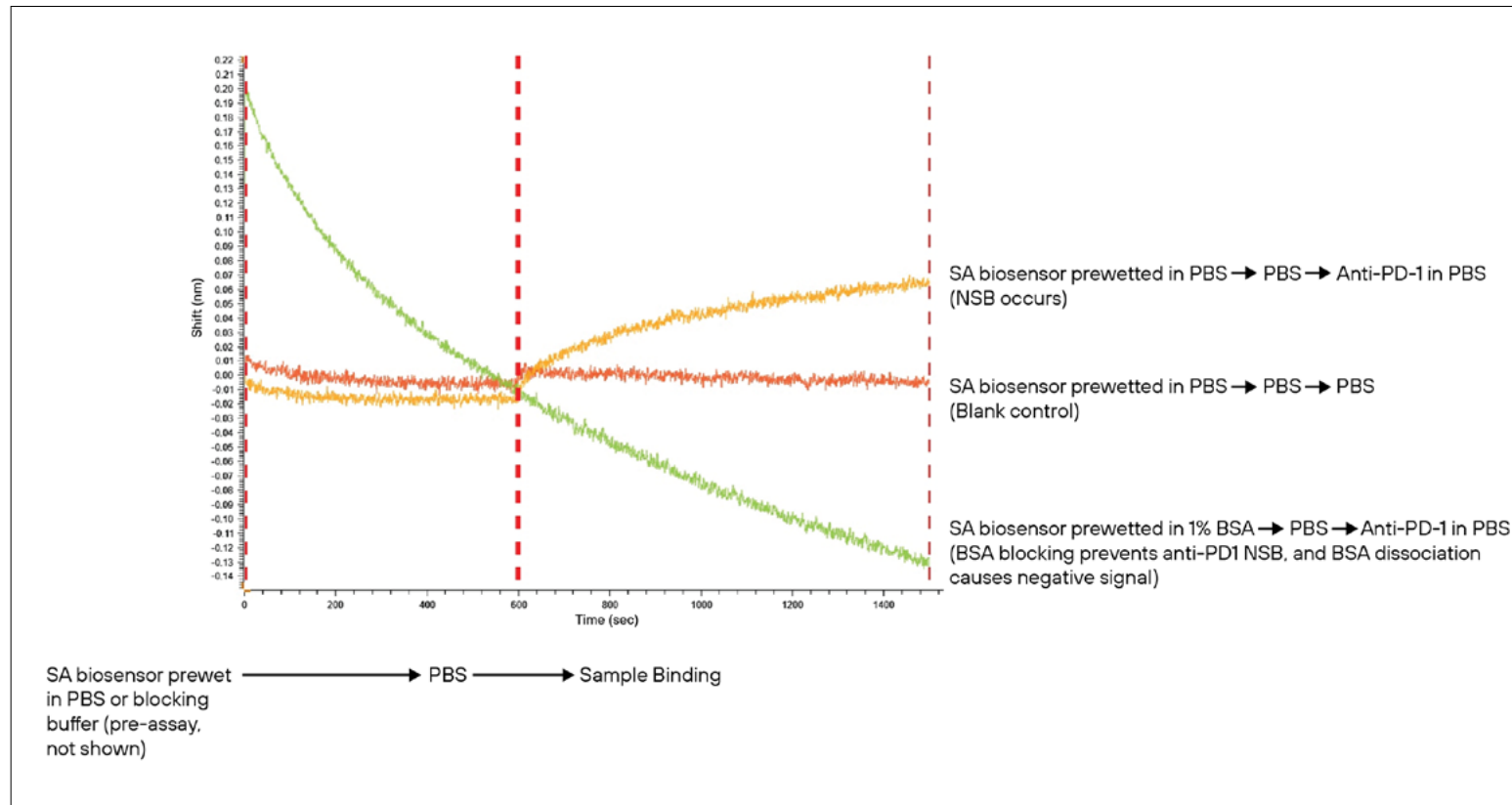


Figure 3: Unloaded SA biosensors undergoing various buffer conditions to investigate NSB. When using 1% BSA (blocking buffer) in the prewet step, no NSB of anti-PD1 is observed, however as BSA is not preserved in the baseline buffer and sample buffer a negative drift is present.

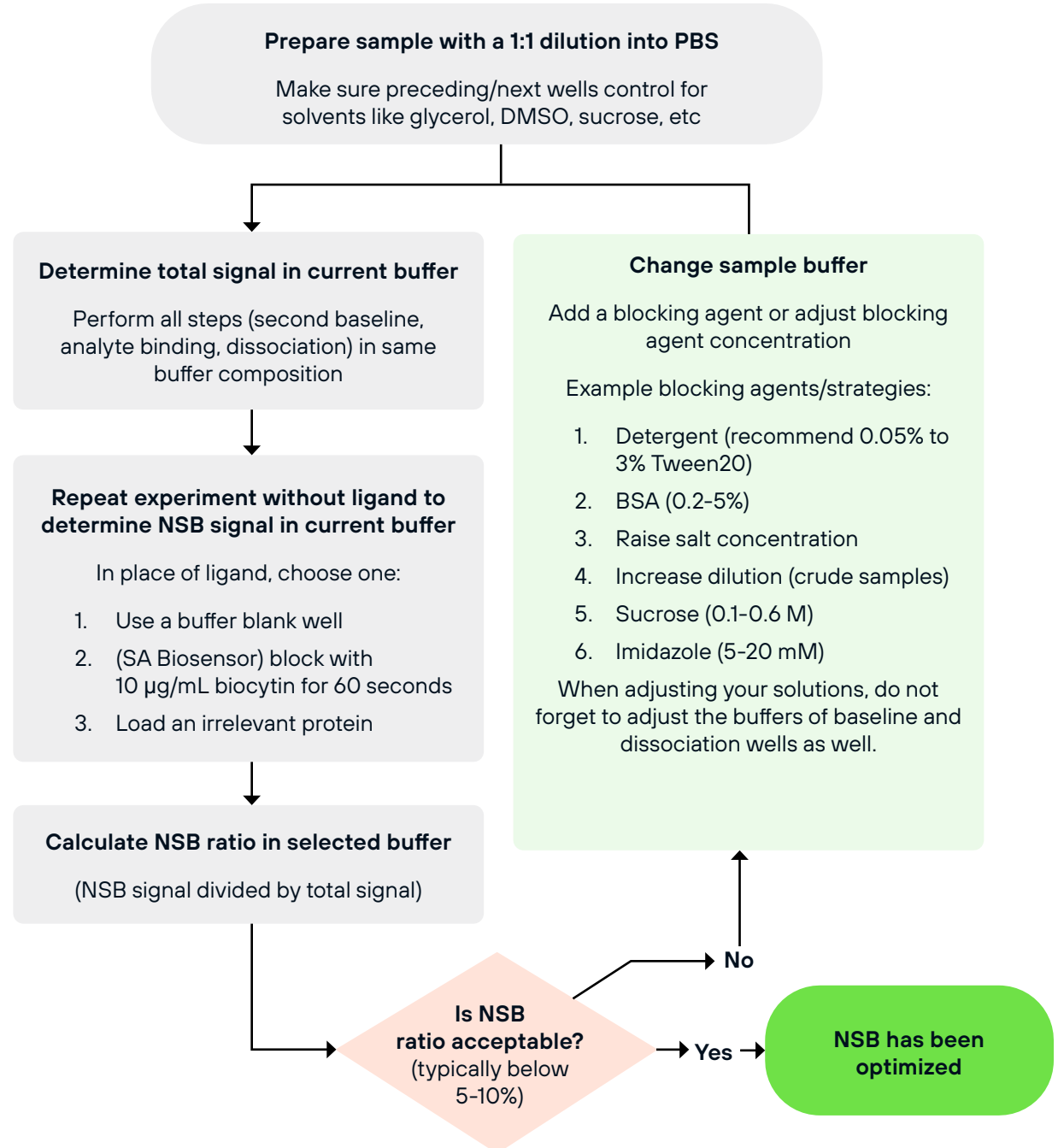
Part 5: Determining the extent of NSB

To determine the extent of non-specific signal, the best approach is to obtain total binding signal without NSB blocking, then add blocking agents one at a time. Figure 4 shows a flowchart for users to identify and minimize NSB for assays with purified and crude samples respectively. Users can follow these guidelines to determine NSB in their assays and determine acceptable NSB levels. In subsequent sections, we demonstrate that incorporating an NSB blocker is a highly effective approach for suppressing unwanted binding.

The general strategy for identifying and eliminating NSB is to start from the total NSB signal in a non-blocking medium such as PBS, only adding blocking agents after determining the starting NSB%. This strategy enables users to know the extent of NSB blocking impact from each element of the blocking buffer, which can help to limit the total blocking agents present in the final solution. Far too commonly, users jump immediately to very strong blocking conditions, which can lead to unusual results without knowing the efficacy of blocking that's actually occurring. In this application note, we primarily focus on the effect of BSA, and diluted many samples 1:1 into a 2% BSA solution (resulting in a 1% BSA working concentration). Additional benefits can be also noted by diluting the sample at a greater ratio than 1:1, provided the concentration is high enough to still reliably detect ligand/analyte binding.

Sometimes using too many blocking agents can affect the specific binding of certain biomolecule partners or cause unwanted data artifacts such as baseline drift. In these scenarios, it is recommended to try either a different buffer system (eg. HBS), a different biosensor type, and/or reversing which binding partner is being immobilized onto the sensor surface.

Figure 4. Flowchart for stepwise troubleshooting of sample NSB. While many users may want to begin with a mild blocking buffer (like Q buffer), it is recommended to begin with PBS in order to determine overall effectiveness of each blocking agent. Using too many or ineffective blocking agents can lead to unusual results or data artifacts.

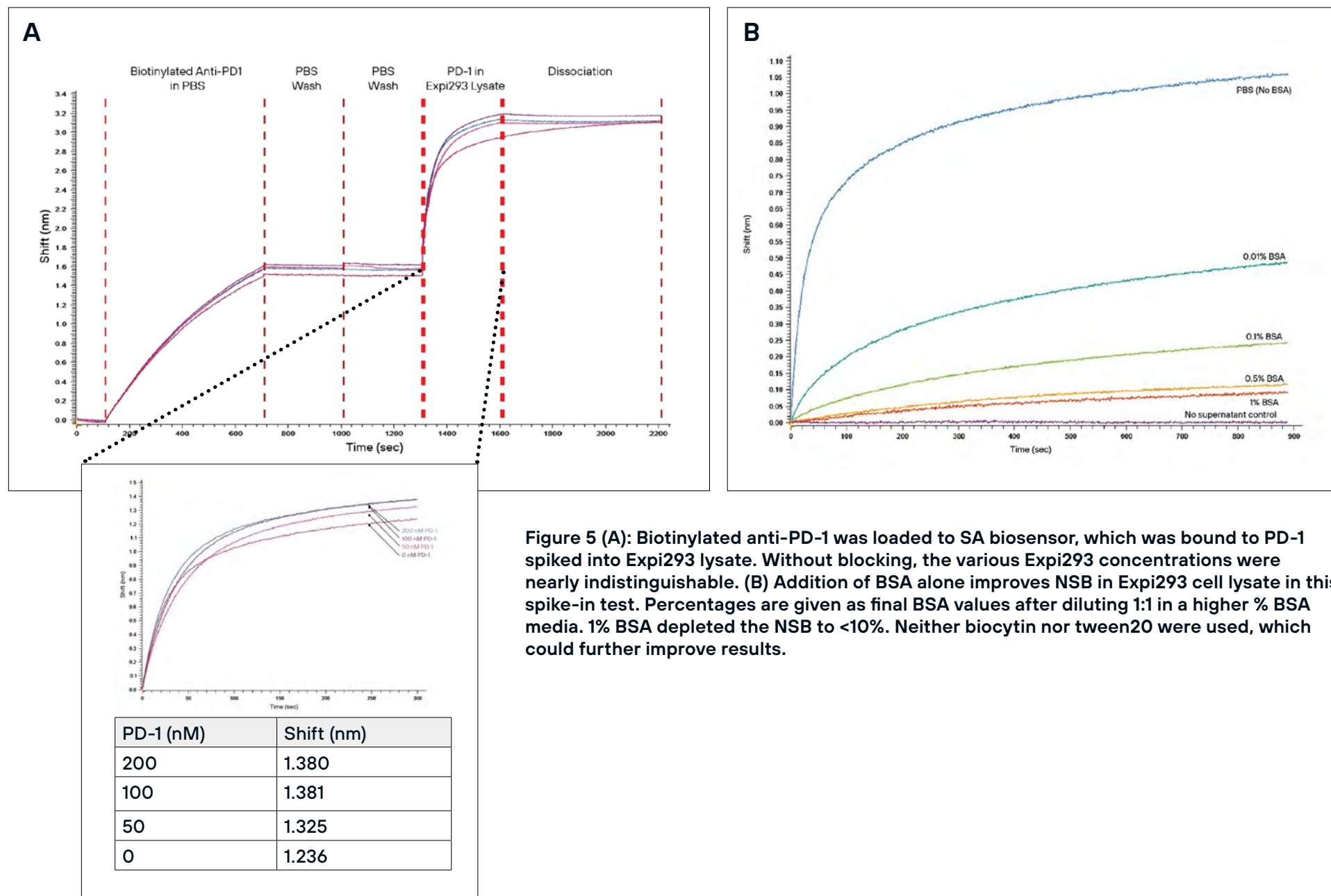


Part 6: Resolving NSB in crude lysates (Expi293 and others)

When screening and using a crude sample for ligand loading, having some media NSB to the sensor is expected, and as long as analyte binding can be detected, it does not matter much. However, when using crude samples as analytes, or when quantitating from a crude sample, blocking is essential for accurate data.

Figure 5 provides an example of the typical NSB response observed when performing quantitation or kinetics where steps are in Expi293 lysate [3]. In this case, we tested the same ligand/analyte pair (biotinylated anti-PD1 and PD-1) using a streptavidin biosensor. Increasing concentrations of BSA yielded a drastic reduction in NSB signal for Expi293 (figure 5, bottom-left), with a 1% BSA concentration driving overall NSB below 10%. Further reduction in NSB could be achieved through combination with other blocking agents, such as Tween20 or biocytin (when using an SA biosensor).

Figure 6 examines calculation of K_D values using analytes in Expi293. To test whether the blocking has a dramatic effect on the intended interaction between the anti-PD-1 and PD-1 analyte, the interaction kinetics were measured in both purified samples, as well as in lysates spike-ins in Expi293, ExpiCHO and E. coli lysates (Table 1). As shown, the K_D of the interactions after adequate blocking (and subtraction of baseline in the appropriate blocking solution) showed a very similar K_D in each assay replicate.



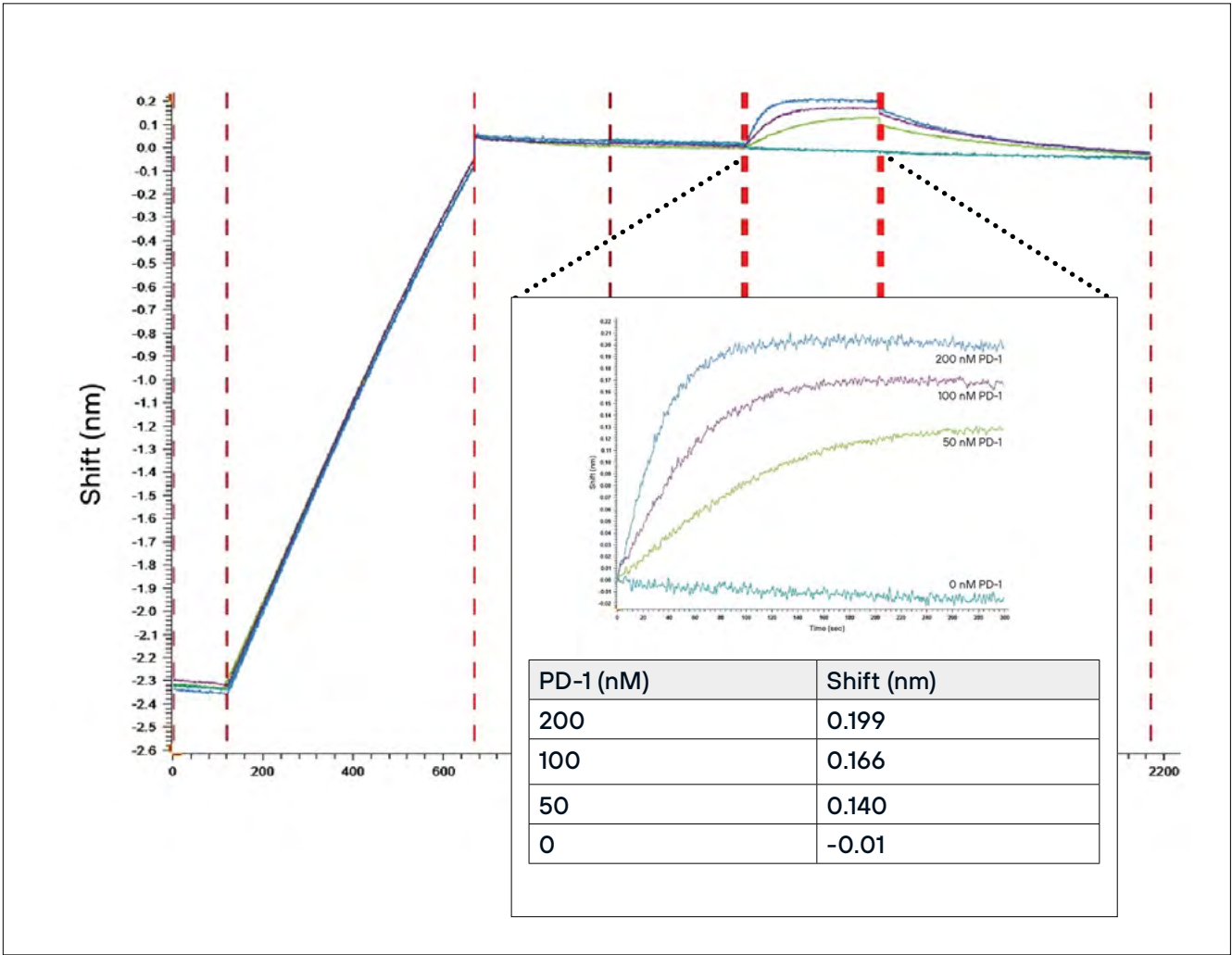


Figure 6: Optimized binding of PD-1 spiked in Expi293 with 1% BSA blocking, removing significant NSB.

	Average Determined K_D (nM, n=3)
K Buffer	16 ± 2
Expi293	17 ± 3
ExpiCHO	14 ± 4
<i>E. coli</i>	22 ± 3

Table 1: Application of effective blocking strategies yield equal K_D results for the interaction of Anti-PD1 and PD-1 comparing purified protein and spike-ins using Expi293, ExpiCHO, and *E. coli* lysates.

Part 7: Conclusion

NSB is a common problem in BLI analysis, caused by biomolecules in a sample interacting with the biosensor components. This can even happen within purified samples, as they interact with sensor components such as the exposed glass, the neutrally-charged polysaccharide on the sensor surface, streptavidin, or other components of the biosensor. These interactions can be prevented thorough competition using blocking agents such as BSA, Tween20, and similar. By following the flow chart provided in Figure 4, users can generate protocols to effectively reduce and optimize for sample NSB in a variety of matrices.

Part 8: Abbreviations

Abbreviation	Full Term
BLI	Biolayer interferometry
BSA	Bovine serum albumin
CV	Coefficient of Variation
DMSO	Dimethyl sulfoxide
<i>E. coli</i>	Escherichia coli
ELISA	Enzyme-Linked Immunosorbent Assay
ExpiCHO	Expressed Chinese Hamster Ovary
Expi293	Expression system based on suspension-adapted human embryonic kidney (293) cells
K_d	Equilibrium dissociation constant
NSB	Nonspecific binding
NTA	Nitrilotriacetic acid
PBS	Phosphate-Buffered Saline
PD-1	Programmed cell death protein 1
SA	Streptavidin
SDS	Sodium dodecyl sulfate

Part 9: References and Recommended Reading

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