Prioritizing Data Quality by Implementing Double Reference Subtraction

Technical Note

Scope
This Technical Note describes the workflow how to apply double reference subtraction to assays on Octet® BLI systems in order to correct for non-specific binding or refractive index artifacts.

Abstract

Non-specific binding (NSB) in Octet® Biolayer Interferometry (BLI) assays refers to the unintended interaction of analytes or other molecules with the biosensor surface during the assessment of a specific binding event to a ligand. This phenomenon can lead to inaccurate data and misinterpretation of results, as it introduces background noise and false positives. Recognizing NSB involves observing unexpected signal responses that do not correlate with the known binding kinetics or specificity of the target interaction. To mitigate NSB, several strategies can be employed, such as optimizing the assay buffer composition, using blocking agents to coat the biosensor surface, and incorporating stringent washing steps. Additionally, selecting appropriate reference sensors and implementing control experiments can help distinguish specific binding events from non-specific interactions, thereby enhancing the accuracy and reliability of the Octet® BLI assay. This Technical Note deals with the additional strategy of performing double reference subtraction using additional biosensors with the specific intent of correcting any observed NSB binding.

Find out more: www.sartorius.com/octet-bli

Introduction

Biological molecules can interact with unintended surfaces based on their biophysical properties; therefore, non-specific binding (NSB) can be a concern with any assay under any conditions. Non-specific binding can occur as a result of many factors, such as cell culture media components, BSA or serum proteins, or charged species in the sample or buffer. A valuable feature of BLI assays is that NSB can often be differentiated from 'true' binding by inspection of the binding plots and with careful consideration, NSB can be minimized using assay optimization in most common scenarios.

In an ideal assay setup that exhibits no NSB inclusion of a reference sample, containing the ligand but is only exposed to the assay buffer, is sufficient to correct for any refractive index (RI) effects observed in the assay as the assay buffer concentration is constant throughout all analyte containing wells and single reference subtraction is therefore sufficient to remove any RI issues across all analyte concentrations.

In a non-ideal assay setup that exhibits NSB or high RI differences (Figure 1) inclusion of a reference sample, containing the ligand but is only exposed to the assay buffer, is not sufficient to correct for any artifacts in the assay. A single reference subtraction is not sufficient to remove NSB issues across all analyte concentrations as the level of refractive index effects or NSB directly correlates to the analyte concentration.

Double Reference Subtraction on Octet® BLI Using Reference Sensor Subtraction

Important: It is highly recommended that new users read Chapter 2 – Editing Step Types and Chapter 4 - Reference Sensor Subtraction of the Octet® Analysis Studio User Guide for a detailed explanation. A simplified walkthrough version tailored to the specific needs of an assay is described here.

In addition to NSB correction, this method is applicable to correct for sample buffers that contain a sample matrix that may cause interference with the assay (e.g., NaCl, sucrose, histidine, and trehalose).

Adding Assay Parameters

In the Octet® BLI Discovery Software, reference biosensors can be added to an existing assay setup through the addition of a new assay step. As shown in Figure 2A, a simple assay plate containing a ligand (Column 1), assay buffer (Column 2) and analyte (Column 3) can be used to create an experiment where assay one contains biosensors loaded with the ligand (Column 1) and assay two, using a separate column of biosensors, which are loaded in the assay buffer (Column 2). Both biosensors are then assessed in the analyte (Column 3). Double reference subtraction therefore, requires a separate column of reference biosensors in addition to those normally used in an assay for reference sample subtraction.

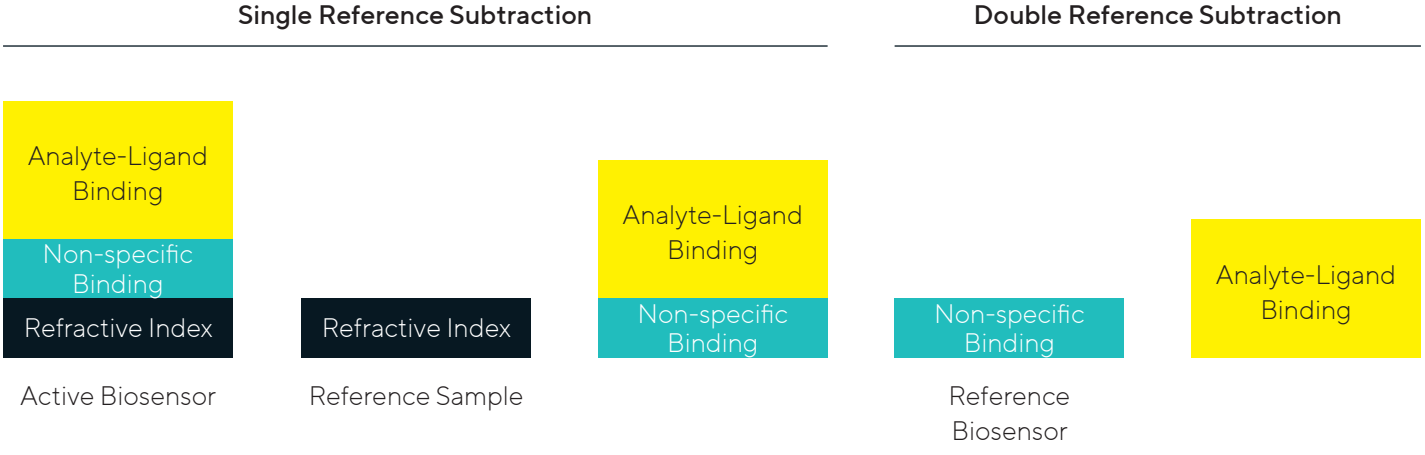
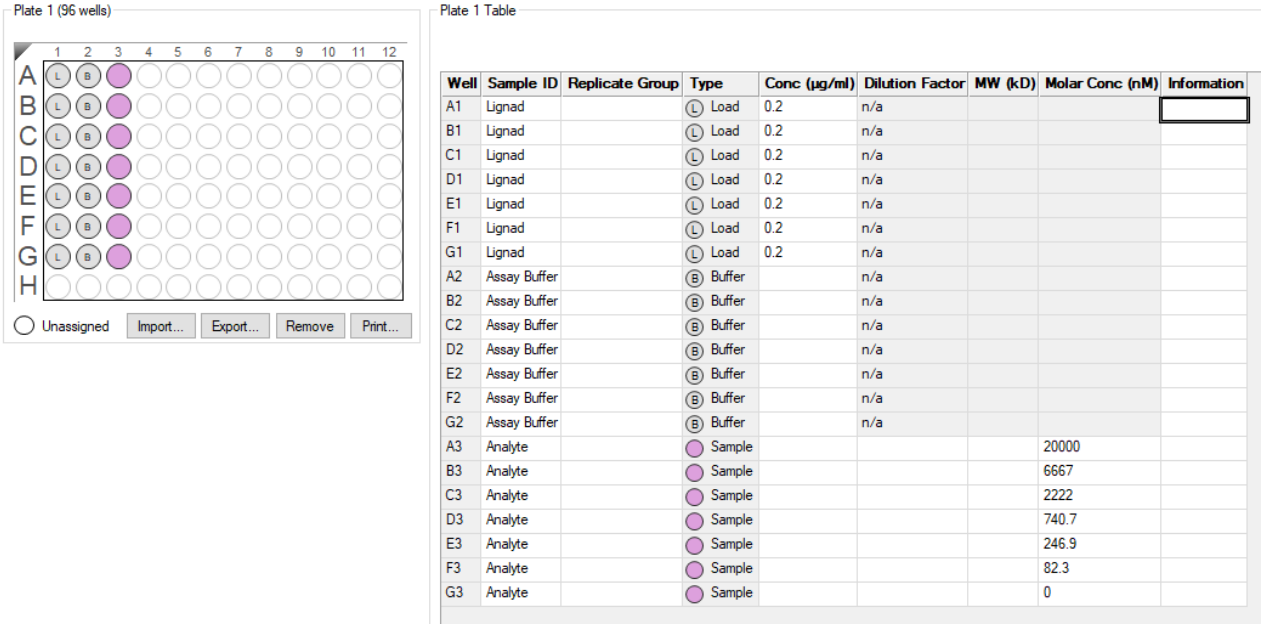


Figure 1: In assays that exhibits NSB, inclusion of a reference sample, containing the ligand but is only exposed to the assay buffer, is not sufficient to correct for any NSB artifacts observed in the assay. Therefore, single reference subtraction is not sufficient to determine accurate kinetics and affinity. Where NSB artifacts remain in the observed data; inclusion of an additional set of reference biosensors allows double reference subtraction to be performed and NSB artifacts to be removed, is required.

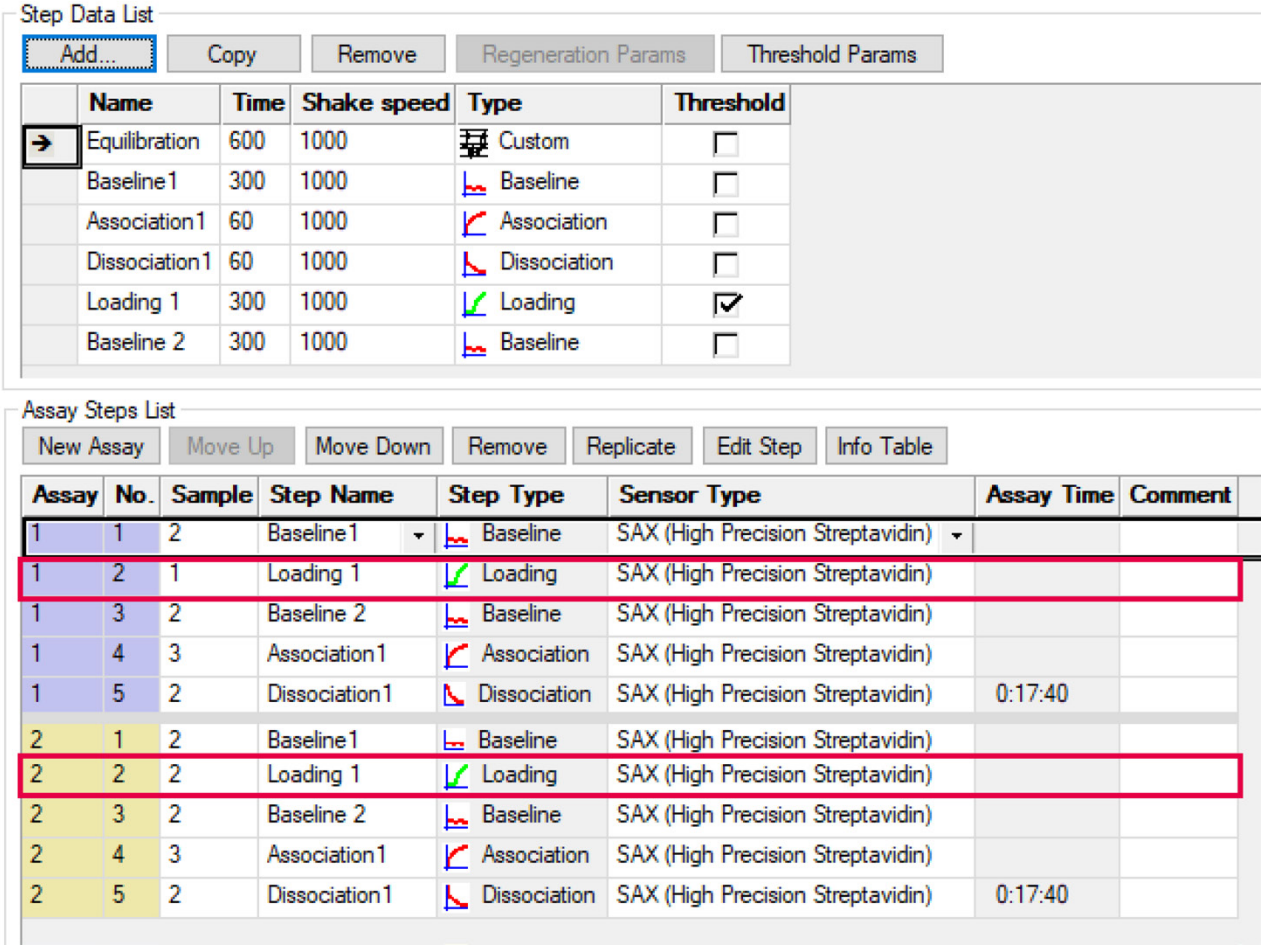
Figure 2: Reference biosensors are added in a new assay step but unlike the biosensors used to assessed binding to the analyte, are not loaded with ligand.

A



Well	Sample ID	Replicate Group	Type	Conc (µg/ml)	Dilution Factor	MW (kD)	Molar Conc (nM)	Information
A1	Lignad		Load	0.2	n/a			
B1	Lignad		Load	0.2	n/a			
C1	Lignad		Load	0.2	n/a			
D1	Lignad		Load	0.2	n/a			
E1	Lignad		Load	0.2	n/a			
F1	Lignad		Load	0.2	n/a			
G1	Lignad		Load	0.2	n/a			
A2	Assay Buffer		Buffer		n/a			
B2	Assay Buffer		Buffer		n/a			
C2	Assay Buffer		Buffer		n/a			
D2	Assay Buffer		Buffer		n/a			
E2	Assay Buffer		Buffer		n/a			
F2	Assay Buffer		Buffer		n/a			
G2	Assay Buffer		Buffer		n/a			
A3	Analyte		Sample				20000	
B3	Analyte		Sample				6667	
C3	Analyte		Sample				2222	
D3	Analyte		Sample				740.7	
E3	Analyte		Sample				246.9	
F3	Analyte		Sample				82.3	
G3	Analyte		Sample				0	

B



	Name	Time	Shake speed	Type	Threshold
→	Equilibration	600	1000	Custom	☐
	Baseline1	300	1000	Baseline	☐
	Association1	60	1000	Association	☐
	Dissociation1	60	1000	Dissociation	☐
	Loading 1	300	1000	Loading	☑
	Baseline 2	300	1000	Baseline	☐

Assay	No.	Sample	Step Name	Step Type	Sensor Type	Assay Time	Comment
1	1	2	Baseline1	Baseline	SAX (High Precision Streptavidin)		
1	2	1	Loading 1	Loading	SAX (High Precision Streptavidin)		
1	3	2	Baseline 2	Baseline	SAX (High Precision Streptavidin)		
1	4	3	Association1	Association	SAX (High Precision Streptavidin)		
1	5	2	Dissociation1	Dissociation	SAX (High Precision Streptavidin)	0:17:40	
2	1	2	Baseline1	Baseline	SAX (High Precision Streptavidin)		
2	2	2	Loading 1	Loading	SAX (High Precision Streptavidin)		
2	3	2	Baseline 2	Baseline	SAX (High Precision Streptavidin)		
2	4	3	Association1	Association	SAX (High Precision Streptavidin)		
2	5	2	Dissociation1	Dissociation	SAX (High Precision Streptavidin)	0:17:40	

Replicating Steps within an Assay

A sample plate can include multiple assays that includes either replicates appended to the end of the current assay or added to a new assay. As each assay utilizes a new set of biosensors, replicates appended to the end of the current assay use the same biosensor and replicates added to a new assay use different biosensors. Here, the new assay format is used.

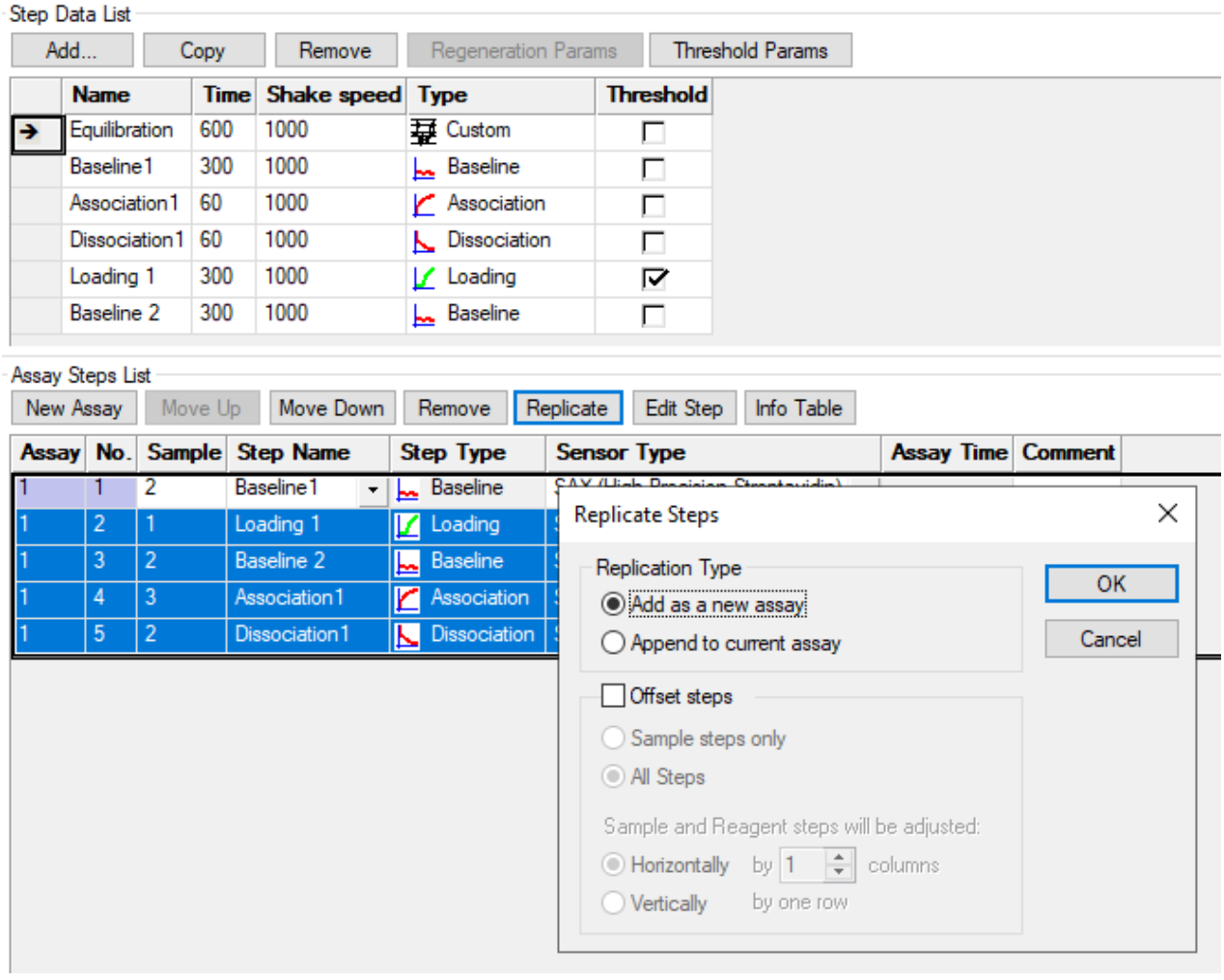
In the Octet® BLI Discovery Software, reference biosensors can be added to a standard assay setup through the addition of a new assay step.

To copy steps and add them to an assay:
In the Assay Steps List, select the step(s) to copy and click Replicate (for example, in Figure 3, step rows 1–5 are selected).

- To select adjacent steps, press and hold the Shift key while you click the first and last step in the selection.
- To select non-adjacent steps, press and hold the Ctrl key while you click the desired steps.

In the Replicate Steps dialog box (Figure 3), click the Add as a new assay option.

Figure 3: Replicating Steps within an Assay

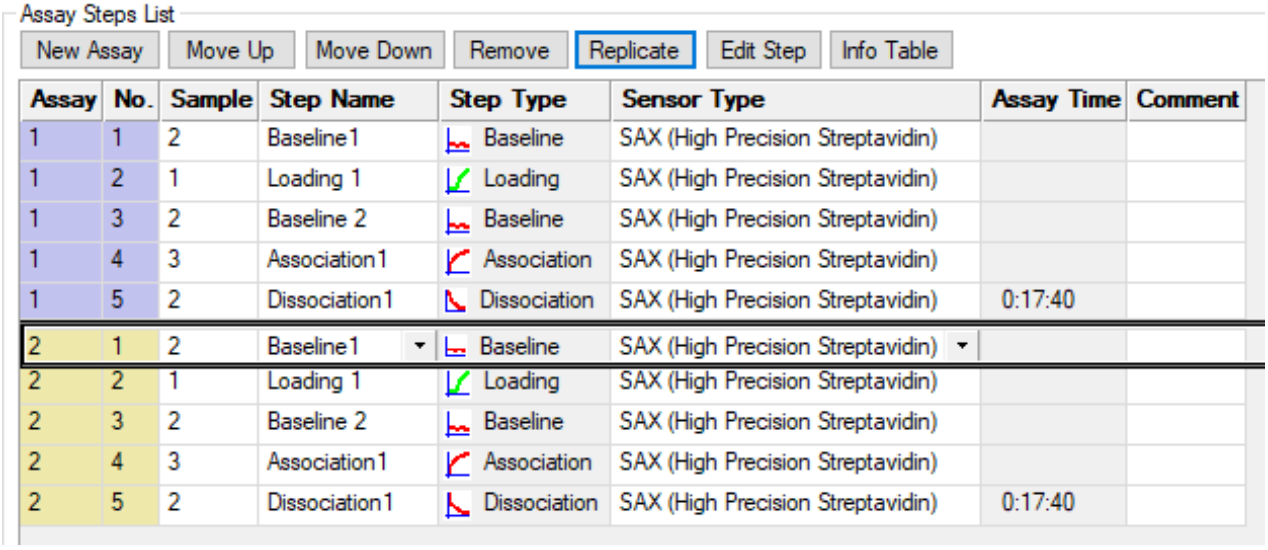


Click OK. The step(s) appear as a new assay at the end of the Assay Steps List (Figure 4).

For the purpose of double reference subtraction, the biosensors used for NSB purposes should either contain a non-specific ligand that has a similar composition to that on the active biosensor or have no ligand present.

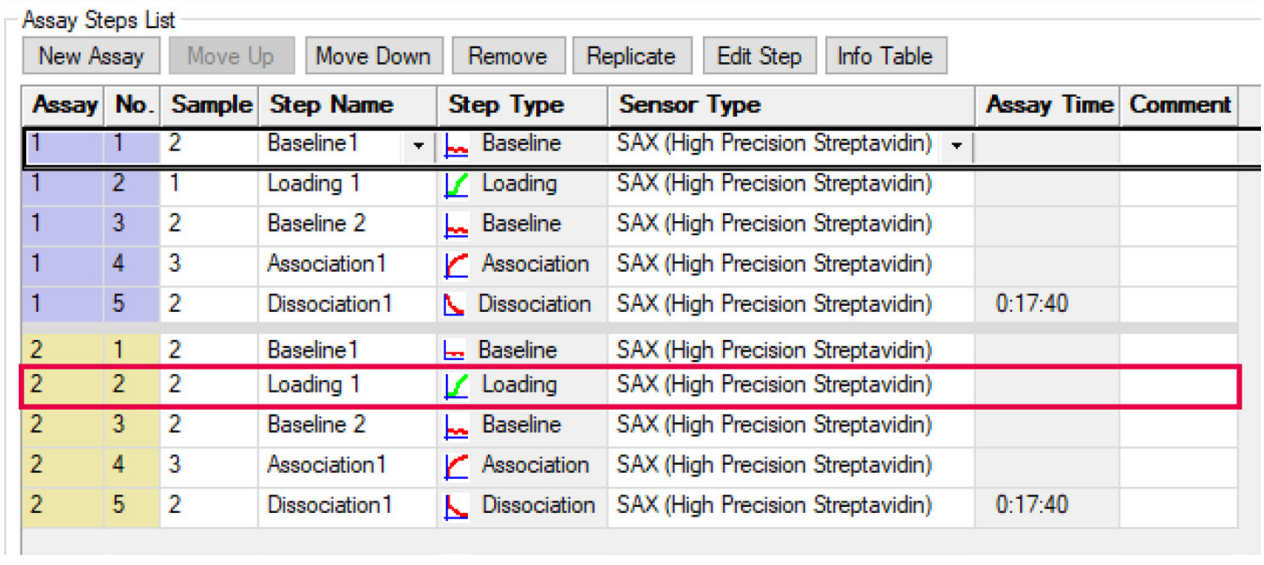
Here, a naive biosensor is used and therefore, the assay step associated with loading of the ligand should be changed to load in buffer. As shown in Figure 5, Assay 2, step 2 (Loading 1) is changed from column 1 (Ligand) to column 2 (Buffer) in the sample plate. It is vital that the NSB biosensor should be exposed to the same assay conditions as the active biosensor.

Figure 4: Replicated Assay Steps (Assay 2) Appear Below the Original Assay (Assay 1).



Assay	No.	Sample	Step Name	Step Type	Sensor Type	Assay Time	Comment
1	1	2	Baseline1	Baseline	SAX (High Precision Streptavidin)		
1	2	1	Loading 1	Loading	SAX (High Precision Streptavidin)		
1	3	2	Baseline 2	Baseline	SAX (High Precision Streptavidin)		
1	4	3	Association1	Association	SAX (High Precision Streptavidin)		
1	5	2	Dissociation1	Dissociation	SAX (High Precision Streptavidin)	0:17:40	
2	1	2	Baseline1	Baseline	SAX (High Precision Streptavidin)		
2	2	1	Loading 1	Loading	SAX (High Precision Streptavidin)		
2	3	2	Baseline 2	Baseline	SAX (High Precision Streptavidin)		
2	4	3	Association1	Association	SAX (High Precision Streptavidin)		
2	5	2	Dissociation1	Dissociation	SAX (High Precision Streptavidin)	0:17:40	

Figure 5: Biosensors used for Double Reference Subtraction Should not Contain any Ligand and the Loading Assay Step Should be Performed in Asay Buffer.



Assay	No.	Sample	Step Name	Step Type	Sensor Type	Assay Time	Comment
1	1	2	Baseline1	Baseline	SAX (High Precision Streptavidin)		
1	2	1	Loading 1	Loading	SAX (High Precision Streptavidin)		
1	3	2	Baseline 2	Baseline	SAX (High Precision Streptavidin)		
1	4	3	Association1	Association	SAX (High Precision Streptavidin)		
1	5	2	Dissociation1	Dissociation	SAX (High Precision Streptavidin)	0:17:40	
2	1	2	Baseline1	Baseline	SAX (High Precision Streptavidin)		
2	2	2	Loading 1	Loading	SAX (High Precision Streptavidin)		
2	3	2	Baseline 2	Baseline	SAX (High Precision Streptavidin)		
2	4	3	Association1	Association	SAX (High Precision Streptavidin)		
2	5	2	Dissociation1	Dissociation	SAX (High Precision Streptavidin)	0:17:40	

After defining the sample plate and assays, click the Sensor Assignment tab, or click the arrow to access the Sensor Assignment window. The color-coded Sensor Tray and Sample Plate Map show the locations of the biosensors associated with the samples Figure 6.

The biosensor types shown in the Sensor Type table column are those designated during the kinetics assay definition. In the example shown in Figure 6, the experiment includes two assays in the same wells. The use of those wells by two different biosensors is indicated by the pie chart colors.

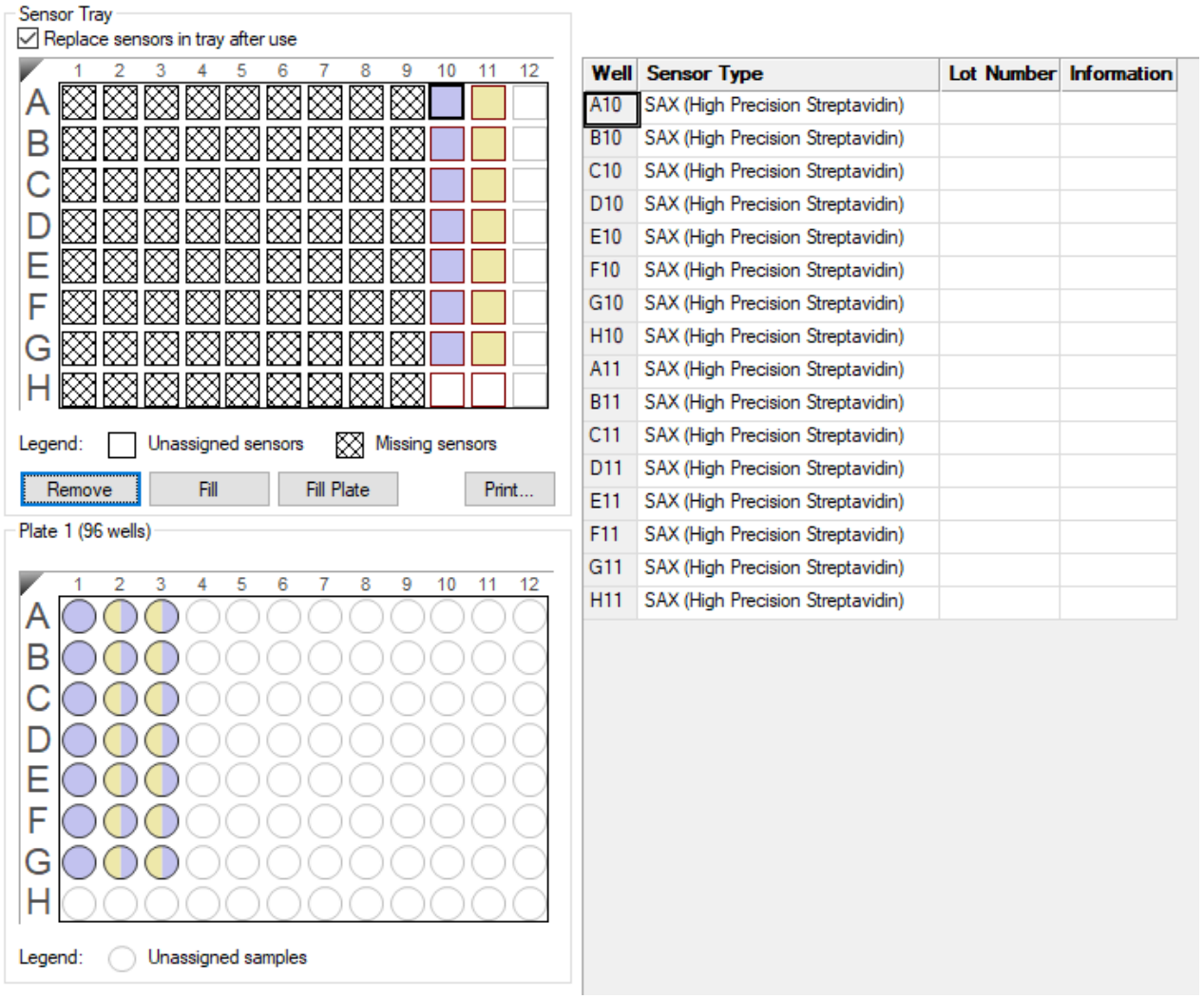
Click through to the Review Experiment tab. Using the assay step arrow, click through the assay to ensure the correct step sequence has been input.

If incorrect, return to the Assay Definition tab and correct the column associated with the assay step. If correct, click through to the Run Experiment tab.

In the Kinetics data repository section, click the three dots and select a suitable location for saving the assay.

To start the experiment, click GO.

Figure 6: Sensor Assignment Window



Well	Sensor Type	Lot Number	Information
A10	SAX (High Precision Streptavidin)		
B10	SAX (High Precision Streptavidin)		
C10	SAX (High Precision Streptavidin)		
D10	SAX (High Precision Streptavidin)		
E10	SAX (High Precision Streptavidin)		
F10	SAX (High Precision Streptavidin)		
G10	SAX (High Precision Streptavidin)		
H10	SAX (High Precision Streptavidin)		
A11	SAX (High Precision Streptavidin)		
B11	SAX (High Precision Streptavidin)		
C11	SAX (High Precision Streptavidin)		
D11	SAX (High Precision Streptavidin)		
E11	SAX (High Precision Streptavidin)		
F11	SAX (High Precision Streptavidin)		
G11	SAX (High Precision Streptavidin)		
H11	SAX (High Precision Streptavidin)		

Data Analysis

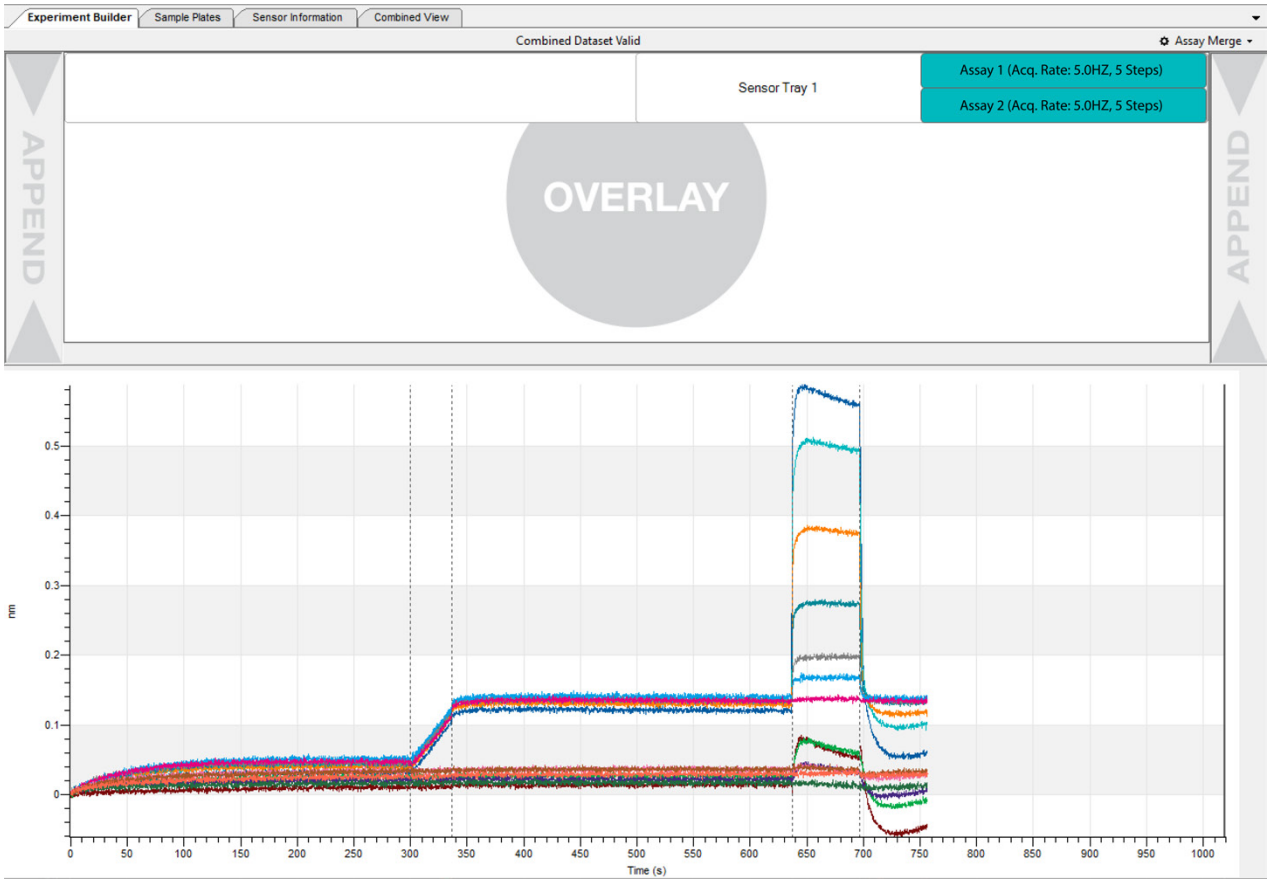
After data generation, the assay is assessed using Octet® Analysis Studio.

To view the assay data, open the data folder by double-clicking the magenta-colored data folder in the Experiment Explorer window. The assay data then appears in the Experiment Builder preview tab and shows both the ligand loaded biosensor data (Assay 1) and ligand unloaded biosensor data (Assay 2) (Figure 7).

In order to use the assay 2 biosensor data for double reference subtraction the assay data must be aligned with the assay 1 data.

Important: Where a signal change threshold parameter has been used for ligand loading it may be necessary to truncate the loading step time(s) so that the assay data aligns. It is recommended that users read Chapter2 - Editing Step for more information on how to Truncate Step Times.

Figure 7: The Experiment Builder tab shows both the ligand loaded biosensor data (Assay 1) and ligand unloaded biosensor data (Assay 2).

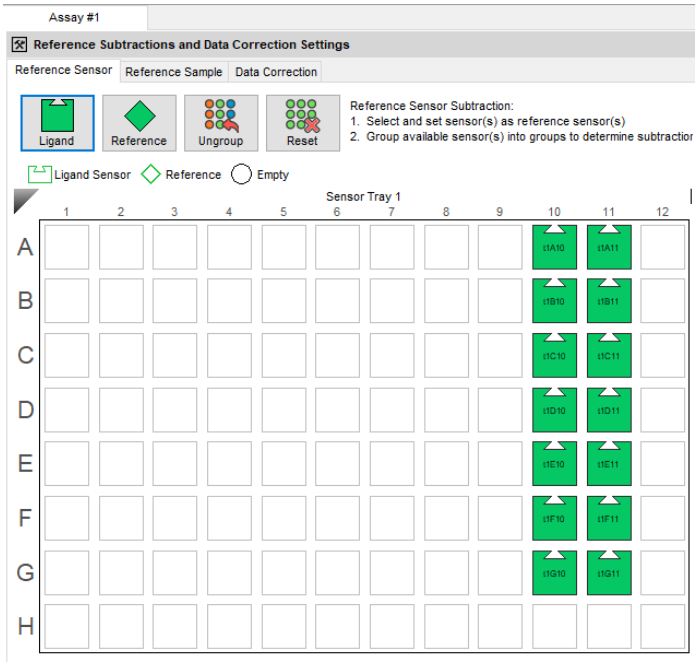


Preprocessing Datasets – Reference Biosensor

Before kinetic or steady state analysis, the Preprocess Data Screen lets you subtract any non-specific binding or baseline drift data and make other data corrections if necessary. Click the Preprocess Data tab to view the Preprocess Data screen.

To subtract non-specific binding in experiments where reference biosensors were used for analyte binding with no ligand present, click the Reference Sensor tab in the Reference Subtractions and Data Corrections window (Figure 8).

Figure 8: Reference Sensor Tab

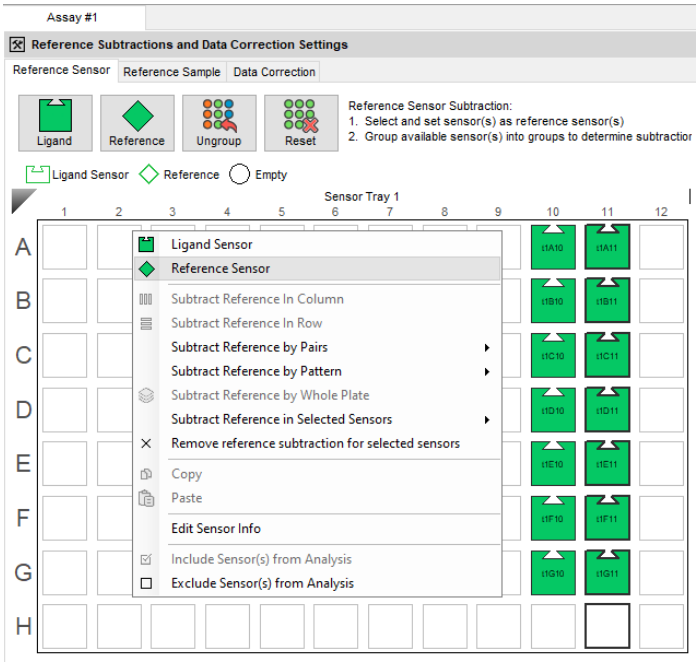


To identify and set reference biosensors, select the biosensors assigned to correct for non-specific binding.

Select Column 11 and right-click on a selected biosensor. In the pop up select Reference Sensor (Figure 9). You can also click the Reference Sensor button to change the sensor type.

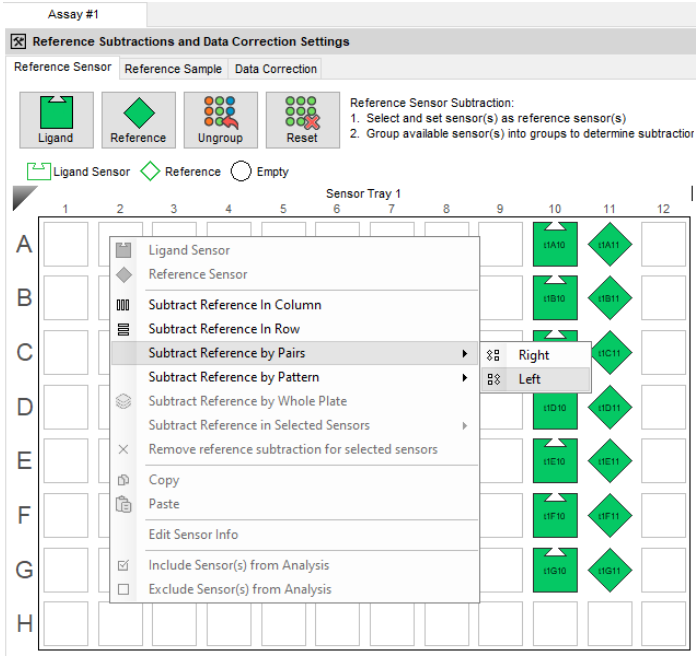
The selected biosensors display as diamonds, indicating they are set as reference biosensors.

Figure 9: Setting Reference Sensors



To subtract specific ligand and reference sensors - select Columns 10 and 11, right-click anywhere on the biosensor tray and select Subtract Reference by Pairs and select Left (Figure 10).

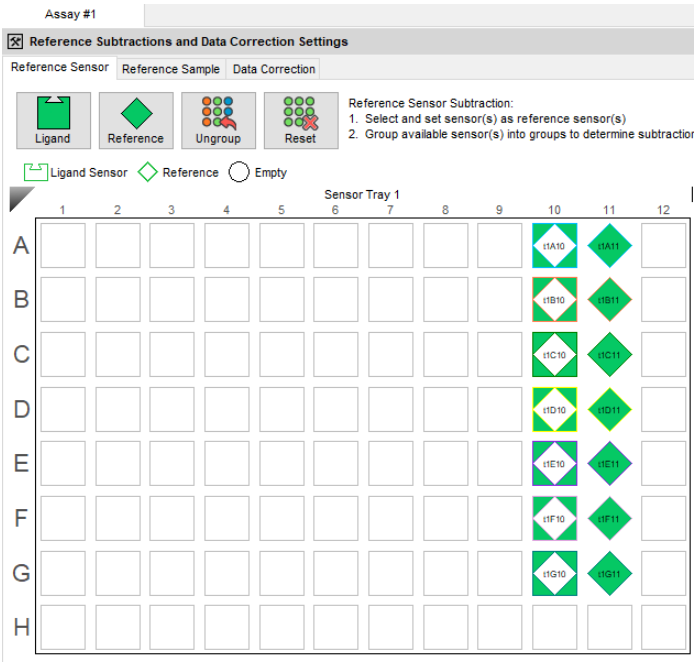
Figure 10: Subtracting Specific Wells



Preprocessing Datasets – Reference Sample

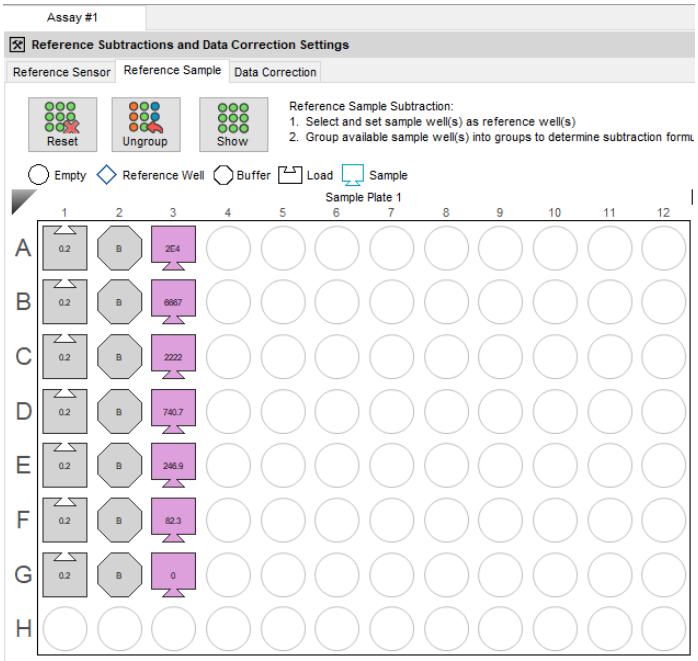
Reference subtracted biosensors are shown as squares with diamond subtraction (Figure 11).

Figure 11: NSB Reference Subtracted Biosensors.



To subtract any baseline drift using reference sample wells that contain ligand but no analyte, click the Reference Sample tab in the Reference Subtractions and Data Corrections window (Figure 12).

Figure 12: Sample Plate Showing Concentrations for Sample/Load Wells.

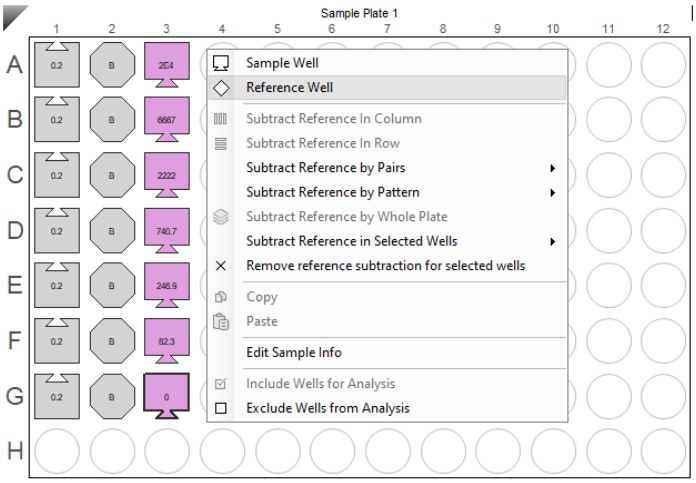


Subtracting Baseline Drift

As shown in Figure 13, the assay uses an analyte concentration of 82.3 – 20,000 nM and well G3 is the reference sample well containing only buffer.

- Select well G3 and then Right Click anywhere on the sample plate and select Reference Well (Figure 13)

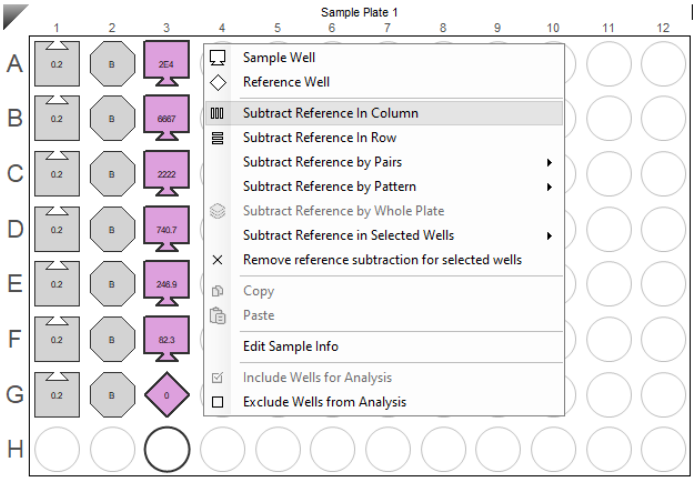
Figure 13: Setting the Reference Sample Well.



The selected well appears as a diamond, to indicate it is set as reference well.

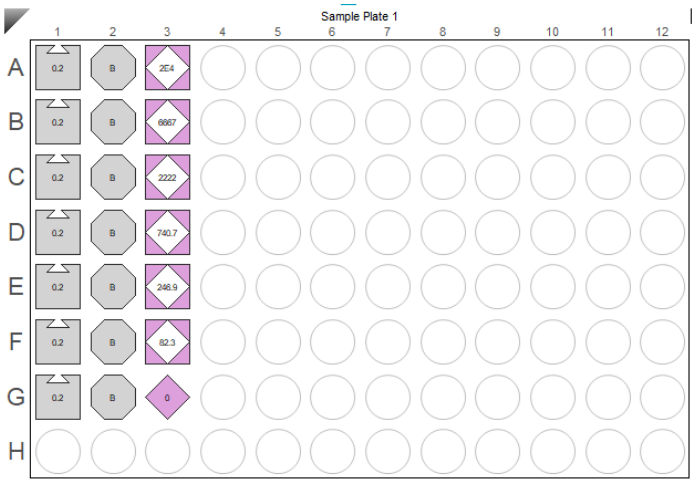
- To subtract the reference well - select the analyte containing column, right click anywhere on the highlighted samples and select Subtract Reference in Column (Figure 14).

Figure 14: Subtracting Reference Well.



Reference sample subtracted biosensors are shown as squares with diamond subtraction (Figure 15).

Figure 15: Reference Sample Subtracted Biosensors.



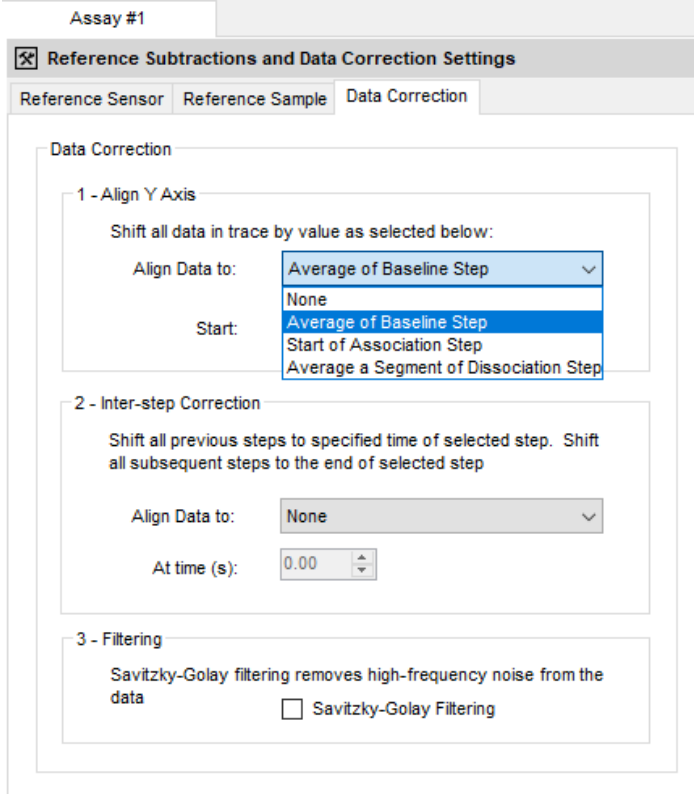
Aligning the Y Axis

To fit curves correctly, they need to be aligned to a common reference point on both the X and Y axes. Curves are aligned to the X-axis automatically during the assay as biosensors move in parallel.

The Align Y Axis function lets you apply a Y-axis adjustment to align the start of the association phase to zero (Figure 16). Most kinetic fitting models require a zero baseline for fitting, so this step is almost always a preparatory step in kinetic evaluation

1. Click the "Align Data" to box to select "Average of Baseline Step".

Figure 16: Align Y-Axis Data To Selections.

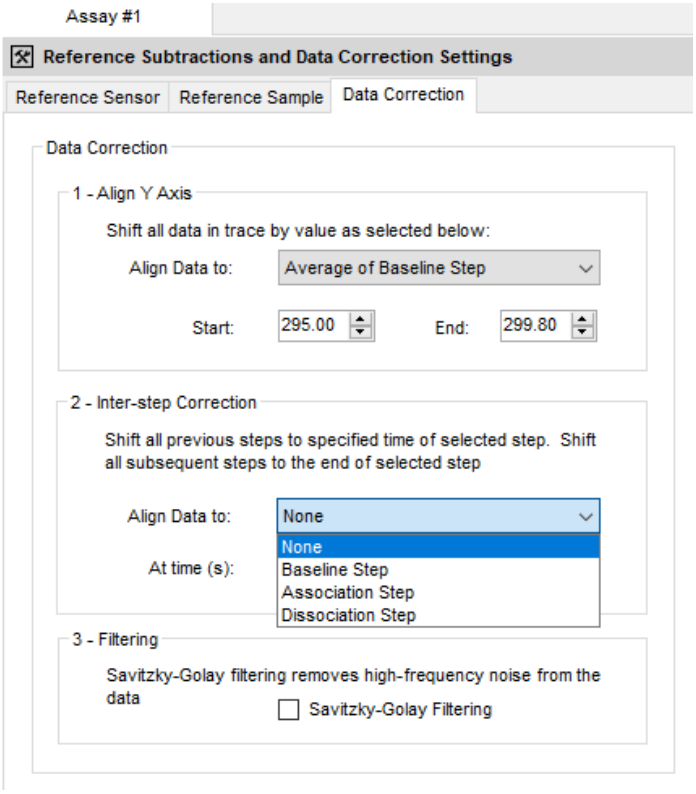


Inter-step Correction

This option lets you correct misalignment between steps that can occur when biosensors move between the wells of the plate. Inter-step correction should only be used for experiments where the baseline and dissociation steps were done in the same well. It is not recommend using the inter-step correction with very fast kinetics as some kinetic information can be lost.

1. Click the "Align Data to" box and select "None".

Figure 17: Align Inter-step correction Data to Selections.



Kinetic Analysis

As shown in Figure 18A, double reference subtraction using reference sensors corrects for NSB and allows the 'true' interaction value to be determined unlike the single reference subtracted data (Figure 18B), which exhibits the hallmarks of NSB.

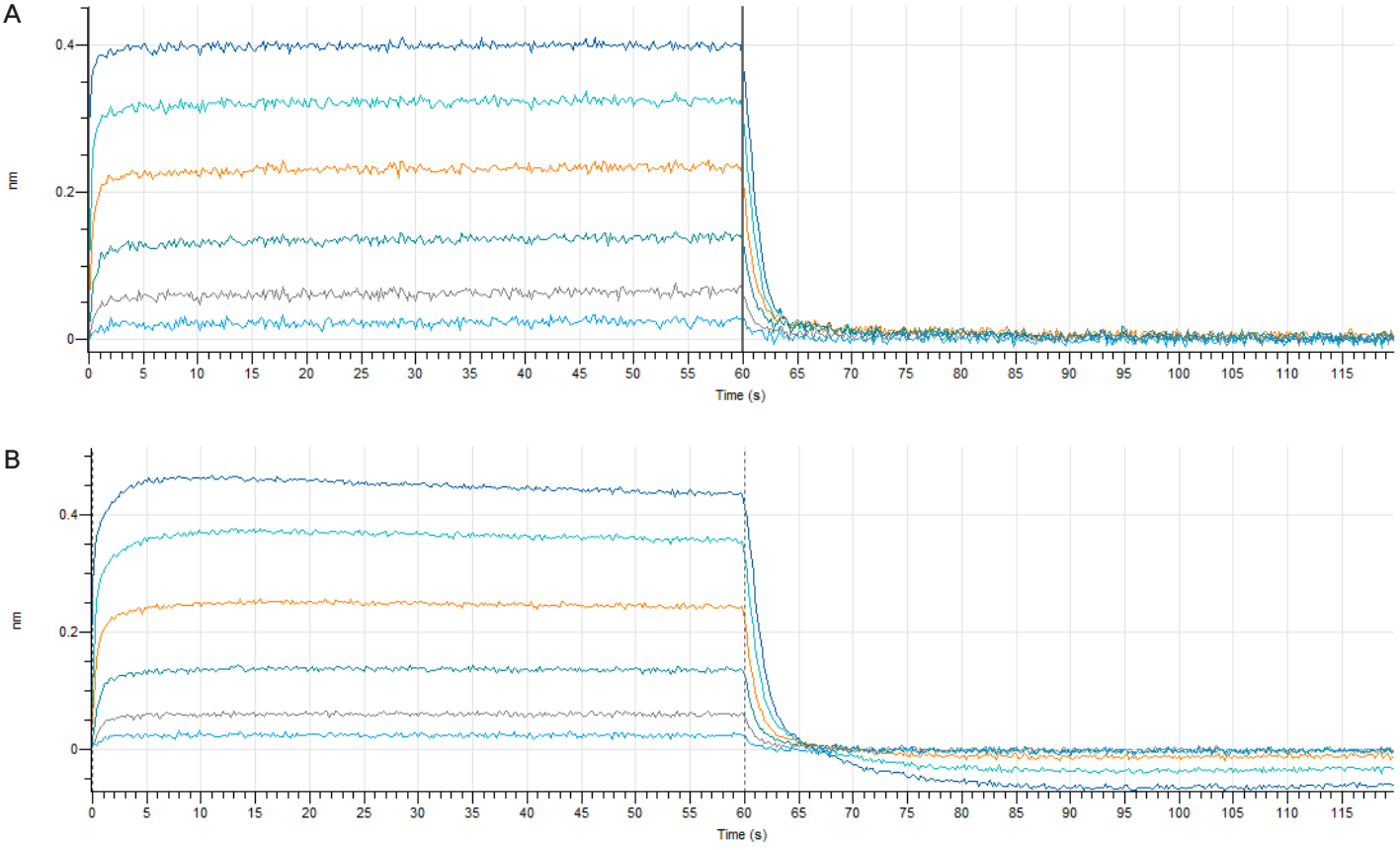


Figure 18: (A) Double reference subtracted data showing NSB corrected data, which exhibits no drift during the association phase and returns to baseline during the dissociation phase. (B) Single/reference sample subtracted data exhibiting artifacts caused by NSB. In general, these may include sensorgrams that show an upward or downward drift during the association phase and during the dissociation phase the binding signal may exhibit a negative signal and not return to baseline.

Conclusion

Double reference subtraction, using additional biosensors, is a particularly effective method to help correct for NSB. This approach is crucial for distinguishing true binding events from non-specific interactions, especially in complex assays or those involving small molecule binding where signal-to-noise ratios are low. By employing both reference samples and reference biosensors, double reference subtraction provides a robust mechanism for correcting NSB.

The strategies outlined in this document are essential for researchers and practitioners working with Octet® BLI biosensors, as they provide practical solutions to enhance the precision of biosensor assays. By minimizing NSB, these techniques ensure that the true binding affinities and kinetics are accurately captured, thereby supporting more reliable and meaningful conclusions from biosensor-based assays.

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