SARTORIUS

Best Practice Guide

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Best Practice Guide: Double Reference Subtraction

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Introduction

Data processing of surface plasmon resonance (SPR) sensorgrams is necessary to eliminate systematic artifacts such as injection noise, non-specific binding, baseline drift, and bulk refractive index (RI) changes between the running buffer and analyte.

After correcting for these artifacts, high-quality data can be generated for further data analysis and fitting to appropriate kinetic models using the Octet® SPR Analysis software.

Whether using low-throughput SPR as a secondary characterization tool to study binding interactions, or high-throughput SPR as a preliminary screening tool to survey hundreds of binding interactions in a single assay, there are some established best practices that aid in improving the quality of biosensor data and minimizing artifacts. When the goal is determining the kinetic rate and affinity constants of a biomolecular binding interaction, a checklist of a few key points should be followed to minimize assay artifacts. Here, we provide a synopsis of those recommendations.

The Purpose of Referencing in Surface Plasmon Resonance

A commonly misunderstood concept of SPR is the importance of double reference subtraction during data analysis. Assay orientation and minimization of assay artifacts using a suitable reference surface contribute to the ability to collect high-quality data on the Octet® SF3 system (see Best Practices Guides on Assay Orientation, Minimizing Artifacts, and Minimizing the Effects of Non-specific Binding for further information).

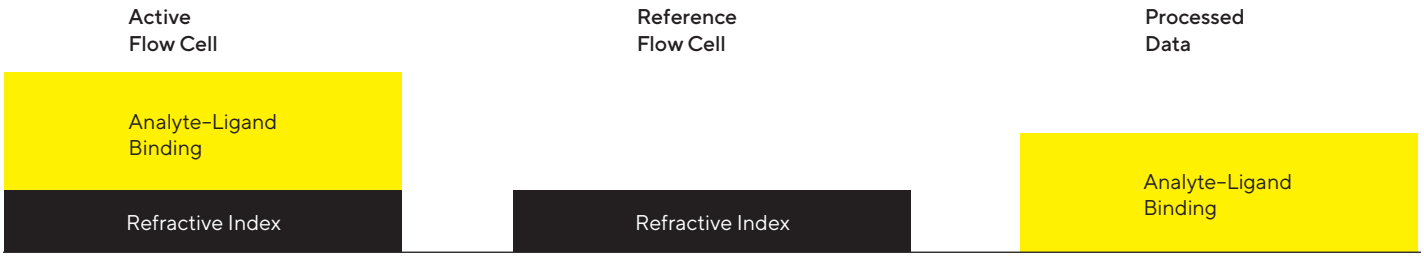
Correcting for these surface-to-surface artifacts between the active (ligand immobilized) and reference flow cells for the analyte-containing sample solution involves a processing step called "referencing." A second referencing step is required to subtract the highly reproducible and systematic deviations in the data collected for buffer blanks, occurring within the active and reference flow cells. Performing the above two referencing steps is referred to as "double referencing." While artifacts related to buffer referencing may be subtle and only appear noticeably in data collected on low-capacity surfaces, it is advisable to apply double referencing routinely regardless of the capacity of the immobilized ligand surface, to ensure the highest quality data input for use in fitting routines.

Double Reference Subtraction

Subtraction of the analyte response to the active and reference surface, and the buffer blank response to the same two surfaces is known as double reference subtraction, and is recommended to be performed to determine the "true" binding response (Figure 1).

In the first step, the reference surface is subtracted from the active surface for the analyte-containing sample solution. This single reference subtraction allows the binding response of the analyte to the ligand on the active flow cell to be determined, as the ligand isn't present on the reference flow cell and the analyte interaction with the sensor chip surface with | without a reference protein is removed by subtracting the reference flow cell. This single subtraction can remove most issues with injection noise. In double reference subtraction, the binding response of the buffer blank is corrected for across the active and reference flow cells and this is subtracted from the analyte-containing sample buffer to correct for any refractive index changes caused by the sample buffer. This double subtraction can remove most issues with refractive index changes commonly referred to as "spiking."

Figure 1



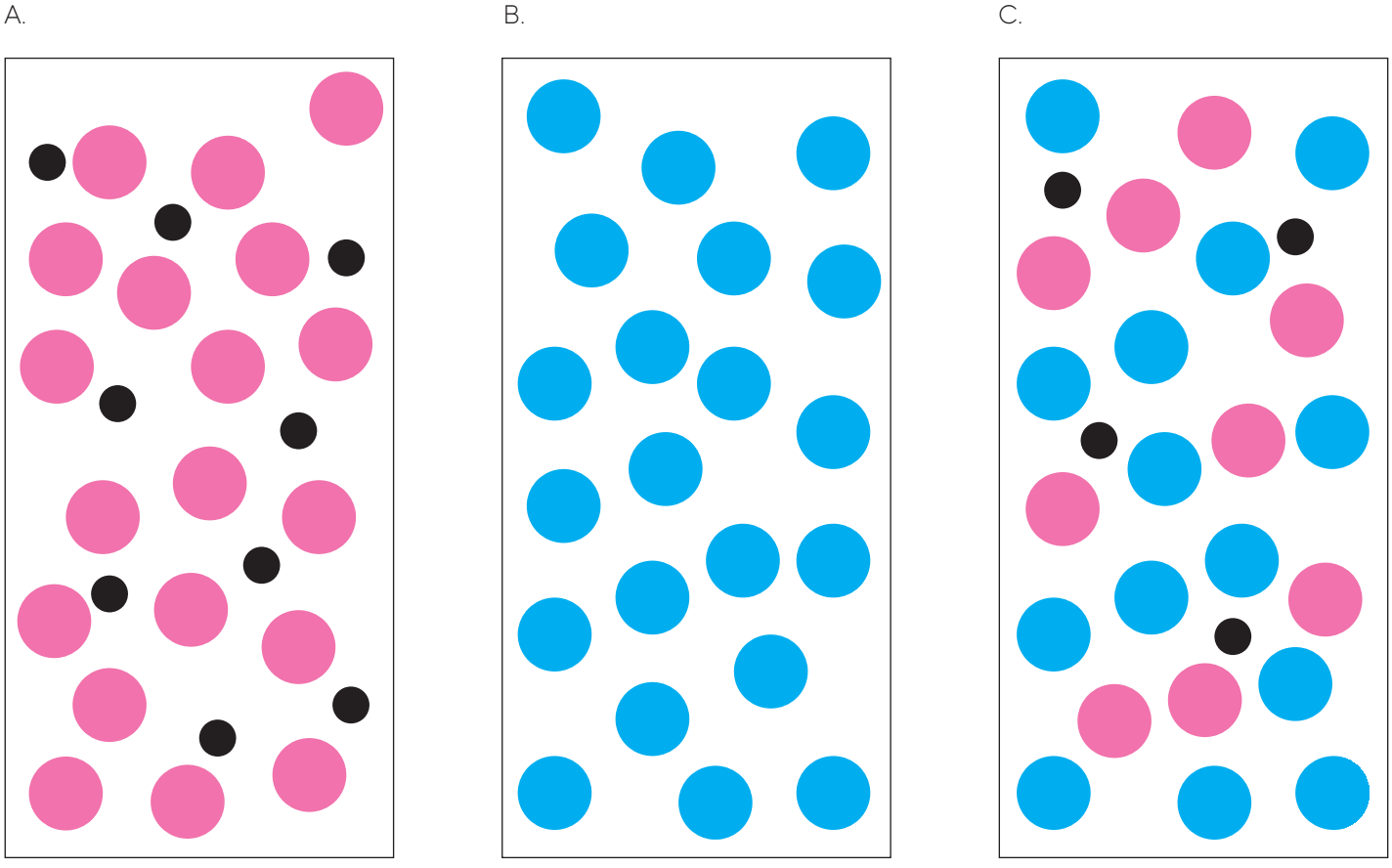
Note. In experiments where the analyte is in a sample buffer that exhibits a low RI, reference subtraction allows the "true" analyte–ligand binding to be assessed.

Solution Types

To understand the necessity of double reference subtraction in SPR the role of the solutions must be considered. In the simplest case, two different solutions are prepared during an SPR assay, the analyte containing sample solution and the running buffer (Figure 2). These solutions are then sequentially injected over an active and reference flow cell (Figure 3).

As shown in Figure 2, the analyte-containing sample solution may contain additional excipients or impurities that are not present in the running buffer (that will subsequently be used for buffer blanks). Therefore, buffer exchange of the sample solution into the running buffer (it is best practice to discard the running buffer that the sample has been exchanged into) ensures that excipients are removed and do not affect the observed SPR data through refractive index bulk effects (See Refractive Index section below). Buffer exchange is especially important when performing multi-cycle kinetics, as the excipient level is diluted along with the sample and can potentially cause a different RI response at each concentration. This cannot be normalised out through reference subtraction, and it is best practice to have no jump present at the start or end of the association phase.

Figure 2



Note. The stock sample solution (A) contains the desired analyte (violet dots) and may also contain excipients or impurities that will be carried over to the sample preparation (black dots). The stock sample solution is mixed into running buffer (B) (blue dots) to create the analyte-containing sample solution at the desired concentration (C).

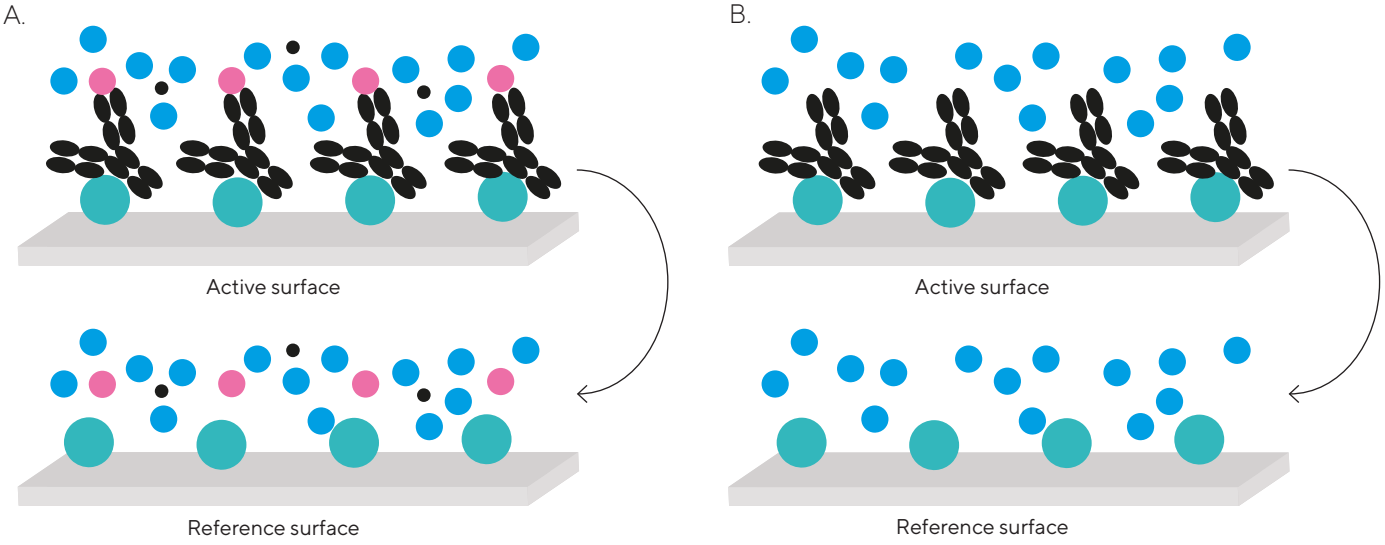
Reference Surface

Inclusion of a suitable reference surface improves the quality of the binding data by correcting for multiple artifacts (refractive index changes [bulk effect], matrix effects, non-specific binding, injection noise and baseline drift). Therefore, to compensate for differences between the analyte-containing sample solution and the buffer solution, reference subtraction is performed (Figure 3).

In the first example the sample solution containing the analyte is injected over the active flow cell that contains the ligand of interest, and a real-time binding response is observed as the analyte binds to the ligand. The same sample solution then passes over a suitable reference surface (Figure 3A), allowing reference subtraction. The same process is then repeated for a buffer blank and the response of the sample buffer to the active and reference is determined (Figure 3B), which allows double reference subtraction for bulk effects (refractive index changes) and non-specific binding.

When designing assays, it is important to note that deviations seen on low-capacity sensor chip surfaces are very consistent (in both the sample solution and buffer) so the buffer blank injections response can be used for all the data in the procedure. As best practice, buffer blank injections should be performed at the beginning, middle and end of a run to ensure that no surface alteration has occurred. In general, variation in buffer blank injections should be very rare and are more likely caused by injection and system issues. Therefore, the buffer blank is passed over the same surfaces as used for the sample solution and the average of the response from the blank injections subtracted from the entire data set.

Figure 3



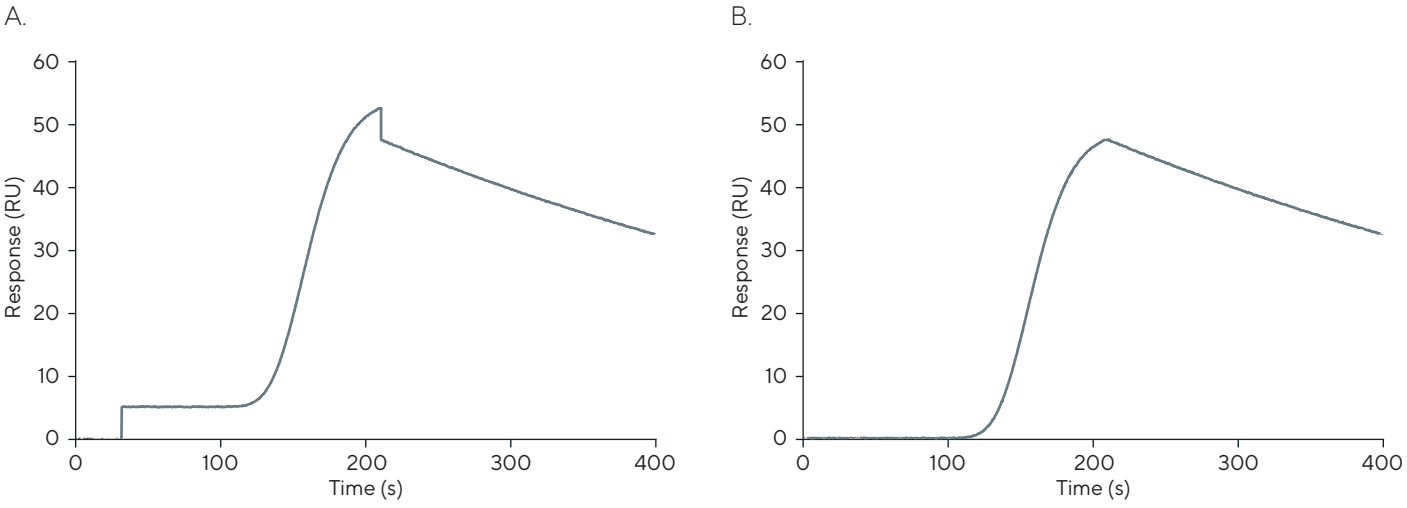
Note. Analyte-containing sample solution is injected across the ligand-containing active surface and a suitable reference surface (A), which allows reference subtraction of the reference surface. Buffer blank is also injected across the same active and reference surfaces (B) and the observed response is used for double reference subtraction, which helps compensate for bulk, non-specific binding and baseline drift.

Refractive Index

Refractive index "spiking" or "jumps" are commonly observed at the start and end of sample solution injections during SPR assays and are caused by a mismatch in the refractive index between the sample solution and the buffer solution (Figure 4).

These RI differences can partially be referenced out during double reference subtraction prior to kinetic data analysis but best practice is to ensure samples are buffer exchanged into the running buffer prior to assessment. This is especially relevant for commercially sourced lyophilised recombinant proteins where excipients that contribute to the observed response such as trehalose are included as cryoprotectants.

Figure 4



Note. (A) Sample OneStep™ sensorgram with a 5 RU RI jump between the sample solution and the running buffer. (B) Buffer exchange of the sample solution into the running buffer can minimize or remove RI jump without the need for double reference subtraction.

Conclusion

Double reference subtraction forms a critical part of processing sensorgrams for subsequent data analysis through the removal of system artifacts and assay artifacts, although it must be noted that double reference subtraction requires high-quality sensorgrams before processing.

Proper assay design is critical to ensure that reference channel (single referencing) and buffer referencing (double referencing) can be performed and should show minimal baseline drift or bulk effects, which is observable by no RI jump between the end of the association phase and the start of the dissociation phase.

It is also best practice to ensure that the parameters for buffer referencing match that of the analyte-containing sample solution; these include the flow rate and association and dissociation times. By following best practices during assay design and development, double referencing allows the user to prepare high-quality sensorgrams that are suitable for data analysis.

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