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Application Note

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Reducing Variability in Small Molecule Screening and Kinetics Applications

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Abstract

Small molecule analysis can be affected by background signals. By minimizing the background variability, smaller positive signals can be resolved, leading to an increase in assay sensitivity. Optimization of the reference biosensor surface increases signal-to-noise levels, which improves the detection of small molecules, including fragments.

This technical note describes the use of a referencing method which results in the standard deviation of signals resulting from buffer controls for the model system being ~1–3 pm lower compared to standard reference methods. Because 1–3 pm of standard deviation translates to 3–9 pm of variances in the detection limit, true signals can be discriminated from those close to background levels.

Introduction

Minimizing the variability of background signals is a key parameter to the success of demanding applications such as small molecule analysis[1]. By reducing the background variability, smaller positive signals can successfully be resolved, providing an increase in assay sensitivity. This application note demonstrates the use of a reference biosensor with biotinylated streptavidin for increasing signal-to-noise levels, thereby improving the detection of small molecules, including fragments.

Small Molecule Kinetics And Fragment Screening on the Octet® Instruments

Sartorius' Super Streptavidin (SSA) Biosensor is the primary type of biosensor used for small molecule applications. SSA Biosensors are typically blocked with biocytin when used as a reference surface (for more details on running a small molecule assay, see Technical Note 16, Small Molecule Binding Kinetics). However, the optical properties of this reference surface are distinct from those of the target surface, presumably due to the increase in optical thickness accompanying an increase in the protein layer of the target biosensor. For high-sensitivity applications, this difference can introduce minor artifacts into the raw data, resulting in slightly higher variability when assessing compounds or negative controls.

To compensate for the difference in the optical properties between a protein-loaded target biosensor and the biocytin-blocked biosensor, a biotinylated protein can be used in place of biocytin. When using the referencing method described here, the standard deviation of the signals resulting from buffer controls for the model system are ~1–3 pm lower when compared to standard reference methods. This result is significant, since 1–3 pm of standard deviation translates to 3–9 pm of variances in the detection limit (where the LLOD = 3 X standard deviation + average background signal), which is important when discriminating true signals from those close to background levels.

Choosing a Protein for the Reference Biosensor

The optimal reference protein is one that is identical to the target protein, but that does not bind the molecules of interest. Some groups are fortunate and have protein mutants that are inactive, and these are ideal reference proteins for creating reference biosensors. If such a protein is available, it should be biotinylated similarly to the target protein (see Technical Note 6, Biotinylation of Protein for Immobilization onto Streptavidin Biosensors and Technical Note 12, Biotinylating Very Small Quantities of Protein for Immobilization onto Streptavidin Biosensors). For best results, the biotinylated reference protein should be loaded onto the SSA Biosensors at a density similar to the target protein. The biotinylated reference protein can be tested as described here.

More often, inactive versions of proteins are not available and a surrogate must be used. A blocked form of streptavidin is a useful surrogate—adding a layer of blocked streptavidin to the SSA Biosensors used as a reference makes the optical properties of the target and reference biosensors more similar. For best results, the loading signal of the reference protein should be similar to the loading signal of the target protein. The following protocol creates biotinylated, blocked streptavidin (SAV-B4) and describes how to use it on reference biosensors.

Preparing Biotinylated, Blocked Streptavidin (SAV-B4)

Materials Needed

Preparing the conjugate

- Streptavidin, 55 kD (Scripps Laboratories part no. S1214)
- Biotin, 244 Da (Pierce Protein Research Products part no 29129)
- Biotin-LCLC-sulfo-NHS, 670 Da (Pierce Protein Research Products part no. 21338)
- PBS (Invitrogen/Gibco or equivalent) 3–12 mL dialysis cassette, 10k MWCO (Pierce Protein Research Products)

Testing the conjugate

- Super Streptavidin Biosensors (Sartorius part no. 18-5057)
- Octet® R8, RH16 or RH96 instrument with software version 6.X or later
- Black, flat-bottom microplates (96-well: Greiner Bio-one part no. 655209; 384-well: Greiner Bio-one part no. 781209; 384-tilted well: Sartorius part no. 18-5080)

Technical resources

Available at www.sartorius.com/en/pr/octet

- Technical Note 6, Biotinylation of Protein for Immobilization onto Streptavidin Biosensors
- Technical Note 12, Biotinylating Very Small Quantities of Protein for Immobilization onto Streptavidin Biosensors
- Poster by C. Wartchow, et al. Small Molecule Fragment Screening on the Octet® Platform

Conjugate Preparation

1. Prepare a 100 µg/mL (400 mM) solution of biotin by adding 2.5 mg to 25 mL PBS.
2. Prepare 1 mg/mL solution of streptavidin (18 mM) by dissolving 6 mg of streptavidin in 6 mL of the 100 µg/mL biotin solution prepared in Step 1.
3. Prepare a 1 mg/mL solution (1.5 mM) of biotin-LCLC-sulfo-NHS by adding 5 mg to 5 mL of PBS buffer.
4. Add 73.2 µL of 1.5 mM biotin-LCLC-sulfo-NHS to 6 mL of the streptavidin solution containing 100 µg/mL biotin. The final biotin-LCLC-sulfo-NHS concentration will be 18 mM. Incubate for 1 hour at room temperature.
5. Place 1 L of PBS in a 1 L beaker and add a stir bar.
6. Transfer the streptavidin solution to the dialysis cassette according to the manufacturer's instructions. Dialyze against PBS buffer (3 X 1 L, 18 hours) in a refrigerator.
7. Transfer to a storage vessel, store at 4°C.
   Note: The stability of this conjugate at 4°C or at –20°C has not been assessed, and may require freezing if use extends beyond one week.

Conjugate Testing

Using the methods described in Technical Note 16, Small Molecule Binding Kinetics, test the loading of the biotin-reference protein on SSA Biosensors using an Octet® R8, RH16 or RH96 instrument. For best results, the signal (nm shift) from loading the biotinylated reference protein should be equivalent to the signal from loading the biotinylated target protein. If loading of the reference protein is higher or lower than that of the target protein, then the conditions (concentration, time of loading, etc.) for immobilizing the reference protein should be further optimized.

Sample Data Using Biotinylated, Blocked Streptavidin (SAV-B4) as a Reference Protein

The buffer control data shown in Figures 1 and 2 was generated during a study screening a small molecule library for binders to immobilized carbonic anhydrase. In this screening study, it was important to minimize the variability of the background signals in order to identify small molecule binding responses. For full details on the screening study, please refer to the poster by C. Wartchow, et al., Small Molecule Fragment Screening on the Octet® Platform.

To determine the baseline variability in this small molecule screening assay, 16 target biosensors and 16 reference biosensors were used on the Octet® RH16 instrument to assay a 384-well microplate filled with buffer. The resulting data was processed using a standard double-reference subtraction technique available in Octet® Analysis Studio Software, which uses the reference biosensors and a set of reference wells to correct for systematic baseline offsets. Ideally, after data processing the baseline will be stable, with no discontinuities. In reality, small artifacts can show up as a result of the optical changes from well to well.

For this example, either the biocytin-blocked SSA Biosensors or the SAV-B4 biosensors (preparation described previously) were used as references for the carbonic anhydrase-coated target biosensor. The typical loading signal for the biotinylated carbonic anhydrase was 6–10 nm and for the SAV-B4 conjugate was 4–7 nm. Figure 1 shows the double-reference subtracted data from the buffer-only plate when using either of these reference biosensors. When comparing the data using the biocytin-blocked biosensors and the SAV-B4 biosensors, both the average baseline signal and the standard deviation of the baselines are significantly lower when using the SAV-B4 biosensors as a reference. This improvement is even more apparent when looking at the calculated LLOD of signal for each assay based on these buffer controls (Table 1).

The experiment was repeated using the Sartorius 384-tilted well plate (384TW) and is shown in Figure 2. Using this plate in conjunction with the SAV-B4 reference biosensors, the LLOD is decreased from 24 to 11 pm (Table 1). This allows for positive signals in the 14–20 pm range to be identified as hits. These signals would have been lost in the baseline noise if the assay were run using the original biocytin-reference biosensors and a standard 384-well microplate.

Table 1: Summary of the data resulting from double reference subtraction processing of 32 target biosensors and 16 reference biosensors assaying a 384-well microplate of buffer only using the Octet® RH16 system (N=648 for each condition). LLOD was calculated as the [average + 3 X standard deviation] of the signals shown in Figures 1 and 2.

Plate	Reference biosensor	Average (pm)	Standard deviation (pm)	LLOD (pm)
384-well (standard)	SSA:Biocytin blocked	7	6	24
	SSA:SAV-B4	4	5	19
384-tilted well	SSA:Biocytin blocked	3	4	14
	SSA:SAV-B4	1	3	11

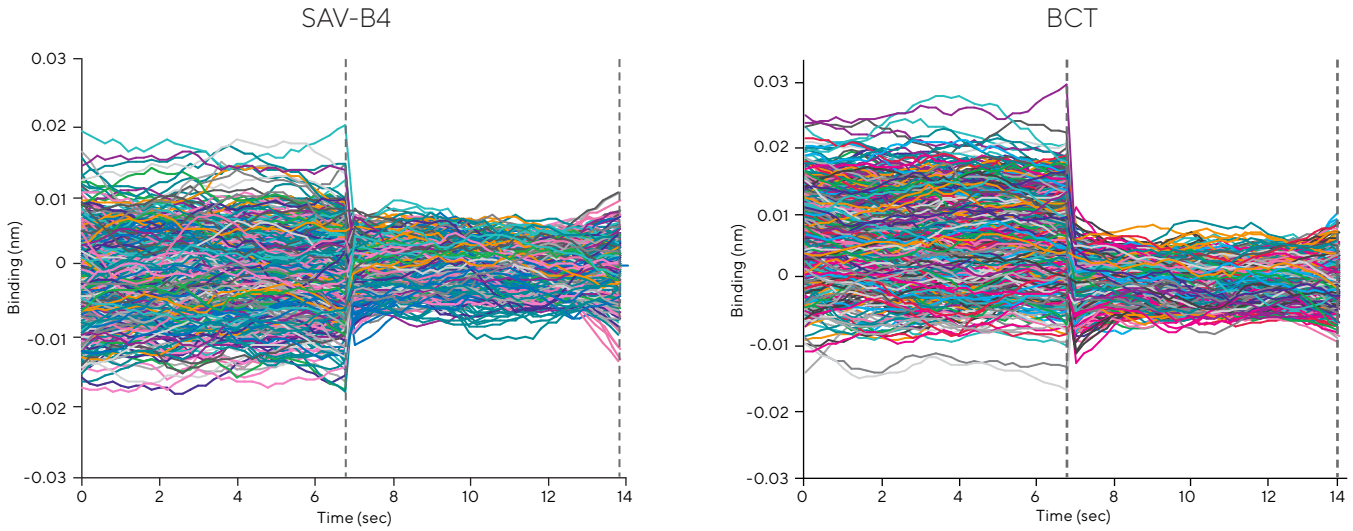

Figure 1: Comparison of the signal responses for the carbonic anhydrase biosensors in PBS buffer (a negative control) with blocked, biotinylated streptavidin coated reference biosensors (SAV-B4, left panel) and biocytin-blocked reference biosensors (BCT, right panel). Assay run using a standard 384-well microplate on the Octet® RH16 system. Data processed using double reference subtraction in Octet® Analysis Studio Software 6.3.

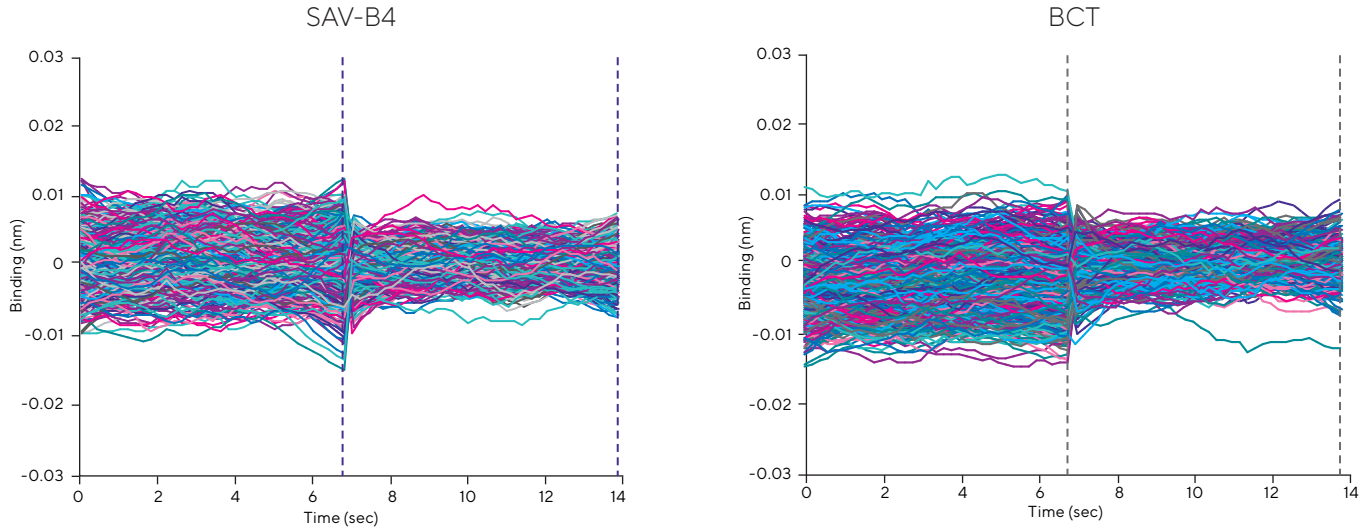

Figure 2: Data from a repeat of the experiment from Figure 1 using the Sartorius 384-tilted well microplate. Use of the 384TW plate further reduces the artifacts in the PBS-only negative controls.

References

1. Wartchow C, Podlaski F, Li S, Rowan K, Zhang X, Mark D and Huang KS. Biosensor-based small molecule fragment screening with biolayer interferometry, J Comput Aided Mol Des. 25 (7): pp 669–76, 2011.

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