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Optimizing Protein-Protein and Protein-Small Molecule Kinetics Assays

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Abstract

Quantitation of protein concentrations, screening antibodies in crude supernatants, characterization of protein-protein interaction kinetics (k_a, k_d and K_D), and screening small molecule fragment libraries are all essential components in the drug discovery process.

Kinetic analysis is a key application for the real-time, label-free Octet® platform. In this application note, strategies to achieve the best possible data, via optimization of target immobilization, analyte binding, regeneration strategies (if required) are discussed. With careful optimization of assay parameters, kinetics analyses can rapidly yield excellent data.

Introduction

Kinetic analysis of protein-protein and protein-small molecule interactions is a key application for real-time, label-free systems such as the Octet® instrument family. The Octet® platform is currently utilized in many segments of the pharmaceutical and biotherapeutic drug development processes such as early discovery, process development, late-stage clinical trials and manufacturing/QC. Applications include quantitation of protein concentrations in crude media, screening antibodies in crude supernatants, characterization of protein-protein interaction kinetics (k_a, k_d and K_D), and screening small molecule fragment libraries.

Tips and tricks for obtaining the best possible kinetic data are presented, including optimization of target immobilization and analyte binding, along with regeneration strategies (if required) and sample plate and method design. Octet® Analysis Studio Software options are also discussed, giving details of the analysis models and their applicability to data interpretation.

Target Molecule Immobilization

The most important consideration in choosing a strategy for target molecule immobilization is retaining biological activity.

Direct Coupling

Whilst direct covalent attachment results in stable, non-reversible target immobilization, a number of factors need to be considered. Direct coupling is usually performed directly to the biosensor surface, such as with amine coupling. This has the potential for loss of target activity due to steric hindrance. In amine coupling procedures, it is difficult to restrict the number of sites on the target molecule which are linked to the biosensor surface. Potentially, any free lysine residue can be involved in the linking, thus if lysines are close to the analyte binding site, loss of target activity can result.

The following requirements should be considered when performing amine coupling:

- The target must be pure, must not contain any extraneous amines, or be diluted in amine-containing buffer.
- The target must also be prepared in low-salt buffer at a pH just below its pI value to maintain a balance between creating enough charge to attract it to the biosensor and retaining as much unprotonated lysine as possible.
- If the target has been lyophilized from buffer, it may be necessary to erform a desalting step to reduce the buffer ionic strength.

For more information on amine coupling, refer to Sartorius Technical Note No. 7 Batch Immobilization of Protein onto Amine-Reactive Biosensors.

Site-Directed Coupling

A site-directed approach is recommended to maximize surface activity. Favorable orientation of the target on the biosensor surface can be achieved by using capture approaches that couple the target via a known position or label. Additionally, steric hindrance can be minimized by including chemical linkers.

Several oriented capture options are available on the Octet® platform. The use of such a capture approach also enables immobilization of targets from crude preparations. A second important consideration in choosing an immobilization technique is minimizing target dissociation from the biosensor. The Amine-Reactive and Streptavidin Biosensors perform best in this respect, but others can be assessed for target capture stability.

The most common, and one of the most favored capture systems employs biotin-labeled target and Streptavidin Biosensors. This approach has a number of advantages over direct amine coupling:

- Biotinylation is performed in solution at neutral pH.
- The ratio of biotins per target molecule can be controlled.
- It is easy to incorporate a long chain linker into the biotin tag to minimize steric effects.
- The biotinylated target can be prepared in batches and re-used for multiple capture experiments. Refer to Sartorius Technical Note No. 10 Batch Immobilization of a Biotinylated Ligand onto Streptavidin Biosensors for more information.

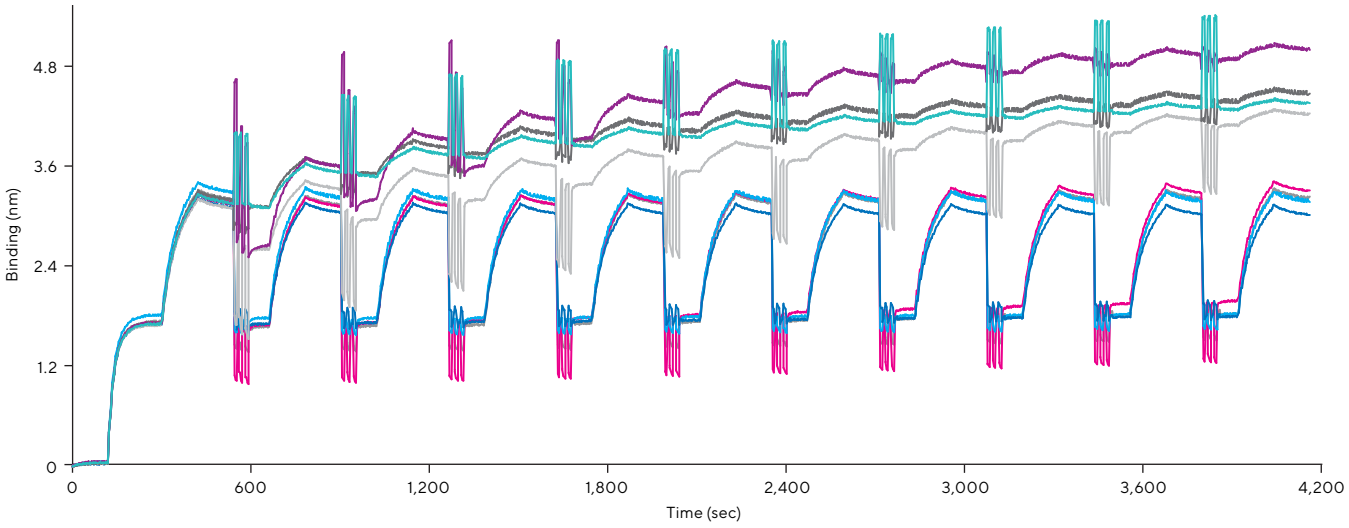

Figure 1: Identifying which of eight solutions is best for regeneration in less than two hours. Eleven cycles were performed with each solution for protein bound to a biotinylated receptor on Streptavidin Biosensors.

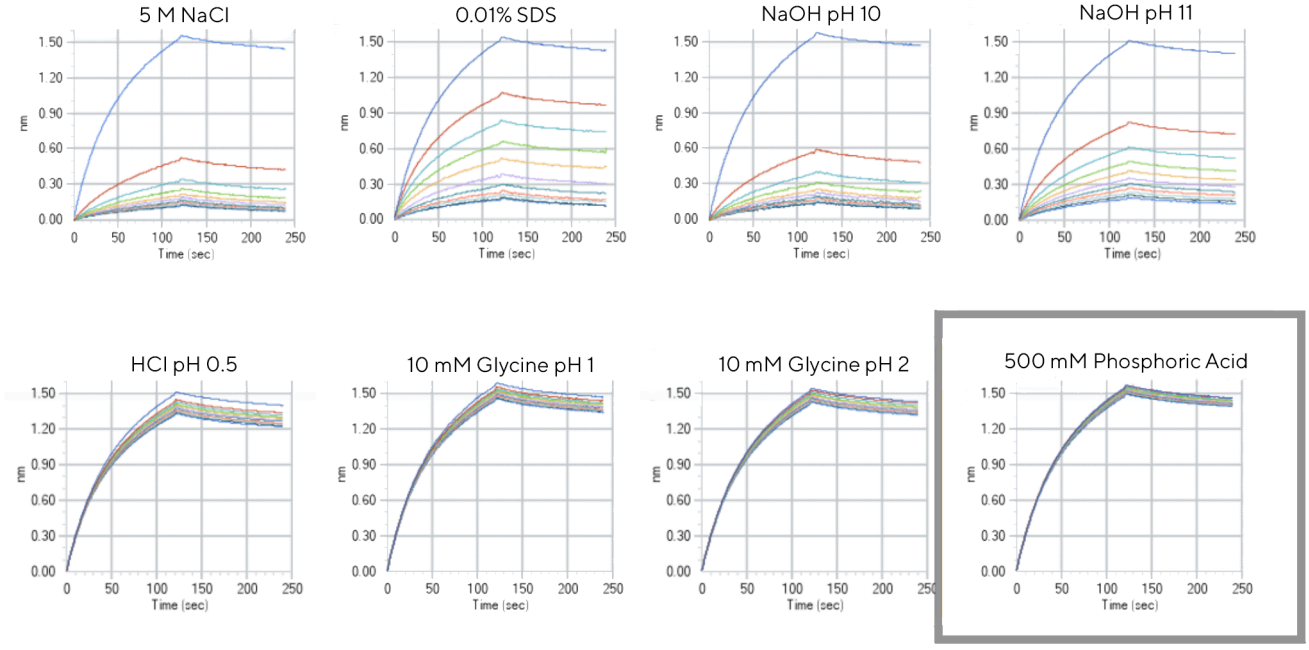

Figure 2: Using the grouping function in Octet® Analysis Studio Software, data for each regeneration solution was overlaid to quickly identify the best regeneration solution, 500 mM phosphoric acid.

Analyte Binding

Analyte can be prepared in most commonly used buffer systems, but it is important that its concentration is known—inaccurate concentration values will produce errors in the calculated affinity.

We recommend Sartorius Kinetics Buffer (available as a 10X solution; Sartorius part no. 18-5032) which contains PBS + 0.1% BSA, 0.02% Tween20 and Kathon as a sample buffer unless the particular interaction calls for another buffer composition.

Optimizing Kinetics Assays

Target Preparation

Biotinylate the target using either NHS-PEG4-Biotin (Pierce, part no. 21329) or NHS-LC-LC-Biotin (Pierce, part no. 21343). Label at a molar ratio of about 1:1 biotin to target. It is important to remove unreacted biotin reagent prior to immobilization. This is usually performed using desalt columns such as Zeba Micro Desalt spin columns (Pierce, part no. 89877). For more information on biotinylating target proteins, refer to the following Sartorius technical notes:

- Technical Note No. 6 Biotinylation of Protein for Immobilization onto Streptavidin Biosensors
- Technical Note No. 11 Biotinylating Antibody in Stocks Containing Carrier Protein
- Technical Note No. 12 Biotinylating Very Small Quantities of Protein for Immobilization onto Streptavidin Biosensors

Target Immobilization

Initially, use the parallel processing capability of the Octet® system to optimize the target concentration. Use a dilution series from 25 µg/mL to 10 µg/mL, aiming for biosensor saturation. Use the 'Extend Current Step' and 'Skip to Next Step' buttons during the experiment to optimize the loading times.

Analyte Binding

Ideally, the analyte dilution series should cover the affinity constants range of 10xK_D to 0.1xK_D. If K_D is unknown, begin with a high concentration and titrate down. For robust kinetics analysis, use at least five analyte concentrations, preferably in duplicate.

If the expected K_D is in the nM range, an association of 15 minutes and a dissociation of 15–30 minutes may be sufficient to obtain kinetic constants with low error. If the K_D is < 1 nM, an association of 15–30 minutes and a dissociation of one hour or more may be necessary to obtain kinetic constants with low error.

If the K_D is unknown, use the 'Extend Current Step' and 'Skip to Next Step' buttons during the experiment to optimize the analyte binding times.

Regenerating Biosensors

The unique 8- or 16-channel simultaneous processing employed in Octet® systems allows accurate kinetics constants to be measured without requiring regeneration. Still, in most cases, biosensors can be regenerated (removal of bound analyte) for re-use, which decreases assay costs. For interactions of modest affinity (around 100 nM or lower), the analyte may often take a long time to fully dissociate. In such cases, if the biosensors are to be re-used, they should be regenerated. Capture biosensors such as Anti-Human Fc Capture (AHC) and Anti-Mouse Fc Capture (AMC) can be regenerated by removal of both analyte and target molecules. This is usually achieved using low pH, but should be optimized for each interacting pair. For more information on regenerating Anti-Human Fc and Anti-Mouse Fc Capture Biosensors, refer to the corresponding datasheet or technical note.

Amine-Reactive and Streptavidin Biosensors are regenerated by removing only the analyte molecule, due to the irreversible nature of target immobilization. For successful regeneration of biosensors, complete removal of bound analyte and retention of target activity are essential.

To optimize regeneration conditions, use the Octet® system's parallel processing to investigate 8 or 16 regeneration conditions in a single experiment. Figure 1 shows how the Octet® system was used to assess eight regeneration conditions over 11 sequential analyte binding cycles (total assay time: 2 hours).

Using the grouping function in the Octet® Analysis Studio Software, rapid evaluation of each regeneration condition is possible, as shown in Figure 2.

Reference Wells and Biosensors

To obtain accurate protein-protein interaction kinetics, it is important to reference out buffer effects by including a reference well (well containing only buffer) in the sample plate. For protein-small molecule interactions, include a reference biosensor to perform double referencing.

Reference biosensors ideally should include an immobilized non-active protein similar to the specific target protein. Avoid BSA as the non-active protein is prone to non-specific binding interactions. If no suitable non-active protein is available, block the active sites on the biosensor (for example, use biocytin to block Streptavidin and Super Streptavidin Biosensors).

For more information on assaying protein-small molecule kinetics on the Octet® platform, refer to Sartorius Technical Note No. 16 Small Molecule Binding Kinetics.

Setting Up the Kinetics Method

A kinetics experiment requires baseline, association and dissociation steps to be set up in consecutive order. Octet® Data Acquisition software simplifies assay setup though the use of experiment templates. Assay optimization for pH scouting and regeneration scouting can be set up quickly using the templates.

Other factors to note:

- Analyte concentrations should be entered in the software in Molar terms (mM, nM, etc).
- Include at least one well of buffer (0 nM sample) for reference subtraction.
- Kinetic measurements under non-mass transport limiting conditions can typically be achieved using a shake speed of 1000 rpm.

Data Analysis

The choice of curve fitting model in the Octet® Analysis Studio Software should be made based on an understanding of the chemistry underlying the binding interaction. As a general rule, use the simplest curve fitting model available (1:1) unless you know that the interaction is more complex. Before trying a curve fit model, get as much information about the interaction as possible, such as stoichiometry of binding, purity of ligand and heterogeneity of analyte.

Use other curve fit models in Octet® Analysis Studio Software to assess experimental optimization. For example, the 2:1 heterogeneous ligand binding model will show if the surface contains multiple affinity binding sites. If this model gives a better fit than the 1:1 model, try to optimize the target loading level further, or consider oriented capture of the target on the biosensor.

The mass transport binding model will show if the analyte is diffusion limited at the surface. If you suspect the presence of mass transport limitation, reduce the target immobilization level and/or increase the shake speed up to 1000 rpm.

Conclusion

By carefully optimizing experimental parameters, kinetics analysis on the Octet® systems will yield excellent data. Oriented, site-specific coupling of target molecule to the biosensor provides the best homogeneous surface. The optimal immobilization level of target on the biosensor should be determined by scouting target solution concentration and incubation time. Ideally, multiple analyte concentrations between the range of 0.1x K_D and 10x K_D should be explored. Use at least one reference sample and perform double-referencing for small molecule assays. Set up the experiment such that the interaction occurring on the biosensor tips follows a 1:1 binding model.

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