Data Sheet
SPR Kinetic Affinity Assay Protocols

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ASSAY PROCESS

SPR-based kinetic affinity measurements are performed using a Biacore T200 instrument capable of automated or manual surface preparation, fully-automated injection of test samples, usually in 96-well format, and output of SPR data as proprietary datafiles for evaluation with Biacore T200 Evaluation software. The process for generation of SPR kinetic affinity data has three stages, as described below.

Receptor Immobilization (Surface Preparation)

This stage is required to coat the biosensor surface with the target receptor (protein) to which the test compound (ligand/analyte) will bind. A variety of different immobilization / capture strategies are available, depending on the nature of the receptor.

Usually, a control receptor (protein) to which the test compound doesn't bind, is also coated onto a reference surface to act as a 'blank'. This allows subtraction of bulk buffer effects, such as changes due to the presence of dimethylsulfoxide (DMSO) or glycerol in the sample during the sample binding phase, that is consistent across all surfaces.

Ligand Binding and Dissociation (Sample Injection and Wash-off Intervals)

After establishing a stable baseline with buffer alone flowing over the sensor surface, sample is passed over the receptor surface for binding to occur. This period defines the binding association rate. After a defined interval, sample flow ceases and flow of buffer alone is resumed. This allows the bound ligand to wash off, defining the dissociation rate. The duration of the ligand binding and ligand dissociation intervals needs to be sufficient to measure binding kinetics but should be as short as practical to conserve reagents and increase sequential sample throughput.

Surface Regeneration (Bound Ligand Removal)

Samples are passed over the sensor surface sequentially. For low-affinity ligands, dissociation can be complete (back to initial baseline level) within a few minutes, allowing the next sample to be injected onto a 'clean' sensor surface after a short delay. For high affinity ligands, complete dissociation back to baseline can take hours, but only a few minutes of dissociation rate data are required for analysis. To remove all bound ligand from the sensor surface before the next sample is injected, a 'regeneration' step is used. This process applies a mild pH change, ionic strength change, or some other biophysical process to rapidly dissociate the ligand from the receptor surface without damaging or denaturing the receptor protein. After returning to baseline with standard buffer flow, the next sample can be injected.

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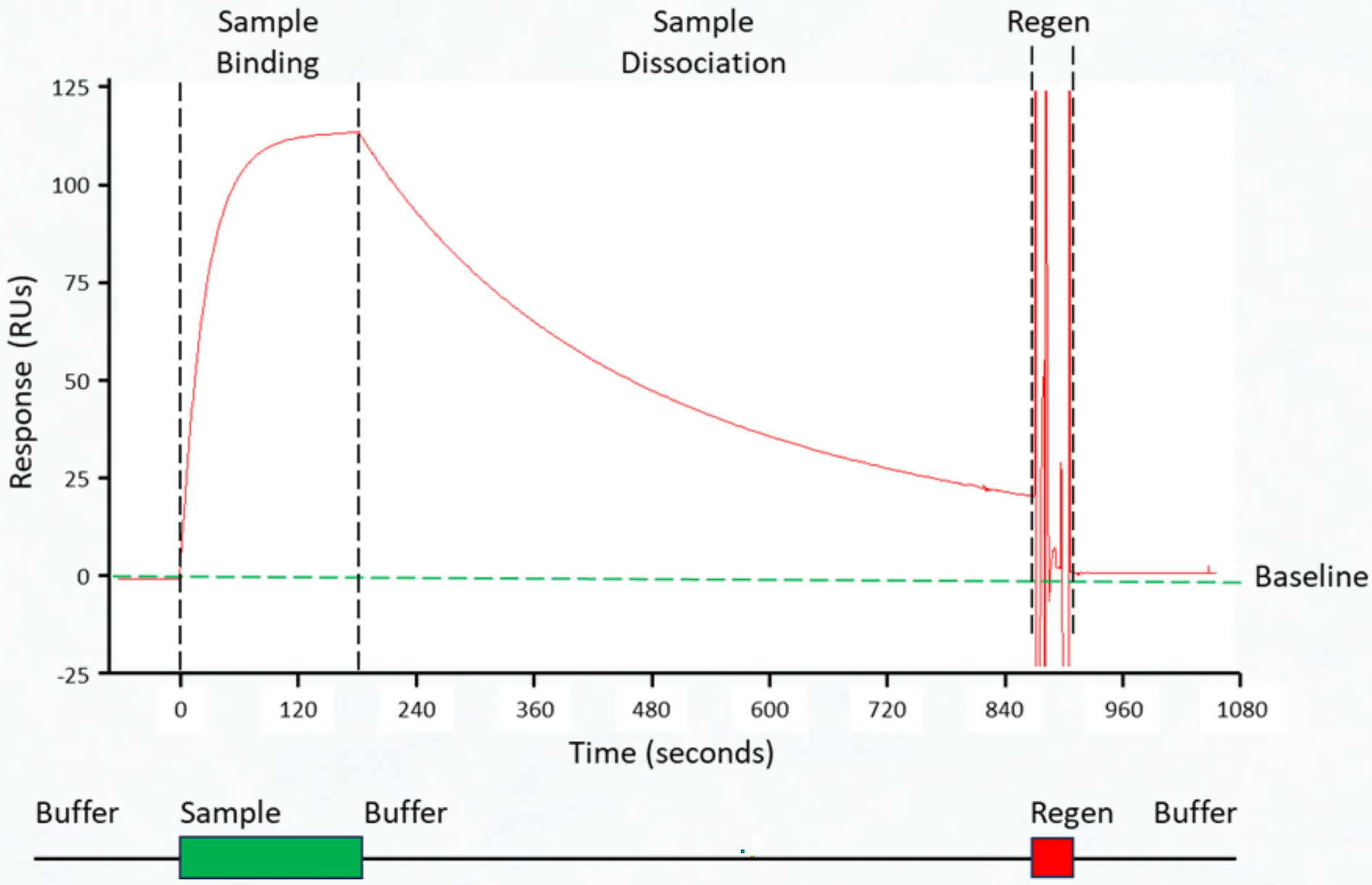

Figure 1: **An example of a typical SPR binding cycle.** After establishing a stable baseline in buffer, the sample is injected (for 180 seconds) to measure Sample Binding. The bound sample is then washed off with buffer (for 600 seconds) to measure Sample Dissociation. At the end of the dissociation period, the surface is regenerated (Regen) to remove any remaining bound sample, to return to the original baseline in order to start the next binding cycle.

It is important to optimize the regeneration conditions at the start of the sample testing process. This ensures that the 'clean' baseline level is achieved before each new sample is tested, avoiding accumulation of traces of previous compounds on the surface. It also ensures that cumulative denaturing of the receptor surface over consecutive binding, dissociation and regeneration cycles is avoided. Together, these ensure that the binding capacity of the surface remains unchanged throughout the assay, allowing meaningful sample to sample comparison.

RECEPTOR IMMOBILIZATION

The immobilization method used to couple the receptor onto the biosensor surface is dependent on the nature of the target receptor and any modification that may have been made to it to facilitate the coupling process. Examples of possible immobilization strategies and the recommended biosensors are given below.

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The amount of receptor coupled / captured onto the biosensor surface determines the amplitude of the sample binding response as a ratio of the molecular masses of the receptor and ligand, i.e.:

MWt of receptor = 50,000 Da; 1,000 RU captured onto biosensor

MWt of ligand = 500 Da; expected binding response = (500/50,000) x 1,000 = 10 RU

Therefore, 1,000 RU of receptor immobilized / captured would be the minimum sufficient to detect a binding response in this case (at a detection threshold of ~0.2 RU). Immobilizing / capturing too little receptor onto the biosensor means that a ligand binding response may not be detected. However, immobilizing / capturing too much receptor can have detrimental effects, such as steric hindrance, ligand depletion at the sensor surface, or rebinding of the ligand to closely adjacent receptors during the dissociation phase. We usually adjust the receptor capture level to give an expected ligand binding response of approximately 100 RUs.

Direct Covalent Coupling

A purified receptor protein can be covalently-coupled onto carboxymethyldextran (CM3, CM4, CM5 or CM7) biosensor surfaces. A range of dextran chain lengths and carboxyl group densities are provided to optimize receptor coupling. Side-chain amine coupling using EDC-NHS chemistry is typical, though thiol and other coupling chemistries are also available. This method produces a stable biosensor surface that can be used for many ligand binding and regeneration cycles. Purified receptor is required to avoid coupling other proteins that may be present in an unpurified mixture onto the surface. The protein must be supplied in a buffer that does not contain Tris/TRIZMA (tris(hydroxymethyl)aminomethane) because this neutralizes the amine-coupling chemistry. Once coupled, the receptor protein does not need to be replenished after each binding cycle. Direct covalent coupling is one of our preferred coupling protocols.

Streptavidin-Biotin Capture

Biotinylated receptor can be captured onto a precoated streptavidin (SA) biosensor surface. Biotinylated proteins may be available from commercial suppliers. Alternatively, purified receptor can be directly-biotinylated using commercially available kits, then repurified by dialysis before injection onto the surface. This method also produces a stable biosensor surface that can be used for many ligand binding and regeneration cycles. Similarly, the biotinylated receptor protein does not need to be replenished after each assay cycle. It is our preferred capture protocol.

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His-tag – Nickel NTA Capture

Recombinant receptor protein incorporating a 4 - 6 x his tag can be captured onto a pretreated nickel-nitrilotriacetic acid (NTA) biosensor surface. The receptor does not need to be purified – only the his-tagged protein will be captured onto the surface. However, this approach can be affected by a significant dissociation rate of the his-tagged receptor from the NTA biosensor surface. The his-tagged receptor protein is removed by the regeneration process, so the protein needs to be replenished at the start of each new cycle. This approach requires use of larger quantities of receptor protein.

His-tag – Anti-His-tag Capture

Alternatively, his-tagged receptor proteins can be captured onto a CM5 surface pre-immobilized with an anti-his-tag antibody. Similarly, the receptor protein does not need to be purified – only the his-tagged protein will be captured onto the surface. This approach is less affected by the his-tagged receptor dissociation rate and is our preferred his-tag capture approach. Similar to the NTA capture approach, the his-tagged receptor protein is removed by the regeneration process, so the protein needs to be replenished at the start of each new cycle, requiring use of larger quantities of receptor protein.

Fc-domain, FLAG, GST or Myc-tag Capture

Similar to the anti-his-tag capture approach, receptor protein incorporating other common purification tags (e.g. Fc-domain, FLAG, GST or Myc) can be captured onto an appropriate anti-tag antibody surface covalently-coupled onto a CM5 sensor. Alternatively, the anti-tag antibody can be captured onto pretreated (Protein A, G or L) biosensors surfaces. The receptor does not need to be purified – only the tagged protein will be captured onto the surface. This approach also has a low tagged receptor dissociation rate and is our preferred capture approach for other tagged proteins. Similar to other capture approaches, the tagged receptor protein is removed by the regeneration process, so the protein needs to be replenished at the start of each new cycle, requiring use of larger quantities of receptor protein.

Specific Antibody Capture

Native receptor without a tag can be captured onto a specific antibody, either covalently-coupled onto a carboxymethyldextran surface (CM5 sensor), or captured onto pretreated (Protein A, G or L) biosensor surfaces. The receptor does not need to be purified – only the native receptor recognized by the specific antibody will be captured onto the surface. As for anti-tag antibody capture, this approach is less affected by the receptor dissociation rate. The untagged receptor protein is removed by the regeneration process, so the protein needs to be replenished at the start of each new cycle, requiring use of larger quantities of receptor protein.

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Transmembrane Receptor Capture

Purified transmembrane protein can be reconstituted into synthetic bilayer lipid membrane liposomes and then captured onto a lipophilic (L1) biosensor surface. Use of recombinant receptors highly-expressed in native membrane micelles prepared from cultured cell membranes is not recommended because receptor expression can still be insufficient to detect a drug binding signal. Additionally, the membrane micelles contain a heterogeneity of unknown proteins which may also bind the test compound.

Nanodisks and Virus-Like Particle (VLPs)

Purified, recombinant transmembrane proteins with an intracellular tag can be reconstituted into bilayer lipid membrane nanodisks and then captured onto a standard CM5 sensor using an immobilised anti-tag antibody. Although this approach ensures only the nanodisks containing the recombinant receptor are captured, there may still be other proteins present in the captured membranes.

Similarly, recombinant transmembrane proteins can be expressed in VLPs and then captured onto the sensor surface using an appropriate antibody. In this case, the capture tag needs to be extracellular because an intracellular tag would be inside the VLP and therefore inaccessible to the capture antibody.

For both these approaches, although the total amount of captured material can be measured, the proportion of receptor protein captured is unknown, so it is not possible to calculate a theoretical binding response amplitude.

LIGAND BINDING AND DISSOCIATION

Samples can be prepared for analysis preferably in 96-well format. Usually five concentrations of the sample analyte, e.g. 1 nM, 3 nM, 10 nM, 30 nM and 100 nM, are tested sequentially, in duplicate. Samples are usually tested against the specific target receptor and a control, reference surface. However, up to three different target receptors and a control surface may be used (up to four sample analysis channels are available, but one always serves as a non-binding reference). Automated sample injection can proceed for up to 48 hours, depending on the number of samples and dilutions required, and the durations of each injection, as described below. It is imperative that the target receptor and test analyte remain stable in aqueous solution over this period. The instrument sample chamber can be held at a lower temperature (e.g. 10 °C) than the sensor (typically 25 °C) to aid sample stability.

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Sample Injection

The buffer flow rate used for protein immobilization and capture is typically 10 µl/minute to conserve on protein use. The buffer flow rate used for sample injection is typically 30 µl/minute, to minimize the transition time from buffer to sample injection, to reduce/prevent ligand depletion, and to reduce/prevent ligand rebinding during the dissociation phase. For small molecule ligands with fast association rates, an injection duration of 60 seconds is usually sufficient to achieve a full binding response. For ligands with slower association rates, a binding duration of up to 5 minutes may be required.

Sample Wash-Off

Immediately after sample injection is complete, buffer flow is resumed to allow the ligand to dissociate. As above, for small molecule ligands with fast dissociation rates, a wash-off duration of 60 – 120 seconds may be sufficient to achieve full dissociation. For ligands with slower dissociation rates, a wash-off duration of up to 15 minutes may be required to be able to measure appreciable dissociation. This affects the duration of each sample injection and dissociation cycle.

Surface Regeneration

For small molecule, low affinity ligands with fast dissociation rates, a regeneration step may not be required to return the sensor to a stable baseline before injection of the next sample. However, for higher affinity, more slowly dissociating ligands (as shown in Fig. 1), we usually regenerate the surface with one or two short injections of mild acid or alkali, high or low salt, or other chemical treatments to rapidly dissociate any remaining bound ligand from the receptor. We usually perform a preliminary study to identify and optimize appropriate regeneration conditions before commencing sample testing. The regeneration conditions are validated by repeating several binding and regeneration cycles using a standard reference compound to show the consistency of binding response amplitude and return to baseline for each cycle. Using additional regeneration steps in each sample binding cycle has further impact on cycle duration.

DATA ANALYSIS

Automated data analysis is performed using proprietary Biacore SPR evaluation software. The continuous sequence of cycles of sample association and dissociation data for each flow channel is automatically segmented into each experimental cycle and then overlaid. Data for each channel are aligned to the start of the sample injection period for each injection cycle, then set to a zero baseline value. Reference channel (control surface) responses are then subtracted from the test channel (target receptor surface) response(s) to remove bulk sample effects (e.g. to correct for DMSO present in the sample). In addition, a blank cycle response (e.g. buffer + DMSO but no test compound) can be subtracted from each flow channel response separately to control for buffer differences.

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After selecting the appropriate association and dissociation regions of the binding responses (eliminating artifacts at the start and end of each injection if necessary), data are globally fitted to a four-parameter non-linear curve fit model using the 'least squares' fit method.

Data Output

Data output for each test compound usually comprises a kinetic affinity chart showing the aligned binding response data for all concentrations tested, and the 'global best fit' line for each concentration plotted through the response data. This provides a visual indication of the quality of the binding response. Kinetic affinity data calculated from the best fit curves are also tabulated for each test compound. These comprise: association rate (k_a); dissociation rate (k_d); kinetic affinity dissociation constant (K_D), and goodness of fit (Chi^2).

For analytes with fast association and dissociation rates, a kinetic analysis fit may not be feasible. In this case, concentration-dependent binding response amplitudes are measured at equilibrium at the end of the sample injection interval, then plotted on either a linear or a log concentration-response plot, from which the equilibrium binding constant, K_D, is calculated as the concentration giving a half-maximal binding response.

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