nature protocols

https://doi.org/10.1038/s41596-024-01079-8

Protocol

Biolayer interferometry for measuring the kinetics of protein–protein interactions and nanobody binding

In the format provided by the authors and unedited

Supplementary Information

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Supplementary information

Expanded definitions:

The term **biosensor** is used to describe the device housing the surface to which a sample biomolecule is bound. For BLI, this is a small disposable biosensor tip (often shortened to "**tip**"), which is typically delivered in trays of 96 tips and generally cost between 5-20 dollars for each sensor, depending on surface chemistry, as of 2024. These tips replace the biosensor chips used for SPR, however SPR chips are generally much more expensive (several hundred dollars each) and include the gold-coated biosensor surface as well as microfluidic channels for directing fluid flow. Fiber optic SPR (FO-SPR) systems are now also available which forgo chips in favor of gold-coated tips, which are handled similarly to BLI tips. By convention for BLI/SPR, the molecule bound to the surface of the biosensor is referred to as the **ligand** (this is notably different from standard biochemical terminology). The ligand may be any type of molecule (protein, nucleic acid, lipid, small molecule, etc.), but is most often a protein such as an antibody or antigen, and it is always attached to the surface of the biosensor prior to binding measurements. Other proteins, such as streptavidin, may be used as capture reagents for the ligand, but generally only the molecule of interest is considered a ligand whereas the streptavidin in this example would simply be considered part of the biosensor. The soluble component which is varied in concentration is referred to as the **analyte**, and this may also be a biomolecule of any type. Selection of which molecule will be the ligand, and which will be the analyte will be discussed further in a later section, but depends on several factors including the size, number of binding sites, and stability over a wide range of protein concentrations/conditions.

Response refers to the magnitude of signal observed at any given moment by the BLI sensor, and a plot of this data over time is called a **sensorgram** (sometimes sensogram). The units of response in BLI are nm and refer to the wavelength shift described in **figure 1**. Critically, the response value is linearly proportional to the mass attachment to the tip, with some limitations such as buffer composition and analyte type. For practical purposes, a protein-protein interaction will show a linear relationship so long as the buffer composition (salinity, pH, solvent/solute admixture) is held constant.

Binding **affinity** describes the strength of a bimolecular interaction. This can be quantified in several different ways including a 50% effective concentration ($\mathbf{EC_{50}}$) from an ELISA or a dissociation constant ($\boldsymbol{K_D}$) from a BLI experiment. EC_{50} values are generally easier to determine because the observed effect can be from any assay that produces a dose-response curve such as an ELISA or cellular assay, but EC_{50} values cannot generally be compared between different assays. K_D measurements, however, directly assess the binding strength of a complex, and are (ideally) completely assay independent. The technical definition of K_D is as follows: given molecules A and B form a complex AB (reversible reaction shown in **Equation 1**), and provided a large excess of molecule A, what is the concentration of B at which half is unbound (A+B) and half is complexed (AB).

Bimolecular binding technically follows second-order kinetics, but by using a large excess of molecule A, we can hold that value constant over time. This allows us to assume pseudo-first-order kinetics, which has a much simpler mathematical description. K_D describes the equilibrium state of a binding pair and can be used to calculate the fraction bound for a given concentration of each component, but it can also be thought of as the point at which the rate of binding is matched by the rate of dissociation. These relationships are shown in **equation 2**.

The equilibrium dissociation constant K_D has units of molarity (M) and can be measured using end-point assays, but it is often useful to know the rate constants, which can only be measured using real-time kinetic assays such as BLI. The dissociation rate constant ($\boldsymbol{k_{OFF}}$) has units of per second (s^{-1}) is frequently also written as ($\boldsymbol{k_d}$), which is distinct from the equilibrium dissociation constant K_D. Likewise, the association rate constant ($\boldsymbol{k_{ON}}$) has units of per molarity second ($M^{-1}s^{-1}$) and is frequently referred to as ($\boldsymbol{k_a}$), which is distinct from the equilibrium association constant ($\boldsymbol{K_A}$). K_A is equal to $1/K_D$. Curve fitting of the BLI data allows direct measurement of k_{ON} and k_{OFF}, which are then used to calculate K_D. Curve fitting and parameter calculation will be discussed further in the analysis section.

Biosensor types:

Antibody capture

Antibody capture tips include a wide array of isotype-specific Fc capture reagents as well as protein A, G, etc. These sensors are set up specifically for antibody ligands, but almost always target the Fc regions which are not present on nanobodies. It would be possible to construct a makeshift nanobody capture biosensor by combining a different biosensor with a high-quality commercial anti-VHH antibody such as Jackson Laboratories' anti-alpaca VHH domain (128-007-232), but this would not be the generally preferred method. Another drawback of these sensors is the finite affinity of the capture antibodies for the ligand, which will lead to measurable dissociation over the course of an experiment. However, the fragility of the capture antibody-ligand interaction can also be exploited to refresh the sensor at the beginning of each cycle by regenerating and re-loading with fresh ligand. Further, affinity capture tips such as these may be used to capture ligand from a complex mixture, potentially allowing the use of unpurified sources of ligand such as whole blood, culture media, or lysates. Because BLI does not contain microfluidic components, there is no concern of clogging, though surface fouling and non-specific binding should still be considered when using unpurified biosamples.

Affinity tag

Affinity tag tips utilize multiple chemistries to target common protein tags such as GST, His, and biotin. These include capture antibodies such as anti-GST, which will behave similarly to Fc capture tips but will have specificity for appropriately tagged recombinant proteins. His-tagged proteins can be targeted with tips either containing a anti-HIS antibody that binds specifically to his tags, or through Nitrilotriacetic acid (NTA) tips which will bind to His tags similarly to Ni-NTA resins commonly used for recombinant protein purifications. We have found that the anti-HIS antibody has much better performance with his-tagged proteins, mainly through greatly decreased background protein binding compared to NiNTA tips. GST and his tag targeting tips may be washed and regenerated similarly to antibody capture tips, with the same benefits and drawbacks. If the GST tag approach is used it is critical that biophysical properties of the tag of interest is taken into account, this is especially critical if there is any potential for oligomerization, as GST tags can drive non-native dimerization.

Also in this category are the streptavidin tips, which are somewhat unique because of the exceptionally high affinity of streptavidin for biotin, about 10^{-15} M [1]. This interaction is highly stable to changes in pH, addition of chaotropes, and extensive washing. This makes the connection behave, for practical purposes, similar to a covalent linkage. One can therefore wash and regenerate the sensor between cycles without needing to re-load with fresh ligand.

The permanence of the streptavidin-biotin interaction also reduces baseline drift due to ligand desorption to negligible levels.
The primary challenge, then, is the addition of biotin to the ligand. This can be easily accomplished with commercial kits using amine-coupling or cystine-labeling, or genetically with an avitag. If using amine-coupling, it is beneficial to use a chromophore-containing reagent, for example Vector Laboratories' ChromaLINK biotinylation kit. This allows quantification of biotin incorporation, which helps minimize batch variation and targeting a specific molar labeling ratio. This method modifies surface lysines as well as the N-terminus, the number of which will vary depending on the ligand. The ideal amount of labeling is one biotin per protein, but as amine-coupling is a random process it is important to acknowledge that the labeling will follow a poisson distribution. This contrasts with avitag labeling which results in site-directed labeling at the location of the tag with exactly one biotin per protein, depending on the efficiency of the biotinylation reaction. Site directed labeling may seem then, to be the preferable option, however one must also consider that labeling of any kind, including at the C- or N-termini can result in alteration of protein structure and function, possibly affecting binding, so one must take into account the pluses and minuses of site directed versus stochastic labeling for any given ligand. Site directed labeling will result in a single population of modified ligands whereas random labeling will give a mixed population with labeling at different locations, unless a large excess of reagent is used to drive the labeling reaction to completion. The site of labeling may even affect the orientation of the ligand on the biosensor surface. Because BLI is a surface adsorption-based method, it is possible for ligands to exhibit bias in which surfaces interact with the surface, and which surfaces are exposed for binding. Therefore, it may be beneficial to perform multiple different antigen labeling methods during pilot experiments to empirically determine the ideal method for each nanobody-antigen pair.

Chemically defined

Chemically defined tips include aminopropylsilane and amine-reactive tips. These tips do not contain capture proteins, and simply have a base layer of chemical moieties. This allows the most flexibility in labeling at the expense of needing to perform substantially more loading optimization. For this reason, chemically defined tips are not recommended for beginning users and should only be used when other options are not available for a particular ligand. For instance, amine-reactive tips are coated with carboxylic acids which can be coupled to a protein using EDC/NHS chemical methods. This is similar to the amine-coupling biotinylation reaction described above, and it is generally easier to perform one large biotinylation reaction and freeze small aliquots of protein with well-defined labeling efficiency than it is to perform a fresh amine-coupling reaction at the start of every BLI experiment. Additionally, it is critical to confirm ligand purity and quality prior to this kind of labeling because general chemical coupling will equally attach the ligand of interest as well as any proteinaceous contaminants or other primary amine-containing molecules.

Experiment types:

Basic (pilot)

Pilot experiments are always the first step to developing a new BLI method. It is often not known what the approximate binding strength is prior to undertaking kinetics experiments, and values like the EC_{50} provide very limited guidance for setting experimental parameters. These experiments provide key information about whether an interaction is detectable using

a given biosensor chemistry, loading conditions, or ligand/analyte identity and orientation. The most important information that needs to be obtained with a pilot experiment is the overall level of signal that is achievable for a given binding pair, the concentration of analyte that results in strong but not overwhelming binding, and approximate k_{ON}, k_{OFF}, and K_D. Spending a few hours and a modest number of biosensors in a pilot experiment can save a significant amount of time and dozens of replicate biosensors from being wasted on full scale tests with suboptimal parameters.

Full kinetics

As stated earlier in the introduction, BLI is one of relatively few methods that can give binding kinetics for a biomolecule pair. Robust determination relies on testing multiple concentrations, and kinetics experiments should be designed to cover a broad concentration range. It bears repeating that only 1:1 interactions can be properly quantified using BLI, or any other currently available technique. However, more complex interactions can be monitored and compared relative to other similar interactions, and these experiments can be quite useful for understanding the biology of complex systems so long as it is correctly reported as non-quantitative.

One step under kinetics is steady-state analysis. BLI can use steady-state analysis to measure the K_D, which represents the equilibrium dissociation constant. This should only be used where interactions are too rapid for proper kinetics experiments, and simply entails plotting the level of steady state binding on a dose-response curve, where 50% signal is equal to the K_D. In general however, protein-protein interactions like nanobody/antibody-antigen interactions are too slow to reach equilibrium for this method to be practical. Fortunately, kinetic binding experiments provide more information than steady-state binding experiments.

Competition

Competition experiments can inform about whether two different analytes bind to the same physical location on a ligand. This is called the epitope when talking about nanobodies/antibodies, and finding multiple non-overlapping antibodies is of significant interest to many researchers. It can also be used to explore the relative binding affinities. Because BLI cannot distinguish the identity of molecules bound to the sensor, just the total mass bound, it is important to know the relative mass of the two competing analytes, and they can be bound consecutively in different steps, with this contribution to the signal able to be distinguished. The primary advantage of BLI competition experiments is the fact that data can be observed in real time. This added time dimension can help distinguish situations whether the second analyte binds because it is non-competitive or because the first analyte already dissociated. When properly set up, BLI is also actually faster than an equivalent ELISA experiment, because there is no need to wait for wash steps, reach an arbitrary end point, or develop signal. Like most other competitive binding assays however, BLI cannot distinguish between orthosteric and allosteric competition the same way that direct epitope mapping (HDX-MS, NMR, and crystallography/Cryo-EM) can.

Epitope binning

Epitope binning is a high-throughput method which is a natural extension of the competition experiments discussed above. Current protocols use an exhaustive round-robin approach to test every possible pairing from a set of analytes. This is most commonly used for screening applications and is named epitope binning because its primary use case at this time is to test a large pool of antibodies and place them into buckets based on whether they bind

competitively. This saves time at later stages of biochemical characterization because one can simply obtain epitope information from one member of each bucket and the data will inform the approximate binding site of a whole class of antibodies. This is particularly useful when a set of commercially available antibodies with known epitopes can be compared to an unknown molecule in order to approximate its binding site. Epitope binning is not quantitative, but this is usually not important and any interactions that do need to be followed up on can be tested individually using more targeted approaches.

Design considerations for individual steps:

General considerations

Some settings apply to the entire experiment and can have a substantial effect on the outcome such as temperature, shaking speed, averaging, sensor offset, and total assay time, and well volumes. Temperature is critical parameter because K_D is temperature dependent due to its relationship with Gibbs free energy [2,3]. For proteins, this relationship is highly complex and non-linear, and is modified by the nature of the non-covalent interactions that drive binding. This will mainly be controlled by the relative entropic and enthalpic contributions to the free energy of complex formation. Hydrophobic interactions tend to decrease at low temperature while charged interactions and hydrogen bonds are less effected, and this leads to increased, decreased, or equivalent K_D over a temperature range depending on the dominant interaction for a particular antibodies; and in fact, measurement of antibody K_D at a range of temperatures can be used as a method of identifying high quality candidates [4]. Most OCTET systems have a very narrow temperature range of ambient plus 4°C up to a total of 40°C, and the default setting is 30°C. This is standard for the field at the time of writing, but may change as device capabilities improve.

Plate shaking is an important feature of BLI which limits the effects of diffusion rate on binding results. We recommend a shake speed of 1000 rpm for every step. Reducing this can slow binding, which can be used for loading high concentration samples, but it is preferable to dilute samples than attempt to slow loading by limiting the diffusion rate.

Averaging is a description of the number of read cycles which the OCTET averages to generate the data points. The inherent measurement frequency varies between equipment, and the number of cycles which is averaged ends up having relatively little impact on the final results in practice because it does not change the actual data acquisition rate, just the amount of averaging that is done in the raw data.

Likewise, the offset parameter does not typically need to be adjusted unless an unusually sized multi-well plate is used. Smaller offsets move the tip closer to the bottom of the well. 4mm is generally recommended, but 3mm can be used for tilted bottom plates. Greater sample volume is needed to adjust for increased offsets, which may slightly reduce the intensity of reflected light from the bottom of a flat plate.

Total assay time is an important consideration, primarily due to evaporation. k_{ON} is linearly proportional to analyte concentration, so excess concentration due to evaporation will tend to increase the apparent k_{ON}, and decrease the apparent K_D by the same factor. In our experience, assays at 30°C should be kept to under 2 hours whenever possible, however increasing the sample volume may allow longer experiments. Little objective data exists for the evaporation, as this is dependent on temperature and humidity, but the authors have observed evaporation sufficient to disrupt data collection after as little as 4 hours using a 384 tilted well plate loaded with 40µL, running at 30°C with a 4mm offset. This includes the full time from pipetting samples into the plate until the final time a biosensor is dipped into a

sample. Another critical feature is determining the sample stability (both of the ligand and the analyte), as assay results will be strongly disrupted if there are any issues with sample fouling over the total assay time. Replicate experiments should be conducted at the beginning and end of any assay window to make sure this is not an issue.
The well volume used for an experiment is dependent primarily on the plate type used. It is impossible to know what the minimum volume is for an arbitrary plate without testing, but generally half of the maximum capacity is a good starting point, however changing sensor offset may alter this slightly. Black plates are recommended to prevent reflections from the well bottom, and a glossy finish on plates is to be avoided. 96 and 384 well plates both work well, but the minimum possible sample volume of 40µL can currently only be achieved using OCTET tilted well 384 plates. Good alternatives plates are the Greiner Bio-One black flat-bottom polypropylene plates (96 or 384 well) 781209 (384), 655209 (96).

Biosensor preparation

OCTET biosensors require extremely careful handling. They should be maintained in their sealed original packaging for as long as possible, primarily to avoid moisture, dust, and unnecessary contact with the biosensor surface. At the time of writing, biosensors are shipped with a proprietary coating (very likely plain sucrose) which must be fully dissolved prior to beginning any experiment. This is accomplished by filling the biosensor soaking plate with 150-250µL of deionized (DI) water or buffer. The authors recommend DI water at this step for streptavidin tips, but a neutral isotonic saline like PBS may be more appropriate for sensors pre-loaded with antibodies or other delicate proteins. Assay running buffer may also be used here, but it is generally unnecessary.
Biosensors can then be loaded carefully into the overlaid sensor tray such that they contact the liquid. Ensure that the tip end of the biosensor does not come into contact with any solid material, including the side of the sensor rack. Tips should be soaked for at least 10 minutes, preferably 20 minutes, prior to starting a run. Only tips being used in the current experiment should be soaked, and they should generally be used the same day that they are prepared.
Tips can be saved for later use by dipping briefly into a 15% sucrose solution, dried at ambient temperature, and returned to a sealed package with desiccant. This technique can be used to pre-load a batch of tips with a particular ligand and stored for later use. However, it is generally preferable to load each experiment separately using a fresh aliquot from a single batch of labeled ligand.

Equilibration

The most common first step of a BLI experiment is to block the tip briefly in the assay running buffer. This step serves two purposes. First, it is useful to observe the behavior of each tip as it is introduced from plain water into the running buffer. The equipment will auto-zero each trace prior to collecting any data, and any defective tips will generally be clearly visible at this step. In our experience, defective tips are very rare, but improper preparation or mishandling may increase the chance of observing unusual results. In our standard running buffer, we generally see 0.4-0.5 nm of signal after 30 seconds. Second, it is useful to pre-bind the sensor with blocking compounds to prevent non-specific binding at the loading step. This step should be delayed until after loading if using a chemically defined tip.
This step is where running buffer is first introduced to the tips, and all tips of an experiment should have the same running buffer. This will require tuning for specific needs, but a good general starting buffer is 10mM HEPES, pH 7.5, 150mM NaCl, 3mM EDTA, 0.005% Tween-20, and 0.1% BSA. Changes to the buffer recipe should be brough forward into every step

that uses running buffer, because relatively small changes can shift the observed signal significantly. Non-neutral pH (up or down) typically shifts the signal lower, while increased salinity typically increases signal. This is likely due to changes in the packing of the silane molecules used to coat the raw biosensor surface. However, because of referencing, it is not usually problematic to change buffer conditions to whatever is required for a particular binding interaction. Streptavidin sensors can handle harsh buffers and wash conditions, but it should be noted that many other tip chemistries will dissociate their ligands, and possibly damage their capture molecules if exposed to overly harsh conditions.

Activation

Activation only applies to amine-coupling tips and involves incubation with a mixture of EDC and sulfo-NHS. This can be acquired as a kit or separately with similar effect, but amine-coupling should not be one's first choice for tip chemistry. One should expect approximately 0.3-0.5 nm of signal at this step, but it will depend on concentration and the exact compounds used for activation.

Loading

Loading is one of the most critical steps of each assay and should be optimized with pilot experiments. Greater loading densities will linearly increase the amount of signal achieved during association steps, but overloading can lead to crowding or mass transport limitation. Proper loading should be approached empirically, but 1-2 nm of binding is often a good starting point. Actual capacity of biosensors will vary substantially based on the tip chemistry, and small variations will also be seen between individual tips, even from the same package. This will result in different rates of loading as well as signal at saturation. A good starting point is 10µg/mL for loading. Results will vary depending on tip chemistry and batch, but an example experiment with streptavidin 16 tips loaded with identical ligand solutions of a biotinylated (~2.5 biotin/protein) 26 kDa protein over 265 seconds reached an average signal of 2.84±0.42 nm (%CV = 5%). This is approximately in line with the manufacturer's certificate of analysis. For streptavidin tips, ligands with excessive biotins per protein will reduce tip capacity, as will proteins contaminated with free biotin.

The signal obtained from a defined amount of mass attached to a biosensor should be theoretically calculable for an arbitrary ligand-analyte pair based on their relative molecular weights, the stoichiometry of binding, and the amount of signal seen during loading. For example, if a 17 kDa nanobody is loaded to 1 nm then one would generally expect to require approximately 5 nm of loading for a 150 kDa bivalent antibody in order to achieve the same level of signal from the same analyte.

For reference, real world examples from experiments we have conducted with streptavidin tips gave roughly the following signals: A biotinylated 26 kDa protein with a single binding site was loaded to 3 nm, a 17 kDa nanobody reached 0.7 nm of binding. A neighboring tip showed a lower capacity and only achieved 2.25 nm of binding, and as a result only reached 0.5 nm of binding with the same concentration of nanobody. Next, an 87 kDa bivalent nanobody construct was tested with the same batch of ligand. In this case 1.8 nm of ligand loading led to 1.1 nm of bivalent nanobody binding. Next, using a 700 kDa trimeric ligand loaded to 0.75 nm led to 0.02 nm of monovalent nanobody binding. These can be compared by correcting the response signal by the molecular weight for each the ligand and the analyte. We can then get a unitless ratio of analyte signal (nm/kDa) per ligand signal (nm/kDa) and we get values between 0.33 and 0.36 after correcting for the stoichiometry of each interaction. The amount of analyte used in each case was over 100 times the K_D. This ratio appears to be

relatively stable over a handful of nanobody-antigen pairs with different stoichiometry in both orientations, but we have not tested it exhaustively and it should be used only as a guide for planning pilot studies, or to help estimate whether a particular ligand-analyte pair is likely to give good results. Similar ratios ranging from 0.33 to 0.45 can be calculated from BLI traces published by other studies where both full sensorgrams and approximate molecular weights are available [5–9]. A physical interpretation of this ratio may be that approximately one third of loaded ligands are able to bind an analyte, but further testing would be required to prove this. It would be reasonable to expect that this ratio will change based on the quality and purity of the loaded ligand as well as the final loading density.

Quenching

Quenching is necessary to fill any remaining binding sites not used during loading. If left active, these extra sites can cause analytes or other compounds present in samples to bind during later steps, confounding the results. This can apply to either chemical or biochemical loading methods. For chemical reactions such as amine-coupling, quenching should chemically inactivate any remaining succinimide esters by reacting with any primary amine-containing small molecule including ethanolamine, tris, or glycine.

For streptavidin tips, loading is rarely complete, and a substantial number of free binding sites will remain. This can be mitigated by filling all remaining sites before proceeding to kinetic measurement cycles. For streptavidin, dilute solutions of free biotin are sufficient to rapidly and permanently saturate the surface. This is only necessary when biotinylated proteins will be present in later samples, but many biosamples do contain trace amounts of biotin and biotinylated proteins. This quenching will persist through regeneration.

This type of quenching/blocking should be approached with caution for other tip chemistries, but may be helpful in some cases. Most other tip types bind to a protein rather than a small molecule such as biotin, and therefore any compound used for this purpose will be similarly bulky as the ligand of interest. Thus, blocking the remaining binding sites could result in overly dense loading of the sensor surface and steric hinderance of the ligand, which must be strictly avoided. In these cases, it would be preferable to ensure that no interfering proteins or compounds are present in the buffer or analyte solutions.

Baseline

The baseline step is beneficial for confirming the level of drift as well as providing a starting point from which to calculate the association and dissociation. A 30 second baseline is generally sufficient. Consistent drift can be removed by reference subtraction, but large amounts of drift will negatively impact the results regardless of proper reference subtraction if it is due to ligand dissociation as the response value at saturation ($\mathbf{R_{max}}$) value will vary over the course of a single cycle. This can be a key step to optimize the type of attachment to the sensor, as if a given approach has significant drift (mainly due to ligand loss from the sensor) this is a good reason to switch approaches. Empirically we have found that the streptavidin tips perform much better compared to anti-GST or anti-HIS approaches in this regard.

Association

The association step is used to calculate k_{ON} and is dependent on accurate knowledge of the analyte concentration, and any underestimation at this stage will lead to falsely low (strong) K_D values. The appropriate concentration range for kinetics measurement is 0.1–10×K_D with at least 5 concentrations over that range. In order to prevent signal spikes due to changes in buffer composition, the stock protein should be sufficiently diluted in the running buffer. For

buffers of similar composition, 100-fold is generally a sufficient dilution factor to avoid buffer spikes. If spike still occur, then dialysis can be used to equalize conditions somewhat, but additives like Tween-20 and BSA are not generally possible to dialyze, and low CMC surfactants and low molecular weight blocking compounds may be substituted, but should be tested. A better strategy is typically to further purify the analyte, exchange into running buffer base without the large molecular weight additives, then dilute at least 100-fold as normal.

As the K_D is often not known prior to BLI, an ELISA EC_{50} is a good starting approximation, and a wider 10- to 100-fold dilution series can be used to home in on the proper range. By definition, at the K_D the curve will reach equilibrium at half of R_{max}, if given enough time. Equilibrium need not be reached, however unless performing equilibrium analysis specifically, which is only ideal for very fast or low affinity analytes. Minimum step times are dependent on k_{ON}, but there must be curvature present in order to make fitting possible. Ideally, the highest concentration tested should be allowed to level out. For nanobodies, typical k_{ON} values range from 10^5 to 10^6 $M^{-1}s^{-1}$, but somewhat counterintuitively the primary factor affecting equilibration time is the dissociation rate k_{OFF}, which typically ranges from 10^{-2} to 10^{-4} [10,11]. Given the slow off rates of many nanobodies, it is often impractical to wait for equilibrium to be reached, however it can be calculated using **Equation 3** [12,13].

$$Eq.3 \qquad t_{\theta} = \frac{-\ln(1-\theta)}{k_{ON}[A] + k_{OFF}}$$

Where θ = 0 represents starting conditions and θ = 1 represents steady state and [A] is the concentration of analyte. This is concentration dependent, and higher concentrations will reach equilibrium faster. Assuming we wish to reach 90% of equilibrium binding for the middle concentration where [A] = K_D, it will take just under 2 minutes for a nanobody with k_{OFF} = 10^{-2} s^{-1}, just under 20 minutes when k_{OFF} = 10^{-3} s^{-1}, and over 3 hours when k_{OFF} = 10^{-4} s^{-1}. Because 10^{-4} s^{-1} is a relatively common k_{OFF} for nanobodies, it is important to consider this factor. Even if we look at the 10×K_D sample, it will take 600 seconds to get halfway to equilibrium. In such cases, it may be prudent to include an even higher concentration sample, because a 100×K_D sample will reach 90% equilibrium in just under 4 minutes. Because a nanobody with k_{OFF} of 10^{-4} s^{-1} is likely to have a K_D between 100pM and 1 nM, it is safe to increase the sample concentration up to 100×K_D = 10-100 nM without needing to worry too much about non-specific binding.

Non-specific binding becomes much more likely as analyte concentration increases. In general, small proteins like nanobodies (12-17 kDa) can be used up to 100-300 nM without substantial effects, but this may not be true for larger macromolecules. In our experience, concentrations around 1μM are where non-specific binding become common. If non-specific binding is observed in pilot studies or otherwise anticipated, the running buffer can be modified, concentrations decreased, or offending substances removed. For severe non-specific binding which might arise when studying low affinity interactions, additives such as 0.6M sucrose may prove useful [14].

Two association steps can be performed back-to-back for competition experiments. This is useful for general competition experiments as well as epitope binning. In either case, it is important to consider whether to include the analyte from step 1 in the buffer for step 2. In the case of high affinity nanobodies it is likely not necessary to include analyte 1 in the second buffer, but for faster off rates it may be obvious that the first analyte is dissociating during the second binding step, complicating the results. If included analyte 1 should be kept at the same concentration as in step 1, to avoid shifting the equilibrium. Further, the shape of the curve for step 2 should be observed carefully. For slow off-rate nanobodies, a rapid increase in signal should always be due to binding at a non-overlapping site, but for fast off-rate

nanobodies/proteins one should consider that analyte 2 is exchanging with analyte 1, but still binding to site 1. Whether this is occurring can be reasoned based on the direction and amount of signal shift compared to the relative molecular weights of the two analytes. Allosteric binding by analyte 2 followed by conformational changes that prevent binding at site 1 is also possible. Prior to performing this type of experiment, one should ensure that analyte 1 is fully saturating and at equilibrium. $100\times K_D$ is usually sufficient for this, and should be continued until the curve flattens.

Dissociation

The dissociation step is always performed immediately following an association step, and it is used to calculate the dissociation rate constant k_{OFF}. This step is generally best performed in the same well that the baseline step was performed in. When possible, a fresh running buffer well should be used for each cycle. Baseline, association, and dissociation should be performed in the same plate with the same well volume, to prevent changes in reflection/refraction from introducing additional noise. If throughput is a concern, then the same buffer well can be re-used for multiple cycles, with the only concern being contamination from previous cycles. However, the amount of carryover is generally minimal, and we have not observed noticeable changes with repeated use. As a precaution, we suggest starting with the lowest concentration and increasing with each cycle. The manufacturer claims that a fully saturated tip will transfer 10^9 molecules, resulting in an 8.3pM solution assuming 100% dissociation and a 200µL well volume. This ignores liquid carryover on the tip, but also likely assumes 100% saturation during the loading step as well. All together, our observations suggest that this is likely accurate, but the primary reason to re-use wells is to save space in crowded layouts and we would generally consider using fresh wells to be best practice.

Dissociation should generally be allowed to reach at least 5% signal reduction in order to get an accurate reading. While the absolute rate of decrease will occur faster for higher concentrations, all concentrations will decrease proportionally, so increasing the concentration during association will not result in reaching 5% reduction faster. The time required at this step can be calculated using **Equation 4**, derived from the equation for one-phase decay, where θ is the fractional progress towards steady state [13].

$$Eq.4 \qquad t_{\theta} = \frac{\ln(\theta^{-1})}{k_{OFF}}$$

Using 0.95 for θ gives the time to dissociate 5% from R_{max}. For a k_{OFF} of 10^{-4}, it will take 8.5 minutes to dissociate 5%, 1.4 hours for 10^{-5}, and 14 hours for 10^{-6}. It is clearly impossible then to definitively show an off rate of 10^{-6} given the time limitations imposed by evaporation, but this is not a hard cutoff and depends on how clean the curves are. The lowest K_D that the OCTET software will assign is 10^{-12} (1pM), which is a k_{ON} of 10^6 $M^{-1}s^{-1}$ and a k_{OFF} of 10^{-6} s^{-1}, below this it will report $>10^{-12}$ M. The time required for this step is unfortunately a physical limitation of dissociation kinetics and cannot be easily circumvented.

One strategy for reducing the total experiment time, is to perform short dissociation steps for the lower concentrations and only perform a full-length dissociation step for the highest concentration tested. This works because the dissociation rate is not dependent on concentration or saturation level of the biosensor. The highest concentration is used because the higher starting point yields a larger absolute change in response over time, and therefore improved signal-to-noise of the resulting drop. However, some software, such as OCTET Data Analysis HT 10, struggles to analyzing data with variable step times for each cycle.

Regeneration

Regeneration is typically a series of steps designed to remove excess analyte from the last completed kinetics cycle and re-set for a new cycle. This is simple for some tips and ligands, but nearly impossible for others. The general strategy is to find conditions which cause the analyte to dissociate rapidly without irreversibly damaging the ligand. The most common regeneration solution is low pH glycine, between pH 1.5 and 2.5, but this also needs to be tested empirically. These conditions work well for most, but not all, nanobodies. We recommend three cycles of 15 second dips each into 10mM glycine pH 1.7, then into running buffer. The reason for repeated short cycles is putatively to limit irreversible unfolding of the ligand, but this is often unnecessary and single stage regeneration is also generally acceptable.

The extent of regeneration is verified by checking the level of baseline after regeneration, which should match very closely the signal of the previous baseline step. Small shifts up or down are common, but not ideal. **Figure S1** compares complete versus incomplete regeneration. Residual ligand-analyte complex will alter the curves as the pre-existing complex is free to dissociate similarly to the newly formed complex. This can be accounted during analysis, and is exploited in a process called kinetic titration (or multi-cycle kinetics), but currently available OCTET software is not capable of this type of analysis. This will be discussed further in the analysis section.

Category	**Example**
Low pH	10 mM Glycine, pH 1.7
High pH	10 mM glycine, pH 10
Ionic	3 M KCl
Chaotropic	6 M Guanidine·HCl
Hydrophobic	50% ethylene glycol
Detergent	0.5% SDS
Chelating	20 mM EDTA
Affinity	Specific peptide

Conversely, some conditions may damage the ligand, reducing the capacity of the tip for analyte binding (**Figure S1**). In theory, this does not alter the curve shapes, but care must be taken during analysis as each cycle will have a different R_{max}, and it is possible that some

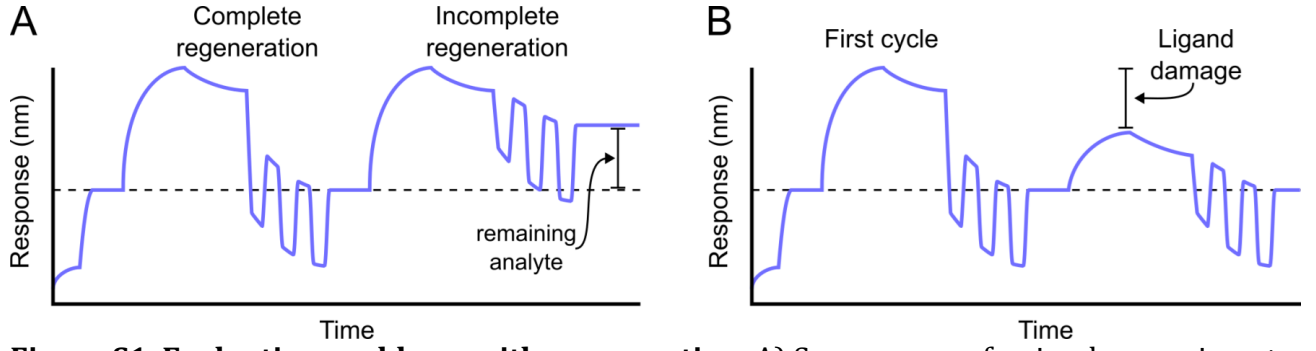


Figure S1. Evaluating problems with regeneration. A) Sensorgram of a simple experiment with a initial blocking step and ligand loading followed by a measurement cycle (baseline, association, and dissociation). The response does not return to baseline following dissociation, so regeneration is required. The first cycle shows complete regeneration, which rises and falls rapidly depending on buffer composition, but returns to the initial baseline value upon completion. Following the second cycle, the response value remains above the initial baseline, indicating that some analyte remains bound to the sensor and regeneration was incomplete. B) A similar first cycle with apparently full regeneration is followed by a measurement cycle with the same analyte at the same concentration, but showing reduced response signal during the association step. This indicates that the ligand was partially damaged during the first regeneration step.

ligand molecules will be partially damaged, confounding analysis even further by creating a heterogeneous surface. Using streptavidin tips, it is unlikely that ligand will dissociate from the biosensor surface, which would present as a decreased baseline response following regeneration. However, other tip chemistries may experience this, and Ni-NTA tips, for example, can be regenerated with imidazole solutions to completely remove the ligand from the sensor, which can then be re-loaded with fresh ligand before the next cycle.

In the case of incomplete regeneration or ligand damage, the time in or composition of regeneration buffer can be adjusted. Several different categories of regenerant exist which act in different ways, depending on the nature of the interaction. Low pH is the most common condition and acts by protonating acidic residues and disrupting hydrogen bonds/electrostatic interactions while high pH deprotonates basic residues and also alters hydrogen bonding/electrostatic interactions. More exotic reagents can be used depending on the biophysical nature of the interaction, and a variety of different compounds can be tested beyond those Table 1. However, more extreme conditions risk damaging not only the ligand but also the biosensor surface, so should be attempted only after milder conditions have failed. Specific molecules should be used whenever possible, such as competitive peptides or small molecules, sugars for lectins, and imidazole for Ni-NTA. A tip may be used until its capacity has degraded, but the tolerance for degradation can be selected on a case-by-case basis depending on target difficulty and signal-to-noise achieved using the damaged ligand. Alternatively, kinetic titration can be used to avoid regeneration entirely.

Appropriate controls:

Artifacts in BLI data are unavoidable, but most common types can be effectively controlled for with minimal additional cost or effort. The most basic control is adding an addition sample with no analyte. This controls for differences in buffer composition between the running buffer in the baseline and association steps. Some buffer components such as DMSO have an outsized impact on signal, but ideally these would be tightly controlled in sample composition rather than relying on referencing to fix. This control can also control somewhat for drift, due to ligand dissociation from the sensor, though imperfectly, and drift is not an issue for streptavidin biosensors. The second, and more important, control is a reference sensor. This sensor gets loaded with either nothing or an irrelevant ligand, and it gets carried alongside the other sensor tips into identical analyte solutions. This helps control for non-specific binding as well as changes in refractive index due to the presence of large quantities of analyte. This control must absolutely be included in every experiment. Testing an analyte such as a nanobody or antibody against multiple different ligands can also help establish specificity. The preferred method, however, is called double-reference subtraction and involves running both of these references simultaneously. Because this requires no additional sensors, double-referencing is usually the right choice even though it provides minimal additional benefit over a reference sensor.

Replicates are also important to include when performing BLI. While pilot experiments may be performed with one sensor per condition, proper kinetic measurements should include at least two parallel sensors. Ideally, separate experiments should also be performed on separate days and with separate batches of protein. When using replicates, take care to keep the exact same experiment design including step lengths so that the analysis software can analyze the replicates together.

Additional controls may also be necessary if pilot experiments indicate complex binding phenomena. In general, if complex (not 1:1) binding is observed, then non-BLI/SPR method

should be used to differentiate between the alternative explanations, but nevertheless there are some methods that can address specific cases. For example, by fully saturating the sensor for different lengths of time prior to dissociation, one can learn whether their interaction is affected by a ligand-induced conformational change [15,16]. Strong mass transport limitation can be detected by observing a linear association phase which saturates without showing much curvature [17,18]. Critically, however, the fitting of more complex models to existing data cannot reveal which model is correct. A more complex model will almost always fit the data better simply because it has more degrees of freedom, so further experiments are always needed to understand the molecular mechanims underlying complex binding.

Data analysis:

Software

BLI data can be easily analyzed using the bundled OCTET software, and this guide will be based on OCTET Data Analysis HT version 10.0. Newer versions are available (version 13 at the time of writing), but most previous versions are sufficient for basic kinetics analysis. No centralized database of software or feature lists exist, but basic kinetic analysis has been possible in every version to date, competition experiments can be performed and analyzed using every version to date (albeit with some manual post-processing), and epitope binning has been available as an officially supported feature since version 8.0 (released around 2016). HT stands for high-throughput and the HT version of the analysis software was released along with version 9.0, and while there is a standard version of the software, there is no notable benefit to its use in its current form. The Data Analysis HT software is not the only option however, and raw or partially processed data can be exported and analyzed in other software such as Graphpad Prism, SPR analysis software like BIAevaluation, or other custom software like TitratonAnalysis [19]. The standard Octet software will be sufficient for most new users, but there are several key features it currently lacks, such as the ability to analyze single-cycle kinetic titration experiments. To summarize, the experiments discussed can be reasonably performed on any BLI equipment using whatever software version is already set up for use with that equipment. The precise methods of analysis may differ slightly, but the only key features necessary for the performance of these protocols are basic reference subtraction and curve fitting. More advanced experiments such as competition and epitope binning are easily interpreted manually, however current generation software features improve the ease and repeatability of these analysis.

It is fortunate that the quantification of binding rate has been made relatively simple by modern computing, and no longer requires log transformed Scatchard plots. Because simple 1:1 binding can be fit using pseudo-first order kinetics, we can use non-linear regression with a single exponential for each the association and dissociation steps. Further, this work happens in the background of most common software. Nevertheless, it is important to understand what calculations are being performed on the data, and there are several stages of data analysis that must take place to get reliable quantification. Critically, all of these steps are identical to the processing steps necessary for SPR analysis, which is the reason why these data are interchangeable during quantification.

Referencing

The raw sensorgram data does a good job of intuitively showing the amount of binding, but the units are relative and need to be converted to a form that can be more easily fit to a model. This starts with background subtraction. Using double reference subtraction, we first

subtract the primary baseline (from a reference tip) over the entire run. This removes artifacts due to slight changes in buffer composition due to the increasing quantities of analyte as well as signal drift which is more common for non-streptavidin tips. Where multiple reference tips are used, they are averaged before subtraction. The second reference in double subtraction is the reference well. This involves performing a blank kinetic cycle using plain buffer during the experiment. To perform this subtraction, each kinetic cycle is first separated into their own trace. Each cycle begins with a baseline step, followed by an association step, and concluded with a dissociation step. These cycles are already primary reference subtracted with the reference tip, and now the blank cycle from each tip is subtracted from each individual kinetic cycle from the same tip. This helps correct for small variations in tip capacity and ligand loading density. Following double referencing, the cycles are then aligned to 0 by subtracting a constant value equal to the average of the last 5 seconds of the baseline step. As a final step, some software like the Octet Data Analysis HT perform a final data smoothing step, Savitzky-Golay filtering in this case. This is typically unnecessary but can help when signal-to-noise is low.

Models

It cannot be overstated that only 1:1 fitting should be relied upon for reliable kinetic parameters for BLI and SPR. Non-quantitative analysis of more complex binding is perfectly acceptable, but as we describe later, for K_D determination the more complex models are much more likely wrong than correct. The standard 1:1 fitting using the Langmuir-Hill equation [20,21], and is a well-established method that provides consistent results. This model was originally designed for modeling the adsorption of ideal gasses to solid surfaces, but it has found widespread use for biochemical kinetics. This involves fitting the reference-subtracted data to the integrated rate equations for the association (**Equation 5**) and dissociation phases (**Equation 6**). Where R_1 is the signal observed during association at time t_1 for analyte concentration [A], and R_2 is the signal observed during dissociation at time t_2.

$$Eq\ 5. \quad R_1 = \left(\frac{R_{max}[A]}{\frac{k_{OFF}}{k_{ON}} + [A]}\right)\left(1 - e^{-t_1(k_{ON}[A]+k_{OFF})}\right)$$

$$Eq\ 6. \quad R_2 = R_1 e^{-k_{OFF}(t_2 - t_1)}$$

These equations can be used directly for fitting reference-subtracted data from a kinetics experiment, and they rely on several simplifying assumptions: 1) Ligands and analytes are homogeneous. 2) Each ligand binds a single analyte at an identical binding site with an identical binding energy. 3) Dynamic reversible equilibrium is established within the time-frame of the experiment. 4) No interaction between ligands or analytes should alter the binding at any other site. Clearly, these assumptions can never be strictly met, much like the assumptions required to use pseudo-first order kinetics in the first place, but for 1:1 binding without cooperativity the violations are not severe enough to warrant the use of a more complex model.

Protein	Model	K_D	k_{ON}^1	k_{OFF}^1	k_{ON}^2	k_{OFF}^2	X^2	R^2
saRBD-1	1:1	471pM	$3.36x10^5$	$1.58x10^{-4}$	N/A	N/A	6.5378	0.9902
Fc-saRBD-1	1:1	<1pM	$1.57x10^5$	$<1x10^{-7}$	N/A	N/A	53.5032	0.9899
saRBD-1	1:2	893pM	$1.92x10^5$	$1.71x10^{-4}$	$1.31x10^2$	$2.32x10^{-2}$	0.8553	0.9936
Fc-saRBD-1	1:2	<1pM	$5.00x10^4$	$<1x10^{-7}$	$2.98x10^0$	$1.15x10^{-2}$	3.7041	0.9965

Issues with more complex models arise due to their increased degrees of freedom allow for improved fitting in many cases where the underlying biology does not support their use. For instance, a 2:1 heterogeneous ligand model and a 1:2 bivalent analyte model will often yield similar results regardless of the nature of the binding interaction. Worse than this, even when there is good justification to use a specific model, the limitations of current generation technology (both software and instrumentation) do not allow for accurate quantification. For example, we tested our nanobody saRBD-1 alongside bivalent Fc-saRBD-1, and then measured their K_Ds using both the standard 1:1, and the 1:2 bivalent analyte model. The results of these fittings are summarized in the following table, but an important note is that this 1:2 model proports to give the K_D of the monovalent interaction by giving the k_{ON}^1 and k_{OFF}^1 equal to the true monovalent interaction while the k_{ON}^2 and k_{OFF}^2 give the amount of extra binding due to avidity. For both proteins, the fit improved, as shown by the X^2 and R^2 both dropping, but the K_D of the bivalent construct was not accurately recovered by using the 1:2 model. The accuracy of this method would be expected to increase with a lower affinity interaction, but the point remains that one cannot simply set up an experiment relying on this method to provide an accurate measurement; particularly when it is relatively easy to swap the bivalent molecule for the ligand to make an effective 1:1 interaction or digest the antibody to make a F(ab). Several kits (Fisher cat no. 44685) and well-established protocols[22] exist for generating F(ab) molecules from traditional antibodies.

Using this data as an example again, the mass transport and heterogenous ligand binding models both also provide improved fits with reduced X^2 and R^2 values, showing that improved fits are generally to be expected any time a more complex model is used, even if inappropriately. This is not to say that it is impossible to use more complex models with BLI data, but one would need to know the stoichiometry prior to designing the experiment, carefully construct the experiment, and validate the findings using other approaches. A far simpler approach is to simply enforce 1:1 binding through experiment design.

For bivalent analytes, we already discussed either flipping the ligand/analyte, such that the monovalent molecule is now the analyte, or producing a monovalent version of the analyte either by digesting or inactivating one binding site. A second approach is to decrease the loading density until ligands are spaced too far apart for a single antibody to bridge the gap, but it can be difficult to tell when this has been achieved.

The mass transport model is designed to address the problem of analyte depletion in the local area of the sensor surface, but this is mainly seen in SPR due to laminar flow in the microfluidics whereas BLI uses turbulent flow to replace the surface layer much more quickly. Regardless, eliminating mass transport by decreasing loading density or increasing shake speed is preferable to using this analytical model. SPR studies and guides generally advocate for low density loading whenever possible as it reduces the likelihood of mass-transport limitation, and it reduces steric hinderance between analytes. For BLI, mass-transport limitation is unlikely due to the turbulent flow within the wells, but steric hinderance between analytes is a legitimate concern which should be considered.

The heterogenous ligand model assumes a mixture of two different binding sites in arbitrary proportions on the biosensor surface. This situation is theoretically possible, but unlikely. The more likely situation is that low quality ligand will contain a mixture functional, non-functional, and partially functional binding sites which may lead to confusing results. This is best remedied by ensuring the quality of protein stocks prior to starting BLI.

In all cases, 1:1 binding is best suited for kinetics measurement, and lack of good fit should be an indication that more complex binding may be occurring. It is not good practice to observe which model fits best and assume that this describes the underlying biology. Instead, after seeing poor fitting in a 1:1 model, one should use alternative methods to determine

which, if any, alternative models are appropriate, or if the protein quality and experimental parameters simply need to be optimized.

Supplementary figures:

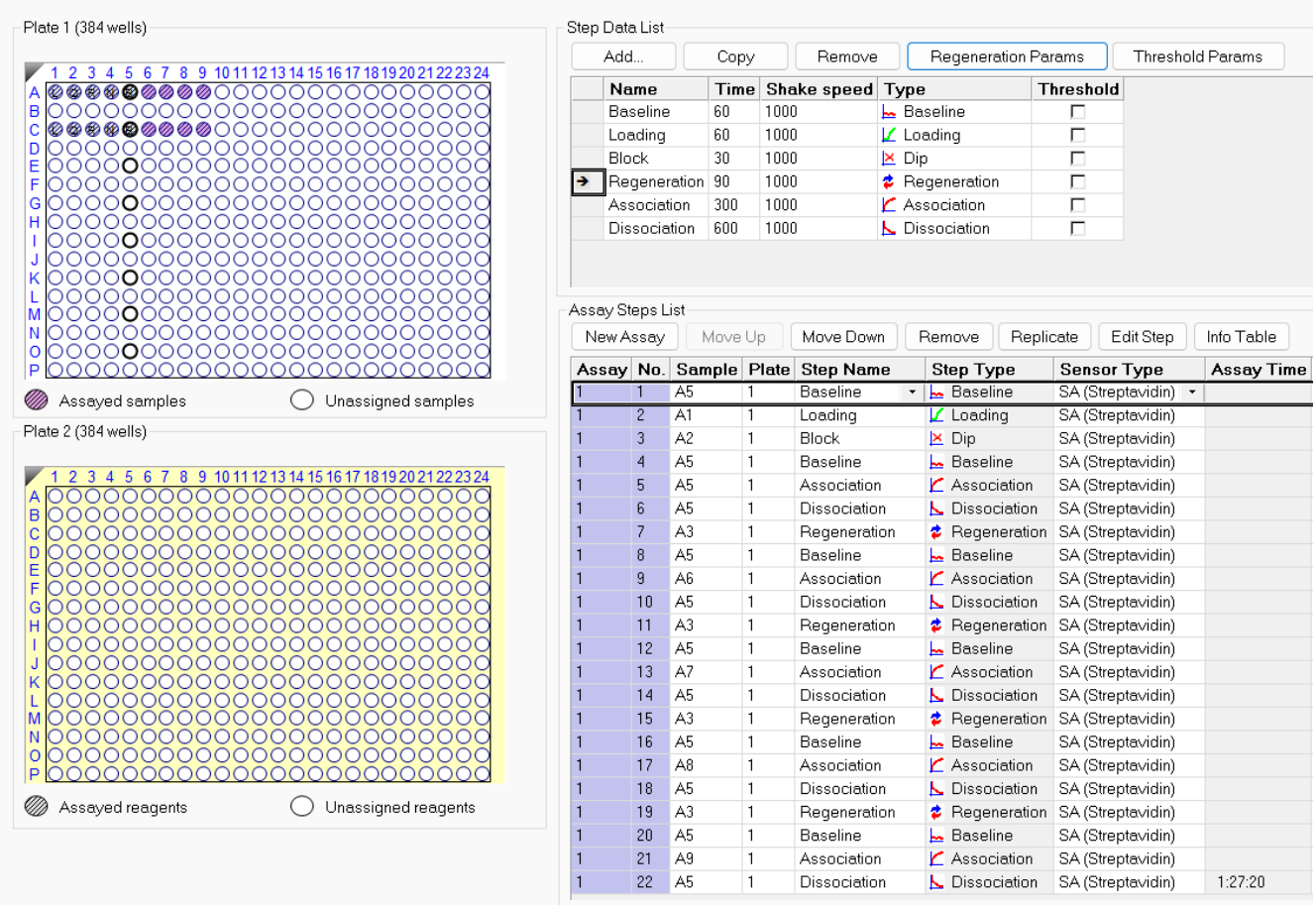


Figure S2: Step definition tab with assay steps set up for a basic kinetics experiment.

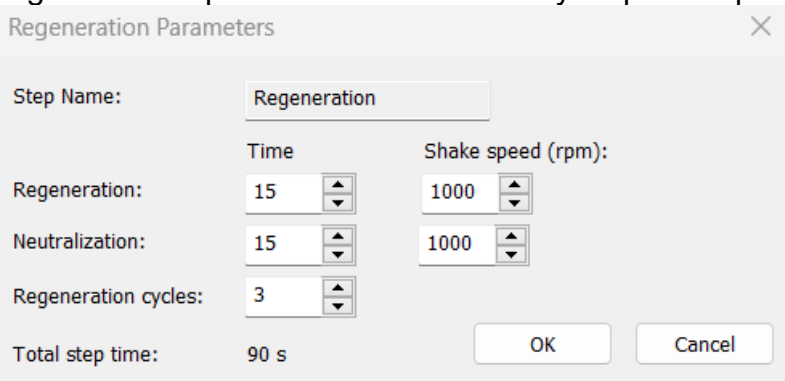


Figure S3: Regeneration parameters window accessible from the step definition tab, showing reasonable starting parameters.

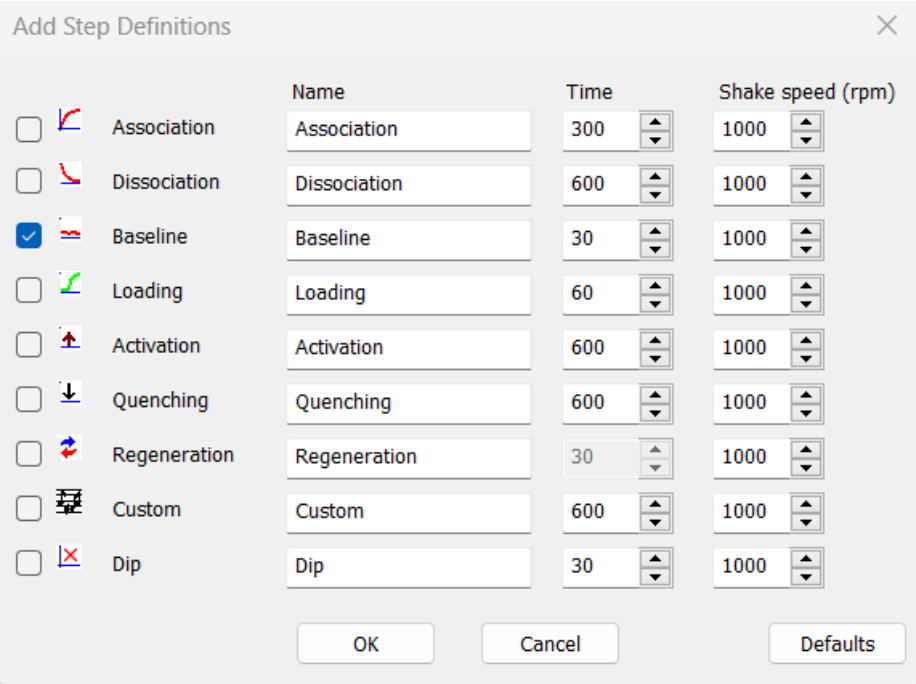


Figure S4: Step addition window selectable from the step definition tab, showing the possible options for adding a new step type to be added to the step data list. If multiple steps of the same type, but with different times, are to be used in the same experiment, then they must be added as separately named steps from this window.

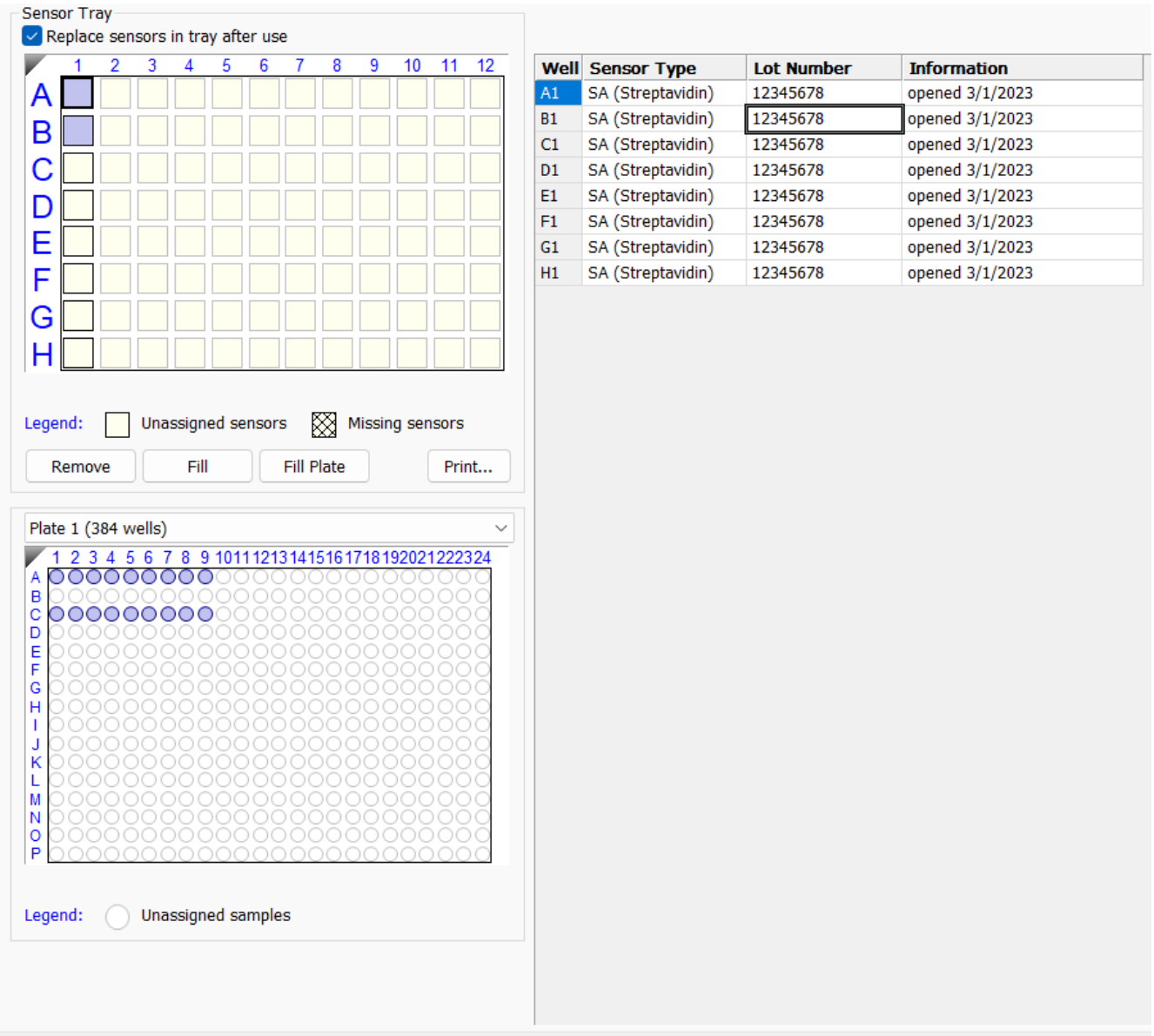


Figure S5: Sensor assignment window showing correctly assigned sensors in column 1. Sensors in purple are slated for use in the current experiment. The replace sensors box should generally be checked.

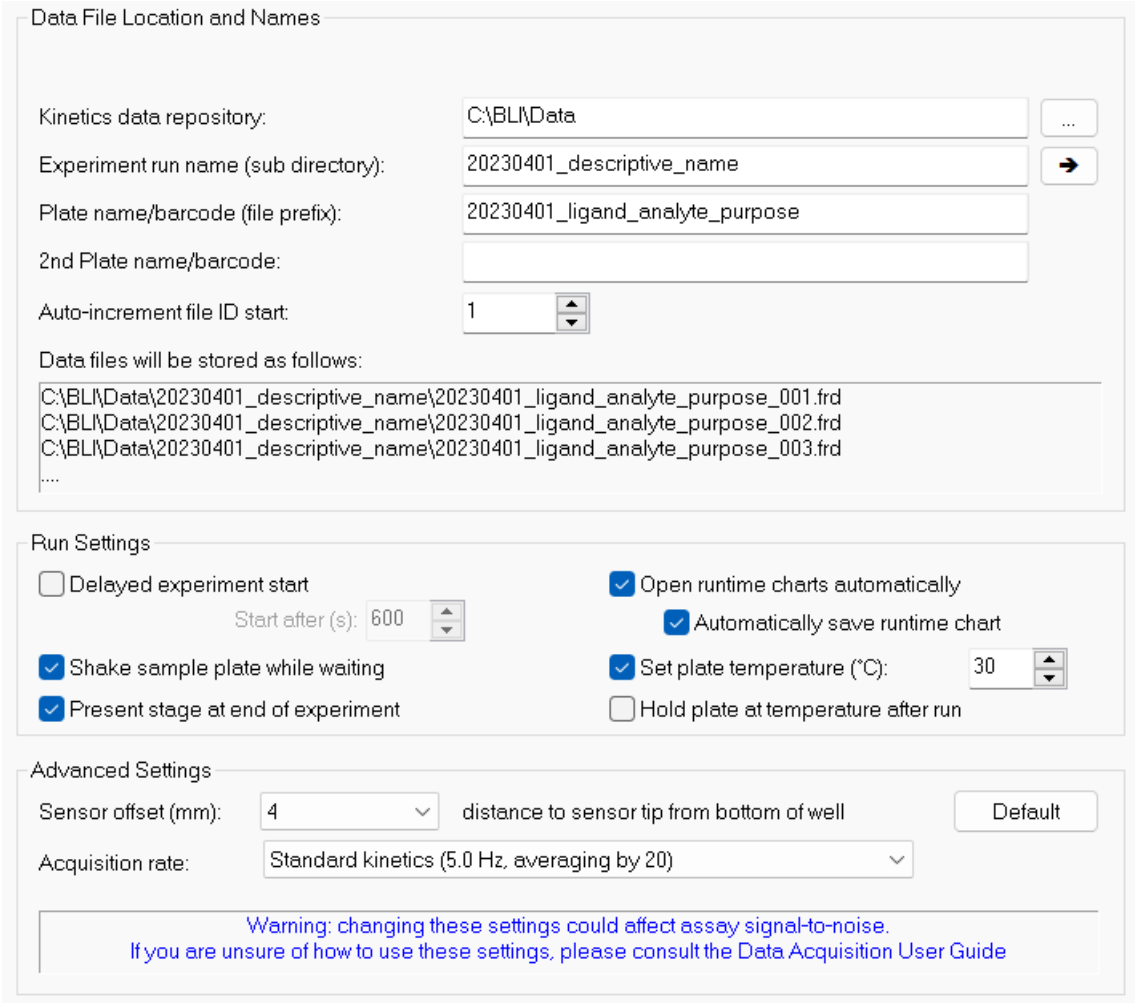


Figure S6: Run information tab showing reasonable average settings. Delay start can be used if sensors have not yet been soaked for a 15 minutes at the time of running.

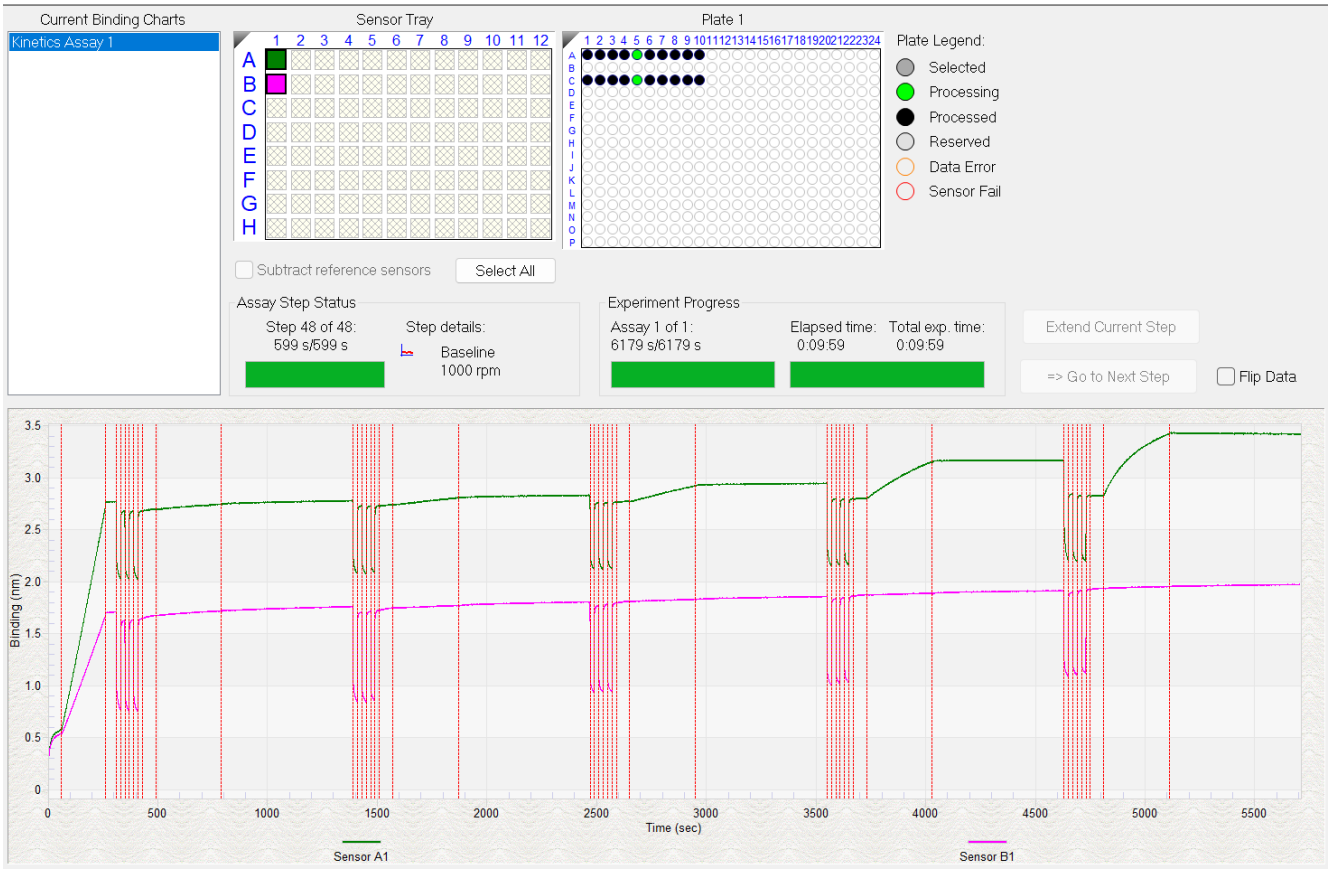


Figure S7: Example runtime display showing data collection and limited controls available during operation.

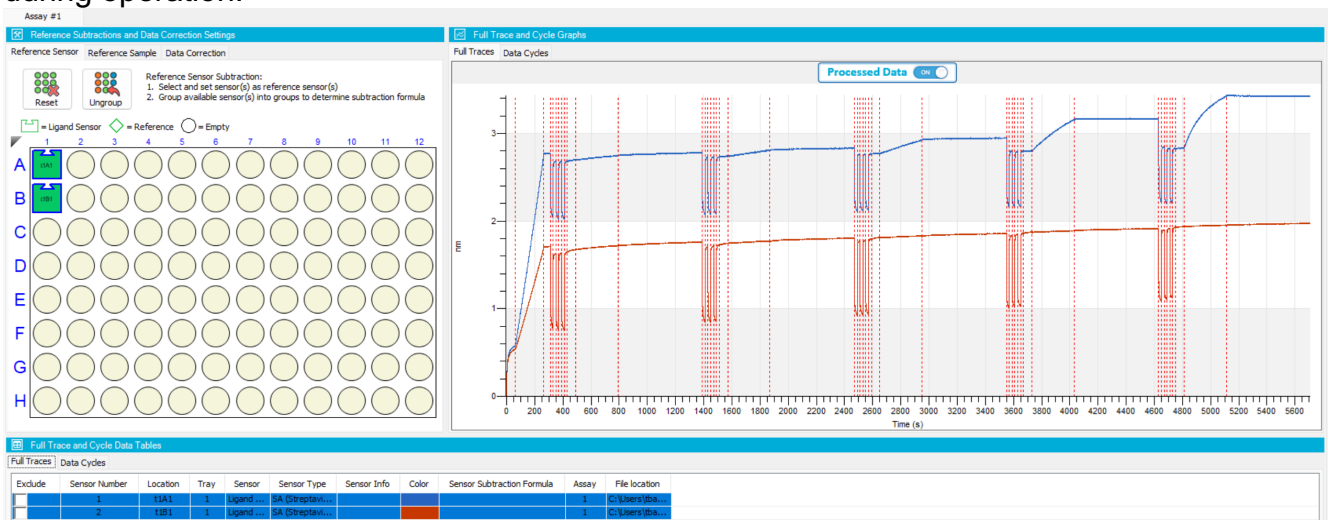


Figure S8: Example raw data in the Data Analysis HT software, for a basic kinetics experiment. Shown is the Pre-process operation tab, and the reference sensor assignment tab within that. Note that no reference information has yet been assigned, but this can be done by right clicking on a sensor in the 96 well plate and selecting “set sensor type”. Then select both the reference sensor and all associated sample sensors, then right click and select “subtract reference in selected wells”.

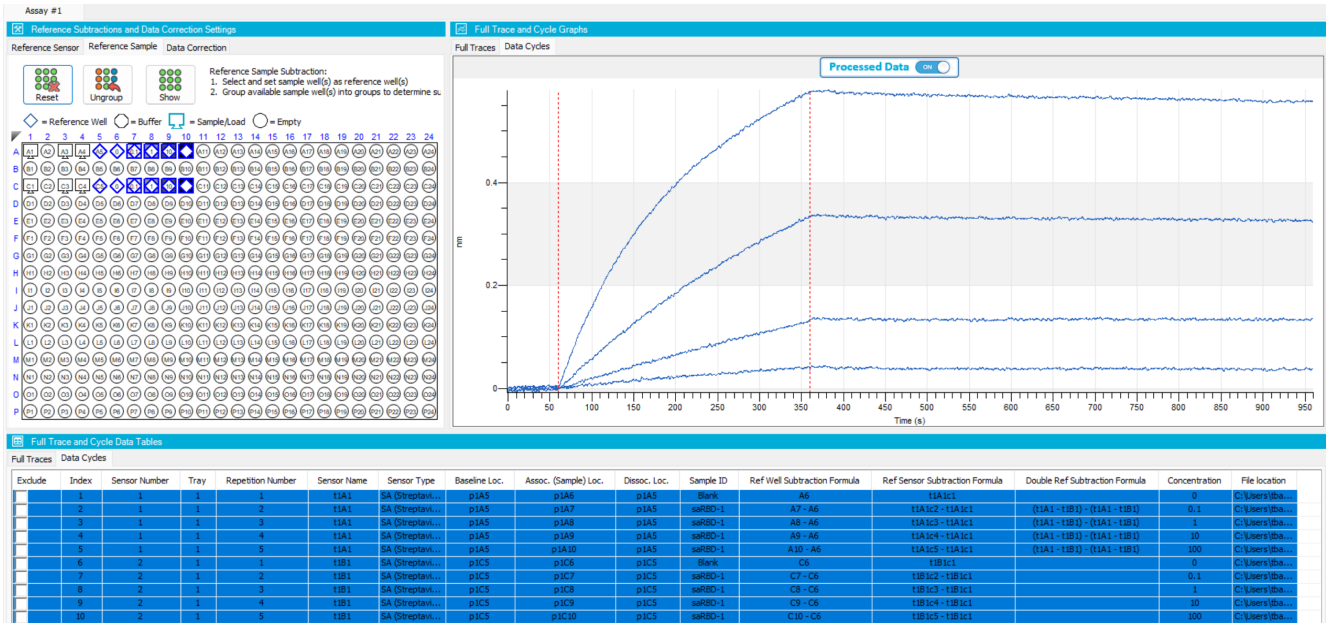


Figure S9: Reference sample selection tab within pre-process operations. Right click on reference wells and select “set reference,” then use control + left click to select both reference and associated sample wells, right click and select “subtract reference in selected wells.”

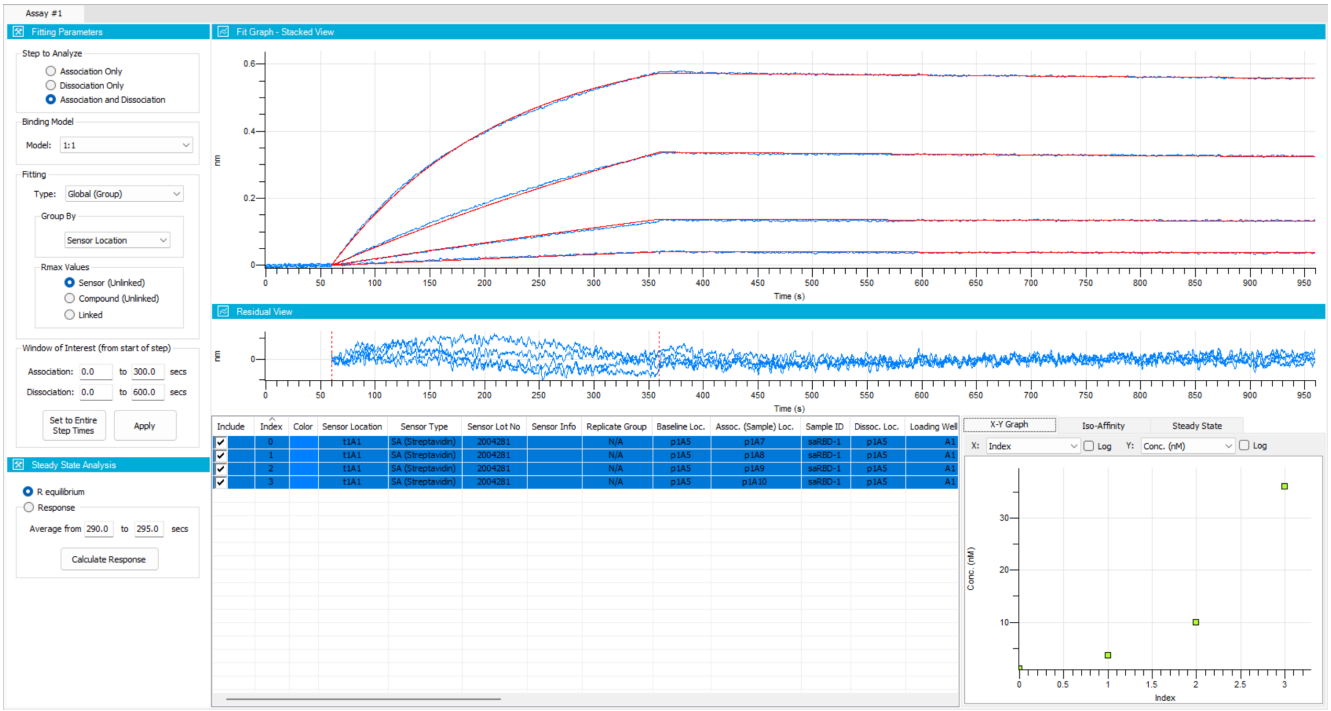
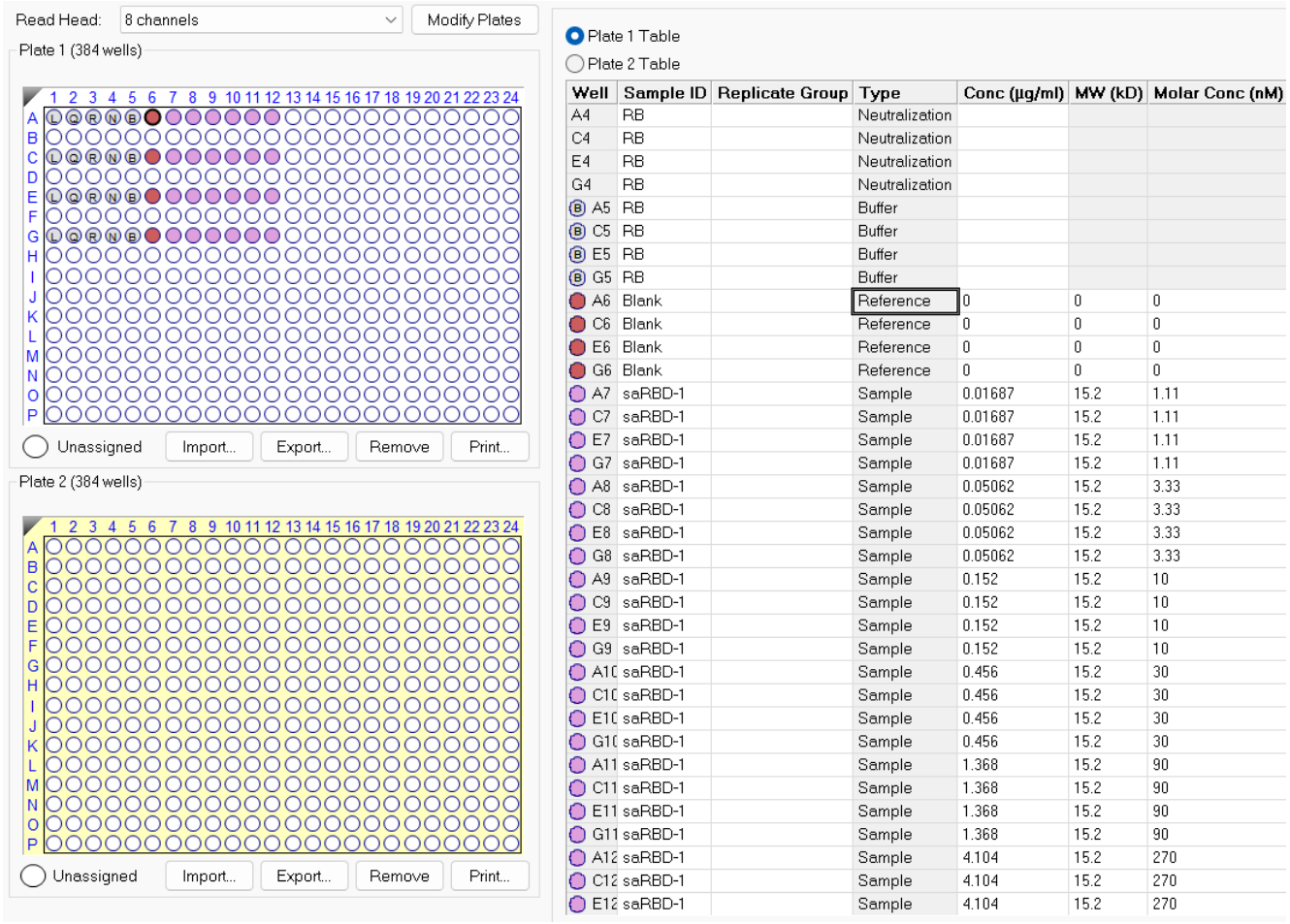
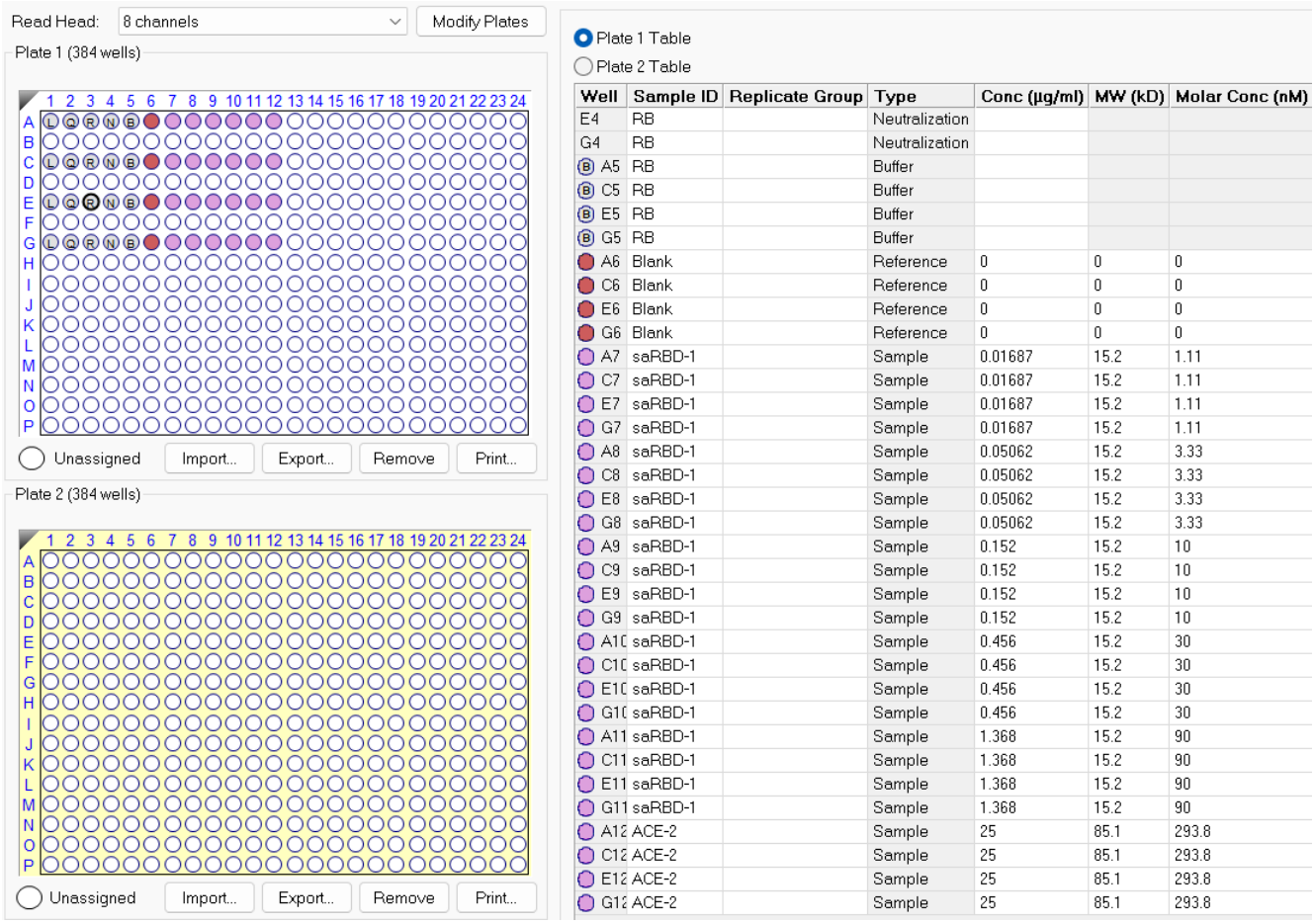


Figure S10: Kinetic operations tab. If not present, then select kinetic from the right hand side of the top bar in pre-process operations. Shown is the appropriate analysis method for a basic kinetics experiment using the 1:1 model and global fitting with Rmax values unlinked by sensor.



Well	Sample ID	Replicate Group	Type	Conc (µg/ml)	MW (kD)	Molar Conc (nM)
A4	RB		Neutralization			
C4	RB		Neutralization			
E4	RB		Neutralization			
G4	RB		Neutralization			
A5	RB		Buffer			
C5	RB		Buffer			
E5	RB		Buffer			
G5	RB		Buffer			
A6	Blank		Reference	0	0	0
C6	Blank		Reference	0	0	0
E6	Blank		Reference	0	0	0
G6	Blank		Reference	0	0	0
A7	saRBD-1		Sample	0.01687	15.2	1.11
C7	saRBD-1		Sample	0.01687	15.2	1.11
E7	saRBD-1		Sample	0.01687	15.2	1.11
G7	saRBD-1		Sample	0.01687	15.2	1.11
A8	saRBD-1		Sample	0.05062	15.2	3.33
C8	saRBD-1		Sample	0.05062	15.2	3.33
E8	saRBD-1		Sample	0.05062	15.2	3.33
G8	saRBD-1		Sample	0.05062	15.2	3.33
A9	saRBD-1		Sample	0.152	15.2	10
C9	saRBD-1		Sample	0.152	15.2	10
E9	saRBD-1		Sample	0.152	15.2	10
G9	saRBD-1		Sample	0.152	15.2	10
A10	saRBD-1		Sample	0.456	15.2	30
C10	saRBD-1		Sample	0.456	15.2	30
E10	saRBD-1		Sample	0.456	15.2	30
G10	saRBD-1		Sample	0.456	15.2	30
A11	saRBD-1		Sample	1.368	15.2	90
C11	saRBD-1		Sample	1.368	15.2	90
E11	saRBD-1		Sample	1.368	15.2	90
G11	saRBD-1		Sample	1.368	15.2	90
A12	saRBD-1		Sample	4.104	15.2	270
C12	saRBD-1		Sample	4.104	15.2	270
E12	saRBD-1		Sample	4.104	15.2	270

Figure S11: Sample plate design for a full kinetics experiment. See the attached importable method file and supplemental methods spreadsheet for additional details.



Well	Sample ID	Replicate Group	Type	Conc (µg/ml)	MW (kD)	Molar Conc (nM)
E4	RB		Neutralization			
G4	RB		Neutralization			
A5	RB		Buffer			
C5	RB		Buffer			
E5	RB		Buffer			
G5	RB		Buffer			
A6	Blank		Reference	0	0	0
C6	Blank		Reference	0	0	0
E6	Blank		Reference	0	0	0
G6	Blank		Reference	0	0	0
A7	saRBD-1		Sample	0.01687	15.2	1.11
C7	saRBD-1		Sample	0.01687	15.2	1.11
E7	saRBD-1		Sample	0.01687	15.2	1.11
G7	saRBD-1		Sample	0.01687	15.2	1.11
A8	saRBD-1		Sample	0.05062	15.2	3.33
C8	saRBD-1		Sample	0.05062	15.2	3.33
E8	saRBD-1		Sample	0.05062	15.2	3.33
G8	saRBD-1		Sample	0.05062	15.2	3.33
A9	saRBD-1		Sample	0.152	15.2	10
C9	saRBD-1		Sample	0.152	15.2	10
E9	saRBD-1		Sample	0.152	15.2	10
G9	saRBD-1		Sample	0.152	15.2	10
A10	saRBD-1		Sample	0.456	15.2	30
C10	saRBD-1		Sample	0.456	15.2	30
E10	saRBD-1		Sample	0.456	15.2	30
G10	saRBD-1		Sample	0.456	15.2	30
A11	saRBD-1		Sample	1.368	15.2	90
C11	saRBD-1		Sample	1.368	15.2	90
E11	saRBD-1		Sample	1.368	15.2	90
G11	saRBD-1		Sample	1.368	15.2	90
A12	ACE-2		Sample	25	85.1	293.8
C12	ACE-2		Sample	25	85.1	293.8
E12	ACE-2		Sample	25	85.1	293.8
G12	ACE-2		Sample	25	85.1	293.8

Figure S12: Sample plate design for a competition experiment. See the attached importable method file and supplemental methods spreadsheet for additional details.

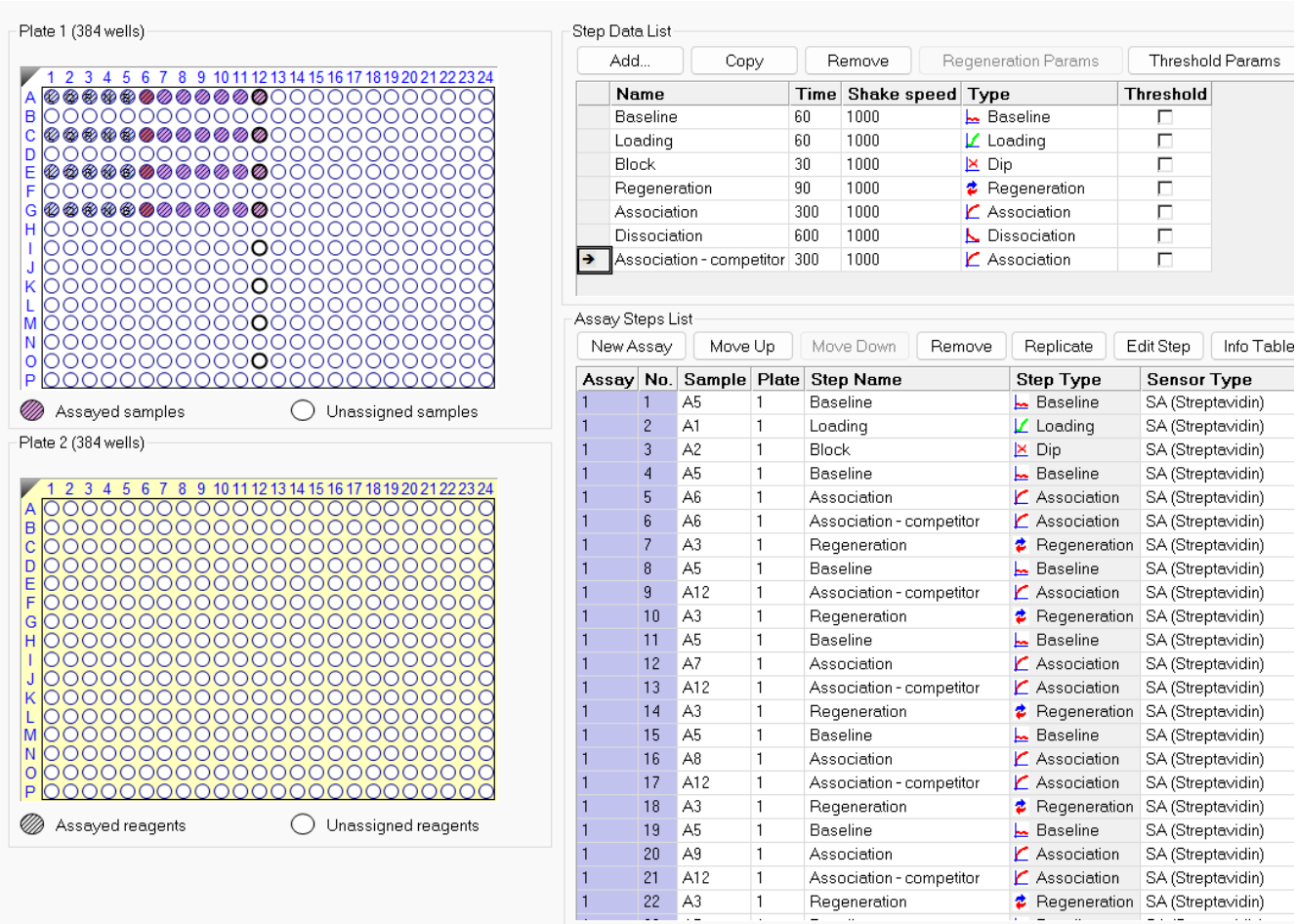
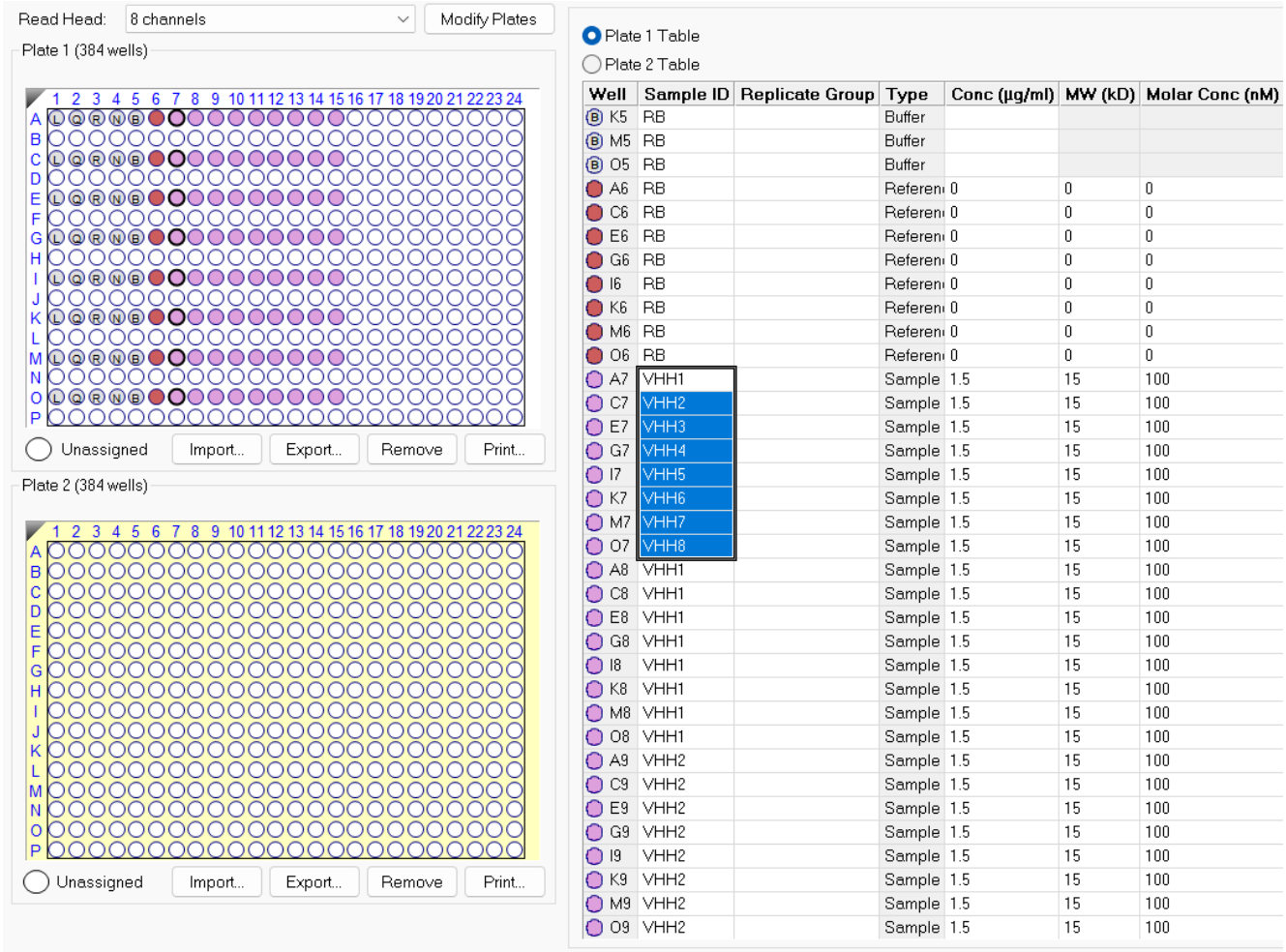


Figure S13: Sample assay design for a competition experiment. See the attached importable method file and supplemental methods spreadsheet for additional details.



Well	Sample ID	Replicate Group	Type	Conc (µg/ml)	MW (kD)	Molar Conc (nM)
K5	RB		Buffer			
M5	RB		Buffer			
O5	RB		Buffer			
A6	RB		Referen	0	0	0
C6	RB		Referen	0	0	0
E6	RB		Referen	0	0	0
G6	RB		Referen	0	0	0
I6	RB		Referen	0	0	0
K6	RB		Referen	0	0	0
M6	RB		Referen	0	0	0
O6	RB		Referen	0	0	0
A7	VHH1		Sample	1.5	15	100
C7	VHH2		Sample	1.5	15	100
E7	VHH3		Sample	1.5	15	100
G7	VHH4		Sample	1.5	15	100
I7	VHH5		Sample	1.5	15	100
K7	VHH6		Sample	1.5	15	100
M7	VHH7		Sample	1.5	15	100
O7	VHH8		Sample	1.5	15	100
A8	VHH1		Sample	1.5	15	100
C8	VHH1		Sample	1.5	15	100
E8	VHH1		Sample	1.5	15	100
G8	VHH1		Sample	1.5	15	100
I8	VHH1		Sample	1.5	15	100
K8	VHH1		Sample	1.5	15	100
M8	VHH1		Sample	1.5	15	100
O8	VHH1		Sample	1.5	15	100
A9	VHH2		Sample	1.5	15	100
C9	VHH2		Sample	1.5	15	100
E9	VHH2		Sample	1.5	15	100
G9	VHH2		Sample	1.5	15	100
I9	VHH2		Sample	1.5	15	100
K9	VHH2		Sample	1.5	15	100
M9	VHH2		Sample	1.5	15	100
O9	VHH2		Sample	1.5	15	100

Figure S14: Sample plate design for an epitope binning experiment. See the attached importable method file and supplemental methods spreadsheet for additional details.

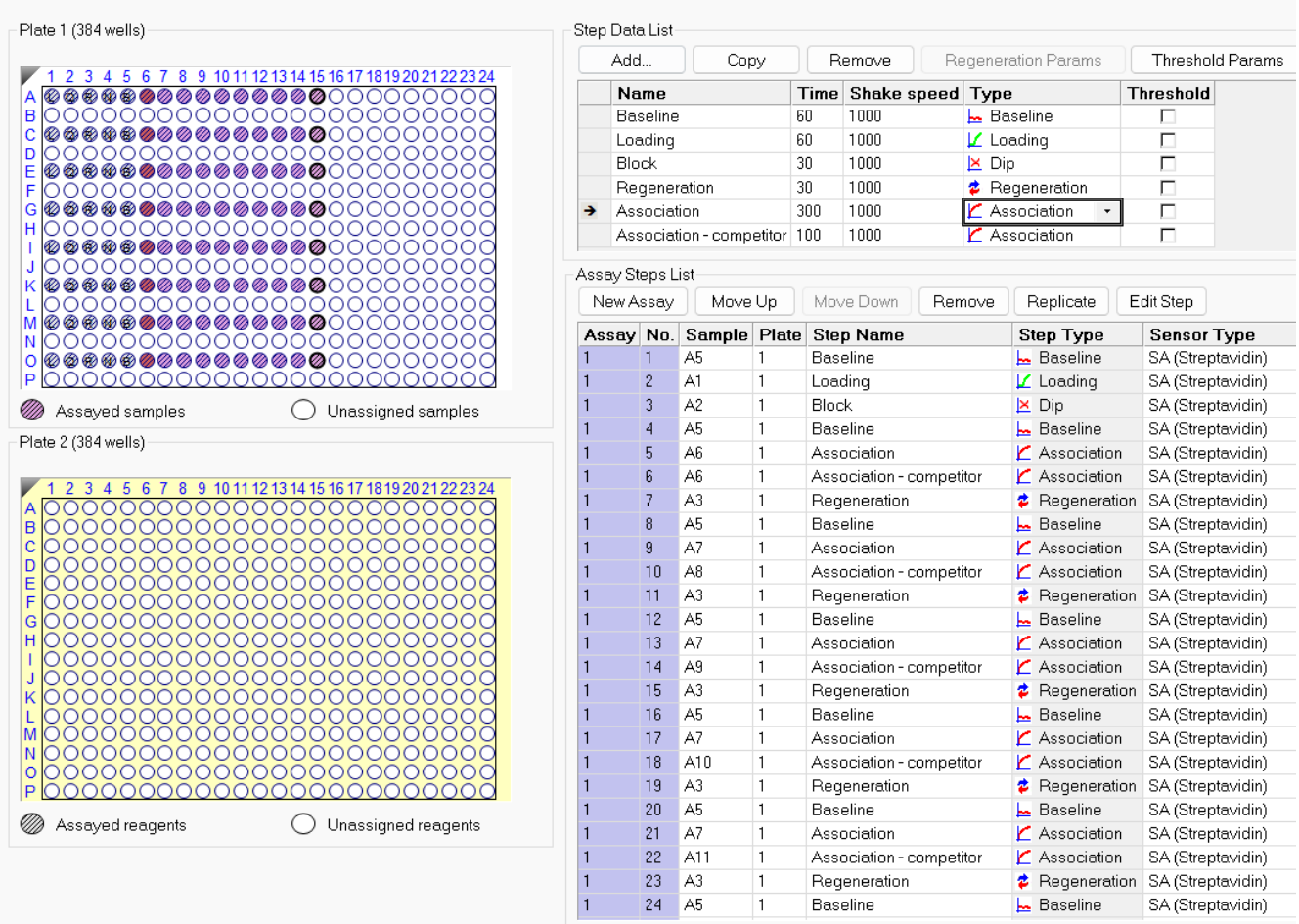


Figure S15: Sample assay design for an epitope binning experiment. See the attached importable method file and supplemental methods spreadsheet for additional details.

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