Gator Bio: Getting Started Note

How to set up a Kinetics Assay with Bio-layer Interferometry (BLI)

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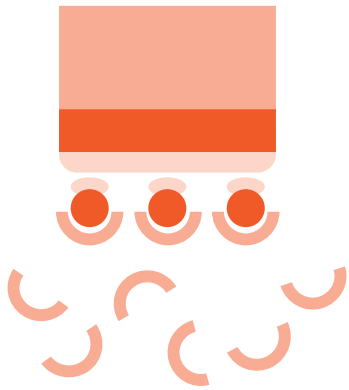
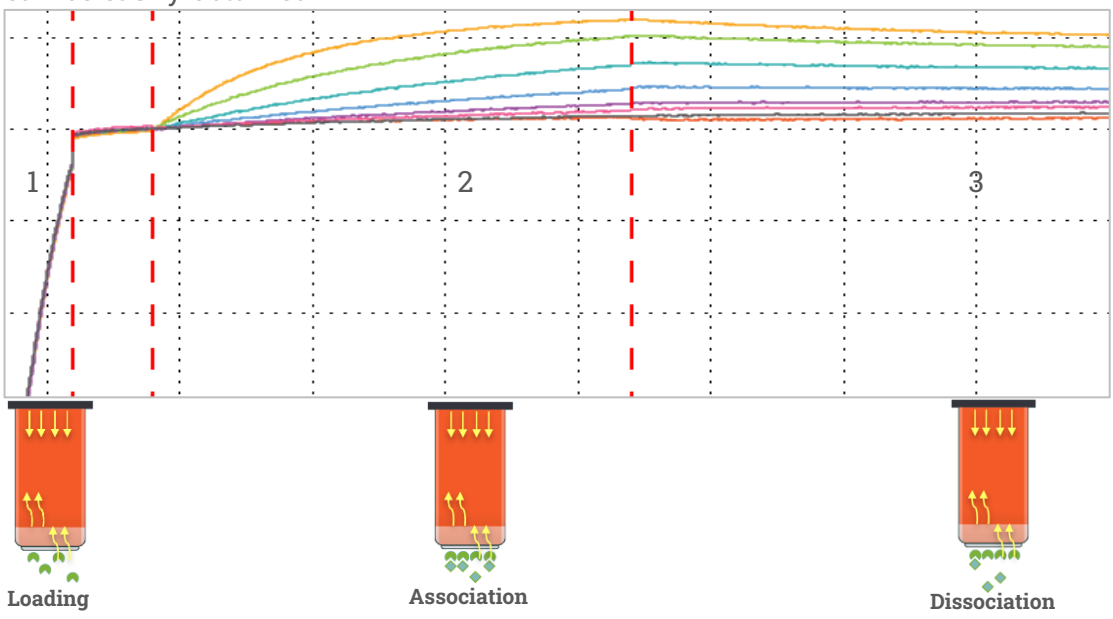
Introduction

Gator® system is a label-free analytical instrument designed for real-time measurement of biological molecules using bio-layer interferometry (BLI). Applications such as titer determination, quantitation of unknown samples, off-rate ranking of antibodies, binding affinity measurement, interrogation of binding to target antigens, antibody affinity maturation, and epitope binning can be readily and accurately performed on the Gator® system.



Measuring kinetics using BLI is achieved by first immobilizing a "ligand" on the biosensor surface (Step 1). If this molecule interacts with a second molecule, known as the "analyte", a change in optical thickness at the biosensor surface will occur. This binding event, also known as association, forms the ligand-analyte complex which results in a shift in the interference pattern, and is recorded in real-time (Step 2). Finally, the analyte dissociates from the complex in the absence of free analyte, leading to a reduction in the optical thickness, and a corresponding reduction in binding signa (Step 3). By fitting the binding event sensor-gram, kinetics values, such as k_{on}, k_{off}, and K_D, can be easily obtained.



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Materials

Plates
To run experiments on Gator® system, users should prepare one black microplate and one **Max plate** (Gator Bio, PN: 130062). Please note: Only Greiner Bio-One black microplates are recommended for the Gator® system.

Buffer
As a start, it is recommended to dilute your samples in the buffer in which your proteins-of-interest behave the best. Surfactants (e.g. 0.02% Tween20) or carrier proteins (e.g. 0.2% BSA) are often added to the assay buffer to minimize non-specific binding. Assay running buffer is added to the black microplate.

Biosensors
Biosensors are placed in the Max plate. Hydrating the biosensors prior to use, for at least 10 minutes in the assay running, in the assay buffer (Q or K buffer recommended) is highly recommended.
The following table shows the wide selection of ready-to-use biosensors available for purchase Custom-Made probes are also available upon request.

Materials

Gator® Probe	Function	Application	Dynamic Range	Regeneration
Protein A (ProA)	Binds IgGs of various species including human and mouse	Q	0.02 - 2000 µg/mL	Yes
Protein G (ProG)	Binds IgGs of various species including human and rat	Q	0.02 - 2000 µg/mL	Yes
Protein L (ProL)	Binds IgGs of various species through the kappa light chain	Q	0.02 - 2000 µg/mL	Yes
Anti-Human IgG Fc (HFC)	Immobilize human Fc-fusion protein or human IgG for quantitative or kinetic analysis	Q/K/QKR/EP	0.05 - 300 µg/mL	Yes
Anti-Human IgG Fc Gen II (HFCII)	Immobilize human Fc-fusion protein or human IgG for quantitative or kinetic analysis	Q/K/QKR/EP	0.3 - 6000 µg/mL	Yes
Anti-Mouse IgG Fc (MFC)	Immobilize mouse Fc-fusion protein or mouse IgG for quantitative or kinetic analysis	Q/K/QKR/EP	0.02 - 2000 µg/mL	Yes
Anti-Human FAB (FAB)	Binds F(ab), F(ab')2, Fc receptor, and full-length Human IgG	Q/K/QKR/EP	0.3 - 3000 µg/mL	Yes
Anti-His (HIS)	Binds His-tagged proteins	Q/K/QKR/EP	Protein-dependent	Yes
Ni-NTA	Tris-NTA and charged with Ni^{2+} ions binding to His-tagged proteins	Q/K/QKR/EP	Protein-dependent typically 0.25 – 1000 ug/mL	Yes
Streptavidin (SA)	Binds biotinylated and Avi-tagged biomolecules	K/EP	Protein-dependent	No
Small Molecule Analysis Probes (SMAP)	Binds biotinylated and Avi-tagged biomolecules and subsequent binding of small molecules and proteins	K	>150 Da	No
Flex SA	Binds biotinylated and Avi-tagged biomolecules with reactivable sensor surface	K	Protein-dependent	Reactivable
Aminopropylsilane (APS)	Binds hydrophobic proteins	K	Protein-dependent	No
Amine-Reactive (AR)	Covalently attach amine group of proteins using EDC/NHS	K/EP	Protein-dependent	No
AAVX	Binds serotypes AAV1-AAV8 and AAV10	Q	10^9 – 10^{13} vp/mL	Yes
AAV9	Binds specifically to AAV9 serotype	Q	3×10^9 – 1×10^{13} vp/mL	Yes
Custom - Made	Custom made biosensors for your specific applications (SARS-CoV-2 RBD, Anti-Rabbit, Anti-Rat and Anti-FLAG)	Varies	Varies	Varies

*For the best performance, it is recommended to regenerate the Gator® HFCII, MFC, FAB, AAVX and AAV9 probes using Regen Buffer - No Salt (Part No. 120063) prior to use

Gator Plates Information:

Max Plates Part. No. 130062
Max Plates with Lids Part. No. 130018
Gator® BLI 96-Flat Plates, Polypropylene (Case of 100) Part No. 130260
Gator® BLI 96-Flat Plates, Polypropylene (Pack of 10) Part No. 130150

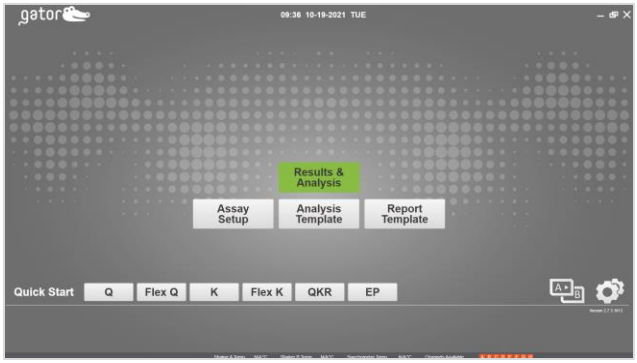
Tips to Design the First Kinetics Experiment

1. Samples and buffers should be equilibrated to room temperature before the start of the assay.
2. New biosensors need to be hydrated for at least 10 minutes before use. Add 250 uL of assay buffer to wells in a **Max plate**. Use tweezers to transfer probes from supply tray to buffer wells.
3. Avoid bubbles when adding buffers and samples to the Max plate and black microplate.
4. Assay buffer is an important component to consider. The optimal buffer is one that helps prevent non-specific binding while allowing proteins-of -interest to behave naturally. The chosen buffer should be used throughout the entire assay to avoid interference changes .
5. Do not over saturate the biosensor. 50%-80% loading capacity is recommended. Optimal ligand concentration can be determined by running a loading optimization assay using high (10-15 ug/mL), medium (5-10 ug/mL), and low (1-5 ug/mL) ligand concentrations. The optimal concentration should reach 50%-80% loading capacity in 120s. This loading density limits analyte rebinding during the dissociation step, steric hindrance, and mass transport limitation.
6. A more accurate estimation of K_D is obtained by performing the experiment using a range of analyte concentrations. The range of concentrations should, ideally, span 0.1x to 10x of anticipated the K_D value. An optimization assay should be performed to obtain an estimate of the K_D before performing the dose response experiments. This optimization assay could also provide insights on the appropriate times for association and dissociation steps.
7. For better data quality, a reference should be included in the experiment, which will be subtracted during analysis. There are four types of references and the reference best suited for your experimental setup, should be included.
    a. Reference well (highly recommended): Immobilized ligand is dipped into a buffer well (reference well) during association step
    b. Non-specific Reference (optional): A probe with NO immobilized ligand ran through highest analyte concentration. If appreciable binding is measured, consider running assay with reference probes included
    c. Reference probe (optional): Analyte series is measured using probes with NO immobilized ligand
    d. Reference assay (optional): An experiment ran with irrelevant molecule to interact with analyte

Kinetics: Assay Setup

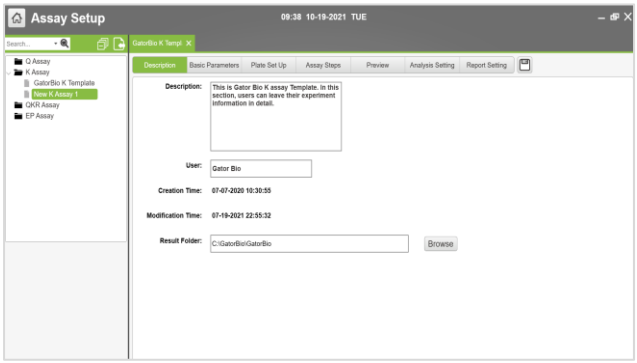
Start the Instrument

- Turn on instrument at least 1 hour before use
- Start the GatorOne Software using the GatorLaunch Shortcut
  - Wait for the software to Launch
  - Allow the instrument to self-check



Setting Up a Kinetics Assay (K Assay)

1. Open a new K assay using Quick Start: K
2. Label the K assay
   - Optionally, the user can include an experimental description and experimenter's name under the Description Tab



3. Select the Basic Parameters Tab

a Select the Data Acquisition frequency

- 2 Hz will collect 2 datapoints a second
- 5 Hz will collect 5 datapoints a second (recommended for most assays)
- 10 Hz will collect 10 datapoints a second

b Select the Plate Type

- The GatorPrime is compatible with the 96-plate format
- The GatorPlus is compatible with the 96-plate and 384-plate formats

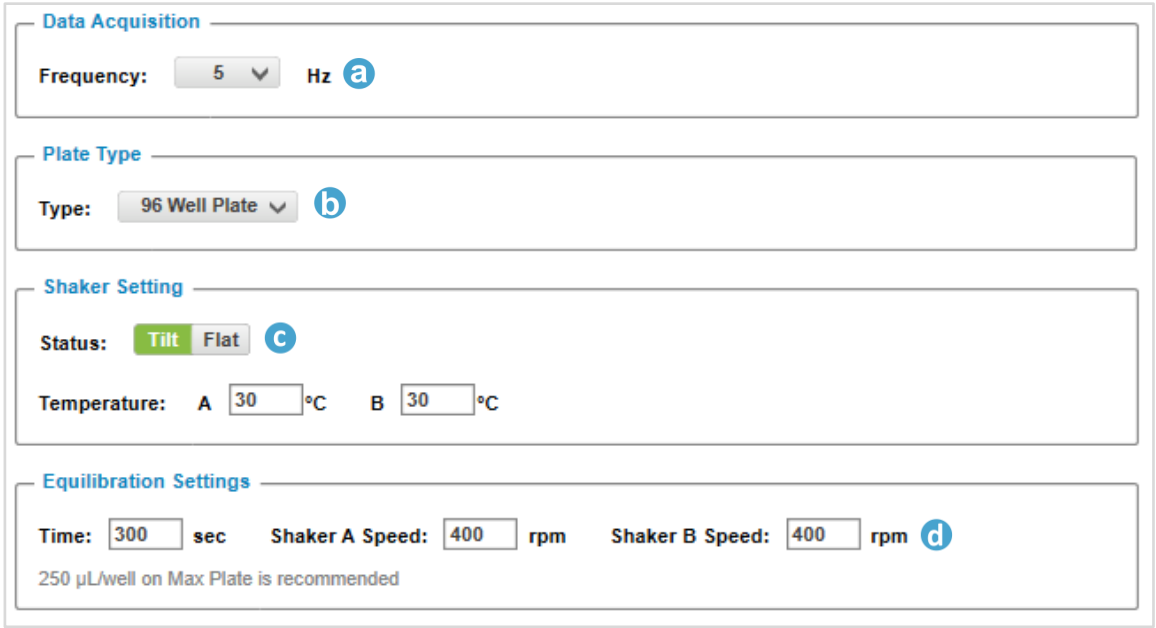
c Select the Shaker Position and Temperatures

- The GatorPrime and GatorPlus shaker temperatures range from ambient temperature to 40°C

d Select the Equilibration time and Shaker Speeds

- The GatorPrime has a max speed of 1500 RPM
- The GatorPlus has a max speed of 2000 RPM

- Entering information in this section, allows the biosensors and samples to be equilibrated to the desired assay running temperature, before the start of the assay



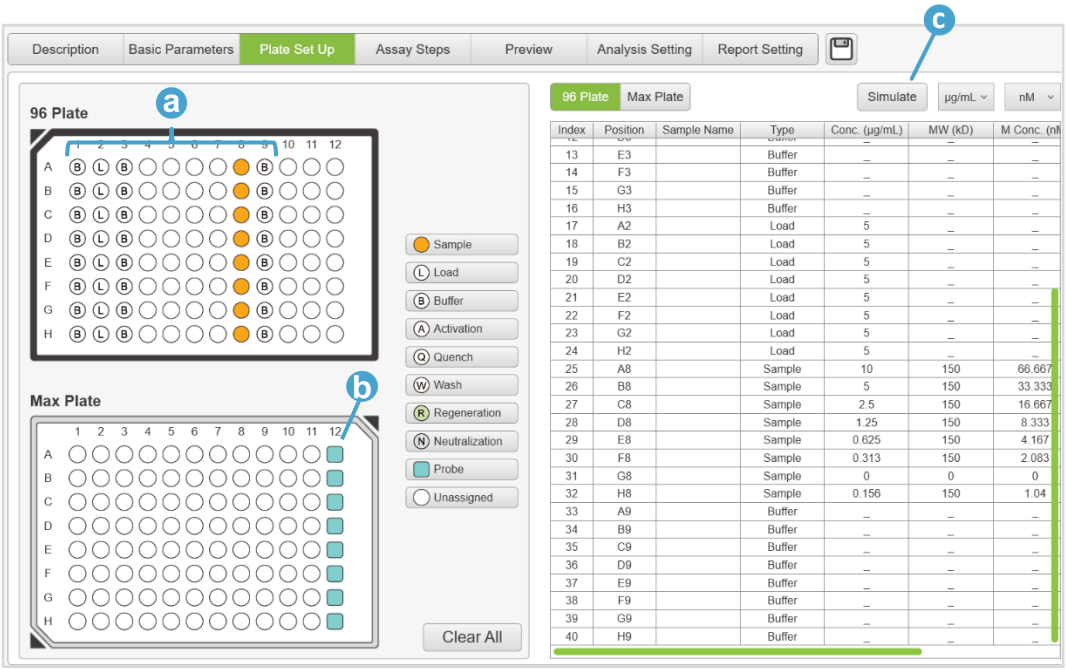
4. Select the Plate Setup Tab

ⓐ Label the 96-plate map with **buffer**, **loading**, and **sample** wells

- In a kinetics experiment, sample wells must be labeled with concentrations in molarity for k_{on} and K_D calculation

ⓑ Label the Max plate map with **Probes**

- If regeneration of the biosensors is desired, add **Regeneration** and **Neutralization** buffers to wells in the black microplate and label them accordingly on the 96-plate map



Additional features:

- Gator will convert ug/mL to molarity for you
- If you highlight sample wells and click ⓒ, you can simulate kinetic curves using inputted values of k_{on} and k_{off}

5. Select the **Assay Steps** Tab

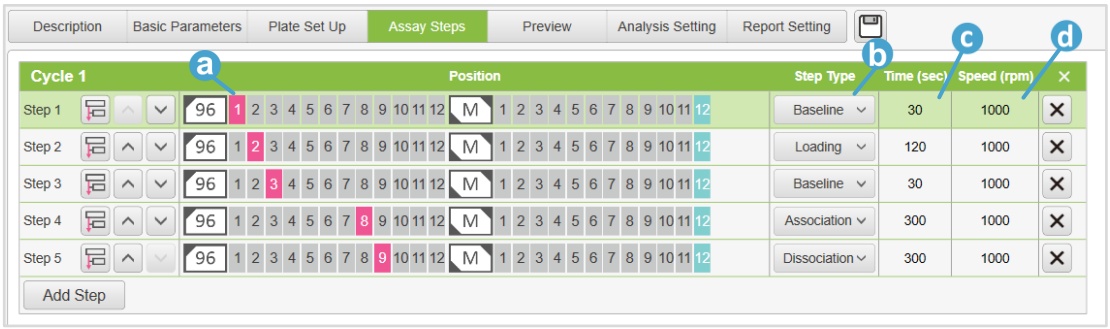
ⓐ Setup your cycle by designating step location

ⓑ Select Step Type

Association and Dissociation steps must be correctly labeled for analysis

ⓒ Input desire step time (in seconds)

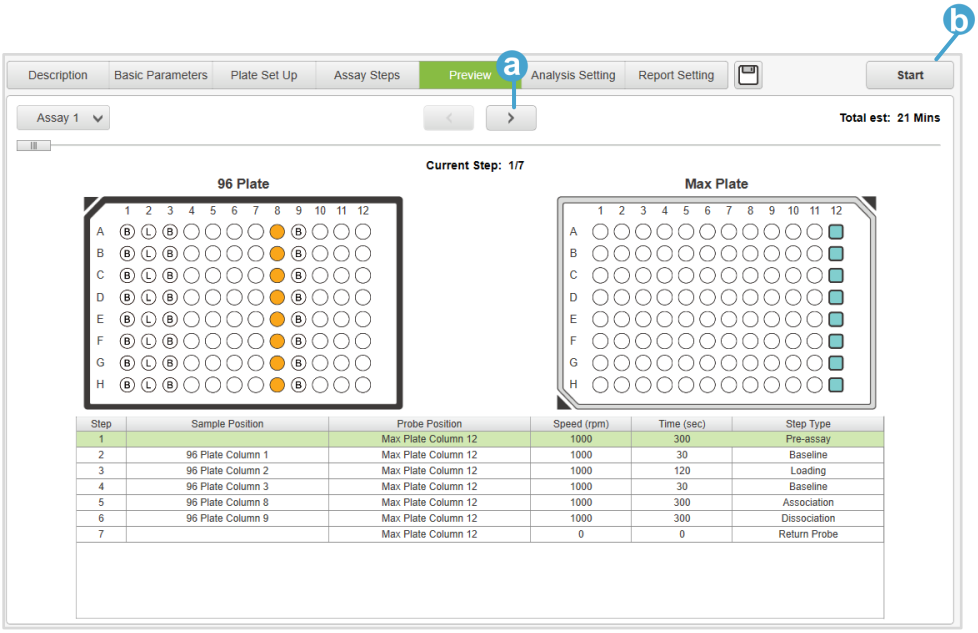
ⓓ Input desire step speed (in RPM)



6. Select the **Preview** Tab

ⓐ Check that the assay is set up correctly by cycling through steps.

ⓑ Start Assay by pressing the Start button



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Kinetics: Data Analysis

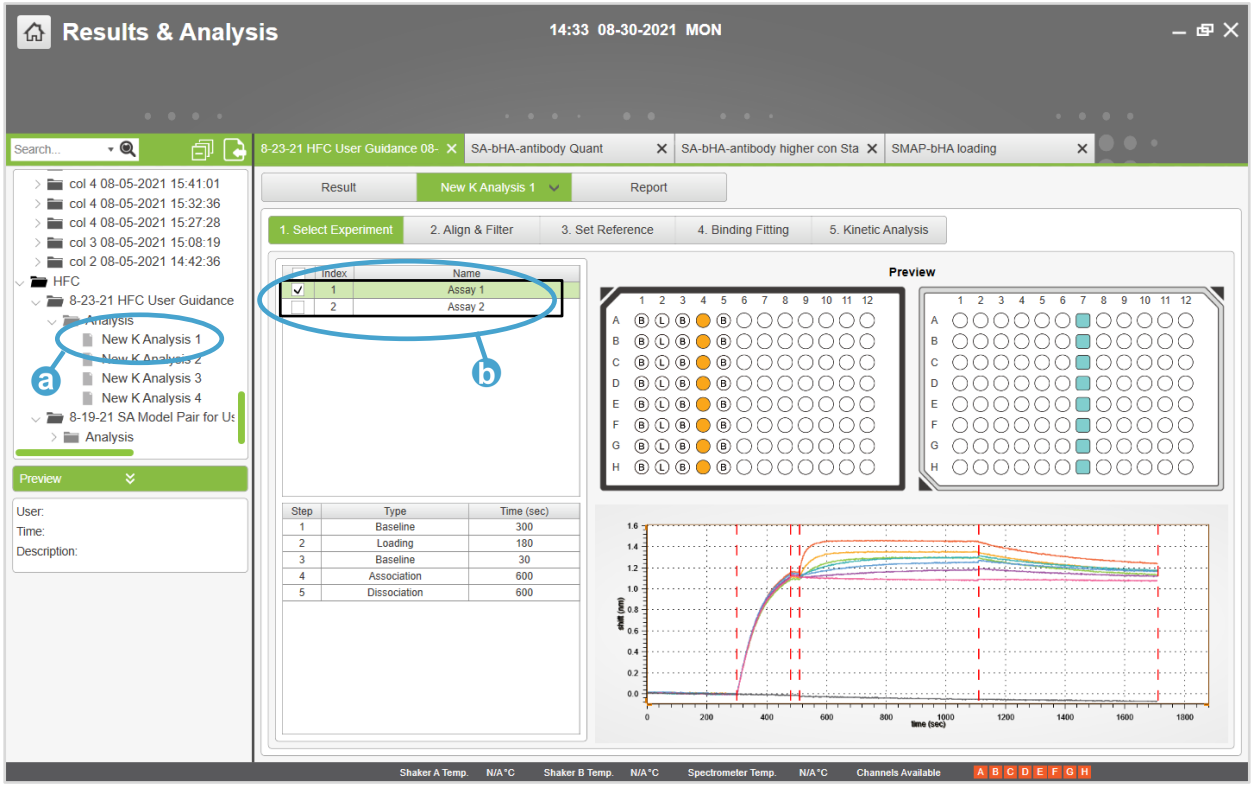
1. Open Results & Analysis from the GatorOne homepage
2. Select Data

   a Locate the experiment in the folder bar

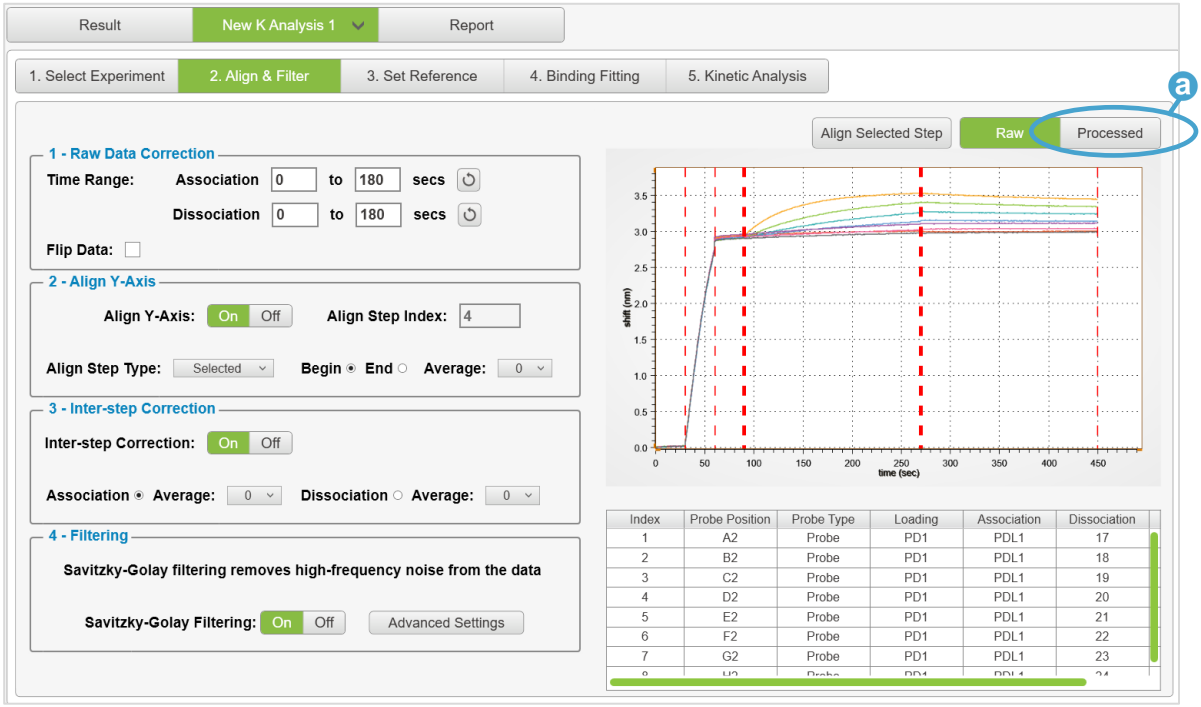
   Click the drop-down arrow to expand the folder

   Double click “New K Analysis 1” (If you want to rename this analysis, right click and go to “rename”)

   b If experiment contains more than 1 assay, you can toggle assays on or off to be analyzed as a group or individually under the “Select Experiment” tab



Align & Filter



1. **Raw Data Correction:** Data range of interest can be adjusted here. Artifacts at beginning or end of steps can be removed here. Data can also be flipped over the x-axis by checking **Flip Data**

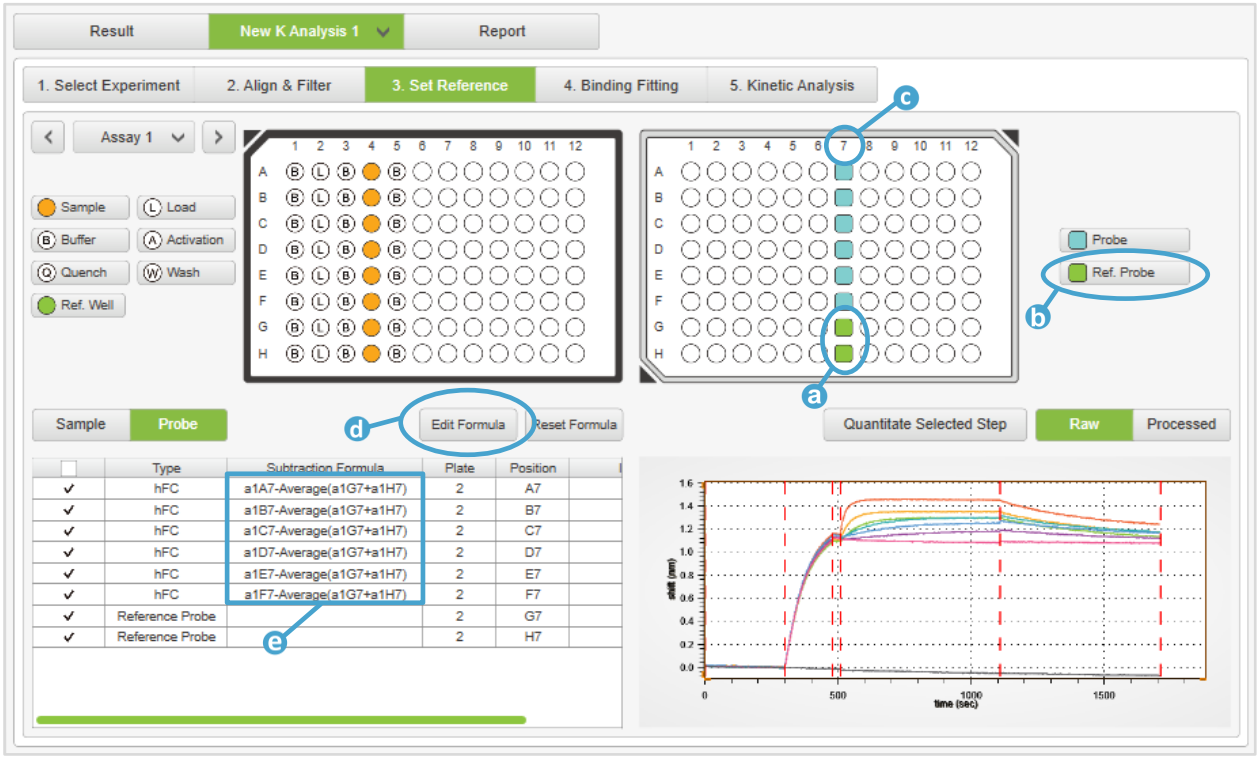
2. **Align Y-Axis:** Kinetics data should be aligned at the beginning of the association step. Under **Align Step Type**, click the drop-down menu and choose association. Be sure that **Begin** is selected

3. **Inter-step Correction:** For typical kinetics assays, **Inter-step Correction** should be on

4. **Filtering:** Smoothing preferences can be selected here

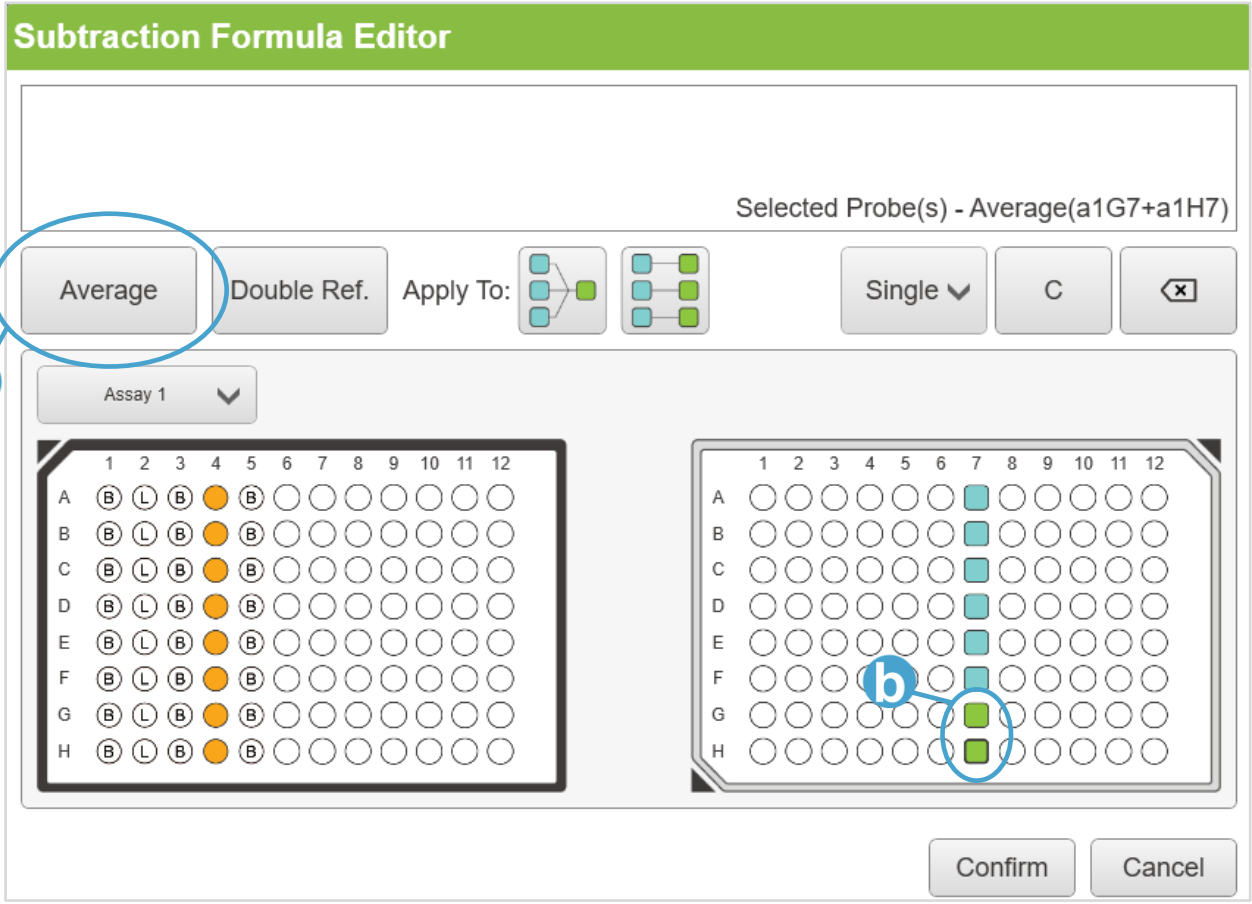
To see Processed data, click a

Set Reference



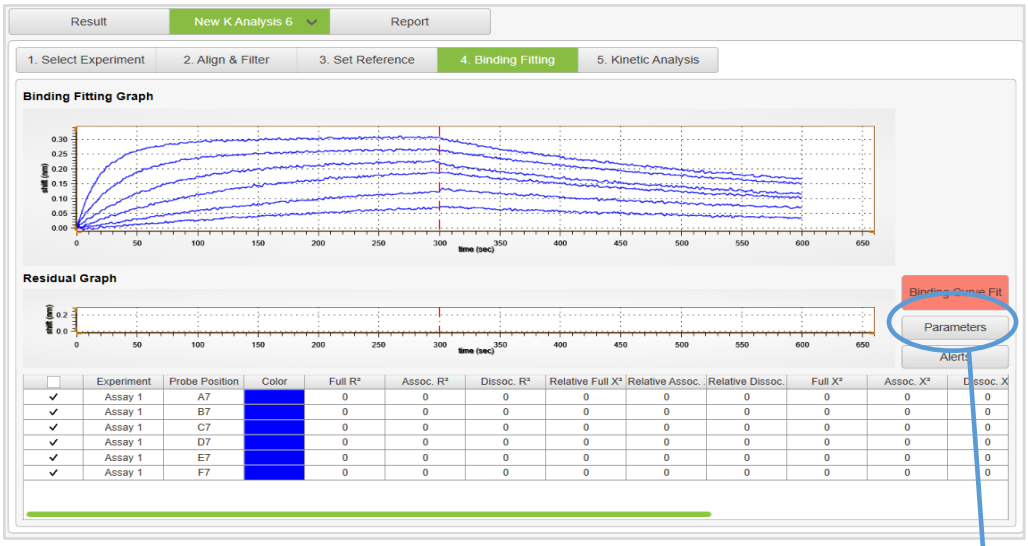
1. Set Reference probe by highlighting appropriate probe (a) and clicking **Ref. Probe** (b).
2. Highlight **sample probes** by clicking column number (c).
3. Click on Edit formula (d) to open the formula editor tab to subtract the reference signals from the sample signals.
4. After doing reference subtraction, check table (e) to confirm sample probes have been appropriately processed.

Subtracting Reference Signals



- For assays with one reference, simply click on the reference probe
- For assays with two references, subtract the average by clicking **Average** (ⓐ), then clicking both reference probes (ⓑ)

Binding Fitting



Click on ⓐ to open the parameters tab

Data to Include: Choose to analyze association and dissociation alone, or both together

Binding Model: Choose appropriate binding model. 1:1 binding is most common

Fitting:

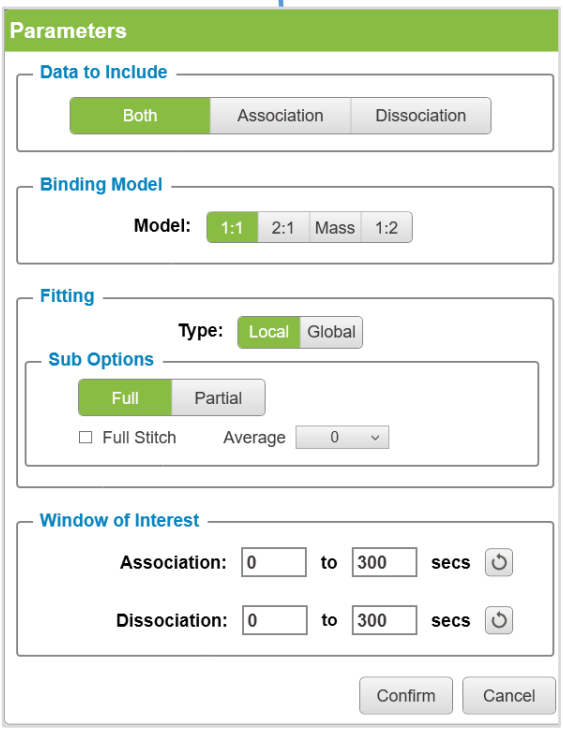
Local type will fit each curve independently

Full fits association and dissociation together

Partial fits association without considering dissociation

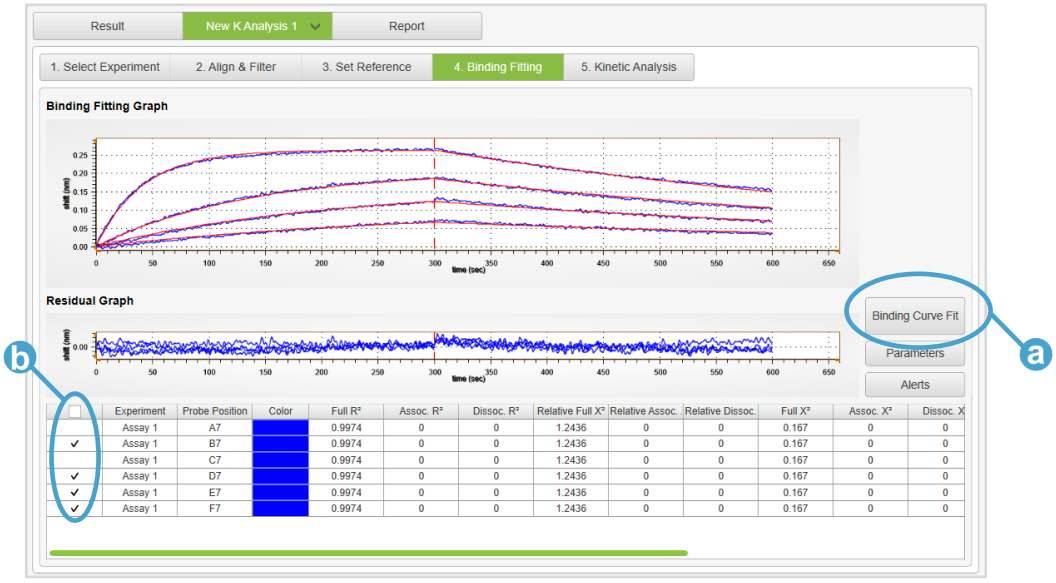
Global fits data based on constants derived simultaneously from all the analyte concentrations

Rmax Linked: This assumes that the calculated Rmax is the same for each curve.
Rmax Unlinked: This allows for the Rmax to vary between the curves.

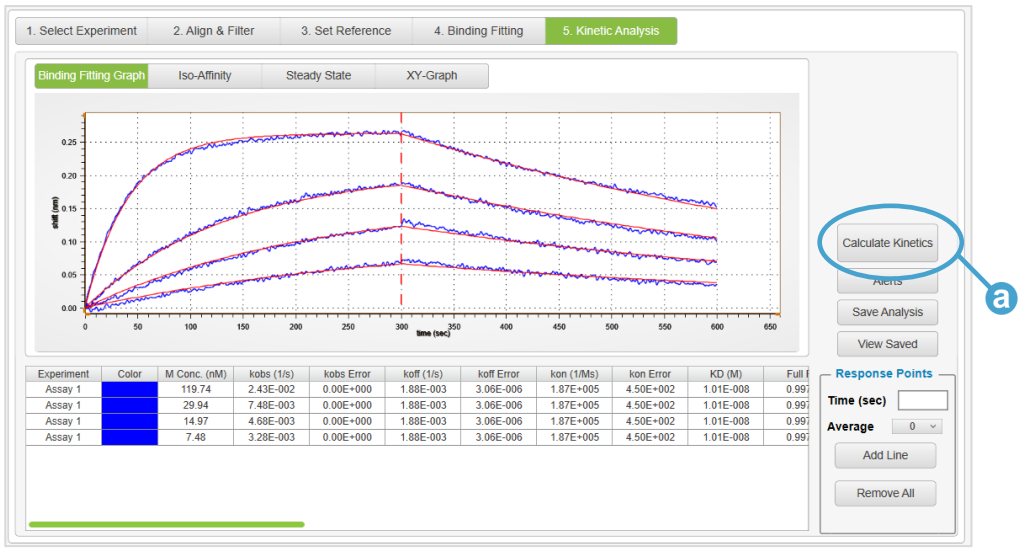


Window of Interest: Fit can be limited to certain windows of interest

Binding Fitting and Kinetic Analysis



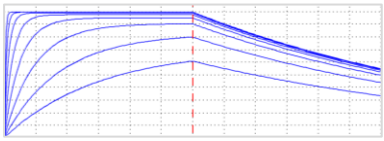
- Click ⓐ to fit all channels
- If certain concentrations (binding curves) could not fit well, they can be removed from the Global fit by unchecking those particular channels (ⓑ)
- More accurate estimate of kinetics values will be obtained if more analyte concentrations are included in the Global Fit setting



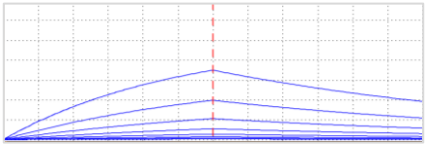
- Click **Calculate Kinetics** (ⓐ) to fill out table with kinetic values from fitting

Troubleshooting

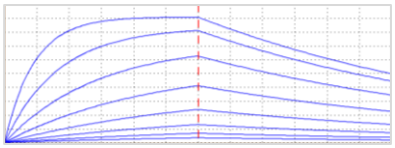
1. Optimizing analyte concentration is important for obtaining accurate kinetic values. Ideally, the concentration series should span $0.1xK_D$ – $10xK_D$.



Concentration series Too high

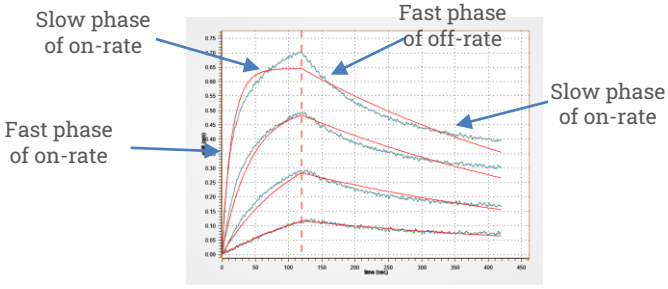


Concentration series too low



Optimized concentration series

2. Non-specific binding (NSB) can lead to poor fitting. Here are typical approaches to prevent NSB:
   a. **Blocking Additives**: Protein blocking additives can drastically minimize NSB. Bovine serum albumin (BSA) is one of the most widely used additives.
   b. **Surfactants:** Non-ionic surfactants, such as Tween20, are used when hydrophobic interactions are causing NSB.
   c. **Adjust pH:** Adjusting the pH of your buffer can affect the overall charge of your molecules, which may be beneficial in preventing NSB.
   d. **Blocking binding sites on probe:** After ligand loading, a blocking step may be necessary to prevent NSB to unoccupied capture molecule binding sites. The molecule used for blocking is probe dependent (e.g. block free streptavidin binding sites on SA probe with Biocytin)



Non-specific binding can create a biphasic response. The sensor-gram above shows the existence of a fast and slow interaction, most obvious at higher concentrations.

After minimizing NSB, consider using a reference to subtract remaining NSB signal. Suggestions for reference can be found on page 3.

Support

Gator Bio Technical Support in the United States is open Monday-Friday, 6:00 am to 5:00 pm, Pacific Standard Time. Phone: 855-208-0743 (U.S. and Canada Only) Technical Support, Ext: 3

E-mail: support@gatorbio.com
Visit our website at www.gatorbio.com

For international users, contact information for the Gator Bio corporate offices is available on the website.
Worldwide technical support is available at support@gatorbio.com

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