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Kinetic Analysis of a Protein-protein Complex to Determine its Dissociation Constant (K_D) and the Effective Concentration (EC_{50}) of an Interplaying Effector Molecule Using Bio-layer Interferometry

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[Abstract] Biolayer interferometry (BLI) is an emerging analytical tool that allows the study of protein complexes in real time to determine protein complex kinetic parameters. This article describes a protocol to determine the K_D of a protein complex using a 6×His tagged fusion protein as bait immobilized on the NTA sensor chip of the FortéBio® Octet K2 System (Sartorius). We also describe how to determine the half maximal effective concentration (EC_{50}, also known as IC_{50} for inhibiting effectors) of a metabolite. The complete protocol allows the determination of protein complex K_D and small molecular effector EC_{50} within 8 h, measured in triplicates.

Graphic abstract:

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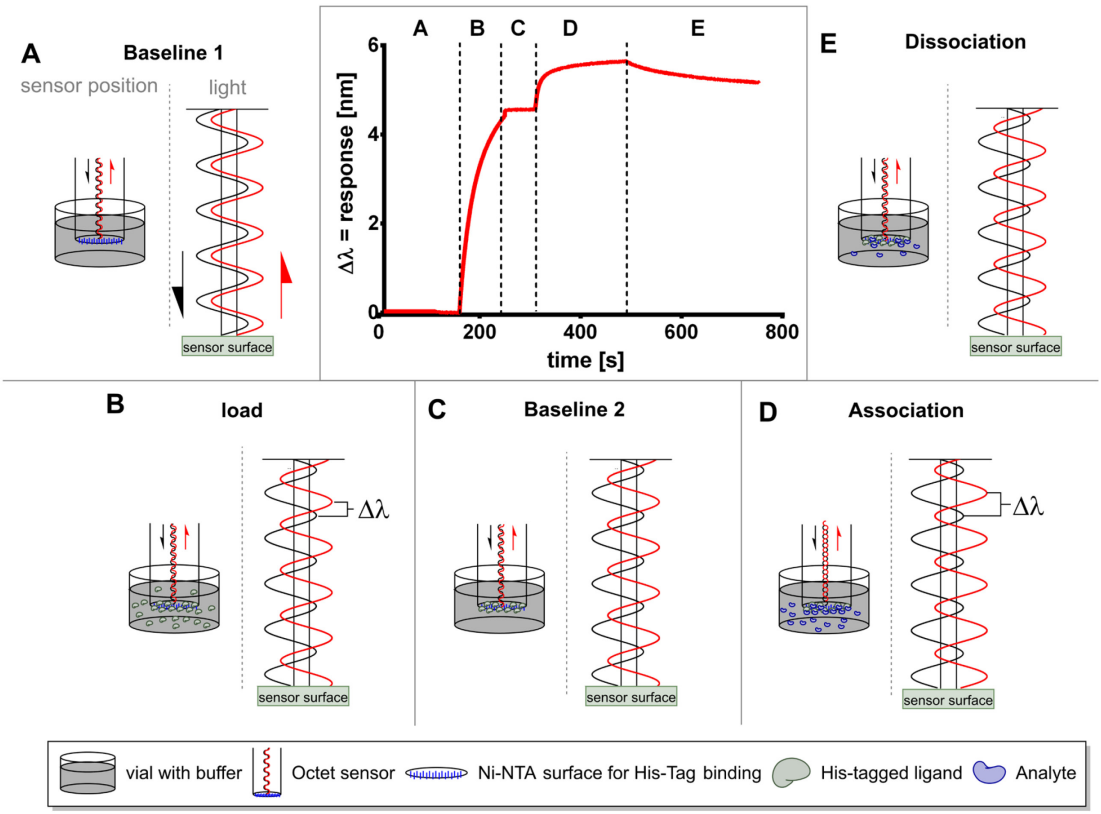

Principle of the Biolayer interferometry measurement. (Middle, top) Exemplary result of the BLI measurement using Octet® (Raw Data). Wavelength shift (Δλ) against time. (A) Baseline 1. Baseline measurement. When the sensor is equilibrated in the kinetics buffer. The light is reflected with no difference. (B) Load. The his-tagged proteins (ligand) are loaded onto the sensor surface. The light is reflected with a shift of the wavelength. (C) Baseline 2. The loaded sensor is equilibrated in the kinetics buffer. No further wavelength shift appears. (D) Association. The loaded sensor is dipped into the analyte solution. The analyte binds to the immobilized ligand along with an increased wavelength shift. (E) Dissociation. Afterward, the sensor is dipped again into the kinetics buffer without the analyte. Some analyte molecules dissociate. The wavelength shift decreases. (Subfigures A-E) The left side shows the position of the sensor during the measurement seen in the representative BLI measurement, marked with the figure label. The right side shows the light path in the sensor. Black waves represent the light emitted to the sensor surface. The red waves show the light reflected from the sensor surface back to the detector.

Keywords: Protein-protein interaction, Biolayer interferometry, Binding kinetics, K_D, IC_{50}, EC_{50}, PII signaling protein

[Background] The Investigation of protein-protein interactions is an important field in life sciences since protein interactions play crucial roles in living organisms. Many biochemical and physiological reactions are under the control of proteins that require physical communication to other proteins within the cell. For example, human cell invasion by SARS-CoV-2 starts with interactions between the viral Spike

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protein and the human ACE2 receptor (Shang *et al.*, 2020). Determination of the kinetic and affinity parameters of specific protein complexes provides important information about their properties and helps to discover their function and regulatory properties. Furthermore, the determination of the half maximal effective concentration (EC_{50}) of a metabolite, which inhibits or enhances protein complex formation, is crucial for the understanding of regulatory mechanisms and/or the development of drugs to combat infectious diseases.

Several methods are available to study protein interactions in real time, such as biolayer interferometry (BLI), surface plasmon resonance (SPR), or isothermal titration calorimetry (ITC). BLI is an emergent optical analytic technique that provides a good trade-off between sensitivity, cost intensity, reproducibility, and the ability for high-throughput measurements. The physics of BLI is based on the principle of a wavelength shift when white light is reflected from two layers, first from an internal reference layer and second from a protein coated layer (FortéBio, 2005-2006). The wavelength shift is detected by the machine and is recorded over time. The length of the shift is proportional to the bound proteins on the layer and can be used to calculate the binding affinities and kinetics. Compared to ITC, BLI measurement requires lower amounts of the target proteins, which is especially important for proteins that are difficult to express and purify, *e.g.*, membrane proteins. BLI has lower sensitivity compared to SPR methods; however, in terms of equipment maintenance, BLI is advantageous compared to the micro-fluidic SPR systems, which are prone to clogging and require high sample purity, buffer degassing, and constant maintenance of the sensor chips and micro-fluidic devices. In addition, BLI is tolerant to the use of analytes in complex matrices (Shah and Duncan, 2014), enabling high-throughput measurements of crude analyte preparations.

Recently, we employed BLI to characterize the interaction between the P_{II} signaling proteins and different binding target proteins, including NAD synthetase (NadE), phosphoenolpyruvate carboxylase (PEPC), P_{II}-**i**nteracting regulator of arginine synthesis (PirA), and P_{II}-**i**nteracting regulator of carbon metabolism (PirC) (Santos *et al.*, 2020; Scholl *et al.*, 2020; Bolay *et al.*, 2021; Orthwein *et al.*, 2021). P_{II} proteins sense the cellular energy state through the competitive binding of ATP and ADP, and sense carbon/nitrogen balance through binding of the citric acid cycle metabolite 2-oxoglutarate (2-OG) (Forchhammer and Selim, 2020; Selim *et al.*, 2020). Here, we describe a robust, simple, and quick protocol for the detection of protein interactions using a His-tagged bait protein (ligand) and a non-His-tagged prey protein (analyte). The BLI measurements were performed using a FortéBio® Octet K2 system (Sartorius). The protocol describes the procedure to determine the dissociation constant (K_D) of the protein complex and also the EC_{50} of small effector molecules (2-OG here), which can promote or disrupt the protein complex. The protocol is exemplified using the P_{II} protein from the unicellular cyanobacterium *Synechocystis* sp PCC 6803 for studying P_{II}-PirC interactions. In cyanobacteria, the interaction between P_{II}-PirC regulates the central carbon metabolism in response to the intracellular concentrations of the carbon/nitrogen indicator 2-OG (Orthwein *et al.*, 2021).

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Materials and Reagents

1. Microplate, 96-well plate, PS, F-Bottom (Chimney well) Black, Non-Binding (Greiner Bio-One, catalog number: 655900)
2. HEPES (Thermo Scientific, Fischer Bioreagents, catalog number: BP-310)
3. KOH (Merck, Millipore, catalog number: 1.05033)
4. Nonident-P40 (Thermo Fischer Scientific, Fluka, catalog number: 74358)
5. KCl (Carl Roth, catalog number: 6781.1)
6. $MgCl_2 \cdot 6H_2O$ (Merck, Millipore, catalog number: 7791-18-6)
7. ATP (store at -20°C) (Carl Roth, catalog number: K054.3)
8. α-ketoglutaric acid disodium salt hydrate (store at 4°C) (Thermo Fischer Scientific, Fluka, catalog number: K3752) – another name for 2-oxoglutarate (2-OG)
9. Glycine (Merck, Sigma-Aldrich, catalog number: 33226)
10. $NiCl_2 \cdot 6H_2O$ (Carl Roth, catalog number: 4489.1)
11. Kinetics buffer (see Recipes)

Equipment

1. Ni-NTA (NTA) Dip and Read™ Biosensors (tray) (Sartorius, FortéBio®, catalog number: 18-5103)
2. FortéBio® Octet K2 System (Sartorius)

Software

1. Data Acquisition 11.0.0.64 (FortéBio®, https://www.sartorius.com/en/products/protein-analysis/octet-systems-software)
2. Data Analysis HT 11.0.0.50 (FortéBio®, https://www.sartorius.com/en/products/protein-analysis/octet-systems-software)
3. Prism, Version 6.01 or higher (GraphPad Software Inc.)

Procedure

For this protocol, we used purified 6×His-tagged P_{II} protein as the bait protein (ligand) and a Strep-tagged PirC protein (analyte), as described previously (Orthwein *et al.*, 2021). It is recommended to use 5-25 µg/ml of ligand per well of a 96-well plate when loading the proteins onto the Ni-NTA biosensors. A purified ligand concentration of 0.5 mg/ml in 500 µl at a minimum is needed to perform 50 measurements if 25 µg/ml is used in the assay. This provides an acceptable dilution of the kinetics buffer with a sufficient amount of protein. A minimum analyte molar concentration that is 5-times higher is needed.

Note: It is possible to store your proteins in a buffer containing glycerol.

An overview of the method: The ligand is loaded specifically onto the Ni-NTA sensor tips via the 6× His-

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tag tail (loading) followed by a washing step in kinetics buffer. The loaded tips are then dipped into the analyte solution to measure the complex formation (association), indicated by an increasing signal. Afterward, the sensors with the complex are dipped back into the kinetic buffer (decreased signal dissociation). Before and after the procedure, the sensors are washed and regenerated with an acidic 10 mM glycine solution (pH 1.7) and a 10 mM $NiCl_2$ solution. The software calculates the response in equilibrium (R_{eq}) using the association and dissociation signal. The values of R_{eq} for each analyte concentration are plotted in a graph (R_{eq} against the analyte concentration), which is used for the determination of the kinetics parameters. For this purpose, the measurement is performed with different concentrations of the analyte to determine the K_D and with different concentrations of the effector to determine the EC_{50}. The R_{eq} values are then plotted against the related analyte concentrations. For EC_{50} determination, the values are transformed and the relative response is plotted against the $\log_{10}$ of the analyte. Afterward, kinetic analysis with a statistical software (*e.g.*, GraphPad Prism) is performed for the calculation of the kinetic parameters.

A. Bio-layer interferometry kinetic binding assay

The assay was performed using the FortéBio® Octet K2 System (Sartorius).

Note: Make sure that other tags used for the analyte do not interact with poly histidine (possibly metalloproteins) or bind non-specifically to Ni-NTA. It is possible to use a His-tagged protein as an analyte after the cleavage removal of the tag; however, it is not recommended. Test this via trial loading experiments of the analyte onto the biosensor. If there is a signal, there is unspecific binding of the analyte. If there is non-specific binding of the analyte, it is recommended to load the remaining loading positions with a His-tagged protein that does not interact with the analyte. For triplicates, measure three times with newly prepared 96-well plates.

1. Switch on the machine and prewarm the sample holder for a minimum of 30 min. Adjust the working temperature of choice.
   Note: We used 30 °C because of the growth temperature of cyanobacteria.
2. Prepare the kinetics buffer, the sensor washing solution, *i.e.*, 10 mM glycine pH 1.7 (adjust pH with HCl), and the regeneration solution, *i.e.*, 10 mM $NiCl_2$.
   Note: The optimal conditions for interaction depend on the proteins under study. A preliminary buffer screening test (e.g., different pH and salts) may be required to determine the optimal conditions for protein-protein interaction. We used the kinetics buffer described in the recipes section.
3. Dilute the ligand in the kinetics buffer for the Ni-NTA sensor tip loading.
   Note: The optimal concentration of the ligand should be tested before the start of the kinetic protein-protein interaction assay. For this, perform a trial loading experiment with different ligand concentrations. The manufacturer protocol recommends a ligand concentration between 5-25 µg/ml. We used concentrations that did not exceed a binding signal of $\Delta\lambda$ = 5 nm.
4. Prepare a dilution series of 7 different concentrations of the analyte.
   Note: We recommend starting the dilution with ~10x the expected K_D, as described in the

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manufacturer protocol.

5. Fill the vials of the 96-well plate with 200 µl of solutions as shown in Figure 1.

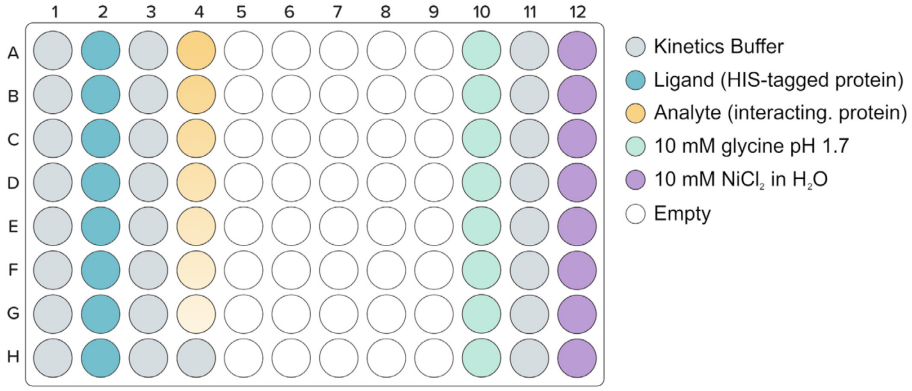

Figure 1. Pipetting scheme of the 96-well plate for a kinetic assay. Column 4 contains the dilution series from the highest (A) to the lowest concentration (G) of the analyte. Vial 4H contains the control without analyte. Adapted from the manufacturer protocol - technical note Ni-NTA biosensor kinetic assays (FortéBio, 2019).

6. Place your plate in the machine for prewarming.
7. Define the experiment in Data Acquisition 11.0.0.64 (Sartorius, FortéBio®) by following the top menu and start the measurement.
   a. Plate definition: Define the plate position and enter additional information in the table, as shown in Figure 2.

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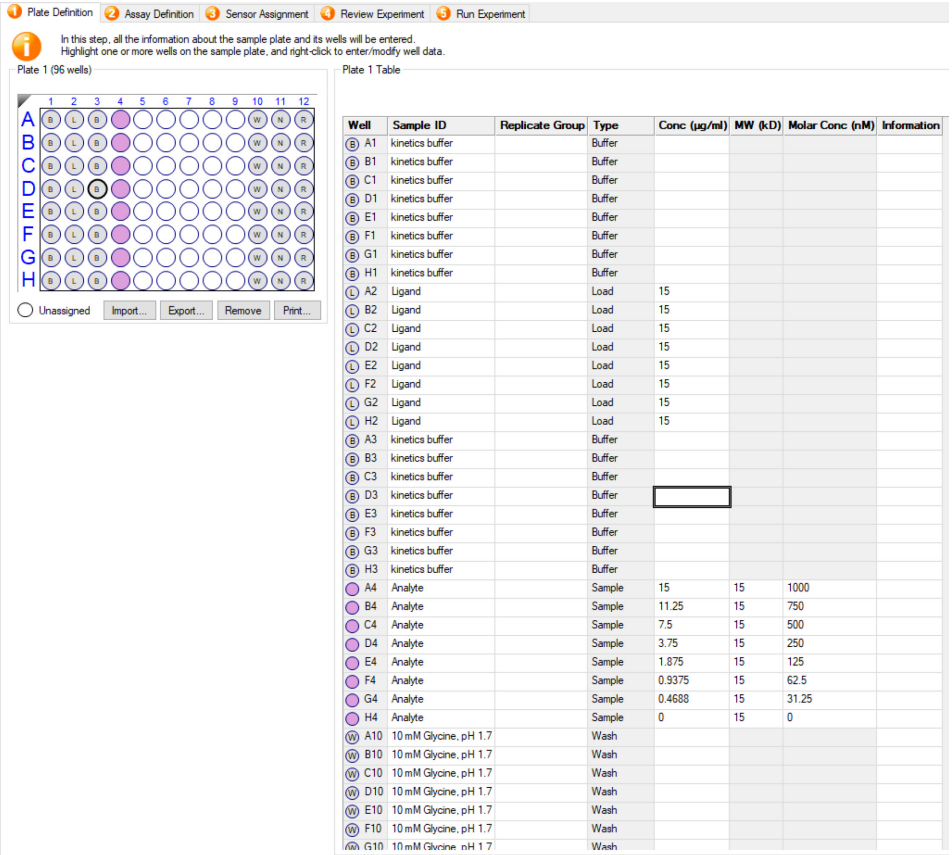

Well	Sample ID	Replicate Group	Type	Conc (µg/ml)	MW (kD)	Molar Conc (nM)	Information
B A1	kinetics buffer		Buffer				
B B1	kinetics buffer		Buffer				
B C1	kinetics buffer		Buffer				
B D1	kinetics buffer		Buffer				
B E1	kinetics buffer		Buffer				
B F1	kinetics buffer		Buffer				
B G1	kinetics buffer		Buffer				
B H1	kinetics buffer		Buffer				
L A2	Ligand		Load	15			
L B2	Ligand		Load	15			
L C2	Ligand		Load	15			
L D2	Ligand		Load	15			
L E2	Ligand		Load	15			
L F2	Ligand		Load	15			
L G2	Ligand		Load	15			
L H2	Ligand		Load	15			
B A3	kinetics buffer		Buffer				
B B3	kinetics buffer		Buffer				
B C3	kinetics buffer		Buffer				
B D3	kinetics buffer		Buffer				
B E3	kinetics buffer		Buffer				
B F3	kinetics buffer		Buffer				
B G3	kinetics buffer		Buffer				
B H3	kinetics buffer		Buffer				
A4	Analyte		Sample	15	15	1000	
B4	Analyte		Sample	11.25	15	750	
C4	Analyte		Sample	7.5	15	500	
D4	Analyte		Sample	3.75	15	250	
E4	Analyte		Sample	1.875	15	125	
F4	Analyte		Sample	0.9375	15	62.5	
G4	Analyte		Sample	0.4688	15	31.25	
H4	Analyte		Sample	0	15	0	
W A10	10 mM Glycine, pH 1.7		Wash				
W B10	10 mM Glycine, pH 1.7		Wash				
W C10	10 mM Glycine, pH 1.7		Wash				
W D10	10 mM Glycine, pH 1.7		Wash				
W E10	10 mM Glycine, pH 1.7		Wash				
W F10	10 mM Glycine, pH 1.7		Wash				
W G10	10 mM Glycine, pH 1.7		Wash				

Figure 2. Plate definition and sample information table. Definitions: (1A-H) Buffer, (2A-H) Loading, (3A-H) Buffer, (4A-H) Sample, (10A-H) Wash, (11A H) Neutralization, and (12A-H) Regeneration. Concentrations are example values.

Note: Use the concentrations and MW of your analyte.

b. Assay Definition. Define the assay as shown in Figure 3.

Note: The time of the association and dissociation steps depends on the proteins under study. For kinetic investigation, we suggest determining the optimal association and dissociation times by performing preliminary assays using different association/dissociation times ranging between 2-10 min and using saturating concentrations of the analyte (~10× the expected K_D). Define the assay steps method for row A and B. Mark the assay step list and click "Replicate" to clone the steps for the other rows (> Replicate> Replication type: add a new assay > Offset step > vertically > OK).

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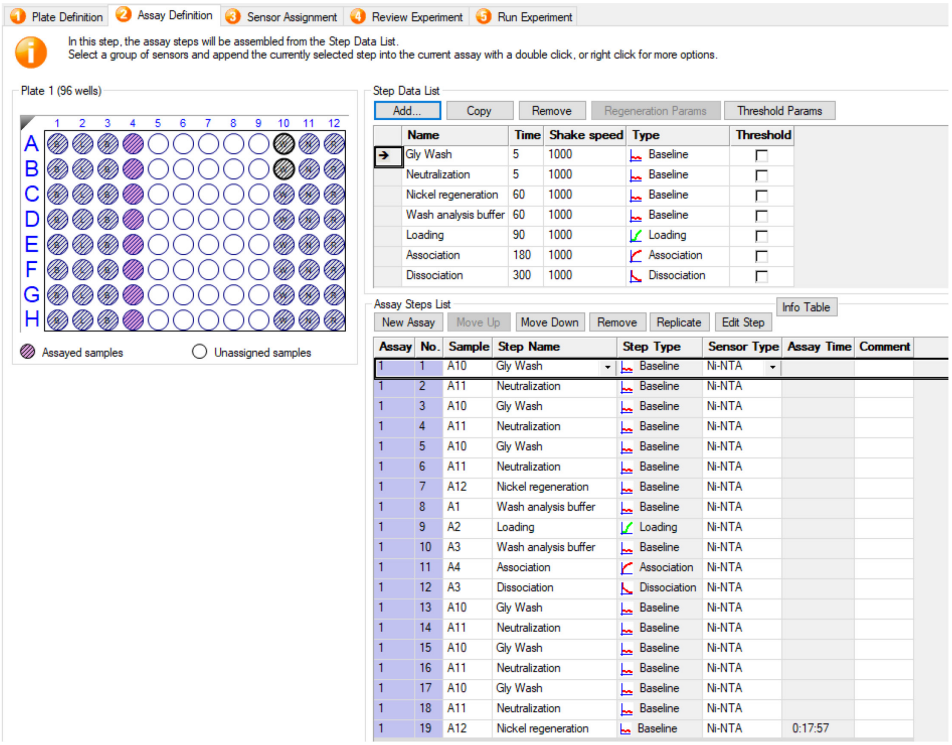

Figure 3. Assay definition for the kinetic binding assay

c. Sensor Assignment. Assign the Sensors in the correct line.

d. Review Experiment. Review your experiment.

e. Run Experiment. Define the localization, name the results and start the experiment.
Note: If you wet your biosensors immediately before, use click on "delayed experiment starts: 600 s". Do this also if you did not prewarm your plates previous to the experiment.

B. Determination of the EC_{50} of a small effector molecule (effector) that interferes in the protein-protein complex

1. Prepare a dilution series of the investigated effector.
Note: In the example, we used the 2-OG metabolite as it inhibits the P_{II}-PirC complex. Prepare a dilution series of the effector without the analyte for the pre-washing step (to be used to fill the wells in column 3) and a dilution series that contains the analyte (constant amount of analyte in each dilution of the effector) for analyte binding (to be used to fill the wells in column 4). The analyte should be used in amounts enabling saturation of the ligand as determined from the previous kinetic data.
2. Fill the vials of the 96-well plate with 200 µl of solutions as shown in Figure 4.

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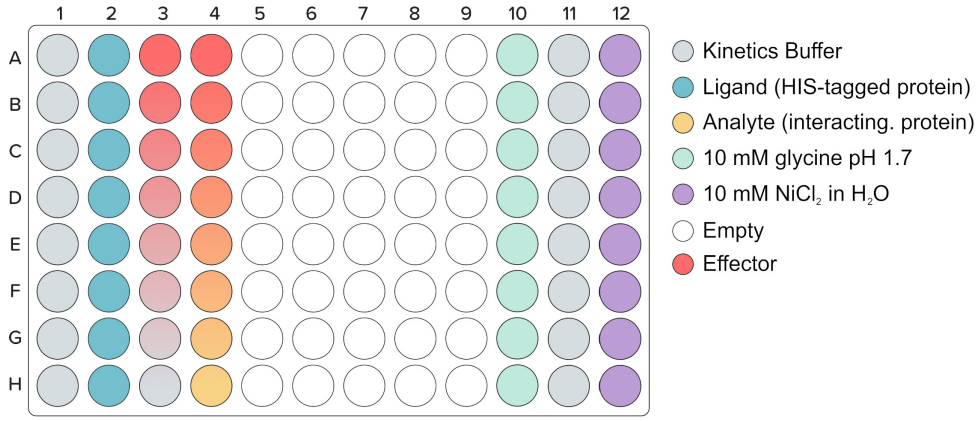

Figure 4. Pipetting scheme of the 96-well plate for a determination of the EC_{50} for an effector that affects the stability of the protein complex. Columns 3 and 4 contain dilution series of the effector from highest (A) to lowest concentrations (G), without and with constant amount of analyte, respectively. Vial 4H contains the control without the effector [adapted from the manufacturer protocol – technical note Ni-NTA biosensor kinetic assays (FortéBio, 2019)].

3. Repeat Steps A6 and A7.

Data analysis

A. Kinetic binding assay
   1. Start the Data Analysis HT 11.0.0.50 (Sartorius, FortéBio®) software and open the results of the measurement.
   2. Go to preprocessed Data > Data Correction and adjust the settings as follows:
      a. Align Axis: Align Data to: Average of Baseline.
      b. Inter-step Correction: Align Data to: Association Step.
      c. Activate Savitky-Golay Filtering.
   3. Go to kinetics analysis and adjust the settings as follows:
      a. Step to Analyze: Association and Dissociation.
      b. Binding Model: depending on protein.

         The 1:1 model is recommended for protein complexes where the analyte binds to one specific binding site of the ligand. If the analyte binds at two independent ligand sites, use the 2:1 (heterogeneous ligand) model. The Mass Transport model is recommended for bindings that are limited by diffusion. The bivalent model fits the binding of one analyte that interacts with two sites of the immobilized ligand. Detailed information is provided in the manufacturer's user manual (Page 114, Pall FortéBio LLC, Octet System Data Analysis User Guide).
      c. Fitting.

         Type: Global (Group)

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Group by: Sensor Type

R_{max} Values: Linked

d. Steady State Analysis: R equilibrium.

The software calculates the response values in the equilibrium and creates a steady-state graph of your measurement, as shown in Figure 5.

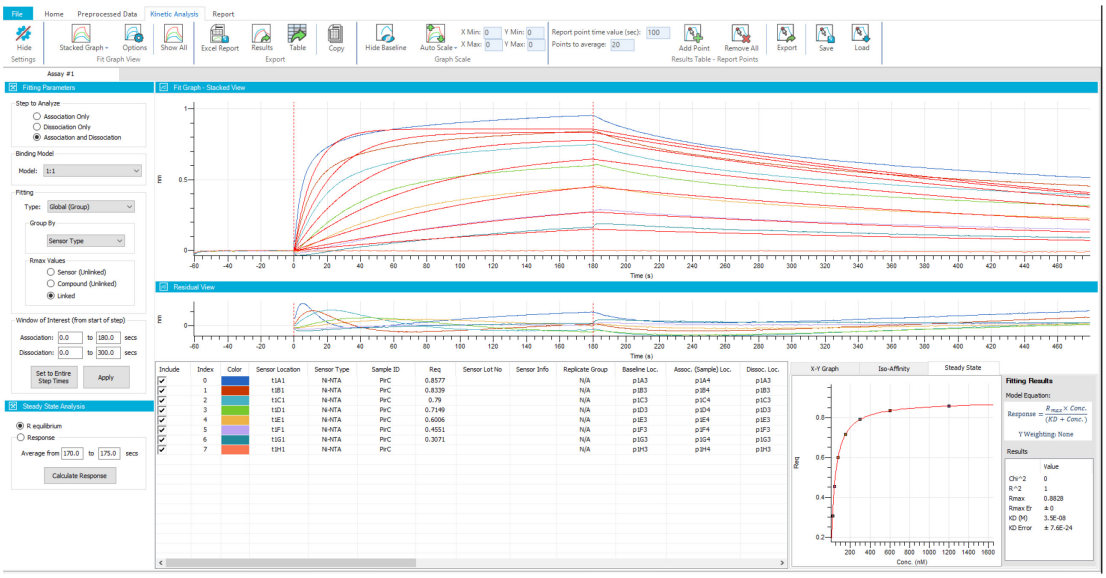

Figure 5. Kinetic Analysis page in the data analysis software. Graphs (fit graph) below the function tab show how the preprocessed data is fitted with the settings in step 3. The list below shows the calculated R_{eq} values and additional information about the sensors. On the right edge, the Steady-State calculation of one measurement row is shown. The calculated K_D is also reported.

Note: You can use this calculation to determine the K_D, but the combination of several measurements is not possible.

4. Export the data by clicking on “Excel Report.”
5. Open Prism and create a new Table and Graph Project.

   Enter and import data:

   a. Click on XY.

   b. Enter three replicate values in side-by-side sub columns.

      Note: It is also possible to use a different software for the calculation and fitting of the binding kinetics. Make sure that the software contains the correct binding kinetic equations.
6. Transfer the R_{eq} values into the GraphPad table as Y-values.
7. Add the interactor concentrations as X Values.
8. Click on the automatically created graph and perform the “Analyze” function.

   a. Nonlinear regression (curve fit).

   b. Binding – Saturation.

   c. One site – Specific binding.

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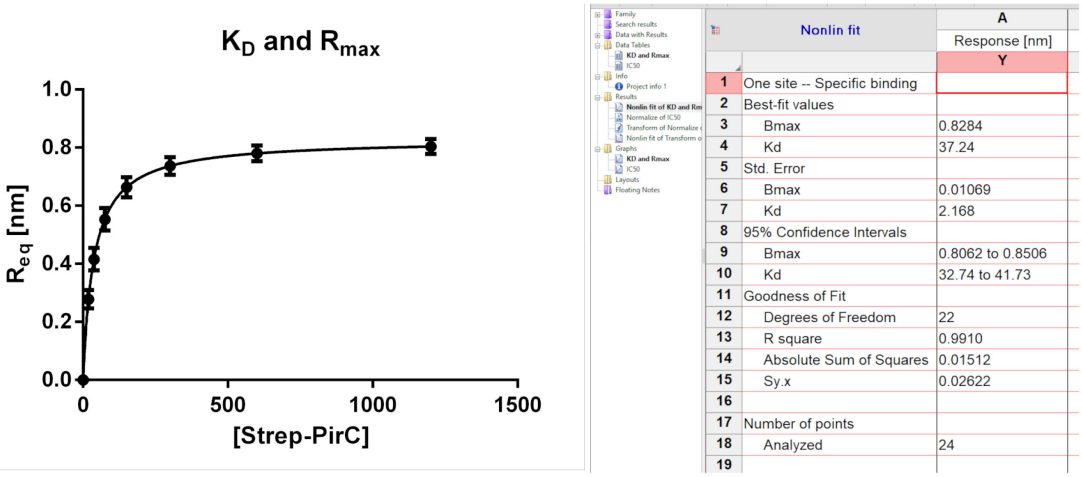
The software fits the graph and creates a results file with the calculated B_{max} (= R_{max}) and K_D, as shown in Figure 6.



	Nonlin fit	A
		Response [nm]
		Y
1	One site -- Specific binding	
2	Best-fit values	
3	Bmax	0.8284
4	Kd	37.24
5	Std. Error	
6	Bmax	0.01069
7	Kd	2.168
8	95% Confidence Intervals	
9	Bmax	0.8062 to 0.8506
10	Kd	32.74 to 41.73
11	Goodness of Fit	
12	Degrees of Freedom	22
13	R square	0.9910
14	Absolute Sum of Squares	0.01512
15	Sy.x	0.02622
16		
17	Number of points	
18	Analyzed	24
19		

Figure 6. Representative Data of Analysis for kinetic analysis of protein interactions for the P_{II}-PirC example. (A) Fit of the binding (R_{eq}) against the concentration of the analyte (Strep-PirC). (B) Prism document (results file) of calculated kinetics in the results folder. The window shows the maximal binding (B_{max}), the calculated K_D, and the standard error of both. Confidence intervals and the quality of the fit are also calculated.

B. EC_{50} determination

1. Repeat the steps of Data analysis A1-A4.
2. Add the effector concentrations as X Values.
3. Export the data by clicking on “Excel Report” and transfer the R_{eq} values into a GraphPad table as Y-values.

   Note: For triplicates, measure three times with newly prepared 96-well plates.
4. Perform the “Analyze” function “Normalize” with y = 0.0 as 0% and the largest value in each data set as 100%.
5. Perform the “Analyze” function “Transform” and transform X values using X = Log[X]. In the original data table, change the concentration from 0 to 1.
6. Perform the “Analyze” function “Nonlinear Regression (curve fit)”
   a. Dose-response – inhibition (negative effector; IC_{50})/Dose-response – Stimulation (positive effector).
   b. Log(inhibitor) vs. normalized response/Log(agonist) vs. normalized response.

   The software fits the graph and creates a results file with the calculated EC_{50}. The example IC_{50} is shown in Figure 7.

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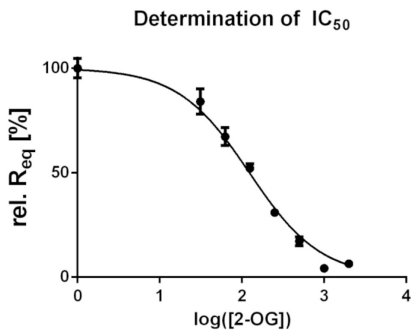

	Nonlin fit	A
		Req
		Y
1	log(inhibitor) vs. normalized response	
2	Best-fit values	
3	LogIC50	2.091
4	IC50	123.4
5	Std. Error	
6	LogIC50	0.02651
7	95% Confidence Intervals	
8	LogIC50	2.035 to 2.148
9	IC50	108.4 to 140.6
10	Goodness of Fit	
11	Degrees of Freedom	15
12	R square	0.9864
13	Absolute Sum of Squares	251.5
14	Sy.x	4.095
15		
16	Number of points	
17	Analyzed	16
18		

Figure 7. Representative Data Analysis for determination of the IC_{50} of 2-OG in the P_{II}-PirC interaction. (A) Fitted data of a triplicate measurement of P_{II}-PirC binding for determination of the IC_{50} of 2-OG. X-Axis logarithm of 2-OG concentration (dilution series of 2 mM, 1 mM, 0.5 mM, 0.25 mM, 0.125 mM, 0.0625 mM, and 0.03125 mM), and Y-Axis: relative response in equilibrium. (B) Prism document (results file) of calculated IC_{50} in the results folder. The window shows the logarithmic IC_{50}, the calculated IC_{50} and the logarithmic standard error of both. Confidence intervals and the quality of the fit are also calculated.

Recipes

1. Kinetics buffer
   20 mM HEPES pH 8
   150 mM KCl
   5 mM $MgCl_2$
   1 mM ATP
   0.005% Nonident P40
   KOH for pH adjustment

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Competing interests

The authors declare no competing interest.

Ethics

K.A.S. conceived and initiated the project. T.O. performed the experiments, wrote the first draft and edited the manuscript. L.F.H., K.F. and K.A.S. commented and edited the manuscript. All authors have read and approved the final version of the manuscript.

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