



Protein Binding Kinetics Simulation tool manual

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1. GETTING STARTED

1.1. Before you start

Some recommendation before you start using the simulation tool:

- When you will enter the values in the input dashboard, it is important to consider that the values of interaction partner 1 will always be depicted in the X-axis of the graphs.
- If actual values are not known such as concentration or affinity, then the simulation tool can be used by considering ranges of these values and be valuable to understand for example what the impact of changes in these values could be and which should ideally be considered.
- In case the legend title and values in the graphs are overlapping, please consider adjusting the scale of your screen



2. EXAMPLES OF USE CASES SUPPORTED BY THE TOOL

2.1. How this tool can help support your work

The in-house developed simulation tool supports mathematical calculations of protein binding interactions occurring in biosamples and bioassays, aimed at understanding the requirements, capabilities and limitations of assays.

For both *in vivo* and *in vitro* conditions, there are distinct example questions the tool can help you with.



BINDING TAB

*Binding interactions in vivo →
in sample*

- How much drug is estimated to be in complex with target in the samples?
- How much target is expected to be occupied by drug at the anticipated C_{max} ?
- What is the impact of dilution on the target-drug complex in the sample?
- Is the free analyte (BM or drug) assay expected to accurately measuring the free fraction?
- What is the required sensitivity for free biomarker target occupancy assay?



BINDING TAB

*Binding interactions in vitro →
in assay plate*

- Will the selected capture reagent with its concentration and affinity have the potential to reach the required sensitivity?
- How long does it take for the interactions between the analyte of interest and the assay capture or detection reagent to be in equilibrium?



COMPETITION TAB

*Interplay between in vivo and
in vitro binding interaction*

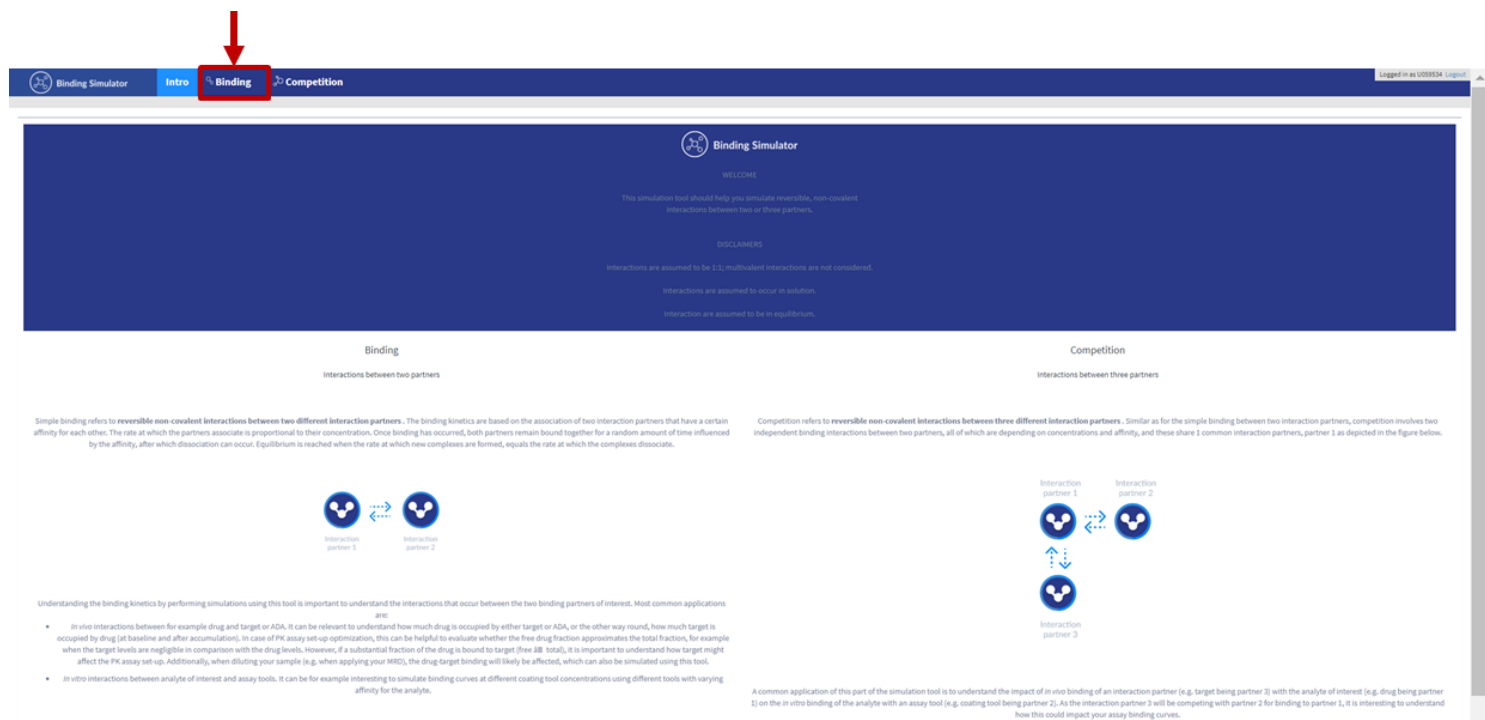
- Is the presence of target or ADA likely to interfere in the PK assay set-up?
- What level of ADA or target are predicted to impact PK levels?
- Will the characteristics of the capture reagent (with a given concentration and affinity) potentially interfere with the drug-target complex in the samples (i.e. outcompete the target from the binding to the drug)?

3. HOW TO USE THE TOOL – THE BINDING TAB

3.1. Getting started with the binding tab

Welcome to the Binding Simulator landing page. The Intro page will give you short explanations for the Binding and Competition tab of the simulation tool. Binding refers to interactions between two binding partners and Competition refers to interactions between three interacting partners.

To get started click on the Binding button on the top header. This will allow you to simulate interactions between two partners.



The screenshot shows the Binding Simulator web application. At the top, there is a navigation bar with three tabs: "Intro", "Binding" (which is highlighted with a red box and a red arrow pointing to it), and "Competition". Below the navigation bar, the main content area is divided into two columns. The left column is titled "Binding" and contains the text "Interactions between two partners". The right column is titled "Competition" and contains the text "Interactions between three partners". Both columns include a diagram illustrating the interaction between partners. The Binding diagram shows two blue circular icons, each with a white dot in the center, connected by a double-headed arrow. The Competition diagram shows three blue circular icons, each with a white dot in the center, arranged in a triangle and connected by double-headed arrows. Below the diagrams, there is a section titled "Understanding the binding kinetics by performing simulations using this tool is important to understand the interactions that occur between the two binding partners of interest. Most common applications are:" followed by a bulleted list. The list includes: "In vivo interactions between for example drug and target or ADA. It can be relevant to understand how much drug is occupied by either target or ADA, or the other way round, how much target is occupied by drug (at baseline and after accumulations). In case of PK assay set-up optimization, this can be helpful to evaluate whether the free drug fraction approximates the total fraction, for example when the target levels are negligible in comparison with the drug levels. However, if a substantial fraction of the drug is bound to target (free $[D]$ total), it is important to understand how target might affect the PK assay set-up. Additionally, when diluting your sample (e.g. when applying your MSD), the drug-target binding will likely be affected, which can also be simulated using this tool." and "In vitro interactions between analyte of interest and assay tools. It can be for example interesting to simulate binding curves at different coating tool concentrations using different tools with varying affinity for the analyte."

Binding Simulator

WELCOME

This simulation tool should help you simulate reversible, non-covalent interactions between two or three partners.

DISCLAIMERS

Interactions are assumed to be 1:1 multivalent interactions are not considered.

Interactions are assumed to occur in solution.

Interactions are assumed to be in equilibrium.

Binding

Interactions between two partners

Simple binding refers to **reversible non-covalent interactions between two different interaction partners**. The binding kinetics are based on the association of two interaction partners that have a certain affinity for each other. The rate at which the partners associate is proportional to their concentration. Once binding has occurred, both partners remain bound together for a random amount of time influenced by the affinity, after which dissociation can occur. Equilibrium is reached when the rate at which new complexes are formed, equals the rate at which the complexes dissociate.

Competition

Interactions between three partners

Competition refers to **reversible non-covalent interactions between three different interaction partners**. Similar as for the simple binding between two interaction partners, competition involves two independent binding interactions between two partners, all of which are depending on concentrations and affinity, and these share 1 common interaction partners, partner 1 as depicted in the figure below.

Understanding the binding kinetics by performing simulations using this tool is important to understand the interactions that occur between the two binding partners of interest. Most common applications are:

- In vivo interactions between for example drug and target or ADA. It can be relevant to understand how much drug is occupied by either target or ADA, or the other way round, how much target is occupied by drug (at baseline and after accumulations). In case of PK assay set-up optimization, this can be helpful to evaluate whether the free drug fraction approximates the total fraction, for example when the target levels are negligible in comparison with the drug levels. However, if a substantial fraction of the drug is bound to target (free $[D]$ total), it is important to understand how target might affect the PK assay set-up. Additionally, when diluting your sample (e.g. when applying your MSD), the drug-target binding will likely be affected, which can also be simulated using this tool.
- In vitro interactions between analyte of interest and assay tools. It can be for example interesting to simulate binding curves at different coating tool concentrations using different tools with varying affinity for the analyte.

A common application of this part of the simulation tool is to understand the impact of in vivo binding of an interaction partner (e.g. target being partner 2) with the analyte of interest (e.g. drug being partner 1) on the in vitro binding of the analyte with an assay tool (e.g. coating tool being partner 2). As the interaction partner 3 will be competing with partner 2 for binding to partner 1, it is interesting to understand how this could impact your assay binding curves.

3.2. How to use the binding tool

Start by adding values at the top section

To start your simulation, insert the values for both interaction partners on the top menu section.

6 Once all set, press "Go" to launch computation

1 Concentration of partner 1 and 2 can be filled in in metric units, but additionally the molecular weight has to be provided as the tool does all calculations using molar units. Note that molar units can also be filled in if preferred.

5 When selecting the **affinity constant**, select 'only KD' when not interested in the time needed to reach equilibrium. One of the other options, i.e. (i) Kon and Koff, (ii) KD and Kon, or (iii) KD and Koff should be selected when interested in the time it takes for the binding to reach equilibrium.

4 Via the advanced option, the number of data points within one log interval can be selected (i.e. a number between 0 and 1). As example, if you enter 0.2, 5 data points will be depicted within the log interval.

3 The concentration range of partner 1 will be depicted in the X-axis in all graphs, which means that all curves represent doseresponse curves in function of partner 1. This slider allows you to choose the range of values across the value entered above, in log scale.

2 Here you need to fill in the starting concentration of your binding partner. Ranges to this concentration can be selected below with the slider bars. Note that when you open the tool, there will be prefilled values for these concentrations which are added by default and thus need to be changed.

3.3. How to extract results from the tool

3.3.1. Output graphs

Here are some insights on the graphs on the binding tab. At the top of the page you will find a dashboard with a summary of calculations made by the tool such as the **free partner 1 and partner 2 at equilibrium**, the **fractional occupancy** of both partners, the **complex concentration** as well as how much **time it will take to reach equilibrium**. Additionally, you will also see all **Values** added on the top section and you can leave **Observations** or notes for other colleagues so they can understand more about the simulation you were running (the comments added on this section will be visible when you download the page).

These calculations are based on the fixed concentrations and affinity constant that are provided in the input dashboard, whereas graphs take into account the ranges provided.

Values		Observations	
Partner 1 name	1e+03 (pM)	<div>Add comments or notes for other colleagues...</div>	
Molecular weight of partner 1	100 (dalton)		
Partner 2 name	1e+03 (pM)		
Molecular weight of partner 2	100 (dalton)		
K _D	100 (pM)		
k _{off}	0.0001 (s ⁻¹)		
k _{on}	1000000 (M ⁻¹ s ⁻¹)		
At equilibrium		In Complex Concentration	
FREE PARTNER 1 NAME	FREE PARTNER 2 NAME	PARTNER 1 NAME PARTNER 2 NAME	
270	270	730	
Fractional Occupancy		Time to equilibrium	
FRACTION PARTNER 1 NAME BOUND TO PARTNER 2 NAME	FRACTION PARTNER 2 NAME BOUND TO PARTNER 1 NAME	COMPLEX FORMATION WILL BE ACHIEVED BY	
73%	73%	1 hr 30 min	

Graphs depict different binding curves

GRAPH 1 and **GRAPH 2** plot different values of interaction partner 2, at the fixed affinity value

GRAPH 3 and **GRAPH 4** plot different affinities, at the fixed concentration of interaction partner 2

GRAPH 5 depicts the time needed to reach equilibrium

Graph axes

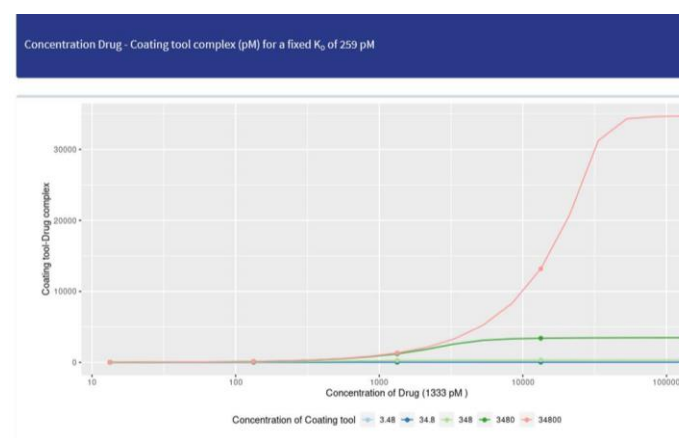
X-axis shows concentrations of partner 1, in log scale

Y-axis shows either complex formation (graphs 1 and 3) or % partner 2 occupancy (graphs 2 and 4), in linear scale (scale can be changed to log in the input dashboard)

3.3.2. Example of drug analyte (partner 1) – coating tool (partner 2) interaction

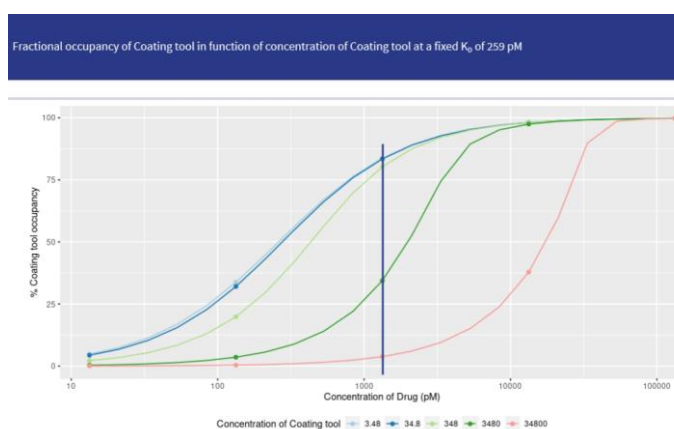
GRAPH 1

The higher the coating tool concentration (colored lines) or drug concentration (X-axis), the more complex formation



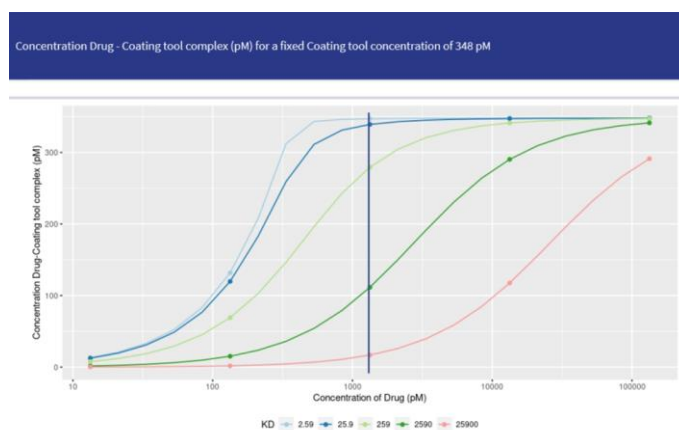
GRAPH 2

The higher the coating tool concentration (colored lines), the less tool will be occupied at a certain drug concentration



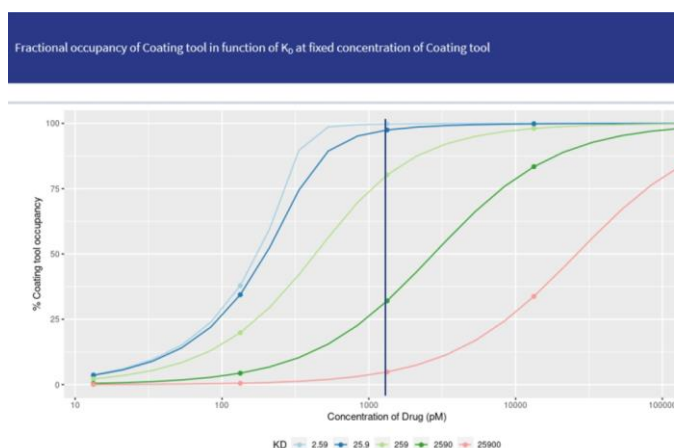
GRAPH 3

The lower the K_D value, i.e. the higher the affinity (colored lines), the more complex formation at a certain drug concentration, thus the more sensitive



GRAPH 4

The lower the K_D value, i.e. the higher the affinity (colored lines), the more tool will be occupied at a certain drug concentration, thus the more sensitive



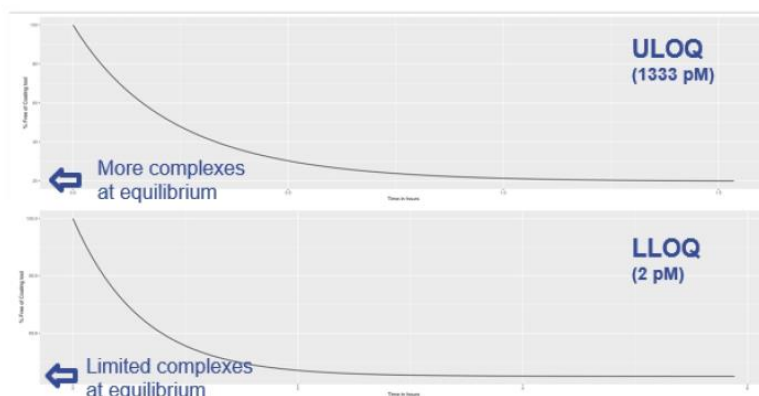
3.3.3. Time to reach equilibrium

Time to equilibrium is [GRAPH 5](#) on the binding tab - you will need to input the Association (K_{on}) or Dissociation (K_{off}) rate constants for the simulation. By default the K_D & K_{on} option is selected and K_{off} is automatically calculated based on the formula below, but you can select another option depending on the known constants.

$$\frac{k_{off}}{k_{on}} = K_D$$

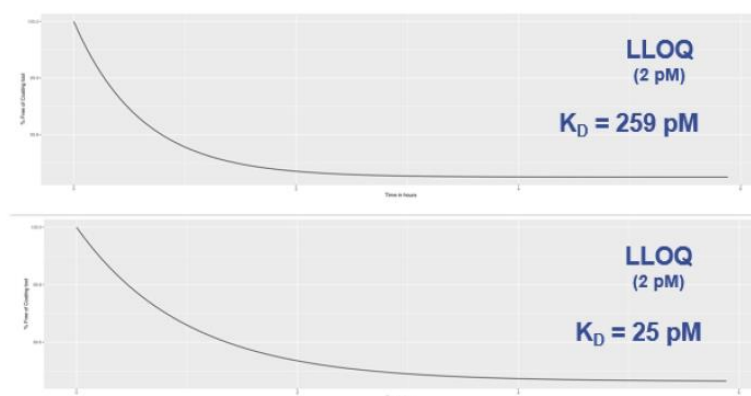
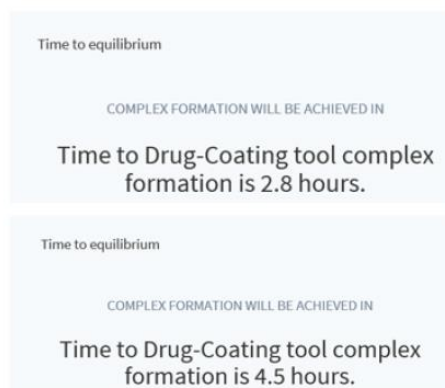
3.3.3.1. Impact of concentrations

At higher concentrations, equilibrium will be reached faster and more complexes will be formed at equilibrium.

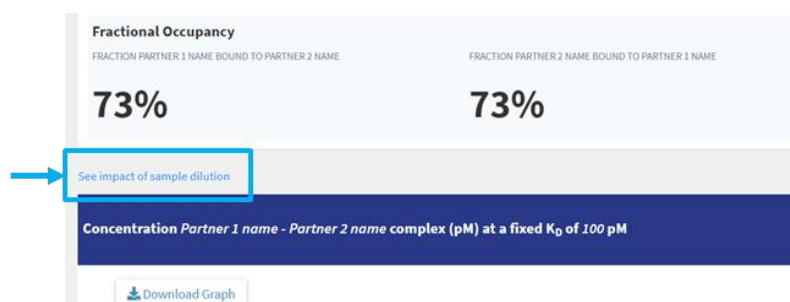


3.3.3.2. Impact of affinity

At lower affinities (higher K_D), equilibrium will be reached faster mostly due to faster dissociation rates.



3.3.4. Simulate impact of sample dilutions on complex formation



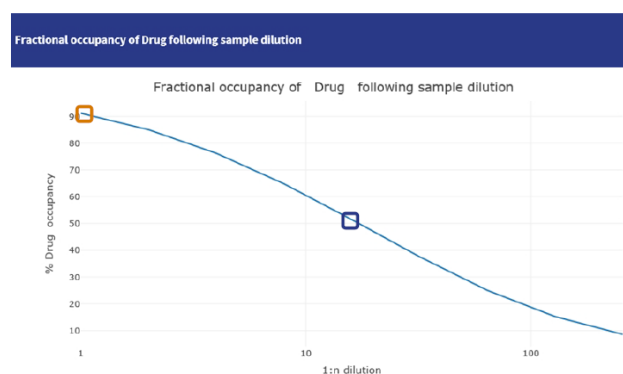
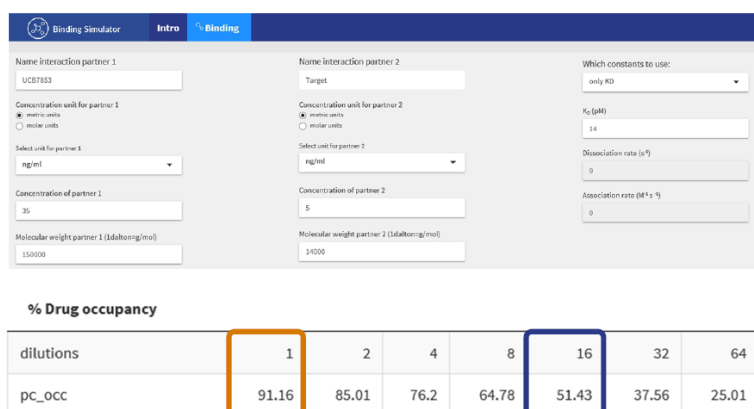
to click the button again.

Example

In the study sample >90% of the drug is occupied by the target (orange boxes)
However, when diluting the sample, the amount of complexes reduces significantly, as exemplified by the blue boxes, showing that at 16-fold dilution, the bound drug fraction is reduced to 51%

To access the Dilution part of the tool you should click on the blue button below the dashboard [See impact of sample dilution](#). After you clicked this button, the graphs in the output section will update to depict a series of graphs where you can see results about the impact of sample dilution.

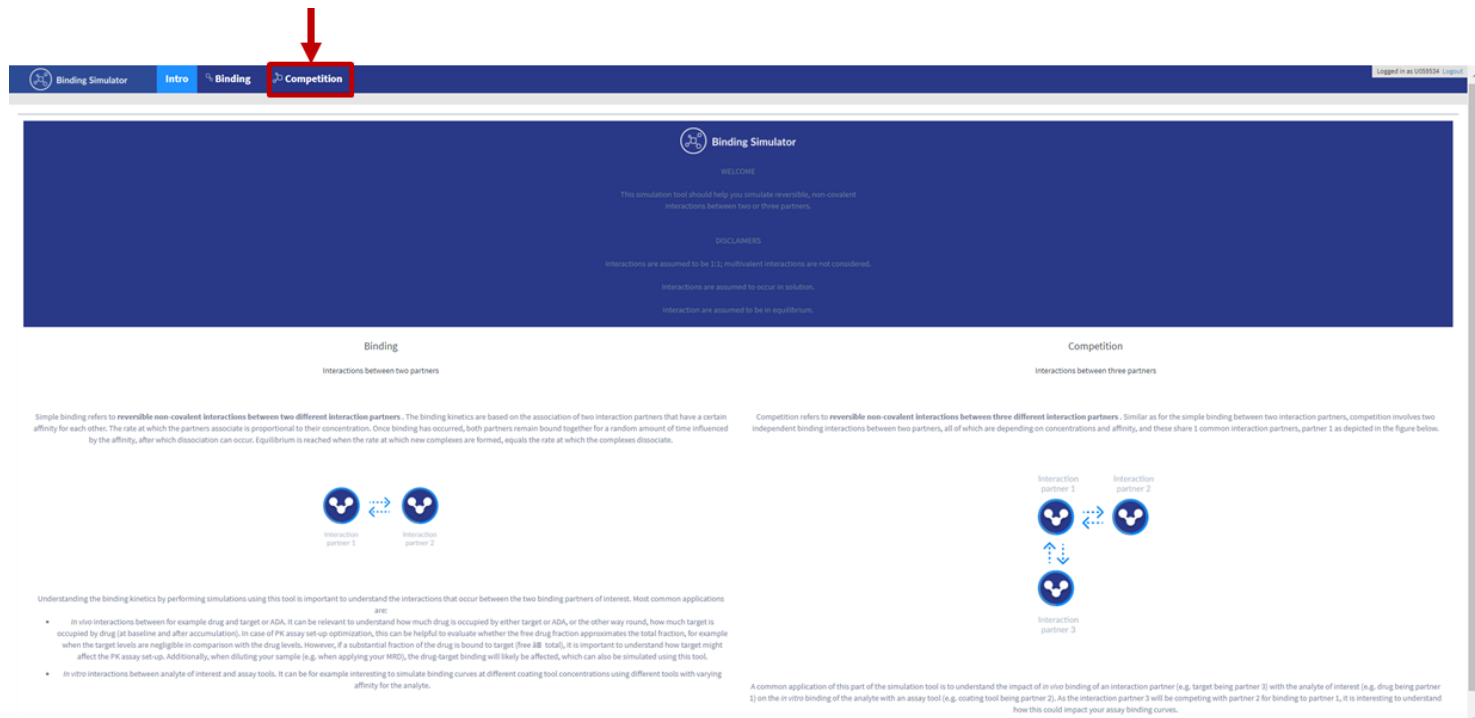
To go back to the Binding output you just need



4. HOW TO USE THE TOOL – THE COMPETITION TAB

4.1. Getting started with the competition tab

To get started click on the Competition button on the top header. This will allow you to simulate interactions between three different interactions partners.



Binding Simulator

WELCOME

This simulation tool should help you simulate reversible, non-covalent interactions between two or three partners.

DISCLAIMERS

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Interactions are assumed to be in equilibrium.

Binding

Interactions between two partners

Simple binding refers to **reversible non-covalent interactions between two different interaction partners**. The binding kinetics are based on the association of two interaction partners that have a certain affinity for each other. The rate at which the partners associate is proportional to their concentration. Once binding has occurred, both partners remain bound together for a random amount of time influenced by the affinity, after which dissociation can occur. Equilibrium is reached when the rate at which new complexes are formed, equals the rate at which the complexes dissociate.




Interaction partner 1 Interaction partner 2

Competition

Interactions between three partners

Competition refers to **reversible non-covalent interactions between three different interaction partners**. Similar as for the simple binding between two interaction partners, competition involves two independent binding interactions between two partners, all of which are depending on concentrations and affinity, and these share 1 common interaction partners, partner 1 as depicted in the figure below.



Interaction partner 1 Interaction partner 2 Interaction partner 3

Understanding the binding kinetics by performing simulations using this tool is important to understand the interactions that occur between the two binding partners of interest. Most common applications are:

- In vivo interactions between for example drug and target or ADA. It can be relevant to understand how much drug is occupied by either target or ADA, or the other way round, how much target is occupied by drug (at baseline and after accumulation). In case of PK assay set-up optimization, this can be helpful to evaluate whether the free drug fraction approximates the total fraction, for example when the target levels are negligible in comparison with the drug levels. However, if a substantial fraction of the drug is bound to target (free AB total), it is important to understand how target might affect the PK assay set-up. Additionally, when diluting your sample (e.g. when applying your MSD), the drug-target binding will likely be affected, which can also be simulated using this tool.
- In vitro interactions between analyte of interest and assay tools. It can be for example interesting to simulate binding curves at different coating tool concentrations using different tools with varying affinity for the analyte.

A common application of this part of the simulation tool is to understand the impact of in vivo binding of an interaction partner (e.g. target being partner 2) with the analyte of interest (e.g. drug being partner 1) on the in vitro binding of the analyte with an assay tool (e.g. coating tool being partner 3). As the interaction partner 3 will be competing with partner 2 for binding to partner 1, it is interesting to understand how this could impact your assay binding curves.

4.2. How to use the competition tool

Start by adding values at the top section

To start your simulation, insert the values for the three different interaction partners on the top menu section.

1 Concentration of partner 1, 2 and 3 can be filled in in metric units, but additionally the molecular weight has to be provided as the tool does all calculations using molar units. Note that molar units can also be filled in if preferred.

2 Here you need to fill in the starting concentration of your binding partner. Ranges to this concentration can be selected below with the slider bars. Note that when you open the tool, there will be prefilled values for these concentrations which are added by default and thus need to be changed.

3 The concentration range of partner 1 will be depicted in the X-axis in all graphs, which means that all curves represent dose-response curves in function of partner 1. This slider allows you to choose the range of values across the value entered above, in log scale.

4 Via the advanced option, the number of data points within one log interval can be selected (i.e. a number between 0 and 1). As example, if you enter 0.2, 5 data points will be depicted within the log interval.

5 Provide the **affinity constant** for both pairs:
 - K_D refers to the affinity constant between interaction partner 1 and 2
 - K_i refers to the affinity constant between interaction partner 1 and 3

6 Once all set, press "Go" to launch computation

4.3. How to extract results from the tool

4.3.1. Output graphs

Here are some insights on the graphs on the competition tab. At the top of the page you will find a dashboard with a summary of calculations made by the tool. Additionally you will also see all **Values** added on the top section and you can leave **Observations** or notes for other colleagues so they can understand more about the simulation you were running (the comments added on this section will be visible when you download the page.)

These calculations are based on the fixed concentrations and affinity constant that are provided in the input dashboard, whereas graphs take into account the ranges provided.

Values Partner 1 name Molecular weight of partner 1 Partner 2 name Molecular weight of partner 2 Competing partner name Molecular weight of the competing partner K_D			Observations Add comments or notes for other colleagues...		
At equilibrium FREE PARTNER 1 NAME 188 (pM)			FREE COMPETITING PARTNER NAME 347 (pM)		
FRACTION OCCUPANCY OF PARTNER 1 NAME AND PARTNER 2 NAME IN PRESENCE OF COMPETITING PARTNER NAME FRACTION PARTNER 1 NAME BOUND TO PARTNER 2 NAME 81.2%			In Complex Concentration PARTNER 1 NAME - PARTNER 2 NAME 653 (pM)		
FRACTIONAL OCCUPANCY OF PARTNER 1 NAME AND COMPETITING PARTNER NAME IN PRESENCE OF PARTNER 2 NAME FRACTION COMPETITING PARTNER NAME BOUND TO PARTNER 1 NAME 81.2%			PARTNER 1 NAME - COMPETITING PARTNER NAME 1580 (pM)		

Graph axes

X-axis shows concentrations of partner 1, in log scale

Y-axis shows either partner 1 – partner 2 complex formation in the graphs in the upper panel, or the % partner 2 occupancy (in the graphs in the lower panel), in linear scale (scale can be changed to log in the input dashboard)

4.3.2. How to change values in the plots

The input dashboard on top of the graphs allows to select the variables that are depicted in the graphs.

Once all set, press the "Go" button to launch computation

1. concentration of Partner 1 name - Partner 2 name in complex in function of concentration of Partner 1 name

1 The fixed variable needs to be selected from the dropdown menu and will be shown as a fixed value in the left and the right graph, and these values need to be provided in the boxes on the right. Note that these values overrule the values that were provided in the first input dashboard

2 The plotted variable needs to be selected from the dropdown menu (but the fixed variable chosen on the left will be removed) and will be shown both graphs similarly as the colored lines. Note that the values provided in the first input dashboard are considered (and are shown in the legend below the graphs, as well as in the tables)

3 The first value for the fixed variable needs to be provided here - this will be shown in the left graph

4 The second value for the fixed variable needs to be provided here - this will be shown in the right graph

Change fixed variable
KD

Change plotted variable
Partner 2 name

Value 1 for KD (pM)
1000

Value 2 for KD (pM)
10000

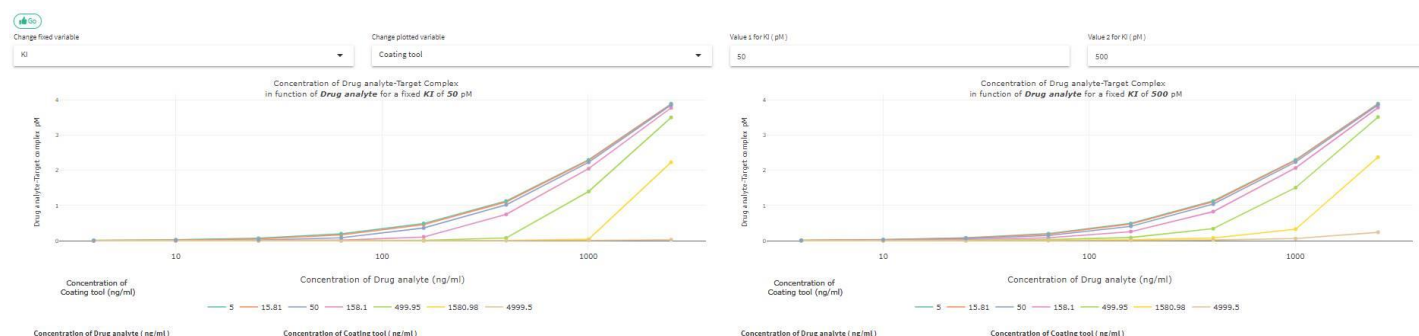
4.3.3. Example of drug analyte (partner 1) – target (partner 2) – coating tool (partner 3) interaction

UPPER PANEL

The example shown below shows how the coating tool can potentially interfere in the drug-target complex in the samples and which concentration and affinity would be most optimal.

The different concentrations of coating tool are depicted by the colored lines. In the left graph, an affinity constant of the coating tool for the drug of 50pM is selected and in the right graph 500pM.

In order not to impact the drug-target complex formed in the samples (in case of free drug measurements), the concentrations of the coating tool should not exceed 50ng/mL (blue curve) as curves drop at higher concentrations reflecting reduced complexes formed. In the right graph, a similar picture is obtained, showing that a tool with lower affinity for the drug would not significantly change the impact on the complex formation for this particular drug-target interaction.



LOWER PANEL

In case same values are selected to be plotted, same conclusions can be drawn. The main difference is what is shown here is the fractional occupancy of target, which increases with increased concentration of drug (as depicted in the X-axis).

Similarly as shown in the upper panel, increasing concentrations of coating tool and higher affinity of the coating tool for the drug will negatively impact the amount of target bound by drug.

