

Manual Kinetics Direct

Some systems reach equilibrium too quickly to be measured using the Kinetics Direct experiment. When using a 4000 instrument or an Autosampler the Manual Kinetics Direct experiment can be used.

One limitation of the Kinetics Direct is that it requires bead handling and time to charge sample lines prior to flowing the first sample. The first point therefore takes 400 to 500 seconds and each subsequent point must wait an entire run (usually 600 to 800 additional seconds) before being sampled. With the 4000 or Autosampler, bead handling can be done prior to mixing the sample and the sample line does not need to be charged. This allows the first sample to be run a few seconds after sample mixing. Furthermore, by mixing each sample individually the successive data points can be much closer together filling in gaps that would otherwise exist in a Kinetics Direct curve.

Figure 1A shows an example of a Kinetics Direct experiment that reached equilibrium quickly and did not clearly define the upper part of the curve. Figure 1B shows the same data with Manual Kinetics Direct time points included. The additional points narrow the confidence interval significantly.

Follow the procedure below to perform the Manual Kinetics Direct experiment:

Prepare the Samples

- The samples are prepared as if the Kinetics Direct will be performed. The number of cycles should be enough to define the upper part of the curve as well as a few points near equilibrium.
- Calculate the final volume for the constant binding partner and titrant mixture.
- Prepare each binding partner separately at half of the final volume needed. For example, if the final volume needed is 6.6 mL, prepare each sample at 3.3 mL. Also, be sure to prepare them at twice the concentration desired because mixing will dilute the sample by half.
- Prepare additional tubes with buffer to use for pushing the sample quickly to the flow cell. Depending on the number of points you will run, the number of tubes will vary.

Note: By using a separate tube for the buffer following the sample, the time it takes for the sample to reach the flow cell is much quicker than if the wash station is used.

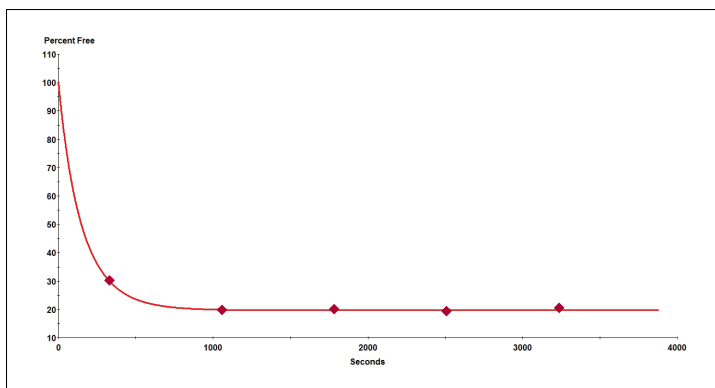


Figure 1A. Kinetics Direct experiment, $k_{on} = 1.72 \times 10^7 / Ms (+2.24, -0.47)$

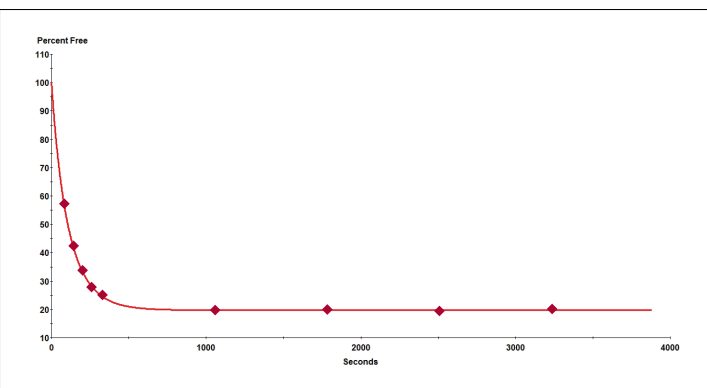


Figure 1B. Manual Kinetics Direct experiment, $k_{on} = 2.66 \times 10^7 / Ms (+0.08, -0.09)$

Prepare the Bead Handling Template

The Bead Handling template will be used to set the bead pack height prior to running each sample. This will eliminate the additional time needed to run each sample.

- In the KinExA Pro software, create a new KinExA Pro experiment.
- Under the Timing Setup Tab, select the appropriate bead type from the tool bar (**Figure 2**).

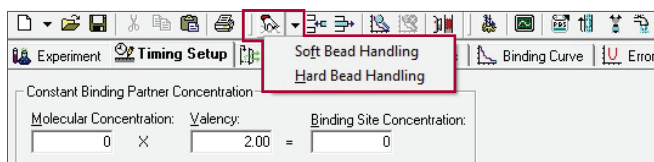



Figure 2. Selecting the bead handling from the tool bar.

- Charge beads and adjust as needed, then save the Bead Handling template. It is not necessary to fill in any other information into this file because it will not be used for analysis.
- Note:** Once the beads have been set, leave them in the flow cell so they are ready for the first sample.

Prepare the Sample Timing Template

The Sample Timing template will be used for samples mixed manually at a specified time point.

- In a new Kinetics Direct template under the *Timing Setup* tab, delete all rows under *Bead Handling*.
 - Note:** The beads are set prior to the experiment so the bead handling is deleted to avoid additional bead handling time.
- To delete the lines, select the first row under *Bead Handling* and select the **Delete Row(s)** button  in the tool bar or select **Delete Row(s)** in the *Edit* menu. Delete all rows until the final row shows zero for the time, volume, and flow rate (**Figure 3A**).
- Make sure to fill in the concentration for the Constant Binding Partner, Titrant, and K_d (**Figure 3B**).
- Select the appropriate positions for the samples, buffer, and label under *Draw Source* (**Figure 3C**).
- Under the *Instrument* tab, the number of cycles should be 1.

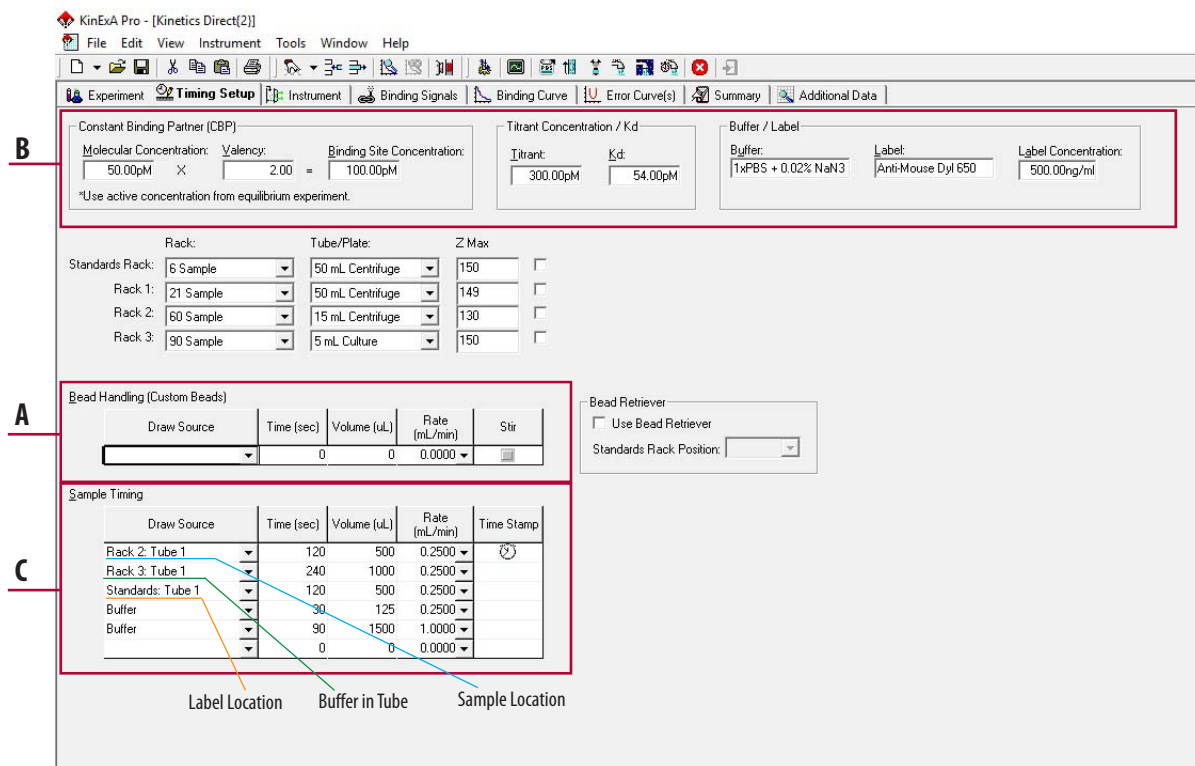



Figure 3A-C. Sample Handling template.

Run the First Data Point

With the beads already set and the sample template prepared, the first data point can be run as soon as the sample is mixed.

Note: Using default volumes, 500 μL will be needed for the sample plus 100 μL for dead volume.*

*The dead volume can be reduced to as little as 10 μL by using the small volume tubes. See Tech Note 206 Minimum Sample Volumes (TN206).

- Aliquot 300 μL of one of the 2x concentrated binding partners into a sample tube. Hold on to this tube as it will soon be mixed with the other binding partner. Have a fresh pipette tip ready to aliquot the other binding partner.
- Under the *Instrument* tab, select the **Clock** button . A count down will initiate until *MIX* appears in the box. When *MIX* appears, aliquot 300 μL of the other 2x concentrated binding partner into the reserved sample tube and mix well.
- Place the sample tube in the appropriate rack and position, then press **Start**. The Autosampler will immediately move to the sample.

Running Subsequent Data Points

Once the first data point is complete a new experiment file will be needed to run any additional data points. All the same parameters will be used for subsequent points with the exception of incubation time. The existing Sample Timing template can be used as a reference. This allows the user to add as many points as necessary to clearly define the upper part of the curve or fill in any gaps that would otherwise exist in the Kinetics Direct experiment.


- To use the existing Sample Timing template for subsequent data points, select the *File* option in the tool bar. Scroll to *New* and select *New from Existing*. This will leave the used file open in the Window. If you select *New from Current*, the used file closes and it will have to be reopened to view later.

Note: Make sure to change the experiment file name and save the file.

- Go back to the Bead Handling template and charge the beads for the next sample.
- Discard the used sample and prepare the next sample the same way the first sample was prepared. Make sure to replace the buffer tube with a fresh tube of buffer to avoid cross contamination. The sipper tip will not move to the sample until **Start** is selected. To set a sample delay, use the incubation delay option in the *Instrument* tab. The units can be changed to minutes and the delay can be changed for each point. After *MIX* appears on the screen, mix the sample, and press **Start**. The incubation delay will count down and once it finishes, the sipper tip will move to the sample.
- Once 3-6 manual points are run, the remaining sample can be used to run the Kinetics Direct experiment.
- It is not necessary to run a signal 100% or NSB sample

Combining and Analyzing Data Points

Each time point run on the Autosampler will be in a separate file. The files need to be combined before the points can be analyzed.

- Open the N-curve Analysis in software version 4.0 or newer and select **Kinetics Direct** from the drop down menu. Use the **Add Experiment** button  to add the first point to the

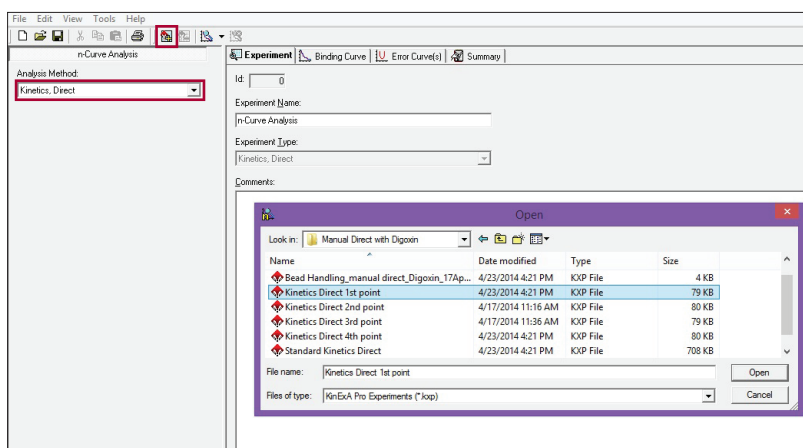




Figure 4A. Adding the first time point to the Kinetics Direct N-curve.

- N-curve (**Figure 4A**). **Add Experiment** can also be found under *Edit* in the tool bar.
- Once the first point is added, use the **Add Experiment** button  associated with Curve 1. Add all additional points (**Figure 4B**).

- Once all additional points are added, select **Analyze**  and the results will be seen under the *Binding Curve* tab (**Figure 4C**).

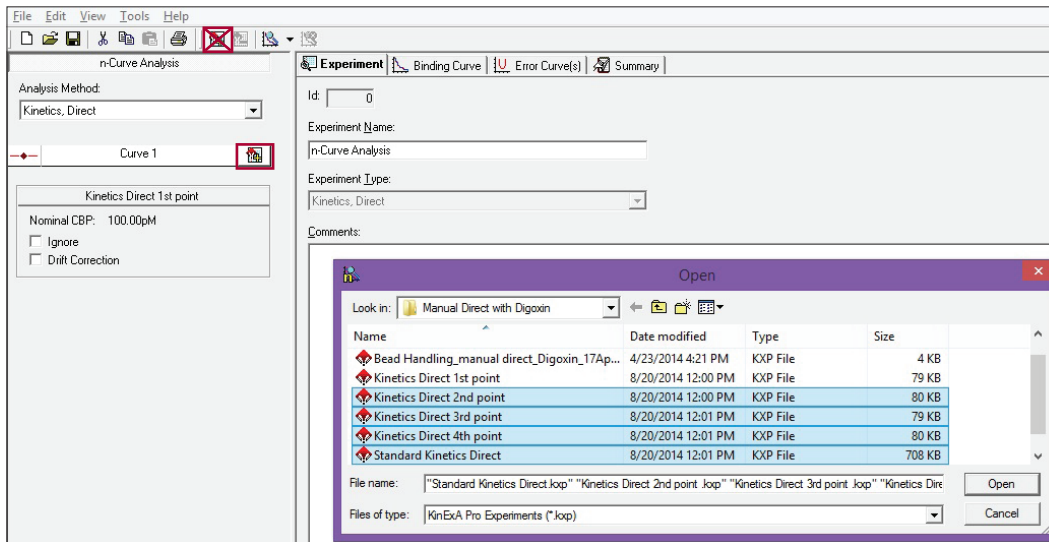


Figure 4B. Using the Add Experiment button associated with Curve 1 to add all additional points.

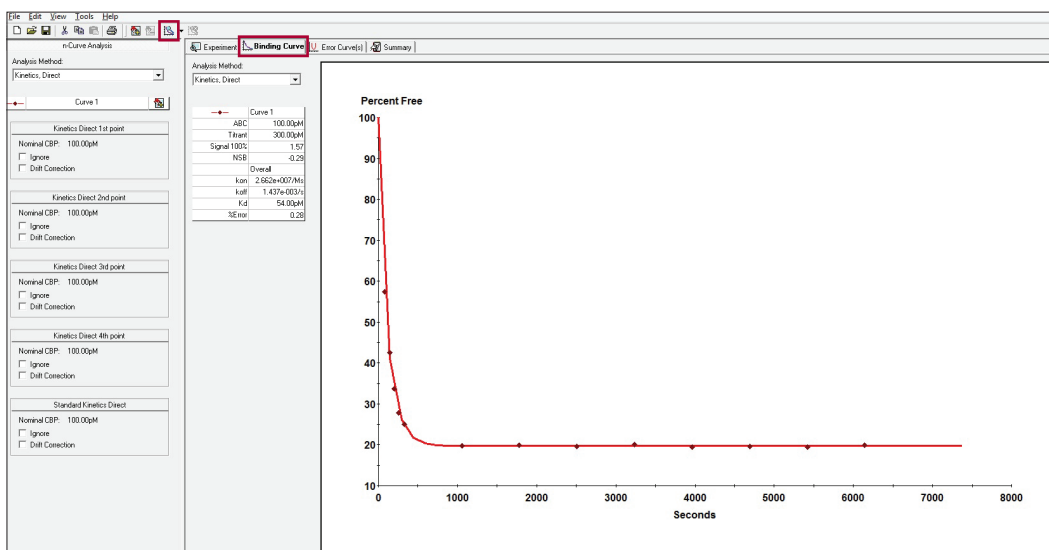


Figure 4C. Results of combined Manual Kinetics Direct and Standard Kinetics Direct.