Protein Ligand Titrations

When analysing a protein-ligand or protein-protein interaction, it is usual to perform a titration of one binding partner (here referred to as the *titrating binding partner*) against a constant concentration of the other binding partner. Examples of techniques available in the Biophysics Facility where this is necessary are fluorescence intensity, fluorescence polarisation and microscale thermophoresis.

This protocol presents two methods to prepare titration series. In each case there are three aims:

1. To make efficient use of your sample, by using as little as possible.
2. To make efficient use of the titration points, by obtaining high data-density in the region of a binding curve that is most important for precise and accurate curve-fitting.
3. To reduce experimental error and scatter in data as far as is possible, by pipetting in a way that is as accurate and consistent as possible.

For both methods, we recommend using serviced and calibrated micropipettes with appropriate well-fitting tips. In general it is best to avoid pipetting volumes smaller than 5 μl and to avoid using very small volume pipettes and tips (e.g. 2 μl maximum volume pipettes), as accuracy is limited. Be sure to work slowly and carefully and use good pipetting technique, to minimise experimental error.

For both methods, the concentrations of both the titrating binding partner and the other binding partner should be known to as high an accuracy as possible. For protein solutions, or ligands that have measurable absorbance in the UV or visible range please read the protocol for measuring protein concentration. Other solutions should be prepared by accurate weighing, and if possible their concentration should be checked (e.g. by amino acid analysis for peptides).

For both methods, the titration will use a highest concentration (top concentration) of the titrating binding partner that is approximately 20-fold above the expected K_d. The titration will use a constant concentration of the other binding partner, where that constant concentration depends on the sensitivity of the technique that is being used to monitor binding.

For both methods, the total sample volume for each titration point will be in the region of 100 ul, as this is a common volume for measurements in microplates. The example will give details for preparation in a microplate, but micro tubes could also be used. In each case, the method will give details for preparing 20 points. This is a good starting point, but can be adjusted to suit the technique.

Titration by Serial Dilution

This protocol is for a serial dilution to cover a narrow range of concentration of the titrating binding partner, to maximise data-density in the region of the binding curve that varies slope for different values of K_d. If the K_d is unknown, it is often useful to make a wider-range serial dilution to look for a signal change over a wider range of concentrations. To do this, one need only change the ratio of mixing in the serial dilution (for instance from 2:1 as shown here, to 1:1).

1. In the first microplate well (Well 1) make a 90 μl of the titrating binding partner in buffer, at a concentration *two-fold higher* than your desired top concentration. If this dilution requires adding a volume of less than 5 μl of your stock solution, make a series of dilutions (e.g. a tenfold dilution, followed by another tenfold dilution). A good starting point is to use a final top concentration approximately 20-fold higher than the expected K_d. To obtain that you would make the first well solution at a concentration of 40-fold higher than the expected K_d.

2. Pipette 30 μl of buffer into each of 19 other microplate wells (Wells 2 to 20).

3. Pipette 60 μl of the 90 μl solution in Well 1 into Well 2 (which already contains 30 μl contains buffer) and mix slowly and carefully by pipetting up and down.

4. Using a fresh pipette tip, pipette 60 μl of the 90 μl solution in Well 2 into Well 3 (which contains buffer) and mix slowly and carefully by pipetting up and down.

5. Repeat this process for the remaining wells so that you have 20 wells with solution in them.

6. The last well (Well 20) will have 90 μl of solution after mixing. Remove 60 μl of this solution, so that all the wells now have only 30 μl of solution in them.

7. Prepare a solution of the other binding partner at a concentration *two-fold higher* than the final concentration you need in the assay. This binding partner will be at constant concentration in the assay, and is usually the binding partner from which you will measure a signal to monitor binding. Therefore the final concentration in the assay will depend upon the sensitivity (limit of detection) of the technique you are using.

8. Pipette 30 ul of this solution of the second binding partner into each well and mix thoroughly by pipetting up and down. This 1:1 mixing of the

serial dilution of the first binding partner, and the solution of the second binding partner will dilute both binding partners by two-fold, giving the desired final concentrations in the assay.

9. You should now have 20 titration points with a varied concentration of one binding partner and a constant concentration of the other binding partner.

10. Incubate the solutions at the desired measurement temperature for as long as is necessary to reach equilibrium and then measure. If in doubt about incubation time, measure a binding curve after various incubation periods (10 minutes - 2 hours is a good starting point) and observe whether there is any change in the curve.

Titration by Linear Dilution

This protocol makes a linear dilution of the titrating binding partner up to a given top concentration. Compared to serial dilution, this method has the disadvantage for binding curves that it gives relatively poor data density at concentrations in the region of the K_d, where good data-density is essential for curve fitting. In general, for manual pipetting, the serial dilution approach is simpler, faster and more accurate. However, since it simply involves the mixing of two solutions from two separate stocks in different ratios, the linear dilution is the only method that can be used in simple automatic titrators for plate readers.

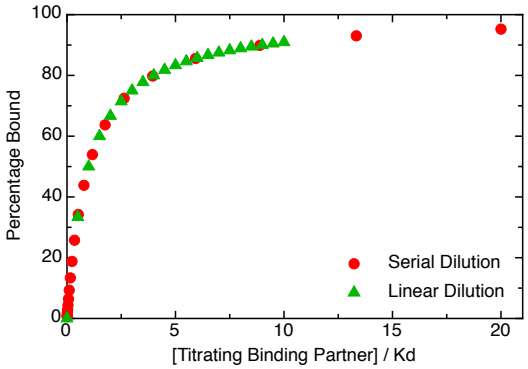

Figure 1: Data density for serial and linear dilutions. The red points are obtained by a 2:1 mixing ratio serial dilution from a concentration 20-fold higher than the K_d. The green points are obtained by a linear dilution from a top concentration 10-fold higher than the K_d. Note that the red data has a higher density at low concentrations and better defines the curve. Both data shown fit to the same parameters, but the red data would be more robust to scatter at low concentration.

This problem of data-density in linear dilutions can be avoided in two ways: (1) using a method that can accurately dispense small volumes (i.e. an automated hamilton syringe driver with small syringes) and measuring more points so that the concentration increment per-point is smaller. (2) Making two sets of linear dilutions from different top concentrations to get more points at lower concentration.

The example below is for a linear dilution of 20 points from a single top concentration. The top concentration should be 10-fold higher than the expected value of K_d. This will give a reasonably well-defined binding curve.

1. Prepare 1200 μl of a solution of the constant concentration binding partner in buffer, at twice the required final concentration, in an eppendorf tube. This is usually the binding partner from which you will measure a signal to monitor binding. Therefore the final concentration in the assay will depend upon the sensitivity (limit of detection) of the technique you are using. Mix the solution well by pipetting up and down.

2. Pipette 600 μl of this solution into a second eppendorf tube.

3. To one of the eppendorfs add the required volume of the stock solution of the titrating binding partner. That volume is determined as follows: *volume A* = 1200 μl x ((10 x K_d / stock concentration). So for a 1 μM K_d, and a 1 mM stock solution, the volume to add would be 12 μl. Mix the solution well by pipetting up and down.

4. To the other eppendorf, add the same volume (*volume A*) of buffer.

5. To each of the two eppendorfs add (600 - *volume A*) μl of buffer. Mix both solutions well by pipetting up and down. The solution that contains only the constant concentration binding partner will be called *Solution A*, and the solution that contains both binding partners will be called *Solution B*.

6. To make the titration points mix the two solutions in different ratios to give a total volume of 100 μl for each point. e.g. Well 1 would contain 100 μl of *Solution A*. Well 2 would contain 95 μl of *Solution A* and 5 μl of *Solution B*. Well 3 would contain 90 μl of *Solution A* and 10 μl of *Solution B*. And so on, for twenty wells.

7. This will give you the desired linear variation in the concentration of the titrating binding partner. You should incubate the solutions at the desired measurement temperature for as long as is necessary to reach equilibrium and then measure. If in doubt about incubation time, measure a binding curve after various incubation periods (10 minutes - 2 hours is a good starting point) and observe whether there is any change in the curve.