Effect of Sample Dilution Method on Incubation Time

Two related questions occasionally arise in conjunction with the time to reach equilibrium in KinExA® studies:

Question 1:

Sapidyne recommends performing my ligand serial dilution in constant receptor solution. To begin, I add concentrated ligand to the first sample then mix and do the serial dilution. Doesn't this mean my first sample (high concentration) may mostly bind up and then I will have to wait for the dissociation (which is slower than association) for subsequent samples to equilibrate?

Question 2:

Sapidyne recommends a large volume serial dilution strategy in which I serial dilute convenient small volumes, again in constant receptor, with much higher ligand. When the serial dilution is done, I dilute the entire sample with constant receptor to the final concentration of ligand and final volumes. Again, I'm diluting solutions that could easily be mostly bound so would I have to wait longer for equilibrium?

The answer to these questions is **NO**. Because the free fraction is always hyperbolic and the dilution curve is always linear, we are guaranteed that immediately following dilution the free fraction will always be too high. In order for the free fraction to decrease to its new equilibrium point, more receptor and ligand have to bind.

It is important to acknowledge, however, that the intuition behind these questions is correct. It can take longer to reach equilibrium if samples start with more material bound than will be bound at equilibrium. ***Figure 1*** shows the percent bound receptor as a function of time for various starting points. Clearly, it does take longer if all the material starts in the bound state. This figure demonstrates an interesting possibility, if we contrived to start with the correct amount bound the time to reach equilibrium would be zero!! This leads to a third question:

Question 3:

Can we equilibrate our samples in a concentrated state to reduce the time to equilibrium?

Before tackling question 3, let's revisit the first two questions. ***Figure 2*** shows the free fraction as a function of total ligand. (Note: both axes are linear instead of the usual semi-log.) The particular curve shown is for a K_d of 1 pM and a receptor concentration of 0.1 pM. The blue line shows the free fraction of receptor at equilibrium. When ligand is diluted in a background of receptor, the total receptor concentration is not changed and the new equilibrium free fraction will lay on the same blue line at a point corresponding to the new (diluted)

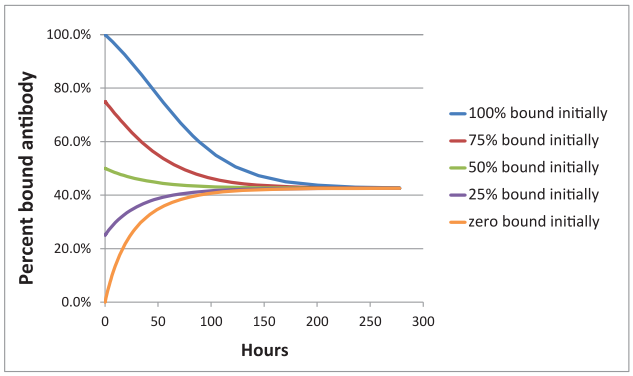

Figure 1. *Percent bound receptor vs time for various starting conditions. Simulation conditions: k_{on} =1e6, k_{off} =1e-6, ABC= 10pM, total ligand = 5pM*

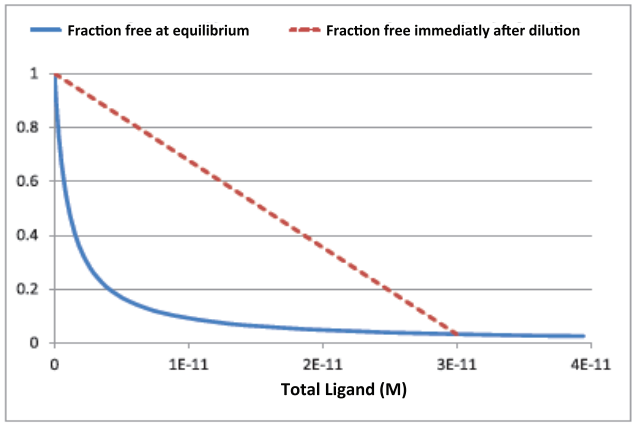

Figure 2. *Free fraction at equilibrium and immediately following dilution.*

total ligand concentration. For example, at 30 pM total ligand, the free receptor is 0.032 or 3.2% of the total receptor. If an equilibrated 30 pM sample is diluted with 0.1 pM receptor, the receptor concentration is unchanged but the bound fraction and total ligand concentration are both reduced. The free fraction is increased because we are adding more free receptor to the mix. The concentration of free receptor immediately after dilution lies on a straight line between the starting point and the end point (red dashed line in ***Figure 2***). Adding a very large volume of free receptor causes the free fraction of receptor to approach 1 immediately following the dilution (as seen in ***Figure 2***). After dilution, the new equilibrium starts from a point on the red line and proceeds to the corresponding point on the blue line. To reduce the free fraction more binding must occur.

Another way to approach questions 1 and 2 is to look at the percentage of total ligand bound as a function of total ligand concentration (***Figure 3***). Again, the receptor concentration is not changed by the dilution, only the total ligand and the bound ligand are diluted. Since each curve in ***Figure 3*** represents the equilibrium of a single receptor concentration, the equilibrium points before and after dilution both lay on the same curve. For example, suppose a concentrated sample contains 1 pM receptor and 10 pM total ligand, this corresponds to approximately 10% bound ligand (≈ 1 pM bound) on the green line below. If that sample is now diluted 10 fold with receptor, the bound concentration drops 10 fold to 0.1 pM and more ligand has to bind to reach the new equilibrium point of 37% of 1 pM or 0.37 pM bound. This just provides another window on the basic fact that we don't have to wait for dissociation to occur because we are in a situation where more ligand has to bind to reach equilibrium.

From the foregoing discussion and figures it *seems* like equilibrium will be reached faster following the protocols referred to in questions 1 and 2 since incubation is starting with at least part of the complex already formed. Waiting for equilibrium can be frustrating, especially for tight binders, so this is potentially great news.

Mathematically speaking, equilibrium is asymptotically approached rather than "reached" at some time. Practically speaking, once the samples are within a few percent of equilibrium, it is close enough to say equilibrium has been reached. Using MathCad's Find function, equations were set up to find the time to get within 1% of the final equilibrium value. When samples are preincubated in a concentrated state for a fixed time, the amount of each component bound immediately after dilution (when the "incubation" starts) can be calculated.

Simulations for a very slow, tight binder show no significant difference in overall equilibrium time between preincubating at a higher concentration and incubating at the final concentration (***Figure 4***).

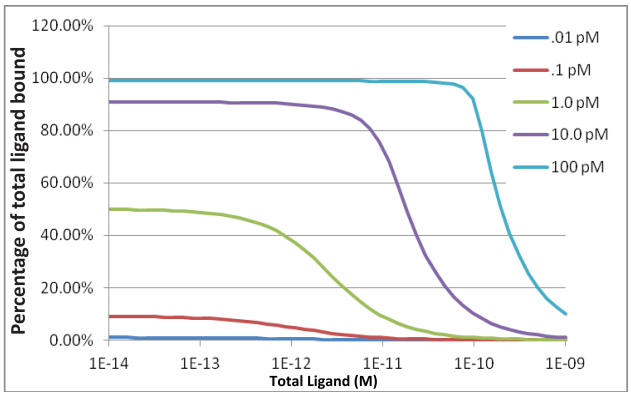

Figure 3. *Percent bound ligand vs total ligand. Five different receptor concentrations are shown, K_d value is 1 pM.*

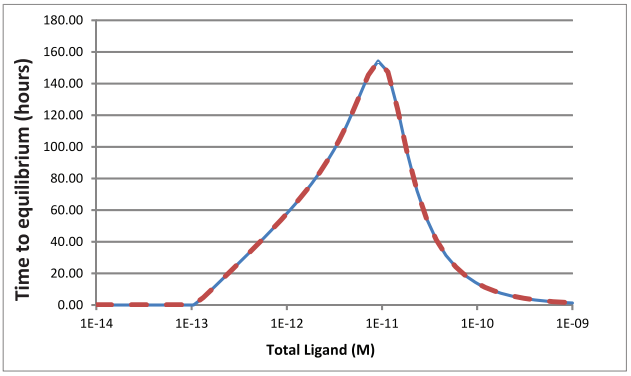

Figure 4. *Time to equilibrium for a tight binder (k_{on} = 1e6, K_d = 1 pM) as a function of total ligand concentration. The receptor concentration is 10 pM. The blue solid line is for samples mixed at the final concentration at time zero. The red dashed line shows the effect of premixing with 10 fold more concentrated ligand for 20 minutes before diluting in receptor solution.*

On the other hand, ***Figure 5*** shows a weaker system that comes to equilibrium faster so the results look somewhat better. The simulation below shows the equilibrium time (measured starting at the time the concentrated samples are diluted) is somewhat shorter. If 20 minutes is added to include the time when the samples were premixed in the concentrated state, the time advantage disappears. The real answer here is that there is no significant difference in the equilibrium time when mixing samples according to the suggested protocols.

It would be great if the 150 hour incubation of ***Figure 4*** could be cut back significantly. This brings us to question 3: can we equilibrate our samples in a concentrated state to reduce the time to equilibrium?

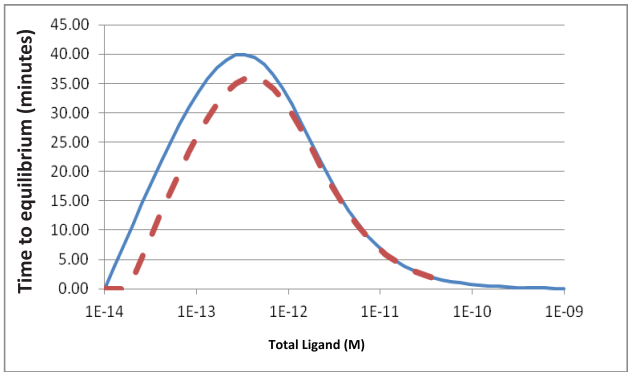

Figure 5. *Time to equilibrium for a weak binder (k_{on} = 1e6, K_d = 1 nM) as a function of total ligand concentration. The receptor concentration is 10 pM. The blue solid line is for samples mixed at the final concentration at time zero. The red dashed line shows the effect of premixing with 10 fold more concentrated ligand for 20 minutes before diluting in receptor solution.*

It *is* possible to dramatically speed things up in certain cases. Refer to ***Figure 6***. The blue line is the same as in ***Figure 4*** and represents the time to equilibrium for 10 pM receptor mixed with the various ligand concentrations indicated on the x-axis. The red dashed line, however, represents the time to reach the same equilibrium after first mixing the samples 100 times concentrated (1 nM receptor mixed with 100 times more concentrated ligand) for 42 minutes before diluting 100 fold. We have saved several days of equilibrium time even if the 42 minutes "preincubation" time is added!

Unfortunately, when both the receptor and ligand are diluted together, we are no longer in a situation where we can be sure we will not have to wait for dissociation. This means the choice of dilution factor and pre-incubation time is critical to achieve the benefit shown in ***Figure 6***. For example, if the off rate increased 100 fold, making the K_d 100 pM, the exact same preincubation time and dilution factor actually make things worse, as shown in ***Figure 7***.

The bottom line is that if the parameters of the system are known beforehand or if we are lucky, the time necessary for equilibrium can be dramatically reduced. Unfortunately, if bad guesses are made, the necessary equilibrium time may be increased, resulting in a failed experiment and wasted samples if they were run prior to reaching equilibrium.

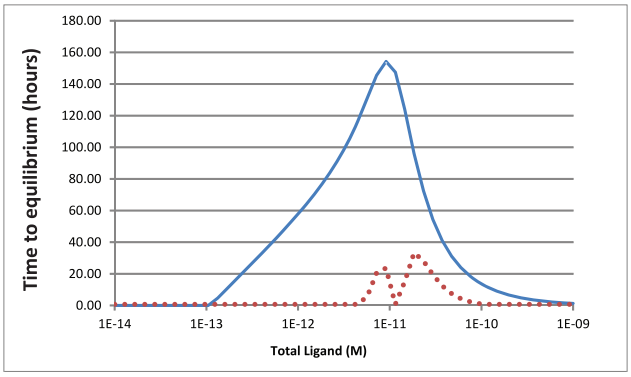

Figure 6. *Time to equilibrium for a tight binder (k_{on} = 1e6, K_d = 1 pM) as a function of total ligand concentration. The receptor concentration is 10 pM. The blue solid line is for samples mixed at the final concentration at time zero. The red dashed line shows the effect of premixing 100 fold more concentrated ligand with 100 fold more concentrated receptor for 42 minutes before diluting in buffer to the final concentrations.*

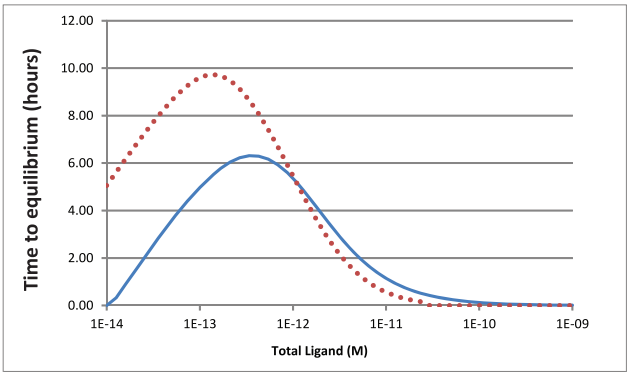

Figure 7. *Time to equilibrium for a moderately tight binder (k_{on} = 1e6, K_d = 100 pM) as a function of total ligand concentration. The receptor concentration is 10 pM. The solid blue line is for samples mixed at the final concentration at time zero. The red dashed line shows the effect of premixing 100 fold more concentrated ligand with 100 fold more concentrated receptor for 42 minutes before diluting in buffer to the final concentrations.*