Sample Preparation for a Whole Cell Equilibrium Experiment

This How to Guide provides sample preparation instructions for the measurement of the K_d and Expression Level (EL) of a cell based system. To become acquainted with the Whole Cell Assay, please review *Whole Cell Assay* (TN211).

Step 1: Prepare Solid Phase

The samples that are run on the instrument should only contain free Constant Binding Partner (CBP). Thus, the solid phase may be coated with any material that binds to the CBP but not the label. If the CBP is an antibody, try an anti-species antibody. Sapidyne commonly recommends using a biotinylated anti-species solid phase. For more information about this and alternative solid phase selections, refer to *Biotinylated PMMA Coating* **(HG208)** and *Solid Phase Selection Guide* **(TN222)**.

Step 2: Conduct a Signal Test

The purpose of a signal test is to help you determine what concentrations and volumes to use in your equilibrium experiments. For whole cell experiments, two curves with at least a 10 fold difference in CBP concentration must be ran in order to accurately determine the K_d and expression level (receptors/cell). Ideal net signals are between 0.5V-2.0V, using the standard red filter set. Signals outside of this range can be used if the linear range is well defined. For more information see *Linear Range* **(TN224)**. A non-specific binding (NSB) point should also be measured during signal testing; this point is sample buffer only.

When the K_d value is known or suspected, use a CBP concentration near that value, one concentration 10 fold higher, and an NSB point for signal testing. If the signals generated by the chosen concentrations are between the recommended net signals, the sample volume should be adjusted to either increase (use more volume) or decrease (use less volume) the signal.

If the K_d is unknown, then it is reasonable to use CBP concentrations of 1 nM, 100 pM, and an NSB point. When a CBP concentration has been measured in the ideal range, it is recommended to use this concentration and one concentration 10 fold higher (using 10 fold less volume) for equilibrium experiments.

When considering sample volumes, it is important to consider how many cells you have available. A smaller sample volume will allow you to decrease the amount of cells you use, but will also reduce your signal. This is especially important to keep in mind when expression levels are unknown or suspected to be low.

Step 3: Prepare a Dual Curve Titration Series

Equilibrium experiments are used to determine the Kd and expression level of a whole cell system to a soluble binding partner. It is recommended to use cells from the same passage number for your experiments (if possible), as expression level can vary between passages.

After identifying two CBP concentrations and volumes to use, it is time to determine your starting cell concentrations. Using the theory curve, we can visualize what starting cell concentrations will achieve adequate inhibition of your CBP. Full inhibition does not need to be achieved, as long as at least 20% inhibition (80% free) can be reached with both curves. If the Kd is completely unknown, then a starting cell concentration can be determined based on convenience. It is reasonable to use anywhere between 5 million cells/mL to 100 million cells/mL.

You will also need to choose the total number of samples and a dilution factor for your titration series. Sapidyne likes to use 12 samples (plus an NSB point) with a two fold dilution series, though these parameters can be adjusted as needed to provide confidence in your data. For more information see *Determining Dilution Series* (**TN203**).

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Prepare your CBP and aliquot the solution among the sample tubes, ensuring that the first tube has extra volume to account for the titration series. Harvest and spin down the total amount of cells you want to start the titration series with and resuspend the pellet using the CBP solution from sample tube one.

Note: If your cell pellet is greater than 5% of the volume of your first sample tube, please contact a Sapidyne representative for more detailed instructions on sample preparation.

Mix the cells and CBP solution thoroughly and perform the serial dilution. The samples should be allowed to reach equilibrium while the cells are in solution, which can be achieved by rotating the samples at the desired temperature. Depending on the K_d of the system, equilibrium may be reached within minutes or hours. If unknown, it is reasonable to incubate the samples overnight.

Once equilibrium has been reached, the cells will need to be spun out of solution. Only the supernatant can be run through KinExA instruments as the cells will clog the flow cell. A recommended spin time and speed is 3 minutes at ~2000 x g.

Below is an example of a whole cell experiment preparation, which will be run on an autosampler and include duplicate points.

(For small volumes and to reduce the dead volume use:
Dual Microtitier Plate Rack - Part # 414106
48 Sample Microcentrifuge Rack - Part # 414148
sapidyne.com)

Part 1:

Choose two CBP concentrations (and associated sample volumes):

A) Curve 1: 1 nM (500 µL)

B) Curve 2: 10 nM (50 µL)

Calculate the minimum supernatant volume needed. In order to extract the desired supernatant volume without disturbing the cell pellet, an additional spacing volume of 100 µL should be included:

A) Curve 1 supernatant volume = (sample volume * 2) + 100 µL dead volume + 100 µL spacing volume = 1.2 mL

B) Curve 2 supernatant volume = (sample volume * 2) + 100 µL dead volume + 100 µL spacing volume = 300 µL

Note: *If using the 3200 alone, 400 µL needs to be added to each sample for the charge volume.*

In order to perform a 2-fold serial dilution double the volume in the 1st tube for each curve. See Tech Note 203 *Determining Dilution Series* (**TN203**) for more info:
A) Curve 1: Tube 1=2.4 mL

B) Curve 2: Tube 1=600 µL

Calculate the total CBP volumes, prepare them and then aliquot among sample tubes:

A) Curve 1 total CBP volume = ((2.4 mL) + (1.2 mL * 11 sample tubes)) = 15.6 mL

B) Curve 2 total CBP volume = ((600 µL) + (300 µL * 11 sample tubes)) = 3.9 mL

Note: *Don't forget to include an NSB sample.*

Part 2:

The same batch of cells will be used to prepare both equilibrium curves. Split 90 million cells into two tubes, one containing 72 million and the other containing 18 million. Centrifuge the cells and remove the supernatant.

A) For Curve 1, resuspend 72 million cells with 2.4 mL of CBP from sample tube 1. The starting concentration of cells will be 30 million cells/mL. Mix the cell solution and perform a 2-fold serial dilution by transferring 1.2 mL down the line.

B) For curve 2, resuspend 18 million cells with 600 µL of CBP from sample tube 1. The starting concentration of cells will be 30 million cells/mL. Mix the cell solution and perform a 2-fold serial dilution by transferring 300 µL down the line.

Rotate both curves so that the cells move in solution while equilibrating.

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Part 3:

After the samples have reached equilibrium, centrifuge all samples at ~2000 x g for 3 minutes (For insight into how long equilibrium may take use the theory curve Tech Note 220 *Theory Curve* (**TN220**)).

A) For Curve 1 transfer 1.1 mL of supernatant from each sample to a corresponding sampling tube.

B) For Curve 2 transfer 200 µL of supernatant from each sample to a corresponding sampling tube.

Note: *When performing this step, it is important to use a new pipette with each sample to avoid cross contamination.*

Analysis

Open the n-curve analysis and select **Equilibrium, Whole Cell** from the drop down menu. Add both of the equilibrium curves into the n-curve program by accessing the edit menu or by selecting the  icon from the toolbar. Navigate to the individual experiment files and open the **Binding Signals** tab. Ensure NSB is displayed under the cells/mL column with the corresponding sample. Select **Analyze** (for software version 4.2.12 and earlier, un-ignore the NSB point and re-select analyze).

Note: *The default unit is millions/mL.*

FAQ:

Q. How much supernatant is needed for KinExA measurements?

A. Enough to get a usable signal. The signal 100% must be large enough to overcome random noise within the system yet stay within the linear range. The best way to determine the volume needed is to perform signal testing.
Sapidyne does not recommend using less than 5 µL for the sample volume (see *Minimum Sample Volumes* (**TN206**)). If 5 µL of volume does not drop the signal within linear range, the supernatant can be diluted after the CBP has been incubated with the cells and spun out of solution. Diluting the supernatant to a concentration that will fall within linear range will not effect the results because the supernatant contains only free CBP. It is important to make sure all samples are diluted by the same factor. Also, when performing the analysis in the KinExA Pro Software, make sure the original concentration is used and not the diluted concentration. KinExA 4000 users can also lower the excitation level, though a new signal test should be completed.

Q: How do I know the signal from the sample buffer accurately represents the NSB of my cellular system?

A: Sapidyne has conducted extensive in-house research and found that the NSB signal associated with complete inhibition of the CBP from a saturating amount of cells almost always matches the NSB signal from plain sample buffer and label. Slight variations in the NSB signal also reported insignificant changes to the measured K_d and EL (always within the true 95% confidence interval bounds).

Q: If I know the expression level of my cells, can I use that as my concentration reference?

A: Yes, as long as you have software version 4.2.13 or newer.