Sample Preparation for a Whole Cell Equilibrium Experiment

This How To Guide explains Sapidyne's recommended procedure to measure the K_d and expression level (EL) of a cell based system. To become acquainted with the Whole Cell Assay please review Tech Note 211 *Whole Cell Assay* (TN211).

Step 1: Prepare Solid Phase

The supernatant 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. For help selecting a solid phase please review Tech Note 222 *Solid Phase Selection Guide* (**TN222**).

Step 2: Conduct a Signal Test

Find a concentration and volume that gives a good net signal for two CBP concentrations ~ 10 fold apart. A reasonable net signal is 0.1V – 2V as described in Tech Note 228 *Signal to Noise* (**TN228**) .

We recommend always measuring two equilibrium curves with enough difference between the CBP concentrations to extrapolate the curve shapes if complete inhibition is not reached (See **TN211** *Whole Cell Assay*). If the K_d is unknown, an initial signal test consisting of 4 nM, 2 nM, 1 nM and 500 pM, plus a non specific binding (NSB) sample will give an idea of the linear binding range as well as concentrations and volumes necessary for a good net signal. For more information on how to determine the linear binding range please see Tech Note 224 *Linear Range* (**TN224**). If a K_d value is known or suspected, use a CBP concentration near that value and one 10 fold higher.

When deciding on a sample volume consider the total amount of cells available. A smaller sample volume will allow you to increase the cells/mL concentration. Working with smaller volumes will also help reduce the total amount of cells needed when expression levels are low.

Step 3: Prepare a Dual Curve Titration Series

After identifying two CBP concentrations, prepare two equilibrium curves. Sapidyne recommends dual curve whole cell experiments be done using the exact same cell passage at the same time to avoid changes in EL from passage to passage.

Determine a dilution factor and sample number that will give you good resolution and span a large concentration range. A two-fold dilution measuring 12 samples plus a non specific binding (NSB) sample is a good start.

The goal is to start the titration series at a high enough cells/mL concentration that the CBP is fully inhibited. A good starting point is ~ 30 million cells/mL. Of course, the amount of inhibition this gives depends on the K_d and EL. Complete inhibition of the CBP is often not practical if working with native cells or measuring K_d s weaker than single digit nanomolar. To overcome this, Sapidyne has developed a dual curve extrapolation that allows one to use a measured NSB in place of a totally inhibited CBP sample. For accurate analysis, the extrapolation requires a dual curve that displays a minimum of 20% inhibition for the high curve and a minimum 5 fold difference in CBP ratio. The NSB sample can be just sample buffer or can include cells without CBP. If you suspect cellular debris may be contributing to the NSB of the system, prepare the NSB sample with cells and sample buffer. Always include a NSB sample when preparing a dual curve whole cell equilibrium experiment. For more information on the extrapolation and how to calculate the molar equivalent of your cell expressed receptor please review **TN211** *Whole Cell Assay*.

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After preparing the CBP, aliquot the solution among the sample tubes ensuring the first tube has extra volume to account for the serial dilution. Spin down the total amount of cells you wish to start the titration series with and resuspend the pellet with the CBP from sample tube one. Ensure the cells are properly mixed and perform a serial dilution. Depending on the K_d of the system equilibrium may be reached within minutes or hours. Equilibrate the cells at the desired temperature keeping the cells moving in suspension. Once equilibrium is reached, spin down all the sample tubes to pellet the cells. Transfer the supernatants to corresponding sampling tubes. Only supernatant is run through the KinExA because cells have been shown to clog the flow cell.

Below is an example of a dual curve preparation, to be run on an Autosampler, starting at 30 million cells/mL and running duplicates of each curve for 12 points plus an NSB.

(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 ~200 x g for 5 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). The expression level is determined from the cells/mL and high ratio curve.

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 100% signal 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 Tech Note 206 *Minimum Sample Volumes* (**TN206**)). If 5 µL of volume is not sufficient to 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.

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.