Saturation Binding Assay

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Contents

Overview

Use 6 columns of 96-well shallow plate, can test 2 membrane/protein per plate. Final Volume in each well is **125 uL**

- 25 uL 3H Hot Ligand
- 25 uL Binding Buffer (BB)/Cold Ligand (Reference)
- 75 uL Membrane (Protein)

Table 1: Plate layout after step 7

	Columns 1/7	Columns 2/8	Columns 3/9	Columns 4/10	Columns 5/11	Columns 6/12
A	|25 uL 3H |**25 uL BB** |75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	|25 uL 3H |**25 uL Ref** |75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem
B	|25 uL 3H |**25 uL BB** |75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	|25 uL 3H |**25 uL Ref** |75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem
C	|25 uL 3H |**25 uL BB** |75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	|25 uL 3H |**25 uL Ref** |75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem
D	|25 uL 3H |**25 uL BB** |75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	|25 uL 3H |**25 uL Ref** |75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem
E	|25 uL 3H |**25 uL BB** |75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	|25 uL 3H |**25 uL Ref** |75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem
F	|25 uL 3H |**25 uL BB** |75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	|25 uL 3H |**25 uL Ref** |75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem
G	|25 uL 3H |**25 uL BB** |75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	|25 uL 3H |**25 uL Ref** |75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem
H	|25 uL 3H |**25 uL BB** |75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	25 uL 3H **25 uL BB** 75 uL Mem	|25 uL 3H |**25 uL Ref** |75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem	25 uL 3H **25 uL Ref** 75 uL Mem

Determining Protein Concentration

1. Resuspend pellet in 12 mL Lysis Buffer (10mM Tris + 5% Sucrose, pH 7.4):
    1. Use 1 mL Lysis Buffer to break up pellet
    2. Fill to ~12 mL with Lysis Buffer
    3. Pool pellets into one tube (if multiple pellets)
2. Perform Bradford Protein Concentration Assay:
    1. Sample Preparation:

- 10 uL Pellet Suspension + 790 uL dH20 + 200 uL Bradford Reagent

2. Blank Preparation:
   - 10 uL Lysis Buffer + 790 uL dH20 + 200 uL Bradford Reagent
3. Incubate @ RT 10 min
4. Measure absorbance @ 595 nm
5. Calculate protein concentration (Refer to Formulas section or Spreadsheet)

Hot Ligand Addition

3. Prepare ~15 mL appropriate BB w/BSA (~30 uL) in trough
4. In an empty 96-well shallow plate (Need one column):
   1. Add **330 uL** BB to well H and **165 uL** BB to well A-G
   2. Add 3H-Ligand to Well H (Refer to Formulas section or Spreadsheet)
   3. Perform a Serial Dilution (1:2) of **165 uL** up from well H to A
   4. Remove **25 uL** from Well A for radioactivity counts
   5. Using a multichanel pipettor, Transfer **25 uL** into 6 columns of the Drug Plate

Tables

Table 2: Step 4.1, in separate 96-well plate

	Empty Column
A	**165 ul** BB
B	**165 ul** BB
C	**165 ul** BB
D	**165 ul** BB
E	**165 ul** BB
F	**165 ul** BB
G	**165 ul** BB
H	**330 ul** BB

Table 3: Step 4.3, Volume after serial dilution completed

	Empty Column
A	**330 ul** BB
B	**165 ul** BB
C	**165 ul** BB
D	**165 ul** BB

	Empty Column
E	|**165 ul** BB
F	|**165 ul** BB
G	|**165 ul** BB
H	|**165 ul** BB

Table 4: Plate layout after 3H-Ligand Addition

	Columns 1/7	Columns 2/8	Columns 3/9	Columns 4/10	Columns 5/11	Columns 6/12
A	|25 uL 3H	25 uL 3H	25 uL 3H	|25 uL 3H	25 uL 3H	25 uL 3H
B	|25 uL 3H	25 uL 3H	25 uL 3H	|25 uL 3H	25 uL 3H	25 uL 3H
C	|25 uL 3H	25 uL 3H	25 uL 3H	|25 uL 3H	25 uL 3H	25 uL 3H
D	|25 uL 3H	25 uL 3H	25 uL 3H	|25 uL 3H	25 uL 3H	25 uL 3H
E	|25 uL 3H	25 uL 3H	25 uL 3H	|25 uL 3H	25 uL 3H	25 uL 3H
F	|25 uL 3H	25 uL 3H	25 uL 3H	|25 uL 3H	25 uL 3H	25 uL 3H
G	|25 uL 3H	25 uL 3H	25 uL 3H	|25 uL 3H	25 uL 3H	25 uL 3H
H	|25 uL 3H	25 uL 3H	25 uL 3H	|25 uL 3H	25 uL 3H	25 uL 3H

Cold Ligand (Reference) Addition

5. Add **25 uL** BB to first 3 columns
6. Prepare Cold Ligand (Reference):
    1. Usually, add **4 uL** Reference compound into **800 uL** BB into an eppendorf tube
    2. Using a single channel pipettor, add **25 uL** Reference Compound into the last 3 columns

Tables

Table 5: Plate layout after cold ligand (reference) addition

	Columns 1/7	Columns 2/8	Columns 3/9	Columns 4/10	Columns 5/11	Columns 6/12
A	|25 uL 3H |**25 uL BB**	25 uL 3H **25 uL BB**	25 uL 3H **25 uL BB**	|25 uL 3H |**25 uL Ref**	25 uL 3H **25 uL Ref**	25 uL 3H **25 uL Ref**

	Columns 1/7	Columns 2/8	Columns 3/9	Columns 4/10	Columns 5/11	Columns 6/12
B	|25 uL 3H |**25 uL BB**	25 uL 3H **25 uL BB**	25 uL 3H **25 uL BB**	|25 uL 3H |**25 uL Ref**	25 uL 3H **25 uL Ref**	25 uL 3H **25 uL Ref**
C	|25 uL 3H |**25 uL BB**	25 uL 3H **25 uL BB**	25 uL 3H **25 uL BB**	|25 uL 3H |**25 uL Ref**	25 uL 3H **25 uL Ref**	25 uL 3H **25 uL Ref**
D	|25 uL 3H |**25 uL BB**	25 uL 3H **25 uL BB**	25 uL 3H **25 uL BB**	|25 uL 3H |**25 uL Ref**	25 uL 3H **25 uL Ref**	25 uL 3H **25 uL Ref**
E	|25 uL 3H |**25 uL BB**	25 uL 3H **25 uL BB**	25 uL 3H **25 uL BB**	|25 uL 3H |**25 uL Ref**	25 uL 3H **25 uL Ref**	25 uL 3H **25 uL Ref**
F	|25 uL 3H |**25 uL BB**	25 uL 3H **25 uL BB**	25 uL 3H **25 uL BB**	|25 uL 3H |**25 uL Ref**	25 uL 3H **25 uL Ref**	25 uL 3H **25 uL Ref**
G	|25 uL 3H |**25 uL BB**	25 uL 3H **25 uL BB**	25 uL 3H **25 uL BB**	|25 uL 3H |**25 uL Ref**	25 uL 3H **25 uL Ref**	25 uL 3H **25 uL Ref**
H	|25 uL 3H |**25 uL BB**	25 uL 3H **25 uL BB**	25 uL 3H **25 uL BB**	|25 uL 3H |**25 uL Ref**	25 uL 3H **25 uL Ref**	25 uL 3H **25 uL Ref**

Membrane (Protein) Addition

7. Prepare ~4 mL membrane receptor:
    1. Add BB volume to new trough (Refer to Formulas section or Spreadsheet)
    2. Add protein to volume new trough (Refer to Formulas section or Spreadsheet)
    3. Using a multichannel pipettor, add **75 uL** protein dilution into all 6 columns
8. Incubate plates @ RT for 1 hour in drawer

Filtering

Formulas

Protein Concentration calculation

Need:

- OD @ 595 nm

Formula:

$$\text{Protein Concentration (ug/uL)} = \frac{\text{OD@595 nm} - 0.094}{0.503}$$

Where:

- 0.094 and 0.503 were determined from experimental procedure, provided by XP.

3H Hot Ligand calculation

Need:

- Starting Concentration (nM)
- 3H-Ligand Specific Activity (Ci/mmol)

Formula:

$$\text{3H-Ligand Vol (uL)} = \frac{330\ \text{(uL)}\ * \text{Starting Concentration (nM)} * 5 * 1.2}{\text{Specific Activity (Ci/mmol)}^{-1} * 1000000}$$

Where:

- 330 uL is double the volume of 165 uL, (25 uL * 6 wells * 1.1 overage = 165 uL), so we can perform a serial dilution
- 5 is a Dilution Factor, the final volume in each well is 125 uL, we add 25 uL from the Hot-Ligand plate, (125/25=5)
- 1.2 is a 20% overage
- 1000000 is for unit conversion

Cold Ligand calculation

Usually need **4 uL** Reference compound and **800 uL** BB

Need:

- Concentration of Cold Ligand (Reference) Stock (most are 10 mM)

Formula:

$$\text{Reference Vol (uL)} = \frac{800\ \text{uL} * 10\ \text{uM Final Concentration} * 5}{10000\ \text{uM Starting Concentration}}$$

Where:

- 800 uL is approixmate volume we need (8 Wells/Column * 3 Columns * 25 uL/Well * 1.1 overage = 660), use 800 so pulling from reference is easier
- Final concentration of Cold Ligand (Reference) in each well is 10 uM
- 5 is a Dilution Factor, the final volume in each well is 125 uL, we add 25 uL from the Hot-Ligand plate, (125/25=5)
- 10000 uM or 10 mM is starting reference concentration, usually

Membrane Receptor calculation

Need:

- Protein Concentration (ug/uL) (Found from "Determining Protein Concentration")
- Protein/Well (ug) (XP will provide this)

Formula:

$$\text{Volume of Protein (uL)} = \frac{\text{Protein/Well (ug)} * 4000\ \text{(uL)}}{\text{Protein Concentration (ug/uL)} * 75\ \text{(uL)}}$$

$$\text{Volume of BB (uL)} = 4000\ \text{(uL)} - \text{Volume of Protein (uL)}$$

Where:

- 75 uL is the volume that will be dispensed into each well
- 4000 uL is approixmate volume we need (8 Wells/Column * 6 Columns * 75 uL/Well * 1.1 overage = 3960 uL), round to 4000 uL for convience

Determining Starting Concentration calculation

After obtaining actual radioactive counts (dpm) starting concentration (concentration of 3H-Ligand in Well H) can be determined.

Need:

- Specific Activity (Ci/mmol)
- Actual Counts (dpm)

Formula:

$$\text{Starting Concentration (nM)} = \frac{\text{Actual Counts (dpm)} * 10^9\ \text{(nM/M)} * 2^7}{2.22 * 10^{12}\ \text{(dpm/Ci)} * \text{Specific Activity (Ci/mmol)} * 0.125\ \text{(mL/Well)}}$$

Where:

- 10^9 in a unit conversion to nM from M
- 2^7 is for the 7 serial 1:2 dilutions
- $2.22 * 10^{12}$ dpm/Ci is a constant
- 0.125 mL/Well is the final volume in each well