Saturation Binding SOP

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Contents

1 Overview

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

- **25 μL** 3H Hot Ligand

- **25 μL** Binding Buffer (BB)/Cold Ligand (Reference)

- **75 μL** Membrane (Protein)

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

Table 1: Contents of drug plate after completion of procedure, adding Membrane (Protein)

2 Determining Protein Concentration

1. Resuspend pellet in 12 mL Lysis Buffer (10mM Tris + 5% Sucrose, pH 7.4):

   (a) Use 1 mL Lysis Buffer to break up pellet

   (b) Fill to 12 mL with Lysis Buffer

   (c) Pool pellets into one tube (if multiple pellets)

2. Perform Bradford Protein Concentration Assay:

   (a) **Sample Prep:** 10 μL *Pellet Suspension* + 790 μL dH20 + 200 μL Bradford Reagent

   (b) **Blank Prep:** 10 μL *Lysis Buffer* + 790 μL dH20 + 200 μL Bradford Reagent

   (c) Incubate @ RT 10 min

   (d) Measure absorbance @ 595 nm

   (e) Calculate protein concentration (Refer to Formulas section or Spreadsheet)

3 Hot Ligand Addition

4. Prepare ~15 mL appropriate BB w/BSA (~30 μL) in trough

5. In an empty 96-well shallow plate (need one column):

   (a) Add **330 μL** BB to well H and **165 μL** BB to well A-G
   (b) Add 3H-Ligand to Well H (Refer to Formulas section or Spreadsheet)
   (c) Perform a Serial Dilution (1:2) of **165 μL** up from well H to A
   (d) Remove **25 μL** from Well A for radioactivity counts
   (e) Using a multichanel pipettor, Transfer **25 μL** into 6 columns of the Drug Plate

3.1 Table

	Empty Column
A	**165 μL**
B	**165 μL**
C	**165 μL**
D	**165 μL**
E	**165 μL**
F	**165 μL**
G	**165 μL**
H	**165 μL**

Table 2: Contents in empty column of 96 well plate, after step 5.(a)

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

Table 3: Contents of drug plate after completion of Hot Ligand Addition

4 Cold Ligand (Reference) Addition

6. Add **25 μL** BB to first 3 columns

7. Prepare Cold Ligand (Reference):

   (a) Usually, add **4 μL** Reference compound into **800 μL** BB in an eppendorf tube
   (b) Using a single channel pipettor, add **25 μL** Reference Compound into the last 3 columns

4.1 Table

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

Table 4: Contents of drug plate after completion of Cold Ligand (Reference) Addition

5 Membrane (Protein) Addition

8. Prepare ~4 mL membrane receptor:

   (a) Add BB volume to new trough (Refer to Formulas section or Spreadsheet)
   (b) Add protein to volume new trough (Refer to Formulas section or Spreadsheet)
   (c) Using a multichannel pipettor, add **75 μL** protein dilution into all 6 columns

9. Incubate plates in drawer @ RT for 1 hour

6 Formulas

6.1 Protein Concentration calculation

Need:

- OD 595 nm

Formula:
$$\text{Protein Concentration } (\mu g/\mu L) = \frac{\text{OD@595 nm} - 0.094}{0.503}$$

Where:

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

6.2 3H Hot Ligand calculation

Need:

- Starting Concentration (nM)

- 3H-Ligand Specific Activity (Ci/mmol)

Formula:

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

Where:

- 330 μL is double the volume of 165 μL, (25 μL * 6 wells * 1.1 overage = 165 μL), so we can perform a serial dilution

- 5 is a Dilution Factor, the final volume in each well is 125 μL, we add 25 μL from the Hot-Ligand plate, (125/25=5)

- 1.2 is a 20% overage

- 1000000 is for unit conversion

6.3 Cold Ligand calculation

Usually need **4 μL** Reference compound and **800 μL** BB
Need:

- Concentration of Cold Ligand (Reference) Stock (most are 10 mM = 10000 μM)

Formula:
$$\text{Reference Vol } (\mu L) = \frac{800\ \mu L * 10\ \mu M \text{ Final Concentration} * 5}{10000\ \mu M \text{ Starting Concentration}}$$

Where:

- 800 μL is approximate volume we need (8 Wells/Column * 3 Columns * 25 μL/Well * 1.1 overage = 660), use 800 so pulling from reference is easier

- Final concentration of Cold Ligand (Reference) in each well is 10 μM

- 5 is a Dilution Factor, the final volume in each well is 125 μL, we add 25 μL from the Hot-Ligand plate, (125/25=5)

- 10000 μM or 10 mM is starting reference concentration, usually

6.4   Membrane Receptor calculation

Need:

- Protein Concentration (μg/μL) (Found from "Determining Protein Concentration")

- Protein/Well (μg) (XP will provide this)

Formula:

$$\text{Volume of Protein } (\mu\text{L}) = \frac{\text{Protein/Well } (\mu\text{g}) * 4000 \ (\mu\text{L})}{\text{Protein Concentration } (\mu\text{g}/\mu\text{L}) * 75 \ (\mu\text{L})}$$

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

Where:

- 75 μL is the volume that will be dispensed into each well

- 4000 μL is approximate volume we need (8 Wells/Column * 6 Columns * 75 μL/Well * 1.1 overage = 3960 μL), round to 4000 μL for convience

6.5   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