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

	Inital	Transfer	Serial Dilution	Actual Counts	Final	3H-Ligand Ratio
A	**165 μL**		**330 μL**	$\rightarrow$ **25 μL**	**305 μL**	1:128
B	**165 μL**	$\uparrow$ **165 μL**	**165 μL**		**165 μL**	1:64
C	**165 μL**	$\uparrow$ **165 μL**	**165 μL**		**165 μL**	1:32
D	**165 μL**	$\uparrow$ **165 μL**	**165 μL**		**165 μL**	1:16
E	**165 μL**	$\uparrow$ **165 μL**	**165 μL**		**165 μL**	1:8
F	**165 μL**	$\uparrow$ **165 μL**	**165 μL**		**165 μL**	1:4
G	**165 μL**	$\uparrow$ **165 μL**	**165 μL**		**165 μL**	1:2
H	**330 μL**	$\uparrow$ **165 μL**	**165 μL**		**165 μL**	1:1

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

	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 *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*
B	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*
C	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*
D	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*
E	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*
F	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*
G	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*
H	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *BB*	25 μL 3H 25 μL *Ref*	25 μL 3H 25 μL *Ref*	25 μL 3H 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 BB in trough (Refer to Formulas section or Spreadsheet)
   (c) Using a multichannel pipettor, add **75 μL** protein dilution into all 6 columns
   (d) Refer to Table 1 for plate contents
9. Incubate plates in drawer @ RT for 1 hour

6 Formulas

6.1 Protein Concentration calculation

Need:

- OD 595 nm

Formula:

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

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:

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

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:

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

Where:

- 800 μL is approixmate 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 approixmate 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