

**Large Scale Purification of Integral Membrane Proteins**  
**December, 2008**  
**Version 1.0**

**Summary:**

The two methods/protocols provided in this document are currently being used by JCIMPT for large-scale production of purified GPCR proteins to be used for crystallization studies. Rather than providing a single fully tested protocol, we provide the methods used in preparing the two GPCR's that were crystallized and structurally determined in the JCIMPT laboratories. The two methods exemplify how steps are modified and/or optimized for different targets. Early evaluation and characterization of expression, protein properties, and design of strategies for large scale production are carried out using results from screening runs using 5-10mL of biomass following JCIMPT's protocol "Microscale Extraction and Purification of Integral Membrane Proteins". This large-scale purification protocol has now been used in preparing samples from a variety of GPCR's with modifications based on results from microscale studies.

Large scale purification normally starts using membrane samples containing over-expressed proteins extracted from lysed cells prepared following JCIMPT's protocol "Large Scale Protocol of Membrane Containing Over-expressed Proteins from SF9 Cells" starting with 1-10 L of biomass of SF9 cells. The final product is purified, deglycosylated protein detergent complex which can then be concentrated for crystallization.

**Overview of Steps:**

1. Membrane treatment and solubilization
2. Talon IMAC purification
3. Ni Sepharose IMAC to concentrate and deglycosylate
4. Quality check and characterization of products

**Materials:**

**1. Reagents:**

Lysis Buffer (10mM HEPES pH 7.5, 10 mM MgCl<sub>2</sub>, 20 mM KCl)

Please note, depending on the susceptibility to proteolyses protease inhibitors may have to be added. At JCIMPT, we have been using the following inhibitors when working with GPCR proteins:

1. Protease Inhibitor Cocktail Set V (calbiochem)
2. Bestatin (Roche)
3. Leupeptin (Roche)
4. Pefabloc (Roche)

Ligands for stabilizing GPCR's if they are available and/or are needed. For  $\beta$ -2 Adrenergic Receptor ( $\beta$ 2AR) we used Timolol and for the Adenosine A2 receptor (A2Ar), we used theophylline

PNGase from New England Biolabs

## 2. Equipment:

Centrifuge

## 3. Step by step methodology

### Protocol used for $\beta$ -2 Adrenergic Receptor construct 157T4

#### *Membrane Extraction and Solubilization*

##### a. Membrane treatment

- i. Dilute to 25mL with lysis buffer
- ii. Add timolol 1mM
- iii. Add iodoacetamide (2mg/mL)
- iv. Add protease inhibitors
  1. 1 vial calbiochem V/50ml tube
  2. Bestatin 20ug/ml
  3. Leupeptin 20ug/ml
  4. Pefablock 20ug/ml
- v. Incubate at 4 degrees 20-30minutes

##### b. Solubilization

- i. Add 25 mL of 2x solubilization buffer
  1. 100mM Hepes pH 7.5; 300mM NaCl; 1% DDM; 0.2% CHS
  2. Final concentration: 50mM Hepes 150mM NaCl 0.5% DDM; 0.1% CHS
- ii. Solubilize for 2.5 hours at 4 deg
- iii. Centrifuge and isolate supernatant

#### *IMAC Purification, First Pass*

##### c. Talon binding (Superflow)

- i. Equilibrate talon resin with water
  1. Use 1mL resin per 50mL solubilization
- ii. Bring NaCl to 800mM
- iii. Add Imidazole to 5mM final concentration
- iv. Add leupeptin
- v. Add 1.5mL of 50% talon slurry in water to each 50mL falcon tube
  1. Rock slowly or stir slowly for 6hr-overnight batch bind in falcon tube or plastic bottle
- vi. Pour resin and supernatant into column



vii. Wash

1. 7CV W1 buffer (25mM Hepes pH7.5; 800mM NaCl; 10% glycerol; 0.1% DDM; 0.01% CHS; 50uM carazolol; 5mM Imid; 10mM MgCl<sub>2</sub>; 8mM ATP)
2. 4 CV W2 buffer (25mM Hepes pH7.5; 150mM NaCl; 10% glycerol; 50uM carazolol; 0.05% DDM; 0.01% CHS; 5mM Imid)

viii. Elute

1. 10mL of W2 buffer (25mM Hepes pH7.5; 150mM NaCl; 10% glycerol; 50uM carazolol; 0.05% DDM; 0.01% CHS; 200mM Imid)

ix. Desalt

1. Equilibrate a PD-10 column with PD-10 buffer (25mM Hepes pH7.5; 150mM NaCl; 10% glycerol; 50uM carazolol; 0.05% DDM; 0.01% CHS)
2. Concentrate or split talon elute to 2.5ml volume add elute to enter the column by gravity
3. Add 3.5ml PD-10 buffer collect elute

*IMAC, Second Pass, Ni Sepherose concentration and PNGase treatment*

- d. Bind PD-10 eluent to 200uL (or less) Ni Seph resin add 20ul PNGase (NEB) batch bind for 4.5hr
- e. Wash with 4 CV W2 buffer (25mM Hepes pH7.5; 150mM NaCl; 50uM carazolol; 0.05% DDM; 0.01% CHS; 10mM Imid)
- f. Elute with 10mL of W2 buffer (25mM Hepes pH7.5; 150mM NaCl; 0.05% DDM; 0.01% CHS; 200mM Imid)

*Quality Check and Protein Characterization*



### Purification protocol for Adenosine 2A Receptor (A2A312)

#### *Membrane Extraction and Solubilization*

- a. Membrane treatment
  - i. Dilute to 25mL with lysis buffer
  - ii. Add Theophylline 4mM
  - iii. Add iodoacetamide (2mg/mL)
  - iv. Add protease inhibitors
    1. 1 vial calbiochem V/50ml tube
    2. Bestatin 20ug/ml
    3. Leupeptin 20ug/ml
    4. Pefablock 20ug/ml
  - v. Incubate at 4 degrees 20-30minutes
- b. Solubilization
  - i. Add 25 mL of 2x solubilization buffer
    1. 100mM Hepes pH 7.5; 1.5M NaCl; 1% DDM; 0.2% CHS
    2. Final concentration: 50mM Hepes 800mM NaCl 0.5% DDM; 0.1% CHS
  - ii. Solubilize for 2.5 hours at 4 deg
  - iii. Centrifuge and isolate supernatant

#### *IMAC Purification, First Pass*

- c. Talon binding (Superflow)
  - i. Equilibrate talon resin with water
    1. Use 1mL resin per 50mL solubilization
  - ii. Bring NaCl to 800mM
  - iii. Add Imidazole to 5mM final concentration
  - iv. Add leupeptin
  - v. Add 1.5mL of 50% talon slurry in water to each 50mL falcon tube
    1. Rock slowly or stir slowly for 6hr-overnight batch bind in falcon tube or plastic bottle
  - vi. Pour resin and supernatant into column
  - vii. Wash
    1. 10CV W1 buffer (25mM Hepes pH7.5; 800mM NaCl; 10% glycerol; 0.1% DDM; 0.01% CHS; 4mM Theophylline; 60mM Imid)
    2. 4 CV W2 buffer (25mM Hepes pH7.5; 800mM NaCl; 10% glycerol; 4mM Theophylline; 0.05% DDM; 0.01% CHS; 25mM Imid, 10mM MgCl<sub>2</sub>; 8mM ATP)
  - viii. Elute
    1. 10mL of W2 buffer (25mM Hepes pH7.5; 800mM NaCl; 10% glycerol; 4mM Theophylline; 0.05% DDM; 0.01% CHS; 10mM MgCl<sub>2</sub>, 10mM ATP)

ix. Desalt

1. Equilibrate a PD-10 column with PD-10 buffer (25mM Hepes pH7.5; 800mM NaCl; 10% glycerol; 4mM Theophylline; 0.05% DDM; 0.01% CHS)
2. Concentrate or split talon elute to 2.5ml volume add elute to enter the column by gravity
3. Add 3.5ml PD-10 buffer collect elute

*IMAC, Second Pass, Ni Sepherose concentration and PNGase treatment*

- d. Bind PD-10 eluent to 200uL (or less) Ni Seph resin add 20ul PNGase (NEB) batch bind for 4.5hr
- e. Wash with 10 CV W2 buffer (25mM Hepes pH7.5; 800mM NaCl; 4mM Theophylline; 10% glycerol, 0.05% DDM; 0.01% CHS; 60mM Imid)
- f. Elute with 10mL of EI buffer (25mM Hepes pH7.5; 800mM NaCl; 4mM Theophylline, 10% glycerol 0.05% DDM; 0.01% CHS; 200mM Imid)

*Quality Check and Protein Characterization*

**References**

1. Cherezov V., Rosenbaum D.M., Hanson M.A., Rasmussen S.G., Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC., High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science*. (2007) **318**(5854):1258-65
2. Hanson MA, Cherezov V, Griffith MT, Roth CB, Jaakola VP, Chien EY, Velasquez J, Kuhn P, Stevens RC.. "A specific cholesterol binding site is established by the 2.8 Å structure of the human beta2-adrenergic receptor.", *Structure*. 2008Jun;**16**:897-905.
3. Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, Ijzerman AP, Stevens RC., "The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist.", *Science*. (2008);**322**:1211-7

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