

**SOP: PP024.2**  
**Modified 5-23-2008**

**Purification of PhoS1 (38kDa protein) from the CFP**

**Materials and Reagents:**

1. Supernatant from 40% ammonium sulfate cut of CFP
2. Ammonium sulfate
3. Endotoxin-free water
4. Dialysis buffer (10mM ammonium bicarbonate, 0.02% NaN<sub>3</sub>)
5. Concanavalin A-Sepharose 4B resin (Sigma, C9017)
6. ConA Binding Buffer: 50mM KH<sub>2</sub>PO<sub>4</sub>, 500mM NaCl, 1mM each of MgCl<sub>2</sub>, CaCl<sub>2</sub>, MnCl<sub>2</sub> and DTT (note 1)
7. ConA Elution Buffer: Binding buffer with 0.5 M Methyl  $\alpha$ -D-mannopyranoside (note 2)
8. 5 ml HiTrap phenyl sepharose column
9. Phen Seph Buffer A: 1M ammonium sulfate, 50mM Sodium Phosphate, 0.02% NaN<sub>3</sub>, 1mM DTT, pH 7.4 (note 3)
10. Phen Seph Buffer B: 50mM Sodium Phosphate, 0.02% NaN<sub>3</sub>, 1mM DTT, pH 7.4 (note 4)
11. 12 x 75 mm disposable culture tubes
12. Sorvall SS34 rotor
13. Polypropylene oak ridge tubes
14. Dialysis tubing, 3500 MWCO
15. Dialysis tank
16. Biorad Econopump chromatography system
17. Sorvall centrifuge

**Protocol:**

1. \_\_\_\_\_ Perform a 70% ammonium sulfate cut on the 40% supernatant (see SOP: PP020, steps 1-3) by adding 0.205 g ammonium sulfate per 1 ml of total volume.
2. \_\_\_\_\_ Stir at room temperature until the ammonium sulfate goes into solution, then incubate at 4°C with stirring overnight.
3. \_\_\_\_\_ Centrifuge at 27,000 xg, 4°C for 1 hour to recover the pellet.
4. \_\_\_\_\_ Resuspend the pellet in 10 mM ammonium bicarbonate, and dialyze against 7L ammonium bicarbonate to remove residual salt.
5. \_\_\_\_\_ Quantitate protein by BCA (see SOP: SP003), and lyophilize.
6. \_\_\_\_\_ Into an open column, pour a volume of Con-A resin which will give a ratio of 1 ml packed resin: 2 mg protein (note 5).
7. \_\_\_\_\_ Pack and equilibrate the column with 3-5 column volumes (CV) ConA Binding Buffer at a flow rate of 2 ml/min using the Biorad Econopump (note 6).
8. \_\_\_\_\_ Resuspend the dried sample in ConA Binding Buffer at a concentration of 1 mg/ml.
9. \_\_\_\_\_ Load the sample onto the column at 1 ml/min and pass the flow through back over the column. Collect as "ConA Flow Thru".
10. \_\_\_\_\_ Run the following gradient at 1.5 ml/min:
  - 3 CV Binding Buffer Wash
  - 3 CV 0→40% Elution Buffer (0.2M Methyl  $\alpha$ -D-mannopyranoside)
  - 1 CV 40% Elution Buffer hold
  - 2 CV 100% Elution Buffer Clean-up

Collect the Wash as “ConA Wash”, then collect 50 fractions starting at the gradient, and collect the final clean-up as “ConA Clean-Up”.

11. \_\_\_\_ Run 10  $\mu$ l of each fraction on a gel.
12. \_\_\_\_ Pool all of the fractions that are predominantly 38kDa (note 7).
13. \_\_\_\_ Concentrate the pool using an amicon ultra-15 and wash three times with 10mM ammonium bicarbonate.
14. \_\_\_\_ Quantitate by BCA and lyophilize.
15. \_\_\_\_ Suspend lyophilized ConA pool in buffer A at a concentration of 1 mg/ml and stir at 4°C overnight.
16. \_\_\_\_ Pump 25 ml (5 CV) of endotoxin-free water through the HiTrap column at 1 ml/min to elute the storage buffer.
17. \_\_\_\_ Pump 25 ml of buffer A through the column at 1 ml/min to equilibrate the column.
18. \_\_\_\_ Centrifuge the resuspended sample at 3000 rpm, 4°C, for 15 minutes to remove any precipitate.
19. \_\_\_\_ Pump the sample (supernatant) onto the column at a flow rate of 1 ml/min, then pass the flow through back over the column. Collect as “Phen Seph Flow Thru”.
20. \_\_\_\_ Run the following gradient:
  - 2 CV Buffer A Wash (10 min)
  - 20 CV A→B Gradient (100 min)
  - 10 CV Buffer B Clean-Up (50 min)Collect the wash as “Phen Seph Wash”, then collect 40 x 2.5 min fractions during the gradient. Collect the final clean up as “Phen Seph Clean-Up” (note 8).
21. \_\_\_\_ Run 10  $\mu$ l of each fraction on a gel.
22. \_\_\_\_ Pool all fractions containing clean 38kDa.
23. \_\_\_\_ Concentrate using amicon ultra-15 centrifugal device and wash three times with 10mM ambic.
24. \_\_\_\_ Run BCA, gel, and blot using IT-23 antibody, for QC.
25. \_\_\_\_ Make aliquots (default quantity = 0.25 mg) and store at -80°C.

**Notes:**

1. Con A Buffer:

To 80 ml of endotoxin-free water stirring on a stirplate, add the following:

KH <sub>2</sub> PO <sub>4</sub>	0.69 g
NaCl	2.92 g
MgCl <sub>2</sub> ·6H <sub>2</sub> O	20.3 mg
CaCl <sub>2</sub> ·2H <sub>2</sub> O	14.7 mg
MnCl <sub>2</sub> ·4H <sub>2</sub> O	19.8 mg
DTT	15.4 mg

After all reagents have gone into solution, add NaOH dropwise until the pH is 5.7. Transfer to a graduated cylinder and bring to final volume of 100 ml with endotoxin-free water.

2. Con A Elution Buffer: Dissolve all the above reagents in 70 ml of endotoxin-free water plus Methyl  $\alpha$ -D-mannopyranoside 9.71 g. Titrate to pH = 5.7 and bring to final volume of 100 ml.

3. Buffer A:

To 70 ml of endotoxin-free water stirring on a stir plate, add the following reagents:

ammonium sulfate	13.22 g
NaH <sub>2</sub> PO <sub>4</sub>	0.114 g
Na <sub>2</sub> HPO <sub>4</sub>	1.0865 g
NaN <sub>3</sub>	20 mg
DTT	15.3 mg

When all reagents have gone into solution, titrate to pH = 7.4. Bring final volume to 100 ml with endotoxin-free water.

#### 4. Buffer B:

To 70 ml of endotoxin-free water which is stirring on a stir plate, add the following reagents:

NaH <sub>2</sub> PO <sub>4</sub>	0.114 g
Na <sub>2</sub> HPO <sub>4</sub>	1.0865 g
NaN <sub>3</sub>	20 mg
DTT	15.3 mg

When all reagents have gone into solution, titrate to pH=7.4. Bring final volume to 100 ml with endotoxin-free water.

5. Measure approximately 1.5 ml resin slurry for every 1 ml of desired packed resin. For large preparations of CFP, it may be necessary to run more than one column, due to the amount of time necessary to run large columns.
6. The column can be packed and equilibrated the day before use, as long as there is sodium azide present in the buffer.
7. It may be necessary to run some fractions on western blots developed with IT-23 as well as  $\alpha$ -1411c. This will tell you which fractions contain 38kDa, but not Rv1411c (which can be difficult to remove after ConA).
8. To clean the column for storage, pump 5 column volumes of water, then 5 column volumes of 20% ethanol. Cap both ends of the column and store at 4°C.