

SOP: PP022**Purification of MPT32 Protocol****Materials and Reagents:**

1. Mannosylated CFP pool or mannosylated flow through from the Ag85 phenyl sepharose (flow through that has been run over a Con-A column, SOP PP019)
2. Biorad Econopump chromatography system with fraction collector
3. Buffer A: 50mM NaH₂PO₄, 1.5M (NH₄)₂SO₄, 1mM DTT, pH 6.8 (note 1)
4. Buffer B: 50mM NaH₂PO₄, 1mM DTT, pH 6.8 (note 2)
5. Buffer C: 10mM Tris, 50% Ethylene Glycol, pH 8.9 (note 3)
6. 12 x 75 mm disposable culture tubes
7. Dialysis tubing, 3500 MWCO
8. Dialysis tank
9. Ammonium bicarbonate
10. 5 ml HiTrap phenyl sepharose column

Protocol:

1. ____ Pump 25 ml (5 column volumes) of Milli-Q water through the column at 1 ml/min to elute the ethanol.
2. ____ Pump 25 ml of buffer A through the column at 1 ml/min to equilibrate the column.
3. ____ Suspend lyophilized starting material in buffer A at a concentration of 1 mg/ml.
4. ____ Pump the sample onto the column at a flow rate of 1 ml/min, collecting the flow through into a plastic container.
5. ____ Pass the flow thru back over the column; collect into a clean container and label as Phenyl Sepharose Flow Thru.
6. ____ Pump 5 column volumes of buffer A onto the column. Collect into a clean container and label as Phenyl Sepharose Wash.
7. ____ Start elution gradient of 100% A to 100% B over 10 minutes at 2.5 ml/min, followed by 100% B for 10 minutes. Collect 2 minute fractions.
8. ____ Flush line A with buffer B and line B with buffer C. Continue elution gradient by running a gradient of 100% B to 100% C over 10 minutes, followed by 100% C for 10 minutes. Use the same flowrate and collection time as in step 7.
9. ____ Take 10 µl aliquots of the unbound material and every fraction collected during the elution gradient and run on SDS-PAGE.
10. ____ Make two pools. Pool one is all fractions containing the 45 kDa doublet, the other is the fractions which are not primarily MPT32 (note 4).
11. ____ Dialyze both pools against 10mM ammonium bicarbonate at 4°C with three buffer exchanges.
12. ____ After dialysis, freeze dry by lyophilization (note 5).
13. ____ Suspend 45kDa pool in the minimal volume of 10mM ammonium bicarbonate.
14. ____ Perform BCA assay, SDS-PAGE, and Western blot. Develop blot using CS93 primary antibody.
15. ____ Make aliquots of the 45kDa (default quantity is 0.5 mg), freeze dry by lyophilization, and store at -80°C.

Notes:

1. Buffer A:

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

ammonium sulfate	19.83 g
NaH ₂ PO ₄	0.69 g
DTT	15.3 mg

When all reagents have gone into solution, titrate to pH = 6.8 with NaOH. Bring final volume to 100ml with Milli-Q water.

2. Buffer B:

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

NaH ₂ PO ₄	0.69 g
DTT	15.3 mg

When all reagents have gone into solution, titrate to pH=6.8 with NaOH. Bring final volume to 100 ml with Milli-Q water.

3. Buffer C:

To 50 ml of ethylene glycol stirring on a stir plate add the following:

Tris Base	0.121 g
Milli-Q water	40 ml

When Tris is in solution, titrate to pH=8.9 with HCl. Bring to final volume of 100 ml with Milli-Q water.

4. This pool can be used for purification of other materials. After lyophilization, freeze this material and save for future use.

5. See SOP SP004 for use of the lyophilizer.

References:

Karen M. Dobos, et al. 1995. Evidence for Glycosylation Sites on the 45-Kilodalton Glycoprotein of *Mycobacterium tuberculosis*. *Infect. Immun.* 63(8): 2846-2853.

Karen M. Dobos, et al. 1996. Definition of the Full Extent of Glycosylation of the 45-Kilodalton Glycoprotein of *Mycobacterium tuberculosis*. *J. Bac* 178(9): 2498-2506.