SOP: PP027

Production of SapM

Materials and Reagents:

1. Crude CFP from *M. tuberculosis* H37Rv
2. Amicon ultrafiltration unit (See SOP: PP006)
3. 25 ml CM Sepharose HPLC column
4. CM Sepharose Buffer A: 20mM Tris, pH 7
5. CM Sepharose Buffer B: 20mM Tris, 1M NaCl, pH 7
6. Acid Phosphatase Assay Reagent: 100mM Sodium Acetate, 20mM Sodium Tartate, pH 6
7. 5 mg p-Nitrophenylphosphate (pNPP) tablets
8. BCA Reagents
9. SDS-PAGE and western blot supplies
10. 96 well Microtiter plates
11. Microplate reader
12. MT3409 – SapM antibody
13. Filter bell funnel with Pall membrane filter
14. Waters HPLC system
15. Amicon ultra-15, 10,000 MWCO centrifugal device
16. Benchtop centrifuge

Protocol:

1._____ Concentrate crude CFP on the amicon ultrafiltration unit as described in steps 1-13 of SOP: PP006.

2._____ When the volume of the concentrate is approximately 75 ml, fill the stirred cell with 800 ml buffer A (note 1).

3._____ Repeat step 2 twice more for a total of three washes.

4._____ When the volume is down to ~75 ml, remove the CFP from the amicon.

5._____ Perform a BCA on the sample to obtain the protein concentration (see SOP: SP003).

6._____ Perform Acid Phosphatase Assay and calculate specific activity (notes 2 and 3)

7._____ Run 5 μg on a silver stain gel and a western blot (note 4).

8._____ Set up HPLC and CM Sepharose column in the 4°C cold room (note 5).

9._____ Wash the column with filtered water, then equilibrate in buffer A (note 6).

10._____ Filter the sample through a 0.2 μm filter.

11._____ With the column running at 2 ml/min, inject the sample 10 ml at a time, allowing approximately 6 minutes between injections. Collect the flow through during the injections (note 7).

12._____ Run the column using the following parameters:

Flow rate = 2 ml/min
Column Volume (CV) = 25 ml

5CV	injection/ buffer A wash	63 min
10CV	A→50% B gradient	125 min
5CV	100% B clean up	63 min

13._____ Collect 63 fractions at 2 min/fraction during the gradient only.

14._____ Run 10 μl of each fraction on a gel.

15._____ Using the gel as a guide, perform the acid phosphatase assay on all of the fractions that appear to contain SapM.

16._____ Pool all fractions with activity.

17._____ Concentrate the pool using the amicon ultra-15 centrifugal device.

18._____ When the pool has concentrated down to approximately 1-2 ml, wash once with buffer A.

19._____ Run BCA, acid phosphatase assay, gel, and blot for QC (note 8).

Notes:

1. At this point, the 20L tank can be disconnected and the stirred cell hooked directly to the nitrogen tank.
2. Acid Phosphatase Assay:
    a. Add a 5mg pNPP tablet to 10ml of assay reagent. This is the substrate.
    b. Pipet 25 µg positive control (CFP), negative control (boiled CFP), and sample into individual wells of a microtiter plate. Be sure to also include a similar volume blank (buffer A).
    c. Add 200µl of substrate to each of those wells.
    d. Incubate at 37°C for 30 minutes.
    e. Read the plate at a wavelength of 405nm.
3. To calculate specific activity, take the absorbance (AU) being sure that the absorbance of the blank has been subtracted, multiply by 18.3 (conversion factor) and the volume (total volume in the well, including assay reagent), then divide by the incubation time and the number of milligrams (based on the BCA).

$$\text{AU} \times \frac{18.3\ \text{nmol}}{\mu\text{l} \times \text{AU}} \times \frac{1}{\text{time (min)}} \times \frac{\text{volume (µl)}}{1} \times \frac{1}{\text{amount (mg)}} = \text{nmol/min/mg}$$

4. See SOPs SP007, SP012, and SP011. Develop the blot using the MT3409 antibody as primary and α-rabbit as secondary.
5. Read SOP SP025 or talk to lab personnel trained in the use of the Waters HPLC before using the equipment.
6. All HPLC buffers MUST be filtered through a 0.45µm filter and degassed for at least 20 minutes before use. Degassing the buffers allows the HPLC to be used without a helium tank to sparge the buffers.
7. This material can be used for purification of Ag85 (SOP PP020 and PP021)
8. With a pure sample, the acid phosphatase assay can be run with 2 µg of sample, instead of the 25µg necessary for the crude material.