CAT ASSAY PROTOCOL

PROTOCOL #1

1. Wash plates 2x in PBS.
2. Scrape into microfuge tube in 1 ml PBS.
3. Spin out cells and aspirate off PBS.
4. Resuspend in 100 µl 0.25 M Tris pH 8. Vortex.
5. Freeze in dry ice/EtOH. *Can be stored at this point*.
6. When ready, freeze/thaw in 37°C (until thawed) to -70°C ice-bath, back and forth 3 times. On third time, freeze again at -70°C.
7. Let thaw at room temp.
8. Spin in cold microfuge 5 min. Leave pellet and extract in tube.
9. For protein determination:
   For each tube mix: 798 µl 50 mM tris pH 8
   2 µl extract
   200 µl Bradford dye
   Vortex.
   Read O.D. at 595 nm vs. blank (as above with no extract).
10. Read concentrations off standard curve.
    On Gregg Williams' standard curve, values are per µl; since we use 2 µl extract, divide number in half to get µg/ml.
11. Calculate amount needed for 100 µg and amount of 0.25 M tris pH 8 to bring total volume to 149 µl.
12. For each reaction: 149 µl extract in 0.25 M Tris 8.0
    1 µl ^{14}C chloramphenicol
    20 µl 4 mM Acetyl CoA T--6.6436 mg in 2 mls
    50 mM Tris pH 8 (or 3.3218 mg in 1ml)

    (MW = 809.6 + 20.85 (Li) = 830.49)

    Make rxn mix of labeled chloramphenicol & acetyl CoA for n + l; add 21 µl to each tube containing the Tris, then add extract last to start the rxn (on ice until start). Incubate at 37°C for 1 hour.
13. Add 1 ml cold ethyl acetate to each; vortex.
14. Spin 5 seconds in cold.
15. Take off top layer carefully (completely avoid interphase) into new tube.
16. Spin vac to dryness, ~1/2 hour.
17. For chromatography tank, mix in a beaker:
    95 ml chloroform
    5 ml methanol
    Carefully place in the bottom of the tank with a 25 ml pipette - do not splash onto sides of tank. Close tightly and let equilibrate while samples are drying down.
18. Resuspend pellets in 20 µl ethyl acetate and spot onto TLC sheet one inch from bottom. Place in tank and allow to chromatograph until liquid is ~2" from top.
19. Remove carefully, let dry to completeness in hood.
20. Place on film at room temperature, no screen.

PROTOCOL #2

1. Transfect cells with 5 µg plasmid DNA encoding activator, 5 µg reporter plasmid containing CAT gene, 2 µg internal control plasmid for transfection efficiency (e.g.plasmid encoding luciferase gene or B-galactosidase gene) and 8 µg of carrier DNA.
2. Harvest cells within 48 hrs after transfection and freeze cell pellets quickly in dry ice. Then store the cell pellets at -80°C overnight or lyse the cells by adding 4 volumes of buffer C to cell pellets, put on ice for 10 min , spin 13,000 rpm 15 min, move the supernatant to a new tube and freeze rapidly in dry ice/methanol. Store at -80°C or do a BioRad protein assay directly.
3. Combine 1 µl 25 µCi/ml [^{14}C] chloramphenicol, 20 µl 4 mM acetyl CoA and 32.5 µl 1M Tris.Cl, pH 7.4. Add 12.5 µg cell extract and add ddH_2O to 150 µl, mix and incubate at 37°C for 1 hr.
4. After incubation add 1 ml ethyl acetate to the reaction and vortex. Spin 1 min at 4°C, transfer top layer to a new tube, dry in Speed-vac for 30 min, and resuspend in 15 ml ethyl acetate.
5. Spot samples evenly on a TLC plate and develop in a chromatography tank containing 19:1 of chloroform : methanol. The tank should be equilibrated 2 hrs prior to developing. Run the sample until the solvent is close to the top of the plate.
6. Remove TLC plate from the tank , air dry and expose to X-ray film at room temperature. No intensifying screen is needed.

Reference: Gorman, C.M., Moffat, L.F., and Howard, B.H. , 1982, MCB 2: 1044-1051