BASIC PROTOCOL

Harvest and Assay for Chloramphenicol Acetyltransferase

Cells are harvested 48 to 72 hr after transfection and extracts are made. The extracts are incubated with radioactive chloramphenicol and acetyl-CoA. The acetylated chloramphenicol is separated from the unacetylated form by thin-layer chromatography.

Materials

Phosphate-buffered saline (PBS; *APPENDIX 2*)
TEN solution
Ice-cold 0.25 M Tris·Cl, pH 7.5
[^{14}C]chloramphenicol (200 μCi/ml, 35 to 55 mCi/mmol)
4 mM acetyl-CoA (store at –20°C for 2 weeks only)
Ethyl acetate
19:1 chloroform/methanol

Policeman
Thin-layer chromatography (TLC) tank
Whatman 3MM filter paper
TLC sheets (J.T. Baker #4-4462, silica gel 1B)
Pen or marker with radioactive ink

Harvesting cells

1. Wash cells in 10-cm dishes twice with 5 ml PBS. Add 1.0 ml TEN solution to each dish. Let cells sit for 5 min on ice.

   The TEN solution helps adherent cells lift off the dish. If cells in suspension are used, simply wash the cells once with PBS and once with TEN solution, collecting each time by centrifugation, then proceed to step 3.

2. Scrape the cells off the dish with a policeman. Transfer them to a microcentrifuge tube on ice.

3. Spin the cells 1 min in a microcentrifuge.

   Do not spin the cells too long or they will form a pellet that is difficult to resuspend.

4. Resuspend the cell pellet in 100 μl ice-cold 0.25 M Tris·Cl, pH 7.5.

   One hundred microliters of solution is sufficient for ~10^7 cells.

5. Freeze the cells in dry ice/ethanol for 5 min. Transfer to 37°C and thaw for 5 min. Repeat this freeze-thaw protocol twice more, for a total of three freezes and thaws.

   Successive freezing and thawing lyses the cell membrane.

6. Cool the cell lysate on ice, then spin 5 min in a microcentrifuge at 4°C. Remove and save the supernatant.

   The supernatant is a cytoplasmic extract and contains the CAT enzyme. The supernatant can be stored by freezing in dry ice/ethanol and storing at –20°C. It is important to freeze the supernatant rapidly to maintain full activity.

Assaying cells

7. To assay 20 μl of cell extract, make the following cocktail (per reaction):

   2 μl [^{14}C]chloramphenicol (200 μCi/ml, 35 to 55 mCi/mmol)
   20 μl 4 mM acetyl-CoA
   32.5 μl 1 M Tris·Cl, pH 7.5
   75.5 μl H_2O

   1 to 50 μl of extract can be assayed. When assaying different amounts of extract, adjust the cocktail so that the final concentration of Tris·Cl is 0.25 M and the final volume of each reaction is 150 μl.

8. For each assay, add 130 µl cocktail to a microcentrifuge tube, add 20 µl extract, and mix gently. Incubate 1 hr at 37°C.

The assay time can be extended to several hours if very little activity is present. If a long assay is performed, it is important to replace the 4 mM acetyl-CoA with 40 mM acetyl-CoA. This is because acetyl-CoA is not stable under the assay conditions. It is critical that the assay be done in the linear time range. This can be verified by setting up a 600-µl reaction, removing 145-µl aliquots at various time points, and analyzing conversion to acetylated chloramphenicol, as in steps 9 to 14. A plot of activity versus time must be linear through the time point chosen for routine assay in order for the assay to be valid.

9. Add 1 ml ethyl acetate to the reaction and vortex. Spin 1 min in a microcentrifuge and remove the top (ethyl acetate) layer.

The chloramphenicol and acetylated chloramphenicol extract into the ethyl acetate.

10. Dry the ethyl acetate down in a Speedvac evaporator (Savant) for 45 min.

Alternatively, the ethyl acetate will evaporate off overnight in a fume hood. This sometimes leads to extra spots on the final TLC because of breakdown products.

11. Resuspend each sample in 30 µl ethyl acetate.

12. Spot the sample 5 µl at a time on a spot 2 cm above the edge of a TLC plate.

13. Develop the thin layer in a chromatography tank containing 200 ml of 19:1 chloroform/methanol (vol/vol). The tank should be equilibrated 2 hr prior to adding the thin layer. Add 190 ml chloroform, 10 ml methanol, and a piece of filter paper (approximately the size of the thin layer) to the tank to equilibrate.

It is important to use fresh chloroform and methanol for each assay.

14. Allow the chromatography to run 2 hr. Remove the TLC sheet and air dry. Mark the TLC sheet with radioactive ink, cover with plastic wrap, and place on film for autoradiography (*APPENDIX 3*).

The final autorad will have up to five spots for each sample. They are, in ascending order, a weak spot at the origin, nonacetylated chloramphenicol, the two forms of acetylated chloramphenicol, and diacetylated chloramphenicol (Fig. 9.6.2). If the diacetylated spot is present, the assay is out of the linear range. The activity of an extract is usually calculated by determining the percentage of counts that are in the monoacetylated chloramphenicol species. To determine the amount of label in each spot, first align the TLC and the autoradiogram using a light box and the marks made with radioactive ink. Next, outline the area of each spot on the TLC using a pencil, cut out the piece of TLC, add the piece to scintillation fluid in a scintillation vial and count in a scintillation counter. The activity is calculated as follows: percent acetylated = (counts in acetylated species)/(counts in acetylated species + counts in nonacetylated chloramphenicol).

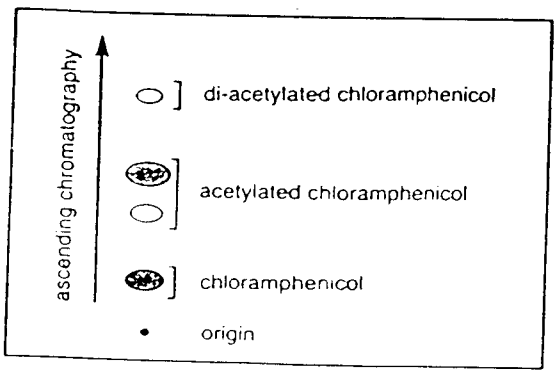

Figure 9.6.2 Schematic depiction of a CAT assay.