

2. The reaction mixture is incubated at 37°. Incubation times of 30–60 min are routine, but longer incubation can give higher signals particularly if an additional aliquot of acetyl CoA solution is added after the first hour. For definitive comparisons of CAT activity between different transfections, it is imperative that a time course study be done on each reaction, since it is the kinetics rather than the final percentage conversion that is the true measure of enzyme activity. This is easily done by removing aliquots from the reaction mixture at set time points and proceeding directly to the extraction procedure (step 3) to stop enzyme activity.

3. At the end of the reaction time, [¹⁴C]chloramphenicol products are extracted with two successive 0.5-ml aliquots of ethyl acetate, which are pooled and evaporated under vacuum. The lower aqueous phase is discarded.

4. The reaction products are redissolved in 30 μ l ethyl acetate immediately before application to a 25-mm silica TLC plate. A 10 μ l aliquot spotted on the plate is usually sufficient.

5. Chromatography is carried out in chloroform–methanol (95:5) in a closed tank.

6. The plate is then dried and autoradiographed. Because chloramphenicol has two potential acetylation sites, two acetylated forms are produced by the enzyme reaction, running faster than the unacetylated form. A third higher spot corresponds to double acetylation and is seen only in conditions of high CAT activity. After autoradiography the spots can be cut out from the plate and counted to obtain exact conversion values.

β -Galactosidase Assay

Extract Preparation. Cells are harvested according to the protocol given for the CAT assay above, but step 5 is eliminated because the 60° incubation inactivates the enzyme. If this assay is to be used as a normalizing control, a portion (10–30%) can be reserved, and the rest of the extract can be frozen at –20° or tested immediately for CAT activity after treatment at 60°.

Enzyme Assay usually 20 μ l

1. The following stocks are prepared: 100 \times magnesium buffer (100 mM MgCl₂, 5 M 2-mercaptoethanol); 0.1 M sodium phosphate buffer (adjust 100 ml of 0.1 M Na₂HPO₄ to pH 7.3 at 37° using 0.1 M NaH₂PO₄); ONPG substrate solution [ONPG (*o*-nitrophenyl- β -D-galactopyranoside), 4 mg/ml in sodium phosphate buffer].

2. The following reagents are combined: 10–150 μ l cell extract, 3 μ l 100 \times magnesium buffer, 66 μ l ONPG solution, and sodium phosphate buffer to a final volume of 300 μ l.

3. The reaction mix is incubated at 37° for 30 min or until a yellow color is visible. The reaction is stopped by adding 0.5 ml of a 1 M Na₂CO₃ solution, and the color reaction is measured in a spectrophotometer at 410 nm.

A positive control for the enzyme assay can be run using commercially available β -galactosidase. The cell lysate in the above reaction is then substituted with 1 μ l of a 100 μ g/ml enzyme solution (in sodium phosphate buffer).

Additional Applications of Expression Assays

Once a promoter and/or enhancer element controlling transcription of a given gene is defined by functional assays in cell culture, the transacting factors required for this activity must be identified. Ideally an *in vitro* assay in which expression could be reconstituted would allow purification of transcription factors from nuclear extracts. Although several *in vitro* expression assays have been reported,^{54,55} none of these studies has succeeded in mimicking the dramatic effects on transcription (up to several orders of magnitude) that enhancers exhibit *in vivo*. To date, various techniques for the detection of protein–DNA binding *in vitro* are available (see this volume [73]). These assays are powerful tools for the isolation of cellular factors which bind with high affinity to specific gene control sequences, yet they can only correlate binding with functional activity. Of obvious importance would be a functional assay in which putative transacting molecules, with specific affinity for known regulatory elements could be tested.

In Vivo Competition

In this approach, the binding of regulatory sequence-specific factors is scored indirectly by an *in vivo* competition assay. The principle of this technique is to coinfect in a transient assay a test construct, usually a reporter gene driven by the regulatory sequences under study, together with an excess of plasmids harboring copies of identical or different regu-

⁵⁴ P. Sassone-Corsi, J. Dougherty, B. Waslyk, and P. Chambon, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 308 (1984).

⁵⁵ H. Schöler, U. Schlokot, and P. Gruss, in "Eukaryotic Transcription," *Cold Spring Harbor Curr. Commun.*, p. 101 (1985).

Include
negative
control \rightarrow

usually 20 μ l