Comparison of ELISA and TLC for the Analysis of Promoter Activity When Using the CAT Reporter Gene

BioTechniques 21:1033-1036 (December 1996)

The chloramphenicol acetyltransferase (CAT) gene is the most commonly used reporter gene for the indirect analysis of promoter activity in transfected cells. Enzyme activity, as opposed to enzyme concentration, has traditionally been measured to quantitate promoter strength. CAT activity is generally measured by incubating lysates from transfected cells with [^{14}C] chloramphenicol and acetyl CoA. The CAT enzyme, present in the lysates, modifies [^{14}C]chloramphenicol by mono- and diacetylation using acetyl CoA as the substrate. The acetylated and non-acetylated forms of chloramphenicol are then separated by thin-layer chromatography (TLC) on silica gels. Although this method is highly sensitive, it involves radioactive materials, organic solvents and time-consuming manipulations. These conditions make the analysis of a large number of samples both tedious and costly.

Enzyme-linked immunosorbent assay (ELISA) kits are available commercially to detect CAT protein, thus making it possible to measure enzyme concentration in lysates from transfected cells. The use of ELISA methodology has several advantages not offered by the standard TLC method. The ELISA procedure does not require radioactive materials or organic solvents, and it can be performed in a 96-well format. These advantages allow the analysis of a large number of samples in a manner that saves cost and time. However, the ELISA systems may not have the sensitivity required for the study of weak promoters as provided by the TLC method. In the study presented here, we directly compared the standard CAT TLC method with a commercially available CAT ELISA kit using both purified CAT protein and lysates from transfected cells.

Various amounts, 1.25–160 pg, of authentic CAT protein (Boehringer Mannheim, Indianapolis, IN, USA) were analyzed in both assays, TLC and ELISA. CAT enzyme activity was determined according to the traditional CAT TLC method of Gorman et al. (2). Briefly, CAT protein was diluted to 225 µL with 0.25 M Tris-HCl, pH 7.8, containing 4 mM acetyl CoA (Boehringer Mannheim) and 0.5 µCi [^{14}C]chloramphenicol (NEN Life Science Products, Boston, MA, USA). After incubating at 37°C overnight, 800 µL of ethyl acetate were added to stop the reaction and to

extract the acetylated and non-acetylated forms of chloramphenicol. The chloramphenicol species were then separated by ascending TLC in a chamber saturated with a chloroform:methanol (95:5) solvent. The chloramphenicol species were quantitated with a PhosphorImager™ SI (Molecular Dynamics, Sunnyvale, CA, USA), and the percentage of acetylation was calculated by dividing the quantity of acetylated chloramphenicol by the sum of all chloramphenicol species. As seen in Figure 1A, the TLC method could not differentiate the activity from 1.25 or 2.5 pg of CAT protein from that of the control sample (buffer only); however, the activity from 5 pg and more CAT protein was significantly greater than that of the control.

The amount of CAT protein was measured with an ELISA kit (Boehringer Mannheim). Each sample of CAT protein (1.25–160 pg) was dissolved in sample buffer to 200 µL, and this solution was then transferred to a microplate well containing CAT antibody and incubated for 2 h at 37°C. The remainder of the procedure was performed according to the manufacturer's recommendations until the final step when substrate and enhancer were added to the wells and incubated at room temperature for 15 min. The

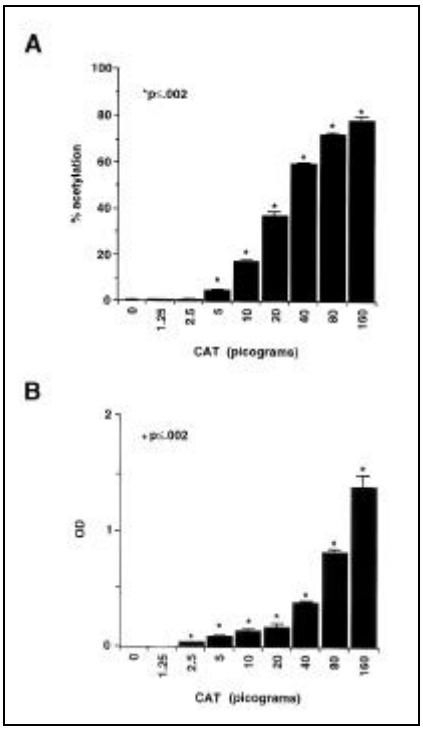

Figure 1. Comparison of the ability of two methods (A) TLC and (B) ELISA to detect authentic CAT protein. Various amounts of CAT protein, 1.25–160 pg, were analyzed. (A) CAT protein samples were diluted in 0.25 M Tris-HCl, pH 7.8, containing 4 mM acetyl CoA and 0.5 µCi [^{14}C]chloramphenicol. Samples were then analyzed according to traditional CAT TLC method of Gorman et al. (2).Values shown represent average (mean +/- SE) of three separate determinations. To determine if a sample had activity significantly greater than that of the control (0 protein), a one-sided Student's *t*-test was performed using the statistical software program JMP Version 3.0 (SAS Institute, Cary, NC, USA). A value equal to or less than 0.002 was considered significant. (B) CAT protein samples were diluted in sample buffer, and the ELISA procedure was performed as described in the text. Values shown represent the average (mean +/- SE) of four separate determinations. Statistical analyses were performed as described above.

absorbance of each sample was then measured at 405 nm with the Model MR5000™ Microplate Reader (Dynatech Laboratories, Chantilly, VA, USA). As seen in Figure 1B, 2.5 pg and more of CAT protein were detected as being significantly greater than the control (buffer only).

To compare the two assays in a more experimental setting, the strength of a human, phorbol ester responsive promoter was analyzed using lysates from transfected cells. Bovine retinal endothelial (BRE) cells were seeded at a density of 10^6 cells/100-mm culture dish. Twenty-four hours after seeding, 8 μg of -1200/+63 CAT (1), a plasmid containing the human collagenase promoter linked to the CAT gene (kindly provided by Hans Jobst Rahmsdorf), was introduced into the cells using the Transfectam® reagent (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Four hours after transfection, fresh culture medium (basal conditions) or medium containing 50 nM 4-β phorbol 12,13-dibutyrate (PDBu) (stimulated conditions) was placed on the cells. Forty-eight hours later, cellular lysates were prepared by three consecutive cycles of freeze/thaw. When the lysates were tested for CAT activity, according to the TLC method, the percentage of acetylation was 4.6 and 16.5 for lysates prepared from cells grown in the absence (basal) and presence of 50 nM PDBu (stimulated), respectively. When these same lysates were analyzed using the ELISA kit, the concentration of CAT protein was 4.2 pg (basal) and 12.4 pg (stimulated) as determined by extrapolation from a standard curve prepared with CAT protein supplied in the kit. The relative fold increases in CAT expression due to PDBu stimulation were determined (results from the stimulated samples were divided by those from the basal samples). The fold increases in CAT expression are very similar, 3.6 (TLC) and 3.0 (ELISA), indicating that the final results are nearly identical when the TLC and ELISA methods are used to analyze promoter strength.

The data presented in this study demonstrate that the traditional TLC method and a commercially available ELISA kit have nearly the same degree of sensitivity when used to analyze authentic CAT protein or CAT protein present in lysates from transfected cells. These findings, in combination with the advantages of the ELISA methodology (absence of radioactive materials and organic solvents and ease of the 96-well format), indicate that a commercially available ELISA kit is a viable alternative to the traditional TLC method for the analysis of promoter strength when using CAT as the reporter gene.

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Received 1 December 1995; accepted 24 May 1996.

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Designing Positive Internal Controls for Mutation Detection Gels

BioTechniques 21:1036-1038 (December, 1996)

Positional cloning of specific genes is ultimately dependent upon finding mutations in order to identify the gene being sought. Mutation detection methods, such as single-strand conformation polymorphism (SSCP) (3) and conformation sensitive gel electrophoresis (CSGE) heteroduplex formation (1), are used to identify mutations. To maximize the sensitivity of these detection methods, the conditions used for screening each DNA fragment must be optimized. This is routinely done when screening known disease genes. However, for unknown gene fragments, it is not possible to determine the best conditions without having a mutant strand as a positive control to show that the resolution of the gel is sensitive enough to detect such mutations. A confident negative result (i.e., no mutations found) is extremely important when screening unknown transcripts for positional cloning of genes.

Here we describe an easy and inexpensive method to introduce subtle mutations into targeted DNA fragments. These mutated DNA fragments can then be used as positive controls to optimize gel conditions. The subtleness of the mutation created directly reflects the degree of resolution attainable by the gel within a specific fragment.

When designing a polymerase chain reaction (PCR) primer pair to amplify the DNA region being screened, a third primer is also designed, which contains a selected point mutation. This mutated primer is then used in conjunction with the normal primer of that set to PCR-amplify normal control DNA. The amplified DNA becomes the positive control to optimize mutation detection for that segment. The altered DNA fragment is a stringent control since the mutation created can be very subtle, such as a single nucleotide change near the edge of the DNA fragment being screened. In fact, it should interfere very little in the three-dimensional folding pattern of SSCP or in the bending created by the flexing of heteroduplex DNA strands.

A pair of primers was designed for a 225-bp region, NS6, using the PRIMERS computer software program by the Whitehead Institute/MIT (Cambridge, MA, USA): NS6F (AGAGGTGGGTAGGGCAGG) and NS6R (TCTCCATGGCACT***C***TCTGG). In addition, mutated primers were synthesized: NS6Rm1 (TCTCCATGGCACT***G***TCTGG), NS6Rm2 (TCTCCATGGCACT***T***TCTGG) and NS6Rm3 (TCTCCATGGCACT***A***TCTGG). All the mutated primers contained a point mutation at position fourteen of the