

FAST CAT[®] (deoxy) Chloramphenicol Acetyltransferase Assay Kits

F-6616 FAST CAT[®] Green (deoxy) Chloramphenicol Acetyltransferase Assay Kit, 100 assays

F-6617 FAST CAT[®] Yellow (deoxy) Chloramphenicol Acetyltransferase Assay Kit, 100 assays

Quick Facts

Storage upon receipt:

- -20°C
- Desiccate
- Protect from light

Abs/Em of reaction products: See Table 1

Introduction

The close correlation between chloramphenicol acetyltransferase (CAT) gene transcript levels and enzymatic activity that is amenable to sensitive quantitation has made it a powerful genetic reporter for investigating transcriptional elements in animal^{1,2} and plant cells.³ The CAT reporter gene system has also found application in transgenic animals,⁴ as well as plant and animal cells infected with recombinant viruses.^{5,6}

Most conventional CAT assays require incubation of cell extracts with radioactive substrates, typically [¹⁴C]-chloramphenicol or [¹⁴C]-acetyl CoA, followed by autoradiography and densitometry or scintillation counting analysis.^{7,8} The fluorescent CAT substrates in Molecular Probes' FAST CAT[®] Green and Yellow (deoxy) Kits (F-6616, F-6617), as well as in our original FAST CAT Kit (F-2900),⁹⁻¹¹ offer safer, less expensive and more convenient alternatives to radioactive methods. In addition, our fluorescent CAT assays provide rapid, easily quantitated results that can be immediately visualized without the use of film or scintillant.

A complicating feature in quantitative analysis of CAT activity using either [¹⁴C]-chloramphenicol or the BODIPY[®] FL chloramphenicol substrate in our original FAST CAT Kit is the multiplicity of reaction products formed by enzymatic reaction. Chloramphenicol contains two acetylation sites, only one of which is acetylated by the CAT enzyme. Once the CAT enzyme acetylates the 3-hydroxyl position, the acetyl group is nonenzymatically transferred to the 1-hydroxyl position, leaving the original site available for another enzymatic acetylation.¹²⁻¹⁵ Thus, CAT-mediated acetylation of our BODIPY FL chloramphenicol substrate produces three products — two monoacetylated and one diacetylated chloramphenicols. Moreover, because the nonenzymatic transacetylation is the rate-limiting step, the rate of product accumulation may not accurately reflect CAT activity.^{12,15,16}

To circumvent this complication, Molecular Probes offers FAST CAT Green and Yellow (deoxy) kits incorporating BODIPY 1-deoxychloramphenicol substrates, which undergo a single acetylation reaction, yielding only one acetylated product (Figure 1 and 2). Thus the rate of acetylation is a direct function of enzymatic activity. The simplified reaction scheme provides a straightforward and reliable measure of transcription and extends the linear range of enzymatic activity detection to over three orders of magnitude.¹⁷ This feature is especially beneficial for measuring high levels of CAT activity that might result, for example, from transcription by a strong promoter. One of these substrates (the BODIPY FL derivative in our FAST CAT Green (deoxy) Kit) is spectrally identical to our original FAST CAT substrate can be excited with the 488 nm spectral line of the argon-ion laser; the second (the BODIPY TMR derivative in our FAST CAT Yellow (deoxy) Kit) can be excited with the 543 nm spectral line of the green He-Ne laser. BODIPY FL deoxychlor-

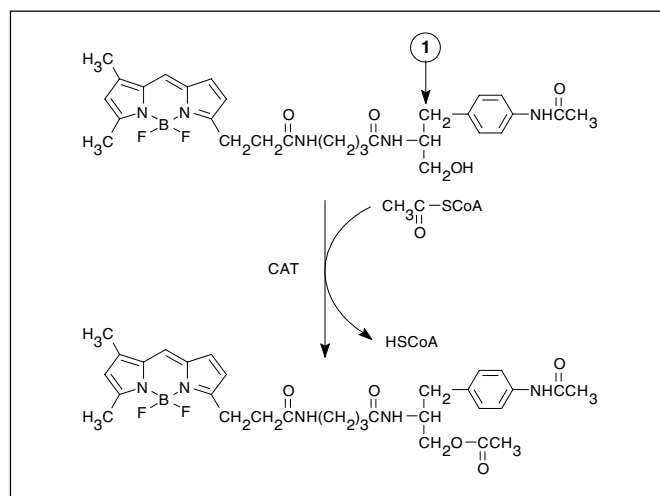


Figure 1. The green fluorescent BODIPY FL 1-deoxychloramphenicol substrate in our FAST CAT Green (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F-6616). CAT-mediated acetylation of this substrate, or the BODIPY TMR 1-deoxychloramphenicol in our FAST CAT Yellow (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F-6617), results in single fluorescent products because these substrates contain only one hydroxyl group that can be acetylated. In contrast, the BODIPY FL chloramphenicol substrate in our original FAST CAT Kit (F-2900) contains a second hydroxyl group at the 1-position (indicated by the labeled arrow). This hydroxyl group undergoes a nonenzymatic transacetylation step, restoring the original hydroxyl for a second acetylation. CAT-mediated acetylation of this chloramphenicol substrate produces three fluorescent products, thus complicating the analysis.

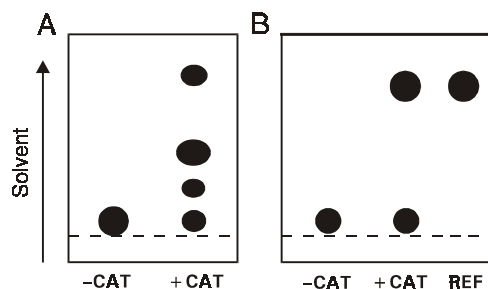


Figure 2. Schematic representation of TLC analysis of CAT-mediated acetylation using A) the original FAST CAT substrate and B) the FAST CAT Green or Yellow (deoxy) substrate. Lane 1 in both TLCs depicts a solution of the substrate prior to the addition of the CAT enzyme; lane 2 represents the reaction mixture following incubation with either purified CAT enzyme or cell extracts containing CAT activity; lane 3 of Plate B represents the reference standard (Component B). The bottom spots in all the lanes are the unacetylated substrate; all other spots represent the acetylated products. The TLC's can be visualized with a long-wavelength UV lamp and photographed using Polaroid Type 667 black-and-white print film. As can be seen, our FAST CAT Green or Yellow (deoxy) substrate yields only one product, providing a method that directly reflects the kinetics of the reaction.

amphenicol and BODIPY TMR deoxychloramphenicol have nearly identical K_M values (17.7 μM and 18.4 μM respectively), which are close to published values for [^{14}C]-1-deoxychloramphenicol.¹⁷ Both of the BODIPY fluorophores have high fluorescence quantum yields, allowing the substrates and their enzymatic products to be easily detected on thin-layer chromatography (TLC) plates using a UV lamp or illuminator.

Materials

FAST CAT Green Kit Contents

- **Substrate reagent** (Component A), BODIPY FL 1-deoxychloramphenicol
- **Reference standard** (Component B), 3-acetyl derivative of the BODIPY FL 1-deoxychloramphenicol substrate

FAST CAT Yellow Kit Contents

- **Substrate reagent** (Component A), BODIPY TMR 1-deoxychloramphenicol
- **Reference standard** (Component B), 3-acetyl derivative of the BODIPY TMR 1-deoxychloramphenicol substrate

Sufficient reagents are supplied for approximately 100 tests using the supplied protocol.

Storage and Handling

Kit contents should be stored desiccated at -20°C . Prepared solutions of both substrate reagent and reference standard (see *Solution Preparation*) should be stored at -20°C . These reagents should be stable for at least six months when stored under these conditions. Presence of particulate matter after prolonged storage in solution may indicate that the reagents no longer meet appropriate standards for use. PROTECT FROM LIGHT

Materials Required but Not Provided

- Methanol, reagent grade
- Ethyl acetate

- Acetyl coenzyme A (acetyl CoA)
- Buffer solutions appropriate for preparing bacterial cell extracts (see *Bacterial Cell Extract Preparation*)
- Buffer solutions appropriate for preparing mammalian cell extracts (see *Mammalian Cell Extract Preparation*)
- Silica gel TLC plates (note A)
- TLC solvent, chloroform:methanol (85:15 v/v, note B)

Experimental Protocols

Overview

The FAST CAT assay is simple, rapid and easily quantitated. Cells expressing the CAT gene are lysed and a cytoplasmic extract is prepared. The extract is then incubated with the fluorescent deoxychloramphenicol substrate and acetyl CoA at 37°C . If there is active CAT enzyme present, the substrate will be acetylated at the 3-hydroxyl position. The reaction is terminated by addition of ice-cold ethyl acetate, which also serves to extract the fluorescent substrate and its acetylated derivative. After drying and dissolution in a small precise volume of ethyl acetate, the reaction substrate and product are resolved by thin-layer chromatography (TLC) on silica gel plates eluted with a chloroform:methanol mixture (85:15 v/v). Once the chromatogram has been developed and dried, the results of the assay are immediately evident from visual inspection using either visible or UV light. Quantitation of the reaction is achieved by scraping the TLC spots corresponding to the unreacted CAT substrate and acetylated derivative, extracting the compounds in methanol, and measuring the absorbance or fluorescence signals using a spectrophotometer or fluorometer. If available, the spots can also be quantitated using a scanner equipped with filters that match the spectra of the dyes (see Table 1). The amount of conversion of substrate to acetylated product is determined from the relative intensities of the signals. If desired, a concentration value can be obtained from these data.

Solution Preparation

1.1 Prepare substrate solution by dissolving the entire amount of substrate reagent (Component A) in 1 mL methanol. This solution should be stored at -20°C in a well sealed container, protected from light.

1.2 Prepare reference standard solution by dissolving the entire amount of reference standard (Component B) in 1 mL of ethyl acetate. This solution should be stored at -20°C in a well-sealed container, protected from light.

1.3 Prepare a 9 mM solution of acetyl coenzyme A (acetyl CoA) in deionized water. For the free acid (MW = 809.6), this corre-

Table 1. Spectral characteristics of the substrates and products in the FAST CAT (deoxy) Chloramphenicol Acetyltransferase Kits.

Kit	Abs* (nm)	Em* (nm)	ϵ ($\text{cm}^{-1} \text{M}^{-1}$) †
FAST CAT Green	504	510	80,000
FAST CAT Yellow	545	570	60,000

* Absorption and fluorescence emission maxima.
† In methanol

sponds to 7.4 mg/mL. This solution should be made fresh before each set of assays.

Bacterial Cell Extract Preparation

2.1 Inoculate the transformed bacterial cells into growth medium and allow to grow until the suspension has an optical density of approximately 0.6 at 600 nm.

2.2 Transfer 0.2 mL of the culture into a centrifuge tube and pellet the cells by centrifugation. Aspirate off the medium and resuspend the pelleted cells in 0.5 mL of 100 mM Tris-HCl, pH 8.0.

2.3 Add 20 μ L of lysis solution (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM dithiothreitol). Add a small drop of toluene from a fine-tip Pasteur pipet.

2.4 Incubate at 30°C for 30 minutes. The extract can be assayed for CAT activity immediately or stored frozen at -70°C.

Mammalian Cell Extract Preparation

3.1 (A) Trypsinization method: Dilute trypsinized cells into medium with serum (phosphate-buffered saline (PBS), 4% (v/v) fetal calf serum, 10 mM HEPES, pH 7.2) or other tissue culture growth medium. Centrifuge the cell suspension at 500–600 rpm and 4°C for 5 minutes. Discard the supernatant. (B) Scraping method: Rinse transfected cells on a confluent tissue culture plate with calcium- and magnesium-free PBS. Then scrape the cells using scraping solution (40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 180 mM NaCl). Transfer the suspension to a 15 mL conical tube and centrifuge at 500–600 rpm and 4°C for 10 minutes. Discard the supernatant.

3.2 Resuspend the cells from either (A) or (B) of step 3.1 in 100 μ L of 0.25 M Tris-HCl, pH 7.4 (at this point, the resuspended cells can be stored for a number of weeks at -70°C if necessary).

3.3 Subject the cells to three freeze-thaw cycles by alternating the suspensions between a dry ice/ethanol bath and a 37°C water bath (3–4 minutes in each bath). Briefly vortex the lysed mixture after each cycle.

3.4 Centrifuge at approximately 12,000 rpm and 4°C for 5 minutes. The supernatant can be assayed for CAT activity immediately or stored frozen at -70°C (note C).

Enzyme Assay

4.1 Mix 60 μ L of cell extract with 10 μ L of FAST CAT substrate solution (prepared in step 1.1; see also note D).

4.2 Incubate at 37°C for 5 minutes.

4.3 Add 10 μ L of 9 mM acetyl CoA (prepared in step 1.3).

4.4 Continue the incubation at 37°C for a fixed period of time between 15 minutes and 5 hours, depending on the level of expected CAT activity (note E).

4.5 Stop the reaction by adding 1 mL of ice-cold ethyl acetate. Vortex each sample for about 20 seconds (note F).

4.6 Centrifuge the samples at top speed for three minutes to separate the liquid phases. Remove the top 900 μ L of ethyl acetate and transfer into a clean tube.

4.7 Evaporate the solvent, leaving a **completely** dry sample (note G).

4.8 Dissolve the residue in a small precise volume (20–30 μ L) of ethyl acetate (note H). The ethyl acetate extracts are stable and may be stored at -20°C, protected from light, for analysis at a later time.

Thin-Layer Chromatography

5.1 Apply 5 μ L of the FAST CAT reference standard solution (prepared in step 1.2) and 5 μ L (or more) of each ethyl acetate extract from steps 4.1 to 4.8 about 1–2 cm above the bottom of a silica gel TLC plate (note I).

5.2 Allow the spots to air dry for 5 minutes or use a hair-dryer set for low heat to accelerate drying.

5.3 Place the plate in a chromatography chamber that is filled to a depth of approximately 0.5 cm with chloroform:methanol (85:15 v/v). Seal the chamber and allow the solvent to ascend the plate (notes B, J). Remove the plate and allow it to air dry.

Semiquantitative Analysis of Results

Although the results of the chromatography can be monitored by visual inspection of the colored product, visualization is greatly enhanced by viewing the plate under UV light. A permanent record of the results can be obtained by photographing the plate while under UV illumination (note K). A typical chromatogram after development is depicted in Figure 2B. Semiquantitative results can be obtained by performing serial dilutions of the cell extracts before adding the substrate reagent, followed by TLC analysis of each dilution. In this manner, an estimate of the endpoint of enzyme activity is obtained.

Quantitative Analysis of Results

6.1 For each sample, lightly circle the spots corresponding to unreacted substrate reagent and the acetylated product using a soft lead pencil. This is best done under an UV illuminator to ensure that all spots are visualized.

6.2 Scrape the acetylated spot for each sample into a clean centrifuge tube. Scrape the unreacted substrate band for each sample into separate tubes.

6.3 Add a precise and constant volume of methanol (note L) to each tube and vortex for approximately one minute to extract the compounds. Centrifuge and withdraw an aliquot from each solution, taking care not to disturb the compacted silica gel.

6.4 For the FAST CAT Green (deoxy) Kit, measure the fluorescence using fluorescein (FITC) bandpass filters or using excitation and emission monochromator settings of 495 nm and 525 nm, respectively. For the FAST CAT Yellow (deoxy) Kit, measure the fluorescence using rhodamine (TRITC) bandpass filters or using excitation and emission monochromator settings of 540 nm and 570 nm, respectively.

6.5 Determine the conversion of substrate into product from the relative intensities of the fluorescence signals (note **M**). The percentage conversion of substrate to acetylated product can be calculated using the following equation:

$$\% \text{ conversion} = \left[\frac{I_{\text{product}}}{I_{\text{substrate}} + I_{\text{product}}} \right] \times 100$$

where I = fluorescence intensity.

The amount of product can then be determined by:

$$\text{amount product} = \text{substrate added} \times \frac{\% \text{ conversion}}{100}$$

If the reaction is performed as described in *Enzyme Assay*, substrate added = 5 nmoles.

Notes

[A] Silica gel TLC plates are available from a number of reliable manufacturers, including EM Science (www.emscience.com), Analtech (www.analtech.com) and Whatman (www.whatman.plc.uk).

[B] We have tested various chloroform:methanol solvent compositions and have found that a mixture of 85:15 (v/v) produces the best chromatographic separation. Note that some variation is to be expected from one laboratory to another depending on the type of silica gel TLC plate used and other factors. It is essential that the chromatography chamber be well sealed to prevent evaporation, which will alter the eluent composition. Placing strips or pads of filter paper in the tank along with the plate will help to keep the atmosphere in the tank saturated with eluent, and consequently improve the reproducibility of the separations.

[C] To inactivate endogenous acetylating enzymes, heat the extracts at 65°C for 10 minutes prior to the assay. The bacterial CAT enzyme is stable at this temperature, whereas most other acetylating enzymes are not.¹⁹

[D] It is recommended that both positive and negative controls be included with each set of assays. The reaction buffer alone serves as a convenient negative control. Likewise, 0.1 units of purified CAT enzyme, which is commercially available, is an appropriate positive control. The reference standard provided contains the 3-acetyl derivative of the BODIPY FL or BODIPY TMR 1-deoxychloramphenicol substrate.

[E] The concentration of substrate in the reaction mixture is approximately 63 μM, which is well above the K_M of ~18 μM for the BODIPY FL and BODIPY TMR 1-deoxychloramphenicol substrates.¹⁷ Since the rate of enzymatic conversion is dependent on the concentration of CAT enzyme present, longer incubation periods will be necessary when assaying cell extracts expressing very low levels of enzyme.

[F] If vortexing is not possible, shake each sample vigorously for at least 30 seconds to ensure complete extraction.

[G] The drying procedure is conveniently carried out using a Savant SpeedVac® Vacuum Concentration System (or other similar device) for a run time of about 90 minutes.

[H] Dissolving the sample in a precise amount of ethyl acetate serves to concentrate the extract and to produce a defined sample volume.

[I] Multiple samples can be analyzed using a 10 × 20 cm silica gel TLC plate. Draw a light pencil line along the long axis of the plate, about 1–2 cm from the edge, and deliver 5 μL (or more) of each sample extract on this line at intervals of at least 1.5 cm. To obtain small spots, repeatedly apply portions of the sample, allowing the solvent spot to partially dry between applications. The spots must be above the solvent when the TLC plate is placed in the chromatography chamber.

[J] The solvent front should be allowed to advance to near the top of the plate for optimum separation of the substrate and product. However, do not allow the plates to remain too long in the chamber after the solvent front reaches the top, as the separated spots will diffuse and be more difficult to visualize.

[K] Plates can be photographed using a variety of cameras and film. Good results have been obtained with a 35 mm camera using Kodak Ektachrome 400 color print film or with a Polaroid camera using Type 667 black-and-white print film.

[L] Any convenient volume that provides enough solution for an accurate measurement in a cuvette will suffice, but the smaller the volume, the more concentrated the solution and hence, the greater the signal.

[M] Alternatively, the conversion can be measured by the relative absorption at 504 nm for the unreacted FAST CAT Green (deoxy) substrate and product or at 545 nm for the unreacted FAST CAT Yellow (deoxy) substrate and product. However, the fluorometric method is inherently more sensitive. In the calculation of percent conversion, I = absorbance.

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