Revised: 02–April–2001

FAST CAT® Chloramphenicol Acetyltransferase Assay Kit (F-2900)

Quick Facts

Storage upon receipt:
- –20°C
- Desiccate
- Protect from light

Abs/Em of reaction products: 504/511 nm

Introduction

The close correlation between chloroamphenicol 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 [^{14}C]-chloramphenicol or [^{14}C]-acetyl CoA, followed by autoradiography and densitometry or scintillation counting analysis.[7,8] Molecular Probes' *FAST* CAT® assay employs a fluorescent BODIPY® FL chloramphenicol substrate (Figure 1) that provides sensitivity comparable to radioactive substrates while avoiding their associated hazards and disposal cost.[9-11] This BODIPY FL chloramphenicol has a K_M for purified CAT of 7.4 µM and a V_{max} of 375 pmol/unit/min, values that are similar to those of ^{14}C-labeled chloramphenicol.[11] Furthermore, our fluorescent CAT assay provides rapid, easily quantitated results that can be immediately visualized without the use of film or scintillant.

The BODIPY FL chloramphenicol *FAST* CAT substrate has been used to measure transcriptional activitation in sensory neurons,[12] to study hormonal regulation of prodynorphin gene expression [13] and to measure the rate of hair growth in single follicles of transgenic mice.[14] In the latter study, fluorescence-detected HPLC using the *FAST* CAT substrate was found to provide 1000-fold greater sensitivity than traditional HPLC-UV detection of CAT products. A complicating feature in quantitative analysis of CAT activity using either [^{14}C]-chloramphenicol or the BODIPY FL chloramphenicol *FAST* CAT substrate is the multiplicity of reaction products formed by enzymatic reaction — two mono-acetylated and one diacetylated chloramphenicols (see Figure 2). For researchers wishing to circumvent this complication, Molecular Probes now offers two *FAST* CAT kits (F-6616, F-6617) incorporating BODIPY deoxychloramphenicol substrates that yield a single acetylated product.[15]

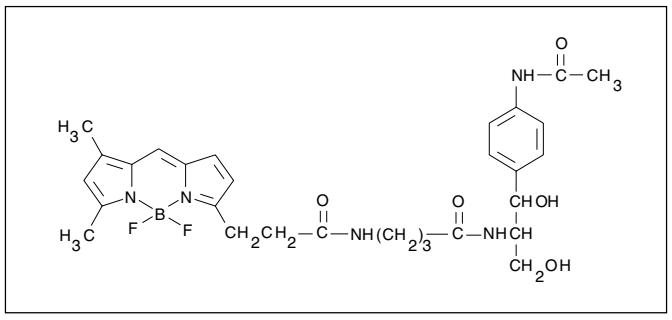

Figure 1. *Structure of BODIPY FL chloramphenicol.*

Materials

Kit Contents

- **Substrate reagent** (Component A), BODIPY FL chloramphenicol
- **Reference standard** (Component B), a mixture of the substrate reagent and its 1- and 3-acetyl and 1,3-diacetyl derivatives

Storage and Handling

Kit contents should be stored desiccated at -20°C. Prepared solutions of the substrate reagent and the reference standard (see *Experimental Protocols*) 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

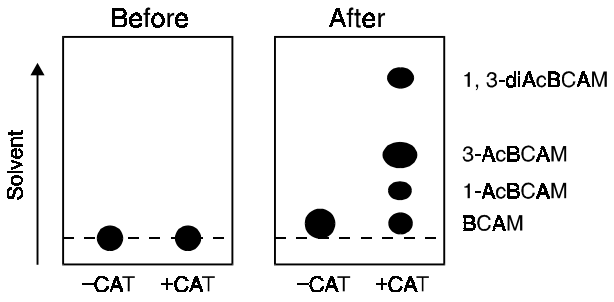

Figure 2. *Schematic representation of TLC analysis of CAT-mediated acetylation using our FAST CAT Kit. Lane 1 in both the "before" and "after" development chromatograms depicts a solution of the substrate prior to the addition of CAT enzyme; lane 2 represents the reaction mixture following incubation with either purified CAT enzyme or cell extracts containing CAT activity. The bottom spots in all the lanes are the unacetylated substrate; all other spots represent the acetylated products. BCAM = BODIPY FL chloramphenicol. AcBCAM = acetylated BODIPY FL chloramphenicol. diAcBCAM = diacetylated BODIPY FL chloramphenicol.*

storage in solution may indicate that the reagents no longer meet appropriate standards for use. PROTECT FROM LIGHT.

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 chloramphenicol substrate and acetyl CoA at 37°C. If there is active CAT enzyme present, the substrate will be acetylated at the 1- and/or 3-hydroxyl positions. The reaction is terminated by addition of ice-cold ethyl acetate, which also serves to extract the fluorescent substrate and its acetylated derivatives. After drying and dissolution in a small volume of ethyl acetate, the reaction substrate and products are resolved by thin-layer chromatography (TLC) on silica gel plates eluted with chloroform:methanol mixtures (about 9:1 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 ultraviolet light. Quantitation of the reaction is achieved by scraping the TLC spots corresponding to the unreacted substrate and acetylated CAT derivatives, extracting the compounds in methanol and measuring the absorbance or fluorescence signals using a spectrophotometer or fluorometer. If available, the spots can be quantitated using a scanner equipped with filters that match the spectra of the BODIPY FL dye (see below) or by HPLC. The substrate and acetylated products, which appear yellow in visible light, have extinction coefficients of 80,000 cm^{-1} M^{-1} in methanol, with maximum absorption and fluorescence emission at 504 nm and 511 nm, respectively. The amount of conversion of substrate to acetylated products is determined from the relative intensities of the signals. If desired, a concentration value can be obtained from this data.

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 (9:1 v/v, note **B**)

Solution Preparation

1.1 Prepare the substrate solution by dissolving the entire amount of substrate reagent (Component A) in 0.15 mL of methanol. Once the substrate is completely dissolved, add 1.35 mL of 0.1 M Tris-HCl, pH 8.0. Store this stock solution at -20°C, protected from light in a well sealed container.

1.2 Prepare reference standard solution by dissolving the entire amount of reference standard (Component B) in 1 mL of ethyl acetate. The 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 corresponds to 7.4 mg/mL. This solution should be made fresh before performing each set of assays.

Bacterial Cell Extract Preparation

2.1 Inoculate the transformed bacterial cells into growth medium and allow them 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 microcentrifuge tube and pellet the cells by centrifugation at 12,000 rpm for 2 minutes. Aspirate the medium and resuspend the pelleted cells in 0.5 mL of 100 mM Tris-HCl, pH 8.0.

2.3 Add 20 µL of lysis medium (100 mM EDTA, 100 mM dithiothreitol (DTT), 50 mM Tris-HCl, pH 8.0). 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 at -70°C.[16]

Mammalian Cell Extract Preparation

3.1 (A) Trypsinization method: Dilute trypsinized cells into media with serum (phosphate-buffered saline, 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, 1 mM EDTA, 180 mM NaCl, pH 7.4), transfer 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) above in 100 µL 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 55 µL of the cell extract with 15 µL of *FAST* CAT substrate reagent (note **D**).

4.2 Incubate at 37°C for 5 minutes.

4.3 Add 10 µL of 9 mM acetyl CoA.

4.4 Continue the incubation 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 µL 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 yellow residue in a small volume (20–30 µL) 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 (TLC)

5.1 Apply 5 µL of the *FAST* CAT Reference Standard and 5 µL (or more) of each ethyl acetate extract from steps 4.1–4.8 about 1 cm above the bottom of a silica gel TLC plate (note **I**).

5.2 Allow the spots to air-dry (allow 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 ~0.5 cm with chloroform:methanol (9:1 v/v). Close the chamber and allow the solvent to ascend the plate (note **B**). 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 bright yellow products, 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, before and after development, is shown in Figure 2. Semi-quantitative 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 all of the acetylated products using a soft lead pencil. This is best done under an UV illuminator to ensure that all the products are visualized.

6.2 Scrape and combine the acetylated spots for each sample into a clean centrifuge tube. Scrape the unreacted substrate band for each sample into a separate tube.

6.3 Add a precise and constant volume of methanol to each tube and vortex for approximately one minute to extract the compounds. 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.

6.4 Centrifuge and then withdraw an aliquot from each solution, taking care not to disturb the compacted silica gel.

6.5 Measure the fluorescence using fluorescein (FITC) bandpass filters or using excitation and emission monochromator settings of 490 nm and 525 nm, respectively.

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

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

where I = fluorescence intensity (or absorbance).

The amount of product can be determined by:

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

If the reaction is performed as in *Enzyme Assay*, substrate added = 10 nmoles.

Notes

[A] Silica gel TLC plates are readily 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 87:13 (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.[17]

[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 approximately equal amounts of all three possible acetylation products.

[E] The concentration of substrate in the reaction mixture is approximately 125 µM, which is well above its K_M of 7.4 µM. 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 small 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, approximately 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 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 the products. However, do not allow the plates to remain too long in the chamber after the solvent front reaches the top, as the separated bands 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] Alternatively, the conversion can be measured by the relative absorption at 504 nm of the products and unreacted substrate. However, the fluorometric method is inherently more sensitive.

[M] Provided that the level of substrate conversion is kept below 50%, the *FAST* CAT assay is quantitatively linear with respect to cell extract dilution.[11]

References

1. Development 109, 577 (1990); **2.** Proc Natl Acad Sci USA 87, 6848 (1990); **3.** Methods Cell Biol 50, 425 (1995); **4.** Science 231, 1574 (1986); **5.** Science 231, 1294 (1986); **6.** EMBO J 7, 1583 (1988); **7.** J Virol 62, 297 (1988); **8.** Anal Biochem 156, 251 (1986); **9.** Methods Enzymol 216, 369 (1992); **10.** Anal Biochem 197, 401 (1991); **11.** Biotechniques 8, 170 (1990); **12.** Neuron 10, 427 (1993); **13.** Mol Endocrinol 6, 2244 (1992); **14.** Eur J Clin Chem Clin Biochem 31, 41 (1993); **15.** Biotechniques 19, 488 (1995); **16.** Cell 40, 527 (1985); **17.** Anal Biochem 163, 88 (1987).

Product List

Current prices may be obtained from our Web site or from our Customer Service Department.

Cat #	Product Name	Unit Size
F-2900	*FAST* CAT® Chloramphenicol Acetyltransferase Assay Kit *100 assays*	1 kit

Contact Information

Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site — **www.probes.com** — for the most up-to-date information

Molecular Probes, Inc.
29851 Willow Creek Road, Eugene, OR 97402
Phone: (541) 465-8300 ● Fax: (541) 335-0504

Customer Service: 6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338 ● Fax: (541) 335-0305 ● order@probes.com

Toll-Free Ordering for USA and Canada:
Order Phone: (800) 438-2209 ● Order Fax: (800) 438-0228

Technical Assistance: 8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353 ● Toll-Free: (800) 438-2209
Fax: (541) 335-0238 ● tech@probes.com

Molecular Probes Europe BV
Poortgebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378 ● Fax: +31-71-5233419

Customer Service: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850 ● Fax: +31-71-5233419
eurorder@probes.nl

Technical Assistance: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431 ● Fax: +31-71-5241883
eurotech@probes.nl

Molecular Probes' products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Several of Molecular Probes' products and product applications are covered by U.S. and foreign patents and patents pending. Our products are not available for resale or other commercial uses without a specific agreement from Molecular Probes, Inc. We welcome inquiries about licensing the use of our dyes, trademarks or technologies. Please submit inquiries by e-mail to busdev@probes.com. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.