



Promega

Technical Bulletin

CAT Enzyme Assay System

INSTRUCTIONS FOR USE OF PRODUCT E1000.



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PRINTED IN USA.
Revised 5/09

Part# TB084

CAT Enzyme Assay System

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1. Description

Chloramphenicol acetyltransferase (CAT), encoded by a bacterial drug-resistance gene, inactivates chloramphenicol by acetylating the drug at one or both of its two hydroxyl groups (1). This gene is not found in eukaryotes, thus eukaryotic cells produce no background CAT activity. This characteristic, along with the ease and sensitivity of the assay for CAT activity, made the CAT gene one of the first reporter genes used for studies of mammalian gene expression (2,3).

Linkage of putative regulatory sequences to the appropriate pCAT[®]3 Reporter Vector (see Related Products, Section 7) and subsequent transfection allows for the efficient assay of CAT activity in cultured cells.

CAT activity may be monitored by two alternative methods in the CAT Enzyme Assay System[®]. The most rapid, sensitive and convenient of these assays is based on liquid scintillation counting (LSC) of CAT reaction products. Cell extracts are incubated in a reaction mix containing ¹⁴C- or ³H-labeled

chloramphenicol and n-Butyryl Coenzyme A (n-Butyryl CoA). CAT transfers the n-butyryl moiety of the cofactor to chloramphenicol. For the LSC assay, the reaction products are extracted with a small volume of xylene. The n-butyryl chloramphenicol partitions mainly into the xylene phase, while unmodified chloramphenicol remains predominantly in the aqueous phase (4). The xylene phase is mixed with scintillant, and radioactive product is measured with a scintillation counter.

This assay can be completed in as little as 2-3 hours, is linear for nearly three orders of magnitude and can detect as little as 3×10^{-4} units of CAT. CAT activity also can be analyzed using thin-layer chromatography (TLC). This method is more time-consuming than the LSC assay but allows visual confirmation of the data.

For peer-reviewed articles that cite the use of the CAT Enzyme Assay System, visit: www.promega.com/citations/

2. Product Components and Storage Conditions

Product	Cat.#
CAT Enzyme Assay System	E1000

This system contains sufficient reagents for 50 reactions. Includes:

- 100u Chloramphenicol Acetyltransferase
- 25ml Tris-HCl, 0.25M (pH 8.0)
- 255µl n-Butyryl CoA, 5mg/ml
- 30ml Reporter Lysis 5X Buffer

CAT Enzyme Unit Definition: One unit is defined as the amount of enzyme required to transfer 1nmol of acetate to chloramphenicol in one minute at 37°C.

Storage Conditions: Store all components of the CAT Enzyme Assay System at -20°C. The Reporter Lysis 5X Buffer may be stored at room temperature.

3. Preparation of Cell Extract

For CAT transient expression assays, cell extracts are typically prepared 48–72 hours post-transfection. Two alternative methods for extract preparation are described below.

The Reporter Lysis Buffer provided with this system allows CAT, β -galactosidase and luciferase assays to be performed from the same cell extract co-transfected with vectors carrying these genes. In cell lines that we have tested, CAT activity is roughly comparable in extracts prepared with Reporter Lysis Buffer and with the freeze-thaw protocol provided.

3.A. Preparation of Cell Extract Using the Reporter Lysis Buffer

Materials to Be Supplied by the User

(Solution compositions are provided in Section 6.)

- PBS buffer (pH 7.4; Mg²⁺- and Ca²⁺-free)
1. Add 4 volumes of water to 1 volume of Reporter Lysis 5X Buffer to produce a 1X stock solution.
 2. Remove the growth medium from the cells to be assayed. Wash the cells three times with PBS buffer, being careful not to dislodge any of the cells. Remove as much of the final PBS wash as possible with a pipette tip.
 3. Add a sufficient volume of 1X Reporter Lysis Buffer to cover the cells (400 μ l for a 60mm culture dish; 900 μ l for a 100mm culture dish). Rock the dish slowly several times to ensure complete coverage of the cells.
 4. Incubate at room temperature for 15 minutes, rocking the dish halfway through the incubation period.
 5. Scrape all areas of the plate surface, then tilt the dish up and scrape the cell lysate to the lower edge of the plate. Take care to scrape down all visible cell debris. Transfer the cell lysate to a microcentrifuge tube with a pipette and place the samples on ice.
 6. Vortex the tubes for 10–15 seconds. Heat only those lysates for use in the CAT assay at 60°C for 10 minutes to inactivate endogenous deacetylase activity (5).
Note: If simultaneously performing reporter assays for other co-transfected vectors, split the lysates before heating, and heat only the lysates intended for use in the CAT assay. This heating step does not inactivate CAT but may inactivate other reporter enzymes such as β -galactosidase or luciferase.
 7. Spin the lysates at top speed in a microcentrifuge for 2 minutes. Transfer the supernatant to a fresh tube.
 8. The extracts may be assayed directly or stored at -70°C.

3.B. Preparation of Cell Extract Using the Tris Buffer Freeze-Thaw Protocol

This protocol is based on the method of Rosenthal (6).

Materials to Be Supplied by the User

(Solution compositions are provided in Section 6.)

- PBS buffer (pH 7.4; Mg²⁺- and Ca²⁺-free)
- TEN buffer
- 0.25M Tris-HCl (pH 8.0)

1. Remove the growth medium from the cells to be assayed. Wash the cells three times with PBS buffer, being careful not to dislodge any of the cells. Remove as much of the final PBS wash as possible with a pipette tip.
2. Add 1ml of TEN buffer (per 60mm or 100mm culture dish), and incubate the cells for 5 minutes at room temperature.
3. Scrape all areas of the plate surface, and transfer the cells to a microcentrifuge tube.
4. Spin the cells at top speed in a microcentrifuge for **1 minute** at 4°C.

Note: Longer centrifugation times can result in compacted cell pellets that are difficult to resuspend.

5. Remove the supernatant and resuspend the pellet in 100µl of 0.25M Tris-HCl (pH 8.0) per 60mm culture dish of cells (or 150µl per 100mm culture dish).
6. Subject the extracts to three rapid freeze-thaw cycles, vortexing vigorously after each thaw cycle. Freeze in dry ice or a dry ice/ethanol bath and thaw rapidly at 37°C.
7. Heat only those lysates for use in the CAT assay at 60°C for 10 minutes to inactivate endogenous deacetylase activity (5).
Note: If simultaneously performing reporter assays for other co-transfected vectors, split the lysates before heating and heat only the lysates intended for use in the CAT assay. This heating step does not inactivate CAT but may inactivate other reporter enzymes such as β-galactosidase or luciferase.
8. Spin the extracts at top speed in a microcentrifuge for 2 minutes. Transfer the supernatant to a fresh tube.
9. The extracts may be assayed directly or stored at -70°C.

4. CAT Enzyme Assay

The standard reaction is performed by adding the cofactor (n-Butyryl CoA) to a microcentrifuge tube containing cell extract and radiolabeled chloramphenicol (see Note 1, Section 4.D) in a final volume of 125 μ l. Following the CAT reaction, samples can be processed for either the LSC assay or the TLC assay.

4.A. Standard Reaction for Cell Extract Prepared with Reporter Lysis Buffer

In this assay, the measured activity is affected not only by the dilution factor but by the actual volume of Reporter Lysis Buffer added. To obtain accurate CAT activity comparisons between samples, use the *same volume of Reporter Lysis Buffer in each reaction*. If a standard curve for CAT activity is prepared, also include the same volume of Reporter Lysis Buffer in each of these reactions.

The protein concentration of the lysate prepared with Reporter Lysis Buffer is approximately 4 times more dilute than the comparable extract prepared by the freeze-thaw method. For samples with a low level of CAT activity, it may be necessary to use the maximum volume of lysate per reaction.

1. Prepare the following reaction mixture in a 1.5ml microcentrifuge tube. A portion of the water may be replaced by Reporter Lysis Buffer to maintain a constant volume of Reporter Lysis Buffer per reaction.

cell extract	50-100 μ l
[¹⁴ C]chloramphenicol (at 0.05mCi/ml) (see Note 1, Section 4.D)	3 μ l
n-Butyryl CoA	5 μ l
distilled water to final volume of	125 μ l

Prepare positive control reactions using the Chloramphenicol Acetyltransferase provided. Also prepare a negative control containing no cell extract. Guidelines for setting up a CAT standard curve are provided in Section 5.

2. Incubate the reactions at 37°C for a fixed period of time from 30 minutes to 20 hours (see Note 2, Section 4.D). We suggest starting with an incubation time of 3 hours.
3. Briefly spin the tubes in a microcentrifuge.
4. Terminate the reactions. For the LSC assay, add 300 μ l of mixed xylenes (Sigma Aldrich Cat.# 247642) to each tube and proceed to Section 4.C. For the TLC assay, add 500 μ l of ethyl acetate and proceed to Section 4.D.

Note: If you wish to perform both assays from a single sample, split the reaction volume into 2 tubes after Step 3. Add 300 μ l of mixed xylenes to one of the tubes and 500 μ l of ethyl acetate to the other tube and proceed with the LSC and TLC assays, respectively.

4.B. Standard Reaction for Cell Extract Prepared with Tris Buffer Freeze-Thaw Protocol

1. Prepare the following reaction mixture in a 1.5ml microcentrifuge tube.

cell extract	10-50 μ l
[¹⁴ C]chloramphenicol (at 0.05mCi/ml)	3 μ l
(see Note 1, Section 4.D)	
n-Butyryl CoA	5 μ l
0.25M Tris-HCl (pH 8.0) to final volume of	125 μ l

Prepare positive control reactions using the Chloramphenicol Acetyltransferase provided. Also prepare a negative control containing no cell extract. Guidelines for setting up a CAT standard curve are provided in Section 5.

2. Incubate the reactions at 37°C for a fixed period of time from 30 minutes to 20 hours (see Note 2, Section 4.D).
3. Briefly spin the tubes in a microcentrifuge.
4. Terminate the reactions. For the LSC assay, add 300 μ l of mixed xylenes (Sigma Aldrich Cat.# 247642) to each tube and proceed to Section 4.C. For the TLC assay, add 500 μ l of ethyl acetate and proceed to Section 4.D.

Note: If you wish to perform both assays from a single sample, split the reaction volume into 2 tubes after Step 3. Add 300 μ l of mixed xylenes to one of the tubes and 500 μ l of ethyl acetate to the other tube and proceed with the LSC and TLC assays, respectively.

4.C. Liquid Scintillation Counting (LSC) Assay

1. Vortex the sample with the mixed xylenes for 30 seconds. Spin at top speed in a microcentrifuge for 3 minutes to achieve good phase separation.
2. Transfer the entire volume or a fixed, known volume of the upper phase (xylenes) to a fresh tube. Add 100 μ l of fresh 0.25M Tris-HCl (pH 8.0) and repeat Step 1.
3. For maximum sensitivity, extract residual substrate from the organic phase a second time by transferring xylenes again to a fresh tube and adding 100 μ l of 0.25M Tris-HCl (pH 8.0). Repeat Step 1. If maximum sensitivity is not required, omit this step and proceed to Step 4.

Note: The chloramphenicol substrate partitions preferentially into the aqueous phase, and the subsequent extractions reduce the amount of chloramphenicol present in the xylene phase.

4. Carefully remove 200 μ l or a fixed, known volume of the upper xylene phase and transfer to a scintillation vial.
5. Add an appropriate scintillation fluid (e.g., Ready-Safe™ scintillant), and measure radioactivity in the samples with a liquid scintillation counter.
6. The cpm measured in each sample represents the butyrylated chloramphenicol products.
7. Subtract the cpm of chloramphenicol in the negative control reactions (no enzyme) from each sample to determine the specific level of n-butyrylated products (see Note 3, Section 4.D).

4.D. Thin-Layer Chromatography (TLC) Assay

1. Vortex the sample with ethyl acetate for 1 minute. Spin at top speed in a microcentrifuge for 3 minutes to achieve good phase separation.
2. Transfer the upper, organic phase to a fresh tube and evaporate to dryness.
3. Resuspend the residue in 30 μ l of ethyl acetate. Spot 10 μ l of each sample onto a silica gel TLC plate and dry. We recommend Baker-flex® silica gel TLC plates SG/IB2, 20 \times 20cm (J.T. Baker, Inc., Cat.# 4448-04). Remaining sample can be dried and stored at -20°C for future use.
4. Place the TLC plate in a developing chamber pre-equilibrated for 1 hour with chloroform/methanol (97:3).
5. Run the silica plate chromatography in a closed chamber for about 1 hour until the solvent is approximately halfway up the plate.
6. Remove the silica plate from the chamber and dry at room temperature.
7. Cover the plate with plastic wrap and expose to X-ray film for an autoradiogram or process the plate for phosphorimaging.

4.D. Thin-Layer Chromatography (TLC) Assay (continued)

8. Develop the autoradiogram 2–7 days after exposing the film. An intensifying screen may be used to reduce the autoradiogram's exposure time to 1 day.
9. Chloramphenicol can be butyrylated at one or two positions, and these multiple forms migrate faster than the unmodified chloramphenicol substrate on the TLC plates in this solvent system. After autoradiography, each of the butyrylated products can be scraped or cut from the plate and counted in a scintillation counter for quantitation of CAT activity.

Notes:

1. [¹⁴C]chloramphenicol is recommended over [³H]chloramphenicol for TLC assays because of the shorter exposure time required for autoradiography. We have used [¹⁴C]chloramphenicol from PerkinElmer Life and Analytical Sciences (Cat.# NEC408050UC). At 50mCi/mmol and 0.05mCi/ml, 3 μ l of this substrate provides a final concentration of 24 μ M chloramphenicol in the 125 μ l standard reaction.

For LSC assays, the use of high-specific-activity [³H]chloramphenicol requires the addition of unlabeled chloramphenicol to ensure an adequate supply of substrate in the CAT assay. [³H]Chloramphenicol (PerkinElmer Cat.# NET928250UC) is supplied in ethanol, and the specific activity is approximately 1,000 times higher than the corresponding [¹⁴C]chloramphenicol. To prepare the [³H]compound, first dilute the specific activity 1:1,000. For example, 250 μ Ci of [³H]chloramphenicol (250 μ l) at 50Ci/mmol is 5×10^{-6} mmol. Add 5×10^{-3} mmol of unlabeled chloramphenicol (MW = 323.14), which is 32 μ l of chloramphenicol at 50mg/ml in ethanol. The resultant specific activity is 50mCi/mmol. Next, bring the volume to 500 μ l with ethanol (in this example, add 218 μ l ethanol) for a concentration of 250 μ Ci/500 μ l. Before performing the assay, dilute this ethanol stock of [³H]chloramphenicol 1:20 with 0.25M Tris-HCl (pH 8.0). The addition of 10 μ l, or 0.25 μ Ci, to the CAT reaction mix with a final volume of 125 μ l results in a concentration of 40 μ M chloramphenicol.

2. Kinetics of the reaction can be monitored by taking aliquots of a reaction mix at defined time points during the first 60 minutes. These data provide an accurate measurement of enzyme activity, particularly in reactions containing high levels of activity. Longer incubations give higher signals and greater sensitivity and may be desirable for extracts containing low levels of CAT activity. For very low levels of CAT activity, the incubation can be extended to 20 hours.
3. If the background of ³H in the xylene phase is high, extract the [³H]chloramphenicol in 0.25M Tris-HCl (pH 8.0) twice with an equal volume of mixed xylenes.

5. Standard Curve

Prepare appropriate dilutions of the CAT enzyme in cold 0.25M Tris-HCl (pH 8.0) before the assay and keep on ice. The following guidelines can be used to establish a standard curve from 0.1 to 0.00625 units, adding 5 μ l of diluted enzyme per reaction.

1. Dilute the CAT enzyme to a concentration of 0.02u/ μ l. For example, dilute 2 μ l of CAT enzyme at 10u/ μ l into a total volume of 1ml. This can be accomplished by serial dilutions. (For this diluted enzyme preparation, 5 μ l = 0.1 unit.)
2. Prepare four serial twofold dilutions of the 0.02u/ μ l CAT solution prepared in Step 1. The relative dilutions will be 1:2, 1:4, 1:8 and 1:16. A 5 μ l aliquot from each of these serial dilutions will contain 0.05u, 0.025u, 0.0125u and 0.00625u of CAT, respectively.

Sample standard curves of CAT activity are shown for the LSC assay and TLC assay in Figure 1, Panels A and B, respectively. See reference 7 for more information on the CAT Enzyme Assay.

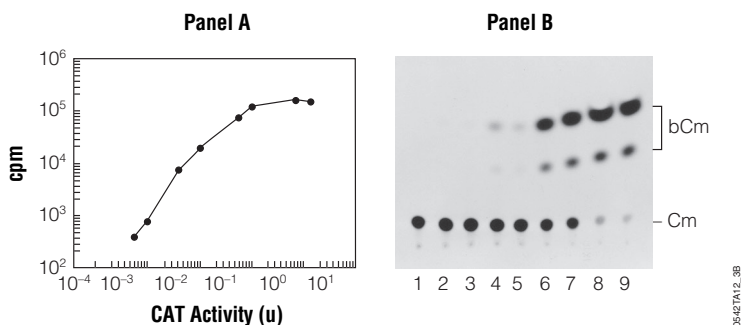


Figure 1. Comparison of sensitivities of CAT enzymatic assays based on liquid scintillation counting (LSC) and thin-layer chromatography (TLC). Panel A. LSC assays of CAT enzyme incubated with [¹⁴C]chloramphenicol and n-Butyryl CoA. Reaction products were extracted with xylene and the cpm determined as described above. **Panel B.** TLC assays of the reaction mixtures shown in Panel A. The mixtures were extracted and run as described above. Lane 1, enzyme blank; lane 2, 5 × 10⁻⁴u CAT enzyme; lane 3, 1 × 10⁻³u; lane 4, 5 × 10⁻³u; lane 5, 1 × 10⁻²u; lane 6, 5 × 10⁻²u; lane 7, 0.1u; lane 8, 0.5u; lane 9, 1u. Cm: chloramphenicol, bCm: butyrylated chloramphenicol.

6. Composition of Buffers and Solutions

PBS buffer (Mg²⁺- and Ca²⁺-free

137mM	NaCl
2.7mM	KCl
8.1mM	Na ₂ HPO ₄ • 7H ₂ O
1.47mM	KH ₂ PO ₄

TEN buffer

40mM	Tris-HCl (pH 7.5)
1mM	EDTA (pH 8.0)
150mM	NaCl

The final pH should be 7.4.

7. Related Products

CAT Assay Systems and Reporter Vectors

Product	Size	Cat.#
Chloramphenicol Acetyltransferase (CAT)	100u	E1051

Product	Size	Cat.#
pCAT [®] 3-Control Vector	20µg	E1851
pCAT [®] 3-Enhancer Vector	20µg	E1881
pCAT [®] 3-Promoter Vector	20µg	E1861
pCAT [®] 3-Basic Vector	20µg	E1871

Other Reporter Systems

Product	Size	Cat.#
Luciferase Assay System With Reporter Lysis Buffer	100 assays	E4030

Product	Size	Cat.#
Dual-Luciferase [®] Reporter Assay System	100 assays	E1910

Product	Size	Cat.#
β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer	65 assays	E2000

Product	Size	Cat.#
Beta-Glo [®] Assay System	10ml	E4720
	100ml	E4740
	10 × 100ml	E4780

Transfection Systems and Reagents

Product	Size	Cat.#
TransFast™ Transfection Reagent	1.2mg	E2431
Tfx™-20 Reagent	4.8mg	E2391
Tfx™-50 Reagent	2.1mg	E1811
Transfectam® Reagent for the Transfection of Eukaryotic Cells	1mg	E1231
	0.5mg	E1232
ProFection® Mammalian Transfection System – Calcium Phosphate		E1200

Plasmid DNA Purification System

Product	Size	Cat.#
PureYield™ Plasmid Miniprep System	50 preps	A1221
	250 preps	A1222
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495
PureYield™ Plasmid Maxiprep System	10 preps	A2392
	25 preps	A2393

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^oCertain applications of this product may require licenses from others.

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