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Not for use in diagnostic procedures.

Luciferase Reporter Gene Assay, high sensitivity

Version 20
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Chemiluminescent assay for the quantitative determination of firefly luciferase activity in transfected cells

Cat. No. 11 669 893 001 200 assays
Cat. No. 11 814 036 001 1000 assays

Store at −15 to −25°C

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Kit Contents

1	**Reaction Buffer,** ready-to-use	15 ml (Cat. No. 11 669 893 001) 105 ml (Cat. No. 11 814 036 001)	blue screw cap
2	**Luciferase Substrate,** Lyophilizate		blue screw cap
3	**Lysis Buffer,** 5× concentrated	1× 50 ml (Cat. No. 11 669 893 001) 4× 50 ml (Cat. No. 11 814 036 001)	black screw cap

Stability/Storage

The kit is stable until the expiration date given on the kit label if stored at or below −15 to −25°C. For stability of kit components upon thawing and of working solutions, see section 5.

Advantages of Luciferase Reporter Gene Assay, high sensitivity

- **linear light emission** — produces a constantly glowing light instead of a short peak kinetic
- **specific** — optimized to the widespread and popular monitor gene encoding firefly *Photinus pyralis* luciferase
- **extremely sensitive** — produces a high light intensity and is up to 1,000 fold more sensitive than CAT assays
- **flexible** — can be performed in all automated or manual microplate-/tube-format luminometers or scintillation counters
- **compatible** — the optimized formulation of the lysis buffer allows evaluation of the sample using other reporters such as β-Gal in cotransfection experiments
- **easy to perform** — the assay is simple to handle and fast

1. Introduction

Gene expression in transfected eukaryotic or prokaryotic cells is generally studied by linking a promoter sequence to an easily detectable “reporter” gene such as that encoding for firefly luciferase (for review see 1). The luciferase gene of the north American firefly *Photinus pyralis* (2, 3), a beetle that is a member of the superfamily of Cantharoidea, is one of the most versatile reporter genes. Monitoring promoter activity using firefly luciferase is described for *e.g.,* cultured animal cells (4, 5), bacteria (6), insects (7), plants (8), yeast (9) and viruses (10). A variety of firefly luciferase expressing vectors are commonly used (3, 11). Besides firefly luciferase, various other luciferases of eukaryotic (12) or prokaryotic (13) origin are also described using different substrates or reaction conditions.

The assay for firefly luciferase activity is an extremely sensitive (1, 14), rapid, easy-to-handle and non-isotopic alternative to other reporter gene assay systems. The luciferase assay is much more sensitive (100- to 1,000-fold) than the standard isotopic CAT assay (3). The detectable linear range of firefly luciferase is approx. 10 fg to 10 ng (10^{-16}–10^{-19} M). The enzyme is composed of a single polypeptide (62 kDa) of 550 amino acids which is active in a monomeric form. Firefly luciferase, *Photinus pyralis* (*EC 1.13.12.7*), catalyzes the adenosine triphosphate (ATP)-dependent oxidative decarboxylation of luciferin producing light emission at a wavelength of 562 nm (15):

$$\text{luciferin} + \text{ATP} + O_2 \xrightarrow[\text{luciferase}]{Mg^{2+}} \text{oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{light}$$

Light emission is measured most sensitively with luminometers in tube and microplate format or alternatively with reduced sensitivity in scintillation counters or with photographic films. The quantum yield 0.88 of the reaction is the highest known for chemiluminescence reactions (14, 16). The kinetics of luciferase catalyzed light emission are characterized by a transient flash which peaks after 0.3–0.5 s and rapidly decays in a biphasic manner. The main flash reaction is finished within approx. 20 s (17). Thus, classical luciferase determination requires rapid mixing and starting of the measurement of light emission.

However, a constantly glowing light emission over a period of several minutes can be achieved, when coenzyme A (CoA) is present in the reaction mix (18–21, see fig.1). CoA apparently enhances light production by promoting the dissociation of oxidized luciferin, a potent inhibitor of the light reaction (18–23). The Luciferase Reporter Gene Assay, high sensitivity thus allows precise quantification of luciferase activity without facing the problems of accurate measurement of short flash kinetics.

2. Application

The Luciferase Reporter Gene Assay, high sensitivity is used to quantitatively measure the expression of firefly luciferase in eukaryotic cells or bacteria transfected with a vector encoding firefly *Photinus pyralis* luciferase. The Luciferase Reporter Gene Assay, high sensitivity can be used in manual or automated luminometers, in microplate or tube format as well as in scintillation counters or with photographic films. In order to achieve maximum sensitivity the use of luminometers operating with ultra-fast photon counters is recommended (*e.g.,* EG&G Berthold luminometers).

3. Test Principle

The Luciferase Reporter Gene Assay, high sensitivity contains all substrate components for the enzymatic determination of firefly luciferase activity as well as a lysis buffer for the mild and rapid extraction of luciferase from eukaryotic cells.

In a first step, transfected cells are lysed by addition of the lysis buffer. Subsequently, the cell extract is transferred to a microplate or tube, depending on the type of equipment used. The enzymatic reaction is then started by adding the luciferase assay reagent which contains all the components required for starting the chemiluminescence reaction. The luciferase assay reagent may be added manually or by automated injector. Photon emission is quantitated by using a luminometer or alternative equipment like a scintillation counter.

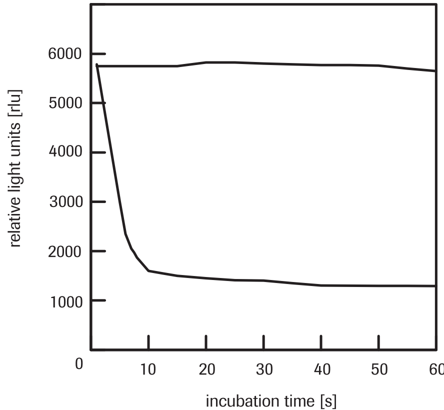

Fig. 1: Influence of coenzyme A (CoA) on luciferase light emission kinetics. The Luciferase Reporter Gene Assay, high sensitivity, which contains CoA (upper curve), in comparison to a standard luciferase assay (24) without CoA (lower curve). The enzyme reaction was performed in black microplates, using purified luciferase, and started by adding the luciferin-containing solution (luciferase assay reagent) or a luciferase reaction buffer with no CoA added, using the injector of an automated microplate luminometer (MicroLumat LB 96, EG&G Berthold). Measurement was started with a delay of 0.5 s after injection. In the reaction with no CoA added, more than 50% of the total light emission is produced within the first 10 s. The use of CoA leads to almost constant light production over 20–30 s which decays with a half-life of approx. 5 min

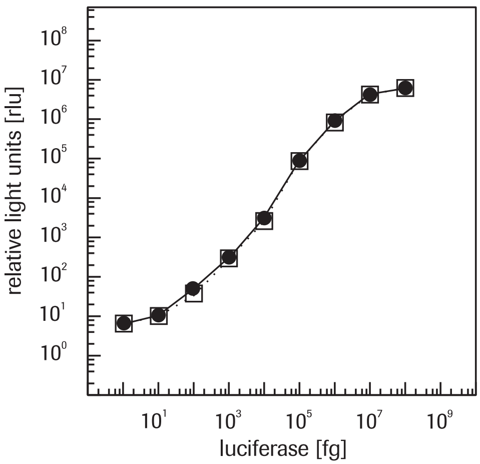

Fig. 2: Influence of the ratio between sample volume and luciferase assay reagent. The Luciferase Reporter Gene Assay, high sensitivity was performed with 100 µl luciferase assay reagent and either 10 µl (●) or 50 µl (❑) of sample containing purified luciferase, as described in Fig. 1.

4. Assay Characteristics

4.1 Sensitivity and Detection Range

Detection limit: 5 fg luciferase
Light emission is linear within a range of approx. 10 fg to 10 ng (see fig. 2).
The exact limits of the detection range depend on the measuring device used.

4.2 Specificity

The Luciferase Reporter Gene Assay, high sensitivity contains reagents for the determination of luciferase activity of the firefly, *Photinus pyralis* (*EC 1.13.12.7*), commonly used in transfection experiments. It is not suitable for the determination of luciferases of bacterial origin.

5. Equipment and Solutions

5.1 Additionally Required equipment and Solutions

The Luciferase Reporter Gene Assay, high sensitivity can be performed in all automated or manual luminometers in tube and microplate formates as well as in scintillation counters, or using photographic films. When using the microplate format, black or white microplates must be used (we recommend using black microplates).

Luciferase, *Photinus pyralis*, Cat. No. 10 411 523 001 (optional, for determination of the linear range of measuring device, see section 6).

5.2 Preparation and Stability of Solutions

Luciferase Assay Reagent

Prepare the luciferase assay reagent by adding 10 ml of the reaction buffer (bottle 1) to the lyophilized luciferase substrate (bottle 2). The luciferase assay reagent is an optimized ready-to-use formulation composed of a tricine buffer at pH 7.8 and containing luciferin, ATP, CoA and supplements, required for starting the enzyme reaction. The Luciferase assay reagent is stable for 12 months at −60°C or below, for 1 month at −15 to −25°C if stored in aliquots, or for 1 week at +2 to +8°C. Avoid repeated freezing and thawing. Under standard conditions 50–100 μl are required per single reaction. Store protected from light, as luciferin is oxidized when exposed to light.

Lysis Buffer

Prepare the lysis buffer by diluting 1 part of lysis buffer, 5× conc. (*e.g.,* 5 ml) with 4 parts redist. water (*e.g.,* 20 ml).
The lysis buffer is stable until the expiration date given on the kit if stored in aliquots at −15 to −25°C. Under standard conditions 250 μl are required per single reaction.

6. General Recommendations

Lysis of Eukaryotic Cells

The lysis buffer, supplied with the kit offers a rapid and gentle method for preparing cell extracts from eukaryotic cells that express firefly luciferase. The use of the lysis buffer achieves full maintenance of enzymatic activity. Another advantage of the lysis buffer is that it is fully compatible with ELISA and chemiluminescence procedures for the determination of CAT or β-Gal levels using the respective, non-isotopic reporter gene assays or ELISAs (see related products).
Mechanical cell lysis by sonication is also possible but may result in a reduction of enzymatic activity and is therefore not recommended. Mechanical lysis of cells is usually done by sonication in 5 cycles with approx. 5 s each. Between the cycles the extract must be kept on ice for several minutes to avoid over-heating, since luciferase is heat-labile.
Disrupting cells by freezing and thawing also leads to a significant reduction in enzymatic activity.

Lysis of Bacteria

Centrifuge a 1ml aliquot of an overnight culture (approx. absorbance $A_{600\ nm}$ = 0.5) for 3 min at 1000 rpm to pellet the bacteria. Discard the supernatant and add 0.5–1 ml of the bacteria lysis buffer (see below) and vortex. Incubate for 10–15 min at +15 to +25°C. Remove cell debris by centrifugation. The extract should be assayed within 20 min according to the basic protocol. If the resulting luciferase activity is too high or too low, alter the culture volume.
The extract may be stored frozen at or below −15 to −25°C for approx. one month.
The bacteria lysis buffer contains: 100 mM potassium phosphate buffer, pH 7.8, 2 mM EDTA, 1% Triton X-100*, 5 mg/ml BSA. Store at −15 to −25°C. Before use add 1 mM DTT and lysozyme (*e.g.,* dissolved in 100 mM potassium phosphate buffer, pH 7.8) to a final concentration of 5 mg/ml and equilibrate the buffer to +15 to +25°C.

Assaying Samples from Transgenic Animals

Blood samples of transgenic animals may be used directly for the determination of luciferase activity using the following protocol: Dilute 1 µl (or more) of the blood sample in 50 µl 100 mM potassium phosphate buffer, pH 7.8 supplemented with 5 mM NaCl, 2 mM DTT, 2 mM EDTA and assay immediately according to the basic protocol.
Alternatively, 1 µl blood can be assayed directly in 100 µl luciferase assay reagent without prior addition of lysis buffer.
Tissue samples must be broken down by rapid freezing in liquid nitrogen, followed by crushing the frozen tissue, *e.g.,* with a pestle in a mortar, to a fine powder. Resuspend the sample in lysis buffer and incubate for 10–15 min at +15 to +25°C. Pellet the debris by centrifugation of the solubilized sample in a centifuge. Assay the supernatant as described in the basic protocol.

Assaying Samples from Plant Tissue

Sample preparation is performed as described for tissue samples of transgenic animals.

Determination of the Linear Range of Light-Detecting Hardware

Adaptation of the Luciferase Reporter Gene Assay, high sensitivity to the detection device used is important because luminometers and scintillation counters experience signal over-flow at high photon intensity. To produce a calibration curve, relative light units (rlu) versus protein concentration, prepare serial dilutions of cell extracts or purified luciferase (see related products) in lysis buffer. When using purified luciferase, it is essential to supplement the lysis buffer with BSA (2.5 mg/ml).

⚠ **All reagents must be fully equilibrated to +15 to +25°C before starting the assay.**

Protein Determination

Results have to be normalized with respect to protein concentration or cell number. For protein determination use copper-based protein assays *e.g.,* according to Lowry (4). Be aware that higher detergent concentrations may interfere with determination methods. Therefore, check for interference or correct the calibration curve by addition of an equal amount of detergent lysis buffer. Protein determination should be performed in the linear range of the calibration curve. If absorbance in the sample is in the non-linear range, we recommend repeating the protein determination to obtain reliable results. Volumes of samples should be adjusted so that the absorbance of the sample falls within the linear range. Do not dilute the cell extracts before performing the protein determination.

Alternatively different methods for determination of cell numbers can be used for normalization *e.g.,* measurement of metabolic activity by cleavage of the tetrazolium salt WST-1*.

How to Use WST-1 Assay

① Perform cell culturing and transfection according to your standard protocol.
② 30-150 min before cell lysis, add 10% WST-1* reagent to the cell medium.
③ Quantify conversion of WST-1 directly from an aliquot, using an ELISA reader.
④ Withdraw reagent/medium and lyse cells for reporter gene assay.
⑤ Normalize reporter results according to the absorbance of the WST-1 assay.

7. Luciferase Assay, Basic Protocol

The protocol below is optimized for the use of eukaryotic cell cultures as sample material. The lysis buffer supplied with the Luciferase Reporter Gene Assay, high sensitivity is compatible with Roche non-isotopic CAT ELISA*, β-Gal ELISA*, and β-Gal Reporter Gene Assay, chemiluminescent* for quantification of cotransfected CAT- or β-Gal-vectors.

Extracts must be assayed immediately or they may be stored at or below −15 to −25°C for up to one month. Do not repeatedly freeze and thaw to avoid a decrease in luciferase activity.

① Aspirate the cell culture supernatant completely and carefully rinse cells twice with phosphate buffered saline (PBS), without calcium and magnesium, *e.g.,* 4 ml for 6 mm dishes. Remove remaining PBS completely *e.g.,* with a fine-tipped pipette.

② Add a minimum volume lysis buffer to cover the cells, *e.g.,* 250 µl for 60 mm dishes, 100 µl for 35 mm dishes. Scrape cells off the plate using a rubber policeman.

③ Transfer solubilized cells to a microcentrifuge tube and incubate at +15 to +25°C for a total of 15 min after addition of lysis buffer to the cells.

④ To remove cellular debris spin (5–10 s) the lysate in a microcentrifuge at maximum speed.

⑤ Transfer the supernatant in microcentrifuge tubes and start reaction immediately.

⑥ Before starting the chemiluminescence reaction, transfer 20–50 μl of the cell extract into an appropriate luminometer or scintillation counter device. We recommend using a 96-well microplate luminescence reader (*e.g.,* MicroLumat LB 96, EG&G Berthold). Start the reaction by injecting 100 μl luciferase assay reagent, **pre-equilibrated to +15 to +25°C**, automatically or by manual pipetting, depending on the equipment used. When using a scintillation counter, gently vortex the assay mixture immediately after addition of the luciferase assay reagent.

⑦ Start measurement of light emission within 0.5–10 s after adding luciferase assay reagent for a period of 1–5 s. Depending on the counting hardware used, or in the case of low luciferase activity, the measuring period can be prolonged (*e.g.,* up to 5 min). After an almost constant light emission over a period of 20 s, the light production decreases with a half-life of about 5 min.

We recommend performing an integrated light analysis, which is the sum of the total light production within a period of time, rather than performing a peak analysis.

If it is not possible to assay the extracts immediately after cell lysis, the samples may be kept on ice for approx. 5 h. Assaying a cold sample using the above protocol will result in a decrease of signal intensity in the range of 5–15%.

In case of high luciferase activity, resulting in a signal overflow, dilute samples with lysis buffer.

We do not recommend to store diluted samples. In case storage of diluted samples cannot be avoided, add 2.5 mg/ml BSA to the diluent to stabilize the diluted sample.

If the sample contains high levels of luciferase activity, a reduction of the sample volume is possible. In this case the volume of luciferase reagent buffer may be reduced correspondingly versus sample volume under standard conditions (100 μl : 20–50 μl) or may be maintained without any effect on the assay kinetics (see fig.2).

Some luminometers require an initial period of 1–2 s for stabilization of the detection system before measuring (for details, see working instructions of the particular luminometer). Therefore, we recommend starting the measurement only after a delay of a few seconds.

Some luminometers may not allow injection of the volume of luciferase reagent buffer given in the basic protocol (50–100 μl). In this case a higher volume of luciferase reagent buffer (*e.g.,* 300 μl) may be added without altering the sample volume (see also fig. 2)

If scintillation counters are used, introduce every sample individually immediately after starting the reaction by adding the luciferase assay reagent to each individual tube.
The automated circuit should be switched off to avoid delays (see brand specific information).

8. Luciferase Assay, Alternative Extraction (freeze and thaw)

This method (24) is more time-consuming and may produce a significant decrease of luciferase activity. It is included, since it is compatible with the isotopic standard CAT-Assay (25) for experiments using cotransfected CAT vectors.

❶ Wash cells as described in basic protocol

❷ Add 1 ml extraction buffer (100 mM potassium phosphate pH 7.8; directly before use add 1 mM DTT) to each dish and scrape cells immediately with a rubber policeman.

❸ Transfer cells to a microcentrifuge tube and spin down for 30 s.

❹ Remove supernatant and resuspend the pellet in 100 µl extraction buffer.

❺ Freeze cells in dry ice/ethanol for 5 min and thaw in a +37°C water bath for three cycles.

❻ Pellet debris by centrifugation in a refrigerated microcentrifuge for 5 min at maximum speed.

❼ For assaying for luciferase activity, further process the supernatant as described in the basic protocol.

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10. Ordering Guide

Product	Pack size	Cat. No.
X-tremeGENE 9 DNA Transfection Reagent	0.4 ml 1 ml 5 × 1 ml	06 365 779 001 06 365 787 001 06 365 809 001
X-tremeGENE HP DNA Transfection Reagent	0.4 ml 1 ml 5 × 1 ml	06 366 244 001 06 366 236 001 06 366 546 001
DOTAP Liposomal Transfection Reagent	2 ml (5× 0.4 ml)	11 202 375 001
CAT ELISA	1 kit (192 tests)	11 363 727 001
β-Gal ELISA	1 kit (192 tests)	11 539 426 001
β-Gal Reporter Gene Assay, chemiluminescence	1 kit (500 assays, MP format 250 assays, tube format)	11 758 241 001
β-Gal Staining Set	1 Set (for 100 tests in 3.5 cm dishes)	11 828 673 001
SEAP Reporter Gene Assay, chemiluminescent	1 kit (500 assays, MP format 250 assays, tube format)	11 779 842 001
hGH ELISA	1 kit (192 tests)	11 585 878 001
G-418	20 ml 100 ml	04 727 878 001 04 727 894 001
Hygromycin B	1 g (20 ml) filtered through 0.2 µm pore size membrane	10 843 555 001

11. Quick reference protocols

11.1 Required solutions

Solution	Content	Volume per reaction	Used for (see: 11.2)
1	**Luciferase assay reagent:** Add 10 ml of the reaction buffer (bottle 1) to the lyophilized luciferase substrate (bottle 2). Store in aliquots, protected from light at or below −15 to −25°C.	50 – 100 µl	step 4
2	**Lysis buffer** (bottle 3, black cap): Dilute 1 part lysis buffer, 5× conc. in 4 parts redist. water Store in aliquots at −15 to −25°C.	250 – 1000 µl	step 6

11.2 Working procedure flow chart: Basic protocol

Steps	Procedure	Volume per sample	Time/ Temperature
	Check the linear detection range of the detection hardware used (single determination)		
	Pre-equilibrate all reagents and samples fully to +15 to +25°C before starting the assay		+20 to +25°C
1	Wash cells twice with PBS, completely remove PBS	4 ml	+15 to +25°C
2	Add lysis buffer	250 – 1000 µl	+15 to +25°C
3	Scrape and transfer cells to a tube		+15 to +25°C
4	Lyse cells		15 min/ +15 to +25°C
5	Centrifuge in a microcentrifuge		5 s/ +15 to +25°C
6	Transfer supernatant to an appropriate counter device	20 – 50 µl	+15 to +25°C
7	Add luciferase reagent buffer	50 – 100 µl	+15 to +25°C
8	Measure light emission within 1–10 s after starting at 562 nm (alternatively measure up to 5 min)		1 – 2 s/ +15 to +25°C

12. Changes to previous version

- Editorial changes

13. Conventions

Text Conventions

To make information consistent and memorable, the following text conventions are used in this document:

Text Convention	Use
Numbered stages labeled ①, ②, *etc.*	Stages in a process that usually occur in the order listed
Numbered instructions labeled ❶, ❷, *etc.*	Steps in a procedure that must be performed in the order listed
Asterisk *	Denotes a product available from Roche Diagnostics

Symbols

In this document, the following symbols are used to highlight important information:

Symbol	Description
(Information Note symbol)	Information Note: Additional information about the current topic or procedure.
(Important Note symbol)	Important Note: Information critical to the success of the procedure or use of the product.

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