FOR RESEARCH USE ONLY

steadylite plus

High Sensitivity
Luminescence Reporter Gene Assay System

PerkinElmer®

DOMINIQUE DUTSCHER SA

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1. Introduction

Luciferase from the North American firefly *(Photinus pyralis)* is one of the most frequently used enzymes for reporter gene assays. Firefly luciferase catalyzes the oxidation of the firefly-specific substrate D-luciferin to produce light. This reaction is extremely efficient and the quantum yield is the highest of any characterized bioluminescent reaction. The bright light produced from the reaction makes firefly luciferase a valuable enzyme for reporting promoter activity.[1,2]

Light emission results from multi-step reactions. The initial reversible reaction step is the activation of D-luciferin in the presence of ATP, Mg^{2+} and luciferase enzyme which leads to enzyme bound adenyl-luciferin with the elimination of inorganic pyrophosphate (PP_i).

Subsequent reaction steps involve the oxidation of adenyl-luciferin with molecular oxygen via adenyl-oxyluciferin to yield AMP, CO_2 and oxyluciferin. The oxyluciferin is generated in an electronically excited state which emits light upon transition to the ground state. The overall reaction is shown in Figure 1.

When light emission is initiated by the addition of luciferase to a reaction mixture containing ATP, Mg^{2+} and D-luciferin in the presence of oxygen, a fast increase in light intensity can be seen followed by a rapid decrease to

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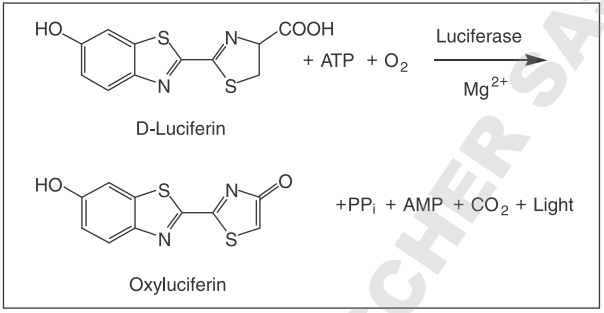


Figure 1: *Reaction scheme*

a low level of sustained light (flash-type kinetics). These flash-type kinetics have been thought to be the result of the formation of intermediate product (adenyl-oxyluciferin) at the enzyme surface which inhibits the enzyme.[4]

Several substances have been described that stimulate the light production by promoting the release of the inhibitor from the enzyme.[3,4] The enhancement in enzyme turnover yields an increase in light output.

The PerkinElmer steadylite plus assay system is a proprietary formulation that modifies the enzymatic reaction to produce a long lasting light output at high signal intensity.

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2. steadylite plus description

steadylite plus is a homogeneous long-lived glow type high sensitivity firefly luciferase reporter gene assay system for the quantification of firefly luciferase expression in mammalian cells. The reagent formulation contains compounds that facilitate both cell lysis as well as the luciferase enzymatic reaction. steadylite plus is designed for batch-processing systems using microplates in high throughput environments. A long signal half-life of the luminescent reaction is important in batch-processing systems where a large number of processed plates can wait in stacks before measurement. In general, steadylite plus has a signal half-life of more than four hours allowing large numbers of plates to be read within several hours without a large change in signal.
steadylite plus does not contain thiol compounds like dithiothreitol (DTT) and is therefore odor free.

steadylite plus offers the following benefits:

- High assay sensitivity
- Designed for batch-processing
- Long lasting signal
- Suitable for higher density microplates such as 384-well and 1536-well plates
- Odor free
- Convenient storage conditions (2 - 8 °C)

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The high light output of steadylite plus is proportional to the firefly luciferase concentration in the sample and allows detection of low levels of luciferase in microplate formats. Figure 2 shows the assay result of a dilution series of firefly luciferase enzyme in Dulbecco's PBS/0.1% BSA (100 µL per well) using steadylite plus in a white 96-well OptiPlate™ (PerkinElmer) measured with the PerkinElmer TopCount® NXT Microplate Scintillation and Luminescence Counter. As can be seen, steadylite plus allows for detection of very low levels of luciferase (femtogram range) with excellent linearity.

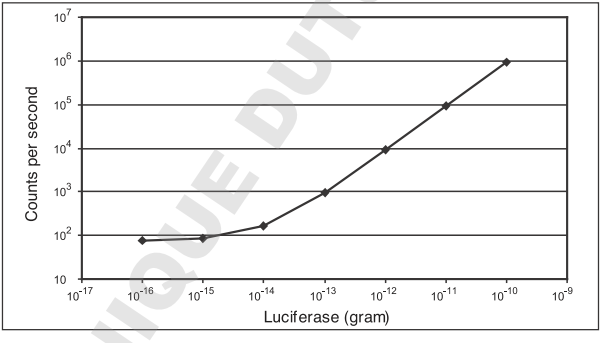


Figure 2: *Sensitivity of steadylite plus*

Figure 3 highlights the time course of the light output of the experiment above for 10^{-10} gram luciferase per well. After steadylite plus addition, an incubation time of 10 to 15 minutes is needed for full signal generation.

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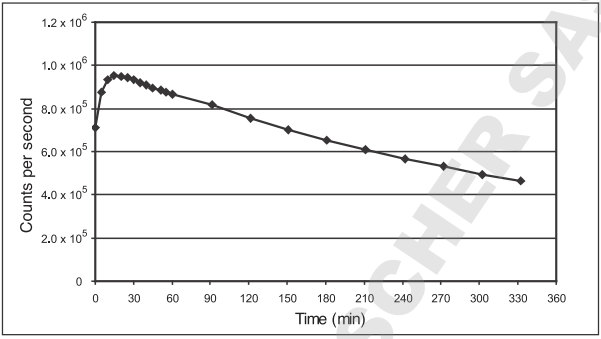


Figure 3: *Time course of the luminescent signal of steadylite plus*

To assess the influence of different culture media on luminescence light output, the following experiment was performed using commercially available basal media in the presence and absence of Fetal Bovine Serum (FBS) and Phenol Red (PR). 100 µL steadylite plus was added to 100 µL samples of the different media in a white 96-well OptiPlate, where each sample contained luciferase at a concentration of 2 x 10^{-9} gram per mL. After shaking the plate, the light output was measured using a TopCount NXT. Figure 4 illustrates the results of the relative luminescence after 15 minutes count delay. Figure 5 shows the results of the stability of the signal as a function of the culture media used.

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The results from Figure 4 show that the light output is to some extent affected by FBS but that Phenol Red has a much larger impact on the light output reflecting the concentrations of Phenol Red in the basal media. Consequently the use of Phenol Red in culture media should be avoided to attain the highest signal. The stability of the luminescent signal is only somewhat dependent on the media as shown in Figure 5.

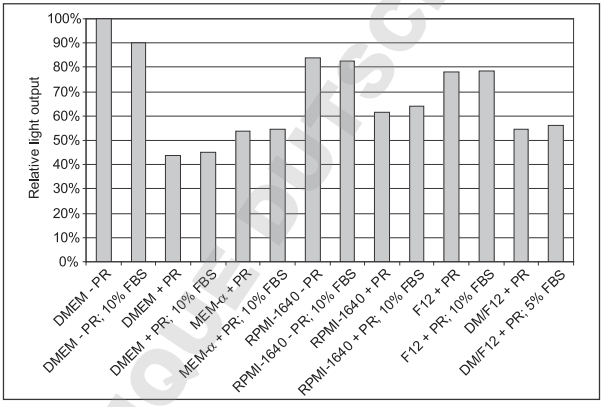


Figure 4: *Relative light output in different media*

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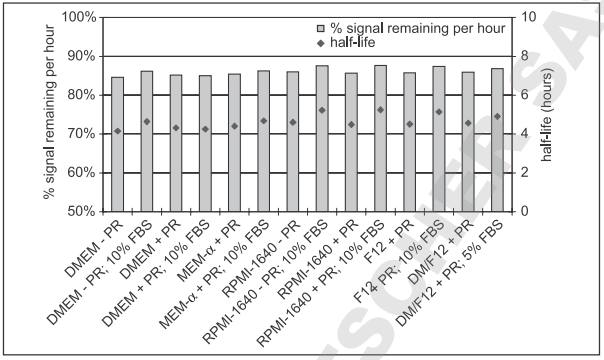


Figure 5: *Signal stability in different media expressed as a percentage of signal remaining per hour and half-life (hours)*

Organic solvents are often used to introduce screening compounds, resulting in the presence of a small percentage of organic solvent in the culture medium. The effect of organic solvents on the light output of steadylite plus was investigated in the following experiment. Luciferase in culture medium (DMEM without Phenol Red, supplemented with 10% FBS) was added at 100 µL per well to a white 96-well CulturPlate™ (PerkinElmer). The medium contained various concentrations of organic solvents (DMSO, ethanol, isopropanol and acetonitrile). Next, 100 µL of steadylite plus was added to the wells. After shaking, the plate was sealed and the luminescence was measured using TopCount NXT. The results

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presented in Figure 6 show that DMSO does not affect the signal at the indicated solvent concentrations. A decrease in signal was shown for the other solvents which was dependent on the specific solvent concentration. The stability of the signal as shown in Figure 7 is not compromised when using these four solvents at the tested concentrations.

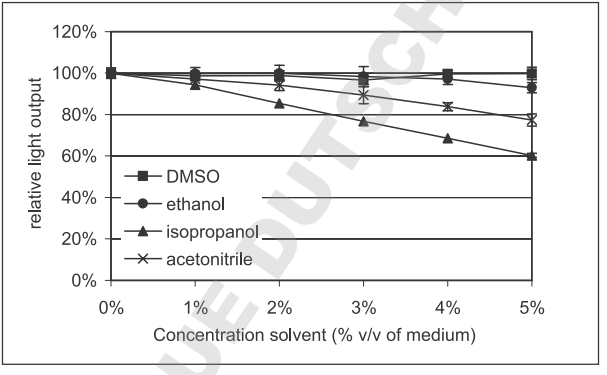


Figure 6: *Relative light output in the presence of organic solvents*

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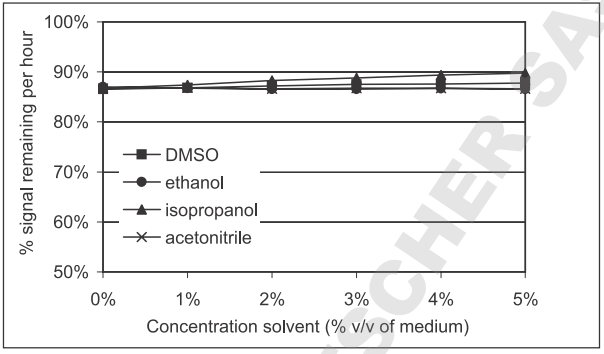


Figure 7: *Signal stability in the presence of organic solvents as a percentage of signal remaining per hour*

3. Contents and storage of steadylite plus

6066756 – steadylite plus 10 mL

Each 10 mL kit contains the following components:

- 1 vial steadylite plus Lyophilized Substrate
- 1 bottle steadylite plus Reconstitution Buffer
- Manual

6066751 – steadylite plus 100 mL

Each 100 mL kit contains the following components:

- 10 vials steadylite plus Lyophilized Substrate
- 1 bottle steadylite plus Reconstitution Buffer
- Manual

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6066759 – steadylite plus 1000 mL

Each 1000 mL kit contains the following components:

- 4 bottles steadylite plus Lyophilized Substrate
- 2 bottles steadylite plus Reconstitution Buffer
- Manual

Number of data points per part number*

steadylite plus part number	kit size	96-well plate	384-well plate	1536-well plate
6066756	10 mL	100	400	3,300
6066751	100 mL	1,000	4,000	33,000
6066759	1000 mL	10,000	40,000	330,000

* Based on recommended volumes per well:

100 µL for 96-well, 25 µL for 384-well and 3 µL for 1536-well plate

Storage conditions:

steadylite plus is shipped at ambient temperature and must be stored at 2 - 8 °C upon receipt.

If stored at the recommended conditions, the kit components are stable through the expiry date found on the kit label.

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4. Additional requirements

A detection instrument such as the PerkinElmer TopCount, MicroBeta®, VICTOR™ Light, VICTOR^3 Multi Label Reader, EnVision™ or EnSpire® is required.
CCD camera systems, such as PerkinElmer ViewLux™ can be used for high throughput applications.

For optimum light yield, low background and minimum well-to-well crosstalk, white microplates should be used. We recommend the use of the PerkinElmer CulturPlate, OptiPlate or ViewPlate® (when visual inspection of cells is preferred). Black plates can also be used when very high signals are expected. Black plates will reduce well-to-well crosstalk but will also quench the light output.

5. Assay procedure

1. Equilibrate the kit components to room temperature (20 - 22 °C) before reconstitution.

2. For the **10 mL** and the **100 mL** kit reconstitute one vial of steadylite plus Lyophilized Substrate with **10 mL** of steadylite plus Reconstitution Buffer.

   For the **1000 mL** kit reconstitute one bottle of steadylite plus Lyophilized Substrate with **250 mL** of steadylite plus Reconstitution Buffer.

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Mix the contents of the vial gently by inversion and leave for 5 minutes. This should result in a clear homogeneous solution.
Keep the steadylite plus reagent at room temperature (20 - 22 °C) before use.

3. Only prepare as much steadylite plus reagent as needed for one day.

4. Add steadylite plus reagent to each well. Equal volumes of cell culture medium and steadylite plus reagent should be used.

   For **96-well** plates: add **100 µL** to each well containing **100 µL** of cells in medium.

   For **384-well** plates: add **25 µL** to each well containing **25 µL** of cells in medium.

   For **1536-well** plates: add **3 µL** to each well containing **3 µL** of cells in medium.

5. Mix the well contents (see section 7).

6. Seal the plate with self-adhesive TopSeal™-A.

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7. Wait 10 to 15 minutes for complete cell lysis and to allow full signal generation.

8. Measure luminescence.

6. Stability of steadylite plus reagent

Stability of reconstituted steadylite plus is approximately:

> 90 % remaining signal after 8 hours at 20 °C
> 85 % remaining signal after 24 hours at 20 °C
> 75 % remaining signal after 48 hours at 20 °C
> 95 % remaining signal after 24 hours at 4 °C
> 90 % remaining signal after 48 hours at 4 °C

Freshly prepared reagents can be aliquoted and stored for one month at -20 °C and for three months at -80 °C.

The reagents can be subjected to at least 10 freeze - thaw cycles without significant loss of activity.

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7. Recommendations for use

- Mixing of culture medium and reagent is vital to obtain low coefficients of variation between replicates. This is especially important with 384- and 1536-well plates. Optimize liquid handling procedures to attain optimal reagent/medium mixing. For detailed background information see reference 5.

- Phenol Red, as well as other colored compounds, will chemically not interfere with the luciferin/luciferase reaction, but will quench some of the emitted light, resulting in lower assay signals (see Figure 4). For optimal light output, the culture medium can be substituted with **Dulbecco's PBS containing calcium and magnesium ions** prior to the addition of steadylite plus.

- The luciferase reaction requires magnesium ions. Although steadylite plus does contain these ions it is strongly recommended that the sample contains sufficient amount of these ions. Normal culture media contain these ions at sufficient levels.

- When handling the plates prior to measurement, work under subdued light conditions and avoid direct sunlight or bright fluorescent light. Bright light may cause plate phosphorescence resulting in higher

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background levels. Plate phosphorescence has a half-life of several minutes.

- If more than one vial of substrate is reconstituted, it is advised to pool all reagents before addition to the plates.

- Optimal room and instrument temperature is 22 °C. Allow plates to adapt to room temperature after removal from the incubator and prior to the addition of the reagent. An adaptation time of 30 minutes is usually sufficient. Stacked plates will need more time to adapt to room temperature.

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8. Ordering Information

steadylite plus	Reorder No.
10 mL steadylite plus assay kit	6066756
100 mL steadylite plus assay kit	6066751
1,000 mL steadylite plus assay kit	6066759

For further information on luminescence readers, microplates, seals and luminescence applications please contact your local PerkinElmer representative or visit our website: **http://www.perkinelmer.com**

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5. Hancock M.K., Medina M.N., Smith B.M. and Orth A.P.. Microplate Orbital Mixing Improves High-Throughput Cell-Based Reporter Assay Readouts. J. Biomol. Screen. 2006 Nov 27. 2 2007; vol. 12: pp. 140 - 144

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U.S. Pat. 7425422 and 7951551; European Pat. 1885874 and foreign equivalent patents.

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