MANUAL

Technology: Luminescence Assay Systems

neolite

High Sensitivity
Luminescence Reporter Gene Assay System

For research only. Not for use in diagnostic procedures

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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 (PPi).

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.

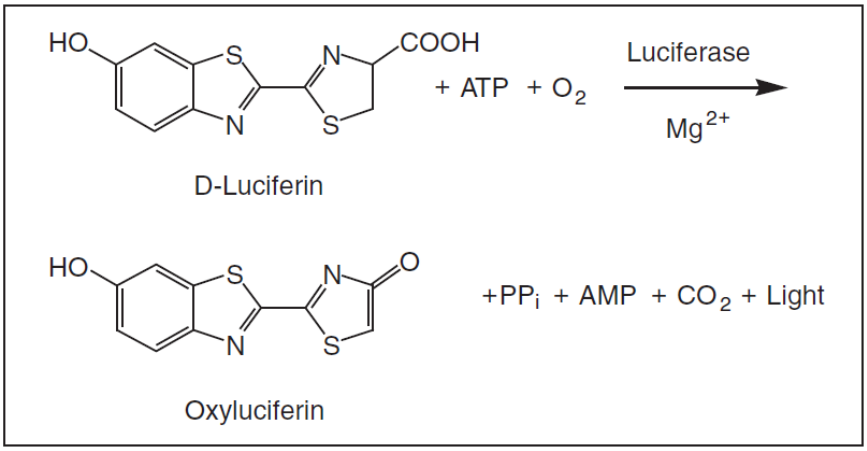

Figure 1: Reaction scheme

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 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 Revvity neolite assay system is a proprietary formulation that modifies the enzymatic reaction to produce a longer lasting light output at high signal intensity.

2 neolite description

neolite is a homogeneous, high sensitivity, long-lived glow type 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. neolite has been designed to cover the applications where a high light intensity is required together with an extended half-life. neolite is designed for systems that require increased sensitivity with the benefit of an extended detection time (Figure 2). neolite generates a luminescent signal that is both bright and stable. In general neolite has a signal half-life of more than 2 hours at high signal intensity. This means that for maximal light intensity the luminescence should be measured within 45 minutes after reagent addition. Nevertheless, the luminescent signal produced by neolite can be even measured after 2.5 hours. At that point the signal will still be brighter than the signal generated by steadylite plus™. In addition, neolite contains no thiol compounds like dithiothreitol (DTT) and is therefore virtually odor free.

In summary, neolite offers the following benefits:

- High assay sensitivity
- Suitable for both batch- and continuous-processing of plates
- Bright and stable signal
- Suitable for higher density microplates such as 384-well and 1536-well plates
- Thiol odor free
- Convenient storage conditions, long term (2-8°C); 1 month at max. 22°C.

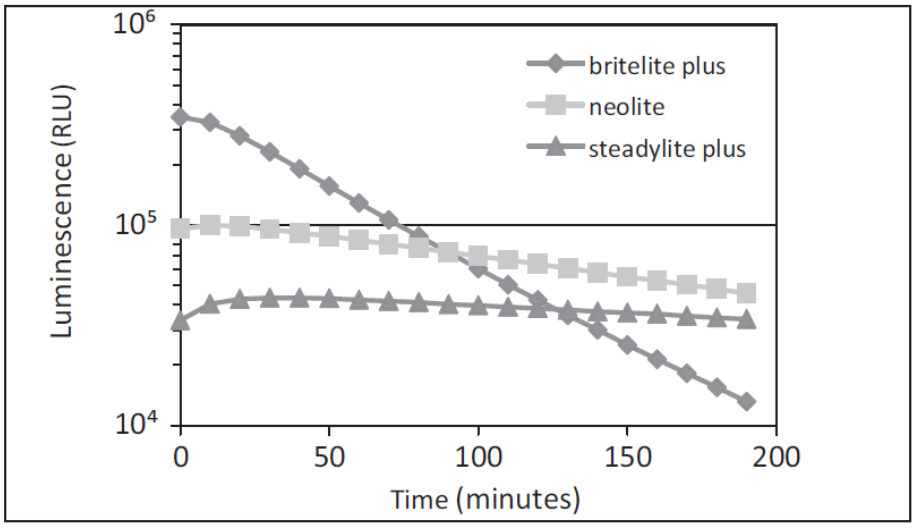

Figure 2: neolite compared to steadylite plus and britelite™ plus.
Samples consisted of 2000 CCL-64 (luciferase expressing) cells per well, incubated for two days in 25 µL DMEM/10%FBS (without phenol red) in a 384-well plate, hereafter combined with 25 µL of the different reagents in a 384-well white CulturPlate. Luminescence was measured on a Revvity EnVision™.

The high light output of neolite is proportional to the firefly luciferase concentration in the sample and allows detection of low levels of luciferase in microplate formats. Figure 3 shows the assay result of a dilution series of firefly luciferase enzyme in Dulbecco's PBS/0.1% BSA (100 µL per well) using neolite in a 96-well OptiPlate™ (Revvity) measured with the Revvity TopCount® NXT Microplate Scintillation and Luminescence Counter. As can be seen, neolite allows for detection of low levels of luciferase (femtogram range) with excellent linearity.

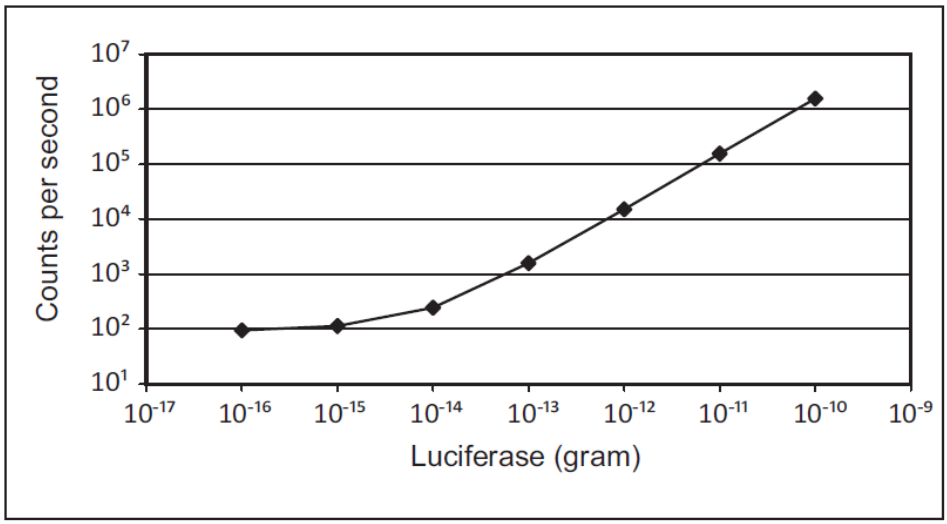

Figure 3: Sensitivity of neolite

Figure 4 highlights the time course of the light output of the described experiment above for 10^{-10} gram luciferase per well. After neolite addition, an incubation time of up to 5 minutes is required for full signal generation.

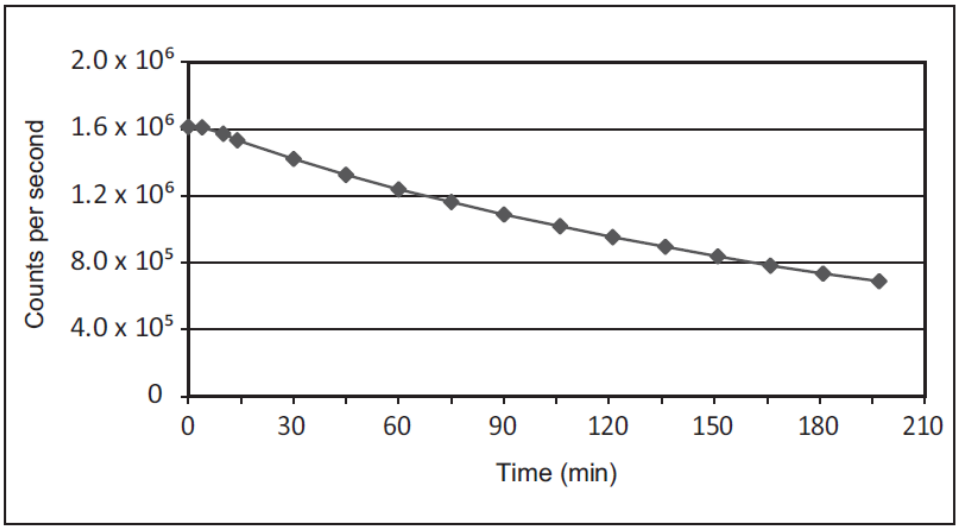

Figure 4: Time course of the luminescent signal of neolite

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 neolite was added to 100 µL samples of the different media in a white 96-well CulturPlate™ (Revvity), where each sample contained firefly luciferase at a concentration of 10-8 gram per mL. After shaking the plate, the light output was measured using an EnVision in Enhanced Luminescence Mode. Figure 5 illustrates

the results of the relative luminescence after 5 minutes count delay. Figure 6 shows the results of the stability of the signal as a function of the culture media tested.

The results as shown in Figure 5 indicate that FBS has a minor effect on the light output. However, the presence of Phenol Red in the basal medium has a larger impact. The reduction in light output reflects the Phenol Red concentration in the media. The stability of the luminescent signal is only slightly dependent on the tested media as shown in Figure 6.

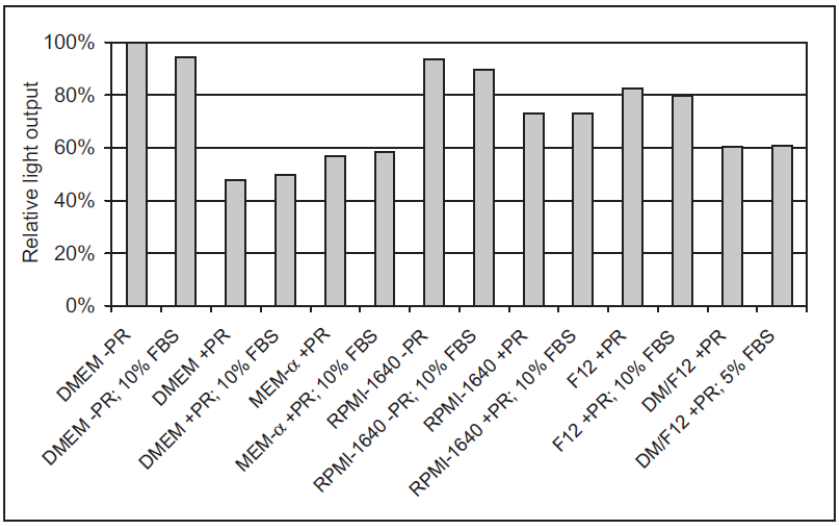

Figure 5: Relative light output in different media

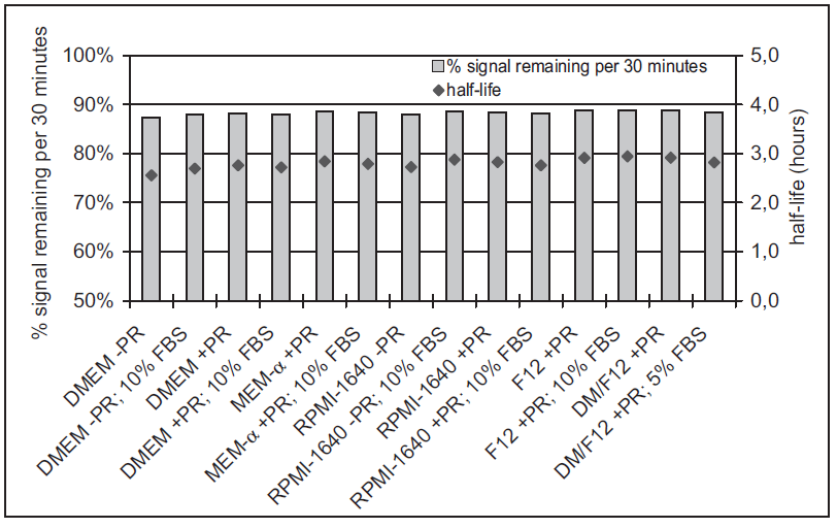

Figure 6: Signal stability in different media expressed as a percentage of signal remaining per 30 minutes and half-life (hours)

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 neolite 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. The medium contained various concentrations of organic solvents (DMSO, ethanol, isopropanol and acetonitrile). Next, 100 µL of neolite was added to the wells. After shaking, the plate was sealed and the luminescence was measured using an EnVision in Enhanced Luminescence Mode.

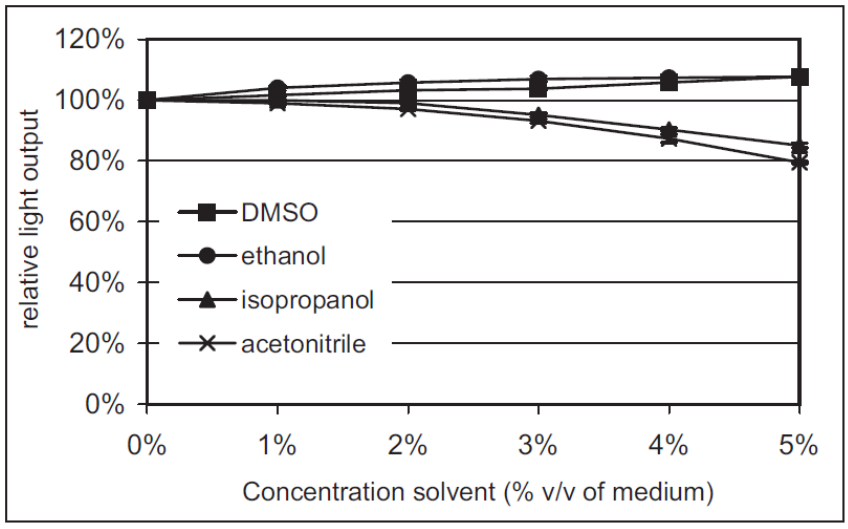

Figure 7: Relative light output in the presence of organic solvents

The results presented in Figure 7 show that DMSO and ethanol do 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 8 is not compromised when using these four solvents at the tested concentrations.

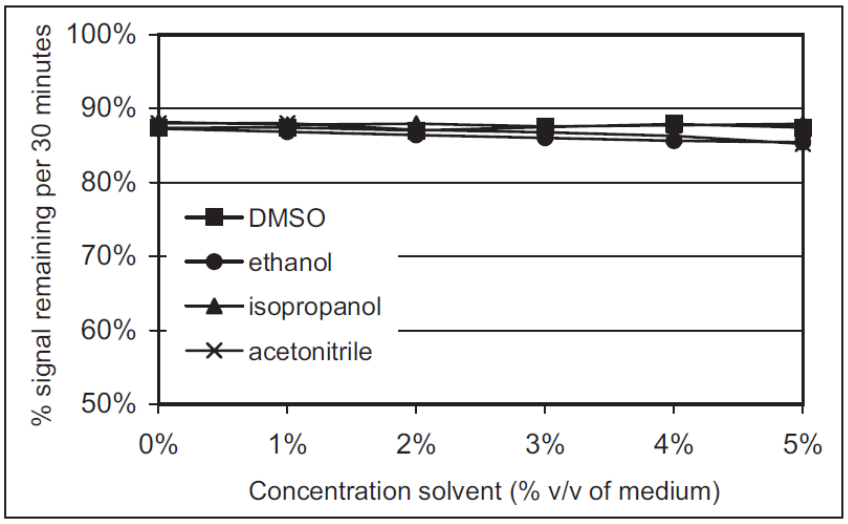

Figure 8: Signal stability in the presence of organic solvents expressed as a percentage of signal remaining per 30 minutes

3 Contents and storage of neolite

6016716 – neolite 10 mL kit

Each 10 mL kit contains the following components:

- 1 vial neolite Lyophilized Substrate
- 1 bottle neolite Reconstitution Buffer
- Manual

6016711 – neolite 100 mL kit

Each 100 mL kit contains the following components:

- 10 vials neolite Lyophilized Substrate
- 1 bottle of neolite Reconstitution Buffer
- Manual

6016719 – neolite 1000 mL kit

Each 1000 mL kit contains the following components:

- 4 bottles neolite Lyophilized Substrate
- 2 bottles neolite Reconstitution Buffer
- Manual

Number of data points per part number*

neolite part number	Kit size	96-well plate	384-well plate	1536-well plate
6016716	10 mL	100	400	3,300
6016711	100 mL	1,000	4,000	33,000
6016719	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

neolite is shipped at ambient temperature and must be stored at 2-8°C upon receipt for long term storage.

For convenience, the neolite Reconstitution Buffer can be stored separately at room temperature (max. 22°C). This allows addition of the Reconstitution Buffer to the Lyophilized Substrate without the need to equilibrate to room temperature. The Lyophilized Substrate can also be stored at room temperature (max. 22°C) for 1 month.

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

4 Additional requirements

A detection instrument such as the Revvity TopCount, MicroBeta®, VICTOR™ Light, VICTOR3 Multi Label Reader, EnVisionTM or EnSpire® is required. CCD camera systems, such as Revvity 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 Revvity 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 neolite Lyophilized Substrate with 10 mL of neolite Reconstitution Buffer.
For the 1000 mL kit reconstitute one bottle of neolite Lyophilized Substrate with 250 mL of neolite Reconstitution Buffer.
Mix the contents of the vial gently by inversion and leave for 5 minutes. This should result in a clear homogeneous solution.
Keep the neolite reagent at room temperature (20 - 22 °C) before use.

3. Only prepare as much neolite reagent as needed for one day.

4. Add neolite reagent to each well. Equal volumes of cell culture medium and neolite 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.

7. For complete cell lysis and full signal generation wait at least 1 minute, but not longer than 15 minutes.

8. Measure luminescence within 15 minutes after reagent addition for maximum sensitivity.

6 Stability of neolite reagent

Stability of reconstituted neolite is approximately:

Stability neolite reagent		
Remaining activity		
Hours	**20°C**	**4°C**
8	> 90%	N.D.
24	> 75%	> 95%
48	> 60%	> 90%
72	N.D.	≈ 90%

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.

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.
- Optimal room and instrument temperature is 20-22°C. Higher temperatures will result in a decrease of the luminescence half-life.
- 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.
- In general, when handling 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 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.
- 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 5). For optimal light output, the culture medium can be substituted with (Dulbecco's) PBS prior to the addition of neolite.

8 Ordering information

neolite	Reorder No
10 mL neolite assay kit	6016716
100 mL neolite assay kit	6016711
1,000 mL neolite assay kit	6016719

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

9 References

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