MANUAL

Technology: Luminescence Assay Systems

twinlite glow

Firefly & *Renilla* Luciferase Dual Reporter Gene Assay System

Part number	6016796	6016799
Test size	10 mL	100 mL

Storage: 2-8°C

Version: 001 **Date:** 2023/09

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1. twinlite glow description

Reporter gene assays have been an important tool in screening and fundamental research since the 1980's. They have been used in applications ranging from gene transcription regulation research, including miRNA analysis, to high-throughput screening for drug development.

Reporter gene assays can be run using a single reporter gene, like firefly luciferase, but the quality of the assay can be greatly improved by the addition of a second reporter gene, like *Renilla* luciferase. The second reporter gene can be used either as a tool for cell number normalization, cell lysis efficiency, transfection efficiency, or as a reporter for a second cellular event.

twinlite glow, a dual reporter gene assay system, has been designed to detect and quantitate both Firefly and *Renilla* luciferase sequentially from cultured cells.

Firefly luciferase from the North American firefly (*Photinus pyralis*), one of the most frequently used enzymes for reporter gene assays, 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.

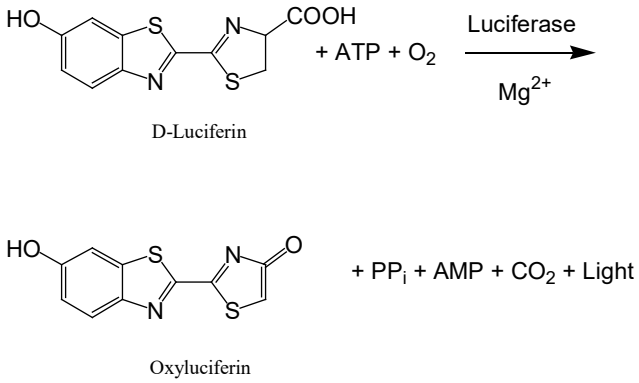


Figure 1. Assay principle for firefly luciferase-based technologies. The firefly luciferase reaction results in emitted light that is quantified with the use of a luminescence plate reader, whereby the measured luminescence signal is directly proportional to the amount of firefly luciferase present in the sample.

Similarly, *Renilla* luciferase catalyzes the oxidation of the substrate coelenterazine (coelenterate luciferin) to produce light (Figure 2). However, unlike the firefly reaction, *Renilla* luciferase luminescence does not require ATP or magnesium.

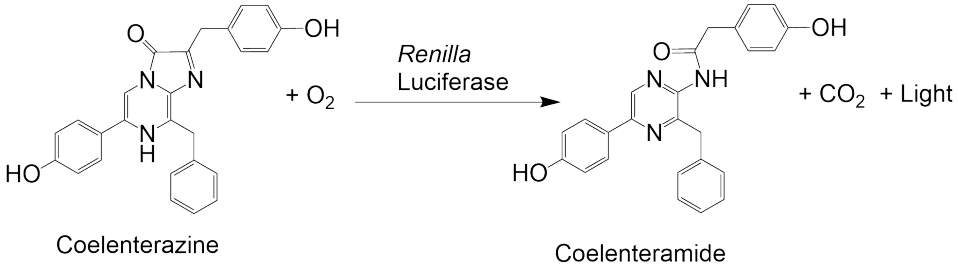


Figure 2. *Renilla* luciferase reaction.

twinlite glow is a dual luminescence reporter gene assay system for use in both high throughput systems as well as on small laboratory scale. The kit is designed to detect and quantitate both firefly (firelite glow) and *Renilla* luciferase (renlite glow) sequentially from cultured mammalian cells in the same well of a microplate allowing for the discrimination between specific and non-specific cellular responses through a control reporter. Also, the kit enables to measure the expression levels of two separate genes in a single assay.

twinlite glow offers the following benefits:

- Homogeneous assay system, no separate lysis steps
- Bright and stable luminescent signals
- Suitable for both batch- and continuous -processing of plates
- Reduced coelenterazine autoluminescence resulting in low background signals
- Stable working reagents, both firelite glow and renlite glow
- User friendly, with long-term storage of the components at 2 – 8°C; no need to thaw reagents before use
- Thiol odor free (no DTT present)
- Environmentally friendly such as ambient shipping conditions

The chemistry of the assay is designed so that the firelite glow reagent can be added directly to an equal volume of culture medium with mammalian cells without washing and separate lysis steps. Addition of the firelite glow reagent to the cells results in the lysis of the cells, release of both firefly and *Renilla* luciferase (the reporter enzymes). At the same time, it activates the firefly luciferase luminescence, which can be read after 10 minutes. The luminescent signal obtained has a half-life of approximately 2 hours.

Next, addition of renlite glow reagent stops the firefly luminescent reaction and quenches the firefly luminescence by at least 40,000-fold. Subsequently, it provides the *Renilla* luciferase substrate coelenterazine and the proper chemical environment for *Renilla* luminescence to start. The reagent contains a proprietary technique to minimize the coelenterazine autoluminescence, resulting in high sensitivity of the *Renilla* assay. The renlite glow luminescence can be read 10 minutes after addition, the half-life of the reaction is quite similar to that of the firelite glow.

2. Contents and Storage of twinlite glow

Contents of twinlite glow assay kits:

Table 1. Contents of twinlite glow assay kits

Kit part number Component	6016796 10 mL kit	6016799 100 mL kit
firelite glow		
Lyophilized Substrate	1 x 10 mL	2 x 50 mL
Reconstitution Buffer	1 x 11 mL	1 x 105 mL
renlite glow		
Lyophilized Substrate	1 x 10 mL	2 x 50 mL
Reconstitution Buffer	1 x 11 mL	1 x 105 mL

Table 2. twinlite glow assay points per kit. *

twinlite glow Part Number	Kit size	96-well plate	384-well plate
6016796	10 mL	100	400
6016799	100 mL	1,000	4,000

** Based on recommended volumes per well: 75 µL for 96-well and 20 µL for 384-well*

Storage conditions:

twinlite glow is shipped at ambient temperature and should be stored at 2 – 8 °C upon receipt for long term storage.

For convenience, both the firelite glow Reconstitution Buffer and the renlite glow Reconstitution Buffer can 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 as printed on the kit label.

Do not store the components and the working reagents in bright light.

3. Stability of twinlite glow working reagents

Table 3. Luminescence potency remaining of the working solutions at different storage conditions.

	firelite glow Reagent	renlite glow Reagent
Room Temperature (22°C)		
24 hours	90%	95%
48 hours	80%	91%
72 hours	73%	89%
Refrigerator (2 – 8°C)		
24 hours	99%	96%
48 hours	98%	95%
1 week	93%	95%
Freeze/Thaw Cycles (-80°C / 22°C)		
10 cycles	98%	97%

Remaining reagents can be stored at -20°C (≤ 6 months) or at -80°C (≤ 12 months).

4. Additional Requirements

A detection instrument such as the Revvity VICTOR® Nivo™, EnSight®, or EnVision® Nexus™ is required for detection of the luminescent signals produced in this assay.

For optimum light yield, low background and minimum well-to-well crosstalk, white microplates should be used. We recommend the use of the Revvity OptiPlate™, CulturPlate™ 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. Reagent preparation

1. Equilibrate the kit components to room temperature (20 - 22°C).
2. firelite glow Reagent

    - For the 10 mL kit, reconstitute one vial of firelite glow Lyophilized Substrate (10mL) with 10 mL of firelite glow Reconstitution Buffer. Mix the contents of the vial gently by inversion and leave for 5 minutes.
    - For the 100 mL kit, reconstitute one vial of firelite glow Lyophilized Substrate (50mL) with 50 mL of firelite glow Reconstitution Buffer. Mix the contents of the vial gently by inversion and leave for 5 minutes.

3. renlite glow Reagent:

    - For the 10 mL kit reconstitute one vial of renlite glow Lyophilized Substrate (10mL) with 10 mL of renlite glow Reconstitution Buffer. Mix the contents of the vial gently by inversion and leave for 5 minutes.
    - For the 100 mL kit reconstitute one vial of renlite glow Lyophilized Substrate (50mL) with 50 mL of renlite glow Reconstitution Buffer. Mix the contents of the vial gently by inversion and leave for 5 minutes.

6. Assay Procedure

1. Remove the microplate with the plated mammalian cells from the incubator. Make sure that the microplate is equilibrated to room temperature before proceeding to the next step. This takes at least 30 minutes.

2. **Firefly luciferase activity measurement:**

   - Add firelite glow reagent to wells containing cells with a volume equal to that of the medium in the wells.

Plate	Medium Volume	firelite glow Reagent
96-well	75 µL	75 µL
384-well	20 µL	20 µL

   - Mix the contents of the wells e.g., by using an orbital plate shaker for 1 minute.

Plate	Suggested Setting
96-well	700 RPM
384-well	1100 RPM

   - Measure firefly luciferase luminescence by reading the plate in a luminometer. Read the luminescence within 2 hours after addition of the **firelite glow** reagent for optimal results.

3. ***Renilla* luciferase activity measurement:**

   - Add renlite glow reagent to wells containing cells with a volume equal to that of the original medium in the wells.

Plate	Medium Volume	renlite glow Reagent
96-well	75 µL	75 µL
384-well	20 µL	20 µL

- Mix the contents of the wells e.g., by using an orbital plate shaker for 1 minute.

Plate	Suggested Setting
96-well	700 RPM
384-well	1100 RPM

- Measure *Renilla* luciferase luminescence by reading the plate in a luminometer. Read the luminescence within 2 hours after addition of the **renlite glow** reagent for optimal results.

twinlite glow assay procedure

Assay using a 96-well plate

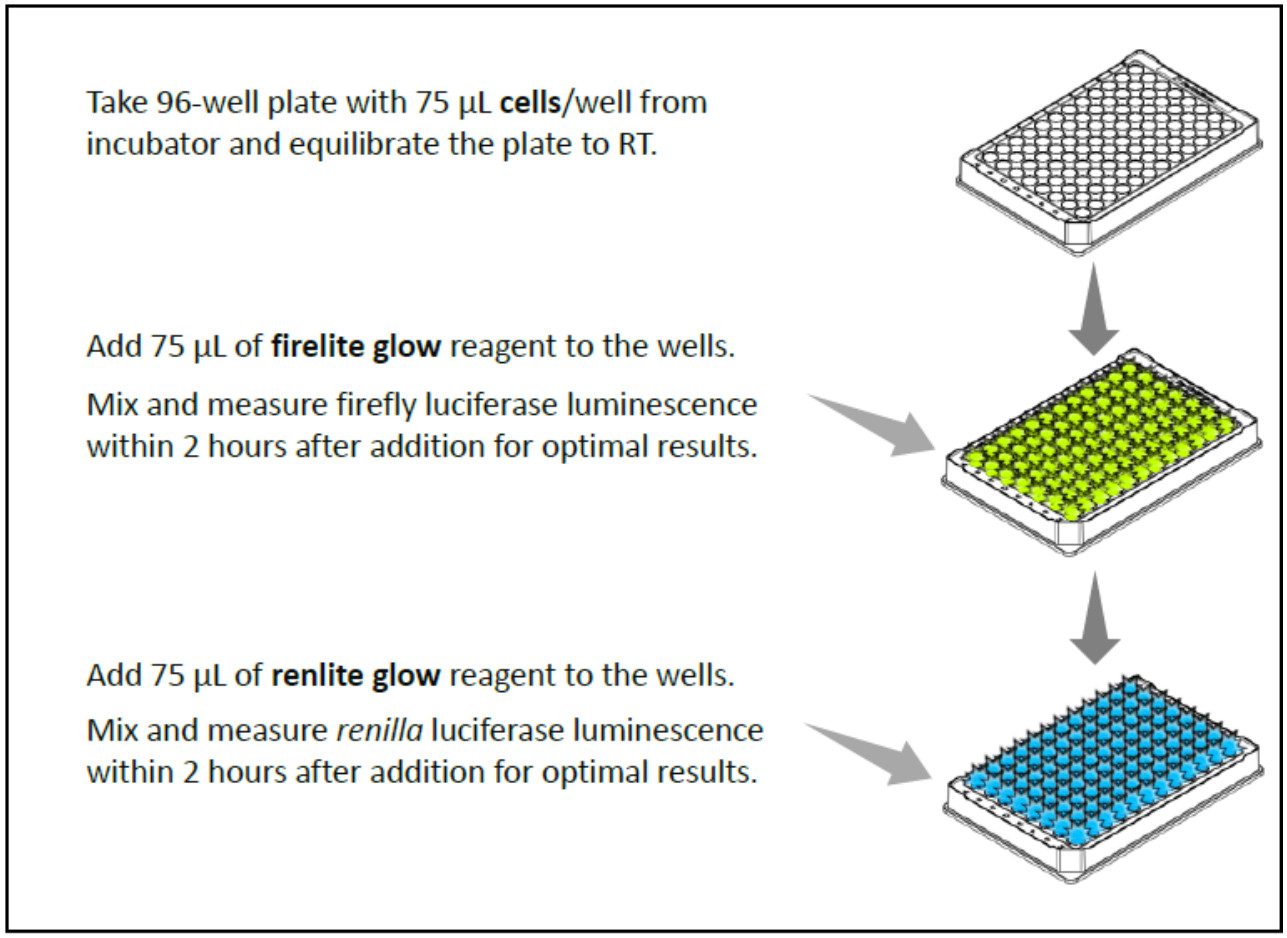


Figure 3. Schematic representation of twinlite glow assay protocol in a 96-well plate.

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-well plates. Optimize liquid handling procedures to attain optimal reagent/medium mixing. For detailed background information see reference 3.

- Phenol red, as well as other colored compounds, will chemically not interfere, but will quench some of the emitted light, resulting in lower assay signals. For optimal light output, the culture medium can be substituted with **Dulbecco's PBS containing calcium and magnesium ions** prior to performing the twinlite glow assay. Alternatively, phenol red free media can be used if available.

- The firefly luciferase reaction requires magnesium ions. Although firelite glow 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 microplates 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.

- 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.

8. twinlite glow Assay Features

Luminescence kinetics:
The luminescent signals for both firefly and *Renilla* decay slowly with a half-life of at least 2 hours each as shown in Figure 4. This means that the assay can be performed with a luminometer without reagent dispensers typically required of flash-type kinetic luminescent reagents.

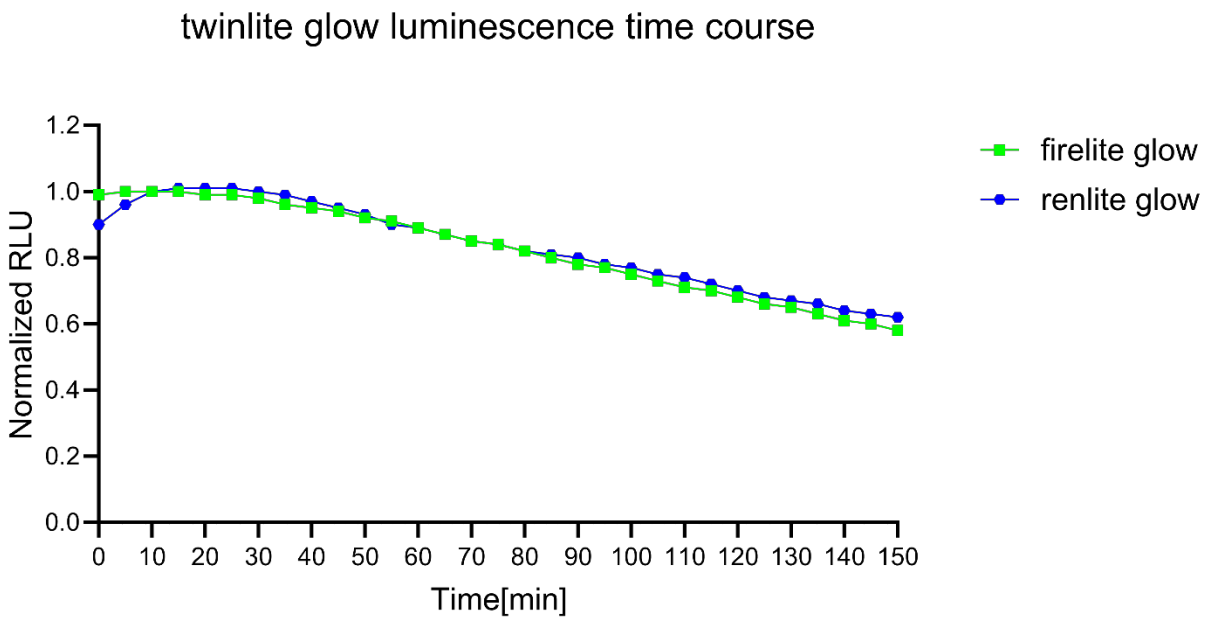


Figure 4. Firefly and *Renilla* luciferase luminescence kinetics of the twinlite glow assay system.

Luminescence spectra:

As seen in Figure 5, the luminescent spectra of firelite glow and renlite glow overlap. This means that it is difficult to measure both reactions simultaneously and to distinguish between the signals.

The twinlite glow dual-luciferase assay system solves this problem by first measuring the firefly luciferase present in the sample by the addition of the firelite glow reagent, followed by the measurement of the *Renilla* luciferase signal after the addition of the renlite glow reagent. To obtain two separate luminescent signals from the same well, the firefly luciferase reaction needs to be quenched to detect the light produced by the *Renilla luciferase* reaction. The quench of the firefly luminescent signal is achieved by the addition of the renlite glow reagent.

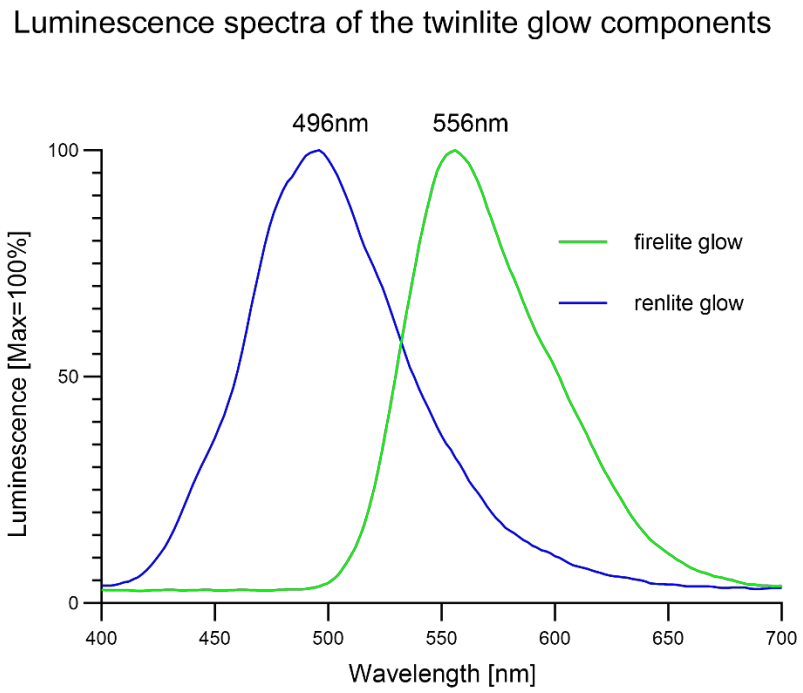


Figure 5. Luminescence spectra of firelite glow and renlite glow luminescence

Firefly luminescence quench:

To confirm the effective quenching of firefly luciferase luminescence by renlite glow reagent, an equal volume of the reagent was added to a firefly luciferase sample which was supplemented with firelite glow reagent. All signals were recorded 10 minutes after each addition of the reagents to the firefly luciferase sample.

As shown in figure 6 the firefly signal was quenched approximately 100,000 times leaving only 0.001% firefly signal. This means that the residual firefly signal will minimally affect the *Renilla* luciferase signal.

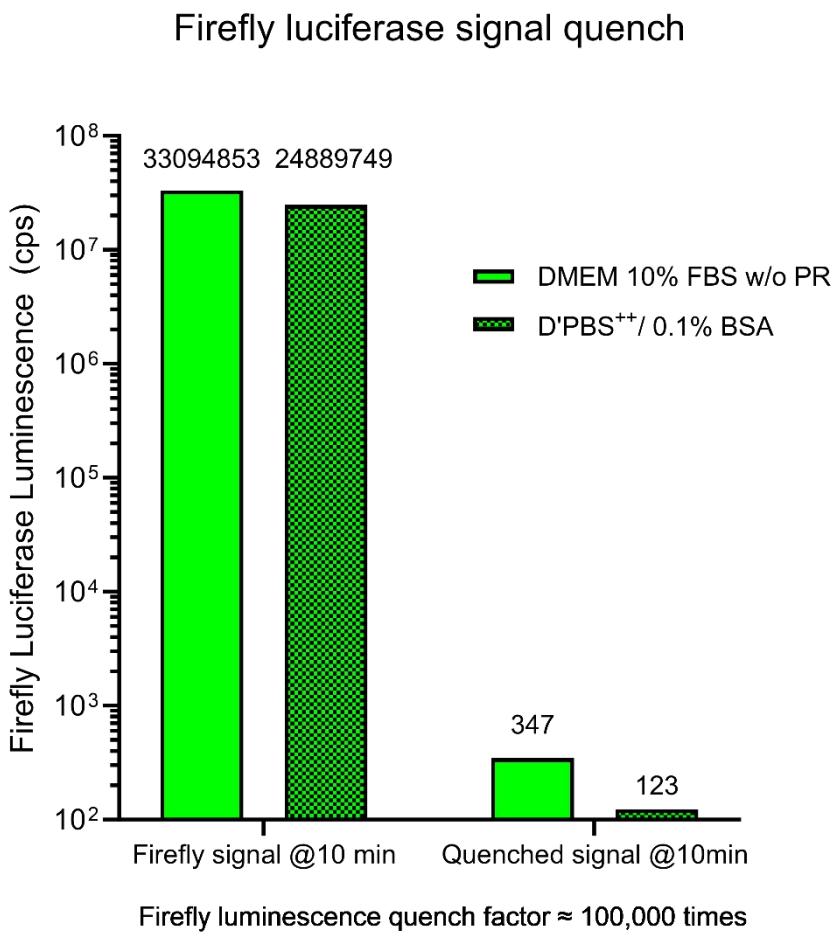


Figure 6. Effect of addition of renlite glow reagents to a purified firefly luciferase sample whose luminescence was activated by firelite glow. The firelite glow and renlite glow substrates were tested in phenol red free DMEM media supplemented with 10% FBS or in Dulbecco's PBS with magnesium and calcium and supplemented with 0.1% BSA. Similarly high quenching was seen in either case.

Organic solvents:

Organic solvents are often used to introduce screening compounds to cells in the experiment, resulting in the presence of a small percentage of organic solvent in medium. The effect of organic solvents on the luminescence of firelite glow and renlite glow reagents was investigated.

The results presented in figure 7 clearly shows that the solvents DMSO, ethanol, methanol and acetonitrile have little effect on the firelite glow luminescence.

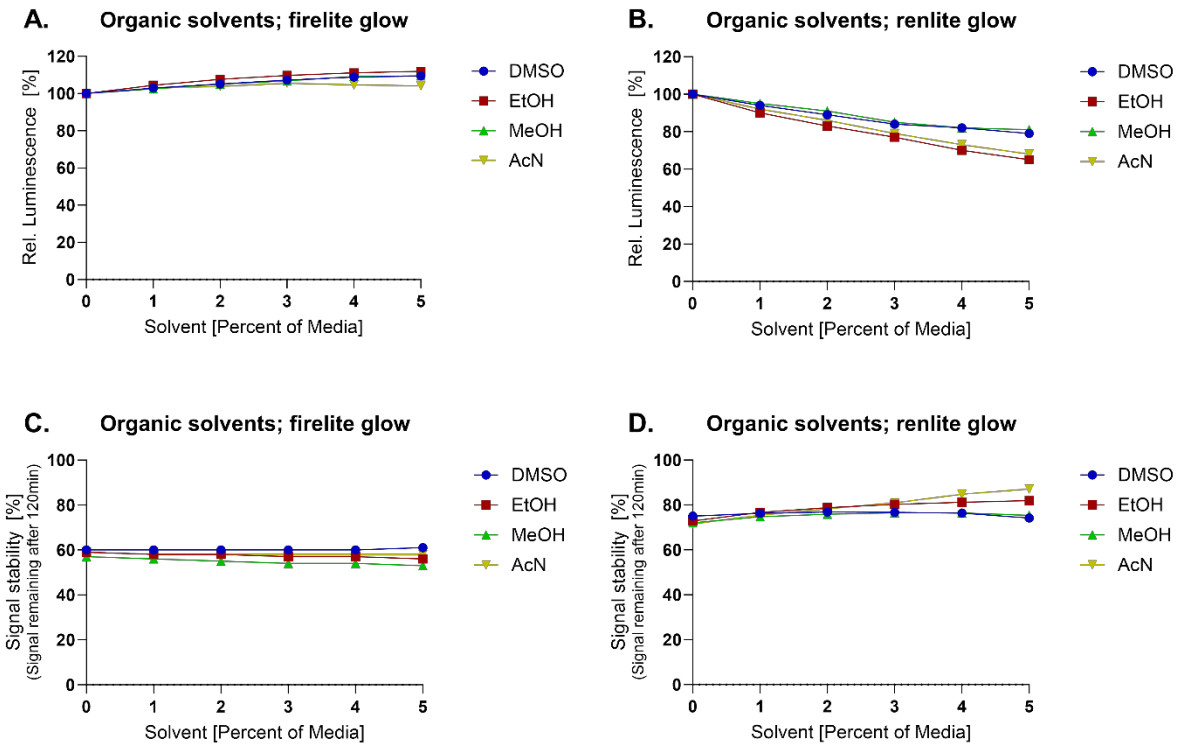


Figure 7. Luminescence potency and signal stability affected by organic solvents.

Firefly or *Renilla* luciferase from cell lysates in Dulbecco's PBS supplemented with Ca/Mg and 0.1% BSA was added to a white 96-well CulturPlate, 75µL per well. The DPBS solution contained a range of concentrations of organic solvents (DMSO, Ethanol, Methanol and Acetonitrile, ranging from 0% to 5%). Either firelite glow reagent (for firefly luciferase) or firelite glow plus renlite glow reagent (for *Renilla* luciferase) was added. After mixing, the luminescence measured after 10 and again after 120 minutes to show the effect of the solvents on signal height (panel A & B) and signal stability (panel C & D) respectively.

9. Data Processing

Background subtraction:

To obtain good data quality, background subtraction of both the firefly and *Renilla* luminescence measurements is advised. Both the *Renilla* as well as the firefly luciferase are not naturally expressed in mammalian cells, so there is no enzymatic background, but there can be substrate induced auto luminescence and luminometer specific interference. Also, the selected assay plate can cause elevated background signals. The firefly luminescent substrate does not suffer from autoluminescence, but the *Renilla* substrate does. Revvity's proprietary technology is used in the renlite glow formulation to diminish this problem significantly, resulting in an increased *Renilla* luciferase detection sensitivity.

The best way to measure background is to grow non-transfected cells as control samples in the assay plate wells, then add and measure firelite glow and renlite glow consecutively.

Normalization:

The *Renilla* luciferase signal is in general a perfect tool for firefly luciferase result normalization and will reduce variability introduced by cell number, transfection efficiency and cytotoxicity of added compounds. When multiple plates are measured, control samples should be added to every plate to correct for plate-to-plate variation caused by differences in temperature and decay between the firefly and *Renilla* luciferase luminescent signals. For the twinlite glow assay, both the firefly and *Renilla* luciferase reactions have similar luminescent half-life (see figure 4) and therefor variation in signal decay is only a minor issue.

10. Ordering Information

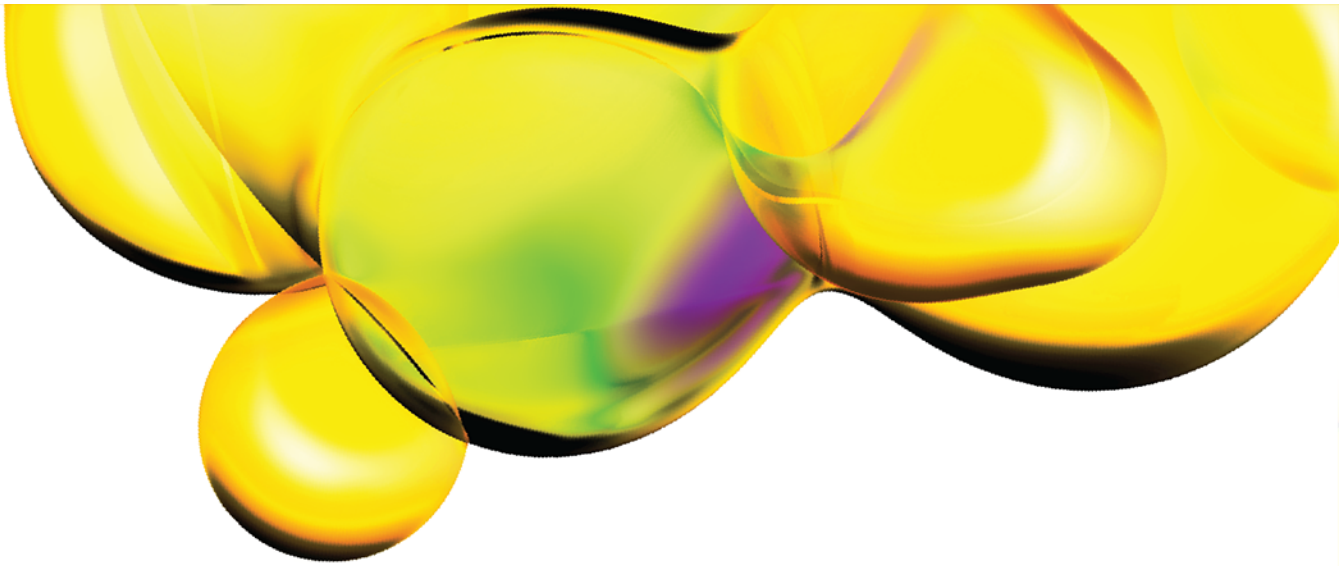
twinlite glow Dual Reporter Gene Assay System

Kit Size	Reorder No.
10 mL	6016796
100 mL	6016799

For further information on luminescence readers, microplates or luminescence applications please contact your local Revvity representative or visit: https://www.revvity.com

11. References

1. Alam, J. and Cook, J.L. (1990). Reporter genes: application to the study of mammalian gene transcription. Anal. Biochem. 1990 Aug 1; 188(2) 245-54
2. Collin Goddard (1994). Cell based screening approaches: advantages of highly automated robotics technology in HTS. Handbook for The 1994 International Forum on Advances in Screening Technologies and Data Management, p.19.
3. 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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