INSTRUCTIONS

Pierce™ Renilla-Firefly Luciferase Dual Assay Kit

16185 16186 2371.2

Number	Description
16185	**Pierce Renilla-Firefly Luciferase Dual Assay Kit,** sufficient reagents to perform 100 assays for *Renilla*-firefly luciferase activity in cultured cell lysate **Kit Contents:** **Renilla-Firefly Dual Assay Buffer,** 5mL, store at 4°C **Coelenterazine (100X),** 50µL, store at -80°C **D-Luciferin, Lyophilized,** 3mg, store at 4°C **2X Cell Lysis Buffer,** 6mL, store at room temperature
16186	**Pierce Renilla-Firefly Luciferase Dual Assay Kit**, sufficient reagents to perform 1000 assays for *Renilla*-firefly luciferase activity in cultured cell lysate **Kit Contents:** **Renilla-Firefly Dual Assay Buffer,** 50mL, store at 4°C **Coelenterazine (100X),** 0.5mL, store at -80°C **D-Luciferin, Lyophilized,** 30mg, store at 4°C **2X Cell Lysis Buffer,** 60mL, store at room temperature

Storage: Upon receipt store kit at -80°C or store individual components as indicated above. Kit is shipped on dry ice.

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Introduction

The Thermo Scientific™ Pierce™ Renilla-Firefly Luciferase Dual Assay provides a highly sensitive system for detecting intracellular luciferase activity from promoter or pathway activation in mammalian cell culture experiments. The activity of green *Renilla* luciferase expressed from green *Renilla* luc reporter plasmid is normalized by the activity of red firefly luciferase from the red firefly luc control plasmid (see Related Thermo Scientific Products). Additionally, red firefly luciferase works as a second experimental reporter for monitoring secondary regulatory element activity.

The green *Renilla* luciferase bioluminescence signal has greater stability and brightness than firefly and native *Renilla* luciferase. The bioluminescent signal (λ_{max} = 535nm) produced by green *Renilla* luciferase protein results from the oxidation of coelenterazine (Figure 1). This reaction does not require adenosine triphosphate (ATP) or other cofactors. The light output correlates with the amount of green *Renilla* protein expressed, which is used to determine promoter activity for green *Renilla* expression.

The second assay uses red firefly luciferase, which is a mutant form of the Italian firefly luciferase from *Luciola cruciata*. This luciferase produces a red-shifted emission spectrum (λ_{max}= 613nm) that results from the oxidation of D-luciferin in the presence of ATP (Figure 2). Red firefly and green *Renilla* luciferases are used in the dual assay because the light output is spectrally resolvable. Green *Renilla* luciferase plasmid acts as an experimental reporter coupled with red firefly expression plasmid (CMV-Red Firefly) as a normalization control. This reporter and control combination enables simultaneous monitoring of experimental reporter and control luciferase activity in a single-read assay without the need for two-step addition of substrate reagents or quenching. Specificity analysis of the two luciferase emissions has less than 5% of green *Renilla* light crossover to the 640nm LP channel (Figure 3).

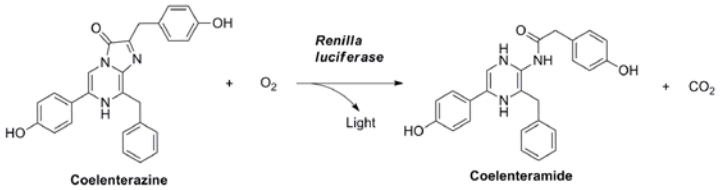


Figure 1. Chemical reaction of coelenterazine and green *Renilla* luciferase. Light, with an emission maximum of 535nm, is produced from the oxidation of coelenterazine and green *Renilla* luciferase.

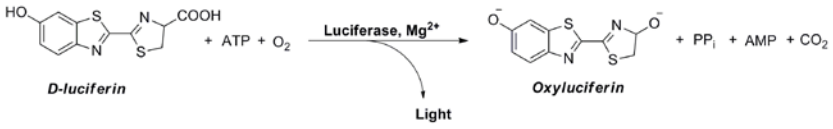


Figure 2. Chemical reaction of luciferin and red firefly luciferase. Light, with an emission maximum of 613nm, is produced from the oxidation of D-luciferin by red firefly luciferase in an ATP-dependent reaction.

Table 1. Filter requirement for the Thermo Scientific Pierce Renilla-Firefly Luciferase Dual Assay Kit.

Luciferase	Emission Range (nm)	Emission Maximum (nm)	Recommended Filter* (nm)
Green *Renilla*	450-650	535	525±20 BP
Red Firefly	560-700	613	640 LP

*The 525±20nm bandpass (BP) is designed to capture light wavelengths ranging from 505 to 545nm (Figure 3). Similarly, the 640nm longpass (LP) filter collects wavelengths above 640nm. Each luminometer requires specific filters; filter availability and dimensions are available from Omega Optical, Inc. or Chroma Technology Corp.

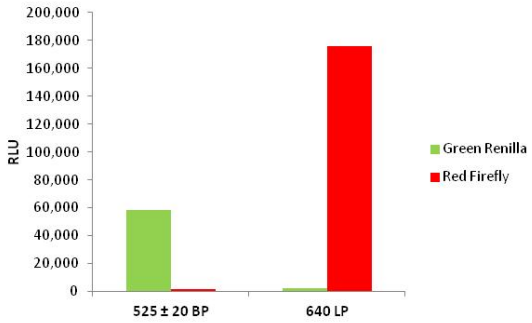
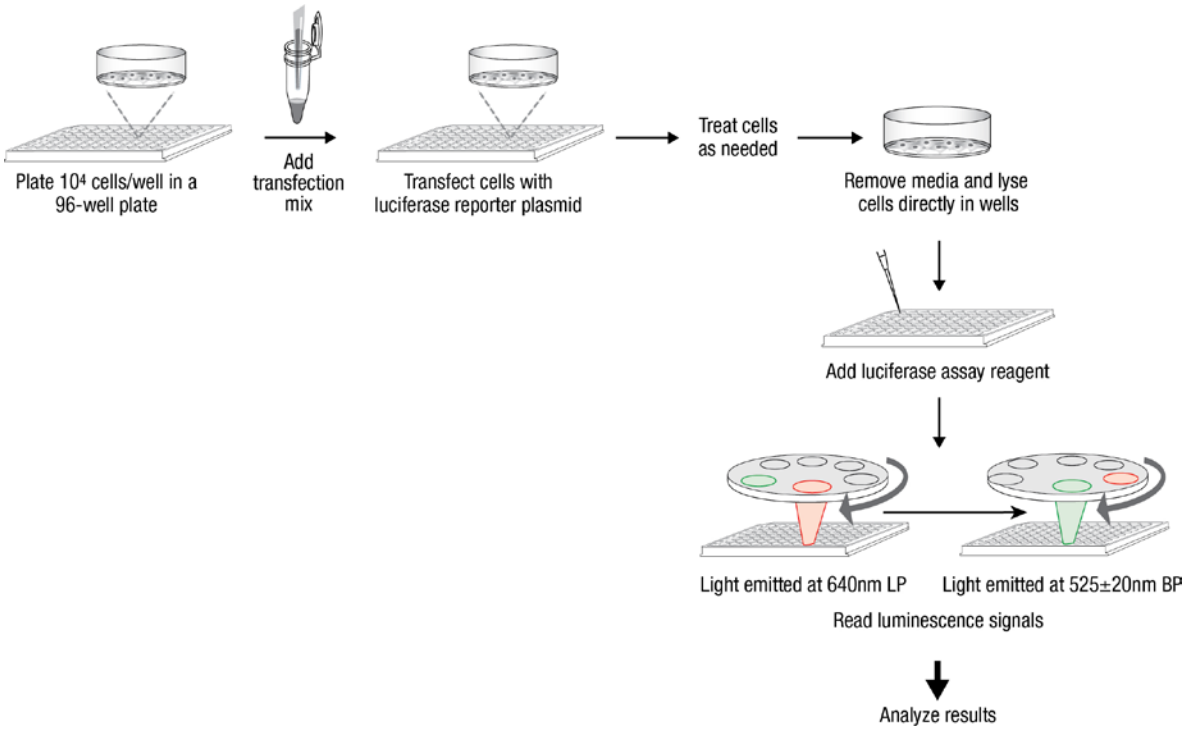


Figure 3. Specificity analysis of the two luciferase emissions. Green *Renilla* luciferase was measured using a 525±20nm BP filter and red firefly luciferase was measured using a 640nm LP filter on a Thermo Scientific™ Varioskan™ Luminometer.

Procedure Summary



Important Product Information

- For long-term use, store Coelenterazine (100X) at -80°C and D-Luciferin (lyophilized) at 4°C.
- Renilla-Firefly Luciferase Dual Assay Working Solution (Working Solution), Coelenterazine (100X) and D-Luciferin must always be stored protected from light.
- Store the Working Solution (see Material Preparation) at -20°C for up to 2 months. Working Solution must be at room temperature (20-25°C) before assay use.
- Green *Renilla* luciferase protein is significantly more stable than red firefly luciferase protein in cell culture media and lysate. Red firefly luciferase in cell lysate is subject to degradation. When working with red firefly luciferase containing cell lysate, store the container on ice and perform luciferase assays immediately following cell lysis. Addition of protease inhibitors (see Related Thermo Scientific Products) to the cell lysis buffer has been shown to increase the stability of red firefly luciferase protein.

- Green *Renilla* protein is ~1000-fold brighter than red firefly protein; for best results, transfect less green *Renilla* plasmid relative to red firefly plasmid and use the CMV (or similar strength) promoter-driven red firefly plasmid as the control for normalization.
- Green *Renilla* luciferase protein displays a broad emission spectral profile, which, at high signal levels (i.e., signals from CMV-Green *Renilla*), can bleed into the red region of the spectrum. Perform a spectral bleed-through test (see Appendix A) before starting the assay; use the experimental system and instrumentation to optimize the assay performance.
- To avoid cross-contamination, use a new disposable pipette tip for each transfer. If using a multichannel pipette, always use a new disposable reagent reservoir.
- Avoid exposing reagents to excessive heat or light during storage and incubation.
- Do not mix reagents from different lots. Do not discard unused working solutions after assay completion. Do not combine leftover reagents with those reserved for additional plates.
- Individual components might contain corrosives and/or preservatives. Wear gloves while performing the assay to avoid contact with samples and reagents. Please follow proper disposal procedures.
- Dispense and equilibrate to room temperature only the reagent volumes needed for the number of plates being used.
- Briefly centrifuge tubes of Coelenterazine (100X) before use.

Additional Materials Required

- Reagents and equipment needed to propagate mammalian cells in culture
- Reagents (e.g., Thermo Scientific™ TurboFect™ Transfection Reagent, Product No. R0533) and equipment needed to transfect plasmid DNA into mammalian cells
- Laboratory platform shaker
- Pipettes and/or liquid handling equipment
- Luminometer or other luminescence-monitoring instrument equipped with reagent injectors and filter wheel
- 525±20nm BP filter and 640nm LP filter (see Figure 3 and Table 1)
- White or black, opaque, 96- or 384-well microplates

Material Preparation

100X D-Luciferin Stock Solution	For 100 reactions, reconstitute lyophilized D-Luciferin pellet in 50µL of Renilla-Firefly Luciferase Dual Assay Buffer. Store at -20°C for up to two months.
Working Solution	For 100 reactions, add 50µL of 100X Coelenterazine and 50µL of 100X reconstituted D-Luciferin to 5mL of Renilla-Firefly Luciferase Dual Assay Buffer. Use 50µL of the Working Solution per reaction. Use within 4 hours or store in single-use volumes at -20°C for up to two months.
1X Cell Lysis Buffer	Dilute 2X Cell Lysis Buffer with an equal volume of ultrapure water.

Assay Protocol

A. Cell Transfection

1. Plate ~10,000 cells/well in a 96-well plate. Incubate cells overnight at 37°C in 5% CO_2. If using a different plate size, adjust the cell number accordingly. Use only cells growing in log phase at a passage number ≤ 15.

   Note: Plate sufficient wells to perform the experiment in triplicate; include appropriate controls, such as nonspecific signal (i.e., non-transfected cells).

2. Use a standard protocol to transfect mammalian cells with two plasmids: green *Renilla* luciferase driven by an experimental promoter and red firefly luciferase driven by a strong constitutive promoter (CMV).

   Note: Empirically determine the optimum ratio for the two plasmids. When using TurboFect Transfection Reagent (Product No. R0533), use an equal molar ratio of two plasmids up to 100ng total per well of a 96-well plate (e.g., 50ng of green *Renilla* luc reporter plasmid: 50ng of red firefly luc control plasmid).

   Note: Optimization of the green *Renilla* to red firefly plasmid ratio might need to be determined. The ratio of green *Renilla* to red firefly needs to remain ≤ 1.0 (see Appendix A).

   Note: For optimal results, use a visual transfection control. For example, transfect cells in a separate well with a constitutively expressed GFP plasmid and observe GFP expression using a fluorescence microscope.

3. Incubate cells for 16-72 hours at 37°C in 5% CO_2 in a cell culture incubator.
4. Proceed with the individual experimental protocol for cell treatment.

B. Cell Lysis

1. Aspirate media from the cells, rinse once with 50-100µL/well of 1X DPBS buffer (Thermo Scientific BupH Modified Dulbecco's PBS, Product No. 28374), aspirate DPBS and add 100µL/well of 1X Cell Lysis Buffer. Do not disturb the cell monolayer during the DPBS rinse.
2. Shake the plate on a platform shaker at moderate speed for 15 minutes. Check for complete cell lysis using a light microscope. If lysis is incomplete, continue shaking the plate for 15 additional minutes.

C. Luciferase Dual Assay

Note: For best results with signals expected to be greater than normal, perform a spectral bleed-through test with each new promoter or treatment used to modulate green *Renilla* expression.

1. Program the luminometer and injector and prime the system with Working Solution.
2. Add 10-20µL of cell lysate to a black flat-bottomed 96-well plate.
3. Using the luminometer's injectors, inject 50µL of Working Solution into each well containing cell lysate.
4. Immediately after adding the reagent, program the luminometer to detect the light output using a 640nm LP filter to capture the red firefly luciferase signal; then immediately read the sample again using a 525±20nm BP filter to capture the green *Renilla* luciferase signal.
5. Repeat Steps 3 and 4 for all samples in the plate. Use the red firefly luciferase signal as a normalization control for the green *Renilla* luciferase signal.

   Note: Follow the manufacturer's recommendations for using injector velocity to obtain a uniform coating of liquid in the well. Adjust the detector's integration time to achieve a signal within the linear range of the instrument.

Troubleshooting

Problem	Possible Cause	Solution
No signal	Low transfection efficiency	Optimize transfection conditions using a visual transfection control (e.g., a plasmid over-expressing a fluorescent protein)
		Verify plasmid DNA quality using restriction digestion and agarose gel electrophoresis **Note:** Most high-quality plasmid DNA should be supercoiled.
		Use actively dividing, low-passage cells
		Use a different cell type
	No promoter induction	Incubate cells using promoter-specific inducing conditions
		Incubate the cells for a longer time after treatment
		Change growth conditions to improve expression
		Use a different promoter
	Coelenterazine or D-Luciferin auto-oxidized	Protect substrate from light and air
		Maintain 100X Coelenterazine at -80°C and D-Luciferin at -20°C
		Prepare new Working Solution and use immediately
Low signal in lysate	Low luciferase expression	Lyse cells in a smaller volume of 1X Cell Lysis Buffer
		Use a different promoter or growth conditions to improve expression
		Increase the integration time on the instrument
		Scale-up the sample volume and reagent per well
High signal	High luciferase expression	Reduce incubation time before collecting samples
		Decrease the integration time on the instrument
		Dilute the sample **Note:** A low sample volume can increase assay variability. Dilute the sample and use the recommended volume of 10-20µL per assay.
High background signal	Nonspecific oxidation of Coelenterazine and D-Luciferin	Use new samples
		Avoid repeated freezing and thawing of the sample
	Control sample was contaminated	Change pipette tips after each well
		Reduce shaker speed during the cell lysis step to avoid contaminating the wells

Appendix A

Protocol for Spectral Bleed-through Testing

1. Prepare sufficient Working Solution (see Material Preparation) for three assays. DO NOT ADD reconstituted D-Luciferin.
2. Add 10-20µL of cell lysate from the experimental sample displaying the strongest green *Renilla* signal to a black flat-bottomed 96-well plate. Add 10-20µL of cell lysate from a non-transfected control sample to a separate, adjacent well.
3. Set the luminometer to detect light output using a 640nm LP filter.
4. Add 50µL of Working Solution (with no D-Luciferin) to each well containing lysate.

5. Immediately read the samples in a luminometer.

   Note: If the green *Renilla* signal detected using the 640nm LP filter is 10% greater than the non-transfected control, then decrease the instrument's integration time or decrease the amount of green *Renilla* plasmid transfected relative to red firefly plasmid. Use Thermo Scientific Pierce Firefly Signal Enhancer (100X) (Product No. 16180) to increase the red firefly signal.

Related Thermo Scientific Products

See our website for a complete list of related luciferase products.

16152	**pMCS-Green Renilla Luc**
16155	**pMCS-Red Firefly Luc**
16156	**pCMV-Red Firefly Luc**
16154	**pTK-Green Renilla Luc**
16157	**pTK-Red Firefly Luc**
16180	**Pierce Firefly Signal Enhancer (100X),** 0.5mL
16189	**Pierce Luciferase Cell Lysis Buffer (2X),** 250mL
28374	**BupH Modified Dulbecco's PBS Packs,** 40 packs
78425	**Halt™ Protease Inhibitor Single-Use Cocktail, EDTA-free (100X),** 24 × 100µL
R0533	**TurboFect Transfection Reagent,** 200µL

General Reference

Osamu, S. (2006). Bioluminescence: Chemical Principles and Methods. World Scientific Publishing Co. Pte. Ltd, Hakensack NJ.