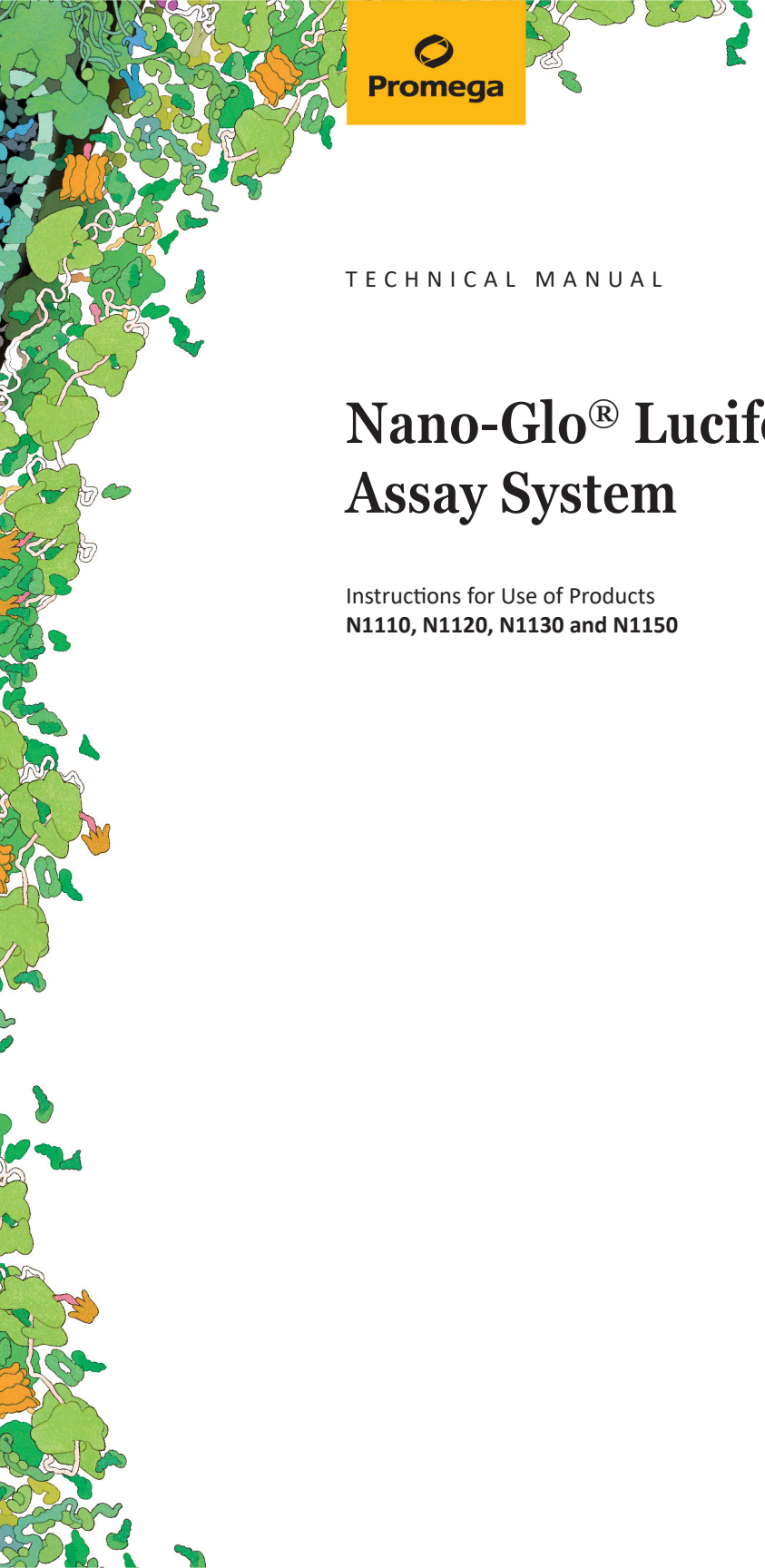

TECHNICAL MANUAL

Nano-Glo® Luciferase Assay System

Instructions for Use of Products
N1110, N1120, N1130 and N1150

Revised 1/22
TM369

Nano-Glo® Luciferase Assay System

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Introduction

NanoLuc® (Nluc) luciferase is a small enzyme (19.1kDa) engineered for optimal performance as a luminescent reporter. The enzyme is about 150-fold brighter than either firefly (*Photinus pyralis*) or *Renilla reniformis* luciferase (Figure 1), using a novel coelenterazine analog (furimazine; Figure 2) to produce high intensity, glow-type luminescence. The luminescent reaction is ATP-independent and designed to suppress background luminescence for maximal assay sensitivity.

For use as a genetic reporter, multiple forms of NanoLuc® luciferase have been configured to meet differing experimental objectives. Unfused Nluc offers maximal light output and sensitivity; NanoLuc®-PEST (NlucP) closely couples protein expression to changes in transcriptional activity and increases signal-to-background ratio; and NanoLuc® luciferase fused to an N-terminal secretion signal (secNluc) is suitable when a secreted reporter is preferred. Luminescence is linearly proportional to the amount of NanoLuc® protein over a 1,000,000-fold concentration range (Figure 1), with a signal half-life of ~120 minutes.

NanoLuc® luciferase possesses a number of physical properties that make it an excellent reporter protein: small, monomeric enzyme (171 amino acids; 513bp); high thermal stability (T_m = 60°C); active over a broad pH range (pH 6–8); no post-translational modifications or disulfide bonds; uniform distribution in cells and an emission spectrum well suited for bioluminescence resonance energy transfer (BRET; λmax = 460nm). The enhanced thermal stability of NanoLuc® luciferase makes the enzyme less susceptible to small molecule inhibitors that can emerge from diverse compound libraries.

Nano-Glo® Luciferase Assay System[a–c] provides a simple, single-addition reagent that generates a glow-type signal in the presence of NanoLuc® luciferase. The reagent is prepared by mixing Nano-Glo® Luciferase Assay Substrate and Nano-Glo® Luciferase Assay Buffer and contains an integral lysis buffer to use directly on cells expressing NanoLuc® luciferase or culture medium when the luciferase is secreted.

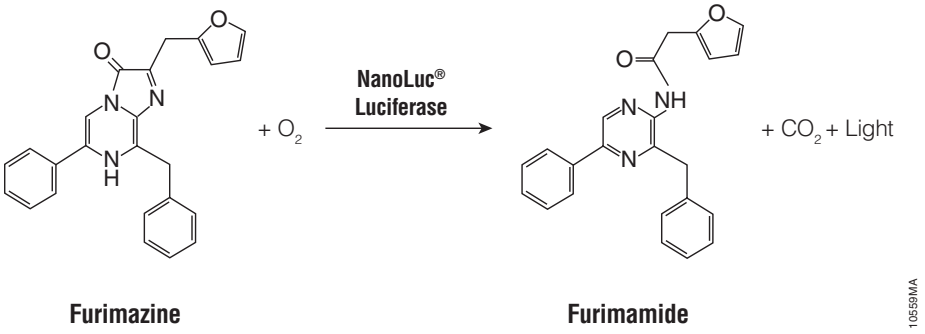

Figure 1. The bioluminescent reaction catalyzed by NanoLuc® luciferase.

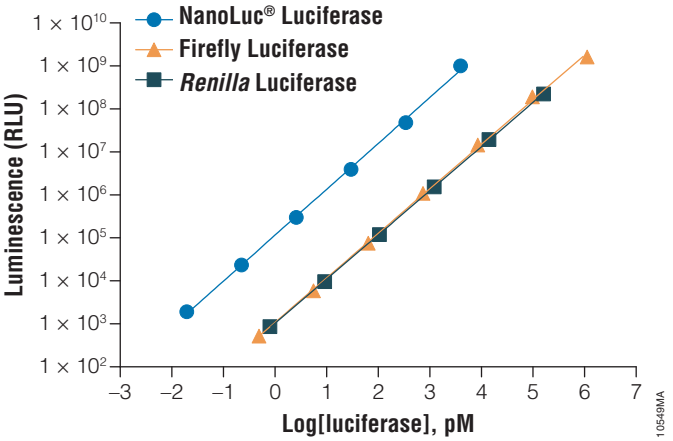

Figure 2. A comparison of the sensitivity of NanoLuc®, firefly and *Renilla* luciferase assays. Luminescence was measured from varying concentrations of purified proteins after mixing the reporter enzyme with its respective detection reagent. NanoLuc® luciferase was approximately 150-fold brighter than firefly or *Renilla* luciferases at equivalent concentrations. Detection reagents used were Nano-Glo® Luciferase Assay Reagent for NanoLuc® luciferase, ONE-Glo™ Luciferase Assay System Reagent for firefly luciferase and *Renilla*-Glo™ Luciferase Assay System Reagent for *Renilla* luciferase.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Nano-Glo® Luciferase Assay System	**10ml**	**N1110**

Each system contains sufficient reagent for 100 assays in 96-well plates. Includes:

- 200µl Nano-Glo® Luciferase Assay Substrate
- 10ml Nano-Glo® Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
Nano-Glo® Luciferase Assay System	**100ml**	**N1120**

Each system contains sufficient reagent for 1,000 assays in 96-well plates. Includes:

- 2 × 1ml Nano-Glo® Luciferase Assay Substrate
- 100ml Nano-Glo® Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
Nano-Glo® Luciferase Assay System	**10 × 10ml**	**N1130**

Each system contains sufficient reagent for 1,000 assays in 96-well plates. Includes:

- 10 × 200µl Nano-Glo® Luciferase Assay Substrate
- 10 × 10ml Nano-Glo® Luciferase Assay Buffer

2. Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
Nano-Glo® Luciferase Assay System	**10 × 100ml**	**N1150**

Each system contains sufficient reagent for for 10,000 assays in 96-well plates. Includes:

- 5 × 4ml Nano-Glo® Luciferase Assay Substrate
- 10 × 100ml Nano-Glo® Luciferase Assay Buffer

Storage Conditions: Store the Nano-Glo® Luciferase Assay Substrate and the Nano-Glo® Luciferase Assay Buffer at –20°C. **Do not** thaw above 25°C. The buffer may be stored at 4°C for 1 year or at room-temperature for 3 months, and it can be frozen and thawed at least ten times without any change in performance. The substrate may be stored at 4°C for up to two weeks.

3. Choice of NanoLuc® Gene Configuration

3.A. Intracellular Lifetime and Effect on Reporter Response

When sensitivity is needed, maximal brightness will be provided by unfused NanoLuc® (Nluc) luciferase, which can accumulate in cells due to the long intracellular half-life of the protein (>6 hours). The bright signal from Nluc and the low autoluminescence of the Nano-Glo® Luciferase Assay Reagent provide maximum sensitivity where expression levels are low, such as in cell lines that are difficult to transfect.

Closer coupling to the cellular transcriptional response can be obtained by using NanoLuc®-PEST (NlucP) reporter, which shows reduced accumulation in cells due to a very short intracellular lifetime (protein half-life approximately 10–30 minutes). The short lifetime of NlucP protein allows expression levels to change rapidly in response to changes in transcription, albeit with reduced overall brightness compared to Nluc (Figure 3). When compared to lucP (firefly luciferase-PEST reporter), NlucP is both brighter and responds more quickly to changes in transcription, consistent with a shorter intracellular protein half-life. As a result, NlucP may be particularly useful for monitoring subtle changes in transcriptional activity, providing the best possible signal-to-background ratio. Moreover, a high signal-to-background ratio can develop sooner when using NlucP compared to more stable forms of luciferase.

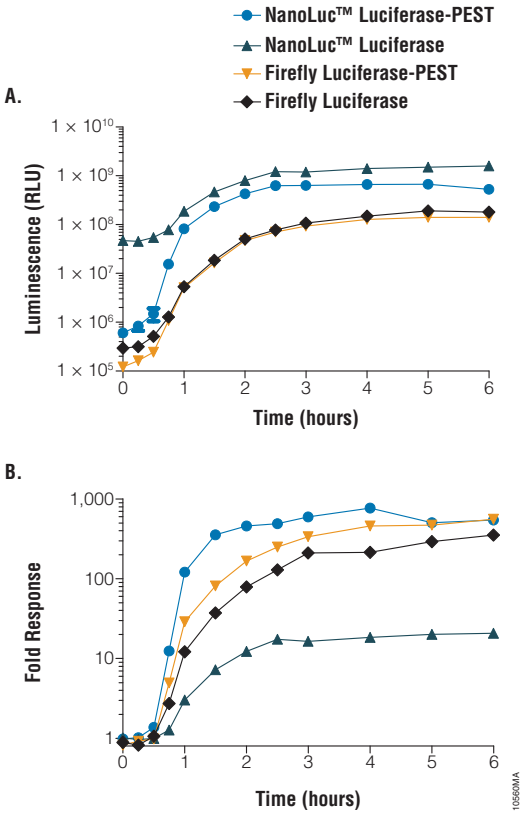

Figure 3. A comparison of light output and response dynamics between NanoLuc® and firefly luciferase constructs. HEK293 cells were transiently transfected with constructs encoding various forms of luciferase under the control of a minimal promoter containing five repeats of a consensus NF-κB response element and replicate plates treated with 100ng/ml of rhTNF-α (Cat.# G5241) for the indicated times. **Panel A.** Luminescence was measured at the indicated time points following the protocol outlined in Section 4.B. **Panel B.** Fold induction was calculated by comparing treated samples to untreated samples at the same time point.

3.B. Use of Secreted NanoLuc® Luciferase

By adding a secretion signal fused to the N-terminus of NanoLuc® luciferase (secNluc), the amount of reporter protein secreted from the cell can be used to monitor transcriptional activation (Figure 4). Removing cell culture medium at multiple time points allows you to perform kinetic studies on the same set of wells. Once secreted, the secNluc protein is extremely stable in cell culture medium with a half-life greater than 4 days at 37°C.

Due to extra sample processing, secretion-based reporter gene assays can be limited by increased data scatter in sample replicates. In general, we have seen similar response dynamics when comparing secNluc and unfused Nluc reporter proteins, including time to develop usable signal-to-background ratio.

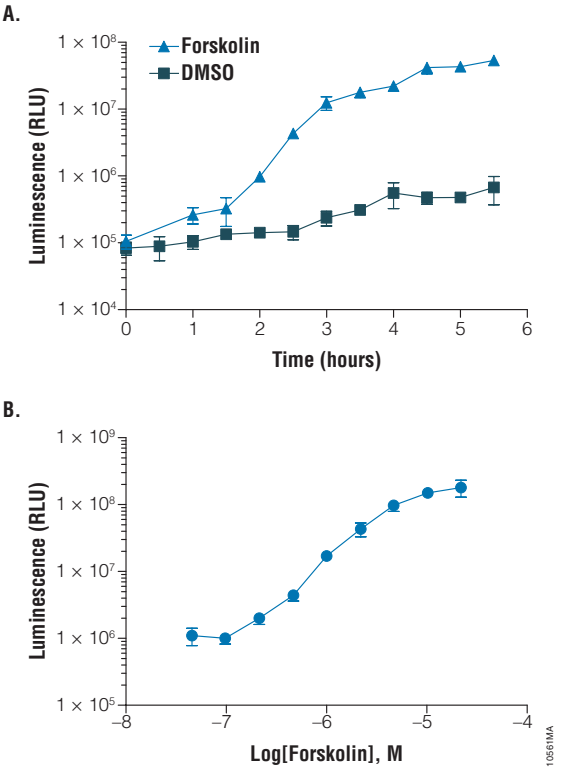

Figure 4. Reporter gene assays using secNluc. HEK293 cells were transiently transfected with a plasmid encoding secNluc under the control of a minimal promoter containing a cAMP response element and treated with forskolin. Cell culture medium (10µl) was removed from replicate plates and luminescence measured following the protocol outlined in Section 4.C. **Panel A.** Cells were treated with 10µM forskolin or vehicle for the indicated times. **Panel B.** Cells were treated with forskolin at the indicated concentrations for 5 hours.

4. Nano-Glo® Luciferase Assay System Protocols

4.A. Preparation of the Nano-Glo® Luciferase Assay Reagent

Remove the Nano-Glo® Luciferase Assay Substrate from storage and mix by pipetting. Thaw the Nano-Glo® Luciferase Assay Buffer if stored at –20°C but do **not** exceed 25°C. Prepare the desired amount of reconstituted Nano-Glo® Luciferase Assay Reagent by combining one volume of Nano-Glo® Luciferase Assay Substrate with 50 volumes of Nano-Glo® Luciferase Assay Buffer. For example, if the experiment requires 10ml of reagent, add 200µl of substrate to 10ml of buffer.

Notes:

1. If the Nano-Glo® Luciferase Assay Substrate has collected in the cap or on the sides of the tube, briefly centrifuge tubes containing 200µl or 1ml of substrate in a microcentrifuge. Place 4ml tubes in a swinging bucket rotor and centrifuge at 200 × *g* for 1 minute.
2. We recommend preparing the Nano-Glo® Luciferase Assay Reagent fresh for each use rather than storing the reagent at any temperature.
3. Once reconstituted, the reagent will lose 10% activity in approximately 8 hours and 50% activity in approximately 2 days at room temperature. The reconstituted reagent may be stored at 4°C with <10% decrease in activity over 2 days.

4.B. Detection of NanoLuc® Luciferase in Mammalian Cells (Lytic Method)

1. Allow all assay components (reagent and sample) to equilibrate to room temperature prior to assay. For example, remove a tissue culture plate from the 37°C, 5% CO_2 incubator and equilibrate it to room temperature for 5–10 minutes.
2. Add a volume of reagent equal to that of the culture medium in each well. Mix for optimal consistency. For 96-well plates, typically 100µl of reagent is added to the cells grown in 100µl of medium. For 384-well plates, typically 30µl of reagent is added to cells grown in 30µl of medium.
3. Wait at least 3 minutes before measuring luminescence. The luminescence intensity will decay gradually, with a signal half-life of approximately 120 minutes at room temperature.

Notes:

1. At high expression levels, the luminescence signal half-life can decrease significantly due to rapid depletion of the furimazine substrate (see Section 5.A). Experimental conditions should be modified to avoid extremely high expression (see Section 5.B).
2. Ensure that the instrument is operating within its linear dynamic range when measuring luminescence (see Section 5.C). Consult with the instrument manufacturer or determine this range empirically. Many instruments will not indicate if values are outside of the linear range.
3. The Nano-Glo® Luciferase Assay System is compatible with a variety of components used in mammalian cell culture experiments, showing minimal differences in luminescence intensity or signal half-life (Figure 5).

4.B. Detection of NanoLuc® Luciferase in Mammalian Cells (Lytic Method; continued)

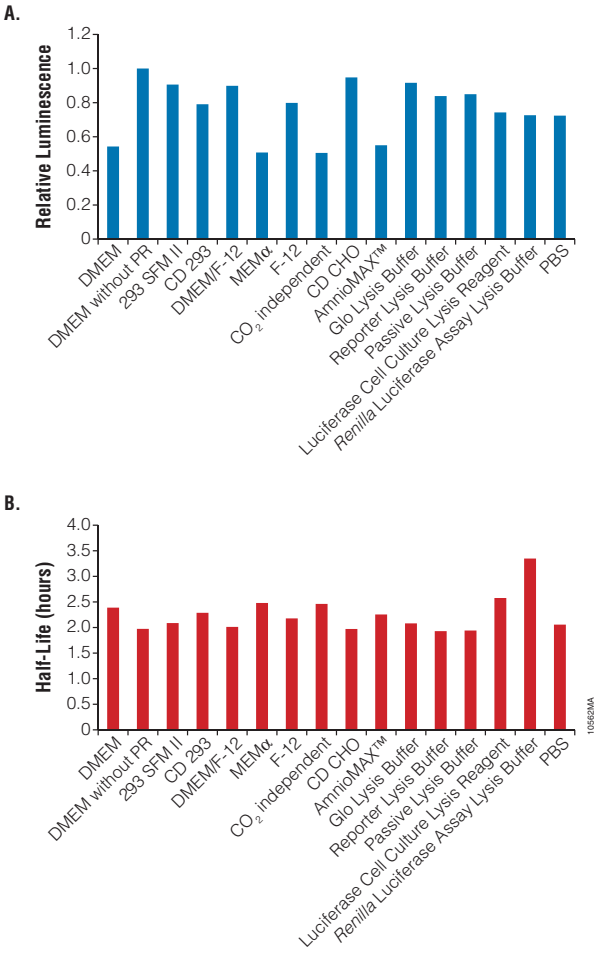

Figure 5. Relative luminescence and signal half-life of NanoLuc® luciferase in a variety of conditions used in mammalian cell culture experiments. The Nano-Glo® Luciferase Assay Reagent was mixed 1:1 with 9pM purified NanoLuc® luciferase. Luminescence was measured 3 minutes after reagent addition and normalized to DMEM without phenol red. The rate of signal decay was monitored every 20 minutes for 2 hours. **Panels A and B.** Nluc was diluted in a variety of media and lysis buffers. (Figure 5 is continued on the next page.)

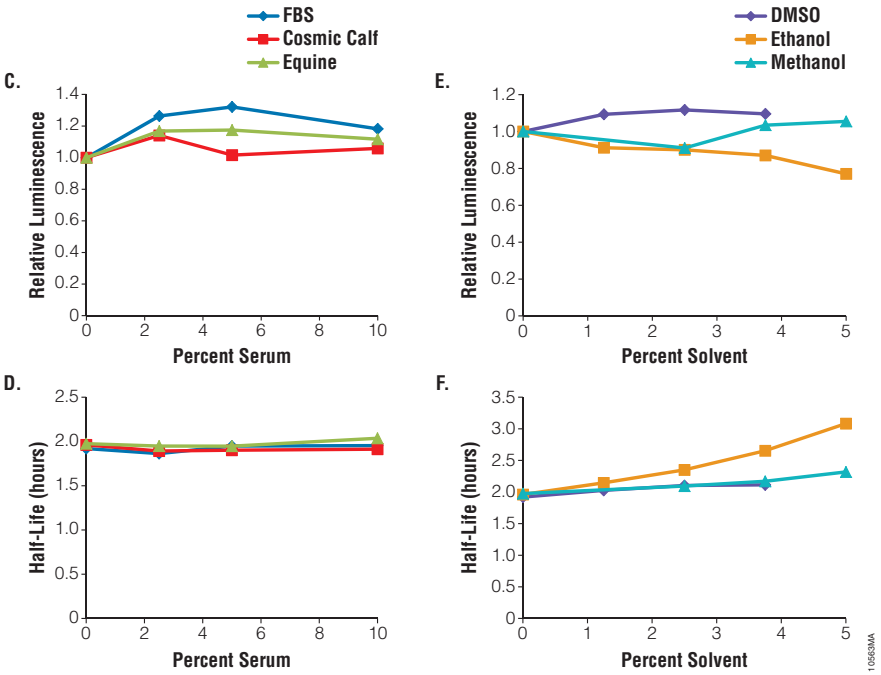

Figure 5. Relative luminescence and signal half-life of NanoLuc® luciferase in a variety of conditions used in mammalian cell culture experiments (continued). Nluc was diluted in DMEM without phenol red containing different amounts of sera (**Panels C and D**) or solvents (**Panels E and F**).

4.C. Detection of NanoLuc® Luciferase Secreted from Mammalian Cells

The following protocol can be used to assay NanoLuc® luciferase secreted from living cells. Prior to adding test or control compounds to induce a physiological response for the pathway of interest, cell culture medium must be replaced to remove any NanoLuc® luciferase that has accumulated as a result of basal or leaky expression, carefully aspirating the medium without disturbing the cell monolayer. NanoLuc® luciferase is extremely stable in cell culture medium with a measured protein half-life of greater than four days.

1. After completing your desired experimental treatment, remove the cells from the tissue culture incubator and gently shake or place on an orbital shaker to remove concentration gradients that may exist in the culture medium. Recommended orbital shaker setting for a 96-well plate (100µl per well): 100rpm for 2 minutes.

   Note: Avoid vigorous shaking conditions that may detach cells from the well.

4.C. Detection of NanoLuc® Luciferase Secreted from Mammalian Cells (continued)

2. Remove an aliquot of culture medium (5–20µl) and transfer to a single well of a 96-well plate without disturbing the cell monolayer. Add distilled or deionized water or cell culture medium to a final volume of 100µl.

   Note: Samples can be removed at multiple time points from the same set of wells if measuring a kinetic response. Apply the following transformation to the measured luminescence of each aliquot to calculate total luminescence at each time point:

   Calculated RLU, time t = (aliquot RLU, time t × dilution factor) + ∑aliquot RLU t–1, t–2, etc.

   Dilution factor = (total volume remaining in well, time t) ÷ (aliquot volume)

   Example calculation:

   Started with 100µl of medium and removed three 10µl aliquots at consecutive time points with readings of 1×10^4, 3×10^4 and 5×10^4 RLUs. The calculated RLUs for the experiment are:

   Calculated RLU, t1 = 1×10^4 RLU × 10 = 1×10^5 RLU

   Calculated RLU, t2 = (3×10^4 RLU × 9) + (1×10^4 RLU) = 2.8×10^5 RLU

   Calculated RLU, t3 = (5×10^4 RLU × 8) + [(1×10^4 RLU) + (3×10^4 RLU)] = 4.4×10^5 RLU

3. Add one volume of Nano-Glo® Luciferase Assay Reagent equal to the volume of the sample and mix for optimal consistency.

4. Wait at least 3 minutes before measuring luminescence. The luminescence intensity will decay gradually, with a signal half-life of approximately 120 minutes at room-temperature.

   Notes:

   1. At high expression levels, the luminescence signal half-life can decrease significantly due to rapid depletion of the furimazine substrate (see Section 5.A). Experimental conditions should be modified to avoid extremely high expression (see Section 5.B).
   2. Ensure the instrument is operating within its linear dynamic range when measuring luminescence (see Section 5.C). Consult with the instrument manufacturer or determine this range empirically. Many instruments will not indicate if values are outside of the linear range.
   3. The Nano-Glo® Luciferase Assay System is compatible with a variety of components used in mammalian cell culture experiments, showing minimal differences in luminescence intensity or signal half-life (Figure 5).

5. Precautions for High Expression Levels of NanoLuc® Luciferase

5.A. NanoLuc® Luciferase Signal Stability and Decay Kinetics

NanoLuc® luciferase is able to maintain a glow-type luminescent signal over a broad concentration range, providing a signal half-life of approximately 120 minutes. However, the signal half-life can decrease significantly at extremely high concentrations of enzyme, likely due to rapid depletion of substrate (Figure 6). Samples in this range will not maintain the same relative levels of light output compared to samples at lower NanoLuc® concentrations in the minutes to hours after adding the Nano-Glo® Luciferase Assay Reagent. If you suspect that you may have an extremely high concentration of NanoLuc® luciferase in your samples, monitor the rate of signal decay starting 3–10 minutes after adding the Nano-Glo® Luciferase Assay Reagent. If the signal half-life is reduced, the initial RLU value at 3–10 minutes can be used in future work to approximate an upper limit of luminescence that will decay with a half-life of approximately 120 minutes under your experimental conditions (luminometer, plate type, etc.). This value is typically near or can often exceed the linear dynamic range of the luminometer used for detection (Section 5.C). If you have samples that exceed the linear dynamic range of the instrument, modify the experimental conditions to lower the final concentration of NanoLuc® protein in the cell lysate (Section 5.B).

5.A. NanoLuc® Luciferase Signal Stability and Decay Kinetics (continued)

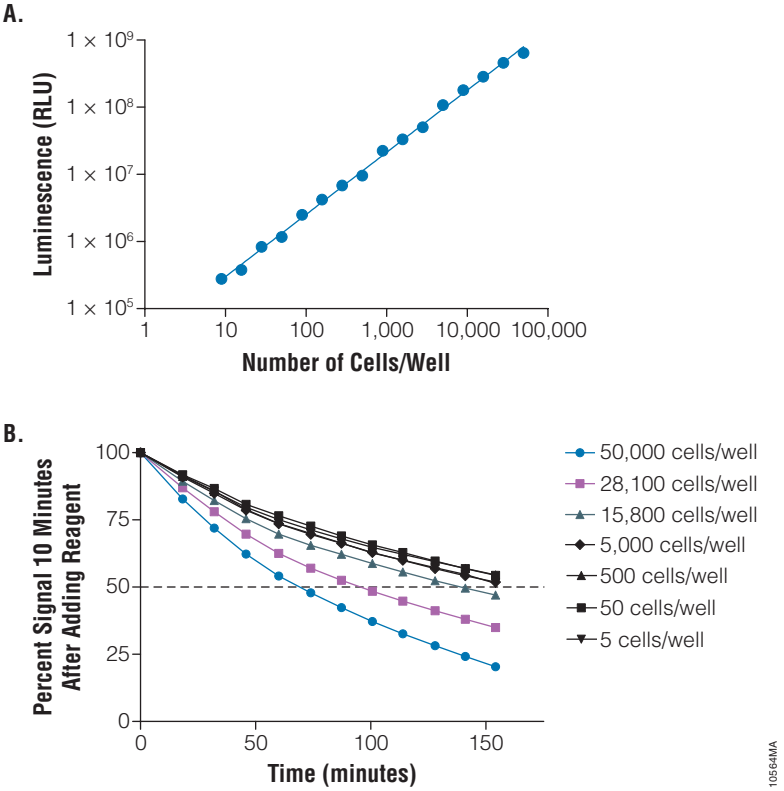

Figure 6. Reduced signal half-life at extremely high concentrations of NanoLuc® luciferase. HEK293 cells were transiently transfected with the pNL1.1[CMV] Vector, trypsinized, serially diluted with complete medium and transferred to a 96-well plate. **Panel A.** Luminescence was measured 10 minutes after adding the Nano-Glo® Luciferase Assay Reagent following the Section 4.B protocol. **Panel B.** Signal decay kinetics were monitored at the indicated time points. At high values of luminescence (≥285,000,000 RLUs corresponding to >15,800 cells lysed/well), signal half-life decreases from the common value seen for the majority of samples in this experiment.

5.B. Modifying Experimental Conditions to Reduce NanoLuc® Luciferase Concentration in the Cell Lysate

NanoLuc® luciferase was designed to provide optimal sensitivity in experimental systems where reporter expression is low. However, if you are using NanoLuc® luciferase in systems where reporter expression levels are much higher, you may experience an increase in signal decay rate or initial luminescence values outside of the linear dynamic range of your luminometer. If confirmed, modify your experimental protocol to reduce the amount of NanoLuc® luciferase present in the cell lysate:

1. Use a less active promoter for constitutive expression. Rather than using full-length CMV promoter, switch to another promoter such as SV40, HSV-TK or a deletion mutant of CMV.
2. For transient transfection assays, transfect less DNA per well (Figure 7). For example, instead of transfecting 50ng of DNA per individual well of a 96-well plate, transfect 5ng + 45ng of carrier DNA, using the same lipid:DNA ratio. Ensure that the carrier DNA plasmid does not express proteins that would affect the experimental results (e.g., pGEM®-3Zf(+) Vector, Cat.# P2271).
3. Plate fewer cells per well (Figure 6).
4. Use a NanoLuc®-PEST expression vector instead of unfused NanoLuc® luciferase (Figure 3).

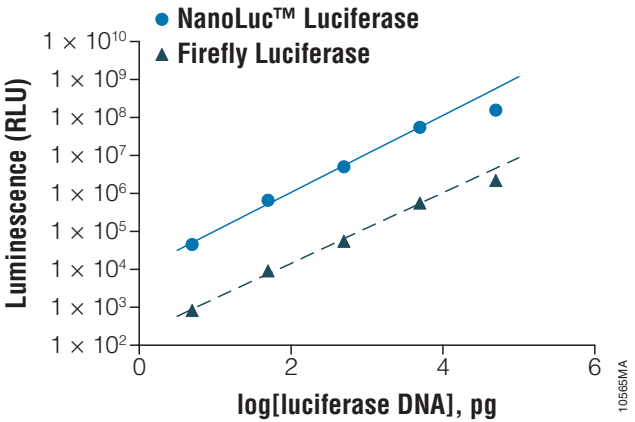

Figure 7. Decreasing NanoLuc® luciferase expression levels by reducing the amount of transfected DNA. Plasmid DNA encoding NanoLuc® or firefly luciferases was serially diluted and combined with pGEM®-3Zf(+) Vector carrier DNA. FuGENE® HD Transfection Reagent was added to DNA in a 3:1 ratio to transiently transfect HepG2 cells in a 96-well plate (10,000 cells and 50ng total DNA per well). After 24 hours, Nano-Glo® Luciferase Assay Reagent or ONE-Glo™ Luciferase Assay Reagent was added to respective wells and luminescence measured.

5.C. Operating Within the Linear Dynamic Range of the Luminometer

All luminometers have a defined linear dynamic range of detection where changes in luciferase concentration give proportional changes in the measured light output. Outside of this range, the relationship between enzyme concentration and luminescence is no longer proportional, which can lead to misinterpretation of experimental results. Although NanoLuc® luciferase was designed to provide maximal sensitivity for experimental systems where reporter expression levels are low, it can be used when expression levels are much higher provided the signal output is within the linear dynamic range of detection. When the concentration of NanoLuc® luciferase is extremely high, light output can exceed the linear dynamic range of the luminometer used for detection, and the signal half-life can decrease significantly (Section 5.A).

The Nano-Glo® Luciferase Assay System was developed using the Promega GloMax® 96 Microplate Luminometer, GloMax®-Multi Detection System and GloMax® Multi+ Detection System with Instinct™ Software. While luminometers from other manufacturers may be used, they may suffer from limitations in sensitivity, dynamic range and/or well-to-well crosstalk. Consult the manufacturer's instructions to determine the operational parameters of your instrument, including any adjustments in gain or sensitivity settings. GloMax® luminometers provide superior sensitivity and linear dynamic range (Figure 8) without gain adjustment, together with very low well-to-well crosstalk.

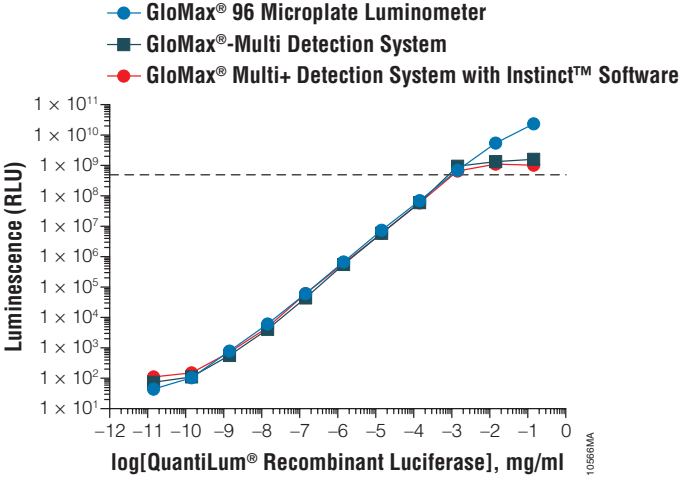

Figure 8. Dynamic range of GloMax® luminometers. QuantiLum® Recombinant Luciferase (Cat.# E1701, 14.37 mg/ml) was diluted in Glo Lysis Buffer, 1X (Cat.# E2661), using tenfold serial dilutions. Fifty microliters of each enzyme dilution was dispensed in triplicate to a white 96-well plate (Costar Cat.# 3355). An equal volume (50µl) of ONE-Glo™ Luciferase Assay Reagent (Cat.# E6110) was added to each well, and luminescence was measured after 3 minutes.

6. Related Products

Table 1. List of NanoLuc® Vectors and Their Characteristics.

Vector (Cat.#)	Multiple Cloning Region	Protein Degradation Sequence	Secretion Signal	Response Element/ Promoter	Mammalian Selectable Marker
pNL1.1[*Nluc*] (N1001)	Yes	No	No	No	No
pNL1.2[*NlucP*] (N1011)	Yes	PEST	No	No	No
pNL1.3[*secNluc*] (N1021)	Yes	No	Yes	No	No
pNL2.1[*Nluc*/Hygro] (N1061)	Yes	No	No	No	Hygromycin
pNL2.2[*NlucP*/Hygro] (N1071)	Yes	PEST	No	No	Hygromycin
pNL2.3[*secNluc*/Hygro] (N1081)	Yes	No	Yes	No	Hygromycin
pNL3.1[*Nluc*/minP] (N1031)	Yes	No	No	minimal promoter	No
pNL3.2[*NlucP*/minP] (N1041)	Yes	PEST	No	minimal promoter	No
pNL3.3[*secNluc*/minP] (N1051)	Yes	No	Yes	minimal promoter	No
pNL1.1.CMV[*Nluc*/CMV] (N1091)	No	No	No	CMV IE promoter	No
pNL1.3.CMV[*secNluc*/CMV] (N1101)	No	No	Yes	CMV IE promoter	No
pNL3.2.NF-κB-RE[*NlucP*/ NF-κB-RE/Hygro] (N1111)	No	PEST	No	NF-κB-RE + minimal promoter	Hygromycin

6. Related Products (continued)

Transfection Reagents

Product	Size	Cat.#
FuGENE® 6 Transfection Reagent	1ml	E2691
	5 × 1ml	E2692
	0.5ml	E2693
FuGENE® HD Transfection Reagent	1ml	E2311
	5 × 1ml	E2312
pGEM®-3Zf(+) Vector	20µg	P2271

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500
GloMax® 96 Microplate Luminometer		E6501
GloMax® 96 Microplate Luminometer with Single Injector		E6511
GloMax® 96 Microplate Luminometer with Dual Injectors		E6521
GloMax® 20/20 Luminometer		E5311
GloMax® 20/20 Luminometer with Single Auto-Injector		E5321
GloMax® 20/20 Luminometer with Dual Auto-Injector		E5331

7. Summary of Changes

The following changes were made to the 1/22 revision of this document:

1. Step 2 in Section 4.B was updated to include examples of the volume of Nano-Glo® Luciferase to use.
2. A disclaimer was added.
3. The cover image was updated.

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(a) use NanoBRET™-branded energy acceptors (e.g., BRET-optimized HaloTag® ligands) for all determinations of energy transfer activity by this product; or
(b) contact Promega to obtain a license for use of the product for energy transfer assays to energy acceptors not manufactured by Promega.

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[b] U.S. Pat. No. 8,809,529, European Pat. No. 2635582 and other patents and patents pending.

[c] Patent Pending.

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