TECHNICAL MANUAL

Nano-Glo® In-Gel Detection System

Instructions for Use of Product
N3020

10/17
TM527

Nano-Glo® In-Gel Detection System

All technical literature is available at: www.promega.com/protocols/
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E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The Nano-Glo® In-Gel Detection System[a,b,c] directly detects the luminescence of NanoLuc® fusion proteins in a polyacrylamide gel matrix, following protein separation by polyacrylamide gel electrophoresis (PAGE) under either native or denaturing SDS conditions (native PAGE or SDS-PAGE). NanoLuc® luciferase is a 19.1kDa engineered enzyme that was developed to be brighter and more versatile than other reporter proteins (1). Because of its stability, small size and high luminescent activity, NanoLuc® luciferase makes an ideal fusion partner for monitoring protein abundance in live cells or cell lysates. Additionally, NanoLuc® fusions can be used as energy donors for bioluminescence resonance energy transfer (BRET) to measure interactions in live cells with proteins (2) or small molecules (3) in NanoBRET™ PPI and NanoBRET™ Target Engagement assays, respectively.

Compared to Western blotting (immunoblotting), the Nano-Glo® In-Gel Detection System offers superior speed, sensitivity and specificity with a simple protocol that does not require transfer to membranes, eliminating the need for blocking or antibodies. Proteins of interest can be tagged at the N or C terminus with NanoLuc® luciferase. Following native PAGE, NanoLuc® activity is maintained, so the gels can be placed directly into Nano-Glo® In-Gel Detection Reagent and imaged. Following SDS-PAGE, the denaturing gels are first washed with isopropanol and water to remove the SDS detergent and allow protein refolding prior to adding detection reagent and imaging the gel.

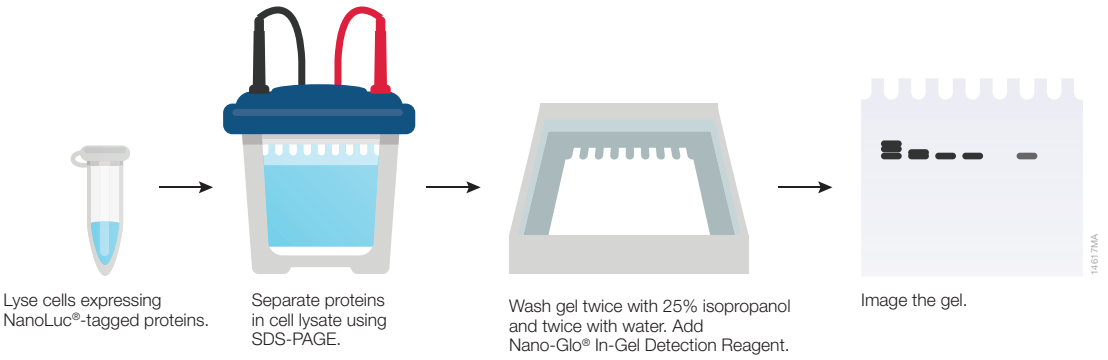

Figure 1. The Nano-Glo® In-Gel Detection System. Lysed samples that include a NanoLuc®-tagged protein are subjected to SDS-PAGE. Gels are washed twice with 25% isopropanol and twice with water to promote refolding of NanoLuc® luciferase. After adding a reagent containing the enzyme substrate, luminescence is detected using an appropriate imager.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Nano-Glo® In-Gel Detection System	100ml	N3020

Each system contains sufficient components to prepare 100ml of reagent. Includes:

- 10ml Nano-Glo® In-Gel Buffer, 10X
- 0.2ml Nano-Glo® Luciferase Assay Substrate

Storage Conditions: Store the Nano-Glo® In-Gel Detection System components at –20°C. The Nano-Glo® Luciferase Assay Substrate solution will not freeze at –20°C. The 10X Nano-Glo® In-Gel Buffer may be stored at 4°C for 6 months. For best results, freshly dilute the 10X Nano-Glo® In-Gel Buffer in deionized water to the 1X final concentration before each use. The 1X Buffer can be stored at 4°C, protected from light, for up to 1 month, but there may be a small decrease in signal stability compared to freshly diluted buffer.

3. Nano-Glo® In-Gel Detection Protocol

Materials to Be Supplied by the User

- isopropanol
- deionized water
- polyacrylamide gels and electrophoresis apparatus
- chemiluminescence imager

3.A. Overview of the Nano-Glo® In-Gel Detection System

The Nano-Glo® In-Gel Detection System is an antibody-free method for detecting and characterizing NanoLuc®-tagged proteins that have been separated by SDS-PAGE or native PAGE. NanoLuc® luciferase refolds spontaneously when SDS is removed to generate a bright, luminescent enzyme. The furimazine substrate readily permeates the gel, producing a luminescent signal that is proportional to the amount of NanoLuc®-tagged protein over multiple orders of magnitude.

3.B. Preparing the Nano-Glo® In-Gel Detection Reagent

We recommend freshly preparing the 1X Nano-Glo® In-Gel Detection Reagent immediately before adding the reagent to gels for the best results (i.e., during the protocol in Section 3.C, Step 6).

1. Determine a volume of Nano-Glo® In-Gel Detection Reagent that completely covers the gel. In a separate container, dilute the 10X Nano-Glo® In-Gel Buffer tenfold into deionized water and mix by inversion (e.g., use 1ml of 10X buffer with 9ml of water in a 15ml conical tube).
2. Dilute Nano-Glo® Luciferase Assay Substrate 500-fold into the 1X buffer and mix by inversion (e.g., add 20µl of substrate into 10ml of 1X buffer).

   Note: If the Nano-Glo® Luciferase Assay Substrate has collected in the cap or on the sides of the tube, briefly spin the tube in a microcentrifuge.

3.C. Detecting NanoLuc® Fusion Proteins after PAGE

1. Generate samples containing NanoLuc® fusion proteins (e.g., by lysing cells transfected with expression constructs for the NanoLuc®-tagged proteins). Combine samples with the appropriate PAGE loading buffer.
2. Separate proteins by SDS-PAGE or native PAGE. We recommend including prestained molecular weight markers for SDS-PAGE.
3. Remove the gel from between the plates, and transfer to the smallest available container that allows the gel some freedom of motion on a rocking platform. Minimize handling of the gel and hold it by the edges. Incubate the gel in water until ready to proceed to Step 5. If native PAGE was performed, you may skip to Step 7, if desired.
4. Determine a volume of liquid that completely covers the gel. Dilute 1 part isopropanol into 3 parts water to create enough 25% (v/v) isopropanol for at least two washes.
5. Perform two 15-minute washes of the gel in 25% isopropanol with gentle rocking.
6. Rinse the gel quickly in water several times, and perform two 15-minute incubations in water with gentle rocking. While the gel is equilibrating in water, prepare the Nano-Glo® In-Gel Detection Reagent as described in Section 3.B.
7. Remove the water rinse on the gel and add sufficient Nano-Glo® In-Gel Detection Reagent to cover the gel. Place the container in an appropriate chemiluminescence imager with a CCD camera. The NanoLuc® signal will generally increase for 10–15 minutes after adding detection reagent (see Section 6.A). If taking a single image, we recommend waiting at least 5 minutes before imaging. If taking multiple exposures that can be integrated (see below), imaging can begin immediately after adding detection reagent. We recommend leaving the gel submerged in the reagent while imaging. If you intend to remove the gel from the detection reagent for imaging, incubate the gel with detection reagent for at least 10 minutes before imaging.
8. Image the blot with appropriate settings for the desired sensitivity, resolution and exposure times. Greater sensitivity will be achieved with longer exposures (e.g., 30 minutes). If compatible with the imager, we recommend using an imaging program that automatically takes increasing exposure times (e.g., 1 second, 3 seconds, 10 seconds, 30 seconds, 100 seconds, 300 seconds, 1,000 seconds) and can integrate the various exposures for maximal signal. In this way, very bright signals are imaged with short exposures that do not exceed the detector limits, while weak signals are visible during longer exposures.

4. Representative Data

The Nano-Glo® In-Gel Detection System enables researchers to verify the expression and size of NanoLuc® fusion proteins and monitor degradation or post-translational modifications. Separating lysates by SDS-PAGE also quantifies a specific species, even when other NanoLuc®-tagged proteins or fragments might be present. Because of the sensitivity of NanoLuc® luciferase, proteins do not need to be overexpressed to be visualized by the Nano-Glo® In-Gel Detection System.

To highlight the ease with which NanoLuc®-tagged proteins can be separated from a complex mixture, HEK293 cells were transiently transfected with CMV expression constructs for five different protein kinases fused to NanoLuc® luciferase, diluted 100-fold into carrier DNA. Proteins were expressed in cells both individually and in combinations. One day after transfection, cell lysates were prepared and separated by SDS-PAGE, and the detection

protocol in Section 3.C was used to visualize the fusion proteins (Figure 2, Panel A). After imaging the NanoLuc® activity, gels were stained with Coomassie® dye and imaged to show total protein bands (Figure 2, Panel B). Cells transfected with carrier DNA alone showed no luminescent background bands, while transfected cells showed fusion proteins of the expected molecular weights. The ability to separate the signals from different NanoLuc® fusions expressed in the same cells highlights the possibility for multiplexing the quantification of multiple proteins (e.g., comparing the levels of a regulated protein and a constitutively expressed control protein).

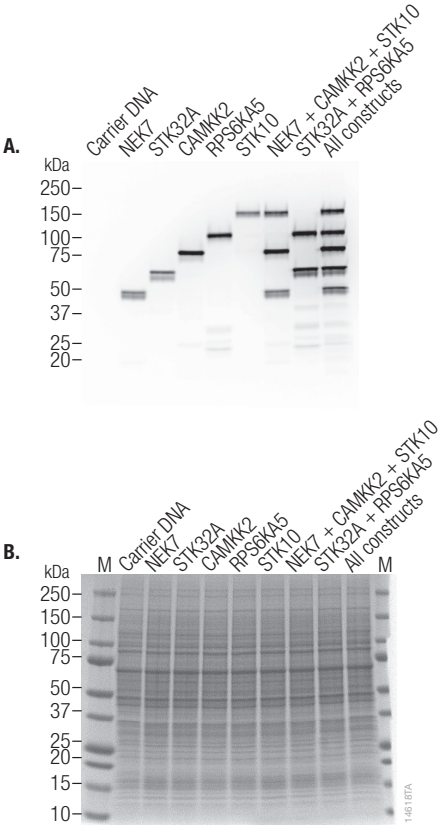

Figure 2. SDS-PAGE of transiently transfected HEK293 cells. HEK293 cells were transiently transfected individually or in combinations with five different fusions of NanoLuc® luciferase to protein kinases: NEK7, STK32A, CAMKK2, RPS6KA5 and STK10. CMV expression constructs were diluted 100-fold into carrier DNA to lower expression levels. After overnight expression, cells were lysed in a ¼ volume of Mammalian Lysis Buffer (Cat.# G9381), supplemented with Protease Inhibitor Cocktail (Cat.# G6521) and RQ1 RNase-Free DNase (Cat.# M6101). For each well, 10µl of lysate was mixed with SDS Loading Buffer, heated and loaded onto a 4–20% gradient gel (Bio-Rad Cat.# 3450033). **Panel A.** Following the protocol in Section 3.C, the NanoLuc® fusions were visualized. The image represents a 30-second exposure immediately after adding reagent. **Panel B.** The gel was subsequently incubated with Coomassie® dye (SimplyBlue™ SafeStain, Thermo Cat.# LC6065) and imaged by transillumination to show total protein in the lysate.

5. Related Products

Detection Reagents

Product	Size	Cat.#
Nano-Glo® Luciferase Assay System	10ml	N1110
	100ml	N1120
	10 × 10ml	N1130
	10 × 100ml	N1150

NanoLuc® Fusion Entry Vectors

Product	Size	Cat.#
NanoBRET™ PPI Starter Systems	1 each	N1811
	1 each	N1821
pNLF1-N [CMV/Hygro] Vector	20µg	N1351
pNLF1-C [CMV/Hygro] Vector	20µg	N1361
pNLF1-secN [CMV/Hygro] Vector	20µg	N1371
pFN31A *Nluc* CMV-Hygro Flexi® Vector	20µg	N1311
pFN31K *Nluc* CMV-neo Flexi® Vector	20µg	N1321
pFC32A *Nluc* CMV-Hygro Flexi® Vector	20µg	N1331
pFC32K *Nluc* CMV-neo Flexi® Vector	20µg	N1341

Transfection Reagents

Product	Size	Cat.#
FuGENE® HD Transfection Reagent	1ml	E2311
	5 × 1ml	E2312
ViaFect™ Transfection Reagent	0.75ml	E4981
	2 × 0.75ml	E4982
Transfection Carrier DNA	5 × 20µg	E4881

Detection Instruments

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System Fully Loaded	1 each	GM3500
GloMax® Explorer System with Luminescence and Fluorescence	1 each	GM3510

6. Appendix

6.A. Overview of the Nano-Glo® In-Gel Detection System

One of the most common ways to characterize protein samples is to separate them by molecular weight using SDS-PAGE, transfer them to a membrane and perform Western blotting. Western blotting depends on the availability of high-quality antibodies for sensitive and selective detection of proteins, and blotting performance can be quite variable, depending on the conditions used (e.g., type of blocking agent, number and duration of washes, antibody concentrations, length of incubations, etc.). Fusing proteins of interest to the NanoLuc® luciferase is a simple and sensitive alternative for protein quantification. Lytic detection of NanoLuc®-tagged proteins using the Nano-Glo® Luciferase Assay System (Cat.# N1110) represents the simplest and most sensitive way to quantify total NanoLuc® levels in a sample. Sometimes you may need to determine the molecular weight of a fusion protein or to separate multiple luminescent species from each other. The Nano-Glo® In-Gel Detection System can specifically image NanoLuc® fusion proteins within the gel matrix after SDS-PAGE or native PAGE without the need for antibodies or membrane transfer.

Following SDS-PAGE, the gel is washed twice in 25% isopropanol to remove the SDS detergent. The small size and stability of NanoLuc® luciferase facilitates its spontaneous refolding within the gel after isopropanol removal to form an active enzyme. The bright luminescent signal and low reagent background cmeans you can detect small amounts of NanoLuc®-tagged proteins (subpicogram amounts of a 50kDa fusion protein). Luminescence in the gel is proportional to the amount of fusion protein over a range of about five orders of magnitude (Figure 3). However, a given exposure of a single gel may only quantify bands over about two orders of magnitude due to imager limitations and carryover of light from surrounding lanes (Figure 3, Panel D). With a titration of purified protein, we observe that the band intensity following SDS-PAGE is nearly as high as after native PAGE (Figure 3, Panel C). This suggests that a high percentage of unfolded NanoLuc® molecules are able to refold into active enzyme following the washes. Comparison studies with a variety of fusion proteins suggest that 10–50% of the NanoLuc® luciferase typically refolds after the wash procedure described in Section 3.C.

The ability of SDS-PAGE to resolve two proteins of different molecular weight means that the Nano-Glo® In-Gel Detection System can multiplex measurements of multiple NanoLuc®-tagged proteins in the same sample (Figure 2, Panel A). For instance, you could monitor changes in the expression level of one NanoLuc®-tagged protein relative to a control protein of different molecular weight that is not expected to change in expression. This could help normalize among samples for differences in cell number, transfection efficiency, cell lysis or total protein loads.

We recommend loading prestained molecular weight markers when running SDS-PAGE. Image the gel by trans- or epi-illumination before or after the luminescence image so that the two images can be overlaid to estimate the molecular weight of your NanoLuc®-tagged protein. Some imagers can perform epifluorescence imaging, which can be particularly useful for overlays because many prestained molecular weight standards are intrinsically fluorescent.

After adding the reagent, luminescence can take 10–15 minutes to reach maximum levels, after which the signal slowly decays with a half-life of about 2 hours (Figure 4). If you wish to quantify and compare the intensities of different bands, incubate gels for 10 minutes with the reagent to saturate the enzyme with substrate before imaging. However, if you do not require maximal initial intensity, you can begin a series of exposures immediately after adding reagent and integrate the images to achieve the desired band intensity.

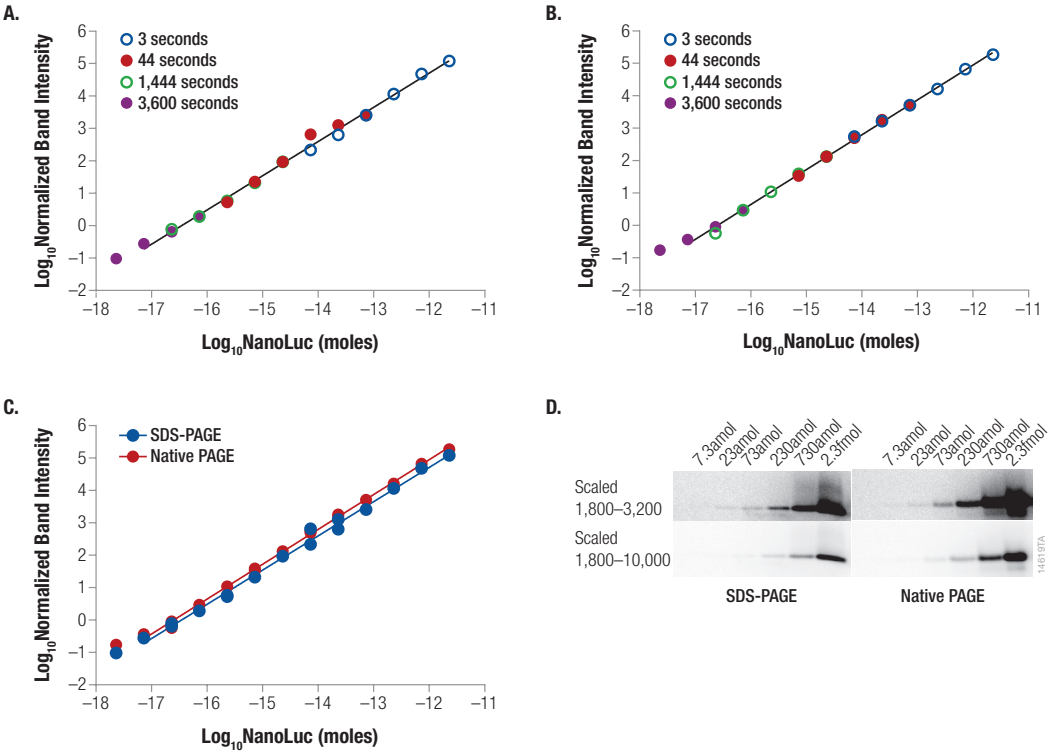

Figure 3. SDS-PAGE and native PAGE of a NanoLuc® luciferase titration. Purified NanoLuc® luciferase was serially diluted in half-log increments into PBS with 0.1mg/ml BSA as carrier. Samples were subjected to either SDS-PAGE or native PAGE, with four separate gels containing overlapping titrations of proteins imaged separately for each type of PAGE to quantify a wide range of protein amounts with varying exposure times. The exposure times used to quantify luciferase activity, from highest to lowest amount, were 3; 44; 1,444 and 3,600 seconds. Background-subtracted band intensities were normalized among different exposure times using bands of equivalent protein amount. Combined normalized intensities for all the exposure times were plotted on a log/log graph, and a best-fit line was generated by linear regression for the data from 7 attomoles to 2 picomoles (150fg–50ng). **Panel A.** Samples were mixed with SDS Loading Buffer, heated, loaded on a 4–20% gel (Bio-Rad Cat.# 3450033), run in SDS running buffer and treated according to the protocol in Section 3.C. **Panel B.** The same samples were mixed with Native Loading Buffer, loaded on the same gel, and run in Native Running Buffer in the absence of SDS. The protocol in Section 3.C was performed, eliminating the isopropanol and water washes. **Panel C.** Comparison of the combined data for SDS and native polyacrylamide (PA) gels shows that the luminescence from NanoLuc® luciferase subjected to SDS-PAGE was within twofold of that subjected to native PAGE, suggesting that >50% of the protein was able to refold within the SDS-PA gel after the washes. The band intensities for SDS and native PA gels displayed excellent proportionality, with slopes on the log/log plot of 1.057 and 1.076 and r^2 values of 0.9942 and 0.9983, respectively. **Panel D.** Representative image of the 1,444-second exposure of the gels with the second-lowest titrations.

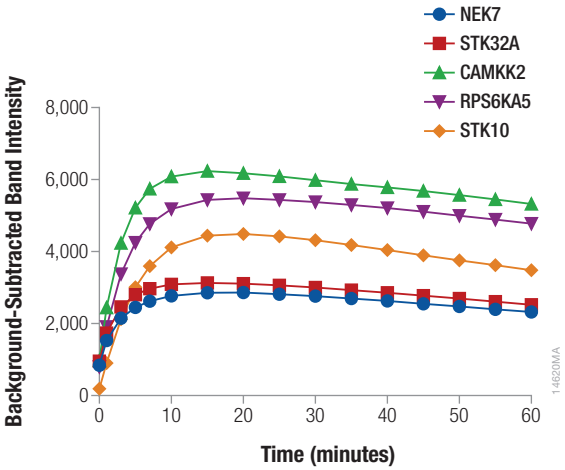

Figure 4. Signal kinetics of various NanoLuc® fusions after adding detection reagent to an SDS-PAGE gel. A duplicate gel to the one shown in Figure 2, Panel A, was imaged repeatedly with 15-second exposures over the course of 1 hour to monitor signal kinetics. The background-subtracted band intensities increased for 10–15 minutes after adding reagent and then declined with a signal half-life of about 2–3 hours.

6.B. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Low band intensity	For maximal intensity, make sure that the gel is incubated with the reagent for at least 10 minutes before imaging.
	If possible, transfect cells with higher amounts of expression constructs, express longer in cells, or load more lysate onto the gel.
	Fusions to particular proteins or use of particular linkers may inhibit refolding of NanoLuc® luciferase. Consider tagging the opposite protein terminus or changing the linker between NanoLuc and the protein.
High background observed	Avoid touching gels on their edges and minimize handling.
Difficult to determine the molecular weight of bands	Molecular weight markers not used or are unmarked. While there are no standards that luminesce with the Nano-Glo® In-Gel Detection Reagent, prestained molecular weight markers can be used. An illuminated image can be taken before or after the luminescent image so that the positions of the molecular weight standards can be determined. If available on the imager, use epifluorescence imaging of prestained markers for particularly easy overlay of images.

6.C. References

1. Hall, M.P. *et al.* (2012) Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem. Biol.* **7**, 1848–57.
2. Machleidt, T. *et al.* (2015) NanoBRET—A novel BRET platform for the analysis of protein-protein interactions. *ACS Chem. Biol.* **10**, 1797–804.
3. Robers, M.B. *et al.* (2015) Target engagement and drug residence time can be observed in living cells with BRET. *Nat. Commun.* **6**, 10091.

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

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