A New Dual Luciferase Assay Using NanoLuc Enables a Second-Generation Coincidence Reporter System to Reduce False Hits in HTS

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Abstract #285

1. Introduction

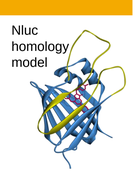
Luciferase-based reporter-gene assays remain a cornerstone of high-throughput screening of compounds because of their high sensitivity and dynamic range. However, a substantial number of non-relevant hits can be generated due to direct interaction of compounds with the luciferase reporter. To help differentiate compounds modulating the biological pathway of interest from those affecting the stability or activity of the reporter enzyme, we have developed a second-generation coincidence reporter system in which transcriptional activation leads to stoichiometric expression of two orthologous reporters that have dissimilar profiles of compound interference. In this system, firefly luciferase (Fluc) and PEST-destabilized NanoLuc® luciferase (NlucP) are expressed off the same promoter using ribosome skipping mediated by the P2A peptide.

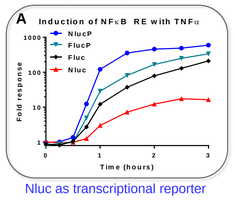
To sensitively measure both Fluc and NanoLuc (Nluc) in the same sample, we have developed the Nano-Glo® Dual-Luciferase® Reporter (NanoDLR™) Assay System, a homogeneous lytic assay performed in an "add-read-add-read" format, in which the Fluc signal is quenched over a million-fold by addition of the Nluc reagent. The increased brightness of Nluc and improved Fluc inhibition means that Nluc can be detected at over 2-3 orders of magnitude lower molar concentration than *Renilla* luciferase in the existing homogenous firefly/*Renilla* dual-luciferase assay (Dual-Glo), allowing both luciferases to be dynamic reporters.

Following single-copy integration of the Fluc-2A-NlucP biocircuit into a gene locus relevant to Parkinson's disease, HTS using NanoDLR easily distinguished compounds affecting one of the reporters from those affecting transcription, yielding a >5-fold decrease in the number of hits.

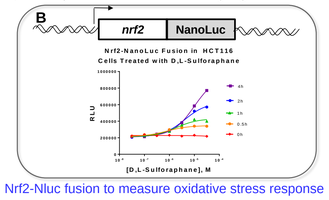
2. NanoLuc: a brighter, smaller luciferase for reporter gene assays and protein fusions

- NanoLuc® luciferase (Nluc) is a 19.1 kDa, **ATP-independent** luciferase that utilizes a novel coelenterazine analog (furimazine) to produce high-intensity, glow-type luminescence (about **150-fold brighter** than firefly or *Renilla*).
- The broad dynamic range and sensitivity of Nluc make it an ideal transcriptional reporter. Adding a PEST sequence to destabilize the protein (NlucP) gives **maximal temporal dynamics and fold induction** (Panel A). Adding a secretion signal (secNluc) allows transcription to be measured without cell lysis.
- Because of their brightness, **Nluc fusions** can enable such applications as 1) measurement of protein stability at endogenous levels (Panel B), 2) **BRET** studies of protein-protein interactions, and 3) bioluminescent imaging.

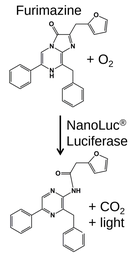




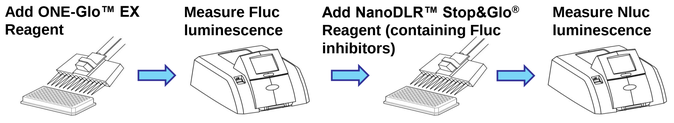
Nluc as transcriptional reporter



Nrf2-Nluc fusion to measure oxidative stress response



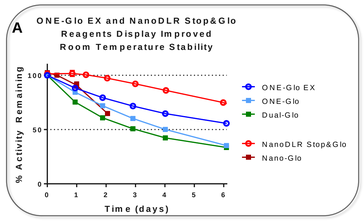
3. The Nano-Glo® Dual-Luciferase® Reporter (NanoDLR™) Assay System

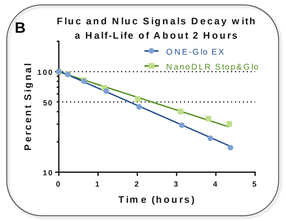


- "Add-read-add-read" **homogeneous format** is conducive to HTS.
- The Fluc signal is quenched over a million-fold upon addition of the NanoDLR™ Stop&Glo® Reagent, which also supplies the substrate for Nluc activity.
- The increased brightness of Nluc compared to *Renilla* luciferase and improved inhibition of the Fluc signal give orders of magnitude **greater sensitivity** than the existing dual assay.
- ONE-Glo™ EX Reagent utilizes new chemistry that provides a two-hour signal half-life for firefly luciferase, **enhanced room temperature stability**, **lack of odor** from DTT, steadier signal decay kinetics, and improved performance in the presence of phenol red.

4. Improved reagent stability and signal stability to facilitate batch processing of plates

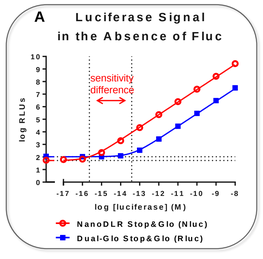
- Both ONE-Glo™ EX and NanoDLR™ Stop&Glo® reagents were designed for improved room temperature stability once reconstituted (Panel A).
  - ➢ Consistent luminescence values throughout long screening runs.
- Both reagents exhibit a steady rate of signal decay with a half-life of about two hours (Panels B).
  - ➢ A stable signal facilitates batch processing and decreases plate-to-plate and well-to-well variability.

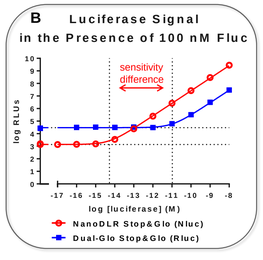




5. Orders of magnitude greater sensitivity for Nluc in NanoDLR™ assay compared to *Renilla* luciferase

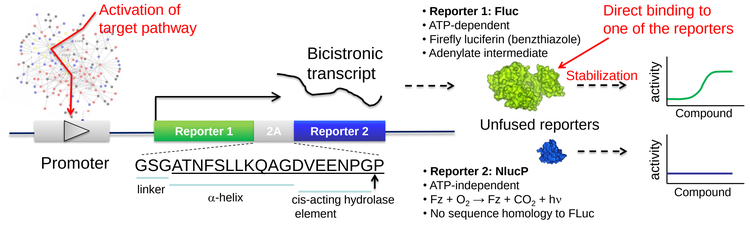
- The inherent brightness of Nluc compared to Rluc makes NanoDLR more than 100-fold more sensitive than Dual-Glo in the absence of Fluc (Panel A).
- With very high Fluc concentrations, uninhibited Fluc signal can increase the background for the second luciferase. The improved Fluc inhibition in NanoDLR yields **over 1000-fold greater sensitivity** than Dual-Glo under these conditions (Panel B).





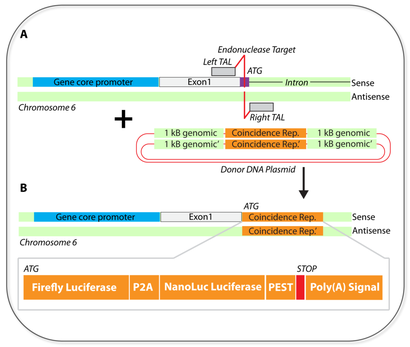
6. Using two different transcriptional reporters can greatly improve confidence in HTS results

- Compounds directly binding the reporter can inhibit and/or stabilize the enzyme, leading to positives not related to altered transcription (Auld, DS, *et al. ACS Chem. Biol.*, 2008; 3: 463-470.)
- A coincidence reporter system, in which transcription is linked to two reporters with dissimilar profiles of compound interference, can dramatically decrease the number of hits arising from direct interactions with the reporter (Cheng, KC & Inglese, J. *Nature Methods*, 2012;9:709–736.)
- Firefly luciferase (Fluc) and PEST-destabilized NanoLuc (NlucP) make a particularly attractive pair of orthologous reporters (Ho, P. *et al. ACS Chem. Biol.*, 2013; 8: 1009-1017.)



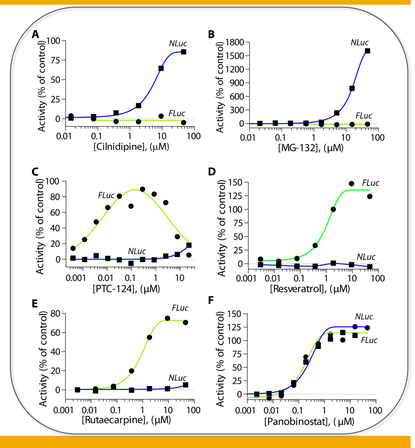
7. Coincidence reporter to screen for increased expression from an endogenous promoter

- The Fluc-P2A-NlucP biocircuit was integrated into a gene locus relevant to Parkinson's disease by TALEN-mediated genome editing in order to screen for compounds increasing transcription (Panels A & B).
- P2A-peptide-mediated ribosome skipping allowed unfused Fluc and NlucP to be expressed stoichiometrically from the same endogenous promoter.
- 315,000 compounds were screened in 5-point qHTS format in 1536-well plates.
- Approximately 6300 compounds (2%) displayed activation of an individual reporter, but only ~950 compounds (0.3%) were positives with coincidence, yielding a >5-fold reduction in hits.



8. Coincidence reporter distinguishes transcriptional activators from compounds stabilizing reporter

- Compounds causing interference with Fluc (Panels C-E) or Nluc (Panel A) alone showed an increase in signal only for that reporter.
- The addition of a PEST sequence to only Nluc allowed for the identification of compounds that interfere globally with protein stability and turnover, such as the proteasome inhibitor, MG-132, which caused an increase in the NlucP, but not Fluc, signal (Panel B).
- Those compounds truly increasing transcription, such as the histone deacetylase inhibitor, panobinostat, showed coincident increases in both reporters (Panel F).



9. Summary

- The Nano-Glo® Dual-Luciferase® Reporter (NanoDLR™) Assay System provides robust and sensitive measurement of firefly luciferase (Fluc) and NanoLuc (Nluc) in a single well.
  - ➢ Homogeneous assay, not requiring cumbersome aspiration, wash, and lysis steps.
  - ➢ "Glow" (not "flash") kinetics, with two-hour signal half-life, allow for batch processing of plates.
  - ➢ Improved stability means reconstituted reagents can be kept at room temperature for long runs with little decrease in luminescence.
  - ➢ The increased brightness of Nluc and improved quenching of the Fluc signal give orders of magnitude greater sensitivity of detection, compared to *Renilla* luciferase in Dual-Glo.
  - ➢ This increased sensitivity enables both luciferases to be used as dynamic reporters, enabling additional formats, like coincidence reporting.
- A coincidence reporter system with Fluc and NlucP can dramatically improve the quality of data coming out of a high-throughput screen.
  - ➢ Luciferase inhibitors can represent a significant fraction of hits in HTS because of their stabilization of the reporter enzyme in cells or their inhibition of reporter activity.
  - ➢ Fluc and Nluc are non-homologous luciferases utilizing different substrates, and they have dissimilar profiles of compound interference.
  - ➢ Performing screens with an Fluc-P2A-NlucP coincidence reporter can eliminate assay artifacts, reducing the number of actives >5-fold and increasing the confidence that hits are biologically relevant.

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January 2014