New Small and Versatile Reporter Technologies for Challenging Applications in Virology

Robert Brazas, Ph.D.
Presentation Overview
Small size of NanoLuc® Luciferase is advantageous

Agenda

✓ Building a better luciferase: NanoLuc® Luciferase
✓ Advantages and performance
✓ NanoLuc® luciferase as a viral reporter
  • Alphavirus and influenza examples
What is NanoLuc® Luciferase?
A highly-engineered small, bright luciferase

Promega Advanced Technologies Group

Promega Corporation

NanoLuc® Luciferase
A modern, engineered luciferase

NanoLuc® (Nluc):
- 19.1kDa (small)
- ATP independent
- Uses a novel coelenterazine analog (furimazine) substrate
- Produces high intensity, glow-type luminescence

Furimazine

2-furanylmethyl-deoxy-coelenterazine

Furimamide

2-furanylmethyl-deoxy-coelenterazine
Nano-Glo® Reagent System
Optimized substrate for NanoLuc® Luciferase

Nano-Glo® Luciferase Assay Reagent
- Furimazine (modified substrate)
  - Maximize NanoLuc® Assay brightness
  - Minimize autoluminescence background
- ATP independent reaction
- Glow kinetics
  - Half-life routinely >2 hours at room temperature
- Stable reconstituted reagent:
  - ~10% decrease in activity over 8 hrs at RT

Add - Mix - Measure format like other Glo Assay Systems
**NanoLuc® Luciferase**  
Smallest luciferase available, ~3x smaller than Fluc

<table>
<thead>
<tr>
<th></th>
<th>Amino acids</th>
<th>M.W. (kD)</th>
<th>Mol. Vol. Å³</th>
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</thead>
<tbody>
<tr>
<td><strong>Nluc</strong></td>
<td>171</td>
<td>19.1</td>
<td>14</td>
</tr>
<tr>
<td><strong>Rluc</strong></td>
<td>312</td>
<td>36.0</td>
<td>32</td>
</tr>
<tr>
<td><strong>Fluc</strong></td>
<td>550</td>
<td>60.6</td>
<td>44</td>
</tr>
</tbody>
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*Firefly (Fluc)*  
*Renilla (Rluc)*  
*NanoLuc® (Nluc)*
NanoLuc® Luciferase is Super Bright
Improves assay sensitivity

Purified Luciferases

NanoLuc® Luciferase is ~100-fold brighter!

![Graph showing luminescence (RLU) vs. luciferase (pM) for NanoLuc, Firefly, and Renilla Luciferases.]

Transfected Luciferase Reporters

NanoLuc® Luciferase is ~100-fold brighter in cells too!

![Graph showing luminescence (RLU) vs. transfected DNA (pg) for HepG2 and HeLa cells transfected with NanoLuc, Firefly, and Renilla Luciferases.]
Decreased Number of False Hits in HTS When Using NanoLuc® Luciferase

LOPAC library (Sigma)
- Library of Pharmaceutically Active Compounds
- 1280 compounds
- Small organic ligands w/ well documented pharmacological activities
- Used to screen for non-specific luciferase activity modulators

Experimental details:
LOPAC library members at 10µM final concentration; incubation with purified NanoLuc® or firefly luciferase for 2 min.; Fluc detection using ONE-Glo™ Luciferase Assay.

<table>
<thead>
<tr>
<th>Inhibition</th>
<th>Stimulation</th>
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<tbody>
<tr>
<td>≥ 10%</td>
<td>≥ 20%</td>
</tr>
<tr>
<td>NanoLuc®</td>
<td>1.2%*</td>
</tr>
<tr>
<td>Firefly</td>
<td>1.9%*</td>
</tr>
</tbody>
</table>

* = % of library compounds
NanoLuc® Luciferase Reporter Genes
Three versions to match your needs

**Intracellular Formats**

- **pNL1.1**
  - NanoLuc® Luciferase
  - *Nluc* (513 bp)
  - Protein destabilization domain

- **pNL1.2**
  - NanoLuc® Luciferase
  - *Nluc*P (636 bp)
  - PEST

- **pNL1.3**
  - **IL6**
  - NanoLuc® Luciferase
  - sec*Nluc* (597 bp)
  - Secretion signal

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Alphavirus-NanoLuc® Reporter Viruses
+ Strand RNA viruses with a subgenomic promoter

Data from Klimstra Lab at Pitt:
Stable, high-level expression of reporter proteins from improved alphavirus expression vectors to track replication and dissemination during encephalitic and arthritogenic disease. Sun et al. (2014) J. Virol. 88:2035-2046
Alphavirus-NanoLuc® Reporter Viruses
Three viral reporter strategies tested

Wild Type

3’ DP

nsP3-Fusion Protein

TaV (Self-cleaving polypeptide)

= NanoLuc® or Firefly luciferase reporter gene
Sindbis-NanoLuc® Reporter Viruses
NanoLuc® reporter viruses are more stable

Firefly

NanoLuc®

Promega Corporation
Leveraging the Small Size of NanoLuc® to Generate an Influenza-NanoLuc® Reporter Virus

- Historically, it’s been difficult to stably integrate reporter genes into influenza
- Mehle lab created influenza recombinant virus stably expressing NanoLuc®
- Data presented here are published in:
  

  Submitted Paper: Multi-modal imaging with a toolbox of influenza A reporter viruses. Tran et. al. (2015 submitted)

Dr. Andrew Mehle’s Lab at UW Madison
Leveraging the Small Size of NanoLuc® to Generate an Influenza-NanoLuc® Reporter Virus

Wild Type Influenza Virus Particle

Influenza-NanoLuc® Virus Particle

NanoLuc® gene inserted into genome
Influenza-NanoLuc® Reporter Virus
Two reporter virus strategies tested

Expressed Protein(s)
- PA
- PA-NLuc Fusion
- PA
- NLuc
- PA-NLuc Fusion
- PA
- NLuc
- PA
- NLuc
- PA
- NLuc
- PA
- NLuc
- PA
- NLuc
- PA
- NLuc
- PA
- NLuc
Influenza-NanoLuc® Reporter Virus
Cell culture-based experiments
Influenza-NanoLuc® Reporter Virus
Measuring viral “titers” with a luminescent assay

Infected cells to be titered → Infect in triplicate w/ 20µl viral supernatants → Fresh MDCK cells → Incubate 1hr → Wash cells → Incubate 8hr → Assay NanoLuc® Activity with Nano-Glo® Luciferase Assay System → ~10 minutes + luminometer read
Influenza-NanoLuc® Reporter Virus
Luciferase activity is a direct readout of viral titer

![Graph showing titer (log RLU) vs. titer (log PFU/ml) over time (hpi). The graph includes a level of detection and a linear regression equation with R² = 0.99017.]

Equation:

\[ y = 0.8658x + 0.3026 \]

R² = 0.99017
Influenza-NanoLuc® Reporter Virus
Replication at nearly native levels
Influenza-NanoLuc® Reporter Virus
Luminescent detection of ribavirin antiviral activity

Significant Inhibition of Viral Replication (100µg/ml, pretreated)
Influenza-NanoLuc® Reporter Virus
PASN virus incorporates PA-Nluc protein into virions

PA (native) 5' UTR PA 3' UTR
PA-SWAP-2A-Nluc50 (PASTN) 5' PA (WSN, PR8, CA04, VN1203, Anhui/01) Nluc (517 nt) 3' Codon swap 2A peptide (73nt) 50nt “repeat”
PASN 5' PA (WSN) Nluc 3'

Expressed Protein(s)
PA & Nluc
PA-Nluc Fusion*

PASN Flu-Nluc Virion
~20 PA-Nluc* Fusion Proteins per Virion

Tran et. al.,Submitted
Influenza-NanoLuc® Reporter Virus
Luminescent detection of virus attachment

~20 PA-Nluc Fusion Proteins per Virion

Add $10^6$ PASN Flu-Nluc viruses to cells
Incubate 0-45 min
Wash cells

MDCK or A549 Cells

Assay bound Nluc Virus with Nano-Glo® Luciferase Assay System
~10 minutes + luminometer read

Tran et. al., Submitted
Influenza-NanoLuc® Reporter Virus
Luminescent detection of influenza virus binding

+RDE = Cells pretreated with neuraminidase to remove sialic acid

Tran et. al., Submitted
Influenza-NanoLuc® Reporter Virus
Highly sensitive detection of influenza binding

*Above background detection of binding with ≥100 pfu

Mehle Lab, Unpublished
Influenza-NanoLuc® Reporter Virus
In vivo experiments
Influenza-NanoLuc® Reporter Virus
Highly similar pathogenicity to wild type virus

weight loss (pathogenicity)

Lung Titers (Viral Load)
Influenza-NanoLuc® Reporter Virus
Real-time imaging of influenza infection
Influenza-NanoLuc® Reporter Virus
Nearly native in vivo pathogenicity

Weight Loss (Pathogenicity)

Flux (Bioluminescent signal) correlates with input virus and weight loss

Mouse in Next Slide
Influenza-NanoLuc® Reporter Virus
Imaging clearance of a sublethal dose in vivo
Real-Time Investigation of Influenza-NanoLuc® Reporter Virus Replication in Ferrets

- Ferrets are the preferred “gold-standard” model of influenza infection
- Schutz-Cherry and Mehle labs created influenza recombinant stably expressing NanoLuc® in the pandemic 2009 H1N1 (A/California/04/2009; CA/09) strain = CA/09-PA Nluc Virus
- Used same construction design as PATSN virus from original Tran et. al. paper
- Data presented here are published in:


Dr. Stacey Schultz-Cherry Lab at St. Jude Children’s Hospital

Dr. Andrew Mehle’s Lab at UW Madison
CA/09-PA Nluc Virus Reporter Virus
In vivo imaging experimental design

Transmission via Direct Contact?

Airborne Transmission?

Direct Group

Donor Group

Respiratory Group
CA/09-PA Nluc Virus Reporter Virus – Donor Group
NanoLuc® brightness makes imaging in ferrets possible

- Intranasally inoculated with $10^5$ TCID$_{50}$ units of virus in 1ml PBS
- 8–10 week-old male ferrets
CA/09-PA Nluc Virus Reporter Virus
Transmission was detected by both routes

- Assayed nasal washes for virus by standard TCID<sub>50</sub> assay
- Direct and respiratory transmission routes resulted in infected ferrets
  - 100% infected by direct route
  - 100% infected by respiratory route
- Viral infection occurred sooner via direct contact than respiratory route
- All infections were eventually cleared

Black = Donor Group Ind.
Blue = Direct Group Ind.
Red = Respiratory Group Ind.

Days post infection
Viral titre (log<sub>10</sub> TCID<sub>50</sub> per ml)

HAU viral titre (log<sub>10</sub> TCID<sub>50</sub> per ml)
CA/09-PA Nluc Virus Reporter Virus

Nluc viral titer TCID$_{50}$ saves time vs. standard method

- Assayed nasal washes for virus by TCID$_{50}$ assay or NanoLuc® TCID$_{50}$ assay
- Both assays correlated extremely well
- NanoLuc® assay takes about 18 hrs vs. 3 days for HUA TCID$_{50}$ assay

Black = Donor Group Ind.
Blue = Direct Group Ind.
Red = Respiratory Group Ind.
CA/09-PA Nluc Virus Reporter Virus

Direct assay of nasal washes for Nluc activity is quick

- Assayed nasal washes for virus by NanoLuc® TCID$_{50}$ assay or by directly assaying for NanoLuc® activity in the nasal washes
- Direct NanoLuc® activity assay correlated very well to TCID$_{50}$ assay
- Direct NanoLuc® assay is instantaneous vs. 18 hrs vs. NanoLuc® TCID$_{50}$ assay
- Bioluminescent detection in vivo correlates with virus titers
CA/09-PA Nluc Virus Reporter Virus
Effective visualization of immunity after challenge

Strong nasal Immunity: 1+
Weak nasal Immunity: 4+
No infection Immunity: 5+
Strong Infection Immunity: (-)

Note: All previously infected ferrets had neutralizing antibodies
A Bright Future for NanoLuc® Luciferase in Viral Research and Drug Development Applications

- Small size and increased brightness make it well suited in the construction of reporter viruses
  - Position in a virus where expression is weak but virus is non-attenuated
  - NanoLuc® Luciferase brightness enables detection even when expressed poorly
A Bright Future for NanoLuc® Luciferase in Viral Research and Drug Development Applications

With these reporter viruses, many experiments are possible:

- Faster titration of viral particles (infection-based assays and direct assay of nasal washes for Nluc activity)
- Fast, sensitive viral binding assays
- In vivo imaging (infectivity, transmission, immunity…)
- Luminescent microneutralization assays that are more sensitive (data not shown)
- Faster, more sensitive immunity testing
- Drug screening both in cell culture and in animal models
Additional References for NanoLuc® Use in Viruses


= Discussed today
Technical Services Scientists are Ready to Help

techserv@promega.com
Looking for information about NanoBiT™ Technology?

We are sorry, we cannot share slides containing NanoBiT™ Technology data at this time.

For more information, please attend the webinar focused on NanoBiT™ Technology later this year:

**Monitoring Protein: Protein Interactions in Living Cells Using a Small, Bright and Reversible Complementation System**

**Tuesday, October 27, 2015**

Presented by Brock F. Binkowski, PhD
Sr. Research Scientist