NanoLuc™ Luciferase: A Smaller, Brighter & More Versatile Luciferase Reporter

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Outline

1. Bioluminescence in Life Science Research
- Reporter gene assays
- Applications using luciferase fusion proteins

2. Introduction to NanoLuc™ Luciferase Assay System
- Enzyme/substrate development
- Key features
- Nano-Glo Luciferase Assay Reagent

3. NanoLuc in reporter gene assays
- Standard or secretion based formats
- NanoLuc genetic constructs
- Performance vs. firefly and Gaussia luciferase

5. NanoLuc as fusion partner
- Results of bioluminescent imaging
- NanoLuc as stability sensor
- NanoLuc in BRET

6. Commercial products for sale
- Plasmid DNA constructs
- Nano-Glo Luciferase Assay Substrate

Luciferase as reporter gene

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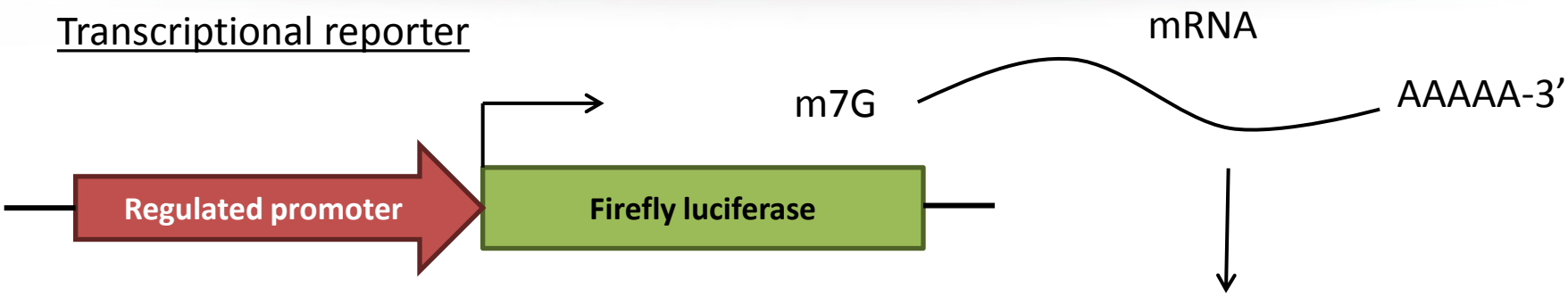
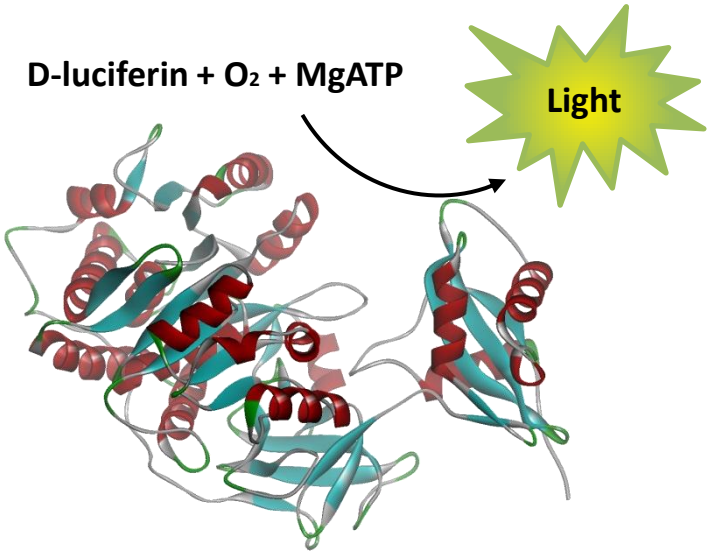
Transcriptional reporter



Desirable characteristics:

- Linear relationship between activity & concentration
  - Preferably over 7-8 logs of [luciferase]
- High sensitivity
  - Bright signal w/ low background
- Single, small subunit
- High structural stability
- Close coupling to the transcriptional response, when desired
- Standard or secretion based formats
- Ease-of-use
  - Single addition detection reagent
  - Glow-type luminescence signal



Model of firefly luciferase based on PDB file 1LCI

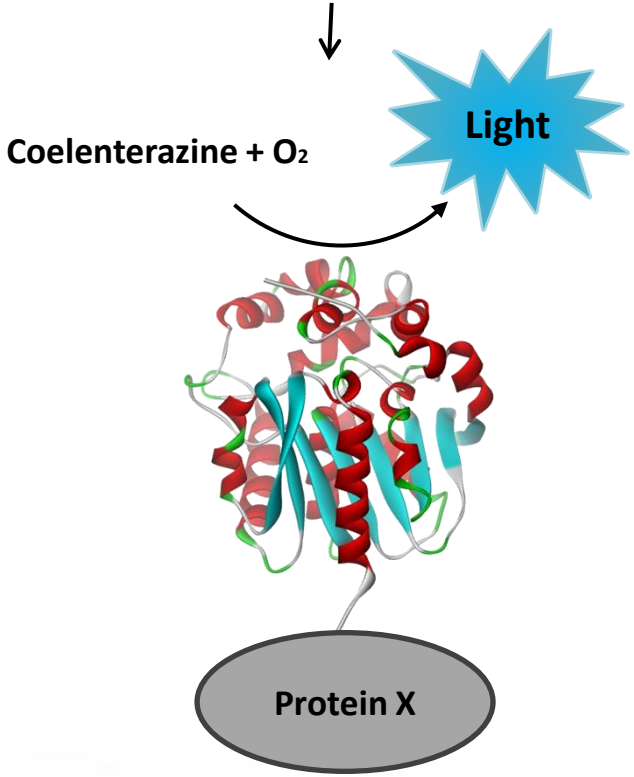
Luciferase as fusion partner

Applications:

- Bioluminescence resonance energy transfer (BRET)
- Protein stability sensors

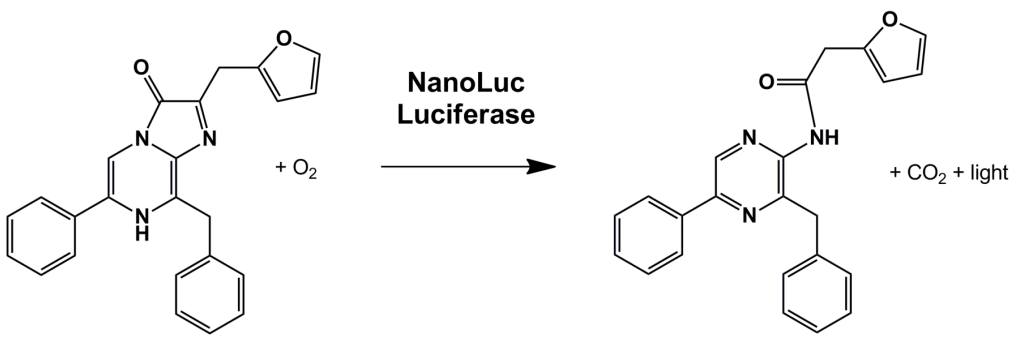
Desirable characteristics:

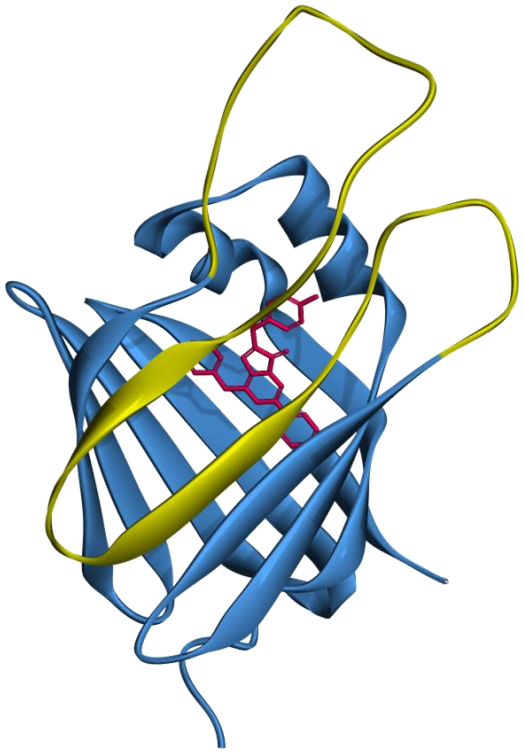
- Linear relationship between activity & concentration
- High sensitivity
  - Bright signal w/ low background
- Single, small subunit
- Emission spectrum compatible w/ fluorescent acceptors
- High structural stability
- Support high levels of activity in any cellular compartment
- No compartmental bias in the absence of targeting sequences
- Ease of use in signal detection
  - Single addition detection reagent
  - Glow-type luminescence signal



NanoLuc™ luciferase

NanoLuc™ (Nluc) is a 19.1 kDa, ATP-independent luciferase that utilizes a novel coelenterazine analog (furimazine) to produce high intensity, glow-type luminescence.





Nluc homology model

Development of NanoLuc™ & furimazine

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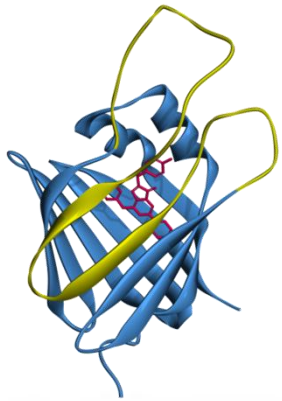
Developed by the Advanced Technology Group at Promega:

- Directed evolution on the 19 kDa subunit of *Oplophorus gracilirostris* luciferase
- Co-development of an optimized substrate
  - Screened numerous coelenterazine analogs for enhanced brightness & increased substrate stability

- Results
  - 2.5 million fold increase in luminescence
  - Large increases in thermal stability
    - Half-life at 37 °C (HEK293 cell lysates): from < 5min to ~7 days
  - Monomeric luciferase
    - Native enzyme is a heterotetramer



NanoLuc™ is very small

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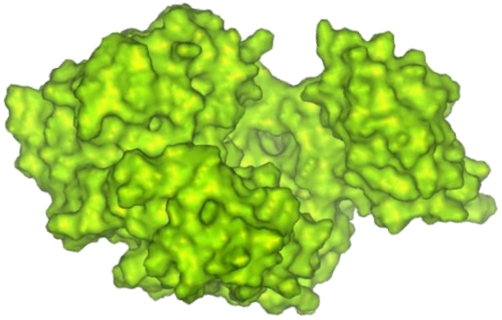
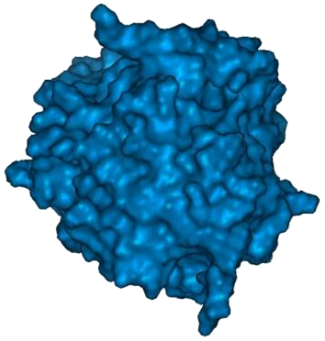
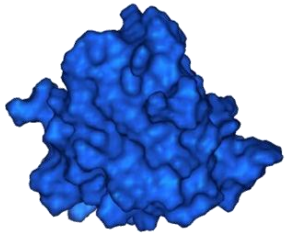
Firefly (Fluc)



Renilla (Rluc)

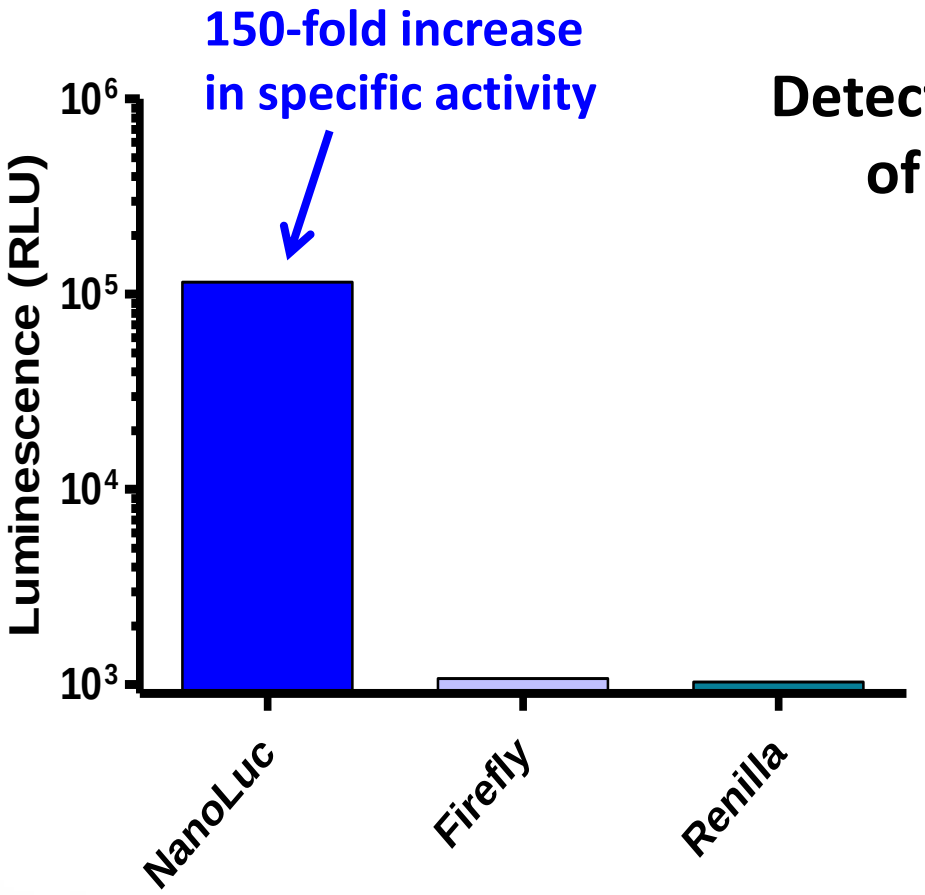


NanoLuc™ (Nluc)



	Amino acids	M.W.	Mol. Vol. Å^3
Nluc	171	19.1	14
Rluc	312	36.0	32
Fluc	550	60.6	44

NanoLuc™ is very bright



Detection of 50 attomoles of purified enzyme

Reagents

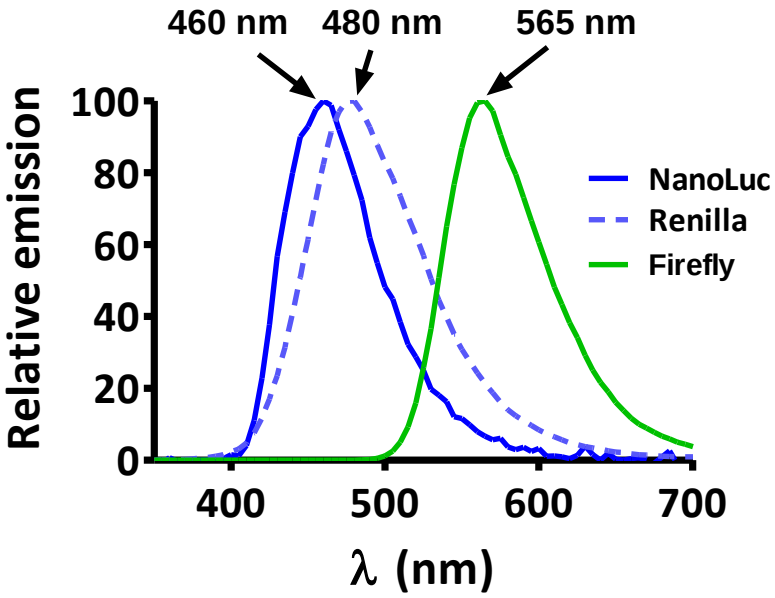
NanoLuc™/Nano-Glo™

Firefly/ONE-Glo™

Renilla/Renilla-Glo™

NanoLuc™ has excellent physical properties

- **Thermal stable enzyme**
  - Retains activity following 30 min incubation at 55 °C
    - Melting temps: Nluc, 58 °C; Fluc, 31 °C
- **Active over broad pH range**
  - Fully active between pH 7-9
  - Retains significant activity at pH 5-7
  - Fluc: sharp decrease in activity below pH = 8
- **Monomeric enzyme**
  - Facilitates use as transcriptional reporter or fusion partner
- **No post-translational modifications detected in mammalian cells**
- **No disulfide bonds**
  - Supports high levels of activity inside living cells
- **Uniform distribution in cells**
  - No apparent compartmental bias in the absence of targeting sequences



Nano-Glo™ Luciferase Assay Reagent

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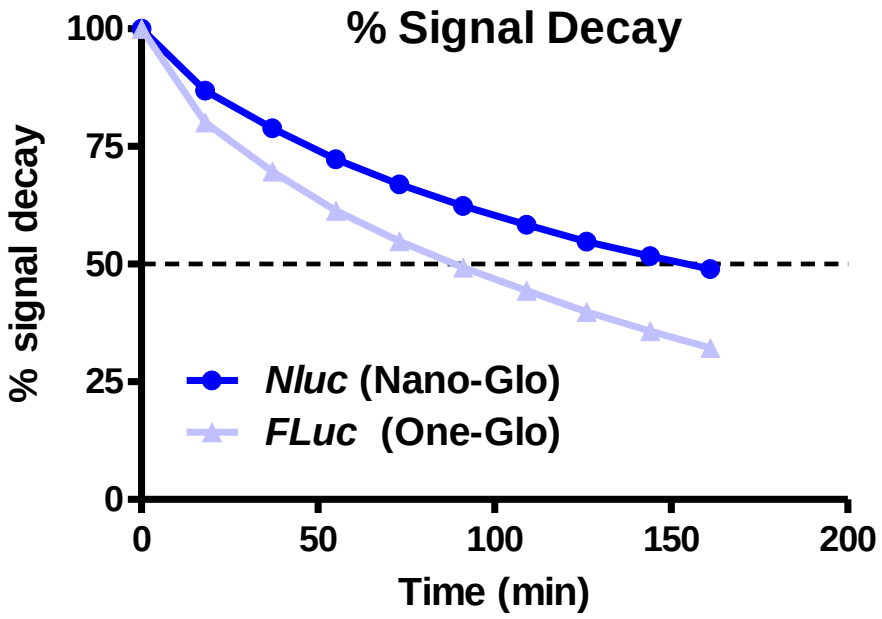
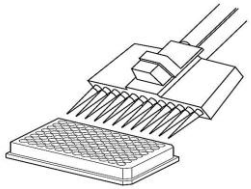
Nano-Glo Luciferase Assay Reagent:

- Furimazine
  - Provides maximal brightness
- ATP independent reaction
- Glow kinetics
  - Half-life routinely >2 hours at room temperature
- Low autoluminescence background
  - Enhances assay sensitivity
- Stable reconstituted reagent:
  - ~10% decrease in activity over 8 hrs at RT



Reporter gene assays

Standard format

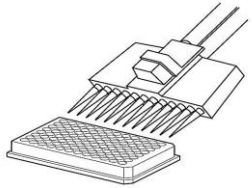


Add Nano-Glo Luciferase Assay Reagent

Measure RLUs after 3’

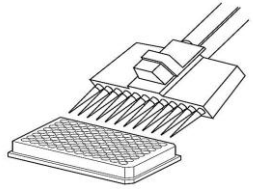
1) Cells lysed
2) Intracellular Nluc released

Secretion format

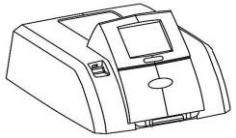


Transfer aliquot of medium

New plate



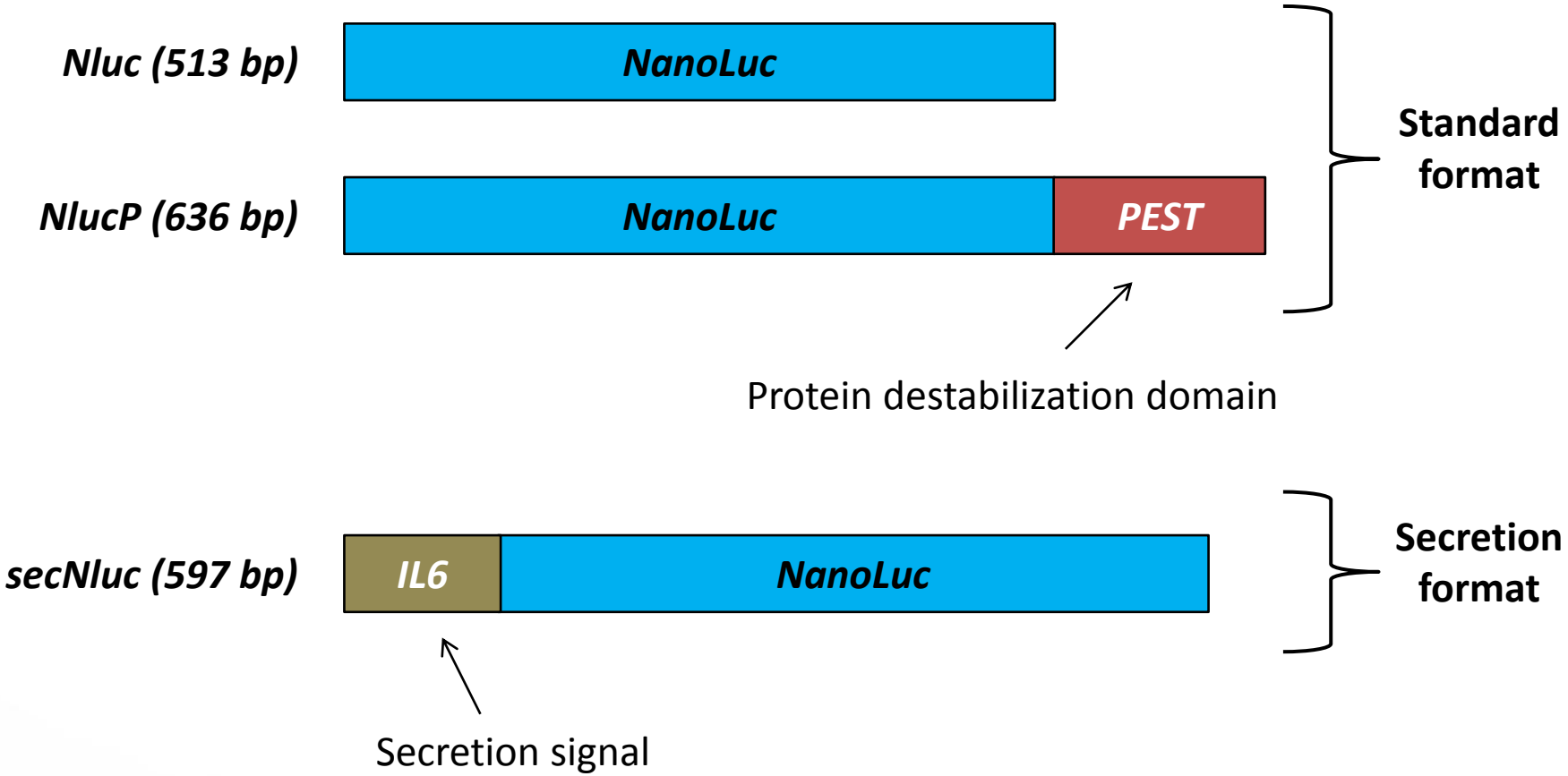
Add Nano-Glo Luciferase Assay Reagent



Measure RLUs after 3’

1) Nluc secreted from cells
2) Sample culture medium (no cell lysis)

NanoLuc™ genetic constructs



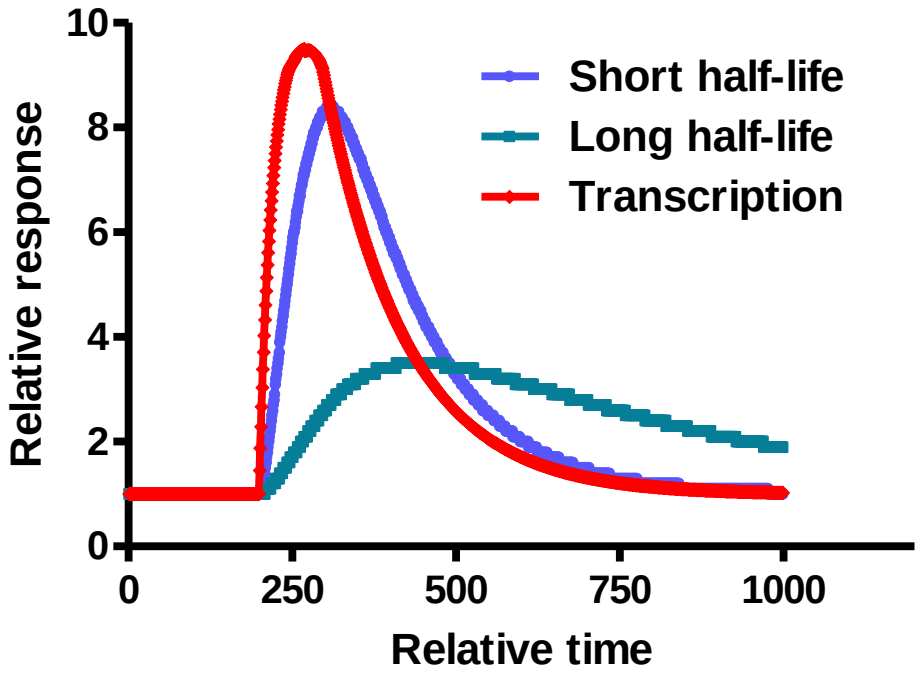
Intracellular stability of NanoLuc™ & firefly

Protein half-life following addition of cycloheximide

Cell line	FLuc	FLucP	NLuc	NLucP
HEK-293	>6 h	2.0 ± 0.4 h	>6 h	18 ± 11 min
HeLa	3.8 ± 1.3 h	1.4 ± 0.2 h	>6 h	20 ± 6 min
U2OS (n=1)	>6 h	2.8 h	>6 h	36 min

Relative protein stability in cells: NlucP < FlucP < Fluc < Nluc

Use of destabilized luciferase reporter proteins

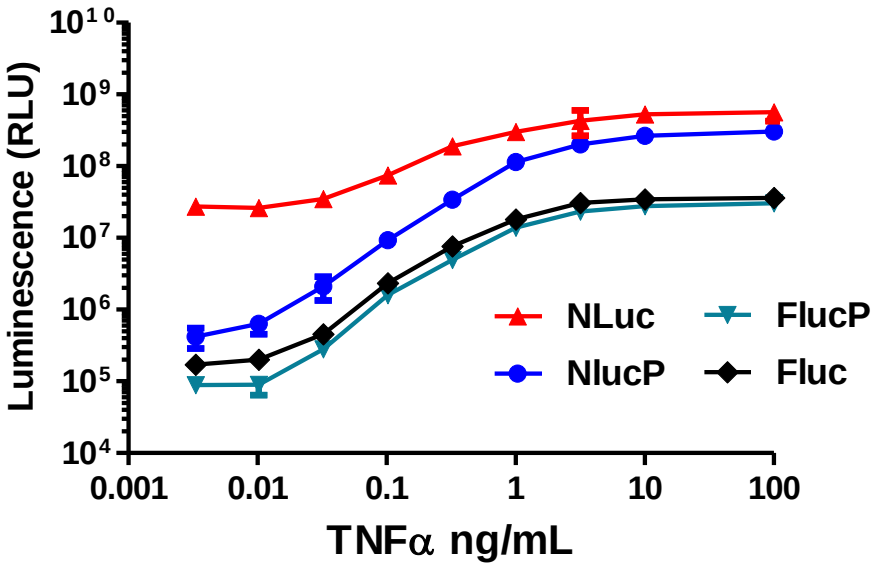


Destabilized luciferase reporters

- Dimmer than unfused constructs
- Respond more quickly to changes in transcription
- Provide greater fold induction
- Basis behind Rapid Response™ Reporters for firefly luciferase

Model assumptions: single component exponential decay; equal rates of synthesis; 10-fold difference in protein half-life.

Relative performance of reporter genes



Experimental details: transient transfection of HEK293 cells with NF-κB inducible constructs. rhTNFα treatment for 5 hours.

Brightness

Nluc > NlucP > Fluc > FlucP

(18 experiments)

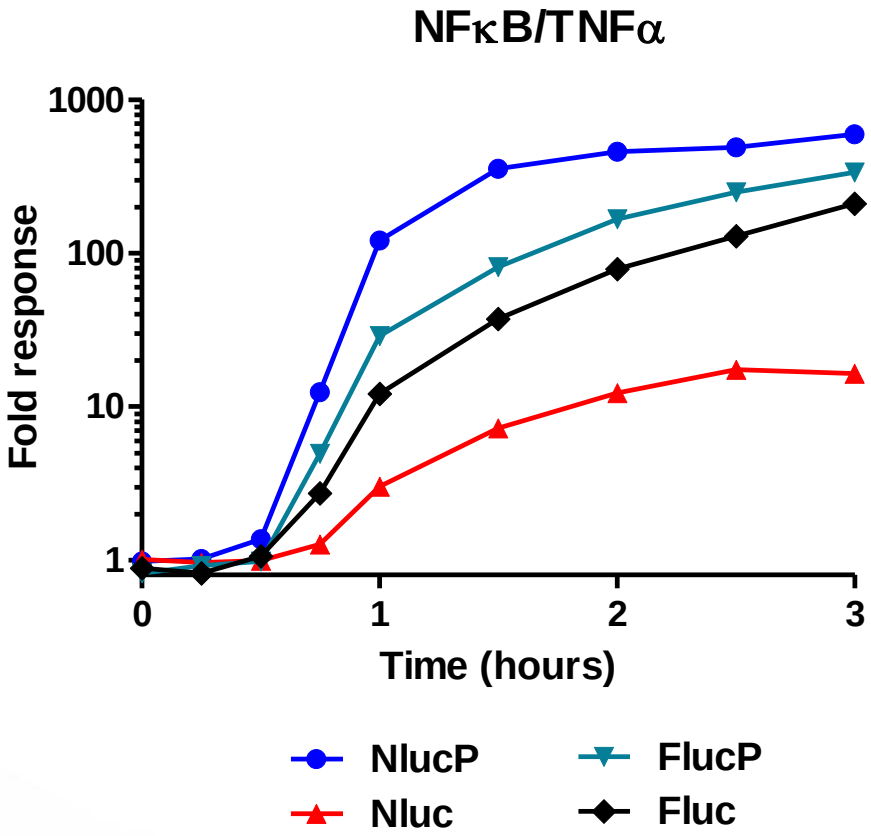
NLuc 13-236 fold brighter than Fluc (79 fold avg.)

NlucP 2-27 fold brighter than FLucP (10 fold avg.)

Nluc 10-78 fold brighter than NlucP (34 fold avg.)

→Very similar pharmacology/EC50s

Relative response of reporter genes: ex. #1

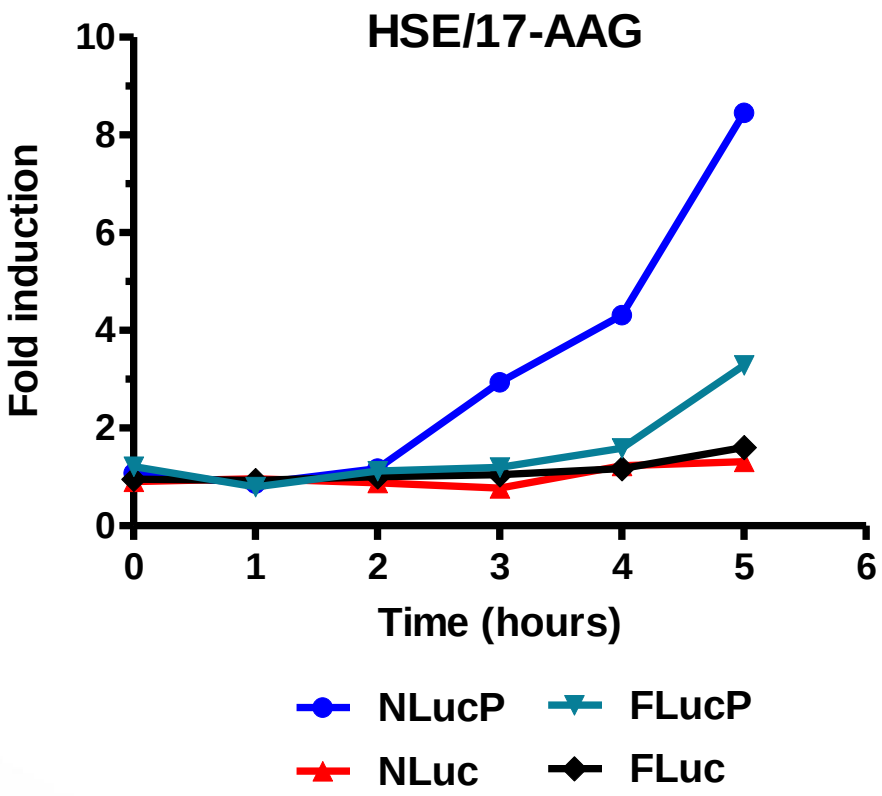


Relative Response

NlucP > FlucP > Fluc > Nluc

Experimental details: transient transfection of HEK293 cells with NFκB inducible constructs; addition of 100 ng/ml rhTNFα at time zero.

Relative response of reporter genes: ex. #2

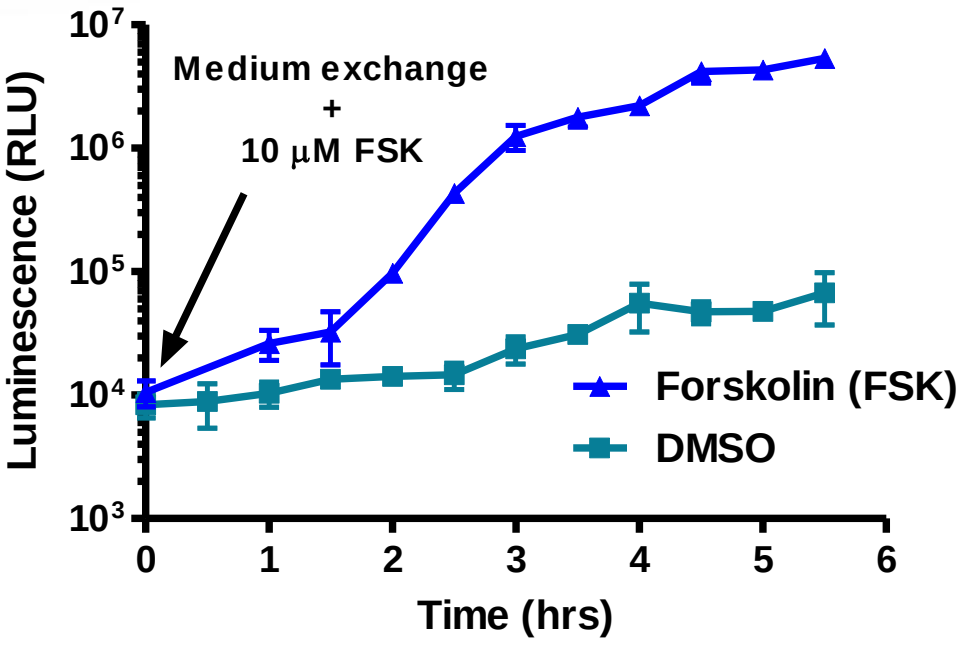


Relative Response

NlucP > FlucP > Fluc,Nluc

Experimental details: transient transfection of Hela cells w/ Hsf1 inducible constructs; addition of 500 nM 17-AAG at time zero.

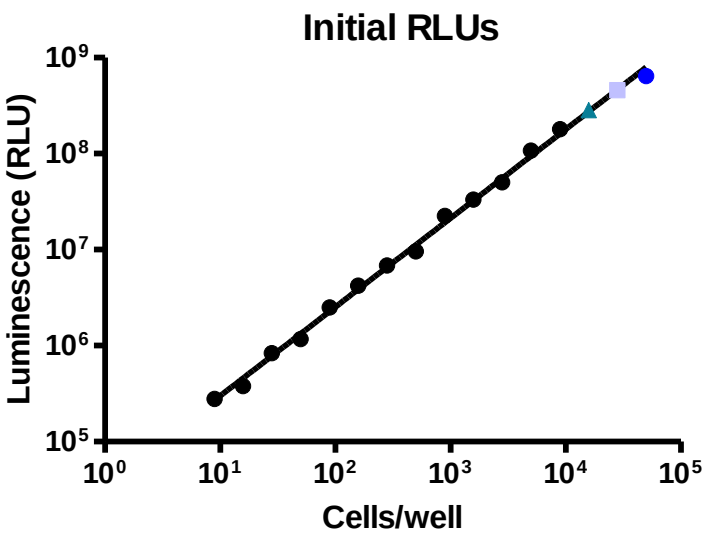
Secretion based format using secNluc

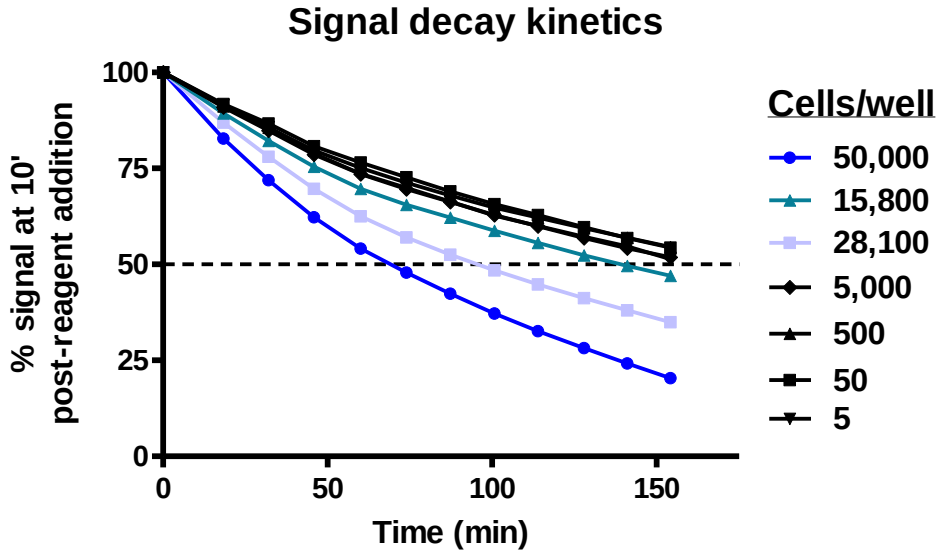


Experimental details: transient transfection of HEK293 cells with CREB inducible construct; addition of 10 µM forskolin at time zero.

- Sample medium at multiple time points without cell lysis
  - Kinetic studies from the same set of wells
- Half-life of secNluc protein > 4 days at 37°C in medium
- Response dynamics similar to unfused Nluc
- Similar pharmacology vs. Nluc/NlucP

Decreased signal half-life at higher RLUs





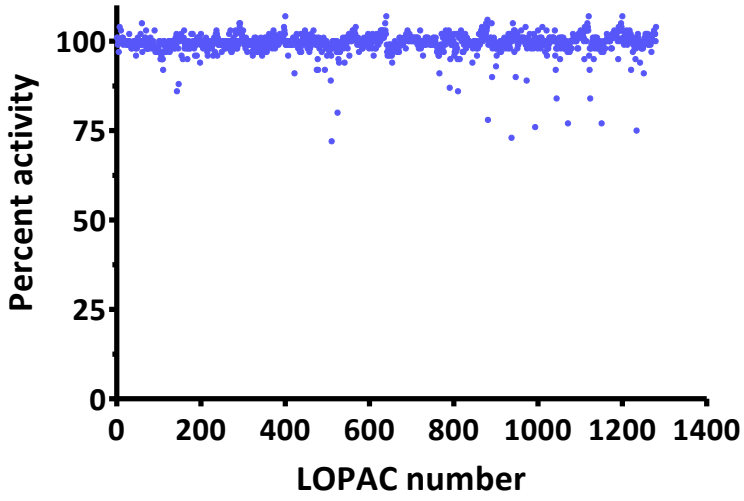
- Example experiment to control [Nluc] in the cell lysate
- At very high RLUs (>~5E8), signal half-life drops

Experimental details: bulk transient transfection of HEK293 cells with pNL1.1.CMV [*Nluc*]; serial dilution with complete medium prior to addition of Nano-Glo Luciferase Assay Reagent.

Reduced false hit rate in HTS

LOPAC library (Sigma)

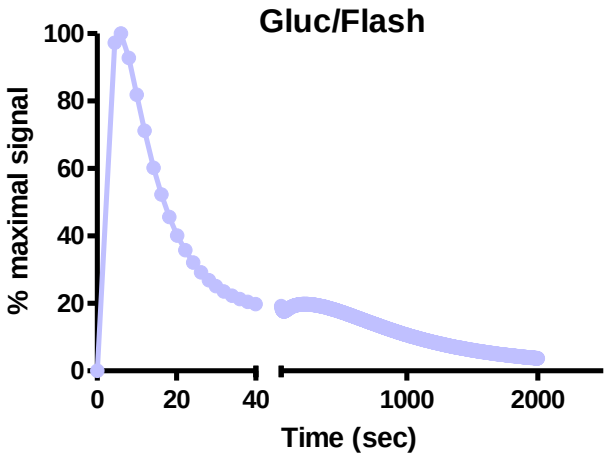
- 1280 compounds
- Small organic ligands w/ well documented pharmacological activities
- Used to screen for non-specific luciferase activity modulators

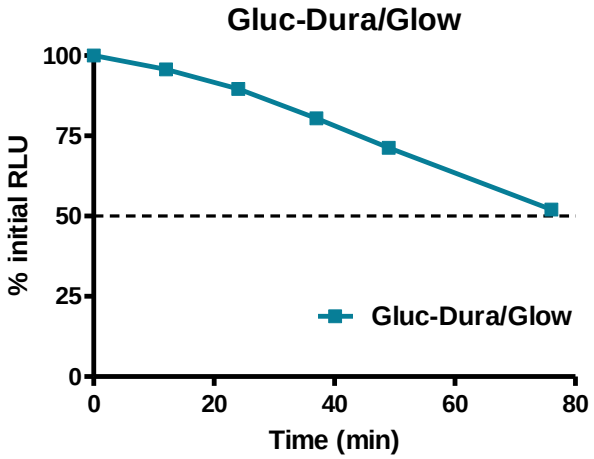


		Level of inhibition			
		≥ 10%	≥ 20%	≥ 30%	≥ 50%
% of library compounds	**NanoLuc**	**1.2%**	**0.5%**	**-**	**-**
	Firefly	**1.9%**	**0.7%**	**0.5%**	**0.3%**

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

Gaussia Luciferase (Gluc): flash or glow





- Gluc: 185 amino acids (19.9 kDa)
- Naturally secreted enzyme
- Coelenterazine substrate

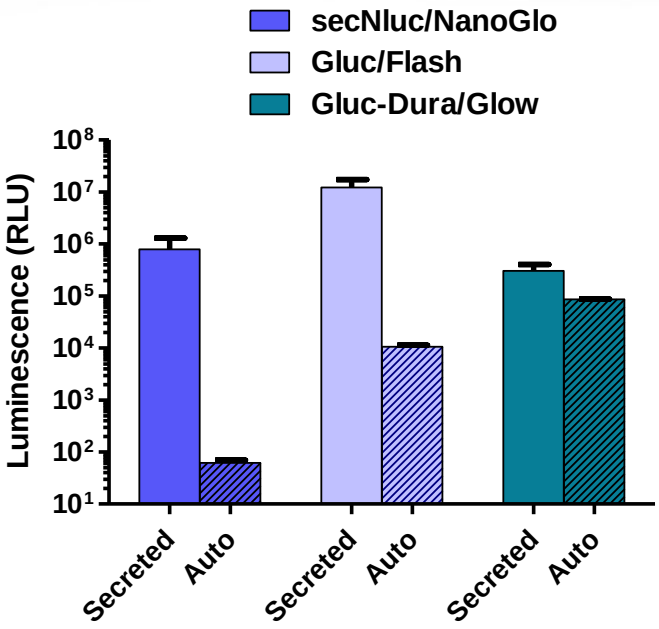
- Numerous disulfide bonds
- Flash assay requires injectors
  - Batch processing of plates is difficult

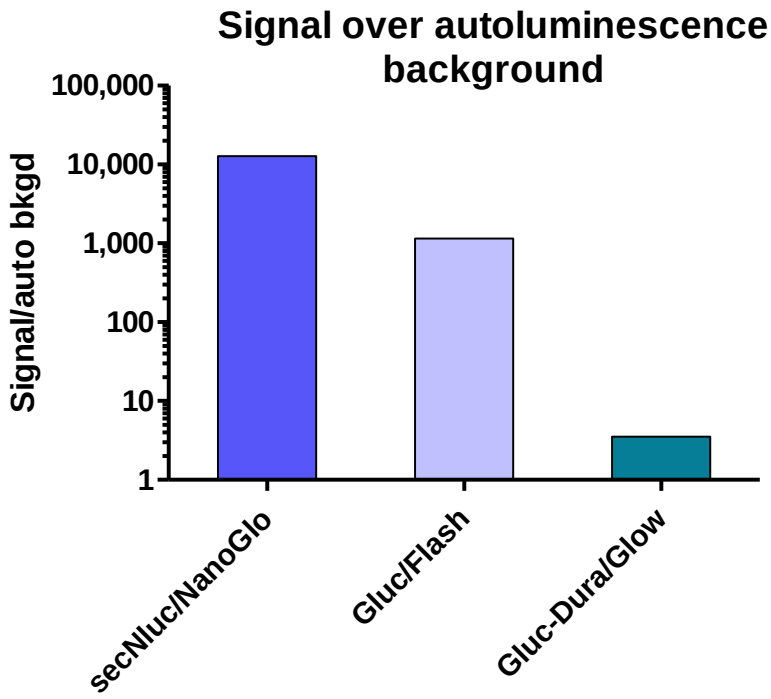
Gluc reagents from Pierce:

pCMV-Gaussia Luc (cat # 16147)
Gaussia Luciferase Flash Assay Kit (cat # 16159)

pCMV-Gaussia-Dura Luc (cat # 16191)
Gaussia Luciferase Glow Assay Kit (cat # 16161)

Gluc kits: bright, but high autoluminescence background

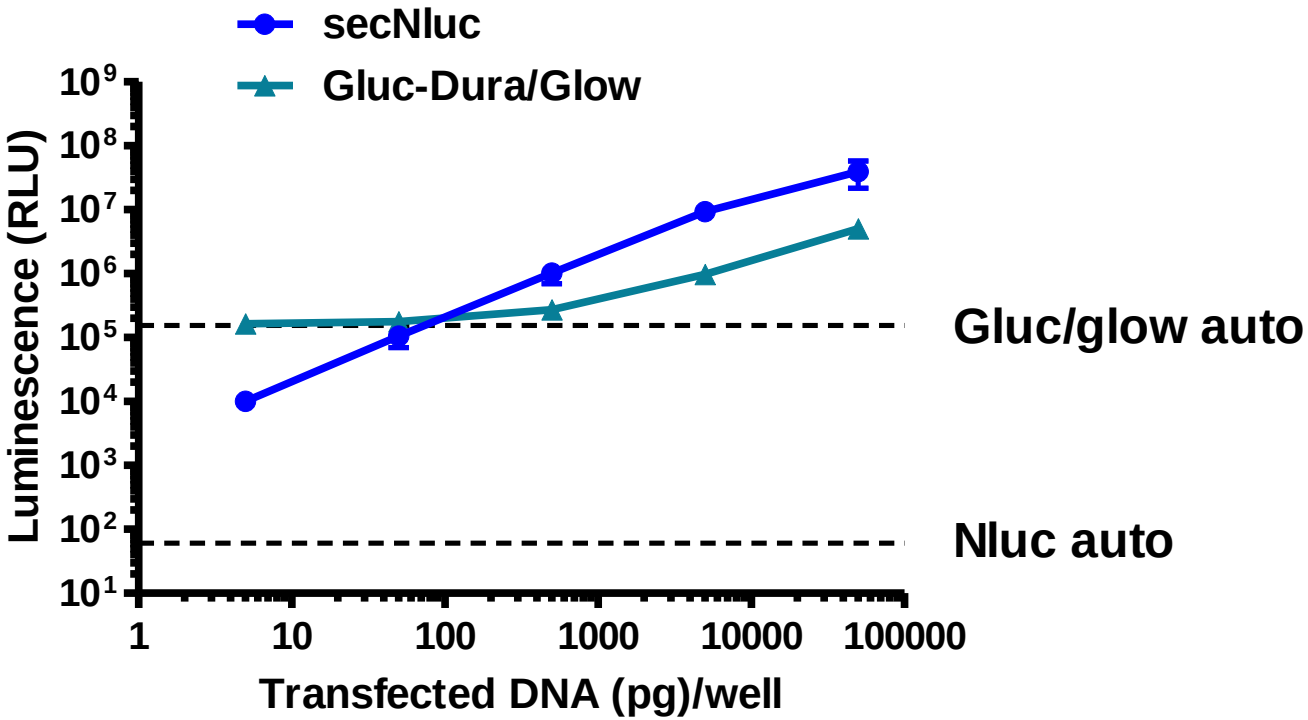




Gluc kits: high background limits sensitivity & dynamic range

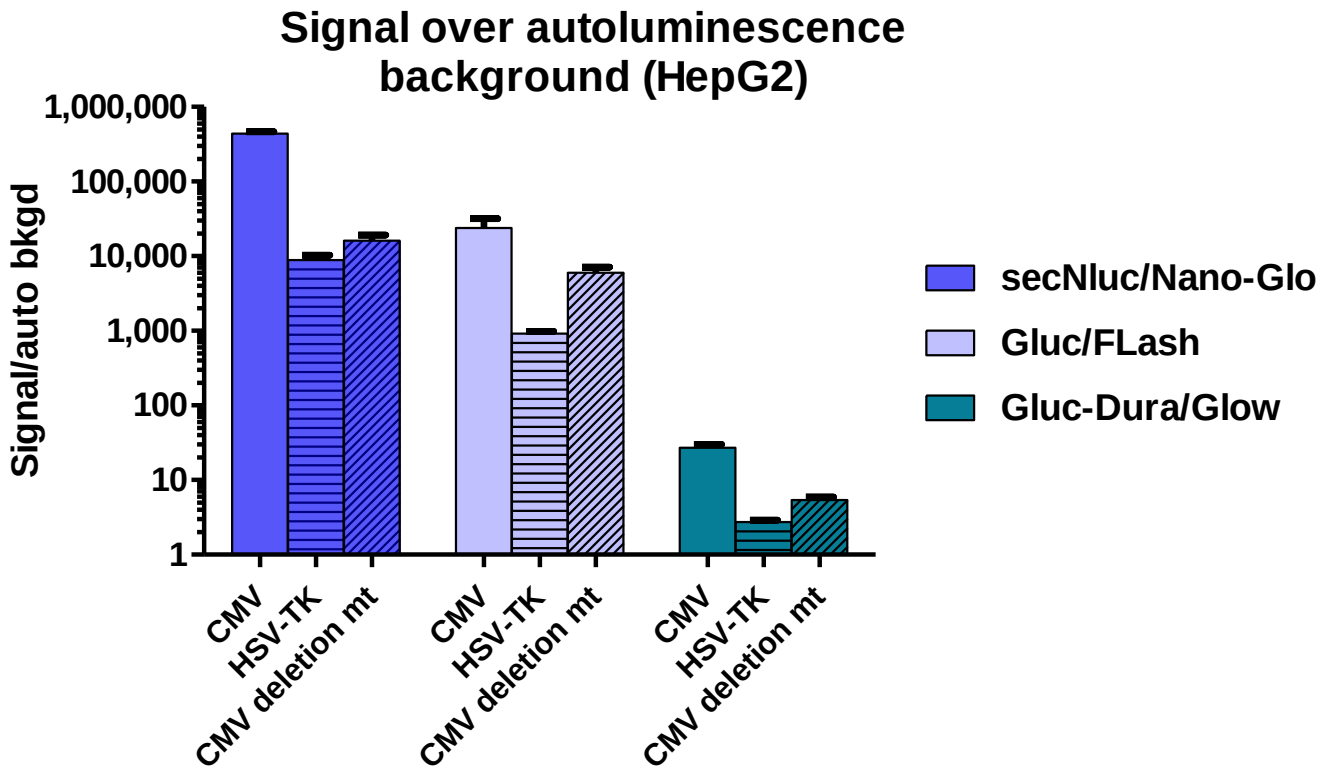
Experimental details: reverse transfection of HepG2 cells (DMEM +10% FBS) w/CMV promoter constructs; removal of aliquots after 22 hrs; n = 12 per treatment.

Gluc glow: high background reduces sensitivity



Experimental details: transfection of HepG2 cells with CMV promoter constructs for ~24 hrs (carrier DNA used to keep total [DNA] constant per well); washed & replaced w/ DMEM + 10% FBS; incubation for 3 hrs prior to aliquot removal to measure RLUs.

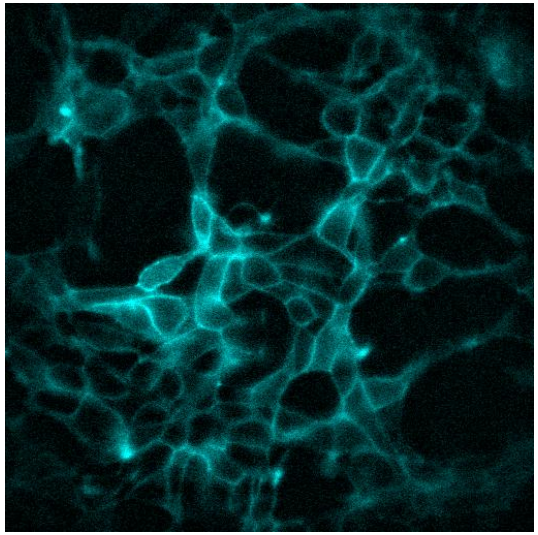
Gluc kits: reduced dynamic range



Experimental details: reverse transfection of HepG2 cells (DMEM +10% FBS) with constitutive promoter constructs; removal of aliquots after 24 hrs; n = 8 per treatment.

NanoLuc™ as fusion partner

Bioluminescence of Nluc-B2AR fusion protein

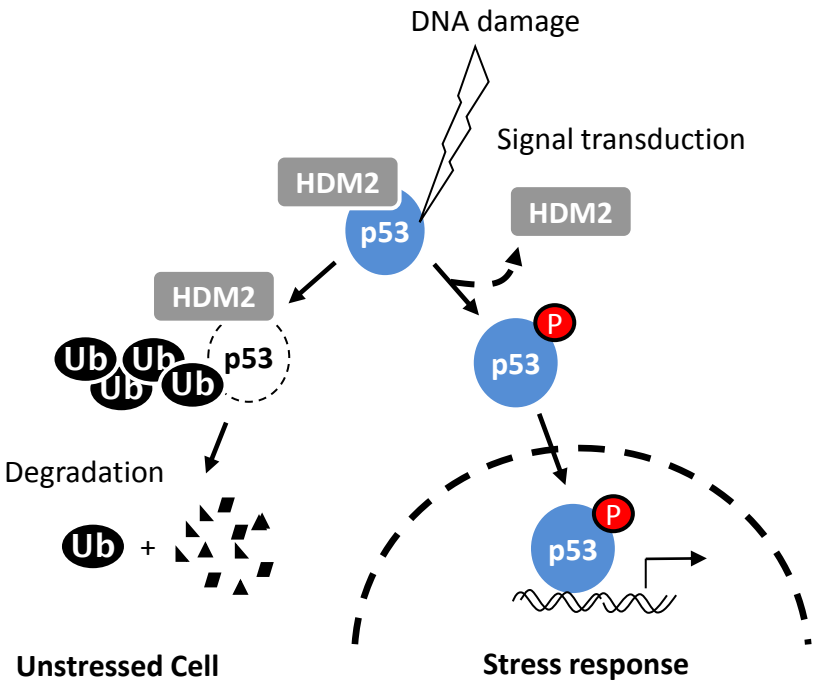


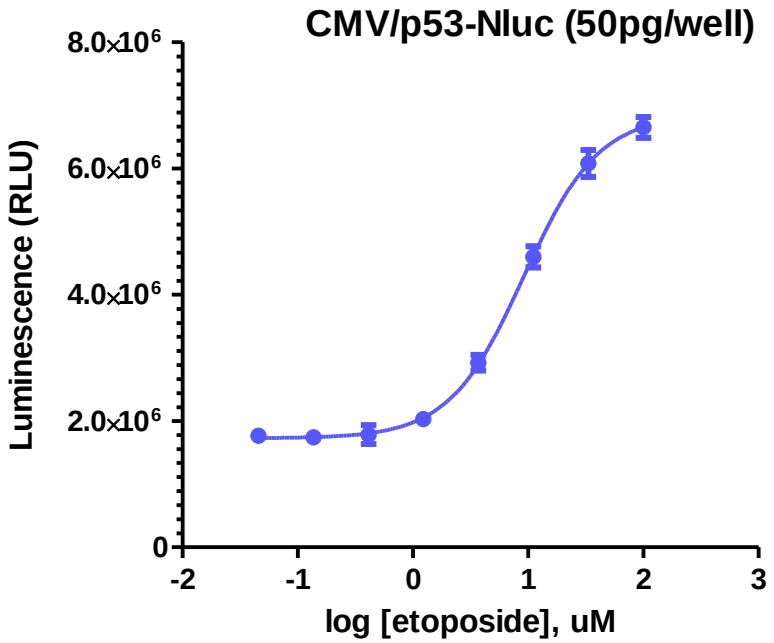
Results of bioluminescence imaging

- Brightness allows short exposure times (1-5 seconds)
- Diffuse signal throughout the cell in the absence of a targeting sequence
- Multiple Nluc fusions showed proper static or dynamic localization patterns
  - No apparent influence on target protein function
- Nluc supports high levels of activity in multiple cellular compartments
  - In contrast to other small luciferase enzymes

Experimental details: stable expression of IL6-Nluc-B2AR in HEK293 cells; image obtained using Olympus LV200 Bioluminescence Imager and a single addition of 20 µM furimazine; 5 second exposure (EMCCD image intensification turned off); CO2 independent medium (no serum).

NanoLuc™ fusions as stability sensors





Experimental details: reverse transfection of HEK293 cells for 24 hrs; treatment with etoposide for 6 hrs followed by addition of Nano-Glo Luciferase Assay Reagent; measurement on GloMax Multi+.

BRET ligand binding assay using NanoLuc™

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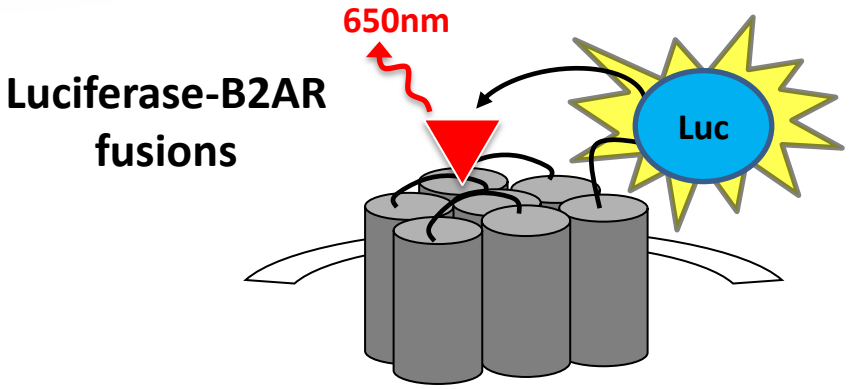
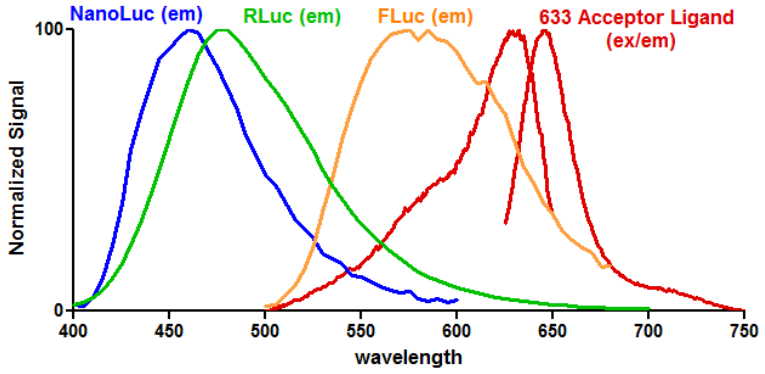
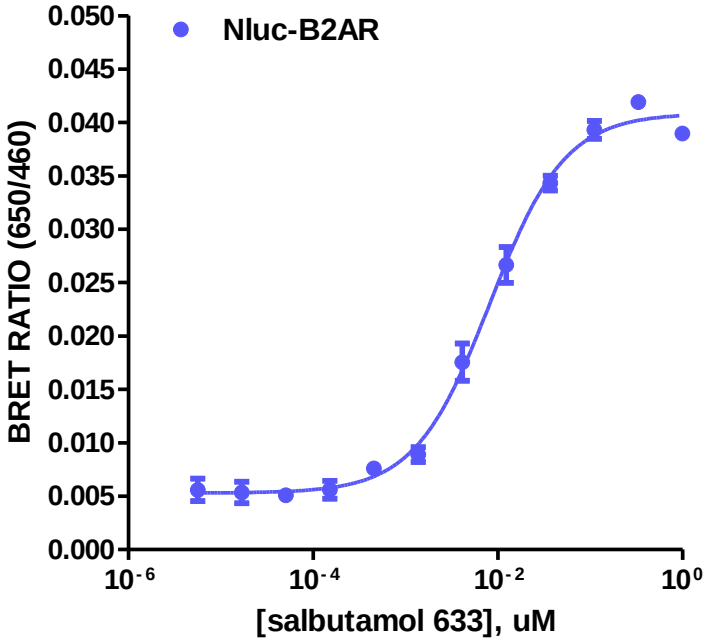


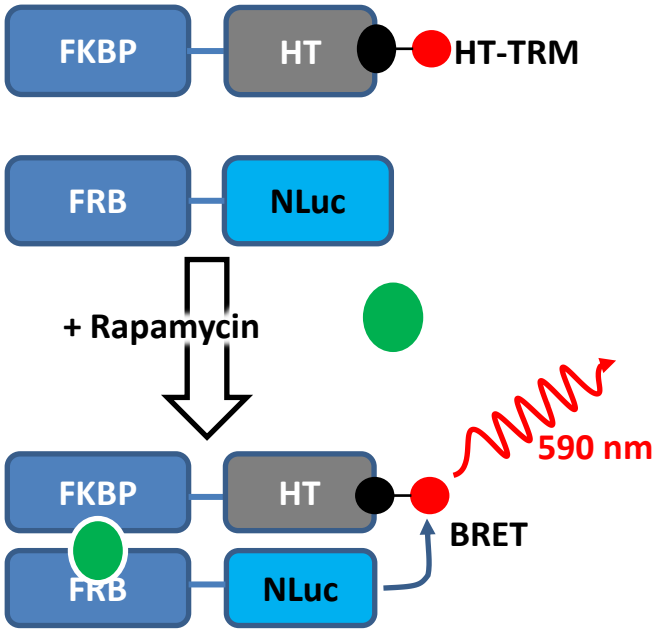


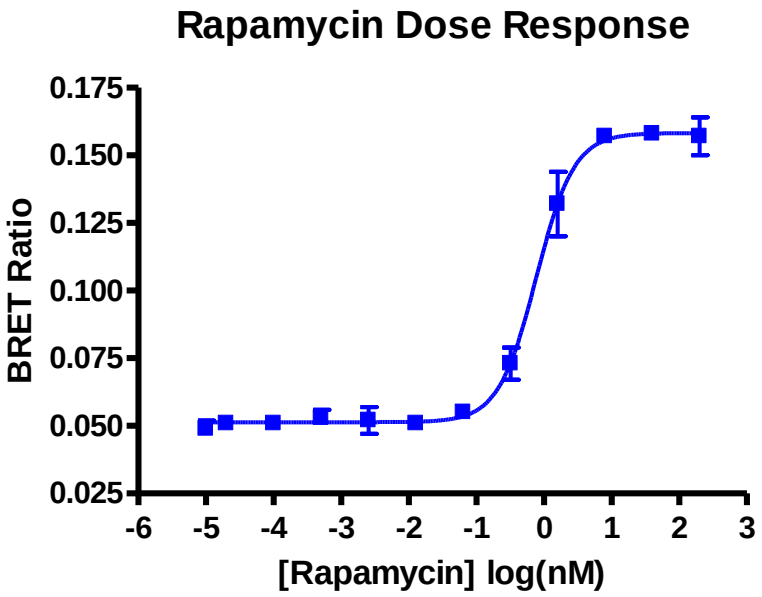


No detectable BRET for Fluc or Rluc

Experimental details: reverse transfection of HEK293 cells w/ IL6-luciferase-B2AR fusions; equilibrate cells with salbutamol-633 tracer; add luciferase substrates; measure BRET using monochromator (Fluc, 650/570; Rluc, 650/480; Nluc, 650/460).

NanoLuc™ & intracellular BRET





Experimental details: reverse transfection of HEK293 cells w/ FKBP-HaloTag v7 & FRB-Nluc constructs; pre-incubation w/ HaloTag TMR ligand; rapamycin added & luminescence measured after addition of 20 µM furimazine to living cells; monochromator measurements of BRET ratio = 590/460.

Summary: NanoLuc™ Luciferase as a Reporter Gene

Promega

Nluc

- Provides maximal sensitivity in detecting low levels of expression
  - ~80-fold brighter than firefly in cell based assays
- Reduced false hit rate in HTS (likely true for NlucP & secNluc as well)

NlucP

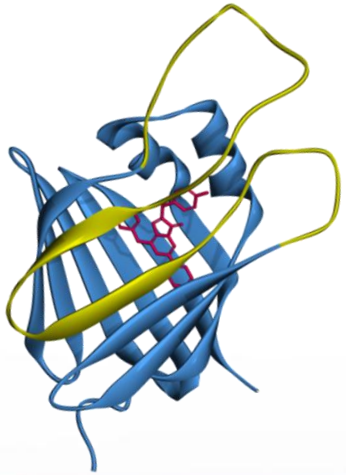
- Best performing Rapid Response™ reporter
  - 10-fold brighter and more responsive than FlucP
    - ~20 min half-life in mammalian cells
- Reporter of choice for a close coupling to the transcriptional response
- Reporter of choice when the transcriptional response is limited (low S/B)

secNluc

- Stable for several days in cell culture medium
- Time course studies on the same set of wells (no cell lysis)

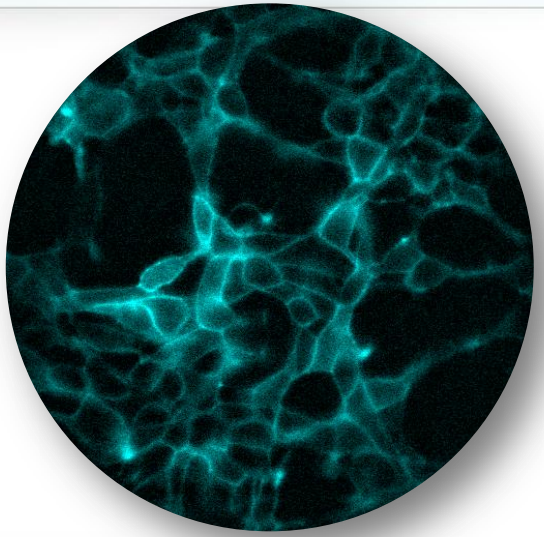
Nano-Glo

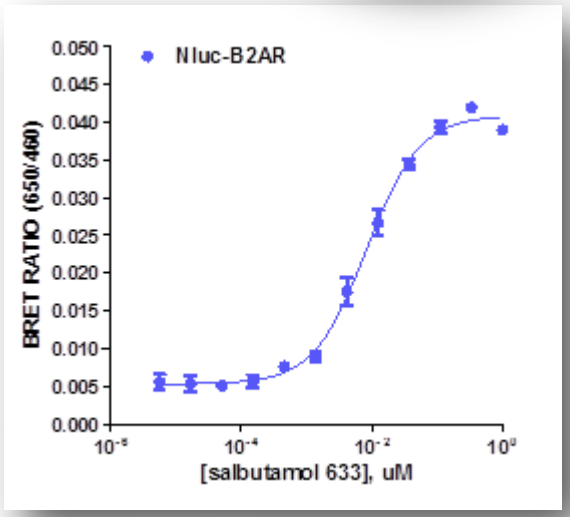
- Glow-type luminescence signal with ~2 hr half-life of signal decay
- Very low autoluminescence background



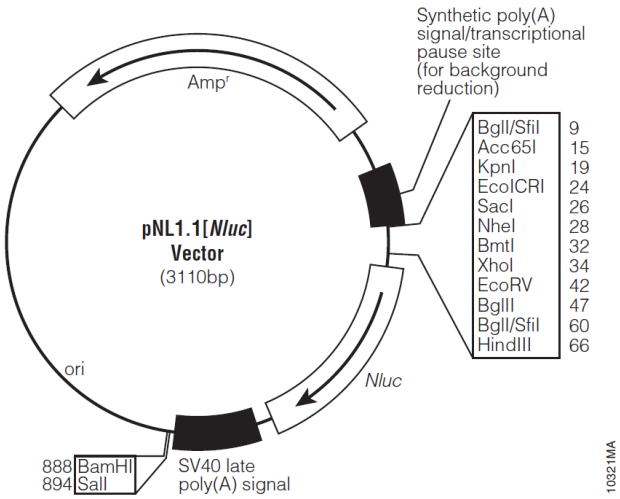
Summary: NanoLuc™ Luciferase as a fusion partner

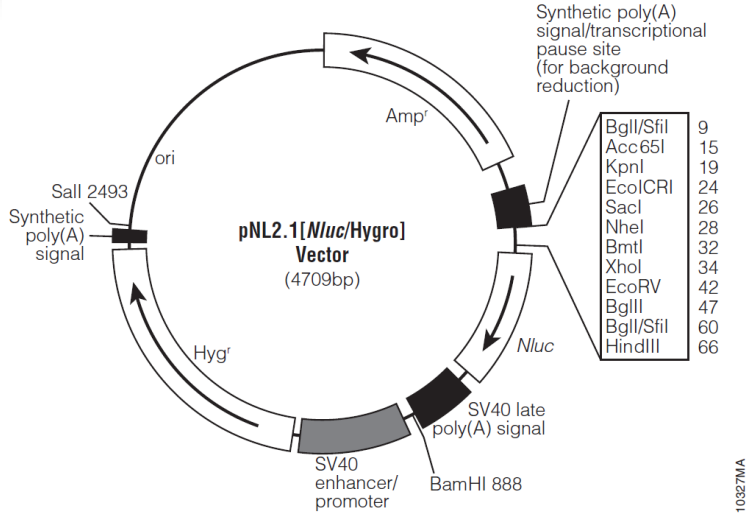
- Supports high levels of activity inside living cells
  - Provides maximal sensitivity in detecting low levels of expression
- Small size (19 kDa) is ideal (minimal impact on fusion partner function)
- No compartmental bias in the absence of a targeting sequence
- Emission spectrum compatible with BRET

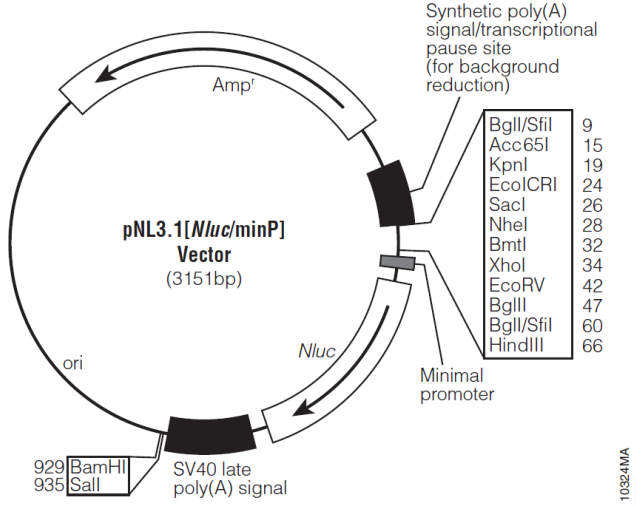




NanoLuc vectors for cloning

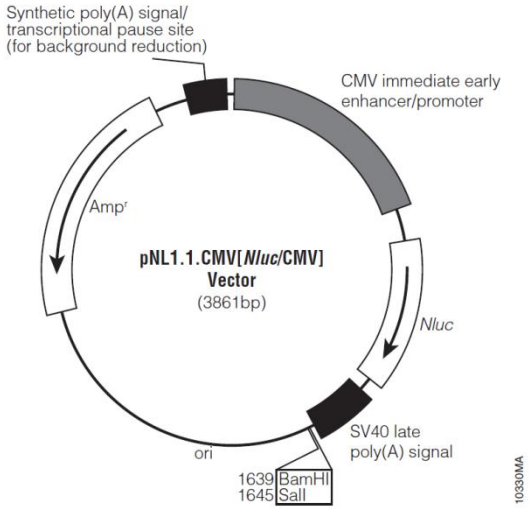


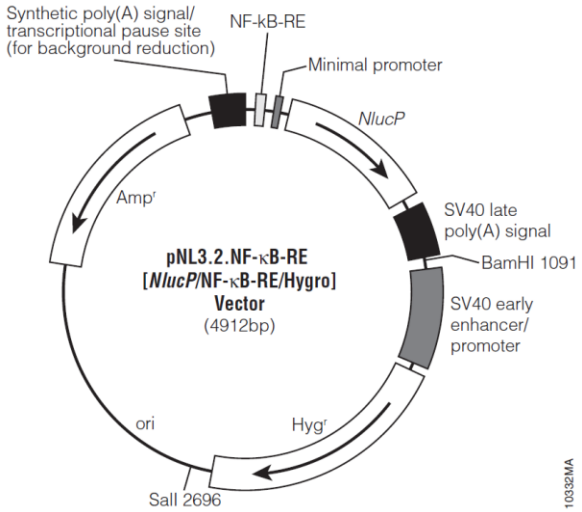


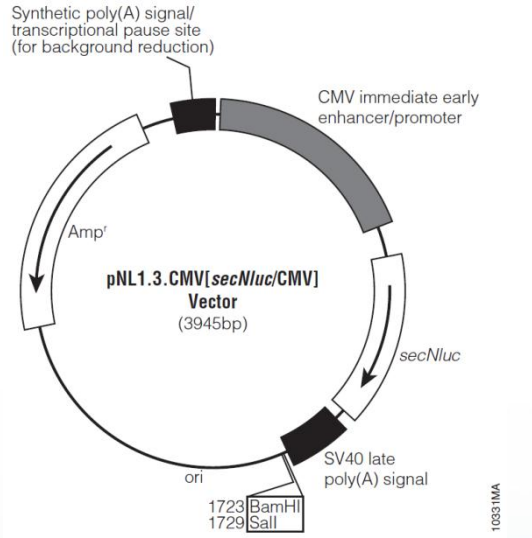


- *Nluc*, *NlucP* & *secNluc* versions for each
  - pNLX.1, pNLX.2 & pNLX.3, respectively
- All *Nluc* vectors possess:
  - Codon optimized ORF for expression in mammalian cells
  - Minimization of consensus transcription factor binding sites throughout the plasmid
  - A parent pGL4 construct (Nluc replaces Fluc)

NanoLuc control and response element vectors







<u>pNL1.1.CMV [*Nluc*/CMV]</u>
- Constitutive expression

<u>pNL1.3.CMV [*secNluc*/CMV]</u>
- Constitutive expression & secretion

<u>pNL.3.2.NF-κB-RE [*NlucP*/NF-κB-RE/Hygro]</u>
- Inducible expression via NFκB

Nano-Glo Luciferase Assay Reagent

Cat # N1110: 10ml (100 assays)

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

Cat # N1130 10x10ml (1,000 assays)

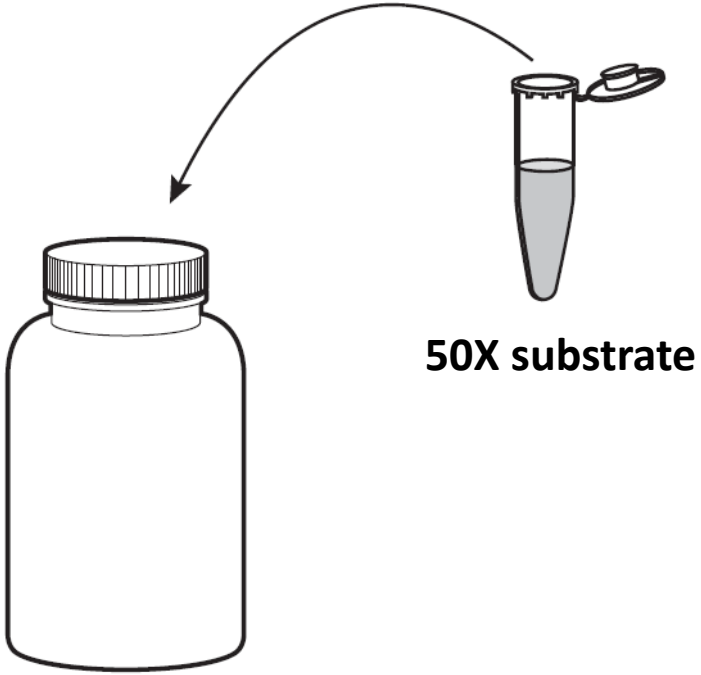
- 10x200µl Nano-Glo™ Luciferase Assay Substrate
- 10x10ml Nano-Glo™ Luciferase Assay Buffer

Cat.# N1120 100ml (1,000 assays)

- 2x1ml Nano-Glo™ Luciferase Assay Substrate
- 100ml Nano-Glo™ Luciferase Assay Buffer

Cat.# N1150 10x100ml (10,000 assays)

- 5x4ml Nano-Glo™ Luciferase Assay Substrate
- 10x100ml Nano-Glo™ Luciferase Assay Buffer



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