Luciferase Reporter Assays
Tips & Techniques

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The reporter gene produces an easy to assay & quantify protein.

Most reporters are enzymes which offer amplification of signal through catalysis.

Reporter assays can be bioluminescent, fluorescent, colorimetric or radioactive.
Reporter Assay Principle

TREATMENT

TRANSCRIPT

TRANFECT REPORTER CONSTRUCT

translation

REPORTER ASSAY
Firefly Luciferase is an ideal reporter.

Beetle Luciferin

\[
\text{Beetle Luciferin} \rightarrow \text{Firefly Luciferase} + \text{Mg}^{2+} \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{PP}_1 + \text{CO}_2 + \text{LIGHT}
\]

Common Firefly

*Photinus pyralis*
## Renilla Luciferase

*Renilla reniformis*  
Sea Pansy

Coelenterazine ➔ *Renilla* Luciferase ➔ Coelenteramide

<table>
<thead>
<tr>
<th>Reporter</th>
<th>Non-Radioactive</th>
<th>Enzymatic</th>
<th>Background</th>
<th>Sensitivity</th>
<th>Active Form</th>
<th>half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firefly Luciferase</td>
<td>Y</td>
<td>Y</td>
<td>–</td>
<td>++++</td>
<td>monomer</td>
<td>3 hours</td>
</tr>
<tr>
<td><em>Renilla</em> Luciferase</td>
<td>Y</td>
<td>Y</td>
<td>–</td>
<td>++++</td>
<td>monomer</td>
<td>5 hours</td>
</tr>
<tr>
<td>CAT</td>
<td>N</td>
<td>Y</td>
<td>–</td>
<td>+</td>
<td>Trimer</td>
<td>50 hours</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>Y</td>
<td>Y</td>
<td>+/-</td>
<td>++</td>
<td>tetramer</td>
<td>20 hours</td>
</tr>
</tbody>
</table>
**Dual-Luciferase® Reporter Assay**

Distinct chemistries allow separate measurement of each luciferase through manipulation of the reaction conditions.

1. **TREATMENT**
   - Firefly Luciferase levels vary with treatment
   - Renilla Luciferase levels vary little with treatment

Remove medium.
Rinse with PBS
Add 1X PLB
Perform lysis.
Briefly centrifuge, then transfer supernatant to new tube.
Mix 20µl of cell lysate and 100µl of Luciferase Assay Reagent II in the tube.
Measure the light produced by firefly luciferase.
Add 100µl of Stop & Glo Reagent to the tube.
Measure the light produced by Renilla Luciferase.
History of Reporter Assay Tools

- **pGL2 Vectors**
- **pGL3 Vectors**
- **pRL Vectors**
- **phRL Vectors**
- **pGL4 Vectors**

**Timeline:**
- 1990: LAR w/ CCLR
- 1992: LAR w/ RLB
- 1994: DLR™ w/ PLB
- 1996: Steady-Glo® Luciferase Assay System
- 1998: Dual-Glo™ Luciferase Assay System
- 2000: Bright-Glo™ Luciferase Assay System
- 2002: EnduRen™ Live Cell Substrate
- 2004: ViviRen™ Live Cell Substrate
- 2006: ONE-Glo™ Luciferase Assay System
- 2008: VivoGlo™ Substrates, EnduRen & ViviRen in vivo Substrates
- 2010:

**Contributors:**
- Keith V. Wood, Ph.D.
Dual-Glo® Luciferase Assay System

**Dual-Glo™ Luciferase Assay:**

**Step 1:**
Dispense **Dual-Glo™ Luciferase Reagent** directly to plates containing cells in culture medium. Wait 10 minutes, then measure firefly luciferase activity for up to 2 hours.

**Step 2:**
Dispense **Dual-Glo™ Stop & Glo® Reagent** to same plates. Wait 10 minutes, then measure Renilla luciferase activity for up to 2 hours.

For multi-well plate assays, Dual-Glo Luciferase System does not require injectors. Long signal half-life allows batch processing.
pGL4 Reporter Vectors

- Brighter luminescence
  *Codon optimized for more efficient expression*

- Improved responsiveness
  *Greater response dynamics by reducing reporter stability*

- Greater sensitivity & Reduced off-target effects
  *Destroyed cryptic transcription factor binding sites*

- Expanded vector options
  *Pick the features you need!*

**Selectable Marker**
- None
- Hygro<sup>r</sup>
- Neo<sup>r</sup>
- Puro<sup>r</sup>

**pGL4 Vectors**
- Synthetic poly(A)
- ori
- Amp<sup>r</sup>

**Upstream Element**
- Poly(A) block (for background reduction)
- SV40 early enhancer/promoter
- Multiple cloning region
- Promoter/response elements

**Luciferase Gene**
- Firefly (luc2)
  - Rapid Response™ (−P, −CP)
- Renilla (hRluc)
  - Rapid Response™ (−P, −CP)
Pre-made pGL4 constructs – RE clones

Pre-designed, ready-to-use response element pGL4 Vectors

<table>
<thead>
<tr>
<th>Response Element</th>
<th>Signaling Pathway</th>
<th>pGL4 Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>cAMP/PKA</td>
<td>pGL4.29</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear Factor of Activated T-cells</td>
<td>pGL4.30</td>
</tr>
<tr>
<td>NF-κB</td>
<td>NF-κB</td>
<td>pGL4.32</td>
</tr>
<tr>
<td>Serum Response</td>
<td>MAP/ERK</td>
<td>pGL4.33</td>
</tr>
<tr>
<td>Serum Response Factor</td>
<td>RhoA</td>
<td>pGL4.34</td>
</tr>
</tbody>
</table>

GloResponse™ HEK 293 cell lines:
- cAMP RE
- NFAT RE
- NF-κB RE

HEK 293 Hyg^R cell line made with the pGL4 vectors

GloResponse NFAT-RE-luc2P HEK293 Cell Line
In vivo applications

**Reporter Assay Applications**

- **Post-Transcription miRNA Control**
- **Protein Interactions**
- **Nuclear Receptors**
- **Signaling Pathway**
- **Promoter Dissection**

**Experimental firefly luciferase construct**
- Expression level varies with treatment

**Control Renilla luciferase construct**
- Expression level varies little with treatment
Variables to Consider in Reporter Assay Design

- Reporter design?
  - Reporter Gene
  - Plasmid DNA

- Transfection parameters?
  - Cell Lysates
  - Enzyme Activity
  - in vivo assay

- Assay choice?
  - Ribosomes

- Timing?
  - RNA

- Culture/Treatment?
  - Nucleus

- Type of cell?
  - Protein

- Controls?
Variables to Consider in Reporter Assay Design

Reporter design?

- Reporter Gene
- Plasmid DNA
- Nucleus
- RNA
- Ribosomes
- Reporter Gene Protein
- Cell Lysates
- Enzyme Activity
- in vivo assay
What sequences should I clone?

*Depends on the question you’re trying to answer…*

Transcriptional fusions typically use the proximal promoter ~1kb upstream of, & including, the transcriptional start site (+1)

Response Element constructs more precisely define the assay
Transcriptional reporter fusions…

…report transcriptional regulatory influences
Transcriptional reporter fusion

...may provide just the right tool!

Period;luc transcriptional fusion

Brain slice explant in culture 4 days

courtesy Dr. Shin Yamazaki, Vanderbilt University
Many regulatory points in gene expression

...any can affect reporter assay signal
...all can be monitored in a reporter assay
What sequences should I clone?

- Distal promoter/enhancer sequences
  - May mask more relevant proximal elements
  - Elements from adjacent genes may be captured

- 5’ or 3’ UTR
  - add post-transcriptional regulatory effects
    - Δ translation – Δ mRNA stability

- Intron
  - Necessitates inclusion of 5’UTR and possibly CDS
  - Introduces splicing artifacts?

- Coding sequence (CDS)
  - Translational fusion may confer post-translational regulation
    - Δ protein stability – Δ localization
  - If out-of-frame, ↓ translation from reporter start codon
Case Study: the Ultimate Genetic Reporter

Yoo, et al. (2004) PNAS 12,1-8

1. Started with genomic clone from gene of interest
2. Reporter inserted; translational fusion made; native 3’UTR preserved
3. “Knock-in” - gene replacement at endogenous locus

Reporter regulated exactly like native gene at all levels!
• More accurate reporter of gene expression
• Better marker for the system
• Enabled new discoveries
Variables to Consider in Reporter Assay Design

Type of cell?

- Fibroblasts (HEK293, Cos)
- Cancer cell lines (HepG2, PC-3)
- Primary cells (HUVECs, hepatocytes)

- Amenable to assay?
- Faithfully represent system?
- Express trans-factors, signaling intermediates?
Variables to Consider in Reporter Assay Design

Cell Culture Variables?

- Reporter Gene
- Plasmid DNA
- RNA
- Ribosomes
- Nucleus
- Reporter Gene Protein
- Cell Lysates
- Enzyme Activity
- in vivo assay
Cell Culture Considerations

• Cell confluence
  • Pre-confluent cultures generally best for transfection…
  • …however, cells may become confluent by treatment
    – how will growth state influence response to treatment?

• Cell health and passage number
  ▪ Use low passage number
  ▪ As passage number increases, cells may change character:
    – differences in transfection efficiency?
    – differences in response to treatments?
  ▪ Usually passage 1-3 days before transfection
  ▪ Media change 1-2 days before transfection
Variables to Consider in Reporter Assay Design

Transfection parameters?
- Method
- Ratio of vectors
- Transient or stable

Diagram:
- Reporter Gene
- Plasmid DNA
- Nucleus
- Reporter Gene Protein
- Ribosomes
- RNA
- Cell Lysates
- Enzyme Activity
- in vivo assay
Ratio of vectors in Dual Luciferase Assay

Q. What ratio of FLuc vs RLuc vectors should I use?
A. No “right” answer – depends on promoters used…
   • Typical ratio is 20:1
   • In some cases ideal ratio may be 1:1 or ≥ 200:1

*Range of DLR assay means optimization usually unnecessary*

Q. What ratio of co-transfected expression vector?
A. No standard answer …
   • For regulatory proteins *use as little as necessary!*
   • ~1:100 relative to primary reporter if driven by a CMV promoter…

*If you need a lot to see an effect, is the effect real?*
Relative Signal in Dual Luciferase Assay

Q. What is the “optimal” signal strength & signal ratio for Fluc and Rluc reporters?

A. Usually don’t have to worry about this, DLR assay is very forgiving…

2 considerations:

- Must be above background & below saturation of the luminometer
- Should be within ~4 logs of each other
  - If Fluc >> RLuc potential for quenching issues
  - If RLuc >> Fluc potential for cross-talk issues
Signal Range in Luciferase Assays

• Luminescence assays have a very broad dynamic range
  • limited mainly by range of detection system
  • >8 log range with GloMax® Luminometers
• Quantitation generally linear over entire range of detector
• Assay doesn’t require standard curve… just need to know:
  - Upper limit -> saturation point of detector
  - Lower limit -> background noise of instrument/assay
Minimum Detectable Level (MDL)

- Measure signal for blanks (non-transfected cells or media only)
- Determine average and standard deviation
- MDL would be Ave + 3×SD

- If Ave = 30 & SD = 8, then MDL is 54
- Signal >54 is significant

Typically, S:B ratio is >10-fold & often 1000-fold or better

Typical Signal (100’s, 1000’s, 10k)

MDL (54)

Average blank reading (30)

Instrument Noise Floor (20)
Transient transfection vs. Stable line

• Transient transfection
  • Versatile
    • *can vary combination of constructs*
    • *different cell types/lines*

• Stable transgenic line
  • Improve process
    • *same reporter needed repeatedly*
    • *cells difficult to transfect*
  • Avoid transient transfection artifacts
    • *induce stress response?*
    • *induce or attenuate target pathway?*
  • Maintain reporter
    • *extended timecourse*
Variables to Consider in Reporter Assay Design

Timing?
- Plating → transfection
- Transfection → treatment
- Treatment → assay
Timing - consider pathway & reporter kinetics

- What are the kinetics of your system?
- Is change state permanent or transient?
- What is the stability of the reporter?

Response kinetic is fast and transient (and using a responsive reporter) - the assay window is sooner & narrower. A time-course may be necessary to identify the peak induction.
Timing - consider pathway & reporter kinetics

- What are the kinetics of your system?
- Is change state permanent or transient?
- What is the stability of the reporter?

Transient response, slower kinetic is (or less responsive reporter) - the assay window occurs later & is broader.

A time-course may or may still be beneficial to identify the optimal timing.
Timing - consider pathway & reporter kinetics

- What are the kinetics of your system?
- Is change state permanent or transient?
- What is the stability of the reporter?
Variables to Consider in Reporter Assay Design

Controls?

- Reporter Gene
- Plasmid DNA
- Nucleus
- RNA
- Ribosomes
- Reporter Gene Protein
- Cell Lysates
- Enzyme Activity
- in vivo assay
Dual Luciferase?

Co-reporter controls for...

1. **Cell number** (starting & ending, e.g. cytotoxic effect of treatment)
2. Transfection efficiency
3. Specificity of effect

When is a co-reporter less important?

- Repeat measures assay (timecourse, live assay)
  - Variation in transfection efficiency & starting cell # don’t matter*
- Stable line
  - no variation in transfection efficiency*

*still advisable to control for cytotoxicity & specificity of effect …

**Multiplex with Cell Viability Assay**
What kind of assay controls do I need?

Q. Should I transfect the unmodified vector as a control?

Q. What if signal from my GOI promoter construct is not higher than the vector without insert?

A. Your construct will probably give higher basal expression than the base vector…but not necessarily.

A. Is this ratio really useful? (No)

• empty vector can be used as a control for specificity treatment.

Q. Do I need a positive control (such as pGL4.13, SV40 promoter)?

A. See above…is the ratio of GOI promoter vs control promoter useful?

• Pos control can be used for specificity of treatment.

• Pos control can be used for transfection optimization.
Normalization signal changes with treatment

Q. My Renilla signal changed in response to my treatment – isn’t this supposed to be constitutive?

A. 3 possibilities:

1. **Promoter effect** - no control promoter is constitutive in all cell types in all circumstances
2. **Vector effect** – through other sequences on vector
3. **General effect** - more basic, e.g., general change in transcriptional state, or mRNA degradation

**How to tell?**

- Try promoterless FLuc vector
- Try different RLuc vector (different promoter)
- Switch to pGL4 vector

If a different promoter doesn’t help, then it is a vector effect or general effect; the “cleaned” pGL4 vector may differentiate these 2
Variables to Consider in Reporter Assay Design

- Reporter design?
- Transfection parameters?
- Assay choice?
- Timing?
- Culture/Treatment?
- Controls?
- Type of cell?
Questions?

Rely on Promega Technical Services

• Experienced & highly trained scientists
  >150 years cumulative bench experience,
  >10 yrs average

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  reporters, cell culture, HTS, etc.

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  model systems, genetics, development, etc.

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