Luciferase Reporter Assays

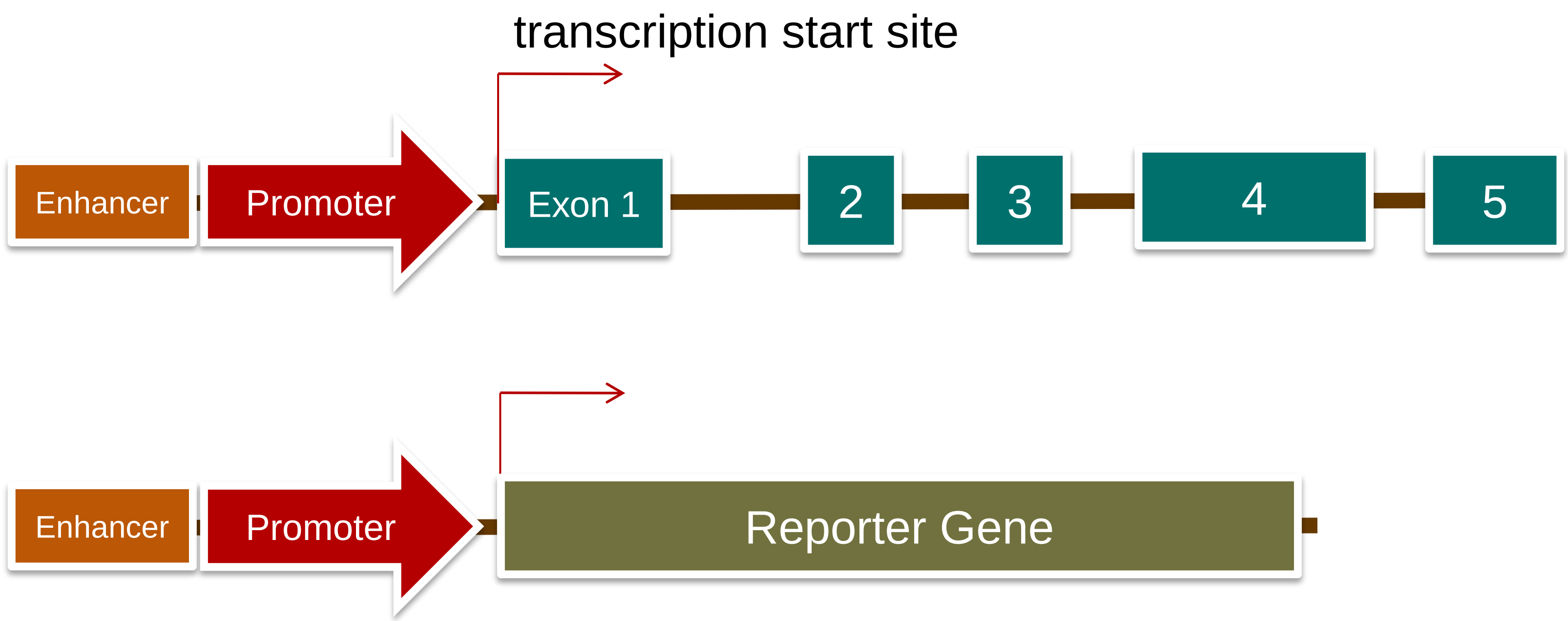
Tips & Techniques

Carl Strayer, Ph.D.

October 11, 2011

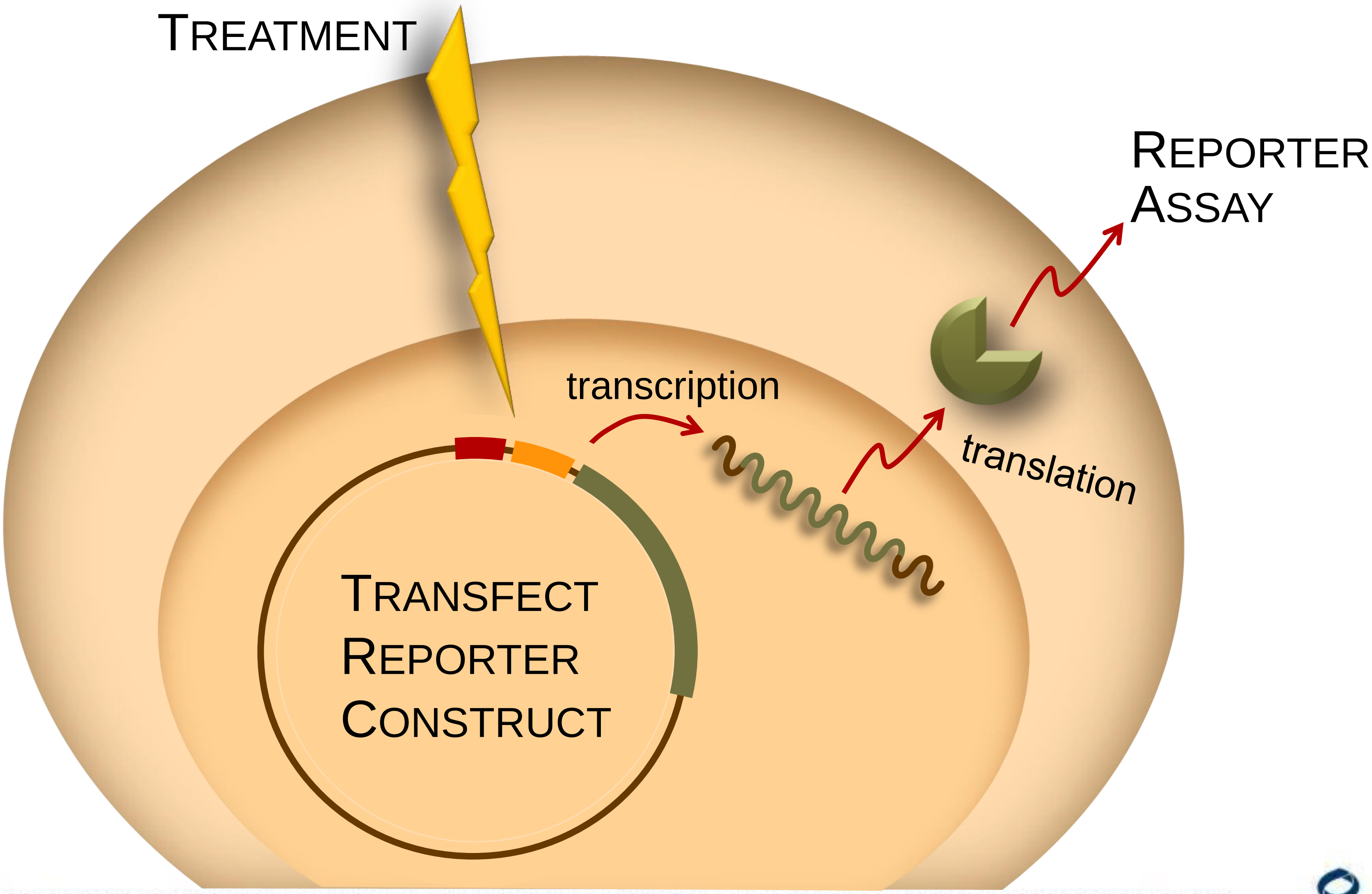
Promega

Reporter Assay Principle

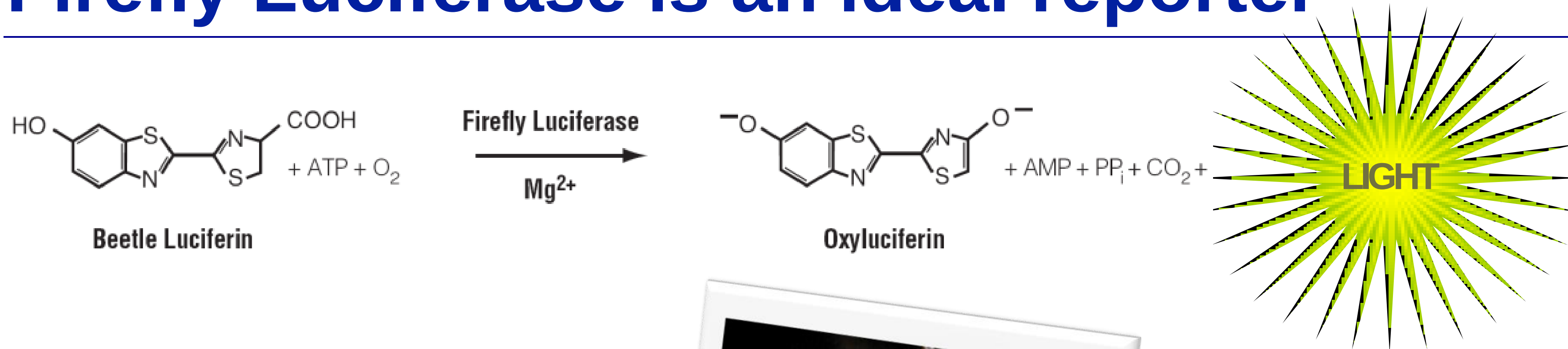


- The reporter gene produces an easy to assay & quantify protein
- Most reporters are enzymes which offer amplification of signal through catalysis
- Reporter assays can be bioluminescent, fluorescent, colorimetric or radioactive

Reporter Assay Principle



Firefly Luciferase is an ideal reporter



Common Firefly
Photinus pyralis

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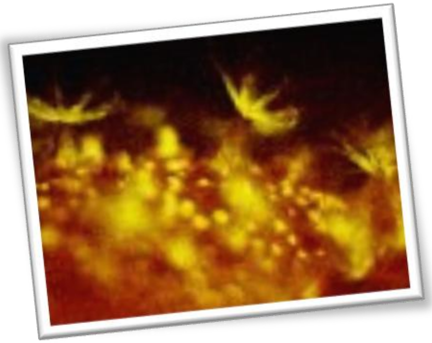
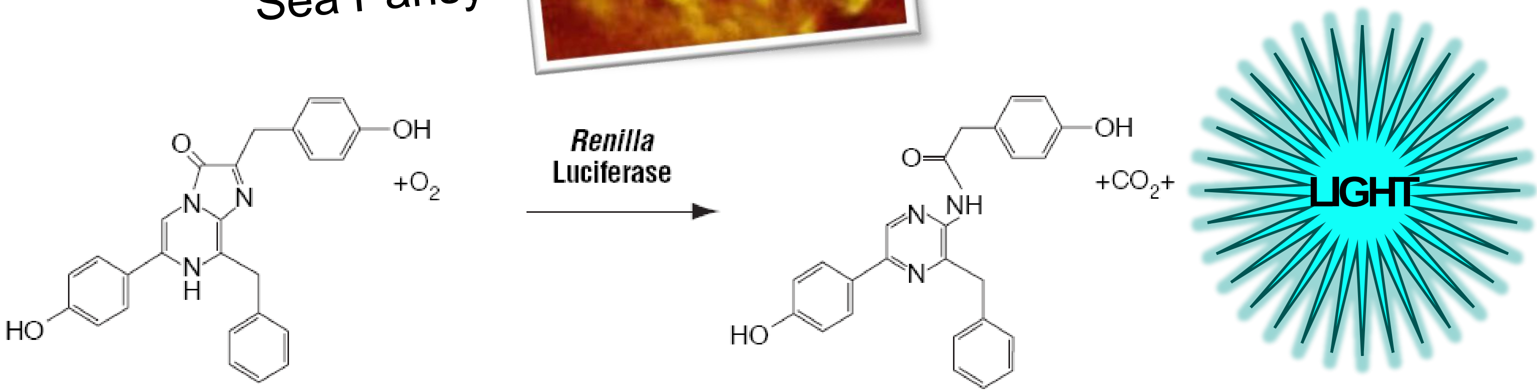
Renilla Luciferase

Renilla reniformis
Sea Pansy



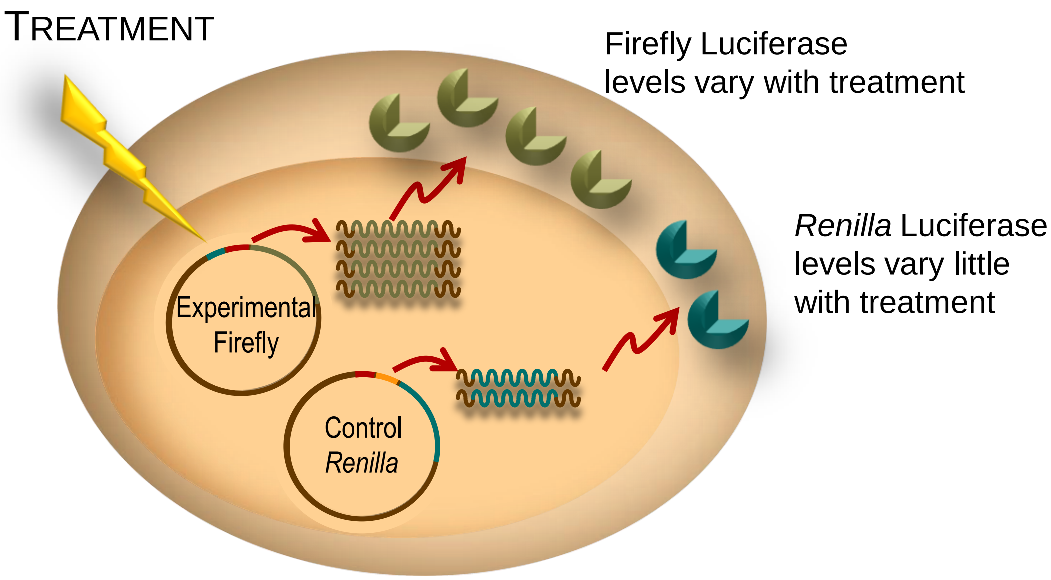


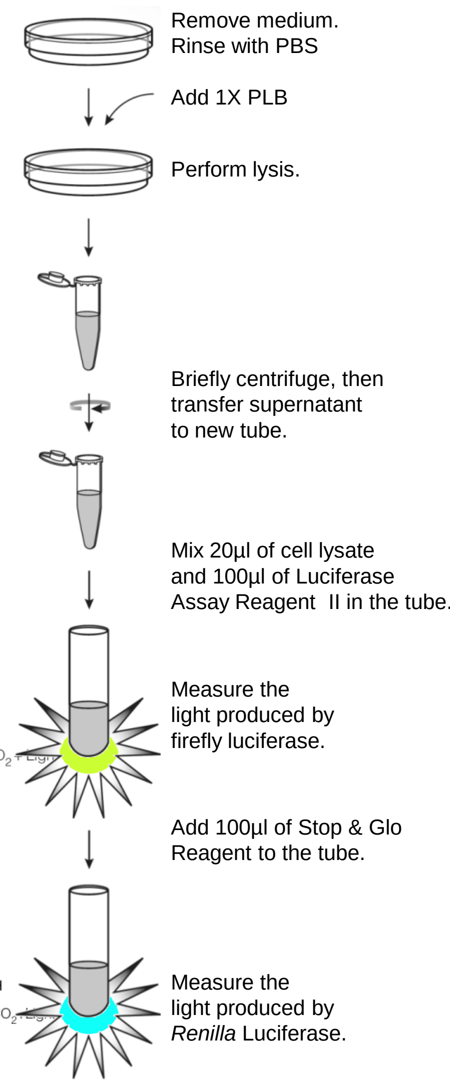
Coelenterazine

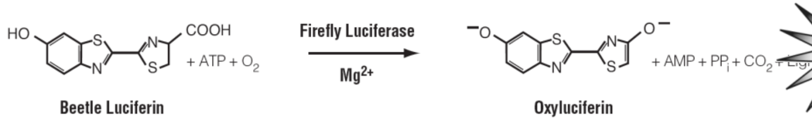
Coelenteramide

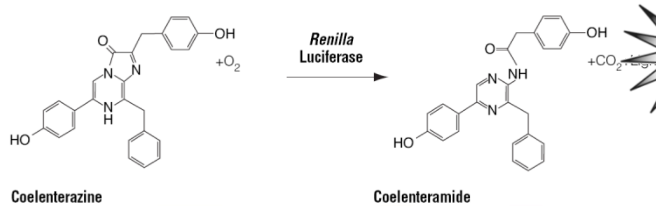
Reporter	Non-Radioactive	Enzymatic	Background	Sensitivity	Active Form	half-life
Firefly Luciferase	Y	Y	–	++++	monomer	3 hours
Renilla Luciferase	Y	Y	–	++++	monomer	5 hours
CAT	N	Y	–	+	Trimer	50 hours
β-Galactosidase	Y	Y	+/–	++	tetramer	20 hours

Dual-Luciferase® Reporter Assay









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

History of Reporter Assay Tools

luc → *luc+* → *luc2*

pGL2 Vectors

pGL3 Vectors

pGL4 Vectors

Rluc → *hRluc* → pGL4 Vectors

pRL Vectors

phRL Vectors

1990 1992 1994 1996 1998 2000 2002 2004 2006 2008 2010

LAR w/ CCLR

LAR w/ RLB

DLR™ w/ PLB

Steady-Glo® Luciferase Assay System

Bright-Glo™ Luciferase Assay System

Dual-Glo™ Luciferase Assay System

EnduRen™ Live Cell Substrate

ViviRen™ Live Cell Substrate

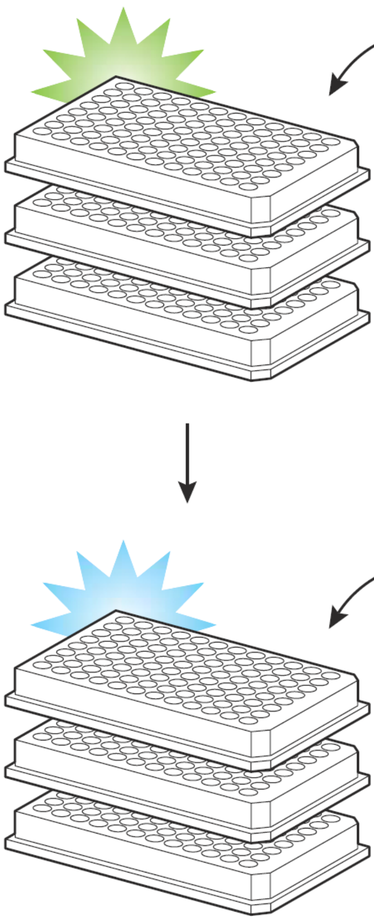
ONE-Glo™ Luciferase Assay System

VivoGlo™ Substrates, EnduRen & ViviRen in vivo Substrates

Keith V. Wood, Ph.D.

Dual-Glo® Luciferase Assay System

Dual-Glo™ Luciferase Assay:



Step 1:

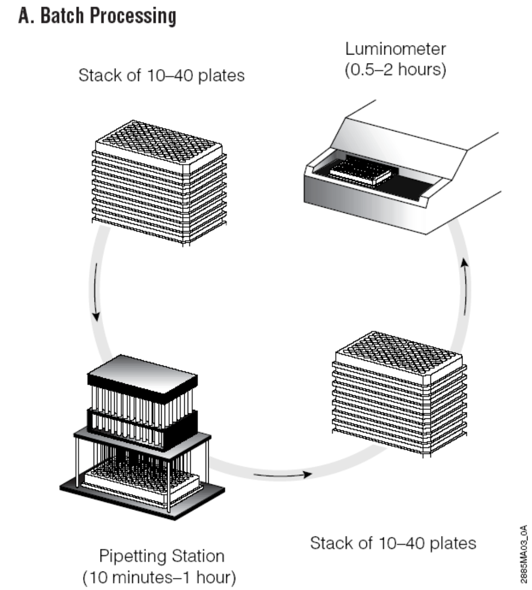
Dispense **Dual-Glo™ Luciferase Reagent** directly to plates containing cells in culture medium. Wait 10 minutes, then measure firefly luciferase activity for up to 2 hours.

2 STEPS

Step 2:

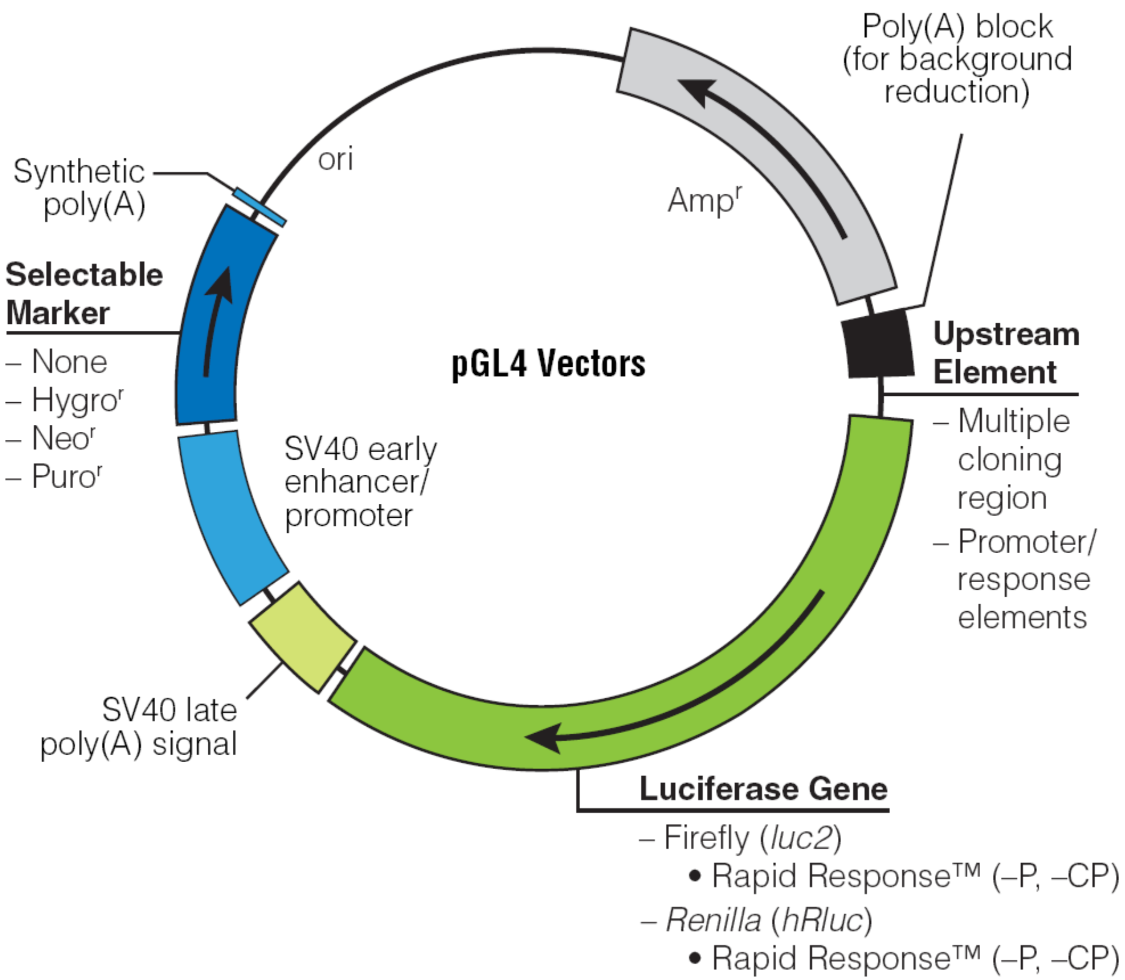
Dispense **Dual-Glo™ Stop & Glo® Reagent** to same plates. Wait 10 minutes, then measure *Renilla* luciferase activity for up to 2 hours.

3606MA01_2A



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!

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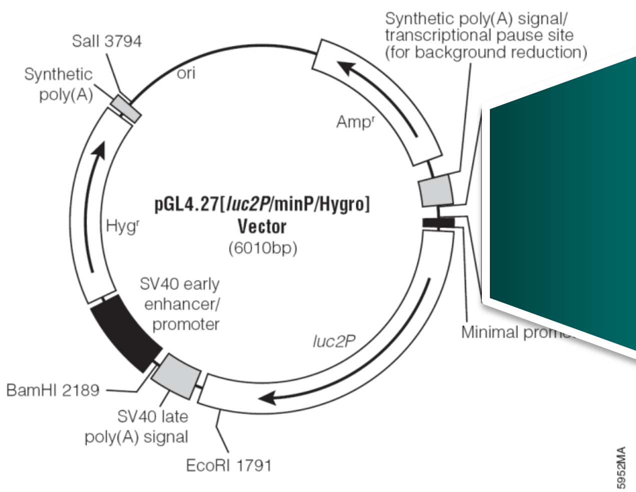
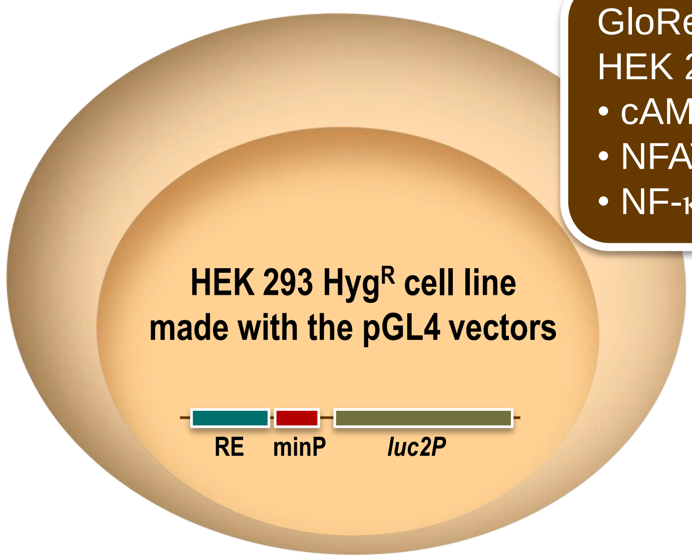
Pre-made pGL4 constructs – RE clones



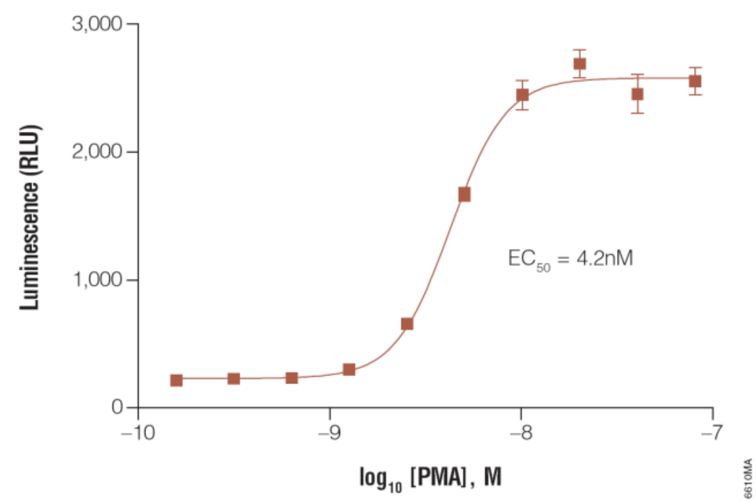
Pre-designed, ready-to-use response element pGL4 Vectors

Response Element	Signaling Pathway	pGL4 Vector
cAMP	cAMP/PKA	pGL4.29
NFAT Nuclear Factor of Activated T-cells	Ca^{2+}/Calcineurin	pGL4.30
NF-κB	NF-κB	pGL4.32
Serum Response	MAP/ERK	pGL4.33
Serum Response Factor	RhoA	pGL4.34



GloResponse™ HEK 293 cell lines:

- cAMP RE
- NFAT RE
- NF-κB RE



GloResponse NFAT-RE-*luc2P* HEK293 Cell Line

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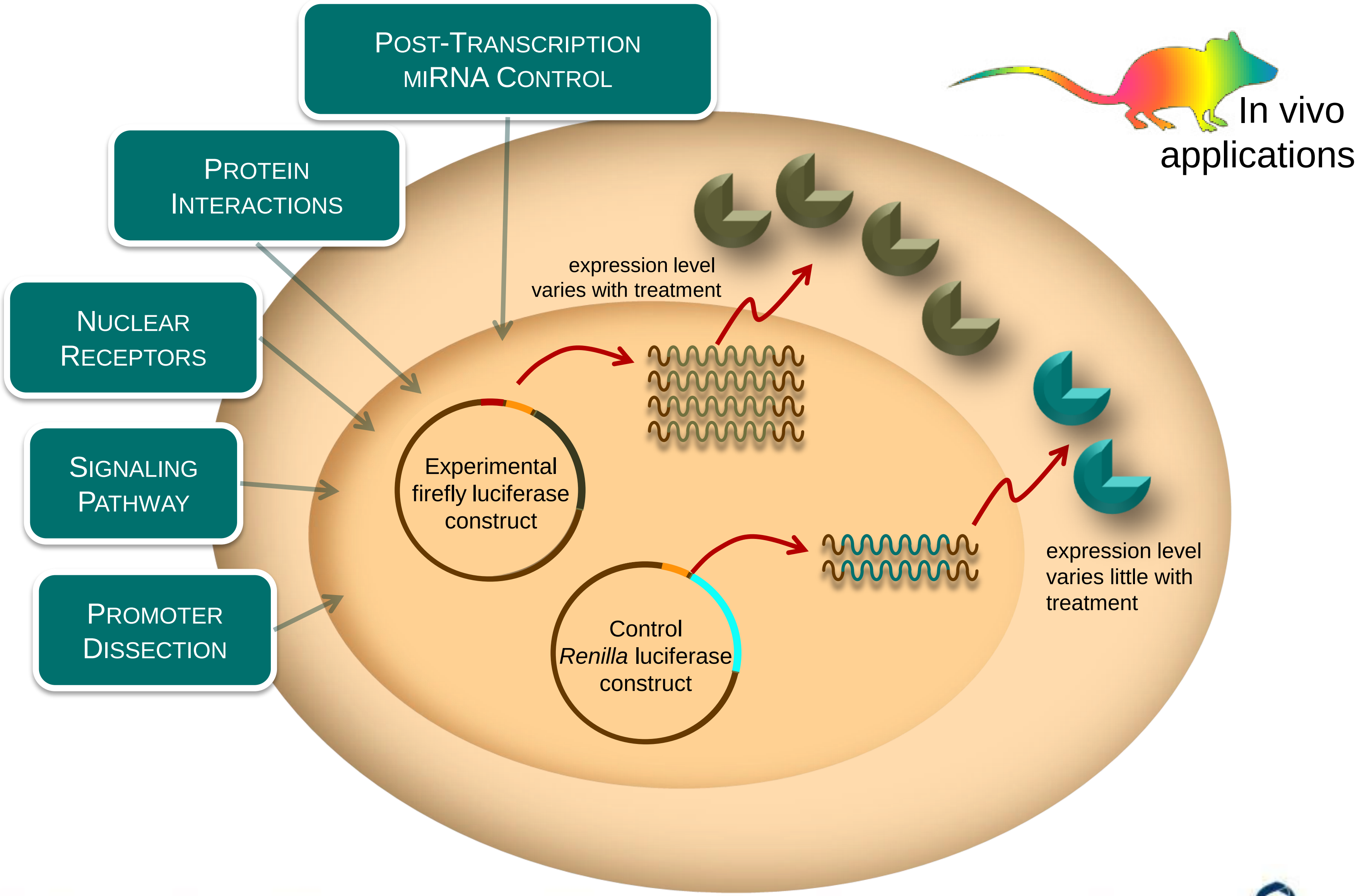
Reporter Assay Applications

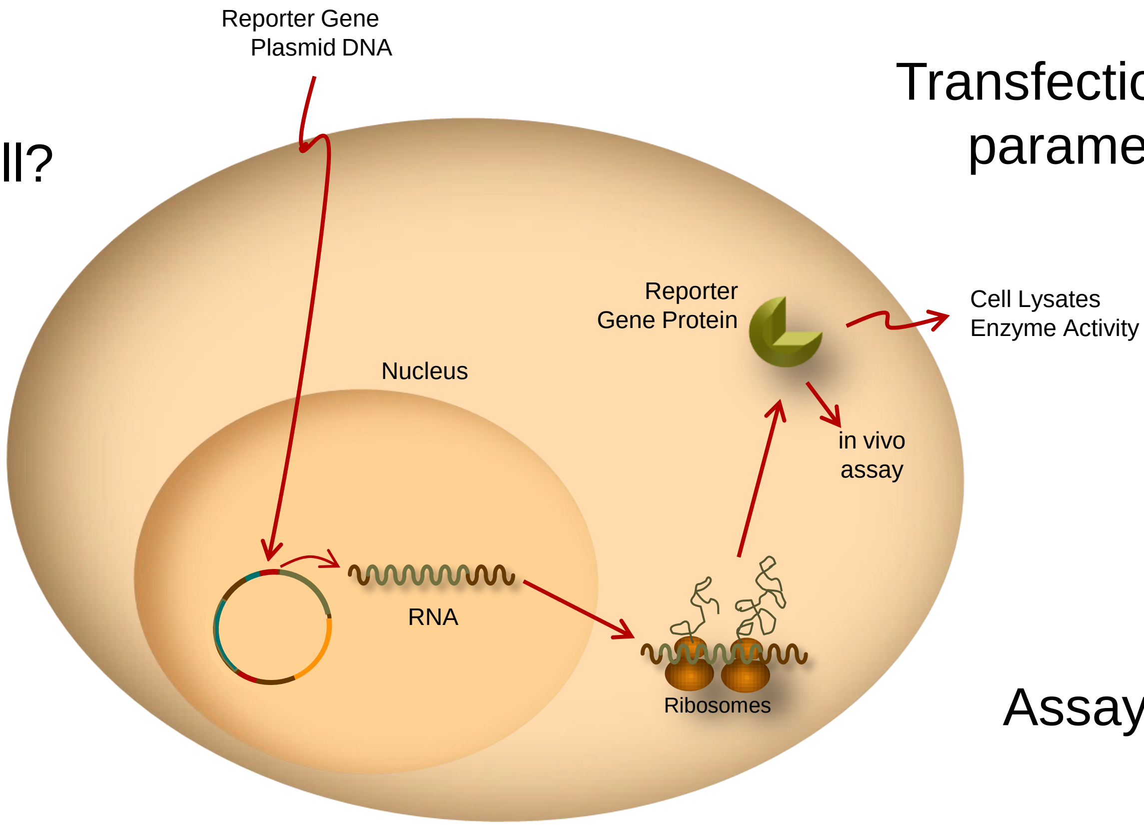


Variables to Consider in Reporter Assay Design

Reporter design?

Type of cell?

Transfection parameters?



Controls?

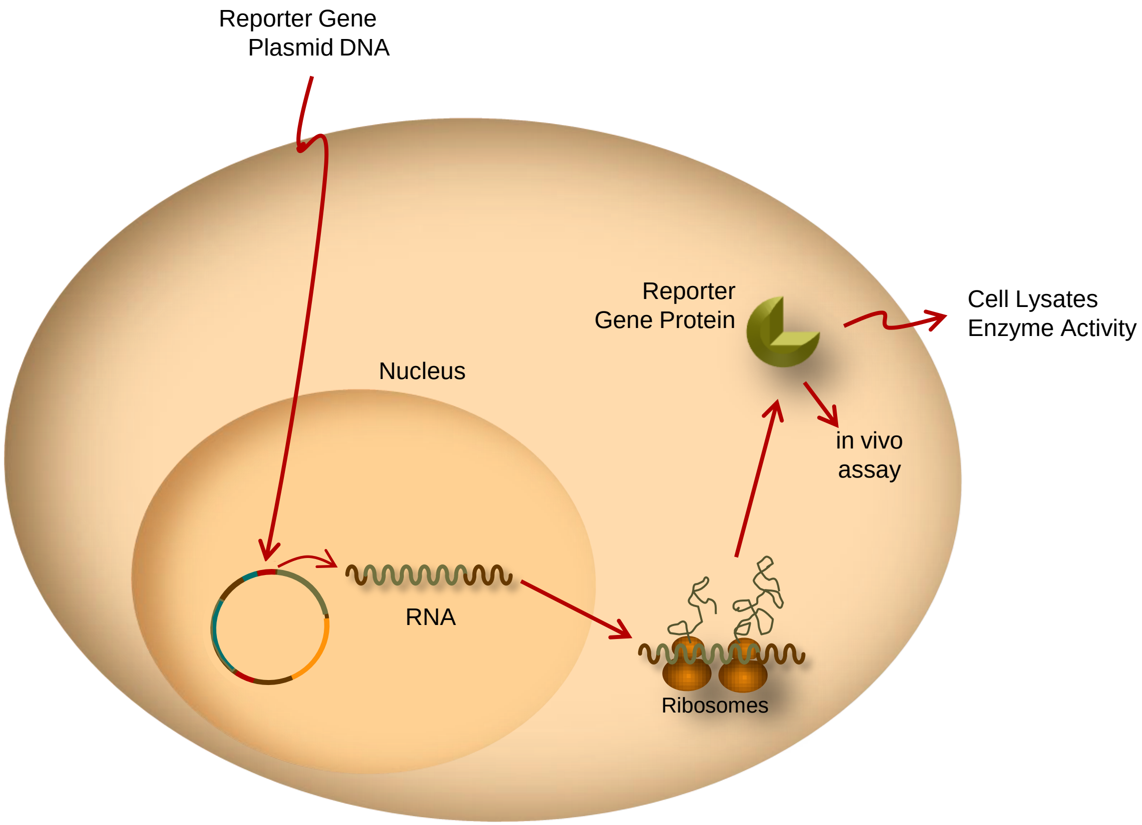
Assay choice?

Culture/Treatment?

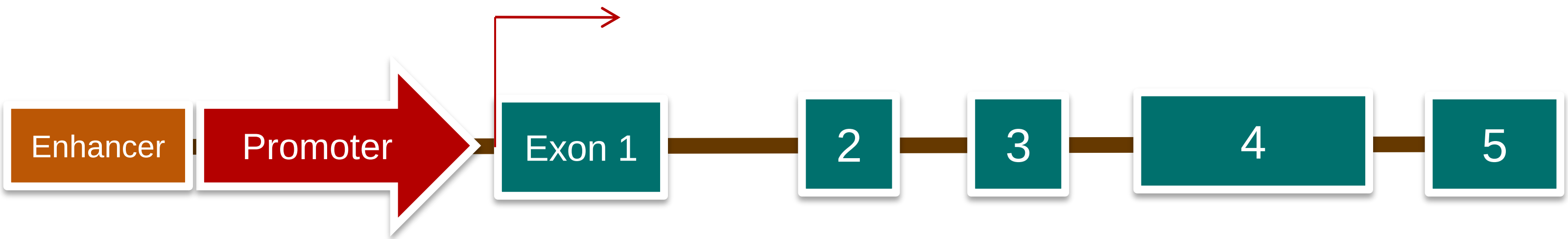
Timing?

Variables to Consider in Reporter Assay Design

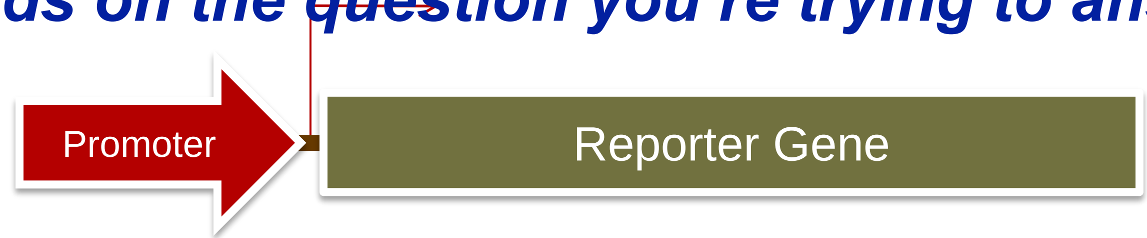
Reporter design?



What sequences should I clone?



Depends on the question you're trying to answer…



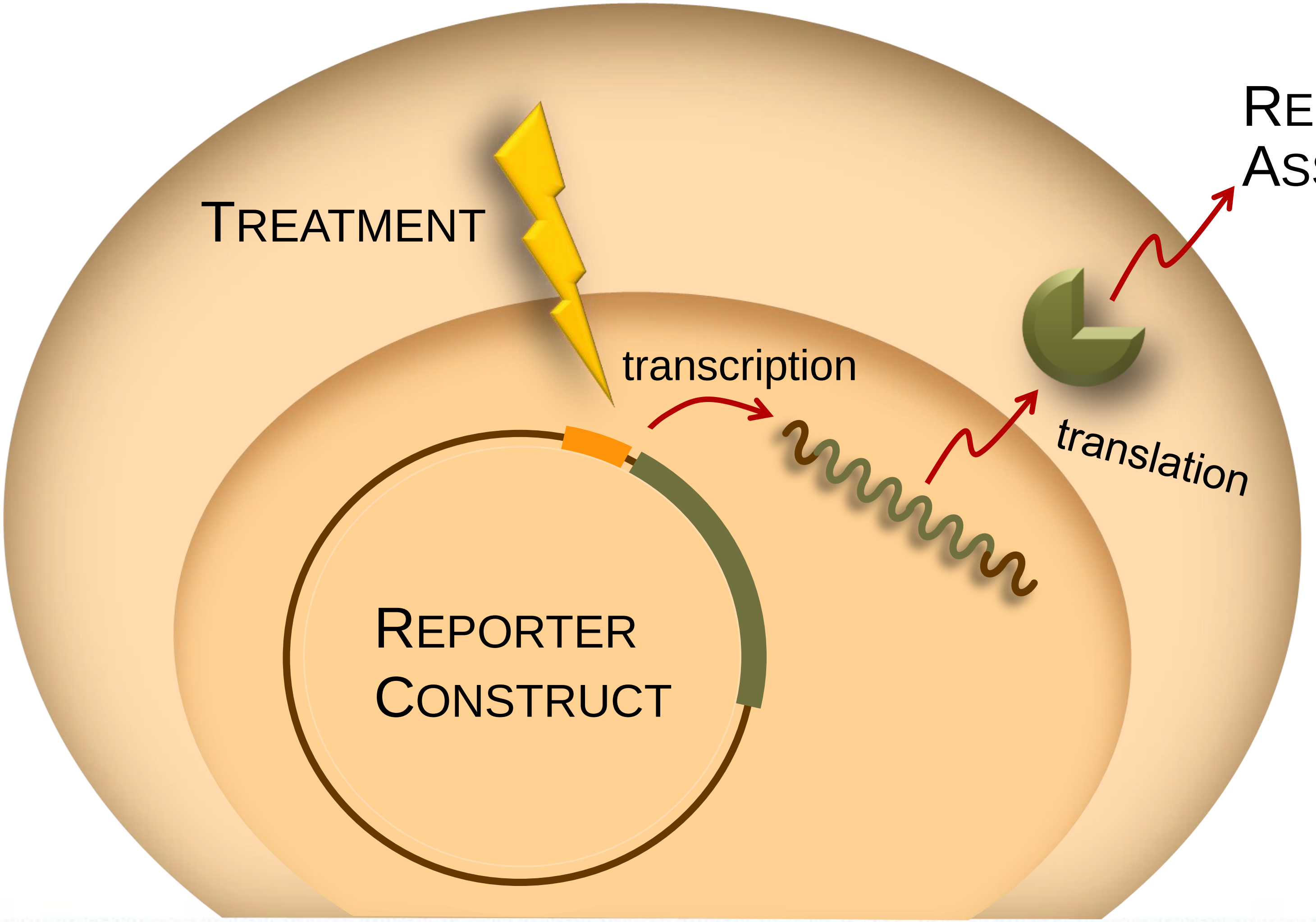
Transcriptional fusions typically use the proximal promoter

~1kb upstream of, & including, the transcriptional start site (+1)

Response Element constructs more precisely define the assay

Transcriptional reporter fusions…

…report transcriptional regulatory influences



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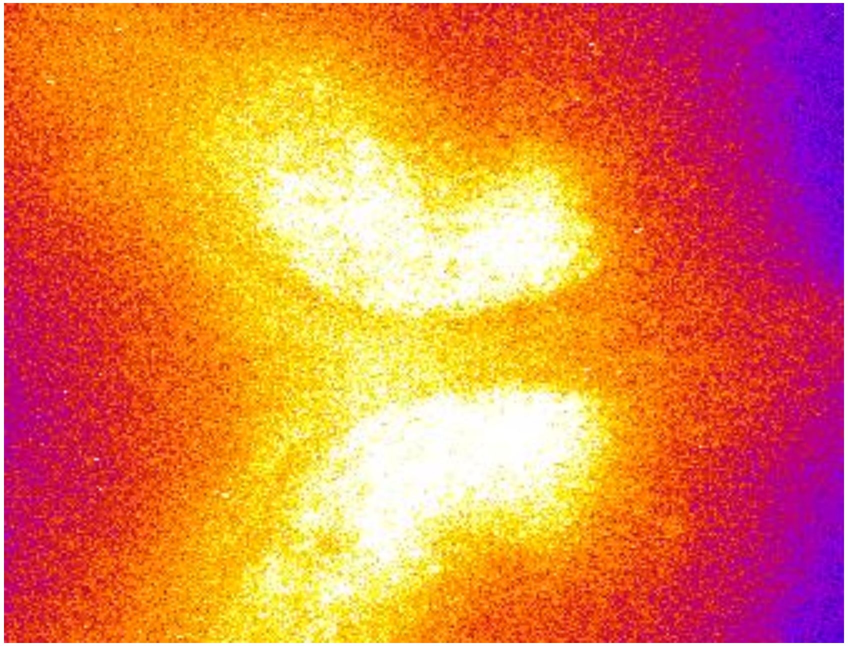
Transcriptional reporter fusion

…may provide just the right tool!

Period:luc
transcriptional
fusion



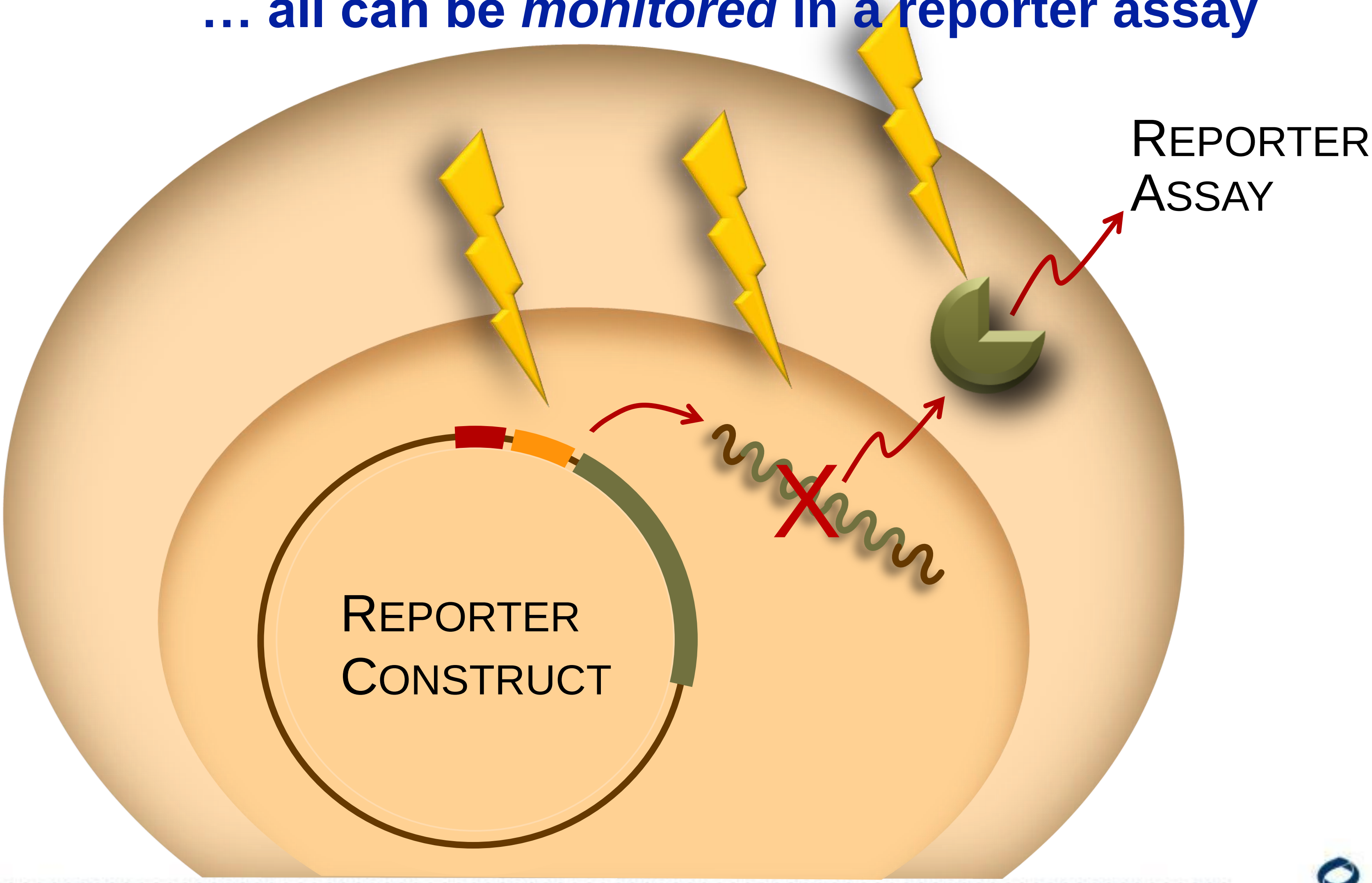
Brain slice
explant
in culture
4 days

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

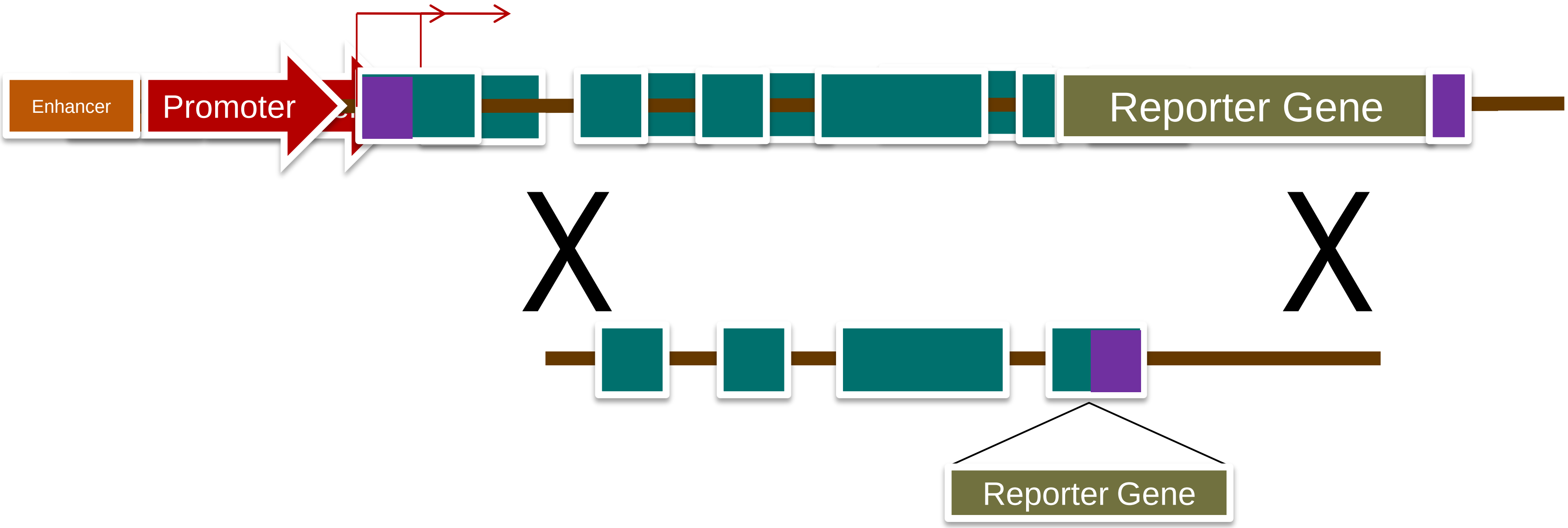


What sequences should I clone?

- 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

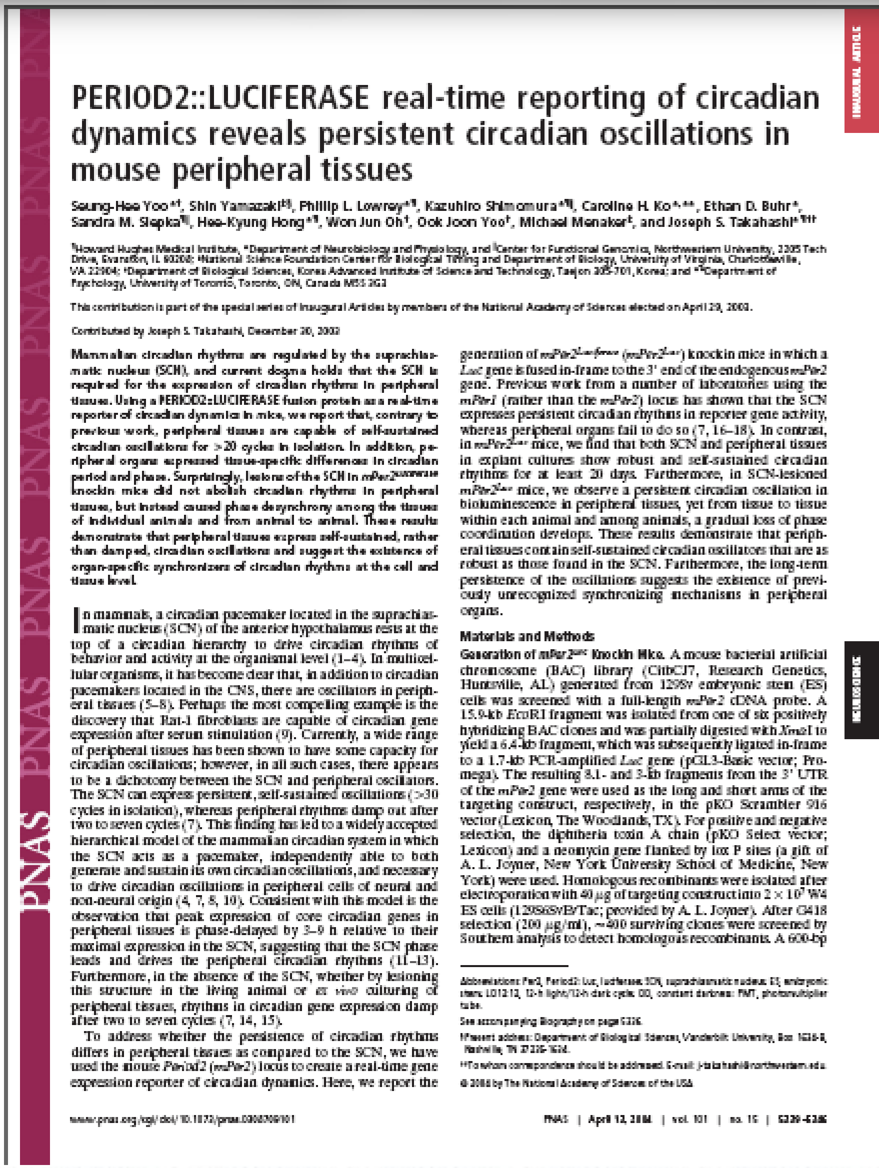
Yoo, *et al.* (2004) *PNAS* **12**,1-8



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!*

Case Study: the Ultimate Genetic Reporter



INAUGURAL ARTICLE

PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues

Seung-Hee Yoo*[†], Shin Yamazaki[‡§], Phillip L. Lowrey*[¶], Kazuhiro Shimomura*[¶], Caroline H. Ko*[,**], Ethan D. Buhr*, Sandra M. Siepka[¶], Hee-Kyung Hong*[¶], Won Jun Oh[†], Ook Joon Yoo[†], Michael Menaker[‡], and Joseph S. Takahashi*[†¶††]

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This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected on April 29, 2003.

Contributed by Joseph S. Takahashi, December 30, 2003

Mammalian circadian rhythms are regulated by the suprachiasmatic nucleus (SCN), and current dogma holds that the SCN is required for the expression of circadian rhythms in peripheral tissues. Using a PERIOD2::LUCIFERASE fusion protein as a real-time reporter of circadian dynamics in mice, we report that, contrary to previous work, peripheral tissues are capable of self-sustained circadian oscillations for >20 cycles in isolation. In addition, peripheral organs expressed tissue-specific differences in circadian period and phase. Surprisingly, lesions of the SCN in *mPer2^Luciferase^* knockin mice did not abolish circadian rhythms in peripheral tissues, but instead caused phase desynchrony among the tissues of individual animals and from animal to animal. These results demonstrate that peripheral tissues express self-sustained, rather than damped, circadian oscillations and suggest the existence of organ-specific synchronizers of circadian rhythms at the cell and tissue level.

In mammals, a circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus rests at the top of a circadian hierarchy to drive circadian rhythms of behavior and activity at the organismal level (1–4). In multicellular organisms, it has become clear that, in addition to circadian pacemakers located in the CNS, there are oscillators in peripheral tissues (5–8). Perhaps the most compelling example is the discovery that Rat-1 fibroblasts are capable of circadian gene expression after serum stimulation (9). Currently, a wide range of peripheral tissues has been shown to have some capacity for circadian oscillations; however, in all such cases, there appears to be a dichotomy between the SCN and peripheral oscillators. The SCN can express persistent, self-sustained oscillations (>30 cycles in isolation), whereas peripheral rhythms damp out after two to seven cycles (7). This finding has led to a widely accepted hierarchical model of the mammalian circadian system in which the SCN acts as a pacemaker, independently able to both generate and sustain its own circadian oscillations, and necessary to drive circadian oscillations in peripheral cells of neural and non-neural origin (4, 7, 8, 10). Consistent with this model is the observation that peak expression of core circadian genes in peripheral tissues is phase-delayed by 3–9 h relative to their maximal expression in the SCN, suggesting that the SCN phase leads and drives the peripheral circadian rhythms (11–13). Furthermore, in the absence of the SCN, whether by lesioning this structure in the living animal or ex vivo culturing of peripheral tissues, rhythms in circadian gene expression damp after two to seven cycles (7, 14, 15).

To address whether the persistence of circadian rhythms differs in peripheral tissues as compared to the SCN, we have used the mouse *Period2* (*mPer2*) locus to create a real-time gene expression reporter of circadian dynamics. Here, we report the generation of *mPer2^Luciferase^* (*mPer2^Luc^*) knockin mice in which a *Luc* gene is fused in-frame to the 3' end of the endogenous *mPer2* gene. Previous work from a number of laboratories using the *mPer1* (rather than the *mPer2*) locus has shown that the SCN expresses persistent circadian rhythms in reporter gene activity, whereas peripheral organs fail to do so (7, 16–18). In contrast, in *mPer2^Luc^* mice, we find that both SCN and peripheral tissues in explant cultures show robust and self-sustained circadian rhythms for at least 20 days. Furthermore, in SCN-lesioned *mPer2^Luc^* mice, we observe a persistent circadian oscillation in bioluminescence in peripheral tissues, yet from tissue to tissue within each animal and among animals, a gradual loss of phase coordination develops. These results demonstrate that peripheral tissues contain self-sustained circadian oscillators that are as robust as those found in the SCN. Furthermore, the long-term persistence of the oscillations suggests the existence of previously unrecognized synchronizing mechanisms in peripheral organs.

Materials and Methods

Generation of *mPer2^Luc^* Knockin Mice. A mouse bacterial artificial chromosome (BAC) library (CitbCJ7, Research Genetics, Huntsville, AL) generated from 129Sv embryonic stem (ES) cells was screened with a full-length *mPer2* cDNA probe. A 15.9-kb *Eco*RI fragment was isolated from one of six positively hybridizing BAC clones and was partially digested with *Xma*I to yield a 6.4-kb fragment, which was subsequently ligated in-frame to a 1.7-kb PCR-amplified *Luc* gene (pGL3-Basic vector; Promega). The resulting 8.1- and 3-kb fragments from the 3' UTR of the *mPer2* gene were used as the long and short arms of the targeting construct, respectively, in the pKO Scrambler 916 vector (Lexicon, The Woodlands, TX). For positive and negative selection, the diphtheria toxin A chain (pKO Select vector; Lexicon) and a neomycin gene flanked by lox P sites (a gift of A. L. Joyner, New York University School of Medicine, New York) were used. Homologous recombinants were isolated after electroporation with 40 μg of targeting construct into 2×10^7 W4 ES cells (129S6SvEvTac; provided by A. L. Joyner). After G418 selection (200 μg/ml), ~400 surviving clones were screened by Southern analysis to detect homologous recombinants. A 600-bp

Abbreviations: Per2, Period2; Luc, luciferase; SCN, suprachiasmatic nucleus; ES, embryonic stem; LD12:12, 12-h light/12-h dark cycle; DD, constant darkness; PMT, photomultiplier tube.

See accompanying Biography on page 5336.

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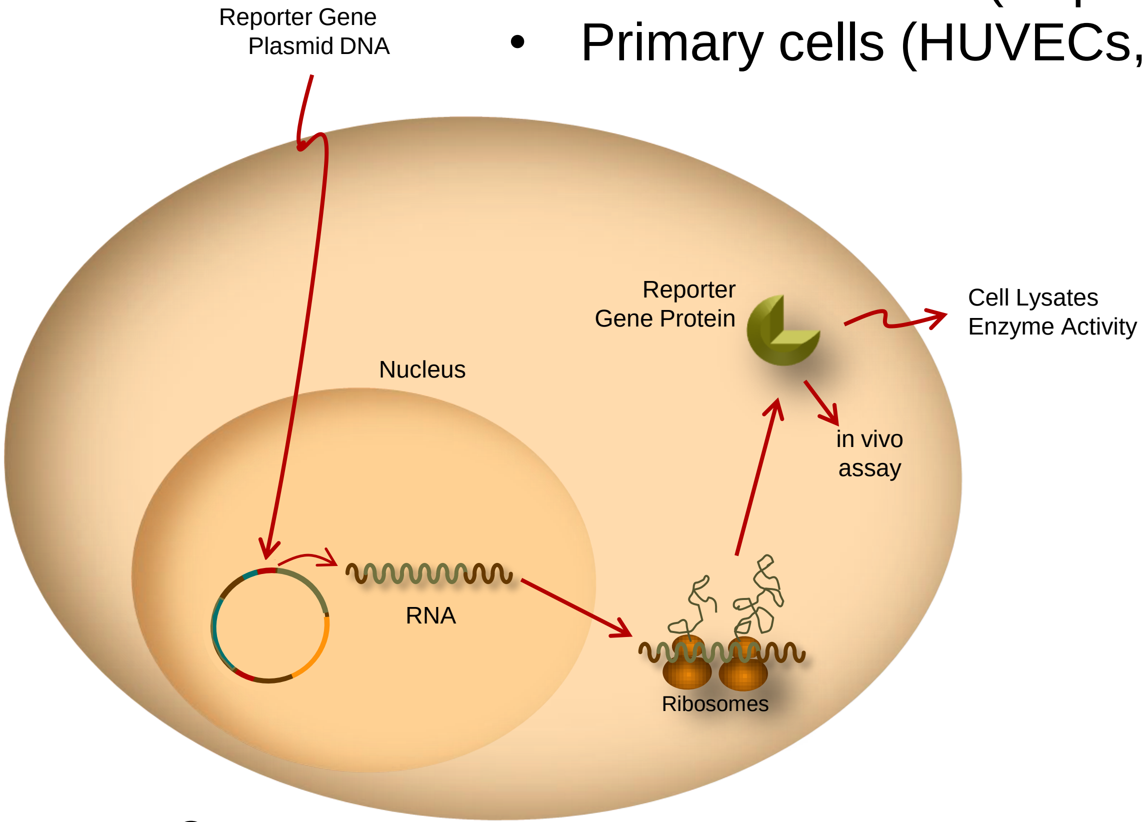
NEUROSCIENCE

- More accurate reporter of *gene expression*
- Better marker for *the system*
- Enabled *new discoveries*

Variables to Consider in Reporter Assay Design

Type of cell?

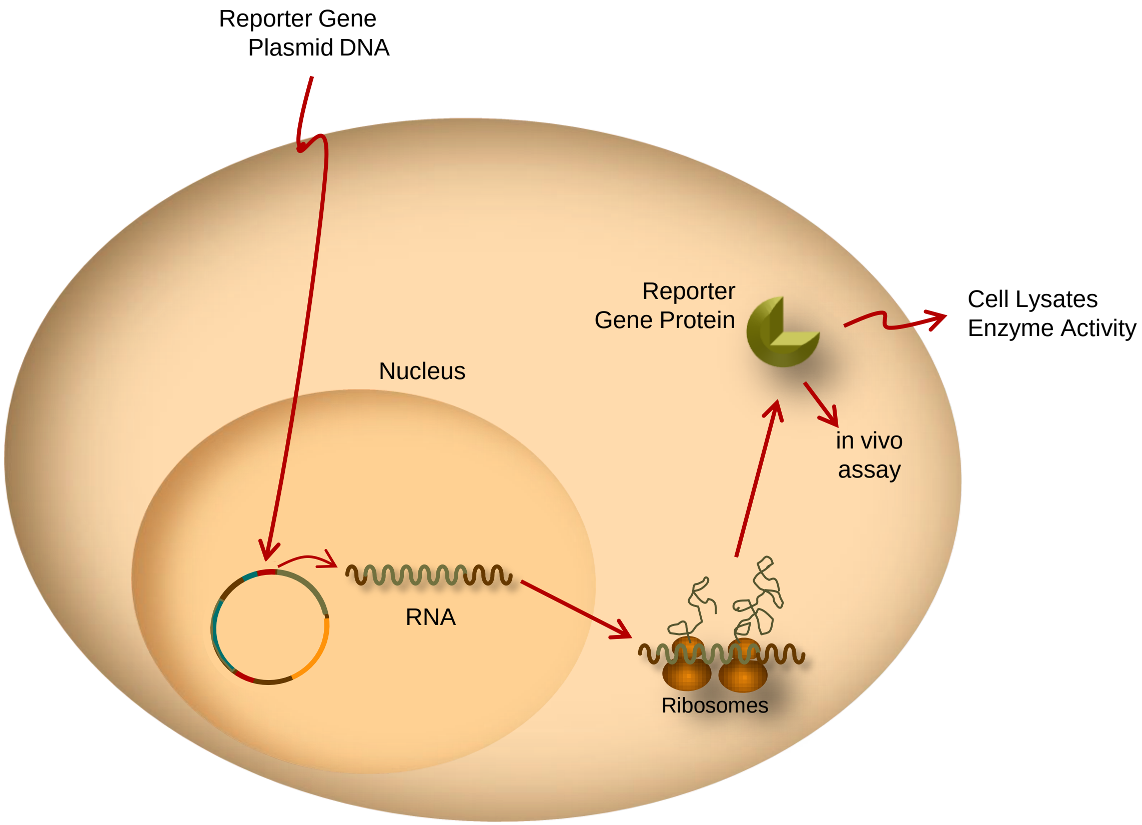
- Fibroblasts (HEK293, Cos)
- Cancer cell lines (HepG2, PC-3)
- Primary cells (HUVECs, hepatocytes)



- Amenable to assay?
- Faithfully represent system?
- Express trans-factors, signaling intermediates?

Variables to Consider in Reporter Assay Design

Cell Culture Variables?



Cell Culture Considerations

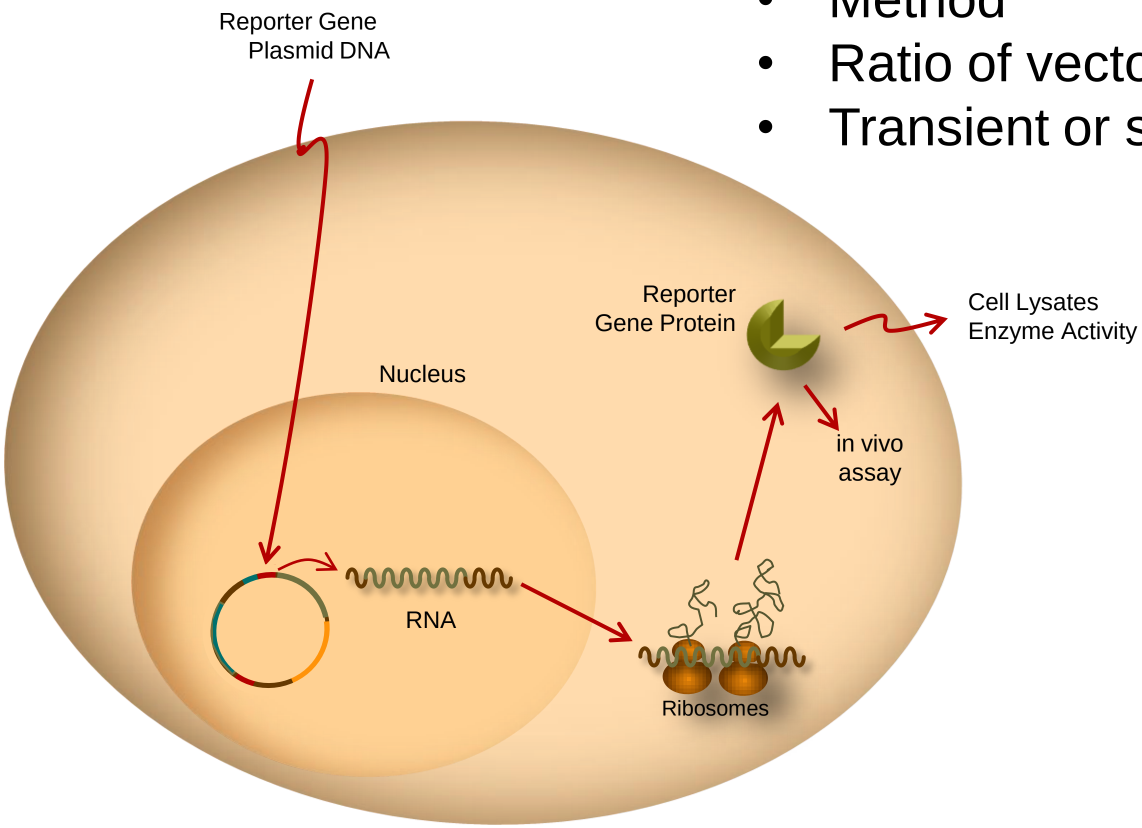
- Cell confluence
    - Pre-confluent cultures generally best for transfection…
    - …however, cells may become confluent by treatment
        - *how will growth state influence response to treatment?*

- 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



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

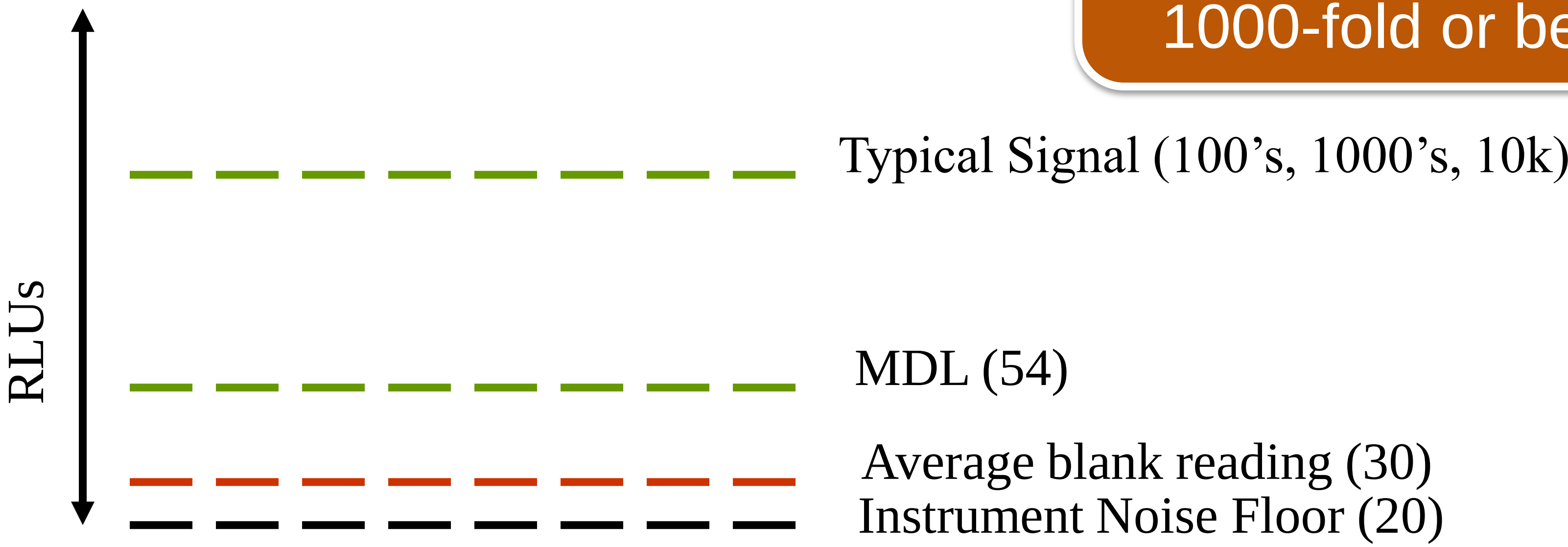
Lower limit -> background noise of instrument/assay

Example Of Background Determination

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



Transient transfection vs. Stable line

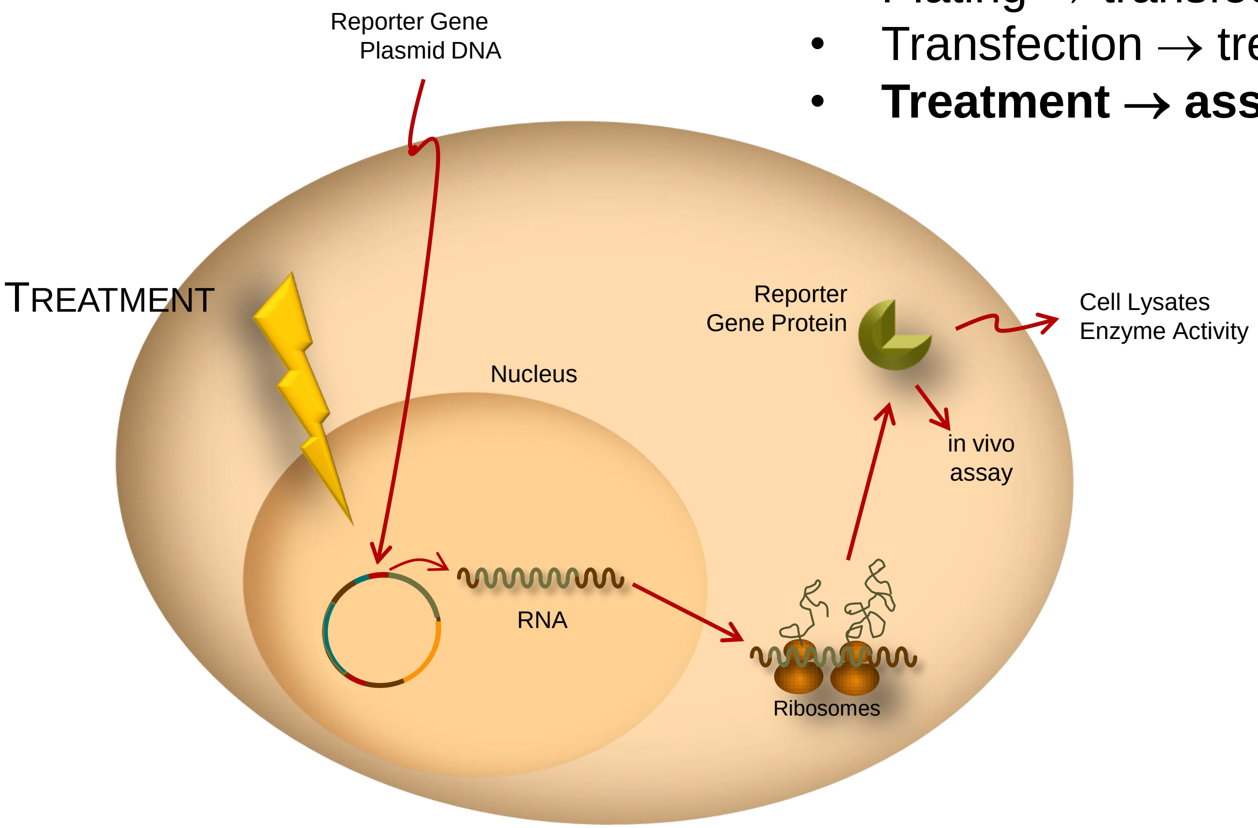
- Transient transfection
  - Versatile
    - *can vary combination of constructs*
    - *different cell types/lines*

- 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*

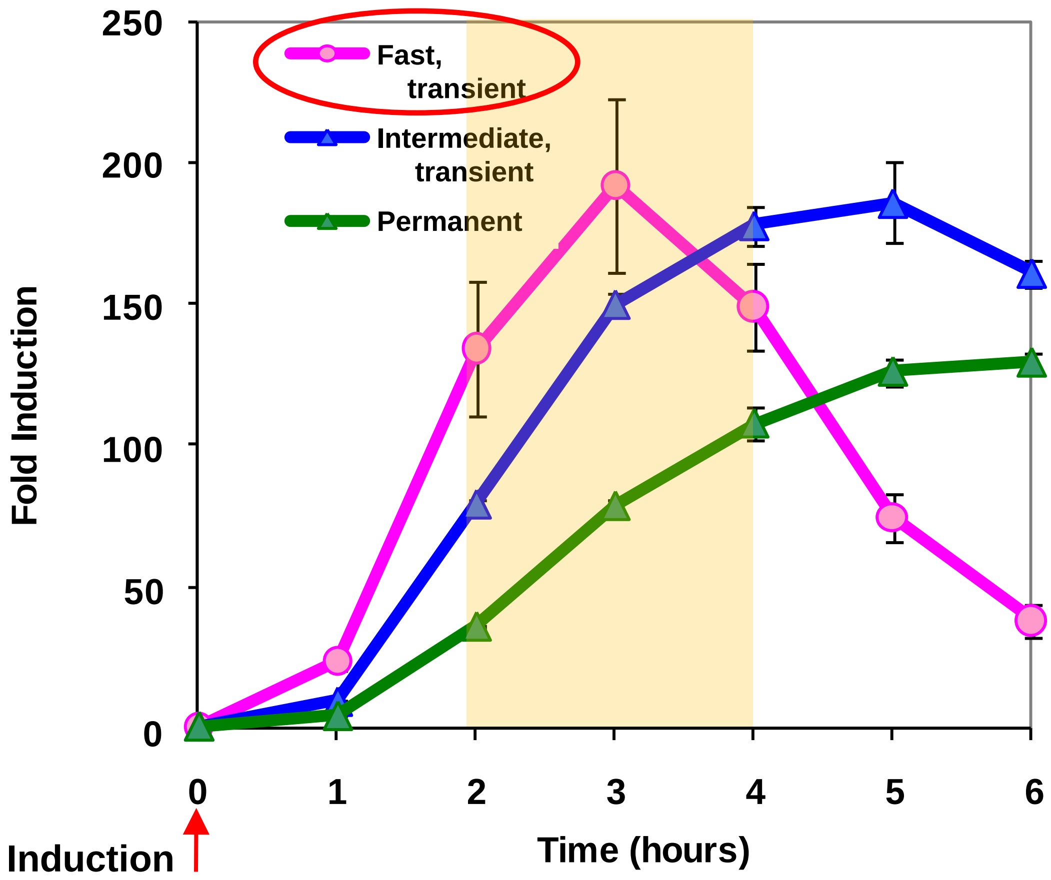
Variables to Consider in Reporter Assay Design

Timing?

- Plating → transfection
- Transfection → treatment
- **Treatment → assay**



Timing - consider pathway &reporter kinetics

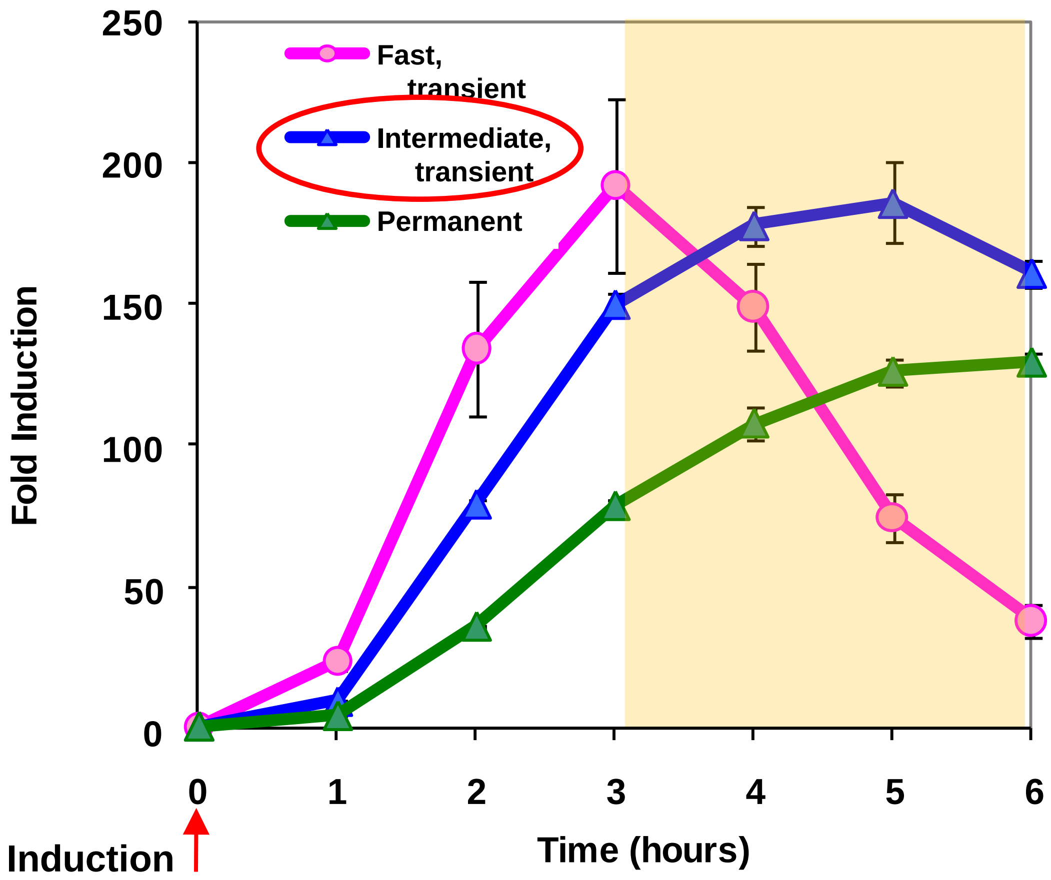


Response kinetic is fast and transient (and using a responsive reporter) - the assay window is sooner & narrower .

A time-course may be necessary to identify the peak induction

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

Timing - consider pathway &reporter kinetics

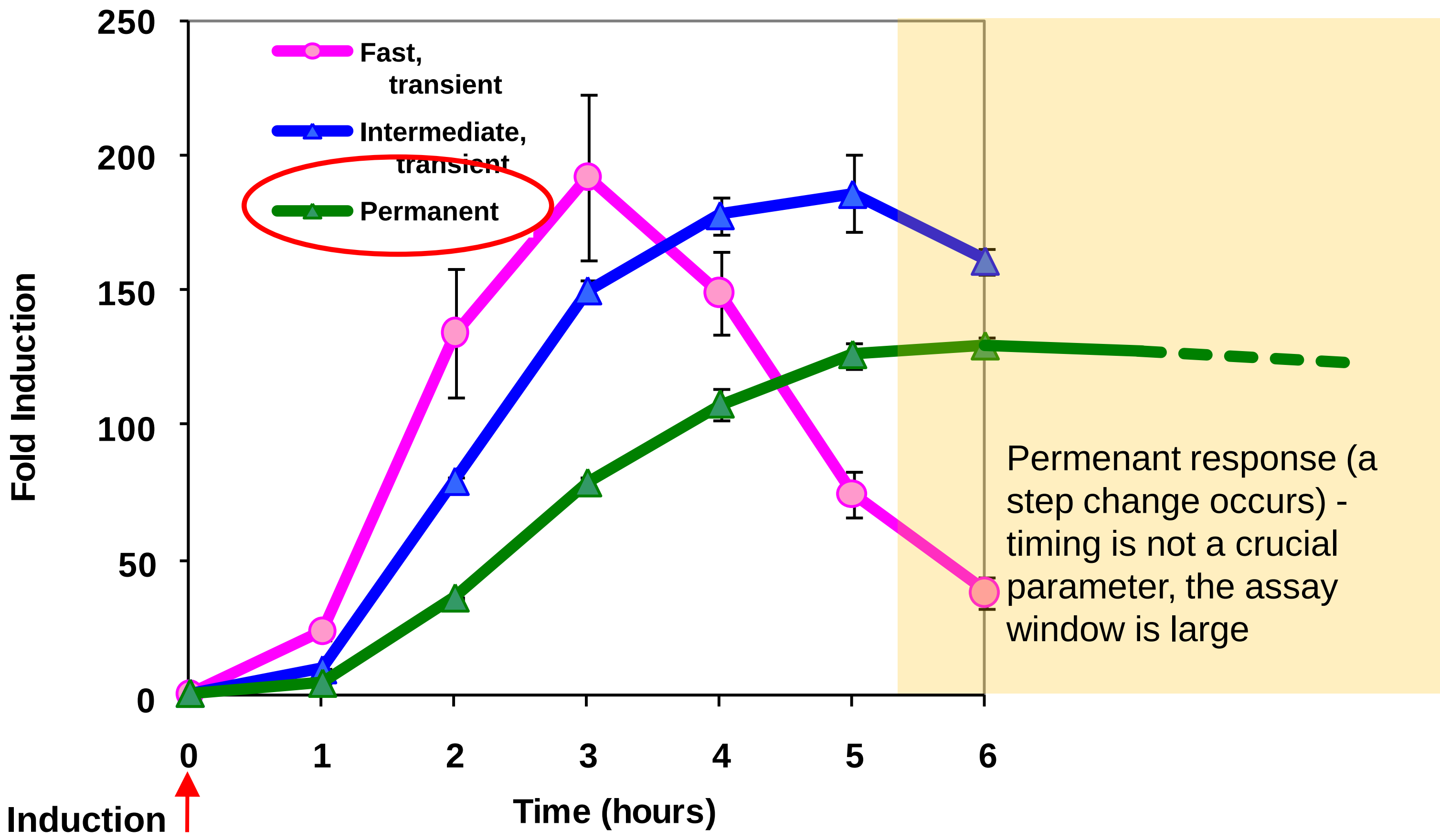


Transient response , slower kinetic is (or less responsive reporter) - the assay window occurs later & is broader.

A time-course may or may still be beneficial to identify the optimal timing

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

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?*

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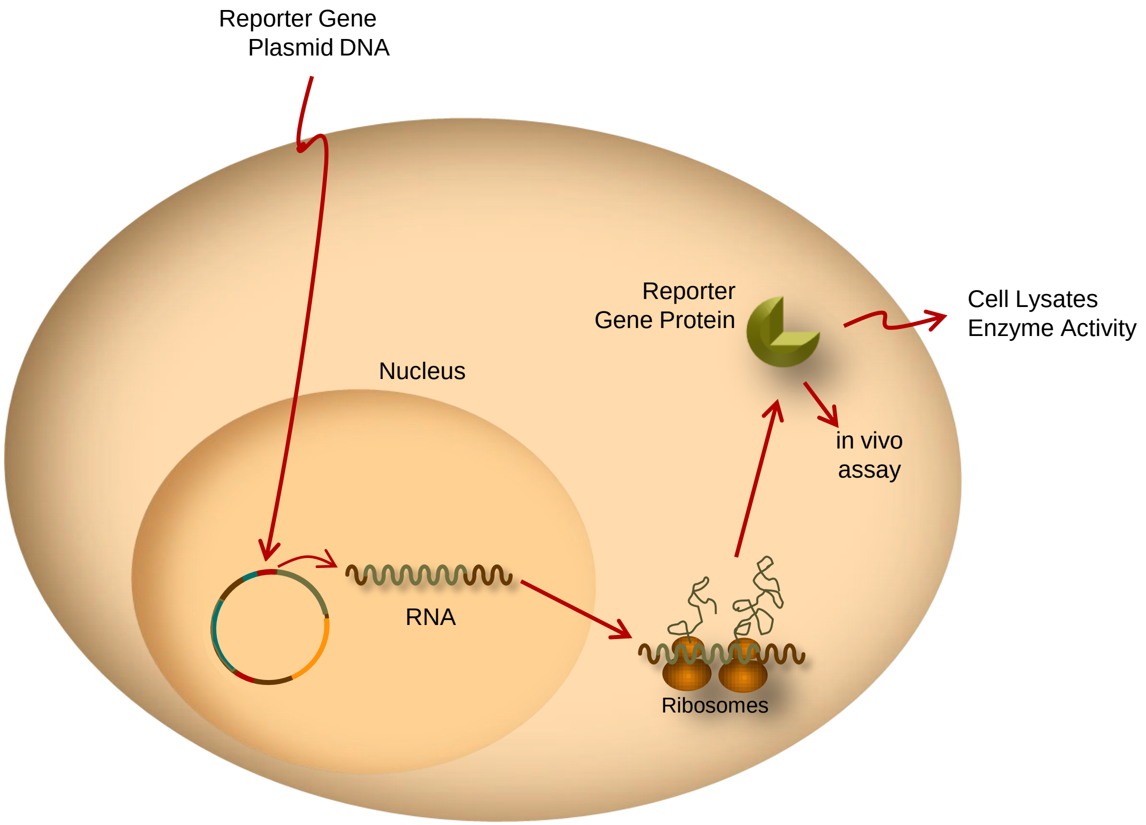
Variables to Consider in Reporter Assay Design

Controls?



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

Promega

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*

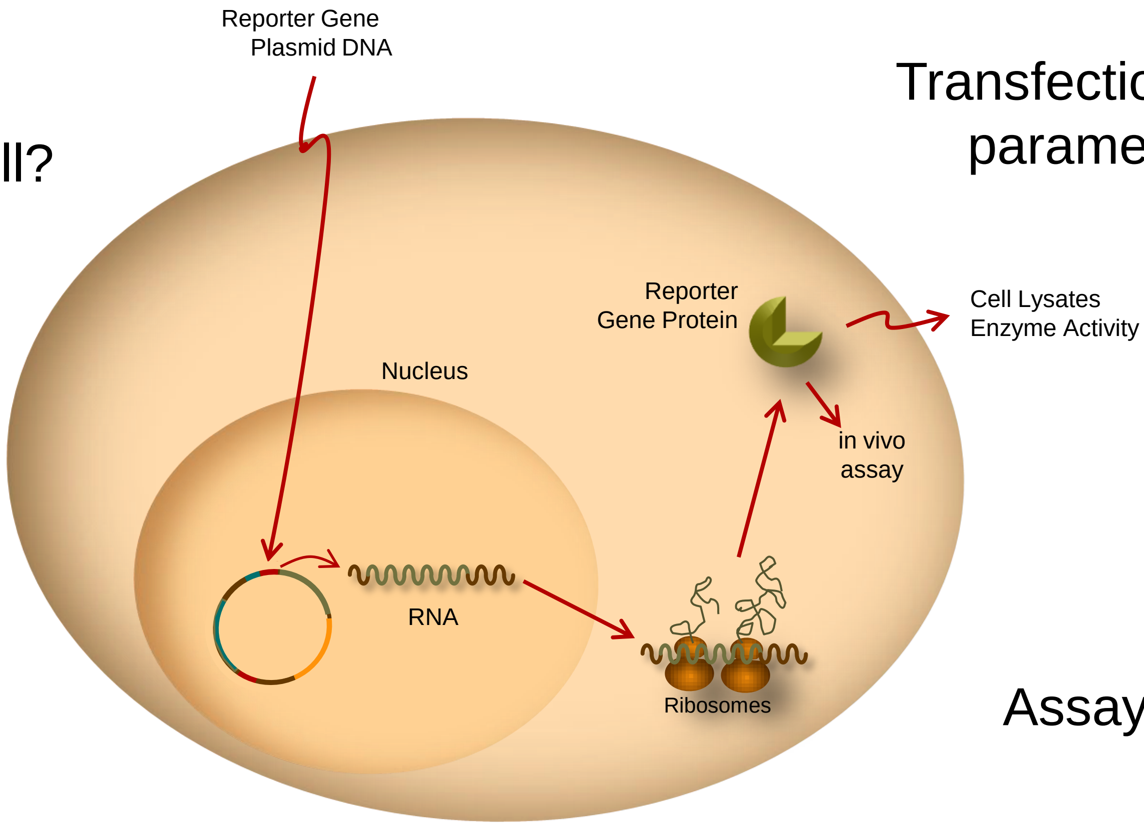
If a different promoter doesn't help, then it is a vector effect or general effect; the "cleaned" pGL4 vector may differentiate these 2

Variables to Consider in Reporter Assay Design

Reporter design?

Type of cell?

Transfection parameters?



Controls?

Assay choice?

Culture/Treatment?

Timing?

Questions?

Rely on Promega Technical Services

- Experienced & highly trained scientists

  >150 years cumulative bench experience,

  >10 yrs average

- Varied technical expertise

  reporters, cell culture, HTS, etc.

- Varied scientific expertise

  model systems, genetics, development, etc.

- ***Easy!*** *– phone, chat, e-mail*