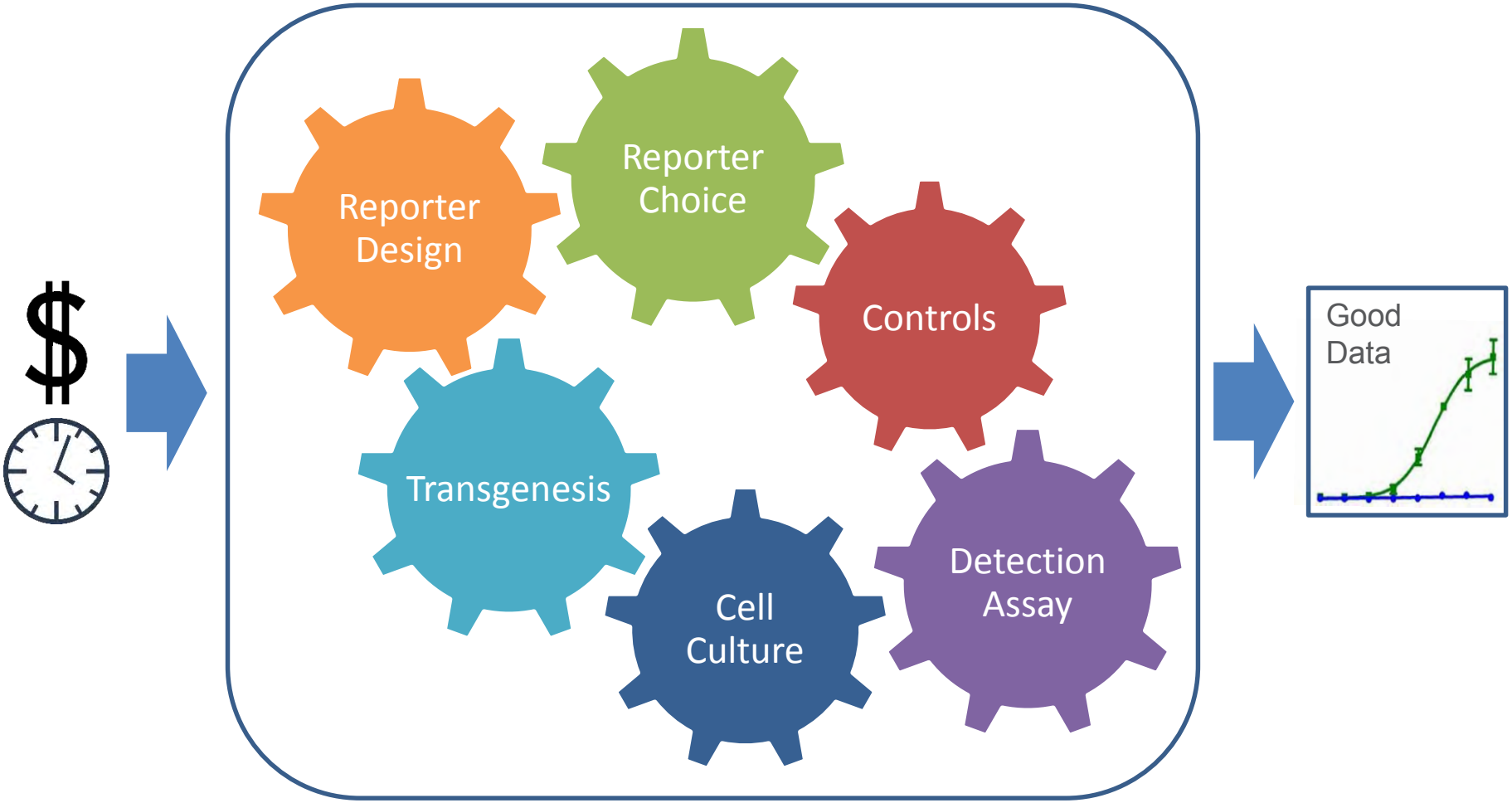
# Understanding Luminescent Reporter Assay Design

Carl Strayer, PhD

Promega Corporation

# Reporter Assay Design Considerations



# Reporter Assay Basic Concepts

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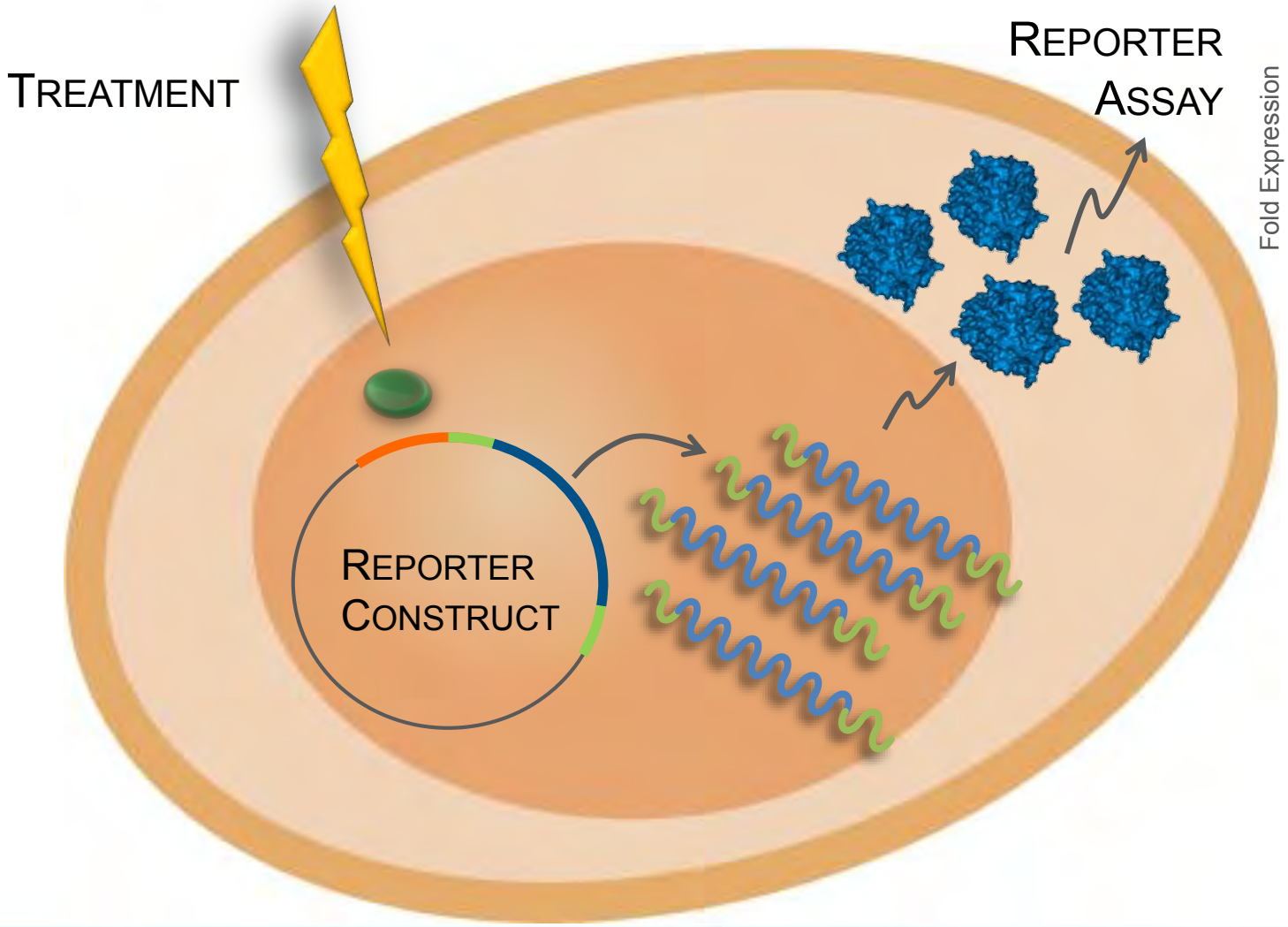
# What is a Reporter?

- ***Genetic reporters*** are ***indicators*** of ***gene expression*** *or* ***cellular events*** *coupled to gene expression*
- Reporters can mark ***any gene product***
  - **Transcriptional Fusion** – *reports on transcriptional and post-transcriptional regulatory inputs & events*
  - **Translational Fusion** – *reports on post-translational regulatory inputs & events*
- Reporters may be used in ***cells, tissues,*** *or* ***whole organisms***

# Basic Reporter Assay Principle



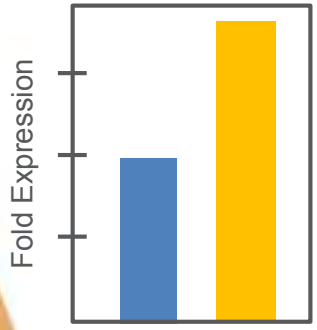
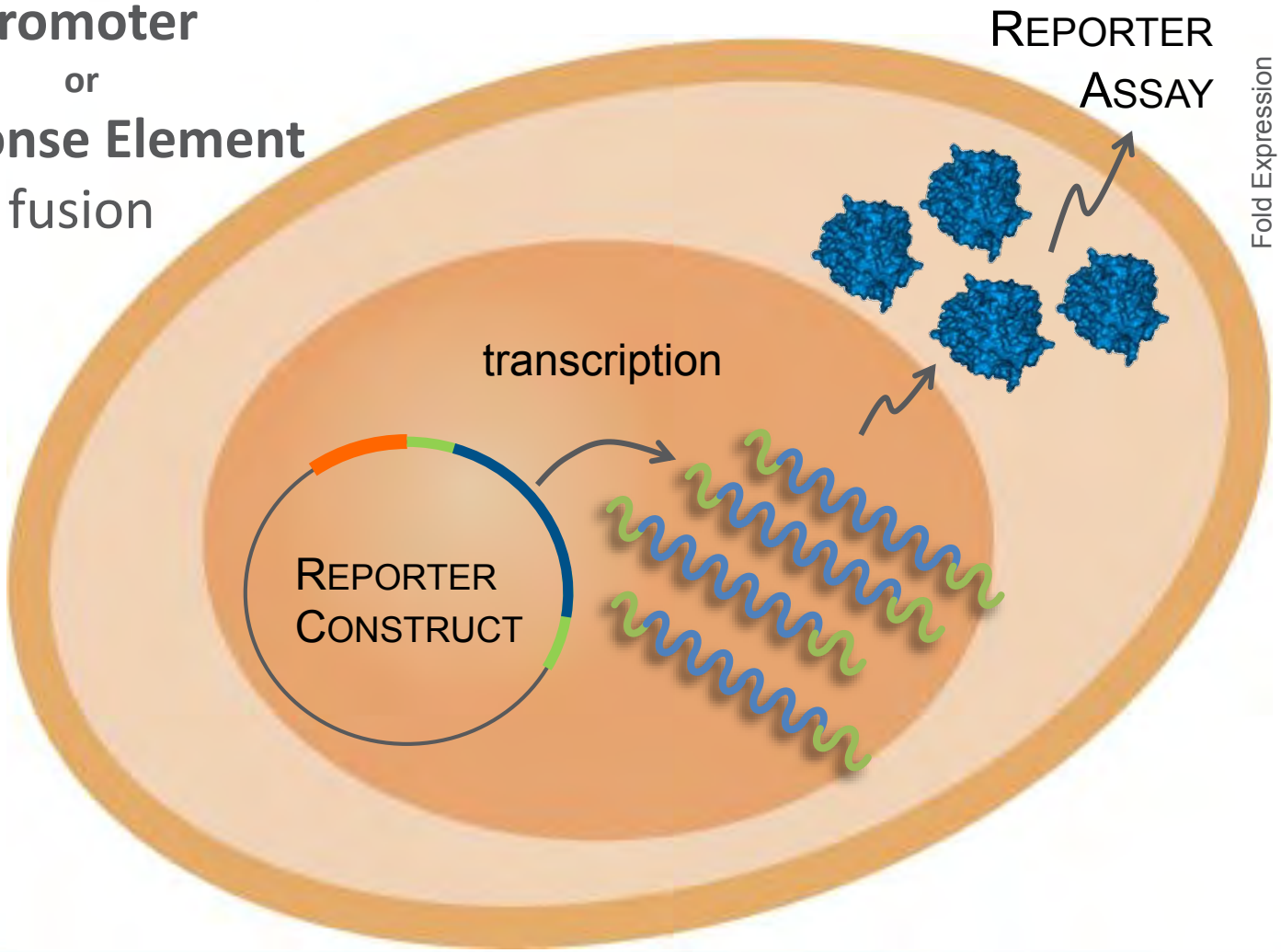




*Figure 1*

# Transcriptional Fusion to the Reporter Gene

**Promoter**
**or**
**Response Element**
fusion



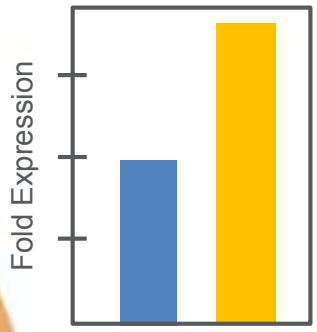




*Figure 1*

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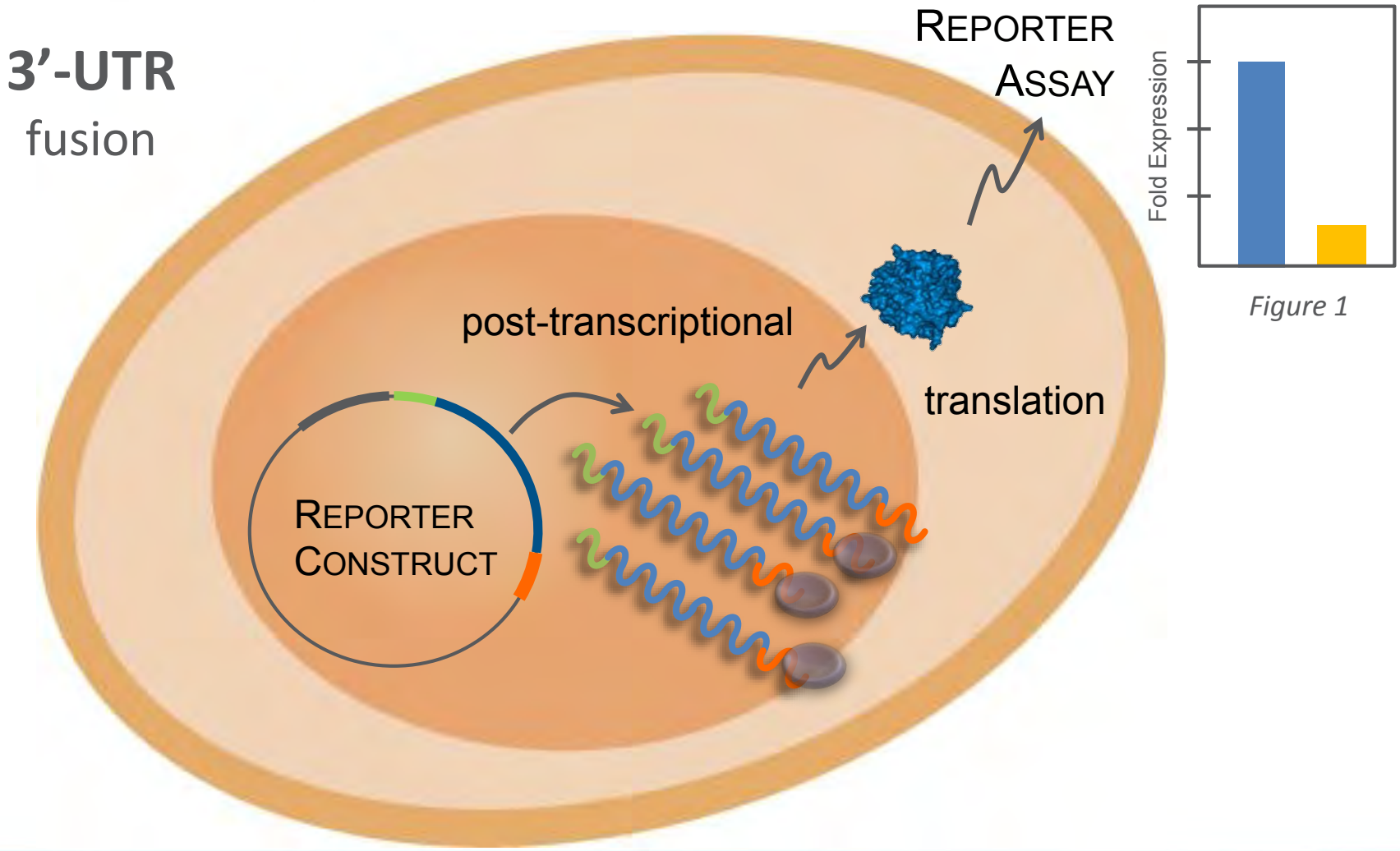
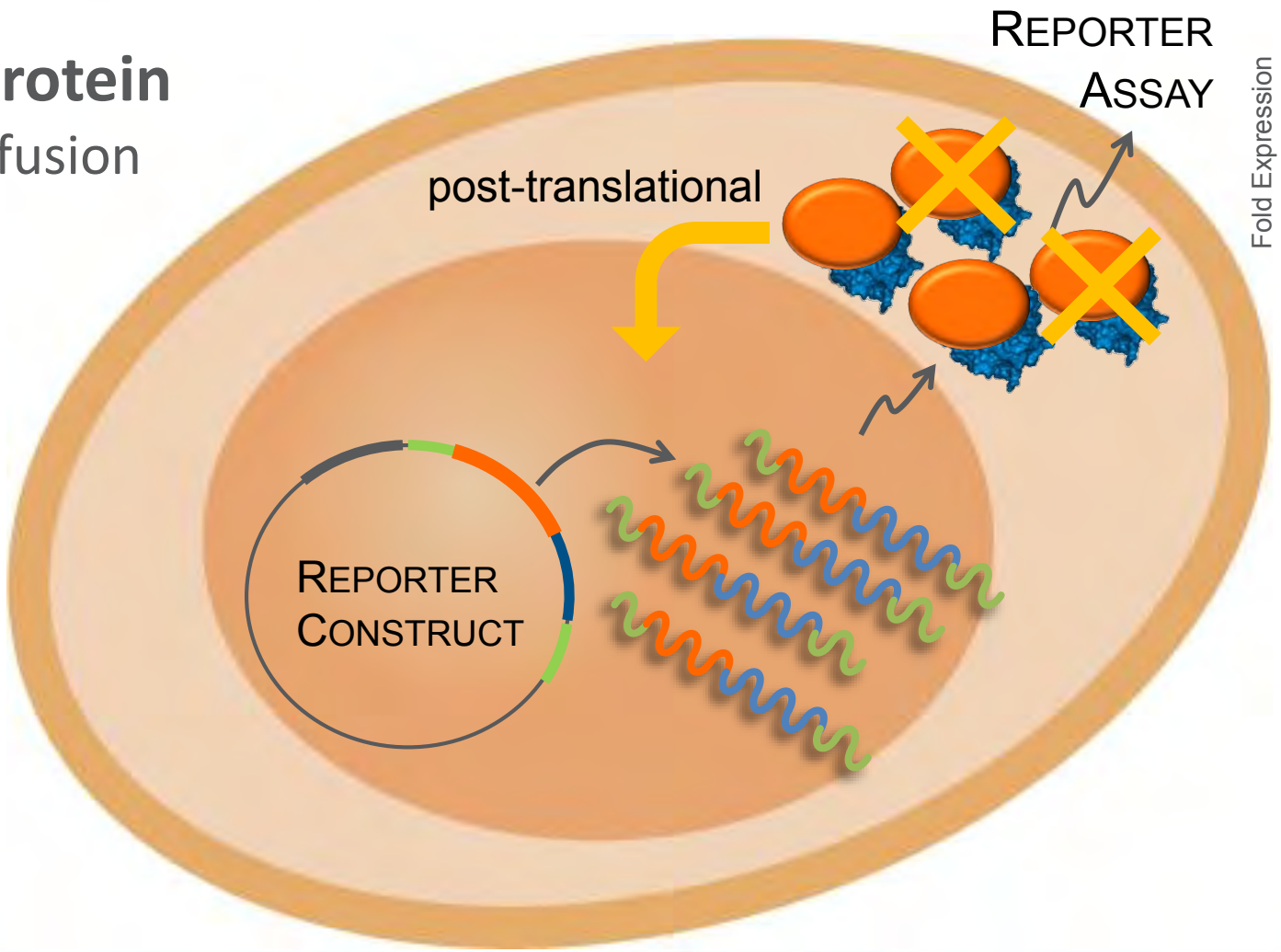



*Figure 1*

# Translational Fusion To The Reporter Gene

**Protein**
fusion



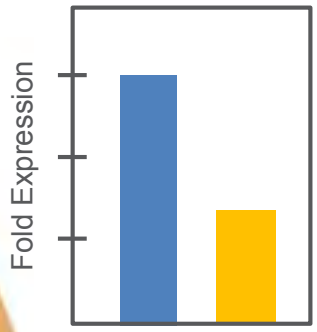




*Figure 1*

# Reporters Can Be Used For A Variety Of Applications

## *Gene expression*:

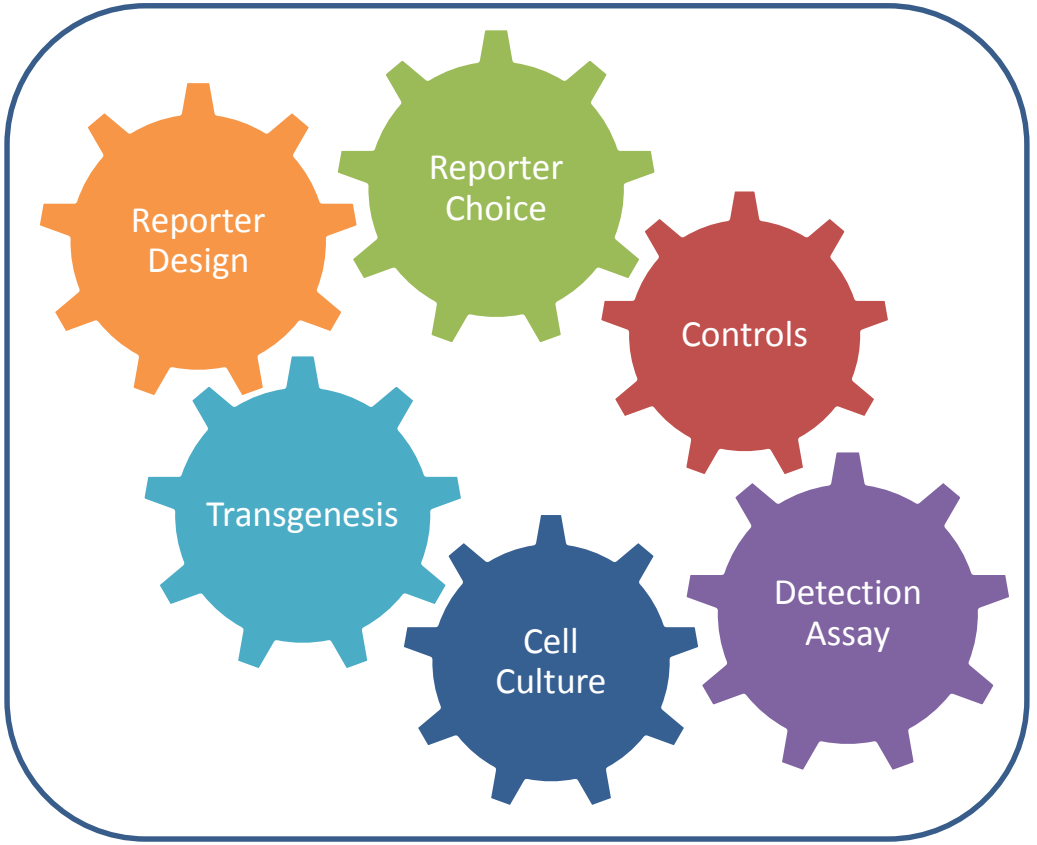
- **Transcription and post-transcriptional regulation**
  - Promoters/response elements
  - Enhancers
  - 5'- and 3'-UTRs
  - Transcriptions factors
  - RNA binding proteins & miRNAs
- **Post-translational regulation**
  - Protein stability
  - Protein localization
  - Protein:protein interactions

## *Cellular Events:*

- **Receptor activation/signaling**
  - Receptor ligands, agonists & antagonists
  - Nuclear receptors
- **Pathway analysis**
  - Defining pathways
  - Protein:protein interactions
- **Disease/Immune responses**
  - Cellular response to infection
  - Cellular response to therapy
  - Infectious agent replication/response to therapy

# Reporter Assay Design Considerations

## Reporter Choice
## &
## Reporter Design

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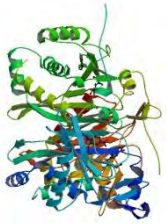
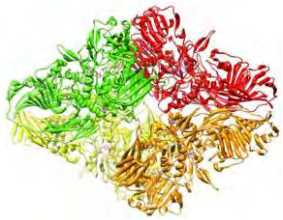
# Properties Of A Good Reporter

- Enzyme - *signal amplification*
- Active upon synthesis - *no processing or assembly*
- No endogenous analog (protein or substrate) – *low background*
- Convenient assay
- Quantitative, sensitive & wide dynamic range

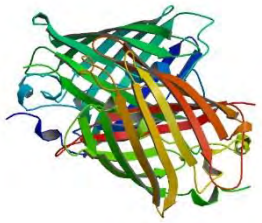
CAT



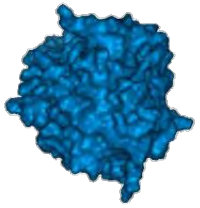
β-gal



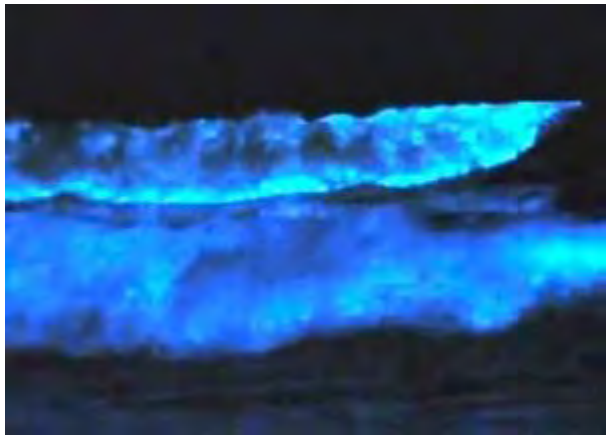
GFP



luciferase

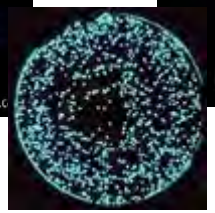


# Bioluminescence In Nature



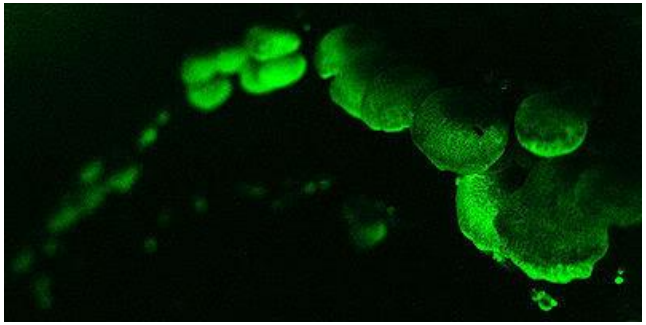
*Marine algae (Gonyaulax)*

*Lantern fish (Lampanyctodes)*



*bacteria (Vibrio)*

*Squid (Euprymna)*



*Mushroom (Armillaria)*

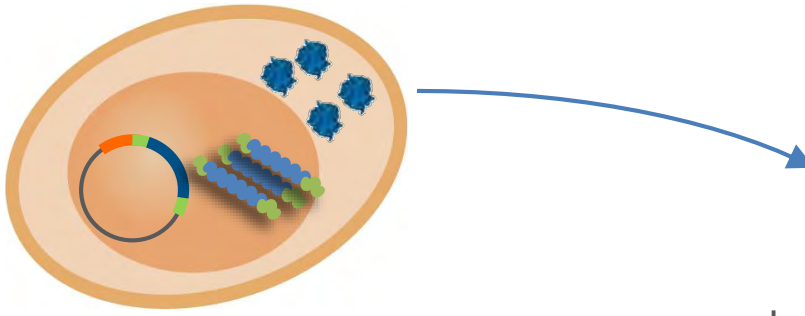
*Glow worms (Arachnocampa)*

*Jellyfish (Aequorea)*

# Bioluminescence In The Lab - Luciferase As A Reporter

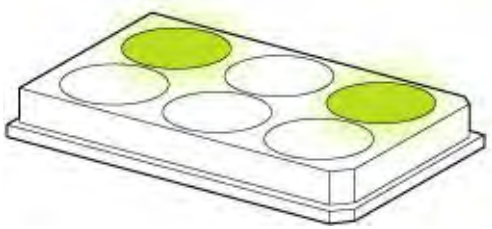
**Luciferase** is the generic name for the class of enzyme

*(the substrate is generically referred to as **luciferin**)*

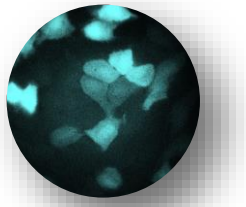


luciferin + $O_2$ —(luciferase, co-factor)→ Oxidized luciferin + LIGHT

Light signal is read by a **luminometer** or a **CCD camera**



**Lytic** *-OR-* **Live** Assay



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## ***Genetic Reporter***

*Reporter as a **transcriptional** fusion*

- Short protein half-life

  *maximally responsive*

## ***Protein Fusion Reporter***

*Reporter as a **translational** fusion*

- Small, compact protein
- Activity is minimally affected by fusion
- Protein half-life & localization determined by fusion partner

# Promega Optimized Luciferase Reporters

## Firefly luciferase - *Luc2*

*61 kDa, ATP-dependent enzyme; utilizes beetle luciferin (D-luciferin)*

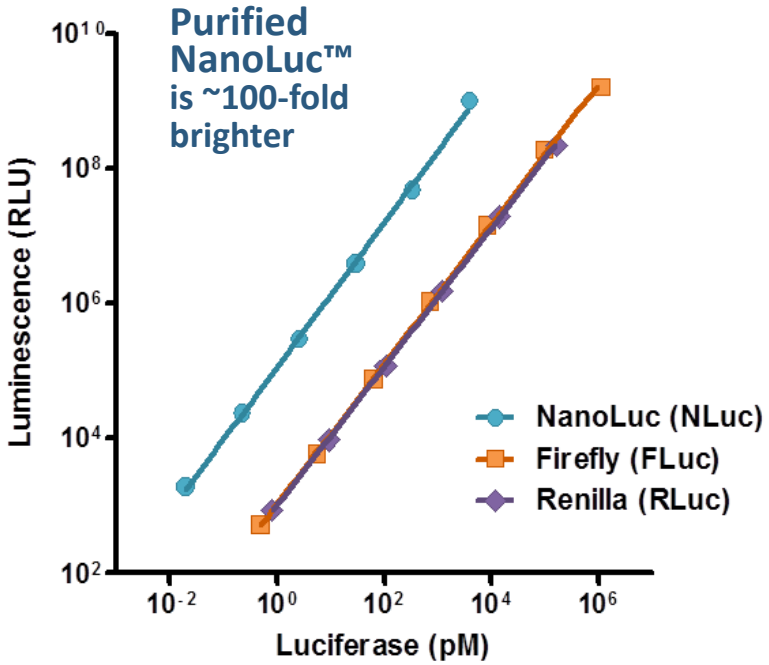
- Sequence optimized
  *codon utilization & "cleaned"*
- ~200-fold increased signal compared to native *luc* *per gene copy number*
- **Luc2** protein half-life ~ 3 hr
- Further destabilized version, **Luc2P**, ~1.5 hr protein half-life

## NanoLuc® - *Nluc*

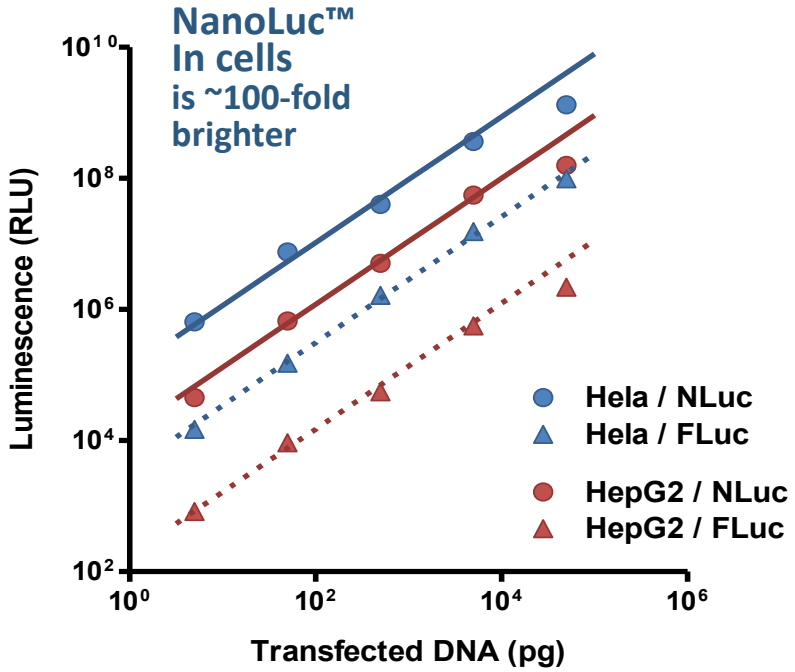
*19 kDa, ATP-independent enzyme; utilizes novel coelenterazine analog (furimazine)*

- Sequence optimized
  *codon utilization & "cleaned"*
- ***Further*** ~100-fold increase in signal compared to *luc/luc2* *per protein copy number*
- **Nluc** protein half-life >6 hr
- **NlucP** destabilized version, ~30 minute protein half-life

# Nanoluc® Provides The Brightest Signal, Best Sensitivity




Recombinant NLuc/Nano-Glo™ Assay
Recombinant FLuc/ONE-Glo™ Assay
Recombinant RLuc/*Renilla*-Glo™ Assay




CMV-driven NLuc/Nano-Glo™ Assay
CMV-driven FLuc/ONE-Glo™ Assay

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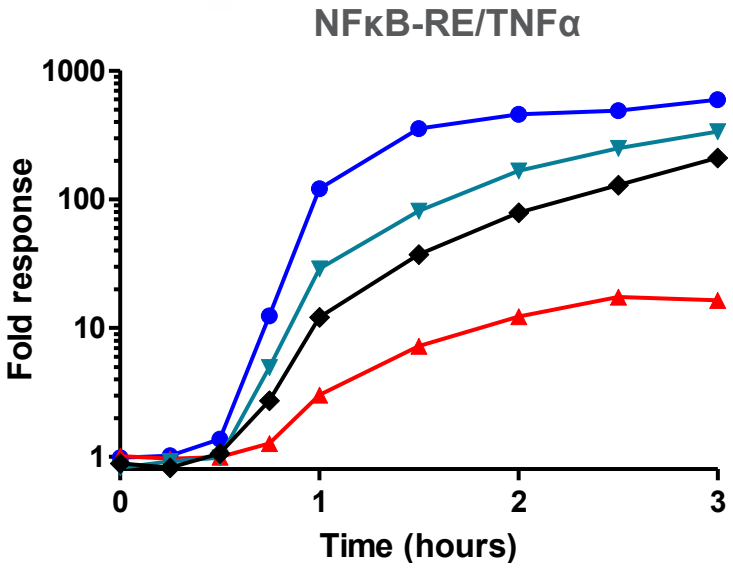
# Destabilized Reporters Gives The Best Dynamic Response




## Protein half-life

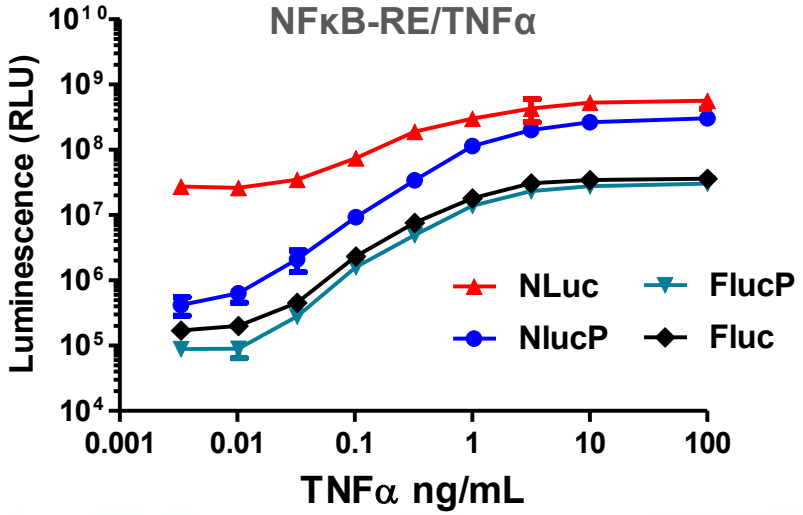
**Nluc > Fluc > FlucP > NlucP**

## Relative Response

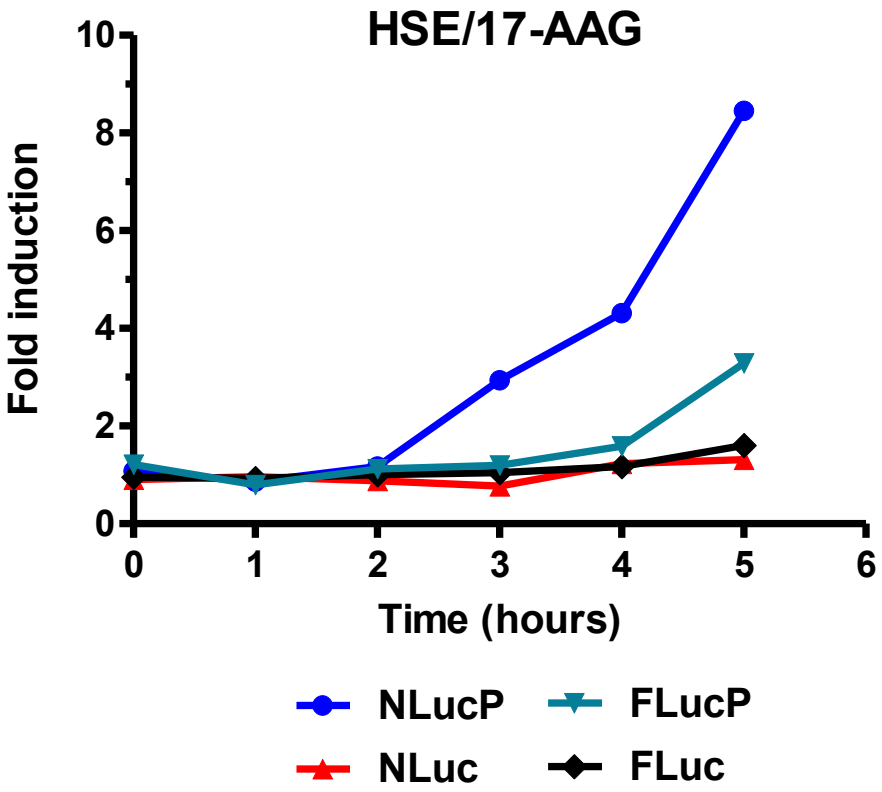
**NlucP > FlucP > Fluc > Nluc**

## Brightness/Sensitivity

**Nluc> NlucP > Fluc > FlucP**



# Nlucp Is Most Sensitive For Weakly Induced Responses




## 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.

# Summary Of Choices For Genetic Reporter

***New users – NanoLuc® is the best choice***

| Brightness/Sensitivity | Relative Response |
|---|---|
| **Nluc > NlucP > Fluc > FlucP** | **NlucP > FlucP > Fluc > Nluc** |

***If I'm already are using Firefly should I switch to NanoLuc?***

**Not necessarily – Fluc is still a *great* reporter!**

- ✓ Excellent signal:background
- ✓ Excellent dynamic range
- ✓ Great *in vivo* reporter
- ✓ Well developed system

**Yes if you are having problems with sensitivity!**

- ✓ Poor transfection
- ✓ Weak promoter
- ✓ Weak responses
- ✓ Detection limitations

**Note that Nluc and Fluc *CAN* be multiplexed as dual primary reporters**

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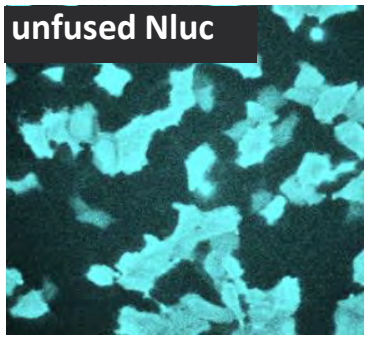
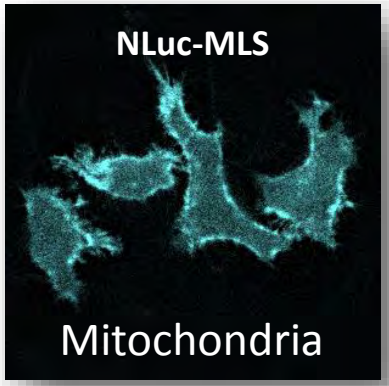
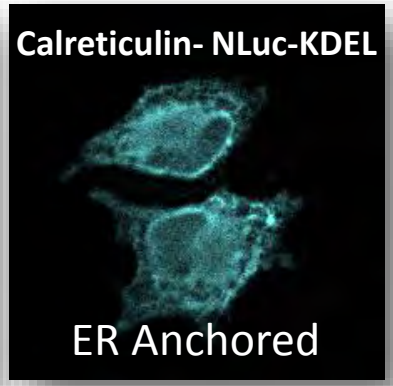
# Nanoluc® Is Localized Based On The Protein Partner

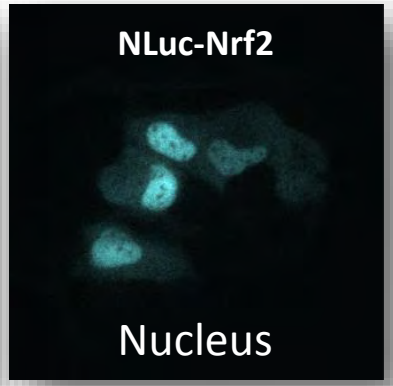


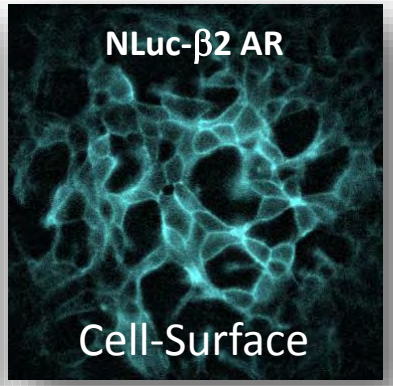

NanoLuc® by itself is uniformly distributed in cells

Fusion of NanoLuc to POI or localization tag confers expected spatial pattern



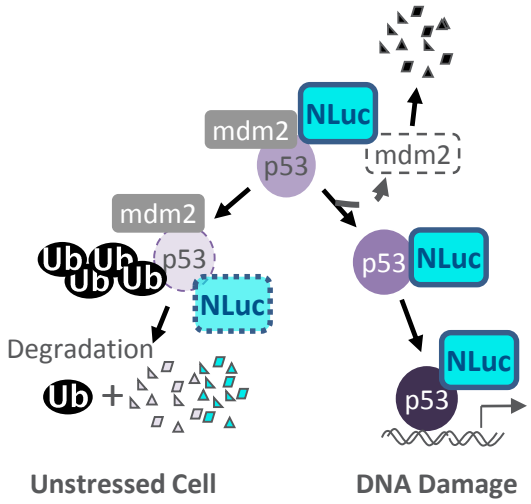



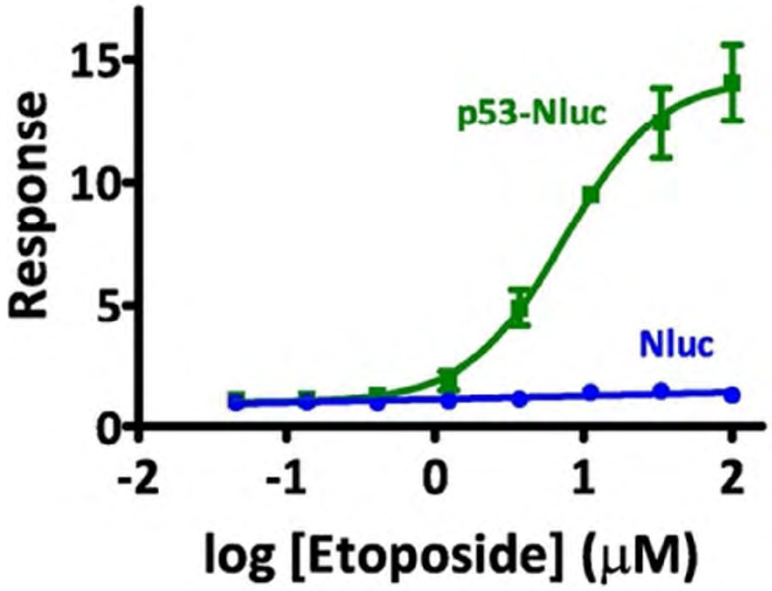



# Nanoluc® As A Protein Fusion Reporter

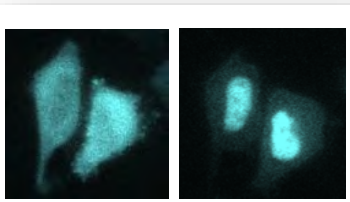
NanoLuc® fused to a protein-of-interest takes on the fusion partner's stability profile




p53 stabilization by MDM2

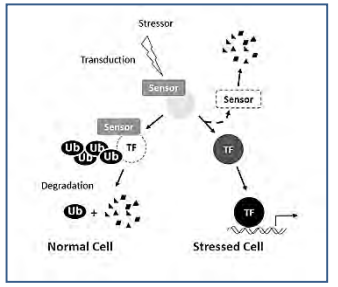


# Nanoluc® Is *The Best Choice* for a Protein Fusion Reporter

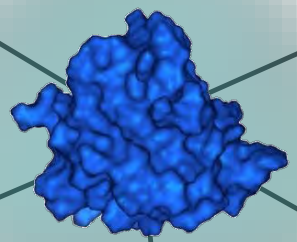


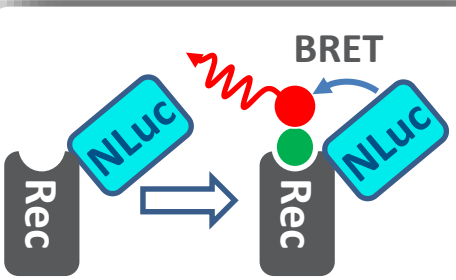
Protein
Re-localization

Protein
Stability

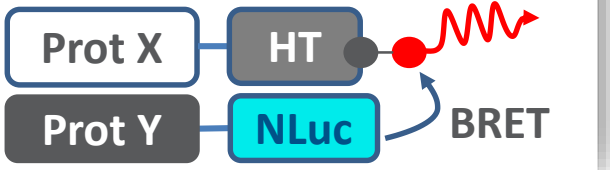








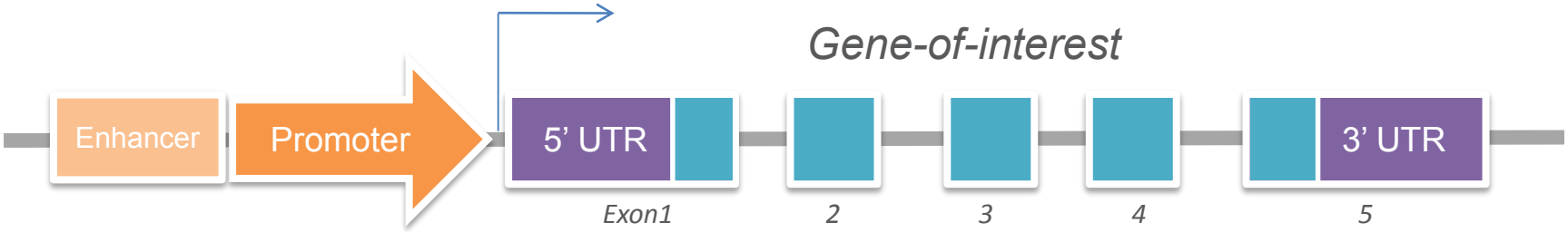

Receptor
Interactions




Protein-Protein Interactions

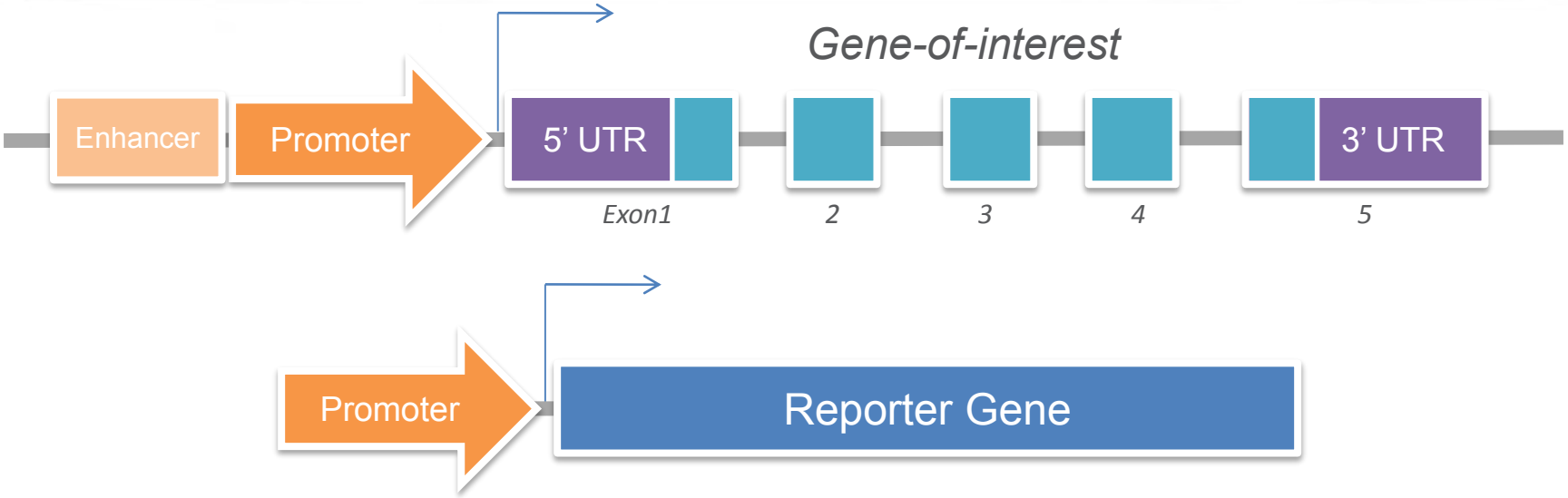

Biosensors

# What Elements To Include In A Genetic Reporter Fusion?




***Elements to include are guided by the experimental question***

# What Elements To Include In A Genetic Reporter Fusion?

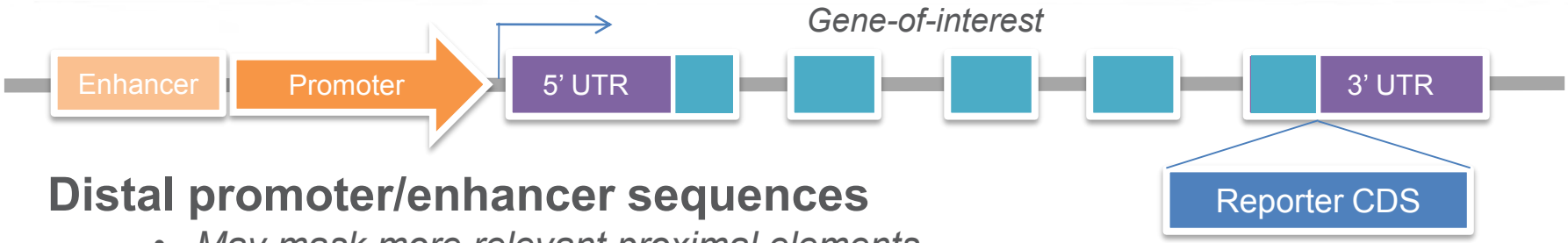



To study transcriptional regulation, you might include *only* the proximal promoter…

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


Response Element constructs more precisely define the experiment

# Including Other Elements From The GOI May Have Other Effects – *Intended Or Not*




## Distal promoter/enhancer sequences

- *May mask more relevant proximal elements*
- *Heterologous regulatory elements from adjacent genes may be captured*

## Untranslated regions (UTR)

- *May add post-transcriptional regulatory effects - change translation efficiency or mRNA stability*

## Intron

- *If spanning from promoter to 1st intron, necessitates inclusion of 5'UTR and possibly CDS - introduces related regulator considerations*
- *May introduce splicing artifacts*

## Coding sequence (CDS)

- *In-frame fusion introduces another level of regulation*
- *Out-of-frame insertion will greatly decrease reporter translation from start codon, and therefore decrease signal*

# Brighter luciferase reporter facilitates knock-ins

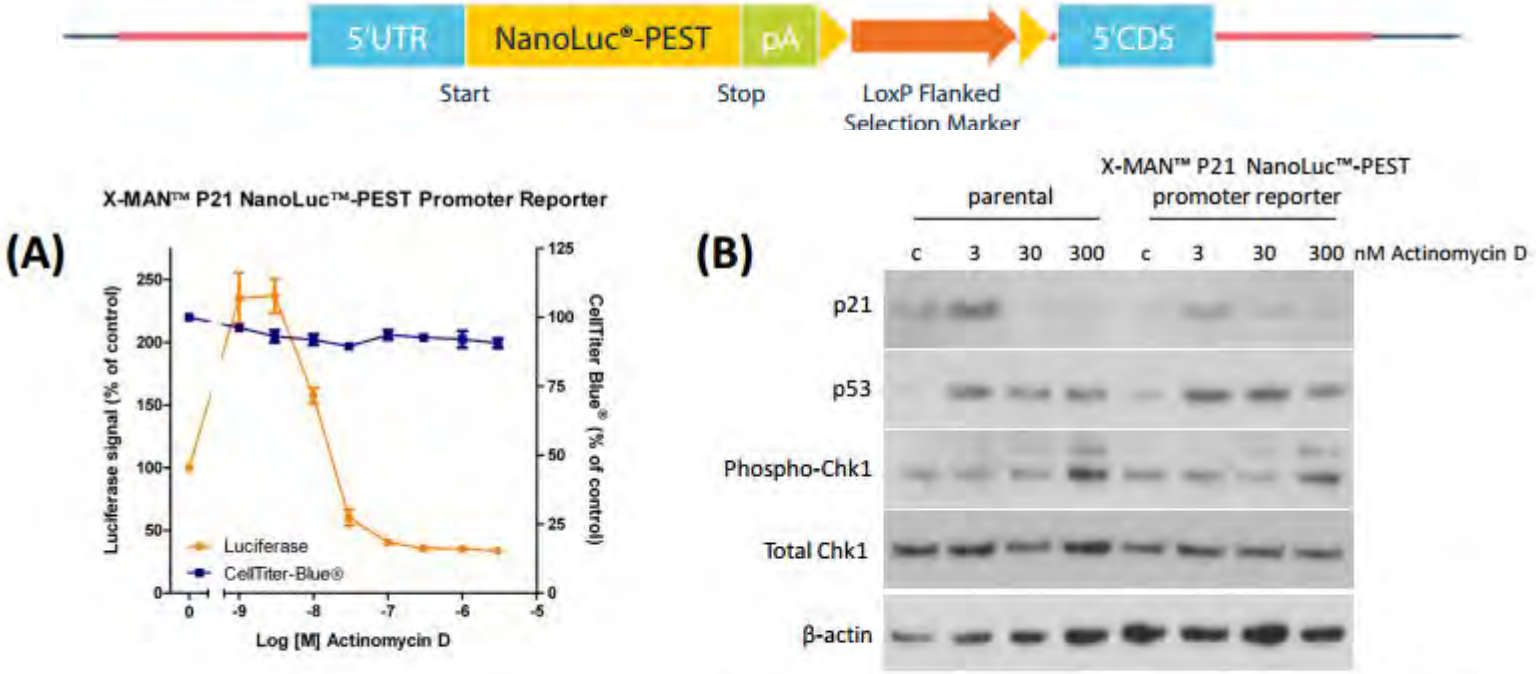



**Figure 4. Treatment of the X-MAN® P21 NanoLuc®-PEST promoter reporter cell line with actinomycin D induces a DDR at low doses.** The X-MAN® P21 NanoLuc®-PEST promoter reporter cell line was treated with actinomycin D for 6h. (A) P21 transcription was measured using Nano-Glo® luciferase and the assays were multiplexed with CellTiter-Blue® to give a measure of cell viability. (B) Western blotting confirmed the induction of a DDR, with results consistent between the unmodified parental and X-MAN® P21 NanoLuc®-PEST promoter reporter cell lines. Abbreviation: c, vehicle control.

horizon

# Reporter Assay Design Considerations

## Controls

*Background*

*Normalization*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# Controls To Determine Assay Background Are *Crucial*

Like any quantitative assay, you need to **determine the background in the assay to know if signal from experimental samples is significant**…

***Background signal is inherent in all instruments***

- ***Electrical noise*** *from current running through the detection device*
- *Varies between instruments; varies depending on gain/sensitivity setting*

***Background signal can be contributed by assay chemistry***

- *Coelenterazines have some chemiluminescence*
- *Varies depending on assay*

***Background Control – Process several replicates of samples without luciferase in them - untransfected cells, medium-only wells, or lysis buffer only – plus detection reagent***

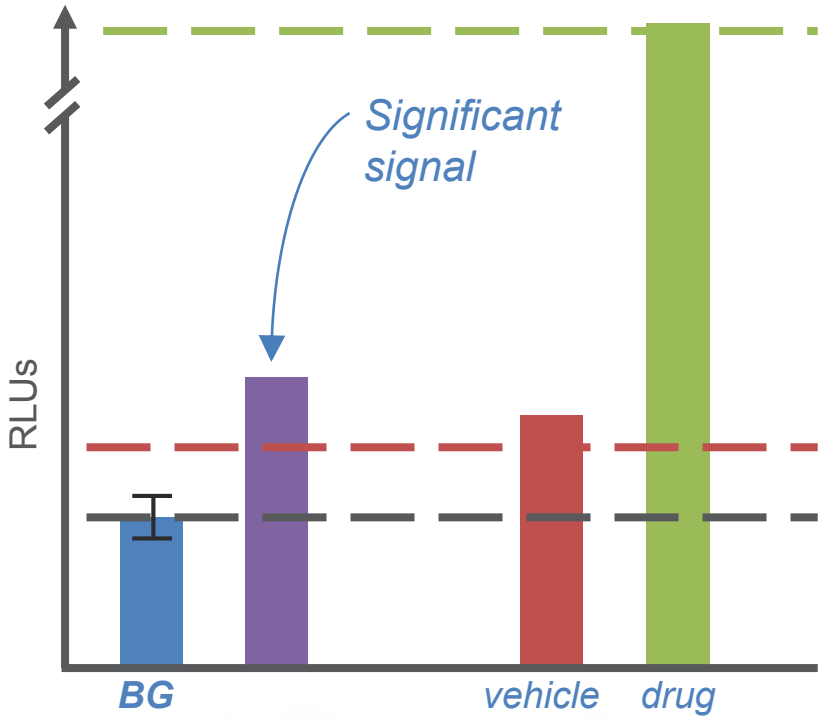
*(This is NOT the same as an untreated reporter control)*

*Background controls are not necessary in every experiment – Perform this control the first time you adopt a new assay or change detection parameters (instrument, gain setting, assay chemistry)*

# Example Of Determining Acceptable Assay Threshold

## Minimum Detectable Level (MDL)

- Measure signal for ***background controls*** (control sample without reporter, plus reagent)
- Determine average and standard deviation
- MDL would be Ave + 3×SD




Typical Signal is often $10^4$ to $10^6$

***Signal from ALL samples must be > MDL!***

MDL = 269

Average background = 200 +/- 23

***Replicates = 223, 189, 220, 166, 200***

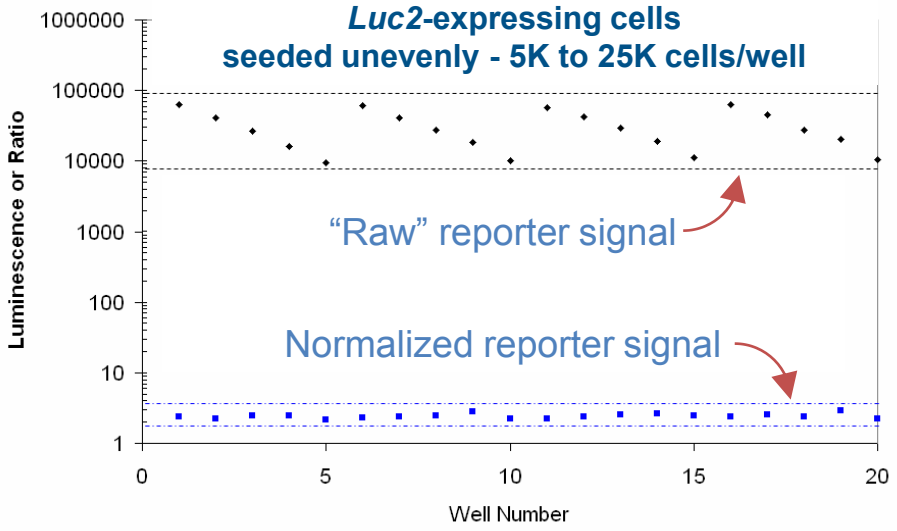
# Normalization Assay Is An Important Control

**Several parameters in a cell-based reporter experiment can cause variation or artifacts in reporter signal**

- **Starting cell number** - *pipetting variation, problems with clumping/dispersion*
- **Transfection efficiency** - *related to cell density*
- **Ending cell number** - *cytotoxic effect of treatment; detached cells lost in media transfers or washing steps*

## Normalization Methods:

- **Co-Reporter**
- **Cell Health Assay**
- **Protein Assay**



# Normalization Using A Co-reporter

***Co-reporter, or normalization reporter:***

- A second, **compatible reporter** gene is **co-transfected** with the primary reporter plasmid. Driven by a **"constitutive" promoter**
- Controls for **cell number** *AND* **transfection efficiency**. Can also serve as a control for **specificity of effect**
- Measured along with primary reporter using a **dual reporter assay**

***When is a co-reporter less important?***

- **Repeat measures assay (timecourse, live assay)**
  Variation in transfection efficiency & starting cell number less important
- **Stable transgenic reporter cell line**
  No variation in transfection efficiency; Variation in seeding density can still be an issue

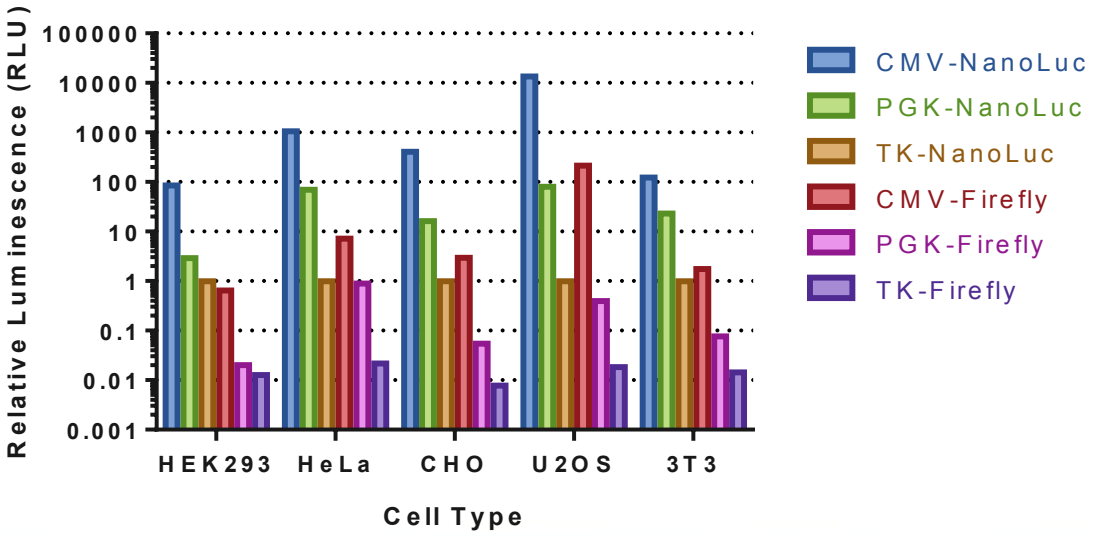
* *Cell Health Assay still may be advisable to control for variation in seeding density & cytotoxicity*

# Co-Reporter Options

- ***Renilla*** luciferase (***Rluc***) is often used as a control with ***Fluc*** as the primary reporter. Use **Dual-Glo®** or Dual-Luciferase® Reporter (**DLR**) assay.
- ***Fluc*** and ***Nluc*** can be combined – either can be used as the primary or co-reporter. Use **NanoDLR®** assay.
- **TK, SV40, CMV, PGK promoters** are most often used. ***TK*** *or* ***PGK*** *are good default choices. Provide low-level expression, least likely to be affected by treatments*.
- **Reporter vector ratios**

  ***(primary:co-reporter)***

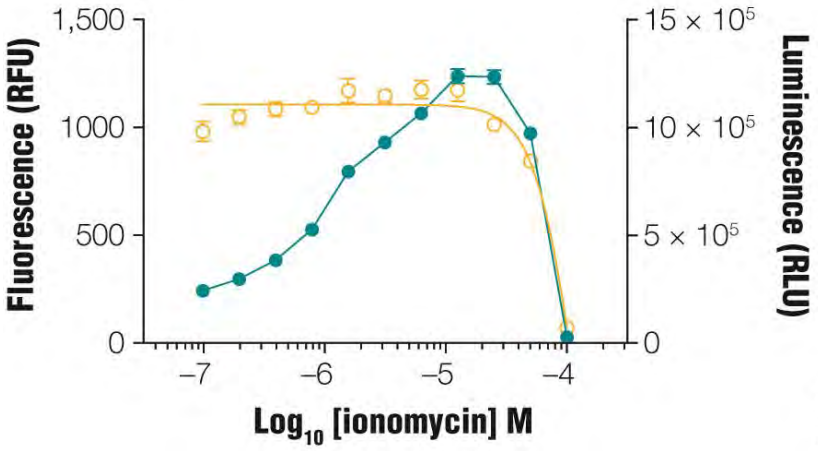
  - **10:1 to 100:1** is typical
  - Depends on promoters and reporters used, etc



# Normalization Using A Cell Health Assay

## *Cell Health Assay*

- Measures cell **viability** &/or **cytotoxicity**
- **Controls for cell number *only***
- Measured using a **compatible assay multiplexed with the reporter assay.** *Sequential assay in same plate. Fluorescent, non-lytic viability assay, performed upstream of reporter assay*
- **Examples:**
  - **CellTiter-Fluor™**
  - **CellTox-Green™**



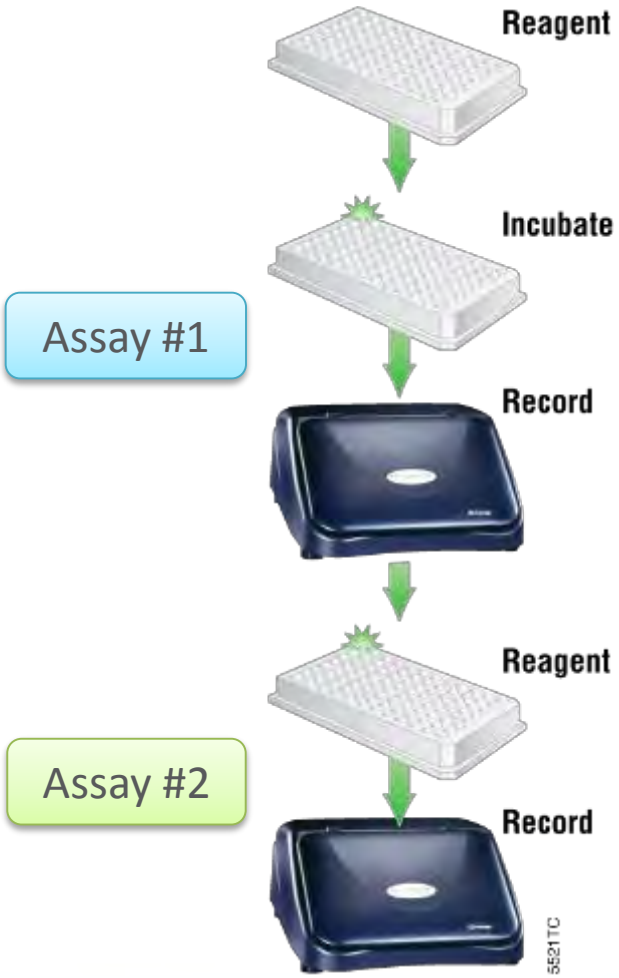

***Luc2*-reporter with Bright-Glo reporter assay multiplexed with CellTiter-Fluor viability assay**

# Multiplexing is…

Gathering more than one set of data from the same sample

*Multiplexing requirements:*

- Assays must be biologically & chemically compatible
- Signals must be spectrally distinct
- Assays must fit in the available volume of the well



# Reporter Assay Design Considerations

## Cell Culture & Transgenesis


Promega Corporation

# Cell Culture Considerations

## Cell confluence

- *Pre-confluent cultures generally best for transfection…*

  *…however, cells may become confluent by the time of treatment*

  – *Pre- and post-confluent cells may have different metabolic states – and respond differently to treatment*

  – *Cell density per se can influence response to treatment*

## Passage number

- *Use a **low passage number** and **minimize variation in passage number** used for experiments*

  *As passage number increases, cells may change character:*

  – *Differences in transfection efficiency*

  – *Differences in response to treatments*

- Consider bulking up a low passage, freeze aliquots, thaw for each experiment

# Transfection – Reporter Gene Dose

*It may be necessary to **titrate** the amount of **reporter gene transfected** – ideally, **to approximate physiological levels.***

***Excessive expression may**…*

- *Overwhelm endogenous regulatory factors or protein partners*
- *Produce a high basal signal that masks small changes in expression*
- *Cause ectopic effects of protein fusion - localization, stability, PTM, protein-protein interactions, etc.*
- *Excess normalization reporter may interfere with expression of the primary reporter*

*To keep the **total DNA mass constant** use a **transfection carrier DNA***

*Transfection carrier DNA* = a standard cloning plasmid, e.g., pGEM, pUC, etc. Avoid a plasmid that has eukaryotic promoters or expression cassettes, e.g., pcDNA3.1. Eukaryotic sequences could interfere with the experiment (e.g., compete for cellular transcriptional machinery).

# Reporter Assay Design Considerations

## Detection Assay


Promega Corporation

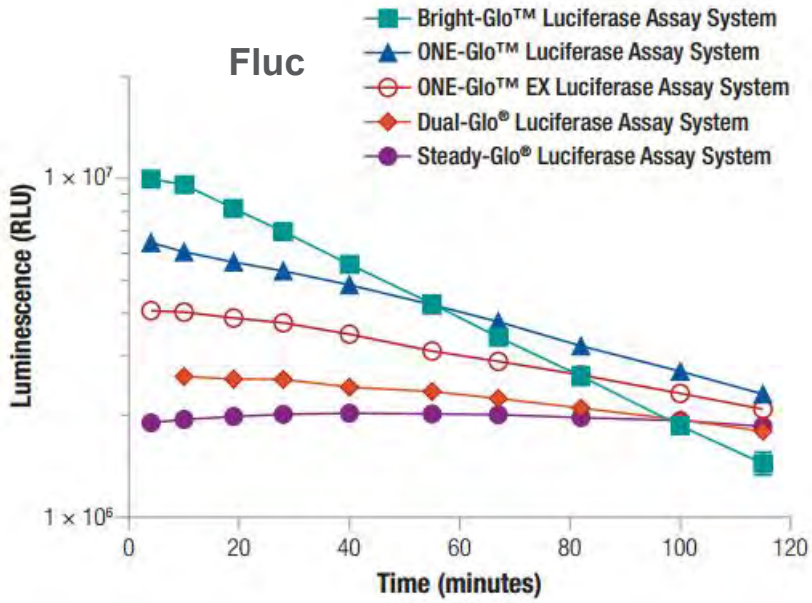
# Reporter Assay Choice – Lytic Assays

For most cell-based, plate-based reporter experiments a lytic, endpoint assay is the best choice:

- 96-well plate a **homogenous, Glo** assay is best – Bright-Glo® or Nano-Glo®
- Low-density plate you'll need to **make a lysate** – you can still use that lysate with a Glo assay.

**Considerations**:

- *Signal brightness*
- *Signal duration*
- *Process # steps; Injectors?*
- *Single vs Dual assay*



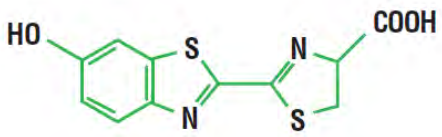
# Lytic vs Live Reporter Assay

| | Lytic | Live |
|---|---|---|
| Sensitivity | +++ | + |
| Repeat-measures | ✗ | ✓ |
| Preserves sample *(propagation)* | ✗ | ✓ |
| Multiplex-capable | ✓ (upstream) | ✓ (upstream or downstream) |
| Compatible with plate-reader | ✓ | ✓ |
| Imaging-compatible | ✗ | ✓ |

# Reporter Assay Choice – Live Assays

## ***Firefly luciferase assays***

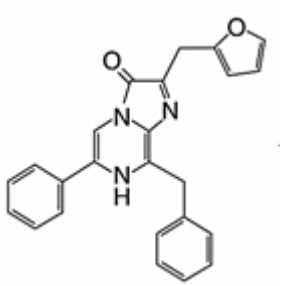
- beetle luciferin, Luciferin-EF, VivoGlo™ Luciferin
- *Chemiluminescence is not an issue*



*D-luciferin*

## ***NanoLuc® luciferase assays***

- Nano-Glo® Live Cell Assay System
- *Chemiluminescence can be a consideration*



*furimazine*

**Substrates** are reasonably **soluble, cell-permeable & stable**

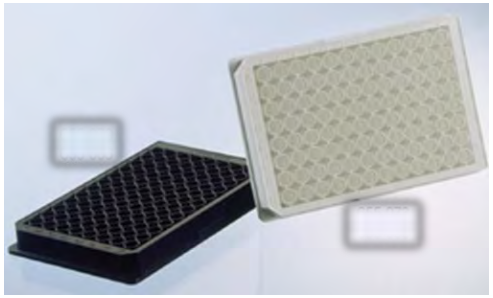
**Several options for timing of addition** - Add substrate to culture at beginning (seeding or media change, with drug, or at end of experiment

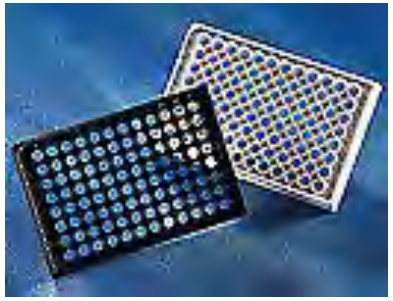
# Luminometer Parameters & Plate Choice

## Luminometer

- **No filters or wavelength setting!**
  - *No need to block excitation light*
  - *Filters reduce signal*
- **0.5 - 1 sec integration for Glo assays**
  - *Increasing integration won't increase sensitivity*

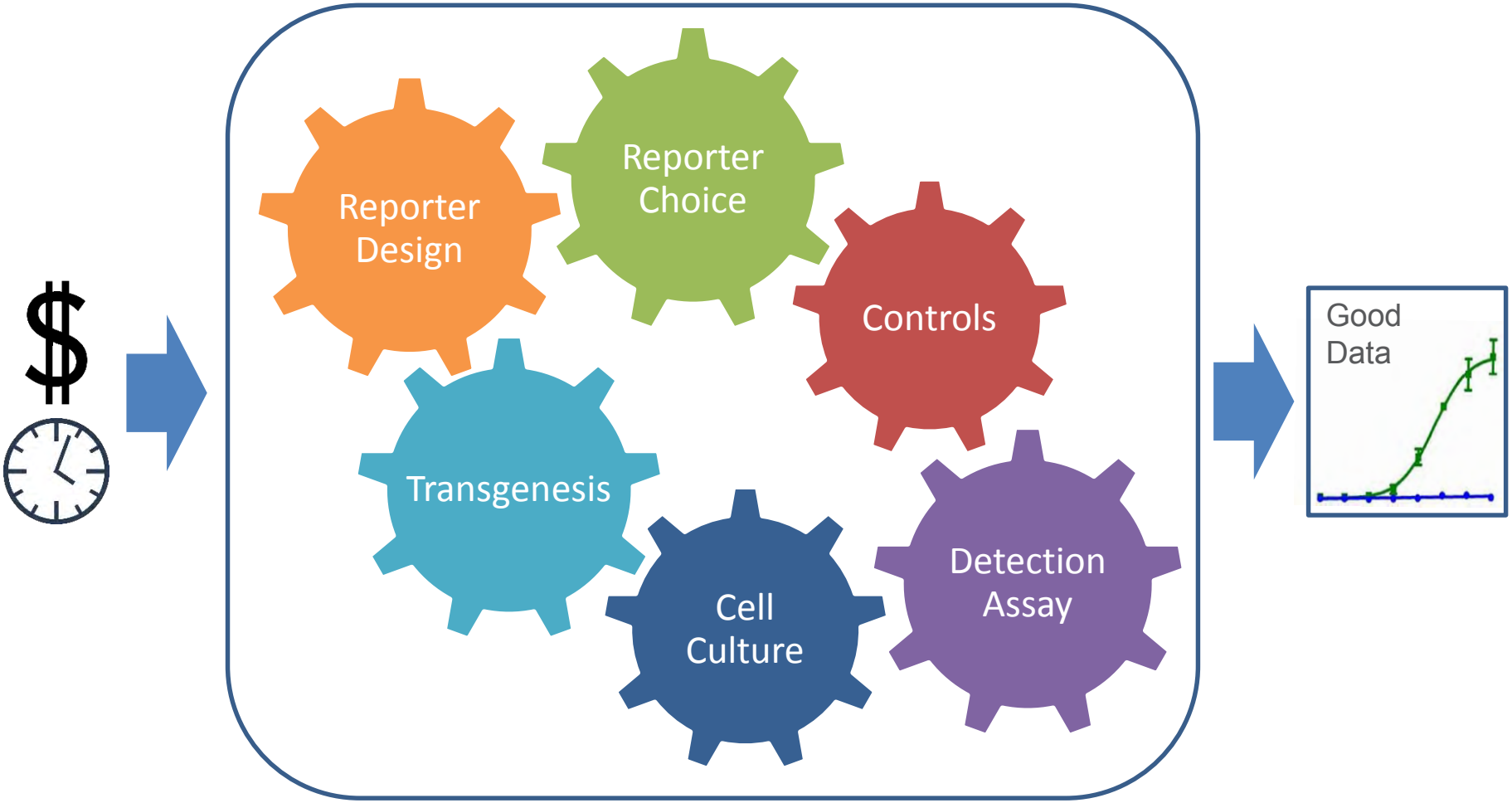
## White or Black Plates? Solid or Clear-bottom?





- **White** is preferred – better signal
- **Black** eliminates cross-talk
- **Clear-bottom** plates can be used for viewing cultures prior to assay
- **Opaque** plates gives better signal (for white), and less cross-talk (both W or B)

# Reporter Assay Design Considerations



# Questions Welcome