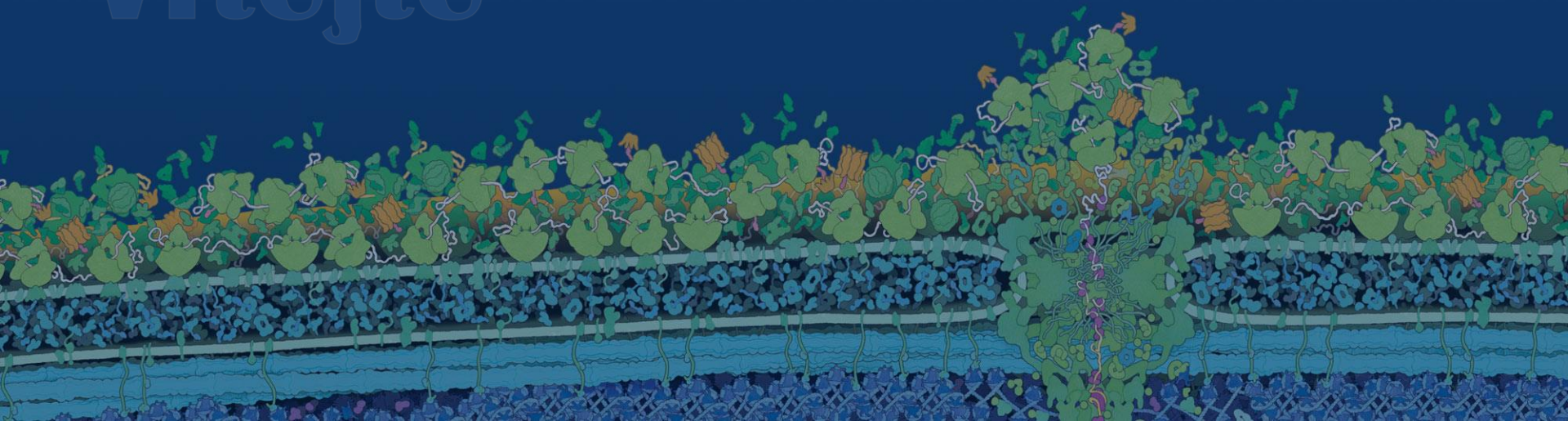
Luciferase Reporter Assays

Illuminating Cellular Protein Biology

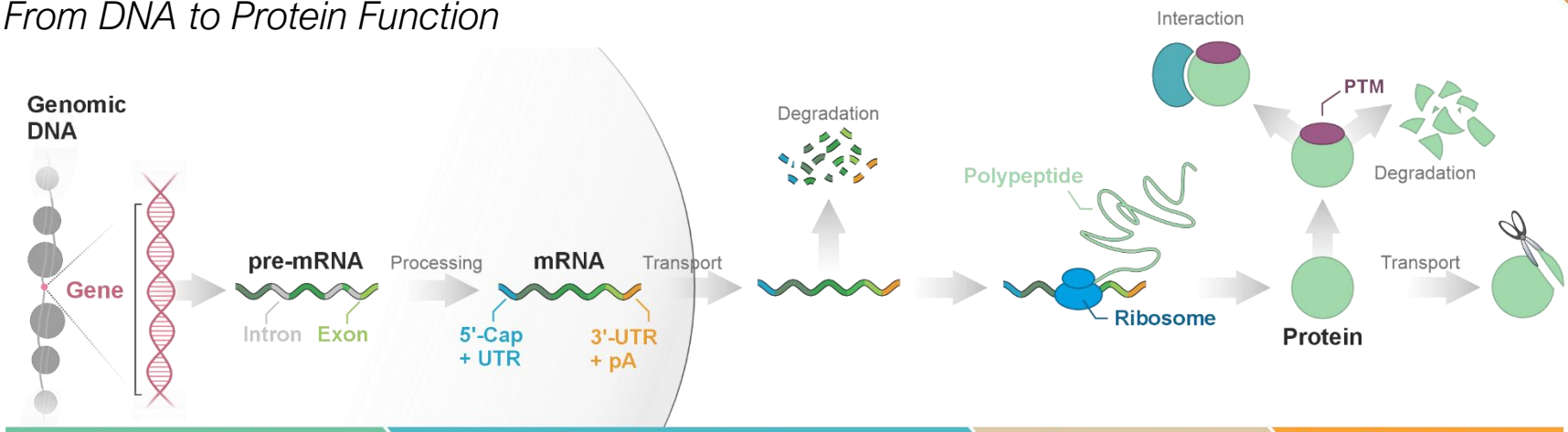
Vítejte

Dr. Erik Bonke | Sr. Sci. Application Specialist | Promega Germany



Regulation of Gene Expression at Multiple Levels

From DNA to Protein Function



TRANSCRIPTIONAL REGULATION

- Cis genetic regulation (enhancer/silencer)
- Trans genetic regulation (activator/repressor)
- Epigenetic regulation (methylation, acetylation)

POST-TRANSCRIPTIONAL REGULATION

RNA processing/stability

- Capping
- Splicing
- Polyadenylation
- Cleavage/Degradation miRNA (siRNA, shRNA)

TRANSLATIONAL REGULATION

- poly-A tail variation

POST-TRANSCRIPTIONAL REGULATION

- Chemical modification
- Proteolytic modification

change of specific activity
change amount of protein (synthesis & degradation)

Promoter — Reporter

- + ~1kb upstream of promoter & the transcriptional start site (+1)

RE RE RE miniP — Reporter

- Response Element (RE) constructs more precisely define the assay
- Constructs need in addition a minimal promoter (TATA-Box)

Promoter — 5'-UTR — Reporter

- integrate target sequence, 5'- or 3'-UTR

Promoter — Reporter — Target Seq si/miRNA

- integrate target sequence or 3'-UTR downstream of the reporter gene
  - suitable vectors: pmirNanoGLO (miRNA) or psiCHECK™ (siRNA)

Promoter — GOI — Reporter

- Translational fusion

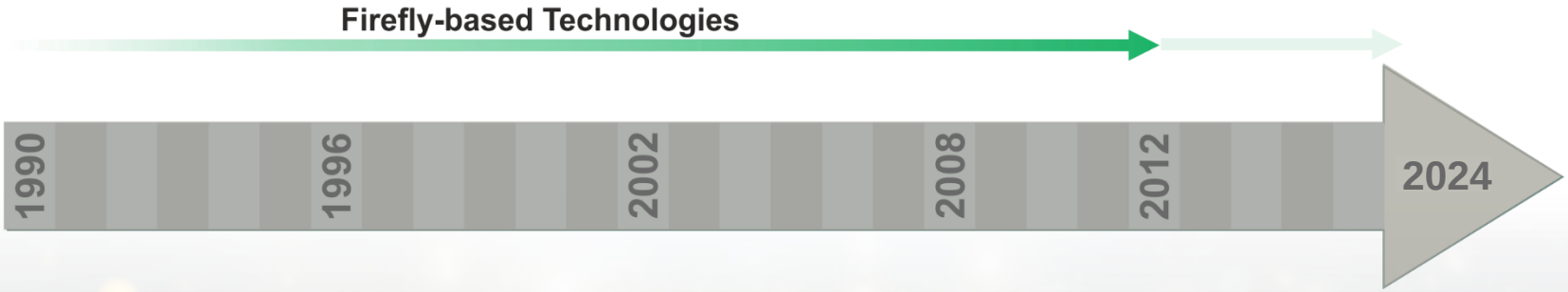
Promoter — Reporter — GOI

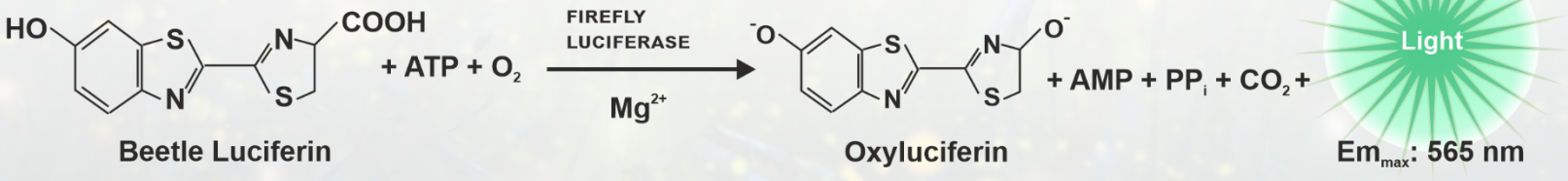
- add protein coding sequence C- or N-terminal in frame with reporter

cloned element

Promega – The Bioluminescent Company

A Continuously Grown Expertise in Luciferase-based Technologies





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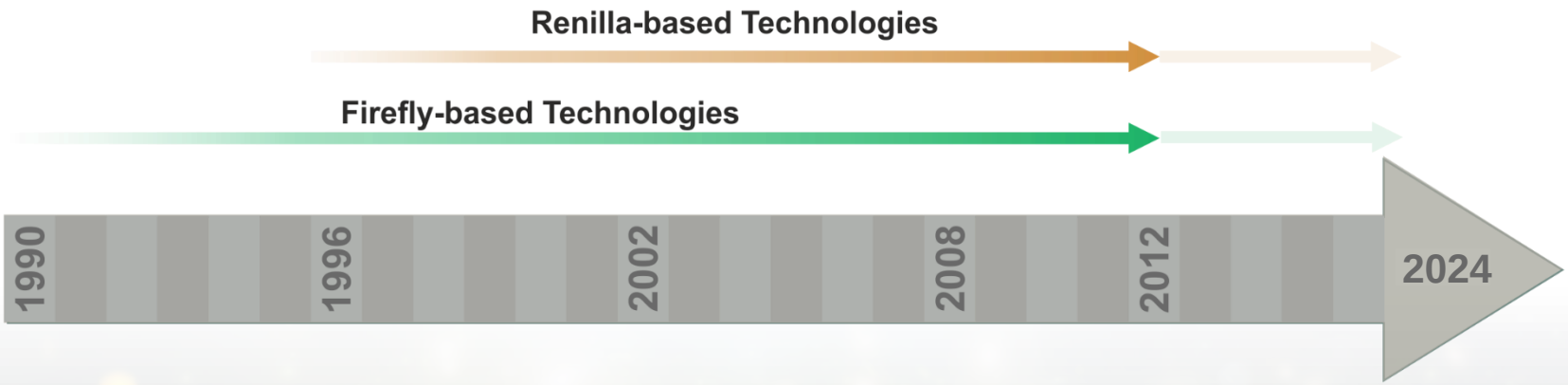

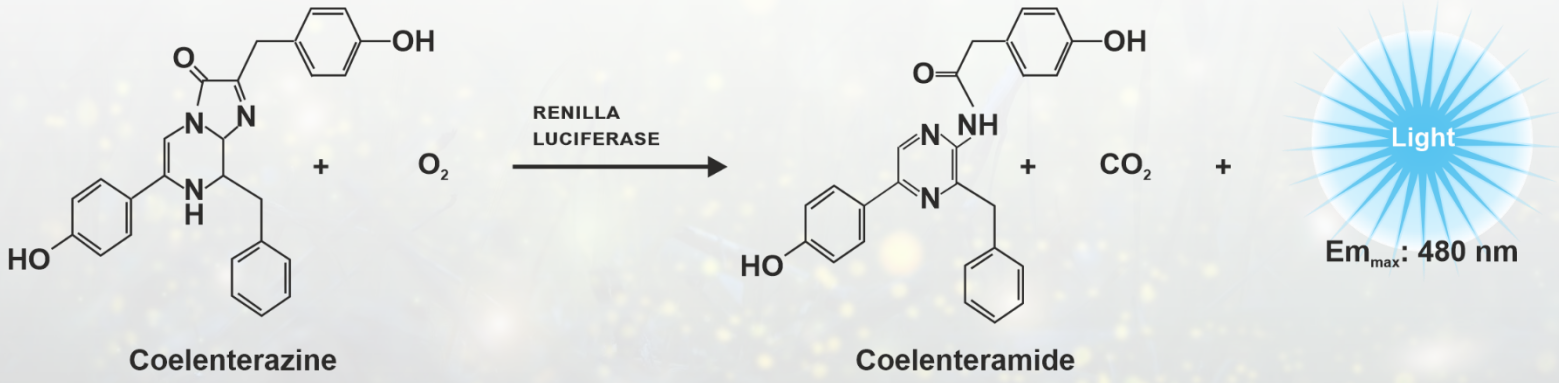

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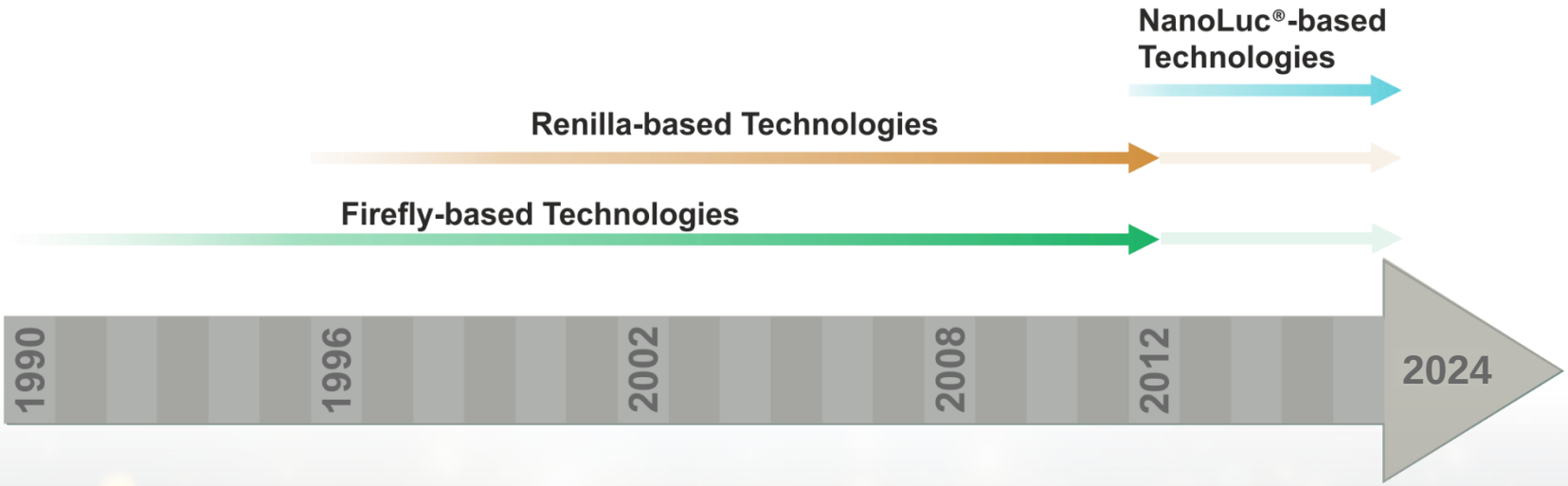
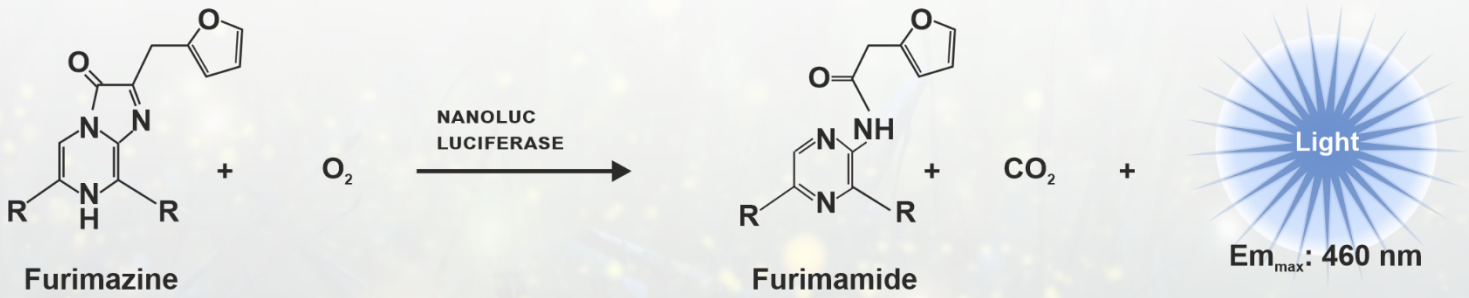
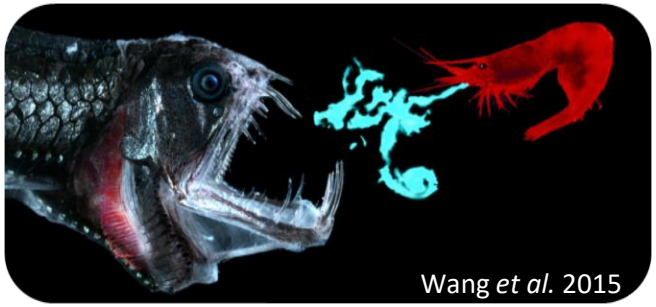


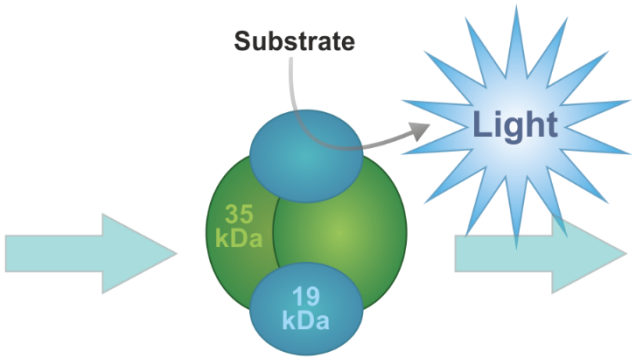


NanoLuc® Luciferase

A Bright & Small Experimental Reporter

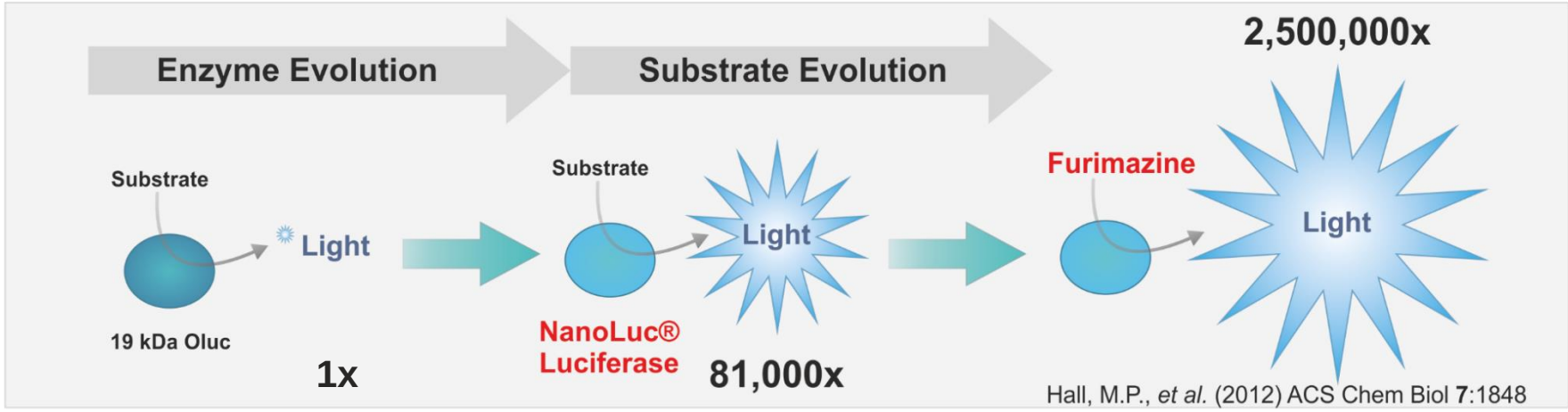


Oplophorus gracilirostris



- *Oplophorus* luciferase 106 kDa
- 7x brighter than Rluc
- Glow luminescence
- Shimomura *et al.* 1978

- Catalytic subunit 19 kDa
- Light output & stability compromised
- Inouye *et al.* 2000 and 2007

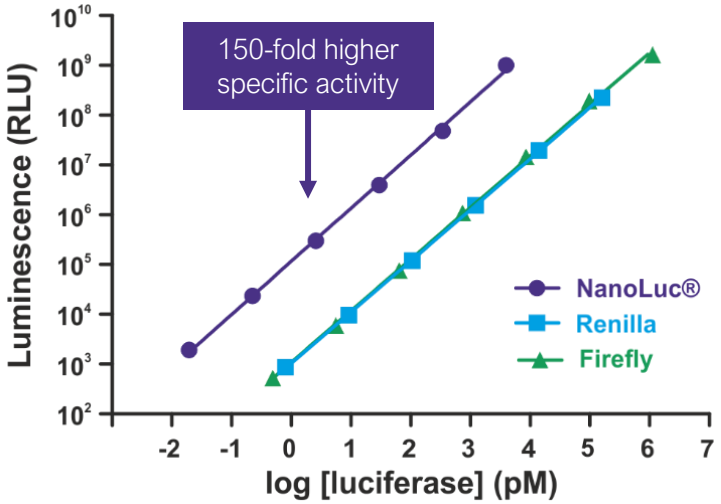


Hall, M.P., *et al.* (2012) ACS Chem Biol **7**:1848

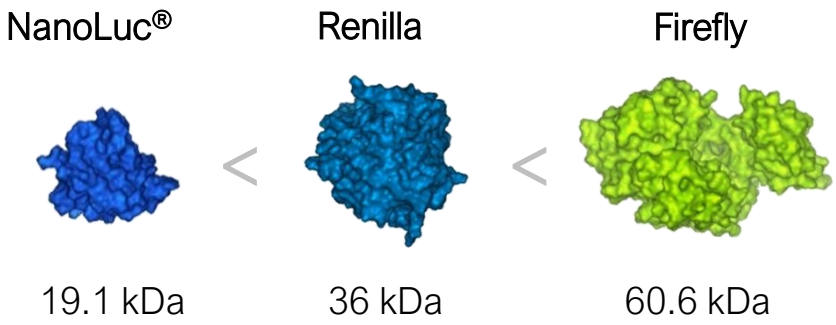
NanoLuc® Luciferase

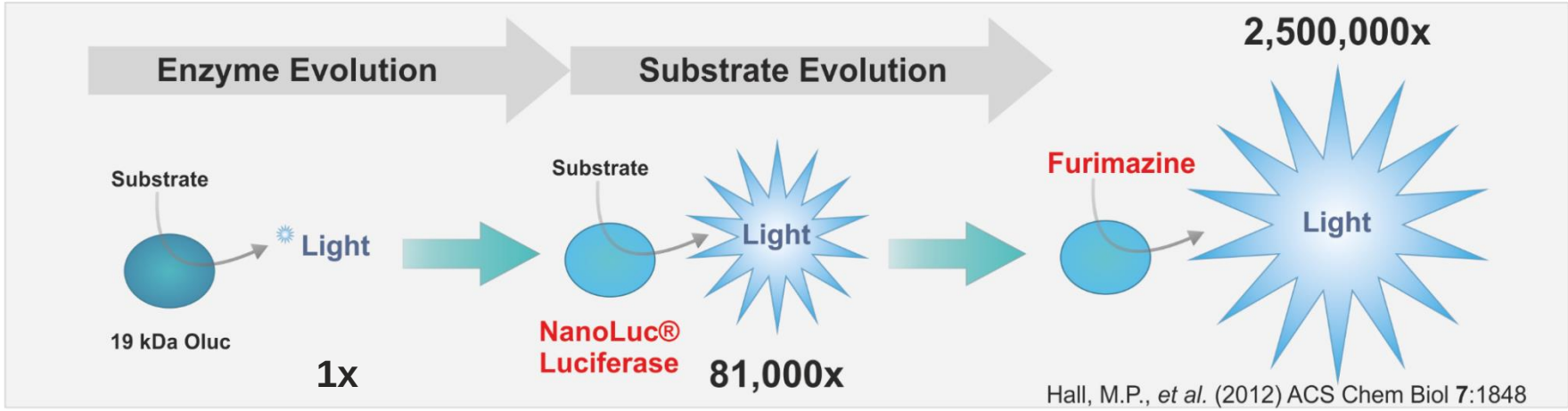
A Bright & Small Experimental Reporter

Bright, Brighter, NanoLuc®



Small, Smaller, NanoLuc®





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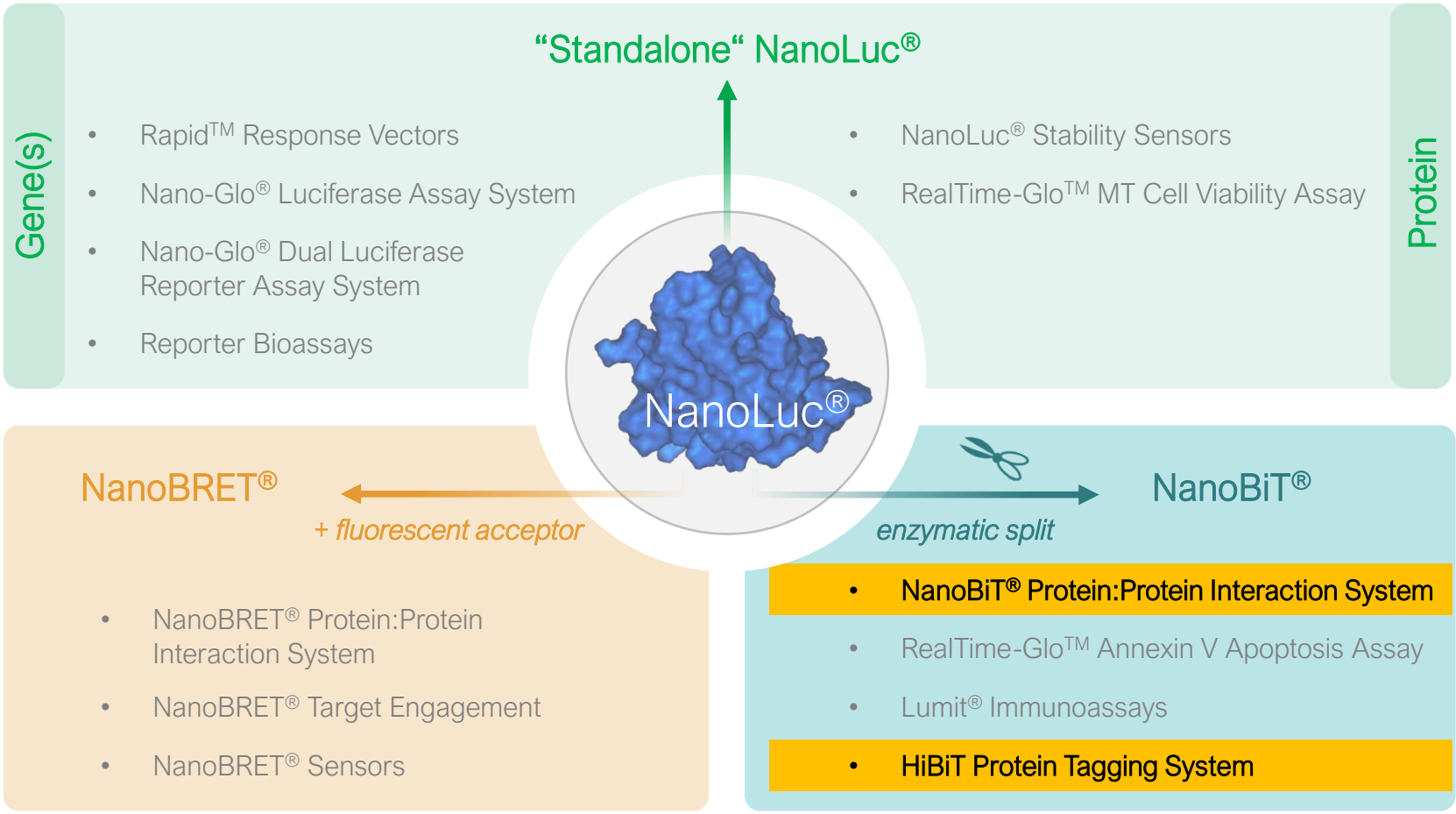
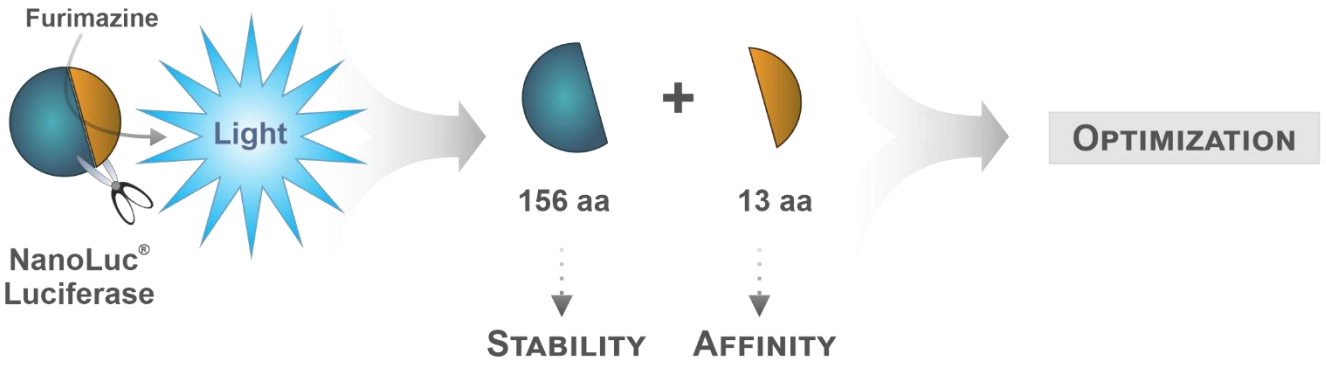
NanoLuc® Luciferase Technology Platform

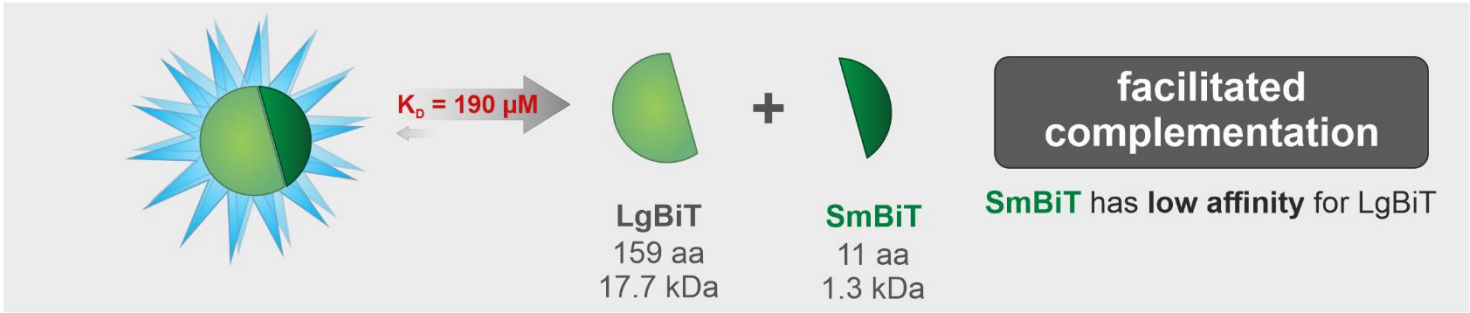
Your Companion to Study Cellular Biology



NanoLuc® Binary Technology (NanoBiT®)

A Structural Complementation Reporter Designed for Biomolecular Interaction Studies





NanoBiT® Protein:Protein Interaction System

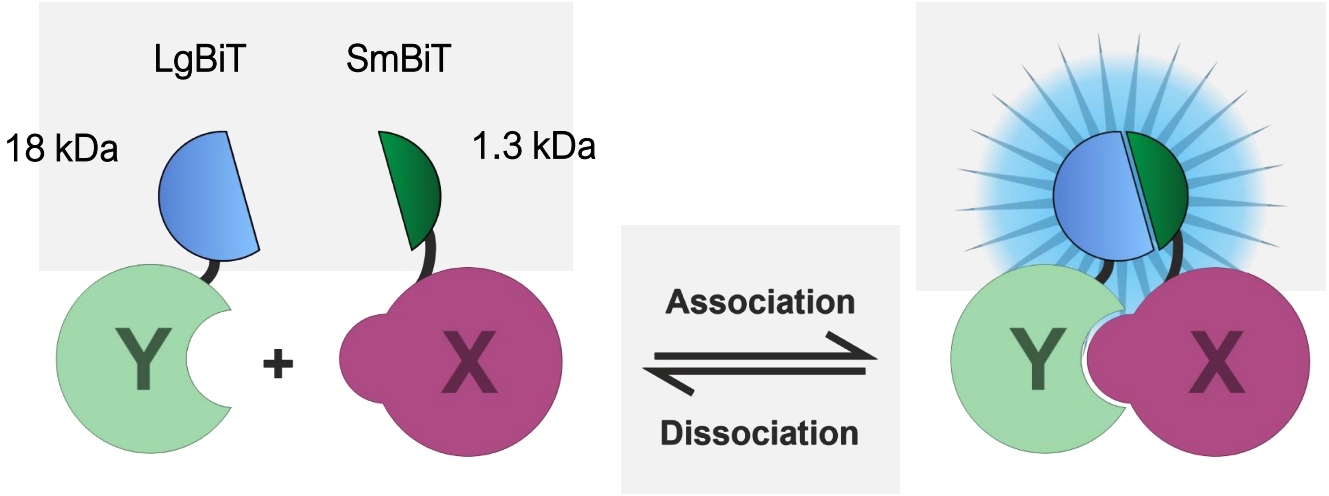
Investigate Interaction Dynamics in Live Cells

Small tag size

minimal influence on fusion partner

Bright signal upon complementation

enables low expression levels



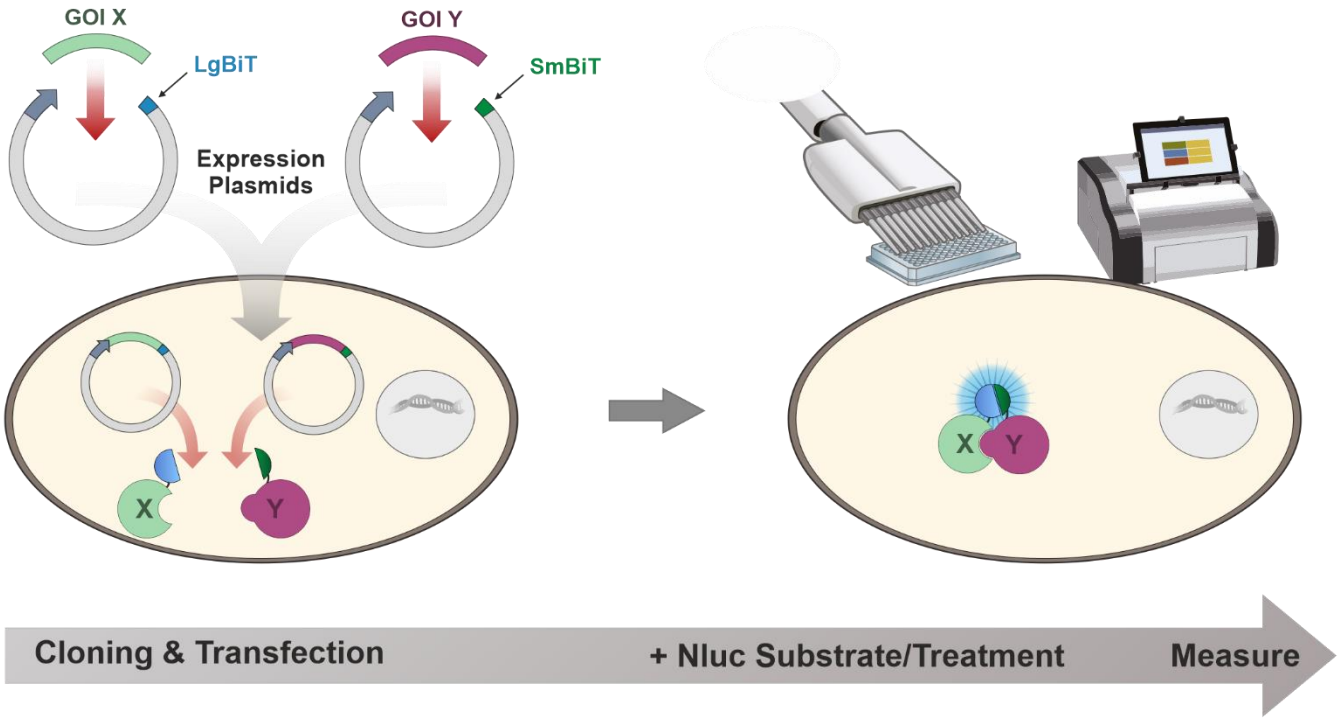
Low intrinsic affinity

reversible to allow investigation of PPI dynamics

increased signal specificity

NanoBiT® PPI Workflow

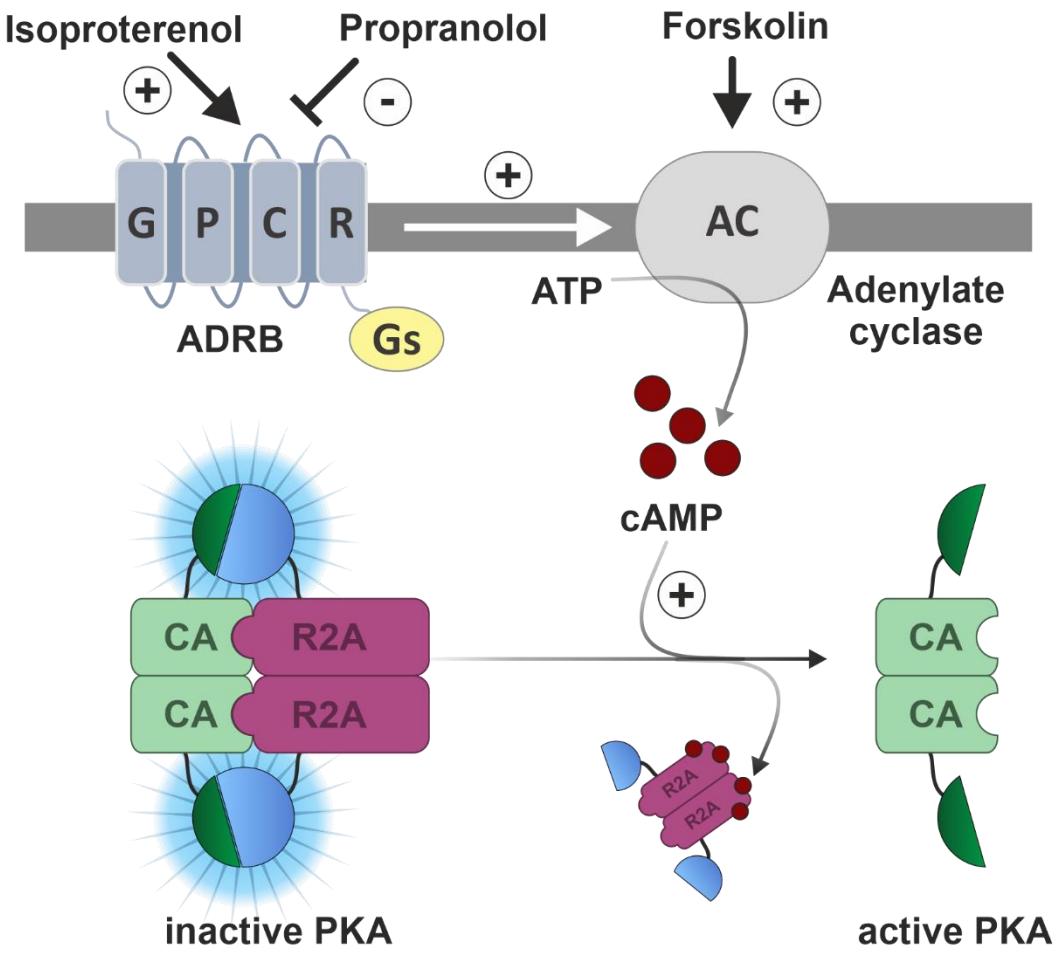
A Simple Transfection-based Experiment



1. Determine optimal LgBiT/SmBiT combinations that shows maximal fold signal change
   tool compound versus vehicle control or in comparison to HaloTag®-SmBiT negative control
2. Check for signal specificity
   expected response to tool compound or signal of SmBiT/LgBiT fusions 10 – 1,000-fold higher than LgBiT fusion co-expressed with HaloTag®-SmBiT (general guideline)

Validation of NanoBiT® PPI

The Protein Kinase A Model



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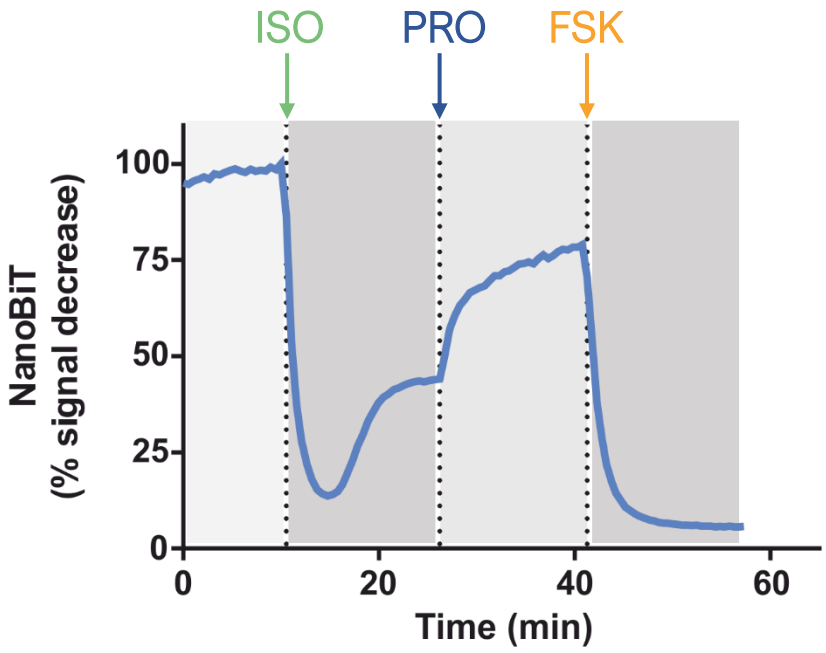


Isoproterenol (ISO)
ADRB agonist (cAMP ↑)

Propranolol (PRO)
ADRB antagonist (cAMP ↓)

Forskolin (FSK)
activator of adenylate cyclase (cAMP ↑)

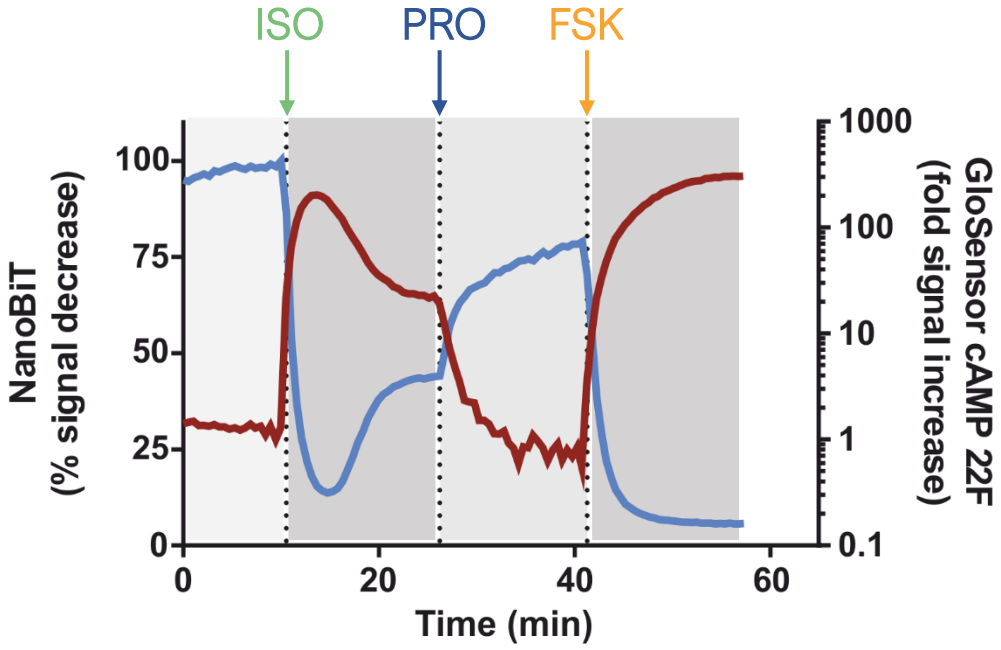
GloSensor™

Conclusions

- Endogenous biology is maintained with the NanoBiT® PPI System
- The NanoBiT® PPI System functions in a reversible manner

Validation of NanoBiT® PPI

The Protein Kinase A Model



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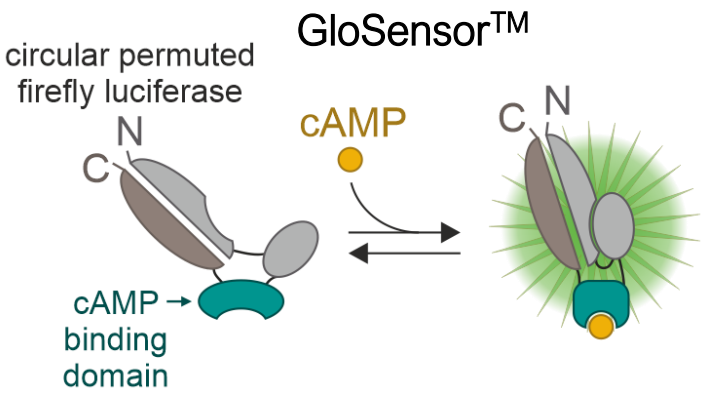
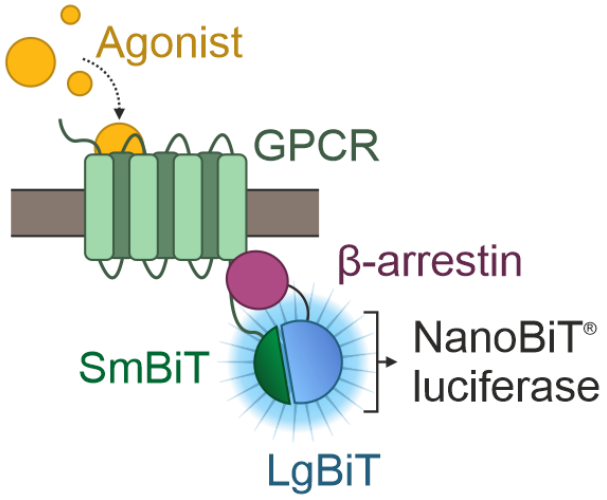
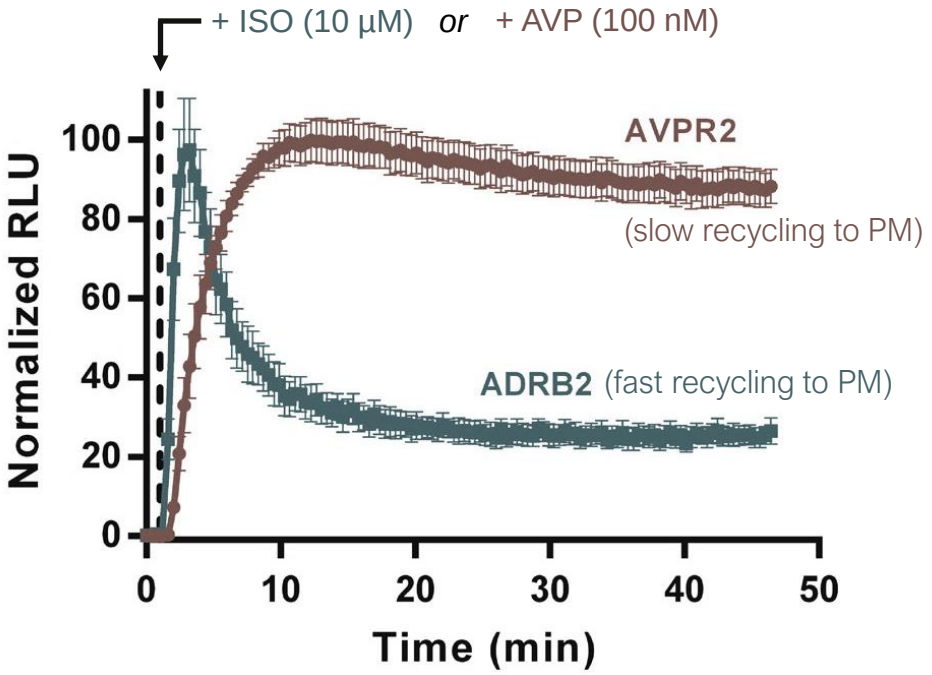


Validation of NanoBiT® PPI

β-Arrestin Recruitment to GPCRs



ADRB2-LgBiT:SmBiT-ARRB2
AVPR2-SmBiT:LgBiT-ARRB2

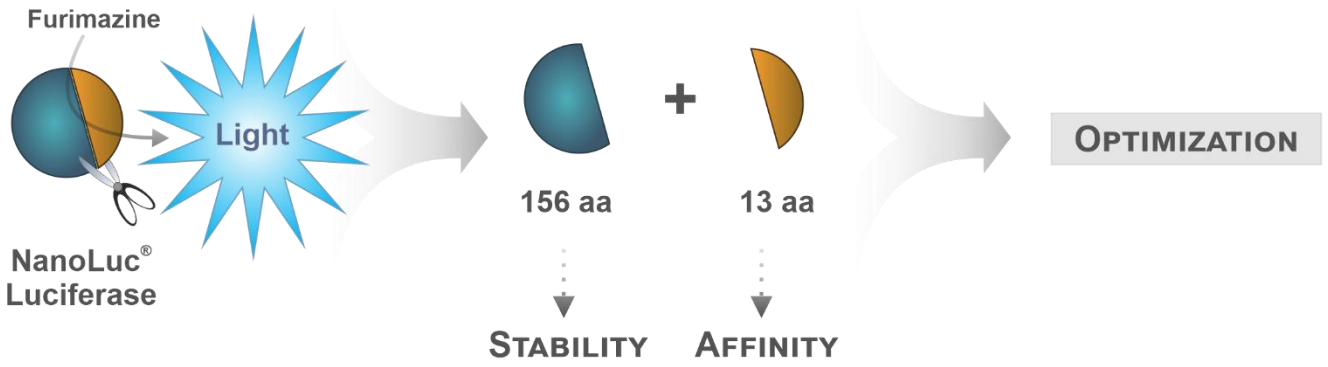


Modified from Dixon, AS. *et al.* (2015) ACS Chem Biol. 11, 2, 400–408

- ADRB2:ARRB2 signal is more transient than AVPR2:ARRB2 signal
- NanoBiT® can be used to monitor transient PPIs in real-time

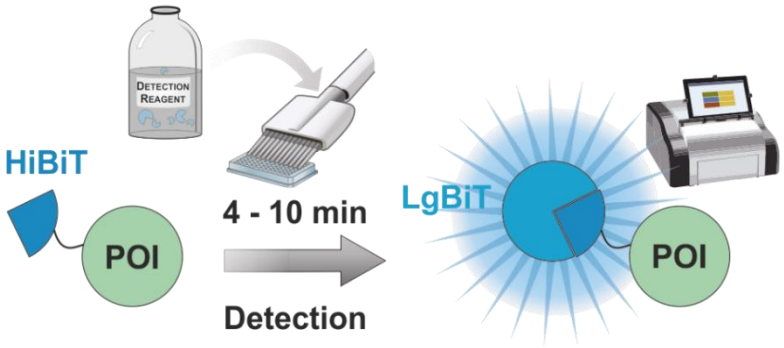
NanoLuc® Binary Technology (NanoBiT®)

A Structural Complementation Reporter Designed for Biomolecular Interaction Studies

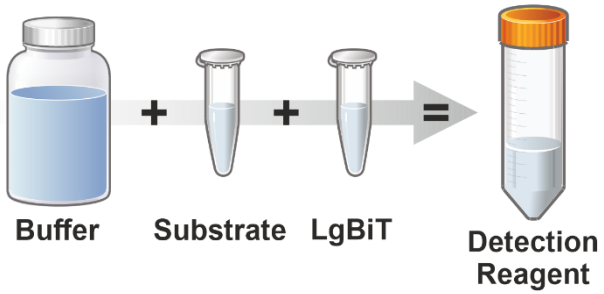


HiBiT Protein Tagging System

Principle & Features



HiBiT Detection Reagent



Small Tag Size (11 aa, 1.3 kDa)

- Low risk to artificially affect fusion partner

Easy Knock-in with CRISPR

- Work at native expression level
- Maintain transcriptional regulation
- Avoid gene dosage effects

Simple, Flexible & Rapid Detection

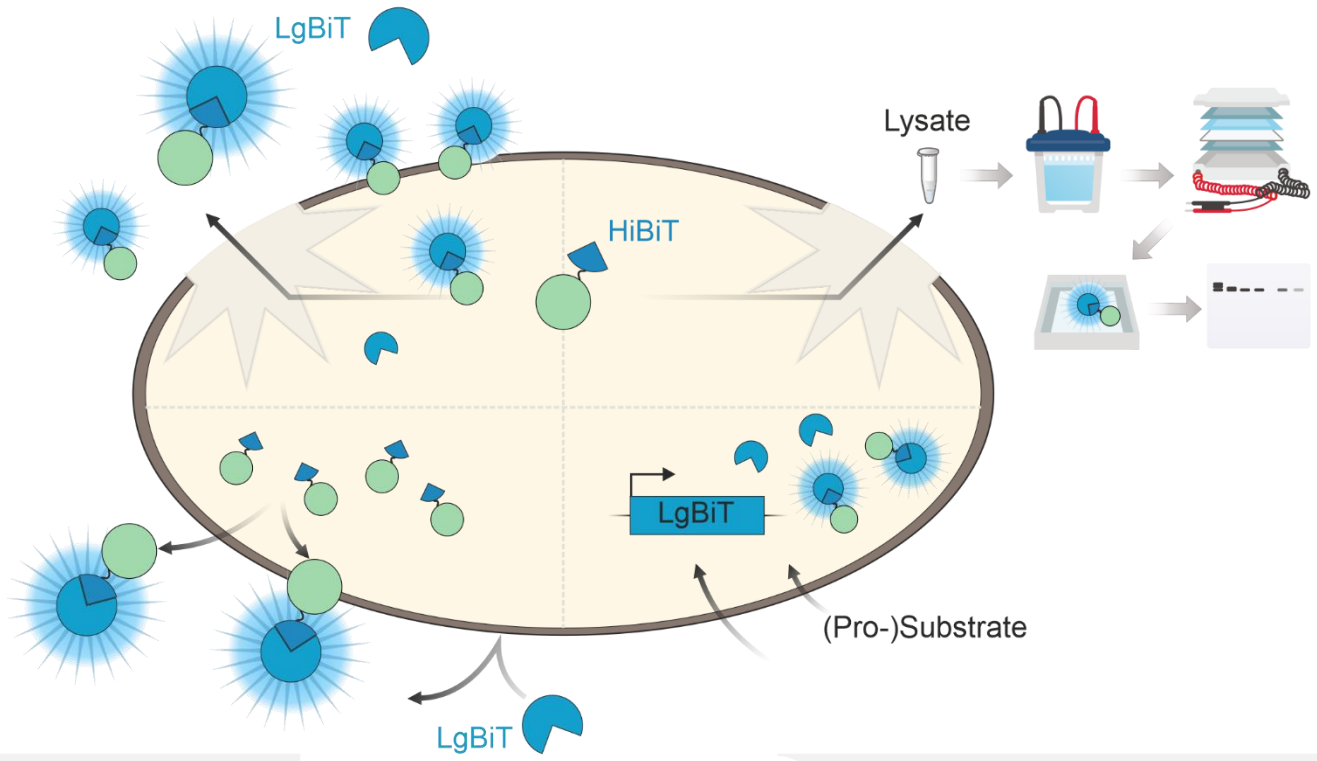
- Homogenous 1-step assay („add only“)
- No antibodies and no washing steps required
- Amenable to HTS
- Easy to automate

Detection of HiBiT Fusion Proteins

Choose From Different HiBiT Detection Strategies

1 Nano-Glo® HiBiT Lytic Detection System

2 Nano-Glo® HiBiT Blotting System



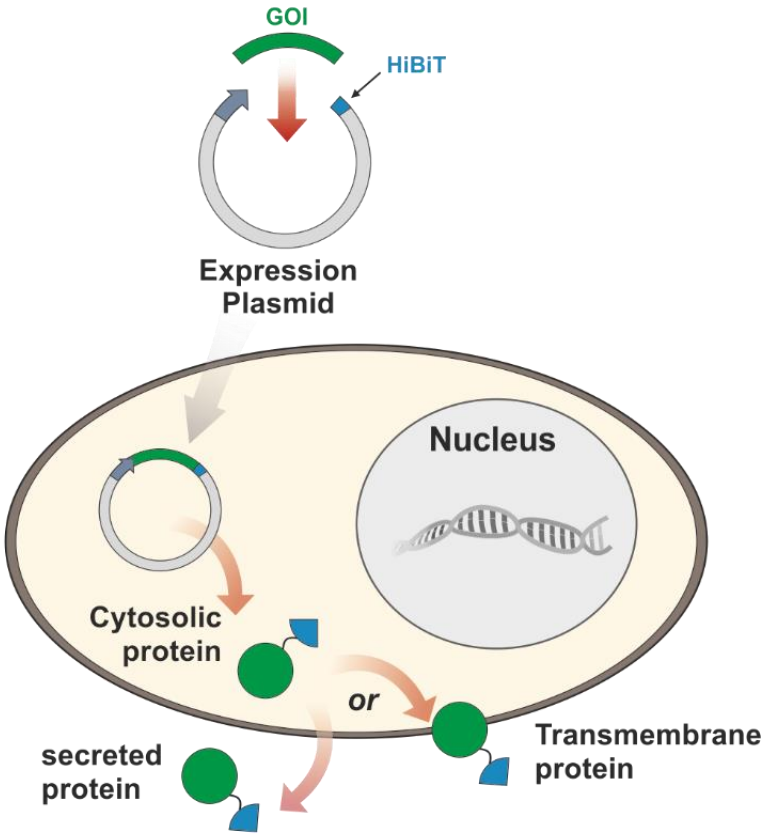
3 Nano-Glo® HiBiT Extracellular Detection System

4 LgBiT co-expression Nano-Glo® Live Cell Substrate

Time course analysis up to 72 hours

Strategies for Tagging with HiBiT

Ectopic Expression Using Constitutive Promoter-driven Plasmid



Your options

1 Promega's HiBiT entry vectors

- N-terminal
- C-terminal
- N-terminal + IL-6 secretion sequence *
- CMV, TK, PGK

* naturally occurring secretion signals shall be removed

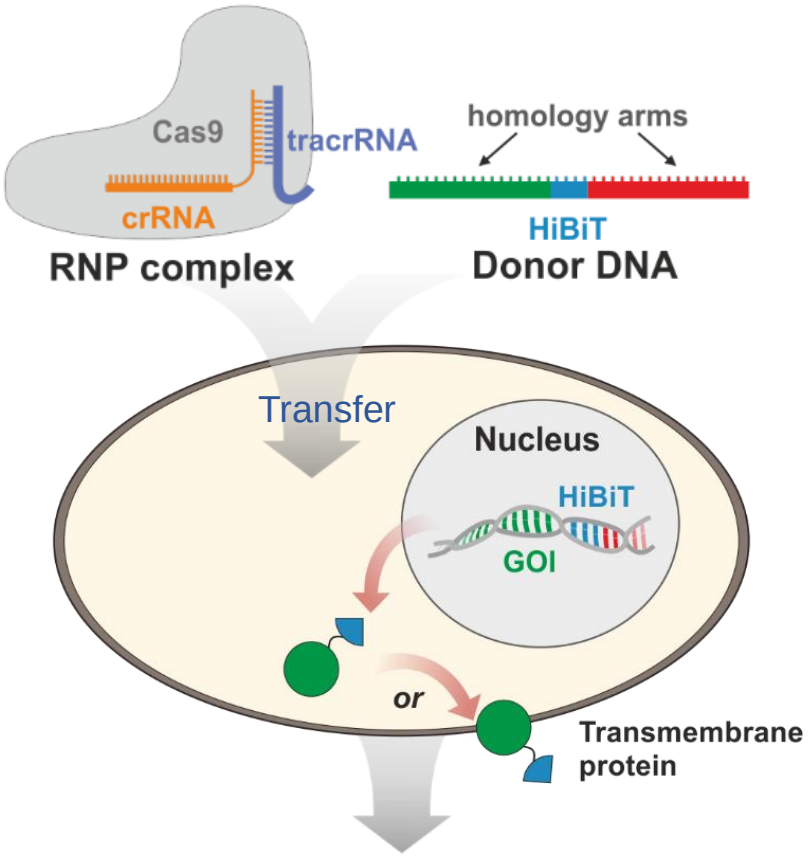
- Bicistronic entry vectors
  (use Fluc for normalization purposes)

2 Use existing vector and
append HiBiT via PCR amplification

(e.g. internal placement of tag)

Strategies for Tagging with HiBiT

Endogenous Expression Following CRISPR-mediated Tagging

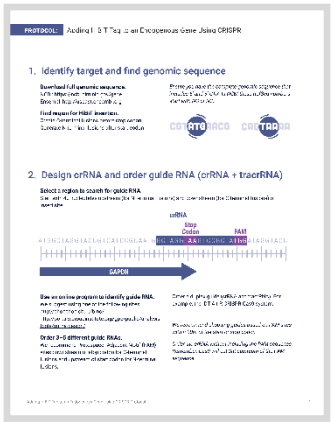


Validate after 24 - 48 h

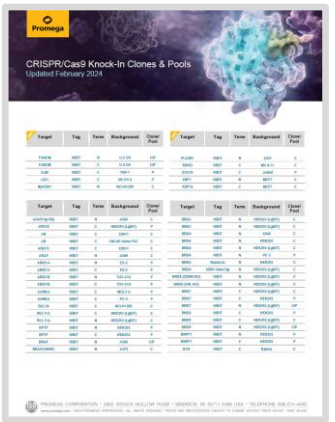
Three key components

1. gRNA (crRNA + tracrRNA)
2. Cas9 endonuclease
3. ssDonor DNA

DIY protocol



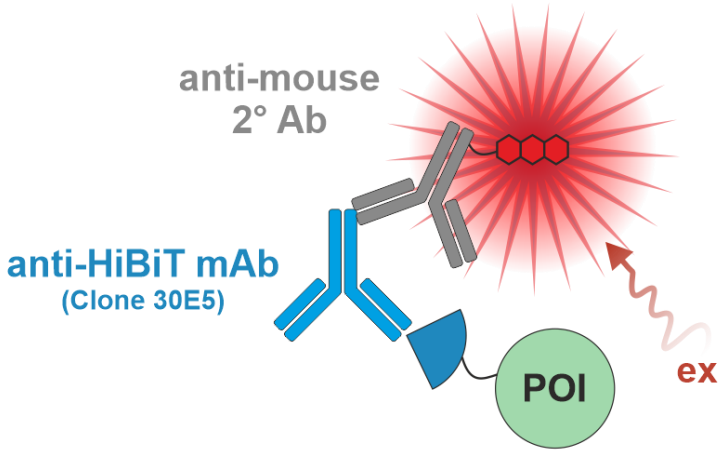
Ready-to-use cell lines

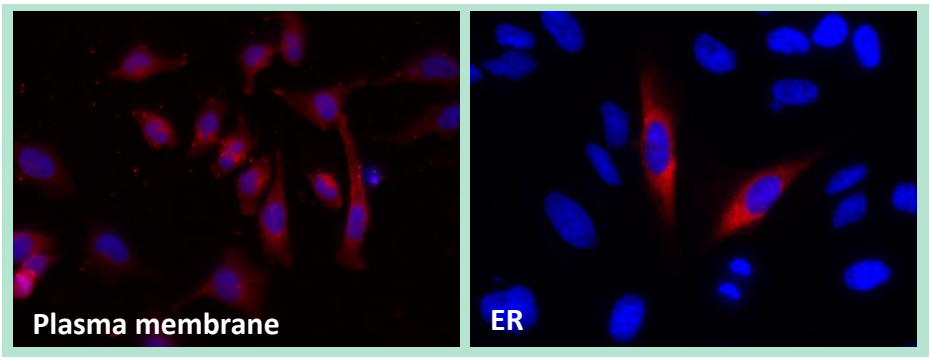


gRNA: guide RNA; **crRNA**: CRISPR RNA; **tracrRNA**: transactivating crRNA

Immunodetection of HiBiT Proteins

Immunofluorescent Imaging and More



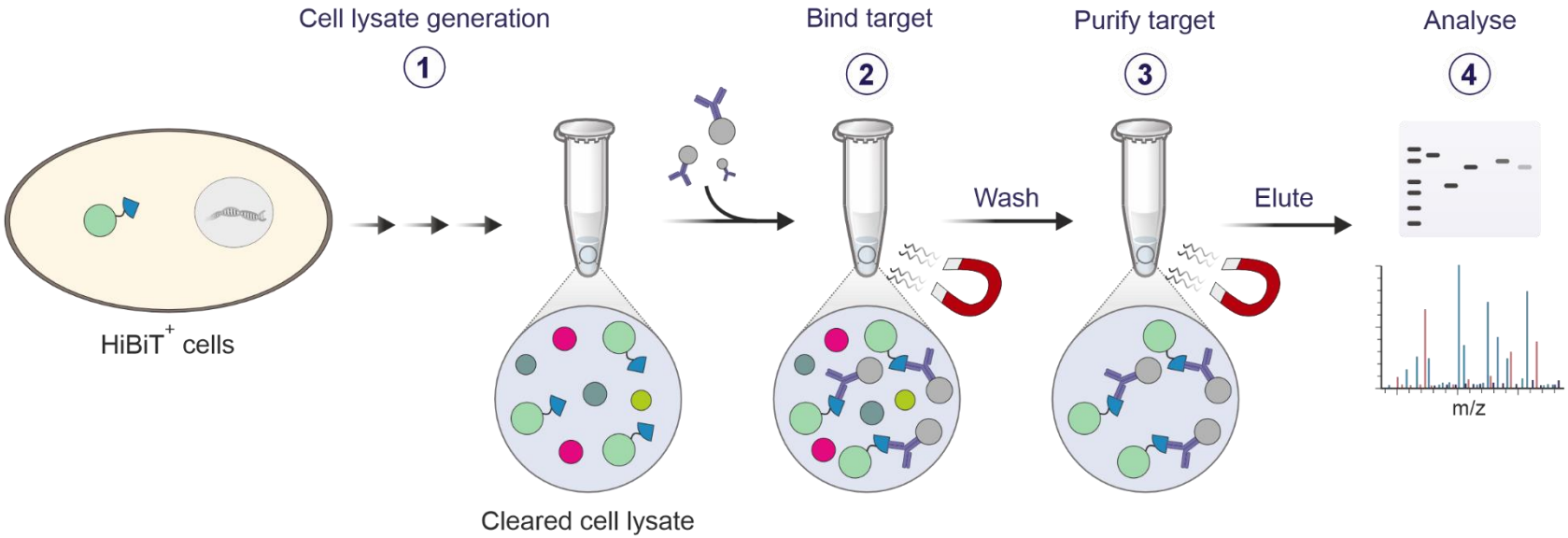


Hoechst dye AlexaFluor® 647

- Potent mAb directed against HiBiT tag
- Validated for various applications including:
  - ✓ Immunofluorescence (**NEW** anti-HiBiT mAb pre-conjugated to Green488 or FarRed647)
  - ✓ Western blotting
  - ✓ Immunoprecipitation (**NEW** Anti-HiBiT Magne® Beads)
  - ✓ FACS

Anti-HiBiT Magne® Beads

Workflow



- A cleared cell lysate is generated from HiBiT$^+$ cells
- Lysate is incubated with Anti-HiBiT Magne® Beads over night at 4°C or > 30 min at RT
- Elution can be performed with

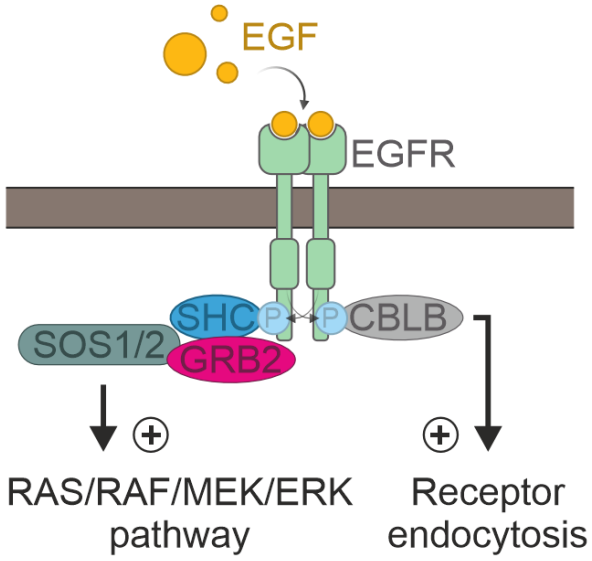
(1) SDS loading buffer and heating to 70 °C for 10 min

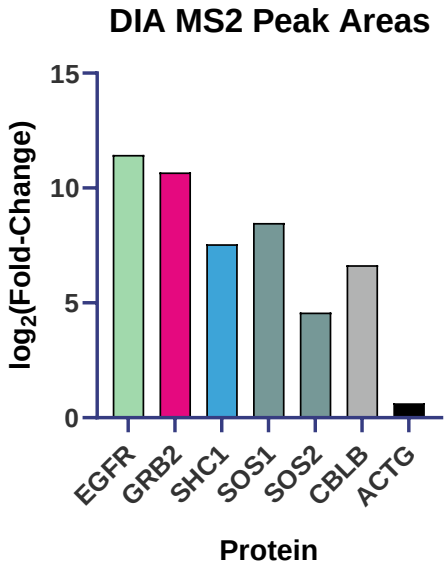
(2) Glycine-HCl (pH 2.5) at RT for 5 – 10 min

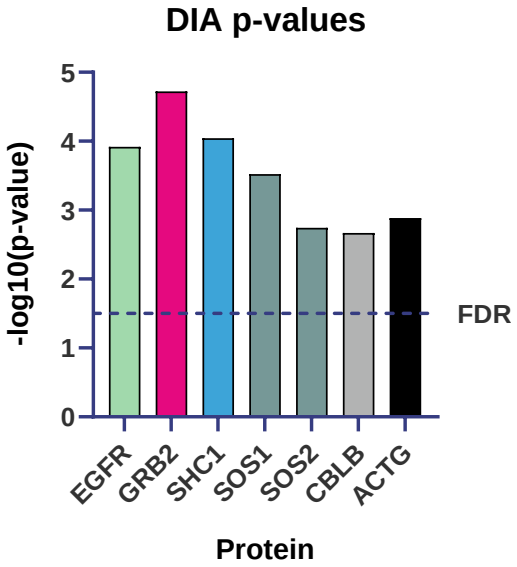
(3) DrkBiT peptide overnight at 4°C

Anti-HiBiT Magne® Beads

Workflow



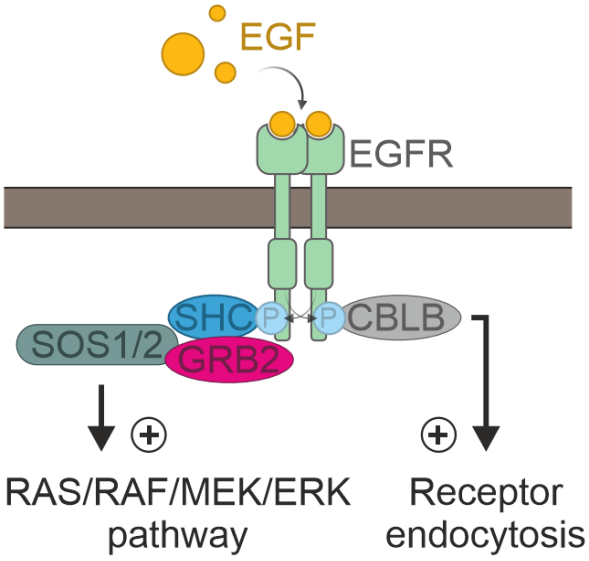


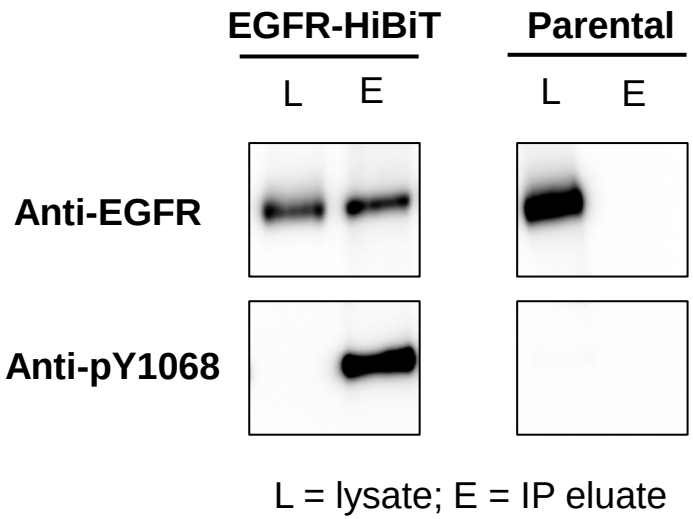


- EGFR-HiBiT HeLa CRISPR knock-in cells
- Upon EGF stimulation, co-IP was performed using the Anti-HiBiT Magne® Beads
  - ✓ DIA MS of IP eluates showed enrichment of EGFR and known direct/indirect interactors
  - ✓ EGFR enrichment and phosphorylation was confirmed by Western blot analysis
  - ✓ FACS

Anti-HiBiT Magne® Beads

Workflow



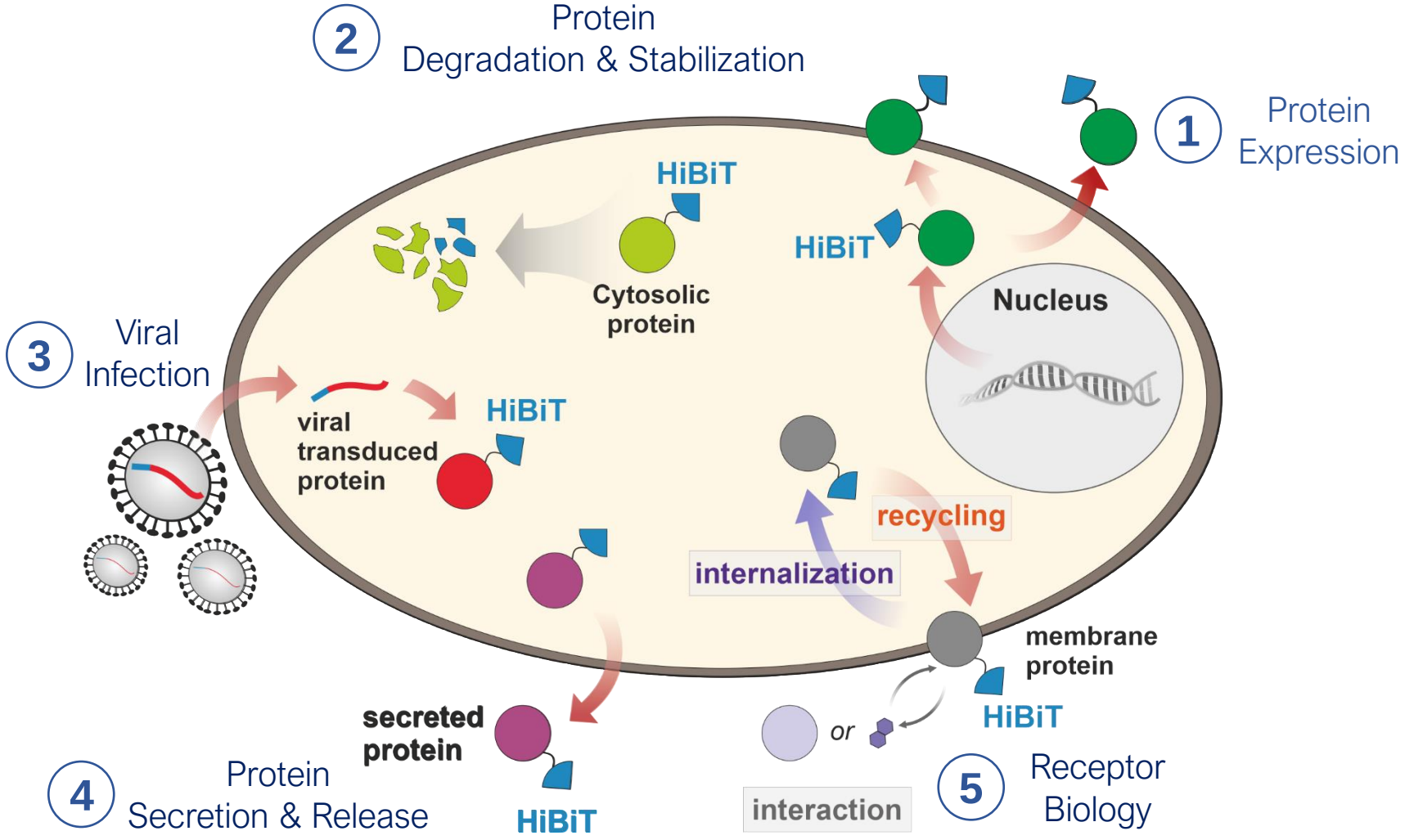


L = lysate; E = IP eluate

- EGFR-HiBiT HeLa CRISPR knock-in cells
- Upon EGF stimulation, co-IP was performed using the Anti-HiBiT Magne® Beads
  - ✓ DIA MS of IP eluates showed enrichment of EGFR and known direct/indirect interactors
  - ✓ EGFR enrichment and phosphorylation was confirmed by Western blot analysis
  - ✓ Phospho-EGFR was also detected by MS (data not shown)

HiBiT Application Portfolio

One Bioluminescent Tag, Endless Possibilities



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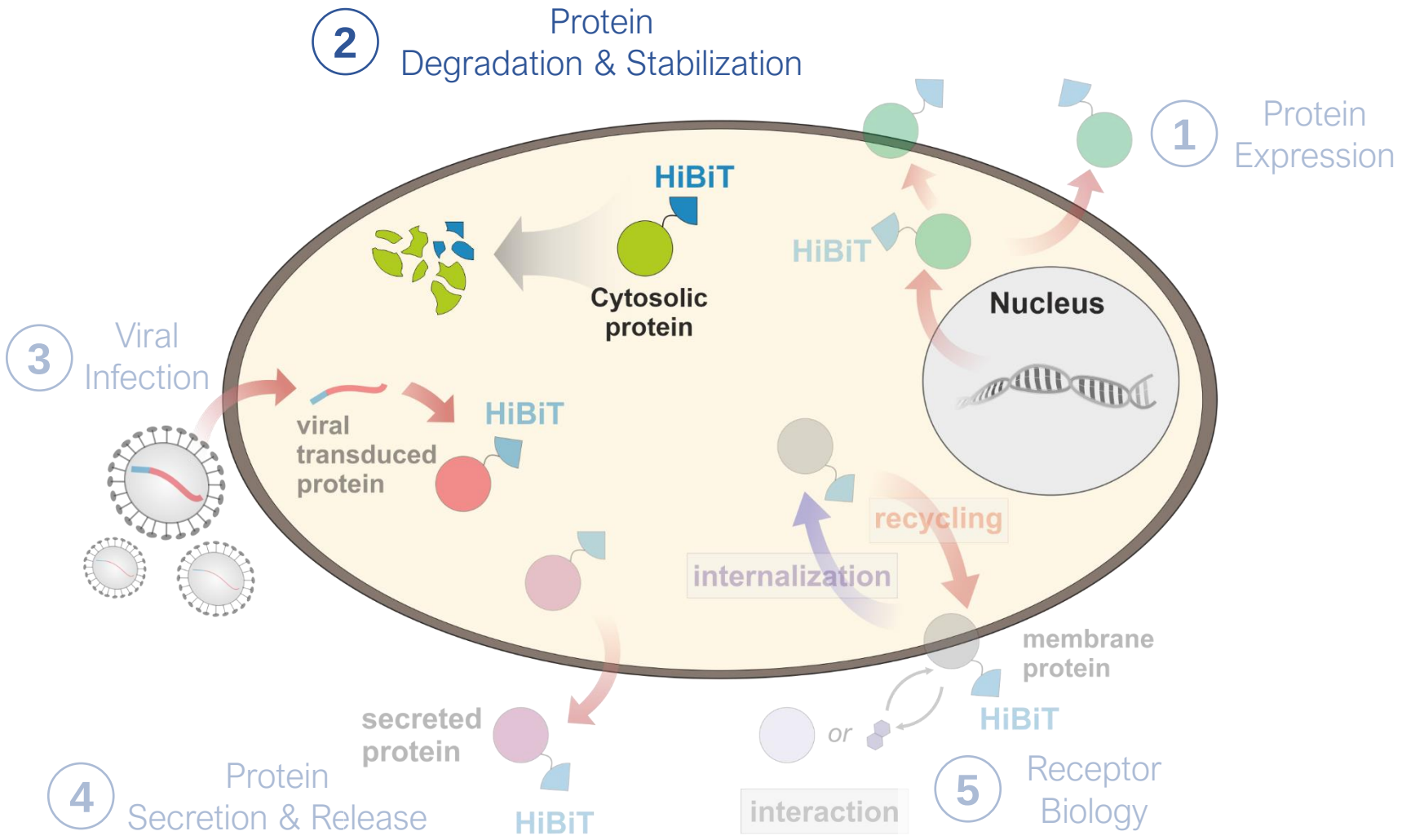
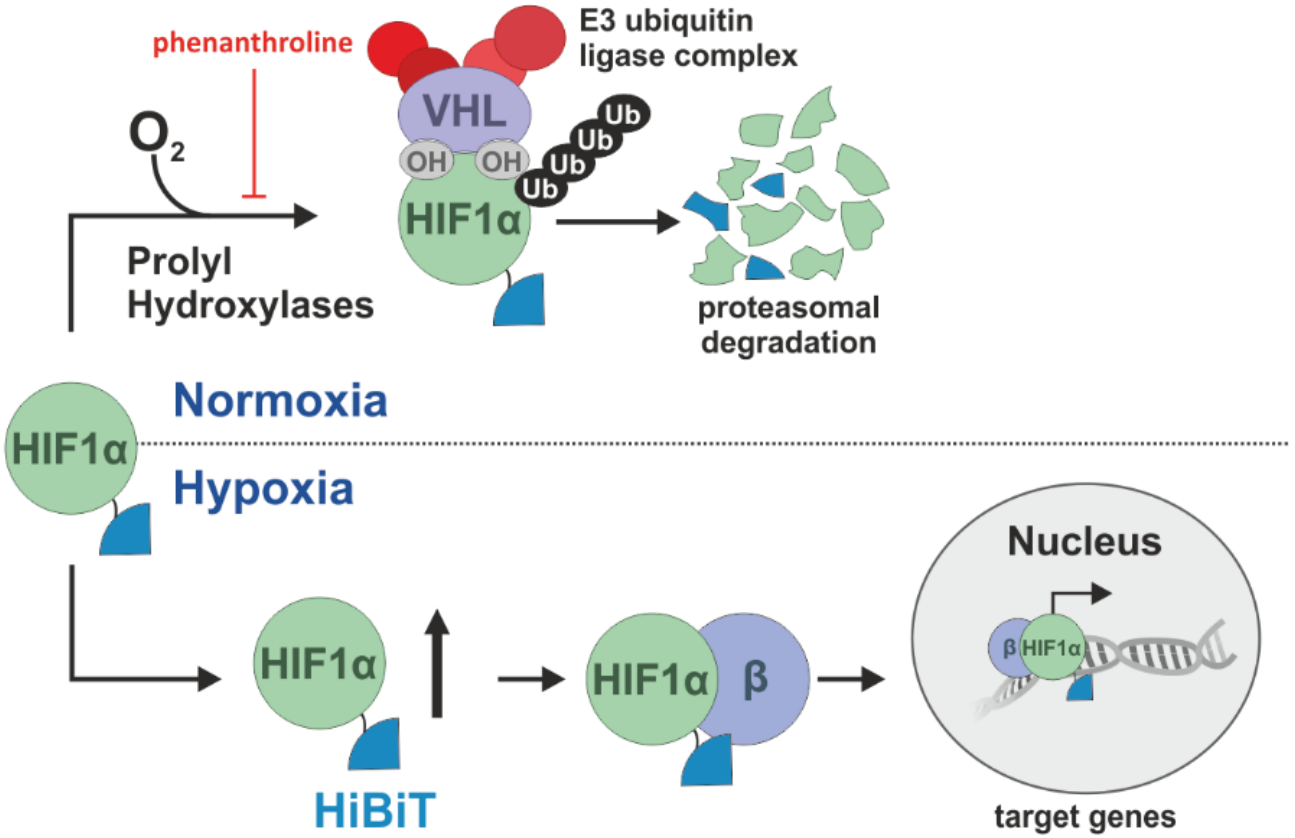


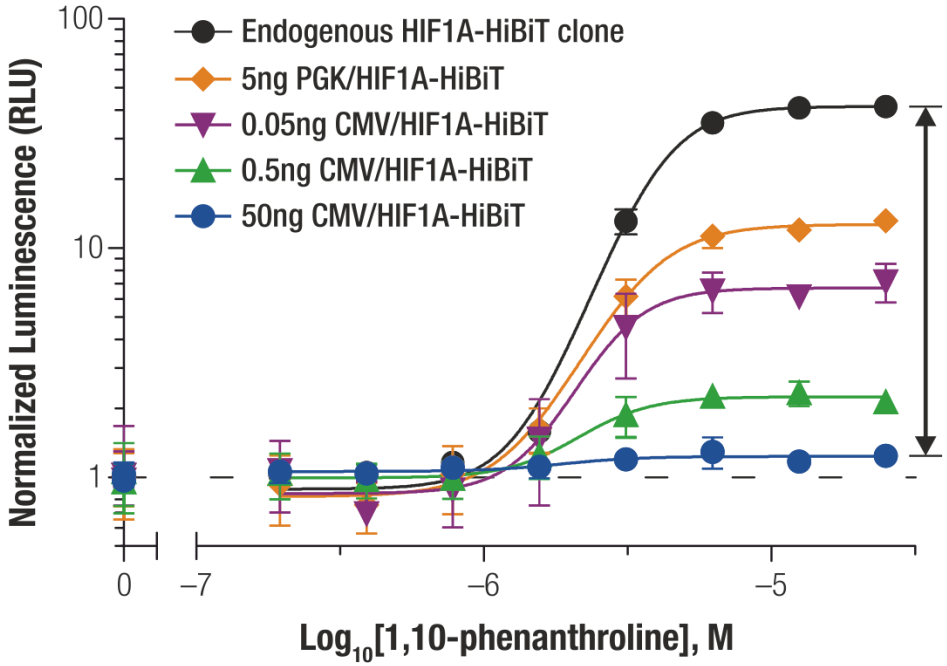
The HIF1α Pathway

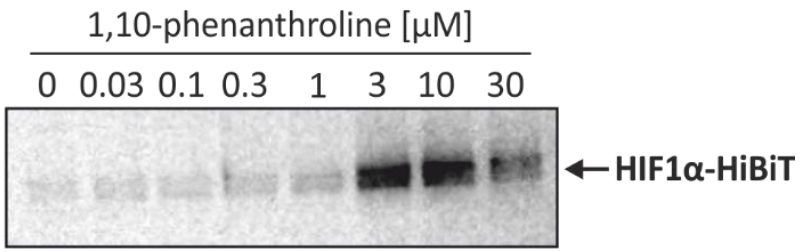
A Model System for Protein Stabilization



Stabilization of HIF1α

The Relevance of Expression Level Protein Stabilization

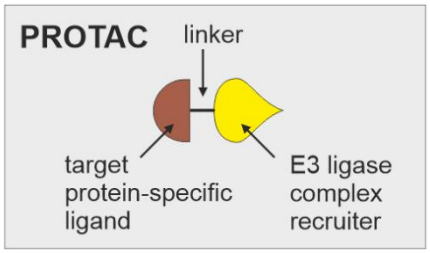


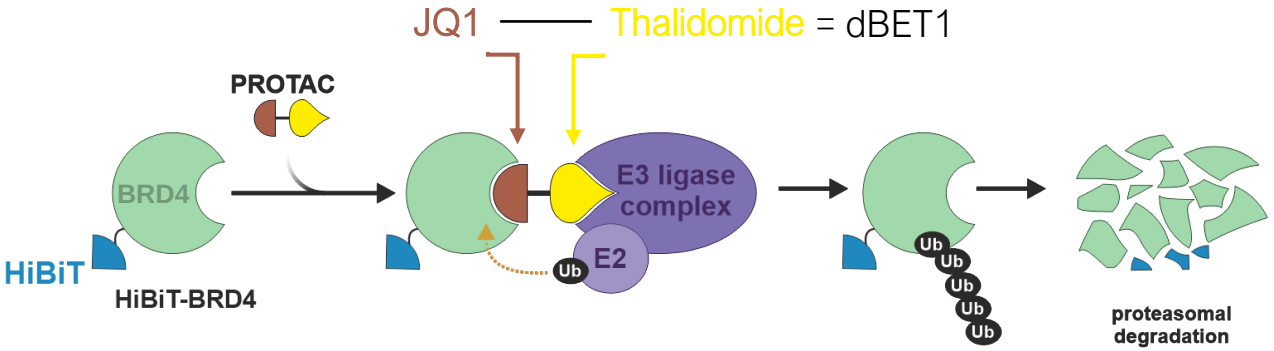


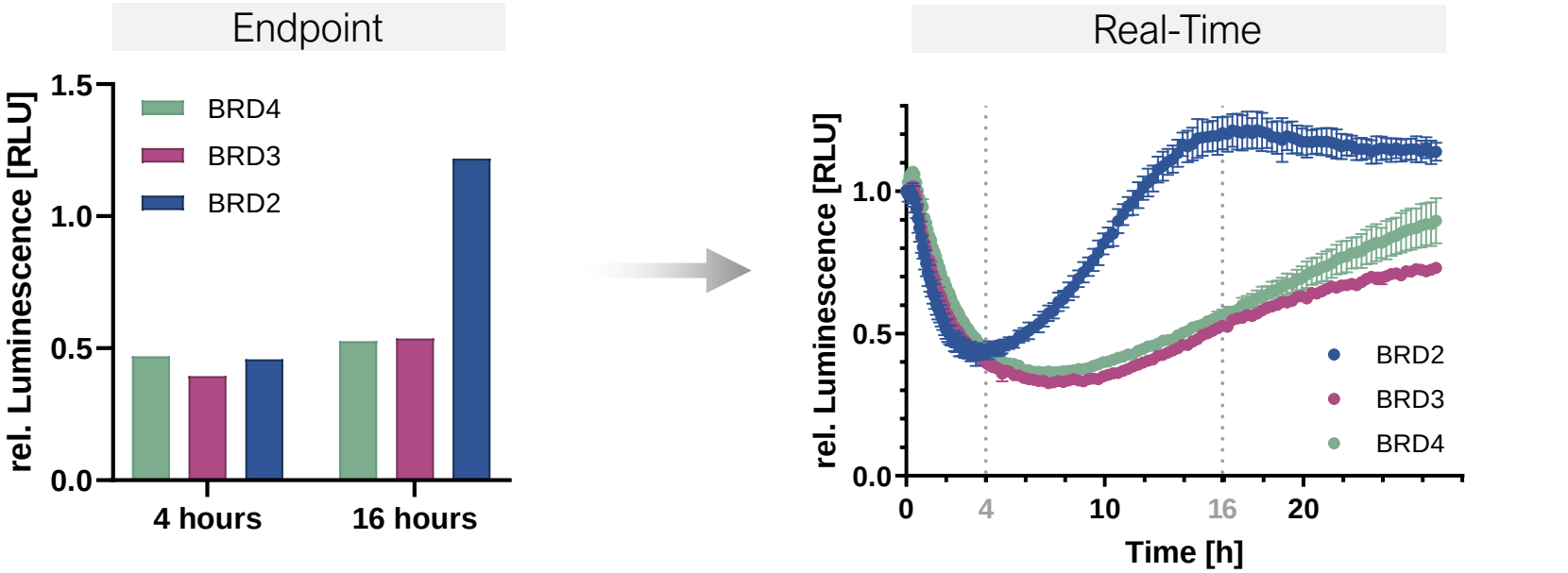
- High expression levels mute the biological response
- Endogenous expression yields highest assay window

Studying (Targeted) Protein Degradation

Proteolysis targeting chimeras (PROTACs)

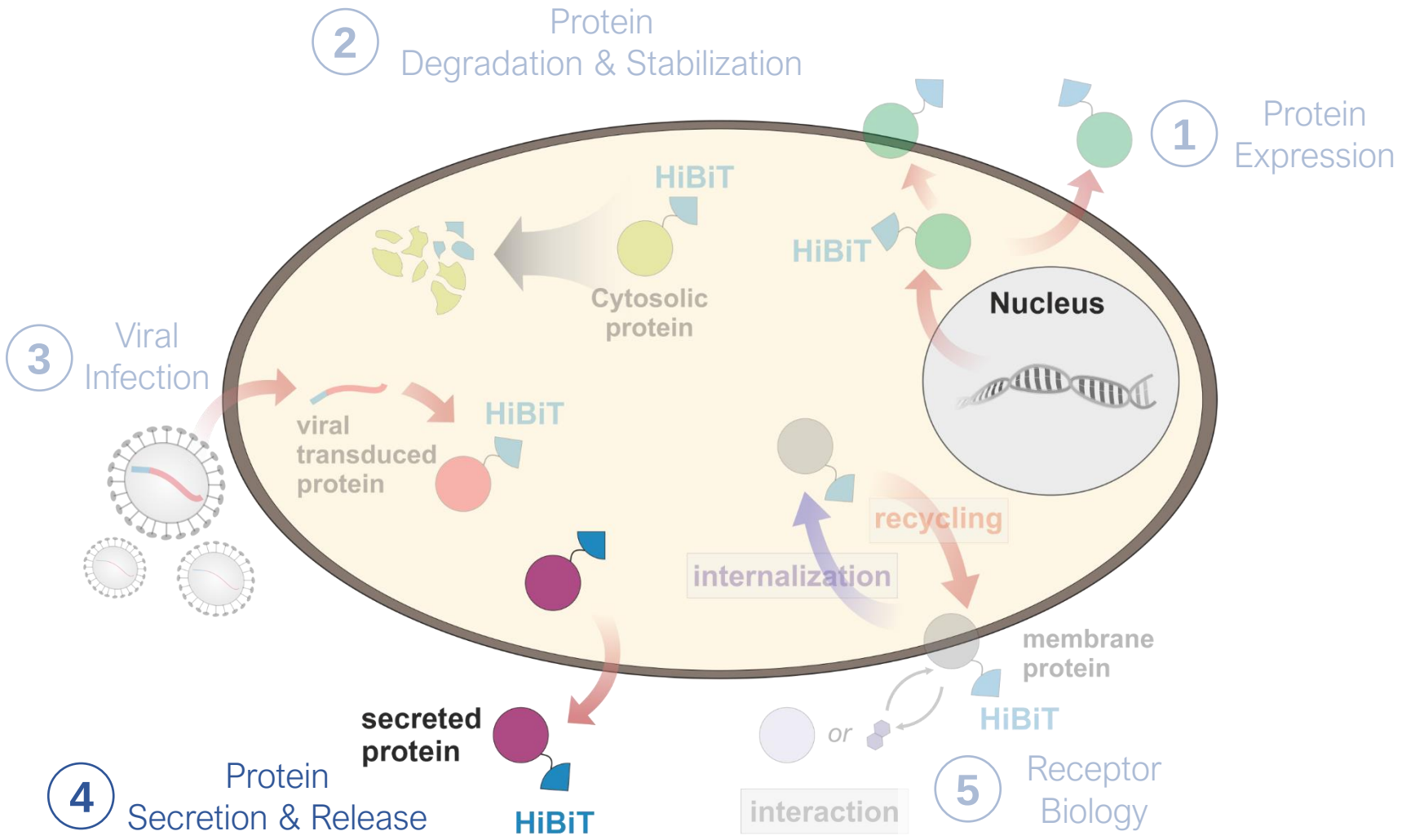






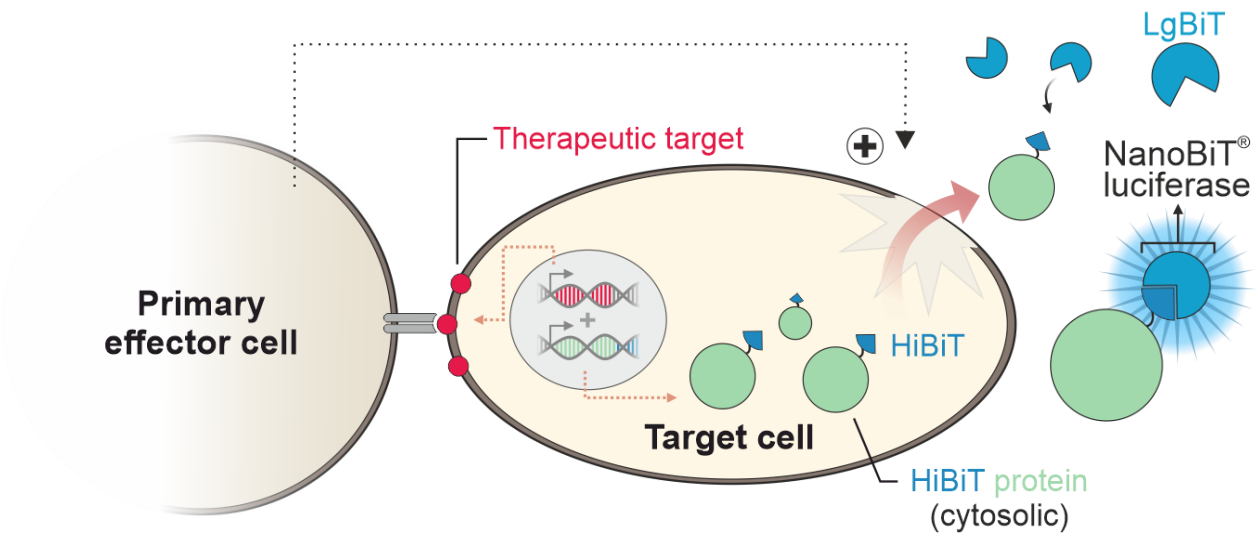
HiBiT Application Portfolio

One Bioluminescent Tag, Endless Possibilities



HiBiT Target Cell Killing Assay

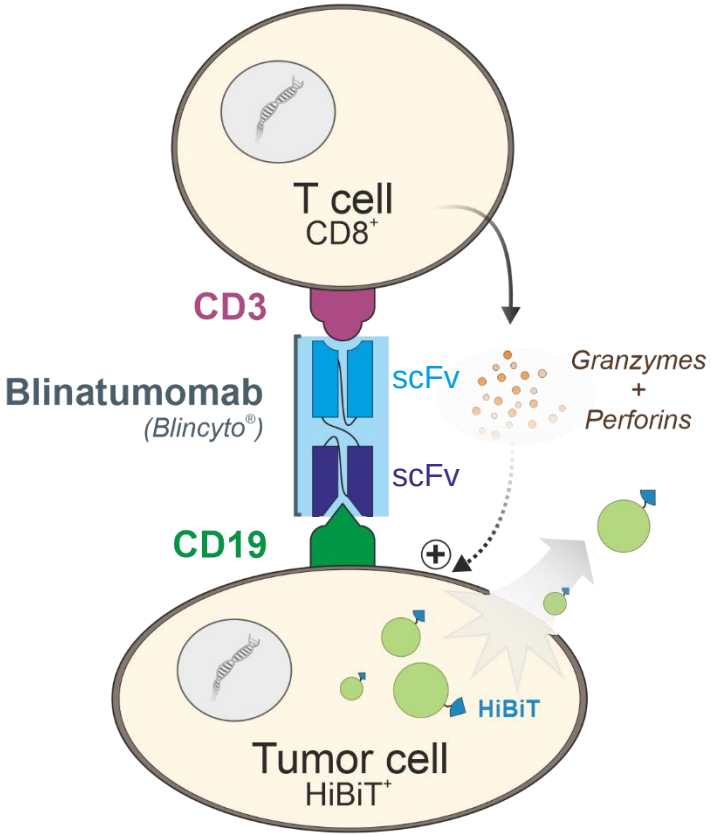
Measure death of a specific cell population within a mixed population of cells

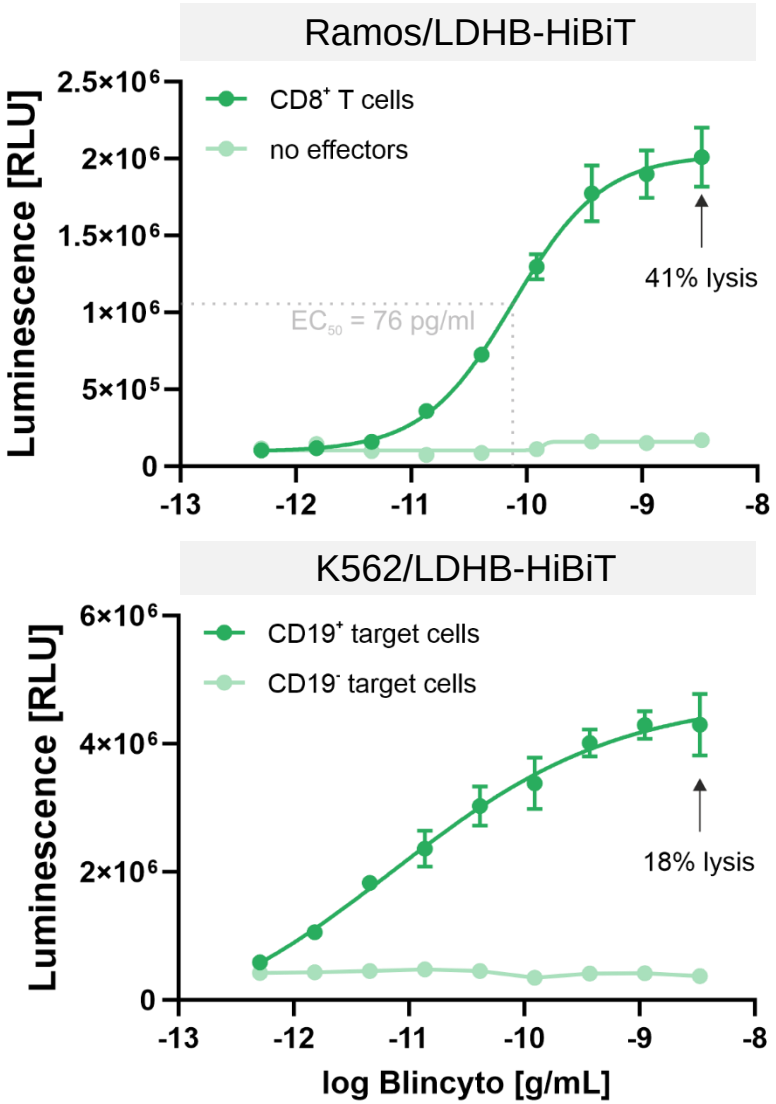


- Target cell with endogenous *or* ectopic expression of target and cytosolic HiBiT fusion protein
- Primary effector cells that mediate TCK and HiBiT release are added
- Released HiBiT is detected by LgBiT and NanoBiT® luciferase substrate addition
  - Endpoint or kinetic analysis possible

T Cell Dependent Cellular Cytotoxicity (TDCC)

Bispecific T-Cell Engager (BiTE)

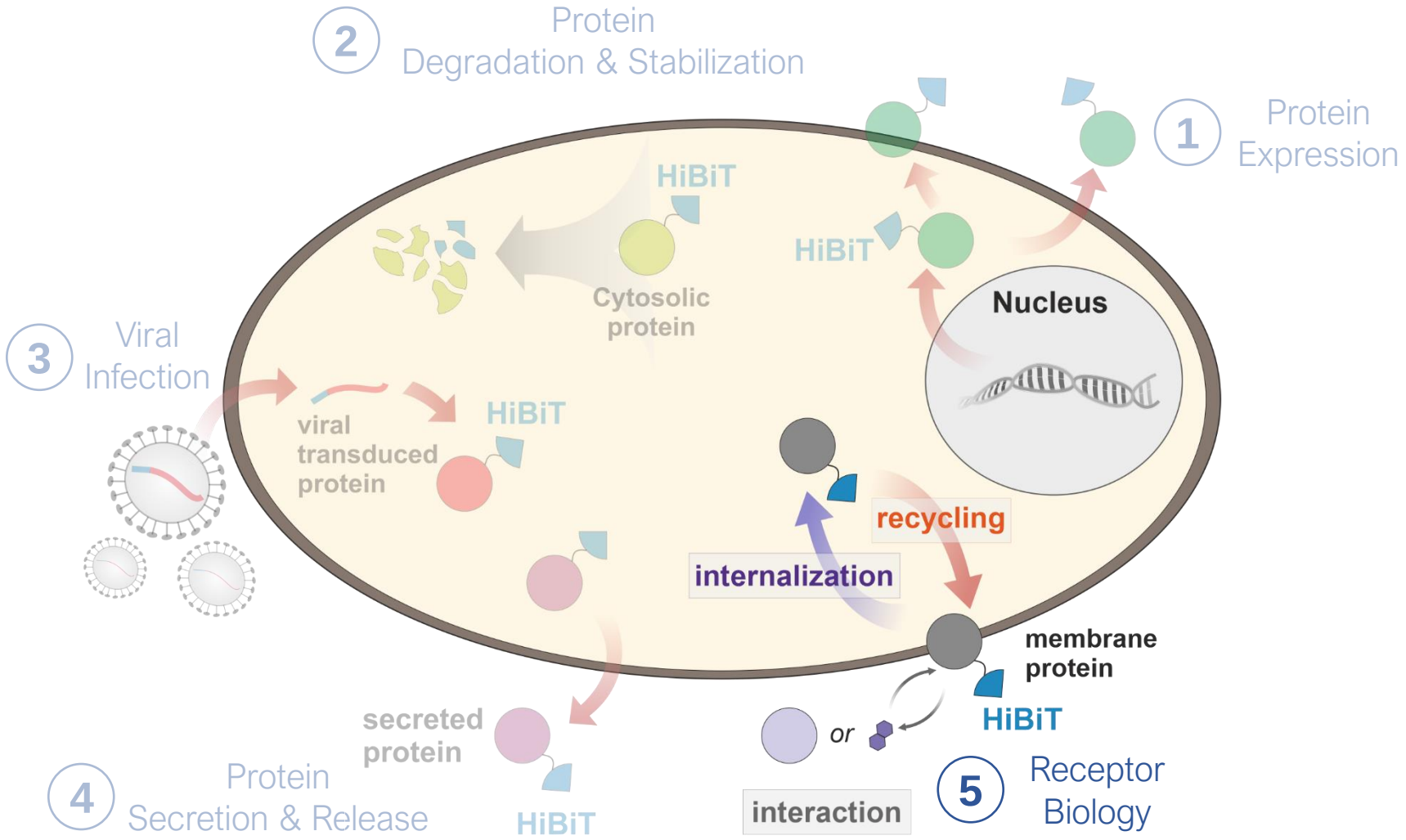




scFv: single-chain variable fragment

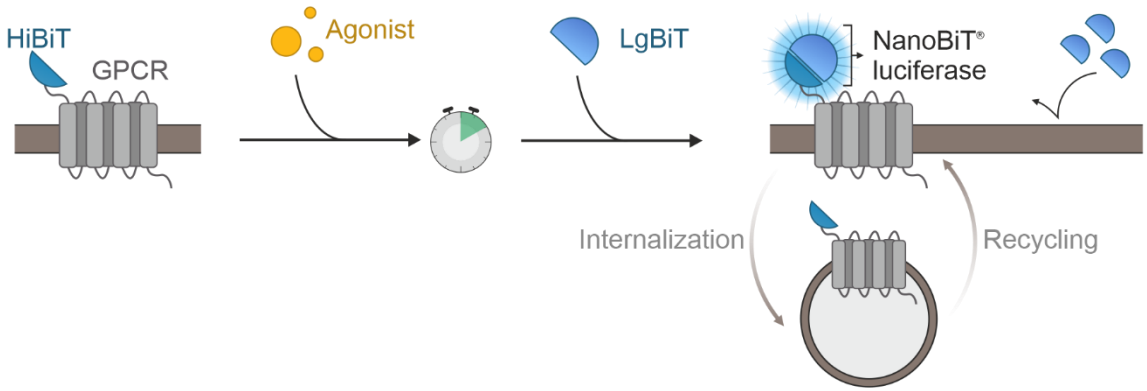
HiBiT Application Portfolio

One Bioluminescent Tag, Endless Possibilities



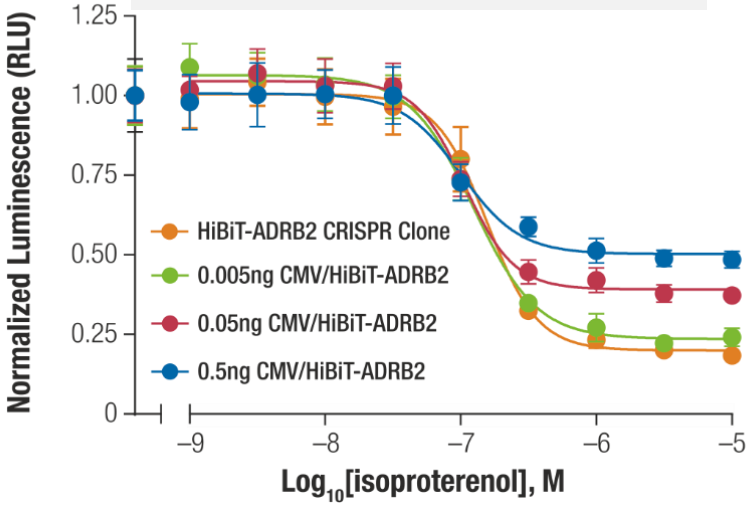
Study Receptor Internalization with HiBiT

GPCRs & RTKs

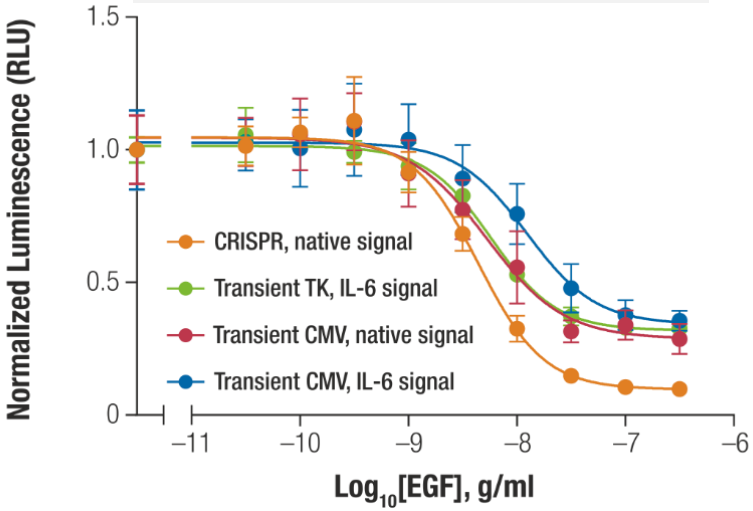


- Ectodomain of receptor tagged HiBiT
- Non-lytic detection with cell-impermeable LgBiT protein
- Measure ligand potency & internalization within minutes

HiBiT-ADBR2



HiBiT-EGFR



Thank You!

- For additional questions please contact:
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- For products & sales-relevant information please contact:

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