NanoLuc® Binary Technology

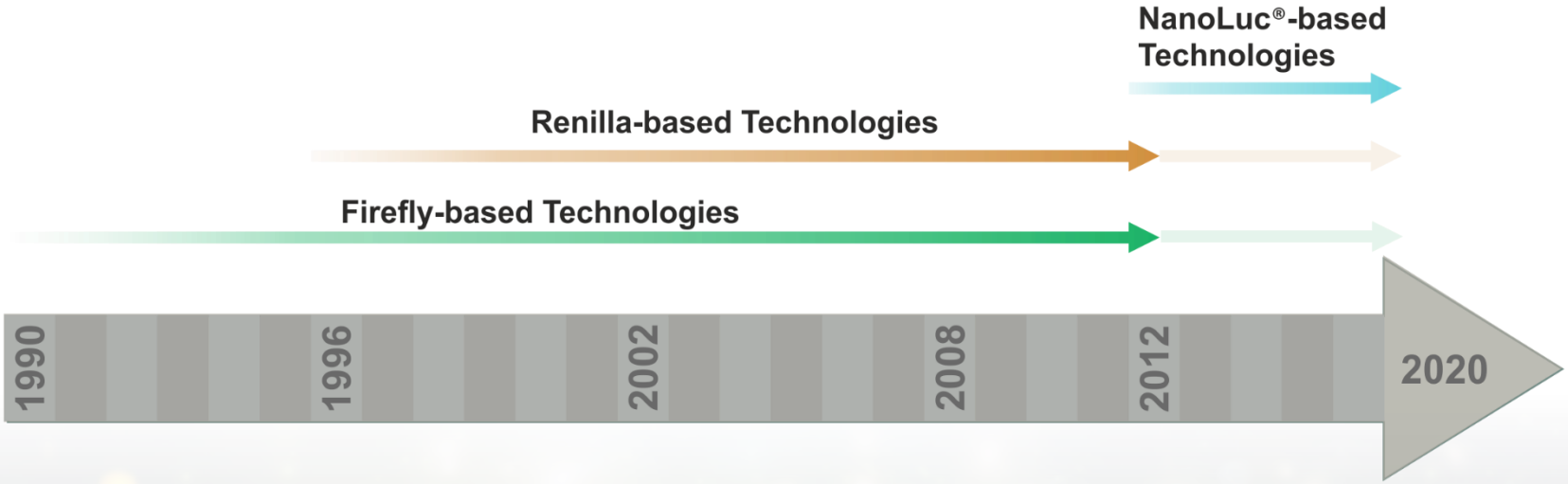
A Structural Complementation Reporter Designed for Cellular Protein Analysis

Dr. Erik Bonke | Application Specialist | Promega GmbH

Welcome

Promega – The Bioluminescent Company

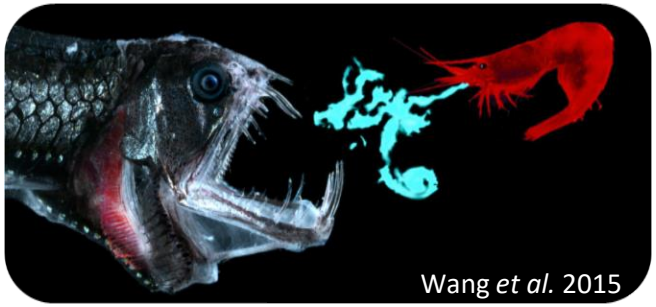
A Continuously Grown Expertise in Luciferase-based Technologies



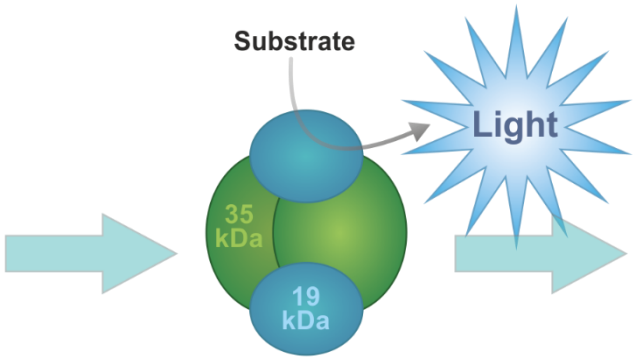
- Reporter Gene Assays
- GloSensor™ (cAMP, Protease Assays)
- GloResponse™ (Signaling Pathways)
- Rapid Response™ (Signaling Pathways)
- Cell-Health Assays
- Bioassays (ADCC, PDL1)
- NanoBRET™ Target Engagement
- NanoBRET™ / NanoBiT® Protein:Protein Interaction
- HiBiT Protein Tagging System
- Lumit™ Immunoassays

NanoLuc® Luciferase

A Bright & Small Experimental Reporter

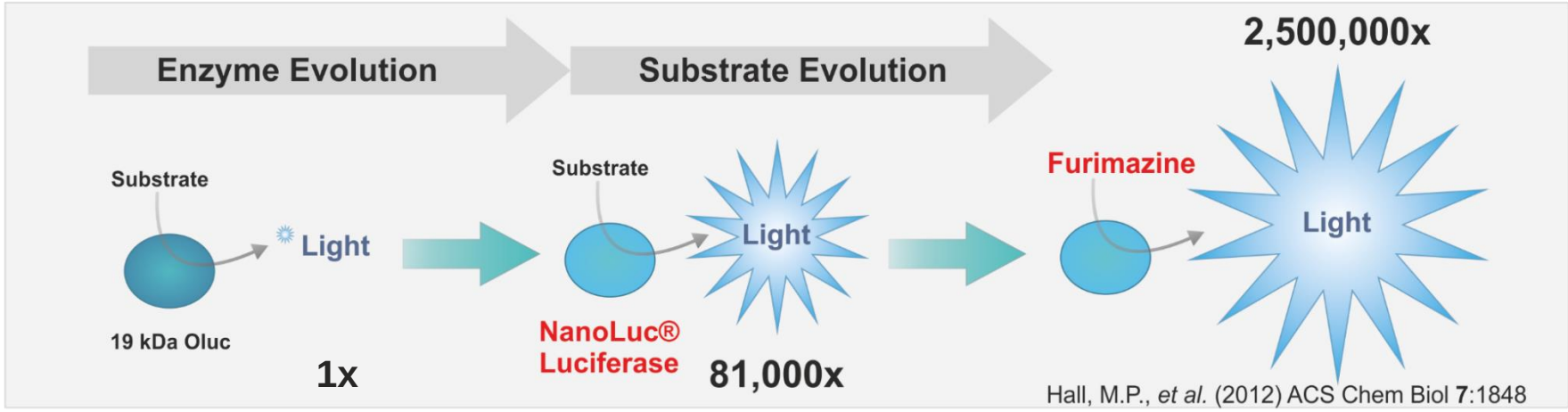


Oplophorus gracilirostris



- *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

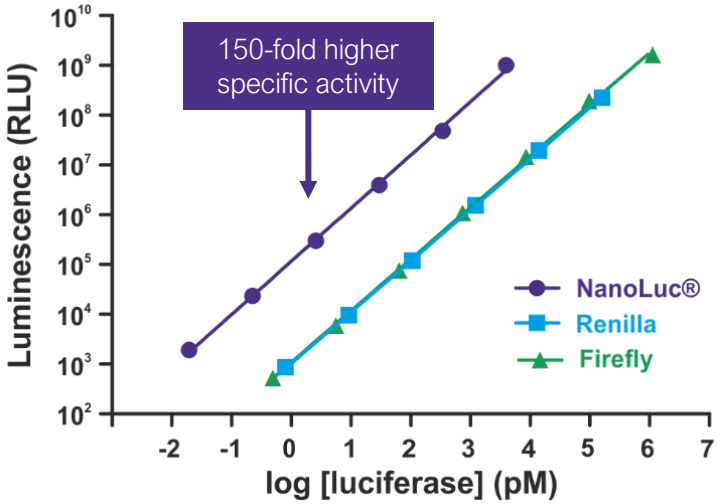


Hall, M.P., *et al.* (2012) ACS Chem Biol **7**:1848

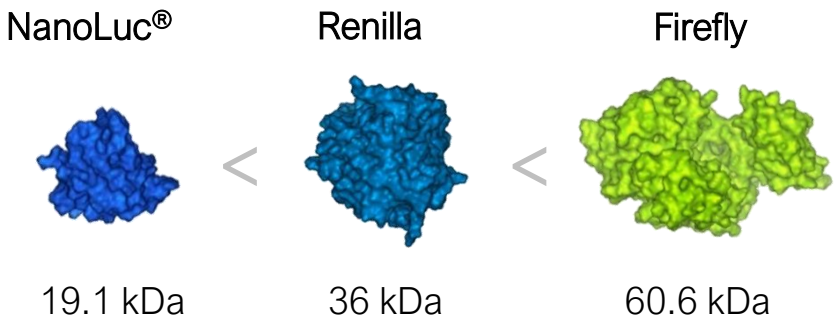
NanoLuc® Luciferase

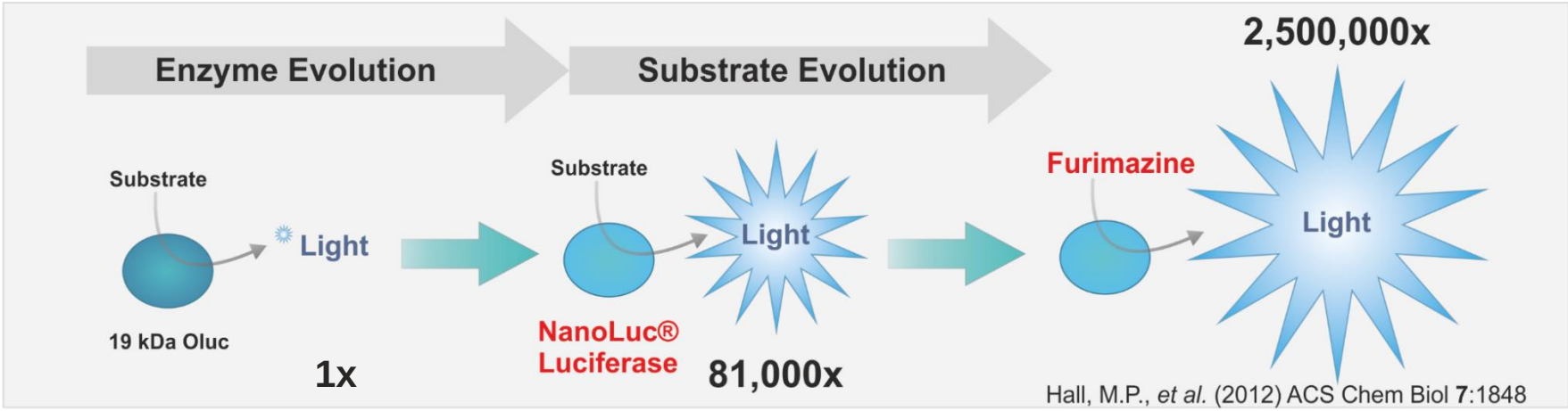
A Bright & Small Experimental Reporter

Bright, Brighter, NanoLuc®



Small, Smaller, NanoLuc®





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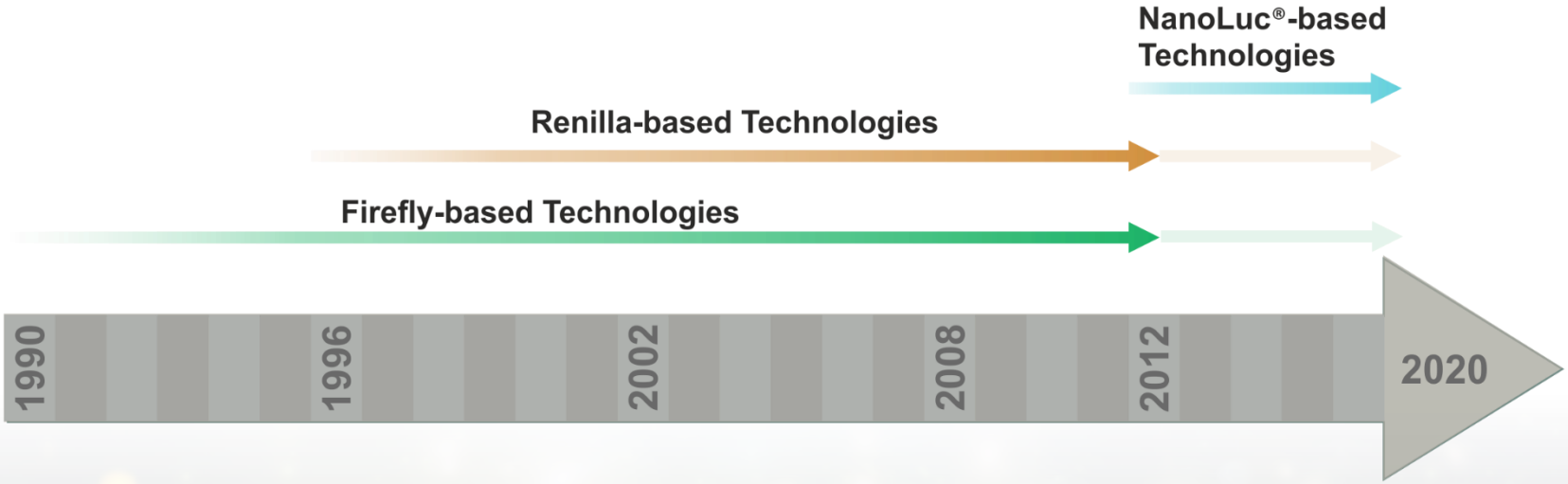
A Continuously Grown Expertise in Luciferase-based Technologies



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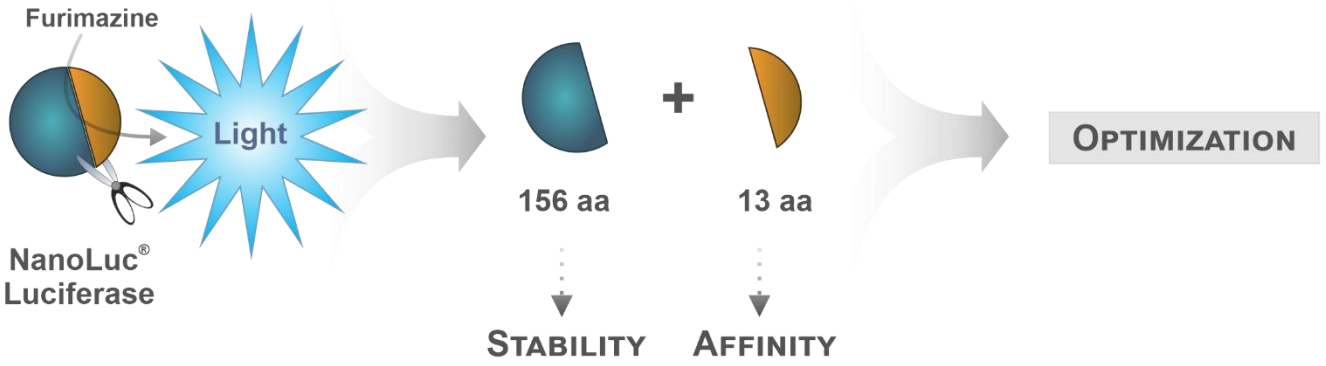
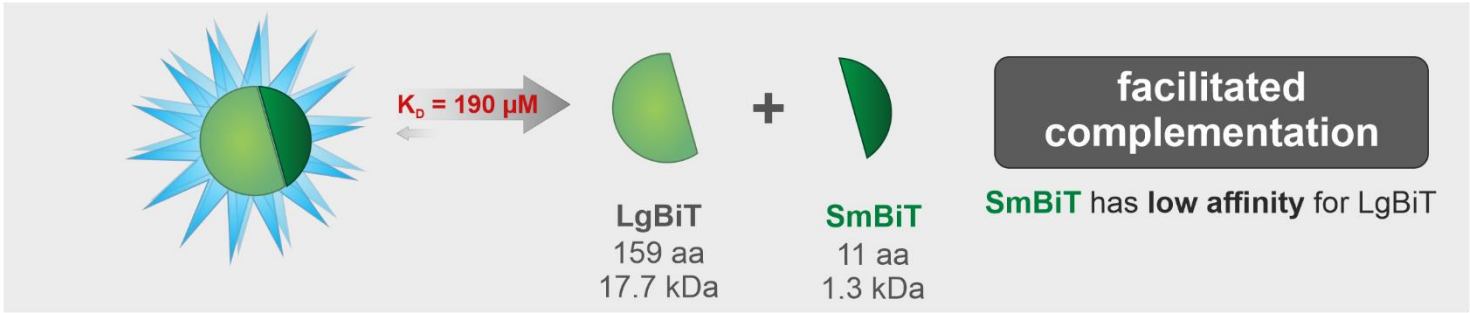
- HiBiT Protein Tagging System
- Lumit™ Immunoassays

NanoBiT® Technology Platform

NanoLuc® Binary Technology (NanoBiT®)

A Structural Complementation Reporter Designed for Biomolecular Interaction Studies





NanoBiT® Protein:Protein Interaction System

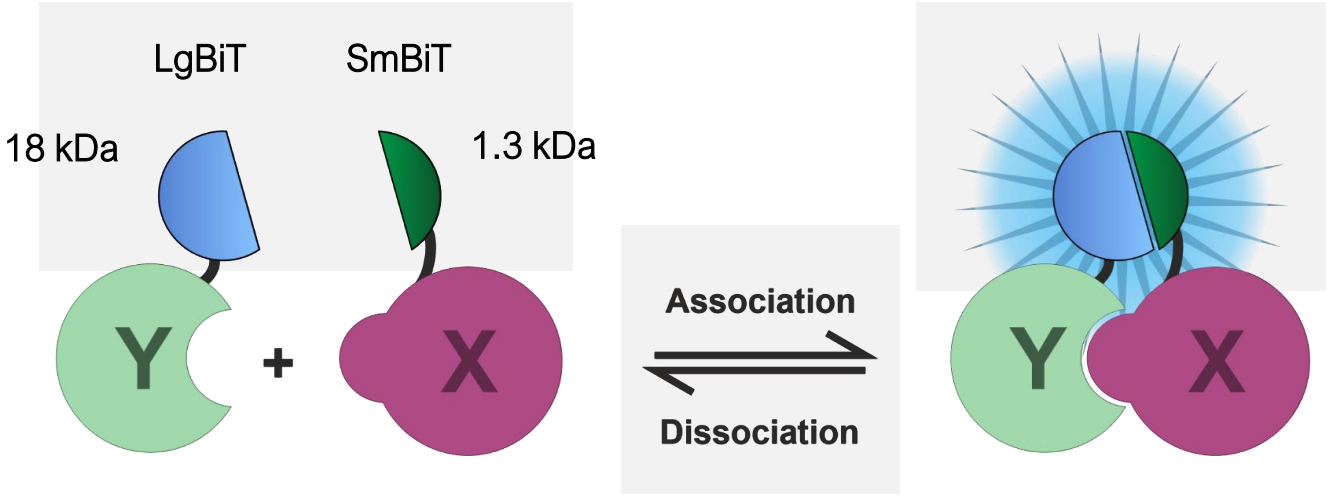
Investigate Interaction Dynamics in Live Cells

Small tag size

minimal influence on fusion partner

Bright signal upon complementation

enables low expression levels



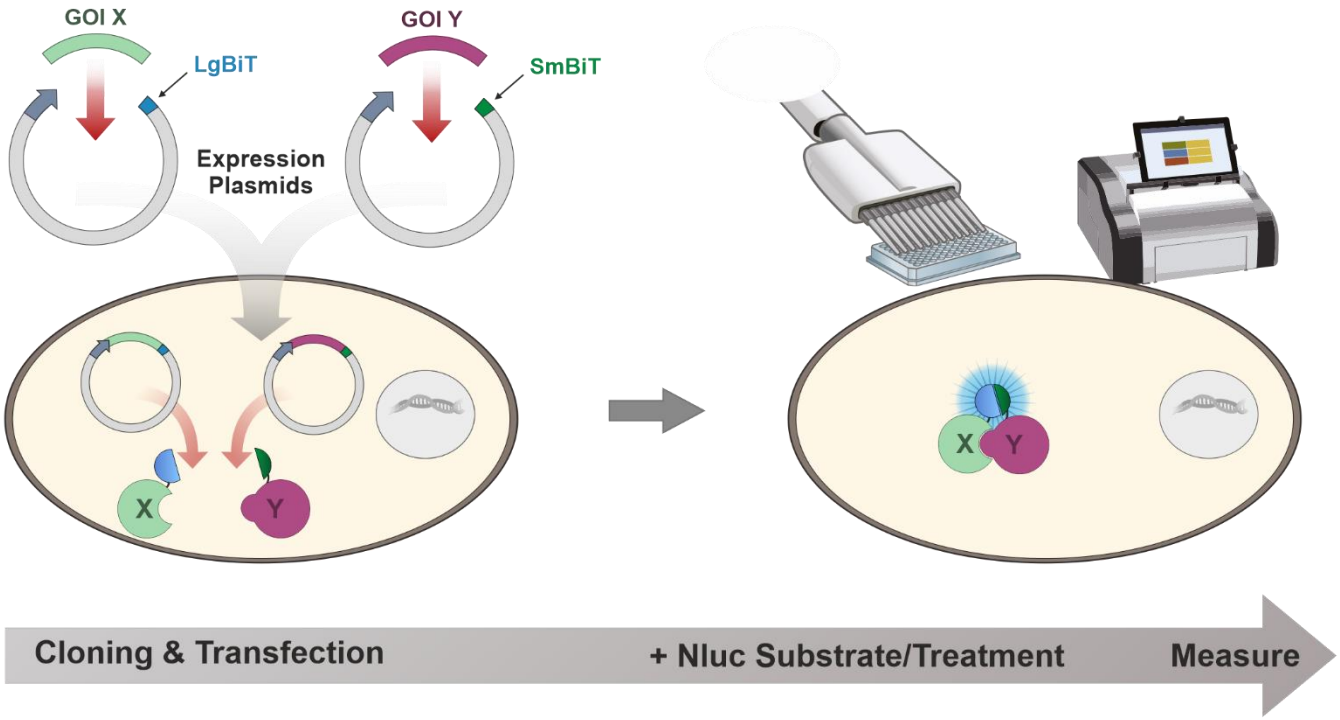
Low intrinsic affinity

reversible to allow investigation of PPI dynamics

increased signal specificity

NanoBiT® PPI Workflow

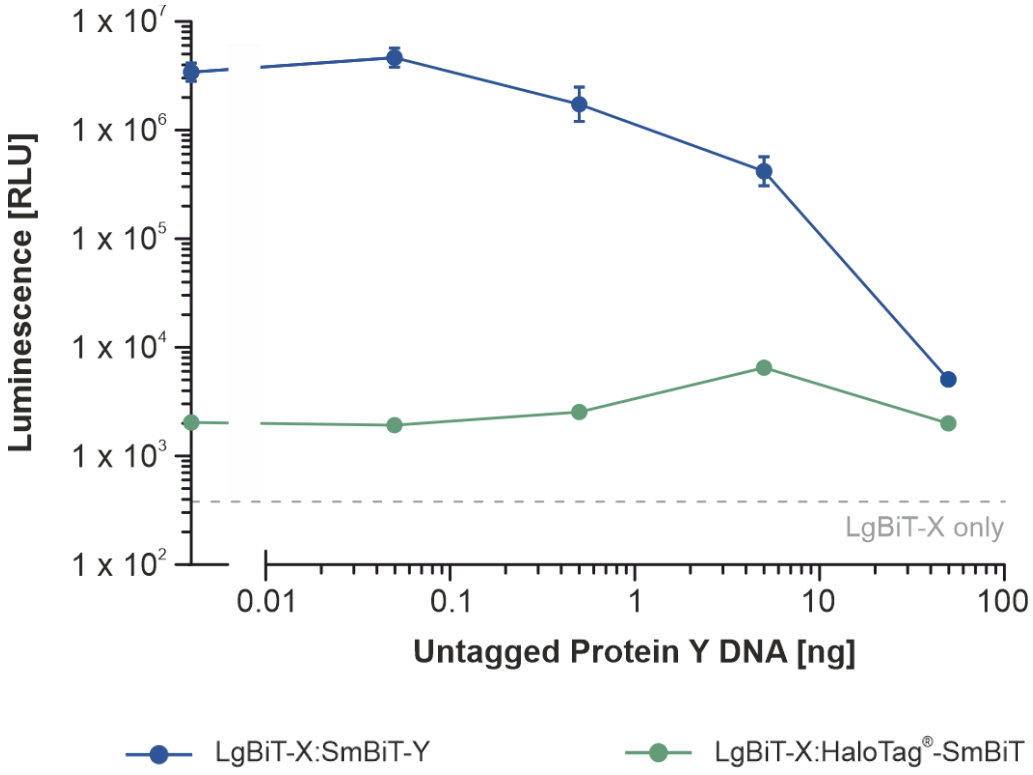
A Simple Transfection-based Experiment



1. Determine optimal LgBiT/SmBiT combinations that shows maximal fold signal increase
   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)

Competition with Untagged Protein

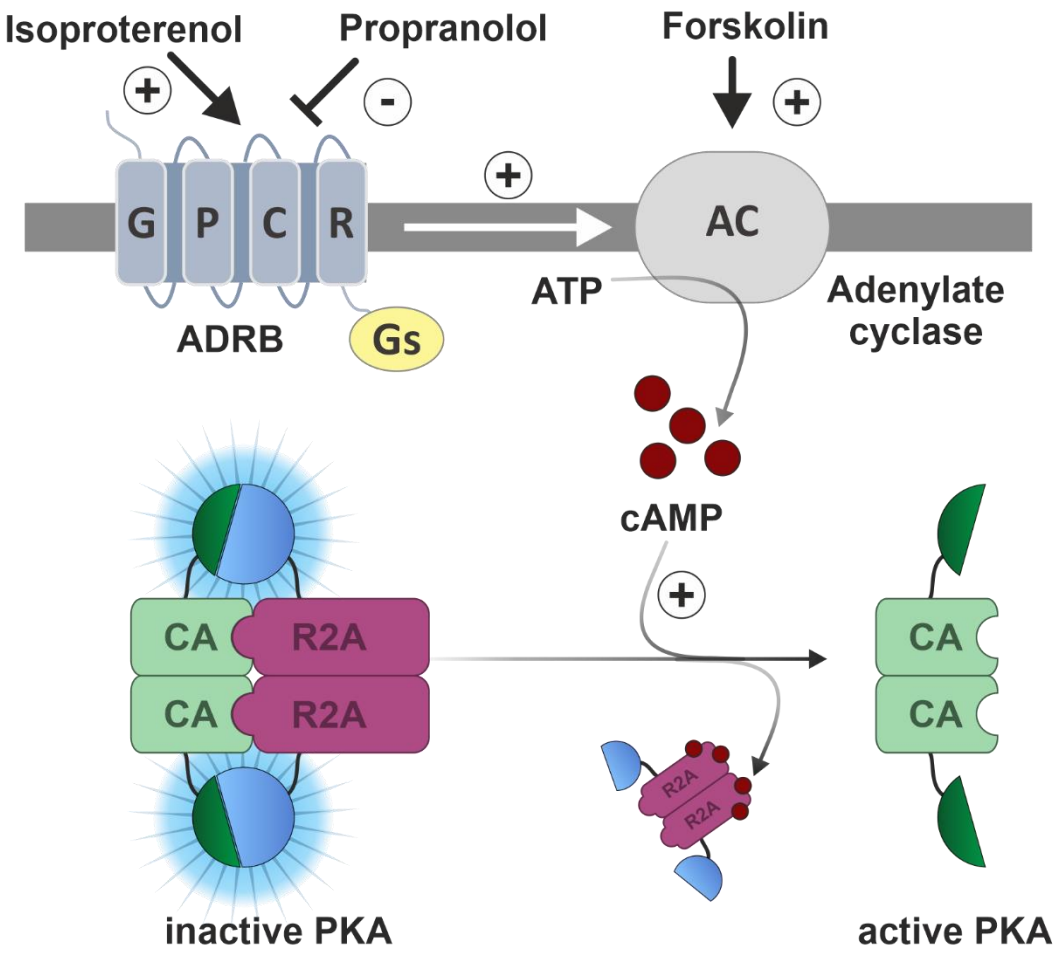
Check for Specificity in NanoBiT® PPI Assays



- Titrate the level of untagged fusion partner
- # of unproductive pairings ↑
- Specific interaction will show decrease in luminescence
- Nonspecific interaction will show no change in luminescence

Validation of NanoBiT® PPI

The Protein Kinase A Model



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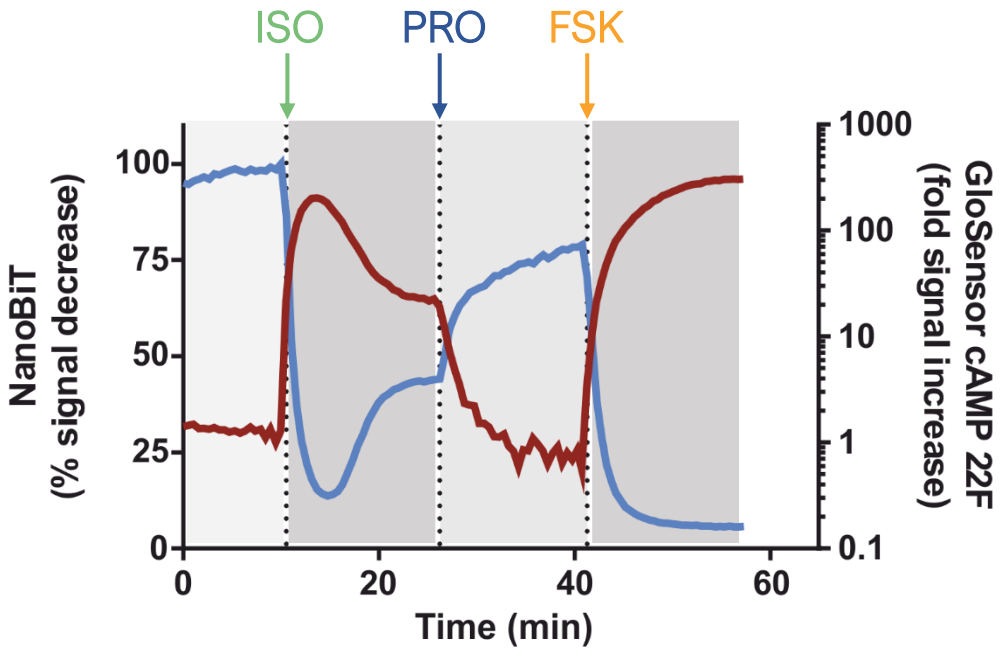


Isoproterenol (ISO)
ADRB agonist (cAMP ↑)

Propranolol (PRO)
ADRB antagonist (cAMP ↓)

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

Conclusions

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

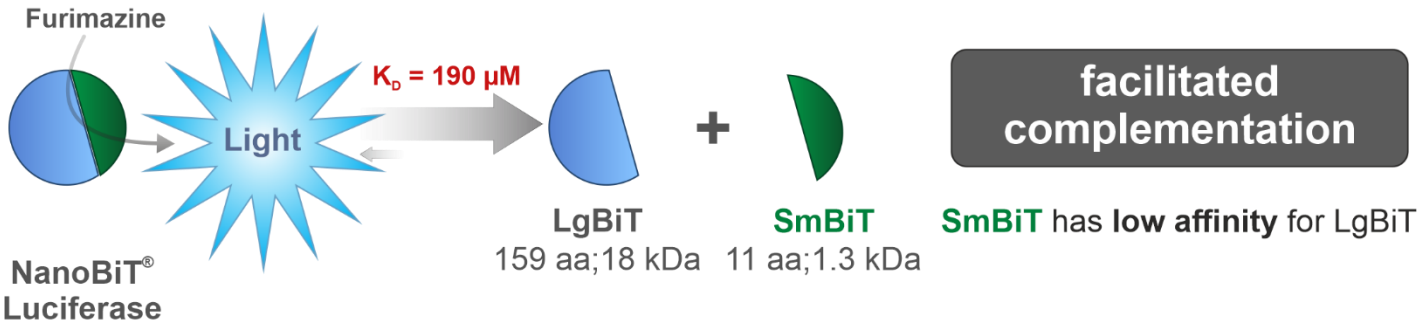
Ready-to-use NanoBiT® PPI Assays

- Pre-optimized *ready-to-use* protein:protein interaction pairs
- *Ready-to-use* stable cell lines
- BiBiT System: Generate your own stable cell line
- Available through **E**lite **A**ccess (**EA**)

AR:AR (NR3C4/NR3C4)	EGFR(1-673):EGFR(1-673)	BRD4:Histone 3.3	L3MBTL3:BCLAF1
MYC:MAX	AR:SRC1	EPOR(1-273):EPOR(1-273)	PDGFRA(1-549):PDGFRA(1-549)
FKBP:FRB	KRAS 2A G12D:CRAF	GR:GR (NR3C1:NR3C1)	PDGFRA(1-549):PDGFRB(1-553)
p53:MDM2	PD-1:SHP1	EGFR:GRB2	PDGFRB(1-553):PDGFRB(1-553)
PRKACA:PRKAR2A	PD-L1:PD-L1	HER1(1-668):HER2(1-675)	RELA:NFKBIA
BRAF:CRAF	PD-L2:PD-L2	HER1(1-668):HER3(1-664)	
CRAF:CRAF	CX3CR1:ARRB2	HER3(1-664):HER3(1-664)	
BRAF:BRAF	ADRB2:ARRB2	KRAS (G12C):CRAF	
HER2(1-657):HER3(1-664)	AVPR2:ARRB2	KRAS (G12C):CRAF	
VEGFR1(1-785):VEGFR1(1-785)	BCL2:BAK	KRAS:CRAF	

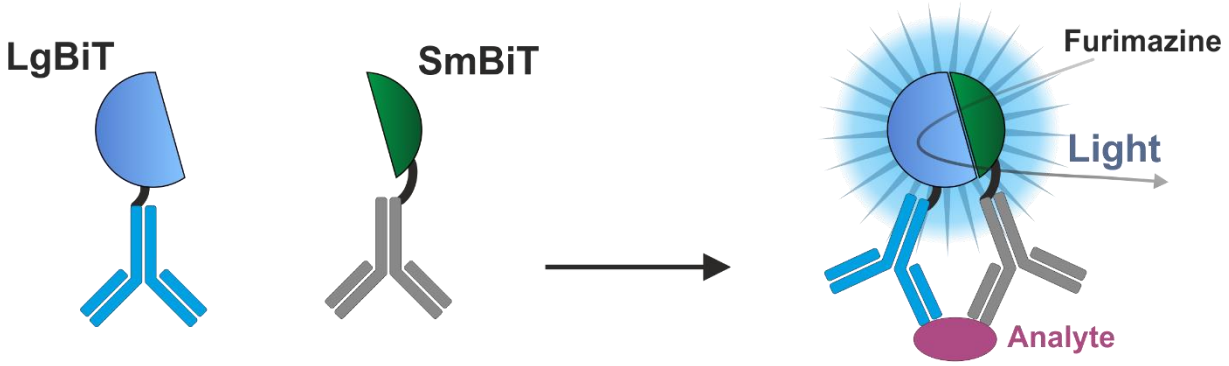
NanoLuc® Binary Technology (NanoBiT®)

A Structural Complementation Reporter Designed for Biomolecular Interaction Studies



Complementation facilitated through … (indirect) Ab:Ab "interaction" → Lumit™ Immunoassays

Hwang *et al*. 2020, *Commun Biol.*



Lumit™ Immunoassays

The Powerful Alternative to Conventional Immunoassay Approaches

Traditional ELISA Workflow

- Traditional ELISA is a heterogenous multistep process involving several wash / incubation steps

Lumit™ Immunoassays

The Powerful Alternative to Conventional Immunoassay Approaches

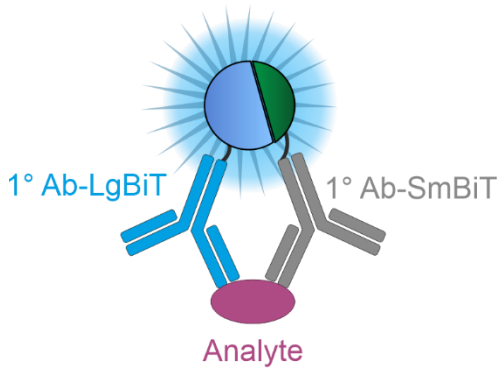
- Traditional ELISA is a heterogenous multistep process involving several wash / incubation steps
- Based on NanoLuc® luciferase we developed *Lumit™ Immunoassays*
  - ✓ Easy and fast (30 – 120 min)
  - ✓ High Sensitivity (low number of cells)
  - ✓ Broad dynamic range (3 – 4 logs)
  - ✓ Flexible formats (96- or 384-well)
  - ✓ Homogenous and HTS compatible

Hwang, B. *et al.* (2020) Commun Biol. 3:8

Lumit™ Immunoassays

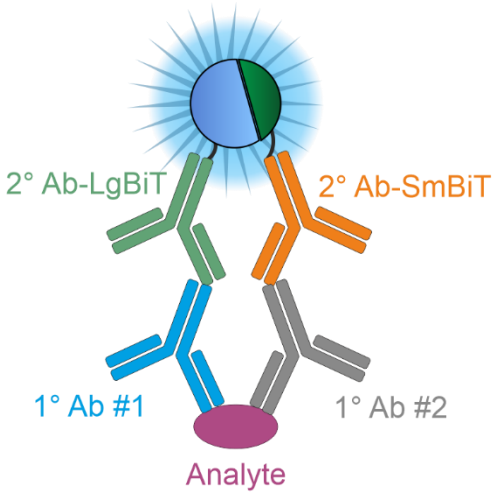
Different Formats for Maximum Flexibility

Direct



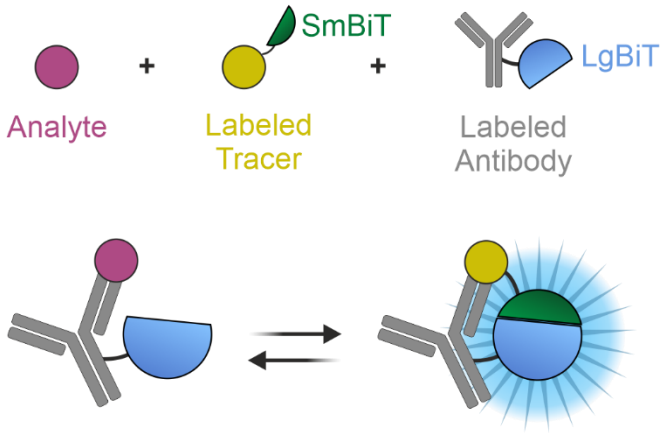
- Requires labeling of 1°Abs
- Validated for cytokines and peptide hormones
- *Ready-to-use* assays for
  - ✓ IL1-β, IFN-γ, IL-2, IL-6, IL-10, IL-4, TNF-α, VEGF, …
  - ✓ Insulin and glucagon

Indirect



- Avoids labeling of 1°Abs
- Generic pre-labeled 2°Abs (different species available)
- Validated for intracellular PTMs, e.g. phosphorylation

Competitive



- Requires antibody and tracer labeling
- Establish competitive antibody binding assays
- *Ready-to-use* assays for hFcRn:Fc protein interactions
  - ✓ Lumit™ FcRn Binding Immunoassay
  - ✓ hFcγ receptor assays *(under development)*

Lumit™ Immunoassays

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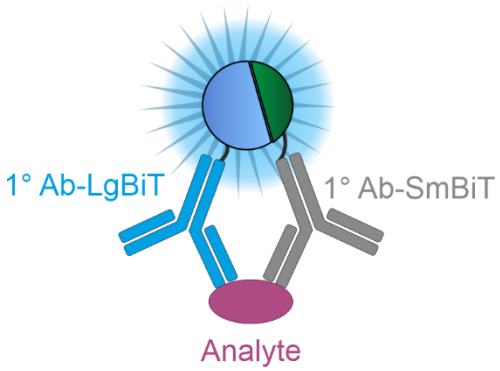

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- Requires antibody and tracer labeling
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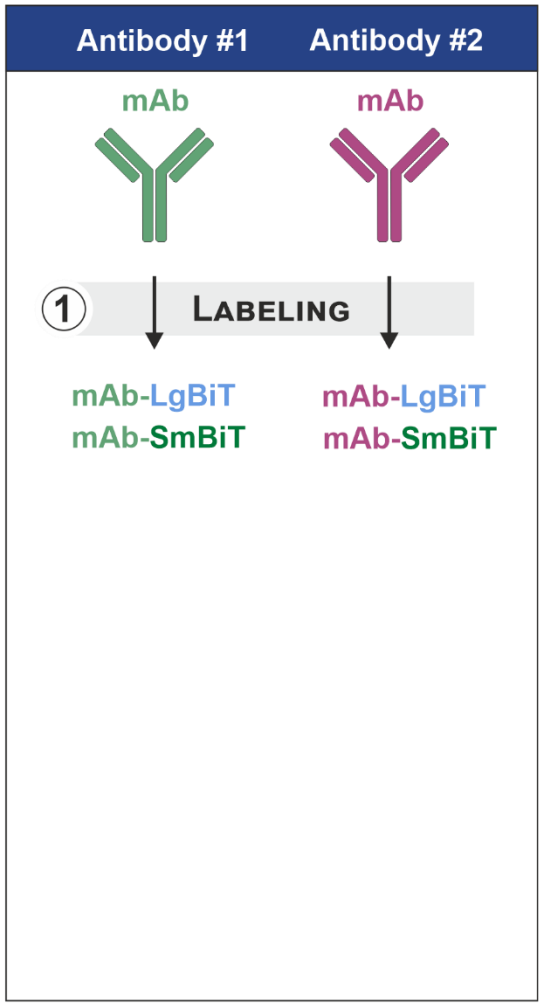
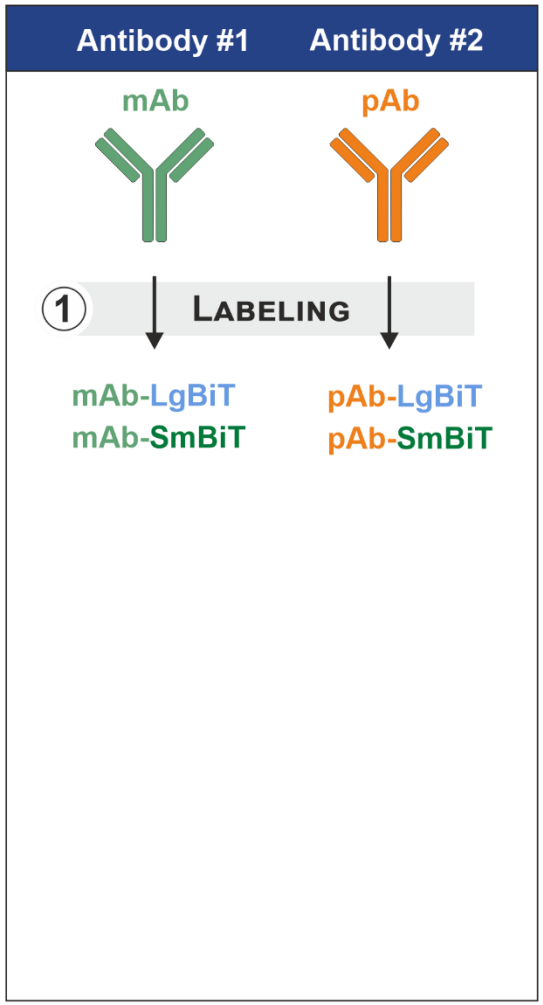
- *Ready-to-use* assays for hFcRn:Fc protein interactions
  - ✓ Lumit™ FcRn Binding Immunoassay
  - ✓ hFcγ receptor assays *(under development)*

Build-Your-Own Direct Lumit™ Immunoassay

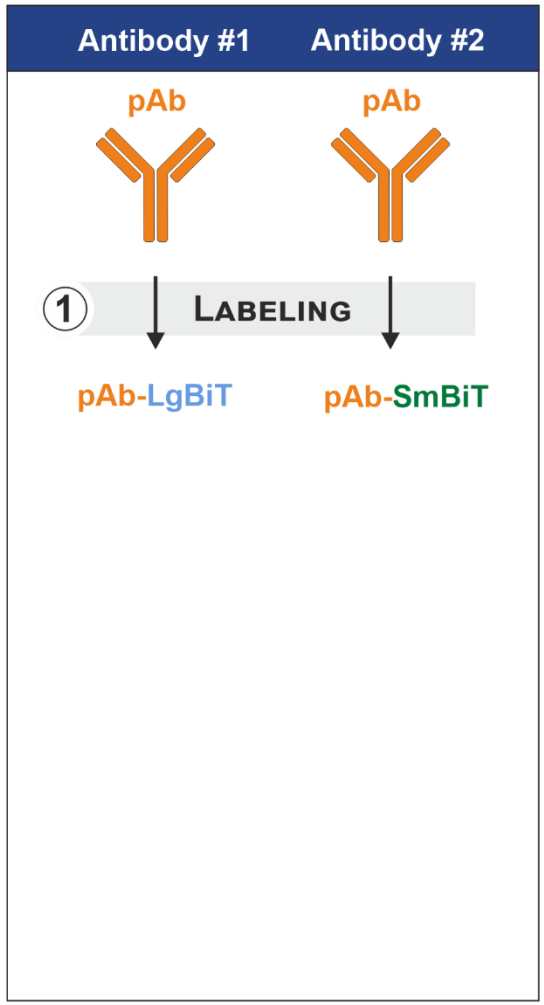
Various Options for Your Convenience



☺ highest specificity and sensitivity



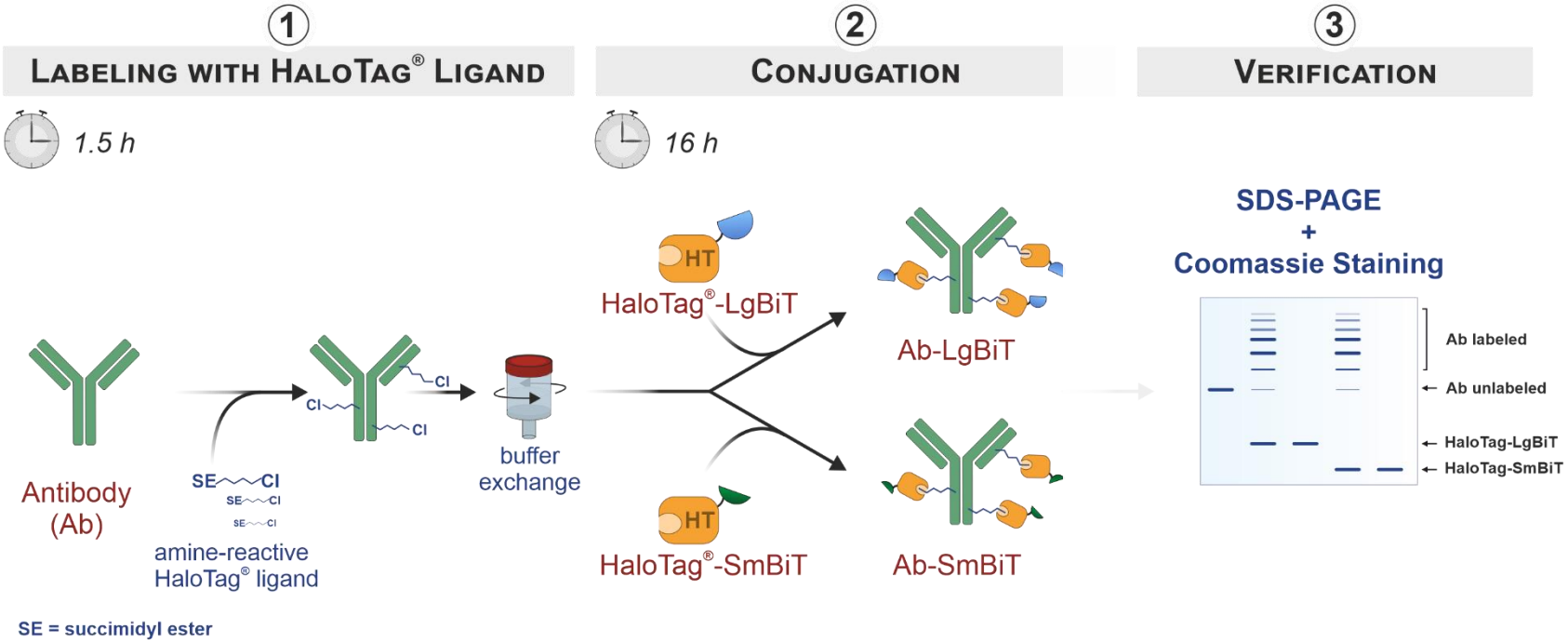
☺ only one mAb needed



☺ most inexpensive variant

Build-Your-Own Direct Lumit™ Immunoassay

Step 1: Labeling of Antibodies with the Lumit™ Immunoassay Labeling Kit



FACTS

- Easy and robust 2-day protocol
- Attachment is highly efficient (> 90%)
- Oriented BiT subunits for maximum activity

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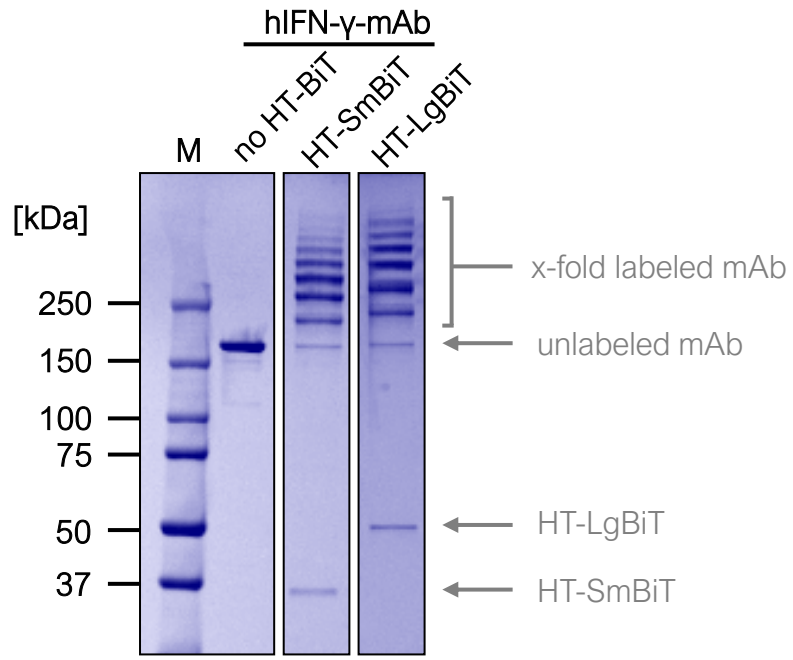
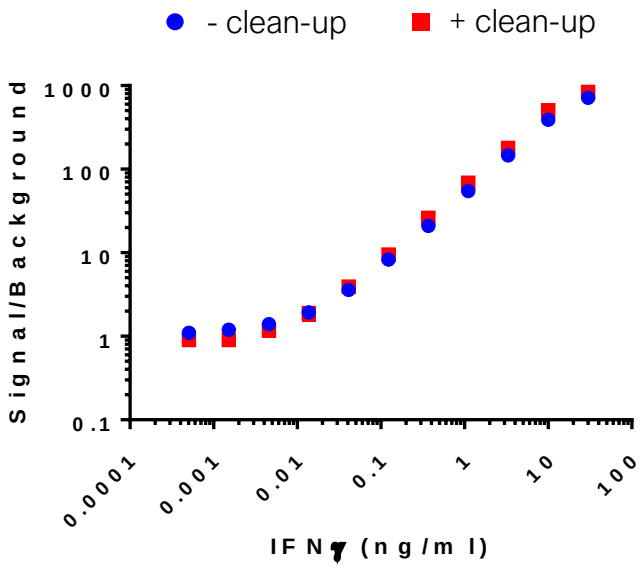


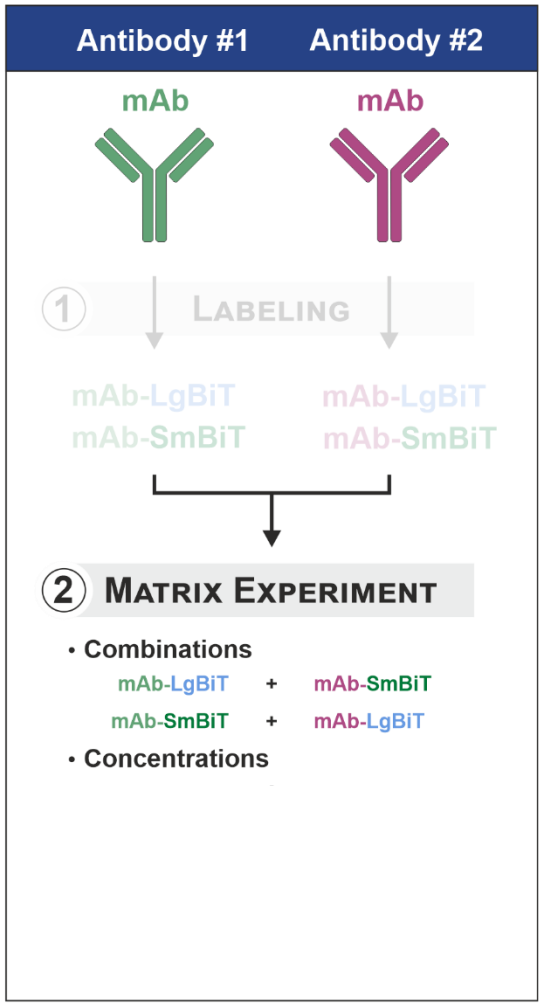


Facts

- Easy and robust 2-day protocol
- Attachment is highly efficient (> 90%)
- Oriented BiT subunits for maximum activity
- Removal of unbound HT-BiTs is not required

Build-Your-Own Direct Lumit™ Immunoassay

Various Options for Your Convenience



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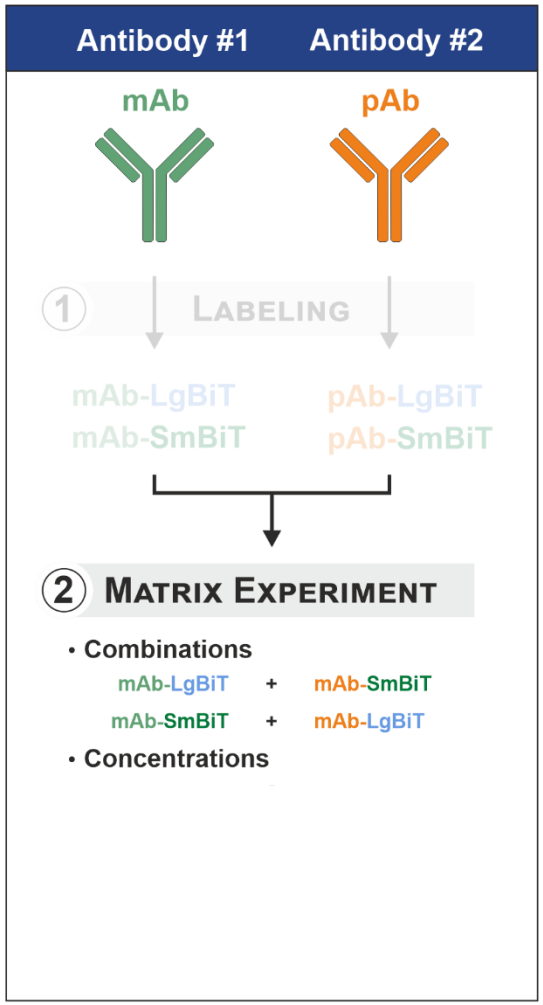

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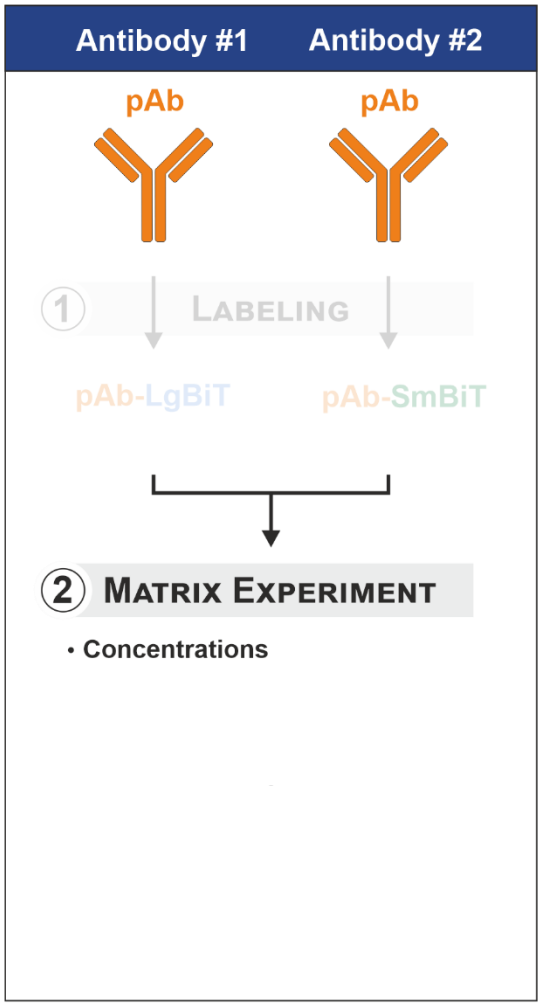
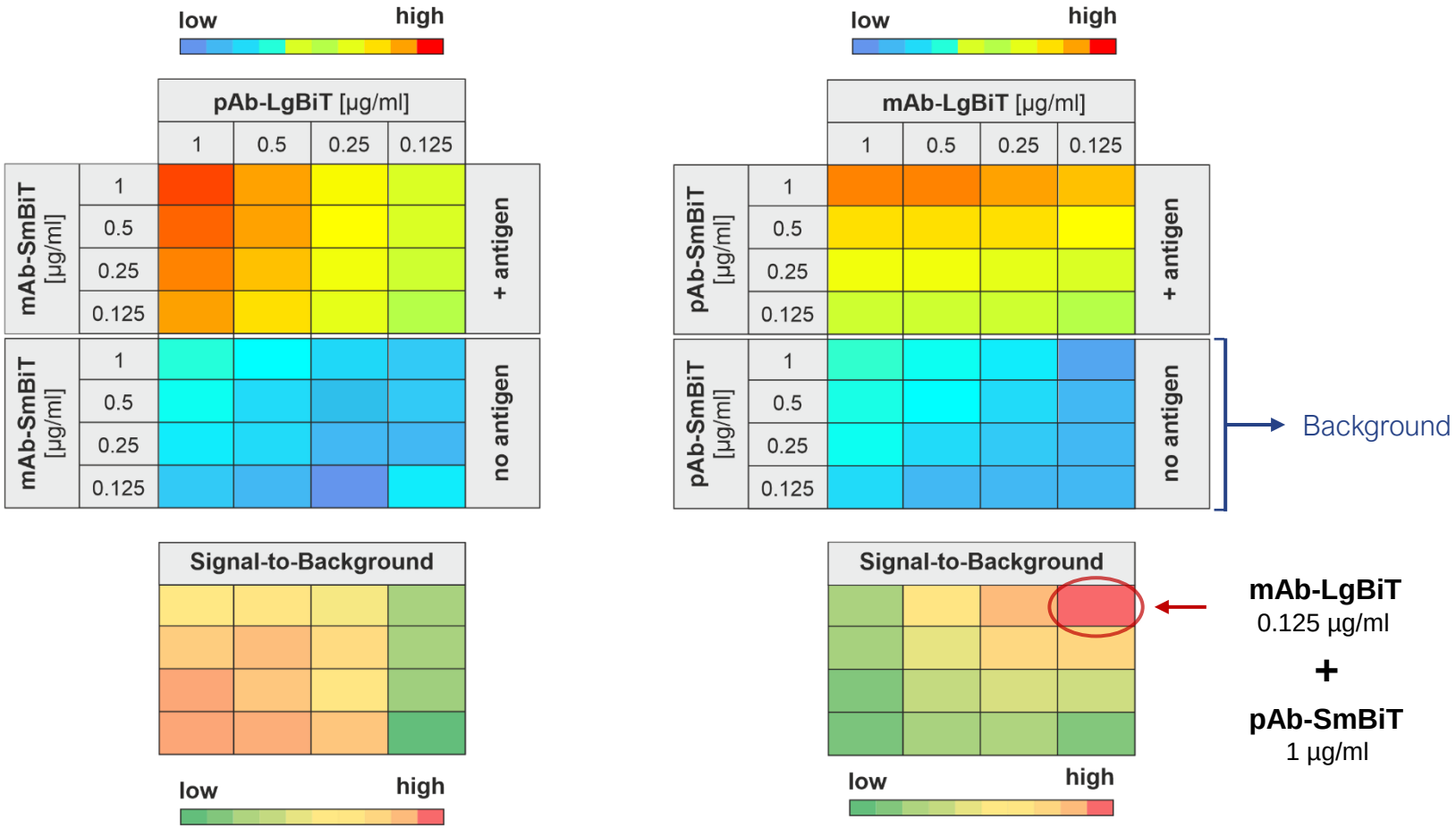


☺ most inexpensive variant

Build-Your-Own Direct Lumit™ Immunoassay

Step 2: Identification of best antibody combination/concentration

Matrix experiment to determine maximal signal-to-background (S/B) ratio



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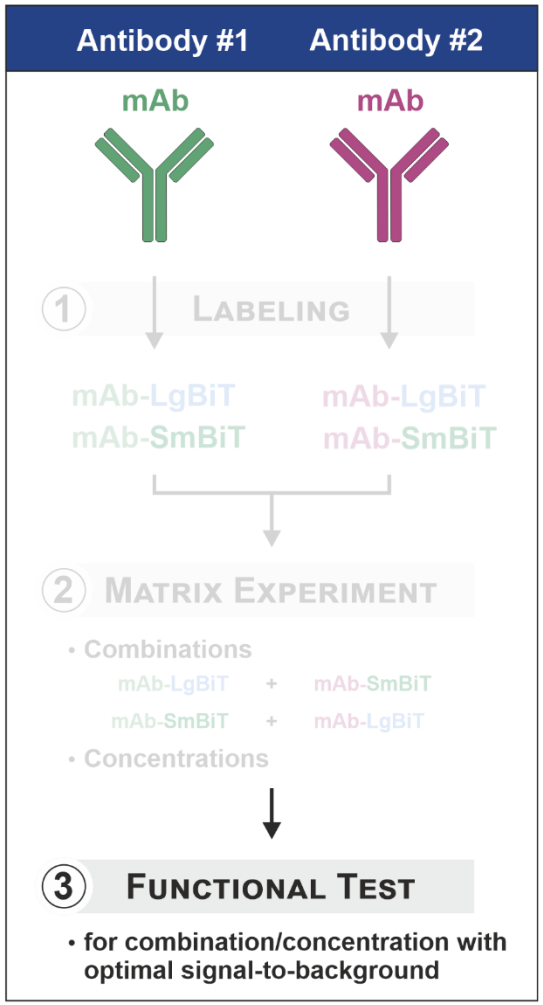

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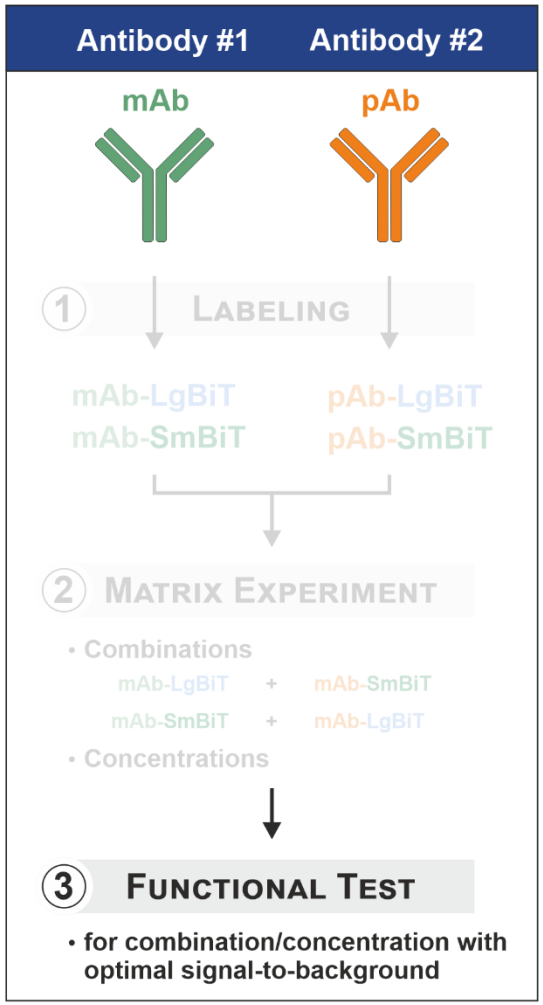

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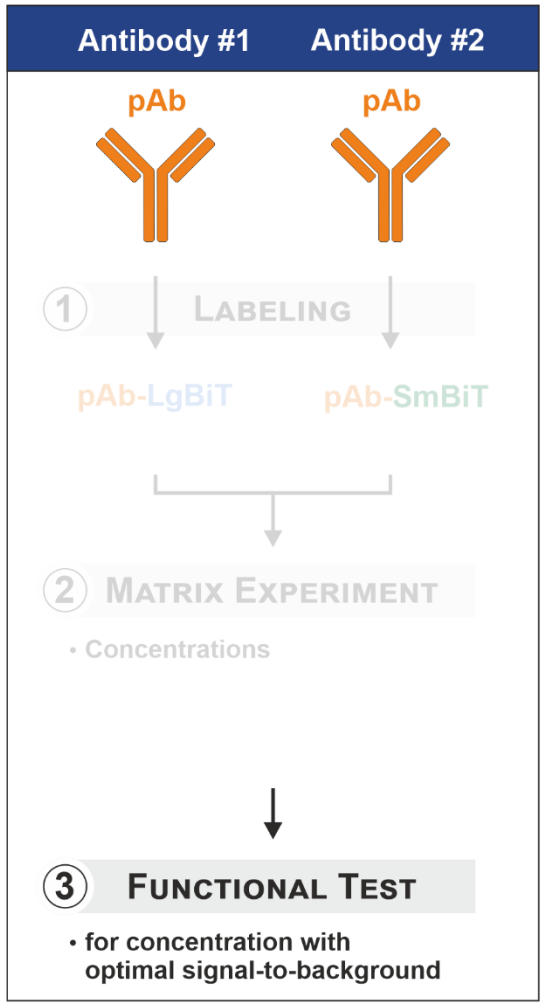


☺ most inexpensive variant

Build-Your-Own Direct Lumit™ Immunoassay

Step 3: Functional Test of Identified Antibody Combination

- **Treatment of cells**
- **Add standard dilutions**

- **Add NanoBiT®-labeled 1° Ab mixture**

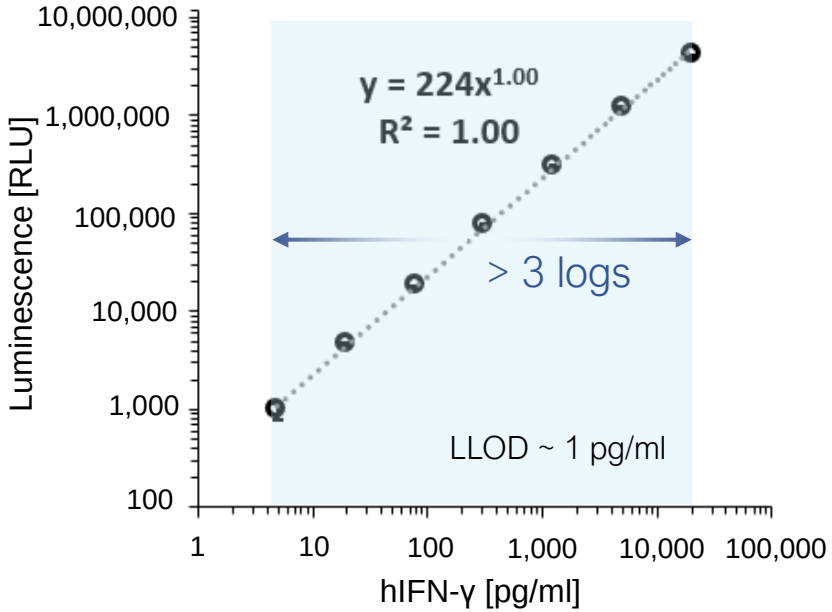
  directly to standard, cells ***or*** *aliquot of supernatant*

- **Incubate**

- **Add Detection Reagent**

- **Measure Luminescence**

Lumit™ (human) IFN-γ Immunoassay



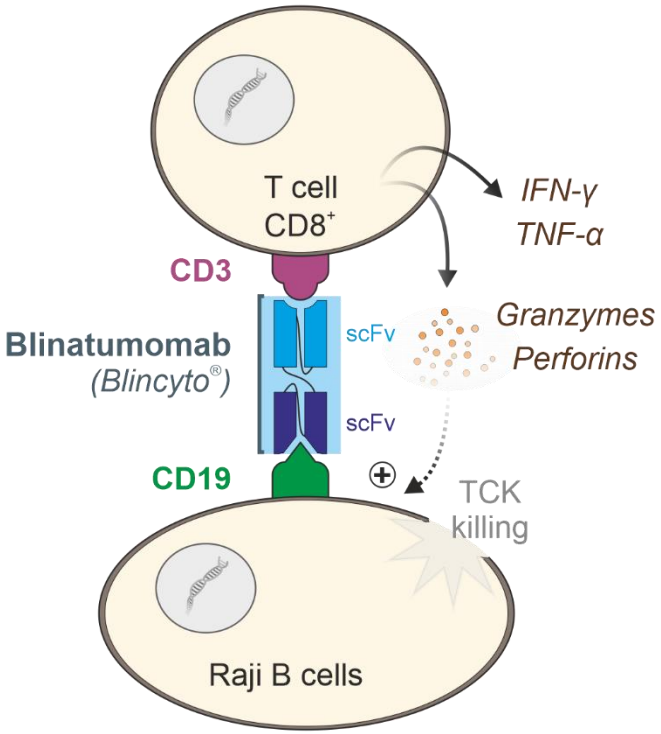
OBSERVATIONS

- Good sensitivity and broad dynamic range (≥ 3 logs)
- No need for sample dilution

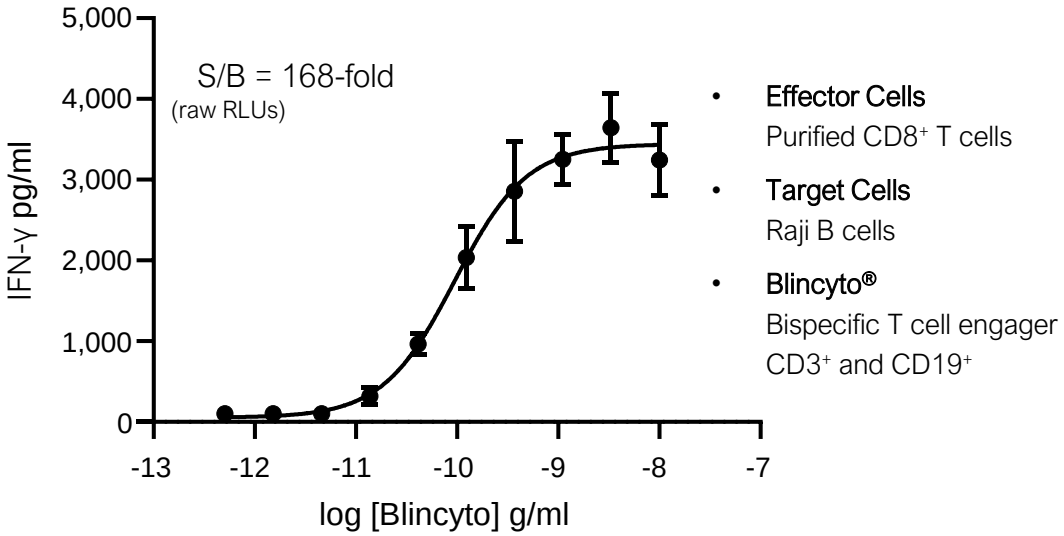
Build-Your-Own Direct Lumit™ Immunoassay

Step 3: Functional Test of Identified Antibody Combination

BiTE-induced IFN-γ release from CD8⁺ T cells



Lumit™ (human) IFN-γ Immunoassay



OBSERVATIONS

- Good sensitivity and broad dynamic range (≥ 3 logs)
- No need for sample dilution
- Excellent cell-based performance (addition to cells)

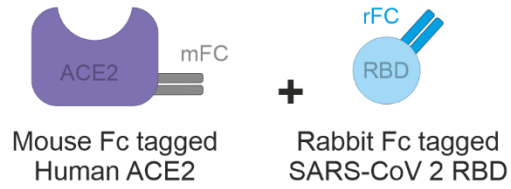
BiTE: Bispecific T cell engager

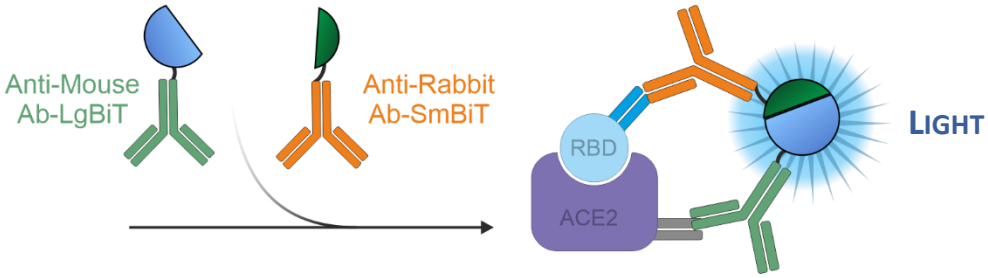
scFv: single-chain variable fragment

Measure Protein:Protein Interactions

Lumit™ SARS-CoV-2 Spike RBD:hACE2 Immunoassay

Detection of neutralizing antibodies and other inhibitory molecules that block the interaction of SARS-CoV-2 and the human surface protein ACE2





Safe

- In contrast to plaque reduction neutralization test (PRNT) no need for high BSL environment

Fast

- Faster than PRNT and other ELISA-based surrogate virus neutralization tests (VNT): hours vs. days

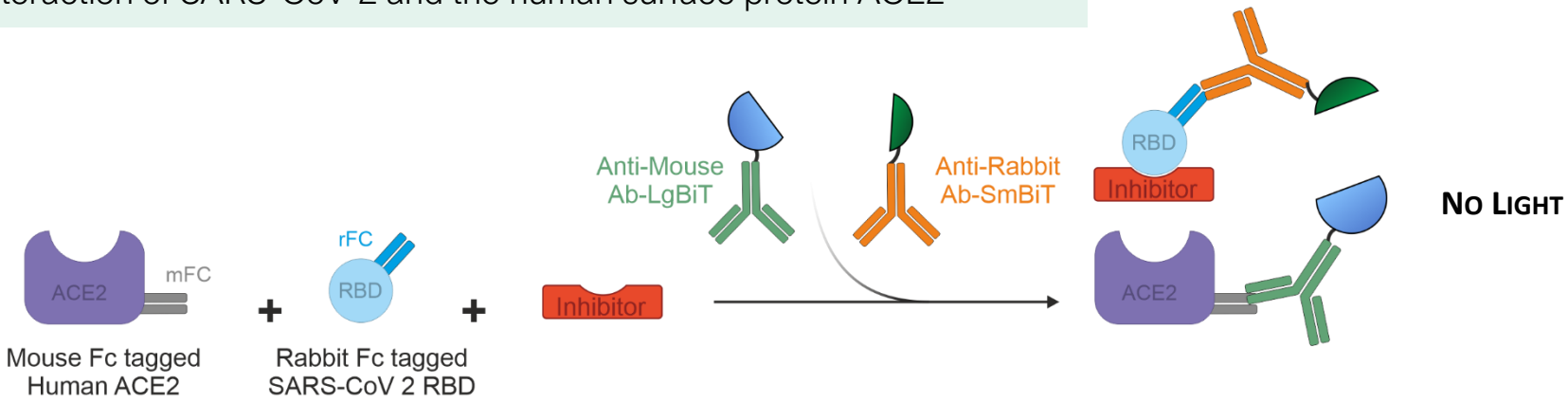
Homogenous, in-solution assay format

- No immobilization, blocking, washing, and transfer steps
- Flexible formats (96- or 384-well)
- Amenable to HTS

Measure Protein:Protein Interactions

Lumit™ SARS-CoV-2 Spike RBD:hACE2 Immunoassay

Detection of neutralizing antibodies and other inhibitory molecules that block the interaction of SARS-CoV-2 and the human surface protein ACE2



Safe

- In contrast to plaque reduction neutralization test (PRNT) no need for high BSL environment

Fast

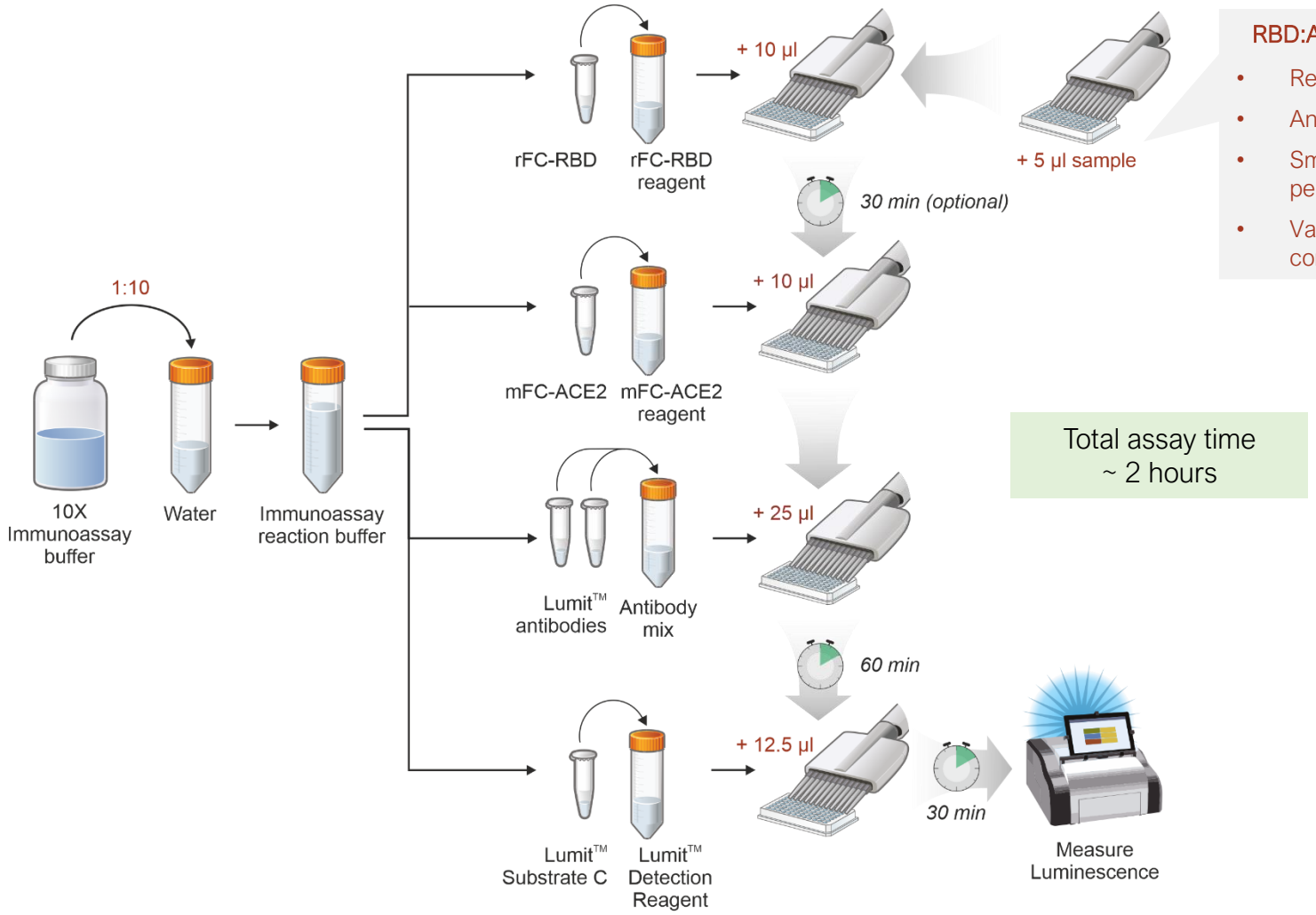
- Faster than PRNT and other ELISA-based surrogate virus neutralization tests (VNT): hours vs. days

Homogenous, in-solution assay format

- No immobilization, blocking, washing, and transfer steps
- Flexible formats (96- or 384-well)
- Amenable to HTS

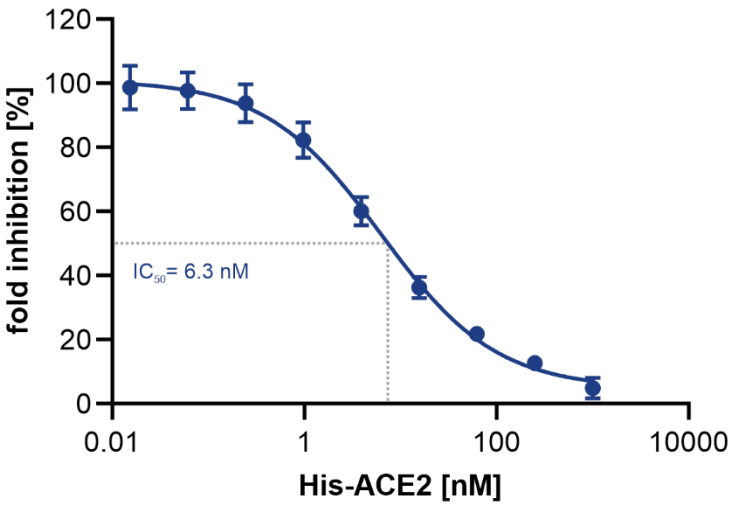
Lumit™ SARS-CoV-2 Spike RBD:hACE2 Immunoassay

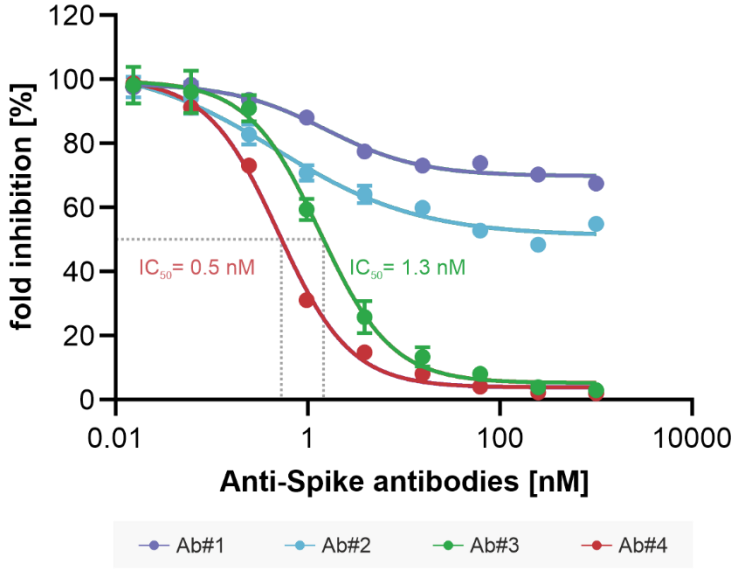
A Homogenous and Fast to Accomplish Workflow



Lumit™ SARS-CoV-2 Spike RBD:hACE2 Immunoassay

Soluble ACE2 & Neutralizing Antibodies





Lumit™ SARS-CoV-2 Spike RBD: hACE2 Immunoassay …

- detects the competition effect of a soluble ACE2 protein on the RBD:ACE2 PPI
- detects neutralizing antibodies that inhibit the interaction
- can be used as a surrogate virus neutralization test (VNT) to analyze patient serum neutralizing antibody levels post SARS-CoV-2 infection and/or vaccination (data not shown)

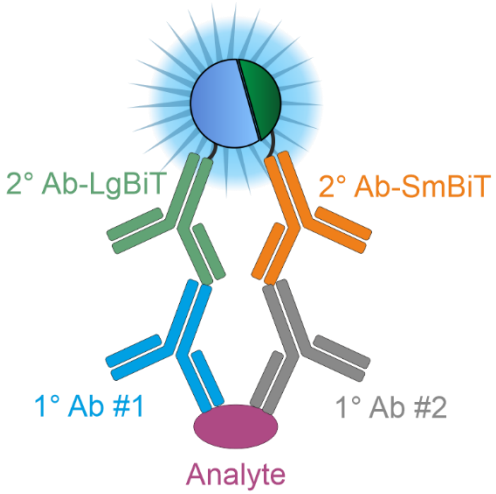
Lumit™ Immunoassays

Different Formats for Maximum Flexibility

Direct

- Requires labeling of 1°Abs
- Validated for cytokines and peptide hormones
- *Ready-to-use* assays for
  - ✓ IL1-β, IFN-γ, IL-2, IL-6, IL-10, IL-4, TNF-α, VEGF, …
  - ✓ Insulin and glucagon

Indirect



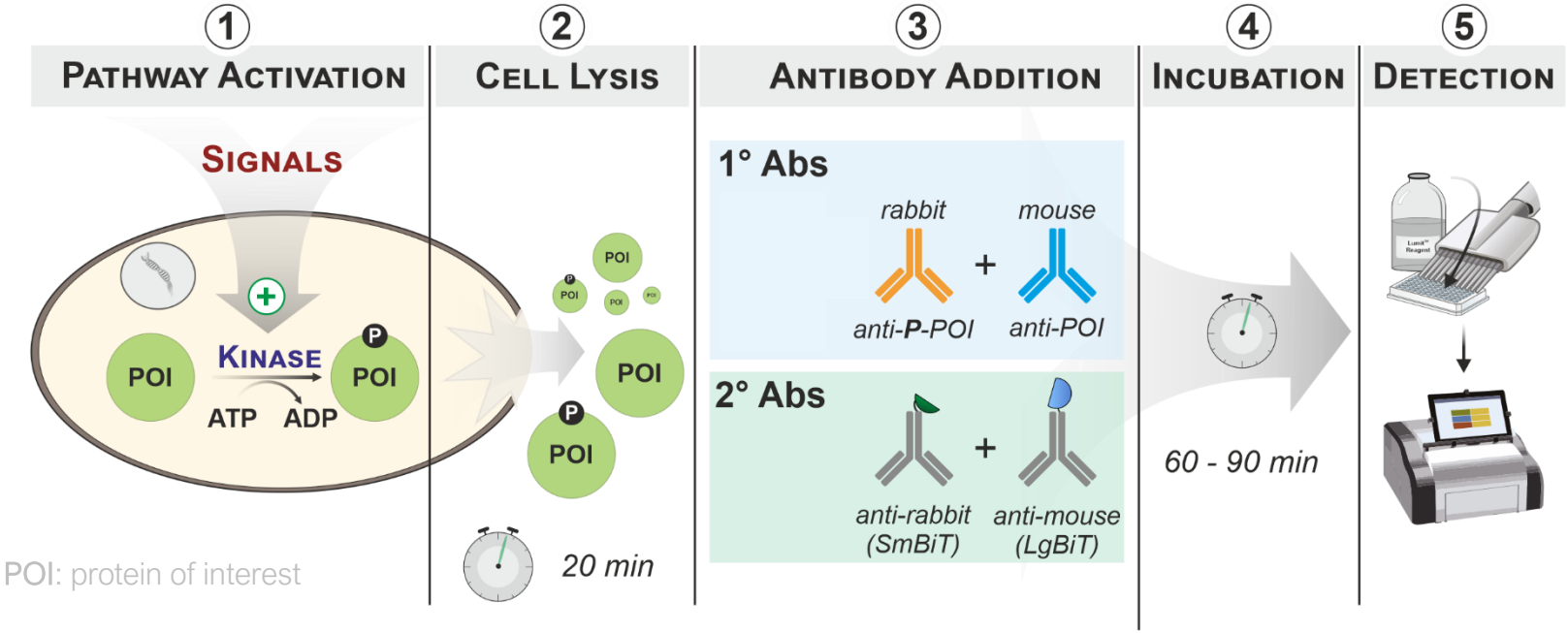
- Avoids labeling of 1°Abs
- Generic pre-labeled 2°Abs (different species available)
- Validated for intracellular PTMs, e.g. phosphorylation

Competitive

- Requires antibody and tracer labeling
- Establish competitive antibody binding assays
- *Ready-to-use* assay for hFcRn:Fc protein interactions, e.g. antibodies
  - ✓ Lumit™ FcRn Immunoassay

Lumit™ Immunoassay Cellular Systems

Study Cellular Signaling Events

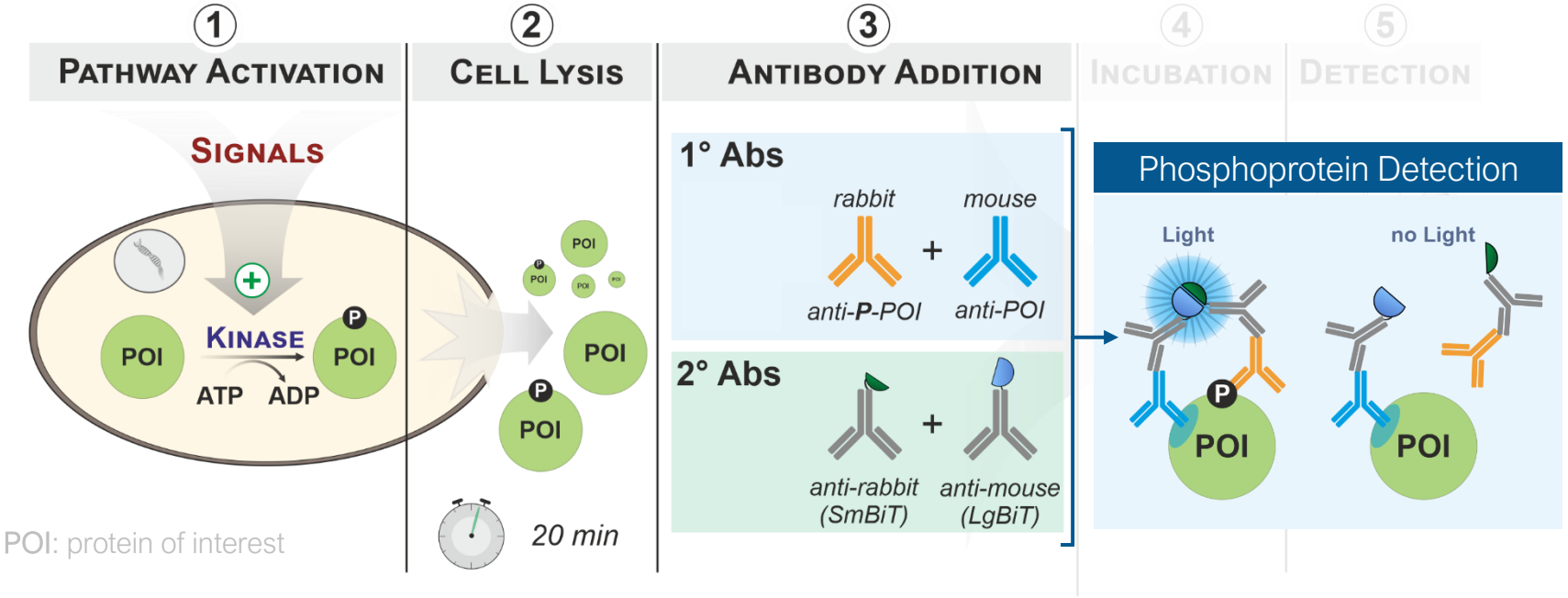


Additionally available pre-labeled 2° Abs:

- anti-rabbit (LgBiT)
- anti-mouse (SmBiT)
- anti-goat (LgBiT)
- anti-goat (SmBiT)

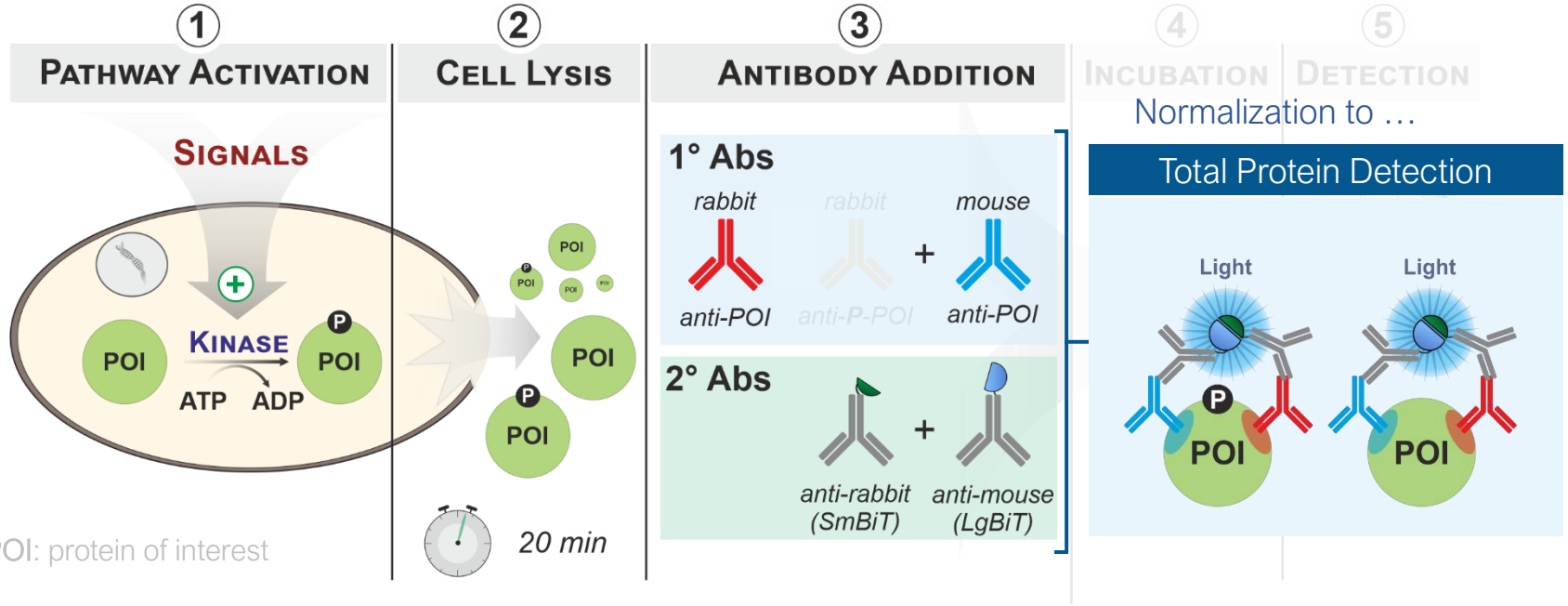
Lumit™ Immunoassay Cellular Systems

Study Cellular Signaling Events

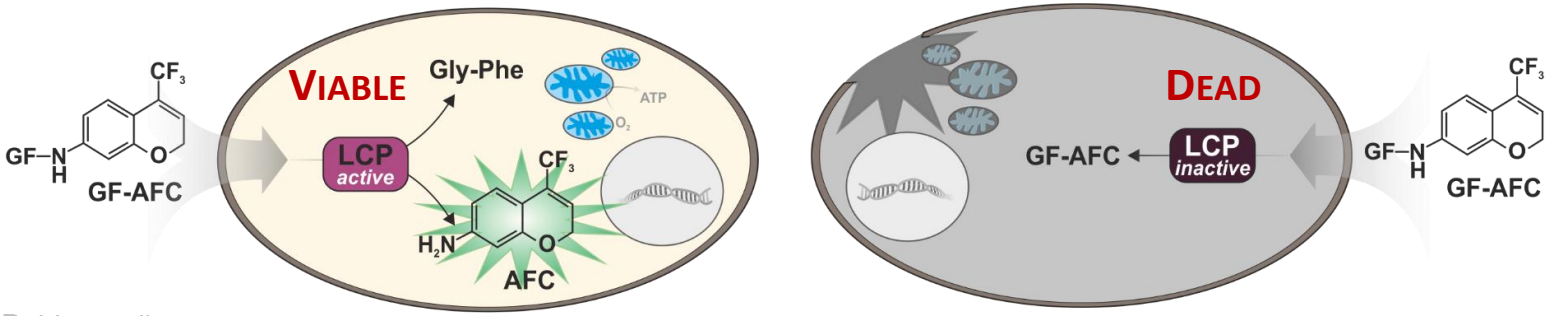


Lumit™ Immunoassay Cellular Systems

Study Cellular Signaling Events



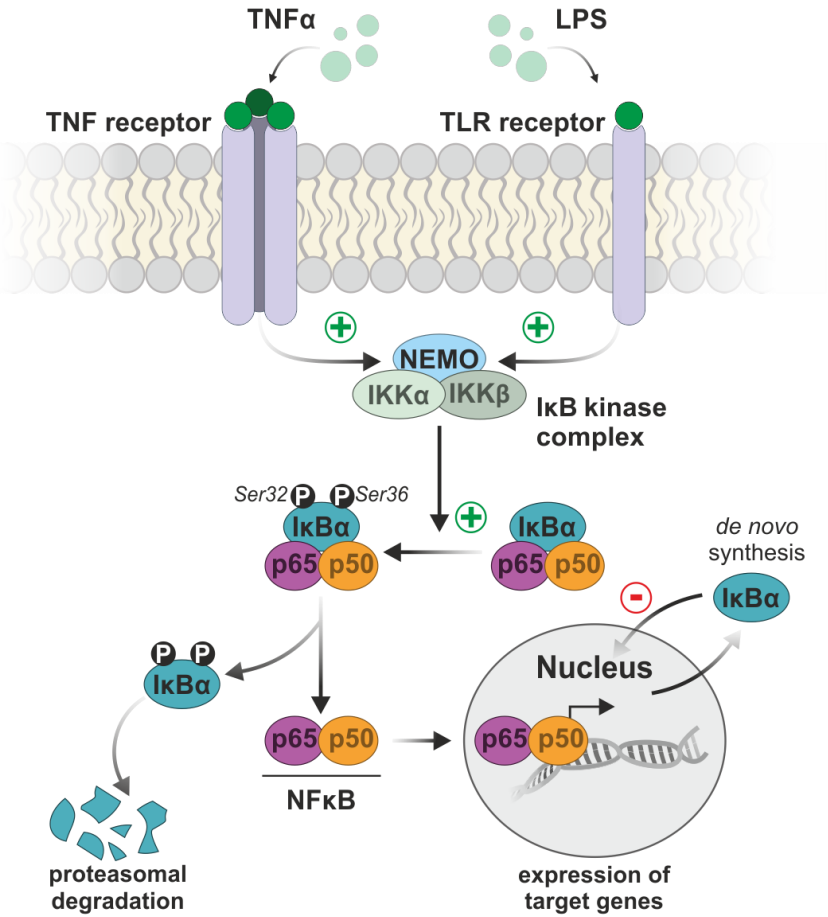
Normalization on number of viable cells using CellTiter-Fluor™

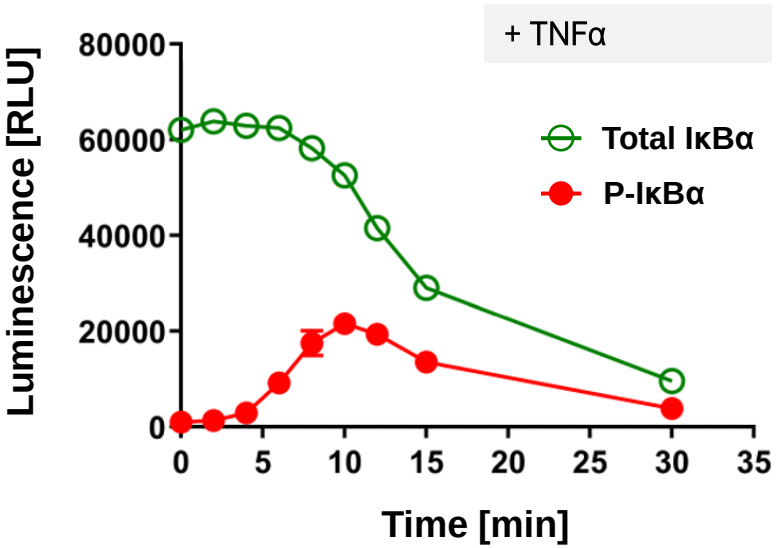


LCP: Live-cell protease

Signaling Pathway and Kinase Activity Analysis

Studying the NFκB Pathway





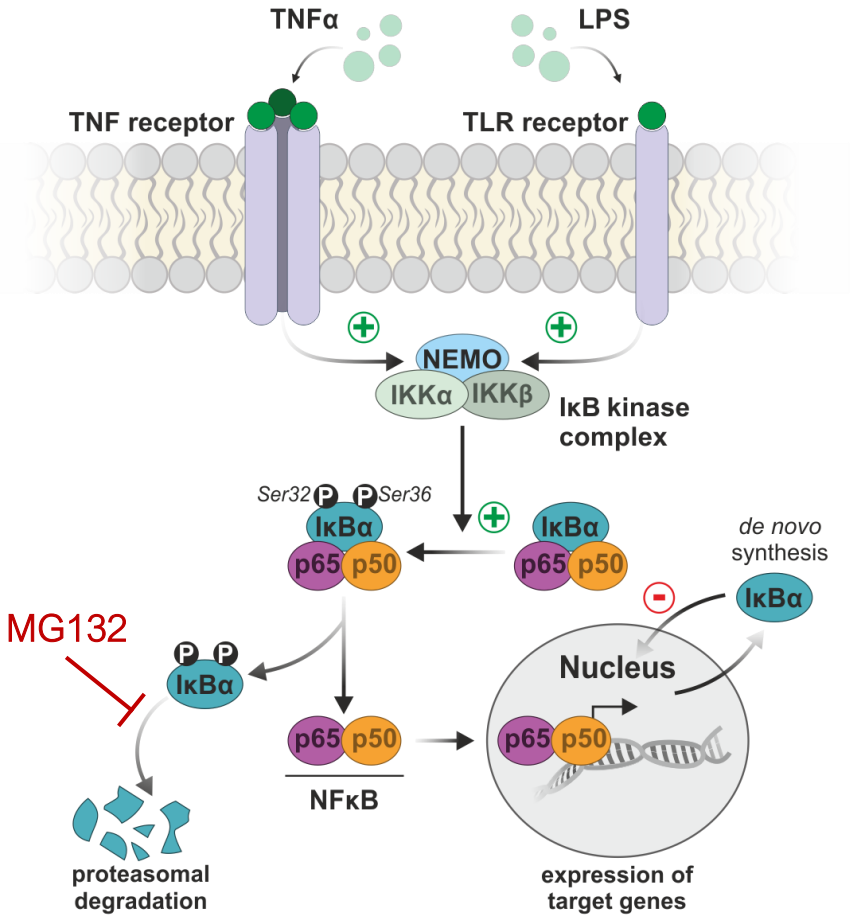
Modified from Hwang, B. *et al.* (2020) Commun Biol. 3:8

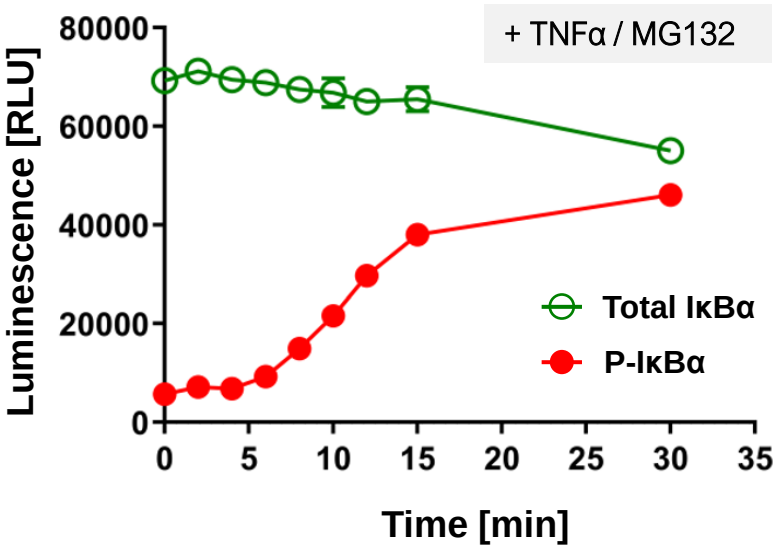
OBSERVATION

- IκBα phosphorylation at Ser32 (pS32)
- Immediately followed by rapid degradation

Signaling Pathway and Kinase Activity Analysis

Studying the NFκB Pathway





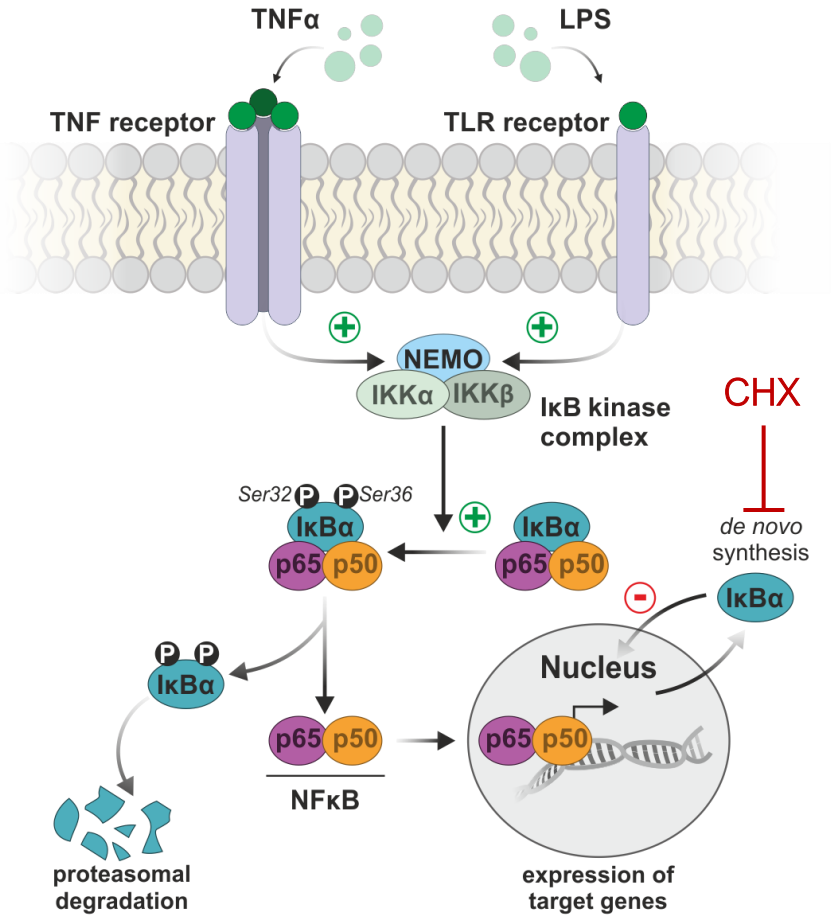
Modified from Hwang, B. *et al.* (2020) Commun Biol. 3:8

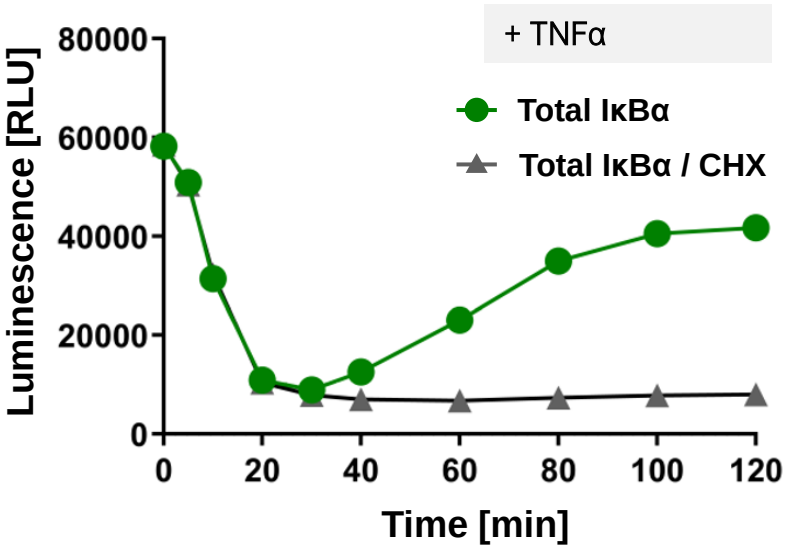
OBSERVATION

- Decrease in IκBα degradation
- Accumulation of phosphorylated IκBα

Signaling Pathway and Kinase Activity Analysis

Studying the NFκB Pathway





Modified from Hwang, B. *et al.* (2020) Commun Biol. 3:8

OBSERVATION

- Cycloheximide (CHX) blocks NFκB-triggered *de novo* IκBα biosynthesis

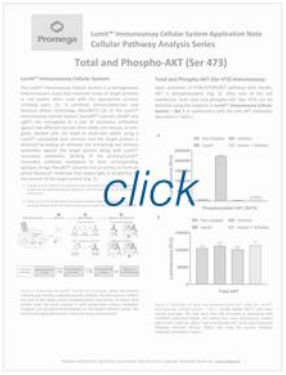
Lumit™ Immunoassay Cellular Systems

A Universal Immunoassay to Study Cellular Signaling

Validated with >20 phospho- and total proteins using 8 cell types, suggesting this universal immunoassay can be adapted for any pathway **with the appropriate antibodies**

- IκBα (phosph-Ser32 and total protein)
- STAT3 (phospho-Tyr705 and total protein)
- BTK (phospho-Tyr223)
- Estrogen receptor (total protein)
- β-Catenin (total protein)
- CREB (phospho-Ser133)
- P38 MAPK (phospho-Thr180/182)
- NFκB p65 (phospho-Ser536 and total protein)
- AKT (phospho-Ser473, phospho-Thr308, and total protein)
- Retinoblastoma tumor suppressor protein (phospho-Ser807/811 and phospho-Ser780)
- S6 ribosomal protein (phospho-Ser235/236, phospho-Ser240/244)
- MEK1/2 (phospho-Ser217/221, phospho-Ser298)

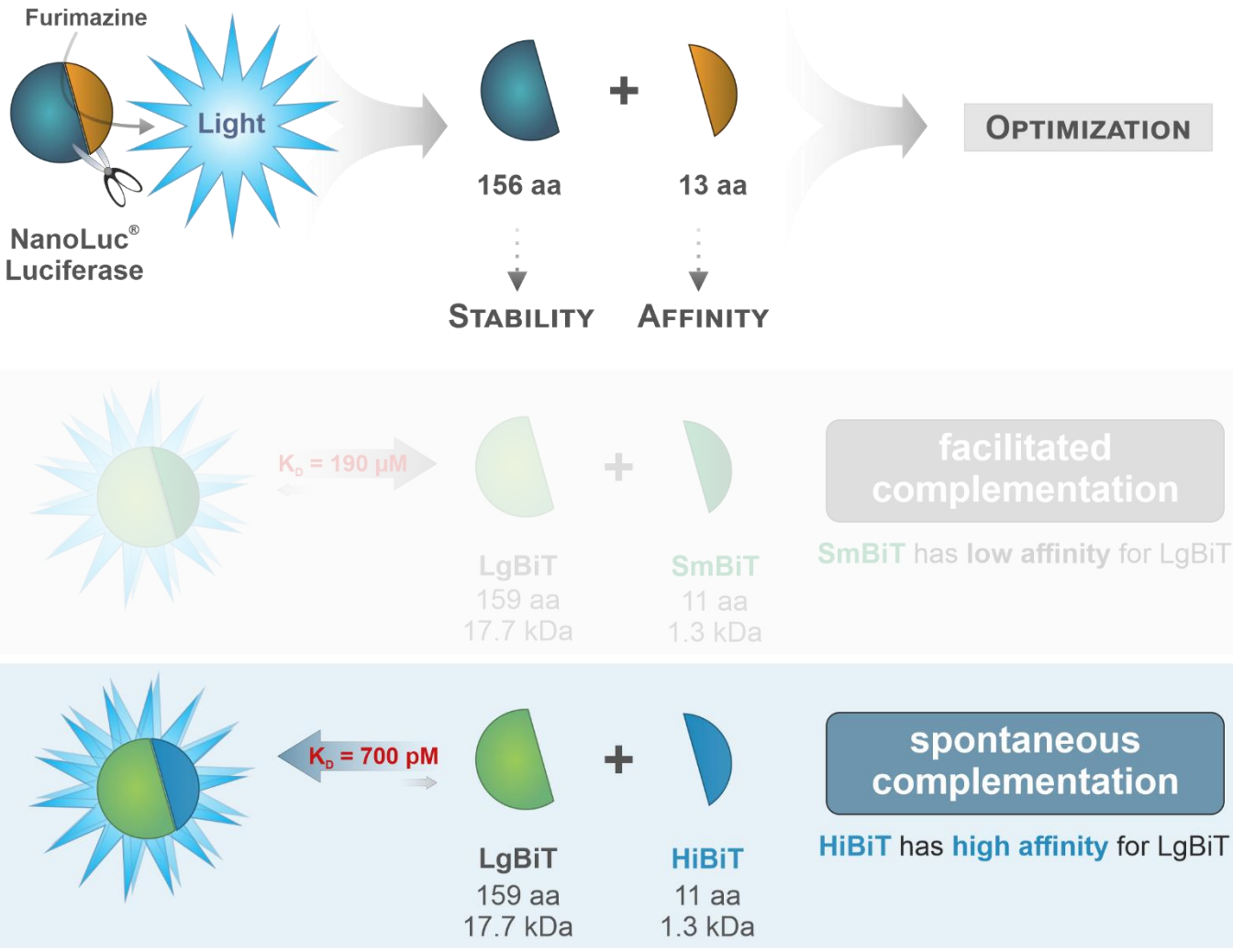
Application Notes



Available on Promega website with information on antibodies used

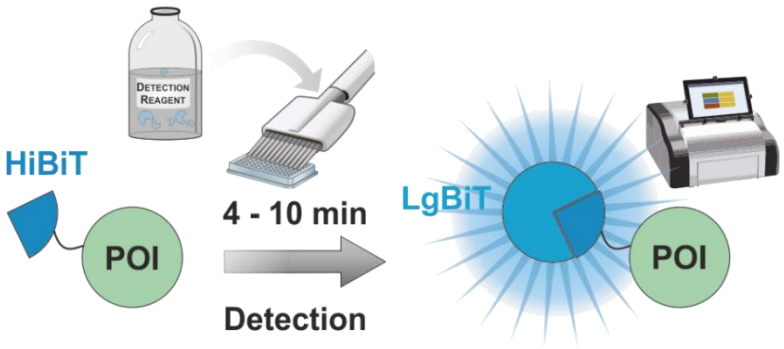
NanoLuc® Binary Technology (NanoBiT®)

A Structural Complementation Reporter Designed for Biomolecular Interaction Studies



HiBiT Protein Tagging System

Principle & Features



- Nano-Glo® HiBiT Lytic Detection System
- Nano-Glo® HiBiT Extracellular Detection System
- Nano-Glo® HiBiT Blotting System

- Nano-Glo® Live Cell Substrates (up to 72 h)
  + co-expression of LgBiT (transient or stable)

Small Tag Size (11 aa, 1.3 kDa)

- Low risk 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

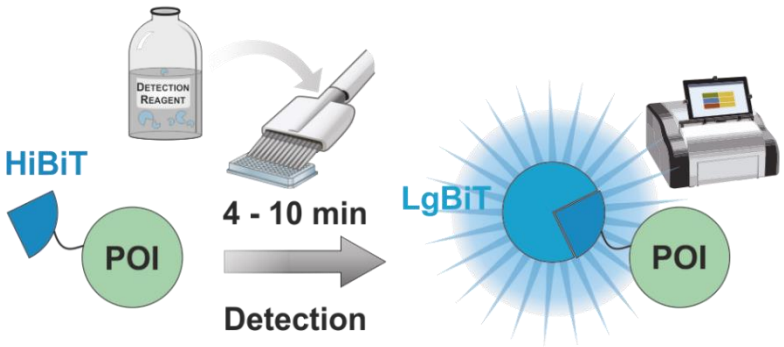
- Homogenous 1-step assay („add only“)
- No antibodies and no washing steps required
- Amenable to HTS and easy to automate

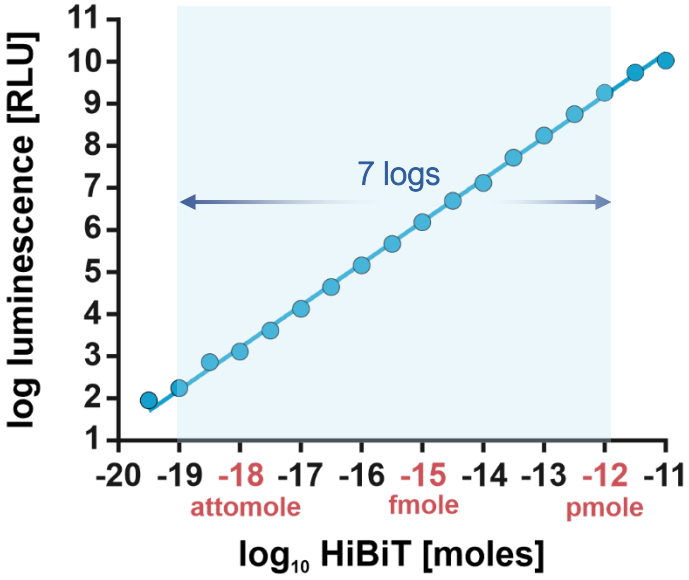
Sensitive & Quantitative

- Sub-attomolar levels can be detected
- High linear range of >7 logs

HiBiT Protein Tagging System

Principle & Features





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Sensitive & Quantitative

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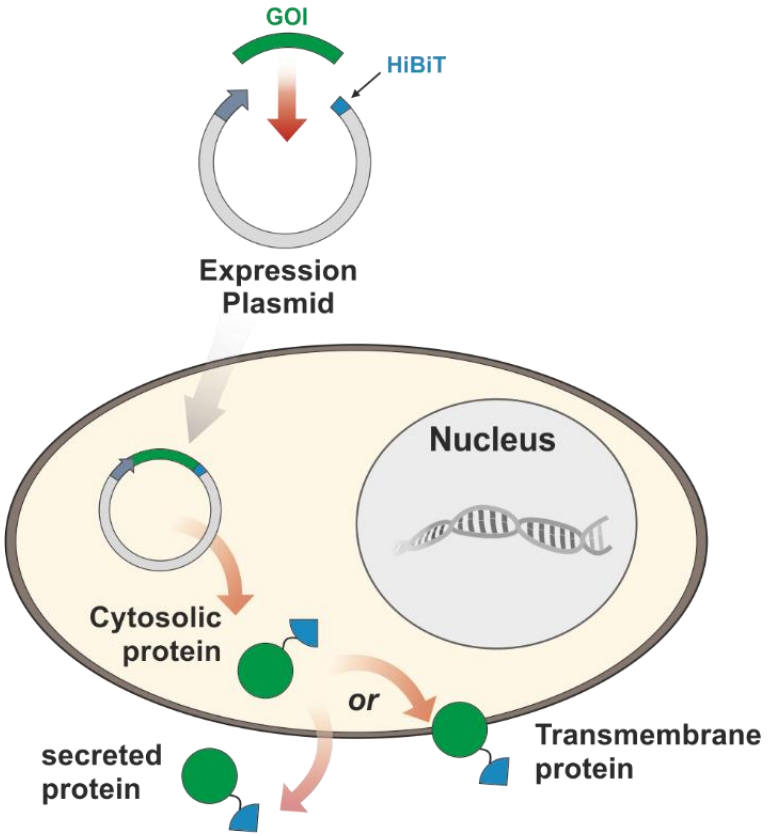
- High linear range of >7 logs

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

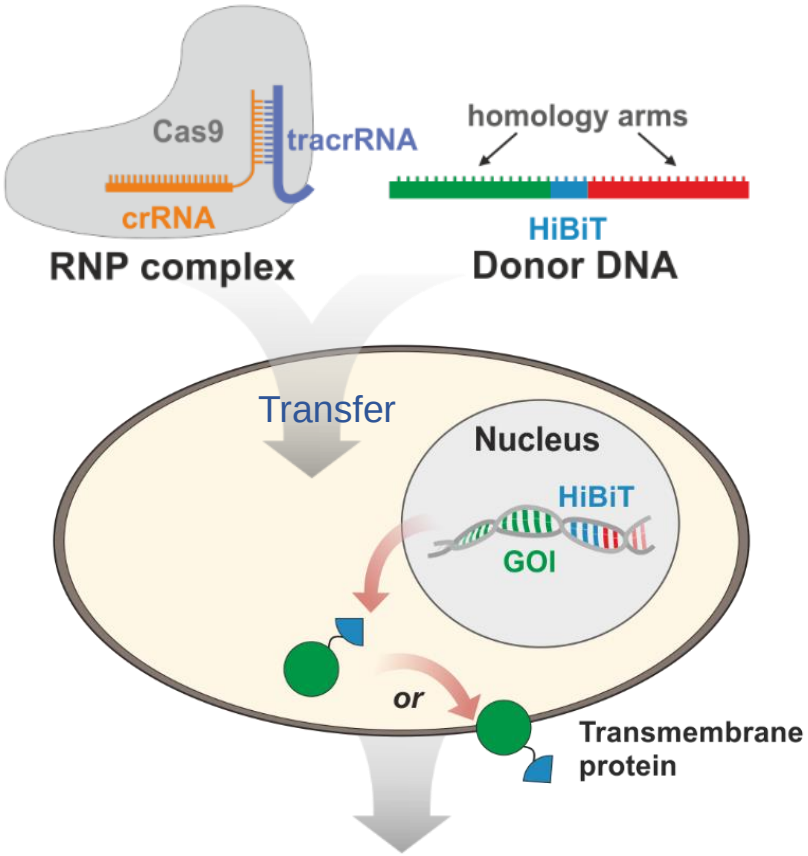
* naturally occurring secretion signals shall be removed

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

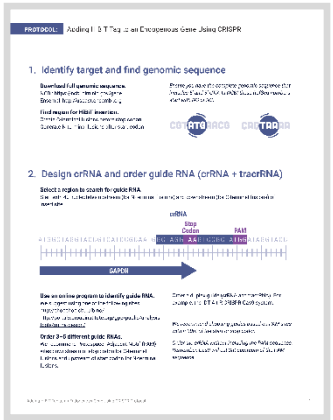


Validate after 24 - 48 h

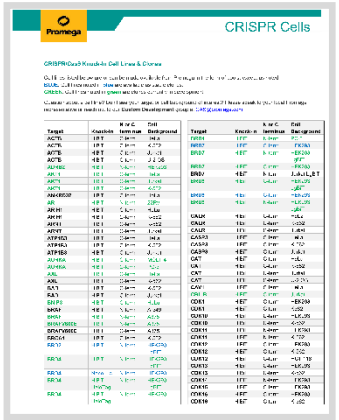
Three key components

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

DIY protocol



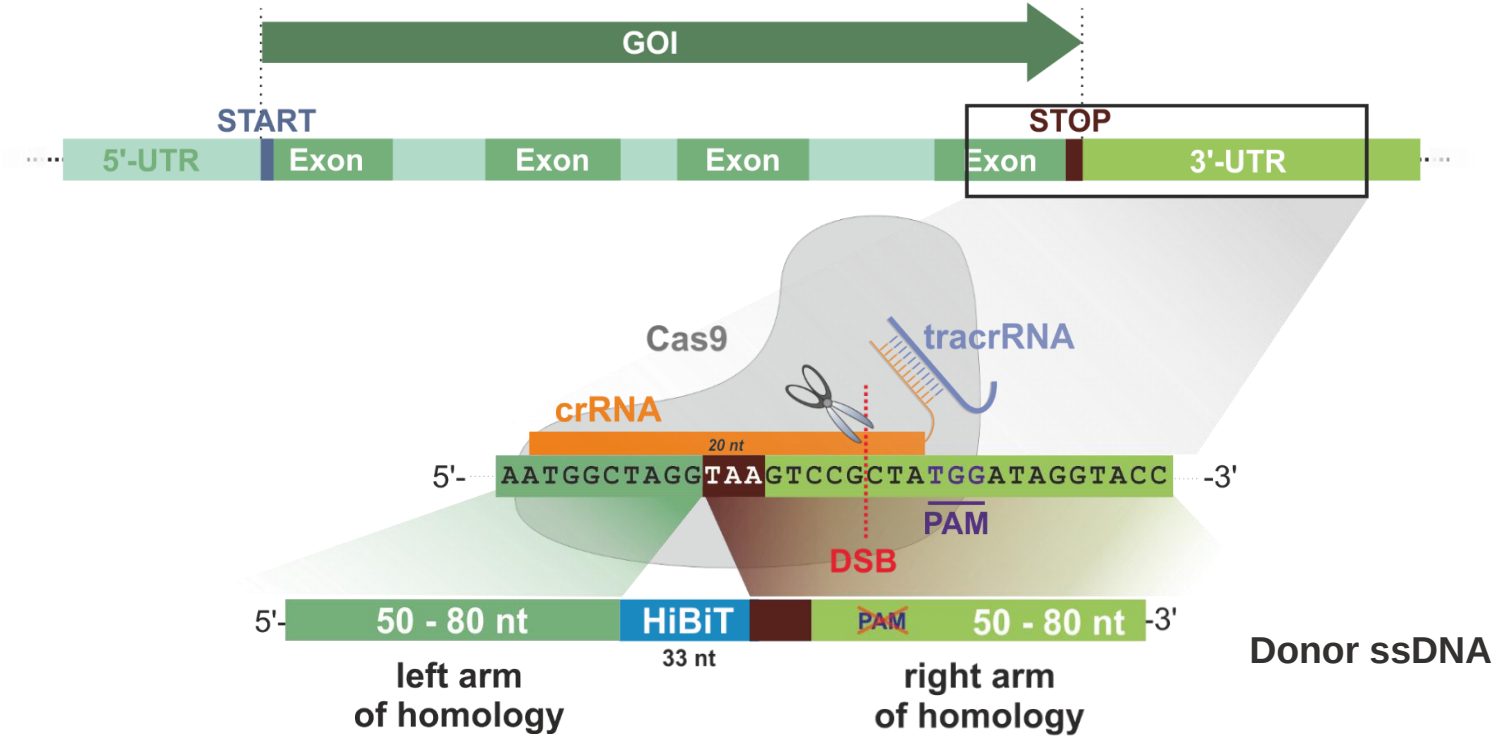
Ready-to-use cell lines



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

Generation of CRISPR/HiBiT Reporter Cells

Design of Target-specific CRISPR Components

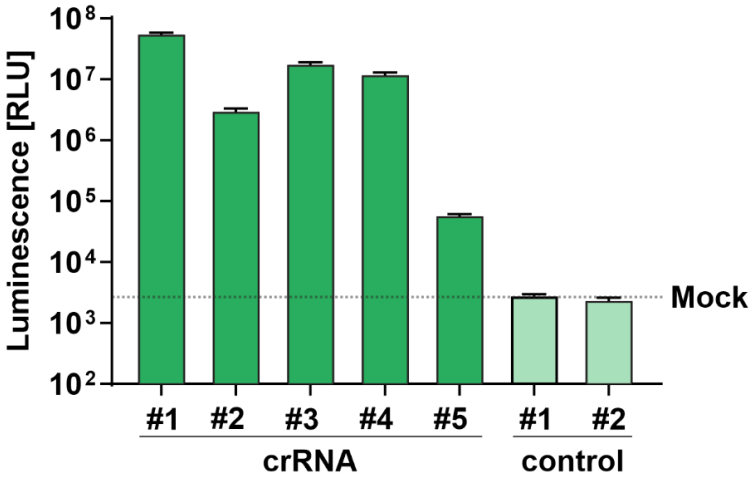


- PAM as close as possible to integration site (knock-in↑)
- Ideally crRNA should span the insertion site
- Choose PAM to avoid cut in coding region
- Mutate PAM within the donor DNA

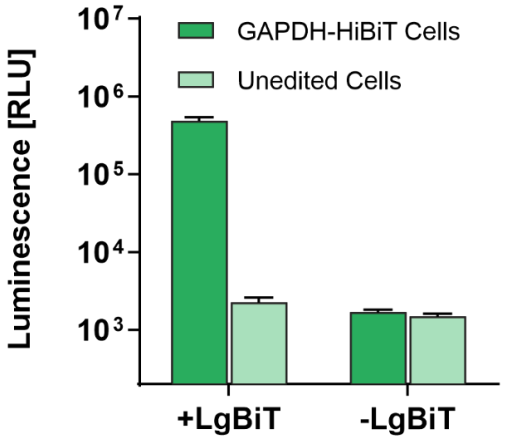
Validation of Genomic Editing in Cell Pools

Determination of Luminescence

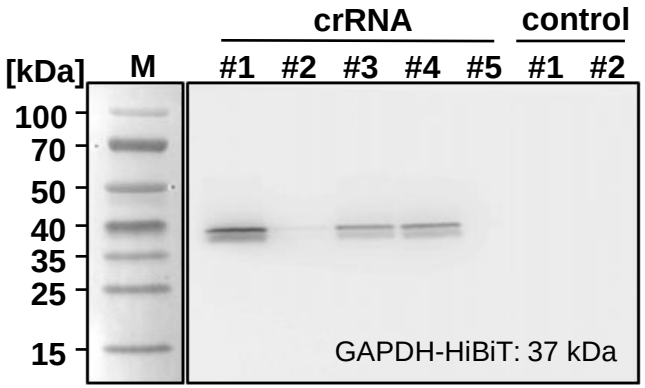
Nano-Glo® HiBiT Lytic Detection System



Nano-Glo® Live-Cell Assay System



Nano-Glo® HiBiT Blotting System



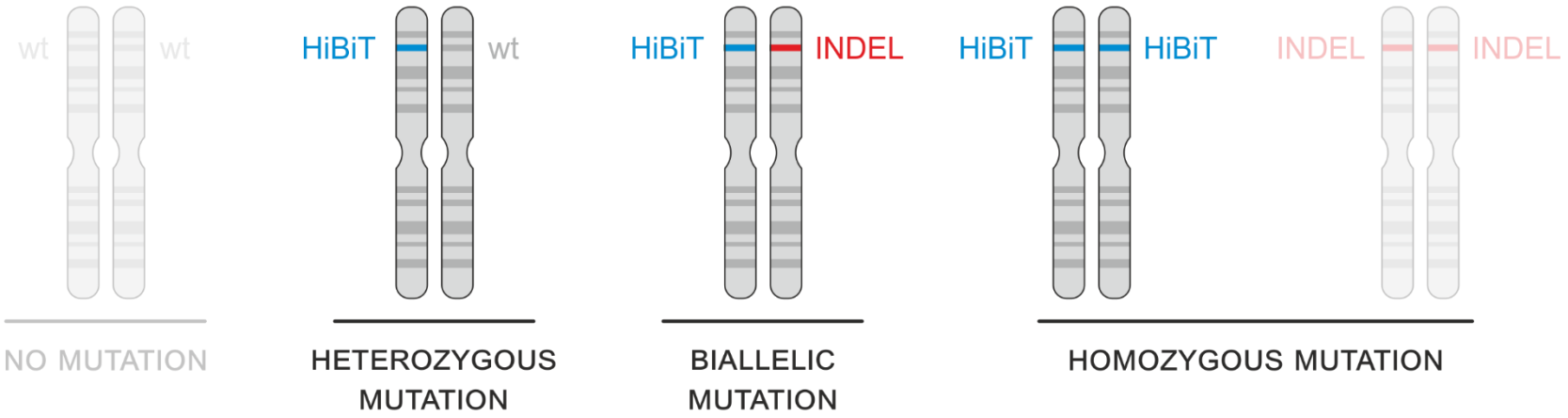
FACTS

- Signal ~ (knock-in %) x (expression level)
- Pick crRNA/donor combination with highest knock-in efficiency
- S/B > 10 is desirable for an assay
- CellTiter-Fluor™ can be used for normalization on viable cell number in plate-based assay

CRISPR Cell Pools *vs.* Clones

Two Powerful Formats

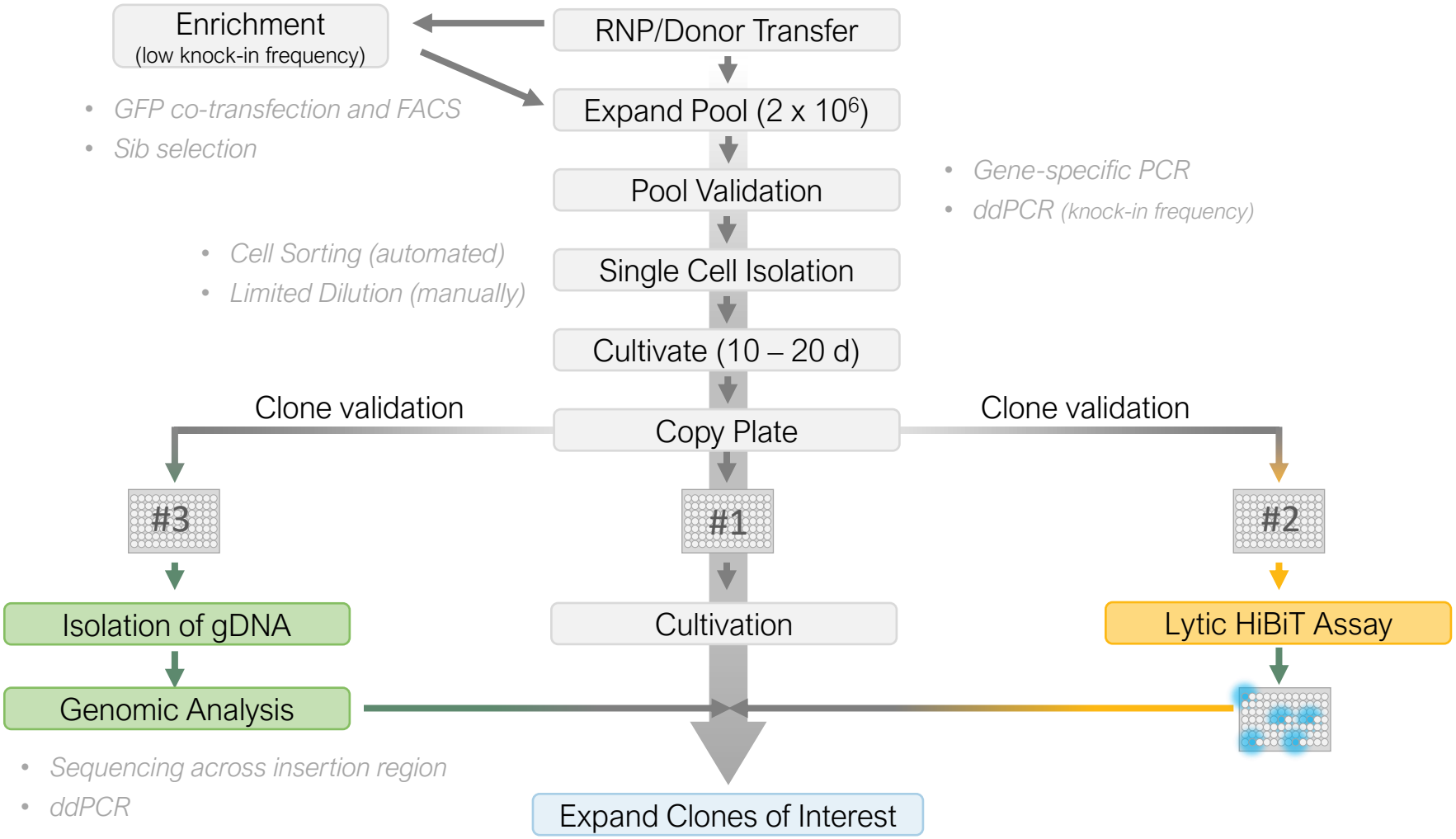
A pool of CRISPR-modified cells may contain cells with a variety of genetic alterations:



- The only HiBiT signal comes from those cells with an appropriate HiBiT knock-in
- In a pool, much of the genetic variability (e.g. unwanted mutations) is averaged out
- Biological response of pools is usually very similar to isolated clones
- Pools are dimmer than clones but are often bright enough to use
  A pool with 5% knock-in efficiency should be approx. 5% as bright as a clone

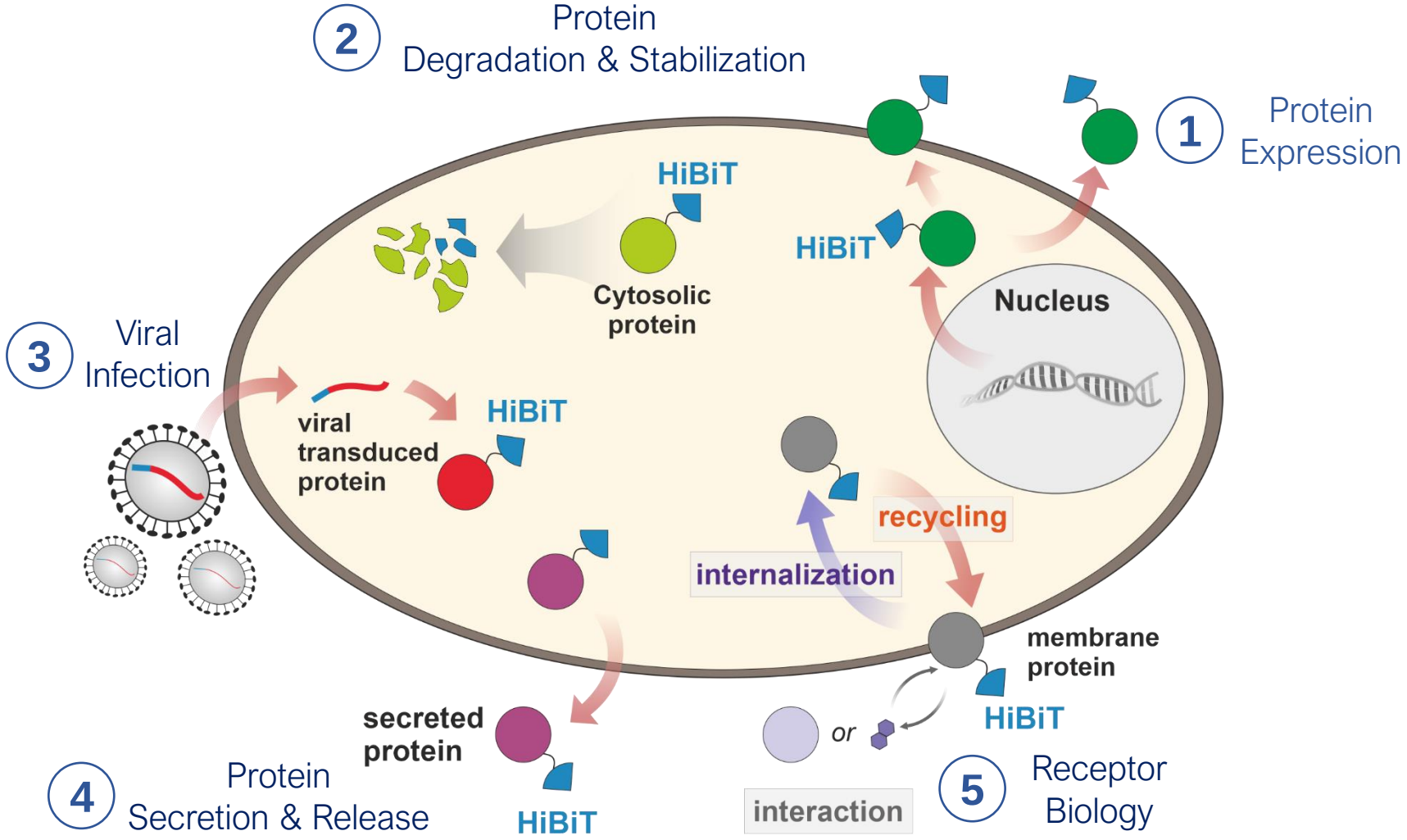
Generation of CRISPR/HiBiT Reporter Cells

Workflow for Edited Cell Clones



HiBiT Application Portfolio

One Bioluminescent Tag, Endless Possibilities



HiBiT Application Portfolio

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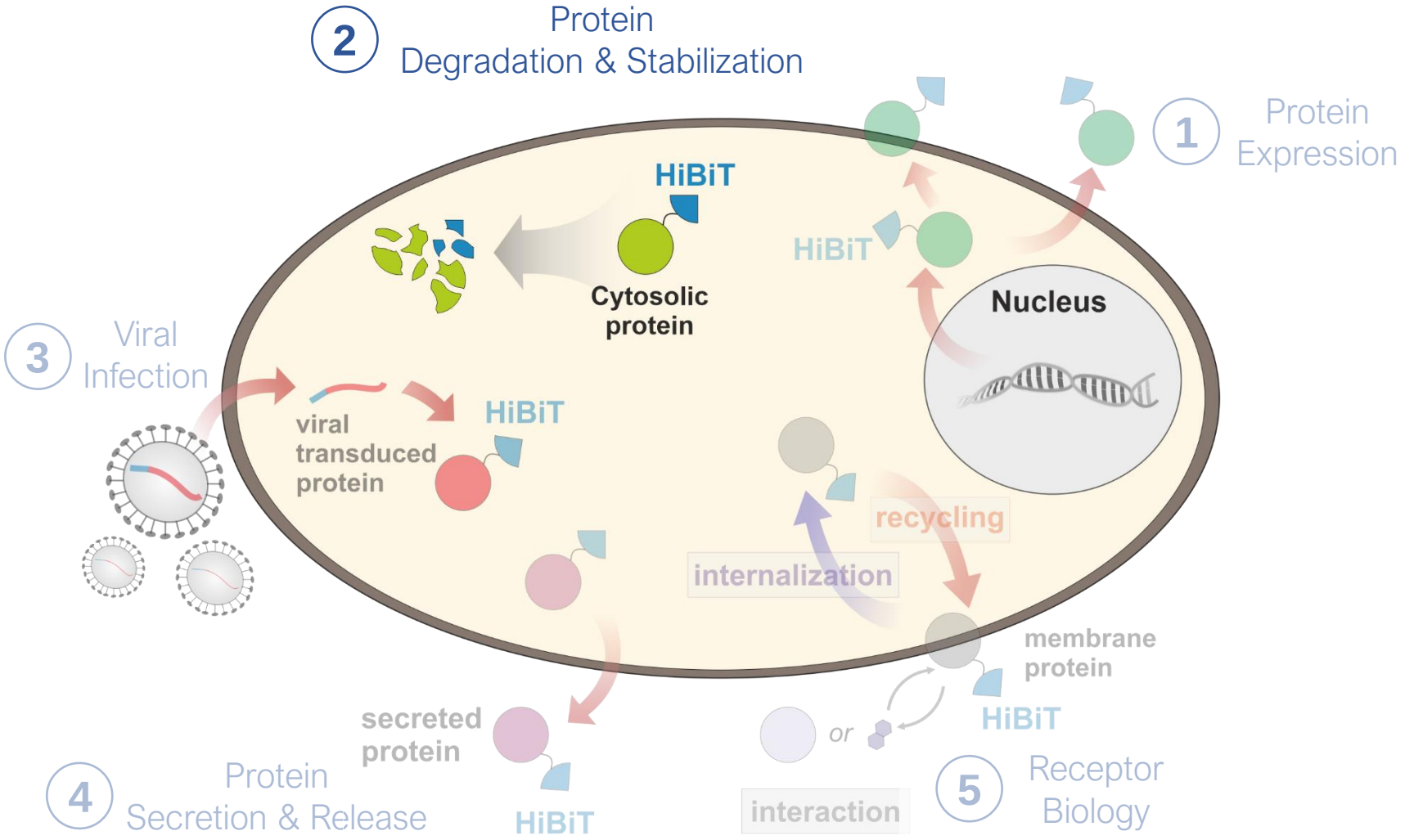
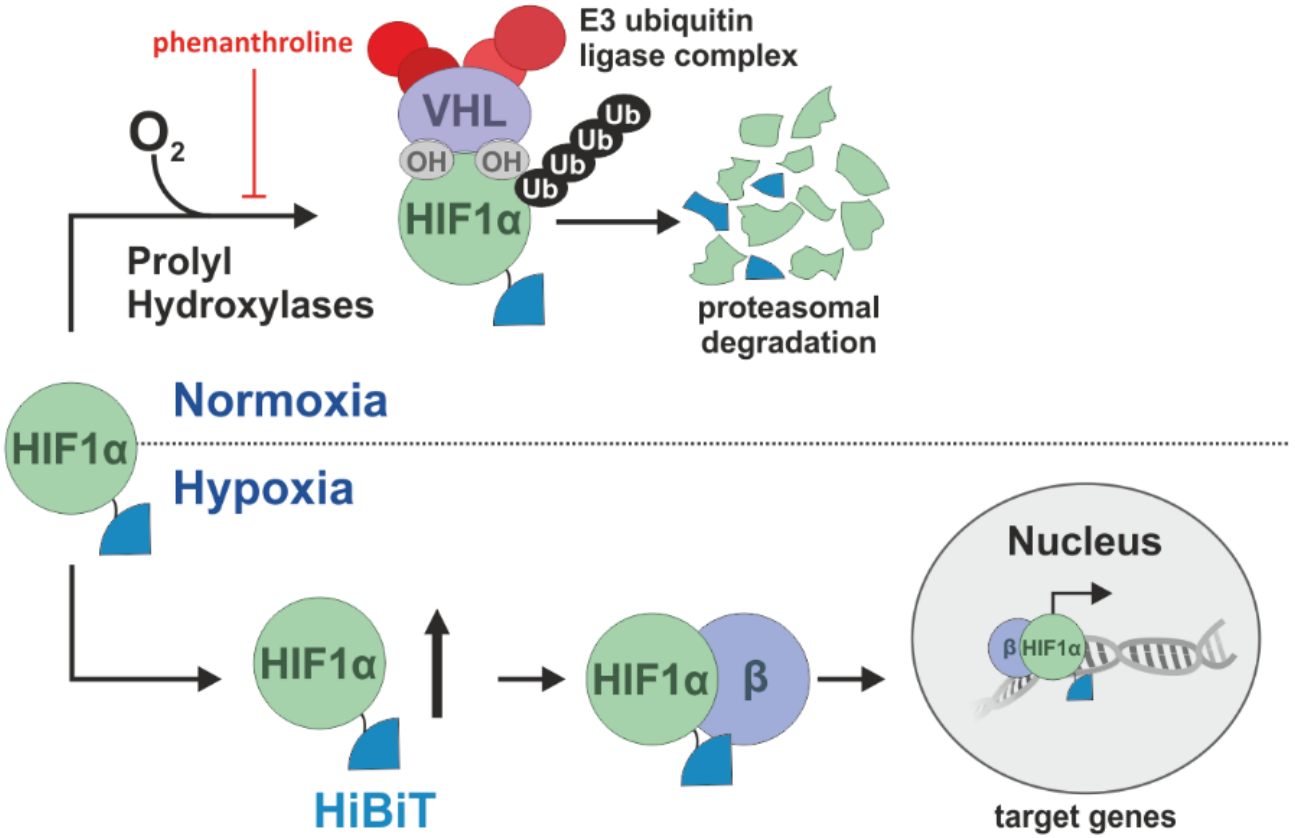


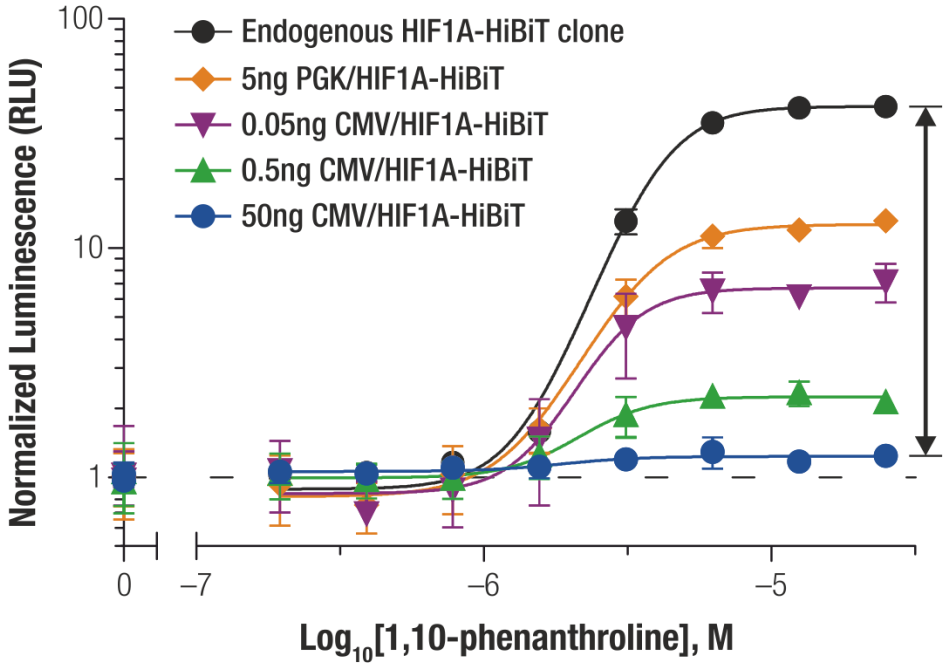
The HIF1α Pathway

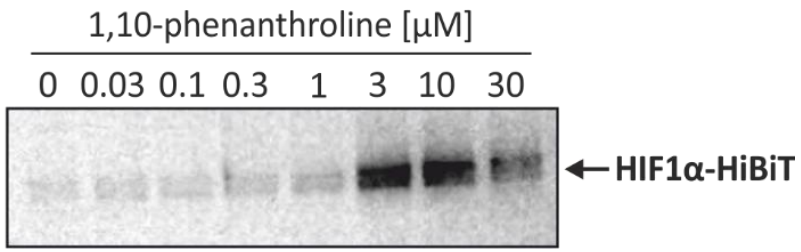
A Model System for Protein Stabilization

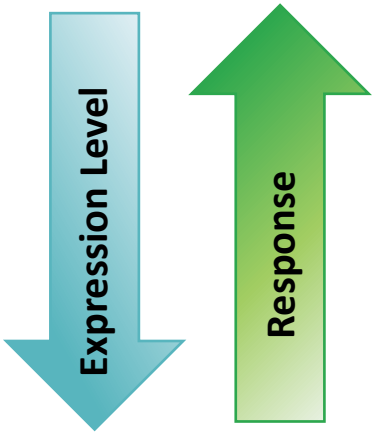


Stabilization of HIF1α

The Relevance of Expression Level Protein Stabilization



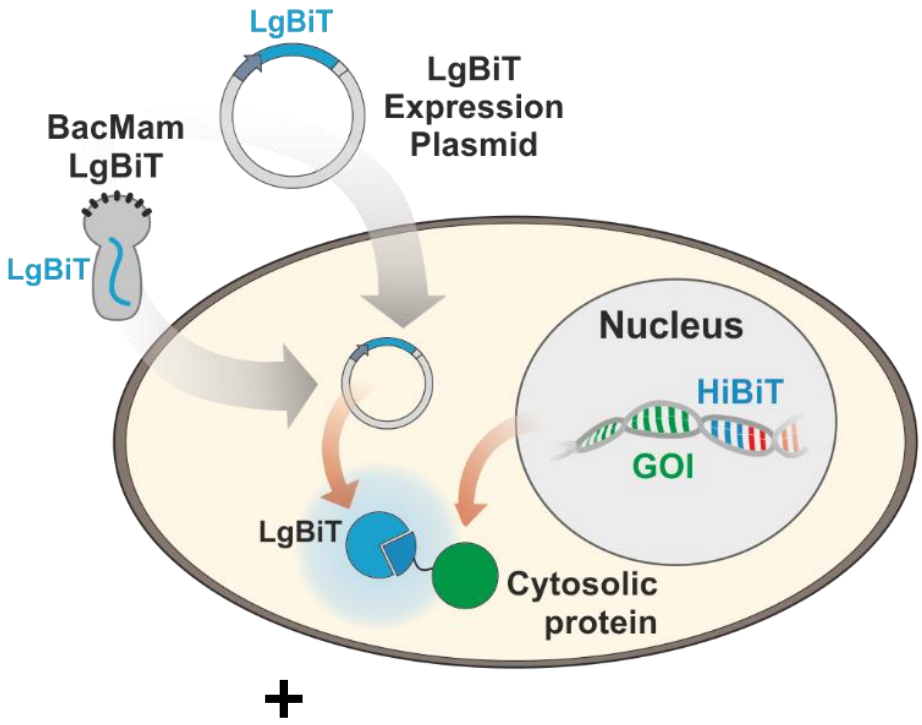




- High expression levels mute the biological response
- endogenous expression yields highest assay window

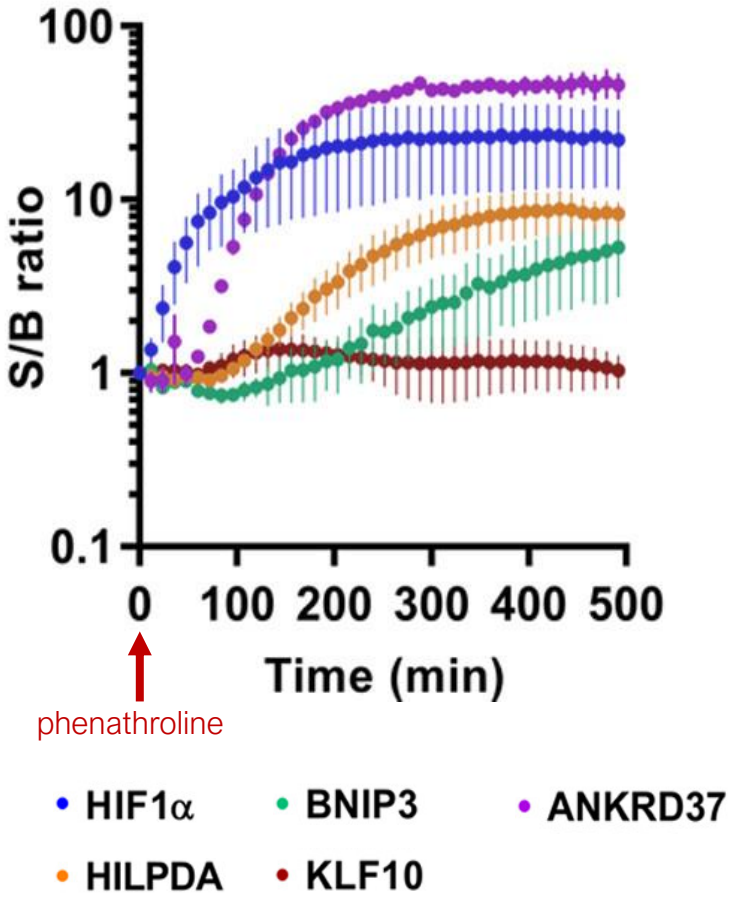
Stabilization of HIF1α

Maximize Information Content by Live-Cell Monitoring in Real-Time



Nano-Glo® Live Cell Substrate

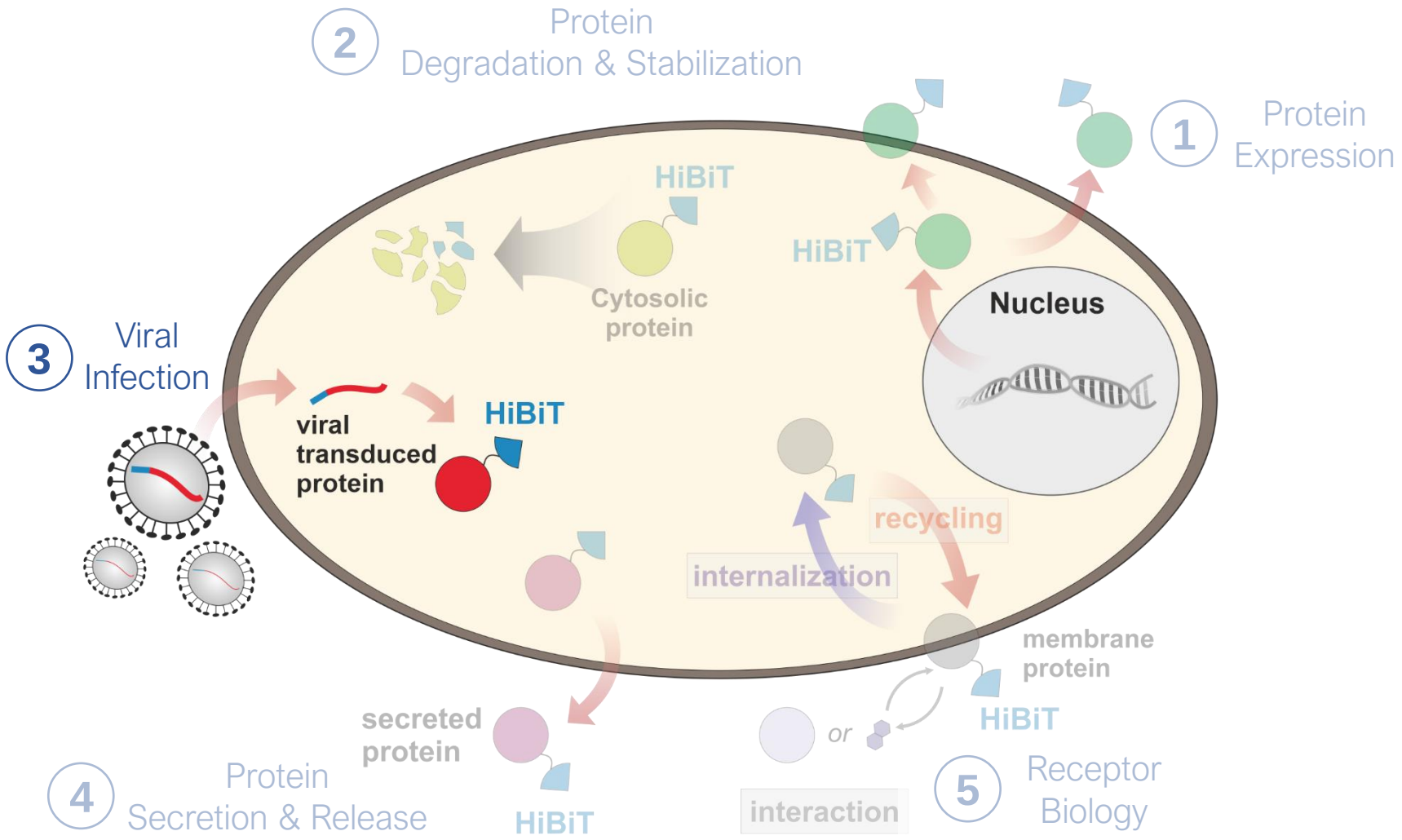
- Nano-Glo® Live Cell Assay System (≤ 2 h)
- Nano-Glo® Vivazine™ (2 - 24 h)
- Nano-Glo® Endurazine™ (24 - 72 h)



Schwinn, M.K., *et al.* (2017). *ACS Chem. Biol*. 13(2):467-474

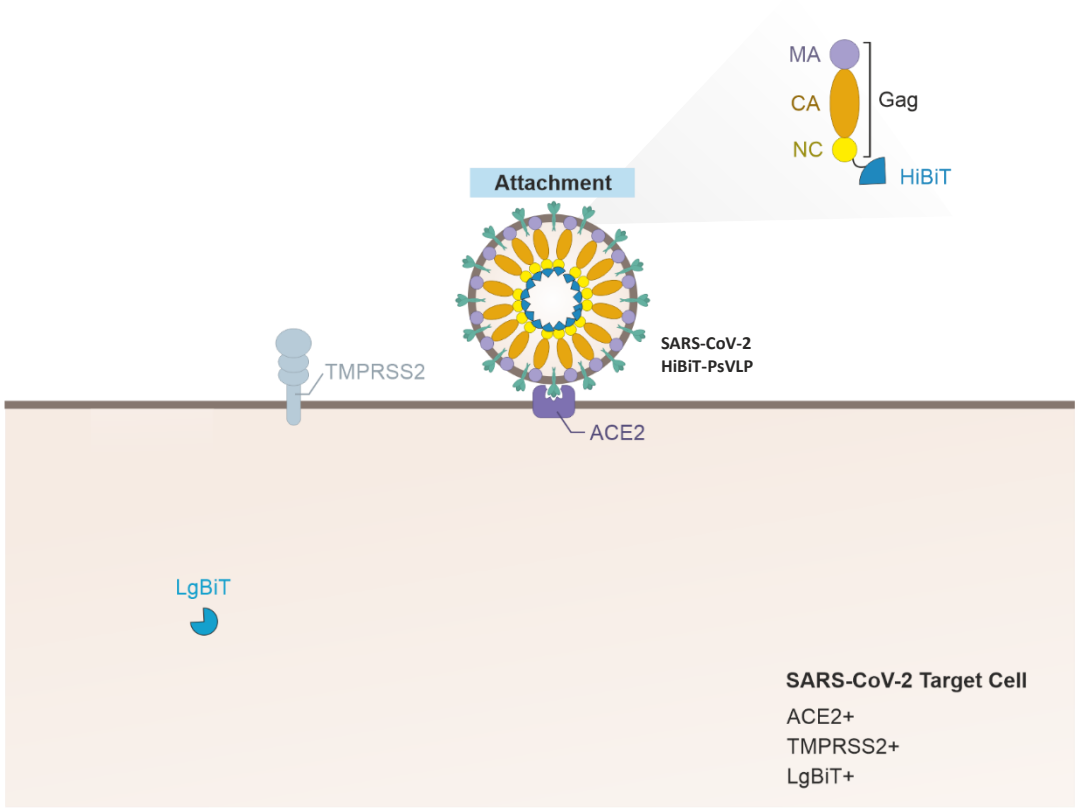
HiBiT Application Portfolio

One Bioluminescent Tag, Endless Possibilities



SARS-CoV-2 HiBiT-PsVLP Assay

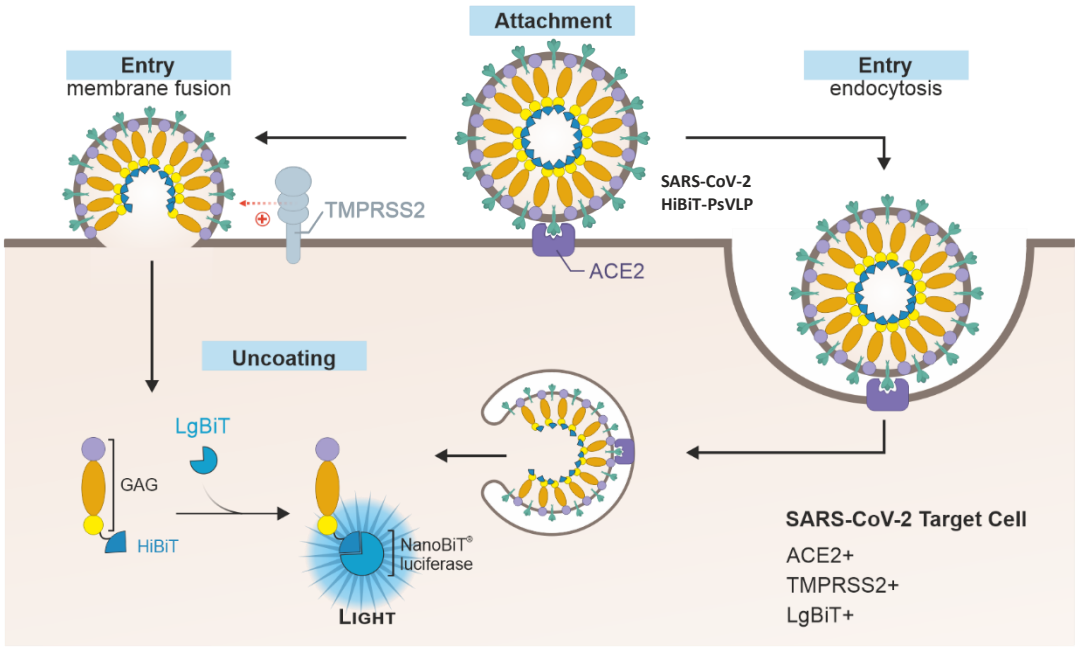
Measure Blocking Activity for Small Molecule Inhibitors and Neutralizing Antibodies



- HiBiT-tagged VLPs pseudotyped with SARS-CoV-2 Spike protein are added to SARS-CoV-2 Target Cells

SARS-CoV-2 HiBiT-PsVLP Assay

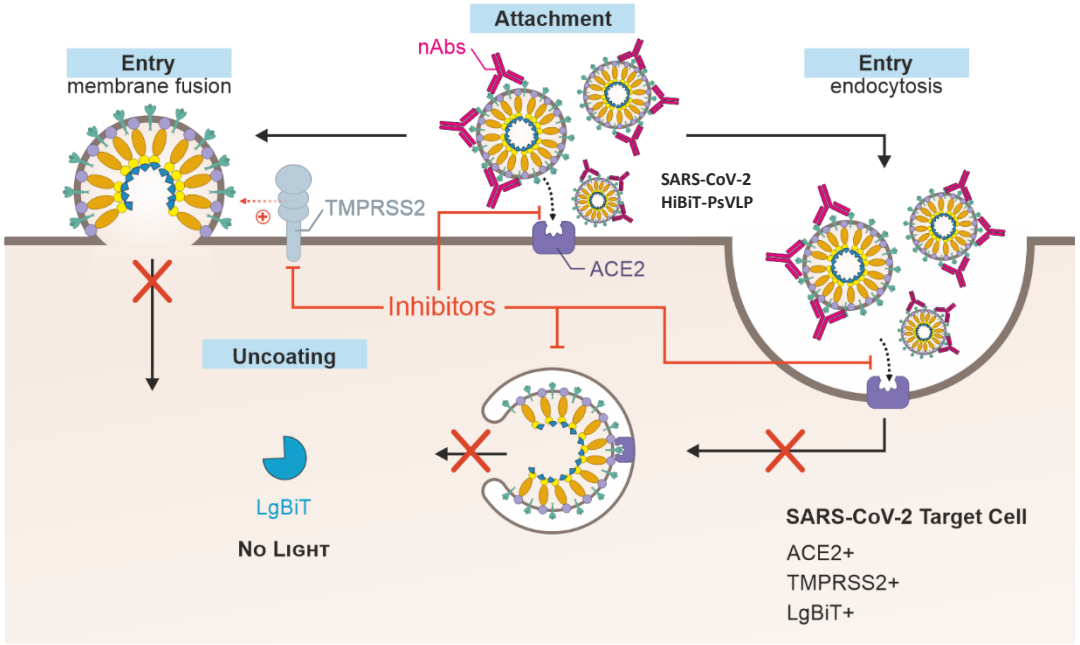
Measure Blocking Activity for Small Molecule Inhibitors and Neutralizing Antibodies



- HiBiT-tagged VLPs pseudotyped with SARS-CoV-2 Spike protein are added to SARS-CoV-2 Target Cells
- Upon entry, GAG-HiBiT is released into target cells
- HiBiT binds to cellular LgBiT to generate a luminescent signal in the presence of luciferase substrate

SARS-CoV-2 HiBiT-PsVLP Assay

Measure Blocking Activity for Small Molecule Inhibitors and Neutralizing Antibodies

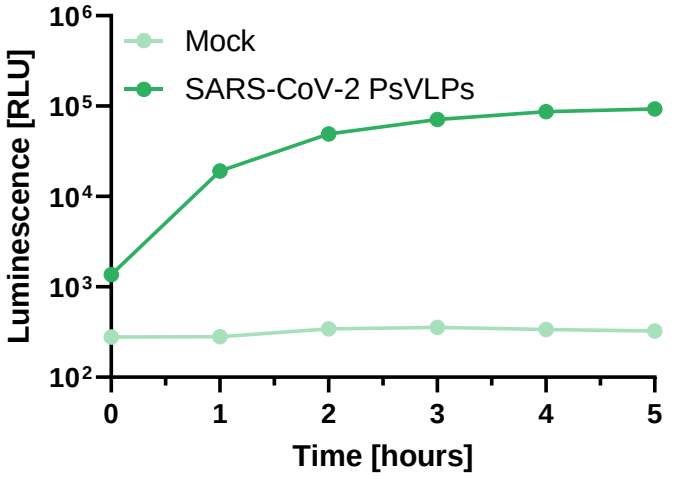


- HiBiT-tagged VLPs pseudotyped with SARS-CoV-2 Spike protein are added to SARS-CoV-2 Target Cells
- Upon entry, GAG-HiBiT is released into target cells
- HiBiT binds to cellular LgBiT to generate a luminescent signal in the presence of luciferase substrate
- Inhibitors that block entry/fusion processes prevent HiBiT release, i.e. no luminescent signal is produced

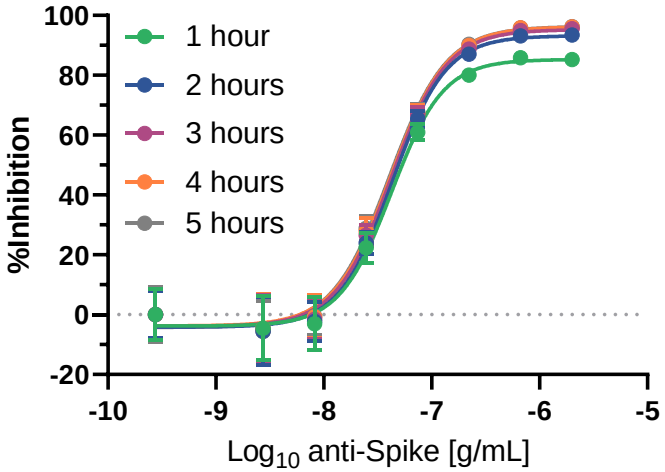
Monitor Infection in Real-Time

Determine Inhibitory Effects of Neutralizing Antibodies

Viral entry kinetic



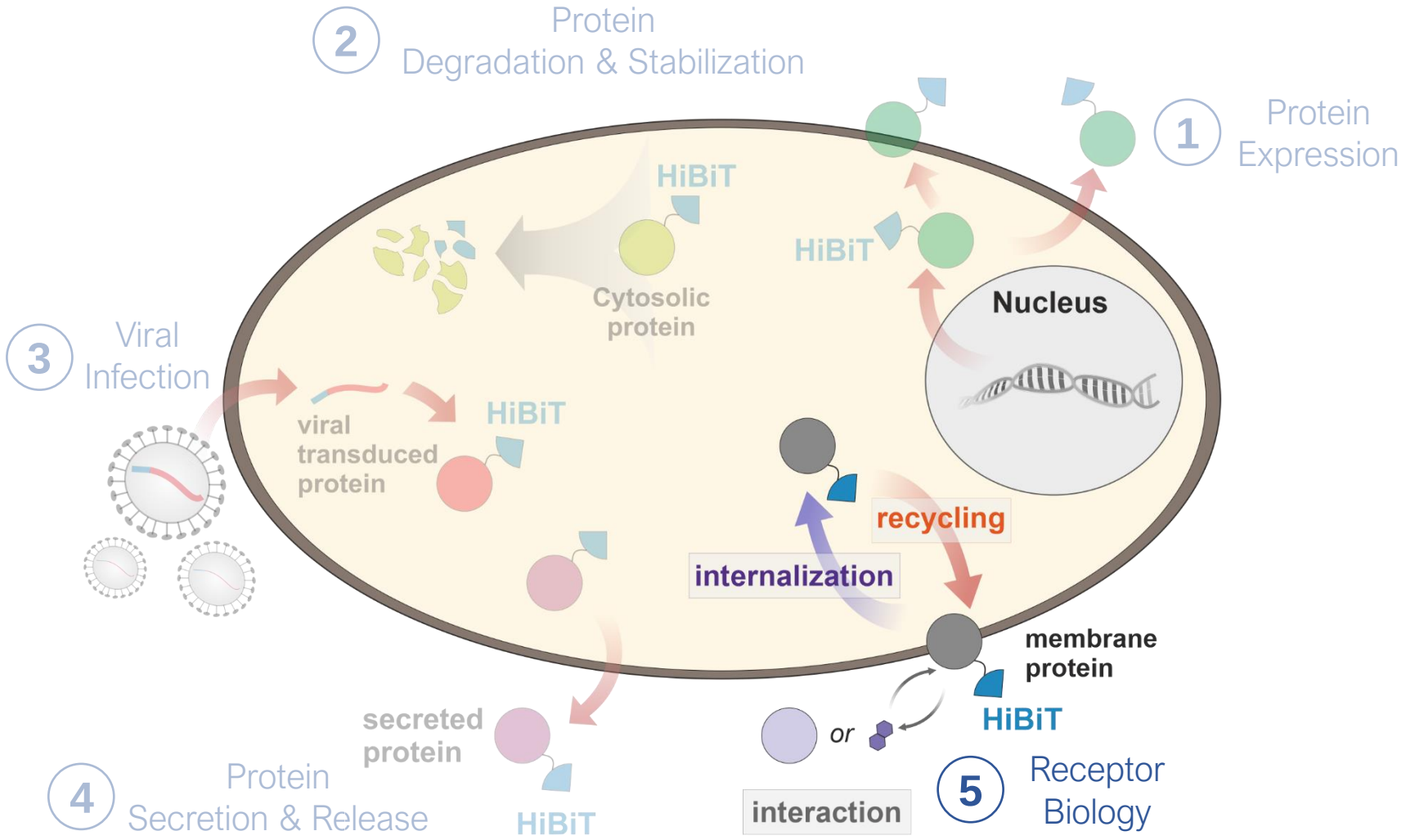
Inhibition of entry by nAbs



- Entry of SARS-CoV-2 HiBiT-PsVLPs increased rapidly from 0 - 2 hours, then continued to increase slowly after 2 hours
- Entry of SARS-CoV-2 HiBiT-PsVLPs is inhibited in a dose-dependent manner by neutralizing antibody (nAb) and can be monitored over time

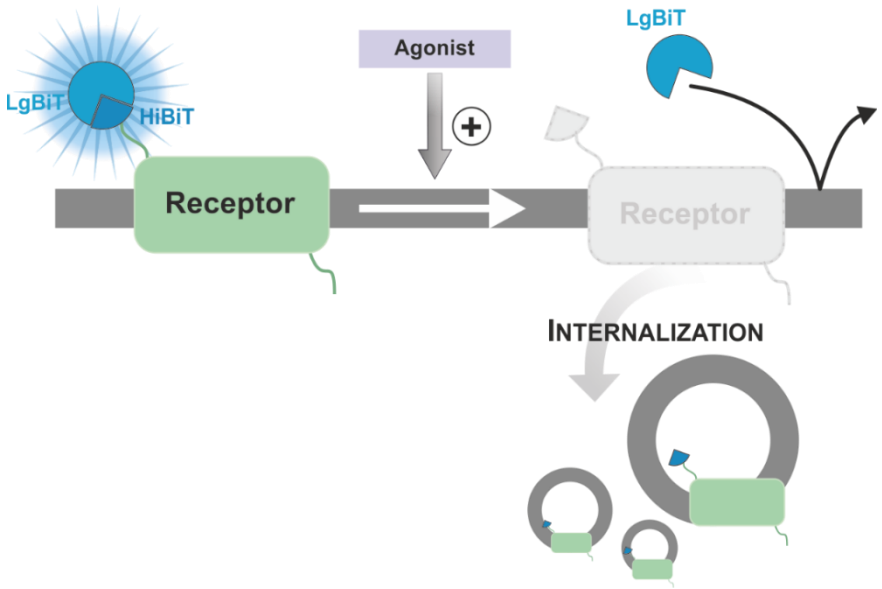
HiBiT Application Portfolio

One Bioluminescent Tag, Endless Possibilities

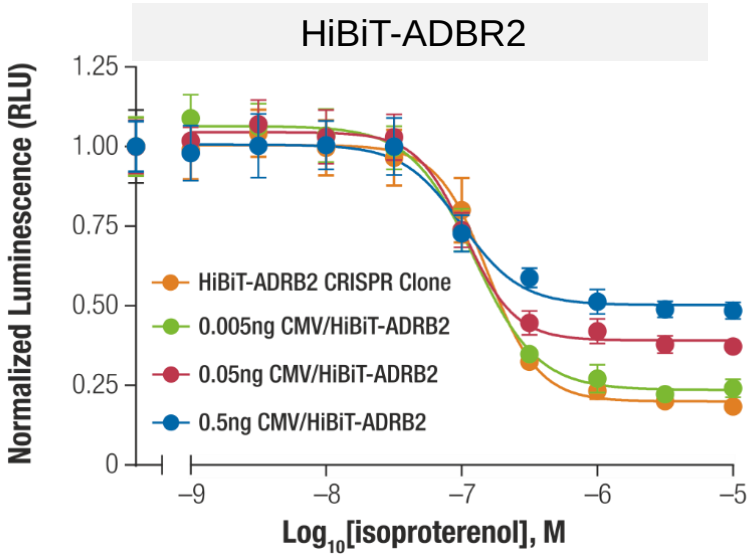


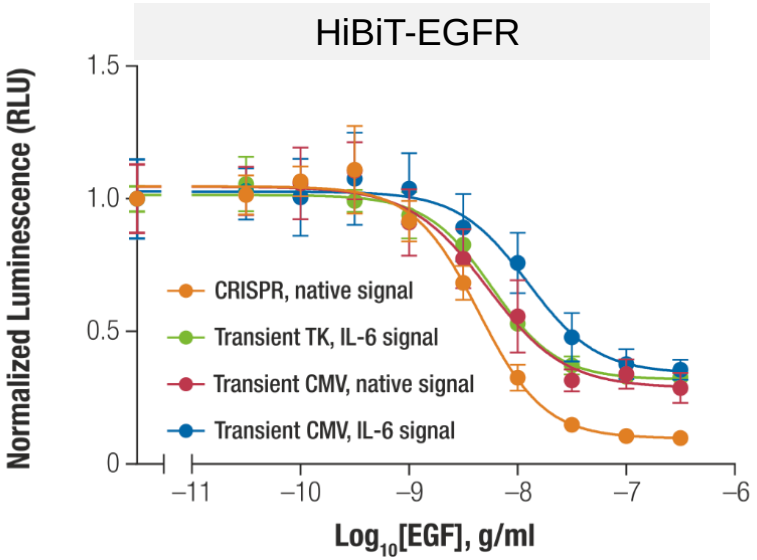
Study Receptor Internalization with HiBiT

GPCRs & RTKs



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





Thank You!

Questions?

- For additional questions please contact: erik.bonke@promega.com