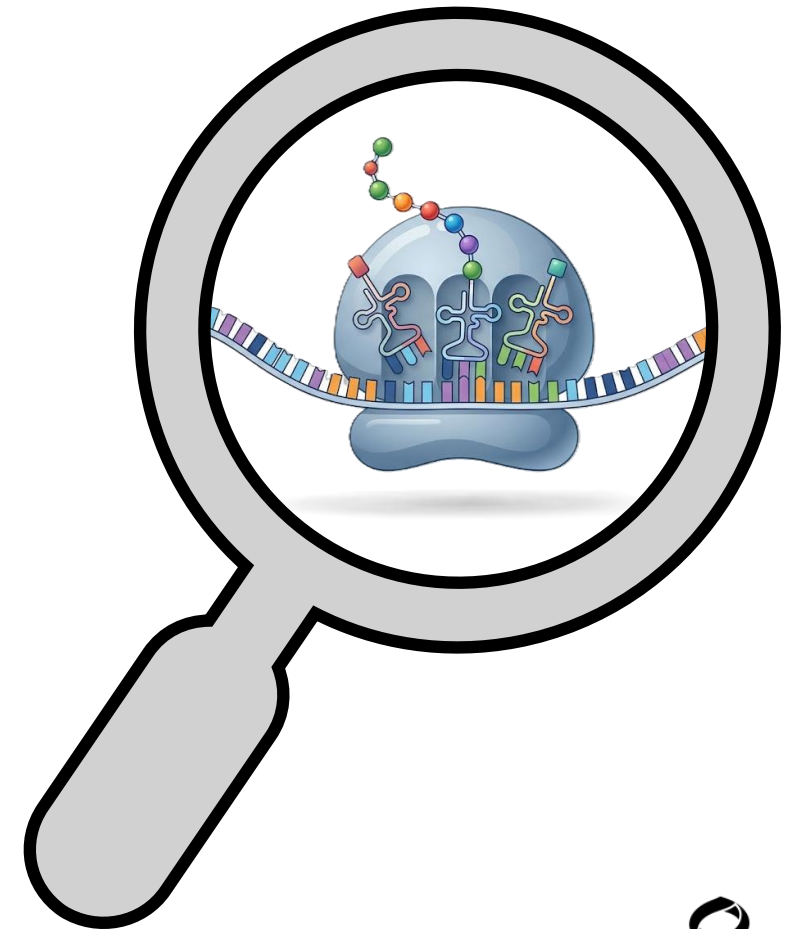


RNA in Focus: From Extraction and Quantification to Protein Expression

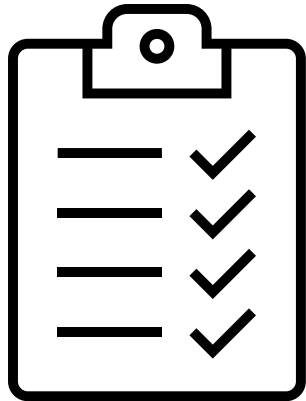
Mgr. Lukáš Išler (East Port)

&

Kerem Yildirim (Promega)



Program



RNA Analysis From the Scratch – Extraction, Protection,
Reverse Transcription (Kerem Yildirim)

Coffee Break and Q&A

Navigating qPCR: Experimental Design, Data Analysis, and
Best Practices (Kerem Yildirim)

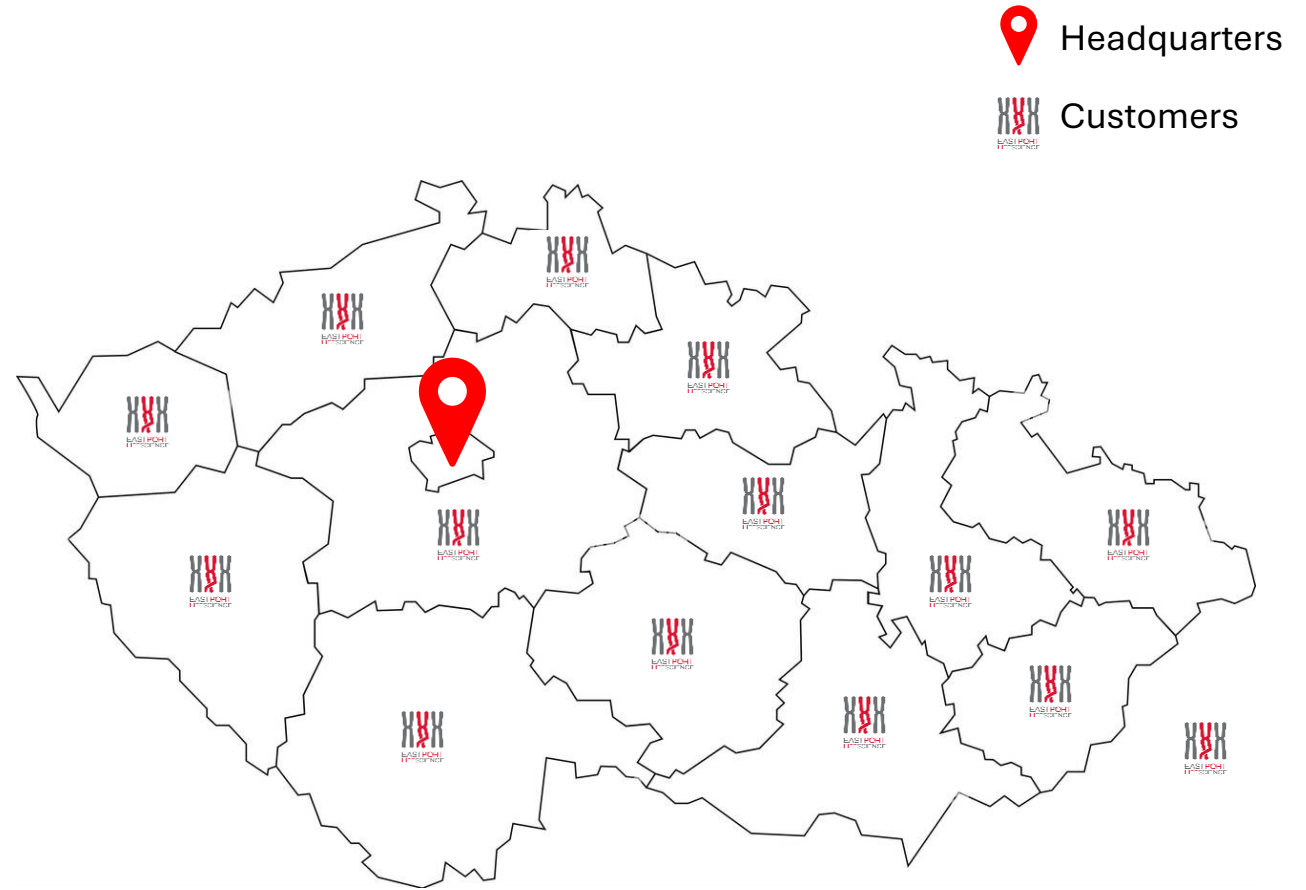
From mRNA to Protein; HiBiT Technology, RNA Interference;
Assessing RNA Therapy Effects on Cells (Lukáš Išler)

Final Thoughts and Discussion

About East Port

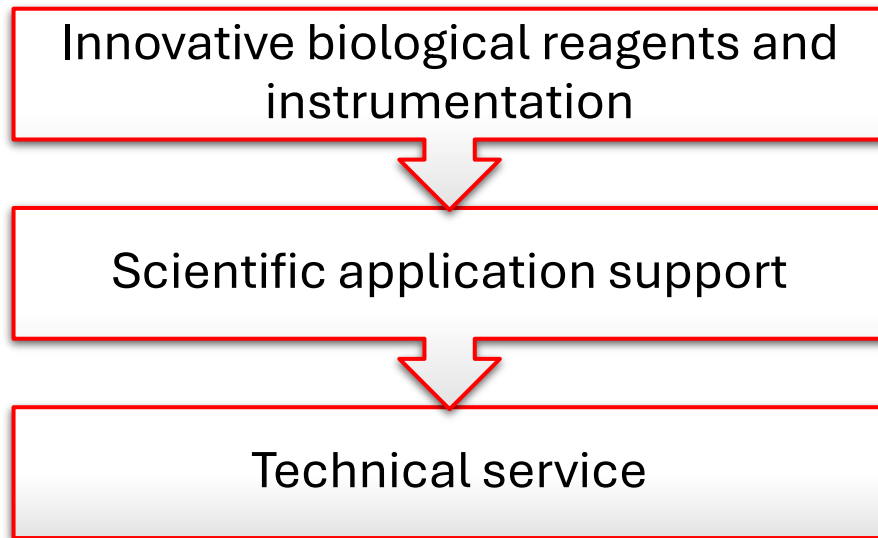
About us

- Over **25 years** supporting life science research in the Czech Republic (+ Slovakia)
- More than **15 partnerships** with leading biotechnology suppliers
- Team with scientific background

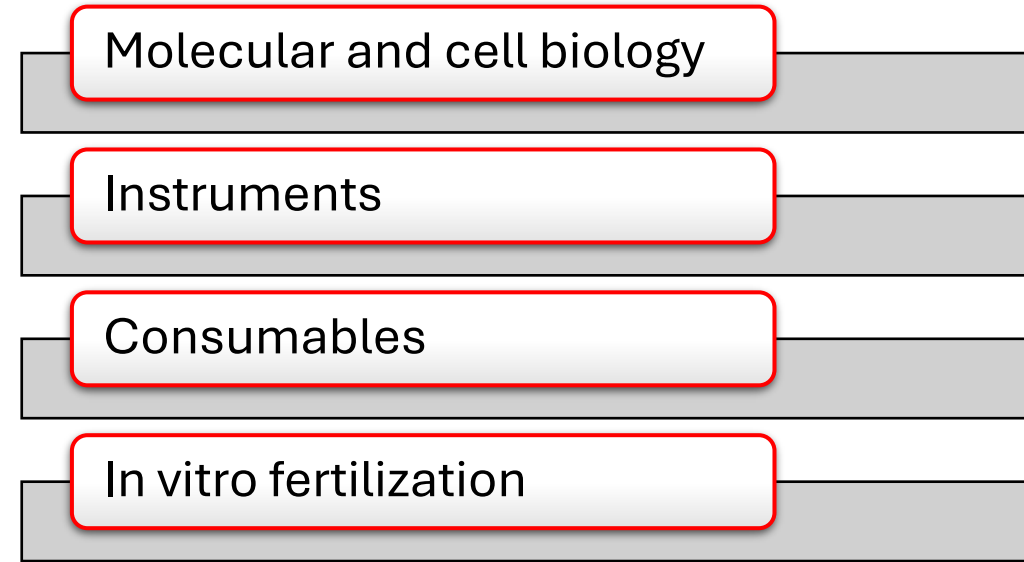


Company Overview

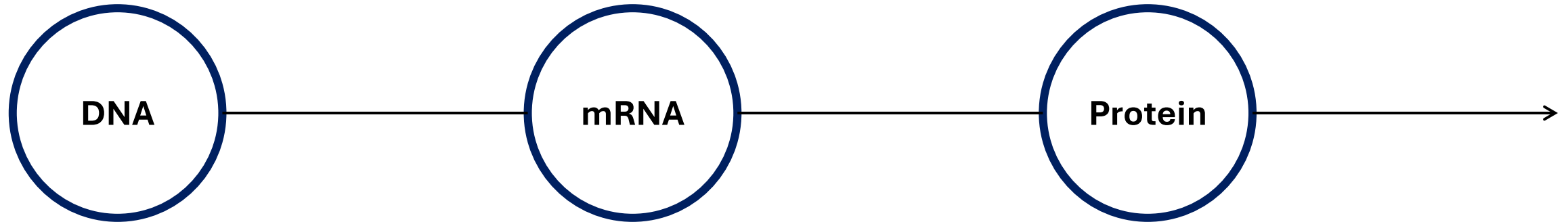
We Provide



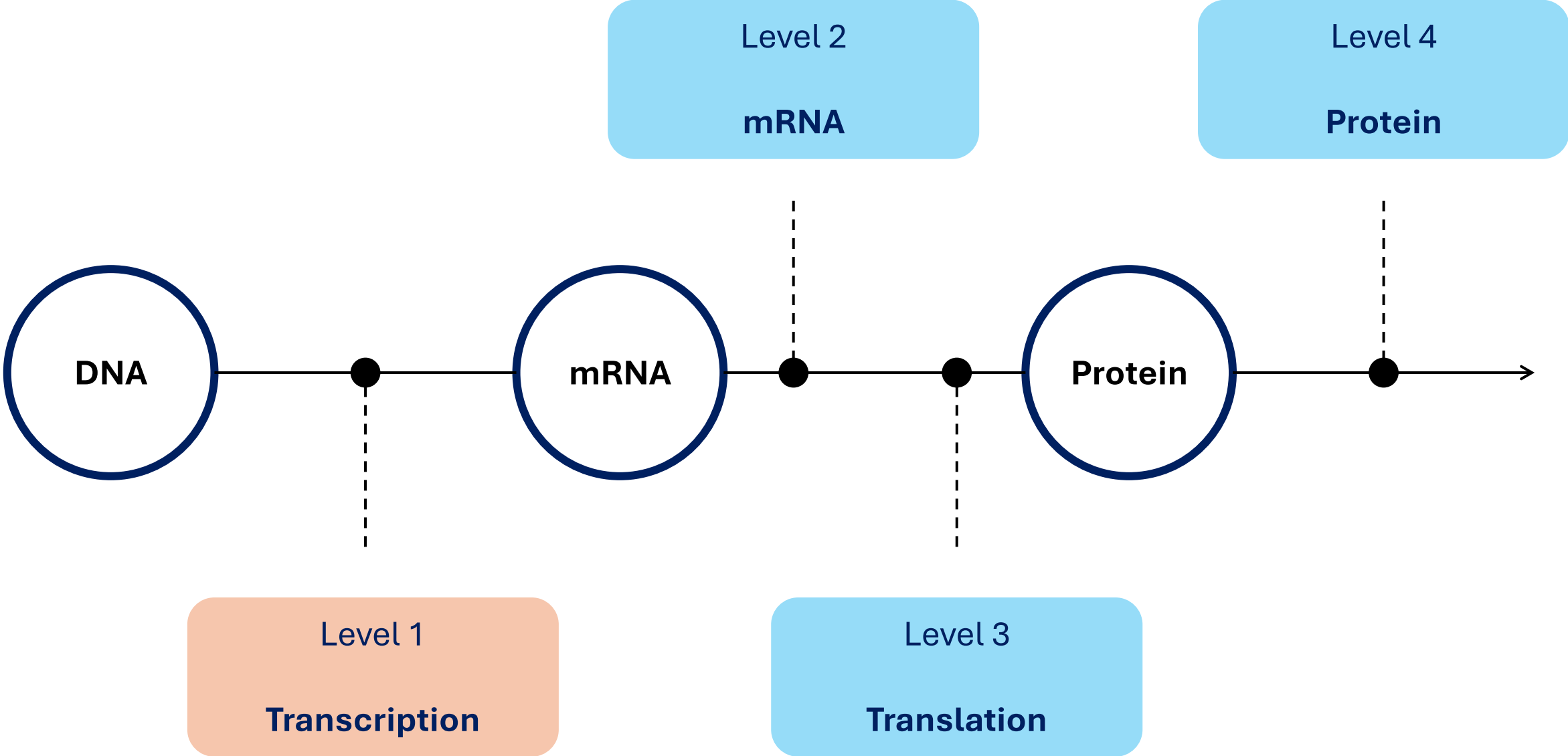
Covered Product Areas



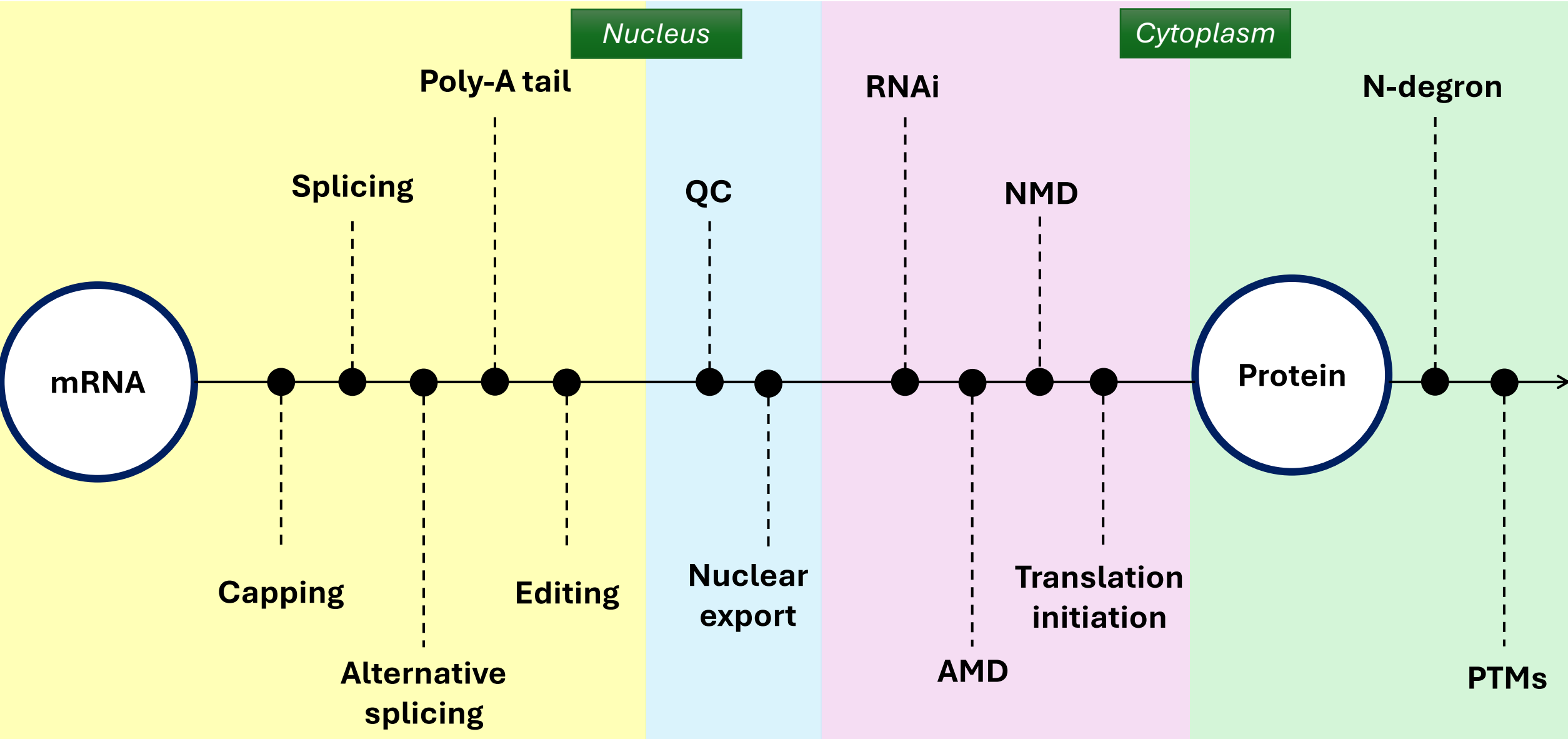
The (un)expected journey



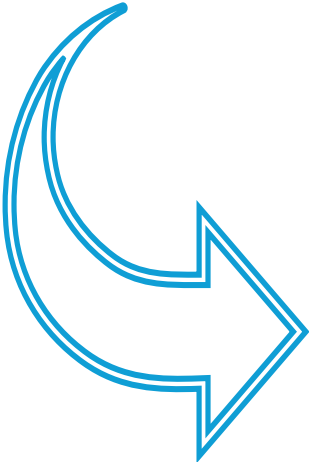
The (un)expected journey



The (un)expected journey



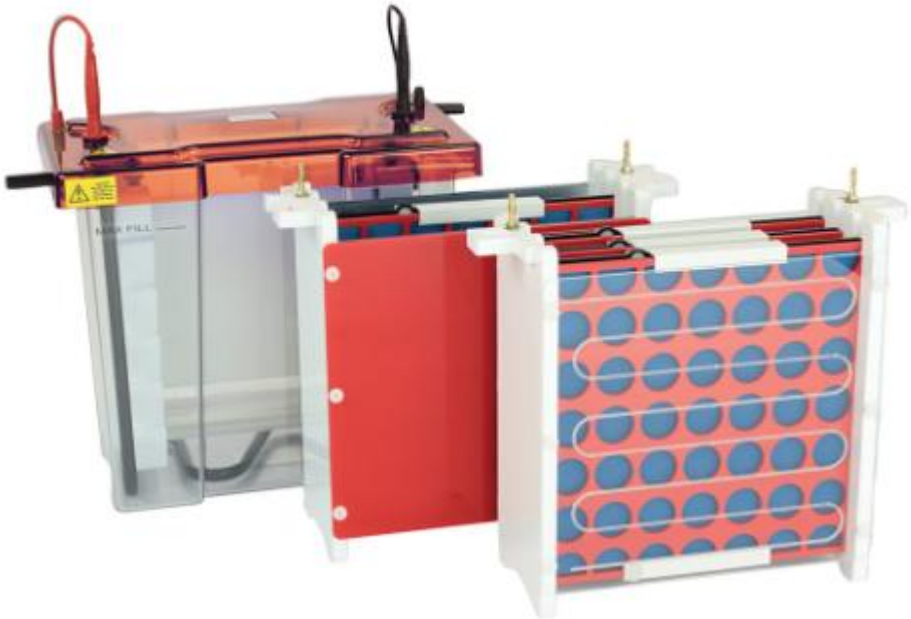
Expression analysis on protein level



Western blot



Nup358



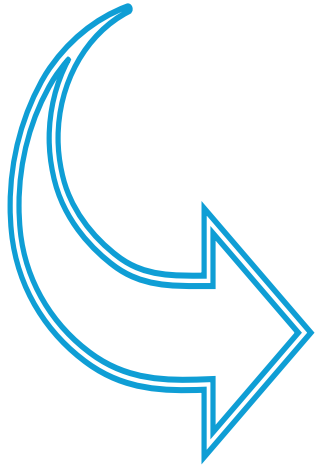
Expression analysis on protein level



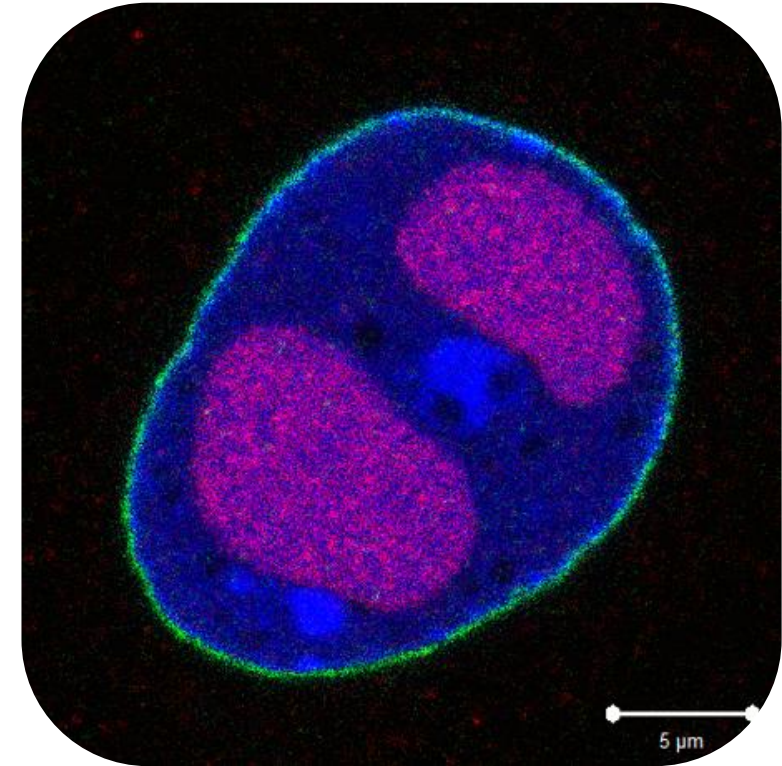
ELISA



Expression analysis on protein level



Immunofluorescence



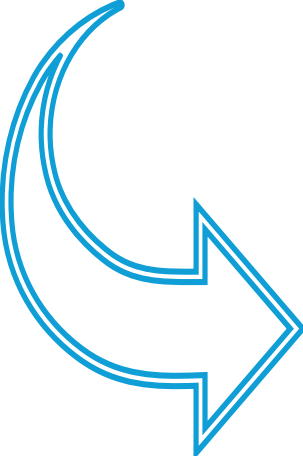
RPTEC 96 hpi with BK virus (BKPyV)

Lamin A/C

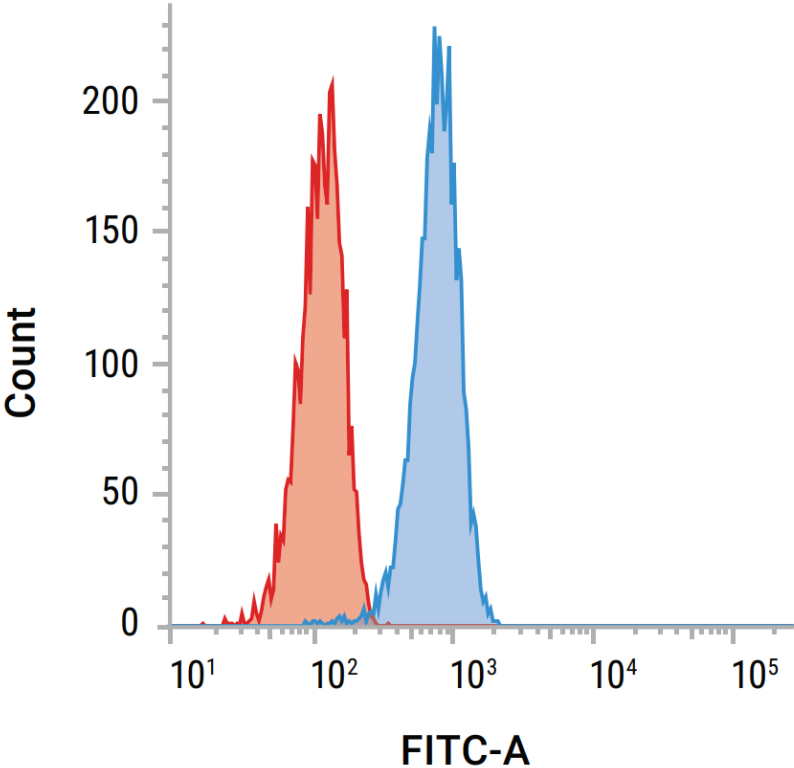
VP1 BKPyV

DNA

Expression analysis on protein level



FACS



All in one with HiBiT tagging system

Do you need?

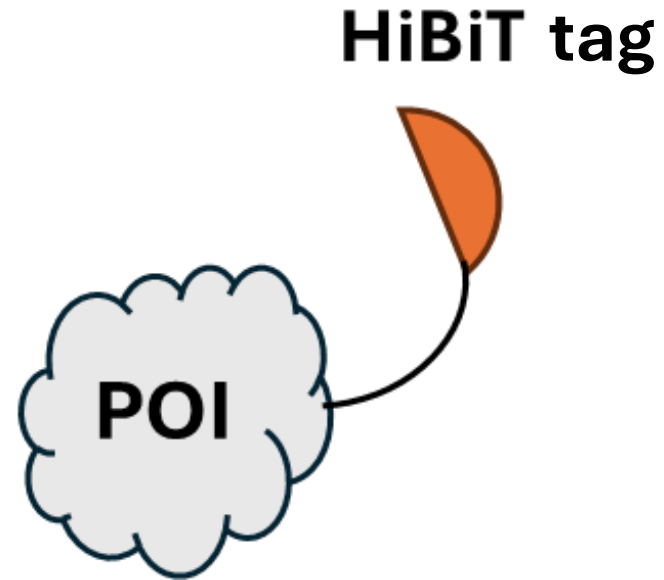
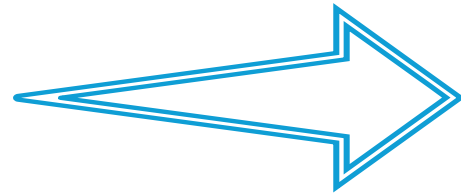
Fast

Simple

Sensitive

Reproducible

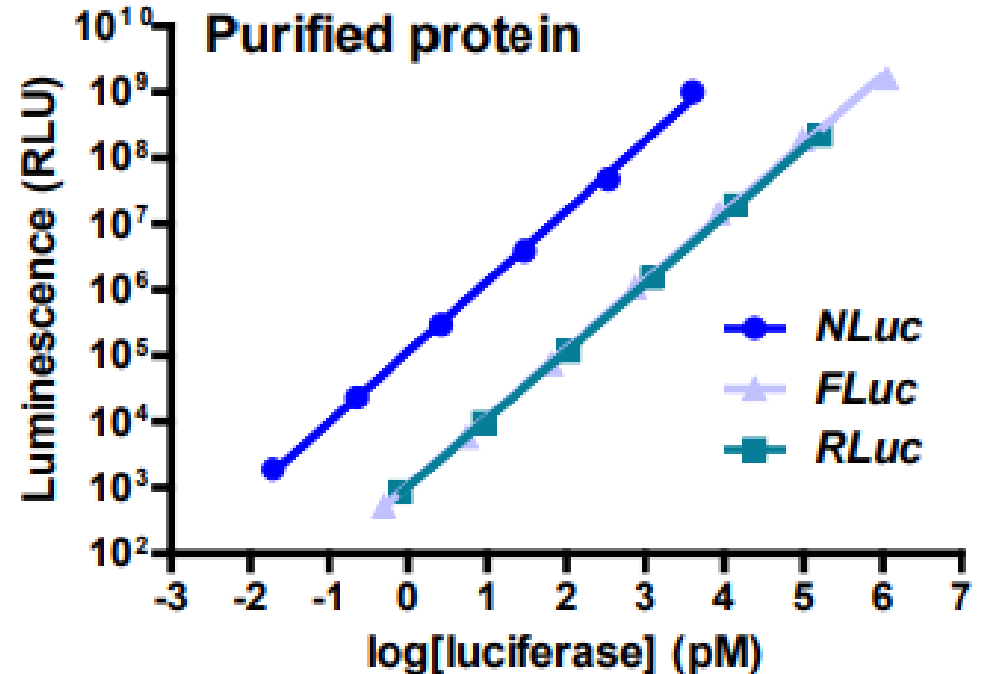
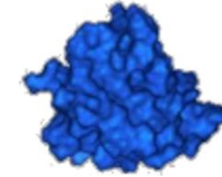
Real Time



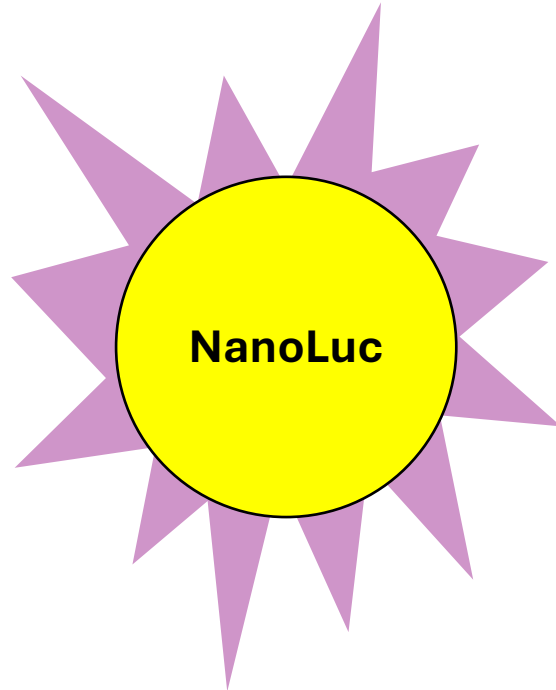
NanoLuc luciferase

- **Small** → 19 kDa
- Very **bright** → over 100-fold then Fluc, RLuc
- Long **glow-type** signal → **2-hour half-life**
- **ATP-independent**
- Improved physical and pH stability

NanoLuc

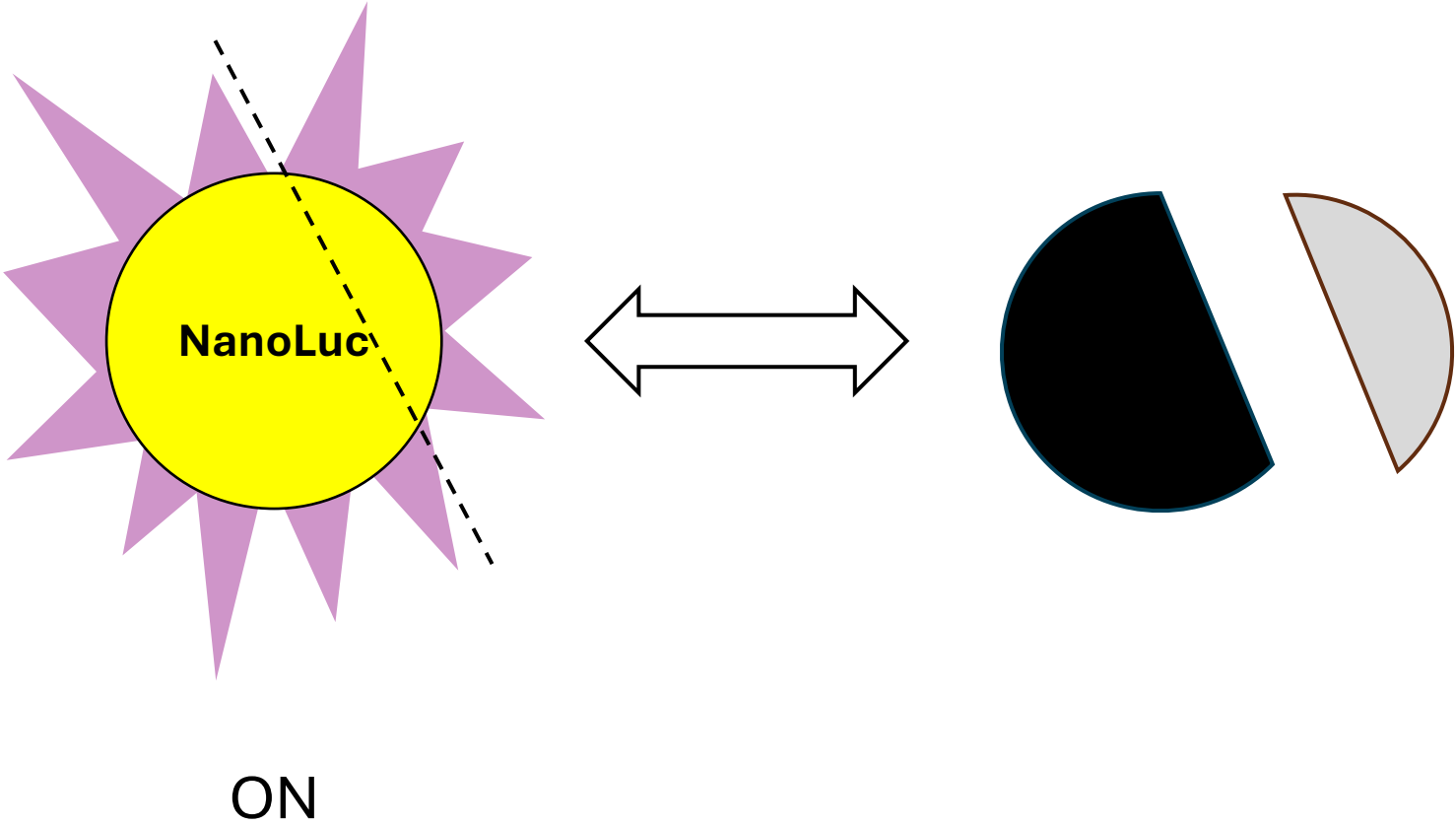


NanoLuc Binary Technology

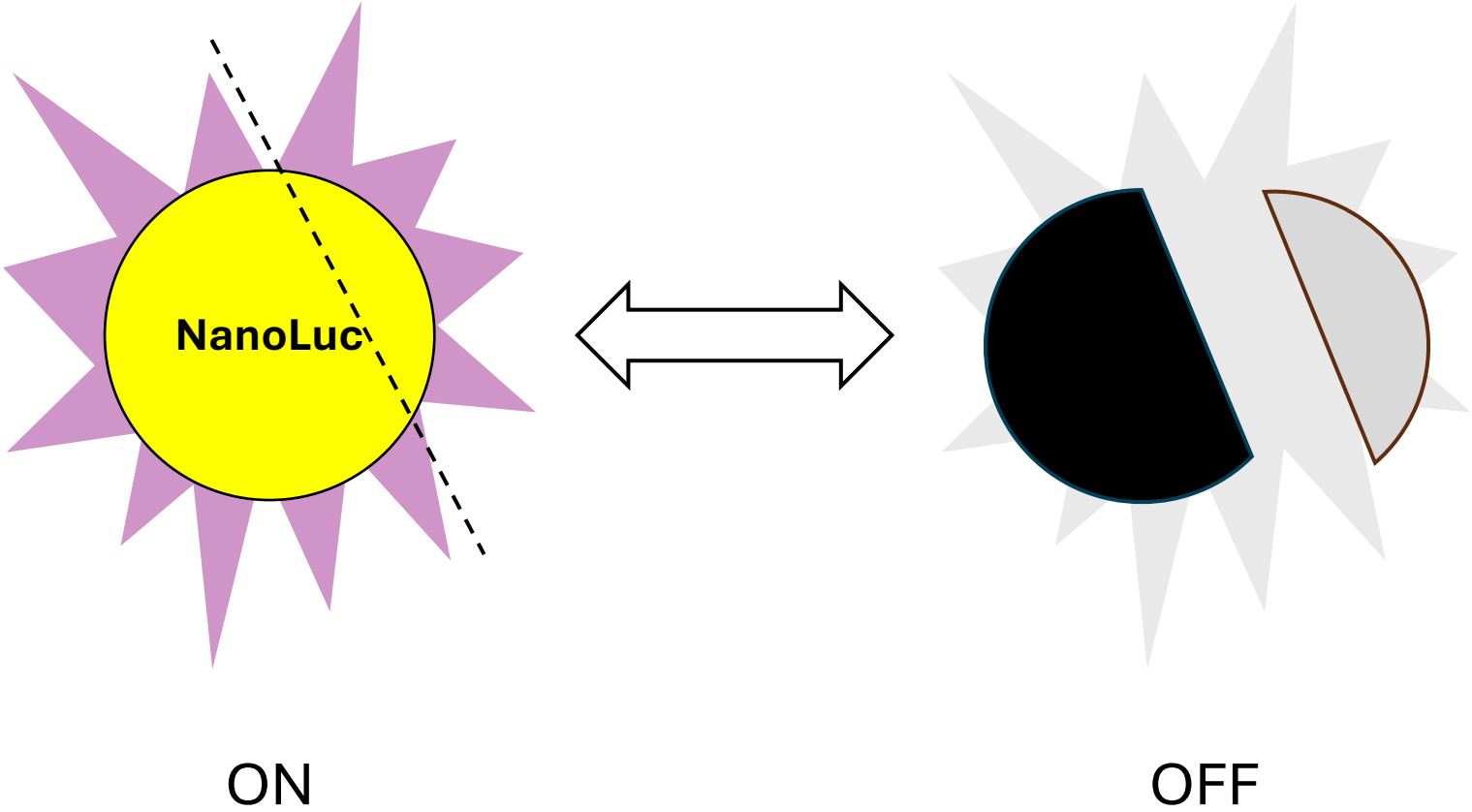


ON

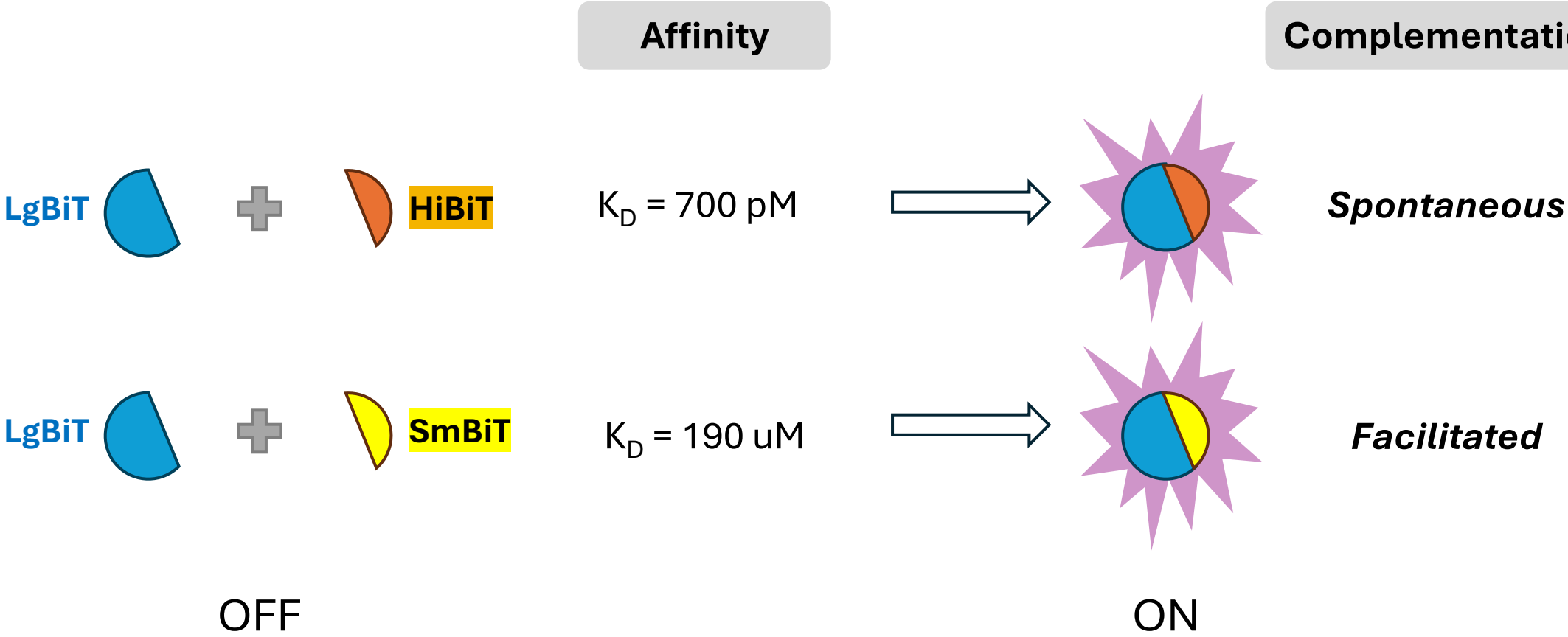
NanoLuc Binary Technology



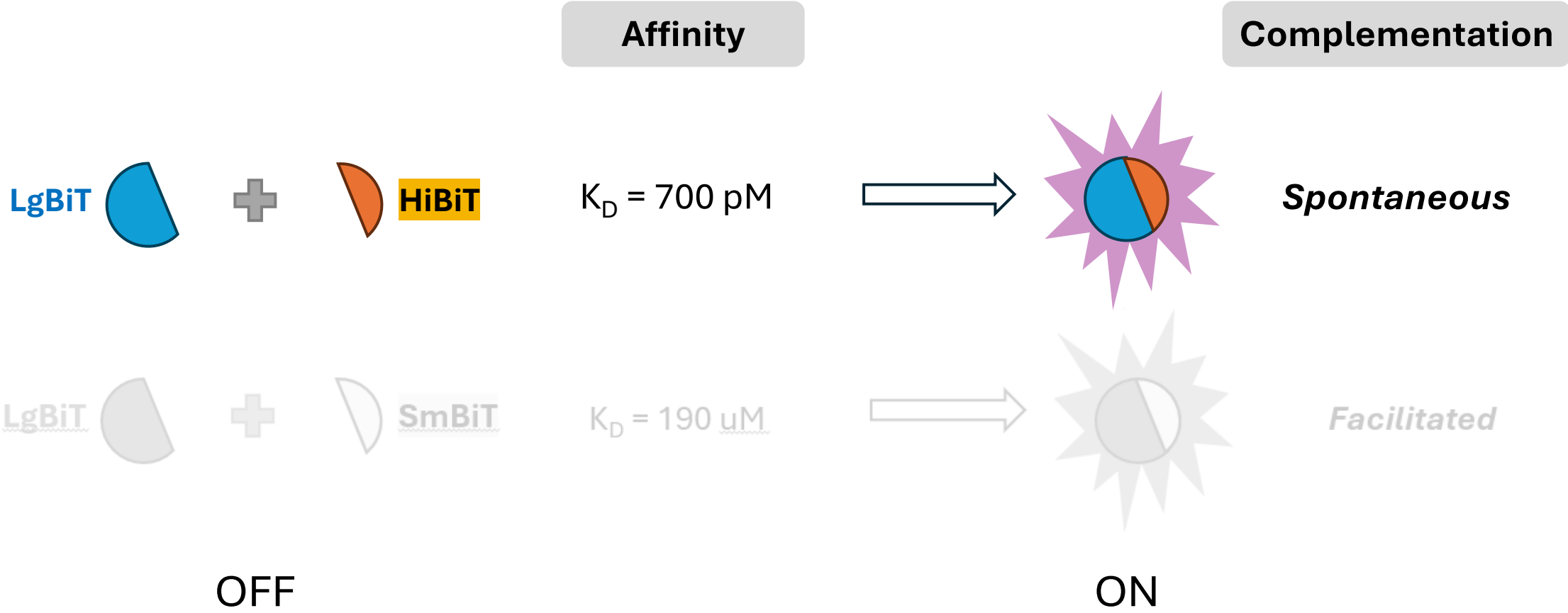
NanoLuc Binary Technology



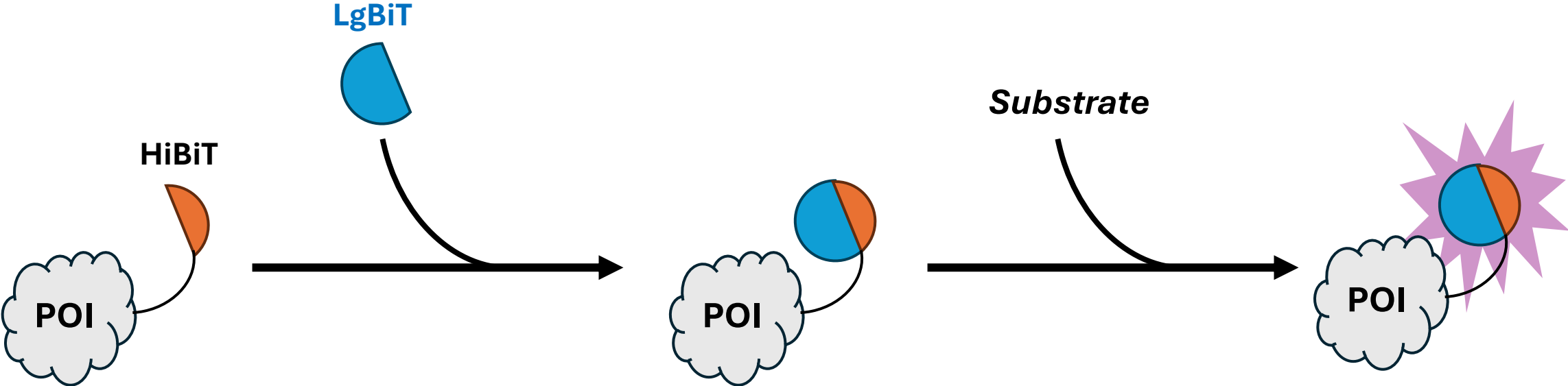
NanoLuc Binary Technology



HiBiT Tagging System – small by size, big by potential



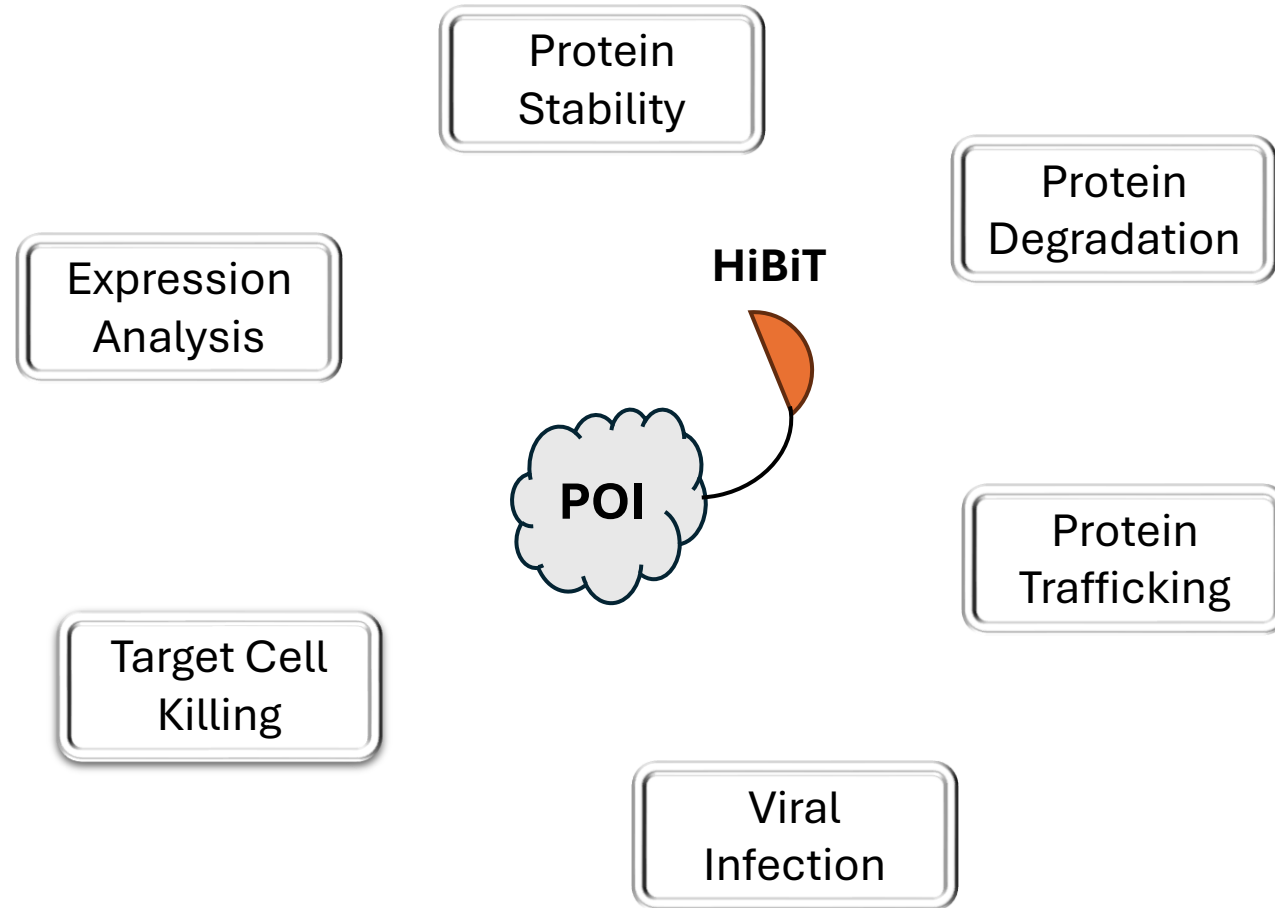
HiBiT Tagging System – small by size, big by potential



POI = protein of interest



HiBiT Tagging System – small by size, big by potential



POI = protein of interest

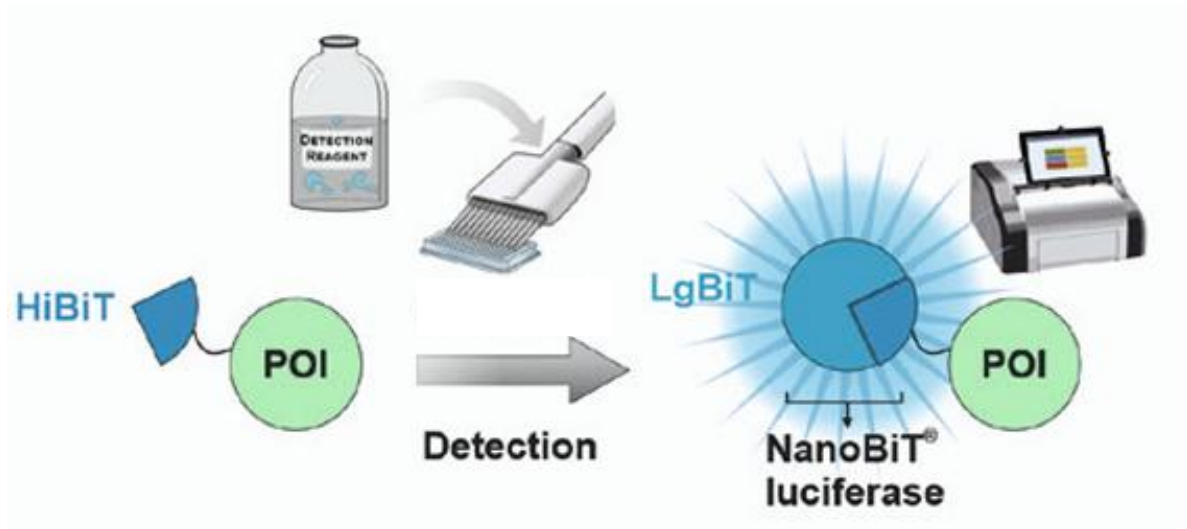
HiBiT Tagging System – small by size, big by potential



Lytic Detection System

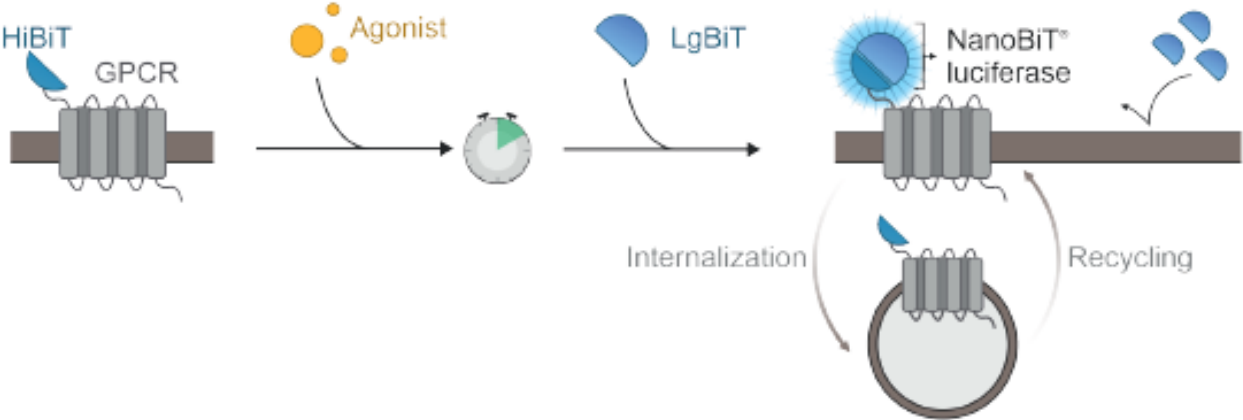
Extracellular Detection System

Live Cell Assay System

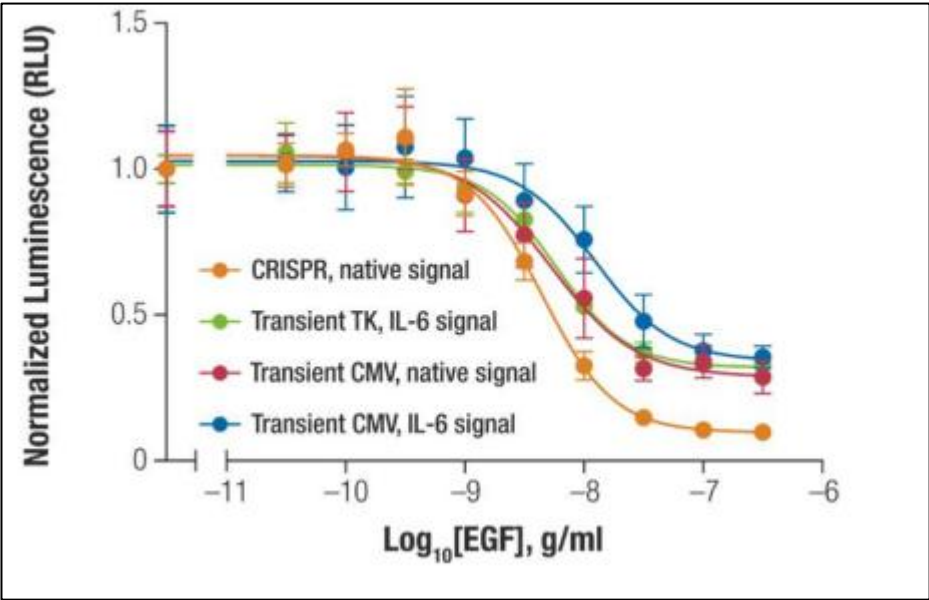


POI = protein of interest

HiBiT Tagging System – small by size, big by potential



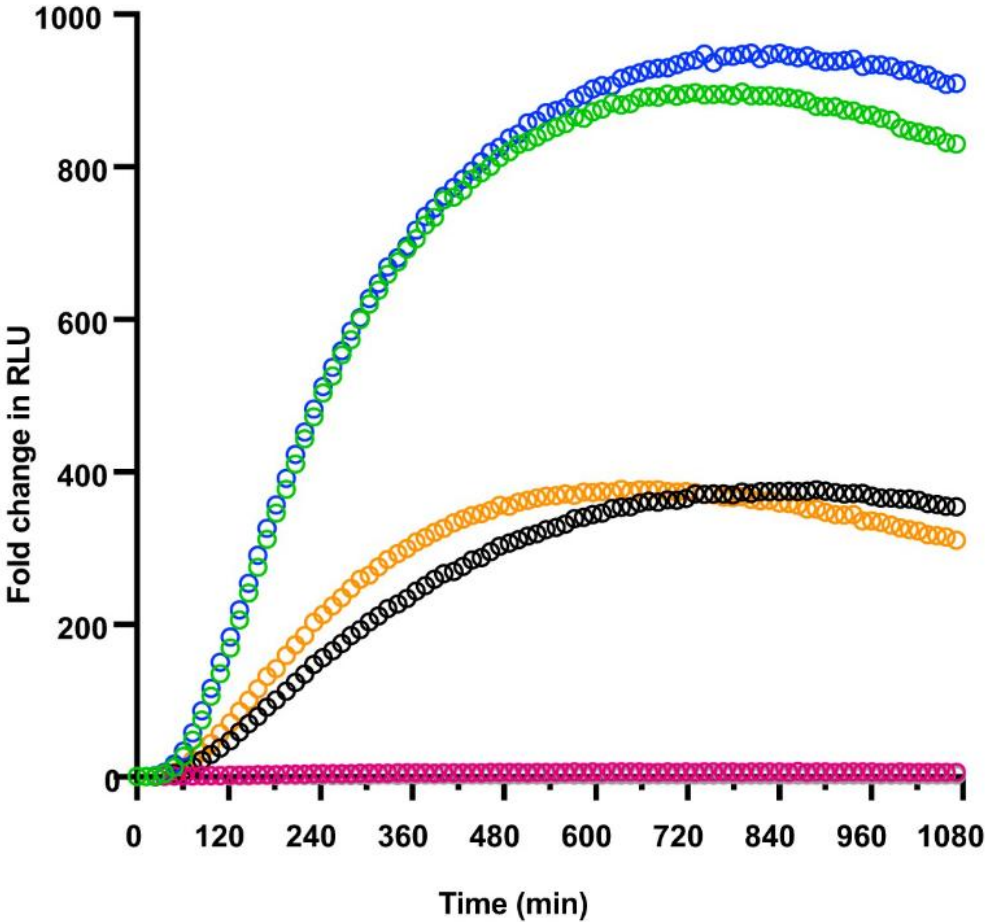
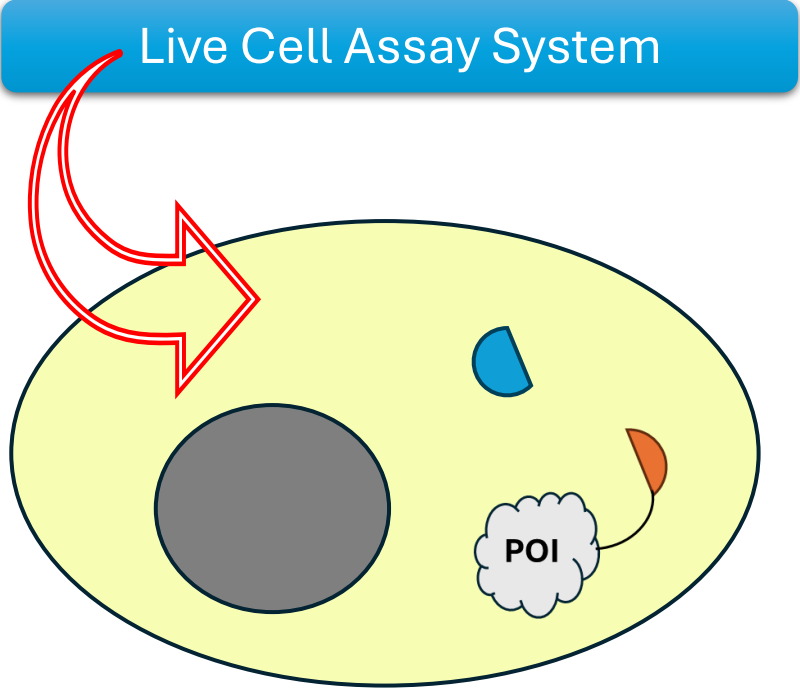
Extracellular Detection System



POI = protein of interest



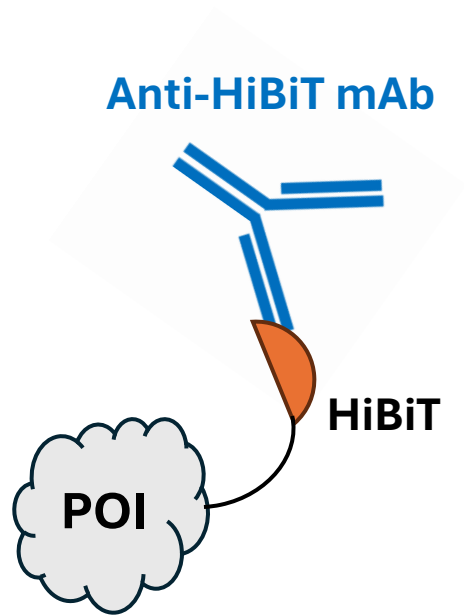
HiBiT Tagging System – small by size, big by potential



POI = protein of interest

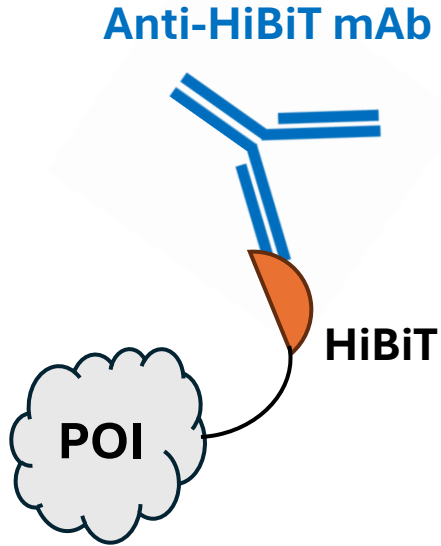


HiBiT Tagging System – small by size, big by potential

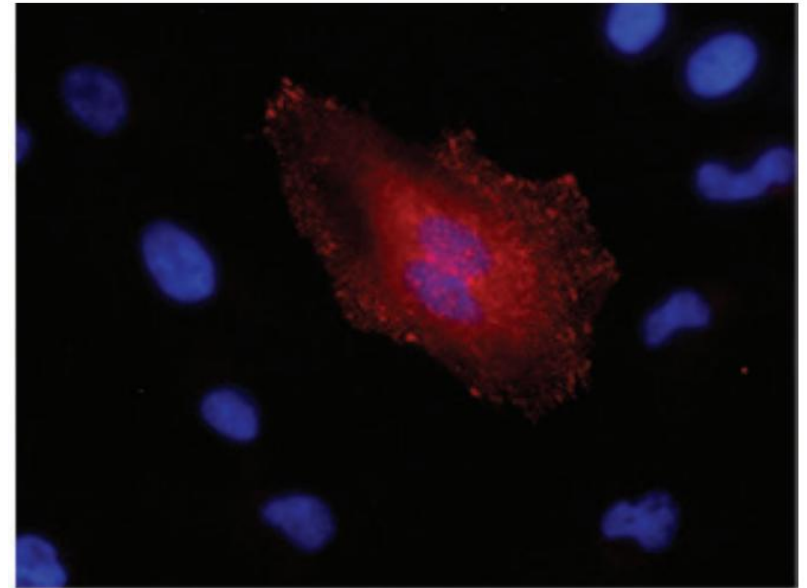


POI = protein of interest

HiBiT Tagging System – small by size, big by potential



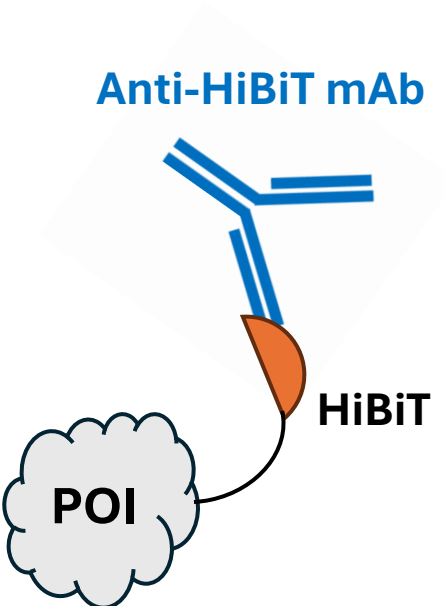
- Immunofluorescence
- Immunoblotting
- Immunoprecipitation
- FACS analysis



Vinculin
mxHiBiT; gxm AF-647; Hoechst

POI = protein of interest, mAb = monoclonal antibody; m = mouse; g = goat

HiBiT Tagging System – small by size, big by potential

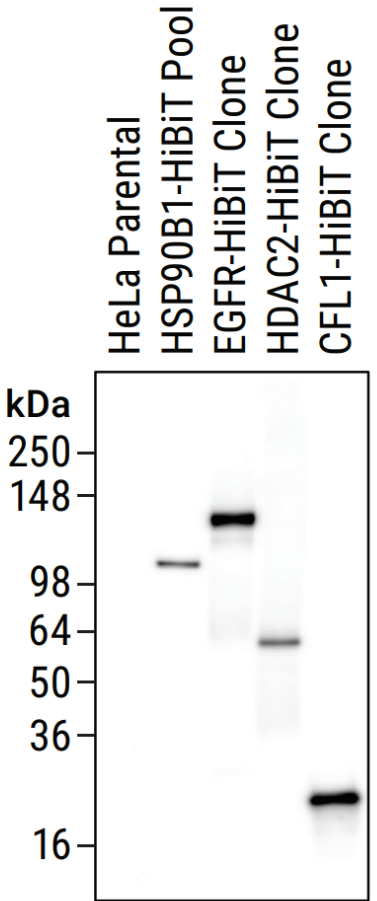


Immunofluorescence

Immunoblotting

Immunoprecipitation

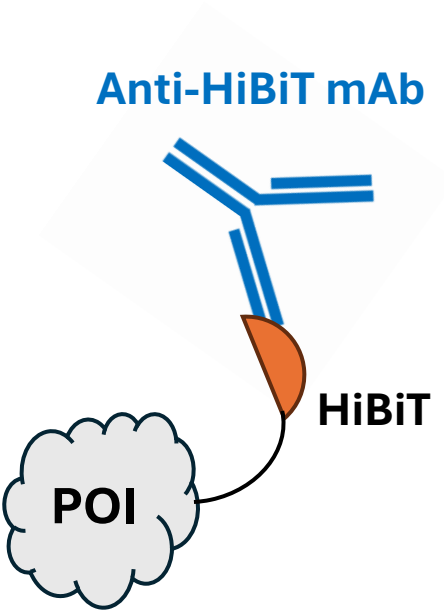
FACS analysis



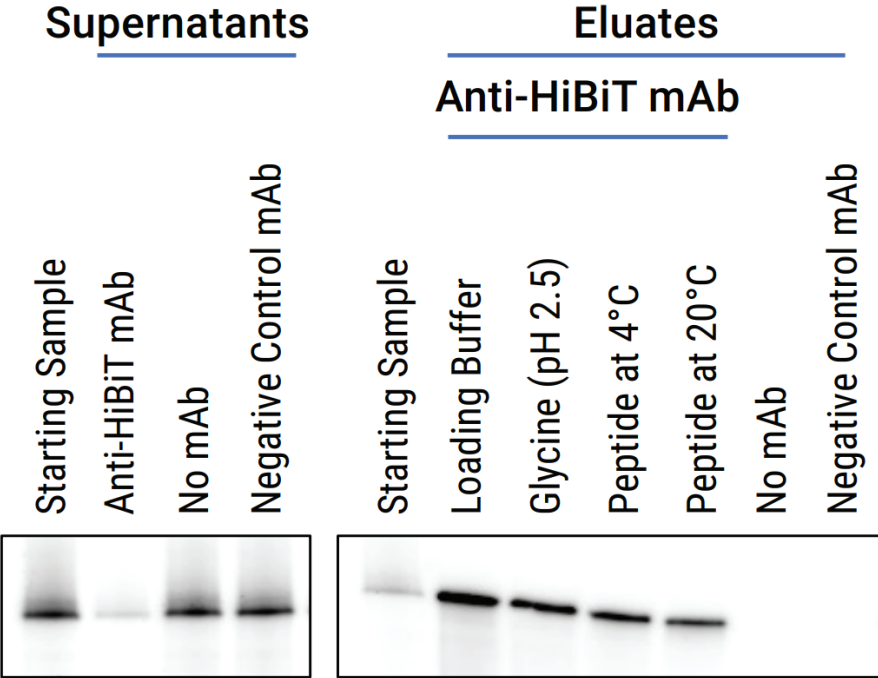
POI = protein of interest; mAb = monoclonal antibody



HiBiT Tagging System – small by size, big by potential



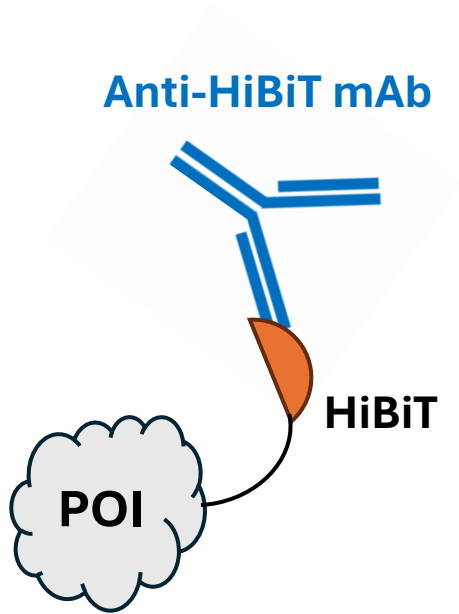
- Immunofluorescence
- Immunoblotting
- Immunoprecipitation**
- FACS analysis



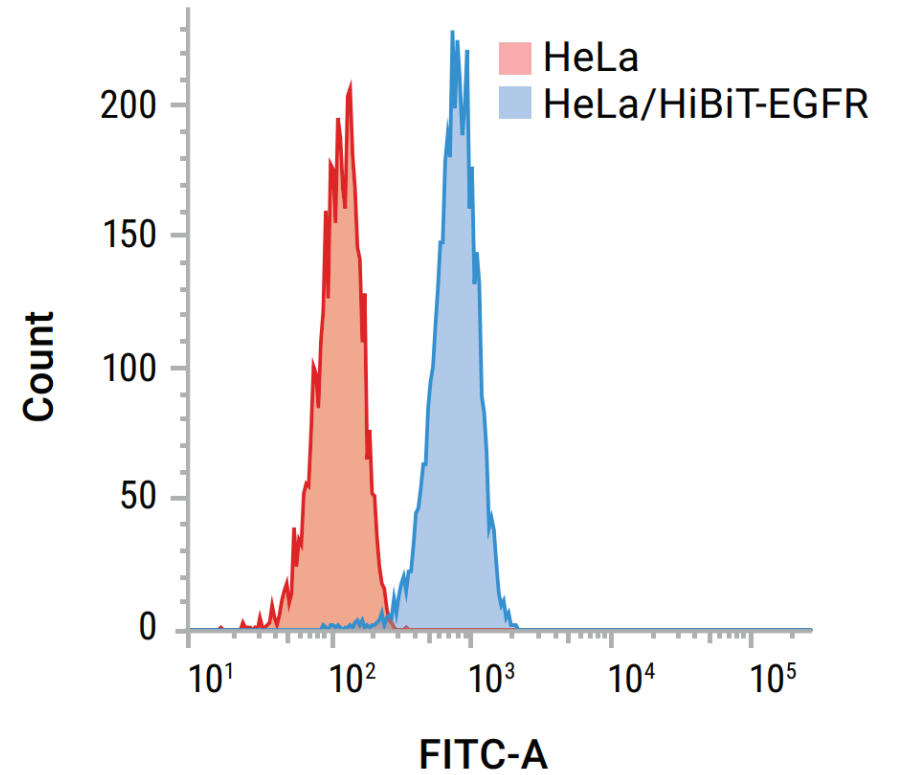
POI = protein of interest; mAb = monoclonal antibody



HiBiT Tagging System – small by size, big by potential



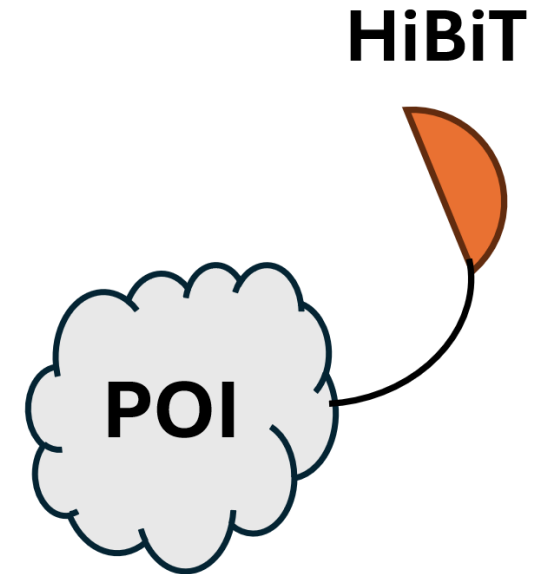
- Immunofluorescence
- Immunoblotting
- Immunoprecipitation
- FACS analysis**



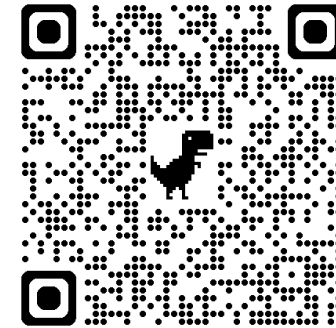
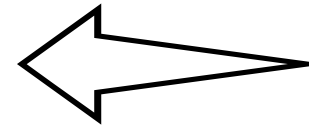
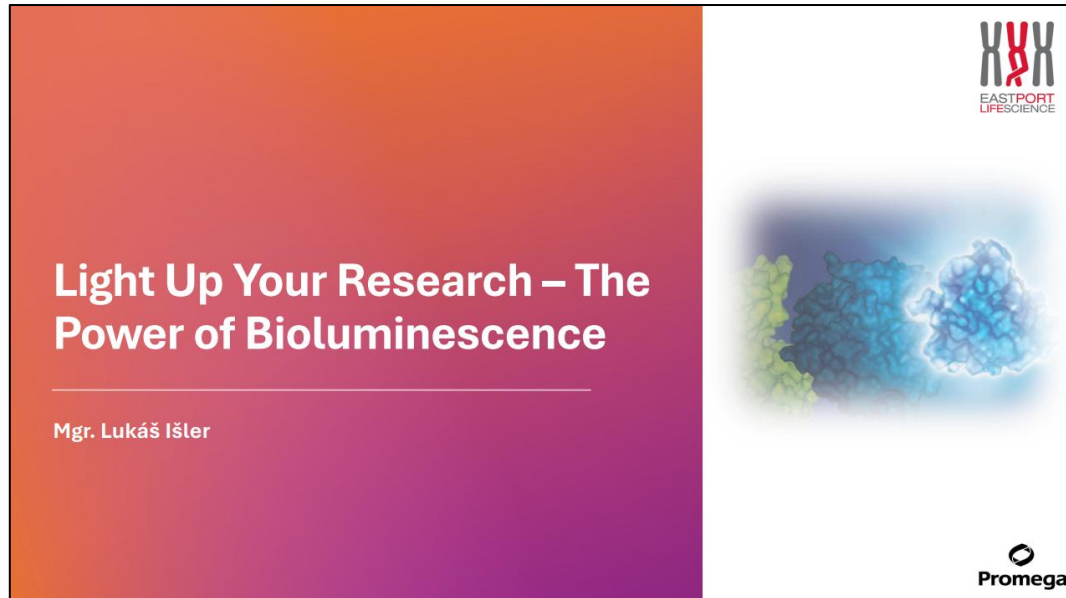
POI = protein of interest; mAb = monoclonal antibody

HiBiT Tagging System – small by size, big by potential

- **Small** (11 aa) → reduced risk on POI function
- Vector or CRISPR/Cas delivery
- Simple, homogenous detection assay → **add-and-read**
- No antibodies, **no washing steps**
- High sensitivity – ideal for **low abundant** proteins
- **HTS** compatibility



HiBiT Tagging System – Application

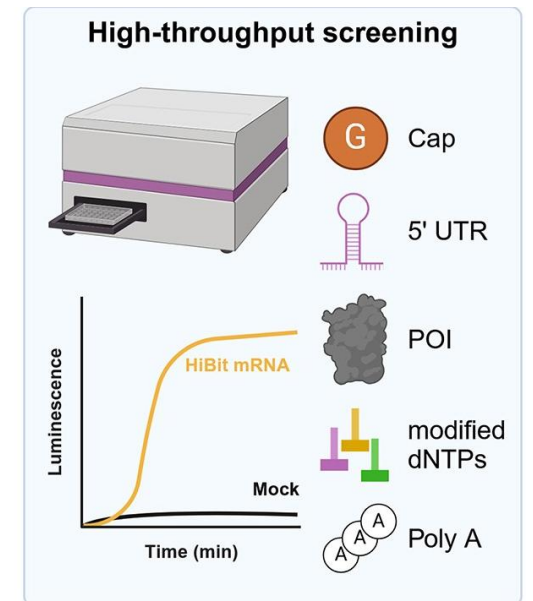
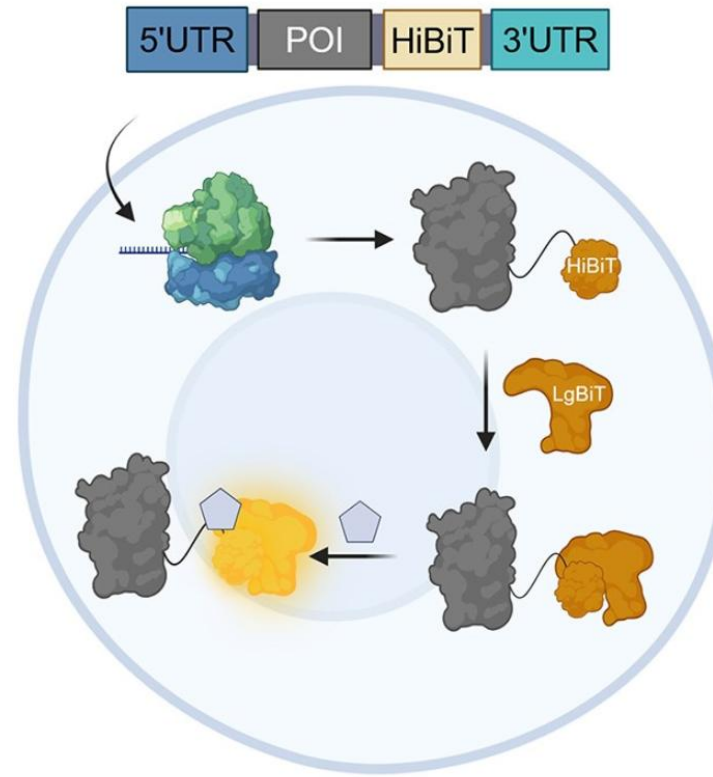


HiBiT for optimization of mRNA therapy

- **HiBiT** used for detection of mRNA modifications:

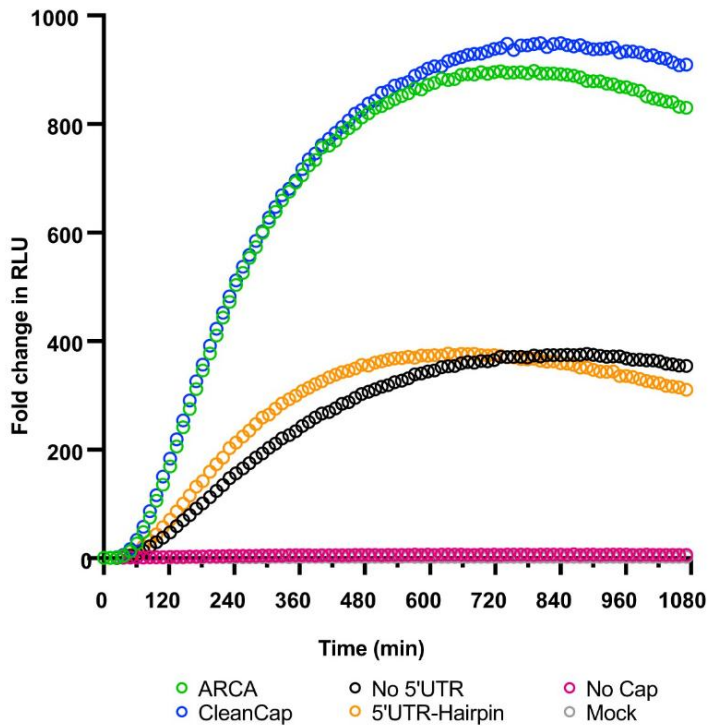
- Cap
- 5' UTR
- Nucleotides
- Coding sequence
- Poly-A length

- **Live Cell Assay System**



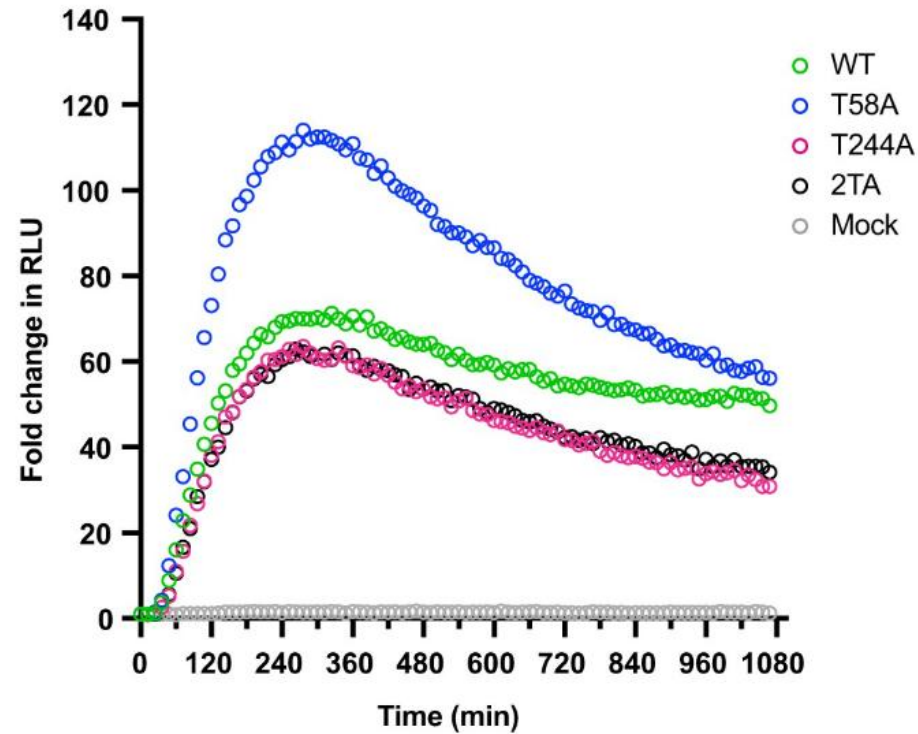
HiBiT for optimization of mRNA therapy

5' alterations



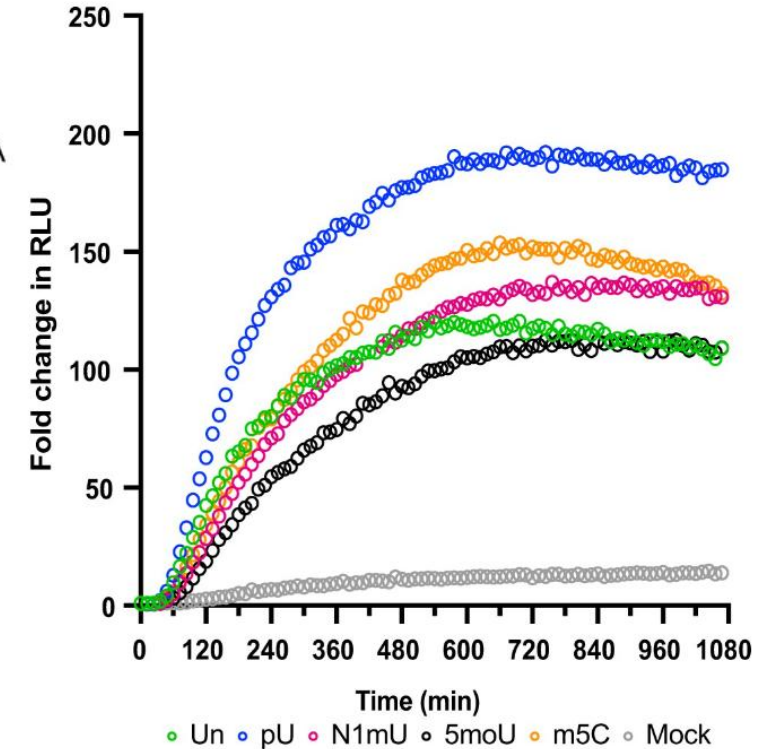
eGFP

Coding sequence



c-Myc

Modified NTPs



c-Myc

Luminescence detection



MyGlo™ Reagent Reader

- ✓ Luminescence



GloMax® Navigator

- ✓ Luminescence
- ✓ +Injectors



GloMax® Explorer

- ✓ Luminescence
- ✓ Fluorescence
- ✓ Vis Absorbance
- ✓ Heating
- ✓ Shaking



GloMax® Discover

- ✓ Luminescence
- ✓ Fluorescence
- ✓ UV/Vis Absorbance
- ✓ BRET / FRET
- ✓ Heating
- ✓ Shaking



GloMax® Galaxy

- ✓ NanoLuc Technology based Bioluminescence Imager

Luminescence detection



MyGlo™ Reagent Reader

- ✓ Luminescence



GloMax® Navigator

- ✓ Luminescence
- ✓ +Injectors



GloMax® Explorer

- ✓ Luminescence
- ✓ Fluorescence
- ✓ Vis Absorbance
- ✓ Heating
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GloMax® Galaxy

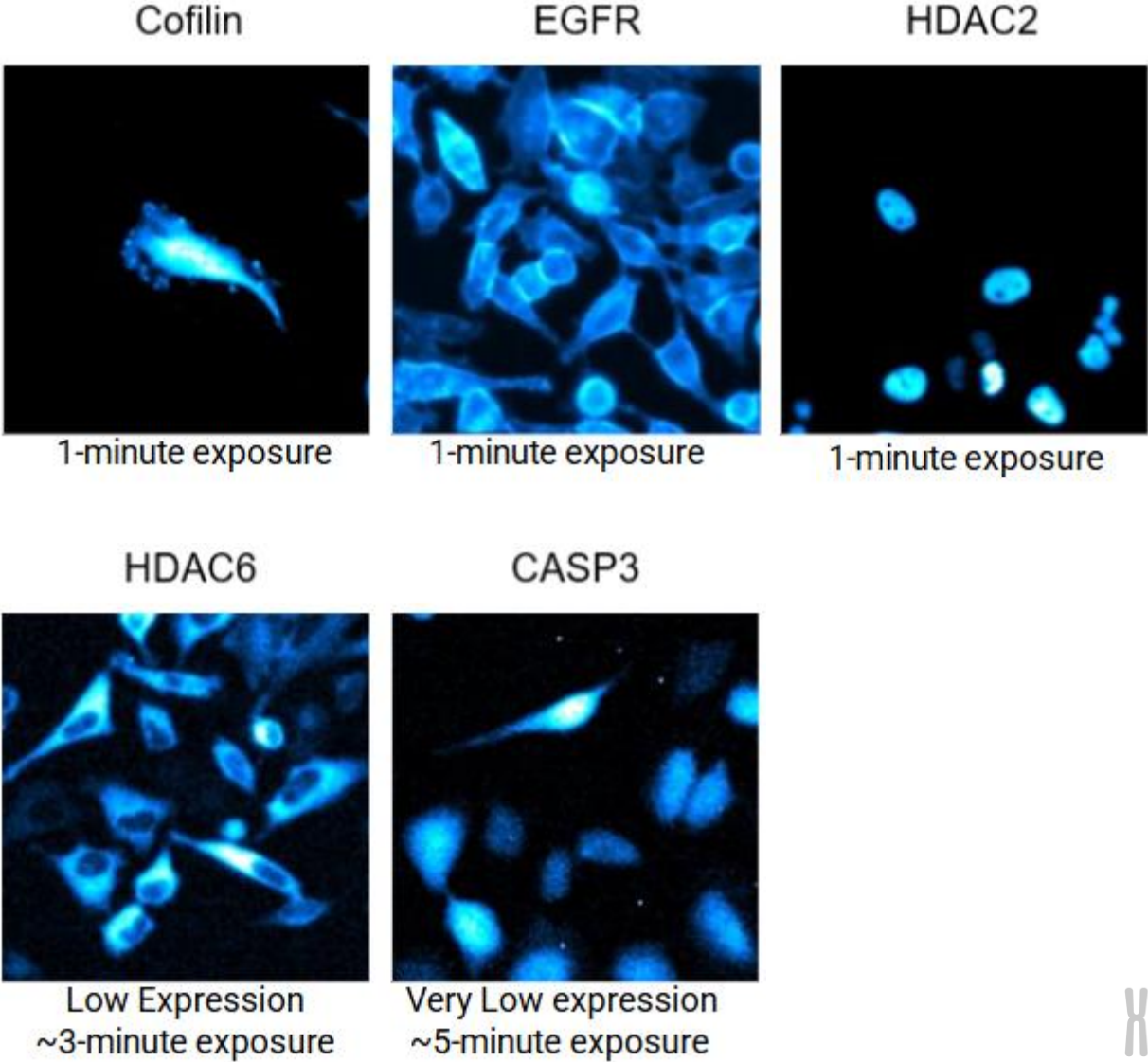
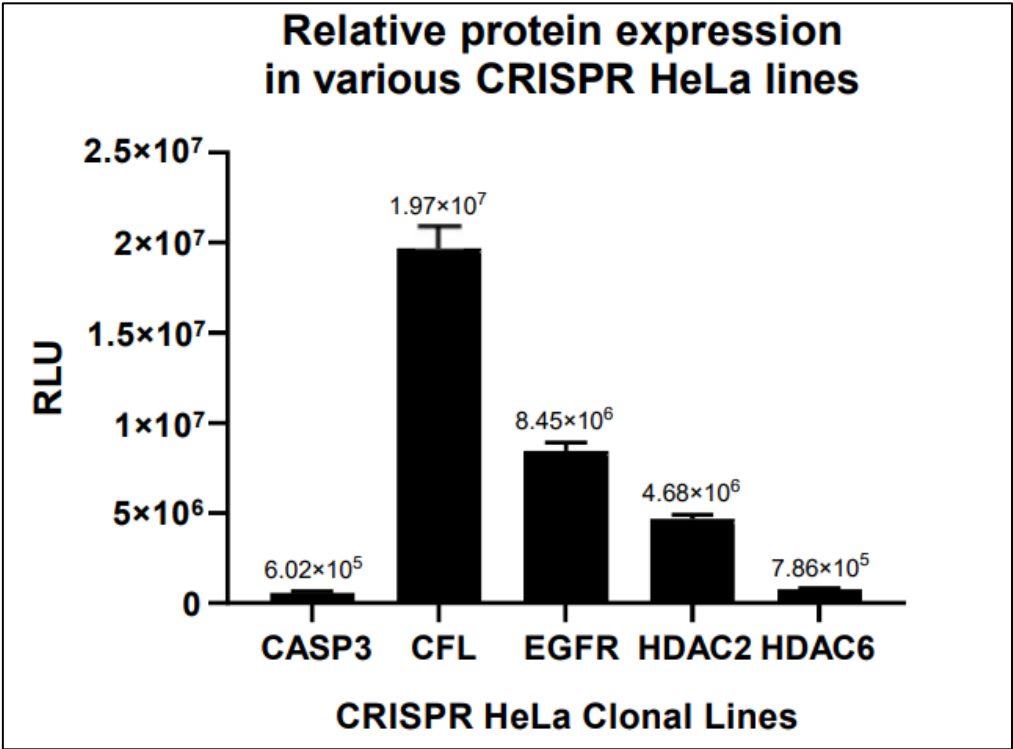
- ✓ NanoLuc Technology based Bioluminescence Imager

Glomax Galaxy – Luminescence under the microscope

- **Bioluminescence** imager system
- Image NanoLuc Technologies – NanoBiT, **HiBiT**, NanoBRET
- Living & fixed cells
- Study Protein Dynamics in **real-time**
- Luminescence, fluorescence, brightfield



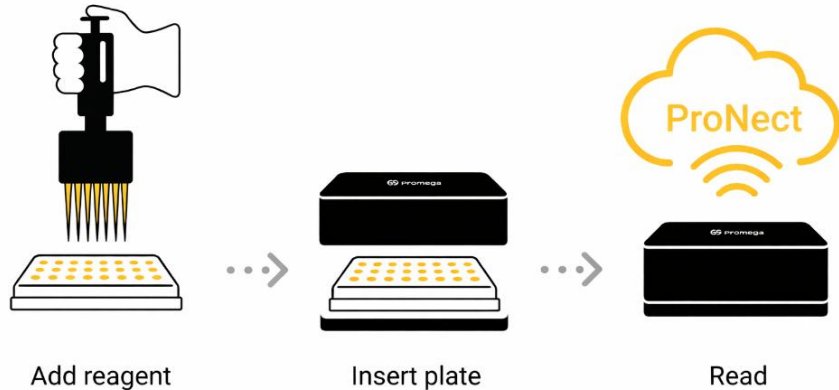
Glomax Galaxy – Luminescence under the microscope



Pocket luminometer? Look at MyGlo!

MyGlo Reagent Reader

- Compact and portable **96-well** plate reader
- Works with **wide range** of luminescent assays



Easy Workflow

- Measurement setup and analysis via **ProNect App**
- Results in less than 30 s (full 96-well, 1s integration time)
- Cloud accesible via any device (laptop, PC, smartphone)

Conclusion

- Gene expression is tightly regulated at multiple levels
- Direct correlation between mRNA and protein levels is not a rule
- **HiBiT tagging system** is a robust, reproducible and sensitive method for analysis of:
 - Protein expression
 - Protein stability
 - Protein localization

RNA vs RNA: RNA interference

- Cellular mechanism to silence gene expression using short RNA molecules
- Present in plants, animals, fungi

Antiviral defense

Degrades dsRNA from viruses

Gene regulation

Fine-tunes endogenous gene expression

Research tool

Experimental gene KD and RNA therapy

KD = knockdown

RNA vs RNA: RNA interference

A light blue rounded square containing a white circle with the text "miRNA" inside.

miRNA

A light blue rounded square containing a white circle with the text "siRNA" inside.

siRNA

A light blue rounded square containing a white circle with the text "piRNA" inside.

piRNA

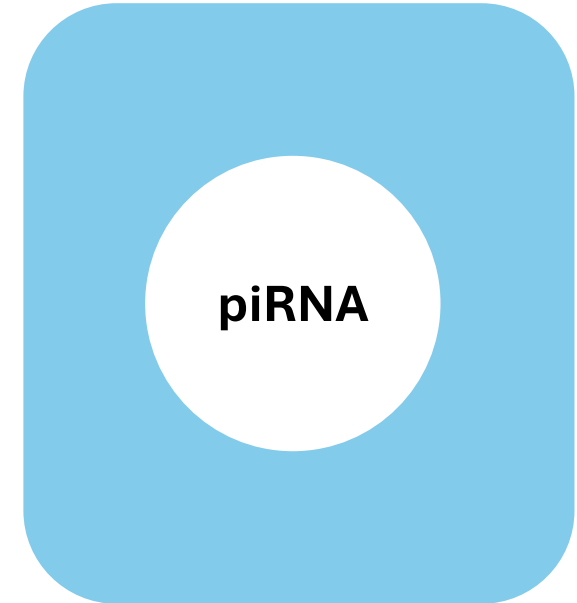
Which type of RNA has the characteristics below?

- Endogenous
- Processed by **Drosha** and **Dicer**
- Hairpin precursor
- Translational repression / mRNA destabilization
- Many targets



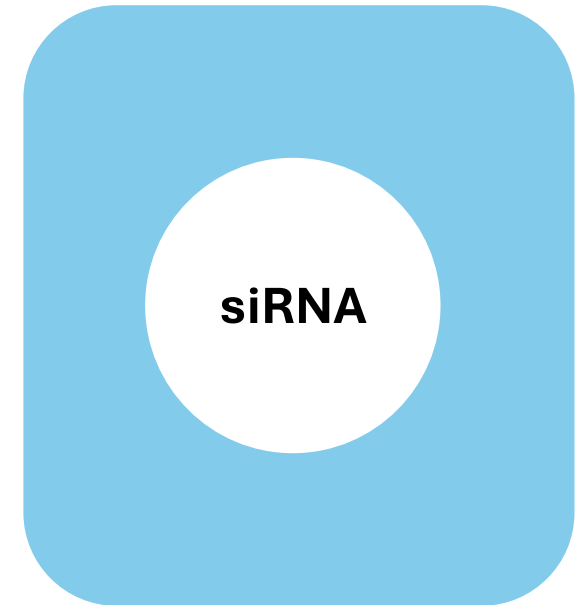
Which type of RNA has the characteristics below?

- Endogenous
- Dicer-independent
- Found in **germline cells**
- Targets transposons
- Couples with **PIWI** proteins



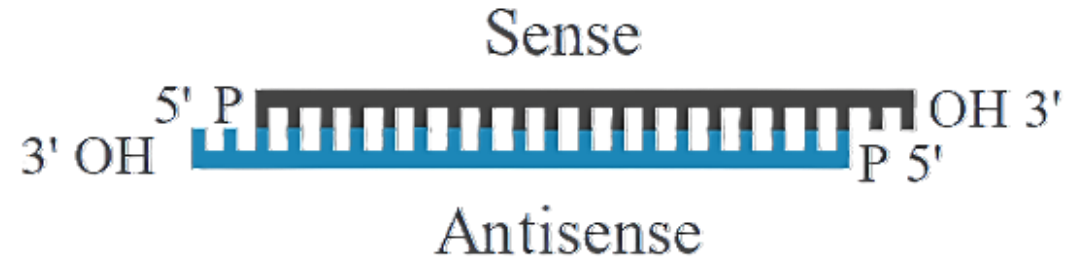
Which type of RNA has the characteristics below?

- Exogenous or endogenous
- Processed by Dicer
- **Antiviral mechanism** in plants
- One specific target
- mRNA cleavage



siRNA

- Small interfering RNA
- 21 – 22 nt long dsRNA
- Discovered in *C. elegans* in
(hint: Nobel prize in 2006)



siRNA

- Small interfering RNA
- 21 – 22 nt long dsRNA
- Discovered in *C. elegans* in **1998**



Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

Andrew Fire^{*}, SiQun Xu^{*}, Mary K. Montgomery^{*}, Steven A. Kostas^{*†}, Samuel E. Driver[‡] & Craig C. Mello[‡]

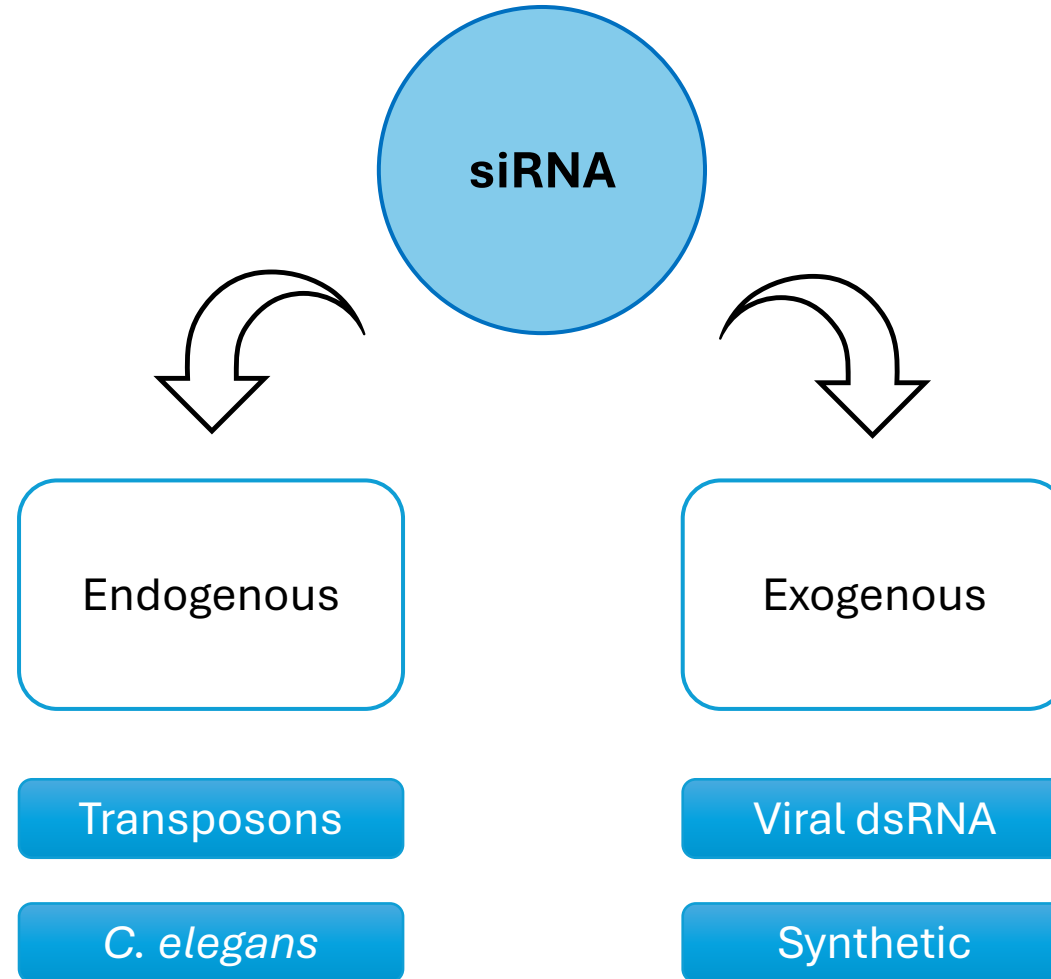
** Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210, USA*

† Biology Graduate Program, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, USA

‡ Program in Molecular Medicine, Department of Cell Biology, University of Massachusetts Cancer Center, Two Biotech Suite 213, 373 Plantation Street, Worcester, Massachusetts 01605, USA

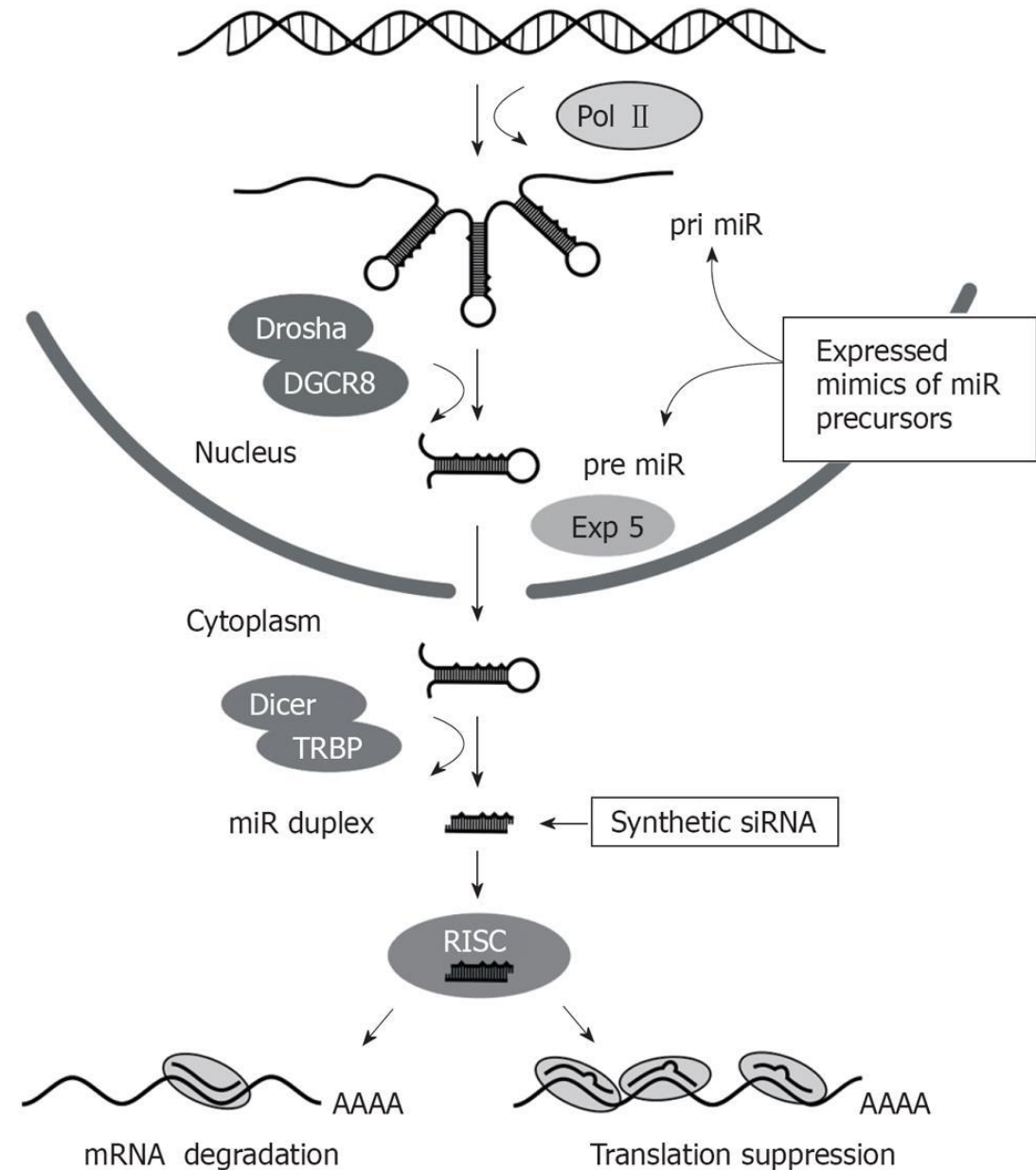
Fire, A., Xu, S., Montgomery, M. et al. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811 (1998). <https://doi.org/10.1038/35888>

siRNA



RNAi silencing mechanism

- Processed miRNA or exogenous siRNA loaded to **RISC**
- RISC = **R**NA-**I**nduced **S**ilencing **C**omplex
- **Argonaut (AGO)** → central RISC component



Role of Ago2 in RNAi pathway



Molecular Cell, Vol. 15, 185–197, July 23, 2004, Copyright ©2004 by Cell Press

Human Argonaute2 Mediates RNA Cleavage Targeted by miRNAs and siRNAs

Gunter Meister, Markus Landthaler, Agnieszka Patkaniowska, Yair Dorsett, Grace Teng, and Thomas Tuschl*
Laboratory of RNA Molecular Biology
The Rockefeller University
1230 York Avenue, Box 186
New York, New York 10021

Ambros, 2003; Lai, 2003) represent other endogenous sources of dsRNA.

Molecules of dsRNA are processed by Drosha and/or Dicer RNase III enzymes into small dsRNA molecules of specific length and structure (Bernstein et al., 2001; Elbashir et al., 2001a; Lee et al., 2002, 2003; Provost et al., 2002; Zamore et al., 2000; Zhang et al., 2002) and can enter into various gene silencing pathways that are collectively referred to as RNA silencing (Grishok et al

[Meister G, Landthaler M, Patkaniowska A...](#)
[Human Argonaute2 Mediates RNA Cleavage Targeted by miRNAs and siRNAs](#)
[Molecular Cell, 15, 185-197](#)

siTOOLS co-founder

Gunter Meister &

Thomas Tuschl 2004

siRNA – the experimental tool



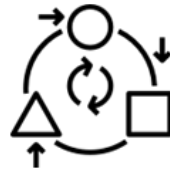
Fast (results in days, not weeks)



Dose-dependent (drug-like)



Simple (no need for engineered cell lines)



Transient (avoids adaptation)



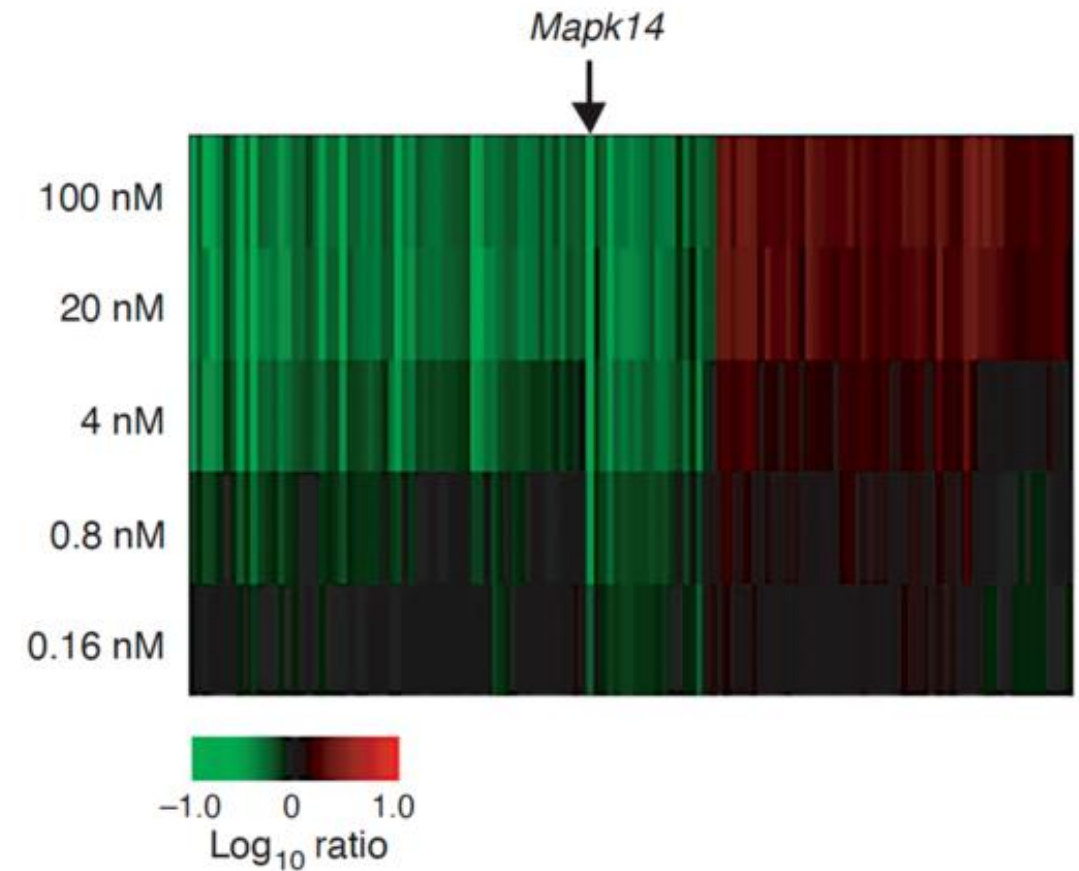
Broadly applicable in various biological context

Off-target effects

- siRNA pitfall
- Silencing of target gene along with many others
- On/off-targets are dose dependent
- ***Between Scylla and Charybdis***

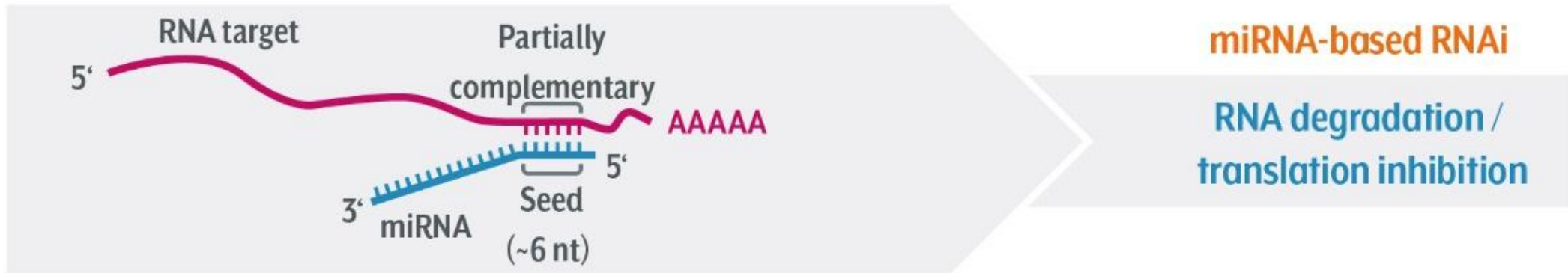
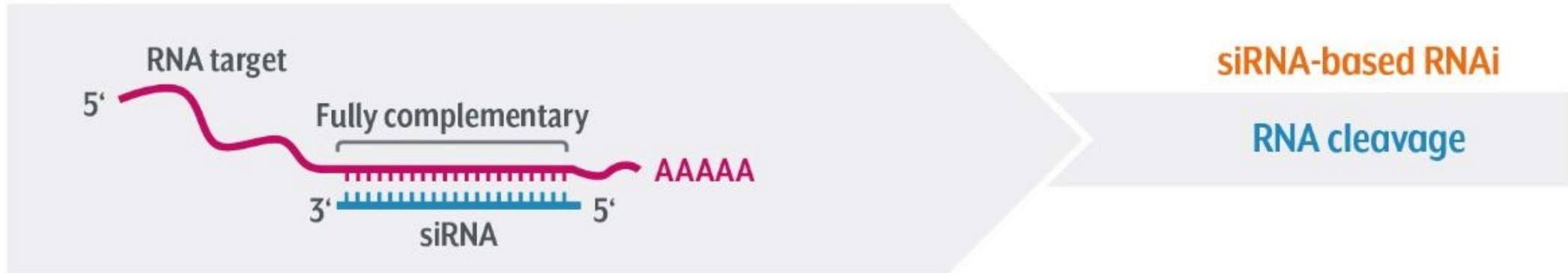
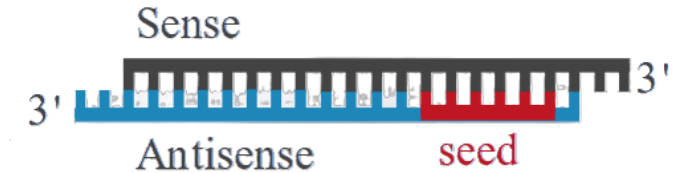


Lower siRNA concentrations decreases both off- and on-targets



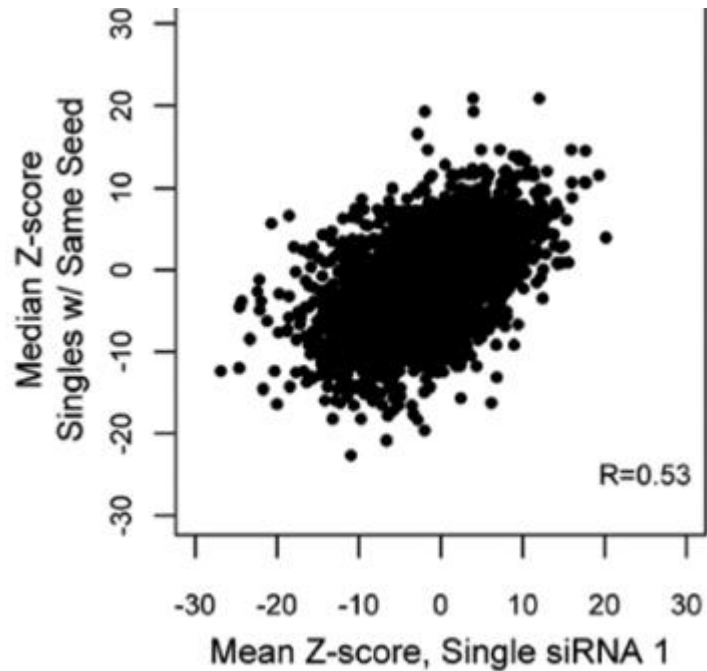
Ups, I missed the target...

- Off-target → siRNA acts as a artificial miRNA



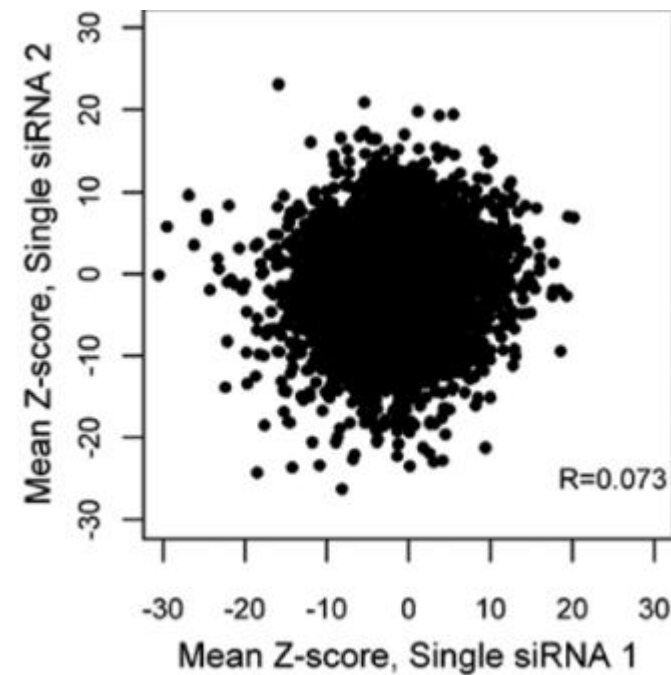
Seed-based off targets

Seed-based off-target effects dominate siRNA-induced phenotypes



Same seed siRNAs but targeting different genes

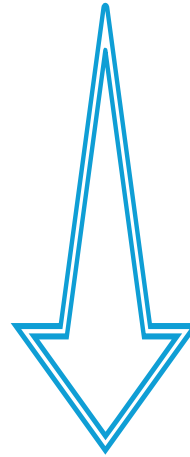
Poor siRNA specificity leads to varying phenotypes



Different siRNAs targeting same gene

Seed-based off targets

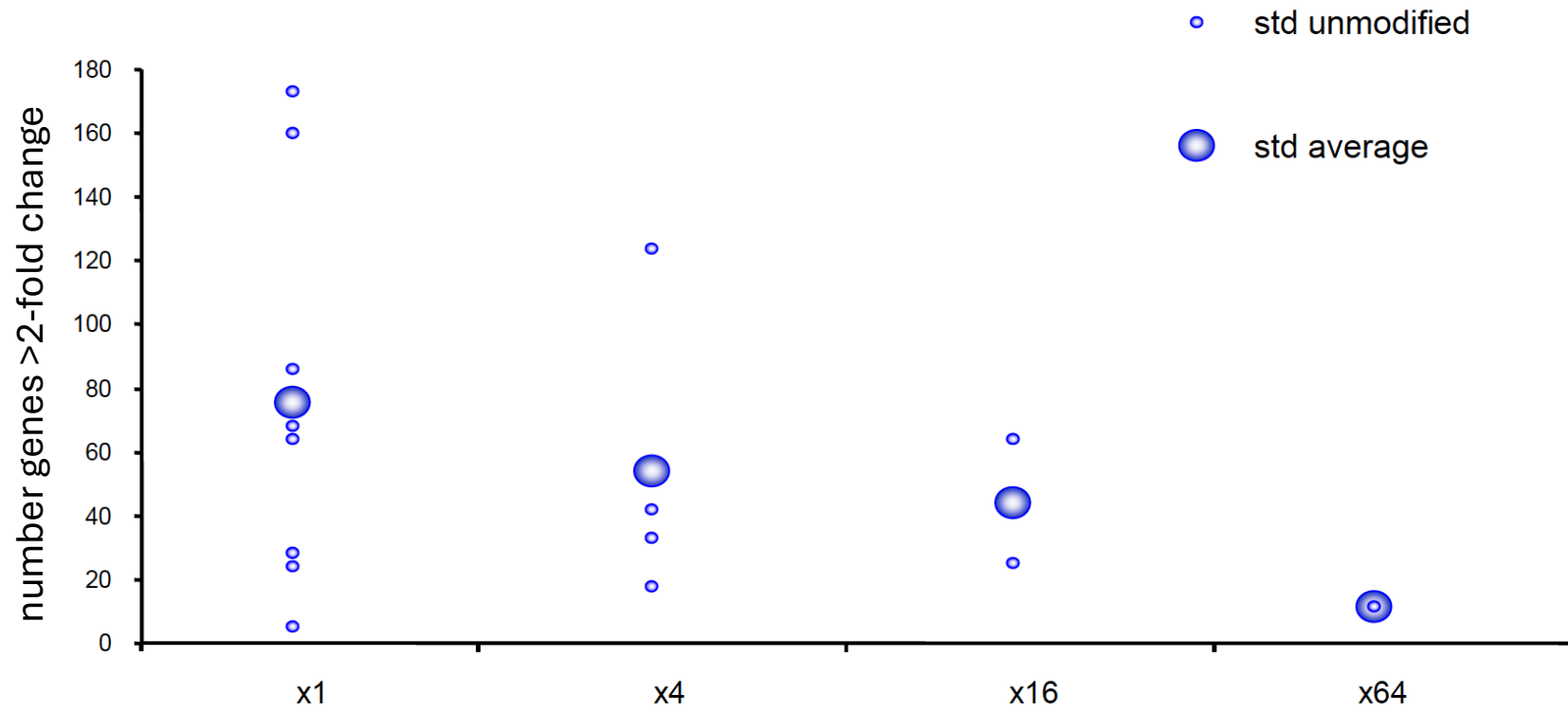
Can we reduce off-targets?



Pooling

Seed-based off targets

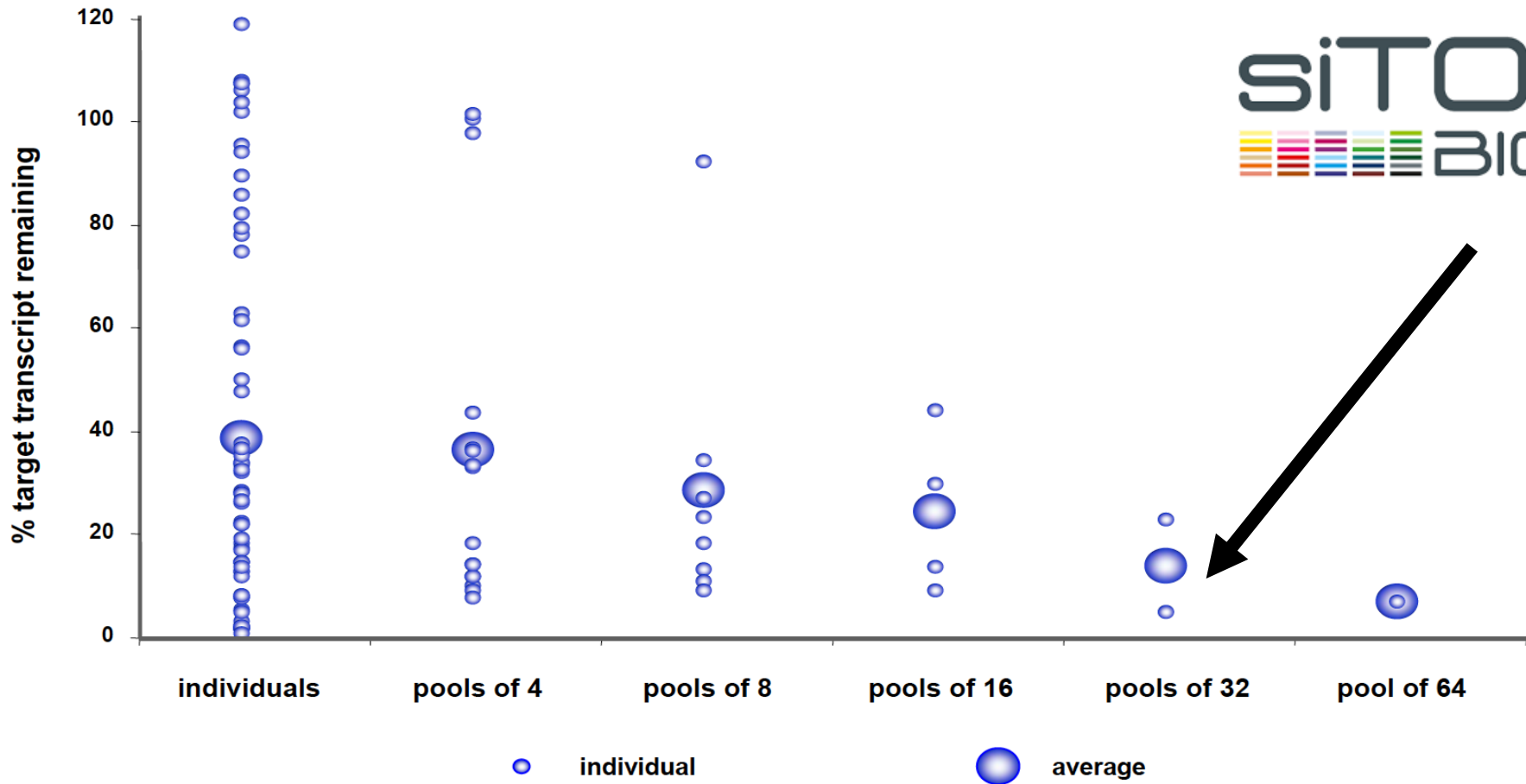
Pooling of siRNA reduces off-targets



=> Specificity improves with complexity

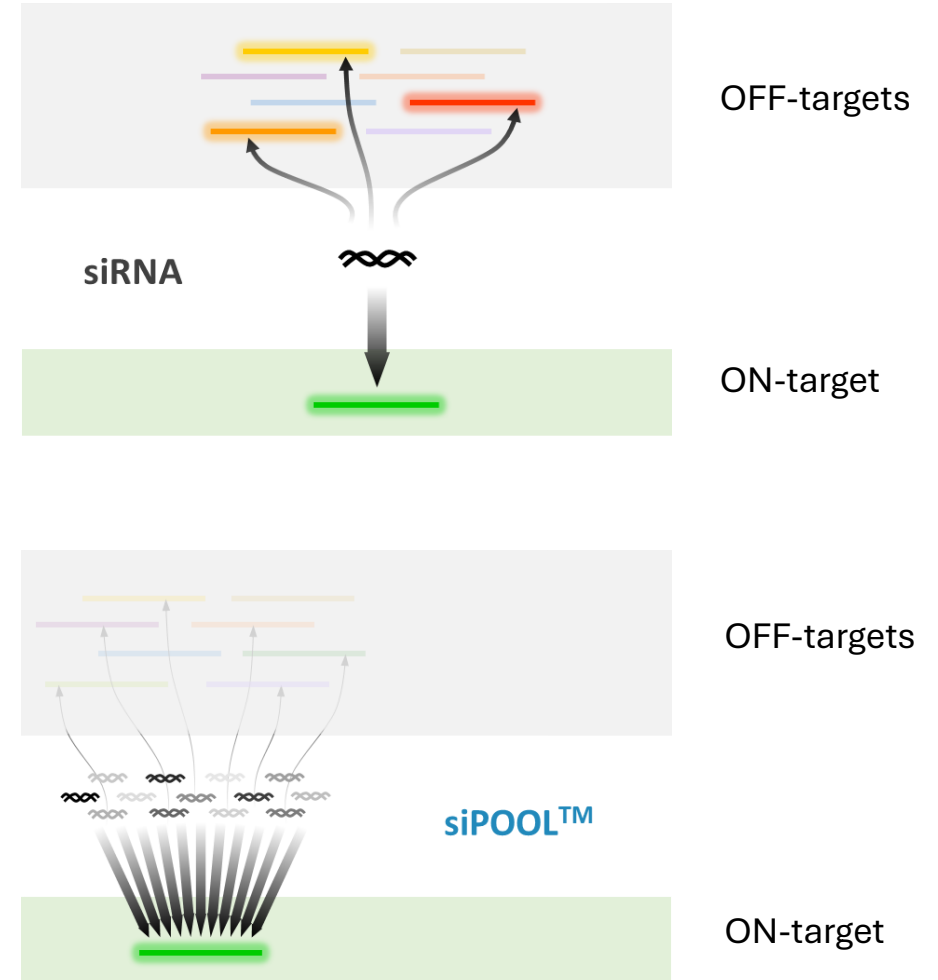
Seed-based off targets

Pooling of siRNA increases on-target silencing



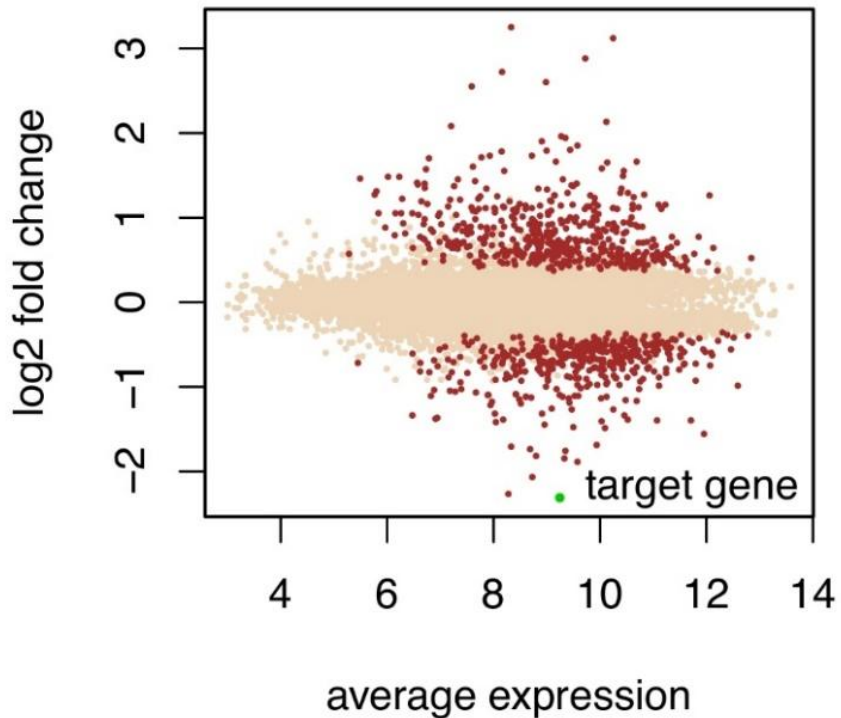
When one siRNA is not enough... Then 30 could help!

- Pooling of 30 individual siRNA → **siPOOLS™**
- **Picomolar** range of individual siRNAs in the pool
- Efficient off-target dilution
- Effective siRNA concentration for on-target silencing is the **sum of all individual siRNAs**

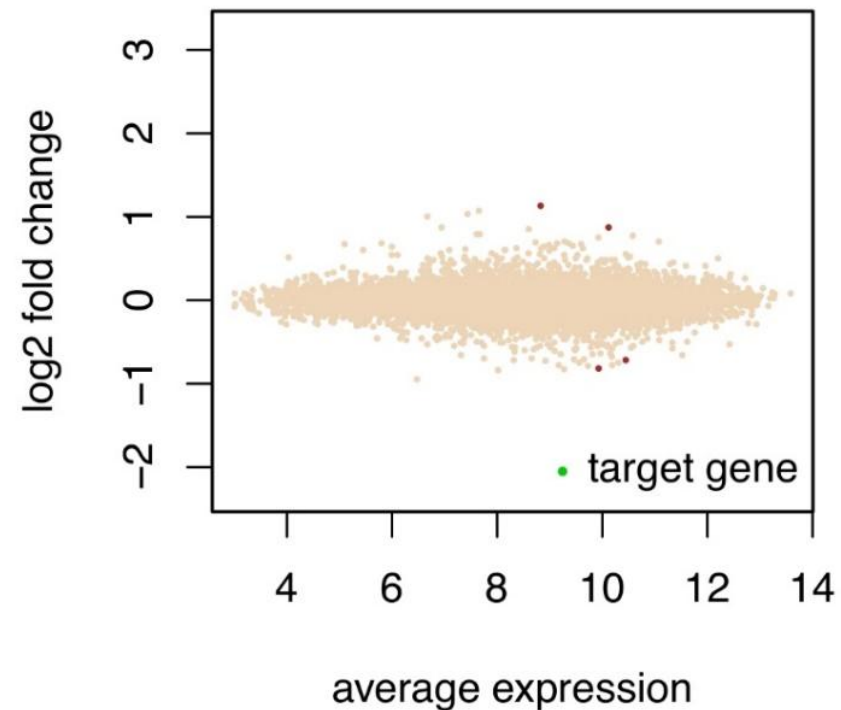


When one siRNA is not enough... Then 30 could help!

siRNA



siPOOL

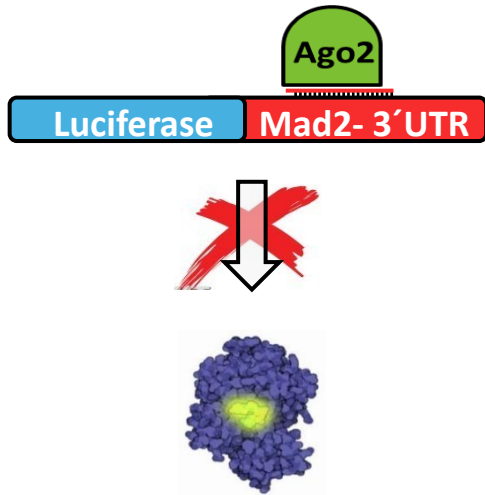


- HeLa cells
- Target: **Scyl1**
- siRNA or siPOOL
- 48 h

Don't fall into that trap... 4 still isn't enough

On-target = PolG

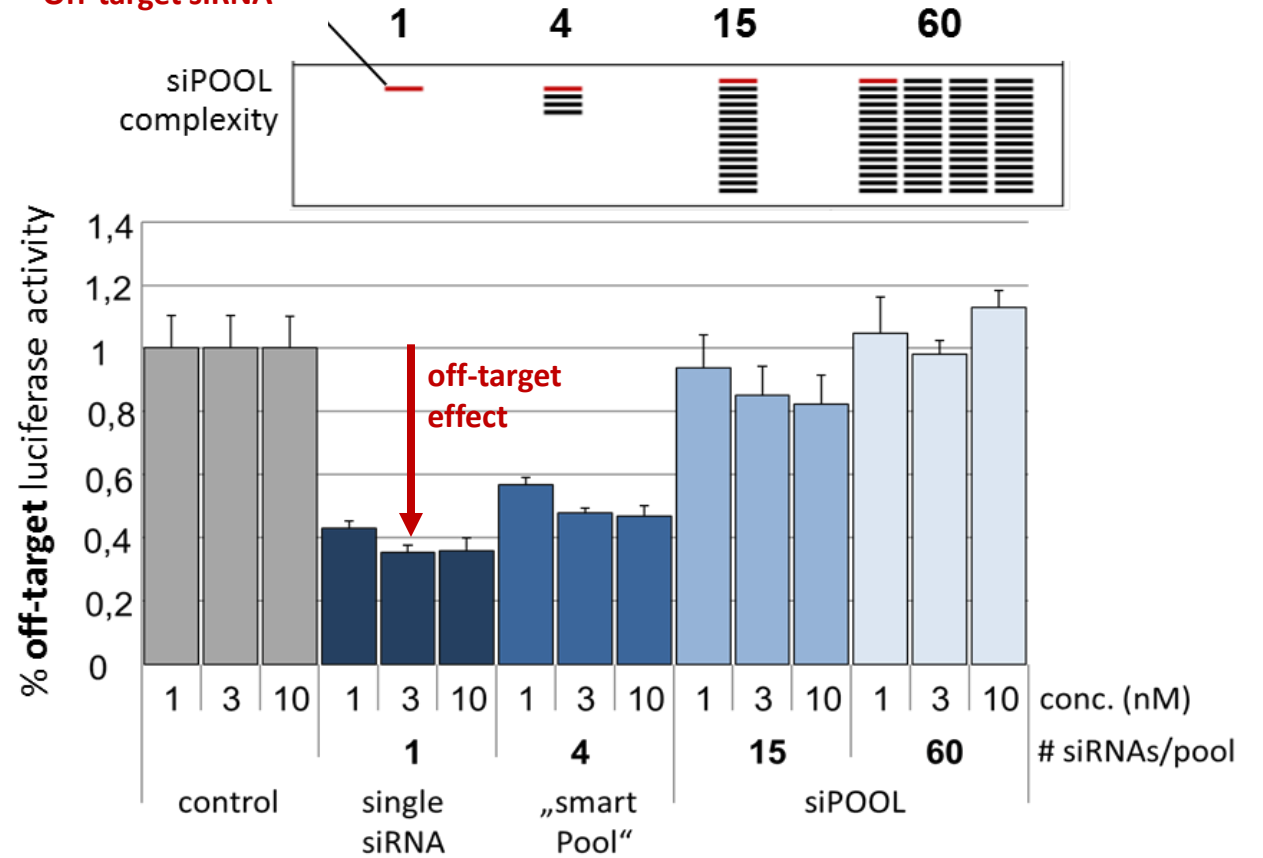
Off-target = Mad2



psiCHECK vector

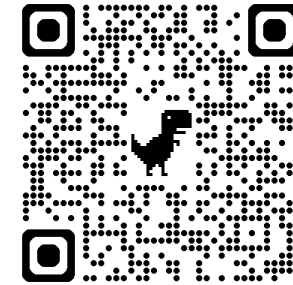


Off-target siRNA



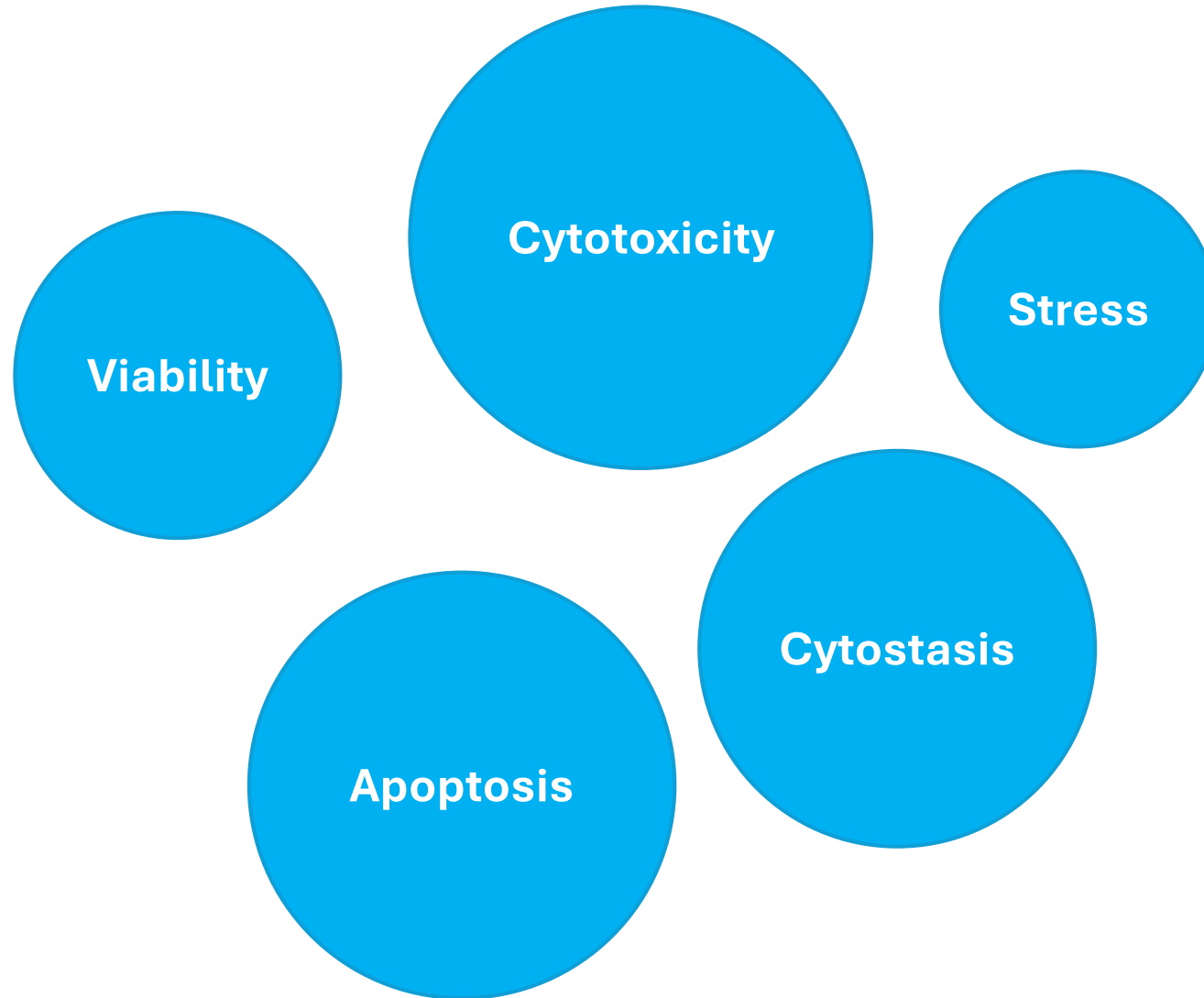
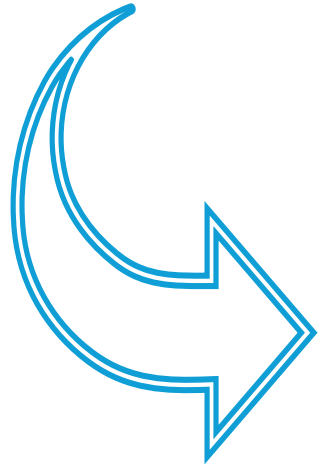
Conclusion

- RNAi is a great tool to study gene function or as tool for RNA therapy
- Seed-based off-targets are frequent in single siRNA experiments and need to be considered
- Complex siRNA pools (**siPOOLS™**) reduce off-target effects by seed dilution
- **siPOOLS™** simultaneously guarantee strong and reproducible target knockdown



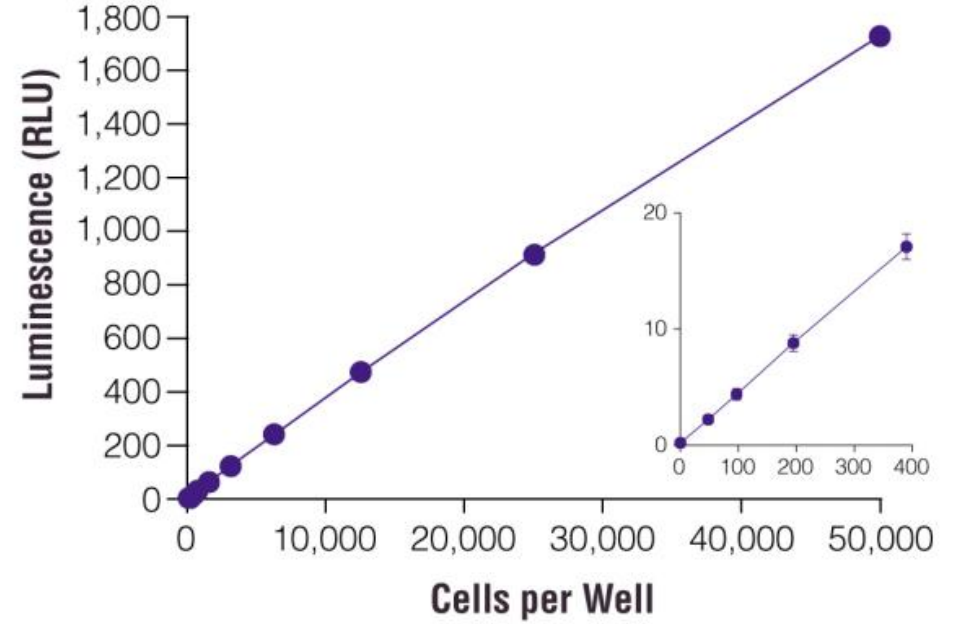
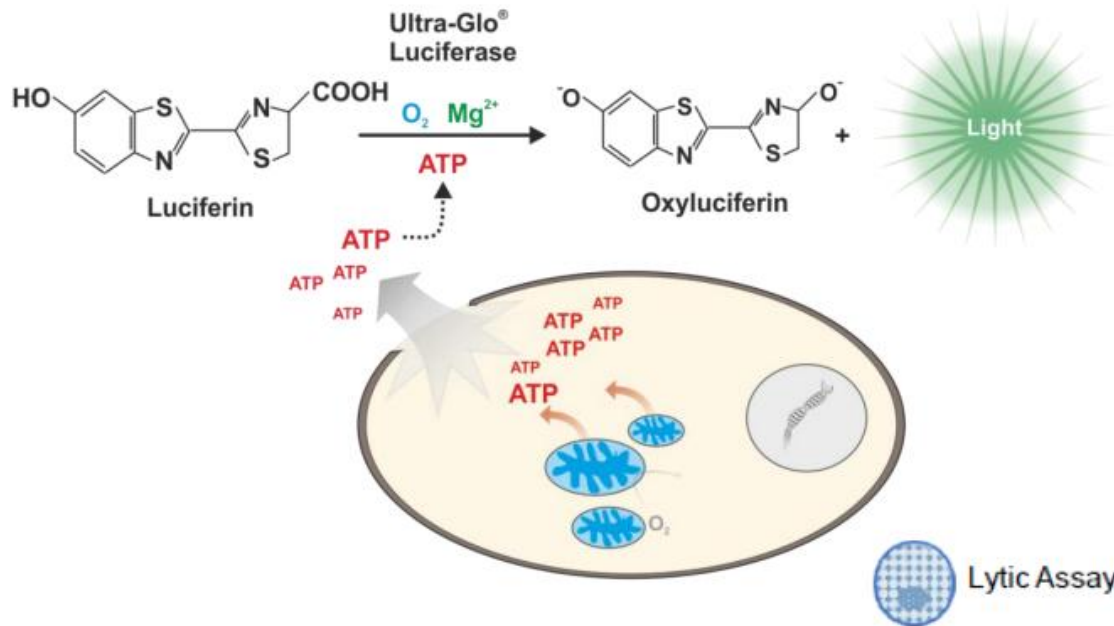
Interested in **siPOOLS™** ?
Send us your gene NCBI ID or symbol

How do cells react on RNA therapy?



Viability

CellTiter-Glo Viability Assay



Linear range: 10 – 50 000 cells

Robust → High Z'-factor

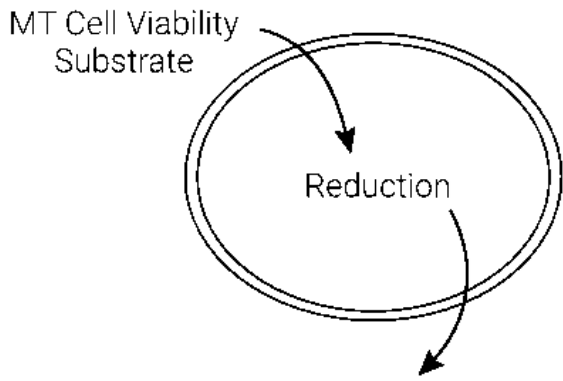
Fast

Viability

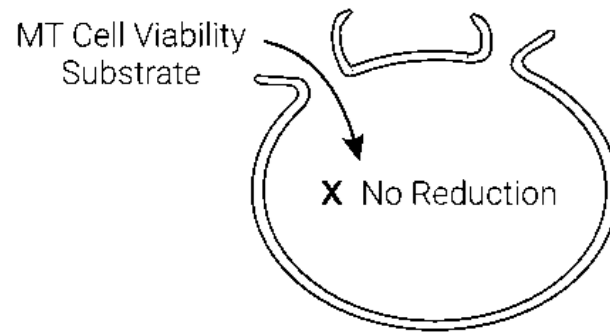


RealTime-Glo MT Cell Viability Assay

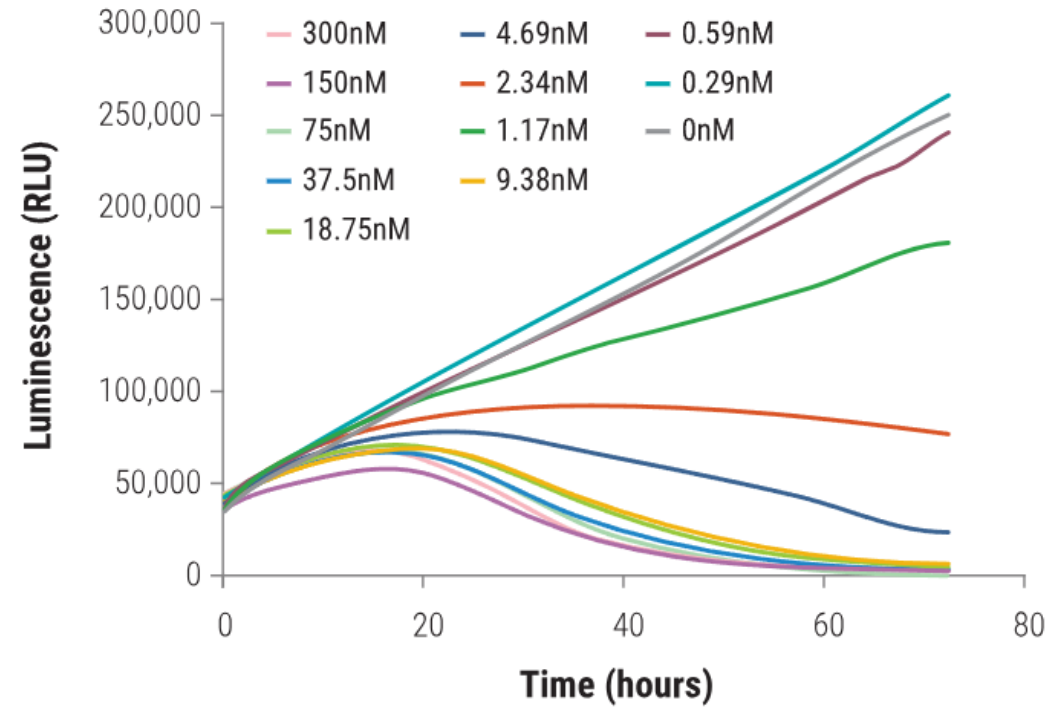
Viable cell



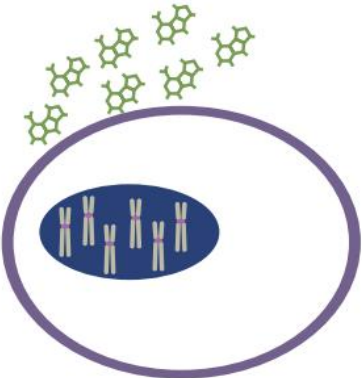
Dead cell



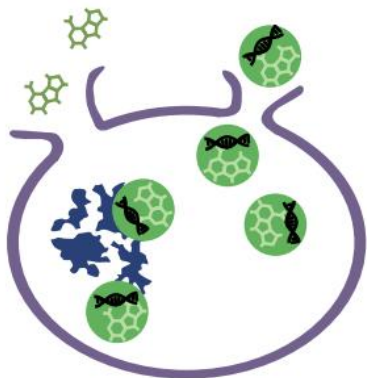
Bortezomib



CellTox Green Cytotoxicity Assay

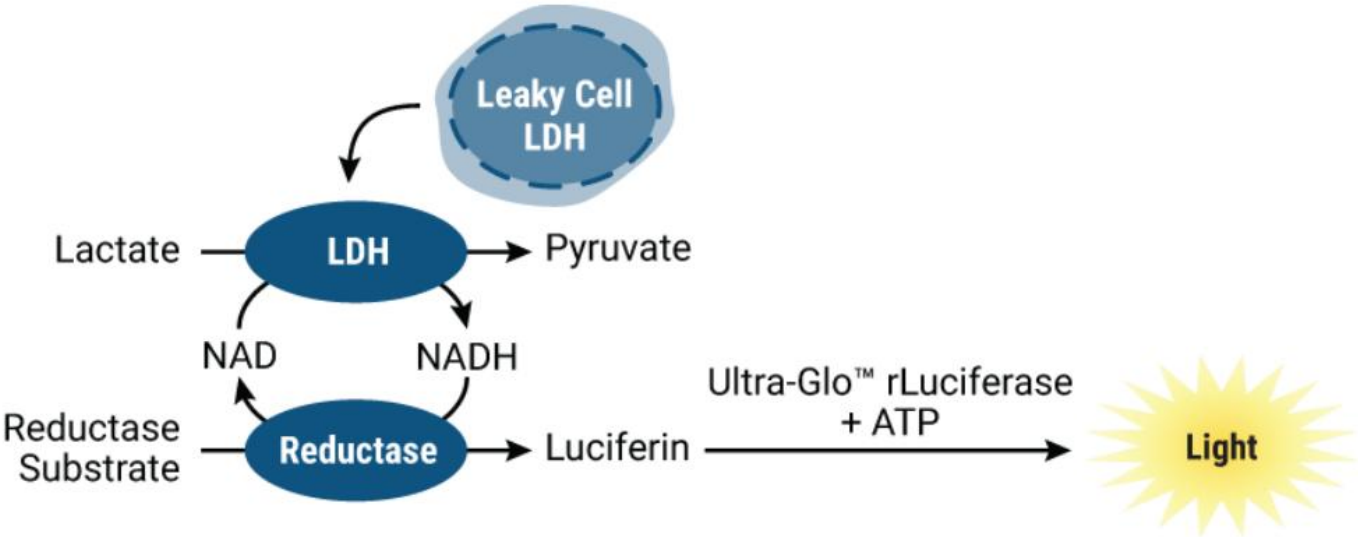


Low Fluorescence
Viable Cells



High Fluorescence
Nonviable Cells

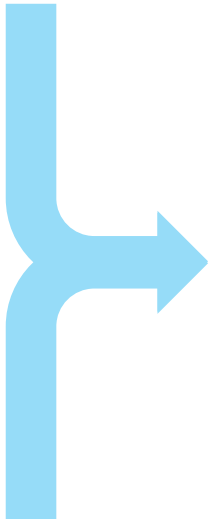
LDH-Glo Cytotoxicity Assay



Cytotoxicity

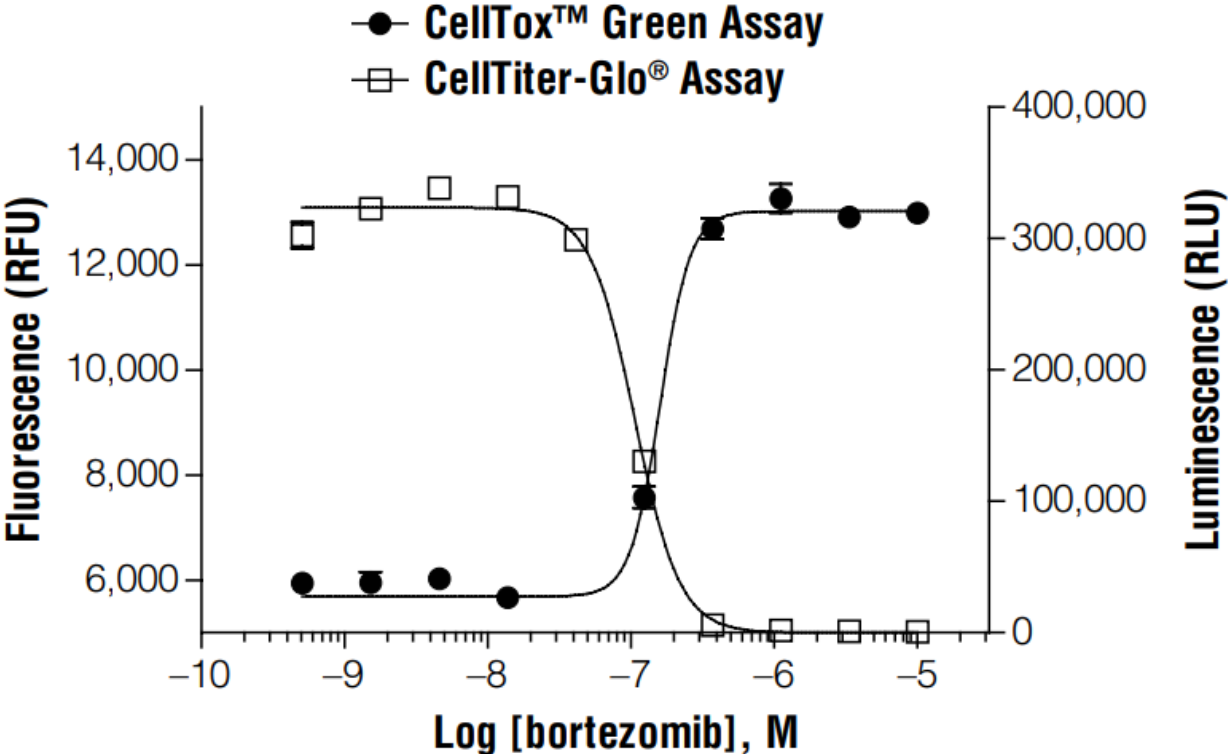


CellTiter-Glo Viability Assay



CellTox Green Cytotoxicity Assay

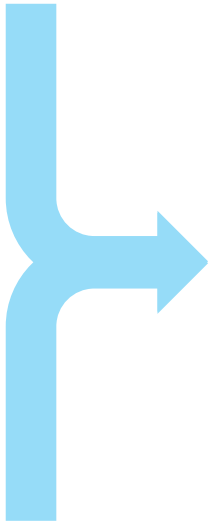
Cytotoxicity



Cytostasis

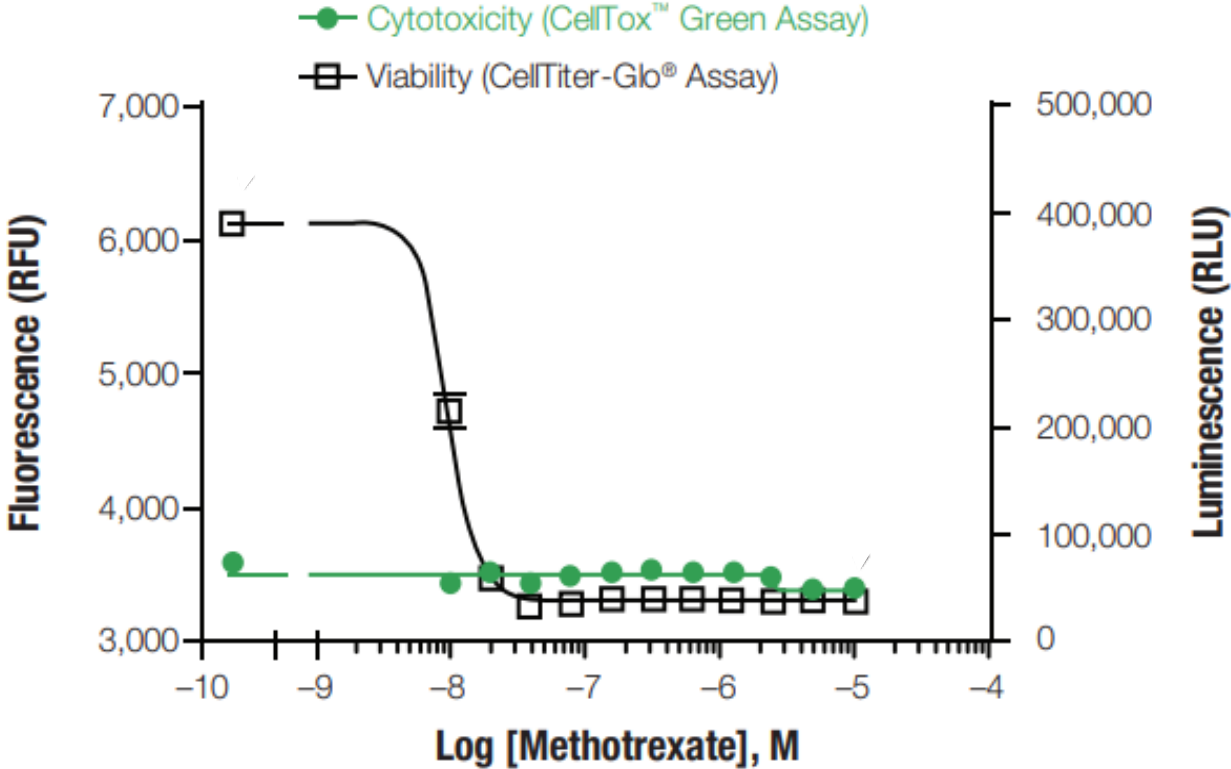


CellTiter-Glo Viability Assay



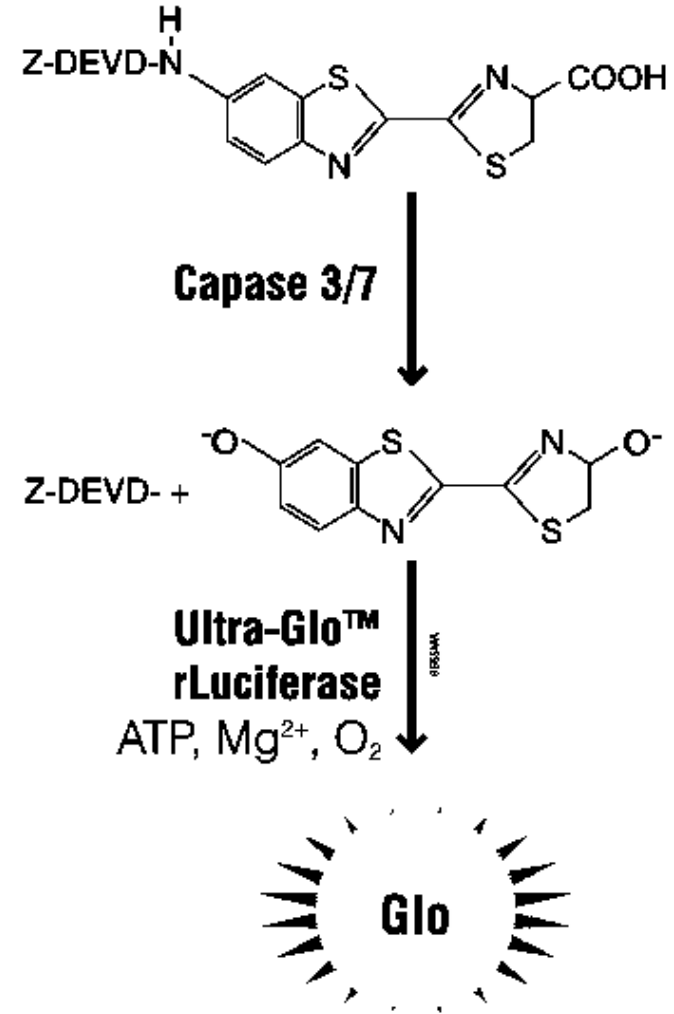
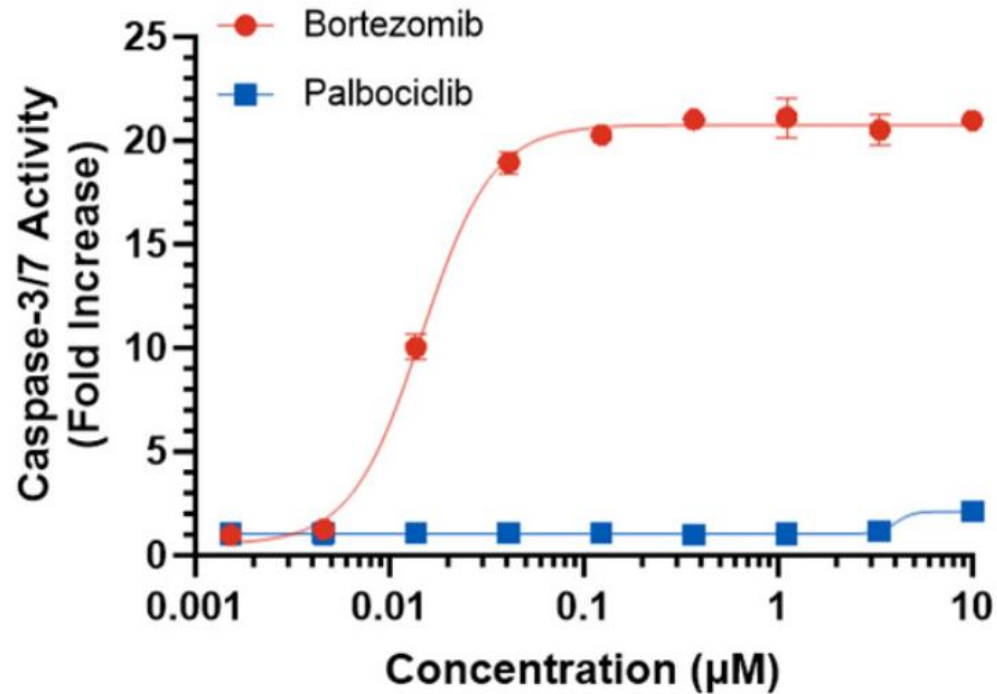
CellTox Green Cytotoxicity Assay

Cytostasis

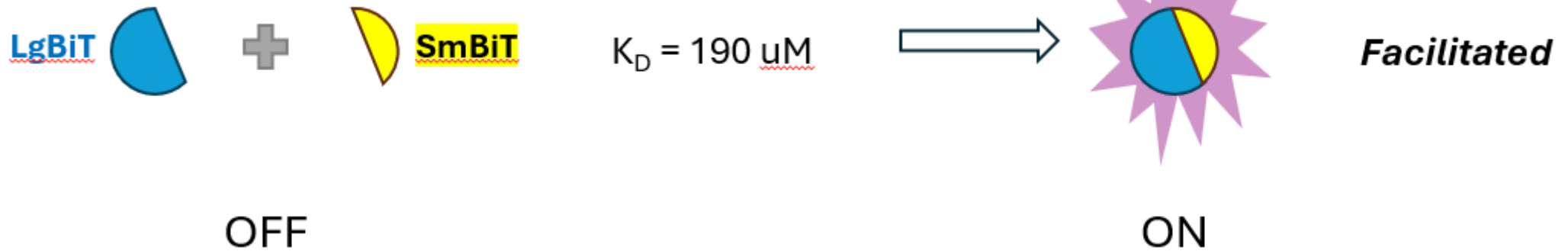


11.669MB

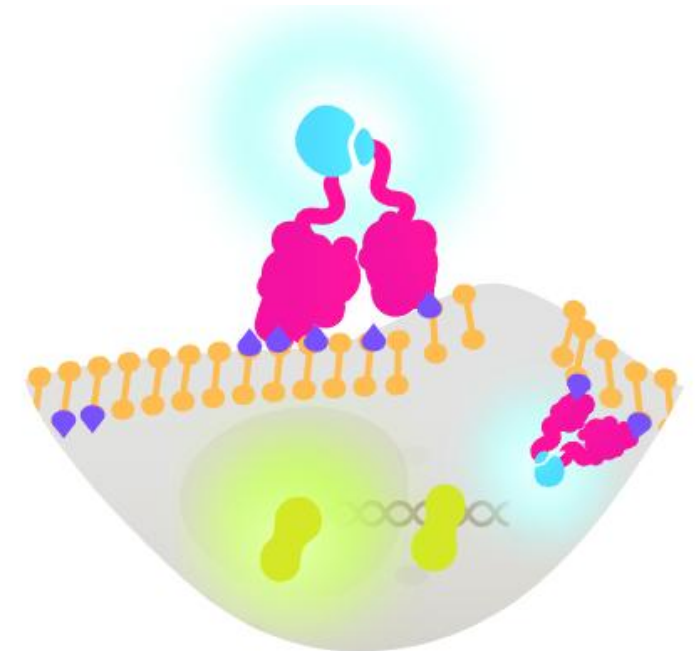
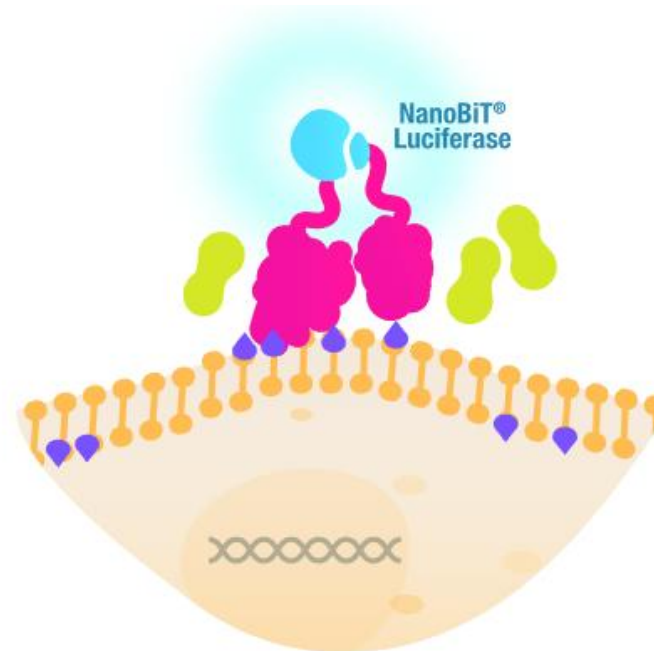
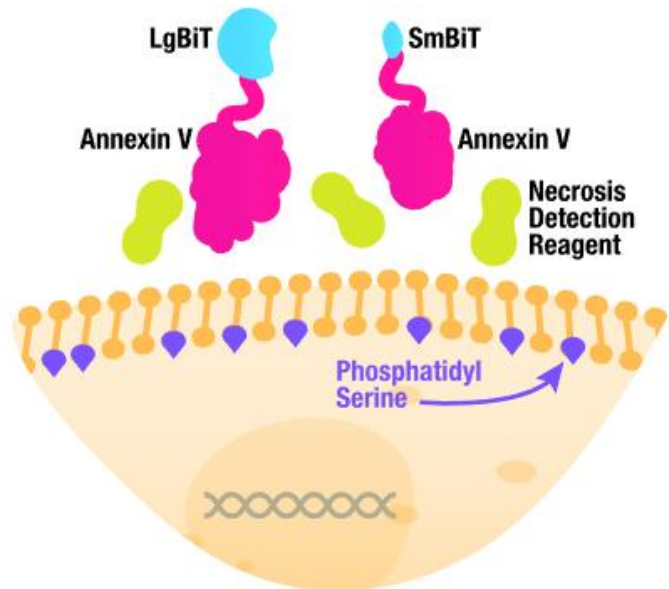
Caspase-Glo 3/7



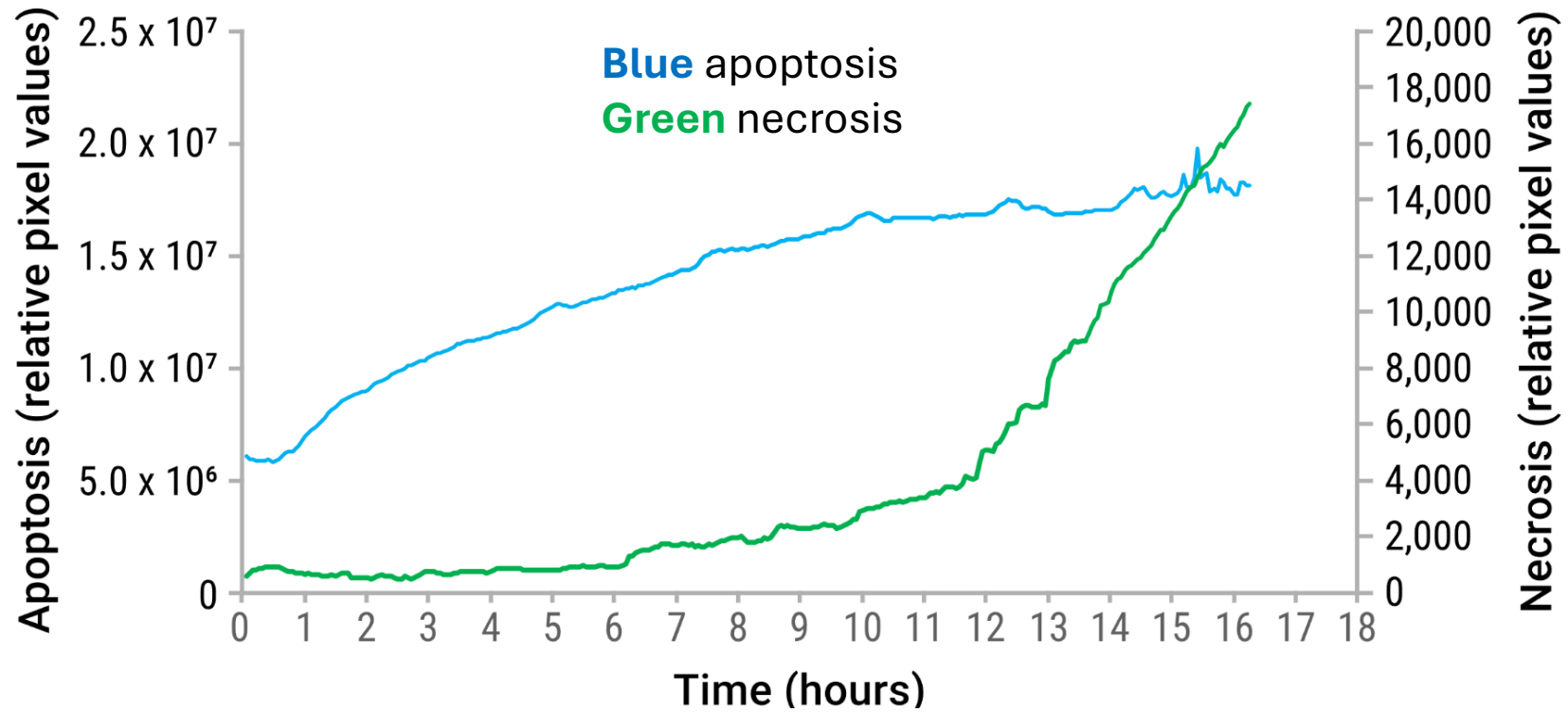
RealTime-Glo Annexin V Apoptosis and Necrosis Assay



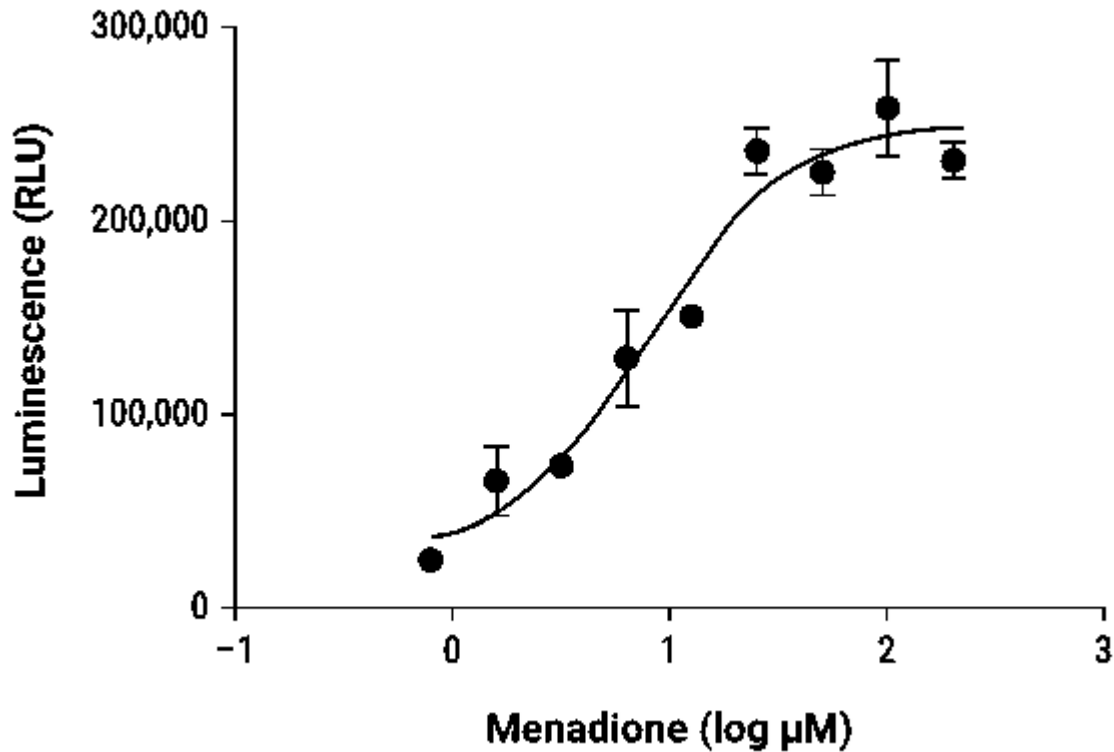
RealTime-Glo Annexin V Apoptosis and Necrosis Assay



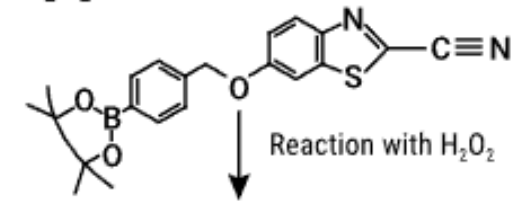
RealTime-Glo Annexin V Apoptosis and Necrosis Assay



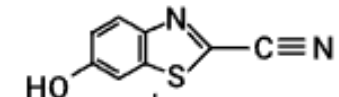
ROS-Glo H_2O_2



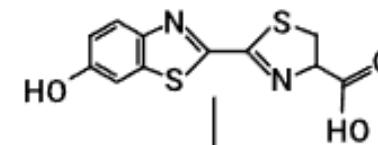
H_2O_2 Substrate



Luciferin Precursor



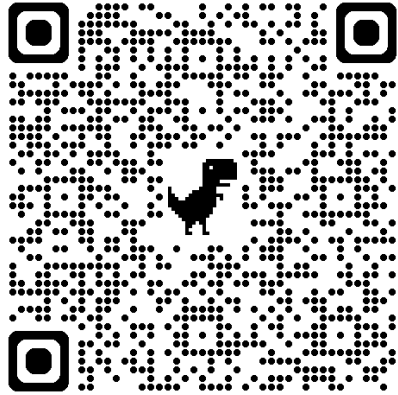
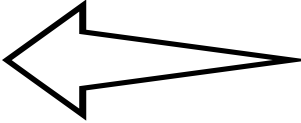
Luciferin



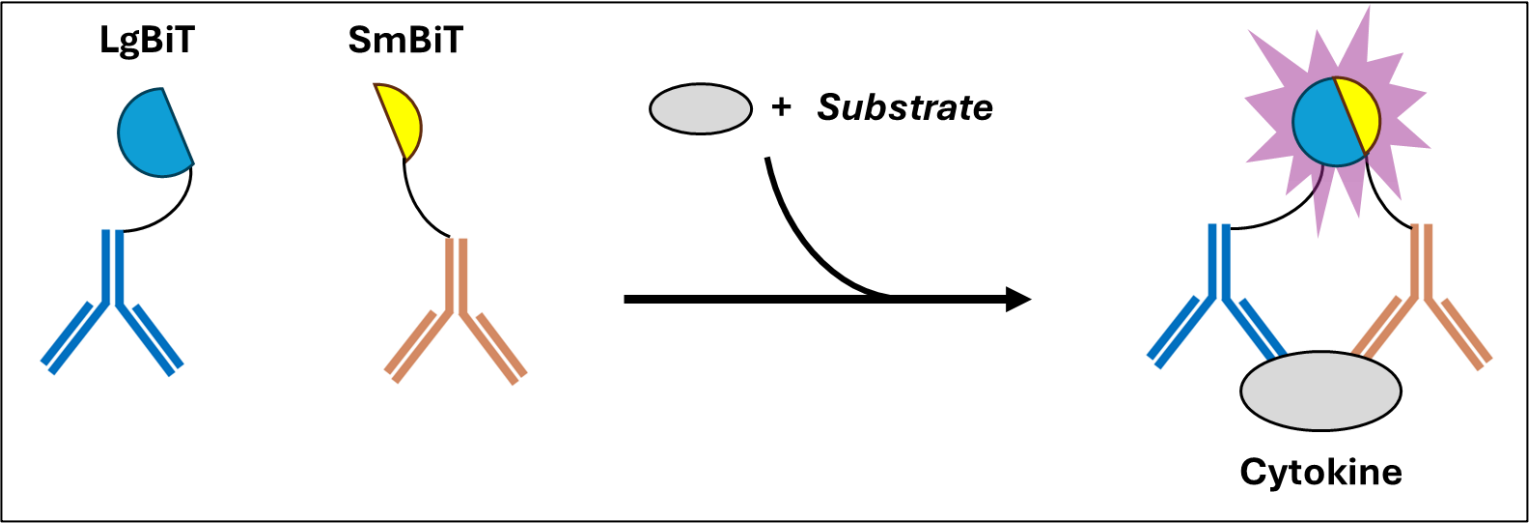
Other Cell Health Assays



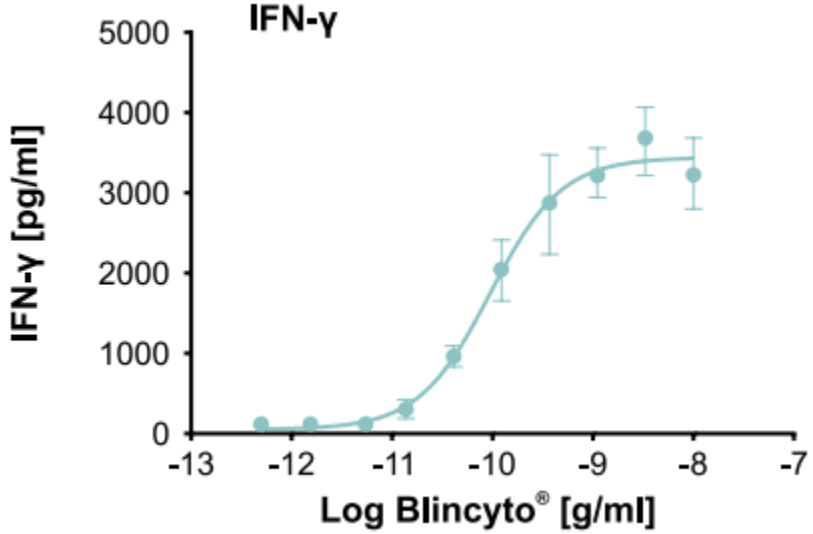
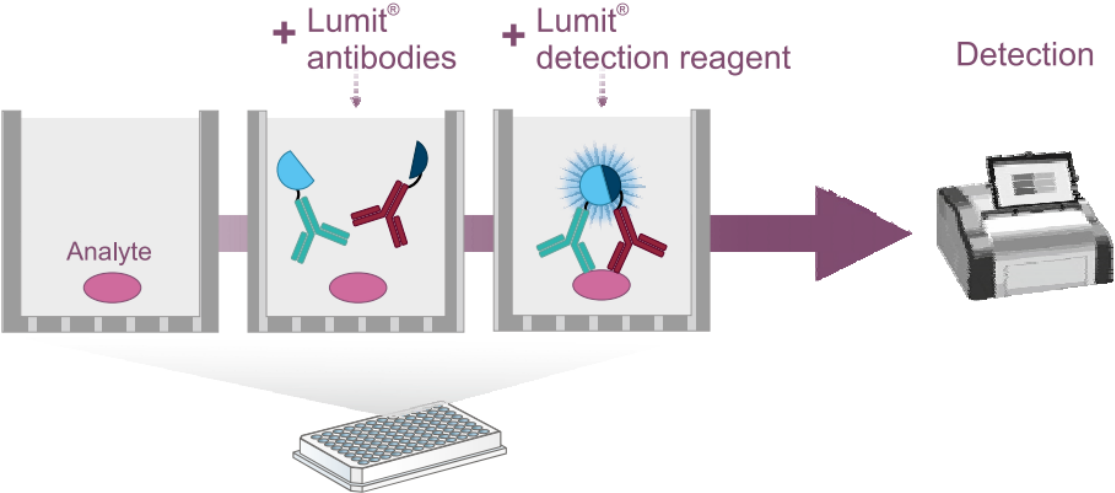
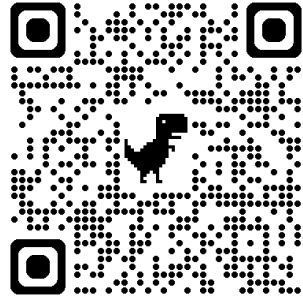
CELL HEALTH ASSAYS														
Product	What is Being Measured?	Cells Detected			Signal Detected				Compatible Plate Format			Minimum Incubation Time	96-Well Sensitivity	Cat.#
		Live Cells	Dead Cells	Apoptotic Cells	Luminescence	Fluorescence	Color Absorbance	Real-Time Deflection	96	384	1536			
RealTime-Glo™ MT Cell Viability Assay	Active metabolism	✓			✓			✓	✓	✓	✓	Real-time monitoring	<100 cells/well	08711
CellTiter-Glo® 2.0 Assay		✓			✓			✓	✓	✓	✓	10 minutes	15 living cells	08241
CellTiter-Glo® Luminescent Cell Viability Assay		✓			✓			✓	✓	✓	✓	10 minutes	10 living cells	07970
CellTiter-Glo® 3D Cell Viability Assay	ATP from viable cells	✓			✓			✓	✓	✓	✓	30 minutes		08681
CellTiter-Glo® One Solution Assay		✓			✓			✓	✓	✓	✓	10 minutes		08461
CellTiter-Fluo™ Cell Viability Assay	Retained live-cell protease activity	✓				AFC 400_/505_			✓	✓	✓	30 minutes	40 living cells	08080
CellTiter-Blue™ Cell Viability Assay	Resazurin reduction	✓				Resazurin 560_/590_	570nm		✓	✓		1-4 hours	400 living cells	08060
CellTiter 96® Non-Radioactive Cell Proliferation Assay	MTT reduction	✓					570nm		✓			4 hours		04000
CellTiter 96® Aqueous One Solution Cell Proliferation Assay	MTS reduction	✓					490nm		✓	✓		1-4 hours	1,000 living cells	03562
CellTiter 96® Aqueous MTS Reagent Powder	MTS reduction	✓					490nm		✓	✓		1-4 hours	1,000 living cells	01112
MultiTos-Fluo Multiplex Cytotoxicity Assay	Live- and dead-cell protease activity	✓	✓			AFC 400_/505_ R110 485_/520_			✓	✓	✓	30 minutes	40 living cells, 10 dead cells	09200
MultiTos-Glo Multiplex Cytotoxicity Assay		✓	✓		✓	AFC 400_/505_			✓	✓	✓	30+15 minutes	40 living cells, 10 dead cells	09270
LDH-Glo™ Cytotoxicity Assay			✓						✓	✓	✓	30 minutes-1 hour	10 dead cells	02380
CytoTos ONE™ Homogeneous Membrane Integrity Assay	LDH release		✓			Resazurin 560_/590_			✓	✓	✓	10 minutes	200 dead cells	07990
CytoTos 96® Non-Radioactive Cytotoxicity Assay			✓				490nm		✓	✓	✓	30 minutes		01780
CytoTos-Fluo™ Cytotoxicity Assay	Released dead-cell protease activity		✓				R110 485_/520_		✓	✓	✓	30 minutes	10 dead cells	08290
CytoTos-Glo™ Cytotoxicity Assay			✓		✓				✓	✓	✓	15 minutes	10 dead cells	08290
CellTox™ Green Cytotoxicity Assay	DNA staining upon membrane disruption		✓			480-500_/520-530_			✓	✓	✓	15 minutes		08741
ApoTos-Glo™ Triplex Assay	Live-cell protease + Dead-cell protease + Caspase 3/7 activity	✓	✓	✓	✓	AFC 400_/505_ R110 485_/520_			✓	✓	✓	30+30 minutes	40 living cells, 100 apoptotic cells	06220
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	Annexin HB11 luciferase binding to phosphatidylserine + DNA staining upon membrane disruption		✓	✓	✓				✓	✓	✓	Real-time monitoring	100 apoptotic cells	J81011
ApoLive-Glo™ Multiplex Assay	Live-cell protease + Caspase 3/7 activity	✓	✓	✓	✓	AFC 400_/505_			✓	✓	✓	30+30 minutes	40 living cells, 100 apoptotic cells	08410
Apo-ONE™ Homogeneous Caspase-3/7 Assay	Caspase-3/7 activity			✓	✓		R110 485_/520_		✓	✓	✓	30 minutes	625 apoptotic cells	07790
Caspase-Glo® 3/7 Assay				✓	✓				✓	✓	✓	20 minutes	100 apoptotic cells	08090
Caspase-Glo® 8 Assay	Caspase-8 activity			✓	✓				✓	✓	✓	20 minutes	1,000 apoptotic cells	08200
Caspase-Glo® 9 Assay	Caspase-9 activity			✓	✓				✓	✓	✓	20 minutes	1,500 apoptotic cells	08210



Innate immunity reaction

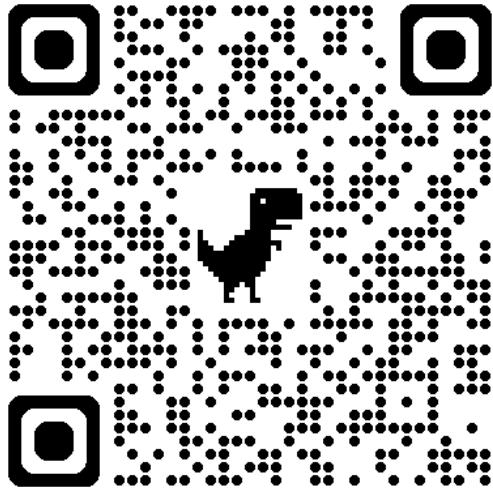


Lumit immunoassays

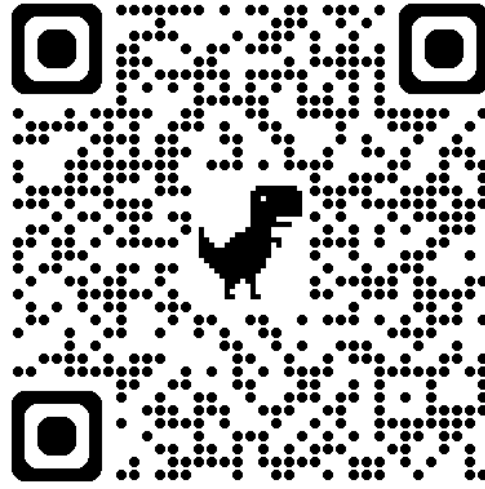


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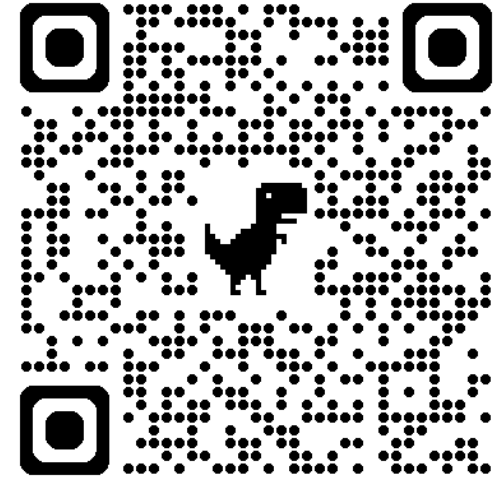
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Technologies We Represent

