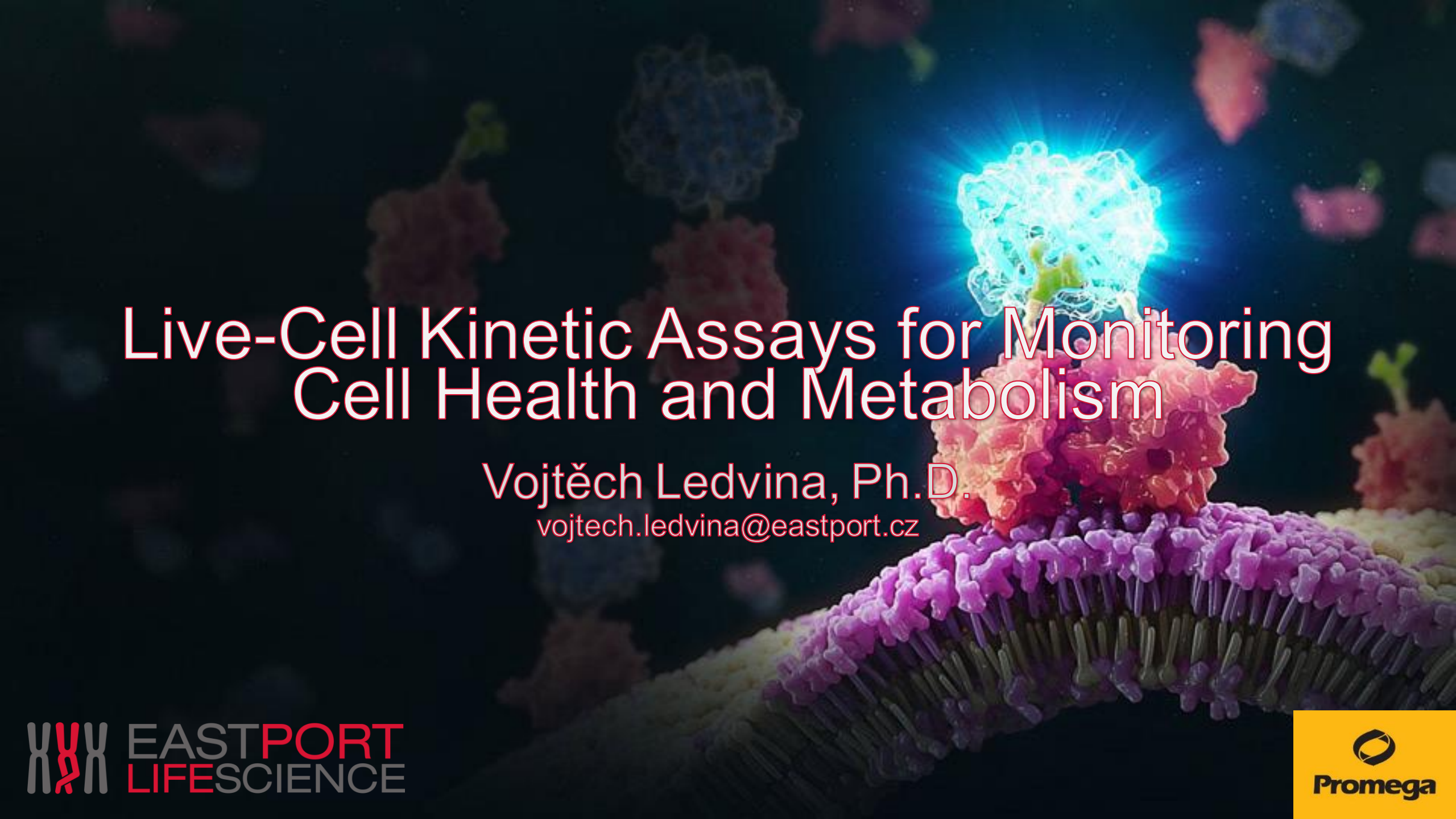

Live-Cell Kinetic Assays for Monitoring Cell Health and Metabolism

Vojtěch Ledvina, Ph.D.
vojtech.ledvina@eastport.cz

EASTPORT LIFESCIENCE

Promega

Today‘s Agenda

1. Bioluminescence
2. Basic modes of detection
3. What Defines a Live-Cell Kinetic Assay?
4. What Are the Benefits of Live-Cell Kinetic Assay?
5. What to Consider When Using Live-Cell Kinetic Assays?
6. Which Live-Cell Kinetic Assay Do We Offer?

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Bioluminescence

- Production of light by living organisms
- Light produced by enzymatic oxidation of a substrate luciferin by an enzyme luciferase
- Common in marine vertebrates and inverebrates, fungi, bacteria
- Most used luciferases are – Firefly, Renilla, NanoLuc and Gaussia luciferases

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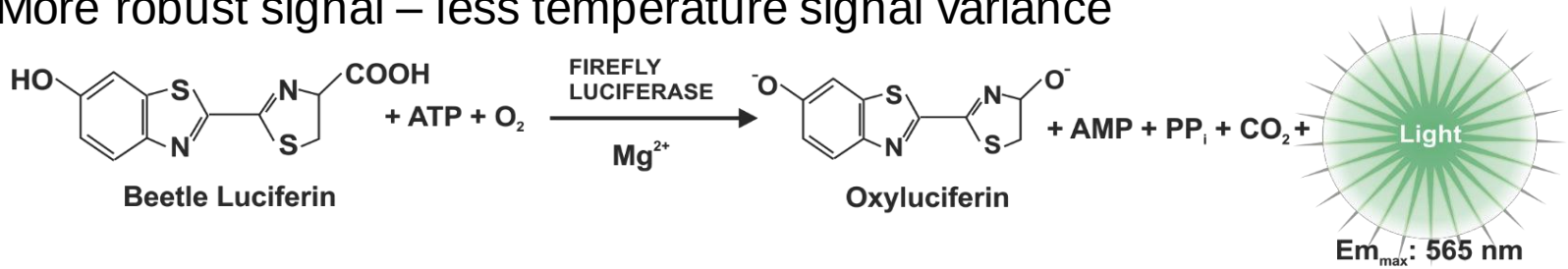
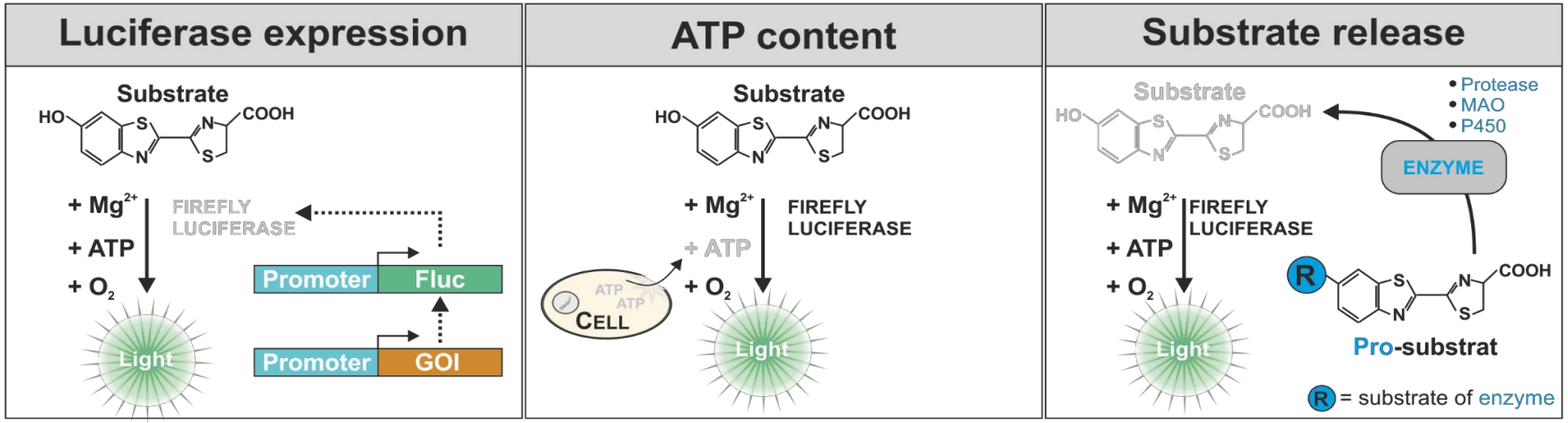
Firefly Luciferase

- ATP-dependent luciferase
- Universal reaction adaptable for various assays
- Ultra-Glo™ rLuciferase
  - Higher stability in the presence of detergent and reducing agents
  - More robust signal – less temperature signal variance

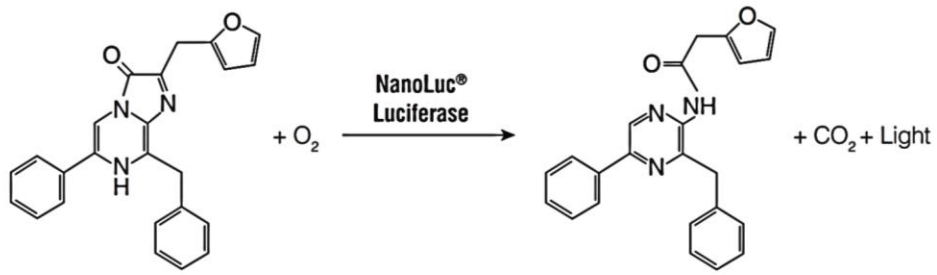




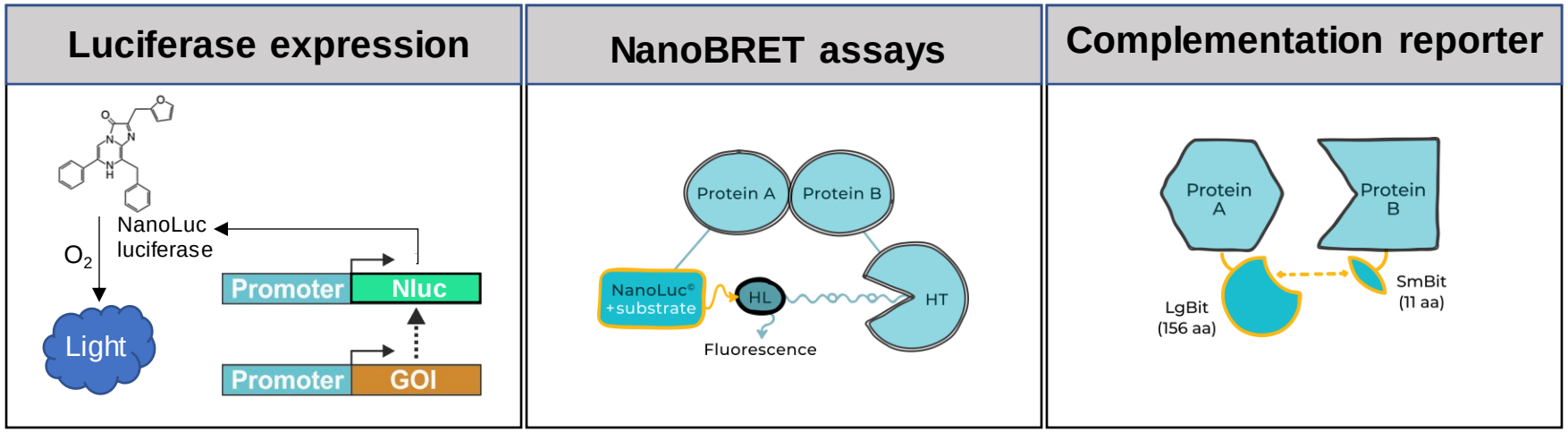
grey = limiting factor

NanoLuc® Luciferase – Novel Experimental Reporter

- ATP-independent luciferase from a deep sea shrimp
- 100x brigther than Rluc and Fluc, glow-type luminescence
- Smallest luciferase – 19,1 kDa



Furimazine Furimamide



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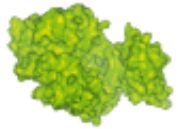
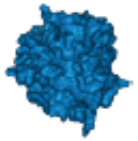
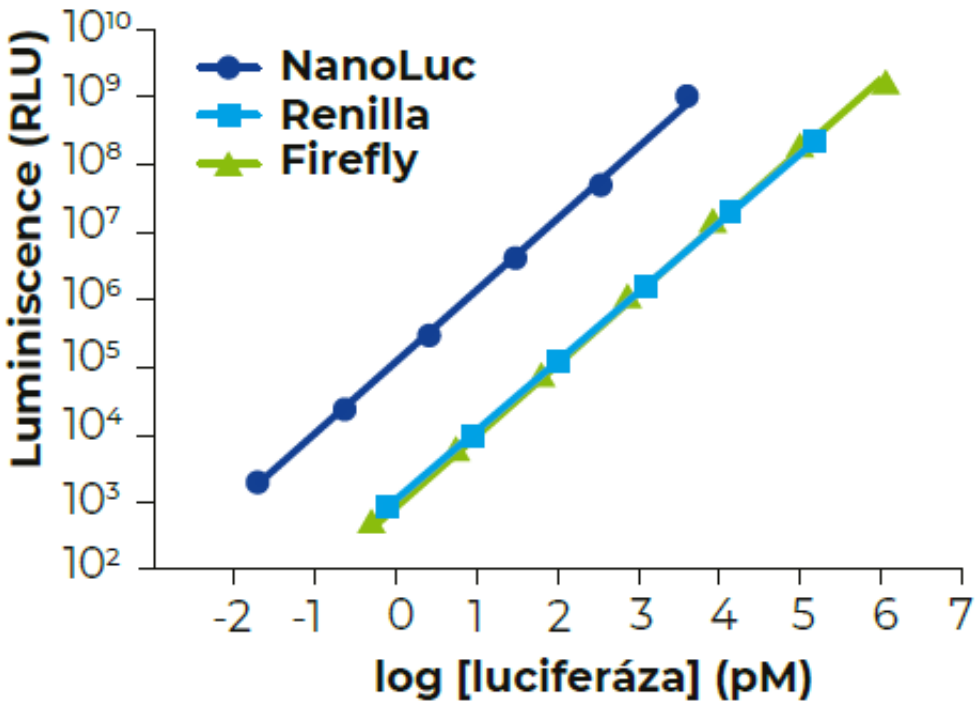
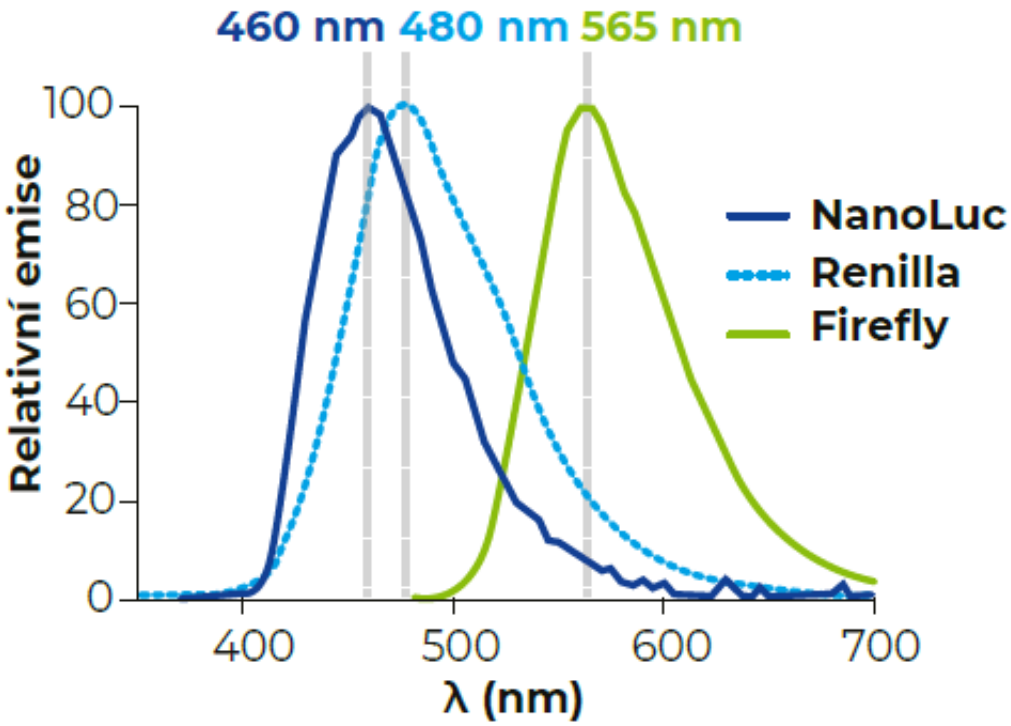
Firefly (Fluc) 60,6 kDa		**Luciferin + ATP + O_2**	**Fluc** **Mg^{2+}**	**Oxyluciferin** **+ AMP + PP_i + CO_2**	**Em_{max} = 565 nm**
Renilla (Rluc) 36,0 kDa		**Coelenterazine + O_2**	**Rluc**	**Coelenteramide** **+ CO_2**	**Em_{max} = 480 nm**
NanoLuc (Nluc) 19,1 kDa		**Furimazine + O_2**	**Nluc**	**Furimamide** **+ CO_2**	**Em_{max} = 460 nm**





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Today‘s Agenda

1. Bioluminescence
2. Other modes of detection
3. What Defines a Live-Cell Kinetic Assay?
4. What Are the Benefits of Live-Cell Kinetic Assay?
5. What to Consider When Using Live-Cell Kinetic Assays?
6. Which Live-Cell Kinetic Assay Do We Offer?

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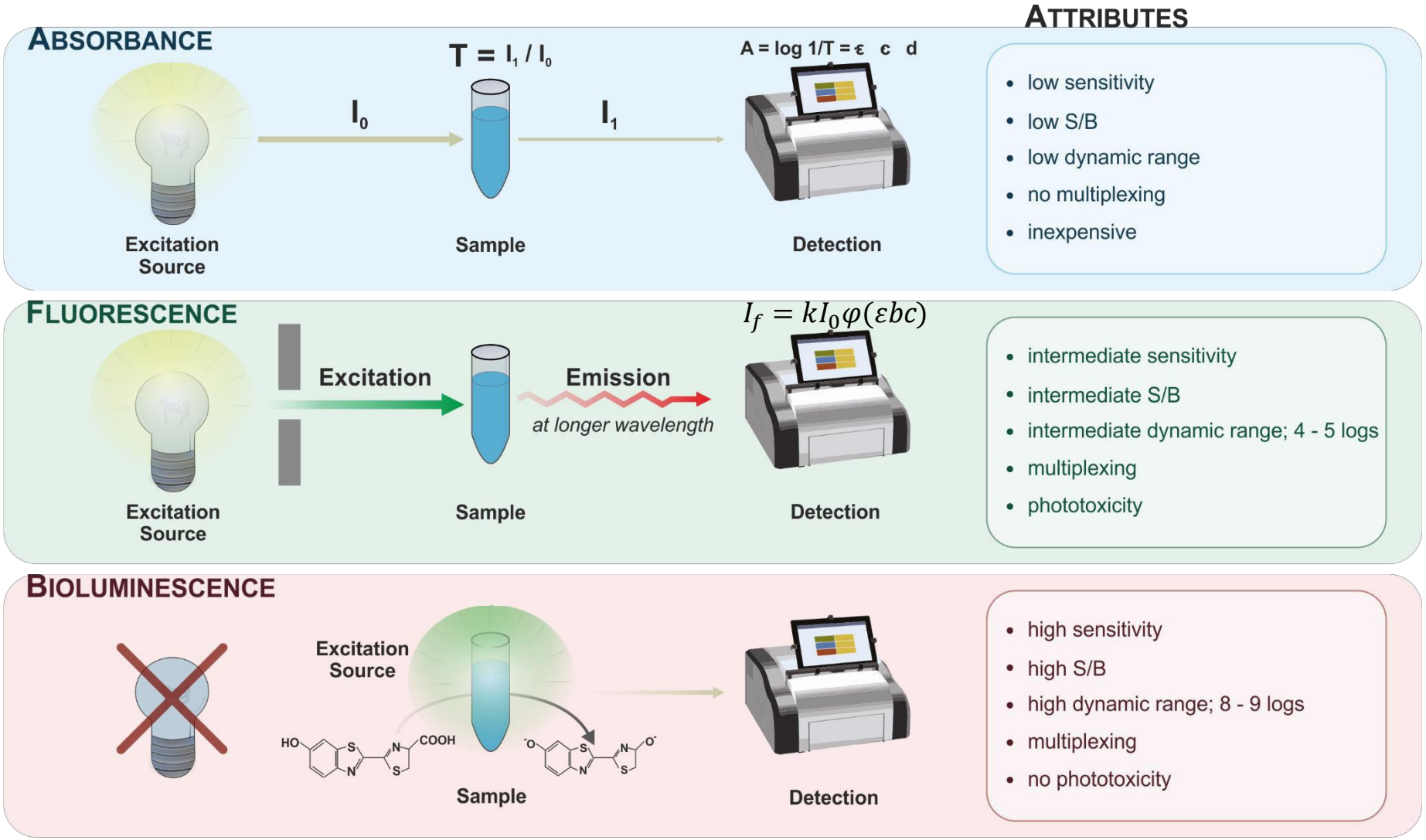
Other Modes of Detection



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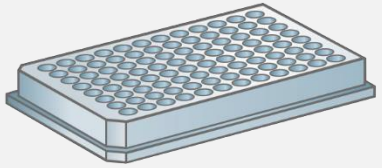
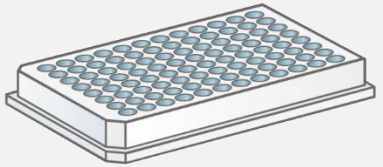
Matching Plate Type and Detection Mode

- *TC treated*
- *Sterile*
- *With lid*

	“clear“	“black”	“white”
			
		“solid or clear bottom”	*„solid or clear bottom“*
ABSORBANCE	**YES**	**(YES)**	
FLUORESCENCE		**YES**	**(YES)**
LUMINISCENCE	**NO!**	**(YES)**	**YES**

- 400 – 800 nm (VIS)
- 200 – 400 nm (UV)
  - ➢ quarz glass
  - ➢ cyclic olefin copolymer (COC)

- plastic autofluorescence (↓)
- background (↓)
- crosstalk (↓)

- maximal reflection
- crosstalk (↓)
- (!) phosphorescence

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3. **What Defines a Live-Cell Kinetic Assay?**
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6. Which Live-Cell Kinetic Assay Do We Offer?

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What Defines a Live-Cell Kinetic Assay?

Live-Cell Kinetic Assays…

- …are non-toxic & maintain cellular integrity
- …allow monitoring a parameter of a sample over extended period of time
- …maximize the information gained from one sample within a given time window
- …typically allow for extended time course analysis (days)

Endpoint assay

Start

End

Kinetic assay

Start

End

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Benefits of Live-Cell Kinetic Assays

Data quantity / reliability

- More data per well
- Easy identification of important time points
- Draw valid conclusions from your experiments

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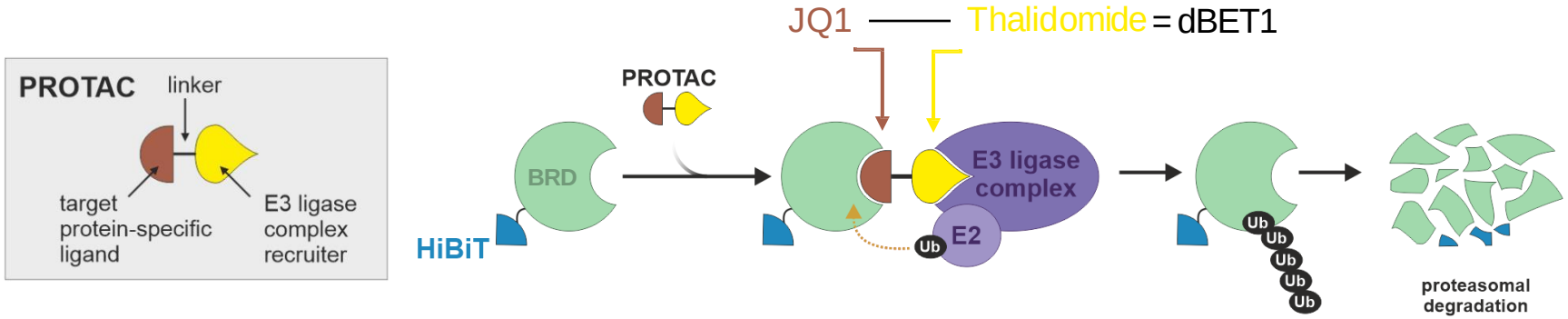
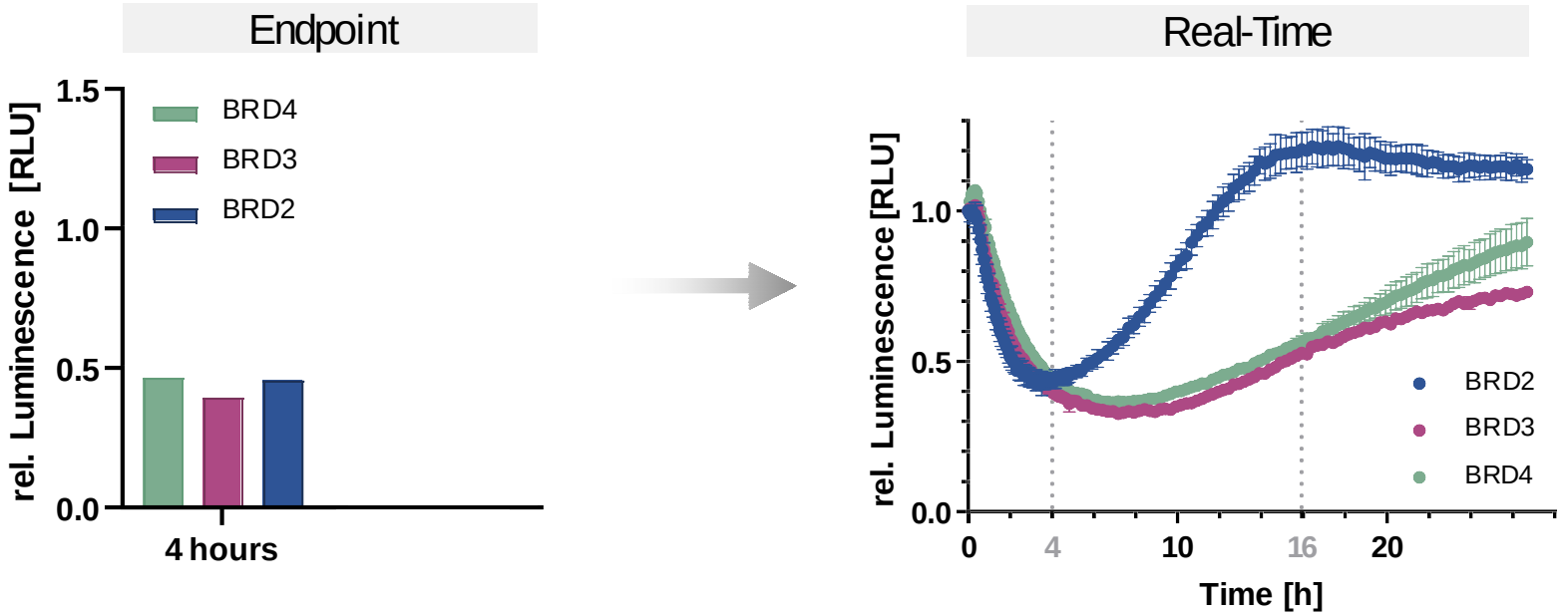
Studying targeted protein degradation with HiBiT

Evaluation of Proteolysis targeting chimeras (PROTACs) Efficiency





Benefits of Live-Cell Kinetic Assays

Data quantity / reliability

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- Easy identification of important time points
- Draw valid conclusions from your experiments

Economical savings/resource efficient

- Saves your precious cells, reagents, consumables…
- Time-efficient identification of important time points

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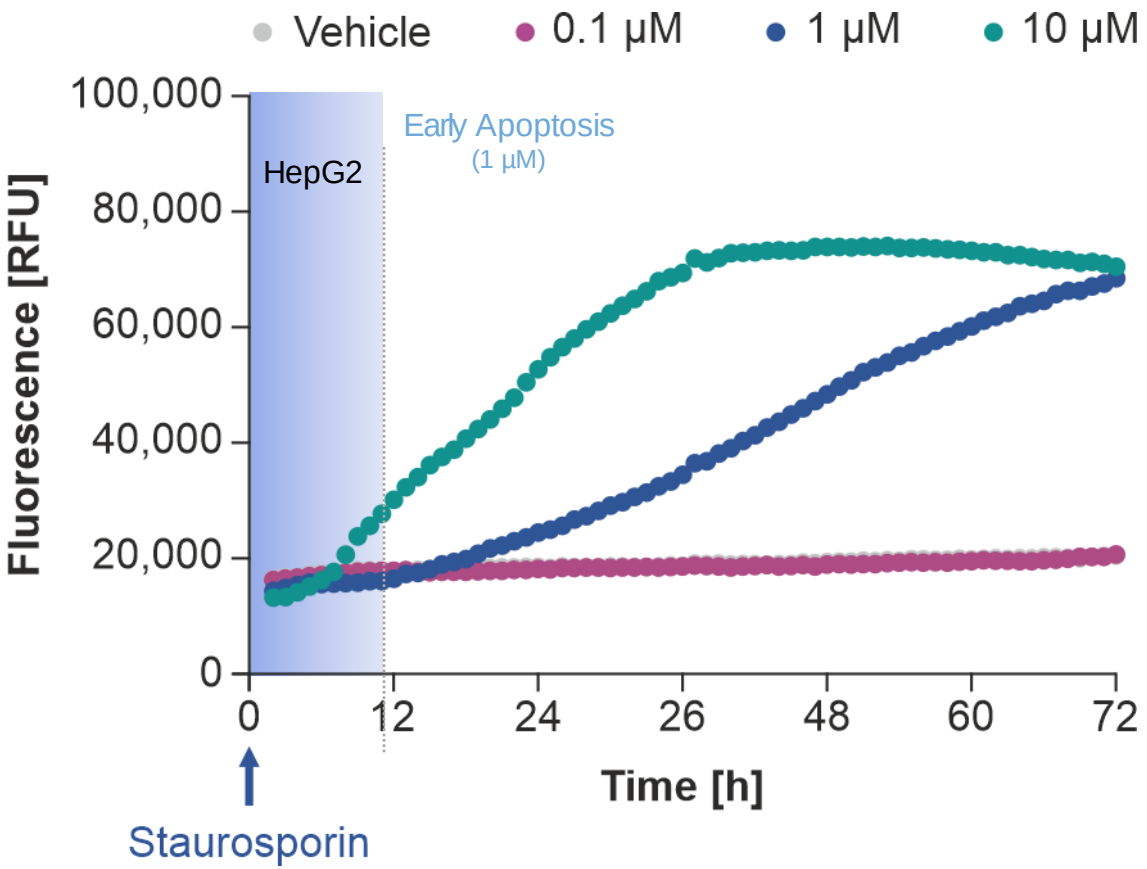
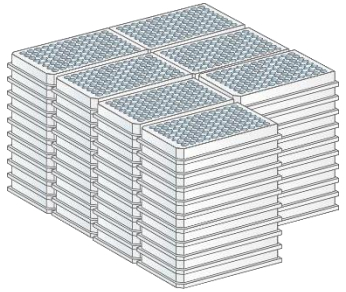
CellTox™ Green Cytotoxicity Assay

Determine Membrane Integrity Using a Fluorescent DNA-binding Dye

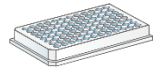


endpoint



VS.

real-time



less medium, cells and reagent

Benefits of Live-Cell Kinetic Assays

Data quantity / reliability

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- Easy identification of important time points
- Draw valid conclusions from your experiments

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Flexibility

- Combine with other assays (multiplexing) for enhanced data reliability and interpretation
- Usually compatible with both 2D and 3D cell culture models
- Compatible with both, time course and endpoint analysis

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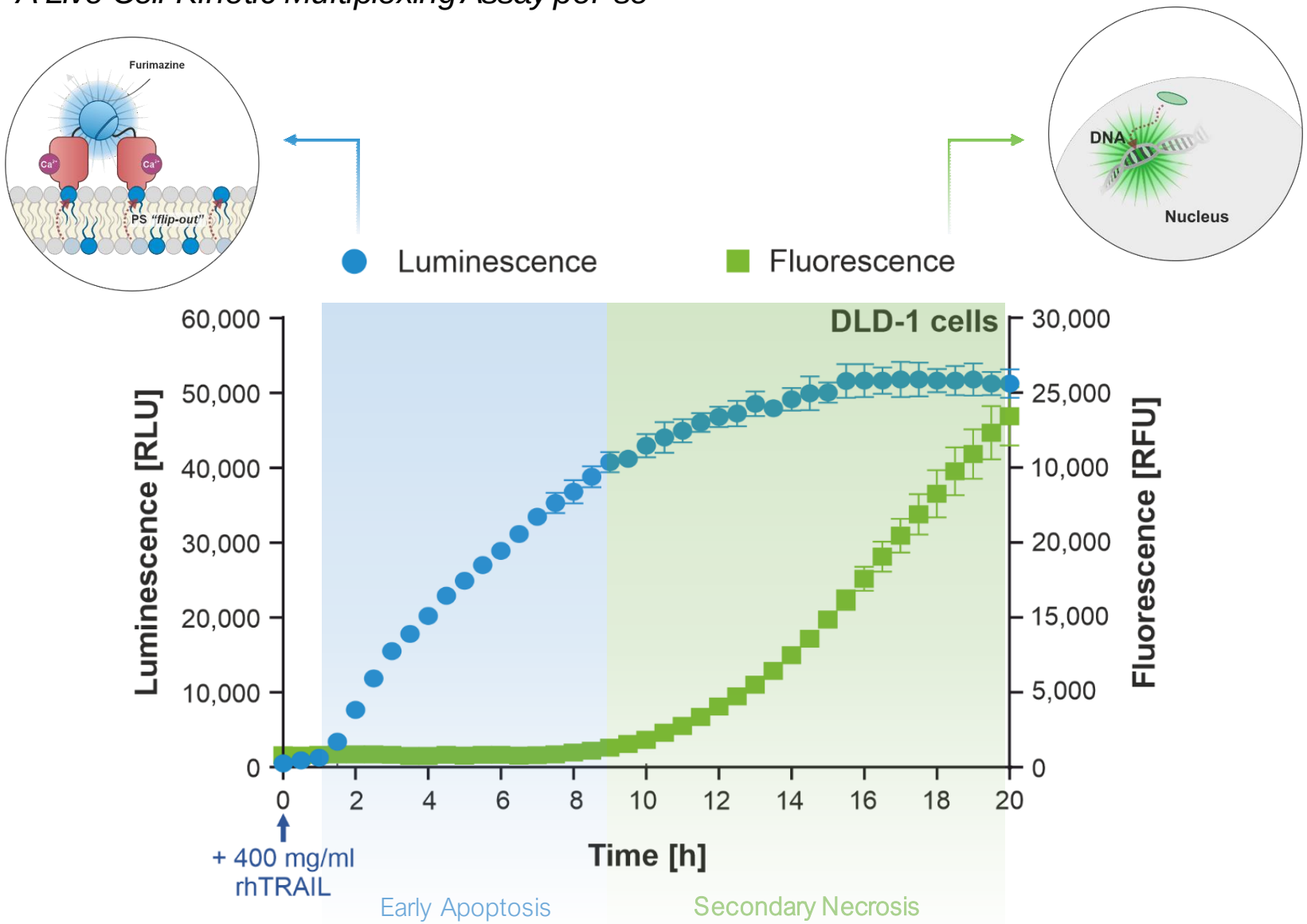
RealTime-Glo® Annexin V Apoptosis and Necrosis Assay

A Live Cell Kinetic Multiplexing Assay per se



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Flexibility

- Combine with other assays (multiplexing) for enhanced data reliability and interpretation
- Usually compatible with both 2D and 3D cell culture models
- Compatible with both, time course and endpoint analysis

No additional instrumentation required

- Heating function and gas control is beneficial but optional
- (Multimode functionality)

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What to Consider When Using Live-Cell Kinetic Assays?

Edge Effect

- Plate layout
- Temperature
- Evaporation

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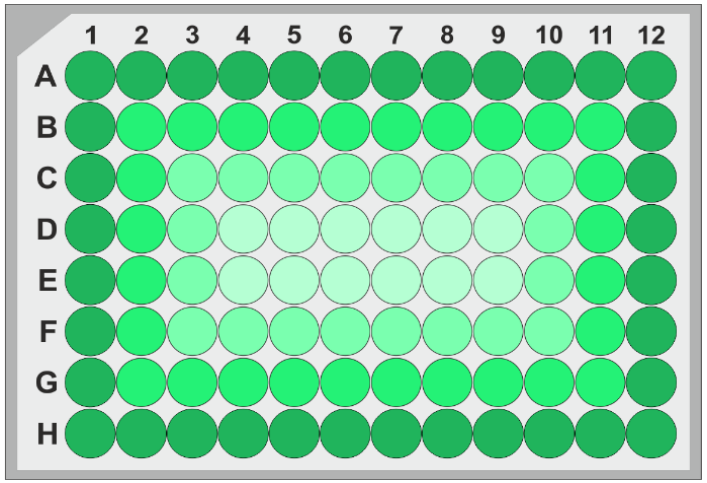
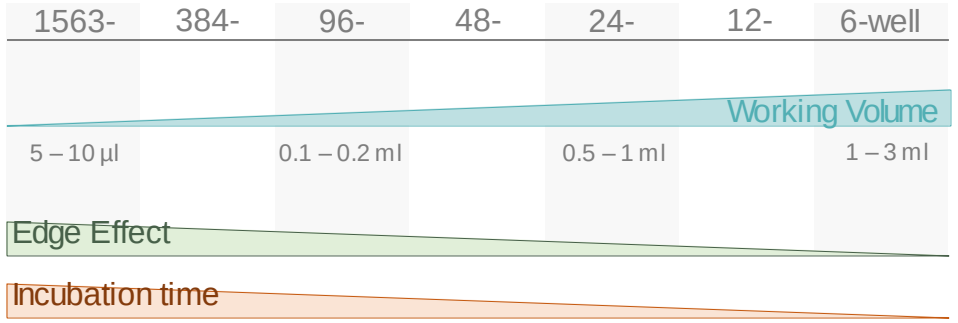
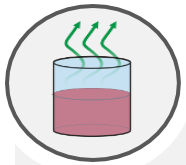
Experimental Plate Layout and Data Variability

The Edge Effect

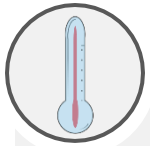






Evaporation

- Use specialized plates/seals for culturing of cells
- Fill outer 36 wells (and inter-well space)
- with pre-equilibrated sterile water or PBS



- Avoid shuttling of plates or hurry up
- Keep temperature constant over time
- Pre-quilibrate to reading temperature

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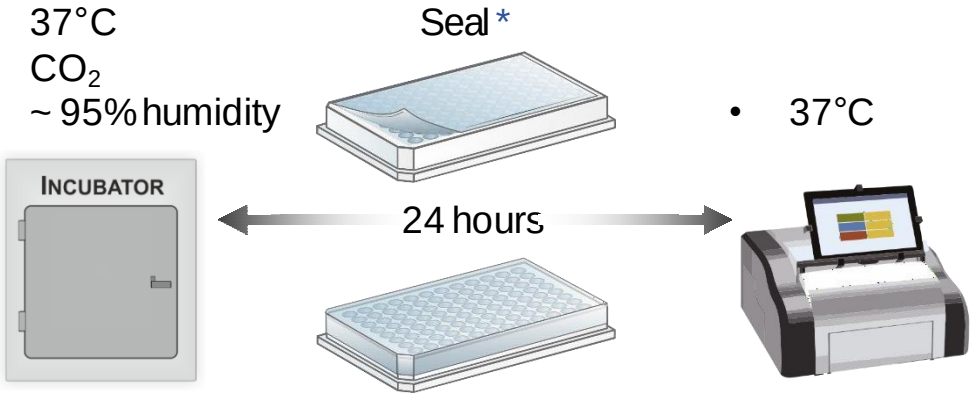
Media Evaporation During Extended Incubations

Counteracting the Edge Effect

- 37°C
- CO_2
- ~ 95% humidity

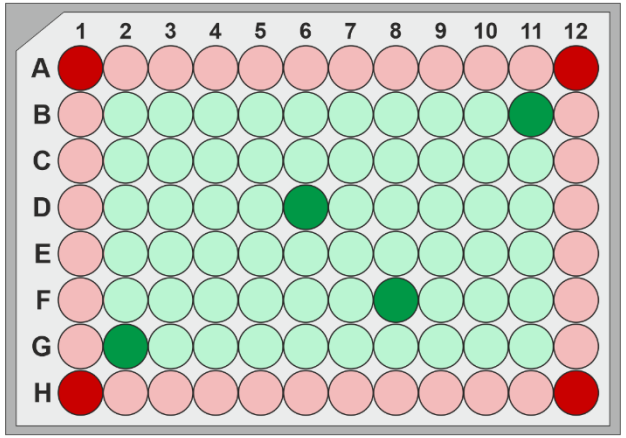


- 37°C

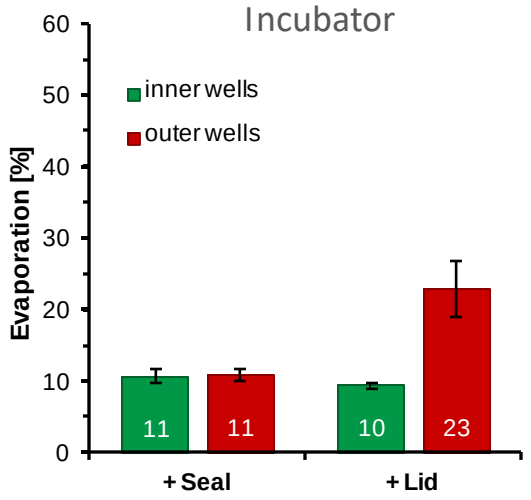
* gas permeable

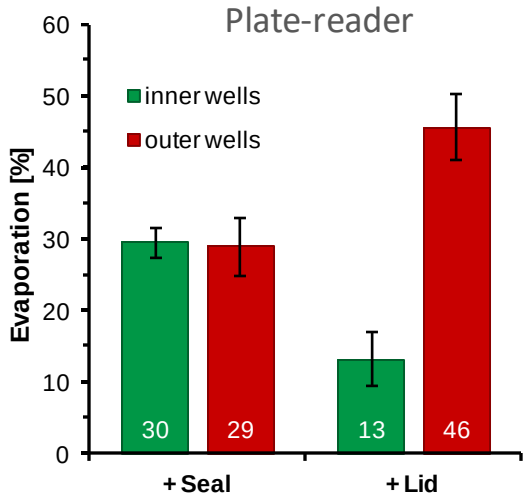
Media Evaporation During Extended Incubations

Counteracting the Edge Effect



- 100 µl/well
- 24 hours
- 37°C
- n = 4





Conclusions

- Plate seals can help to alleviate the edge effect
- If one can spare the outer wells, a lid is an excellent strategy too
- Even under non-humidified conditions, a lid ensures a relatively low degree of evaporation in the inner wells

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What to Consider When Using Live-Cell Kinetic Assays?

Edge Effect

- Plate layout
- Temperature
- Evaporation

Instrumentation

- No CO_2 control? → CO_2-independent medium
- Heating?
- Condensation on the lid
- Software and protocol setup

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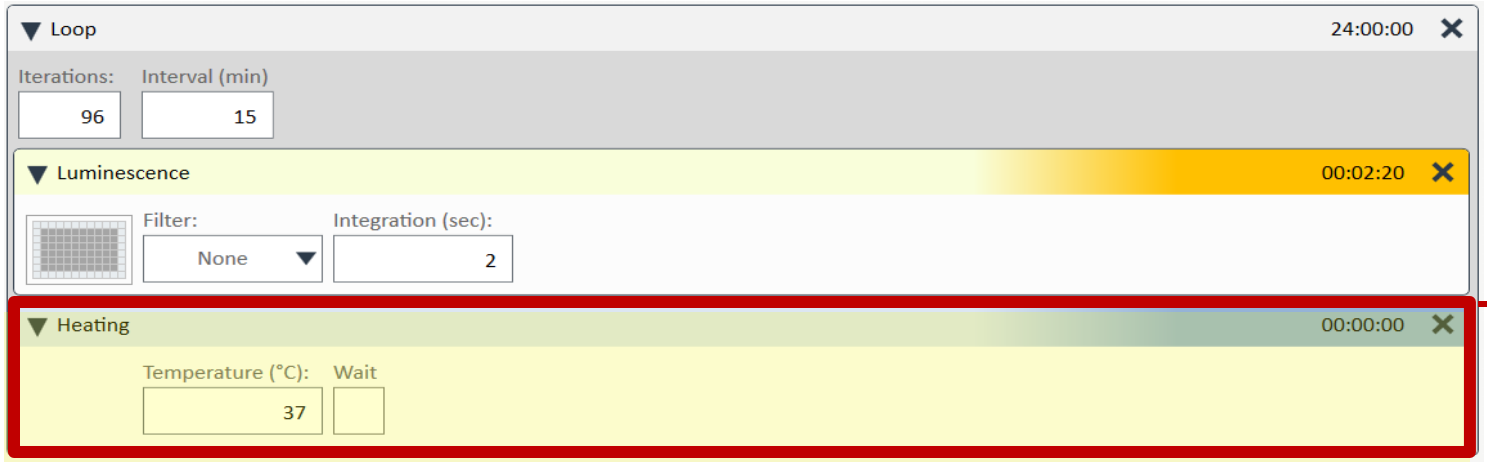
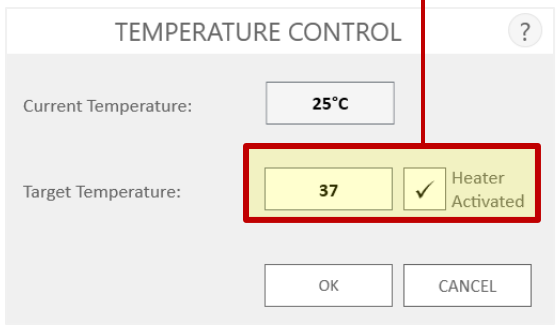
Instrumentation for Kinetic Assays

- No additional instrumentation needed
- Temperature and CO_2 control are beneficial but not required
- No CO_2 control? → use CO_2-independent medium
- No heating? → shuttle the plate between the incubator and luminometer
- Condensation on the lid?
  - Cover the inside of the lid with 1-2 ml of 0,5% Triton X-100 in sterile water or PBS
  - Remove the liquid and let dry for a while
  - Return the lid on the plate



Heating step needs to be incorporated in the "Loop"

Heating needs to be activated



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What to Consider When Using Live-Cell Kinetic Assays?

Edge Effect

- Plate layout
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Instrumentation

- No CO_2 control? → CO_2-independent medium
- Software and protocol setup
- Condensation on the lid

Length of measurements

- Always check the manual for the actual length of measurement – 24, 48, 72 hours

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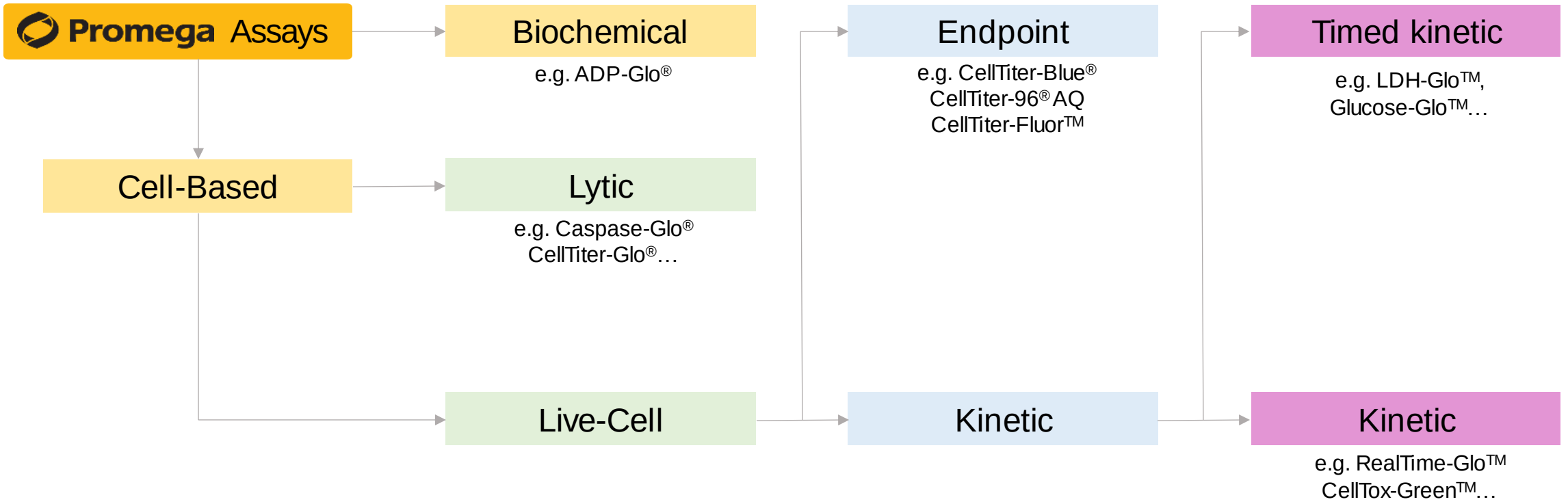
Which Live-Cell Kinetic Assay Do We Offer?



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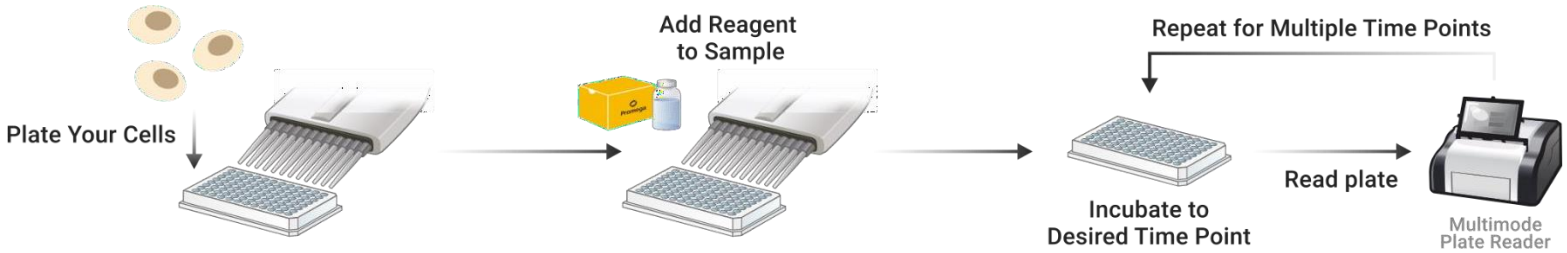
Live-Cell Kinetic Assays

Real-Time Kinetic

- RealTime-Glo™ MT Cell Viability Assay
- RealTime-Glo™ Annexin V Apoptosis / Necrosis Assay
- RealTime-Glo™ Extracellular ATP Assay
- CellTox-Green™ Cytotoxicity Assay
- Nano-Glo® Live Cell Assay System
- Nano-Glo® Vivazine™ Live Cell Substrate
- Nano-Glo® Endurazine™ Live Cell Substrate

- Continuous reading from one well
- Less hands-on time (no transfer steps required)
- Non-toxic for the cells
- Number of data points is limited only by instrument/software

Workflow



Cytotoxicity | Metabolic phenotype | Apoptosis | Cell Viability | Protein:Protein Interactions | Target Engagement | Reporter Expression

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Timed Kinetic Live-Cell Assays

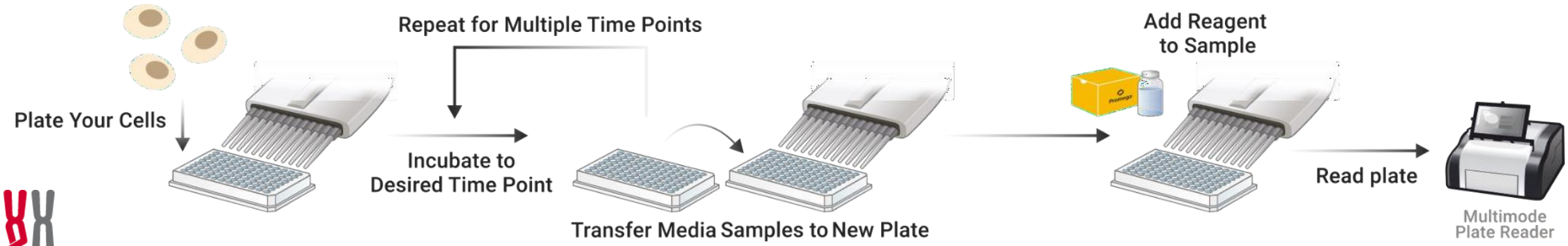
Timed Kinetic

- LDH-Glo™ Cytotoxicity Assay
- Glucose-Glo™ Assay
- Lactate-Glo™ Assay
- Glutamate/Glutamine-Glo™ Assay
- Glutamate-Glo™ Assay

Cytotoxicity Metabolic phenotype

- Require periodic sampling of supernatant
- Higher hands on time (sampling, dillution, transfer, assay…)
- High sensitivity
- Number of data points is limited by sampling volume/assay sensitivity

Workflow



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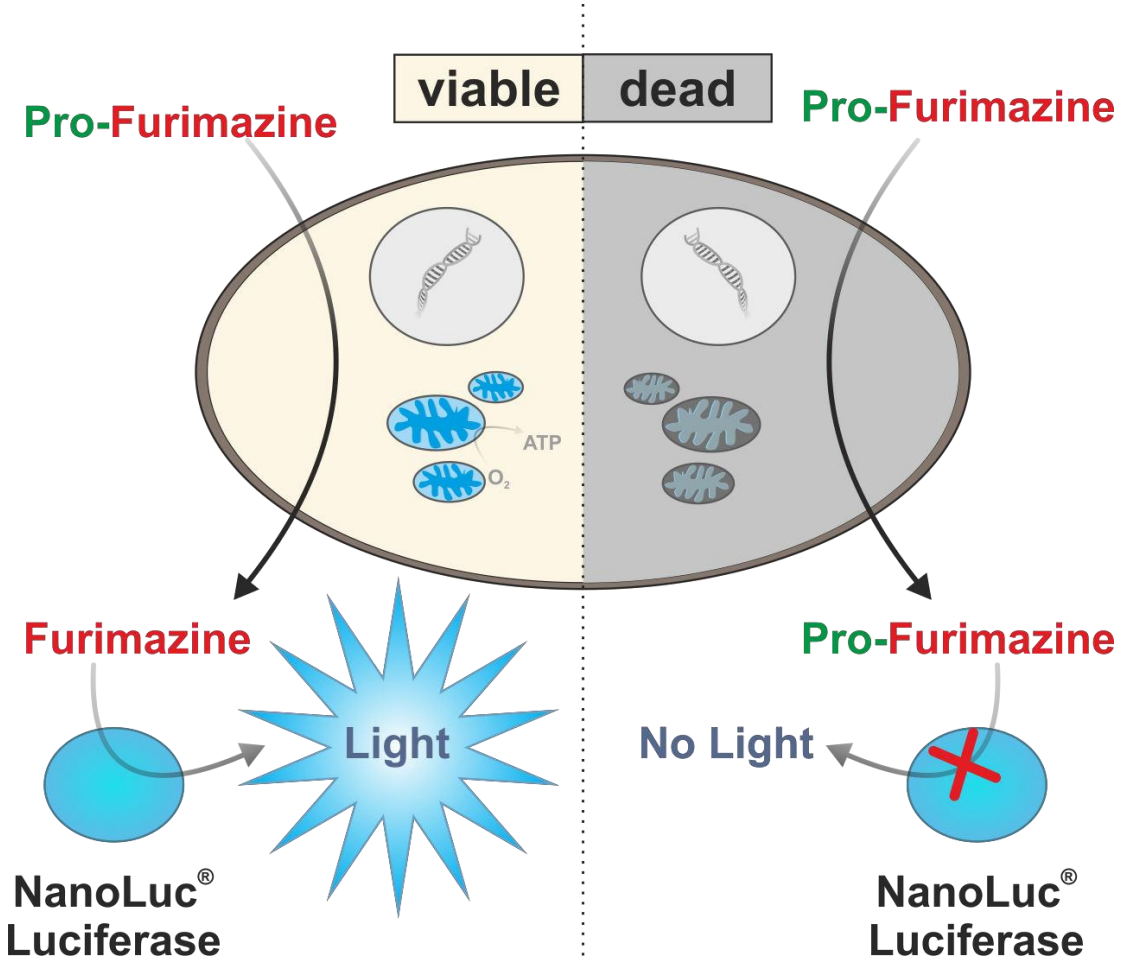
Real Time-Glo MT Cell Viability Assay



- True kinetic nonlytic assay based on the NanoLuc luciferase
- NanoLuc and prosubstrate pro-furimazine added to the medium
- Pro-furimazine diffuses into the cells where it is reduced
- Reduced furimazine is released into the medium and processed by the NanoLuc which generates bioluminescence
- Suitable for kinetic measurements up to 72 hours

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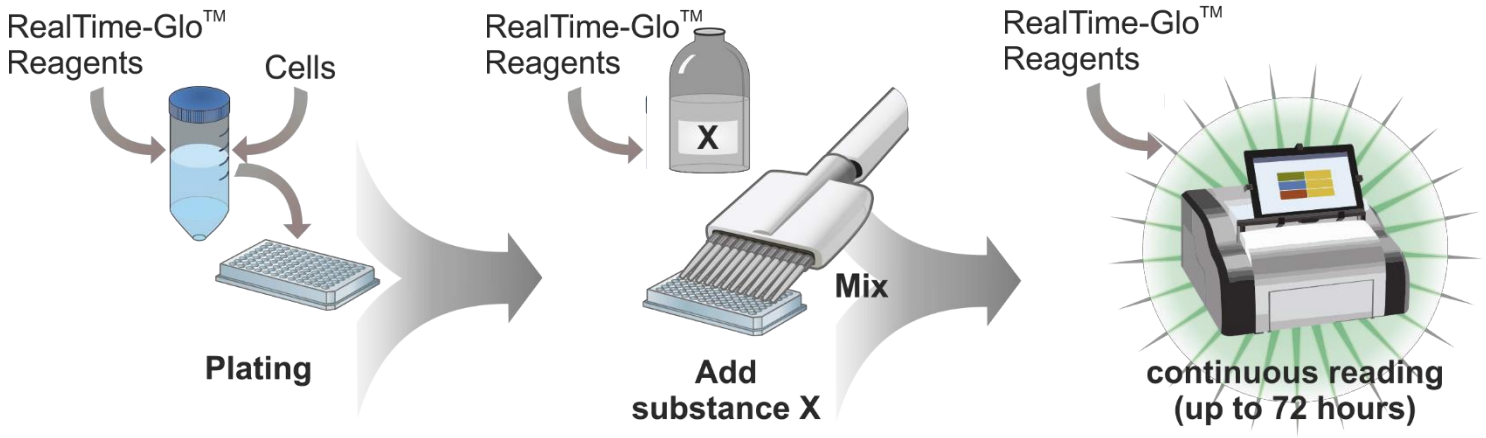
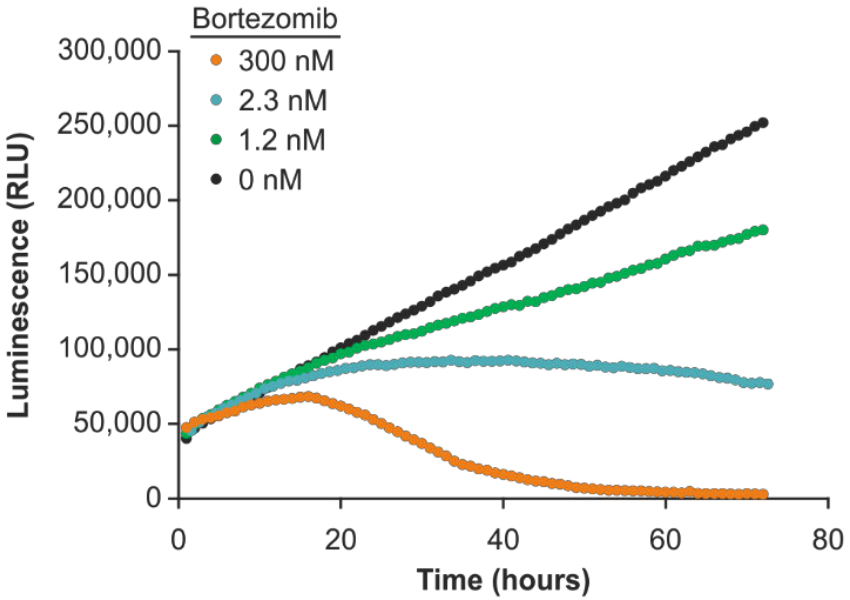
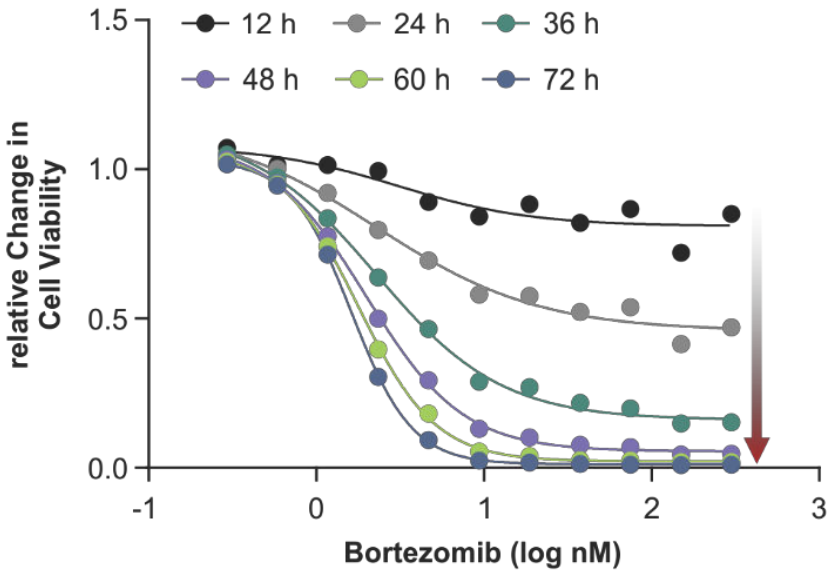
Real Time-Glo MT Assay – workflow a data

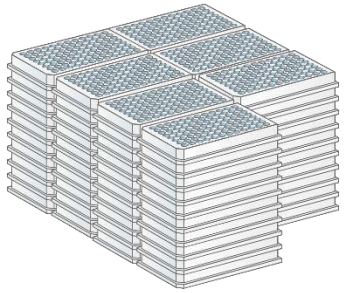


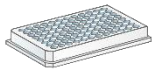




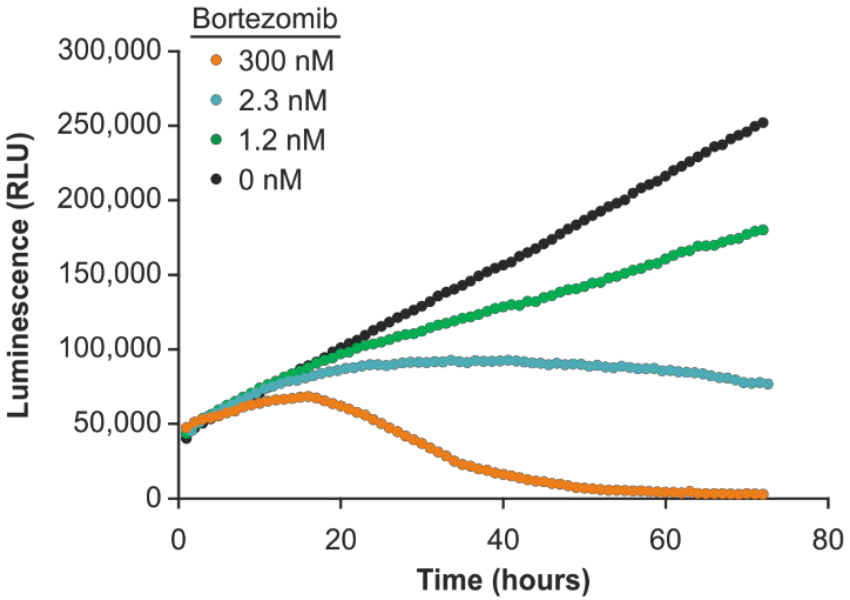
Real Time-Glo MT Assay – Workflow and Data

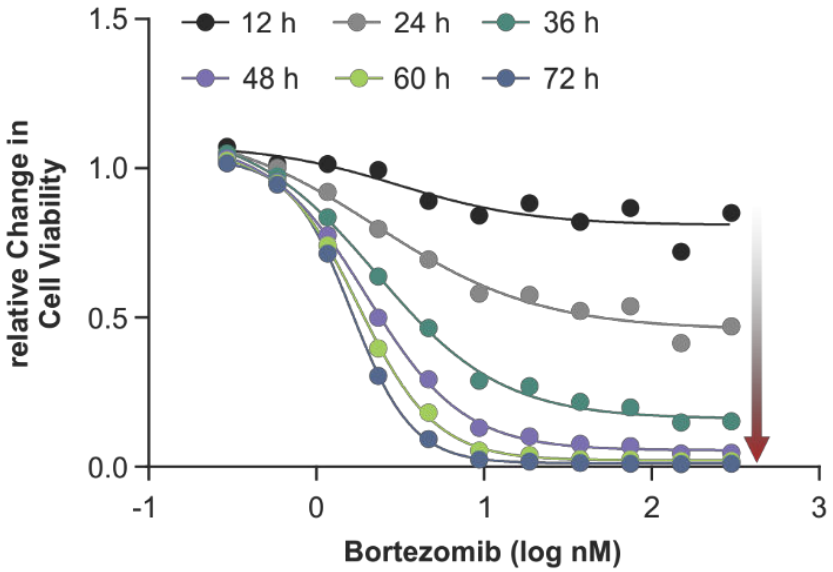
„endpoint“ vs. „real-time“





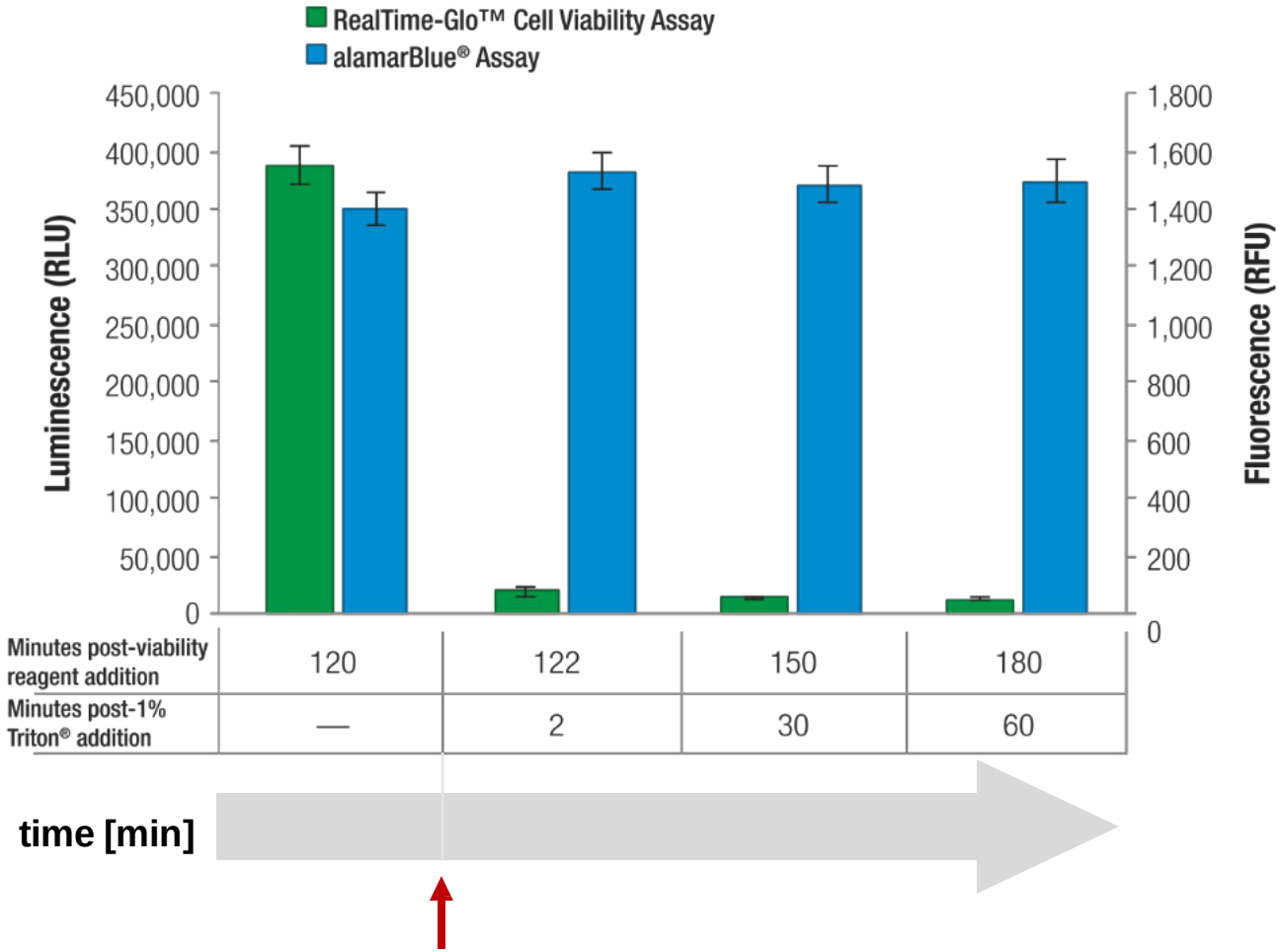
and much less medium, cells and reagent





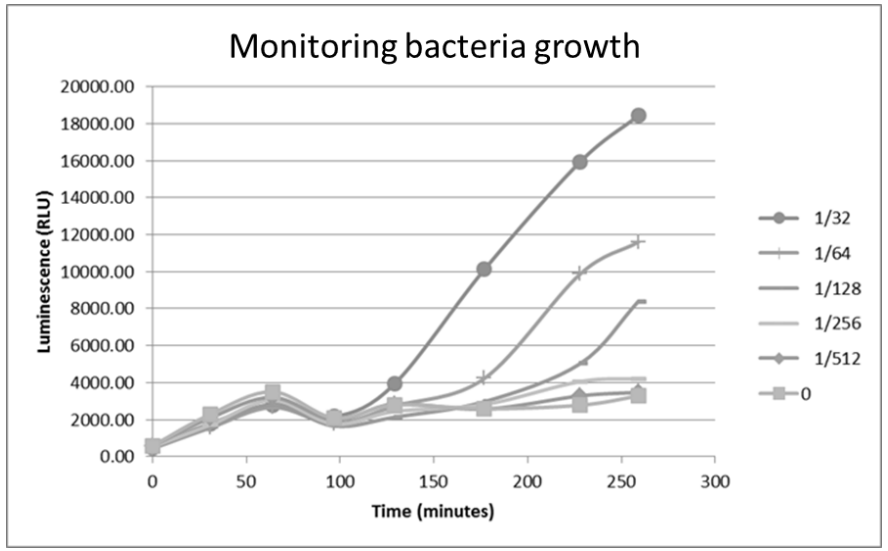
EASTPORT LIFESCIENCE

Furimazine does not accumulate in cells

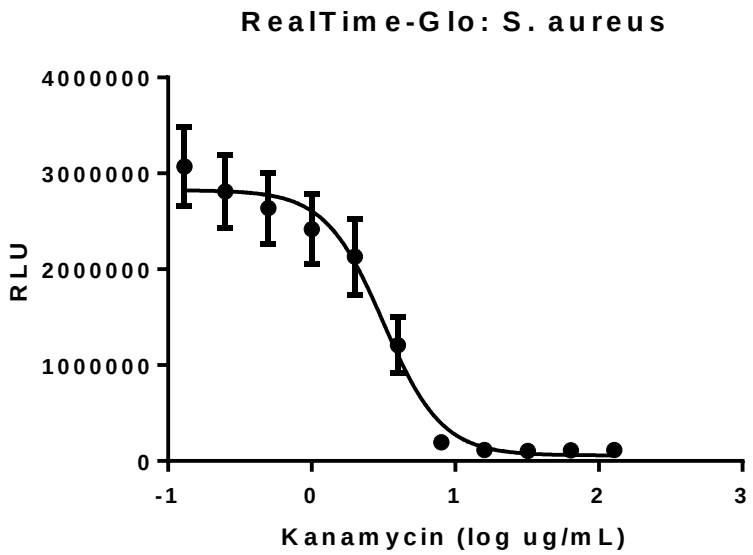


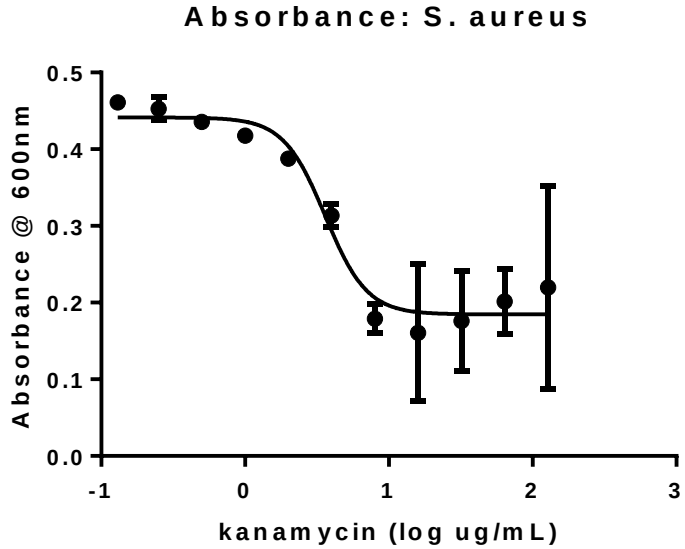
Real Time-Glo MT Assay – Bacterial Viability

- Allows measuring viability of both gram-negative and gram-positive bacteria



- Serial dillution of G- bacteria *E. coli* (ATCC25922) cultured in the presence of the RealTime-Glo MT Assay



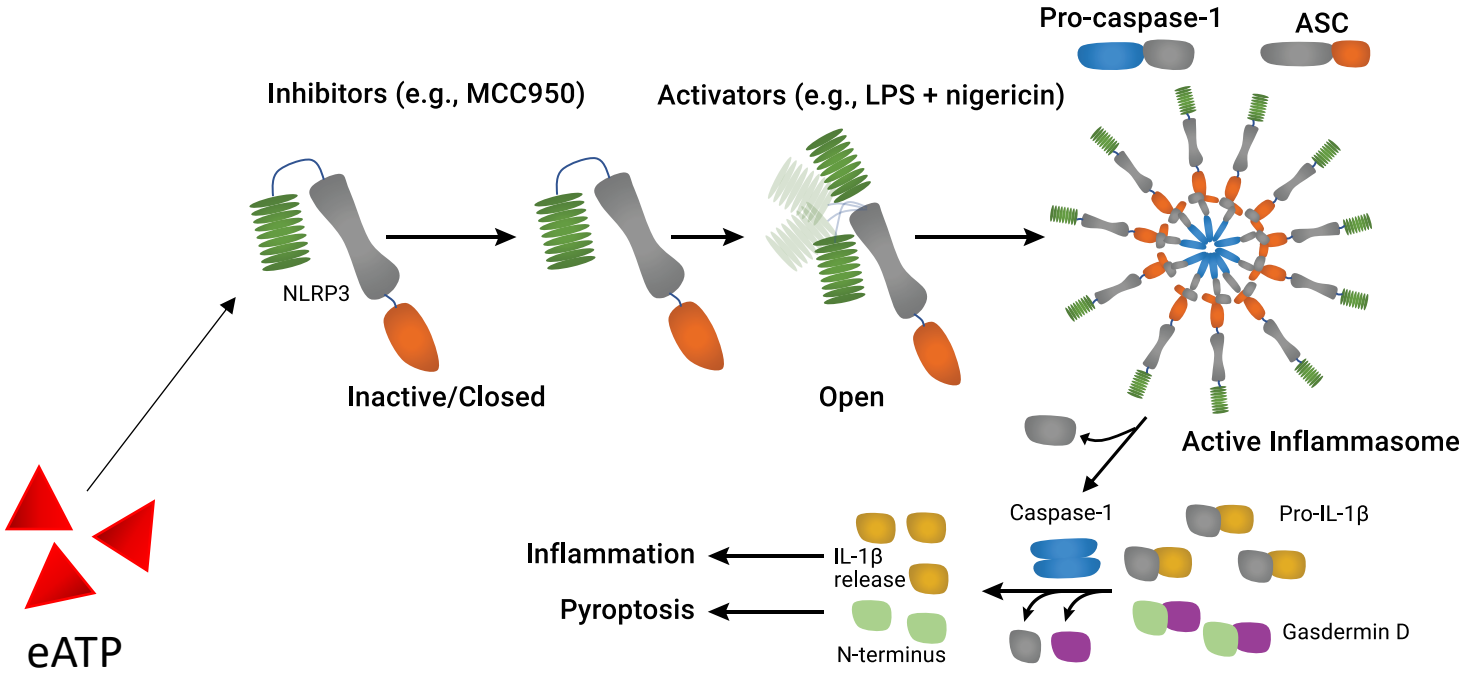


Assay	EC50 (ug/ml)	S/B
RealTime-Glo	3.6	30.6
Absorbance	3.2	2.4

- Comparison of kanamycin EC50 value determination using the the bioluminescence and absorbance assays
- G+ bacteria *Staphylococcus aureus* was treated with various concentrations of kanamycin
- EC50 values in good correlation but BL assay provides superior sensitivity

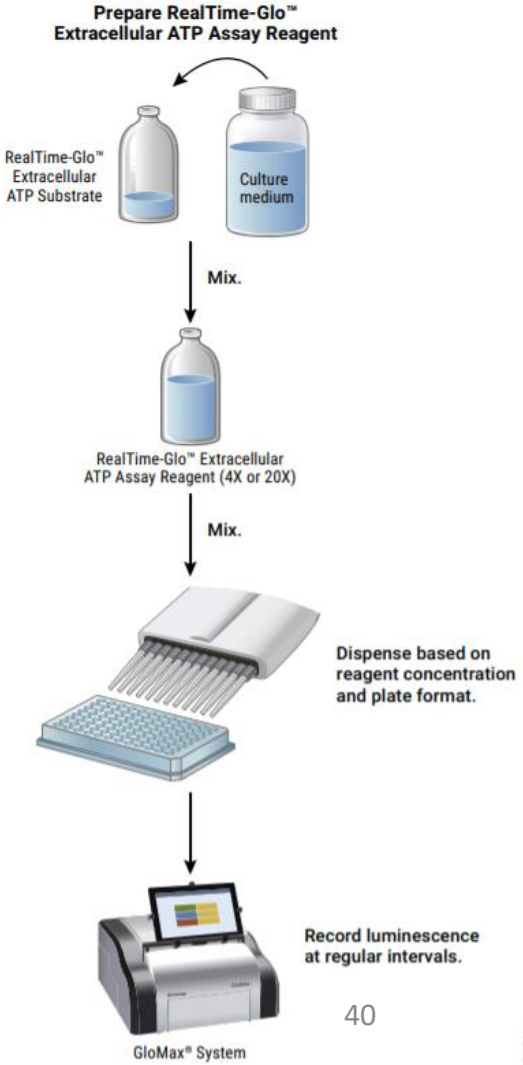
RealTime-Glo Extracellular ATP Assay

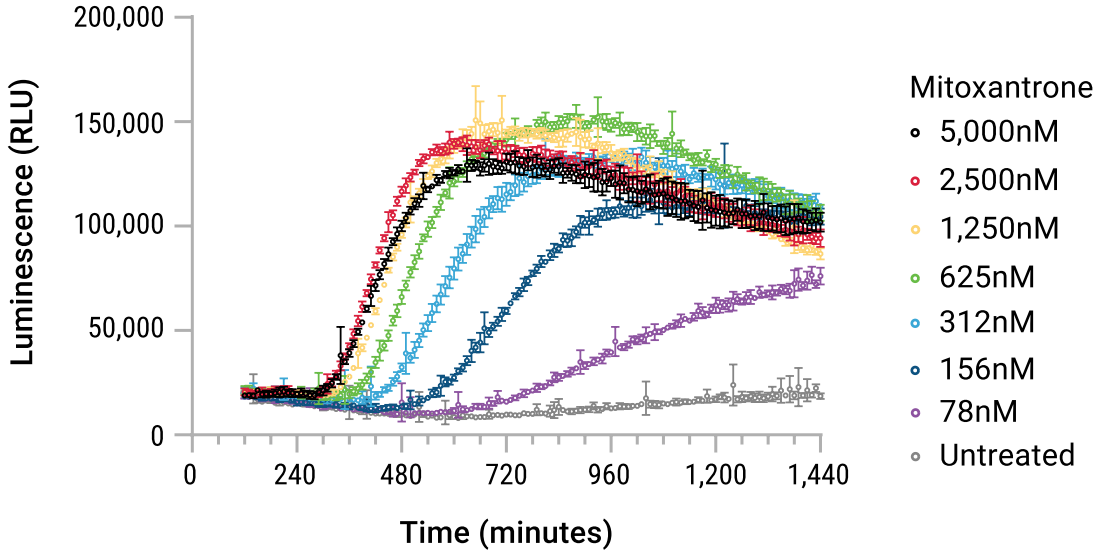
- Damage-associated molecular patterns (DAMPs) are molecules released into extracellular space as an indicator of cell damage or infection
- eATP can function as one of DAMPs and trigger inflammasome activation



RealTime-Glo Extracellular ATP Assay

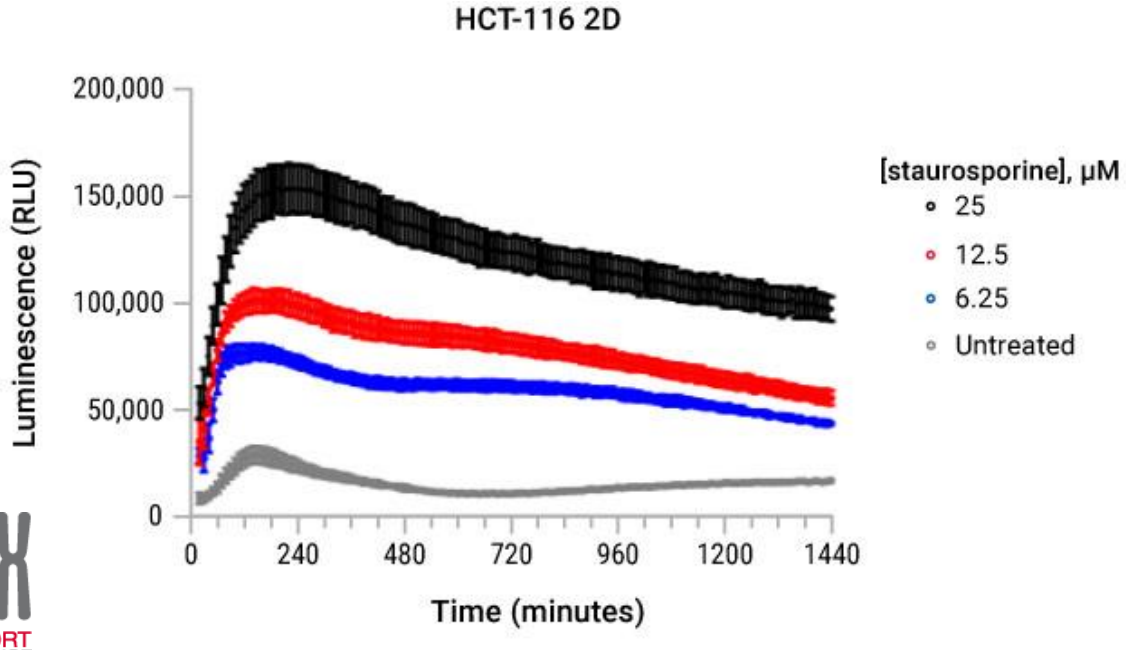
- Bioluminescent assay for kinetic monitoring of ATP release from dying, stressed or activated cells
- Assay suitable for detecting immunogenic cell death or inflammasome activation
- Based on the modified Firefly luciferase
- Enzyme and substrate are added to the cells and ATP is supplied from the medium
- Compatible with 2D and 3D models

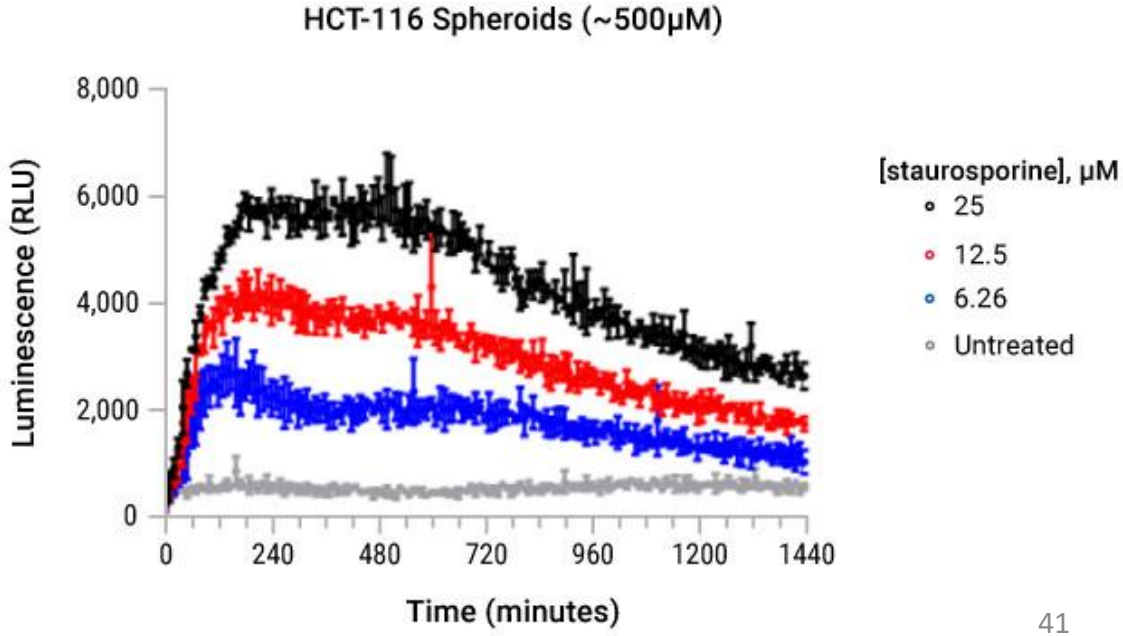




- U937 cells treated with a dilution of mitoxantrone and anthracycline to induce immunogenic cell death
- ATP release measured every 10 minutes for 24 hours at 37°C

Comparison of 2D and 3D cell culture model





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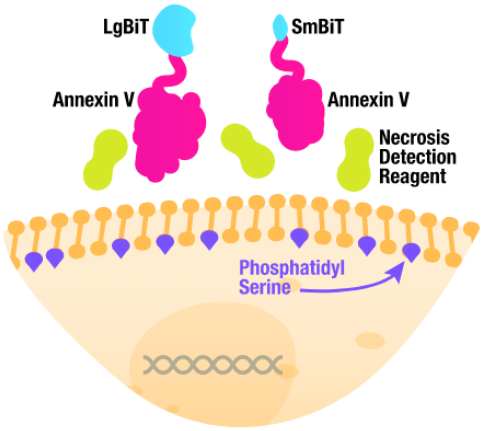
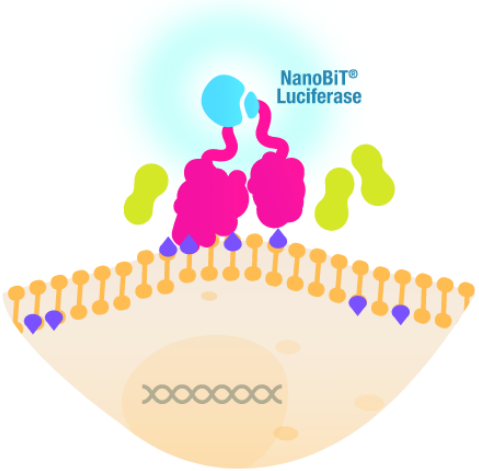
RealTime-Glo Annexin V Assay

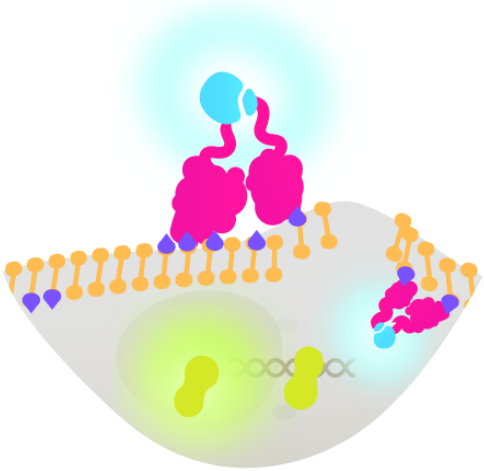
- Detects onset of apoptosis by measuring the phosphatidylserin (PS) exposure on surface of the cell membrane
- Based on the NanoBiT split NanoLuc luciferase technology
- Annexin V conjugated with LgBiT and SmBiT subunits of NanoLuc
- Annexin V-LgBiT/SmBiT bind to PS on the membrane → subunits brought to close proximity recombine and restore the NanoLuc luminescent activity
- Easily combined with CellTox Green



PS confined to inner leaflet
Cell membrane intact
Luminescence (RLU) negative
Fluorescence (RFU) negative



PS translocation to outer leaflet
Cell membrane intact
Luminescence (RLU) POSITIVE
Fluorescence (RFU) negative



PS on inner and outer leaflet
Cell membrane compromised
Luminescence (RLU) POSITIVE
Fluorescence (RFU) POSITIVE

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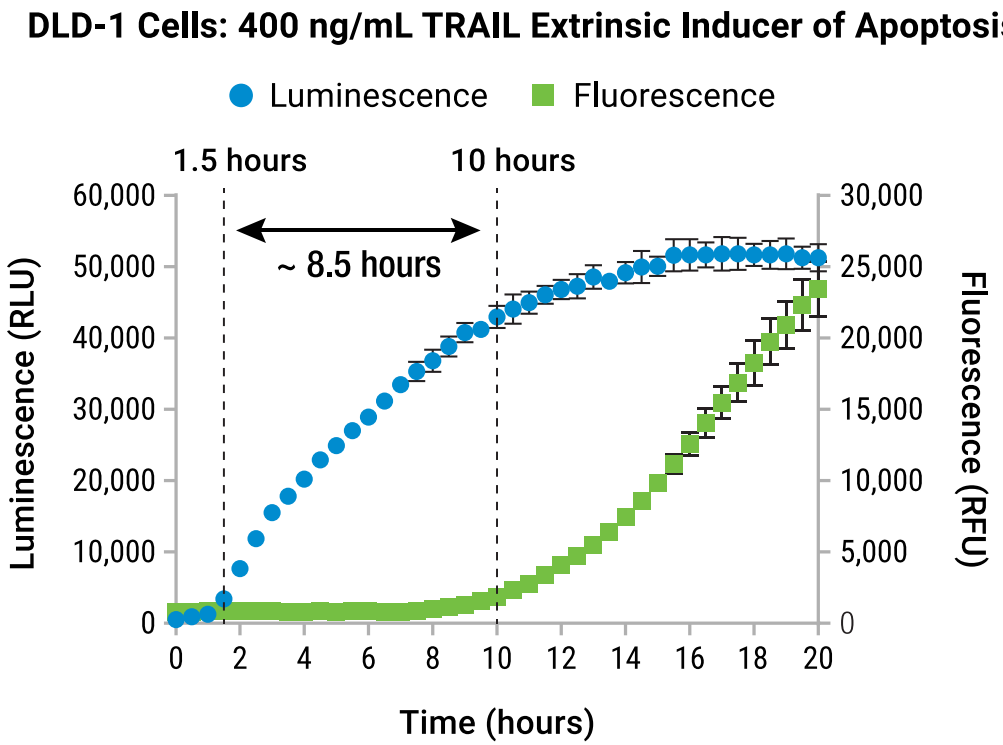
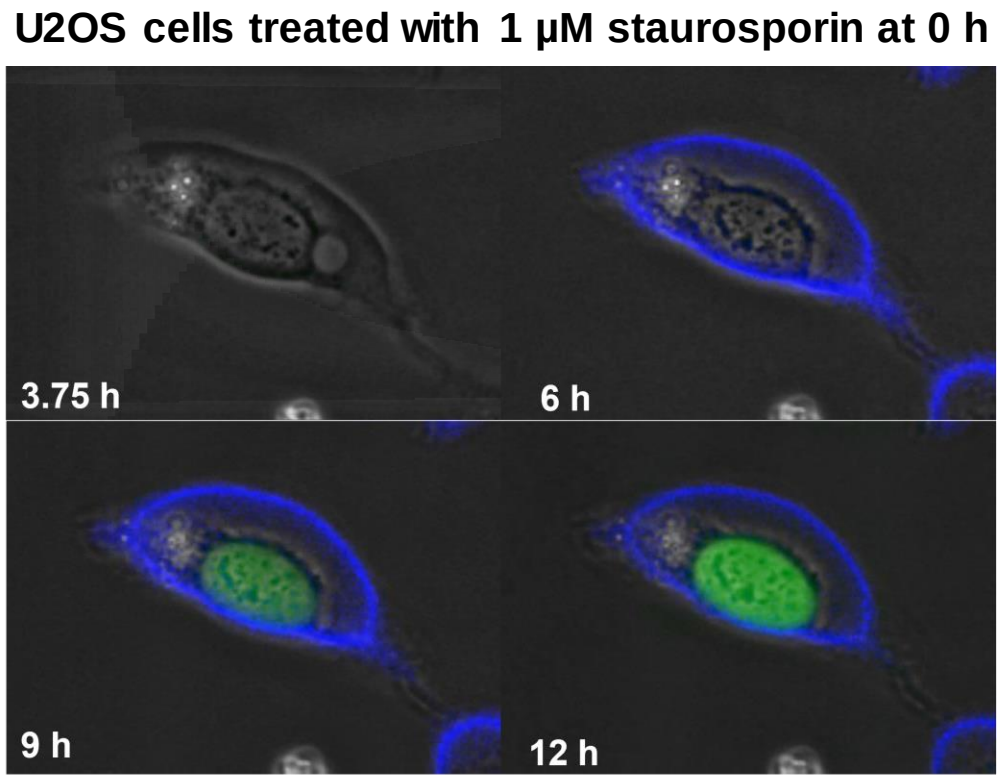
RealTime-Glo Annexin V Assay - Advantages

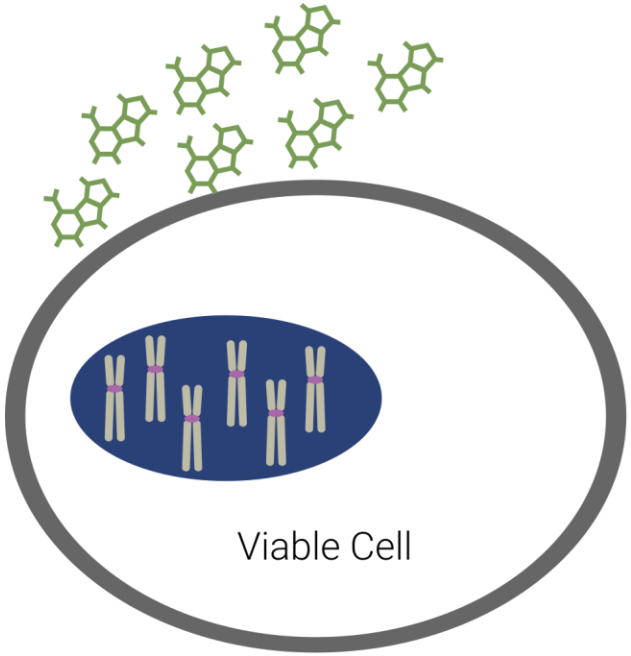
- Different compounds trigger apoptosis with various kinetics in different cell lines
- Easily identify the onset of apoptosis caused by your treatment
- More information from a single well
- Simple no-wash protocol, saves reagents, cells and plates





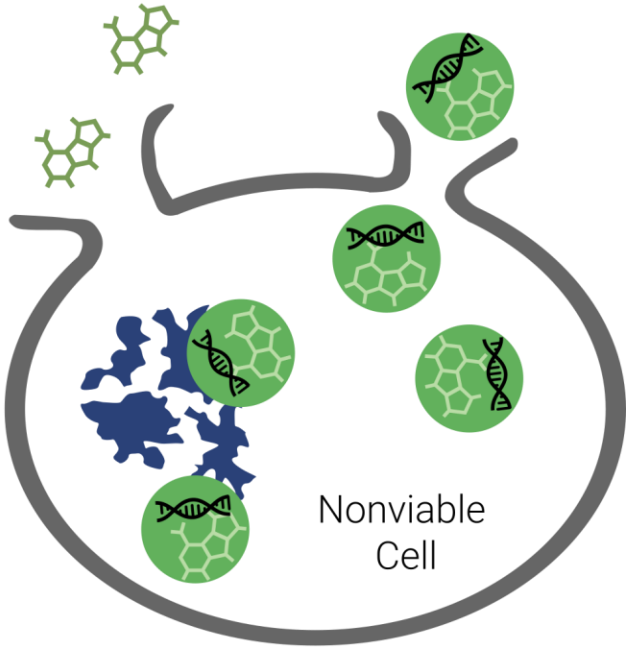
CellTox™ Green Cytotoxicity Assay

- Dying cells form pores in the membrane and have compromised membrane integrity
- Determines membrane integrity by a non-permeable DNA-binding dye
- The dye enters the compromised cells, binds to nuclear DNA and enhances its fluorescence



Low Fluorescence

Excluded dye yields very low background fluorescence



High Fluorescence

Penetrated dye significantly enhances its fluorescence

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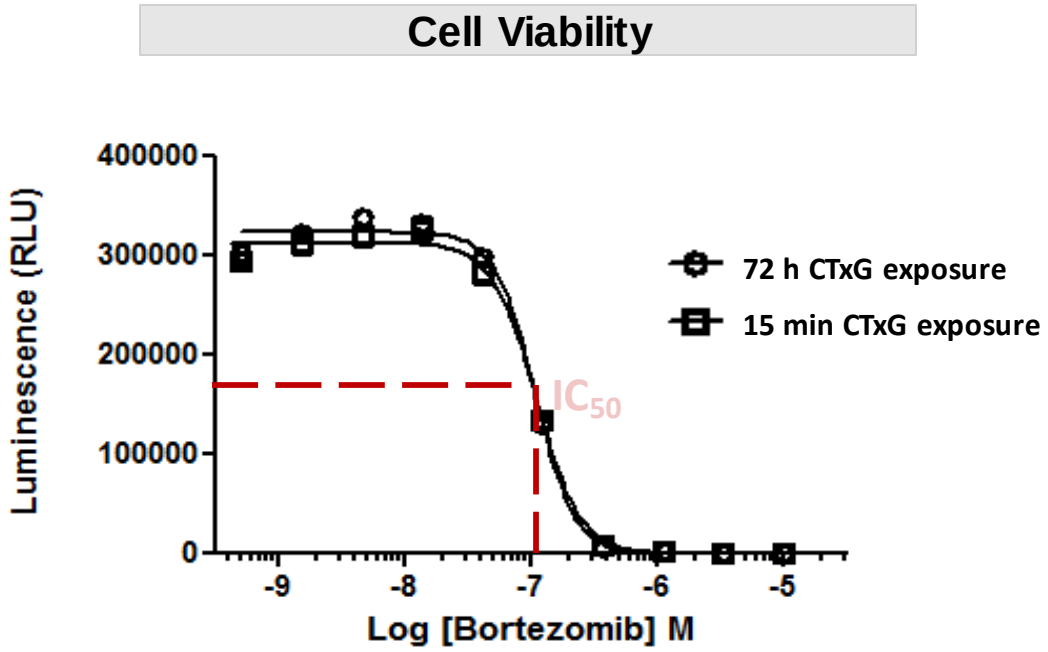
CellTox Green Advantages

- Well tolerated by the cells
- True kinetic assay – measurements for up to 72 hours
- Easily multiplex with other fluorescent and BL assays
- Suitable for 3D cultures, easily diffuses inside the spheroids
- Compatible with standard GFP/FITC filters
- Suitable for microplate readers, flow cytometry and fluorescence microscopy
- Low price – 1000x dillution for the experiments



Dye is nontoxic for at least 72 hodin

= does not affect the IC_{50} of tested compound (bortezomib)

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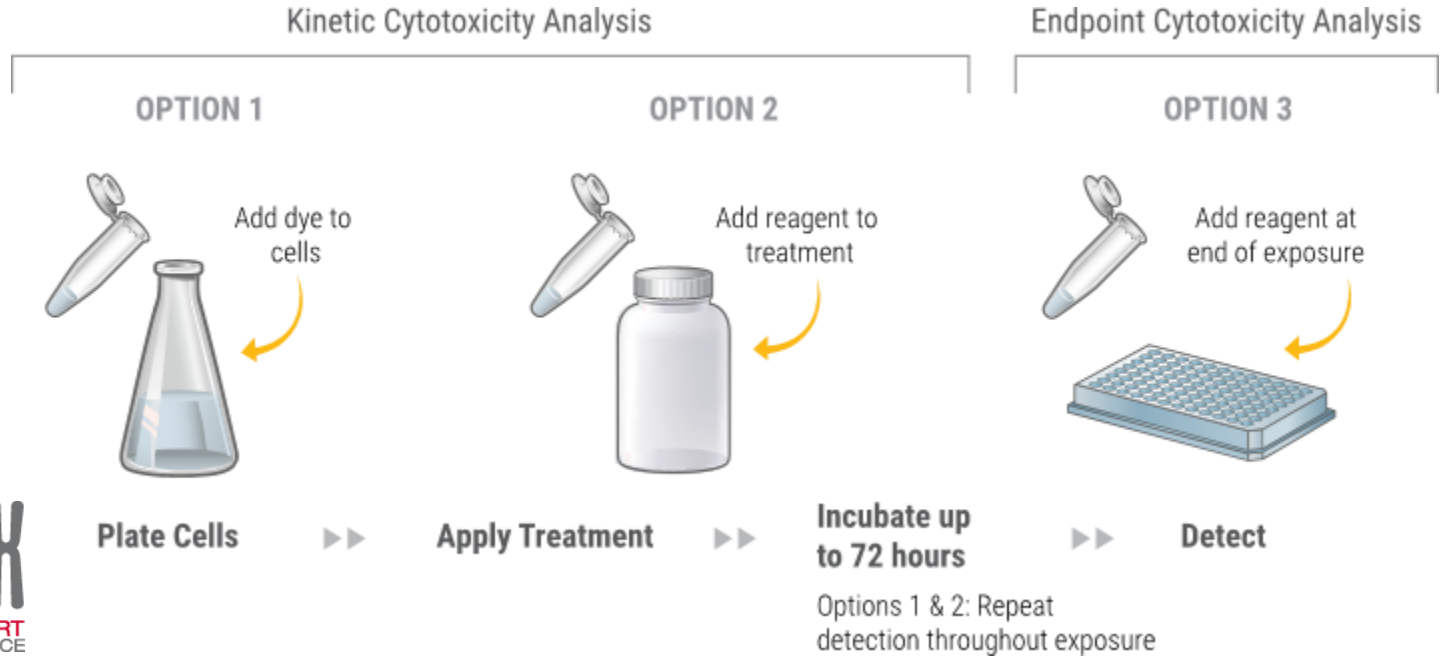
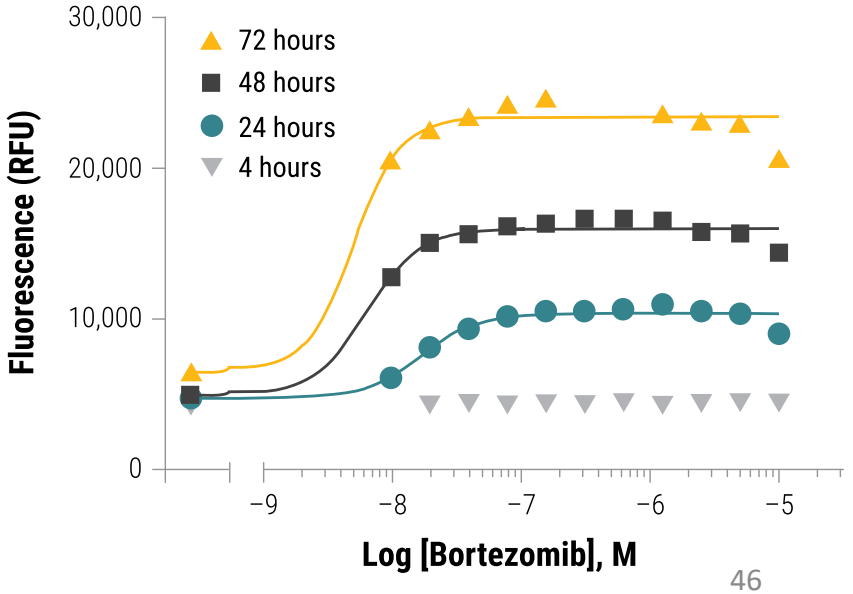
Flexible Protocol

The reagent can be added:

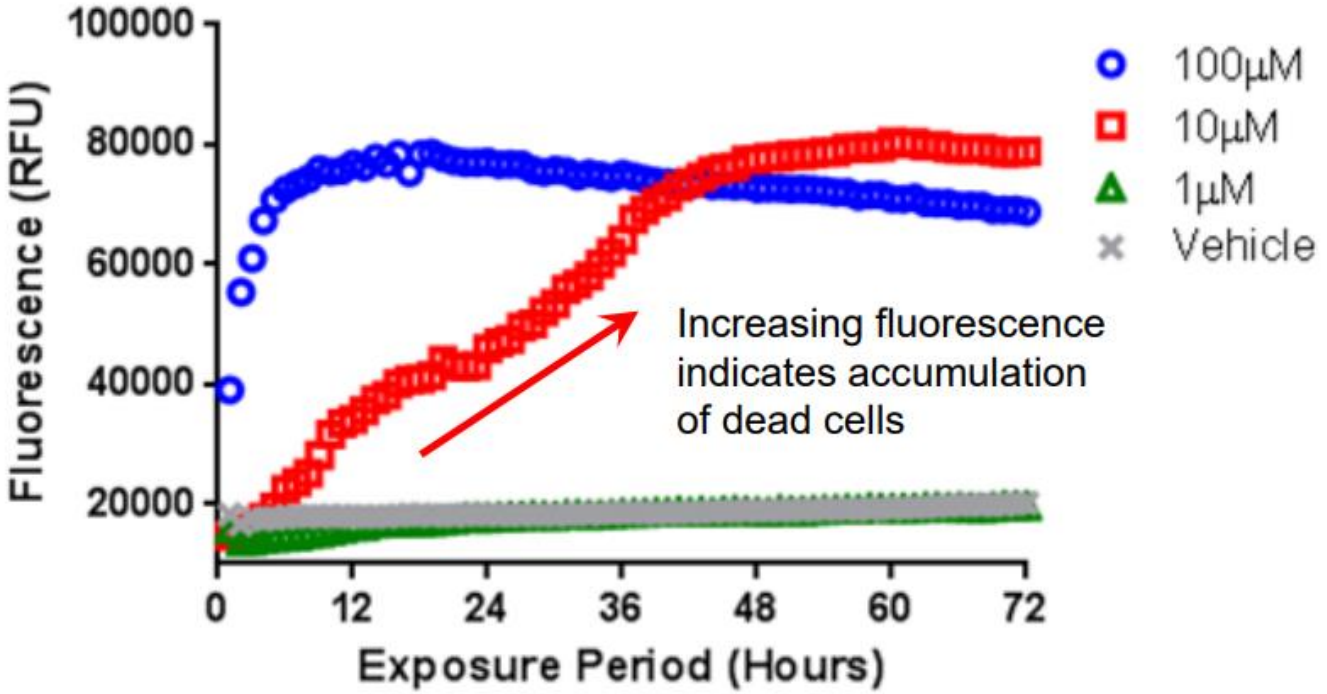
- During cell seeding
- With the treatment addition
- At the end of the experiment as an end-point assay

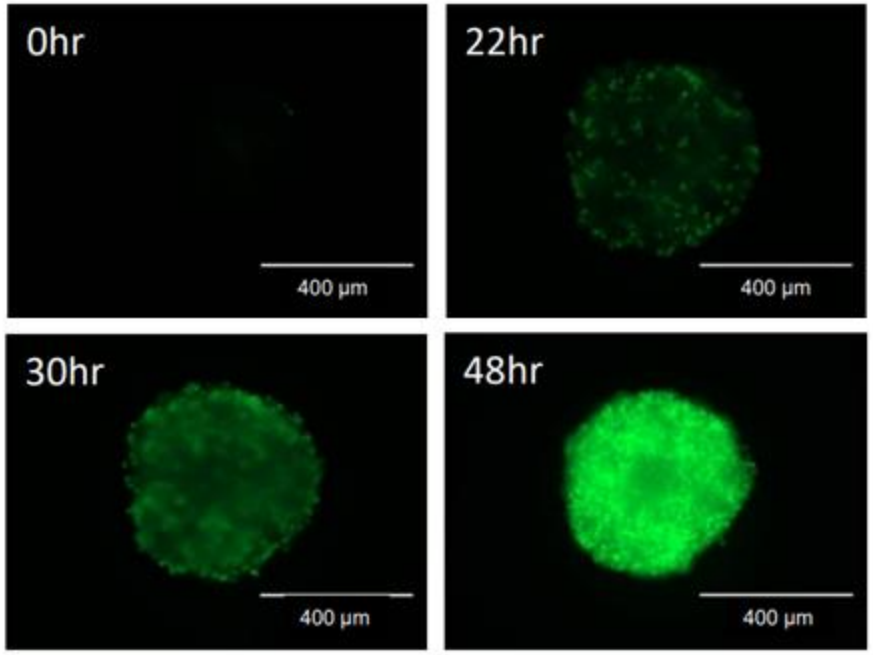




EASTPORT LIFESCIENCE

HepG2 cells treated with various doses of terfenadine. Fluorescence measured every hour from the same sample wells. ▶



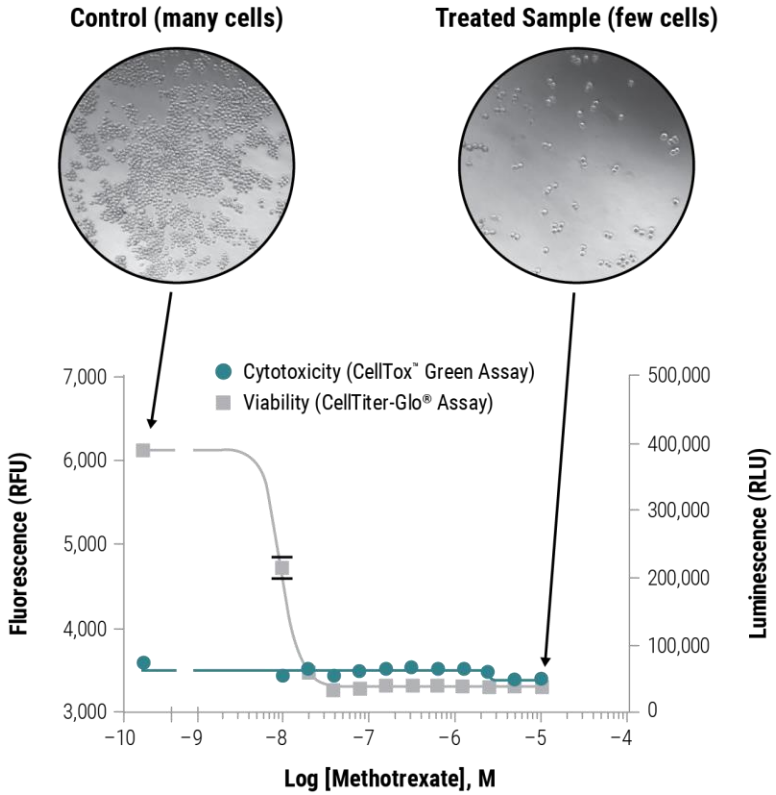


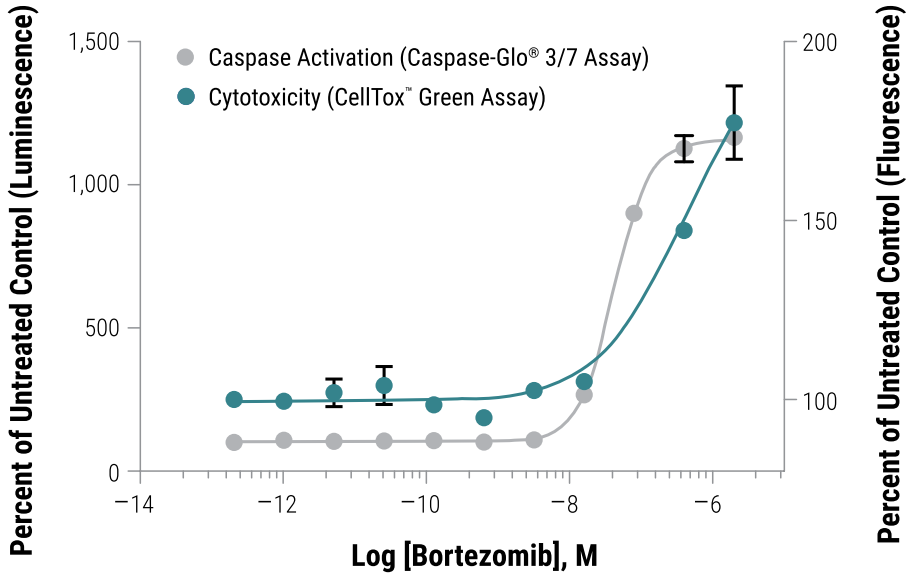
◀Live cell kinetic imaging of CellTox™ staining of a paclitaxel treated HepG2 cell spheroid photographed at different times over 2 days. Green fluorescence shows the accumulation of dead cells.

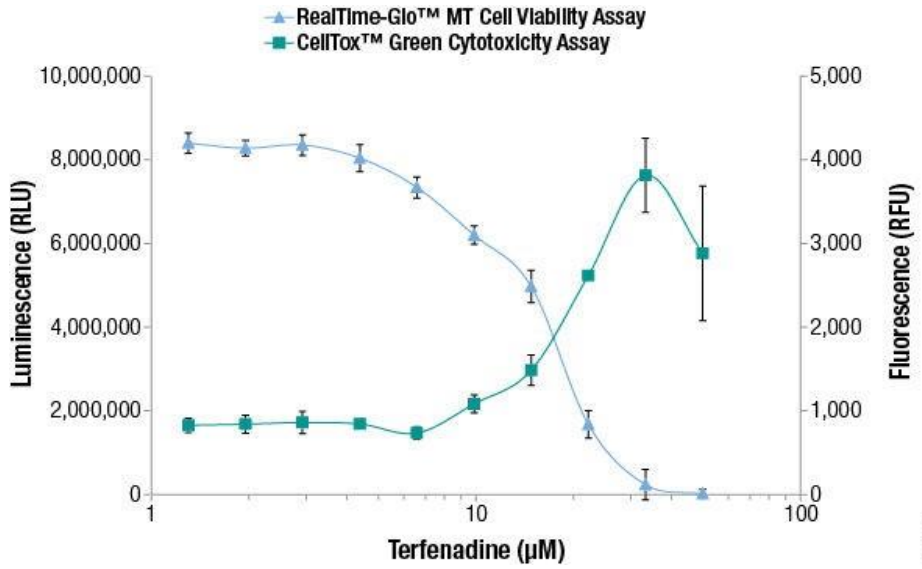
EASTPORT LIFESCIENCE

Assay Designed for Multiplexing

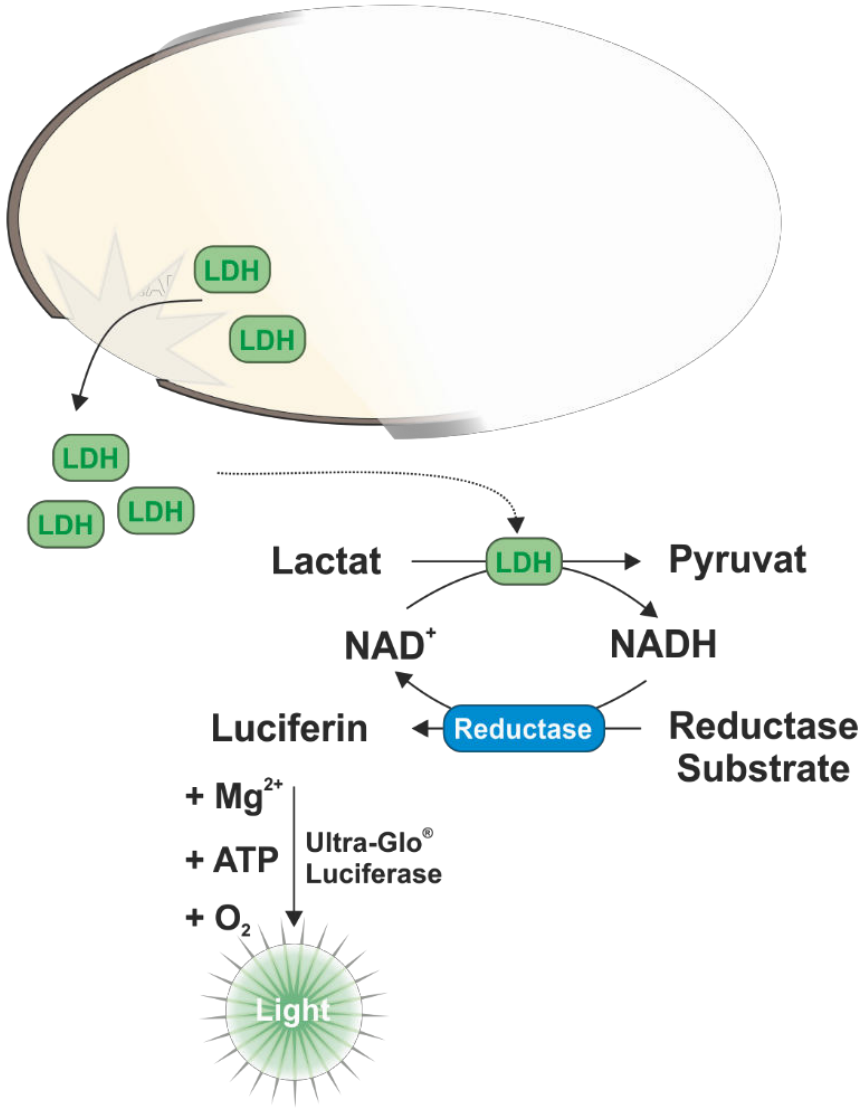
- Can be multiplexed with other kinetic/endpoint assays
- Allows differentiation between cytotoxic and cytostatic effect
- Allows determining the dose that triggers apoptosis or cytotoxicity







LDH-Glo® Assay – measures LDH leaking from cells

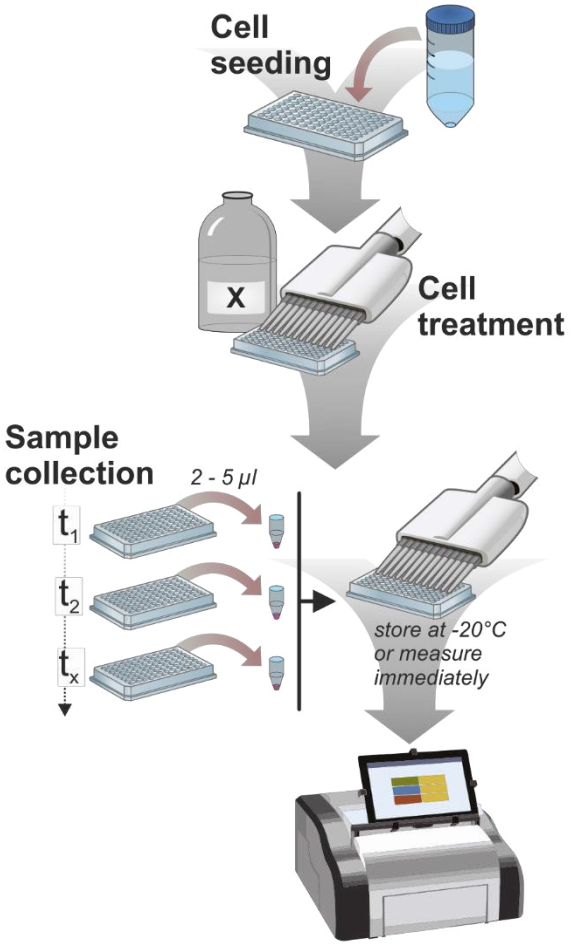


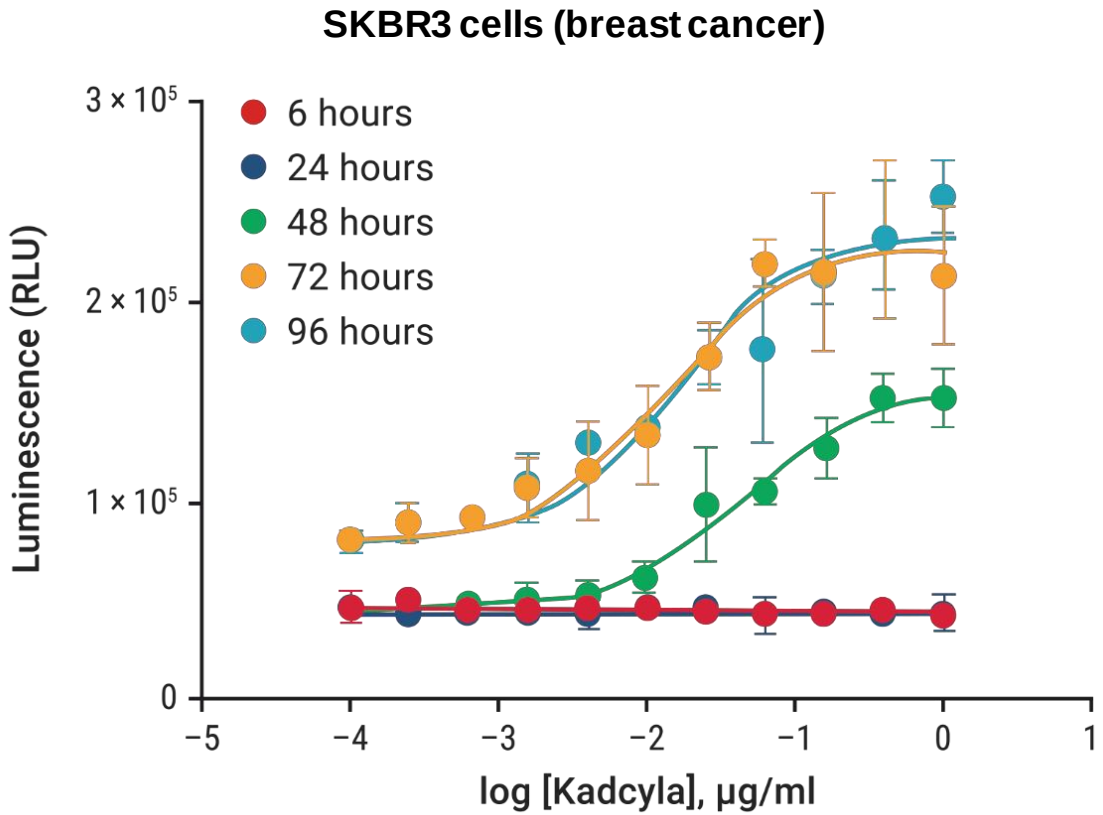
- Lactate dehydrogenase leaks from damaged cells
- Its activity is detected in supernatant in a coupled enzymatic reaction
- Pro-luciferin is reduced by a proprietary reductase to luciferin and processed by firefly luciferase in the medium
- Timed kinetic assay
- Suitable for 3D cell cultures
- Suitable for measuring antibody-dependent-cell-mediated cytotoxicity (ADCC)

EASTPORT LIFESCIENCE

LDH-Glo® Can be Used as a Kinetic Assay

Experimental workflow



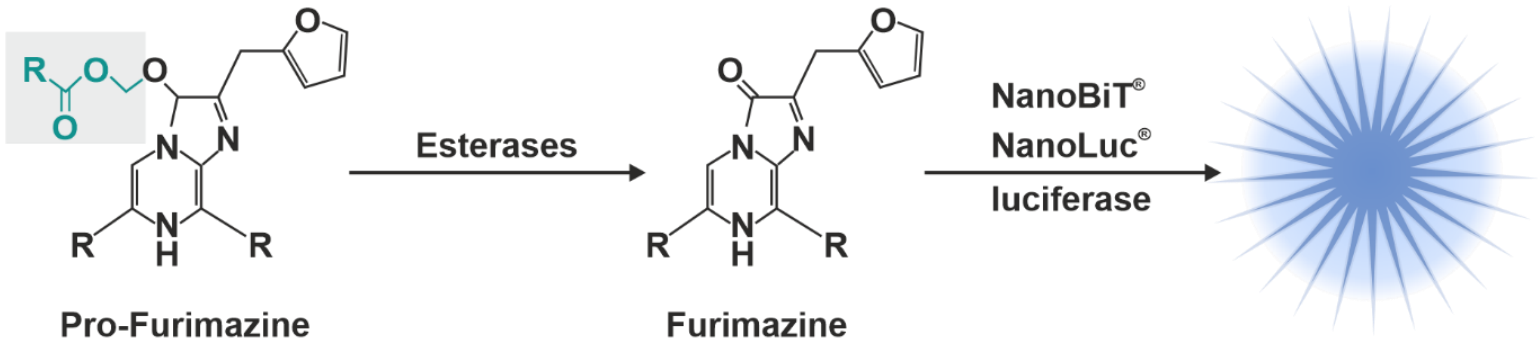


- Enzymatic marker cytotoxicity
- Suitable for timed kinetic analysis
- For ideal results, use inactivated FBS – higher sensitivity

EASTPORT LIFESCIENCE

NanoLuc®/NanoBiT® Live Cell Substrates

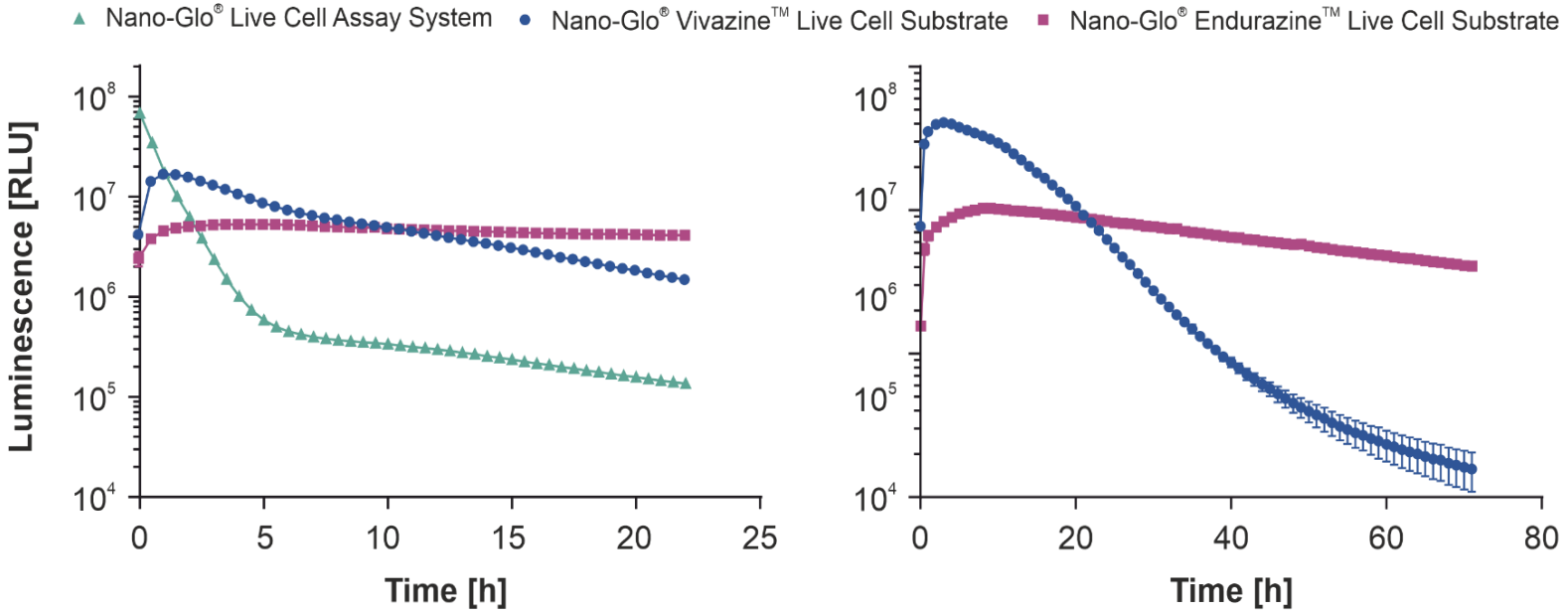
Different Substrates for Different Purposes



Nano-Glo® Live Cell Assay System
Endpoint / up to 2 h

Nano-Glo® Vivazine™ Substrate
2-24 h

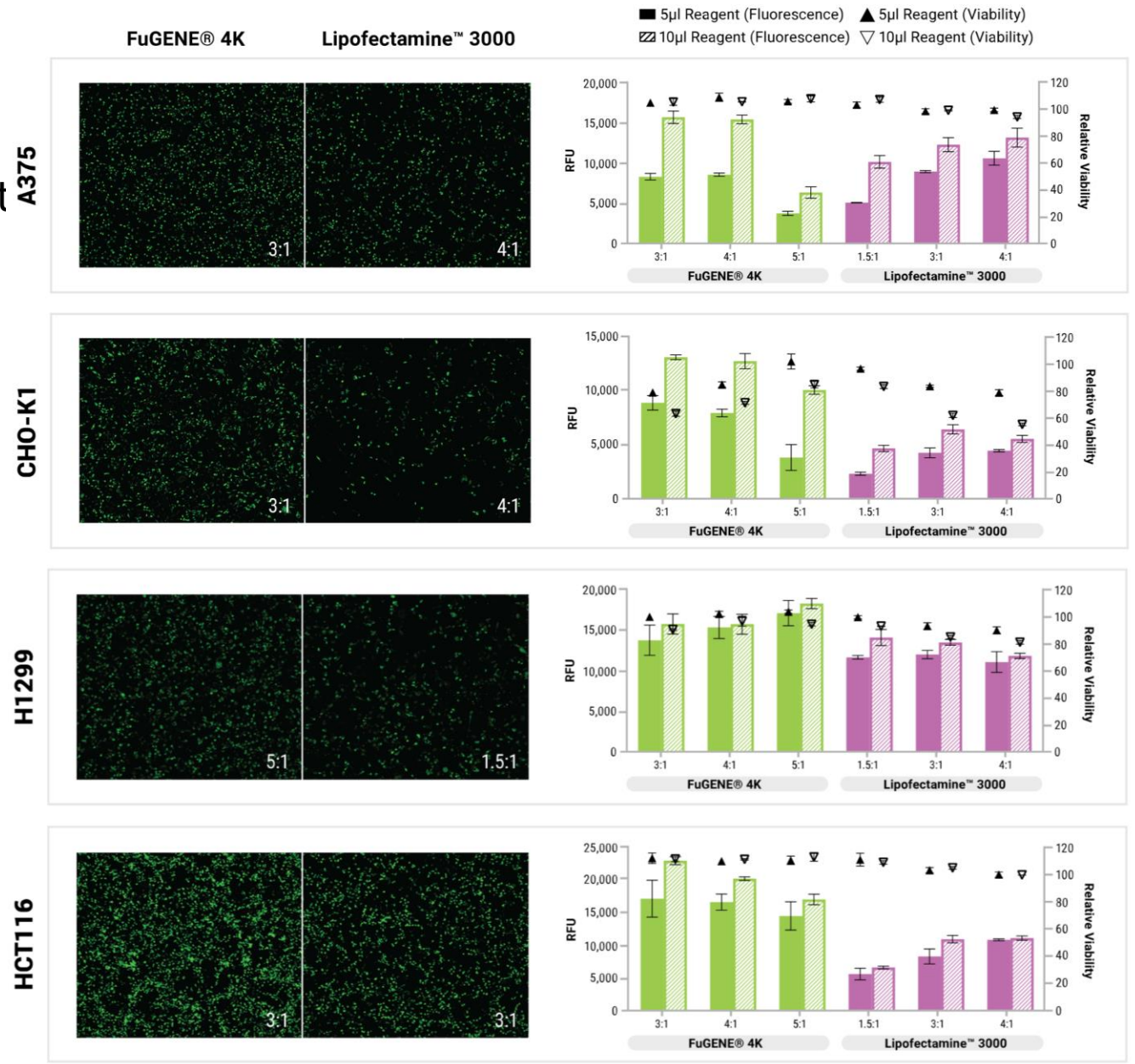
Nano-Glo® Endurazine™ Substrate
2 - 72 h



EASTPORT LIFESCIENCE

Fugene 4K – Novel Transfection Reagent

- Designed for the delivery of DNA into challenging and routine mammalian cell lines
- Higher transfection efficiencies with minimal impact on viability
- Simple protocol
- Strong performance in HEK293 and CHO cells → ideal for protein and viral production
- 100% synthetic and animal-free

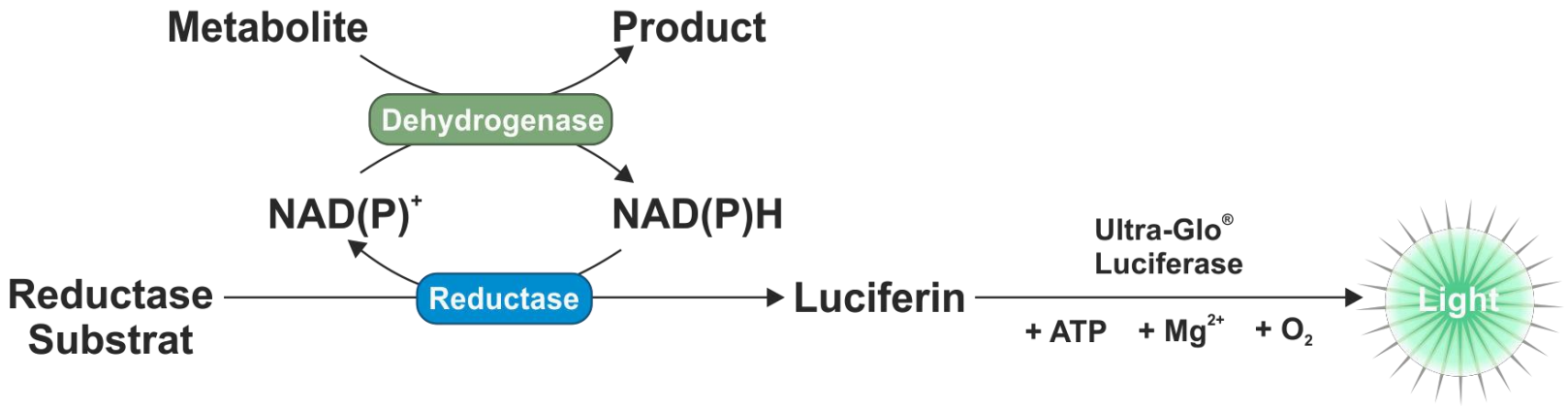


▶ Cells transfected with a GFP expression construct using FuGENE® 4K or Lipofectamine™ 3000 with varying reagent:DNA ratios. After 48 hours, cells in a clear-bottom plate were measured for total GFP fluorescence. Cell viability was measured on a duplicate plate using the CellTiter-Glo® Luminescent Cell Viability Assay.

EASTPORT LIFESCIENCE

Metabolite Assays – One Reaction to Rule Them All

Metabolite-selective dehydrogenases coupled to bioluminescent NAD(P)H detection



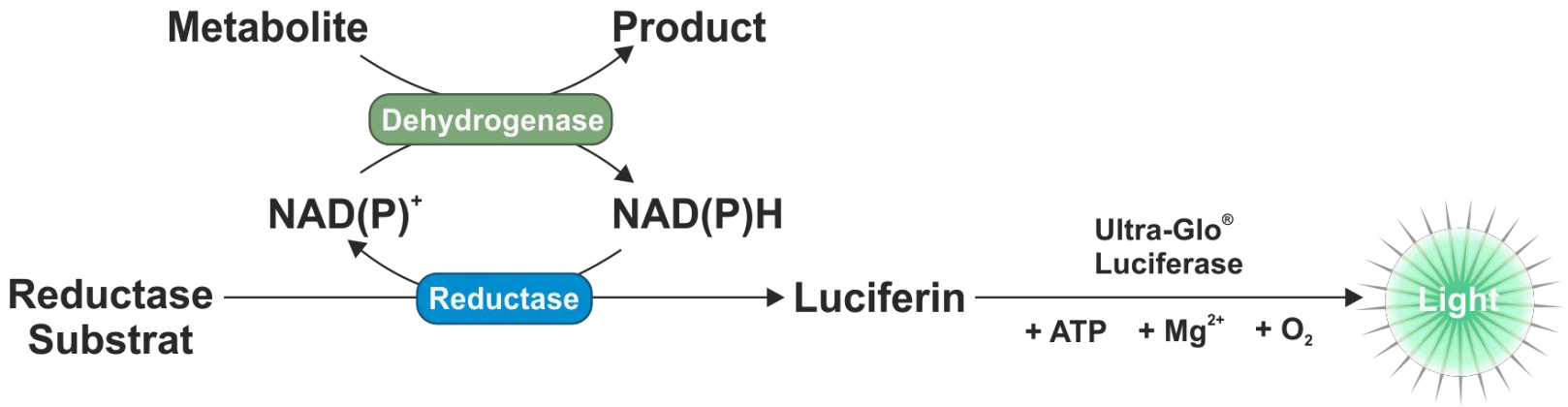
"*With the aid of auxiliary enzymes nearly every substance of biological interest could be measured with a pyridine nucleotide system*" Oliver Lowry JBC (1961) 236, 2746

- Broad linear range of 3 logs (0,1-100 µM)
- Wide dynamic range S/B < 100
- High sensitivity, requiring only a small sample volume
- Simplified protocol applicable to many sample types – homogenized tissue, 2D & 3D culture, serum, medium
- Can be used as timed kinetic assays

EASTPORT LIFESCIENCE

Metabolite Assays – One Reaction to Rule Them All

Metabolite-selective dehydrogenases coupled to bioluminescent NAD(P)H detection



BCAA-Glo™ Assay (NEW)
Rapid, sensitive method for BCAA detection based on bioluminescent technology.

Glycogen-Glo™ Assay (NEW)
Rapid, sensitive method for detection of glycogen in biological samples.

Glucose-Glo™ Assay
Easily detect glucose from a variety of biological samples.
J6021, J6022

Lactate-Glo™ Assay
Bioluminescent assay to quickly detect lactate from a variety of sample types.
J5021, J5022

Glutamate-Glo™ Assay
Quickly measure glutamate from many sample types.
J7021, J7022

Glutamine/Glutamate-Glo™ Assay
Detect glutamine and glutamate in biological samples.
J8021, J8022

Glucose Uptake-Glo™ Assay
Non-radioactive assay for measuring glucose uptake.
J1341, J1342, J1343

Glycerol-Glo™ Assay
Measures glycerol using the enzymes glycerol kinase and glycerol-3-phosphate dehydrogenase.
J3150, J3151

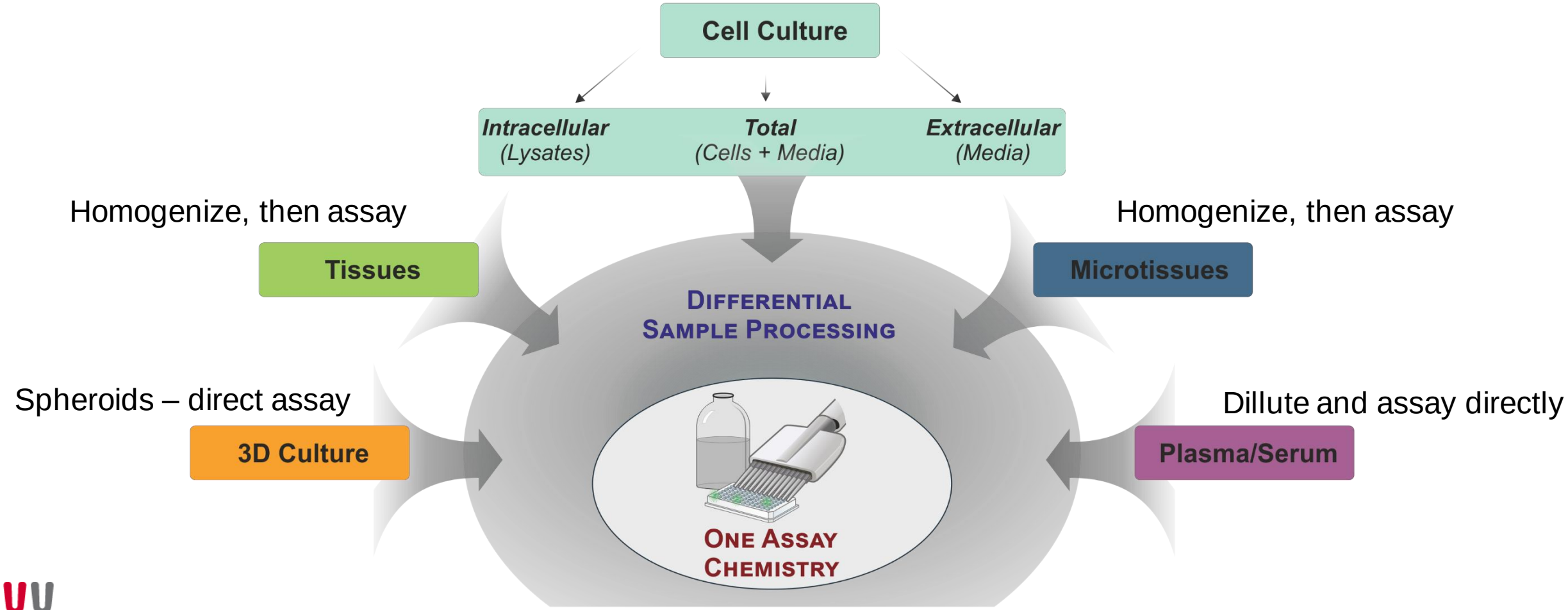
Triglyceride-Glo™ Assay
Detects triglyceride levels by measuring glycerol that is released from an enzymatic reaction with a lipase.
J3160, J3161

Cholesterol/Cholesterol Ester-Glo™ Assay
Measures cholesterol by association with NADH production and proluciferin activation that produces light with a luciferase enzyme.
J3190, J3191

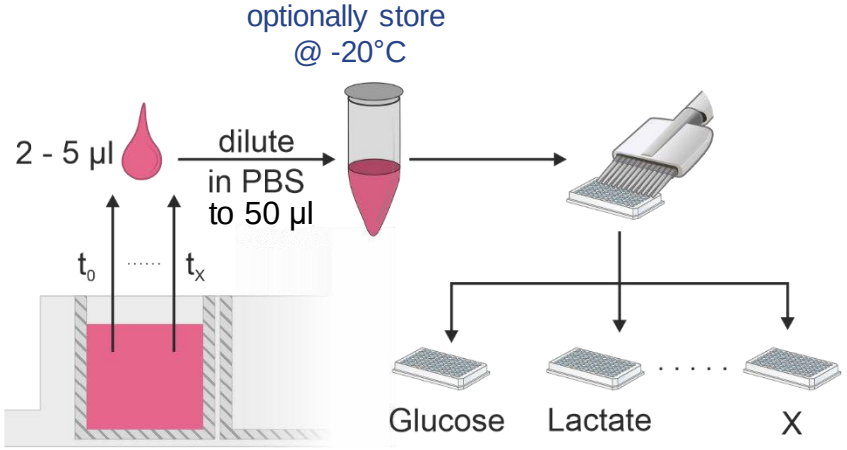
→ Can be used in a timed kinetic format to measure the signal from supernatant

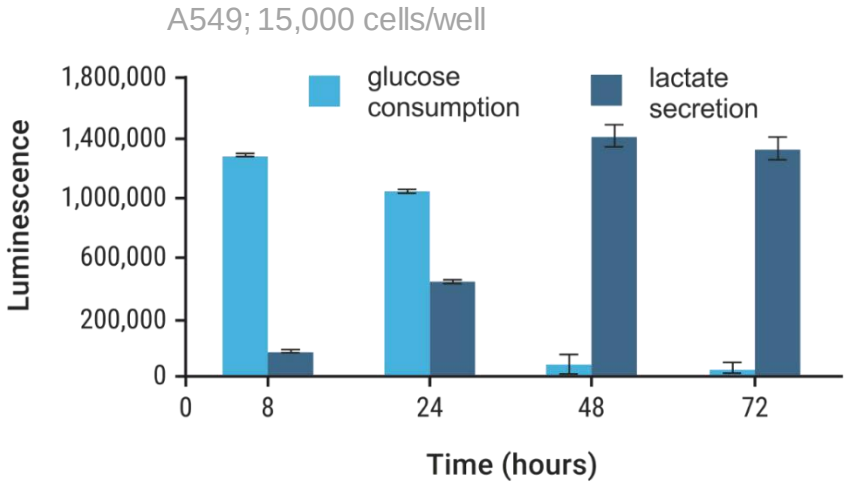
EASTPORT LIFESCIENCE

Metabolite Assays – Compatible Sample Types



Glucose and Lactate-Glo Assays

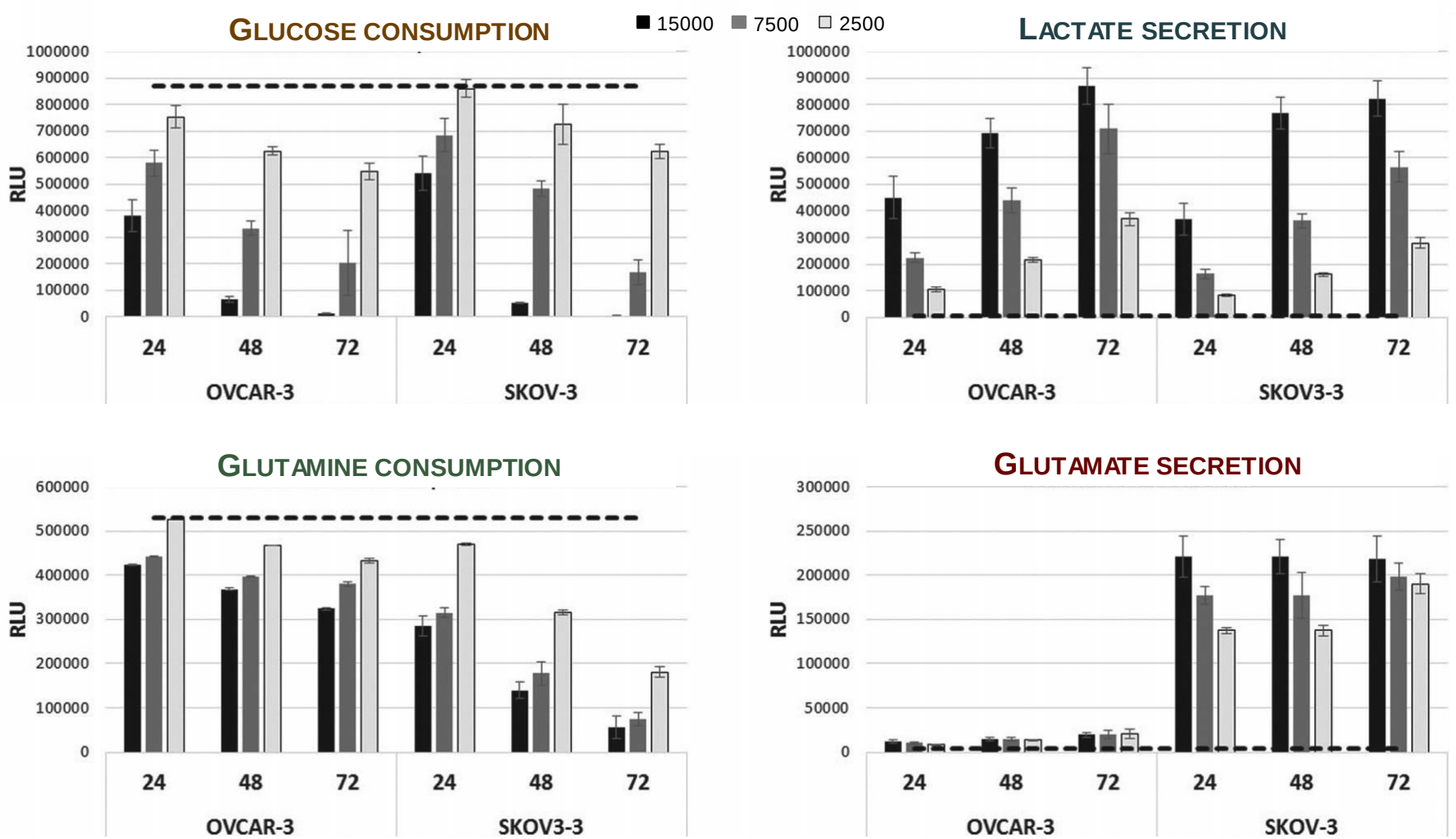




- Only 2–5 µl sample for measurement
- Multiple metabolites are easily measured from one sample
- Samples can be frozen and measured at the end of the experiment - kinetic information
- Investigate glycolysis, a central pathway for providing energy and precursors for biosynthesis
- Metabolic profiling of cancer cells, identify vulnerabilities for anti-cancer treatment

EASTPORT LIFESCIENCE

Multiple Metabolites Offer a More Complete Image



Adapted from Leippe, D *et al.* (2017), SLAS Discov. 22(4):366-377

EASTPORT LIFESCIENCE

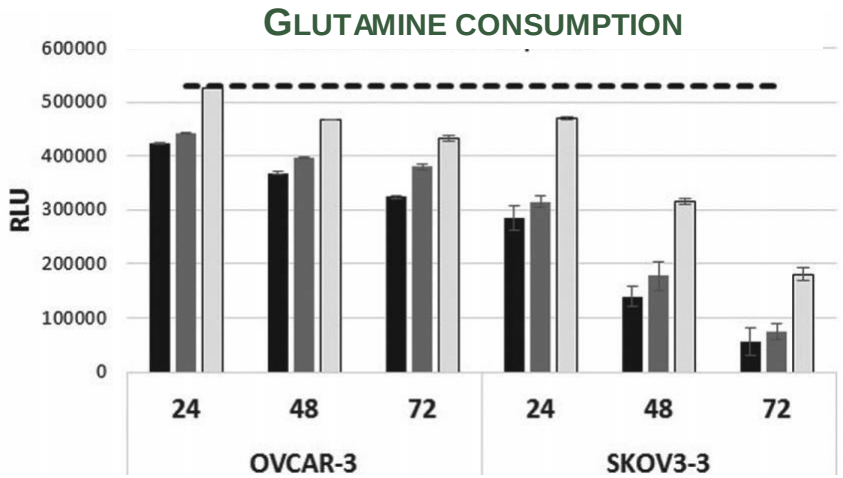
Multiple Metabolites Offer a More Complete Image

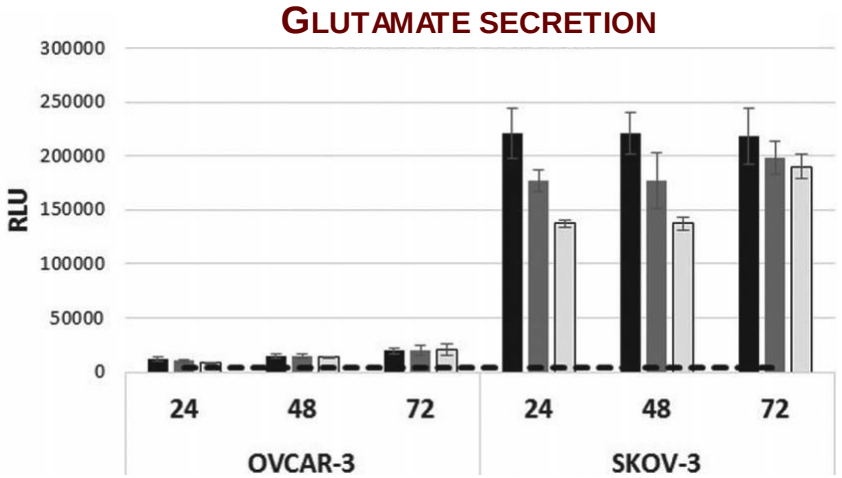
OVCAR-3

- Moderate glutamine consumption
- Low glutamate secretion
- Low invasiveness

SKOV-3

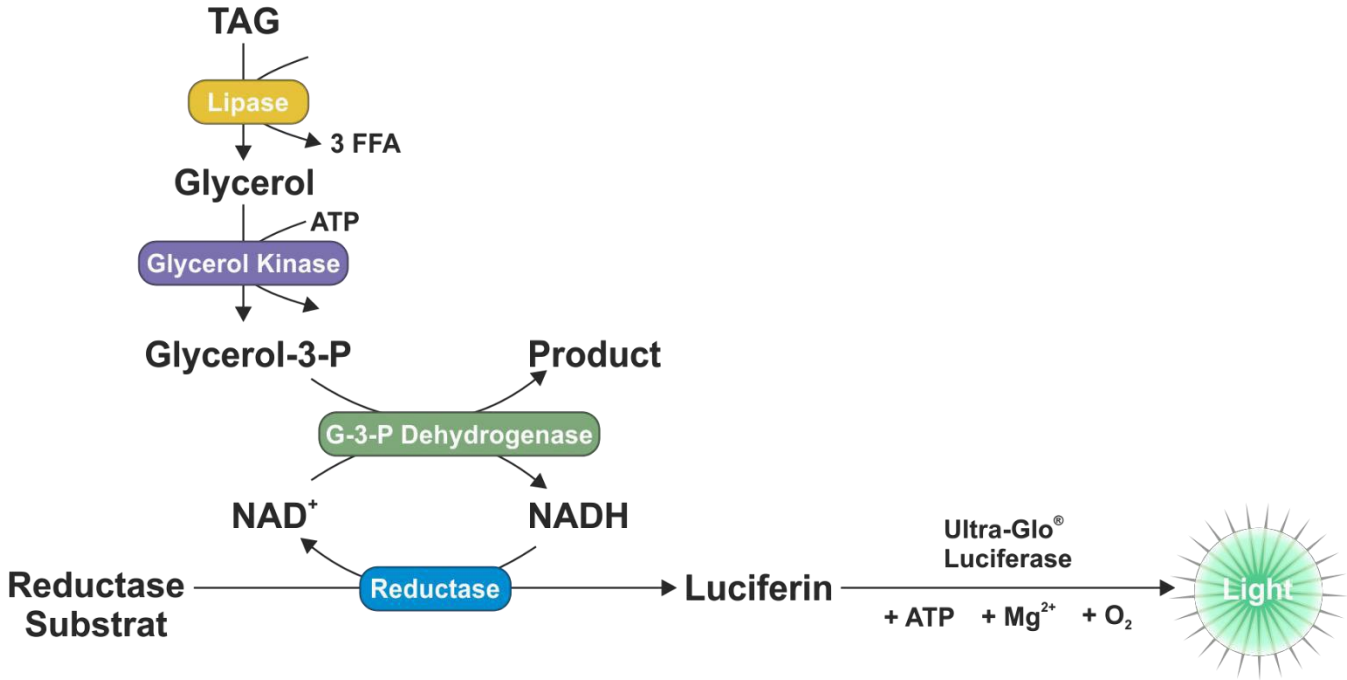
- High glutamine consumption (Glutamine “addicted”)
- High glutamate secretion
- Highly invasive





Adapted from Leippe, D *et al.* (2017), SLAS Discov. 22(4):366-377

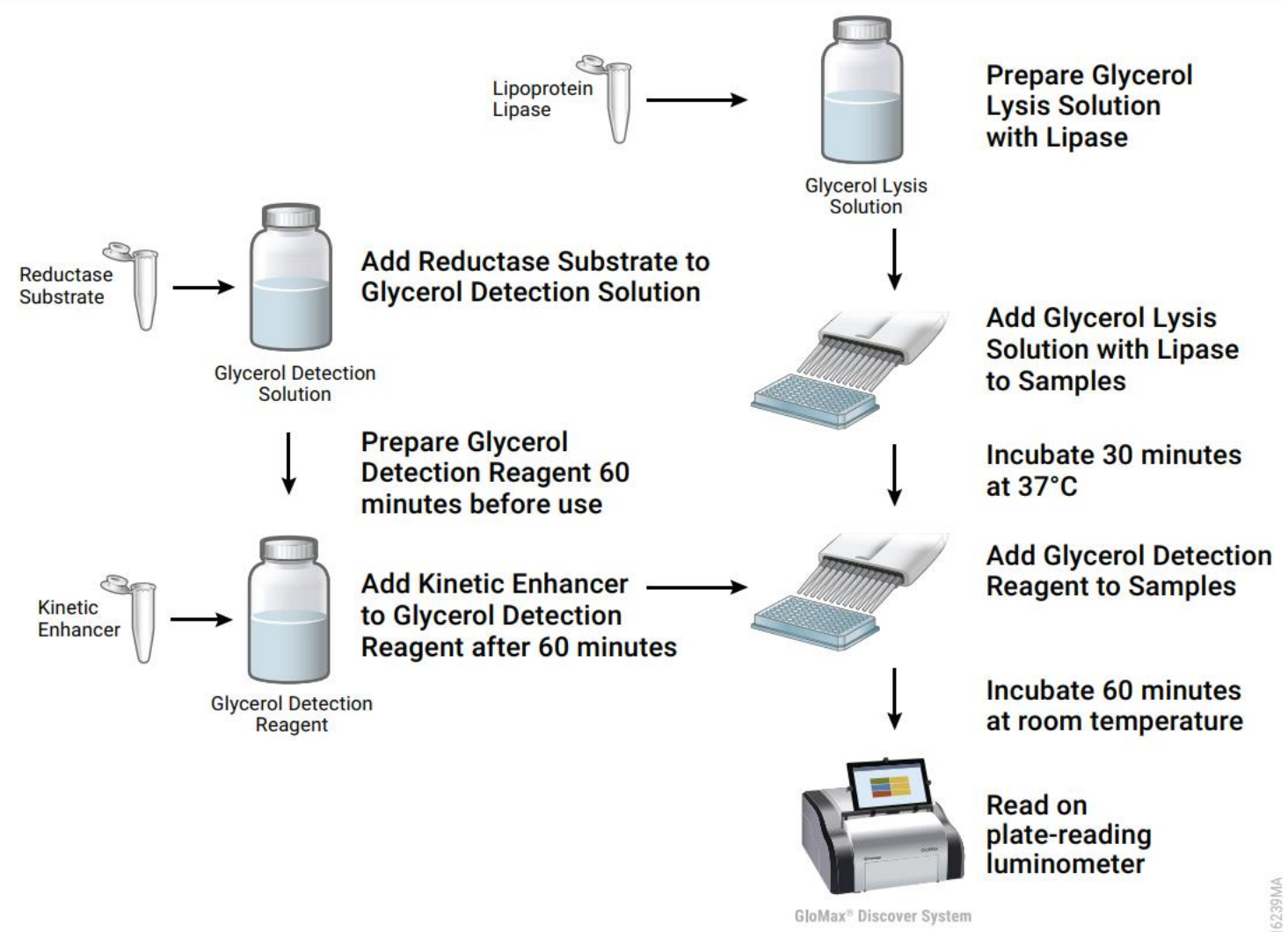
Glycerol/TAG Assay - Principle



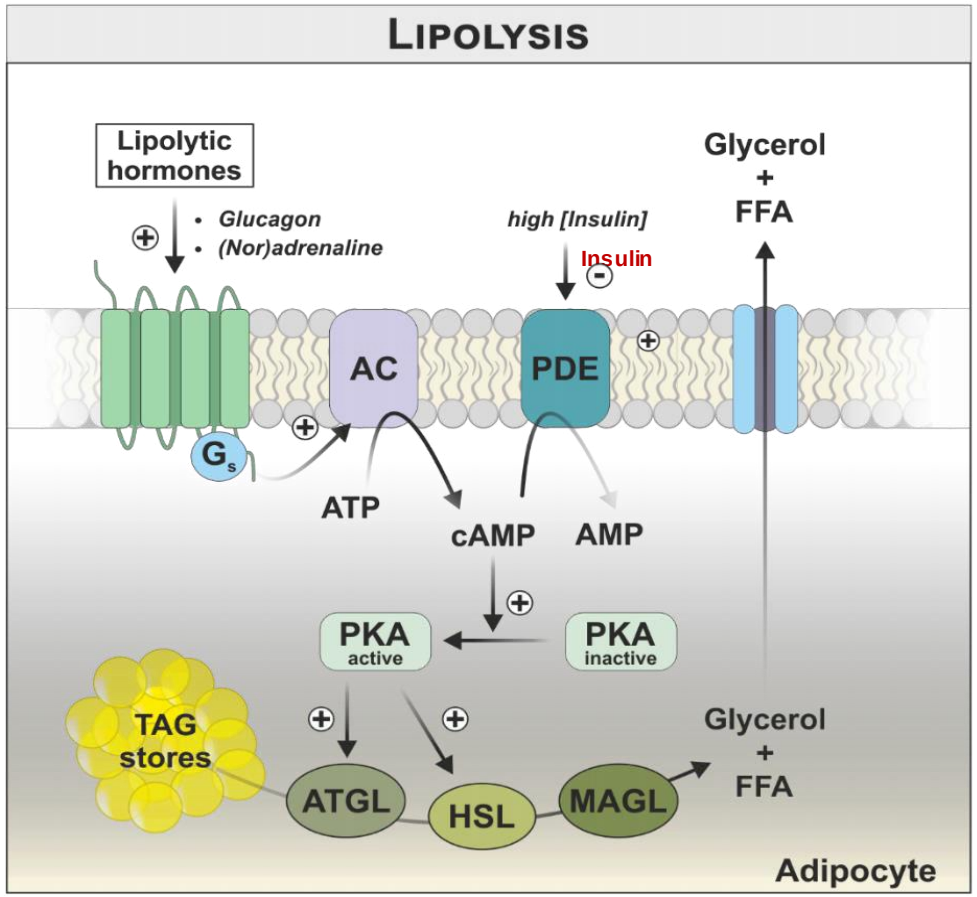
Benefits

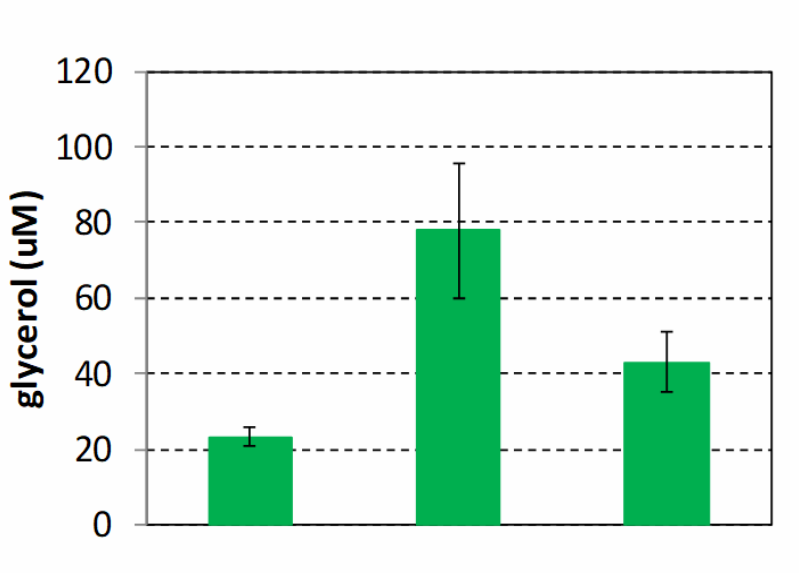
- Quantitative results
- No extraction steps, only cell lysis
- BL assay = high sensitivity
- Simple and fast protocol

Glycerol/TAG Assay – Experimental Workflow



Stimulation and Inhibition of Lipolysis





Isoproterenol 25 nM	-	+	+
Insulin 150 nM	-	-	+

- 3T3L1-MBX fibroblasts plated at 20,000 cells/well and differentiated for 2 weeks to form adipocytes
- Treatment with Isoproterenol and Insulin for 90 min

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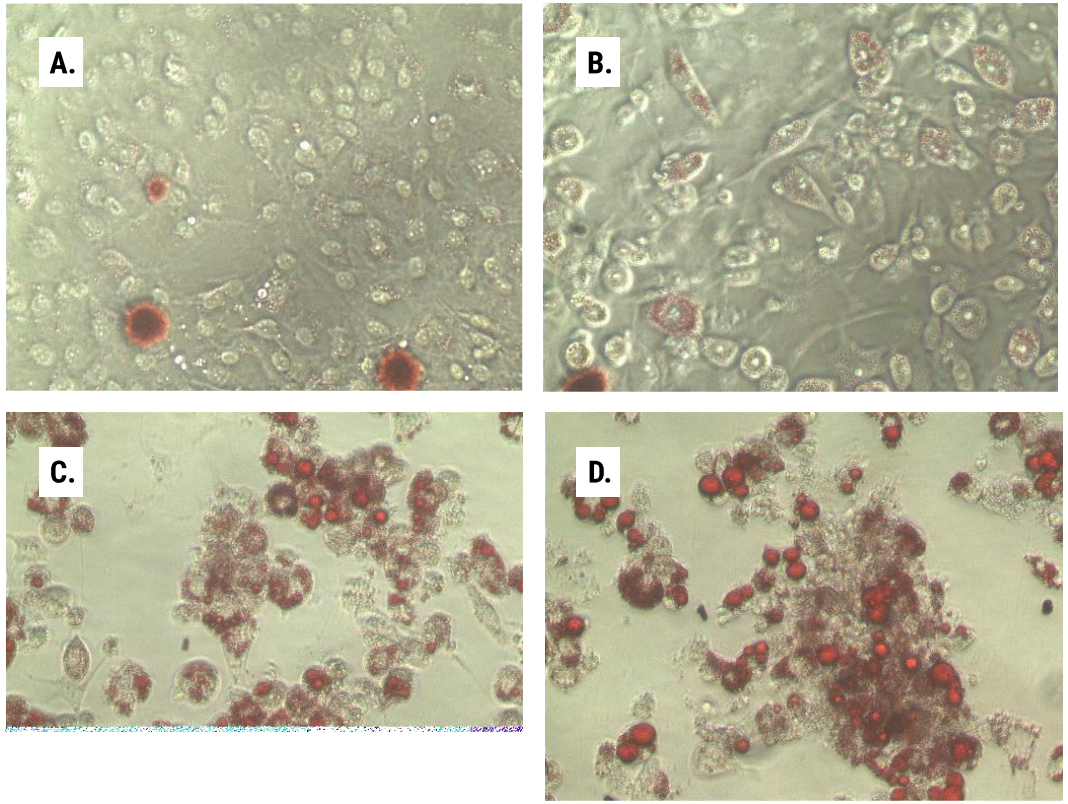
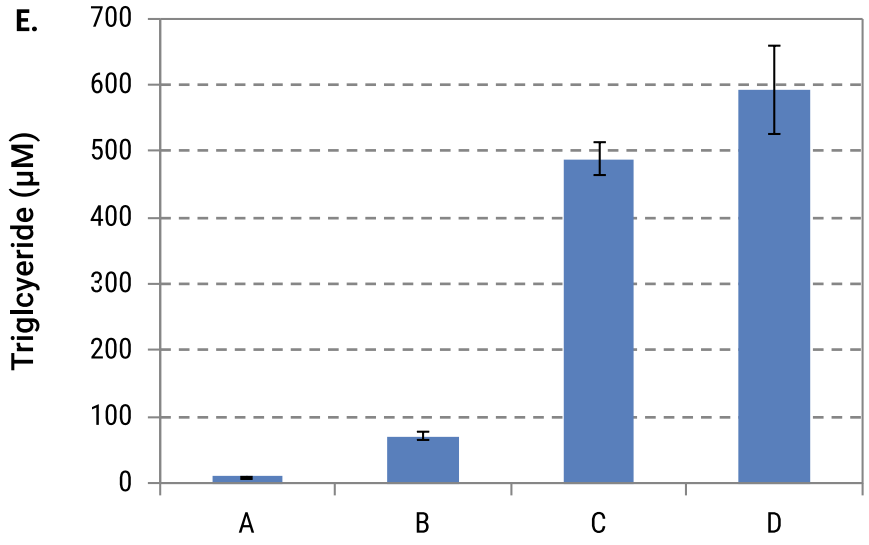
- Quantification of lipogenesis in differentiated 3T3L1-MBX fibroblasts
- Lipid content observed at days 5, 12, 14 and 21 of differentiation (A-D respectively)
- The cells were either stained with Oil Red O dye or the lipid content was measured by the Glycerol/TAG-Glo assay from Promega
- Simple quantification and complementary information to microscopic techniques and flow cytometry





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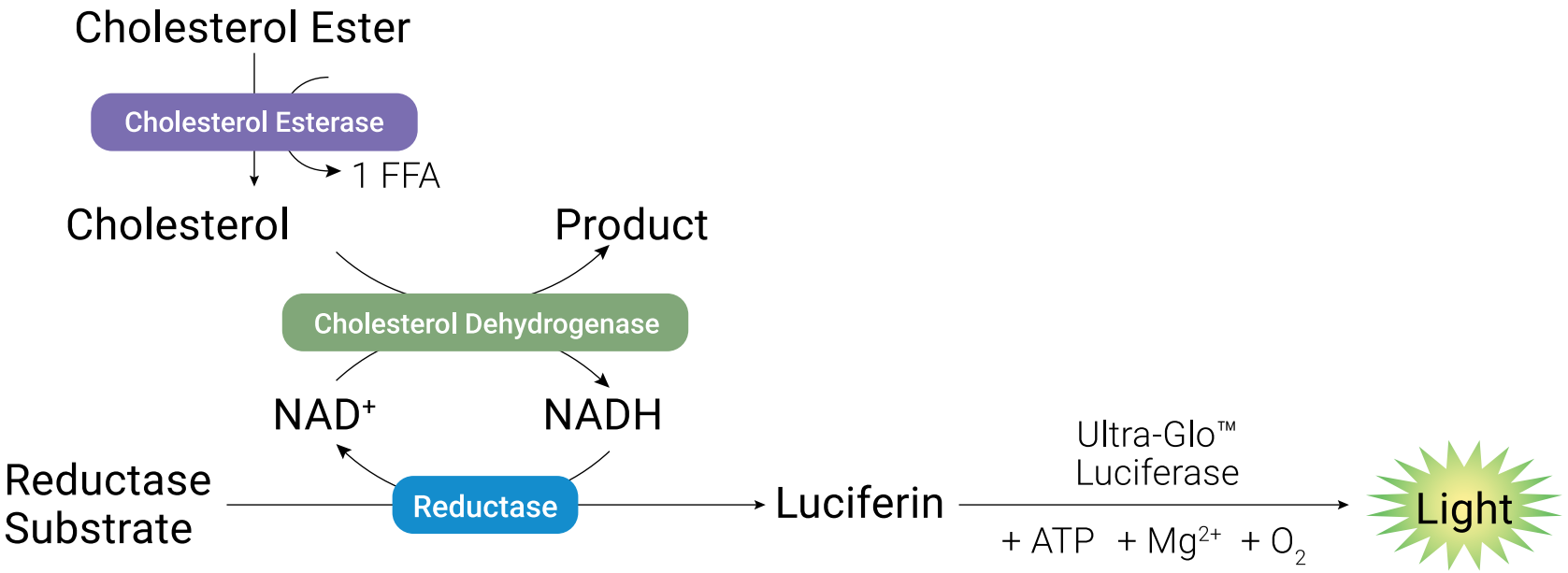
Cholesterol/Cholesterol Ester Assay

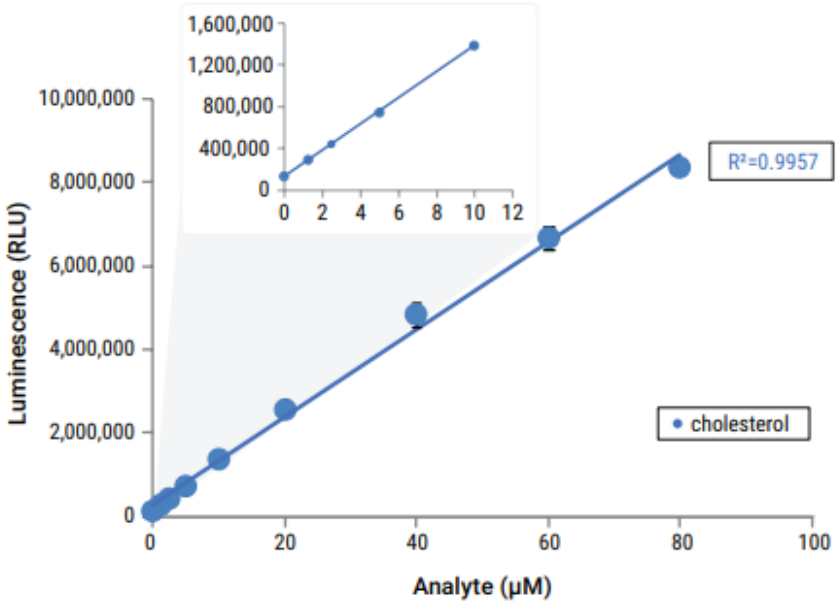


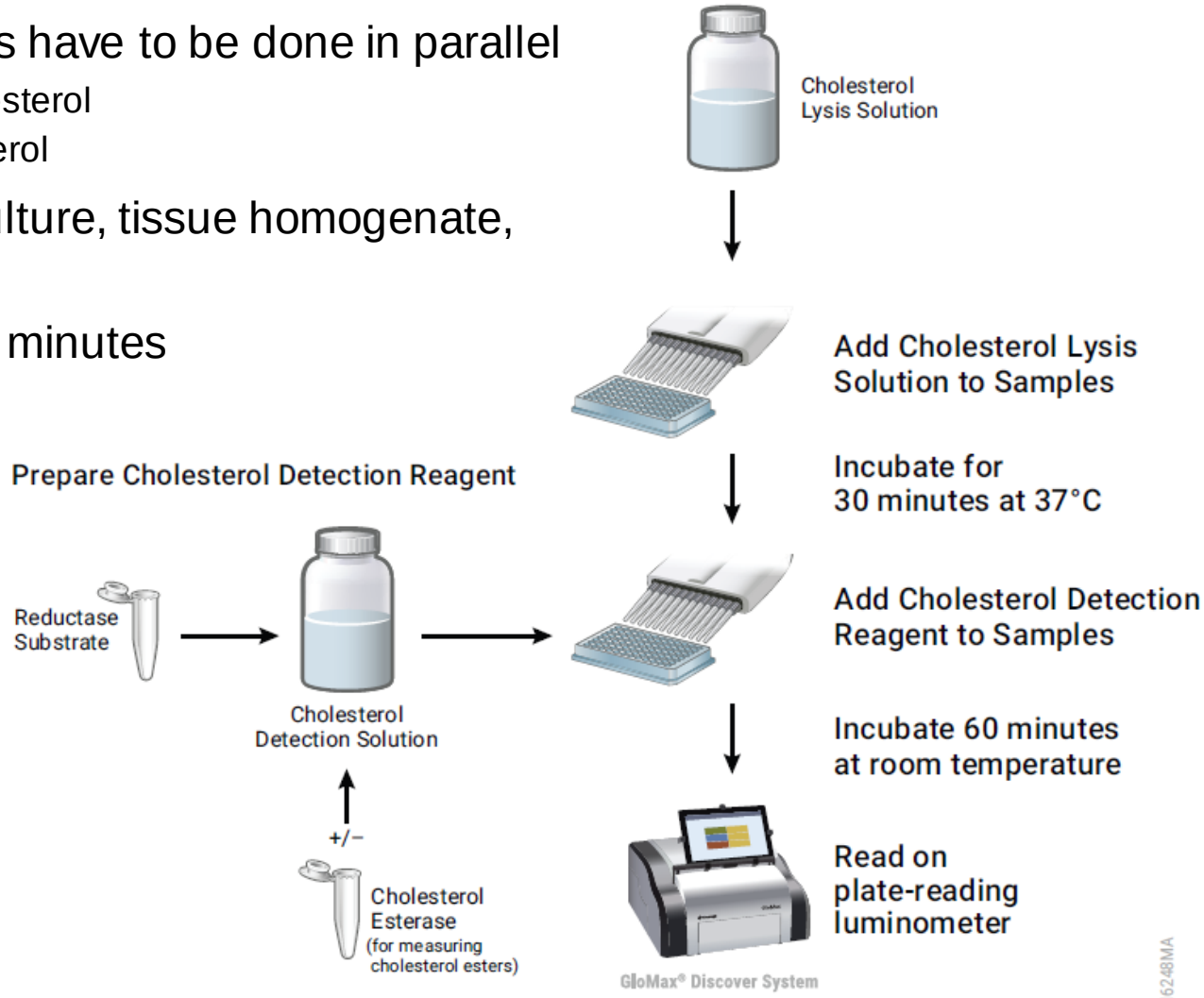
Benefits

- Quantitative measurement
- No extraction steps, only cell lysis
- BL assay = high sensitivity
- Simple and quick workflow

Cholesterol Assay - Protocol

- Measures cholesterol – two measurements have to be done in parallel
  - Sample without cholesterol estarase – free cholesterol
  - Sample with cholesterol esterase – total cholesterol
- Add lytic buffer to the sample (2D or 3D culture, tissue homogenate, serum), incubate at 37 °C
- Add detection reagent and incubate for 60 minutes
- Detect bioluminescence





Instruments – Features and Configurations

GloMax® Discover

6-, 12-, 24-, 48-, 96- and 384-well

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

GloMax® Explorer

6-, 12-, 24-, 48-, 96- and 384-well

- ✓ Heating
- ✓ Shaking
- ✓ Luminescence
- ✓ Fluorescence

Available Upgrades

- ✓ Vis Absorbance
- ✓ UV/Vis Absorbance
- ✓ BRET / FRET

GloMax® Navigator

96-well

- ✓ Luminescence

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Primary Cells, Stem Cells & Media

Lonza

- Primary cells - over 150 human and animal cell types available
- Clonetics media a growth factors for wide spectrum of primary cells
- Stem cells together with media
- Blood and immune cells from vast collection of donors and sources
  - Specialized X-Vivo™ media

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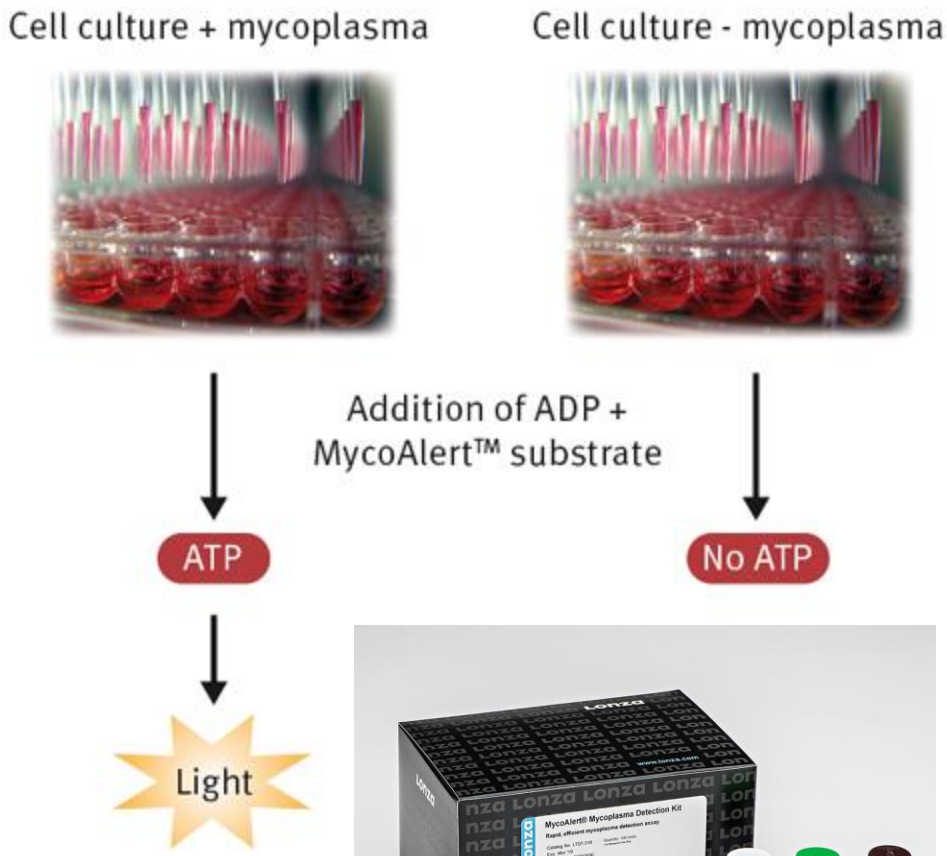
Mycoplasma Testing

- Mycoplasma = small bacteria lacking cell wall, not susceptible to penicillin and other antibiotics
- Widespread contamination in a variety of cell culture systems
- Size below 1 µm, hardly visible in optical microscope
- BL MycoAlert™ kit:
  - Mycoplasma from cell culture supernatant are lysed and their enzymes released into the medium
  - Enzymes convert added ADP to ATP
  - ATP is processed by firefly luciferase from the detection reagent and in case of contamination bioluminescence is produced



High Quality Cell Culture Media and Sera

- German company established in 2013
- Specialises on the production of high quality sera and cell culture media and reagents
- Possibility of custom manufacturing from 20 liters

Sera
- FBS
- Other bovine and animal sera
- Human sera

Cell Culture Media
- Classic liquid media
- Classic powdered media
- Special culture media
- Cryopreservation

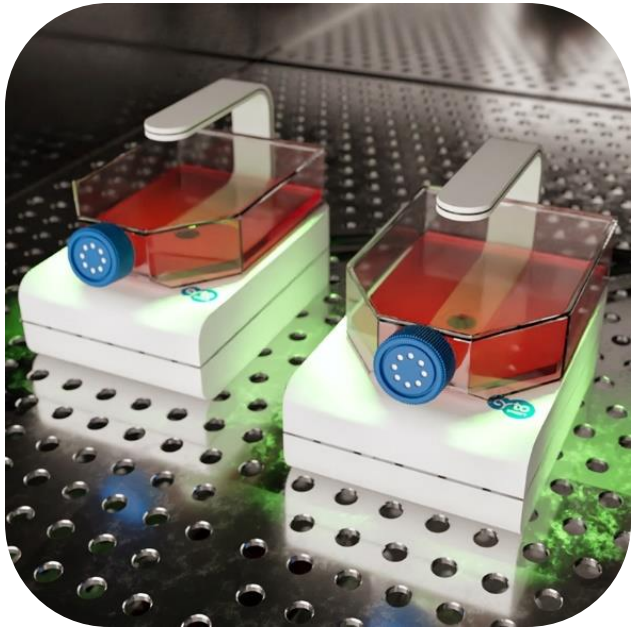
Cell Culture reagents
- Supplements and additives
- Antibiotics
- BSA
- Trypsin
- Cell separation

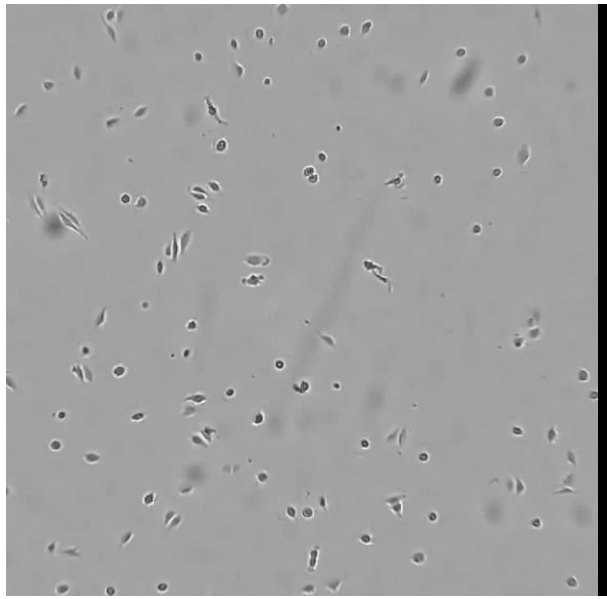
Balanced Salt Solutions
- Liquid buffers
- Powdered buffers

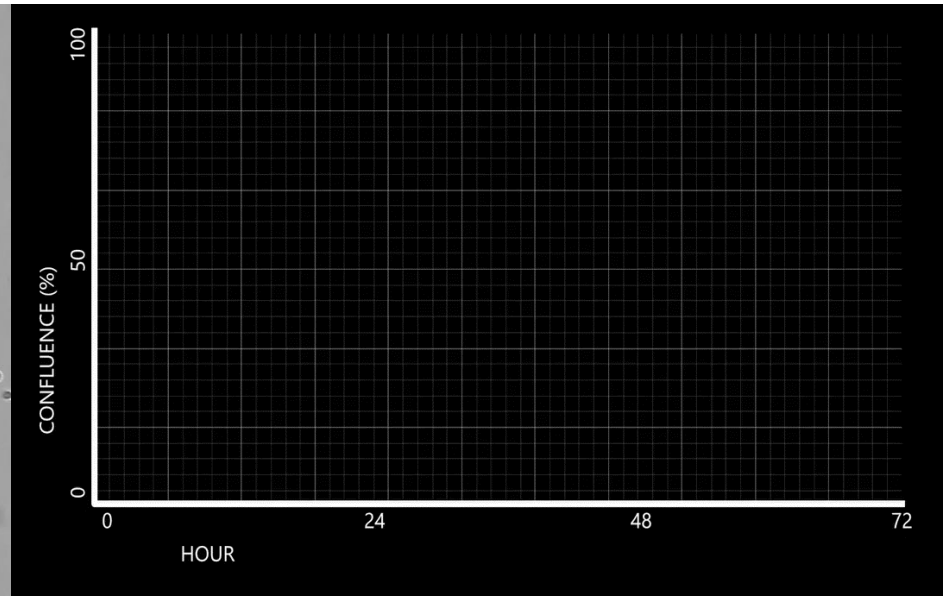
Diagnostics
- Virology media
- Cytogenetics

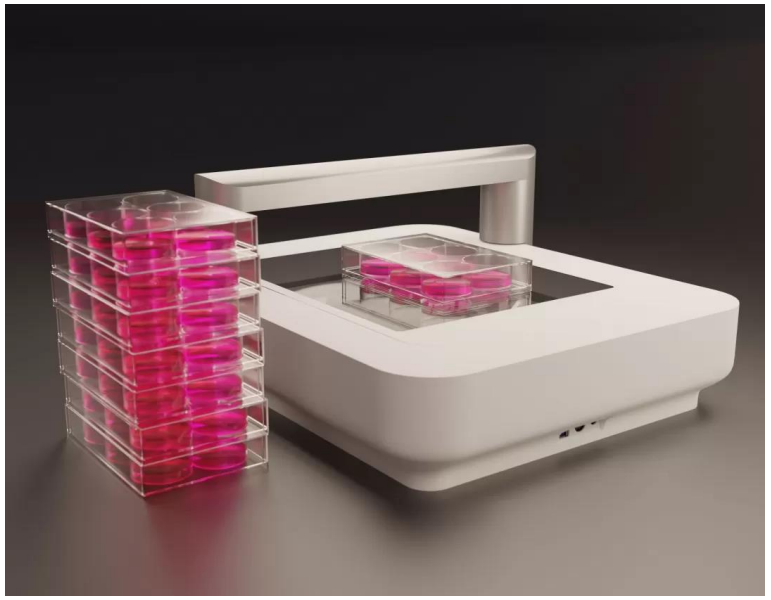
Label-Free Cell Monitoring

- Live-cell monitoring of cells in individual culture vessels (Lux microscopes) or 6 to 384 well plates (Omni microscopes)
- Compact microscopes placed directly in the incubator
- Confluency measurements, scratch assays, colony counting
- Fluorescence versions – fluorescent object counting









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Thank you for your attention!

Questions?

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