Cell-Based Assays to Detect Mechanisms of Toxicity

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Overview

Real-time assays for multi-well plates:

- cell viability
- cell death

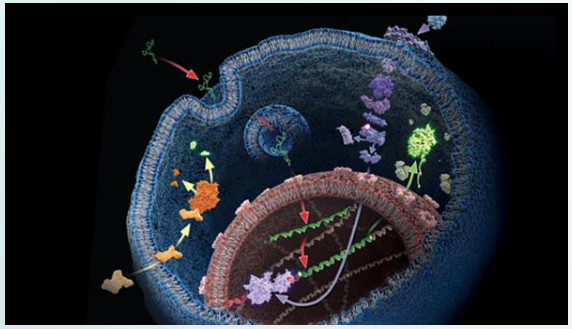
Cytotoxicity assays:

- biomarkers of apoptosis, necrosis or cell stress

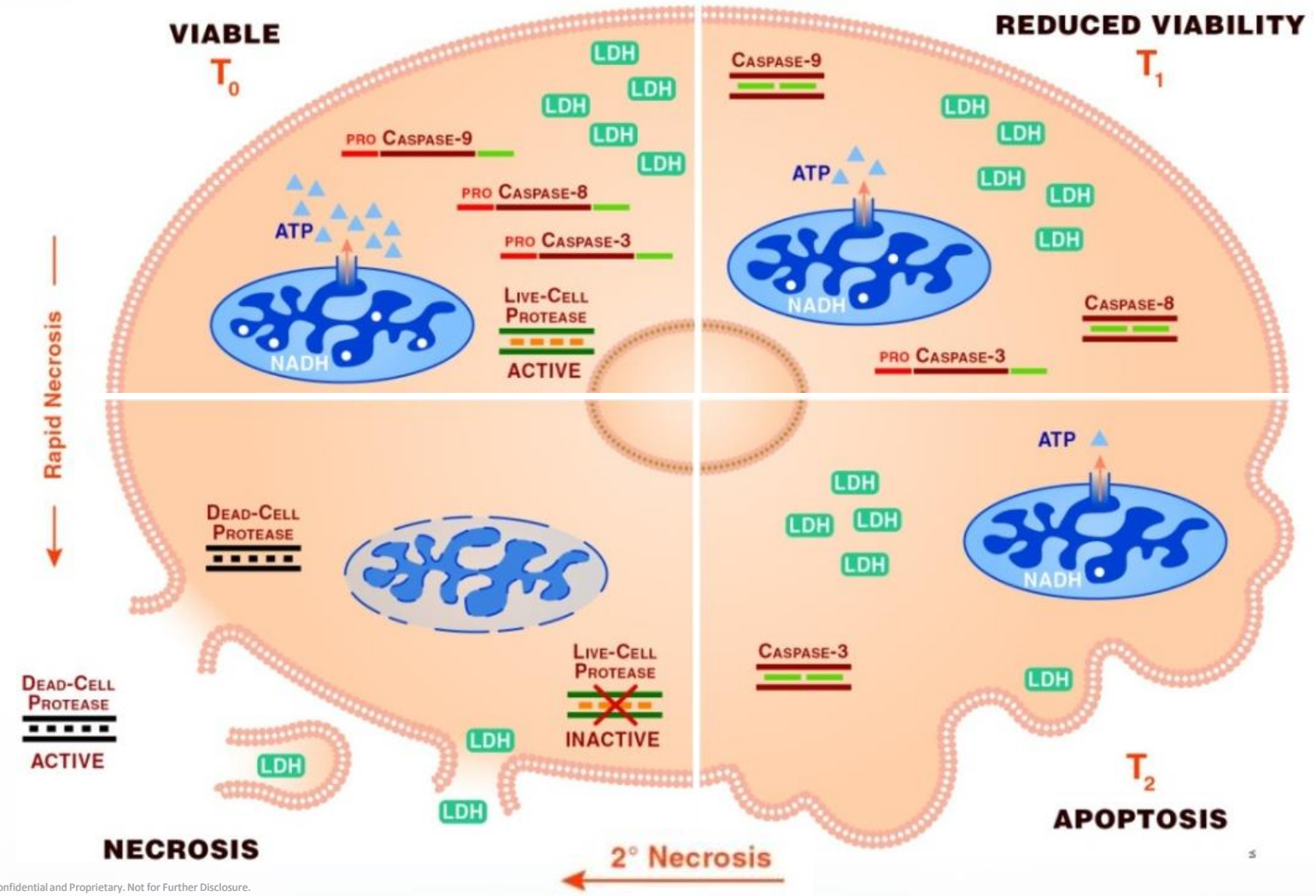
Multiplexing:

- advantages and examples

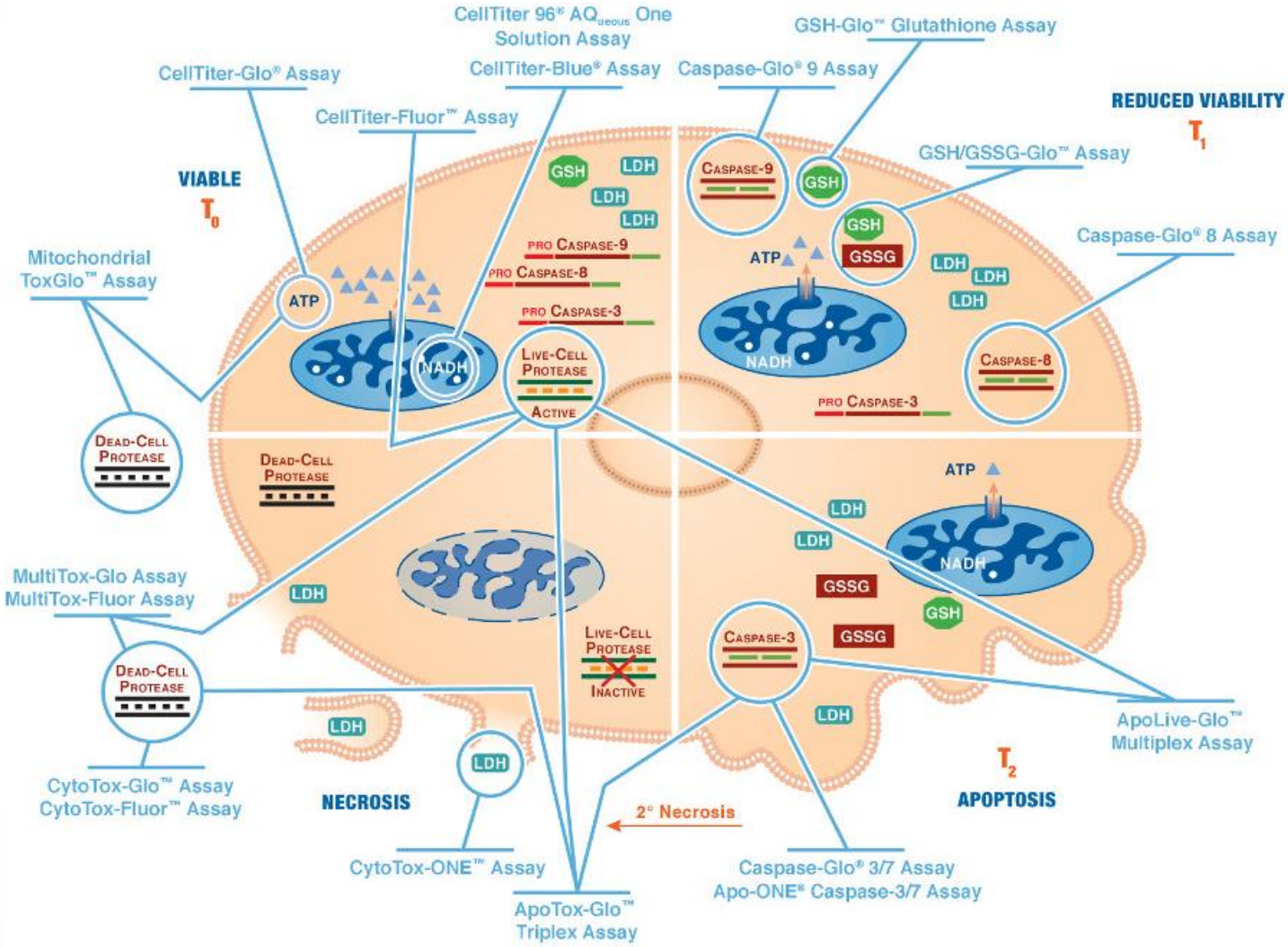
Introduction



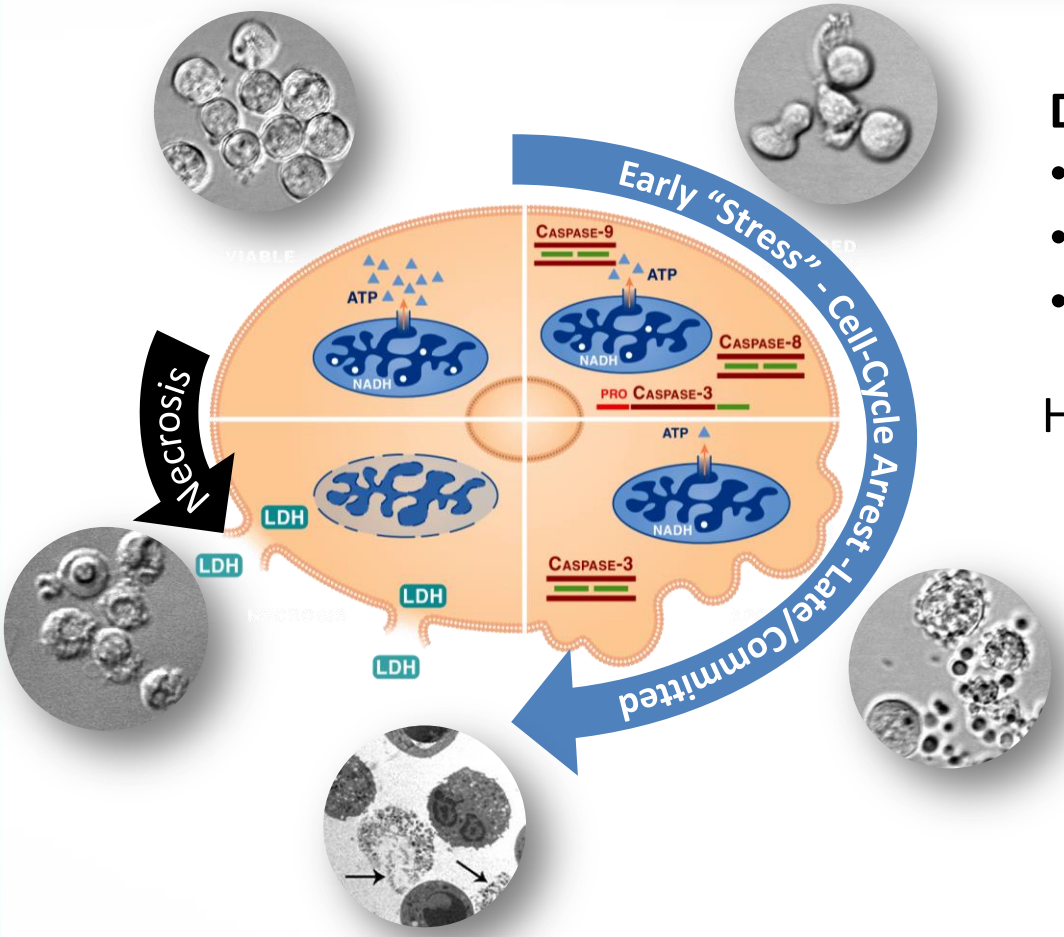
Monitoring Cytotoxicity



Monitoring Cytotoxicity



Cytotoxicity and Biological Complexity



Did the treatment affect viability:

- yes/ no?
- how?
- When?

How **potent** was the treatment?

Cytotoxicity depends on:

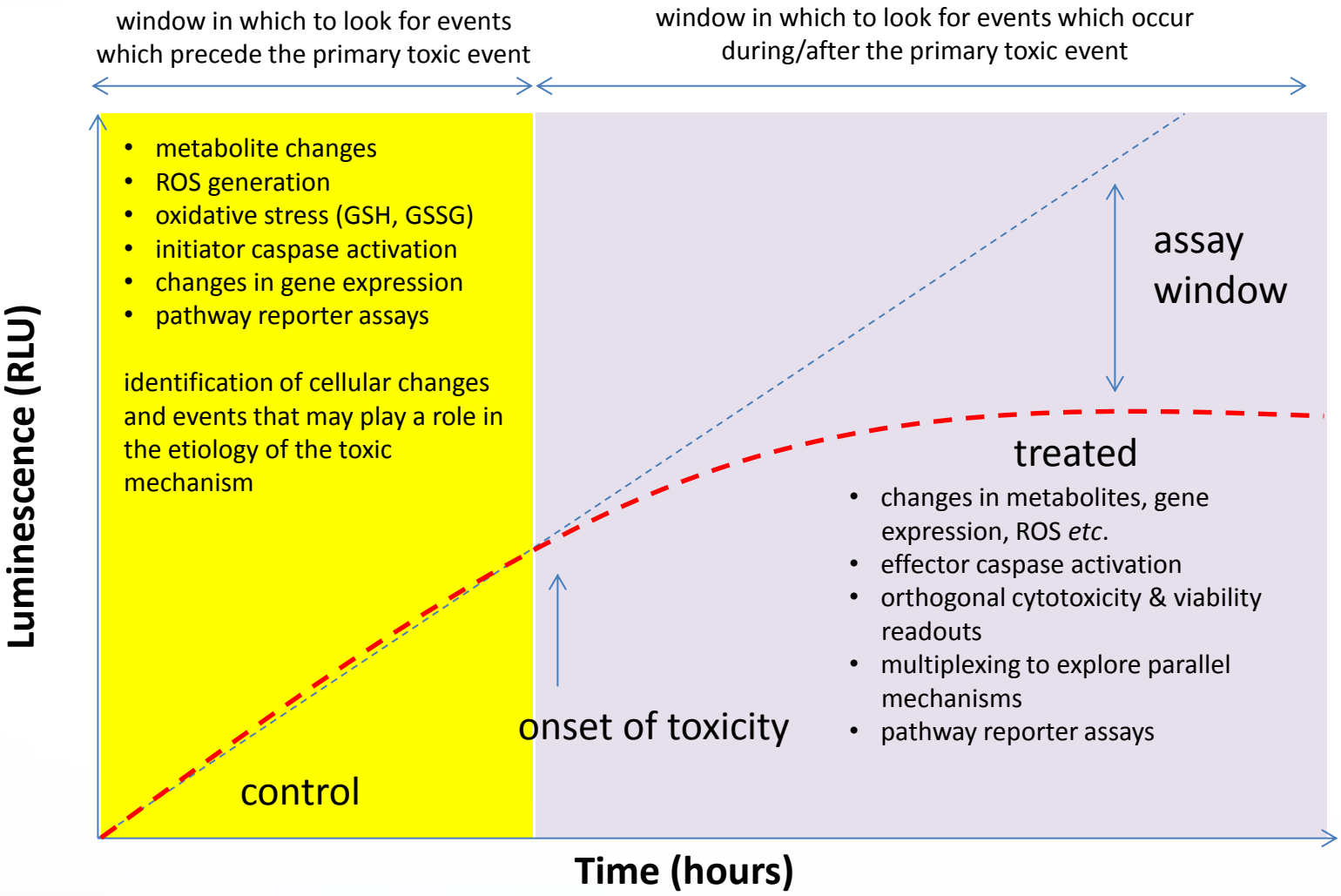
- Dosage
- Exposure time
- Mechanism of action
- Cell type and susceptibility

Cytotoxicity Determination

Cellular response to treatment	Viability	Cytotoxicity	Apoptosis
Proliferation	↑	→	→
Cell cycle arrest/ senescence	→	→	→
Cytotoxicity (necrosis)	↓	↑	→
Cytotoxicity (apoptosis)	↓	↑	↑

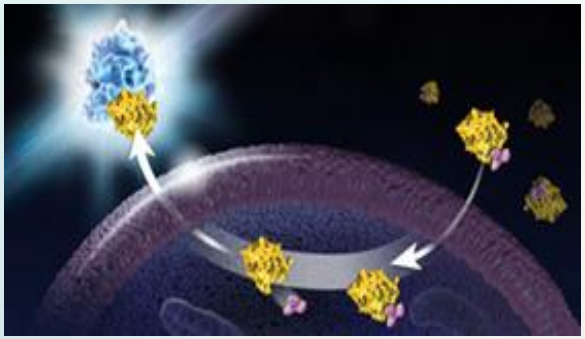
No single parameter can fully characterise cytotoxicity

Timing of Cytotoxicity is Critical!

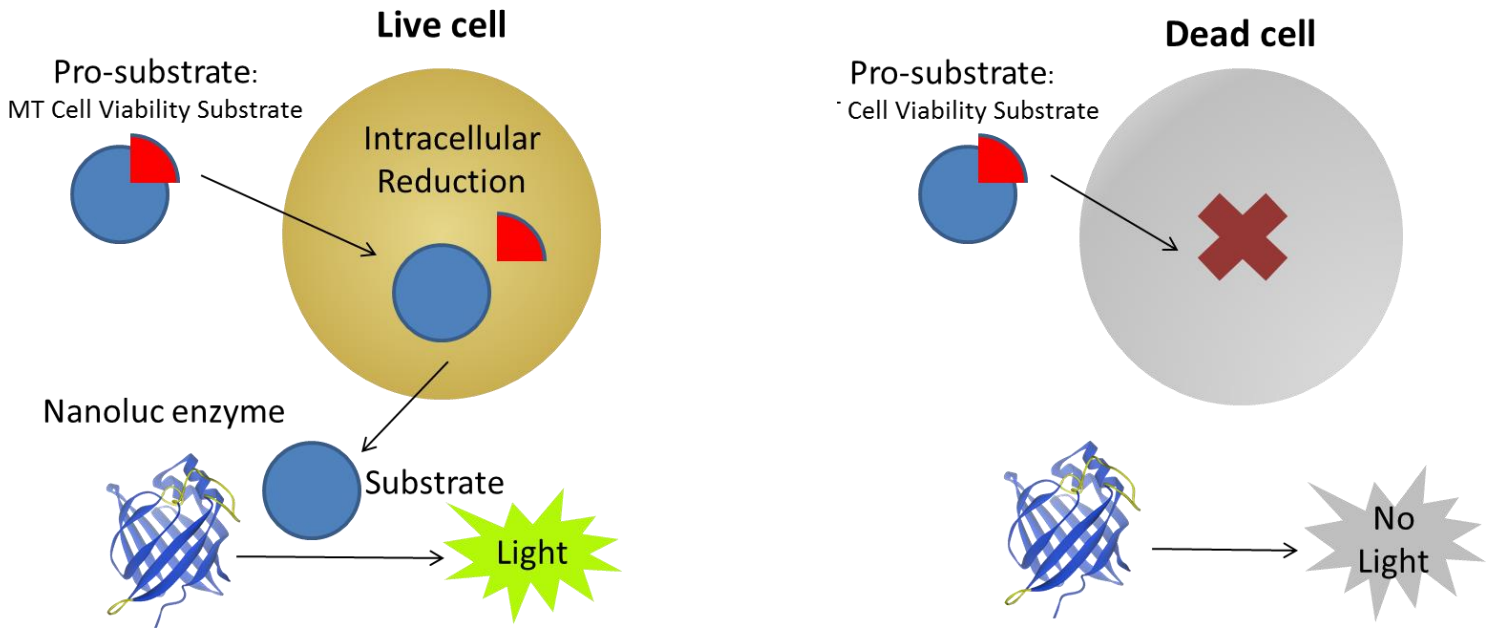


Real-Time Assay:

RealTime-Glo™ MT Cell Viability Assay

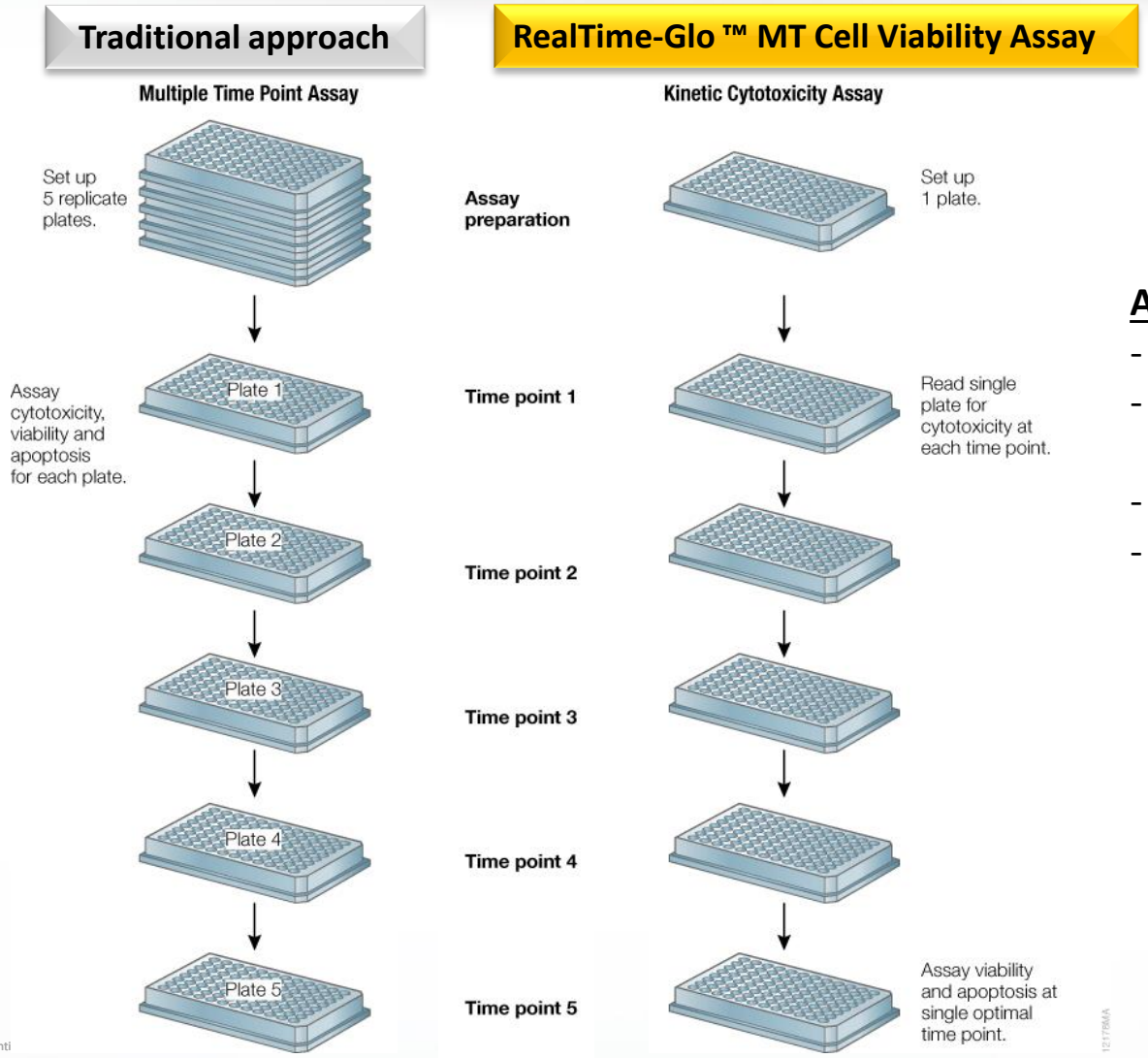


RealTime-Glo™ MT Cell Viability Assay Measures Reducing Potential of Cells



- NanoLuc® enzyme is present outside of the cells
- Pro-substrate enters the cell and is reduced by the cell to form the NanoLuc® substrate
- The NanoLuc® substrate diffuses from the cell and is rapidly used by NanoLuc® to produce light

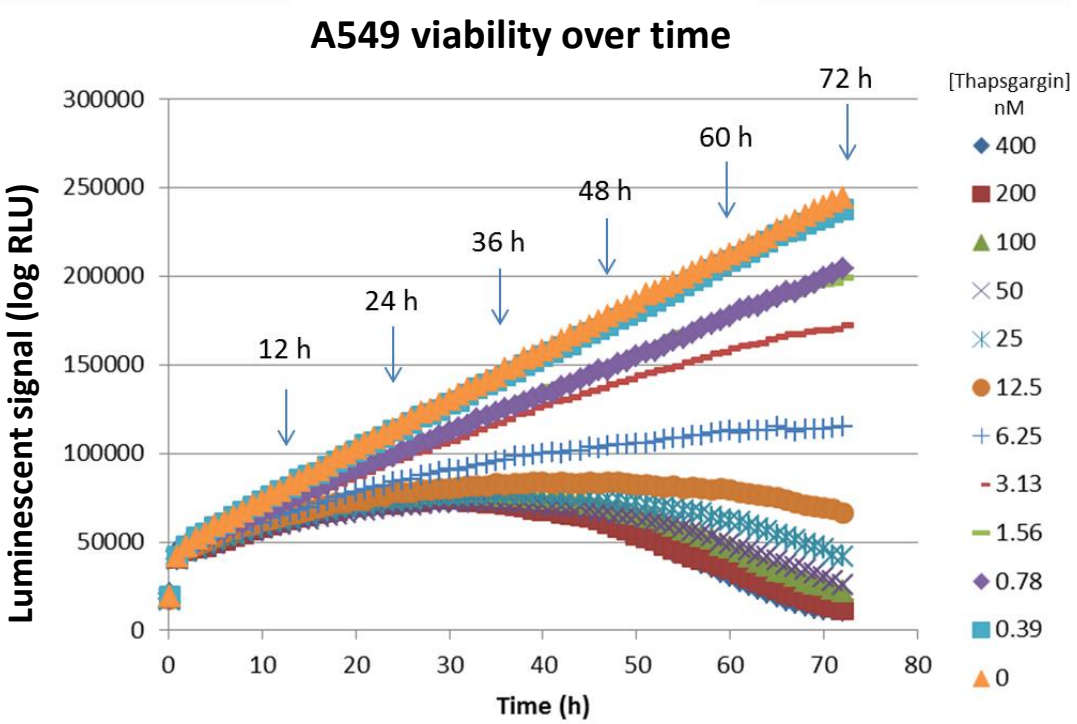
Why is RealTime-Glo™ MT Cell Viability Assay Different?



Advantages:

- Multiple measurements
- Reduced variation & data points
- Cost- effective
- Multiplexing

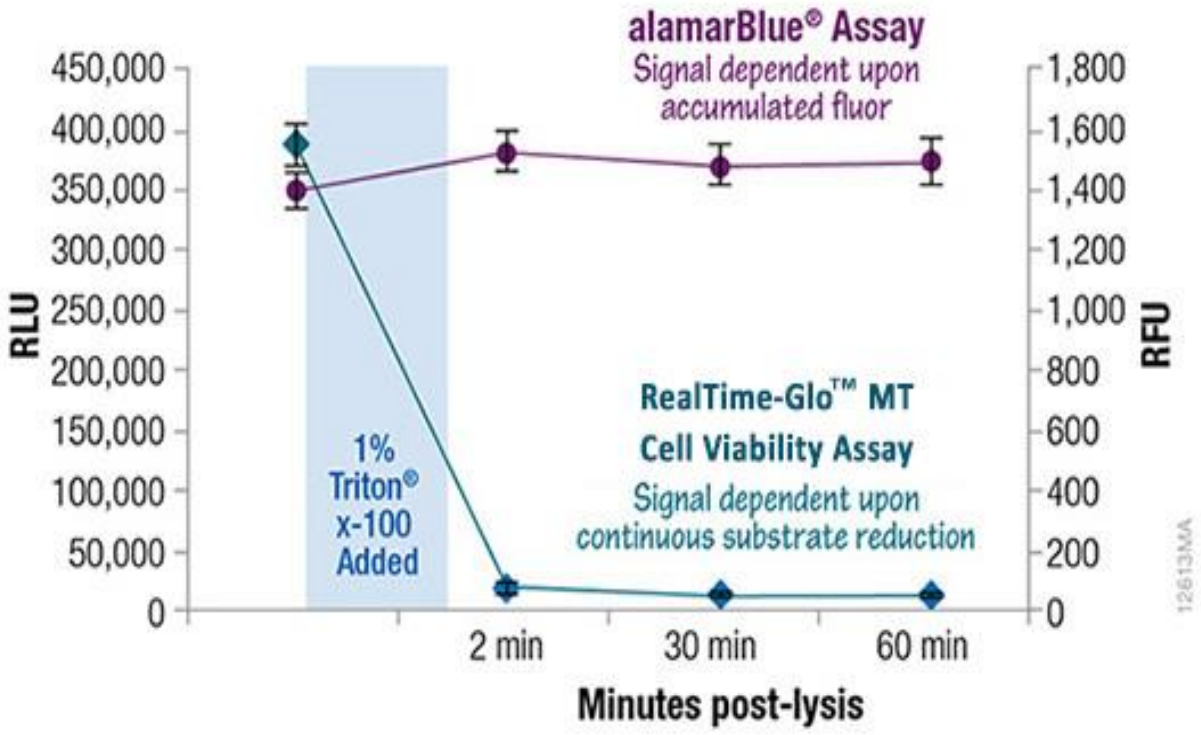
RealTime-Glo™ Measures Signal in Real-time



A549 cells were treated with thapsigergin dose curve. Luminescence measured continuously every 1h over 72h in Tecan M200 plate reader, with gas control module (37°C/5% CO_2).

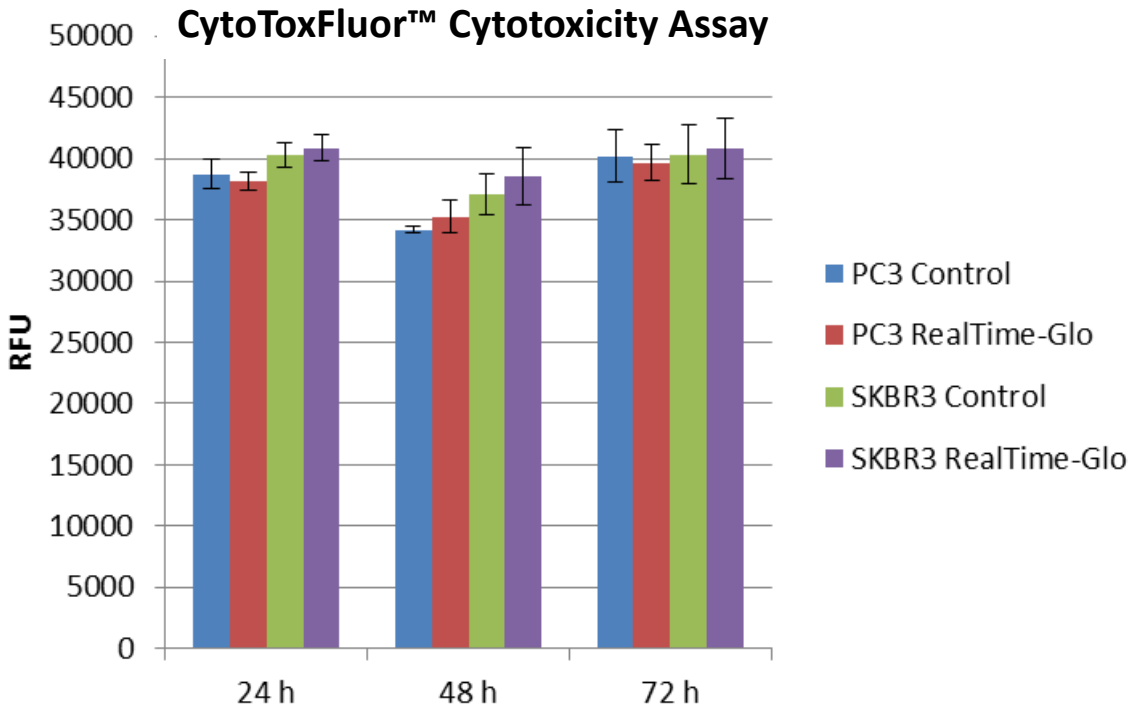
RealTime-Glo™ is More Responsive

RealTime-Glo™ vs. AlamarBlue



A549 cells treated with 1% Triton ®x-100. Monitored for 1h post- treatment.

RealTime-Glo™ Reagents are Not Toxic

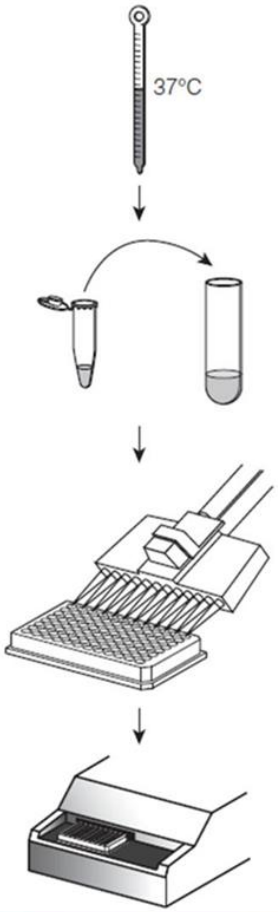


PC3 and SKBR3 cells were incubated in the presence or absence of RealTime-Glo™ reagents for 72h. Samples were tested for membrane integrity using **CytoTox-Fluor™ Cytotoxicity Assay**.

Different Assay Set up Methods

RealTime-Glo™ is flexible

Continuous Read Format, Addition at Seeding



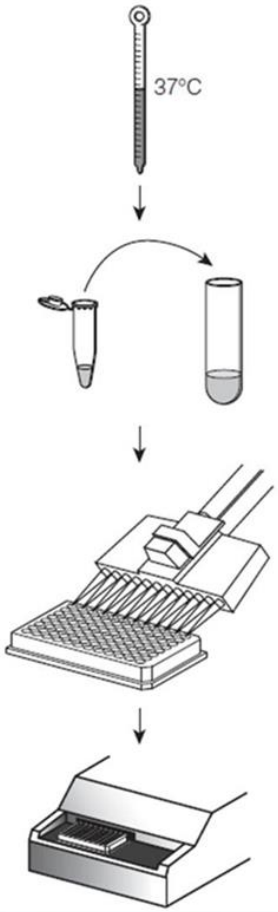
Thaw RealTime-Glo reagents in 37C water bath.
Harvest cells and adjust to desired density.

Transfer 10 µl of NanoLuc Enzyme and 10 µl of Metabolic Cell Viability Substrate per 5 ml of cell suspension.

Pipet RealTime-Glo/cell suspension into plate.
Add test compound at 2x concentration.
Incubate.

Measure luminescence

Continuous Read Format, Addition at Dosing



Thaw RealTime-Glo reagents in 37C water bath.

Transfer 10 µl of NanoLuc Enzyme and 10 µl of Metabolic Cell Viability Substrate per 5 ml of compound diluent.

Pipet RealTime-Glo/compound diluent into plate and perform serial dilutions. Pipet cell suspension into wells at 2x and incubate.

Measure luminescence

Endpoint Method, 2X or 5X Reagent Addition

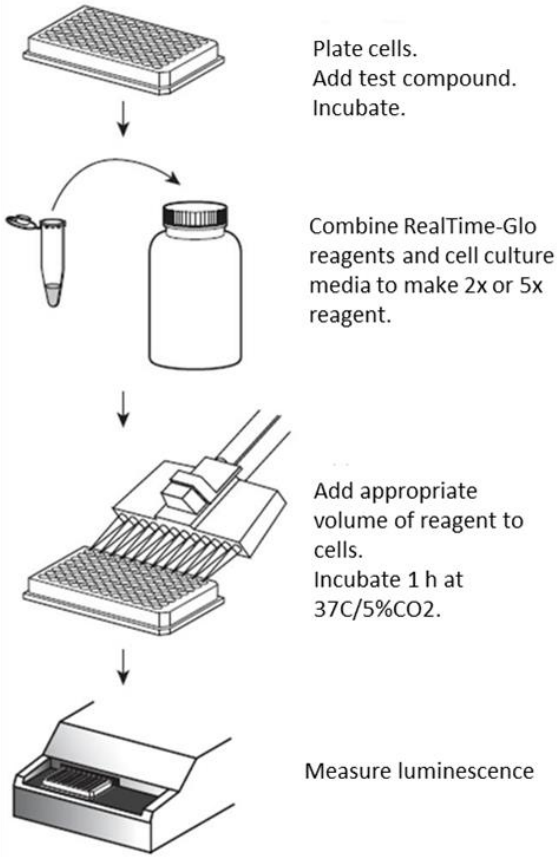


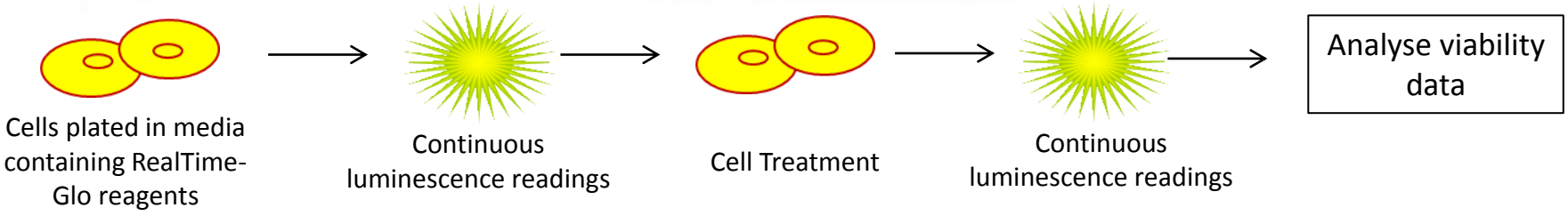
Plate cells.
Add test compound.
Incubate.

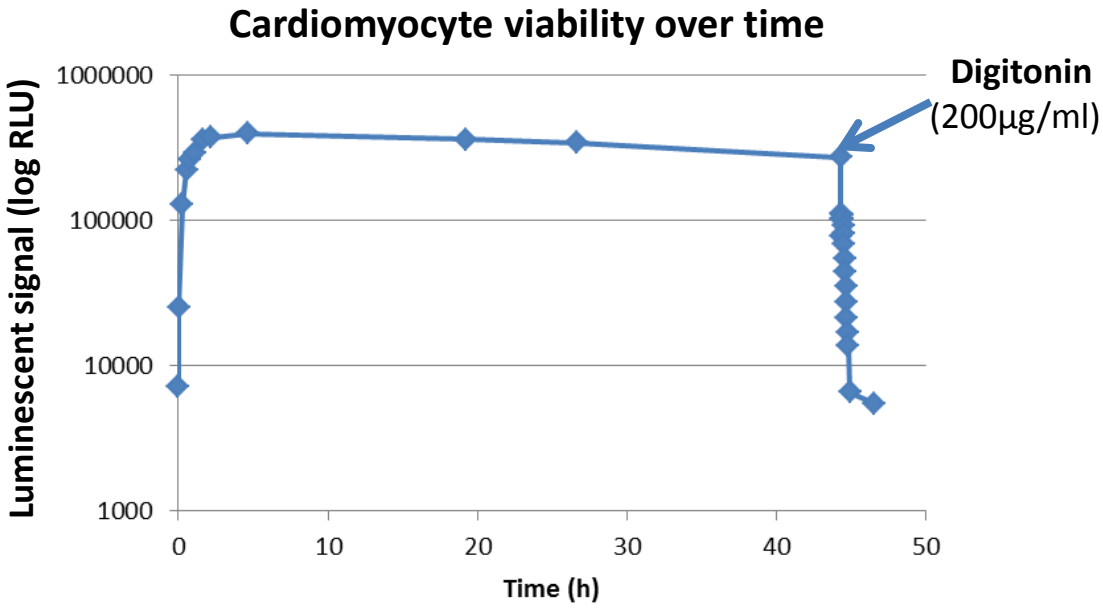
Combine RealTime-Glo reagents and cell culture media to make 2x or 5x reagent.

Add appropriate volume of reagent to cells.
Incubate 1 h at 37C/5%CO2.

Measure luminescence

Continuous Read Format and Responsiveness

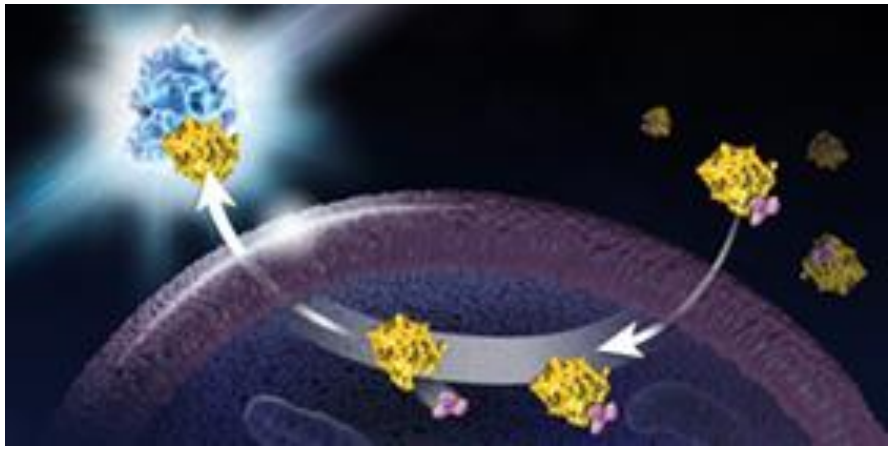




RealTime-Glo™ Summary

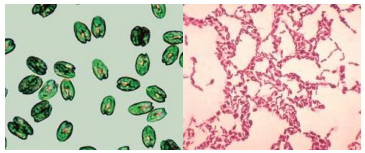
Features:

- **Real-time** measurements
- Superior **responsiveness**
- **Non- toxic**
- Cost-effective
- Reduces variation & data points
- **Multiplex** opportunities



Applications:

- Mammalian cells
- Bacteria (gram+ and gram-)
- Yeast and fungus
- Algae

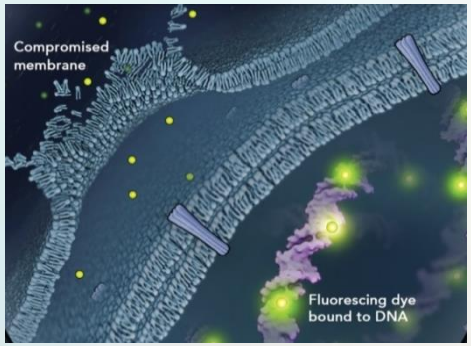


Kit components:

- NanoLuc® enzyme
- MT Cell Viability Substrate

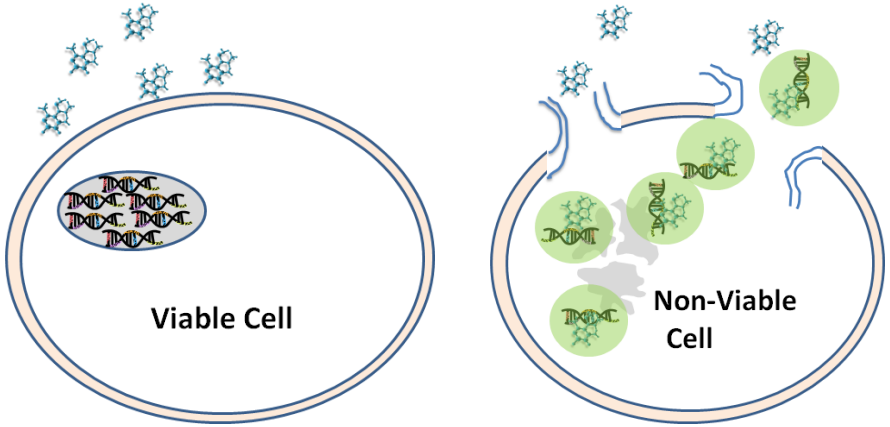
Real- Time Assay:

CellTox™ Green Cytotoxicity Assay



CellTox™ Green Cytotoxicity Assay

CellTox™ Green Dye

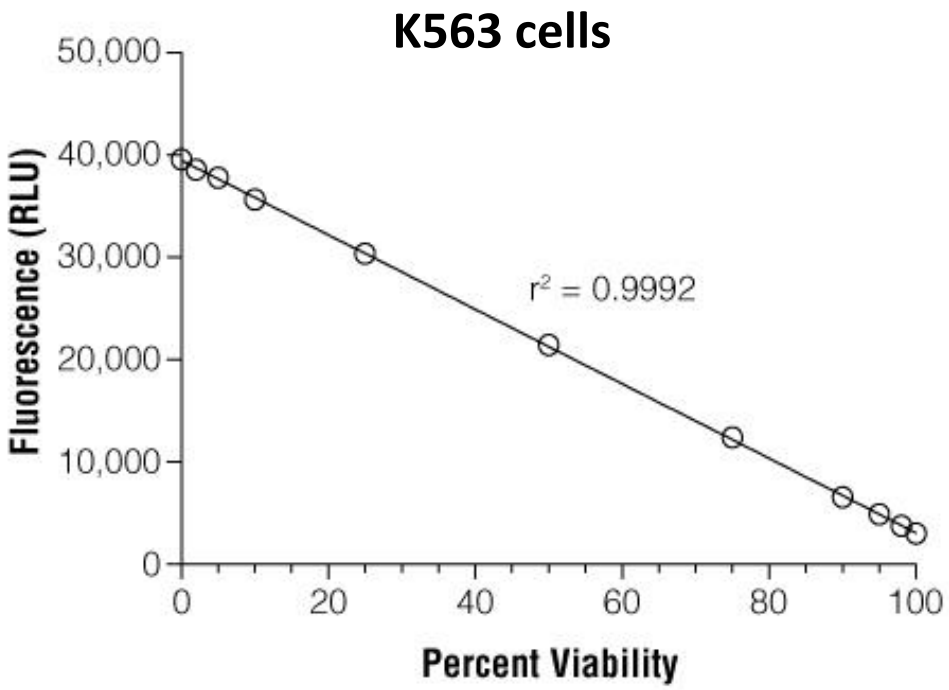


excluded dye = low fluorescence

non-excluded dye= high fluorescence

CellTox™ Green Dye spectra
Ex: 512nm
Em: 523nm
Detection: GloMax Multimode 'blue filter'

CellTox™ Green Signal is Directly Proportional to Number of Dead Cells

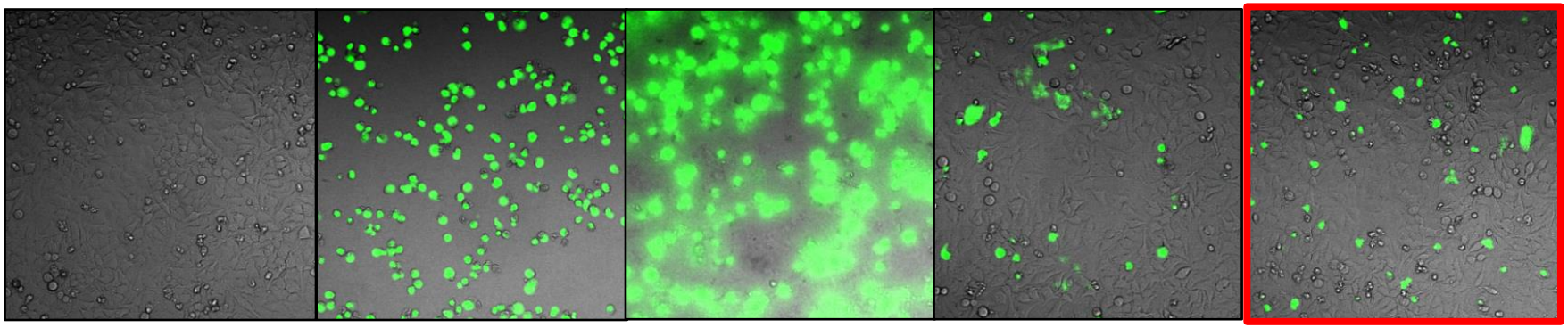


To create a range of prototypical cytotoxic response a population of non-viable K562 cells was mixed with a population of viable K56 cells at various ratios to represent 0- 100% viability. Each ratio contained a total of 10K cells/ 100µl/ well in 96- well plate.

CellTox™ Green is Not Toxic

Fluorescence (imaging)

HeLa Cells (72h incubation)

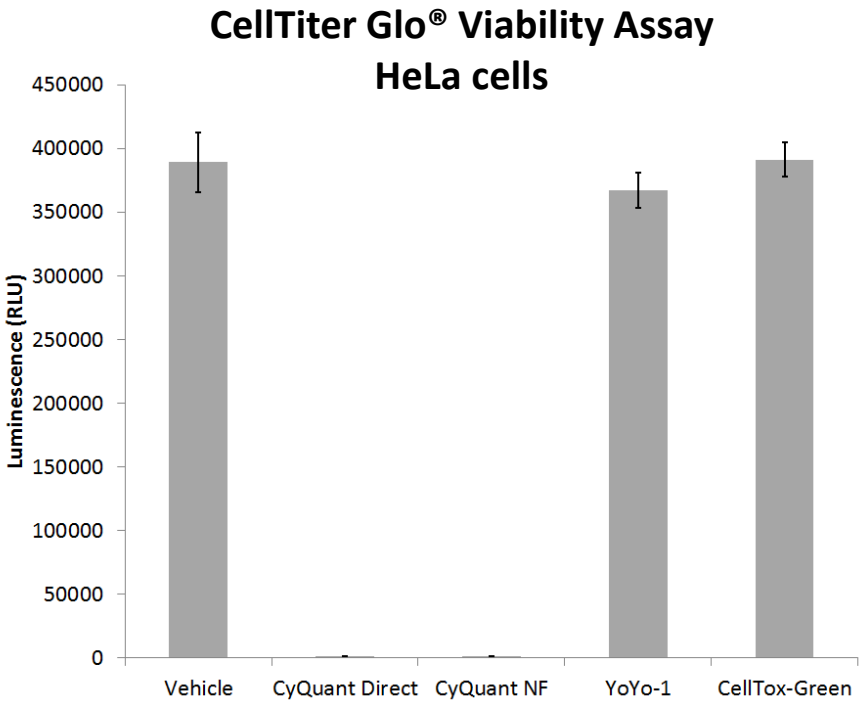


Control (DMSO)	CyQuant Direct ® Life Technologies	CyQuant®NF Life Technologies	YoYo®-1 Life Technologies	CellTox™ Green

Dyes diluted 1:500 in DMEM and added to **HeLa cells.** Images collected after 72h of dye exposure with PMT= 575.

CellTox™ Green is Not Toxic

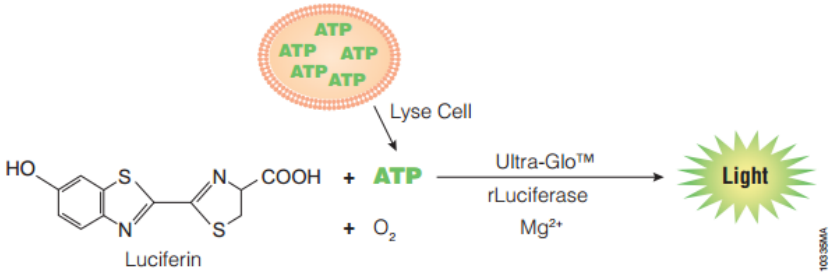
Luminescence : CellTiter-Glo® Viability Assay



CellTiter-Glo®

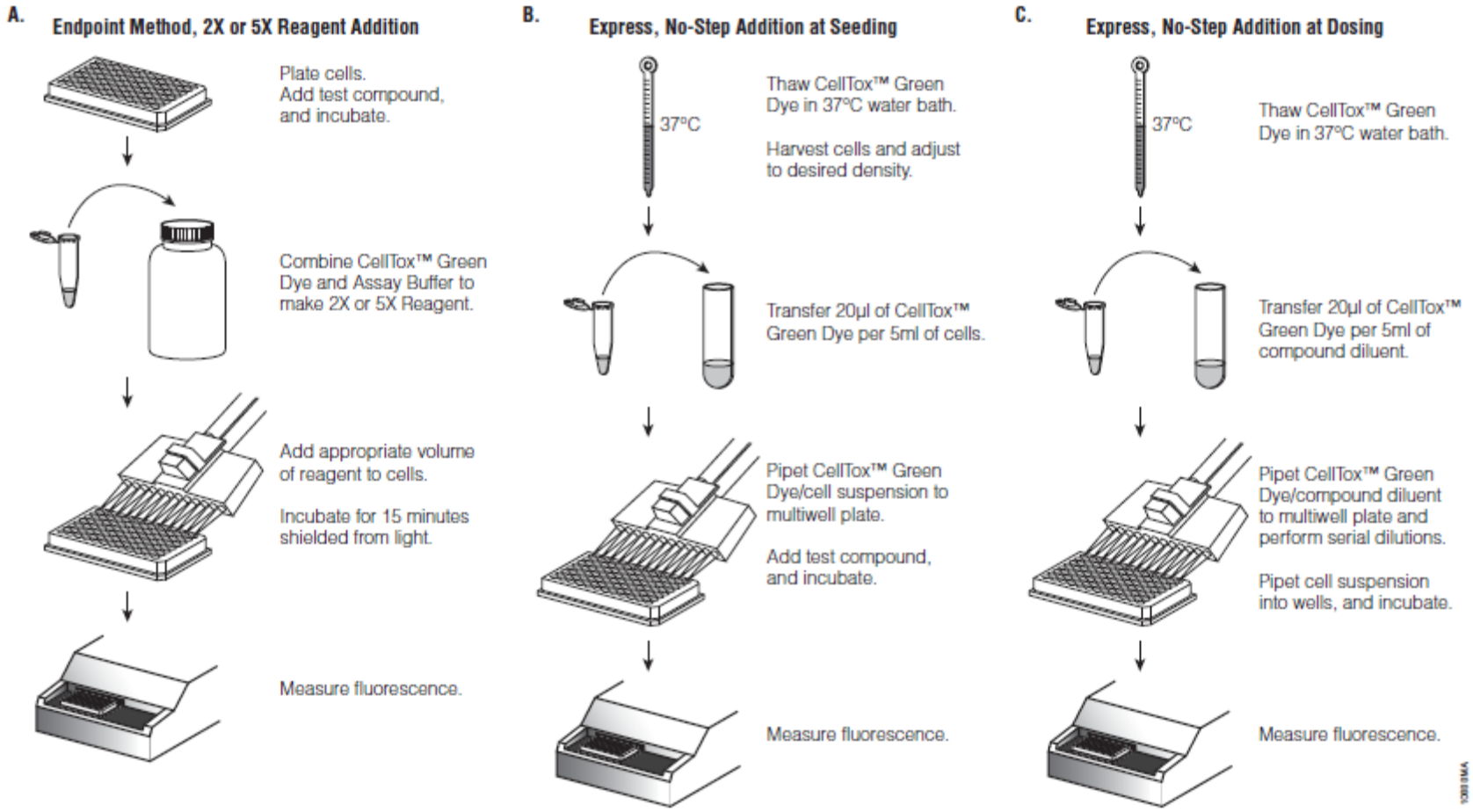
- luminescent assay
- Lytic
- measures ATP
- 'Add-mix- measure'

CellTiter Glo®



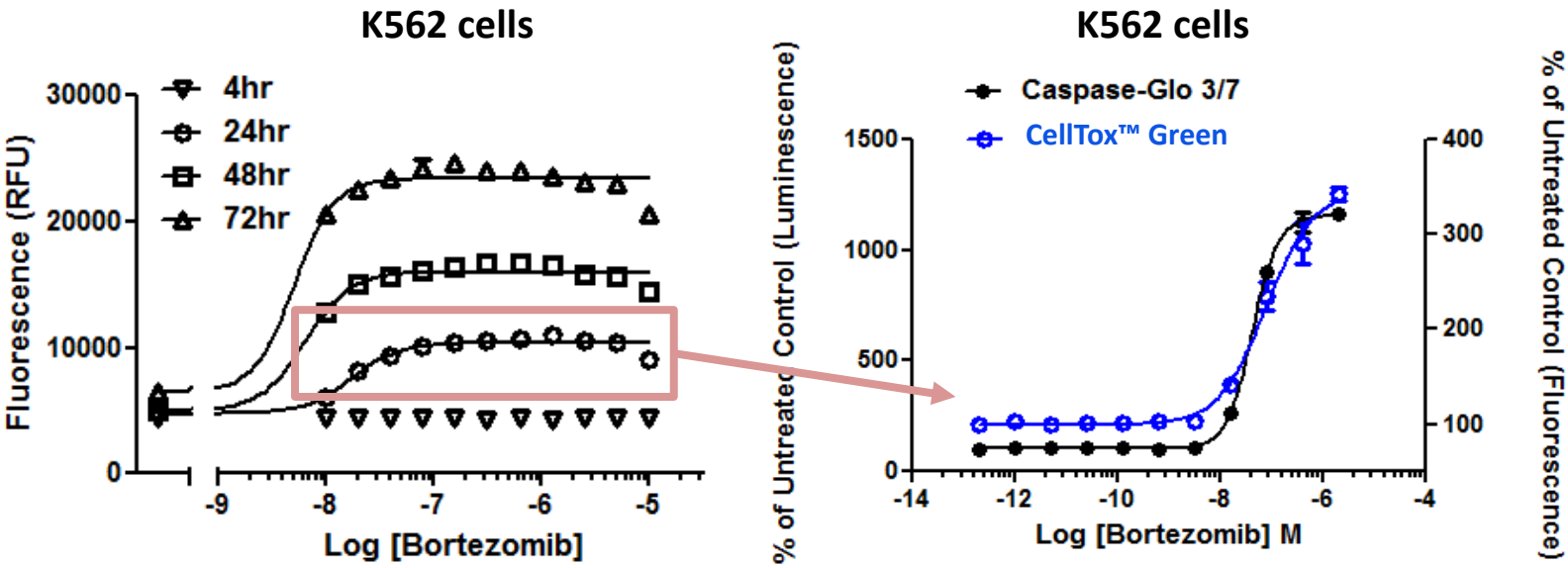
Different Assay Set up Methods

CellTox Green™ is flexible



CellTox™ Green Can Tell You When to Measure Caspase Activity!

CellTox™ Green Cytotoxicity Assay → **Caspase-Glo® 3/7Assay**



add CellTox™ Green at dosing and monitor for cytotoxicity

at first emergence of cytotoxicity, add Caspase-Glo® 3/7 Assay reagent

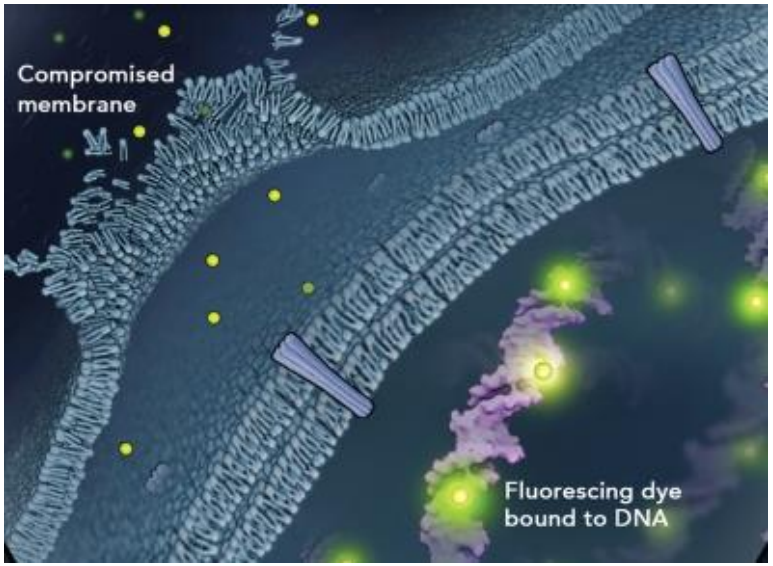
K562 cells treated with Bortezombid for 72h.

K562 cells treated with Bortezombid for 24h. CellTox Green and Caspase-Glo 3/7 assays.

CellTox-Green™ Summary

Features:

- **Real-time** cell death assay
- **Non- toxic**
- No enzyme involved-→no half-life issues
- Cost-effective
- Reduces variation & data points
- **Multiplex** opportunities



Applications:

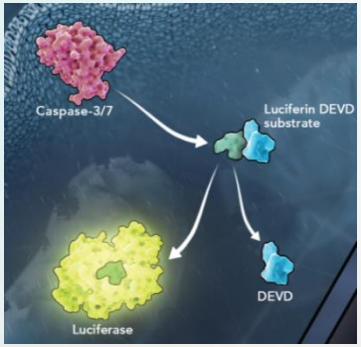
- Mammalian cells

Kit components:

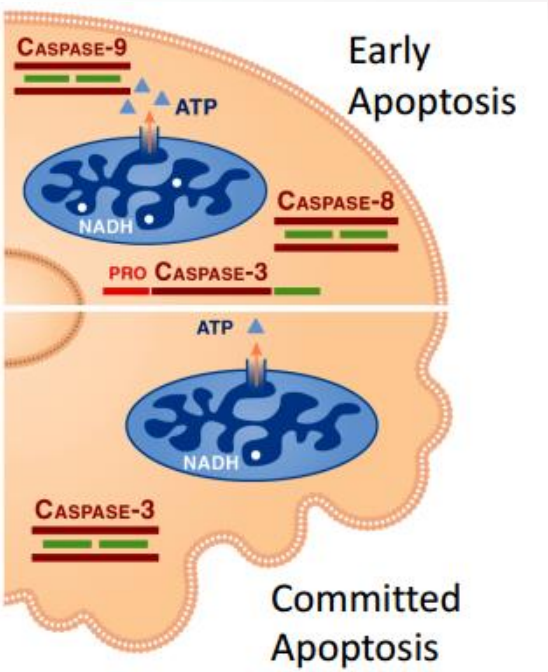
- CellTox™Green Dye
- Assay Buffer
- Lysis Buffer

Cytotoxicity Detection:

Apoptosis and Caspase-Glo® Assay



Apoptosis and Caspase-Glo® Assay Range



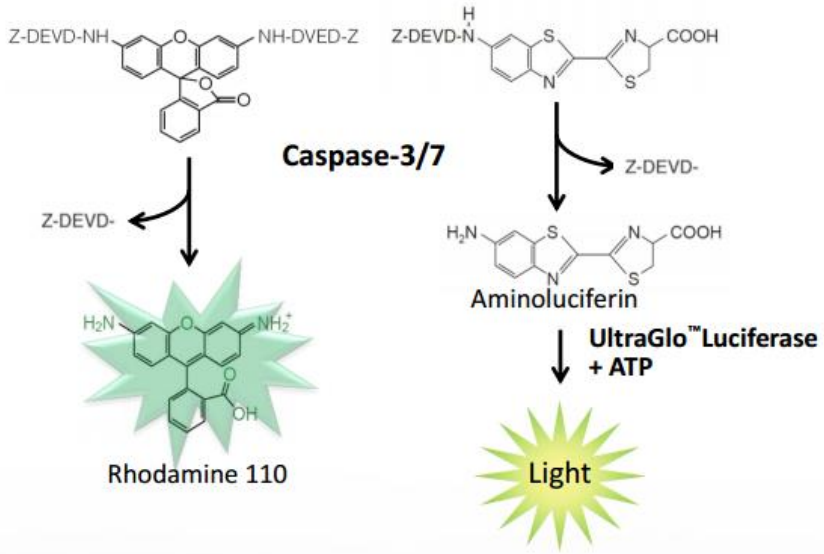
Promega Assays:

- Caspase-Glo® 2
- **Caspase-Glo® 3/7**
- Caspase-Glo® 6
- Caspase-Glo® 8
- Caspase-Glo® 9

luminescence

- **Apo-ONE® Caspase 3/7** fluorescence

Apo-ONE® Caspase3/7 | **Caspase-Glo® Caspase 3/7**

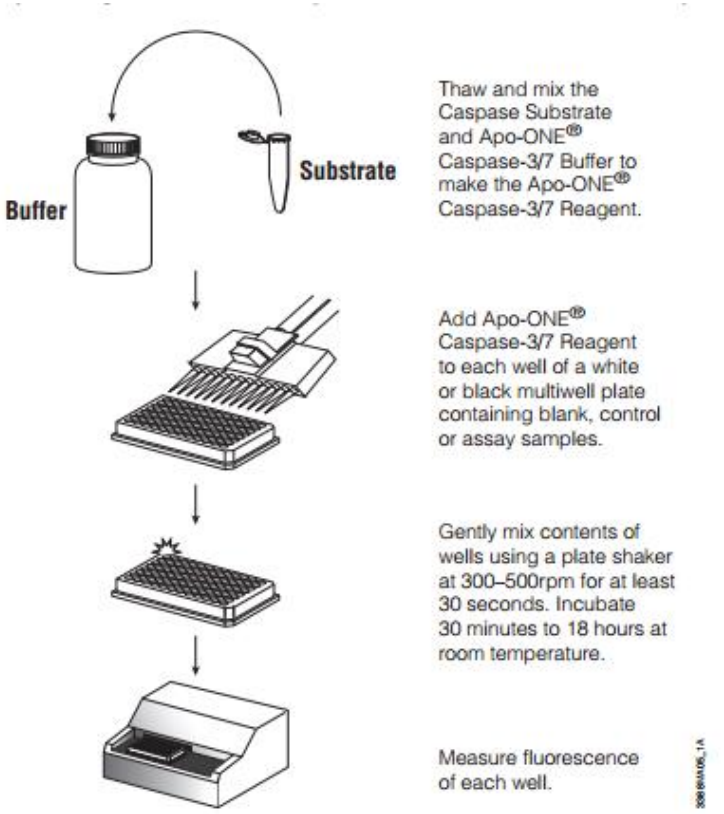


Consensus tetrapeptides:

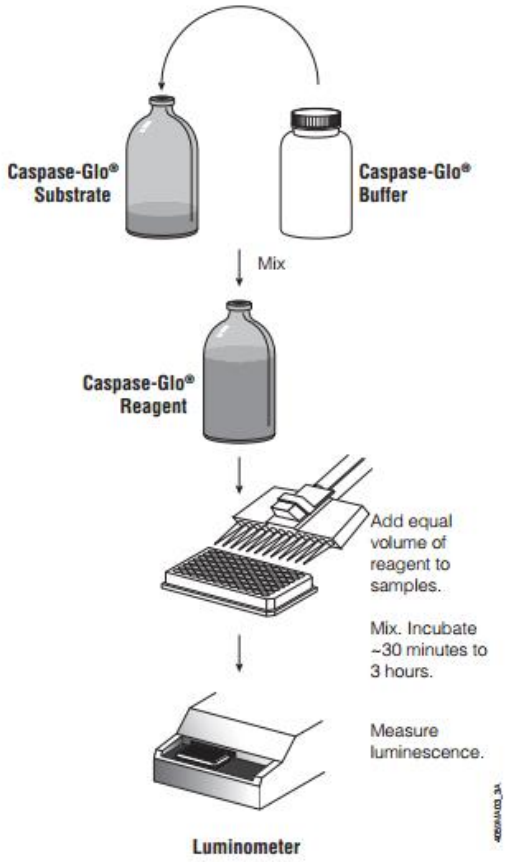
- VDVAD Caspase 2
- **DEVD Caspase 3/7**
- VEID Caspase 6
- LETD Caspase 8
- LEHD Caspase 9

Apoptosis and Caspase-Glo® Assay Range

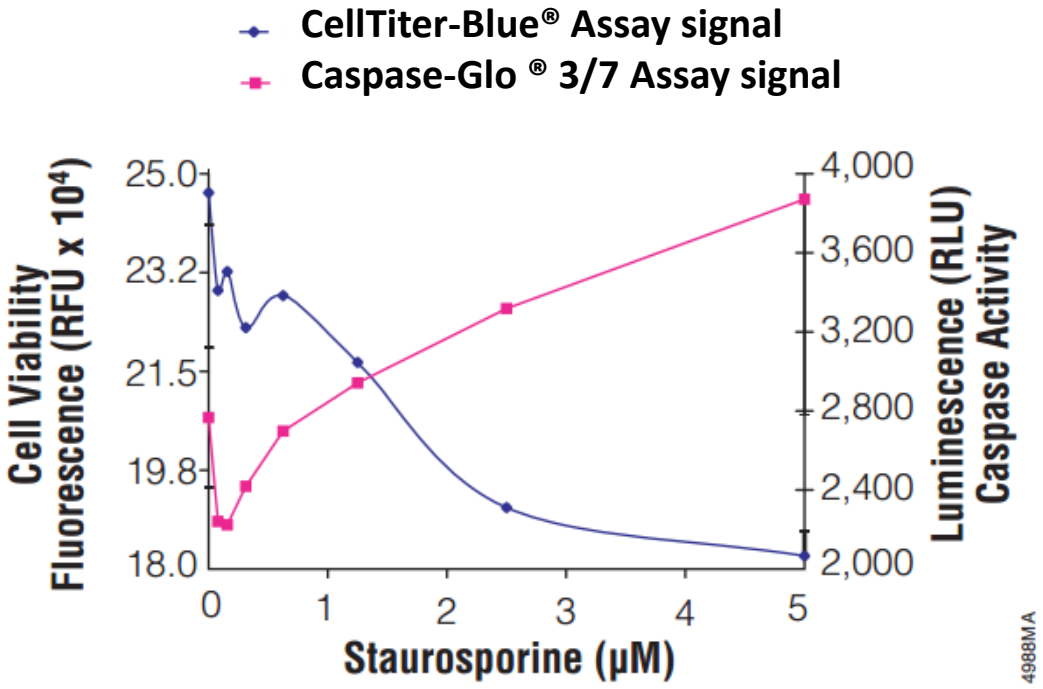
Apo-ONE® Caspase3/7



Caspase-Glo® 3/7 Assay

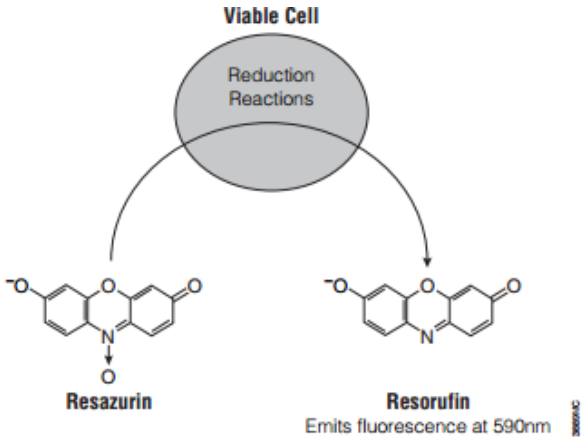


Apoptosis Detection and Caspase-Glo® Assay



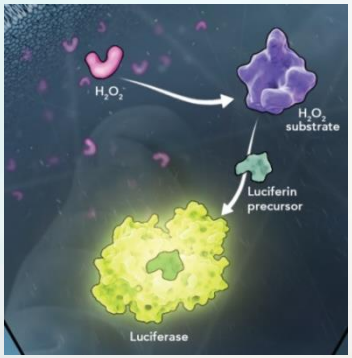
HEK293 cells

CellTiter-Blue® Viability Assay



Cytotoxicity Detection:

Oxidative Stress and ROS-Glo® H_2O_2 Assay

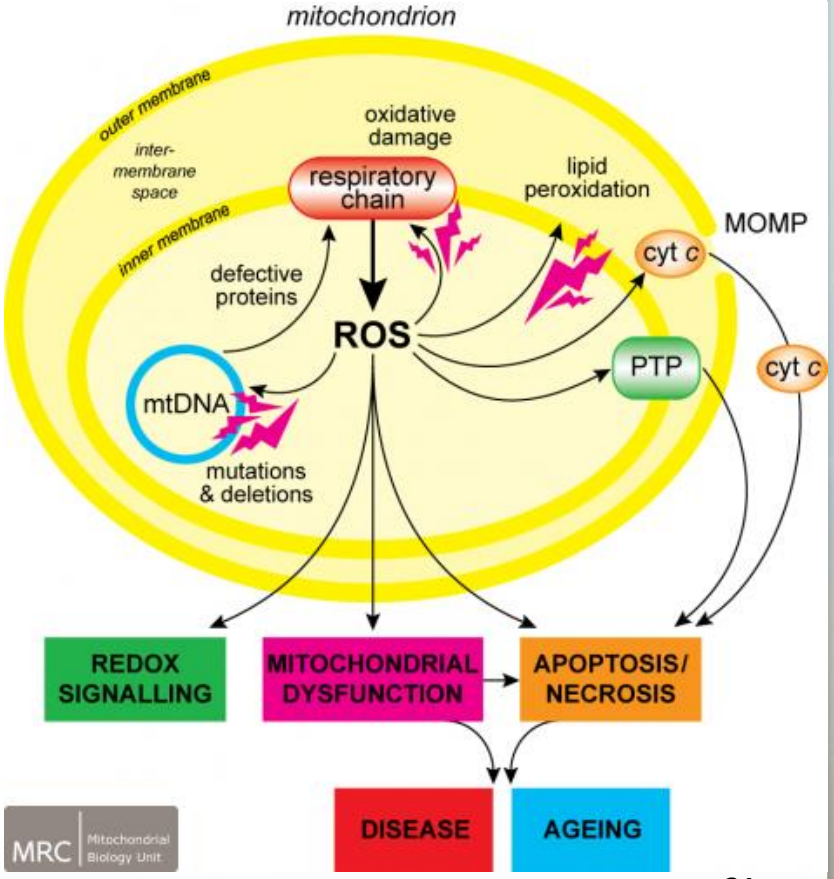


Cytotoxicity and Oxidative Stress

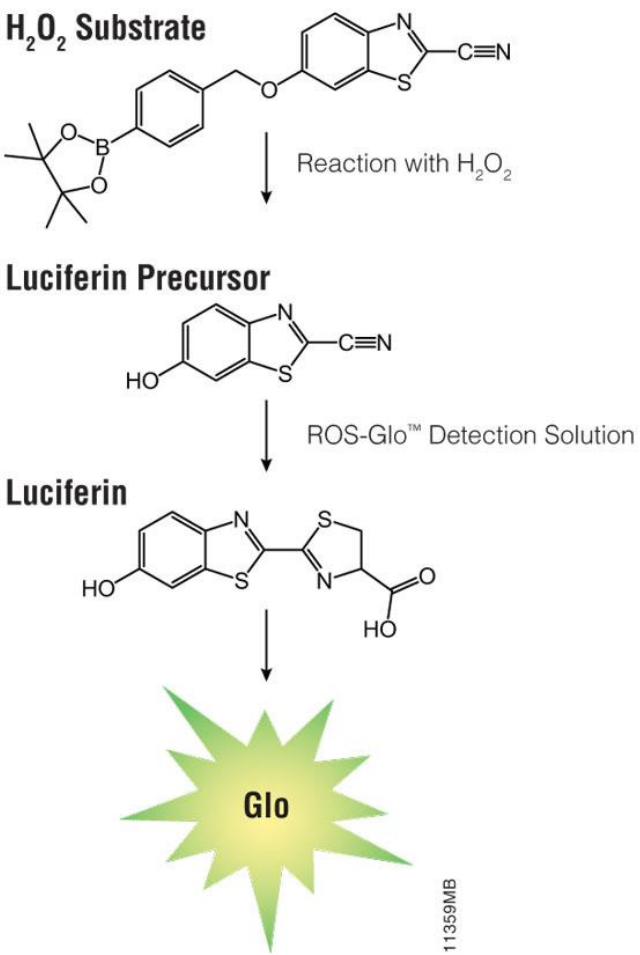
Oxidative stress: an imbalance between the production of reactive oxygen species (**ROS**) and the cell's capacity to detoxify the ROS or to repair the oxidative damage.

Markers of oxidative stress:

- Altered GSH:GSSG ratio (lowered GSH, increased GSSG)
- **ROS** (super oxide, hydroxyl radical, nitric oxide, hypochlorite convert to more stable H_2O_2)

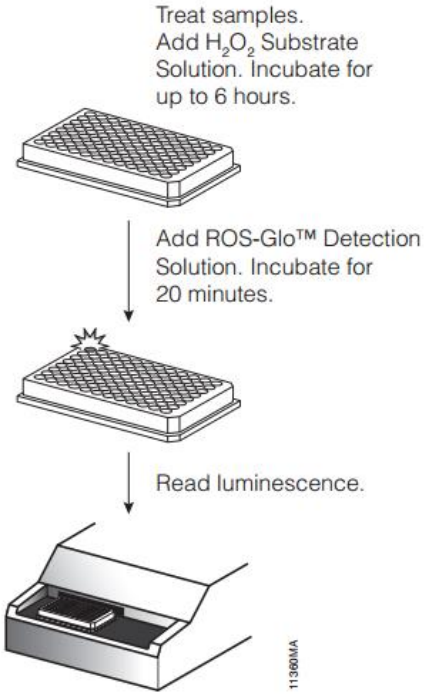


ROS-Glo™ Measures H_2O_2 Level

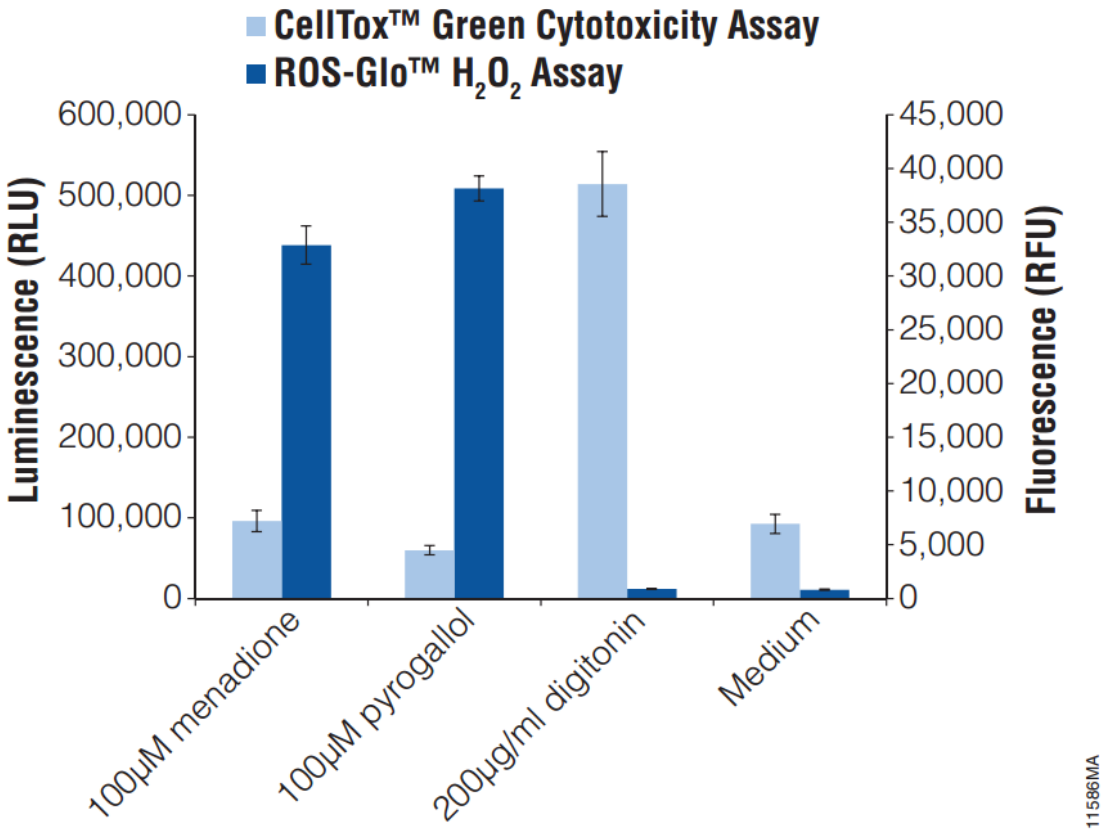


ROS- Glo™ H_2O_2 Assay:

- Direct H_2O_2 detection
- Homogeneous bioluminescent assay ('add-mix-read')



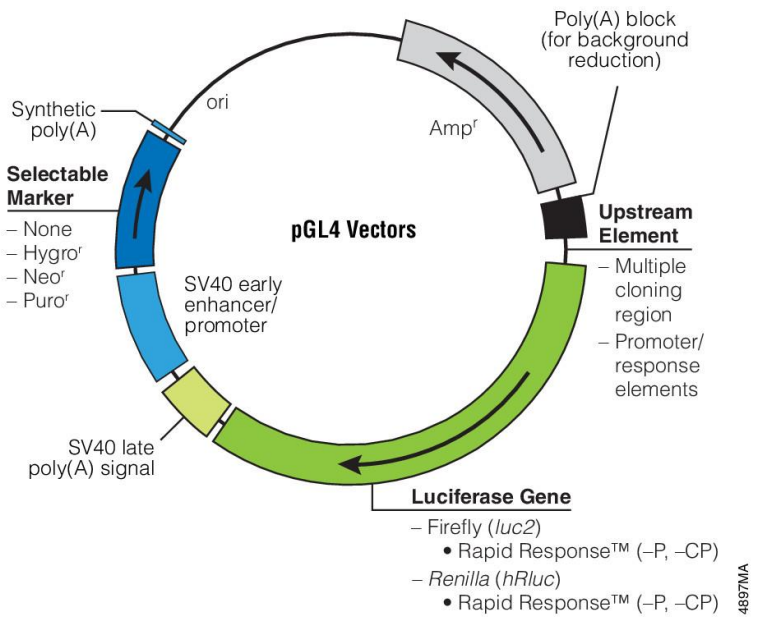
ROS-Glo™ H_2O_2 Assay



HepG2 cells

pGL4 Firefly Vectors for Signalling Pathway Analysis

- Vectors used to create a cell line with an indicator of a functional signaling pathway
- Useful for deciphering the components in a signaling pathway
- Made by insertion of multiple repeats of a response element upstream of a minimal promoter (minP)
- Collection of vectors that report the activity of a variety of pathways using the optimized ***luc2* firefly luciferase gene** in the **pGL4 backbone**



- **Hygromycin resistance** selectable marker, allowing use either in transient transfection experiments or for selection of a stable cell line

pGL4 Firefly Vectors for Signaling Pathway Analysis

Activator/Pathway	Transcription Factor	Binding Site	Vector Name	Catalogue
Oxidative Stress	Nrf2	Antioxidant Response Element (ARE)	pGL4.37[*luc2P*/ARE/Hygro]	E3641
DNA Damage	p53	p53 Response Element (p53 RE)	pGL4.38[*luc2P*/p53 RE/Hygro]	E3651
Heavy Metal Stress	MTF1	Metal Regulatory Element (MRE)	pGL4.40[*luc2P*/MRE/Hygro]	E4131
Heat Shock	HSF1	Heat Shock Element (HSE)	pGL4.41[*luc2P*/HSE/Hygro]	E3751
Hypoxia	Hif1α	Hypoxia Response Element (HRE)	pGL4.42[*luc2P*/HRE/Hygro]	E4001
Xenobiotic Stress	AhR	Xenobiotic Responsive Element (XRE)	pGL4.43[*luc2P*/XRE/Hygro]	E4121
Endoplasmic Reticulum Stress/ Unfolded Protein Response	ATF6	Activating Transcription Factor 6 Response Element (ATF6 RE)	pGL4.39[luc2P/ATF6 RE/Hygro]	E3661

Cytotoxicity Assays

Cell Viability Assays:
- RealTime-Glo™ MT
- CellTiter-Glo®2.0
- CellTiter-Glo® 3D
- CellTiter-Fluor™
- CellTiter-Blue ™
- CellTiter96®-AQueous
- BacTiter-Glo® Microbial

Apoptosis Assays:
- Caspase-Glo® 2
- Caspase-Glo® 3/7
- Caspase-Glo® 6
- Caspase-Glo® 8
- Caspase-Glo® 9
- Apo-ONE® Caspase 3/7
- CaspACE™ Assay System
- DeadEnd™ Fluorometic TUNEL
- DeadEnd™ Colorimetric TUNEL

Cytotoxicity Assays:
- CellToxGreen™
- CytoTox-Glo™
- CytoTox-Fluor™
- CytoTox-ONE™
- CytoTox™ 96

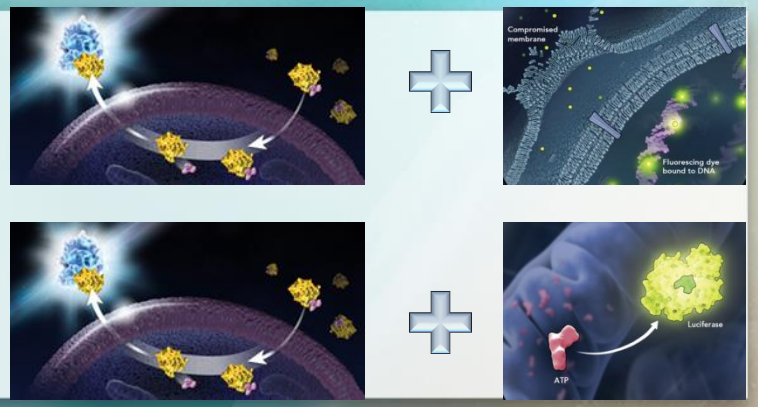
Metabolism Assays:
- NAD/NADH-Glo™
- NADP/NADPH-Glo ™
- NAD(P)H-Glo™ Detection System

Oxidative Stress Assays:
- ROS-Glo™ H_2O_2 Assay
- GSH-Glo™ Glutathione Assay
- GSH-GSSG-Glo™ Assay

Mitochondrial Toxicity Assays:
- Mitochondrial ToxGlo™

Viral Toxicity Assays:
- Viral-ToxGlo™ Assay

Multiplexing



Multiplexing Set-up

continuous assay #1
and
continuous assay #2

Assay #1

Assay #2

RealTime-Glo™ MT Cell Viability Assay

CellTox™ Green Cytotoxicity Assay

luminescence + fluorescence

continuous assay
and
end-point assay

Assay #1

luminescence
+
fluorescence

Assay #2

RealTime-Glo™ MT Cell Viability Assay

CytoTox-Fluor™ Viability Assay

Multiplexing Set-up

continuous assay #1
and
continuous assay #2

Assay #1

Assay #2

RealTime-Glo™ MT Cell Viability Assay

CellTox™ Green Cytotoxicity Assay

luminescence + fluorescence

continuous assay
and
end-point assay

Assay #1

luminescence
+
luminescence

Assay #2

RealTime-Glo™ MT Cell Viability Assay

CellTiter-Glo® Cell Viability Assay

Multiplexing - Examples

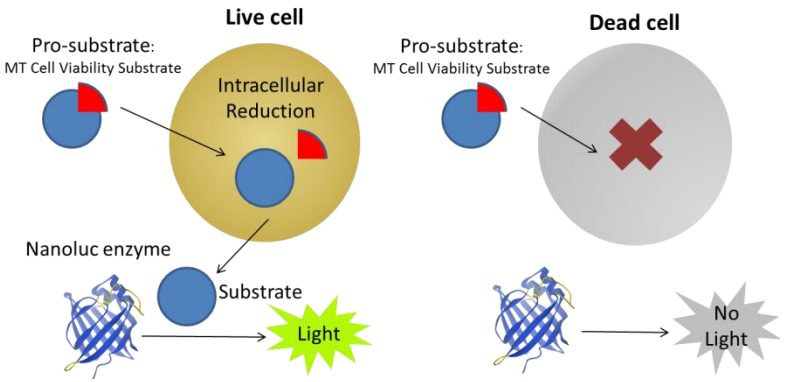
Table 1. Assays that can be Multiplexed with the RealTime-Glo™ MT Cell Viability Assay.

First Assay	Second Assay	Information Obtained by Multiplexing
RealTime-Glo™ MT Cell Viability Assay	CellTox™ Green Cytotoxicity Assay[1]	Cell viability and cytotoxicity (membrane integrity)
RealTime-Glo™ MT Cell Viability Assay	CytoTox-Fluor™ Cytotoxicity Assay	Cell viability and cytotoxicity (protease release)
RealTime-Glo™ MT Cell Viability Assay	CellTiter-Glo® Luminescent Cell Viability Assay	Two independent markers of cell viability (reducing potential and ATP)
RealTime-Glo™ MT Cell Viability Assay	NAD/NADH-Glo™ Assay	Cell viability and measurement of NAD^+ and NADH
RealTime-Glo™ MT Cell Viability Assay	NADP/NADPH-Glo™ Assay	Cell viability and measurement of $NADP^+$ and NADPH
RealTime-Glo™ MT Cell Viability Assay	Reporter assays[2]	Cell viability and reporter gene activity
RealTime-Glo™ MT Cell Viability Assay	RNA isolation	Cell viability and RNA analysis

Multiplexing and Mechanism of Cytotoxicity (1)

RealTime-Glo ™ MT Cell Viability Assay

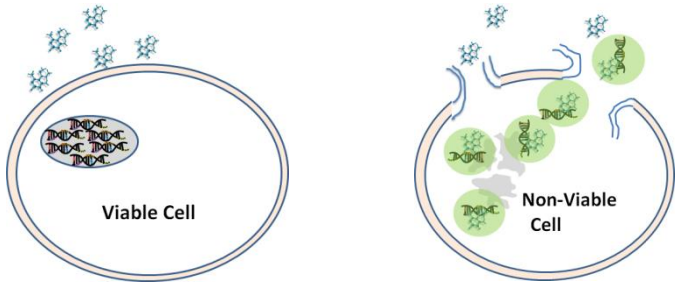
continuous assay #1



- **Luminescent**
- Metabolically active cells
- **Viable cells → high signal**
- Dead cells→ low signal

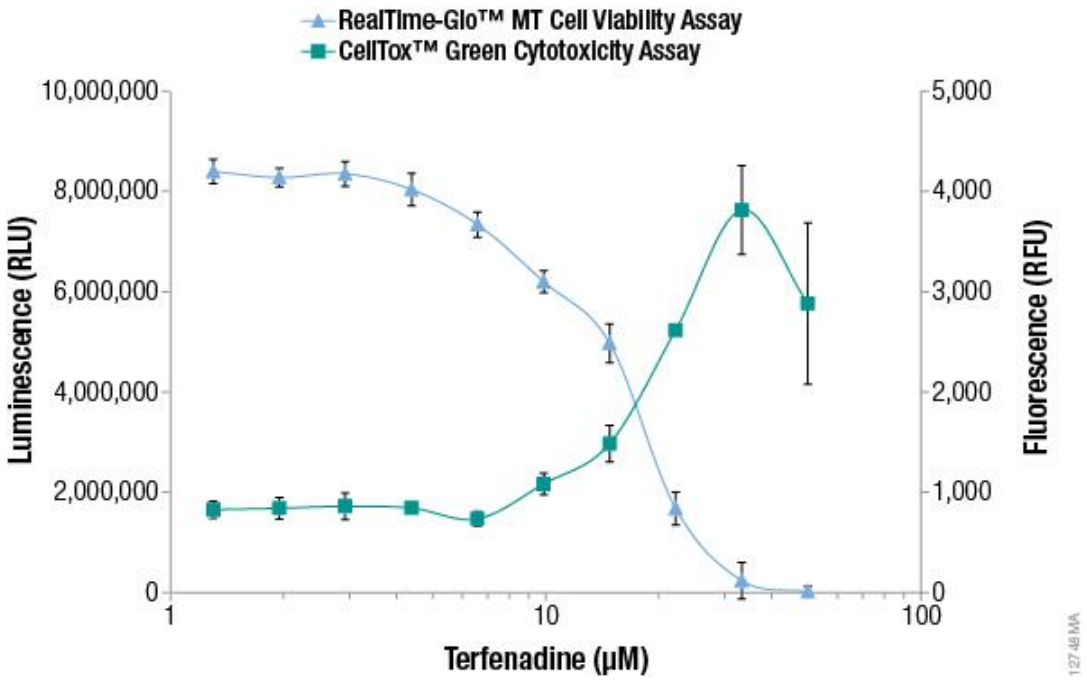
CellTox™ Green Cytotoxicity Assay

continuous assay #2



- **Fluorescent**
- DNA released from dead cells
- **Viable cells →low signal**
- Non- viable cells → high signal

Multiplexing and Mechanism of Cytotoxicity (1)

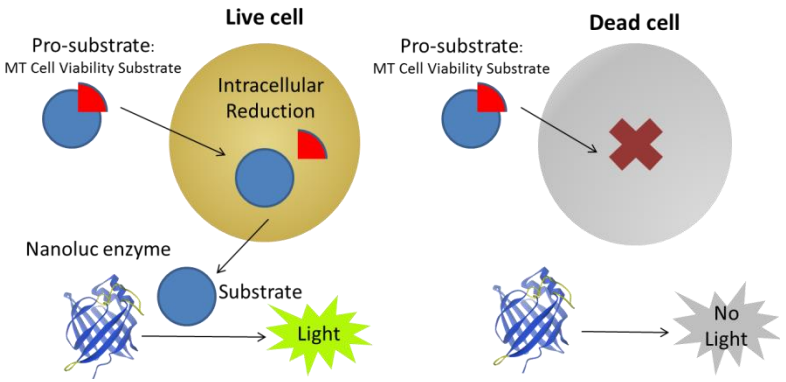


Hep2G frozen instant cells; 10,000 cells/ well
96-well white plate; 6h time- point

Multiplexing and Mechanism of Cytotoxicity (2)

RealTime-Glo ™ MT Cell Viability Assay

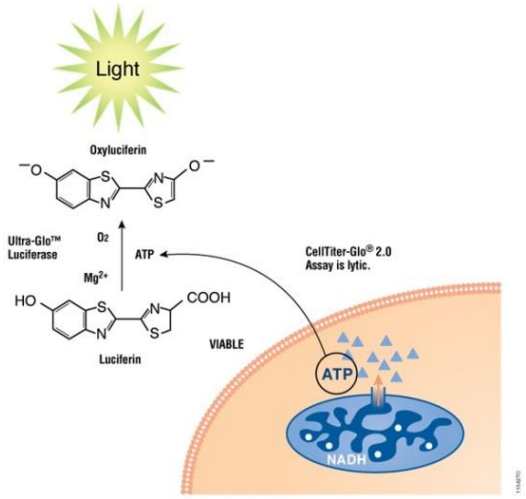
continuous assay #1



- **Luminescent**
- Metabolically active cells
- **Viable cells → high signal**
- Dead cells→ low signal

CellTiter-Glo® Cell Viability Assay

end point assay #1

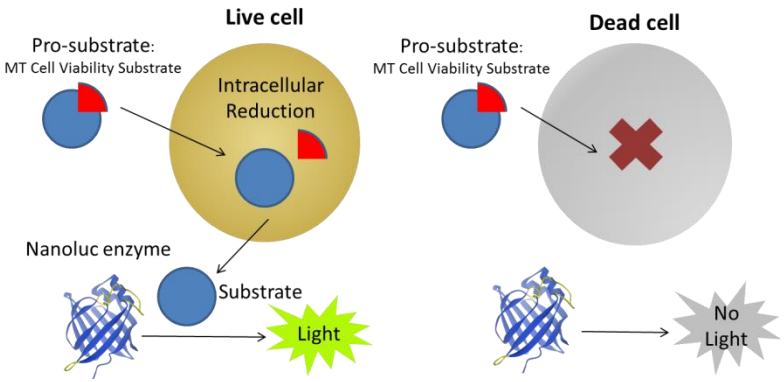


- **Luminescent**
- ATP
- **Viable cells → high signal**
- Dead cells→ low signal

Multiplexing and Mechanism of Cytotoxicity (3)

RealTime-Glo ™ MT Cell Viability Assay

continuous assay #1



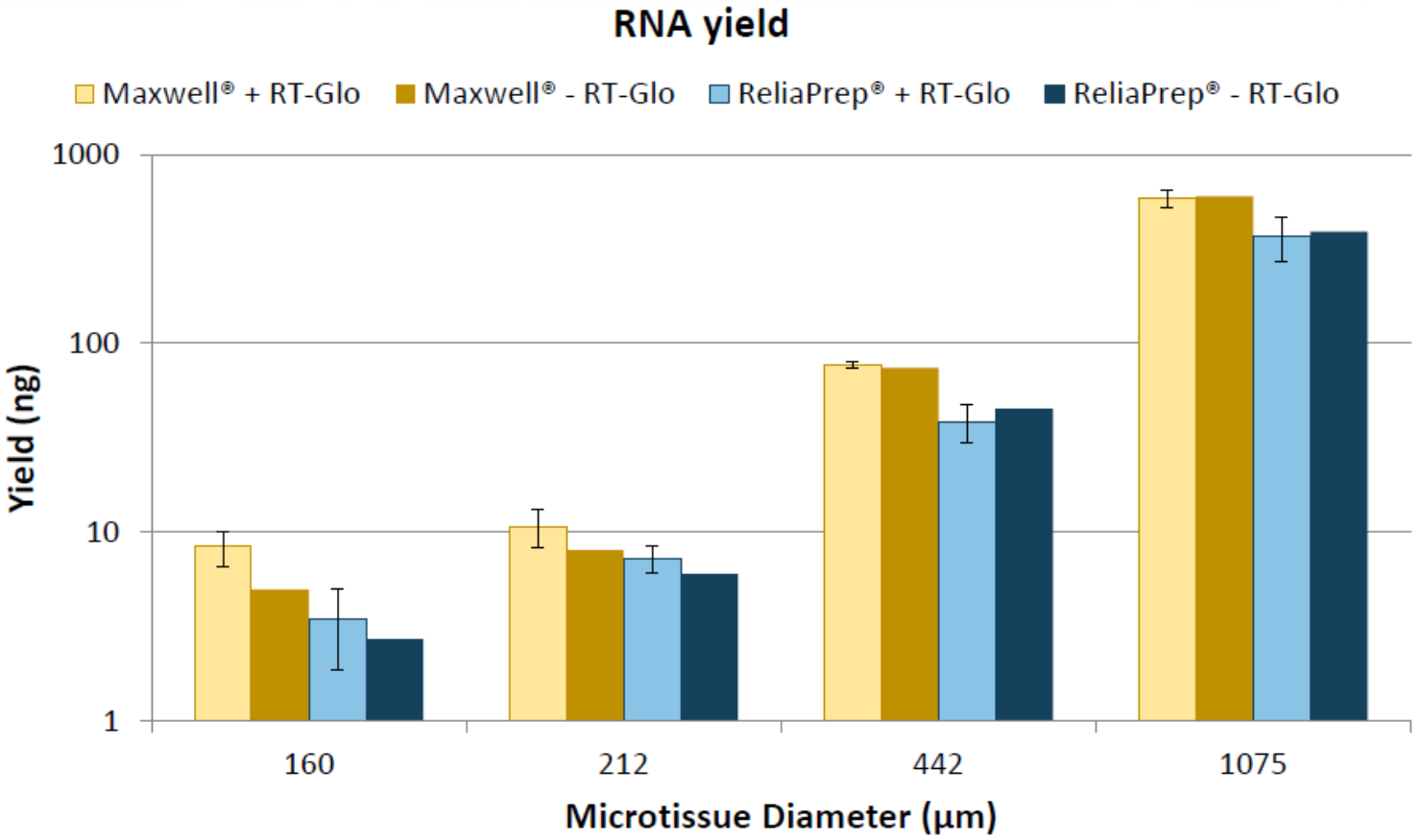
- **Luminescent**
- Metabolically active cells
- **Viable cells → high signal**
- Dead cells→ low signal

RNA Isolation

end point assay #1

- Extract RNA using **ReliaPrep™ Assay**
- Perform qPCR
- Gene expression microarray
- RNA Seq

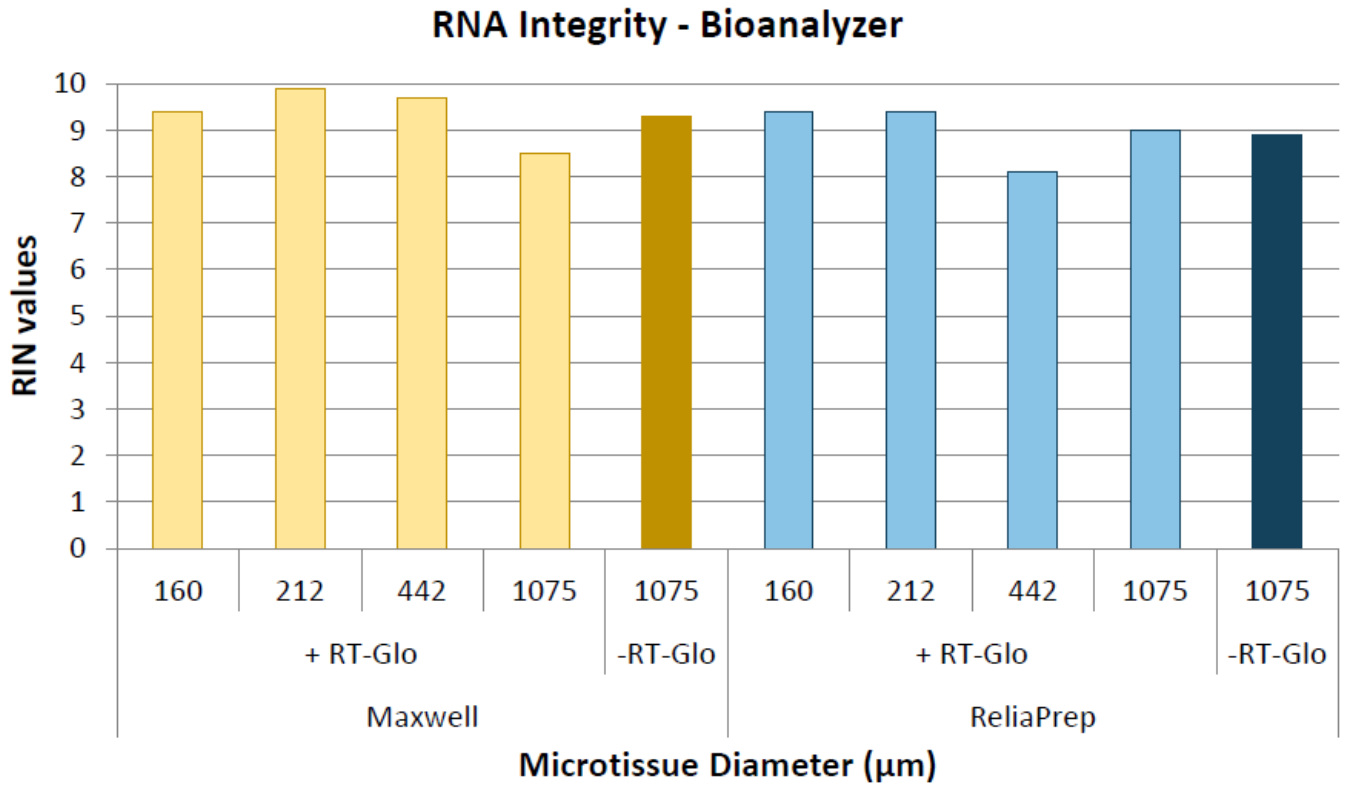
Multiplexing and Mechanism of Cytotoxicity (3)



RealTime-Glo™ reagents do NOT effect RNA yield using QuantiFluor® RNA System

Multiplexing and Mechanism of Cytotoxicity (3)

RNA integrity is NOT affected by presence of RealTime-Glo™ reagents!



Multiplexing and Mechanism of Cytotoxicity (3)

RealTime-Glo™ Reagent does NOT affect cycle threshold of extracted RNA

Table 2. C_t Values Generated from RNA Isolated from Different Cell Types With or Without the RealTime-Glo ™ Reagent.

	C_t Value	
Cell Type	With Medium Only	With RealTime-Glo ™ Reagent
A549	30.9	30.3
K562	31.3	30.2
MCF7	30.9	30.1
THP-1	29.9	29.9

Summary

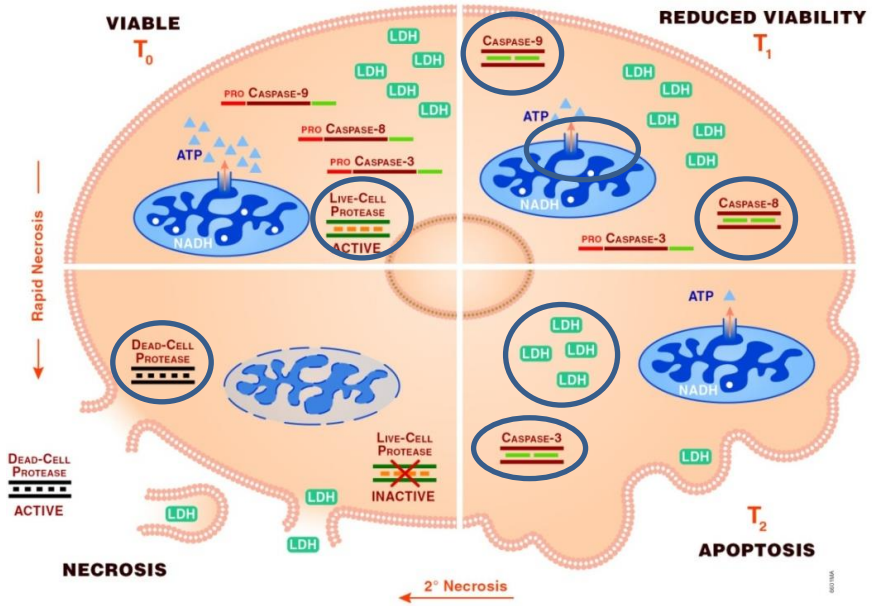
Multiplexing gives a more complete picture of what's happening in the cell

Requirements :

- Assays must be biologically & chemically compatible
- Signals must be spectrally different

Advantages:

- Reduces ambiguity
- Eliminates variables
- Normalises data
- Increases data content/well/ aliquot



Conclusions

Real-Time Glo™ /CellToxGreen™:

- Provides kinetic information about viable/ dead cell number during the course of the experiment that enables 'on the go' decision making
- Reagents are non- toxic to the cells
- Optional protocols enables adding the reagents at different stages of the experiment
- **Multiplexing** opportunities with other assays to determine **the mechanism of cytotoxicity** and possible involvement of other processes such as oxidative stress