Plate-Based Assay Methods for the Assessment of Cellular Health

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Biological Outcomes in Cell Culture

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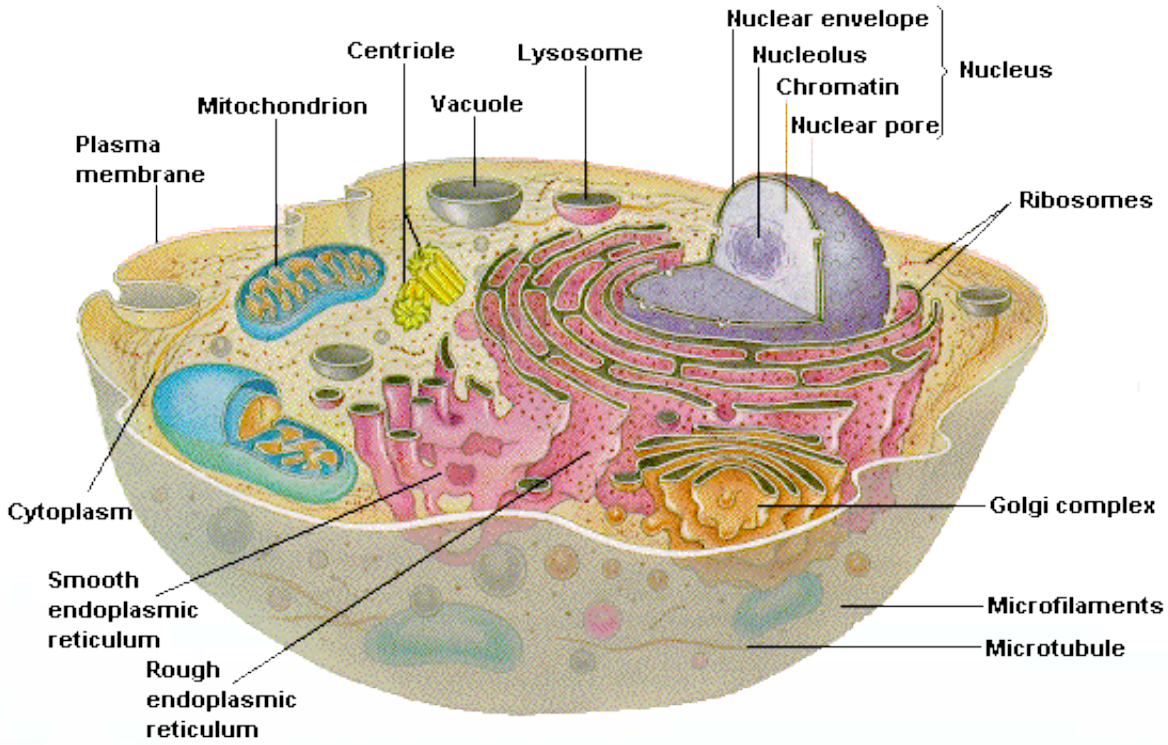
Treatment
-Small molecule
-Bio-molecule
-Transgene
-Physical insult

Cause → **Effect**



Normal Proliferation

Enhanced Proliferation

Cell-cycle Arrest

Oxidative Stress

1° Necrosis

Apoptosis

2nd Necrosis

Which response is it?

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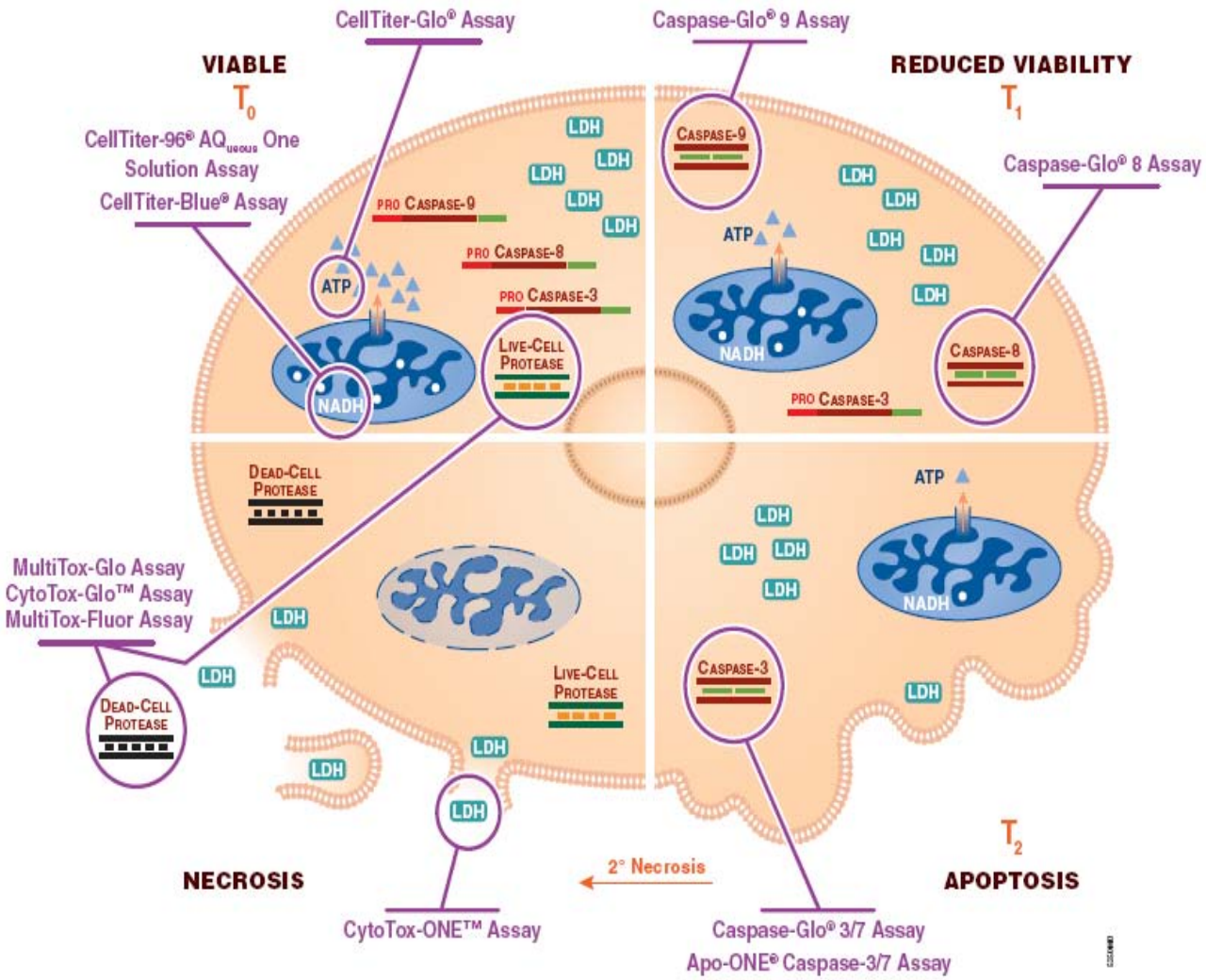
Rarely a simple answer. Often subject to experimental qualification.

The biological **"profile"** of any treatment is dependent upon:

1. Dosage
   - Addressed through serial dilution series
2. Exposure Time (cells with compound contact)
3. Mechanism of action of the test compound
4. Cell Type
   -specific target
   -off target

Biomarkers and the Cytotoxic Response



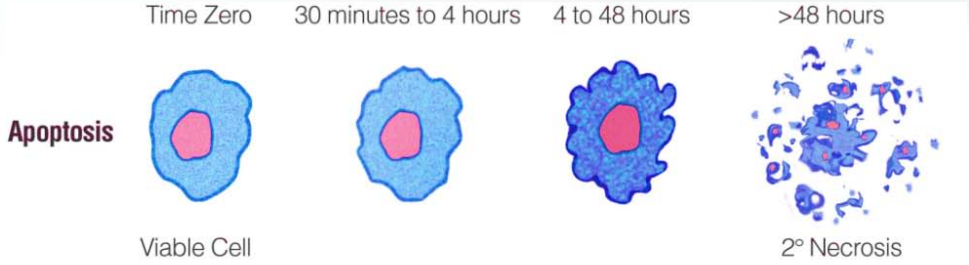
Biomarkers of cell health:

Can decrease due to cytotoxicity

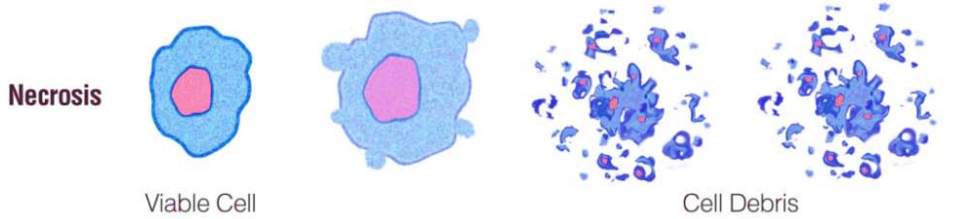
Can increase due to cytotoxicity

No single parameter assay can fully characterize cytotoxicity

Kinetics of Cell Death Affect Assay Results



Enzyme Release	0	+	++++	+
Caspase	0	++	++++	0
ATP	++++	+++	+	0
Tetrazolium	++++	+++	+	0
Resazurin	++++	+++	+	0
Enzyme Retention	++++	+++	+	0



Enzyme Release	0	++++	++	0
Caspase	0	0	0	0
ATP	++++	0	0	0
Tetrazolium	++++	0	0	0
Resazurin	++++	0	0	0
Enzyme Retention	++++	0	0	0

7495TA

Choosing an appropriate biomarker and appropriate cell model is critical

Primary Necrosis occurs quickly, apoptosis may take up to 48hrs to affect toxicity (secondary necrosis)

Cytotoxicity and caspase activities are transient but definitive

Viability assays always report the number of viable cells remaining but offer little information about mechanism (anti-proliferative vs cytotoxic effects.

Tangible Examples of In Vitro Cytotoxicity

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Typical Cell Health Work-Flow

Different mechanistic toxins
- serially diluted for potency calculations

+

Cell model(s)
- On target
- Off target

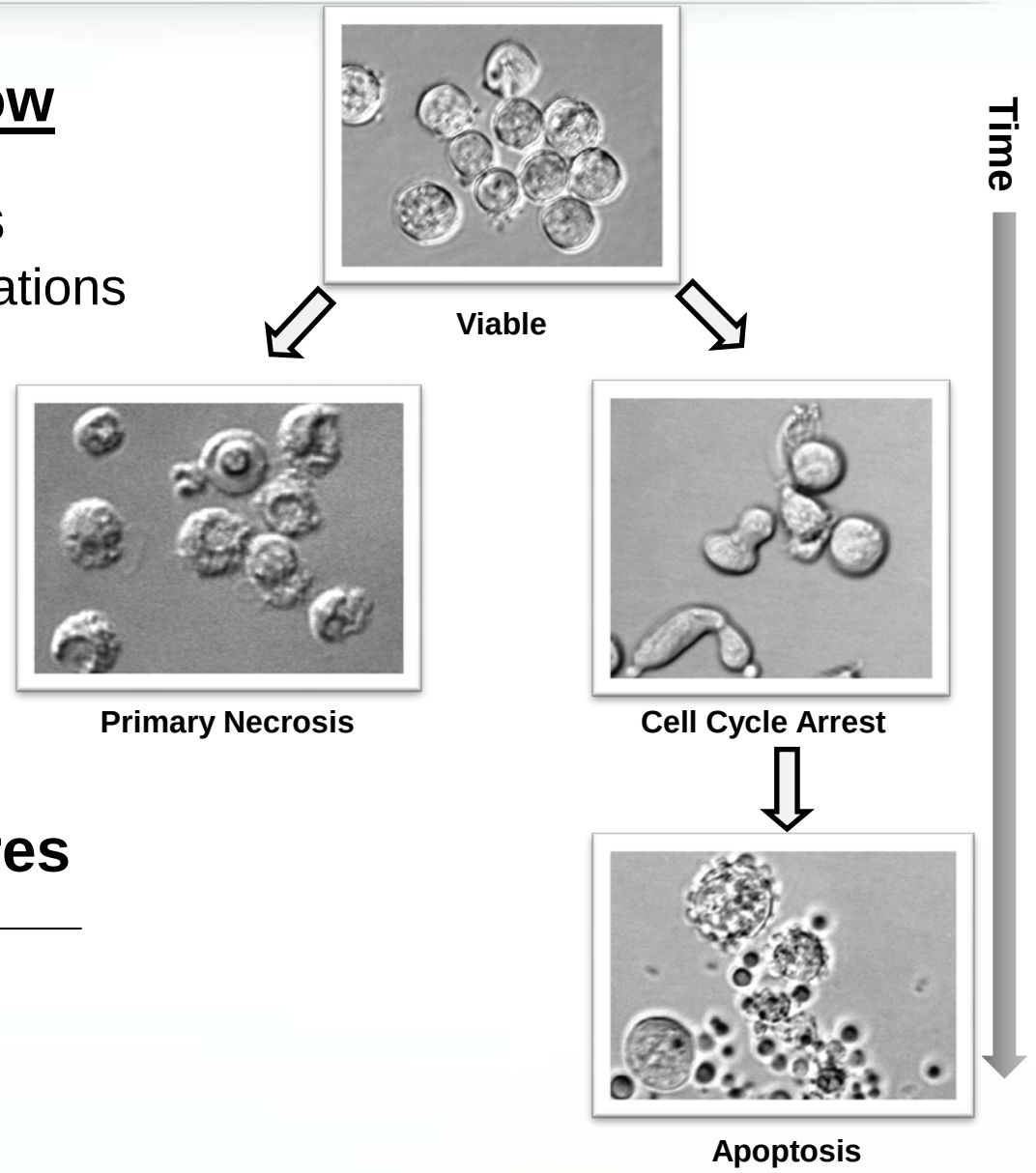
+

Time Course Exposures

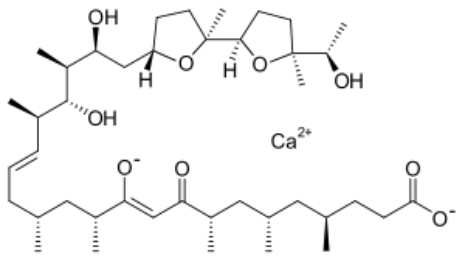
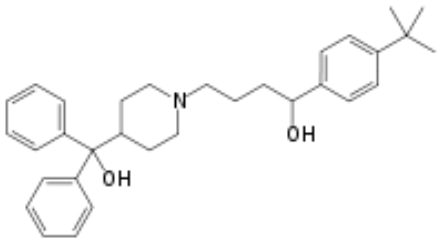
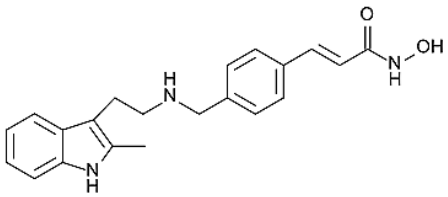
+

Different Cell Health Measures

= Data Interpretation(s)



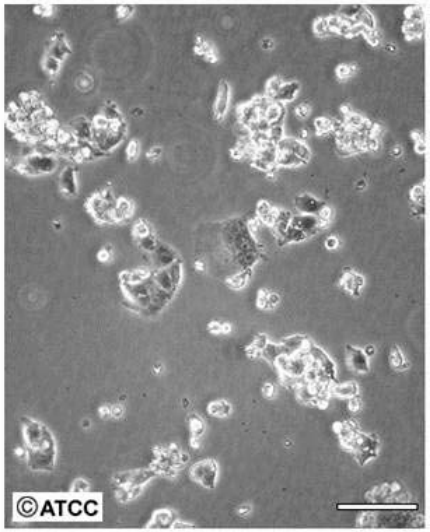
Three Model Toxins…

Compound	Structure	Mode of Action	Cytotoxic Mechanism
Ionomycin		Ionophore Ca^{++} flux	Primary necrosis
Terfenadine		Incompletely characterized pro-drug toxicity	Apoptosis (fast On-set)
Panobinostat		Histone deacetylase Inhibitor (HDACi)	Apoptosis (late On-set)

The Cell Model…

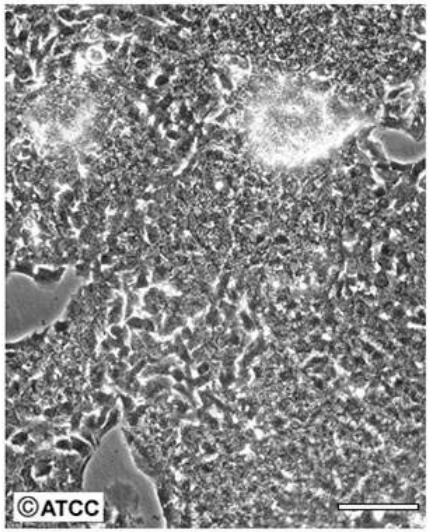
ATCC Number: **HB-8065**
Designation: **Hep G2**

Untreated at "zero time"



Low Density Scale Bar = 100µm

Untreated with normal proliferation



High Density Scale Bar = 100µm

HepG2 cells are a human hepatocellular carcinoma (epithelial morphology) commonly used as a model system for studies of liver metabolism and toxicity of xenobiotics.

The Experiment

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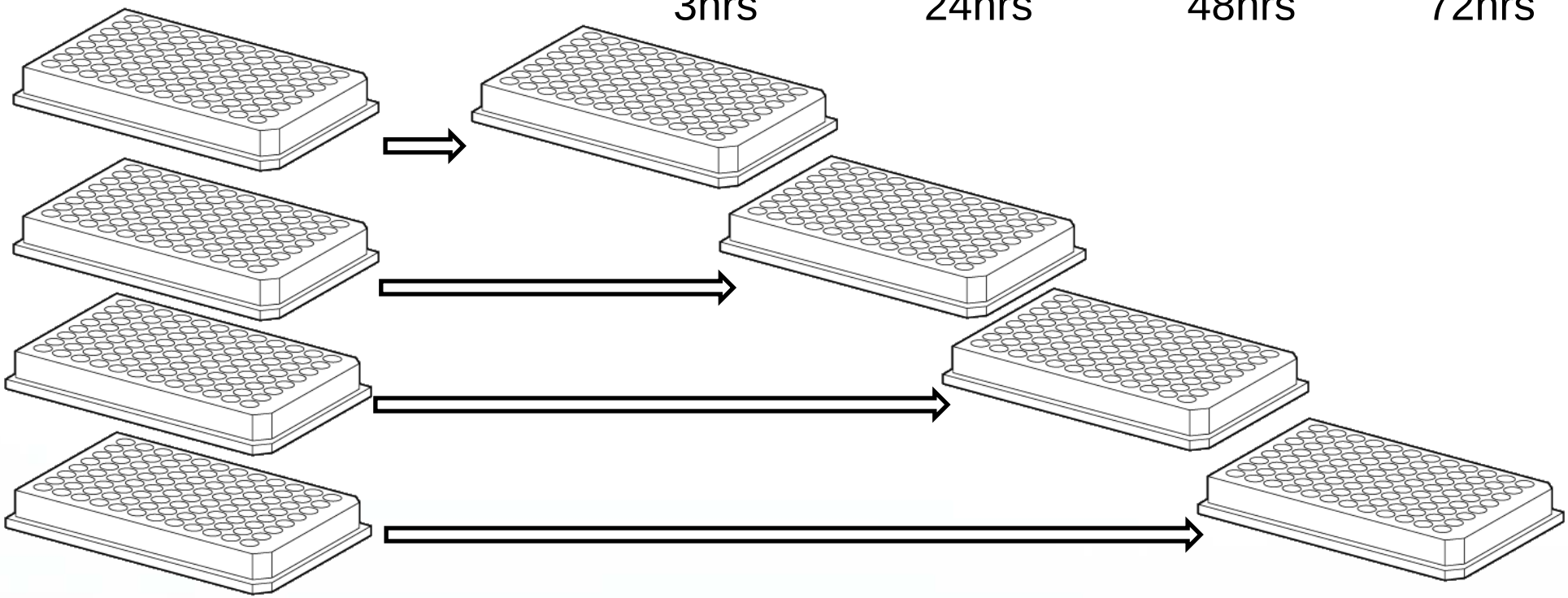
Compounds diluted
in 10-fold dilutions of medium
and added in equal volumes
of sub-confluent HepG2 cells.
Vehicle control served as
“Untreated control”

Hours of Compound Exposure with Cells

3hrs 24hrs 48hrs 72hrs

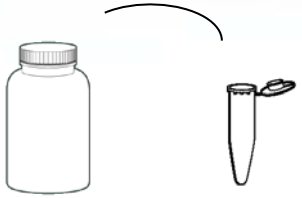


Three Model Cell Health Assays…

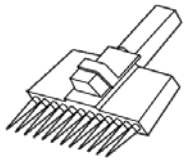
Assay Name	**Assay Type**	**Biomarker**	**Measurement**
CellTiter-Fluor™	Viability	Live Cell Protease	Fluorescence (AFC, $400_{ex}/505_{em}$)
CytoTox-Fluor™	Cytotoxicity	Dead Cell Protease	Fluorescence (R110, $485_{ex}/530_{em}$)
Caspase-Glo® 3/7	Cytotoxicity	Caspase Activity	Luminescence

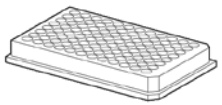
Homogenous, “Add-Mix-Measure” Formats

CellTiter-Fluor™: A Protease Viability Assay

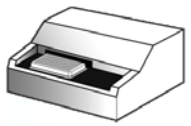


Create CellTiter-Fluor Reagent





Incubate 30 min

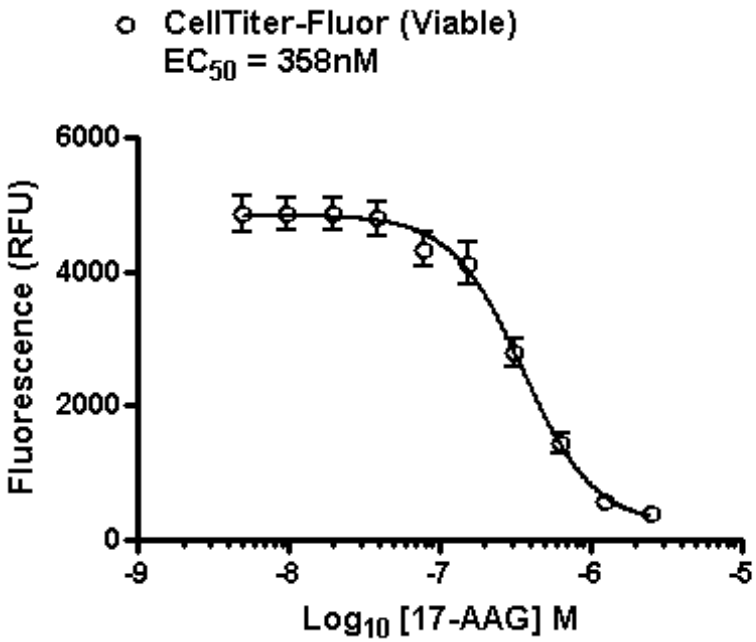


Fluorescence

CellTiter-Fluor Reagent (cell-permeable profluorogenic peptide substrate)

Viable cell

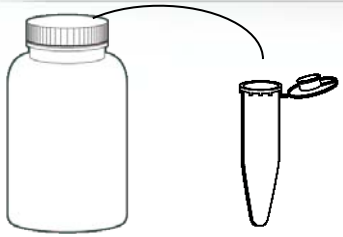
Constitutive "live cell protease" liberates AFC fluorophore



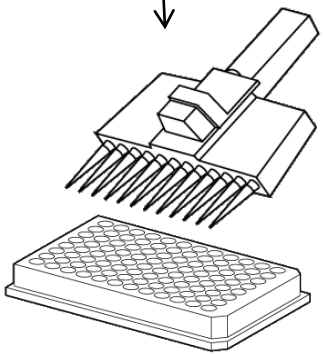
Advantages:

- Sensitive
- Scalable
- Compatible for multiplexing

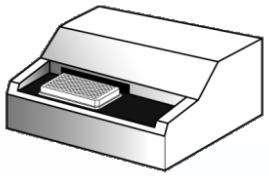
CytoTox-Fluor: Protease Cytotoxicity Assay



Create CytoTox-Fluor Reagent

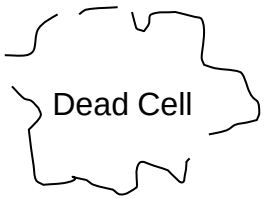


Incubate 30 min



Fluorescence

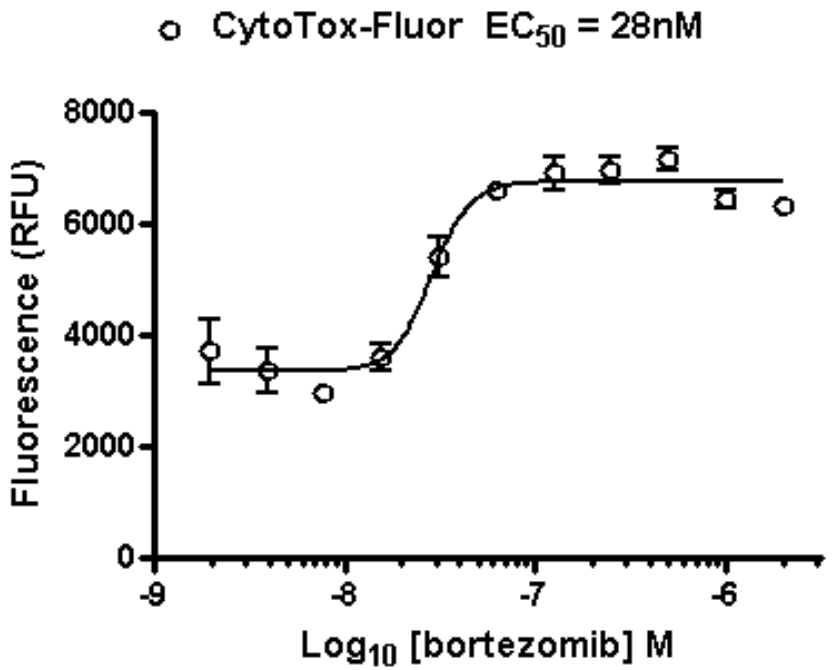
CytoTox-Fluor Reagent (non-cell permeable, profluorigenic Substrate)



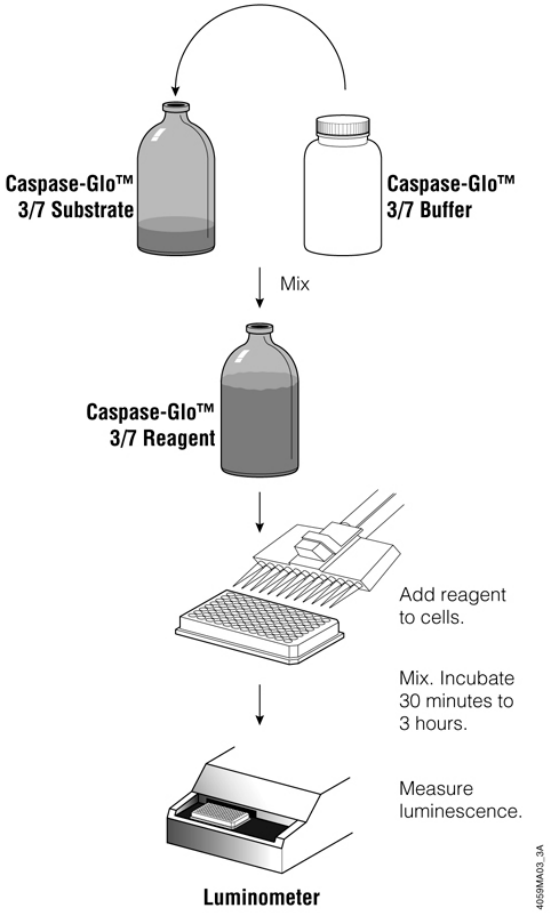
Leaked “Dead cell protease”

R110 is liberated

485ex/520em



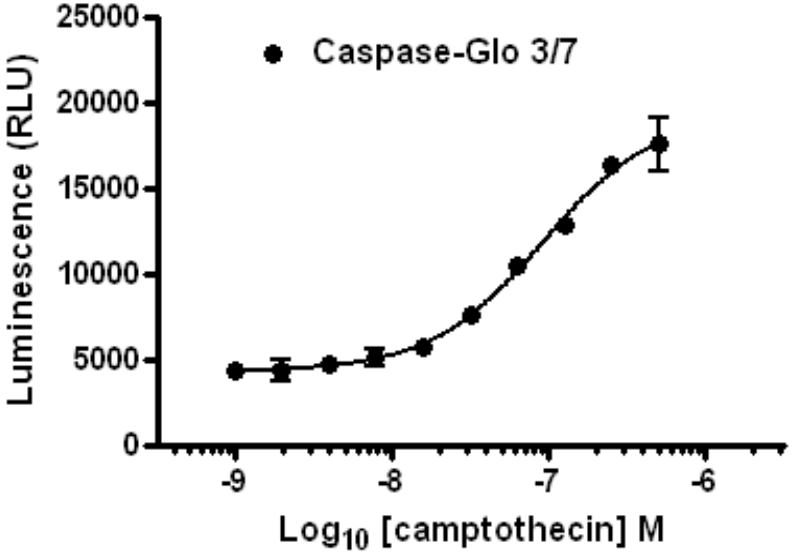
Caspase Activity Assay



Caspase-Glo 3/7 Reagent
(Z-DEVD-luciferin + Ultra-Glo
+ ATP in lytic buffer)

Activated caspase
cleaves substrate

Aminoluciferin is liberated
and consumed by
luciferase reaction



Three Assays Applied in Sequential, Same Well Multiplex = ApoTox-Glo™

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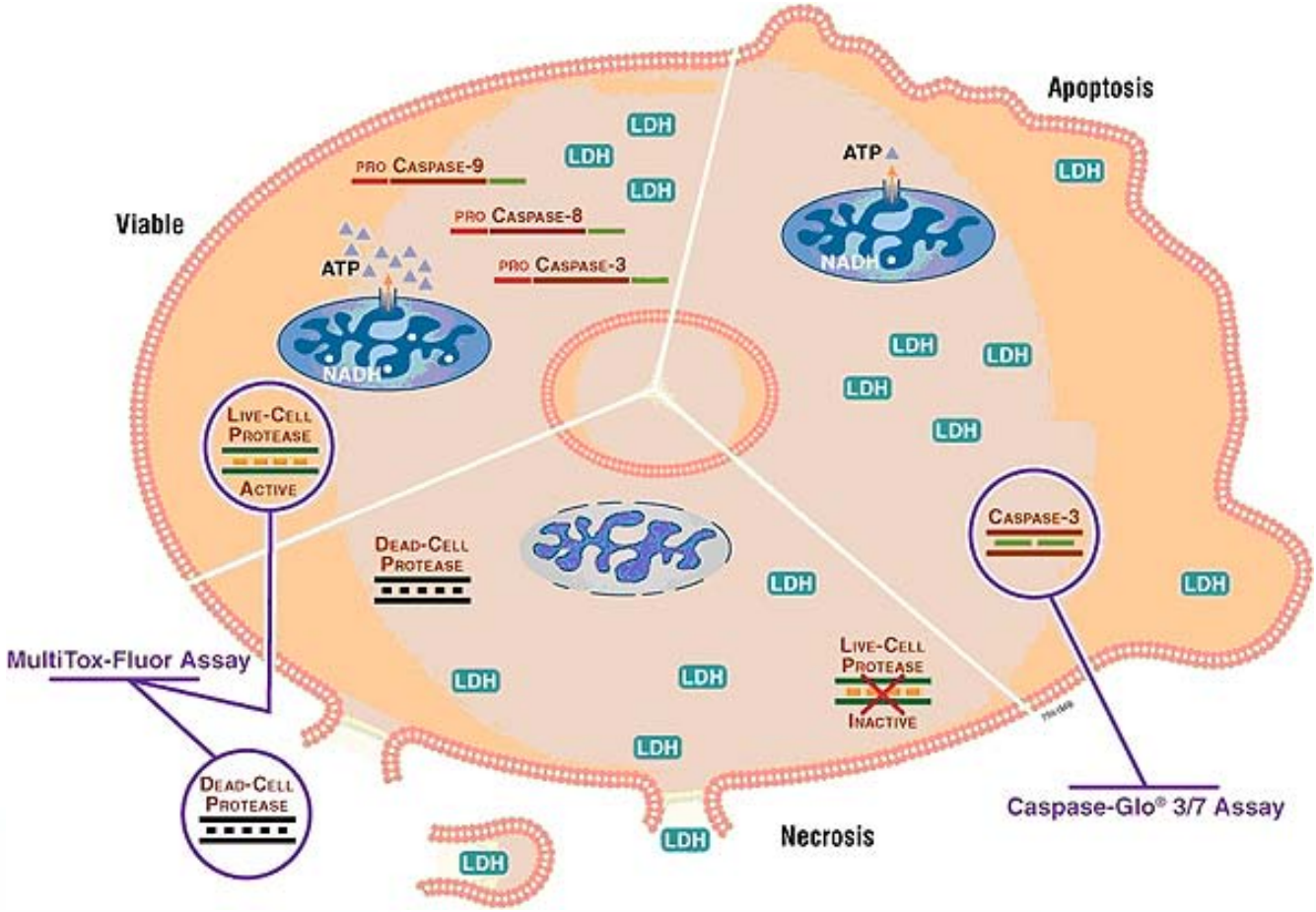
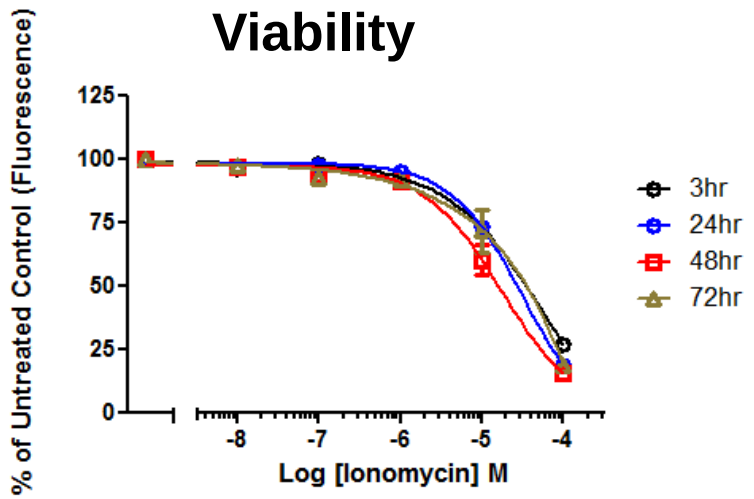
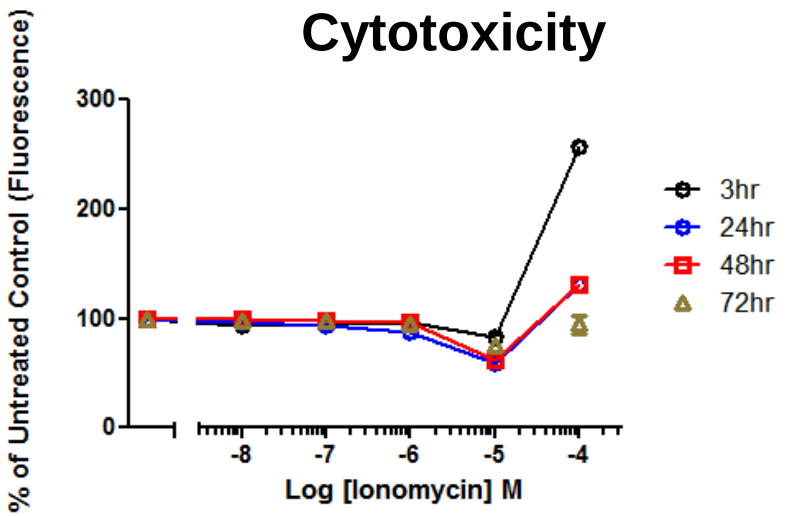
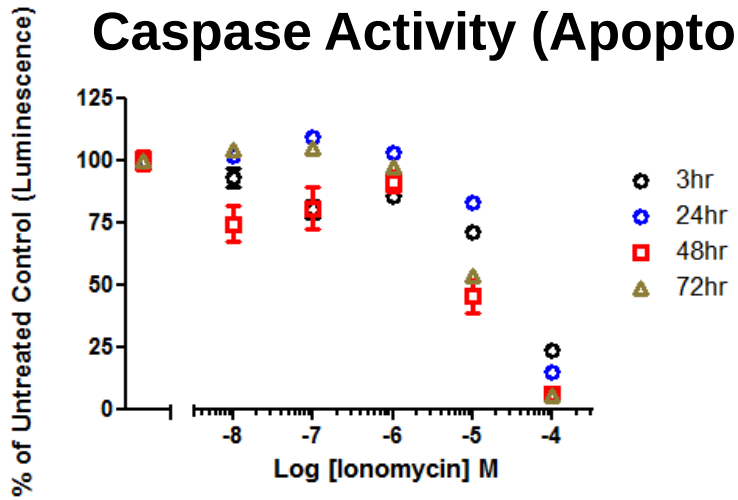
Multi-parametric analysis facilitates dissection of the cytotoxic process



Ionomycin (Fast-acting, 1° Necrosis Inducer)







Viability assays always tell you the relative number of cells left after treatment.

Activity-based cytotoxicity markers are definitive for cell death, but subject to degradation as a function of time.

All cell populations have a basal caspase activity. In the absence of apoptosis, this activity declines with necrotic cell death.

Terfenadine (Fast-acting, Apoptosis Inducer)

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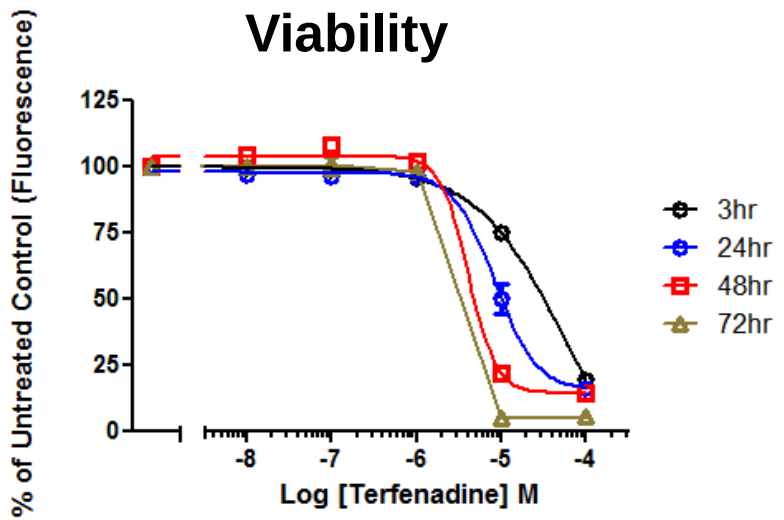
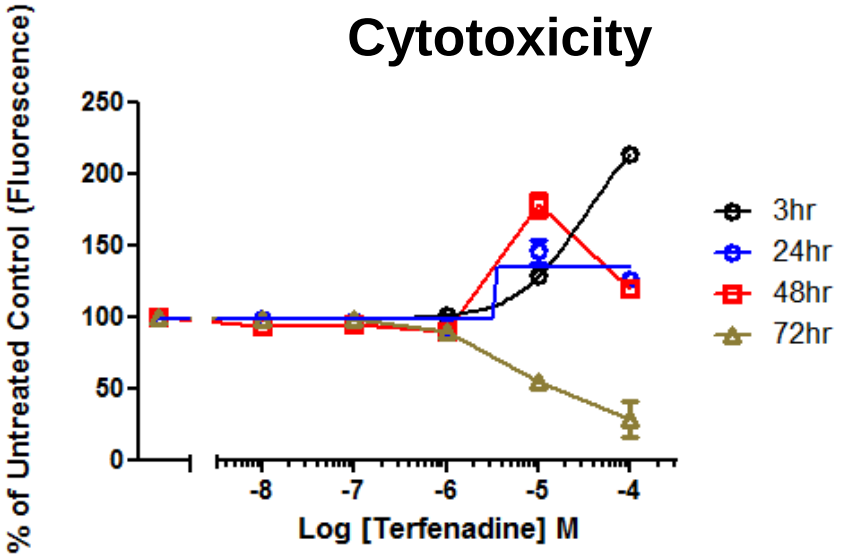
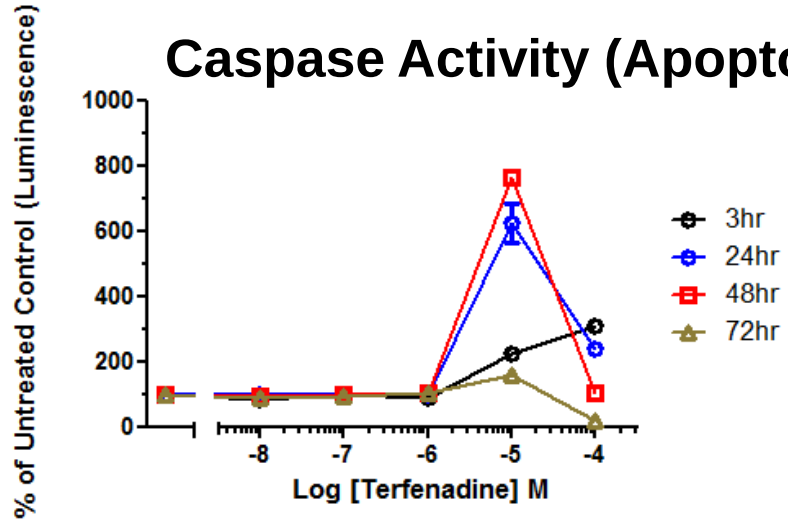




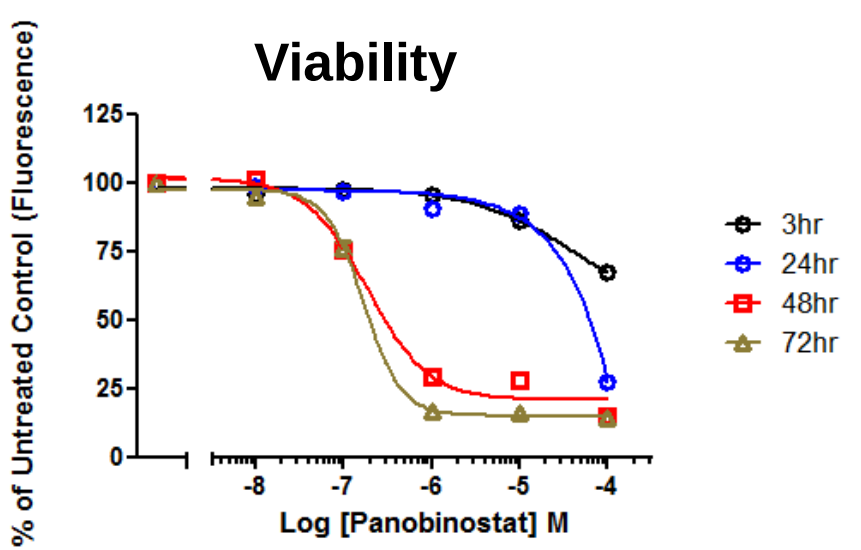


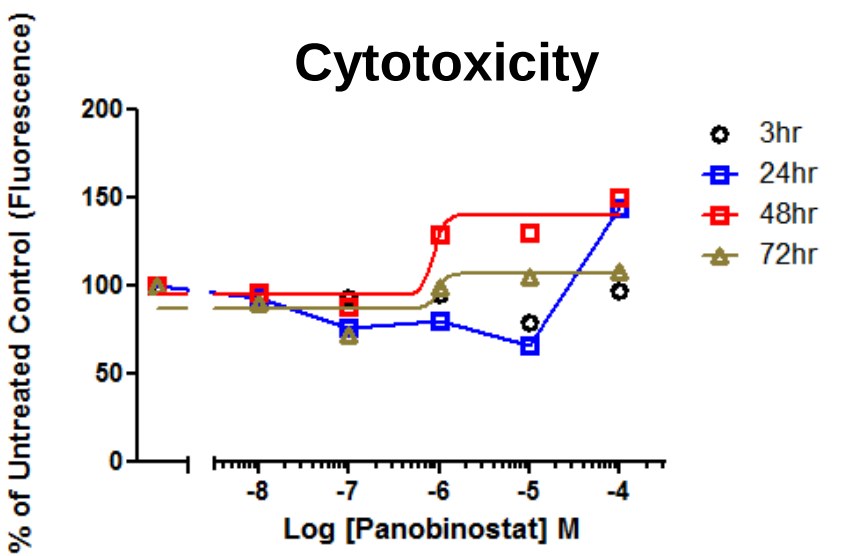
Viability assays always tell you the relative number of cells left after treatment.

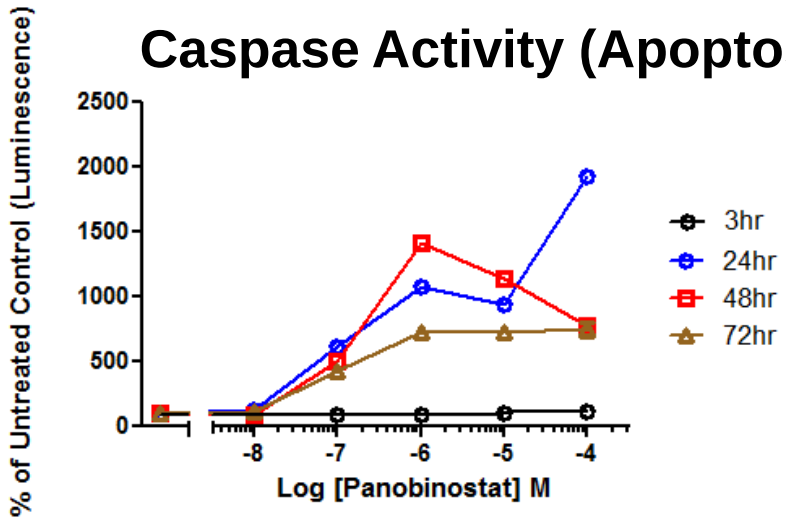
Activity-based cytotoxicity markers are definitive for cell death, but subject to degradation as a function of time.

Caspase activity above basal levels is definitive for apoptosis. Caspase activity declines when cells reach secondary necrosis (natural enzyme degradation).

Panobinostat (Slow-acting, Apoptosis)







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Activity-based cytotoxicity markers are definitive for cell death, but subject to degradation as a function of time.

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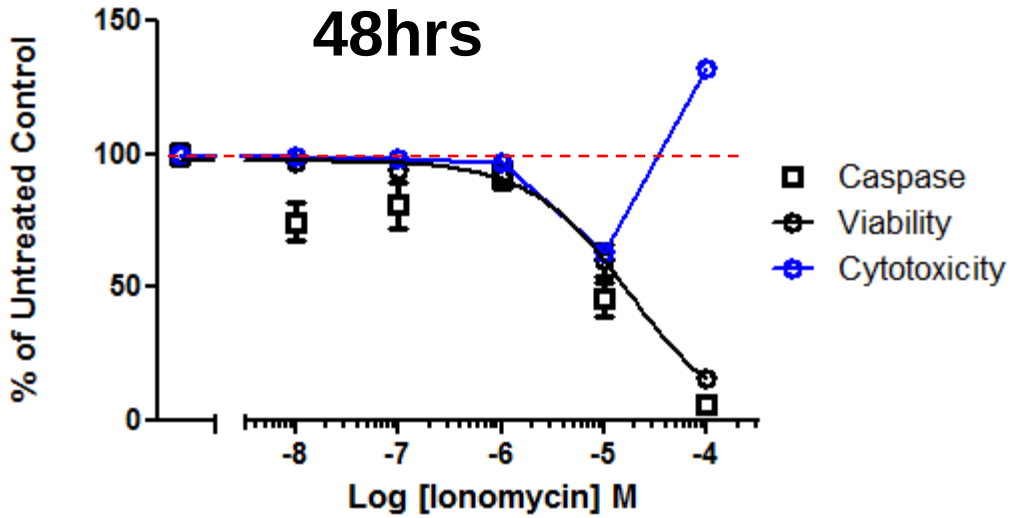
Cytotoxic Time Courses Preferred… Endpoints Can be Meaningful!

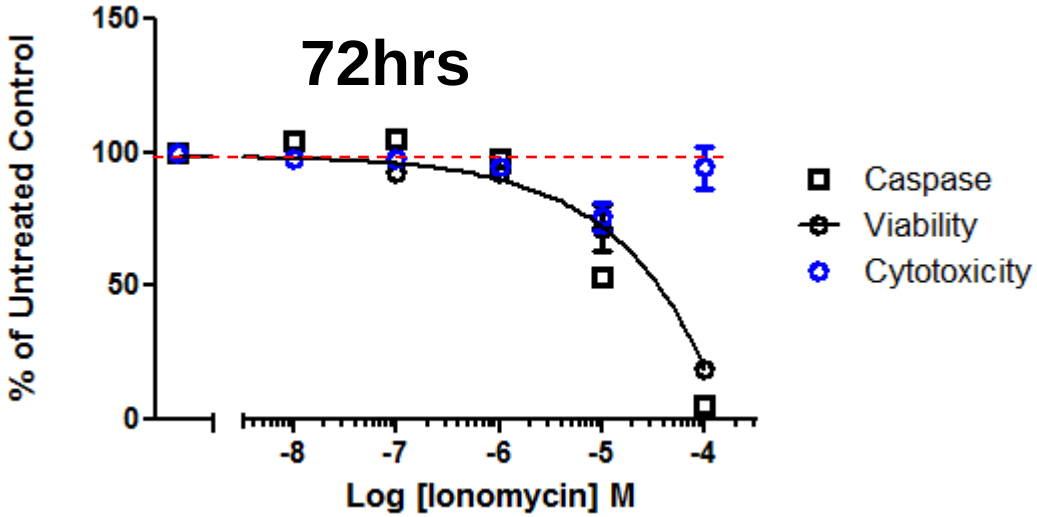


Caspase ⇩

Viability ⇩

Cytotoxicity ⇧

-Consistent with 1º necrosis



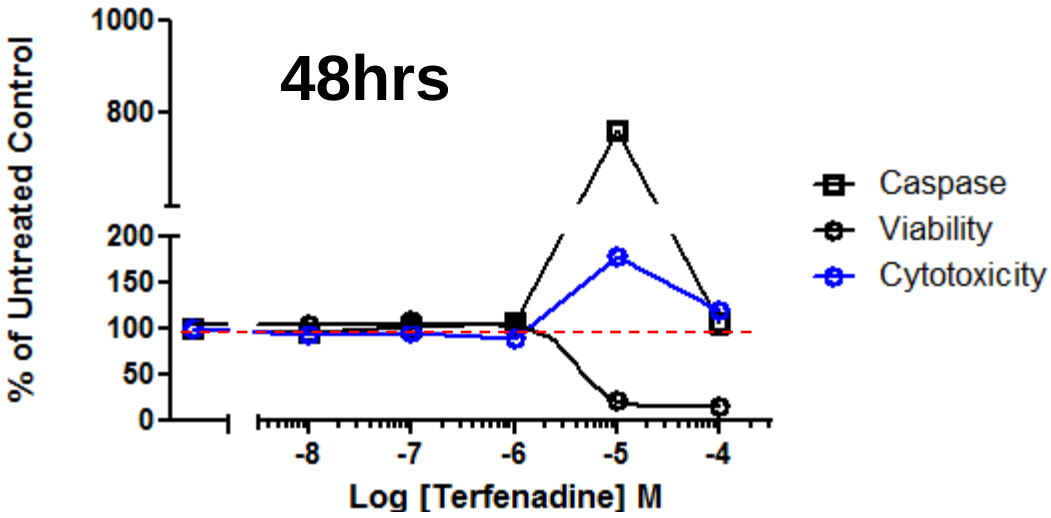
Caspase ⇩

Viability ⇩

Cytotoxicity ⇨

-Consistent with 1º necrosis
-Inconsistent with cell cycle arrest due to exposure period

Cytotoxic Time Courses Preferred… Endpoints Can be Meaningful!

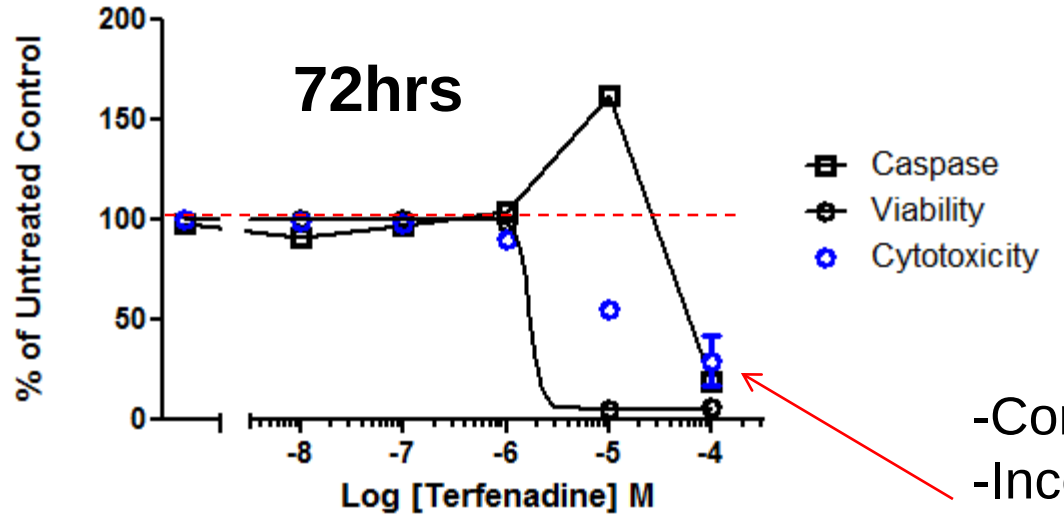


Caspase ⇧

Viability ⇩

Cytotoxicity ⇧

- Consistent with late apoptosis



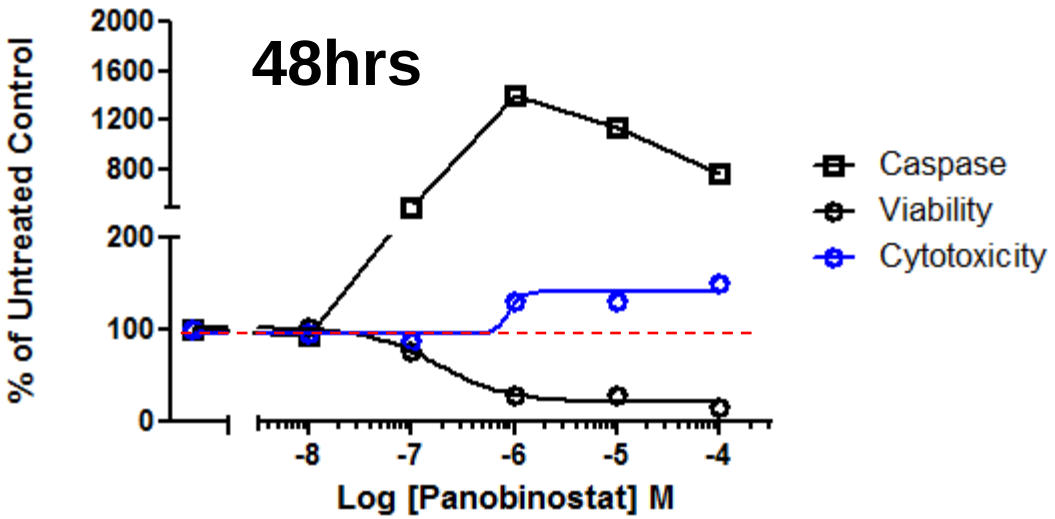
Caspase ⇧

Viability ⇩

Cytotoxicity ⇩

-Consistent with late(r) apoptosis
-Inconsistent with early cell cycle arrest (due to cytotoxicity and exposure)

Cytotoxic Time Courses Preferred… Endpoints Can be Meaningful!

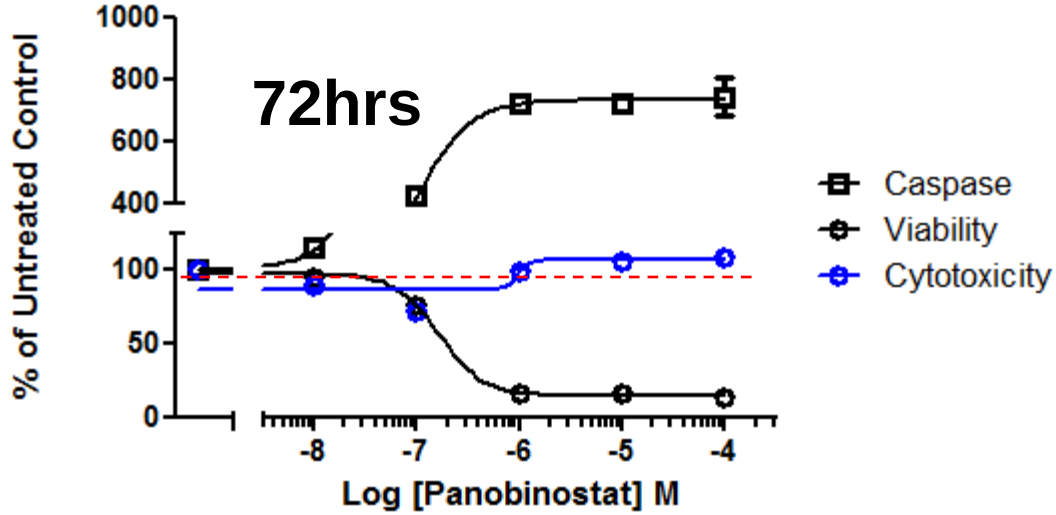


Caspase ⇧

Viability ⇩

Cytotoxicity ⇧

-Consistent with apoptosis



Caspase ⇧

Viability ⇩

Cytotoxicity ⇧

-Consistent with apoptosis

ApoTox-Glo™ Multiplexed MOA Assay

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Advantages

- Provides a detailed profile of the cytotoxic response
  - Primary Necrosis
  - Cell Cycle Arrest
  - Apoptosis
  - Secondary Necrosis
- Convenient, same-well multiplex
- Measures are inversely correlated, reducing optical interferences and providing "flagging" opportunities

Disadvantages

- Best employed in time course experiments requiring multiple times and data points per test compound
- Can be applied at specified endpoints…but biomarker degradation may complicate data interpretation

Other complimentary and orthogonal assay options for cytotoxicity determination?

Non-Activity Based Cytotoxicity Measures? Differential DNA Dye Excludability?

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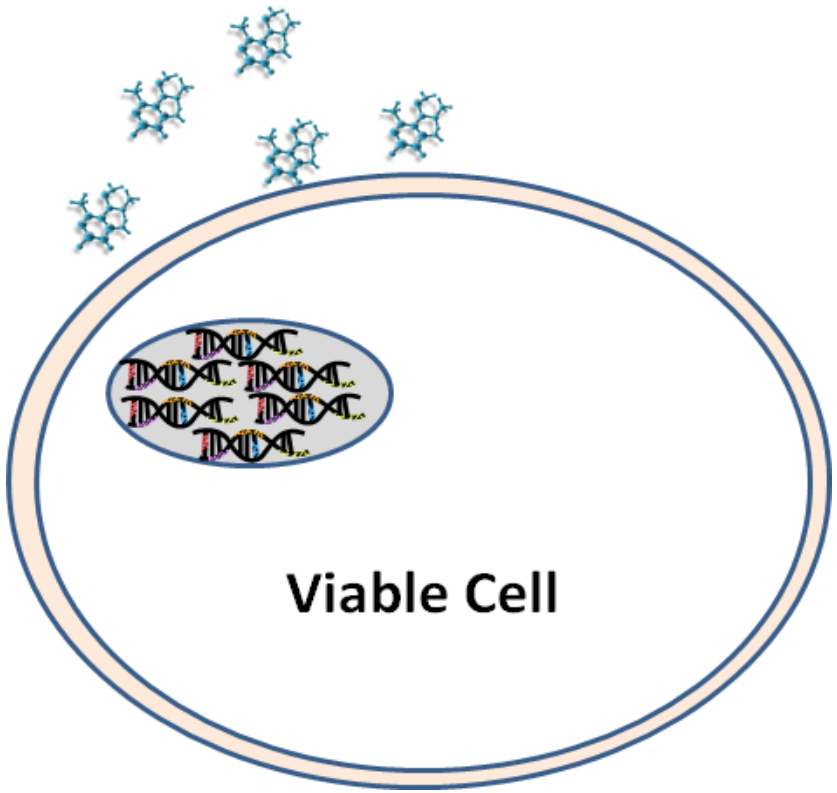
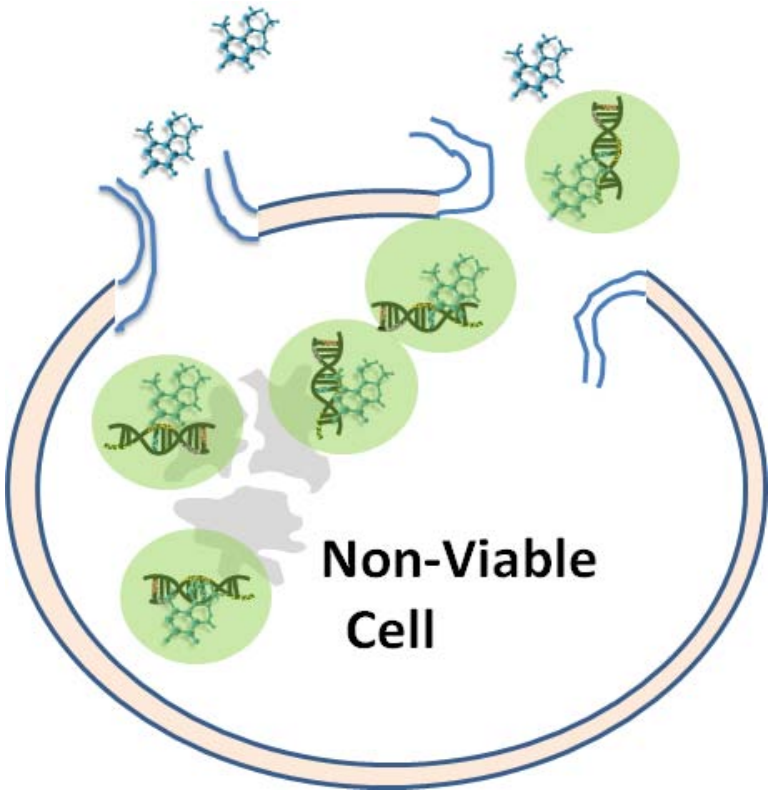
Membrane integrity "sensed" by environmental dye.



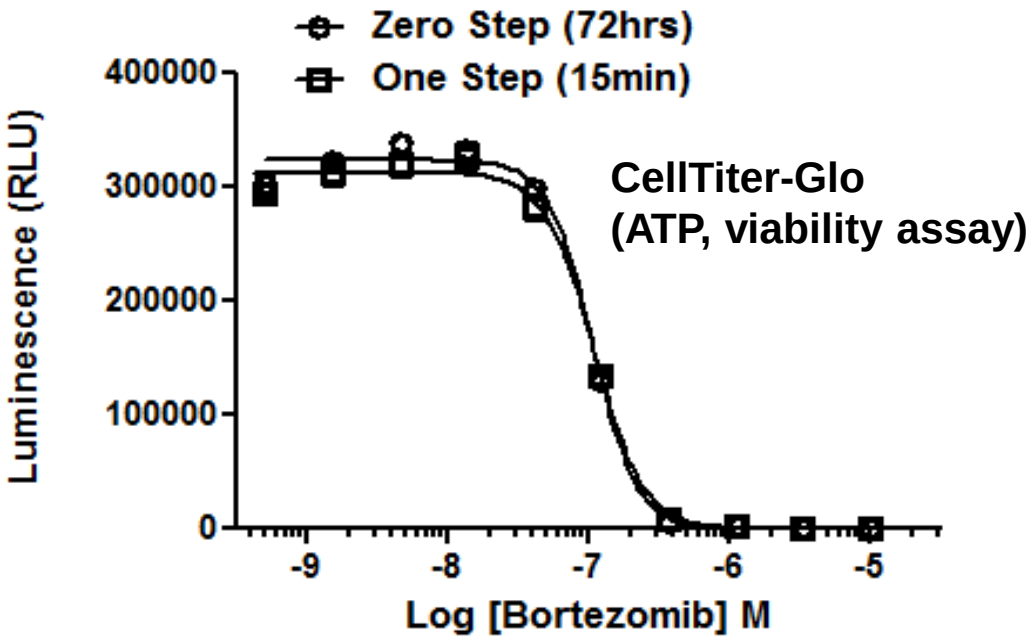
Excluded dye yields no increase in fluorescence.



Non-excluded dye yields increase in fluorescence

Probe "Inertness" and "No Step Format"*

*No Step means adding the dye at cell dosing… either in drug dilutions or with cells.



CellTox-Green™ does not alter viability or impact dose-dependence of cytotoxic model compounds in extended co-incubations. HeLa at 72hrs shown.

Kinetic Assay Format

Add DNA probe at cell seeding

↓

Add diluted test article

↓

Measure fluorescence at 4hrs

↓

Measure fluorescence at 24hrs

↓

Measure fluorescence at 48hr

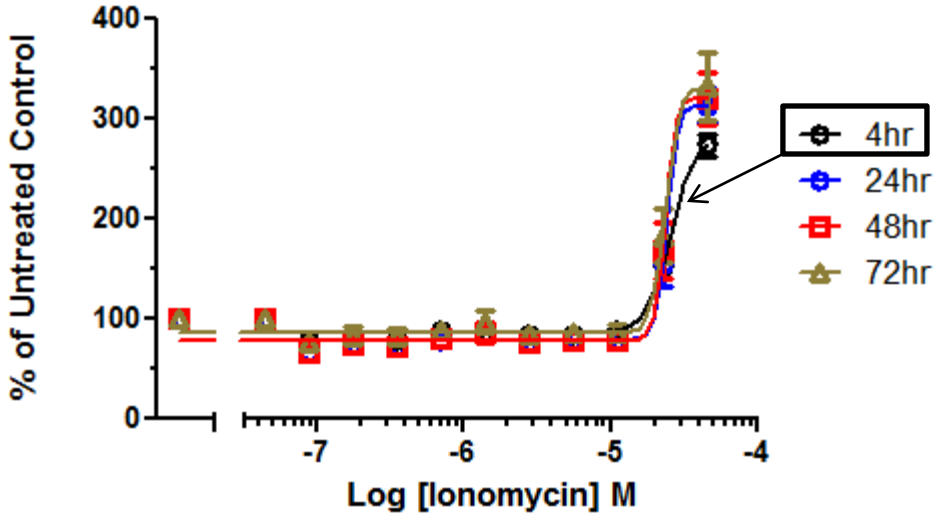
↓

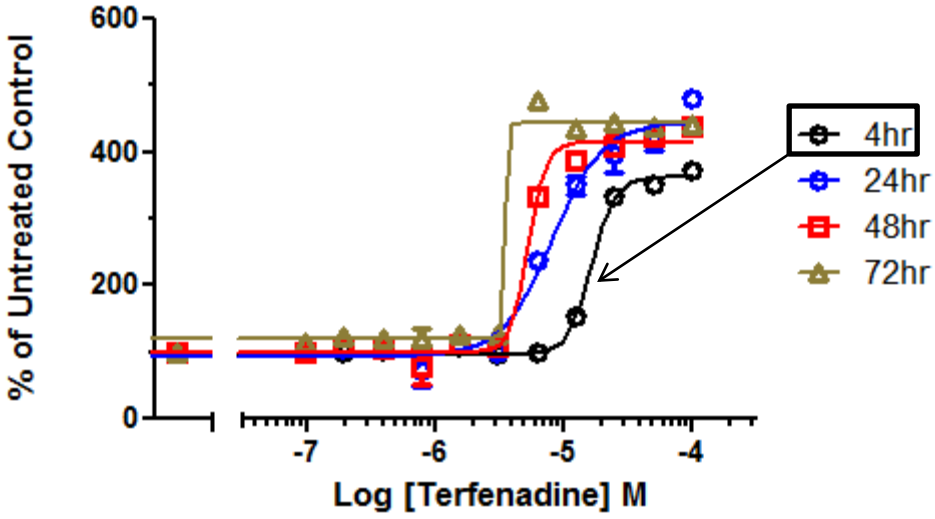
Measure fluorescence at 72hr

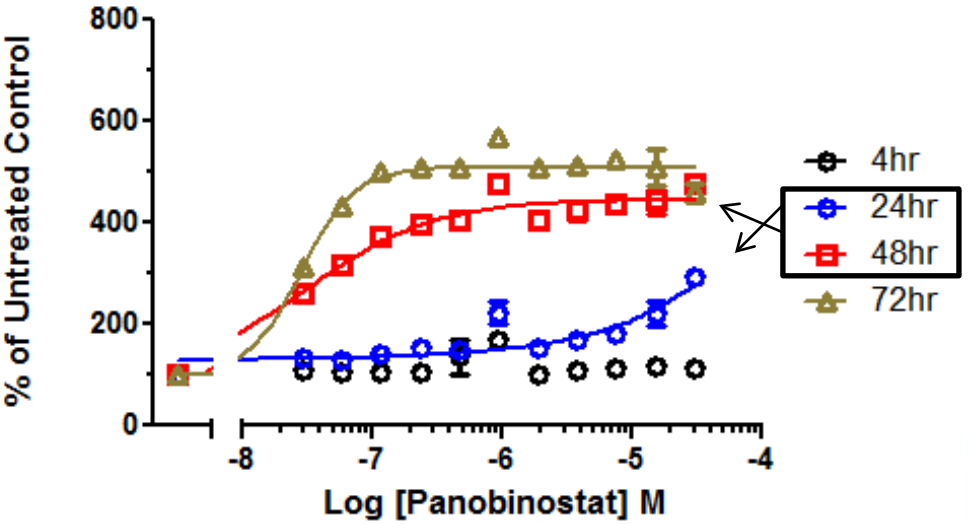
↓

Optional sequential multiplex with viability assay (CellTiter-Glo, CellTiter-Blue or CellTiter-Fluor)

CellTox-Green™: A Kinetic Cytotox Assay



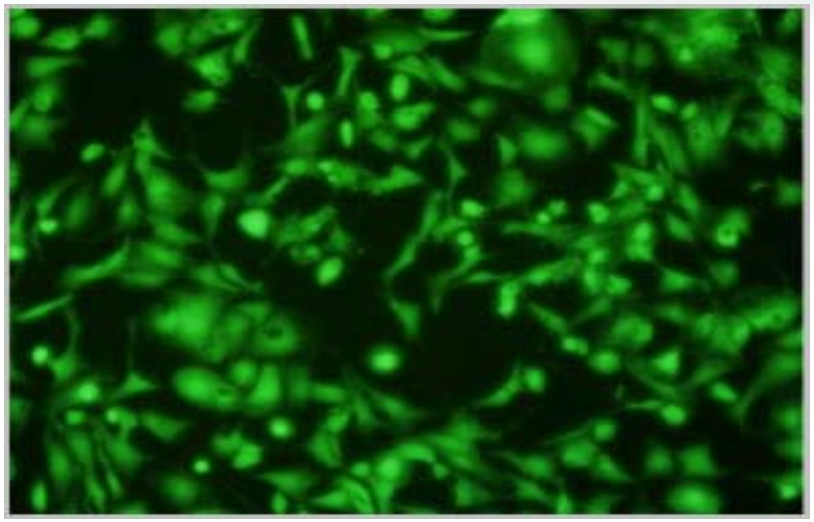




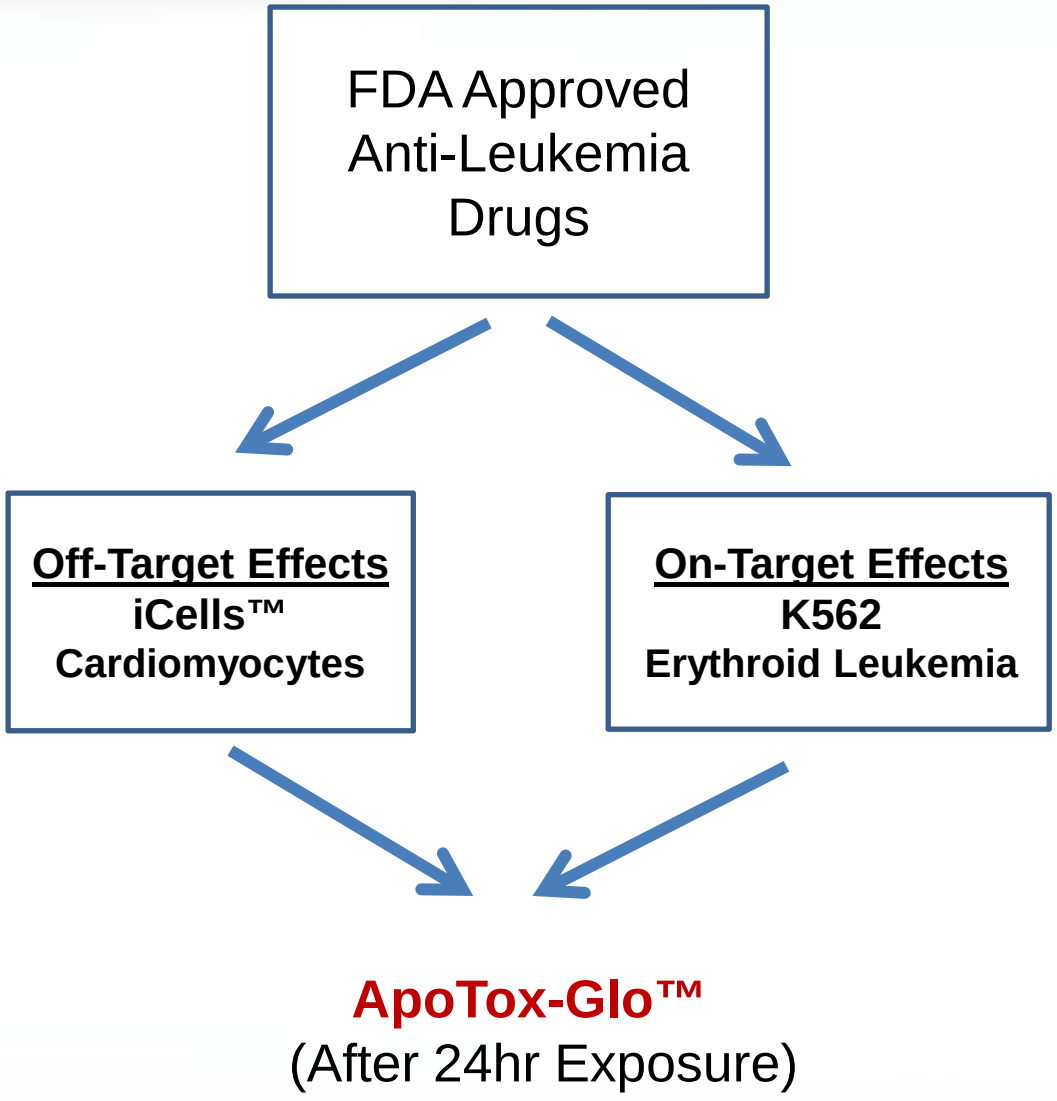
- Provides flexible, non-duplicative time course data for cytotoxicity
- Can be sequentially multiplexed at first emergence of cytotoxicity
- Data can be used to explore the complete cytotoxic response with a subsequent ApoTox-Glo assay

Potency and Safety Evaluation: Validation of ApoTox-Glo™ with Clinical Cancer Therapeutics

Promega



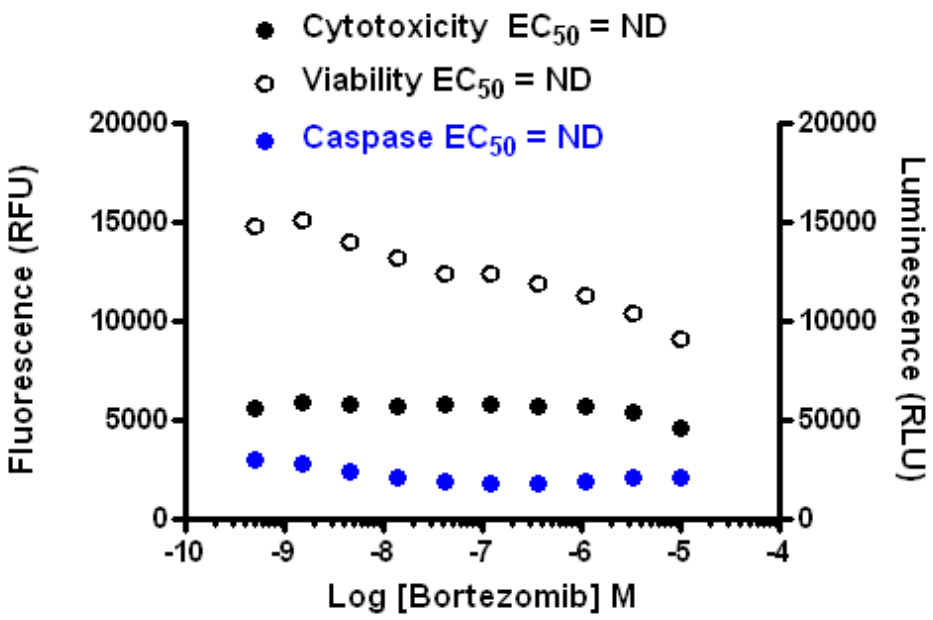
*"iCells™ are specifically designed to aid drug discovery and **improve the predictability of drug efficacy and toxicity** screens, weeding out ineffective and potentially toxic compounds early in the pharmaceutical pipeline process before significant time and resources have been invested".*
-Cellular Dynamics International



Proteasome Inhibitor (Velcade™)

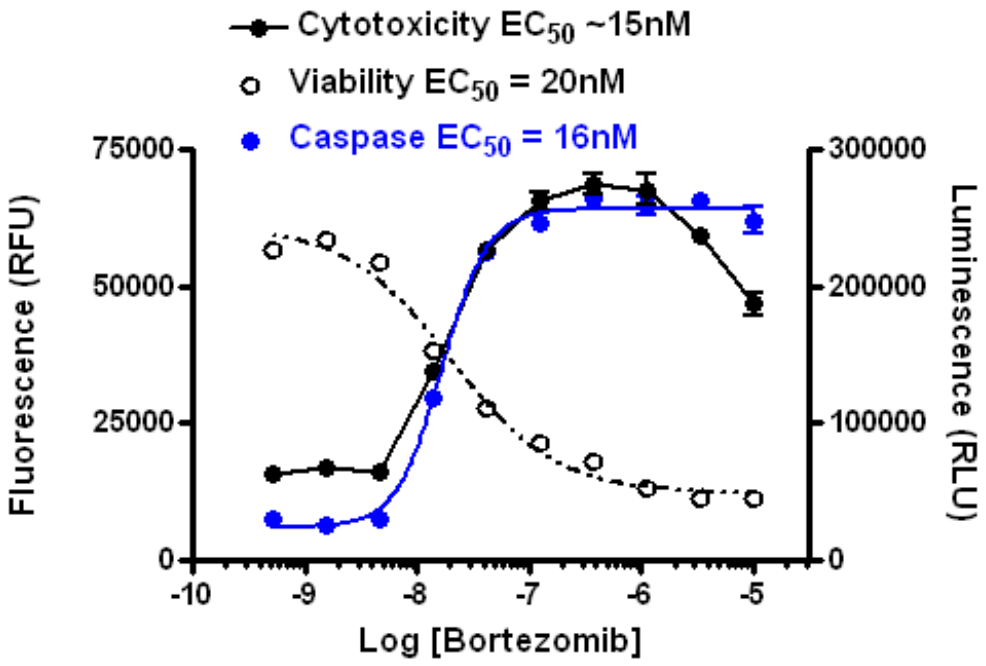
Promega

iCell™



**No apparent cytotoxicity*
or caspase activation**

K562



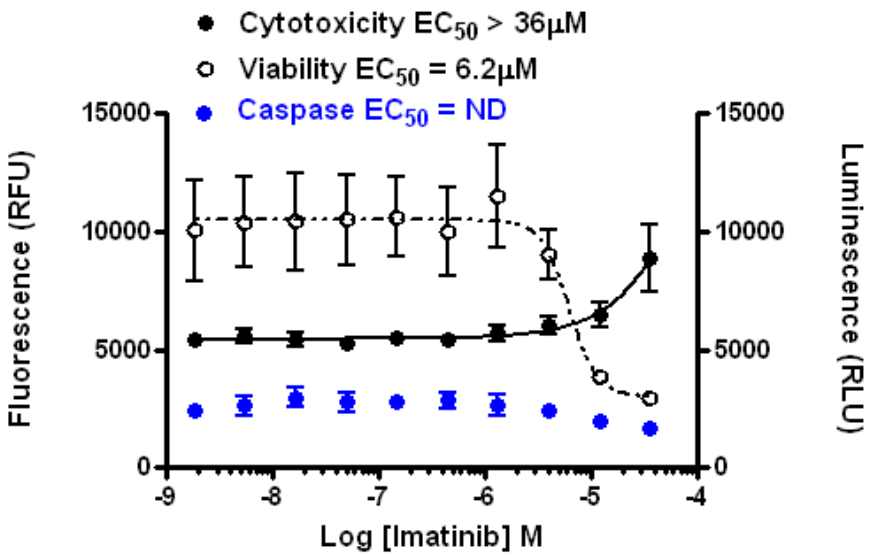
Cytotoxicity by apoptosis

*Bortezomib (and other proteasome inhibitors) are known to partially inhibit the viability assay protease biomarker at concentrations greater than 1µM

Tyr Kinase Inhibitor (Gleevec™)

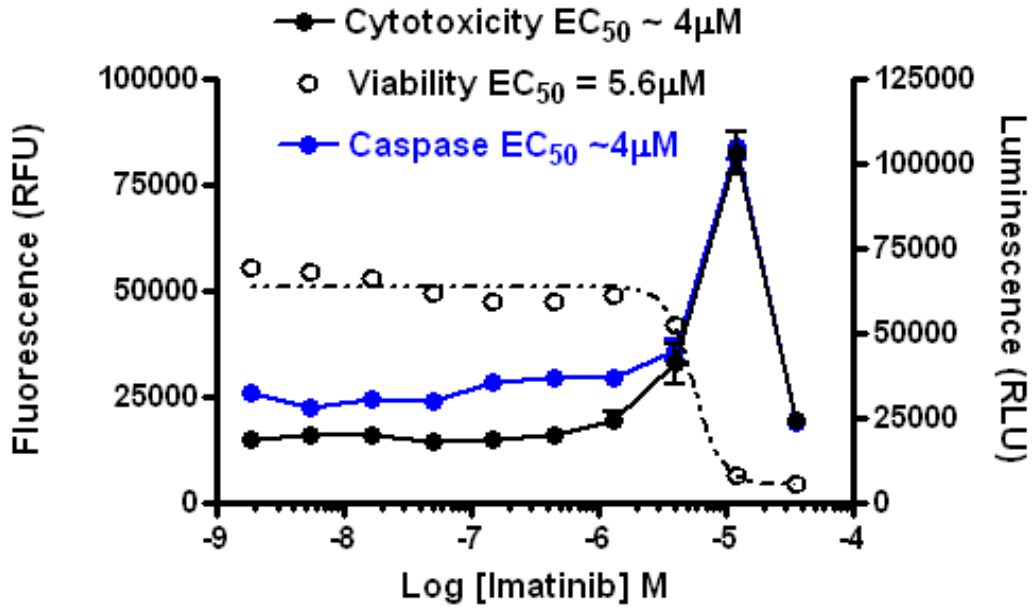
Promega

iCell™



Caspase-independent cytotoxicity at concentrations greater than 1µM

K562



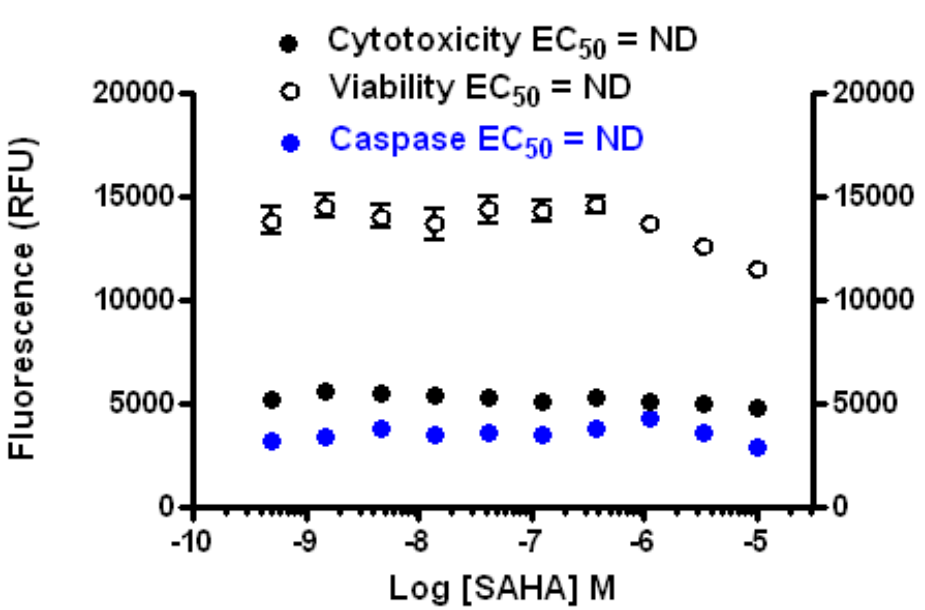
Cytotoxicity by apoptosis

Note: Diminution of caspase and cytotoxicity biomarker signals at highest doses of imatinib (with K562) are consistent with activation kinetics and time-dependent biomarker decay.

Histone Deacetylase Inhibitor (Vorinostat™)

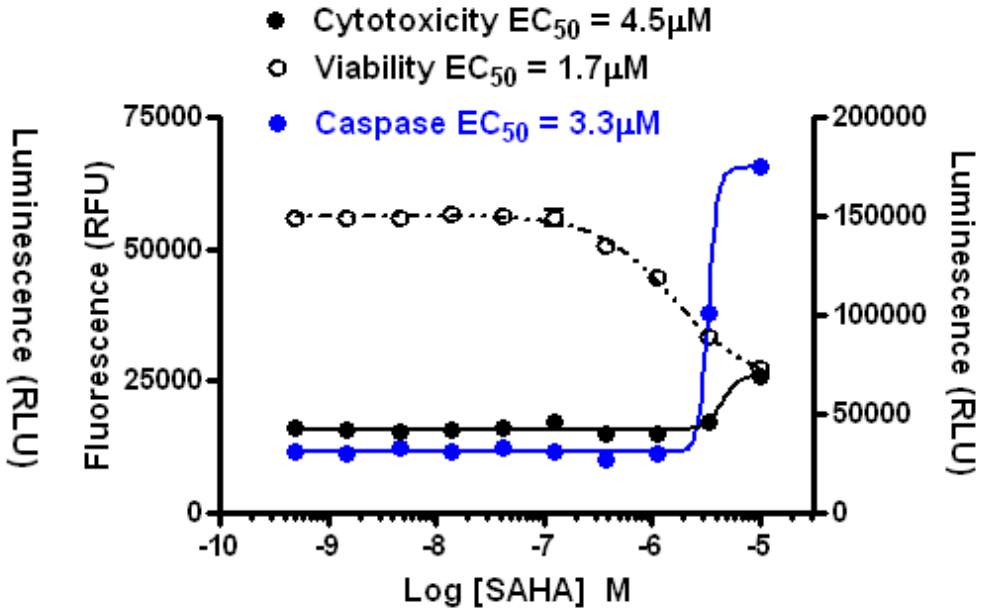
Promega

iCell™



No apparent cytotoxicity or caspase activation.

K562



Cytotoxicity by apoptosis

Targeted Activities…Differential Cytotoxicity

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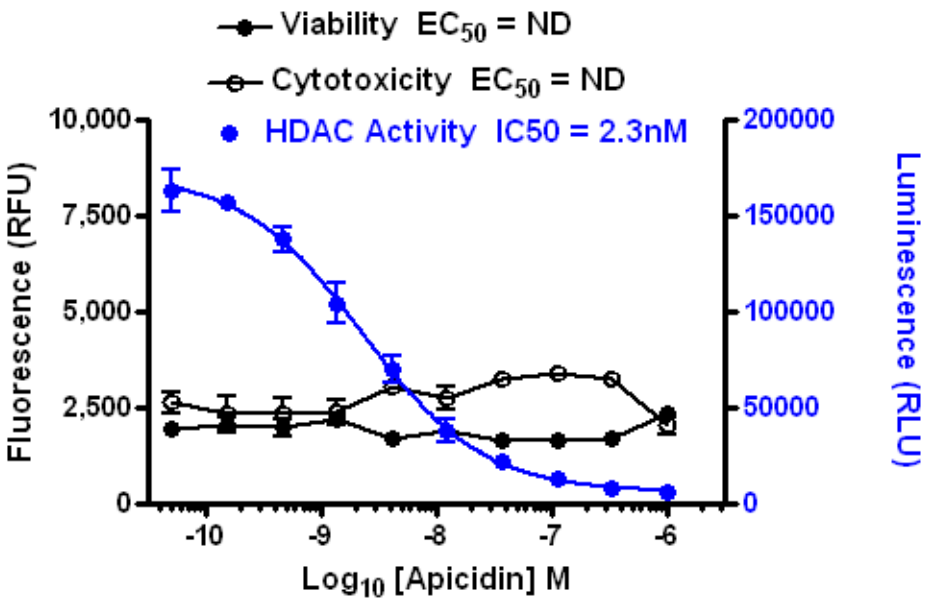
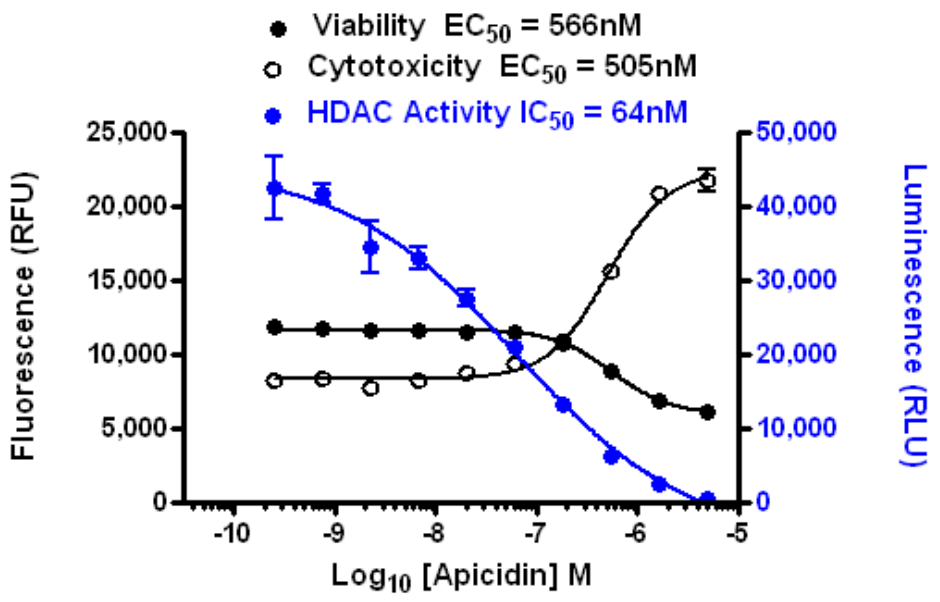


U937 (Cancer Cells)



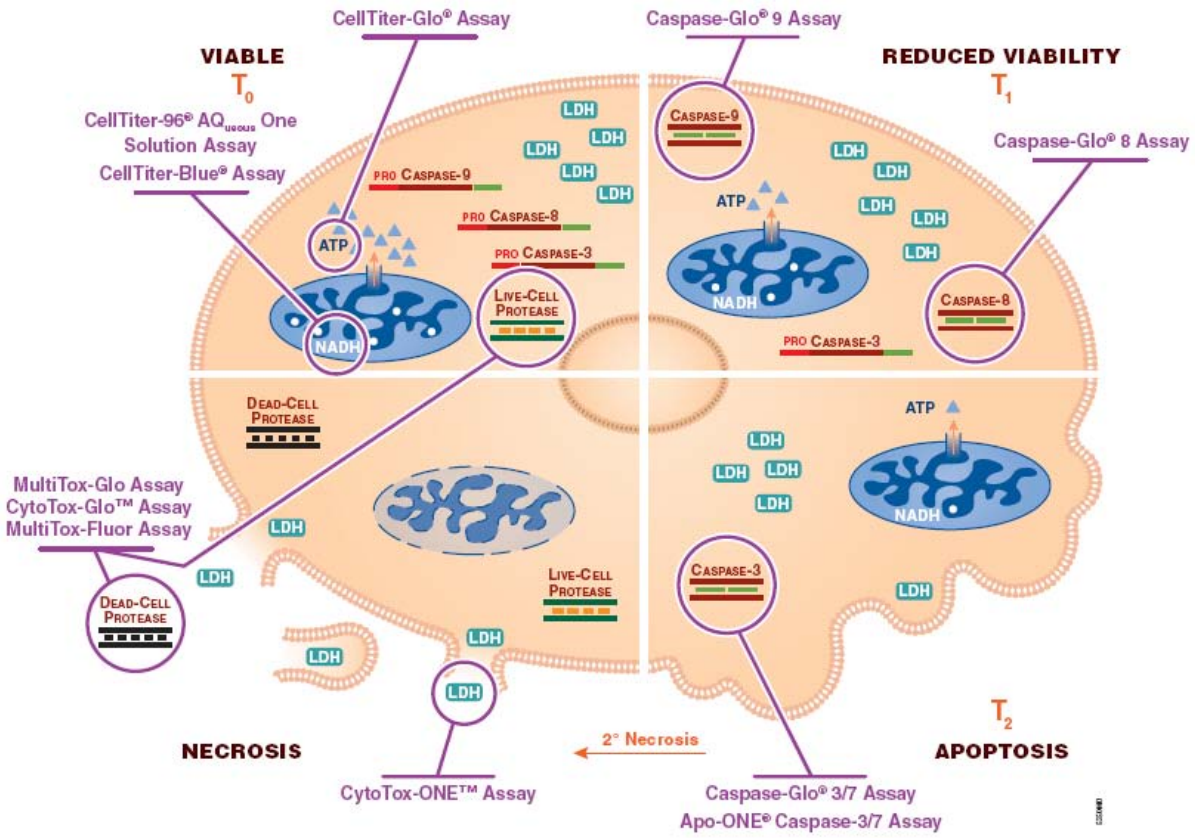
[24hr compound exposure]

MultiTox-Fluor followed by HDAC-Glo™ I/II

Summary

In vitro cell health data is influenced by:

- Dosage
  - Addressed through serial dilution series
- Exposure Time (cells with compound contact)
- Mechanism of action of the test compound
- Cell Type
  - -specific target
  - -off target



…and ApoLive-Glo™ , ApoTox-Glo™, HDAC-Glo™ I/II Assays!

For more information contact:
andrew.niles@promega.com

Acknowledgements

Promega

Promega Scientists and Support Staff Worldwide!