How to Measure Live and Dead Cells in Real Time using a Plate Reader

Terry Riss
June 2015
Presentation Outline

Traditional endpoint viability assay technologies

Measuring viable cell number in real time
  • How the assay works
  • Example data including multiplexing

Measuring accumulation of dead cells in real time
  • How the assay works
  • Example data including multiplexing

Advantages & disadvantages of each method

Summary
How Live and Dead Cell Assays Work

Viable cells maintain active metabolism
Dead cells lose membrane integrity

Viable

Dead

Substrate → Product

Dye

Enzyme Marker
Metabolic Indicators of Cell Viability

Reagent

Tetrazolium Reagents
- MTT, MTS, XTT

Redox Indicators
- Resazurin

RealTme-Glo™
- Pro-substrate

Viable Cell
- Active Metabolism
- Incubation Period
- Substrate
- Product

Dead Cell
- No Rxn
- Substrate

Substrate
Why Not Use MTT or Resazurin Assays?

- MTT and resazurin have been shown to be toxic to cells
- Sensitivity of MTT assay limits signal to background ratio
- Compounds interference resulting in background signal
- Formazan or resorufin product accumulates resulting in misinterpretation of rapid cell death events
MTT is Toxic to Balb 3T3 Cells

Images captured by Tracy Worzella using Incucyte instrument from Essen Biosciences
Resazurin is Toxic to Balb 3T3 Cells

Time Zero 4 hours

MTT Assay Sensitivity is Not Adequate for Low Cell Numbers

HCT116 cells were cultured in the InSphero GravityPLUS™ 3D Cell Culture system for 4 days to form 340 and 640 µm microtissues. Spheroids were processed to measure ATP (CellTiter-Glo® 3D), resazurin reduction (alamarBlue), or MTT reduction following standard procedures.
Compound Interferences with Tetrazolium Assays

https://www.promega.com/resources/pubhub/is-your-mtt-assay-really-the-best-choice/

Is Your MTT Assay Really the Best Choice?

ABSTRACT

The MTT assay was the first widely accepted method that replaced the radioactive tritiated thymidine incorporation assay to measure cell proliferation. However, there are several limitations associated with using the MTT assay. A better understanding of these limitations has influenced experienced assay development scientists to choose assay technologies that are better suited for their applications and have properties superior to MTT assays. This article briefly describes some of the disadvantages of the MTT assay method as well as highlighting alternative methods.

Terry Riss
Promega Corporation
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Introduction

Use of the MTT tetrazolium compound to measure the number of viable cells in culture was first described by Mosmann in 1983 (1). The state of the art for high throughput screening (HTS) in the 1980s was transitioning into 96-well plates and the MTT assay represented the first homogeneous assay method that was useful for HTS. The broad adoption of this method was based on the simplicity of the homogeneous protocol, which includes adding two reagents to the assay wells, but does not require extra steps such as removing liquid or washing the cells that were necessary for radioisotope incorporation assays. For these reasons, the MTT assay was the first widely accepted method that replaced the radioactive tritiated thymidine incorporation assay used to measure cell proliferation.
Comparison of RealTime-Glo™ Assay to alamarBlue Endpoint Approach

Resorufin from alamarBlue assay accumulates in medium resulting in misinterpretation of rapid cell death events. Substrate from real time assay is rapidly used by NanoLuc and does not accumulate.
**Viable Cell Protease Assay (CellTiter-Fluor™)**

- **Cell Permeable Protease Substrate (GF-AFC)**
  - Viable cells retain protease activity and generate signal
  - “Viable” Cell protease becomes inactive upon cell death

- **Viable Cell**
  - “Viable” Protease
  - GF-AFC
  - AFC Fluorescence 400/505nm

- **Dead Cell**
  - “Viable” Protease Inactive
  - Inactive “Viable” Protease
GF-AFC Exposure for 4 hours is Not Toxic to Balb 3T3 Cells

Images captured by Tracy Worzella using Incucyte instrument from Essen Biosciences.
ATP Assay for Cell Viability

CellTiter-Glo® Assay Reagent

- Lysis Solution
- ATPase Inhibitors
- Luciferin
- UltraGlo Luciferase

Viable Cell

ATP

Luciferin + Luciferase

Light

Dead Cell

ADP

No Reaction

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CellTiter-Glo® (ATP) Assay

Advantages:

- Speed (read signal in 10 minutes: Immediate cell lysis)
- Sensitivity (can detect 4-15 cells)
- Addition of reagent “stops” reactions
- No fluorescence interference
- Stable “glow-type” signal (5 hour half-life)

Disadvantages:

- ATP/cell may change with some treatments
- Potential for luciferase inhibitor effects
- Other multiplexed assays must be run first
## Advantages & Disadvantages of Traditional Endpoint Viability Assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>MTT / MTS</td>
<td>Widely used</td>
<td>2 step protocol (MTT)</td>
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<tr>
<td></td>
<td>Inexpensive</td>
<td>1-4 hour incubation</td>
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<td></td>
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<td>Limited sensitivity</td>
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<td>Interference by reducing compounds</td>
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<td></td>
<td>Toxic to cells</td>
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<td></td>
<td>Endpoint assay</td>
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<tr>
<td>Resazurin</td>
<td>Inexpensive</td>
<td>1-4 hour incubation</td>
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<tr>
<td></td>
<td>Fluorescent readout</td>
<td>Interference by reducing compounds</td>
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<tr>
<td></td>
<td>Good sensitivity</td>
<td>Toxic to cells in some cases</td>
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<tr>
<td></td>
<td></td>
<td>Fluorescence interference</td>
</tr>
<tr>
<td>Protease</td>
<td>Faster 30 min protocol</td>
<td>Fluorescence interference</td>
</tr>
<tr>
<td></td>
<td>Cells remain viable</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Better sensitivity than resazurin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Good choice for multiplexing</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>Reagent stops reaction immediately</td>
<td>Lytic protocol dictates sequence for multiplexing</td>
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<tr>
<td></td>
<td>10 min protocol</td>
<td>Endpoint assay</td>
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<tr>
<td></td>
<td>Best sensitivity</td>
<td></td>
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<td></td>
<td>No fluorescence interference</td>
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RealTime-Glo™ MT Cell Viability Assay
What is a Real Time Assay and What are the Advantages?

• Designed to provide information on the same population of cells by recording data at various times
• Reagents have little or no effect on the viable population of cells which enables multiplexing secondary assays on the same sample
• Reduces error from preparing replicate samples
• Improves efficiency by reducing the number of assay plates and reagents needed for development activities
RealTime-Glo™ MT Cell Viability Assay

- Monitor viable cells continuously over 72 hours, saving time, cell samples, culture and reagent costs
- Option to add reagent before seeding, with dosing of compound, or at the end
- Sensitivity is greater than colorimetric or fluorometric cell viability assays
- Multiplex with other assays and downstream applications
The “RealTime” Viability Assay Measures Reducing Potential of Cells

- **Luciferase** and **Pro-substrate** are added as reagents to culture medium
- Pro-substrate enters the cell and is reduced to form a substrate for luciferase
- Substrate diffuses from the cell and is used by luciferase to produce light
RealTime-Glo™ Assay Protocol

Seed cells in medium containing RealTime-Glo™ Reagent

↓

Add test compound

↓

Record luminescence (continually for up to 3 days)
Measure Changes in Viability Over Time

The luminescence signal was determined every hour for 72 h in a Tecan M200 plate reader with gas control module (37°C/5%CO₂).
iCell cardiomyocytes were plated and grown in medium containing pro-substrate and NanoLuc luciferase. After 2 days, digitonin was added to a final concentration of 200 µg/ml.
RealTime-Glo™ Reagents are Not Toxic

PC3 or SKBR3 cells cultured in the presence or absence of RealTime-Glo™ Reagent for 3 days. Samples were tested for membrane integrity using CytoTox-Fluor™ Cytotoxicity Assay.
RealTime-Glo™ Reagent May Affect ATP Levels After 3 Days in Culture

ATP assay of cells with and without RealTime-Glo™ Reagent
Determining Optimal Cell Number is Recommended for Long Term Assays

• Large numbers of metabolically active cells will eventually deplete the RealTime-Glo™ Pro-substrate from culture medium and result in reduced signal intensity

• Pro-substrate depletion is dependent on cell type, cell number and length of incubation

• Testing a range of cell number per well and length of incubation is recommended to confirm linearity
RealTime-Glo™ Assay Signal Linearity Over 72h is Dependent on Seeding Density
RealTime-Glo™ Assay Signal Linearity Over 72h is Dependent on Seeding Density
Real Time Assays Improve Efficiency

Viability assay development using an endpoint method requires multiple plates.

Assay development using a “real time” method uses one plate...

...and much less reagent.
Multiplexing Examples Using RealTime-Glo™ MT Cell Viability Assay
Multiplexing with Real Time Assays

A major advantage of using non-toxic reagents in the real time assays is the population of cells remains viable and available for subsequent multiplexing.

Examples:

- Orthogonal cell viability assay measuring a different marker
- Cell death / membrane integrity
- Apoptosis
- Luciferase reporter
- RNA extraction
HCT116 cells (1000/well) were seeded and RealTime-Glo™ signal monitored at 1, 24, 48 and 72 hours. CellTiter-Glo® Reagent was added to the same plate after 72 hours and luminescence recorded. Values represent mean ± SD of n= 5.
HepG2 cells were plated at 10,000 cells/well in the presence of the RealTime-Glo™ Reagent and CellTox™ Green Dye. Cells were treated with various concentrations of terfenadine for 6 hours, then luminescence and fluorescence recorded using a GloMax® Discover multimode reader. Each point represents the mean ±SD from 4 samples.
THP1 cells were grown in medium containing the RealTime-Glo™ Assay reagents and treated 1 μM doxorubicin. Cell viability was monitored every 4 hours and Caspase-Glo® 3/7 multiplexed at indicated times.
Seed HEK293 cells expressing luciferase in 384 well plate

- Incubate overnight

Add RealTime-Glo™ Reagent

- Incubate 2 hours

Record luminescence

Add firefly luciferase reagent

- Incubate 10min

Record luminescence

Firefly luciferase reporter assay signal is not affected by the presence (red squares) or absence (green triangles) of RealTime-Glo™ Reagent
RealTime-Glo™ MT Cell Viability Assay Applied to 3D Microtissues

Hanging Drop Spheroids of HEK293 Cells

Signal/Background vs. Microtissue Diameter (µm)
RealTime-Glo™ Reagents Do Not Effect RNA Yield using QuantiFluor® RNA System

RNA yield

- Maxwell® + RT-Glo
- Maxwell® - RT-Glo
- ReliaPrep® + RT-Glo
- ReliaPrep® - RT-Glo

![Graph showing RNA yield vs. Microtissue Diameter (µm) for different sample preparation methods.](image)
RNA Integrity is Not Affected by Presence of RealTime-Glo™ Reagent

RNA Integrity - Bioanalyzer

<table>
<thead>
<tr>
<th>Microtissue Diameter (µm)</th>
<th>RIN values</th>
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<tbody>
<tr>
<td>160</td>
<td>9</td>
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<tr>
<td>212</td>
<td>9</td>
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<tr>
<td>442</td>
<td>9</td>
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<td>1075</td>
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<td>1075</td>
<td>9</td>
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<tr>
<td>1075</td>
<td>9</td>
</tr>
</tbody>
</table>

+ RT-Glo

Maxwell

-RT-Glo

ReliaPrep

-RT-Glo
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.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>With Medium Only</th>
<th>With RealTime-Glo™ Reagent</th>
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<tbody>
<tr>
<td>A549</td>
<td>30.9</td>
<td>30.3</td>
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<tr>
<td>K562</td>
<td>31.3</td>
<td>30.2</td>
</tr>
<tr>
<td>MCF7</td>
<td>30.9</td>
<td>30.1</td>
</tr>
<tr>
<td>THP-1</td>
<td>29.9</td>
<td>29.9</td>
</tr>
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</table>
Advantages of RealTime-Glo™ Assay

• Provides kinetic information on viable cell number during the course of experiments that enables “on the fly” decision making

• Optional protocols enables reagent to be added when cells are plated, when test compound is added, or at any time point when cell viability measurements are needed

• Sensitivity is better than colorimetric or fluorometric viability assays that measure reducing potential of cells

• Viable cells remain after applying RealTime-Glo™ Reagent (i.e. the reagent is not toxic)

• Remaining viable cells enable a variety of opportunities for multiplexing with other assay chemistries
Real Time Detection of Dead Cells
Detecting Dead Cells

Cell viability is defined based on membrane integrity

Viable

Dead

Dye

Enzyme Marker
LDH-Release Assay Time Course Showing Loss of Enzymatic Activity After 24 Hours

Tamoxifen-treated HepG2 Cells

Fluorescence ( Thousands )

0  2  4  6  8  10  12

0  40  80  120  160

Tamoxifen (µM)

0hr  0.5hr  2hr  6hr  24hr

Assay & Drug Devel Tech 2(1): 51, 2004
DNA Dye Staining to Detect Dead Cells
(Overcomes some limitations of short half-life markers)

CellTox™ Green non-permeable DNA dye

Staining of dead cells results in a fluorescent signal that is stable.

- Dye is excluded from live cells
- DNA dye only stains nucleus of “dead” cells or debris
HepG2 cells were treated with various doses of Terfenadine. CellTox™ Green Dye was added and fluorescence was measured every hour for 3 days. Increasing fluorescence indicates an increase in the number of dead cells.
CellTox™ Green Dye is Not Toxic to Cells
...and does not affect response to other toxins

ATP assay data showing viability of cells exposed to DNA binding dye for 15 minutes or 72 hours.
- Dye is non-toxic for at least 72 hours
- No effect on IC$_{50}$ value of test compounds
Reading the Same Plate Multiple Times to Detect the Onset of Cell Death

5000 K562 cells in 96 well plate

First appearance of cell death may trigger further experimentation with the same sample.
Multiplexing Examples Using CellTox™ Green Cytotoxicity Assay
Samples Stained with DNA Dye can be Multiplexed with Cell Viability and Apoptosis Assays

Add DNA dye when seeding cells

Record fluorescence from dead cells

Add GF-AFC Reagent

Incubate

Record fluorescence from live cells

Add Caspase Reagent

Record luminescence from apoptotic cells

24hr

% of Untreated Control (Fluorescence)

% of Untreated Control (Luminescence)

Log [Bortezomib] M

CellTox Green

CellTiter Fluor

Caspase Glo 3/7

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Multiplexing DNA Staining and ATP Assays

Add CellTox™ Green Dye when seeding cells

- Incubate 72hr
- Record fluorescence from dead cells
- Add CellTiter-Glo® Reagent
- Record luminescence from live cells

![Graph showing fluorescence and luminescence](image)

<table>
<thead>
<tr>
<th></th>
<th>Membrane Integrity</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IC50</strong></td>
<td>2.177e-006</td>
<td>3.950e-006</td>
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</tbody>
</table>
CellTox™ Green Assay: Multiplexing with Luminescent Assays

Glo Reporter Assay
Multiplexes include:
Nano-Glo®, One-Glo™, Bright-Glo™, Steady-Glo®

All possible with GloMax® Detection Systems

CellTox Fluo™ Viability Assay
Will work

CellTiter-Fluo™ Viability Assay
Will work

CellTiter-Glo® Viability Assay
Will work

CellTiter-Glo™ Cell Viability Assay
Will work

NAD(P) / NAD(P)H-Glo™ Assay
Will work

P450-Glo™ Cell-Based Assays
Will work

NAD(P) / NAD(P)H-Glo™ Assay
Will work

Caspase-Glo® Assays
Will work

HDAC-Glo™ Assays
Will Work

Glo Reporter Assays
Will work

CytoTox-Glo™ Assay
Probable

cAMP-Glo™ Max Assays
Will Work

GSH/GSSG-Glo™ Assays
Will Work

ROS-Glo™
Will work

BacTiter-Glo™ Assay
Probable

CellTox Green™ Cytotoxicity Assay

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Advantages of CellTox™ Green DNA Staining

Advantages:

• “Real Time” DNA staining of dead cells produces a fluorescent signal that lasts much longer than the signal from enzyme release

• DNA staining dye overcomes the major disadvantage of enzyme release assays

• Numerous multiplex opportunities because dye is non-toxic

• Stained cells can be detected using imaging or flow cytometry

Disadvantage:

• Signal window is not as great as enzyme marker assays where signal is amplified by enzymatic generation of product
Overall Summary of Real Time Assays

A novel assay has been developed to measure viable cell number in “real time”:
• Repeated kinetic luminescent measurements indicate viable cell number over time
• Reagents are not toxic, thus cells remain viable for subsequent multiplexing assays

A non-toxic non-permeable DNA dye can measure dead cell number in “real time”:
• Repeated fluorescence measurements indicate appearance of dead cells
• DNA dye is non-toxic, thus cells remain viable for subsequent multiplexing assays

Real time detection methods provide flexibility during assay development:
• Kinetic measurements of cell health from the same plate eliminates the need for multiple parallel plates during development and optimization of phenotypic assays
• Multiplexing real time assay methods can provide an internal control to verify viable cell number simultaneously with a variety of other phenotypic assays
Acknowledgments

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Questions Welcome

terry.riss@promega.com