Multiplexing Cell-Based Assays Using 3D Culture Models

Terry Riss, Sarah Duellman, Mike Valley, Kevin Kupcho, Brad Hook and Andrew Niles

Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711

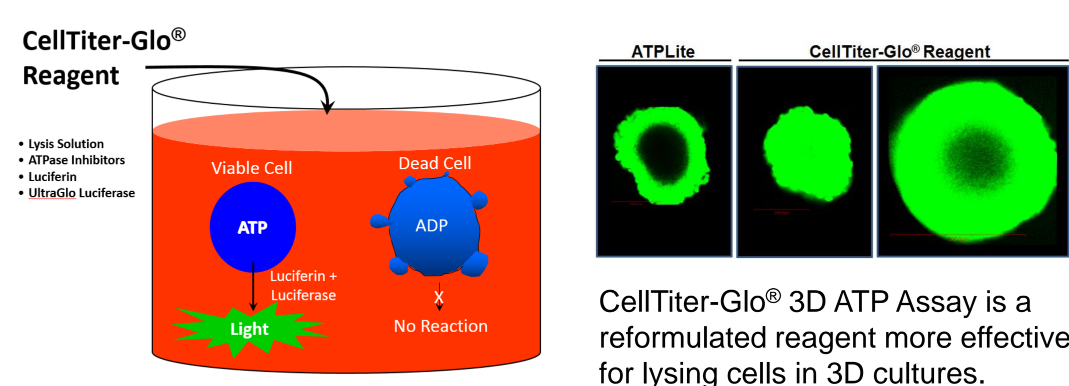
P1.1.44

1. Introduction

The physical nature and size of 3D cell culture models can be much different than cells grown as a monolayer on a plastic surface. Applying off the shelf commercial assay reagents that were originally designed for use with monolayers of cells can lead to artifacts if incomplete cell lysis or incomplete reagent penetration occurs in the larger 3D structures. Those problems may be further amplified when attempting to combine (multiplex) more than one assay chemistry to interrogate the same sample comprised of 3D cell structures. We have designed modified cell health assay formulations and protocols to overcome some of the problems encountered with assaying 3D culture models. We have also tested combining/multiplexing different assay combinations on the same samples of individual spheroids formed using the hanging drop method. We have combined a novel real time cell viability assay with: measuring cell death using a DNA binding dye, measuring firefly luciferase reporter activity to detect cell stress events, and extraction of RNA to perform gene expression analysis. The parameters necessary to validate each multiplex assay combination and the advantages and disadvantages of each method will be described.

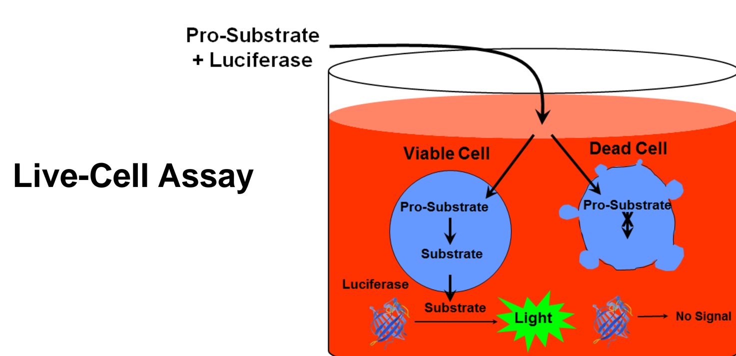
2. Cell Viability Assays for 3D Cultures

ATP assays have been adapted to measure viable cell number of cells in 3D cultures



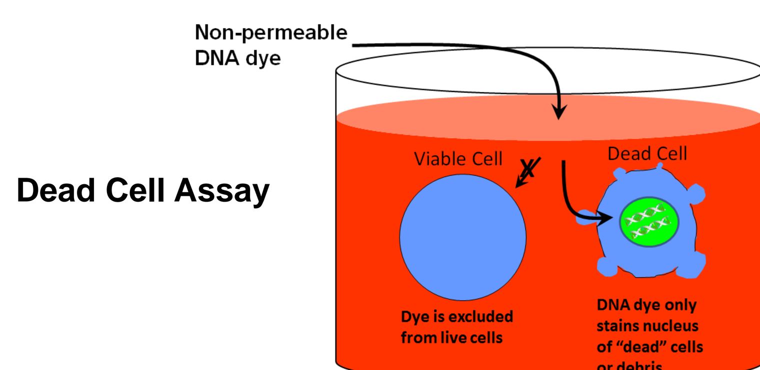
CellTiter-Glo® 3D ATP Assay is a reformulated reagent more effective for lysing cells in 3D cultures.

RealTime-Glo™ MT Cell Viability Assay contains a pro-substrate and shrimp-derived luciferase added as reagents directly to cell culture. Only live cells convert pro-substrate to luciferase substrate and generate light. Luminescence is proportional to the number of live cells.



3. Use of DNA-Binding Dye to Detect Dead Cells and Help with Assay Development

CellTox-Green™ DNA binding dye is added directly to cell culture. The dye is non-toxic and not permeable to live cells, but penetrates dead cells to stain DNA. Fluorescence is proportional to the number of dead cells that accumulate over time in culture.

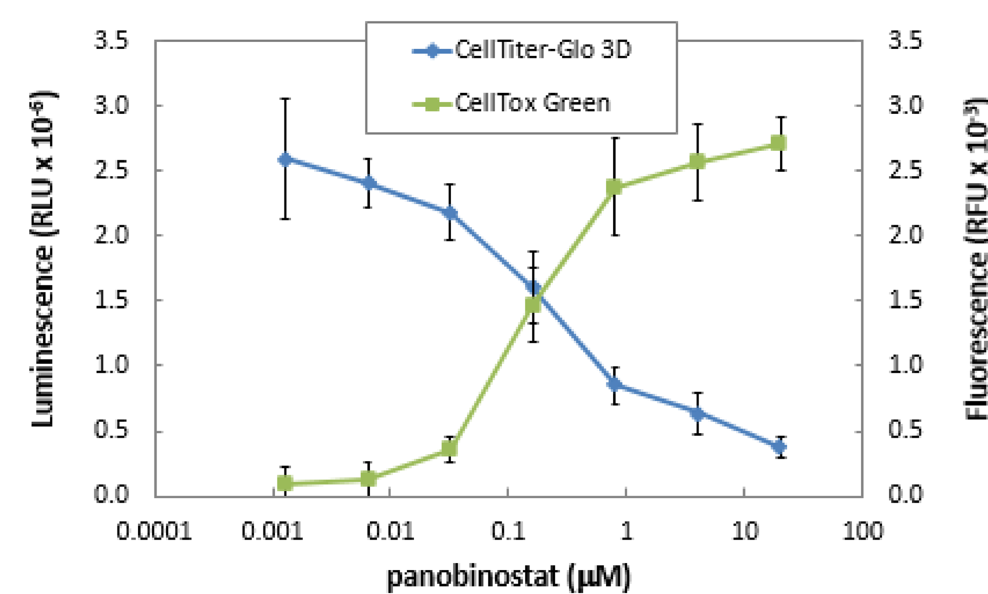


5' shake **30' shake**

Use of CellTox™ Green for assay validation. CellTox™ Green dye was combined with Caspase-Glo® 3/7 reagent prior to adding to ~330µm HCT116 microtissues. Samples were shaken for 5 min (then incubated for 25 min) or 30 min prior to confocal imaging.

4. Multiplexing Live and Dead Cell Assays

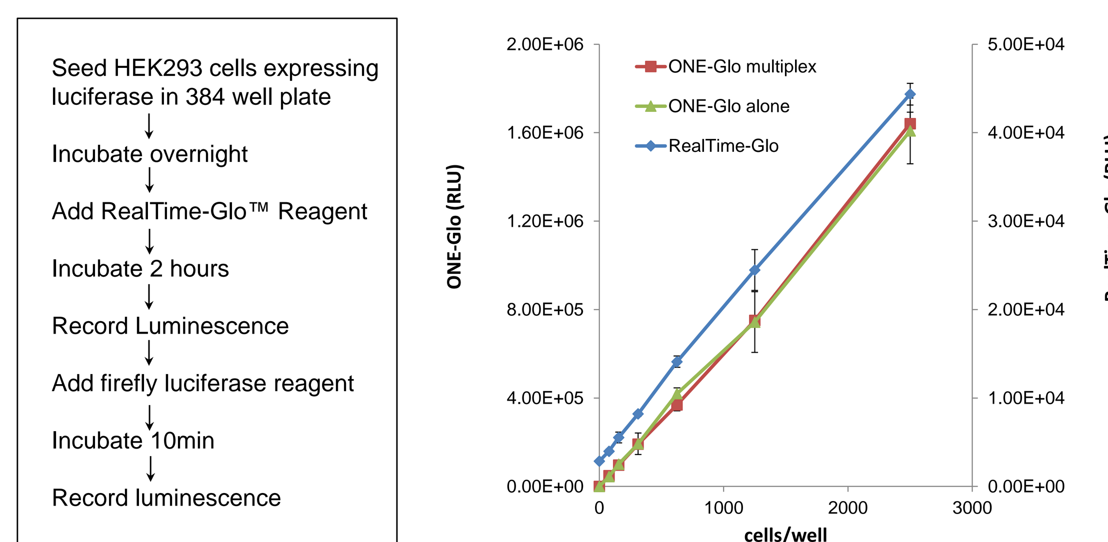
CellTox™ Green is a membrane impermeable DNA-binding dye that selectively stains dead cells. CellTox™ Green can be multiplexed with CellTiter-Glo® 3D ATP Assay.



HCT116 cells were cultured in InSphero GravityPLUS™ 3D Cell Culture system for 4 days to form ~350 µm microtissues. Samples were treated for 48 hr with CellTox™ Green and panobinostat. After recording fluorescence, an equal volume of CellTiter-Glo® 3D Reagent was added, the plate was shaken for 5', and luminescence was recorded after 30 min.

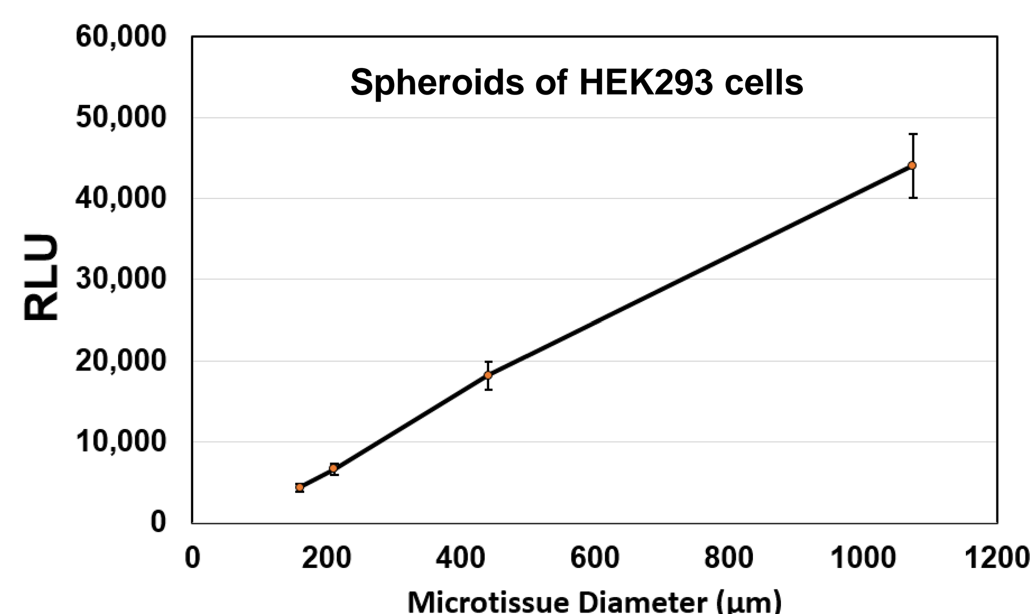
5. Multiplexing RealTime-Glo™ Cell Viability and Firefly Luciferase Reporter Assays

Luminescent signal from the RealTime-Glo™ Assay decreases immediately after cell death (not shown) enabling multiplexing of a different luciferase assay chemistry. Firefly luciferase reporter assay signal is not affected by the presence (red squares) or absence (green triangles) of RealTime-Glo™ Reagent.



6. RealTime-Glo™ Cell Viability Assay of Spheroids

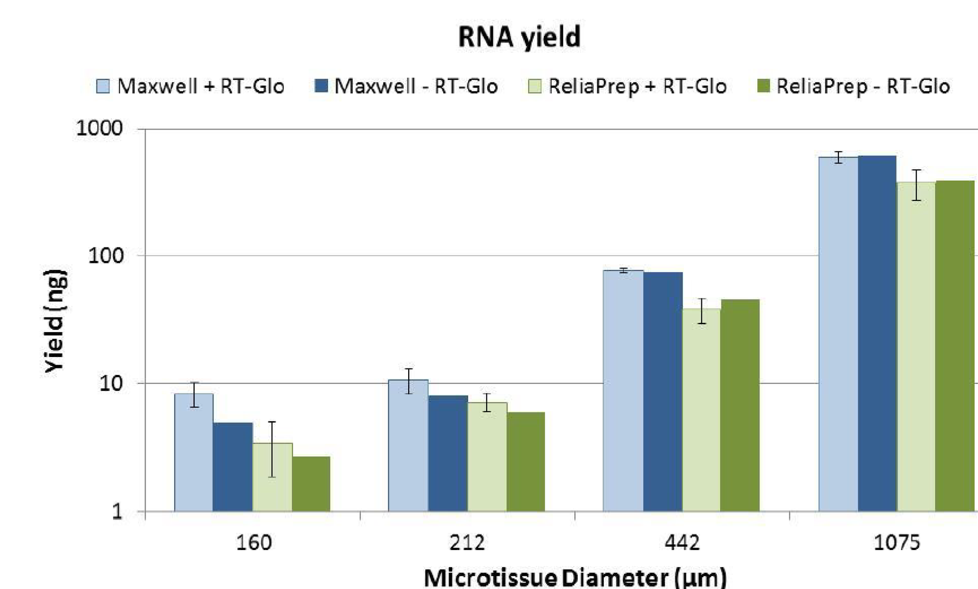
RealTime-Glo™ Cell Viability Assay signal is proportional to spheroid diameter. Cells remain viable following RealTime-Glo™ Assay enabling multiplexing with a variety of methods.



HEK293 cell spheroids of different sizes were produced using the InSphero GravityPlus 3D platform.

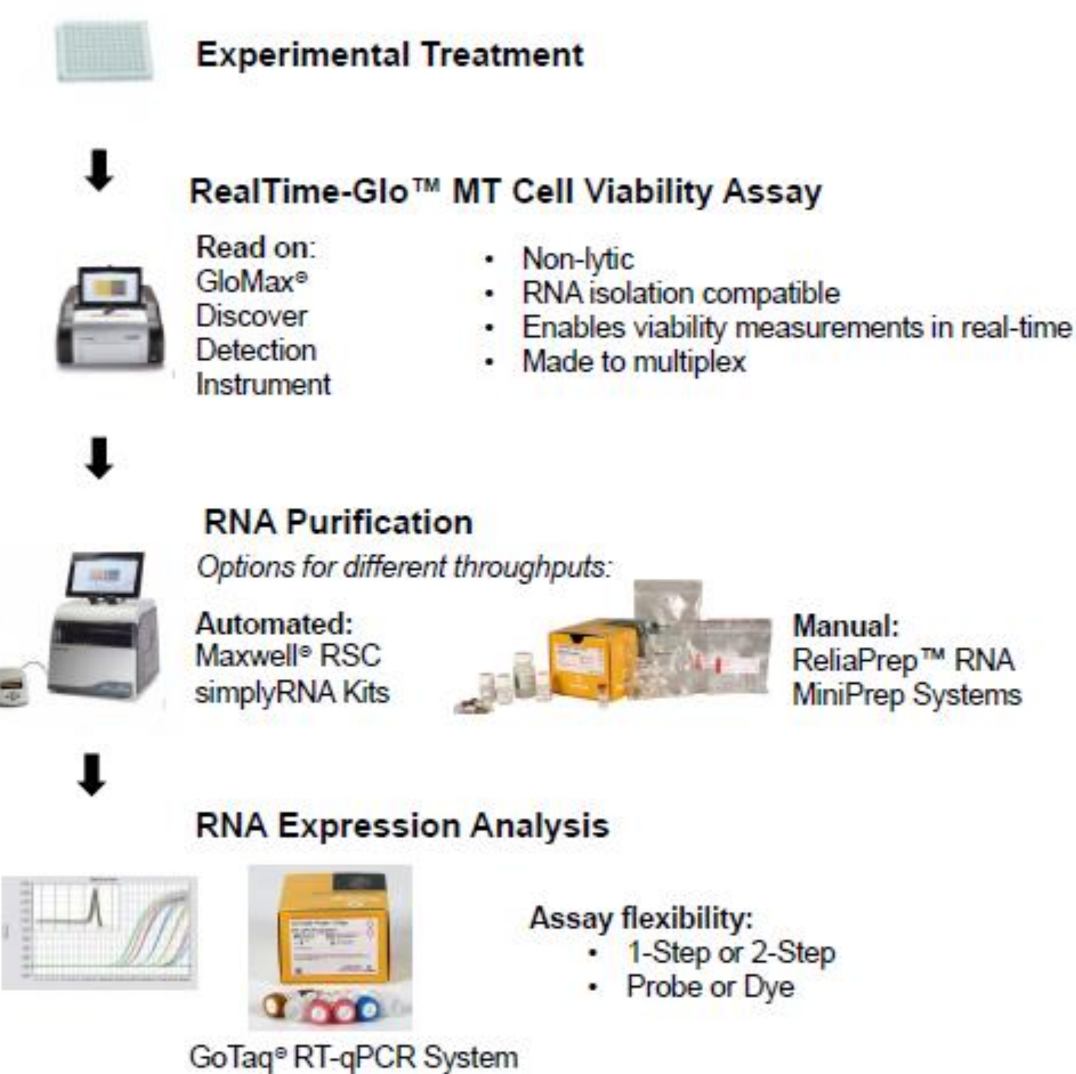
7. Multiplexing RNA Extraction After Real Time Viability Assay of 3D Spheroids

RealTime-Glo™ Assay was used to measure viability of HEK293 cell spheroids (panel 6) followed by RNA extraction of the same samples using ReliaPrep™ RNA Tissue Miniprep System or Maxwell® 16 LEV simplyRNA Tissue Kit. The presence of RealTime-Glo™ Reagent had no effect on the recovery or quality (PCR cycle threshold or RIN values ~8.1 - 9.8) of RNA extracted from cells.



Cell Type	C_t Value 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

8. Example Workflow for Multiplexing RNA Extraction After the RealTime-Glo™ Assay



9. Conclusions

Multiplexing cell-based assays is possible using 3D culture models that are larger and more complex than monolayers.

Real-time detection methods to measure live or dead cells provide much flexibility for multiplexing:

- Non-toxic reagents enable cells to remain viable for subsequent multiplexing of other assays
- Kinetic measurements of cell viability from the same plate eliminates the need for multiple parallel plates during assay development
- Multiplexing real time assays for live or dead cells provides an internal control to verify cell health during experiments to analyze gene expression using firefly luciferase reporter assays or extraction of RNA for expression analysis using RT-qPCR

All multiplexed assay combinations should be verified using appropriate controls for each 3D cell culture model.

Corresponding author: Terry.Riss@promega.com