Modifications Required for ATP and Caspase Detection Assays Applied to 3D Cell Spheroids

Terry Riss, Michael P. Valley, Kevin R. Kupcho, Chad A. Zimprich, Donna Leippe, Andrew Niles, Jolanta Vidugiriene, Brad Hook, James J. Cali, & Dan F. Lazar

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

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1. Introduction

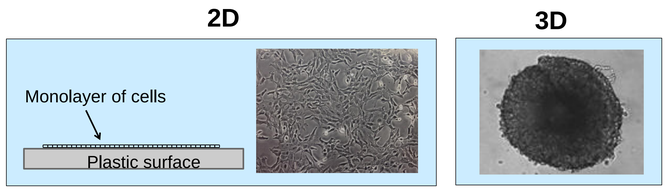
We investigated whether cell viability and apoptosis assays designed for 2D monolayers of cells would work effectively with 3D spheroid models. Cell viability assay reagents that measure ATP contain detergent to lyse cells and release ATP. Some commercial reagents were found to have a low recovery of ATP from spheroids compared to acid extraction accepted as the gold standard. Reformulation of ATP assay reagent to contain higher amounts of detergent (CellTiter-Glo® 3D Assay), increasing mixing time using a plate shaker, and using a longer incubation time in the presence of lytic reagents resulted in improvements in extraction of ATP from large 3D cell spheroids. Although caspase-3 assay reagents (Caspase-Glo® 3/7 Assay) contain detergent to lyse cells, large increases in detergent concentration to achieve lysis of large 3D spheroids was not possible because of damage to the caspase-3 enzymatic activity. Modification of the caspase-3 assay protocol to include 5 minutes of plate shaking followed by 25 minutes incubation resulted in improved recovery of active caspase from 3D spheroids.

2. Rationale for Study

Rationale was to investigate whether cell viability and apoptosis assays designed for measuring 2D monolayers of cells could be applied to 3D spheroid models.

Questions:

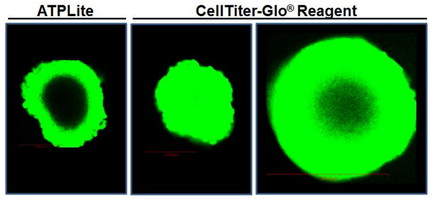
- What is different between 2D and 3D cell culture models?
- Will reagents penetrate to center of spheroid to effectively lyse all cells?
- Will light scattering or quenching by 3D structures block signal before it reaches detector?
- How can these problems be overcome?



3. Standard Reagents and Protocols May be Insufficient to Lyse Cell in Large Spheroids

Using “off the shelf” commercially available reagents and protocols designed for cells cultured in 2D monolayers may not result in lysing all cells in center of large spheroids.

- HEK293 cell spheroids grown to ~350 or 600µm using InSphero hanging drop method
- ATP assay reagents & nonpermeable DNA dye (to indicate dead cells) were added
- Photographed using laser confocal microscopy
- Dark (unstained) center suggest ineffective cell lysis of large spheroids with some reagent formulations

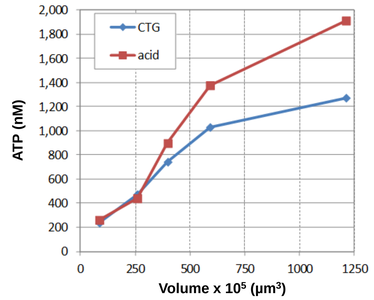


4. Percent Recovery of ATP is Related to Spheroid Size

Original formulation of detergent-containing CellTiter-Glo® ATP Assay Reagent used with protocol designed for 2D monolayer of cells shows lower % recovery of ATP from large HCT116 cell spheroids compared to acid extraction used as the gold standard.

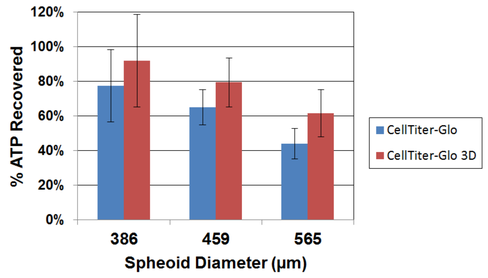
HCT116 Cells Grown for 4 Days using InSphero Hanging Drop Plates

# Cells Seeded	Spheroid Diameter (µm)	% Recovery of ATP
10,000	614	66.6 ± 0.7
5000	483	74.8 ± 3.3
2500	424	82.8 ± 10.3
1250	367	107.7 ± 14.0
625	254	90.9 ± 10.6



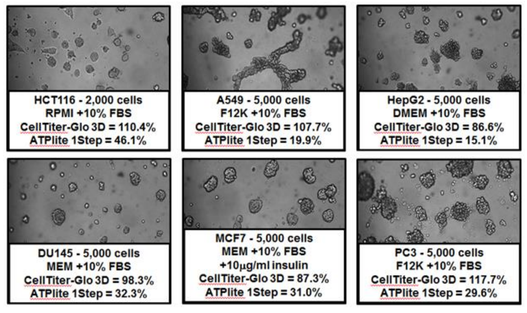
5. Increasing Detergent Content and Lengthening Incubation Time Improved Assay Performance

The ATP assay formulation was improved by optimizing detergent content to create the CellTiter-Glo® 3D Assay. The recommended protocol was modified to include 5 min mixing on a standard plate shaker and incubation for a total of 30 min before recording luminescence. The average % recovery of ATP from triplicate spheroids is shown compared to acid extraction.



6. Re-formulated ATP Assay Reagent Effectively Extracts ATP From 3D Clusters of Cells Grown on Matrigel

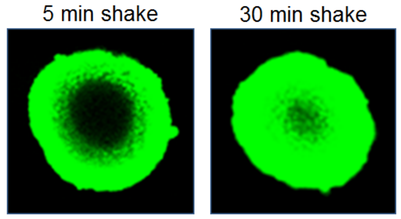
The new ATP assay formulation (CellTiter-Glo® 3D Assay) shows an average of 100% recovery of ATP from 6 cell lines grown in 3D compared to 29% recovery using ATPLite. Note: Cells that grow as larger structures have poorer % recovery of ATP with ATPLite.



7. Protocol Modification is Necessary for Cell Lysis with Caspase-3 Detection Reagent

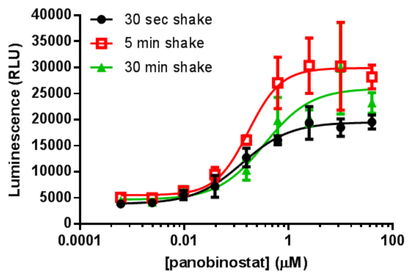
Increasing detergent concentration was not an option because of instability of caspase-3 enzymatic activity. A modified protocol for Caspase-Glo® 3/7 Assay shows a longer shake time more effectively lyses cells in large microtissues.

- CellTox™ Green dye (DNA stain) was combined with Caspase-Glo® 3/7 Reagent prior to adding to ~330µm HCT116 spheroids.
- Samples were shaken for 5 min or 30 min prior to confocal imaging.
- Increased shaking and incubation time is necessary to improve cell lysis (green stained cells).



8. Caspase-3 Activity Measurement Using Modified Protocol to Increase Shaking and Incubation Time

HCT116 cell spheroids were treated with panobinostat for 24 hr. An equal volume of Caspase-Glo® 3/7 was added and samples shaken for indicated times prior to recording luminescence. Shaking time should be optimized for each 3D model system.



9. Summary

Assay reagents designed for monolayers of cells may not work with 3D spheroids

- Standard reagents and protocols designed for 2D monolayers of cells may not effectively lyse all cells in 3D spheroids
- Re-formulation of reagents to contain improved lysis conditions may be needed

ATP assay reagent formulation was improved to create CellTiter-Glo® 3D Assay

- Detergent content was optimized to improve cell lysis in 3D structures
- Protocol was modified to include 5 min mixing on plate shaker and total of 30 min incubation before recording luminescence

Caspase-3 assay was improved with protocol modifications

- Because stability of the caspase-3 enzyme was a limiting factor, reformulation to increase detergent concentration was not an option
- Physical disruption and increased incubation time were used to improve lysis of cells and recovery of activity from 3D spheroids

Corresponding author: Terry.Riss@promega.com