Intracellular uptake of chitosan nanoparticle-based vaccines

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Intracellular uptake of chitosan nanoparticle-based vaccines

An Undergraduate Honors College Thesis

in the

Department of Biomedical Engineering
College of Engineering
University of Arkansas
Fayetteville, AR

by

Katherine E. Wilson
This thesis is approved.

Thesis Advisor:

Thesis Committee:
Intracellular uptake of chitosan nanoparticle-based vaccines

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Abstract

Particle-based delivery systems are a promising approach to enhance antigen-specific immunity for use in vaccines. Chitosan, a natural polysaccharide derived from the exoskeletons of crustaceans, shows great potential as a vaccine carrier due to its favorable properties as a biomaterial, including biocompatibility and biodegradability. In this study, the intracellular uptake of chitosan nanoparticles is explored and compared to that of other commonly used particle-based delivery vehicles, such as poly (lactic-co-glycolic acid) (PLGA). Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) was used as a model protein antigen and encapsulated in both chitosan particles via precipitation-coacervation and PLGA particles via double emulsion solvent evaporation. Fluorescence microscopy images showed that, when co-incubated with dendritic cells, all cells were found to internalize FITC-BSA-chitosan nanoparticles. Additionally, punctate fluorescence in the cytoplasm indicated that at least some of the particles were within endosomes at early (less than 24 hour) time points. Furthermore, flow cytometry results showed chitosan particles to have a greater uptake (74%) by dendritic cells compared to PLGA particles (32%) or soluble protein (43%) at early time points (1 hour).
1. Introduction

Vaccines play a vital role in modern medicine. While vaccines have been responsible for the near elimination of some major diseases, such as smallpox and poliomyelitis, as well as significant control over many other illnesses, there remain several diseases that require new and improved vaccine formulations to enhance current treatments [1]. Presently, vaccine development studies focus on using subunit antigens to elicit an immune response, which is a safer but less effective method than delivering whole pathogens. Subunit antigens are rapidly degraded by proteases upon injection. Furthermore, subunit antigens lack the requisite immunostimulatory signals required for a robust adaptive immune response [2]. A delivery system, therefore, is needed to increase the persistence of antigens and enhance the immune response. Particle-based delivery systems in which antigens are encapsulated in polymeric nano- or microparticles are a promising approach and under intense investigation.

Several advantages of antigen encapsulation by nano- and/or micro particles have been established. For one, encapsulation prevents antigen degradation. Two, polymeric particles can be engineered to certain specifications, such as amount of adjuvant loading. Finally, antigen-presenting cells (APC) like macrophages and dendritic cells (DCs) phagocytize and process the antigen-containing particles [2]. These properties demonstrate the potential of particle-based vaccine delivery systems to enhance antigen-specific immunity.

The most commonly used material for particle-based delivery is poly (lactic-co-glycolic acid) (PLGA), but another polymer, chitosan, shows great potential as a vaccine carrier. Chitosan, a natural polysaccharide derived from the exoskeletons of crustaceans, has recently been introduced in several biomedical applications due to its favorable properties and versatility as a biomaterial, including biocompatibility and biodegradability. Chitosan is mucoadhesive,
which has led to its exploration as a nasal and oral vaccine carrier. In addition, chitosan is highly hydrophilic and positively charged, indicating its high protein loading capacity [1, 2]. Thus, this remarkable natural polymer has shown great potential as a particle-based vaccine delivery system, and may have advantages over PLGA.

Previous research in our lab, the Laboratory of Vaccine and Immunotherapy Delivery at the University of Arkansas, has demonstrated that chitosan’s properties support its potential as an effective vaccine carrier. Chitosan particles efficiently deliver encapsulated antigens and enhance the activation of both macrophages and DCs [2]. Furthermore, antigen-encapsulated chitosan particles outperformed soluble antigens in eliciting both humoral and cell-mediated antigen-specific immune responses [2].

To supplement these findings, my research focused on characterizing the uptake of chitosan nanoparticle-based vaccines and comparing the uptake to other nanoparticles commonly used in particle-based vaccine delivery, such as PLGA. Fluorescence microscopy was used to determine the time course of internalization of nanoparticles by DCs. Flow cytometry was then used to further quantify uptake. Cytotoxicity became of interest in the study, so cell viability assays were performed and PI stain was used with flow cytometry as a measure of cell viability. Finally, scanning electron microscopy (SEM) images were taken of both chitosan particles and PLGA particles in the interest of comparing their morphologies. Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) was used as a model protein antigen and encapsulated in both chitosan particles via precipitation-coacervation and PLGA particles via double emulsion solvent evaporation.
2. Materials and Methods

2.1 Cell Culture

A murine immature dendritic cell line, JAWS II cells, were cultured in complete media consisting of DMEM supplemented with 20% FBS, 1% penicillin/streptomycin, 1% L-glutamine, and 5ng/ml GM-CSF.

2.2 Particle synthesis

Chitosan particles encapsulating FITC-BSA were prepared via precipitation-coacervation using low molecular weight chitosan [Batch #: MKBD0020, 448869-50G, CAS 9012-76-4]. For full protocol, see appendix A. PLGA particles encapsulating FITC-BSA were prepared via double emulsion solvent evaporation using PLGA (50/50). For full protocol, see appendix B. Particles were lyophilized before further use. Particle size was measured via dynamic light scattering (DLS) (Nano ZS90, Malvern Instruments, Malvern, UK).

2.3 Fluorescence Microscopy

Phase contrast images and fluorescence images were taken with a Nikon eclipse Ti fluorescence microscope. Prior to imaging, cells were seeded in a 6-well plate at 2 million cells per well and incubated overnight. Particle suspensions, chitosan or PLGA at 200 µg/ml, or FITC-BSA solution at 20 µg/ml, were then added to different wells. Cells alone was used as a negative control. The plate was incubated for the desired period of time. The samples were washed three times with phosphate buffered saline (PBS) prior to imaging.
2.4 Flow Cytometry

Flow cytometry was used to quantify cell-by-cell uptake of particles or FITC-BSA. Briefly, cells were plated 1 million cells per well in a 6-well plate. Chitosan or PLGA suspension (200 µg/ml) or FITC-BSA solution (20 µg/ml) were added to appropriate wells. The plate was incubated for the desired time, either 1 hour or 24 hours. Samples were harvested after the incubation period and were washed and re-suspended in PBS prior to measuring. PI stain (2µl) was added just before measuring.

2.5 Viability Assays

Two different viability assay kits were used: 1) CytoTox-Glo Cytotoxicity Assay (see appendix C for product instructions) and 2) CellTiter-Glo Luminescent Cell Viability Assay (see appendix D for product instructions). Briefly, JAWS II cells were seeded in a 96-white wall plate. Particle suspensions or FITC-BSA solution was added to appropriate wells and the plate was incubated for 1 hour or 24 hours. Plates were prepared for measurement after the incubation period according to the respective assay protocol. Luminescence was read with a plate reader and reported in relative light units (RLU).

2.6 Scanning Electron Microscopy

Lyophilized particles were sputter coated with gold prior to imaging. SEM images were acquired at a beam voltage of 10-15 kV.
3. Results and Discussion

Chitosan particles had a mean diameter size of 521.5nm ± 123.4 (n=18) and PLGA particles had a mean diameter of 281.1nm ± 52.3 (n=4) as determined via dynamic light scattering (DLS). Fluorescence images verified the successful encapsulation of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) by chitosan nanoparticles (Figure 1).

![Figure 1. Chitosan nanoparticles encapsulating FITC-BSA (green). 40x.](image)

The time course of internalization of chitosan particles by dendritic cells was determined using fluorescence microscopy. When co-incubated with JAWS II cells, all cells were found to internalize FITC-BSA-chitosan nanoparticles. Additionally, punctate fluorescence in the cytoplasm indicated that at least some of the particles were within endosomes at early (~1 hour) time points (Figure 2). Cells alone showed no fluorescence, which was expected for the negative control. Cells co-incubated with chitosan particles appear to have the highest intensity of FITC fluorescence signal. Cells co-incubated with FITC-BSA or with PLGA particles showed some fluorescence but less than that of cells with chitosan particles. This suggests that the dendritic cells more readily took up chitosan particles than PLGA particles or soluble protein antigens.
To quantify intracellular uptake, cell-by-cell analysis was performed using flow cytometry. An increase in intensity, or right shift, in the FITC channel (P3) denotes an increase in protein (FITC-BSA) or particle uptake. Likewise, an increase in intensity in the PE channel (P2) denotes an increase in percentage of the population stained with propidium iodide (PI). PI stain was included as a measure of cell viability.

It was found that intracellular uptake is greater with chitosan particles than PLGA particles or FITC-BSA (Figure 3). After a 1-hour incubation period, cellular uptake of chitosan was 74% compared to only 32% with PLGA and 43% with FITC-BSA. Uptake showed an increase at 24 hours compared to 1 hour with both particles and FITC-BSA, but chitosan particles retained the highest value, 77%, compared to 45% with PLGA and 59% with FITC-BSA. These results, together with fluorescence microscopy images, support that chitosan particles stimulate greater intracellular uptake than PLGA particles or soluble protein antigens.

The PE channel showed that cell viability appears to decrease more with chitosan particles than PLGA particles after 24-hour incubation. At 1 hour, there was little decrease in cell
viability with either particle. At 24 hours, however, cell viability had decreased by 31% with chitosan particles and only 11% with PLGA particles. There were a few things to consider when analyzing this result. First, since uptake of chitosan particles was significantly higher than that of PLGA, the larger decrease in cell viability with chitosan particles may be due to a higher concentration (dose) of particles rather than increased cytotoxicity. It is also important to note that while the percentage values provide a good quantitative measure of the increase in signal intensity, the visual shift in intensity provides the best comparison. In other words, it is difficult to affirm that, for example, there was precisely a 31% decrease in cell viability at 24 hours of cells co-incubated with chitosan particles. With this in mind, the shifts in the PE channel of cells co-incubated with chitosan particles or PLGA particles at 24 hours are visually more similar than the percentages suggest. The shift in the FITC channel, on the other hand, is obviously more dramatic with chitosan particle uptake compared to PLGA particle uptake. Additionally, there may be some interaction between chitosan and PI causing the shift in intensity. Repeat studies are necessary to verify that PI and chitosan are not interacting.
Figure 3. Flow cytometry of cells alone (A), cells co-incubated with FITC-BSA for 1 hour (B) or 24 hours (C), cells co-incubated with chitosan particles for 1 hour (D) or 24 hours (E), and cells co-incubated with PLGA particles for 1 hour (F) or 24 hours (G). P1 (left) is the selected population, P2 (middle) is a PE channel gate, and P3 (right) is a FITC channel gate. The percentage in the top right corner of each image is the percent of the parent population that corresponds to the selected region or positive channel signal. Performed 3 times (only once with PI stain) with similar results.
Viability assays were used to further investigate cytotoxicity of both polymeric particles. Results with the CytoTox-Glo Cytotoxicity Assay showed that there was no significant decrease in cell viability after 1 hour of incubation with either chitosan particles (P=0.158) or PLGA particles (P=0.347) (Figure 4). After 24 hours of incubation, however, there was a significant decrease in cell viability in cells co-incubated with PLGA particles (P=0.0047). There was not a significant decrease in cell viability in cells co-incubated with chitosan particles (P=0.129) (Figure 5).

Results with the CellTiter-Glo Luminescent Cell Viability Assay showed that as particle concentration increases, viable cell luminescence decreases. There was a significant decrease in cell viability with 25µg and 50µg of PLGA particles and with 50µg of chitosan particles (Figure 6). Table 1 shows the measured RLU values and Figure 7 shows the percent difference calculations of cell viability as particle concentration increases between cells alone and cells co-incubated with chitosan particles or between cells alone and cells co-incubated with PLGA particles.

It appears that chitosan particles are less toxic than PLGA particles. It is noteworthy, however, that particle concentrations in this study are relatively low, so results may vary at higher concentrations. Also, results could not yet be reproduced, so further experimentation is necessary to validate these results or draw more concrete conclusions.
Figure 4. Viable cell luminescence. JAWS II cells were incubated alone or co-incubated with chitosan particles or PLGA particles for 1 hour. Particle concentration was 200µg/ml. Bars indicate relative light units (RLU) as determined by CytoTox-Glo Cytotoxicity Assay. (n=1)

Figure 5. Viable cell luminescence. JAWS II cells were incubated alone or co-incubated with chitosan particles or PLGA particles for 24 hours. Particle concentration was 200µg/ml. Bars indicate relative light units (RLU) as determined by CytoTox-Glo Cytotoxicity Assay. *p<0.05 compared to cells alone. (n=1)
Table 1. Viable cell luminescence measured in relative light units (RLU) as determined by CellTiter-Glo Luminescent Cell Viability Assay. Values were used in Figures 6 and 7.

<table>
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<tr>
<th>Particle Concentration (µg)</th>
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<tr>
<td>cells only</td>
<td>923,201</td>
<td>75,106</td>
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<tr>
<td>cells + PLGA</td>
<td>841,314</td>
<td>40,041</td>
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<tr>
<td>cells + Chitosan</td>
<td>878,638</td>
<td>39,478</td>
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<td>16.7 µg</td>
<td>750,247</td>
<td>48,279</td>
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<tr>
<td>cells + PLGA</td>
<td>839,215</td>
<td>61,636</td>
</tr>
<tr>
<td>cells + Chitosan</td>
<td>645,229</td>
<td>33,616</td>
</tr>
<tr>
<td>25 µg</td>
<td>755,567</td>
<td>15,444</td>
</tr>
<tr>
<td>cells + PLGA</td>
<td>50 µg</td>
<td></td>
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</table>

Figure 6. Viable cell luminescence. JAWS II cells were incubated alone or co-incubated with either chitosan particles or PLGA particles for 24 hours at increasing concentrations. Bars indicate relative light units (RLU) as determined by CellTiter-Glo Luminescent Cell Viability Assay. *p<0.05 compared to cells alone. **p<0.01 compared to cells alone. Samples were plated in triplicate, so error bars represent standard deviation of three wells of repeated samples in the same study. (n=1)
Lastly, I investigated the morphology of chitosan particles and PLGA particles with scanning electron microscopy (SEM). Representative SEM images are shown in Figure 6. The images I acquired with PLGA did not match those found in literature, so I cannot be sure that the chitosan images are accurate either. It may be that a different sample preparation method is required to obtain better images.

Figure 7. Percent difference in cell viability in cells co-incubated with chitosan particles or PLGA particles compared to cells alone at increasing amounts of particles. Calculated using values from Table 1.

Figure 8. Representative SEM images of chitosan particles (A) and PLGA particles (B).
There are several avenues of future research that could be pursued. For one, repeat studies using the CellTiter-Glo Luminescent Cell Viability Assay kit need to be performed in order to draw conclusions about the differences in cytotoxicity between chitosan particles and PLGA particles. Moreover, repeat studies using PI stain with flow cytometry could be used to support the viability assay results. It would be worthwhile to experiment with a higher and wider range of particle concentrations, as well.

Furthermore, multiphoton microscopy would provide better, clearer images than the fluorescence microscopy used in this study. Multiphoton images would show greater detail and more qualitative information could probably be determined by analyzing the intracellular uptake of particles more closely.

Additionally, performing flow cytometry with samples incubated for periods greater than 24 hours may be useful in determining intracellular retention rate of particles. This data could indicate the time period between uptake by DCs and DC maturation and antigen presentation.

Another study to pursue is SEM imaging. Morphology of the particles may play a role in their intracellular behavior, so knowledge of the differences in morphology between chitosan and PLGA particles may be valuable in analyzing the differences in the cellular mechanisms of uptake and processing of either particle.

4. Conclusions

In conclusion, dendritic cells internalized FITC-BSA-encapsulated chitosan nanoparticles at early (~1 hour) time points. When co-incubated with JAWSII cells, all cells were found to internalize FITC-BSA-chitosan nanoparticles. Punctate fluorescence in the cytoplasm indicated
that at least some of the particles were within endosomes at early (less than 24 hour) time points.

Cellular uptake of particles appears to be greater with chitosan particles compared to PLGA particles at early time points (1 hour). Intracellular uptake increased from 1 hour to 24 hours with both chitosan and PLGA particles, but chitosan particle uptake remained significantly higher (77% compared to 45% with PLGA).

Additional data is required, but there may be a significant difference in cytotoxicity between PLGA and chitosan particles. My results show that chitosan particles are somewhat less toxic than PLGA particles after 24-hour incubation with cells.

Tunable release kinetics is a key advantage of polymeric nanoparticle delivery systems. The increased uptake with chitosan particles compared to PLGA particles suggest chitosan particles may provide greater sustained release of loaded molecules, and therefore could present the greatest immunostimulatory response.

**Acknowledgments**

Funding provided by a University of Arkansas Honors College Research Grant. I would like to thank the Arkansas Nano & Bio Materials Characterization Facility for providing training with and access to the SEM imaging machine. There are no conflicts of interest to disclose.

**References**


Appendix A: Chitosan particle synthesis

Appendix B: PLGA particle synthesis

Appendix C: CytoTox-Glo Cytotoxicity Assay product instructions

Appendix D: CellTiter-Glo Luminescence Cell Viability Assay product instructions
Appendix A

Protocol: Chitosan Nanoparticles

(Bhanu, Wilson et al 2013)

1. Prepare 2% v/v acetic acid solution by adding 200 µl acetic acid in 9.8 ml of DI water

2. Add 10 mg chitosan (Sigma Low MW) to the acetic acid solution on a magnetic stirrer and mix for 1 hour (~850 RPM).

3. Add 100 µl (or 1 mg) of FITC-BSA and mix for 10 min in the dark (~800 RPM)

4. Prepare 10% w/v sodium sulfate solution by adding 1 g sodium sulfate to 10 ml DI water and mix.

5. Use infusion pump to add 1 ml sodium sulfate solution to chitosan solution drop wise until particle formation is observed

6. Add 50 mg Pluronic F-68 to the solution and stir for 5 min.

7. Stir the nanoparticle solution for another 30 minutes while sonicating (30-40 W) every 15 min for 60 sec to obtain better dispersion.

8. Use the nanosizer to determine the size of the chitosan nanoparticles
   - 1 ml DI water + 100 µl chitosan solution in cuvette
   - Remove any bubbles before measuring.

9. Centrifuge at 15000 RPM for 8 min.

10. Keep supernatant (wrap in foil and store in 4°C fridge to use in drug loading efficiency calculations) and resuspend the pellet in ~10 ml DI water and sonicate for 1 min in water bath sonicator.

11. Centrifuge again at 15000 RPM for 8 min.

12. Dispose of the supernatant and resuspend the pellet in 5 ml of DI water.

13. Transfer the solution to a 15 ml conical tube, wrap in foil, and store in -80°C freezer.
Appendix B

**Protocol: PLGA nanoparticles (300nm)**

*(Bhanu et al, 07/14/13)*

PLGA nanoparticles encapsulating OVA antigen were prepared using a double emulsion solvent evaporation method.

Note: Make sure all the components used in the reactions including beakers, pipettes etc. are of glass. Do not use plastic containers or pipettes to handle the solutions.

1. Prepare 12ml 2% w/v PLA solution by taking 240mg of PVA into 12ml DI water
2. Mix the solution at room temperature for 1 hour
3. Prepare protein solution by dissolving 2 mg of FITC-BSA in 200µl DI water (use black eppindorf tube to protect from light)
4. Prepare 3% PLGA solution by dissolving 90mg of PLGA (50/50) in 3ml of dichloromethane (DCM) in a 10ml beaker
5. Cover the beaker mouth tightly with aluminum foil
6. Mix the solution at 600 RPM for 10 – 15 minutes
7. After 15 minutes, increase the stirring speed of PLGA mixture to 800 RPM and slowly add the protein solution drop wise
8. Transfer the mixture to an ice bath and sonicate at 40W for 30 seconds
9. Keep the PVA solution on the stirrer mixing at 800 RPM
10. Slowly add the PLGA mixture to PVA solution using a glass transfer pipette
11. Let the solution mix for 2 minutes
12. Transfer the beaker to an ice bath and sonicate the solution at 50W for 2 minutes
13. Transfer the beaker back to stirrer
14. Close the mouth of the beaker with aluminum foil
15. Poke 3-4 holes in the aluminum foil lid using a pipette tip

16. Leave the beaker on stirrer overnight to allow DCM to evaporate

17. After stirring overnight, transfer PLGA nanoparticles solution into centrifuge tubes

18. Weigh balance the centrifuge tube and centrifuge for 10 minutes at 20,000 RPM using high speed centrifuge

19. Collect the supernatant – Can be used to determine the loading efficiency

20. Re-suspend the pellet in 10ml DI water – Make sure the particles are mixed well into the solution

21. Centrifuge the particle solution for 10 minutes at 20,000 RPM using high speed centrifuge

22. Discard the supernatant and re-suspend the protein loaded PLGA nanoparticles in 10ml DI water

23. Mix well and freeze at -80°C before freeze drying
CytoTox-Glo™ Cytotoxicity Assay
INSTRUCTIONS FOR USE OF PRODUCTS G9290, G9291 AND G9292
1. Description

The CytoTox-Glo™ Cytotoxicity Assay is a single-reagent-addition, homogeneous, luminescent assay that allows measurement of the number of dead cells in cell populations (Figure 1). The CytoTox-Glo™ Assay shows excellent correlation with other methods of assessing cell viability (Figure 2). The CytoTox-Glo™ Assay measures a distinct protease activity associated with cytotoxicity (1). The assay uses a luminogenic peptide substrate (alanyl-alanyl-phenylalanyl-aminoluciferin; AAF-Glo™ Substrate) to measure “dead-cell protease activity”, which is released from cells that have lost membrane integrity (Figure 3). The AAF-Glo™ Substrate cannot cross the intact membrane of live cells and does not generate any appreciable signal from the live-cell population. The assay selectively detects dead cells (Figure 4). The CytoTox-Glo™ Assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which uses aminoluciferin as a substrate to generate a stable "glow-type" luminescent signal and is formulated to improve performance across a wide range of assay conditions.

With the addition of Lysis Reagent (provided), the CytoTox-Glo™ Cytotoxicity Assay also can deliver the luminescent signal associated with the total number of cells in each assay well. Viability can be calculated by subtracting the luminescent signal resulting from experimental cell death from total luminescent values (Section 5).
Step 1. Measure dead-cell number.

Add AAF-Glo™ Reagent. Mix. Incubate for 15 minutes at room temperature.

Measure luminescence to determine number of dead cells.

Step 2. Measure total cytotoxicity (optional).

Add Lysis Reagent. Mix. Incubate for 15 minutes at room temperature.

Measure luminescence to determine total cell number.

Total Cell Number – Dead-Cell Number = Viable Cell Number

Figure 1. Schematic diagram of the CytoTox-Glo™ Cytotoxicity Assay.
Assay Advantages

Measure the Relative Number of Dead Cells in Culture: Measure cytotoxicity by adding a single reagent with the homogeneous “add-mix-measure” protocol.

Normalize Data for Cytotoxicity: Data normalization for dead-cell number makes results more comparable well-to-well, plate-to-plate and day-to-day.

Measure the Relative Number of Remaining Viable Cells Using a Total Lysis Protocol: Correlate increased cytotoxicity with a reduction in viable cells.

Improve Your Data: Reduce statistical probability of false positives (or negatives), and eliminate fluorescence interference issues with a stable luminescence readout.

Figure 2. The CytoTox-Glo™ Cytotoxicity Assay shows strong correlation with established methods for measuring cytotoxicity. Panel A. The CytoTox-Glo™ Cytotoxicity Assay signal from serial twofold dilutions of dead cells plotted against results from the CytoTox-ONE™ Homogeneous Membrane Integrity Assay, which measures LDH release. Panel B. The CytoTox-Glo™ Assay signal from serial dilutions of dead cells plotted against results achieved using ethidium homodimer (DNA quantitation).
Figure 3. Cleavage of the luminogenic AAF-Glo™ Substrate by dead-cell protease activity. Following cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase-mediated production of light.

Figure 4. The CytoTox-Glo™ Cytotoxicity Assay signal derived from lysed cells is extremely sensitive and proportional to cell number and demonstrates selective detection of dead cells.
2. **Product Components and Storage Conditions**

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G9290 contains sufficient reagents for 100 assays at 100µl per assay in a 96-well plate format or 400 assays at 25µl per assay in a 384-well plate format. Includes:

- 1 bottle AAF-Glo™ Substrate
- 2 × 5ml Assay Buffer
- 1 × 40µl Digitonin

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G9291 contains sufficient reagents for 500 assays at 100µl per assay in a 96-well plate format or 2,000 assays at 25µl per well in a 384-well format. Includes:

- 5 bottles AAF-Glo™ Substrate
- 10 × 5ml Assay Buffer
- 1 × 175µl Digitonin

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G9292 contains sufficient reagents for 1,000 assays at 100µl per assay in a 96-well plate format or 4,000 assays at 25µl per well in a 384-well format. Includes:

- 2 bottles AAF-Glo™ Substrate
- 4 × 25ml Assay Buffer
- 2 × 175µl Digitonin

**Storage Conditions**: Store the CytoTox-Glo™ Cytotoxicity Assay components at -20°C, protected from light. See product label for expiration date information.
3. **Reagent Preparation and Storage**

1. Thaw the CytoTox-Glo™ Cytotoxicity Assay components in a 37°C water bath. Mix the components to ensure homogeneity.

2. Prepare the CytoTox-Glo™ Cytotoxicity Assay Reagent by transferring the contents of one bottle of Assay Buffer to the AAF-Glo™ Substrate bottle.  
   **Note:** The appearance of the AAF-Glo™ Substrate prior to addition of Assay Buffer may vary from lot to lot. This variation does not affect assay performance.

3. Prepare the Lysis Reagent one of two ways, depending on the protocol used.  
   a. For Section 4.A, transfer Assay Buffer (2.75ml for Cat.# G9290 and 13.75ml for Cat. G9292) to a conical tube. Prepare Lysis Reagent by transferring Digitonin (13µl for Cat.# G9290 and G9291; 65µl for Cat. G9292) to the conical tube containing Assay Buffer. Mix to ensure homogeneity.  
   
   b. For Sections 4.B and 4.C, transfer Digitonin (33µl for Cat.# G9290 and G9291; 162µl for Cat.# G9292) to the Assay Buffer (5ml for Cat.# G9290 and G9291; 25ml for Cat.# G9292). Mix well to ensure homogeneity.  
   **Note:** Because the Lysis Reagent can be added before or after the CytoTox-Glo Cytotoxicity Assay Reagent, the volume of digitonin added to Assay Buffer differs. The difference in the volume of cells means that the volume of digitonin has to be adjusted to be delivered at the same lytic concentration.

**Storage:** For optimal results, use freshly prepared CytoTox-Glo™ Cytotoxicity Assay Reagent (rehydrated AAF-Glo™ Substrate). Use within 12 hours if stored at room temperature. The CytoTox-Glo™ Cytotoxicity Assay Reagent and Lysis Reagent can be stored at 4°C for up to 7 days with no appreciable loss of performance. The CytoTox-Glo™ Cytotoxicity Assay Reagent may be stored in single-use aliquots for up to 4 months at –70°C. Freezing and thawing will damage the reagent and must be avoided.
4. Protocols

Materials to be Supplied by the User
• 96-, 384- or 1536-well, white-walled tissue culture plates compatible with luminometer and fluorometer (clear or solid bottom)
• multichannel pipettor or liquid-dispensing robot
• reagent reservoirs
• luminescence plate reader or multimode reader
• orbital plate shaker
• positive control cytotoxic compound

If you have not performed this assay with your cell line previously, we strongly recommend that you determine the assay sensitivity for your cells using one of the two methods described below (Section 4.A or 4.B). Use Method 1 to determine the linear range of the assay for your cell type. Use Method 2 to determine the practical sensitivity once you have already chosen the number of cells to use in the assay. If you do not need to determine assay sensitivity for your cells, proceed to Section 4.C.

4.A. Determining Assay Sensitivity, Method 1

In this method, the Lysis Reagent is added as the first reagent to simulate experimental cytotoxicity. Lysis Reagent is added as the first reagent to effect 100% cytotoxicity in the presence of a limited number of cells to determine assay sensitivity. The luminescence measured reflects total cell luminescence.

In all other assay applications, Lysis Reagent is added after the CytoTox-Glo™ Reagent. CytoTox-Glo™ Reagent is first added, and luminescence is measured to determine dead cell luminescence. Lysis Reagent is then added, and luminescence is measured to determine total cell luminescence. The final concentration of the Lysis Reagent used in this method is different than that when Lysis Reagent is added after the CytoTox-Glo™ Reagent. These two concentrations of Lysis Reagent cannot be used interchangeably.

1. Harvest adherent cells (by trypsinization, etc.), wash with fresh medium to remove residual trypsin and resuspend in fresh medium.
   
   **Note:** For cells growing in suspension, proceed to Step 2.

2. Determine the number of viable cells by trypan blue exclusion using a hemacytometer, and then dilute to 100,000 viable cells/ml in at least 3.0ml of fresh medium.
   
   **Note:** Concentrate cells by centrifugation if the cell suspension is less dense than 100,000 cells/ml.

3. Add 100µl of the 100,000 cell/ml suspension (10,000 cells/well) to all wells of row A and B in a 96-well plate.

4. Add 100µl of fresh medium to all wells in rows B–H.
4.A. Determining Assay Sensitivity, Method 1 (continued)

5. Using a multichannel pipettor, mix the cell suspension in row B by pipetting, being careful not to create foam or bubbles. Transfer 100µl from each well of row B to row C. Repeat mixing, and transfer 100µl from row C to row D. Continue this process to row G. After mixing the diluted suspension in row G, aspirate 100µl from the wells, and discard. This procedure creates dilutions of 10,000 cells/well in row A to 156 cells/well in row G. Row H will serve as the no-cell background control (Table 1).

Table 1. Schematic Diagram of 96-Well Plate Layout.

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6. Prepare Lysis Reagent according to instructions in Section 3, Step 3.a.

7. Using a multichannel pipettor, add 50µl of Lysis Reagent to all wells of columns 7–12 to lyse cells; these are the treated samples. Add 50µl of the remaining Assay Buffer to all wells in columns 1–6 so that all wells have equal volumes; these are the untreated samples. Mix by orbital shaking at 700–900rpm for at least 1 minute.

8. Prepare the CytoTox-Glo™ Cytotoxicity Assay Reagent as described in Section 3, and add 50µl to each well. Mix briefly by orbital shaking, and incubate at room temperature for 15 minutes.

   Note: Maximal sensitivity is achieved within 15 minutes. The signal intensity will remain largely unchanged for approximately 30 minutes to 1 hour, depending on plate-well density. The signal half-life is approximately 5 hours in a 96-well plate but shorter (about 2 hours) in a 1536-well plate.

9. Measure luminescence.

   Note: You may need to adjust instrument gain setting (applied photomultiplier tube energy).
10. Calculate the signal-to-noise (S:N) ratios to determine practical sensitivity for your cell type for each dilution of cells (10,000 cells/well; 5,000 cells/well, etc.).

Cytotoxicity S:N = \frac{(Average \ Treated \ RLU - Average \ Untreated \ RLU)}{S.D. \ of \ No-cell \ Background \ Controls \ (H1 \ through \ H6)}

**Note:** The practical level of assay sensitivity for either assay is a signal-to-noise ratio of greater than 3 standard deviations (derived from reference 2).

4.B. Determining Assay Sensitivity, Method 2

1. Harvest adherent cells (by trypsinization, etc.), wash with fresh medium to remove residual trypsin and resuspend in fresh medium.

   **Note:** For cells growing in suspension, proceed to Step 2.

2. Determine the number of viable cells by trypan blue exclusion using a hemacytometer, then dilute to 100,000 viable cells/ml in at least 20ml of fresh medium.

   **Note:** Concentrate the cells by centrifugation if the cell suspension is less dense than 100,000 cells/ml.

3. Divide the volume of diluted cells into two separate tubes. Subject one tube to “moderate” sonication, which is empirically determined by post-sonication morphological examination, to disrupt cell membrane integrity and to simulate a 100% population of dead cells. The second tube of untreated cells will serve as the maximum viable population.

4. Create a spectrum of viability by blending sonicated and untreated populations in 1.5ml tubes as described in Table 2.

**Table 2. Spectrum of Viability Generated by Blending Sonicated and Untreated Cells.**

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4.B. Determining Assay Sensitivity, Method 2 (continued)

5. After mixing each blend of sonicated and untreated cells by gentle vortexing, pipet 100μl of each blend into eight replicate wells of a 96-well plate. Add the 100% viable cell sample to column 1, 95% viable to column 2, etc. Add 100μl of cell culture medium only to column 10 to serve as the no-cell background control.

6. Add 50μl of CytoTox-Glo™ Cytotoxicity Assay Reagent to all wells, mix briefly by orbital shaking and then incubate for 15 minutes at room temperature.

Note: Maximal sensitivity is achieved within 15 minutes. The signal intensity will remain largely unchanged for approximately 30 minutes to 1 hour, depending on plate-well density. The signal half-life is approximately 5 hours in a 96-well plate but shorter (about 2 hours) in a 1536-well plate.

7. Measure luminescence.

Total Cell Number (optional)

8. Add 50μl of Lysis Reagent prepared according to instructions in Section 3, Step 3.b. Mix by orbital shaking, and then incubate at room temperature for 15 minutes.

9. Measure luminescence.

10. Calculate the signal-to-noise (S:N) ratios to determine practical sensitivity for your cell type for each blend of cell viability (X = 95%, 90%, etc.).

Note: The practical level of assay sensitivity for either assay is a signal-to-noise ratio of greater than 3 standard deviations (derived from reference 2).

\[
\text{Cytotoxicity S:N} = \frac{\text{Average luminescence for } X\% - \text{Average luminescence for 100\% viable cells}}{\text{S.D. of 100\% viable cells}}
\]

\[
\text{Viability S:N} = \frac{\text{Average total luminescence for } X\% - \text{Average luminescence for 0\% viable cells}}{\text{S.D. of 0\% viable cell sample}}
\]


Representative data are shown in Figure 5.

1. Set up 96-well assay plates containing cells in culture medium at the desired density.

2. Add test compounds and vehicle controls to appropriate wells so that the final volume is 100μl in each well (25μl for a 384-well plate).
3. Culture cells for the desired test exposure period.

**Note:** All enzymatic markers for cytotoxicity have finite activity half-lives (Figure 6). Although the protease marker(s) measured in this assay demonstrate an improved stability profile compared to other enzymatic markers under most circumstances, we recommend exposing the cells to the test compound for 24 hours or less to ensure that cytotoxicity is not underestimated. If longer exposures are desired, reducing the initial compound concentration may influence the kinetics of cytotoxicity and benefit the assessment of cell death.

4. Add 50 µl of CytoTox-Glo™ Cytotoxicity Assay Reagent to all wells (12.5 µl for a 384-well plate). Mix briefly by orbital shaking, and incubate for 15 minutes at room temperature.

**Note:** Maximal sensitivity will be achieved within 15 minutes. The signal intensity will remain largely unchanged for approximately 1 hour. The signal half-life is approximately 5 hours.
4.C. Example Cytotoxicity Assay Protocol and Viability (by Lysis) Assay Protocol (continued)

5. Measure luminescence.

6. Add 50µl of Lysis Reagent (prepared according to instructions in Section 3, Step 3.b) to all wells (12.5µl for a 384-well plate). Mix, and incubate at room temperature for 15 minutes.

7. Measure luminescence.

8. Calculate the luminescent contribution of previously viable cells (after lysis) by subtracting the luminescent signal resulting from experimental cell death (Step 5) from total luminescence death (Step 7).

Viable cell luminescence = Total luminescence – Experimental dead cell luminescence

Figure 6. Half-life of enzymatic markers for cytotoxicity. Jurkat cells were plated in white-walled plates in 100µl at a density of 10,000 cells per well in RPMI 1640 medium + 10%FBS. Digitonin was added to appropriate replicate wells in 10µl (30µg/ml final concentration) every hour for 10 hours to facilitate maximal cytotoxicity. The plate was incubated at 37°C during this period. CytoTox-Glo™ Reagent and reagents to measure glyceraldehyde-3-phosphate dehydrogenase and adenylate kinase activities were prepared and added as directed by the manufacturer. Luminescence was measured after 15 minutes using a BMG POLARstar luminometer. CytoTox-ONE™ Reagent was prepared and added as directed. Fluorescence was measured using a Labsystems Fluoroskan Ascent plate reader. All data were background-subtracted and plotted as a percentage of the last lysis time point.
4.D. Recommended Controls

**No-Cell Background Control:** Prepare triplicate wells without cells to serve as the negative control to determine background signal.

**Untreated Cells Control:** Prepare triplicate wells with untreated cells to serve as a vehicle control. Add the same solvent used to deliver the test compounds to the untreated-cells control wells.

**Test Compound Control:** Prepare triplicate wells without cells and containing the vehicle and test compound to test for possible interference with the CytoTox-Glo™ Assay chemistry.

**Positive Control for Cytotoxicity:** Prepare triplicate wells containing cells treated with a compound known to be toxic to the cells used in your model system (staurosporine, ionomycin, etc.).

5. General Considerations

**Assay Principle**

The CytoTox-Glo™ Assay detects both the dead and live cell populations found in a sample using sequential luminescent measures. Data are collected as relative light units (RLU). The CytoTox-Glo™ Assay achieves a signal steady-state between the dead-cell protease and firefly luciferase from 2,500 dead cells after about 15 minutes. The same number of live cells in the control well contribute a small, but measurable, background signal. After addition of digitonin to lyse remaining viable cells, a new steady-state signal is reached that represents a proportional increase in the number of dead cells (5,000 total) in the sample well (Figure 7). Therefore, cells that were initially viable contribute to the dead cell values after lysis. Because pre- and postlysis signals are stable after reaching steady-state, the viable cell contribution of any sample can be determined by a subtractive method:

\[
\text{Signal from Viable Cells} = \text{Total Cytotoxicity Signal} - \text{Initial Cytotoxic Signal}
\]

The Lysis Reagent has the same lytic efficiency as sonication.

**Background Luminescence and Inherent Serum Activity**

Tissue culture medium supplemented with animal serum may contain detectable levels of the protease marker used for dead-cell measurement. The level of this protease activity can vary between different lots of serum. To correct for variability, background luminescence should be determined using samples that contain medium plus serum without cells (i.e., the no-cell background control).
5. General Considerations (continued)

Assay Controls
In addition to a no-cell background control to establish background luminescence, we recommend including an untreated-cell control to measure maximum viability and a positive control to measure maximum cytotoxicity in the experimental design. The maximum viability control is established by adding the vehicle used to deliver the test compound to test wells. In most cases, this consists of a buffer system or medium and the equivalent amount of solvent used to deliver the test compound. Maximum cytotoxicity can be determined using the Lysis Reagent. See Section 4.A.

Figure 7. CytoTox-Glo™ Assay principle. Jurkat cells were washed and adjusted to 50,000 cells/ml in fresh RPMI 1640 medium + 10% FBS. The pool was split: One fraction was treated by mild sonication to cause cell death; the other was untreated. Equal volumes of treated and untreated cells were added to the same replicate wells (100µl final volume, 5,000 cell equivalents/well) to represent a sample population with 50% viable and 50% dead cells. Viability control wells received 50µl containing 2,500 untreated cells and 50µl of RPMI 1640 + 10% FBS. The AAF-Glo™ reagent was delivered to sample and control wells at 50µl per well. Luminescence was measured on a BMG POLARstar luminometer in kinetic mode for 25 minutes to collect the signal from the control and 50% dead populations. The plate was removed, and Lysis Reagent was added. After mixing by orbital shaking at 700rpm for 1 minute, luminescence from the total cytotoxicity was measured in kinetic mode for an additional 25 minutes.

Assay Controls
In addition to a no-cell background control to establish background luminescence, we recommend including an untreated-cell control to measure maximum viability and a positive control to measure maximum cytotoxicity in the experimental design. The maximum viability control is established by adding the vehicle used to deliver the test compound to test wells. In most cases, this consists of a buffer system or medium and the equivalent amount of solvent used to deliver the test compound. Maximum cytotoxicity can be determined using the Lysis Reagent. See Section 4.A.
Temperature
The generation of luminogenic product is proportional to the protease activity of the marker associated with cytotoxicity. The activity of this protease is influenced by temperature. For best results, we recommend incubating reactions at a constant temperature to ensure uniformity across the plate. After adding the CytoTox-Glo™ Cytotoxicity Assay Reagent and mixing briefly, we suggest one of two methods:

1. Incubate at room temperature in a water-jacketed incubation module (Me’Cour, etc.).
2. Incubate at room temperature with or without orbital shaking.

Instrumentation
Luminescence mode: Luminescence chemistry does not require an external light source to generate photons but generally collects unrestricted/unfiltered light output.

Cytotoxicity Marker Half-Life
The activity of the protease marker released from dead cells has a half-life estimated to be greater than 10 hours (Figure 6). In situations where cytotoxicity occurs rapidly such as necrosis and the incubation time is greater than 24 hours, the degree of cytotoxicity may be underestimated. When using extended incubation times, adding a lytic detergent such as digitonin may be useful to determine the total cytotoxicity marker activity remaining (from remaining live cells).

Luminescent Signal Half-Life
The CytoTox Glo™ Assay chemistry uses a thermostable, recombinant luciferase to generate a “glow-type” luminescent signal that is proportional to the number of dead cells in the sample. After a short signal ramping period (0–15 minutes), the signal reaches a plateau and is relatively stable for a period of approximately 1 hour (Figure 8). Although the signal half-life is greater than 5 hours, we suggest measuring the luminescent signal at steady-state (15 minutes to 1 hour).

Light Sensitivity
Although the AAF-Glo™ Substrate demonstrates good general photostability, aminoluciferin can degrade with prolonged exposure to ambient light sources. We recommend shielding the plates from ambient light at all times.

Cell Culture Medium
The AAF-Glo™ Substrate is introduced into the test well using an optimized buffer system that mitigates differences in pH from treatment with your test compound. In addition, the buffer system supports protease activity in a host of different culture media with varying osmolarities. With the exception of medium formulations with very high serum content or phenol red indicator, no substantial performance differences should be observed among media.
6. References


Figure 8. CytoTox-Glo™ Assay signal.
7. Related Products

## Cell Viability and Cytotoxicity Assays

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7. Related Products (continued)

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8. Summary of Changes

The following change was made to the 2/15 revision of this document:

1. The legal disclaimers were updated.
TECHNICAL BULLETIN

CellTiter-Glo® Luminescent Cell Viability Assay

Instructions for Use of Products G7570, G7571, G7572 and G7573
1. Description

The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells. The CellTiter-Glo® Assay is designed for use with multiwell-plate formats, making it ideal for automated high-throughput screening (HTS) and cell proliferation and cytotoxicity assays. The homogeneous assay procedure (Figure 1) involves adding a single reagent (CellTiter-Glo® Reagent) directly to cells cultured in serum-supplemented medium. Cell washing, removal of medium or multiple pipetting steps are not required.

The homogeneous “add-mix-measure” format results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present (Figure 2). The amount of ATP is directly proportional to the number of cells present in culture in agreement with previous reports (1). The CellTiter-Glo® Assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which generates a stable “glow-type” luminescent signal and improves performance across a wide range of assay conditions. The luciferase reaction for this assay is shown in Figure 3. The half-life of the luminescent signal resulting from this reaction is greater than five hours (Figure 4). This extended half-life eliminates the need for reagent injectors and provides flexibility for continuous or batch-mode processing of multiple plates. The unique homogeneous format reduces pipetting errors that may be introduced during the multiple steps required by other ATP-measurement methods.
1. Description (continued)

Figure 1. Flow diagram showing preparation and use of CellTiter-Glo® Reagent.
System Advantages

- **Homogeneous:** "Add-mix-measure" format reduces the number of plate-handling steps to fewer than that required for similar ATP assays.

- **Fast:** Data can be recorded 10 minutes after adding reagent.

- **Sensitive:** Measures cells at numbers below the detection limits of standard colorimetric and fluorometric assays.

- **Flexible:** Can be used with various multiwell formats. Data can be recorded by luminometer or CCD camera or imaging device.

- **Robust:** Luminescent signal is very stable, with a half-life >5 hours, depending on cell type and culture medium used.

- **Able to Multiplex:** Can be used with reporter gene assays or other cell-based assays from Promega (2,3).

Figure 2. Cell number correlates with luminescent output. A direct relationship exists between luminescence measured with the CellTiter-Glo® Assay and the number of cells in culture over three orders of magnitude. Serial twofold dilutions of HEK293 cells were made in a 96-well plate in DMEM with 10% FBS, and assays were performed as described in Section 3.B. Luminescence was recorded 10 minutes after reagent addition using a GloMax®-Multi+ Detection System. Values represent the mean ± S.D. of four replicates for each cell number. The luminescent signal from 50 HEK293 cells is greater than three times the background signal from serum-supplemented medium without cells. There is a linear relationship ($r^2 = 0.99$) between the luminescent signal and the number of cells from 0 to 50,000 cells per well.
1. Description (continued)

![Chemical Structure: Beetle Luciferin and Oxyluciferin](image)

**Figure 3. The luciferase reaction.** Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg\(^{2+}\), ATP and molecular oxygen.

![Graph: Relative Luminescence vs. Time](image)

**Figure 4. Extended luminescent half-life allows high-throughput batch processing.** Signal stability is shown for three common cell lines. HepG2 and BHK-21 cells were grown and assayed in MEM containing 10% FBS, while CHO-K1 cells were grown and assayed in DME/F-12 containing 10% FBS. CHO-K1, BHK-21 and HepG2 cells, at 25,000 cells per well, were added to a 96-well plate. After an equal volume of CellTiter-Glo® Reagent was added, plates were shaken and luminescence monitored over time with the plates held at 22°C. The half-lives of the luminescent signals for the CHO-K1, BHK-21 and HepG2 cells were approximately 5.4, 5.2 and 5.8 hours, respectively.
2. Product Components and Storage Conditions

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**Storage Conditions:** For long-term storage, store the lyophilized CellTiter-Glo® Substrate and CellTiter-Glo® Buffer at −20°C. For frequent use, the CellTiter-Glo® Buffer can be stored at 4°C or room temperature for 48 hours without loss of activity. See product label for expiration date information. Reconstituted CellTiter-Glo® Reagent (Buffer plus Substrate) can be stored at room temperature for up to 8 hours with <10% loss of activity, at 4°C for 48 hours with ~5% loss of activity, at 4°C for 4 days with ~20% loss of activity or at −20°C for 21 weeks with ~3% loss of activity. The reagent is stable for up to ten freeze-thaw cycles, with less than 10% loss of activity.
3. Performing the CellTiter-Glo® Assay

Materials to Be Supplied by the User

- opaque-walled multiwell plates adequate for cell culture
- multichannel pipette or automated pipetting station for reagent delivery
- device (plate shaker) for mixing multiwell plates
- luminometer, CCD camera or imaging device capable of reading multiwell plates
- optional: ATP for use in generating a standard curve (Section 3.C)

3.A. Reagent Preparation

1. Thaw the CellTiter-Glo® Buffer, and equilibrate to room temperature prior to use. For convenience the CellTiter-Glo® Buffer may be thawed and stored at room temperature for up to 48 hours prior to use.

2. Equilibrate the lyophilized CellTiter-Glo® Substrate to room temperature prior to use.

3. Transfer the appropriate volume (10ml for Cat.# G7570 and G7571, or 100ml for Cat.# G7572 and G7573) of CellTiter-Glo® Buffer into the amber bottle containing CellTiter-Glo® Substrate to reconstitute the lyophilized enzyme/substrate mixture. This forms the CellTiter-Glo® Reagent.

4. Mix by gently vortexing, swirling or inverting the contents to obtain a homogeneous solution. The CellTiter-Glo® Substrate should go into solution easily in less than 1 minute.

3.B. Protocol for the Cell Viability Assay

We recommend that you perform a titration of your particular cells to determine the optimal number and ensure that you are working within the linear range of the CellTiter-Glo® Assay. Figure 2 provides an example of such a titration of HEK293 cells using 0 to 50,000 cells per well in a 96-well format.

1. Prepare opaque-walled multiwell plates with mammalian cells in culture medium, 100µl per well for 96-well plates or 25µl per well for 384-well plates.

Multiwell plates must be compatible with the luminometer used.

2. Prepare control wells containing medium without cells to obtain a value for background luminescence.

3. Add the test compound to experimental wells, and incubate according to culture protocol.

4. Equilibrate the plate and its contents at room temperature for approximately 30 minutes.
5. Add a volume of CellTiter-Glo® Reagent equal to the volume of cell culture medium present in each well (e.g., add 100µl of reagent to 100µl of medium containing cells for a 96-well plate, or add 25µl of reagent to 25µl of medium containing cells for a 384-well plate).

6. Mix contents for 2 minutes on an orbital shaker to induce cell lysis.

7. Allow the plate to incubate at room temperature for 10 minutes to stabilize luminescent signal. 
   **Note:** Uneven luminescent signal within standard plates can be caused by temperature gradients, uneven seeding of cells or edge effects in multiwell plates.

8. Record luminescence. 
   **Note:** Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

3.C. Protocol for Generating an ATP Standard Curve (optional)

It is a good practice to generate a standard curve using the same plate on which samples are assayed. We recommend ATP disodium salt (Cat.# P1132, Sigma Cat.# A7699 or GE Healthcare Cat.# 27-1006). The ATP standard curve should be generated immediately prior to adding the CellTiter-Glo® Reagent because endogenous ATPase enzymes found in sera may reduce ATP levels.

1. Prepare 1µM ATP in culture medium (100µl of 1µM ATP solution contains 10−10 moles ATP).
2. Prepare serial tenfold dilutions of ATP in culture medium (1µM to 10nM; 100µl contains 10−10 to 10−12 moles of ATP).
3. Prepare a multiwell plate with varying concentrations of ATP standard in 100µl medium (25µl for a 384-well plate).
4. Add a volume of CellTiter-Glo® Reagent equal to the volume of ATP standard present in each well.
5. Mix contents for 2 minutes on an orbital shaker.
6. Allow the plate to incubate at room temperature for 10 minutes to stabilize the luminescent signal.
7. Record luminescence.
4. Appendix

4.A. Overview of the CellTiter-Glo® Assay

The assay system uses the properties of a proprietary thermostable luciferase to enable reaction conditions that generate a stable "glow-type" luminescent signal while simultaneously inhibiting endogenous enzymes released during cell lysis (e.g., ATPases). Release of ATPases will interfere with accurate ATP measurement. Historically, firefly luciferase purified from *Photinus pyralis* (LucPpy) has been used in reagents for ATP assays (1,4–7). However, it has only moderate stability in vitro and is sensitive to its chemical environment, including factors such as pH and detergents, limiting its usefulness for developing a robust homogeneous ATP assay. Promega has successfully developed a stable form of luciferase based on the gene from another firefly, *Photuris pennsylvanica* (LucPpe2), using an approach to select characteristics that improve performance in ATP assays. The unique characteristics of this mutant (LucPpe2m) enabled design of a homogeneous single-reagent-addition approach to perform ATP assays with cultured cells. Properties of the CellTiter-Glo® Reagent overcome the problems caused by factors, such as ATPases, that interfere with ATP measurement in cell extracts. The reagent is physically robust and provides a sensitive and stable luminescent output.

**Sensitivity and Linearity:** The ATP-based detection of cells is more sensitive than other methods (8–10). In experiments performed by Promega scientists, the luminescent signal from 50 HEK293 cells is greater than three standard deviations above the background signal from serum-supplemented medium without cells. There is a linear relationship ($r^2 = 0.99$) between the luminescent signal and the number of cells from 0 to 50,000 cells per well in the 96-well format. The luminescence values in Figure 2 were recorded after 10 minutes of incubation at room temperature to stabilize the luminescent signal as described in Section 3.B. Incubation of the same 96-well plate used in the experiment shown in Figure 2 for 360 minutes at room temperature had little effect on the relationship between luminescent signal and number of cells ($r^2 = 0.99$).

**Speed:** The homogeneous procedure to measure ATP using the CellTiter-Glo® Assay is quicker than other ATP assay methods that require multiple steps to extract ATP and measure luminescence. The CellTiter-Glo® Assay also is faster than other commonly used methods to measure the number of viable cells (such as MTT, alamarBlue® or Calcein-AM) that require prolonged incubation steps to enable the cells' metabolic machinery to convert indicator molecules into a detectable signal.
4.B. Additional Considerations

**Temperature:** The intensity and decay rate of the luminescent signal from the CellTiter-Glo® Assay depends on the luciferase reaction rate. Environmental factors that affect the luciferase reaction rate will change the intensity and stability of the luminescent signal. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates to a constant temperature before performing the assay. Transferring eukaryotic cells from 37°C to room temperature has little effect on ATP content (5). We have demonstrated that removing cultured cells from a 37°C incubator and allowing them to equilibrate to 22°C for 1–2 hours had little effect on ATP content. For batch-mode processing of multiple assay plates, take precautions to ensure complete temperature equilibration. Plates removed from a 37°C incubator and placed in tall stacks at room temperature will require longer equilibration than plates arranged in a single layer. Insufficient equilibration may result in a temperature gradient effect between wells in the center and at the edge of the plates. The temperature gradient pattern also may depend on the position of the plate in the stack.

**Chemicals:** The chemical environment of the luciferase reaction affects the enzymatic rate and thus luminescence intensity. Differences in luminescence intensity have been observed using different types of culture media and sera. The presence of phenol red in culture medium should have little impact on luminescence output. Assaying 0.1µM ATP in RPMI medium without phenol red resulted in ~5% increase in luminescence output (in relative light units [RLU]) compared to assays in RPMI containing the standard concentration of phenol red, whereas assays in RPMI medium containing twice the normal concentration of phenol red showed a ~2% decrease in luminescence.

Solvents for the various test compounds may interfere with the luciferase reaction and thus the light output from the assay. Interference with the luciferase reaction can be detected by assaying a parallel set of control wells containing medium without cells. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations of up to 2% in the assay and only minimally affects light output.

**Plate Recommendations:** We recommend using standard opaque-walled multiwell plates suitable for luminescence measurements. Opaque-walled plates with clear bottoms to allow microscopic visualization of cells also may be used; however, these plates will have diminished signal intensity and greater cross talk between wells. Opaque white tape may be used to decrease luminescence loss and cross talk.

**Cellular ATP Content:** Different cell types have different amounts of ATP, and values reported for the ATP level in cells vary considerably (1,4,11–13). Factors that affect the ATP content of cells may affect the relationship between cell number and luminescence. Anchorage-dependent cells that undergo contact inhibition at high densities may show a change in ATP content per cell at high densities, resulting in a nonlinear relationship between cell number and luminescence. Factors that affect the cytoplasmic volume or physiology of cells also will affect ATP content. For example, oxygen depletion is one factor known to cause a rapid decrease in ATP (1).

**Mixing:** Optimal assay performance is achieved when the CellTiter-Glo® Reagent is mixed completely with the cultured cells. Suspension cell lines (e.g., Jurkat cells) generally require less mixing to achieve lysis and extract ATP than adherent cells (e.g., L929 cells). Tests were done to evaluate the effect of shaking the plate after adding the CellTiter-Glo® Reagent. Suspension cells cultured in multiwell plates showed only minor differences in light output whether or not the plates were shaken after adding the CellTiter-Glo® Reagent. Adherent cells are more difficult to lyse and show a substantial difference between shaken and nonshaken plates.
4.B. Additional Considerations (continued)

Several additional parameters related to reagent mixing include the force of delivery of CellTiter-Glo® Reagent, sample volume and dimensions of the well. All of these factors may affect assay performance. The degree of reagent mixing required may be affected by the method used to add the CellTiter-Glo® Reagent to the assay plates. Automated pipetting devices using a greater or lesser force of fluid delivery may affect the degree of subsequent mixing required. Complete reagent mixing in 96-well plates should be achieved using orbital plate shaking devices built into many luminometers and the recommended 2-minute shaking time. Special electromagnetic shaking devices that use a radius smaller than the well diameter may be required to efficiently mix contents of 384-well plates. The depth of medium and geometry of the multiwell plates may have an effect on mixing efficiency. We recommend that you take these factors into consideration when performing the assay and empirically determine whether a mixing step is necessary for the individual application.

Luminometers

For highly sensitive luminometric assays, the luminometer model and settings greatly affect the quality of data obtained. Luminometers from different manufacturers will vary in sensitivities and dynamic ranges. We recommend the GloMax® products because these instruments do not require gain adjustments to achieve optimal sensitivity and dynamic range. Additionally, GloMax® instruments are preloaded with Promega protocols for ease of use.

If you are not using a GloMax® luminometer, consult the operating manual for your luminometer to determine the optimal settings. The limits should be verified on each instrument before analysis of experimental samples. The assay should be linear in some portion of the detection range of the instrument used. For an individual luminometer there may be different gain settings. We recommend that you optimize the gain settings.
4.C. References


### 4.D. Related Products

#### Cell Proliferation Products

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#### Cytotoxicity Assays

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U.S. Pat. No. 7,700,310, European Pat. No. 1546374 and other patents pending.


The method of recombinant expression of Coleoptera luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.