Chapter 12

High-Throughput Cell Death Assays

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Abstract

High-throughput screens (HTS) are powerful tools that permit the rapid evaluation of thousands of samples in a cost-effective manner and minimize sample and reagent consumption. Such assays have recently begun to be utilized to evaluate cell death modalities and also the cytoprotective efficacy of compounds against a wide variety of stresses. Here we describe the design, preparation, and undertaking of HTS-appropriate assays that utilize simple and cost-effective fluorophore- and luminescence-based functional readouts of cell viability. These assays permit the examination of 96–384 compounds in a single multiwell plate with highly robust statistical significance at a fraction of the financial and work cost of traditional approaches.

Key words Cell line, Fluorophore, Ischemia, Luciferase, Microplate, Z′-factor

1 Introduction

Cell death assays are widely used both in the examination of cell death modalities induced by a given insult and also in the search for cytoprotective candidate compounds against a wide variety of clinically relevant stresses. Most commonly used cell death assays are not suitable for large-scale screening approaches and this has led to the development of a specialized field of study focused on the design of assays optimized for the rapid analysis of large numbers of candidate compounds [1, 2]. Such high-throughput screens (HTS) are assays designed to rapidly evaluate very large numbers of compounds for a biological interaction of interest and recently they have begun to inform the study of cell death modalities and the efficacy of cytoprotective compounds against a wide variety of stressors [3]. HTS rely on simple, cost-effective cell viability assays that are easily "scaled up" to evaluate cells in multiwell plates and permit the examination of compounds on a scale that vastly exceeds traditional single-sample approaches at a far more cost-effective price point. HTS rely on outstanding statistical separation between

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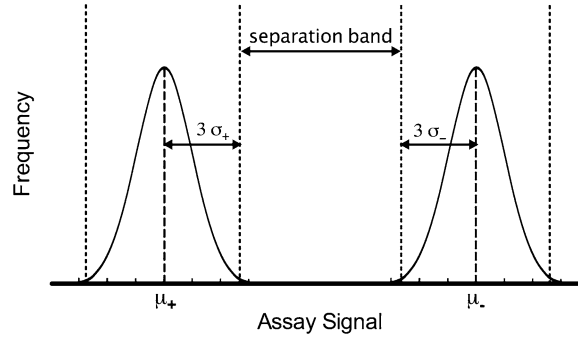

Fig. 1 Illustration of data variation and separation between positive and negative controls in a typical HTS assay. σ_+ and σ_- are the standard deviations of positive and negative controls, and μ_+ and μ_- are the mean values of positive and negative controls. Modified from [4]

positive and negative controls such that the means of these two groups are typically separated by >12 standard deviations. At this degree of separation, a single replicate is sufficient to infer statistical significance, permitting researchers to assay upward of 100,000 compounds in a few weeks. The robustness of an HTS is determined by a Z′-factor score, which is an evaluation of the separation of the means between positive and negative controls and the tightness of the standard deviations of the means (Fig. 1) [4]. Typically the positive controls are the insult-treated cells, and the negative controls are cells treated in serum-free culture media (i.e., healthy, growth-arrested cells).

HTS are typically designed to incorporate microplate-based assays that can be read on a standard laboratory spectrophotometer or luminometer. Such assays are usually developed initially in 96- or 384-well plates but can be scaled up to automated systems that support up to 9,600-well plates and utilize volumes of solution so minute that sonic impulses are required to accurately dispense nano-volumes into the wells [5]. Each sample well should contain an approximately equal number of cells that behave in a homogenous fashion, and therefore immortalized cell lines are typically used for HTS because such cells are strongly adherent to the growth matrix (i.e., the bottom of the wells), hardy, and divide rapidly [6]. These properties allow for cultures to be quickly scaled up, producing sufficient cells to screen a large number of compounds in a short period of time. Lastly, HTS of cell viability usually rely upon simple cell death assays to act as "readouts" of cell state. Typically fluorophore- or luciferase-based assays are utilized for such screens [7, 8], as these assays require only simple reagent addition to the wells (cf. as opposed to many molecular assays of cell viability that require multiple rinsing or incubation steps before reading).

HTS offer remarkable advantages over traditional single-replicate cell viability assays such as flow cytometry, fluorescence microscopy, and immunohistochemistry. In particular, HTS are far cheaper and simpler than such traditional cell viability assays, and thus permit far more compounds to be evaluated in a short period of time while minimizing reagent consumption. For example, evaluating the efficacy of a 1,000 candidate compounds at inhibiting the expression of the commonly used apoptotic markers using traditional microscopy approaches would require months of effort and considerable materials and manpower costs to treat and evaluate samples, while evaluation of 100,000 compounds would take an entire research career using traditional methods. Conversely, the same large number of compounds can be evaluated using an HTS on multiwell plates in days to weeks by a single researcher. HTS are limited somewhat in that they are generally restricted to evaluations in cell lines, which are considerably removed from more physiologically relevant in vivo experiments and results from HTS must therefore eventually be scaled down and validated in more complex systems. However, HTS approaches can be used in smaller replicates to rapidly assay primary cell cultures and may eventually be adaptable to evaluate isolated tissues. In this chapter we describe the basic steps of preparing an HTS, including (1) cell selection and cell culture preparation, (2) scaling up of cell cultures and setting up multiwell microplates, (3) assay selection and experimentation, and (4) data evaluation.

2 Materials

2.1 Cell Culture Media and Treatment Media Components

Prepare all solutions using ultrapure water and analytical grade reagents. The choice of cell culture media will vary between cell lines and a neuronal cell line culture media is utilized as an example for our protocol. Prepare all reagents at room temperature and store at 4 °C unless otherwise indicated.

1. Complete cell culture media: Dulbecco's modified Eagle medium (DMEM), 10 % bovine calf serum (BCS), 100 U/mL penicillin/streptomycin. Filter using standard cell culture filters.
2. Serum-free cell culture media: DMEM, 100 U/mL penicillin/streptomycin. Filter using standard cell culture filters.
3. Artificial cerebral spinal fluid (ACSF): 129 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 21 mM $NaHCO_3$, 10 mM glucose, 315 mOsM, pH 7.4.
4. Ischemic solution (IS): 64 mM K^+, 51 mM Na^+, 77.5 mM Cl^-, 0.13 mM Ca^{2+}, 1.5 mM Mg^{2+}, 3 mM glucose, 0.1 mM glutamate, 315 mOsM, pH 6.5, 1.5 % O_2, 15 % CO_2, balance N_2 [9, 10].

2.2 Other Materials and Hardware Components

1. Standard sterile cell culture facilities (laminar flow fume hood, incubator, media-filtration apparatus, low-temperature centrifuge, etc.).
2. Standard cell culture flasks.
3. Sterile 96- or 384-well microplates. Solid white or black with clear bottoms (for fluorescence-based assays) or solid bottoms (for luminescence-based assays).
4. 8-Channel 100 μL (for 384-well plates) and 1,000 μL (for 96-well plates) multi-pipetters.
5. Plate spectrophotometer with fluorescence or luminescence reading capabilities (as appropriate for the chosen cell viability assay). For fluorescence-based assays ensure that the plate reader scans in the required wavelength specified for the chosen fluorescent probe. For repeated measures in real time, plate readers that maintain physiological temperatures are required.
6. 0.05 % Trypsin-EDTA solution (commercially available).
7. Propidium iodide (PI).
8. ATP luciferase assay: PerkinElmer ATPlite Luminescence Assay System kits (PerkinElmer, Waltham, MA).
9. 4 % paraformaldehyde (optional).
10. Orbital plate shaker.
11. Light microscope.
12. Vortex.

3 Methods

3.1 Preparation of Culture Media and Cell Cultures

For the purposes of demonstration we have chosen the HT22 murine hippocampal neuronal cell line, which is grown in DMEM and split with 0.05 % trypsin. These cells divide extremely rapidly and are an excellent subject for HTS assays (Fig. 2). Feeding rate, cell growth, desired confluence, etc. will vary depending on the cell line used and researchers should refer to the suppliers' instructions for information specific to their chosen cell type.

1. Prepare complete cell culture media (normal growth media) and filter sterilize. Sterile-filtered media may be stored at 4 °C for several weeks.
2. Thaw an aliquot of frozen cell suspension. *See* **Note 1**.
3. Suspend the contents of the cryovial in an appropriate volume of pre-warmed culture media (approximately 5, 10, or 20 mL of media for 25, 75, or 150 cm^2 flasks, respectively) and add this suspension to the appropriate cell culture flask. *See* **Note 2**.

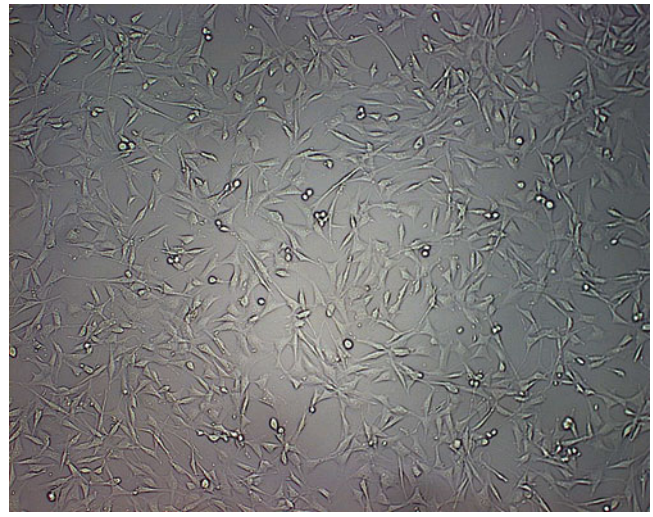

Fig. 2 Representative image of ideal cell density of HT22 neurons in a single well of a 96-well plate prior to experimentation

4. Place the flask in a 5 % CO_2 incubator at 37 °C and allow cells to grow and divide.
5. Feed cells 2–3 times per week and split cells when they reach approximately 70–80 % confluence.

3.2 Seeding Cells onto Microplates

1. On the day before the experiment, detach cells from the growth matrix. Use 0.05 % trypsin with EDTA for 10 min to split HT22 neurons. The appropriate lysis agent and concentration will vary between cell types. *See* **Note 3**.
2. Resuspend cells in ~5 volumes of complete cell culture media in a 15 mL Falcon tube. *See* **Note 4**.
3. Inspect the cell suspension under a light microscope to ensure that cells are isolated and not clumped.
4. Centrifuge the resulting cell suspension at 125 × *g* for 5–7 min at room temperature.
5. Aspirate the supernatant and resuspend the cell pellet in complete cell culture media diluted to the desired seeding density. *See* **Note 5**.
6. Add cell suspension to each well of the microplate (use ~30–40 μL of the cell suspension for 384-well plates and 200–300 μL of the cell suspension for 96-well microplates) (*see* **Notes 6** and **7**). Be sure to leave several blank wells and also several wells with media only (no cells) to serve as on-plate controls.
7. Replace the lids of the microplates and incubate the cells and cover slips overnight in the normal tissue culture incubator.

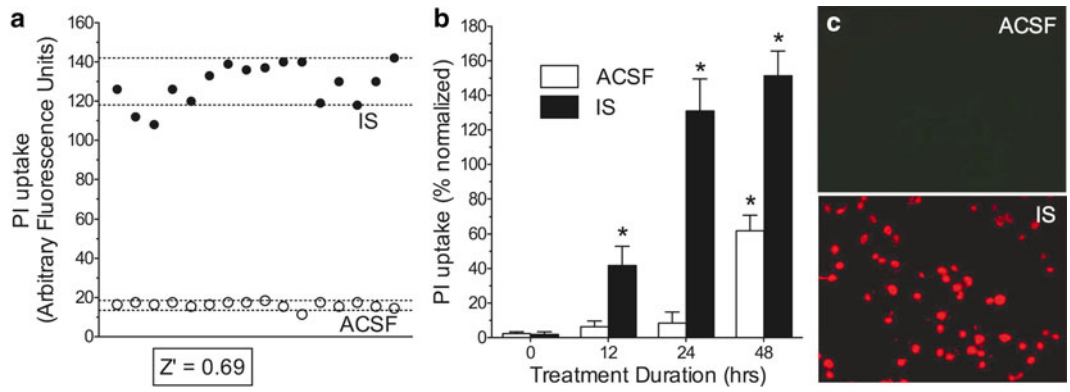

Fig. 3 (**a**) Representative PI exclusion measurements from HT22 neurons treated with ACSF (cell death negative control) or IS (cell death positive control) for 24 h in a 96-well microplate. *Dashed lines* are coefficients of variance for each treatment. (**b**) Effect of experimental duration on PI exclusion. (**c**) Sample images of PI uptake (red fluorescence) into HT22 neurons treated with ACSF (*top panel*) or IS (*bottom panel*) for 24 h. Data are mean ± SD from 20 wells for each treatment condition on a single plate. *Asterisks* indicate significant difference from control at $t = 0$ min ($P < 0.001$)

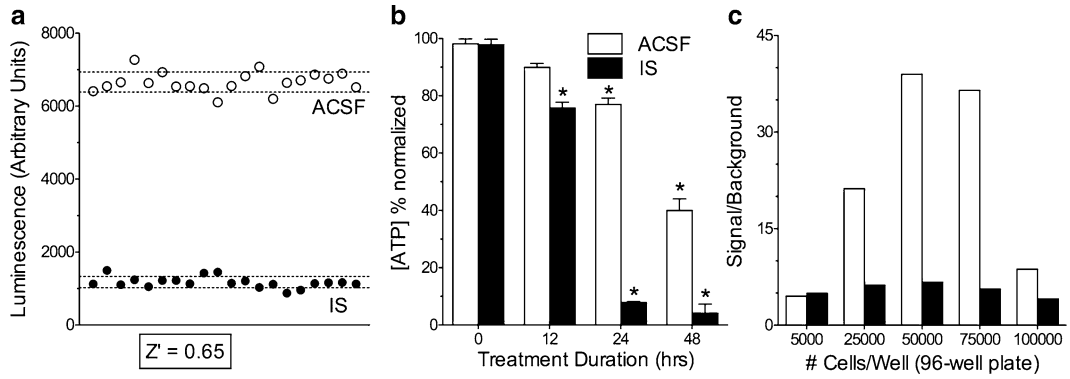

Fig. 4 (**a**) Representative total (ATP) measurements from HT22 neurons treated with ACSF (cell death negative control) or IS (cell death positive control) for 24 h in a 96-well microplate. *Dashed lines* are coefficients of variance for each treatment. (**b**) Effect of experimental duration on [ATP]. (**c**) Effect of initial cell seeding density on signal-to-background noise ratio of measurements of [ATP]. Data are mean ± SD from 20 wells for each treatment condition on a single plate. *Asterisks* indicate significant difference from control at $t = 0$ min ($P < 0.001$)

3.3 Treating Cells for Experimentation

For the purposes of demonstration we have chosen to treat HT22 neurons with either ACSF (cell death negative control) or an in vitro mimic of the ischemic mammalian penumbra (IS—cell death positive control) [11]. We assessed cell viability using both a fluorescence-based assay (PI exclusion; Fig. 3) and a luminescence-based assay (ATP luciferase; Fig. 4). PI is a bulky molecule that is too large to penetrate the plasma membrane of healthy cells but which readily permeate dying cells with damaged plasma membranes. Upon penetrating a cell, PI binds to nuclear DNA and fluoresces brightly in the red wavelength.

ATP luciferase measures the total ATP content of a cell and requires lysis of cells to free cellular ATP before measurement. If multiple time points are to be examined then care should be taken in determining the appropriate number of plates to seed with cells. Fluorescence-based assays can be read multiple times during an experiment; however, luminescence-based assays can only be read once. Therefore for these later assays, separate plates will be required for each time point, including controls (i.e., $t = 0$ min).

1. On the day of experimentation, view the microplates under a microscope to ensure that cells are evenly distributed in the wells and appear healthy (Fig. 2). *See* **Note 8**.
2. For fluorescence-based assays only: Prepare serum-free treatment media (preferably pre-equilibrated to 37 °C and 5 % CO_2 overnight) with the appropriate concentration of the fluorescent probe. For PI assays use 5 μg PI per mL media. Protect from light.
3. Remove the culture media and rinse cells to remove all residual serum. *See* **Note 9**. Inspect wells to ensure that cells have not detached during the rinsing process. Note any wells that have been compromised.
4. Add an equal volume of the treatment media. *See* **Notes 10** and **11**.
5. Optional: For fluorescence-based assays: read pretreatment fluorescence on the plate spectrophotometer to determine baseline fluorescence if desired (*see* Subheading 3.4).
6. Optional: For luminescence-based assays: read control plates on the plate spectrophotometer to determine baseline luminescence if desired (*see* Subheading 3.4).
7. Return the microplates to the incubator and incubate samples for the desired experimental period.

3.4 Data Acquisition and Analysis

Fluorescence-based assays of live cells should be performed at physiological temperatures. Luminescence-based assays may be performed at room temperature.

1. For fluorescence-based assays: Following treatment, read fluorescence at the appropriate wavelength. For our PI assay these wavelengths are excitation/emission = 485/630 nm (Fig. 3). *See* **Note 12**.
2. For luciferase-based assays: Add cell lysis reagent to each well and shake plate on an orbital plate shaker at 700 rpm for 5 min. The volume of lysis reagent added will vary depending on the specific assay kit chosen.
3. Add luciferase reagent to each well and shake plate on an orbital shaker at 700 rpm for 5 min. Protect plates from light once this reagent has been added.

4. Let plate sit in the dark for 15–30 min.
5. Read luminescence on a plate reader (Fig. 4).
6. Determine Z′ score according to the following equation: $Z' = 1 - |(3\sigma_+ + 3\sigma_-)/(\mu_+ - \mu_-)|$, where σ_+ and σ_- are the standard deviations of positive and negative controls, and μ_+ and μ_- are the mean values of positive and negative controls. A Z′-factor of >0.5 is considered to be an excellent and robust HTS [4].

4 Notes

1. Cells are typically stored at −80 to −160 °C and can be significantly damaged if warmed up too rapidly. To minimize temperature shock, thaw an aliquot of frozen cell suspension by rapidly rubbing the cryovial between gloved hands.
2. Cultured cells are often a relatively fragile preparation and cell death can result from the handling of cells prior to experimentation and examination, which can confound results. To help ensure that cells are healthy during the initial experimental setup, equilibrate media in a cell culture incubator in vented-top sealed cell culture flasks overnight. This will allow the media to reach the temperature and pH of the incubator (typically 37 °C and 5 % CO_2) without compromising the sterility of the solution, and thereby reduce the stress to cells during setup.
3. Use 1–2 mL trypsin for 75 and 150 cm^2 cell culture flasks. Care should be taken to make sure that trypsin is evenly distributed over all cells. Cells treated with trypsin should be kept in the incubator at 37 °C for the duration of the treatment to speed enzymatic cleavage of adherent proteins.
4. The serum in the media inactivates the trypsin and prevents unwanted cell lysis. Add ~4–5 volumes of complete cell culture media directly to each cell culture flask for each volume of trypsin. Repeatedly sluice this media around the bottom of the flask to detach all cells. Repeated pipetting of the media may be used to detach cells that are more adherent. Removal of cell clumps is important to ensure homogenous cell distribution in the multiwell plates and holding the tip of a 5 or 10 mL pipette against the wall of the flask and repeatedly pipetting the cell suspension can remove cell clumps. The sheer force of the culture media being forced out between the pipette tip held flush to the flask wall will facilitate clump breakage.
5. The desired seeding density will vary depending on the growth rate and desired confluency of the cells at the time of experimentation. Typically, it is ideal to seed cells at a density such that following 24 h (overnight growth) they will be at the desired density. If cells are seeded too thinly the resulting signal will be weak. Thin seeding can also result in uneven growth

between wells. If cells are seeded too densely then they may become overgrown and more easily detach in "sheets" from the bottoms of the wells. Detached cells may be washed away during rinse stages, leading to minimal signal from a given well and experimental error. It is necessary to determine the ideal seeding density for each cell type used. A good starting point is to seed ~50,000 cells per well in 96-well plates, and ~10,000 cells per well in 384-well plates.

6. Adherent cells will begin to clump rapidly in a cell suspension and care should be taken to resuspend the cells regularly by gentle pipette mixing while they are being dispensed into microplates. With 384-well plates in particular, this step can be time consuming and so 8-well multi-pipette dispensers should be used where possible to facilitate even cell distribution between wells. If available, automated liquid handling and cell dispensing machines are preferable as they permit the rapid seeding of numerous plates and reduce human errors in pipetting. However, liquid-handling systems typically require a large dead volume of cell suspensions, making them less ideal for experiments where the volume of available cells is limited.
7. It is important to consider the design of the microplate in setting up your assay. In general it is best to minimize exposure to light sources and light transmission between wells. Therefore black or white microplates are desirable over clear microplates. Fluorescence-based assays require plates with bottoms that permit light transmission (i.e., clear bottom plates), while luminescence-based assays require solid-bottom plates to reduce light contamination. If using a luminescence-based assay with solid-bottom plates then cells should also be seeded into additional clear-bottomed plates at the same seeding density to permit visual examination of cell density and health prior to experimentation. These "test" plates should be rinsed and treated in the same fashion as the solid-bottom experimental plates in order to control for treatment permutations and ensure that cell density is maintained throughout washing and experimentation.
8. (Optional) It may be desirable to halt the growth of cells at some point prior to experimentation. If so, 2–16 h prior to experimentation, replace normal growth media with serum-free media to halt cell division and phase-lock cells.
9. Effective removal and rinsing of culture medium in a consistent fashion between wells are critical to maximizing the signal-to-noise ratio and minimizing the coefficient of variance of the results. Serum can protect cells from a given stress and may also interact with fluorophores or luciferase probes to quench the signal and interfere with measurements. Numerous options are available to researchers to rinse cells. The most time consuming of these is to carefully remove the growth media

and rinse the cells by manual pipetting. This is a reasonable approach if working with up to a few dozen wells in a single plate but is overly time consuming for larger sample sizes. A better manual option that works well particularly in 384-well plates is to hold the plate upside down and whip the plate downwards before abruptly stopping this motion. This "whiplash-like" action will effectively remove most cell culture media from all wells of the plate and adherent cells will not detach. Multiwell pipetters can then be used to pipette a rinse media (usually pre-warmed serum-free culture media) into the wells and rinses can be repeated using the same approach. If available, cells seeded into multiwell microplates can be more gently washed with 4–5 volumes of rinse media using a plate washer such as the TECAN PW96/384 Washer (TECAN, San Jose, CA).

10. Assay consistency is critical to developing a robust HTS and researchers should be aware of the time bottlenecks of their procedure. Often the biggest bottlenecks occur at the readout stage as most plate spectrophotometers will take several minutes to read a single plate. Thus the setup of the initial assay should be staggered by 15–30 min between each plate to account for this and ensure that samples are treated for the same duration.
11. Luminescence-based assays require the addition of cell lysis reagents and luciferase reagents following treatment. Plan ahead to ensure that adequate space remains in the wells of each plate to permit these additions, and such that vortexing the plate to mix the wells does not result in liquid spillover, and contamination of adjacent wells.
12. (Optional) Fluorophore-treated cells can also be fixed by addition of 4 % paraformaldehyde before measurement. This approach permits experimental plates to be preserved for future examination as desired.

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