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High-throughput cytotoxicity screening: hit and miss

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It has long been established that there is a need for toxicity testing in drug discovery. As the number of lead compounds continues to expand, the problem facing the industry is where to position the toxicity tests in the drug discovery pipeline. To save time and effort, the tests should be performed as early in the process as possible. Historically, the assays available have not lent themselves well to the needs of HTS. This review discusses some of the pitfalls associated with toxicity testing, and covers the advances in technology that enable provision of accurate and reproducible data.

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▼ Historically, toxicity testing has been performed before Phase I clinical trials and often involves the use of animal models in addition to a battery of other tests involving *in vitro* models. There are several reasons why drugs fail during development, including poor biopharmaceutical properties, such as solubility, stability and permeability[1]. A large proportion of the drugs fail as a result of toxicity (22%), highlighting the importance of toxicity testing in the drug discovery process. Moving toxicity tests to an earlier stage of the process should reduce the drop-out rate at the late pre-clinical stages. On average, every new molecule requires 12–15 years to proceed to market, and can cost anywhere between US$400 million and US$650 million to develop[2]. As a compound identified in primary HTS moves further down the drug discovery pipeline, the costs associated with its failure increase dramatically. According to the Pharmaceutical Research and Manufacturers of America[3], US pharmaceutical companies spend 32.3% of total compound development on the pre-clinical phase (up to and including toxicology and safety testing). By moving toxicity testing to an earlier stage, a large part (62%) of the R&D expenditure that is associated with biological screening, pharmacological testing, toxicology and safety testing could be saved. This could result in a potential saving of US$80–US$130 million (the cost of the late detection of toxicity) for each toxic compound. It has been suggested that linking toxicity with compound structure could lead to the design of compound libraries with less potential for toxicity[4]. The determination of cytotoxicity has, therefore, become a major focus in drug discovery[5].

Whichever strategy is used for hit identification, whether diversity-based or focussed screening or both[6], cytotoxicity determination is still required as part of the lead optimization process to reduce development failures. A wide variety of established cytotoxicity end-points have been tried on HTS systems with limited success. Problems with sensitivity, reproducibility and simplicity are often encountered when methods such as the tetrazolium assay and other dye-based procedures are used[7,8]. Any scientist working in HTS understands the paramount importance of optimizing these parameters and knows the difficulties that are encountered during assay miniaturization for high-density formats. Cytotoxicity assays are complicated further by the simple fact that they require living cells.

New technologies generate new problems

Over the past decade, the speed of screening has increased significantly. For example, with the technologies available in 1992, it would have taken the equivalent of 20 years to screen one million compounds[9]. By 1998, it was possible to screen 55,000 wells per week and this is anticipated to increase to 350,000 by 2003 (Ref. 10). In certain areas, some companies hope to screen 100,000 or more microplates per day as a result of the emerging ultra-HTS platforms[10]. This significant progress has been achieved through

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assay miniaturization, parallel processing, and innovations in hardware and assay technologies.

The most common detection systems in HTS are fluorescence, scintillation proximity assays (SPA) and luminescence[11]. All these techniques provide excellent sensitivity (which can facilitate miniaturization), and by using luminescence or fluorescence imaging devices such as the ViewLux (PerkinElmer Life Sciences; Turku, Finland) or the CLIPR (Molecular Devices; Sunnyvale, CA, USA), ultra-high speed measurements can be achieved where an entire 384-well microplate can be read in 1 sec. However, the key to employing a good cytotoxicity screen is to ensure reproducibility during all stages of the assay.

Cytotoxicity screening assays – current status

The parameters for a successful HTS assay are now well established. Assays should facilitate miniaturization to 96-, 384- or 1536-well formats without detrimental effects on reproducibility, robustness and statistical significance of the results. To function within these parameters on laboratory robots, homogeneous 'mix and measure' assays are required because they avoid filtration, separation and washing steps, which are time consuming and difficult to automate[12]. Although sensitivity is vital for early-phase detection of cell toxicity, an assay should also be simple, rapid, efficient, reliable, safe and cost-effective[13].

A successful cell-based assay is not wholly dependent upon the end-point detection system used; standardization of every part of the assay set-up is also important. This ranges from the supply and maintenance of cells and every aspect of the tissue culture procedure, through to the quality of the tissue culture/detection plates used, the hardware, software and data management.

Overcoming the problems of cell supply

Cell-based screening requires not only large quantities of an individual cell type, but also parallel processing of multiple, smaller batches of a wide range of different cell lines derived from various tissue origins. Scale and flexibility are absolute requirements, and having large quantities of different cell types available on demand requires continuous maintenance of many cell cultures. Problems can arise with the use of cells resuscitated from frozen stocks, owing to the unforeseen delays that can occur when scheduling the assays on the laboratory robots. These problems have been addressed by a number of cell suppliers through optimization of cell culture media so that growth and performance of the cells is more reliable. Many cells are maintained in cultures containing foetal calf serum (FCS), and different batches of FCS can cause differing effects upon cells owing to their varied compositions. This has resulted in the drive towards the use of serum-free media.

Cell lines and media are expensive, and this cost can be a limiting factor if cell-based assays are ever to be used at the primary screening level. The good news is that the problem of cell supply, and recognition of this as a potentially limiting factor in the use of cell-based assays early on in the screening process, is being recognized by commercial suppliers of cells. A consortium of six major pharmaceutical companies, together with a cell-culture robot manufacturer has been established, with the objective of having automated bulk cell-culture systems in operation by the end of this year. This has been called the 'SelecT' consortium and represents Automation Partnership's answer to parallel processing of multiple cell lines[14], the aim of which is to maximize throughput and improve consistency of results.

Variation in the performance of microtitre plates

All of the culture plastics used in the cell-based assays need to be critically assessed for performance. Comparisons of microtitre plates from different manufacturers have shown variations in the growth properties of cells. Having established the optimal plates for cell growth and performance, the plates used at the point of detection of cytotoxicity (if different from those used for the initial culture) should also be compatible with the assay being performed. The plates should not interfere with assay performance and need be tested to ensure there is no adverse effect on signal-to-noise ratio, which will affect the sensitivity of the assay. Ideally, the same plates that are used to set up the culture should be used at the detection stage to reduce the number of manipulation steps.

The importance of microplate standardization has been highlighted by the Society of Biomolecular Screening[15]. However, these guidelines relate only to the dimensions of wells and plates and not to the use of different moulding materials and sterilization procedures. Variations in plate performance have been recognized for a number of years, particularly with respect to bioassays. One study, carried out in nine countries by twenty different laboratories, looked at the performance of one cytotoxicity assay (involving L929 cells and tumour necrosis factor) and found that variation between the microtitre plates used was one of the contributory factors[16]. At LumiTech (Nottingham, UK), we have also tested the performance of a number of different standard 96-well tissue culture plates for luminescence detection (white walled, clear bottomed), and found wide variations in performance (Fig. 1). The only changed variable in the assays was the plates used; all of the plates were seeded simultaneously at the same cell density. The performance of the non-parametric statistics (Wilcoxon signed rank test) revealed significant differences in performance ($p<0.001$) when plates 1 and 2 were compared (Fig. 1). However, although the plates were from the same manufacturer, the plate design was different. The differences in performance could be attributed to differences in growth rates of the cells and effects on signal detection, which, in some cases, gave a lack of reproducibility in the data.

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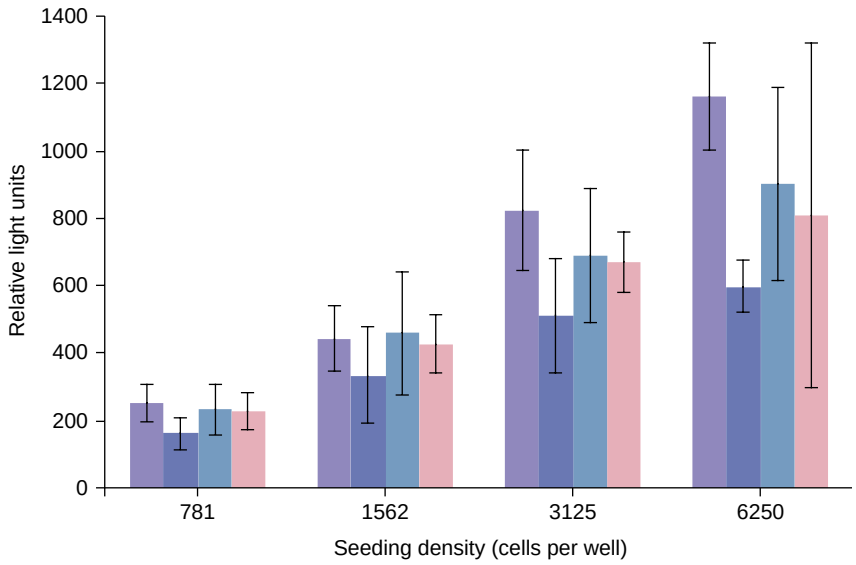

Figure 1. Comparison of 96-well tissue culture plates using an ATP bioluminescence assay. Swiss 3T3 cells were seeded at the concentrations shown and then cultured for 48 h before detection of ATP levels. All the plates were clear-bottomed white-walled 96-well tissue culture treated plates supplied by different manufacturers. Purple: Krystal 2000 plates (Porvair Science; Middlesex, UK) – injection-grade polystyrene, with a high percentage of titanium dioxide, and patented design to prevent 'piping' across the clear part of the plate. Dark blue: Krystal Plate 1 from Porvair Science (as above but without patented design). Light blue: Isoplate™ (PerkinElmer Life Sciences; Turku, Finland) – 'cups' of transparent polystyrene fixed into an opaque frame. Pink: Flat-bottomed polystyrene plate (60% thinner than conventional plates). The results shown represent the means of six separate experiments (performed in triplicate) ±SD.

One statistical test that should be routinely performed when determining the applicability of a cytotoxicity assay for scale up to HTS is Z′ analysis, which analyses the robustness of the assay at the HTS level[17]. To be accepted for scale-up, an assay system must achieve a Z′ value of >0.7. Table 1 shows Z′ statistics obtained in a luminescence detection assay using 96-well microtitre plates from different manufacturers. The calculation of Z′ accounts for all factors, such as cross-talk between wells and reproducibility. All ATP assays were performed with the same reagents and detection system, with the plates as the only changed variable. However, a source of variability between plates could be the percentage of titanium dioxide in the manufacture of the white plates; this is added to increase the opacity and reduce cross-talk. The reproducibility of the ATP bioluminescence assay gave a CV of <3% for the replicate experiments using an ATP standard of 1 μM.

Historically, detection systems have not been sensitive enough to miniaturize cell-based toxicity tests to 384-wells plates. However, with the new generation of luminescent and fluorescent detection systems, it is now possible to perform these assays in smaller volumes. The improvement in sample handling and low-volume liquid handling has improved the reliability of using 384-well plates. As the trend for miniaturization in HTS continues, the 384-well microplate gains increasing prevalence. Aside from the theoretical difficulties of growing cells in the small volumes demanded by 384-well (and higher density) microplates (owing to issues of gaseous exchange resulting from the reduced surface area/volume ratio), many pharmaceutical companies are now culturing cells routinely in this format.

The use of the higher-density plate formats (≥1536-well) is starting to pay dividends for cell-free assays. However, cell-based cytotoxicity assays performed in 1536-well plates result in great variation according to seeding density and culture conditions. Although many of the new technology platforms are capable of detecting low cell numbers, the greatest difficulty is maintaining cell viability in the control (untreated) population before any compounds have been added. Futhermore, although single-cell analyses can be performed, one has to question the relevance of determining toxicity, or lack of it, when extrapolating to animals and humans.

Detection systems

Historically, *in vitro* cytotoxicity assays have relied on a number of end-points:

- Chemical dyes, such as crystal violet or sulphorhodamine B, which stain specific cell components and measure residual cellular material following incubation with the test compound.
- Detection of the release of a constitutive cellular component, such as lactate dehydrogenase, and subsequent measurement of enzyme activity in the culture supernatant.
- Measurement of cellular metabolic function using tetrazolium salts, such as [4,5-dimethyl(thiazol-2-yl)-3,5-diphenyl] tetrazolium bromide (MTT); 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) and sodium [2,3-bis [2-methoxy-4-nitro-5-sulphophenyl]-2*H*-tetrazolium-5-carboxanilide, inner salt (XTT). These salts are reduced to intensely-coloured formazan dyes by mitochondrial activity.
- The introduction of radiolabels such as ^{51}[Cr]. Cells are labelled with the radioisotope, which is then released upon cytolysis.

As regards hazard identification, DNA damage can be detected using the micronucleus assay. These assays identify substances that are potentially carcinogenic to humans[18] and have

been essential in the study of genetic toxicology. However, even with the use of rapid flow cytometric systems, this type of assay does not lend itself well to HTS.

With the exception of the newer tetrazolium salts MTS and XTT, none of these assays can be considered to be homogeneous and therefore robot friendly, as they require pre-incubation and washing steps. Over recent years, the focus has tended to be towards either fluorescent or luminescent end-points, which facilitate significantly more-simple and -convenient assay procedures, and considerably higher levels of sensitivity can be achieved using these light-emitting chemistries.

A wide range of different fluorescent dyes are available for use in cell viability studies. Many of these dyes rely on the observation that viable cells possess intact plasma-membrane structures that exclude the dye from the cell, whereas cells treated with cytotoxic agents exhibit plasma membrane disruption, which facilitates ingress of the dye into the cytosol. However, in a very detailed review of fluorescent dyes used in the measurement of cell killing[19], King points out that there are a number of exceptions to this observation and care should be taken in interpreting data obtained using some of the fluorescent dyes that are now available. For example, although a cell might be severely damaged by a drug, it could still retain membrane integrity for a relatively long time. King raises an interesting point by highlighting that healthy, surviving cells within a population could proliferate following drug treatment. It is the absolute number of surviving cells that is the most important parameter and not the percentage of dead cells (usually determined by flow cytometry).

Despite recent developments with high-speed machines[20], flow cytometry is currently not suited to the demands of high-throughput cytotoxicity testing. High-throughput cytotoxicity assays need to be performed in microplates where incubation and analysis can be achieved in the same plate. To this end, a number of fluorescent microplate assays have been developed. Wodnicka *et al.* have used a ruthenium dye [tris 4,7-diphenyl-1, 10-phenanthroline ruthenium (II) chloride] to act as a biosensor[21]. The fluorescence produced by this dye is substantially quenched in the presence of oxygen. Thus, as cells grow, the oxygen present in the medium diminishes over time. This is detected by an increase in fluorescence resulting from a reduction in the quenching effect. As the ruthenium was non-toxic to the cell types tested, it was possible to monitor the growth of cells during the culture period by measuring increase in fluorescence with a microplate fluorimeter. Addition of cytotoxic agents to the cultures reversed this effect, thus providing a very simple and rapid microplate cytotoxicity assay. However, this assay requires at least 50,000 cells.

Another fluorescent dye, Alamar Blue (resazurin), lends itself well to the demands of high-throughput cytotoxicity testing.

Table 1. Comparison of Z′ data from a luminescence detection assay using opaque 96-well plates from different manufacturers[a]

Plate type	Manufacturer	Z′ value[b]
Black polystyrene, flat-bottomed wells		0.412
Black polystyrene, flat-bottomed wells		0.611
Black polystyrene, flat-bottomed wells Microfluor® 1	Dynex Technologies; Chantilly, VA, USA	0.842
Standard white polystyrene opaque plate	PerkinElmer Life Sciences; Turku, Finland	0.696
White polystyrene, flat-bottomed wells Microfluor® 1	Dynex Technologies	0.946
White polystyrene, flat-bottomed wells		0.659
White polystyrene, flat-bottomed wells	Porvair Science; Middlesex, UK	0.920

[a]Plates were tested using a concentration of 1 μM ATP against Tris-acetate buffer controls.

[b]Z′ values are given as the mean of five separate experiments.

Alamar Blue is a blue non-fluorescent dye that is reduced to a pink fluorescent dye in cell culture medium as a result of cell activity. The exact mechanism of this dye reduction remains to be elucidated, although it is thought to be induced by mitochondrial enzymes[13]. The test is very simple to perform, requiring the addition of only one reagent to the cell culture supernatant. It does not kill the cells, thus enabling additional tests on the same sample. Most importantly, Alamar Blue can provide a high degree of sensitivity, detecting as few as 80 cells. Although Alamar Blue can be co-cultured with the cells, thereby giving the opportunity for kinetic analysis of cytotoxicity, O'Brien noted that addition of compounds such as nickel to the cultures resulted in a reduction of the dye even when the cells were dead, therefore giving an overestimation of cell survival[13]. He concluded that kinetic experiments could be unreliable unless strictly controlled. Underestimation of toxic effects using the Alamar Blue assay have also been reported elsewhere[8].

Bioluminescent detection of ATP using the firefly luciferase enzyme provides a very simple, highly reproducible, and extremely sensitive assay. ATP plays a central role in energy exchanges in biological systems (both eukaryotic and prokaryotic) and has

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Table 2. Properties of 96-well microplate assays available for apoptosis detection

Product	Company	Technology platform	Principle	Differentiates apoptosis/necrosis?	Total assay time	Lower detection limit	Assay preparation
ApoGlow™	Biowhittaker Cambrex[a]	Luminescence	ADP:ATP ratio	Yes	20 min	100	Dispense and read
CaspACE™	Promega[b]	Fluorimetric/ colourimetric	Caspase detection	No	>2 h	$>1 \times 10^6$	Multiple steps
ApoAlert®	Clontech[c]	Fluorimetric/ colourimetric	Caspase detection	No	>2 h	1×10^6–2×10^6	Multiple steps
ApoAlert®	Clontech[c]	Fluorimetric	Glutathione detection	No	>1 h	5×10^6 (recommended cell number)	Multiple steps
Cell Death Detection ELISA^{Plus}	Roche Molecular Biochemicals[d]	Colourimetric	DNA fragmentation	No	3–4 h	125	Multiple steps

[a]Walkersville, MD, USA.

[b]Madison, WI, USA.

[c]Palo Alto, CA, USA.

[d]Mannheim, Germany.

been used as a tool to assess the functional integrity of living cells[22] because all cells require ATP to remain alive. The benefits of ATP bioluminescence compared with the widely used MTT assay are clearly demonstrated in a paper by Petty *et al*. (Ref. 7). The principal advantages of ATP detection were shown to be sensitivity and reproducibility. The performance of the MTT assay can also be affected by altered properties of the culture medium including changes in pH and reduced glucose, which affect the reproducibility of this detection system. However, this lack of sensitivity has not stopped MTT from being incorporated into some screening systems[4]. It should be noted that not all cells metabolize MTT (Ref. 23) and there are a number of conditions that need to be controlled in order to assure performance with this type of assay[24].

The move towards high-content screening

High-content screening (HCS) was a phrase adopted by Cellomics (Pittsburgh, PA, USA) for their multi-colour fluorescence detection system[25]. The concept of HCS requires gaining as much information from one sample as possible. This can be achieved either through performing a number of complementary assays on each well of the plate, or by performing one assay that gives more than one piece of information on the status of those cells within the well. In addition to the Cellomics detection system, the term also lends itself well to a number of other assays and therefore opens a whole new chapter for HTS. The high cost of obtaining cells for HTS, which facilitates multiple analyses on cell samples, is fuelling the drive towards HCS. Green fluorescent protein (GFP) provides an excellent tool for HCS assay systems. It involves labelling individual cell constituents with GFP-variants that emit at different wavelengths[26]. By imaging each cell individually, it becomes possible to treat cells as 'wells' within a 96-well microplate.

Differentiating between modes of cell death (apoptosis and necrosis) is another form of high-content assay. The socio-economic importance of degenerative diseases and cancer, where apoptosis is a key factor, is fuelling a considerable research effort into cell death in both academia and the pharmaceutical industry.

Flow cytometric analysis remains the method of choice in the study of apoptosis and necrosis. The full power of flow cytometry in the analysis of the multifarious events that occur during apoptosis is reviewed by Vermes *et al*. (Ref. 27). However, as already discussed, flow cytometry is not currently an ideal tool for screening large sample numbers. Although not in widespread use at the moment, there are a number of microplate assays that are capable of detecting apoptosis (Table 2), but many of these require a number of manipulation steps and cannot reliably detect differences between apoptosis and necrosis.

A considerable body of work on apoptosis has focussed on the mitochondria and it is now widely accepted that this organelle is fundamental to the biochemistry of apoptosis in a wide variety of cell types. Bioluminescence can be used to monitor mitochondrial function through a drop in oxidative phosphorylation (reduced ATP) together with an increase in cellular ADP. The ratio of ADP:ATP can then be used to determine the induction of apoptosis, necrosis, growth arrest and cell proliferation within a cell population[28]. Although flow cytometry can quantify the percentage of cells undergoing apoptosis within the selected population, bioluminescence offers a rapid screen for induction of apoptosis/necrosis within the entire population of a microtitre plate well.

Making the right choice

Providing all of these parameters are determined, and adverse effects are controlled, the performance of cell-based assays is becoming far simpler, and the data more robust than in the past. In terms of assay reagents and detection equipment, present technologies enable implementation of cytotoxicity assays at a very high-throughput level.

There are a large number of technologies currently available at the predictive level that are complementary to cell-based assays with respect to determining the toxic potential of compounds. One example is toxicogenomics: applying genomics to toxicology[29]. Information from these systems, used together with cytotoxicity data, should not only lead to the identification and characterization of new drug actions, but also increase our knowledge of toxic mechanisms. Although this will hopefully give valuable information on the probable response of individuals to pharmaceutical agents, there is still the need to screen for toxicity at the cell-response level. Investments in new technologies for drug discovery will only prove themselves if they genuinely reduce the development time and failure rate.

Prediction of toxicity from the genome or proteome gives information on how a compound is likely to behave, but in the complex world of cell biology does it really tell us whether the agent is toxic when taken up by a number of different cell types? If information from *in vitro* cytotoxicity assays are used in combination with profiling information for the compounds, a powerful pedigree for each product can be attained, which can be built upon as it passes through the development stages and onto final release as an effective therapeutic. Present technology, in terms of assay reagents and detection equipment, enables the implementation of cytotoxicity assays at a very high-throughput level, and all parameters (including cost) should be assessed carefully in order to determine the most appropriate choice of technology.

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