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Review

Biomarkers for Drug Discovery: Important Aspects of *in vitro* Assay Design for HTS and HCS Bioassays

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High throughput screening (HTS) is the foundation of current drug discovery to assay drug candidates for toxicity and biological effects (*i.e.* off-target and on-target responses, respectively). HTS is typically based on measuring thousands of drug candidates per day with a single endpoint assay on a limited number of doses or even a single dose of compound. The assays can be either based on absorbance or fluorescence measurements (*i.e.* Alamar Blue, MTT, Fluo-4 for calcium, *etc.*). Conversely, high content screening (HCS) is based on measuring a limited number of drugs per day, but measuring up to eight different assays simultaneously with multiple drug doses and even kinetic measurements. HCS assays typically are based on fluorescence microscopy and automatic image analysis algorithms. With HCS technology, tedious and time consuming assays can now be automated (*i.e.* nuclear size, micronucleus assay, lysosomal mass, mitochondrial membrane potential, neurite outgrowth, *etc.*). Multi-channel FACS (fluorescence activated cell sorting) can also be considered to be »high content« analysis. The purpose of this essay is to review important aspects of *in vitro* assay design common to both HTS and HCS screening technologies.

Keywords
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INTRODUCTION

High Content Screening (HCS) is an advanced fluorescence microscopy technique utilizing sophisticated image analysis algorithms to perform multiple bioassays simultaneously. As many as eight different assays can be performed at the same time on live or fixed cells in various well plates, using multiple fluorescent probes or endogenous fluorophores. Most HCS systems are essentially an automated inverted fluorescent microscope with computer control, some containing integral CO_2 incubators for monitoring live cells over entended intervals. Some important bioassays for toxicology and drug discovery applications include nuclear area, cytosolic calcium, mitochondrial membrane potential, cell proliferation, cell cycle, cell motility, oxidative stress, cell morphology, *etc.* Mixed cell cultures can be measured with HCS methods and the data for each cell type can be analyzed separately based on cell morphology. Labor intensive methods such as the micronucleus assay, neurite outgrowth and transcription factor translocation assays can be automated with HCS technology. Literally hundreds of distinct assays are possible with antibody-based protocols. HCS bioassay applications include research areas such as toxicology, cancer, infectious diseases, CNS, cardiopulmonary, and endocrine disorders. HCS assays are typically based on primary cell or tumor cell cultures, but the flexible HCS image analysis software can also measure

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fluorescently-labelled histology slides. HCS algorithms allow the monitoring of specific organelles within cells, allowing important drug mechanism information. Individual cells can be monitored *versus* time, providing important sequence of events information for various cellular parameters. By measuring multiple biomarkers simultaneously, several cellular mechanisms of toxicity can be monitored at the same time providing drug safety predictivity data rivaling animal testing.[1] Various HCS platforms are available from several manufacturers, including Becton Dickinson Biosciences, General Electric Healthcare, Cellomics, Molecular Devices, *etc.*

CELL CULTURE

The most important reagent of *in vitro* assays is the cells used for the assays, upon which the assays are based. Consistent and careful cell culture is vital to *in vitro* assay reproducibility. Strict adherence to cell culture protocols is critical to consistent results (*i.e.* trypsinization methods, serum levels, media type, *etc.*).

The choice of primary cells *versus* tumor cells or human cells *versus* animal *in vitro* models depends on the end goal of the assay. Is the assay data intended to model human responses to drugs, or is the data to predict animal responses to drugs to prioritorize drug candidates for animal testing. Primary cells tend to have more metabolic potential than tumor cell lines (*i.e.* cytochrome P450 activity), valuable for metabolic assays or for compounds which require metabolic activation for toxicity (*i.e.* acetaminophen, diclofenac, *etc.*). However, primary cells tend to have more inherent variability due to derivation from distinct animal donors and often are actually a mixture of cells types with differing characteristics (*i.e.* fibroblasts with muscle cells or periportal, perivenous and Kupffer cell mixtures in hepatocyte isolations, *etc.*). Tumor cells are by nature more consistent due to their clonal expansion from a single cell. For example, the coefficient of variation (*i.e.* 100 × SD/mean) for acetaminophen IC_{50} values is 22.9 % for primary rat hepatocytes and only 6.0 % for HepG2 cells in the same WST-1 tox assay.[2] The HCS toxicology results utilizing HepG2 cells as the model has very good reproducible results for compound IC_{50} values.[1] However, sub-clones of tumor cell lines can occur after prolonged propagation, with altered biological characteristics. It is recommended that cells be propagated to a limited extent beyond that obtained from the supplier such as ATCC (*i.e.* a maximum of 20 passages in the current laboratory). Frozen stocks of the cell lines should be stored and regularly thawed/passaged.

Primary hepatocytes have been assumed to be more sensitive cells than tumor cells for toxicology assays due to their higher metabolic capability. However, hepatocytes also loose metabolic capacity with time in culture, plus hepatocytes are difficult and expensive to culture for multiple days. HepG2 cells can have more sensitive IC_{50} values than hepatocytes if the assay is extended to three days for the HepG2 cells. For example, the IC_{50} toxicology values for several drugs are as follows: amiodarone, 38.3 μmol dm^{-3} *vs.* 9.8 μmol dm^{-3}; chlorpromazine, 45.5 μmol dm^{-3} *vs.* 6.4 μmol dm^{-3}; ketoconazole, 62.3 μmol dm^{-3} *vs.* 62.2 μmol dm^{-3}; and quinidine, 244 μmol dm^{-3} *vs*. 14.7 μmol dm^{-3}, for primary hepatocytes[2] (one day incubation) and HepG2 cells[1] (three days incubation), respectively. Thus, tumor cells with longer incubation times can produce more sensitive assay results than primary cells. Tumor cell lines are much cheaper, more convenient to propagate and store as frozen stocks than primary cells as well.

Cell lines should be regularly assayed for bacterial and mycoplasma contamination to prevent artifacts in the assay results, an often overlooked, but important aspect. Mycoplasma contamination could alter the normal biochemistry of the cell lines and induce karytype changes to the cells.[3] The addition of antibiotics to culture media (*i.e.* penicillin and/or streptomycin) typically inhibits bacterial growth. Primary cell lines can be prone to fungal growth from hair or dander contamination, so agents such as amphotericin may also be required. Rinsing the animals with 70 % ethanol or detergent prior to tissue removal can be useful to minimize microbial contamination to primary cell cultures. However, the effects of these media anti-microbial compounds on the assays must be considered (*i.e.* amphotericin generates pores in the plasma membrane). Evaluation of test antibiotic compounds effects on cells can be altered by the presence of similar compounds in the culture media. Media antibiotics may allow adaption of the cells to these class of compounds and alter the responses of the test compounds in the assays. Control experiments with cell lines not adapted to antibiotics should be considered for some compounds.

PLATE LAYOUT

The use of the outer wells of assay plates as valid data sources needs to be considered carefully. The outer wells tend to have the highest coefficients of variation and cell growth tends to be inhibited relative to the wells in the center of the plates. Idealists tend to exclude outer wells from data analysis, with the loss of a major portion of plate testing areas and decreasing assay throughput (*i.e.* 18–38 % decrease for 384 well and 96 well plates, respectively). The outer wells often have the highest evaporation rates and the high variation of outer wells results is almost certainly due to increases of media osmolality above physiological levels (*i.e.* 288 mmol/kg). For example, the outer wells of a 96-well plate lose 34.8 % in volume and the inner wells lose only 16.7 % in volume over a three day period in a humidified 37 °C incubator (see Figure 1). Depending on plate design, even the outer

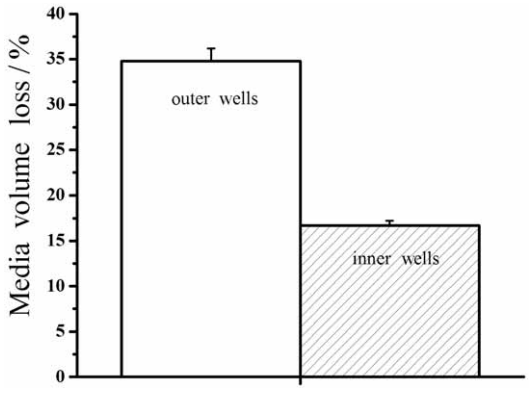

Figure 1. Outer wells *versus* inner wells evaporation. A 96-well plate was loaded with 100 µL of media per well and incubated at 37 °C for three days at 90–95 % relative humidity and the volume of all outer wells and all inner wells was determined.

wells can have large variations in evaporation rates. Corner wells can have high evaporation losses approaching 60 % in some cases, suggesting osmolality levels 250 % of normal, certainly stressful to cells. These varying culture conditions between outer wells and inner wells can have significant effects on assay outputs. A 25 % or more increase of media osmolality (or decrease of media volume) over the testing period can inhibit growth of some cell types.[1] The actual effect of hormesis or biphasic dose response curves needs to be evaluated with unbias when the effect is relative to outer control wells (*i.e.* increases in cell growth at low compound doses). When doubt exists, the testing should be repeated with the same drug doses at the center of the plate where edge effects are absent. Trend analysis on entire negative control plates is a useful technique to determine the effect of edge wells horizontally and vertically across the assay plate. This trend data can be useful to design the plate layout for reproducible assay results and valid conclusions being derived.[4]

CELL DENSITY

Cell density selection is an important consideration to optimize signal-to-noise ratios. However, if tumor cell lines are utilized, then the cell seeding density which produces a sub-confluent field of cells is often desirable to prevent cellular metabolism senescence by cell-cell contact inhibition. For HCS and imaging-based assays, over-confluent cell densities complicates accurate focusing since cells can be growing in several planes/layers. Titration of seeding density is recommended to determine the optimum cell density for the particular assay conditions at the specific time point. Cellular organelle cross section areas are certainly inversely affected by cell culture densities. Nuclear diameter correlates inversely linearly with cell density after cells come into contact with each other, but not at sub-confluent cell densities (unpublished observations). Toxicology investigations utilizing HCS techniques have shown that nuclear diameter is a very robust and sensitive parameter to judge compound effects on cells.[1] Typically, nuclear diameter decreases with increasing drug dose. However, if nuclear diameter is biphasic with cell density, then the data is more complicated to interpret. For example, if cell density is over confluent, the nuclei are compressed and the toxic effect of drugs to decrease cell numbers relieves this constraint and nuclear size can initially increase, followed by a decrease in nuclear size at higher toxic compound doses. Therefore, calculation of IC_{50} values in biphasic curves are more complicated in over-confluent cell models. High cell densities can also quickly deplete media nutrients as well, complicating cell culture models. Cytosolic organelle cross sections are increased at lower cell densities, making imaging these organelles much easier. However, some primary cell lines prefer confluent cell densities, so optimum cell density depends on the cell type and particular assay.

CULTURE SUBSTRATE

The selection of culture substrate is also a very important factor to bioassay results. Many cell types prefer biological substrates which are more similar to their normal environment (*i.e.* collagen I, collagen IV, fibronectin, MatriGel, laminin, *etc.*). Cells can grow on plastic cell culture surfaces (*i.e.* polystyrene), but there is some skepticism whether the cell biology is normal. Moreover, different cell types prefer different culture substrates. Plastics treated with plasma discharge can make the surfaces with a positive charge more accommodating to the cells, but still not natural. Poly-D-lysine (PDL) is often used as a substrate since its poly-cation nature binds well to the negatively charged phospholipids and carbohydrates in cell membranes. The unnatural D-isomer polymer of lysine seems to be less degraded by cellular proteases than the L-isomer polymer. HepG2 cells tolerate PDL substrates well, however, primary hepatocytes prefer collagen I as a substrate and skeletal muscles prefer laminin.[1,12,13] Non-native culture substrates may be more convenient for assays, however, they may make cells more sensitive to apoptosis or alter cellular biochemistry. It seems accepted that natural extra-cellular matrix (ECM) proteins are optimal for cell growth, however, these materials tend to be relatively expensive and can be slowly degraded by the cells. Natural proteins can engage membrane receptors and have anti-apoptotic and proliferative effects to the cells.[5,12] The choice of culture substrate may depend on the length of the assay. Short assays of a few hours may not be significantly affected by the growth substrate, while assays lasting several days can be greatly affected by the culture substrate. It is recommended that various cell substrates be evaluated to find the optimal substance

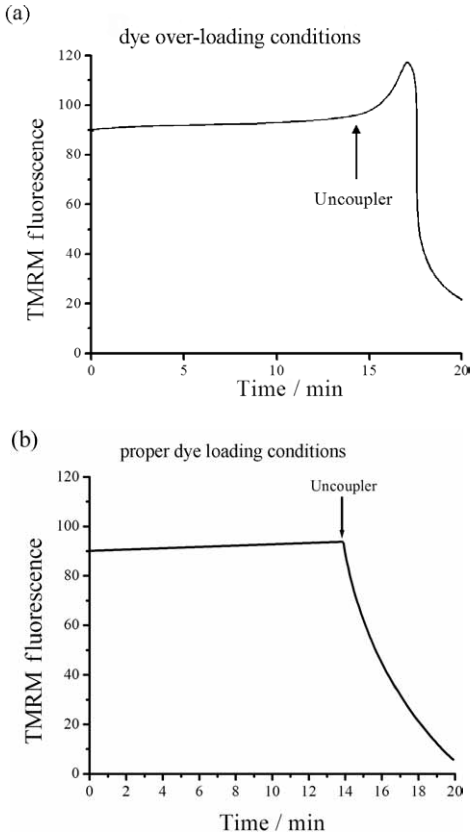

Figure 2. Proper dye and dye over-loading conditions. Figure 2a demonstrates cells over-loaded with TMRM by the addition of the uncoupler FCCP induces an initial increase of fluorescence. Figure 2b demonstrates cells correctly loaded with the Nernstian dye TMRM by the addition of FCCP causes an immediate decrease in mitochondrial fluorescence.

for the particular assay and cell type. In some conditions, mixtures of ECM proteins may be the most physiological condition (*i.e.* MatriGel, co-culture with fibroblasts to secrete ECM protein mixtures, *etc.*).

ASSAY CONDITIONS

Various assay parameters are important to minimize assay coefficient of variation and maximize reproducibility, such as assay duration, dye concentrations, dye loading times, washing protocols, temperature, *etc.* Sufficient assay dye concentration is required for optimum signal-to-noise ratios, however dyes can alter cell physiology and even be toxic to the cells (*i.e.* calcium dyes such as Fluo-4 AM bind calcium, altering free calcium levels and increasing calcium diffusion rates). Excessive dye concentrations can quench the signals of fluorescent dyes, actually decreasing signal-to-noise ratios and causing difficult to interpret dual response curves. This is particulary true of Nernstian dyes which are accumulated in cells according to the Nernst equation (defined by membrane potential and dye concentrations terms).[11] The dye TMRM is used to monitor mitochondrial membrane potential and can be concentrated up to 10 000 times the buffer levels inside the mitochondria. The quenching limit for this dye is about 20 μmol dm^{-3}, therefore only nanomolar dye levels are required for appropriate loading conditions. A decrease in the TMRM signal is accepted as a toxicity marker at controlled dye loading conditions. Excessive TMRM levels can actually decrease the fluorescence signal due to dye quenching. Titration of dye levels is recommended for optimal responses and monitored kinetically with control addition (*i.e.* mitochondrial uncoupler) to ensure dye quenching is not occurring.[6] Different cell types (*i.e.* liver, heart, fibroblasts, *etc.*) may require different dye levels due to dye metabolism, cell morpology or dye extrusion by drug export pumps often upregulated in tumor cell lines. Dyes can also have multiple responses depending on the concentration. It is reported that dihydroethidium (DHE) monitors free radical production at concentrations below 1 μmol dm^{-3} levels, however, the DHE signal monitors mitochondrial membrane potential above 1 μmol dm^{-3} levels.[7] Relevant assay control compounds are also important to verify that the parameter desired to be monitored is actually being measured. Ionomycin is a good acute non-fluorescent control compound for Fluo-4 calcium measurements. For example, FCCP is a protonophore which serves as an excellent control compound for mitochondrial membrane potential assays.[12,13] An initial increase in the TMRM signal after FCCP uncoupler addition indicates dye de-quenching is occurring and the cells are over-loaded with dye (see Figure 2a) and the resulting dual-response data is not reliable. Figure 2b shows cells properly loaded with TMRM, since the uncoupler addition immediately causes a decrease in mitochondrial fluorescence, producing reliable results. Compound absorbance and inherent fluorescence must also be considered in the data analysis. Control experiments must be designed to evaluate these effects on the final results so that valid conclusions can be achieved.

Titration of the duration of the incubation time of the compounds with the cells and the reporter dye loading time can be very useful information to optimize responses while minimizing detrimental effects of either reagent. Longer incubation times of cells with drugs often tend to increase assay sensitivity by lowering IC_{50} values, however it can also increase coefficient of variation values due to the detrimental effects on the cells. Plotting assay coefficient of variation and/or IC_{50} values *versus* compound incubation time can be used to determine the optimum assay time (*i.e.* low CV values and sensitive IC_{50} values). Also, for image-based assays, the number of fields acquired per well is an important consideration. Too many fields imaged is not efficient and decreases assay throughput, but too few images can produce unacceptable assay variability. Plotting assay coefficient of variation *versus* fields imaged is very useful in determining the optimum

amount of data to collect for a particular assay. Drug toxicity can manifest itself by altering cell morphology (*i.e.* cell rounding), which complicates image-based assays due to effects on focus accuracy vital to these methods. When to add compounds to the cells is an important consideration. Adding the compounds to the cells within a few hours of seeding them can save overall assay time. However, the cells must be firmly attached to the culture substrate before compound addition to ensure that the loss of cell numbers is not merely due to interference of cell adhesion rather than cytotoxicity. Overnight culturing of cells before adding compounds increases assay times, but allows the cells to recover from trypsinization or isolation stresses (*i.e.* receptor degradation), helping to make sure that the assay results are predominantly from the compounds alone.

Reporter dyes can be photo-toxic to cells, so increased levels can increase signal levels but paradoxically decrease assay reliability or even to induce the event that they are intended to measure. MitoTracker Orange used to measure mitochondrial membrane potential can actually induce the loss of membrane potential at high loading concentrations (*i.e.* the chlormethoxy moiety reacting with key mitochondrial thiol proteins). Covalent membrane potential dyes of this type do not measure continuously and data must be analyzed accordingly to avoid invalid conclusions being derived. For instance, initial labeling of cells with covalent dyes will not measure subsequent loss of membrane potential caused by added modulators.[8] Several solvents are possible for organic fluorescent dyes. Some of these solvents can modify assay results or are not very compatible with biological systems, so care must be taken to chose the solvent wisely. For example, DMSO has anti-oxidant properties so may alter free radical assay responses and dimethylformamide is toxic to some cell lines.[1] The overall goal of assay optimization is to maximize assay S/N ratios while also minimizing coefficient of variation values, while considering possible assay artifacts.

DRUG TRANSPORTER INHIBITION CONSIDERATIONS

Cells can adapt to stresses caused by culture conditions or drug exposure by upregulating various drug transporters, cytochrome P450 isozymes, mitochondrial ATP synthase, *etc.* Under conditions of respiratory chain inhibition, these mitochondria can still maintain a membrane potential by the reverse action of the ATP synthase. Detection of these mitochondrial defects by the TMRM method require »sensitization methods« by inhibition of the ATP synthase by the co-addition of oligomycin to detect hidden mitochondrial pathologies.[12]

Drug inhibition of plasma membrane transporters such as multi-drug resistance pumps (*i.e.* MDR, Pgp, *etc.*) or organic anion transporters (*i.e.* OAT) can be utilized to increase dye indicator levels inside cells. For instance, inhibition of OAT by probenicid aids in loading cells with FURA-2 AM and cyclosporin H inhibits MDR pumps to increase cell loading of TMRM. However, test compounds can also inhibit these transporters and alter dye loading of the cells (perhaps causing results misinterpretation), causing dye quenching artifacts or increasing the phototoxicity of the dyes. Normalizing dye loading of cells with transporter inhibitors (*i.e.* probenicid, verapamil, reversin, *etc.*) is a possible method to keep dye levels inside cells constant from compound to compound.[9] An increase in assay signal with drug dose is not necessarily a real response of the dye, but merely could be an increase in the cellular dye concentration due to transporter inhibition.[11] Cyclosporin A and ketoconazole are excellent inhibitors of MDR pumps and an increase in the TMRM signal with dose is merely an increased cellular accumulation of TMRM and not a true hormesis drug effect, an indicator of toxicity nor an indication of mitochondrial proliferation.[1] In effect, TMRM is acting as an MDR pump assay probe. TMRM signal intensity is strictly a measure of mitochondrial membrane potential and the presence of a true mitochondrial proliferation should be verified by other methods (*i.e.* fixed mitochondrial protein immunostaining such as cytochrome oxidase). In fact, most increases in TMRM signal with drug dose seems to correlate with their ability to inhibit drug transporters. Some drugs require extended metabolism periods in order to inhibit drug transporters and this effect of a drug may not be present in acute drug exposure models. Diaz *et al.* concluded that MDR inhibitors represent a serious risk of error in the evaluation of mitochondrial potential.[16]

COMPOUND TESTING CONCENTRATIONS

If IC_{50} values are to be utilized for assay outputs, then the accuracy of the curve fitting will depend on the concentrations tested. If the assay response is less than 50 % change of maximum, then inaccurate IC_{50} values will be determined (*i.e.* poor sigmoidal curve definition). For more accurate IC_{50} value calculations, one compound dose which is above the IC_{50} value is recommended to be tested. If the IC_{50} value is over 100 times the maximum serum concentration (*i.e.* c_{max}), then no higher testing levels is suggested for the sake of relevance. Dilution factor between doses is also an important consideration. A dilution factor of 1:3 produces a wide range of dosing concentrations with the same number of doses, however, a lower dilution factor (*i.e.* 1:2) produces smoother curves, perhaps with better correlation values.

If the assay is to cover multiple days, then re-dosing the cells on a daily basis with compound needs to be considered. Replacing drugs each day with fresh solu-

tions may reflect regular patient dosing, however, it also removes potentially important drug metabolites from the media as well and increases the chances of cell layer detachment the more physical manipulations of the assay plates. Increased reagent, compound synthesis and labor costs due to regular drug changes must also be considered. Adding media/nutrients to each well at day one sufficient for multiple days of growth is an alternative method to ensure good cell culture for several day assays.

Drug serum concentrations vary widely by a factor of a million or more for various drugs, from nanomolar levels up to millimolar levels (*i.e.* 3 nmol dm^{-3} for trifluoperazine and 1.65 mmol dm^{-3} for aspirin). Therefore, a fixed dosing scheme for drug evaluations is not always relevant to physiological drug concentrations in human blood plasma. Some hormones (*i.e.* estradiol) can even have picomolar plasma concentrations.[1] For example, testing a compound up to 100 mmol dm^{-3} would be 33,333 times the c_{max} (*i.e.* maximum serum concentration) value for trifluoperazine and only 0.06 c_{max} for aspirin. Therefore, trifluoperazine would be tested to many times its normal levels and aspirin would be evaluated at a fraction of its normal levels. A comparison of drugs to rank toxicity would not be normalized or uniform with a fixed dose testing scheme. Conversely, testing drugs to an equal therapeutic index level (*i.e.* TI or testing concentration / c_{max} value) would normalize drugs to the same biological effect scale and make drug toxicity comparisons more standardized. A TI value of 30 or more could be considered to be a safe drug, since a patient probably would not achieve a dose 30 times the normal dose, except during intentional overdose situations. Obviously, there are exceptions where the TI of some currently marketed drugs are five or less since there are no less risky drugs available for serious conditions absolutely requiring treatment (*i.e.* phenytoin, quinidine, *etc.*). These drugs typically require regular blood monitoring to ensure that toxic doses are not being achieved in the patient. Testing drugs to 30 times the serum levels is a guideline for safety evaluations, or where the c_{max} value is not available, testing to 50–100 times the 50 % efficacy concentration (*i.e.* EC_{50} value) for an *in vitro* efficacy assay might be considered. Some compounds are not soluble to these levels in an aqueous system, so lower levels must be utilized in some situations for practical reasons. For compounds with high c_{max} values such as aspirin, millimolar levels of drug are present and dilution of the osmolality down to iso-isomotic levels needs to be considered (*i.e.* 290 mmol/kg). DMSO (dimethylsulfoxide) is utilized up to 1 % concentrations in assays, but this is equivalent to 141 mmol dm^{-3} levels and increases osmolality by 48 %. DMSO has been reported to have anti-oxidant properties, so the effect of this solvent on each particular assay is recommended (*i.e.* free radical assays). Basic or acidic compounds can alter the pH of culture media. Media pH after compound additions should be tested and sterilely adjusted back to the original media pH if significant deviations are measured, or the inclusion of a good buffer such as HEPES to media should be considered.

The higher the top testing dose, the more likely drug safety concerns are to be revealed and toxicity assays tend to have more concordance with human clinical experiences. HCS-based toxicity assays which utilized a testing format of this type achieved concordance with human toxicity for various target organs of about 70 % -results rivaling animal testing responses. The toxicity of cerivastatin, a drug recalled from the market due to a number of deaths, was revealed by every *in vitro* HCS assay utilizing HepG2 cells as the model.[1]

IC_{50} VALUE RELEVANCE

A common method to express assay results is to present the concentration at which there exists a 50 % change of the range (*i.e.* IC_{50} value). This is a relatively typical method to characterize data curves using most graphics programs. In some cases the data is not sigmoidal in shape or the full curve is not well defined, thus IC_{50} values are not easy to calculate. However, in other cases a 50 % change is an extreme one physiologically. For example, only a 2.2 % change in serum osmolality levels is considered abnormal (typical range 282–290 mmol/kg), or only a 0.04 change in blood pH is considered to be abnormal (typical range 7.36–7.44). When using Fluo-4 for monitoring calcium levels, a 50 % change corresponds to a calcium level of approximately 350 nmol dm^{-3}, or over four times the typical cytosolic levels of about 50–80 nmol dm^{-3} (*i.e.* the K_d of Fluo-4 is about 350 nmol dm^{-3}).[14] Sustained cytosolic calcium levels only twice that of normal has been considered to be pathological.[10] The IC_{50} value for a plasma membrane permeability indicator (*i.e.* propidium iodide or TOTO-3) corresponds to 50 % cell death, not a sensitive indicator of *in vitro* cellular pathology. The coefficient of variation (*i.e.* SD/mean) for an HCS nuclear size assay is very robust (about 1–4 %). Therefore, a highly statistically significant difference for nuclear area is only about a 15 % change. Obviously, accepted statistical methods, such as a student's *t*-test to obtain *p* values, are recommended to determine significance between control and test results, plus biologically relevant changes need to be considered to determine cut off limits for each particular assay. For example, a cell count assay can have a coefficient of variation of approximately 15 %. Therefore, a cell count change of at least 30 % should be considered for a significant change, or at a 95 % confidence level.[1] Therefore, more biologically relevant changes for each particular assay needs to be considered on an individual basis and an IC_{50} value to determine toxicity may not be suitable for every assay (*i.e.* IC_{50}, IC_{30}, IC_{15}, *etc.* may be more appropriate).

The application of the *Z'* equation to evaluate the robustness of HTS assays is common, when only one or two doses for each compound are tested and thousands of compounds are tested each day. The *Z'* equation is $Z' = 1 - [(3 \times SD1 + 3 \times SD2) / (Mean1 - Mean2)]$, where SD1, Mean1, SD2 and Mean2 are the standard deviations and means of the high and low standards, respectively.[15] This equation implies that when $Z' = 0$ that the difference in the means is an average of six standard deviations, a very strict criteria when only a couple of doses are tested per sample. However, in HCS assays, each well is often measured multiple times and many doses are tested, providing more confidence and degrees of freedom in the collected data (*i.e.* over a hundred data points define each compound's results). In assays which determine IC_{50} values, the number of doses, the correlation and standard error of the resulting curve fit also provides confidence in the data, whether it is reliable or not. Thus, for HTS assays with only a few data points upon which to make decisions, the very strict *Z'* equation is often applied. However, in HCS assays which provide IC_{50} values derived from dozens, or even hundreds of measurements, the *Z'* equation may not be applicable and the coefficient of variation and curve fit statistics may be all that is required to perform quality control of this data.

Compounds can have either cytostatic or cytotoxic effects on cells (*i.e.* inhibition of cell growth *versus* actual cell death, respectively). Cytostatic effects on cell growth (*i.e.* inhibition of the cell cycle) primarily occurs in dividing cells such as tumor cells, peripheral blood mononuclear cells (*i.e.* PBMCs), *etc.* Conversely, primary tissue cultures have limited cell division (*i.e.* hepatocytes, skeletal muscle, *etc.*). Tumor cells approximately double in number every day in culture, while non-dividing cells have relatively constant numbers with time. Therefore, cell counts at 50 % of control wells in tumor cells could be entirely due to cytostatic effects of drugs, while a 50 % loss of cells in non-dividing cells is almost certainly due to cytotoxic effects of compounds. Cytotoxic effects on dividing cells is a decrease in the cell count below the number of cells seeded at time zero. Data analysis has to be considered based on these facts, whether the compound is merely inhibiting cell growth or actually directly killing cells. Cell cycle analysis measurements are useful to decide which event is predominating.

CONCLUSION

The previous discussion reveals that *in vitro* assay development is a complicated and multi-factorial exploration with many challenges. Multiple components of the entire assay system need to be considered to produce a robust, reproducible and reliable assay. Cell culture, cell type, temperature, dye loading conditions, data analysis methods, drug dosing protocols, *etc.* must be carefully evaluated to produce accurate and dependable results.

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SAŽETAK

Biološki obilježivači u istraživanju novih lijekova: Važni čimbenici prilagodbe pokusa *in vitro* tehnologijama HTS i HCS

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Tehnologija visokoproduktivnog probira (HTS, engl. *High Throughput Screening*) osnova je modernog istraživanja lijekova, čime se uvelike ubrzava otkriće novih aktivnih spojeva, ali i ispituju različiti toksični i biološki učinci već u ranoj fazi istraživanja. HTS se uglavnom temelji na mjerenju aktivnosti stotina ili tisuća spojeva dnevno – promatranjem samo jedne koncentracije ili ograničenog broja različitih doza spojeva. Testovi se uglavnom provode mjerenjem absorbancije ili fluorescencije. S druge strane, tehnologija visokosadržajnog probira (HCS, engl. *High Content Screening*) temelji se na mjerenju manjeg broja spojeva dnevno, pri čemu se istovremeno mjeri nekoliko različitih bioloških testova u isto vrijeme uz različite doze ispitivanih spojeva, uzimanjem u obzir čak i kinetičkih parametara. Automatizirani testovi HCS uglavnom ovise o fluorescencijskoj mikroskopiji i automatiziranim algoritmima analize slika, čime se istovremeno mjere različiti parametri u stanici poput veličine jezgre, mase lizosoma, mitohondrijskoga membranskog potencijala i slično. No, stanični parametri mogu se uspješno mjeriti i u mikrotitracijskim pločicama od 96 jažica korištenjem različitih fluorescentnih obilježivača ili endogenih fluorofora. Važni čimbenici uspješnih pokusa u ovom području jesu izbor stanične kulture i odabir supstrata u kulturi, zatim uvjeti pokusa koji obuhvaćaju raspored i koncentracije spojeva u jažicama pločica, te na posljetku predviđanje uvjeta u stanicama, poput uzimanja u obzir inhibicije transportera lijekova. Razvoj staničnih testova *in vitro* složen je proces koji ovisi o mnogim čimbenicima. Samo pažljiva priprema i odabir najboljih uvjeta za određenu metodu može dovesti do kvalitetnog, reproducibilnog i biološki značajnog staničnog testa.