Assay Ready Cryopreserved Cells Can be Used for Cytotoxicity Testing Instantly after Thawing without Prior Passaging or Extended Cultivation

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introduction

To avoid animal experiments, in vitro assays using mammalian cell lines are an indispensable tool for toxicology testing. For many applications, e.g. genotoxicity, phototoxicity or skin sensitation, cell based alternatives have been accepted by national or international authorities. According to a guidance document from the US national institute of health (NIH Publication No 01-4500; 2001) cells can be used to estimate the starting dose of chemicals that are tested for acute oral toxicity in vivo.

Although the use of in vitro cell cultures is well established, the handling and cultivation of mammalian cells still entail a lot of variability due to differences in cell passage and culture conditions. To prepare fresh cells from a continuously growing culture on time, in right quantities and in reproducible quality is sophisticated and tedious.

In drug discovery and high-throughput screening the use of assay ready cryopreserved cells has been implemented since 2005. These frozen cells can be instantly used after thawing without prior passaging or extended cultivation - basically like any other reagent in a cell based assay. In drug discovery this process helped a lot to improve not only the convenience of the user but also the reproducibility of the assay. We here directly compare assay ready frozen cells with passaged cells from a growing culture for the use in cytotoxicity assays.

preparation of assay ready frozen cells

To prepare high quality assay ready cryopreserved cells, the cells have to be expanded under optimal culture conditions and strict passage control and need to be kept in the logarithmic growth phase. The cells are harvested enzymatically with enzyme formulations which are more gentle than regular trypsin, e.g. Accutase® or TryLE™ Express. For cryopreservation the cells are suspended in freezing medium containing 5% DMSO or less for cryoprotection. For improved reproducibility the cells are automatically dispensed into vials and frozen in a controlled-rate freezer to minimize the impact of the crystallisation heat. For long-term storage the assay ready frozen cells are kept in vapour phase of liquid nitrogen.

large scale cell production | automatic dispensing into cryo vials | controlled rate cryopreservation | long term storage in liquid nitrogen | aliquots of assay ready cells

Fig. 1: Production of assay ready frozen cells

batch to batch comparison of assay ready frozen cells

To investigate the reproducibility, five batches of assay ready frozen NIH-3T3 cells (CLS Cell Line Services) have been produced on different days. From each batch 10% of the vials have been thawed for quality control. Cell count, amount of debris (Fig. 2A) and viability (Fig.2B) have been measured with a CASY TT cell counter. Because the viability is usually quite good directly after thawing but can collapse within the first hours of culture, the viability was also analyzed 24 hours after thawing. In all five batches the expected number of cells could be recovered (average of 4,3 million cells) with a standard deviation of 6%. The frozen stocks contain an average of 1,2 million particles of debris per 4 million cells. Only batch 3 showed a slightly elevated level (1,6 million particles per 4 million cells).

Viability directly after thawing was above 96 % in four of five batches with an SD below 0,5 %. In continuously passaged cultures viability was 95,8 % and varies by 1,8 %. After 24 hours in culture the consolidated viability of attached cells and cells in the supernatant was still greater than 90% in all batches. In none of the batches a strong drop in viability was observed after thawing. The morphology and confluence of fresh versus assay ready frozen cells did not look different 24 or 48 hours after seeding (Fig.3).

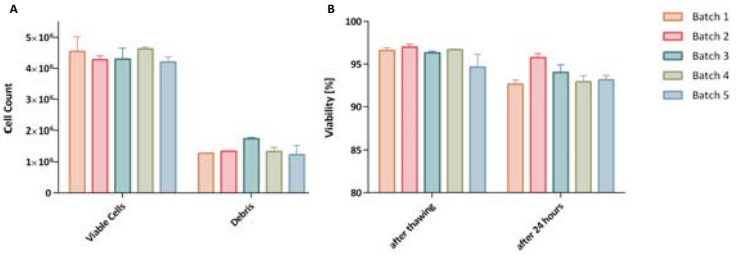
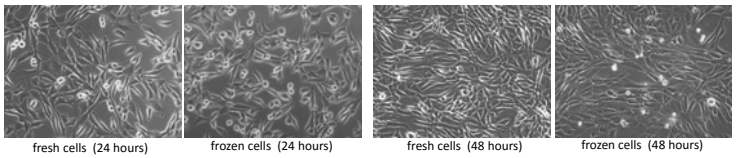


Fig. 2: Batch to batch comparison of assay ready frozen cells



fresh cells (24 hours) | frozen cells (24 hours) | fresh cells (48 hours) | frozen cells (48 hours)

Fig. 3: Morphology of assay ready cryopreserved NIH3T3 cells at different times after seeding

use of assay ready frozen cells in cytotoxicity testing

To see if assay ready prepared cells can be used lika a standard reagent for cytotoxicity testing, cryopreserved NIH-3T3 cells were compared with fresh cells from a continuously growing culture using a set of reference compound of different toxicity. In a direct comparison (Fig.4) the same IC50s were determined for sodium selenite, antipyrine and glycerol. Top and bottom levels, as well as the slope was almost identical, when using assay ready cryopreserved cells.

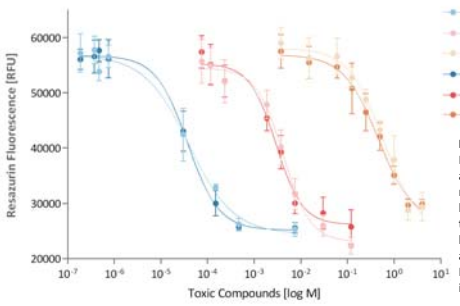


Fig. 4: Cytotoxicity assay fresh versus frozen
NIH3T3 cells from a growing culture and from a frozen stock (batch 2) were seeded at 20 million cells per well into a 96-well plate. 24 hours after seeding compounds were added in triplicates of eight serial dilutions. After 24 hours at 37°C Resazurin, a metabolic dye, was added. 4 hours later the amount of fluorescent Resofurin produced by live cells was quantified in a multi-plate reader at Ex560/Em590.

The reproducibility in cytotoxicity assays was tested in three independent experiments performed by different users at different days (Fig. 5). Assay ready cryopreserved NIH-3T3 cells (batch 1) and fresh cells from a growing culture were used. While top and bottom values of the experiments with fresh cells varied by 22% and 6%, respectively, with frozen cells more consistent results could be achieved (top: 5%, bottom: 2%). IC50 can vary two-fold with fresh cells but only by 50% with the assay-ready cryopreserved cells.

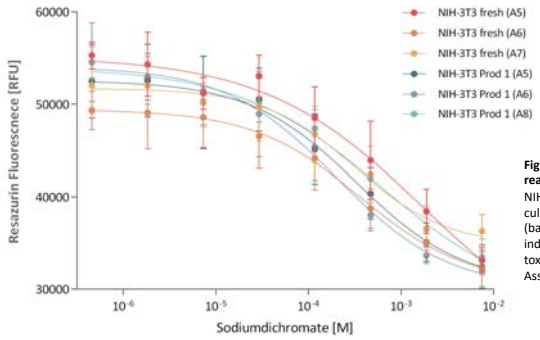


Fig. 5: Reproducibility of assay ready frozen cells
NIH-3T3 cells from a growing culture and from a frozen stock (batch 1) where used in three independent assays to test the toxicity of sodium dichromate. Assay protocol see Fig. 4

In vitro cytotoxicity assays can be used if not as a complete replacement for animal testing but to estimated the starting dose for experiments in rat (NIH Publication No 01-4500; 2001). According to a recommendation by Spielmann et al. (ALTA 27; 957, 1999) ten reference compounds were tested for cytotoxicity on assay ready cryopreserved NIH-3T3 cells (Fig. 6). The acquired IC50 values (blue) correlate with acute oral LD50 of rat and/or mice (R^2=0,93) and the correlation lays within the 95% confidence interval (red) of the correlation between IC50 value taken from the Register of Chemistry.

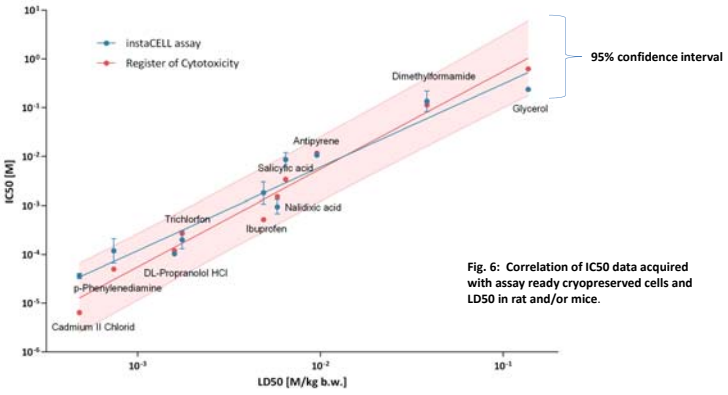


Fig. 6: Correlation of IC50 data acquired with assay ready cryopreserved cells and LD50 in rat and/or mice.

discussion

The comparison of assay ready cryopreserved cells with cells from a continuously growing culture showed, that frozen cells which are instantly used after thawing without any prior cultivation or passing, perform as good as fresh cells in cytotoxicity assays. Because individual vials of assay ready frozen cells derive from a single batch the reproducibility performed with assay ready frozen cells can be even improved.

Moreover, with assay ready frozen cells a user gains additional flexibility, because the cells are always available from the frozen stock in a constant and pre-validated quality.

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