Validation of a Cytotoxicity Bioassay Kit Based on Assay Ready Frozen Cells for the Biocompatibility Testing of Medical Devices

Christopher Rösch[1], Oliver Wehmeier[2], Alexander Loa[2], Hermann Benedikter[1]
1: BBF Sterilisationsservice GmbH; Germany; 2: acCELLerate GmbH, Germany

introduction

A medical device or material that comes in contact with the human body has to be tested for its biocompatibility and cytotoxicity which is regulated by the ISO guideline 10993 "Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity ". Parts of the medical device or extracts prepared thereof are brought in contact with a sensitive test cell line to determine the viability of the cells after a certain incubation time. The guideline does no specify a particular cell line or viability assay but recommends the use murine fibroblasts L-929 and formazan based dyes which are metabolized by living cells.

For improved reproducibility and better convenience in cell handling, we established and validated the use of an instaCELL® cytotoxicity assay kit (acCELLerate) which includes assay ready frozen L-929 cells. The cells don't have to be cultivated to recover from cryopreservation but can be used like a reagent instantly after thawing. Extracts from reference materials of different toxicity were tested in comparison with a formazan based reagent EZ4U (Biomedica) and Resazurin, a fluorescent metabolic dye, provided with the kit.

preparation of extracts from medical device materials

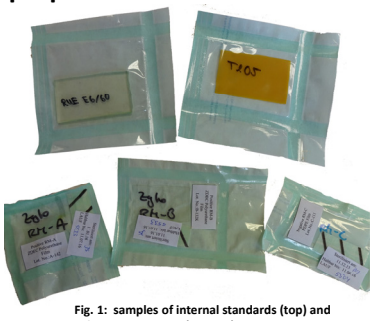


Fig. 1: samples of internal standards (top) and reference materials (bottom)

Three reference materials (RM-A, RM-B, RM-C) provided by the Food & Drug Safety Center of the Hatano Research Institute (Japan) and an internal standard (T105) were used for the validation. From each sample pieces of 30 cm^2 (3 x 5 cm), were cut, sealed and autoclaved. To extract potential toxic ingredients or contaminants the samples were incubated in culture medium (RPMI 1640) for 24 hours @ 37°C while careful agitation. The extracts were applied directly to the cells or stored at -20°C.

Sample	Material	Extraction Vol.	Toxicity
T105	Latex-Mixture T105 (1mm)	5 ml	strongly toxic
RM-A	Polyurethan, 0.1% ZDEC, < 0.5 mm	2.5 ml	moderately toxic
RM-B	Polyurethan, 0.25% ZDEC, <0.5 mm	2.5 ml	slightly toxic
RM-C	HDPE, <0.5 mm	2.5 ml	non-toxic

performance of cytotoxicity testing

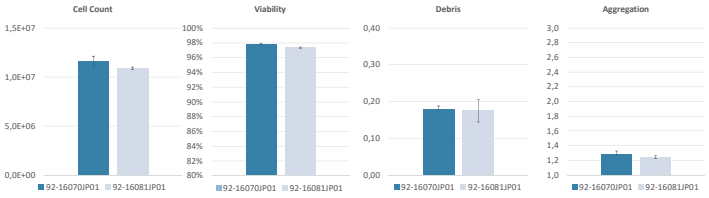
Assay ready L-929 cells, delivered on dry ice with the instaCELL® cytotoxicity assay kit, were thawed quickly in a water bath (37°C) and washed once in recovery buffer. The cell pellet was completely resuspended in the provided amount of assay medium and 80 µl were dispensed into each well of a provided 96-well plate. A counting of the cell was not required according to the manufacturer's protocol.

Sparing any pre-cultivation of the cells serial dilutions of the sample extracts (80 µl) were added directly to the cells and incubated for 24 hours at 37°C. Different from the assay protocol of the instaCELL kit the cells have been incubated in normal atmosphere and not in a CO_2 cell culture incubator.

After the incubation time two alternative read-outs performed in a Synergy HTX multifunctional reader have been compared to determine the viability of the cells: a) 20 µl of Resazurin solution, provided with the instaCELL kit were added to the cells and incubated at 37°C for 4 hours. The fluorescence of the metabolite was measure at 540_{EX} / 590_{EM}; b) 20 µl of the formazan derivate EZ4U was added to the cells and incubated at 37°C for 4 hours. The absorbance of the blue formazan was measured at 450nm with a reference at 630nm.

quality control of assay ready L-929 cells

For quality control of the assay ready L-929 cells provided with the kit the number of cryopreserved cells provided per vial, the viability of the cells, the amount of debris and the grade of aggregation was determined in CASY TT cell counter.



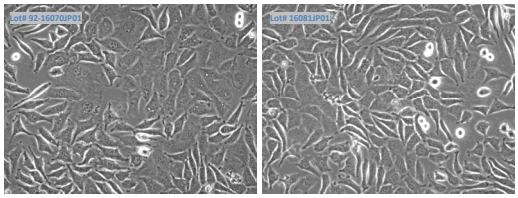


Fig. 2.: quality parameters of assay ready L-929 cells of two different batches. Cell count, viability, debris and aggregation (top). Morphology and confluence 24 hours after plating (left)

cytotoxicity testing using assay ready L-929 cells

Extracts from reference materials representing different levels of toxicity were used to investigate if assay ready cryopreserved L-929 cells which are challenged instantly after thawing without pre-cultivation, provide the expected sensitivity and classify the samples correctly. To determine if the recommendation in the guide-line to use a formazan derivate is obligatory an established formazan based detection reagent (EZ4U) was used in comparison with the fluorescent dye Resazurin which is provided with the instaCELL cytotoxicity assay kit.

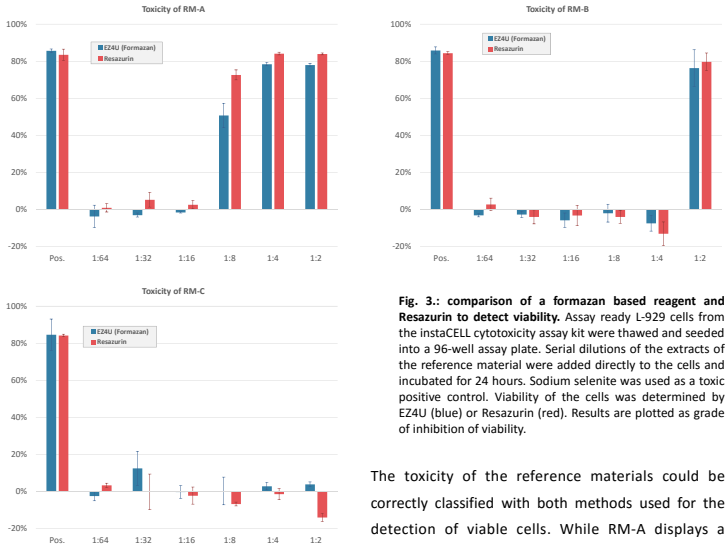


Fig. 3.: comparison of a formazan based reagent and Resazurin to detect viability. Assay ready L-929 cells from the instaCELL cytotoxicity assay kit were thawed and seeded into a 96-well assay plate. Serial dilutions of the extracts of the reference material were added directly to the cells and incubated for 24 hours. Sodium selenite was used as a toxic positive control. Viability of the cells was determined by EZ4U (blue) or Resazurin (red). Results are plotted as grade of inhibition of viability.

The toxicity of the reference materials could be correctly classified with both methods used for the detection of viable cells. While RM-A displays a moderate toxicity, RM-B is only slightly toxic at the highest concentration. RM-C does not show a cytotoxic effect at all. The assay ready L-929 provided a strong signal of viable cells in the untreated controls generating a S/B ratio between 13 and 15 and a Z' of at least 0.8 with both detection reagents. With Resazurin the extracts provide a slightly higher fluorescence as the untreated control which is most likely due to an auto-fluorescence of the material.

lab to lab comparison

To investigate the robustness of the assay instaCELL cytotoxicity assay kit when used from different operators the testing was performed in two different laboratories. RM-B a slightly toxic material and T105 which is strongly toxic were tested. In both laboratories the assay ready cells were prepared according to the protocol and incubated with serial dilutions of the reference extracts for 24 hours @ 37°C. While laboratory A was using a humidified CO2 incubator the assay was performed at humidified normal atmosphere in laboratory B.

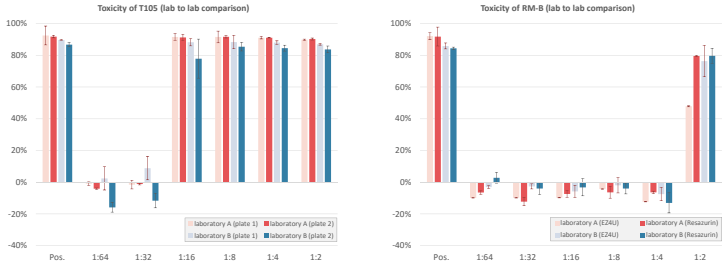


Fig. 4: comparison of assay ready L-929 cells for cytotoxicity testing in two different laboratories. assay ready L-929 cells were thawed and seeded into a 96-well assay plate. Serial dilutions of the reference extracts were added directly to the cells and incubated for 24 hours. Viability of the cells was determined by EZ4U or Resazurin.

The toxicity of the reference materials was correctly classified by both laboratories. The results were very reproducible when performed in the different laboratories by different operators. The fact that a non-carbonated atmosphere was used in laboratory B does not negatively influence the performance of the assay or results in higher variability.

discussion

assay ready L-929 cells which are cryopreserved at a high quality provide a very robust and reproducible tool when used for cytotoxicity testing of medical devices according ISO 10993-5. Although the cells are not supposed to be cultivated or even let adhere before the sample extracts are added, they generate a strong viability signal when treated with non-toxic concentrations of the extracts but display the expected sensitivity to toxic concentrations. Different detection reagents (e.g. formazan based or Resazurin) can be used equally in combination with the assay ready cells.

The one-day protocol and the release from preparatory cell culture work save a lot of resources. The handling is very convenient and enables any operator to perform a cytotoxicity assay without being an expert in cell culture techniques or have proper cell culture equipment like a sterile hood or a CO_2 incubator available. Because the assay ready cells are prepared and validated externally in homogenous batches, a big factor which generates inter laboratory variability is eliminated.

SAFETY

contact: Dr. Oliver Wehmeier, acCELLerate GmbH, oliver@accellerate.me