Cryopreserved Human Hepatocyte High-Throughput Screening Protocol: 96-Well ATP Cytotoxicity Assay

Introduction

Cryopreserved human hepatocytes represent a relevant experimental model for the evaluation of acute hepatotoxic potential of test articles in man.[1, 2]

Purpose

This assay is designed to screen for the cytotoxic potential of test articles in human hepatocytes.

Principle of the Procedure

Cellular adenosine triphosphate (ATP) content is a marker of cellular energy status and viability. When cells undergo necrosis or apoptosis, their ATP levels decline rapidly. In combination with luciferase, the addition of D-luciferin to cells generates light in the presence of ATP. The intensity of the luminescence is proportional to the intracellular ATP content.[3, 4]

Materials*

Item	Manufacturer	Name/Catalog/Model #
ATPLite	Perkin Elmer Life Sciences	6016941
Cryopreserved human hepatocytes	BioreclamationIVT	M00995, F00995
Orbital shaker	Bellco Glass Inc.	7744-01000
96-well opaque plastic plates	Costar	3915
Chlorpromazine	Sigma Chemical Co.	C-8138
Wallac Victor^2 Multilabel Counter	Wallac	1420-040
Solvents/Solubilizers		
Methanol	Fisher Scientific	A-452-4
Buffers		
*InVitro*GRO™ HI Medium	BioreclamationIVT	Z90009
Krebs-Henseleit Buffer powder (KHB)	Sigma Chemical Co.	K-3753
Amikacin sulfate	Sigma Chemical Co.	A-2324
Calcium chloride dihydrate	Sigma Chemical Co.	C-3881
Gentamicin sulfate	Sigma Chemical Co.	G-3632
N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonate) (HEPES)	Sigma Chemical Co.	H-3375
Heptanoic acid	Sigma Chemical Co.	H-9378
Hydrochloric acid (HCl)	JT Baker	9535-01
Sodium bicarbonate	Sigma Chemical Co.	S-5761
Sodium hydroxide (NaOH)	Sigma Chemical Co.	S-9625

*Items listed in this Materials section are for convenience; suitable materials and equipment from other manufacturers may be substituted as appropriate. Contact information for vendors used by BioreclamationIVT are listed in the Notes section at the end of this document.

Procedure

Reagent Preparation

(To be completed in advance of assay.)

1. Prepare KHB by supplementing it with
   - amikacin sulfate (84 µg/mL)
   - calcium chloride (1 mM)
   - HEPES (20 mM)
   - gentamicin sulfate (84 µg/mL)
   - heptanoic acid (4.2 µM)
   - sodium bicarbonate (2.2 g/L).

Keep KHB at a pH of 7.3. Use 1N NaOH to raise pH. Use 1N HCl to lower pH.

2. Prepare stock solutions of test articles and positive and negative controls. Stock solutions should be made at 100X, using an appropriate organic solvent (e.g., DMSO). Chlorpromazine is recommended as the positive control (final concentration = 100 µM). For a negative control, use KHB alone for water-soluble test articles or KHB with 1% solvent for lipophilic test articles.

Assay

3. Thaw cryopreserved human hepatocytes according to instructions provided by BioreclamationIVT (see Storing and Thawing Cryopreserved Hepatocytes).

4. Re-suspend hepatocytes in KHB at a density of 1.0×10^6 cells/mL.

5. Add 50 µL of 2X test article solution per well (dissolved in KHB with 1% solvent; final cell density = 50,000 cells/100 µL; final test article concentration = 1X) to a 96-well opaque plate pre-warmed to 37 °C.

6. Load 50 µL of hepatocytes per well.

7. Place the plate in an incubator at 37 °C, 5% CO_2 for 2-4 hours.

8. Add 50 µL of Mammalian Cell Lysis Solution per well. Shake for 2 minutes at 700 rpm on a room temperature orbital shaker.

9. Add 50 µL of reconstituted substrate solution (see kit instructions) per well. Shake at 700 rpm for 2 minutes. Keep the plate in the dark for 10 minutes.

10. Measure the luminescence in each well.

11. Compare luminescence of the treated samples to the untreated controls to determine the percent viability.

Note: The ATPLite kit contains standards that can also be used to determine µM ATP in each well based on a standard curve.

References

1. Li, A. P.; Gorycki, P. D.; Hengstler, J. G.; Kedderis, G. L.; Koebe, H. G.; Rahmani, R.; de Sousas, G.; Silva, J. M.; Skett, P. Present status of the application of cryopreserved hepatocytes in the evaluation of xenobiotics: consensus of an international expert panel. *Chem. Biol. Interact*. **1999**, *121*, 117–123.

2. Li, A. P.; Lu, C.; Brent, J. A.; Pham, C.; Fackett, A.; Ruegg, C. E.; and Silber, P. M. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. *Chem. Biol. Interact*. **1999**, *121*, 17–35.

3. Crouch, S. P. M.; Kozlowski, R.; Slater, K. J.; Fletcher, J. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J. Immunol. Methods* **1993**, *160*, 81–88.

4. Cree, I. A.; Andreotti, P. E. Measurement of cytotoxicity by ATP-based luminescence assay in primary cell cultures and cell lines. *Toxicology In Vitro* **1997**, *11*, 553–556.