NANOTECHNOLOGY CHARACTERIZATION LABORATORY

NCL Method GTA-1
Version 1.0

LLC-PK1 Kidney Cytotoxicity Assay

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

Method is written by _____ 3/13/06 .

Stephan T. Stern, Ph.D. Date

_____ 3/13/06 .

Timothy M. Potter, B.S. Date

Method validation was conducted on: 9/20/05 .

Date

Validation report is complete on: 9/30/05 .

Date

Testing facility: NCL, NCI-Frederick, Bldg. 469, Room 250 .

1. Introduction

This protocol describes the cytotoxicity testing of nanoparticle formulations in porcine proximal tubule cells (LLC-PK1), as part of the *in vitro* NCL preclinical characterization cascade. The protocol utilizes two methods for estimation of cytotoxicity, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) release.

2. Principles

2.1 MTT Assay: MTT is a yellow water-soluble tetrazolium dye that is reduced by live cells to a water-insoluble purple formazan. The amount of formazan can be determined by solubilizing it in DMSO and measuring it spectrophotometrically. Comparisons between the spectra of treated and untreated cells can give a relative estimation of cytotoxicity. (Alley *et al.* (1988) *Cancer Res.* 48:589-601).

2.2 LDH Assay: LDH is a cytoplasmic enzyme that is released into the cytoplasm upon cell lysis. The LDH assay, therefore, is a measure of membrane integrity. The basis of the LDH assay: (1) LDH oxidizes lactate to pyruvate, (2) Pyruvate reacts with the tetrazolium salt INT to form formazan, and (3) the water-soluble formazan dye is detected spectrophotometrically. (Decker, T. & Lohmann-Matthes, M.L. (1988) *J. Immunol Methods* 15:61-69; Korzeniewski, C. & Callewaert, D.M. (1983) *J. Immunol Methods* 64:313-320).

3. Required Materials and Equipment

3.1 Materials

3.1.1 MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma # M5655)

3.1.2 Acetaminophen (Sigma Cat. # A7085)

3.1.3 Dimethyl sulfoxide (Aldrich# 154938)

3.1.4 Glycine (Sigma# G7403)

3.1.5 Sodium Chloride (Sigma# S7653)

3.1.6 Triton-X-100 (Sigma# 93443)

3.1.7 M199 Cell Culture Media (Cambrex Cat. #12-109-F)

3.1.8 Fetal Bovine Serum (Hyclone SH30070.03)

3.1.9 Biovision LDH-Cytotoxicity Assay Kit (Biovision Cat. # K311-400)

3.1.10 Costar 96 well flat bottom cell culture plates, Cat. No. 3598

3.1.11 Nanoparticle

3.2 Cell Lines

3.2.1 LLC PK1 (pig kidney cells) (ATCC#CL-101)

3.3 Equipment

3.3.1 Plate reader (Safire^2 –Tecan or equivalent)

3.3.2 Centrifuge set at 700-800g (Allegra X-15R- Beckman Coulter) with 96-well plate adapter

Note: The NCL does not endorse any of the material/instrument suppliers listed below, their inclusion is for informational purposes only

4. Reagent and Control Preparation

4.1 Positive control

4.1.1 Acetaminophen (APAP) positive control: Add 19 mg to a total volume of 5 mL Maintanence Media to make a 25 mM solution. Sterile filter using a 0.2 μm filter.

4.1.2 1% Triton-X-100 positive control: Add 1 mL of Triton-X-100 to 99 mL of media. Sterile filter using a 0.2 μm filter.

4.2 MTT Assay

4.2.1 MTT solution: 5mg/mL MTT in PBS, store for up to one month at 4°C in dark

4.2.2 Glycine Buffer: 0.1M glycine (MW 75.07), 0.1 M NaCl (MW 58.44), pH 10.5, store at room temperature.

4.3 LDH Assay

4.3.1 Reconstitute catalyst in 1mL dH_20 for 10 min and vortex (stable for 2 weeks at 4°C).

4.3.2 Reaction mixture (for 1 96-well plate): Add 250uL of reconstituted catalyst solution to 11.25mL of dye solution (stable for 2 weeks at 4°C).

5. Experimental Procedure

5.1 Cell Preparation (or as recommended by supplier)

5.1.1 Harvest cryopreserved cells from prepared flasks **(limit to 20 passages)**.

5.1.2 Count cell concentration using a coulter counter or hemocytometer.

5.1.3 Dilute cells to a density of 2.5 X 10^5 cells/mL in M199 (3% FBS) cell culture media.

5.1.4 Plate 100 uL cells/well as per plate format for 4 96-well plates (time zero, 6 hour sample exposure, 24 hour sample exposure, 48 hour sample exposure).

5.1.5 Incubate plates for 24 hours at 5% CO_2, 37°C and 95% humidity (**cells are grown to approximately 80% confluence**).

5.2 Time Zero Plate (MTT Assay)

5.2.1 Remove time zero plate from incubator and replace media from Triton-X positive control wells (see plating format in Appendix A) with 1% Triton-X. Add 100 μL of media to the remaining wells. Let the plate set for 10 minutes at room temperature. Spin plate at 700 g for 3 minutes.

5.2.2 Remove 100 μL of media from each well and transfer it to another plate on ice, maintaining plate format. Use this plate immediately for the LDH assay (see below).

5.2.3 Add 50 μL of MTT to all wells.

5.2.4 Cover in aluminum foil and incubate at 37°C for 4 hours.

5.2.5 Remove plate from incubator and spin at 700g for 3 minutes.

5.2.6 Aspirate media and MTT.

5.2.7 Add 200 μL of DMSO to all wells.

5.2.8 Add 25 μL of glycine buffer to all wells

5.2.9 Read absorbance at 570nm on plate reader

5.3 Test sample and positive control addition

5.3.1 The highest concentration of nanoparticle tested should be at the limit of solubility. The test sample should be at physiological pH. Neutralization of acidic/basic test samples may be required.

5.3.2 Dilute test compound in media, making a total of 9 1:4 dilutions.

5.3.3 Add 100 μL of each dilution and positive control to 6 hour, 24 hour and 48 hour exposure plates as per the plate format.

5.4 Test Plates - 6, 24 and 48 hour exposures (MTT Assay)

5.4.1 Remove appropriate test plate from incubator and replace media from Triton-X positive control wells (see plating format in Appendix A) with 1% Triton-X. Let the plate set for 10 minutes at room temperature. Spin plate at 700 g for 3 minutes.

5.4.2 Remove 100 μL of media from each well and transfer it to another plate on ice, maintaining plate format. Use this plate immediately for the LDH assay (see below).

5.4.3 Remove remaining media from original plate.

5.4.4 Add 200 μL l of fresh media to all wells.

5.4.5 Add 50 μL of MTT to all wells.

5.4.6 Cover in aluminum foil and incubate for 37°C for 4 hours.

5.4.7 Remove plate from incubator and spin at 700g for 3 minutes.

5.4.8 Remove media and MTT.

5.4.9 Add 200 μL of DMSO to each well.

5.4.10 Add 25 μL of glycine buffer to each well

5.4.11 Read absorbance at 570nM on plate reader using a reference wavelength of 680nm.

5.5 Test Plates – 0, 6, 24 and 48 hour exposures (LDH Assay) Adapted from Biovision LDH Cytotoxicity Assay Kit (cat# K311-400)

5.5.1 Add 100 μL of the Reaction Mixture to each well of transfer plate. Shake plate on an orbital shaker.

5.5.2 Incubate at room temperature for up to 20 minutes in the dark.

5.5.3 Read the plate on plate reader at 490nM using a reference wavelength of 680nm.

6. Calculations

LDH Assay: % Total LDH leakage= ((sample abs-media control abs)/ (triton X positive control abs – media control abs))*100

MTT Assay: % Cell Viability= (sample abs/media control abs)*100

Mean, SD and %CV should be calculated for each blank, positive control, negative control and unknown sample.

7. Acceptance Criteria

7.1 The 48 hour % cell viability and % total LDH leakage for the APAP positive control should be less than 75% and greater than 15%, respectively.

7.2 The positive and sample replicate coefficient of variations should be within 50%.

7.3 The assay is acceptable if condition 7.1 and 7.2 are met. Otherwise, the assay should be repeated until acceptance criteria are met.

References

ISO 10993-5 Biological evaluation of medical devices: Part 5 Tests for *in vitro* cytotoxicity.

F1903 – 98 Standard Practice for Testing for Biological Responses to Particles *in vitro.*

Appendix A.

Example of Plate format

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cells Media	Cells Sample 1 Dilution 9	Cells Sample 1 Dilution 8	Cells Sample 1 Dilution 7	Cells Sample 1 Dilution 6	Cells Sample 1 Dilution 5	Cells Sample 1 Dilution 4	Cells Sample 1 Dilution 3	Cells Sample 1 Dilution 2	Cells Sample 1 Dilution 1	Cells APAP 25 mM	Cells Triton X 1%
B	Cells Media	Cells Sample 1 Dilution 9	Cells Sample 1 Dilution 8	Cells Sample 1 Dilution 7	Cells Sample 1 Dilution 6	Cells Sample 1 Dilution 5	Cells Sample 1 Dilution 4	Cells Sample 1 Dilution 3	Cells Sample 1 Dilution 2	Cells Sample 1 Dilution 1	Cells APAP 25 mM	Cells Triton X 1%
C	Cells Media	Cells Sample 1 Dilution 9	Cells Sample 1 Dilution 8	Cells Sample 1 Dilution 7	Cells Sample 1 Dilution 6	Cells Sample 1 Dilution 5	Cells Sample 1 Dilution 4	Cells Sample 1 Dilution 3	Cells Sample 1 Dilution 2	Cells Sample 1 Dilution 1	Cells APAP 25 mM	Cells Triton X 1%
D	Media Media	Media Sample 1 Dilution 9	Media Sample 1 Dilution 8	Media Sample1 Dilution 7	Media Sample 1 Dilution 6	Media Sample 1 Dilution 5	Media Sample 1 Dilution 4	Media Sample 1 Dilution 3	Media Sample 1 Dilution 2	Media Sample 1 Dilution 1	Media APAP 25 mM	Media Triton X 1%
E	Media Media	Media Sample 2 Dilution 9	Media Sample 2 Dilution 8	Media Sample 2 Dilution 7	Media Sample 2 Dilution 6	Media Sample 2 Dilution 5	Media Sample 2 Dilution 4	Media Sample 2 Dilution 3	Media Sample 2 Dilution 2	Media Sample 2 Dilution 1	Media APAP 25 mM	Media Triton X 1%
F	Cells Media	Cells Sample 2 Dilution 9	Cells Sample 2 Dilution 8	Cells Sample 2 Dilution 7	Cells Sample 2 Dilution 6	Cells Sample 2 Dilution 5	Cells Sample 2 Dilution 4	Cells Sample 2 Dilution 3	Cells Sample 2 Dilution 2	Cells Sample 2 Dilution 1	Cells APAP 25 mM	Cells Triton X 1%
G	Cells Media	Cells Sample 2 Dilution 9	Cells Sample 2 Dilution 8	Cells Sample 2 Dilution 7	Cells Sample 2 Dilution 6	Cells Sample 2 Dilution 5	Cells Sample 2 Dilution 4	Cells Sample 2 Dilution 3	Cells Sample 2 Dilution 2	Cells Sample 2 Dilution 1	Cells APAP 25 mM	Cells Triton X 1%
H	Cells Media	Cells Sample 2 Dilution 9	Cells Sample 2 Dilution 8	Cells Sample 2 Dilution 7	Cells Sample 2 Dilution 6	Cells Sample 2 Dilution 5	Cells Sample 2 Dilution 4	Cells Sample 2 Dilution 3	Cells Sample 2 Dilution 2	Cells Sample 2 Dilution 1	Cells APAP 25 mM	Cells Triton X 1%

Appendix B.

Example of LLC-PK1 Cell Culture Appearance

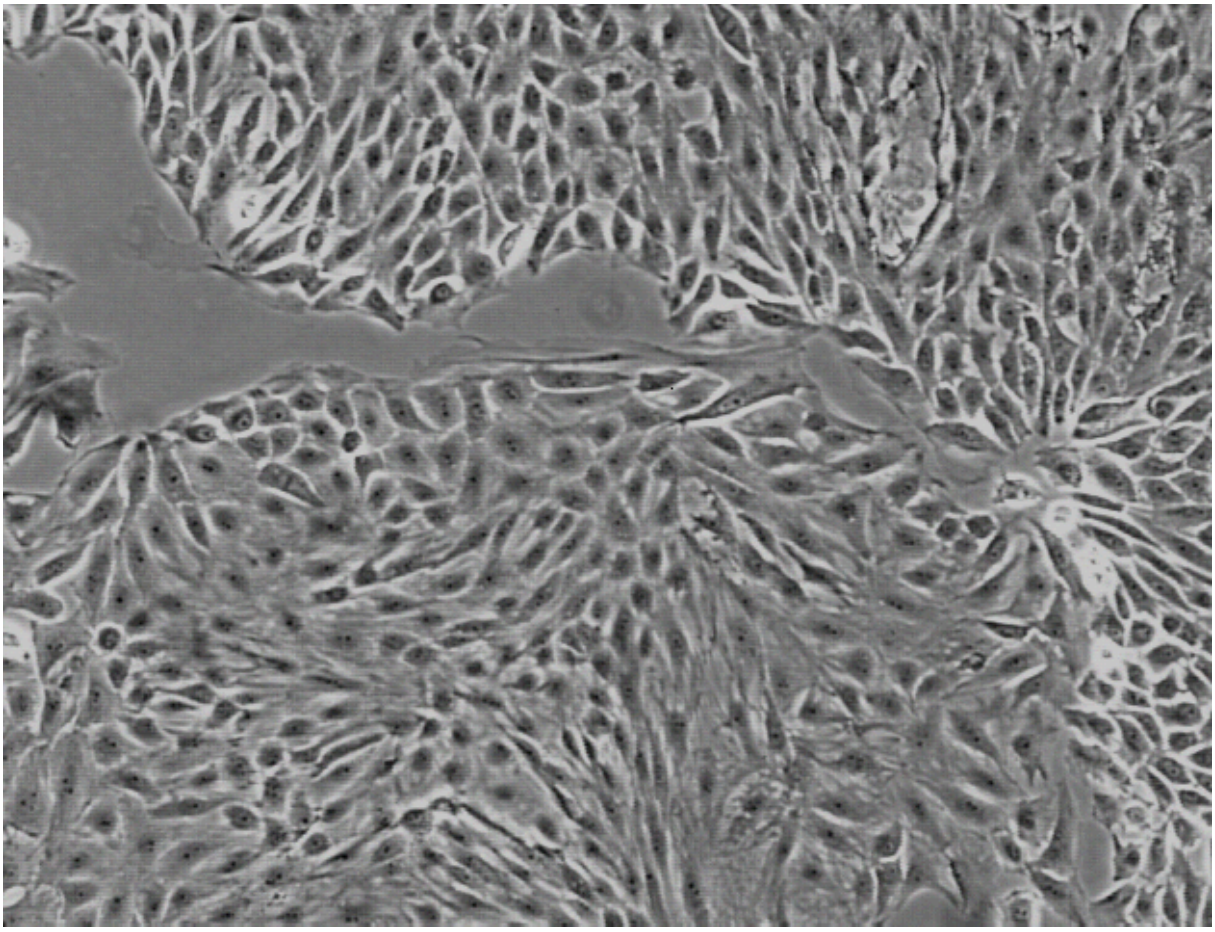

Image was taken with a phase contrast microscope at 225x magnification. LLC-PK1 cells are approximately 80% confluent at this stage.