NCL Method GTA-2

Hep G2 Hepatocarcinoma 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.

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1. Introduction

This protocol describes the cytotoxicity testing of nanoparticle formulations in human hepatocarcinoma cells (Hep G2), 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 [1-2].

2. Principles

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 [3].

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: a) LDH oxidizes lactate to pyruvate, b) pyruvate reacts with the tetrazolium salt INT to form formazan, and c) the water-soluble formazan dye is detected spectrophotometrically [4, 5].

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Reagents

1. Hep G2 (human hepatocarcinoma) (ATCC, HB-8065)
2. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma, M5655)
3. Acetaminophen (Sigma, A7085)
4. Dimethyl sulfoxide (Sigma, S5879)
5. Glycine (Sigma, G7126)

6. Sodium chloride (Sigma, S7653)
7. 10% Triton-X-100 (Sigma, 93443)
8. RPMI 1640 (Hyclone, SH30096.01)
9. L-Glutamine (Hyclone, SH30034.01)
10. Pen/Strep solution (Invitrogen, 15140-148)
11. Fetal bovine serum (Hyclone, SH30070.03)
12. Biovision LDH-cytotoxicity assay kit (Biovision, K311-400)

3.2 Materials

1. Costar 96 well flat bottom cell culture plates (3598)

3.3 Equipment

1. Plate reader (Safire^2 –Tecan or equivalent)
2. Centrifuge, 700-800x*g* with 96-well plate adapter (Allegra X-15R, Beckman Coulter)
3. Orbital plate shaker
4. Incubator, 37˚C with 5% CO_2 and 95% humidity

4. Reagent and Control Preparation

4.1 Positive Control

1. Acetaminophen (APAP)

   Add 15.2 mg to a total volume of 5 mL RPMI 1640 cell culture media (with 2 mM L-glutamine and 10% FBS) to make a 20 mM solution. Sterile filter using a 0.2 µm filter.

2. 0.1% Triton-X-100

   Prepare a 20X stock (2%) of Triton X-100 by adding 2 mL of Triton-X-100 to 8 mL.

4.2 MTT Assay

1. MTT Solution

   Prepare 5 mg/mL MTT in PBS. Store for up to one month at 4˚C in the dark.

2. Glycine Buffer

   Prepare 0.1 M glycine (MW 75.07) with 0.1 M NaCl (MW 58.44), pH 10.5. Store at room temperature.

4.3 LDH Assay

1. Reconstitute catalyst in 1 mL dH_2O for 10 min with occasional vortexing. Stable for 2 weeks at 4˚C.
2. Reaction mixture (for one 96-well plate): Add 250 µL of reconstituted catalyst solution to 11.25 mL of dye solution. Once thawed, the kit components are stable for 2 weeks stored at 4C. Reconstituted catalyst solution should be added to the dye solution immediately before use.

4.4 Media

1. Maintenance media: used to maintain cells prior to experiment

    RPMI 1640, 10% Fetal Bovine Serum, 2mM L-Glutamine, 100U/mL penicillin, and 100 µg/mL streptomycin sulfate

2. Assay media: used for assay procedure

    RPMI 1640, 10% Fetal Bovine Serum, 2mM L-Glutamine

5. Cell Culture

Grow cells in maintenance medium. Renew growth media twice a week. A subcultivation ratio of 1:4 or 1:6 is recommended. To split the cells, remove and discard culture medium; briefly rinse the cell layer with 0.25% (w/v) Trypsin, 0.53 mM EDTA solution to remove all traces of serum (contains trypsin inhibitor). Next, add 2.0 to 3.0 mL of Trypsin-EDTA solution to the flask and observe cells under an inverted microscope until the cell layer is dispersed (usually within 2 to 5 minutes).

To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37ºC to facilitate dispersal. Add 6.0 to 8.0 mL of maintenance medium and aspirate cells by gently pipetting. Add appropriate aliquots of the cell suspension to new culture flasks and incubate cultures at 37ºC in a 5% CO_2 environment. **Note**: Limit to 20 passages.

6. Experimental Procedure

6.1 Cell Preparation (or as recommended by supplier)

1. Harvest cells from prepared flasks.
2. Count cell concentration using a coulter counter or hemocytometer.

3. Dilute cells to a density of 5 x 10^5 cells/mL in assay media.
4. Plate 100 µL cells/well as per plate format (Appendix) for four 96-well plates (time zero, 4, 24, and 48 hr sample exposure). The format indicates no cells in rows D and E as they serve as particle blanks to be subtracted from cell treatment wells. Each plate accommodates two samples (Rows A–C and F–H). Each nanoparticle is tested at nine dilutions. Column 11 receives the paclitaxel injection positive control and column 12 receives Triton X-100 at the end of the relevant time point.
5. Incubate plates for 24 hr at 5% CO_2, 37˚C and 95% humidity. Cells are grown to approximately 80% confluence (Figure 1).

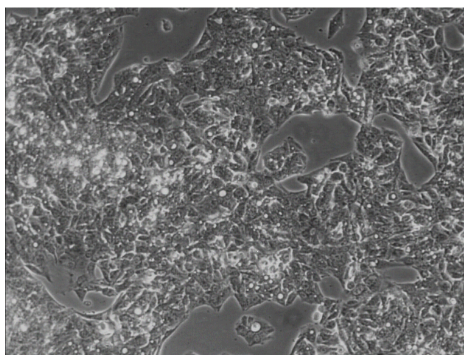

Figure 1. Hep G2 Cell Culture. Image was taken with a phase contrast microscope at 225X magnification. Hep G2 cells are approximately 80% confluent at this stage.

6.2 Time Zero Plate (MTT Assay)

1. Remove time zero plate from incubator and add 10 μL of 20X Triton-X to positive control wells (see plate format in Appendix) for a final concentration of 0.1% Triton X-100 (step 4.1.2). Add 100 μL media to the remaining wells. Let the plate set for 10 minutes at room temperature.
2. Remove 100 μL of media from each well and transfer it to another plate, maintaining plate format. Use this plate immediately for the LDH assay (see Section 5.5).
3. Remove remaining media from original plate and discard.
4. Add 200 μL fresh media to all wells.
5. Add 50 μL MTT (step 4.2.1) to all wells.
6. Cover with aluminum foil and incubate at 37˚C for 4 hr.
7. Aspirate media.
8. Add 200 μL DMSO to all wells to solubilize the MTT formazan crystals.
9. Add 25 μL glycine buffer (step 4.2.2) to all wells. Place on shaker to mix.
10. Read absorbance at 570 nm on plate reader using a reference wavelength of 680 nm.

6.3 Test Sample and Positive Control Addition

1. The highest concentration of nanoparticle tested should be at the limit of solubility or determined by the potency of the test material.
2. Dilute test compound in media, making a total of nine 1:4 dilutions at 2X the desired final concentration. Different dilution series (i.e., 1:2, 1:10) can be done depending on test material potency and availability.
3. Add 100 μL of each sample dilution and positive control to 4, 24 and 48 hr exposure plates as per the plate format (Appendix)), and place in 37˚C incubator with 5% CO_2 and 95% humidity for indicated time. Alternatively, sample and positive control can be made at desired final concentration. Media is aspirated from wells on the test plates, and sample and positive control are added at 200 μL per well as per the plate format (Appendix).

6.4 Test Plates, 4, 24 and 48 hr Exposures (MTT Assay)

1. Remove plate from incubator and add 10 µL of 20X Triton-X to positive control wells (see plate format in Appendix) for a final concentration of 0.1% Triton X-100 (step 4.1.2). Let the plate set for 10 minutes at room temperature.
2. Remove 50 µL of media from each well and transfer it to another plate, maintaining plate format. Use this plate immediately for the LDH assay (see Section 5.5).
3. Remove remaining media from original plate and discard.
4. Add 200 µL fresh media to all wells.
5. Add 50 µL MTT to all wells.
6. Cover with aluminum foil and incubate for 37˚C for 4 hr.
7. Aspirate media.
8. Add 200 µL of DMSO to each well.
9. Add 25 µL of glycine buffer to each well. Place on shaker to mix.
10. Read absorbance at 570 nm on plate reader using a reference wavelength of 680 nm.

6.5 Test Plates: 0, 4, 24 and 48 hr Exposures (LDH Assay)

(Adapted from Biovision LDH Cytotoxicity Assay Kit, K311-400)

1. Add 50 µL of the Reaction Mixture (step 4.3.2) to each well of transfer plate. Shake plate on an orbital shaker briefly to mix samples.
2. Incubate at room temperature for up to 20 minutes in the dark.
3. Read the plate on plate reader at 490 nm using a reference wavelength of 680 nm.

7. Calculations

All samples, positive, negative, and media controls are run in triplicate (e.g., rows A-C or F-H). Each well will be subtracted from its respective cell-free blank (e.g., A2-D2 or C3-D3) in the following calculations. The average of these three values should be used in the equations below for the positive and negative controls (e.g., [(A1-D1) + (B1-D1) + (C1-D1)]/3 = mean media control absorbance for sample 1, or [(A12-D12) + (B12-D12) + (C12-D12)]/3 = mean TritonX positive control absorbance for sample 1; Sample 2 uses the blank row E for subtractions.).

7.1 MTT Assay

$$\% \text{ Cell Viability} = \frac{\text{sample absorbance} - \text{cell free sample blank}}{\text{mean media control absorbance}} \times 100$$

7.2 LDH Assay

$$\% \text{ Total LDH Leakage} = \frac{(\text{sample absorbance} - \text{cell free sample blank}) - \text{mean media control absorbance}}{\text{mean TritonX positive control absorbance} - \text{mean media control absorbance}} \times 100$$

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

8. Acceptance Criteria

1. The 48 hr % cell viability and % total LDH leakage for the APAP positive control should be less than 50% and greater than 50%, respectively.
2. The positive and sample replicate coefficient of variations should be within 50%.
3. The assay is acceptable if criteria 1 and 2 are met. Otherwise, the assay should be repeated until acceptance criteria are met.
4. If the acceptance criteria are met, determine the highest concentration of the nanoparticulate material that does not interfere with the assay system indicated in rows D and E.
5. The concentration–response curves for the 48 hr MTT and LDH data should be classified as having complete (two observed asymptotes) or incomplete (second asymptote not obtained) curves, single point activity (activity at the highest concentration only), or no activity. For all complete 48 hr concentration–response curves, a nonlinear fit of the sigmoidal hill equation should be performed, and an estimate of potency (EC_{50}), efficacy (E_{max}), minimum response (E_0), and hill slope (γ) from the Hill equation (below) fit should be reported. Any excluded points (excluded by outlier analysis) should also be reported.

$$E = E_0 + [\ (E_{max} - E_0)C\gamma \ /\ EC\gamma_{50} \cdot C\gamma]$$

9. References

1. ISO 10993-5, Biological evaluation of medical devices: Part 5, Tests for *in vitro* cytotoxicity.
2. F1903 – 98, Standard Practice for Testing for Biological Responses to Particles *in vitro*.
3. Alley, et al. (1988) *Cancer Res.* 48:589-601.
4. Decker, T. & Lohmann-Matthes, M.L. (1988) *J. Immunol Methods* 15:61-69.
5. Korzeniewski, C. & Callewaert, D.M. (1983) *J. Immunol Methods* 64:313-320.

10. Abbreviations

APAP	acetaminophen
CV	coefficient of variation
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
Hep G2	human hepatocarcinoma cells
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride
LDH	lactate dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
PBS	phosphate buffered saline
SD	standard deviation

11. Appendix

Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cells Media	Cells TS1, D9	Cells TS1, D8	Cells TS1, D7	Cells TS1, D6	Cells TS1, D5	Cells TS1, D4	Cells TS1, D3	Cells TS1, D2	Cells TS1, D1	Cells APAP 20 mM	Cells TritonX 0.1 %
B	Cells Media	Cells TS1, D9	Cells TS1, D8	Cells TS1, D7	Cells TS1, D6	Cells TS1, D5	Cells TS1, D4	Cells TS1, D3	Cells TS1, D2	Cells TS1, D1	Cells APAP 20 mM	Cells TritonX 0.1 %
C	Cells Media	Cells TS1, D9	Cells TS1, D8	Cells TS1, D7	Cells TS1, D6	Cells TS1, D5	Cells TS1, D4	Cells TS1, D3	Cells TS1, D2	Cells TS1, D1	Cells APAP 20 mM	Cells TritonX 0.1 %
D	Media Media	Media TS1, D9	Media TS1, D8	Media TS1, D7	Media TS1, D6	Media TS1, D5	Media TS1, D4	Media TS1, D3	Media TS1, D2	Media TS1, D1	Media APAP 20 mM	Media TritonX 0.1 %
E	Media Media	Media TS2, D9	Media TS2, D8	Media TS2, D7	Media TS2, D6	Media TS2, D5	Media TS2, D4	Media TS2, D3	Media TS2, D2	Media TS2, D1	Media APAP 20 mM	Media TritonX 0.1 %
F	Cells Media	Cells TS2, D9	Cells TS2, D8	Cells TS2, D7	Cells TS2, D6	Cells TS2, D5	Cells TS2, D4	Cells TS2, D3	Cells TS2, D2	Cells TS2, D1	Cells APAP 20 mM	Cells TritonX 0.1 %
G	Cells Media	Cells TS2, D9	Cells TS2, D8	Cells TS2, D7	Cells TS2, D6	Cells TS2, D5	Cells TS2, D4	Cells TS2, D3	Cells TS2, D2	Cells TS2, D1	Cells APAP 20 mM	Cells TritonX 0.1 %
H	Cells Media	Cells TS2, D9	Cells TS2, D8	Cells TS2, D7	Cells TS2, D6	Cells TS2, D5	Cells TS2, D4	Cells TS2, D3	Cells TS2, D2	Cells TS2, D1	Cells APAP 20 mM	Cells TritonX 0.1 %

TS, test sample; D, dilution