NCL Method GTA-14

Hep G2 Hepatocarcinoma Homogeneous Apoptosis 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 monitoring of nanoparticle treated human hepatocarcinoma cells (HepG2) for apoptosis, as part of the *in vitro* NCL preclinical characterization cascade **(1, 2)**. The protocol utilizes a fluorescent method to measure Caspase-3/7 activation. This is a homogeneous assay that does not require cell isolation, as required for HepG2 Hepatocarcinoma Apoptosis Assay (NCL Method GTA-6).

2. Principles

Caspase-3/7 Homogeneous Fluorometric Assay:
Apoptosis in mammalian cells is initiated by activation of the Caspase family of cysteine proteases. This assay quantifies Caspase-3/7 activation *in vitro* by measuring the cleavage of the non-fluorescent substrate Z-DEVD-R110 (rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide)) to free rhodamine 110, which emits green fluorescence (λ_{max} = 521 nm) **(3)**. This free rhodamine 110 is measured using a microtiter plate reader.

3. Reagents, Materials, Cell Lines, 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

- 3.1.1 Acetaminophen (APAP) (Sigma, A7085)
- 3.1.2 RPMI 1640 (Hyclone, SH30096.01)
- 3.1.3 L-glutamine (Hyclone, SH30034.01)
- 3.1.4 Fetal Bovine Serum (Hyclone, SH30070.03)
- 3.1.5 Nanoparticle
- 3.1.6 Apo-ONE® Homogeneous Caspase-3/7 Assay Kit (Promega, G7790)

3.2 Materials

- 3.2.1 Costar 96 well flat bottom cell culture plates (Costar, 3598)

3.3 Cell Lines

- 3.3.1 HepG2 (human hepatocarcinoma cells) (ATCC, HB-8065)

3.4 Equipment

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

3.4.2 Plate Shaker

4. Reagent and Control Preparation

4.1 Positive control

4.1.1 Acetaminophen (APAP) positive control: Add 8 mg to a total volume of 5 mL RPMI 1640 cell culture media (with 2 mM L-glutamine and 10% FBS) to make a 10 mM solution. Sterile filter using a 0.2 μm filter.

4.2 Solutions to make up fresh for each run (can be stored at 4˚C for 24 h)

4.2.1 Retrieve Apo-ONE® Assay kit from -20˚C.

4.2.2 Thaw the 100X substrate and buffer to room temperature.

4.2.3 Mix by inversion or vortexing. Dilute substrate 1:100 in buffer.

4.2.4 Apo-ONE® Reagent is light sensitive; store protected from light.

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)** (Figure 1).

5.1.2 Count cell concentration using a coulter counter or hemocytometer.

5.1.3 Dilute cells to a density of 2.0 x 10^5 cells/mL in RPMI 1640 cell culture media (2 mM L-glutamine, 10% FBS).

5.1.4 Plate 100 μL/well of diluted cells as per plate format (see Appendix) for each 96 well plate (2 particles/96 well plate).

5.1.5 Incubate plates for 24 h at 5% CO_2, 37˚C and 95% humidity (**cells should be approximately 80% confluent**).

5.1.6 Replace cell culture media with 100 μL media containing test material or positive control. Desired test sample concentration is determined from HepG2 Hepatocarcinoma Cytotoxicity Assay (NCL Method GTA-2). Treat cells for 24 h.

5.2 Caspase Activation Assay

5.2.1 Add 100 µL of Apo-ONE® reagent to each well for a final volume of 200 µL/well.

5.2.2 Cover plate with foil and place on plate shaker (300-500 rpm).

5.2.3 Incubate for 1 h at room temperature.

5.2.4 Read fluorescence on plate reader with an excitation of 499 nm and an emission of 521 nm.

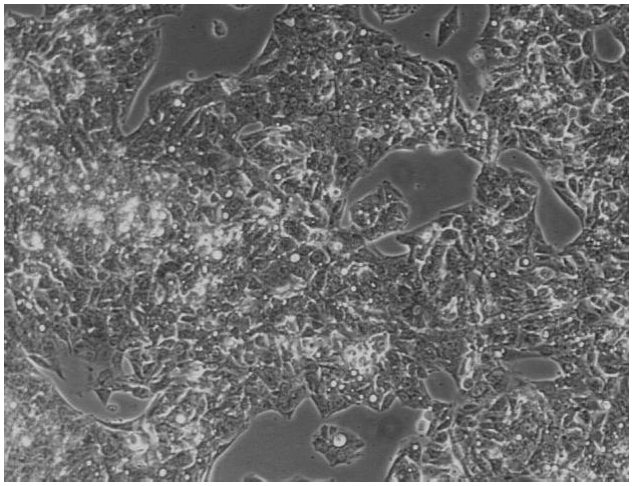

Figure 1. Example of HepG2 Cell Culture Appearance.

Image was taken with a phase contrast microscope at 225X magnification. Human hepatocarcinoma cells (HepG2) are approximately 80% confluent at this stage.

6. Calculations

Total Caspase Activity =

$$\left(\frac{\text{(Sample Fluorescence – Sample Cell Free Blank Fluorescence)}}{\text{mean of (Media Fluorescence – Media Blank Fluorescence)}} \right) \times 100$$

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

7. Acceptance Criteria

7.1 If a concentration responsive increase or decrease in sample cell-free blank fluorescence is observed, then the nanoparticle is interfering with the assay fluorescence and the non-homogeneous apoptosis assay; NCL Method GTA-6 Hepatocarcinoma Apoptosis Assay should be used instead.

7.2 The fold increase in caspase activity at 24 h for the APAP positive control versus media negative control should be at least 3 (300% of negative control).

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

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

8. 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. Promega Technical Bulletin #295: Apo-ONE® Homogeneous Caspase-3/7 Assay.

9. Abbreviations

APAP	acetaminophen
CV	coefficient of variation
FBS	fetal bovine serum
rpm	revolutions per minute
RPMI	Rosewell Park Memorial Institute
SD	standard deviation
Z-DEVD-R110	Rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide

10. Appendix

Example of a 96-well plate template.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cells Media	Cells Samp. 1 Dilution 10	Cells Samp. 1 Dilution 9	Cells Samp. 1 Dilution 8	Cells Samp. 1 Dilution 7	Cells Samp. 1 Dilution 6	Cells Samp. 1 Dilution 5	Cells Samp. 1 Dilution 4	Cells Samp. 1 Dilution 3	Cells Samp. 1 Dilution 2	Cells Samp. 1 Dilution 1	Cells APAP 10 mM
B	Cells Media	Cells Samp. 1 Dilution 10	Cells Samp. 1 Dilution 9	Cells Samp. 1 Dilution 8	Cells Samp. 1 Dilution 7	Cells Samp. 1 Dilution 6	Cells Samp. 1 Dilution 5	Cells Samp. 1 Dilution 4	Cells Samp. 1 Dilution 3	Cells Samp. 1 Dilution 2	Cells Samp. 1 Dilution 1	Cells APAP 10 mM
C	Cells Media	Cells Samp. 1 Dilution 10	Cells Samp. 1 Dilution 9	Cells Samp. 1 Dilution 8	Cells Samp. 1 Dilution 7	Cells Samp. 1 Dilution 6	Cells Samp. 1 Dilution 5	Cells Samp. 1 Dilution 4	Cells Samp. 1 Dilution 3	Cells Samp. 1 Dilution 2	Cells Samp. 1 Dilution 1	Cells APAP 10 mM
D	Media Media	Media Samp. 1 Dilution 10	Media Samp. 1 Dilution 9	Media Samp. 1 Dilution 8	Media Samp. 1 Dilution 7	Media Samp. 1 Dilution 6	Media Samp. 1 Dilution 5	Media Samp. 1 Dilution 4	Media Samp. 1 Dilution 3	Media Samp. 1 Dilution 2	Media Samp. 1 Dilution 1	Media APAP 10 mM
E	Media Media	Media Samp. 2 Dilution 10	Media Samp. 2 Dilution 9	Media Samp. 2 Dilution 8	Media Samp. 2 Dilution 7	Media Samp. 2 Dilution 6	Media Samp. 2 Dilution 5	Media Samp. 2 Dilution 4	Media Samp. 2 Dilution 3	Media Samp. 2 Dilution 2	Media Samp. 2 Dilution 1	Madia APAP 10 mM
F	Cells Media	Cells Samp. 2 Dilution 10	Cells Samp. 2 Dilution 9	Cells Samp. 2 Dilution 8	Cells Samp. 2 Dilution 7	Cells Samp. 2 Dilution 6	Cells Samp. 2 Dilution 5	Cells Samp. 2 Dilution 4	Cells Samp. 2 Dilution 3	Cells Samp. 2 Dilution 2	Cells Samp. 2 Dilution 1	Cells APAP 10 mM
G	Cells Media	Cells Samp. 2 Dilution 10	Cells Samp. 2 Dilution 9	Cells Samp. 2 Dilution 8	Cells Samp. 2 Dilution 7	Cells Samp. 2 Dilution 6	Cells Samp. 2 Dilution 5	Cells Samp. 2 Dilution 4	Cells Samp. 2 Dilution 3	Cells Samp. 2 Dilution 2	Cells Samp. 2 Dilution 1	Cells APAP 10 mM
H	Cells Media	Cells Samp. 2 Dilution 10	Cells Samp. 2 Dilution 9	Cells Samp. 2 Dilution 8	Cells Samp. 2 Dilution 7	Cells Samp. 2 Dilution 6	Cells Samp. 2 Dilution 5	Cells Samp. 2 Dilution 4	Cells Samp. 2 Dilution 3	Cells Samp. 2 Dilution 2	Cells Samp. 2 Dilution 1	Cells APAP 10 mM