



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 ($\lambda_{\max} = 521 \text{ nm}$) (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 (Safire²–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 2.0×10^5 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% CO₂, 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.

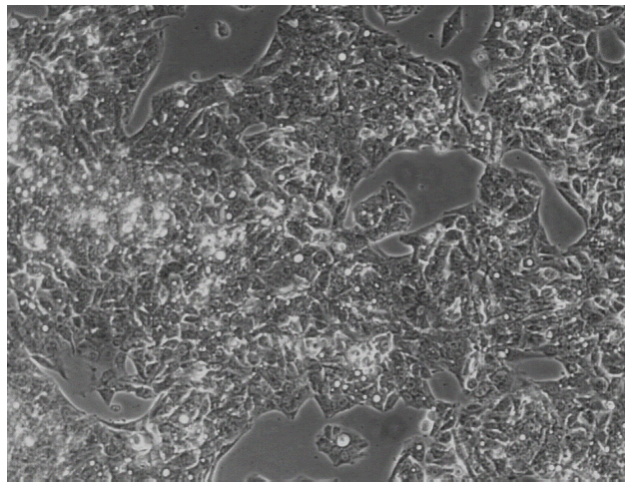


Figure 1. Example of HepG2 Cell Culture Appearance.

Image was taken with a phase contrast microscope at 225X magnification.

Human hepatocarcinoma cells (HepG2) are approximately 80% confluent at this stage.

6. Calculations

$$\text{Total Caspase Activity} = \frac{(\text{sample fluorescence} - \text{sample cell free blank fluorescence})}{\text{mean of (media fluorescence} - \text{media blank fluorescence)}} * 100$$

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. 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 |
| 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 |