NCL Method GTA-6

HepG2 Hepatocarcinoma 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. The protocol utilizes a fluorescent method to determine the degree of caspase-3 activation.

2. Principles

Caspase-3 Fluorometric Protease Assay:
Apoptosis in mammalian cells is initiated by activation of the caspase family of cysteine proteases. This assay quantifies caspase-3 activation *in vitro* by measuring the cleavage of caspase-3 substrate DEVD-7-amino-4-trifluoromethyl coumarin (AFC) to free AFC, which emits yellow-green fluorescence (λ_{max} = 505 nm). This free AFC is measured using a microtiter plate reader (**1-3**).

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 (Sigma, A7085)
- 3.1.2 RPMI 1640 (Hyclone, SH30096.01)
- 3.1.3 Quick Start Bradford Dye Reagent, 1X (Bio-Rad Lab., Inc., Cat. #500-0205)
- 3.1.4 Biovision Caspase-3 Fluorometeric Assay Kit (Biovision Cat. # K105-25)
- 3.1.5 Nanoparticle
- 3.1.6 Fetal Bovine Serum (FBS) (Hyclone, SH30070.03)
- 3.1.7 L-glutamine (Hyclone, SH30034.01)

3.2 Materials

- 3.2.1 Costar 6 well flat bottom cell culture plates (Costar, 3506)
- 3.2.2 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 Fluorescent Plate reader (Safire^2 –Tecan or equivalent)

3.4.2 Microfuge 22R Centrifuge

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 Maintenance Media (RPMI 1640 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 prior (from the Assay kit, Step 3.1.5) (can be stored at 4˚C for 6 months)

4.2.1 Add 10 μL of the DTT solution to 1 mL of the 2X Reaction Buffer.

4.2.2 Thaw the Cell Lysis buffer and store at 4˚C.

4.2.3 DEVD-AFC 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 7.5 x 10^5 cells/mL in RPMI 1640 cell culture media (2 mM L-glutamine, 10% FBS).

5.1.4 Plate 2 mL of diluted cells to each well of a 6-well plate (1.5 x 10^6 cells/ well). Test samples, positive controls, and media controls are run in triplicate. Timepoints are 0 h (media control), 6 h (sample and media control), 24 h (sample, positive control, and media control), and 48 h (sample and media control) for a total of 24 wells (see Appendix A).

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 2 mL 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 designated time period.

5.2 Caspase Activation Assay

5.2.1 Wash well with 1 mL of room temperature PBS.

5.2.2 Add 200 µL ice cold lysis buffer to the well, scrape cells and collect in 0.6 mL eppendorf tubes.

5.2.3 Incubate on ice for 10 minutes and centrifuge at 2000 x g for 5 minutes.

5.2.4 Transfer 50 µL of supernatant to a 96 well plate reader using the plating format described in Appendix B. Add 50 µL of 2X Reaction Buffer (with DTT) to each sample well. Retain remaining cell lysate for protein determination.

5.2.5 Add 5 µL of DEVD-AFC substrate (50 µM final concentration) and incubate at 37˚C for 1-2 hours.

5.2.6 Read at fluorescence at ex. 415 nm and em. 505 nm on a microtiter plate reader.

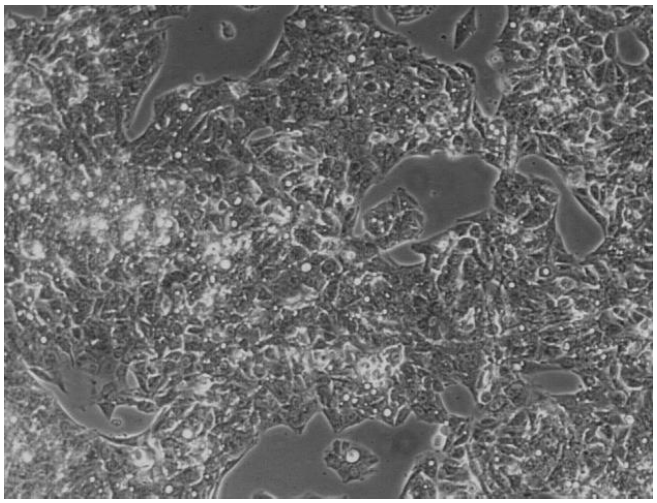

Figure 1. Example of HepG2 Cell Culture Appearance.

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

5.3 Protein Determination (Bradford Assay)

5.3.1 Dilute the 2 mg/mL BSA standard to make a standard curve from 0.125-1.0 mg/mL in 0.5 N NaOH.

5.3.2 Add 5 µL of standard (from step 5.3.1), cell lysate supernatant (from step 5.2.3), or water blank to each well of a microtiter plate in duplicate according the template in Appendix C.

5.3.3 Add 250 µL of 1X Dye Reagent to each well of the plate.

5.3.4 Incubate at room temperature for at least 5 min and not longer than 1 h.

5.3.5 Read absorbance on a microtiter plate reader at 595 nm.

6. Calculations

6.1 Protein concentration is determined from the BSA standard curve following linear regression analysis (y = x(slope) + y int). Total protein is determined from the equation:

Total lysate protein (mg) = ((mg protein/mL) * 0.05 mL sample volume)

6.2 Total Protein Normalized Caspase-3 Activity as a percentage of the control:

% Control activity =

$$\left(\frac{\text{Sample Fluorescence}}{\text{Total Sample Lysate Protein}}\right) \div \left(\frac{\text{Mean of Media Control Fluorescence}}{\text{Mean of Total Media Control Lysate Protein}}\right) * 100$$

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

7. Acceptance Criteria

7.1 The fold change in caspase activity at 24 hours for the APAP positive control versus media negative control should be at least 3.

7.2 The positive, media control, 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.

7.4 If statistical analysis determines that the total protein normalized control and treated fluorescence are significantly different from one another, then the fold change in fluorescence can be considered meaningful. This result would indicate that sample treatment significantly affected cell apoptosis.

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. Wang et al. (2005) Cell Bio. Int. 29: 489-496.

9. Abbreviations

AFC	7-amino-trifluoromethyl coumarin
APAP	acetaminophen
BSA	bovine serum albumin
CV	coefficient of variation
DEVD	aspartic acid-glutamic acid-valine-aspartic acid
DTT	dithiothreitol
em.	emission
ex.	excitation
FBS	fetal bovine serum
HepG2	human hepatocarcinoma cells
λ_{max}	maximal wavelength
PBS	phosphate buffered saline
RPMI	Rosewell Park Memorial Institute
SD	standard deviation

10. Appendices

Appendix A

Example of 6-well plate templates.

	1	2	3
A	Media 0 h # 1	Media 0 h # 2	Media 0 h # 3
B			

	1	2	3
A	Media 6 h # 1	Media 6 h # 2	Media 6 h # 3
B	Sample 6 h # 1	Sample 6 h # 2	Sample 6 h # 3

	1	2	3
A	Media 24 h # 1	Media 24 h # 2	Media 24 h # 3
B	Sample 24 h # 1	Sample 24 h # 2	Sample 24 h # 3

	1	2	3
A	Positive Control 24 h # 1	Positive Control 24 h # 2	Positive Control 24 h # 3
B			

	1	2	3
A	Media 48 h # 1	Media 48 h # 2	Media 48 h # 3
B	Sample 48 h # 1	Sample 48 h # 2	Sample 48 h # 3

All samples are run in triplicate. The following timepoints are recommended: 0 h (media control), 6 h (sample and media control), 24 h (sample, positive control, and media control), and 48 h (sample and media control) in which case 5 plates will be necessary.

Appendix B

Example of a 96-well plate template.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Media # 1**	**Media # 2**	**Media # 3**									
B	**Sample # 1**	**Sample # 2**	**Sample # 3**									
C	**Positive Control # 1**	**Positive Control # 2**	**Positive Control # 3**									
D												
E												
F												
G												
H										**Blank**	**Blank**	**Blank**

Legend: Row (A), Media Negative Control; Row (B), Samples; Row (C), Acetaminophen Positive Control. Each timepoint will be measured individually, as it is harvested.

Appendix C

Example of a 96-well plate template.

			Set A			Set B						
	1	2	3	4	5	6	7	8	9	10	11	12
A	1 mg/mL	1 mg/mL	Media 0 h #1	Media 0 h #2	Media 0 h #3	Media 0 h #1	Media 0 h #2	Media 0 h #3				
B	0.75 mg/mL	0.75 mg/mL	Media 6 h #1	Media 6 h #2	Media 6 h #3	Media 6 h #1	Media 6 h #2	Media 6 h #3				
C	0.5 mg/mL	0.5 mg/mL	Sample 6 h #1	Sample 6 h #2	Sample 6 h #3	Sample 63 h #1	Sample 6 h #2	Sample 6 h #3				
D	0.25 mg/mL	0.25 mg/mL	Media 24 h #1	Media 24 h #2	Media 24 h #3	Media 24 h #1	Media 24 h #2	Media 24 h #3				
E	0.125 mg/mL	0.125 mg/mL	Sample 24 h #1	Sample 24 h #2	Sample 24 h #3	Sample 24 h #1	Sample 24 h #2	Sample 24 h #3				
F			Positive Control 24 h #1	Positive Control 24 h #2	Positive Control 24 h #3	Positive Control 24 h #1	Positive Control 24 h #2	Positive Control 24 h #3				
G			Media 48 h #1	Media 48 h #2	Media 48 h #3	Media 48 h #1	Media 48 h #2	Media 48 h #3				
H			Sample 48 h #1	Sample 48 h #2	Sample 48 h #3	Sample 48 h #1	Sample 48 h #2	Sample 48 h #3			Blank	Blank

Legend: Pale Yellow, BSA Standards; Green, Media Controls; Blue, Samples; Bright Yellow, Positive Controls. Each sample is run in duplicate.