NCL Method GTA-4

Hep G2 Hepatocyte Lipid Peroxidation 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 analysis of human hepatocarcinoma cells (Hep G2) for lipid peroxidation products such as malondialdehyde (MDA), following treatment with nanoparticle formulations, as part of the *in vitro* NCL preclinical characterization cascade **(1-2)**.

2. Principles

Lipid peroxidation is an indicator of oxidative stress. The thiobarbituric acid reactive substances (TBARS) assay measures lipid hydroperoxides and aldehydes, such as malondialdehyde (MDA), in the cell culture media and cell lysate. MDA combines with thiobarbituric acid (TBA) in a 1:2 ratio to form a fluorescent adduct that is measured at ex. 530 nm and em. 550 nm. TBARS are expressed as MDA equivalents **(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 Trichloroacetic Acid (TCA) (Sigma-Aldrich, T9159)
- 3.1.2 Thiobarbituric Acid (TBA) (Sigma-Aldrich, T5500)
- 3.1.3 Malondialdehyde tetraethylacetal (1,1,3,3-Tetraethoxypropane) (MDA) (Sigma-Aldrich, T9889)
- 3.1.4 Diethyl maleate, 97% (DEM) (Aldrich Catalog, D97703-1006)
- 3.1.5 1-Butanol, spectrophotometric grade (Sigma-Aldrich, 154679)
- 3.1.6 RPMI 1640 (Hyclone, SH30096.01)
- 3.1.7 Quick Start Bradford Dye Reagent, 1X (Bio-Rad Lab. Inc., 500-0205)
- 3.1.8 L-glutamine (Hyclone, SH30034.01)
- 3.1.9 Fetal Bovine Serum (FBS) (Hyclone, SH30070.03)
- 3.1.10 Butylated hydroxytoluene (BHT) (Sigma-Aldrich, B1378)
- 3.1.11 Sodium Hydroxide, SigmaUltra (NaOH) (Sigma, S8045)
- 3.1.12 Dulbecco's phosphate buffered saline (PBS), Ca/Mg free (Sigma, D8537)

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 Hep G2 (human hepatocarcinoma) (ATCC, HB-8065)

3.4 Equipment

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

3.4.2 Centrifuge (Microfuge 22R Centrifuge and Allegra X-15R- Beckman Coulter, or equivalent)

4. Reagent and Control Preparation

4.1 Solutions to make up in advance (stable for 2 months at -20˚C)

4.1.1 15% TCA (w/v) (for cell media TBARS):
Add 7.5 g TCA to a total volume of 50 mL in ddw. This can be prepared in advanced and stored at 4˚C.

4.1.2 2.5% TCA (w/v) (for cell media TBARS):
Add 1.25 g TCA to a total volume of 50 mL in ddw. This can be prepared in advanced and stored at 4˚C.

4.2 Solutions to make up prior (use within one day)

4.2.1 DEM Positive Control: prepare 5 mM DEM treatment solution in RPMI 1640 media. Use within 24 hours.

4.2.2 0.67% TBA (w/v) /0.01% BHT (w/v):
Add 0.335 g TBA and 0.005 g BHT to a total volume of 50 mL in ddw. This solution should be used within 24 hours and stored on ice.

4.3 MDA Standard Curve

Note: Media MDA standard curve is diluted in ddw. The cell lysate MDA standard curve is diluted in 2.5% TCA.

4.3.1 (**Standard A**) 400 nmol/mL MDA Standard: *QS* 50 µL of MDA to 500 mL with ice cold ddw (or 2.5% TCA for cell lysate std curve) and vortex

4.3.2 (**Standard B**) 4 nmol/mL MDA Standard: *QS* 1 mL of **Standard A** to 100 mL with ice cold ddw (or 2.5% TCA for cell lysate std curve) and vortex

4.3.3 (**Standard C**) 2 nmol/mL MDA Standard: 1 mL of **Standard B** + 1 mL of ddw (or 2.5% TCA for cell lysate std curve) and vortex

4.3.4 (**Standard D**) 1 nmol/mL MDA Standard: 1 mL of **Standard C** + 1 mL of ddw (or 2.5% TCA for cell lysate std curve) vortex

4.3.5 (**Standard E**) 0.5 nmol/mL MDA Standard: 1 mL of **Standard D** + 1 mL ddw (or 2.5% TCA for cell lysate std curve) vortex

4.3.6 (**Standard F**) 0.25 nmol/mL MDA Standard: 1 mL of **Standard E** + 1 mL ddw (or 2.5% TCA for cell lysate std curve) vortex

4.3.7 (**Standard G**) 0.125 nmol/mL MDA Standard: 1 mL of **Standard F** + 1 mL ddw (or 2.5% TCA for cell lysate std curve) vortex

4.3.8 (**Standard H**) 0.063 nmol/mL MDA Standard: 1 mL of **Standard G** + 1 mL 2.5% TCA (for cell lysate std curve) vortex

4.3.9 (**Standard I**) 0.031 nmol/mL MDA Standard: 1 mL of **Standard H** + 1 mL 2.5% TCA (for cell lysate std curve) vortex

4.3.10 (**Standard J**) 0.015 nmol/mL MDA Standard: 1 mL of **Standard I** + 1 mL 2.5% TCA (for cell lysate std curve) vortex

4.3.11 (**Standard K**) 0.007 nmol/mL MDA Standard: 1 mL of **Standard J** + 1 mL 2.5% TCA (for cell lysate std curve) vortex

4.3.12 (**QC 1)** 0.8 nmol/mL: 1 mL of **Standard B** + 4 mL ddw vortex

4.3.13 (**QC 2)** 0.025 nmol/mL: 1 mL of **Standard G** + 4 mL 2.5% TCA vortex

4.3.14 Prepare standards using the cell media and cell lysate procedures below, Section 5.2.2 and 5.3.5. This is for measurement of MDA equivalents in cell media and cell lysate, respectively.

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). All samples and controls are run in triplicate, 21 wells total (3 hour sample exposure + 3 hour media control + 3 hour positive control, 6 hour sample exposure + 6 hour media control, and 24 hour sample exposure + 24 hour media control) (Appendix A).

5.1.5 Incubate plates for 24 hours at 5% CO_2, 37˚C and 95% humidity (**cells should be approximately 80% confluent**) (Figure 1).

5.1.6 Replace cell culture media with media containing test nanomaterial or positive control. Desired test nanomaterial concentration is determined from Hep G2 Hepatocyte Cytotoxicity Assay (NCL Method GTA-2). Treat cells for designated time period, as described in Section 5.1.4.

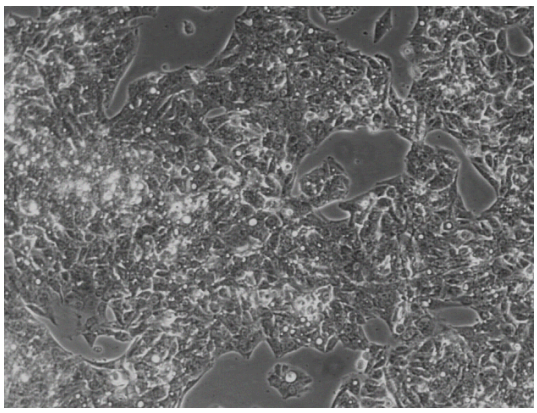

Figure 1. Human hepatocarcinoma (Hep G2) cells

Image was taken with a phase contrast microscope at 200X magnification. Hep G2 cells are approximately 80% confluent at this stage.

5.2 Cell media sample preparation

5.2.1 Collect cell media following treatment.

5.2.2 Add 500 µL of sample media (or appropriate MDA stock for standard curve sample preparation, or blank media) to 400 µL 15% TCA and 800 µL of 0.67% TBA/0.01% BHT in a 5 mL amber vial. Vortex and heat for 20 min in 95˚C water bath. Allow to cool and add 3 mL butanol, gently mix phases, and transfer 200 µL of the butanol phase (top) to 96 well plate as per Appendix B.

5.3 Cell lysate sample preparation

5.3.1 Wash 96 well plates with ice cold PBS.

5.3.2 Scrape cells into 1 mL 2.5% TCA.

5.3.3 Centrifuge cells at 13,000 x g for 2 min.

5.3.4 Retain pellet for determination of cellular protein by Bradford Assay (Section 6); can be frozen at -20˚C until analysis.

5.3.5 Remove 500 µL of lysate supernatant (or appropriate MDA stock for standard curve sample preparation, or blank media) and add to 400 µL 15% TCA and 800 µL of 0.67% TBA/0.01% BHT in a 5 mL amber vial. Vortex and heat for 20 min in 95˚C water bath. Allow to cool and add 3 mL butanol, gently mix phases, and transfer 200 µL of the butanol phase (top) to 96 well plate as per Appendix B.

5.4 Read cell media and cell lysate plate in fluorescence mode, ex. = 530 nm, em. = 550 nm.

6. Protein Determination (Bradford Assay)

6.1 Dilute the 2 mg/mL BSA standard to make a standard curve from 0.125-1.0 mg/mL in 0.05 N NaOH.

6.2 Resuspend pellets from Section 5.3.4 in 0.5 mL of 0.05 N NaOH.

6.3 Add 5 µL of standard, resuspended protein pellet, or 0.05 N NaOH blank to each well of a microtiter plate in duplicate according to the template in Appendix C.

6.4 Add 250 µL of 1X Dye Reagent to each well of the plate. Incubate at room temperature for at least 5 min, but not longer than 1 hour.

6.5 Read on a microtiter plate at 595 nm.

7. Calculations

7.1 TBARS assay concentrations are determined by comparison to an MDA standard curve following linear regression analysis (y = x(slope) + y int), expressed as MDA equivalents and normalized to total protein.

7.2 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 Protein = (mg/mL protein x 0.5 mL).

7.3 Total lysate or media MDA equivalents normalized to total protein =

[(MDA, ng/mL) x 1 mL / total protein, mg] = ng MDA/mg protein

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

8. Acceptance Criteria

8.1 The fold change at 3 hours for the total protein normalized media and lysate DEM positive control versus media negative control MDA equivalents should be at least 2.

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

8.3 The assay is acceptable if condition 8.1 and 8.2 are met. Otherwise, the assay should be repeated until acceptance criteria are met.

8.4 If statistical assays determine 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 effected cellular lipid peroxidation.

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. Adapted from: Dubuisson ML, de Wergifosse B, Trouet A, Baguet F, Marchand-Brynaert J, Rees JF., 2000. Antioxidative properties of natural coelenterazine and

synthetic methyl coelenterazine in rat hepatocytes subjected to tert-butyl hydroperoxide-induced oxidative stress. Biochem. Pharmacol. 60, 471-478.

10. Abbreviations

BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CV	coefficient of variation
ddw	deionized distilled water
DEM	diethyl maleate
em.	emission
ex.	excitation
FBS	fetal bovine serum
Hep G2	human hepatocarcinoma cells
MDA	malondialdehyde
PBS	phosphate buffered saline
QS	*Quantum sufficiat*
RPMI	Roswell Park Memorial Institute
SD	standard deviation
STD	standard
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
w/v	weight to volume ratio

11. Appendices

Appendix A

Example of 6-well plate templates.

	1	2	3
A	Media 3 hr # 1	Media 3 hr # 1	Media 3 hr # 1
B	Sample 3 hr # 1	Sample 3 hr # 1	Sample 3 hr # 1

	1	2	3
A	Positive Control 3 hr # 1	Positive Control 3 hr # 1	Positive Control 3 hr # 1
B			

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

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

All samples are run in triplicate. The following timepoints are recommended: 3 hr (sample, positive control, and media control), 6 hr (sample and media control), and 24 hr (sample and media control) in which case 4 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	Std B 4.0 nmol/mL	Std B 4.0 nmol/mL	Media 3 hr # 1	Media 3 hr # 2	Media 3 hr # 3	Std F 0.25 nmol/mL	Std F 0.25 nmol/mL	Media 3 hr # 1	Media 3 hr # 2	Media 3 hr # 3		Blank Cell Media
B	Std C 2.0 nmol/mL	Std C 2.0 nmol/mL	Sample 3 hr # 1	Sample 3 hr # 2	Sample 3 hr # 3	Std G 0.125 nmol/mL	Std G 0.125 nmol/mL	Sample 3 hr # 1	Sample 3 hr # 2	Sample 3 hr # 3		Blank Cell Media
C	Std D 1.0 nmol/mL	Std D 1.0 nmol/mL	Positive Control 3 hr # 1	Positive Control 3 hr # 2	Positive Control 3 hr # 3	Std H 0.063 nmol/mL	Std H 0.063 nmol/mL	Positive Control 3 hr # 1	Positive Control 3 hr # 2	Positive Control 3 hr # 3		Blank Cell Media
D	Std E 0.5 nmol/mL	Std E 0.5 nmol/mL	Media 6 hr # 1	Media 6 hr # 2	Media 6 hr # 3	Std I 0.031 nmol/mL	Std I 0.031 nmol/mL	Media 6 hr # 1	Media 6 hr # 2	Media 6 hr # 3		Blank Cell Lysate
E	Std F 0.25 nmol/mL	Std F 0.25 nmol/mL	Sample 6 hr # 1	Sample 6 hr # 2	Sample 6 hr # 3	Std J 0.015 nmol/mL	Std J 0.015 nmol/mL	Sample 6 hr # 1	Sample 6 hr # 2	Sample 6 hr # 3		Blank Cell Lysate
F	Std G 0.125 nmol/mL	Std G 0.125 nmol/mL				Std K 0.007 nmol/mL	Std K 0.007 nmol/mL					Blank Cell Lysate
G	QC 1	QC 1				QC 2	QC 2					
H												

Legend: Columns 1-2: Cell Media Standard Curve; Columns 3-5: Cell Media Samples; Columns 6-7: Cell Lysate Standard Curve; Columns 8-10: Cell Lysate Samples; Column 12: Blanks

The 3 hr and 6 hr samples can be run on the same day (3 hr samples sit on ice until ready for analysis). The 24 hr samples will be set up the next day and run in an identical manner.

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 3 hr # 1	Media 3 hr # 2	Media 3 hr # 3	Media 3 hr # 1	Media 3 hr # 2	Media 3 hr # 3				Blank
B	0.75 mg/mL	0.75 mg/mL	Sample 3 hr # 1	Sample 3 hr # 2	Sample 3 hr # 3	Sample 3 hr # 1	Sample 3 hr # 2	Sample 3 hr # 3				Blank
C	0.5 mg/mL	0.5 mg/mL	Positive Control 3 hr # 1	Positive Control 3 hr # 2	Positive Control 3 hr # 3	Positive Control 3 hr # 1	Positive Control 3 hr # 2	Positive Control 3 hr # 3				
D	0.25 mg/mL	0.25 mg/mL	Media 6 hr # 1	Media 6 hr # 2	Media 6 hr # 3	Media 6 hr # 1	Media 6 hr # 2	Media 6 hr # 3				
E	0.125 mg/mL	0.125 mg/mL	Sample 6 hr # 1	Sample 6 hr # 2	Sample 6 hr # 3	Sample 6 hr # 1	Sample 6 hr # 2	Sample 6 hr # 3				
F			Media 24 hr # 1	Media 24 hr # 2	Media 24 hr # 3	Media 24 hr # 1	Media 24 hr # 2	Media 24 hr # 3				
G			Sample 24 hr # 1	Sample 24 hr # 2	Sample 24 hr # 3	Sample 24 hr # 1	Sample 24 hr # 2	Sample 24 hr # 3				
H												

All Samples are run in duplicate.

Legend: Columns 1-2: BSA Standard Curve; Columns 3-5: Samples; Columns 6-8: Duplicate of Samples; Column 12: Blanks