NANOTECHNOLOGY CHARACTERIZATION LABORATORY

NCL Method GTA-4
Version 1.0

HEP G2 Hepatocyte Lipid Peroxidation Assay

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February 2006

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.

Method is written by _____ 8/18/06.

Stephan T. Stern, Ph.D. Date

_____ 8/21/06.

Timothy M. Potter, B.S. Date

_____ 8-21-06.

Barry W. Neun, B.S. Date

Method validation was conducted on: 2-6-06.

Date

Validation report is complete on: 2-6-06.

Date

Testing facility: NCL, NCI-Frederick, Bldg. 469, Room 250.

1. Introduction

1.1 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.

2. Principles

2.1 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.521nm and em. 552nm. TBARS are expressed as MDA equivalents (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.)

3. Required Materials and Equipment

3.1 Materials:

3.1.1 Trichloroacetic Acid (TCA) (Sigma-Aldrich Cat. # T 9159)

3.1.2 Thiobarbituric Acid (TBA) (Sigma-Aldrich Cat. # T 5500)

3.1.3 Malondialdehyde tetraethylacetal (1,1,3,3 Tetraethoxypropane) (MDA) (Sigma-Aldrich Cat. # T9889)

3.1.4 Diethyl maleate, 97% (DEM) (Aldrich Catalog #D97703-1006)

3.1.5 1-Butanol, Spec. grade (Sigma-Aldrich Cat. # 154679)

3.1.6 RPMI 1640 (Hyclone # SH30096.01)

3.1.7 Costar 6 well flat bottom cell culture plates, (Costar, Cat. No. 3506)

3.1.8 Quick Start Bradford Dye Reagent, 1X (Bio-Rad Lab., Inc., Cat. #500-0205)

3.1.9 L-glutamine (Hyclone #SH30034.01)

3.1.10 Fetal Bovine Serum (Hyclone SH30070.03)

3.2 Cell Lines

3.2.1 Hep G2 (human hepatocarcinoma) (ATCC # HB-8065)

3.3 Equipment

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

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

Note: The NCL does not endorse any of the material/instrument suppliers listed. Their inclusion is for informational purposes only.

4. Reagent and Control Preparation

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

4.1.1 DEM Positive Control: prepare 5 mM DEM treatment solution in RPMI 1640.

4.1.2 15% TCA (w/v) (for cell media TBARS):

Add 7.5 TCA to a total volume of 50 mL in DDW

4.1.3 2.5% TCA (w/v) (for cell media TBARS):

Add 1.25 g TCA to a total volume of 50 mL in DDW

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

4.2.1 0.67% TBA (w/v):

Add 0.335 g TBA to a total volume of 50 mL in DDW

4.3 MDA Standard Curve

Note: Media MDA Std. curve is diluted in ddw, the cell lysate MDA std. curve is diluted in 2.5% TCA

4.3.1 (**Stock A**) 400 nmol/mL MDA Stock: *QS* 50 µL of MDA to 500 mL with ice cold ddw and vortex

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

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

4.3.4 (**Stock D**) 1 nmol/mL STD: 1 mL of **Stock C** + 1 mL of dddw (or 2.5% TCA for cell lysate std. curve) vortex

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

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

4.3.7 (**Stock G**) 0.125 nmol/mL STD: 1 mL of **Stock F** + 1 mL DDW (or 2.5% TCA for cell lysate std. curve) vortex

4.3.8 (**Stock H**) 0.063 nmol/mL STD: 1 mL of **Stock G** + 1 mL DDW (or 2.5% TCA for cell lysate std. curve) vortex

4.3.9 (**Stock I**) 0.031 nmol/mL STD: 1 mL of **Stock H** + 1 mL DDW (or 2.5% TCA for cell lysate std. curve) vortex

4.3.10 (**Stock J**) 0.015 nmol/mL STD: 1 mL of **Stock I** + 1 mL DDW (2 or.5% TCA for cell lysate std. curve) vortex

4.3.11 (**Stock K**) 0.007 nmol/mL STD: 1 mL of **Stock J** + 1 mL DDW (or 2.5% TCA for cell lysate std. curve) vortex

4.3.12 (**QC 1)** 0.8 nmol/mL: 1 mLof **Stock B** + 4 mL DDW vortex

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

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

5. Experimental Procedure

5.1 Cell Preparation

5.1.1 Cell Preparation (or as recommended by supplier)

5.1.2 Harvest cryopreserved cells from prepared flasks **(limit to 20 passages)**.

5.1.3 Count cell concentration using a coulter counter or hemocytometer.

5.1.4 Dilute cells to a density of 7.5×10^5 cells/mL in RPMI 1640 cell culture media (2mM L-glutamine, 10% FBS).

5.1.5 Plate 2 mL of diluted cells to each well of a 6-well plate (1.5×10^6 cells/ well). Test samples and controls are run in triplicate, 24 wells total (time zero, 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).

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

5.1.7 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.

5.2 Cell media sample preparation

5.2.1 Collect media.

5.2.2 Add 500 μL of media to 400 μL 15% TCA and 800 μL of 0.67% TBA. Vortex and heat for 20 min in 95ºC water bath. Add 3 mL butanol, mix and transfer 200 μL to 96 well plate as per Appendix A.

5.3 Cell lysate sample preparation

5.3.1 Wash 6 well plates with ice cold PBS.

5.3.2 Scrape cells into 2 mL 2.5% TCA.

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

5.3.4 Retain pellet for determination of cellular protein by Bradford Assay. Can be frozen at -20ºC until analysis.

5.3.5 Remove 500 μL of supernatant and add to 400 μL 15% TCA and 800 μL of 0.67% TBA. Vortex and heat for 20 min in 95ºC water bath. Add 3 mL butanol, mix and transfer 200 μL to 96 well plate as per Appendix A.

5.3.6 Read plate in fluorescence mode @ ex.= 530 nm, em.=550nm using the TBAR method.

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 0.5 N NaOH.Resuspend pellets in 0.5 mL of 0.5 N NaOH. Add 5 μL of standard, resuspended protein pellet, or water blank to each well of a microtiter plate in duplicate according the template in appendix B. Add 250 μL

of 1X Dye Reagent to each well of the plate. Incubate at room temperature for at least 5 min and not longer than 1 hr. 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 2 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 and sample replicate coefficient of variations should be within 50%.

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

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.

References

ISO 10993-5 Biological evaluation of medical devices: Part 5 Tests for *in vitro* cytotoxicity.

F1903 – 98 Standard Practice for Testing for Biological Responses to Particles *in vitro*.

Appendix A

96-Costar Well Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	4.0 nmoles/mL	4.0 nmoles/mL	Smple 1A	Smple 1B	Smple 1C	0.25 nmoles/mL	0.25 nmoles/mL	Smple 1A	Smple 1B	Smple 1C		Blank Cell Media
B	2.0 nmoles/mL	2.0 nmoles/mL	Smple 2A	Smple 2B	Smple 2C	0.125 nmoles/mL	0.125 nmoles/mL	Smple 2A	Smple 2B	Smple 2C		Blank Cell Media
C	1.0 nmoles/mL	1.0 nmoles/mL	Smple 3A	Smple 3B	Smple 3C	0.063 nmoles/mL	0.063 nmoles/mL	Smple 3A	Smple 3B	Smple 3C		Blank Cell Media
D	0.5 nmoles/mL	0.5 nmoles/mL	Smple 4A	Smple 4B	Smple 4C	0.031 nmoles/mL	0.031 nmoles/mL	Smple 4A	Smple 4B	Smple 4C		Blank Cell Lysate
E	0.25 nmoles/mL	0.25 nmoles/mL	Smple 5A	Smple 5B	Smple 5C	0.016 nmoles/mL	0.016 nmoles/mL	Smple 5A	Smple 5B	Smple 5C		Blank Cell Lysate
F	0.125 nmoles/mL	0.125 nmoles/mL	Smple 6A	Smple 6B	Smple 6C	0.007 nmoles/mL	0.007 nmoles/mL	Smple 6A	Smple 6B	Smple 6C		Blank Cell Lysate
G	QC 1	QC 1	Smple 7A	Smple 7B	Smple 7C	QC 2	QC 2	Smple 7A	Smple 7B	Smple 7C		
H			Smple 8A	Smple 8B	Smple 8C			Smple 8A	Smple 8B	Smple 8C		

Legend: Columns (1-2): Cell Media Std. Curve, Columns (3-5): Cell Media Samples, Columns (6-7): Cell Lysate Std. Curve, Columns (8-10): Cell Lysate Samples, Columns (12): Blanks

Appendix C.
Example of Hep G2 Cell Culture Appearance.

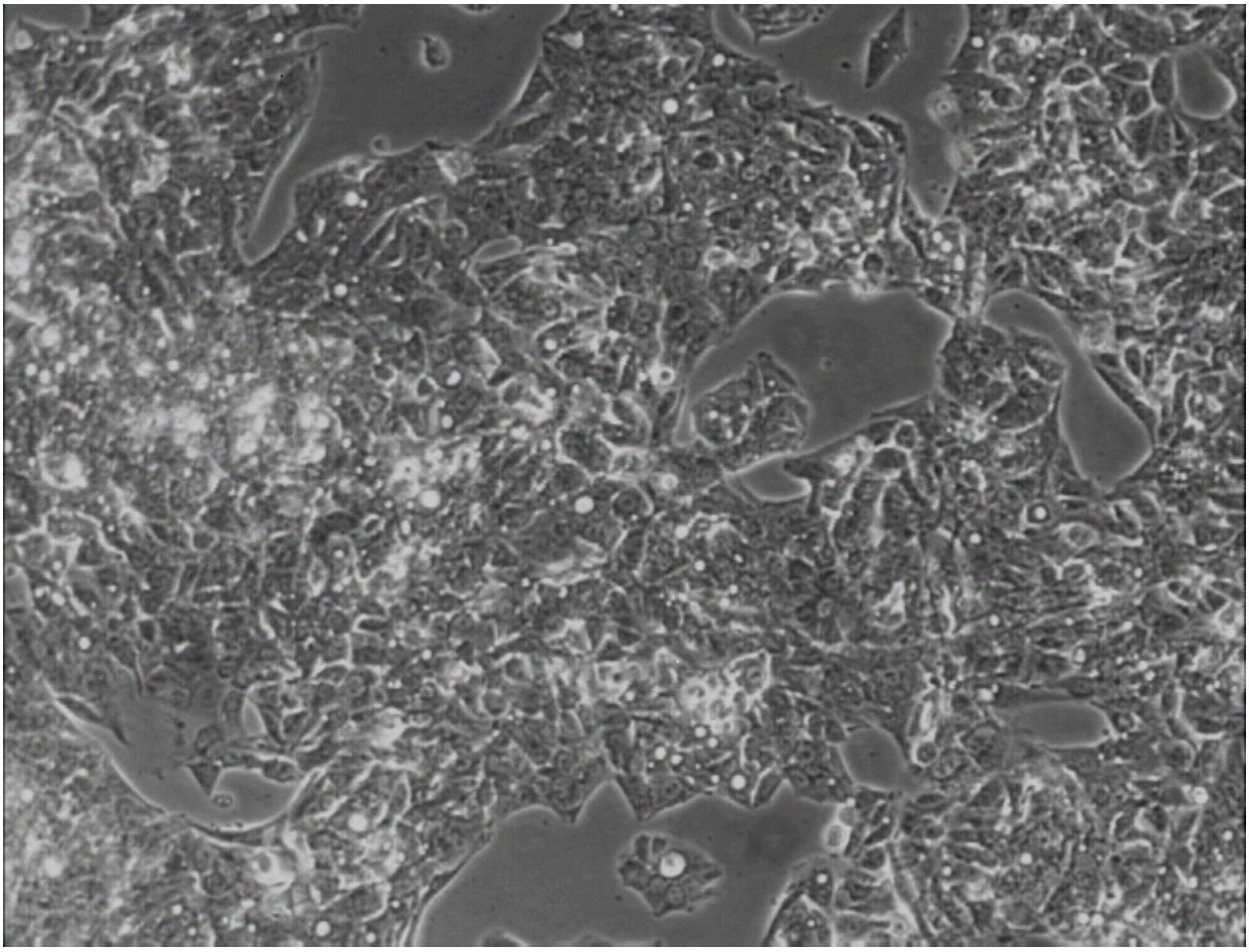

Image was taken with a phase contrast microscope at 200x magnification. human hepatocarcinoma cells (HEP G2) are approximately 80% confluent at this stage.