NANOTECHNOLOGY CHARACTERIZATION LABORATORY

NCL Method GTA-3
Version 1.0

HEP G2 Hepatocyte Glutathione Assay

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May 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.

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Method validation was conducted on: 5/10/06 Date

Validation report is complete on: 5/10/06 Date

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

1. Introduction

This protocol describes the analysis of human hepatocarcinoma cells (Hep G2) for reduced and oxidized glutathione, following treatment with nanoparticle formulations, as part of the NCL *in vitro* preclinical characterization cascade. A shift in the ratio of reduced to oxidized glutathione is indicative of oxidative stress, while a decrease in the overall GSH pool is indicative of conjugative metabolism or impaired synthesis (Deleve L.D., Kaplowitz N., 1991. Glutathione metabolism and its role in hepatotoxicity. Pharmacol. Ther. 52, 287-305). This protocol utilizes a colorimetric method for glutathione determination.

2. Principles

2.1 Reduced glutathione (GSH) interacts with 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB) to form the colored product 2-nitro-5-thiobenzoic acid, which is measured at 415 nm, and oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase to form reduced glutathione GSH, which is again measured by the preceding method. Preincubation of the sample with the thiol masking agent 1-Methyl-4vinyl-pyridinium prevents measurement of GSH, resulting in measurement of GSSG only (I. H. Shaik and R. Mehvar. Rapid determination of reduced and oxidized glutathione levels using a new thiol-masking reagent and the enzymatic recycling method: application to the rat liver and bile samples. *Anal Bioanal Chem* 1618-2642 (March 18, 2006 (Published Online))).

3. Required Materials and Equipment

3.1 Materials

3.1.1 5-5’-Dithiobis(2-nitrobenzoic acid) (**DTNB**) (Sigma # D8130)

3.1.2 β-Nicotinamide adenine dinucleotide 2’-phophate reduced tetrasodium salt (**NADPH**) (Sigma # N7505)

3.1.3 Ethylenediaminetetraacetic acid tetrasodium salt dihydrate (**EDTA**) (Sigma #ED4SS)

3.1.4 5-Sulfosalicylic acid dihydrate (**SSA**) (Sigma # S7422)

3.1.5 Sodium phosphate (Sigma #342483)

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

3.1.7 RPMI 1640 (Hyclone # SH30096.01)

3.1.8 Costar 6 well flat bottom cell culture plates, Cat. No. 3527

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

3.1.10 Sodium Carbonate, SigmaUltra (Sigma # S7795)

3.1.11 1-Methyl-4vinyl-pyridinium (OXIS International, Inc. # 26513)

3.1.12 Oxidized Glutathione (GSSG), SigmaUltra (Sigma # G6654)

3.1.13 L-glutamine (Hyclone #SH30034.01)

3.1.14 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)

3.4 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 above, their inclusion is for informational purposes only.

4. Reagent and Control Preparation

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

4.2 DEM Positive Control: Prepare 0.5 mM DEM treatment solution in RPMI 1640.

4.3 Phosphate-EDTA Dilution Buffer (100 mM Na_3PO_4 – 1 mM EDTA, pH 7.4):
QS 8.2 g of sodium phosphate and 208 mg of EDTA to 500 mL with ddw. Adjust pH to 7.4.

4.4 10 ng/µL GSSG Standard:

(A) *QS* 10 mg GSSG to 10 mL in 5% SSA

(B) Add 990 µL of to 5% SSA 10 uL A

4.5 400 mM Sodium Carbonate:

QS 21 g to 500 mL ddw (400 mM sodium carbonateSolution)

Dilute 1:2 with 5% SSA (200mM sodium carbonate-2.5% SSA for GSSG curve)

5. Solutions to make up prior (Use within 1 day)

5.1 Reaction buffer (1.9 units/mL glutathione reductase, 0.4 mM NADPH):

QS 38 units of glutathione reductase and 7 mg NADPH in 20 mL in Phosphate Dilution Buffer

5.2 DTNB Substrate (4.5 mM):

QS 9 mg DTNB to 5 ml in Phosphate-EDTA Dilution buffer.

5.3 SSA (5%):

QS 500 mg SSA to 10 mL with ddw to make 5% solution.

5.4 0.5 mM 1-Methyl-4vinyl-pyridinium (M4VP) GSH masking reagent:

QS 7 mg M4VP in 5 mL 5% SSA

1:10 dilution in 5% SSA

6. GSH Standards

6.1 GSH Assay Std. Curve (Use within 1 day)

Add 50, 40, 30, 20, 10 and 5 μL of the 10 ng/μL GSSG stock to micro-eppendorf tubes and *QS* to 100 μL w/200 mM sodium carbonate-2.5% SSA buffer (to generate 5, 4, 3, 2, 1 and 0.5 ng/μL GSH standards, respectively).

6.2 GSSG QC Standard Preparation (8 ng/mL GSSG + 8 ng/mL GSH)

(A) *QS* 10 mg GSH and 10 mg GSSG to 10 mL in 5% SSA (1 μg/μL)

(B) Dilute A 1:12.5 in 5% SSA (80 ng/μL)

(C) Dilute B 1:10 in 5% SSA (8 ng/μL)

6.3 Total GSH QC Standard Preparation (3 ng/mL GSSG)

(A) *QS* 10 mg GSSG to 10 mL in 200mM sodium carbonate-2.5% SSA (1 μg/μL)

(B) Dilute A 1:30 in 200mM sodium carbonate-2.5% SSA (30 ng/μL)

(C) Dilute B 1:10 in 200mM sodium carbonate-2.5% SSA (3 ng/μL)

7. Experimental Procedure

7.1 Cell Preparation (or as recommended by supplier)

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

7.1.2 Count cell concentration using a coulter counter or hemocytometer.

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

7.1.4 Plate 2 mL of diluted cells(1.5 x 10^6 cells/ well) to each well of a 6-well plate. Test samples and positive controls are run in duplicate, 14 wells total (time zero, 3 hour sample exposure + media control, 6 hour sample exposure + control, and 24 hour sample exposure + media control).

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

7.1.6 Replace cell culture media with media containing test nanomaterial or positive control. Desired test nonmaterial concentration is determined from HEP G2 Hepatocyte Cytotoxicity Assay (NCL Method GTA-2). Treat cells for designated time period.

7.1.7 Wash well with 1 ml of room temp. PBS.

7.1.8 Remove PBS, add 100 μL ice-cold 5% SSA to the plate and scrape cells. Transfer lysed cells to 0.6 mL eppendorf tubes and incubate for 10 min on ice. Centrifuge @ 8000 x g for 5 min.

7.1.9 Total GSH Assay: Dilute 5 μL of supernatant 1:2 with 5% SSA dilution, further dilute to 1:2 with 400mM sodium carbonate, then further dilute 1:8 with Phosphate-EDTA Dilution Buffer (total dilution 1:32).

7.1.10 GSSG Assay: Dilute 5 μL of supernatant 1:2 with 5μL of M4VP masking reagent in eppendorf tube, then further dilute to 1:2 with 400 mM sodium carbonate (total dilution 1:4).

7.1.11 Incubate GSSG assay samples for 2 min at room temperature.

7.1.12 Transfer supernatant for assay according to template in Appendix A.

7.1.13 Retain pellet for determination of cellular protein by Bradford Assay. Pellet can be frozen at -20ºC until analysis.

7.2 Glutathione Assay Protocol

7.2.1 Add 170 μL of reaction mixture to each standard and sample well of a 96 well plate (see Appendix A). Incubate for 10 min at RT

7.2.2 Add 20 µL of GSH standard, QC 2.0 GSSG-2.0 GSH ng/uL Std., sample or blank to each well.
Incubate for 10 min at RT

7.2.3 Add 10 µL of DTNB Substrate Solution to each well. Incubate for 5 to 10 min @ room temperature.

7.2.4 Read absorbance at 415 nm on a microplate reader.

7.3 Protein Determination (Bradford Assay)

7.3.1 Dilute the 2 mg/mL BSA standard to make a standard curve from 0.125-1.0 mg/mL.

7.3.2 Resuspend pellets in 0.5 mL of PBS.

7.3.3 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.

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

7.3.5 Incubate at room temperature for at least 5 min and not longer than 1 hr.

7.3.6 Read on a microtiter plate at 595 nm.

8. Calculations

Assay total and reduced GSH concentrations are determined from their respective GSH standard curves following linear regression analysis (y=x(slope) + y int) and then normalized to total protein.

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).

9. Acceptance Criteria

9.1 The protein normalized total GSH concentration of the DEM positive control should be at least 40 % lower than the media control at 6 hours.

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

9.3 The assay is acceptable if condition 9.1 and 9.2 are met. Otherwise, the assay should be repeated until acceptance criteria are met.

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	0.5 ng/µL	0.5 ng/µL	Sample 1A	Sample 1B	Sample 9A	Sample 9B	Sample 17A	Sample 17B				
B	1 ng/µL	1 ng/µL	Sample 2A	Sample 2B	Sample 10A	Sample 10B	Sample 18A	Sample 18B				
C	2 ng/µL	2 ng/µL	Sample 3A	Sample 3B	Sample 11A	Sample 11B	Sample 19A	Sample 19B				
D	3 ng/µL	3 ng/µL	Sample 4A	Sample 4B	Sample 12A	Sample 12B	Sample 20A	Sample 20B				
E	4 ng/µL	4 ng/µL	Sample 5A	Sample 5B	Sample 13A	Sample 13B	Sample 21A	Sample 21B				
F	5 ng/µL	5 ng/µL	Sample 6A	Sample 6B	Sample 14A	Sample 14B	Sample 22A	Sample 22B				
G	QC	QC	Sample 7A	Sample 7B	Sample 15A	Sample 15B	Sample 23A	Sample 23B				
H	QC+ M4VPP	QC+ M4VP	Sample 8A	Sample 8B	Sample 16A	Sample 16B	Sample 24A	Sample 24B		Blank	Blank	Blank

Legend: Columns (1-2): Total GSSG Std. Curve and quality control samples; Columns (3-8): Samples; Columns (10-12): Blanks

Appendix B
96-Costar Well Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 mg/mL	1 mg/mL	Sample 1A	Sample 1B	Sample 9A	Sample 9B						
B	0.75 mg/mL	0.75 mg/mL	Sample 2A	Sample 2B	Sample 10A	Sample 10B						
C	0.5 mg/mL	0.5 mg/mL	Sample 3A	Sample 3B	Sample 11A	Sample 11B						
D	0.25 mg/mL	0.25 mg/mL	Sample 4A	Sample 4B	Sample 12A	Sample 12B						
E	0.125 mg/mL	0.125 mg/mL	Sample 5A	Sample 5B	Sample 13A	Sample 13B						
F			Sample 6A	Sample 6B	Sample 14A	Sample 14B						
G			Sample 7A	Sample 7B	Sample 15A	Sample 15B						
H			Sample 8A	Sample 8B	Sample 16A	Sample 16B					Blank	Blank

Legend: Columns (1-2): BSA Std. Curve, Columns (3-6): Samples, Columns (10-12): Blanks

Appendix C.

Example of human hepatocarcinoma cells (HEP G2) cell culture appearance.

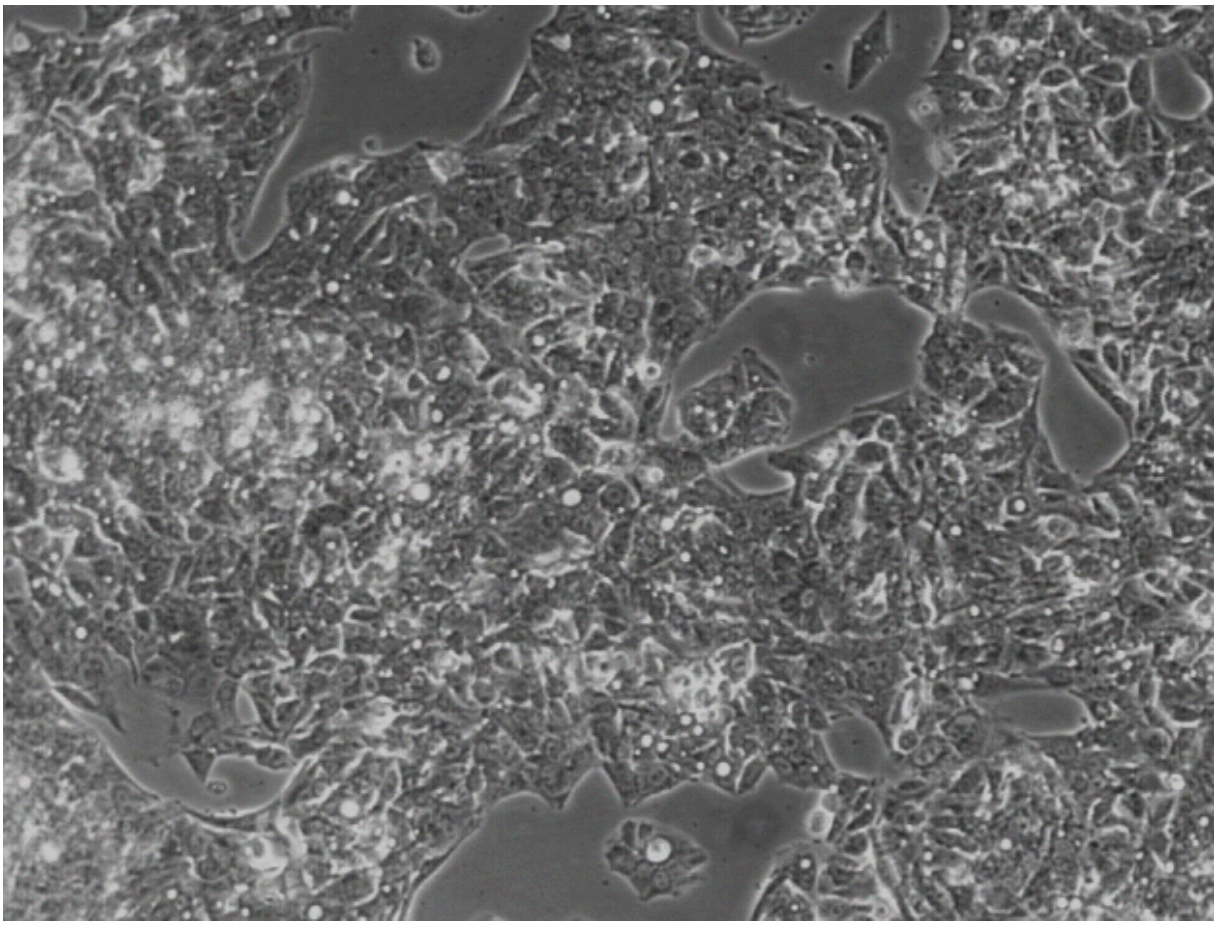

Image was taken with a phase contrast microscope at 200x magnification. Human hepatocarcinoma cells (HEP G2) are approximately 70% confluent at this stage.