



NCL Method GTA-3

Hep G2 Hepatocyte Glutathione 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 reduced and oxidized glutathione, following treatment with nanoparticle formulations, as part of the NCL *in vitro* preclinical characterization cascade. A shift from reduced to oxidized glutathione is indicative of oxidative stress, while a decrease in the overall reduced glutathione pool is indicative of conjugative metabolism or impaired synthesis (1). This protocol utilizes a colorimetric method for glutathione determination.

2. Principles

Reduced glutathione (GSH) reduces with 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB) to form the colored product 2-nitro-5-thiobenzoic acid, which is measured kinetically 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-4-vinyl-pyridinium prevents measurement of GSH, resulting in measurement of GSSG only (2).

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 5-5'-Dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma, D8130)
- 3.1.2 β -Nicotinamide adenine dinucleotide 2'-phosphate 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)

- 3.1.9 Quick Start Bradford Dye Reagent, 1X (Bio-Rad Lab., Inc., 500-0205)
- 3.1.10 Sodium Carbonate, SigmaUltra (Sigma, S7795)
- 3.1.11 1-Methyl-4-vinyl-pyridinium (M4VP) (Abcam, ab145627)
- 3.1.12 Oxidized Glutathione (GSSG), SigmaUltra (Sigma, G6654)
- 3.1.13 Reduced Glutathione (GSH), SigmaUltra (Sigma, G6529)
- 3.1.14 L-glutamine (Hyclone, SH30034.01)
- 3.1.15 Fetal Bovine Serum (Hyclone, SH30070.03)
- 3.1.16 Sodium Hydroxide, SigmaUltra (Sigma, S8045)
- 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 (Safire²–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)
 - 4.1.2 DEM Positive Control: Prepare 0.5 mM DEM treatment solution in RPMI 1640.
 - 4.1.3 Phosphate-EDTA Dilution Buffer (100 mM Na₃PO₄ – 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.
 - 4.1.4 10 ng/μL GSSG Standard:
 - (A) *QS* 10 mg GSSG to 10 mL using 5% SSA
 - (B) Add 990 μL of 5% SSA to 10 μL of (A)
 - 4.1.5 400 mM Sodium Carbonate:
 - (A) *QS* 21 g sodium carbonate to 500 mL with ddw (400 mM sodium carbonate solution)

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

4.2 Solutions to make up prior (Use within 1 day)

4.2.1 Reaction Buffer (1.9 units glutathione reductase/mL, 0.4 mM NADPH):
QS 38 units of glutathione reductase and 7 mg NADPH to 20 mL in Phosphate-EDTA Dilution Buffer (from step 4.1.3)

4.2.2 DTNB Substrate (4.5 mM):
QS 9 mg DTNB to 5 mL in Phosphate-EDTA Dilution buffer (from step 4.1.3).

4.2.3 SSA (5%):
QS 500 mg SSA to 10 mL with ddw to make a 5% solution.

4.2.4 0.5 mM 1-Methyl-4-vinyl-pyridinium (M4VP) GSH masking reagent:
(A) *QS* 7 mg M4VP to 5 mL with 5% SSA
(B) Dilute 1:10 with 5% SSA

4.3 GSH Standards

4.3.1 GSH Assay Standard Curve (Use within 1 day)
Add 50, 40, 30, 20, 10 and 5 μ L of the 10 ng/ μ L GSSG stock (step 4.1.4) to micro-ependorf tubes and *QS* to 100 μ L with 200 mM sodium carbonate-2.5% SSA buffer (to generate 5, 4, 3, 2, 1 and 0.5 ng/ μ L GSH standards, respectively).

4.3.2 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 μ g/ μ L)
(B) Dilute A 1:12.5 in 5% SSA (80 ng/mL GSSG + 80 ng/mL GSH)
(C) Dilute B 1:10 in 5% SSA (8 ng/mL GSSG + 8 ng/mL GSH)
(This standard is treated with M4VP in parallel with samples in step 5.1.10)

4.3.3 Total GSH QC Standard Preparation (3 ng/mL GSSG)
(A) *QS* 10 mg GSSG to 10 mL in 200 mM sodium carbonate-2.5% SSA (1 μ g/ μ L)
(B) Dilute A 1:30 in 200 mM sodium carbonate-2.5% SSA (30 ng/ μ L)
(C) Dilute B 1:10 in 200 mM sodium carbonate-2.5% SSA (3 ng/ μ L)

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**).
 - 5.1.2 Count cell concentration using a coulter counter or hemocytometer.
 - 5.1.3 Dilute cells to a density of 7.5×10^5 cells/mL in RPMI 1640 cell culture media (2 mM L-glutamine, 10% FBS).
 - 5.1.4 Plate 2 mL of diluted cells (1.5×10^6 cells/ well) to each well of a 6-well plate. Test samples and positive controls are run in triplicate, 21 wells total (3 hour sample exposure + media control, 6 hour sample exposure + media control + DEM positive control, and 24 hour sample exposure + media control; see Appendix A).
 - 5.1.5 Incubate plates for 24 hours at 5% CO₂, 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.
 - 5.1.7 Remove media and wash well with 1 mL of room temperature PBS.
 - 5.1.8 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 at 8000 x g for 5 min. Prepare supernatants as described below. Retain pellet for determination of cellular protein by Bradford Assay (Section 6). Pellet can be frozen at -20°C until analysis.
 - 5.1.9 Total GSH Assay: Dilute 5 µL of supernatant 1:2 with 5% SSA dilution, further dilute to 1:2 with 400 mM sodium carbonate, then further dilute 1:8 with Phosphate-EDTA Dilution Buffer (total dilution 1:32). Transfer supernatant to 96-well plate as depicted in Appendix B.
 - 5.1.10 GSSG Assay: Dilute 10 µL of supernatant 1:2 with 10 µL of M4VP masking reagent in eppendorf tube, then further dilute to 1:2 with 400 mM sodium carbonate (total dilution 1:4). Incubate samples for 2 min at room temperature. GSSG QC Standard (step 4.3.2) is treated in the same manner

as the samples; total dilution will be 1:4. Transfer samples to 96-well plate as depicted in Appendix B.

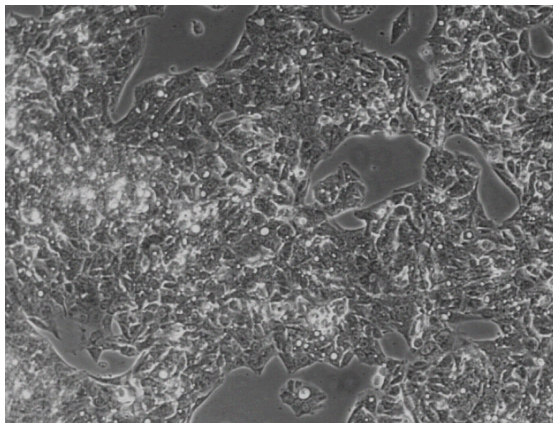


Figure 1. Human hepatocarcinoma (Hep G2) cells

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

5.2 Glutathione Assay Protocol

5.2.1 Add 170 μL of reaction buffer (step 4.2.1) to each standard and sample well of a 96 well plate (Appendix B). Incubate for 10 min at RT.

5.2.2 Add 20 μL of each GSH standard (step 4.3.1), total GSH QC Standard (step 4.3.3), GSSG QC Standard (step 4.3.2), sample (steps 5.1.9 and 5.1.10), DEM positive control, or blank to each well according to plate template in Appendix B. Mix and incubate for 10 min at RT.

5.2.3 Add 10 μL of DTNB Substrate Solution to each well. Vortex.

5.2.4 Read 415 nm absorbance kinetically, 5 cycles at 300 second intervals on a microplate reader.

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 step 5.1.8) in 0.5 mL of 0.05 N NaOH.

- 6.3 Add 5 μ L of BSA standard, resuspended protein pellet, or 0.05 N NaOH blank to each well of a microtiter plate in duplicate according the template in Appendix C. (Each sample is analyzed in duplicate.)
- 6.4 Add 250 μ L of 1X Dye Reagent to each well of the 96-well plate and mix.
- 6.5 Incubate at room temperature for at least 5 min and not longer than 1 h.
- 6.6 Read on a microtiter plate at 595 nm.

7. Calculations

- 7.1 Assay total GSH and GSSG concentrations are determined from their respective GSH standard curves following linear regression analysis ($y = x(\text{slope}) + y \text{ int}$) and then normalized to total protein.
- 7.2 Protein concentration is determined from the BSA standard curve following linear regression analysis ($y = x(\text{slope}) + y \text{ int}$). Total protein is determined from the equation:

$$\text{Total protein (mg)} = \left(\frac{\text{mg protein}}{\text{mL}} \right) * 0.05\text{mL}$$

8. Acceptance Criteria

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

9. References

1. Deleve L.D., Kaplowitz N. (1991) *Pharmacol Ther* 52, 287-305.
2. Shaik, I. H. and Mehvar, R. (2006) *Anal Bioanal Chem* 385, 105-113.

10. Abbreviations

BSA bovine serum albumin

ddw	deionized distilled water
DEM	Diethyl maleate
DTNB	5-5'-Dithiobis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid tetrasodium salt dihydrate
FBS	fetal bovine serum
GSH	reduced glutathione
GSSG	oxidized glutathione
Hep G2	human hepatocarcinoma cells
M4VP	1-Methyl-4vinyl-pyridinium
NADPH	β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt
<i>QS</i>	<i>Quantum sufficiat</i>
PBS	phosphate buffered saline
RPMI	Roswell Park Memorial Institute
SSA	5-Sulfosalicylic acid dehydrate

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	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	Positive Control 6 hr #1	Positive Control 6 hr #1	Positive Control 6 hr #1
B			

	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 and media control), 6 hr (sample, positive control, and media control), and 24 hr (sample and media control) in which case 5 plates will be necessary.

Appendix B

Example of 96-well plate template.

			GSH			GSSG						
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.5 ng/ μ L	0.5 ng/ μ L	Media 3 hr #1	Media 3 hr #2	Media 3 hr #3	Media 3 hr #1	Media 3 hr #2	Media 3 hr #3				
B	1 ng/ μ L	1 ng/ μ L	Sample 3 hr #1	Sample 3 hr #2	Sample 3 hr #3	Sample 3 hr #1	Sample 3 hr #2	Sample 3 hr #3				
C	2 ng/ μ L	2 ng/ μ L	Positive Control 6 hr #1	Positive Control 6 hr #2	Positive Control 6 hr #3	Positive Control 6 hr #1	Positive Control 6 hr #2	Positive Control 6 hr #3				
D	3 ng/ μ L	3 ng/ μ L	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	4 ng/ μ L	4 ng/ μ L	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	5 ng/ μ L	5 ng/ μ L										
G	GSH QC	GSH QC										
H	GSSG QC+ M4VP	GSSG QC+ M4VP									Blank	Blank

96-well Costar Plate Template. Legend: Columns (1-2): Total GSSG Std. Curve and quality control samples; Columns (3-5): Samples for total GSH Assay; Columns (6-8): Samples for GSSG Assay; Column (11-12): 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 96-well plate template.

	1	2	Set A			Set B			9	10	11	12
	1	2	3	4	5	6	7	8				
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				
B	0.75 mg/mL	0.75 mg/mL	Sample 3 h #1r	Sample 3 hr #2	Sample 3 hr #3	Sample 3 hr #1	Sample 3 hr #2	Sample 3 hr #3				
C	0.5 mg/mL	0.5 mg/mL	Positive Control 6 hr #1	Positive Control 6 hr #2	Positive Control 6 hr #3	Positive Control 6 hr #1	Positive Control 6 hr #2	Positive Control 6 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 6 hr #1	Media 6 hr #2	Media 6 hr #3	Media 6 hr #1	Media 6 hr #2	Media 6 hr #3				
G			Sample 6 hr #1	Sample 6 hr #2	Sample 6 hr #3	Sample 6 hr #1	Sample 6 hr #2	Sample 6 hr #3				
H											Blank	Blank

96-well Costar Plate Template. Legend: Columns (1-2): BSA Std. Curve, Columns (3-8): Samples, Columns (11-12): Blanks.

All samples are run in duplicate.