NCL Method GTA-7

Hepatocyte Primary ROS 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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Protocol adapted from:

Zolnik, B.; Potter, T. M.; and Stern, S. T., Detecting reactive oxygen species in primary hepatocytes treated with nanoparticles, in *Characterization of nanoparticles intended for drug delivery*, S. McNeil, Editor. **2011**, Humana Press. p. 173-179. doi: 10.1007/978-1-60327-198-1_18

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1. Introduction

This protocol describes the testing of nanoparticle formulations for reactive oxygen species (ROS) generation in male Sprague-Dawley (SD) primary hepatocytes, as part of the *in vitro* NCL preclinical characterization cascade. The protocol utilizes a fluorescent redox active probe. Primary hepatocytes were chosen since they have a greater metabolic activity than hepatocyte cell lines.

2. Principles

Dichlorofluorescein diacetate (DCFH-DA) is a ROS probe that undergoes intracellular deacetylation, followed by ROS mediated oxidation to a fluorescent species (ex. 485 nm and em. 530 nm). DCFH-DA can be used to measure ROS generation in the cytoplasm and cellular organelles, such as the mitochondria. Fluorescence intensity is quantified in a microplate spectrophotometer **(1)**.

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 2',7'-Dichlorodihydrofluoroscein Diacetate (DCFH-DA) (Molecular Probes, D399)
- 3.1.2 Dimethyl sulfoxide (DMSO) (Aldrich, 154938)
- 3.1.3 HyQ Phosphate Buffered Saline (PBS) (1X) (HyClone, SH30256.01)
- 3.1.4 Diethyl maleate, 97% (DEM) (Aldrich, D97703-1006)
- 3.1.5 Williams Media E (Sigma, W1878)
- 3.1.6 L-glutamine (HyClone, SH30034.01)
- 3.1.7 Penicillin/Streptomycin (Invitrogen, 15140-122)
- 3.1.8 Fetal bovine serum (FBS) (HyClone, SH30070.03)
- 3.1.9 Insulin (Sigma, I-1882)
- 3.1.10 Dexamethasone (Sigma, D4902)

3.1.11 ITS + Premix (insulin, human transferrin, and selenous acid) (BD Biosciences, 354352)

3.2 Materials

3.2.1 Black Costar 96 well plates (Sigma, CLS3603)

3.3 Cell Lines

3.3.1 Cryopreserved Male Sprague-Dawley primary heptocytes (Cellzdirect, RTCH-M).

3.4 Equipment

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

3.4.2 Centrifuge set at 70 x g (Microfuge 22R Centrifuge-Beckman Coulter)

4. Reagent and Control Preparation (Prepare immediately prior to use)

4.1 DEM Positive Control: prepare 5 mM DEM treatment solution in William's Medium E Maintenance Media (described in Section 5.1.2).

4.2 ROS Fluorescent Probe reagent (Prepare in dark room, protect solutions from light!)

4.2.1 DCFH-DA Stock (10 mM): 5 mg in 1 mL of DMSO.

4.2.2 DCFH-DA Working Stock (40 µM): *QS* 200 µL of 10 mM Stock to 50 mL in PBS buffer.

5. Experimental Procedure

5.1 Prepare the two required media for the hepatocytes, as follows:

5.1.1 Thaw Media:

5.1.1.1 Add 100 µL of insulin stock (4 mg/mL) (stored at -20˚C) and 10 µL of 10 mM dexamethasone stock (stored at -20˚C) to 100 mL of William's Medium E with serum (2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin and 5% FBS).

5.1.2 Maintenance Media:

5.1.2.1 Add 1 mL of ITS+ (stored at +4˚C) and 1 µL dexamethasone to 100 mL of William's Medium E (2 mM L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin)

5.2 Cell Preparation:

5.2.1 Warm the Thaw Media to 37˚C in the water bath and thaw the vial containing hepatocytes as follows:

5.2.1.1 Add a few milliliters of warm Thaw Media to a 50 mL conical tube, swirl the media, and aspirate off supernatant.

5.2.1.2 Wipe the vial with 70% EtOH, loosen and retighten the cap.

5.2.1.3 Swirl the vial containing cryopreserved cells in the water bath until only a small ice pellet remains (about 1 minute, 45 seconds).

5.2.1.4 Wipe the vial with 70% EtOH and transfer the contents to the 50 mL conical tube.

5.2.1.5 Add Thaw Media to the 50 mL conical tube as follows:

- Add 1 mL by adding 200 µL at a time, swirling between additions.
- Add 5 mL by adding 500 µL at a time, swirling between additions.
- Add 5 mL by adding 1 mL at a time, swirling between additions.
- *QS* the tube to 50 mL.
- Spin the cells at room temperature for 4 min at 70 x g.
- Carefully aspirate the supernatant and add 5 mL of Thaw media. Gently resuspend by pipetting.
- Count viable cell density using a hemocytometer.
- Dilute cells to a density of 7.5 x 10^5 cells/mL in Maintenance Media.
- Plate 150 µL cells/well as per plate format for time zero, 0.5, 1, 1.5, 2, 2.5 and 3 hour sample exposures (Appendix).
- Incubate plates for 4 hours at 5% CO_2, 37˚C and 95% humidity (Figure 1).

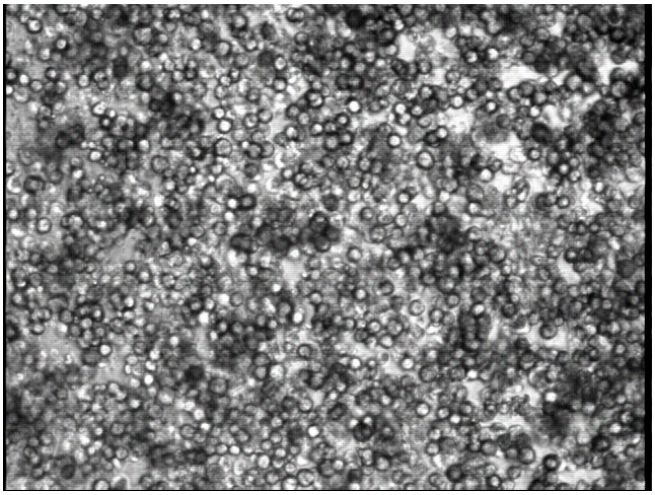

Figure 1. Example of SD Primary Hepatocytes Cell Culture Appearance.
Image was taken with a phase contrast microscope at 250X magnification.

6. Test Nanomaterial Addition

6.1 The highest concentration of nanomaterial tested should be at the limit of solubility. The test sample should be at physiological pH. Neutralization of acidic/basic test samples may be required.

6.2 Dilute test compound in Maintenance Media, making a total of nine 1:4 dilutions.

6.3 **(Work in the dark!)** Add 150 µL of 40 µM DCFH-DA to ~~t~~est sample exposure plate containing 150 µL of Maintenance Media (Final concentration of DCFH-DA is 20 µM) and incubate cells for 30 min under standard culture conditions. Centrifuge the plates at 70 x g for 4 min without brake. Remove DCFH-DA and wash plate with 200 µL of Maintenance Media at 70 x g for 4 minutes with no deceleration. Read time zero measurement, then add 200 µL of each sample dilution to each plate as per plate format (Appendix).

6.4 ROS Assay Experimental Procedure **(do not expose plates to light!)**

6.4.1 Remove test plate at 0.5, 1, 2, and 3 h post exposure from the incubator and read at ex. 485 nm and em. 530 nm.

7. Calculations

7.1 Rows D and E are used as cell-free blanks, which are subtracted from the corresponding sample and control columns (e.g., A1-D1 or B2-D2; see Appendix).

7.2 Wells 1(A-C) and 12(A-C) are the media controls, and wells 11(A-C) are the DEM positive controls for samples in wells 2(A-C) - 10(A-C). Wells 1(F-H) and 12(F-H) are the media controls, and wells 11(F-H) are the positive controls for samples in wells 2(F-H) - 10(F-H) (see Appendix).

$$\%\ Control\ fluorescence = \frac{sample\ fluorescence}{media\ control\ fluorescence} * 100$$

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

8. Acceptance Criteria

8.1 DCFH-DA fluorescence for the DEM positive control should be at least 140 % of media control at 2 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. Black, M.J. and Brandt, R.B., Spectrofluorometric analysis of hydrogen peroxide, *Anal. Biochem.,* 58, 246, 1974.

10. Abbreviations

APAP	acetaminophen
CV	coefficient of variation
DCFH-DA	dichlorofluorescein diacetate
DEM	diethyl maleate
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride
LDH	lactate dehydrogenase
LLC-PK1 cells	renal epithelial cell line, porcine kidney
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate buffered saline
ROS	reactive oxygen species
SD	standard deviation

11. Appendix

Example of a 96-well plate template.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cells Media	Cells Samp. 1 Dilution 9	Cells Samp. 1 Dilution 8	Cells Samp. 1 Dilution 7	Cells Samp. 1 Dilution 6	Cells Samp. 1 Dilution 5	Cells Samp. 1 Dilution 4	Cells Samp. 1 Dilution 3	Cells Samp. 1 Dilution 2	Cells Samp. 1 Dilution 1	Cells DEM 0.5 mM	Cells Media
B	Cells Media	Cells Samp. 1 Dilution 9	Cells Samp. 1 Dilution 8	Cells Samp. 1 Dilution 7	Cells Samp. 1 Dilution 6	Cells Samp. 1 Dilution 5	Cells Samp. 1 Dilution 4	Cells Samp. 1 Dilution 3	Cells Samp. 1 Dilution 2	Cells Samp. 1 Dilution 1	Cells DEM 0.5 mM	Cells Media
C	Cells Media	Cells Samp. 1 Dilution 9	Cells Samp. 1 Dilution 8	Cells Samp. 1 Dilution 7	Cells Samp. 1 Dilution 6	Cells Samp. 1 Dilution 5	Cells Samp. 1 Dilution 4	Cells Samp. 1 Dilution 3	Cells Samp. 1 Dilution 2	Cells Samp. 1 Dilution 1	Cells DEM 0.5 mM	Cells Media
D	Media Media	Media Samp. 1 Dilution 9	Media Samp. 1 Dilution 8	Media Samp. 1 Dilution 7	Media Samp. 1 Dilution 6	Media Samp. 1 Dilution 5	Media Samp. 1 Dilution 4	Media Samp. 1 Dilution 3	Media Samp. 1 Dilution 2	Media Samp. 1 Dilution 1	Media DEM 0.5 mM	Media Media
E	Media Media	Media Samp. 1 Dilution 9	Media Samp. 1 Dilution 8	Media Samp. 1 Dilution 7	Media Samp. 1 Dilution 6	Media Samp. 1 Dilution 5	Media Samp. 1 Dilution 4	Media Samp. 1 Dilution 3	Media Samp. 1 Dilution 2	Media Samp. 1 Dilution 1	Media DEM 0.5 mM	Media Media
F	Cells Media	Cells Samp. 2 Dilution 9	Cells Samp. 2 Dilution 8	Cells Samp. 2 Dilution 7	Cells Samp. 2 Dilution 6	Cells Samp. 2 Dilution 5	Cells Samp. 2 Dilution 4	Cells Samp. 2 Dilution 3	Cells Samp. 2 Dilution 2	Cells Samp. 2 Dilution 1	Cells DEM 0.5 mM	Cells Media
G	Cells Media	Cells Samp. 2 Dilution 9	Cells Samp. 2 Dilution 8	Cells Samp. 2 Dilution 7	Cells Samp. 2 Dilution 6	Cells Samp. 2 Dilution 5	Cells Samp. 2 Dilution 4	Cells Samp. 2 Dilution 3	Cells Samp. 2 Dilution 2	Cells Samp. 2 Dilution 1	Cells DEM 0.5 mM	Cells Media
H	Cells Media	Cells Samp. 2 Dilution 9	Cells Samp. 2 Dilution 8	Cells Samp. 2 Dilution 7	Cells Samp. 2 Dilution 6	Cells Samp. 2 Dilution 5	Cells Samp. 2 Dilution 4	Cells Samp. 2 Dilution 3	Cells Samp. 2 Dilution 2	Cells Samp. 2 Dilution 1	Cells DEM 0.5 mM	Cells Media