Neutral Red Uptake Cytotoxicity Assay

Introduction

The Neutral Red Uptake (NRU) assay is used as a cytotoxicity test to assess the potential toxicity of a test material. The cytotoxicity caused by the test material is measured by a concentration-dependent reduction in NRU by the cells after exposure to a test material. The assay is based on the ability of healthy cells to incorporate and bind the dye neutral red. The dye is later chemically extracted from the viable cells and the absorbance of the solubilized dye is measured and quantified using a spectrophotometer.

When viable cells are exposed to a toxic material leading to cell death, the dye neutral red cannot be retained by the dead cells resulting in a reduction of NRU. The decrease in NRU can be measured and compared to a control to determine the relative toxicity pf a test material.

Components and Recommended Storage

Component	Catalog No.	Format	Storage
Neutral red solution 0.33 g/L in ultra-pure water	115-341-061	100 ml	4-8°C

Protocol

1. Material:

- Primary cells or establish cell lines
- Neutral red solution (**Quality Biological #115-341-061**)
- DPBS without calcium and magnesium (**Quality Biological #114-057-131**).
- Trypsin-EDTA solution (**Quality Biological Trypsin (0.25%)-EDTA (0.02%) #118-093-721EA**).
- Cell maintenance medium: the medium in which the cells are regularly passaged and expanded
- FBS (**Quality Biological FBS, USDA Approved Origin #110-001-101**) and other cell culture supplements adequate for cells requirements.
- Glacial acetic acid
- Ethanol 96%
- Ultrapure water (**Quality Biological #351-029-101**).

2. Seed cells on Day 1

- Maintain cells in cell culture flasks containing complete cell maintenance medium in an incubator at 37°C with 5% CO_2.
- When ready to seed 96-well plates, discard medium from the flask, wash cells with DPBS, then add trypsin solution to detach cells. Resuspend cells in complete cell maintenance medium, count cells and check that viability is at least 95%.
- Prepare 20 mL of cell suspension in maintenance medium of an adequate density for the cell type used (typically, 5-50x10^4 cells/mL is an adequate density for most cells).
- Seed a 96-well plate with 0.2 mL of cell suspension per well. Incubate in an incubator at 37°C with 5% CO_2 overnight to achieve at least 50% confluency.

3. Treatment with test material on Day 2

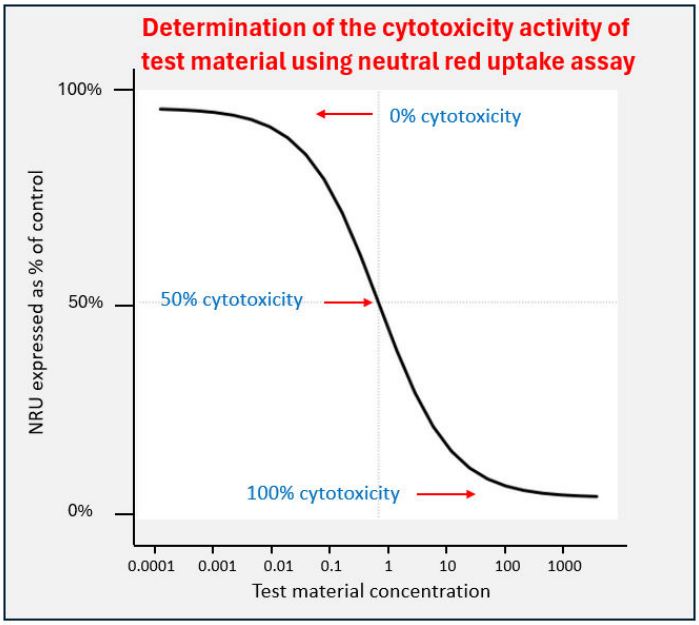
- Prepare a test material solution in maintenance medium under sterile conditions; filter solutions through a 0.22 μm filter if necessary.
- Serial dilute the test material solution in a range a doses, *e.g.* 0.0001, 0.001, 0.01, 0.1, 1.0, 10, 100, 1000 μg/mL in a range-finding experiment.
- Dispense 0.2 mL maintenance medium only in the control wells and 0.2 mL of the serial diluted test material in the test wells. It is recommended to include triplicate wells for each control and test dilutions.
- Incubate the plate in an incubator at 37°C with 5% CO_2 for 24 hours.

4. Neutral red uptake assay on Day 3

- Observe cells and record morphological changes due to cytotoxic effect of test material compared to control conditions (growth inhibition, vacuolization, rounding, detachment and lysis are potential consequences of cytotoxic effect).
- Discard the medium from all wells and rinse cells with DPBS to remove residual test material.
- Add 100 μL of neutral red solution to each well.
- Incubate the plate in an incubator at 37°C with 5% CO_2 for 1-2 hours, up to 4 hours.
- Prepare the neutral red destain solution (50% ethanol 96%, 49% ultrapure water, 1% glacial acetic acid).
- Inspect the plate for confirm NRU difference between control and test wells.
- Discard the neutral red solution from wells, rinse wells with 150 μL of DPBS, discard.
- Add 150 μL of destain solution to extract the neutral red contained withing the cells.
- Shake the plate on a plate shaker for at least 10 minutes, or until the neutral red has been extracted and forms a homogeneous suspension.
- Measure the OD at 540 nm in a microplate spectrophotometer.

3. Results

Analyze data drawing a dose-response curve, express OD as percentage of control NRU from control wells set up at 100%. The cytotoxicity is expressed as percentage inhibition of NRU at a specific test material concentration.



Notes

- Always wear appropriate personal protective equipment including lab coat, gloves, and safety glasses when handling the neutral red solution.
- This protocol consists of general guidelines to perform the neutral red uptake assay for determining the cytotoxicity of a test material; it may be necessary to optimize conditions, such as optimal test material concentrations, cells density, and incubation times.
- After extended storage of the neutral red solution, a precipitate might occur in the form of small particles. If the neutral red solution contains precipitates, use the supernatant only, or filter the solution to remove the precipitates.
- The Quality Biological neutral red solution is for research or further manufacturing use only, not for diagnostics or therapeutic use.
    - The NRU assay can be performed with non-adherent cells in a suspension culture; dispense nonadherent cells in a round-bottomed 96-well plate and include a 5-min centrifugation step (400g) before each change of reagents.

References

Borenfreund E. & Puerner J.A. A simple quantitative procedure using monolayer cultures for cytotoxicity assays. (1984) *J. Tissue Cult. Methods* 9, 7-9.

Inayat-Hussain S., Rajab N.F, & Siew E.L. In vitro testing of biomaterials toxicity and biocompatibility. *Cellular response to Biomaterials*. Woodhead Publishing Series in Biomaterials. (2009) pp 508-537.

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