

NEUTRAL RED RELEASE BIOASSAY

Theory: An increase in the release of pre-incorporated neutral red dye (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) by NHEK (Normal Human Epidermal Keratinocytes) cell cultures following exposure to a test material is used as an indication of potential eye and or skin irritancy.

Applications and Use

- Used to assess cell membrane activity of test articles.
- The target cells are epithelial in origin and derived from human skin. The assay system may be used to predict ocular irritation of dilution-based materials.
- The test system uses a serum-free medium, which eliminates the possibility of serum protein and test article interaction (Shopsis and Eng, 1988).
- Test materials are dilution based. Therefore, topical application can not be modeled.
- The assay is suited for water-soluble formulations, but formulations may also be dissolved in intermediate solvents.
- The neutral red release bioassay has been demonstrated in a multi-assay system validation to be particularly effective at predicting ocular irritation due to surfactant formulations.
- The assay system has been demonstrated to be quite reproducible, and provided a high level of resolution between similar formulations.

Experimental Procedure

Target Cell Preparation

- Stock cell cultures are maintained at $37 \pm 1^\circ\text{C}$ in a humidified atmosphere containing $5 \pm 1\%$ CO_2 .
- Cells are subcultured when the stock culture is 50 to 80% confluent.
- A cell suspension is prepared to yield 1.0 to 1.4×10^4 cells/ml, depending on when cells need to be at confluent dosing level.
- 250 μl of the cell suspension are added to designated wells of the 96-well bioassay plate.
- The cultures are incubated until the cells become 75 to 90% confluent (~48-120 hours) prior to test chemical dosing.

Sample and Positive Control Preparation

- On the day of dosing, the test chemical is suspended in KGM (or another appropriate solvent).
- A total of 8 dilutions of the test chemical are made for the dose range finding assay and a minimum of 6 doses for the definitive assays.
- Triton X-100 is used as the positive control. Four concentrations (1,0.3, 0.1, and 0.03 mg/ml) are tested.

Assay Procedure

- Neutral Red Pre-loading: 72 to 120 hours after seeding, KGM is removed from the wells and 250 μl of neutral red solution (50 $\mu\text{g/ml}$) in KGM is added to each well and the plates are returned to the incubator for 3 hours.
- The neutral red solution is removed and refed with 250 μl of fresh KGM. Cells must be treated within 2 hours after neutral red removal. This medium will be decanted immediately before dosing.
- 100 μl of test or control article dilutions are added to the appropriate wells immediately after the KGM is decanted.
- The plates are incubated for 5 minutes at room temperature.
- After incubation, the treatment solution is removed and the cultures are rinsed at least once with 125 μl of KGM.
- 200 μl of wash/fix solution are added to each well. After two minutes, the wash/fix is removed and 100 μl of neutral red solvent added.
- The neutral red is extracted from the cultures for at least 20 minutes at room temperature while shaking.
- The absorbance of the neutral red at 550 nm (OD_{550}) is measured with a 96-well plate reader.

Data Evaluation

- The relative survival of each treatment group is determined by comparing the mean corrected OD_{550} of the test article-treated wells to the mean corrected OD_{550} of the negative control-treated wells.
- Dose response curves may be plotted with the % of control on the ordinate and the test article doses on the abscissa.
- The NRR_{50} (the concentration of the test article that increases the release of pre-incorporated neutral red by 50%) is determined by interpolation from the dose response curves.