



IN VITRO TOXICOLOGY ASSAY KIT NEUTRAL RED BASED

Stock No. LiT-NRed-AK

Store at 2-8 °C

IT IS RECOMMENDED THAT THE ENTIRE PROTOCOL BE REVIEWED BEFORE STARTING THE ASSAY.

Product Description

Traditionally, the in vitro determination of toxic effects of unknown compounds has been performed by counting viable cells after staining with a vital dye. Alternative methods used are measurement of radioisotope incorporation as a measure of DNA synthesis, counting by automated counters and others which rely on dyes and cellular activity. The neutral red assay system is a means of measuring living cells via the uptake of the vital dye neutral red.

The neutral red method, as originally developed by Borenfreund and Puerner (1) is simple, accurate and yields reproducible results. The key component is the vital dye, neutral red (Basic Red 5, Toluyene Red). Viable cells will take up the dye by active transport and incorporate the dye into lysosomes, whereas non-viable cells will not take up the dye. After the cells have been allowed to incorporate the dye they are briefly washed or fixed. The incorporated dye is then liberated from the cells in an acidified ethanol solution. An increase or decrease in the number of cells or their physiological state results in a concomitant change in the amount of dye incorporated by the cells in the culture. This indicates the degree of cytotoxicity caused by the test material.

REAGENT

For Research Use Only.

Not for Use in Diagnostic Procedures.

Kit Components

<u>Prod. No.</u>	<u>Item</u>	<u>Quantity</u>
LiT-NRed	Neutral Red Solution 0.33% in DPBS	20 ml
LiT-NRed-Fix	Neutral Red Assay Fixative, 0.1% CaCl ₂ in 0.5% Formaldehyde	125 ml
LiT-NRed-Sol	Neutral Red Assay Solubilization Solution, 1% Acetic acid in 50% Ethanol	125 ml

WARNING: Components in this kit should be carefully handled when using. **Neutral Red Assay Fixative** may be harmful if swallowed, inhaled, or absorbed through skin. This product contains low levels of formaldehyde which is toxic and may cause heritable genetic changes. **Neutral Red Assay Solubilization Solution** is flammable.

Product Storage

Kit components should be stored at 2-8 °C.

Procedure

The neutral red method of monitoring in vitro cytotoxicity is well suited for use with multiwell plates. For best results, cells in the log phase of growth should be employed and final cell number should not exceed 10⁶ cells/cm². Each test should include a blank containing complete medium without cells.

1. Remove cultures from incubator into laminar flow hood or other sterile work area.
2. Add 0.33% Neutral Red Solution [LiT-NRed] in an amount equal to 10% of the culture medium volume.

3. Return cultures to incubator for 2-4 hours depending on cell type and maximum cell density. An incubation period of 2 hours is generally adequate but may be lengthened for low cell densities or cells with low metabolic activity. Incubation times should be consistent when making comparisons.
4. At the end of the incubation period, the medium is carefully removed and the cells quickly rinsed with Neutral Red Assay Fixative [LiT-NRed-Fix]. Extended fixation times can result in leaching of the dye into the fixative solution. Alternatively the cells can be washed in an osmotically balanced saline solution such as PBS or HBSS.
5. The fixative or wash solution is removed and the incorporated dye is then solubilized in a volume of Neutral Red Assay Solubilization Solution [LiT-NRed-Sol] equal to the original volume of culture medium. The cultures are allowed to stand for 10 minutes at room temperature. Gentle stirring in a gyratory shaker or pipetting up and down (trituration) will enhance mixing of the solubilized dye.
6. Spectrophotometrically measure absorbance at a wavelength of 540 nm. Measure the background absorbance of multiwell plates at 690 nm and subtract from 540 nm measurement. Tests performed in multiwell plates can be read using an appropriate type of plate reader or the contents of individual wells may be transferred to appropriate size cuvetts for spectrophotometric measurement.

Possible Sources of Error

1. Neutral red may precipitate in solution upon storage. If precipitated dye crystals interfere with the assay, it may be desirable to filter the dye solution using a syringe filter prior to addition to the cell cultures.
2. Prolonged exposure of the cells to the fixative can result in leaching of the dye into the fixative solution.
3. Uneven evaporation of culture fluid in wells of multiwell plates may cause erroneous results.

References

1. Borenfreund, E. and Puerner, J. [1985] Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicol. Lett.* 24:119-124.
2. Triglia, D. et al. [1991] In vitro toxicity of various classes of test agents using the neutral red assay on a human three-dimensional physiologic skin model. *In Vitro Cell Develop. Biol.* 27A:239-244.
3. Babich, H. et al. [1991] In vitro cytotoxicity studies with the fish hepatoma cell line, PLHC-1 (*Poeciliopsis lucida*). *Ecotox. Environ. Safety* 21:327-336.
4. Fautz, R. et al. [1991] Application of the neutral red assay (NR assay) to monolayer cultures of primary hepatocytes: rapid colorimetric viability determination for the unscheduled DNA synthesis test (UDS). *Mutation Research* 253:173-179.
5. Morgan, C. et al. [1991] An improved colorimetric assay for tumor necrosis factor using WEHI 164 cells cultured on novel microtiter plates. *J. Immunol. Methods* 145:259-262.

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