CELLBIO™
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Technical Datasheet

HiPer® Neutral Red Cell Assay Teaching Kit

Product Code: CCK033

Contents

1. Introduction

Cell proliferation and death are essential processes for tissue generation and regeneration, organ development etc. in mammals and are usually under stringent control of extra and intracellular factors. Non-physiological alterations in levels of these factors lead to anomalous cytogenetic behavior of cells which in turn leads to cell transformation, uncontrolled cell growth - the initiating event for cancer development. Pharmaceutical research is hence largely focused on effects of drugs, cytotoxic agents and biologically active compounds which affect cytogenetics.

Multiple procedures are available for determination of cell proliferation and cytotoxicity. Simple and cheap methods for estimating cell viability (or death) are Trypan Blue exclusion and Erythrocin B staining. However, these methods are not sensitive enough and cannot be used for high throughput screening. Measuring the uptake of radioactive substances, usually tritium-labeled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances. Neutral red uptake assay is a simple, accurate and reproducible assay for determining cellular proliferation and cytotoxicity. The assay detects living cells and the signal generated is dependent on the metabolic state of the cells. This method can therefore be used to measure cytotoxicity, proliferation or activation. The results can be read on a multi-well scanning spectrophotometer or a standard ELISA reader and show a high degree of precision.

2. About the Assay

The HiPer® Neutral Red Cell Assay Teaching Kit is designed for determination of cell viability and cell proliferation and/or effect of cytotoxic agent. This kit is based on the quantitative measurement of uptake of neutral red by cellular lysosomes. Neutral red is a weak cationic dye that preadily penetrates the cell membrane by non-ionic diffusion. In intact cells it binds to anionic sites in lysosomes, becomes charged and gets trapped. Neutral red trapped in lysosomes does not freely pass out in the cytoplasm. Damage of the cells causes release of neutral red or prevents its accumulation in lysosomes. Alterations of cell surface or sensitive lysosome membrane leads to lysosomal fragility, resulting in decreased uptake and binding of dye. Damaged or dead cells lose their ability to retain the dye. This property of neutral red is utilized for determining cytotoxic effects of test compounds.

3. Applications

- Antibody dependent complement cell damage.
- Tumoral cell growth and chemotherapy effectiveness.
- Toxicity of physical agents either alone or in combination with chemical and in particular phototoxicity.
- Ranking chemicals according to their toxic potencies or hazard.
- Identification of organ-specific and cell-specific toxicity.
- Comparison of species toxicity.
- Establishment of chemical structure-toxicity relationships.
- Elucidation of antagonistic / synergistic interactions between chemicals.
- Toxicity prevention and treatment effectiveness.
- The investigation of metabolism mediated cytotoxicity (detoxification and toxification process).
- The study of temperature-dependent toxicity.

4. Kit contents

The reagents supplied in this kit are sufficient for 100 assays (for 1 microwell plates).

Code	Description	CCK033 -100
CCK033(A)	Neutral Red Solution	1ml
CCK033(B)	Neutral Red Fixative	10ml
CCK033(C)	Neutral Red Solubilization Solution	10ml

Note: Quantities supplied in excess to compensate operational losses

5. Materials required but not provided in the kit

- Cells in appropriate medium.
- Adjustable pipettes and a repeat pipettor.
- Flat-bottom 96-well microtiter plate for culturing the cells.
- 96-well plate reader capable of measuring the absorbance.

6. General guidelines

It is important to optimize experimental factors like cell density, incubation time, media composition and concentration of the agents under investigation prior to use of EZcount™ Neutral Red Cell Assay Kit. Procedure for optimizing cell density is outlined in section 7.2. **Assay controls**

Include appropriate assay controls i.e.

1. Medium control (medium without cells).
2. Cell control (medium with cells but without the experimental drug/ compound).
3. Vehicle control (medium containing the experimental drug or compound but no cells).

Accuracy

- To obtain statistically significant data, perform the assay in triplicates or more.
- Accuracy of the assay depends on pipetting skills of the personnel. Inappropriate addition and mixing practices may result in erroneous and false-positive or false-negative results.
- Use of a repeating pipettor is recommended to pipette reagents. This saves time and helps maintain more precise incubation times.
- Pipette tip should be equilibrated with the reagent before use. This is carried out by slowly filling up the tip with reagent and gently expelling the contents several times.
- Care should be taken so that no bubbles are introduced into the wells during pipetting or mixing of the reagents.

Incubation period

- Different cell lines may have different properties such as metabolic activity and doubling time and hence respond to Neutral Red differently. For this reason, plating density and incubation period for every cell line should be optimized to obtain results in linear range.
- Staining of cellular lysosomes with neutral red can be checked microscopically as intracellular red coloured dots. Longer incubation period may be required if adequate amount of neutral red has not bound to lysosomes.

Temperature

- It is crucial to run the assay at a uniform temperature to ensure reproducibility across a single plate or among stacks of several plates. Since absorbance is measured at room temperature, it is important to ensure adequate equilibration of assay plates after removal from a 37ºC incubator to avoid differential temperature gradients. Stacking large numbers of assay plates in close proximity should be avoided to ensure complete temperature equilibration.

Measurement of absorbance

- Absorbance should be read with a filter at the wavelength of 540nm (primary wavelength).
- Reference wavelength (for non-specific readings) should be >600nm.

7. Directions for use

Users are advised to review entire procedure before starting the assay

7.1 Preparation of cells

Always use freshly harvested cells for assay. Seed the cells in a cell culture flask or dish in an amount appropriate for the assay and incubate at 37ºC in a 5% CO_2 environment. Allow the cells to grow for up to 24 hours or till confluence is reached. Harvest the cells and use for assay.

*(**Note**: The quantity of the cell suspension to be seeded in the medium depends on doubling time of individual cell lines and seeding density to be used in the assay.)*

7.2 Pre-assay optimization procedure

Pre-assay optimization procedure needs to be performed once for each cell line to determine optimum plating density and incubation time.

1. Harvest the cells as explained in section 7.1.
2. Adjust the cell density to 1 x 10^6 cells /ml.
3. Serially dilute the cell suspension from 1 x 10^6 to 1 x 10^3 cells/ml using appropriate culture medium.
4. Seed 100µl of each dilution in 96-well microtiter plate in triplicate.
   Note: *Non-adherent cells should be preferably cultured in round bottom 96 well plate. Adherent cells can be cultured in flat bottom plate.*
5. Add medium control in triplicate.
6. Incubate the cells under appropriate conditions depending on the cell line under study.
   Note: It is recommended to incubate the adherent cells for 24 hours to allow their attachment to the plate. Non-adherent cells can be used immediately.
7. Add 10µl of Neutral Red Solution CCK033 (A) to each well, including controls.
8. Wrap the plate with aluminium foil to avoid exposure to light.
9. Incubate the in incubator at 37ºC and 5% CO_2 for 2 to 4 hours.
10. Observe the cells at periodic intervals under an inverted microscope for presence of stained lysosomes.
    Note: Refer figure 1.

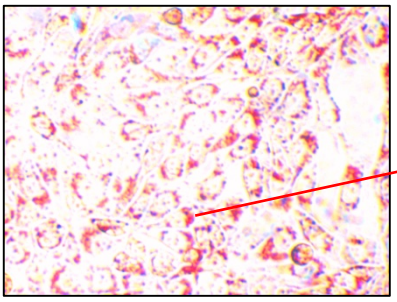


Fig 1: Intracellular lysosomes stained by Neutral red

11. a) <u>For adherent cell lines</u>:
    i. After incubation with neutral red, discard the entire medium and add 100µl of Neutral
    ii. Red Fixative [CCK033 (B)] to each well. Swirl the plate gently and quickly to wash the monolayer and remove unbound neutral red.
    iii. Discard the fixative solution.
    Note: Avoid prolonged exposure of the cells to fixative. This may cause leaching of neutral red into the solution. Alternatively phosphate buffered saline can also be used for washing the monolayer. For loosely attached cell lines it is recommended to centrifuge the plate at 1000rpm for 5 minutes.
    iv. Add 100µl of Neutral Red Solubilization Solution CCK033(C) in each well and incubate the plate at room temperature for 10 minutes.

    b) <u>For non-adherent (suspension) cell lines</u>:
    i. After incubation with neutral red, centrifuge the plate at 1000rpm for 5 minutes.
    ii. Discard the supernatant carefully without disturbing the pellet.
    iii. Add 100µl of phosphate buffered saline to the wells.
    iv. Swirl the plate gently and quickly to wash the monolayer and remove unbound neutral red.
    Note: It is not recommended to use fixative solution CCK033 (B) for washing non-adherent cells. This may cause leaching of incorporated neutral red into the solution during centrifugation step.
    v. Centrifuge the plate at 1000rpm for 5 minutes.
    vi. Discard the supernatant.
    vii. Add 100µl of Neutral Red Solubilization Solution CCK033(C) and incubate the plate at room temperature for 10 minutes.

12. Gently stirring on gyratory shaker OR pipetting up and down may enhance solubilization of the dye in solution.
13. Read the absorbance on a spectrophotometer or an ELISA reader at 540nm with a reference wavelength at >600nm.
14. Determine the average values from triplicate readings at 540nm and subtract from this value the average value for blank (i.e. medium control) and average value at the reference wavelength. Specific absorbance = [Absorbance $_{(540nm)}$ (test) – Absorbance $_{(>600nm)}$ (test) – [Absorbance $_{(540nm)}$ (blank) - Absorbance $_{(>600nm)}$ (blank)]
15. Plot absorbance against cell density.
16. Number of cells to be used in the cell proliferation assay should lie within linear portion of the plot.

7.3 <u>Assay procedure</u>

1. Seed 100µl of cell suspension in a 96-well microtiter plate at the required cell density, with or without the cell growth modifying agent.
   *(**Note**:*
   a) If the cell growth modifying agent is a cytokine, metabolite, growth factor or any other compound, add its required quantity in the culture system.
   b) If the cell growth modifying agent is any kind of radiation or waves, treat the cells with them for required period of time.)

2. Incubate the cells at 37ºC in a 5% CO_2 atmosphere for the required period of time.
3. After the incubation period, follow the procedure given in section 7.2, step 7 onwards.
4. Plot the absorbance values on the Y-axis and your experimental parameters on the X-axis.

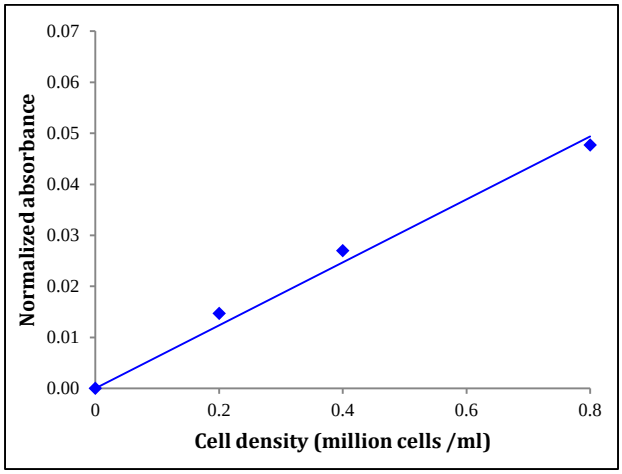
7.4 Interpretation of Data

1. The linear portion of the neutral red curve depicts maximum sensitivity to changes induced by experimental parameters.
2. Test values higher than control values indicate increase in cell proliferation and viability and *vice versa*.

8. Storage and shelf life

Store the kit at 15-30ºC away from bright light.
Use before expiry date given on the product label.

9. Performance characteristics



Relationship between cell number and the absorbance at 580nm/ 630nm after neutral red uptake assay. The sensitivity of Neutral Red to detect changes in cell number has been determined by plotting the graph of normalized absorbance values versus cell number.
CHO were seeded at different densities in 96-well plate and assayed for neutral red uptake using HiPer® Neutral Red Cell Assay Teaching Kit. After incubation for 4 hours in a humidified incubator at 37ºC, 5% CO_2, absorbance was read at 580nm / 630nm using an ELISA plate reader.

10. Advantages

- **Time saving**: Ready to use solutions supplied in the kit eliminate the need to prepare the solutions and save time.
- **Reproducibility**: Entire assay can be performed in a single plate. Cells and reagents need not be transferred. This facilitates reproducibility of the results.
- **Sensitivity and accuracy:** Binding of neutral red to lysosomes strongly correlates with the lysosomal activity and metabolic activity of the cells. This allows use of very low cell densities.
- **Safety**: No radioisotopes are involved.
- **Fast**: Use of multi-well ELISA plates allows the processing of large number of samples.
- **Flexibility**: Neutral Red works on adherent as well as suspension cell lines.

11. Troubleshooting points

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
Very high absorbance values	Too much uptake of Neutral Red due to high cell densities	Repeat the assay with reduced cell densities
	Too much uptake of neutral red due to long incubation period	Repeat the assay with reduced incubation period
Very low absorbance values	Very low cell density	Repeat the assay with high cell densities
	Short incubation period	Repeat the assay with longer incubation period. Certain cell types require longer incubation period of up to 24 hours
	Cell detachment during washing	Add and remove the reagents gently from the wells without disturbing the monolayer
	Improper selection of filter for reading the absorbance	Choose appropriate filters within the range of 540-600nm
	Precipitation of the dye	Centrifuge the neutral red solution and use supernatant for assay
	Precipitation of dye due to test compound	Decant the medium containing test compound after treatment and wash the cells with PBS before adding neutral red
Random absorbance values/ poor consistency of replicates	Incomplete solubilization of incorporated neutral red	Allow the incorporated neutral red to dissolve in solubilization solution completely, by mixing with a pipette or on gyratory shaker
	Inaccurate pipetting technique or inaccurate equipment	Perform the assay using automated electronic pipettes for seeding the cell suspension and adding the reagents
	Test compound under study is responsible for improper response of the cells to neutral red	Refer to the pharmacological properties of the compound
	Precipitate in the neutral red solution	Neutral red may precipitate upon storage. Precipitation does not affect performance of the kit. Centrifuge the solution and filter the supernatant through 0.22micron filter before use.

HiMedia Laboratories Pvt. Ltd.
Plot No. C40, Road No. 21Y, MIDC, Wagle Industrial Area, Thane (West) 400604, Maharashtra, India.Tel No.022-69034800
Email: atc@himedialabs.com Website: www.himedialabs.com.