# Chapter 2

# Assaying Cellular Viability Using the Neutral Red Uptake Assay

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## Abstract

The neutral red uptake assay is a cell viability assay that allows in vitro quantification of xenobiotic-induced cytotoxicity. The assay relies on the ability of living cells to incorporate and bind neutral red, a weak cationic dye, in lysosomes. As such, cytotoxicity is expressed as a concentration-dependent reduction of the uptake of neutral red after exposure to the xenobiotic under investigation. The neutral red uptake assay is mainly used for hazard assessment in in vitro toxicology applications. This method has also been introduced in regulatory recommendations as part of 3T3-NRU-phototoxicity-assay, which was regulatory accepted in all EU member states in 2000 and in the OECD member states in 2004 as a test guideline (TG 432). The present protocol describes the neutral red uptake assay using the human hepatoma cell line HepG2, which is often employed as an alternative in vitro model for human hepatocytes. As an example, the cytotoxicity of acetaminophen and acetyl salicylic acid is assessed.

**Key words** Viability assay, Neutral red uptake, HepG2

## 1 Introduction

The neutral red uptake (NRU) assay is a viability assay based on the ability of living cells to incorporate and bind neutral red (NR) [1]. This weak cationic eurhodine dye can penetrate cells by nonionic diffusion at physiological pH. Once NR is in the cell, it accumulates intracellularly in lysosomes, where a proton gradient assures a more acidic pH and the dye becomes charged [2]. Xenobiotics can lead to alterations of the cell surface or lysosomal membrane, which results in a decreased uptake and binding of NR. As such, the NRU assay allows to assess membrane permeability and lysosomal activity, making it possible to differentiate viable, damaged, or dead cells. Cytotoxicity is expressed as a concentration-dependent reduction of the uptake of NR after exposure to the xenobiotic, thus providing a sensitive, integrated signal of both cell integrity and cell growth inhibition [1]. The NRU has miscellaneous biological applications and is commonly used to evaluate the cytotoxicity

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of a variety of chemical substances such as pharmaceuticals and cosmetics [3, 4]. Several validation studies have been set up for the NRU as a test for cytotoxicity [5]. In 2000 a NRU test on Balb/c 3T3 mouse fibroblasts to assess phototoxicity, was regulatory accepted in all EU member states and in 2004 it was adopted as an official Organisation for Economic Co-operation and Development (OECD) test guideline (TG 432) [6]. In 2013, the European Commission Joint Research Centre has published a recommendation on the use of the 3T3 NRU assay in which it stresses the validity of the NRU in a weight-of-evidence approach to predict acute oral toxicity of chemicals in a regulatory setting [7]. The facility of the NRU assay permits automation, which improves throughput and allows fast and reliable screening of a large amount of test compounds in a relatively short time span [4, 8].

For the purpose of this book chapter, the NRU is described on HepG2 cells. This human hepatoma cell line originates from a 15-year-old Caucasian male and is widely employed in hepatotoxicity studies. Under proper culture conditions, HepG2 cells display (limited) hepatocyte-like features and are therefore often utilized as an alternative in vitro model for human hepatocytes [9–11].

## 2 Materials

### 2.1 General Equipment

1. Incubator: 37 ± 1 °C, 90 ± 5% humidity, 5.0 ± 1% $CO_2$/air.
2. Laminar flow clean bench/cabinet (standard: "biological hazard").
3. Water bath: 37 ± 1 °C.
4. Inverse-phase contrast microscope.
5. Laboratory balance.
6. 96-Well plate spectrophotometer (i.e., plate reader) equipped with 540 ± 10 nm filter.
7. Shaker for microtiter plates.
8. Cell counter or hemocytometer.
9. Pipettes, pipettors (multichannel and single channel; multichannel repeater pipette).
10. 96-Well flat-bottom tissue culture microtiter plates.
11. Multichannel reagent reservoir.
12. Vortex mixer.
13. Filters/filtration devices.

### 2.2 Reagents

1. HepG2 cells.
2. Dulbecco's modification of Eagle's medium (DMEM) with L-glutamine (preferably) and high glucose (4.5 g/l).

3. 200 mM L-Glutamine (in case DMEM does not contain L-glutamine).
4. Fetal bovine serum (FBS) (*see* **Note 1**).
5. TripLE™ Express Enzyme.
6. Phosphate-buffered saline (PBS) without $Ca^{2+}$ and $Mg^{2+}$.
7. Penicillin/streptomycin solution.
8. Neutral Red (NR) Dye liquid form.
9. Dimethyl sulfoxide (DMSO, cell culture grade).
10. Ethanol (EtOH), U.S.P. analytical grade (100%, non-denatured for test chemical preparation; 95% can be used for the desorption solution).
11. Glacial acetic acid, analytical grade.
12. Distilled water or any purified water suitable for cell culture (sterile).
13. Test compounds (acetaminophen and acetyl salicylic acid were used in this protocol).

## 3 Methods

All solutions, glassware, pipettes, etc. have to be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

### 3.1 HepG2 Cell Culture

HepG2 cells are routinely grown as a monolayer in tissue culture-grade flasks (e.g., 75 $cm^2$) at 37 ± 1 °C, 90 ± 5% humidity, and 5.0 ± 1% $CO_2$/air (*see* **Note 2**). When cells exceed 50% confluence (but do not reach 80% confluence) they should be passaged by removing them from the flask using TripLE™ Express Enzyme as follows:

1. Prepare Routine Culture Medium by supplementing DMEM with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 4 mM glutamine (if not already present in media) (*see* **Note 3**).
2. Pre-warm the routine culture medium and PBS in a water bath at 37 °C.
3. Aspirate routine culture medium from the flask, briefly rinse cultures with 10 ml PBS, and add 5 ml TripLE™ Express Enzyme.
4. Incubate flask in the incubator for 2–5 min. Tap the flask on the side, to make sure that all cells are detached (check with microscope).
5. Carefully resuspend the cells and transfer the cell suspension into a Falcon tube (*see* **Note 4**).

6. Rinse the culture flask two times with 10 ml pre-warmed routine culture medium and add to the Falcon tube. Centrifuge the cell suspension at 385 × *g* for 5 min.
7. Remove supernatant. To avoid aspiration of cells, leave some routine culture medium on top of the pellet. Resuspend pellet in 5 ml routine culture medium and count the cells using a cell counter or a hemocytometer.
8. Prepare a cell suspension of 2.0–3.0 × $10^4$ cells/ml in routine culture medium. Using a multichannel pipette, dispense 100 µl routine culture medium only into the peripheral wells (blanks) of a 96-well tissue culture microtiter plate. In the remaining wells, seed between 7.5 and 10.0 × $10^3$ cells/well (*see* **Note 5**).
9. Incubate cells for 24 ± 2 h (37 ± 1 °C, 90 ± 5% humidity, 5.0 ± 1% $CO_2$/air). This incubation period assures cell recovery and adherence and progression to the exponential growth phase.
10. Refresh routine culture medium every 2–3 days (*see* **Note 6**).
11. Examine each plate under a phase-contrast microscope to assure that cell growth is relatively even across the microtiter plate. This check is performed to identify experimental and systemic cell seeding errors.

### 3.2 Preparation of Test Chemicals

1. Prepare test chemical immediately prior to use. Test chemical solutions should not be prepared in bulk for use in subsequent tests. The test chemical should be completely soluble and the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have a minimal volume of at least 1–2 ml.
2. For chemicals dissolved in DMSO or EtOH, the final DMSO or EtOH concentration for application to the cells must not exceed 0.5% (v/v). All test concentrations and vehicle controls should contain the same concentration of DMSO or EtOH.
3. The stock solution for each test chemical should be prepared at the highest concentration found to be soluble. The lower concentrations in a range-finding experiment would then be prepared by successive dilutions that decrease by, e.g., one log unit each. Once the toxicity range for a compound is found, smaller concentration intervals should be tested.
4. Prior to exposure of the test chemicals, the stock solutions must be diluted in pre-warmed (37 °C) routine culture medium.
5. Table 1 shows an example of the concentration gradients for acetaminophen and acetylsalicylic acid. Hereby a dilution factor of 2.15 was used to prepare the serial dilutions.

### 3.3 Cell Culture Treatment

1. Aspirate the routine culture medium from the plates.
2. Add 100 µl of each concentration according to a predefined plate layout. An example of a plate layout is given in Fig. 1 (*see* **Note 7**).

**Table 1**
**Test concentration gradients of acetaminophen and acetylsalicylic acid**

| Concentration (μg/ml) | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 |
|---|---|---|---|---|---|---|---|---|
| Acetaminophen | 5000 | 2325.6 | 1081.7 | 503.1 | 234.0 | 108.8 | 50.6 | 23.5 |
| Acetylsalicylic acid | 2000 | 930.2 | 432.7 | 201.2 | 93.6 | 43.5 | 20.2 | 9.4 |

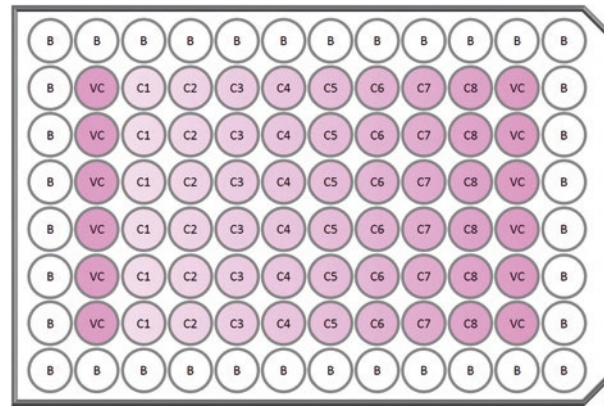


**Fig. 1** Plate layout for cell exposure. B blank, VC vehicle control, C1–C8 test concentration in descending order

3. Incubate the plate at proper conditions, for 24 ± 1 h. Longer incubation times, e.g., 48 or 72 h, can also be used.

### *3.4 Neutral Red Uptake Assay*

1. Prepare the NR medium (25 μg/ml) in routine culture medium (pre-warmed to 37 °C) (*see* **Note 8**).
2. Carefully aspirate the routine culture medium with test chemical and rinse the cells very carefully with 250 μl pre-warmed PBS. Aspirate the rinsing solution (*see* **Note 9**).
3. Add 250 μl NR medium (to all wells including the blanks) and incubate (37 ± 1 °C, 90 ± 5% humidity, and 5.0 ± 1% $CO_2$/air) for 3 ± 0.1 h.
4. After incubation, remove the NR medium, and carefully rinse the cells with 250 μl pre-warmed PBS. Figure 2 illustrates HepG2 cells with intracellularly bound NR.
5. Prepare the desorption solution (1% glacial acetic acid solution, 50% EtOH, 49% $H_2O$) and add exactly 100 μl desorption solution to all wells, including blanks.
6. Shake microtiter plate on a microtiter plate shaker (e.g., 80 rpm, Stuart mini orbital shaker SSM1) for 20–45 min to extract

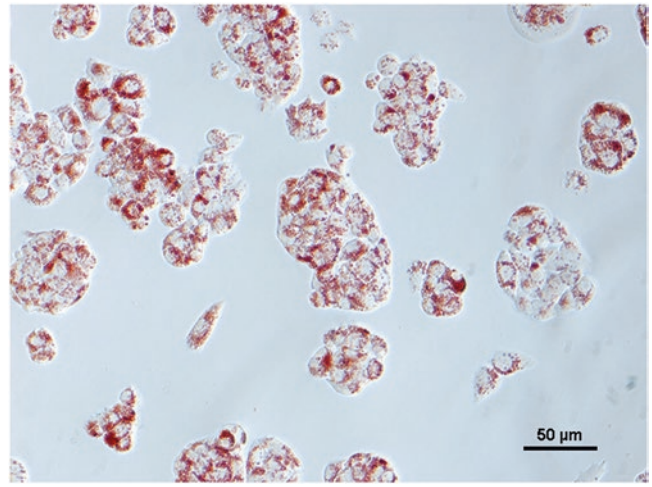


**Fig. 2** HepG2 cells with intracellularly bound neutral red

NR from the cells and form a homogeneous solution. Protect plates from light by covering them, e.g., with aluminum foil.

7. Plates should be still for at least 5 min after removal from the plate shaker. Measure the absorption (within 60 min of adding the desorption solution) of the resulting colored solution at 540 ± 10 nm in a microtiter plate reader.

### 3.5 Data Analysis

The obtained spectrophotometric data is mostly presented as a concentration-response curve (often referred to as dose-response curve) in which the effect caused by the xenobiotic can be visualized following a concentration gradient. Analysis of this curve provides information on the cytotoxic effect caused in the cells exposed for a determined period of time. This analysis is based on four-parameter logistic nonlinear regression that can be conducted using several mathematical software packages. Some of those, e.g., MasterPlex® (MiraiBio Group of Hitachi Solutions America, Ltd.), have been specifically designed for analysis of life sciences assays. Figure 3 illustrates the concentration-response of HepG2 cells exposed during 24 h to a concentration gradient of acetaminophen and acetylsalicylic acid.

The main endpoint readout of concentration-response curves is the determination of the concentration at which a particular percentage of the cells show a decrease in viability. Hereby, the $IC_{50}$ (inhibitory concentration 50) or the concentration of test substance at which 50% of cell death is observed is conventionally used as a parameter of in vitro cytotoxicity. The $IC_{10}$, representing the concentration that induces 10% cell death, or lower concentrations are on the other hand often referred to as subcytotoxic concentrations.

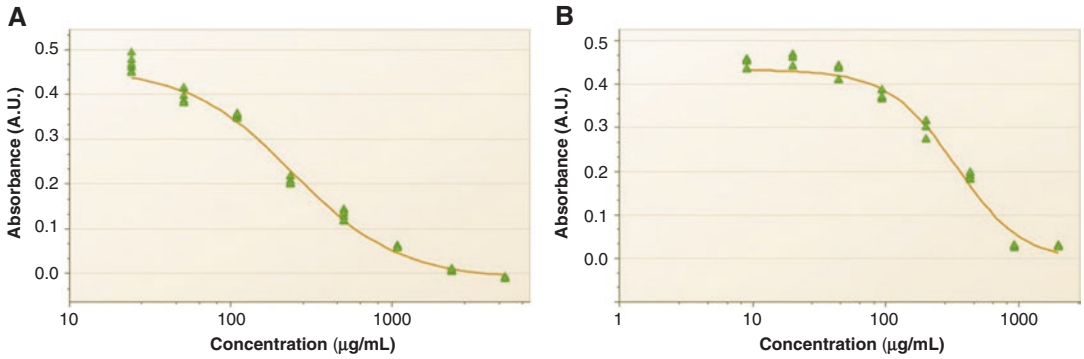


**Fig. 3** Concentration-response curves of HepG2 cells exposed for 24 h to (**a**) acetaminophen and (**b**) acetylsalicylic acid

## 4 Notes

1. Due to the inconsistency of different sera, the cytotoxicity of different batches of FBS should be investigated. A sufficient amount of the same batch FBS should be reserved and used within the same experiments.
2. Completed media formulations should be kept at approximately 2–8 °C and stored for no longer than 1 month.
3. For the purpose of this book chapter, the NRU assay is documented using the hepatic human cell line HepG2. This assay can, however, be applied using other animal or human cell systems.
4. Trypsinization is inhibited by the presence of serum in cell culture media. Therefore, the dissociation of the cells should be complete before adding routine culture medium.
5. Other plate formats than 96-well plates might also be used for the NRU assay. The incubation volumes should be adapted according to each recipient.
6. At higher cell densities, slight acidification of the routine culture medium may occur (observed by an orange color shift of phenol red indicator). In this case the frequency of routine culture medium refreshment should be increased and/or the cells should be passaged.
7. In the presented plate layout each concentration is tested in sixfold. These technical repeats can be decreased to three, which makes it possible to test two different compounds in the same plate.
8. The NR medium can be filtered to reduce potentially formed NR crystals (e.g., Millipore filtering, 0.2–0.45 µm pore size).
9. To reduce the time of this step, the aspiration of NR medium and PBS can be replaced by "dumping" the content of the

plate by a flip movement into a recipient with large opening or the sink. Eventual liquid at the edges of the plates can be dried by pressing the plate to a pile of paper cloths. This procedure can only be performed if no further culturing of the cells is envisaged.

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