In-vitro 3T3 Neutral Red Uptake Phototoxicity Test Method for Chemicals used in Cosmetics

化妆品用化学原料体外 3T3 中性红摄取光毒性试验方法

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In-vitro 3T3 neutral red uptake phototoxicity test method for chemicals used in cosmetics

1 Scope

This method specifies the scope, normative references, terms and definitions, test principle, test materials and reagents, test procedures and result judgment criteria of in-vitro 3T3 neutral red uptake phototoxicity test for chemicals used in cosmetics.

It is recommended that this method applies to the evaluation of the potential phototoxicity of chemicals used in cosmetics.

2 Normative References

The following referenced documents contain provisions which, through reference in this text, constitute provisions of this standard. For dated references, subsequent amendments (excluding the corrigendum) to or revisions of any of these publications shall not apply to this standard. However, the users are encouraged to study the references of revised part, make comments and investigate the possibility of applying the most recent editions of the standards indicated below. For undated references, the latest edition of the publication referred to applies to this standard.

OECD Guidelines for the testing of chemicals: 3T3 NRU phototoxicity test. NO. 432

3 Terms and Definitions

The following terms and definitions apply to this method.

3.1 Phototoxicity

A kind of skin toxic reaction that is elicited after the first exposure of skin to chemicals and subsequent exposure to long wave ultraviolet irradiation.

3.2 Cell viability

Parameter measuring total viability of a cell population (e.g., uptake of the vital dye Neutral Red into cellular lysosomes), which, depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of the cells.

3.3 Relative cell viability

Cell viability expressed in relation to solvent (negative) controls which have been taken through the whole test procedure (either +/-Rr or -Irr) but not treated with test chemical.

3.4 Photo irritation factor; PIF

Factor generated by comparing two equally effective cytotoxic concentrations (IC_{50}) of the test chemical obtained in the absence (-Irr) and in the presence (+Irr) of a non-cytotoxic irradiation with ultraviolet/visible (UV/vis) light.
3.5 IC₅₀

The concentration of the test chemical by which the cell viability is reduced by 50%.

3.6 Mean photo effect; MPE

Measurement derived from mathematical analysis of the two concentration response curves obtained in the absence (-Irr) and in the presence (+Irr) of a non-cytotoxic irradiation with ultraviolet/visible (UV/vis) light.

3.7 Prediction model

An algorithm converting the results of toxicity test to the prediction of toxicity potential. In this method, PIF and MPE can be used to convert the results of in-vitro 3T3 neutral red uptake phototoxicity test to a prediction of phototoxicity potential.

4 Test Principle

Phototoxicity is defined as a toxic response from a substance applied to the body which is either elicited or increased (apparent at lower dose levels) after subsequent exposure to light, or that is induced by skin irradiation after systemic administration of a substance. Neutral red is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion, accumulating intracellularly in lysosomes. The reaction of certain chemicals and external conditions can cause alterations of cell surface or lysosomal membrane sensitivity and such alterations lead to an increased lysosomal fragility and other irreversible cytotoxic changes, thus resulting in a decreased ability of cells to absorb neutral red.

This test method is an attempt to judge whether a chemical is phototoxic by measuring the ability of 3T3 fibroblast cell to absorb neutral red after a combined action of chemical and ultraviolet irradiation or the change of cytotoxicity.

5 Test Materials and Reagents

(1) Type of light source

Criteria for the choice of an appropriate light source must include the requirement that the light source emits wavelengths can be absorbed by the test chemical (absorption spectrum) and that the dose of light (achievable in a reasonable exposure time) shall be sufficient for the detection of known photocytotoxic chemicals. Furthermore, all the wavelengths and doses employed shall not be deleterious to the test system, for example, the emission of heat (infrared region) or the interference of high cytotoxicity like UVB wavelengths, so the light source is required to stably release UVA and visible wavelengths.

Because all solar simulators emit significant quantities of UVB, suitable filtration shall be made to enable UVB<0.1 J/cm². The light intensity through the 96-well tissue culture plate lid is recommended to be 1.7 mW/cm² light intensity (i.e., 5 J/cm²) dose.

(2) Cell line

A permanent mouse fibroblast cell line-Balb/c 3T3 fibroblast cell shall be selected. It is required that the cells must source from the credible institution and a stable cell quality can be guaranteed.

Because the UVA sensitivity of cells may increase with the number of passages, it is recommended that...
Balb/c 3T3 fibroblast cells of the passage number, preferably less than 100, shall be used in the test.

If the test unit cultures the cell line by itself, the cell line shall be checked regularly about its sensitivity to the UV light for an absence of mycoplasma contamination.

(3) Cultures

Adopt DMEM culture medium, 10% new-born calf serum, 4 mmol/L glutamine and antibiotics [penicillin (100 IU), and streptomycin (100 μg/mL)], and incubate at 36.5°C-37.5°C and 5%-7.5% CO₂.

(4) Selection of solvents

Before the measurement, the solubility of the test substance shall be evaluated first for the purposes of selecting the optimal solvent system. The solvent shall not have a chemical reaction with the test substance and shall not affect the cell viability.

The test substance capable of being soluble in water and having a concentration of 1000 μg/mL can be dissolved in the pre-warmed (37°C) and sterilized phosphate buffer (EBSS or PBS). Test chemicals (<1000 μg/mL) of limited solubility in water can be dissolved in dimethylsulphoxide (DMSO) or ethanol (ETOH) and other solvents. When DMSO or ETOH is used as the solvent, its final concentration shall not exceed 1% (v/v) of the total volume. The negative control group shall have the same volume ratio of solvent as that of the test group.

(5) Preparation of test substances

The test substance must be freshly prepared before use. It is recommended that all chemical handling and the initial treatment of cells be performed under light conditions that would avoid photoactivation or degradation of the test substance.

The blank control, solvent control, and positive control (chlorpromazine is recommended) shall be set for each experiment. See Annex B for the schematic diagram of sample injection.

(6) Setting of the dose of the test substance

The concentration ranges of a chemical tested in the presence (+Irr) and in the absence (-Irr) of light shall be determined through the pre-experiment. The test substance stock solution is diluted to 8 concentrations using the same constant dilution factor (for example,√10 = 3.16) with a solvent, and the relevant concentration range shall include the concentrations from maximum cytotoxicity to almost no cytotoxicity (range of 20%-100% cell viability).

If the pre-experimental results indicate that the cytotoxicity is still absent at a concentration of 1000 μg/mL, then the highest concentration is recommended to be 1000 μg/mL; if the cytotoxicity is present at the concentrations (<3 in number) of less than 1000 μg/mL, repeated test or a smaller dilution factor is required until at least three concentrations show the cytotoxicity. If the highest concentration cannot reach 1000 μg/mL depending on the solubility of the test substance, then the concentration at the maximum solubility is taken as the highest concentration to prevent precipitation of the test substance at any concentration.

If the test substance is still not cytotoxic at the highest concentration in the absence (-Irr) of light but strongly cytotoxic in the presence (+Irr) of light, different test concentrations can be used in the -Irr test and the +Irr test.
(7) Neutral Red (NR)

The chemical name: 3-amino-7-dime-thylamino-2-methylphenazine hydrochloride, CAS No.: 553-24-2; Or national drug reference material, No.: 100460.

(8) Neutral red solution

1. Neutral red stock solution. Weigh 0.4 g of neutral red dye and dissolve it in 100 mL of distilled water (available for 2 months at room temperature)

2. Neutral red working solution. Add 1 mL of neutral red stock solution to 79 mL of DMEM culture solution, the resulting solution is the working solution. The final concentration of neutral red is 50 μg/mL.

(9) Neutral red desorption solution

Distilled water, ethanol, and acetic acid are prepared in a ratio of 49:50:1 (freshly prepared before use, be stored for no more than 1 h).

6 Test Procedures

(1) Add 100 μL of culture medium into the peripheral wells of a 96-well tissue culture plate (blank control), and add 100 μL of cell suspension of 1x10⁵ cells/mL in the remaining wells (1x10⁴ cells/well). Two plates shall be prepared for each test, including the same test substance concentration series, solvent control, blank control and positive control, one plate for the determination of cytotoxicity (-Irr) and the other plate for the determination of phototoxicity (+Irr).

(2) Incubate cells for 24 h (5%-7.5% CO₂, 37°C) until they form a monolayer half-saturated cell. This incubation period allows for cell recovery and adherence.

(3) Decant culture medium, and gently rinse the cells once or twice with 150 μL of EBSS or PBS. Add 100 μL of the buffer containing the appropriate concentration of test chemical or solvent into the wells. Incubate cells for 1 h (5%-7.5% CO₂, 37°C).

(4) Perform the +Irr exposure for one of the plates at room temperature, irradiate the cells for 50 min through the lid of the 96-well plate at a light intensity of 1.7 mW/cm². Keep non-irradiated plate (-Irr) at room temperature in the dark for 50 min.

(5) Decant test solution and carefully rinse the cells twice with 150 μL of EBSS or PBS. Add 100 μL of the culture medium and incubate overnight (18-22 h, 5%-7.5% CO₂, 37°C).

(6) Examine the cells with the phase contrast microscope and record the changes in cell morphology caused by the cytotoxicity of test substance so as to eliminate the test error.

(7) Neutral red uptake (NRU) test. The cell uptake of neutral red into lysosomes and vacuoles in viable cells can be used as quantitative indicators of cell number and viability.

1. Wash the cells once or twice with 150 μL of the pre-warmed EBSS or PBS. Remove the washing solution by gentle tapping of plates. Add 100 μL of the culture medium containing 50 μg/mL neutral red and incubate the cells for 3 h under the conditions of 5%-7.5% CO₂, 37°C and proper humidity.

2. Remove the NR medium, and wash cells once or twice with 150 μL of EBSS or PBS.
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