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Indian Standard

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प्रसाधन सामग्री के सुरक्षा मूल्यांकन के लिए परीक्षण की पद्धतियाँ

(तीसरा पुनरीक्षण)

Methods of Test for Safety Evaluation of Cosmetics

(Third Revision)

ICS 71.100.40

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FOREWORD

This Indian Standard (Third Revision) was adopted by the Bureau of Indian Standards, after the draft finalized by the Cosmetics Sectional Committee, had been approved by the Petroleum, Coal and Related Products Division Council.

In this revision, the standard has been divided into three sections. Section 1 provides guidelines for skin irritation testing of cosmetics and Section 2 covers guidelines for assessing contact hypersensitivity and photosensitization. It includes tests to enable the diagnosis of contact allergy and photosensitization. Section 3 includes an Informative Annexure covering inclusion-exclusion criteria and latest updates on alternatives to in-vivo safety testing on animals.

Depending on the products, prior human participant studies may be carried out in order to confirm the safety of the cosmetic finished product and to evaluate its tolerance in humans. Any human testing must be scientifically justified, as unjustified testing is unethical. The test should be conducted on selected panels of human volunteers, using good clinical practices, under the supervision of dermatologist, and/or ophthalmologist and/or pediatrician (depending on the nature of the evaluation). These tests are not intended to be carried out on a batch-to-batch basis or random samples as a quality control tool.

Many new cosmetic products are variations on the existing ones. It is the task and responsibility of the qualified safety assessor in-charge of product safety evaluation, to decide whether each new formula represents a minor adaptation without toxicological significance, a toxicologically important modification, or a new technological concept.

Safety assessor's decision is key in the choice of the subsequent safety-testing programme, which is constructed on the case-by-case basis. Examination of the degree of innovation of the new formula in relation to the known formula requires considerable experience in the field of cosmetic toxicology. The outcome of safety assessor's conclusion is the decision on the necessity to carry out or not, additional clinical safety testing and extent of the test, in order to confirm formulas tolerance in humans.

There is sufficient published scientific evidence to support the concept of universality of human skin reactions to chemicals. In this respect, a qualified safety assessor applies his or her knowledge to predict, from the available data, the market place tolerance of the new product. It will be upto the safety assessor, allowing for the state of knowledge, to conclude that the product should not present unacceptable risks of damage to human health under normal or reasonable foreseeable conditions of use anywhere in the world.

Prior to conducting any tests on human volunteers one needs to check each ingredient in the formulation for potential effects through available literature or on the basis of Quantitative Risk Assessment (QRA) techniques. *In vitro, in chemico* and Quantitative Structure-Activity Relationship Models (QSAR) should be used in a Weight of Evidence (WoE) approach prior to testing in humans.

Indian Standard

METHODS OF TEST FOR SAFETY EVALUATION OF COSMETICS

(Third Revision)

1 SCOPE

This standard covers methods of test for safety evaluation of cosmetics.

2 REFERENCES

The following standards contain provisions, which through reference in this text, constitute provisions of this standard. At the time of publication the conditions indicated were valid. All standards are subject to revision and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below.

IS No.	*Title*
4707	Classification of cosmetics raw materials and adjuncts:
(Part 1) : 2001	Dyes, colours and pigments
(Part 2) : 2017	List of raw materials generally not recognized as safe for use in cosmetics

3 TERMINOLOGY

For the purpose of this standard, the following definition and explanations shall apply.

3.1 Allergic Reactions — Symptoms/Sign caused by exposure to allergens.

3.2 Bullae — Bullae are blisters on the skin each of which is more than 5 mm in diameter. These may be of any colour and shape.

3.3 Carcinogenicity — Carcinogen is any substance that is an agent either directly involved in causing cancer or increasing its incidence or promotes the activity of other carcinogens in causing cancer. Since genetic events are central in the overall process of cancer development, evidence of mutagenic activity may indicate that a chemical has a potential for carcinogenic effects. Carcinogenicity studies are performed for substances where there is concern about their carcinogenic potential.

3.4 Cauterizing Agents — Chemical substances which burn the skin on local application.

3.5 Cauterizing Reaction — The change in the skin produced by the local application of a cauterizing agent.

3.6 Cross Sensitization — Sensitization to a primary allergen spreading to one or more allergens which are of such closely similar chemical constitution to the primary allergen that the sensitized cells are unable to distinguish between them. This has to be distinguished from false cross sensitization which occurs when the same chemical substance is present in different products.

3.7 Dermatitis — Inflammation of the skin.

3.8 Epidermal Barrier — Epidermal barrier pertaining to the most superficial layer of the skin (stratum corneum) which allows only certain substances to enter the skin from outside.

3.9 Erythema — Redness of the skin due to dilation of the blood vessels.

3.10 Eschar Formation — A dry scab or slough formed on the skin as a result of a burn or by the action of a corrosive or caustic substance.

3.11 Exudation — The discharge of fluids (serum or pus) on the diseased skin surface.

3.12 Fissuring — Cracks on the surface of the skin.

3.13 Genotoxicity — Genotoxicity describes the property of chemical agents that damages the genetic information within a cell causing mutations, which may lead to cancer. While genotoxicity is often confused with mutagenicity, all mutagens are genotoxic; however, not all genotoxic substances are mutagenic.

3.14 Hyperaemia — Increased blood circulation.

3.15 Hypersensitivity (Sensitization) — A process by which an individual develops the capability of reacting in an abnormal (allergic) manner to an external agent.

3.16 Milliaria — A papular or vesicular eruption on the skin which accompanies profuse sweating and is due to the blockage of the ducts of the sweat glands.

3.17 Mutagenicity — Mutagenicity refers to the induction of permanent transmissible changes in the structure of the genetic material of cells or organisms. These changes (mutations) may involve a single gene or a block of genes.

3.18 Necrosis — Death of a portion of a tissue.

3.19 Occlusion Area — An area of skin which is isolated from the environment.

3.20 Oedema — The swelling of soft tissues (skin) as a result of excess fluid accumulation.

3.21 Papules — A small circumscribed solid elevation of the skin not larger than 5 mm in diameter. It may be of any shape and colour.

3.22 Percutaneous Absorption — Transfer of an agent (usually a chemical) through the skin from outside.

3.23 Photopatch Test — A test where the skin is exposed to sunlight or any other equivalent source of light after contact with a particular chemical agent.

3.24 Pustular Reaction — A reaction in the skin characterized by appearance of several lesions (eruptions) containing pus.

3.25 Repeated — Insult Irritant — An agent which causes an irritant reaction only after repeated applications to the surface of the skin.

3.26 Skin Sensitization — An allergic reaction to a particular irritant that results in the development of skin inflammation and itchiness. Unlike skin irritation, the skin becomes increasingly reactive to the substance as a result of subsequent exposures. A skin sensitizer is a substance that will induce an allergic response following skin contact.

SECTION 1 GUIDELINES ON SKIN IRRITATION TESTS

4 GENERAL

4.1 Raw Material Purity

The purity of raw materials used in a formulation must be established by physicochemical analysis and specifications established for use, before the products containing them are subjected to safety testing.

4.2 Facilities

The testing should be carried out by the manufacturer in-house or in reputed laboratories both national and international, which maintain high standards including compliance to national regulatory guidelines for human participant studies.

The test should be carried out by trained personnel under the supervision of toxicologists (for example, persons having qualifications and/or competence in biomedical research methodology in the case of human volunteer studies).

4.3 Tests

The type of tests to be performed for particular type of cosmetics is given in Table 1.

Additional data may be generated on skin sensitization and/or skin photosensitization, if the product contains any suspect ingredients, which are above the recommended levels that are indicated by the international agencies.

Carcinogenicity, Genotoxicity and Mutagenicity testing may be necessary using various methods as part of an integrated testing strategy or weight of evidence approach. Human carcinogens identified by IARC monograph 1992 (and updated later) must never be directly added into the cosmetic formulations. Naturally occurring chemicals also need to be safety tested.

4.3.1 *Skin Irritation Test (Patch Test in Humans)*

4.3.1.1 *Principle*

Irritants are substances that may damage the skin. The damage will depend upon the nature, concentration and duration of exposure. Irritation is manifested as erythema (redness), oedema (swelling), vesiculation and finally to an intense suppurate reaction without the involvement of immune system. The irritation potential of a substance can be assessed in human patch test. This patch test is carried out on human volunteers.

This test with the product may be carried out in humans, only after ensuring that all the ingredients used have acceptable toxicological end points based on available literature or by carrying out alternate evaluation techniques. No product with unknown ingredient should be directly tested in humans. The same is also applicable for new/novel ingredients.

Table 1 Tests to be Carried Out on Different Types of Cosmetics
(*Clause* 4.3)

Sl No.	Test	Type of Product
(1)	(2)	(3)
i)	Skin irritation test	Skin and hair products and lip products.
ii)	Photo irritation tests	Skin and hair products and lip products showing a potential of causing photo-irritation.
iii)	Relevant *in-vitro*/*ex-vivo* tests from those listed in Annex B	Skin and hair products, lip products and oral care products prior to human participant studies.

Table 2 Method of Product Application*
(*Clause* 4.3.1.2)

Type of Patch	Products
(1)	(2)
Open	Paints, glues, essential oils, perfumes
Semi occluded (keep open for up to half an hour post application and then occlude	Nail lacquers, colognes, after shave lotion, alcohol based products, solvent based products
Occluded	Skin powder for infants, shampoos (8%), surfactant based products (8%), skin creams, hair creams, hair oils, pomades, brilliantine, cosmetic pencils, lipstick, lip salve, bindi-liquid, kum kum powder, liquid foundation make up, sindoor, cold wax, face pack, kajal, herbal cosmetics, leave-on and rinse-off conditioners, mascaras
Short duration open patch test (30 min)	Dyes, colours, pigments, oxidation hair dyes, henna powder, depilatories, bleaching creams, permanent wave solutions

* The above list is not exhaustive but indicative of the categories. Any new category or new product should be similarly considered, depending on its nature and end use.

4.3.1.2 *Procedure*

Apply the neat cosmetic product as such (or diluted solution where defined in Table 2) on the upper arms or back of human subjects as follows:

The skin should essentially be non-hairy and free from any type of skin lesions or other dermatological problems. In case the back is hairy, the hair can be cut off by clipper or shaver before applying the patches, taking care to avoid any abrasions, cuts or lesions. In case of rinse off products, rinse the treated sites with water to remove any residue after removal of the patch (or the specified time of contact in case of open patch).

If the volunteer experiences unbearable discomfort with any of the patches the volunteer is instructed to remove such patches any time prior to the targeted 24 h contact (where applicable). Mark such sites with a blue/ black skin marker to facilitate evaluation later.

The volunteer is also requested to note down the sign and symptoms of the discomfort and the time of removal of the patch and hand it over to the investigator. Assess the skin reactions subjectively using the Draize scale given in Table 3, 24 h after removal of the patches. Follow up the reactions if any, after one week to confirm recovery and later if necessary at the discretion of the investigator.

4.3.1.2.1 *Human subjects*

Select 24 healthy adult subjects, preferably equal number of males and females who do not have any previous history of adverse skin conditions and are not under medication likely to interfere with the results. Pregnant ladies and breast-feeding mothers should be excluded. The typical inclusion /exclusion criteria to be kept in mind are as listed in Annex A. Explain the test procedure to volunteers and obtain a signed informed consent from each of them.

4.3.1.2.2 *Test patches for topical treatment*

Ideally use ready-made standard test patches (Finn chambers/IQ chambers or such suitable equivalent) measuring about 1 cm diameter or 1 sq cm. Take 0.04 ml or 0.04 g of the sample using a micropipette or weighing balance (as applicable) on the patch and apply the patch on the upper arm or back as mentioned in **4.3.1.2**. Alternatively if such patches are not available, use 1 cm diameter discs made out of chromatography paper (Whatman No. 3) taken on a slightly bigger polythene sheet having about 0.25 cm hole punched at the centre and fixed on the adhesive tape. Keep about 2.5 cm distance between the two adjacent test patches (filter paper discs).

4.3.1.2.3 *Volatile substances*

Volatile substances can act as primary irritant if tested by the standard occluded patch test technique. Products having volatile substances, where the volatile substances are likely to be in contact with the skin for a longer duration of time should, therefore, be tested by the cup technique. In this method, the volatile agent is soaked into a small piece of absorbent cotton filter paper which is placed at the bottom of specially designed cup (*see* Fig. 1). This cup is inverted on the forearm skin of the individual and bandaged. Care should be taken to ensure that the piece of filter paper remains sticking to the bottom of the cup and does not fall on the skin, and the skin is exposed only to the vapours of the agent.

4.3.1.2.4 *Positive and negative control*

Use Sodium Lauryl Sulphate (SLS) analytical grade at 1 percent (*w/w*) concentration in distilled water in the form of occluded patch as the positive control.

Use 0.9 percent Isotonic Saline Solution in the form of occluded patch as negative control.

4.3.1.3 *Observation and scoring*

Assess the skin reaction under a constant artificial daylight source, 24 h after the removal of the patches (or 24 h after product contact in the case of open

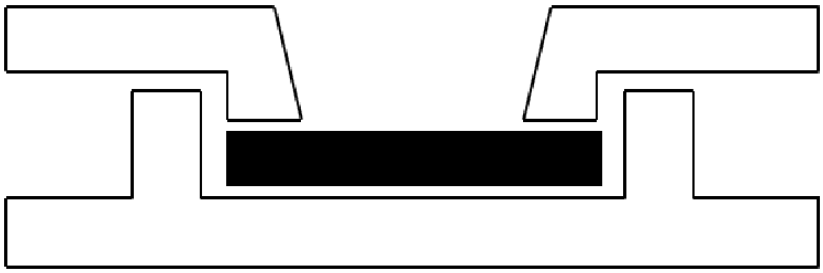

FIG. 1 CUP FOR VOLATILE SUBSTANCES

patches). Score the reaction, namely, erythema (including dryness, scaliness and wrinkles) on a 0-4 point scale and oedema on another 0-4 points scale as per the Draize Scale given in Table 3.

4.3.1.4 *Results*

The combined mean scores and standard deviation of the 24 subjects are calculated:

a) Positive control must give a combined mean score of < 2.0. Negative control must give a combined mean score of < 2. If positive control gives a combined mean score less than 2.0 and/ or negative control gives a combined mean score of < 2.0, then the test need to be repeated on another group of newly recruited volunteers.
b) A combined mean score of up to 2.0/8.0 will mean that product is non-irritant.
c) Usage of cosmetic product with a score up to 4.0/8.0 which is mildly irritating may be reviewed by manufacturer for safety of the formulation.
d) No cosmetic product should be marketed which has irritation score above 4.0/ 8.0.

4.3.2 *Photopatch Test*

4.3.2.1 In case a substance is considered to produce skin irritation by photocontact-sensitivity, it will be necessary to undertake photopatch tests rather than the standard patch tests. This evaluates the potential of a product to induce phototoxicity (photo-irritation). This test is of particular significance if the suspected photo-irritant product (or ingredient) shows absorbance in the UV-A and UV-B region (290-400 nm).

4.3.2.2 Phototoxicity occurs due to the formation of toxic photoproducts and results in tissue damage.

4.3.2.3 Sample size for the evaluation is typically 24 subjects.

4.3.2.4 For this, each substance is applied in duplicate patches in the same manner as for standard patch tests, but after 24 h, one of the patches in each pair is removed and the skin at the test site is exposed to sunlight for 30 min or appropriate sources of ultraviolet light (UVA 5 J/cm^2). These patches are covered again. The other patches in each of the pairs are left undisturbed.

4.3.2.5 One additional site in the adjoining skin is exposed to sunlight or ultraviolet light for the same period to act as a control for the ultraviolet exposure.

4.3.2.6 After a further period of 24 h, all the patches are removed and the test sites are examined for evidence of skin irritation. Scoring is carried out using Draize scales (Table 3) and results are reported as combined mean scores. The photopatch test is

Table 3 Draize Scale for Scoring the Treatment Sites
(*Clauses* 4.3.1.3 *and* 4.3.2.6)

Score for Erythema/ Dryness/ Wrinkles	Reaction	Score for Oedema	Reaction
(1)	(2)	(3)	(4)
0	No reaction	0	No reaction
1	Very slight erythema/dryness with shiny appearance	1	Very slight oedema
2	Slight erythema/dryness/wrinkles	2	Slight oedema
3	Moderate erythema/dryness/wrinkle	3	Moderate oedema
4	Severe erythema/wrinkle/scale	4	Severe oedema

considered to be positive if there is no reaction at the site of the standard patch (not exposed to light), but the site of the exposed patch (exposed to sunlight or UV light) shows evidence of skin irritation. The skin area taken as control and exposed only to sunlight or ultraviolet light should also not develop any skin irritation for the results to be valid.

4.4 Use Test

4.4.1 After the cosmetic has passed appropriate tests, 15 human volunteers shall be asked to use the cosmetics as normally used for 15 days. If there is no adverse reaction, the cosmetic may be released for consumer tests.

4.5 Restricted Consumer Test

4.5.1 The cosmetic may be then released into the market on a limited scale and should be closely monitored for any adverse consumer response. It is customary to release between 5 000 and 10 000 units of the cosmetic. In case adverse reactions are reported/collected during this period, they should be studied adopting appropriate tests.

NOTES

1 It is important to realize that all tests are experimental laboratory based and therefore, the results cannot be considered to represent exactly what is likely to happen when the cosmetic is actually released for mass use. It will, therefore, be necessary to keep a watch on the reactions if any, which may occur following general release of the cosmetics when the agent is used by a much larger number of individuals and for much longer periods.

2 It is also known that there are some differences in the reactivity of the skin of various racial groups, the two sexes and various age groups. It will therefore, be appropriate to perform the above mentioned tests on the same type of individuals who are targeted to be the main consumers of that particular cosmetic.

SECTION 2 GUIDELINES ON ASSESSING CONTACT HYPERSENSITIVITY AND PHOTOSENSITIZATION

5 GENERAL INFORMATION ABOUT CONTACT HYPERSENSITIVITY

5.1 Before an individual can manifest an allergic reaction to a locally applied agent, he normally develops hypersensitivity to the agent. In biological terms, this means that some lymphocytes in the blood acquire the capability of recognizing and reacting with the same chemical whenever and wherever that chemical is applied on the skin. An individual may start developing hypersensitivity following the very first exposure but generally it does not happen this way. Nevertheless, every exposure to the allergen has a chance of sensitizing the individual. Therefore, the more the number of exposures, the greater the chances of developing hypersensitivity. This also means that individual develops allergy to only those agents which he or she has already been using for several days, months or even years. Once, however, an individual becomes allergic to a substance, he is likely to develop a reaction following every subsequent exposure provided the exposure is adequate.

5.2 If an individual is exposed to a chemical agent for the first time, he is not likely to be allergic to that agent and, therefore, he cannot develop an allergic reaction. Sometimes, however, if the individual had already been allergic to another chemical agent, whose chemical structure resembles that of the present chemical, then even the so called first exposure will be able to produce an allergic reaction. This is called a reaction of cross-sensitivity. It is, however, important to be aware that many times, a person may get exposed to an agent without being aware of it. Thus he may become allergic during one such exposure and develop an allergic reaction during the subsequent exposure.

5.3 Some substances may cause sensitization more easily than others. The chemical basis of this characteristic of these agents is not known, but such substances are considered to be more potent sensitizers than the others.

5.4 Stratum corneum, the most superficial layer of skin is a very selective barrier which allows only some substances to pass through. It is quite obvious that if a substance cannot penetrate this barrier and enter the skin, it cannot cause contact allergy. In case, however this barrier is defective due to disease or injury, there is a greater change of an allergic reaction.

5.5 The process of sensitization takes a minimum of 5 days during which there are no clinical signs or symptoms. Thus even if an individual starts developing hypersensitivity following the very first exposure, there will be no clinical signs or symptoms during the first 5 days. However, after the individual has become allergic, a subsequent exposure will result in signs and symptoms within a day or two.

6 CLINICAL SIGNS AND SYMPTOMS

6.1 The earliest indication of an allergic reaction is itching. This is soon followed by redness. If the reaction is mild, it may subside soon, but generally, because of continued exposure, the reaction progresses through papules, papulo-vesicles, vesicles and bullae, exudation and crusting. These reactions are called acute reactions. Subacute reactions generally result in erythema and scaling, while chronic reactions due to continued exposures may lead to darkening and thickening of the skin. On the lips and palms, the reaction frequently manifests as scaling and fissuring.

6.2 The reaction is generally limited to the area where the causative substance is applied. Sometimes, however, the agent may get applied on the other areas of the body

by mean of the fingers, clothes, pillows, etc. In such instances, the reaction will be sent at other areas as well.

6.3 If further applications of the agent are continued, the reaction keeps on increasing in severity and extent, but in case further applications are stopped, the reaction tends to subside even without any treatment.

6.4 The degree of allergy varies in different individuals. Some individuals are more severely allergic and, therefore, they will react to even smaller amounts of substance. Moreover, an allergic individual will develop a severe reaction if exposed to a larger amount of substance. Conversely, an allergic individual may not develop any signs or symptoms if he is not exposed to an adequate amount of the substance.

6.5 Photocontact Dermatitis

Some agents, particularly dyes and perfumes, cause dermatitis only after the skin area on which they have been applied is exposed to sunlight or ultraviolet rays. Sunlight alone or the agent alone (without exposure to sunlight) does not result in dermatitis. This type of reaction often consist of darkening of the skin, though in acute cases, itching, papular, papulo-vesicular or scaly lesions may also be present.

7 DIAGNOSTIC TESTS FOR CONTACT HYPERSENSITIVITY

Prior to conducting any tests on human volunteers for contact hypersensitivity, one needs to check each ingredient in the formulation for potential to produce contact allergy through available literature or on the basis of dermal sensitization Quantitative Risk Assessment (QRA) techniques. In vitro methods and Quantitative Structure–Activity Relationship models (QSAR models) should be used in a Weight of Evidence (WoE) approach prior to the HRIPT. If all the ingredients do not show any contact allergy potential, HRIPT with the final product is recommended as a confirmatory test to rule out any ingredient-ingredient interactions. HRIPT should not be carried out directly on human volunteers with the product, if it contains unknown ingredients or ingredients with moderate/ strong sensitization potential. The same is also applicable for new/novel ingredients.

7.1 Human Repeat Insult Patch Test (*HRIPT/RIPT*)

7.1.1 This test is based on the principle that in an allergic individual, the whole skin is capable of reacting with the allergen. Therefore, if the substance is applied on the skin on any part of the body, it will result in dermatitis at the site. Although this test is simple, yet a little experience is essential for accurate interpretation of results.

7.1.2 The test should not be applied if the volunteer is already having acute dermatitis because there is risk of aggravation of the dermatitis and occurrence of false positive reactions. The test should also be postponed if the volunteer is being given systemic corticosteroid or other cyclophosphamide, methotrexate, azathioprine, etc, because these drugs can suppress the reaction and lead to false negative results. Further, it should be ensured that the volunteer is not under any form of medication immediately before or during the study, which might impact the outcome of the study; in which case the volunteer should be excluded or dropped from the study as the case may be.

7.1.3 The substance to be used as allergen for human repeat insult patch test may be either the finished product as such or preferably each of the suspect ingredient used in the product.

7.1.4 When using ingredients, it is important to use each agent in a specified concentration and also in a specified vehicle (or base). This is necessary to avoid false positive or false negative results, because if the concentration of the agent is too high, it may result in irritant dermatitis at the test site, while if the concentration is too low, it may not evoke any reaction. Similarly, if the vehicle used for dissolving the agent does not allow the agent to penetrate the epidermal barrier, it may result in a false negative reaction. In the case of cosmetic, it will be especially useful if agents which may be used as substitutes of the standard ingredients are also included for patch tests. This will help the industry to know the substitutes which may be employed in the manufacture of the cosmetic in place of the ingredient causing contact dermatitis.

7.1.5 Normal saline (0.9 percent aqueous solution of sodium chloride) or any other agent(s) used as a vehicle for dissolving the ingredient should also be applied as controls for the human repeat insult patch test. It is necessary to ensure that the controls do not produce any reaction at the site of patch test.

7.1.6 *Procedure*

Prior to conducting the HRIPT, a preliminary Skin Irritation Test is carried out (as per methodology described in **4.3.1**) on about 5-10 volunteers using different concentrations of the test product or ingredient to be tested, prepared by using suitable diluents (where necessary) as explained in **7.1.4** in order to arrive at the highest non-irritant concentration to be used for conducting the HRIPT study. Apply the neat cosmetic product as such (or diluted solution where defined in Table 4) or the highest non-irritant concentration as determined through the Preliminary Patch Test (if the highest non-irritant concentration is found to be lower as compared to the neat product or the dilution where

Table 4 Method of Product Application*
(*Clause* 7.1.6)

Type of Patch (1)	Products
Open	Paints, glues, essential oils, perfumes
Semi-occluded (keeps open for up to half an hour post application and then occlude.)	Nail lacquers, colognes, after shave lotion, alcohol based products, solvent based products
Occluded	Skin powder for infants, shampoos (8%), surfactant based products (8%), skin creams, hair creams, hair oils, pomades, brilliantine, cosmetic pencils, lipstick, lip salve, bindi-liquid, kum kum powder, liquid foundation make up, sindoor, cold wax, face pack, kajal, herbal cosmetics, leave-on and rinse-off conditioners, mascaras
Short duration open patch test (30 min)	Dyes, colours, pigments, oxidation hair dyes, henna powder, depilatories, bleaching creams, permanent wave solutions

* The above list is not exhaustive but indicative of the categories. Any new category or new product should be similarly considered, depending on its nature and end use.

specified in Table 4) on the upper arms or back of human subjects as follows:

The skin should essentially be non-hairy and free from any type of skin lesions or other dermatological problems. In case the back is hairy, the hair can be cut off by clipper or shaver before applying the patches, taking care to avoid any abrasions, cuts or lesions.

In case of rinse off products, rinse the treated sites with water to remove any residue after each patch removal (or the specified time of contact in case of open patch).

If the volunteer experiences unbearable discomfort with any of the patches the volunteer is instructed to remove such patches any time prior to the targeted 24 h contact (where applicable). Mark such sites with a blue/black skin marker to facilitate evaluation later.

The volunteer is also requested to note down the sign and symptoms of the discomfort and the time of removal of the patch and hand it over to the investigator.

7.1.6.1 Select 200 healthy adult subjects, preferably equal number of males and females who do not have any previous history of adverse skin conditions or allergic reaction and are not under medication likely to interfere with the results. Pregnant ladies and breast-feeding mothers should be excluded. The typical inclusion / exclusion criteria to be kept in mind are as listed in Annex A. Explain the test procedure to volunteers and obtain a signed informed consent from each of them.

7.1.6.2 Ideally use ready-made standard test patches (Finn Chambers/IQ Chambers or such suitable equivalent) measuring about 1 cm diameter or 1 cm^2. Take 0.04 ml or 0.04 g of the sample using a micropipette or weighing balance (as applicable) on the patch and apply the patch on the upper arm or back as mentioned in **4.3.1.2**. Alternatively if such patches are not available, use 1 cm diameter discs made out of chromatography paper (Whatman No. 3) taken on a slightly bigger polythene sheet having about 0.25 cm hole punched at the centre and fixed on the adhesive tape. Keep about 2.5 cm distance between the two adjacent test patches (filter paper discs).

For volatile substances, refer to **4.3.1.2.3** for sample application.

7.1.6.3 Various allergens should be incorporated into different patches and all these patches should be applied on the skin of the volunteer so that the allergen is in direct contact with the skin. Various patches should be numbered and an accurate account of the allergens put on these patches should be entered into a register for records.

7.1.6.4 The patches are generally applied on the back or the upper arm. The skin should essentially be non-hairy and free from any type of skin lesions. In case the back is hairy, the hair can be cut off by clipper or shaver before applying the patches, taking care to avoid any cuts or lesions.

7.1.6.5 *Timing and location of the patch test*

7.1.6.5.1 *Induction phase*

There is first induction phase where patches as described above, will be applied on thrice a week basis for three weeks (single patch of the sample on each volunteer). The patch is removed after 24 h of contact each time and clinical examination is carried out 24 h after patch removal (or 24 h after product contact in the case of open patches). In case the original site shows a reaction of >2 on the erythema scale, then an adjacent area will be patched.

7.1.6.5.2 *Rest phase*

After the three week induction phase, there will no more patch application, this is called as rest phase. The rest phase involves two weeks (14 days) of no application of any patch in order to develop (if at all) sensitization to the test product.

7.1.6.5.3 *Challenge phase*

During challenge phase, single patch for each product

or ingredient (applied during induction phase) will be put on a new site on upper arm or back (naïve skin site) at approximately a similar position.

Patch is removed at 24 h and scoring done at 24 h, 48 h, and 72 h after removal (or 24 h, 48 h, and 72 h after product contact in the case of open patches) (120 h optional).

The Draize scale (erythema and oedema) is used for evaluation during induction phase (*see* Table 5).

The International Contact Dermatitis Research Group Scale (ICDRG Scale) is used for evaluation during challenge phase (*see* Table 6).

7.1.6.5.4 Reactions produced by solid substances are generally milder, as the amount of the allergen that can enter the skin is usually much less. Thus even milder reactions produced by solid agents should be considered significant.

7.1.6.5.5 Sometimes, when the hypersensitivity in a volunteer is very mild, there may be no reaction at the patch test site after 48 h, though after an additional 24 h, there may be evidence of dermatitis. It is, therefore, preferable to take another reading after approximately 72 h.

7.1.6.5.6 Reaction during induction phase suggests skin irritation or pre-existing sensitization to the test material. Prominent reactions in the challenge phase suggest allergenicity to the ingredients of the test material.

7.1.6.5.7 Some individuals develop dermatitis reaction to the adhesive plaster which may spread to involve the entire test site, making it difficult to observe any reaction to the allergen. In such an instance the patch can be applied on the upper arm using only small strips of adhesive plaster or suitable hypo allergenic plaster.

7.1.6.5.8 Sometimes particularly in summer, the volunteer may develop a miliaria (prickly heat) — like reaction at the patch test site due to occlusion. This reaction is not reproducible and, therefore, it should be regarded as false positive reaction.

7.1.6.5.9 Some chemicals particularly nickel sulphate can at times lead to a pustular reaction which also is non-specific.

NOTE — A report of a Human Repeat Insult Patch Test is not complete unless it mentions the following data:

a) Concentration of the chemical/ agent;
b) Amount used;
c) Areas of skin contacted;
d) Site of application;
e) Number of days the patch was left on the skin; and
f) Period after removal of the patch that the readings were made.

7.2 Exposure and Withdrawal Test

7.2.1 Hypersensitivity of an individual to an agent can also be confirmed by asking the individual to stop the suspected agent for a week or so. If the reaction subsides during this period, it suggests that the agent was probably responsible for the dermatitis. Then the individual should be asked to use the agent again. In

Table 5 Draize Scale for Scoring the Treatment Sites
(*Clause* 7.1.6.5.3)

Score for Erythema/ Dryness/Wrinkles	Reaction	Score for Oedema	Reaction
(1)	(2)	(3)	(4)
0	No reaction	0	No reaction
1	Very slight erythema/dryness with shiny appearance	1	Very slight oedema
2	Slight erythema/dryness/wrinkles	2	Slight oedema
3	Moderate erythema/dryness/wrinkle	3	Moderate oedema
4	Severe erythema/wrinkle/scale	4	Severe oedema

Table 6 ICDRG Scale for Scoring the Treatment Sites
(*Clauses* 7.1.6.5.3 *and* 7.3.5)

Symbol	Morphology	Interpretation
(1)	(2)	(3)
-	No reaction	Negative
?	Erythema only, no infiltration	Doubtful reaction
+	Erythema, infiltration, possibly discrete papules	Weak positive reaction
++	Erythema, infiltration, papules, vesicles	Strong positive reaction
+++	Erythema, infiltration, confluent vesicles	Extreme positive reaction
Ir	Different types of reactions (soap effect, vesicles, blister, necrosis)	Irritant reaction
nt		Not tested

case the reaction reappears within the next 2-3 days, it is suggestive of the causal relationship of the dermatitis with the agent. For further confirmation, withdrawal of the agent and exposure can be repeated. In case multiple agents are suspected, it will be necessary to re-expose to these agents one by one and to add every new substance after an interval of at least 2 or 3 days and preferably after 7 days.

7.3 Human Repeat Insult Photo Patch Test (HRIPPT/RIPPT)

7.3.1 This is a common methodology for photo allergenicity testing and is usually carried out after conducting an HRIPT. This test is of particular significance if the suspected photo-allergenic product (or ingredient) shows absorbance in the UV-A and UV-B region (290- 400 nm).

7.3.2 In induction phase, test material neat cosmetic product as such (or diluted solution where defined in Table 4) or the highest non-irritant concentration as determined through the Preliminary Patch Test (if the highest non-irritant concentration is found to be lower as compared to the neat product or the dilution where specified in Table 4)] is applied twice a week, for three weeks on the same site on the back or upper arm (six consecutive applications of the product/s). Patches are kept in place for 24 h. Skin reaction (if any) is checked after patch removal and the test site is exposed to UV (UVA, 5 J/cm^2). Reactions if any are observed immediately and after 24 h.

7.3.3 The rest phase involves one week of no application of any patch in order to develop (if at all) sensitization to the test product.

7.3.4 In the challenge phase only one patch is applied to a naïve skin site on the back or upper arm. Patch is removed after 24 h. Skin reaction (if any) is checked after patch removal and the test site is exposed to UV (UVA, 5 J/cm^2). Reactions (if any) are observed immediately and after 24 h, 48 h and 72 h.

7.3.5 Scoring is carried out as per the ICDRG scales (Table 6). A positive reaction in even one subject indicates that the product is likely to be photo allergenic.

SECTION 3 INFORMATIVE ANNEX COVERING INCLUSION-EXCLUSION CRITERIA AND LATEST UPDATES ON ALTERNATIVES TO IN-VIVO SAFETY TESTING ON ANIMALS

ANNEX A

(*Clauses* 4.1.3.2.1 *and* 7.1.6.1)

TYPICAL INCLUSION / EXCLUSION CRITERIA TO BE FOLLOWED WHILE CONDUCTING SKIN IRRITATION / SKIN SENSITIZATION STUDIES ON HUMAN VOLUNTEERS (INCLUDING PHOTO-IRRITATION / PHOTO-SENSITIZATION)

A-1 INCLUSION CRITERIA

1. Voluntary mn/women beetween 18 and 65 years.
2. Photo type III to V.
3. Having apparently healthy skin on test area
4. For whom the investigator considers that the compliance will be correct.
5. Cooperating, informed of the need and duration of the examinations and ready to comply with protocol procedures.
6. Having signed a Consent Form.
7. Willingness to avoid intense UV exposure on test site (sun or artificial UV), during the course of the study.
8. Willingness to avoid excessive water contact (for example swimming) or activity which causes excessive sweating (that is exercise, sauna…), during the course of the study.
9. Should be able to read and write (in English, Hindi or local language).
10. Having valid proof of identity and age.

A-2 EXCLUSION CRITERIA

1. Pregnant/nursing mothers
2. Scars, excessive terminal hair or tattoo on the studied area.
3. Henna tattoo anywhere on the body (in case of studies involving hair dyes).
4. Dermatological infection/pathology on the level of studied area.
5. Hypersensitivity, allergy antecedent (to any

cosmetic product, raw material or hair dye).

6. Any clinically significant systemic or cutaneous disease, which may interfere with study treatment or procedures.
7. Chronic illness which may influence the outcome of the study.
8. Subjects on any medical treatment either systemic or topical which may interfere with the performance of the study treatment (presently or in the past 1 month).
9. Subject in an exclusion period or participating in another food, cosmetic or therapeutic trial.

ANNEX B

[*Table* 1, *Sl No.* (iii)]

ALTERNATE METHODS FOR SAFETY TESTING

(Source Reference — OECD Guidelines, EURL ECVAM Recommendations)

B-1 TESTS THAT MAY BE USED FOR THE TOXICITY ASSESSMENT OF COSMETIC INGREDIENTS AND PRODUCTS

B-1.1 Skin Corrosion — Test No. 431 — OECD Guideline[1)] — To be used in the frame of a testing strategy for corrosion/irritation.

The test described in this Test Guideline allows the identification of corrosive chemical substances and mixtures and discriminates corrosives from non-corrosive substances and mixtures. The test protocol may also provide an indication of the distinction between severe and less severe skin corrosives. This Test Guideline does not require the use of live animals or animal tissue for the assessment of skin corrosivity.

The test material (solid or liquid) is applied uniformly and topically to a three-dimensional human skin model, comprising at least a reconstructed human epidermis (RhE) with a functional stratum corneum. At least two tissue replicates should be used for each test chemical and controls for each exposure time. For liquid as well as solid chemicals, sufficient amount of test chemical should be applied to uniformly cover the epidermis surface while avoiding an infinite dose,that is a minimum of 70 µl/cm^2 or 30 mg/cm^2 should be used. Depending on the methods, the epidermis surface should be moistened with deionized or distilled water before application of solid chemicals, to improve contact between the test chemical and the epidermis surface. Whenever possible, solids should be tested as a fine powder. The application method should be appropriate for the test chemical. At the end of the exposure period, the test chemical should be carefully washed from the epidermis with an aqueous buffer, or 0.9 percent NaCl. Depending on which of the four validated RhE test methods is used, two or three exposure periods are used per test chemical (for all four valid RhE models: 3 min and 1 h; for EpiSkin™ an additional exposure time of 4 h). Depending on the RhE test method used and the exposure period assessed, the incubation temperature during exposure may vary between room temperature and 37°C.

Concurrent negative and positive controls (PC) should be used in each run to demonstrate that viability (with negative controls), barrier function and resulting tissue sensitivity (with the PC) of the tissues are within a defined historical acceptance range. The suggested PC chemicals are glacial acetic acid or 8N KOH depending upon the RhE model used. It should be noted that 8N KOH is a direct MTT reducer that might require adapted controls. The suggested negative controls are 0.9 percent (*w/v*) NaCl or water.

The principle of the human skin model assay is based on the hypothesis that corrosive chemicals are able to penetrate the stratum corneum by diffusion or erosion, and are cytotoxic to the underlying cell layers. Corrosive materials are identified by their ability to produce a decrease in cell viability below defined threshold levels at specified exposure periods.

Suitable Models for the test include:

a) *In Vitro Skin Corrosion*: Human Skin Model Test (HSM): EpiSkin™ (SM)
b) *In Vitro Skin Corrosion*: Human Skin Model Test (HSM): EpiDerm™ SCT (EPI-200)
c) *In Vitro Skin Corrosion*: Human Skin Model Test (HSM): SkinEthic™ RhE
d) *In Vitro Skin Corrosion*: Human Skin Model Test (HSM): epiCS®

The optical density (OD) values obtained for each test chemical should be used to calculate percentage of viability relative to the negative control, which is set at 100 percent. The cut-off percentage cell viability values distinguishing corrosive from non-corrosive test chemical (or discriminating between different corrosive sub-categories) depend on the RhE Model adopted as specified in the Guideline.

For each tissue, OD values and calculated percentage cell viability data for the test material, positive and negative controls, should be reported in tabular form, including data from replicate repeat experiments as appropriate, mean and individual values.

Also kindly refer to *OECD Guidance Document 203* — New Guidance Document on An Integrated Approach on Testing and Assessment (*IATA*) for Skin Corrosion and Irritation

B-1.2 Skin Irritation — Test No. 439 — OECD Guidelines[1] — *To be used in the frame of a testing strategy for corrosion/irritation*.

Also kindly refer to *OECD Guidance Document 203* — New Guidance Document on An Integrated Approach on Testing and Assessment (*IATA*) for Skin Corrosion and Irritation:

This Test Guideline describes an in vitro procedure that may be used for the hazard identification of irritant chemicals (substances and mixtures) in accordance with the UN Globally Harmonized System of Classification and Labelling (GHS) Category 2 and discriminates Category 2 chemicals from those not classified for skin irritation. It is based on reconstructed human epidermis (RhE), which in its overall design closely mimics the biochemical and physiological properties of the upper parts of the human skin. Cell viability is measured by enzymatic conversion of the vital dye MTT into a blue formazan salt that is quantitatively measured after extraction from tissues. Irritant test chemicals are identified by their ability to decrease cell viability below defined threshold levels (below or equal to 50 percent for UN GHS Category 2). This Test Guideline also includes a set of Performance Standards for the assessment of similar and modified RhE-based test methods. Depending on the regulatory framework and the classification system in use, this procedure may be used to determine the skin irritancy of test substances as a stand-alone replacement test for in vivo skin irritation testing, or as a partial replacement test, within a tiered testing strategy.

The Test Guideline is applicable to solids, liquids, semi-solids and waxes. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Whenever possible, solids should be ground to a fine powder before application; no other pre-treatment of the sample is required. The current Test Guideline does not allow testing of gases and aerosols. It should also be noted that highly coloured chemicals may interfere with the cell viability measurements and need the use of adapted controls for corrections.

A single testing run composed of three replicate tissues should be sufficient for a test chemical when the classification is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements and/or mean percent viability equal to 50 ± 5 percent, a second run should be considered, as well as a third one in case of discordant results between the first two runs.

The test chemical is applied topically to a three-dimensional RhE model, comprised of non-transformed human-derived epidermal keratinocytes, which have been cultured to form a multilayered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayered stratum corneum containing intercellular lamellar lipid layers representing main lipid classes analogous to those found in vivo.

a) *In Vitro Skin Irritation*: Reconstructed Human Epidermis Model Test (RhE). EpiSkin™
b) *In Vitro Skin Irritation*: Reconstructed Human Epidermis Model Test (RhE). Modified EpiDerm™ SIT
c) *In Vitro Skin Irritation*: Reconstructed Human Epidermis Model Test (RhE). SkinEthic™ RHE

B-1.3 Skin Sensitization

B-1.3.1 *In Chemico Skin Sensitisation*: *Direct Peptide Reactivity Assay (DPRA) — Test No. TG 442C - OECD Guidelines* [1), 3)]

There is general agreement regarding the key biological events underlying skin sensitisation. The existing knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised in the form of an Adverse Outcome Pathway (AOP), from the molecular initiating event through the intermediate events to the adverse effect namely allergic contact dermatitis in humans. Within the skin sensitisation AOP, the molecular initiating event is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins.

The Direct Peptide Reactivity Assay (DPRA) is proposed to address the molecular initiating event of the skin sensitisation (AOP), namely protein reactivity, by quantifying the reactivity of test chemicals towards model synthetic peptides containing either lysine or cysteine. Cysteine and lysine percent peptide depletion values are then used to categorise a substance in one of four classes

of reactivity for supporting the discrimination between skin sensitisers and non-sensitisers.

The test method described in this Test Guideline can be used, in combination with other complementary information, to support the discrimination between skin sensitisers (that is UN GHS Category 1) and non-sensitisers in the context of an Integrated Approach on Testing & Assessment (IATA). This Test Guideline cannot be used on its own, neither to sub-categorise skin sensitisers into sub-categories 1A and 1B as defined by UN GHS (1), for authorities implementing these two optional sub-categories, nor to predict potency for safety assessment decisions. However, depending on the regulatory framework, a positive result with the DPRA may be used on its own to classify a chemical into UN GHS category 1.

The DPRA is an *in chemico* method which quantifies the remaining concentration of cysteine- or lysine-containing peptide following 24 h incubation with the test chemical at 25 ± 2.5°C. The synthetic peptides contain phenylalanine to aid in the detection. Relative peptide concentration is measured by high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 220 nm. Cysteine- and lysine peptide percent depletion values are then calculated and used in a prediction model which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitisers and non-sensitisers.

B-1.3.2 *In Vitro Skin Sensitisation*: *ARE-Nrf2 Luciferase Test Method* - *Test No. TG 442D - OECD Guidelines* [1), 3)]

There is general agreement regarding the key biological events underlying skin sensitisation. The existing knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised in the form of an Adverse Outcome Pathway (AOP), going from the molecular initiating event through the intermediate events up to the adverse health effect, that is. allergic contact dermatitis in humans. The molecular initiating event is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as gene expression associated with specific cell signaling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells, typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell proliferation.

The test method described in this Test Guideline (ARE-Nrf2 luciferase test method) is proposed to address the second key event as explained above. Skin sensitisers have been reported to induce genes that are regulated by the antioxidant response element (ARE). Small electrophilic substances such as skin sensitisers can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1), by e.g. covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2). The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes.

B-1.4 Eye Irritation

B-1.4.1 *Bovine Cornea Opacity Test (BCOP) — Test No. 437 — OECD Guidelines*[1), 2)]

Useful for the identification of ocular corrosives and severe irritants (chemicals inducing serious eye damage) as well as chemicals not requiring a classification for eye irritation (non-irritants) but not of mild/moderate ocular irritants. If combined with other alternative test methods in the frame of a testing strategy (for example Bottom-Up and Top-Down Approach, Scott L. *et al*. 2009), can lead to full characterization of the eye irritation and corrosion potential of an ingredient or product.

The BCOP test method is an organotypic model that provides short-term maintenance of normal physiological and biochemical function of the bovine cornea in vitro. In this test method, damage by the test chemical is assessed by quantitative measurements of changes in corneal opacity and permeability with an opacity meter and a visible light spectrophotometer, respectively. Corneal opacity is measured quantitatively with the aid of an opacity meter as the amount of light transmission through the cornea, resulting in opacity values measured on a continuous scale. Permeability is measured quantitatively as the amount of sodium fluorescein dye that passes across the full thickness of the cornea, as detected in the medium in the posterior chamber. Test chemicals are applied to the epithelial surface of the cornea by addition to the anterior chamber of the corneal holder. Both measurements are used to calculate an IVIS, which is used to assign an in vitro irritancy hazard classification category for prediction of the in vivo ocular irritation potential of a test chemical.

Once the opacity and mean permeability (OD490) values have been corrected for background opacity and the negative control permeability OD490 values, the mean opacity and permeability OD490 values for each treatment group should be combined in an empirically-derived formula to calculate an in vitro irritancy score (IVIS) for each treatment group as follows:

IVIS = mean opacity value + (15 × mean permeability OD490 value)

The IVIS cut-off values for identifying test chemicals as inducing serious eye damage (UN GHS Category 1) and test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category) are given hereafter:

IVIS	*UN GHS*
≤ 3	No category
< 3; ≤ 55	No prediction can be made
> 55	Category 1

B-1.4.2 *The Isolated Chicken Eye Test (ICE) — Test No. 438 — OECD Guidelines*[1), 2)]

The Isolated Chicken Eye Test (ICE) Method is an in vitro test method that can be used to identify chemicals (substances or mixtures) as either (a) causing "serious eye damage" [category 1 of the Globally Harmonized System for the Classification and Labelling of chemicals (GHS)], or (b) not requiring classification for eye irritation or serious eye damage according to the GHS.

The ICE test method is an organotypic model that provides short-term maintenance of the chicken eye in vitro. In this test method, damage by the test chemical is assessed by determination of corneal swelling, opacity, and fluorescein retention. While the latter two parameters involve a qualitative assessment, analysis of corneal swelling provides for a quantitative assessment. Each measurement is either converted into a quantitative score used to calculate an overall Irritation Index, or assigned a qualitative categorization that is used to assign an in vitro ocular hazard classification, either as UN GHS Category 1 or as UN GHS non-classified. Either of these outcomes can then be used to predict the potential in vivo serious eye damage or no requirement for eye hazard classification of a test chemical. However, no classification can be given for chemicals not predicted as causing serious eye damage or as not classified with the ICE test method.

B-1.5 Photo Toxicity

B-1.5.1 *In Vitro 3T3 NRU Phototoxicity Test — Test No. 432 — OECD Guidelines*[1)]

The in vitro 3T3 NRU phototoxicity test is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of simulated solar light. Cytotoxicity in this test is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red when measured 24 h after treatment with the test chemical and irradiation. Neutral Red is a weak cationic dye that readily penetrates cell membranes by non-diffusion, accumulating intra cellularly in lysosomes. Alterations of the cell surface of the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged or dead cells, which is the basis of this test.

Balb/c 3T3 cells are maintained in culture for 24 h for formation of monolayers. Two 96-well plates per test chemical are pre-incubated with eight different concentrations of the test substance for 1 h. Thereafter one of the two plates is exposed to the highest non-cytotoxic irradiation dose whereas the other plate is kept in the dark. In both plates the treatment medium is then replaced by culture medium and after another 24 h of incubation cell viability is determined by Neutral Red uptake. Cell viability is expressed as percentage of untreated solvent controls and is calculated for each test concentration. To predict the phototoxic potential, the concentration responses obtained in the presence and in the absence of irradiation are compared, usually at the IC_{50} level, that is, the concentration reducing cell viability to 50 percent compared to the untreated controls.

Test chemicals shall be dissolved in buffered salt solutions, for example Earle's Balanced Salt Solution (EBSS), or other physiologically balanced buffer solutions, which must be free from protein components and light absorbing components (for example, *p*H-indicator colours and vitamins) to avoid interference during irradiation. Since during irradiation cells are kept for about 50 minutes outside of the CO_2 incubator, care has to be taken to avoid alkalisation. If weak buffers like EBSS are used this can be achieved by incubating the cells at 7.5 percent CO_2. If the cells are incubated at 5 percent CO_2 only, a stronger buffer should be selected.

Test chemicals of limited solubility in water should be dissolved in an appropriate solvent. If a solvent is used it must be present at a constant volume in all cultures, that is in the negative (solvent) controls as well as in all concentrations of the test chemical, and be noncytotoxic at that concentration. Test chemical concentrations should be selected so as to avoid precipitation or cloudy solutions.

Dimethylsulphoxide (DMSO) and ethanol (EtOH) are the recommended solvents. Other solvents of low cytotoxicity may be appropriate. Prior to use, all solvents should be assessed for specific properties, for example, reaction with the test chemical, quenching of the phototoxic effect, radical scavenging properties and/or chemical stability in the solvent.

The highest concentration of the test substance should be within physiological test conditions, for example osmotic and *p*H stress should be avoided.

A test substance with a PIF < 2 or an MPE < 0.1 predicts: "no phototoxicity". A PIF >2 and < 5 or an MPE > 0.1 and < 0.15 predicts: "probable phototoxicity" and a PIF > 5 or an MPE > 0.15 predicts: "phototoxicity".

B-1.6 Skin Absorption

B-1.6.1 *Skin Absorption: In Vitro Method — Test No. 428 — OECD Guidelines*[1)]

This Test method has been designed to provide information on absorption of a test substance, (ideally radiolabelled), applied to the surface of a skin sample separating the two chambers (a donor chamber and a receptor chamber) of a diffusion cell. Static and flow-through diffusion cells are both acceptable.

Skin from human or animal sources can be used. Although viable skin is preferred, non-viable skin can also be used. Either epidermal membranes (enzymically, heat or chemically separated) or split thickness skin (typically 200-400 ìm thick) prepared with a dermatome, are acceptable. Full thickness skin may be used but excessive thickness (DB. > 1 mm) should be avoided unless specifically required for determination of the test chemical in layers of the skin. The selection of species, anatomical site and preparative technique must be justified. Acceptable data from a minimum of four replicates per test preparation are required.

The skin has been shown to have the capability to metabolize some chemicals during percutaneous absorption. In this case, metabolites of the test chemical may be analyzed by appropriate methods. Normally more than one concentration of the test substance is used in typical formulations, spanning the realistic range of potential human exposures. The application should mimic human exposure, normally 1-5 mg/cm^2 of skin for a solid and up to 10 µl/cm^2 for liquids. The temperature must be constant because it affects the passive diffusion of chemicals. The absorption of a test substance during a given time period (normally 24h) is measured by analysis of the receptor fluid and the distribution of the test substance chemical in the test system and the absorption profile with time should be presented.

The test substance is the entity whose penetration characteristics are to be studied. Ideally, the test substance should be radiolabelled. The test substance preparation

(for example, neat, diluted or formulated material containing the test substance which is applied to the skin) should be the same (or a realistic surrogate) as that to which humans or other potential target species may be exposed. Any variation from the 'in-use' preparation must be justified.

Normally more than one concentration of the test substance is used in typical formulations, spanning the realistic range of potential human exposures. Likewise, testing a range of typical formulations should be considered.

In all studies adequate recovery should be achieved (the aim should be a mean of

100 ±10 percent of the radioactivity and any deviation should be justified). The amount of test substance in the receptor fluid, skin preparation, skin surface washings and apparatus rinse should be analysed, using a suitable technique.

The analysis of receptor fluid, the distribution of the test substance chemical in the test system and the absorption profile with time, should be presented. When finite dose conditions of exposure are used, the quantity washed from the skin, the quantity associated with the skin (and in the different skin layers if analysed) and the amount present in the receptor fluid (rate, and amount or percentage of applied dose) should be calculated. Skin absorption may sometimes be expressed using receptor fluid data alone. However, when the test substance remains in the skin at the end of the study, it may need to be included in the total amount absorbed. When infinite dose conditions of exposure are used the data may permit the calculation of a permeability constant (Kp). Under the latter conditions, the percentage absorbed is not relevant.

B-1.7 Others

B-1.7.1 *Stably Transfected Human Estrogen Receptor-alpha Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals (STTA). STTA for EDs — Test No. 455 - OECD Guidelines*[1)]

This Test Guideline describes an in vitro assay, which provides mechanistical information, and can be used for screening and prioritization purposes. The test system utilises the hERalpha-HeLa-9903 cell line derived from a human cervical tumor and stably transfected. This cell line can measure the ability of a test chemical to induce hERalpha-mediated transactivation of luciferase gene expression. The cells are exposed to 7 non-cytotoxic concentrations of the test chemical for 20-24 h to induce the reporter gene products. Four reference chemicals should be included in each experiment: a strong estrogen (17beta-estradiol), a weak estrogen (17alpha-estradiol), a very weak estrogen (17alpha-methyltestosterone) and a negative control (corticosterone). The activity of the luciferase enzyme is measured in a luminometer. A test chemical is considered to be positive if the maximum response induced is equal to or exceeds 10 percent of

the response of the positive control (1 nM 17alpha-estradiol) in at least two of two or two of three runs.

B-1.7.2 *3T3 Neutral Red Uptake Cytotoxicity Assay for Acute Oral Toxicity Testing – EURL ECVAM Recommendations - April 2013*[1)]

3T3 NRU test method shows a high sensitivity (ca 95 percent) and, consequently, a low false negative rate (ca 5 percent) when employed in conjunction with a prediction model to distinguish potentially toxic versus non-toxic (that is classified *versus* non-classified) substances. However, chemicals not exhibiting significant cytotoxicity but which act through mechanisms specific only to certain cell types and tissues (for example of the heart or central nervous system) may not be indicated as potentially acutely toxic by this method. Moreover, chemicals requiring metabolic activation to induce toxicity may also go undetected since the cell model lacks significant metabolic capacity. Care must be taken therefore in interpreting negative results derived from this assay. Results derived from the 3T3 NRU test method should be always used in combination with other information sources to build confidence in the decision not to classify a substance for acute oral toxicity. Possible complementary information sources include chemical analogues, physico-chemical properties, structural alerts, structure–activity relationships, and toxicokinetic data. The 3T3 NRU method would fit well therefore within a Weight of Evidence (WoE) approach or as a component of an Integrated Testing Strategy (ITS).

The 3T3 NRU cytotoxicity assay employs the BALB/c3T3 mouse fibroblast cell line and is based on the ability of viable cells to incorporate and bind the dye Neutral Red (NR). The uptake of NR is measured spectrophotometrically (Stokes *et al*., 2008). The basic concept of basal cytotoxicity assays is that chemicals exert their toxic effects by disrupting structures and functions universal to all cells, such as cell membranes

(Gennari et al, 2004). With the basal cytotoxic assays it is possible to quantify the cytotoxicity of a compound by its IC_{50} value, that is the concentration of the tested substance that decreases cell viability by 50 percent in the cell culture.

B-1.8 Genotoxicity

The following OECD Guidelines may be considered –

a) OECD 471 — Bacterial Reverse Mutation Test
b) OECD TG 473 — In vitro Mammalian Chromosomal Aberration Test
c) OECD 476 — In Vitro Mammalian Cell Gene Mutation Test
d) OECD TG 487 — In vitro Mammalian Cell Micronucleus Test

NOTES

1 The test method described in this Annex is based on the official guidelines/recommendations as mentioned. Kindly refer to the latest updated version of the Guideline/Recommendation for complete information on the scope, methodology and limitations.

2 This test can also be used for evaluating oral mucosal irritation in the absence of a suitable alternate methodology.

3 For skin sensitization, an Integrated Testing Strategy needs to be adopted and the results cannot be interpreted on the basis of only one test. Prediction should be considered in the framework of an IATA and at least 2 out of 3 tests need to show absence of skin sensitization.

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Amendments Issued Since Publication

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