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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test Method (TER)

INTRODUCTION

1. Skin corrosion refers to the production of irreversible damage to the skin manifested as visible necrosis through the epidermis and into the dermis, following the application of a test chemical [as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS)] (1). This updated Test Guideline 430 provides an *in vitro* procedure allowing the identification of non-corrosive and corrosive substances and mixtures in accordance with UN GHS (1).

2. The assessment of skin corrosivity has typically involved the use of laboratory animals (OECD Test Guideline 404 (TG 404); adopted in 1981 and revised in 1992 and 2002) (3). In relation to animal welfare concerns, TG 404 recommends the use of a tiered testing strategy for the determination of skin corrosion and irritation which includes the use of validated *in vitro* or *ex vivo* test methods avoiding pain and suffering of animals. In addition to TG 430 (originally adopted in 2004)(4), several other *in vitro* test methods for testing of corrosivity have been validated and adopted as OECD Test Guidelines 431 (5) and 435 (6). Several validated *in vitro* test methods have been adopted as OECD TG 439 (7), to be used for the skin irritation part of the tiered testing strategy recommended in supplement to TG 404 (3).

3. This Test Guideline addresses the human health endpoint skin corrosion. It is based on the rat skin transcutaneous electrical resistance (TER) test method, which utilizes skin discs to identify corrosives by their ability to produce a loss of normal *stratum corneum* integrity and barrier function. This Test Guideline also includes a set of Performance Standards (PS) (Annex 1) for the assessment of similar and modified TER-based test methods (8), in accordance with the principles of Guidance Document No. 34 (9).

4. In order to evaluate *in vitro* skin corrosion testing for regulatory purposes, pre-validation studies (10) followed by a formal validation study of the rat skin TER test method for assessing skin corrosion were conducted (11) (12) (13) (14). The outcome of these studies led to the recommendation that the TER test method (designated the Validated Reference Method – VRM) could be used for regulatory purposes for the assessment of *in vivo* skin corrosivity (15) (16) (17).

5. Before a proposed similar or modified *in vitro* TER test method for skin corrosion other than the VRM can be used for regulatory purposes, its reliability, relevance (accuracy), and limitations for its proposed use should be determined to ensure its similarity to the VRM, in accordance with the requirements of the PS set out in this Test Guideline (Annex 1). The Mutual Acceptance of Data will only be guaranteed after any proposed new or updated test method following the PS of this Test Guideline have been reviewed and included in this Test Guideline.

DEFINITIONS

6. Definitions used are provided in Annex 2.

INITIAL CONSIDERATIONS

7. A validation study (12) and other published studies (18) (19) have reported that the rat skin TER test method is able to discriminate between known skin corrosives and non-corrosives with an overall sensitivity of 94% (51/54) and specificity of 71% (48/68) for a database of 122 substances.

8. This Test Guideline addresses the *in vitro* skin corrosion component of the tiered testing strategy recommended in supplement to TG 404 on dermal corrosion/irritation (3) (20). It allows the identification of non-corrosive and corrosive substances and mixtures in accordance with the UN GHS (1). A limitation of this Test Guideline, as demonstrated by the validation studies (11) (12) (13) (14), is that it does not allow the sub-categorization of corrosive substances and mixtures in accordance with the UN GHS (1). The regulatory framework in member countries will decide how this Test Guideline will be used. While this Test Guideline does not provide adequate information on skin irritation, it should be noted that OECD TG 439 specifically addresses the health effect skin irritation *in vitro* (7). For a full evaluation of local skin effects after a single dermal exposure, it is recommended to follow the sequential testing strategy supplementing TG 404 (3) (20). This testing strategy includes the conduct of *in vitro* tests for skin corrosion (such as described in this Test Guideline) and skin irritation before considering testing in live animals.

9. A wide range of chemicals representing mainly substances has been tested in the validation underlying this Test Guideline and the empirical database of the validation study amounted to 60 substances covering a wide range of chemical classes (11) (12). On the basis of the overall data available, the Test Guideline is applicable to a wide range of chemical classes and physical states including liquids, semi-solids, solids and waxes. However, since for specific physical states test items with suitable reference data are not readily available, it should be noted that a comparably small number of waxes and corrosive solids were assessed during validation. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. In cases where evidence can be demonstrated on the non-applicability of the Test Guideline to a specific category of substances, the Test Guideline should not be used for that specific category of substances". In addition, this Test Guideline is assumed to be applicable to mixtures as an extension of its applicability to substances. However, due to the fact that mixtures cover a wide spectrum of categories and composition, and that only limited information is currently available in the public domain on the testing of mixtures, in cases where evidence can be demonstrated on the non-applicability of the Test Guideline to a specific category of mixtures (*e.g.* following a strategy as proposed by Eskes *et al.*, 2012) (21), the Test Guideline should not be used for that specific category of mixtures. Gases and aerosols have not been assessed yet in validation studies (11) (12). While it is conceivable that these can be tested using the TER test method, the current Test Guideline does not allow testing of gases and aerosols.

10. This Test Guideline also includes a set of Performance Standards (PS) (Annex 1) for determining the validation status (reliability and relevance) of similar and modified skin corrosion test methods that are structurally and mechanistically similar to the rat skin TER test method (8), in accordance with the principles of Guidance Document No. 34 (9). These PS include a list of 24 Reference Chemicals by which to evaluate assay performance, the essential test method components by which to evaluate the structural, mechanistic and procedural similarity of a new proposed test method, and the minimum reliability and accuracy values necessary for the test method to be considered comparable to the VRM. Within the Reference Chemical list, a subset of 12 Proficiency Chemicals (Table 1) is provided that can be used by laboratories to demonstrate proficiency in using the rat skin TER test method (see paragraph13).

PRINCIPLE OF THE TEST

11. The test chemical is applied for up to 24 hours to the epidermal surfaces of skin discs in a two-compartment test system in which the skin discs function as the separation between the compartments. The skin discs are taken from humanely killed rats aged 28-30 days. Corrosive chemicals are identified by their ability to produce a loss of normal *stratum corneum* integrity and barrier function, which is measured as a reduction in the TER below a threshold level (18) (see paragraph 34). For rat skin TER, a cut-off value of 5kΩ has been selected based on extensive data for a wide range of substances where the vast majority of values were either clearly well above (often > 10 kΩ), or well below (often < 3 kΩ) this value (18). Generally, chemicals that are non-corrosive in animals but are irritant or non-irritant do not reduce the TER below this cut-off value. Furthermore, use of other skin preparations or other equipment may alter the cut-off value, necessitating further validation.

12. A dye-binding step is incorporated into the test procedure for confirmation testing of positive results in the TER including values around 5 kΩ. The dye-binding step determines if the increase in ionic permeability is due to physical destruction of the *stratum corneum*. The TER method utilizing rat skin has shown to be predictive of *in vivo* corrosivity in the rabbit assessed under OECD guideline 404 (3).

DEMONSTRATION OF PROFICIENCY

13. Prior to routine use of the rat skin TER test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly classifying the twelve Proficiency Chemicals recommended in Table 1.

Table 1: List of Proficiency Chemicals

Chemical[1]	CASRN	Chemical Class[2]	UN GHS Cat. Based on *In Vivo* Results[3]	VRM Cat. Based on *In Vitro* Results	Physical State	pH[4]
***In Vivo* Corrosives**						
N,N'-Dimethyl dipropylenetriamine	10563-29-8	organic base	1A	6 x C	L	8.3
1,2-Diaminopropane	78-90-0	organic base	1A	6 x C	L	8.3
Sulfuric acid (10%)	7664-93-9	inorganic acid	(1A/)1B/1C	5 x C 1x NC	L	1.2
Potassium hydroxide (10% aq.)	1310-58-3	inorganic base	(1A/)1B/1C	6 x C	L	13.2
Octanoic (Caprylic) acid	124-07-2	organic acid	1B/1C	4 x C 2 x NC	L	3.6
2-tert-Butylphenol	88-18-6	phenol	1B/1C	4 x C 2 x NC	L	3.9
***In Vivo* Non-corrosives**						
Isostearic acid	2724-58-5	organic acid	NC	6 x NC	L	3.6
4-Amino-1,2,4-triazole	584-13-4	organic base	NC	6 x NC	S	5.5

Chemical[1]	CASRN	Chemical Class[2]	UN GHS Cat. Based on *In Vivo* Results[3]	VRM Cat. Based on *In Vitro* Results	Physical State	pH[4]
Phenethyl bromide	103-63-9	electrophile	NC	6 x NC	L	3.6
4-(Methylthio)-benzaldehyde	3446-89-7	electrophile	NC	6 x NC	L	6.8
1,9-Decadiene	1647-16-1	neutral organic	NC	6 x NC	L	3.9
Tetrachloroethylene	127-18-4	neutral organic	NC	6 x NC	L	4.5

Abbreviations: aq = aqueous; CASRN = Chemical Abstracts Service Registry Number; UN GHS = United Nations Globally Harmonised System (1); VRM = Validated Reference Method; ND = Not Determined.
[1]These substances, sorted first by corrosives versus non-corrosives, then by corrosive subcategory and then by chemical class, were selected from the substances used in the ECVAM validation study of the rat skin TER test method (11) (12). Unless otherwise indicated, the substances were tested at the purity level obtained when purchased from a commercial source (11). The selection included, to the extent possible, substances that: (i) are representative of the range of corrosivity responses (*e.g.* non-corrosives; weak to strong corrosives) that the VRM is capable of measuring or predicting; (ii) are representative of the chemical classes used in the validation study; (iii) reflect the performance characteristics of the VRM; (iv) have chemical structures that are well-defined;; (v) induce definitive results in the *in vivo* reference test method; (vi) are commercially available; and (vii) are not associated with prohibitive disposal costs.
[2]Chemical class assigned by Barratt *et al.* (1998) (11).
[3]The corresponding UN Packing groups are I, II and III, respectively, for the UN GHS 1A, 1B and 1C.
[4]The pH values were obtained from Fentem *et al.* (1998) (12) and Barratt *et al.* (1998)(11).

PROCEDURE

14. Standard Operating Procedures (SOP) for the rat skin TER skin corrosion test method are available (2). The rat skin TER test methods covered by this Test Guideline should comply with the following:

Animals

15. Rats should be used because the sensitivity of their skin to substances in this test method has been previously demonstrated (15) and is the only skin source that has been formally validated (11) (12). The age (when the skin is collected) and strain of the rat is particularly important to ensure that the hair follicles are in the dormant phase before adult hair growth begins.

16. The dorsal and flank hair from young, approximately 22 day-old, male or female rats (Wistar-derived or a comparable strain), is carefully removed with small clippers. Then, the animals are washed by careful wiping, whilst submerging the clipped area in antibiotic solution (containing, for example, streptomycin, penicillin, chloramphenicol, and amphotericin, at concentrations effective in inhibiting bacterial growth). Animals are washed with antibiotics again on the third or fourth day after the first wash and are used within 3 days of the second wash, when the *stratum corneum* has recovered from the hair removal.

Preparation of the skin discs

17. Animals are humanely killed when 28-30 days old; this age is critical. The dorso-lateral skin of each animal is then removed and stripped of excess subcutaneous fat by carefully peeling it away from the skin. Skin discs, with a diameter of approximately 20-mm each, are removed. The skin may be stored before discs are used where it is shown that positive and negative control data are equivalent to that obtained with fresh skin.

18. Each skin disc is placed over one of the ends of a PTFE (polytetrafluoroethylene) tube, ensuring that the epidermal surface is in contact with the tube. A rubber 'O' ring is press-fitted over the end of the tube to hold the skin in place and excess tissue is trimmed away. The rubber 'O' ring is then carefully sealed to the end of the PTFE tube with petroleum jelly. The tube is supported by a spring clip inside a receptor chamber containing $MgSO_4$ solution (154 mM) (Figure 1). The skin disc should be fully submerged in the $MgSO_4$ solution. As many as 10-15 skin discs can be obtained from a single rat skin. Tube and 'O' ring dimensions are shown in Figure 2.

19. Before testing begins, the TER of two skin discs are measured as a quality control procedure for each animal skin. Both discs should give electrical resistance values greater than 10 kΩ for the remainder of the discs to be used for the test method. If the resistance value is less than 10 kΩ, the remaining discs from that skin should be discarded.

Application of the test and control chemical

20. Concurrent positive and negative controls should be used for each run (experiment) to ensure adequate performance of the experimental model. Skin discs from a single animal should be used in each run (experiment). The suggested positive and negative control test chemicals are 10M hydrochloric acid and distilled water, respectively.

21. Liquid test chemicals (150 μL) are applied uniformly to the epidermal surface inside the tube. When testing solid materials, a sufficient amount of the solid is applied evenly to the disc to ensure that the whole surface of the epidermis is covered. Deionised water (150 μL) is added on top of the solid and the tube is gently agitated. In order to achieve maximum contact with the skin, solids may need to be warmed to 30^0 C to melt or soften the test chemical, or ground to produce a granular material or powder.

22. Three skin discs are used for each test and control chemical in each testing run (experiment). Test chemicals are applied for 24 hours at 20-23^0 C. The test chemical is removed by washing with a jet of tap water at up to room temperature until no further material can be removed.

TER measurements

23. The skin impedance is measured as TER by using a low-voltage, alternating current Wheatstone bridge (19). General specifications of the bridge are 1-3 Volt operating voltage, a sinus or rectangular shaped alternating current of 50 - 1000 Hz, and a measuring range of at least 0.1 -30 kΩ. The databridge used in the validation study measured inductance, capacitance and resistance up to values of 2000H, 2000 μF, and 2 MΩ, respectively at frequencies of 100Hz or 1kHz, using series or parallel values. For the purposes of the TER corrosivity assay measurements are recorded in resistance, at a frequency of 100 Hz and using series values. Prior to measuring the electrical resistance, the surface tension of the skin is reduced by adding a sufficient volume of 70% ethanol to cover the epidermis. After a few seconds, the ethanol is removed from the tube and the tissue is then hydrated by the addition of 3 mL $MgSO_4$ solution (154mM). The databridge electrodes are placed on either side of the skin disc to measure the resistance in kΩ/skin disc (Figure 1). Electrode dimensions and the length of the electrode exposed below the crocodile

clips are shown in Figure 2. The clip attached to the inner electrode is rested on the top of the PTFE tube during resistance measurement to ensure that a consistent length of electrode is submerged in the $MgSO_4$ solution. The outer electrode is positioned inside the receptor chamber so that it rests on the bottom of the chamber. The distance between the spring clip and the bottom of the PTFE tube is maintained as a constant (Figure 2), because this distance affects the resistance value obtained. Consequently, the distance between the inner electrode and the skin disc should be constant and minimal (1-2 mm).

24. If the measured resistance value is greater than 20 kΩ, this may be due to the remains of the test chemical coating the epidermal surface of the skin disc. Further removal of this coating can be attempted, for example, by sealing the PTFE tube with a gloved thumb and shaking it for approximately 10 seconds; the $MgSO_4$ solution is discarded and the resistance measurement is repeated with fresh $MgSO_4$.

25. The properties and dimensions of the test apparatus and the experimental procedure used may influence the TER values obtained. The 5 kΩ corrosive threshold was developed from data obtained with the specific apparatus and procedure described in this Test Guideline. Different threshold and control values may apply if the test conditions are altered or a different apparatus is used. Therefore, it is necessary to calibrate the methodology and resistance threshold values by testing a series of Proficiency Chemicals chosen from the substances used in the validation study (11) (12), or from similar chemical classes to the substances being investigated. A set of suitable Proficiency Chemicals is identified in Table 1 of Annex 1.

Dye Binding Methods

26. Exposure of certain non-corrosive materials can result in a reduction of resistance below the cut-off of 5 kΩ allowing the passage of ions through the *stratum corneum*, thereby reducing the electrical resistance (12). For example, neutral organics and substances that have surface-active properties (including detergents, emulsifiers and other surfactants) can remove skin lipids making the barrier more permeable to ions. Thus, if TER values produced by such chemicals are less than or around 5 kΩ in the absence of visually perceptible damage of the skin discs, an assessment of dye penetration should be carried out on the control and treated tissues to determine if the TER values obtained were the result of increased skin permeability, or skin corrosion (10) (12). In case of the latter where the *stratum corneum* is disrupted, the dye sulforhodamine B, when applied to the skin surface rapidly penetrates and stains the underlying tissue. This particular dye is stable to a wide range of substances and is not affected by the extraction procedure described below.

Sulforhodamine B dye application and removal

27. Following TER assessment, the magnesium sulfate is discarded from the tube and the skin is carefully examined for obvious damage. If there is no obvious major damage (*e.g.* perforation), 150 µL of a 10% (w/v) dilution in distilled water of the dye sulforhodamine B (Acid Red 52; C.I. 45100; CAS number 3520-42-1), is applied to the epidermal surface of each skin disc for 2 hours. These skin discs are then washed with tap water at up to room temperature for approximately 10 seconds to remove any excess/unbound dye. Each skin disc is carefully removed from the PTFE tube and placed in a vial (*e.g.* a 20-mL glass scintillation vial) containing deionised water (8 mL). The vials are agitated gently for 5 minutes to remove any additional unbound dye. This rinsing procedure is then repeated, after which the skin discs are removed and placed into vials containing 5ml of 30% (w/v) sodium dodecyl sulphate (SDS) in distilled water and are incubated overnight at 60^0 C.

28. After incubation, each skin disc is removed and discarded and the remaining solution is centrifuged for 8 minutes at 21^0 C (relative centrifugal force ~175 x g). A 1mL sample of the supernatant is diluted 1 in 5 (v/v) [*i.e.* 1mL + 4mL] with 30% (w/v) SDS in distilled water. The optical density (OD) of the solution is measured at 565 nm.

Calculation of dye content

29. The sulforhodamine B dye content per disc is calculated from the OD values (12) (sulforhodamine B dye molar extinction coefficient at 565nm = 8.7 x $l0^4$; molecular weight = 580). The dye content is determined for each skin disc by the use of an appropriate calibration curve and mean dye content is then calculated for the replicates.

Acceptability Criteria

30. The mean TER results are accepted if the concurrent positive and negative control values fall within the acceptable ranges for the method in the testing laboratory. The acceptable resistance ranges for the methodology and apparatus described above are given in the following table:

Control	Substance	Resistance range (kΩ)
Positive	10M Hydrochloric acid	0.5 - 1.0
Negative	Distilled water	10 - 25

31. The mean dye binding results are accepted on condition that concurrent control values fall within the acceptable ranges for the method. Suggested acceptable dye content ranges for the control substances for the methodology and apparatus described above are given in the following table:

Control	Substance	Dye content range (µg/disc)
Positive	10M Hydrochloric acid	40 - 100
Negative	Distilled water	15 - 35

Interpretation of results

32. The cut-off TER value distinguishing corrosive from non-corrosive test chemicals was established during test method optimization, tested during a pre-validation phase, and confirmed in a formal validation study.

33. The prediction model for rat skin TER skin corrosion test method (12) (2), associated with the UN GHS (1) classification system, is given below:

The test chemical is considered to be non-corrosive to skin:

i) if the mean TER value obtained for the test chemical is greater than (>) 5 kΩ, or
ii) the mean TER value obtained for the test chemical is less than or equal to (≤) 5 kΩ, and
   - the skin discs show no obvious damage(*e.g.* perforation), and
   - the mean disc dye content is less than (<) the mean disc dye content of the 10M HCl positive control obtained concurrently (see paragraph 31 for positive control values).

The test chemical is considered to be corrosive to skin:

if the mean TER value obtained for the test chemical is less than or equal to (≤) 5 kΩ and the skin discs are obviously damaged(*e.g.* perforated), or

the mean TER value obtained for the test chemical is less than or equal to (≤) 5 kΩ, and
- the skin discs show no obvious damage(*e.g.* perforation), but
- the mean disc dye content is greater than or equal to (≥) the mean disc dye content of the 10M HCl positive control obtained concurrently (see paragraph 31 for positive control values).

34. A testing run (experiment) composed of at least three replicate skin discs 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 TER equal to 5 ± 0.5 kΩ, a second independent testing run (experiment) should be considered, as well as a third one in case of discordant results between the first two testing runs (experiments).

DATA AND REPORTING

Data

35. Resistance values (kΩ) and dye content values (µg/disc), where appropriate, for the test chemical, as well as for positive and negative controls should be reported in tabular form, including data for each individual replicate disc in each testing run (experiment) and mean values ± SD. All repeat experiments should be reported. Observed damage in the skin discs should be reported for each test chemical.

Test report

36. The test report should include the following information:

Test and Control Chemicals:

- Substance name(s) such as IUPAC or CAS name, and CAS number, if known;
- Purity and composition of the substance or mixture (in percentage(s) by weight);
- Physical-chemical properties relevant to the conduct of the study (*e.g.* physical state, stability, volatility, pH, water solubility, if known);
- Treatment of the test/control chemicals prior to testing, if applicable (*e.g.* warming, grinding);
- Storage conditions;

Test Animals:

- Strain and sex used;
- Age of the animals when used as donor animals;
- Source, housing condition, diet, etc.;
- Details of the skin preparation;

Test Conditions:

- Calibration curves for test apparatus;
- Calibration curves for dye binding test performance, band pass used for measuring OD values, and OD linearity range of measuring device (*e.g.* spectrophotometer), if appropriate;
- Details of the test procedure used for TER measurements;
- Details of the test procedure used for the dye binding assessment, if appropriate;
- Test doses used, duration of exposure period(s) and temperature(s) of exposure;
- Details on washing procedure used after the exposure period;
- Number of replicate skin discs used per test chemical and controls (positive and negative control);
- Description of any modification of the test procedure;
- Reference to historical data of the model. This should include, but is not limited to;
    i) Acceptability of the positive and negative control TER values (in kΩ) with reference to positive and negative control resistance ranges

ii) Acceptability of the positive and negative control dye content values (in µg/disc) with reference to positive and negative control dye content ranges

iii) Acceptability of the test results with reference to historical variability between skin disc replicates

– Description of decision criteria/prediction model applied;

Results:

– Tabulation of data from the TER and dye binding assays (if appropriate) for individual test chemicals and controls, for each testing run (experiment) and each skin disc replicate (individual animals and individual skin samples), means, SDs and CVs;
– Description of any effects observed;
– The derived classification with reference to the prediction model/decision criteria used;

Discussion of the results

Conclusions

LITERATURE

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Figure 1: Apparatus for the rat skin TER assay

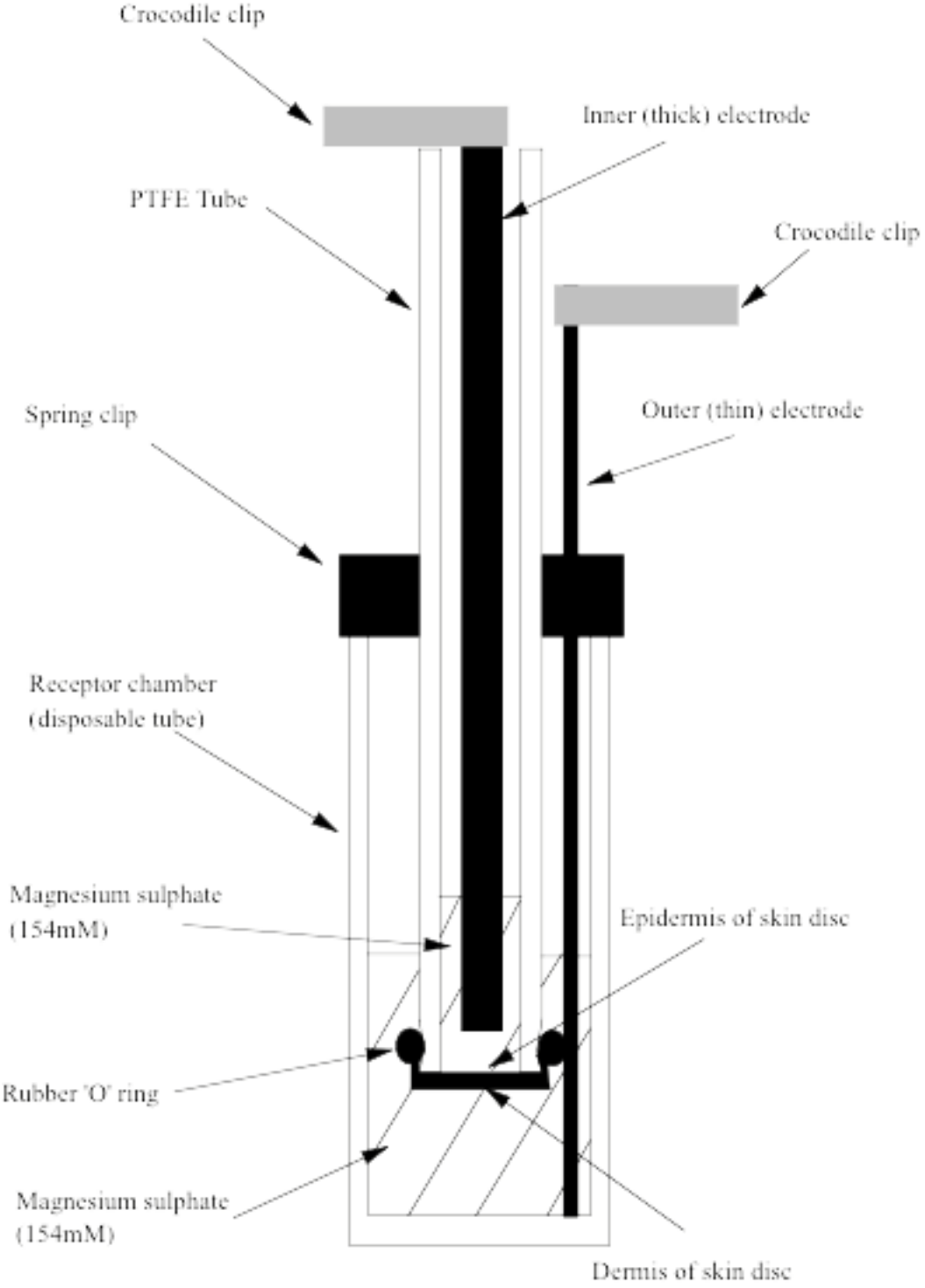
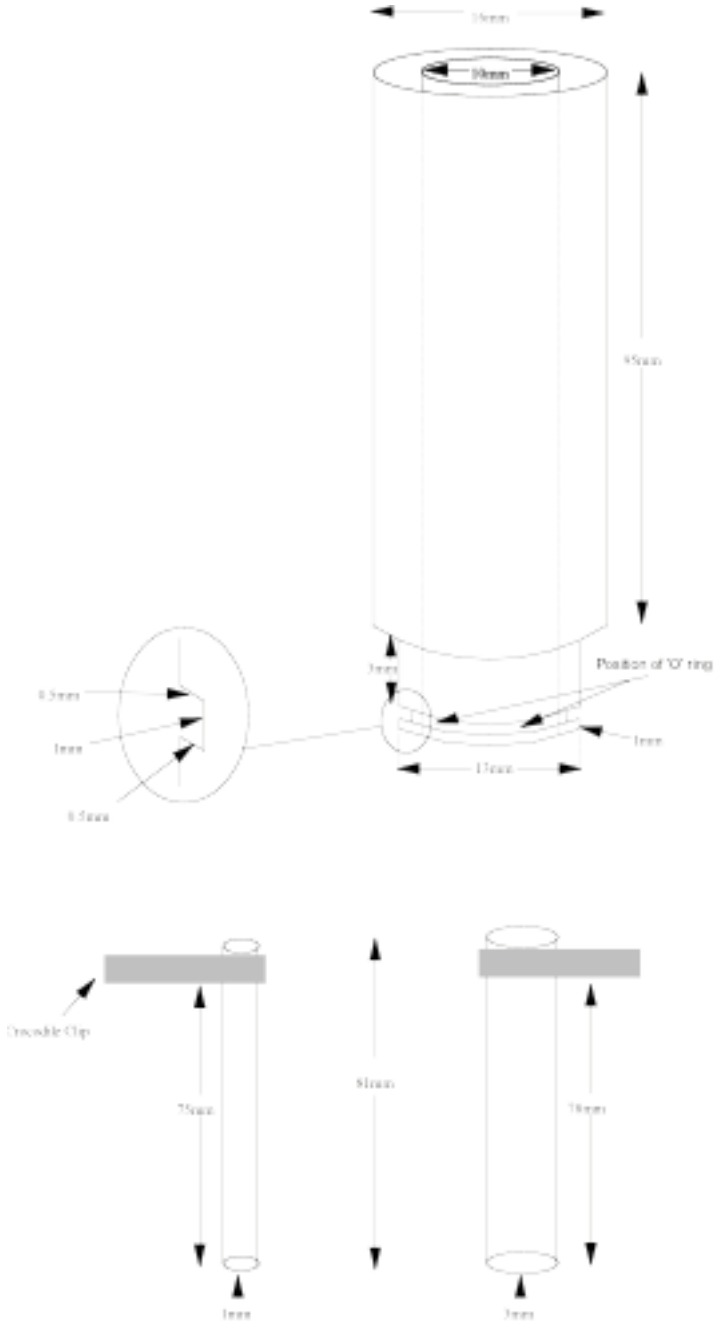


Figure 2: Dimensions of the polytetrafluoroethylene (PTFE) and receptor tubes and electrodes used



Critical factors of the apparatus shown above:

- The inner diameter of the PTFE tube,
- The length of the electrodes relative to the PTFE tube and receptor tube, such that the skin disc should not be touched by the electrodes and that a standard length of electrode is in contact with the $MgSO_4$ solution,
- The amount of $MgSO_4$ solution in the receptor tube should give a depth of liquid, relative to the level in the PTFE tube, as shown in Figure 1,
- The skin disc should be fixed well enough to the PTFE tube, such that the electrical resistance is a true measure of the skin properties.

ANNEX 1

PERFORMANCE STANDARDS FOR ASSESSMENT OF PROPOSED SIMILAR OR MODIFIED *IN VITRO* TRANSCUTANEOUS ELECTRICAL RESISTANCE (TER) TEST METHODS FOR SKIN CORROSION[1]

INTRODUCTION

1. The purpose of Performance Standards (PS) is to provide the basis by which new similar or modified test methods, both proprietary (*i.e.* copyrighted, trademarked, registered) and non-proprietary can demonstrate to have sufficient reliability and relevance for specific testing purposes. These PS, based on a scientific valid and accepted test method, can be used to evaluate the reliability and relevance of other analogous test methods (colloquially referred to as "me-too" test methods) that are based on similar scientific principles and measure or predict the same biological or toxic effect (9). In addition, modified test methods which propose potential improvements to an approved test method, should be evaluated to determine the effect of the proposed changes on the test method's performance and the extent to which such changes affect the information available for the other components of the validation process. Depending on the number and nature of the proposed changes, the generated data and supporting documentation for those changes, they should either be subjected to the same validation process as described for a new test method, or, if appropriate, to a limited assessment of reliability and relevance (accuracy) using established PS (9).

2. Similar (me-too) or modified test methods proposed for use under this Test Guideline should be evaluated to determine their reliability and accuracy using Reference Chemicals (Table 1) representing the full range of the TG 404 *in vivo* corrosivity scores, *i.e.*, Corrosive (UN GHS Category 1A, 1B, and 1C) and non-corrosive chemicals (1). The proposed similar or modified test methods should have reliability, sensitivity, specificity and accuracy values which are comparable or better than those derived from the VRM and as described in paragraphs 6 to 10 of this Annex (12). The reliability of the new similar or modified test method, as well as its ability to correctly identify UN GHS Category 1 corrosive chemicals and non-corrosive chemicals should be determined prior to its use for testing new chemicals.

3. These PS are based on the US-ICCVAM PS (8) for evaluating the validity of new or modified TER test methods. The PS consists of (9): (i) essential test method components; (ii) recommended reference chemicals, and; (iii) defined reliability and accuracy values that the proposed test method should meet or exceed.

ESSENTIAL TEST METHOD COMPONENTS

4. These consist of essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed, mechanistically and functionally similar or modified test method. These components include unique characteristics of the test method, critical procedural details, and quality control measures. Adherence to essential test method components will help to assure that a

[1] Proposed new similar or modified test method following the PS of this Test Guideline should be submitted to the OECD for adoption and inclusion into the Test Guideline before being used for regulatory purposes.

similar or modified proposed test method is based on the same concepts as the corresponding VRM (9). The essential test method components are described in detail in paragraphs 15 to 34 of the Test Guideline:

- Procedures connected to the use of laboratory animals, species, strain (paragraphs 15 and 16)
- The physical components of the test method including the apparatus for measuring skin impedance, the skin disc construct (paragraphs 17 to 19)
- Application of test and control chemicals (paragraphs 20 to 22)
- Measurement of membrane barrier penetration (paragraphs 23 to 25)
- Dye binding procedures (paragraphs 26 to 29)
- Acceptability criteria (paragraphs 30 and 31)
- Interpretation of results (paragraphs 32 to 34)

For specific parameters, adequate values should be provided for any new similar or modified test method; these specific values may vary depending on the specific test method. For the TER test method, the cut-off value distinguishing corrosive from non-corrosive test chemical s is highly dependent on the nature of the skin preparations (source animals) and the equipment used. (2) (22).

MINIMUM LIST OF REFERENCE CHEMICALS

5. Reference Chemicals are used to determine if the reliability and relevance of a proposed similar or modified test method, proven to be structurally and functionally sufficiently similar to the VRM, or representing a minor modification of the VRM, are comparable or better than those of the VRM (12). The 24 recommended Reference Chemicals listed in Table 1 include substances representing different chemical classes (*i.e.* chemical categories based on functional groups), and are representative of the full range of TG 404 *in vivo* scores. The substances included in this list comprise 5 UN GHS Category 1A, 7 UN GHS Category 1B/1C (the *in vivo* data do not permit distinction between the two categories) and 12 non-corrosive substances. The substances listed in Table 1 are selected from the substances used in the validation study of the VRM, with regard to chemical functionality and physical state (11)(12). These Reference Chemicals represent the minimum number of chemicals that should be used to evaluate the reliability and relevance of a proposed similar or modified test method. The use of these Reference Chemicals for the development/optimization of new similar test methods should be avoided to the extent possible. In situations where a listed substance is unavailable, other substances for which adequate *in vivo* reference data are available could be used, primarily from the substances used in the validation study of the VRM. If desired, additional substances representing other chemical classes and for which adequate *in vivo* reference data are available may be added to the minimum list of Reference Chemicals to further evaluate the accuracy of the proposed test method.

Table 1: Minimum list of Reference Chemicals for determination of reliability, sensitivity, specificity and accuracy values for similar or modified *in vitro* TER skin corrosion test methods

Chemical[1]	CASRN	Chemical Class[2]	UN GHS Cat based on *In Vivo* Results[3]	VRM Cat. based on *In Vitro* Results	Physical State	pH[4]
***In Vivo* Corrosives**						
Phosphorus tribromide	7789-60-8	inorganic acid	1A	6 x C	L	1.0
Boron trifluoride dihydrate	13319-75-0	inorganic acid	1A	6 x C	L	1.5
Phosphorus pentachloride	10026-13-8	inorganic acid	1A	6 x C	S	ND
N,N'-Dimethyl dipropylenetriamine	10563-29-8	organic base	1A	6 x C	L	8.3
1,2-Diaminopropane	78-90-0	organic base	1A	6 x C	L	8.3
Sulfuric acid (10%)	7664-93-9	inorganic acid	(1A/)1B/1C	5 x C 1x NC	L	1.2
Potassium hydroxide (10% aq.)	1310-58-3	inorganic base	(1A/)1B/1C	6 x C	L	13.2
Hexanoic acid	142-62-1	organic acid	(1A/)1B/1C	6 x C	L	3.9
Octanoic (Caprylic) acid	124-07-2	organic acid	1B/1C	4 x C 2 x NC	L	3.6
N,N-Dimethyl isopropylamine	996-35-0	organic base	1B/1C	6 x C	L	8.3
n-Heptylamine	111-68-2	organic base	1B/1C	6 x C	L	8.4
2-tert-Butylphenol	88-18-6	phenol	1B/1C	4 x C 2 x NC	L	3.9

Chemical[1]	CASRN	Chemical Class[2]	UN GHS Cat. based on *In Vivo* Results.[3]	VRM Cat. based on *In Vitro* Results	Physical State	pH[4]
***In Vivo* Non-corrosives**						
Sulfamic acid	5329-14-6	inorganic acid	NC	5 x C 1 x NC	S	1.5
Sodium carbonate (50% aq.)	497-19-8	inorganic base	NC	6 x C	L	11.7
Isostearic acid	2724-58-5	organic acid	NC	6 x NC	L	3.6
Dodecanoic acid (Lauric acid)	143-07-7	organic acid	NC	6 x NC	S	ND
4-Amino-1,2,4-triazole	584-13-4	organic base	NC	6 x NC	S	5.5

Chemical[1]	CASRN	Chemical Class[2]	UN GHS Cat. based on *In Vivo* Results.[3]	VRM Cat. based on *In Vitro* Results	Physical State	pH[4]
Eugenol	97-53-0	phenol	NC	1 x C 5 x NC	L	3.6
2-Methoxyphenol	90-05-1	phenol	NC	6 x NC	L	3.9
Phenethyl bromide	103-63-9	electrophile	NC	6 x NC	L	3.6
4-(Methylthio)-benzaldehyde	3446-89-7	electrophile	NC	6 x NC	L	6.8
1,9-Decadiene	1647-16-1	neutral organic	NC	6 x NC	L	3.9
Tetrachloroethylene	127-18-4	neutral organic	NC	6 x NC	L	4.5
Sodium lauryl sulfate (20% aq.)	151-21-3	surfactant	NC	6 x C	L	3.9

Abbreviations: aq = aqueous; CASRN = Chemical Abstracts Service Registry Number; UN GHS = United Nations Globally Harmonised System (1); VRM = Validated Reference Method; ND = Not Determined.
[1]These substances, sorted first by corrosives versus non-corrosives, then by corrosive subcategory and then by chemical class, were selected from the substances used in the ECVAM validation study of the rat skin TER test method (11)(12). Unless otherwise indicated, the substances were tested at the purity level obtained when purchased from a commercial source (11). The selection included, to the extent possible, substances that: (i) are representative of the range of corrosivity responses (*e.g.* non-corrosives; weak to strong corrosives) that the VRM is capable of measuring or predicting; (ii) are representative of the chemical classes used in the validation study; (iii) reflect the performance characteristics of the VRM; (iv) have chemical structures that are well-defined; (v) induce definitive results in the *in vivo* reference test method; (vi) are commercially available; and (vii) are not associated with prohibitive disposal costs.
[2]Chemical class assigned by Barratt *et al.* (1998)(11).
[3]The corresponding UN Packing groups are I, II and III, respectively, for the UN GHS 1A, 1B and 1C.
[4]The pH values were obtained from Fentem *et al.* (1998)(12) and Barratt *et al.* (1998)(11).

DEFINED RELIABILITY AND ACCURACY VALUES

6. For purposes of establishing the reliability and relevance of proposed similar or modified TER test methods to be used by several independent laboratories, all 24 Reference Chemicals listed in Table 1 should be tested in at least three laboratories. It is however essential that all PS-based validation studies are independently assessed by internationally recognized validation bodies, in agreement with international guidelines (9). In each laboratory, all 24 Reference Chemicals should be tested in three independent runs performed with skin discs obtained from different animals and at sufficiently spaced time points. Each testing run should consist of at least three concurrently tested skin discs for each test chemical, negative control and PC, all obtained from the same animal.

7. The calculation of the reliability, sensitivity, specificity and accuracy values of the proposed test method should be done according to the rules described below to ensure that a predefined and consistent approach is used:

1. Within-laboratory reproducibility (WLR) should be calculated based on concordance of classifications using at least two qualified testing runs from Reference Chemicals.
2. For the calculation of between-laboratory reproducibility (BLR) the final classification for each Reference Chemical in each participating laboratory should be obtained by using the arithmetic mean TER and dye binding values over the different qualified testing runs performed. BLR should be calculated based on concordance of classifications using only qualified testing runs obtained with theReference Chemicals for which at least one qualified testing run per laboratory is available.
3. The calculation of the sensitivity, specificity and accuracy values should be done using all qualified testing runs obtained for each Reference Chemical in each laboratory. The calculations should be based on the individual predictions of each qualified testing run for each Reference Chemical in each laboratory and not on the arithmetic mean TER and dye binding values over the different qualified tests performed.

In this context, a qualified testing run consists of at least three replicates tested concurrently within a qualified run that meets the acceptance criteria for the negative and positive control, as defined in the corresponding SOP. Otherwise, the testing run is considered as non-qualified.

Within-laboratory reproducibility

8. An assessment of within-laboratory reproducibility should show a concordance of classifications (corrosive or non-corrosive) obtained in different, independent runs of the 24 Reference Chemicals within one single laboratory equal or higher (≥) than 90% (actual for rat skin TER: 87.5%, 91.7% and 100% in each laboratory, respectively).

Between-laboratory reproducibility

9. For similar or modified test methods , the concordance of classifications (corrosive or non-corrosive) between a minimum of three laboratories, obtained for the 24 Reference Chemicals, should be equal or higher (≥) than 80% (actual for rat skin TER: 95.8 to 79.2% - 1 to 5 chemicals non-concordant –).

Predictive capacity

10. The sensitivity, specificity and accuracy of the proposed similar or modified TER test method should be comparable or better to that of the VRM. The sensitivity and specificity obtained with the 24 relevant Reference Chemicals listed in Table 1 should be equal or higher (≥) than 90% and 75%, respectively, and the accuracy should be equal or higher (≥) than 82.5% (Table 2).

Table 2: Required sensitivity, specificity and accuracy for similar or modified TER skin corrosion test methods to be considered valid to discriminate corrosive from non-corrosive chemicals (C vs. NC) but not able to subcategorize corrosive chemicals

Sensitivity	Specificity	Accuracy
≥ 90% (actual for rat skin TER: 93.1%)	≥ 75% (actual for rat skin TER:75%)	≥ 82.5% (actual for rat skin TER: 84%)

ANNEX 2

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with "concordance" to mean the proportion of correct outcomes of a test method (9).

C: Corrosive

Chemical: means a substance or a mixture

Concordance: This is a measure of test method performance for test methods that give a categorical result, and is one aspect of relevance. The term is sometimes used interchangeably with accuracy, and is defined as the proportion of all chemicals tested that are correctly classified as positive or negative. Concordance is highly dependent on the prevalence of positives in the types of test chemical being examined (9).

GHS (Globally Harmonized System of Classification and Labelling of Chemicals (UN)): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1)

Me-too test: A colloquial expression for a test method that is structurally and functionally similar to a validated and accepted reference test method. Such a test method would be a candidate for catch-up validation. Interchangeably used with similar test method (9).

Mixture: means as a mixture or solution composed of two or more substances in which they do not react.

NC: Non corrosive

OD: Optical Density

PC: Positive Control

Performance standards (PS): Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are; (i) essential test method components; (ii) a minimum list of Reference Chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the similar levels of reliability and accuracy, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of Reference Chemicals.

Qualified run (experiment): A run that meets the acceptance criteria for the negative and positive controls, as defined in the corresponding SOP. Otherwise, the run is considered as non-qualified.

Reference chemicals: Chemicals selected for use in the validation process, for which responses in the *in vitro* or *in vivo* reference test system or the species of interest are already known. These chemicals should be representative of the classes of chemicals for which the test method is expected to be used, and should represent the full range of responses that may be expected from the chemicals for which it may be used, from strong, to weak, to negative. Different sets of reference chemicals may be required for the different stages of the validation process, and for different test methods and test uses (9).

Relevance: Description of relationship of the test method to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test method correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (9).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility (9).

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (9).

Skin corrosion *in vivo*: The production of irreversible damage of the skin; namely, visible necrosis through the epidermis and into the dermis, following the application of a test chemical for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discoloration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (9).

Substance: means chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

(Testing) run: A single test chemical concurrently tested in a minimum of three replicate skin discs.

Test chemical: means what is being tested

Tiered testing strategy: Testing which uses test methods in a sequential manner; the test methods selected in each succeeding level are determined by the results in the previous level of testing (9).

Transcutaneous Electrical Resistance (TER): is a measure of the electrical impedance of the skin, as a resistance value in kilo Ohms. A simple and robust method of assessing barrier function by recording the passage of ions through the skin using a Wheatstone bridge apparatus.