

## OECD GUIDELINE FOR THE TESTING OF CHEMICALS

### Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA

#### INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in light of scientific progress, changing regulatory needs, and animal welfare considerations. The first Test Guideline (TG) for the determination of skin sensitization in the mouse, the Local Lymph Node Assay (LLNA; TG 429) was adopted in 2002, and has since then been revised (1). The details of the validation of the LLNA and a review of the associated work have been published (2) (3) (4) (5) (6) (7) (8) (9). In the LLNA, radioisotopic thymidine or iodine is used to measure lymphocyte proliferation and therefore the assay has limited use in regions where the acquisition, use, or disposal of radioactivity is problematic. The LLNA: BrdU-ELISA [Enzyme-Linked Immunosorbent Assay] is a non-radioactive modification to the LLNA test method, which utilises non-radiolabelled 5-bromo-2-deoxyuridine (BrdU) (Chemical Abstracts Service [CAS] No 59-14-3) in an ELISA-based test system to measure lymphocyte proliferation. The LLNA: BrdU-ELISA has been validated and reviewed and recommended by an international independent scientific peer review panel as considered useful for identifying skin sensitizing and non-sensitizing test substances, with certain limitations (10) (11) (12). This Test Guideline is designed for assessing skin sensitization potential of chemicals in animals. TG 406 utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (13). The LLNA (TG 429) and the two non-radioactive modifications, LLNA: BrdU-ELISA (TG 442 B) and LLNA: DA (TG 442 A), all provide an advantage over the guinea pig tests in TG 406 (13) in terms of reduction and refinement of animal use.

2. Similar to the LLNA, the LLNA: BrdU-ELISA studies the induction phase of skin sensitization and provides quantitative data suitable for dose-response assessment. Furthermore, an ability to detect skin sensitizers without the necessity for using a radiolabel for DNA eliminates the potential for occupational exposure to radioactivity and waste disposal issues. This in turn may allow for the increased use of mice to detect skin sensitizers, which could further reduce the use of guinea pigs to test for skin sensitization potential (*i.e.* TG 406) (13).

#### DEFINITIONS

3. Definitions used are provided in Annex 1.

#### INITIAL CONSIDERATIONS AND LIMITATIONS

4. The LLNA: BrdU-ELISA is a modified LLNA method for identifying potential skin sensitizing test substances, with specific limitations. This does not necessarily imply that in all instances the LLNA: BrdU-ELISA should be used in place of the LLNA or guinea pig tests (*i.e.* TG 406) (13), but rather that the assay is of equal merit and may be employed as an alternative in which positive and negative results generally no longer require further confirmation (10) (11). The testing laboratory should consider all available information on the test substance prior to conducting the study. Such information will include the identity and chemical structure of the test substance; its physicochemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the test substance; and toxicological data on structurally related test substances. This information should be considered in order to determine whether the LLNA: BrdU-ELISA is

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appropriate for the test substance (given the incompatibility of limited types of test substances with the LLNA: BrdU-ELISA [see paragraph 5]) and to aid in dose selection.

5. The LLNA: BrdU-ELISA is an *in vivo* method and, as a consequence, will not eliminate the use of animals in the assessment of allergic contact sensitizing activity. It has, however, the potential to reduce the animal use for this purpose when compared to the guinea pig tests (TG 406) (13). Moreover, the LLNA: BrdU-ELISA offers a substantial refinement of the way in which animals are used for allergic contact sensitization testing, since unlike the TG 406, the LLNA: BrdU-ELISA does not require that challenge-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA: BrdU-ELISA does not require the use of an adjuvant, as is the case for the guinea pig maximisation test (13). Thus, the LLNA: BrdU-ELISA reduces animal distress. Despite the advantages of the LLNA: BrdU-ELISA over TG 406 (13), there are certain limitations that may necessitate the use of TG 406 (*e.g.* the testing of certain metals, false positive findings with certain skin irritants [such as some surfactant-type substances] (6) (1), solubility of the test substance). In addition, test substance classes or substances containing functional groups shown to act as potential confounders (15) may necessitate the use of guinea pig tests (*i.e.* TG 406 (13)). Limitations that have been identified for the LLNA (1) have been recommended to apply also to the LLNA: BrdU-ELISA (10). Other than such identified limitations, the LLNA: BrdU-ELISA should be applicable for testing any test substances unless there are properties associated with these substances that may interfere with the accuracy of the LLNA: BrdU-ELISA. In addition, consideration should be given to the possibility of borderline positive results when Stimulation Index (SI) values between 1.6 and 1.9 are obtained (see paragraphs 31-32). This is based on the validation database of 43 substances using an  $SI \geq 1.6$  (see paragraph 6) for which the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers, but incorrectly identified two of 11 LLNA non-sensitizers with SI values between 1.6 and 1.9 (*i.e.* borderline positive) (10). However, as the same dataset was used for setting the SI-values and calculating the predictive properties of the test, the stated results may be an over-estimation of the real predictive properties.

## PRINCIPLE OF THE TEST

6. The basic principle underlying the LLNA: BrdU-ELISA is that sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of test substance application. This proliferation is proportional to the dose and to the potency of the applied allergen and provides a simple means of obtaining a quantitative measurement of sensitization. Proliferation is measured by comparing the mean proliferation in each test group to the mean proliferation in the vehicle treated control group (VC). The ratio of the mean proliferation in each treated group to that in the concurrent VC group, termed the SI, is determined, and should be  $\geq 1.6$  before further evaluation of the test substance as a potential skin sensitizer is warranted. The methods described here are based on the use of measuring BrdU content to indicate an increased number of proliferating cells in the draining auricular lymph nodes. BrdU is an analogue of thymidine and is similarly incorporated into the DNA of proliferating cells. The incorporation of BrdU is measured by ELISA, which utilises an antibody specific for BrdU that is also labelled with peroxidase. When the substrate is added, the peroxidase reacts with the substrate to produce a coloured product that is quantified at a specific absorbance using a microtiter plate reader.

## DESCRIPTION OF THE ASSAY

### *Selection of animal species*

7. The mouse is the species of choice for this test. Validation studies for the LLNA: BrdU-ELISA were conducted exclusively with the CBA/JN strain, which is therefore considered the preferred strain (10) (12). Young adult female mice, which are nulliparous and non-pregnant, are used. At the start of the study, animals should be between 8-12 weeks old, and the weight variation of the animals should be minimal and

not exceed 20% of the mean weight. Alternatively, other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA: BrdU-ELISA response do not exist.

#### *Housing and feeding conditions*

8. Mice should be group-housed (16), unless adequate scientific rationale for housing mice individually is provided. The temperature of the experimental animal room should be  $22 \pm 3^\circ\text{C}$ . Although the relative humidity should be at least 30% and preferably not exceed 70%, other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

#### *Preparation of animals*

9. The animals are randomly selected, marked to permit individual identification (but not by any form of ear marking), and kept in their cages for at least five days prior to the start of dosing to allow for acclimatisation to the laboratory conditions. Prior to the start of treatment all animals are examined to ensure that they have no observable skin lesions.

#### *Preparation of dosing solutions*

10. Solid test substances should be dissolved or suspended in solvents/vehicles and diluted, if appropriate, prior to application to an ear of the mice. Liquid test substances may be applied neat or diluted prior to dosing. Insoluble substances, such as those generally seen in medical devices, should be subjected to an exaggerated extraction in an appropriate solvent to reveal all extractable constituents for testing prior to application to an ear of the mice. Test substances should be prepared daily unless stability data demonstrate the acceptability of storage.

#### *Reliability check*

11. Positive controls (PC) are used to demonstrate appropriate performance of the assay by responding with adequate and reproducible sensitivity to a sensitizing test substance for which the magnitude of the response is well characterised. Inclusion of a concurrent PC is recommended because it demonstrates competency of the laboratory to successfully conduct each assay and allows for an assessment of intra-, and inter-laboratory reproducibility and comparability. Some regulatory authorities also require a PC for each study and therefore users are encouraged to consult the relevant authorities prior to conducting the LLNA: BrdU-ELISA. Accordingly, the routine use of a concurrent PC is encouraged to avoid the need for additional animal testing to meet such requirements that might arise from the use of a periodic PC (see paragraph 12). The PC should produce a positive LLNA: BrdU-ELISA response at an exposure level expected to give an increase in the  $SI \geq 1.6$  over the negative control (NC) group. The PC dose should be chosen such that it does not cause excessive skin irritation or systemic toxicity and the induction is reproducible but not excessive (*e.g.*  $SI > 14$  would be considered excessive). Preferred PC test substances are 25% hexyl cinnamic aldehyde (CAS No 101-86-0) and 25% eugenol (CAS No 97-53-0) in acetone: olive oil (4:1, v/v). There may be circumstances in which, given adequate justification, other PC test substances, meeting the above criteria, may be used.

12. While inclusion of a concurrent PC group is recommended, there may be situations in which periodic testing (*i.e.* at intervals  $\leq 6$  months) of the PC test substance may be adequate for laboratories that conduct the LLNA: BrdU-ELISA regularly (*i.e.* conduct the LLNA: BrdU-ELISA at a frequency of no less than once per month) and have an established historical PC database that demonstrates the laboratory's ability to obtain reproducible and accurate results with PCs. Adequate proficiency with the LLNA: BrdU-

ELISA can be successfully demonstrated by generating consistent positive results with the PC in at least 10 independent tests conducted within a reasonable period of time (*i.e.* less than one year).

13. A concurrent PC group should always be included when there is a procedural change to the LLNA: BrdU-ELISA (*e.g.* change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test animals), and such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of the previously established historical database in determining the necessity for establishing a new historical database to document consistency in the PC results.

14. Investigators should be aware that the decision to conduct a PC study on a periodic basis instead of concurrently has ramifications on the adequacy and acceptability of negative study results generated without a concurrent PC during the interval between each periodic PC study. For example, if a false negative result is obtained in the periodic PC study, negative test substance results obtained in the interval between the last acceptable periodic PC study and the unacceptable periodic PC study may be questioned. Implications of these outcomes should be carefully considered when determining whether to include concurrent PCs or to only conduct periodic PCs. Consideration should also be given to using fewer animals in the concurrent PC group when this is scientifically justified and if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used (17).

15. Although the PC test substance should be tested in the vehicle that is known to elicit a consistent response (*e.g.* acetone: olive oil; 4:1, v/v), there may be certain regulatory situations in which testing in a non-standard vehicle (clinically/chemically relevant formulation) will also be necessary (18). If the concurrent PC test substance is tested in a different vehicle than the test substance, then a separate VC for the concurrent PC should be included.

16. In instances where test substances of a specific chemical class or range of responses are being evaluated, benchmark test substances may also be useful to demonstrate that the test method is functioning properly for detecting the skin sensitization potential of these types of test substances. Appropriate benchmark test substances should have the following properties:

- structural and functional similarity to the class of the test substance being tested;
- known physical/chemical characteristics;
- supporting data from the LLNA: BrdU-ELISA;
- supporting data from other animal models and/or from humans.

## TEST PROCEDURE

### *Number of animals and dose levels*

17. A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus a concurrent NC group treated only with the vehicle for the test substance, and a PC group (concurrent or recent, based on laboratory policy in considering paragraphs 11- 15). Testing multiple doses of the PC should be considered especially when testing the PC on an intermittent basis. Except for absence of treatment with the test substance, animals in the control groups should be handled and treated in a manner identical to that of animals in the treatment groups.

18. Dose and vehicle selection should be based on the recommendations given in the references 2 and 19. Consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%,

25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. Adequate scientific rationale should accompany the selection of the concentration series used. All existing toxicological information (*e.g.* acute toxicity and dermal irritation) and structural and physicochemical information on the test substance of interest (and/or structurally related test substances) should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximises exposure while avoiding systemic toxicity and/or excessive local skin irritation (19)(20). In the absence of such information, an initial pre-screen test may be necessary (see paragraphs 21-24).

19. The vehicle should not interfere with or bias the test result and should be selected on the basis of maximising the solubility in order to obtain the highest concentration achievable while producing a solution/suspension suitable for application of the test substance. Recommended vehicles are acetone: olive oil (4:1 v/v), *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulphoxide (6) but others may be used if sufficient scientific rationale is provided. In certain situations it may be necessary to use a clinically relevant solvent or the commercial formulation in which the test substance is marketed as an additional control. Particular care should be taken to ensure that hydrophilic substances are incorporated into a vehicle system, which wets the skin and does not immediately run off, by incorporation of appropriate solubilisers (*e.g.* 1% Pluronic® L92). Thus, wholly aqueous vehicles are to be avoided.

20. The processing of lymph nodes from individual mice allows for the assessment of inter-animal variability and a statistical comparison of the difference between test substance and VC group measurements (see paragraph 33). In addition, evaluating the possibility of reducing the number of mice in the PC group is only feasible when individual animal data are collected (17). Further, some national regulatory authorities require the collection of individual animal data. Regular collection of individual animal data provides an animal welfare advantage by avoiding duplicate testing that would be necessary if the test substance results originally collected in one manner (*e.g.* via pooled animal data) were to be considered later by regulatory authorities with other requirements (*e.g.* individual animal data).

#### ***Pre-screen test***

21. In the absence of information to determine the highest dose to be tested (see paragraph 18), a pre-screen test should be performed in order to define the appropriate dose level to test in the LLNA: BrdU-ELISA. The purpose of the pre-screen test is to provide guidance for selecting the maximum dose level to use in the main LLNA: BrdU-ELISA study, where information on the concentration that induces systemic toxicity (see paragraph 24) and/or excessive local skin irritation (see paragraph 23) is not available. The maximum dose level tested should be a concentration of 100% of the test substance for liquids or the maximum possible concentration for solids or suspensions.

22. The pre-screen test is conducted under conditions identical to the main LLNA: BrdU-ELISA study, except there is no assessment of lymph node proliferation and fewer animals per dose group can be used. One or two animals per dose group are suggested. All mice will be observed daily for any clinical signs of systemic toxicity or local irritation at the application site. Body weights are recorded pre-test and prior to termination (Day 6). Both ears of each mouse are observed for erythema and scored using Table 1 (20). Ear thickness measurements are taken using a thickness gauge (*e.g.* digital micrometer or Peacock Dial thickness gauge) on Day 1 (pre-dose), Day 3 (approximately 48 hours after the first dose), and Day 6. Additionally, on Day 6, ear thickness could be determined by ear punch weight determinations, which should be performed after the animals are humanely killed. Excessive local irritation is indicated by an erythema score  $\geq 3$  and/or ear thickness of  $\geq 25\%$  on any day of measurement (21) (22). The highest dose selected for the main LLNA: BrdU-ELISA study will be the next lower dose in the pre-screen concentration series (see paragraph 18) that does not induce systemic toxicity and/or excessive local skin irritation.

**Table 1.** Erythema Scores

| Observation   | Score |
|---|-------|
| No erythema   | 0     |
| Very slight erythema (barely perceptible)   | 1     |
| Well-defined erythema   | 2     |
| Moderate to severe erythema   | 3     |
| Severe erythema (beet redness) to eschar formation preventing grading of erythema | 4     |

23. In addition to a 25% increase in ear thickness (21) (22), a statistically significant increase in ear thickness in the treated mice compared to control mice has also been used to identify irritants in the LLNA (22) (23) (24) (25) (26) (27) (28). However, while statistically significant increases can occur when ear thickness is less than 25% they have not been associated specifically with excessive irritation (25) (26) (27) (28) (29).

24. The following clinical observations may indicate systemic toxicity (30) when used as part of an integrated assessment and therefore may indicate the maximum dose level to use in the main LLNA: BrdU-ELISA: changes in nervous system function (*e.g.* pilo-erection, ataxia, tremors, and convulsions); changes in behaviour (*e.g.* aggressiveness, change in grooming activity, marked change in activity level); changes in respiratory patterns (*i.e.* changes in frequency and intensity of breathing such as dyspnea, gasping, and rales), and changes in food and water consumption. In addition, signs of lethargy and/or unresponsiveness and any clinical signs of more than slight or momentary pain and distress, or a >5% reduction in body weight from Day 1 to Day 6 and mortality should be considered in the evaluation. Moribund animals or animals showing signs of severe pain and distress should be humanely killed (31).

#### ***Main study experimental schedule***

25. The experimental schedule of the assay is as follows:

- Day 1:  
Individually identify and record the weight of each animal and any clinical observation. Apply 25 µL of the appropriate dilution of the test substance, the vehicle alone, or the PC (concurrent or recent, based on laboratory policy in considering paragraphs 11-15), to the dorsum of each ear.
- Days 2 and 3:  
Repeat the application procedure carried out on Day 1.
- Days 4:  
No treatment.
- Days 5:  
Inject 0.5 mL (5 mg/mouse) of BrdU (10 mg/mL) solution inter-peritoneally.

- Day 6:

Record the weight of each animal and any clinical observation. Approximately 24 hours (24 h) after BrdU injection, humanely kill the animals. Excise the draining auricular lymph nodes from each mouse ear and process separately in phosphate buffered saline (PBS) for each animal. Details and diagrams of the lymph node identification and dissection can be found in reference (17). To further monitor the local skin response in the main study, additional parameters such as scoring of ear erythema or ear thickness measurements (obtained either by using a thickness gauge, or ear punch weight determinations at necropsy) may be included into the study protocol.

### ***Preparation of cell suspensions***

26. From each mouse, a single-cell suspension of lymph node cells (LNC) excised bilaterally is prepared by gentle mechanical disaggregation through 200 micron-mesh stainless steel gauze or another acceptable technique for generating a single-cell suspension (*e.g.* use of a disposable plastic pestle to crush the lymph nodes followed by passage through a #70 nylon mesh). The procedure for preparing the LNC suspension is critical in this assay and therefore every operator should establish the skill in advance. Further, the lymph nodes in NC animals are small, so careful operation is important to avoid any artificial effects on SI values. In each case, the target volume of the LNC suspension should be adjusted to a determined optimised volume (approximately 15 mL). The optimised volume is based on achieving a mean absorbance of the NC group within 0.1- 0.2.

### ***Determination of cellular proliferation (measurement of BrdU content in DNA of lymphocytes)***

27. BrdU is measured by ELISA using a commercial kit (*e.g.* Roche Applied Science, Mannheim, Germany, Catalogue Number 11 647 229 001). Briefly, 100 µL of the LNC suspension is added to the wells of a flat-bottom microplate in triplicate. After fixation and denaturation of the LNC, anti-BrdU antibody is added to each well and allowed to react. Subsequently the anti-BrdU antibody is removed by washing and the substrate solution is then added and allowed to produce chromogen. Absorbance at 370 nm with a reference wavelength of 492 nm is then measured. In all cases, assay test conditions should be optimised (see paragraph 26).

## **OBSERVATIONS**

### ***Clinical observations***

28. Each mouse should be carefully observed at least once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. All observations are systematically recorded with records being maintained for each mouse. Monitoring plans should include criteria to promptly identify those mice exhibiting systemic toxicity, excessive local skin irritation, or corrosion of skin for euthanasia (31).

### ***Body weights***

29. As stated in paragraph 25, individual animal body weights should be measured at the start of the test and at the scheduled humane kill.

## **CALCULATION OF RESULTS**

30. Results for each treatment group are expressed as the mean SI. The SI is derived by dividing the mean BrdU labelling index/mouse within each test substance group and the PC group by the mean BrdU labelling index for the solvent/VC group. The average SI for the VCs is then one.

The BrdU labelling index is defined as:

$$\text{BrdU labelling index} = (\text{ABS}_{\text{em}} - \text{ABS blank}_{\text{em}}) - (\text{ABS}_{\text{ref}} - \text{ABS blank}_{\text{ref}})$$

Where; em = emission wavelength; and ref = reference wavelength.

31. The decision process regards a result as positive when  $SI \geq 1.6$  (10). However, the strength of the dose-response relationship, the statistical significance and the consistency of the solvent/vehicle and PC responses may also be used when determining whether a borderline result (*i.e.* SI value between 1.6 and 1.9) is declared positive (3) (6) (32).

32. For a borderline positive response between an SI of 1.6 and 1.9, users may want to consider additional information such as dose-response relationship, evidence of systemic toxicity or excessive irritation, and where appropriate, statistical significance together with SI values to confirm that such results are positives (10). Consideration should also be given to various properties of the test substance, including whether it has a structural relationship to known skin sensitizers, whether it causes excessive skin irritation in the mouse, and the nature of the dose-response observed. These and other considerations are discussed in detail elsewhere (4).

33. Collecting data at the level of the individual mouse will enable a statistical analysis for presence and degree of dose-response relationship in the data. Any statistical assessment could include an evaluation of the dose-response relationship as well as suitably adjusted comparisons of test groups (*e.g.* pair-wise dosed group versus concurrent solvent/vehicle control comparisons). Statistical analyses may include, *e.g.* linear regression or Williams's test to assess dose-response trends, and Dunnett's test for pair-wise comparisons. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequalities of variances and other related problems that may necessitate a data transformation or a non-parametric statistical analysis. In any case, the investigator may need to carry out SI calculations and statistical analyses with and without certain data points (sometimes called "outliers").

## DATA AND REPORTING

### *Data*

34. Data should be summarised in tabular form showing the individual animal BrdU labelling index values, the group mean BrdU labelling index/animal, its associated error term (*e.g.* SD, SEM), and the mean SI for each dose group compared against the concurrent solvent/vehicle control group.

### *Test report*

35. The test report should contain the following information:

Test substance and control test substance:

- identification data (*e.g.* CAS number, if available; source; purity; known impurities; lot number);
- physical nature and physicochemical properties (*e.g.* volatility, stability, solubility);
- if formulation, composition and relative percentages of components;



## Solvent/vehicle:

- identification data (purity; concentration, where appropriate; volume used);
- justification for choice of vehicle;

## Test animals:

- source of CBA mice;
- microbiological status of the animals, when known;
- number and age of animals;
- source of animals, housing conditions, diet, etc.;

## Test conditions:

- source, lot number, and manufacturer's quality assurance/quality control data (antibody sensitivity and specificity and the limit of detection) for the ELISA kit;
- details of test substance preparation and application;
- justification for dose selection (including results from pre-screen test, if conducted);
- vehicle and test substance concentrations used, and total amount of test substance applied;
- details of food and water quality (including diet type/source, water source);
- details of treatment and sampling schedules;
- methods for measurement of toxicity;
- criteria for considering studies as positive or negative;
- details of any protocol deviations and an explanation on how the deviation affects the study design and results;

## Reliability check:

- a summary of results of latest reliability check, including information on test substance, concentration and vehicle used;
- concurrent and/or historical PC and concurrent negative (solvent/vehicle) control data for testing laboratory;
- if a concurrent PC was not included, the date and laboratory report for the most recent periodic PC and a report detailing the historical PC data for the laboratory justifying the basis for not conducting a concurrent PC;

## Results:

- individual weights of mice at start of dosing and at scheduled humane kill; as well as mean and associated error term (*e.g.* SD, SEM) for each treatment group;
- time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal;
- a table of individual mouse BrdU labelling indices and SI values for each treatment group;
- mean and associated error term (*e.g.* SD, SEM) for BrdU labelling index/mouse for each treatment group and the results of outlier analysis for each treatment group;
- calculated SI and an appropriate measure of variability that takes into account the inter-animal variability in both the test substance and control groups;
- dose-response relationship;
- statistical analyses, where appropriate;

## Discussion of results:

## 442B

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- a brief commentary on the results, the dose-response analysis, and statistical analyses, where appropriate, with a conclusion as to whether the test substance should be considered a skin sensitizer.

**LITERATURE**

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## ANNEX 1

**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 (33).

**Benchmark test substance:** A sensitizing or non-sensitizing substance used as a standard for comparison to a test substance. A benchmark substance should have the following properties: (i) a consistent and reliable source(s); (ii) structural and functional similarity to the class of substances being tested; (iii) known physical/chemical characteristics; (iv) supporting data on known effects; and (v) known potency in the range of the desired response.

**False negative:** A test substance incorrectly identified as negative or non-active by a test method, when in fact it is positive or active (33).

**False positive:** A test substance incorrectly identified as positive or active by a test, when in fact it is negative or non-active (33).

**Hazard:** The potential for an adverse health or ecological effect. The adverse effect is manifested only if there is an exposure of sufficient level.

**Inter-laboratory reproducibility:** A measure of the extent to which different qualified laboratories, using the same protocol and testing the same test substance, can produce qualitatively and quantitatively similar results. Inter-laboratory reproducibility is determined during the pre-validation and validation processes, and indicates the extent to which a test can be successfully transferred between laboratories, also referred to as between-laboratory reproducibility (33).

**Intra-laboratory reproducibility:** A determination of the extent that qualified people within the same laboratory can successfully replicate results using a specific protocol at different times. Also referred to as within-laboratory reproducibility (33).

**Outlier:** An outlier is an observation that is markedly different from other values in a random sample from a population.

**Quality assurance:** A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures, and the accuracy of data transfer, are assessed by individuals who are independent from those performing the testing.

**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 (33).

**Skin sensitization:** An immunological process that results when a susceptible individual is exposed topically to an inducing chemical allergen, which provokes a cutaneous immune response that can lead to the development of contact sensitization.

**Stimulation Index (SI):** A value calculated to assess the skin sensitization potential of a test substance that is the ratio of the proliferation in treated groups to that in the concurrent vehicle control group.

**Test substance:** Any material tested using this TG, whether it is a single compound or consists of multiple components (*e.g.* final products, formulations). When testing formulations, consideration should be given to the fact that certain regulatory authorities only require testing of the final product formulation. However, there may also be testing requirements for the active ingredient(s) of a product formulation.