



Section 4
Health effects

Test Guideline No. 442B

Skin Sensitisation

Local Lymph Node Assay: BrdU-ELISA or –FCM

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OECD Guidelines for the Testing
of Chemicals



OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Local lymph node assay: BRDU-ELISA or –FCM

GENERAL INTRODUCTION

1. A skin sensitiser refers to a substance that will lead to an allergic response following repeated skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1).

2. There is general agreement regarding the key biological events underlying skin sensitisation. The current knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised in the form of an Adverse Outcome Pathway (AOP) (2), starting with the molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. This AOP focuses on chemicals that react with thiol (i.e. cysteine) and primary amines (i.e. lysine) such as organic chemicals. In this instance, the molecular initiating event (i.e. the first key event) is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The first key event can be addressed using the in chemico Direct Peptide Reactivity Assay (DPRA) TG 442C (3). The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. This key event can be addressed using the in vitro ARE-Nrf2 Luciferase Test Methods (KeratiSens™ or LuSens) TG 442D (4). The third key event is the activation of dendritic cells (DC), typically assessed by expression of specific cell surface markers, chemokines and cytokines, and can be addressed using either the in vitro Human Cell Line Activation Test (h-CLAT), the in vitro U937 Cell Line Activation Test (U-SENS™) or the Interleukin-9 Reporter Gene assay (IL-8 Luc assay) as described in TG 442E (5). The fourth key event is T-cell proliferation, which is indirectly assessed in the in vivo murine Local Lymph Node Assays (LLNA) (6).

3. The first Test Guideline (TG) for the determination of skin sensitisation in the mouse, the Local Lymph Node Assay (LLNA; TG 429) was adopted in 2002, and has since then been revised (7). The details of the validation of the LLNA and a review of the associated work have been published (8) (9) (10) (11) (12) (13) (14) (15). 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.

4. This Test Guideline describes two non-radioactive modifications to the LLNA test method, which utilise non-radiolabelled 5-bromo-2-deoxyuridine (BrdU) (Chemical Abstracts Service [CAS] No 59-14-3) in an ELISA [Enzyme-Linked Immunosorbent Assay] - or FCM [Flow Cytometry Method]-based test system to measure lymphocyte proliferation:

The Local Lymph Node Assay: BrdU-ELISA (Appendix II), and

The Local Lymph Node Assay: BrdU-FCM (Appendix II).

5. Similar to the LLNA, the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM study the induction phase of skin sensitisation and provide quantitative data suitable for dose-response assessment. The LLNA: BrdU-ELISA provides the UN GHS category 1 of skin sensitisation or no classification (1), and also provides sub-categories of 1A and 1B (17) (18) (19) (20). The LLNA: BrdU-FCM can only be used to conclude on either classification in category 1 or no classification. Furthermore, an ability to detect skin sensitisers 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 sensitisers, which could further reduce the use of guinea pigs to test for skin sensitisation potential (i.e. TG 406) (16).

6. This Test Guideline is designed for assessing skin sensitisation potential of chemicals in animals. TG 406 utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (16). The LLNA (TG 429) (7) and the non-radioactive modifications, LLNA: BrdU-ELISA and FCM (TG 442 B) and LLNA: DA (TG 442 A) (21), all provide an advantage over the guinea pig tests in TG 406 (16) in terms of reduction and refinement of animal use. Currently defined approaches that use the combination of non-animal methods described in Test Guideline 497 (20) or the stand-alone kDPRA described in TG 442C (3) should be used as the first choice for hazard identification and GHS subcategorization, if applicable.

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Annex I – 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 (12).

AOP (Adverse Outcome Pathway): sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an in vivo outcome of interest (2).

Benchmark test chemical: A sensitising or non-sensitising substance used as a standard for comparison to a test chemical. A benchmark chemical 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.

EC1.6: Estimated concentrations of the chemical need to produce a stimulation index of 1.6.

False negative: A test chemical incorrectly identified as negative or non-active by a test method, when in fact it is positive or active (12). The false negative rate is one indicator of the test method performance.

False positive: A test chemical incorrectly identified as positive or active by a test, when in fact it is negative or non-active (12). The false positive rate is one indicator of the test method performance.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

Inter-laboratory reproducibility: A measure of the extent to which different qualified laboratories, using the same protocol and testing the same test chemical, 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 (12).

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 (12).

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

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration $\geq 10\%$ (w/w) and $< 80\%$

(w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

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

Performance standards: 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 comparable levels of accuracy and reliability, 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 (12).

Proficiency chemicals (substances): A subset of the Reference Chemicals included in the Performance Standards that can be used by laboratories to demonstrate technical competence with a standardised test method. Selection criteria for these substances typically include that they represent the range of responses, are commercially available, and have high quality reference data available.

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.

Reference chemicals (substances): A set of chemicals to be used to demonstrate the ability of a new test method to meet the acceptability criteria demonstrated by the validated reference test method(s). 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.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (12).

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 and intra-laboratory repeatability (12).

Reproducibility: The agreement among results obtained from testing the same substance using the same test protocol (see reliability) (12).

Receiver operating Characteristic (ROC) analysis: An analysis to set an optimal cut-off value for the prediction model. The prediction models using cut-off values allow test chemical to be categorized as positive or negative. Any variation of the cut-off value will

result in changes of the sensitivity and specificity, in opposite directions. ROC analysis is commonly used to obtain optimal cutoff values for diagnostic tests.

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 (12).

Skin sensitisation: 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 sensitisation.

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 (12).

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

Substance: 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 (1).

Test chemical: The term "test chemical" is used to refer to what is being tested. It is not related to the applicability of the test methods to the testing of mono-constituent substances, multi-constituent substances and/or mixtures.

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.

Appendix I: In Vivo Skin Sensitisation: The Local Lymph Node Assay: BrdU-ELISA

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. 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 sensitising and non-sensitising test chemicals, with certain limitations (1) (2) (3).
2. The LLNA: BrdU-ELISA is a modified non-radioactive LLNA method for identifying potential skin sensitising test chemicals, with specific limitations. This does not necessarily imply that in all instances the LLNA: BrdU-ELISA should be used in place of the radioactive LLNA (TG 429) or guinea pig tests (i.e. TG 406) (4), when the use of an in vivo method is deemed necessary, 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 (1) (2). The testing laboratory should consider all available information on the test chemical prior to conducting the study. Such information will include the identity and chemical structure of the test chemical; its physicochemical properties; the results of any other in vitro or in vivo toxicity tests on the test chemical; and toxicological data on structurally related test chemicals. This information should be considered in order to determine whether the LLNA: BrdU-ELISA is appropriate for the test chemical (given the incompatibility of limited types of test chemicals with the LLNA: BrdU-ELISA [see paragraph 3]) and to aid in dose selection.
3. 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 sensitising activity. Therefore, consideration should be given to the applicability domain of suitable in vitro, in chemico and in silico methods and consequently, the possibility of using these approaches rather than testing on animals. Like other LLNA test methods, the LLNA: BrdU-ELISA has, however, the potential to reduce the animal use for this purpose when compared to the guinea pig tests (TG 406) (4). Moreover, the LLNA: BrdU-ELISA offers a substantial refinement of the way in which animals are used for allergic contact sensitisation testing, since unlike 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 (4). Thus, the LLNA: BrdU-ELISA reduces animal distress. The positive result of the LLNA: BrdU-ELISA provides the UN GHS category 1 of skin sensitisation, and also provides sub-categories of 1A and 1B (5) (6) (7) (8). When the EC1.6 value is 6% or less, the chemical is subcategorized as 1A, and above 6% as 1B. Despite the advantages of the LLNA: BrdU-ELISA over TG 406 (4), there are certain limitations applicable to the LLNA test, 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] (9) (10), solubility of the test chemicals [such as rarely soluble or non-soluble substances]). In addition, test chemical classes or substances containing functional groups shown to act as potential confounders (e.g. fatty acid glutamate, oleic acid, oleic acid ester, fatty alcohol 1, fatty alcohol 2, polyaminofunctional

siloxane (11)) may necessitate the use of guinea pig tests (i.e. TG 406 (4)). Other limitations that have been identified for the LLNA (10) have also been recommended to apply to the LLNA: BrdU-ELISA (1). Other than such identified limitations, the LLNA: BrdU-ELISA should be applicable for testing any test chemicals 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) in the LLNA: BrdU-ELISA. 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 sensitiser, but incorrectly identified two of 11 LLNA non-sensitisers with SI values between 1.6 and 1.9 (i.e. borderline positive) (1). 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.

4. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically.

5. Definitions are provided in the Annex 1 of the General Introduction.

PRINCIPLE OF THE TEST

6. The basic principle underlying the LLNA: BrdU-ELISA is that sensitiser induce proliferation of lymphocytes in the lymph nodes draining the site of test chemical 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 sensitisation. 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 ≥ 1.6 before further evaluation of the test chemical as a potential skin sensitiser 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 (1) (3). 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 or 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 (12) on solid-bottomed cages (13) with suitable substrate and nesting material (14) (15) (16) (17), unless adequate scientific rationale for alternative 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, humanely marked to permit individual identification preferably by non-invasive hair clipping (18) (19), 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. During all examinations, the mice should be handled using non-aversive methods such as cupping or tunnel handling (20).

Preparation of dosing solutions

10. Solid test chemicals should be dissolved or suspended in solvents/vehicles and diluted, if appropriate, prior to application to an ear of the mice. Liquid test chemicals may be applied neat or diluted prior to dosing. Insoluble chemicals, such as those generally seen in medical devices (39), 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 chemicals 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 sensitising test chemical 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 ≥ 1.6 over the VC 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 chemicals 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 chemicals, 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 ≤ 6 months) of the PC test chemical 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 chemical 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 (21).

15. Although the PC test chemical 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 (22). If the concurrent PC test chemical is tested in a different vehicle than the test chemical, then a separate VC for the concurrent PC should be included.

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

- structural and functional similarity to the class of the test chemical 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 chemical, plus a concurrent VC group treated only with the vehicle for the test chemical, 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 chemical, 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 27. Three 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 chemical of interest (and/or structurally related test chemicals) 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 (23) (24). 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 chemical. Recommended vehicles are acetone: olive oil (4:1 v/v), N,N-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulphoxide (5) 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 chemical 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 chemical and VC group measurements (see paragraph 34). In addition, evaluating the possibility of reducing the number of mice in the PC group is only feasible when individual animal data are collected (22). 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 chemical 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 chemical 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 (24). 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 ≥ 3 and/or ear thickness of $\geq 25\%$ on any day of measurement (25) (26). The highest dose selected for the main LLNA: BrdU-ELISA study will be the highest dose used in the pre-screen concentration series (see paragraph 18) that did 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 (25) (26), a statistically significant increase in ear thickness in the treated mice compared to solvent/vehicle control mice has also been used to identify irritants in the LLNA (26) (27) (28) (29) (30) (31) (32). However, while statistically significant increases can occur when ear thickness is less than 25%, they have not been associated specifically with excessive irritation (29) (30) (31) (32) (33).

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 (35).

Main study experimental schedule

The experimental schedule of the assays 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 chemical, 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.
- Day 4:

No treatment.
- Day 5:

Inject 0.5 mL (5 mg/mouse) of BrdU (10 mg/mL) solution intra-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 (21). 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

25. 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 VC 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 VC group within 0.1-0.2.

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

26. BrdU is measured by ELISA using a commercial kit (e.g. in the validation study the Roche Applied Science, Mannheim, Germany, was used). Other BrdU ELISA kits may be used if they provide consistent results. 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, peroxidase-conjugated 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

27. 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 (35).

Body weights

28. 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

29. 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 chemical 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.

30. The BrdU labelling index is defined as:

$$\text{BrdU labelling index} = (\text{ABSem} - \text{ABS blankem}) - (\text{ABSref} - \text{ABS blankref})$$

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

31. The decision process regards a result as positive when $SI \geq 1.6$ (1). 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 (5) (36) (37). When the positive result is obtained, the EC1.6 value can be calculated using following formula:

$$EC1.6 = c + (1.6 - d)/(b - d) \times (a - c)$$

Where; a = dose concentration immediately above SI = 1.6, b = SI immediately above 1.6, c = dose concentration immediately below SI = 1.6, and d = SI immediately below 1.6.

32. Also, when all the SIs exceeded 1.6, extrapolate EC 1.6 value (EC1.6 ex) can be calculated with the two lowest SI values above 1.6 and their corresponding concentrations using following formula:

$$EC1.6ex = 2^{\log_2(c) + (1.6 - d)(b - d) \times [\log_2(a) - \log_2(c)]}$$

Where; a = dose concentration for next to lowest SI above 1.6, b = next to lowest SI above 1.6, c = dose concentration for lowest SI above 1.6, d = lowest SI above 1.6.

33. 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 (1). Consideration should also be given to various properties of the test chemical, 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 (38).

34. 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

35. 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

36. The test report should contain the following information:

Test chemical

source, lot number, limit date for use, if available;

stability of the test chemical, if known;

Mono-constituent substance

physical appearance, water solubility, and additional relevant physicochemical properties;

chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures

characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Controls

identification data (e.g. CAS number, if available; source; purity; known impurities; lot number);

physical nature and physicochemical properties (e.g. volatility, stability, solubility);

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 chemical preparation and application;

justification for dose selection (including results from pre-screen test, if conducted);

vehicle and test chemical concentrations used, and total amount of test chemical 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;

report whether (yes/no) blinding was applied in the study.

Reliability check

- a summary of results of latest reliability check, including information on test chemical, concentration, PC, VC and benchmark test chemical used, as appropriate;
- concurrent and/or historical PC and concurrent VC 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 chemical and control groups;
- dose-response relationship;
- statistical analyses, where appropriate;
- EC1.6 or EC1.6ex value, if available

Discussion of results:

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

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Appendix II: In Vivo Skin Sensitisation: The Local Lymph Node Assay: BrdU-FCM

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The LLNA: BrdU-FCM has been validated and recommended, following an international independent scientific peer review, as useful for identifying skin sensitising and non-sensitising test chemicals, with certain limitations (1) (2) (3) (4). The validation study for the LLNA: BrdU-FCM was performed in compliance with the performance standards (PS) for assessment of proposed similar or modified LLNA test methods for skin sensitisation in Annex 1 of the OECD Guideline for the testing of chemicals, Skin sensitisation: Local lymph node assay (TG 429).

2. The LLNA: BrdU-FCM is a modified non-radioactive LLNA method for identifying potential skin sensitising test chemicals, with specific limitations. This does not necessarily imply that in all instances the LLNA: BrdU-FCM should be used in place of the radioactive LLNA (TG 429) or guinea pig tests (i.e. TG 406) (5), when the use of an *in vivo* method is deemed necessary, 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 (1) (2). The testing laboratory should consider all available information on the test chemical prior to conducting the study. Such information will include the identity and chemical structure of the test chemical; its physicochemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the test chemical; and toxicological data on structurally related test chemicals. This information should be considered in order to determine whether the LLNA: BrdU-FCM is appropriate for the test chemical (given the incompatibility of limited types of test chemicals with the LLNA: BrdU-FCM [see paragraph 3]) and to aid in dose selection.

3. The LLNA: BrdU-FCM is an *in vivo* method and, as a consequence, will not eliminate the use of animals in the assessment of allergic contact sensitising activity. Therefore, consideration should be given to the applicability domain of suitable *in vitro*, *in chemico* and *in silico* methods and consequently, the possibility of using these approaches rather than testing on animals. Like other LLNA test methods, the LLNA: BrdU-FCM has, however, the potential to reduce the animal use for this purpose when compared to the guinea pig tests (TG 406) (5). Moreover, the LLNA: BrdU-FCM offers a substantial refinement of the way in which animals are used for allergic contact sensitisation testing, since unlike TG 406, the LLNA: BrdU-FCM does not require that challenge-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA: BrdU-FCM does not require the use of an adjuvant, as is the case for the guinea pig maximisation test (5). Thus, the LLNA: BrdU-FCM reduces animal distress. Despite the advantages of the LLNA: BrdU-FCM over TG 406 (5), there are certain limitations applicable to the LLNA test, 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) (7), solubility of the test chemicals [such as practically insoluble or insoluble substances]). In addition, test chemical classes or substances containing functional groups shown to act as potential confounders (e.g. fatty acid glutamate, oleic acid, oleic acid

ester, fatty alcohol 1, fatty alcohol 2, polyaminofunctional siloxane (8)) may necessitate the use of guinea pig tests (i.e. TG 406 (5)). Other limitations that have been identified for the LLNA (7) have also been recommended to apply to the LLNA: BrdU-FCM (1). Other than such identified limitations, the LLNA: BrdU-FCM should be applicable for testing any test chemicals unless there are properties associated with these substances that may interfere with the accuracy of the LLNA: BrdU-FCM. According to the validation study, the LLNA: BrdU-FCM correctly identified 20 among the 22 reference substances listed in the TG 429 PS on the basis of the LLNA results (1). One moderate skin sensitiser, 2-mercaptobenzothiazole, and one weak skin sensitiser, methyl methacrylate for which the other LLNA variants have limitation in prediction, were misclassified in the LLNA: BrdU-FCM (1) (2) (9). However, as the same dataset was used for setting the Stimulation Index (SI)-values and calculating the predictive properties of the test, the stated results may be an over-estimation of the real predictive properties.

4. Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.
5. Definitions are provided in the Annex 1 of the General Introduction.

PRINCIPLE OF THE TEST

6. The basic principle underlying the LLNA: BrdU-FCM is that sensitisers induce proliferation of lymphocytes in the lymph nodes draining the site of test chemical 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 sensitisation. 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 ≥ 2.7 before further evaluation of the test chemical as a potential skin sensitiser 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 FCM, which utilises an antibody specific for BrdU that is also labelled with fluorescein isothiocyanate (FITC). The FCM quantifies the number of BrdU-positive viable cells using a flow cytometer, which is widely employed in analysing lymphocyte population.

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-FCM were conducted exclusively with the BALB/c strain, which is therefore considered the preferred strain (1) (2). The CBA/J strain can also be used in the LLNA: BrdU-FCM. CBA/J strain responses are highly correlated with and more sensitive than BALB/c strain responses (2) (10) (11) (12). However, different cut-off SI values may have to be adopted for each strain to maximize sensitivity after Receiver Operating Characteristic (ROC) analysis. 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 or males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA: BrdU-FCM response do not exist.

Housing and feeding conditions

8. Mice should be group-housed (13) on solid-bottomed cages (34) with suitable substrate and nesting material (35) (36) (37) (38), unless adequate scientific rationale for alternative 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, humanely marked to permit individual identification preferably by non-invasive hair clipping (39) (40), 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. During all examinations, the mice should be handled using non-aversive methods such as cupping or tunnel handling (41).

Preparation of dosing solutions

10. Solid test chemicals should be dissolved or suspended in solvents/vehicles and diluted, if appropriate, prior to application to an ear of the mice. Liquid test chemicals may be applied neat or diluted prior to dosing. Insoluble chemicals, such as those generally seen in medical devices (33), 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 chemicals 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 sensitising test chemical 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-FCM. 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-FCM response at an exposure level expected to give an increase in the SI ≥ 2.7 over the VC 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 > 27 would be considered excessive). Preferred PC test chemicals 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 chemicals, 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 ≤ 6 months) of the PC test chemical may be adequate for laboratories that conduct the LLNA: BrdU-FCM regularly (i.e. conduct the LLNA: BrdU-FCM 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-FCM 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-FCM (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 chemical 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 (14).

15. Although the PC test chemical 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 (15). If the concurrent PC test chemical is tested in a different vehicle than the test chemical, then a separate VC for the concurrent PC should be included.

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

- structural and functional similarity to the class of the test chemical being tested;
- known physical/chemical characteristics;
- supporting data from the LLNA: BrdU-FCM;
- 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 chemical, plus a concurrent VC group treated only with the vehicle for the test chemical, 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 chemical, 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. Three 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 chemical of interest (and/or structurally related test chemicals) 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 (16) (17). 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 chemical. 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 chemical 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 chemical 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 (14). 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 chemical 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-FCM. The purpose of the pre-screen test is to provide guidance for selecting the maximum dose level to use in the main LLNA: BrdU-FCM 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 chemical 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-FCM 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 (17). 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 ≥ 3 and/or ear thickness of $\geq 25\%$ on any day of measurement (18) (19). The highest dose selected for the main LLNA: BrdU-FCM study will be the highest dose used in the pre-screen concentration series (see paragraph 18) that did 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 (18) (19), a statistically significant increase in ear thickness in the treated mice compared to solvent/vehicle control mice has also been used to identify irritants in the LLNA (19) (20) (21) (22) (23) (24) (25). However, while statistically significant increases can occur when ear thickness is less than 25%, they have not been associated specifically with excessive irritation (22) (23) (24) (25) (26).

24. The following clinical observations may indicate systemic toxicity (27) when used as part of an integrated assessment and therefore may indicate the maximum dose level to use in the main LLNA: BrdU-FCM: 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 (28).

Main study experimental schedule

The experimental schedule of the assays 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 chemical, 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.
- Day 4:
 - No treatment.
- Day 5:
 - Inject 0.1 mL (2 mg/mouse) of BrdU (20 mg/mL) solution intra-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 (14). 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

25. 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 VC animals are small, so careful operation is important to avoid any artificial effects on SI values. The LNC are harvested with an appropriate volume of cold PBS (e.g. 2 mL) and, if necessary, the LNC suspension can be diluted (e.g. 1/10 dilution). The number of LNC should be counted and then 1.5×10^6 LNC are needed for the next step.

Determination of cellular proliferation (measurement of BrdU-positive lymphocytes)

26. BrdU-positive lymphocytes are counted through the FCM using a commercially available kit (e.g. in the validation study the BD Pharmingen, Franklin Lakes, NJ, USA, was used). Other anti-BrdU antibody kits may be used if they provide consistent results. Briefly, the LNC suspension (1.5×10^6) is washed once with PBS by centrifugation and then re-suspended. Cells are permeabilised with the buffer supplied with the kit and then treated with DNase. After washing, FITC-conjugated anti-BrdU antibody is added and after another wash, 7-aminoactinomycin D (7-AAD) solution is added. The number of BrdU-positive cells within the viable 7-AAD-expressing cell population (10^4 cells) is counted with a flow cytometer.

OBSERVATIONS

Clinical observations

27. 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 (28).

Body weights

28. 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

29. Results for each treatment group are expressed as the mean SI. The SI for the LLNA: BrdU-FCM is derived by dividing the number of BrdU-positive LNCs/mouse of test chemical group or the PC group by the mean number of BrdU-positive LNCs in the solvent/VC group. The average SI for the VCs is then one.

The number of BrdU-positive LNCs is defined as (See Appendix IB-Annex 1 paragraph 7):
Number of BrdU-positive LNCs = % of BrdU-positive cells (% of Q2¹) × number of LNCs

¹ The gated percentage data (Q2 region %) from 'Quadrant Statistics' in the flow cytometer analysis.

30. The decision process regards a result as positive when $SI \geq 2.7$ (1) (2) (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 is declared positive (6) (29) (30).

31. If it is necessary to clarify the results obtained, consideration should also be given to various properties of the test chemical, including whether it has a structural relationship to known skin sensitisers, 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 (31).

32. 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

33. Data should be summarised in tabular form showing the number of BrdU-positive LNCs for the individual animal, the group mean number of BrdU-positive LNCs/animal, or, 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

34. The test report should contain the following information:

Test chemical:

source, lot number, limit date for use, if available;
stability of the test chemical, if known;

Mono-constituent substance:

physical appearance, water solubility, and additional relevant physicochemical properties;
chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Controls:

identification data (e.g. CAS number, if available; source; purity; known impurities; lot number);
physical nature and physicochemical properties (e.g. volatility, stability, solubility);

Solvent/vehicle:

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

Test animals:

source of BALB/c mice or 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 FCM kit;
details of test chemical preparation and application;
justification for dose selection (including results from pre-screen test, if conducted);
vehicle and test chemical concentrations used, and total amount of test chemical 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 chemical, concentration, PC, VC and benchmark test chemical used, as appropriate;
concurrent and/or historical PC and concurrent VC 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 number of BrdU-positive LNCs, and SI values of individual mouse for each treatment group;

mean and associated error term (e.g. SD, SEM) for number of BrdU-positive LNCs/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 chemical and control groups;

dose-response relationship;

statistical analyses, where appropriate;

Discussion of results:

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

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APPENDIX IB - ANNEX I: MEASUREMENT OF BrdU-POSITIVE LNCs WITH FLOW CYTOMETRY

This method is based on the LLNA: BrdU-FCM protocol, which was used for the KoCVAM- coordinated validation study (1). It is recommended that this protocol is used when implementing and using the LLNA: BrdU-FCM in the laboratory.

Preparation prior to measurement

1. To measure incorporated BrdU, the following samples should be prepared prior to the measurement.

Blank sample (n=1): LNCs from the mouse not injected with BrdU.

Non-treatment sample (n=1): LNCs from the mouse not treated with any substances, but received a BrdU injection.

Vehicle control-treatment sample (n≥4): LNCs from the mouse treated with the vehicle control and received a BrdU injection.

Test chemical-treatment sample (n≥4, a minimum of three concentrations): LNCs from the mouse treated with test chemicals and received a BrdU injection.

Positive control-treatment sample (n≥4): LNCs from the mouse treated with the positive control and received a BrdU injection.

Analysis of flow cytometric results

A flow cytometer should be calibrated using appropriate tools (e.g. 'BD FACSCComp' for FACSCalibur™ or 'Beckman coulter FlowCheck' for Cytomics FC500) prior to testing or regularly.

Forward scatter-side scatter (FSC-SSC) graph

- 1) Both the X axis (FSC) and Y axis (SSC) should be on a linear scale.
- 2) Set up a zone (gate) with a flock of viable lymph nodes at its centre in the FSC-SSC graph.
- 3) Outline the gate such that it has at least 10,000 cells.

7-AAD-BrdU graph

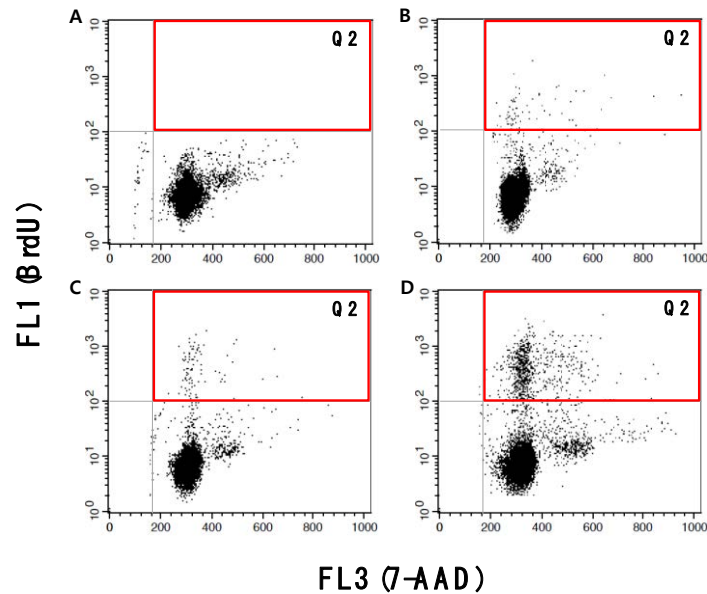
- 1) The X axis (7-AAD, FL3) should be on a linear scale, whereas the Y (BrdU, FL1) axis should be a log scale (Figure 1).

* Compensation should be set using unstained, only BrdU-stained, only 7-AAD stained samples, and double stained with both anti-BrdU and 7-AAD at the time of beginning this assay. The compensation can be saved for future use.

Set up Q2 following the steps below

- 1) Using the blank sample, set up Q2 (upper right) where no cells are present (Figure 1A).
- 2) Using the non-treatment sample, set up Q2 so that % BrdU-positive cells are about 1% of all cells (Figure 1B).
- 3) The Q2 region percentage indicates the proportion of FITC conjugated anti-BrdU-Antibody positive live lymphocyte in 10,000 LNCs.

- Figure 1. Flow cytometry configuration for the calculation of % of BrdU-positive cells (% of Q2)



Note: A, blank sample; B, non-treatment sample; C, vehicle control-treatment sample; D, test chemical or positive control-treatment sample

Count of % BrdU-positive cells

Perform flow cytometric operation for the vehicle control-treatment samples (Figure 1C), the test chemical-treatment samples and the positive control-treatment samples (Figure 1D). Obtain the gated percentage data (Q2 region %) from ‘Quadrant Statistics’ for each sample.

Calculation of the SI and the EC2.7

The number of BrdU-positive LNCs in the LNs of the vehicle control-treatment group is obtained by multiplying the number of LNCs in the LNs by the ratio of cells expressing BrdU in 10,000 LNCs (obtained by flow cytometry). The number of BrdU-positive LNCs in the LNs of the test chemical-treatment group is obtained by the method described above. Individual SIs are calculated by dividing the number of BrdU-positive LNCs/mouse in the test chemical-treatment group by the mean number of BrdU-positive LNCs in the vehicle control-treatment group. The mean SI of each test chemical group is calculated based on individual SIs.

$$\text{Stimulation Index (SI)} = \frac{\text{Number of BrdU-positive LNCs/mouse exposed to a test chemical}}{\text{Mean number of BrdU-positive LNCs in the vehicle control group}}$$

For the positive results, the EC2.7 value, i.e. an estimated concentration showing 2.7 of SI, could be calculated by linear regression method using the following equation.

$$Y \text{ (SI)} = aX \text{ (concentration)} + b \rightarrow \text{EC2.7} = (2.7-b)/a$$

* Parameters a (slope) and b (y-intercept) can be derived using linear least squares method.

Other estimation methods (e.g. linear interpolation or extrapolation formulas) could be utilized to calculate EC2.7 value (32).