

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR AN UPDATE TO TEST GUIDELINE 429

Skin Sensitisation: Local Lymph Node Assay

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress, changing regulatory needs, and animal welfare considerations. The first Test Guideline (TG) for the determination of skin sensitisation in the mouse, the Local Lymph Node Assay (LLNA) TG (i.e., TG 429) was adopted in 2002 (1), after sufficient validation studies. 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). The updated LLNA is based on the evaluation of experience and scientific data (9). This is the second Test Guideline to be promulgated for assessing skin sensitisation potential of chemicals in animals. The other Test Guideline (i.e., TG 406) utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (10). This updated Test Guideline includes a set of Performance Standards (PS) (Annex 1) that can be used to more efficiently evaluate the validation status of new and/or modified test methods that are functionally and mechanistically similar to the LLNA, in accordance with the principles of Guidance Document No. 34 (11).

2. The LLNA studies the induction phase of skin sensitisation and provides quantitative data suitable for dose response assessment. It should be noted that the mild/moderate sensitisers, which are recommended as suitable positive control substances for guinea pig test methods, are also appropriate for use with the LLNA (6)(8)(12). The LLNA provides certain advantages with regard to animal welfare and a reduced LLNA (rLLNA) for hazard classification of skin sensitising substances can be performed under this Test Guideline (13)(14).

DEFINITIONS

3. Definitions used are provided in Annex 2.

INITIAL CONSIDERATIONS

4. The LLNA provides an alternative method for identifying skin sensitising chemicals and for confirming that chemicals lack a significant potential to cause skin sensitisation. This does not necessarily imply that in all instances the LLNA should be used in place of guinea pig tests, 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.

5. The LLNA is an *in vivo* method and, as a consequence, will not eliminate the use of animals in the assessment of allergic contact sensitising activity. It has, however, the potential to reduce the number of animals required for this purpose. Moreover, the LLNA offers a substantial refinement of the way in which animals are used for allergic contact sensitisation testing. The LLNA is based upon consideration of immunological events

38 stimulated by chemicals during the induction phase of sensitisation. Unlike guinea pig tests
39 (i.e., TG 406) (10) the LLNA does not require that challenge-induced dermal hypersensitivity
40 reactions be elicited. Furthermore, the LLNA does not require the use of an adjuvant, as is
41 the case for the guinea pig maximisation test, as described in reference (10). Thus, the LLNA
42 reduces animal distress. Despite the advantages of the LLNA over TG 406, it should be
43 recognised that there are certain limitations that may necessitate the use of TG 406 (e.g., false
44 negative findings in the LLNA with certain metals, false positive findings with certain skin
45 irritants) (15).

46 **PRINCIPLE OF THE TEST**

47 6. The basic principle underlying the LLNA is that sensitisers induce proliferation of
48 lymphocytes in the lymph nodes draining the site of chemical application. This proliferation
49 is proportional to the dose and to the potency of the applied allergen and provides a simple
50 means of obtaining a quantitative measurement of sensitisation. The LLNA assesses this
51 proliferation as the proliferation in test groups compared to that in vehicle treated controls.
52 The ratio of the proliferation in treated groups to that in the concurrent vehicle control group,
53 termed the Stimulation Index (SI), is determined, and should be ≥ 3 before a test substance
54 can be further evaluated as a potential skin sensitiser. The methods described here are based
55 on the use of *in vivo* radioactive labelling to measure an increased number of proliferating
56 cells in the draining auricular lymph nodes. However, other endpoints for assessment of the
57 number of proliferating cells may be employed provided there is justification and appropriate
58 scientific support, including full citations and description of the methodology.

59 **DESCRIPTION OF THE ASSAY**

60 **Selection of animal species**

61 7. The mouse is the species of choice for this test. Young adult female mice of
62 CBA/Ca or CBA/J strain, which are nulliparous and non-pregnant, are used. At the start of
63 the study, animals should be between 8-12 weeks old, and the weight variation of the animals
64 should be minimal and not exceed 20% of the mean weight. Other strains and males may be
65 used when sufficient data are generated to demonstrate that significant strain and/or gender-
66 specific differences in the LLNA response do not exist.

67 **Housing and feeding conditions**

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

74 **Preparation of animals**

75 9. The animals are randomly selected, marked to permit individual identification (but
76 not by any form of ear marking), and kept in their cages for at least five days prior to the start

77 of dosing to allow for acclimatisation to the laboratory conditions. Prior to the start of
78 treatment all animals are examined to ensure that they have no observable skin lesions.

79 **Preparation of dosing solutions**

80 10. Solid test substances should be dissolved in appropriate solvents/vehicles and
81 diluted, if appropriate, prior to application to an ear of the mice. Liquid test substances may
82 be applied neat or diluted prior to dosing. Insoluble materials, such as those generally seen in
83 medical devices, should be extracted in an appropriate solvent and, if appropriate, further
84 processed prior to application to an ear of the mice. The test substance should be prepared
85 daily unless stability data demonstrate the acceptability of storage.

86 **Reliability check**

87 11. Positive controls are used to demonstrate appropriate performance of the assay by
88 responding with adequate and reproducible sensitivity to a sensitising substance for which
89 the magnitude of the response is well characterised. Inclusion of a concurrent positive control
90 is recommended because it demonstrates competency of the laboratory to successfully
91 conduct each assay and allows for an assessment of intra- and inter-laboratory reproducibility
92 and comparability. The positive control should produce a positive LLNA response at an
93 exposure level expected to give an increase in the SI > 3 over the negative control group. The
94 positive control dose should be chosen such that the induction is reproducible but not
95 excessive. Preferred positive control substances are hexyl cinnamic aldehyde (Chemical
96 Abstracts Service [CAS] No 101-86-0) and mercaptobenzothiazole (CAS No 149-30-4).
97 There may be circumstances in which, given adequate justification, other positive control
98 substances, meeting the above criteria, may be used.

99 12. While inclusion of a concurrent positive control group is recommended, there may
100 be situations in which periodic testing (i.e., at intervals ≤ 6 months) of the positive control
101 substance may be adequate for laboratories that conduct the LLNA regularly (i.e., conduct
102 the LLNA at a frequency of no less than once per month) and have an established historical
103 positive control database that demonstrates the laboratory's ability to obtain reproducible and
104 accurate results with positive controls. Adequate proficiency with the LLNA can be
105 successfully demonstrated by generating consistent results with the positive control in at least
106 10 independent tests conducted within a reasonable period of time (i.e., less than one year).

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

114 14. Investigators should be aware that the decision to conduct a positive control on a
115 periodic basis instead of concurrently has ramifications on the adequacy and acceptability of
116 negative study results generated without a concurrent positive control during the interval

117 between each periodic positive control study. For example, if a false negative result is
118 obtained in the periodic positive control study, all negative test substance results obtained in
119 the interval between the last acceptable periodic positive control study and the unacceptable
120 periodic positive control study will be questioned. Any study reports associated with these
121 negative test substance results should immediately be amended to report the failed positive
122 control test. In order to demonstrate that the prior negative test substance study results are
123 acceptable, a laboratory would be expected to repeat all negative studies, which would
124 require additional expense and increased animal use. Simply repeating a failed periodic
125 positive control study is not scientifically valid. These implications should be carefully
126 considered when determining whether to include concurrent positive controls or to only
127 conduct periodic positive controls. Consideration should also be given to using fewer animals
128 in the concurrent positive control group when this is scientifically justified and if the
129 laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be
130 used without substantially increasing the frequency with which studies will need to be
131 repeated.

132 15. Although the positive control substance should be tested in the vehicle that is known
133 to elicit a consistent response (e.g., acetone: olive oil), there may be certain regulatory
134 situations in which testing in a non-standard vehicle (clinically/chemically relevant
135 formulation) will also be necessary. In such situations the possible interaction of a positive
136 control with this unconventional vehicle should be tested. If the concurrent positive control
137 substance is tested in a different vehicle than the test substance, then a separate vehicle
138 control for the concurrent positive control should be included.

139 16. In instances where substances of a specific chemical class or range of responses are
140 being evaluated, benchmark controls may be useful to demonstrate that the test method is
141 functioning properly for detecting the skin sensitisation potential of a test substance.
142 Appropriate benchmark controls should have the following properties:

- 143 • structural and functional similarity to the class of the substance being tested;
- 144 • known physical/chemical characteristics;
- 145 • supporting data on known effects in animal models;
- 146 • known potency for sensitisation response.

147 **TEST PROCEDURE**

148 **Number of animals and dose levels**

149 17. A minimum of four animals is used per dose group, with a minimum of three
150 concentrations of the test substance, plus a concurrent negative control group treated only
151 with the vehicle for the test substance, and a concurrent positive control (see paragraphs 11-
152 15). Except for absence of treatment with the test substance, animals in the control groups
153 should be handled and treated in a manner identical to that of animals in the treatment
154 groups.

155 18. Dose and vehicle selection should be based on the recommendations given in
156 references (3) and (5). Doses are selected from the concentration series 100%, 50%, 25%,
157 10%, 5%, 2.5%, 1%, 0.5%, etc. Existing acute toxicity and dermal irritation data should be
158 considered, where available, in selecting the three consecutive concentrations so that the
159 highest concentration maximises exposure whilst avoiding systemic toxicity and excessive
160 local skin irritation (3)(17). In the absence of such information, an initial prescreen test may
161 be necessary (see paragraphs 21-24).

162 19. The vehicle should not interfere with or bias the test result and should be selected on
163 the basis of maximising the solubility in order to obtain the highest concentration achievable
164 whilst producing a solution/suspension suitable for application of the test substance. In order
165 of preference, recommended vehicles are acetone: olive oil (4:1 v/v), *N,N*-
166 dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulphoxide (3)(8),
167 but others may be used if sufficient scientific rationale is provided. In certain situations it
168 may be necessary to use a clinically relevant solvent or the commercial formulation in which
169 the test substance is marketed as an additional control. Particular care should be taken to
170 ensure that hydrophilic materials are incorporated into a vehicle system, which wets the skin
171 and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided.

172 20. The processing of lymph nodes from individual mice allows for the assessment of
173 interanimal variability and a statistical comparison of the difference between test substance
174 and vehicle control group measurements. In addition, evaluating the possibility of reducing
175 the number of mice in the positive control group is only feasible when individual animal data
176 are collected.

177 **Prescreen test**

178 21. The purpose of the prescreen test is to provide guidance for selecting the maximum
179 dose level to use in the main LLNA study. The maximum dose level tested should be a
180 concentration of 100% (i.e., neat substance) for liquid substances or the maximum soluble
181 concentration (for solids), unless available information suggests that this concentration
182 induces systemic toxicity or excessive local irritation after topical application in the mouse.

183 22. In the absence of such information, a prescreen test should be performed using three
184 dose levels of the test substance, in order to define the appropriate dose level to test in the
185 LLNA. Six mice (two per concentration) are used, and the prescreen test is conducted under
186 identical conditions as the main LLNA study, except there is no assessment of lymph node
187 proliferation. All mice will be observed daily for any clinical signs of systemic toxicity or
188 local irritation at the application site. Body weights are recorded pre-test and prior to
189 termination (Day 6). Both ears of each mouse are observed for erythema and scored using
190 Table 1. Ear thickness measurements are taken using a thickness gauge (e.g., digital
191 micrometer or Peacock Dial thickness gauge) on Day 1 (pre-dose), Day 3 (approximately 48
192 hours after the first dose), and Day 6. Excessive local irritation is indicated by an erythema
193 score ≥ 3 and/or ear swelling of $\geq 25\%$ (18)(19).

194 **Table 1 Erythema Scores**

Observation	Value
No visual effect	0
Slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema (beet redness)	3
Eschar (i.e., piece of dead tissue that is cast off from the surface of the skin)	4

195 23. In addition to a 25% increase in ear swelling (18)(19), a statistically significant
196 increase in ear swelling in the treated mice compared to control mice has also been used to
197 identify irritants in the LLNA (20)(21)(22)(23)(24)(25)(26). While statistically significant
198 increases can occur when ear swelling is less than 25%, they have not been associated
199 specifically with excessive irritation (22)(24)(25)(26). Additionally, an adequately robust
200 statistical comparison would require that a vehicle control group be included and that more
201 than two mice per group be tested. Both of these requirements would substantially increase
202 the number of mice used in a prescreen test. For this reason, a threshold increase in ear
203 swelling above pre-dosing levels is recommended for this prescreen test.

204 24. Test guidelines for assessing acute dermal toxicity recommend a number of clinical
205 observations for assessing systemic toxicity (27)(28). The following clinical observations,
206 which are based on test guidelines and current practices (29), may indicate systemic toxicity
207 when used as part of an integrated assessment and therefore may indicate the maximum dose
208 level to use in the main LLNA:

- 209 • Changes in nervous system function (e.g., piloerection, ataxia, tremors, and
210 convulsions)
- 211 • Changes in behavior (e.g., aggressiveness, change in grooming activity,
212 marked change in activity level)
- 213 • Changes in respiratory patterns (i.e., changes in frequency and intensity of
214 breathing such as dyspnea, gasping, and rales)
- 215 • Changes in food and water consumption
- 216 • Lethargy and/or unresponsiveness
- 217 • Any clinical signs of more than slight or momentary pain and distress
- 218 • Reduction in body weight >10% from Day 1 to Day 6
- 219 • Mortality

220 **Reduced LLNA**

221 25. Use of a rLLNA protocol (13)(14) has the potential to reduce the number of animals
222 used in the LLNA by omitting the middle and low dose groups. This is the only difference
223 between the LLNA and the rLLNA and thus, the test substance concentration evaluated in
224 the rLLNA should be the maximum concentration that does not induce overt systemic
225 toxicity and/or excessive local irritation in the mouse. The rLLNA should be used for the
226 hazard classification of skin sensitising substances if dose-response information is not
227 needed, provided there is adherence to all other LLNA protocol specifications, as described
228 in this Test Guideline. To further reduce animal use, the rLLNA should be used routinely as
229 an initial test to determine allergic contact dermatitis potential of chemicals and products
230 before conducting the LLNA. Negative substances can be classified as nonsensitisers and
231 positive substances can be classified as sensitisers.

232 **Main study experimental schedule**

233 26. The experimental schedule of the assay is as follows:

- 234 • *Day 1:*
235 Individually identify and record the weight of each animal and any clinical
236 observations. Apply 25 µL of the appropriate dilution of the test substance,
237 the vehicle alone, or the concurrent positive control (see paragraphs 11-15), to
238 the dorsum of each ear.
- 239 • *Days 2 and 3:*
240 Repeat the application procedure carried out on Day 1.
- 241 • *Days 4 and 5:*
242 No treatment.
- 243 • *Day 6:*
244 Record the weight of each animal. Inject 250 µL of sterile phosphate-buffered
245 saline (PBS) containing 20 µCi (7.4e+5 Bq) of tritiated (³H)-methyl thymidine
246 into all test and control mice via the tail vein. Alternatively, inject 250 µL
247 sterile PBS containing 2 µCi (7.4e + 4 Bq) of ¹²⁵I-iododeoxyuridine and 10⁻⁵M
248 fluorodeoxyuridine into all mice via the tail vein. Five hours (5 h) later,
249 humanely kill the animals. Excise the draining auricular lymph nodes from
250 each mouse ear and process separately in PBS for each animal. Details and
251 diagrams of the node identification and dissection can be found in reference
252 (9).

253 **Preparation of cell suspensions**

254 27. A single cell suspension of lymph node cells (LNC) excised bilaterally from each
255 mouse is prepared by gentle mechanical disaggregation through 200 micron-mesh stainless
256 steel gauze or another acceptable technique for generating a single-cell suspension. LNC are
257 washed twice with an excess of PBS and the DNA is precipitated with 5% trichloroacetic
258 acid (TCA) at 4°C for 18h (3). Pellets are either re-suspended in 1 mL TCA and transferred
259 to scintillation vials containing 1.0 mL of scintillation fluid for ³H-counting, or transferred
260 directly to gamma counting tubes for ¹²⁵I-counting.

261 **Determination of cellular proliferation (incorporated radioactivity)**

262 28. Incorporation of ³H-methyl thymidine is measured by β-scintillation counting as
263 disintegrations per minute (DPM). Incorporation of ¹²⁵I-iododeoxyuridine is measured by
264 ¹²⁵I-counting and also is expressed as DPM. The incorporation is expressed as DPM/mouse.

265 **OBSERVATIONS**

266 **Clinical observations**

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

272 **Body weights**

273 30. As stated in paragraph 26, individual animal body weights should be measured at
274 the start of the test and at the scheduled kill.

275 **CALCULATION OF RESULTS**

276 31. Results for each treatment group are expressed as the mean SI. The SI is derived by
277 dividing the mean DPM/mouse within each test substance group and the concurrent positive
278 control group by the mean DPM/mouse for the solvent/vehicle control group. The average SI
279 for vehicle treated controls is then one.

280 32. Collecting radioactivity data at the level of the individual mouse will enable a
281 statistical analysis for presence and degree of dose response in the data. Any statistical
282 assessment should include an evaluation of the dose response relationship as well as suitably
283 adjusted comparisons of test groups (e.g., pair-wise dosed group versus concurrent vehicle
284 control comparisons). Statistical analyses may include, for instance, linear regression or
285 William's test to assess dose-response trends, and Dunnett's test for pairwise comparisons. In
286 choosing an appropriate method of statistical analysis, the investigator should maintain an
287 awareness of possible inequalities of variances and other related problems that may
288 necessitate a data transformation or a non-parametric statistical analysis. In any case, the
289 investigator should be alert to possible "outlier" responses for individual mice within a group
290 that may necessitate analysis both with and without outliers.

291 33. The decision process with regard to a positive response includes a $SI \geq 3$, together
292 with consideration of dose response and, where appropriate, statistical significance
293 (4)(5)(6)(28).

294 34. If it is necessary to clarify the results obtained, consideration should be given to
295 various properties of the test substance, including whether it has a structural relationship to
296 known skin sensitizers, whether it causes excessive skin irritation in the mouse, and the
297 nature of the dose response seen. These and other considerations are discussed in detail
298 elsewhere (7).

299 **DATA AND REPORTING**

300 **Data**

301 35. Data should be summarised in tabular form showing the individual animal DPM
302 values, the group mean DPM/animal, its associated error term, and the mean SI for each dose
303 group compared against the concurrent vehicle control group.

304 **Test report**

305 36. The test report should contain the following information:

306 Test substance and control substances:

- 307 – identification data (e.g. CAS number, if available; source; purity; known
308 impurities; lot number);
- 309 – physical nature and physicochemical properties (e.g. volatility, stability,
310 solubility);
- 311 – if mixture, composition and relative percentages of components.

312 Solvent/vehicle:

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

315 Test animals:

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

320 Test conditions:

- 321 – details of test substance preparation and application;
- 322 – justification for dose selection (including results from range finding study, if
323 conducted);
- 324 – vehicle and test substance concentrations used, and total amount of substance
325 applied;
- 326 – details of food and water quality (including diet type/source, water source);
- 327 – details of treatment and sampling schedules;
- 328 – methods for measurement of toxicity;
- 329 – criteria for considering studies as positive or negative;
- 330 – details of any protocol deviations and an explanation on how the deviation
331 affects the study design and results.

332 Reliability check:

- 333 – a summary of results of latest reliability check, including information on
334 substance, concentration and vehicle used;
335 – concurrent and/or historical positive and negative control data for testing
336 laboratory;
337 – if a concurrent positive control was not included, the date and laboratory
338 report for the most recent periodic positive control and a report detailing the
339 historical positive control data for the laboratory justifying the basis for not
340 conducting a concurrent positive control.

341 Results:

- 342 – individual weights of mice at start of dosing and at scheduled kill; as well as
343 mean and associated error term for each treatment group.
344 – time course of onset and signs of toxicity, including dermal irritation at site of
345 administration, if any, for each animal;
346 – a table of individual mouse DPM values and SIs for each treatment group;
347 – mean and associated error term for DPM/mouse for each treatment group and
348 the results of outlier analysis for each treatment group;
349 – calculated SI and an appropriate measure of variability that takes into account
350 the interanimal variability in both the test substance and control groups;
351 – dose response relationship;
352 – statistical analysis, where appropriate.

353 Discussion of results:

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

357 Quality assurance statement for Good Laboratory Practice compliant studies:

- 358 – statement should indicate all inspections made during the study and the dates
359 any results were reported to the Study Director. The statement should also
360 confirm that the final report reflects the raw data.

361

361 **LITERATURE**

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472

ANNEX 1

473 PERFORMANCE STANDARDS FOR ASSESSMENT OF PROPOSED SIMILAR OR 474 MODIFIED LOCAL LYMPH NODE ASSAY TEST METHODS FOR SKIN 475 SENSITISATION

476 INTRODUCTION

477 1. The purpose of Performance Standards (PS) is to communicate the basis by which
478 new test methods, both proprietary (i.e., copyrighted, trademarked, registered) and non-
479 proprietary can be determined to have sufficient accuracy and reliability for specific testing
480 purposes. These PS, based on validated and accepted test methods, can be used to evaluate
481 the reliability and accuracy of other analogous test methods (colloquially referred to as “me-
482 too” tests) that are based on similar scientific principles and measure or predict the same
483 biological or toxic effect (11).

484 2. Prior to adoption of modified test methods (i.e., proposed potential improvements to
485 an approved test method), there should be an evaluation to determine the effect of the
486 proposed changes on the test’s performance and the extent to which such changes affect the
487 information available for the other components of the validation process. Depending on the
488 number and nature of the proposed changes, the generated data and supporting
489 documentation for those changes, they should either be subjected to the same validation
490 process as described for a new test, or, if appropriate, to a limited assessment of reliability
491 and relevance using established PS (11).

492 3. Similar (me-too) or modified test methods proposed for use under this Test
493 Guideline should be evaluated to determine their reliability and accuracy using chemicals
494 representing the full range of the LLNA scores.

495 4. These PS are based on the US-ICCVAM PS (9), for evaluating the validity of new
496 or modified versions of the LLNA. The PS consist of essential test method components,
497 recommended reference substances, and standards for accuracy and reliability that the
498 proposed test method should meet or exceed.

499 I. Essential test method components

500 5. To ensure that a modified LLNA test method is functionally and mechanistically
501 similar to the LLNA and measures the same biological effect, the following components
502 should be included in the test method protocol:

- 503 1. The test substance should be applied topically to both ears of the mouse.
- 504 2. Lymphocyte proliferation should be measured in the lymph nodes draining from
505 the site of test substance application.
- 506 3. Lymphocyte proliferation should be measured during the induction phase of skin
507 sensitisation.
- 508 4. For test substances, the highest dose selected should be the maximum soluble
509 concentration that does not induce systemic toxicity and/or excessive local

510 irritation in the mouse. For positive control substances, the highest dose selected
511 should exceed the known EC3 values of the reference substances without
512 producing systemic toxicity and/or excessive local irritation in the mouse.

513 5. A concurrent vehicle control should be included in each study and, where
514 appropriate, a concurrent positive control should also be used.

515 6. A minimum of four animals per dose group is required.¹

516 If any of these criteria are not met, then these performance standards cannot be used for
517 validation of the modified test method.

518 **II. Minimum list of reference substances**

519 6. ICCVAM identified 18 minimum required reference substances and four optional
520 reference substances (i.e., substances that produced either false positive or false negative
521 results in the LLNA, when compared to human and guinea pig results, and therefore provide
522 the opportunity to demonstrate equal to or better performance than the LLNA) that are
523 included in the LLNA performance standards. The selection criteria for identifying these
524 substances were:

525 • The list of reference substances represented the types of substances typically tested
526 for skin sensitisation potential and the range of responses that the LLNA is capable
527 of measuring or predicting;

528 • The substances had well-defined chemical structures;

529 • LLNA data from guinea pig tests and (where possible) data from humans were
530 available for each substance; and

531 • The substances were readily available from a commercial source.

532 The recommended reference substances are listed in Table 1. Studies using the proposed
533 reference substances should be evaluated in the vehicle with which they are listed in Table 1.
534 In situations where a listed substance may not be available, other substances that meet the
535 selection criteria mentioned may be used, with adequate justification.

¹ The ICCVAM-recommended performance standards for the LLNA (9), which were developed in order to harmonise with the procedures described in OECD TG 429 to ensure international applicability, indicate that either pooled or individual animal data may be collected. However, because the proposed updated OECD TG 429 specifies the need for collecting individual animal data from a minimum of four animals per dose group, the provision to pool animals has been removed from this document.

536 **TABLE 1. RECOMMENDED REFERENCE SUBSTANCES FOR THE LLNA PERFORMANCE STANDARDS**

Number	Substance	CASRN	Form	Veh	EC3 (%) ¹	N ²	0.5x - 2.0x EC3	Actual EC3 Range	LLNA vs. GP	LLNA vs. Human
1	5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	Liq	DMF	0.009	1	0.0045-0.018	NC	+/+	+/+
2	DNCB	97-00-7	Sol	AOO	0.049	15	0.025-0.099	0.02-0.094	+/+	+/+
3	4-Phenylenediamine	106-50-3	Sol	AOO	0.11	6	0.055-0.22	0.07-0.16	+/+	+/+
4	Cobalt chloride	7646-79-9	Sol	DMSO	0.6	2	0.3-1.2	0.4-0.8	+/+	+/+
5	Isoeugenol	97-54-1	Liq	AOO	1.5	47	0.77-3.1	0.5-3.3	+/+	+/+
6	2-Mercaptobenzothiazole	149-30-4	Sol	DMF	1.7	1	0.85-3.4	NC	+/+	+/+
7	Citral	5392-40-5	Liq	AOO	9.2	6	4.6-18.3	5.1-13	+/+	+/+
8	HCA	101-86-0	Liq	AOO	9.7	21	4.8-19.5	4.4-14.7	+/+	+/+
9	Eugenol	97-53-0	Liq	AOO	10.1	11	5.05-20.2	4.9-15	+/+	+/+
10	Phenyl benzoate	93-99-2	Sol	AOO	13.6	3	6.8-27.2	1.2-20	+/+	+/+
11	Cinnamic alcohol	104-54-1	Sol	AOO	21	1	10.5-42	NC	+/+	+/+
12	Imidazolidinyl urea	39236-46-9	Sol	DMF	24	1	12-48	NC	+/+	+/+
13	Methyl methacrylate	80-62-6	Liq	AOO	90	1	45-100	NC	+/+	+/+
14	Chlorobenzene	108-90-7	Liq	AOO	NA	1	NA	NA	-/-	-/*
15	Isopropanol	67-63-0	Liq	AOO	NA	1	NA	NA	-/-	-/+
16	Lactic acid	50-21-5	Liq	DMSO	NA	1	NA	NA	-/-	-/*
17	Methyl salicylate	119-36-8	Liq	AOO	NA	9	NA	NA	-/-	-/-
18	Salicylic acid	69-72-7	Sol	AOO	NA	1	NA	NA	-/-	-/-

Number	Substance	CASRN	Form	Veh	EC3 (%) ¹	N ²	0.5x - 2.0x EC3	Actual EC3 Range	LLNA vs. GP	LLNA vs. Human
Optional Substances to Demonstrate Improved Performance Relative to the LLNA										
19	Sodium lauryl sulfate	151-21-3	Sol	DMF	8.1	5	4.05-16.2	1.5-17.1	+/-	+/-
20	Ethylene glycol dimethacrylate	97-90-5	Liq	MEK	28	1	14-56	NC	+/-	+/+
21	Xylene	1330-20-7	Liq	AOO	95.8	1	47.9-100	NC	+/**	+/-
22	Nickel chloride	7718-54-9	Sol	DMSO	NA	2	NA	NA	-/+	-/+

537 Abbreviations: AOO = acetone: olive oil (4:1); CASRN = Chemical Abstracts Service Registry Number; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB =
538 2,4-dinitrochlorobenzene; EC3 = estimated concentration needed to produce a stimulation index of 3; GP = guinea pig test result (TG 406) (10); HCA = hexyl cinnamic aldehyde;
539 Liq = liquid; LLNA = murine local lymph node assay result (TG 429) (1); MEK = methyl ethyl ketone; NA = not applicable since stimulation index <3; NC = not calculated since
540 data was obtained from a single study; Sol = solid; Veh = test vehicle.

541 ¹ Mean value where more than one EC3 value was available

542 ² Number of LLNA studies from which data were obtained

543 * = Presumed to be a nonsensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of
544 human sensitisation were located.

545 ** = GP data not available.

546 **III. Defined reliability and accuracy standards**

547 7. The accuracy of a modified LLNA test method should meet or exceed that of the
548 LLNA PS when it is evaluated using the 18 minimum required reference substances. The
549 new or modified test method should result in the correct classification based on a “yes/no”
550 decision. However, the new or modified test method might not correctly classify all of the
551 minimum required reference substances. If, for example, one of the weak sensitizers were
552 misclassified, a rationale for the misclassification and appropriate additional data (e.g., test
553 results that provide correct classifications for other substances with physical, chemical, and
554 sensitising properties similar to those of the misclassified reference substance) could be
555 considered to demonstrate equivalent performance. Under such circumstances, the validation
556 status of the new or modified LLNA test method would be evaluated on a case-by-case basis.

557 **Intra-laboratory reproducibility**

558 8. To determine intra-laboratory reproducibility, a new or modified LLNA test method
559 should be assessed using a sensitising substance that is well characterised in the LLNA.
560 Therefore, the LLNA PS is based on the variability of results from repeated tests of hexyl
561 cinnamic aldehyde (HCA). To assess intra-laboratory reliability, threshold estimated
562 concentration (EC_t) values for HCA should be derived on four separate occasions with at
563 least one week between tests. Acceptable intra-laboratory reproducibility is indicated by a
564 laboratory’s ability to obtain, in each HCA test, EC_t values between 5% and 20%, which
565 represents the range of 0.5-2.0 times the mean EC₃ specified for HCA (10%) in the LLNA.

566 **Inter-laboratory reproducibility**

567 9. Inter-laboratory reproducibility of a new or modified LLNA test method should be
568 assessed using two sensitising substances that are well characterised in the LLNA. The
569 LLNA PS is based on the variability of results from tests of HCA and 2,4-
570 dinitrochlorobenzene (DNCB) in different laboratories. EC_t values should be derived
571 independently from a single study conducted in at least three separate laboratories. To
572 demonstrate acceptable inter-laboratory reproducibility, each laboratory should obtain EC_t
573 values of 5% to 20% for HCA and 0.025% to 0.1% for DNCB, which represents the range of
574 0.5-2.0 times the mean EC₃ concentrations specified for HCA (10%) and DNCB (0.05%),
575 respectively, in the LLNA.

576

577

ANNEX 2

578 **Accuracy:** The closeness of agreement between test method results and accepted reference
579 values. It is a measure of test method performance and one aspect of relevance. The term is
580 often used interchangeably with “concordance” to mean the proportion of correct outcomes
581 of a test method.

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

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

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

588 **Inter-laboratory reproducibility:** A measure of the extent to which different qualified
589 laboratories, using the same protocol and testing the same substances, can produce
590 qualitatively and quantitatively similar results. Inter-laboratory reproducibility is determined
591 during the prevalidation and validation processes, and indicates the extent to which a test can
592 be successfully transferred between laboratories, also referred to as between-laboratory
593 reproducibility.

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

597 **Me-too test:** A colloquial expression for a test method that is functionally and
598 mechanistically similar to a validated and accepted reference test method. Such a test method
599 would be a candidate for catch-up validation. Interchangeably used with similar test method.

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

608 **Proprietary test method:** A test method for which manufacture and distribution is restricted
609 by patents, copyrights, trademarks, etc.

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

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

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

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

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

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

633 **Estimated concentration threshold (EC_t):** Estimated concentration of a substance needed
634 to produce a stimulation index that is indicative of a positive response.

635 **Estimated concentration three (EC₃):** Estimated concentration of a substance needed to
636 produce a stimulation index of three.

637 **Validated test method:** A test method for which validation studies have been completed to
638 determine the relevance (including accuracy) and reliability for a specific purpose. It is
639 important to note that a validated test method may not have sufficient performance in terms
640 of accuracy and reliability to be found acceptable for the proposed purpose.