Commission Regulation (EU) No 640/2012 of 6 July 2012 amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (Text with EEA relevance)

COMMISSION REGULATION (EU) No 640/2012

of 6 July 2012

amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

(Text with EEA relevance)

THE EUROPEAN COMMISSION,

Having regard to the Treaty on the Functioning of the European Union,

Having regard to Regulation (EC) No 1907/2006 of 18 December 2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC[(1)], and in particular Article 13(3) thereof,

Whereas:

(1) Commission Regulation (EC) No 440/2008[(2)] contains the test methods for the purposes of the determination of the physico-chemical properties, toxicity and eco-toxicity of substances to be applied for the purposes of Regulation (EC) No 1907/2006.

(2) It is necessary to update Regulation (EC) No 440/2008 to include with priority new and updated alternative test methods recently adopted by the OECD, in order to obtain a reduction of the number of animals to be used for experimental purposes, in accordance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes[(3)] and Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes[(4)]. Stakeholders have been consulted on this draft.

(3) Regulation (EC) No 440/2008 should therefore be amended accordingly.

(4) The measures provided for in this Regulation are in accordance with the opinion of the Committee established under Article 133 of Regulation (EC) No 1907/2006,

Changes to legislation: *There are currently no known outstanding effects for the Commission Regulation (EU) No 640/2012. (See end of Document for details)*

HAS ADOPTED THIS REGULATION:

Article 1

The Annex to Regulation (EC) No 440/2008 is amended in accordance with the Annex to this Regulation.

Article 2

This Regulation shall enter into force on the third day following that of its publication in the *Official Journal of the European Union*.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 6 July 2012.

For the Commission

The President

José MANUEL BARROSO

***Changes to legislation:** There are currently no known outstanding effects for the Commission Regulation (EU) No 640/2012. (See end of Document for details)*

ANNEX

The Annex to Regulation (EC) No 440/2008 is amended as follows:

1. Chapter B.42 is replaced by the following:

B.42. SKIN SENSITISATION: LOCAL LYMPH NODE ASSAY

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals and EU Test Methods based on them are periodically reviewed in light of scientific progress, changing regulatory needs, and animal welfare considerations. The original Test Method (TM) for the determination of skin sensitisation in the mouse, the Local Lymph Node Assay (LLNA; OECD Test Guideline 429; Chapter B.42 of this Annex) was adopted previously (1). The details of the validation of the LLNA and a review of the associated work have been published (2) (3) (4) (5) (6) (7) (8) (9) (10) (11). The updated LLNA is based on the evaluation of experience and scientific data (12). This is the second TM to be designed for assessing skin sensitisation potential of chemicals (substances and mixtures) in animals. The other TM (*i.e.* OECD Test Guideline 406; Chapter B.6 of this Annex) utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (13). The LLNA provides advantages over B.6 and OECD Test Guideline 406 (13) with regard to animal welfare. This updated LLNA TM includes a set of Performance Standards (PS) (Appendix 1) that can be used to 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 OECD Guidance Document No 34 (14).

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 chemicals (PC) for guinea pig test methods (*i.e.* B.6; OECD Test Guideline 406) (13) are also appropriate for use with the LLNA (6) (8) (15). A reduced LLNA (rLLNA) approach, which could use up to 40 % fewer animals is also described as an option in this TM (16) (17) (18). The rLLNA may be used when there is a regulatory need to confirm a negative prediction of skin sensitising potential, provided there is adherence to all other LLNA protocol specifications, as described in this TM. Prediction of a negative outcome should be made based on all available information as described in paragraph 4. Before applying the rLLNA approach, clear justifications and scientific rationale for its use should be provided. If, against expectations, a positive or equivocal result is obtained in the rLLNA, additional testing may be needed in order to interpret or clarify the finding. The rLLNA should not be used for the hazard identification of skin sensitising test substances when dose-response information is needed such as sub-categorisation for Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures and UN Globally Harmonised System of Classification and Labelling of Chemicals.

DEFINITIONS

3. Definitions used are provided in Appendix 2.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. The LLNA provides an alternative method for identifying potential skin sensitising chemicals. This does not necessarily imply that in all instances the LLNA should be used in place of guinea pig tests (*i.e.* B.6; OECD Test Guideline 406) (13), but rather that the assay is of equal merit and may be employed as an alternative in which positive and negative results generally no longer require further

Changes to legislation: *There are currently no known outstanding effects for the Commission Regulation (EU) No 640/2012. (See end of Document for details)*

confirmation. The testing laboratory should consider all available information on the test substance prior to conducting the study. Such information will include the identity and chemical structure of the test substance; its physicochemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the test substance; and toxicological data on structurally related chemicals. This information should be considered in order to determine whether the LLNA is appropriate for the substance (given the incompatibility of limited types of chemicals with the LLNA — see paragraph 5) and to aid in dose selection.

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 (less pain and distress) of the way in which animals are used for allergic contact sensitisation testing. The LLNA is based upon consideration of immunological events stimulated by chemicals during the induction phase of sensitisation. Unlike guinea pig tests (*i.e.* B.6; OECD Test Guideline 406) (13) the LLNA does not require that challenge-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA does not require the use of an adjuvant, as is the case for the guinea pig maximisation test (13). Thus, the LLNA reduces animal pain and distress. Despite the advantages of the LLNA over B.6 and OECD Test Guideline 406, it should be recognised that there are certain limitations that may necessitate the use of B.6 or OECD Test Guideline 406 (13) (*e.g.* false negative findings in the LLNA with certain metals, false positive findings with certain skin irritants (such as some surfactant type chemicals) (19) (20), or solubility of the test substance). In addition, chemical classes or substances containing functional groups shown to act as potential confounders (21) may necessitate the use of guinea pig tests (*i.e.* B.6; OECD Test Guideline 406) (13). Further, based on the limited validation database, which consisted primarily of pesticide formulations, the LLNA is more likely than the guinea pig test to yield a positive result for these types of test substances (22). However, when testing formulations, one could consider including similar substances with known results as benchmark substances to demonstrate that the LLNA is functioning properly (see paragraph 16). Other than such identified limitations, the LLNA should be applicable for testing any substances unless there are properties associated with these substances that may interfere with the accuracy of the LLNA.

PRINCIPLE OF THE TEST

6. The basic principle underlying the LLNA is that sensitisers induce proliferation of lymphocytes in the lymph nodes draining the site of test substance application. This proliferation is proportional to the dose and to the potency of the applied allergen and provides a simple means of obtaining a quantitative measurement of sensitisation. Proliferation is measured by comparing the mean proliferation in each test group to the mean proliferation in the vehicle treated control (VC) group. The ratio of the mean proliferation in each treated group to that in the concurrent VC group, termed the Stimulation Index (SI), is determined, and should be ≥ 3 before classification of the test substance as a potential skin sensitiser is warranted. The procedures described here are based on the use of *in vivo* radioactive labelling to measure an increased number of proliferating cells in the draining auricular lymph nodes. However, other endpoints for assessment of the number of proliferating cells may be employed provided the PS requirements are fully met (Appendix 1).

DESCRIPTION OF THE ASSAY

Selection of animal species

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

Housing and feeding conditions

8. Mice should be group-housed (23), unless adequate scientific rationale for housing mice individually is provided. The temperature of the experimental animal room should be 22 ± 3 °C. Although the relative humidity should be at least 30 % and preferably not exceed 70 %, other than during room cleaning, the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Preparation of animals

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

Preparation of dosing solutions

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

Reliability check

11. Positive control chemicals (PC) are used to demonstrate appropriate performance of the assay by responding with adequate and reproducible sensitivity as a sensitising test substance for which the magnitude of the response is well characterised. Inclusion of a concurrent PC is recommended because it demonstrates competency of the laboratory to successfully conduct each assay and allows for an assessment of intra- and inter-laboratory reproducibility and comparability. A PC for each study is also required by some regulatory authorities and therefore users are encouraged to consult the relevant authorities prior to conducting the LLNA. 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 response at an exposure level expected to give an increase in the SI > 3 over the negative control (NC) group. The PC dose should be chosen such that it does not cause excessive skin irritation or systemic toxicity and the induction is reproducible but not excessive (*i.e.* a SI > 20 would be excessive). Preferred PC are 25 % hexyl cinnamic aldehyde (Chemical Abstracts Service (CAS) No 101-86-0) in acetone: olive oil (4:1, v/v) and 5 % mercaptobenzothiazole (CAS No 149-30-4) in *N,N*-dimethylformamide (see Appendix 1, Table 1). There may be circumstances in which, given adequate justification, other PC, meeting the above criteria, may be used.