

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW TEST GUIDELINE

In Vitro Skin Sensitisation: IL-8 Luc assay

INTRODUCTION

1. A skin sensitizer refers to a substance that will lead to an allergic response following skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS)(1). This Test Guideline (TG) describes the *in vitro* procedure called the Interleukin-8 Reporter Gene Assay (IL-8 Luc assay), to be used for supporting the discrimination between skin sensitizers and non-sensitizers in accordance with the UN GHS (1).

2. There is general agreement on the key biological events underlying skin sensitization. The current knowledge of the chemical and biological mechanisms associated with skin sensitization has been summarised as an Adverse Outcome Pathway (AOP) (2), starting with the molecular initiating event through intermediate events to the adverse effect, allergic contact dermatitis. In this instance, the molecular initiating event (i.e. the first key event) is covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in 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. The third key event is the activation of dendritic cells (DC), typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell proliferation, which is indirectly assessed in the murine Local Lymph Node Assay (LLNA) (3).

3. The assessment of skin sensitization has typically involved the use of laboratory animals. The classical methods using guinea-pigs, i.e. the Guinea Pig Maximisation Test (GPMT) and the Buehler Test (TG 406) (4) assess both the induction and elicitation phases of skin sensitization. The murine tests, the LLNA (TG 429) (3) and its two non-radioactive modifications, LLNA: DA (TG 442A) (5) and LLNA: BrdU-ELISA (TG 442B) (6), all assess the induction response exclusively and have also gained acceptance, since they provide an advantage over the guinea pig tests in terms of animal welfare together with an objective measurement of the induction phase of skin sensitization.

4. Recently, mechanistically based *in chemico* and *in vitro* methods have been adopted to contribute to evaluation of the skin sensitization hazard potential of chemicals: OECD TG 442C: Direct Peptide Reactivity Assay addresses the first key event of the skin sensitization AOP (7); OECD TG 442D: ARE-Nrf2 Luciferase Test Method addresses the second key event (8), OECD TG 442E: human Cell

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Line Activation Test (h-CLAT) addresses the third key event of the AOP (9). However, a combination of non-animal methods (*in silico*, *in chemico*, *in vitro*) within Integrated Approaches to Testing and Assessment (IATA) will be needed to replace fully current *in vivo* tests, given the restricted AOP mechanistic coverage of each of the currently available non-animal test methods (2, 10-16).

5. The IL-8 Luc assay addresses the third key event (dendritic cell activation) of the skin sensitisation AOP. In contrast to the h-CLAT, which follows the expression of cell surface markers, it quantifies changes in IL-8 expression, a cytokine associated with the activation of DC. In the THP-1-derived IL-8 reporter cell line (THP-G8, established from the human acute monocytic leukemia cell line THP-1), IL-8 expression is measured following exposure to sensitisers (17). The expression of luciferase is then used to aid discrimination between skin sensitisers and non-sensitisers.

6. The IL-8 Luc method has been evaluated in a validation study (18) conducted by the Japanese Centre for the Validation of Alternatives Methods (JaCVAM), the Ministry of Economy, Trade and Industry (METI), and the Japanese Society for Alternatives to Animal Experiments (JSAAE) and subsequently subjected to independent peer review (19) under the auspices of JaCVAM and the Ministry of Health, Labour and Welfare (MHLW) with the support of the International Cooperation on Alternative Test Methods (ICATM). Considering all available evidence and input from regulators and stakeholders, the IL-8 Luc assay is considered useful as part of IATA to discriminate sensitisers from non-sensitisers for the purpose of hazard classification and labelling. Examples of the use of IL-8 Luc assay data in combination with other information is reported in the literature (16, 20, 21).

7. Definitions are provided in Annex I.

INITIAL CONSIDERATIONS AND LIMITATIONS

8. It is well known that DCs maintain immature phenotype in the absence of hapten stimulation. After hapten stimulation, they change drastically, to mature phenotype (Aiba and Katz, 1990; Aiba et al., 1997). Mature DCs increase expression of CD40, CD54, CD80, CD83, and CD86 in addition to induction of proinflammatory cytokines, such as IL-1 β and TNF- α , and several chemokines including IL-8 (CXCL8) and CCL3 (22-25). Therefore, biomarkers of DC activation need not be restricted to the expression of CD54 and CD86. Other surface molecules (e.g. CD40 and CD80) and the production of proinflammatory cytokines IL-1 β and TNF- α and chemokines IL-8 and CCL3 can also be good biomarkers for DC activation. Recently, it has been demonstrated that neutrophils and neutrophil chemokine receptor, CXCR2, which are receptors for murine IL-8 homologues, are critically involved in the induction and elicitation phase of contact hypersensitivity (26, 27).

9. Methods such as the IL-8 Luc assay are therefore relevant for assessment of the skin sensitisation

potential of chemicals. However, as DC activation is only one key event of the AOP, information generated with methods measuring markers of DC activation alone may not be sufficient to confirm the absence of skin sensitisation potential of chemicals. Therefore, data generated with the IL-8 Luc assay should be considered in the context of integrated approaches, such as IATA, and combined with complementary information derived from *in vitro* assays addressing other key events of the skin sensitisation AOP, as well as non-testing methods, including read-across from chemical analogues.

10. The method described in this Test Guideline can be used in an IATA to support the discrimination between skin sensitisers (UN GHS Category 1) and non-sensitisers (16). It should not be used alone to place skin sensitisers into subcategories 1A and 1B as defined by UN GHS (1) nor to predict potency for safety assessment. However, depending on the regulatory framework, a positive result with the IL-8 Luc assay may be used on its own to classify a chemical into UN GHS Category 1.

11. The IL-8 Luc assay proved transferable to laboratories experienced in cell culture and luciferase measurement. Within and between laboratory reproducibility is approximately 80% (28). Data generated in the validation study (28) and other published work (17, 21) show that versus the LLNA, the accuracy of the IL-8 Luc assay in distinguishing skin sensitisers (UN GHS Cat. 1) from non-sensitisers (UN GHS No Cat.) is 80% (n = 143) with a sensitivity of 86% (92/107) and specificity of 64% (23/36). Excluding substances used in the validation study, but outside the applicability domain described below (paragraph 13), the accuracy of the IL-8 Luc assay is 85% (115/136), sensitivity is 88% (92/105) and specificity is 74% (23/31) (n = 136). Using human data from Urbisch et al. (13), the IL-8 Luc assay accuracy is 78% (70/90), sensitivity is 86% (54/63) and specificity is 59% (16/27) (n = 90). Excluding substances outside the applicability domain, assay accuracy is 82% (69/84), sensitivity is 86% (54/63) and specificity is 71% (15/21) for 84 chemicals with human data. False negative predictions with the IL-8 Luc assay are more likely to occur with chemicals showing low/moderate skin sensitisation potency (UN GHS subcategory 1B) than those with high potency (UN GHS subcategory 1A) (21). Together, the information supports a role for the IL-8 Luc assay in the identification of skin sensitisation hazards. The accuracy given for the IL-8 Luc assay as a standalone test method is only for guidance, as the method should be considered in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraph 10 above. Furthermore, when evaluating non-animal methods for skin sensitisation, it should be remembered that the LLNA and other animal tests may not fully reflect the situation in humans.

12. The term "test chemical" is used in this Test Guideline to refer to what is being tested¹ and is not

¹ In June 2013, the Joint Meeting agreed that where possible, a more consistent use of the term "test chemical" describing what is being tested should be applied in new and updated Test Guidelines.

related to the applicability of the IL-8 Luc assay to the testing of mono-constituent substances, multi-constituent substances and/or mixtures. On the basis of the data currently available, the IL-8 Luc assay was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, skin sensitisation potency (as determined in *in vivo* studies) and physicochemical properties (21, 28). Limited information is currently available on the applicability of the IL-8 Luc assay to multi-constituent substances/mixtures. The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. However, before use of this 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 purpose². Such considerations are not needed when there is a regulatory requirement for the testing of the mixture. Moreover, when testing multi-constituent substances or mixtures, consideration should be given to possible interference of cytotoxic constituents with the observed responses.

13. A high false negative rate for anhydrides was seen in the validation study. Furthermore, because of the limited metabolic capability of the cell line (29) and the experimental conditions, pro-haptens (substances requiring metabolic activation) and pre-haptens (substances activated by air oxidation) might give negative results in the assay. However, although negative results for suspected pre/prohaptens should be interpreted with caution, a comprehensive review has shown that almost all are correctly detected by validated *in vitro* methods (30). The IL-8 Luc assay correctly evaluated several chemicals with a Log $K_{o/w}$ >8 and those with a water solubility of around 0.1 µg/ml as calculated by EPI SuiteTM. Test chemicals with a water solubility of less than 100 µg/ml (obtained by EPI SuiteTM) in combination with Inh-GAPLA of more than 0.8 in every examined concentration (the latter indicating that the cells have not been sufficiently “stressed” by the exposure) tend to produce false negative results as it may not dissolve in X-VIVOTM 15. Therefore negative results with these chemicals should not be considered. However, positive results obtained with test chemicals with a water solubility of less than 100 µg/mL in combination with Inh-GAPLA of more than 0.8 in every examined concentration could still be used to support the identification of the test chemical as a skin sensitiser. Surfactants tested so far gave (false) positive results irrespective of their type (e.g., cationic, anionic or on-ionic). Finally, chemicals that interfere with luciferase can confound its activity/measurement, causing apparent inhibition or increased luminescence (31). For example, phytoestrogen concentrations higher than 1µM were reported to interfere with luminescence signals in other luciferase-based reporter gene assays due to over-activation of the luciferase reporter gene. Consequently, luciferase expression obtained at high concentrations of phytoestrogens or compounds suspected of producing phytoestrogen-like activation of the luciferase reporter gene needs to be examined carefully (32). Based on the above, surfactants, anhydrides and chemicals interfering with

² This sentence was proposed and agreed at the April 2014 WNT meeting

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luciferase are outside the applicability domain of this assay. In cases where there is evidence demonstrating the non-applicability of the IL-8 Luc assay to other specific categories of test chemicals, the method should not be used for those specific categories.

14. As described above, the IL-8 Luc assay supports discrimination of skin sensitisers from non-sensitisers. It may also potentially contribute to the assessment of sensitising potency (13, 14, 18) when used in integrated approaches such as IATA. However, further work, preferably based on human data, is required to determine whether IL-8 Luc results can inform potency assessment.

PRINCIPLE OF THE TEST

15. The IL-8 Luc assay makes use of a human monocytic leukemia cell line THP-1 that was obtained from the American Type Culture Collection (Manassas, VA, USA). Using this cell line, the Dept. of Dermatology, Tohoku University School of Medicine, established a THP-1-derived IL-8 reporter cell line, THP-G8, that harbours the Stable Luciferase Orange (SLO) and Stable Luciferase Red (SLR) luciferase genes under the control of the IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters, respectively (17). This allows quantitative measurement of luciferase gene induction by detecting luminescence from well-established light producing luciferase substrates as an indicator of the activity of the IL-8 and GAPDH in cells following exposure to sensitising chemicals.

16. The dual-colour assay system comprises an orange-emitting luciferase (SLO; $\lambda_{\max} = 580 \text{ nm}$) (33) for the gene expression of the IL-8 promoter as well as a red-emitting luciferase (SLR; $\lambda_{\max} = 630 \text{ nm}$) (34) for the gene expression of the internal control promoter, GAPDH. The two luciferases emit different colours upon reacting with firefly D-luciferin and their luminescence is measured simultaneously in a one-step reaction by dividing the emission from the assay mixture using an optical filter (35) (Annex II).

17. THP-G8 cells are treated for 15-16 hours with test chemical, after which SLO luciferase activity (SLO-LA) reflecting IL-8 promoter activity and SLR luciferase activity (SLR-LA) reflecting GAPDH promoter activity are measured. To make the abbreviations easy to understand, SLO-LA and SLR-LA are designated as IL8LA and GAPLA, respectively. Table 1 gives a description of the terms associated with luciferase activity in the IL-8 Luc assay. The measured values are used to calculate the normalised IL8LA (nIL8LA), which is the ratio of IL8LA to GAPLA; the induction of nIL8LA (Ind-IL8LA), which is the ratio of the arithmetic means of quadruple-measured values of the nIL8LA of THP-G8 cells treated with a test chemical and the values of the nIL8LA of untreated THP-G8 cells; and the inhibition of GAPLA (Inh-GAPLA), which is the ratio of the arithmetic means of quadruple-measured values of the GAPLA of THP-G8 cells treated with a test chemical and the

values of the GAPLA of untreated THP-G8 cells.

18. Performance standards (PS) (40) are available to facilitate the validation of modified *in vitro* IL-8 luciferase test methods similar to the IL-8 Luc assay and allow for timely amendment of this Test Guideline for their inclusion. Mutual Acceptance of Data (MAD) will only be guaranteed for test methods validated according to the PS, if these test methods have been reviewed and included in this Test Guideline by the OECD (36).

Table 1. Description of terms associated with the luciferase activity in the IL-8 Luc assay

Abbreviations	Definition
GAPLA	SLR luciferase activity reflecting GAPDH promoter activity
IL8LA	SLO luciferase activity reflecting IL-8 promoter activity
nIL8LA	IL8LA / GAPLA
Ind-IL8LA	nIL8LA of THP-G8 cells treated with chemicals / nIL8LA of untreated cells
Inh-GAPLA	GAPLA of THP-G8 treated with chemicals / GAPLA of untreated cells
CV05	Minimum concentration at which chemicals show less than 0.05 of Inh-GAPLA

DEMONSTRATION OF PROFICIENCY

19. Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency, using the 10 Proficiency Substances listed in Annex III. Moreover, test method users should maintain a historical database of data generated with the reactivity checks (see paragraph 22) and with the positive and solvent/vehicle controls (see paragraphs 28-31), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

PROCEDURE

20. Currently, the only test method covered by this Test Guideline is the IL-8 Luc assay (17, 21). The Standard Operating Procedure (SOP) for the IL-8 Luc assay is available and should be employed when performing the test (36). Laboratories willing to perform the test can obtain the recombinant cell line used from GPC Lab. Co. Ltd., Tottori, Japan, by establishing a licence agreement with the test method developer. The following paragraphs provide a description of the main components and procedures of the assay.

Preparation of cells

21. A transgenic cell line with stable insertion of luciferase reporter genes SLO and SLR should be used (e.g. the THP-G8 cell line) for performing the IL-8 Luc assay. On receipt, cells are propagated (2-4 passages) and stored frozen as a homogeneous stock. Cells from this stock can be propagated up to a

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maximum of 12 passages or a maximum of 6 weeks. They are employed for routine testing using a culture medium of RPMI-1640 containing 10% foetal bovine serum (FBS), antibiotic/antimycotic solution (100U/ml of penicillin G, 100µg/ml of streptomycin and 0.25µg/ml of amphotericin B in 0.85% saline) (e.g. GIBCO Cat#15240-062), 0.15µg/ml Puromycin (e.g. CAS:58-58-2) and 300µg/ml G418 (e.g. CAS:108321-42-2).

22. Prior to use for testing, the cells should be qualified by conducting a reactivity check. This check should be performed 1-2 weeks after thawing, using the positive control, 4-nitrobenzyl bromide (4-NBB) (CAS:100-11-8, ≥ 99% purity) and the negative control, lactic acid (LA) (CAS:50-21-5, ≥85% purity). 4-NBB should produce a positive response to Ind-IL8LA (≥1.4), while LA should produce a negative response to Ind-IL8LA (<1.4). Only cells that pass the reactivity check are used for the assay. The check should be performed according to the procedures described in paragraphs 29-31.

23. For testing, THP-G8 cells are seeded at a density of 2.5×10^5 cells/ml, and pre-cultured in culture flasks for 48-96 hours. On the day of the test, cells harvested from the culture flask are washed with RPMI-1640 containing 10% FBS without any antibiotics, and then, resuspended with RPMI-1640 containing 10% FBS without any antibiotics at 1×10^6 cells/ml. Then, cells are distributed into a 96 well flat-bottom black plate with 50µL (5×10^4 cells/well) (e.g. a 96-well µclear-black plate (Greiner Bio-one Cat#655090 Costar Cat#3603 or Nunc Cat#165305)).

Preparation of the test chemical and control substances

24. The test chemical and control substances are prepared on the day of testing. For the IL-8 Luc assay, test chemicals are dissolved in X-VIVO™ 15, a commercially available serum-free medium (Lonza, 04-418Q), to the final concentration of 20 mg/ml. X-VIVO™ 15 is added to 20 mg of test chemical (regardless of the chemical's solubility) in a microcentrifuge tube and brought to a volume of 1ml and then vortexed vigorously and shaken on a rotor at a maximum speed of 8 rpm for 30 min at an ambient temperature of about 25°C. Furthermore, if solid chemicals are still insoluble, the tube is sonicated until the chemical is dissolved or completely and stably dispersed. For test chemicals soluble in X-VIVO™ 15, the solution is diluted by a factor of 5 with X-VIVO™ 15 and used as an X-VIVO™ 15 stock solution of the test chemical (4 mg/ml). For test chemicals not soluble in X-VIVO™ 15, the mixture is rotated again for at least 30 min, then centrifuged at 15,000 rpm (≈20,000g) for 5 min; the resulting supernatant is used as an X-VIVO™ 15 stock solution of the test chemical. Visualisation of insoluble chemicals can be done by the use of Sudan black B for liquids and from observation of the pellets for solids (see Annex VI). The concentration of a stock solution of test chemical insoluble in X-VIVO™ 15 at 20 mg/ml can be obtained by the procedure shown in Annex VI, but this is not a requirement for use of the assay for hazard identification purposes. A scientific rationale should be provided for the use of solvents other than X-VIVO™ 15, such as

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dimethyl sulfoxide (DMSO), water, or the culture medium.

25. It is worth noting that in using the above procedure, the actual concentration of partially soluble test substances remains uncertain. Thus for test chemicals that are soluble in X-VIVO™ 15, the final concentrations of the test chemicals are from 0.002 to 2 mg/ml in the X-VIVO™ 15 solutions: culture medium=1:1. For test chemicals that are not soluble in X-VIVO™ 15, the final concentrations of the test chemicals are dependent on the saturated concentration of the test chemicals in the X-VIVO™ 15 stock solution.

26. In the first test run, using X-VIVO™ 15, a total of 11 serial dilutions of the X-VIVO™ 15 stock solutions of the test chemicals are made at a dilution factor of two using a 96-well Assay Block (e.g., a 96-well assay block (Corning Cat#07-2000-700, Costar Cat#EW-01729-03 or Thermo Scientific Cat#4222). Next, 50 µl/well of diluted solution is added to the cell suspension in a 96-well flat-bottom black plate.

27. In subsequent test runs (i.e., the second, third, and fourth replicates), the X-VIVO™ 15 stock solution is made at the concentration 4 times higher than the concentration of cell viability 05 (CV05; the lowest concentration at which the Inh-GAPLA becomes <0.05) in the first experiment. The concentration of CV05 is calculated by dividing the concentration of the stock solution in the first run by dilution factor for CV05 (dilution factor CV05; the dilution factor required to dilute stock solution to CV05) (see fig. 1). The stock solutions are then diluted at a factor of 1.5 using X-VIVO™ 15. Next, 50 µl/well of diluted solution is added to a cell suspension in the wells of a 96-well flat-bottom black plate. Each concentration of each test chemical should be tested in 4 wells. The samples are then mixed on a plate shaker and incubated for 15-16 hours at 37°C and 5% CO₂, after which the luciferase activity is measured as described below. The IL-8 Luc assay requires at most 4 test runs to determine whether a chemical is positive or negative. No sterile filtration is needed. In the assay, cell suspensions are made using RPMI-1640 containing 10% FBS without any antibiotics. The detailed procedure for dissolving chemicals is shown in the scheme of Annex V.

28. The solvent control is the mixture of 50 µL/well of X-VIVO™ 15 and 50 µL/well of cell suspension in RPMI-1640 containing 10% FBS.

29. The positive control is 4-NBB. 20 mg of 4-NBB is prepared in a 1.5-ml microfuge tube, to which X-VIVO™ 15 is added up to 1 ml. The tube is vortexed vigorously and shaken on a rotor a maximum speed of 8 rpm for more than 30 min. After centrifugation at 20,000g for 5 min, the supernatant (0.2 mg/ml by calculation) is diluted by a factor of 4 with X-VIVO™ 15 (0.05 mg/ml), and 500 µl of the diluted supernatant is transferred to a well in a 96-well assay block. The diluted supernatant (0.05

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mg/ml) is further diluted with X-VIVO™ 15 at factors of 2 (0.025 mg/ml) and 4 (0.0125 mg/ml), and 50 µl of the solution (0.05 mg/ml, 0.025 mg/ml, 0.0125 mg/ml, and 0.0 mg/ml) is added to 50 µl of THP-G8 cell suspension in the wells of a 96-well plate. The final concentrations are 0.025 mg/ml, 0.0125 mg/ml, and 0.0063 mg/ml. Each concentration of the positive control should be tested in 4 wells. The plate is agitated on a plateshaker, and incubated in a CO₂ incubator for 15-16 hours (37°C, 5% CO₂), after which the luciferase activity is measured as described in paragraph 36.

30. The negative control is 20 mg of LA prepared in a 1.5-ml microfuge tube, to which X-VIVO™ 15 is added up to 1 ml (20 mg/ml). Twenty mg/ml of LA solution is diluted by a factor of 5 with X-VIVO™ 15 (4 mg/ml); 500 µl of this 4 mg/ml LA solution is transferred to a well of a 96 well assay block. This solution is diluted by a factor of 2 with X-VIVO™ 15 and then diluted again by a factor of 2 to produce 2 mg/ml and 1 mg/ml solutions. 50 µl of these 3 solutions and vehicle control (X-VIVO™ 15) are added to THP-G8 in a 96 well plate. Each concentration of the negative control is tested in 4 wells. The plate is agitated on a plateshaker and incubated in a CO₂ incubator for 15-16 hours (37°C, 5% CO₂), after which the luciferase activity is measured as described in paragraph 36.

31. Other suitable positive or negative controls may be used if historical data are available to derive comparable run acceptance criteria.

32. The X-VIVO™ 15 solutions described in paragraphs 26-30 are mixed 1:1 (v/v) with the cell suspensions prepared in a 96-well flat-bottomed plate (see paragraph 23). The treated plates are incubated for 15 to 16 hours at 37°±1C in the presence of 5% CO₂. Care should be taken to avoid evaporation of volatile test chemicals and cross-contamination between wells by test chemicals, e.g. by sealing the plate prior to the incubation with the test chemicals.

33. Each test chemical and solvent control requires from 2-4 runs to derive a positive or negative prediction (see Table 2). Each run is performed on a different day with fresh X-VIVO™ 15 stock solution of test chemicals and independently harvested cells. Cells may come from the same passage.

Luciferase activity measurements

34. Luminescence is measured using a 96-well microplate luminometer equipped with optical filters, e.g. Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany) and the ARVO series (PerkinElmer, Waltham, MA, USA). The luminometer must be calibrated for each test to ensure reproducibility (38). Recombinant orange and red emitting luciferases are available for this calibration.

35. 100µL of pre-warmed Tripluc® Luciferase assay reagent (Tripluc) is transferred to each well of the plate containing the cell suspension treated with or without chemical. The plate is shaken for 10 min at

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an ambient temperature of about 25°C. The plate is placed in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter. Justification should be provided for the use of alternative settings, e.g. depending on the model of luminometer used.

36. Parameters for each concentration are calculated from the measured values, e.g. IL8LA, GAPLA, nIL8LA, Ind-IL8LA, Inh-GAPLA, the mean \pm SD of IL8LA, the mean \pm SD of GAPLA, the mean \pm SD of nIL8LA, the mean \pm SD of Ind-IL8LA, the mean \pm SD of Inh-GAPLA, and the 95% confidence interval of Ind-IL8LA. Definitions of the parameters used in paragraph 36 are provided in Annexes I and IV, respectively.

37. Prior to measurement, colour discrimination in multi-colour reporter assays is generally achieved using detectors (luminometer and plate reader) equipped with optical filters, such as sharp-cut (long-pass or short-pass) filters or band-pass filters. The transmission coefficients of the filters for each bioluminescence signal colour should be calibrated prior to testing, per Annex II.

DATA AND REPORTING

Data evaluation

38. Criteria (see annex IV) for a positive/negative decision require that in each run:

- an IL-8 Luc assay prediction is judged positive if a test chemical has a Ind-IL8LA \geq 1.4 and the lower limit of the 95% confidence interval of Ind-IL8LA \geq 1.0
- an IL-8 Luc assay prediction is judged negative if a test chemical has a Ind-IL8LA $<$ 1.4 and/or the lower limit of the 95% confidence interval of Ind-IL8LA $<$ 1.0

Prediction model

39. Test chemicals that provide two positive results from among the 1st, 2nd, 3rd or 4th runs are identified as positives whereas those that give three negative results from among the 1st, 2nd, 3rd or 4th runs are identified as negatives. Once a decision can be made, no further run is necessary (Table 2).

Table 2. Criteria for identifying positives and negatives

1st run	2nd run	3rd run	4th run	Final prediction	
Positive	Positive	-	-	Positive	
	Negative	Positive	-	Positive	
		Negative	Positive	Positive	Positive
			Negative	Negative	Negative
Negative	Positive	Positive	-	Positive	
		Negative	Positive	Positive	
		Negative	Negative	Negative	
	Negative	Positive	Positive	Positive	
		Negative	Positive	Negative	Negative
			Negative	-	Negative

Acceptance criteria

40. The following acceptance criteria should be met when using the IL-8 Luc assay:

- Ind-IL8LA should be more than 5.0 at least in one concentration of the positive control, 4-NBB, in each run.
- Ind-IL8LA should be less than 1.4 at any concentration of the negative control, lactic acid, in each run.
- Data from plates for which the SLR-LA of control wells with cells and Tripluc but without chemicals is less than 5 times of that of well containing test medium only (50 µl/well of RPMI-1640 containing 10% FBS and 50 µl/well of X-VIVO™ 15) should be rejected.
- Data from plates for which the II-SLR-LA of all concentrations of the test or control chemicals is less than 0.05 should be rejected. In this case, the first test should be repeated so the highest final concentration of the repeated test is the lowest final concentration of the previous test.

Test report

41. The test report should include the following information:

Test chemicals

Mono-constituent substance:

- Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
- Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
- Purity, chemical identity of impurities as appropriate and practically feasible, etc;

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- Treatment prior to testing, if applicable (e.g. warming, grinding);
- Solubility in X-VIVO™ 15. For chemicals that are insoluble in X-VIVO™ 15, whether precipitation or flotation are observed after centrifugation;
- Concentration(s) tested;
- Storage conditions and stability to the extent available;
- Justification for choice of solvent/vehicle for each test chemical if X-VIVO™ 15 has not been used.

Multi-constituent substance, UVCB and mixture:

- Characterisation by chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
- Physical appearance, water solubility, and additional relevant physicochemical properties, to the extent available;
- Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant to testing;
- Treatment prior to testing, if applicable (e.g. warming, grinding);
- Solubility in X-VIVO™ 15. For chemicals that are insoluble in X-VIVO™ 15, whether precipitation or flotation are observed after centrifugation;
- Concentration(s) tested;
- Storage conditions and stability to the extent available;
- Justification for choice of solvent/vehicle for each test chemical, if X-VIVO™ 15 has not been used.

Controls

Positive control:

- Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
- Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available and where applicable;
- Purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Treatment prior to testing, if applicable (e.g. warming, grinding);
- Concentration(s) tested;
- Storage conditions and stability to the extent available;
- Reference to historical positive control results demonstrating suitable acceptance criteria, if applicable.

Negative control:

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- Chemical identification, such as IUPAC or CAS name(s), CAS number(s), and/or other identifiers;
- Purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Physical appearance, molecular weight, and additional relevant physicochemical properties in the case other negative controls than those mentioned in the Test Guideline are used and to the extent available;
- Storage conditions and stability to the extent available;
- Justification for choice of solvent for each test chemical.

Assay conditions

- Name and address of the sponsor, test facility and study director;
- Description of test method used;
- Cell line used, its storage conditions, and source (e.g. the facility from which it was obtained);
- Lot number and origin of FBC, supplier name, lot number of 96 multi-well plate, and lot number of Tripluc reagent;
- Passage number and cell density used for testing;
- Cell counting method used for seeding prior to testing and measures taken to ensure homogeneous cell number distribution;
- Luminometer model used including instrument settings, luciferase substrate used, and demonstration of appropriate luminescence measurements based on the control test described in Annex II;
- The procedure used to demonstrate proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or to demonstrate reproducible performance of the test method over time.

Test procedure

- Number of runs performed;
- Application procedure and exposure time (if different from the those recommended);
- Description of evaluation and decision criteria used;
- Description of study acceptance criteria used;
- Description of any modifications of the test procedure.

Results

- Measurements of IL8LA and GAPLA;
- Calculations for nIL8LA, Ind-IL8LA, and Inh-GAPLA;

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- The 95% confidence interval of Ind-IL8LA;
- A graph depicting dose-response curves for induction of luciferase activity and viability;
- Description of any other relevant observations, if applicable.

Discussion of the results

- Discussion of the results obtained with the IL-8 Luc assay;
- Consideration of the assay results in the context of an IATA, if other relevant information is available.

Conclusion

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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 (OECD, 2005).

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 (OECD, 2012a).

CV50: Cell viability 05. Minimum concentration at which chemicals show less than 0.05 of II-SLR-LA

FInSLO-LA: The fold induction of normalised SLO-LA. It is obtained by dividing the nSLO-LA of THP-G8 cells treated with chemicals by that of non-stimulated THP-G8 cells and represents the induction of IL-8 promoter activity by chemicals. This is the same meaning with Ind-IL8LA, and used in the validation report and the previous publications regarding the IL-8 Luc assay.

GAPLA: Luciferase Activity of Stable Luciferase Red (SLR) ($\lambda_{\text{max}} = 630 \text{ nm}$), regulated by GAPDH promoter and demonstrates cell viability and viable cell number.

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.

IATA (Integrated Approach to Testing and Assessment): A structured approach used for hazard identification (potential), hazard characterisation (potency) and/or safety assessment (potential/potency and exposure) of a chemical or group of chemicals, which strategically integrates and weights all relevant data to inform regulatory decision regarding potential hazard and/or risk and/or the need for further targeted and therefore minimal testing.

II-SLR-LA: The Inhibition Index of SLR-LA. It is obtained by dividing SLR-LA of THP-G8 treated with chemicals with SLR-LA of non-treated THP-G8 and represents cytotoxicity of chemicals. This is the same meaning with Inh-GAPLA, and used in the validation report and the previous publications

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regarding the IL-8 Luc assay.

IL-8 (Interleukin-8): A cytokine derived from endothelial cells, fibroblasts, keratinocytes, macrophages, and monocytes that causes chemotaxis of neutrophils and T-cell lymphocytes.

IL8LA: Luciferase Activity of Stable Luciferase Orange (SLO) ($\lambda_{\text{max}} = 580 \text{ nm}$), regulated by IL-8 promoter.

Ind-IL8LA: Induction of IL8LA. It is obtained by dividing the nIL8LA of THP-G8 cells treated with chemicals by that of non-stimulated THP-G8 cells and represents the induction of IL-8 promoter activity by chemicals.

Inh-GAPLA: Inhibition of GAPLA. It is obtained by dividing GAPLA of THP-G8 treated with chemicals with GAPLA of non-treated THP-G8 and represents cytotoxicity of chemicals.

Minimum induction threshold (MIT): the lowest concentration at which a chemical satisfies the positive criteria

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 of the main constituents 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.

nIL8LA: The SLO luciferase activity reflecting IL-8 promoter activity normalised by the SLR luciferase activity reflecting GAPDH promoter activity. It represents IL-8 promoter activity after considering cell viability or cell number.

nSLO-LA: The SLO luciferase activity (SLO-LA) normalised by SLR luciferase activity (SLR-LA). It represents IL-8 promoter activity after considering cell viability or cell number. This is the same meaning with nIL8LA, and used in the validation report and the previous publications regarding the IL-8 Luc assay.

Positive control: A replicate containing all components of a test system and treated with a substance

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known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Pre-haptens: Chemicals which become sensitisers through abiotic transformation.

Pro-haptens: Chemicals requiring enzymatic activation to exert skin sensitisation potential.

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 (OECD, 2005).

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 (OECD, 2005).

Run: A run consists of one or more test chemicals tested concurrently with a solvent/vehicle control and with a positive control.

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test. 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 (OECD, 2005).

SLO-LA: Luciferase Activity of Stable Luciferase Orange (SLO) ($\lambda_{\text{max}} = 580 \text{ nm}$), regulated by IL-8 promoter. This is the same meaning with IL8LA, and used in the validation report and the previous publications regarding the IL-8 Luc assay.

SLR-LA: Luciferase Activity of Stable Luciferase Red (SLR) ($\lambda_{\text{max}} = 630 \text{ nm}$), regulated by GAPDH promoter demonstrating cell viability and viable cell number. This is the same meaning with GAP8LA, and used in the validation report and the previous publications regarding the IL-8 Luc assay.

Solvent/vehicle control: An untreated sample containing all components of a test system except of the test chemical, but including the solvent/vehicle that is used. It is used to establish the baseline response for the samples treated with the test chemical dissolved or stably dispersed in the same solvent/vehicle. When tested with a concurrent medium control, this sample also demonstrates whether the solvent/vehicle interacts with the test system.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test. It

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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 (OECD, 2005).

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.

Surfactant: Also called surface-active agent, this is a substance, such as a detergent, that can reduce the surface tension of a liquid and thus allow it to foam or penetrate solids; it is also known as a wetting agent. (TG437)

Test chemical: The term "test chemical" is used to refer to what is being tested.

THP-G8: An IL-8 reporter cell line used in IL-8 Luc assay. The human macrophage-like cell line THP-1 was transfected with the SLO and SLR luciferase genes under the control of the IL-8 and GAPDH promoters, respectively.

United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (UN, 2013).

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

Valid test method: A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose.

ANNEX II

Determination of the transmission coefficients of optical filter for SLO and SLR**Principle of measurement of luciferase activity**

MultiReporter Assay System -Tripluc- can be used with a microplate-type luminometer with a multi-colour detection system, which can equip an optical filter (e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)). The optical filter used in measurement is 600–620 nm long or short pass filter, or 600–700 nm band pass filter.

(1) Measurement of two-colour luciferases with an optical filter.

This is an example using Phelios AB-2350 (ATTO). This luminometer is equipped with a 600 nm long pass filter (R60 HOYA Co.), 600 nm LP, Filter 1) for splitting SLO ($\lambda_{\max} = 580$ nm) and SLR ($\lambda_{\max} = 630$ nm) luminescence.

To determine transmission coefficients of the 600 nm LP, first, using purified SLO and SLR luciferase enzymes, measure i) the intensity of SLO and SLR bioluminescence intensity without filter (F0), ii) the SLO and SLR bioluminescence intensity that passed through 600 nm LP (Filter 1), and iii) calculate the transmission coefficients of 600 nm LP for SLO and SLR listed below.

Transmission coefficients		Abbreviation	Definition
SLO	Filter 1 Transmission coefficients	κO_{R60}	The filter's transmission coefficient for the SLO
SLR	Filter 1 Transmission coefficients	κR_{R60}	The filter's transmission coefficient for the SLR

When the intensity of SLO and SLR in test sample are defined as O and R, respectively, i) the intensity of light without filter (all optical) F0 and ii) the intensity of light that transmits through 600 nm LP (Filter 1) F1 are described as below.

$$F0=O+R$$

$$F1=\kappa O_{R60} \times O + \kappa R_{R60} \times R$$

These formulas can be rephrased as follows:

$$\begin{pmatrix} F0 \\ 1 \\ F1 \end{pmatrix} = \begin{pmatrix} 1 & 1 \\ & 1 \\ \kappa O_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} O \\ 1 \\ R \end{pmatrix}$$

Then using calculated transmittance factors (κO_{R60} and κR_{R60}) and measured F0 and F1, you can calculate O and R-value as follows:

$$\begin{pmatrix} O \\ 1 \\ R \end{pmatrix} = \begin{pmatrix} 1 & 1 \\ & 1 \\ \kappa O_{R60} & \kappa R_{R60} \end{pmatrix}^{-1} \begin{pmatrix} F0 \\ 1 \\ F1 \end{pmatrix}$$

Materials and methods for determining transmittance factor

(1) Reagents

· Single purified luciferase enzymes:

Lyophilised purified SLO enzyme

Lyophilised purified SLR enzyme

(which for the validation work were obtained from GPC Lab. Co. Ltd., Tottori, Japan with THP-G8 cell line)

· Assay reagent:

Tripluc[®] Luciferase assay reagent (for example from TOYOBO Cat#MRA-301)

· Medium: for luciferase assay (30 ml, stored at 2 – 8°C)

Reagent	Conc.	Final conc. in medium	Required amount
RPMI-1640	-	-	27 ml
FBS	-	10 %	3 ml

(2) Preparation of enzyme solution

Dissolve lyophilized purified luciferase enzyme in tube by adding 200 µl of 10 ~ 100 mM Tris/HCl or Hepes/HCl (pH 7.5 ~ 8.0) supplemented with 10% (w/v) glycerol, divide the enzyme solution into 10 µl aliquots in 1.5 ml disposable tubes and store them in a freezer at -80°C. The frozen enzyme solution can be used for up to 6 months. When used, add 1 ml of medium for luciferase assay (RPMI-1640 with 10% FBS) to each tube containing the enzyme solutions (diluted enzyme solution) and keep them on ice to prevent deactivation.

(3) Bioluminescence measurement

Thaw Tripluc[®] Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Power on the luminometer 30 min before starting the measurement to allow the photomultiplier to stabilize. Transfer 100 µl of the diluted enzyme solution to a black 96 well plate (flat bottom) (the SLO reference sample to #B1, #B2, #B3, the SLR reference sample to #D1, #D2, #D3). Then, transfer 100 µl of pre-warmed Tripluc to each well of the plate containing the diluted enzyme solution using a pipetman. Shake the plate for 10 min at room temperature (about 25°C) using a plate shaker. Remove bubbles from the solutions in wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter.

Transmission coefficient of the optical filter was calculated as follows:

Transmission coefficient (SLO (κO_{R60}))= (#B1 of F1+ #B2 of F1+ #B3 of F1) / (#B1 of F0+ #B2 of

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F0+ #B3 of F0)

Transmission coefficient (SLR (κR_{R60}))= (#D1 of F1+ #D2 of F1+ #D3 of F1) / (#D1 of F0+ #D2 of F0+ #D3 of F0)

Calculated transmittance factors are used for all the measurements executed using the same luminometer.

Quality control of equipment

In order to confirm the detector stability as the quality control, the reference luciferase sample, optical property, the protocol described hereafter should be performed at the beginning of the experiments every day using reference LED light source plates equipped with stabilised LEDs. LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than $3 \times \sigma$ (= 4.5%). Here describes an example using a commercially available reference light source, TRIANT® (wSL-0001) by ATTO (Tokyo, Japan).

Start LED plate and select “PMT” mode.

Select three-color (BRG) mode and adjust light intensity to 1/10 (10E-1).

Place the LED plate into the luminometer. Light intensity is measured for 3 sec each in the absence (F0) and presence (F2) of the optical filter.

Read and record the data at the position of #F6, #E6, and #D6 that are LEDs of blue, green, and red LEDs, respectively.

LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than $3 \times \sigma$ (= 4.5

ANNEX III
PROFICIENCY SUBSTANCES

Prior to routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by obtaining the expected IL-8 Luc assay prediction for the 10 substances recommended in Table 1 and by obtaining values that fall within the respective reference range for at least 8 out of the 10 proficiency substances (selected to represent the range of responses for skin sensitisation hazards). Other selection criteria were that the substances are commercially available, and that high-quality *in vivo* reference data as well as high quality *in vitro* data generated with the IL-8 Luc assay are available. Also, published reference data are available for the IL-8 Luc assay (Kimura et al., 2015; Takahashi et al., 2011).

Table 1: Recommended substances for demonstrating technical proficiency with the IL-8 Luc assay

Proficiency substances	CAS no.	State	Solubility in X-VIVO15 at 20 mg/ml	<i>In vivo</i> prediction ¹	IL-8 Luc prediction ²	Reference range (µg/ml) ³	
						CV05 ⁴	IL-8 Luc MIT ⁵
2,4-Dinitrochlorobenzene	97-00-7	Solid	Insoluble	Sensitiser (Extreme)	Positive	2.3-3.9	0.5-2.3
Glyoxal	107-22-2	Liquid	Soluble	Sensitiser (Strong)	Positive	1200-2000	500-1000
2-Mercaptobenzothiazole	149-30-4	Solid	Insoluble	Sensitiser (Moderate)	Positive	>2000	60-250
Ethylenediamine	107-15-3	Liquid	Soluble	Sensitiser (Moderate)	Positive	500-700	0.1-0.4
Imidazolidinyl Urea	39236-46-9	Solid	Soluble	Sensitiser (Weak)	Positive	31-37	16-31
4-Allylanisole (Estragol)	140-67-0	Liquid	Insoluble	Sensitiser (Weak)	Positive	>2000	0.01-0.07
4-Aminobenzoic acid	150-13-0	Solid	Insoluble	Non-sensitiser	Negative	>2000	>2000
4-Hydroxybenzoic acid	99-96-7	Solid	Insoluble	Non-sensitiser	Negative	>2000	>2000
Glycerol	56-81-5	Liquid	Soluble	Non-sensitiser	Negative	>2000	>2000
Isopropanol	67-63-0	Liquid	Soluble	Non-sensitiser	Negative	>2000	>2000

Abbreviations: CAS no. = Chemical Abstracts Service Registry Number

¹ The *in vivo* potency is derived using the criteria proposed by ECETOC (38).

² Based on historical observed values (17, 21).

³ CV05 and IL-8 Luc MIT were calculated using water solubility given by EPI Suite™.

⁴ CV05: the minimum concentration at which chemicals show less than 0.05 of Inh-GAPLA.

⁵ MIT: the lowest concentrations at which a chemical satisfies the positive criteria.

ANNEX IV

Indexes and judgment criteria

nSLO-LA (nIL8LA)

The j-th repetition ($j = 1-4$) of the i-th concentration ($i = 0-11$) is measured for SLO-LA (IL8LA) and SLR-LA (GAPLA) respectively. The normalised SLO-LA, referred to as nSLO-LA (nIL8LA), and is defined as:

$$\text{nSLO-LA}_{ij} = \text{SLO-LA}_{ij} / \text{SLR-LA}_{ij}.$$

This is the basic unit of measurement in this assay.

FInSLO-LA (Ind-IL8LA)

The fold increase of the averaged nSLO-LA (nIL8LA) for the repetition on the i-th concentration compared with it at the 0 concentration, FInSLO-LA, is the primary measure of this assay. This ratio is written by the following formula:

$$\text{FInSLO-LA}_i = \left\{ (1/4) \times \sum_j \text{nSLO-LA}_{ij} \right\} / \left\{ (1/4) \times \sum_j \text{nSLO-LA}_{0j} \right\}.$$

The lead laboratory has proposed that a value of 1.4 corresponds to a positive result for the tested chemical. This value is based on the investigation of the historical data of the lead laboratory. Data management team then used this value through all the phases of validation study. The primary outcome, FInSLO-LA, is the ratio of 2 arithmetic means as shown in equation.

95% confidence interval (95% CI)

The 95% confidence interval (95% CI) based on the ratio can be estimated to show the precision of this primary outcome measure. The lower limit of the 95% CI ≥ 1 indicates that the nSLO-LA with the i-th concentration is significantly greater than that with solvent control. There are several ways to construct the 95% CI. We used the method known as Fieller's theorem in this study. This 95% confidence interval theorem is obtained from the following formula:

$$\left[\frac{-B - \sqrt{B^2 - 4AC}}{2A}, \frac{-B + \sqrt{B^2 - 4AC}}{2A} \right],$$

where $A = \bar{x}_0^2 - t_{0.975(v)}^2 \times \frac{\text{sd}_0^2}{n_0}$, $B = -2 \times \bar{x} \times \bar{y}$, $C = \bar{y}_i^2 - t_{0.975(v)}^2 \times \frac{\text{sd}_{y_i}^2}{n_{y_i}}$, and

$$\bar{x}_0^2 = \left\{ (1/4) \times \sum_j \text{nSLO-LA}_{0j} \right\}^2, \quad \text{sd}_0^2 = (1/3) \times \sum_j (\text{nSLO-LA}_{0j} - \bar{x}_0)^2, \quad n_0 = 4,$$

$$\bar{y}_i^2 = \left\{ (1/4) \times \sum_j \text{nSLO-LA}_{ij} \right\}^2, \quad \text{sd}_{y_i}^2 = (1/3) \times \sum_j (\text{nSLO-LA}_{ij} - \bar{y}_i)^2, \quad n_{y_i} = 4,$$

$t_{0.975(v)}$ is 97.5 percentile of the central t distribution with the v of the degree of freedom.

II-SLR-LA (Inh-GAPLA)

The II-SLR-LA is a ratio of the averaged SLR-LA (GAPLA) for the repetition of the i -th concentration compared with that with solvent control, and this is written by

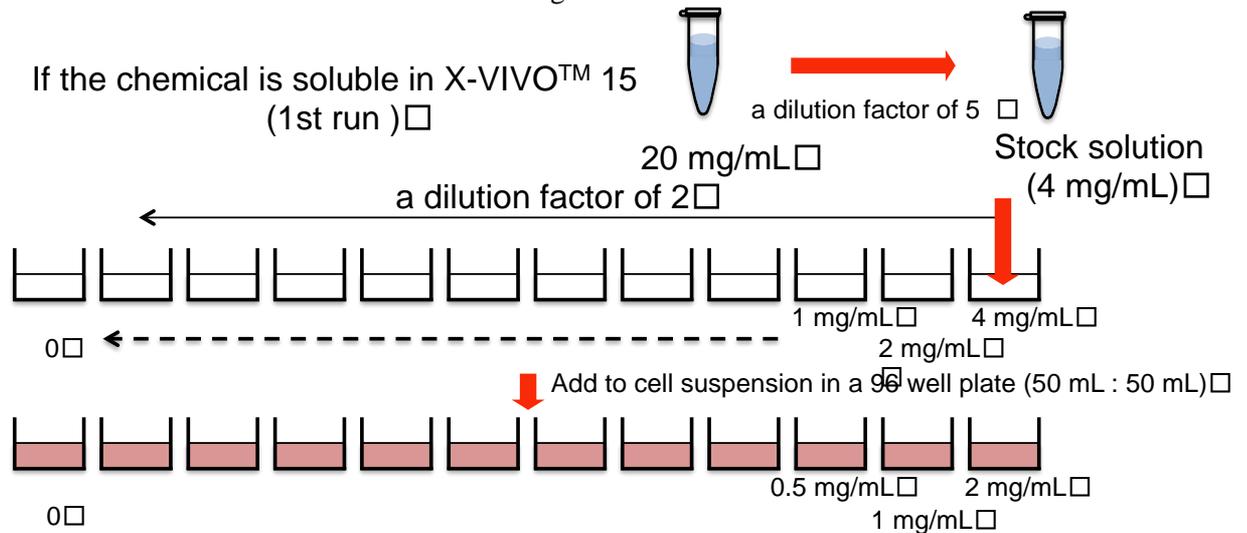
$$\text{II-SLR-LA}_i = \left\{ (1/4) \times \sum_j \text{SLL-LA}_{ij} \right\} / \left\{ (1/4) \times \sum_j \text{SLL-LA}_{0j} \right\}.$$

Since the SLR-LA is the denominator of the nSLO-LA, an extremely small value causes large variation in the nSLO-LA. Therefore, the i -th FInSLO-LA value with an extremely small value of II-SLR-LA might be considered poor precision.

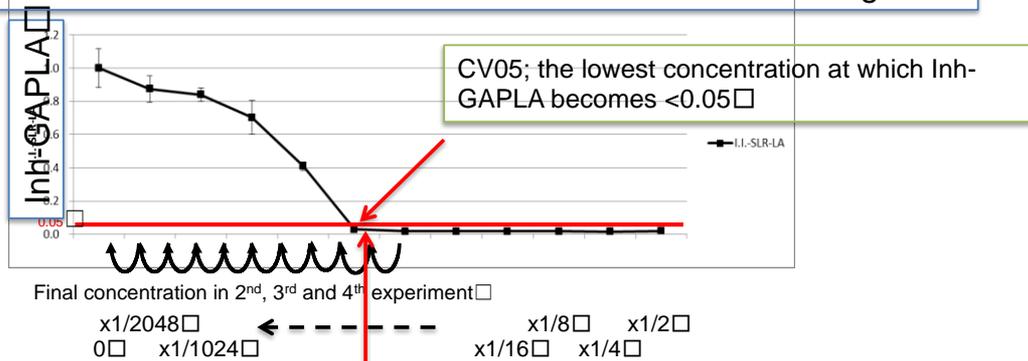
Annex V

The scheme of the methods to dissolve chemicals for the IL-8 Luc assay.

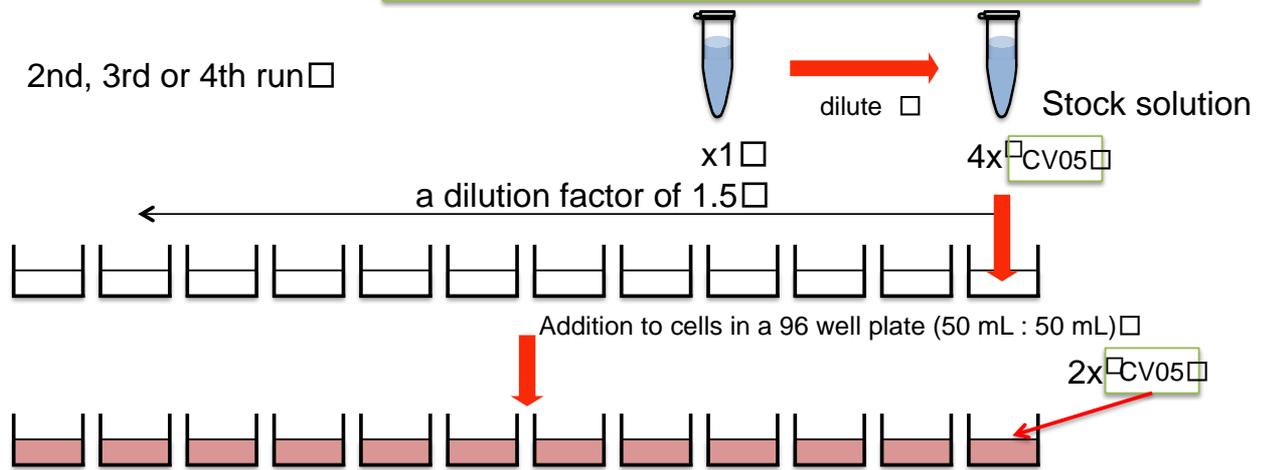
(a) For chemicals dissolved in X-VIVO™ 15 at 20 mg/ml



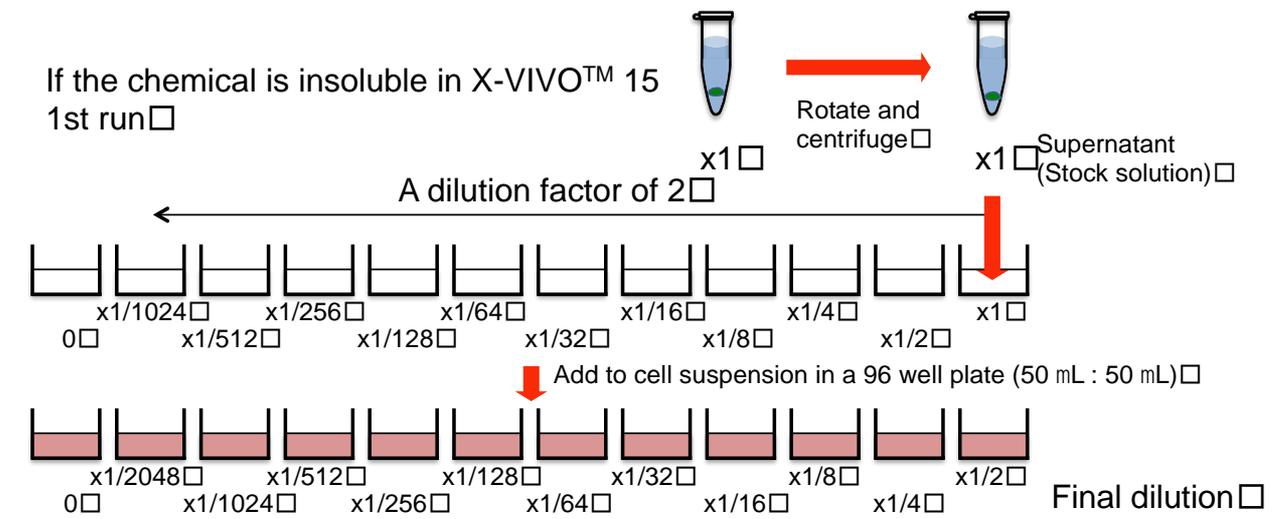
Determine the concentration of the stock solution of the following runs □



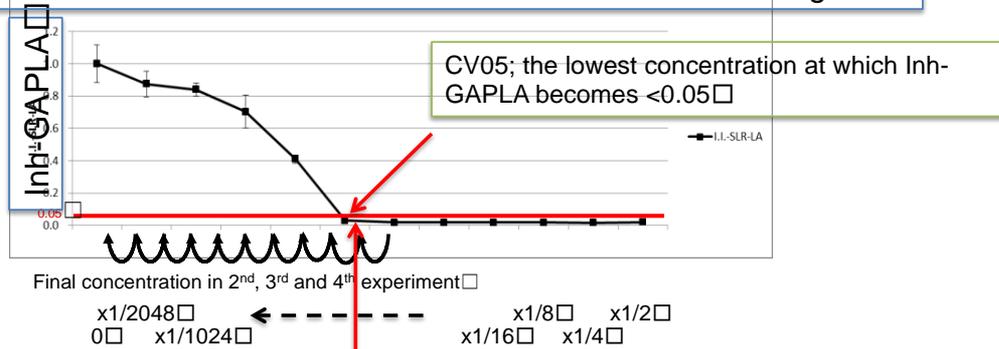
1/dilution factor for CV05 (dilution factor for CV05; the dilution factor required to dilute stock solution to CV05) □



(b) For chemicals insoluble in X-VIVO™ 15 at 20 mg/ml

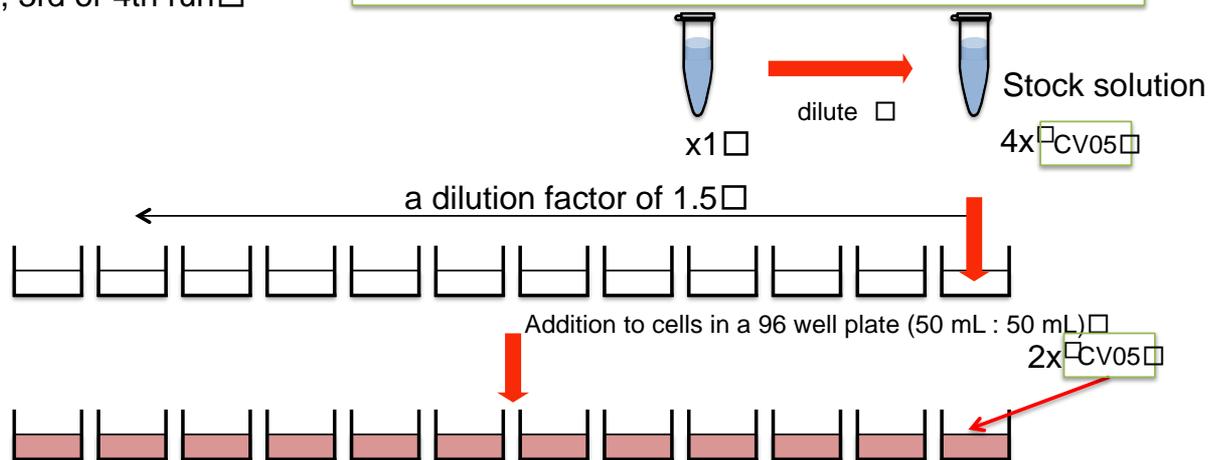


Determine the concentration of the stock solution of the following runs □



2nd, 3rd or 4th run □

1/dilution factor for CV05
(dilution factor for CV05; the dilution factor required to dilute stock solution to CV05) □



Annex VI**Determination of stock solution concentrations of chemicals not dissolved at 20 mg/ml X-VIVO™ 15**

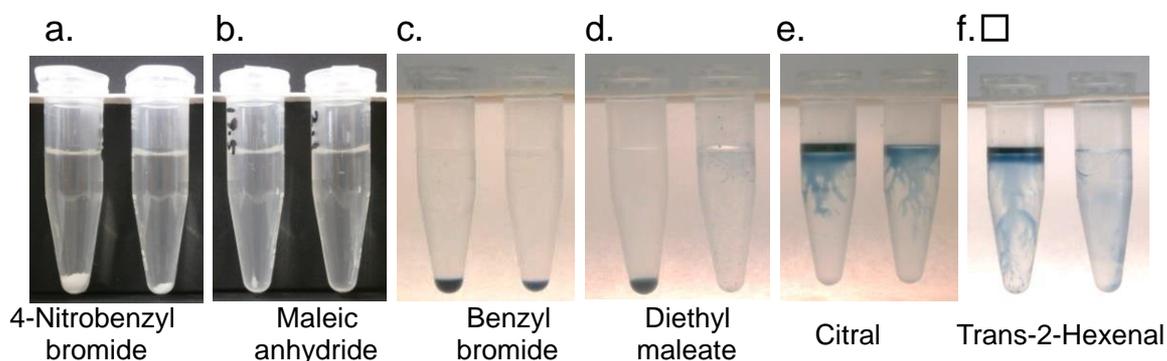
In the IL8 Luc assay, the information on the concentration of a test chemical is not a requirement for use in hazard identification IATA. However, when the information on the concentration of test chemicals is used, for example to consider their potency, the following procedure can be employed to determine the concentration of the stock solution of chemicals that are insoluble in X-VIVO™ 15 at the concentration of 20 mg/ml.

1. Using EPI Suite™

The procedure attempts by various methods to dissolve chemicals to their limit of water solubility, thus the concentration of the stock solution is very close to maximum water solubility. Therefore, the water solubility given by EPI Suite™ is substituted as the concentration of the stock solution.

2. Determining the solubility of chemicals at 2 mg/ml

X-VIVO™ 15 is added to 2 mg of the chemical up to 1 ml in a 1.5-ml microfuge tube. If dissolved after vigorous vortexing and rotation at a maximum speed of 8 rpm for 30 min, the concentration of the stock solution is estimated as 2 mg/ml, and if not dissolved, that is estimated as 0.2 mg/ml. For liquid chemicals, 1 mg of Sudan black B (Merck, Dalmstadt, Germany) is added to 1 ml of the chemical substance (0.1 % [w/v]) to permit visualization of insoluble chemical, while 1 mg/ml insoluble solid chemicals are recognised as pellets. After determining the concentration of the stock solution, the concentrations of diluted chemicals are calculated by dividing the concentration of the stock solution by the dilution factor. Practical experience has shown that the solubility of chemicals can be determined visually at concentrations >1 mg/ml. If necessary, a more accurate concentration of the stock solution can be obtained by determining the solubility of chemicals at concentrations from 1 - 20 mg/ml. The representative figures showing the solubility of chemicals in X-VIVO™ 15 is shown in Fig .1 – the determination of the solubility of test chemicals at 2 mg/ml.

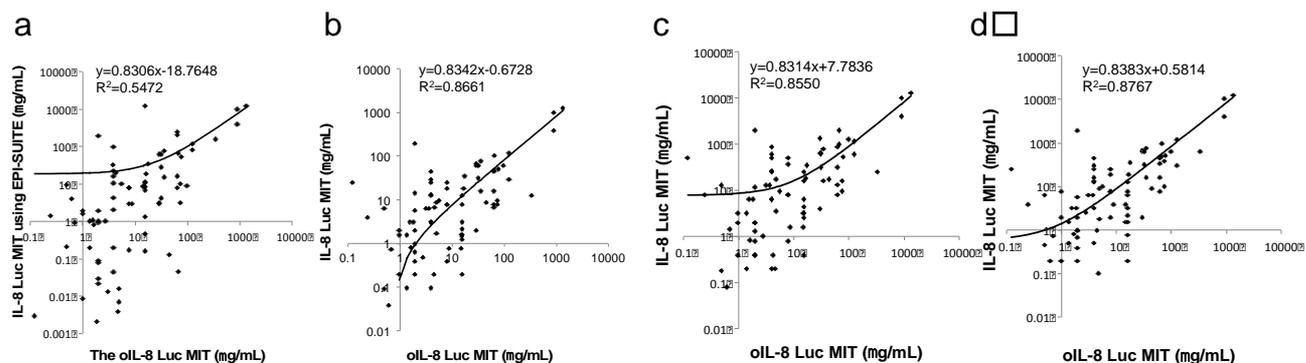


15 December 2016

(a) solid chemical (4-nitrobenzyl bromide)(insoluble at both 20 mg/ml and 2 mg/ml), (b) solid chemical (maleic anhydride) (insoluble at 20 mg/ml and soluble at 2 mg/ml), (c) liquid chemical (benzyl bromide)(insoluble at both 20 mg/ml and 2 mg/ml), (d) liquid chemical (diethyl maleate)(insoluble at 20 mg/ml and soluble at 2 mg/ml), (e) liquid chemical (citral)(insoluble at both 20 mg/ml and 2 mg/ml), and (f) liquid chemical (trans-2-hexenal)(insoluble at 20 mg/ml and soluble at 2 mg/ml). The figures for the solubility of chemicals at 20 mg/ml is shown to clearly visualize the difference of solubility of chemicals at 20 mg/ml and 2 mg/ml.

Next, correlation was examined between the IL-8 Luc minimum induction concentrations (MITs) (the lowest concentration at which a chemical satisfies the positive criteria using X-VIVOTM 15 as a solvent or mL8 Luc MIT) and the IL-8 Luc MITs using DMSO as a solvent (oIL8 Luc MIT). After estimating the concentrations of the stock solutions based on measured solubility of chemicals, the minimum induction thresholds (MITs: the lowest concentrations at which the chemicals satisfy the positive criteria) of sensitizers was determined by dividing the estimated concentration of the stock solution by the dilution factor required to obtain the lowest concentration at which the chemical would satisfy the positive criterion. The correlation between the MITs determined using the IL-8 Luc assay using X-VIVOTM 15 as a solvent (IL-8 Luc assay) and those determined using the IL-8 Luc assay used DMSO as a solvent (original IL-8 Luc assay) was then assessed. A strong correlation was observed when the IL-8 Luc MITs using EPI SuiteTM water solubility data was calculated (Fig. 2a). The correlation was much stronger when the IL-8 Luc MITs using the estimated concentration of the stock solution based on measured solubility was calculated (Fig. 2b, 2c, and 2d), thus validating the calculations of the prepared stock solutions. (Kimura et al. J Toxicol Sci, submitted).

Fig. 1. Correlation between the IL-8 Luc MITs and the original IL-8 Luc MITs using DMSO and the estimated concentration of stock solutions with the margin of error toward high concentration.



	Measured solubility	Estimated concentration of stock solution (mg/mL)				The margin of error toward high concentration	
a	EPI-SUIT						
b	20 and 2 (mg/mL)	20	2	0.2		< 10 times	
c	20 and 2 (mg/mL)	20	4	0.4		< 5 times	
d	20, 10, 2, and 1 (mg/mL)	20	10	2	1	0.2	< 2 times or < 5 times