Section 4
Health effects

Test No. 444A

In vitro immunotoxicity

IL-2 Luc and Il-2 Luc LTT Assays



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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In Vitro Immunotoxicity: Combination Of Il-2 Luc Il-2 Luc Ltt Assays

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INITIAL CONSIDERATIONS AND LIMITATIONS

1. Immunotoxicology is defined as the study of adverse effects on any part of the immune system as the result of exposure to drugs or chemicals (Corsini and Roggen, 2009). Immunotoxicology can also include adverse effects on different organs and tissues mediated by cells and molecules of the immune system. The immune system is susceptible to toxic insults, in part because of: 1) the need to maintain the delicate balance between activation, regulation and silencing; 2) its dependence on regeneration of cells from hematopoietic stem cells in the bone marrow; 3) its requirement of clonal expansion of T cells and B cells by cellular proliferation during the adaptive response; and 4) the required maintenance of appropriate levels of lymphocytes, including effector, memory and naïve subsets.

2. Immunotoxicity by drugs or chemicals can be manifested in various ways, including dysregulation of the immune response, which could lead to immunosuppression or inappropriate immune stimulation. The latter can include exaggerated immune stimulation, sustained inflammation, hypersensitivity reactions and autoimmune diseases. With reference to chemical-induced immunotoxicity, the effect may not be exclusively in one direction and the same substance can produce immunosuppression or immune stimulation, depending on the dose and the cellular target. Thus, it may be more appropriate to define an immunotoxic substance as any agent that can alter one or more immune functions resulting in an adverse effect for the host. In this way, the focus is not on the direction of the effect, but on its consequence. For this reason, the term immunotoxicant/immunotoxicity is used in this Test Guideline (TG), which is consistent with the Detailed Review Paper on immunosuppression that was developed by OECD (OECD, 2022).

3. Currently, the assessment of chemical immunotoxicity relies mainly on animal models and assays that characterise immunosuppression and sensitization. However, animal studies have many drawbacks, such as high cost, ethical concerns, and have varying ability to predict effects on human health (Adler et al. 2011). In addition, current *in vivo* models do not always provide a mechanistic understanding of the data. Overcoming these problems requires the development of *in vitro* methods to detect immunotoxicity. A workshop hosted by the European Centre for the Validation of Alternative Methods in 2003 focused on state-of-the-art *in vitro* systems for evaluating immunotoxicity (Gennari et al. 2005; Galbiati et al. 2010; Lankveld et al. 2010) and a tiered approach was proposed.

4. From the OECD Adverse Outcome Pathway (AOP) development programme, AOP 154 “Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response” was approved in 2021 (OECD, 2021). This AOP describes calcineurin (CN) activity inhibition by binding of stressors, i.e., CN inhibitors (CNIs). CNIs bind to CN with their respective immunophilins, which interferes with the nuclear localization of nuclear factor of activated T cells (NFAT), a substrate of CN. As a result, the formation of functional NFAT complexes with activator protein-1 (AP-1) that bind at the site of IL-2, IL-4 and other T cell-derived cytokine promoters, is reduced, thereby suppressing production of these cytokines. Among the affected cytokines from each of the helper T cell subsets, reduced production of IL-2 and IL-4 negatively affects the proliferation and differentiation of B cells to suppress the T cell–dependent antibody response (TDAR).

5. IL-2 exerts pleiotropic actions on CD4+ T cell differentiation via its modulation of cytokine receptor expression. IL-2 promotes Th1 differentiation by inducing IL-12Rβ2 (and IL-12Rβ1), promotes Th2 differentiation by inducing IL-4Rα, inhibits Th17 differentiation by inhibiting gp130 (and IL-6Rα), and drives Treg differentiation by inducing IL-2Rα. IL-2 also potently represses IL-7Rα, which decreases survival signals that normally promote cell survival and memory cell development (Liao et al., 2011). It is therefore conceivable that chemicals that affect IL-2 release by T cells can significantly impact immune function.

6. The IL-2 luciferase assay (IL-2 Luc assay) and the IL-2 luciferase leukocyte toxicity test (IL-2 Luc LTT assay) both use 2H4 cells to identify the effects of chemicals on the IL-2 and IFN-γ promoters in the presence of the stimulants phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io) (Saito et al., 2011). 2H4, derived from Jurkat cells, contains stable luciferase green (SLG) regulated by the IL-2 promoter, stable luciferase

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orange (SLO) regulated by the interferon (IFN)-γ promoter, and stable luciferase red (SLR) regulated by the glyceraldehyde 3-phosphate dehydrogenase **(**GAPDH) promoter. Although these two assays use the same cell line, the IL-2 Luc assay and the IL-2 Luc LTT assay evaluate immunosuppression by different mechanisms. In other words, the IL-2 Luc assay can detect the effects of chemicals on IL-2 production (Kimura et al., 2020), i.e., their effects on T cell activation, while the IL-2 Luc LTT assay can identify the antimitotic effects of chemicals (Kimura et al., 2021 and Kimura et al., 2023), i.e. it can evaluate myelotoxicity.

7. The validation studies of the IL-2 Luc and the IL-2 Luc LTT assays were conducted by a validation management team (VMT) composed of a lead laboratory, three independent laboratories, and four international expert members coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM). The validation study of the IL-2 Luc assay met the acceptance criteria regarding within- (80.0%,4/5) and between-laboratory reproducibility (80.0%, 16/20) which could satisfy the acceptance criteria of 80% (Kimura et al. 2020). To determine the predictivity, the lead laboratory collected immunotoxicological information and selected the reference data by classifying the chemical into immunotoxic compounds targeting T cells or others according to previously reported criteria. The predictivity of the bidirectional Il-2 Luc assay (i.e. measuring both increase or decrease of IL-2 production) is presented in the validation study report (Kimura et al., 2020. These results were reviewed by an international peer review panel. While the predictive capacity was not satisfactory for a stand-alone method, the IL-2 Luc assay is acceptable for use in combination with other assays (JaCVAM, 2020).

8. Similarly, the validation study of the IL-2 Luc LTT assay met the acceptance criteria regarding within- and between-laboratory reproducibilities which could satisfy the acceptance criteria of 80% (Kimura et al., 2023). To determine the predictivity, the lead laboratory collected immunotoxicological information and constructed the reference data by classifying the chemical into immunosuppression possibly leading to increased susceptibility to infection or cancer or others according to previously reported criteria. The average within-laboratory reproducibility was 100.0% (15/15). The between laboratory reproducibility was 92.0% (23/25). If the performance of IL-2 Luc LTT assay is determined by referring to the classification of chemical substances with immunosuppressive effects, it was 71.4 % (15/21) for mean sensitivity, 100.0 % (4/4) for mean specificity, and 76.0 % (19/25) for mean prediction of the combined data from the Phase I and Phase II trials (Kimura et al., 2023). To further characterize the IL-2 Luc LTT assay, the lead laboratory assessed a total of 83 test compounds including 46 pharmaceutical drugs that were composed of 12 anti-cancer drugs, 8 immunosuppressive drugs with antimitotic effects, 8 immunosuppressive drugs without antimitotic effects, 3 JAK inhibitors, and 15 non-immunosuppressive drugs; and 37 non-pharmaceutical chemicals (23 immunosuppressive and 14 non-immunosuppressive). Eight of the 23 immunosuppressive chemicals have antimitotic properties. The exact names of pharmaceutical and non-pharmaceutical chemicals and their evaluation by IL-2 Luc and IL-2 Luc LTT assays were given in previous publications (Kimura et al., 2021 and Kimura et al., 2023). The result demonstrated that the performance of the IL-2 Luc LTT assay alone for immunosuppressive effects was 35.8 % (19/53) for sensitivity, 88.0 % (22/25) for specificity, and 52.6 % (41/78) for predictivity. (Kimura et al., 2023).

9. Similar to the IL-2 Luc LTT assay, the performance of the IL-2 Luc assay alone for immunosuppressive effects (only the decrease of Il-2 production) was 63.0 % (34/54) for sensitivity, 58.6 % (17/29) for specificity, and 61.4 % (51/83) for predictivity. On the other hand, by the binary combination, in which chemicals were judged as immunosuppressive when either one of the assays judged as immunosuppressive, the performance of the combined assay for immunosuppressive effects was 88.7 % (47/53) for sensitivity, 53.8 % (14/26) for specificity, and 77.2 % (61/79) for predictivity. After considering the applicability domain, the performance of the combined IL-2 Luc and IL-2 Luc LTT assays was 90.0 % (45/50) for sensitivity, 53.8 % (14/26) for specificity, and 77.6% (59/76) for predictivity (Kimura et al., 2023). While the predictive capacity was not satisfactory for a stand-alone method, the IL-2 Luc LTT assay is acceptable for use in combination with other assays (OECD, 2024a) (OECD, 2024b).

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10. The purpose of this Test Guideline is to describe the procedures for evaluating the potential immunotoxicity of chemicals that may lead to increased susceptibility to infection or cancer. The combination of the IL-2 Luc assay and the IL-2 Luc LTT assay is a stepwise approach for evaluating the potential immunotoxicity of chemicals. In other words, chemicals that are assessed for immunosuppressive effects are tested using both the IL-2 Luc assay and the IL-2 Luc LTT assay. If a chemical is determined to have immunosuppressive effects in either assay, it is identified as an immunosuppressive chemical *in vitro*.

Specific limitations

11. The following limitations should be noted:

1) because PMA/Io stimulates bypasses of the intracellular signalling events from T-cell receptor stimulation to phospholipase C activation, either the IL-2 Luc assay or the IL-2 Luc LTT assay alone cannot evaluate the effects of chemicals on molecules involved in these signals. (Ohtsuka et al., 1996). (Ohtsuka et al., 1996) (Kimura et al., 2014; Kimura et al., 2020);

2) although the Jurkat T cell line (from which 2H4 cells are derived) are demonstrated to be suitable for examining the molecular mechanism underlying immunotoxicity (Shao et al., 2013), they may lack several key proteins involved in the activation of normal T cells in response to TCR stimulation, and therefore may not be able to detect effects of chemicals that act on those key proteins;

3) 2H4 cells are not supposed to express the enzymes required to metabolize and activate chemicals and thus chemicals that need metabolic activation are out of the applicability domain;

4) both assays share limitations common to many suspension cell-based techniques when testing highly hydrophobic substances;

5) test chemicals that interfere with luciferase can confound their activity/measurement, causing apparent inhibition or increased luminescence (Thorne et al. 2010);

6) the immunosuppressive effect of chemicals with LOELs of 200 µg/L or higher cannot be identified in either the IL-2 Luc assay or the IL-2 Luc LTT to the TG.

12. Definitions are provided in Appendix I.

PRINCIPLE OF THE TEST

13. This Test Guideline describes two consecutive assays, using the same test system except for the exposure time of chemicals, the seeding cell density and the prediction models, that generate data interpreted separately and the resulting predictions are used in combination.

14. The IL-2 Luc assay and the IL-2 Luc LTT assay make use of a human acute T lymphoblastic cell line Jurkat. Using this cell line, Department of Dermatology, Tohoku University School of Medicine and Tsuruga Institute of Biotechnology, TOYOBO Co., Ltd, established a Jurkat-derived IL-2 reporter cell line, 2H4, that harbors SLG, SLO and SLR luciferase genes under the control of the IL-2, IFN-γ, and GAPDH promoters, respectively (Saito et al., 2011). Laboratories willing to perform the test can obtain the recombinant 2H4 cell line from GPC Lab. Co. Ltd., Tottori, Japan, upon signing a Material Transfer Agreement (MTA). This cell line allows quantitative measurement of luciferase gene induction by detecting luminescence from well-established light producing luciferase substrates as indicators of the promoter activity of IL-2, IFN-γ and GAPDH genes in cells following exposure to immunotoxic chemicals.

15. Mainly the IL-2 promoter driven luciferase activity (IL2LA) and GAPDH promoter driven luciferase activity have been used based on the following observations described in the literature (Kimura et al., 2018). Most of chemicals examined by 2H4 cells showed similar suppressive effects on IL2LA and IFN promoter-

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driven luciferase activity (IFNLA). In addition, when the lowest observed effect level (LOELs) of these chemicals was plotted against their effects on IL-2LA and those on IFNLA, they showed a significant correlation between them. Therefore, a decision was made to only use IL2LA.

16. The multicolour assay system (Nakajima et al. 2005, Nakajima et al. 2010) comprises a green-emitting luciferase (SLG; λmax = 550 nm) (Ohmiya et al. 2000) for the gene expression of the IL-2 promoter, an orange-emitting luciferase (SLO; λmax = 580 nm) (Viviani et al., 2001) for the gene expression of the IFN-γ, promoter as well as a red-emitting luciferase (SLR; λmax = 630 nm) (Viviani, et al., 1999) for the gene expression of the internal control promoter, GAPDH. The three 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 two optical filters (Nakajima et al., 2005) (see Appendix II). For accurate luminescence measurements, a highly sensitive luminescence meter (e.g. a luminescence meter dedicated to luminescence measurements as described in Appendix II) should be used.

IL-2 Luc assay

17. 2H4 cells are treated for 1 hour with the test chemical and then treated for 6 hours with PMA and Io after which SLG luciferase activity (SLG-LA) reflecting IL-2 promoter activity, SLO luciferase activity (SLO-LA) reflecting IFN-γ promoter activity and SLR luciferase activity (SLR-LA) reflecting GAPDH promoter activity are measured. To make the abbreviations easier to understand, SLG-LA, SLO-LA and SLR-LA are designated as IL2LA, IFNLA and GAPLA, respectively. Table 1 provides a description of the terms associated with luciferase activity in the IL-2 Luc assay. The measured values are used to calculate the normalised IL2LA (nIL2LA) and IFNLA (nIFNLA), which is the ratio of IL2LA and IFNLA to GAPLA, respectively, and the inhibition of GAPLA (Inh-GAPLA), which is the ratio of the arithmetic means of quadruple-measured values of the GAPLA of 2H4 cells treated with a test chemical and the values of the GAPLA of untreated 2H4 cells, and used as an indicator for cytotoxicity. The % suppression, calculated as shown in the table, indicates the effect of tested chemicals on IL-2 and IFN-γ promoter.

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Table 1. Description of terms associated with the luciferase activity in the IL-2 Luc assay

Abbreviations	Definition
IL2LA	SLG luciferase activity reflecting IL-2 promoter activity
IFNLA	SLO luciferase activity reflecting IFN-γ promoter activity
GAPLA	SLR luciferase activity reflecting GAPDH promoter activity
nIL2LA	IL2LA / GAPLA
nIFNLA	IFNLA / GAPLA
Inh-GAPLA	GAPLA of 2H4 treated with chemicals / GAPLA of untreated cells
% suppression	(1-(nIL2LA of 2H4 treated with chemicals) / (nIL2LA of non-treated 2H4)) x 100
CV05	The lowest concentration of the test chemical at which Inh-GAPLA becomes <0.05.
Min Inh-GAPLA	Minimum value of the Inh-GAPLA of each experiment

18. The IL-2 Luc assay can simultaneously examine GAPLA and IL2LA. GAPDH mRNA is ubiquitously expressed at moderately abundant levels. It is frequently used as an endogenous control for quantitative real time polymerase chain reaction in several experimental systems because its expression is constant at different times and after experimental manipulation (Edwards and Denhardt, 1985; Mori et al., 2008; Winer et al., 1999). In addition, the lead laboratory demonstrated that Inh-GAPLA is more sensitive in detecting dying cells than the percentage of propidium iodide (PI)-excluding cells and that cells showing Inh-GAPLA $\geq$ 0.05 maintain more than 75% of the PI-excluding cells. (Kimura et al., 2015). Therefore, the results were evaluated using only data obtained in the concentration at which Inh-GAPLA is $\geq$ 0.05.

IL-2 Luc LTT assay

19. The 2H4 cells are treated for 24 hour with the test chemical, and treated for 6 hours with PMA and Io after which IL2LA, IFNLA and GAPLA are measured. The terms used in the IL-2 Luc LTT assay are the same as those used in the IL-2 Luc assay, as shown in Table 1.

20. Both the IL-2 Luc assay and the IL-2 Luc LTT assay can measure GAPLA, which correlates with GAPDH mRNA expression. GAPDH mRNA is ubiquitously expressed at moderately abundant levels. It is frequently used as an endogenous control for quantitative real time polymerase chain reaction in some experimental systems, because its expression is constant at different times and after experimental manipulation (Edwards and Denhardt, 1985; Mori et al., 2008; Winer et al., 1999). It has been demonstrated that Inh-GAPLA is a good marker for cell viability, with a strong correlation with propidium iodide (PI)-excluding cells, and that Inh-GAPLA below 0.8 indicates cytotoxicity of the test chemical (Kimura et al., 2021). In the IL-2 Luc assay, because the incubation period with the chemical substance is only 1 hour, there is no significant change in cell count, so GAPLA becomes a marker for cell viability. In the IL-2 Luc LTT assay, 2H4 cells are treated with chemicals for 24 hours. This is slightly longer than the doubling time of Jurkat cells (20.7 ± 2.2 hours) (Schoene et al., 1999). Since GAPLA in 2H4 cells shows a good correlation with the number of viable cells (Kimura et al., 2015; Takahashi et al., 2011), GAPLA in the IL-2 Luc LTT assay reflects changes in cell number.

DEMONSTRATION OF PROFICIENCY

21. Prior to routine use of the test methods described in this Test Guideline, laboratories should demonstrate technical proficiency, using Proficiency Substances listed in Appendix III (Table 1 for the IL-2 Luc assay and Table 2 for the IL-2 Luc LTT assay) in compliance with the Good in vitro Method Practices (OECD, 2018). Moreover, test method users should maintain a historical database of data generated with the reactivity

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checks and with the positive and solvent/vehicle controls, and use these data to confirm that the reproducibility of the test method in their laboratory is maintained over time.

PROCEDURE

22. The Standard Operating Procedures (SOP) for the IL-2 Luc assay and the IL-2 Luc LTT assay are available and should be employed when performing the test (JaCVAM, 2020c) (JaCVAM, 2024b). The following paragraphs provide a description of the main components and procedures of the assays.

IL-2 Luc assay

Preparation of cells

23. On receipt, 2H4 cells are propagated (2-4 passages) and stored frozen as a homogeneous stock. Cells from this stock can be propagated up to a maximum of 12 passages or a maximum of 6 weeks. The medium used for propagation is the RPMI-1640 culture medium containing 10% foetal bovine serum (FBS), antibiotic/antimycotic solution (100 U/mL of penicillin G, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B in 0.85% saline) (e.g. GIBCO Cat#15240-062), 0.15 µg/mL Puromycin (e.g. CAS:58-58-2), 300 µg/mL G418 (e.g. CAS:108321-42-2) and 200 µg/mL hygromycin B (e.g. CAS:31282-04-9).

24. Prior to use for testing, the cells should be qualified by conducting a reactivity check. This check should be performed 1-2 weeks or 2-4 passages after thawing, using the positive controls, dexamethasone (100 µg/mL) (CAS:50-02-2, ≥ 98% purity) and cyclosporine A (100 ng/mL) (CAS:59865-13-3, ≥ 95% purity). Dexamethasone and cyclosporine A should produce a positive response to % suppression (≥35). Only cells that pass the reactivity check are used for the assay.

25. For testing, 2H4 cells are seeded at a density of 1 to 3 × 10^5 cells/mL, and pre-cultured in culture flasks for 72 to 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 4 × 10^6 cells/mL. Then, cells are distributed into a 96-well flat-bottom black plate (e.g. Corning Costar Cat#3603) with 50 µL (2 × 10^5 cells/well).

Preparation of the test chemical and control substances

26. The test chemical and control substances are prepared on the day of testing. For the IL-2 Luc assay, the test chemical is dissolved in distilled water or dimethyl sulfoxide (DMSO) (stock solution). The test chemical is first dissolved in distilled water.

- If the chemical is soluble at 10 mg/mL, 10 mg/mL is the concentration of the stock solution.
- If the test chemical is not soluble at 10 mg/ml in distilled water, the test chemical is dissolved in DMSO at 200 mg/mL. If the test chemical is not soluble at 200 mg/mL, the highest concentration is determined as the maximum dissolved concentration after dilution with DMSO at a dilution factor of 2 (see scheme in Appendix IV).
- If the chemical is not soluble in distilled water, prepare the highest concentration possible in DMSO. Sonication and vortex may be used if needed. Centrifuge at 20.000x g for 5 minutes and confirm that it is soluble by the absence of precipitates. The test chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

27. The first test run is aimed at determining the cytotoxic concentration and examining the immunotoxic potential of chemicals. Serial dilutions of distilled water or DMSO stock solutions of the test chemicals are made at a dilution factor of 2 (see Appendix IV) using a 96-well round-bottom clear plate. When the chemical is

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prepared in distilled water, dilute 20 µL of the diluted stock solution further with 480 µL of medium in a 96-well assay block (e.g. Corning Costar Cat#3960) and add 50 µL of the diluted solution to 50 µL of the cell suspension in a 96-well flat-bottom black plate. When the chemical is prepared in DMSO, dilute 10 µL of the diluted stock solution with 90 µL of the medium in a 96-well round-bottom clear plate, then, dilute 10 µL of the diluted solution with 490 µL of the medium in a 96-well assay block and then, add 50 µL of the diluted solution to 50 µL of the cell suspension in a 96-well flat-bottom black plate.

28. Thus, when the chemical is prepared as 10 mg/mL distilled water solution, the final concentrations of the test chemicals range from 0.00039 to 0.2 mg/mL, and when the chemical is prepared as 200 mg/mL DMSO solution, the final concentrations of the test chemicals range from 0.0039 to 0.2 mg/mL (Appendix IV).

29. In subsequent test runs (i.e., the second and third run), the distilled water stock solution or the DMSO stock solution is made at the concentration 100 times or 2000 times higher than the concentration of cell viability 05 (CV05; the lowest concentration at which the Inh-GAPLA becomes <0.05) in the first run, respectively (see Appendix IV, Fig. 4). If Inh-GAPLA does not decrease below 0.05 at the any concentration in the first run, the concentration of the stock solution in subsequent test runs is same as that of the first run. In addition, if the chemical gives Inh-GAPLA < 0.7 at the lowest concentration, the distilled water stock solution or the DMSO stock solution is made at the concentration 200 times or 4000 times higher than the lowest concentration of the first run, respectively.

30. Each concentration of test chemical should be tested in 4 wells. The samples are then mixed on a plate shaker and incubated for 1 hour at 37±0.5ºC and 5±0.5% CO_2.

31. After 1-hour incubation with the test chemical, the cells are stimulated with 25 nM PMA and 1 µM Io for 6 hours. For example, x10 PMA/ionomycin solution is made by diluting 2 mM PMA DMSO solution and 2 mM ionomycin ethanol solution using the medium, and 10 µL of the x10 solution PMA/ionomycin is added to 90 µL of the cell suspension containing the test chemical. Subsequently, the luciferase activity is measured as described in following paragraph 36-39.

32. The recommended positive controls are dexamethasone and cyclosporine A. For example, add 10 µL of 100 mg/mL dexamethasone DMSO solution or 10 µL of 100 µg/mL cyclosporine A DMSO solution to 90 µL of the medium in a 96-well round-bottom clear plate, dilute 10 µL of the diluted solution with 490 µL of the medium in a 96-well assay block and then, add 50 µL of the diluted solution to 50 µL of the cell suspension in a 96-well flat-bottom black plate. The final concentrations of dexamethasone and cyclosporine A are 100 µg/mL and 100 ng/mL, respectively. Each concentration of the positive control should be tested in 4 wells. The samples are then mixed on a plate shaker and incubated for 1 hour at 37°C and 5% CO_2. The cells are stimulated with 25 nM PMA and 1 µM Io for 6 hours and subsequently the luciferase activity is measured as described in paragraph 36-39.

33. The solvent and negative control is RPMI-1640 containing 10% FBS containing 2% of distilled water or 1% DMSO. Other suitable positive or negative controls may be used if historical data are available to derive comparable run acceptance criteria.

34. 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.

35. The test chemicals and solvent control require 2 to 3 runs to derive data evaluation and a prediction model (see paragraph 60). Each run is performed on a different day or on the same day provided that for each run fresh stock solution of test chemicals and independently harvested cells. Cells may come from the same passage.

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Luciferase activity measurements

36. 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) or the ARVO series (PerkinElmer, Waltham, MA, USA). Examples of the optical filters are sharp-cut (long-pass or short-pass) filters or band-pass filters. The luminometer can be qualified to ensure reproducibility by light emitting diode (LED) reference light source (Yasunaga et al. 2017).

37. Prior to testing, the transmission coefficients of the filters to discriminate each bioluminescence signal colour should be determined using recombinant green, orange and red emitting luciferases (Niwa et al. 2010), per Appendix II.

38. One hundred µL 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 and with or without PMA/Io. The plate is shaken for 10 min at an ambient temperature of about 20°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, F2) of the optical filters. Justification should be provided for the use of alternative settings, e.g. depending on the model of luminometer used.

39. Parameters for each concentration are calculated from the measured values, e.g., IL2LA, GAPLA, nIL2LA, Inh-GAPLA, the mean ±SD of IL2LA, the mean ±SD of GAPLA, the mean ±SD of nIL2LA, the mean ±SD of Inh-GAPLA, and % suppression using the excel-based spreadsheet available for the IL-2 Luc assay (see https://www.oecd.org/en/topics/sub-issues/testing-of-chemicals/software-test-guidelines.html). Definitions and calculations of the parameters used in this paragraph, i.e. luciferase activity and suppression index are provided in Appendices I and V, respectively.

IL-2 Luc LTT assay

Preparation of cells

40. The 2H4 cell line should be used for performing the IL-2 Luc LTT assay. On receipt, 2H4 cells are propagated (2-4 passages) and stored frozen as a homogeneous stock. Cells from this stock can be propagated up to a maximum of 12 passages or a maximum of 6 weeks. The medium used for propagation is the RPMI-1640 culture medium 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), 300µg/mL G418 (e.g. CAS:108321-42-2) and 200 µg/mL hygromycin B (e.g. CAS:31282-04-9).

41. Prior to use for testing, the cells should be qualified by conducting a reactivity check. This check should be performed 1-2 weeks or 2-4 passages after thawing, using the positive controls, bleomycin sulfate (CAS:9041-93-4) and mizoribine (CAS:50924-49-7) and the negative control, cyclosporine A (CAS:59865-13-3). Bleomycin sulfate and mizoribine should produce a positive response and cyclosporine should produce a negative response based on the criteria of the IL-2 Luc LTT assay to judge positive (antimitotic) or negative (non-antimitotic). Only cells that pass the reactivity check are used for the assay.

42. For testing, 2H4 cells are seeded at a density of 1 to 3 × 10^5 cells/mL, and pre-cultured in culture flasks for 72 to 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 2 × 10^5 cells/mL. Then, cells are distributed into a 96-well flat-bottom black plate (e.g. Greiner Bio-one Cat#655090) with 50µL (1 × 10^4 cells/well).

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Preparation of the test chemical and control substances

43. The test chemical and control substances are prepared on the day of testing. For the IL-2 Luc LTT assay, the test chemical is dissolved in distilled water or dimethyl sulfoxide (DMSO) (stock solution). The test chemical is first dissolved in distilled water.

- If the chemical is soluble at 10 mg/mL, 10 mg/mL is the concentration of the stock solution.
- If the test chemical is not soluble at 10 mg/ml in distilled water, the test chemical is dissolved in DMSO at 200 mg/mL. If the test chemical is not soluble at 200 mg/mL, the highest concentration is determined as the maximum dissolved concentration after dilution with DMSO at a dilution factor of 2 (see scheme in Appendix IV).
- If the chemical is not soluble in distilled water, prepare the highest concentration possible in DMSO. Sonication and vortex may be used if needed. Centrifuge at 15,000 rpm (≈20.000x g) for 5 minutes and confirm that it is soluble by the absence of precipitates. The test chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

44. The first test run is aimed at determining the cytotoxic concentration and examining the immunotoxic potential of chemicals. Serial dilutions of distilled water or DMSO stock solutions of the test chemicals are made at a dilution factor of 2 (see Appendix IV) using a 96-well round-bottom clear plate.

- When the chemical is prepared in distilled water, dilute 20 µL of the diluted stock solution further with 480 µL of medium in a 96-well assay block (e.g. Coring Costar Cat#3960) and add 50 µL of the diluted solution to 50 µL of the cell suspension in a 96-well flat-bottom black plate.
- When the chemical is prepared in DMSO, dilute 10 µL of the diluted stock solution with 90 µL of the medium in a 96-well round-bottom clear plate, then, dilute 10 µL of the diluted solution with 490 µL of the medium in a 96-well assay block and then , add 50 µL of the diluted solution to 50 µL of the cell suspension in 96-well flat-bottom black plate.

45. Thus, when the chemical is prepared as 10 mg/mL distilled water solution, the final concentrations of the test chemicals range from 0.00039 to 0.2 mg/mL, and when the chemical is prepared as 200 mg/mL DMSO solution, the final concentrations of the test chemicals range from 0.00039 to 0.2 mg/mL (Appendix IV).

46. In subsequent test runs (i.e., the second and third run), the distilled water stock solution or the DMSO stock solution is made at the concentration 100 times or 2000 times higher than the concentration of cell viability 05 (CV05; the lowest concentration at which the Inh-GAPLA becomes <0.05) in the first run, respectively (Appendix IV, Fig. 4). If Inh-GAPLA does not decrease below 0.05 at the any concentration in the first run, the concentration of the stock solution in subsequent test runs is the same as that of the first run.

In addition, if the chemical gives Inh-GAPLA < 0.7 at the lowest concentration, the distilled water stock solution or the DMSO stock solution is made at the concentration 200 times or 4000 times higher than the lowest concentration of the first run, respectively.

47. Each concentration of each test chemical should be tested in 4 wells. The samples are then mixed on a plate shaker and incubated for 24 hours at 37±0.5ºC and 5±0.5% CO_2.

48. After 24-hour incubation with the test chemical, the cells are stimulated with 25 nM PMA and 1 µM Io for 6 hours. For example, x10 PMA/ionomycin solution is made by diluting 2 mM PMA DMSO solution and 2 mM ionomycin ethanol solution using the medium, and 10 µL of the x10 solution PMA/ionomycin is added to 90 µL of the cell suspension containing the test chemical. Subsequently, the luciferase activity is measured as described in paragraph 53-56.

49. The recommended positive controls are bleomycin. Prepare 10 mg/mL distilled water solution as the stock solution. The recommended negative controls are dexamethasone. Prepare 100 mg/mL DMSO solution as the stock solution. Then this stock solution is diluted as described in paragraph 44. The samples are then

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mixed on a plate shaker and incubated for 24 hour at 37±0.5ºC and 5±0.5% CO_2. The cells are stimulated with 25 nM PMA and 1 µM Io for 6 hours and subsequently the luciferase activity is measured as described in paragraph 53-56.

50. The solvent and negative control is RPMI-1640 containing 10% FBS. Other suitable positive or negative controls may be used if historical data are available to derive comparable acceptance criteria.

51. 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.

52. The test chemicals and solvent control require 2 to 3 runs to derive a positive (antimitotic) or negative (non antimitotic) prediction. Each run is performed on a different day with fresh stock solution of test chemicals and independently harvested cells. Cells may come from the same passage.

Luciferase activity measurements

53. 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) or the ARVO series (PerkinElmer, Waltham, MA, USA). Examples of the optical filters are sharp-cut (long-pass or short-pass) filters or band-pass filters. The luminometer can be qualified to ensure reproducibility by light emitting diode (LED) reference light source (Yasunaga, Nakajima and Ohmiya, 2014).

54. Prior to testing, the transmission coefficients of the filters to discriminate each bioluminescence signal color should be determined using recombinant green, orange and red emitting luciferases as described in Appendix II (Niwa et al., 2010).

55. One hundred µL 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 and with or without PMA/Io. The plate is shaken for 10 min at an ambient temperature of about 20°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, F2) of the optical filters. Justification should be provided for the use of alternative settings, e.g. depending on the model of luminometer used.

56. Parameters for each concentration are calculated from the measured values, e.g., IL2LA, GAPLA, nIL2LA, Inh-GAPLA, the mean ±SD of IL2LA, the mean ±SD of GAPLA, the mean ±SD of nIL2LA, the mean ±SD of Inh-GAPLA, and % suppression using the excel-based spreadsheet available for the IL-2 Luc LTT assay (see https://www.oecd.org/en/topics/sub-issues/testing-of-chemicals/software-test-guidelines.html). Definitions and calculations of the parameters used in this paragraph, i.e. luciferase activity and suppression index are provided in Appendices I and V, respectively.

DATA AND REPORTING

Data evaluation

57. IL-2 Luc assay: criteria to determine immune suppression in the IL-2 Luc assay. In each run, the test chemical is judged positive (immune-suppressive) when all three following criteria are fulfilled:

1. The mean of % suppression is ≥ (equal to or more) than 35 (immune-suppressive) and statistically significant. The statistical significance is judged by its 95% confidence interval.
2. The outcome shows two or more consecutive statistically significant results (increase or decrease); alternatively one statistically significant result (increase or decrease) with the same trend for at least 3 consecutive data points (i.e. dose dependent trend); in this case the trend can cross the zero line,

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but the data point on the other side of the 0 line does not become statistically significant for the opposite effect. (A figure illustrating criteria 2 is available in Fig.5 of Appendix V.)

3. The results are judged using only data obtained in the concentration range at which Inh-GAPLA is ≥ 0.05.

In all other cases the test chemical is judged as negative (not active).

58. IL-2 Luc LTT assay: In each run, the test chemical is judged positive (i.e. antimitotic) when all three following criteria are fulfilled in addition to giving Min Inh-GAPLA less than 0.7.

a. The mean of % suppression is ≤ (equal to or less) than -35 (stimulatory or antimitotic) with statistical significance. The statistically significant is judged when the upper limit of 95% confidence interval of % suppression is under 0.

b. The outcome shows two or more consecutive statistically significant stimulatory results; alternatively one statistically significant stimulatory result with the same trend for at least 3 consecutive data points (i.e. dose dependent trend); in this case the trend can cross the zero line, but the data point on the other side of the 0 line does not become statistically significant for the opposite effect. The statistically significant is judged when the upper limit of 95% confidence interval of % suppression is under 0. (A figure illustrating criteria 2 is available in Fig. 6 of Appendix V.)

c. The results are judged using only data obtained in the concentration range at which Inh-GAPLA is equal to or more than 0.05.

In all other cases the test chemical is judged as negative (i.e. not antimitotic).

59. If a test chemical produces a negative result and is insoluble at 10 mg/mL in distilled water, the test result is considered inconclusive because it cannot dissolve in the vehicle at the concentration sufficient to show potential effects in the culture medium (a figure illustrating criteria 2 is available in Fig.7 of Appendix V.)

Prediction model

60. The runs are repeated until two consistent positive (suppressive) runs are obtained. A maximum of three runs is possible. Identification of immunotoxicants is evaluated by the mean inhibition rate and its 95% simultaneous confidence interval in the IL-2 Luc assay, and by MinInh-GAPLA in addition to these in the IL-2 Luc LTT assay. Chemicals that are assessed for immunosuppressive effects are tested using both the IL-2 Luc assay and the IL-2 Luc LTT assay. If a chemical is determined to have suppressive effects in the IL-2 Luc assay or antimitotic effects in the IL-2 Luc LTT assay, it is identified as an immunosuppressive chemical in vitro.

Acceptance criteria

61. The following acceptance criterion applies for these two assays: For each set of experiments, a control experiment examining nIFNLA of 2H4 cells treated with PMA/Io and nIFNLA of non-treated 2H4 cells must be conducted. If the fold induction of nIFNLA of 2H4 cells treated with PMA/Ionomycin compared to nIFNLA of non-treated 2H4 cells is less than 3.0, the results obtained from these experiments should be rejected.

TEST REPORT

62. The test report should include the following information for each of the assays:

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Test Chemical

- 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.
  - Treatment prior to testing, if applicable (e.g., warming, grinding);
  - Concentration(s) tested.
  - Storage conditions and stability to the extent available;
  - Justification for choice of solvent/vehicle for each test chemical if distilled water or DMSO has not been used.

- Multi-constituent substance, UVCB and mixture:
  - Characterisation as far as possible by e.g., 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 for the conduct of the study;
  - Treatment prior to testing, if applicable (e.g., warming, grinding);
  - Concentration(s) tested.
  - Storage conditions and stability to the extent available.
  - Justification for choice of solvent/vehicle for each test chemical, if distilled water or DMSO has not been used.

Controls in each of the assays

- 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);
  - Justification for choice of solvent vehicle for each test chemical (if distilled water or DMSO has not been used).
  - 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:
  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), and/or other

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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.

Test method conditions for each of the assays

- 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 FBS, supplier name, lot number of 96-well flat-bottom black 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 used (e.g., model), including instrument settings, luciferase substrate used, and demonstration of appropriate luminescence measurements based on the control test described in Appendix 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 for each of the assays

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

Results for each of the assays

- Measurements of IL2LA, IFNLA and GAPLA.
- Calculations for nIL2LA, nIFNLA, Inh-GAPLA and % suppression;
- The 95% confidence interval of % suppression.
- 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-2 Luc assay and the Il-2 Luc assay separately.
- Consideration of the assay results in combination.

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Any modification to the Test Guideline

Conclusion

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APPENDIX I- DEFINITIONS

2H4: An IL-2 reporter cell line used in IL-2 Luc assay. The human acute T lymphoblastic leukaemia cell line Jurkat was transfected the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFN-γ and GAPDH promoters, respectively.

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.

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.

CV05: Cell viability 05. Minimum concentration at which chemicals show less than 0.05 of Inh-GAPLA.

GAPLA: Luciferase activity of stable luciferase red (SLR) (λmax = 630 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.

IFNLA: Luciferase activity of stable luciferase orange (SLO) (λmax = 580 nm), regulated by interferon (IFN)-γ promoter.

Il-SLR-LA: Abbreviation used in the validation report and in previous publications regarding the refer to Inh-GAPLA. See Inh-GAPLA for definition.

IL-2 (Interleukin-2): A cytokine derived from T lymphocytes that causes activation of T lymphocytes, B lymphocytes, monocyte and natural killer cells.

IL2LA: Luciferase activity of stable luciferase green (SLG) (λmax = 550 nm), regulated by IL-2 promoter.

Inh-GAPLA: Inhibition of GAPLA. It is obtained by dividing GAPLA of 2H4 treated with chemicals with GAPLA of non-treated 2H4 and represents cytotoxicity of chemicals.

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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 ≥ 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.

nIL2LA: The SLG luciferase activity reflecting IL-2 promoter activity (IL2LA) normalised by the SLR luciferase activity reflecting GAPDH promoter activity (GALPA). It represents IL-2 promoter activity after considering cell viability or cell number.

nSLG-LA: Abbreviation used in previous publications regarding the IL-2 Luc assay to refer to nIL2LA. See nIL2LA for definition.

nSLO-LA: Abbreviation used in previous publications regarding the IL-2 Luc assay to refer to nIFNLA. See nIFNLA for definition.

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

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.

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.

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.

SLG-LA: Abbreviation used in previous publications regarding the IL-2 Luc assay to refer to IL2LA. See IL2LA for definition.

SLO-LA: Abbreviation used in previous publications regarding the IL-2 Luc assay to refer to IFNLA. See IFNLA for definition.

SLR-LA: Abbreviation used in previous publications regarding the IL-2 Luc assay to refer to GAPLA. See

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GAPLA for definition.

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 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.

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, inducing 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 it composition.

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

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.

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APPENDIX II - PRINCIPLE OF MEASUREMENT OF LUCIFERASE ACTIVITY AND DETERMINATION OF THE TRANSMISSION COEFFICIENTS OF OPTICAL FILTER FOR SLG, SLO AND SLR

Multi Reporter Assay System -Tripluc- can be used with a microplate-type luminometer with a multi-colour detection system, which can equip at least two kinds of optical filters (e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)). Examples of the optical filters used in measurement are a 560 nm long-pass filter and a 600 nm long-pass filter.

(1) Measurement of three-color luciferase with two optical filters.

This is an example using Phelios AB-2350 (ATTO). This luminometer equips a 560 nm long-pass (LP) filter (560 nm LP, Filter 1) and a 600 nm long pass filter (600 nm LP, Filter 2) for optical isolation.

First, using recombinant luciferase enzyme of SLG (λmax = 550 nm), SLO (λmax = 580 nm) and SLR (λmax = 630 nm), measure i) the intensity of light without filter (all optical), ii) the intensity of 560 nm LP (Filter 1) transmitted light iii) the intensity of 600 nm LP (Filter 2) transmitted light, and calculate the transmission coefficient factor listed below.

Table 1. Definition of the parameters in the luciferase assay

Transmission coefficient factor		Abbreviation	Definition
SLG	Filter 1 transmittance factor	κG_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLG / the intensity of SLG without filter (all optical)
	Filter 2 transmittance factor	κG_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLG / the intensity of SLG without filter (all optical)
SLO	Filter 1 transmittance factor	κO_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLO / the intensity of SLO without filter (all optical)
	Filter 2 transmittance factor	κO_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLO / the intensity of SLO without filter (all optical)
SLR	Filter 1 transmittance factor	κR_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLR / the intensity of SLR without filter (all optical)
	Filter 2 transmittance factor	κR_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLR / the intensity of SLR without filter (all optical)

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When the intensity of SLG, SLO and SLR in test sample are defined as G, O and R, respectively, i) the intensity of light without filter (all optical): F0, ii) the intensity of 560 nm LP (Filter 1) transmitted light and iii) the intensity of 600 nm LP (Filter 2) transmitted light are described as below.

$$F0=G+O+R$$

$$F1=\kappa G_{R56} \times G + \kappa O_{R56} \times O + \kappa R_{R56} \times R$$

$$F2=\kappa G_{R60} \times G + \kappa O_{R60} \times O + \kappa R_{R60} \times R$$

These formulas can be rephrased as follows.

$$\begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} G \\ O \\ R \end{pmatrix}$$

Then using calculated transmission coefficient factors and measured F0, F1 and F2, you can calculate G, O and R-value as follows.

$$\begin{pmatrix} G \\ O \\ R \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix}^{-1} \begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix}$$

Materials and methods for determining transmittance factor

(1) Reagents

· Single purified recombinant luciferase enzymes:

Lyophilised purified SLG enzyme

Lyophilised purified SLO enzyme

Lyophilised purified SLR enzyme

(which for the validation work were obtained from Biospecimen Laboratories, Inc.,(https://reporter-assay.com/) Japan with 2H4 cell line).

· Assay reagent:

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

· Medium: RPMI-1640 with 10% FBS for luciferase assay (30 ml, stored at 2 – 8°C)

(2) Preparation of enzyme solution

Dissolve lyophilised 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 stabilise. Transfer 100 µL of the diluted enzyme solution to a black 96 well plate (flat bottom) (the SLG reference sample to #B1, #B2, #B3, the SLO reference sample to #D1, #D2, #D3, the SLR reference sample to #F1, #F2, #F3). Then, transfer 100 µL of pre-warmed Tripluc to each well of the plate containing the diluted enzyme solution using a pipette. 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, F2) of the optical filter.

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Transmission coefficient of the optical filter was calculated as follows:

Transmission coefficient (SLG (κG_{R56})) = (#B1 of F1+ #B2 of F1+ #B3 of F1) / (#B1 of F0+ #B2 of F0+ #B3 of F0)

Transmission coefficient (SLO (κO_{R56})) = (#D1 of F1+ #D2 of F1+ #D3 of F1) / (#D1 of F0+ #D2 of F0+ #D3 of F0)

Transmission coefficient (SLR (κR_{R56})) = (#F1 of F1+ #F2 of F1+ #F3 of F1) / (#F1 of F0+ #F2 of F0+ #F3 of F0)

Transmission coefficient (SLG (κG_{R60})) = (#B1 of F2+ #B2 of F2+ #B3 of F2) / (#B1 of F0+ #B2 of F0+ #B3 of F0)

Transmission coefficient (SLO (κO_{R60})) = (#D1 of F2+ #D2 of F2+ #D3 of F2) / (#D1 of F0+ #D2 of F0+ #D3 of F0)

Transmission coefficient (SLR (κR_{R60})) = (#F1 of F2+ #F2 of F2+ #F3 of F2) / (#F1 of F0+ #F2 of F0+ #F3 of F0)

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

Quality control of equipment

The procedures described in the IL-2 Luc protocol should be used (JaCVAM, 2020c).

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APPENDIX III - PROFICIENCY SUBSTANCES

Table 1. Recommended substances for demonstrating technical proficiency with the IL-2_Luc assay

No.	Chemical name	CAS No.	T cell targeting	Physical state	Reference range (µg/mL) CV05[1]	Reference range (µg/mL) MIT[2]
1	Dexamethasone	50-02-2	Yes	Solid	>2000	16-63
2	Cyclosporine	59865-13-3	Yes	Solid	>1	0.002-0.006
3	Lead(II) acetate trihydrate	6080-56-4	Yes	Solid	>2000	31-63
4	Indomethacin	53-86-1	Yes	Solid	500-2000	16-63
5	Perfluorooctanoic acid	335-67-1	Yes	Solid	250-1000	8-31
6	Tributyltin chloride	1461-22-9	Yes	Liquid	0.5-1.0	0.12-0.24
7	Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	No	Solid	1-4	>2000
8	Mannitol	69-65-8	No	Solid	>2000	>2000
9	Acetonitril	75-05-8	No	Liquid	>2000	>2000

Abbreviations: CAS no. = Chemical Abstracts Service Registry Number

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

[2] MIT: the lowest concentrations at which a chemical satisfies the positive criteria.

Table 2. Recommended substances for demonstrating technical proficiency with the IL-2 Luc LTT assay

No.	Chemical name	CAS No.	T cell targeting	Antimitotic	Physical state	Reference range (µg/mL) CV051	Reference range (µg/mL) MIT2
1	Mycophenolic acid	24280-93-1	Yes	Yes	Solid	>200	0.05-2
2	Indomethacin	53-86-1	Yes	Yes	Solid	100->200	6-25
3	5-FU	51-21-8	Yes	Yes	Solid	>100	0.4-1.6
4	Colchicine	64-86-8	Yes	Yes	Solid	200->200	0.003-0.012
5	Perfluorooctanoic Acid	335-67-1	Yes	Yes	Solid	100-200	6.3-25

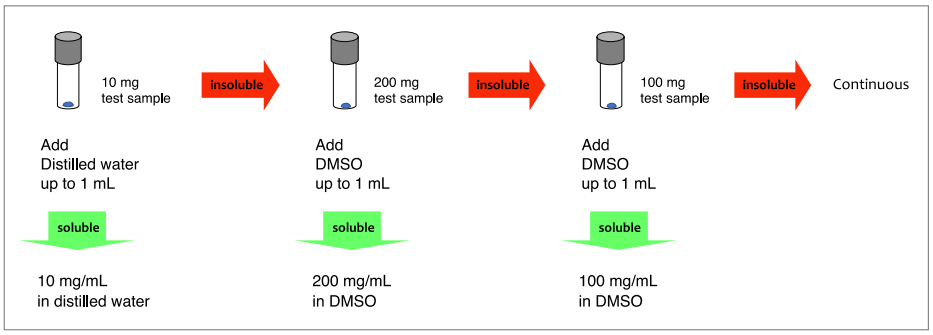
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6	Etoposide	33419-42-0	Yes	Yes	Solid	>200	0.4-1.6
7	Cyclosporin A	59865-13-3	Yes	No	Solid	>200	>200
8	Methyl carbamate	598-55-0	Yes	No	Solid	>200	>200
9	Nicotinamide (NA)	98-92-0	No	No	Solid	>200	>200
10	Dichloroacetic acid	79-43-6	No	No	Liquid	>200	>200

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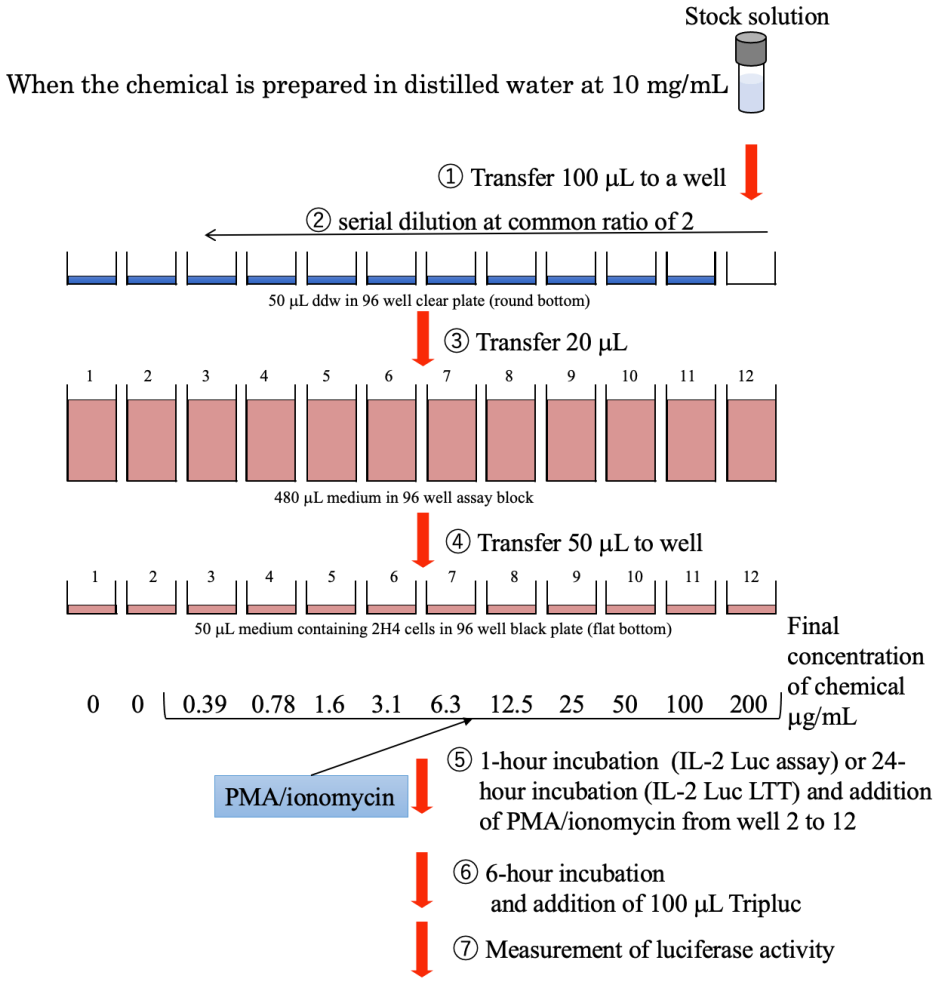
APPENDIX IV – TEST CHEMICALS DISSOLUTION IN ASSAYS.

Figure 1. Dissolution by vehicle



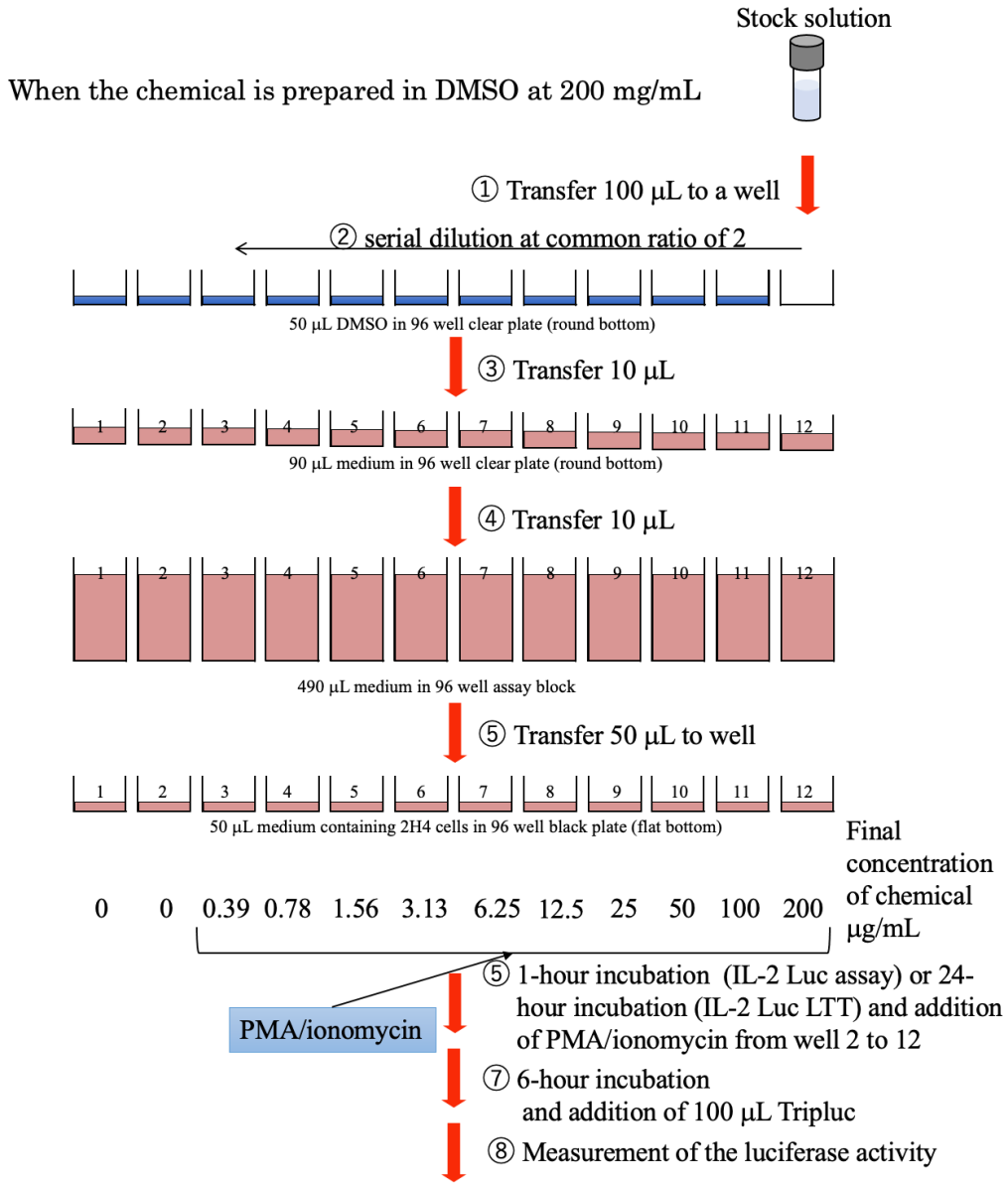
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Figure 2. The scheme of the procedure for the assays when the chemical is prepared in distilled water at 10 mg/mL.



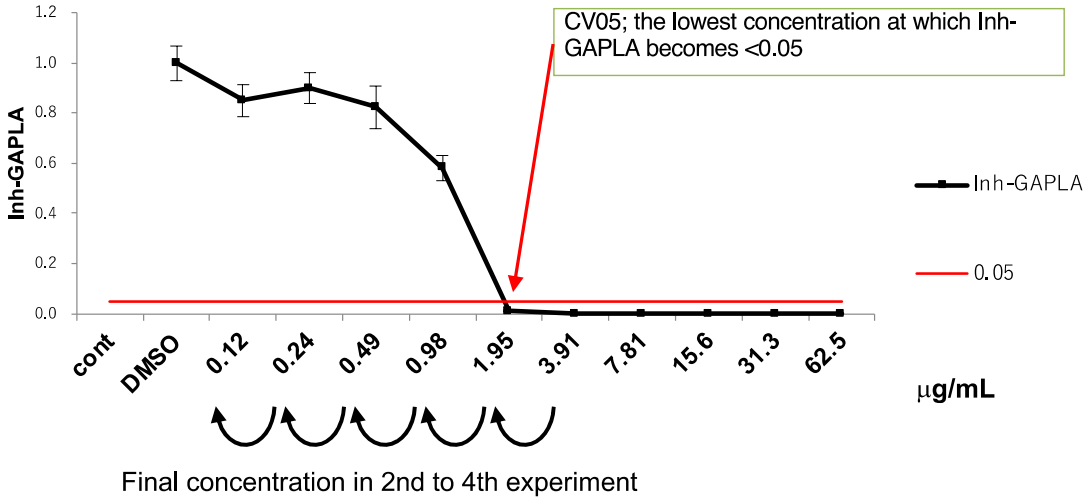
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Figure 3. The scheme of the procedure for the assays when the chemical is prepared in DMSO at 200 mg/mL.



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Figure 4. Determination of the concentration of the test chemicals in subsequent test runs (i.e., the second, and third run)



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APPENDIX V – CALCULATION OF THE SUPPRESSION INDEXES

IL2LA

The j-th repetition (j= 1 to 4) of the i-th concentration (i = 0 to 10) is measured for IL2LA and GAPLA respectively. The normalized IL2LA is referred as nIL2LA, and is defined as

$nIL2LA_{ij} = IL2LA_{ij} / GAPLA_{ij}$

This is the basic unit of measurement in this assay.

% suppression

The % suppression is an index for the averaged nIL2LA for the repetition on the i-th concentration compared with it on the 0 concentration, it is the primary measure of this assay. The % suppression is able to write by the following formula,

$$\%\ \text{suppression}_{\text{i}} = \left\{1 - \frac{\left(\frac{1}{4}\right)\sum_{\text{i}} \text{nIL2LA}_{\text{ij}}}{\left(\frac{1}{4}\right)\sum_{\text{i}} \text{nIL2LA}_{0\text{j}}}\right\} \times 100 \qquad (1)$$

The lead laboratory has proposed that ±35 of the value suggests suppressive and stimulatory for a tested chemical. This value is based on the investigation of the historical data of the lead laboratory. Data management team followed to use the value through all the phase of present validation study.

The primary outcome measure, % suppression, is basically the ratio of 2 arithmetic means of nIL2LA as shown in equation (1). The 95% confidence interval (95% CI) of the % suppression for the i-th concentration can be estimated.

The lower limit of the 95% CI above 0 is interpreted as that the nIL2LA with the i-th concentration is statistical-significantly greater than it with the 0-concentration, whereas the upper limit of the 95% CI blow 0 is interpreted as that the nIL2LA with the i-th concentration is statistical-significantly lesser than it with the 0-concentration.

There are several ways to construct the 95% CI. We used the method kwon as the Delta method in this study. This 95% confidence interval theorem is obtained from the following formula.

$$\%\text{suppression} \pm 100 \times \left\{z_{0.975} \times \sqrt{\frac{\text{sd}_{\text{i}}^2}{\text{mean}_0^2} + \frac{\text{mean}_{\text{i}}^2 \times \text{sd}_0^2}{\text{mean}_0^4}}\right\},$$

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where mean_i is the mean of nIL2LA at the i-th concentration, mean_0 is the mean of nIL2LA at 0 concentration, sd_i is the standard deviation of nIL2LA at the i-th concentration and sd_0 is the standard deviation of nIL2LA at 0 concentration. $z_{0.975}$ is 97.5 percentile of the standard normal distribution.

Inh-GAPLA

The Inh-GAPLA is a ratio of the averaged GAPLA for the repetition of the i-th concentration compared with it of the 0 concentration, and this is written by

$$\text{Inh-GAPLA}_i = \left\{(1/4)\times\sum \text{GAPLA}_{ij}\right\} / \left\{(1/4)\times\sum \text{GAPLA}_{0j}\right\}$$

Since the GAPLA is the denominator of the nIL2LA, the extremely smaller value of this is considered to cause the large variation of the nIL2LA. Therefore, the i-th %suppression value with extremely smaller value of the Inh-GAPLA might be poor precision.

Judgment for "Suppressive" or "No effect" in each experiment of the IL-2 Luc assay

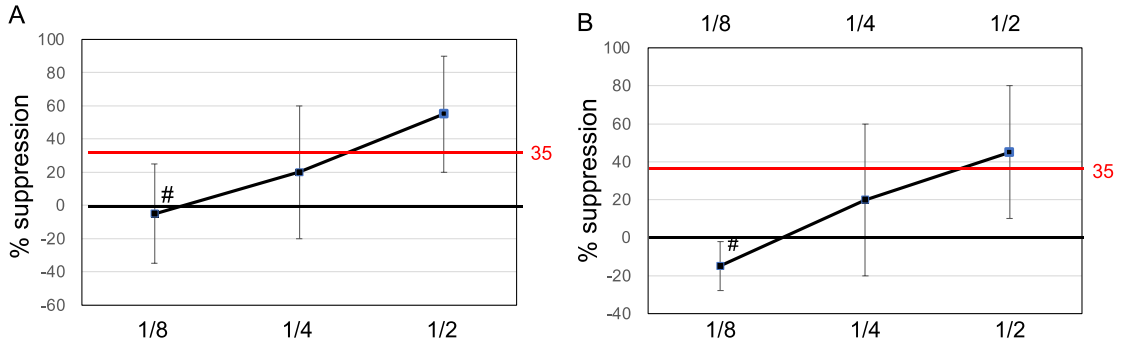
Criteria to judge a positive effect (either suppressive or stimulatory) are provided in the section "Data evaluation", paragraph 57, and illustrated through Figure 5 below, extracted from Appendix 17 of the validation report.

Paragraph 57 says that an experiment is judged positive when all three following criteria are fulfilled:

1. The mean of % suppression is ≥ 35 (suppressive) and statistically significant. The statistical significance is judged by its 95% confidence interval.
2. The outcome shows two or more consecutive statistically significant results (increase); alternatively one statistically significant result (increase) with the same trend for at least 3 consecutive data points (i.e. dose dependent trend); in this case the trend can cross the zero line, but the data point on the other side of the 0 line does not become statistically significant for the opposite effect.
3. The results are judged using only data obtained in the concentration range at which Inh-GAPLA is ≥ 0.05.

The following representative graphs are judged as positive (either suppressive or stimulatory).

Figure 5. Two patterns showing one positive data point (increase) with the same trend for at least 3 consecutive data points



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The x-axis represents the chemical concentration and the y-axis represents % suppression. Each plot is the % suppression value from quadruple assays with 95% confidence intervals. The red lines indicate 35. All graphs show one positive data point (increase or decrease) with the same trend for at least 3 consecutive data points (i.e. concentration dependent trend) and the trends cross the zero line. A is judged as suppression, because the 95% confidence interval of the data point indicating the opposite response (#) crosses 0. In contrast, B is judged no effect because the 95% confidence interval of the data point indicating the opposite response (#) does not cross 0.

Judgment for "antimitotic", "indeterminate" or "non-antimitotic" in each experiment of the IL-2 Luc LTT assay

In each run, the test chemical is judged antimitotic when all three following criteria are fulfilled in addition to giving Min Inh-GAPLA less than 0.7. Otherwise, they are judged as provisional non-antimitotic.

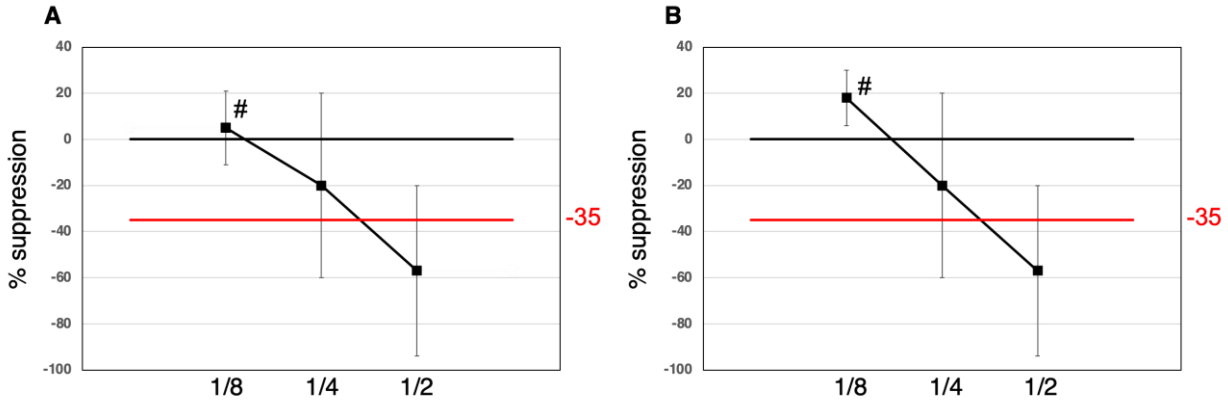
a. The mean of % suppression is equal to or less than -35 (stimulatory) with statistical significance. The statistically significant is judged when the upper limit of 95% confidence interval of % suppression is under 0.

b. The outcome shows two or more consecutive statistically significant stimulatory results; alternatively one statistically significant stimulatory result with the same trend for at least 3 consecutive data points (i.e. dose dependent trend); in this case the trend can cross the zero line, but the data point on the other side of the 0 line does not become statistically significant for the opposite effect. The statistically significant is judged when the upper limit of 95% confidence interval of % suppression is under 0.

c. The results are judged using only data obtained in the concentration range at which Inh-GAPLA is equal to or more than 0.05.

The following two representative graphs explain the judgment when the data shows only one statistically significant stimulatory result with the same trend for at least 3 consecutive data points.

Figure 6. Two patterns showing one positive data point with the same trend for at least 3 consecutive data points in the IL-2 Luc LTT assay.



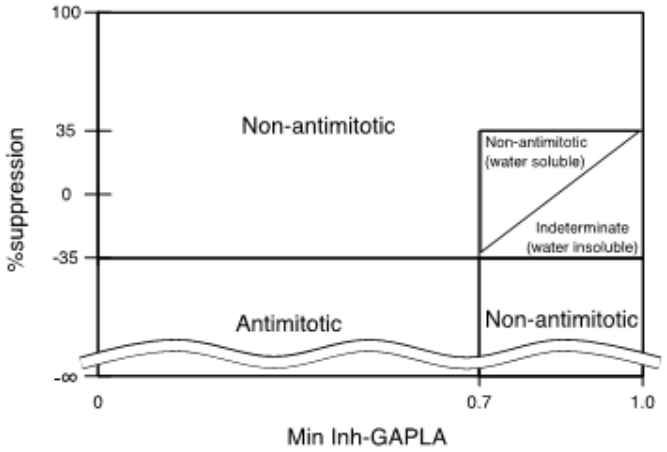
The x-axis represents the chemical concentration and the y-axis represents % suppression. Each plot is the % suppression value from four technical replicates with 95% confidence intervals. The red lines indicate -35. Two graphs show one positive data point with the same trend for at least 3 consecutive data points (i.e. concentration dependent trend) and the trends cross the zero line. A is judged as positive, because the 95% confidence interval

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of the data point indicating the opposite response (#) crosses 0. In contrast, B is judged negative because the 95% confidence interval of the data point indicating the opposite response (#) does not cross 0.

Of chemicals that are judged as provisional non-antimitotic, if chemicals do not demonstrate statistically significant suppressive data point (the mean of %suppression is equal to or more than 35) or stimulatory data points (the mean of %suppression is equal to or less than -35), show Min Inh-GAPLA $\geq$ 0.7, and they are insoluble at 10 mg/mL in distilled water, they are judged as indeterminate because they may be not dissolved in the vehicle at the concentration sufficient to show the effects in the culture medium. Otherwise, they are judged as non-antimitotic. Fig. 7 show the graphic presentation of the criteria.

Figure 7. Criteria to determine antimitotic, non-antimitotic or indeterminate.



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