8-1 Cells

- 2H4 (IL2-SLG、IFNγ-SLO、G3PDH-SLR)

  The human acute T lymphoblastic leukemia cell line Jurkat was obtained from the ATCC. A Jurkat-derived IL-2 and IFN-γ reporter cell line, 2H4, that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFN-γ and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd., Fukui, Japan. (Saito R. et al. Nickel differentially regulates NFAT and NF-κB activation in T cell signaling *Toxicology and Applied Pharmacology,* 254, 245–255, 2011)

8-2. Protocol for the IL-2 Luc assay

8-2-1. Reagents and equipment

The following reagents and equipment were used.

For maintenance of 2H4 cells

- RPMI-1640 (GIBCO Cat#11875-093, 500 mL)
- FBS (Biological Industries Cat#04-001-1E Lot: 715004)
- Antibiotic-Antimycotic (GIBCO Cat#15240-062)
- HygromycinB (CAS:31282-04-9, Invitrogen Cat#10687-010)
- G418 (CAS:108321-42-2, Nacalai Tesque Cat#16513-84)
- Puromycin (CAS:58-58-2, InvivoGen Cat#ant-pr-1)

For chemical exposure, stimulation and solvents

- Ionomycin (CAS:56092-82-1, Sigma Cat#I0634)
- Phorbol 12-myristate 13-acetate (PMA) (CAS:16561-29-8, Sigma Cat#P8139)
- Ethanol (e.g., Wako Cat#057-00456)
- Dimethyl sulfoxide (DMSO) (CAS:67-68-5, Sigma Cat#D5879)
- Distilled water (GIBCO Cat#10977-015)

For measurement of luciferase activity

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

8-2-2. Culture medium

Various culture media were used depending on the purpose of the cell culture.

Table 8. A medium: for maintenance of 2H4 cells (500 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	440 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	50 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	5 mL
Puromycin	InvivoGen #ant-pr-1	10 mg/mL	0.15 μg/mL	7.5 μL
G418	Nacalai Tesque #16513-84	50 mg/mL	300 μg/mL	3 mL
HygromycinB	Invitrogen #10687-010	50 mg/mL	200 μg/mL	2 mL

Table 9. B medium: for luciferase assay (30 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

Table 10. C medium: for thawing 2H4 cells (30 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	26.7 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	0.3 mL

8-2-3. Cell line

The Jurkat human acute T lymphoblastic leukemia cell line (ATCC, Manassas, VA, USA), was cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) with Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA, USA) and 10% Hyclone™ fetal calf serum (Thermo Fisher Scientific, Wilmington, NC, USA) (Jurkat growth medium) at 37 °C with 5% CO_2. The luciferase reporter assay system was constructed using three different luciferases, SLG, SLO and SLR, that emit green, orange, and red light, respectively, with a single substrate. In brief, we constructed three luciferase vectors, pSLG-test/Hygr, pSLO-test/Neor, and pSLR-test/Purr, by ligating the BamHI/SacI site of resistant gene vectors containing one of the three resistant genes, hygromycin (SLG), neomycin (SLO) or puromycin (SLR), the SV40 promoter, and HSVtk polyA into the luciferase gene vectors, pSLG-test, pSLO-test and pSLRtest (Toyobo, Osaka, Japan), respectively.

The activities of the luciferases can be measured simultaneously and quantitatively using optical filters. This system can rapidly and easily monitor multiple gene expression (Nakajima et al., 2005; Noguchi et al., 2008). Promoter cloning was carried out as follows. The IL-2 promoter construct containing nt −3006 to +286, the IFN-γ promoter construct containing nt −4971 to +111, and the G3PDH promoter construct containing nt −1373 to +128 from transcription initiation sites that were identified using DBTSS (http://dbtss.hgc.jp/), were amplified from genomic DNA by PCR using KOD-Plus- ver. 2 (Toyobo) for the IL-2 promoter or KOD-Plus- (Toyobo) for the IFN-γ and G3PDH promoters and specific primers. The IL-2 promoter, IFN-γ promoter, or G3PDH promoter was ligated into pSLG-test/Hygr, pSLOtest/Neor or pSLR-test/Purr vectors that had been digested with MluI and XhoI, MluI and SalI, or MluI and EcoRI, respectively. Before transfection, we confirmed the sequence of the 5' and 3' regions of each promoter using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). IL-2, IFN-γ and G3PDH reporter plasmids (1 μg) were transfected into Jurkat T cells ($5x10^5$ cells) using SuperFect (Qiagen, Valencia, CA, USA). After transfection, cells were cultured in Jurkat growth medium containing 200 μg/ml hygromycin (Invitrogen), 300 μg/ml G418 (Nacalai tesque, Kyoto, Japan) and 0.15 μg/ml puromycin (InvivoGen, San Diego, CA, USA) for selection. After repeated limiting dilution, we established a stable cell line (2H4 cells).

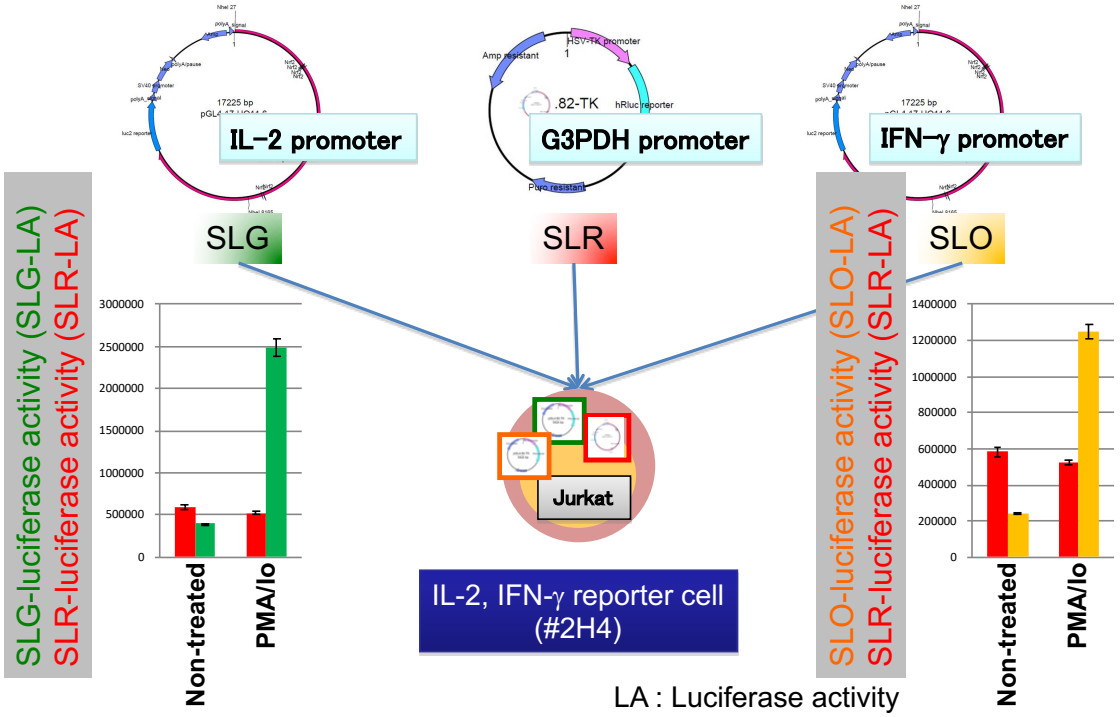

Fig. 12. IL-2 reporter cell, 2H4

8-2-4. Thawing of 2H4 cells

Pre-warm 9 mL of C medium in a 15 mL polypropylene conical tube in a 37°C water bath (for centrifugation) and 15 mL of C medium in a T-75 flask at 37°C in a 5% CO_2 incubator (for culture).

Thaw frozen cells ($2x10^6$ cells/0.5 mL of freezing medium) in a 37°C water bath, then add to a 15 mL polypropylene conical tube containing 9 mL of pre-warmed C medium. Centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in 15 mL of pre-warmed C medium in a T-75 flask. Cells are incubated at 37°C, 5% CO_2.

8-2-4. Maintenance of 2H4 cells

Pre-warm the A medium in a T-75 Flask at 37°C in a 5% CO_2 incubator. The culture medium should be changed to the pre-warmed A medium 3 or 4 days after thawing. At that time, count the number of cells, centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in pre-warmed the A medium in a T-75 Flask. Cells are passaged at $3x10^5$/mL and incubated at 37°C, 5% CO_2.

The interval between subcultures should be 3~4 days. Cells can be used between one and six weeks after thawing.

The lead laboratory has examined how long 2H4 cells could be cultured without losing their reactivity to PMA/Io. 2H4 cells maintained their response to PMA/Io up to 16 weeks or 35 passages.

8-2-5. Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.0×10^7 cells for two chemicals are required, but to have some leeway, 3.0×10^7 cells for two chemicals should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir (Thermo Scientific), and add 50 μL of cell suspension to each well of a 96 well μclear black plate (flat bottom) using an 8 channel or 12 channel pipetman (Gison, Inc, Middleton, WI, USA). (cf. Figure 13)

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

Fig. 13. Components in each well of 96-well plates after cell preparation.

8-2-6. Preparation of chemicals and cell treatment with chemicals

Water soluble chemicals were dissolved in distilled water at a concentration of 25 mg/mL. If the chemicals were soluble at 25 mg/mL, then 50 mg/mL solutions were prepared if their solubility was sufficient. If they were not soluble at 50 mg/mL, then 25 mg/mL was judged the highest soluble concentration. If the chemicals were soluble at 50 mg/mL, then 100 mg/mL solutions were prepared if their solubility was sufficient. If they were not soluble at 100 mg/mL, then 50 mg/mL was judged the highest soluble concentration. If they were soluble at 100 mg/mL, then 100 mg/mL was judged the highest soluble concentration.

Chemicals not soluble in water were dissolved in DMSO at 500 mg/mL. If they were not soluble at 500 mg/mL, the highest soluble concentration was determined by diluting the suspension from 500 mg/mL by a factor of 2 with DMSO. Sonication and vortex mixing were used if needed and the attempt to dissolve the chemical continued for at least 5 minutes. All dissolved chemicals were used within 4 hours of being dissolved in distilled water or DMSO.

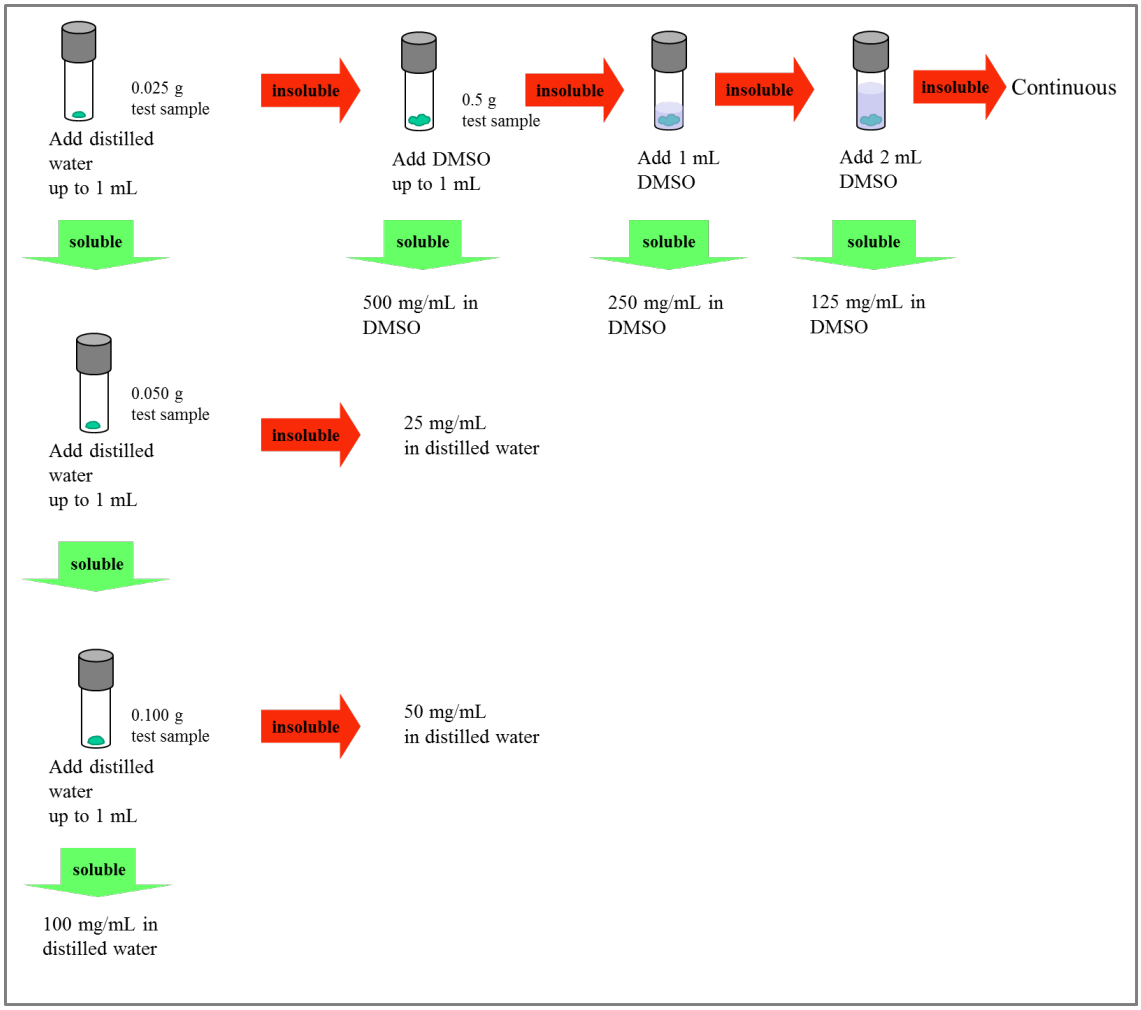

Fig. 14. Dissolution by vehicle

8-2-7. Dilution of chemicals

For water soluble chemicals, 11 serial dilutions were conducted using B medium, diluting by a factor of 2, in the 1st experiment. In the 2nd, 3rd, or 4th experiment, 11 serial dilutions were conducted, diluting by a factor of 1.5. For water insoluble chemicals, 11 serial dilutions were conducted using DMSO as the solvent, diluting by a factor of 2 in the 1st experiment and by a factor of 1.5 in the 2nd, 3rd, and 4th experiments. The diluted chemicals are added to 2H4 cells in a 96 well plate. After one-hour incubation at 37°C in a 5% CO_2 incubator, 2H4 cells are added 10 µL of PMA/Io solution and incubated again at 37°C in a 5% CO_2 incubator for 6 hours.

8-2-8. Measurements

After incubation with the chemical and PMA/Io for 6 h at 37°C in a 5% CO_2 incubator, 100 µL of pre-warmed Tripluc is added to each well in the plate containing reference samples using a pipetman and the plate is shaken for 10 min at room temperature (about 25°C) using a plate shaker. Surface bubbles are removed if present and bioluminescence in each well is measured using

a microplate-type luminometer with a multi-color detection system (Phelios; Atto Co., Tokyo, Japan) for 3 sec each in the absence (F0) and presence (F1, F2) of the optical filter. The F0, F1 and F2 data (values are expressed as counts) are processed using an Excel-based data sheet (Appendix 12). SLG-LA, SLO-LA and SLR-LA are calculated for each well based on the algorithm to calculate SLG-LA, SLO-LA and SLR-LA from the raw luminescence data reported previously (Nakajima et al., 2005; Noguchi et al., 2008). In addition to being used to calculate SLG-LA, SLO-LA and SLR-LA, this data sheet can automatically generate final graphs showing the correlation between %suppression and the concentration of chemicals, and between II-SLR-LA and the concentration of the chemical.

8-2-9. Luminometer apparatus

Multi-color detection systems such as microplate-type luminometers are available and include Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany), and the ARVO series (PerkinElmer, Waltham, MA). The luminometer detectors must have high sensitivity and low background noise and are usually equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmission coefficients of these filters for each bio-luminescence signal color must be calibrated prior to all experiments following the manufacturer's recommended protocol because the transmittance of the optical filter or the sensitivity of the detector are dependent on the measurement conditions.

8-2-10. Positive control

In each experimental set, dexamethasone and cyclosporine A are used as positive controls.

8-2-11. Calculation and definition of parameters for the IL-2 Luc assay

In the IL-2 Luc assay, the lead laboratory defined nSLG-LA to represent IL-2 promoter activity by the SLG luciferase activity (SLG-LA) normalized by SLR luciferase activity (SLR-LA). The suppression index of SLR-LA (I.I.-SLR-LA) was obtained by dividing SLR-LA of 2H4 treated with chemicals with SLR-LA of non-treated 2H4. %suppression reflects the effect of chemicals on IL-2 promoter. (Table 19).

Table 19. Abbreviations used in the 2H4 luciferase assay protocol

Parameter	Definition
SLG-luciferase activity (SLG-LA)	Luciferase activity of stable luciferase green (Under the control of IL-2 promoter)
SLO-luciferase activity (SLO-LA)	Luciferase activity of stable luciferase orange (Under the control of IFN-γ promoter)
SLR-luciferase activity (SLR-LA)	Luciferase activity of stable luciferase red (Under the control of G3PDH promoter)
Normalized SLG-LA (nSLG-LA)	=(SLG-LA)/(SLR-LA)
Normalized SLO-LA (nSLO-LA)	=(SLO-LA)/(SLR-LA)
Inhibition index of SLR-LA (I.I.-SLR-LA)	=(SLR-LA of #2H4 treated with chemicals)/(SLR-LA of untreated #2H4) (The cytotoxic effect of chemicals)
%suppression	=(1-(nSLG-LA of #2H4 treated with chemicals) /(nSLG-LA of non-treated #2H4)) x 100 (The effect of chemicals on IL-2 promoter)

8-2-11 Acceptance criteria

The following acceptance criteria should be satisfied when using the IL-2 Luc Assay method.

- If Fold induction of nSLO-LA of PMA/Io wells without chemicals (=(nSLO-LA of 2H4 cells treated with PMA/Ionomycin) / (nSLO-LA of non-treated 2H4 cells)) demonstrate less than 3.0, the results obtained from the plate containing the control wells should be rejected.

8-2-12 Criteria

The experiments are repeated until 2 consistent positive (or negative) results or 2 consistent "no effect results" are obtained. When 2 consistent results are obtained, the chemicals are judged as indicated by the obtained consistent results.

An immunotoxicant is identified by the mean of %suppression and its 95% simultaneous confidence interval.

In each experiment, when the chemicals clearly meet the following 3 criteria, they are judged as suppressive or stimulatory. Otherwise, they are judged as 'no effect' chemicals.

1. The mean of %suppression is ≥35 (suppressive) or ≤-35 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.
2. The result shows 2 or more consecutive statistically significant positive (negative) data points or 1 statistically significant positive (negative) data point with a trend in which at least 3 consecutive data points increase (decrease) in a dose-dependent manner. In the latter case, the trend can cross 0, as long as only 1 data point shows the opposite effect without statistical significance.

3. The results are judged using only data obtained at the concentration at which I.I.-SLR-LA is ≥0.05.

8-3. Data collection

8-3-1. Operating procedure

The operating procedure for Phase I is described in protocol version 008.5E, and that for Phase II is described in protocol version 009.1E. These protocols differ slightly regarding the decision rules for identifying immunotoxicants but are the same regarding the operating procedure.

The result of each experiment is judged “Positive” or “Negative”, and the final judgment for “Immunosuppression”, “Immunoaugmentation” or “No effect” of a test chemical is based on the results of 2-4 experiments. This rule was decided after discussion of the Phase II study at the VMT meeting, and the details are described in protocol ver. 0.11E.

8-3-2. Chemicals

The main aim of the Phase I study was to evaluate the within- and between-laboratory reliability of the assay. Three sets of 5 chemicals were distributed to 3 laboratories. A different code for each set of chemicals was used for each laboratory. The chemicals are re-coded in the present document. Each set of chemicals is indicated by a suffix, providing code names such as MIB012A, MIB014A, MIB017A, and so on.

The main aim of the Phase II study was to evaluate predictively and thus only 1 set of 20 chemicals was used in this phase. The Table 20 shows the chemical codes used throughout this document.

Table 20. The chemical codes

Phase		Chemical		
		Lab A	Lab B	Lab C
I	set A	MIB012A MIB014A MIB017A MIB018A MIB110A	MIC021A MIC023A MIC025A MIC027A MIC029A	MID034A MID036A MID037A MID038A MID310A
	set B	MIB011B MIB013B MIB015B MIB017B MIB019B	MIC024B MIC026B MIC027B MIC028B MIC210B	MID031B MID033B MID035B MID037B MID039B
	set C	MIB014C MIB016C MIB017C MIB018C MIB110C	MIC021C MIC023C MIC025C MIC027C MIC029C	MID032C MID034C MID037C MID038C MID310C
II		MIB501 MIB502 MIB503 MIB504 MIB505 MIB506 MIB507 MIB508 MIB509 MIB510 MIB511 MIB512 MIB513 MIB514 MIB515 MIB516 MIB517 MIB518 MIB519 MIB520	MIC601 MIC602 MIC603 MIC604 MIC605 MIC606 MIC607 MIC608 MIC609 MIC610 MIC611 MIC612 MIC613 MIC614 MIC615 MIC616 MIC617 MIC618 MIC619 MIC620	MID701 MID702 MID703 MID704 MID705 MID706 MID707 MID708 MID709 MID710 MID711 MID712 MID713 MID714 MID715 MID716 MID717 MID718 MID719 MID720

8-3-3. Data handling

The Excel data sheet developed for this study was distributed to the laboratories. The data management team received data files from the 3 laboratories. Since the Excel data sheet is able to display a concentration-response plot for %suppression with its 95% confidence interval, we were able to judge “Suppression”, “Stimulation” or “Negative” for each experiment by seeing the plot.

8-3-4. Index from each experiment and decision criteria for judgment

The j-th repetition (j = 1 to 4) of the i-th concentration (j = 0 to 11) is measured for SLG-LA and SLR-LA respectively. The normalized SLG-LA is referred as nSLG-LA, and is defined as $nSLG\text{-}LA_{ij} = SLG\text{-}LA_{ij} / SLR\text{-}LA_{ij}$. This is the basic unit of measurement in this assay.

8-3-4-1. %suppression

The %suppression is an index for the averaged nSLG-LA 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,

$$\%\text{suppression}_i = \left\{1 - \frac{\left(\frac{1}{4}\right)\sum_i \text{nSLG} - \text{LA}_{ij}}{\left(\frac{1}{4}\right)\sum_i \text{nSLG} - \text{LA}_{0j}}\right\} \times 100$$

The lead laboratory has proposed that ± 35 of the value suggest the suppression and stimulation 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 nSLG 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 nSLG-LA with the i-th concentration is greater than it with the 0 concentration statistical-significantly, whereas the upper limit of the 95% CI blow 0 is interpreted as that the nSLG-LA with the i-th concentration is lesser than it with the 0 concentration statistical-significantly.

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.

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

where mean_i is the mean of nSLG-LA at the i-th concentration, mean_0 is the mean of nSLG-LA at 0 concentration, sd_i is the standard deviation of nSLG-LA at the i-th concentration and sd_0 is the standard deviation of nSLG-LA at 0 concentration.

$z_{0.975}$ is 97.5 percentile of the standard normal distribution.

8-3-4-2. I.I.-SLR-LA

The I.I.-SLR-LA is a ratio of the averaged SLR-LA for the repetition of the i-th concentration compared with it of the 0 concentration, and this is written by

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

Since the SLR-LA is the denominator of the nSLG-LA, the extremely smaller value of this is considered to cause the large variation of the nSLG-LA. Therefore, the i-th %suppression value with extremely smaller value of the I.I.- SLR-LA might be considered to be poor precision.

8-3-4-3. Judgment for “Suppression”, “Stimulation” or “Negative” in each experiment

In each experiment, when the following 3 criteria are satisfied, they are judged as “suppression” or “stimulation”. Otherwise, they are judged as no effect chemicals.

1. %suppression is $\geq$ 35 (suppressive) or ≤ -35 (stimulatory) at any dose and statistically significant.

2. The result shows two or more consecutive statistically significant positive (negative) data or one statistically significant positive (negative) data with a trend in which at least 3 consecutive data increase (decrease) in a dose dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.

3. The results are judged using only data obtained in the concentration at which I.I.-SLR-LA is $\geq$ 0.05

For 1, 2, the statistically significant is judged by the lower limit of 95%

confidence interval of %suppression is over 0 or the upper limit of it is under 0.

8-3-4-4. Final judgment for “Suppression” “Stimulation” or “No effect” using this assay

In this assay, “Suppression” or “Stimulation” is defined as in case that the 2 same judgments were found in a set of experiments; “No effect” is defined as in case that the 3 “Negative” judgments were found in a set of experiments.

8-3-5. Reliability

8-3-5-1. Within-laboratory reproducibility for 5 common chemicals

Within-laboratory reproducibility was determined by whether or not tables of 3 sets for the final judgment for each chemical by each laboratory were concordant. The concordance rate was then calculated as a proportion of the concordance of each laboratory.

The concordance rate for within-laboratory reproducibility was based on the results of 3 sets.

To summarize, the concordance rate for within-laboratory reproducibility from the 3 laboratories were used to calculate the averaged concordance rate.

8-3-5-2 Between-laboratory reproducibility

Between-laboratory reproducibility was determined using the results from the final judgment from the 3 laboratories for 25chemicals, this is, 5 chemicals in Phase I study and 25 chemicals in Phase II study. These judgements were tabulated, then the concordance rate was calculated as a proportion of the concordance in each laboratory.

To summarize, the concordance rate for between-laboratory reproducibility from the 3 laboratories were used to calculate the averaged concordance rate.

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8-3-6. Predictivity

8-3-6-1. Definition of concordance, sensitivity and specificity

In the evaluation of predictivity, we did not distinguish suppression and stimulation, because both of these indicate modulation of immune function. Then, we dealt as “Positive” in case of “suppression” or “stimulation”, and “Negative” in case of “No effect” for each chemical judgement.

The concordance, sensitivity and specificity were estimated as the indexes of predictivity. These indexes were estimated using the frequency results obtained from the 2 by 2 contingency table for T cell targeting. The definitions of these indexes are summarized in Table 21 below. This calculation was based on the results decided by a majority for the between-laboratory results for each chemical.

Table 21. Definition of the concordance, sensitivity and specificity

Sensitivity = $100 \times a / (a+c)$
Specificity = $100 \times d / (b+d)$
Accuracy = $100 \times (a+d) / N$

8-4. Quality assurance

Assays and quality assurance were carried out in the spirit of GLP, although not all the participating laboratories routinely worked under GLP certification. The participating laboratories conducted the experiments in accordance with the protocol provided by the VMT. All raw data and data analysis sheets were pre-checked for quality by each laboratory and then were reviewed by the VMT quality assurance team. The results accurately reflect the raw data.

9. Results

We conducted Phase I and II studies in this validation. The assay procedure and criteria used to judge immunotoxicants in the validation studies are summarized in Fig. 15.

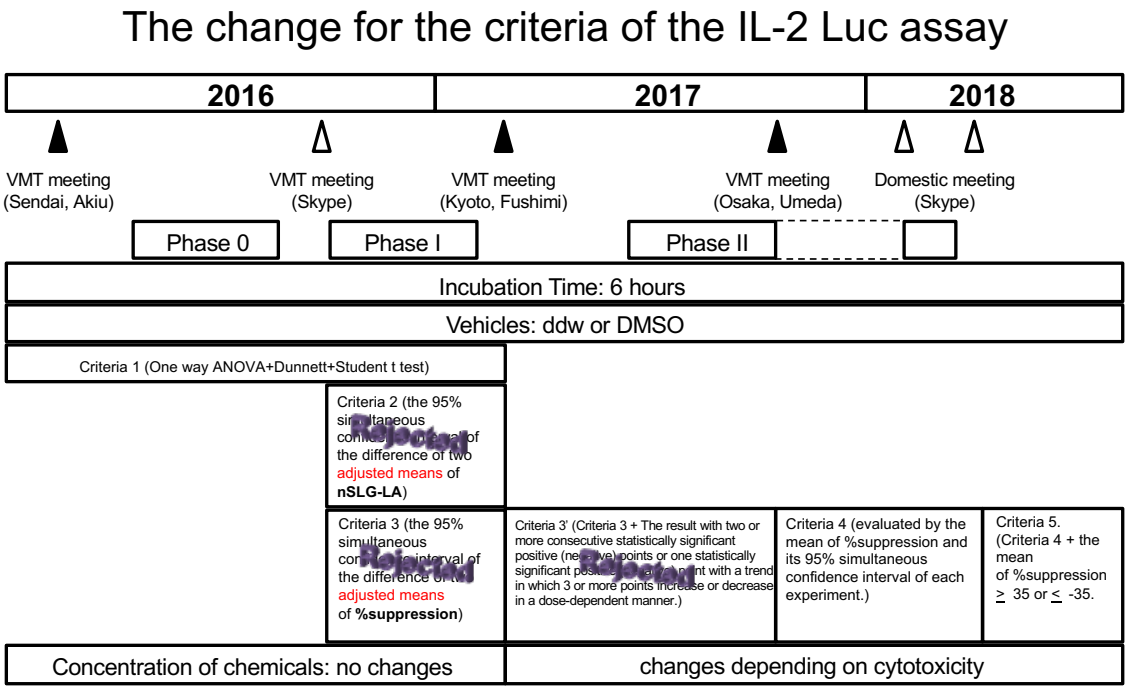


Fig. 15. The modification of the protocols of the IL-2 Luc assay.

9-1. The final criteria

9-1-1. Acceptance criteria

The following acceptance criteria should be satisfied when using the MITA method.

In each time of the experiments, a control experiment examining nIL2LA of 2H4 cells treated with PMA/Io and nIL2LA of non-treated 2H4 cells must be conducted. Then, the fold induction of nIL2LA of PMA/Ionomycin wells without chemicals (= (nIL2LA of 2H4 cells treated with PMA/Ionomycin)/(nIL2LA of non-treated 2H4 cells)) is calculated. If the fold induction is less than 3.0, the results obtained from these experiments should be rejected.

9-1-2. Criteria

The experiments are repeated until 2 consistent positive (or negative) results or 2 consistent "no effect results" are obtained. When 2 consistent results are obtained, the chemicals are judged as indicated by the obtained consistent results.

An immunotoxicant is identified by the mean of %suppression and its 95% simultaneous confidence interval.

In each experiment, when the chemicals clearly meet the following 3 criteria, they are judged as suppressive or stimulatory. Otherwise, they are judged as 'no effect' chemicals.

1. The mean of %suppression is ≥35 (suppressive) or ≤-35 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.
2. The result shows 2 or more consecutive statistically significant positive (negative) data points or 1 statistically significant positive (negative) data point with a trend in which at least 3 consecutive data points increase (decrease) in a dose-dependent manner. In the latter case, the trend can cross 0, as long as only 1 data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained at the concentration at which I.I.-SLR-LA is ≥0.05.

9-2. Phase 0 study (for technical transfer)

The preliminary test trial (Phase 0) was performed by the participating laboratories following explicit explanations of the IL-2 Luc assay procedures and protocol Ver. 008.1E by the lead laboratory, Tohoku University. Three laboratories participated in the Phase 0 study of the IL-2 Luc assay using 5 open labeled chemicals (2-aminoantracene, citral, chloroquine, dexamethasone, methyl mercuric chloride, 1 set (3 experiments) for each chemical). Most response patterns for the 5 chemicals were similar among the 3 laboratories except for 2 early experiments conducted by the naïve laboratory. Based on these results, VMT judged that technical and protocol transfer of the IL-2 Luc assay is acceptable.

After the Phase 0 study, we amended the protocol as follows:

- We changed the speed of centrifugation of the cells, and the preparation method for the selection antibiotics and PMA/Io.
- We set nSLO-LA >3 as an acceptance criterion.
- Because nSLG-LA is dependent on the properties of the specific luminometer used, we expressed the results of the data by %suppression, which is determined by dividing nSLG-LA of the chemically treated cells by nSLGLA of the vehicle-treated cells.
- Volatile chemicals were to be sealed.

- We determined the criteria to judge chemicals from a statistical standpoint (Criteria 2).

9-3. Phase I study (for within and between-laboratory reproducibility)

9-3-1. Test conditions

A total of 5 coded chemicals (4 T cell targeting and 1 non-T cell targeting) were evaluated by 3 experimental sets in the Phase I study based on the MITA protocol Ver. 008.5E.

In each experimental set, 3 or more experiments were conducted for each chemical.

Chemicals that satisfied criteria 5 were judged as positive. Chemicals that provided 2 positive results were judged as immunotoxicants.

9-3-2. Within-laboratory variation assessments in the Phase I study

Lab A	80.0% (4/5)
Lab B	100% (5/5)
Lab C	80.0% (4/5)
Average	86.7% (13/15)

9-3-3. Between-laboratory variation assessments in the Phase I study

Between-Lab reproducibility (Based on Majority)

80.0% (4/5)

9-3-4. Predictivity in the Phase I study (Based on Majority)

Accuracy of Lab A	80.0% (4/5)
Accuracy of Lab B	100% (5/5)
Accuracy of Lab C	100% (5/5)
Average	93.3% (14/15)

Table 21. Results of the Phase I study

Chemical	CAS	Set	Lab. A	Lab. B	Lab. C	concordance	T cell targeting

Dibutyl phthalate	84-74-2	1st round	S	S	S	1	Yes
		2nd round	S	S	S		
		3rd round	S	S	S		
Hydrocortisone	50-23-7	1st round	S	S	S	0	Yes
		2nd round	N	S	S		
		3rd round	N	S	N		
Lead(II) acetate	6080-56-4	1st round	S	S	S	1	Yes
		2nd round	S	S	S		
		3rd round	S	S	S		
Nickel(II) sulfate	10101-97-0	1st round	S	S	S	1	Yes
		2nd round	S	S	S		
		3rd round	S	S	S		
Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	1st round	N	N	N	1	No
		2nd round	N	N	N		
		3rd round	N	N	N		
Within-laboratory reproducibilities (%)			80.0 (4/5)	100 (5/5)	80.0 (4/5)		
			Average 86.7 (13/15)				
Between-laboratory reproducibilities (%) (Based on Majority)						80 (4/5)	
Sensitivity (%) (Based on Majority)			75.0 (3/4)	100 (4/4)	100 (4/4)		
			Average 91.7 (11/12)				
Specificity (%) (Based on Majority)			100 (1/1)	100 (1/1)	100 (1/1)		
			100 (3/3)				
Accuracy (%) (Based on Majority)			80.0 (4/5)	100 (5/5)	100 (5/5)		
			Average				

93.3 (14/15)

S : Immunosuppression, A : Immunoaugmentation, N : No effect, A/S : Immunoaugmentation/suppression

9-2-5. Contingency tables for the Phase I study

Lab A		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	10	2	12
	-	0	3	3
Total		10	5	15

Sensitivity : 83.3% (10/12)

Specificity : 100% (3/3)

Accuracy : 86.7% (13/15)

Lab B		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	12	0	12
	-	0	3	3
Total		12	3	15

Sensitivity : 100% (12/12)

Specificity : 100% (3/3)

Accuracy : 100% (15/15)

Lab C		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	11	1	12
	-	0	3	3
Total		11	4	15

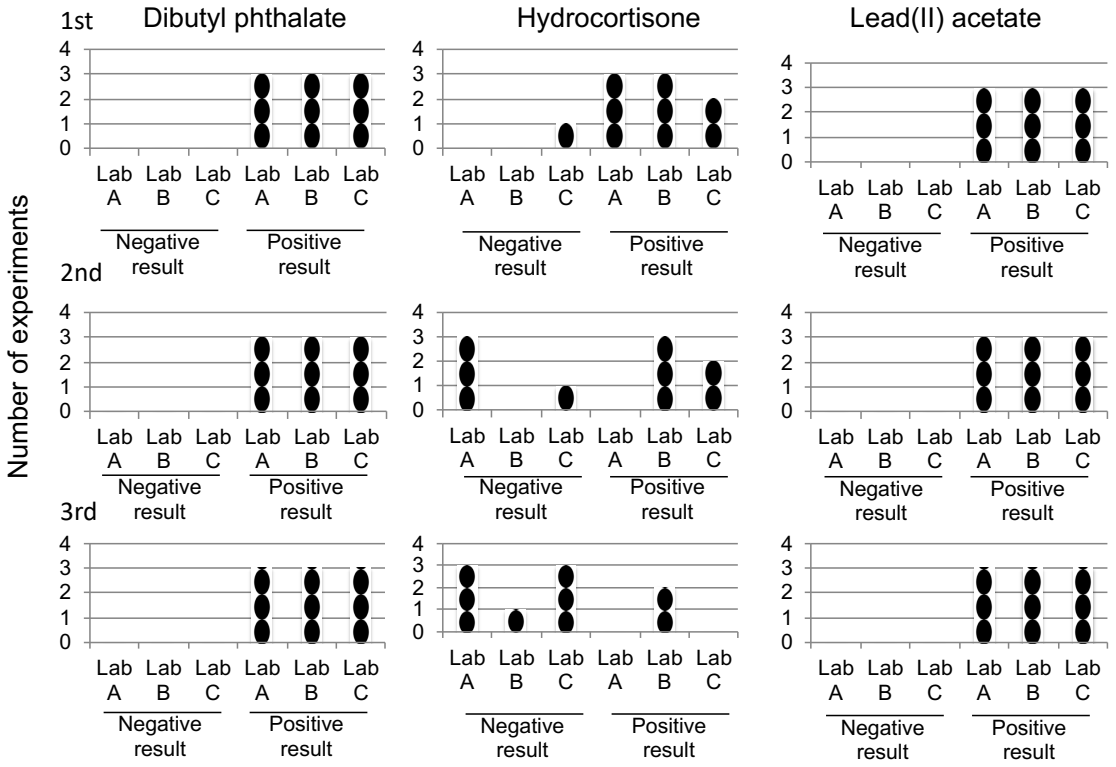
Sensitivity : 91.7% (11/12)

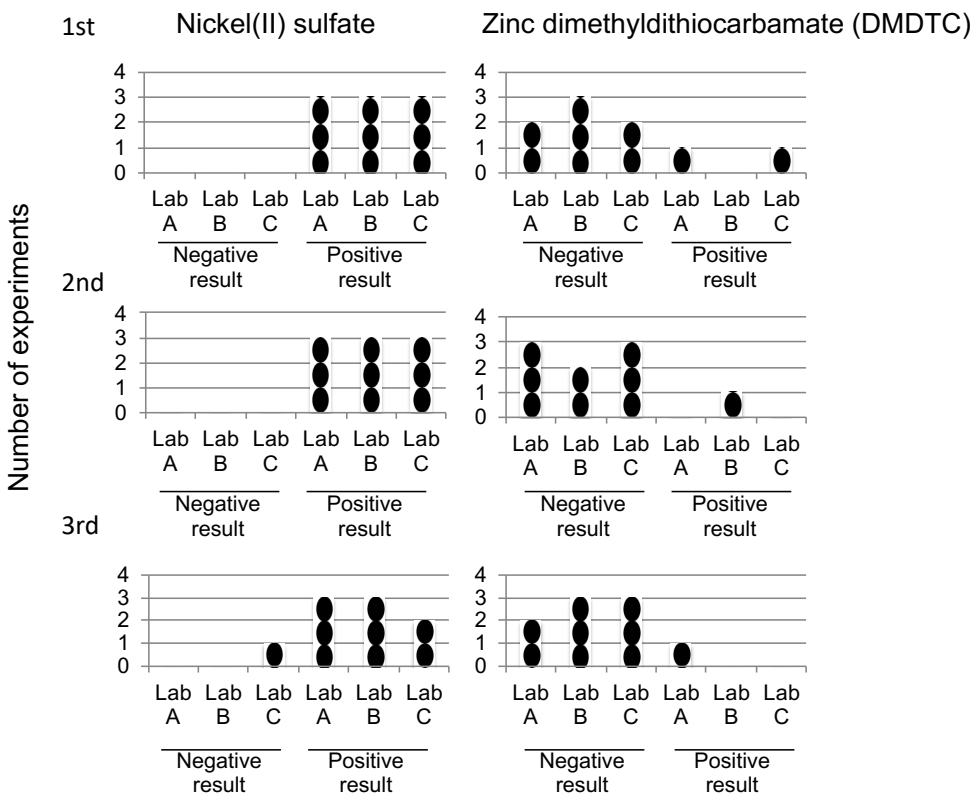
Specificity : 100% (3/3)

Accuracy : 93.3% (14/15)

A graphical presentation of between- and within-laboratory variation in Phase I study is shown in Fig. 15.

Within-laboratory reproducibility





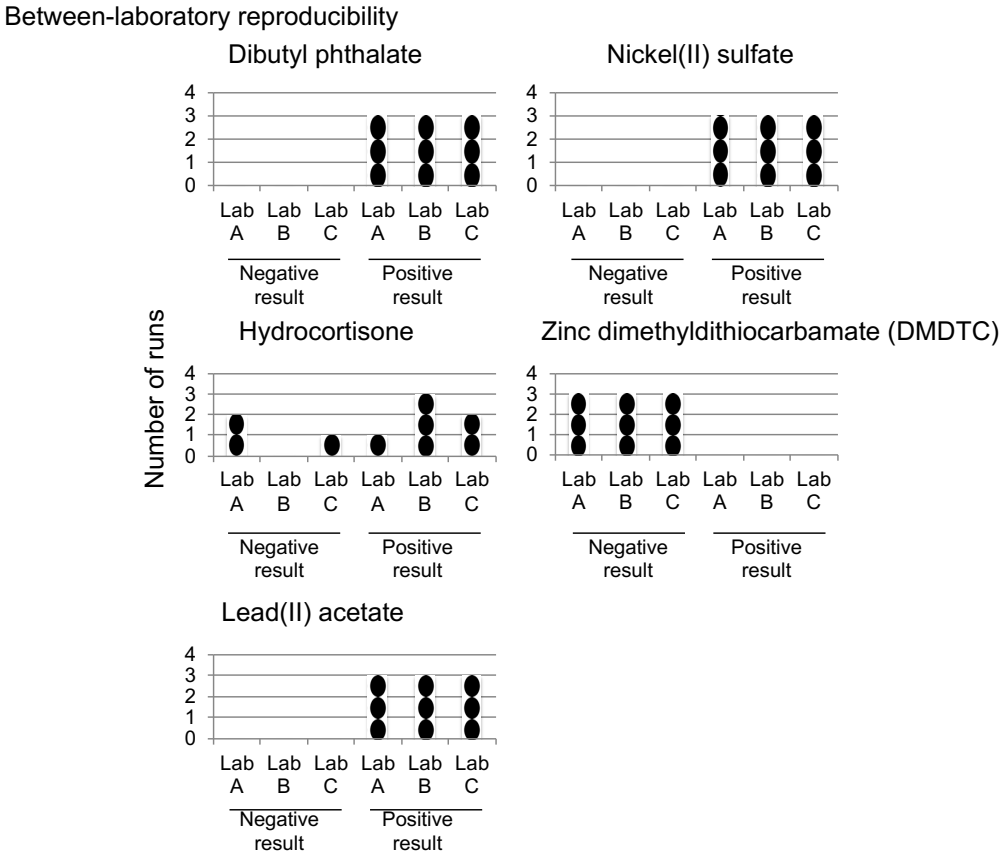


Fig. 15. Between- and within- laboratory variation assessments in Phase I study

The Phase I study examined within-and between-laboratory reproducibilities using a total of 5 coded chemicals (4 T cell targeting and 1 non-T cell targeting) evaluated by 3 experimental sets based on the MITA protocol Ver. 008.5E. Closed circles represent the judgments for individual experiments for within-laboratory reproducibility or the judgments in individual experimental sets for between-laboratory reproducibility.

9-4. Phase II study (for between-laboratory reproducibility and predictivity)

9-4-1. Test conditions

The Phase II study for between-laboratory reproducibility and predictivity was conducted using a total of 20 coded chemicals (13 T cell targeting, 6 non-T cell targeting and 1 undetermined) and evaluated by 1 experiment set based on the IL-2 Luc assay protocol Ver. 009.1E.

9-4-2. Between-laboratory variation assessments in the Phase II study

Between-Lab reproducibility 80% (16/20)

9-4-3. Predictivity in the Phase II study

Accuracy of Lab A	57.9% (11/19)
Accuracy of Lab B	57.9% (11/19)
Accuracy of Lab C	63.2% (12/19)
Average	59.6% (34/57)

Table 22. Results of the Phase II study

Chemical	CAS	Lab.A	Lab.B	Lab.C	concordance	T cell targeting
2,4-Diaminotoluene	95-80-7	N	N	N	1	No
Benzo(a)pyrene	50-32-8	S	S	S	1	Yes
Cadmium chloride	10108-64-2	N	N	N	1	Yes
Dibromoacetic acid	631-64-1	A/S	A	N	0	No
Diethylstilbestol	56-53-1	S	S	S	1	Yes
Diphenylhydantoin	630-93-3	N	N	N	1	Yes
Ethylene dibromide	106-93-4	N	N	N	1	Yes
Glycidol	556-52-5	A	A	A	1	Yes
Indomethacin	53-86-1	A	A	A	1	Yes
Isonicotinic Acid Hydrazide	54-85-3	S	N	S	0	Yes
Nitrobenzene	98-95-3	N	S	N	0	Yes
Urethane, Ethyl carbamate	51-79-6	A	A	A	1	No
Tributyltin chloride	1461-22-9	S	S	S	1	Yes
Perfluorooctanoic acid	335-67-1	A	A	A	1	Yes
Dichloracetic acid	79-43-6	A	S	S	0	Yes
Toluene	108-88-3	N	N	N	1	No
Acetonitril	75-05-8	N	N	N	1	undetermined
Mannitol	69-65-8	N	N	N	1	No

Vanadium pentoxide	1314-62-1	N	N	N	1	Yes
o-Benzyl-p-chorolophenol	120-32-1	S	S	S	1	No
Between-laboratory reproducibilities (%)					80 (16/20)	
Sensitivity (%)		61.5 (8/13)	61.5 (8/13)	61.5 (8/13)		
Specificity (%)		50.0 (3/6)	50.0 (3/6)	66.7 (4/6)		
Accuracy (%)		57.9 (11/19)	57.9 (11/19)	63.2 (12/19)		

S : Immunosuppression, A : Immunoaugmentation, N : No effect, A/S : Immunoaugmentation/suppression

9-4-4. Contingency tables for the Phase II study

Lab A		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	8	5	13
	-	3	3	6
Total		11	8	19

Sensitivity : 61.5% (8/13)

Specificity : 50.0% (3/6)

Accuracy : 57.9% (11/19)

Lab B		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	8	5	13
	-	3	3	6
Total		11	8	19

Sensitivity : 61.5% (8/13)

Specificity : 50.0% (3/6)

Accuracy : 57.9% (11/19)

Lab C	IL-2 Luc assay	Total

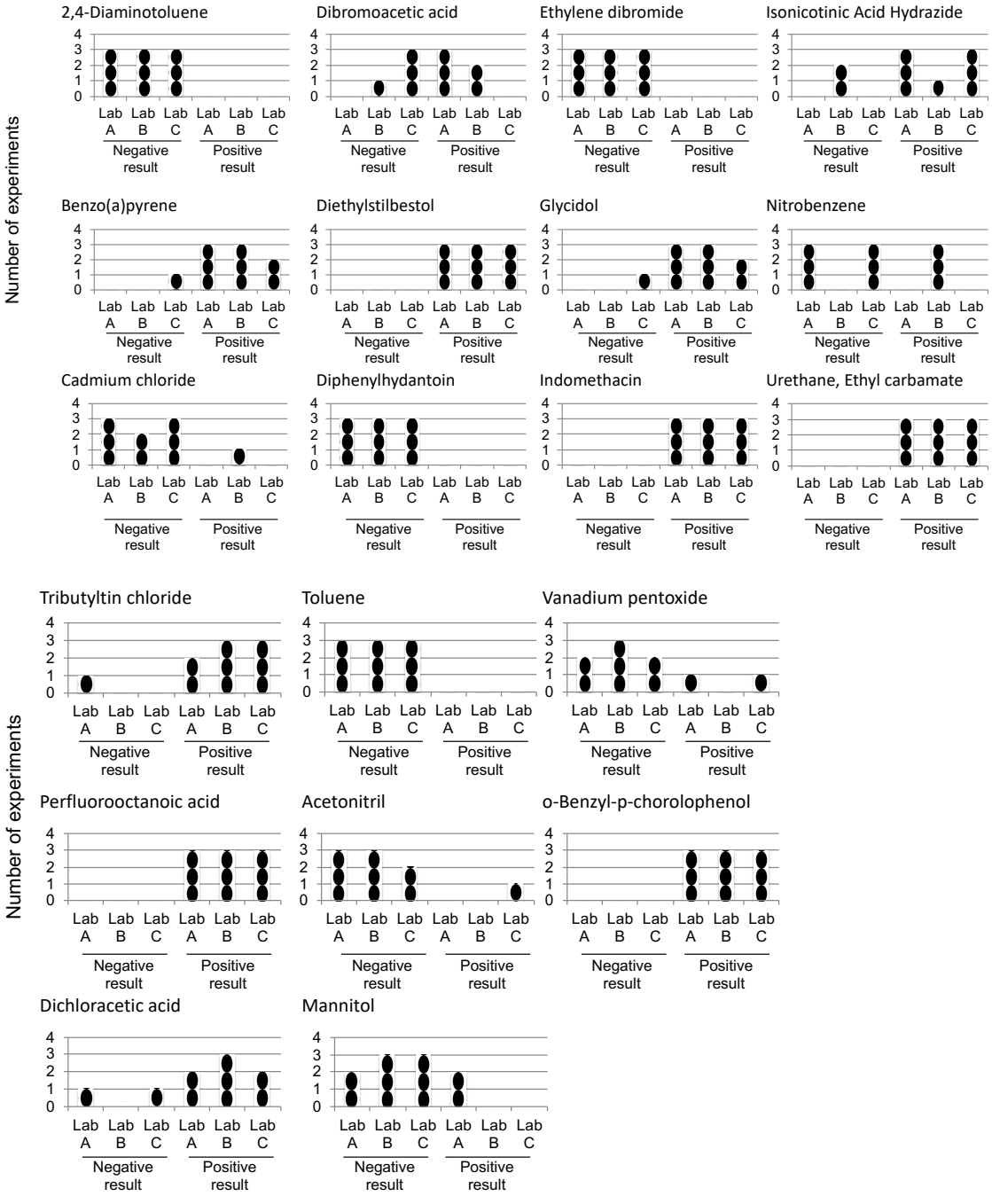
		+	-	
T cell targeting	+	8	5	13
	-	2	4	6
Total		11	4	19

Sensitivity : 61.5% (8/13)

Specificity : 66.7% (4/6)

Accuracy : 63.2% (12/19)

The graphical presentation of between- and within-laboratory variation in Phase II study is shown in Fig. 16.



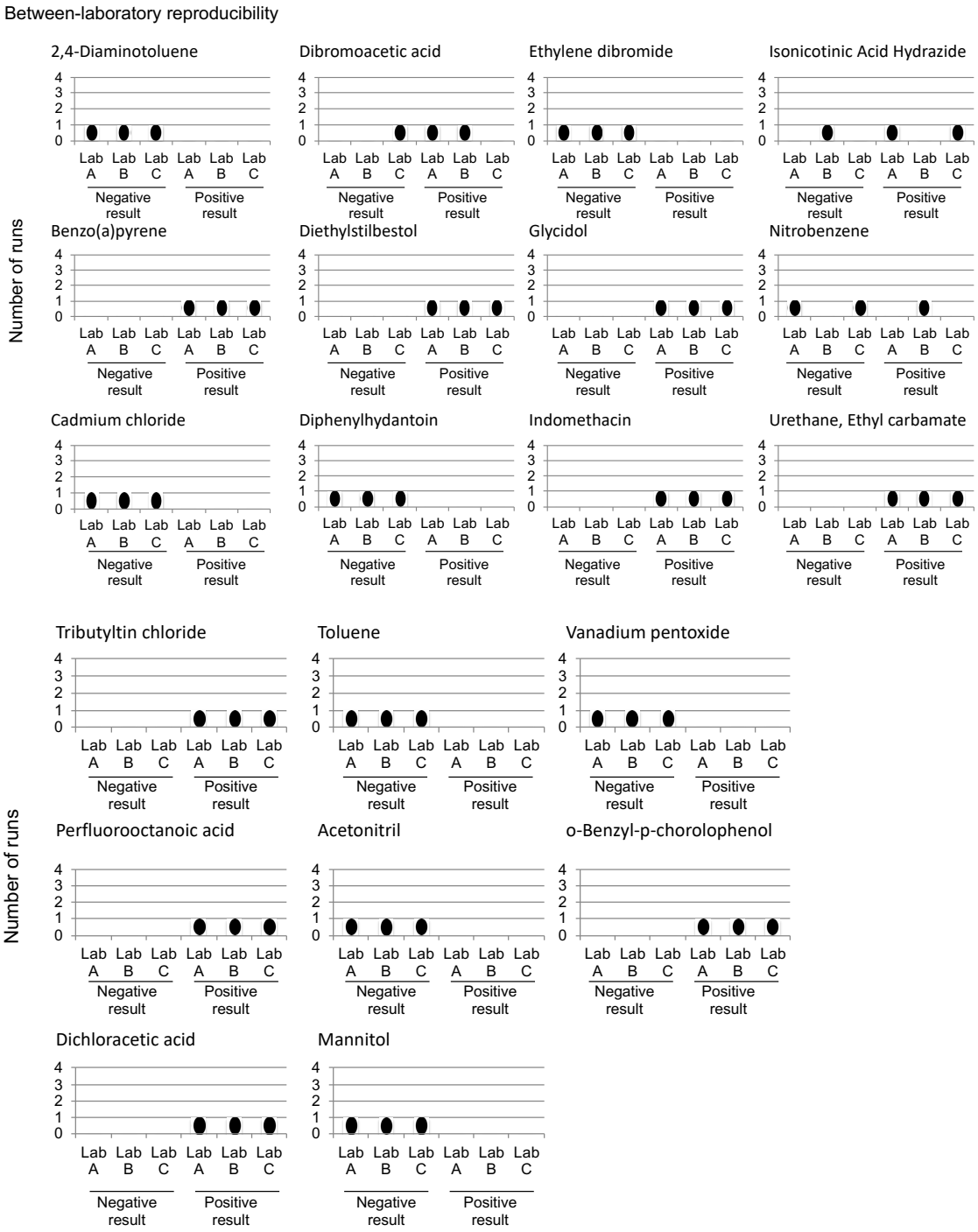


Fig. 16. Between variation assessments in Phase II study

The Phase II study examined between-laboratory reproducibilities using a total of 20 coded chemicals (13 T cell targeting, 6 non-T cell targeting and 1 undetermined) evaluated by 1 experiment sets based on Multi-Immuno Tox Assay protocol Ver. 009.1E. Closed circles represent the judgments for individual experiments in within-laboratory reproducibility or represent the judgments in individual experimental sets for between-laboratory reproducibility.

9-5. Quality assurance

All the records (data sheets and record sheets) from the participating laboratories were checked by Kobe univ. and JaCVAM, As a result, all record sheets on the maintenance of measuring instruments, the culture of cell line and the preparation and application of test chemicals were completed. JaCVAM considered these records had no effect on quality of data in the validation study.

9-6. Combined results of the Phase I and II studies (for between- and within- laboratory reproducibility and predictive capacity)

9-6-1. Test conditions

The within- and between-laboratory reproducibilities, and the predictivity of the IL-2 Luc assay, were evaluated using all the results from Phases I and II.

9-6-2. Within- and between-laboratory variation assessments from the Phase I and II studies.

Between-Lab reproducibility	80% (20/25)
Within-Lab reproducibility	Lab. A 80.0% (4/5)
	Lab. B 100% (5/5)
	Lab. C 80.0% (4/5)
	Average 86.7% (13/15)

9-6-3. Predictivity in the Phases I and II studies

Accuracy of Lab. A	62.5% (15/24)
Accuracy of Lab. B	66.7% (16/24)
Accuracy of Lab. C	70.8% (17/24)
Average	66.7% (48/72)

Table 23. Combined results of the Phase I and II studies

Chemical	CAS	Lab.A	Lab.B	Lab.C	concordance	T cell targeting
		Phase I				
Dibutyl phthalate	84-74-2	SSS	SSS	SSS	1	Yes

Hydrocortisone	50-23-7	SNN	SSS	SSN	0	Yes
Lead(II) acetate	6080-56-4	SSS	SSS	SSS	1	Yes
Nickel(II) sulfate	10101-97-0	SSS	SSS	SSS	1	Yes
Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	NNN	NNN	NNN	1	No
		Phase II				
2,4-Diaminotoluene	95-80-7	N	N	N	1	No
Benzo(a)pyrene	50-32-8	S	S	S	1	Yes
Cadmium chloride	10108-64-2	N	N	N	1	Yes
Dibromoacetic acid	631-64-1	A/S	A	N	0	No
Diethylstilbestol	56-53-1	S	S	S	1	Yes
Diphenylhydantoin	630-93-3	N	N	N	1	Yes
Ethylene dibromide	106-93-4	N	N	N	1	Yes
Glycidol	556-52-5	A	A	A	1	Yes
Indomethacin	53-86-1	A	A	A	1	Yes
Isonicotinic Acid Hydrazide	54-85-3	S	N	S	0	Yes
Nitrobenzene	98-95-3	N	S	N	0	Yes
Urethane, Ethyl carbamate	51-79-6	A	A	A	1	No
Tributyltin chloride	1461-22-9	S	S	S	1	Yes
Perfluorooctanoic acid	335-67-1	A	A	A	1	Yes
Dichloracetic acid	79-43-6	A	S	S	0	Yes
Toluene	108-88-3	N	N	N	1	No
Acetonitril	75-05-8	N	N	N	1	undetermined
Mannitol	69-65-8	N	N	N	1	No

Vanadium pentoxide	1314-62-1	N	N	N	1	Yes
o-Benzyl-p-chorolophenol	120-32-1	S	S	S	1	No
Within-laboratory reproducibilities (%)		80 (4/5)	100 (5/5)	80 (4/5)		
			Average 86.7 (13/15)			
Between-laboratory reproducibilities (%) (Based on majority for Phase I)					80 (20/25)	
Sensitivity (%)		64.7 (11/17)	70.6 (12/17)	70.6 (12/17)		
			Average 68.6 (35/51)			
Specificity (%)		57.1 (4/7)	57.1 (4/7)	71.4 (5/7)		
			Average 61.9 (13/21)			
Accuracy (%)		62.5 (15/24)	66.7 (16/24)	70.8 (17/24)		
			Average 66.7 (48/72)			

9-8-4. Contingency tables for the Phase II study

Lab A		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	11	6	17
	-	3	4	7
Total		15	9	24

Sensitivity : 64.7% (11/17)

Specificity : 57.1% (4/7)

Accuracy : 62.5% (15/24)

Lab B		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	12	5	17
	-	3	4	7
Total		15	9	24

Sensitivity : 70.6% (12/17)

Specificity : 57.1% (4/7)

Accuracy : 66.7% (16/24)

Lab C		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	12	5	17
	-	2	5	7
Total		14	10	24

Sensitivity : 70.6% (12/17)

Specificity : 71.4% (5/7)

Accuracy : 70.8% (17/24)

10. Discussion

10-1. Reliability

The IL-2 Luc assay is based on the modulation of PMA + ionomycin-induced luciferase activity in the IL-2 reporter cell line, 2H4. Therefore, it is crucial that 2H4 cells maintain their ability to induce luciferase activity following stimulation by PMA/Io. Before and during this validation study, the response of 2H4 cells to PMA/Io was carefully observed. We confirmed that a frozen stock of 2H4 cells can be cultured without losing luciferase activity for at least 16 weeks or 35 passages.

The culture of 2H4 cells is relatively simple and does not require the use of trypsin or EDTA because 2H4 cells do not adhere to the culture dishes. First, chemicals at graded concentrations are added to the wells of a 96-well culture plate. Then, cells adjusted to the optimum concentration are seeded into each well. After 6 h incubation, 100 μL of pre-warmed Tripluc is added to each of the 96 wells. The subsequent process is completely automated, except for calculating the results using the predesigned Excel spreadsheet. Therefore, the IL-2 Luc assay is a test method that can significantly reduce human error.

Moreover, the IL-2 Luc assay does not require the determination of cell viability after chemical treatment. 2H4 cells can present IL-2 promoter activity as well as promoter activity of GAPDH, a well-known housekeeping gene; therefore, information regarding the effects of the chemical on both IL-2 induction and cell viability is obtained in each experiment. Furthermore, a single experiment takes only 8 h, including the time required for chemical preparation and cell plating, making the IL-2 Luc assay a true high-throughput method.

10-2. Between- and within-laboratory reproducibility

We examined within-laboratory reproducibility in the Phase I study. Lab A, Lab B, and Lab C demonstrated 80%, 100%, and 80% reproducibility, respectively. On the other hand, Lab A, Lab B, and Lab C demonstrated 80% between-laboratory reproducibility in the combined data of the Phase I and Phase II studies. These results satisfied the acceptance criteria for the validation study with a within-laboratory reproducibility of at least 80% and a between-laboratory reproducibility of at least 80%.

10-3. Predictivity

To determine the predictivity of this assay, it is necessary to classify chemicals used in the validation study as immunotoxic or non-immunotoxic. In addition, since the IL-2 Luc assay focused on T cell response, we further classified immunotoxic chemicals into those that affect T cell function and those that do not. We therefore collected immunotoxicological information regarding the 25 test chemicals with the support of National Toxicology Program (NTP). The information for each chemical is provided in Appendix 5. The summarized data are shown in Appendix 6. The information for each chemical is composed of *in vivo*, *ex vivo*, and *in vitro* data. The *in vivo* data present the change in weight of immune system organs such as spleen and thymus, delayed hypersensitivity response, and the susceptibility to infection and resistance to transplanted tumors. The *ex vivo* data contain the effects of chemicals on cytokine production, T cell-dependent antibody response *in vitro*, as well as cytotoxic T cell response, mixed lymphocyte reaction, T cell mitogen-induced proliferation using cells from animals treated with the chemicals *in vivo*. The *in vitro* data provide the effects of the chemicals on cytokine production or on T cell proliferation after mitogen stimulation *in vitro*.

From these data we extracted the effects of the chemicals on the change of thymus weight in vivo, and T cell function ex vivo and in vitro. We also used their reported mode of action on T cell function as criteria to determine the immunotoxic effects of the chemicals on T cells. Based on these criteria, the 25 chemicals were classified into 16 immunotoxic chemicals, 8 non-immunotoxic chemicals, and 1 chemical with undetermined effects. According to this classification, the sensitivities of the assays as conducted by Lab A, Lab B, Lab C, and their average in the combined data of the Phase I and II studies are 64.7%, 70.6%, 70.6%, and 68.6%, respectively. The specificities of the assays as conducted by Lab A, Lab B, Lab C, and their average are 57.1%, 57.1%, 71.4%, and 61.9%, respectively. The accuracies of the assays as conducted by Lab A, Lab B, Lab C, and their average are 62.5%, 66.7%, 70.8%, and 66.7%, respectively.

10-4. IL-2 Luc assay data set for 60 chemicals

Based on the IL-2 Luc assay protocol (version 011E) and the Criteria 5, the lead laboratory reevaluated the data of 60 chemicals reported previously (Table 24). Similar to the classification by the criteria used in our published paper (Kimura et al., 2018), TAC, CyA, and Dex significantly suppressed IL-2 luciferase activity (IL-2 LA), although average LOEL of TAC and CyA was significantly lower that of DEX. The off-label immunosuppressive drugs, chloroquine, minocycline, and dapsone significantly suppressed IL-2 LA. Anti-cancer drugs, actinomycin D and cisplatin also significantly suppressed IL-2LA. In addition, azathioprine and colchicine were

demonstrated to suppress IL-2LA by the Criteria 5. Again, the suppressive effects on the IL-2 LA was not demonstrated by some of immunosuppressants the mechanism of which is inhibition of DNA synthesis or anti-proliferative effects on T cells, such as mitomycin C, cyclophosphamide, methotrexate or mizoribine by the Criteria 5.

If we calculated the predictivity of this assay based on the reported effects of the chemicals on the change of thymus weight in vivo, and T cell function ex vivo and in vitro that were obtained from the literature (Viora et al., 1996, Haley et al., 1990, Guo et al., 2001, Ulrich et al., 2004, Kobayashi et al., 2006, Wagner et al., 2006, Li et al., 2013, Kimura et al., 2014, Kimura et al., 2018), the sensitivity, specificity and accuracy (predictivity) are 84%, 56%, and 78%, respectively. In this calculation, we considered whether chemicals are targeting T cells or not irrespective of suppression or augmentation.

Table 24. Data set of the IL-2 Luc assay based on Criteria 5.

Chemical name	Judge	Ave.LOEL(35%)	Ave.LOEL(-35%)	Immunotoxicity in references
FK506	S	0.0002		S
Cyclosporine A	S	0.0041		S
Actinomycin D	S	0.0156		S
Digoxin	S	0.0686		N
Colchichine	S	0.2743		S
FR1667953	S	1.3021		S
Benzethonium chloride	S	1.6276		U
Mercuric chloride	S	1.9531		S
Chlorpromazine	S	1.9531		U
Amphoterycin B	S	2.6042		U
Dibutyl phthalate	S	2.6042		S
2-Aminoanthracene	S	5.8594		U
Formaldehyde	S	7.8125		S
Pyrimethamine	S	7.8125		S
Isophorone diisocyanate	S	15.6250		U
Cisplatin	S	16.9271		S
Cobalt chloride	S	16.9271		S
Chloroquine	S	17.8326		S
Minocycline	S	18.5185		S
Mitomycin C	S	20.0000		S
Hydrogen peroxide	S	23.4375		U
Citral	S	25.0000		U
Dexamethasone	S	41.1692		S
Pentamidine isethionate	S	52.0833		U
Lead(II)acetate	S	57.2917		S
Azathioprine	S	58.4778		S
Diesel exaust particle	S	62.5000		S
Sodium dodecyl sulfate	S	62.5000		N
Dapsone	S	72.9167		S
Nitrofurazone	S	83.3333		U
p-Nitroaniline	S	83.3333		U
Sulfasalazine	S	92.9444		S
Aluminium chloride	S	104.1667		S
Nickel sulfate	S	104.1667		S
Diethanolamine	S	250.0000		U
Chloroplatinic acid	S	250.0000		U
Sodium bromate	S	500.0000		S
Histamine	S	750.0000		S
Isoniazid	N			N
Triethanolamine	N			U
Magnesium sulfate	N			U
Hydrocortisone	N			S
Rapamycin	N			S
Mizoribine	N			S
Warfarin	N			N
2,4-Diaminotoluene	N			U
Cyclophosphamide	N			S
Dibenzopyrene	N			U
Ethanol	N			N
Hexachlorobenzene	N			S
Lithium carbonate	N			U
Methanol	N			N
Methotrexate	N			S
Dimethyl sulfoxide	N			N
Trichloroethylene	N			U
Mycophenolic acid	A		0.395061728	S
2-Mercaptobenzothiazole	A		16.11328125	N
Ribavirin	A		26.04166667	U
Nicotinamide	A		288.0658436	N
Acetaminophen	A		100	S

S: Suppression; N: No effects on T cells, U: Undetermined

U	Unclassified
S	Suppression, correctly judged
S	Suppression, misjudged
N	No effects, correctly judged
N	No effects, misjudged

Luster et al (Luster et al., 1993) proposed a screening battery using a 'tier' approach for detecting potential immunotoxic compounds in mice. In their study, they conducted the following immune test for approximately 30 to 50 chemicals, such as IgM plaque forming cell (PFC) response to sheep red blood cells, NK cell activity against YAC-I tumor cells, mixed leucocyte response (MLR) to allogeneic leucocytes, cytotoxic T-lymphocyte (CTL) response to P815 tumor

cells, T-cell mitogenic response to concanavalin A, B-Cell mitogenic response to lipopolysaccharide, delayed hypersensitivity response to keyhole limpet hemocyanin, surface marker expression including SIg, Thyl.2, CD4 and CD8, peripheral leucocyte counts, spleen cellularity (nucleated cells), thymus:body weight ratio, and spleen : body weight ratio. The conclusions derived from their study were as follows: (1) A good correlation exists between changes in the immune tests and altered host resistance, in that there were no instances where host resistance was altered without affecting one or more immune test(s). However, in some instances immune changes occurred without corresponding changes in host resistance. (2) No single immune test could be identified that was fully predictive for altered host resistance, although most assays were relatively good indicators (i.e. > 70%). (3) The ability to resist infectious agent challenge is dependent on the degrees of immunosuppression and the quantity of infectious agent administered. (4) Logistic and standard regression modelling using one extensive chemical data set from the immunosuppressive agent, cyclophosphamide, indicated that most immune function-host resistance relationships followed linear rather than linear~luadratic (threshold-like) models.

In their test, they obtained cells or organs from animals. Therefore, these tests are classified into ex vivo study. Using even these ex vivo studies, the top three predictivity values were 82% by delayed hypersensitivity, 76% by thymus/BW ration, and 74% by spleen/BW.

Considering these predictivity values by ex vivo tests and the limited target of the IL-2 Luc assay that is a high-throughput *in vitro* test, the performance of the IL-2 Luc assay might be acceptable.

10-5. Factors responsible for false negative results in the IL-2 Luc assay

Although the within- and between-laboratory reproducibilities satisfied the acceptance criteria for the validation study, the predictivity results were not satisfactory. We considered at least 2 reasons for the poor predictivity of the assay.

1) We collected as much immunotoxicological information on the chemicals as possible and determined whether or not the chemicals exhibited T-cell dependent immunotoxicity or not based on information in the peer reviewed literature. The criteria used for classification were the effects of the chemicals on the production of cytokines predominantly produced by T cells, *in vitro* or *ex vivo*, and their reported mode of action on T cell function. However, the information available was very limited for most chemicals and very little data had been reproduced by different laboratories. Therefore, the reliability of the criteria is sometimes uncertain.
2) It is reasonable to consider that the IL-2 Luc assay does not cover every aspect of the effects of the chemicals on T cell function. Using PMA + ionomycin de facto limits effects to chemicals

targeting calcium and PKC-mediated T cell activation. Other assays targeting T cell functions may be mandatory.

10-6. Limitations and drawback, and applicability domain of the modified IL-8 Luc assay

Since the 2H4 cell line used in the IL-2 Luc assay is derived from Jurkat cells, it is conceivable that this cell line is more resistant to the cytotoxic effects of chemicals than bone marrow cells. Indeed, our study demonstrated that the IL-2 Luc assay cannot evaluate the immunotoxic effects of some immunosuppressive drugs which act by inhibiting DNA synthesis leading to myelotoxicity (Kimura et al., 2014). Thus, these chemicals in addition to chemicals that need metabolic activation need to be outside the applicability domain. To overcome this drawback at present, the IL-2 Luc assay must be combined with assays capable of detecting myelotoxicity, such as the conventional 28-day subacute toxicity test (Investigators, 1998) or *in vitro* myelotoxicity tests (Pessina et al., 2003).

10-7. Potential of the MITA

The MITA can evaluate the effects of chemicals on IL-2, IFN-γ, IL-1β, and IL-8 promoter activities. The induction of these cytokines is mediated by a wide range of signaling pathways, including the MAP kinase, NF-kB, and calcium/calmodulin pathways. It is also well known that the induction of different immune-related molecules such as cytokines or chemokines commonly use the same signaling pathways. Therefore, although MITA evaluate only the effects of chemicals on the transcription of 4 cytokines, it may be able to cover the effects of chemicals on much wider range of immune response.

Furthermore, the combination of the MITA with the IL-8 Luc assay can evaluate the effects of chemicals on T cells and macrophages, and on the sensitizing potential of chemicals. The data obtained from these assays can be used by both industry and regulatory agencies to assess the immunotoxicity risks of chemicals. At the least, the IL-2 Luc assay and the IL-8 or IL-1β Luc assay should be officially validated and a larger number of chemicals must be evaluated using the MITA to fully determine the potential and limits of this technique.

11. Conclusion

Using 3 luciferase reporter cell lines, we established the MITA in which the effects of chemicals on the IL-2 luciferase activity of 2H4 cells are evaluated in the presence of PMA/Io. In

addition, the effect of chemicals on the IL-1β and IL-8 luciferase activity of THP-G1b and THP-G8 cells, respectively, were examined in the presence of the stimulant LPS. Our final goal is to officially validate the MITA for within- and between-laboratory reproducibility and predictivity. In this validation study, we first conducted a validation study of the IL-2 luciferase reporter assay (IL-2 Luc assay). After confirming the transferability of the IL-2 Luc assay among different laboratories, we conducted Phase I and II studies. These studies produced satisfactory within- and between-laboratory reproducibilities. However, the predictivity was below 80%, possibly due to inadequate immunotoxicological data for several chemicals used in the validation, leading to misjudgment, and possibly due to the IL-2 Luc assay covering limited aspects of the effects of chemicals on T cell function.

12. Acknowledgement

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14. List of abbreviations.

95% CI : the 95% confidence interval

AIST : National Institute of Advanced Industrial Science and Technology

AOP : Adverse outcome pathway

ARE: Antioxidant response element

CAS No. : Chemical Abstract Service Number

CMV : Cytomegalovirus

CSC : the Chemical Selection Committee

DMSO : Dimethyl sulphoxide

DPRA : the Direct Peptide Reactivity Assay

ECVAM : the European Centre for Validation of Alternative Methods

EDTA : Ethylenediaminetetraacetic acid

EGFR : Epidermal growth factor receptor

EGR-1 : Early growth response-1

EU : European Union

FBS : Fetal bovine serum

FN : False Negative Rate

GLP : Good laboratory Practice

GSH : Glutathione

HRI/FDSC : Hatano Research Institute, Food & Drug Safety Center

HSV : Herpes simplex viruses

ICCVAM : Interagency Coordinating Committee on the Validation of Alternative Methods

ID : Identification

IFN-γ : Interferon-γ

I.I.-SLR-LA : Inhibition index of SLR-LA

IL-2 : Interleukin-2

IL-8 : Interleukin-8

JaCVAM : the Japanese Center for the Validation of Alternative Methods

Keap-1 : Kelch-like ECH-associated protein 1

KoCVAM : Korean Center for the Validation of Alternative Methods

LLNA : Local lymph node assay

LPS : Lipopolysaccharide

MIT : Minimum induction threshold

MITA : Multi-Immuno Tox Assay

mMUSST : modified myeloid U937 dendritic cell activation test

MoDCs : Monocyte-derived dendritic cells

MOVS: Management Office of Validation Study

mRNA : messenger ribonucleic acid

MSDS : Material safety data sheet

NICEATM : the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

NIHS : National Institute of Health Sciences

NPV : Negative predictive value

Nqo1 : NADPH-quinone oxidoreductase 1

Nrf2 : Nuclear factor (erythroid-derived 2)-like factor 2

nSLG-LA : normalized SLG luciferase activity

nSLO-LA : normalized SLO luciferase activity

OECD : the Organization for Economic Co-operation and Development

PCR : Polymerase chain reaction

PI : Propidium iodide

PMA/Io : Phorbol 12-myristate 13-acetate/Ionomycin

PN : False Positive Rate

PPV : Positive Predictive Value

QC : Quality Control

REACH : Registration, Evaluation, Authorization and Restriction of CHemicals

RFI : Relative fluorescence intensity

RT : Ring trial

SLG : Stable luciferase green

SLG-LA : SLG luciferase activity

SLO : Stable luciferase orange

SLO-LA : SLO luciferase activity

SLR : Stable luciferase red

SLR-LA : SLR luciferase activity

SLS : Sodium lauryl sulfate

SLR : Stable luciferase red

SLR-LA : SLR luciferase activity

SV40 : Simian virus 40

TG : Test Guideline

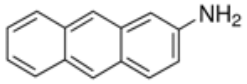
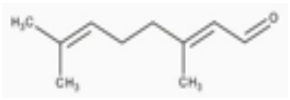
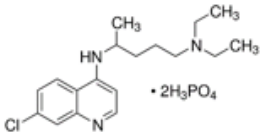
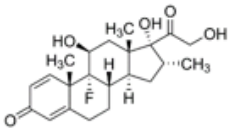
TNF-α : Tumor necrosis factor-α

UN GHS : the United Nations Globally Harmonized System of Classification and Labeling of Chemicals

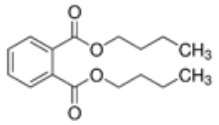
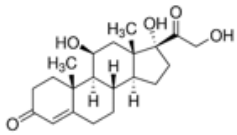
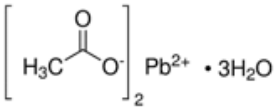
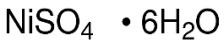
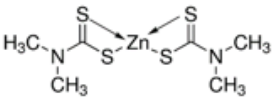
VMT : Validation Management Team

15. Appendixes

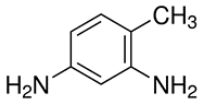
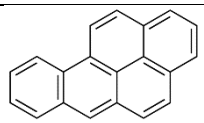
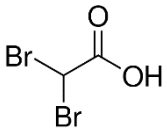
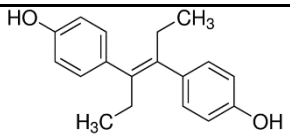
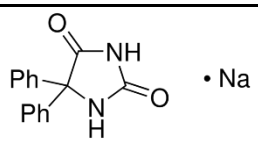
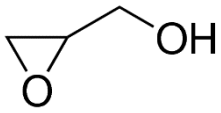
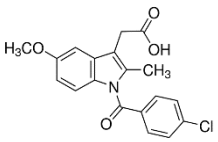
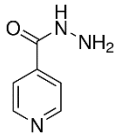
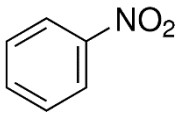
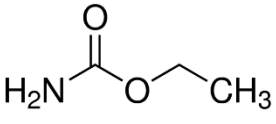
Appendix 1. Chemical structure of the test chemicals for Phase 0 study

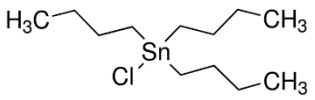
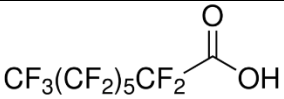
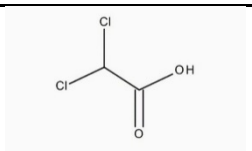
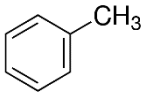
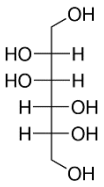
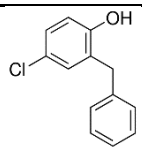
No.	Chemical name	CAS No.	Molecular weight	Chemical structure
0-1	2-Aminoanthracene	613-13-8	193.24	
0-2	Citral	5392-40-5	152.23	
0-3	Chloroquine diphosphate salt	50-63-5	515.86	
0-4	Dexamethasone	50-02-2	392.46	
0-5	Methylmercury(II) chloride	115-09-3	251.08	CH_3HgCl

Appendix 2. Chemical structure of the test chemicals for the Phase I study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
I-1	Dibutyl phthalate	84-74-2	278.34	
I-2	Hydrocortisone watersoluble	50-23-7	362.46	
I-3	Lead(II)acetate	6080-56-4	379.33	
I-4	Nickel sulfate hexahydrate	10101-97-0	262.85	
I-5	Dimethyldithiocarbamate	137-30-4	305.82	

Appendix 3. Chemical structure of the test chemicals for the Phase II study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
II-1	2,4-Diaminotoluene	95-80-7	122.17	
II-2	Benzo(a)pyrene	50-32-8	252.31	
II-3	Cadmium chloride	10108-64-2	183.32	$CdCl_2$
II-4	Dibromoacetic acid	631-64-1	217.84	
II-5	Diethylstilbestrol	56-53-1	268.35	
II-6	Diphenylhydantoin	630-93-3	274.25	
II-7	Ethylene dibromide	106-93-4	187.86	$BrCH_2CH_2Br$
II-8	Glycidol	556-52-5	74.08	
II-9	Indomethacin	53-86-1	357.79	
II-10	Isoniazid	54-85-3	137.14	
II-11	Nitrobenzene	98-95-3	123.11	
II-12	Urethane, Ethyl carbamate	51-79-6	89.09	

II-13	Tributyltin chloride	1461-22-9	325.51	
II-14	Perfluorooctanoic acid	335-67-1	414.07	
II-15	Dichloroacetic acid	79-43-6	128.94	
II-16	Toluene	108-88-3	92.14	
II-17	Acetonitrile	75-05-8	41.05	CH_3CN
II-18	Mannitol	69-65-8	182.17	
II-19	Vanadium pentoxide	1314-62-1	181.88	V_2O_5
II-20	o-Benzyl-p-chlorophenol	120-32-1	218.68	

Multi-Immuno Tox Assay protocol ver. 011E

May. 10th, 2018

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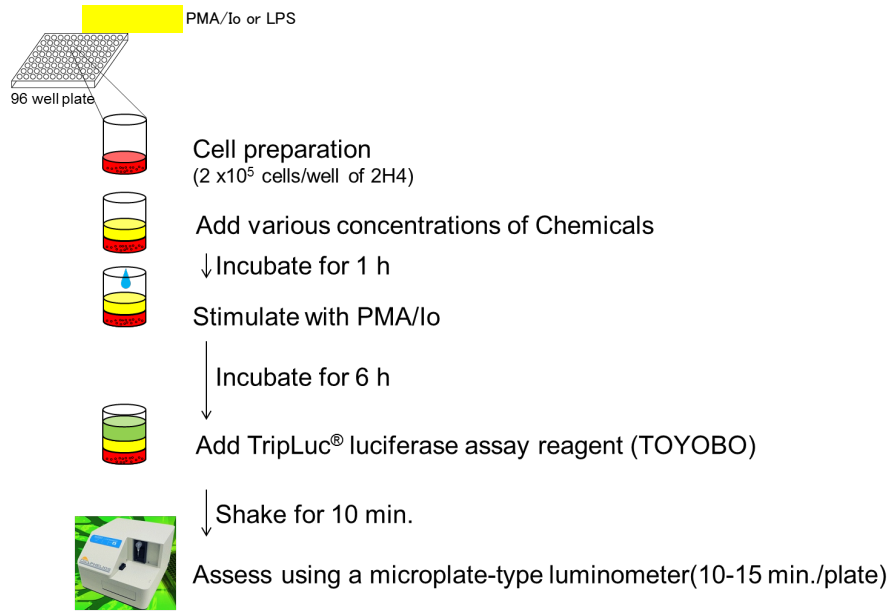
1. Introduction

This protocol describes how to maintain the cells, how to prepare the test chemicals, and how to measure the luciferase activity of 2H4 cells transfected with 3 luciferase genes, stable luciferase green (SLG), stable luciferase orange (SLO) and stable luciferase red (SLR), under the control of IL-2, IFNγ and G3PDH promoters, respectively, for the Multi-Immuno Tox Assay.
(Kimura Y. et al. Evaluation of the Multi-Immuno Tox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs *Toxicol in Vitro*, 28, 759-768, 2014)

Figure 1

Assay design (2 chemicals per one plate)

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	cont (distilled water or DMSO)	PMA/Io only	$A/2^9$ μg/ml	$A/2^8$ μg/ml	$A/2^7$ μg/ml	$A/2^6$ μg/ml	$A/2^5$ μg/ml	$A/2^4$ μg/ml	$A/2^3$ μg/ml	$A/2^2$ μg/ml	$A/2^1$ μg/ml	A μg/ml
B												
C												
D			Chemical A (common ratio of 2、10 concentrations、n=4)									
E	cont (distilled water or DMSO)	PMA/Io only	$B/2^9$ μg/ml	$B/2^8$ μg/ml	$B/2^7$ μg/ml	$B/2^6$ μg/ml	$B/2^5$ μg/ml	$B/2^4$ μg/ml	$B/2^3$ μg/ml	$B/2^2$ μg/ml	$B/2^1$ μg/ml	B μg/ml
F												
G												
H			Chemical B (common ratio of 2、10 concentrations、n=4)									



2. Materials

2-1 Cells

2H4 (IL2-SLG、IFNγ-SLO、G3PDH-SLR)
The human acute T lymphoblastic leukemia cell line Jurkat was obtained from the American Type Culture Collection (Manassas, VA, USA). A Jurkat-derived IL-2 and IFNγ reporter cell line, 2H4, that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFNγ and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd.
(Saito R. et al. Nickel differentially regulates NFAT and NF-κB activation in T cell signaling *Toxicology and Applied Pharmacology*, 254, 245–255, 2011)

2-2 Reagents and equipment

2-2-1 For maintenance of the 2H4 cells
RPMI-1640 (GIBCO Cat#11875-093, 500 mL)
FBS (Biological Industries Cat#04-001-1E Lot: 715004)
Antibiotic-Antimycotic (GIBCO Cat#15240-062)
HygromycinB (CAS:31282-04-9, Invitrogen Cat#10687-010)
G418 (CAS:108321-42-2, Nacalai Tesque Cat#16513-84)
Puromycin (CAS:58-58-2, InvivoGen Cat#ant-pr-1)

2-2-2 For chemical exposure, stimulation and solvents
Ionomycin (CAS:56092-82-1, Sigma Cat#I0634)
Phorbol 12-myristate 13-acetate (PMA) (CAS:16561-29-8, Sigma Cat#P8139)
Ethanol (e.g., Wako Cat#057-00456)
Dimethyl sulfoxide (DMSO) (CAS:67-68-5, Sigma Cat#D5879)
Distilled water (GIBCO Cat#10977-015)

2-2-3 For measurement of the luciferase activity
Tripluc® Luciferase assay reagent (TOYOBO Cat#MRA-301)

2-2-4 Expendable supplies
T-75 flask tissue culture treated (e.g., Corning Cat#353136)
96 well μ clear black plate (flat-bottom, for measurement of the luciferase activity, e.g. Greiner Bio-one Cat#655090)
96 well clear plate (round-bottom, for preparation of chemicals and stimulants)
96 well assay block, 2 mL (e.g., Costar Cat#3960)
Seal for 96 well plate (e.g., Perkin Elmer TopSeal-A PLUS Cat#6050185, EXCEL Scientific SealMate Cat#SM-KIT-SP)
Reservoir
Pipette

2-2-5 Equipment for measurement of luciferase activity
Measuring device: a microplate-type luminometer with a multi-color detection system that can accept two optical filter
e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)
Optical filter: 560 nm long-pass filter and 600 nm long-pass filter
Measuring time: set at 1〜5 sec/well measuring time

2-2-6 Others
Pipetman
8 channel or 12 channel pipetman (optimized for 10~100 μL)
Plate shaker (for 96 well plate)
CO_2 incubator (37°C, 5% CO_2)
Water bath
Cell counter: hemocytometer, trypan blue

2-3 Culture medium

2-3-1 A medium: for maintenance of 2H4 cells (500 mL, stored at 2-8° C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	440 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	50 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	5 mL
Puromycin	InvivoGen # ant-pr-1	10 mg/mL	0.15 μg/mL	7.5 μL
G418	Nacalai Tesque #16513-84	50 mg/mL	300 μg/mL	3 mL
HygromycinB	Invitrogen #10687-010	50 mg/mL	200 μg/mL	2 mL

2-3-2 B medium: for luciferase assay (30 mL, stored at 2-8° C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

2-3-3 C medium: for thawing 2H4 cells (30 mL, stored at 2-8° C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	26.7 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	0.3 mL

2-4 Preparation of the stimulant of 2H4

2-4-1 Phorbol 12-myristate 13-acetate (PMA)

Reagent	Company	Concentration of the stock solution	Final concentration
Phorbol 12-myristate 13-acetate (PMA)	Sigma #P8139	2 mM	25 nM
DMSO	Sigma #D5789		

Dissolve 1 mg PMA using DMSO 811 μL, dispend at 5 μL/tube and store at freezer at -30°C. Use these stocks within 6 months after dissolution.

2-4-2 Ionomycin

Reagent	Company	Concentration of the stock solution	Final concentration
Ionomycin	Sigma # I0634	2 mM	1 μM
Ethanol	Wako #057-00456		

Dissolve 1mg Ionomycin using ethanol 669.3 μL, dispend at 30 μL/tube and store at freezer at -30°C. Use these stocks within 6 months after dissolution.

3. Cell culture

3-1 Thawing of 2H4 cells

Pre-warm 9 mL of C medium in a 15 mL polypropylene conical tube in a 37°C water bath (for centrifugation) and 15 mL of C medium in a T-75 Flask at 37°C in a 5% CO_2 incubator (for culture).

Thaw frozen cells ($2x10^6$ cells / 0.5 mL of freezing medium) in a 37°C water bath, then add to a 15 mL polypropylene conical tube containing 9 mL of pre-warmed C medium. Centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in 15 mL of pre-warmed C medium in a T-75 Flask. Cells are incubated at 37°C, 5% CO_2.

3-2 Maintenance of 2H4 cells

Pre-warm the A medium in a T-75 Flask at 37°C in a 5% CO_2 incubator. The culture medium should be changed to the A medium 3 or 4 days after thawing. At that time, count the number of cells, centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in pre-warmed the A medium in a T-75 Flask. Cells are passaged at $3x10^5$/mL and incubated at 37°C, 5% CO_2.

The interval between subcultures should be 3~4 days. Cells can be used between one and six weeks after thawing.

4. Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.
Use cells between 1 and 6 weeks after thawing.
Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.0 x 10^7 cells for two chemicals are required, but to have some leeway, 3.0 x 10^7 cells for two chemicals should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 2)

Figure 2

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

5. Preparation of chemicals and cell treatment with chemicals

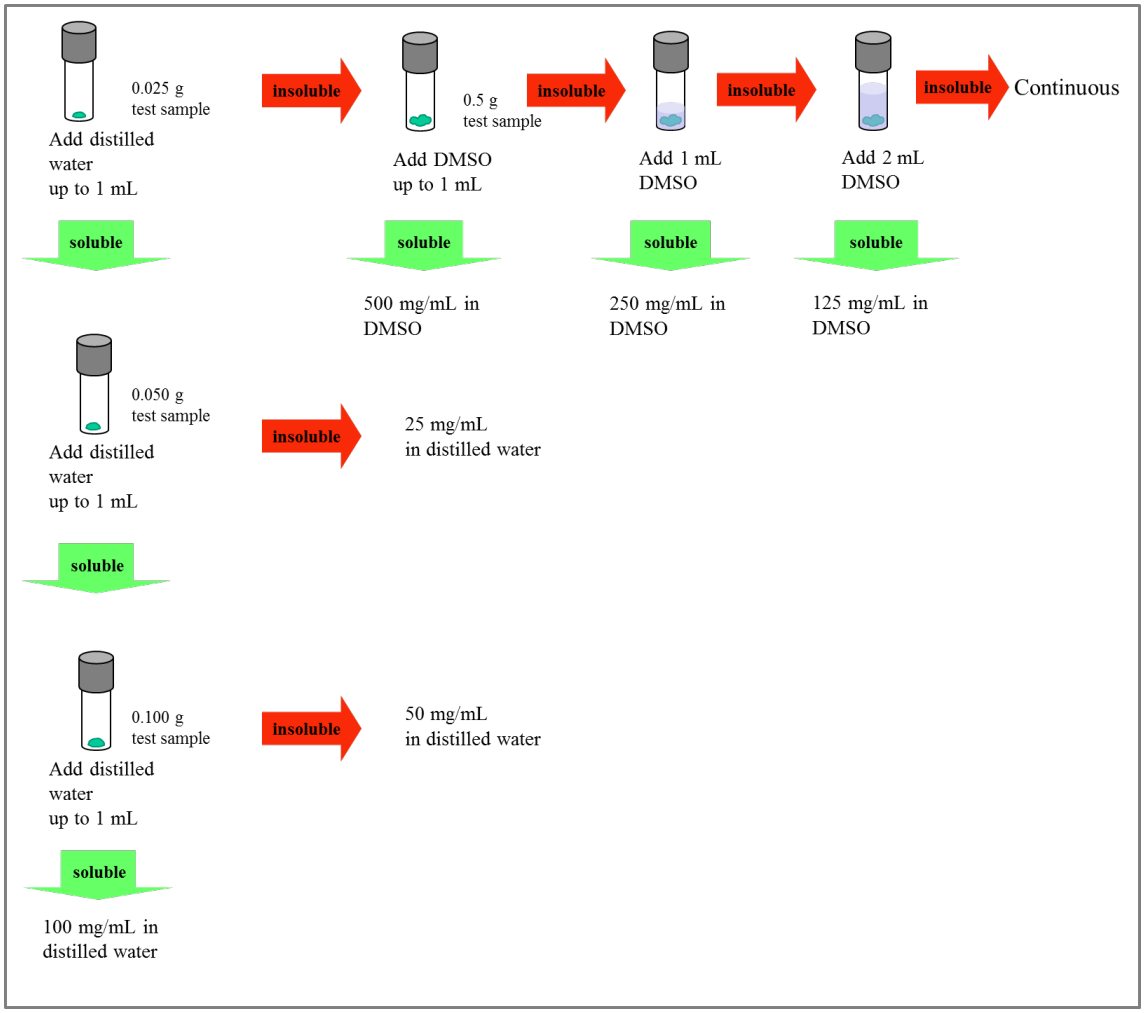
5-1 Dissolution by vehicle (cf. Figure 3)

Dissolve the chemical first in distilled water. Namely, weigh 0.025 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is soluble at 25 mg/mL, weigh 0.050 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is not soluble at 50 mg/mL, 25 mg/mL is the highest soluble concentration. If the chemical is soluble at 50 mg/mL, weigh 0.100 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is not soluble at 100 mg/mL, 50 mg/mL is the highest soluble concentration. If the chemical is soluble at 100 mg/mL, 100 mg/mL is the highest soluble concentration.

If the chemical is not soluble in water, the chemical should be dissolved in DMSO at 500 mg/mL. Namely, weigh 0.5 g of the test chemical in volumetric flask and add DMSO up to 1 mL.
If the chemical is not soluble at 500 mg/mL, the highest soluble concentration should be determined by diluting the solution from 500 mg/mL at a common ratio of two (250 mg/mL → 125 mg/mL → continued if needed) with DMSO.

Sonication and vortex may be used if needed, and attempt to dissolve the chemical for at least 5 minutes. Being soluble should be confirmed by centrifugation at 15,000 rpm (≈20,000 x g) for 5 min and absence of precipitation. The chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

Figure 3



In the first experiment (1st experiment), when the chemical is prepared in distilled water, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration using distilled water. When the chemical is prepared as a DMSO solution, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration using DMSO.

In the second to fourth experiment (2nd to 4th experiment), determine the minimum concentration at which I.I.-SLR-LA (mentioned later in **10**) became lower than 0.05 in the 1st experiment, use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 10 serial dilutions at a common ratio of 2 from the highest concentration. If I.I.-SLR-LA did not become lower than 0.05 or became lower than 0.05 at the highest concentration in the 1st experiment, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration in the 1st experiment.
For example, in Figure 3 below, the minimum concentration at which I.I.-SLR-LA became lower than 0.05 is 1.95 μg/ml. The highest concentration of the chemical to examine is the concentration one step (2-times) higher than 1.95 μg/ml, which is 3.91 μg/ml.
In Figure 4 below, I.I.-SLR-LA did not become lower than 0.05. In such a case, the highest concentration of the chemical to examine is the highest concentration in the 1st experiment, namely 125 μg/ml.

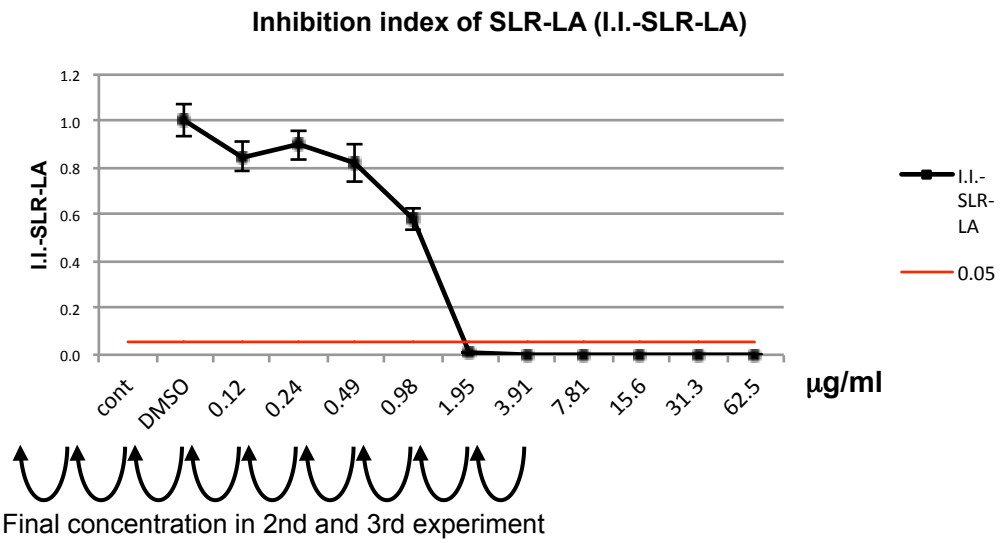


Figure 3.

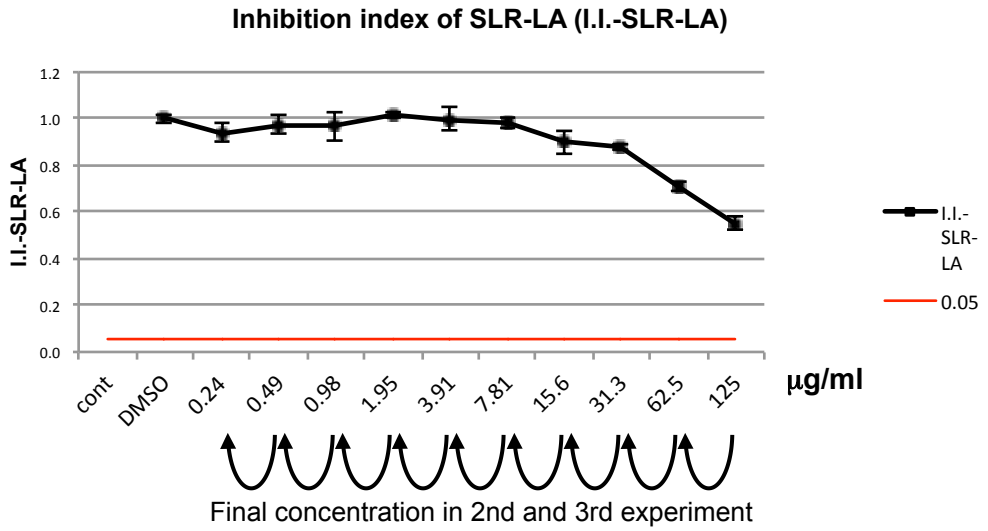


Figure 4

5-2 When the chemical is prepared in distilled water

If the chemical is prepared at a lower concentration, use the prepared concentration instead of the 100 mg/mL distilled water solution.

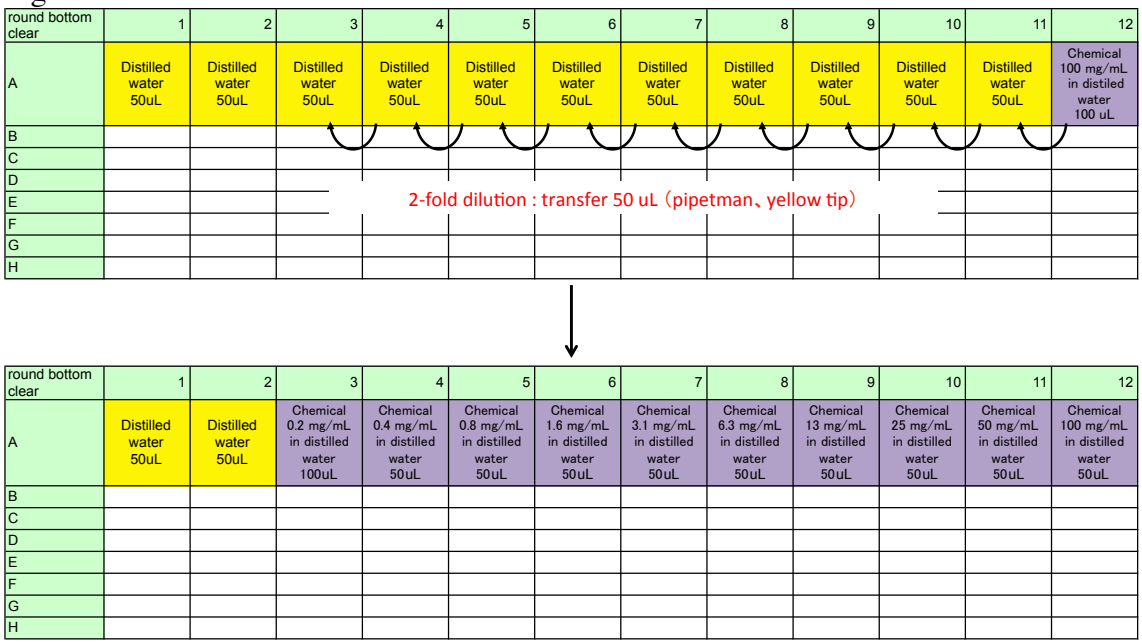
5-2-1 Arrangement of chemicals and vehicle

Add 100 µL of the 100 mg/mL distilled water solution of the chemical to well #A12, and 50 µL of the distilled water to wells #A1-#A11 of the 96 well clear plate (round bottom).

5-2-2 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 4 from well #A11 to well #A3. Transfer 50 µL to the next (left) well. (cf. Figure 4)

Figure 4



5-2-3　2 step dilution

Add 20 µL of the diluted chemical to 480 µL of the B medium prepared in the assay block. And add 50 µL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%) (cf. Figure 5-7).

Figure 5

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Chemical 0.2 mg/mL in distilled water 100uL	Chemical 0.4 mg/mL in distilled water 50uL	Chemical 0.8 mg/mL in distilled water 50uL	Chemical 1.6 mg/mL in distilled water 50uL	Chemical 3.1 mg/mL in distilled water 50uL	Chemical 6.3 mg/mL in distilled water 50uL	Chemical 13 mg/mL in distilled water 50uL	Chemical 25 mg/mL in distilled water 50uL	Chemical 50 mg/mL in distilled water 50uL	Chemical 100 mg/mL in distilled water 50uL
B												
C												
D												
E												
F												
G												
H												

20uL

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL
B												
C												
D												
E												
F												
G												
H												

Figure 6

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500uL	B medium 500uL	Chemical 0.008 mg/uL in B medium 500uL	Chemical 0.02 mg/mL in B medium 500uL	Chemical 0.03 mg/mL in B medium 500uL	Chemical 0.06 mg/mL in B medium 500uL	Chemical 0.1 mg/mL in B medium 500uL	Chemical 0.3 mg/mL in B medium 500uL	Chemical 0.5 mg/mL in B medium 500uL	Chemical 1 mg/mL in B medium 500uL	Chemical 2 mg/mL in B medium 500uL	Chemical 4 mg/mL in B medium 500uL
B												
C												
D												
E												
F												
G												
H												

50uL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

Figure 7 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
B	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
C	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
D	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

5-3 When the chemical is prepared as a DMSO solution

If the chemical is prepared at a lower concentration, use the prepared concentration instead of 500 mg/mL DMSO solution.

5-3-1 Arrangement of chemicals and vehicle

Add 100 µL of the 500 mg/mL DMSO solution of the chemical to well #A12, 50 µL of DMSO to wells #A1-#A11, and 90 µL of the B medium to wells #B1-#B12 of the 96 well clear plate (round bottom)

5-3-2 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 8 from well #A11 to well #A3. Transfer 50 µL to the next (left) well. (cf. Figure 8)

Figure 8

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 500 mg/mL in DMSO 100uL
B	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
C												
D												
E												
F												
G												
H												

2-fold dilution : transfer 50 uL（pipetman、yellow tip）

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 1.0 mg/mL in DMSO 100uL	Chemical 2.0 mg/mL in DMSO 50uL	Chemical 3.9 mg/mL in DMSO 50uL	Chemical 7.8 mg/mL in DMSO 50uL	Chemical 16 mg/mL in DMSO 50uL	Chemical 31 mg/mL in DMSO 50uL	Chemical 63 mg/mL in DMSO 50uL	Chemical 125 mg/mL in DMSO 50uL	Chemical 250 mg/mL in DMSO 50uL	Chemical 500 mg/mL in DMSO 50uL
B	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
C												
D												
E												
F												
G												
H												

5-3-3 Dilution of DMSO solution with the B medium

Dilute 10 µL of the DMSO solution of the chemical in wells #A1-#A12 with 90 µL of the B medium using an 8-12 channel pipetman. (cf. Figure 9)

Figure 9

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 1.0 mg/mL in DMSO 100uL	Chemical 2.0 mg/mL in DMSO 50uL	Chemical 3.9 mg/mL in DMSO 50uL	Chemical 7.8 mg/mL in DMSO 50uL	Chemical 16 mg/mL in DMSO 50uL	Chemical 31 mg/mL in DMSO 50uL	Chemical 63 mg/mL in DMSO 50uL	Chemical 125 mg/mL in DMSO 50uL	Chemical 250 mg/mL in DMSO 50uL	Chemical 500 mg/mL in DMSO 50uL
B	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
C												
D												
E												
F												
G												
H												

10uL

↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 40uL	DMSO 100% 40uL	Chemical 1.0 mg/mL in DMSO 90uL	Chemical 2.0 mg/mL in DMSO 40uL	Chemical 3.9 mg/mL in DMSO 40uL	Chemical 7.8 mg/mL in DMSO 40uL	Chemical 16 mg/mL in DMSO 40uL	Chemical 31 mg/mL in DMSO 40uL	Chemical 63 mg/mL in DMSO 40uL	Chemical 125 mg/mL in DMSO 40uL	Chemical 250 mg/mL in DMSO 40uL	Chemical 500 mg/mL in DMSO 40uL
B	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0.10 mg/mL DMSO 10% in B medium 100uL	Chemical 0.20 mg/mL DMSO 10% in B medium 100uL	Chemical 0.39 mg/mL DMSO 10% in B medium 100uL	Chemical 0.78 mg/mL DMSO 10% in B medium 100uL	Chemical 1.6 mg/mL DMSO 10% in B medium 100uL	Chemical 3.1 mg/mL DMSO 10% in B medium 100uL	Chemical 6.3 mg/mL DMSO 10% in B medium 100uL	Chemical 12.5 mg/mL DMSO 10% in B medium 100uL	Chemical 25 mg/mL DMSO 10% in B medium 100uL	Chemical 50 mg/mL DMSO 10% in B medium 100uL
C												
D												
E												
F												
G												
H												

5-3-4　2 step dilution

Add 10 µL of the diluted chemical to 490 µL of the B medium prepared in the assay block. And add 50 µL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 5-3-3 to 5-3-4 as quickly as you can, and do not leave a long time at step after 5-3-3 or Figure 10. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%) (cf. Figure 10-12).

Figure 10

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 40uL	DMSO 100% 40uL	Chemical 1.0 mg/mL in DMSO 90uL	Chemical 2.0 mg/mL in DMSO 40uL	Chemical 3.9 mg/mL in DMSO 40uL	Chemical 7.8 mg/mL in DMSO 40uL	Chemical 16 mg/mL in DMSO 40uL	Chemical 31 mg/mL in DMSO 40uL	Chemical 63 mg/mL in DMSO 40uL	Chemical 125 mg/mL in DMSO 40uL	Chemical 250 mg/mL in DMSO 40uL	Chemical 500 mg/mL in DMSO 40uL
B	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0.10 mg/mL DMSO 10% in B medium 100uL	Chemical 0.20 mg/mL DMSO 10% in B medium 100uL	Chemical 0.39 mg/mL DMSO 10% in B medium 100uL	Chemical 0.78 mg/mL DMSO 10% in B medium 100uL	Chemical 1.6 mg/mL DMSO 10% in B medium 100uL	Chemical 3.1 mg/mL DMSO 10% in B medium 100uL	Chemical 6.3 mg/mL DMSO 10% in B medium 100uL	Chemical 12.5 mg/mL DMSO 10% in B medium 100uL	Chemical 25 mg/mL DMSO 10% in B medium 100uL	Chemical 50 mg/mL DMSO 10% in B medium 100uL
C												
D												
E												
F												
G												
H												

10uL

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL
B												
C												
D												
E												
F												
G												
H												

Figure 11

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0ug/mL DMSO 0.2% in B medium 500uL	Chemical 0ug/mL DMSO 0.2% in B medium 500uL	Chemical 2.0ug/mL DMSO 0.2% in B medium 500uL	Chemical 3.9ug/mL DMSO 0.2% in B medium 500uL	Chemical 7.8ug/mL DMSO 0.2% in B medium 500uL	Chemical 15.6ug/mL DMSO 0.2% in B medium 500uL	Chemical 31.3ug/mL DMSO 0.2% in B medium 500uL	Chemical 62.5ug/mL DMSO 0.2% in B medium 500uL	Chemical 125ug/mL DMSO 0.2% in B medium 500uL	Chemical 250ug/mL DMSO 0.2% in B medium 500uL	Chemical 500ug/mL DMSO 0.2% in B medium 500uL	Chemical 1000ug/mL DMSO 0.2% in B medium 500uL
B												
C												
D												
E												
F												
G												
H												

50uL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

Figure 12 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
B	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
C	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
D	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

6. Preparation of the stimulant (PMA/ionomycin) and addition to 2H4

6-1 Material

・2 mM PMA stock
・2 mM Ionomycin stock
・B medium
・Ethanol

6-2 Preparation of 100 μM PMA

Dilute 2 mM PMA stock with the B medium as follows (20 times, final concentration is 100 μM).

2 mM PMA	B medium	Total	final concentration
5 μL	95 μL	100 μL	100 μM

6-3 Preparation of control and x10 PMA/ionomycin solution

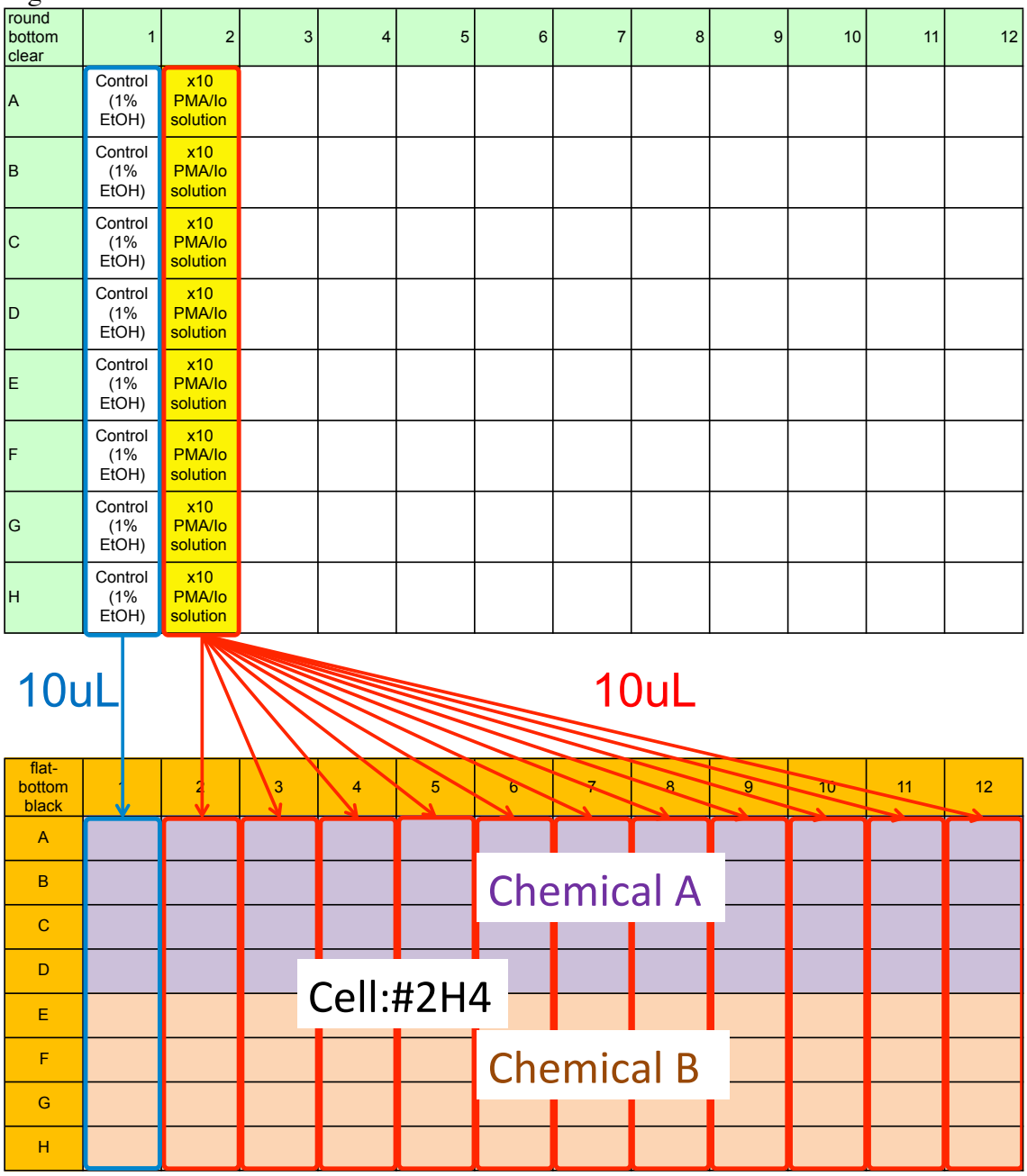
Dilute ethanol, 2 mM ionomycin and 100 μM PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

	B medium	2 mM Ionomycin	100 μM PMA	Ethanol	Total
Control	995 μL	-		5 μL	1000 μL
x10 PMA/ionomycin solution	2382 μL	12 μL	6 μL	-	2400 μL

6-4 Addition of PMA/ionomycin to 2H4

One hour after the addition of chemicals, add 10 µL of control or PMA/ionomycin solution to the cells (#A1-#H1 or #A2-#H12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hour (37°C, CO_2, 5%). (cf. Figure 13)

Figure 13



7. Control

7-1 Preparing control chemical (dexamethasone, cyclosporine A)

7-1-1 Preparing dexamethasone stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
Dexamethasone-water soluble	Sigma #D2915-100MG	2.5 mg/mL	2.5 mg/mL	50 μg/mL
Distilled water	GIBCO Cat#10977-015			

Dissolve 100 mg of Dexamethasone-water soluble with distilled water 40 mL, dispend at 50 μL/tube and store a freezer at -30°C.

7-1-2 Preparing cyclosporine A stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
Cyclosporine A	Sigma #C1832-5MG	100 μg/mL	100 μg/mL	100 μg/mL
DMSO	Sigma #D5789			

Dissolve 5 mg of cyclosporine A with DMSO 50 mL, dispend at 50 μL/tube and store a freezer at -30°C.

7-2 Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.
Use cells between 1 and 6 weeks after thawing.
Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (5.0 x 10^6 cells are required, but to have some leeway, 7.5 x 10^6 cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 14)

Figure 14

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
E												
F												
G												
H												

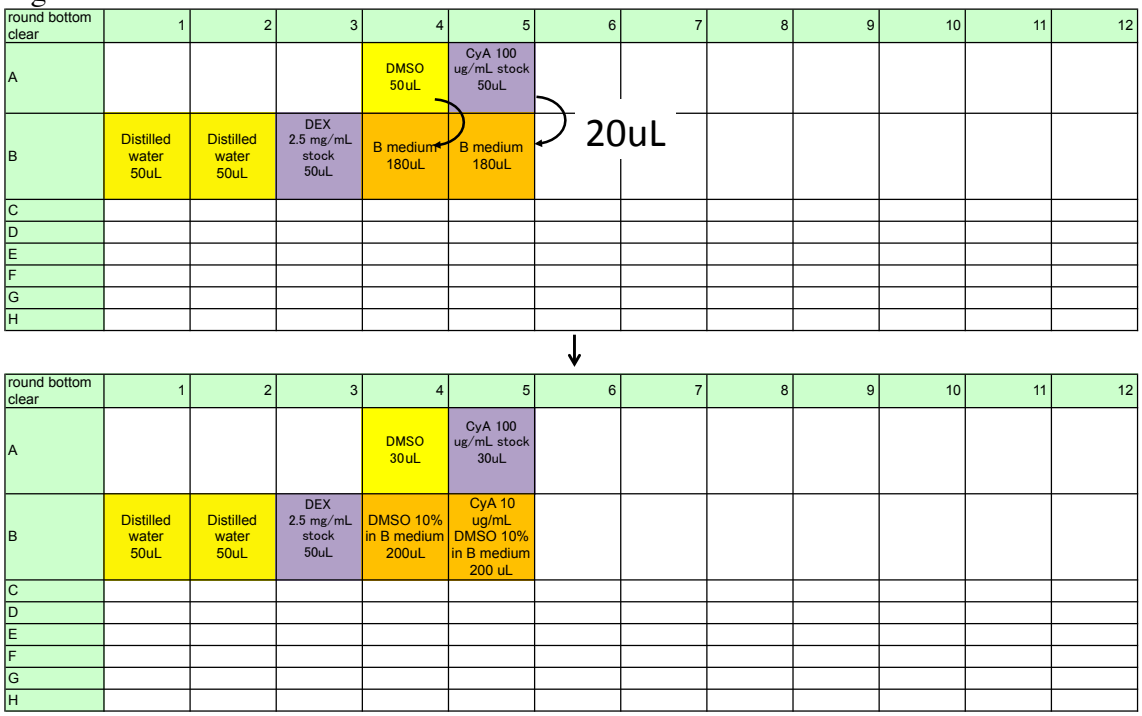
7-3 Arrangement of chemicals and vehicle

Add DMSO 50 µL to #A4, 100 µg/mL cyclosporine A stock 50 µl to #A5, distilled water 50 µl to #B1 and #B2, 2.5 mg/ml dexamethasone stock 50 µl to #B3 and the B medium 180 µl to #B4 and #B5 of the 96 well clear plate (round bottom). (cf. Figure 15)

7-4 Dilution with the B medium

Dilute DMSO in #A4 and cyclosporine A DMSO solution in #A5 by adding 20 µL to the B medium in #B4 and #B5, respectively. (cf. Figure 15)

Figure 15



7-5 2 step dilution

Add 20 µL of the diluted chemical or vehicle to 480 µL (1-3 lanes) or 980 µL (4-5 lanes) of the B medium prepared in the assay block. And add 50 µL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 7-4 to 7-5 as quickly as you can, and do not leave a long time at step after 7-4 or Figure 16. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%). (cf. Figure 16-18)

Figure 16

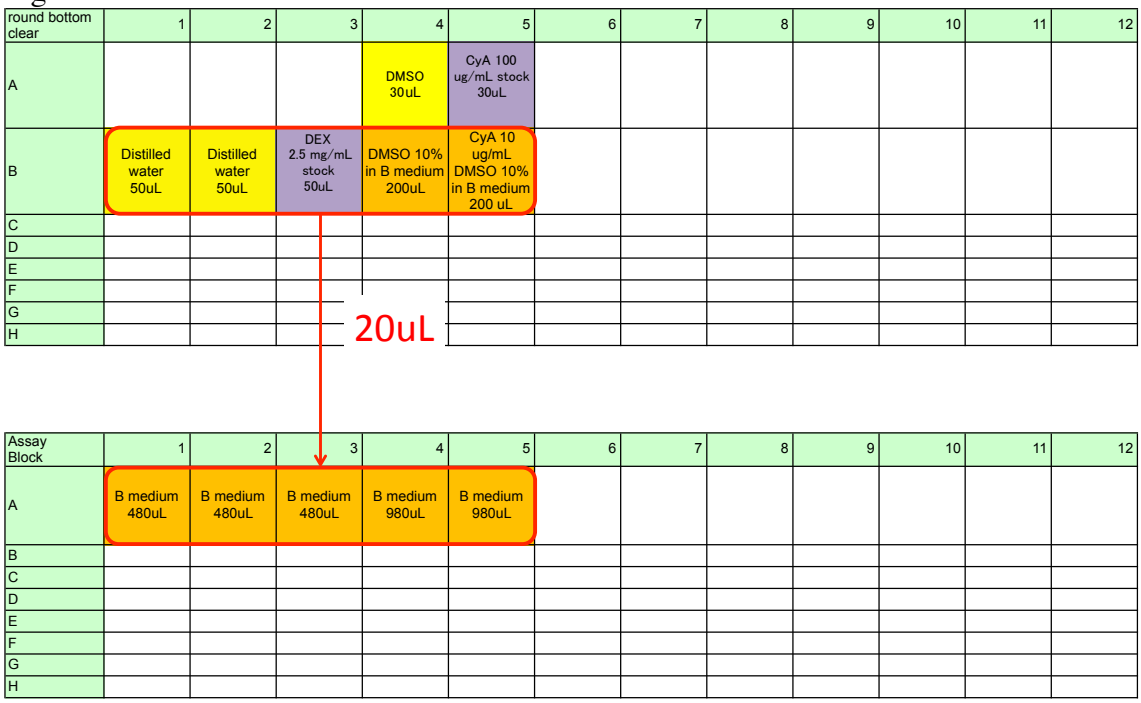


Figure 17

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500uL	B medium 500uL	DEX 100 ug/mL B medium 500uL	DMSO 0.2% B medium 1000uL	CyA 200 ng/mL DMSO 0.2% B medium 1000uL							
B												
C												
D												
E												
F												
G												
H												

50ul

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
E												
F												
G												
H												

Figure 18 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
B	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
C	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
D	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
E												
F												
G												
H												

7-6 Addition of PMA/ionomycin to 2H4

One hour after the addition of dexamethasone and cyclosporine A, add 10 µL of control or PMA/ionomycin solution prepared in §6-3 to the cells (#A1-#D1 or #A2-#D5, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hour (37°C, CO_2, 5%). (cf. Figure 19)

Figure 19

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Control (1% EtOH)	x10 PMA/Io solution										
B	Control (1% EtOH)	x10 PMA/Io solution										
C	Control (1% EtOH)	x10 PMA/Io solution										
D	Control (1% EtOH)	x10 PMA/Io solution										
E	Control (1% EtOH)	x10 PMA/Io solution										
F	Control (1% EtOH)	x10 PMA/Io solution										
G	Control (1% EtOH)	x10 PMA/Io solution										
H	Control (1% EtOH)	x10 PMA/Io solution										

10uL

10uL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
B	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
C	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
D	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
E												
F												
G												
H												

8. Calculation of the transmittance factors

Color discrimination in multi-color reporter assays is generally achieved using detectors (luminometer and plate reader) equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmittance factors of these filters for each bio-luminescence signal color must be calibrated prior to all experiments by following the protocols below.

8-1 Reagents

Single reference samples:
Lyophilized luciferase enzyme reagent for stable luciferase green (SLG)
Lyophilized luciferase enzyme reagent for stable luciferase orange (SLO)
Lyophilized luciferase enzyme reagent for stable luciferase red (SLR)
Assay reagent:
Tripluc® Luciferase assay reagent（TOYOBO Cat#MRA-301）
B medium: for luciferase assay (30 mL, stored at 2- 8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

8-2 Preparation of luminescence reaction solution

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.
Add 200 µL of 100 mM Tris-HCl (pH8.0) contains 10 % glycerol to each tube of lyophilized reference sample to dissolve the enzymes, divide into 10 µL aliquots in 1.5 mL disposable tubes and store in a freezer at -80°C. The stored frozen solution of the reference samples can be used for up to 6 months.
Add 1 mL of the B medium to each tube of frozen reference sample (10 µL sample per tube). Keep the reference samples on ice to prevent deactivation.

8-3 Bioluminescence measurement

Transfer 100 µL of the diluted reference samples to a black 96 well plate (flat bottom) as shown below.
Figure 20

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLG 100 µL	SLG 100 µL	SLG 100 µL									
C												
D	SLO 100 µL	SLO 100 µL	SLO 100 µL									
E												
F	SLR 100 µL	SLR 100 µL	SLR 100 µL									
G												
H												

Transfer 100 µL of pre-warmed Tripluc to each well of the plate containing the reference sample using a pipetman. Shake the plate for 10 min at room temperature (about 25°C) using a plate shaker. Remove bubbles in 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 filters. An example of the raw output data is shown below.

Figure 22

Measurement without Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	3757015	3716611	3810382									
C												
D	1202691	1210208	1122295									
E												
F	2465453	2207572	2077689									
G												
H												

Measurement with Filter 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	1269950	1257268	1289562									
C												
D	808550	813160	754174									
E												
F	2193723	1968240	1853873									
G												
H												

Measurement with Filter 2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	236478	234079	240876									
C												
D	235121	235878	217432									
E												
F	1585258	1420099	1339265									
G												
H												

Six transmittance factors of the optical filters were calculated as follow:

Transmittance factor (κG_{R56})=$\frac{\#B1 \text{ of } F1+ \#B2 \text{ of } F1+ \#B3 \text{ of } F1}{\#B1 \text{ of } F0+ \#B2 \text{ of } F0+ \#B3 \text{ of } F0}$

Transmittance factor (κO_{R56})=$\frac{\#D1 \text{ of } F1+\#D2 \text{ of } F1+\#D3 \text{ of } F1}{\#D1 \text{ of } F0+\#D2 \text{ of } F0+\#D3 \text{ of } F0}$

Transmittance factor (κR_{R56})= $\frac{\#F1 \text{ of } F1+\#F2 \text{ of } F1+\#F3 \text{ of } F1}{\#F1 \text{ of } F0+\#F2 \text{ of } F0+\#F3 \text{ of } F0}$

Transmittance factor (κG_{R60})=$\frac{\#B1 \text{ of } F2+ \#B2 \text{ of } F2+ \#B3 \text{ of } F2}{\#B1 \text{ of } F0+ \#B2 \text{ of } F0+ \#B3 \text{ of } F0}$

Transmittance factor (κO_{R60})=$\frac{\#D1 \text{ of } F2+\#D2 \text{ of } F2+\#D3 \text{ of } F2}{\#D1 \text{ of } F0+\#D2 \text{ of } F0+\#D3 \text{ of } F0}$

Transmittance factor (κR_{R60})=$\frac{\#F1 \text{ of } F2+\#F2 \text{ of } F2+\#F3 \text{ of } F2}{\#F1 \text{ of } F0+\#F2 \text{ of } F0+\#F3 \text{ of } F0}$

In the case shown above,

Transmittance factors (κG_{R56})=$\frac{1269550+1257268+1289562}{3757015+3716611+3810382}$=0.338

Transmittance factors (κO_{R56})=$\frac{808550+813160+754174}{1202691+1210208+1122295}$=0.672

Transmittance factors (κR_{R56})=$\frac{2193723+1968240+1853873}{2465453+2207572+2077689}$=0.891

Transmittance factors (κG_{R60})= $\frac{236478+234079+240876}{3757015+3716611+3810382}$=0.06

Transmittance factors (κO_{R60})=$\frac{235121+235878+217432}{1202691+1210208+1122295}$=0.195

Transmittance factors (κR_{R60})=$\frac{1585258+1420099+1339265}{2465453+2207572+2077689}$=0.644

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

Input the transmittance factors to #C6-#E7 of the “Data Input” sheet of the Data sheet as follow.

Figure 23

	A	B	C	D	E	F
1	MultiReporter Assay System -Tripluc®- Calculation Sheet					
2						
3		Transmittance Data				
4			SLG	SLO	SLR	
5		F0	1	1	1	
6		F1	κG_{R56}	κO_{R56}	κR_{R56}	
7		F2	κG_{R60}	κO_{R60}	κR_{R60}	
8						

9. Measurement

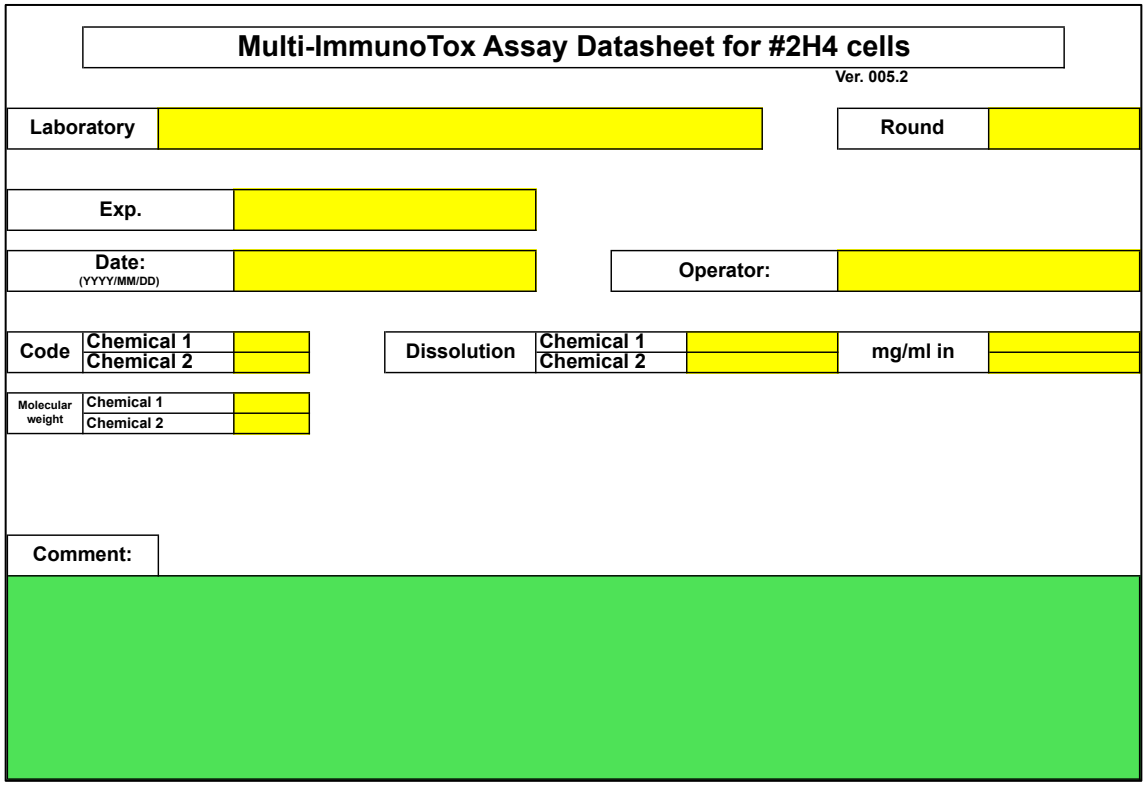
Please refer Appendix 1 for the principle of measurement of luciferase activity.

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 pre-warmed Tripluc from the reservoir to each well of the plate containing the reference sample using an 8 channel or 12 channel pipetman. Shake the plate for 10 min at room temperature (about 25°C) on a plate shaker. Remove bubbles in the solutions in the 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 of (F0) and presence (F1, F2) of the optical filters.

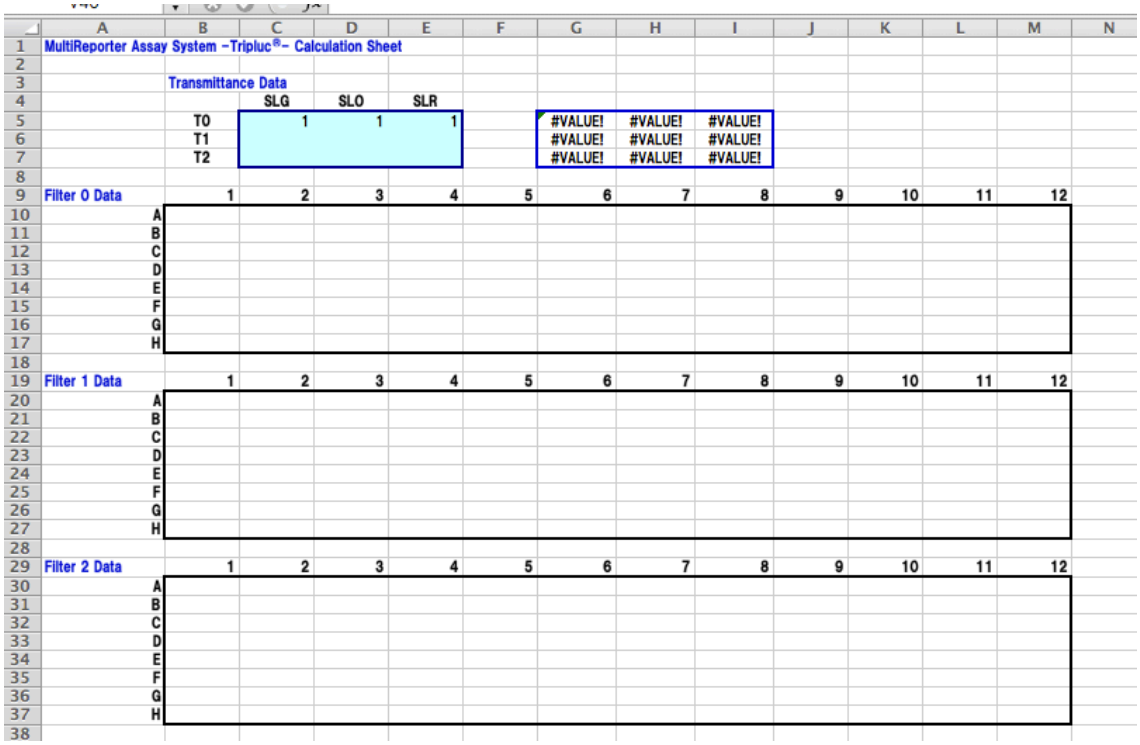
1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration, molecular weight of the chemicals and comments if any to Face Sheet of the data sheet.

Figure 24 “Face Sheet” of the data sheet



2nd. Copy the results of the F0, F1 and F2 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below. In addition, input the transmittance factors calculated in "§5. Calculation of the transmittance factors" to #C6-#E7 of the “Data Input” sheet.

Figure 25 “Data Input” sheet of the data sheet



	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	MultiReporter Assay System –Tripluc®– Calculation Sheet													
2														
3		Transmittance Data												
4			SLG	SLO	SLR									
5		T0	1	1	1		#VALUE!	#VALUE!	#VALUE!					
6		T1					#VALUE!	#VALUE!	#VALUE!					
7		T2					#VALUE!	#VALUE!	#VALUE!					
8														
9	Filter 0 Data	1	2	3	4	5	6	7	8	9	10	11	12	
10	A													
11	B													
12	C													
13	D													
14	E													
15	F													
16	G													
17	H													
18														
19	Filter 1 Data	1	2	3	4	5	6	7	8	9	10	11	12	
20	A													
21	B													
22	C													
23	D													
24	E													
25	F													
26	G													
27	H													
28														
29	Filter 2 Data	1	2	3	4	5	6	7	8	9	10	11	12	
30	A													
31	B													
32	C													
33	D													
34	E													
35	F													
36	G													
37	H													
38														

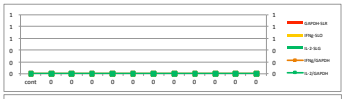
Next, the calculated results for the parameters of the Multi-Immuno Tox assay for each concentration, e.g., SLG-LA, SLO-LA, SLR-LA, nSLG-LA, nSLO-LA, the mean ± SD of SLG-LA, the mean ± SD of SLO-LA, the mean ± SD of SLR-LA %suppression and graphs will automatically appear on the "Result Format" sheet of the data sheet.

Figure 26 "Result Format" sheet of the data sheet

MultiReporter Assay System -Tripluc®- Calculation Sheet

SLG-LA	1	2	3	4	5	6	7	8	9	10	11	12
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Chemical 1



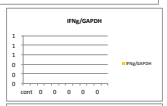
SLO-LA	1	2	3	4	5	6	7	8	9	10	11	12
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Chemical 2

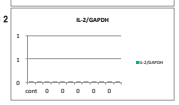
SLR-LA	1	2	3	4	5	6	7	8	9	10	11	12
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

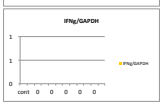
Chemical 1


IL-2/GAPDH


IFNg/GAPDH

Chemical 2


IL-2/GAPDH


IFNg/GAPDH

nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
I.I.-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input "*"												
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input "*"												
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Chemical 1													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml
Chemical 2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml

%Suppression (IL-2)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
%Suppression (IFNg)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

10. Data analysis

Definition of the parameters used in the Multi-Immuno Tox assay

SLG-luciferase activity (SLG-LA): Luciferase activity of stable luciferase green (Under the control of IL-2 promoter)
SLO-luciferase activity (SLO-LA): Luciferase activity of stable luciferase orange (Under the control of IFN-γ promoter)
SLR-luciferase activity (SLR-LA): Luciferase activity of stable luciferase red (Under the control of G3PDH promoter)
Normalized SLG-LA (nSLG-LA): =(SLG-LA)/(SLR-LA)
Normalized SLO-LA (nSLO-LA): =(SLO-LA)/(SLR-LA)
Inhibition index of SLR-LA (I.I.-SLR-LA): The cytotoxic effect of chemicals
=(SLR-LA of 2H4 treated with chemicals)/(SLR-LA of untreated 2H4)
%suppression: The effect of chemicals on IL-2 or IFN-γ promoter
=(1-(nSLG-LA or nSLO-LA of 2H4 treated with chemicals)
/(nSLG-LA or nSLO-LA of non-treated 2H4)) x 100

11. Criteria

11-1 Acceptance criteria

The following acceptance criteria should be satisfied when using the Multi-Immuno Tox Assay method.

If Fold induction of nSLO-LA of PMA/Ionomycin wells without chemicals (=(nSLO-LA of 2H4 cells treated with PMA/Ionomycin) / (nSLO-LA of non-treated 2H4 cells)) demonstrate less than 3.0, the results obtained from the plate containing the control wells should be rejected.

11-2 Criterion

The experiments are repeated until two consistent positive (negative) results or two consistent "no effect results" are obtained. When two consistent results are obtained, the chemicals are judged as the obtained consistent results.

Identification of immunotoxicant is evaluated by the mean of %suppression and its 95% simultaneous confidence interval.

In each experiment, when the chemicals clear the following 3 criteria, they are judged as suppressive or stimulatory. Otherwise, they are judged as no effect chemicals.
1. The mean of %suppression is $\geq$ 35 (suppressive) or $\leq$ -35 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.
2. The result shows two or more consecutive statistically significant positive (negative) data or one statistically significant positive (negative) data with a trend in which at least 3 consecutive data increase (decrease) in a dose dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained in the concentration at which I.I.-SLR-LA is $\geq$ 0.05

12. Update record

Ver. 0011.0E 2018.5.10
Change the criteria

Ver. 0010.0E 2018.1.15 distribution
Change the criteria

Ver. 009.1E 2017.5.8 distribution
Change the criteria

Ver. 009.0E 2017.4.7 distribution
Change the preparation of chemicals
Change the acceptance criteria
Change the criteria

Ver. 008.5E 2016.9.14 distribution
Change the criteria

Ver. 008.4E 2016.9.9 distribution
Change the criteria

Ver. 008.3E 2016.8.1 distribution
Correction of the preparation of PMA and ionomycin
Change the preparation of PMA and ionomycin
Change the preparation of controls
Addition of Acceptance criteria

Ver. 008.1E 2016.2.2 distribution
Changes after the VMT meeting

Ver. 008.0E 2016.1.19
Translation to English
Addition of appendix

Ver. 006.0J 2015.8.17
Change the preparation of chemicals (same method to the IL-8 Luc assay)
Delete the alteration in Ver. 005.0J

Ver. 005.0J 2015.1.9 distribution
Change to use SLR-LA of THP-G8 at the calculation of nSLG-LA of TGCHAC-A4

Ver. 004.1J 2014.12.10 distribution
Change the cellar concentration at cell passage
Modify figure 16, 17

Ver. 004.0J 2014.11.17 distribution
For the validation study at AIST, FDSC and Tohoku university (chemicals: Sodium Bromate ($NaBrO_3$), Nickel (II) sulfate ($NiSO_4$), Dibutyl phthalate (DP), 2-Mercaptobenzothiazole (2-MBT))
Change THP-G1b cells to TGCHAC-A4 cells
Change cell number of THP-G8 and TGCHAC-A4 5x10^4/well to 1x10^5/well
Change concentration of chemicals 11 steps to 10 steps

Change final concentration of LPS (THP-G8 : 25 ng/mL, TGCHAC-A4 : 1 ng/mL)
Change the way of addition of LPS (2 mL/well to 10 mL/well)
Change the criteria

Ver. 002.0J 2013.08.19 distribution
For the validation study at AIST and FDSC (chemicals: $CoCl_2$, $NiSO_4$, Isophorone diisocyanate, 2-Mercaptobenzothiazole)
Change the common ratio 3 to 2
Change the concentration of LPS 100 ng/mL to 25 ng/mL
Add description about the control (dexamethasone)
Delete the appendix about THP-G8 cell

Ver. 001.1J 2012. Nov. 13 distribution
Add the appendix about THP-G8 cell

Ver. 001J 2012. Nov. 09 distribution

Appendix 1 Principle of measurement of luciferase activity

MultiReporter Assay System -Tripluc- can be used with a microplate-type luminometer with a multi-color detection system, which can equip two optical filters (e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)). 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 filter (560 nm LP, Filter 1) and a 600 nm long pass filter (600 nm LP, Filter 2) for optical isolation.

First, using luciferase enzyme reagent 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 coefficient factor listed below.

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)

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

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

This calculation can be performed using the functions "MININVERSE" and "MMULT" in Microsoft Excel. These calculations are integrated in the Data Sheet.

Appendix 2　　Validation of reagents and equipment

5-1 Measurement of transmittance of optical filter for multicolor measurement

For color discriminations in the multi-color reporter assay, detectors (luminometer and plate reader) are usually equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmittance factors of these filters for each bioluminescence signal color have to be calibrated prior to all experiments by following the protocols below.

5-1-1　Reagents

・Single reference samples:

Lyophilized luciferase enzyme reagent of SLG

Lyophilized luciferase enzyme reagent of SLO

Lyophilized luciferase enzyme reagent of SLR

・Assay reagent:

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

・B medium: for luciferase assay (30 mL, stored at 2 – 8°C)

Reagent	Company	Conc.	Final conc. in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

5-1-2 Calibration

5-1-2-1 Preparation of luminescence reaction solution

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature by bathing in water or ambient air. Start the luminometer 30 min before starting the measurement for stabilization of the photomultiplier.

Add 200 μL of 100 mM Tris-HCl (pH8.0) contains 10% glycerol to each tube of the lyophilized reference samples to dissolve the enzymes, followed by separating them into 1.5 mL disposable tubes at 10 μL each and storing in a freezer at -80°C. The stored frozen solution of the reference samples can be used for one half year.

Add 1 mL of the B medium to each tube of the frozen reference sample (10 μL in a tube) and label them as SLG1/1, SLO1/1 and SLR1/1. Keep the reference samples on ice to prevent deactivation.

Prepare dilution series of the single reference samples of SLG, SLO and SLras follows. Dilute 0.3 mL of each 1/1 solution with 0.9 mL of the B medium to make SLG1/4, SLO1/4 and SLR1/4. In the same manner, prepare 1/16 and 1/64 solution of each. Keep diluted reference samples on ice.

5-1-2-2 Bioluminescence measurement

Transfer 100 μL of the diluted reference samples to a black 96 well plate (flat bottom) as shown below.

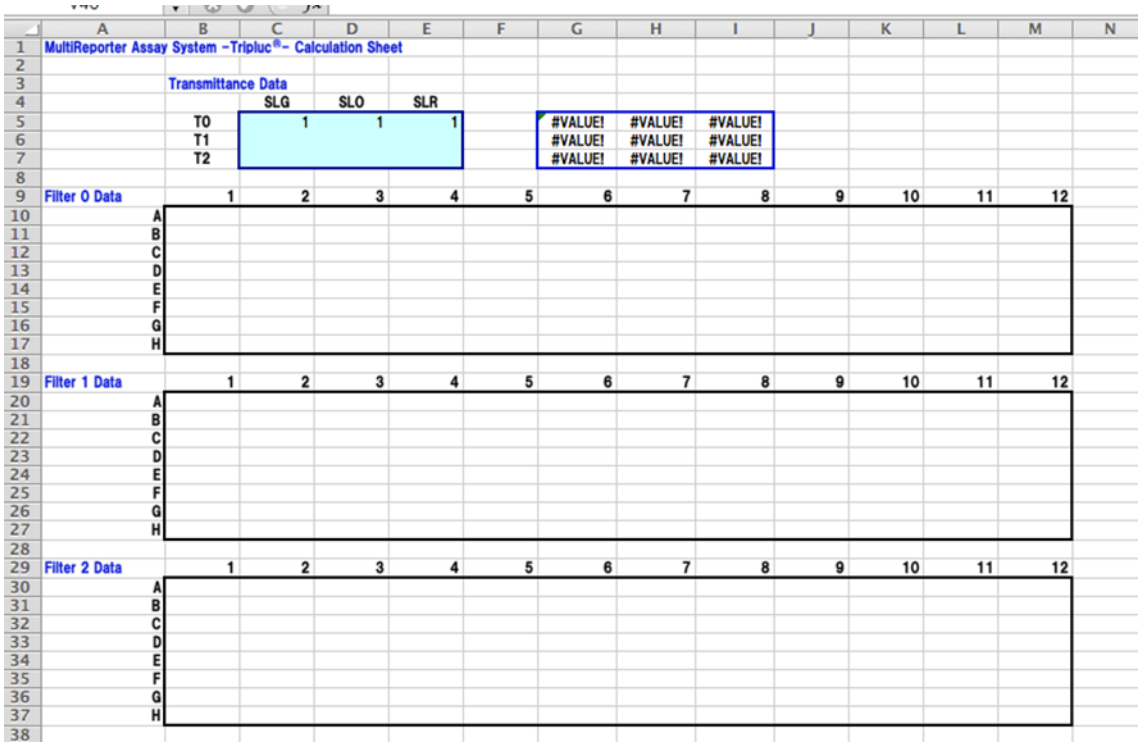
Figure 27

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLG 1/1	SLG 1/1	SLG 1/1	SLG 1/4	SLG 1/4	SLG 1/4	SLG 1/16	SLG 1/16	SLG 1/16	SLG 1/64	SLG 1/64	SLG 1/64
C												
D	SLO 1/1	SLO 1/1	SLO 1/1	SLO 1/4	SLO 1/4	SLO 1/4	SLO 1/16	SLO 1/16	SLO 1/16	SLO 1/64	SLO 1/64	SLO 1/64
E												
F	SLR 1/1	SLR 1/1	SLR 1/1	SLR 1/4	SLR 1/4	SLR 1/4	SLR 1/16	SLR 1/16	SLR 1/16	SLR 1/64	SLR 1/64	SLR 1/64
G												
H												

Transfer 100 μL of pre-warmed Tripluc to each well containing the reference samples of the plate using a pipetman. Shake the plate for 10 min at room temperature (about 25°C) with a plate shaker. Remove bubbles on the solutions in wells if they appear. Place the plate into 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.

Copy the results of the F0, F1 and F2 measurement (values are expressed as counts) and paste it to the appropriate area in the “Data Input” sheet of the data sheet for data analyses shown below.

Figure 28



MultiReporter Assay System –Tripluc®– Calculation Sheet

Transmittance Data

	SLG	SLO	SLR			
T0	1	1	1	#VALUE!	#VALUE!	#VALUE!
T1				#VALUE!	#VALUE!	#VALUE!
T2				#VALUE!	#VALUE!	#VALUE!

Filter 0 Data	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Filter 1 Data	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Filter 2 Data	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Record all the results for quality control.

5-2 Quality control of equipment

In order to confirm the detector stability as the quality control, the reference luciferase sample, optical property, the protocol described here should be performed at the beginning of the experiments every day.

5-2-1 Light source

LED Plate: Reference LED light source plates equipped with stabilized red, green, and blue LEDs are commercially available. For example,

TRIANT® (wSL-0001) by ATTO (Tokyo, Japan)

L12367 by Hamamatsu Photonics (Shizuoka, Japan)

5-2-2 Data collection (an example using TRIANT® by ATTO)

1) Start luminometer 30 min before starting the measurement for stabilization of the photomultiplier.

2) Start LED plate and select “PMT” mode.

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

4) 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.
5) Blue, green, and red LEDs are located at the position of #F6, #E6, and #D6, respectively. Copy the collected data of each position to the appropriate area on Sheet "LED" in the excel file of the data sheet.
6) Check the photo-detector performance by comparing with old data of the LED plate. For quality control purpose, every collected data should be recorded.
7) LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than $3\times\sigma$ (= 4.5%).

Appendix 5. Immunotoxicological information of 25 chemicals used in the validation study

Table of Contents

2,4-Diaminotoluene (DAT) [CASRN 95-80-7]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female B6C3F1 mice were orally dosed with 25, 50, or 100 mg/kg DAT for 14 days. Absolute and relative (to body weight and to brain weight) liver weights (LOAEL = 100 mg/kg) were increased compared to controls. No effect on absolute or relative spleen weights were reported. However, trend analyses indicated significant changes in relative spleen weight and spleen/brain ratio in treated mice. Leukocyte and lymphocyte numbers also were increased (LOAEL =
100 mg/kg). The percentage of lymphocytes and polymorphonuclear leukocytes also were increased (LOAEL = 50 mg/kg). No changes in serum chemistry parameters (e.g., ALT levels) and bone marrow parameters (e.g., number of cells in the femur) were noted. The number of spleen cells, and percentage of T- and B-cells (LOAELs = 100 and 25 mg/kg, respectively) were altered in treated animals. While the number of spleen cells was decreased 18% at the highest dose tested, the percentage of T-cells and B-cells were increased 75% and 15%, respectively.
Peak IgM and IgG responses (in response to sheep erythrocytes) were observed on days 4 and 5 after immunization, respectively. DAT produced a dose-dependent decrease in IgM (46% at 100 mg/kg) and IgG (56% at 100 mg/kg) AFC responses based on total spleen activity. DAT exposure also produced a dose-dependent increase in delayed hypersensitivity response to keyhole limpet hemocyanin (2.2-fold increase at 100 mg/kg). Serum CH50 and C3 levels were not significantly affected in mice treated with DAT. The activity of the reticuloendothelial system was increased in the liver (LOAEL = 100 mg/kg), decreased in the spleen (LOAEL = 50 mg/kg) and kidney (LOAEL = 100 mg/kg), and not affected in the lung or thymus of treated mice. Decreased host resistance (LOAEL = 100 mg/kg) to *Streptococcus pneumoniae* and *Listeria monocytogenes*. However host resistance to B16F10 fibrosarcoma and PYB6 melanoma were not affected (Burns et al. 1994).

In vitro data with cells or cell lines

Spleen cells from female B6C3F1 cells treated with 25, 50, or 100 mg/kg DAT for 14 days were evaluated for response to mitogens and DBA/2 spleen cells. DAT exposure did not affect cell

responses to T-cell mitogens PHA and ConA. An increase in responsiveness to LPS was reported in cells obtained from mice treated with 25 or 50 mg/kg, but not those treated with 100 mg/kg.
Spleen cellularity was decreased 20% and 15% at 50 and 100 mg/kg DAT. In response to DBA/2 cells, an enhanced response was observed in responder cells (LOAEL = 100 mg/kg) while no mixed lymphocyte response was noted (Burns et al. 1994).
Peritoneal exudate cells from female B6C3F1 cells treated with 25, 50, or 100 mg/kg DAT for 14 days were allowed to adhere to plastic and the percentage of cells phagocytizing fluorescent Covaspheres or chicken erythrocytes was measured. No significant change in the percentage of phagocytosis was noted at any of the doses (Burns et al. 1994).
Splenic NK cell activity was decreased in cells obtained from mice exposure to DAT for 14 days. A dose-dependent decrease was observed at all effector/target ratios tested (100/1, 50/1, and 25/1). The LOAEL was 50 mg/kg (Burns et al. 1994).
Spleen cell suspensions from female NMRI mice were evaluated to determine whether DAT could modulate luminol-dependent chemiluminescence of phagocytotic cells. Cells were treated with 0.01, 0.1, 1.0, 10, or 100 mg/L DAT. At concentrations greater than 1 mg/L, a dose-dependent decrease in response was observed. When compared to control levels, chemiluminescence was decreased 43%, 90%, and 100% at 1.0, 10, and 100 mg/kg, respectively (Thierfelder and Masihi 1995).

Mode of action information
Based on the combined effects, Burns and colleagues (1994) proposed that DAT affects differentiation and maturation of leukocytes.

References
Burns LA, Bradley SG, White KL, McCay JA, Fuchs BA, Stern M, et al. 1994. Immunotoxicity of 2,4-diaminotoluene in female B6C3F1 mice. Drug and chemical toxicology 17:401-36; doi:10.3109/01480549409017865.
Thierfelder W, Masihi KN. 1995. Effects of trinitrotoluene (TNT) metabolites on chemiluminescence response of phagocytic cells. International journal of immunopharmacology 17: 453-6.

5,5-Diphenylhydantoin (DPH) [CASRN 57-41-0]

Human Data

Data from epidemiology studies
In a study of 51 epileptic patients, 20 of whom had not received anticonvulsant treatment for at least two years and 31 of whom had received DPH at 300 mg/24 hours for at least 4 months, the DPH treated group had decreased serum levels of IgA (156 ± 65 mg/100 mL) and IgM (121 ± 43 mg/100 mL) as compared to untreated epileptics (IgA, 179 ± 70 mg/100 mL; IgM, 133 ± 50 mg/100 mL) or control subjects (n= 15; IgA, 223 ± 49 mg/100 mL; IgM, 163 ± 48 mg/100 mL). Serum IgG levels were not statistically significantly different among the groups. The authors concluded that DPH treatment suppresses the normal function of the humoral immune response and that epilepsy may be a contributing factor (Badawy et al. 1991).
Peripheral blood lymphocytes, serum immunoglobulins and complement (C3 and C4) protein concentrations were determined in 20 patients with idiopathic epilepsy who were receiving 200- 300 mg DPH treatment and 30 healthy controls. A significant decrease in T-suppressor cells (28%) and subsequently higher T-helper to T-suppresor lymphocyte ratio (36%) were observed in DPH treated patients. A significant increase in B-lymphocytes (39%) and in serum IgM levels (data in graph) was also observed in DPH treated patients as compared to controls. No significant changes in serum concentrations of IgG, IgA or complement proteins was observed (Basaran et al. 1989).
Peripheral blood lymphocyte subsets, serum immunoglobulins and complement (C3 and C4) protein concentrations were determined in 40 healthy subjects, 30 DPH treated patients (200-300 mg/day), 22 carbamazepine treated patients, and 38 untreated epilepsy patients. Subjects receiving drug therapy had been taking the drug for 3 months up to 20 years. The DPH treated group had decreased IgA (19% and 24%, respectively) and IgG (16% and 14%, respectively) as compared to both healthy subjects and untreated epileptic patients. Significantly lower T-suppressor lymphocyte counts (23% decrease) was observed when compared to healthy controls. Significantly higher T-helper to T-suppressor lymphocyte ratio was observed when compared to healthy subjects and untreated epileptic patients. No significant differences in C3 or C4 protein levels were observed in DPH treated patients as compared to controls (Basaran et al. 1994).
Serum IgA values were determined in 191 patients taking DPH (dosage not provided). A reduction in serum IgA levels was observed in up to 20% of the patients. Cellular immune status was assessed in the 11% of patients with IgA values lower than two standard deviations below the mean and included: lymphocyte counts, lymphocyte population studies and responses to *in vitro* mitogen stimulation. No significant variations from control values were observed in any of the evaluated endpoints (Burks et al. 1989).

In vitro data with cells or cell lines
No data were located.

Mode of action information
DPH (20 µg/mL) induced IL-1 activity and potentiated LPS-induced IL-1 production in human PMBC and in U-937 cells, a stable monocytic cell line (Modeer et al. 1989).
DPH treatment can lead to a decrease of suppressor T cells and a reversible IgA deficiency in patients with epilepsy. Gingival overgrowth, which often develops in patients taking DPH, is hypothesized to be due to increased production of both IL-6 and IL-8, combined with elevations of basic fibroblast growth factor as observed *in vitro* using human gingival fibroblasts (Beghi and Shorvon 2011; Godhwani and Bahna 2016).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Male Balb/C mice were given DPH at doses of 0, 25, 50 or 100 mg/kg via oral gavage for 7 days. DPH significantly increased cellularity in the spleen (LOAEL = 25 mg/kg), however, both the direct and indirect plaque-forming cells responses following intraperitoneal injection with sheep erythrocytes, were significantly depressed (LOAELs = 25 mg/kg). A significant decrease in the delayed type hypersensitivity in response to sheep erythrocytes was also observed (LOAEL = 25 mg/kg) (Andrade-Mena et al. 1994).
Pregnant Balb/C mice were treated with DPH at doses of 0, 20, 40, and 60 mg/kg via oral gavage on days 9 through 18 of gestation. A dose-related suppression of humoral immune function (measured as the antibody response to type III pneumococcal polysaccharide) was observed in male and female offspring at 25 days, but not at 15 weeks of age (NOAEL = 20 mg/kg). Female offspring of dams treated with 20 or 60 mg/kg DPH had greater antibody levels than controls. No difference was noted in female offspring of dams treated with 40 mg/kg DPH when compared to controls. Cell-mediated immune function (as measured by delayed-type hypersensitivity response to oxazolone) was not affected in offspring of treated dams. Immunosuppressive effects also were greater in offspring born with an open eye defect, also attributed to DPH treatment (Chapman and Roberts 1984).
Female B10.s, B10.d2 and DBA/2 mice were injected with 2 mg DPH and received a single injection of 10 µg TNP-OVA subcutaneously into the right hind footpad. Popliteal lymph nodes (PLN) were isolated 7 days after injection. DPH increased the number of cells in all three strains (B10.s>B10.d2>DBA/2) (data in graph). IgG1 production to TNP-OVA was increased in all three mouse strains (in B10.d2 about 850-fold; and in B10.s and DBA/2 about 120-fold). DPH treatment did not facilitate immune complex deposition in any of the mouse strains, six days after challenge (Albers et al. 1999).
DPH (administered subcutaneously) produced a significant, dose-dependent response in the PLN assay at 0.5 mg (mean PLN index =1.60 ± 0.18) and 1.0 mg (mean PLN index = 2.79 ± 0.30) as compared to control (mean PLN index = 1.11 ± 0.24) in C3Hf mice. The maximal response occurred at 6-8 days post treatment and returned to normal after 3-4 weeks. The observed response was proposed to be T-lymphocyte dependent since only heterozygous C3H +/nu mice developed PLN enlargement whereas their congenitally athymic C3H nu/nu counterparts did not.

The PLN response to DPH was significantly amplified in thymectomized C57BL/10 mice (PLN index = 6.73 ± 0.83 vs. control PLN index = 2.93 ± 0.53). Proliferation of B lymphocytes was considered a major contributor to the PLN enlargement. A marked increase in IgM and IgG secreting cells was observed following inoculation of BALB/c mice with 1 mg DPH. A maximal increase was observed 10 days after treatment (Gleichmann et al. 1982).
Male C3H/HeN mice were given intraperitoneal injections of DPH (10 mg/mL, once per day) for 28 days and immunized with 100 µg KLH on day 14 and 21. Serum levels of anti-KLH IgG and IgE antibodies were determined on day 28. The KLH-specific IgE response was significantly increased compared to control (data in graph); the IgG response was not changed. Plasma ACTH and corticosterone were significantly higher in DPH-treated mice as compared to controls (data not provided) (Okada et al. 2001).

In vitro data with cells or cell lines
Splenocytes from DPH-treated mice (10 mg/mL for 28 days) immunized with KLH were cultured for 3 days with 50 or 100 µg/mL KLH. No effect on proliferation was noted in splenocytes from DPH-treated mice at either concentration of KLH. Comparatively, splenocytes from control mice immunized with KLH showed a potent proliferative response to stimulation with 50 or 100 µg/mL KLH. T cell function was also impaired in splenocytes from DPH-treated mice, in response to nonspecific mitogens (ConA and LPS) and in response to cross-linking of CD3. The accessory cell function (e.g. macrophages) was also impaired in spleen cells from DPH-treated mice. IL-4 production was significantly enhanced, while IFN-γ and IL-2 production, and NK cell activity were significantly reduced in spleen cells from DPH-treated mice (data in graphs or not provided). IL-1α production was decreased in spleen adherent cells from DPH-treated mice stimulated with *S. aureus*. No effect on IL-6 or IL-12 levels was reported (Okada et al. 2001).
The offspring of female C3H Orleans mice treated with 25 mg/kg diphenylhydantoin 2 times/day throughout gestation, exhibited a reduced thymic cortex and low mitotic activity in the lymphoid population. The reticuloepithelial tissue was enlarged. In the spleen, the white pulp was enlarged due to lymphocyte accumulation. The dams did not exhibit any changes in the thymus or spleen following treatment (Kohler et al. 1987).

Mode of action information
Heat shock proteins were not induced in the PLNs in female BALB/c mice injected subcutaneously with 2 mg DPH (Albers et al. 1996).
Male ICR mice injected intraperitoneally with 60 mg diphenylhydantoin for 3, 8 and 30 days exhibited elevated levels of serum glucocorticoids and thymic atrophy throughout the experiment (Hirai and Ichikawa 1991).

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Modeer T, Karsten J, Weintraub A, Gidlund M, Sundqvist KG. 1989. Phenytoin induces interleukin-1 production in vitro. Life sciences 44: 35–40.
Okada K, Sugiura T, Kuroda E, Tsuji S, Yamashita U. 2001. Phenytoin promotes Th2 type immune response in mice. Clinical and experimental immunology 124: 406–13.

Acetonitrile [CASRN 75-05-8]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
In F344/N rats exposed to acetonitrile by inhalation for 13 weeks, gross and histophathologic changes were evaluated in males (800 and 1600 ppm) and females (1600 ppm) that died during the study. Changes reported included thymic atrophy and splenic lymphoid depletion. Decreased absolute and relative thymus weights also were reported in male and female rats (LOAEL =
800 ppm). In F344/N rats exposed to 100, 200, or 400 ppm acetonitrile for 2 years, no immune related effects were reported (National Toxicology Program 1996)
In B6C3F1 mice exposed to acetonitrile by inhalation for 13 weeks, lymphoid depletion and lymphocytolysis in the thymus, spleen and bone marrow was reported in animals that died. A lack of immune effects were reported in mice exposed to acetonitrile for 2 years (NOAEL = 200 ppm) (National Toxicology Program 1996).
Based on a 14-day inhalation study in B6C3F1 mice (doses not provided), acetonitrile was not identified as an immunotoxicant (Luster et al. 1992).
Male Wistar rats were subcutaneously injected with acetonitrile at a dose of 0.8 LD50 (dose not provided). Antibody titer to sheep erythrocytes was decreased by 43%. Additionally, the number of antibody producing cells against sheep erythrocytes and Vi-Ag (no further information provided in article) were decreased by 52% and 27%, respectively. Thymus T-cell count, percentage of natural cytotoxicity (used as a surrogate for NK cell activity), and antibody- dependent cell cytotoxicity also were significantly decreased after acetonitrile exposure. The percentage decreases were calculated as 31%, 52%, and 41%, respectively (Zabrodskii et al.
2002).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

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Benzo(a)pyrene [B(a)P] [CASRN 50-32-8]

Human Data

Data from epidemiology studies
No data were located

In vitro data with cells or cell lines
B(a)P (1 μM) and related metabolites significantly increased IgE-mediated histamine release from human basophils, but did not induce cell death. Additionally, a B(a)P metabolite significantly increased IgE-mediated IL-4 production in human basophils (Kepley et al. 2003). In primary human macrophages, 10 μM B(a)P increased expression of TNF-α and IL-1β and produced no effect on IFNγ, IL-6, or IL-12 expression (Lecureur et al. 2005). Comparatively, B(a)P did not modulate IL-6 or IL-8 production in BEAS-2B cells at concentrations ranging from 0.1 to 10 μM (Chowdhury et al. 2017).
B(a)P inhibited anti-CD3 antibody stimulation of human lymphocyte proliferation (IC50 = 12.82 μM) (Carfi et al. 2007).
Six breast epithelial cell strains were incubated with 4μM B(a)P for 24 hours. Gene expression studies (using Hu-Gene 133A arrays) showed that signal log ratio (SLR) was altered by ≥1.5 for 5 immune-related genes in at least one of the tested cell strains. Four genes were upregulated, while one was down regulated. Up regulated genes were IL1B, MAL, HTLF, and SECTM1. CXCL14 gene expression was down-regulated (John et al. 2009).
PBMCs were exposed to ConA and B(a)P and assessed after 3 days. B(a)P dose-dependently decreased DNA synthesis and cell viability in treated cells (LOAELs = 0.01 and 0.1 μM, respectively). The number of cells recovered during the same period also was decreased (LOAEL = 0.01 μM). B(a)P did not affect IL-2 activity or expression of CD25 on small cells or blasts at concentrations up to 1 μM. B(a)P decreased the percentage of blasts that were CD71+ by 13% at 1 μM. Cell cycle analysis indicated that B(a)P increased the percentage of cells in S- phase and decreased the percentage in G0/G1 phase (Mudzinski 1993).

Mode of action information
Calcium mobilization in human T-cells is a proposed mode of action for B(a)P (Krieger et al. 1994). Additionally, Ah receptor activation by B(a)P is proposed to inhibit differentiation of monocytes to macrophages and cell growth of B-cells which may contribute to immunotoxic effects (Allan and Sherr 2005, 2010; van Grevenynghe et al. 2003).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Lactating C3H/HeJ dams were dosed with 0.25, 5.0, or 100 pmol/week B(a)P via oral gavage on PND 1, 8, and 15. Pups (5-weeks old) were treated with OVA via intratracheal instillation every

2 weeks for 6 weeks. B(a)P had no effect on the number of macrophages or lymphocytes in BAL from male or female offspring not treated with OVA. Additionally, no effect was noted on the number of macrophages or lymphocytes in B(a)P-treated offspring that were immunized with OVA (when compared with offspring only treated with OVA). IL-4, IL-5, IL-13, IL-33, and IFN-γ levels in the BAL were not affected in offspring not treated with OVA. Increased IL-33 and IFN-γ levels were observed in OVA-sensitized female offspring lactationally exposed to 5.0 and 100 pmol/week B(a)P, respectively. Lactational exposure to 0.25 B(a)P increased the total number of mediastinal lymph node cells in males. Lactational 100 pmol/week B(a)P increased numbers of TCRβ+ and CD86+ cells compared with vehicle in non-sensitized male offspring. In non-sensitized female offspring, lactational exposure to 100 pmol/week B(a)P increased numbers of CD11c+ PDCA-1-, CD28+, TCRβ+CD28+, MHC Class II+, and MHC Class II+CD86+ cells.
In OVA-sensitized female offspring, a significant increase in CD11c+PDCA-1+ and CD11c+PDCA-1- cells was observed after exposure to 0.25 and 5.0 pmol/week, respectively (Yanagisawa et al. 2018).
Pregnant C3H/HeB mice were administered 150 mg/kg B(a)P via intraperitoneal injection on GD 11; immune effects were assessed at parturition and again one week after parturition. A significant reduction in newborn CD4+CD8+ (46%), CD4+CD8+Vγ2+ (60%), and CD4+CD8+Vβ2+ (53%) thymocytes were noted. Additionally, CD4+ splenocytes from 1-week-old offspring were significantly reduced (50%) (Rodriguez et al. 1999).
B6C3F1 mice were administered 0.4, 4.0, or 40 mg/kg B(a)P by intratracheal instillation for seven days and immunized with sheep erythrocytes after the last B(a)P exposure. Decreased formation of antigen-specific AFC (by 60%) was observed at 40 mg/kg B(a)P in LALN. When sheep erythrocytes were administered by intraperitoneal injection, an increase in antigen-specific AFC was observed at 40 mg/kg B(a)P in LALN. However, the levels of AFC in the spleen were decreased (Schnizlein et al. 1987).
B6C3F1 mice (3-6 months, 13-16 months, and 23-26 months) were administered 40 mg/kg B(a)P for 8 days by intraperitoneal injection. Mice also were immunized with sheep erythrocytes after day 4 of the B(a)P treatment. Spleens were removed and splenocytes assessed for formation of AFCs. Decreased formation of AFCs was noted in splenocytes from all three age groups. In two sets of experiments, the observed decreases were 23%-43% in mice ages 3-6 months, 63%- 84% in mice ages 16-18 months, and 93% in mice ages 23-26 months (Lyte and Bick 1985).
B6C3F1 mice were administered 5, 20, or 40 mg/kg B(a)P for 14 days by subcutaneous injection. Spleens were removed and ConA-induced production of IL-2 and IL-3 were assessed. While splenocyte IL-2 production was decreased in a dose dependent manner, no effect on splenocyte IL-3 production was noted. As shown in other studies, B(a)P decreased responses to sheep erythrocytes (>95% inhibition). Addition of exogenous IL-2 to the treated splenocytes, reversed the B(a)P-induced inhibition of responses to sheep erythrocytes (Lyte et al. 1987; Lyte and Bick 1986).
Female B6C3F1 mice were administered 10 subcutaneous injections of B(a)P over a 14-day period at doses of 5, 20, or 40 μg/g. KLH-sensitization did not affect delayed hypersensitivity

responses at the tested doses. Additionally, B(a)P treatment did not induce rejection to DBA mice skin that was grafted onto mice. Proliferative responses to PHA were dose-dependently decreased (LOAEL = 20 µg/g B(a)P). Spontaneous and LPS-induced proliferative responses were increased at 5 µg/g B(a)P and significantly decreased at 40 µg/g B(a)P. MLC responses, and the percentage of spleen cells with T- and B-cell surface markers were not significantly affected at any of the tested doses. Additionally, NK cell activity against YAC- I target cells was not impacted in mice treated with 40 µg/g B(a)P (data not provided). Serum IgG levels were dose-dependently decreased in treated mice (18-24%). A reduction in the number of antibody plaque forming cells to sheep erythrocytes and LPS were noted (LOAELs = 20 and 5 µg/g B(a)P, respectively). B(a)P exposure decreased response to TNP-Ficoll without effects on TNP-LPS response. Host resistance studies showed that B(a)P had no effect on PYB6 tumor incidence or susceptibility to *L. monocytogenes* (Dean et al. 1983).

In vitro data with cells or cell lines
Rat and mouse spleen cells were treated with B(a)P for 24 hours and then LPS or PHA for 48 hours. Then, mitogen stimulation was assessed by 5-bromo-2'-deoxyuridine incorporation.
B(a)P inhibited cellular proliferation for both species at similar concentrations (data in graphs).
B(a)P also inhibited rat spleen proliferation that was stimulated by ConA (data provided in graph).
B(a)P inhibited anti-CD3 antibody stimulation of mouse lymphocyte proliferation (IC50 > 160 µM) (Carfi et al. 2007).
B(a)P decreased viability of mouse antigen presenting cells (APC) and increased expression of CD86 expression on APC (LOAELs = 0.1 µM). In murine splenocytes, B(a)P decreased cell viability and proliferation (LOAELs = 0.1 and 1.0 µM, respectively). B(a)P did not modulate the expression of T-cell receptors or CD19 at any of the tested concentrations in murine splenocytes (Chowdhury et al. 2017).
B(a)P decreased ConA induced cellular proliferation of mouse splenic T-cells in a dose-dependent manner (LOAEL = 0.1 µg/mL). Inhibition of IL-2, IL-4, and IFN-γ also was observed in ConA-stimulated splenic T-cells (LOAELs = 0.1, 0.2, and 0.1 µg/mL, respectively) (Guan et al. 2017).
B(a)P inhibited spleen cell response to sheep erythrocytes in a concentration dependent manner (LOAEL = 0.01 µM). B(a)P also inhibited one-way mixed lymphocyte response with a maximal inhibition of 19% (Urso et al. 1986). Similar response of murine spleen cell response to sheep erythrocytes was reported by Kawabata and White (1987) (LOAEL = 1 nM) after incubation for 5 days.
Splenocytes from B6C3F1 mice (3-6 months and 23-26 months) were exposed to 1, 10, or 50 µg/mL B(a)P and sheep erythrocytes for 4-5 days. After end of exposure period, the number of AFCs was determined. Dose-dependent decrease in the number of cells was observed in splenocytes from both age groups (data in graphs) (Lyte and Bick 1985).
B(a)P (in PVP-NaCl) dose-dependently increased LPS-induced IL-1 production by peritoneal exudate macrophages isolated from B6C3F1 mice; tested concentrations ranged from 25 to 800

μg/mL. A concurrent decrease in cell viabilities was noted at the same test concentrations. Comparatively, when B(a)P was dissolved in corn oil no effect on IL-1 production or cell viabilities was noted (Lyte and Bick 1986).

Mode of action information

Disruption of T-cell activities has been associated with B(a)P induced immunotoxic effects (Urso et al. 1986). Modulation of mouse splenic T-cell effects was associated with modulation of calcium levels; which was associated with suppression of the NF-□B and NFAT pathways (Guan et al. 2017).

In addition to T-cell effects, modulation of B-cell population or responses, or macrophage functions also have been implicated in B(a)P mode of action (Saxena et al. 2018; Urso et al. 1986). Hardin and colleagues (1992) proposed that B(a)P-induced suppression of B-cell lymphopoiesis was, partially, produced through induction of programmed cell death. Ah-receptor dependent- and/or independent-pathways could produce the observed effects.

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Cadmium Chloride [CASRN 10108-64-2]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
Cadmium chloride (10–100 µM) inhibited NK (against K562 cells) and antibody-dependent cellular (against P815 cells) cytotoxicity (ADCC) in peripheral blood lymphocytes in a concentration-dependent manner. The estimated 50% inhibition doses (ID50) for NK and ADCC activities were 50 and 100 µM, respectively. NK and ADCC activities were not significantly affected by changing the effector cell:target cell ratios. Cadmium chloride also inhibited cytotoxic activity against K562 or Daudi cells in activated IL-2 cells (data in graph). Time-course studies showed that a significant decrease in NK and ADCC activities was observed when added at 90 minutes after the start of the experiment (Cifone et al. 1990).
Viability of A549 cells was decreased (44.5% of control) after exposure to 75 µM cadmium chloride. At the same concentration, cadmium chloride increased select cytokine levels (e.g., IFN-γ, IL-3, IL-5, IL-10, IL-15, and IL-16). Comparatively, cadmium chloride decreased TGF-β3 levels (Odewumi et al. 2016).

Mode of action information
In vitro studies suggest that in peripheral blood lymphocytes, cadmium chloride modulated phosphoinositide hydrolysis induced by a target molecule. This modulation is proposed to lead to inhibited NK activity (Cifone et al. 1990).
Proposed direct action of cadmium on immunocompetent cells stimulates production and release of cytokines, which may produce proinflammatory effects (Marth et al. 2000).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female BDF1 mice were provided drinking water containing 5, 10, or 50 µg/mL cadmium (as cadmium chloride) for 3 weeks. Antibody response to sheep erythrocytes was decreased in a dose-dependent manner. Splenic plaque-forming cell number decreased by 16% to 28%. A dose-dependent increase in LPS-induced proliferation also was observed (LOAEL = 10 µg/mL). In the absence of a mitogen, cadmium chloride also increased lymphocyte proliferation (LOAEL = 10 µg/mL). No effect was observed when ConA mitogen was used to stimulate proliferation (Blakley 1985).
Female CD1 mice were provided drinking water containing 5, 10, or 50 µg/mL cadmium (as cadmium chloride) for 3 weeks. *In vivo* T-lymphocyte independent (against DNP-Ficoll) and T-

lymphocyte and macrophage independent (against *E. coli*) responses were increased by cadmium exposure (Blakley and Tomar 1986).
Female BDF1 mice were provided drinking water containing 50 µg/mL cadmium (as cadmium chloride) for 3 weeks. Spleen cell suspensions from pooled spleens were separated by adherence techniques and antibody production against sheep erythrocytes was assessed. Suppressed antibody production (26%–34%) was noted in cultures that contained cadmium–exposed T– lymphocytes. Antibody production was similar to controls in cultures that contained cadmium– exposed macrophages (Blakley and Tomar 1986).
Male Sprague–Dawley rats were administered 0.7 or 6 mg/kg cadmium (as cadmium chloride) by oral gavage for 28 days. Splenocyte proliferation was significantly decreased (76% of control) in rats that were administered 6 mg/kg cadmium. Splenocyte IL–2 production also was increased after administration of 6 mg/kg cadmium, when production was normalized with cell number.
No effect was noted on splenocyte IFN–γ production (Wang et al. 2017).
Immunotoxic effects in offspring were noted after exposure to cadmium chloride *in utero* or through milk. In ICR mice administered 2.5 or 5.0 mg/kg cadmium chloride on GD 16, a significant increase in offspring spleen weight was reported (LOAEL = 2.5 mg/kg). Unstimulated spleen lymphocyte proliferation was significantly increased at both tested doses (1.5– to 2–fold). Additionally, ConA, PHA, and LPS stimulation was increased in treated animals (LOAELs = 5.0, 5.0, and 2.5 mg/kg). No effect on delayed–type hypersensitivity to sheep erythrocytes was reported, but an increase in total Ig and IgM antibody titer was noted at 2.5 mg/kg (Soukupova et al. 1991). In offspring that were exposed to cadmium chloride through maternal milk (dams received 5 ppm or 10 ppb cadmium chloride in water until weaning) decreased spleen weights were observed in females, but not males (data in graphs). The effect was greater in lower dosed females. Effects on organ weight did not persist to adulthood. In adult and juvenile rats, effects on cytotoxic activity of splenic NK–cells was noted (data in graphs).
Additionally, cadmium chloride inhibited ConA–induced thymocyte proliferation in both male and female adult rats (Pillet et al. 2005).
Female C57BL/6 mice were exposed (nose–only) to aerosolized cadmium chloride (60–minute exposure to 0.88 mg Cd/m^3) and examined 5–18 days later. Decreased splenic cell viability was observed (data in graph). Significant decreases of proliferative responses to LPS and PHA, and inhibition of IgM secretion in response to sheep erythrocytes were observed. Comparatively, oral chronic exposure (5, 100, or 300 ppm cadmium chloride in water for 12–16 weeks) suppressed IgM response to sheep erythrocytes, without effects on cell viability (Krzystyniak et al. 1987).

In vitro data with cells or cell lines
Splenocytes isolated from male Sprague–Dawley rats were treated with ConA for 24 hours, followed by incubation with 5, 10, or 20 µM cadmium chloride for 4 or 24 hours. After exposure for 4 hours, decreased IL–2 (LOAEL = 5 µM) and IFN–γ (LOAEL = 10 µM) production was observed in the absence of effects on cell proliferation. After exposure for 24 hours, decreased IL–2 (LOAEL = 5 µM) production and cell proliferation (LOAEL = 10 µM) were observed.

When cytokine production after 24-hour exposure was normalized based on cell number,

increased IFN-γ production (LOAEL = 10 μM) was noted. For IL-2 production, a significant decrease was noted at 5 μM and an increase was noted at 20 μM (Wang et al. 2017).

Mode of action information

In RAW264.7 cells, cadmium chloride upregulation of COX-2 and MIP-2 was associated with activation of the phosphatidylinositol 3-kinase/Akt signaling pathway (Huang et al. 2014). Cadmium chloride also may induce overstimulation of nuclear factors of activated T-cells to activate Jurkat T cells (Colombo et al. 2004).

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Dibromoacetic Acid (DBAA) [CASRN 631-61-1]

Human Data

Data from epidemiology studies
No studies were located.

In vitro data with cells or cell lines
In cultured PBMCs collected from healthy, non-smoking volunteers and cultured in DBAA for four hours, DBAA increased the percentage of necrotic human PBMC and decreased PBMC cell size (LOAEL = 5 mM). Increases in the percentage of apoptotic cells and PBMC granulation also was reported (LOAEL = 1 and 5mM, respectively). Caspase-8, -9, and -3 expression were upregulated at 1 and 5 mM. Increased transmembrane mitochondrial potential and levels of reactive oxygen species (ROS) also were noted with DBAA exposure (LOAEL = 1 and 0.1 mM) (Michalowicz et al. 2015).

Mode of action information
DBAA may increase ROS levels and transmembrane mitochondrial potentials (Michalowicz et al. 2015).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
In female F344/N rats exposed to DBAA for 3 months (0-2000 mg/L in drinking water), minimal to mild hematopoietic cell proliferation was noted at the highest dose. A similar effect was not observed in males. While no spleen effects were noted in B6C3F1 mice exposed to DBAA for 3 months (0-2000 mg/L in drinking water), thymus atrophy was reported in males and females (LOAEL = 1000 and 2000 mg/L, respectively) (National Toxicology Program 2007).
In male and female BALB/c mice orally gavaged with 5, 20, or 50 mg/kg DBAA for 28 days altered spleen and thymus weights, and splenic and thymic cellularity were reported. DBAA also inhibited B-cell proliferation (LOAEL = 20 mg/kg). DBAA increased T-cell mitogenesis (value not provided) at 20 mg/kg. DBAA increased apoptosis in spleen and thymus in a dose-dependent manner (values not provided). Additionally, DBAA exposure altered the expression of apoptosis- related genes in spleens and thymus of treated mice. In the thymus, expression of Fas and TRAF2 were altered (2-2.5 fold). In spleens of treated mice, expression of Fas and TRAF2 were increased 5-fold while bcl-2 expression was decreased 1.5-fold. Increased protein expression of Fas and FasL also were observed in spleen and thymus of treated mice (LOAEL = 5 mg/kg) (Gao et al. 2008).

Table 1. Data from Gao et al. (2008)

Endpoint	0 mg/kgs	5 mg/kg	20 mg/kg	50 mg/kg
Male				
Spleen weight (mg)	80.5 ± 2.7	88.7 ± 3.7	91.2 ± 3.4**	94.0 ± 2.5***
Thymus weight (mg)	43.1 ± 3.4	37.6 ± 2.0	33.3 ± 2.8**	33.4 ± 2.3**
Relative spleen weight (mg/g)	3.68 ± 0.14	3.87 ± 0.22	4.18 ± 0.20*	4.23 ± 0.09*
Relative thymus weight (mg/g)	1.85 ± 0.14	1.65 ± 0.09	1.50 ± 0.13**	1.50 ± 0.11**
Splenic cellularity ($x10^7$)	9.00 ± 0.44	9.09 ± 0.28	7.63 ± 0.65	6.11 ± 0.38***
Thymic cellularity ($x10^7$)	8.88 ± 1.06	9.16 ± 0.28	7.39 ± 0.47	5.37 ± 0.82**
Female				
Spleen weight (mg)	78.9 ± 2.2	100.1 ± 7.7**	102.4 ± 5.0**	101.2 ± 4.8**
Thymus weight (mg)	46.9 ± 3.7	47.3 ± 3.9	35.8 ± 2.3*	29.5 ± 3.3***
Relative spleen weight (mg/g)	3.99 ± 0.18	5.33 ± 0.45**	5.29 ± 0.27**	5.22 ± 0.19**
Relative thymus weight (mg/g)	2.39 ± 0.17	2.41 ± 0.18	1.87 ± 0.11*	1.55 ± 0.17***
Splenic cellularity ($x10^7$)	8.60 ± 0.55	8.28 ± 1.19	6.14 ± 1.27	4.65 ± 0.43**
Thymic cellularity ($x10^7$)	7.97 ± 0.53	7.08 ± 0.74	5.42 ± 0.79*	4.28 ± 0.39***

Data are presented as mean ± SEM.

***p < 0.001, **p < 0.01, *p < 0.05, significance assessed by ANOVA when compared with control group (DBA 0 mg/kg).

Increased neuronal expression of immune factors was noted in Sprague-Dawley rats administered 20, 50, or 125 mg/kg DBAA via intragastric injection for 4-weeks. mRNA expression of Iba-1, NK-□B, IL-6, IL-1β, and TNF-α were increased in the pre-frontal cortex and hippocampus of treated rats (LOAEL = 50 mg/kg for all brain regions). Protein levels of Iba-1, NK-□B, IL-6, IL-1β, and TNF-α also were significantly increased in the same brain regions. Protein expression LOAEL in the pre-frontal cortex for Iba-1, NK-□B, IL-6, IL-1β, and TNF-α was 50 mg/kg. Protein expression LOAEL In the hippocampus was 100 mg/kg for NK-□B and 50 mg/kg for other evaluated cytokines (Jiang et al. 2017).

Female B6C3F1 mice were given drinking water with 125, 500, or 1000 mg/L for 28 days. A significant decrease in thymus weight was noted at 500 and 1000 mg/L (19%). No effect on absolute or relative spleen weight, or relative thymus weight was reported. A non-dose response decrease (19%) in total spleen cell number and number of CD+CD- T-lymphocytes

(13%) was observed at 500 and 125 mg/L, respectively. A significant decrease in absolute (38%) and percent (22%) NK1.1+CD3- cells was noted at 500 mg/L. Significant decreases in absolute and percent splenic macrophages also were observed (LOAEL = 500 and 1000 mg/L, respectively). No effects on absolute or percent Ig+, CD3+, CD4-CD+, or CD4+CD8+ markers were noted. No effects on AFC response or IgM antibody titers in response to exposure to sheep red blood cells were noted. Additionally, no impact on response to allogeneic spleen cell stimulation was noted. A significant decrease in cytotoxicity was only observed after splenocyte NK cell activity was augmented with poly-IC; the effect was only observed at 125 mg/L. Host resistance to *Streptococcus pneumoniae*, *Plasmodoium yoelii*, and B16F10 melanoma tumors was not affect by treatment (Smith et al. 2010).

In vitro data with cells or cell lines

DBAA decreased thymocyte (obtained from BALB/c mice) proliferation at exposure lengths of at least 6 hours. At 6 hours, a significant decrease in proliferation was only observed at 40 μM. Comparatively, at 12, 24, and 48 hour exposure periods a significant decrease in proliferation was observed at 5, 10, 20 and 40 μM. DBAA also decreased IL-2 and IL-4 secretion (LOAEL = 10 and 5 μM, respectively). DBAA also increased late and early apoptosis (LOAEL = 5 and 10 μM), without effects on the percentage of necrotic cells. DBAA induced an increase in the percentage of cells in the G0/G1 phase and decreased the percentage of cells in the S phase. Increased intracellular thymocyte calcium levels (LOAEL = 5μM) and thymocyte Fas and FasL protein levels were reported (LOAELs = 10 μM for both proteins). Additionally, bcl-2 protein level was significantly decreased at all tested concentrations (LOAEL = 5 μM) (Gao et al. 2016).

Peritoneal exudate cells, obtained from B6C3F1 mice treated with 125, 500, or 1000 mg/L DBAA for 28 days, were evaluated for their ability to suppress B16F10 melanoma tumor cell proliferation *in vitro.* Treatment did not affect the ability of macrophages obtained from treated animals to suppress proliferation (Smith et al. 2010).

In Cl.Ly1 + 2/-9 cells, non-adherent cloned T-cell line derived from spleen cells from C57BL/6TL+ mice, DBAA (1-40 μM) decreased cell viability after exposure for 24, 48, or 72 hours (LOAEL = 1 μM). An increase in the mean percentage of early, late and total apoptotic cells also was noted (LOAEL = 5 μM) (Zhou et al. 2018).

Mode of action information

Overall, studies suggest that DBAA produces immunotoxic effects through modulation of T-cell mediated cell immunity. T-cell apoptosis, through extrinsic and intrinsic pathways, are proposed to play a role in the mode of action. Apoptosis may occur through a variety of pathways including modulation of transmembrane potential, the Fas/FasL pathway, modulation of intracellular calcium, and cell cycle arrest (Gao et al. 2008; Gao et al. 2016).

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Dibutyl phthalate (DBP) [CASRN 84-74-2]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
DBP significantly decreased phagocytotic capacity of differentiated THP-1 cells at all tested concentrations (LOAEL = 0.001μM). DBP also increased TNF-α secretion (LOAEL = 0.1 μM). Comparatively, DBP had no effect on IL-1β or IL-8 secretion from differentiated THP-1 cells (NOAEL = 0.001 μM) (Couleau et al. 2015).
High-density microarray studies were conducted using normal human mammary epithelial cell strains obtained from discarded tissues; cells were treated with 1 μM DBP for 10 hours. Gene expression of 29 genes were increased in all four isolated cell strains. Gene expression of 28 genes were decreased in all four isolated cell strains including genes involved in the immune response (TNF-α-induced protein 3; values not provided) (Gwinn et al. 2007).
DBP (tested at 0.1 and 100 μM) increased IL-6, CXCL8, and IL-10 secretion from monocytes/macrophages, isolated from blood of healthy individuals. The cells were, stimulated with *E. coli* lipopolysaccharide (LPS) for 1 hour. Comparatively, DBP did not affect IL-1β and decreased TNF-α secretion from the cells. For all affected cytokines the LOAEL was 100 μM. For phytohemagglutinin-P (PHA-P) stimulated T cells, DBP decreased IL-2, IL-4, TNF-α and IFN-γ secretion (LOAEL for all cytokines = 100 μM). No effect on IL-6 or IL-10 secretion was observed in the PHA-P stimulated T cells treated with DBP. Metabolism studies indicated that DBP was metabolized to monobutyl phthalate *in vitro*. Additionally, secretion patterns of monobutyl phthalate was similar to those observed for DBP (Hansen et al. 2015).
DBP increased IL-1β gene expression (as assessed by RT-PCR) in human corneal endothelial cell line B4G12 at all tested concentrations (LOAEL = 1 μM). IL-8 gene expression was increased at 1 and 10 μM (values not provided). IL-β, IL-8, and IL-6 secretion from cells also was increased. IL-6 and IL-8 LOAEL values were 10 and 5 μM, respectively. Significant IL-1β secretion was only observed at 1 μM. [Note: The authors note that secretion for IL-1β and IL-6 was low and quantification was approximate] (Kruger et al. 2012).
In THP-1 cells, DBP did not induce release of IL-18 (doses tested not provided) or IL-8 (NOAEL = 250 μM), or expression of CD86 (NOAEL = 250 μM). However, DBP did induce IL-8 mRNA expression at 500 μM after exposure for 3 hours (values not provided in paper) (Lourenco et al. 2015).
In HepG2 and L02 (normal human liver) cell lines, DBP (10 μM and 25 μM, respectively) significantly increased levels of mature caspase-1, IL-1β, and nucleotide oligomerization domain (NOD) like receptor family, pyrin domain containing 3 (NLRP3) (values not provided). KN-62,

a P2X7 receptor inhibitor, attenuated DBP-induced effects on caspase-1, IL-1β, and NLRP3 (Ni et al. 2016).
In primary human keratinocytes cultured on an amorphous pseudodermis, DBP increased TSLP (thymic stromal lymphopoietin) mRNA expression (Schuepbach-Mallepell et al. 2013).

Mode of action information
Studies suggest that innate and adaptive immune system is impacted by DBP exposure (Hansen et al. 2015). DBP is proposed to be metabolized to the monoester *in vitro*. This compound then is proposed to modulate cytokine secretion from both monocytes/macrophages and T cells.
The results from Couleau and colleagues (2015) suggest that some effects may occur through activation of the endocrine pathway. DBP also may regulate gene and protein expression of a variety of immune factors (e.g., cytokines) without impacting cell viability.
Immunomodulation by DBP also may occur through receptor-mediated effects on the inflammasome.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
DBP did not increase proliferative responses in lymph nodes of BALB/c mice at concentrations up to 20% (v/v in acetone; dermal route of exposure). Additionally, 10% DBP did not increase dendritic cell accumulation in draining lymph nodes (Dearman et al. 1996).
Wistar rats were fed a diet containing 0.5% or 5% DBP for 34-36 days. While no effect on absolute spleen weight was reported, a significant increase (1.8-fold) in relative spleen weight was reported at 5% DBP (Murakami et al. 1986).
Female BALB/cJ mice were subcutaneously exposed to ovalbumin (antigen) and 2-2000 µg/mL DBP. After the primary immunization, one or two booster shots were given to the mice. No effects on the IgG1 or IgE serum levels after either one or two booster shots were noted. A dose- dependent effect was observed on IgG1 serum levels; maximum responses were observed at 200 µg/ml (value not provided). No effect was noted on IgE serum levels (data not provided) (Larsen et al. 2002).
Thymic stromal lymphopoietin (TSLP) mRNA expression was significantly increased in BALB/c mouse ears 24 hours after exposure to DBP (in acetone, 1:1) (values not provided). An increase in TSLP protein levels was also measured at 24 hours (values not provided) (Larson et al. 2010; Schuepbach-Mallepell et al. 2013). DBP-induced induction of TSLP was strain dependent (BALB/c was more sensitive than C57Bl/6 mice). DBP also produced effects on TSLP in IL-1 receptor or apoptosis-associated speck-like protein containing a caspase recruitment domain deficient mice (Schuepbach-Mallepell et al. 2013).

In vitro data with cells or cell lines

DBP was cytotoxic to murine peritoneal exudate macrophages (PEM) after exposure to 50 or 100 μM for 24 hours. Annexin V and PI double stained cells (markers of apoptosis) were significantly increased after treatment with 100 μM DBP for 24 hours. Additionally, using trypan blue exclusion, a significant decrease in viable cells was reported after DBP exposure (LOAEL = 50 μM). Using two-color flow cytometry, DBP was shown to decrease expression of CD80, CD36, and major histocompatibility-II molecules on F4/80+ macrophages at 1 and 10 μM. Cytokine expression (IL-1β, IL-6, IL-12, and TNF-α) also were decreased at the same concentrations. Phagocytotic capacity of PEM to apoptotic thymocytes and *E. coli* was decreased after exposure to DBP when compared to controls (LOAEL = 1 μM). DBP exposure also decreased PEM immunogenicity to allogenic T cells (LOAEL = 1 μM) (Li et al. 2013).

DBP decreased cell viability of RAW 264.7 macrophages (LOAEL = 100 μM for 60 minutes) but did not increase cellular apoptosis (NOAEL = 1 mM for 60 minutes) (Naarala and Korpi 2009).

In RBL-2H3 mast cells sensitized with anti-dinitrophenyl monoclonal IgE, DBP potentiated β-hexosaminidase activity, which was used as a measurement of degranulation (LOAEL = 50 μM for 10 minutes). DBP did not induce degranulation in the cells that were not sensitized (NOAEL = 500 μM for 10 minutes) (Nakamura et al. 2002).

In PAM212 keratinocytes, 1% DBP increased relative expression of TSLP; maximal effect (values not provided) was observed at 36 hours post treatment (Larson et al. 2010). DBP-induced TSLP expression was associated with epidermal mouse skin and human abdominal skin transplanted on mice (Schuepbach-Mallepell et al. 2013).

Mode of action information

In vivo rodent studies suggest that DBP impacts the Th2 response. Inflammasome activation by DBP impacts TSLP expression and Th2 response.

In vitro studies suggest that while high doses of DBP induced macrophage apoptosis, moderate doses induced protein expression and production of cytokines. DBP also impacted the antigen-presenting capacity of macrophages.

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Dichloroacetic Acid (DCAA) [CASRN 79-43-6]

Human Data

Data from epidemiology studies
No studies were identified.

In vitro data with cells or cell lines
A single study suggested that DCAA may produce immunosuppressive effects. Using a two-way mixed lymphocyte reaction, DCAA (LOAEL = 0.33 mM; lowest dose tested) increased IL-10 production and FOX P3 expression 11.4- and 4.5-fold, respectively (Eleftheriadis et al. 2013).
DCAA (3.0 mM and 0.5 mM, respectively) increased IL-2 production after incubation for 16 hours and expression of the T-cell activation marker CD25 in Jurkat cells. Comparatively, no effect on CD69 expression (0.5 and 3.0 mM) was noted. IL-2 and IFN-γ mRNA expression was significantly increased after DCAA treatment (3.0 and 0.5 mM, respectively) (Pan et al. 2015).
DCAA (N/LOAEL = 0.1/1.0 mM) induced statistically significant increases in necrosis in PBMC, as shown by a decrease in PBMC cell size combined with an increase in cellular granulation. Statistically significant increases in the percentage of apoptotic cells were observed at similar concentrations of DCAA (N/LOAEL = 1.0/2.0 mM) (Michalowicz et al. 2015).

Mode of action information
T-cell activation was one proposed mode of action for DCAA. Increased IL-10 production, combined with increased FOX P3 expression, is proposed to increase regulatory T-cell differentiation which may lead to increased IL-10 production. Additionally, DCAA increased expression of T-cell activation markers in Jurkat cells.
Apoptosis was proposed be associated with a variety of mechanisms including ROS generation, alterations in mitochondrial transmembrane potential, and activation of caspase activity.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
In a 90-day drinking water study (0, 50, 500 and 5000 ppm (w/v) DCAA) with male Sprague-Dawley rats, a significant increase in relative spleen weight was noted at 5000 ppm (0.25% vs. 0.21%). No consistent effects on T cell-dependent anti-keyhole limpet hemocyanin IgG antibody production (measured by ELISA), delayed hypersensitivity to bovine serum albumin, NK cell cytotoxicity, or production of peritoneal macrophage-derived PGE_2 or spleen lymphocyte-derived IL-2 were detected at tested doses (data not shown in paper) (Exon et al. 1986; Mather et al. 1990).
In autoimmune-prone MRL +/+ female mice, 0.5 mg/mL DCAA (provided *ad libitum* in drinking water for 12 weeks) significantly increased serum IgG (32%) and IgM (30%) levels. DCAA significantly decreased IL-10 (34%) and KC chemokine (31%) in liver extracts from

MRL +/+ mice. Comparatively, a significant increase in serum IgG3 levels (27%) was observed in wild-type B6C3F1 after DCAA exposure. In liver extracts from treated B6C3F1 mice, DCAA significantly increased IL-4 (400%), IL-5 (33%), IL-6 (53%), IL-10 (25%), IL-12 (32%), KC chemokine (18%), GM-CSF (42%), G-CSF (56%), and IFN-γ (45%) compared to controls. Compared to isolated MRL +/+ splenic lymphocytes from controls, DCAA decreased IL-4 and IL-10 secretion in MRL+/+ treated mice. DCAA decreased IL-4 and increased IFN-γ secretion from splenic lymphocytes from treated B6C3F1 mice when compared to controls (values not provided). DCAA also significantly decreased IL-4 and IL-2 secretion and significantly increased IL-5, IFN-γ, and GM-CSF secretion from B6C3F1 isolated splenic lymphocytes when compared to secretion from MRL +/+ isolated splenic lymphocytes from treated animals (values not provided) (Cai et al. 2007).

In vitro data with cells or cell lines
No studies were identified.

Mode of action information
DCAA-induced increase of p53 accumulation has been proposed to lead to increased formation of cells in G2-M phase (Staneviciute et al. 2016).

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Diethylstilbestrol (DES) [CASRN 56-53-1]

Human Data

Data from epidemiology studies

Male and female offspring of pregnant women given DES doses from the seventh week to 34th week of pregnancy, were interviewed about immune-related health problems. A total of 549 DES-exposed offspring and 487 placebo-exposed offspring participated in the study. Rates of allergy-related health problems (e.g., asthma, drug allergy, hives) were similar between DES- and placebo-offspring. Infection (e.g., shingles, flu) and autoimmune disease (e.g., diabetes, rheumatoid arthritis) also were similar between the two groups (Baird et al. 1996).
The frequency of any autoimmune disease in women exposed to DES *in utero* (n = 1711) was higher than the frequency observed in control women. The overall frequency was 28.6 per 1000 women compared to 16.3 per 1000 women. Hashimoto's thyroiditis was significantly more prevalent (relative prevalence = 5.4) in exposed women compared to controls (Noller et al. 1988).
Increased incidence of asthma, arthritis, and diabetes mellitus was reported in sons and daughters exposed to DES *in utero* when compared to unexposed individuals. Additionally, the number of respiratory tract conditions (e.g., colds) was increased in the exposed population vs. the unexposed population (Wingard and Turiel 1988).

In vitro data with cells or cell lines
Lymphocyte NK activity (assessed using chromium release from K562 cells) from 12 patients exposed to DES *in utero* was greater than observed from controls; however, effects were not significant. No effects on adherent cells were noted (Ford et al. 1983). Comparatively, DES dose-dependently inhibited lysis of K562 cells in PBMCs obtained from 12 patients. At the highest concentration tested (100 µM), an 82% reduction in activity was observed compared to control samples (Ablin et al. 1988b, 1988a)
Responses to 0.125 µg/mL PHA (as measured by uptake of radiolabeled thymidine) was significantly greater in peripheral blood monocytes from women exposed to DES *in utero* compared to controls (88.6×10^3 vs. 44.0×10^3 cpm; $p < 0.002$). Maximal blastogenic response to PHA in lymphocytes from DES-exposed women was observed at 0.125 µg/mL while it was observed at 0.25-0.50 µg/mL in controls (Ways et al. 1987).

Mode of action information
DES inhibits the lytic activity of human NK cells (Kalland and Campbell 1984).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
C57BL/6 dams were orally administered 48 µg/kg DES from GD 14–16 and then sacrificed on GD 18. Fetal thymic weight and cellularity were significantly decreased (44% and 51%, respectively) in treated animals. Relative fetal thymic weight was decreased 28% when compared to controls. The percentage of thymocytes in the CD4–8– and CD4–8+ populations were increased 87% and 138%, respectively. Comparatively, CD4+8+ thymocyte population was decreased 12%. Increased apoptosis of CD4+8+, CD4–8+, and CD4–8– thymocytes also was observed (Besteman et al. 2005).
Male and female CD–1 mice were subcutaneously injected with 5, 15, or 30 µg/kg DES, four times on alternate days. Relative thymic weight (LOAEL = 30 µg/kg) was decreased, and absolute and relative splenic weights (LOAELs = 15 µg/kg) were increased in female mice. A similar effect in male mice was not observed. Relative expression of thymocyte populations (e.g., CD4–8–) were not affected in males or females. However, an increase in the number of total apoptotic and decrease in the number of live CD4+8+ and CD4+8– thymocytes was observed in male and female mice. Additionally, an increase in the number of total apoptotic and decrease in the number of live CD4–8– cells were observed in females. Increased proliferative response to ConA, LPS, or PMA was observed in splenic lymphocytes isolated from female mice treated with 5 µg/kg DES. At higher doses, a trend for decreased proliferation was observed in female splenic lymphocytes. Proliferative responses by splenic lymphocytes were only modulated in response to ConA at 15 µg/kg DES (Calemine et al. 2002).
Female mice (strain not provided) were administered (route not provided) 0.2, 2.0, or 8.0 mg/kg DES for 5 days. Antibody response to sheep erythrocytes and LPS were decreased 15% to 45% (LOAELs = 2.0 mg/kg). Delayed hypersensitivity response to keyhole lymphet hemocyanin was similar to controls when mice were exposed to DES before sensitization. However, when mice were exposed to DES after sensitization and before challenge a decrease in response was observed (LOAEL = 2 mg/kg). The percentage of splenic T lymphocytes was decreased 25% at the highest dose tested. No effect on the percentage of splenic B lymphocytes was observed. Splenic lymphoproliferative response to PHA and ConA were decreased (>30%) at all tested doses. Responses to *Staphylococcus* enterotoxin A were increased at 0.2 mg/kg and decreased at higher doses, while responses to LPS were increased at 0.2 and 2.0 mg/kg and decreased at 8.0 mg/kg. MLC responses also were decreased (LOAEL = 2 mg/kg). Suppressor cell activity was decreased after exposure to 8 mg/kg DES (Luster et al. 1980).
Differential effects on the immune system were observed in female NMRI mice depending on the time of DES exposure. Thymus weights were increased in 56–day–old mice that were subcutaneously injected with 5 µg from PND 1–5, 6–10, or 30–34 (1.2– to 1.4–fold). Comparatively, thymus weight was decreased in mice subcutaneously injected with DES from PND 48–52 (29%). A dose–related effect on thymus weight was observed in mice treated from PND 1–5; no effects on absolute or relative spleen weight were noted. Differences in thymus weight also were noted depending on when the mice were killed after treatment. Four days after treatment, thymus weights were decreased in all test groups. However, 4 to 8 weeks after

treatment showed an increase in thymus weight in mice treated on PND 1–5 and weights similar to controls in other treatment groups. DES treatment on PND 1–5 also reduced the number of cells in S–phase in the thymus (Forsberg 1996).
C57BL/6 mice were treated with DES once *in utero* and/or once at 12–16 months of age via subcutaneous injection. Increased secretion of IFN γ was observed in splenic lymphocytes obtained from mice exposed to DES *in utero* and as adults. Increased IFN γ also was observed when splenocytes were stimulated with anti–CD3 antibodies. This increase was not observed in other treatment conditions (data in graphs). An increase in IFN γ production also was observed in T–cells from mice exposed to DES *in utero* and as adults (Karpuzoglu–Sahin et al. 2001).

In vitro data with cells or cell lines
DES stimulated IL–1 production from peritoneal exudate macrophages at concentrations ranging from 0.01 to 1 µM; the maximal response was observed at 0.1 µM. DES (0.1 µM) also significantly increased production of IL–6 (1.7–fold), IL–12 (9.5–fold) TNF–α (3.1–fold), and macrophage chemotactic protein 1 (7.2–fold), and surface expression of CD86 (1.6–fold). DES also increased proliferative responses (8.6–fold) and IL–2 production (5.6–fold) observed when macrophages were incubated with purified T cells. Anti–MHC–II, –CD–80, and –CD86 blocked effects produced by DES (Yamashita et al. 2005).
DES increased IgE levels in male BALB/c mouse splenocytes at concentrations greater than 1 µM. Comparatively, DES had no effect on IgM, IgG, or IgA levels at concentrations up to 1 mM (Han et al. 2002).

Mode of action information
DES–induced thymic atrophy was proposed to be due, in part, to estrogen–related thymocyte apoptosis (Besteman et al. 2005; Fenaux et al. 2004). Brown and colleagues suggested that DES exposure upregulates TNF family members, which leads to altered T–cell development. This alteration was suggested to lead to thymic atrophy (Brown et al. 2006). Direct effects on T lymphocytes also may occur (Luster et al. 1980).
In mice, DES exposure was associated with down–regulation of gene expression in the TCR complex, and the TCR and CD28 signaling pathways. Genes in the B–cell receptor signaling pathway, antigen presentation, and dendritic cell pathways also was altered by DES exposure. It was proposed that DES dysregulation of T–cell development plays a role in thymus effects (Frawley et al. 2011). Alterations of microRNA expression also has been proposed as playing a role in the immunotoxic effects produced by DES (Singh et al. 2015).
Additional proposed modes of action on the immune system include effects on adherent suppressor cells, modulation of NK activity by interfering with bone marrow lymphoid precursors, and modulation of the mononuclear phagocyte system (Dean et al. 1986; Forsberg 1984; Kalland 1984; Luster et al. 1980).

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Ethylene Dibromide (EDB) [CASRN 106-93-4]

Human Data

Data from epidemiology studies
The prevalence of adult-onset asthma, in relation to lifetime pesticide use, were assessed using data from the Agricultural Health Study (19,704 male farmers). Adult-onset asthma was reported in 441 individuals; 127 classified as allergic and 314 classified as non-allergic. EDB exposure was positively associated with allergic asthma (OR: 2.07 [1.02-4.20]) (Hoppin et al. 2009).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female B6C3F1 mice were intragastrically treated with 100, 125, 160, or 200 mg/kg EDB for 14 days. Relative thymus and spleen weights were decreased in a dose-related manner (LOAEL = 200 mg/kg). Comparatively, relative liver and kidney weights were increased at higher doses (LOAEL = 125 and 160 mg/kg, respectively). Significant increases in white blood cells (LOAEL = 200 mg/kg) and neutrophils (LOAEL = 160 mg/kg) were noted. Host resistance to influenza A2, *Listeria monocytogenes*, and herpes simplex virus types 1 and 2 was not significantly affected by EDB exposure. The total number of resident peritoneal exudate cells were significantly increased in EDB-treated mice (LOAEL = 160 mg/kg). However, the percentage of cell types present in the exudates were similar to those observed in control exudates (macrophages: 53%; lymphocytes: 47%). Phagocytosis of radiolabeled chicken red blood cells was increased in peritoneal macrophages obtained from EDB-treated mice (187% of control; LOAEL = 125 mg/kg). Splenic NK cell activity was evaluated in animals treated with 100, 125, or 160 mg/kg; a significant decrease in activity was observed at 160 mg/kg. The number of viable cells in the spleen decreased at 125, 160, and 200 mg/kg (not significant), while a significant increase in the number of anti-SE PFC/10^6 viable spleen cells was significantly increased at 160 mg/kg. Splenic lymphocyte responses to allogenic spleen cells, PHA and ConA, but not LPS, were significantly decreased at 125 and 160 mg/kg (Ratajczak et al. 1994). Female B6C3F1 were intragastrically treated with 31.25, 62.5, or 125 mg/kg EDB for 5 days per week for 12 weeks. No effect on white blood cell numbers, or the percentage of neutrophils or lymphocytes were noted at the doses tested. Splenic lymphocyte responses to PHA and LPS were significantly decreased at the highest dose tested (data not provided) (Ratajczak et al. 1995).

Relative spleen weights were not significantly affected in male Sprague-Dawley rats inhalationally exposed to EDB 7 hours per day, 5 days per week, for 30 days. However, relative liver weights were increased at the highest dose tested (LOAEL = 455 ppm) (Igwe et al. 1986).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

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Glycidol [CASRN 556-52-5]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Of 10 F344/N female rats that received 400 mg/kg glycidol for 13-weeks (via gavage), lymphoid necrosis of the thymus was observed in nine (Irwin et al. 1996; National Toxicology Program 1990). Enlarged spleen were observed in haploinsufficient $p16^{Ink4a}/p19^{Arf}$ male mice treated with 200 mg/kg glycidol for 40 weeks via gavage (National Toxicology Program 2007). Increased splenic fibrosis incidence was reported in male and female F344/N rats gavaged with 37.5 and 75 mg/kg glycidol for 2 years. In males, splenic fibrosis incidences were 26% in controls, 68% in rats treated with 37.5 mg/kg, and 56% in rats treated with 75 mg/kg. In females, splenic fibrosis incidences were 6%, 29%, and 40% for control, 37.5 mg/kg rats and 75 mg/kg rats, respectively (National Toxicology Program 1990).
In female B6C3F1 mice administered glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days, no effect on spleen or thymus weights, or leukocyte or lymphocytes counts were reported. To assess AFC response, treated mice were intravenously exposed to sheep erythrocytes on day 11 and spleen IgM AFC response was measured 4 days later. At the highest treatment dose, there was a 31% reduction in specific activity. When expressed as total spleen activity, significant decreases were noted at 125 and 250 mg/kg (29% and 41%, respectively).
Splenic T-cell proliferation, in response to 10 μg/mL ConA was significantly decreased (16% and 26%, respectively) in splenocytes obtained from mice treated with 125 and 250 mg/kg glycidol. B-cell proliferation, in response to Il-4 or Il-4 and goat anti-mouse IgM F(ab')2, was only decreased in splenocytes obtained from mice treated with 125 mg/kg glycidol (13% and 16%, respectively). Comparatively, proliferation in response to goat anti-mouse IgM F(ab')2 was decreased in splenocytes from mice treated with 125 and 250 mg/kg glycidol (30-32%). While glycidol had no effect on lymphocyte blastogenesis (as assessed by splenocyte proliferative response) alone, in the presence of allogenic DBA/2 spleen cells a 25% decrease in response was noted at the middle dose only. NK cell activity in spleens was decreased at two ratios of effector:target ratios (100:1 and 50:1); the LOAELs at both ratios were 125 and 250 mg/kg, respectively. Using flow cytometry, the number and percent of B lymphocytes, T-lymphocytes,

$CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ cells from spleens isolated from treated mice were quantified. The total number of spleen cells, B lymphocytes, and $CD4^+CD8^-$ were significantly decreased at 250 mg/kg. The LOAEL also was 250 mg/kg when the percent values of B and T lymphocytes were assessed (Guo et al. 2000).

In vitro data with cells or cell lines

To further assess the effect of glycidol on the immune function, Guo and colleagues (2000) conducted a set of *ex vivo* assays. Glycidol inhibited cytotoxic T cell activity in spleens obtained from mice administered glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days.

Splenocytes were sensitized with mitomycin C–exposed P815 mastocytoma cells, and co-cultured with labeled P815 cells at a variety of effector:target ratios. At an effector:target ratio of 25:1 and 0.75:1, glycidol inhibited CTL activity at a 25 mg/kg when compared to vehicle (53.8 vs. 31.5, and 8.8 vs. 2.1, respectively). At a ratio of 12.5:1, CTL activity was decreased significantly (39%) in spleens from mice treated with 125 mg/kg glycidol (Guo et al. 2000).

Resident macrophage activity (in the presence of macrophage stimulators) was assessed in mice treated with glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days. Increased cytotoxicity was only observed after treatment with 25 mg/kg glycidol in the presence or absence of macrophage stimulators (1.7- to 2.5-fold increase) (Guo et al. 2000).

Host resistance to B16F10 melanoma cells, *Listeria monocytogenes* and *Streptococcus pneumoniae* were assessed in mice treated with glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days. Glycidol increased pulmonary tumor formation in mice treated with B16F10 melanoma cells (LOAEL = 125 mg/kg). No effect on host resistance was noted at the three challenge levels of *Listera monocytogenes* (1, 2, or 4×10^4 CFU/mouse). At the challenge level 5.52×10^7 CFU *Streptococcus phenumoniae*/mouse, increased host resistance was observed in the 250-mg/kg glycidol treated mice (Guo et al. 2000).

Mode of action information

Studies suggest that glycidol modulates B-cell function, and NK cell and macrophage activities (Guo et al. 2000).

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Hydrocortisone (HC) [CASRN 50-23-7]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
The effect of HC on IL-4-induced IgE production was measured in PBMCs isolated from healthy volunteers. HC induced an ~20 fold increase in IgE production at a LOEL of 1×10^{-7} M. HC did not have any effect on IgE production in the absence of IL-4 (data not shown) (Nüsslein et al.
1994).
Blood samples from healthy adults were pre-treated with 30 µg/dL HC (identified as cortisol); INF production was then stimulated with Newcastle disease virus. HC decreased IFN-α response by 50-60% (data in graph) (Reissland and Wandinger 1999).

Mode of action information
Keh and colleagues (2003) reported that in septic shock patients, HC attenuated inflammatory and anti-inflammatory responses without inducing immunosuppression.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Swiss inbred mice were intraperitoneally injected with 0, 1.5, 5 or 15 mg/kg of HC. Forty-eight hours later, there was a significant decrease in thymus weight at 15 mg/kg (data in graph). To test the effect of HC on delayed hypersensitivity, mice were immunized with sheep erythrocytes in FCA, challenged in the footpad on day 5, and treated simultaneously with increasing amounts of HC. The mice received another injection of HC two hours before measuring 24-hour footpad swelling. 5 and 15 mg/kg HC suppressed footpad swelling (data in graph). Glucocorticoid- induced leukopenia and monocytopenia was evaluated in mice 2.5 hours after intravenous injection with HC. The numbers of circulating nucleated and monocytic cells was maximally decreased at the lowest dose tested (1.5 mg/kg), with the number of both cell types increasing with increasing dose (data in graph). A plasma transfer study found that 2.5 hours after transfer, the plasma of HC treated mice raised the number of nucleated cells in saline treated acceptor mice by 46%. To evaluate feedback-inhibition, mice were injected (route not specified) with 5 mg/kg HC for four days and examined 7 or 11 days (data not shown) after the last injection. At day 7, HC had no effect on delayed hypersensitivity, serum corticosterone, or numbers of circulating nucleated and monocytic cells (data in graphs) (Van Dijk et al. 1979).
In a trio of studies by El Fouhil and colleagues (El Fouhil et al. 1993a, 1993b; El Fouhil and Turkall 1993), immunologically immature rats were treated subcutaneously with 400 mg/M^2/day HC, administered on alternate days from PND 7 to PND 19. At two days after the last

treatment (PND 21), thymus and spleen weights were decreased (71 and 28%) compared to vehicle control,

but at PND 42 organ weights were increased (18 and 7%). Leucocytosis was increased in PND 21 and 42 rats (12 and 24%), with a decrease in IgM concentration in serum (45 and 15%). At PND 21 there was a 46% decrease in the percentage of lymphocytes, which resolved by PND 42 (El Fouhil and Turkall 1993). On PND 21, splenic white pulp was largely depleted of small lymphocytes. There were no distinct periartiolar lymphoid sheaths and no primary follicles. The number of T cells surrounding the central arteriole was decreased (data not shown). By PND 42, the pulp appeared normal (El Fouhil et al. 1993a). On PND 21, the outer cortex of mesenteric lymph nodes was found to be depleted of small lymphocytes and primary follicles, and neither cortical expansion nor capsular indentations were detected. There was a marked depletion of B lymphocytes, which were more or less discrete and did not aggregate to form follicles. There was no apparent change in T lymphocytes. On PND 42, the lymph nodes were comparable between HC treated and control rats (El Fouhil et al. 1993b).
HC (1.5 mg intraperitoneally administered) decreased formation of splenic anti-sheep erythrocyte (4×10^7 sheep erythrocytes) PFC in female BALB/c mice (data in graph). HC did not affect IgM-PFC or IgG-PFC response or serum antibody titers (data in graphs) (Jokay et al.
1980).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

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Indomethacin [CASRN 53-86-1]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
Heparinized whole blood, from healthy adult volunteers, was incubated with 1 to 50 µM indomethacin for 1 hour prior to stimulation with LPS. A significant increase in IL-6 expression was only noted at 50 µM indomethacin (129.7%). Comparatively, a dose-dependent increase in TNF-α was observed, and at 50 µM the number of TNF-α positive cells had doubled (204.7%) (Hartel et al. 2004).
Human PBMC were treated with 1, 10 or 100 µM indomethacin. At all tested doses, indomethacin decreased LPS-induced PGE2 synthesis to near 0%; the calculated IC50 was 0.039 µM (data in graph). Indomethacin also decreased IgG and IgM production (data in graph) at all doses tested. Indomethacin up-regulated IL-2 production and down-regulated IL-6 production in treated PBMC (data not shown). Increased PHA-, anti-CD3, and IL-2-induced lymphocyte proliferation was reported after indomethacin exposure. NK activity (against K562 target cells) was increased at 1 (1.5-fold) and 10 (1.5-fold) µM. A significant effect on LAK cell activity was not observed at 50 µM. Co-incubation of PBMCs with IL-2 and indomethacin caused an increase in IFN-γ production by LAK cells at 1, 10 or 100 µM (data not shown) (Tanaka et al. 1998).
Indomethacin (5.6 µM) increased proliferation of PHA- and ConA-stimulated lymphocytes (in mononuclear cell cultures) (data in graph). The effect was only observed at suboptimal concentrations of PHA and ConA. The observed increased proliferation was lost at optimal and supraoptimal concentrations. Additional testing showed indomethacin increased PHA-stimulated lymphocyte proliferation in a dose-dependent manner (LOEL = 0.04 µM). Removal of adherent cells from the culture negated the stimulatory effect produced by indomethacin. Indomethacin did not affect cell viability, but increased incorporation of tritiated thymidine in a dose- dependent manner (Jawad and Rogers 1984).

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female C57BL/6 mice were orally administered 5 mg/kg indomethacin for 4 days. Animals were then immunized with sheep erythrocytes and then serum hemagglutination and AFC titers were assessed 4 and 8 days, or 6 days later, respectively. Indomethacin decreased both titers by approximately 40% (data in graphs). Indomethacin also decreased ConA- and LPS-induced

stimulation of lymphocyte proliferation (data in graphs). Incubation of indomethacin (3 μM) with LPS-stimulated lymphocytes isolated from indomethacin-treated mice decreased proliferation (Barasoain et al. 1980).

Female B6C3F1 mice were subcutaneously injected with 1, 2, or 4 mg/kg indomethacin for 6 days. Studies were conducted on mice 3 days after final treatment. No effect on thymus weight was reported, while 4 mg/kg indomethacin caused a 38% increase in spleen weight. A dose-dependent increase in splenic lymphocyte proliferation (10%-80%) was observed in non-stimulated cultures. Increased proliferation was observed in LPS-stimulated cultures from mice treated with 1 or 2 mg/kg indomethacin (13% and 22%, respectively), while a decrease was observed at the highest indomethacin dose. Comparatively, decreased proliferation was observed in PHA-, ConA-, or MLC-treated splenic cultures from indomethacin treated mice. Increased formation of PFC/10^6 splenocytes also were observed in treated mice (149% increase at 4 mg/kg). Indomethacin did not affect macrophage-induced inhibition of tumor cell growth (MBL- 2), but did increase phagocytosis of sheep erythrocytes. Host resistance to *Listeria* was increased in treated mice. No effect on delayed hypersensitivity was noted (Boorman et al. 1982).

Oral exposure to indomethacin (2.5, 5, or 10 mg/kg/day for 3 days) decreased formation of PFC in C57BL/6 mice after immunization to sheep erythrocytes. Studies showed a dose-dependent decrease in the number of PFC/10^6 spleen cells; decrease ranged from 43% to 97%. Similar inhibition was observed at 5 mg/kg/day indomethacin and various concentrations of sheep erythrocytes (2.5×10^8 and 5×10^8); decreases ranged from 47% to 68%. Indomethacin also inhibited antibody response to *P. aeruginosa* LPS; total response was decreased by 44% (Rojo et al. 1981).

Oral administration of 6 mg/kg/day indomethacin for 4 days produced a 32% decrease in total number of lymphocytes in Swiss male mice. No effect was noted at earlier time points (i.e., 2 or 3 days). An increase in the number of colonies/10^5 bone marrow cells (2.7- to 3.9-fold) also was noted in mice that were administered indomethacin for 4 days. Indomethacin also decreased PGE2 (25-43%) and PGF2α (41-56%) levels in bone marrow cells after 4 days of administration (Fontagne et al. 1980).

Male CBA mice were intraperitoneally injected with 0.7, 4, or 8 mg/kg indomethacin. Two to 24 hours after exposure, mice were euthanized and spleens removed. A dose-dependent increase in splenocyte proliferation was noted after 2 hours, with a 14.3-fold increase in proliferation at the highest dose tested. A time-dependent increase in proliferation was also noted when mice were treated with 4 mg/kg indomethacin, with a maximal fold change of 31.4-fold at 24 hours. Distribution of T-cell phenotypes was not affected by indomethacin administration (Gonzalez-Cabello et al. 1987).

Kushima and colleagues (2007, 2009) evaluated effects of indomethacin in young Sprague-Dawley rats after *in utero* exposure. In 3-week old pups from dams treated with 0.25, 0.5, or 1.0 mg/kg indomethacin on GD 18-21, a significant increase (31%) in the number of spleen cells was observed in males from the highest dose group. Immunophenotyping of splenocytes showed a dose-dependent increase in the proportion of CD45RA+ cells in male pups. However, a similar

increase in peripheral blood lymphocytes was noted. No effect on serum IgM or IgG levels was reported in males or females. A significant decrease in anti-KLH IgG titers, but not IgM titers was reported in males from the highest dose group tested (Kushima et al. 2007). When doses of 0.5, 1.0, or 2.0 mg/kg indomethacin were used, a significant decrease in splenocyte IL-10 levels were reported in males; no effects on IL-6, IL-2, IL-4, TNF, or IFN-γ levels were noted in either sex (Kushima et al. 2009).
Indomethacin (1 or 2 mg/kg administered twice daily for 3 days to adjuvant induced arthritic Sprague-Dawley rats) reduced PHA-induced lymphocyte proliferation in a dose-dependent manner (data in graph). LPS-stimulated proliferation was also inhibited at both doses, however the response was partially recovered at the higher tested indomethacin dose (data in graph) (Seng et al. 1990).
Indomethacin increased the total number of cells, and number of T- and B-cells up to 14 days after birth, in newborn ddy mice intraperitoneally injected with 5 μg/g every 2 days from birth (data in graphs) (Shibuya et al. 1986).

In vitro data with cells or cell lines
Indomethacin (3 μM) inhibited proliferation of lymphocytes isolated from C57BL/6 mice (data in graph). Additionally, dose-dependent inhibition LPS-induced proliferation of isolated lymphocytes was noted (Barasoain et al. 1980).
Indomethacin dose-dependently increased male rat (strain not provided) ConA-induced lymphocyte proliferation after an 18-hour incubation (LOAEC = 1 μM). A time-course evaluation with 1 μM indomethacin showed that ConA-inducted lymphocyte proliferation was enhanced at incubation times up to 30 hours. Proliferation at exposure times ranging from 36 to 66 hours were not different from controls (Calder et al. 1991).
Indomethacin (50 nM to 50 μM) dose dependently increased LAK activity in BALB/c mouse splenocytes that were cocultured with recombinant IL-2. Increased lysis of JC tumor cells was observed, reaching a maximum response of 123.6 lytic units at 50 μM compared to 43.6 lytic units for IL-2 alone. Studies also showed that the increased response, compared to addition of IL-2 alone, was observed when culture conditions were maintained for up to 4 days. Addition of nylon wool to the culture, abrogated the induction of LAK response observed in the presence of indomethacin (Chao et al. 1989).
Increased time-dependent proliferation was observed in lymphocytes, from CBA mice, treated with 10 μg/mL indomethacin. After 6 and 24 hours, proliferation was increased 4.3- and 46.6-fold, respectively (Gonzalez-Cabello et al. 1987).
Indomethacin decreased IL-4 levels in ConA-stimulated splenocytes isolated from 3-week old male rats (LOAEL = 50 μM). No effect was noted in splenocytes from females. Decreased IL-6 splenocyte levels was observed in cells obtained from females and treated with 2.0 μM indomethacin. No effect on IL-2, IL-10, IFN-γ, and TNF-α were noted (data not shown or in graph) (Kushima et al. 2009).

Mode of action information

Indomethacin induced effects on prostaglandin synthesis was associated with several immune effects. Lala and Parhar (1988) suggested that indomethacin effects are associated with suppression of prostaglandin synthesis. Rojo and colleagues (1981) and Franceschi and colleagues (1988) proposed that indomethacin inhibition of prostaglandin synthesis leads to altered T-cell function, and NK- and antibody-dependent cytotoxicity.

Differential effects on T-cell and B-cell-induced lymphocyte proliferation were reported. A dose- dependent effect on T-cell function was reported, while an inverse effect on B-cell function was noted (Seng et al. 1990).

Indomethacin has been postulated to produce immune effects through inhibition of Th1, and to a lesser extent Th2, responses (Yamaki et al. 2003). Studies conducted by Jaramillo and colleagues (1992) supported this proposed mode of action.

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Isonicotinic Acid Hydrazide (IAH) [CASRN 54-85-3]

Human Data

Data from epidemiology studies
In 19 cases of INH-induced liver failure, antibodies were present in sera of 15 patients. Anti-INH antibodies were present in 8 patients. Additionally, anti-cytochrome P450 antibodies were identified in up to 14 patients. Antibodies were not detected in patients that were treated with INH but did not have significant liver injury (Metushi et al. 2014c). In eight INH-induced liver failure patients, the dominant serum immunoglobulin isotype of anti-INH antibodies was IgG. A low titer of IgM was observed in two patients, while IgA and IgE antibodies were not detected. Phenotyping the IgG antibody indicated that the isotype was IgG3 (Metushi et al. 2014b).
INH (1.25 µg/mL) did not stimulate PGE3 production in polymorphonuclear leukocytes or modulate PHA-stimulated mononuclear leukocytes transformations. No effect on PG2 production was observed at 5 µg/mL (Zeis 1987).

In vitro data with cells or cell lines
In a series of studies, Kucharz and colleagues studies the immunomodulatory effects of IAH. In 5 µg/mL PHA-stimulated T-cells, IAH increased cellular proliferation (16% to 27%) at concentrations ranging from 0.01 to 0.0001 mM. (Kucharz and Sierakowski 1990a). In PBMC stimulated with 5 ng/mL anti-CD3 antibody, IAH produced a biphasic response. At 1 and 10 mM IAH decreased (53.6% and 24.4%, respectively) cell proliferation. Increased cellular proliferation (18-47%) was observed at concentrations ranging from 0.0001 to 0.1 mM. A similar biphasic pattern was observed when 10 ng/mL anti-CD3 antibody was used. In T-cells stimulated with anti-CD3 antibody, PHA, or PHA with PMA, IAH also modulated proliferation in a biphasic manner (Kucharz and Sierakowski 1990a). In cells stimulated with 5 µg/mL PHA and 20 ng/mL PMA, 0.1 to 10 mM IAH decreased T-cell proliferation 17% to 46%. At 0.001 mM IAH, at significant increase (21%) in T-cell proliferation was observed (Kucharz and Sierakowski 1990d). In cells stimulated with IL-2, IAH decreased cell proliferation 0.1 and 1 mM (71% and 47%, respectively) and increased proliferation at 0.01 to 0.001 mM (8% to 12%, respectively (Kucharz 1995).
IAH also decreased T-cell IL-2 production at 0.1 and 1 mM (44.7% and 71.6%, respectively) and increased T-cell IL-2 production at 0.01 to 0.0001 mM (105% to 115%). No effect on IL-2 receptor expression in T-cells was observed (Kucharz and Sierakowski 1990b).
IAH decreased IL-1 production from human monocytes in a dose-dependent manner in the absence or presence of lipopolysaccharide (LOAEL = 0.001 mM) (Kucharz and Sierakowski 1992).
In the absence of PHA, IAH stimulated proliferation of Jurkat cells (LOAEL = 0.01 mM). In the presence of PHA (2 or 5 µg/mL), IAH stimulation was observed at higher concentrations (1 and 10 mM) while at lower concentrations no effect was observed (Kucharz and Sierakowski 1990c).

When PMA (20 ng/mL) or PMA (20 ng/mL) and PHA (5 µg/mL) were added to the media, increased Jurkat cellular proliferation was observed at 0.001 mM (32%) and 0.01 and 0.001 mM (8% and 18%, respectively) (Kucharz and Sierakowski 1990d).
INH (5 µg/mL) did not have any effect on the phagocytic activity or intracellular killing activity on polymorphonuclear leukocytes obtained from healthy volunteers (Okuyan et al. 2005).

Mode of action information
Metushi and colleagues proposed that INH produced an immune response that leads to liver injury (Metushi et al. 2014c, 2014b).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female *Nat1/2(-/-)* mice were treated with INH either by oral gavage (100 mg/kg/day) for up to 7 days or by feed (0.2%) for 35 days. In mice treated by gavage, significant decrease in M1 macrophages and increase in M2a and M2b macrophages in cervical lymph nodes was noted. No effect on the M2c macrophages was observed. Comparatively, no effect was noted in the macrophage phenotypes obtained from mice that were exposed by feed (Metushi et al. 2014a).
INH (0.1 to 1.0 mg/10 µL) did not alter the weight of popliteal lymph nodes from C57BL/10 mice 7 days after subcutaneous injection (Kammuller et al. 1989). A lack of effect on popliteal lymph nodes from Brown Norway rats also was observed when exposed to 5 mg/50 µL INH (Verdier et al. 1990).
Four female Cbl-b-/-, C57BL/6 background that lack an E3 ubiquitin ligase, were provided diets containing 0.2% w/w INH for 5 weeks. Blood was collected to assess serum cytokine levels.
Significant decreases in serum IL-12 and IL-1α was noted in female Cbl-b-/- mice (data provided in graph). No effects on IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-13, IL- 17A, eotaxin, GCSF, GMCSF, IFNγ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α were
observed (data provided in supplementary materials) (Metushi and Uetrecht 2014).

In vitro data with cells or cell lines
In HT-2 cells, stimulated with IL-2 (3 or 30 U/mL), increased proliferation was observed at 1 and 10 mM IAH at 30 U/mL and only at 1 mM at 3 U/mL. No effect on proliferation was observed in cells stimulated with 60 U/mL IL-2 (Kucharz and Sierakowski 1990c). Additionally, no effect on proliferation by IAH was observed in HT-2 cells stimulated with PMA (data not provided) (Kucharz and Sierakowski 1990d).

Mode of action information
No data were located.

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Lead (II) Acetate Trihydrate [CASRN 6080-56-4]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
Lead (lead acetate 5.0 mg – 1.5 ng/mL, or lead chloride 0.5 mg – 0.15 ng/mL for 24 hours) significantly reduced cell vitality and/or proliferation and affected secretion of proinflammatory, TH1 and TH2 cytokines in human peripheral mononuclear blood cells that were stimulated with either heat-killed *Salmonella enteritidis* (hk-SE) or monoclonal antibodies. At lower lead levels, expression of IFN-γ, IL-1β and TNF-α were reduced. Monoclonal antibody induced IL-4, IL-6 and IL-10 and hk-SE induced IL-10 and IL-6 levels were increased in the presence of lower lead levels. The authors suggest that lower dose lead suppresses the TH1 cytokine and the proinflammatory cytokines while the increased IL-4 and/or IL-10 production can induce and maintain a TH2 immune response (Hemdan et al. 2005).
Thirty male lead-exposed (battery recycling industry) workers with a blood lead level > 10 µg/dL and 27 unexposed healthy volunteers without any history of occupational exposure to lead were selected for this study. The serum level of IgA was found to be significantly increased in the lead-exposed group as compared to controls. No differences were observed in serum IgG and IgM levels. Both the level of nitric oxide production after stimulation with zymosan-A and the neutrophil respiratory burst as measured by nitroblue tetrazolium reduction were comparable in neutrophils from lead-exposed and unexposed volunteers (Mishra et al. 2006).

Mode of action information
Lead acetate (1 µM) induced activation of NF-κB in primary human CD4+ T lymphocytes. This lead induced activation was blocked by antibodies for p65 and p50 subunits (indicating that the p65:p50 heterodimer (NF-κB) is involved), but not by cRel. Lead acetate (100 pM – 100 µM) did not activate NF-κB in 4 different T cell lines, suggesting that these cell lines may not be a reliable system for studying transcriptional activation in human T cells (Pyatt et al. 1996).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Lead acetate suppressed macrophage-dependent immune responses in cells taken from female BDF1 mice exposed to lead in drinking water at concentrations from 0 to 1000 ppm for 3 weeks. The T-cell dependent sheep erythrocyte primary immune response was suppressed by approximately 40-50% in all lead-exposed groups. In contrast, the *E. coli* lipopolysaccharide (T-cell and macrophage-independent) induced response, was not suppressed (Blakley and Archer 1981). Lead did not alter the ability of T-cell mitogens to induce interferon (Blakley et al. 1982).

Lead acetate effects were modulated by maternal protein intake. Fischer 344 rats were exposed to lead acetate (250 ppm) in the drinking water during breeding and pregnancy until parturition and were fed isocaloric diets (either 20% casein or 10% casein). Offspring exposed to lead and high maternal dietary protein had significantly elevated levels of both IL-4 and TNF-α (values not provided). Offspring exposed to lead and low maternal dietary protein had significantly reduced IL-4 levels compared to the lead control group (values not provided). No other changes were observed, and immune parameters measured in the dams were not affected by treatment (Chen et al. 2004).

In a study comparing immunotoxic effects of various lead salts, Balb/c mice were treated for five consecutive days between immunization and elicitation with intraperitoneal injections of 0.5 or 6 mg/kg of a lead salt. A statistically significant increase in delayed hypersensitivity (as measured by footpad swelling) was observed following administration of lead acetate (55.0% increase in footpad thickness as compared to controls; LOAEL = 6 mg/kg) (Descotes et al. 1984).

Exposure to lead acetate resulted in a decreased ability of mice to survive a sublethal dose of a virulent strain of *S. typhimurium*. C3H/HeN mice were exposed to lead acetate (5 or 10 mM) in the drinking water for up to 18 weeks. At week 16, mice were infected with *S. typhimurium*. 40% of the mice exposed to 5 mM lead acetate survived the infection with a median survival of 26 days. None of the mice treated with 10 mM lead acetate survived, with death occurring within three weeks of becoming infected. In contrast, 80% of control mice survived with a median survival of 60 days. The ability of splenocytes, cultured from the lead-treated and control mice showed a marked reduction in the production of IFN-γ (27% and 35% in mice treated with 5 and 10 mM lead acetate, respectively) and IL-12p40 (42-45% in mice treated with 5 and 10 mM lead acetate, respectively, as compared to induced control). Secretion of IL-4 by splenocytes from lead-treated mice was 3 to 3.6-fold higher than in control mice (Fernandez-Cabezudo et al.
2007).

Adult Sprague-Dawley females were treated with 500 ppm lead acetate via drinking water either early in gestation (days 3-9) or late in gestation (days 15-21). Offsping were assessed as adults. Significantly depressed DTH responses as well as increased IL-10 production, relative monocyte numbers and relative thymic weights were reported in female offspring exposed to lead during late gestation. Male offspring exposed during late gestation had significantly increased IL-12 production and decreased IL-10 production while the DTH response, relative monocyte numbers and thymic weights were unchanged compared to controls. The authors found that adherent splenocytes (likely macrophages) and T lymphocytes are the primary immune cells affected during fetal lead exposure and that gender may influence immunotoxicity due to lead exposure (Bunn et al. 2001).

Lead acetate increased IL-4 production in mice at 40 and 400 mg/L and decreased IFN-γ levels in mice at 400 mg/L. Adult Swiss mice were administered lead acetate in drinking water for 14 days. The authors concluded that low level lead exposure enhances a Th2 response while high lead levels can either stimulate Th2 immune activity or reduce Th1 activity, thus resulting in an imbalance between Th1 and Th2 activation (Iavicoli et al. 2004).

Lead acetate (100 or 1000 ppm in drinking water) did not alter the ability of splenocytes isolated from exposed male Alderly Park rats to mediate native and interferon activated natural cytotoxicity at 2,4,6 and 8 weeks following commencement of exposure. Splenic T-cell function of treated rats as determined by phytohaemagglutinin induced proliferation was comparable to control values (Kimber et al. 1986).
Lead acetate (10 mM in the drinking water for 8 weeks) did not suppress the primary direct humoral immune response to T-dependent antigen (sheep erythrocyte) and T-independent antigens (TNP-LPS, TNP-Ficoll) in several inbred (A, BALB/c, C57Bl/6, DBA/1, SJL, and NZW/NZB F1) and an outbred (CFW) strains of mice (Mudzinski et al. 1986).
Lead acetate (200 ppm either in the drinking water or given intraperitoneally for 4 weeks) decreased the number of lymphocyte cells and cellularity (i.e., number of cells per mg tissue) in the thymus, but no significant changes in either parameter were reported for the submaxillary lymph nodes. Proliferation of T cells stimulated by ConA and proliferation of B cells stimulated by LPS was increased by lead in the thymus by both routes of exposure. In the submaxillary lymph nodes, there was a decrease in the proliferation of T cells following treatment by either route (Teijon et al. 2010).

In vitro data with cells or cell lines
RAW 264.7 cells were treated with 100 ppm lead acetate for 24 hours in the presence or absence of LPS. Lead produced a statistically significant inhibition of the level of LPS-induced nitric oxide (data not provided). No effect on cytotoxicity was observed (Mishra et al. 2006).

Mode of action information
C3H/HeN mice were exposed to lead acetate (0, 5 or 10 mM in drinking water for periods of up to 18 weeks) and inoculated with a virulent strain of *S. typhimurium*. Sera were collected on days 15 and 38 post infection. The authors report that the IgG2a antibodies were elevated in control mice by day 38 post infection (0.09 ± 0.05 on day 15 vs. 0.30 ± 0.03 on day 38; an increase of 300% from day 15), but were only slightly increased in lead-exposed mice (0.11 ± 0.01 on day 15 vs. 0.16 ± 0.02 on day 38). IgG1 isotype antibodies (an isotype induced by IL-4) were significantly elevated in lead exposed mice on day 38, as compared to control mice. The authors conclude that lead acetate induces a subtle but substantial shift toward a Th2-type immune response to infection with Salmonella organism (Fernandez-Cabezudo et al. 2007).
A single intraperitoneal exposure to lead acetate (12 mg/kg) in B6C3F1 mice produced changes in cell surface markers on discrete subpopulations of lymphoid cells from the spleen and bone marrow. The authors concluded that while the changes may not correlate with functional activity of the cells, they seemed to predict a shift to immature cell types, which correlated with the increase in progenitor cells observed (Burchiel et al. 1987).

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Mannitol [CASRN 69-65-8]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
Increased urinary excretion of 9α,11β-prostaglandin F2 and leukotriene-4 were reported in association with mannitol-induced bronchoconstriction in 14 asthmatic patients. Urinary excretion of 9α,11β-prostaglandin F2 and leukotriene-4 increased from 61 to 92 and 19 to 31 ng
× mmol/creatinine, respectively (Brannan et al. 2006). A separate study reported that repeated challenge with mannitol induced refractoriness in asthma patients. The mannitol refractoriness was associated with maintained release of 9α,11β-prostaglandin F2 and leukotriene-4 (Larsson et al. 2011).
Increased proportion of submucosal MCTC was observed in asthmatic individuals with airway hyperresponsiveness to mannitol compared to asthmatic individuals without responses to mannitol. The percentage MCTC increased from 18.7% to 40.3%, but the increase in the numbers of MCTC between the two groups was not significantly increased. Increased gene expression of thymic stromal lymphopoietin and carboxypeptidase AM also were reported (Sverrild et al.
2016).
Mannitol significantly increased 9α,11β-prostaglandin F2, leukotriene-C4, and histamine release from cord blood-derived mast cells (LOAEL = 0.7 M for all endpoints). At the same tested mannitol concentrations (0.3-1.0 M), no concordant increase in lactate dehydrogenase release was observed suggesting cell viability was not affected. The ratio of 9α,11β-prostaglandin F2 to leukotriene-C4 was 156-1 (Gulliksson et al. 2006).
Mannitol did not induce DNA damage in human leukocytes at concentrations from 1.25 to 10 mM (Frenzilli et al. 2000).
Mannitol (22 mmol/L) did not increase IL-6 or TNF-α secretion from monocytes treated with glucose (11 mmol/glucose) for 24 hours. A similar lack of effect was observed when cells were incubated for 48 hours (Morohoshi et al. 1996).
At the highest concentration tested (100,000 μM), mannitol did not reduce cell viability in human LCLs or PBMCs. Mannitol (50,000 μM) did not modulate TNF-α, IL-6, IL-2, IL-4, IL-10, or IFNγ release in LCLs pre-treated with phorbol 12-myristate 13-acetate/ionomycin stimulated cells (Markovic et al. 2015).
Mannitol did not inhibit growth of human granulocyte precursor cells at a concentration up to 5 mM (Holdener et al. 1983).

Mode of action information
Mannitol is shown to narrow the airway in asthmatic, but not healthy, test subjects (Brannan et al. 2001, 2003, 2000). Mannitol is proposed to increase osmolarity of airway surface liquid, leading to an increase in mediator release (e.g., histamine, prostaglandins, and leukotrienes) from inflammatory cells which induces bronchoconstriction (Brannan et al. 2006; Sverrild et al. 2016). One mediator that is proposed to be released is prostaglandin D2 from mast cells (Brannan et al. 2003, 2006).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

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Nickel (II) Sulfate Hexahydrate ($NiSO_4$) [CASRN 10101-97-0]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
In PBMC from five nickel allergic individuals 0.1 mM $NiSO_4$ increased IL-4 and IFN-γ production. The peak effect was lower than when PBMC were incubated with PHA (data shown in graph) (Thomas et al. 2003).
$NiSO_4$ (85 µg/mL) significantly upregulated expression of CD40, CD83, CD86, and CD54 markers on THP-1 cells. $NiSO_4$ also significantly increased production of TNF-α and IL-8 in a dose-dependent manner. IL-6 production was significantly increased after exposure to 170 µg/mL (Miyazawa et al. 2007). Ade and colleagues noted that $NiSO_4$ induced CD83, CD86, HLA-DR, and CD40 in a dose dependent manner in dendritic cells (Ade et al. 2007).

Mode of action information
$NiSO_4$ was shown to alter dendritic cell phenotypes by activation of MAPKs and NF-□B. Additionally, $NiSO_4$ induced IL-8, IL-6, and IL-12 p40 production (Ade et al. 2007; Antonios et al. 2009). Activation of the MAPK pathway may lead to upregulation of the Cys-Cys chemokine receptor, CCR7, which allows dendritic cells to migrate to the draining lymph nodes (Boisleve et al. 2004).
$NiSO_4$ has a similar capacity to stimulate polyclonal CD+4 in Ni-allergic and -nonallergic individuals. Differences in clonal expansion or presence of Ni-binding motifs in MHC class II complexes could be involved in the development of allergic contact dermatitis (Lisby et al. 1999).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Male Wistar rats were intratracheally instilled with 1, 2, 4, or 8 µmole $NiSO_4$ per rat. The rats were euthanized up to 7 days after treatment. Treatment decreased the percentage of lymphocytes in pulmonary lymphoid cells (~55% to ~40%). NK activity in lymphoid lung cells was dependent on concentration and effector:target cell ratio. NK activity was decreased 1 day after treatment of 4 and 8 µmole $NiSO_4$ at the effector to target cell ratio of 6:1. Two days after treatment suppression of NK activity was significant at doses ≥2 µmole $NiSO_4$ and at the effector to target cell ratio of 6:1. After 7 days, a significant decrease was only observed at 8 µmole.
$NiSO_4$ did not significantly modulate alveolar macrophage cytotoxic activity towards 3T12 target cells. Decreased levels of TNF-α was reported at all time points, while increased IFN-

γ level was only noted after exposure to 8 μmole/rat on day 2 (data in graph) (Goutet et al. 2000).

Female B6C3F1 mice were exposed to $NiSO_4$ aerosol for 6 hours per day, 5 days per week for 65 days. The actual exposure concentrations tested were 0.027, 0.11, or 0.45 mg Ni/m3. No change in thymic weight was reported. A significant increase in the number of nucleated cell numbers from lung-associated lymph nodes (LALN) and lavage fluid, after mice were immunized with sheep red blood cells, was noted at the highest dose tested (1.72- and 3.86-fold, respectively). Nonsignificant increase in the total antibody-forming cells (AFC)/(LALN) and nonsignificant decrease in AFC/spleen, after immunization with sheep red blood cells, were also noted after $NiSO_4$ exposure. $NiSO_4$ had no effect on mixed lymphocyte response of spleen cells after exposure to mitomycin C-treated spleen cells from DBA/2 mice. No effect in mitogen- stimulation assays also were noted by $NiSO_4$ exposure. $NiSO_4$ modulated pulmonary alveolar macrophage function, as measured by phagocytosis of opsonized erythrocytes; activity was significantly increased at 0.11 mg Ni/m3 (data not provided). Comparatively, $NiSO_4$ had no effect on peritoneal macrophage phagocytosis activity at any tested dose. The highest dose of $NiSO_4$ was associated with a significant two-fold increase in the number of B16F10 tumor nodules in the lungs of treated animals. However, incorporation of radiolabeled uridine was not considered biologically significant. $NiSO_4$ did not affect splenic NK cell cytolytic activity (Haley et al. 1990).

Histopathological lesions in lungs, liver, thymus, kidneys, spleen, and lymph nodes were noted in male F344 rats intramuscularly injected with 125 μmole/kg $NiSO_4$ over 26 days. Thymus glands from rats treated with the highest dose were much smaller than controls.

Corticomedullary junction was not distinct and extensive degeneration and depletion of lymphocytes in the thymic cortex were noted. Additional tissues from these rats were evaluated further. In the lungs, large alveolar macrophages and polymorphonuclear leukocytes were noted in alveolar spaces and exudate. In the spleen and lymph nodes, lymphocytes were focally depleted in the white and red pulp (Knight et al. 1991).

Male Sprague-Dawley rats were exposed to 0.02, 0.05, and 0.1% $NiSO_4$ in drinking water for 13 weeks. Effects on splenic lymphocyte and thymocyte subpopulations were evaluated. In splenic lymphocytes, increases in the total number of T-cells (LOAEL = 0.05%) and CD8+ T-cells (LOAEL = 0.02%) were reported. For CD4+ T-cells, the number of cells increased at 0.05% $NiSO_4$ and then decreased at 0.1% dose. An increase in the total number of B cells was noted at 0.05% $NiSO_4$. Subchronic exposure to 0.02% $NiSO_4$ also increased the percentage and absolute number of thymocyte CD8+ cells. Exposure to 0.05% $NiSO_4$ increased the total number of thymocyte cells, the percentage and absolute number of CD8+ cells, and absolute numbers of both CD4+ and B-cell populations. Exposure to 0.1% $NiSO_4$ decreased the total number of thymocytes, the percentage and absolute number of CD4+ T cells, and absolute numbers of CD8+ T cells and of B cells (Obone et al. 1999).

Male C3H/He mice were provided 0.01, 0.05, 0.1, 0.25, 0.5, or 1% $NiSO_4$ for 7 or 10 weeks. Mice were then sensitized with $NiSO_4$ for 7 days and the footpad thickness was measured. The mice were then challenged with 0.4% $NiSO_4$ and footpad swelling was measured 24 hours later. After 7 weeks of oral exposure, footpad swelling was not reduced at any of the tested doses.

However, after 10 weeks of exposure swelling was decreased (LOAEL = 0.1%) (Ishii et al. 1993). Lymph nodes from C3H/He mice sensitized to $NiSO_4$ were incubated with various monoclonal antibodies and then injected into naïve mice. After challenging with $NiSO_4$, footpad swelling was measured. Cells treated with CD4-, Thy1.2-, or Ig-specific antibodies showed reduced swelling while cells treated with CD8 antibodies induced footpad swelling (Ishii et al. 1993). Macrophage and PMN chemotactic activities in bronchoalveolar fluid were increased at 2 days after intratracheal instillation of 50 μg Ni per male Wistar rat. Activity then decreased until end of the experiment (14 days). Comparatively, LTB4 were maximally decreased at day 1 and then increased to control levels by day 14 (Hirano et al. 1994).

In vitro data with cells or cell lines

Spleen cells from C57BL/6 and Rag-1 deficient mice were stimulated with varying concentrations of $NiSO_4$ (concentrations not provided). Using the ELISPOT assay, IL-2, IL-4 and IFN-γ secreting cells were identified in splenic cells from C57BL/6 mice. The number of IFN-γ cells were greater than the IL-2 and IL-4 cells. At higher concentrations (≥400 μM), the numbers of IL-2 and IL-2 secreting cells decreased while those secreting IFN-γ remained high. The number of IFN-γ cells did not increase due to previous immunization of $NiSO_4$. In splenic cells from Rag-1 deficient mice, $NiSO_4$ also contained IFN-γ secreting cells. However, at higher concentrations the cell levels decreased (in comparison to wild-type). Addition of NK1.1 antibodies produced a partial depletion in the cells. Further studies showed that addition of NKG2D antibodies reduced the number of IFN-γ secreting cells in wild-type and RAG-1 deficient mice (Kim et al. 2009).

Mode of action information

No data were located.

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Nitrobenzene [CASRN 98-95-3]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female B6C3F1 mice were exposed to 30, 100, or 300 mg/kg nitrobenzene for 14 days via gastric intubation. Hepatomegaly and splenomegaly were observed in mice that received 100 and 300 mg/kg nitrogenzene. Mild congestion in the red pulp areas of the spleen was noted in mice that received 100 mg/kg, while the spleen was dark red in those that received 300 mg/kg. Absolute and relative spleen weight were significantly increased (LOAEL = 100 mg/kg). Comparatively, absolute and relative thymus weights were increased only at 100 mg/kg. The number of bone marrow cells increased in a dose-dependent manner (LOAEL = 30 mg/kg). At the highest dose tested the increase was 60% above controls. DNA synthesis and the number of CFU-GM per femur also were increased (LOAELs = 30 mg/kg). In response to sheep erythrocytes, a significant increase in spleen weight (62%) and spleen cell number (29%) was observed at 300 mg/kg, when animals were sensitized four days after nitrobenzene exposure. Comparatively, a decrease in IgM AFCs were decreased (LOAEL = 100 mg/kg). When responses to sheep erythrocytes were observed (sensitization occurred 5 days after nitrobenzene exposure), spleen weight and cells were increased at 100 and 300 mg/kg. However, no effects on IgG AFC were noted. When 20 days lapsed between nitrobenzene exposure and sensitization to sheep erythrocytes, no effects were reported. No effect on delayed hypersensitivity was reported at any of the tested doses. Splenic proliferation responses induced by PHA and ConA were suppressed by exposure to nitrobenzene (LOAEL = 100 mg/kg). No effect on LPS-induced proliferation were reported. Responses to DBA/2 mice spleen cells also were decreased (LOAEL
= 100 mg/kg). Using radiolabeled sheep erythrocytes, the phagocytic index was shown to be increased in a dose-dependent manner. The phagocytic activity of peritoneal cells also was increased in a dose-dependent manner (LOAEL = 300 mg/kg). The ability of spleen cells to lyse radioactivity from YAC-1 target cells also was evaluated. Nitrobenzene exposure produced a decrease in lysis capacity at 100 and 300 mg/kg at effector:target ratios of 100:1 and 30:1.

Nitrobenzene did not affect host resistance to *Streptococcus pneumonia, Plasmodium berghei,* herpes simplex 2, or B16F10 melanoma. Comparatively, host resistance to *Listeria monocytogenes* was decreased. A challenge of 6 x 10^3 *L. monocytogenes* per mouse killed 13%

of control animals and 57% of animals treated with 300 mg/kg nitrobenzene. A challenge with 1.2×10^4 *L. monocytogenes* increased animal death from 19% in controls to 100% at 100 mg/kg nitrobenzene and 86% at 300 mg/kg nitrobenzene (Burns et al. 1994).

In vitro data with cells or cell lines
No data were located.

Mode of action information
Two proposed targets of nitrobenzene are: (1) erythrocytes and (2) precursors to erythrocytes and other cells (e,g., granulocytes). The site of action is proposed to be the bone marrow. Additionally, effects on T-cell function may play a role in increased susceptibility to *L. monocytogenes* (Burns et al. 1994).

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o-Benzyl-p-chlorophenol (BCP) [CASRN 120-32-1]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
B6C3F1 mice were orally administered 100, 300, or 500 mg/kg BCP for 14 days. No effect on spleen or thymus weight were reported. No effect on delayed hypersensitivity response (to keyhold limpet hemocyanin), antibody response to sheep erythrocytes, serum IgM, IgA, or IgG levels, or splenic lymphocyte proliferation were noted. Absolute and relative liver weights were increased at the highest dose group. Additionally, BCP-treated mice did not develop tumors after challenge with PYB6 tumor cells (vs. controls which had a 15% tumor incidence) (Birnbaum et al. 1986).
BCP produced contact hypersensitivity in female B6C3F1 mice (Stern et al. 1991).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

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Stern ML, Brown TA, Brown RD, Munson AE. 1991. Contact hypersensitivity response to o-benzyl-p-chlorophenol in mice. Drug and chemical toxicology 14:231-42; doi:10.3109/01480549109002186.

Perflouorooctanoic Acid (PFOA) [CASRN 335-67-1]

Human Data

Data from epidemiology studies
Several studies have suggested that prenatal PFOA exposure is linked to immunosuppressive and immunotoxicant effects observed in offspring. Granum and colleagues (2013) reported that maternal PFOA blood levels, collected at birth, were positively associated with decreased rubella antibody-levels (β = -0.40) and an increased number of common cold episodes in children from 0-3 and 2-3 years old. Cord blood IgE levels also were suppressed in female infants with high maternal PFOA levels. However, no effects on number of 18 month-old infants with allergies (e.g., food allergy, eczema) or infections (e.g., otitis media, pneumonia, skin infections, chicken pox) were noted (Okada et al. 2012). Okada and colleagues noted that while the correlation between fetal PFOA levels and the evaluated endpoints were not available, the results suggest that PFOA produced immunosuppressive effects after prenatal exposure.
A positive association between serum PFOA in adults and development of ulcerative colitis also was reported. However, a positive association with other autoimmune diseases, such as Type 1 diabetes, lupus, multiple sclerosis, Chron's disease, and rheumatoid arthritis, was not observed (Steenland et al. 2013).
Chang and colleagues (2016) conducted a systematic review to summarize and evaluate epidemiological literature on PFOA and perfluorooctanesulfonate (PFOS) with relation to evaluated immune endpoints. Endpoints evaluated included immune biomarker levels (e.g., IgE levels, white blood cell count, and C-reactive protein), immune gene expression patterns, atopic or allergic disorders (e.g., asthma, eczema, and food allergy), infectious disease (e.g., common cold), vaccine response, and autoimmune and inflammatory conditions (e.g., ulcerative colitis, rheumatoid arthritis, and osteoarthritis). The authors stated that the totality of the data limited development of a conclusion on the causal relationship between PFOA and/or PFOS exposure and evaluated endpoints due to inconsistent results and confounding factors.

In vitro data with cells or cell lines
Studies with human cells or human-derived cell lines indicate that PFOA modulates cell activation and cytokine production. In human PBMC, PFOA significantly increased the percentage of viable cells at concentrations <125 µg/mL. At higher concentrations (250 and 500 µg/mL), a significant decrease in cell viability was reported (values not reported). No effects on T-cell proliferation (NOAEL = 1 µg/mL) or, TNF-α or IL-6 release (NOAEL = 1 µg/mL) were noted. PFOA also increased monocyte differentiation in HL-60 cells (LOAEL = 100 µg/mL) (Brieger et al. 2011). Comparatively, PFOA decreased TNF-α, IL-4, and IL-10 (LOAEL
= 1 µg/mL, 10 and 10 pg/mL, respectively) in peripheral leukocytes. PFOA also decreased TNF- α (LOAEL = 10 µg/mL) production in THP-1 cells (value not reported). PFOA did not affect IL- 2 production in Jurkat cells (value not reported) (NOAEL = 0.005 µg/mL) (Corsini et al. 2011, 2012; Midgett et al. 2015).

Mode of action information

Direct modulation of NF-□B has been implicated in modulation of cytokine production and secretion (Corsini et al. 2012). PFOA interaction with the PPARα receptor also was implicated in immunomodulatory effects in human cells. Receptor interaction was associated with reduced p65 phosphorylation and NF-□B-mediated transcription (Corsini et al. 2011). The extent the role of PPARα receptor activation plays in human effects is unclear given the low level of human receptor expression (Corsini et al. 2014).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Animal studies suggest that PFOA exposure can affect innate and adaptive immune functions *in vivo*. Dietary exposure to PFOA (0.02% w/w) for 7 days significantly decreased spleen and thymus weight, and splenocyte and thymocyte levels in wild-type C57Bl/6 mice. Spleen weight and splenocyte numbers were not affected in PPARα-null mice (Yang et al. 2002).

Table 1. Data from Yang et al. (2002)

Group and Treatment	Body weight (g)	Spleen weight (g)	Splenocyte number (x 10^6)	Thymus weight (g)	Thymocyte number (x 10^6)
Wild-type mice					
None	24.5 ± 1.58	0.082 ± 0.006	68.8 ± 16.8	0.061 ± 0.014	81.0 ± 28.2
PFOA	21.0 ± 0.74**	0.050 ± 0.001***	15.3 ± 5.84***	0.013 ± 0.001***	12.8 ±7.98***
PPARα-null mice					
None	23.6 ± 2.9	0.064 ± 0.021	84.0 ± 19.3	0.054 ± 0.006	88.3 ± 7.04
PFOA	23.5 ± 1.0†	0.054 ± 0.015	73.8 ± 26.2†††	0.033 ± 0.005***†††	54.0 ± 12.7**†††

All values are means ± SEM for four animals in each group. **P<0.01, ***P<0.001 compared to the corresponding control.

†P<0.05, †††P<0.001 compared to the corresponding wild-type group.

Dose response studies in C57Bl/6N mice reported that PFOA decreased absolute and relative spleen weights (LOAEL = 7.5 and 15 mg/kg/day, respectively) and absolute and relative thymus weights (LOAEL =15 mg/kg/day for both endpoints). Organ weight effects were generally reversed by 15 days after exposure was terminated (DeWitt et al. 2008). No effect on organ weights was reported in PPARα knockout mice treated with 7.5 or 30 mg/kg/day PFOA for 15 days (DeWitt et al. 2016).

PFOA exposure in drinking water was associated with reduced IgM antibody titers in C57Bl/6J and C57Bl/6N mice (DeWitt et al. 2008, 2016). Removal of the adrenal glands in C57Bl/6N mice did not reverse reductions in IgM antibody titer levels, suggesting that the observed suppression was not in response to corticosterone production (DeWitt et al. 2009). Modulation of the complement system was observed in C57Bl/6 mice administered PFOA-treated diets. In mice provided diets containing PFOA for 10 days, activity of the classical and alternative pathways of the complement system was decreased (N/LOAEL = 0.01%/0.02%, respectively). Serum C3 levels also was decreased by PFOA (N/LOAEL = 0.01%/0.02%, respectively). Results showed that PFOA-induced hepatotoxicity was associated with activation of the complement system (Botelho et al. 2015).

Dietary PFOA (0.02% w/w) for 10 days significantly decreased total white blood cell count (72%) and number of macrophages in the bone marrow (12.2%) (Qazi et al. 2009). Exposure of mice to 0.002% PFOA for 10 days modulated levels of intrahepatic immune cells. The total number of all leukocytes (CD45+) was increased 2-fold in treated mice. Additionally, changes in cell numbers other cell types also were noted (e.g., granulocytes and myeloid suppressor cells). Hepatic levels of TNF-α (33%), IFN-γ (37%), and IL-4 (31%) were decreased in treated mice; IL-6 levels were not affected (Qazi et al. 2010).
Hu and colleagues reported effects in offspring of dams exposed to PFOA. Dams were gavaged with 0.02, 0.2, or 2 mg/kg PFOA from before pregnancy to PND 21. Splenic CD4+CD25+Foxp3+ T cells was decreased by 22% in exposed offspring (LOAEL = 2 mg/kg) (Hu et al. 2012).

In vitro data with cells or cell lines
Reduced lymphocyte proliferation was observed in cells isolated from C57Bl/6 mice treated with diets containing 0.02% PFOA for 7 days. No effect was observed in lymphocytes isolated from PPARα-null mice also provided diets containing 0.02% PFOA (values not provided) (Yang et al. 2002). Increased *ex vivo* production of TNF-α in cells isolated from peritoneal cavity (2.2-fold) and bone marrow (1.7-fold), and IL-6 in cells isolated from peritoneal cavity (2.6-fold) was observed in mice treated with 0.02% dietary PFOA for 10 days. Comparatively, TNF-α production was decreased (0.8-fold) in cells isolated from spleen of treated animals (Qazi et al. 2009). IgM or IFN-γ production levels were not modulated in intrahepatic immune cells isolated from male C57Bl/6 mice provided diets with 0.002% (w/w) PFOA for 10 days (Qazi et al. 2010).
Ex vivo co-cultures of splenic CD4+CD25+ and CD4+CD25- T cells offspring gestationally and lactationally exposed to PFOA were assessed for effects on IL-10 production. Results showed IL-10 produced was significantly decreased at all doses 61%-75% in cells obtained from male offspring (LOAEL = 0.02 mg/kg). *Ex vivo* measurement of autoreactivity antibodies in female mice gestationally and lactationally exposed to 0.02 and 2 mg/kg PFOA showed an decrease (26%) in anti-ssDNA (Hu et al. 2012).

Mode of action information
PFOA suppresses T-cell-dependent and T-cell-independent antibody responses (DeWitt et al. 2012). The role of PPARα in PFOA-induced immunosuppression may be strain dependent (Corsini et al. 2014). PFOA-induced effects on humoral immunity may occur through effects on B-cell/plasma cell function (DeWitt et al. 2016). Direct effects on immune cells also are a proposed mode of action of PFOA (Corsini et al. 2014).
The lack of impact of removal of the adrenal gland on PFOA-induced inhibition of IgM antibody titer levels suggests that the observed effects are not dependent on elevated corticosterone levels in mice (DeWitt et al. 2009).
Effects on lymphoid organ weights and measures of immune function (i.e., thymus and spleen) indicate that they are differentially sensitive to PFOA effect. The biological basis for this difference is not known (DeWitt et al. 2016).

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Human Data

Data from epidemiology studies
No difference was noted in lymphocyte counts between individuals with or without toluene exposure (Akbas et al. 2004).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Male C3H mice were exposed to 9 ppm toluene (nose-only inhalation exposure) for 30 minutes on study days 0, 1, 2, 7, 14, 21, and 28. Mice also were immunized with ovalbumin. Toluene exposure significantly increased total cell (3-fold) and macrophage (3.1-fold) count in BAL 24 hours after final exposure. No effect on lymphocyte count was noted. BDNF level in BAL was increased in toluene-exposed mice that were immunized with ovalbumin (data in figure). Splenic ratio of CD4 and CD8 cells in control and toluene-exposed mice were not significantly different;
3.95 and 4.14, respectively. Treatment with ani-CD4 antibody decreased the ratios to 0.65 and 0.49, respectively. Toluene exposure significantly increased plasma levels of nerve growth factor (data in figure), but did not increase plasma BDNF levels (data not provided) (Fujimaki et al. 2009).
Male C57BL/10 and B10.BR/Sg mice were inhalationally exposed to 0, 5, and 50 ppm toluene for 6 hours per day, 5 days per week for 6 weeks. Subgroups of control and treated mice were administered ovalbumin prior to exposure. Toluene exposure did not impact ConA- or LPS-induced proliferation of spleen cells from C57BL/10 mice. While no effect of ConA was noted in B10.BR/Sg (not treated with ovalbumin) mice spleen cells, 50 ppm toluene significantly increased the LPS-induced proliferation of spleen cells. Comparatively, 50 ppm toluene significantly decreased spleen cell proliferation in B10.BR/Sg mice treated with ovalbumin (data in graphs). Toluene did not alter expression of CD3, CD19, and CD11b (data not provided). Forkhead box P3 (Foxp3) transcription was significantly increased in spleen cells from B10.BR/Sg mice exposed to 5 ppm toluene and ovalbumin, when compared to controls and those not treated with ovalbumin. No effect on GATA3 or T-bet expression was noted (Fujimaki et al. 2010).
Pregnant C3H/HeN mice were exposed to 50 ppm toluene via inhalation on GD 14-18. Additionally, male offspring of unexposed dams were exposed to 50 ppm toluene on PND 2-6 or 8-12. The following table summarizes the effects observed in male offspring on PND 21.

Table 1. Summary of effects in male offspring

Origin	Biomarker	GD 14–18	PND 2–6	PND 8–12
Plasma	IgG2a	No effect	Decrease	Increase
	IgG1	Decrease	Decrease	Decrease
Spleen	CD4+ lymphocyte subset	No effect	Decrease	Decrease
	CD8+ lymphocyte subset	No effect	No effect	Decrease
	T-bet mRNA	No effect	Decrease	Decrease
	Foxp3 mRNA	No effect	Decrease	Decrease
	GATA3 mRNA	No effect	No effect	No effect

On PND 42, IgG2a levels were decreased in mice exposed to 50 ppm toluene on PND 8–12. No effect on IgG1 was noted. CD19+ B-lymphocytes and CD4+ T-lymphocytes were significantly decreased, while CD3+ T-lymphocytes were increased at PND 42 after exposure on PND 8–12. Additionally, T-bet expression was significantly decreased, while no effects on GATA3 or Foxp3 mRNA expression were reported (Win-Shwe et al. 2012a).
Pregnant C3H/HeN mice were exposed to 5 or 50 ppm toluene via inhalation on GD 14–18. Additionally, male offspring of unexposed dams were exposed to 5 or 50 ppm toluene on PND 2–6 or 8–12. In the hippocampus of PND 21 male offspring, TNF-α and NF-κB mRNA were significantly increased in mice exposed to 50 ppm on PND 2–6 when compared to controls (data in graphs). TNF-α, CCL3, and NF-κB were increased in mice exposed to 5 ppm on PND 8–12 (data in graphs) (Win-Shwe et al. 2012b).

In vitro data with cells or cell lines
Toluene (500 μM) exposure significantly increased ConA- (1.8-fold) and LPS- (2.1-fold) induced proliferation of spleen cells from female C57BL/6 mice. However, at the same concentration toluene did not modulate NK activity or suppress CTL formation (Grayson and Gill 1986).

Mode of action information
Low-level (5 ppm) inhalational exposure to toluene activates the STAT6, STAT5, and Foxp3 signaling pathway to enhance Th2-related and T_{reg}-related responses in B10.BR/Sg mice treated with ovalbumin (Fujimaki et al. 2010). Toluene also enhanced NF-□B, STAT5, and NF-AT in thymus cells of C3H/HeN mice inhalationally exposed to toluene (Liu et al. 2010). Toluene modulation of IL-2 synthesis, after oral exposure, may play a role in observed immunotoxic effects (Hsieh et al. 1989).

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Tributyltin Chloride (TBTC) [CASRN 1461-22-9]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
TBTC dose-dependently decreased the percentage of colony forming unit-granulocyte macrophage (CFU-GM) colonies at concentrations ranging from 0.001 to 3.3 µM (data not provided) (Carfi et al. 2007).
Cytokine release from human lymphocytes was assessed in whole blood obtained from three donors. IFN-γ was evaluated in blood from two donors after PHA stimulation for 72 hours. Comparatively, TNF-α was evaluated in blood from three donors after LPS stimulation for 72 hours. Overall, IFN-γ and TNF-α was modulated (i.e., either increase or decrease release) in all tested samples (Carfi et al. 2007).
Long-term cultures of human bone marrow cells were incubated with 0.001 µM TBTC in the presence or absence of a cytokine mixture for 7 or 14 days. A significant decrease in the percentage of CD19+CD22+ cells, in the absence of effects on the total lymphocyte population or percentage of T-cell subsets was reported after 7 and 14 days. Addition of cytokine mixture had no effect on TBTC effects. TBTC also induced cell death in CD19+ lymphocytes, in the absence of PPAR-γ receptor expression (Carfi et al. 2010).
The IC50s for cell viability in human LCLs or PBMCs were 0.25 and 0.33 µM, respectively. TBTC (0.1 µM) did not modulate TNF-α, IL-2, IL-4, or IL-10 release in LCLs pre-treated with phorbol 12-myristate 13-acetate/ionomycin stimulated cells. Comparatively, TBTC significantly decreased IL-6 and IFN-γ release (Markovic et al. 2015).

Mode of action information
In vitro toxicogenomic studies in Jurkat cells (human lymphoblastic T-cell line) showed that TBTC activated cellular stress response and retinoic-acid mediated response genes (Shao et al. 2013).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
In a 2-week study, male Wistar rats were provided diets containing 15, 50, or 150 ppm TBTC. A dose-related decrease in relative and absolute spleen and thymus weights were reported (LOAEL
= 50 and 15 ppm, respectively). Concurrent to the change in thymus weight, a decrease in thymic cell counts also was observed (LOAEL = 50 ppm). However, no signs of increased lymphocyte destruction in the spleen was observed. A dose-related increase in relative liver weight was reported (LOAEL = 50 ppm). Decreased thymus weight also was observed in rats fed 100 ppm

TBTC for 4-weeks (43% of control weight); no effects on spleen or liver weight were noted (Snoeij et al. 1985).

In utero and lactational exposure effects of TBTC (0.025, 0.25, or 2.5 mg/kg/day) were evaluated in Sprague-Dawley rats. Dams were orally dosed with TBTC from GD 8 until weaning. After weaning, pups were orally exposed to the same dose as the dam until sacrifice (up to PND 90). In males, a significant decrease in spleen weight was only observed in pups treated with 0.25 mg/kg/day on PND 30. A significant decrease in thymus weight also was noted on PND 30 (LOAEL = 2.5 mg/kg/day) (Cooke et al. 2004). Serum IgM levels were increased in 30- and 60-day old female offspring, while IgA, IgM, IgG, and IgG2a levels were increased in 90-day old male rats (Tables 1 and 2) (Tryphonas et al. 2004).

Table 1. Serum IgM levels in 30- and 60-day old females†.

	30-day old females				60-day old females			
	Control	0.025 mg/kg/day	0.25 mg/kg/day	2.5 mg/kg/day	Control	0.025 mg/kg/day	0.25 mg/kg/day	2.5 mg/kg/day
IgM	51.6 ± 8.8	41.2 ± 6.8	39.6 ± 5.8	66.5 ± 9.9	34.0 ± 3.2	63.8 ± 5.8	68.1 ± 16.4	73.0 ± 15.0

†Values provided as pg Ig/mL serum × 10^4 (standard error of the mean ± standard error).

Table 2. Serum immunoglobulin levels in 90-day old males†

	Control	0.025 mg/kg/day	0.25 mg/kg/day	2.5 mg/kg/day	Pearson product moment correlation
IgA	32.0 ± 8.8	13.9 ± 3.3	9.7 ± 3.4*	11.9 ± 1.6	>0.05
IgM	46.2 ± 8.9	65.1 ± 4.9	69.6 ± 5.8	232.5 ± 90.1*	0.00168
IgG	96.8 ± 9.6	184.2 ± 86.7	194.6 ± 25.7*	314.1 ± 57.5*	0.0134
IgG1	41.5 ± 8.6	77.2 ± 28.6	85.2 ± 18.6	58.1 ± 15.9	>0.05
IgG2a	53.1 ± 5.7	59.1 ± 6.3	50.8 ± 4.6	31.3 ± 4.4*	0.00041
IgG2b	34.1 ± 3.3	39.1 ± 5.6	39.3 ± 3.5	31.6 ± 4.2	>0.05
IgG2c	13.6 ± 1.7	20.6 ± 3.6	41.0 ± 19.4	20.9 ± 2.2	>0.05

†Values provided as pg Ig/mL serum × 10^4 (standard error of the mean ± standard error).
* Significantly different from control.

The number and percentage of NK cells was increased in 30-day female and male offspring (LOAEL = 2.5 mg/kg/day). A dose-dependent increase in the number and percentage of NK cells also was noted in 90-day male rats. In 60-day female offspring an increase in the percentage of CD4+8+ T lymphocytes (LOAEL = 0.25 mg/kg/day). No anti-sheep erythrocyte IgM response or lymphoproliferative activity of splenocytes in response to mitogen stimulation

was noted in 60- day old female rats or 90-day old male rats (data not provided). Delayed-type hypersensitivity to oxazolone was increased in 90-day old male rats at 0.025 and 0.25 mg/kg/day and decreased at
2.50 mg/kg/day. Mean colony forming *L. monocytogenes* bacteria was non-linearly increased at 48 hours post-infection and statistically significant in pairwise comparisons (0.25 mg/kg/day) in 60-day old females. In 90-day old males, a non-linear dose-response trend 3 days after infection was reported. No effects in serum levels of IL-2, TNF-α, IFN-γ, and IL-1β were reported in males or females. A non-linear dose-response increase in NK activity in 60-day females was reported (Tryphonas et al. 2004).

Lactational exposure in mice to TBTC also impaired innate immunodefenses in offspring. C57BL/6 pregnant mice were given drinking water with 15 or 50 µg/mL TBTC from parturition to weaning. Clearance of *Escherichia coli* K-12 from the peritoneal cavity and spleen of offspring treated with 15 µg/mL TBTC was significantly decreased (Kimura et al. 2005).
ICR mice were orally dosed with 0.5, 4, or 20 mg/kg TBTC for 28 days. Relative spleen and thymus weights were significantly decreased at the highest dose tested (46% and 59% decrease, respectively). TBTC also decreased the number of plaque forming cells in response to exposure to sheep red blood cells (LOAEL = 4 mg/kg). TBTC also suppressed delayed-type hypersensitivity response to sheep red blood cells when assessed 24 and 48 hours after injection (LOAEL = 4 mg/kg). TBTC suppressed T-lymphocyte proliferation in a dose dependent manner (LOAEL = 20 mg/kg). Increased percentage of early- and late-stage thymocyte apoptosis, and expression of Fas protein expression in proteins also were noted (LOAEL = 4 mg/kg) (Chen et al. 2011).
Esophageal tubing of male C3H/Hen mice with 10 or 100 ppm TBTC for 1 week was associated with decreased NK activity. NK activities were inhibited 36% to 46% at effector:target (YAC-1 cells) ratios of 25:1 and 50:1, respectively. A significant decrease in the percentage of large granular lymphocytes (~60%) also was noted 1 week after end of treatment (Ghoneum et al. 1990).

In vitro data with cells or cell lines
Neutrophils and macrophages from mice lactationally exposed to TBTC (15 or 50 µg/mL) were isolated from peritoneal exudates. Bacterial binding to isolated neutrophils from offspring treated with 50 µg/mL TBTC was significantly decreased (data not provided). Comparatively, bacterial binding was increased in macrophages isolated from offspring treated with 50 µg/mL TBTC. Decreased phagocytosis (LOAEL = 15 µg/mL) and killing activities (15 µg/mL) only were observed in neutrophils. No effect on IL-1β, IL-6, or TNF-α production was noted from macrophages or neutrophils. MCP-1 production was significantly increased in neutrophils isolated from offspring treated with 50 µg/mL TBTC (Kimura et al. 2005).
Rat and mouse spleen cells were treated with TBTC for 24 hours and then LPS or PHA for 48 hours. Then, mitogen stimulation was assessed by 5-bromo-2'-deoxyuridine incorporation. TBTC inhibited cellular proliferation for both species; the inhibitory response was more potent in mice cells vs. rat cells (IC50 with LPS: 0.0025 vs. 0.007 µM, IC50 with PHA: 0.002 vs. 0.007 µM). TBTC also inhibited rat spleen proliferation that was stimulated by ConA (no data provided). TBTC also inhibited anti-CD3 antibody stimulation of mouse lymphocyte proliferation (IC50 > 0.1 µM) (Carfi et al. 2007).
NK activity was dose-dependently inhibited in splenic lymphocytes incubated with 0.01 to 1 ppm TBTC. The LOAEL values at effector:target ratios of 25:1 and 50:1 were 0.05 and 0.01 ppm, respectively. Decreased viability of splenic lymphocytes also was reported after exposure to TBTC (LOAEL = 0.1 ppm) (Ghoneum et al. 1990).

Mode of action information

In vivo effects of TBTC on the thymus of orally treated rats are proposed to be due to the metabolite dibutyltin chloride (Snoeij et al. 1988).

The role of apoptosis is not clear. In one study the authors indicated that apoptosis does not appear to be involved in inhibition of immature thymocyte proliferation, which may lead to thymus atrophy (Gennari et al. 1997). In a separate study, the authors proposed oxidative stress plays a role in TBTC-caspase-dependent apoptosis in murine thymocytes (Sharma and Kumar 2014).

In vitro studies suggest that TBTC promotes Th2 polarization via depletion of glutathione in antigen-presenting cells, which leads to modulation of IL-10 and IL-12 production (Kato et al. 2006).

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Urethane [CASRN 51-79-6]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
The IC50s for cell viability in human LCLs or PBMCs were 82,329 and 140,768 μM, respectively. Urethane (5000 μM) did not modulate TNF-α, IFN-γ, IL-2, IL-4, IL-6, or IL-10 release in LCLs pre-treated with phorbol 12-myristate 13-acetate/ionomycin stimulated cells (Markovic et al. 2015).
Urethane dose-dependently decreased the percentage of CFU-GM colonies at concentrations greater than 1000 μM (data provided in graph) (Carfi et al. 2007).
Cytokine release from human lymphocytes was assessed in whole blood obtained from four donors. IFN-γ was evaluated in blood from three donors after PHA stimulation for 72 hours. Comparatively, TNF-α was evaluated in blood from four donors after LPS stimulation for 72 hours. IFN-γ was modulated (i.e., either increase or decrease release) in a single tested sample. TNF-α was not modulated any of the tested samples (Carfi et al. 2007).

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Inbred A/J mice were administered urethane (1 mg/g) via intraperitoneal injection. A biphasic response on splenic NK cell activity was noted. At one day after the injection activity was decreased 60%, activity then increased (decreased 35%), and then remained decreased until 14 days after exposure (decreased 98%). Spleen size was initially reduced, but then increased to control levels. Mitogen response (against YAC-1 or RL♂1 target cells) was initially depressed after urethane exposure and then returned to control levels (Gorelik and Herberman 1981a).
Inbred A/J mice (5-24 days old) were administered urethane (0.5 mg/g or 1 mg/g) up to 24 days old. In all tested groups, splenic NK activity was inhibited without effects on cellularity on spleens. Decreased NK activity remained until at least 8-10 weeks of age (Gorelik and Herberman 1981a).
Inbred A/J, CBA/J, and C57BL/6 mice were administered urethane (1 mg/g) via intraperitoneal injection. One day after injection, cytotoxicity (against YAC-1 target cells) of A/J and CBA/] spleen cells was significantly decreased (63% and 25%, respectively). Activity was similar to control levels at day 4. Activity then decreased in splenic cells from A/J mice (58%), while a

similar effect in cells from CBA/J mice was not observed. No effect on activity was observed in C57BL/6 mice (Gorelik and Herberman 1981b).
Female B6C3F1 mice were administered 1, 2, or 4 mg/g urethane over a 14-day period via intraperitoneal injection. Decreased spleen weight (decreased 47%) and thymic atrophy (decreased 40%) were observed at 4 mg/g. Splenic lymphoproliferative response to ConA was decreased at 4 mg/g (42%). Responses to PHA and spleen cells from DBA mice were similar to controls. Delayed hypersensitivity responses also were not affected by exposure to urethane. Serum immunoglobulin levels and antibody responses to sheep erythrocytes and LPS were decreased in mice administered 4 mg/kg (decreased 61% and 46%, respectively). Macrophage cytostasis of MBL-2 target cells was decreased (LOAEL = 1 mg/g). However, phagocytosis and bactericidal activity against *S. aureus* was not affected. Pluripotent stem cells proliferation was inhibited at all doses. Urethane decreased NK activity against all YAC-1 target to cell ratios at all doses (Luster et al. 1982).
C57BL/6J dams were subcutaneously injected with 0.05 or 0.1 mg/g urethane on GD 7-17. Offspring were evaluated 8 weeks after parturition. Increased relative spleen weight was reported for the litter at 0.05 mg/g urethane. When evaluated based on sex, only an increase in relative thymus weight was observed at 0.05 and 0.1 mg/g. Decreased white blood cell count was also observed (LOAEL = 0.05 mg/g). No effect on lymphoproliferative responses or NK cell activity was noted. However, a decrease in the levels of plaque forming cells in response to sheep erythrocytes was noted (LOAEL = 0.1 mg/g) (Luebke et al. 1986).
C57BL/6J offspring were subcutaneously injected with 0.2 mg/g urethane on PND 5-14. No effects on organ weight or lymphoproliferative responses were noted. NK cell activity was decreased at an effector:target (YAC-1) ratio of 50:1. Splenic cellularity was increased in female offspring and decreased in male offspring (Luebke et al. 1986).
Female C57BL/6J mice were subcutaneously injected with 1, 2, or 4 mg/g urethane. Significant reduction in absolute (LOAEL = 1 mg/g) and relative (data not provided) spleen weights were observed. Additionally, absolute thymus weight was decreased (LOAEL = 4 mg/g). Dose-dependent reduction in leukocyte number was noted, but differential counts of white blood cells were not altered. Lymphoproliferative responses, induced by ConA, PHA, and LPS, were suppressed by urethane (LOAELs = 1, 1, and 4 mg/g, respectively). were noted. Lymphoproliferative responses to allogenic cells (mitomycin C treated CBA/J mouse spleen cels) were not affected by urethane exposure. NK cell activity was not affected at any effector:target (YAC-1) ratio. Splenic cellularity of mice treated with urethane and sheep erythrocytes was decreased (LOAEL = 2 mg/g) without effects on PFC/spleen or PFC/splenocytes. Decreased DTH index (to keyhole limpet hemocyanin) was decreased in urethane treated mice (LOAEL = 4 mg/g) (Luebke et al. 1987).
mRNA expression of interleukins and TNF-α were evaluated in spleens of male Wistar rats exposed to 1500 mg/kg urethane. Increased expression of IL-6 was noted, while decreased expression of IL-1β and TNF-α were reported. No effects on IL-2 expression were observed (Bette et al. 2004).

Urethane (10%) did not deplete ear epidermis Ia-positive LCs after male BALB/c mice were treated with topical application. Similarly, urethane did not alter the density of β-glucuronidase- positive LC in C57BL mouse tails topically treated for 1 or 3 weeks (Halliday et al. 1988).
Urethane administration to pregnant ICR mice (1.5 mg/g subcutaneous injection on GD 10) produced a transient decrease in dam thymocyte cell count. At 3 days after treatment, a significant decrease in cell count was noted. By 5 days after treatment, the cell count had recovered to control levels (data in graph). A similar phenomenon was noted with thymocyte phenotypes; decrease in CD4+8+ thymocytes (88%) at day 3 after treatment was recovered by day 5. Transient changes in dam splenocyte cell count and splenocyte phenotype CD4+8-, CD4- 8+, and CD4-8- also were reported. Gene expression analyses identified changes in spleen gene expression due to urethane exposure with or without immune stimulation (FCA). Increased expression of TGFβ3 was observed in the presence or absence of immune stimulation one day after treatment. IGF-I, IGF-II and IL-2 were also differentially expressed (Sharova et al. 2002).

In vitro data with cells or cell lines
Rat and mouse spleen cells were treated with urethane for 24 hours and then LPS or PHA for 48 hours. Then, mitogen stimulation was assessed by 5-bromo-2'-deoxyuridine incorporation. Urethane did not inhibit cellular proliferation in either species (data not provided). Urethane also did not modulate rat spleen proliferation that was stimulated by ConA or inhibit anti-CD3 antibody stimulation of mouse lymphocyte proliferation (data not provided) (Carfi et al. 2007).

Mode of action information
In vitro and *in vivo* studies suggest that urethane metabolism by cytochrome P450 is needed to produce the observed immunomodulatory effects (Cha et al. 2000). Macrophage effects are based on urethane effects on the inductive phase of immune responses (Foris et al. 1983).

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Vanadium Pentoxide [CASRN 1314-62-1]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
Vanadium pentoxide (25 – 400 μM) inhibited cell proliferation and induced cell apoptosis in a dose and time-related manner in the IL-2-independent human NK cell line, NK-92MI. Cell proliferation was maximally inhibited (78%) at 400 μM vanadium pentoxide, and the percentage of cells undergoing apoptosis increased at 12 and 24 hours of exposure (51.2 and 64.7%, respectively) as the concentration of vanadium pentoxide increased. IL-2, IL-10 and IFN γ secretion were all inhibited by vanadium pentoxide after 24 hours at the highest concentration tested. IL-2 secretion also was inhibited after 12 hours. Expression of CD25 significantly increased above background starting at 50 μM, reaching a maximal migration inhibitory factor (MIF) of 47.4% at 400 μM. A similar pattern was observed for IL-15R α, with a maximal MIF of 55.2% at 400 μM. Fas expression began to increase at 100 μM and reached a maximal MIF of 48.9% at 400 μM, while FasL peaked at 200 μM (62.1%). Jak3 phosphorylation was increased at 12 and 24 hours after treatment with 200 and 400 μM vanadium pentoxide (data in figure), and intracellular staining showed a strong presence of pJak3 in the internal cell membranes after treatment. (Gallardo-Vera et al. 2016).

Mode of action information
Vanadium in the +2, +3, and +4 (but not the +5) valence states interacted with human FMLP-activated neutrophils and statistically significantly increased the formation of hydroxyl radicals, with additional augmentation observed in the presence of sodium azide (values not provided) (Fickl et al. 2006).
Vanadium pentoxide induced toxic effects on the IL-2-independent human NK cell line, NK-92MI, through dysregulation of signaling pathways mediated by IL-2 via increased PTEN and decreased SHP1 expression (Gallardo-Vera et al. 2018).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Male F344 rats were exposed to vanadium pentoxide (100 μg V/m^3) via inhalation, 5 hours per day for 5 days. The animals were infected with Listeria following the 5-day exposure and the bacterial burden assessed at 24, 48 and 72 hours, post-infection. Vanadium pentoxide did not have any significant effect on Listeria burdens at any of the timepoints observed (Cohen et al. 2007).

Male CD-1 mice were exposed to vanadium pentoxide (0 or an average of 1436 μg/m^3) via inhalation, 1 hour per day, 2 times per week over 12 weeks. An increase in the number

(3.8±0.12 vs. 2.1±0.12 μm per field) and the size (36±0.52 vs. 25±0.35 μm) of megakaryocytes in the

spleen was observed in vanadium pentoxide exposed mice, as compared to controls. These same types of changes were also observed in the bone marrow (values not provided). No statistical difference was observed in spleen weight between treated and control mice (Fortoul et al. 2008). When male and female CD-1 mice were exposed to vanadium pentoxide (0 or 1.4 mg/m^3) using the same protocol as in Fortoul et al., 2008, a sex difference was observed in the expression of Ki-67, a specific proliferation marker for lymphocytes. The percentage of Ki-67 immunopositive lymphocytes increased in male mice (38.86, 41.75 and 41.91%) after 4, 8 and 12 weeks of exposure, respectively, with both cytoplasmic and nuclear expression of Ki-67 observed. In female mice, the percentage of proliferating lymphocytes increased only after the first week of exposure (34.87%) and the signal was observed only in the nucleus. Subsequent exposures did not produce significant changes in the percentage of proliferating cells in females. The authors concluded there is a role for sex hormones in potential protection against vanadium immunoxicity (Rodriguez-Lara et al. 2016).

Male CD-1 mice were exposed to vanadium pentoxide (0 or an average of 1.4 mg/m^3) via inhalation, 1 hour per day, 2 times per week over 12 weeks. Spleen weight of vanadium exposed animals peaked at 9 weeks (546 ± 45 vs. 274 ± 27 mg in controls) and progressively decreased afterwards (321 ± 39 mg at 12 weeks vs. 298 ± 35 mg in controls). The spleens of vanadium exposed animals had histological changes that included increased numbers of lymphocytes and megakaryocytes as compared to controls. The number of $CD19^+$ cells was also increased within the hyperplastic germinal node (values not provided) and the mean hepatitis B surface antigen levels in immunized control mice was greater than in the exposed hosts (OD=0.39 ± 0.03 vs.
0.11 ± 0.05). The authors concluded that vanadium pentoxide induces functional changes in the spleen which appear to result in effects on the humoral immune response (Pinon-Zarate et al. 2008).

Male CD-1 mice were exposed to vanadium pentoxide (0 or an average of 1.4 mg/m^3) via inhalation, 1 hour per day, 2 times per week over 4 weeks. The expression of CD11c in the thymic medulla was decreased in vanadium pentoxide exposed mice, as compared to controls (values not provided), based on immunohistochemistry. Flow cytometry also demonstrated a decrease in $CD11c^+$ and $MHC\text{-}II^+$ cells in vanadium pentoxide exposed mice, as compared to controls (values not provided). The decrease was both, in terms of number and in mean fluorescence intensity values (Ustarroz-Cano et al. 2012).
Male F344/N rats and female B6C3F1 mice were exposed to 0, 4,8, or 16 mg/m^3 vanadium pentoxide, via inhalation, 6 hours per day, 5 days per week for 16 days. Pulmonary inflammation was assessed via analysis of BAL fluid. Significant alterations in the percentage of recoverable macrophages and neutrophils (NOAEL 4 mg/m^3), and increased lung protein and lysozyme in male rats (LOAEL 4 mg/m^3) were observed. In female mice, an increase in lymphocytes, protein and lysozymes was observed (LOAEL 4 mg/m^3). No effects were observed on systemic immunity as evidenced by a normal response to *Klebsiella pneumoniae* (National Toxicology Program 2002).

The induction of pulmonary inflammation was examined in three different strains of mice [A/J (sensitive strain for pulmonary inflammation and carcinogenesis), BALB/c (intermediate

sensitivity), and C57Bl/6J (resistant)]. Mice were aspirated with vanadium pentoxide (4 mg/kg) or phosphate-buffered saline, four times per week, with BALF collected at 6 hours, and 1, 3, 6 and 21 days. In A/J mice, vanadium pentoxide increased BALF levels of total cells (95.7%) inflammatory markers (PMNs, macrophages and lymphocytes, 74.6, 99.5, and 623.8%, respectively). Levels of inflammatory chemokines (keratinocyte-derived chemokine, macrophage inflammatory protein-2 and monocyte chemoattractant protein 1), transcription factor activity (NF κB and c-Fos) and signaling pathway activation (MAPK) were increased with highest levels observed in A/J mice followed by BALB/c and then C57BL/6J mice (data in graphs). All results returned to baseline 21 days post exposure (Rondini et al. 2010).

In vitro data with cells or cell lines
No data were located.

Mode of action information
Rondini and colleagues (2010) reported that vanadium pentoxide impacts pulmonary levels of inflammatory markers, induction of chemokines, and modulation of transcription factors
Alterations in macrophage mediated functions have been associated with vanadium exposure (Cohen et al. 1996).
The ability of several vanadium compounds to increase mRNA levels of cytokines in BALF was investigated in female CD rats. Rats received 42 or 420 µg of vanadium pentoxide or phosphate- buffered saline by intratracheal instillation. BALF was collected at times ranging from 1 hour to 10 days. Influx of neutrophils was significantly increased 24 hours after exposure to vanadium pentoxide and peaked 24-48 hours post exposure (data in graph). Macrophage inflammatory protein-2 mRNA expression levels were significantly elevated in vanadium pentoxide treated rats at 1 to 48-hour timepoints, as compared to controls (Pierce et al. 1996).

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Zinc Dimethyldithiocarbamate (ZDMDC) [CASRN 137-30-4]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
ZDMDC induced cytotoxicity in purified NK cells from healthy donors. Exposure to 2.5 μM ZDMDC for 24 hours produced a 99% decrease in lytic function (against K562 target cells) and at 1 μM for 6 days produced a 96% decrease. When a preparation containing T- and NK-cells were exposed to 2.5 μM for 24 hours a 41% decrease in function was observed. Comparatively, a 6-day exposure to 1 μM ziram did not inhibit lytic function. (Whalen et al. 2003). Wilson and colleagues showed that concentrations as low as 125 nM decreased cytotoxic function of purified NK cells (Wilson et al. 2004).
ZDMDC significantly inhibited NK-92MI activity (against K562 target cells) in a dose- and concentration-dependent manner (LOAEL = 0.125 μM at 2 hours incubation). A similar dose- and concentration-dependent inhibition of NK activity was observed with human lymphokine activated killer cells (LOAEL = 0.125 μM at 2 hours incubation) (Li et al. 2012a).
Purified, human NK cells were exposed to ZDMDC (0.5-5 μM) for 1 hour. Then the cells were incubated for 24 or 48 hours, or 6 days in ZDMDC-free media. A decrease in NK activity was observed at 2.5 and 5 μM. The loss of activity lasted up to 6 days after exposure (Taylor et al. 2005).
ZDMDC (5 μg/mL) decreased LPS-induced TNF-α production in THP-1 cells (data in graph). ZDMDC (5 μg/mL) also blocked LPS-induced degradation of I□B (data in Western blot) (Corsini et al. 2006).
ZDMDC induced apoptosis and necrosis in U937, NK-92MI, NK-92CI, Jurkat, and human T cells. Of U937 cells treated with 2 μM ZDMDC, 49.3% were apoptotic and 18.5% were necrotic (Li et al. 2011). In Jurkat cells treated with 0.5 μM ZDMDC, 52.5% were apoptotic and 7.9% were late apoptotic/necrotic (Li et al. 2012c). In NK-92MI cells treated with 0.5 μM ZDMDC, 47.4% were apoptotic and 12.2% were late apoptotic/necrotic (Li et al. 2012b). In NK-92CI cells treated with 0.5 μM ZDMDC, 28.7% were apoptotic and 38.5% were necrotic (Li et al. 2014). Increased apoptosis and late apoptosis/necrosis also was observed in a time- and dose-dependent manner in isolated primary T-cells (data in graph) (Li et al. 2012c).
At concentrations ranging from 0.1 to 10 μg/mL, ZDMDC was not cytotoxic to lymphocyte cultures obtained from peripheral blood from healthy volunteers (Zenzen et al. 2001).

Mode of action information
Effects in U937, NK-92MI, and Jurkat cells were dose- and time-dependent. Increased DNA fragmentation, level of active caspase-3, and level of cytochrome c release from U937 and Jurkat

cells also were noted after ZDMDC exposure (Li et al. 2011, 2012c, 2012b, 2015). Increased levels of caspase-7, -8, and -9 also were detected in NK-92MI and Jurkat cells (Li et al. 2012c, 2012b).
ZDMDC-induced inhibition of NK and LAK activity was mediated, in part, by decreases in intracellular levels of Gr3/K, granulysin, perforin, granzyme (Gr) A, and GrB (Li et al. 2012a). Decreased levels of GrB was associated with activation of p38 while activation of p44/42 was associated with decreased levels of perforin (Taylor and Whalen 2011).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
The EC3 in the local lymph node assay was between 1.0% and 5.0% in female BALB/c mice (De Jong et al. 2002). ZDMDC also was identified as a skin sensitizer in the guinea pig maximization test (TS_5 = 0.01%) (van Och et al. 2001).

In vitro data with cells or cell lines
ZDMDC inhibited murine (C57BL/6J) cytotoxic T lymphocyte activity in a dose- and concentration-dependent manner (LOAEL = 0.125 μM) (Li et al. 2012a).
ZDMDC (10 μM) decreased expression of pro-caspase-1 and NLRP3 in J774A.1 cells. Studies in RAW264.7 cells showed that 10 μM ZDMDC increased pro-caspase-1 degradation and not protein cleavage. ZDMDC also decreased LPS-induced production of IL-18 and IL-1β in bone marrow macrophages. Inhibition of LPS-induced IL-1β production occurred in a dose-dependent manner in J774A.1 (LOAEL = 5 μM) (Muroi and Tanamoto 2015).
J774A.1 cells were infected with *S. typhimurium* TA98 and then treated with ZDMDC. ZDMDC (1-10 μM) increased the number of infected bacteria in a concentration-dependent manner (LOAEL = 5 μM) (Muroi and Tanamoto 2015).

Mode of action information
ZDMDC increased intracellular level of zinc in rat thymic lymphocytes, which may be associated with induction of apoptosis (Kanemoto-Kataoka et al. 2015).

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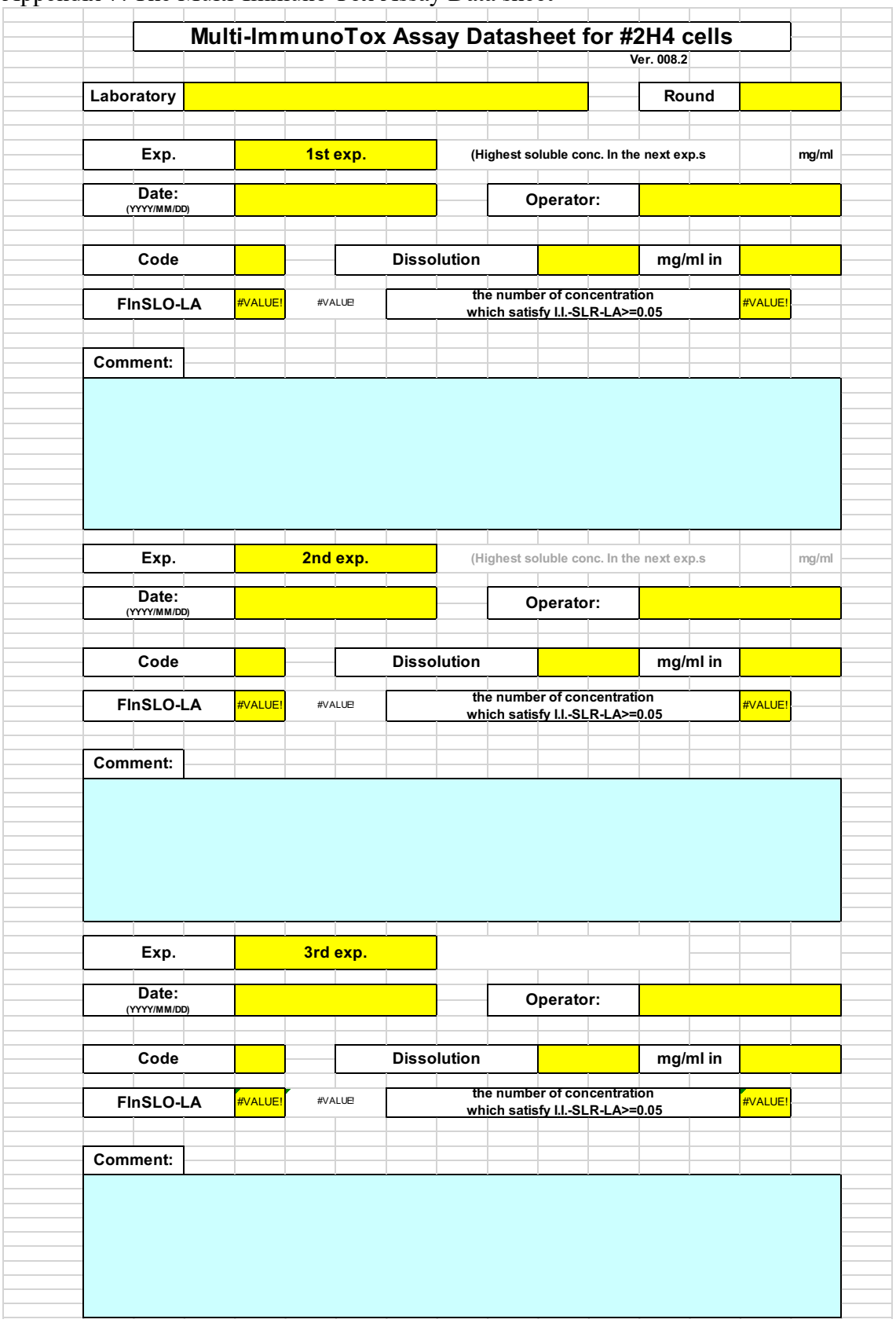
Appendix 6. The summary of immunotoxicological data of 25 chemicals.

Chemicals	In vivo				Ex vivo			In vitro		T cell targeting	Mode of action
	immune sytem organ weight	DTH	infection	tumor	cytokine production	NK activity	TDAR	cytokine production	cell proliferation		
Phase I study											
Dibutyl phthalate	A (spleen)							S (IL-2, 4, IFN-g)(H) A (IL-1b)(H) x 3 S (IL-1b)		YES	This compound then is proposed to modulate cytokine secretion from both monocytes/macrophages and T cells.
Hydrocortisone	S (thymus) x 2 S (spleen)	S					N	S (IFN-a)		YES	
Lead(II) acetate	A(thymus)	S A	S				S N	S (IFN-g, IL-1b)(H) A (IL-4)(H)	S(H)	YES	
Nickel(II) sulfate	N S (thymus)	S		S		S N	N	A (IL-4, IFN-g)(H) S (IL-2) S (IFN-g)		YES	
dimethyldithiocarbamate (DMDTC)		A x 2						S (IL-1b)	N(H)	NO	
Phase II study											
2.4-diaminotoluene	N (spleen) A (spleen)	A	S	N		S	S	-	-	NO	
Benzo(a)pyrene		N	N		S(IL-2)	N	S x 5 A	A (IL-4)(H) N (IFNγ)(H) N (IL-2)(H)	S (H) x 2 S x 6	YES	Disruption of T-cell activities has been associated with B(a)P induced immunotoxic effects (Urso et al. 1986).
Cadmium Chloride	A (spleen) S (spleen)	N			A (IL-2) N (IFN-γ)	N	S x 4	S (IL-2, 4, IFN-g) A (IFN-g)(H) S (IL-2, IFN-g) A (IFN-g) S (IL-2) A (IL-2)	S	YES	
Dibromoacetic acid (DBAA)	A (spleen) S (thymus) x 2		N	N		S	N	S (IL-2, 4)	S	YES	Overall, studies suggest that DBAA produces immunotoxic effects through modulation of T-cell mediated cell immunity. T-cell apoptosis, through extrinsic and intrinsic pathways, are proposed to play a role in the mode of action.

Diethylstilbestrol (DES)	**S (thymus) x 4 A (thymus) x 2 A (spleen)**	**N S**	**N(H) A(H)**		**A (IFN-g) x 3**	**A(H)**	**S**	**A (IL-1) A (IL-2)**		**YES**	DES exposure was associated with down-regulation of gene expression in the TCR complex, and the TCR and CD28 signaling pathways.
Diphenylhydantoin		**S N**			**A (IL-4) S (IFN-γ, IL-2) S (IL-1α) N (IL-6, 12)**	**S**	**S A x 2**	-	-	**YES**	DPH treatment can lead to a decrease of suppressor T cells
Ethylene Dibromide (EDB)	**S (thymus) S (spleen) N**		**N**			**S**	**A**	-	-	**NO**	
Glycidol	**N**		**A**	**S**		**S**	**S**	-	-	**NO**	
Indomethacin	**N A (spleen)**		**A**				**S x 3 A x 1**	**A (IL-2)(H) A (IFN-g)(H)**	**A (H) x 4 S A x 3**	**YES**	indomethacin inhibition of prostaglandin synthesis leads to altered T-cell function,
Isonicotinic Acid Hydrazide (IAH)	**N x 2**							**S (IL-2)(H) A (IL-2)(H) S (IL-1)(H)**	**S (H) x 3 A (H) x 6 A N**	**YES**	
Nitrobenzene	**A (spleen) x 3 A (thymus) x 2**		**N S**	**N**			**S N**	-	-	**NO**	effects on T-cell function may play a role in increased susceptibility to L. monocytogenes (Burns et al. 1994).
Urethane, Ethyl carbamate	**S (thymus) x2 S (spleen) x 2 N A (thymus) A (spleen)**	**S**			**N (IL-2)**	**S x 4 N x 3**	**S x 2 N**	**N (IL-2, 4, IFN-g)(H) A (IFN-g)(H) S (IFN-g)(H)**	**N x 2**	**YES**	
Tributyltin Chloride (TBTC)	**S (thymus) x4 S (spleen) x 3**	**N S**	**S x 2**			**S**	**N S**	**A (INF-g)(H) N (IL-2, 4)(H) S (IFN-g)(H)**	**S (H) S x 3**	**YES**	
Perflouorooctanoic Acid (PFOA)	**S (thymus) x2 S (spleen) x 2**		**S(H) N(H)**		**N (IFN-g)**			**S (IL-4)(H) N (IL-2)(H)**	**A (H) S (H) N (H)**	**YES**	Direct modulation of NF-kB has been implicated in modulation of cytokine production and secretion (Corsini et al. 2012).
Dichloroacetic Acid (DCAA)	**A(spleen)**	**N**			**N (IL-2) A (IFN-γ) x 3 S (IL-4) x 2 S (IL-2)**	**N**	**N**	**A (IL-2)(H) A (IL-2, IFN-g)**		**YES**	T-cell activation was one proposed mode of action for DCAA.
Toluene									**A**	**NO**	Toluene also enhanced NF-kB, STAT5, and NF-AT in thymus cells of C3H/HeN mice inhalationally (Liu et al. 2010). Toluene modulation of IL-2 synthesis, after oral exposure, may play a role in observed immunotoxic effects (Hsieh et al. 1989).
Acetonitrile	**S(thymus)**					**S**	**S S**	-	-	**Undetemined**	No data were located.
Mannnitol									**N (H)**	**NO**	
Vanadium Pentoxide	**N A (spleen)**		**N x 2**					**S (IL-2, IFN-g)(H)**	**S (H)**	**YES**	
o-Benzyl-p-chlorophenol (BCP)	**N**	**N A**		**N**			**N**	-	-	**NO**	

Appendix 7. The Multi-Immuno Tox Assay Data sheet



Multi-ImmunoTox Assay Datasheet for #2H4 cells

Ver. 008.2

Laboratory		Round	

Exp.	1st exp.	(Highest soluble conc. In the next exp.s	mg/ml
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

Comment:

Exp.	2nd exp.	(Highest soluble conc. In the next exp.s	mg/ml
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

Comment:

Exp.	3rd exp.		
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

Comment:

Exp.	4th exp.		
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

Comment:

Exp.	5th exp.		
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

Comment:

Exp.	6th exp.		
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

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MultiReporter Assay System -Tripluc® - Calculation Sheet

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MultiReporter Assay System -Tripluc® - Calculation Sheet

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	F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

SLR-LA		1	2	3	4	5	6	7	8	9	10	11	12
	A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
II-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input"*"												
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

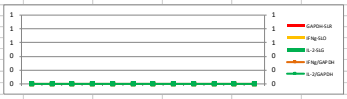
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input"*"												
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

FInSLO-LA	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chemical1													
													ug/ml
Chemical2		0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml

%Suppression（L-2）		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
%Suppression（FNg）		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
濃度の数(n)		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

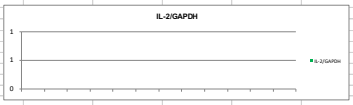
1st exp.

Chemical1



Chemical2

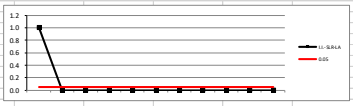
Chemical1



Chemical2

%suppression
Chemical1

Chemical2



MultiReporter Assay System -Tripluc® - Calculation Sheet

2nd exp.													2nd exp.
SLG-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLO-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLR-LA	1	2	3	4	5	6	7	8	9	10	11	12	Chemical1
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
													Chemical2
nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	%suppression
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
II-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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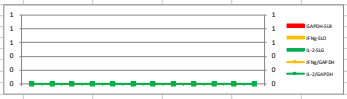
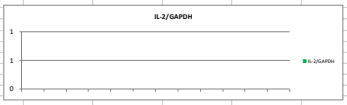
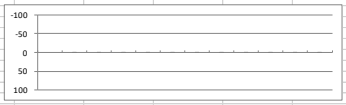
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
FInSLO-LA	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chemical1													
													ug/ml
Chemical2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml
%Suppression （L-2）		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
%Suppression （FNg）		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
濃度の数(n)		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	







MultiReporter Assay System -Tripluc® - Calculation Sheet

3rd exp.													3rd exp.
SLG-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chem ical1
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLO-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chem ical2
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLR-LA	1	2	3	4	5	6	7	8	9	10	11	12	Chem ical1
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
													Chem ical2
nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chem ical1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chem ical2
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
II-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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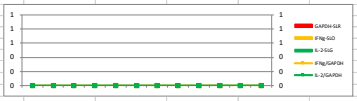
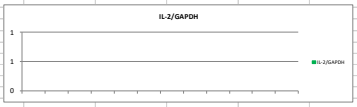
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
FInSLO-L/	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chem ical1													
													ug/m l
Chem ical2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/m l
%Suppression (L-2)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
%Suppression (FNg)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
濃度の数(n)		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	





MultiReporter Assay System -Tripluc® - Calculation Sheet

4th exp.													4th exp.
SLG-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLO-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLR-LA	1	2	3	4	5	6	7	8	9	10	11	12	Chemical1
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
													Chemical2
nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	%suppression
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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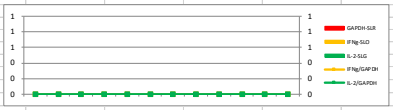
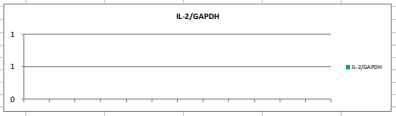
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
II-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
FInSLO-LA	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chemical1													
													ug/ml
Chemical2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml
%Suppression (IL-2)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
%Suppression (IFNg)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
濃度の数(n)		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	





MultiReporter Assay System -Tripluc® - Calculation Sheet													
5th exp.													5th exp.
SLG-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLO-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLR-LA	1	2	3	4	5	6	7	8	9	10	11	12	Chemical1
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B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
													Chemical2
nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	%suppression
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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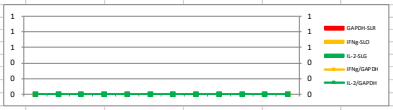
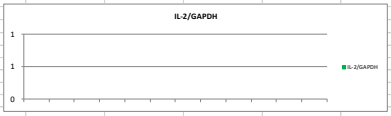
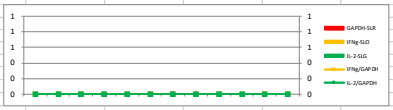
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
II-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
FInSLO-LA	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chemical1													
													ug/ml
Chemical2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml



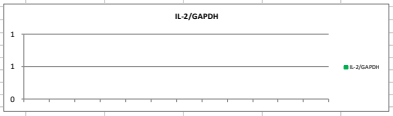


MultiReporter Assay System -Tripluc® - Calculation Sheet

6th exp.													6th exp.
SLG-LA		1	2	3	4	5	6	7	8	9	10	11	12	
	A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
	D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLO-LA		1	2	3	4	5	6	7	8	9	10	11	12	
	A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
	C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	



SLR-LA		1	2	3	4	5	6	7	8	9	10	11	12	Chemical1
	A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
														Chemical2



nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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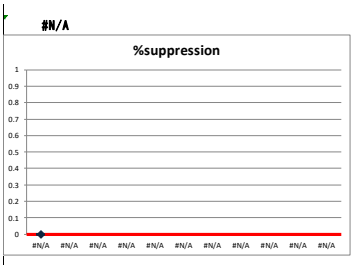
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	%suppression
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
II-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
FInSLO-LA	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chemical1													
													ug/ml
Chemical2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml

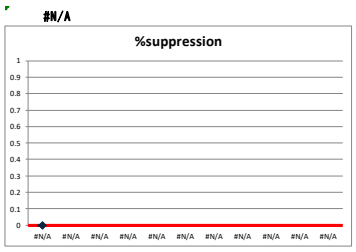
#N/A



#N/A

a Comparison Conc.	%supp	Lower Limit	Upper Limit
#N/A	0	0	0
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

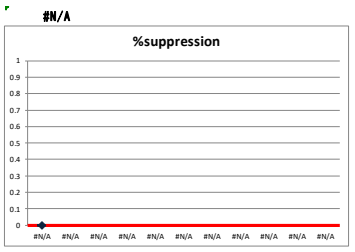
#N/A



#N/A

a Comparison Conc.	%supp	Lower Limit	Upper Limit
#N/A	0	0	0
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

#N/A



#N/A

a Comparison Conc.	%supp	Lower Limit	Upper Limit
#N/A	0	0	0
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

1st exp. Chemical 1

conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
	0	#VALUE!	#VALUE!	1	1	1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
cont.		#VALUE!				

2nd exp. Chemical 1

conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
	0	#VALUE!	#VALUE!	1	1	1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!				

3rd exp. Chemical 1

conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
	0	#VALUE!	#VALUE!	1	1	1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!				

Accept Exp.

#N/A
#N/A
#N/A

%suppression

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

dummy

#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A

Accept %suppression

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

#N/A

a Comparison Conc.	1-(ave/Dave)	Low er Limit	Upper Limit	ACCEPT Dummy*n
#N/A	0	0	0	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
ACCEPTV（併合分散）	#N/A	ACCEPTdunnet 棄却域	#N/A	

average

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

Accept average

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

#N/A

a Comparison Conc.	1-(ave/Dave)	Low er Limit	Upper Limit	ACCEPT Dummy*n
#N/A	0	0	0	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
ACCEPTV（併合分散）	#N/A	ACCEPTdunnet 棄却域	#N/A	

sd^2

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

Accept sd^2

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

#N/A

a Comparison Conc.	1-(ave/Dave)	Low er Limit	Upper Limit	ACCEPT Dummy*n
#N/A	0	0	0	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
ACCEPTV（併合分散）	#N/A	ACCEPTdunnet 棄却域	#N/A	

ave/Dave

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

ave/Dave

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

		4th exp.	Chemical 1			
conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
	0	#VALUE!	#VALUE!	1	1	1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!				

		5th exp.	Chemical 1			
conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
	0	#VALUE!	#VALUE!	1	1	1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!				

		6th exp.	Chemical 1			
conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
	0	#VALUE!	#VALUE!	1	1	1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!				

EXP	Dummycount	V 併合分散)	dunnetDF(全例数－対象群を含めた群の数)	dunnet比較数（対照群を含めた群の数－1)	dunnet棄却域
1st exp.	1	#VALUE!	#VALUE!	0	FALSE
2nd exp.	1	#VALUE!	#VALUE!	0	FALSE
3rd exp.	1	#VALUE!	#VALUE!	0	FALSE
4th exp.	1	#VALUE!	#VALUE!	0	FALSE
5th exp.	1	#VALUE!	#VALUE!	0	FALSE
6th exp.	1	#VALUE!	#VALUE!	0	FALSE

1st exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!

2nd exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!

3rd exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!

4th exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!

5th exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!

6th exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!

ダネット棄却値

	1	2	3	4	5	6	7	8	9	10
3	3.18245	3.86634	4.26254	4.5382	4.74793	4.91629	5.05639	5.17604	5.28024	5.37237
4	2.77631	3.31035	3.61766	3.83152	3.99447	4.12549	4.23469	4.32808	4.4095	4.48158
5	2.57058	3.03015	3.29305	3.47589	3.61512	3.72711	3.8205	3.90042	3.97015	4.0319
6	2.44692	2.86275	3.09929	3.26336	3.38838	3.48878	3.57273	3.64448	3.70709	3.76256
7	2.36464	2.75171	2.97085	3.12264	3.23813	3.33099	3.40834	3.47465	3.53251	3.58376
8	2.30603	2.67282	2.87967	3.02271	3.13145	3.21885	3.29171	3.35406	3.40847	3.45667
9	2.26218	2.61395	2.81166	2.94817	3.05188	3.13519	3.20463	3.26403	3.31587	3.36178
10	2.22817	2.56837	2.75902	2.8905	2.9903	3.07044	3.13722	3.19433	3.24416	3.2883
11	2.20102	2.53204	2.71709	2.84456	2.94126	3.01887	3.08352	3.1388	3.18703	3.22974
12	2.17885	2.50241	2.68291	2.80712	2.90128	2.97683	3.03975	3.09354	3.14045	3.182
13	2.16041	2.4778	2.65452	2.77602	2.86809	2.94193	3.0034	3.05595	3.10177	3.14234
14	2.14483	2.45702	2.63057	2.74979	2.84009	2.91248	2.97273	3.02423	3.06913	3.10889
15	2.13149	2.43926	2.6101	2.72737	2.81615	2.88731	2.94652	2.99712	3.04124	3.08029
16	2.11994	2.42389	2.59239	2.70799	2.79546	2.86555	2.92387	2.97369	3.01712	3.05556
17	2.10985	2.41047	2.57694	2.69107	2.7774	2.84656	2.90408	2.95323	2.99606	3.03397
18	2.10096	2.39865	2.56332	2.67617	2.7615	2.82983	2.88667	2.93521	2.97752	3.01496
19	2.09306	2.38815	2.55115	2.66285	2.74738	2.81499	2.87121	2.91923	2.96107	2.99809
20	2.086	2.37878	2.54035	2.65103	2.73468	2.80164	2.85731	2.90486	2.94628	2.98303
21	2.07965	2.37035	2.53064	2.64041	2.72334	2.78972	2.8449	2.89202	2.93307	2.96939
22	2.07391	2.36264	2.52188	2.63081	2.7131	2.77895	2.83369	2.88042	2.92113	2.95715
23	2.06869	2.35572	2.51391	2.6221	2.7038	2.76918	2.82351	2.86989	2.91029	2.94604
24	2.06393	2.34941	2.50665	2.61415	2.69532	2.76026	2.81422	2.86029	2.90041	2.9359
25	2.05957	2.34363	2.5	2.60688	2.68756	2.7521	2.80572	2.8515	2.89136	2.92662
26	2.05556	2.33831	2.49389	2.60019	2.68042	2.7446	2.79791	2.84342	2.88304	2.91809
27	2.05186	2.33341	2.48825	2.59402	2.67384	2.73768	2.79071	2.83596	2.87537	2.91023
28	2.04843	2.32888	2.48304	2.58832	2.66776	2.73128	2.78404	2.82907	2.86828	2.90296
29	2.04526	2.32467	2.4782	2.58302	2.66211	2.72534	2.77786	2.82267	2.8617	2.89621
30	2.0423	2.32075	2.47369	2.5781	2.65685	2.71981	2.77211	2.81673	2.85557	2.88993
31	2.03954	2.3171	2.46949	2.57351	2.65195	2.71466	2.76674	2.81118	2.84986	2.88407
32	2.03696	2.31368	2.46557	2.56921	2.64737	2.70985	2.76173	2.80599	2.84452	2.8786
33	2.03454	2.31048	2.46189	2.56519	2.64308	2.70533	2.75703	2.80113	2.83952	2.87347
34	2.03227	2.30748	2.45844	2.56141	2.63905	2.7011	2.75262	2.79657	2.83483	2.86866

1st exp.	nSLG-LA		**2nd exp.**	nSLG-LA		**3rd exp.**	nSLG-LA		**4th exp.**	nSLG-LA		**5th exp.**	nSLG-LA		**6th exp.**	nSLG-LA	
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Appendix 8 Study plan

Study plan

for the validation trial on multicolor reporter assay using IL-2 Luc (IL-2 Luc assay) as a test evaluating the immunotoxic potential of chemicals

Version 1.4 February, 2017

Conducted by:

IL-2 Luc assay Validation Management Team

INDEX

1. Background

The multicolor reporter assay using IL-2 Luc in Jurkat cells (IL-2 assay) is important for evaluating the immunotoxic potential of chemicals. This assay forms part of the Multi-ImmunoTox assay (MITA) and has the advantages of technical simplicity and a short test period, and the accuracy of the test result is based on the mechanism underlying immunotoxicity.

The aim of this trial is to (pre)validate the IL-2 Luc assay method to assess its transferability and inter-laboratory variability so that this test can be used to screen for immunotoxic chemicals. The IL-2 Luc assay for the validation trial was undertaken i) in accordance with the principles and criteria documented in the OECD No. 34 Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment [OECD, 2005], ii) according to the Modular Approach to validation [Hartung et al., 2004], iii) according to the concept discussed in the validation trials with the participation of GLP Test Facilities [Cooper-Hannan et al., 1999] where the whole concept of validation trials is described in the context of GLP, and iv) in line with the ISO procedure JRC.I.03.GP.01v.01 (http://ihcpnet.jrc.it/quality-safety/quality-documents/unit-03-ivm/doc/JRC.I.03.GP.01v.01.pdf).

The studies comprising a validation trial should ideally be performed in accordance with GLP [OECD, 1998-2007; FDA, 1999; EPA, 1998a&b; JSQA, 2010; SCC, 2010]. As a minimum, use of standard operating procedures (SOP) and adequate data recording, reporting and record keeping are essential.

A general conceptual framework [Hartung et al., 2004; OECD, 2005] will be used for documenting the entire study to assess the validation status of the test method. This is called a "modular approach" to validation. In this approach, the information needed to support the validity of the method is organized into modules that provide the following information:

Module 1: Test Definition
Module 2: Within-laboratory repeatability and reproducibility
Module 3: Between-laboratory transferability
Module 4: Between-laboratory reproducibility
Module 5: Predictive capacity
Module 6: Applicability domain
Module 7: Performance standards

The modular approach as introduced by Hartung et al. allows the use of datasets from various data sources and studies. This advantage is used in the following proposal to assess the scientific validity of the IL-2 Luc assay. This IL-2 Luc assay for the validation trial has been performed under GLP principles.

2. Objective of the trial

The validation trial will assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of the IL-2 Luc assay with a challenging set of test substances (test items) for which high quality *in vitro* and *in vivo* data are available.

3. Validation Management Team (VMT)

The VMT encompasses collective expertise with the test, in the underlying science, and the scientific design, management and evaluation of a validation trial.

The VMT, which plays a central role overseeing the conduct of the validation trial, includes:

Table 1. Members for IL-2 Luc assay Validation Management Team

Name	Role and expertise	Affiliation
Trial Coordinator Hajime Kojima	VMT trial coordinator, Chemical supplier and Management of quality control	JaCVAM, NIHS, Japan (JaCVAM representative)
Lead Lab Yutaka Kimura* Setsuya Aiba*	*Developer of this assay Test method, expertise underlying science	Tohoku Univ., Japan
Takashi Omori	Data analysis, biostatistics dossier	Kobe Univ., Japan
International expert members		
EU liaison Emanuela Corcini	Test system expertise, validation expertise, immunotoxicity expertise	Milan Univ., Italy
EU liaison Erwin L. Roggen	Test system expertise, validation expertise, immunotoxicity expertise	3Rs Management and Consulting ApS, Denmark
ICCVAM liaison Dori Germolec	Immunotoxicity expertise	NTP/NIEHS, USA
JSIT liaison Tomoaki Inoue	Immunotoxicity expertise	Chugai Pharmaceutical Co., Ltd.

Participating Test Facilities

The laboratories participating in the trial are defined as follow:

Test Facility 1: Hatano Res. Inst., FDSC. Study Director (SD): Kohji Yamakage

Test Facility 2: AIST, Tsukuba SD: Yoshihiro Ohmiya

Test Facility 3: AIST, Takamatsu SD: Yoshihiro Nakajima

Information relevant for Modules 1, 2, 3 performed by all laboratories. Data obtained by these laboratories have demonstrated that the IL-2 Luc assay is transferable and reproducible between experienced laboratories. All laboratories participating in this validation trial will act as unexperienced laboratories to assess between-laboratory transferability, reliability, and relevance of the IL-2 Luc assay method under non-GLP conditions (GLP principle).

Trial management structure

1) Chemical management group

The members of the chemical management group are elected by recommendation of the IL-2 Luc assay VMT. The members prepare a tentative list of test chemicals and work with the VMT to make a final decision on the test chemicals to be used in the validation trial. The coded test chemicals listed in Table 6 and 7 are distributed by the JaCVAM.

2) Data analysis group

The members of the data analysis group are elected by recommendation of the IL-2 Luc assay VMT and check and analyze the data obtained in this validation trial from a third-party standpoint. The members also take charge of statistical processing in this validation trial.

3) Quality assurance group

The members of the record management group are elected by recommendation of the IL-2 Luc assay VMT. The members prepare the protocol, the test chemical preparation record forms, blank data sheets, etc., and distribute them to the research laboratories participating in this validation trial. The members also collect completed forms and data sheets after completion of the experiments, and point out omissions or flaws in recording, if any, and request corrections of such errors.

4) Lead laboratory

The lead laboratory representing the test method is responsible for providing the test method protocol and the necessary data recording or calculation templates. The Trial Coordinator must ensure that such data recording or calculation templates have been validated before distribution to the test facilities involved in the validation trial. The lead laboratory is also responsible for providing, if necessary, new versions of the protocols during the entire validation trial. The lead laboratory and the other participating test facilities might be contacted by the VMT regarding technical issues.

Sponsor

The validation trial for assessing the validity of IL-2 Luc assay will be financed by the Ministry of Health, Labour and Welfare (MHLW), Japan.

The lead laboratory will support the IL-2 Luc assay validation trial by assuring that reliability is assessed. At the same time, preliminary results of the test method can be evaluated. For this purpose, Lead laboratory will support:

- financial aspects related to the coordination of a validation trial (e.g. organization of VMT meetings where also the involved test facilities can be invited for technical clarifications to the VMT, the publication of the validation trial results)
- test, reference and control item purchase, coding and distribution to the test facility
- availability of the test systems to the participating laboratories by supporting the lead laboratory with the logistics for delivering the test system to the facility
- independent analysis of data and statistical support (biostatistician) based on the study reports generated
- other costs incurred by the participating laboratories

Trial coordination

Dr. Hajime Kojima was appointed as the Trial Coordinator with well-defined roles and responsibilities to coordinate the trial and to establishment of a VMT by supporting of JaCVAM. The name and location of the Trial Coordinator should be identified in each individual study plan. For the IL-2 Luc assay validation trial, the Trial Coordinator has direct access to the test item coding.

The Trial Coordinator's responsibilities include:
a) Establishment of/support to lead laboratory, including meeting organization
b) Trial communication and coordination with test facilities
c) Recording of document and data flow between test facilities
d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial
e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis
f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratories and other test facilities involved in the validation trial, and should reflect the overall trial
g) Approval with date and signature of all protocols, Study Plans and Study Reports
h) The communication of the results of the trial into the public domain
The Trial Coordinator's responsibilities include:
a) Establishment/support of the lead laboratory, including meeting organization
b) Trial communication and coordination with the test facilities
c) Recording of documents and data flow between the test facilities
d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial
e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis
f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratory and other test facilities involved in the validation trial, and should reflect the overall trial
g) Approval with date and signature of all protocols, study plans and study reports
h) Communication of the results of the trial to the public domain
The role of the Trial Coordinator (as the formal representative of the VMT and the single contact point with the SDs) is of fundamental importance. The Trial Coordinator is the single critical point of trial control and must ensure clear lines of communication between the involved test facilities in the trial. The communication line of the Trial Coordinator is with the SDs of the different test facilities. The SDs are the single point of contact with the Trial Coordinator (unless otherwise communicated by the participating test facilities) to assure a transparent and recorded documentation flow during the trial. The Trial Coordinator should also ensure that appropriate arrangements have been made for the supply of the test systems, and test, control and reference items, which meet the requirements of the trial, and that there are appropriate test method protocols (dated signature by the Trial Coordinator and the lead laboratory) and, if appropriate, validated data recording, data analysis, and data reporting sheets for the test method.
It is the responsibility of the Trial Coordinator to approve the study plans sent for approval by the test facilities, and any amendments to the study plan, by dated signature.

Training

The lead laboratory will be responsible for issuing a training agenda to the Trial Coordinator for further distribution to all test facilities, giving details of what training aspects will be covered during the training of the other SDs and study personnel at the lead laboratory. Furthermore, after the training during the Phase 0 study, the lead laboratory will issue to the Trial Coordinator a training report and indicate if critical observations are made by the other test facilities regarding the IL-2 Luc assay protocols. In case any critical observations are made, a new version of the IL-2 Luc assay protocols might need be issued to the other test facilities before initiating the between-laboratory transferability test.

[Module 2] Within-laboratory reproducibility

The within-laboratory reproducibility of all test facilities has been done by an independent biostatistical analysis using 5 coded chemicals under the VMT. The concordance should be equal to or greater than 80% as a tentative acceptance criterion for the Phase I study.

3.7 [Module 3] Between-laboratory transferability

This between-laboratory transferability (Module 3) study is performed in order to assess the successful transfer of the assay to a test facility unexperienced with that particular test method but having knowledge of similar test systems and endpoint detection methods.

Transfer of the IL-2 Luc assay to all test facilities in the Phase 0 study using 5 coded five chemicals was achieved. A few concentrations of each test item were tested in triplicate in 3 independent runs according to the IL-2 Luc assay protocol describing the details of the experimental design.

The 5 test items selected for the Phase I study are coded as A, B, C, D, and E. The facilities will prepare a study according to internal GLP principles. This plan will be submitted to the Trial Coordinator and lead laboratory for approval.

The results of the between-laboratory transferability study will be reviewed before progressing with module 4 in the between-laboratory reproducibility study. If the transferability data do not meet the test acceptance criteria, the Trial Coordinator representing the VMT will try to identify the problems and make corrections where needed. At the end of testing, the test facilities will submit a QC certified copy of the entire study dossier to the Trial Coordinator (study plan adhering to GLP principles, raw data, records and data analysis, study report adhering to GLP principles).

3.8 [Module 4] Between-laboratory reproducibility

Twenty-five coded test items have been selected to confirm the between-laboratory reproducibilities in the Phase I and II studies. Several concentrations of each test item will be tested in triplicate according to the IL-2 Luc assay method protocol describing the details of the experimental design.

At the end of testing, the test facilities will submit a QC certified copy of the entire study dossier to the Trial Coordinator (study plan adhering to GLP principles, raw data, records and data analysis, study report adhering to GLP principles). The concordance for between-laboratory reproducibility should be equal or greater than 80% to meet the acceptance criteria.

[Module 5] Predictive capacity

The necessity for further chemical analysis will be subject to a VMT decision once the data for between-laboratory reproducibility has been assessed. Depending on the statistical analysis, lean design for validation as well as automatisation of the test leading to an increased dataset will be considered.

Protocol
In this validation trial, the protocols ver. 0.08E, Phase I and 0.1E, Phase II will be used. These protocols will be drafted by the lead laboratory and will be finalized by the VMT. The criteria to identify immunotoxicants by the MITA are provisionally fixed in protocol ver. 0.08E prior to the Phase I study. There are 2 temporary criteria to identify imunotoxicants. The VMT adopted these criteria after the Phase I validation study.
A measurement of bioluminescence intensity induced by chemical treatment will be measured by a luminometer (Phelios: ATTO, Cat #:AB-2350) calibrated using stabilized SLG, SLO and SLR enzymes in this validation trial.

Chemicals
5.1 Chemical Selection
Test chemicals have been selected from a chemical repository based on published papers on *in vivo* immunotoxicity.
The applied selection criteria were:
information on mode/site of action
coverage of range of relevant chemical classes and product classes
quality and quantity of reference data (*in vivo* and *in vitro*)
high quality data derived from animals and (if available) also humans
knowledge of interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)
coverage of range of toxic effects/potencies
chemicals that do not need metabolic activation
appropriate negative and positive controls
physical and chemical properties (feasibility of use in the experimental set-up as defined by the CAS No.)
single chemical entities or formulations of known high purity
availability
cost

In the first phase of the selection procedure, the chemical management group identified and collected several existing lists of potential chemical sensitizers in order to establish a primary database. These chemicals had originally been compiled by international experts for various purposes, such as reference compounds for validation studies. An extensive literature research was performed by the chemical management group, insuring that the preselected chemicals fulfilled the selection criteria described above.

Emphasis was laid on the fact that different potencies (strong, weak and no activity) have been chosen. In addition, it was decided that at least 20% of the total substances to be tested should be negative in order to increase the statistical power of the data analysis.

In the first phase of the IL-2 Luc assay validation trial using data generated at the test facilities, 5 chemicals will be tested 3 times for each test chemical for between-laboratory reproducibility and to confirm transferability. After discussion of the Phase I results, detailed test planning for Phase II will be established. Currently, it is planned that 20 chemicals will be tested in the Phase II trial to establish predictive capacity (Table 2).

Table 2. Outline of test planning at each study in the validation trial.

Study	Chemicals	Test Number	Information obtained
Phase I	5 non- coded	1	Between-lab transferability
Phase I	5 coded	3	Within & between-lab reproducibility
Phase II	20 coded	1	Between-lab reproducibility & predictability

5.2 Chemical Acquisition, Coding and Distribution

The within-laboratory reproducibility (Module 2) and between-laboratory transferability (Module 3) in all test facilities have been assessed with coded chemicals. This IL-2 Luc validation trial plan describes generation of the missing data sets under coded test item. If the results obtained are not highly similar to the previously obtained sets, the VMT must assess if coded chemicals need to be tested in all the test facilities.

Coding will be supervised by the Trial Coordinator, in collaboration with the chemical repository responsible for coding and distribution of the test, reference and control items for the validation trial.

5.3 Handling

Each test facility shall receive through the Trial Coordinator essential information about the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions). Moreover, the SD should receive safety information concerning hazards identification and exposure controls/personal protection.

Records and archiving

At the end of the trial, the IL-2 Luc assay validation trial report is prepared by the Trial Coordinator or the VMT personnel who appointed by the Trial Coordinator. The trial report summarizes the trial goals, procedures, results and conclusions of the validation trial. This represents the whole validation trial, including archiving and, as such, will cover several study reports, as well as reports for test item supply, data management and statistics. The Trial Coordinator oversees the preparation of the trial report. The Trial Coordinator will be representing the VMT discussions responsible for preparation of the scientific conclusions. Signatories to the trial report include the Trial Coordinator, the statistician, and the SDs of the involved test facilities. Although the SDs may not be involved with the preparation of the trial report, their signatures confirm that the trial report is an accurate reflection of the management and study events. The trial report should contain a statement, signed by the Trial Coordinator,

commenting on the accuracy and completeness of the trial report and identifying any significant issues which could have affected the integrity of the trial, including matters of GLP compliance. A QC statement will be included in the trial report, in order to identify what QC monitoring was done and to confirm whether or not the trial report is an accurate reflection of the validation trial data.

Study timeline

An approximate schedule for IL-2 Luc assay validation trial is shown in Table 3. The duration of this validation trial is around 20 months, from May 2016 to December 2017.

Table 3. Schedule of IL-2 Luc assay validation trial

Month	Activity
January 2016	Establish the VMT
	Selection of participating research laboratories
	Deliberation, decision and read-through of draft study plan
	Deliberation and decision of protocol
	Preparation of a tentative list of test chemicals
	Distribution of test chemicals, standard chemicals and positive control chemicals
February,2016	Technical transfer using five known chemicals (non-coded) Start of technical transfer **to know between laboratory transferability**
	Data collection of technical transfer (**Phase 0 study**)
Phase I study	
September 2016	Coding and distribution of five coded test chemicals
September, 2016	Start of Phase I study
December, 2016	End of Phase I study
February, 2017	**2nd VMT Meeting** / Phase I results and planning of Phase II study
Phase II study to know between- and within-laboratory reproducibility	
April, 2017	Coding and distribution of coded test chemicals and positive chemicals
May, 2017	Start of Phase II study using 20 coded test chemicals
August,2017	End of Phase II study
November-December, 2017	**3rd VMT Meeting** /reviewing of Phase II study results
2018	Completed validation report

Abbreviations

CAS: Chemical Abstracts Service
GLP: Good Laboratory Practice
HRI: Hatano Research Institute
FDSC: Food and Drug Safety Center
JaCVAM: Japanese Centre for the Validation of Alternative Methods
NIHS: National Institute of Health Sciences
OECD: Organization for Economic Co-operation and Development
QC: Quality Control
TG: Test Guideline
VMT: Validation Management Team

Appendix 9. The list of proficiency chemicals

Undetermined yet

Appendix 10. MITA QC confirmation table

MITA(P1) confirmation tabale

	LabB (AIST, Tsukuba)			LabC (FDSC)			LabD (AIST, Takamatsu)		
setA-1 (run 1)	date	2016.9.12	(document 4 - 5)						
	Cell culture records	○							
	Weighting records	○							
	Test records	○							
	Datasheet	×							
	Graph	○							
setA-1 (run 2)	date	2016.10.4	(document 4 - 6)						
	Cell culture records	○							
	Weighting records	○							
	Test records	○							
	Datasheet	×							
	Graph	○							
setA	Weighting records	○		Weighting records	○		Weighting records	○	
	Cell culture records	○		Cell culture records	×		Cell culture records	○	wake up 2016.8.26~ Last culture 2016.9.29
setA-1	date	2016.10.26		date	2016.10.4.		date	2016.9.9	
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	○	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph			Graph	○	
setA-2	date	2016.11.1		date	2016.10.17		date	2016.9.12	
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph			Graph	○	
setA-3	date	2016.11.4		date	2016.10.21		date	2016.9.15	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph			Graph	○	
setA-4	date			date			date	2016.9.20	(3rd re trial)
	Cell culture records			Cell culture records			Cell culture records	○	
	Weighting records			Weighting records			Weighting records	×	
	Test records			Test records			Test records	○	
	Datasheet			Datasheet			Datasheet	○	
	Graph			Graph			Graph	○	
setB	Weighting records	○		Weighting records	○		Weighting records	○	
	Cell culture records	○		Cell culture records	×		Cell culture records	○ Newly starting cell culture on the way	continue from SetA + (wake up from 20160923 cell culture till 20161014)
setB-1	date	2016.11.8		date	2016.10.27		date	2016.9.23	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	○	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setB-2	date	2016.11.12		date	2016.10.28		date	2016.9.26	(1st re-trial)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setB-3	date	2016.11.16		date	2016.10.31		date	2016.9.29	(2nd trial)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setB-4	date			date			date	2016.10.3	(3rd trial)
	Cell culture records			Cell culture records			Cell culture records	○	
	Weighting records			Weighting records			Weighting records	×	
	Test records			Test records			Test records	○	
	Datasheet			Datasheet			Datasheet	○	
	Graph			Graph			Graph	○	

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