Hand1-Luc Embryonic Stem Cell Test (Hand1-Luc EST)

Draft protocol ver. 09 E

March, 9th, 2016

Environmental Health Science Laboratory,
Sumitomo Chemical Co., Ltd.

Noriyuki Suzuki, Ph.D.
Florian Le Coz, M.Sc.
Hirohisa Nagahori, Ph.D.
Koichi Saito, Ph.D.

TABLE OF CONTENTS

INTRODUCTION

Hand1-Luc Embryonic Stem Cell Test (Hand1-Luc EST) is a novel short term test for predicting embryonic toxic chemicals using transgenic engineering mouse embryonic stem (ES) cells with consideration of pharmacokinetic property of test chemicals. This protocol describes how to prepare, determine the pharmacokinetic property and analyze cytotoxicity and differentiation toxicity of chemicals.

MATERIALS AND METHODS

~~EXPERIMENT1~~

~~1-1 Measurement of actual value of pKa or in silico methods~~

~~Actual value can be measured by titration, spectrophotometric and conductometric methods. Detailed protocol is shown in OECD Test Guideline (112, http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm)~~

~~The value can be calculated by commercial software or available tools on the internet such as ADMET Predictor, ACD/labs or Mervin (http://www.chemaxon.com/marvin/sketch/index.jsp).~~

~~EXPERIMENT 2~~

~~2~~ 1 Measurement of ID_{50} and IC_{50} values in the Hand1-Luc EST

Schema of the Hand1-Luc EST procedure is described in Figure 1.

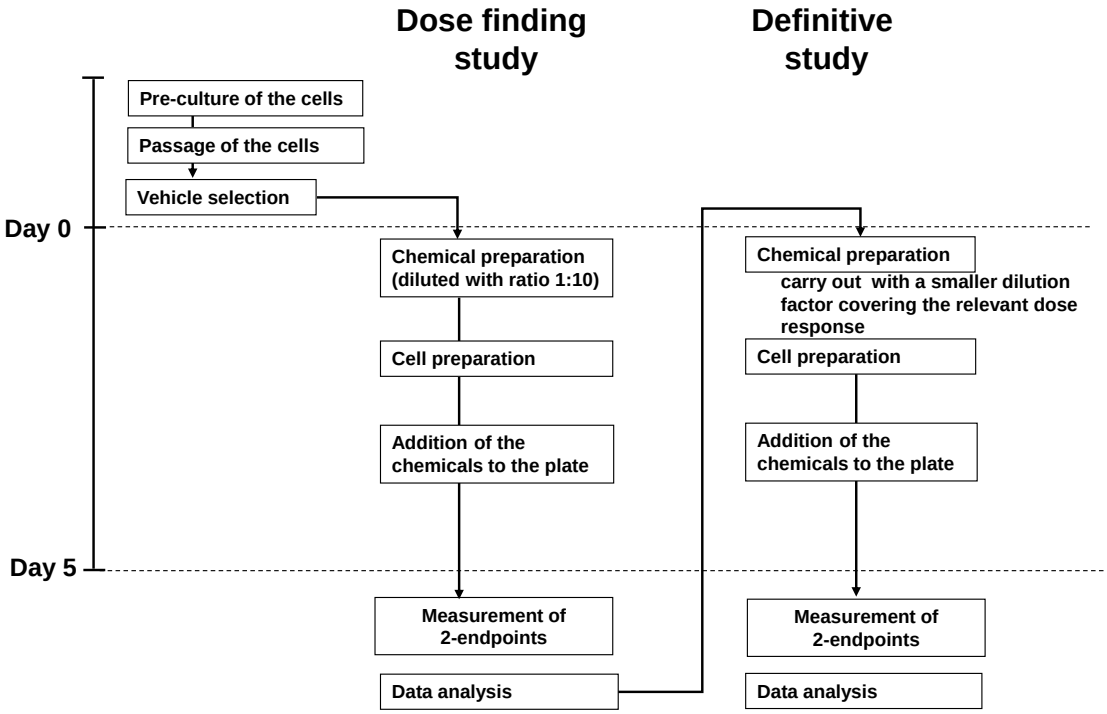


Figure.1 Outline of the Hand1-Luc EST

A. Materials

Cells (Hand1-ES)

Transgenic mouse embryonic stem (ES) cell, named as Hand1-ES, containing firefly luciferase genes under the control of Hand1 (heart and neural crest derivatives expressed transcript 1) promoters were established by Sumitomo Chemical Co., Ltd. as a previous method with minor modification (Suzuki et al., Toxicol. Sci., 124, 460–471, 2011). An embryonic stem cell line (named as KOB1-ES) from C57/B6 mouse established by Sumitomo Chemical Co. Ltd. was used for development of the Hand1-ES cells.

Reagents and preparation methods

For maintenance of Hand1-ES cells

- StemMedium (Serum Free Media for Mouse ES Cell) (DS Pharma Biomedical, Cat. DSRK100)
  ▲**Note** ~~Dissolved~~ Dispensed medium is stored at -20 °C (in an appropriate volume in tubes).
- Gelatin from porcine, type A (SIGMA, Cat. G2500)
- ESGRO mLIF (10^7 units/mL) (Millipore, Cat. ESG1107)

- Distilled water (SIGMA, Cat. W3500-500mL)
- 0.25 % Trypsin /1mM EDTA solution (Nacalai tesque, Cat.35554-64)
- 50 mg/mL Geneticin Disulfate (G418) solution (Nacalai tesque, Cat. 16513-84)
- Trypan blue solution (SIGMA, Cat.T8154-20ML or equivalents, regardless of makers, are acceptable)

For chemical exposure, positive control, solvents

- 5-Fluorouracil (5-FU) (SIGMA, Cat. F6627-1G, purity 99 %)
- Dimethyl sulfoxide (DMSO)（MP Biomedicals, Inc. (WAKO Pure Chemicals), Cat. 594-03771）
- PBS(-) (Invitrogen, Cat No. 10010-023 (500mL))

For differentiation or cytotoxicity assay

- DMEM (Invitrogen, Cat. 11965-092)
- FBS (Hyclone, Cat 533-90935, Lot. KTC30729)
  ▲**Note** The quality of the serum is very important for mESC growth and differentiation. Several lots of serum have to be tested before performing the assay.
- GlutaMaxI solution (100×conc.) (Invitrogen, Cat. 35050-061)
- 100 mM Non-Essential Amino Acid solution (NEAA) (Invitrogen, Cat. 11140-050)
- Penicillin-Streptomycin, liquid (PS solution) (Nacalai tesque, Cat. 26252-94)
- 2-Mercaptoethanol (2-ME) (Nacalai tesque, Cat. 21438-82)
- Steady-Glo® Luciferase Assay System（Promega, Cat No. E2510, E2520 and E2550）
  ▲**Note** Dissolved reagents stored at -80 °C (in an appropriate volume in tubes).
- CellTiter-Fluor™ Cell Viability Assay（Promega, Cat No. G6080, G6081 and G6082）

Maintenance medium (Culture medium for maintenance of Hand1-ES cells)

Content	Volume
StemMedium 100mM 2-ME solution ESGRO (10^7unit/mL) 50 mg/mL G418 solution	10 mL 10 μL 1 μL 20 μL
Note	
- Supplemented medium is stored for no longer than 1 week at 4 °C. - Before use, supplemented medium should be prewarmed to 37 °C in a water bath or incubator.	

Assay medium

Content	Volume

DMEM FBS (Heat inactivated) GlutaMaxI solution (100× conc.) 100mM NEAA solution PS solution 100mM 2-ME solution	82 mL 15 mL 1 mL 1 mL 1 mL 100 μL
Note	
- Supplemented medium is stored for no longer than 2 weeks at 4 °C. - Before use, supplemented medium should be prewarmed to 37 °C in a water bath or incubator.	

0.1% Gelatin solution

Content	Volume
Gelatin Distilled water	0.5 g 500 mL
Note	
- Autoclaved solution is stored for no longer than 2 months at 4 °C. - 60mm dishes should be coated with gelatin solution (37°C, over 30min).	

2-Mercaptoethanol (2-ME) solution

Content	Volume
2-ME (14.3 M solution) Distilled water	70 μL 10 mL
Note	
Stock solutions are stored at -80 °C or prepared at time of use.	

Fetal Bovine Serum, Heat-Inactivated

Content
FBS
Note
Incubate at 56 °C for 30 min and store at -20 °C (appropriate volume in tubes). Dissolved FBS is kept at 4 °C and use within 1 month.

Equipments

- Measuring device of micro-plate type luminometer
- Measuring device of micro-plate type fluorometer
- Plate shaker
- Water bath
- CO_2 incubator (5%, 37°C)
- Single and 8 channel micropipette
- Appropriate cell counter (hemocytometer)

Expendable supplies

- 60 mm Cell culture dish (BD Falcon, Cat.353004)
- Storage Plate 96 well Round Bottom (Corning, Cat.3359)

- 96 well Assay Blocks 2mL/well (Corning, Cat.3960)
- PrimeSurface® 96well white plate (Sumitomo Bake Lite, Co., Ltd. MS-9096W）
- Plate seal (for PrimeSurface® 96well white plate) (Watson, Cat. 547-KTS-HC)
- Plate seal “TopSeal-A+” (PerkinElmer No. 6050185)
- Cell strainer (40μm, BD Falcon, Cat.352340)
- Reservoir
- Pipette

B. Experimental Methods

Thawing of Hand1-ES cells

1. Coat the 60 mm dishes with the 0.1 % gelatin solution. Add 5 ml of the gelatin solution in the dishes and incubate at 37 °C during 30 min.
2. Thaw frozen cells in 37 °C water bath, and add to the 9 mL of maintenance medium.
3. Centrifuge the tube at 900 to 1,400 rpm for 5 min at room temperature, discard supernatant and resuspend the cells in 5 mL of maintenance medium.
4. Count cell number using aliquot of solution.
5. Remove the gelatin solution from the dishes (step 1) and seed the cells at a concentration of approx. 0.5 to 1.0 $\times 10^6$ cells/ gelatin-coated 60 mm dish in 5mL of maintenance medium.
6. Incubate the cells at 37°C in a humidified atmosphere with 5% CO_2.

Maintenance of cells

Grown cells should be passaged 2 or 3 days after thawing.

1. Prepare the 60 mm dishes coated with the 0.1 % gelatin solution before maintenance of cells.
2. Remove the supernatant from culture dish, wash with 5 mL of PBS(-).
3. Add 2 mL of 0.25% trypsin/1 mM EDTA and remove immediately. Then, cells are incubated at 37 °C for 1 to 2 min.
4. Add to 2 mL of maintenance medium to the dish, suspend the cells using micropipette (1000 μL).
5. Count cell number
6. Remove the gelatin solution from the dishes (step 1)
7. For assay, seed the cells at a concentration of 2.0×10^6cells/ gelatin-coated 60 mm dish in 5mL of maintenance medium. Incubate the cells at 37°C with 5% CO_2 for 1 day.
8. For passage, seed the cells at a concentration of 0.2, 0.5 and 1.0×10^6cells/ gelatin-coated 60 mm dish in 5mL of maintenance medium. Incubate the cells at 37°C with 5% CO_2 for 2 or 3 days. Cells should be used until the cells reach 80–90% confluence.

▲Note;

- Cells should be passed until the cells reach 80–90% confluence.
- Cells should be used within 2 passages.

Preparation of test chemicals, choice of solvent and precipitation evaluation

Test chemicals are dissolved in an appropriate solvent such as PBS(-) and DMSO. The recommended maximum final concentrations are 1 % (v/v) for PBS(-) or 0.1 % (v/v) for DMSO. The chemicals must be weighed and dissolved in solvent before each experiment, including 5-FU for the positive-reference control. The highest test concentration of any chemicals is 1000 μg/mL.

Dilution series of 7 concentrations of 5-FU (0.0003, 0.0006, 0.0013, 0.0025, 0.005, 0.01 and 0.02 mg/mL; with a common ratio of 1:2) as positive-reference control is dissolved from a 0.02 mg/mL prepared stock solution in PBS(-).

A flowchart for selection of appropriate vehicle, preparation of chemicals and precipitation evaluation is shown in Figure 2.

Dissolve the test chemical first in PBS(-) to obtain a concentration of 100 mg/mL. For example, weigh 10 mg of the test chemical in an appropriate tube and add PBS(-) up to 100 μL. If the chemical is not soluble at 100 mg/mL, dilute with a common ratio of 1:2 to obtain a concentration of 50 mg/mL. If the chemical is not soluble at 50 mg/mL, dilute with a common ratio of 1:2 to obtain a concentration of 25 mg/mL. Other final volumes are also possible but the minimum required one should be 100 μL depending on the needs of test chemical.

If the chemical is not soluble at 25 mg/mL in PBS(-), the chemical should be dissolved in DMSO at 1000 mg/mL. For example, weigh 100 mg of the test chemical in an appropriate tube and add DMSO up to 100μL. At this step, a precipitation test should be undertaken as followed: add 1μl of the DMSO-dissolved chemical in the tube containing 999μl of assay medium. Mix the medium with the chemical thoroughly by vortex. If the precipitate is not observed at 1000μg/ml, ~~check water solubility over 1000 μg/ml.~~ then 1000 μg/ml should be the highest concentration tested.

If the chemical is not soluble at 1000 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 if needed) with DMSO. If the chemical is not soluble at 250 mg/mL in DMSO, prepare solution of test chemical using PBS(-) or DMSO with 2-fold dilution factor (ex; 125 mg/mL in DMSO or 12.5 mg/mL in PBS(-) if needed). Other final volumes are also possible but the minimum required one should be 100 μL depending on the needs of test chemical.

Sonication and vortexing may be used if needed, and attempt to dissolve the chemical for at least 5 minutes.

For each highest concentration where the chemical is soluble, the precipitation test should be provided. For example, if the chemical is soluble in DMSO at 500 mg/mL, then from this concentration and with a 2-fold dilution factor, the possibility of precipitation in the assay medium should be verified. For this purpose, add 1 μL of the DMSO dissolved chemical in the tube containing 999 μl of assay medium. Mix the medium with the chemical thoroughly by vortex.

If a precipitate ~~appears on the tips of the 8 channel micropipette or~~ remains in

the medium after mixing, then the corresponding concentrations should not be taken into account for the experiment. For example, if the last precipitate is observed at 250µg/mL then the highest concentration used for the experiment will be 125µg/mL.

For chemicals that do not dissolve in PBS, the solubility in DMSO should be carefully evaluated especially for liquid chemicals where small and difficult to see bubbles may appear.

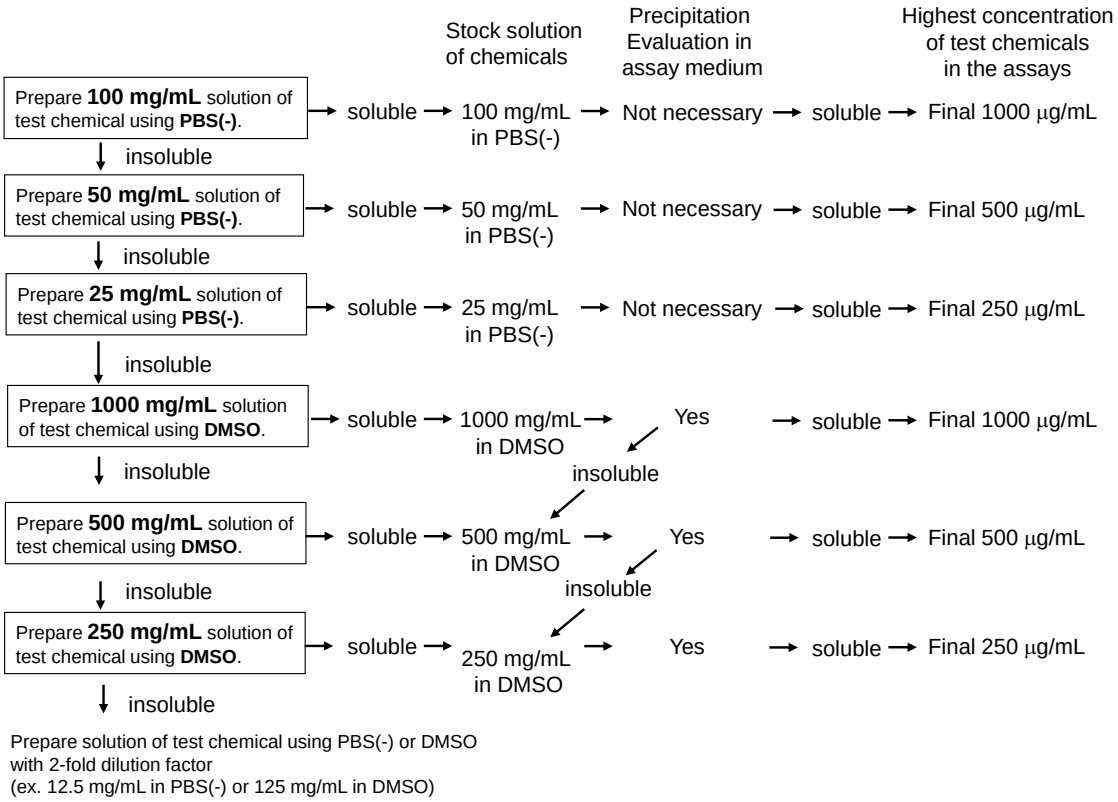


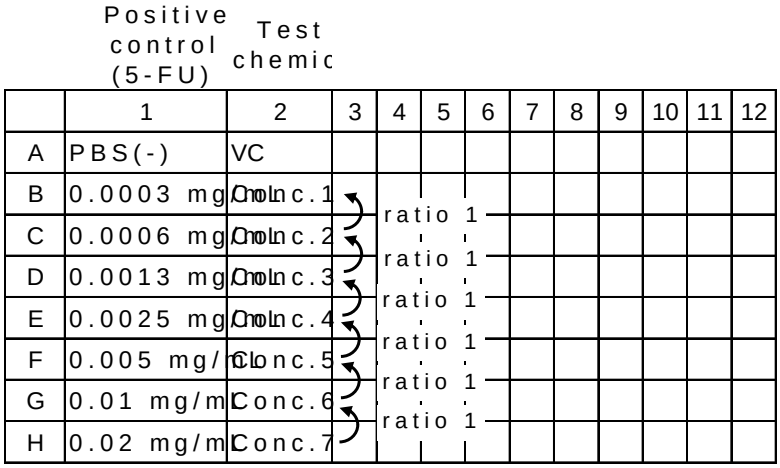
Figure 2 Vehicle selection and preparation of test chemicals

Serial dilution of test chemicals and arrangement of plate format

Generally, the test chemicals should be prepared as follows;

- Maximum concentration (final concentration): 1000 µg/mL (depend on the solubility of test chemicals)
- Serially dilute with the same solvent at a common ratio of 1:10, basically in the dose finding assay. Carry out the definitive study with smaller dilution factor (ex; 1:1.5, 1:2, 1:3 or 1:5) covering the relevant range of dose response and as much as possible and if the gap between the IC_{50} and ID_{50} allows it, the lowest common ratio should be chosen. If IC_{50} and ID_{50} values are close to each other, the IC_{50} value should be situated in the middle of the new concentration range.

1. Prepare dissolved 5-FU as positive-reference control and test chemicals according to the procedure (described in section “Preparation of test chemicals”) using 96 well microplates (Corning, Cat. 3359) (cf. Figure 3, row 1A - 1H, 2A - 2H) before assay.



VC: Vehicle control (DMSO or PBS(-))

Figure 3 Serial dilution and arrangement of test chemicals (1)

2. Add 20 μL of diluted 5-FU to the well of 96 well Assay Block containing 980 μL of assay medium using an 8 channel micropipette (cf. Figure 4, row 1A – 1H).
3. Add 20μL of chemicals to the well of 96 well Assay Block containing 980 μL of assay medium using an 8 channel micropipette if chemicals are dissolved in PBS(-) (cf. Figure 4, row 2A – 2H).

   Add 2 μL of chemicals to the well of 96 well Assay Block containing 998 μL of assay medium using an 8 channel micropipette if chemicals are dissolved in DMSO. Store solution at room temperature (20 - 30°C) until next steps.

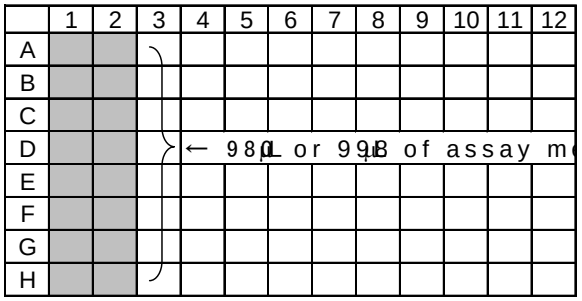


Figure 4 Serial dilution and arrangement of test chemicals (2)

Dose finding study

Preparation procedure of cells for the assays.

1. Dispense 100 μL of PBS(-) into the peripheral wells and 100 μL of assay medium into row 11B – 11G (cf. Figure 5).
2. Prewarm assay medium and PBS(-) at 37 °C. 0.25% Trypsin /1mM EDTA solution is kept at room temperature (20 - 30°C) by bathing in water or ambient air.

3. Remove the supernatant from culture dish, wash with 5 mL of PBS(-).
4. Add 1 - 2 mL of 0.25% trypsin/1 mM EDTA and remove immediately. Then, cells are incubated at 37 °C for 1 - 2 min.
5. Add to 2 mL of assay medium to the dish, and suspend the cells using a micropipette (1000μL).
6. Use cell strainer with 50mL centrifuge tubes to obtain more uniform single-cell suspensions.
7. Check viability by staining an aliquot of the cell suspension with Trypan blue.
   ▲**Note** A viability of 90 % or higher is acceptable.
8. Count cell number.
9. Dilute the cells in assay medium at cell density of 15,000 cells/mL (750 cells /50 μl/well).
10. Transfer the cells to a reservoir, and dispense 50 μL of cell suspension to PrimeSurface® 96well white plate (Sumitomo Bake Lite, Co. Ltd., MS-9096W） (cf. Figure 5; row 2B – 10G in the plates for 5-FU or test chemicals).
11. Incubate the cells at 37°C with 5% CO_2.

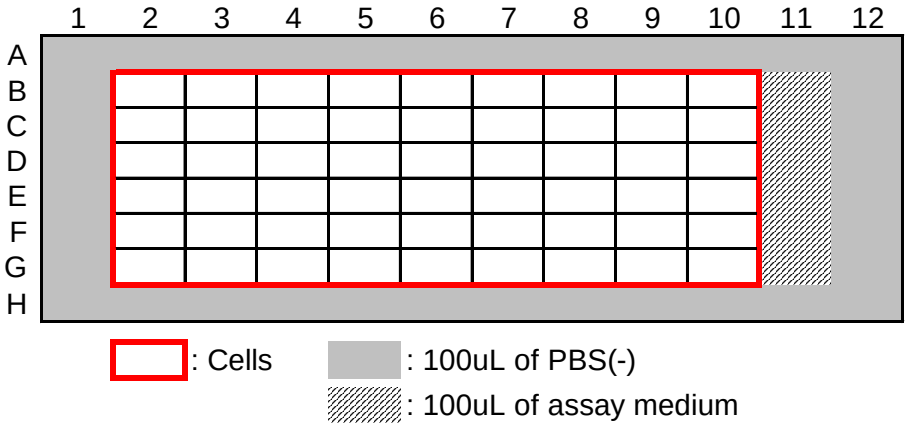


Figure 5　Preparation of the cells for the assay

Treatment of the test chemicals for the assays

1. After more than 2 hrs of incubation, mix prepared solution of 5-FU and test chemicals by pipetting thoroughly.
2. Add 50 μL of assay medium to a plate (cf. Figure 6; row 2B –2G).
3. Dispense 50 μL of solution of chemicals to 96 well-plate containing the cells using an 8 channel micropipette (cf. Figure 6; row 3B –10G).
4. Shake the plate with a plate shaker for a few seconds.
5. Incubate the cells at 37°C with 5% CO_2.

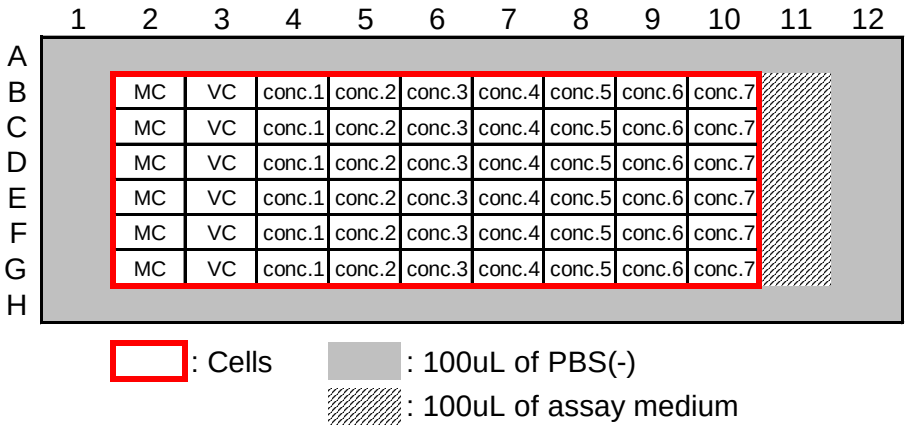


Figure 6 Arrangement of chemicals and vehicles for assay

Measurement of fluorescence and luminescence

▲**Note** Switch on measuring equipments 30 min before starting the measurement.

120 hours after seeding the Hand1-ES cells, measure the two following endpoints as described below:

For cytotoxicity assay

1. Completely thaw the CellTiter-Fluor™ Cell Viability Assay components in a 37°C water bath. Vortex the GF-AFC substrate (▲**Note** avoid exposure to light) to ensure homogeneity, then briefly centrifuge for complete substrate volume recovery.
2. Transfer the GF-AFC Substrate into the Assay Buffer container to form a complete reagent (cf; Assay buffer 1mL : GF-AFC substrate 10 μL) . Mix by vortexing the contents until the substrate is thoroughly dissolved.
3. Add 10 μL of CellTiter-Fluor™ complete reagent to each well (cf. Figure 6; row 2B - 11G).
4. Mix briefly by orbital shaking, then incubate for at least 30 minutes at 37°C. Seal the surface of the plates.
   ▲**Note** Don't incubate longer than 1hour, and be sure to shield plates from ambient light.
5. Measure resulting fluorescence using a fluorometer (380–400nmEx /505nmEm).
   ▲**Note** You may need to adjust instrument gains.

For differentiation assay

1. Thaw the Steady-Glo® Luciferase Assay System and equilibrate to 25 °C prior to use (▲**Note** avoid exposure to light).
2. Dispense 100 μL of Steady-Glo® Luciferase Assay System to each well of 96 well plates by an 8 channel micropipette (cf: Figure 6; row 2B – 11G).
3. Shake the plates gently for over 30 min at room temperature (20 – 30 °C) with a plate shaker. Seal the surface of the plates.

▲**Note** Don't incubate longer than 1hour, and be sure to shield plates from ambient light.

4. Place the plate in luminometer. Luminescence is measured with the appropriate time (cf. 1sec for validation study) according to the manufacturer's instructions.

Definitive study

Calculate endpoints according to Steps "Data analysis". For the definitive study, choose seven dilutions. Carry out the definitive study with a smaller dilution factor covering the relevant range of dose response according to dose finding study (see Section of Experimental validity of definitive studies)

Data analysis

Use an Excel (Microsoft) spreadsheet for data recording (Result format ver. 08E). Calculation method and the acceptance criteria for these assays are as follows;

Determination of ID_{50} and IC_{50} values

Relative viabilities (surviving rate) or relative activities for each concentration of test chemicals against vehicle control can be calculated using Excel (Microsoft) spreadsheet. Calculation formulas are described as follows;

For differentiation assay
a : Mean values of vehicle control
b : Mean values of test chemicals
c : Mean values of background
d : NET values of vehicle control : a-c
e : NET values of test chemicals : b-c
A : Relative activity of vehicle control : 100 (%)
B : Relative activity of test chemicals : e/d X 100 (%)

For cytotocxicity assay
a : Mean values of vehicle control
b : Mean values of test chemicals
c : Mean values of background
d : NET values of vehicle control : a-c
e : NET values of test chemicals : b-c
A : Relative viability of cells for vehicle control : 100 (%)
B : Relative viability of cells for test chemicals : e/d X 100 (%)

Inhibition of differentiation is expressed as the concentration of the test chemical that reduced the luminescence by 50% ($ES\text{-}ID_{50}$, calculated from the concentration-response curve). Cytotoxicity is expressed as the concentration of the chemical reducing the viability of cells to 50% of the control level ($ES\text{-}IC_{50}$ determined from concentration-response curves).

The IC_{50} and ID_{50} values relative to solvent controls were derived from

computational regression analysis of concentration response curves with a three parameter logistic function using an Excel (Microsoft) spreadsheet for data recording (Result format ver.08E).

The full concentration-response curve is required for the calculation of the ID_{50} and IC_{50} values, but this may not always be achievable or practical due to limitations of the test concentration range (for example due to cytotoxicity or solubility problems).

Acceptance criteria

Quality control

i) Quality control of cell condition

Check viability of the cells by staining an aliquot of the cell suspension with Trypan blue. A viability of 90% or greater is acceptable.

ii) Quality control of differentiation and cytotoxicity assays

To verify the cell growth and cell differentiation, the comparison of the medium control (MC) and background (BG) is used.

- The Lower limit of the 95% confidence intervals of the ratio of MC / BG should be above 1 for the cytotoxicity assay
- The Lower limit of the 95% confidence intervals of the ratio of MC / BG should be above 10 for differentiation assay

iii) Performance standard of the assay

The quality of the assay must be controlled using 5-FU as a positive-reference chemical.

-The range of ID_{50} for 5-FU should be within **0.003 and 0.067 μg/mL** (according to phase 0 study).

-The range of IC_{50} for 5-FU should be within **0.003 and 0.065 μg/mL** (according to phase 0 study).

iv) Quality control for effect of vehicle

To verify the effect of the vehicle, the comparison of the medium control (MC) and vehicle control (VC) is used.

- The Lower limit of the 95% confidence intervals of the ratio of VC / MC should be above 0.2 for the cytotoxicity and differentiation assays.
- In addition to the above criterion

  CV (SD/mean X 100%) of VC should be below 100%.

EXPERIMENT 3 (for optional prediction with PM2)

3-1 Measurement of metabolic stability of test chemicals

Liver S9 fractions

Rat liver S9 fractions are commercially available (Male, pool of 400 livers, XenoTech). The brief procedure for preparation is as follows. A portion of liver from male SD rats is homogenized in 4 volumes of Tris/HCl buffer (pH7.4) with a homogenizer. Liver homogenate is centrifuged at 9,000 × g for about 20 min at 4 °C to separate S9 fractions. The protein level in the S9 fraction can be measured by commercially available protein assay kit.

Metabolic stability

Substrate solution is prepared by mixing 100 µM substrate in acetonitrile with 100 fold volume of 6 mM β-NADPH (Oriental Yeast) in 125 mM potassium phosphate buffer (pH7.4). The reaction is initiated by mixing 50 µL of substrate solution with 50 µL of diluted liver S9 fractions (0.5 mg protein/mL) in 125 mM potassium phosphate buffer (pH7.4). After incubation for 35 min at 37 ºC, the reaction was stopped by addition of 400 µL methanol. After centrifugation at 3,000 g for 10 min, the supernatant is analyzed by the triple quadrupole LC/MS and the peak area of the target ion is calculated. The control sample is prepared without liver S9 fractions. The metabolic stability is expressed as the ratio of peak area with liver S9 to that of control.

JUDGMENT

In the Hand1-Luc EST, the embryotoxic potency of test chemicals was predicted using following equation. ~~a biostatistically based prediction model shown in Figure 7.~~

Discriminant equation

Prediction Model 1(PM1)

Score = 1.474 × log (1) + 0.714 × log (2) − 1.109

1, $\log(IC_{50}/ID_{50})$; 2, $\log(\text{Maximum dose}/IC_{50})$

Probability: inverse logit transform of the score

Positive : probability ≥ 0.52
Negative : probability < 0.52

Majority judgment of 3 replicates ~~(Phase 2a)~~

	Dose finding	Definitive study1	Definitive study2	Definitive study3	Final judgment
Case 1	-	**P**	**P**	-	**P**
Case 2	-	**N**	**P**	**P**	**P**
Case 3	-	**N**	**P**	**N**	**N**
Case 4	-	**N**	**N**	-	**N**
Case 5	-	**P**	**N**	**P**	**P**
Case 6	-	**P**	**N**	**N**	**N**

The dose finding is not considered for the evaluation. If both first definitive studies conclude negativity or positivity, then only two definitive studies are required.
However, if definitive study 1 and definitive study 2 have 2 different conclusions, then a 3rd definitive study will be required.

Experimental validity of definitive studies:
If the IC_{50} and ID_{50} values of DS1 compared to the IC_{50} and ID_{50} values of DS2 are different from a factor 7, then one more test should be done and the 2 closest results should be taken into account.
(Example1: If DS1, IC_{50}=10 µg/ml, DS2, IC_{50}=125 µg/ml and DS3, IC_{50}=28 µg/ml then only the results of DS1 and DS3 should be analyzed.
Example 2: If DS1, IC_{50}=10 µg/ml, DS2, IC50=12 µg/ml and DS3, IC_{50}=128 µg/ml then only the results of DS1 and DS2 should be analyzed)
If, again, IC_{50} and ID_{50} values of DS3 are higher or lower than a factor 7 compared to DS1 and DS2, then one more last test should be performed. This evaluation should be done disregarding the positivity or negativity judgment.

OPTION (Experiment 2)

In the Hand1-Luc EST, prediction of the embryotoxic potency of test chemicals can be improved optionally by measuring metabolic stability (to be submitted).

UPDATE RECORD

Ver.01E, 2013, Feb, 20th distributions

Ver.02E, 2013, April, 30th distributions
Major modification
- Plate format for the assays
- Positive reference chemicals
- Preparation of test chemicals
- Maximum final concentrations of vehicle (PBS(-) and DMSO)
- Quality control and acceptability criteria

Ver.03E, 2013, July, 18th distributions
- Plate format for the assays
- Detection method for cytotoxicity

Ver.04E, 2013, July 23th distributions
- Minor modification
- Tentative acceptance criteria

Ver.05-1 E, 2013, November 6th distributions
Major modification
- Measurement at 120 hr
- Tentative acceptance criteria or phase 1 study.

Ver.06-1 E, 2014, March 10th distributions
Major modification:
- Measurement of the water solubility, suppression of the measurement of the logP
- Inclusion of a precipitation evaluation step
- Modification of the prediction model
-Tentative acceptance criteria modified for phase 2.

Ver.07 E, 2014, May 14th distributions
Minor modification:
- Measurement of the water solubility
- Modification of the prediction model

Ver.08 E, 2015, June 26th distributions
Minor modification:
- According the suggestion of VMT member, calculation method of IC_{50} and ID_{50} values using a three parameter logistic function instead of 2-parameter
- Modification of the prediction model

Ver.09 E, 2016, March 9th distribution
Minor modifications:
- Correction English figure 1
- Correction on the top seal product reference number (expendable supplies section), that has been updated by the supplier.

- Precisions about dissolution of liquid chemicals in solvent
- Figure 2 corrected with insertion of evaluation of precipitation in the assay medium
- Precision on dilution ratio that should be chosen for the definitive studies
- Definitive studies' quality control: IC_{50} and ID_{50} of DS1 and the ones in DS2 are significantly different.
- Correction of the prediction model.
- Correction of optional experiment for PM2