

Human Pluripotent Stem Cell Test for Assessing the Potential Teratogen Risk

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Abstract

In order to reduce the reliance on animal studies, both the pharmaceutical and chemical industries have been investigating new human cell-based in vitro assays capable of predicting a chemical's potential harm to humans. Here we describe a human pluripotent stem cell test (hPST) capable of identifying compounds that pose teratogenic risk to humans such as thalidomide. Given the complexity of human development and the broad spectrum of compounds correctly classified based on their known in vivo teratogenic risk, the hPST is a remarkably simple assay. Following 3 days of directing the differentiation of a monolayer of human pluripotent stem cells toward the mesendoderm fate, the nuclear localization of a single transcription factor, SOX17, is quantitated. The reduction of SOX17 in the presence of test compounds is used to stratify the compounds' teratogenic risk. However, as is the case with many human pluripotent stem cell experimental systems, careful optimization of the differentiation conditions is required in order to obtain reproducible results. Therefore, in addition to outlining the methodology of the hPST, we describe in detail various techniques we have used to optimize the assay.

Key words Teratogen, Embryotoxicity, Developmental toxicity, Human pluripotent stem cell

1 Introduction

Teratogens disturb the normal development of the embryo or fetus. Assessing the risk of drug- or chemical-induced teratogenicity in animals is both costly and imperfect [1–4]. In the 1970s, an unrecognized teratogen, thalidomide, was given to pregnant women as a treatment for morning sickness. Although thalidomide had been tested in pregnant rodent models and failed to exhibit teratogenic risk, in humans it proved to cause debilitating birth defects [5, 6]. In recent years, several in vitro methods for assessing compound- and chemical-driven teratogenic risk have been developed [7–19]. One widely used model uses mouse embryonic stem cells differentiated toward the cardiac lineage, the mouse embryonic stem cell test (mEST) [20]. Disruption of the number of beating cardiomyocytes

is used as a marker of teratogenicity. In 2013, our group sought to refine this method with the goal of developing a higher-throughput, more quantitative assay using human embryonic stem cells that would ideally be capable of detecting human teratogens such as thalidomide that may be missed by rodent models [21].

The human pluripotent stem cell test, hPST, is a 3-day monolayer differentiation of human pluripotent stem cells toward the mesendoderm lineage. The degree of disruption of the germ layer specification, as judged by cell loss and loss of nuclear protein localization of the transcription factor SOX17, is compared to the effects seen at similar concentrations with known teratogens, thereby extrapolating the risk of an unknown compound's potential teratogenicity.

Conceptually, two primary hypotheses guided our assay development. The first hypothesis was that if we could examine the specification of pluripotent stem cells into the three primary germ layers, endoderm, ectoderm, and mesoderm, we would capture more teratogenic compounds than a single lineage differentiation protocol such as the cardiomyocyte differentiation used in the mEST. Much to our surprise, at least based on our training set of 70 compounds, a single differentiation protocol toward the mesendoderm lineage was able to provide sufficient predictive value.

The second hypothesis was that maximal sensitivity could be achieved if we were able to quantify the protein expression of key developmental regulators at the very onset of lineage specification—the stage where approximately half of the cells express the pluripotency markers such as Oct4 and NANOG and the other half express the mesendoderm markers SOX17 and EOMES. The theory was that, at this stage, multiple developmentally important pathways would be active, and increases or decreases in expression of the regulators may be easier to detect at this stage compared to later when lineage specification had already occurred and expression of signaling pathways may be more restricted. While not formally proven, we believe that this conjecture is key to the assay performance. In our hands, we have found that assay performance is suitable as long as around 30–60% of the cells express SOX17, but we observed reduced sensitivity when we tested compounds at a stage where 80–100% of the cells in the well expressed the mesendoderm markers at the conclusion of the differentiation. Unfortunately, determining the conditions where all wells in the plate exhibit 50% expression of the lineage marker is perhaps the most difficult aspect of the assay. Conditions for achieving this can vary between individual human pluripotent stem cell lines. Nevertheless, by carefully optimizing seeding densities and growth factor concentrations, we have been able to reproduce the assay with multiple human ES cell lines and a human iPSC line.

2 Materials

2.1 Reagents for Pluripotent Stem Cell Culture

1. mTESR1 (05850, STEMCELL Technologies). Store the 5× supplement in -80°C . Once the supplement is mixed with media, store it at 4°C and use it within 7 days. Do not warm the entire bottle in the 37°C water bath. Warm only the amount of media needed each day.
2. hESC-qualified Matrigel Matrix, LDEV-Free (354277, BD Biosciences). Matrigel should be stored at -80°C and thawed on ice overnight in 4°C refrigerator before use.
3. PBS without calcium and magnesium (10010-023, Thermo Fisher).
4. 100 mm dishes. Polystyrene, clear, sterile, and tissue culture-treated surface. We used Corning 430167, but a similar product from different company should work.
5. Accutase cell detachment solution (07920, STEMCELL Technologies).
6. Falcon disposable polystyrene serological pipettes (5, 10, 25 ml) (13-668-2, 13-675-20, 13-675-22, Thermo Fisher). Individually wrapped.
7. Y-27632 dihydrochloride (1254, Tocris). Dissolve it to 10 mM for 2000× stock solution in DMSO or PBS. It should be aliquoted into multiple tubes and stored at -20°C for up to 3 years.

2.2 Reagents for Mesendoderm Differentiation

1. TC-coated, polystyrene, black, clear flat bottom 96-well plates. We have used Corning (3603) and BD Falcon (353948, 353219) plates successfully.
2. Advanced RPMI Medium 1640, no glutamine (12633-012, Thermo Fisher). Store at 4°C . Once glutamine is added, use it within 3 months.
3. Recombinant human/mouse/rat activin A (338-AC-005, R&D Systems). Aliquot at $40\text{ }\mu\text{g/ml}$ and store at -80°C . Avoid multiple freeze-thaw cycles to minimize protein degradation.
4. Recombinant human Wnt-3a (5036-WN-010, R&D Systems). Aliquot at $40\text{ }\mu\text{g/ml}$ and store at -80°C . Avoid multiple freeze-thaw cycles to minimize protein degradation.
5. Fetal bovine serum (FBS) (ES009B, ES cell qualified, Millipore). FBS should be aliquoted into smaller volumes and stored at -80°C . The same lot should be used for all studies for consistency.
6. L-Glutamine, 200 mM (25030-081, Thermo Fisher). Store at -20°C . Once it is thawed, store at 4°C and use it within 3 months.
7. SB 431542 hydrate (S4317, Sigma).

8. Thalidomide (T144-100MG, Sigma).
9. Imatinib mesylate (SML1027, Sigma).
10. All-trans retinoic acid (554720, Calbiochem).

2.3 Reagents for Immunofluorescence Staining

1. Human SOX17 NorthernLights NL557-conjugated Antibody, Goat IgG, polyclonal (NL1924R, R&D Systems). Store at 4 °C. We tested six different SOX17 antibodies from different sources; this antibody was most specific and showed highest signal-to-noise ratio. Dilute 1:20 in blocking solution right before use. We occasionally found small precipitated particles which resulted in strong background. To prevent this, make sure to filter antibody with 0.22 µm syringe filter (SLGP033RB, Millipore).
2. Human Brachyury NorthernLights NL557-conjugated Antibody, Goat IgG, polyclonal (NL2085R, R&D Systems).
3. Human SOX2 NorthernLights NL493-conjugated Antibody, Goat IgG, polyclonal (NL20181G, R&D Systems). Brachyury and SOX2 antibodies are only used for supplementary experiment to optimize the hPST for new cell lines.
4. Formaldehyde solution, 16%, 10×10 ml ampule (28908, Thermo Fisher). Store at room temperature (RT). Open ampule on the day of experiment.
5. 30% albumin solution from bovine serum (A9576-50ML, Sigma). Store at 4 °C.
6. Donkey serum (S30-100ML, Millipore). Store at -20 °C.
7. Sheep serum (S22-100ML, Millipore). Store at -20 °C.
8. Image-iT FX Signal Enhancer (I36933, Thermo Fisher). Store at 4 °C.
9. Triton X-100, Sigma (T8787, Sigma). Store at RT.
10. SlowFade Gold antifade reagent with DAPI (S36938, Thermo Fisher). Store at 4 °C.
11. DPBS with calcium and magnesium (14040-133, Thermo Fisher). Store at RT.

2.4 Blocking Solution for Immunostaining

Mix the following reagents and store at 4 °C up to 3 days.

1. DPBS.
2. 10% donkey serum.
3. 2% sheep serum.
4. 0.2% Triton X-100.
5. 1% BSA.

2.5 Mesendoderm Differentiation Medium I

Make fresh media on the day of experiment. Final concentrations are shown below:

1. Advanced RPMI Medium 1640.

2. 2 mM L-glutamine.
3. 80 ng/ml human activin A.
4. 20 ng/ml human Wnt-3a.
5. 80 units/ml (0.08 mg/ml) penicillin-streptomycin (P0781, Sigma).

2.6 Mesendoderm Differentiation Medium II

1. Advanced RPMI Medium 1640.
2. 2 mM l-glutamine.
3. 80 ng/ml human activin A.
4. 0.1 % FBS.
5. 80 units/ml (0.08 mg/ml) penicillin-streptomycin (P0781, Sigma).

3 Methods

3.1 General Technical Guidelines

1. Routine stem cell culture
Pluripotent stem cells were maintained in mTESR1 media on Matrigel-coated 100 mm plates. Passaging was performed by nonenzymatic dissociation with 2 mM EDTA in PBS. Antibiotics (penicillin, streptomycin) or antifungal reagents were not used during pluripotent stem cell maintenance. We primarily used the human ES cell line H9 (from WiCell) for our studies.
2. Mycoplasma
Absence of mycoplasma contamination was regularly confirmed using the MycoAlert Detection Kit (LT07-118, Lonza).
3. Karyotype analysis
Karyotype analysis should be performed around every 10–20 passages of pluripotent stem cell culture. For most experiments, only cells between passages 20 and 40 were used. An abnormal karyotype can affect proliferation rate and mesendoderm differentiation efficiency.
4. Cell culture
All cell culture procedures were performed at 37 °C, 5 % CO₂. It is recommended that one use a CO₂ incubator capable of on-demand, high-temperature decontamination cycle (e.g., Forma Steri-Cycle CO₂ incubator, 201370, Thermo Fisher). All manipulations of cells were performed inside a Class II biosafety cabinet using standard sterile technique.
5. Pipetting
Pipetting for 96-well plates should be performed with 12-channel electronic pipettes. We use E4 Multi Pipette Multi E12-200XLS+ (17013800, Thermo Fisher). In general, reverse pipetting is recommended to increase the accuracy. Use pipette tips with filters, as it is difficult to sterilize inside the pipette

and it becomes an easy source of contamination. An automated liquid handler can be used for the hPST assay except when performing serial dilutions with DMSO, as low surface tension of DMSO makes the handling of liquid significantly inaccurate due to the adhesion of solution to the pipette tips.

6. Adapting pluripotent stem cells to mTESR1 on Matrigel

If the stem cell line was maintained on feeder fibroblast cells in media other than mTESR1, it is recommended that the line be adapted to feeder-free conditions with mTESR1 media for several passages prior to beginning the differentiation protocol.

When pluripotent stem cell colonies are sufficiently large on feeder cells, scrape the small pieces off and transfer to a Matrigel-coated dish with media composed of 20 % mTESR1, 80 % old media, and 10 μ M Y-27632.

Next day (Day 2), change media to 40 % mTESR1 and 60 % old media without Y-27632.

On Day 3, change media to 60 % mTESR1 and 40 % old media.

On Day 4, change media to 80 % mTESR1 and 20 % old media.

On Day 5, change media to 100 % mTESR1.

When cells reach about 80 % confluence, wash with PBS once; incubate cells in 5 ml of dissociation buffer (2 mM EDTA in PBS) for 3 min at room temperature. Aspirate dissociation buffer and add mTESR1 media. Manually dissect uniform pluripotent regions from the colonies using either a sterile pipette tip, sterile scalpel or pulled glass Pasteur pipette. Transfer small pieces to a new Matrigel-coated plate, and culture with mTESR1 with 5 μ M Y-27632. Y-27632 is only required for cell attachment, not subsequent feedings.

Repeat this dissociation process for an additional two passages.

7. Preparation of cell bank

To prevent batch-to-batch effects across differentiations, large banks of pluripotent stem cells should be generated. We usually make a bank from 8×100 mm dishes of pluripotent stem cell (approximately 80 million cells), dissociated with 2 mM EDTA in PBS, suspended in mFreSR (05854, STEMCELL Technologies), and dispensed at a concentration of 1.5 million cells per tube in a total of 50 tubes. These can be stored in liquid nitrogen indefinitely.

8. Preparation of stock solution of compounds

All hydrophobic and hydrophilic small chemical compounds should be dissolved in DMSO and PBS, respectively, and stored as stock solutions in amber glass bottles or 1.5 ml polypropylene tubes at -20 °C for up to 2 years. If the compound is known to be unstable in these forms, it should be kept as a lyophilized form. Repeated freeze-thaw should be avoided. If cLogP data is available, compounds with cLogP larger than 5

should not be used due to potential solubility problems in the aqueous media across the full dose-range study. Concentration of stock solution should be 20 mM. After dissolving a compound in DMSO or PBS, it should be sonicated for 5–30 min at 30–37 °C, until compound is dissolved completely. We use Branson Ultrasonics Bransonic MH Series Ultrasonic Baths 1800 (15-336-110, Thermo Fisher), but any alternative sonicator should work. The standard dose range we used for the hPST assay encompassed six doses with 1:2 dilution (100, 50, 25, 12.5, 6.3, and 3.1 μ M).

3.2 Standard Protocol

We delineate the day that we switch from mTESR to “Differentiation Medium I” with test compound as Day 0 of differentiation. At Day 0, the cells should be at the predetermined optimal density (see the Notes section below for optimization tips) that results in approximately 50% SOX17-positive cells at the end of the experiment. For our stocks of the H9 human ES cell line, the optimal starting density at Day 0 was approximately 30–60% confluency. For the standard protocol outlined in Fig. 1, we initiate the differentiation 48 h after plating and growing the pluripotent stem cells in the 96-well dish.

3.2.1 Day 2: Plate Pluripotent Stem Cells on 96-Well Plates

1. Preparation of Matrigel-coated 96-well plates.

Thawing

Thaw hESC-qualified Matrigel on ice inside the refrigerator overnight. After thawing, swirl the bottle and make sure the liquid solution does not contain any solid granules or precipitation.

Dilution of Matrigel

Add prechilled 300 ml PBS to a prechilled 500 ml storage bottle (we use 455-0500, Thermo Fisher). Add 5 ml Matrigel to this (1:60 dilution) and gently swirl the bottle until the Matrigel is uniformly dissolved.

Transfer to 96-well plate

Dispense 70 μ l Matrigel per well on all wells of black/clear 96 plate using a 12-channel electronic pipette. Make sure to perform this process quickly, as Matrigel solidifies at room temperature.

Matrigel solidification

Incubate plates at 37 °C in CO₂ incubator for 60–90 min. Do not incubate too long, as the coated surface may become too thick and could inhibit optimal cell attachment.

Washing

Discard the Matrigel by flipping the plate to eject the Matrigel solution into a waste container. Dispense 100 μ l of PBS into each well. Be careful not to pipette directly on the surface, as

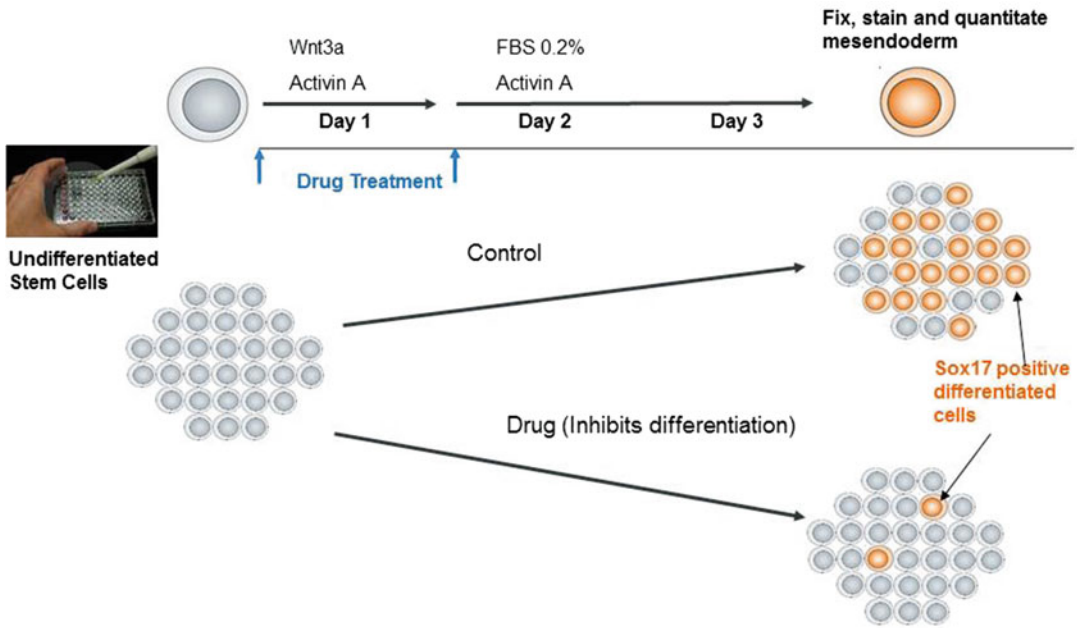


Fig. 1 Differentiation protocol in a 96-well plate

Matrigel forms a very fragile coat on the surface which can be disrupted easily by pipetting. Shake the plate at 650 rpm for 2 min. Discard PBS again by flipping the plate.

Storage

The Matrigel-coated 96-well plates can be prepared up to 5 days prior to plating the PSCs. If prepared ahead of time, dispense 100 μ l of PBS per well, replace the lid, and store plates at 4 $^{\circ}$ C.

- Seed pluripotent stem cells onto Matrigel-coated 96-well dishes
Our standard method requires approximately 3×100 mm pluripotent stem cell plates to test three drugs in duplicate wells in duplicate plates.

Dissociate cells

When pluripotent stem cells in 100 mm plate become about 60–70% confluent, remove media, rinse with PBS once, and then add 3 ml of Accutase per 100 mm plate. Cells should detach within 3–5 min. Immediately add 17 ml mTESR1 with 5 μ M Y-27632 to stop the reaction, transfer it to a 50 ml tube, and centrifuge at $400 \times g$ for 5 min.

Count cells

Remove supernatant. Add 10 ml mTESR1 with 5 μ M Y-27632. Pipette up and down gently to suspend cell pellet. Perform cell

counting and check cell viability. This can be done by automated machine such as Vi-CELL cell counter or manual counting with Trypan Blue method using a hemocytometer (we use INCYTO C-Chip DHC-N01). Note that it is not unusual for cell numbers obtained by two methods to differ significantly (>50%).

Dilute cells

To obtain uniform conditions for the assay, one must first optimize the plating cell density for each cell line, since the doubling times of individual cell lines may vary. The optimal seeding density is empirically determined by finding the density that results in 30–70% SOX17+ cells at the end of the differentiation (*see* Sect. 4.1). In our hands with the H9 hESC line, the ideal plating density resulted in 40–50% confluence 48 h after plating. Dilute the optimized cell number per well so that 100 µl of media contain the appropriate number of cells and plate the cells as described below.

Plate cells

Bring the Matrigel-coated 96-well plates prepared in the previous section from 4 °C to room temperature. Pipette up and down to resuspend the cell mixture. Swirl 50 ml tubes gently and transfer cells to 50 ml reagent reservoirs (individually wrapped, 2321–2530, USA Scientific) using a 30 µm strainer (SmartStrainer, 130-098-458, Miltenyi) to remove potential cell clumps. It is preferable to use a 50 ml tube instead of a 15 ml tube, as cell mixing can be done more gently by swirling. Set the multichannel electronic pipette to 8 × 100 µl multi-dispense mode and dispense 100 µl of cells to eight rows. Make sure that all 12 pipette tips dispensed equal volume of media at same rate, as air leakage due to loose contact between pipette and pipette tips may cause uneven pipetting.

Incubate the plate at 37 °C in CO₂ incubator for 24 h. If necessary place four water-filled trays surrounding the plates to further reduce evaporation-mediated edge effects.

3.2.2 Day 1: Change Media

1. Change mTESR media

Twenty-four hours after seeding, observe the plate under a phase-contrast microscope and record the confluency. Discard media by flipping the plate quickly and pressing it on a kimwipe gently to remove liquid from the top of the plate. Add 100 µl mTESR1 media without Y27632 per well to the side wall of 96-well plate.

3.2.3 Day 0: Initiation of Differentiation and First Compound Treatment

1. Preparation of compounds

A typical plate map layout is shown in Fig. 2. Rows A and H are not used as they are most affected by edge effects. We use 12 wells (columns 2, 7) of DMSO controls as vehicle control and also to quantify the edge effect, 6 wells of the TGF- β inhibitor SB-431542 as a positive control to inhibit mesendoderm differentiation, and 6 wells of imatinib as an internal reference compound, as its SOX17-IC₅₀ is 10–30 μ M, near the safety threshold of hPST. It is important to always have these three internal controls on the same plate as the experimental test compounds.

The example in Fig. 2 investigates compounds in dose ranged from 3 to 100 μ M in duplicates, but one can modify the doses and the number of replicates depending on the goals of the study. In addition to this, column 1 contains DMSO controls which can be specifically used to confirm the quality of immunofluorescence staining. This edge column is not used for IC₅₀ calculation, as peripheral wells are often affected by an edge effect, but are still suitable as control wells that can be used to optimize image acquisition and analysis without worrying about photobleaching.

Prepare a 200 \times compound dilution series master plate

Dispense 50 μ l of DMSO or PBS in rows C to G (2nd to 7th) of a 96-well plate except column 12. Dispense 100 μ l of a 20 mM stock compound solution into top row B (second row) according to the plate map (Fig. 2). Transfer 50 μ l of row B to row C and mix ten times with an electronic pipette. Repeat this process for row C to row G. For column 12, add 50 μ l of 5 μ M SB-431542 for all wells, which serves as a positive control for the inhibition of SOX17 expression and mesendoderm formation.

Aliquot into 2 ml deep 96-well plates for final dilutions

Transfer 2.5 μ l from the wells of the compound plate to two new sterile 2 ml deep 96-well plates (e.g., 1896–2110, USA Scientific). Make two plates: plate 1 for Differentiation Medium I and plate 2 for Differentiation Medium II. For plate 1, proceed to next step immediately. For plate 2, seal it with sealing foil (2923–0110, USA Scientific) and keep it at room temperature for 24 h. Do not put this plate in 4 °C or –20 °C as low temperature may cause precipitation of some compounds.

Add Differentiation Medium I to the aliquoted compounds in the 2 ml deep 96 -well plates

Add 497.5 μ l of warm Differentiation Medium I media (1:200 dilution) to all 96 wells of the compound plate and gently mix

a

	1	2	3	4	5	6	7	8	9	10	11	12
A	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty
B	DMSO	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	SB-431542
C	DMSO	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	SB-431542
D	DMSO	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	SB-431542
E	DMSO	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	SB-431542
F	DMSO	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	SB-431542
G	DMSO	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	DMSO	Imatinib	Drug 1	Drug 2	Drug 3	SB-431542
H	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty

b

	1	2	3	4	5	6	7	8	9	10	11	12
A	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty
B	0	0	100	100	100	100	100	100	100	100	100	5
C	0	0	50	50	50	50	50	50	50	50	50	5
D	0	0	25	25	25	25	25	25	25	25	25	5
E	0	0	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	5
F	0	0	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	5
G	0	0	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	5
H	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty

Fig. 2 Example of plate map and corresponding result. **(a)** 96-well plate map 1, compound plate; **(b)** map 2, drug dose (μM); **(c)** result (raw data) (number of SOX17+ cells/well, % of average of DMSO control); and **(d)** result (summary)

10 times immediately. Since both Differentiation Media contain high amount of proteins, care should be taken while pipetting to avoid foaming.

2. Treat cells with compound

Remove the cell plate from incubator. Discard the media by flipping the plate over a waste container to expel the media.

c

	1	2	3	4	5	6	7	8	9	10	11	12
A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
B	NA	100.6	0.0	0.0	0.5	3.3	97.7	0.0	0.0	0.0	4.9	0.0
C	NA	101.8	0.0	0.0	0.0	85.4	98.8	0.0	0.7	0.0	69.1	0.0
D	NA	91.8	0.2	0.0	0.0	91.5	89.1	2.3	0.3	0.0	88.1	0.0
E	NA	103.6	59.2	0.0	0.5	93.5	100.6	89.0	0.0	0.4	83.0	0.0
F	NA	101.5	88.2	1.2	11.8	108.2	98.5	107.5	0.0	39.3	93.0	0.0
G	NA	109.6	92.9	0.0	79.4	107.4	106.4	103.1	0.0	69.8	121.5	0.0
H	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

d

Compound	SOX17-IC50 (uM)	Call
Imatinib	16.5	toxic
Drug 1	< 3.1	toxic
Drug 2	4.7	toxic
Drug 3	68.0	safe

Fig. 2 (continued)

Wash plate with 100 µl/well DPBS (with calcium and magnesium) four times. It is important to remove mTESR completely as it may inhibit differentiation. Each wash was done by shaking the plate at 650 rpm for 3 min using an Eppendorf MixMate or similar plate shaker. Do not pipette directly on the surface of cells, as that may disrupt the fragile monolayer. Ideally, the entire washing steps should not exceed 30 min, since maintaining cells in PBS at room temperature for a prolonged duration is not optimal.

Following the final wash, replace the PBS with 100 µl media containing compounds in Differentiation Medium I from the compound plate. Shake the plate at 650 rpm for 3 min. Place the plate in 37 °C CO₂ incubator overnight.

*3.2.4 Day 4: Media
Change and Second
Compound Treatment*

1. Second compound treatment

Prepare the second compound plate by warming the Differentiation Media II to 37 °C and adding 497 µl to the compound plate made in the previous section, gently mixing by pipetting up and down ten times without bubble formation.

Take the cell plate from the incubator, discard the media by flipping the plate, and transfer 100 µl media containing compounds in Differentiation Media II. Place the plate in 37 °C CO₂ incubator for next 48 h.

*3.2.5 Day 6: Terminate
Differentiation and Fix
and Stain Cells*

1. Stop the differentiation

Seventy-two hours after the start of differentiation, discard the media by flipping the plate. Add 200 µl DPBS per well and discard immediately. Repeat this two more times. This washing step is needed to remove dead cell debris and potential compound precipitation on the top of monolayer. At this point, cells are not fixed and the monolayer can be disrupted very easily. Care must be taken not to pipette PBS directly on the cell surface. Discard DPBS at the last step.

2. Fix cells

Add 100 µl of freshly prepared 3% formaldehyde in DPBS per well, shake the plate at 650 rpm for 1 min, and incubate for 15 min at room temperature. Fixing cells for extended periods should be avoided as this may damage the antigen epitope. Incomplete fixation may also be problematic, as it may result in incomplete attachment of cells on the plate, leading to the loss of cells during the multiple washing steps of subsequent immunostaining.

Discard formaldehyde by flipping the plate, adding 200 µl DPBS and then discarding it. Repeat this washing step two times, leaving a final 100 µl of DPBS in each well until ready to proceed with the antibody staining.

Although at this stage the fixed cell plate can be stored up to 3 days at 4 °C, performing immunofluorescence staining immediately usually produces better results.

3. Immunofluorescence staining of cells.

Permeabilize cells

Discard DPBS from the plate and add 100 µl of DPBS with 0.1% Triton X-100 per well. Shake the plate at 650 rpm for 1 min. Leave the plate for 15 min at room temperature.

Pre-blocking

Discard the DPBS/Triton X-100 solution from the plate and add 30 µl Image-iT FX solution per 96 well. Shake the plate at 650 rpm

for 1 min. Incubate with the pre-block solution for 30 min at room temperature. Discard the solution by flipping the plate.

Blocking

Add 100 μ l blocking solution per well and shake the plate at 650 rpm for 1 min. Block for 1 h and then discard the blocking solution by flipping the plate over a waste container.

Antibody binding

Filter the antibody solution as described in the reagent section. Add 60 μ l of SOX17 antibody solution per well and shake the plate at 650 rpm for 3 min. Incubate the plate for 1 h at room temperature. Then shake the plate at 650 rpm for 3 min again. Incubate the plate for another 1–2 h at room temperature.

Wash

Discard the antibody by flipping the plate and add 100 μ l of antibody blocking solution. Shake the plate at 650 rpm for 3 min to wash. Repeat this one more time and wash with DPBS two more times.

Discard DPBS and add 35 μ l of SlowFade with DAPI per well very slowly as it is very viscous. The plate can be immediately used for fluorescence microscopy or stored at 4 °C for 1–2 weeks.

3.2.6 Microscopic Image Analysis to Quantify SOX17-IC₅₀

Note on plate reader use

We found that once the assay conditions had been optimized, the high content image analysis (Cellomics ArrayScan VTI HCS Reader, Thermo Fisher) readout (number of SOX17+ cells per well) and plate reader (PerkinElmer Envision) readout (580 nm Rfu per well) were highly associated ($R=0.89$, $P<0.0001$) and the IC₅₀ values determined by both methods were similar. However, the plate reader reading is more easily affected by background fluorescence noise generated by non-specific binding of SOX17 antibody, cell debris, and autofluorescence of compounds. Because of this, it is recommended to use the plate reader as a first quick scan tool and microscope-based image analysis as a more robust investigative tool.

Excitation and emission filters for the anti-SOX17 antibody conjugated to NL557

For immunofluorescence of NL557 fluorochrome (557/574 nm), use Krypton (568 nm) or HeNe (543 nm) for laser excitation and use filter sets for phycoerythrin (565/575), Rhodamine Red (570/590), or Cy3 (548/562).

3.2.7 Analyzing the Data and Calculating Compound IC₅₀ Values

1. Quality assessment of the differentiation run
First, check the DMSO control wells in column 1 with a manual fluorescence microscope. This column is not used for IC₅₀

quantitation, but is specifically designed for quality assessment of the plate. Do not observe any well except column 1 at this stage, as fluorescence excitation causes photobleaching that may affect the proper quantitation of test sample wells. Since SOX17 is a transcription factor, only the nucleus should be stained, while the cytoplasm is free of signal. All column 1 wells should have 30–70 % SOX17+ cells, although the column may contain non-optimized wells due to edge effects. If there is a significant aberration from the ideal 30–70 % SOX17+ cells (e.g., less than 10 % SOX17+ cells or near 100 % SOX17+ cells), it is likely that the differentiation or immunostaining was sub-optimal and may yield inconsistent results.

2. Capture images of all 96 wells and quantify the SOX17+ cell number and DAPI+ cell number

When setting up the parameters such as excitation strength, exposure time, z-axis focusing, and intensity histogram threshold, use wells in the column 1 to prevent photobleaching of other wells. After setting these benchmarks, quantify SOX17+ cell numbers and DAPI+ cell numbers for all other wells.

To define the background level for SOX17 signal, six wells in column 12 (Fig. 2) on each plate were treated with 5 μ M SB-431542 which inhibits mesendoderm differentiation. These wells should show 0 % SOX17+ cells. Columns 2 and 7 are all DMSO vehicle controls, and the average of these wells should be used to normalize against the test compounds when calculating the IC_{50} values (i.e., % inhibition = ((compound)/(DMSO)) \times 100). An example of the number calculated is shown in Fig. 2c.

3. Calculate IC_{50} values

Determine SOX17- IC_{50} and DAPI- IC_{50} using a curve fit program such as GraphPad Prism or TableCurve (Fig. 2d). In the example shown in Fig. 2d, where we used H9 cells and 30 μ M as a safety threshold, drug 1 and drug 2 were determined as potentially toxic and drug 3 as nontoxic. One should set a safety threshold based on IC_{50} values of the reference compounds and the individual goals of the experiment. As with any in vitro safety assessment, one should interpret the data while taking into account the appropriate risk tolerance given the investigation context. For instance, if an investigator is interested in identifying only chemicals that exhibit the highest risk of teratogenicity in humans, one could flag only those compounds that exhibit the most complete inhibition of SOX17, without reducing the number of DAPI positive cells.

3.2.8 Interpreting the Quality of Each hPST Experiment

The following points should be considered in judging whether the particular run of the assay is successful:

DMSO negative control

Standard deviation of all 12 wells of DMSO control should be lower than 15–20%, and averages of columns 2 and 6 should be similar. If this is not the case, an edge effect during cell culture or uneven immunostaining may be the cause.

TGF-beta inhibitor SB-431542 positive control

SOX17-IC₅₀ for SB-431542 is less than 1 μ M (about 0.1 μ M in H9 ES cell) and DAPI-IC₅₀ is known to be higher than 50 μ M. All wells in column 12 should have no cell death (near 100% DAPI+ cell numbers) and 0% SOX17+ cell number.

Internal reference compound control

SOX17-IC₅₀ of imatinib, which is an internal reference compound in Fig. 2a, in our batch of H9 cell is 10–30 μ M. Each run of the hPST assay should produce a result consistent with this. SOX17-IC₅₀ of imatinib may vary depending on cell line. Therefore it may be desirable to choose additional reference compounds that better suit the goals of your study.

4 Notes

4.1 Optimization of the hPST Assay with a New Cell Line

Many parameters have to be optimized before running the first hPST using a new cell line, as the efficiency of mesendoderm differentiation and the responsiveness to growth factors vary among cell lines.

Differentiation should be stopped at the time when about 30–70% of cells are SOX17+ mesendoderm. To achieve this, the duration of differentiation may vary between cell lines and needs to be determined empirically. In addition, ideally cells should not reach 100% confluence during the assay since contact inhibition may affect the differentiation.

When differentiation is stopped, well-to-well variance of SOX17+ cell number should be minimum. Coefficient of variance (CV) of SOX17+ cells should be less than 20%.

To optimize conditions for a new cell line, the seeding cell density, differentiation duration period, and growth factor concentrations should be optimized. We optimize our assay by running the standard protocol without test compounds with the following parameters modified as described below.

Differentiation duration

Stop the differentiation at Days 1, 2, 3, 4, and 5 from the start of differentiation to do time course analysis to determine the optimal duration that results in 30–70% SOX17+ cells.

Cell density

Test five different cell seeding densities: 4k, 8k, 16k, 32k, and 48k/well. Choose the cell density that results in about 20–70 % confluence at Day 0. Some cell lines proliferate significantly after the start of differentiation. Observe cells under a phase-contrast microscope every day to record the confluence and identify the starting density that results a sub-confluent density at the end of the differentiation.

Activin concentration

One problem we observed frequently was that the number of SOX17-positive cells reaches too high a level (nearly 100 %) by Day 3. This is usually solved by decreasing activin concentration and/or stopping the differentiation at Day 2.5.

Like many recombinant growth factors, the specific biological activity of activin at a given concentration may vary between individual productions lots or different commercial sources. Therefore, one may need to test a range of activin concentrations when switching to a new lot or new vendor. We typically tested three concentrations of activin: 20 ng, 80 ng, and 200 ng/ml.

Run Each hPST Assay with the Selected Conditions and with Multiple Standard Compounds

After setting up the baseline differentiation parameters, perform the hPST with multiple compounds in triplicate over a full dose range (usually 3.1–100 μ M). At least three replicate wells for each condition are required to determine reproducibility. The following compounds are recommended as useful internal controls.

Caffeine or saccharin

These compounds serve as a negative control and should not show any toxicity; both SOX17-IC₅₀ and DAPI-IC₅₀ should be higher than 200 μ M.

All-trans retinoic acid (RA) and SB-431542

These compounds should serve as positive controls for differentiation inhibition. SOX17-IC₅₀ of both should be less than 1 μ M. Cytotoxicity of RA is usually higher than that of SB-431542. DAPI-IC₅₀ of RA and SB-431542 is usually between 30 and 200 μ M.

Thalidomide

Also a positive control for differentiation inhibition. SOX17-IC₅₀ is around 1 μ M, while it shows no cytotoxicity up to 100 μ M. However, unlike retinoic acid or SB-431542, even relatively high doses of 20–50 μ M do not inhibit differentiation completely, and about 5–25 % cells remain SOX17+. This is a unique feature of thalidomide that is most probably due to its

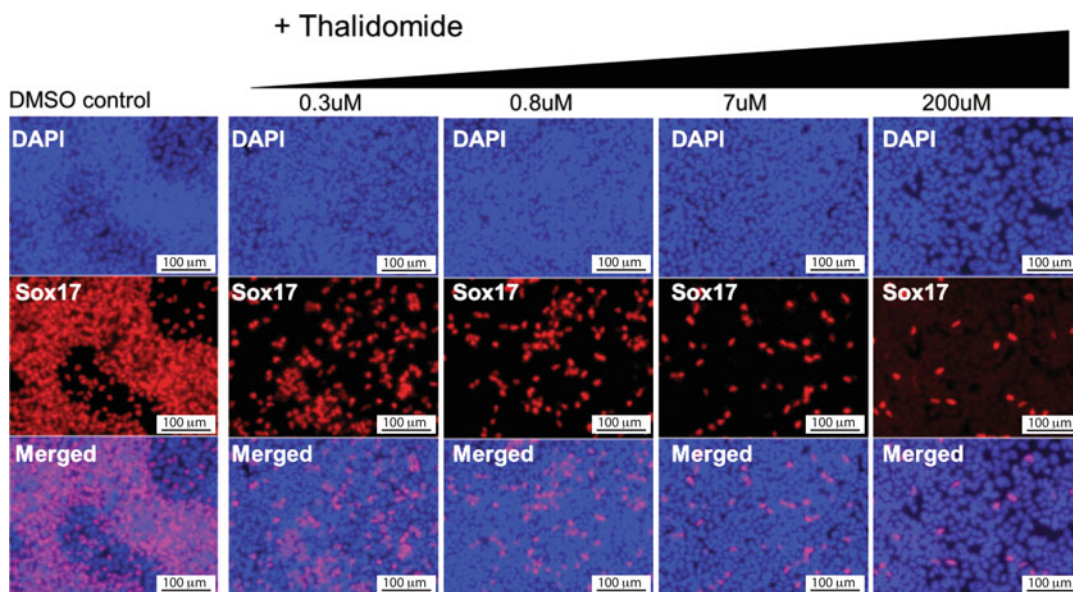


Fig. 3 Thalidomide dose response illustrating the reduction of SOX17-positive cells

toxic mechanism being very different from that of SB-431542 or retinoic acid. An example of a thalidomide dose response experiment is shown in Fig. 3.

Imatinib

This serves as a convenient reference compound for us, as it is commercially available and both SOX17-IC₅₀ and DAPI-IC₅₀ fall within the full dose range (3.1–100 μM).

5 Future Directions

The hPST described above is capable of correctly identifying human teratogens including thalidomide, which had not been identified using rodent systems. Furthermore, this assay holds the promise of being adapted to an HTS format that could assist in efforts to triage chemicals and rank order compounds during drug development. Adapting the assay to a higher density plate format is possible but would certainly require optimizing the plating density of the cells and the duration of the assay. In order to eliminate the requirement for immunostaining the cells, it may be possible to engineer a human pluripotent stem cell line to carry a reporter capable of measuring SOX17 nuclear localization. If validated, such a system may significantly increase the assay throughput.

Although the field clearly could benefit from novel in vitro human cellular systems, given the extraordinarily high stakes involved when making risk assessments of potential human

teratogens, new assays should be rigorously validated by independent laboratories. Driven largely by the breakthrough for creating human-induced pluripotent stem cells [22], the increased availability of human pluripotent stem cell lines and recent advances allowing routine growth of the cells have lowered the barrier researchers previously faced when considering initiating new human pluripotent stem cell studies in their labs. Hopefully, this will facilitate greater adaptation of this technology into the field of toxicology and the broader investigation and validation of human pluripotent stem cell-based assays such as the one described here.

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