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Human Pluripotent Stem Cell Based Developmental Toxicity Assays for Chemical Safety Screening and Systems Biology data generation

--Manuscript Draft--

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RE: Submission of the revised manuscript entitled:

Human Pluripotent Stem Cell Based Developmental Toxicity Assays for Chemical Safety Screening and Systems Biology data generation

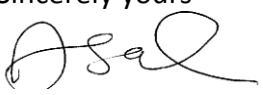
Dear Allisson,

Please find enclosed our manuscript.

We have responded all the comments of the reviewers accordingly.

Once again, thank you very much for invitation.

Sincerely yours



Agapios

TITLE:

Human Pluripotent Stem Cell Based Developmental Toxicity Assays for Chemical Safety Screening and Systems Biology data generation

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KEYWORDS:
Human embryonic stem cells, developmental toxicity, neurotoxicity, neuroectodermal progenitor cells, immunoprecipitation, differentiation, cytotoxicity, embryopathy, embryoid body

Short Abstract:
The protocols describe two *in vitro* developmental toxicity test systems (UKK and UKN1) based on human embryonic stem cells and transcriptome studies. The test systems predict human developmental toxicity hazard, and may contribute to reduce animal studies, costs and the time required for chemical safety testing.

ABSTRACT:
Efficient protocols to differentiate human pluripotent stem cells to various tissues in combination with –Omics technologies opened up new horizons for *in vitro* toxicity testing of potential drugs. To provide a solid scientific basis for such assays, it will be important to gain quantitative information on the time course of development and on the underlying regulatory mechanisms by systems biology approaches. Two assays have therefore been tuned here for these requirements. In the UKK test system, human embryonic stem cells (hESC) (or other pluripotent cells) are left to spontaneously differentiate for 14 days in embryoid bodies, to allow generation of cells of all three germ layers. This system recapitulates key steps of early human embryonic development, and it can predict human-specific early embryonic toxicity/teratogenicity, if cells are exposed to chemicals during differentiation. The UKN1 test system is based on hESC differentiating to a population of neuroectodermal progenitor (NEP) cells for 6 days. This system recapitulates early neural development and predicts early developmental neurotoxicity and epigenetic changes triggered by chemicals. Both systems, in combination with transcriptome microarray studies, are suitable for identifying toxicity biomarkers. Moreover, they may be used in combination to generate input data for systems biology analysis. These test systems have advantages over the traditional toxicological studies requiring large amounts of animals. The test systems may contribute to a reduction of the costs for drug development and chemical safety evaluation. Their combination sheds light especially on compounds that may influence neurodevelopment specifically.

INTRODUCTION:

The ability of human embryonic stem cells (hESC) to differentiate into various types of cells opened up a new era of *in vitro* toxicity testing[1], disease modelling and regenerative medicine[2]. The stem cells are endowed with the capacity to self-replicate, to keep their pluripotent state, and to differentiate into specialized cells[3,4]. The properties of hESC (capacity to differentiate to all major cell types) are also found in other human pluripotent stem cells, such as human induced pluripotent stem cells (hiPSC) or cells generated by nuclear transfer[5],. For instance, many different hESC lines have been differentiated into neurons[6], renal cells[7], neural crest cells[8], cardiomyocytes[9-12], or hepatocytes like cells[13,14]. Moreover, hESC can spontanously differentiate into cells of all three germ layers[15-18] in embryoid bodies (EBs)[19,20]. Early embryonic development is regulated by differential expression of various genes related to the different germ layers which has been captured at mRNA level by transcriptomics using microarray technology[15]. These efforts resulted in the establishment of organ specific toxicological models based on hESC/hiPSC and transcriptomics analysis (for review see [21,22]). These models have advantages over the traditional use of laboratory animals for toxicological studies, as preclinical studies using laboratory animals are not always predictive of human safety. The drug induced toxicities encountered in patients are often related to metabolic or signaling processes that differ between humans and experimental animals. The species difference has prevented the reliable early detection of developmental toxicity in humans, and for instance drugs such as thalidomide[23,24] and diethylstilbestrol[25,26] were withdrawn from the market due to teratogenicity. Thalidomide has not shown any developmental toxicity in rats or mice. Environmental chemicals such as methyl mercury[27] resulted in prenatal developmental toxicity with respect to the nervous system in various species, but human manifestations have been hard to model in animals. To address the problem of species specificity issues, scientists working under different projects based on stem cells like ReProTect, ESNATS, DETECTIVE etc. are engaged in the development of different models for embryonic toxicity, neurotoxicity, cardiotoxicity, hepatotoxicity and nephrotoxicity using human toxicants suspected to affect humans. Under the European consortium project ´Embryonic Stem cell-based Novel Alternative Testing Strategies (ESNATS)´ five test systems have been established. One test system the so called UKK (**U**niversitäts**k**linikum **K**öln) test system partially captures early human embryonic development. In this system human embryonic H9 cells are differentiated in to three germ layers (ectoderm, endoderm and mesoderm)[15] and germ layer specific signatures have been captured by transciptomics profile using the Affymetrix microarray platform. Various developmental toxicants like thalidomide[28], valproic acid, methyl mercury[16,17], or cytosine arabinoside[15] have been tested in this system, and toxicant specific gene signatures have been obtained. In a second test system, the so called the UKN1 (**U**niversity of **K**onsta**n**z) test system 1, H9 cells are differentiated to neuroectodermal progenitor cells (NEP) for 6 days. This is evidenced by high expression of neural gene markers such as *PAX6* and *OTX2*. During differentiation for 6 days, NEP cells have been exposed to developmental neuro-toxicants such as VPA, methyl mercury. Toxicant-specific de-regulated transcriptomics profiles have been obtained as well by using the Affymetrix microarray platform[16,29].

The new vision for toxicology of the 21st century envisages that test systems do not only yield phenotypic descriptions like histopathology *in vivo*, or transcriptome changes at the end of long-

term toxicant incubations. It rather suggests that assays provide mechanistic information[3], and that this information can be mapped to so-called adverse outcome pathways (AOP) that provide a scientific rationale for hazardous effects[30]. To provide such information, the test systems applied have to be highly quality controlled[31], as for instance documented by robust standard operation procedures. Moreover, time-dependent changes need to be mapped with high resolution. This requires test systems with synchronized changes[32]. The UKN1 and UKK test systems described here have been optimized for these requirements.

PROTOCOL:

The following protocol was performed using human Embryonic Stem Cell line (hESC) H9. This cell line was routinely cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) in hESC culture media supplemented with bFGF and then cultured in stem cell media on 6 cm petri-plates coated with basement membrane matrix such as matrigel, to get rid of MEFs. The H9 cells from >80% confluent plates were used for further passage. H9 cells cultured on basement membrane matrix plates were used for EBs formation. All procedures mentioned in the following protocol have been performed using standard methods for aseptic and good cell culture practices.

PART 1 - UKK test System:

1. Human Embryonic Stem Cell Culturing

1.1. Splitting and maintenance of H9 on feeder cells

1.1.1) Pipette 2ml 0.1% gelatin into each 6 cm plate and incubate for 30 min in cell culture incubator (37^0C and 5% CO_2). Aspirate gelatin solution with sterile pasteur pipette.

1.1.2) Add 2 ml MEF medium containing 0.1 x 10^6 MEF cells/ml into the two gelatin coated plates and incubate them in cell culture incubator (37^0C and 5% CO_2) overnight.

1.1.3) On next day, remove the H9 cells vial from the liquid nitrogen storage tank using forceps and thaw the vial in a 37^0C water bath using long forceps.

1.1.4) Remove the vial from water bath, bath it with 70% ethanol, air dry in the biosafety cabinet for 15 to 30 seconds and transfer the cells to 15 ml falcon tube.

1.1.5) Add 9 ml of H9 culture medium slowly on the inner wall and centrifuge the cells at 200 x g for 5 min.

1.1.6) Aspirate the supernatant and re-suspend the cells in 6 ml culture medium containing ROCK inhibitor (10µM, Y27632) and gently pipette to mix. Aspirate MEF medium from the 6 cm plate and add 3 ml cell suspension in each plate. Change the medium on day3 and then every other day. Subculture >80% confluent plate cells with split ratio 1:3.

Note: Usually in 5 to 7 days plate becomes confluent. Feeders used are obtained from CF1 mice

embryo and inactivated by exposure to γ radiation.

1.2. H9 cell culturing on basement membrane matrix coated plates

1.2.1) Thaw stem cell medium (5x) supplement at room temperature and add 100 ml into 400 ml basal medium in biosafety cabinet.

1.2.2) Thaw basement membrane matrix on ice. Add suggested volume of basement membrane matrix (refer certificate of analysis for each batch) in 24ml chilled DMEM/F-12 basal medium for 12 number of 6 cm plates. Mix by pipetting up and down.

1.2.3) Add 2 ml in each 6 cm plate. Keep the plate at room temperature for 1 hour. Remove the medium and add 2 ml of stem cell medium.

1.2.4) Take out four confluent H9 plates on MEFs. Remove the differentiated colonies with 1ml pipette tip under stereomicroscope kept in biosafety cabinet.

1.2.5) Aspirate the medium and wash the cells with 4 ml PBS and add 2 ml stem cell medium in each plate. Cut the undifferentiated colonies with 26 G needle in to 6 to 9 pieces each.

1.2.6) Gently collect the cells in 50 ml falcon tube. Centrifuge at 200 x g for 5 min.

1.2.7) Aspirate the supernatant and re-suspend in 12 ml stem cell medium. Count the clumps by putting 20 µl on glass slide under the microscope and adjust the volume for 150 clumps per ml. Add 2ml of suspension in each 6 cm plate.

1.2.8) Move the plates back-and-forth and side –to-side motions for uniform clump distribution and incubate the plates in cell culture incubator (37^0C and 5% CO_2).

1.2.9) Remove the differentiated colonies and give medium change every alternate day.

2. Embryoid Bodies (EBs) formation

Perform all procedure mentioned below as per aseptic precautions and in the biosafety cabinet.

2.1. Day 0 – Plating of H9 cells on V bottom plates

2.1.1) Prepare 5% block copolymer such as Pluronic F 127 in PBS and filter through vacuum driven filtration system using 0.22 µm sterile filter.

2.1.2) Coat V bottom 96 well plates with 40 µl of 5% block copolymer per well and incubate at room temperature for 45 min.

2.1.3) Remove the confluent basement membrane matrix plates with H9 cells from incubator and remove the differentiated colonies with 1 ml pipette tip under stereomicroscope in biosafety cabinet.

2.1.4) Aspirate the medium and wash the cells with 4 ml PBS. Add 2 ml random differentiation medium (H9 culture medium without bFGF, RD medium) in each plate. Use passage tool and cut the H9 cell colonies in clumps of uniform size and shape by observing under stereomicroscope in biosafety cabinet and then gently scrape with the cell scraper.

2.1.5) Collect the clumps in 50 ml falcon tube and centrifuge at 200 x g for 5 min. Aspirate the supernatant and re-suspend the cell in RD medium to get 1000 clumps per ml.

2.1.6) Aspirate the block copolymer from V bottom plates. Pour the clumps in sterile square plate and with help of multichannel pipette add 100 µl of suspension to each well of v bottom plate.

2.1.7) For the force aggregation of clumps, centrifuge the v bottom plates at 4^0C for 4 min at 400 x g. Incubate plates in cell culture incubator (37^0C and 5% CO_2) for four days.

2.2. Day 4- Collection of EBs

2.2.1) Collect the EBs in the sterile square plate from v bottom plates using multichannel pipette and wide bore 200µl tips.

2.2.2) Collect the EBs from sterile square plate in to 15 ml falcon tube with 10 ml sterile serological pipette. Allow EBs to settle for 2 min. Aspirate the supernatant and wash the EBs with 5 ml PBS.

2.2.3) Allow EBs to settle for 2 min and aspirate the supernatant. Re-suspend EBs in 5 ml RD medium.

2.2.4) Pipette out 10ml RD medium in 10 cm bacteriological plates. Transfer the EBs in 10 cm bacteriological plates.

2.2.5) Incubate bacteriological plates on horizontal shaker (reciprocation motion 50/ min) kept in cell culture incubator (37^0C and 5% CO_2) for required time period. Give medium change (15 ml RD medium) every alternate day.

Note: Gentle handling is required while culturing hESCs. The size of EBs varies on day 4. Select the uniform size EBs (± 20%) by observing under stereomicroscope for further experiment. Approximately 50% EBs formed with this method are of uniform in size. The transfer of EBs on shaker results in uniform shape.

3. Cytotoxicity Assay for IC_{10} determination

3.1. Transfer of EBs on optical bottom plates

3.1.1) Thaw 0.1% gelatin in water bath at 37^oC for 15 min and coat optical bottom plates with 50µl of 0.1% gelatin per well using multichannel pipette. Incubate the plates at room temperature for 45 min. After incubation aspirate the gelatin from optical bottom plates.

3.1.2) Take out the EBs collected on day 4 in 10 cm bacteriological plate containing RD medium.

3.1.3) Keep optical bottom plates in slanted position in biosafety cabinet. Transfer two uniform size of EBs in 100µl RD medium per well in optical bottom 96 well plate by observing under stereomicroscope. Keep 12^{th} column empty.

3.1.4) Incubate plates in cell culture incubator (37^0C and 5% CO_2) for 24 hrs.

3.2. Drug exposure from day 5 to day 14

3.2.1) Weigh the test compound and make highest concentration in known solvent.

3.2.2) Perform half-logarithmic dilution of the test compound serially till 8 dilutions in the solvent containing falcon tubes numbered with A to H, Keep tube no. I as vehicle control, tube no. J as negative control (RD medium) and tube no. K as positive (70% ethanol) control.

3.2.3) Thaw the RD medium in water bath at 37^0C for 15 min. Take out 5 ml RD medium each in 11 sterile falcon tubes labelled from 1 to 11.

3.2.4) Transfer 5µl of solution from tube A to tube K in to tube 1 to 11 respectively and vortex the tubes. Take out the optical bottom plate from the incubator and carefully remove the media with use of multichannel pipette.

3.2.5) Add 200 µl of media from tube number 1 to 11 into the respective columns of the optical bottom plate. Give medium/ drug change every alternate day.

Note: For half-logarithmic dilutions take 6.48 µl solvent in 7 tubes labeled from 2 to 8. From highest concentration tube no.1 transfer 3 µl to tube no.2, vortex and serially transfer 3µl to next tube. Keep tube no.9 for vehicle control and tube no.10 for negative control. Tube no. 11 is 70% ethanol.

3.3. Day 14 : Resazurin exposure and fluorescence measurement

3.3.1) Thaw RD medium in water bath at 37^0C for 15 min. Perform all procedure mention below in absence of light in the biosafety cabinet.

3.3.2) Take 10 ml RD medium in 15 ml tube (A) and add recommended volume of resazurin reagent and mix by pipetting. Take out the optical bottom plate from the incubator and carefully remove all medium with multichannel pipette.

3.3.3) Add 100 µl of medium from tube A in each well. Incubate the plate in cell culture incubator (37^0C and 5% CO_2) for 90 mins.

3.3.4) Measure the fluorescence using spectrophotometer ($560_{Ex}/590_{Em}$).

3.4. IC_{10} value determination

3.4.1) Import the values in graph pad prism after subtracting the blank values. Set x axis as a dose and y- axis as a fluorescence units.

3.4.2) Normalize the values to obtain percentage on y axis and transform the values (x- axis as log scale). Calculate IC_{50} value by using sigmoidal-dose response (variable slope) parameter. Calculate log IC_{10} values by using following equation Equation

F=10 logEC_{50}=logECF - (1/HillSlope)*log(F/(100-F))

Y=Bottom + (Top-Bottom)/(1+10^((LogEC_{50}-X)*HillSlope))

3.4.3) Determine the IC_{10} values to be taken for further studies.

4. Biomarker study based on microarrays

4.1. Day 0 to day 5:

4.1.1) Embryoid body formation and transfer to 10 cm bacteriological plates-Follow the steps mentioned in point 2 for embryoid body formation.

Note: Use three biological replicates for each study. Divide each biological replicate in to two parts – Drug treatment at IC_{10} concentration and vehicle control. Prepare drug concentration 10000 fold above the IC_{10} conc. in vehicle and from this add 10 µl to 100 ml RD medium with H9 cell clumps in 50 ml Eppendorf tube mix well and seed it on V bottom plates. Follow the same procedure for vehicle control group.

4.2. For drug exposure on Day 5 to 14, collect the EB´s and transfer them in 10 cm bacteriological plates on day 4 as per the steps mentioned in point 2. Transfer the plates on horizontal shaker (reciprocation motion 50/ min) in cell culture incubator (37^0C and 5% CO_2) for 14 days. Give medium change every alternate day.

4.3. For sample collection, on day 14, collect the EBs from 10 cm plates in to 15 ml falcon tube with sterile serological pipette. Allow EBs to settle for 2 min. Aspirate the supernatant and wash the EBs with 5 ml PBS. Allow EBs to settle for 2 min and aspirate the supernatant. Re-suspend EBs in 1 ml RNAlater solution or TRIzol reagent, vortex and store the sample at -80^0C till further processing.

Note: Perform all procedure in biosafety cabinet as per good laboratory practices. Rotate the plates in circular motion around the center to bring all EBs in center, aspirate the medium from surrounding with the help of sterile glass pasture pipette, add 15 ml RD medium and then add 15µl of drug / vehicle for respective group.

5. RNA Isolation and integrity testing

5.1. RNA Isolation:

Most of the steps mentioned below are to be performed for RNA purification using RNeasy Mini Kit as per the instruction manual. Always use nuclease free tubes, pipette tips and water. While working with TRIzol carry out all procedure in chemical safety hood and wear protective glasses as well as chemical protective gloves.

5.1.1) Thaw the samples on ice. If samples are stored in RNAlater solution, centrifuge the tubes at 12000 x g for 5 min at 4^0C. Discard the supernatant and add 1 ml TRIzol reagent.

5.1.2) Triturate the samples using 24 G needle and 1 ml syringe. Approximately 15 times trituration is sufficient for disruption of EBs, cell wall and plasma membranes.

5.1.3) Add 200 µl of chloroform in each sample. Vortex to mix the contents uniformly. Centrifuge at 12000 x g for 15 min at 4^0C. Remove the RNeasy mini spin columns, 1.5ml tubes and label them properly.

5.1.4) Collect the supernatant in 1.5 ml tubes (While collecting supernatant do not disturb the middle or bottom layer). Add equal volume of chilled 70% ethanol. Mix the contents by gentle shaking.

5.1.5) Apply 700 µl from the tubes to respective mini spin columns and centrifuge them at 12000 x g for 20 seconds at room temperature. Perform all further steps at room temperature.

5.1.6) Discard the filtrate and apply remaining solution to the respective columns and centrifuge them at 12000 x g for 20 seconds. Discard the filtrate.

5.1.7) Apply 350 µl of RW1 buffer to the column and centrifuge them at 12000 x g for 20 seconds. Discard the filtrate and apply 10 µl of DNAse and 70 µl RDD buffer to the column.

5.1.8) Incubate at room temperature for 15 min. Apply 350 µl of RW1 buffer to the column and centrifuge them at 12000 x g for 20 seconds. Discard the filtrate. Apply 500 µl of RPE wash buffer to the column and centrifuge them at 12000 x g for 20 seconds. Discard the filtrate. Again Apply 500 µl of RPE wash buffer to the column and centrifuge them at 12000 x g for 2 min. Discard the filtrate.

5.1.9) Shift the columns to new 2 ml collection tubes and centrifuge them at 12000 x g for 1 min. Transfer the columns to labelled 1.5 ml collection tube and apply 22 µl of nuclease free water. Centrifuge the tubes at 12000 x g for 1 min.

5.1.10) Remove the collection tube and put them on ice. Quantify RNA using automated electrophoresis system.

5.2. RNA concentration, purity and integrity testing

For RNA purity and integrity testing use automated electrophoresis system and respective kit[33].

6. Microarray studies

6.1. Perform transcriptional profiling using commercial available Human array chips. For RNA target preparation, fragmentation, hybridization[34] and array chip staining, washing[35] use commercial available kits.

6.2. Perform array chip scanning and quality control check by using standard fluidics station, array scanners and standard operating softwares[36]. For gene expression analysis import the files generated from scanners to the standard commercial available software[37], perform background correction, summarization and normalization with Robust Multi-array Analysis (RMA).

6.3. For obtaining list of differentially expressed genes (DEG´s) perform one way ANOVA analysis. From this list filter out the genes based on the fold change (± 2) and FDR- controlled p-value (< 0.05). Obtain the Principal Component Analysis (PCA), Heat Map, etc. using this software.

PART 2 - UKN 1 Test System:

1. Maintenance of hESC

1.1. Seeding of MEFs

1.1.1) For the differentiation use the NSCB#8534 (H9) cell line. Culture cells on mouse embryonic fibroblasts (MEFs) as feeder cells. Coat T25 flask with 4 ml of 0.1% gelatin and incubate for 30 min at 37°C.

1.1.2) **Thaw** MEFs in 37°C water bath and transfer the cells into pre-warmed DMEM/10%FBS.

1.1.3) Spin 3.5 min with 500 x g, remove supernatant and re-suspend cells to obtain 1 x 10^7 cells/ml. Plate MEFs 4 x $10^4/cm^2$ in T25 flasks on gelatin. Optionally, use the MEFs for the next two days. Quality of MEF batches are a very critical issue for hESC maintenance. Therefore it is advisable to elucidate the best company and preparation method for the H9 cells. We use PMEF P3.

1.2. Splitting and maintenance of H9

1.2.1) Add 1 ml pre-warmed dispase per T25 flask H9 and incubate 9 min at 37°C.

1.2.2) Add 2 ml wash medium to dispase treated cells and pipet 5 times up and down with 5 ml pipet and transfer cell solution to a falcon tube.

1.2.3) Wash the flask with 9 ml wash medium and add cells to the others. Spin 3.5 min with 500 x g, remove supernatant and re-suspend cells in 10 ml hESC medium.

1.2.4) Spin 3.5 min with 500 x g, remove supernatant and re-suspend cells in 4 ml hESC medium. Add 0.5 ml cell suspension and 4.5 ml hESC medium and plate in a new (PBS washed) T25 flask with MEFs. Change entire hESC medium (5 ml) of the flask every day.

2. Differentiation of hESC towards neuroectodermal progenitor cells (NEP)

2.1. Prepare hESC medium and KCM medium. Coat one 10 cm dish with gelatine (0.1% in PBS) per T25 flask and incubate for 30 min at 37°C. Remove medium from hESC and add enough accutase to cover the whole bottom of the flask (1 ml per T25 flask) and incubate 25 to 30 min at 37°C.

2.2. Prepare basement membrane matrix coated plates during accutase incubation. Add cold DMEM/F12 to frozen basement membrane matrix pellet and resolve it 1:20. Filter basement membrane matrix solution through a 40 µm cell strainer. Add filtered solution to plate, the whole bottom has to be covered (1 ml per 6-well is required) and incubate for 2 h at room temperature.

2.3. After incubation period remove the basement membrane matrix supernatant and seed cells on the coated wells. After accutase step (2.1) stop reaction by addition of 1.5 ml HES medium. Scrape cells from the flask, add 8 ml hESC medium and produce a single cell solution by pipetting with 10 ml pipet thoroughly. Filter cells through a 40 µm cell strainer.

2.4. Spin cells 3 min with 500 x g, remove supernatant and re-suspend cells in 10 ml of hESC. Spin cells again 3 min with 500 x g, remove supernatant and re-suspend cells in hESC containing ROCK inhibitor Y-27632 at a final concentration of 10 µM.

2.5. Remove supernatant of gelatin coated dish. Plate cell suspension on gelatin coated dish to remove the MEFs and leave in the incubator for exactly 1 h.

NOTE: During this step the MEFs will settle onto the gelatine coated plate, whereas the hESC cannot attach to gelatin. Therefore this is crucial to obtain a feeder-free differentiation. It is a critical step as too long incubation results in hESC clumps and too short incubation in unefficient removal of MEFs. After 45 min of incubation the plate should be investigated for already setteled MEFs and hESC clumps.

2.6. When the MEFs have attached, gently wash non-adherent cells (hESC) off after incubation with the medium already in the plate. If several T25 were used to get more cells, single cells now can be combined. Wash plate once with hESC medium.

2.7. Spin cells 3 min with 500 x g, remove supernatant and re-suspend cells in approximately 4 ml KCM containing 10 µM ROCK inhibitor Y-27632 and 10 ng/ml FGF-2.

2.8. Count cells in a hemocytometer using Trypan blue. Plate 18 x 10^3 cells/cm^2 on basement membrane matrix coated plates in KCM containing 10 µM ROCK inhibitor Y-27632 and 10 ng/ml FGF-2 (for 6-well use 1.5 ml per well). It is crucial to plate the cells in the right density to differentiate them successfully into NEPs.

2.9. After 24h change medium to fresh KCM containing 10 µM ROCK inhibitor Y-27632 and 10 ng/ml FGF-2. After further 24h change medium to fresh KCM containing 10 ng/ml FGF2.

2.10. 72h after seeding the cells, differentiation starts by medium change towards KSR medium. This time point is referred to as day of differentiation 0 (DoD0). The addition of test substances is possible now.

2.11. On DoD1 and DoD2 the medium is changed exactly as on DoD0. Next medium change is at DoD4 containing 25% N2-S and 75% KSR. At DoD6 the differentiation is stopped and cells are harvested for analysis

3. Chromatin Immunoprecipitation (ChIP) of hESC and NEP

3.1. Preparation of nuclei

3.1.1) Add 500 µl accutase to each 6 well which should be analyzed and incubate for 25 to 30 min. Count cells in a Neubauer chamber using Trypan blue.

3.1.2) Resuspend cells in 1% formaldehyde in DMEM/F12 for crosslink. Add Tris pH7.5 to a final concentration of 125 mM after 10 mins to stop the crosslink.

3.1.3) Spin cells 3 min with 500 x g at 4°C, remove supernatant and re-suspend cells in cold PBS.

3.1.4) Spin cells 3 min with 500 x g at 4°C, remove supernatant and re-suspend cells in 1 ml L1-buffer/ 1 x 10^6 cells.

3.1.5) Incubate for 5 min on ice. Spin 5 min with 800 x g at 4°C, remove supernatant and re-suspend nuclei in 1 ml L2- buffer/ 2 x 10^6 cells.

3.2. Sonication and quality control

3.2.1) Sonicate so that DNA fragments of 300 – 700 bp length are generated. Spin 1 min with 10000 x g at 4°C. Transfer supernatant to a new tube. The fragments need to have the correct size, otherwise the immunoprecipitation will be inefficient as well as the followed qPCR.

3.2.2) Remove 50 µl and mix with 50 µl L2 buffer to check efficiency of sonication by running an agarose gel.

3.2.3) Reverse crosslink by incubation at 65°C for 4 h and 500 rpm. Load samples 1:5 with Orange G loading dye on a 1.5% agarose gel and run 45 min at 110V in 1x TBE buffer. Control fragment size (should be between 300 – 700 bp).

3.3. Chromatin Immunoprecipitation

3.3.1) Dilute samples 1:5 in dilution buffer and aliquot 1 ml per IP in siliconised tubes.

3.3.2) Remove 5% (volume) from diluted chromatin sample (step 3.3.1) and store at 4°C as “input”.

3.3.3) Incubate samples with antibodies of your choice and with unspecific IgG over night at 4°C

on a rotator.

3.3.4) Add 50 µl Protein-A/G sepharose beads to each sample after immunoprecipitation. Incubate samples 3h at 4°C on a rotator. Spin 1 min with 1500 x g at 4° C and remove supernatant.

3.3.5) Wash with 1 ml washing beads. Spin 1 min with 1500 x g at 4°C and remove supernatant. Repeat step g to h. Wash with 1 ml final washing buffer. During the washing steps you should not lose any of the beads, because this alters the amount of eluate directly.

3.3.6) Centrifuge 1 min with 1500 x g at 4°C and remove supernatant. Add 125 µl elution buffer and incubate 15 min with 65°C at 1000 rpm on a shaker.

3.3.7) Spin 1 min with 1500 x g and transfer supernatant to a new tube Repeat step k and l. Add 200 µl elution buffer to input (3.3.2). Add Proteinase K and RNase to each sample and incubate 30 min with 37°C at 500 rpm on a shaker and afterwards 4 h with 65°C at 500 rpm on a shaker.

NOTE: For DNA extraction use commercial available ChIP DNA Clean and Concentrator Kit[38].

REPRESENTATIVE RESULTS:

Methyl mercury exposure in UKK test system:

The cytotoxicity assay was performed with H9 EBs to obtain an IC_{10} value (reduction of viability by 10%) for the cytotoxicityof methyl mercury (Figure 1). We also performed a microarray based (affymetrix platform) biomarker study. The H9 EBs have been exposed to methyl mercury (0.25 and 1 µM) for 14 days. On day 14, samples have been collected using TRIzol and RNA was isolated. Transcriptional profiling was performed using Human Genome U133 plus 2.0 array chips. The data have been analyzed with Partek Genomic Suite™ 6.6. First data overview was obtained by Principle Component Analysis (Figure 2A), generation of Venn diagrams (Figure 2B) and construction of heat maps (Figure 2C). The principle component analysis represents the overall distribution of gene expression and it clearly visualized segregation of MeHg 1 µM from the vehicle control and MeHg 0.25 µM groups (PC # 25.2) (Figure 2A). A list of differentially-expressed genes (DEG) was obtained after statistical treatment (one-way ANOVA) and filtering of the data using a fold change cut-off of ± 2 and a multiplicity-corrected (Benjamini-Hochberg method) p-value <0.05 (Table 1). The 1 µM MeHg treatment resulted in 276 DEGs and 0.25 µM in 31 DEGs (Figure 2B). The heat map showed that MeHg 1 µM treatment mainly reduced gene expression (Figure 2C). Information on overrepresented gene ontology terms was obtained by using the DAVID bioinformatics tool. Table 2 represents the significantly overrepresented GO gene categories that contained more than 5 genes. The down-regulated transcription factors related to the nervous system development were identified. *SEPP1, DDIT4, AK4, FRZB* (brain development), *PITX* (neural nucleus development) and *ERBB3, UGT8, APOB, APOA1* (nervous system development) were down-regulated in a dose dependent manner for methyl mercury treatment (Table 3).

UKN 1 test system:

This differentiation protocol uses dual SMAD inhibiton[6] to generate a pure population of NEP within six days of differentiation. The resultant cells are characterized by an up-regulation of the neural precursor genes *PAX6* and *OTX2*. The stem cell markers *OCT4* and *Nanog* are down regulated during the differentiation towards NEP (Figure 3A). Due to the highly synchronous and homogenous differentiation, it is also possible to get information on the histone modifications during this early stage of development. We adapted the protocol for chromatin immuno-precipitation (ChIP) using the cells either at the beginning of differentiation or after 6 days of differentiation. A switch of methylation sites on the promoter regions of *PAX6* and *OTX2* was evident from these studies (Figure 3B). The investigated methylation sites histone 3 lysine 4 trimethylation (H3K4me3) and histone 3 lysine 27 trimethylation (H3K27me3) were highly dynamic during the differentiation. Also on protein level a down regulation of *Oct4* could be observed (Figure 4). The up-regulation of *Pax6* and the neural stem cell marker *Nestin* was observed by immunofluorescence microscopy on protein level (Figure 4). The cell population showed a homogeneous and pure differentiation after six days of differentiation. Therefore the cultures can be easily used for analysis of RNA and protein. The system provides also the possibility to test substances and the effect they have on early neural development [16,29].

FIGURE LEGENDS:

Figure 1: Cytotoxicity Assay (H9 differentiation) for MeHg.

The assay has been performed as per the protocol to define the IC_{10} value for methyl mercury.

Figure 2: Representative analysis of the differential expressed genes induced by 0.25 and 1 µM MeHg after application of the UKK test system.

The hESCs were treated with 0.25 and 1 µM MeHg according to the UKK test system. Analysis of the differential expressed transcripts in 14-days differentiated EBs has been performed using the Partek Genomic Suite™ 6.6 software. (A) Principal component analysis (3-Dimenional) of the microarray data. (B) Venn diagram obtained from microarray analysis of gene expression. The diagram shows the number of genes modulated by the MeHg treatment (fold change > ± 2, p value < 0.05). (C) Hierarchical clustering of the gene expression data (fold change > ± 2, p value < 0.05). The highly expressed genes in vehicle control group are repressed by 1 µM MeHg treatment. The 1 µM MeHg treatment resulted in 233 transcripts with lower expression and 43 probes with higher expression as compare to vehicle control group.

Figure 3: Gene expression and histone methylation pattern during differentiation from hESC towards NEP.

For all experiments, hESC were differentiated to neuroectodermal precursor cells (NEP). (A) Samples were taken at day 6 of differentiation, and transcript levels of marker genes of neural differentiation were determined by RT-qPCR. Data (gene expression relative to hESC) are means ± SEM of 5 experiments. (B) Samples for chromatin immunoprecipitation (ChIP) were prepared at day 6 of differentiation. ChIP was performed with antibodies specific for H3K4me3 or H3K27me3 or control IgG. The enrichment factors of promoter sequences are given as % input for H3K4me3 (grey) and H3K27me3 (black). Data are means ± SEM of 3 independent cell preparations.

Figure 4: Protein expression during differentiation from hESC towards NEP.
Cells were fixed and stained for the stem cell marker *Oct4* (green) at day 0 of differentiation (DoD0) and for NEP markers *Pax6* (red) and *Nestin* (green) at day 6 of differentiation (DoD6). Scale bar indicates 50 µm.

Table 1: List of differentially expressed genes (> ± 2 fold, p value <0.05) of MeHg treatment versus vehicle control in 14 day old EBs.

Table 2: List of significantly enriched and selected GO categories (p value <0.05, > 5 genes) with dysregulated transcripts for MeHg versus vehicle control in 14 day old EBs.

Table 3: List of significantly down-regulated transcripts related to the developmental nervous system with MeHg treatment in 14 day old EBs.

Table 4: Composition of culture media.

DISCUSSION:
Traditional approaches to toxicological testing involve extensive animal studies thus making testing costly and time-consuming. Moreover, due to the interspecies differences the preclinical animal safety studies are not always valid to predict toxicity effects of potential drugs relevant for humans. Although non-human primates are most predictable, still strong ethical, and socioeconomical demands are rapidly raising by modern societies for developing sensitive and robust *in vitro* test system relevant to human safety.

The unique ability of hESCs to differentiate into all somatic cell types, therefore recapitulating in vivo human developmental processes in the combination with sensitive toxicogenomics approaches has been proposed as an alternative to the traditional approaches for drug safety testing[6,21]. Under the ´ESNATS´ project the ´UKK´ test system has been developed to predict the developmental embryonic toxicity based on transcriptomics profiling. In this system hESC have been differentiated in to the embryoid bodies for 14 days. The time kinetic transcriptomics profile obtained shows high expression of differentiation marker specific to the three germ layers ectoderm, endoderm and mesoderm on day 14 which partially recapitulate early human embryonic development. Based on these results, known teratogenic drugs have been exposed during differentiation for 14 days and differential expressed gene profile have been obtained. Impressively, gene signatures associated with the teratogenic effects of thalidomide observed in humans, could be predicted by this test system[28]. The representative results for methyl mercury in UKK system show concentration-dependent down regulation of the transcription factors related to the nervous system development. The other developmental neuro-toxicants were also tested in this system and efforts are going on to identify the common toxico-markers across the compounds at mRNA level and validate them at the protein level. The UKK test protocol provided here gives basic guideline for conducting the experiment with human embryonic stem cell H9 to identify the transcriptomic signature for developmental toxicant.

The optimised standard operation procedure (SOP) for differentiation of pluripotent stem cells according to the UKN1 protocol allows a robust and synchronized differentiation of hESC to NEP. Already after six days of differentiation, a homogeneous cell population with high PAX6 expression levels is generated. The cells grow in adherent cultures, which allow analysis by immunostaining. Immunocytochemical analysis with high resolution and by confocal microscopy requires that cells are grown on thin glass surfaces. This is possible for these cultures if the glass is coated optimally, but it needs to be mentioned that the cells grow very dense, in more than one single layer after six days. Therefore, routine analysis of lineage-specific markers is more easily performed by RT-qPCR, ChIP or western blot. A big advantage for the biochemical analysis of the cultures is the high yield of cells which can be achieved by this differentiation protocol from a small starting population of hESC. One drawback of this protocol is the high cost of the medium supplements (e.g. noggin) required to force the homogeneous neural differentiation. Another drawback for some applications may be that some small molecules (kinase inhibitors) need to be present in the culture medium as part of the protocol. Thus, certain signal pathways cannot be examined toxicologically, as the change of the culture conditions also changes the differentiation[29].

The advantage of test system combination is the better understanding of DNT. Whereas UKK covers a broader range and adverse effect on early germ layer formation can be investigated, UKN1 allows to investigate more neural-specific mechanisms such as epigenetics. Although the two culture systems presented here have been shown to predict developmental neurotoxicity for few model toxicants[16], there is still a need for higher throughput versions of the protocols that allow screening of a large number of potential developmental neurotoxicants. Moreover, more work is required to identify and validate common markers of toxicity at the mRNA or protein level, and to establish them as a part of preclinical drug safety evaluation.

More than 20 billion US dollars per year are invested by the pharmaceutical industries for drug discovery[39]. As a proof of concept, we have developed *in vitro* toxicity test systems based on hESC and transcriptomics that may be suitable to predict human relevant toxicity effects of potential drug compounds in a cost-effective and less-time consuming manner.

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DISCLOSURES:
The authors have nothing to disclose.

REFERENCE:

1. Liu, W.W., Deng, Y.G., Liu, Y., Gong, W.R., & Deng, W.B. Stem Cell Models for Drug Discovery and Toxicology Studies. *Journal of Biochemical and Molecular Toxicology.* **27** (1), 17-27, doi:10.1002/jbt.21470, (2013).

2. Zuba-Surma, E.K., Jozkowicz, A., & Dulak, J. Stem Cells in Pharmaceutical Biotechnology. *Current Pharmaceutical Biotechnology.* **12** (11), 1760-1773, (2011).

3. Leist, M., Hartung, T., & Nicotera, P. The dawning of a new age of toxicology. *ALTEX.* **25** (2), 103-114, (2008).

4. Kuegler, P.B. *et al.* Markers of murine embryonic and neural stem cells, neurons and astrocytes: reference points for developmental neurotoxicity testing. *ALTEX.* **27** (1), 17-42, (2010).

5. Yamada, M. *et al.* Human oocytes reprogram adult somatic nuclei of a type 1 diabetic to diploid pluripotent stem cells. *Nature.* doi:nature13287 [pii];10.1038/nature13287 [doi], (2014).

6. Chambers, S.M. *et al.* Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nature Biotechnology.* **27** (3), 275-280, doi:10.1038/nbt.1529, (2009).

7. Takasato, M. *et al.* Directing human embryonic stem cell differentiation towards a renal lineage generates a self-organizing kidney. *Nature cell biology.* **16** (1), 118-126, doi:Letter, (2014).

8. Zimmer, B. *et al.* Evaluation of Developmental Toxicants and Signaling Pathways in a Functional Test Based on the Migration of Human Neural Crest Cells. *Environmental Health Perspectives.* **120** (8), 1116-1122, doi:10.1289/ehp.1104489, (2012).

9. Bosman, A. *et al.* Molecular and Functional Evidence of HCN4 and Caveolin-3 Interaction During Cardiomyocyte Differentiation from Human Embryonic Stem Cells. *Stem Cells and Development.* **22** (11), 1717-1727, doi:10.1089/scd.2012.0247, (2013).

10. Sartiani, L. *et al.* Developmental changes in cardiomyocytes differentiated from human embryonic stem cells: A molecular and electrophysiological approach. *Stem Cells.* **25** (5), 1136-1144, doi:10.1634/stemcells.2006.0466, (2007).

11. Xu, X.Q. *et al.* Chemically defined medium supporting cardiomyocyte differentiation of human embryonic stem cells. *Differentiation.* **76** (9), 958-970, doi:10.1111/j.1432-0436.2008.00284.x, (2008).

12. Pal, R., Mamidi, M.K., Das, A.K., & Bhonde, R. Comparative analysis of cardiomyocyte differentiation from human embryonic stem cells under 3-D and 2-D culture conditions. *Journal of Bioscience and Bioengineering.* **115** (2), 200-206, doi:10.1016/j.jbiosc.2012.08.018, (2013).

13. Subramanian, K. *et al.* Spheroid Culture for Enhanced Differentiation of Human Embryonic Stem Cells to Hepatocyte-Like Cells. *Stem Cells and Development.* **23** (2), 124-131, doi:10.1089/scd.2013.0097, (2014).

14. Sivertsson, L., Synnergren, J., Jensen, J., Bjorquist, P., & Ingelman-Sundberg, M. Hepatic Differentiation and Maturation of Human Embryonic Stem Cells Cultured in a Perfused Three-Dimensional Bioreactor. *Stem Cells and Development.* **22** (4), 581-594, doi:10.1089/scd.2012.0202, (2013).

15. Jagtap, S. *et al.* Cytosine arabinoside induces ectoderm and inhibits mesoderm expression in human embryonic stem cells during multilineage differentiation. *British Journal of Pharmacology.* **162** (8), 1743-1756, doi:10.1111/j.1476-5381.2010.01197.x, (2011).

16. Krug, A.K. *et al.* Human embryonic stem cell-derived test systems for developmental neurotoxicity: a transcriptomics approach. *Archives of Toxicology.* **87** (1), 123-143, doi:10.1007/s00204-012-0967-3, (2013).

17. Leist, M. *et al.* Test systems of developmental toxicity: state-of-the art and future perspectives. *Archives of Toxicology.* **87** (12), 2037-2042, doi:10.1007/s00204-013-1154-x, (2013).

18. Itskovitz-Eldor, J. *et al.* Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Molecular medicine.* **6** (2), 88-95, (2000).

19. Khoo, M.L.M. *et al.* Growth and differentiation of embryoid bodies derived from human embryonic stem cells: Effect of glucose and basic fibroblast growth factor. *Biology of Reproduction.* **73** (6), 1147-1156, doi:10.1095/biolreprod.104.036673, (2005).

20. Son, M.Y., Kim, H.J., Kim, M.J., & Cho, S. Physical Passaging of Embryoid Bodies Generated from Human Pluripotent Stem Cells. *Plos One.* **6** (5), doi:10.1371/journal.pone.0019134, (2011).

21. Winkler, J., Sotiriadou, I., Chen, S., Hescheler, J., & Sachinidis, A. The potential of embryonic stem cells combined with -omics technologies as model systems for toxicology. *Current medicinal chemistry.* **16** (36), 4814-4827, (2009).

22. Gunaseeli, I., Doss, M.X., Antzelevitch, C., Hescheler, J., & Sachinidis, A. Induced pluripotent stem cells as a model for accelerated patient- and disease-specific drug discovery. *Current medicinal chemistry.* **17** (8), 759-766, doi:BSP/CMC/E-Pub/ 047 [pii], (2010).

23. Miller, M.T. & Stromland, K. Teratogen update: Thalidomide: A review, with a focus on ocular findings and new potential uses. *Teratology.* **60** (5), 306-321, doi:10.1002/(SICI)1096-9926(199911)60:5<306::AID-TERA11>3.0.CO;2-Y, (1999).

24. Newman, C.G.H. Teratogen Update - Clinical Aspects of Thalidomide Embryopathy - A Continuing Preoccupation. *Teratology.* **32** (1), 133-144, doi:10.1002/tera.1420320118, (1985).

25. Stern, L. In vivo assessment of the teratogenic potential of drugs in humans. *Obstetrics and gynecology.* **58** (5 Suppl), 3S-8S, (1981).

26. Lynch, H.T. & Reich, J.W. Diethylstilbestrol, Genetics, Teratogenesis, and Tumor Spectrum in Humans. *Medical Hypotheses.* **16** (3), 315-332, doi:10.1016/0306-9877(85)90014-3, (1985).

27. Satoh, H. Behavioral teratology of mercury and its compounds. *Tohoku Journal of Experimental Medicine.* **201** (1), 1-9, doi:10.1620/tjem.201.1, (2003).

28. Meganathan, K. *et al.* Identification of Thalidomide-Specific Transcriptomics and Proteomics Signatures during Differentiation of Human Embryonic Stem Cells. *Plos One.* **7** (8), doi:10.1371/journal.pone.0044228, (2012).

29. Balmer, N.V. *et al.* Epigenetic changes and disturbed neural development in a human embryonic stem cell-based model relating to the fetal valproate syndrome. *Human Molecular Genetics.* **21** (18), 4104-4114, doi:10.1093/hmg/dds239, (2012).

30. Smirnova, L., Hogberg, H.T., Leist, M., & Hartung, T. Developmental neurotoxicity - Challenges in the 21st Century and In Vitro Opportunities. *ALTEX.* **31** (2), 129-156, doi:http://dx.doi.org/10.14573/altex.1403271 [doi], (2014).

31. Leist, M., Efremova, L., & Karreman, C. Food for thought ... considerations and guidelines for basic test method descriptions in toxicology. *ALTEX.* **27** (4), 309-317, (2010).

32. Zimmer, B. *et al.* Coordinated waves of gene expression during neuronal differentiation of embryonic stem cells as basis for novel approaches to developmental neurotoxicity testing. *Cell Death and Differentiation.* **18** (3), 383-395, doi:cdd2010109 [pii];10.1038/cdd.2010.109 [doi], (2011).

33. Experion RNA StdSens Analysis Kit for RNA analysis by Electrophoresis. http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000976B%20%20RNA%20STDSENS%20MANUAL.PDF. 2014. Ref Type: Online Source

34. Affymetix GeneChip 3 IVT Express Kit for Target RNA preparation, Fragmentation and hybridization. http://microarray.csc.mrc.ac.uk/downloads/3'%20IVT%20Express%20Manual.pdf. 2014. Ref Type: Online Source

35. Affymetrix GeneChip Hybridization, Wash and Stain Kit. Source: http://www.affymetrix.com/catalog/131467/AFFY/Hybridization,-Wash,-and-Stain-Kit#1_1. 2014. Ref Type: Online Source

36. Affymetrix GeneChip Operating Software. Source: http://www.affymetrix.com/estore/partners_programs/programs/developer/gcos_sdk/gcos_sdk_overview.affx#1_1. 2014.Ref Type: Online Source

37. Partek Genomics Suite. Source http://www.partek.com/pgs. 2014. Ref Type: Online Source

38. ChIP DNA Clean and Concentrator. Source: http://www.zymoresearch.com/dna/dna-clean-up/pcr-dna-clean-up-concentration/chip-dna-clean-concentrator. 2014. Ref Type: Online Source

39. Kraljevic, S., Stambrook, P.J., & Pavelic, K. Accelerating drug discovery. *EMBO Reports.* **5** (9), 837-842, doi:7400236 [pii];10.1038/sj.embor.7400236 [doi], (2004).

Figure 1
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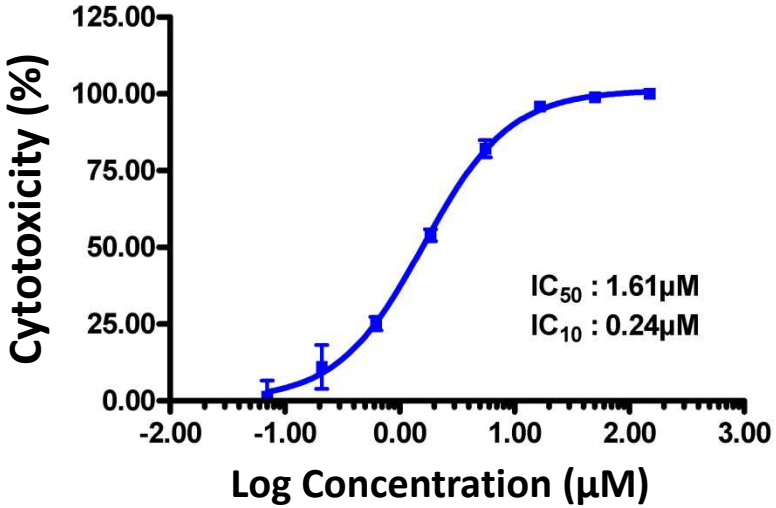

Figure 1

Figure 2

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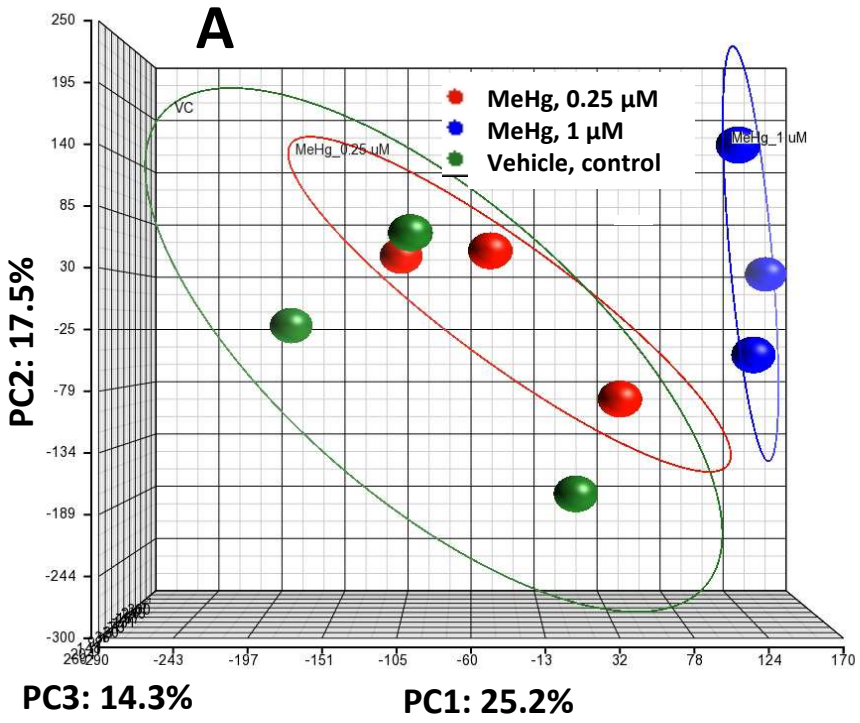

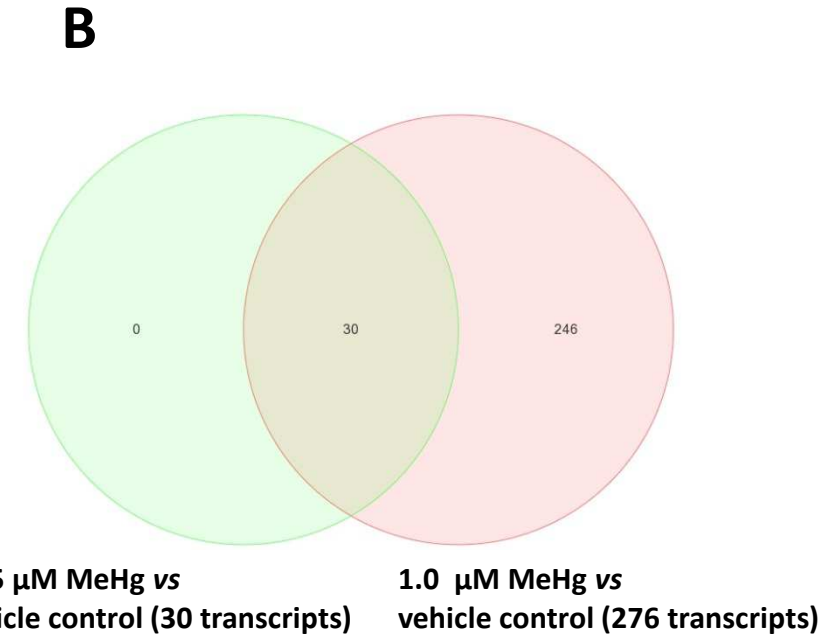

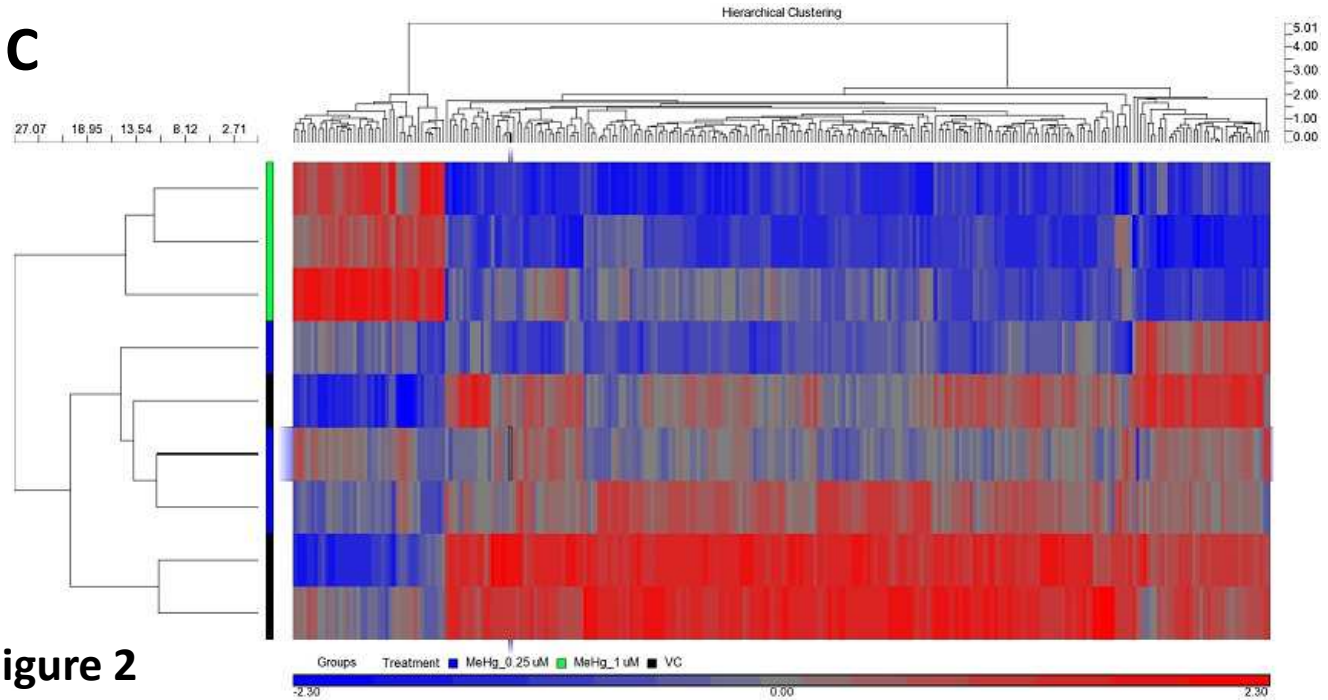

Figure 2

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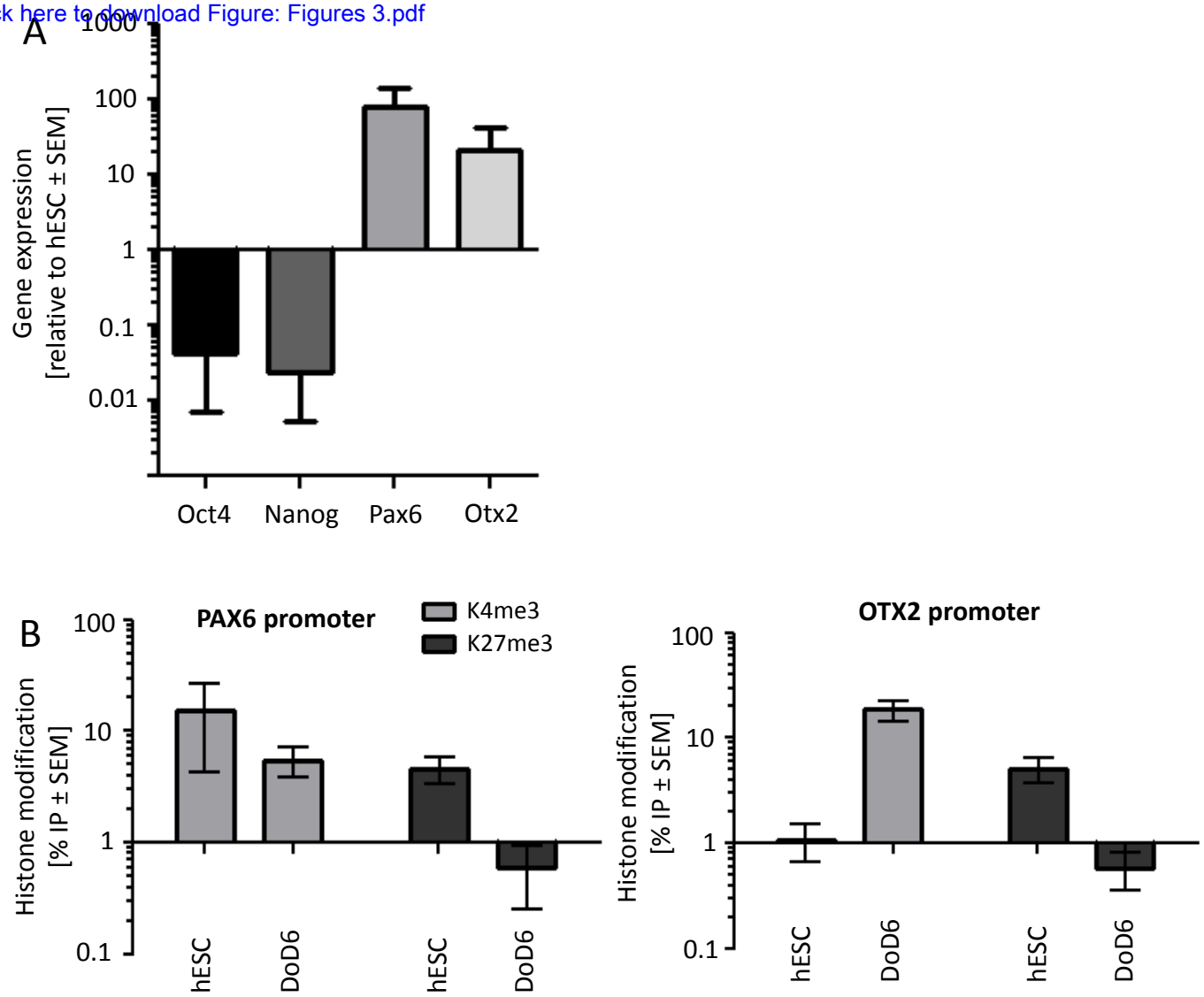

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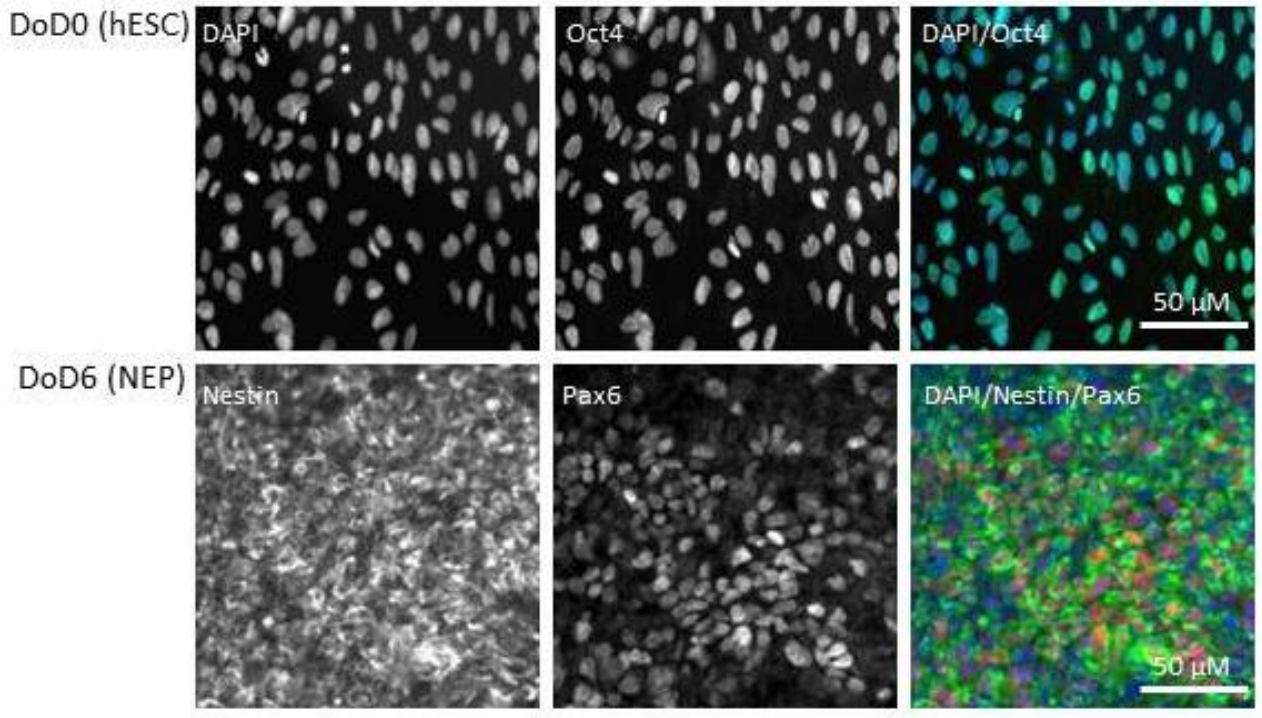

Figure 4

Table 1

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Column #	Probeset ID	Entrez Gen	Gene Symb	Gene Title	RefSeq Tra	UniGene ID	p-value(Gr	p-value(Tre
15391	205935_at	2294	FOXF1	forkhead b	NM_00145	Hs.155591	0.002325	7.49E-05
15388	205932_s_	4487	MSX1	msh home	NM_00244	Hs.424414	0.002373	0.013386
36431	227167_s_	283349	RASSF3	Ras associa	NM_17816	Hs.643605	0.004033	0.000755
27038	217744_s_	64065	PERP	PERP, TP53	NM_02212	Hs.201446	0.007204	0.001527
28399	219106_s_	10324	KLHL41	kelch-like f	NM_00606	Hs.50550 /	0.008174	0.057882
6175	1561657_a	---	---	---	---	Hs.659667	0.008198	0.006101
34100	224833_at	2113	ETS1	v-ets eryth	NM_00114	Hs.369438	0.008792	0.011056
23603	214295_at	57235	KIAA0485	uncharacte	---	Hs.604754	0.010116	0.007968
13153	203697_at	2487	FRZB	frizzled-rel	NM_00146	Hs.128453	0.010132	0.018799
22451	213139_at	6591	SNAI2	snail family	NM_00306	Hs.360174	0.010327	0.044398
13488	204032_at	8412	BCAR3	breast can	NM_00126	Hs.36958	0.010798	0.003734
13795	204339_s_	5999	RGS4	regulator o	NM_00110	Hs.386726	0.011381	0.013651
13390	203934_at	3791	KDR	kinase inse	NM_00225	Hs.479756	0.011647	0.004847
14822	205366_s_	3216	HOXB6	homeobox	NM_01895	Hs.652929	0.012705	0.016451
32884	223599_at	117854	TRIM6	tripartite m	NM_00100	Hs.729048	0.012945	0.002643
27456	218162_at	56944	OLFML3	olfactomed	NM_02019	Hs.9315	0.013039	0.016785
16993	207543_s_	5033	P4HA1	prolyl 4-hy	NM_00091	Hs.500047	0.013041	0.000131
18431	209008_x_	3856	KRT8	keratin 8	NM_00125	Hs.533782	0.013129	0.004397
11306	201849_at	664	BNIP3	BCL2/aden	NM_00405	Hs.144873	0.014202	0.000172
35847	226582_at	400043	LOC40004	uncharacte	NR_02665	Hs.19193 /	0.014329	0.0249
42522	233261_at	1879	EBF1	early B-cell	NM_02400	Hs.573143	0.014337	0.006463
17008	207558_s_	5308	PITX2	paired-like	NM_00032	Hs.643588	0.014885	0.010086
10194	200737_at	5230	PGK1	phosphogl	NM_00029	Hs.567505	0.015279	0.00014
43340	234081_at	---	---	---	---	Hs.677088	0.016045	0.005653
15887	206432_at	3037	HAS2	hyaluronan	NM_00532	Hs.159226	0.016345	0.015683
52159	242901_at	---	---	---	---	Hs.658773	0.016655	0.002902
13793	204337_at	5999	RGS4	regulator o	NM_00110	Hs.386726	0.016895	0.005333
30312	221019_s_	81035	COLEC12	collectin su	NM_03078	Hs.464422	0.017361	0.019019
13154	203698_s_	2487	FRZB	frizzled-rel	NM_00146	Hs.128453	0.017695	0.017467
14564	205108_s_	338	APOB	apolipopro	NM_00038	Hs.120759	0.019656	0.000833
32158	222870_s_	10678	B3GNT2	UDP-GlcNA	NM_00657	Hs.173203	0.021705	0.00114
171	1552487_a	646	BNC1	basonuclin	NM_00171	Hs.459153	0.021819	0.009307
52176	242918_at	4678	NASP	nuclear aut	NM_00119	Hs.319334	0.023692	0.027777
46563	237305_at	---	---	---	---	Hs.674599	0.024621	0.008817
26371	217073_x_	335	APOA1	apolipopro	NM_00003	Hs.93194	0.025081	0.001059
33817	224549_x_	---	---	---	---	---	0.027994	0.009765
11084	201627_s_	3638	INSIG1	insulin indu	NM_00554	Hs.520819	0.029914	0.001853
35478	226213_at	2065	ERBB3	v-erb-b2 er	NM_00100	Hs.118681	0.030134	0.004048
3171	1556682_s	---	---	---	---	Hs.655881	0.030427	0.001649
29431	220138_at	9421	HAND1	heart and r	NM_00482	Hs.152531	0.030608	0.001574
12190	202733_at	8974	P4HA2	prolyl 4-hy	NM_00101	Hs.519568	0.031839	0.005528
35083	225817_at	84952	CGNL1	cingulin-lik	NM_00125	Hs.148989	0.032416	0.007087
40383	231120_x_	5570	PKIB	protein kin	NM_00127	Hs.595503	0.032642	0.005892
12760	203304_at	25805	BAMBI	BMP and a	NM_01234	Hs.533336	0.032917	0.02538
36636	227372_s_	55971	BAIAP2L1	BAI1-assoc	NM_01884	Hs.656063	0.033449	0.001265
3119	1556606_a	89797	NAV2	neuron nav	NM_00111	Hs.502116	0.035056	0.0022

26650	217356_s_	5230	PGK1	phosphogl	NM_00029	Hs.567505	0.035763	0.000759
43203	233944_at	---	---	---	---	Hs.663099	0.035961	0.016577
3147	1556650_a	---	---	---	---	Hs.677181	0.037056	0.008095
45258	236000_s_	---	---	---	---	Hs.656096	0.037251	0.041633
13366	203910_at	9411	ARHGAP29	Rho GTPas	NM_00481	Hs.483238	0.037556	0.034848
49589	240331_at	---	---	---	---	Hs.658892	0.037739	0.023594
37991	228728_at	79974	CPED1	cadherin-lil	NM_00110	Hs.189652	0.039137	0.01494
11866	202409_at	3481	IGF2	insulin-like	NM_00061	Hs.272259	0.039855	0.019386
39635	230372_at	3037	HAS2	hyaluronan	NM_00532	Hs.159226	0.04024	0.027887
46265	237007_at	---	---	---	---	---	0.040477	0.003543
4706	1559282_a	---	---	---	---	Hs.667050	0.041008	0.001199
28703	219410_at	55076	TMEM45A	transmemb	NM_01800	Hs.658956	0.041021	0.000513
11083	201626_at	3638	INSIG1	insulin indu	NM_00554	Hs.520819	0.041054	0.002399
10161	200704_at	9516	LITAF	lipopolysac	NM_00113	Hs.459940	0.041292	0.015633
19140	209723_at	5272	SERPINB9	serpin pept	NM_00415	Hs.104879	0.042241	0.016107
13473	204017_at	11015	KDELR3	KDEL (Lys-A	NM_00685	Hs.528305	0.042687	0.004007
22707	213397_x_	6038	RNASE4	ribonuclea	NM_00293	Hs.283749	0.043995	0.000339
23145	213836_s_	55062	WIPI1	WD repeat	NM_01798	Hs.463964	0.047405	0.003122
36601	227337_at	353322	ANKRD37	ankyrin rep	NM_18172	Hs.508154	0.048145	0.011535
28758	219465_at	336	APOA2	apolipopro	NM_00164	Hs.237658	0.04878	0.001058
20532	211161_s_	1281	COL3A1	collagen, ty	NM_00009	Hs.443625	0.049424	0.065268
29409	220116_at	3781	KCNN2	potassium	NM_00127	Hs.606380	0.049678	0.016025
10588	201131_s_	999	CDH1	cadherin 1,	NM_00436	Hs.461086	0.052725	0.007964
38817	229554_at	4060	LUM	lumican	NM_00234	---	0.055878	0.038496
12392	202934_at	3099	HK2	hexokinase	NM_00018	Hs.591588	0.057055	0.000744
6168	1561642_a	---	---	---	---	Hs.684746	0.057221	0.006128
11309	201852_x_	1281	COL3A1	collagen, ty	NM_00009	Hs.443625	0.059083	0.07292
36561	227297_at	3680	ITGA9	integrin, al	NM_00220	Hs.113157	0.059363	0.024538
10494	201037_at	5214	PFKP	phosphofru	NM_00124	Hs.26010	0.060763	0.001648
42499	233238_s_	---	CTB-12O2.1	NULL /// N	---	Hs.549665	0.061365	0.008878
24379	215076_s_	1281	COL3A1	collagen, ty	NM_00009	Hs.443625	0.06171	0.084176
11201	201744_s_	4060	LUM	lumican	NM_00234	Hs.406475	0.062426	0.087494
14588	205132_at	70	ACTC1	actin, alpha	NM_00515	Hs.118127	0.062567	0.047208
12117	202660_at	3709	ITPR2	inositol 1,4	NM_00222	Hs.512235	0.06371	0.021426
11035	201578_at	5420	PODXL	podocalyxi	NM_00101	Hs.744213	0.064904	0.005084
7239	1563494_a	---	---	---	---	Hs.675516	0.06653	0.004164
33621	224345_x_	26355	FAM162A	family with	NM_01436	Hs.584881	0.068144	0.00031
14522	205066_s_	5167	ENPP1	ectonucleo	NM_00620	Hs.453381	0.068963	0.015542
6475	1562235_s	---	---	---	---	Hs.493096	0.069008	0.012522
51280	242022_at	---	---	---	---	---	0.069796	0.003545
22568	213258_at	7035	TFPI	tissue factc	NM_00103	Hs.516578	0.07089	0.015092
41549	232286_at	---	---	---	---	Hs.656220	0.071196	0.054966
22126	212812_at	256987	SERINC5	serine inco	NM_00117	Hs.288232	0.072406	0.013298
33533	224254_x_	---	---	---	---	---	0.074264	0.007678
19309	209894_at	3953	LEPR	leptin rece	NM_00100	Hs.258228	0.076277	0.007693
11296	201839_s_	4072	EPCAM	epithelial c	NM_00235	Hs.542050	0.077869	0.015402
46150	236892_s_	404266	HOXB-AS3	HOXB clust	NR_033201	Hs.660088	0.078233	0.055085

16197	206742_at	2277 /// 10	FIGF /// PIF	c-fos induc	NM_00446	Hs.11392 /	0.080812	0.026216
31680	222392_x_	64065	PERP	PERP, TP53	NM_02212	Hs.201446	0.082648	0.007625
27795	218501_at	50650	ARHGEF3	Rho guanin	NM_00112	Hs.476402	0.084484	0.015589
26911	217617_at	---	---	---	---	Hs.663061	0.08522	0.017951
674	1553185_a	158158	RASEF	RAS and EF	NM_15257	Hs.129136	0.086522	0.031935
11350	201893_x_	1634	DCN	decorin	NM_00192	Hs.156316	0.087329	0.107095
11255	201798_s_	26509	MYOF	myoferlin	NM_01345	Hs.602086	0.087851	0.017445
43407	234148_at	---	---	---	---	Hs.677340	0.088371	0.004464
27647	218353_at	8490	RGS5	regulator o	NM_00119	Hs.24950 /	0.089643	0.009791
32480	223193_x_	26355	FAM162A	family with	NM_01436	Hs.584881	0.093956	0.000166
28016	218723_s_	28984	RGCC	regulator o	NM_01405	Hs.507866	0.094759	0.020388
19417	210002_at	2627	GATA6	GATA bindi	NM_00525	Hs.514746	0.095394	0.015766
10329	200872_at	6281	S100A10	S100 calciu	NM_00296	Hs.143873	0.096273	0.012447
10884	201427_s_	6414	SEPP1	selenoprot	NM_00108	Hs.275775	0.096939	0.003545
675	1553186_x	158158	RASEF	RAS and EF	NM_15257	Hs.129136	0.099205	0.024899
11107	201650_at	3880	KRT19	keratin 19	NM_00227	Hs.654568	0.099928	0.015851
45376	236118_at	1E+08	LOC100128	uncharacte	NR_10276	Hs.514745	0.100809	0.00931
18858	209436_at	10418	SPON1	spondin 1,	NM_00610	Hs.623673	0.102108	0.028601
19667	210258_at	6003	RGS13	regulator o	NM_00292	Hs.497220	0.102364	0.026796
22816	213506_at	2150	F2RL1	coagulatior	NM_00524	Hs.744181	0.104426	0.030196
40158	230895_at	1404	HAPLN1	hyaluronan	NM_00188	Hs.2799	0.104995	0.045037
12076	202619_s_	5352	PLOD2	procollager	NM_00093	Hs.477866	0.105576	0.000362
28767	219474_at	79669	C3orf52	chromoson	NM_00117	Hs.434247	0.105966	0.014541
28975	219682_s_	6926	TBX3	T-box 3	NM_00599	Hs.129895	0.107422	0.034389
37021	227758_at	85004	RERG	RAS-like, es	NM_00119	Hs.199487	0.108621	0.03172
13343	203887_s_	7056	THBD	thrombom	NM_00036	Hs.2030	0.108783	0.037778
13570	204114_at	22795	NID2	nidogen 2 (	NM_00736	Hs.369840	0.111689	0.020972
7178	1563357_a	---	---	---	---	---	0.11289	0.017373
31049	221760_at	4121	MAN1A1	mannosida	NM_00590	Hs.102788	0.112952	0.041256
13872	204416_x_	341	APOC1	apolipopro	NM_00164	Hs.110675	0.114236	0.010433
7609	1564378_a	---	---	---	---	Hs.677321	0.11523	0.055625
30235	220942_x_	26355	FAM162A	family with	NM_01436	Hs.584881	0.116683	0.00034
8764	1568609_s	57234 /// 3	FLJ39739 /	uncharacte	NM_20740	Hs.456578	0.117524	0.05357
28380	219087_at	54829	ASPN	asporin	NM_00119	Hs.435655	0.119457	0.01363
26133	216834_at	5996	RGS1	regulator o	NM_00292	---	0.119975	0.013813
17211	207761_s_	25840	METTL7A	methyltran	NM_01403	Hs.744021	0.124516	0.01208
32332	223044_at	30061	SLC40A1	solute carri	NM_01458	Hs.643005	0.126036	0.021067
28759	219466_s_	336	APOA2	apolipopro	NM_00164	Hs.237658	0.128222	0.002302
14980	205524_s_	1404	HAPLN1	hyaluronan	NM_00188	Hs.2799	0.129169	0.054409
17167	207717_s_	5318	PKP2	plakophilin	NM_00100	Hs.164384	0.133095	0.012306
11911	202454_s_	2065	ERBB3	v-erb-b2 er	NM_00100	Hs.118681	0.134655	0.005243
10925	201468_s_	1728	NQO1	NAD(P)H d	NM_00090	Hs.406515	0.137982	0.001856
51351	242093_at	94122	SYTL5	synaptotag	NM_00116	Hs.625148	0.13801	0.002091
21852	212538_at	23348	DOCK9	dedicator c	NM_00113	Hs.596105	0.140396	0.04345
28366	219073_s_	114884	OSBPL10	oxysterol b	NM_00117	Hs.150122	0.141164	0.046049
29116	219823_at	79727	LIN28A	lin-28 hom	NM_02467	Hs.628226	0.143555	0.084367
1628	1554507_a	10003	NAALAD2	N-acetylate	NM_00546	Hs.503560	0.144929	0.00012

41407	232144_at	---	---	---	---	Hs.661311	0.147717	0.012168
25539	216238_s_	2244	FGB	fibrinogen	NM_00118	Hs.300774	0.148102	0.00802
31397	222108_at	347902	AMIGO2	adhesion m	NM_00114	Hs.121520	0.149422	0.027529
12003	202546_at	8673	VAMP8	vesicle-ass	NM_00376	Hs.714302	0.149731	0.07736
38715	229452_at	92162	TMEM88	transmemb	NM_20341	Hs.389669	0.151529	0.033443
54334	39248_at	360	AQP3	aquaporin	NM_00492	Hs.234642	0.151723	0.004095
2573	1555778_a	10631	POSTN	periostin, c	NM_00113	Hs.136348	0.1543	0.101941
35932	226668_at	151525	WDSUB1	WD repeat	NM_00112	Hs.20848	0.161309	0.008254
35717	226452_at	5163	PDK1	pyruvate d	NM_00261	Hs.470633	0.163754	0.003751
20059	210665_at	7035	TFPI	tissue factc	NM_00103	Hs.516578	0.163759	0.010859
35568	226303_at	5239	PGM5	phosphoglu	NM_02196	Hs.307835	0.16573	0.041857
18494	209071_s_	8490	RGS5	regulator o	NM_00119	Hs.24950 /	0.169049	0.019478
39065	229802_at	8840	WISP1	WNT1 indu	NM_00120	Hs.492974	0.171907	0.05532
35331	226066_at	4286	MITF	microphtha	NM_00024	Hs.166017	0.172687	0.025249
15488	206032_at	1825	DSC3	desmocolli	NM_00194	Hs.41690	0.175939	0.056507
328	1552711_a	124637	CYB5D1	cytochrom	NM_14460	Hs.27475 /	0.179079	0.018403
24127	214823_at	7754	ZNF204P	zinc finger	NR_00272	Hs.8198	0.17997	0.002925
39467	230204_at	1404	HAPLN1	hyaluronan	NM_00188	Hs.2799	0.180624	0.091252
16992	207542_s_	358	AQP1	aquaporin	NM_00038	Hs.704201	0.182224	0.030454
39346	230083_at	54532	USP53	ubiquitin sp	NM_01905	Hs.431081	0.185629	0.041027
45069	235811_at	---	---	---	---	---	0.185789	0.024399
21458	212143_s_	3486	IGFBP3	insulin-like	NM_00059	Hs.450230	0.186241	0.041029
18373	208949_s_	3958	LGALS3	lectin, gala	NM_00117	Hs.531081	0.187102	0.004442
10806	201349_at	9368	SLC9A3R1	solute carri	NM_00425	Hs.724482	0.18826	0.006929
17534	208096_s_	81578	COL21A1	collagen, ty	NM_03082	Hs.47629 /	0.188606	0.030147
10063	200606_at	1832	DSP	desmoplak	NM_00100	Hs.519873	0.189721	0.029313
7201	1563414_a	---	---	---	---	Hs.553077	0.191159	0.028185
11053	201596_x_	3875	KRT18	keratin 18	NM_00022	Hs.406013	0.191188	0.101139
19508	210095_s_	3486	IGFBP3	insulin-like	NM_00059	Hs.450230	0.193481	0.044473
20058	210664_s_	7035	TFPI	tissue factc	NM_00103	Hs.516578	0.197709	0.02747
13098	203642_s_	22837	COBLL1	cordon-ble	NM_00127	Hs.470457	0.198493	0.019608
40227	230964_at	341640	FREM2	FRAS1 rela	NM_20736	Hs.253994	0.199838	0.034196
36081	226817_at	1824	DSC2	desmocolli	NM_00494	Hs.607260	0.204779	0.007572
13906	204450_x_	335	APOA1	apolipopro	NM_00003	Hs.93194	0.206179	0.005797
15106	205650_s_	2243	FGA	fibrinogen	NM_00050	Hs.351593	0.21564	0.0063
26682	217388_s_	8942	KYNU	kynurenina	NM_00103	Hs.470126	0.217683	0.055081
12082	202625_at	4067	LYN	v-yes-1 Yar	NM_00111	Hs.491767	0.219907	0.030706
20198	210809_s_	10631	POSTN	periostin, c	NM_00113	Hs.136348	0.220158	0.069739
18470	209047_at	358	AQP1	aquaporin	NM_00038	Hs.704201	0.220835	0.070321
7742	1564753_a	---	---	---	---	Hs.680646	0.221559	0.007443
23302	213994_s_	10418	SPON1	spondin 1,	NM_00610	Hs.623673	0.22347	0.052368
11807	202350_s_	4147 /// 10	LOC100506	uncharacte	NM_00238	Hs.189445	0.223608	0.072825
11305	201848_s_	664	BNIP3	BCL2/aden	NM_00405	Hs.144873	0.223924	0.000243
28588	219295_s_	26577	PCOLCE2	procollager	NM_01336	Hs.598034	0.226166	0.011359
24749	215446_s_	4015	LOX	lysyl oxidas	NM_00117	Hs.102267	0.226886	0.011292
15105	205649_s_	2243	FGA	fibrinogen	NM_00050	Hs.351593	0.231518	0.009557
14979	205523_at	1404	HAPLN1	hyaluronan	NM_00188	Hs.2799	0.23326	0.110822

31451	222162_s_	9510	ADAMTS1	ADAM met	NM_00698	Hs.643357	0.23418	0.061526
51038	241780_at	---	---	---	---	Hs.660113	0.235429	0.022735
12890	203434_s_	4311	MME	membrane	NM_00090	Hs.307734	0.240637	0.081887
19375	209960_at	3082	HGF	hepatocyte	NM_00060	Hs.396530	0.240821	0.080122
1275	1554012_a	340419	RSPO2	R-spondin 2	NM_17856	Hs.444834	0.258504	0.05427
9008	1569041_a	---	---	---	---	Hs.655987	0.259871	0.022172
36917	227654_at	140876	FAM65C	family with	NM_08082	Hs.372578	0.262752	0.017208
27195	217901_at	1829	DSG2	desmogleir	NM_00194	Hs.412597	0.263917	0.007132
32288	223000_s_	50848	F11R	F11 recept	NM_01694	Hs.517293	0.264717	0.048756
33827	224559_at	378938	MALAT1	metastasis	NR_00281	Hs.605347	0.268814	0.020194
21900	212586_at	831	CAST	calpastatin	NM_00104	Hs.436186	0.269229	0.007086
12083	202626_s_	4067	LYN	v-yes-1 Yar	NM_00111	Hs.491767	0.269731	0.007597
23301	213993_at	10418	SPON1	spondin 1,	NM_00610	Hs.623673	0.270229	0.061975
19911	210512_s_	7422	VEGFA	vascular en	NM_00102	Hs.644747	0.274346	0.003167
12344	202887_s_	54541	DDIT4	DNA-dama	NM_01905	Hs.523012	0.275928	0.0037
45399	236141_at	79804	NBLA00301	Nbla00301	NR_00367	Hs.508117	0.277939	0.088915
45771	236513_at	---	---	---	---	Hs.655060	0.286361	0.009862
11317	201860_s_	5327	PLAT	plasminoge	NM_00093	Hs.491582	0.287625	0.046275
15622	206167_s_	395	ARHGAP6	Rho GTPas	NM_00117	Hs.435291	0.291841	0.057256
42197	232935_at	---	---	---	---	Hs.659578	0.297701	0.00572
10107	200650_s_	3939	LDHA	lactate deh	NM_00113	Hs.2795 //	0.30459	0.00073
51030	241772_at	---	---	---	---	Hs.670424	0.309209	0.008058
51279	242021_at	7494	XBP1	X-box bindi	NM_00107	Hs.437638	0.312799	0.010005
14150	204694_at	174	AFP	alpha-fetop	NM_00113	Hs.518808	0.319982	0.001741
15787	206332_s_	3428	IFI16	interferon,	NM_00120	Hs.380250	0.323927	0.03461
12121	202664_at	7456	WIPF1	WAS/WASI	NM_00107	Hs.128067	0.324274	0.004362
10335	200878_at	2034	EPAS1	endothelial	NM_00143	Hs.468410	0.326688	0.070153
18791	209369_at	306	ANXA3	annexin A3	NM_00513	Hs.480042	0.33114	0.050039
12143	202686_s_	558	AXL	AXL recept	NM_00169	Hs.590970	0.332508	0.072095
14080	204624_at	540	ATP7B	ATPase, Cu	NM_00005	Hs.492280	0.337366	0.032455
14207	204751_x_	1824	DSC2	desmocolli	NM_00494	Hs.607260	0.34218	0.028523
12532	203074_at	244 /// 653	ANXA8 ///	annexin A8	NM_00103	Hs.535306	0.344948	0.0857
10924	201467_s_	1728	NQO1	NAD(P)H d	NM_00090	Hs.406515	0.348069	0.003029
2629	1555851_s	6415	SEPW1	selenoprot	NM_00300	Hs.603350	0.349105	0.000146
13680	204224_s_	2643	GCH1	GTP cycloh	NM_00016	Hs.604206	0.349413	0.001961
12055	202598_at	6284	S100A13	S100 calciu	NM_00102	Hs.516505	0.350025	0.013849
20305	210929_s_	197	AHSG	alpha-2-HS	NM_00162	Hs.324746	0.356202	0.037038
16557	207102_at	6718	AKR1D1	aldo-keto r	NM_00119	Hs.201667	0.358721	0.006678
12077	202620_s_	5352	PLOD2	procollager	NM_00093	Hs.477866	0.363469	0.00174
14830	205374_at	6588	SLN	sarcolipin	NM_00306	Hs.334629	0.370504	0.069647
29524	220231_at	10842	PPP1R17	protein pho	NM_00114	Hs.227011	0.374523	0.01832
35177	225911_at	255743	NPNT	nephronec	NM_00103	Hs.518921	0.378612	0.016082
13973	204517_at	5480	PPIC	peptidylpro	NM_00094	Hs.110364	0.390505	0.10718
13804	204348_s_	205 /// 10(	AK4 /// LO	adenylate k	NM_00100	Hs.10862 /	0.393069	0.000755
25028	215726_s_	1528	CYB5A	cytochrom	NM_00119	Hs.465413	0.401441	0.002525
11956	202499_s_	6515	SLC2A3	solute carri	NM_00693	Hs.419240	0.41043	0.024015
18788	209366_x_	1528	CYB5A	cytochrom	NM_00119	Hs.465413	0.423791	0.006166

614	1553105_s	1829	DSG2	desmogleir	NM_00194	Hs.412597	0.423808	0.021188
33975	224707_at	84418	CYSTM1	cysteine-ri	NM_03241	Hs.529798	0.424151	0.007508
11528	202071_at	6385	SDC4	syndecan 4	NM_00299	Hs.632267	0.428663	0.016443
37845	228582_x_	378938	MALAT1	metastasis	NR_00281	Hs.621695	0.43548	0.053837
15276	205820_s_	345	APOC3	apolipopro	NM_00004	Hs.73849	0.438468	0.037393
54004	244745_at	85004	RERG	RAS-like, e	NM_00119	Hs.199487	0.438821	0.058963
14051	204595_s_	6781	STC1	stanniocalc	NM_00315	Hs.25590 /	0.443082	0.016454
41192	231929_at	22807	IKZF2	IKAROS fan	NM_00107	Hs.159115	0.445053	0.015515
12370	202912_at	133	ADM	adrenomec	NM_00112	Hs.441047	0.449217	0.046062
19918	210519_s_	1728	NQO1	NAD(P)H d	NM_00090	Hs.406515	0.45124	0.000764
20145	210755_at	3082	HGF	hepatocyte	NM_00060	Hs.396530	0.464026	0.049977
28433	219140_s_	5950	RBP4	retinol binc	NM_00674	Hs.50223	0.470808	0.023982
45108	235850_at	26355	FAM162A	family with	NM_01436	Hs.594920	0.476579	0.001779
11098	201641_at	684	BST2	bone marro	NM_00433	Hs.118110	0.478732	0.053079
22406	213094_at	57211	GPR126	G protein-c	NM_00103	Hs.743302	0.480266	0.01457
28905	219612_s_	2266	FGG	fibrinogen	NM_00050	Hs.727584	0.520975	0.008372
13221	203765_at	25801	GCA	grancalcin,	NM_01219	Hs.377894	0.522222	0.007203
14444	204988_at	2244	FGB	fibrinogen	NM_00118	Hs.300774	0.524757	0.018244
15348	205892_s_	2168	FABP1	fatty acid b	NM_00144	Hs.380135	0.529694	0.014326
31377	222088_s_	6515 /// 14	SLC2A14 //	solute carri	NM_00693	Hs.419240	0.531506	0.061262
33006	223721_s_	56521	DNAJC12	DnaJ (Hsp4	NM_02180	Hs.260720	0.535099	0.002517
13775	204319_s_	6001	RGS10	regulator o	NM_00100	Hs.501200	0.535673	0.00667
13380	203924_at	2938	GSTA1	glutathione	NM_14574	Hs.446309	0.550769	0.085465
36363	227099_s_	387763	C11orf96	chromoson	NM_00114	Hs.530443	0.570669	0.002867
12313	202856_s_	9123	SLC16A3	solute carri	NM_00104	Hs.500761	0.581187	0.013672
11955	202498_s_	6515	SLC2A3	solute carri	NM_00693	Hs.419240	0.622711	0.047022
14053	204597_x_	6781	STC1	stanniocalc	NM_00315	Hs.25590 /	0.632782	0.031751
22882	213572_s_	1992	SERPINB1	serpin pept	NM_03066	Hs.381167	0.669842	0.011339
35566	226301_at	116843	SLC18B1	solute carri	NM_05283	Hs.347144	0.672191	0.000746
17291	207843_x_	1528	CYB5A	cytochrom	NM_00119	Hs.465413	0.708574	0.003824
34608	225342_at	205 /// 10(	AK4 /// LO	adenylate k	NM_00100	Hs.10862 /	0.728067	0.00392
28269	218976_at	56521	DNAJC12	DnaJ (Hsp4	NM_02180	Hs.260720	0.752432	0.00042
38219	228956_at	7368	UGT8	UDP glycos	NM_00112	Hs.144197	0.799037	0.003522
26972	217678_at	23657	SLC7A11	solute carri	NM_01433	Hs.390594	0.800743	0.004924
10651	201194_at	6415	SEPW1	selenoprot	NM_00300	Hs.603350	0.809903	0.009879
11954	202497_x_	6515	SLC2A3	solute carri	NM_00693	Hs.419240	0.827867	0.01422
19336	209921_at	23657	SLC7A11	solute carri	NM_01433	Hs.390594	0.850049	0.014975
18690	209267_s_	64116	SLC39A8	solute carri	NM_00113	Hs.288034	0.862991	0.018124
20815	211478_s_	1803	DPP4	dipeptidyl-	NM_00193	Hs.368912	0.910259	0.014531
32010	222722_at	4969	OGN	osteoglycin	NM_01405	Hs.109439	0.913626	0.014706
12448	202990_at	5836	PYGL	phosphoryl	NM_00116	Hs.282417	0.977304	0.002518
16920	207469_s_	8544	PIR	pirin (iron-l	NM_00101	Hs.495728	0.979834	0.001339

p-value(Me	Ratio(MeH	Fold-Chang	Fold-Chang	p-value(Me	Ratio(MeH	Fold-Chang	Fold-Chang	F(Groups)
0.000127	0.588692	-1.69868	MeHg_0.25	3.13E-05	0.469762	-2.12874	MeHg_1 uM	39.4807
0.02765	0.638977	-1.565	MeHg_0.25	0.005403	0.484389	-2.06446	MeHg_1 uM	39.0536
0.002476	0.652562	-1.53242	MeHg_0.25	0.00029	0.473821	-2.1105	MeHg_1 uM	29.4933
0.010823	0.651483	-1.53496	MeHg_0.25	0.000583	0.389429	-2.56787	MeHg_1 uM	21.5644
0.134696	0.654361	-1.52821	MeHg_0.25	0.023764	0.447046	-2.2369	MeHg_1 uM	20.1209
0.052027	1.3218	1.3218	MeHg_0.25	0.002408	2.00509	2.00509	MeHg_1 uM	20.089
0.0227	0.581063	-1.72098	MeHg_0.25	0.004462	0.418813	-2.3877	MeHg_1 uM	19.3292
0.076896	1.30534	1.30534	MeHg_0.25	0.003202	2.03628	2.03628	MeHg_1 uM	17.8852
0.187731	0.776571	-1.28771	MeHg_0.25	0.007955	0.456899	-2.18867	MeHg_1 uM	17.8693
0.06785	0.619562	-1.61404	MeHg_0.25	0.018895	0.479719	-2.08455	MeHg_1 uM	17.6808
0.010905	0.651849	-1.5341	MeHg_0.25	0.00145	0.475031	-2.10513	MeHg_1 uM	17.2468
0.018386	0.567983	-1.76061	MeHg_0.25	0.005978	0.456697	-2.18963	MeHg_1 uM	16.7472
0.007865	0.603581	-1.65678	MeHg_0.25	0.002035	0.481407	-2.07725	MeHg_1 uM	16.5324
0.061203	0.660323	-1.51441	MeHg_0.25	0.006454	0.432525	-2.312	MeHg_1 uM	15.744
0.007154	0.533709	-1.87368	MeHg_0.25	0.001031	0.346851	-2.88308	MeHg_1 uM	15.5784
0.032325	0.629217	-1.58928	MeHg_0.25	0.006845	0.477959	-2.09223	MeHg_1 uM	15.515
0.001383	0.668458	-1.49598	MeHg_0.25	4.99E-05	0.38904	-2.57043	MeHg_1 uM	15.5135
0.009938	0.577437	-1.73179	MeHg_0.25	0.001749	0.412697	-2.42309	MeHg_1 uM	15.4546
0.026018	0.748903	-1.33529	MeHg_0.25	8.01E-05	0.252184	-3.96536	MeHg_1 uM	14.7828
0.125244	0.679743	-1.47114	MeHg_0.25	0.010023	0.399148	-2.50534	MeHg_1 uM	14.7077
0.021762	1.54859	1.54859	MeHg_0.25	0.002502	2.2468	2.2468	MeHg_1 uM	14.7035
0.050638	0.479776	-2.0843	MeHg_0.25	0.00393	0.204139	-4.89862	MeHg_1 uM	14.3928
0.010944	0.822717	-1.21548	MeHg_0.25	6.13E-05	0.465187	-2.14967	MeHg_1 uM	14.1802
0.045095	1.37032	1.37032	MeHg_0.25	0.002217	2.14658	2.14658	MeHg_1 uM	13.7894
0.034939	0.475405	-2.10347	MeHg_0.25	0.006285	0.287936	-3.473	MeHg_1 uM	13.6436
0.021339	1.36076	1.36076	MeHg_0.25	0.001119	2.01626	2.01626	MeHg_1 uM	13.4974
0.015745	0.534228	-1.87186	MeHg_0.25	0.002074	0.331072	-3.02049	MeHg_1 uM	13.387
0.02591	0.586415	-1.70528	MeHg_0.25	0.008293	0.472278	-2.1174	MeHg_1 uM	13.1791
0.198941	0.731293	-1.36744	MeHg_0.25	0.007476	0.361273	-2.76799	MeHg_1 uM	13.0351
0.001202	0.278849	-3.58617	MeHg_0.25	0.000361	0.174824	-5.72005	MeHg_1 uM	12.2652
0.004065	0.639528	-1.56365	MeHg_0.25	0.000436	0.44682	-2.23804	MeHg_1 uM	11.5755
0.019156	0.56533	-1.76888	MeHg_0.25	0.003753	0.402639	-2.48361	MeHg_1 uM	11.5398
0.056468	1.65949	1.65949	MeHg_0.25	0.011302	2.33162	2.33162	MeHg_1 uM	10.9935
0.031192	1.47203	1.47203	MeHg_0.25	0.003421	2.09053	2.09053	MeHg_1 uM	10.7462
0.000861	0.379994	-2.63162	MeHg_0.25	0.000592	0.344262	-2.90476	MeHg_1 uM	10.6288
0.04469	1.61178	1.61178	MeHg_0.25	0.003794	2.71354	2.71354	MeHg_1 uM	9.95367
0.760578	0.970031	-1.03089	MeHg_0.25	0.001138	0.460086	-2.17351	MeHg_1 uM	9.5636
0.011017	0.53711	-1.86181	MeHg_0.25	0.001581	0.346448	-2.88644	MeHg_1 uM	9.52125
0.019951	1.58482	1.58482	MeHg_0.25	0.000647	3.26741	3.26741	MeHg_1 uM	9.46578
0.005629	0.542396	-1.84367	MeHg_0.25	0.000602	0.329741	-3.03269	MeHg_1 uM	9.4318
0.027074	0.679632	-1.47138	MeHg_0.25	0.002128	0.449542	-2.22448	MeHg_1 uM	9.20855
0.013819	0.548529	-1.82306	MeHg_0.25	0.002877	0.393397	-2.54196	MeHg_1 uM	9.10846
0.196913	0.843375	-1.18571	MeHg_0.25	0.002687	0.481808	-2.07552	MeHg_1 uM	9.0698
0.049904	0.48152	-2.07676	MeHg_0.25	0.010354	0.301513	-3.3166	MeHg_1 uM	9.0235
0.002143	0.495609	-2.01772	MeHg_0.25	0.000528	0.363074	-2.75426	MeHg_1 uM	8.93555
0.035452	1.33125	1.33125	MeHg_0.25	0.000886	2.25763	2.25763	MeHg_1 uM	8.68193

0.070025	0.858716	-1.16453	MeHg_0.25	0.00035	0.496243	-2.01514	MeHg_1 uM	8.57581
0.071528	1.48578	1.48578	MeHg_0.25	0.00652	2.32825	2.32825	MeHg_1 uM	8.54664
0.066133	1.43572	1.43572	MeHg_0.25	0.003213	2.48561	2.48561	MeHg_1 uM	8.38972
0.041288	1.76074	1.76074	MeHg_0.25	0.020083	2.04147	2.04147	MeHg_1 uM	8.36249
0.0459	0.599218	-1.66884	MeHg_0.25	0.015306	0.483133	-2.06982	MeHg_1 uM	8.32029
0.241373	1.23198	1.23198	MeHg_0.25	0.010224	2.00284	2.00284	MeHg_1 uM	8.29524
0.019135	0.59243	-1.68796	MeHg_0.25	0.006634	0.490088	-2.04045	MeHg_1 uM	8.10972
0.052956	0.54138	-1.84713	MeHg_0.25	0.007678	0.326307	-3.0646	MeHg_1 uM	8.01821
0.049128	0.45141	-2.21528	MeHg_0.25	0.011553	0.284485	-3.51513	MeHg_1 uM	7.97012
0.067262	1.2708	1.2708	MeHg_0.25	0.001469	2.11328	2.11328	MeHg_1 uM	7.94089
0.010269	1.37142	1.37142	MeHg_0.25	0.00046	2.07068	2.07068	MeHg_1 uM	7.87635
0.010638	0.631424	-1.58372	MeHg_0.25	0.000206	0.268235	-3.72807	MeHg_1 uM	7.8748
0.512064	0.934579	-1.07	MeHg_0.25	0.001345	0.472389	-2.1169	MeHg_1 uM	7.87083
0.090218	0.728509	-1.37267	MeHg_0.25	0.006215	0.472116	-2.11812	MeHg_1 uM	7.84234
0.044652	0.505062	-1.97996	MeHg_0.25	0.006356	0.289811	-3.45053	MeHg_1 uM	7.73116
0.007172	0.622542	-1.60632	MeHg_0.25	0.00165	0.493217	-2.02751	MeHg_1 uM	7.68018
0.007944	0.767652	-1.30267	MeHg_0.25	0.000136	0.461895	-2.16499	MeHg_1 uM	7.53517
0.0414	0.717699	-1.39334	MeHg_0.25	0.001249	0.402884	-2.48211	MeHg_1 uM	7.18578
0.217173	0.777564	-1.28607	MeHg_0.25	0.005118	0.384396	-2.60149	MeHg_1 uM	7.115
0.005828	0.339126	-2.94876	MeHg_0.25	0.000401	0.110981	-9.01053	MeHg_1 uM	7.0554
0.148636	0.610265	-1.63863	MeHg_0.25	0.026953	0.389168	-2.56959	MeHg_1 uM	6.99622
0.081228	0.613291	-1.63055	MeHg_0.25	0.006332	0.331162	-3.01967	MeHg_1 uM	6.97319
0.031636	0.464746	-2.15171	MeHg_0.25	0.003081	0.220819	-4.52859	MeHg_1 uM	6.71004
0.060764	0.478744	-2.0888	MeHg_0.25	0.016271	0.321368	-3.1117	MeHg_1 uM	6.46075
0.09286	0.825081	-1.212	MeHg_0.25	0.000354	0.373309	-2.67875	MeHg_1 uM	6.37306
0.072011	1.36193	1.36193	MeHg_0.25	0.002479	2.36591	2.36591	MeHg_1 uM	6.36091
0.176433	0.60959	-1.64045	MeHg_0.25	0.030264	0.370582	-2.69846	MeHg_1 uM	6.22809
0.04622	0.637415	-1.56884	MeHg_0.25	0.010061	0.484194	-2.06529	MeHg_1 uM	6.20863
0.0124	0.659725	-1.51578	MeHg_0.25	0.000631	0.393267	-2.5428	MeHg_1 uM	6.11355
0.083722	0.76812	-1.30188	MeHg_0.25	0.003576	0.493417	-2.02668	MeHg_1 uM	6.07368
0.182868	0.637636	-1.56829	MeHg_0.25	0.035254	0.416981	-2.39819	MeHg_1 uM	6.05104
0.113151	0.595103	-1.68038	MeHg_0.25	0.038792	0.459591	-2.17585	MeHg_1 uM	6.00477
0.031511	0.376406	-2.65671	MeHg_0.25	0.029162	0.367171	-2.72353	MeHg_1 uM	5.99572
0.02287	0.576452	-1.73475	MeHg_0.25	0.010099	0.494813	-2.02097	MeHg_1 uM	5.92366
0.029132	0.519093	-1.92644	MeHg_0.25	0.001961	0.241652	-4.13819	MeHg_1 uM	5.85044
0.011314	1.65911	1.65911	MeHg_0.25	0.001627	2.37162	2.37162	MeHg_1 uM	5.7539
0.006693	0.696236	-1.4363	MeHg_0.25	0.000124	0.355894	-2.80982	MeHg_1 uM	5.66157
0.128064	0.775976	-1.2887	MeHg_0.25	0.006354	0.499703	-2.00119	MeHg_1 uM	5.61594
0.061823	1.57926	1.57926	MeHg_0.25	0.004905	2.71734	2.71734	MeHg_1 uM	5.61345
0.149186	1.23086	1.23086	MeHg_0.25	0.001609	2.424	2.424	MeHg_1 uM	5.57032
0.040685	0.369948	-2.70308	MeHg_0.25	0.005958	0.168918	-5.92003	MeHg_1 uM	5.51167
0.373594	1.22967	1.22967	MeHg_0.25	0.02472	2.06413	2.06413	MeHg_1 uM	5.49551
0.024485	0.602466	-1.65985	MeHg_0.25	0.005453	0.45519	-2.19689	MeHg_1 uM	5.43263
0.05396	1.35882	1.35882	MeHg_0.25	0.003014	2.07336	2.07336	MeHg_1 uM	5.33908
0.016509	0.608009	-1.64471	MeHg_0.25	0.003083	0.449287	-2.22575	MeHg_1 uM	5.2416
0.022884	0.495196	-2.0194	MeHg_0.25	0.006579	0.362698	-2.75711	MeHg_1 uM	5.16719
0.047113	0.409698	-2.44082	MeHg_0.25	0.028432	0.3478	-2.87521	MeHg_1 uM	5.15047

0.119187	0.695496	-1.43782	MeHg_0.25	0.010515	0.434567	-2.30114	MeHg_1 uM	5.03546
0.020006	0.616396	-1.62233	MeHg_0.25	0.002996	0.435477	-2.29633	MeHg_1 uM	4.95686
0.04264	0.666259	-1.50092	MeHg_0.25	0.006153	0.481265	-2.07786	MeHg_1 uM	4.88088
0.155267	1.31719	1.31719	MeHg_0.25	0.007441	2.20172	2.20172	MeHg_1 uM	4.8511
0.099768	1.46219	1.46219	MeHg_0.25	0.012771	2.14553	2.14553	MeHg_1 uM	4.79935
0.197917	0.672423	-1.48716	MeHg_0.25	0.045709	0.478415	-2.09023	MeHg_1 uM	4.76784
0.026566	0.510244	-1.95985	MeHg_0.25	0.00741	0.373731	-2.67572	MeHg_1 uM	4.74772
0.504707	1.08983	1.08983	MeHg_0.25	0.002432	2.22545	2.22545	MeHg_1 uM	4.72782
0.010381	0.451279	-2.21593	MeHg_0.25	0.004662	0.369024	-2.70985	MeHg_1 uM	4.67995
0.004939	0.717471	-1.39378	MeHg_0.25	6.75E-05	0.362465	-2.75889	MeHg_1 uM	4.52482
0.115135	0.728305	-1.37305	MeHg_0.25	0.008189	0.463042	-2.15963	MeHg_1 uM	4.4971
0.086805	0.364692	-2.74204	MeHg_0.25	0.006253	0.095442	-10.4776	MeHg_1 uM	4.47545
0.052754	0.40667	-2.459	MeHg_0.25	0.004857	0.155108	-6.44712	MeHg_1 uM	4.44582
0.012218	0.459949	-2.17415	MeHg_0.25	0.001364	0.241935	-4.13335	MeHg_1 uM	4.42364
0.082588	1.4728	1.4728	MeHg_0.25	0.009877	2.17383	2.17383	MeHg_1 uM	4.34986
0.059827	0.300748	-3.32504	MeHg_0.25	0.006213	0.087871	-11.3804	MeHg_1 uM	4.32685
0.019021	0.600745	-1.6646	MeHg_0.25	0.003758	0.444532	-2.24956	MeHg_1 uM	4.29914
0.397683	0.844492	-1.18414	MeHg_0.25	0.013437	0.470204	-2.12673	MeHg_1 uM	4.25894
0.069984	0.284624	-3.51341	MeHg_0.25	0.010701	0.099278	-10.0727	MeHg_1 uM	4.25111
0.02849	0.499703	-2.00119	MeHg_0.25	0.014956	0.428891	-2.33159	MeHg_1 uM	4.18909
0.075552	0.448005	-2.23212	MeHg_0.25	0.018883	0.277003	-3.61007	MeHg_1 uM	4.17228
0.008312	0.699017	-1.43058	MeHg_0.25	0.000146	0.35261	-2.836	MeHg_1 uM	4.15528
0.014037	0.493133	-2.02785	MeHg_0.25	0.007191	0.424165	-2.35757	MeHg_1 uM	4.14392
0.06806	0.589535	-1.69625	MeHg_0.25	0.014073	0.411896	-2.4278	MeHg_1 uM	4.10216
0.086101	0.554576	-1.80318	MeHg_0.25	0.012704	0.327192	-3.05631	MeHg_1 uM	4.06837
0.166194	0.635009	-1.57478	MeHg_0.25	0.015434	0.336614	-2.97076	MeHg_1 uM	4.06386
0.063427	0.392667	-2.54669	MeHg_0.25	0.00829	0.168264	-5.94305	MeHg_1 uM	3.98446
0.023567	0.566166	-1.76627	MeHg_0.25	0.007586	0.45117	-2.21646	MeHg_1 uM	3.95254
0.160225	0.668234	-1.49648	MeHg_0.25	0.016809	0.39654	-2.52181	MeHg_1 uM	3.9509
0.030597	0.548591	-1.82285	MeHg_0.25	0.004082	0.338088	-2.95781	MeHg_1 uM	3.91738
0.288733	1.28791	1.28791	MeHg_0.25	0.023936	2.08259	2.08259	MeHg_1 uM	3.8918
0.008384	0.717288	-1.39414	MeHg_0.25	0.000137	0.373791	-2.67529	MeHg_1 uM	3.855
0.114987	0.659864	-1.51546	MeHg_0.25	0.022006	0.470976	-2.12325	MeHg_1 uM	3.83401
0.86775	0.965217	-1.03604	MeHg_0.25	0.009498	2.53974	2.53974	MeHg_1 uM	3.7866
0.01377	0.50367	-1.98543	MeHg_0.25	0.006733	0.430542	-2.32265	MeHg_1 uM	3.77409
0.069705	0.441755	-2.2637	MeHg_0.25	0.004757	0.151854	-6.58529	MeHg_1 uM	3.66784
0.106606	0.465765	-2.147	MeHg_0.25	0.008415	0.16844	-5.93682	MeHg_1 uM	3.63356
0.030389	0.278597	-3.58942	MeHg_0.25	0.000914	0.032381	-30.8825	MeHg_1 uM	3.58534
0.065047	0.486021	-2.05753	MeHg_0.25	0.024528	0.366033	-2.732	MeHg_1 uM	3.56481
0.040716	0.370945	-2.69582	MeHg_0.25	0.004805	0.152137	-6.57301	MeHg_1 uM	3.48213
0.024638	0.664551	-1.50478	MeHg_0.25	0.002016	0.434757	-2.30014	MeHg_1 uM	3.45027
0.104071	0.76156	-1.31309	MeHg_0.25	0.002301	2.45422	2.45422	MeHg_1 uM	3.38418
0.003405	0.38217	-2.61664	MeHg_0.25	0.000878	0.25213	-3.96621	MeHg_1 uM	3.38362
0.038059	0.501887	-1.99248	MeHg_0.25	0.022224	0.440447	-2.27042	MeHg_1 uM	3.33769
0.073282	0.591112	-1.69173	MeHg_0.25	0.019469	0.439104	-2.27736	MeHg_1 uM	3.32314
0.09506	0.418907	-2.38717	MeHg_0.25	0.038736	0.297755	-3.35847	MeHg_1 uM	3.27863
0.005313	0.805539	-1.24141	MeHg_0.25	5.03E-05	0.483495	-2.06828	MeHg_1 uM	3.25355

0.113085	1.32647	1.32647	MeHg_0.25	0.004967	2.18823	2.18823	MeHg_1 uM	3.20373
0.049852	0.464451	-2.15308	MeHg_0.25	0.003133	0.172919	-5.78306	MeHg_1 uM	3.19696
0.057541	0.539109	-1.85491	MeHg_0.25	0.011169	0.352297	-2.83851	MeHg_1 uM	3.17396
0.129091	0.555138	-1.80135	MeHg_0.25	0.032821	0.372314	-2.6859	MeHg_1 uM	3.16862
0.053296	0.537872	-1.85918	MeHg_0.25	0.014098	0.386219	-2.5892	MeHg_1 uM	3.13786
0.017579	0.609015	-1.642	MeHg_0.25	0.00157	0.377878	-2.64636	MeHg_1 uM	3.13456
0.326027	0.618413	-1.61704	MeHg_0.25	0.044321	0.288163	-3.47025	MeHg_1 uM	3.09152
0.057599	0.715521	-1.39758	MeHg_0.25	0.003246	0.449851	-2.22296	MeHg_1 uM	2.97967
0.228972	0.756502	-1.32187	MeHg_0.25	0.001799	0.234279	-4.26841	MeHg_1 uM	2.94235
0.014881	0.416614	-2.4003	MeHg_0.25	0.004737	0.297005	-3.36695	MeHg_1 uM	2.94228
0.079661	0.61956	-1.61405	MeHg_0.25	0.017256	0.447986	-2.23221	MeHg_1 uM	2.9128
0.021148	0.390266	-2.56235	MeHg_0.25	0.009129	0.298956	-3.34498	MeHg_1 uM	2.86434
0.066968	0.600938	-1.66407	MeHg_0.25	0.02485	0.489589	-2.04253	MeHg_1 uM	2.82373
0.043532	0.62891	-1.59005	MeHg_0.25	0.010487	0.485341	-2.06041	MeHg_1 uM	2.81283
0.094678	0.589583	-1.69612	MeHg_0.25	0.023782	0.423028	-2.36391	MeHg_1 uM	2.76814
0.017893	0.561204	-1.78188	MeHg_0.25	0.009044	0.493529	-2.02622	MeHg_1 uM	2.72615
0.158085	0.804585	-1.24288	MeHg_0.25	0.001354	0.368741	-2.71193	MeHg_1 uM	2.71444
0.157075	0.381445	-2.62161	MeHg_0.25	0.038821	0.186563	-5.36012	MeHg_1 uM	2.70589
0.085489	0.605581	-1.65131	MeHg_0.25	0.012173	0.382997	-2.61098	MeHg_1 uM	2.68519
0.134919	0.701495	-1.42553	MeHg_0.25	0.016591	0.471329	-2.12166	MeHg_1 uM	2.64203
0.057817	1.50597	1.50597	MeHg_0.25	0.009779	2.05386	2.05386	MeHg_1 uM	2.64002
0.189372	0.572436	-1.74692	MeHg_0.25	0.016925	0.248473	-4.02458	MeHg_1 uM	2.63439
0.004903	0.375249	-2.66489	MeHg_0.25	0.002093	0.29104	-3.43596	MeHg_1 uM	2.62371
0.022112	0.547173	-1.82757	MeHg_0.25	0.002689	0.332839	-3.00445	MeHg_1 uM	2.60947
0.100471	0.574948	-1.73929	MeHg_0.25	0.012036	0.321456	-3.11084	MeHg_1 uM	2.60524
0.118286	0.459542	-2.17608	MeHg_0.25	0.011751	0.178714	-5.59553	MeHg_1 uM	2.59168
0.111036	1.43948	1.43948	MeHg_0.25	0.011268	2.21329	2.21329	MeHg_1 uM	2.57438
0.161251	0.553617	-1.8063	MeHg_0.25	0.04353	0.366518	-2.72838	MeHg_1 uM	2.57403
0.248928	0.511234	-1.95605	MeHg_0.25	0.018867	0.14979	-6.676	MeHg_1 uM	2.54685
0.056761	0.367662	-2.71989	MeHg_0.25	0.011157	0.186041	-5.37517	MeHg_1 uM	2.49797
0.03816	0.513895	-1.94592	MeHg_0.25	0.007992	0.342064	-2.92343	MeHg_1 uM	2.48908
0.066057	0.581918	-1.71846	MeHg_0.25	0.014029	0.406759	-2.45846	MeHg_1 uM	2.47395
0.019133	0.575272	-1.73831	MeHg_0.25	0.002984	0.391383	-2.55504	MeHg_1 uM	2.41965
0.010033	0.46128	-2.16788	MeHg_0.25	0.002402	0.316916	-3.15541	MeHg_1 uM	2.40462
0.01403	0.428402	-2.33426	MeHg_0.25	0.002512	0.253602	-3.94319	MeHg_1 uM	2.30691
0.155362	0.609556	-1.64054	MeHg_0.25	0.022537	0.359674	-2.7803	MeHg_1 uM	2.28665
0.087559	0.65384	-1.52943	MeHg_0.25	0.012272	0.44093	-2.26793	MeHg_1 uM	2.26491
0.211201	0.620594	-1.61136	MeHg_0.25	0.029044	0.343335	-2.91261	MeHg_1 uM	2.26249
0.105451	0.640385	-1.56156	MeHg_0.25	0.030206	0.494893	-2.02064	MeHg_1 uM	2.25595
0.090163	1.27659	1.27659	MeHg_0.25	0.003044	2.02104	2.02104	MeHg_1 uM	2.24898
0.38316	0.752756	-1.32845	MeHg_0.25	0.023716	0.356524	-2.80486	MeHg_1 uM	2.23078
0.140972	0.544512	-1.83651	MeHg_0.25	0.030432	0.336427	-2.97242	MeHg_1 uM	2.22948
0.044272	0.7753	-1.28982	MeHg_0.25	0.000116	0.268243	-3.72796	MeHg_1 uM	2.22648
0.037781	0.67724	-1.47658	MeHg_0.25	0.004429	0.478105	-2.09159	MeHg_1 uM	2.20549
0.018854	0.604493	-1.65428	MeHg_0.25	0.004709	0.471975	-2.11876	MeHg_1 uM	2.19881
0.027038	0.340864	-2.93372	MeHg_0.25	0.003743	0.147649	-6.77283	MeHg_1 uM	2.15659
0.281558	0.348895	-2.86619	MeHg_0.25	0.047611	0.091484	-10.9309	MeHg_1 uM	2.14104

0.126735	0.674635	-1.48228	MeHg_0.25	0.025453	0.491095	-2.03627	MeHg_1 uM	2.1329
0.041358	0.639645	-1.56337	MeHg_0.25	0.009361	0.493013	-2.02834	MeHg_1 uM	2.12192
0.16964	0.638129	-1.56708	MeHg_0.25	0.034284	0.428415	-2.33418	MeHg_1 uM	2.07708
0.222783	0.527801	-1.89465	MeHg_0.25	0.033585	0.244476	-4.09038	MeHg_1 uM	2.07552
0.14615	0.610361	-1.63838	MeHg_0.25	0.022178	0.369688	-2.70498	MeHg_1 uM	1.93366
0.198386	1.31847	1.31847	MeHg_0.25	0.009376	2.32097	2.32097	MeHg_1 uM	1.92329
0.027996	0.618416	-1.61703	MeHg_0.25	0.007212	0.486594	-2.0551	MeHg_1 uM	1.90173
0.026323	0.546153	-1.83099	MeHg_0.25	0.002757	0.314148	-3.18321	MeHg_1 uM	1.8931
0.096048	0.572866	-1.74561	MeHg_0.25	0.020097	0.38232	-2.61561	MeHg_1 uM	1.88722
0.658449	0.838382	-1.19277	MeHg_0.25	0.011041	0.191195	-5.23026	MeHg_1 uM	1.85748
0.061417	0.755514	-1.3236	MeHg_0.25	0.002812	0.490708	-2.03787	MeHg_1 uM	1.85451
0.113783	0.742903	-1.34607	MeHg_0.25	0.003179	0.39337	-2.54214	MeHg_1 uM	1.85092
0.335939	0.773747	-1.29241	MeHg_0.25	0.027225	0.44993	-2.22257	MeHg_1 uM	1.84737
0.158073	0.779815	-1.28236	MeHg_0.25	0.001459	0.326618	-3.06168	MeHg_1 uM	1.81839
0.303379	0.832663	-1.20097	MeHg_0.25	0.001854	0.321173	-3.11359	MeHg_1 uM	1.80743
0.193448	0.545143	-1.83438	MeHg_0.25	0.037361	0.303569	-3.29414	MeHg_1 uM	1.79363
0.182734	0.818641	-1.22154	MeHg_0.25	0.004309	0.484373	-2.06453	MeHg_1 uM	1.73743
0.150659	0.580246	-1.72341	MeHg_0.25	0.018824	0.310088	-3.22489	MeHg_1 uM	1.72921
0.077118	0.608477	-1.64345	MeHg_0.25	0.025043	0.480272	-2.08215	MeHg_1 uM	1.70217
0.055931	1.44995	1.44995	MeHg_0.25	0.002275	2.62539	2.62539	MeHg_1 uM	1.66555
0.073669	0.840939	-1.18915	MeHg_0.25	0.000339	0.440915	-2.26801	MeHg_1 uM	1.62387
0.110944	1.37942	1.37942	MeHg_0.25	0.003351	2.67697	2.67697	MeHg_1 uM	1.5967
0.135385	1.37308	1.37308	MeHg_0.25	0.004204	2.71095	2.71095	MeHg_1 uM	1.576
0.016797	0.250582	-3.9907	MeHg_0.25	0.000675	0.035408	-28.2423	MeHg_1 uM	1.53563
0.04156	0.536144	-1.86517	MeHg_0.25	0.015601	0.427039	-2.34171	MeHg_1 uM	1.51404
0.030719	0.697527	-1.43364	MeHg_0.25	0.00169	0.43795	-2.28336	MeHg_1 uM	1.51216
0.211215	0.599875	-1.66701	MeHg_0.25	0.02922	0.318876	-3.13602	MeHg_1 uM	1.49916
0.071919	0.444963	-2.24738	MeHg_0.25	0.021566	0.295381	-3.38546	MeHg_1 uM	1.47556
0.163582	0.664662	-1.50452	MeHg_0.25	0.029919	0.453309	-2.206	MeHg_1 uM	1.4684
0.177539	0.707786	-1.41286	MeHg_0.25	0.013367	0.408769	-2.44637	MeHg_1 uM	1.44333
0.070752	0.653819	-1.52948	MeHg_0.25	0.011434	0.463307	-2.1584	MeHg_1 uM	1.41903
0.175178	0.673243	-1.48535	MeHg_0.25	0.035982	0.473814	-2.11053	MeHg_1 uM	1.40528
0.21299	0.836357	-1.19566	MeHg_0.25	0.003204	2.14541	2.14541	MeHg_1 uM	1.38998
0.012641	0.828914	-1.2064	MeHg_0.25	6.48E-05	0.469035	-2.13204	MeHg_1 uM	1.38495
0.01236	0.707659	-1.41311	MeHg_0.25	0.000749	0.47636	-2.09925	MeHg_1 uM	1.38346
0.092465	0.741735	-1.34819	MeHg_0.25	0.005531	0.477786	-2.09299	MeHg_1 uM	1.38049
0.095895	0.525476	-1.90304	MeHg_0.25	0.014921	0.296847	-3.36873	MeHg_1 uM	1.35106
0.005191	0.396576	-2.52159	MeHg_0.25	0.003789	0.364792	-2.74129	MeHg_1 uM	1.33927
0.026564	0.63601	-1.5723	MeHg_0.25	0.000695	0.286682	-3.48818	MeHg_1 uM	1.31739
0.281738	0.693369	-1.44223	MeHg_0.25	0.029754	0.377548	-2.64867	MeHg_1 uM	1.28574
0.061704	0.657088	-1.52187	MeHg_0.25	0.007207	0.438414	-2.28095	MeHg_1 uM	1.26806
0.097983	0.500872	-1.99652	MeHg_0.25	0.006423	0.186557	-5.3603	MeHg_1 uM	1.25037
0.147364	0.633921	-1.57748	MeHg_0.25	0.047176	0.486655	-2.05485	MeHg_1 uM	1.20049
0.023189	0.707315	-1.4138	MeHg_0.25	0.000314	0.324971	-3.0772	MeHg_1 uM	1.19004
0.007511	0.610769	-1.63728	MeHg_0.25	0.000978	0.425418	-2.35063	MeHg_1 uM	1.1566
0.112794	0.56917	-1.75694	MeHg_0.25	0.00961	0.273666	-3.65408	MeHg_1 uM	1.12184
0.026864	0.66184	-1.51094	MeHg_0.25	0.002376	0.437078	-2.28792	MeHg_1 uM	1.07223

0.065753	0.582253	-1.71747	MeHg_0.25	0.008373	0.352723	-2.83509	MeHg_1 uM	1.07217
0.096331	0.786558	-1.27136	MeHg_0.25	0.003089	0.492661	-2.02979	MeHg_1 uM	1.07093
0.062266	0.698752	-1.43112	MeHg_0.25	0.006451	0.482606	-2.07208	MeHg_1 uM	1.05472
0.925279	0.964332	-1.03699	MeHg_0.25	0.032906	0.312204	-3.20303	MeHg_1 uM	1.03072
0.066304	0.542754	-1.84246	MeHg_0.25	0.015521	0.372859	-2.68198	MeHg_1 uM	1.02037
0.097436	0.635055	-1.57467	MeHg_0.25	0.024882	0.478275	-2.09085	MeHg_1 uM	1.01916
0.255521	0.806383	-1.24011	MeHg_0.25	0.007334	0.442042	-2.26223	MeHg_1 uM	1.00461
0.455213	0.886636	-1.12786	MeHg_0.25	0.007768	0.486335	-2.0562	MeHg_1 uM	0.997949
0.333481	0.718225	-1.39232	MeHg_0.25	0.020418	0.325858	-3.06882	MeHg_1 uM	0.984023
0.328305	0.903105	-1.10729	MeHg_0.25	0.00064	2.4253	2.4253	MeHg_1 uM	0.977324
0.138559	0.601552	-1.66237	MeHg_0.25	0.020346	0.358461	-2.7897	MeHg_1 uM	0.936019
0.061402	0.439772	-2.2739	MeHg_0.25	0.009564	0.226372	-4.41751	MeHg_1 uM	0.914796
0.165526	0.778426	-1.28464	MeHg_0.25	0.000855	0.265467	-3.76695	MeHg_1 uM	0.897094
0.058969	0.476376	-2.09918	MeHg_0.25	0.024481	0.369012	-2.70994	MeHg_1 uM	0.890572
0.01563	0.468685	-2.13363	MeHg_0.25	0.006879	0.382452	-2.61471	MeHg_1 uM	0.885953
0.107789	0.340869	-2.93368	MeHg_0.25	0.003464	0.039754	-25.155	MeHg_1 uM	0.770905
0.077137	0.710759	-1.40695	MeHg_0.25	0.002909	0.392096	-2.55039	MeHg_1 uM	0.767594
0.092623	0.295158	-3.38802	MeHg_0.25	0.007245	0.060863	-16.4305	MeHg_1 uM	0.760903
0.026007	0.279848	-3.57337	MeHg_0.25	0.005889	0.13898	-7.1953	MeHg_1 uM	0.748004
0.205265	0.623799	-1.60308	MeHg_0.25	0.025413	0.337591	-2.96217	MeHg_1 uM	0.743317
0.015246	0.688111	-1.45325	MeHg_0.25	0.000963	0.449769	-2.22336	MeHg_1 uM	0.734091
0.010077	0.590058	-1.69475	MeHg_0.25	0.002844	0.472521	-2.11631	MeHg_1 uM	0.732626
0.210297	0.65117	-1.5357	MeHg_0.25	0.035845	0.408526	-2.44782	MeHg_1 uM	0.694915
0.01309	0.649052	-1.54071	MeHg_0.25	0.001095	0.425783	-2.34861	MeHg_1 uM	0.647512
0.055698	0.539148	-1.85478	MeHg_0.25	0.005343	0.280648	-3.56318	MeHg_1 uM	0.623446
0.129286	0.582048	-1.71807	MeHg_0.25	0.01909	0.339927	-2.94181	MeHg_1 uM	0.534467
0.378184	0.8062	-1.24039	MeHg_0.25	0.014677	0.4085	-2.44798	MeHg_1 uM	0.514217
0.053393	0.65465	-1.52753	MeHg_0.25	0.004423	0.404854	-2.47003	MeHg_1 uM	0.443677
0.024009	0.773498	-1.29283	MeHg_0.25	0.000311	0.429764	-2.32686	MeHg_1 uM	0.439404
0.014054	0.642111	-1.55736	MeHg_0.25	0.001469	0.437252	-2.28701	MeHg_1 uM	0.37595
0.152676	0.739873	-1.35158	MeHg_0.25	0.001772	0.282026	-3.54577	MeHg_1 uM	0.343928
0.00425	0.588206	-1.70009	MeHg_0.25	0.000161	0.286761	-3.48723	MeHg_1 uM	0.305667
0.006345	0.599662	-1.6676	MeHg_0.25	0.001449	0.466463	-2.14379	MeHg_1 uM	0.237415
0.501609	0.890205	-1.12334	MeHg_0.25	0.00412	2.53623	2.53623	MeHg_1 uM	0.235031
0.129416	0.781394	-1.27976	MeHg_0.25	0.004133	0.465996	-2.14594	MeHg_1 uM	0.222356
0.059565	0.665052	-1.50364	MeHg_0.25	0.005565	0.427429	-2.33957	MeHg_1 uM	0.198111
0.904541	0.977085	-1.02345	MeHg_0.25	0.010235	2.29361	2.29361	MeHg_1 uM	0.169242
0.156348	0.774288	-1.29151	MeHg_0.25	0.007516	0.48034	-2.08186	MeHg_1 uM	0.152915
0.026379	0.62433	-1.60172	MeHg_0.25	0.005973	0.481728	-2.07586	MeHg_1 uM	0.096272
0.818416	1.04006	1.04006	MeHg_0.25	0.008774	2.15122	2.15122	MeHg_1 uM	0.092405
0.007355	0.656961	-1.52216	MeHg_0.25	0.000976	0.484664	-2.06328	MeHg_1 uM	0.02309
0.718233	0.964573	-1.03673	MeHg_0.25	0.000966	2.2458	2.2458	MeHg_1 uM	0.020476

SS(Groups)	F(Treatmer	SS(Treatme	SS(Error)	F(Error)
0.323635	229.153	1.87843	0.016395	1
4.26852	15.2867	1.67082	0.218598	1
0.730844	70.7686	1.75365	0.04956	1
1.22096	49.1782	2.78443	0.113238	1
6.45594	6.313	2.02557	0.641714	1
1.3027	23.6059	1.53076	0.129693	1
2.74045	17.021	2.41319	0.283555	1
1.4128	20.4051	1.61185	0.157985	1
2.83322	12.587	1.9957	0.317105	1
4.09772	7.49175	1.73629	0.463521	1
0.978208	30.7283	1.74285	0.113436	1
2.26365	15.1179	2.04343	0.270332	1
1.08205	26.7278	1.74934	0.1309	1
2.5401	13.5933	2.19311	0.322675	1
1.49472	36.903	3.54076	0.191896	1
2.00718	13.4372	1.73838	0.258741	1
0.251289	173.02	2.80257	0.032396	1
1.36798	28.1627	2.49284	0.177031	1
0.647785	150.383	6.58983	0.087641	1
3.65921	10.6744	2.65574	0.49759	1
1.31777	22.877	2.05031	0.179246	1
6.34496	17.9146	7.89749	0.881682	1
0.167585	167.113	1.97498	0.023637	1
1.03163	24.5997	1.84037	0.149626	1
4.78585	13.9703	4.90043	0.70155	1
0.592858	35.1241	1.54279	0.087848	1
2.02384	25.3866	3.83794	0.302359	1
1.96256	12.5024	1.86179	0.297828	1
3.37106	13.1329	3.39633	0.517227	1
1.85488	67.3056	10.1787	0.302462	1
0.411397	57.2398	2.03433	0.071081	1
1.62596	18.7315	2.63927	0.2818	1
2.49142	10.0002	2.26631	0.453253	1
0.946054	19.2996	1.69906	0.176072	1
0.77518	59.4487	4.33572	0.145864	1
1.6989	18.2396	3.11315	0.341361	1
0.519359	44.4599	2.41443	0.108612	1
1.146	29.4358	3.54297	0.240726	1
0.891274	47.2471	4.44867	0.188315	1
0.751213	48.4188	3.8564	0.159294	1
0.738342	24.9003	1.99651	0.16036	1
1.16888	21.7577	2.79214	0.256658	1
0.687202	24.055	1.8226	0.151536	1
3.8984	10.554	4.55963	0.864054	1
0.554311	54.222	3.36362	0.124069	1
0.455271	40.6425	2.13125	0.104878	1

0.206035	70.5951	1.69606	0.04805	1
1.41002	13.5337	2.23279	0.329959	1
1.08856	20.2297	2.62479	0.259499	1
1.8991	7.80196	1.7718	0.454194	1
1.66513	8.71377	1.74387	0.400257	1
1.19394	11.0205	1.58619	0.287862	1
0.962097	14.3627	1.70392	0.23727	1
2.5471	12.3643	3.92771	0.635329	1
4.03398	9.97647	5.04947	1.01228	1
0.458155	31.6004	1.8232	0.115391	1
0.23503	55.7543	1.66371	0.05968	1
0.508206	86.3013	5.56952	0.129072	1
0.435541	38.8368	2.14908	0.110672	1
0.9934	13.996	1.77289	0.253343	1
2.70053	13.7588	4.80603	0.69861	1
0.420453	29.5951	1.62019	0.10949	1
0.136041	106.593	1.92445	0.036108	1
0.562036	33.7931	2.64313	0.15643	1
1.31296	16.622	3.06732	0.369068	1
1.79005	59.4764	15.09	0.507428	1
3.34015	5.82855	2.78268	0.954846	1
1.93542	13.7992	3.83	0.555104	1
2.34168	20.4107	7.12297	0.697963	1
3.26609	8.19343	4.142	1.01105	1
0.304513	71.3019	3.40689	0.095562	1
0.642027	23.5497	2.37695	0.201866	1
3.54412	5.40639	3.07653	1.13811	1
0.965391	10.7676	1.67427	0.310983	1
0.353071	47.2702	2.72996	0.115504	1
0.502662	19.2261	1.59117	0.165522	1
2.95466	4.89345	2.38942	0.976579	1
2.46875	4.76148	1.9576	0.822263	1
3.39337	7.20497	4.07777	1.13193	1
0.868707	11.6634	1.71045	0.293301	1
1.41709	26.0506	6.30994	0.484437	1
0.466664	28.9923	2.35139	0.162208	1
0.173976	111.674	3.43165	0.061459	1
0.615383	14.0426	1.53876	0.219156	1
1.10606	15.8728	3.12754	0.394074	1
0.472118	31.5902	2.67746	0.169512	1
3.82865	14.2801	9.91957	1.38929	1
1.46463	6.53071	1.74052	0.533027	1
0.703611	15.3437	1.98725	0.259031	1
0.429159	20.8252	1.67395	0.160761	1
0.513543	20.8031	2.03817	0.195949	1
1.2339	14.1153	3.37069	0.477593	1
3.1865	6.52147	4.03472	1.23737	1

1.06059	10.3522	2.18041	0.421247	1
0.516231	20.9032	2.17696	0.20829	1
0.583771	14.0183	1.67664	0.239207	1
0.751932	12.9275	2.00379	0.310005	1
0.949983	9.19179	1.81942	0.39588	1
1.97185	4.11146	1.70039	0.827147	1
1.14166	13.1425	3.16031	0.480931	1
0.40759	27.9341	2.40823	0.172422	1
0.891791	18.2122	3.47043	0.381112	1
0.098735	153.221	3.34339	0.043641	1
0.700379	12.007	1.86998	0.31148	1
5.57335	13.9282	17.345	2.49063	1
3.02809	15.9263	10.8476	1.36222	1
0.883055	31.5896	6.30597	0.399243	1
0.767059	10.6749	1.88242	0.352682	1
5.75383	13.8855	18.465	2.65959	1
0.481464	18.7275	2.0973	0.223982	1
0.848791	9.82597	1.95828	0.398592	1
6.94806	10.2178	16.7002	3.26882	1
1.11976	9.50951	2.54193	0.534608	1
2.95211	7.42422	5.25303	1.41511	1
0.141167	103.116	3.50313	0.067946	1
0.743931	14.5856	2.61846	0.359047	1
1.16096	8.78507	2.48627	0.566022	1
1.71945	9.2296	3.90079	0.845278	1
1.83108	8.28986	3.73521	0.901152	1
3.34818	11.8106	9.92457	1.68062	1
0.629863	13.1739	2.09934	0.318713	1
1.35238	7.84657	2.68585	0.684592	1
0.821283	17.5803	3.68572	0.419302	1
1.0415	6.47999	1.73414	0.53523	1
0.11333	106.454	3.12955	0.058796	1
1.02555	6.64108	1.7764	0.534974	1
0.940703	15.1307	3.75891	0.496859	1
0.630404	15.0169	2.50834	0.334069	1
2.52649	16.1971	11.1569	1.37764	1
3.0759	11.7794	9.97158	1.69305	1
3.39056	39.686	37.5301	1.89135	1
1.81823	6.57425	3.35319	1.0201	1
2.40701	16.029	11.08	1.38249	1
0.291755	25.6203	2.16645	0.16912	1
0.356722	44.4202	4.68228	0.210817	1
0.505597	41.74	6.237	0.29885	1
1.0653	7.59476	2.42404	0.638346	1
0.984709	7.32009	2.16908	0.592637	1
3.27024	4.88564	4.87315	1.99489	1
0.031356	180.432	1.7389	0.019275	1

0.39008	16.1306	1.96404	0.243516	1
1.51984	20.3326	9.66616	0.950804	1
1.08492	10.0541	3.43666	0.683637	1
1.88314	5.19071	3.08488	1.18862	1
1.02267	8.93652	2.91254	0.651829	1
0.316878	29.2529	2.95722	0.202183	1
3.56464	4.26406	4.91663	2.30608	1
0.2992	20.0145	2.00973	0.200827	1
0.71076	30.6568	7.40553	0.483124	1
0.838825	17.193	4.90162	0.570187	1
0.763426	7.77562	2.03794	0.524186	1
1.16734	12.3304	5.02513	0.815083	1
0.733293	6.50332	1.68885	0.51938	1
0.445072	10.5867	1.67513	0.316458	1
1.01467	6.41355	2.3509	0.733105	1
0.377989	12.7431	1.76687	0.277306	1
0.26673	34.9784	3.4371	0.196527	1
5.19149	4.62078	8.86537	3.83717	1
0.816742	9.46068	2.87761	0.60833	1
0.593363	7.87404	1.7684	0.449172	1
0.397677	10.804	1.62745	0.301268	1
2.05191	7.87377	6.13283	1.55779	1
0.496936	28.0091	5.30497	0.378804	1
0.448997	22.0267	3.79	0.344129	1
1.10079	9.51877	4.02197	0.84506	1
2.48591	9.68158	9.28643	1.91837	1
0.512942	9.91298	1.97515	0.398497	1
1.90766	4.28885	3.17853	1.48223	1
3.93934	7.48378	11.5755	3.0935	1
2.21749	10.067	8.93664	1.77543	1
0.7422	12.2829	3.66253	0.596364	1
0.718773	8.81547	2.56122	0.581073	1
0.320165	20.9842	2.77661	0.264638	1
0.424864	24.2675	4.28775	0.353373	1
0.595234	23.1982	5.98566	0.516046	1
1.14497	6.52179	3.2656	1.00144	1
0.503934	9.41354	2.09448	0.444992	1
1.45402	5.57342	3.58183	1.28533	1
0.643778	5.54204	1.58153	0.570738	1
0.169223	21.1823	1.59384	0.150488	1
1.17312	6.73975	3.5443	1.05176	1
1.53337	5.41122	3.72168	1.37554	1
0.107332	126.192	6.08331	0.096414	1
0.223874	16.7659	1.70186	0.203015	1
0.238978	16.8209	1.82819	0.217371	1
1.3418	18.4579	11.4842	1.24437	1
9.585	4.00782	17.9422	8.95358	1

0.557511	6.06307	1.58481	0.522773	1
0.30097	11.2643	1.59771	0.283677	1
0.935045	4.98914	2.24598	0.900346	1
2.54547	5.0657	6.2127	2.45285	1
0.907796	6.5852	3.09156	0.938942	1
0.387015	11.4315	2.3003	0.402451	1
0.241236	13.2463	1.68031	0.253702	1
0.365711	21.6821	4.18857	0.386361	1
0.778274	7.05771	2.91054	0.824783	1
1.58604	12.0741	10.3097	1.70773	1
0.136882	21.7594	1.60608	0.147621	1
0.250708	20.9454	2.83708	0.270901	1
0.635719	6.0338	2.07636	0.688242	1
0.233755	33.5364	4.31112	0.257101	1
0.271806	30.8791	4.64367	0.300765	1
1.69089	4.7072	4.43756	1.88544	1
0.167642	18.1398	1.75029	0.192977	1
1.01597	7.29728	4.2874	1.17507	1
0.468417	6.35837	1.74974	0.550375	1
0.201691	24.4441	2.96008	0.242191	1
0.052454	72.0058	2.32592	0.064604	1
0.24788	20.2803	3.14843	0.310491	1
0.283968	17.9955	3.24248	0.360366	1
1.17622	45.9391	35.1871	1.5319	1
0.419302	8.75057	2.42341	0.553886	1
0.114407	28.2828	2.13982	0.151316	1
1.10556	5.55107	4.09368	1.47492	1
1.02246	6.94083	4.80951	1.38586	1
0.526863	5.44867	1.95499	0.717604	1
0.403057	9.1017	2.54169	0.558508	1
0.267413	9.84227	1.85476	0.376897	1
0.507189	4.83187	1.7439	0.721833	1
0.126543	34.3397	3.12626	0.182079	1
0.016464	163.296	1.94116	0.023775	1
0.055119	43.1601	1.71956	0.079683	1
0.158707	14.9947	1.72385	0.229927	1
0.742293	8.39217	4.61079	1.09883	1
0.233093	22.4745	3.91157	0.34809	1
0.143286	45.9408	4.99674	0.217529	1
0.696744	5.57841	3.02295	1.0838	1
0.210715	12.7765	2.12309	0.332342	1
0.807422	13.7712	8.89272	1.29149	1
0.484259	4.10904	1.65752	0.806769	1
0.069561	70.7958	4.1382	0.116905	1
0.070329	37.8012	2.29858	0.121614	1
0.542321	10.9061	5.27222	0.966842	1
0.097703	23.4699	2.13859	0.182241	1

0.309774	11.7399	3.39192	0.577843	1
0.082224	21.0825	1.61867	0.153556	1
0.128558	13.597	1.6573	0.243775	1
0.851788	6.61968	5.47051	1.6528	1
0.378718	8.34274	3.09646	0.742312	1
0.282501	6.23644	1.72868	0.554381	1
0.165241	13.592	2.23565	0.328966	1
0.132215	14.0566	1.86232	0.264974	1
0.557295	7.31878	4.14494	1.13269	1
0.051223	70.3384	3.6865	0.104822	1
0.442816	6.94629	3.28618	0.946169	1
0.579638	10.9149	6.91597	1.26725	1
0.122479	45.4153	6.20047	0.273056	1
0.446404	6.68096	3.34887	1.00251	1
0.194845	14.5693	3.20417	0.439853	1
1.30704	19.8586	33.6696	3.39093	1
0.099786	21.5655	2.80349	0.259997	1
1.46129	12.8072	24.5957	3.84093	1
0.635678	14.71	12.501	1.69966	1
0.452633	6.08044	3.7026	1.21787	1
0.0387	37.8615	1.99598	0.105436	1
0.060325	22.4881	1.85168	0.164681	1
0.359336	4.84128	2.5034	1.03419	1
0.041693	35.35	2.27615	0.128778	1
0.208107	15.1048	5.04202	0.667603	1
0.268966	7.22318	3.635	1.00648	1
0.152008	9.22419	2.72677	0.591222	1
0.067575	16.7822	2.55605	0.304615	1
0.01444	71.2217	2.34061	0.065727	1
0.026518	30.341	2.14012	0.141071	1
0.062712	29.9424	5.4597	0.36468	1
0.015687	95.6301	4.90793	0.102644	1
0.014125	31.6995	1.886	0.118992	1
0.036473	26.501	4.11251	0.310367	1
0.023268	18.1218	1.89631	0.209285	1
0.030266	14.7717	2.25672	0.305546	1
0.034819	14.3435	2.95094	0.411467	1
0.020574	12.8561	1.72976	0.269096	1
0.011296	14.5917	1.71213	0.234672	1
0.014817	14.4925	2.32389	0.320702	1
0.001007	37.8586	1.65185	0.087264	1
0.001109	52.6656	2.85175	0.108297	1

Table 2
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Table 2

GO Term	Count	P value	Genes
Regulation of Apoptosis	18	0,0068	ARHGEF3, TBX3, ERBB3, MITF, BNIP3, CDH1, IGF2, IFI16, HGF, GCH1, AMIGO2, SERPINB9, KRT18, MSX1, ETS1, VEGFA, PERP, IGFBP3
Regulation of Cell Proliferation	17	0,0123	RBP4, LYN, TBX3, ERBB3, MITF, IGF2, KDR, RERG, MSX1, ADM, ETS1, VEGFA, BNC1, ADAMTS1, FABP1, IGFBP3, FIGF
Vasculature Development	12	0,0001	PLAT, APOB, HAND1, TBX3, EPAS1, FOXF1, LEPR, VEGFA, COL3A1, LOX, FIGF, KDR
Skeletal System Development	12	0,0008	RBP4, MSX1, LGALS3, TBX3, HOXB6, COL3A1, STC1, IGF2, POSTN, FRZB, IGFBP3, AHSG
Heart Development	11	0,0001	RBP4, ACTC1, MSX1, HAND1, TBX3, ADM, PKP2, ERBB3, GATA6, COL3A1, ADAMTS1
Glucose Metabolic Process	9	0,0003	PDK1, RBP4, LDHA, PGM5, PYGL, HK2, PFKP, IGF2, PGK1
Lung Development	7	0,0008	RBP4, EPAS1, GATA6, FOXF1, VEGFA, LOX, KDR
Epithelium Development	7	0,0386	F11R, FREM2, GATA6, FOXF1, VEGFA, DSP, KDR
Mesoderm Development	5	0,0088	HAND1, TBX3, FOXF1, VEGFA, SNAI2

Table 3
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Table 3

Term	Gene Symbol	Fold Change*	
		0.25 µM MeHg vs VC	1 µM MeHg vs VC
Brain Development	SEPP1	-2,17	-4,13
	DDIT4	-1,20	-3,11
	AK4	-1,41	-3,08
	FRZB	-1,29	-2,19
Neuronal nucleas development	PITX2	-2,08	-4,90
Nervous system development	ERBB3	-1,86	-2,89
	UGT8	-1,67	-2,14
	APOB	-3,59	-5,72
	APOA1	-2,63	-2,90
	VEGFA	-1,28	-3,06

* p value < 0.05

Table 4

Click here to download Table: Table 4.pdf

Sr. No.	Medium / Buffer Name	Composition	
		Contents	Amount
1	MEF Medium	DMEM High glucose	
		FCS	10%
		Penicillin	100 units/ml
		Streptomycin	100 µg / ml
		L- Glutamine	2 mM
2	H9 Culture Medium	DMEM F12	
		KOSR	20%
		NEAA	1%
		Glutamax	1 X
		ß Mercaptoethanol	0.1 mM
		Penicillin	100 units/ml
		Streptomycin	100 µg / ml
		bFGF	4 ng /ml
3	RD Medium	H9 culture medium without bFGF	
4	Wash Medium	DMEM/F12	
		Knockout Serum Replacment	20%
		1x GlutaMAX	1 X
		MEM non-essential amino acids	
		HEPES	15mM
		β-Mercaptoethanol	90 µM
5	KCM Medium	DMEM	
		FBS	10%
		incubated for 24h on MEFs	
6	Knockout Serum Replacement (KSR)	Knockout DMEM/F12	
		Knockout serum replacement	15%
		1x GlutaMAX	
		1x MEM non-essential amino acids	
		β-Mercaptoethanol	15 µm
		Noggin	35 ng/ml
		Dorsomorphin	600 nM
		SB431542	10 µM
7	N2-S	DMEM/F-12	
		Apotransferin	100 µg/ml
		Glucose	1.55 mg/ml
		Putrescine	10 mM
		Selenium	500 µM
		Progesteron	20 µM
		GlutaMAX	200 µM
		Insulin	25 µg/ml
8	L 1 Buffer	Tris pH 8	50 mM
		EDTA	2 mM
		NP-40	0.10%
		Glycerol	10%
9	L2 Buffer	Tris pH 8	50 mM
		EDTA	10 mM
		SDS	1%
10	Elution Buffer	$NaHCO_3$	100 mM
		SDS	1%
11	Wash Buffer	Tris	20 mM
		EDTA	2 mM
		SDS	0.10%
		NP-40	0.50%
		NaCl	150 mM
12	Final Wash Buffer	Tris	20 mM
		EDTA	2 mM
		SDS	0.10%
		NP-40	0.50%
		NaCl	500 mM
13	Stem Cell Medium	mTESAR™ basal medium	400 ml
		mTESAR™ supplement	100 ml

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number
DMEM/F-12	Life Technologies	11320082
KOSR	Life Technologies	10828028
GlutaMAX	Life Technologies	35050061
NEAA	Life Technologies	11140050
DPBS	Life Technologies	14190-0144
mTeSR medium	Stemcell Technologies	5850
Pluronic F-127	Sigma	P2443-250G
V bottom plate	VWR	734-0483
Vbottom plate lid	VWR	634-0011
Pen/Strep	Life Technologies	15140-122
Distilled Water	Life Technologies	15230-089.
Human FGF-2 (bFGF)	Millipore	GF003AF-100UG
Filter 0.22 µm	Millipore	SCGPU02RE
StemPro EZPassage™ Disposablte	Invitrogen	23181010
BD MatrigelTM, hESC qualified Matrix	Stemcell Technologies	354277
DMSO	Sigma	D-2650
RNA*later* Stabilization Solution	Life Technologies	AM7020
70 µm Cell Strainer	Becton Dickinson	352350
35 µm Lid cell strainer, 5 ml tube	Becton Dickinson	352235
50 ml sterile Polypropylene tube	Greiner Bio-One	227261
T75 flask	Greiner Bio-One	658175
TRIzol	Life Technologies	10296010
96 well optical bottom plates	Thermo Scientific	165305
CellTiter-Blue	Promega	G8081
Accutase	PAA	L11-007
Apotransferin	Sigma-Aldrich	T-2036
Dispase	Worthington Biochemicals	LS002104
Dorsomorphin	Tocris Bioscience	3093
EDTA	Roth	8043.2
FBS	PAA	A15-101
FGF-2	R&D Systems	233-FB
Gelatine	Sigma-Aldrich	G1890-100G
Glucose	Sigma-Aldrich	G7021-100G
GlutaMAX	Gibco Invitrogen	35050-038
HEPES	Gibco Invitrogen	15630-056
Insulin	Sigma-Aldrich	I-6634
Knockout DMEM	Gibco Invitrogen	10829-018
Matrigel	BD Biosciences	354234
Noggin	R&D Systems	719-NG
PBS	Biochrom AG	L1825
Progesteron	Sigma-Aldrich	P7556
Putrescine	Sigma-Aldrich	P-5780

ROCK inhibitor Y-27632	Tocris Biosciences	1254
SB431542	Tocris Biosciences	1614
SDS	Bio-Rad	161-0416
Selenium	Sigma-Aldrich	S-5261
β-Mercaptoethanol	Gibco Invitrogen	31350-010

List of Kits

RNeasy Mini Kit (250)	QIAGEN	74106
GeneChip Hybridization, Wash, and Stain Kit	Affymetrix	900721, 22, 23
Rnase-Free DNase Set	QIAGEN	79254

List of equipment.

Inverted microscope	Olympus	
Genechip Hybridisation Oven - 645	Affymetrix	
Genechip Fluidics Station-450	Affymetrix	
Affymetrix Gene-Chip Scanner-3000-7 G	Affymetrix	
Spectramax M5	Molecular Devices	

List of softwares

Prism 4
Affymetrix GCOS
Partek Genomic Suite 6.25
Online tools for Functional annotation DAVID Onto-tools Intelligent Systems and Bioinformatics Laboratory

Comments/Description
Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12
Knockout Serum Replacement
GlutaMAX supplement
MEM Nonessential Amino Acids Solution
Dulbecco's Phosphate-Buffered Saline, without calcium, without magnesium
Plate,Microwell,V BTTM,96 Well,Sterile 1 * 50 ST
Lid, Microtitre plates, Cond. Ring 1 * 50 ST
Penicillin- Streptomycin, Liquid
Sterile Distilled Water
Fibroblast Growth Factor basic, human recombinant, animal-free
Stericup-GP, 0.22 μm, polyethersulfone, 250 ml, radio- sterilized
5 ml vial
It stabilizes and protect the RNA integrity in unfrozen samples.
Cell strainer with 70 μm Nylon mesh
5 ml polystyrene round bottom test tube, with a cell strainer cap (35 μm)
50 ml Polypropylene tube with conical bottom, Sterile
CELLSTAR Filter Cap Cell Culture 75 cm2 Flasks

This kit provides all reagents required for hybridization wash and staining of microarrays.

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Editorial comments:

1) Please ensure that references 33-38 are in the correct format and that the links are live.

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2) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

Done

3) If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.

The figures and tables are original and not published previously.

Reviewer #1

1.) The title is misleading - neither drug safety (only data are for an environmental toxicant) nor systems biology are addressed. The claims are repeated in abstract and discussion and need to be substantiated or dropped.

Thank you for this comment. The title was changed to "Human Pluripotent Stem cell Based Developmental Toxicity Assays for chemical Safety Screening and Systems Biology data generation" and the specific parts in abstract and discussion has been changed.

2) It is not clear, why the two methods are put into one paper; the transcriptomics approaches appear to be standard and do not really require a detailed protocol. The authors should consider splitting. If not separated, the added value of the combination and a comparison of the systems should be added.
We agree with the referee that we have not sufficiently explained this. Therefore we have introduced a sentence in the discussion (see page 16).

3) Most reagents lack sources. The descriptions are very brief, more detail and precision are needed. It is advised to reread each and every step to eliminate ambiguities.
The details are included in the excel table named material list and table 4 composition of culture media.

4) Information on critical steps and possible protocol variants should be added. Documentation according to Good Cell Culture Practices is advised.
We appreciate this advice and have added information on critical steps directly in the protocol section.

5) The acknowledgement refers to German funding while the work was apparently EU funded.

Our acknowledgements are correct as previous papers were founded as ESNATS but the efforts for this paper were all by BMBF.

Reviewer #2:
The authors describe standardized pluripotent stem cell-based assays for developmental toxicity. Examples applying these tests to methyl mercury toxicity are provided. This is a sound scientific work, I have no major scientific concerns.

Thank you very much for the work appreciation.

The impact of methyl mercury on gene expression in the assay system is very nicely described (Fig. 2). It would have been of interest for the reader to see the impact of methyl mercury on histone methylation pattern (Fig. 3) and protein expression pattern (Fig.4)
We agree that reader will be interested to see the impact methyl mercury on histone methylation pattern- as primary aim of this manuscript is to explain the protocols in detail we will focus this point in the upcoming manuscript.

Reviewer #3:
Manuscript Summary:
This manuscript is well written and the topic is relevant as well as important to the field of pluripotent stem cells and drug development. However there are several concerns.
Thanks for the appreciation.

Major Concerns:
There is no novelty. Several similar papers are available in the literature even in form of protocols.
We totally agree that there is no novelty in this manuscript, but please note that under the ESNATS project the novel systems (UKK and UKN1) have been established to investigate developmental toxicity and developmental neurotoxicity. This system is validated with the known developmental toxicants and developemental neurotoxicants and several papers have been published by our labs (we already provided reference in current manuscript). But the detail protocol has not been discussed in any of these publications. So we took opportunity in this manuscript to put down detail protocols of UKK and UKN1 system according to the aims of the JoVE and as requested from us by the journal.

Minor Concerns:
Authors have mentioned about both feeder (MEF) and feeder free(Matrigel) culture systems EB's from which culture system have been used for this study and why?

For cost reduction H9 cells are routinely maintained on MEF feeder cells. These H9 cells are transferred on matrigel plates to get rid of mouse embryonic fibroblast.

The EB's are formed from H9 cells cultured on the Matrigel plates.

The corrections have been made in the protocol section.

Selecting uniform size EB's is not possible with this protocol based on the visual screening. Authors need to address this point.

All EBs formed with the method are not in uniform size that's why we need to select uniform size EBs for further experiment. In this protocol we seed equal number of H9 cell clumps in V bottom plate. Approximately 50% EBs formed with this method are uniform in size (± 20%).

The corrections have been made in the note section of point 2.

Is it not necessary to see the drug effect on the germ layer development also since, the title says developmental toxicity?

We totally agree with this point. The H9 microarray time kinetics data of EBs till day 21 have been obtained. On day 14, > 90% of genes related to ectoderm, mesoderm and endoderm have shown the peak expression and for this reason day 14 has been chosen as stop point. The various known developmental toxicants such as thalidomide, valproic acid, methyl mercury, cytosine arabinoside have been tested in this system and results have been published by our laboratories (references included in current manuscript). Other than these we have also tested belinostat, entinostat, panbinostat, mercury chloride, etc. in these system and the results will be soon published.

The authors need to expose the cells from day 0 to see the drug effect on the germ layer formation.

Yes we start the drug exposure from day 0. Please refer the note in point number 4.1.

The number of compounds tested is very less to reach a logical conclusion.

Yes we totally agree with this point. We have tested around 12 compounds in these systems. As this manuscript is designed to explain the protocol in detail and just provide one representative example, we have provided one example of each system.

The reviewer would like to see images of EBs post drug treatment.

Unfortunately at this time point of time we don't have images post EB treatment for methyl mercury. The video will capture the EB formation method as well as the images of the EBs formed. That time we can also expose EBs to methyl mercury and include them in video.

The images post thalidomide treatment using this system has been already published by our lab (Meganathan et al., *Plos One.* 7 (8), doi:10.1371/journal.pone.0044228, (2012)).