Editorial comments:

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

**These are Online seurces and rthe journal advise us to put them in a such way; Minor deletions are made and the live functioning of the links have been tested.**

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)).**