We thank the reviewers for their comments. Our responses are in **red**.

**Editorial comments:**  
  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you, corrected.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Our figures were adapted from images published in *Cell Reports*. According the policy of *Cell Reports*, we are permitted to use such images in other journals as long as we cite the original reference.

3. Please provide an email address for each author.

L.T.A. [layteng@stanford.edu](mailto:layteng@stanford.edu)

A.P. apalaria@stanford.edu

K.M.L. [kyleloh@stanford.edu](mailto:kyleloh@stanford.edu)

4. Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

Thank you for the comment. First, we have now stated advantages of our approaches over alternative techniques. Second, we added references to previous studies that we compared our approach with. These references can be found in line 78.

5. Please spell out each abbreviation the first time it is used.

Thank you, corrected.

6. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Thank you, corrected. Please refer to line 190.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Geltrex, Accutase, etc.

Thank you, corrected.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Thank you, corrected.

9. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

There are no personal pronouns in the protocol text now.

10. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

We have made these changes.

11. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:  
Line 84: Please specify the pore size of the filter.  
Line 85: Listing an approximate volume to prepare would be helpful.  
Line 91: This step is unclear. Please specify the small molecules and growth factors used in this step.  
Line 94: Please specify centrifugation parameters.  
Line 99: How to mix the medium? Vortex?  
Line 138: Instead of writing “see above”, please state the specific step.  
Line 145: Please specify the volume of mTeSR1 used.

Thank you, corrected.

12. Please consider describing FACS in the protocol because FACS data are presented in the Representative Results section.

The FACS protocol for intracellular FACS has been added in Section 5.

13. Please include single-line spaces between all paragraphs, headings, steps, etc.

Thank you, corrected.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Yes, we have highlighted sections detailing essential steps for the video.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

This has been done.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

This has been done.

17. Figure 1: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend. Please define all abbreviations in the figure legend.

This has been done.

18. Tables 1 and 2: Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

This has been done.

19. Please provide a table of the essential supplies, reagents, and equipment. It should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.  
This has been done.

**Reviewers' comments:**  
  
Reviewer #1:  
  
Manuscript Summary:  
The paper of Ou et al. describes the differentiation of pluripotent stem cells into hepatocyte-like cells by using a multistep protocol. The paper is well designed and written and it is really interesting for its possible application for regenerative medicine purposes and also for drug discovery and screening.  
  
Minor Concerns:  
1. It would be really useful to have the manufacturers and references of the products used as supplementary material in order researcher can exactly reproduce the protocol.

This has been done.

2. Since the authors have a wide experience in differentiating and characterizing the cells, they should include more detailed characterization (i.e. immunofluorescence, mRNA, enzymatic activities) in the figures and show them in the video.

This has been done.

3. ALthough the authors have included important references at the end of the introduction, maybe it could be useful to document some of the statements with a specific reference rather than all the references at the end.

We are not sure what the reviewer is asking here. All references have been listed at the end and they have been referred to in the text at the appropriate locations.  
  
  
Reviewer #2:  
  
Manuscript Summary:  
In their manuscript, Ou et al, described an "Efficient differentiation of human pluripotent stem cells into liver cells". As it is explained in the introduction of the manuscript, it is important to develop alternatives to primary hepatocytes for the treatment of liver failure or for drug development studies. Methods published until now have described differentiation of hPSCs with various outcomes for the cells generated. This manuscript complements a previously published manuscript from the same group entitled "A Roadmap for Human Liver Differentiation from Pluripotent Stem Cells" and described in more details the method of differentiation. Overall the manuscript is well written and provides sufficient information for any reader who wants to differentiate hPSCs into hepatocytes-like cells.  
  
Major Concerns:  
This manuscript guides well the author through the differentiation but supplementary information should be provided to help better the readers to generate hepatocytes-like cells from hPSCs and to be able to track their progress.

1. Together with the manuscript, the authors should provide phase-contrast images that will guide better the readers during the process. Like it is mentioned several times in the manuscript, cell density plays an important role in the success of the differentiation, therefore some more details should be given. The authors provide a wide range of plating ratio (40.000-80.000 cells/well of a 12 well plate). This information is important but as cell proliferation vary between cell lines, it would also be valuable to give the optimal number of cells on the 1st day of APS treatment.

This is a good point. Images will be incorporated.

2. Related to the density and to better illustrate, it is necessary that the authors provide pictures of lower magnification to show the optimal density at the beginning of each stage; it would also be informative to show pictures of suboptimal cells density at each stage that would lead to less efficient differentiation. Such information would provide valuable help to readers who want to reproduce the method described in the manuscript.

Images showing suboptimal and optimal densities at the start of the differentiation protocol have been added (Figure 4). The first few steps are critical as there is quite a bit of cell death. A lower magnification image at day 3 (Figure 4) has been provided to check oneself for optimal cell densities. Death at other steps is very less and is variable in different hands, hence, it is difficult to put an exact quantification to it. However, lower magnification images have been provided at critical steps as an example of optimal densities (Figure 4).

Lower magnification images have been provided.

3. Together with the FACS in figure 2, the authors should add immunostaining for each stage using 1 or 2 typical markers that would define best each stage.

This has been done.

4. The authors should provide the brand and catalogue number of each reagents (medium, small molecules…) used in the described method. Different activities can be observed between different suppliers, and for a better start, these details should be provided.

This has been done.

5. The pictures in Figure1 are not providing enough information on cell morphology. These pictures should be replaced.

Additional lower magnification images have been added (Figure 1).  
  
Minor Concerns:  
In the chapter: "Passaging and seeding of hPSCs for liver differentiation", the 4th bullet point should clarify the type of pipette such as serological (5 or 10 ml) or 1ml pipette tip, used to detach the cells as it is also described in the 9th bullet.  
In the same chapter, in the 9th bullet point, it is said that cells are dissociated into single cells and in the 12th point, cells are forming clumps, unless otherwise the authors should correct.

This has been corrected.  
  
Reviewer #3:  
  
Manuscript Summary:  
The manuscript "Efficient differentiation of human pluripotent stem cells into liver cells" by Ou et al is an invited submission which describes in detail the generation of PSC derived hepatocyte-like cells. The investigators provide a detailed protocol to describe and delineate a platform that supports rapid differentiation into hepatocyte-like cells. Overall the submission is well crafted and detailed. Overall the manuscript should be accepted with minor revision to fill in a few details which would be helpful in reproducing the author's protocol and differentiation methodology.  
  
Major Concerns:  
1. The authors describe the culture of human induced pluripotent stem cells GelTrex and mTeSR. As many labs use a variety of culture matrixes (e.g. Matrigel (similar) or Laminin 511 as the backbone) of their mTESR iPSC cultures, do these other iPSC culture models support the homogeneity required for the rest of the differentiation platform? Has that been tested? If so that should be offered as an alternative for pluripotent stem culture. In addition have the authors examined whether the use of E8 or more traditional media conditions using SRKO produce PSC cells of enough quality and/or homogeneity to support the use of this differentiation protocol.

We thank the reviewer for the questions. We have maintained hPSCs using E8 and tested their differentiation efficiencies up to the endoderm stage. We could obtain highly pure populations of SOX17+ endoderm cells. However, we have not tested SRKO.

As for the matrices used, we have largely used growth factor reduced Geltrex, which is also derived from sarcoma, like Matrigel. Thus, we speculate that cells may differentiate fine on Matrigel. Apart from Geltrex, we have also successfully maintained hPSCs on vitronectin.

Minor Concerns:  
1. The authors should include a library of antibodies for both IF and FACS characterization to determine the efficiency of differentiation at each step of the bifurcations of their differentiation that they describe in their manuscript. They show figures of them but do not include this information. Are there certain steps that the readers who are trying to reproduce this protocol should stop and characterize the efficiency and if below a certain threshold that they should first focus on those steps (i.e. Endoderm or liver bud). What specific tools (IF, FACS, qRTPCR) should be used at each step of the protocol for characterization and the authors should include that information with specific details to evaluate (with protocols). Which specific antibodies or PCR combinations (company/number/concentration and protocols of use) do the authors recommend?

This will be added as a reference to our previous publication.

2. The authors similarly give the media information and describe the preparation of the base media but include abbreviations that are nor defined (e.g. PVA, KOSR) and do include which company and the catalog number of the reagents they use for all media combinations. This should be included and be clearly spelled out.  
3. The authors in instruction step 11 recommended a desired cell concentration and recommend a cell number for use in a 12-well plate. They should include at this step the surface area of the plate as there is a small variability in surface area based on the manufacture (i.e. X cells/cm2). To make this easier for the reader additional info for different well sizes (that the authors have been successful using their protocol) should be included. In addition does this protocol work similarly well across well-sizes as most stem cell differentiation protocols have peak efficiency for the 12-well-6-well size. Does this protocol have similar efficiency levels down to 96 or 384 well sizes?

The differentiation protocol ahs been tested for 12 well and 6 well plates and works well on them. We have not tested the efficiencies in 96 or 384 well plates but we surmise that as we scale down the cell numbers for differentiation, the efficiencies are comparable.

4. The authors use thiazovivin (ROCK inhibitor) to prevent PSC loss during replating as single cells. Have the authors tested other ROCK inhibitors such as Y-27632 (more commonly used) to see if this is compatible with their platform. Each ROCK inhibitor likely leads to different levels of cell loss and subsequent cell density. How many hours after initial plating do you wait before differentiation initiation (as overnight as you describe is pretty variable in timing) and may impact ultimately the final concentration of PSC at the initiation of differentiation. Pictures of the plate at this stage would be very helpful in giving readers an idea of the ideal final density and spacing between PSC and PSC islands given the known importance of cell density in differentiation outcomes (as described as well as by the authors in their discussion). Moreover pictures of homogeneity versus heterogeneity would also add value for the readers given the importance of this step.

5. For step 2 (line 173) please define APS before using the first time. Similarly for figure 1 the authors should define the abbreviations for all steps.

This has been done.

6. When media is being transitioned from cocktail X to cocktail Y besides aspirating the media do you wash the well with anything to remove dead cells as well as lingering cytokines which may differ from the new combination. If so how many times?

Yes, cells are washed once with DMEM/F12. This has been added to the protocol in Step 3.2.

7. At the end of the differentiation process what protocols or methodologies do the authors use to validate robust hepatocyte-like cell differentiation (e.g. IF, functional characterization, or qRT-PCR) to ensure during setup that uniformity of differentiation is obtained. As an example, the authors denote albumin and CYP3A4 marker expression. Which specific antibodies (company/number) do the authors recommend and consideration for addition of the protocol will make the protocol more complete. Moreover the addition of functional assays such as examination of protein production (via ELISA) or other assays would strengthen the protocol as well as help readers who use this protocol validate that they are truly producing bona fide hepatocyte-like cells in vitro.  
We use IF and qRT-PCR assays to validate the formation of HLCs. We have also used these cells in survival studies of mice. Please refer to our publication (Ang et al. 2018; Cell Reports). We have provided IF and qRT-PCR data with our manuscript with a reference to the publication it was taken from.  
  
  
  
Reviewer #4:  
  
Manuscript Summary:  
Based on their recently published studies, Ou et al. described in this manuscript a highly efficient, convenient and reliable method to achieve induced differentiation of human pluripotent stem cells (PSCs), i.e, ES cells and iPS cells, into hepatocyte-like cells. Below, a list of several concerns and comments are provided that the authors will hopefully consider to improve the clarity of their protocol.  
  
Major Concerns:  
- For the readers' reference, the list of specific names and/or clones of hPSC lines with which the present protocol exactly worked should be provided, including those described in line 203 [14 hESC and hiPSC lines], line 211 [3 hESC lines] and line 258 [3 hPSC lines].

Examples of these lines have been provided in the text.

- Table 2. The names for each of the differentiation media should be specified here and used consistently throughout the manuscript. The "Time point" shown in this table seems confusing and a bit misleading, as those described here (i.e., D1, D2, …) indicate the timing when the differentiation is completed, but not started by applying the corresponding medium: for instance, the "day 1 (D1) medium" is applied on day 0 in the time course shown in Figure 1.

The table has been edited.

- Line 192. "Primitive streak cells should generally have ragged borders and will become more spread and less compact". It should be better to include in Figure 1 a picture corresponding to this notion.

We have a representative picture for this but it does not have a scale bar.

- Line 213. Can the authors provide any evidence showing that bile canaliculi are indeed formed, for instance, by immuno-staining for canalicular markers (e.g., P-glycoprotein, MRP2) or dye efflux assays?

We have not performed these assays except for a preliminary study showing MRP2 protein expression by immunostaining.   
  
Minor Concerns:  
- Abbreviations used in the manuscript should be defined, such as CDM, PVA, and APS. The same applies to those used in Figure 1 (e.g., DE, PFG, LB, …)

This has been corrected (Line 445)

- Line 131. It would be better to clarify whether the cells should be resuspended as complete "single cells" at this step, or dissociation into clumps is also acceptable. In the latter case, typical and/or maximal size of the clumps amenable for the following differentiation steps should also be described.

This has been corrected to say ‘single cells’ at all places.

- Line 148. Description on thiazovivin should go to line 145.

We placed the description on Thiazovivin right after the paragraph it was mentioned.

- Line 176. What is "APS differentiation medium" ? (Maybe the same as the "day 1 medium" written in the following sentence)

This has been described line 259

- Line 180. Isn't is necessary to replace media with fresh ones everyday or at any time during each of the differentiation steps (i.e., on day 4 and 5, days 7-11, or days 13-17)?

Yes, this has been added in the protocol Step 3.6

- Line 220. "MIXL" should be "MIXL1+".

The errors are corrected (see lines 273 and 299).

- Table 3, Cells in center of colony do not differentiate, iii), Proffered Solution. Description here should be revised by taking what is written in lines 184-186 into account.

This has been corrected