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TITLE:

A Simplified Method for Generating Kidney Organoids from Human Pluripotent Stem Cells

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KEYWORDS:

kidney organoids, CHIR99021, knock-out serum replacement, embryoid bodies, renal tissue, pluripotent stem cells, suspension culture

SUMMARY:

Here we describe a protocol to generate kidney organoids from human pluripotent stem cells (hPSCs). This protocol generates kidney organoids within two weeks. The resulting kidney organoids can be cultured in large-scale spinner flasks or multi-well magnetic stir plates for parallel drug-testing approaches.

ABSTRACT:

Kidney organoids generated from hPSCs have provided an unlimited source of renal tissue. Human kidney organoids are an invaluable tool for studying kidney disease and injury, developing cell-based therapies, and testing new therapeutics. For such applications, large numbers of uniform organoids and highly reproducible assays are needed. We have built upon our previously published kidney organoid protocol to improve the overall health of the organoids. This simple, robust 3D protocol involves the formation of uniform embryoid bodies in minimum component medium containing lipids, insulin-transferrin-selenium-ethanolamine supplement and polyvinyl alcohol with GSK3 inhibitor (CHIR99021) for 3 days, followed by culture in knock-out serum replacement (KOSR)-containing medium. In addition, agitating assays allows for reduction in

clumping of the embryoid bodies and maintaining a uniform size, which is important for reducing variability between organoids. Overall, the protocol provides a fast, efficient, and cost-effective method for generating large quantities of kidney organoids.

INTRODUCTION:

In recent years, a number of protocols to differentiate human pluripotent stem cells into kidney organoids have been developed[1-5]. Kidney organoids have provided an important tool to aid research into new regenerative medicine approaches, model kidney-related diseases, perform toxicity studies and therapeutic drug development. Despite their wide applicability, kidney organoids have limitations such as lack of maturation, limited long-term culture capacity *in vitro*, and a paucity of several cell types found in the human kidney[6-8]. Recent work has focused on improving the level of organoid maturation, extending the culture periods and expanding the complexity of kidney cell populations by modifying the existing protocols[9-12]. In this present iteration of our established protocol[5,13], we have modified the medium components in the first stage of the protocol to a serum-free base medium supplemented with insulin-transferrin-selenium-ethanolamine (ITSE), lipids, polyvinyl alcohol (E5-ILP) and CHIR99021 (**Figure 1**). These changes provide a fully-defined, serum-free, low-protein medium, with less components than our previous medium composition[5,13] and without additional growth factors. As a result, the first stage medium is less labor-intensive to prepare than our previously published version, and may reduce batch-to-batch variability[5]. Previous studies have shown that both insulin and transferrin are important in serum-free culture[14,15], however, high levels of insulin can be inhibitory to mesoderm differentiation[16]. We have maintained the low insulin levels as provided in the original protocol, and further reduced levels of KOSR (containing insulin) in second stage of the assay. In line with other protocols for kidney organoid formation, lower levels of KOSR are beneficial to maintaining a balance between proliferation and differentiation of the kidney tissue[17]. In addition, we have lowered the glucose concentration in our Stage II medium[13].

Our method describes a setup for suspension assay of kidney organoids, yielding up to ~1,000 organoids from an initial ~60% confluent hPSC 100 mm culture plate as described in the original publication[5,13]. This protocol can be easily scaled up to starting with multiple 100 mm or 150 mm plates to further increase the organoid numbers.

PROTOCOL:

All experiments using hPSCs were performed in compliance with institutional guidelines, and were carried out in a Class II biosafety hood with appropriate personal protective equipment. All reagents are cell culture-grade unless stated otherwise. All cultures are incubated at 37 ˚C, 5% CO_2 air atmosphere. At all stages of the assay, embryoid bodies or kidney organoids can be collected, and fixed or prepared for analysis. The hPSC lines used to generate this data have been fully characterized and published[18].

1. Preparing culture plates

NOTE: Approximately 1 h prior to splitting hPSCs, coat 2 x 100 mm tissue culture plates with a stem cell qualified basement membrane matrix extract (BME). One may pre-coat the plates, seal them with a paraffin film and store at 4 ˚C according to manufacturers' instructions.

1.1. Prepare 2 x 100 mm tissue culture-treated plates (1 for kidney organoid assay, 1 to maintain the cell line) and a 15 mL conical tube in the Class II biosafety hood.

1.2. Aliquot 8 mL of cold, serum-free Dulbecco's Modified Eagle Medium (DMEM) into a 15 mL conical tube and ~4 mL into each of the 100 mm plates, enough to cover the bottom of each plate with medium.

1.3. Take a 100 µL aliquot of BME out of the freezer (-20 ˚C). Using a 2 mL serological pipette, take ~1 mL of cold DMEM from the 15 mL conical tube. Slowly thaw the BME aliquot by gently pipetting up and down with the cold DMEM, avoiding making bubbles.

NOTE: Do not let BME aliquot sit at room temperature. Use immediately.

1.4. Transfer the thawed DMEM/BME back into the 15 mL conical tube with the remaining DMEM. With a 10 mL serological pipette, gently mix the diluted BME by pipetting up and down at least 8 times to evenly disperse the BME, avoiding making bubbles.

1.5. Transfer 4 mL of the diluted BME into each plate with DMEM and gently swirl the plate so that the BME is evenly distributed. Incubate the coated plate for 1 h at room temperature or 30 min at 37 ˚C.

NOTE: Use 50 µL of BME per 100 mm plate. Use of other hPSC culture media and cell lines may require different concentrations of BME.

2. Passaging hPSCs

NOTE: For routine hPSC culture, passage cell lines at 70-80% confluency.

2.1. Aspirate the culture medium from the hPSC plate to be passaged. Add ~ 8 mL of Dulbecco's phosphate-buffered saline (DPBS) to the hPSC plate and gently swirl to wash the cells.

2.2. Aspirate DBPS and add 2 mL of gentle cell dissociation reagent (GCDR) to the 100 mm plate, drop by drop on top to cover the cells.

NOTE: Other dissociation reagents may also be used. Adjust accordingly.

2.3. Incubate at room temperature for ~6-8 min until the colonies are breaking up and cells are refractive under phase contrast (**Figure 2A**).

NOTE: The timing may vary between cell lines. Adjust accordingly.

2.4. While incubating, prepare a 50 mL conical tube. Add 16 mL of hPSC medium (8 mL per 100 mm plate) and add Rho-associated kinase inhibitor (ROCKi) to a final concentration of 5 µM.

2.5. Aspirate DMEM from the BME-coated plates, and add 8 mL of hPSC medium plus ROCKi to each plate.

2.6. When the cells are ready (as described in point 0, **Figure 2A**), aspirate the GCDR and tilt the plate ~45˚ towards the experimenter and scrape the cells with a cell lifter.

NOTE: If cells are detatching, omit aspirating GCDR and proceed.

2.7. Turn the plate ~90˚ and scrape again to lift the remaining cells. Keep the plate ~45˚ and wash the cells down with 3 mL of hPSC medium using a 10 mL serological pipette.

2.8. Gently pipette up and down to break up large clumps (no more than 2-3 times) and seed the cells at the appropriate ratio for the cell line of interest onto the prepared plates. Place the plate with the cells in the incubator and move the plate gently in figure eight motions to distribute the cells evenly.

NOTE: In this experiment hPSC lines were split at a ratio of 1:5, this may vary for other cell lines and conditions. Leave the plate undisturbed over night.

2.9. After ~ 24 h, examine the cells for attachment. Look for small individual colonies attached. Aspirate the spent medium and replenish with 8 mL of fresh hPSC medium (no ROCKi added).

2.10. Continue observing and feeding daily until the cells reach ~60% confluency to start the kidney organoid assay (usually reached 48 to 72 h post passaging). The colonies will ideally be discrete and not merging (**Figure 2B**).

NOTE: It is very important to limit the cells to no more than 80% confluency in order to maintain their pluripotency state. Confluent cultures, rough handling or higher passages may lead to unwanted spontaneous differentiation or low efficiency of kidney organoid formation.

3. Day 0 - Setting up the kidney organoid assay

3.1. Before starting, prepare both the E5-ILP and Stage II media as per formulations (**Table 1** and **Table 2**).

NOTE: The media can be stored for up to 14 days at 4 ˚C.

3.2. For one kidney organoid assay (one 100 mm culture plate is needed for one 6-well plate), prepare complete E5-ILP medium in a 50 mL conical tube: 18 mL of E5-ILP supplemented with 8 µM CHIR99021 (14.4 µL), 3.3 µM ROCKi (6 µL), 0.1 mM beta-mercaptoethanol (32.7 µL).

3.3. Place 2 mL of complete E5-ILP medium into each well of a 6 well ultra-low attachment plate.

3.4. Wash hPSCs at ~60 % confluency (**Figure 2B**) twice with ~ 8 mL of DPBS. Aspirate DPBS then add 2 mL of dispase per 100 mm plate, drop by drop to cover the cells and incubate for 6 min at 37 ˚C.

NOTE: After 6 min, the edges of the colonies will start to curl up (**Figure 2C**, red arrows) while the rest of the colony remains attached. If this is not obtained after 6 min, place the cells back into the incubator for additional 30 s. Other hPSC media and matrix may not be compatible with this timing. Laminin based BME coating is not compatible with dispase. If laminin based BME are the standard hPSC matrix, coat one of the plates in section 0 with the BME described in this method to be used for the kidney organoid assay.

3.5. Wash cells 3x with ~10 mL of DPBS. Aspirate DPBS then tilt the plate ~45˚ and scrape down with a cell lifter.

NOTE: Dispase is not deactivated, hence it needs to be washed out thoroughly. Do not reduce the number of washes.

3.6. Wash the colonies down from the top with 6 mL of complete E5-ILP medium using a 10 mL serological pipette. Pipette up and down gently to break up any large colonies (2 or 3 times is usually enough).

3.7. Distribute the colony clusters evenly by adding 1 mL per well into the 6-well plate. Place the plate on an orbital shaker (settings: orbital = 30, reciprocal = 330˚, vibration = 5˚ - 2 s) that is placed in the 37 ˚C incubator (**Figure 2D**).

NOTE: The vibration feature is important for adequate distribution of organoids and to prevent clumping.

4. Day 2 - Feeding by half-medium change

NOTE: Within the 48 h, colony clusters will form embryoid bodies.

4.1. Prepare the complete medium: For one 6-well plate prepare 12 mL of E5-ILP medium + 8 µM CHIR99021 in a 15 mL conical tube.

NOTE: Beta-mercaptoethanol and ROCKi are not required.

4.2. Let the embryoid bodies settle at the bottom of the plate, tilt the plate ~45˚ then aspirate the medium slowly from the top, leave ~1 mL per well.

NOTE: Embryoid bodies at this stage clump rapidly. Do not leave them to settle for > 5 min.

4.3. Add 2 mL of prepared complete medium (section 0) per well. Return the plate back onto the shaker.

5. Day 3 - Transfer of embryoid bodies to Stage II medium

5.1. Prepare a 50 mL conical tube and DMEM (low glucose). Let the embryoid bodies settle at the bottom of the plate. Tilt the plate ~45˚ and aspirate the medium from the top slowly, leave ~1 mL per well.

5.2. Collect all the embryoid bodies carefully from each well using a 10 mL serological pipette and transfer them to the 50 mL conical tube.

5.3. Wash each well to collect any remaining embryoid bodies with ~ 1 mL of DMEM (low glucose) and add them to the same 50 mL conical tube.

5.4. Leave the embryoid bodies to settle to the bottom of the tube, ~5 min. While waiting, add 2 mL of Stage II medium to each well of the 6-well plate. Seive out large embryoid bodies (>300 µm) using a 200 µm cell strainer (**Figure 2E**).

5.4.1. Use a new 50 mL conical tube and place the 200 µm cell strainer on top. Pipette all of the embryoid bodies using a 10 mL serological pipette carefully over the cell strainer.

5.4.2. Rinse the cell strainer with an additional ~5 mL of DMEM (low glucose) to collect any embryoid bodies stuck in the cell strainer. Allow the embryoid bodies to settle to bottom of the conical tube.

5.5. When the embryoid bodies are settled, aspirate the supernatant and wash with ~10 mL of DMEM (low glucose).

5.6. Aspirate DMEM and re-suspend the embryoid bodies in 6 mL of Stage II medium.

5.7. Transfer the embryoid bodies back into the 6 well ultra-low attachment plate, distributing them evenly among the 6 wells.

5.8. Carry out half medium changes as described in steps 4.2 and 0 every other day.

NOTE: From day 3 onwards, the embryoid bodies will have a 'golden' and smooth, spherical appearance (**Figure 2F**). From ~ day 6, tubule formation in individual embryoid bodies will become apparent, with increasing numbers over the following days reaching optimum numbers and growht by day 14 (**Figure 2G,H**). To eliminate occasional clumping forming, upon visually observing the kidney organoids, or very small embryoid bodies without tubules, sieve out the <200 and large >500 µm organoids with a 500 and 200 µm cell strainers as described in steps 0 and 0.

6. Transfer to spinner flask and feeding

NOTE: A spinner flask may be used anytime from day 3 onwards for experiments that require large numbers of organoids. Routine transfer organoids between days 6-8 happens in our lab. Please see the **Discussion** section for alternatives if equipment is not available.

6.1. Transfer embryoid bodies into a 125 mL spinner flask with 45 mL of Stage II medium. Set magnetic stirrer speed to 120 rpm and place into the incubator (**Figure 2I**).

6.2. To feed embryoid bodies or kidney organoids, let the kidney organoids settle briefly to the bottom of the spinner flask. Lift the lid from one side arm of the flask and place the aspirating pipette inside, with the tip touching the opposite inside wall.

6.3. Slowly angle the aspirating pipette down and aspirate approximately half of the medium. Replenish with 20 mL of fresh Stage II medium by pipetting it through the same opening.

7. Setting up 6-well magnetic stir plate (6MSP)

NOTE: The 6MSP format may be used in place of spinner flasks if multiple conditions need to be tested. Use the 6MSP for compound or nephrotoxin treatments. This saves the amount of medium used in the second stage while maintaining nutrient availability through diffusion.

7.1. Clean the oval magnetic stir bars in a 50 mL conical tube by washing in a tissue culture suitable detergent briefly (if never used) or soak for > 1 h if previously used.

7.2. Briefly wash 3x in sterile DPBS.

7.3. Wash 1x for 5 min in 70% ethanol, 1x in sterile DPBS.

7.4. Rinse with anti-adherence solution and wash 1x in sterile DPBS and aspirate.

7.5. Carefully, using long sterile forceps place one magnetic stir bar into each well of the 6-well plate with embryoid bodies or kidney organoids.

7.6. Place the plate onto the 6MSP and set the speed to 120 rpm (**Figure 2J**). Maintain kidney organoids with half medium change as per section 4.2 and 4.3.

NOTE: In order for the magnetic stir bars to snap into position and start spinning, you may need to first put the power level to 100 briefly, then once they are all spinning, bring the power level down to 25.

RESULTS:

In this most recent version of our protocol, kidney organoid differentiation is initiated in a defined, low protein medium. The assays are performed entirely in suspension and rely on the innate ability of hPSCs differentiation and organization for initiation of tubulogenesis. A single assay originating from a 100 mm ~60% confluent hPSC culture plate routinely yields 500-1,000 kidney organoids, as shown in our previous publication[5]. Due to such high numbers of organoids generated, this protocol is well suited for compound testing. We routinely use a 6-well format for compound testing however, this protocol can easily be scaled in the second stage (day 3 onwards) to other multi-well formats for higher-throughput compound testing. Immunofluorescence of paraffin sections shows presence of nephron segments in the organoids, i.e. renal tubules expressing Hepatocyte Nuclear Factor-1 beta (HNF1B) and Lotus Tetragonolobus Lectin (LTL) (**Figure 3A** - HNF1B, LTL), and podocyte clusters expressing V-maf Musculoaponeurotic Fibrosarcoma oncogene homolog B (MAFB) and nephrin (NPHS1) (**Figure 3A** - MAFB, **Figure 3B** - NPHS1). Furthermore, the modifications in this protocol can support expansion of endothelial cells as seen in **Figure 3B** showing staining with Platelet and Endothelial Cell Adhesion Molecule 1 (PECAM1) at day 26 of culture.

FIGURE AND TABLE LEGENDS:

Table 1: E5-ILP medium composition. Pipette all the reagents except the chemically defined lipids and anti-mycoplasma reagent directly into the upper chamber of a 0.22 µm Stericup filtration unit. After filtration, add the lipids and anti-mycoplasma reagent. Store at 4 °C for up to two weeks.

Table 2: Stage II medium composition. Pipette all the reagents except and anti-mycoplasma reagent directly into the upper chamber of a 0.22 µm Stericup filtration unit. Once filtered, add anti-mycoplasma reagent. Store at 4 °C for up to two weeks.

Figure 1: Protocol overview. Schematic overview of the protocol showing timing of the two stages and use of spinner flasks and 6MSP.

Figure 2: Stages of the protocol. (**A**) Bright-field image of hPSC colony treated with GCDR. (**B**) Optimal confluency, and colony size to begin a kidney organoid assay. (**C**) hPSCs treated with dispase for 6 minutes. Red arrows point to edges of the colonies curling up. (**D**) Organoid assays on an orbital shaker. (**E**) Use of 200 µm cell strainer to sieve out large embryoid bodies. (**F**) Embryoid bodies at day 3 (D3) before transferring to Stage II medium. (**G**) Emergence of tubule formation can be observed at day 8 (D8) and (**H**) optimal timepoint for organoid harvesting and

treatment at day 14 (D14). (**I**) Spinner flask used for bulk culture on a multi-position magnetic plate. (**J**) Assay on a multi-well magnetic stir plate. Scale bars, 200 µm.

Figure 3: Expected results. (**A**) Representative confocal images of immunofluorescently labeled paraffin sections of day 14 kidney organoids showing positive staining for tubule epithelia (HNF1B and LTL) and podocyte clusters (MAFB). (**B**) Day 26 kidney organoid sections labeled for podocyte clusters (NPHS1) and endothelial cells (PECAM1). Scale bars, 100 µm (A); 200 µm (B).

DISCUSSION:

Previous studies have shown that the initial protocol steps are critical for intermediate mesoderm differentiation[5,19,20] and, therefore, it is essential to implement a stringent medium composition at this stage. Removing undefined components such as serum, albumin, protein free hybridoma medium II from the first stage of the protocol may help to improve consistent differentiation efficiency between assays[21].

The metabolic state of kidney cells is critical to their function, and glucose changes can lead to altered metabolic state[22]. Previous studies have described that high levels of glucose (up to 25 mM) can induce endothelial cell dysfunction and alter growth and oxidant capacity of kidney cells[22-24]. High levels of glucose have also been described to alter mitochondrial function[24], which may be unfavorable when investigating kidney disease and nephrotoxicity or performing drug discovery using kidney organoids. We have, therefore, reduced the level of glucose in our protocol to promote a more *in vivo*-like metabolic state of the organoid kidney cells. As a result, the modifications to the kidney organoid assay provide a consistent, robust protocol, while maintaining its simplicity.

Kidney organoids are immature, and extended culture (>20 days) may lead to incidence of pro-fibrotic and non-renal cell types as previously described[5,25], leaving organoids less representative of healthy human kidney tissue. Based on our experience, the optimal treatment window, where the kidney organoids are at their healthiest is between days 14-18. Use of spinner flasks and multi-well magnetic stirrers as described above will enhance uniform nutrient availability as opposed to static culture[21,26]. If the equipment for suspension culture such as the shaker or magnetic stirrers are not available, this protocol can still be carried out completely in the ultra-low attachment plates in static culture. There may however be increased incidence of embryoid bodies/organoid merging, leading to large specimens with necrotic cores due to hypoxia. Any organoids larger than 500 µm can be removed by using the cell strainers described. To reduce the chance of merging of the organoids in those cases, we suggest not seeding more than 100 organoids per 6-well. In addition, following feeding, the organoids should be evenly distributed by performing figure eight motions with the plate.

Low efficiency (<50%) of organoid formation may be observed. This usually occurs when the hPSC cultures have reached high confluency (>80%) during standard passaging. It is critical that hPSC maintenance is consistent and cells are not left to become over-confluent. High confluency and inconsistent passaging technique may also lead to spontaneous differentiation and increased cell death. If differentiation is present in the hPSC culture, we recommend removing the

differentiated areas by aspirating with a fine pipette tip if it does not exceed 5% of the cell population, prior to starting the assay. If the differentiation areas exceed 5%, we recommend that a new batch of hPSCs is thawed and split at least once before starting a new assay.

We have observed that some hPSC lines are more prone to form non-renal cell types, such as cardiac or neural tissue. If this occurs, size filtration using the cell strainers may help to remove those organoids that contain non-renal outgrowths. Alternatively, changing the hPSC medium and/or matrix may help to reduce the non-renal outgrowths. From our experience, alternative hPSC media containing minimum components, and BME such as vitronectin, provide a more stringent pluripotent niche and thus help generate more homogeneous hPSC cultures.

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DISCLOSURES:

Authors have nothing to disclose.

REFERENCES:

1. Takasato, M. et al. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. *Nature.* **526** (7574), 564-568, (2015).
2. Freedman, B. S. et al. Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. *Nature Communications.* **6**, 8715 (2015).
3. Morizane, R. et al. Nephron organoids derived from human pluripotent stem cells model kidney development and injury. *Nature Biotechnology.* **33** (11), 1193-1200 (2015).
4. Taguchi, A. et al. Redefining the in vivo origin of metanephric nephron progenitors enables generation of complex kidney structures from pluripotent stem cells. *Cell Stem Cell.* **14** (1), 53-67 (2013).
5. Przepiorski, A. et al. A simple bioreactor-based method to generate kidney organoids from pluripotent stem cells. *Stem Cell Reports.* **11** (2), 470-484 (2018).
6. Freedman, B. S. et al. Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. *Nature Communication.* **6**, 8715 (2015).
7. Morizane, R. et al. Nephron organoids derived from human pluripotent stem cells model kidney development and injury. *Nature Biotechnology.* **33** (11), 1193-1200 (2015).
8. Takasato, M. et al. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. *Nature.* **526** (7574), 564-568 (2015).
9. Taguchi, A., Nishinakamura, R. Higher-order kidney organogenesis from pluripotent stem cells. *Cell Stem Cell.* **21** (6), 730-746 e736 (2017).
10. Uchimura, K., Wu, H., Yoshimura, Y., Humphreys, B. D. Human pluripotent stem cell-derived kidney organoids with improved collecting duct maturation and injury modeling. *Cell Reports.* **33** (11), 108514 (2020).

11. Howden, S. E., Little, M. H. Generating kidney organoids from human pluripotent stem cells using defined conditions. *Methods in Molecular Biology.* **2155**, 183-192 (2020).
12. Tanigawa, S. et al. Activin is superior to BMP7 for efficient maintenance of human iPSC-derived nephron progenitors. *Stem Cell Reports.* **13** (2), 322-337 (2019).
13. Sander, V. et al. Protocol for large-scale production of kidney organoids from human pluripotent stem cells. *STAR Protocols.* **1** (3), 100150 (2020).
14. Ekblom, P., Thesleff, I., Miettinen, A., Saxen, L. Organogenesis in a defined medium supplemented with transferrin. *Cell Differentiation.* **10** (5), 281-288 (1981).
15. Thesleff, I., Ekblom, P. Role of transferrin in branching morphogenesis, growth and differentiation of the embryonic kidney. *Journal of Embryology and Experimental Morphology.* **82**, 147-161 (1984).
16. Freund, C. et al. Insulin redirects differentiation from cardiogenic mesoderm and endoderm to neuroectoderm in differentiating human embryonic stem cells. *Stem Cells.* **26** (3), 724-733 (2008).
17. Nishikawa, M. et al. An optimal serum-free defined condition for in vitro culture of kidney organoids. *Biochemistry and Biophysics Research Communication.* **501** (4), 996-1002 (2018).
18. Oh, J. K. et al. Derivation of induced pluripotent stem cell lines from New Zealand donors. *Journal of the Royal Society of New Zealand.* 1-14, (2020).
19. 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 (2013).
20. Lam, A. Q. et al. Rapid and efficient differentiation of human pluripotent stem cells into intermediate mesoderm that forms tubules expressing kidney proximal tubular markers. *Journal of American Society of Nephrology.* **25** (6), 1211-1225 (2014).
21. Bratt-Leal, A. M., Carpenedo, R. L., McDevitt, T. C. Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. *Biotechnology Progress.* **25** (1), 43-51 (2009).
22. Imasawa, T. et al. High glucose repatterns human podocyte energy metabolism during differentiation and diabetic nephropathy. *FASEB Journal.* **31** (1), 294-307 (2017).
23. Kim, K. A. et al. High glucose condition induces autophagy in endothelial progenitor cells contributing to angiogenic impairment. *Biological and Pharmaceutical Bulletin.* **37** (7), 1248-1252 (2014).
24. Piwkowska, A., Rogacka, D., Audzeyenka, I., Jankowski, M., Angielski, S. High glucose concentration affects the oxidant-antioxidant balance in cultured mouse podocytes. *Journal of Cellular Biochemistry.* **112** (6), 1661-1672 (2011).
25. Wu, H. et al. Comparative analysis and refinement of human PSC-derived kidney organoid differentiation with single-cell transcriptomics. *Cell Stem Cell.* **23** (6), 869-881 e868 (2018).
26 Lei, X., Deng, Z., Duan, E. Uniform embryoid body production and enhanced mesendoderm differentiation with murine embryonic stem cells in a rotary suspension bioreactor. *Methods in Molecular Biology, Clifton, N.J.*(2016).

Figure 1

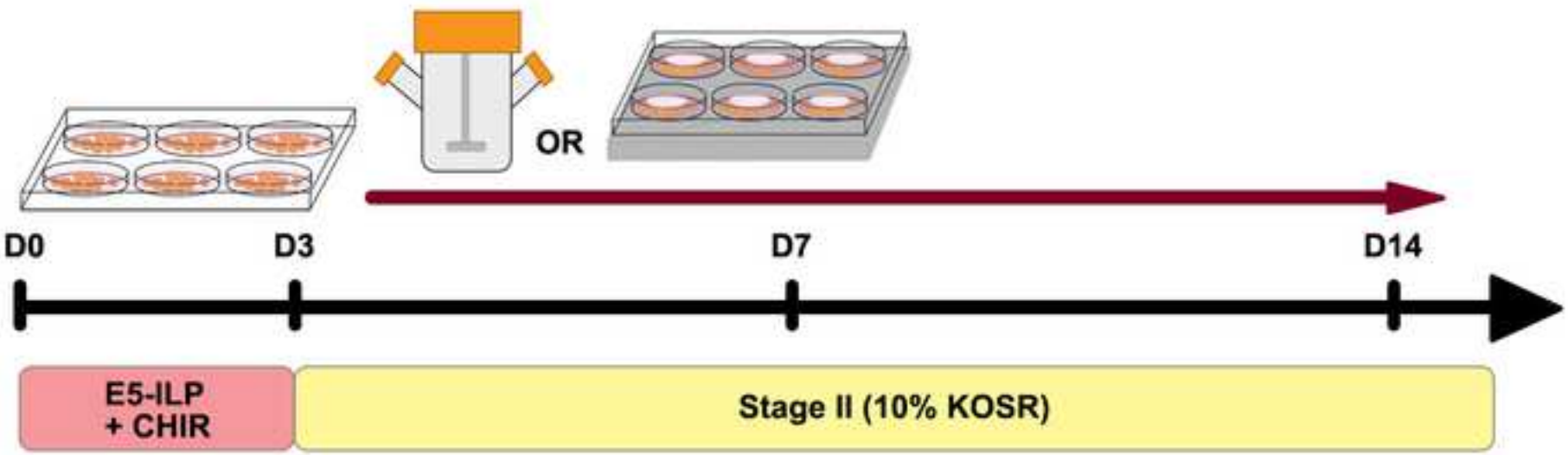

Figure 2

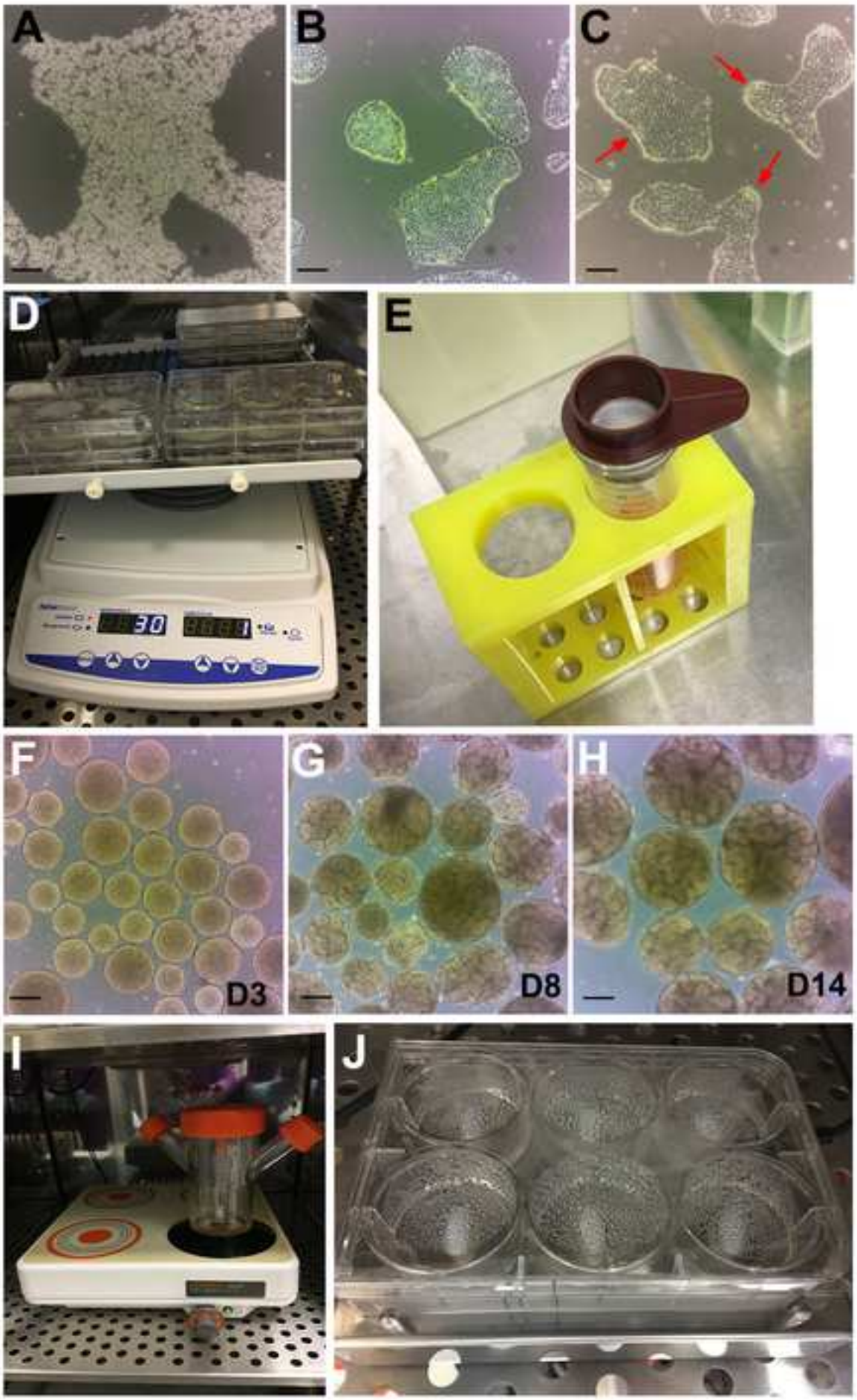

Figure 3

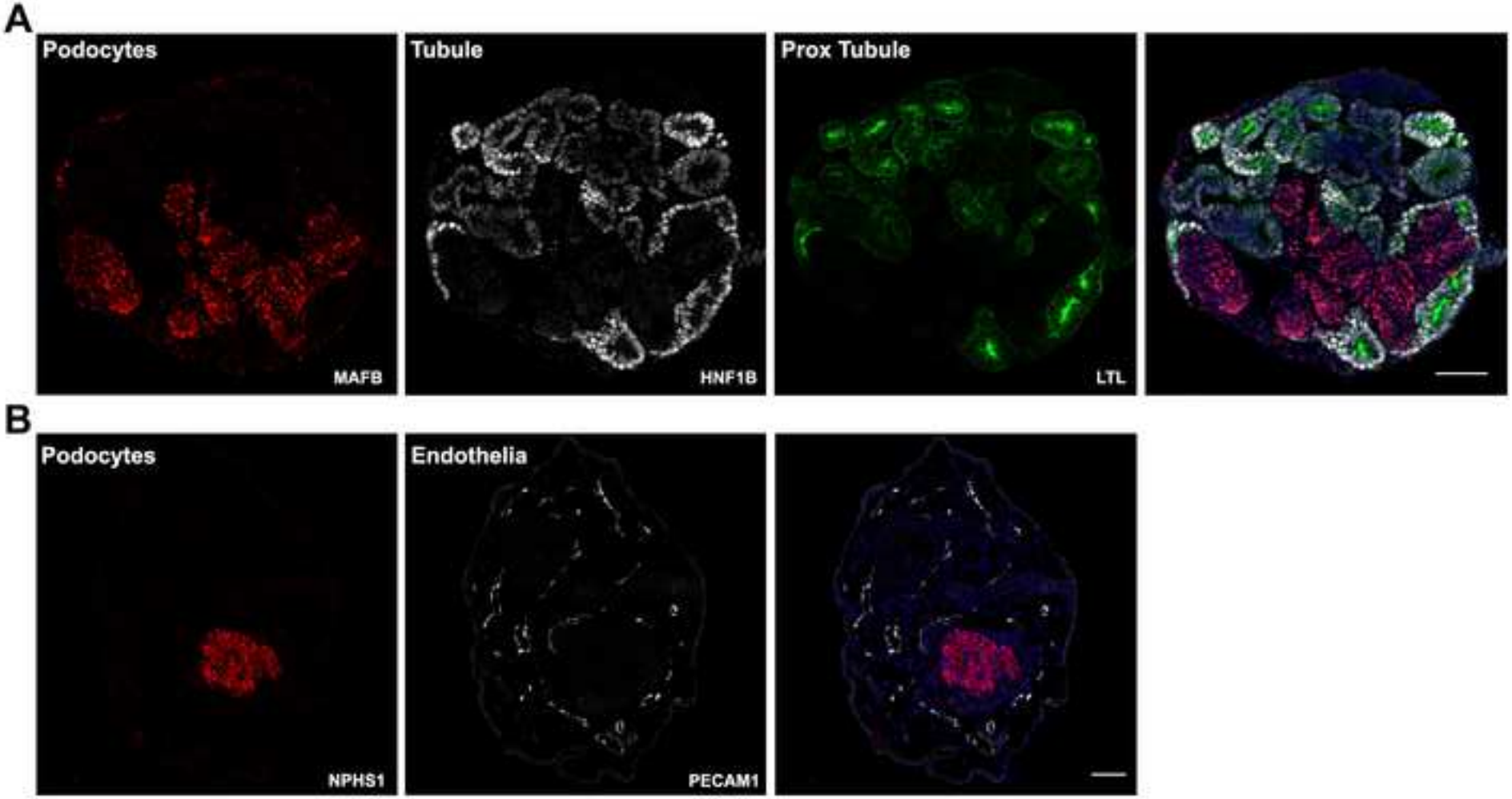

Table 1

Reagent	Stock conc.	Working conc.	Amount per 250 mL
TeSR-E5	n/a	n/a	238.48 mL
PVA	10%	0.25%	6.25 mL
Pen-Strep	100x	1x	2.5 mL
ITSE	100x	0.1x	250 µL
Chemically defined Lipids	100x	1x	2.5 mL
Plasmocin	25 mg/mL	2.5 µg/mL	25 µL

Table 2

Reagent	Stock conc.	Working conc.	Amount per 500 mL
DMEM (Low Glucose)	n/a	n/a	417.5 mL
KOSR	n/a	10%	50 mL
PVA	10%	0.25%	12.5 mL
Pen-Strep	100x	1x	5 mL
MEM-NEAA	100x	1x	5 mL
GlutaMAX	100x	1x	5 mL
HEPES	100x	1x	5 mL
Plasmocin	25 mg/mL	2.5 µg/mL	50 µL

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials (4).xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2-Mercaptoethanol	Thermo Fisher	21-985-023	
Anti-adherence rinsing solution	STEMCELL Technologies	7010	
CHIR99021	STEMCELL Technologies	72054	10 mM stock in DMSO
Corning disposable spinner flasks	Fisher Scientific	07-201-152	
Corning Ultra-Low Attachment 6-well plates	Fisher Scientific	07-200-601	
Corning Slow-Speed Stirrers	Fisher Scientific	11-495-03	Multi plate magnetic stirrer for spinner flask culture
Dispase	STEMCELL Technologies	7923	Aliquot and freeze
DMEM, low glucose, pyruvate, no glutamine, no phenol red	Thermo Fisher	11054020	
DPBS 1x, no calcium, no magnesium	Thermo Fisher	14-190-250	
Egg / Oval Stirring Bars	2mag	PI20106	
Excelta General-Purpose Tweezers	Fisher Scientific	17-456-103	Keep sterile in the cell culture hood
EZBio Single Use Media Bottle, 250mL	Foxx Life Sciences	138-3211-FLS	Used to make PVA 10%
Falcon Standard Tissue Culture Dishes (100 mm)	Thermo Fisher	08-772E	
Fisherbrand Sterile Aspirating Pipet 2mL	Fisher Scientific	14-955-135	
Fisherbrand Cell Lifters - Cell lifter	Fisher Scientific	08-100-240	
Fisherbrand Multi Function 3D Rotators	Fisher Scientific	88-861-047	Orbital shaker
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	Thermo Fisher	A1413302	**BME**. Aliquot on ice and freeze. Another suitable matrix alternative is Matrigel or Cultrex.
Gentle Cell Dissociation Reagent	STEMCELL Technologies	7174	**GCDR**
GlutaMAX Supplement	Thermo Fisher	35-050-061	L-glutamine supplement.
HEPES (1M)	Thermo Fisher	15-630-080	
Insulin-Transferrin-Selenium-Ethanolamine	Thermo Fisher	51-500-056	**ITSE**
KnockOut Serum Replacement - Multi-Species	Thermo Fisher	A3181502	**KOSR**. Aliquot and freeze
Lipid Mixture 1, Chemically Defined	Millipore-Sigma	L0288-100ML	
MEM Non-Essential Amino Acids Solution	Thermo Fisher	11140-050	

MilliporeSigma Stericup Quick Release-GP Sterile Vacuum Filtration System 500mL	Fisher Scientific	S2GPU05RE	
MilliporeSigma Stericup Quick Release-GP Sterile Vacuum Filtration System 250mL	Fisher Scientific	S2GPU02RE	
MIXcontrol MTP / Variomag TELEcontrol MTP Control Unit	2mag	VMF 90250 U	
MIXdrive 6 MTP / Variomag TELEdrive 6 MTP Microplate Stirring Drive	2mag	VMF 40600	**6MSP**
MP Biomedicals 7X Cleaning Solution	Fisher Scientific	MP0976670A4	**Tissue culture suitable detergent.** Make a 5% solution in water
mTeSR1	STEMCELL Technologies	85850	**hPSC medium.**TeSR-E8, NutriStem XF, and mTeSR Plus medium have also been tested and are suitable alternatives.
Nunc 50 mL Conical, Sterile Centrifuge Tubes	Fisher Scientific	12-565-270	
Nunc 15mL Conical Sterile Centrifuge Tubes	Fisher Scientific	12-565-268	
Penicillin-Streptomycin	Thermo Fisher	15-140-122	Aliquot and freeze
Plasmocin	Invivogen	ant-mpt	**Anti-mycoplasma reagent.** Aliquot and freeze
pluriStrainer® 200 µm	Fisher Scientific	NC0776417	Cell strainer
pluriStrainer® 500 µm	Fisher Scientific	NC0822591	Cell strainer
Poly(vinyl alcohol) 87-90% hydrolyzed (PVA)	Millipore-Sigma	P8136-250G	10% in DPBS stirring at 98 degrees C until disolves, make in 138-3211-FLS
ROCK inhibitor Y-27632 (ROCKi)	STEMCELL Technologies	72304	10 mM stock in DPBS
Sterile Disposable Serological Pipets - 10mL	Fisher Scientific	13-678-11E	
Sterile Disposable Serological Pipets - 25mL	Fisher Scientific	13-678-11	
Sterile Disposable Serological pipette - 5 mL	Fisher Scientific	13-678-12D	
TeSR-E5	STEMCELL Technologies	5916	Serum-free, low protein base medium for **E5-ILP**

Variomag distriBOX 2 Distributor	2mag	VMF 90512	If you use more than one MIXdrive

Submission JoVE62452 - RESPONSE TO REVIEWERS

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please provide an email address for each author.

The corresponding authors were added.

3. Please define all abbreviations before use. E.g. DPBS

Corrected.

4. Use “h” instead of “hrs”, “µL” instead of “µl”, “ ° “ instead of “degrees”, etc. Include a space between the quantity and its unit (e.g., “1 mL” instead of “1mL”).

Corrected.

5. Line 120: What do you mean by “cells look ready”?

We added clarification and a corresponding image.

6. Line 172: What is the speed of rotation? Added
7. Please highlight up to 3 pages 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. Please include all relevant details that are required to perform the highlighted step. 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted the steps of the protocol for the video that we think are necessary, however we are happy to adjust based on your experience.

8. Consider combining shorter protocol steps so that individual steps contain 2-3 actions and a maximum of 4 sentences per step. E.g. in section 1.

We have combined several of the steps.

9. Consider labeling section 1 as “Preparing culture plates”, as the steps do not actually deal with cells. Also, there is no section 1.2.

We have modified the section 1 label.

10. 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. E.g. Geltrex, MIXdrive

We changed Geltrex to BME, Mixdrive to 6-well magnetic stir plate, mTeSR1 to hPSC medium and referenced in the table and materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This protocol manuscript describes how to generate kidney organoids from human pluripotent stem cells. There was a minor change from the protocol published in Stem Cell Reports by Alan Davidson lab.

We would like to thank the reviewer for their detailed comments and suggestions. We have addressed their concerns below under each point.

Major Concerns:
1. The speed of shaker/rotator is missing in line 172.

We have now added this to the manuscript.

2. In lines 260-262, authors claimed the protocol is suited for compound screening, but the well format was 6-well plates which is not suited for compound screening. It would be better tone down the claim.

We have adjusted the manuscript to read "compound testing" as we routinely test compounds in our lab using this set up. We have additionally clarified this in the results section stating **"We routinely use a 6-well format for compound testing however, this protocol can easily be scaled in the second stage (day 3 onwards) to other multi-well formats for higher-throughput compound testing."**

3. It is unclear how many kidney cells can be generated from 10 cm dish. Please include the cell number or the volume of total kidney organoids. The medium volume in the reactor is quite high compared to other differentiation protocols. In the abstract, authors claimed this is a cost-effective method, but I feel this is an over claim. Efficiency is also unclear since there is no quantitative result.

The cell numbers per dish, volume of organoids generated and quantitative data are described in our original publication in Stem cell reports 2018. These parameters have not changed in this current iteration and we have not drawn direct comparisons to the original protocol. We have included more citations to the original paper in the manuscript to guide readers to examine the original data.

We have utilized the use of spinner flasks based on Doug Melton's pancreatic organoid publication and other neural organoid labs which use substantially higher volumes of medium in the flasks (>75mL) and culture for longer periods of time. In addition, they incorporate many different growth factors adding to the cost of the total assay. Based on lack of growth factors in our protocol, shorter culture time, and smaller volumes it becomes more cost-effective.

4. In figure 1A, 6-well plate culture is not included, making it difficult to understand the overall protocol. Please revised the cartoon.

Thank you for pointing this out. Figure 1A has been modified to include the 6-well culture on the magnetic stir plate.

5. In table 1 and 2, there are 6-8 components for stage 1 and 2 media, which are not that simple. Authors claimed this protocol was simplified from the original published from Davidson lab, but I feel this is not the case.

We agree with your assessment, as the original protocol was "simplified", we were trying to make clear the protocol has evolved but is still simple. There are some simplifications from original protocol, such as the first stage media, which originally consisted of 12 components, and now consists of 6-8 components. However, we have changed the language in the manuscript from "simplified" to "modified". We have not changed the title, since this is based upon the simplified protocol, but are happy to adjust.

Minor Concerns:

1. The protocol is very similar to the original and there is no direct comparison in the manuscript. I feel this paper should be published by the original group of Davidson lab. Inclusion of some original authors (at least the last author) might be better to avoid future troubles after publication.

Thank you for the comment, Alan Davidson, who is a close collaborator, has agreed to be an author and has been added to the manuscript. In addition, the first author was a member of the Davidson lab (now in Hukriede lab) and also the lead author on the original 2018 publication.

Reviewer #2:

Manuscript Summary:

This work describes a protocol to generate kidney organoids from pluripotent human stem cells. It is a refinement of a procedure published by the first author in 2018. It provides a clear experimental guide to the procedure and I think it will be of interest to JOVE readers.

We would like to thank the reviewer for their thoughtful comments.

Major Concerns:

There is no information on pluripotency testing, for example is there a lower limit of Oct4/Tra-1-60 positivity at which the directed differentiation should not be attempted?

Thank you for this question, we should have included more details. The iPSC lines that we use have been fully characterized in a separate publication and we have added the citation to the manuscript and a sentence that reads "**The hPSC lines used to generate this data have been fully characterized and published**". We feel that testing the levels of Oct4/Tra-1-60 and correlating to the directed differentiation is beyond the scope of this manuscript and would require weeks of data collection. We have added clarification on how to proceed if there is differentiation present in the hPSC cultures, "**If differentiation is present in the hPSC culture, we recommend removing the differentiated areas by aspirating with a fine pipette tip if it does not exceed 5 % of the cell population, prior to starting the assay. If the differentiation areas exceed 5 %, we recommend that a new batch of hPSCs is thawed and split at least once before starting a new assay**."

Why is an anti-mycoplasma drug included in the culture conditions? For downstream applications it is often important to know that the cultures are mycoplasma-free. The maintenance-dose proposed is not curative but would probably suppress mycoplasma growth, potentially making it undetectable.

Thank you for pointing this out. We do test our hPSC batches for mycoplasma presence on a regular basis. This is done by culturing them in Plasmocin-free, antibiotic-free medium for at least 7-10 days before testing, using MycoAlert assay (Lonza), which is highly sensitive. We include a prophylactic dose of Plasmocin in the media to prevent mycoplasma infection in routine culture.

The culture method appears sound, and can be useful in compound screening. But the data falls short here. What degree of variability is seen in size and composition between the organoids in a single batch? This could be a major influence on drug uptake and confounding hypoxic death. In my opinion this needs to be addressed with data so that prospective users can assess if these organoids will be appropriate for their experiment.

We agree that size and presence of hypoxia is a confounding factor. This has been addressed in the original Stem cell reports 2018 paper with detailed data showing variability and size of the organoids

and how this has been addressed. The use of cell strainers as described limit the size of the organoids keeping them below 500 µm in diameter, which is the appropriate size for adequate nutrient and oxygen diffusion.

Minor Concerns:
2.9 needs better guidance - include a picture here.

An image is now included.

Figure 2 needs labels to identify structures in the organoids.

Labels have been added.

Reviewer #3:
Przepiorski et al have written a protocol for generating kidney organoids in suspension. They have updated their protocol that was published in 2018 in Stem Cell Reports. They clearly write each step of maintaining PSCs in culture and differentiating the cells. They provide technical advise and notes on how to perform the protocol and handle the plates with the organoids. I am interested to see the movie.

We would like to thank the reviewer for the positive comments.

A few minor concerns:
* I think the paper would benefit if more details on the scientific background of the protocol would be included. How are the steps in the developing kidney recapitulated in the protocol?

Thank you, we have added more references to the original publication (2018 Stem cell reports), which provides detailed background and compares kidney organoids from the protocol to the human fetal kidney.

* Should differentiated areas be removed in maintenance of the PSCs in mTeSR1?

Thank you for this question. This has been clarified and expanded in the Discussion section. "**If differentiation is present in the hPSC culture, we recommend removing the differentiated areas by aspirating with a fine pipette tip if it does not exceed 5 % of the cell population, prior to starting the assay. If the differentiation areas exceed 5 %, we recommend that a new batch of hPSCs is thawed and split at least once before starting a new assay**."

* It would be informative to include pictures of stem cells in maintenance and how they look in culture.

Images have now been included in Figure 1.

* Should the medium for the differentiation always be freshly prepared or can you also store (part of) the medium long term in the freezer?

We have added a note callout in Section 3 "The media can be stored for up to 14 days at 4 °C" and additionally to the materials table.

* In the overview, it would be informative to include a picture of the sieving of the organoids of step 5.8

An image has been added, and this will be featured in the video portion.

* The authors should elaborate on the use of the multi-well magnetic stir plate? Is that an alternative for the spinner flask or is it a follow up of the spinner flask?

Thank you for pointing this out. We have added an opening sentence to section 6 and 7 to provide context for use of the equipment and guide readers to the discussion for alternatives if equipment is not available.