TECHNICAL MANUAL

Generation of Human Kidney Organoids Using STEMdiff™ Kidney Organoid Kit

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1.0 Introduction

Kidney organoids are self-organizing 3D structures containing functional renal cell types resembling some aspects of the in vivo counterpart. Kidney organoid cultures are established by guiding human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs) in a stepwise manner through stages of late primitive streak, intermediate mesoderm, and metanephric mesoderm to give rise to pretubular aggregates, then renal vesicles that ultimately form kidney organoids (Freedman et al.; Morizane et al.; Takasato et al.). Because kidney organoids recapitulate the cellular components and structure of the developing nephron, they are able to overcome the limitations of monolayer culture systems, including insufficient modeling of cellular interactions. Kidney organoids offer new opportunities for modeling patient-specific kidney disease, studying kidney development, and performing nephrotoxic compound screening.

STEMdiff™ Kidney Organoid Kit was developed based on the protocol published by Freedman et al. It was further optimized to standardize the differentiation of human pluripotent stem cells (hPSCs) into kidney organoids that are composed of podocytes, proximal and distal tubules, and an associated endothelium and mesenchyme. These kidney organoids are particularly amenable to high-throughput phenotypic screening assays in 96- or 384-well plate formats.

2.0 Materials, Reagents, and Equipment Required

2.1 STEMdiff™ Kidney Organoid Kit (Catalog #05160)

The following components are sold as a complete kit and are not available for individual sale.

Refer to the Product Information Sheet (PIS) for STEMdiff™ Kidney Organoid Kit (Document #10000005418) for component storage and stability information; the PIS is also available at www.stemcell.com, or contact us to request a copy.

COMPONENT #	COMPONENT NAME	SIZE
05161	STEMdiff™ Kidney Basal Medium	100 mL
05162	STEMdiff™ Kidney Supplement SG (100X)	200 µL
05163	STEMdiff™ Kidney Supplement DM (50X)	1.6 mL

2.2 Additional Materials and Reagents

PRODUCT	CATALOG #
37 µm Reversible Strainer, Large	27250
50 mL conical tubes	e.g. 38010
96-well flat-bottom microplate, tissue culture-treated	e.g. 38022
ACCUTASE™	07920
CloneR™2	100-0691
Corning® 96-Well Half-Area High-Content Imaging Microplate*	100-0367
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free	Corning 356231
DMEM/F-12 with 15 mM HEPES	36254
D-PBS (Without Ca++ and Mg++)	37350
Hausser Scientific™ Bright-Line Hemocytometer	100-1181
µ-Plate 96-well, black*	Ibidi 89626
mTeSR™1	85850

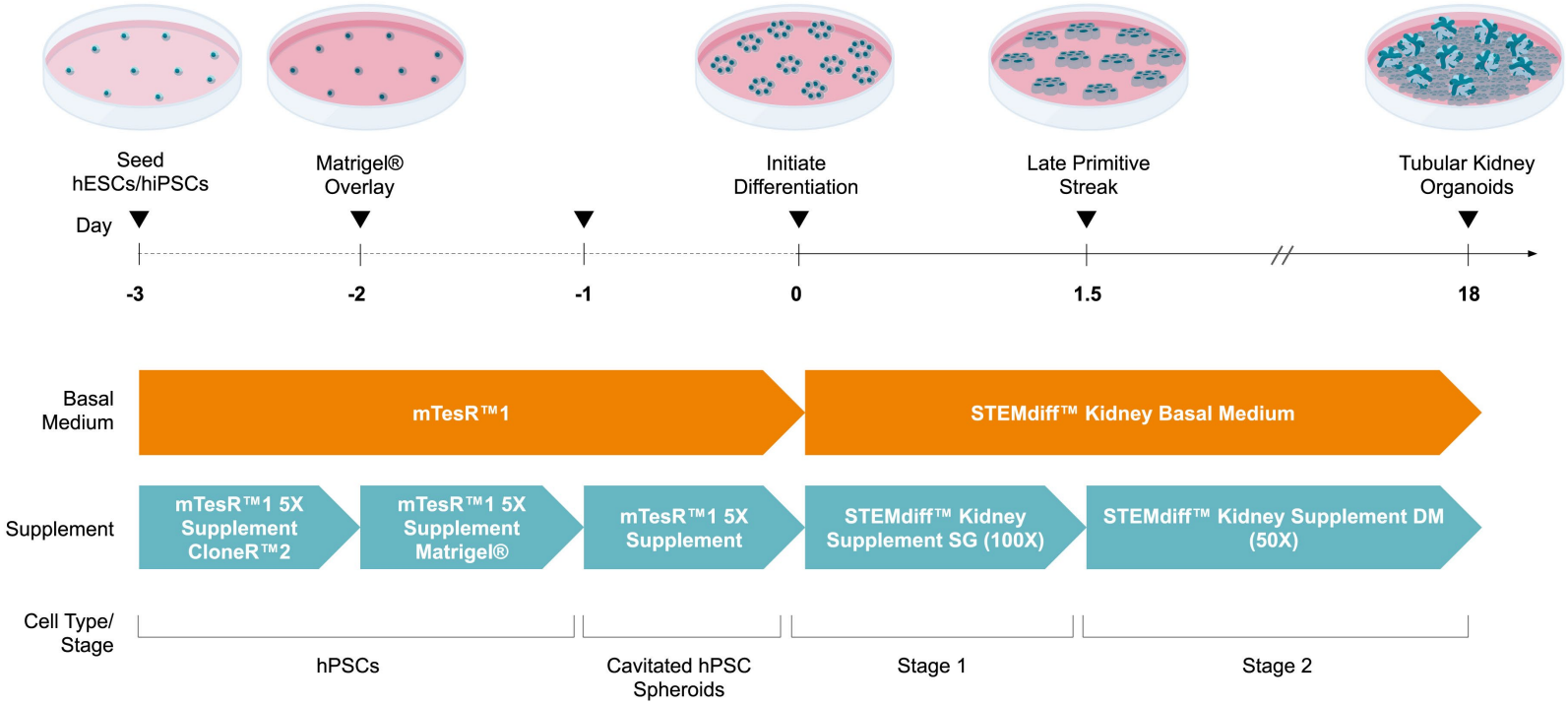
*Use this plate if high-quality imaging acquisition and analysis is required.

For a complete list of products available from STEMCELL Technologies Inc., visit www.stemcell.com.

2.3 Equipment

- Biosafety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Low-speed centrifuge with a swinging bucket rotor
- Pipette-aid with appropriate serological pipettes (e.g. Catalog #38002)
- Hemocytometer or Nucleocounter®
- Pipettor with appropriate tips (e.g. Catalog #38058)
- Multi-channel pipettor (20 - 200 µL; e.g. Catalog #38064) and reagent reservoir (e.g. Catalog #38080)
- Inverted microscope
- -20°C freezer
- Refrigerator (2 - 8°C)

3.0 Protocol Diagram



Experimental outline for differentiating hPSCs into kidney organoids, with illustrations from critical stages of differentiation. hPSCs, previously maintained in mTeSR™1 or mTeSR™ Plus, are plated into Corning® Matrigel®-coated plates on Day -3 in mTeSR™1. After 24 hours (Day -2), adherent cells are overlaid with an additional layer of Matrigel®, which results in the formation of cavitated PSC spheroids within the next 48 hours. On the following day (Day 0), differentiation of cavitated PSC spheroids is initiated by changing medium from mTeSR™1 to STEMdiff™ Kidney Organoid Kit. During the next 18 days, the cells are directed through stages of the late primitive streak, intermediate mesoderm, and metanephric mesoderm to give rise to kidney organoids that are composed of podocytes, proximal and distal tubules, and an associated endothelium and mesenchyme.

4.0 Coating Cultureware with Corning® Matrigel®

Corning® Matrigel® matrix should be aliquoted and frozen. Consult the Matrigel® Quality Certificate for the protein concentration. Always keep Matrigel® on ice when thawing and handling to prevent it from gelling.

The following instructions are for coating one well of a tissue culture-treated 96-well plate. For multiple wells or other cultureware, adjust volumes accordingly.

1. Thaw one aliquot of Matrigel® on ice.
2. Dilute Matrigel® in **cold** DMEM/F-12 with 15 mM HEPES to a protein concentration of 0.5 mg/mL.

   Example: For Matrigel® with a protein concentration of 10 mg/mL, add 2.5 µL Matrigel® to 47.5 µL DMEM/F-12 with 15 mM HEPES.
3. Immediately add 50 µL of the diluted Matrigel® to one well of a 96-well plate (25 µg protein/well). Swirl the plate to spread the Matrigel® solution evenly across the surface.
4. Incubate at room temperature (15 - 25°C) for at least 1 hour. Do not let the Matrigel® solution evaporate.

   Note: If not used immediately, cultureware must be sealed to prevent evaporation of the Matrigel® solution (e.g. with Parafilm®) and can be stored at 2 - 8°C for up to 7 days after coating. Allow stored coated cultureware to come to room temperature for 30 minutes before proceeding to step 5.
5. Immediately before use, gently tilt the cultureware on one side and allow the excess Matrigel® solution to collect at the edge. Remove the excess solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.

5.0 Differentiation of Kidney Organoids From hPSCs

5.1 Seeding hPSCs as Single Cells and Formation of Cavitated Spheroids

The following protocol is for single-cell seeding of high-quality hPSCs from a 6-well plate to a 96-well plate (Day -3), followed by the formation of cavitated PSC spheroids (Day -2 to Day 0), which become visible on Day -1.

Day -3

1. Use a microscope (4X magnification) to visually identify regions of differentiation in the hPSC culture. Mark these using a felt tip or lens marker on the bottom of the plate.
2. Remove regions of differentiation by scraping with a pipette tip or by aspiration. Wash with 2 mL/well D-PBS (Without Ca++ and Mg++).

   Note: It is critical that the cells are of high quality (less than 5% differentiation). Removal of differentiated cells will result in better organoid formation efficiency.
3. Aspirate D-PBS and add 0.5 mL ACCUTASE™ per well. Incubate at 37°C for 10 minutes.
4. During incubation, add 2 mL DMEM/F-12 per well harvested to a 50 mL conical tube.
5. After incubation, use a 1 mL pipettor to transfer cells into the tube containing DMEM/F-12.
6. Break up colonies into single cells by pipetting up and down 3 - 4 times.
7. Centrifuge cells at 300 x g for 5 minutes.
8. Aspirate supernatant and resuspend in 1 mL mTeSR™1 + CloneR™2 (1 in 10 dilution) per well harvested.
9. Filter cell suspension through a 37 µm Reversible Strainer and retain the flow-through.
10. Count single cells in the flow-through using a Nucleocounter® or hemocytometer.
11. Add the cell suspension to a sterile reagent reservoir. Using a multi-channel pipettor, seed 750 to 3000 cells into each well of a Matrigel®-coated 96-well plate (prepared in section 4.0). This should be ~20 - 140 µL of cell suspension per well. If the seeding volume is < 200 µL, top up to 200 µL with mTeSR™1 + CloneR™2 (1 in 10 dilution). A maximum of 200 µL of cell suspension can be seeded per well. Adjust seeding densities accordingly for alternative well formats.

    Note: An initial experiment is recommended to determine the optimal single-cell seeding density for the cell line being used. Seed a range of single-cell densities (e.g. 750, 1000, 2000, and 3000 single cells per well), and initiate differentiation of each density on the same day.
12. Ensure cells are evenly distributed by pipetting up and down several times with the multi-channel pipettor. After mixing, allow cells to settle by leaving the plate undisturbed inside the safety cabinet for 5 - 10 minutes.
13. Transfer the plate and incubate at 37°C and 5% CO_2 with 95% humidity overnight. See Figure 1 for representative images.

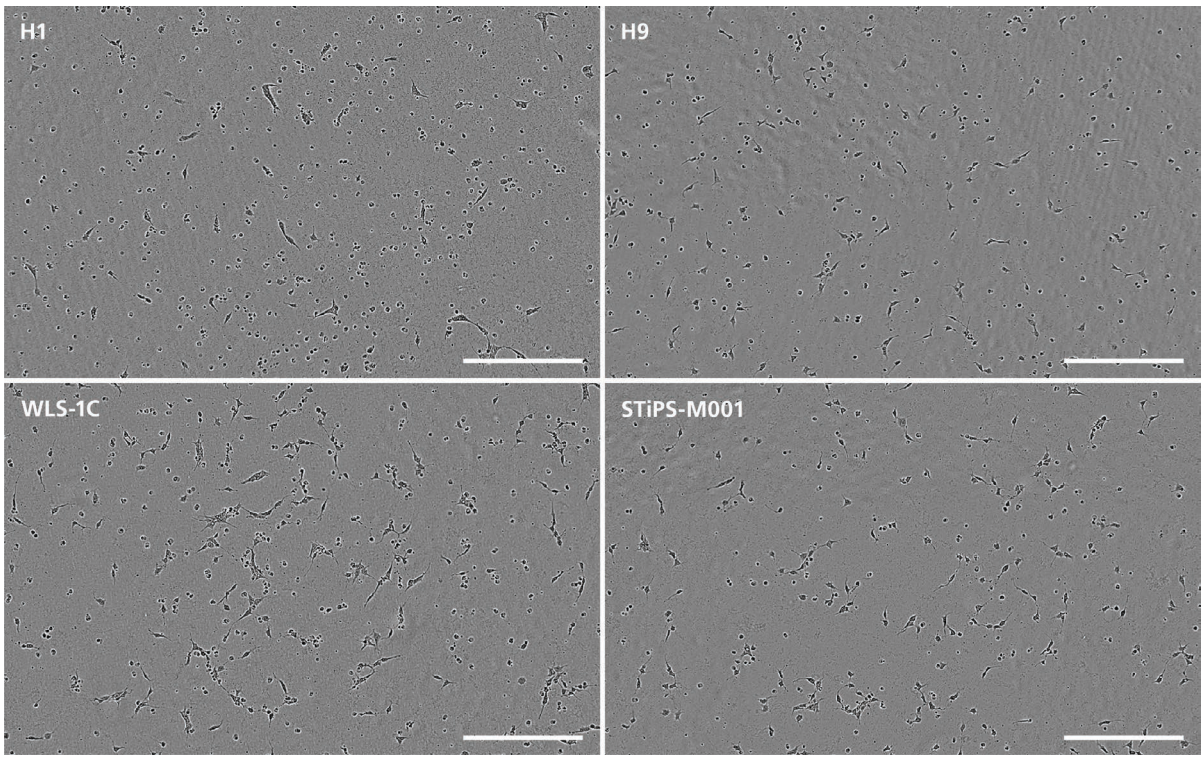


Figure 1. Single Cells After Seeding hPSCs (Day -2)

H1 (WA01) and H9 (WA09) hESCs and WLS-1C and StiPS-M001 hiPSCs on Day -2. H1, H9, and StiPS-M001 cells were seeded at 3000 cells/well; WLS-1C cells were seeded at 5000 cells/well. Scale bars: 450 µm.

Day -2 (hPSCs to cavitated spheroids)

14. Perform a full-medium change to overlay cells with Matrigel® as follows:
    a. Thaw one aliquot of Matrigel® on ice.
    b. Add Matrigel® to **cold** (2 - 8°C) mTeSR™1 (without CloneR™2) to give a protein concentration of 0.25 mg/mL.

       Example for one well: For Matrigel® with a protein concentration of 10 mg/mL, add 2.5 µL Matrigel® to 97.5 µL mTeSR™1.
    c. **Immediately** remove medium from wells, then add 100 µL **cold** mTeSR™1 + Matrigel® per well (25 µg protein/well).

       Note: At this stage cold mTeSR™1 + Matrigel® should be added directly to the cells to prevent Matrigel® from gelling. Medium changes can be performed with a multi-channel pipettor. Remove medium with sterile tips, ensuring tips do not touch the bottom of the well, then discard in a waste reservoir.
    d. Incubate at 37°C for 24 hours.

Day -1

15. Perform a full-medium change with 100 µL/well of mTeSR™1 (room temperature). Incubate at 37°C for 1 day.

    Note: At Day 0, hPSC colonies should begin to form round, cavitated PSC spheroids. See Figure 2 for representative images.

16. Proceed to section 5.2 for Stage 1 of kidney organoid differentiation.

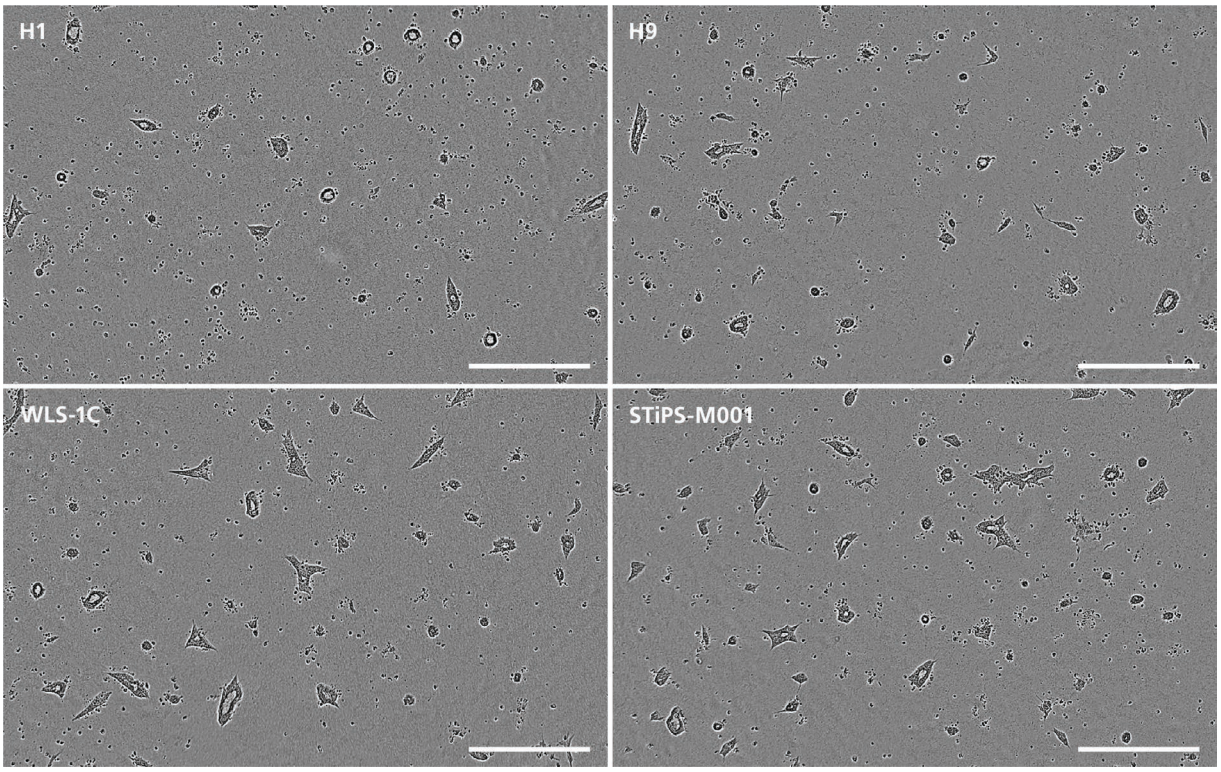


Figure 2. hESCs (H1 and H9) and hiPSCs (WLS-1C and StiPS-M001) on Day 0

Overlaying hPSCs (seeded as single cells) with Corning® Matrigel® results in the efficient formation of cavitated PSC spheroids. Scale bars: 450 µm.

5.2 Kidney Organoid Differentiation Stage 1

After initiation of differentiation (steps 1 - 2 below), an incubation period of 36 hours is optimal (up to 40 hours is acceptable). Plan to perform the following steps at the end of Day 0, based on when you would like to reach the 36- to 40-hour timepoint (Day 1.5, section 5.3).

Day 0 (Initiate kidney organoid differentiation)

1. Use sterile technique to prepare **Stage 1 Medium** (STEMdiff™ Kidney Basal Medium + STEMdiff™ Kidney Supplement SG). The following example is for preparing 200 µL of medium (one well of a 96-well plate). If preparing other volumes, adjust accordingly.
   a. Thaw STEMdiff™ Kidney Basal Medium at room temperature (15 - 25°C) or overnight at 2 - 8°C. Mix thoroughly.

      Note: If not used immediately, store at 2 - 8°C for up to 2 months. Alternatively, aliquot and store at -20°C. Do not exceed the shelf life of the medium. After thawing aliquots, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.

   b. Thaw STEMdiff™ Kidney Supplement SG on ice. Mix thoroughly.

      Note: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. After thawing aliquots, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.

c. For one well of a 96-well plate, add 2 µL Supplement SG to 198 µL Basal Medium. Mix thoroughly. Warm to room temperature before use.

2. Carefully remove medium from each well of the 96-well plate from section 5.1. Add 200 µL of Stage 1 Medium (room temperature) to each well. Incubate at 37°C for 36 - 40 hours.
3. Proceed to section 5.3 for Stage 2 of kidney organoid differentiation.

5.3 Kidney Organoid Differentiation Stage 2

See Figure 3 for representative images of cells at 36 hours after induction of late primitive streak (Day 1.5).

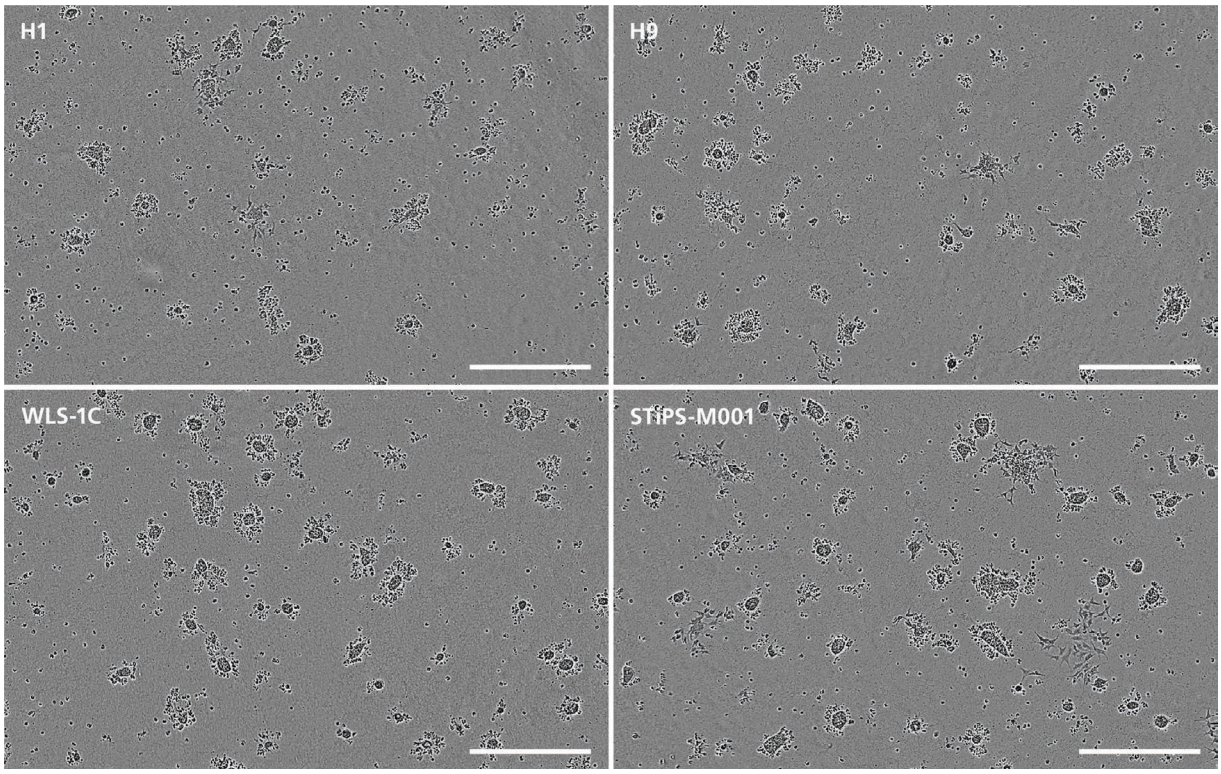


Figure 3. hESCs (H1 and H9) and hiPSCs (WLS-1C and StiPS-M001) on Day 1.5

During Stage 1 differentiation (Day 0 - Day 1.5), late primitive streak is induced. Cells undergo a significant amount of cell death, which results in partial cell detachment and reduced colony sizes. Scale bars: 450 µm.

Day 1.5 (36 hours after initiation of differentiation)

1. Use sterile technique to prepare **Stage 2 Medium** (STEMdiff™ Kidney Basal Medium + STEMdiff™ Kidney Supplement DM). The following example is for preparing 500 µL of medium (sufficient for 4 - 5 medium changes in one well). If preparing other volumes, adjust accordingly.
   a. Thaw STEMdiff™ Kidney Supplement DM at room temperature (15 - 25°C). Mix thoroughly.

      Note: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. After thawing aliquots, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.

b. For one well of a 96-well plate, add 10 µL Supplement DM to 490 µL Basal Medium. Mix thoroughly. Warm to room temperature before use.

Note: This volume is sufficient for 4 - 5 medium changes. If tubular kidney organoids begin to form on the monolayer at ~Day 8 - 10, continue differentiation and prepare medium for the remaining days of culture.

2. Perform a full-medium change with 200 µL/well of Stage 2 Medium (room temperature). Incubate at 37°C for 2 days.

Note: Carefully remove Stage 1 Medium, then slightly tilt the plate and add Stage 2 Medium dropwise against the plate wall. Cells are delicate and can easily lift off at this stage.

3. Store remaining Stage 2 Medium at 2 - 8°C for up to 2 weeks.

Day 4

4. Carefully perform a full-medium change with 100 µL/well of Stage 2 Medium (room temperature). Incubate at 37°C for 2 - 3 days.

Note: See Figure 4 for representative images from Day 4.

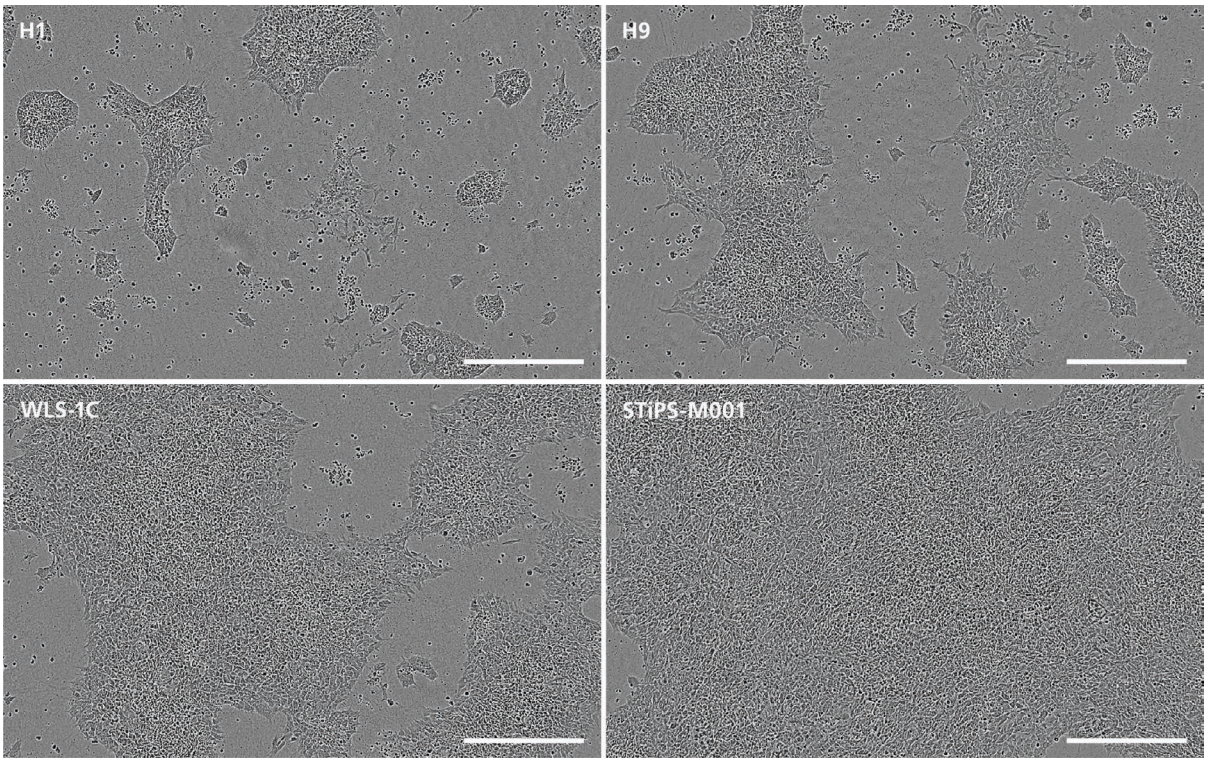


Figure 4. Differentiation Cultures Begin to Form a Confluent Monolayer on Day 4

Day 4 differentiation cultures derived from hESCs (H1 and H9) and hiPSCs (WLS-1C and StiPS-M001) show increased cell recovery and expansion compared to the end of Stage 1. Cells begin to form confluent monolayers, but cell densities are highly dependent on the intrinsic variability between different cell lines. Scale bars: 450 µm.

Day 6 - 18

5. Every 2 - 3 days until Day 18, perform a full-medium change with 100 µL/well of Stage 2 Medium (room temperature). Incubate at 37°C.

   Note: We recommend performing full-medium changes on Monday, Wednesday, and Friday each week. At Day 8 - 10, if no tubular kidney organoid structures are visible (see Figure 5) the cultures should be restarted, as no kidney organoids will develop.

6. See Figure 5 and Figure 6 for representative images from Day 8 and Day 15, respectively. On Day 18, tubular kidney organoids will be fully established and can be used in standard assays or harvested for flow cytometry. Kidney organoids can be cultured for up to 1 week beyond Day 18, with full-medium changes every 2 - 3 days; however, no further development will occur.
7. For kidney organoid fixation and immunostaining, proceed to section 6.0.

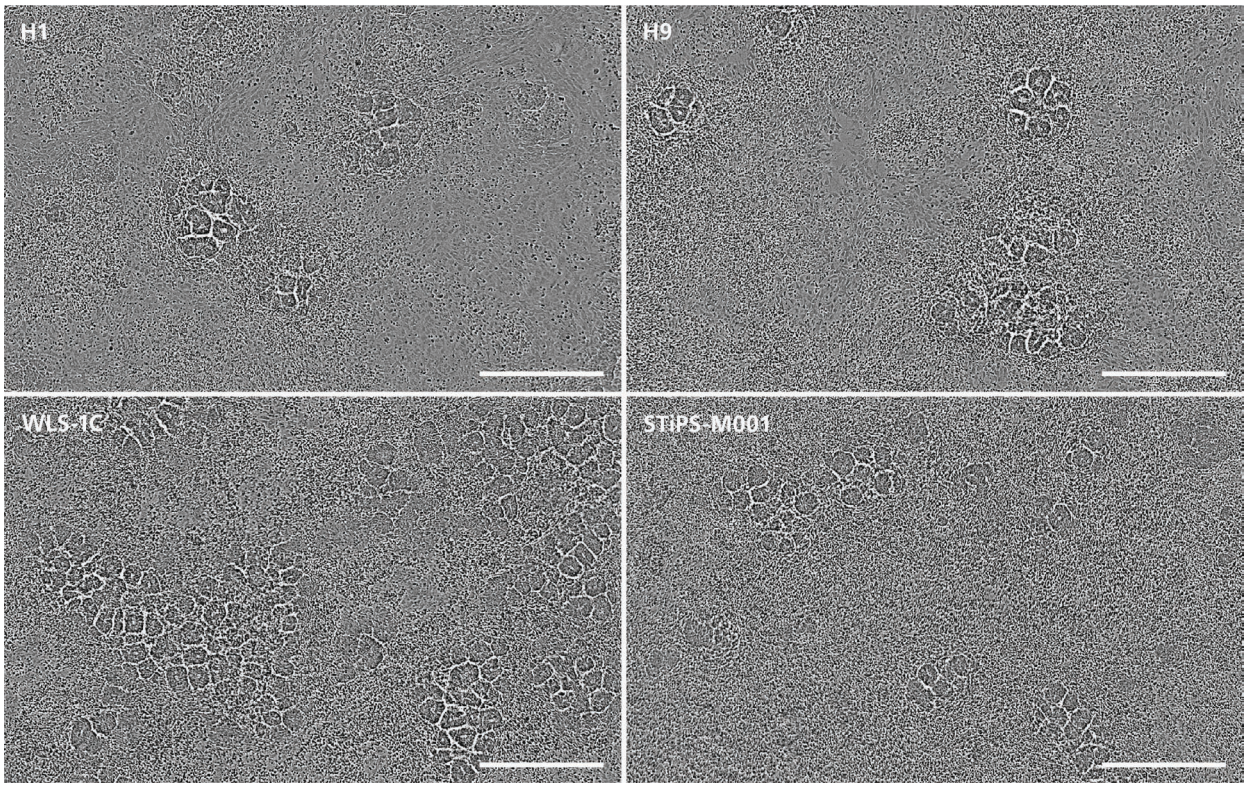


Figure 5. Early Tubular Kidney Organoid Structures on Day 8

Emergence of early organoid structures derived from hESCs (H1 and H9) and hiPSCs (WLS-1C and StiPS-M001). Self-organizing convoluted tubular organoid structures arise from the underlying monolayer culture. Scale bars: 450 µm.

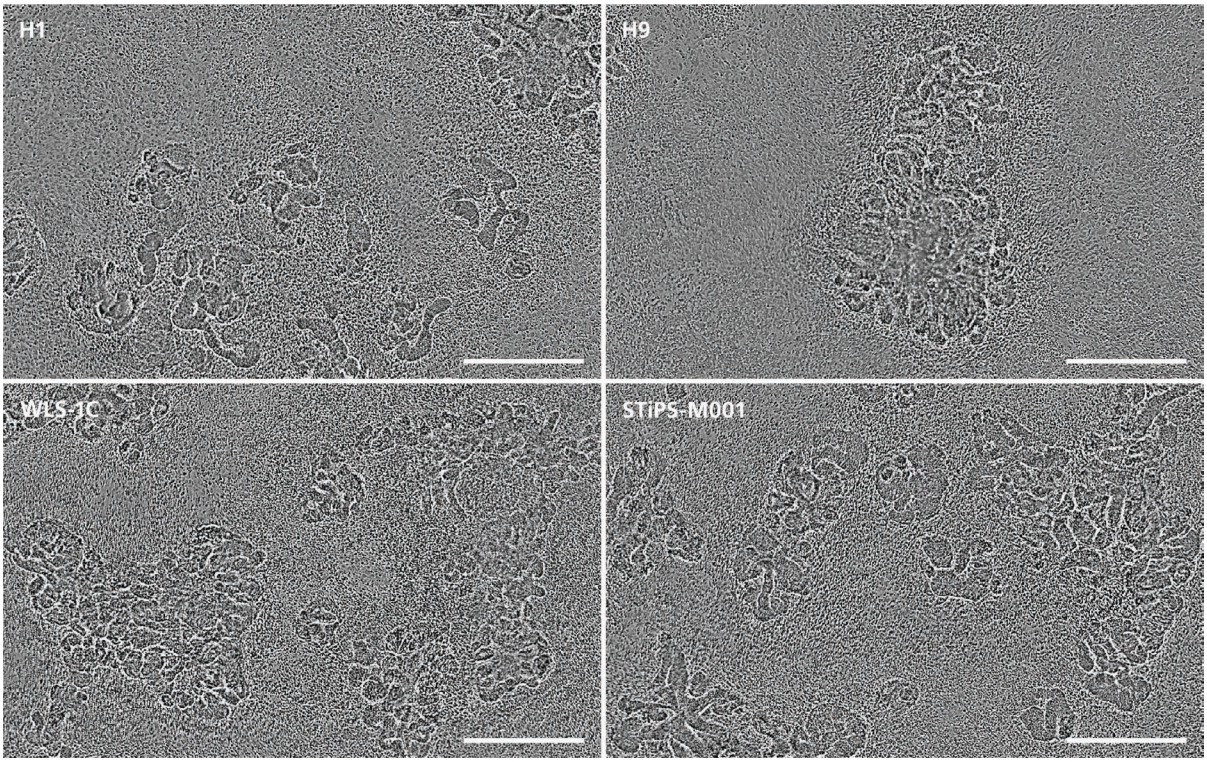


Figure 6. Late Tubular Kidney Organoids on Day 15

Kidney organoids derived from hESCs (H1 and H9) and hiPSCs (WLS-1C and StiPS-M001) have a densely packed, multi-tubular morphology that protrudes from the underlying monolayer culture. Scale bars: 450 µm.

6.0 Kidney Organoid Fixation and Immunostaining

6.1 Materials Required

PRODUCT	CATALOG #
D-PBS (Without Ca++ and Mg++)	37350
Paraformaldehyde	e.g. Affymetrix 19943 1 LT
Triton™ X-100	e.g. Millipore Sigma X100-100ML
Donkey Serum	Millipore Sigma D9663-10ML
DAPI (Hydrochloride)	75004

6.2 Preparation of Reagents

Prepare the following reagents before proceeding to section 6.3:

4% Paraformaldehyde Solution

Prepare a 4% solution of paraformaldehyde in D-PBS. Mix thoroughly and store at 2 - 8°C.

PBS-T (permeabilization buffer)

Dilute Triton™ X-100 in D-PBS to a final concentration of 0.2%. Mix thoroughly.

Note: Prepare fresh for each experiment.

Blocking Buffer

Dilute Donkey Serum in PBS-T to a final concentration of 10%. Mix thoroughly. Store on ice or at 2 - 8°C while in use.

Note: Prepare fresh for each experiment.

DAPI Solution

Add DAPI to Blocking Buffer to a final concentration of 1 µg/mL. Mix thoroughly.

6.3 Fixation and Immunostaining Protocol

The following protocol is for one well of a 96-well plate. If using other cultureware, adjust volumes accordingly.

1. Aspirate medium from the well containing kidney organoids. Carefully add 200 µL D-PBS to the well. Remove D-PBS.
2. Add 85 µL 4% Paraformaldehyde Solution to the well (fixation). Incubate at room temperature (15 - 25°C) for 15 minutes. Remove the solution.
3. Wash the well 3X with 200 µL D-PBS. Remove D-PBS.

   Note: For later immunostaining, keep 200 µL D-PBS in the well, wrap plate with Parafilm®, and store at 2 - 8°C.
4. Remove D-PBS and add 200 µL PBS-T to the well. Incubate at room temperature for 15 minutes. Remove PBS-T.
5. Add 200 µL Blocking Buffer to the well. Incubate at room temperature for at least 30 minutes.
6. While incubating Blocking Buffer in the well, prepare primary antibody mix by diluting each primary antibody in Blocking Buffer. Refer to Table 1 for recommended dilutions and antibodies.
7. Remove Blocking Buffer from the well and add 80 µL primary antibody mix. Incubate at 2 - 8°C overnight (> 16 hours) with low shaking. Remove primary antibody mix.

8. Prepare secondary antibody mix by diluting each secondary antibody 1 in 1000 in Blocking Buffer + DAPI (1 µg/mL final concentration). Refer to Table 1 for recommended antibodies.
9. Add 200 µL D-PBS to the well and incubate at room temperature for 5 minutes. Remove D-PBS. Repeat this wash step 5X for a total of 6 washes.
10. Add 80 µL secondary antibody mix to the well. Incubate in the dark at room temperature overnight with low shaking. Remove secondary antibody mix.
11. Add 200 µL D-PBS to the well and incubate at room temperature for 5 minutes. Remove D-PBS. Repeat this wash step 5X for a total of 6 washes.
12. Add 200 µL D-PBS to stained cells; they are now ready for immunofluorescent imaging.

    Note: If not used immediately for imaging, wrap plate with Parafilm® and store in the dark at 2 - 8°C.

Table 1. Recommended Primary and Secondary Antibodies for Immunostaining Kidney Organoids

PRIMARY ANTIBODY	DILUTION	CATALOG #	SECONDARY ANTIBODY (1 in 1000 dilution)	JACKSON IMMUNORESEARCH CATALOG #
PODXL	1 in 100	R&D Systems AF1658	Alexa Fluor® 594-conjugated Donkey Anti-Goat IgG (H+L)	705-585-147
LTL	1 in 200	Vector Laboratories B-13225	Alexa Fluor® 488-conjugated Streptavidin	016-540-084
ECAD	1 in 100	BD Biosciences 610182	Alexa Fluor® 647-conjugated Donkey Anti-Mouse IgG (H+L)	715-605-151
WT1	1 in 200	Cell Signaling Technology 83535	Alexa Fluor® 647-conjugated Donkey Anti-Rabbit IgG (H+L)	711-605-152i
VIM	1 in 400	abcam ab92547	Alexa Fluor® 647-conjugated Donkey Anti-Rabbit IgG (H+L)	711-605-152i
CD31	1 in 200	Dako M082329-2	Alexa Fluor® 594-conjugated Donkey Anti-Mouse IgG (H+L)	715-585-151
NPHS1	1 in 300	R&D Systems AF4269-SP	Alexa Fluor® 594-conjugated Donkey Anti-Sheep IgG (H+L)	713-585-147
PAX8	1 in 300	VWR 10091-826	Alexa Fluor® 647-conjugated Donkey Anti-Rabbit IgG (H+L)	711-605-152i
SIX2	1 in 100	proteintech 11562-1-AP	Alexa Fluor® 647-conjugated Donkey Anti-Rabbit IgG (H+L)	711-605-152i

7.0 Troubleshooting

PROBLEM	POSSIBLE CAUSE	SOLUTION
Low efficiency of kidney organoid formation	• Low-quality hPSC starting culture • Not optimal seeding density • mTeSR™ Plus is used for the three days prior to the start of differentiation (Day -3 – 0)	• Ensure high-quality, undifferentiated hPSC starting culture by removing all spontaneously differentiated hPSC colonies (section 5.1 step 2). • Determine optimal seeding density for the cell line being used by testing a range of single-cell densities (e.g. 1000, 3000, 5000, and 7000 single cells per well of a 96-well plate) in an initial experiment. • Seed cells maintained in mTeSR™ Plus into mTeSR™1 + 10 µM CloneR™2 on day -3 and continue subsequent medium changes with mTeSR™1 until the start of differentiation.
Cells completely detach after Stage 1 differentiation	• Single-cell seeding density is too low • Insufficient Matrigel® coating on plate or insufficient overlay with Matrigel®	• Start differentiation with higher single-cell seeding density. • Calculate optimal Matrigel® concentration based on the examples provided in section 4.0 step 2 and section 5.1 step 14. Work carefully but quickly to avoid gelling of Matrigel®.
Uneven distribution of kidney organoids across the well	hESCs/hiPSCs were not plated evenly	• Ensure single cells are evenly distributed by pipetting up and down several times with the multi-channel pipettor prior to plating. After mixing the cell suspension, leave the plate undisturbed inside the biosafety cabinet for 5 - 10 minutes (section 5.1 step 12). • Carefully transfer plate into an incubator at 37°C and 5% CO_2 with 95% humidity overnight.
Medium turns yellow quickly and formed organoids darken and lose tubular morphology	• Cultures are over-confluent • Medium change is not performed frequently enough, resulting in insufficient nutrient supply.	• Perform medium changes every 3 days until Day 10, then switch to performing medium changes every 2 days as cultures grow denser and organoids form. • Over-confluent wells can be rescued by doubling the medium volume or changing medium daily. Note that this will reduce the number of total wells that can be differentiated with STEMdiff™ Kidney Organoid Kit. • Reduce the number of cells seeded on Day -3 to avoid overgrowth.

8.0 References

Freedman BS et al. (2015) Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. Nature Communications 6: 8715.

Morizane R et al. (2015) Nephron organoids derived from human pluripotent stem cells model kidney development and injury. Nature Biotechnology 33(11): 1193–200.

Takasato M et al. (2015) Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. Nature 526(7574): 564–8.

TECHNICAL MANUAL

Generation of Human Kidney Organoids Using STEMdiff™ Kidney Organoid Kit

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