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## Generation of human kidney tubuloids from tissue and urine

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**TITLE:**

Generation of Human Kidney Tubuloids from Tissue and Urine

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**SUMMARY:**

Human kidney tubuloid cultures represent a valuable *in vitro* model to study kidney physiology and disease. Tubuloids can be established from kidney tissue (healthy and diseased) as well as urine, the latter representing an easily obtainable and less invasive source of research material.

**ABSTRACT:**

Adult stem cell (ASC)-derived human kidney epithelial organoids, or tubuloids, can be established from healthy and diseased kidney epithelium with high efficiency. Normal kidney tubuloids recapitulate many aspects of their tissue of origin. They represent distinct nephron segments—most notably of the proximal tubule, loop of Henle, distal tubules, and collecting duct—and can be used to study normal kidney physiology. Furthermore, tubuloid technology facilitates disease modeling, *e.g.*, for infectious diseases as well as for cancer. Obtaining kidney epithelial cells for tubuloid generation is, however, dependent on leftover surgical material (*e.g.*, (partial) nephrectomies) or needle biopsies. The ability to grow tubuloids from urine would provide an alternative, less invasive source of healthy kidney epithelial cells. It has been previously shown that tubuloid cultures can be successfully generated from only a few milliliters of freshly collected urine. This article describes the protocols to generate and propagate ASC-derived human kidney tubuloid cultures from tissue and urine samples.

**INTRODUCTION:**

Kidneys perform the function of systemically controlling the balance of body fluids. The impairment of their physiological function can be caused by different factors, including diabetes, hypertension, and drug-induced toxicity<sup>1</sup>. For a better understanding of normal kidney physiology as well as the development of renal diseases, the use of representative preclinical models is crucial. In recent years, several *in vitro* kidney models have been generated based on the so-called organoid technology<sup>2</sup>. Organoids are three-dimensional, multicellular structures resembling the morphology and physiology of the tissue (normal or diseased) from which they originate. They can be generated from pluripotent (PSCs) or adult stem cells (ASCs), each with their own characteristics and applications.



PSC-derived kidney organoids mimic nephrogenesis<sup>3-5</sup>. They can also be established from patient-derived committed cells by forced dedifferentiation (induced pluripotency or iPSC). iPSCs can subsequently be differentiated into the different cell types of the nephron, the functional unit of the kidney, by timely exposure to specific growth factor cocktails. While creating a rather complete mini-organ in a dish, their establishment remains time-consuming, and due to the reprogramming protocol, iPSCs can be susceptible to undesired genetic instability<sup>6</sup>. Furthermore, iPSC organoids are not able to fully mature into adult kidney cells, revealing a transcriptome profile that resembles the fetal kidney at an early development stage<sup>7</sup>.

ASC-derived human kidney tubuloids have been shown to recapitulate the renewal of adult kidney epithelium. They primarily represent the proximal tubule, loop of Henle, distal tubules, and collecting duct, as confirmed by the expression of different transporter proteins<sup>8-10</sup>. The tubuloid culture protocol allows for the rapid expansion of patient-derived kidney tissue, while retaining a stable genome. Research applications include studying normal kidney physiology, nephrotoxicity, drug testing, as well as disease modelling<sup>8,10-12</sup>. A potential limitation of the establishment of patient-derived organoid cultures, including tubuloids, is the availability of fresh tissue. However, several reports have shown that urine can serve as a source for kidney epithelial cells, thereby providing a much simpler, less invasive strategy to obtain patient material for tubuloid cultures<sup>8,13,14</sup>. Indeed, it has been recently shown that tubuloids can be grown from urine<sup>8</sup>. This article describes the establishment and maintenance of tubuloid cultures from kidney tissue and urine.

## **PROTOCOL:**

NOTE: The experiments described herein were approved by the medical ethical committee of the Erasmus Medical Center (Rotterdam, the Netherlands) and Princess Máxima Center for Pediatric Oncology (Utrecht, the Netherlands).

### **1. Generation of human kidney tubuloids from tissue**

#### **1.1. Materials**

1.1.1. Prewarm multiwell tissue culture plates (6, 12, 24, and 48 wells) overnight at 37 °C.

1.1.2. Prepare basal medium (AdDF+++) by adding 1x L-alanine/L-glutamine supplement, 1% w/v 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES, 10 mM), and 1% Penicillin/Streptomycin antibiotics to Advanced DMEM/F12.

1.1.3. Prepare culture medium by adding 1.5% B27 supplement, 10% R-spondin-conditioned medium, epidermal growth factor (50 ng/mL), fibroblast growth factor-10 (100 ng/mL), *N*-acetylcysteine (1.25 mM), Rho-kinase inhibitor Y-27632 (10 μM), broad-spectrum antibiotics (0.1 mg/mL), and A83-01 (5 μM) to AdDF+++. Warm to 37 °C before use.

1.1.4. Prepare the basement membrane extract (BME) by thawing an aliquot overnight at 4 °C. Keep the BME on ice during the procedure.

1.1.5. Prepare collagenase solution by diluting collagenase to a final concentration of 1 mg/mL in AdDF+++, with the addition of 10 µM Y-27632. Warm up to 37 °C.

1.1.6. Prepare 10 cm Petri dishes, scalpels, and tweezers. Disinfect the utensils by applying an ultraviolet lamp for 20 min, followed by washings with disinfectant, demi water, and 70% v/v ethanol. Air-dry the utensils.

1.1.7. Prewarm a horizontal shaker to 37 °C.

## 1.2. Procedure

1.2.1. Collect kidney tissue in a 50 mL tube filled with 30–40 mL of AdDF+++ medium. Place the piece of tissue in ~1 mL of medium in a 10 cm Petri dish. Mince the tissue into pieces of ~1 mm<sup>3</sup> size using scalpels (**Figure 1A**).

1.2.2. Transfer the minced tissue to a 15 mL tube using forceps and scalpels. Add 5 mL of AdDF+++ to the Petri dish, wash, and collect the medium with a 10 mL sterile pipette. Transfer all these contents to the same tube.

1.2.3. Centrifuge at 300 × *g* for 5 min at room temperature, and remove the supernatant. Add 3–4 mL of collagenase solution to the 15 mL tube. Move the tube to a horizontal shaker set at 37 °C and a speed of 250 rpm.

1.2.4. After the first 15 min of incubation, check the sample and vigorously shake the tube. Repeat until the suspension is homogeneous and most pieces of tissue have disappeared, with a maximum incubation time of 45–60 min. (**Figure 1B**).

1.2.5. Fill up the tube with AdDF+++, and mix by inverting the tube 5–10x. Centrifuge at 300 × *g* for 5 min at 4 °C, and remove the supernatant. If the pellet is red, proceed with step 1.2.6. Otherwise, go to step 1.2.8.

1.2.6. Add 1 mL of red blood cell lysis buffer (see the **Table of Materials**) on top of the cell pellet. Gently tap the tube to resuspend the pellet (do not resuspend with pipette tip). Incubate at room temperature for 5 min.

1.2.7. Fill up the tube with AdDF+++. Centrifuge at 300 × *g* for 5 min at 4 °C, and remove the supernatant. If blood cells are still visible, repeat the procedure once more, starting from step 1.2.6. Otherwise, proceed with step 1.2.8.

1.2.8. If chunks of tissue are visible at this point of the protocol, proceed with step 1.2.8. Otherwise, proceed with the measurement of the pellet volume in step 1.2.9. Add 5 mL of

AdDF+++ to the tube, and resuspend with a 10 mL sterile pipette. Filter the suspension through a 100  $\mu$ m nylon cell strainer placed on a 50 mL tube.

1.2.9. Transfer the filtered suspension to a new 15 mL tube. Centrifuge at  $300 \times g$  for 5 min at 4  $^{\circ}$ C, and remove the supernatant. Measure the volume of the cell pellet using a p1000 pipette set to a known volume. Carefully pipette up and down to resuspend without creating air bubbles. Transfer the tube to ice for 1 min.

1.2.10. Add 70–75% volume of BME to the pellet. Resuspend using a p1000 or p200 pipette, and plate 15  $\mu$ L droplets in a prewarmed, multiwell cell culture plate (6, 12, or 24 wells, based on total plating volume (<100  $\mu$ L, 24 wells; 100–200  $\mu$ L, 12 wells; >200  $\mu$ L, 6 wells)).

1.2.11. Turn the plate upside down, and let the plate rest in the incubator for 15–20 min at 37  $^{\circ}$ C (**Figure 1C**). Add prewarmed culture medium, and inspect the cultures (**Figure 1C,D**).

## 2. Generation of human kidney tubuloids from urine

NOTE: Urine is a hostile environment for cells. It is important for a successful execution of this protocol that urine samples are processed as soon as possible, preferably within 4 h from excretion. In the meantime, urine samples should be stored at 4  $^{\circ}$ C.

### 2.1. Materials

2.1.1. Prewarm multiwell tissue culture plates (6, 12, 24, and 48 wells) overnight at 37  $^{\circ}$ C.

2.1.2. Prepare washing medium: AdDF+++ supplemented with broad-spectrum antibiotics (0.1 mg/mL) and 10  $\mu$ M Y-27632.

2.1.3. Prepare culture medium as described above. Warm up at 37  $^{\circ}$ C before use.

2.1.4. Prepare BME matrix. Keep on ice during the procedure.

### 2.2. Procedure

2.2.1. Collect a urine sample, and divide it equally into 50 mL tubes (**Figure 2A–I**). Add 10–20 mL of washing medium to each of the tubes. Centrifuge at  $300 \times g$  for 5 min at 4  $^{\circ}$ C (**Figure 2A–II**), and remove the supernatant carefully.

2.2.2. Add 10 mL of washing medium to each of the tubes. Carefully resuspend the pellets using a 10 mL sterile pipette. Centrifuge at  $300 \times g$  for 5 min at 4  $^{\circ}$ C (**Figure 2A–III**), and remove the supernatant carefully.

2.2.3. Resuspend the pellets, and transfer the contents of all the tubes into one 15 mL tube. Fill the tube with washing medium. Centrifuge at  $300 \times g$  for 5 min at 4  $^{\circ}$ C (**Figure 2A–IV**), and remove

the supernatant.

2.2.4. Measure the volume of the cell pellet using a p1000 pipette set to a known volume. Carefully pipette up and down to resuspend without creating air bubbles. Transfer the tube to ice for 1 min.

2.2.5. Add 70–75% volume of the BME to the pellet. Resuspend using a p1000 or p200 pipette, and plate 15  $\mu$ L droplets in a prewarmed, multiwell cell culture plate (6, 12, or 24 wells, based on total plating volume (<100  $\mu$ L, 24 wells; 100–200  $\mu$ L, 12 wells; >200  $\mu$ L, 6 wells)).

2.2.6. Turn the plates upside down in the incubator for 15–20 min at 37 °C. Add prewarmed culture medium, and inspect the cultures (**Figure 2B**).

### 3. Expansion of tubuloid cultures

NOTE: Tubuloid cultures can be passaged approximately every 1–2 weeks with a split ratio of 1:2–1:3. They can be typically expanded for a maximum of 15 passages, with line-specific variations.

#### 3.1. Materials

3.1.1. Prewarm multiwell tissue culture plates (6, 12, 24, and 48 wells) overnight at 37 °C.

3.1.2. Prepare basal medium (AdDF+++), and keep it on ice during the procedure.

3.1.3. Prepare culture medium as previously described. Warm up to 37 °C before use.

3.1.4. Prepare BME matrix, and keep on ice during the procedure.

3.1.5. Prepare trypsin replacement agent with the addition of Y-27632 to a concentration of 10  $\mu$ M. Warm up to 37 °C before use.

3.1.6. Autoclave non-filtered p10 tips.

3.1.7. Cool the centrifuge to 4 °C.

#### 3.2. Procedure

3.2.1. Disrupt the droplets containing the tubuloids by pipetting up and down with a p1000 pipette using the medium present in the well. Use the tip to scrape the bottom of the well, making sure to collect all cells attached to the bottom.

3.2.2. Collect the contents in a 15 mL tube. Add 10 mL of AdDF+++. Centrifuge at 300 x *g* for 5 min at 4 °C, and remove the supernatant.

3.2.3. Based on the size of the pellet, add trypsin replacement agent supplemented with 10  $\mu$ M Y-27632. Use 1 mL of trypsin replacement agent for 200  $\mu$ L of BME droplets containing tubuloids. Incubate at 37 °C for 5 min.

3.2.4. Use a p1000 pipette with tip, and insert a non-filtered, sterile p10 tip over the 1000  $\mu$ L tip. Pipette up and down for 20–30x to mechanically disrupt the organoids.

3.2.5. Check under the microscope to see if many intact organoids are still present in the tube (**Figure 3A**). If more than 10% of intact organoids are present at this point, repeat from the 5 min incubation in step 3.2.3 to the microscopic observation for intact organoids in step 3.2.5 once more. Otherwise, proceed with step 3.2.6.

3.2.6. Fill up the tube with AdDF+++. Centrifuge at 300  $\times g$  for 5 min at 4 °C, and remove the supernatant. Measure the volume of the cell pellet using a p1000 pipette set to a known volume. Carefully pipette up and down to resuspend without creating air bubbles. Transfer the tube to ice for 1 min.

3.2.7. Add 70–75% volume of BME to the pellet. Resuspend using a p1000 or p200 pipette, and plate 15  $\mu$ L droplets in a prewarmed, multiwell cell culture plate (6, 12, or 24 wells, based on total plating volume (<100  $\mu$ L, 24 wells; 100–200  $\mu$ L, 12 wells; >200  $\mu$ L, 6 wells)).

3.2.8. Turn the plates upside down, and let the plate rest in the incubator for 15–20 min at 37 °C. Add prewarmed culture medium, and inspect the cultures (**Figure 3B**).

#### REPRESENTATIVE RESULTS:

For kidney tissue, tubuloid structures typically appear within 7 days after establishment (**Figure 1D**). Lack of apparent growth within the first 7 days indicates an unsuccessful outcome of the protocol. Generally, tubuloid cultures need to be passaged within 1–2 weeks after first plating. For urine, cell growth becomes apparent approximately 14–21 days after establishment, with the appearance of compact tubuloid structures and/or adherent cells on the bottom of the plate (**Figure 2B**). Culture establishment most likely has failed if no growth can be detected with a brightfield microscope within 4 weeks after plating. For urine-derived cultures, the first passaging generally occurs between 3 and 4 weeks. While in the first passages, kidney tubuloid cultures consist mainly of compact structures, the presence of cystic epithelial tubuloids increases with the increase of passage number (**Figure 1D** and **Figure 2B**). The successful generation of human kidney tubuloid cultures can be assessed by performing immunohistochemical staining for markers expressed in tubular kidney epithelium, such as paired box gene 8 protein (PAX8) (**Figure 4A,B**).

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Tissue-derived kidney tubuloid cultures.** (A) Overview of the procedure to mince kidney tissue. Tissue is minced to a size of  $\sim 1 \text{ mm}^3$  using scalpels. (B) Example of correct

enzymatic digestion of healthy kidney tissue. Tissue is shown before (left) and after (right) 45 min of enzymatic digestion with collagenase. Few pieces of tissue are still visible at the bottom of the tube, and the solution should become cloudy, indicating the presence of cells in the suspension. (C) Representative image of cell culture plates after plating of BME droplets containing the processed kidney tissue. After plating the droplets, culture plates are turned upside down (left) and placed in the incubator at 37 °C. After 15–20 min, prewarmed culture medium is added to the well (right). (D) Representative brightfield images of tissue-derived tubuloid cultures. The first tubuloid structures become visible 2–3 days after first seeding. With increasing passage numbers, tubuloids typically change morphology to a more cystic phenotype. Scale bars = 300 µm. Abbreviations: BME = basement membrane extract.

**Figure 2: Urine-derived kidney tubuloid cultures.** (A) Overview of urine sample processing. As soon as possible after collection (I), urine samples are divided into 50 mL tubes and diluted in washing medium (II). After a second washing step (III), the content of the tubes is pooled and a third and final wash step (IV) is performed before plating. (B) Representative brightfield images of urine-derived tubuloid cultures. The first tubuloid structures and adherent cells should be visible within 21 days after first plating. Scale bars = 300 µm.

**Figure 3: Passaging of kidney tubuloid cultures.** (A) Representative image of kidney tubuloid cultures after enzymatic digestion. After completing digestion, no more than 10% of intact tubuloid structures should remain. Scale bar = 300µm. (B) Representative image of kidney tubuloid cultures after plating. Inspection of the cultures confirms that the majority of the tubuloids have been disrupted. Scale bar = 300 µm

**Figure 4: Histological characterization of tubuloid cultures.** (A) H&E staining of healthy kidney tissue and tubuloids. Scale bars = 100 µm. (B) Immunohistochemistry of PAX8 in normal kidney tissue, tubuloids, MRTK tissue, and MRTK organoids. PAX8 positivity of the organoid structures confirms their kidney epithelial origin. Healthy kidney tissue shows both positive (tubules) and negative structures (glomeruli). MRTK tissue and organoids were included as negative control. Scale bars = 100 µm. Abbreviations: H&E = hematoxylin and eosin; PAX8 = paired box gene 8; MRTK = malignant rhabdoid tumor of the kidney.

## DISCUSSION:

Organoids are considered avatars of the tissue from which they are derived. They allow for rapid expansion of patient material while retaining the genotypic and phenotypic characteristics of the tissue of origin<sup>15</sup>. Organoid technology has recently opened the doors for the development of more representative preclinical models, which can be used as important tools to translate findings from the bench to the bedside. Kidney tubuloids are promising *in vitro* models for testing drug-induced nephrotoxicity, a common side effect of many chemotherapeutic drugs<sup>2,8,12</sup>. As such, patient-derived tumor organoid cultures were demonstrated to be predictive for patient response to treatment<sup>16-18</sup>. Testing drugs in a high-throughput manner on tubuloids therefore potentially allows better definition of therapeutic windows and decreases the risk of drug-induced nephrotoxicity in patients.

Commonly used antibiotics have been described to exert a toxic effect on the kidneys<sup>19</sup>. Although the presence of broad-spectrum antibiotics is necessary for the successful establishment of the cultures by preventing contamination, it is important to consider their potential nephrotoxicity. To date, although no negative effects of antibiotics have been observed on the establishment of tubuloid cultures, further investigation is needed to thoroughly evaluate their effects. Tubuloids can be exploited for studying and modeling diseases<sup>8</sup>. Ciliopathies (pathological dysfunction of cilia) as well as other genetic syndromes affecting the kidney could be studied by either generating tubuloid lines directly from affected subjects, or by using healthy cultures in which disease-specific driver mutations can be introduced via CRISPR/Cas9 genome editing<sup>20</sup>.

Although tubuloids are multicellular kidney cultures, they lack several renal cell types, including podocytes and endothelial cells<sup>8</sup>. Moreover, in contrast to some other ASC-derived organoid models, tubuloids have a limited replicative potential as they can be cultured for up to 15 passages. This limited lifespan can, however, be significantly extended by the addition of Wnt to the culture medium<sup>21</sup>. Further optimization of the tubuloid protocol is required to make tubuloids truly representative of the kidney. Although the efficiency of tubuloid establishment from tissue samples is very high (>95%), it can, in rare cases, fail. There can be different causes including: 1) poor quality of the starting material (*e.g.*, necrotic tissue as a consequence of drug treatment), 2) overdigestion of the tissue sample, or 3) contamination of the primary sample.

To make sure that the quality of the tissue samples received is sufficient to proceed with the protocol, it is important to maintain close contact with the pathology staff performing the evaluation of the tissue upon surgery. If sufficient material is available, its viability must be confirmed by histological examination (*e.g.*, hematoxylin and eosin staining). Furthermore, to prevent cell lysis during enzymatic digestion, it is important that the incubation procedure is no longer than 1 h. Lastly, to prevent contamination, antibiotics and antifungal agents should be added to the washing and culture media. Urine can contain exfoliated epithelial cells that are not derived from the kidney<sup>22</sup>, which can contaminate the tubuloid cultures. These include, for instance, urothelium cells that are, in contrast to renal tubular cells, positive for tumor protein P63 and negative for PAX8<sup>8</sup>.

It is therefore recommended to test the cultures for PAX8 positivity to confirm the purity of established kidney tubuloid lines before proceeding with follow-up experiments (**Figure 4B**). Urine represents a hostile environment for cells due to high osmotic pressure and low pH. It is therefore crucial for the success of the protocol that samples are processed as soon as possible upon urine collection. As such, the collected urine should be diluted and extensively washed with buffered solution as soon as possible to ensure presence of viable cells at the time of seeding. The success rate of tubuloid establishment will significantly decrease if urine is stored for several hours before processing. Lastly, although sterile, urine has a high risk of contamination associated with the collection process. It is therefore important to supplement washing and growth medium with antibiotics and antifungal agents. When taking all the above into account, a success rate of approximately 50% (3/6 samples) can be achieved.

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#### DISCLOSURES:

The authors declare no conflict of interest.

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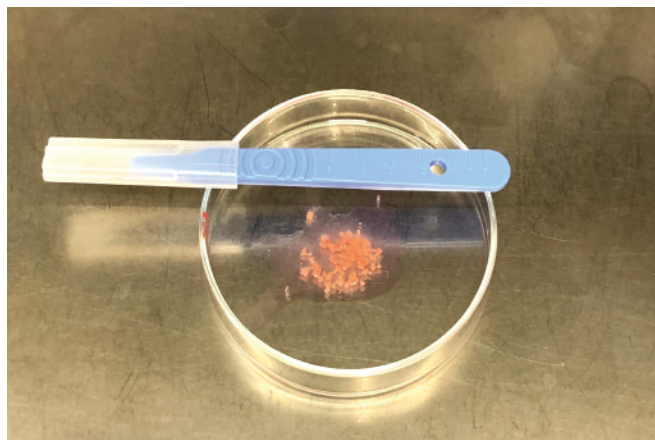
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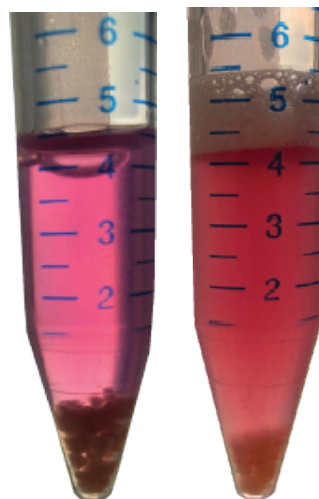
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## Figure 1

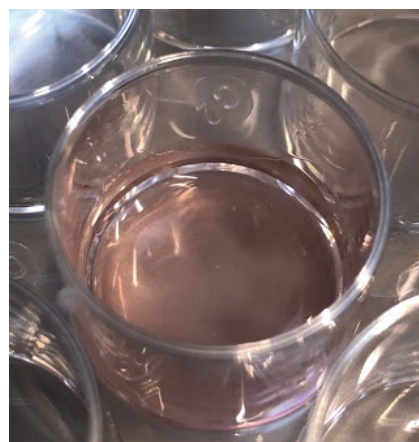
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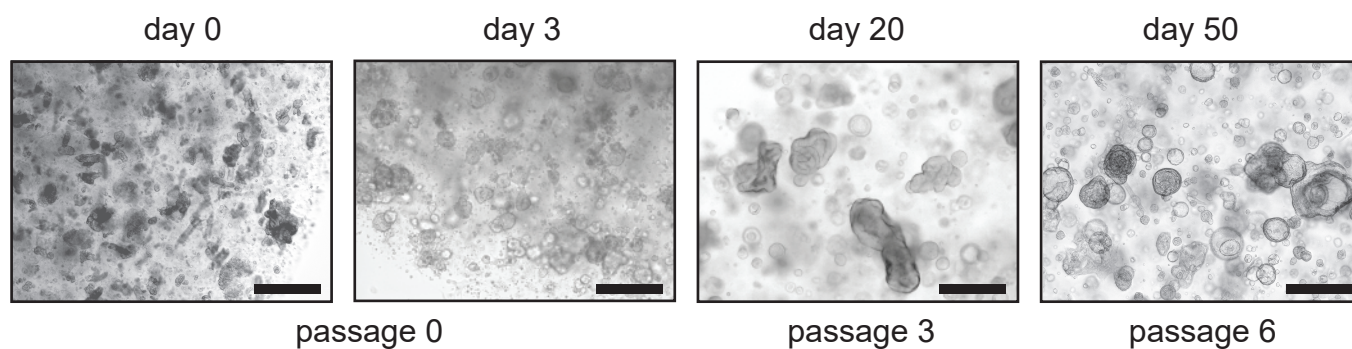
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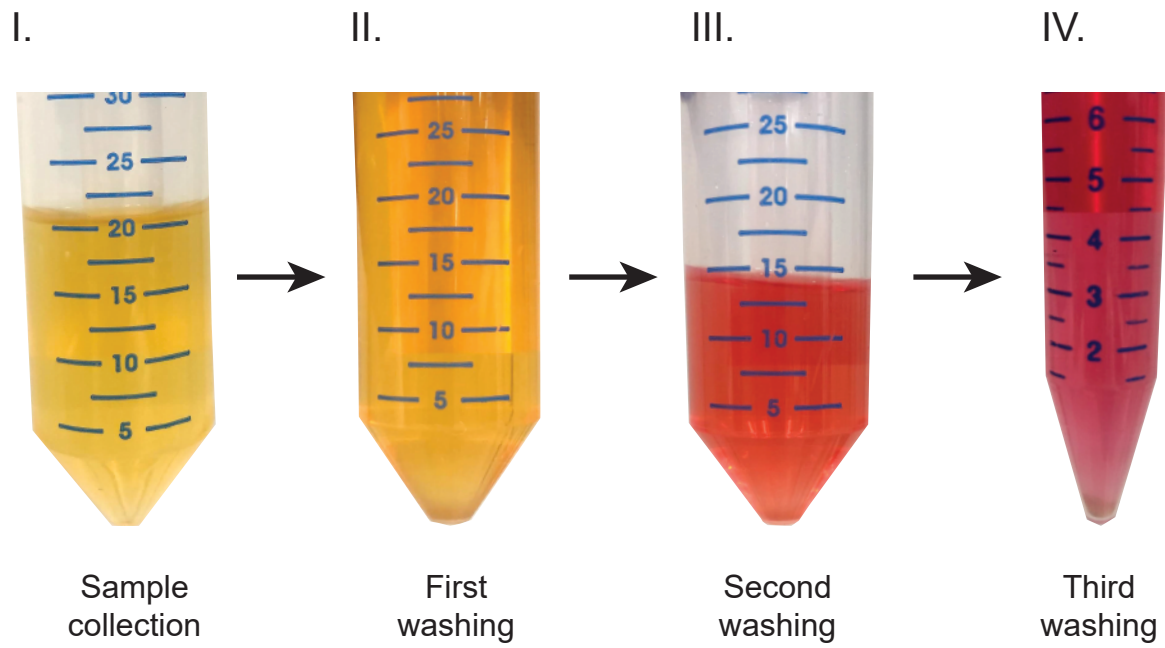
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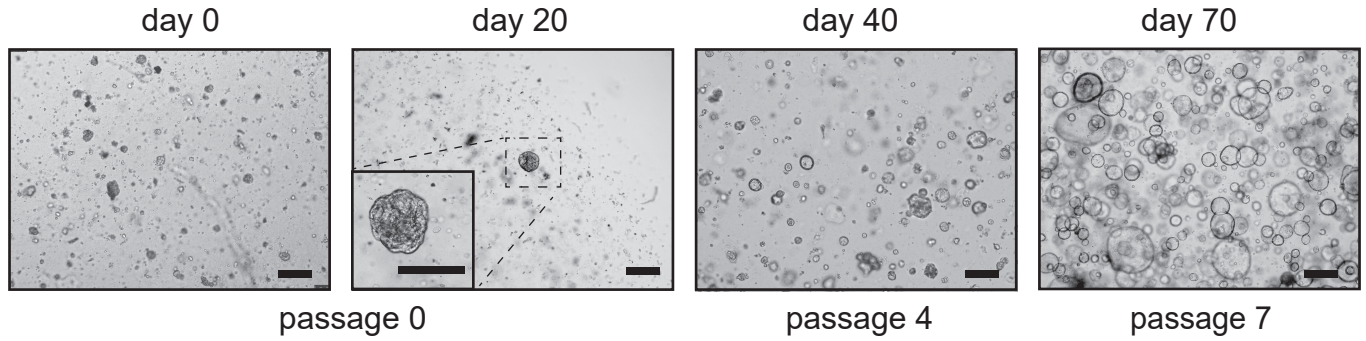


## Figure 2

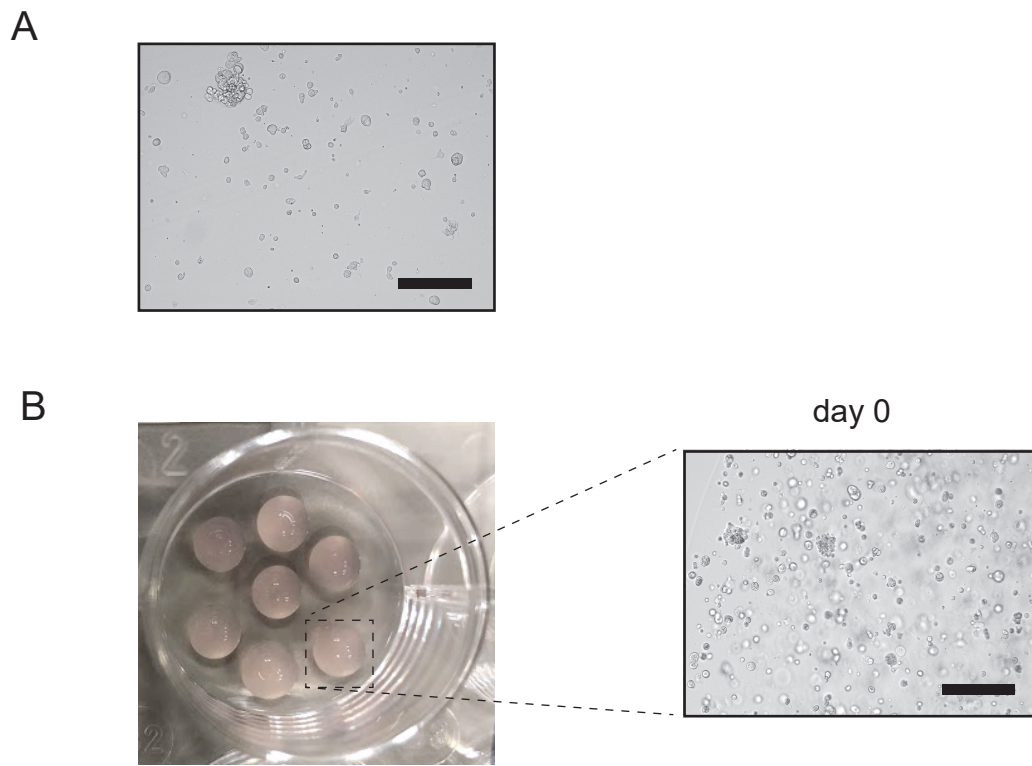
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B



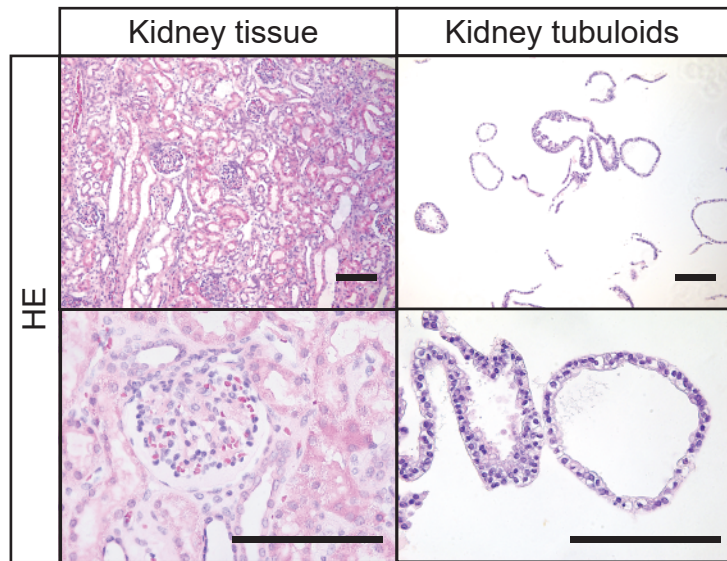
## Figure 3



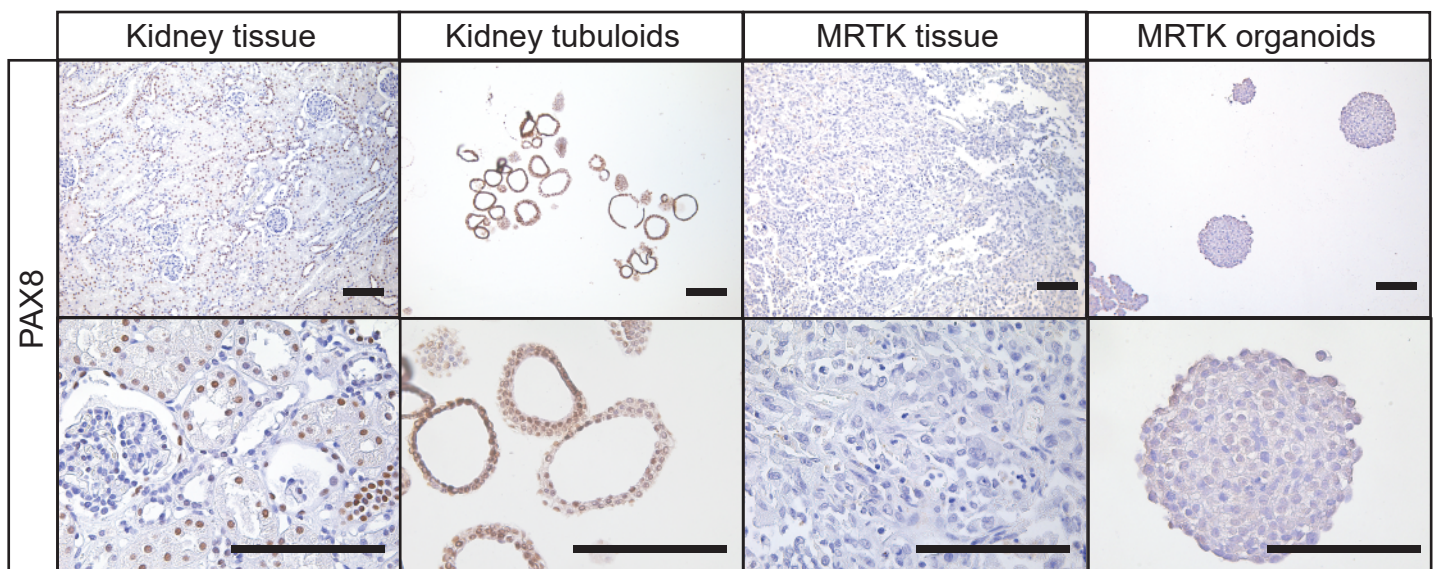


## Figure 4

A



B



<b>Name of Material/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
A83-01	Tocris	2939/10
Advanced DMEM/F12	ThermoFisher Scientific	12634010
antiPAX8 antibody	LSBio	LS-B13466
B27 supplement	ThermoFisher Scientific	17504044
BME	Trevigen	3533-010-02
Collagenase	Sigma Aldrich	C9407
EGF	Peprtech	AF-100-15
FGF10	Peprtech	100-26
GlutaMAX - L-alanine/L-glutamine	Gibco	35050061
Hepes	Gibco	15630106
Multiwell tissue culture plates 12 wells	CELLSTAR	665180
Multiwell tissue culture plates 24 wells	CELLSTAR	662160
Multiwell tissue culture plates 6 wells	CELLSTAR	657160
N-acetylcysteine	Sigma Aldrich	A9165
Penicillin/Streptomycin	Gibco	15140163
Primocin - broad-range antibiotics	Invivogen	ant-pm-1
Red blood cells lysis buffer	Roche	11814389001
RhoKinase inhibitor Y-27632	Abmole Bioscience	M1817
R-spondin conditioned medium		
TrypLe Express/ trypsin replacement agent	ThermoFisher Scientific	12605010

### Comments/Description

Stock of 5 mM, final concentration of 5  $\mu$ M, activin receptor-like kinase 5 inhibitor

Stock of 50x, final concentration of 1x

Stock of 10 mg/mL, final concentration of 1 mg/mL

Stock of 0.5 mg/mL, final concentration of 50 ng/mL

Stock of 0.1 mg/mL, final concentration of 100 ng/mL

Stock of 100x, final concentration of 1x

Stock of 1 M, final concentration of 10 mM

Stock of 500 mM, final concentration of 1.25 mM

Stock of 10.000 U/mL, final concentration of 100 U/mL

Stock of 50 mg/mL, final concentration of 0.1 mg/mL

Stock of 100 mM, final concentration of 10  $\mu$ M

Produced in house with the use of stable cell lines generated by Calvin Kuo lab. Final concentration is 10%



**To** Vidhya Iyer, PhD  
Review Editor, *Journal of Visualized Experiments*  
**Date** 27 February 2021  
**Subject** Manuscript re-submission

Dear Vidhya,

Please find the revised manuscript "*Generation of human kidney tubuloids from tissue and urine*" for publication in the *Journal of Visualized Experiments*.

In the new version, we made the requested changes and included the requested additional information. We highlighted these changes in yellow and/or used track changes. We hope that, with these changes and additions, *Journal of Visualized Experiments* will be able to publish our manuscript.

We look forward to hearing from you.

With best wishes,

A handwritten signature in blue ink, appearing to read "Jarno Drost", with a stylized flourish.

Jarno Drost, PhD  
Principal Investigator  
Princess Máxima Center for Pediatric Oncology and Onco Institute



**Manuscript JoVE62404 - Generation of human kidney tubuloids from tissue and urine-****Response to the reviewers and editorial comments**

We thank the Editor and Reviewers for their time and helpful comments. The constructive feedback significantly improved our manuscript. In our revised manuscript, we addressed the reviewer's comments. You can find our point-by-point rebuttal below.

**Editorial comments:**

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

We have revised the full manuscript and corrected for spelling and grammar issues.

2. Please provide an institutional email address for each author.

An institutional email address has been provided for each author.

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

We have revised the full manuscript and exclude all personal pronouns.

4. 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.  
For example: Glutamax, Primocin, TrypLe, etc.

We have revised the full manuscript and substitute company names with generic names.

5. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

As requested, we have included an ethics statement (line 74-76).

6. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks (Line: 90, 101, 108, 114, 123, 125, 129, 152, 156, etc.

Durations of less than one day have been abbreviated throughout the full manuscript.

7. Line 130: Please specify the temperature to which the medium is pre-warmed.

The temperature of medium before use has been specified in lines 92, 189, 241.

8. Please include a one-line space between each protocol step and 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

The requested changes have been applied to the manuscript.

9. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

The requested change has been applied to the manuscript.

10. Please sort the Table of Materials in alphabetical order.

The requested change has been inserted in the Table of Materials.

### **Reviewers' comments:**

#### **Reviewer #1:**

Manuscript Summary:

The manuscript describes a clear and concise protocol of the generation of adult stem cell derived kidney organoid cultures, also named tubuloids. The protocols are straightforward and readily to be implemented. Experimental details are sufficiently explained.

Major Concerns:

Lines 51-55: Authors should mention that one of the important limitations of iPSC use is their limited maturation revealing mostly tissues/organoids that resemble embryonal stages of the kidney.

We thank the reviewer for pointing us to this. We included this limitation of the use of iPSC cells in the text (lines 55-57):

*“Furthermore, iPSC organoids are not able to fully mature into adult kidney cells, revealing a transcriptome profile that resembles fetal kidney at early development stage”.*

Lines 61-62 and 290-295: Note that only 10% of the urine samples reveal viable cultures and are not necessarily of tubular epithelial origin. These limitations should be mentioned (see PMID 19902259; PMID 11201669)

Well taken. We now addressed these points as follows:

Regarding the efficiency of establishment for urine-derived cultures, Zhou et al. (Nature Protocols, 2012) recently demonstrated an efficiency of establishment of up to 82%, suggesting that more than 10% of urine samples reveal presence of viable cells. With our protocol, we are able to reach up to 50% establishment efficiency, achieved with extensive washing steps and use of broad-spectrum antibiotics in the culture medium. We do acknowledge that urine is a hostile environment for cells and that extensive washing needs to be performed as soon as possible to ensure presence of viable cells at the moment of seeding. We elaborate on this subject in the discussion section (lines 402-407):

*“Urine represents a hostile environment for cells due to high osmotic pressure and low pH. It is therefore crucial for the success of the protocol that samples are processed as soon as possible upon urine collection. As such, the collected urine should be diluted and extensively washed with buffered solution as soon as possible to ensure presence of viable cells at the moment of seeding. The success rate of tubuloid establishment will significantly decrease if urine is stored for several hours before processing.”*

Regarding the different epithelia cells present in urine samples, we elaborated on this topic in the discussion section (lines 397-401):

*“Urine can contain exfoliated epithelial cells that are not derived from the kidney<sup>22</sup>, which can contaminate the tubuloid cultures. These include, for instance, urothelium cells which are, in contrast to renal tubular cells, positive for TP63 and negative for PAX8<sup>8</sup>. It is therefore recommended to test the cultures for PAX8 positivity to confirm the purity of established kidney tubuloid lines before proceeding with follow up experiments (Figure 4B).”*

Lines 267-268 and 295-297: Cultures are maintained in antibiotics-containing media. These drugs are nephrotoxic too and this reviewer wonders whether that may affect the tissue culture, eg in rendering it less sensitive to treatments. How will this affect nephrotoxic potential predictions?

The addition of antibiotics to the culture medium is essential to prevent bacterial and/or fungal growth after tissue and urine processing. The protocol described in this manuscript allows for an establishment efficiency of kidney tubuloids from tissue greater than 95% (Schutgens et al., 2019; Calandrini et al., 2020). Therefore, the presence of antibiotics seems not to interfere with the successful generation of these cultures. However, we cannot exclude that the presence of antibiotics in the culture medium could induce some nephrotoxicity, which should indeed be taken into consideration. To address this, we have added the following sentences to the Discussion section (lines 371-376):

*“Commonly used antibiotics have been described to exert a toxic effect in the kidneys (Morales-Alvarez et al., 2020). While the presence of broad-spectrum antibiotics is necessary for the successful establishment of the cultures by preventing contaminations, we cannot exclude that their presence in the culture medium could potentially have some nephrotoxic effects. So far, however, no negative effects of antibiotics have been observed on the establishment of tubuloid cultures. Yet, further investigations are needed to thoroughly evaluate this.”*

#### **Reviewer #2:**

Manuscript Summary:

Relevant protocol for researchers in the kidney organoid field. In general well described, though some clarifications are necessary.

Major Concerns:

None

Minor Concerns:

> line 77, 127, 139: specify type of culture plate; number of wells? brand? coated or not? Consider adding the culture plates to the list of materials/ equipment at the end

*We now included the requested information in the revised manuscript.*

> line 101, 108, 148, 152, 156: specify temperature for centrifugation.

*We now specified the temperatures for all centrifugation steps.*

> line 124, 157: specify how you measure the volume of the cell pellet

*Well taken. We describe how to measure the cell pellets volume in our revised manuscript.*

> line 126: specify how you resuspend. Using 1mL pipette?

*We now added these specific details to the text.*

> line 128 and 130: it could be useful to add a macroscopic picture of the culture plates at these stages (the upside down plates with droplets and after the addition of medium)

*Following the reviewer's suggestion, we now added **Figure 1C** and **3B** to illustrate these steps of the protocol.*

>Line 193, step 3.2.9. 'check under microscope if many intact organoids are still present in the tube' -

-> be more specific, should there be no intact organoids left or is a certain percentage OK? Is it possible to repeat steps 3.2.6 - 3.2.9 more than once or is that detrimental to the culture? Add a picture of what the cells / organoid fragments are supposed to look like at this stage.

We thank the reviewer for this suggestion. We now specify in step 3.2.9 what is the percentage of intact organoids that can be tolerated. We do suggest repeating steps 3.2.6 – 3.2.9 only once more as longer enzymatic digestion can be detrimental for the cultures. We also included **Figure 3A** and **3B** to illustrate the cultures at different stages of the protocol (A illustrates step 3.2.9, B illustrates step 3.2.15).

In general: are there steps in the protocol that are difficult / can easily go wrong? If so, specify this and add advice on how to troubleshoot these problems.

Yes, some steps are crucial for the successful completion of the protocols describe. We do elaborate on these crucial steps/requirements in the discussion section (lines 387-396; lines 402-410).