MethodsX 9 (2022) 101843

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Protocol Article

FACS-Free isolation and purification protocol of mouse prostate epithelial cells for organoid primary culture

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A B S T R A C T

The prostate is a gland that contributes to men's fertility. It is highly responsive to androgens and is often the site of carcinogenesis, as prostate cancer is the most frequent cancer in men in over a hundred countries. To study the normal prostate, few *in vitro* models exist, and most of them do not express the androgen receptor (AR). To overcome this issue, prostate epithelial cells can be grown in primary culture *ex vivo* in 2- and 3-dimensional culture (organoids). However, methods to purify these cells often require flow cytometry, thus necessitating specialized instruments and expertise. Herein, we present a detailed protocol for the harvest, purification, and primary culture of mouse prostate epithelial cells to grow prostate organoids *ex vivo*. This protocol does not require flow cytometry approaches, facilitating its implementation in most research laboratories, and organoids grown with this protocol are highly responsive to androgens. In summary, we present a new simple method that can be used to grow prostate organoids that recapitulate the androgen response of this gland *in vivo*.

A R T I C L E I N F O

Method name: FACS-Free isolation and purification protocol of mouse prostate epithelial cells for organoid primary culture
Keywords: *Ex vivo* prostate model, Prostate model, Prostate cancer model, Three-dimensional prostate model, Organoids, Androgens, Citrate, Fertility
Article history: Received 14 April 2022; Accepted 29 August 2022

DOI of original article: 10.1016/j.molmet.2022.101516
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https://doi.org/10.1016/j.mex.2022.101843

Specifications table

Subject Area:	Medicine and Dentistry
More specific subject area:	Prostate and prostate cancer laboratory models
Protocol name:	Isolation and purification of mouse prostate epithelial cells for organoid primary culture
Reagents/tools:	• HBSS 1X (Wisent, 311-513-CL) • HEPES 100 mM (Wisent, 330-050-EL) • Penicillin-streptomycin solution (Wisent, 450-200-EL) • EpiCult B Medium Mouse Kit (Stemcell Technologies, 05610) • Human recombinant EGF (Stemcell Technologies, 78006) • Human recombinant bFGF (Stemcell Technologies, 78134) • Heparin sodium salt from porcine intestinal mucosa (Sigma Aldrich, H3393) • Gentle collagenase/hyaluronidase (StemCell Technologies, 07919) • Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free (Corning, 356231) • 0.25% trypsin-EDTA (Wisent, 325-043-EL) • Dispase 5U/mL (Stemcell Technologies, 07913) • DNase I (Sigma Aldrich, 11284932001) • Falcon 40 µm Cell Strainer (Fisher Scientific, 08-771-1) • EasySep Mouse PE Positive Selection Kit II (StemCell Technologies, 17666) • PE Rat Anti-Human CD49f (BD Biosciences, 555736)
Experimental design:	First, mouse prostates are harvested and subjected to sequential enzymatic digestions. Next, epithelial cells are purified using specific antibodies coupled to magnetic beads. Finally, purified epithelial cells are grown in 2- or 3-dimensional culture *ex vivo*.
Trial registration:	NA
Ethics:	Université Laval's Animal Research and Ethics Committee's guidelines and regulations were followed for all mouse work.
Value of the Protocol:	• This protocol enables the generation of mouse prostate organoids without using flow cytometry, facilitating its implementation in most research laboratories. • Prostate organoids are an *ex vivo* study model that recapitulate the glandular structure as well as the specialized metabolic program of the prostate. • Usage of prostate organoids allows the study of complex *in vivo* phenotypes, beyond what can be done with immortalized cell lines.

Description of protocol

The prostate is a gland involved in male fertility, notably by secreting citrate and zinc in the semen. Unfortunately, few laboratory models of the normal prostate exist to study its functions in fertility. The prostate is also extensively studied in the context of prostate cancer, the most frequent cancer in more than 100 countries [1,2]. However, the lack of normal prostate models limits our ability to clearly understand the initial carcinogenic events occurring in prostate cancer. To overcome such limitations, epithelial cells from the prostate can be purified to grow organoids *ex vivo* [3–9]. Organoids are a complex 3-dimensional (3D) structure that recapitulate the prostate gland's morphology, such as developing internal lumen [3–12]. These organoids are composed of several cell types, such as luminal and basal epithelial cells, that properly communicate in a cell-cell dependent manner and organize themselves in 3D in the same structured manner as *in vivo* [5,13]. To date, most protocols require flow cytometry and fluorescence-activated cell sorting (FACS). Here, we describe an isolation and purification protocol of prostate epithelial cells to grow organoids *ex vivo*, adapted from the protocol by Lacouture *et al.* [14] for purification of mammary epithelial cells and recently used to study the unique metabolic program of the prostate [15]. This protocol is simple and thus does not require FACS, facilitating its implementation in most research laboratories. In brief, prostates are enzymatically digested with collagenase/hyaluronidase, trypsin, and dispase, to obtain a single cell suspension (a critical step for subsequent purification). Epithelial cells are then purified using magnetic beads coupled to an antibody that recognizes a specific epithelial cell marker, CD49f, before being plated for

primary organoid culture. To ensure viability of the cells throughout the protocol, cell counting and trypan blue viability test can be used at the different steps of tissue digestion and cell resuspension.

Purification and isolation protocol of mouse prostate epithelial cells

For this protocol, mice (C57BL/6) were bred, housed in a 12h light:12h dark cycle at 22°C, and handled at the animal care facility of the Centre de recherche du CHU de Québec – Université Laval. All volumes described are for purification of epithelial cells using four mice of at least 12 weeks of age. If using a different number of prostates, volumes can simply be adjusted accordingly.

1. The mice are sacrificed by cervical dislocation under isoflurane anesthesia, and the different prostate lobes are harvested. For the current protocol, all the prostate lobes are pooled, but they could be kept separated to study their specific biology *ex vivo*.
2. Once harvested, prostates are maintained in 1X complete HBSS solution (HBSS + 2% FBS + 10 nM HEPES + 100 U/mL penicillin + 100 µg/mL streptomycin) until transfer to a biological hood.
3. Under a biological hood, prostates are minced with scissors and transferred to a tube containing 4 mL of a 1X complete EpiCult-B mouse medium with 5 % FBS (Epicult basal medium + 10 ng/mL EGF + 10 ng/mL bFGF + 4 µg/mL heparin + 100 U/mL penicillin + 100 µg/mL streptomycin + 10% EpiCult proliferation supplement) with 1 mL of Gentle Collagenase/Hyaluronidase solution.
4. The solution is incubated 2 hours at 37°C without agitation. During the incubation, the culture plates that will be used for plating the Matrigel drops at the end of the protocol are heated in an incubator at 37°C. The Matrigel is thawed on ice at 4°C and the pipette tips that would be used for plating are put at -20°C (cold tips ease the pipetting of Matrigel). The 0.25% trypsin-EDTA solution for the next digestion is thawed at room temperature.
5. After incubation, the solution is gently mixed by pipetting with 2 or 3 ups and downs to ensure tissue dissociation. The solution is then centrifuged 5 minutes at 350*g* at room temperature. If large pieces of tissue remain, incubation with a diluted collagenase/hyaluronidase solution (500 μL instead of 1 mL) can be performed overnight.
6. The supernatant is discarded and the pellet is resuspended with 1 mL of warm 0.25% trypsin-EDTA. After 1 minute of gentle pipetting, 2 mL of 0.25% trypsin-EDTA are added followed by another 1 minute of gentle pipetting. Again, 2 mL of 0.25% trypsin-EDTA are added followed by another 1 minute of gentle pipetting. If more than 4 mice are used at the beginning of the protocol, up to 8 prostates can be pooled together in the same tube at this stage.
7. The solution is then incubated on ice for 1 hour. During the incubation, the dispase solution for the next digestion is thawed at room temperature. Note that, if time is limited, this digestion can be performed at 37°C for 5 min instead of 1 hour on ice, but we recommend incubation on ice for maximal digestion efficiency and cell recovery.
8. At the end of the incubation, 10 mL of 1X complete HBSS solution are added and the tube is inverted 2 or 3 times to ensure homogenization. The solution is centrifugated 5 minutes at 350*g* at room temperature.
9. The supernatant is then removed and the pellet is resuspended by pipetting for 1-3 minutes in 2 mL of warm dispase and 200 µL of 1 mg/mL DNase I solution. At this step, no more clumps or tissue aggregates should be visible; it is important to obtain a single cell suspension after this last digestion. If digestion is incomplete, longer incubation time can be optimized if required. Generally, as clumps are due to DNA release, if needed repeat the DNase I treatment. If large tissue aggregates remains at these steps, the collagenase/hyaluronidase step should be adjusted first (see details in step 5).
10. After resuspension, 10 mL of cold 1X complete HBSS solution are added and the tube is inverted 2-3 times. The solution is then filtered with a 40 µm Cell Strainer.
11. The cell suspension is centrifugated for 5 minutes at 350*g* at room temperature. The supernatant is removed and the cells are resuspended in 1 mL of 1X complete EpiCult-B mouse medium with 5% FBS for cell counting with a hemocytometer. We recommend using trypan blue during cell counting to ensure that viability is >95%. Note that if the different digestion

steps are changed, cell viability should be assessed at the different steps of the protocol to ensure maximal efficiency and minimal cell death throughout the protocol.

12. After cell counting, the suspension is centrifuged 5 minutes at 350*g* at room temperature. The isolation of cells from 4 prostates typically provides about 2.5×10^6 to 3×10^6 cells. The supernatant is removed and the cells are resuspended in a minimal volume of 100 µL of kit media (PBS + 0.02 % FBS + 2 µM EDTA), with a maximal concentration of 1×10^8 cells/mL.
13. Prostate epithelial cells are then purified using a specific antibody and magnetic beads. To do so, add FcR Blocker from the EasySep Mouse PE Positive Selection Kit II in the cell suspension at a concentration of 10 µL/mL and CD49f-PE antibody at a concentration of 10 µL for 20.3×10^6 cells. After mixing and incubation of 15 minutes at room temperature, selection cocktail is added at a concentration of 100 µL/mL, followed by mixing and 15 minutes of incubation at room temperature. RapidSpheres are vortexed for 30 seconds before being added to the cell sample at a final concentration of 50 µL/mL. After 10 minutes of incubation at room temperature, the cells are resuspended in 2.5 mL of the media and put on a magnet for 5 minutes. The supernatant is then gently discarded by a smooth inversion movement and the cells are purified two more times using the magnet and 2.5 mL of the media.

- Note that CD49f is a plasma membrane marker highly expressed by basal epithelial cells, which are the most potent cells to generate prostate organoids *ex vivo* [3]. Note that CD49f is also expressed at intermediate levels by luminal epithelial cells, as shown by flow cytometry [3,4,7,15]. Cell sorting using flow cytometry allows purification of individual cell types, but we found that magnetic-based purification enriches for both epithelial cell types [15]. The current protocol thus allows generation of prostate organoids from a combination of basal and luminal epithelial cells. If more specific isolation of luminal epithelial cells is required, an antibody against CD24, expressed at higher levels by luminal epithelial cells, could be used instead of the anti-CD49f. In addition, if purification of other cell types from the prostate needs to be performed, other antibodies could be used for positive selection of these cells.

14. The purified cells, attached to the magnetic beads, are then resuspended in 1 mL of 1X complete EpiCult-B mouse medium with 5% FBS for cell counting with a hemocytometer. We recommend using trypan blue during cell counting to ensure that viability is >95%.

Plating of Matrigel drops for organoid culture

Each Matrigel drop contains between 30,000 to 45,000 cells depending on the foreseen experiment. For example, 30,000 cells or less per drop are more suitable for organoid quantification and microscopic study because the organoids are less likely to be superimposed. If RNA or protein extraction is foreseen, drops containing 45,000 cells provide more biological material after 2 weeks in culture.

Each Matrigel drop has a volume of 40 µL, is made of 75% Matrigel and 25% of 1X complete EpiCult-B mouse medium with 5% FBS in which the cells are resuspended.

1. After cell counting (step 14 of previous section), the suspension is divided to provide the right number of cells for every experimental condition. The tubes are then centrifuged at 350*g* for 5 minutes at room temperature.
2. The supernatant is removed, and the cells are resuspended in the right volume of media for the number of drops produced.
3. The thawed Matrigel volume for the number of drops required is added to one condition with cold pipette tips. The solution is mixed well with 2-3 ups and downs.
4. Using a cold pipette tip, 40 µL drops are laid in the warm culture plates. The culture plates are put upside down for 15 minutes in an incubator at 37°C with a continual movement to avoid spread out drops.
5. Steps 2-4 are repeated for every other experimental condition.
6. After 15 minutes of incubation, the plates are reversed again and 1X complete EpiCult-B mouse medium with 5% FBS is added to each well to cover the Matrigel drop.

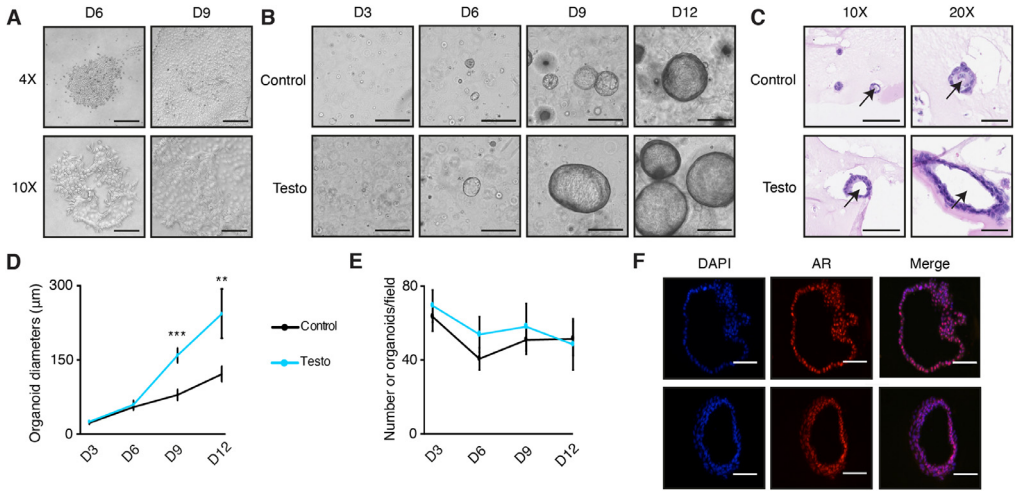

Fig. 1. Mouse prostate organoid are responsive to androgens in *ex vivo* culture. **A)** Brightfield images of mouse prostate epithelial cells cultured in two dimensions. Scale bars of 375 μm and 150 μm for 4X and 10X views, respectively, after 6 and 9 days (D) in culture. **B)** Brightfield visualization of organoids over 12 days in three-dimensional culture with and without testosterone (testo), an androgen treatment. Scale bars = 300 μm. **C)** H&E visualization of mouse prostate organoids after 14 days in culture, with and without testo. Arrows show the presence of internal lumen in these organoids; these structures are characteristics of the prostate gland. Scale bars = 125 μm and 50 μm, respectively, for 10X and 20X views. Note that during the fixation process, bigger organoids tend to lose their circular architecture. Measurement of organoid diameters **(D)** in μm (n = 45 organoids per condition) and organoid counts (**E**) over time in three-dimensional culture. Results are shown as the average and SEM of one out of three independent experiments. **F)** Immunofluorescence visualisation of AR in organoids after 14 days in culture with testo. DAPI is shown for nuclear staining, indicating a nuclear localization of AR. Scale bars = 75 μm. $^{***}p<0.001$; $^{**}p<0.01$.

7. After 24 hours, 1X complete EpiCult-B mouse medium with 5% FBS is replaced with 1X complete EpiCult-B mouse medium (Epicult basal medium + 10 ng/mL EGF + 10 ng/mL bFGF + 4 µg/mL heparin + 100 U/mL penicillin + 100 µg/mL streptomycin + 10% EpiCult proliferation supplement).
8. Every 2 or 3 days (3 times/week), 1X complete EpiCult-B mouse medium is changed. The organoids continuously grow for about 12 days and they can be harvested for study up to 2- or 3-weeks in culture.

Prostate epithelial cells purified with this protocol grow well in 2D (Fig. 1A) and 3D (Fig. 1B). Organoids are responsive to androgens, as shown by their increased growth when cultured with testosterone (Fig. 1B-D). Interestingly, androgens do not change the number of organoids (Fig. 1E), but specifically stimulate organoid growth (Fig. 1D). Finally, AR expression in organoids can be detected in most cells using immunofluorescence (Fig. 1F), consistent with the presence of luminal (secretory) epithelial cells in these organoids. In the article by Frégeau-Proulx et al., mouse prostate organoids were used to study the unique citrate-secretory phenotype of the prostate *ex vivo* with gas chromatography – mass spectrometry (GC-MS) metabolomics [15] . These organoids were shown to recapitulate the glandular structure of the prostate, with the presence of internal lumen for secretion, as well as to produce and secrete citrate, as the prostate gland does *in vivo*. These organoids thus recapitulate these important hallmarks of the prostate.

Data Availability

Data will be made available on request.

Acknowledgments

This work was supported by funding to E.A.W. from the Canadian Institutes of Health Research (CIHR). L.F.P. received scholarships from the Fonds de soutien à la recherche Joseph-Demers de la Faculté de médecine - Université Laval, the Fondation du CHU de Québec - Université Laval, the Centre de recherche sur le cancer de l'Université Laval, and the CIHR. A.L. received scholarships from the Fondation du CHU de Québec - Université Laval, the Fonds de recherche et d'enseignement de la Faculté de médecine – Université Laval and the Citoyenne du monde scholarship from Université Laval and the Fonds de Recherche du Québec – Santé (FRQS). E.A.W. holds a Tier 2 Canada Research Chair from CIHR on targeting metabolic vulnerabilities in hormonal-dependent cancers. The graphical abstract was created with BioRender.com.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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