

SOP #

Subject **Preparation of Colon Organoids**Sheet **1** of **3**

Rev

Effective
Date

Author

07/26/20 Ver 1	SOP for the BioDRoid Biospecimen Repository	Jan 2020	Chakrabarti/Zavros
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1. PURPOSE & SCOPE

To ensure proper collection, handling and preservation of stomach tissue samples which are entered into the UACC Biology Development and Research of organoids (BioDRoid).

2. DEFINITIONS

TissueMetrix (AIM) is the biospecimen information management system administered through the University of Arizona Cancer Center.

3. REFERENCES

- Steps to Enroll Subjects and Bank Biospecimens
- Information Management

4. RESPONSIBILITIES

- 4.1. BioDRoid Laboratory research personnel who preparing samples for banking are responsible for following the procedures in the SOP and confirming that each step has been executed properly
- 4.2. Deviations are to be documented on the Collection Event Form
- 4.3. Unexpected events are to be reported to the Lab Manager or Lab Director

5. SAFETY AND CAUTIONARY NOTES

- 5.1. Universal precautions and sterile technique should be followed. At minimum this should include the use of gloves, eye protection and lab coat. All processing of specimens should take place under a laminar flow hood.
- 5.2. Any spills or drops of blood should be immediately cleaned up by first spraying the spill area with 10% bleach, followed by 70% ethanol.
- 5.3. All spent tubes and pipet tips are to be discarded in a red biohazard container.
- 5.4. Blood should be processed immediately.
- 5.5. If processing is to be delayed, store blood in the specimen refrigerator in room 0936. If needed blood can be stored overnight in the refrigerator and processed the following morning. This is not preferred however.
- 5.6. Complete the corresponding collection event form.

6. MATERIALS AND EQUIPMENT

- 6.1. Advanced Dulbecco's modified Eagle medium/F12 medium (Thermo Fisher Scientific, 12634010).
- 6.2. GlutaMAX # 350-50-061 (Fisher Scientific)
- 6.3. Penicillin/Streptomycin # SV30010 (Thermo Fisher Scientific)
- 6.4. Amphotericin B / Gentamicin # R-01510 (Thermo Fisher Scientific)
- 6.5. Kanamycin Sulfate # 11815024 (Thermo Fisher Scientific)
- 6.6. HEPES Buffer # BP299-100 (Fisher Scientific)
- 6.7. n-Acetylcystine # A7250 (Sigma Aldrich)
- 6.8. N2 # 17502048 (Thermo Fisher Scientific)
- 6.9. B27 # 12587010 (Thermo Fisher Scientific)
- 6.10. Epidermal Growth Factor # 236-EG-01M (EGF, R&D Systems)
- 6.11. Nicotinamide # N0636 (Sigma Aldrich)
- 6.12. Y-27632 ROCK inhibitor # 125410 (Tocris)

- 6.13. SB202190 # 12-641-0 (Tocris)
- 6.14. Fetal bovine serum # SI2450H (FBS, Atlanta Biologicals)
- 6.15. Matrigel™ # CB40230C (Corning)
- 6.16. Ca²⁺/Mg²⁺ -free Dulbecco's Phosphate Buffered Saline # 14190-144 (DPBS, Fisher Scientific)
- 6.17. Collagenase Type 3 # 150704 (MP Biomedicals)
- 6.18. 5ml Round bottom polystyrene tubes # 14956-3C (Fisher Scientific)
- 6.19. TZV (THIAZOVIVIN) # SML1045 (SIGMA)
- 6.20. CHIR99021 # 4423 (Tocris)
- 6.21. A8301 # A2939 (Tocris)
- 6.22. T-175 Tissue culture flask # 431038 (Fisher Corning)
- 6.23. 0.25% Trypsin/EDTA # 25200056 (Thermo Fisher)
- 6.24. Freezing media # 12648-010 (Thermofisher)
- 6.25. 150 mm Tissue culture Petri dish # 430599 (Fisher)
- 6.26. Bottle-Top Filters with 0.22µm Membrane # 430513 (Fisher)
- 6.27. 500 mL Sterile Collection bottles # 430282 (Fisher Sci)
- 6.28. L-WRN cells # L-3276 (ATCC)
- 6.29. G418 # 15710064 (Thermofisher Scientific)
- 6.30. Hygromycin # 10687010 (Thermofisher Scientific)
- 6.31. Biohazard containers

7. TISSUE COLLECTION

- 7.1. Obtain the TissueMetrix Collection Event form and the sequential Specimen ID barcodes that are linked to the PTID. Refer to *SOP : Information Management* for details regarding the TissueMetrix labeling system.
- 7.2. The Biorepository requests normal or tumor biopsies/resected tissues from consented study participants, in 50ml tube containing collection media.
- 7.3. Pre-op is alerted by Biorepository personnel the evening before of the next day's consented patients.
- 7.4. The Biorepository is responsible for stocking Pre-op with blood collection kits. Each kit (biospecimen bag) includes two 50ml tube, 10ml collection media and a Tissue Bank index card with the Biorepository phone number (626-7319) on it.
(Collection Media contains, Advanced DMEM/F12 media supplemented with 2 mM GlutaMAX, 1% Penicillin/Streptomycin, 10 mM HEPES Buffer, 0.25 mg/mL Amphotericin B /10 mg/mL Gentamicin, 1% Kanamycin, 1x N2, 1x B27, 1 mM N-acetyl-L-cysteine, 10nM Nicotinamide, 100nM A8301, 4µM CHIR, and 2.5 µM Thiazovivin).
- 7.5. When called, Biorepository personnel proceeds to Pre-op and retrieve the tissue. Pre-op puts the tissue on a ice bucket labeled tumor bank on the main desk.
- 7.6. Record the patient information on the collection event form: name, MRN, DOS, DOB, sex, race/ethnicity, attending physician.
- 7.7. Record the time and date of tissue collection as well as the date and time of processing.

8. CRYOPRESERRATION OF TISSUE

- 8.1 Wash Tissue with DPBS + Antibiotics (DPBS supplemented with 1% Penicillin/Streptomycin, 0.25 mg/mL Amphotericin B /10 mg/mL Gentamicin, 1% Kanamycin)
- 8.2 Cut the tissue into smaller pieces with razor blades in a petri dish
- 8.3 Transfer tissue to a cryovial with freezing media

(Freezing media composition: 70% colon organoid growth media, supplemented with 20% FBS, 10% DMSO, 100nM A8301, 4 μ M CHIR, and 2.5 μ M Thiazovivin)

- 8.4 Store overnight at -80°C inside Mr. Frosty containing 250mL Isopropanol, 24hrs
- 8.5 Transfer the vial to liquid nitrogen for long term storage
- 8.6 Dispose all waste and the pipet tip in the biohazard container.

9. THAWING OF CRYOPRESERVED TISSUE

- 9.1 Thaw tissue at 37°C water bath, leaving one ice crystal left
- 9.2 Slowly add 1ml pre-warmed thawing media dropwise to the vial
(Thawing media composition: colon organoid growth media, supplemented with 100nM A8301, 4 μ M CHIR, and 2.5 μ M Thiazovivin).
- 9.3 Remove the media
- 9.4 Place tissue in a petri dish containing thawing media
- 9.5 Mince to smaller pieces with razor blades in a petri dish, proceed to digestion
- 9.6 Dispose all waste and the pipet tip in the biohazard container.

10. GENERATION OF HUMAN-DERIVED TUMOR COLON ORGANOIDS

- 10.1 Mince the tissues using surgical scalpel blades on a cell culture Petri dish. Wash the fragmented tissues with 5-10 ml of DPBS supplemented with antibiotics.
- 10.2 Transfer the tissue into a 15 ml Falcon® tube.
- 10.3 Add 5-10 ml of pre-warmed digestion buffer to the tissues, depending on the tissue size.
(Composition of digestion buffer: DPBS supplemented with 1mg/mL Collagenase Type 3).
- 10.4 Incubate the tissue at 37°C for 15-30 minutes in an orbital shaker, depending on the size and consistency of the tissues. Check for the appearance of cell clusters under the microscope, every 10 minutes.
- 10.5 Dilute the digestion buffer by adding twofold DPBS-antibiotics.
- 10.6 Centrifuge at 400 \times g for 5 minutes at 4°C to pellet cells.
- 10.7 Discard the supernatant and resuspend the pellet with cold DPBS plus antibiotics.
- 10.8 Centrifuge at 400 \times g for 5 minutes at 4°C.
- 10.9 Carefully remove the supernatant and store the cells on ice.
- 10.10 Resuspend cell pellet in an appropriate volume of Matrigel® Seed 50 μ l cell-Matrigel® droplets in 24 – 12 well cell culture treated plates.
- 10.11 Incubate the cell-Matrigel™ droplets at 37°C for 15 minutes to solidify as a dome.
- 10.12 Add 500 ml-1 ml pre-warmed 3D colon organoid culture medium to overlay cell-Matrigel™ dome.
(Composition of 3D Colon Organoid Culture Media: 50% of 2x Basal media (Advanced Dulbecco's modified Eagle medium/F12 medium supplemented with 4mM L-glutamine, 1% Penicillin/Streptomycin, 20 mM HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid), 2 mM N-acetyl-L-cysteine (Sigma-Aldrich), 2 \times N-2 supplement, 2x B27 supplement), 50% LWRN-conditioned medium, 100 μ g/mL Epidermal Growth Factor (EGF), 10 nM Nicotinamide, and 10 μ M Y-27632 ROCK inhibitor, 500nM A83-01, 10 μ M SB 202190, 2.5 μ M CHIR99021).
- 10.13 Maintain the 3D organoid cultures at 37°C in 5% CO₂. Replace with 50% fresh medium every 1-2 days depending on the organoid growth.
- 10.14 Passage organoids once every 7-10 days in 1:3 ratio, based on the organoid density.
- 10.15 Dispose all waste and the pipet tip in the biohazard container.

11. PASSAGING AND EXPANSION OF ORGANOIDS

- 11.1 Carefully aspirate media from each well
- 11.2 Add 1mL cold DPBS (-Ca & -Mg)/well

- 11.3 Harvest organoids and transfer them to round bottom 5mL tube (3wells/tube)
- 11.4 Centrifuge @ 400 x g, 5 min
- 11.5 Carefully remove the supernatant.
- 11.6 Add 1mL prewarmed TrypLE and 10mM Y-27632/ tube
- 11.7 Incubate @ 37°C, 6 min
- 11.8 Pipette up and down for 10 times in medium speed.
- 11.9 Check under a microscope for small clusters
- 11.10 Add 4ml DPBS/tube
- 11.11 Centrifuge @ 400 x g, 5 min
- 11.12 Carefully remove the supernatant.
- 11.13 Resuspend the pellet with the desired amount of Matrigel™
- 11.14 Carefully add 50µL Matrigel™ per well of a 12 well tissue culture plate, 30µL per well of an 8 well chamber slide, 40µL per well of a 24 well chamber slide, 20µL per well of a 48 well chamber slide
- 11.15 Add pre warmed growth media in each well
- 11.16 Change media every 3-4 days and continue culture with organoid growth media.
- 11.17 Dispose all waste and the pipet tip in the biohazard container.

12. CRYOPRESERVATION OF ORGANOIDS

- 12.1 Carefully aspirate media from each well
- 12.2 Add 1mL cold DPBS (-Ca & -Mg)/well
- 12.3 Harvest organoids and transfer them to round bottom 5mL tube (3wells/tube)
- 12.4 Centrifuge @ 400 x g, 5 min
- 12.5 Carefully remove the supernatant.
- 12.6 Add 1mL prewarmed TrypLE and 10mM Y-27632/ tube
- 12.7 Incubate @ 37°C, 6 min
- 12.8 Syringe 5 times with 26g needle (medium speed)
- 12.9 Check under a microscope for small clusters
- 12.10 Add 4ml cold DPBS/tube
- 12.11 Centrifuge @ 400 x g, 5 min
- 12.12 Carefully remove the supernatant.
- 12.13 Resuspend the pellet with the freezing media (1ml/vial) (Section 8.3).
- 12.14 Store overnight at -80°C inside Mr. Frosty containing 250mL Isopropanol, 24hrs
- 12.15 Transfer the vial to liquid nitrogen for long term storage
- 12.16 Dispose all waste and the pipet tip in the biohazard container.

13. THAWING AND CULTURING OF CRYOPRESERVED ORGANOIDS

- 13.1 Quickly thaw 1vial of frozen vial of organoid in 37°C water bath, leaving a small crystal of ice.
- 13.2 Slowly add 1mL pre warmed thawing media (Section 9.2) dropwise to each cryovial
- 13.3 Transfer the cells to a 5mL round bottom tube
- 13.4 Add 1mL thawing media
- 13.5 Centrifuge @ 400 x g, 5 min
- 13.6 Carefully remove the supernatant.
- 13.7 Resuspend the pellet with required volume of cold matrigel.
- 13.8 Carefully add matrigel bubble to each plate
- 13.9 Incubate @ 37°C, 13 min
- 13.10 Add pre warmed thawing media in each well.

- 13.11 After 48hrs, remove media and continue culture with organoid growth media.
- 13.12 Dispose all waste and the pipet tip in the biohazard container.

14. CULTURE OF LWRN CONDITIONED MEDIA PRODUCING CELLS

- 14.1 Quickly thaw 1vial of frozen vial of organoid in 37°C water bath, leaving a small crystal of ice.
- 14.2 Transfer the thawed cells to a 15 mL tube and add 9 mL growth media.
- 14.3 Centrifuge at 500xg, 5 min
- 14.4 Remove supernatant
- 14.5 Plate L-WRN cells in to a 150mm cell culture petridish, containing 30mL complete growth media (500mL Advanced DMEM/F12 + 20% FCS+ 1% Penicillin/Streptomycin).
- 14.6 The next day, depending on cell recovery, change media with dual selection (0.5mg/ml G418 and 0.5 mg/ml Hygromycin) and grow to about 80-90% confluency (usually ~ five days) without media change.
- 14.7 Passage cells into 10 new plates without antibiotic selection.
- 14.8 Remove media and wash with 10 mL warm DPBS, without Ca/Mg
- 14.9 Add 10 mL 0.25% trypsin/EDTA, incubate at 37°C, 5 min
- 14.10 Collect cells in to a 50mL tube
- 14.11 Centrifuge at 500xg, 5 min
- 14.12 Resuspend pellet in 10 mL media (1ml cell/ dish)
- 14.13 Add 25 mL of Growth Media per dish.
- 14.14 Once cells are about 80% confluent (usually ~2days), collect the conditioned media and replace with fresh media on each plate every day for 12 consecutive days.
- 14.15 Save 500 µL of media into a centrifuge tube from each day for testing activity using the TOPflash Wnt Reporter testing. Store at 4°C.
- 14.16 Centrifuge the conditioned media every day at 500xg for 10 minutes, to allow debris to settle at the bottom.
- 14.17 Filter sterilize the conditioned media every day and store the media in 500mL sterile bottle.
- 14.18 Combine conditioned media from day 1-4, 5-8 and 9-12.
- 14.19 Aliquot into 25- or 50-mL volumes and store aliquots at -80° C.
- 14.20 Dispose all waste and the pipet tip in the biohazard container.