Rev		Effective Date	Author
07/26/20 Ver 1	SOP for the BioDRoid Biospecimen Repository	Jan 2020	Chakrabarti/Zavros

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-Acteylcystine # A7250 (Sigma Aldrich)
6.8. N2 # 17502048 (Thermo Fisher Scientific)
6.9. B27 # 12587010 (Thermo Fisher Scientific)
6.10. Bone morphogenetic protein inhibitor # 250-38 (Noggin, Peprotech)
6.11. Gastrin # 30061 (Tocris Biosciences)
6.12. Epidermal Growth Factor # 315-09 (EGF, Peprotech)

6.13. Fibroblast growth factor 10 # 100-26 (FGF10, Peprotech)
6.14. Nicotinamide # N0636 (Sigma Aldrich)
6.15. Y-27632 ROCK inhibitor # Y0503 (Sigma Aldrich)
6.16. L cells, a Wnt3a producing cell line (Hubrecht Institute for Developmental Biology and Stem Cell Research, Netherlands).
6.17. Modified HEK-293T R-spondin secreting cell line
6.18. Dulbecco's Modified Eagle Medium (DMEM) # 12634-010 (Thermo Fisher Scientific)
6.19. Fetal bovine serum # SI2450H (FBS, Atlanta Biologicals)
6.20. OPTIMEM # 51985-034(Thermo Fisher Scientific)
6.21. Zeocin # R25001 (Thermo Fisher)
6.22. Matrigel™ # CB40230C (Corning)
6.23. Ca2+/Mg2+ -free Dulbecco's Phosphate Buffered Saline # 14190-144 (DPBS, Fisher Scientific)
6.24. EDTA # E6758 (Sigma Aldrich)
6.25. Hyaluronidase Type IV-S # H3884 (Sigma-Aldrich).
6.26. Collagenase Type 1A # C9891 (Sigma-Aldrich)
6.27. BSA # A7906 (Sigma Aldrich)
6.28. 5ml Round bottom polystyrene tubes # 14956-3C (Fisher Scientific)
6.29. HBSS # 14175095 (Thermo Fisher Scientific)
6.30. 40micron filter # 352340 (Fisher Scientific)
6.31. TZV (THIAZOVIVIN) # SML1045 (SIGMA)
6.32. CHIR99021 # SML1046 (SIGMA)
6.33. A8301 # A2939 (Tocris)
6.34. DMEM+ Glutamax-1 # 10569-010 (Thermo Fisher)
6.35. Optimem + Glutamax 1 # 51985-034 (Thermo Fisher)
6.36. Zeocin (Thermo Fisher # R25001)
6.37. T-175 Tissue culture flask # 431038 (Fisher Corning)
6.38. 0.25% Trypsin/EDTA # 25200056 (Thermo Fisher)
6.39. Freezing media # 12648-010 (Thermofisher)
6.40. 150 mm Tissue culture Petri dish # 430599 (Fisher)
6.41. Bottle-Top Filters with 0.22µm Membrane # 430513 (Fisher)
6.42. 500 mL Sterile Collection bottles # 430282 (Fisher Sci)
6.43. 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, 10mM Nicoinamide, 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. CRYOPRESERRATTION 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% gastric organoid growth media, supplemented with 20% FBS, 10% DMSO, 100nM A8301, 4μM CHIR, and 2.5 μM 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: gastric organoid growth media, supplemented with 100nM A8301, 4μM CHIR, and 2.5 μM 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, and proceed to digestion

9.6 Dispose all waste and the pipet tip in the biohazard container.

10. GENERATION OF HUMAN-DERIVED TUMOR GASTRIC 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 50 ml Falcon® tube. Add 5-10 ml of pre-warmed EDTA stripping buffer, depending on the size of the minced tissues.
(Composition of EDTA stripping buffer: Hank's Balanced Salt Solution (HBSS) supplemented with 5 mM EDTA, 25 mM HEPES, and 10% heat FCS).

10.3 Incubate the tissue at 37°C for 10 minutes. Carefully remove the EDTA buffer and add fresh 10 ml EDTA buffer.

10.4 Continue incubation at 37°C for another 5 minutes.

10.5 Take off the EDTA buffer, wash twice with 10 mL DMEM-antibiotics (no centrifugation needed).

10.6 Add 5-10 ml of pre-warmed digestion buffer to the tissues, depending on the tissue size.
(Composition of digestion buffer: Gastric organoid culture media supplemented with 1.5 mg/mL Collagenase Type 1A and, 0.4 mg/mL Hyaluronidase Type IV-S).

10.7 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.

10.8 Dilute the digestion buffer by adding twofold cold DMEM-antibiotics.

10.9 Filter undigested tissues through 40micron filters and collect the flow through.
10.10 Centrifuge the flow through at 400 × g for 5 minutes at 4oC to pellet cells.
10.11 Discard the supernatant and resuspend the pellet with cold DPBS plus antibiotics.
10.12 Centrifuge at 400 × g for 5 minutes at 4°C.
10.13 Carefully remove the supernatant and store the cells on ice.
10.14 Resuspend cell pellet in an appropriate volume of Matrigel® Seed 50 µl cell-Matrigel® droplets in 24 – 12 well cell culture treated plates.
10.15 Incubate the cell-Matrigel™ droplets at 37°C for 15 minutes to solidify as a dome.
10.16 Add 500 ml-1 ml pre-warmed 3D gastric organoid culture medium to overlay cell-Matrigel™ dome.
(Composition of 3D Gastric Organoid Culture Media: Advanced Dulbecco's modified Eagle medium/F12 medium supplemented with 2mM L-glutamine, 1% Penicillin/Streptomycin, 10 mM HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid), 1 mM N-acetyl-L-cysteine (Sigma-Aldrich), 1× N-2 supplement, 1 x B27 supplement, 50% Wnt3a-conditioned medium, 10% R-spondin-conditioned medium, 100 ng/mL bone morphogenetic protein inhibitor (Noggin), 1 nM gastrin 1, 50 ng/mL Epidermal Growth Factor (EGF), 200 ng/mL Fibroblast growth factor 10 (FGF10), 10 mM Nicotinamide, and 10 µM Y-27632 ROCK inhibitor).
10.17 Maintain the 3D organoid cultures at 37°C in 5% CO2. Replace with fresh medium every 3-4 days depending on the organoid growth.
10.18 Passage organoids once every 7-10 days in 1:2 or 1:3 ratio, based on the organoid density.
10.19 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 Accutase/ tube
11.7 Incubate @ 37°C, 6 min
11.8 Syringe 5 times with 26g needle (medium speed)
11.9 Check under a microscope for small clusters
11.10 Add 4ml cold 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 Accutase/ 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 Wnt CONDITIONED MEDIA PRODUCING L CELLS

14.1 Quickly thaw 1vial of frozen vial of organoid in 37°C water bath, leaving a small crystal of ice.
14.2 Plate L cells in T-175 flasks, containing 40mL complete Media (500mL DMEM+ 10% FCS+ 1% Pen/Strep) plus 50uL Zeocin.
14.3 Let the cells grow up to 90% confluency (5-7 days)
14.4 Passage cells in to 7 x T175 culture flasks
14.5 Remove media and wash with 10 mL warm DPBS, without Ca/Mg
14.6 Add 10 mL 0.25% trypsin/EDTA, incubate at 37°C, 5 min
14.7 Collect cells in to a 50mL tube
14.8 Centrifuge at 800xg, 5 min
14.9 Resuspend pellet in 7 mL media
14.10 Add 1 mL cells/1 T175 containing 40 mL media
14.11 2xT175 will get zeocin rests are not
14.12 When 2, + zeocin T175 becomes 70% confluent, they can be passaged (1x T175 to 7x T175) or frozen down (1x T175 to 8 aliquots).
14.13 The other 5x T175s washed with 10 mL warm DPBS/flask, without Ca/Mg
14.14 Add 10 mL 0.25% trypsin/EDTA, incubate at 37°C / flask
14.15 Add 10 mL media / flask and collect all cells in to 50mL tubes
14.16 Centrifuge at 800xg, 5 min

14.17 Resuspend pellet in 10 mL media
14.18 Add 10 mL cells into 500mL complete media bottle
14.19 Add 23 mL cells in to each of 25 x 150mm petri dishes
14.20 Let them grow for 1 week
14.21 Collect Wnt condition media, filter, aliquot, label and store at -80°C
14.22 Dispose all waste and the pipet tip in the biohazard container.

15. CULTURE OF R spondin CONDITIONED MEDIA PRODUCING HEK293T CELLS

15.1 Quickly thaw 1vial of frozen vial of organoid in 37°C water bath, leaving a small crystal of ice.
15.2 Plate L cells in T-175 flasks, containing 40mL complete Media (500mL DMEM+ 10% FCS+ 1% Pen/Strep) plus 50uL Zeocin.
15.3 Let the cells grow up to 90% confluency (5-7 days)
15.4 Passage cells in to 7 x T175 culture flasks
15.5 Remove media and wash with 10 mL warm DPBS, without Ca/Mg
15.6 Add 10 mL 0.25% trypsin/EDTA, incubate at 37°C, 5 min
15.7 Collect cells in to a 50mL tube
15.8 Centrifuge at 800xg, 5 min
15.9 Resuspend pellet in 7 mL media
15.10 Add 1 mL cells/1 T175 containing 40 mL media
15.11 2xT175 will get zeocin rests are not
15.12 When 2, + zeocin T175 becomes 70% confluent, they can be passaged (1x T175 to 7x T175) or frozen down (1x T175 to 8 aliquots).
15.13 The other 5x T175s washed with 10 mL warm DPBS/flask, without Ca/Mg
15.14 Add 10 mL 0.25% trypsin/EDTA, incubate at 37°C / flask
15.15 Add 10 mL media / flask and collect all cells in to 50mL tubes
15.16 Centrifuge at 800xg, 5 min
15.17 Resuspend pellet in 10 mL media
15.18 Add 10 mL cells into 500mL complete media bottle
15.19 Add 23 mL cells in to each of 25 x 150mm petri dishes
15.20 After 24hrs, change media of all 25 dishes with 500 mL Optimem + 1% Pen/Step (no FCS), 20mL/dish
15.21 Let them grow for 1 week
15.22 Collect R spondin condition media, filter, aliquot, label and store at -80°C
15.23 Dispose all waste and the pipet tip in the biohazard container.