SOP #	Subject **Preparation of Pancreatic Cancer Organoids**	Sheet **1** of **3**

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. B27 # 12587010 (Thermo Fisher Scientific)

6.6. Fibroblast growth factor 10 # 100-26 (FGF10, Peprotech)

6.7. Y-27632 ROCK inhibitor # Y0503 (Sigma Aldrich)

6.8. Ascorbic acid # 4055-50 (R&D Systems)

6.9. Insulin # 3435/10 (R&D Systems)

6.10. Hydrocortisone # H0888-1G (Sigma Aldrich)

6.11. Fibroblast growth factor-basic #100-18B (FGF2, Peprotech)

6.12. All trans Retinoic Acid # R2625 (ATRA, Sigma)
6.13. Bovine pituitary extract # P1476 (BPE, Sigma,),
6.14. L cells, a Wnt3a producing cell line (Hubrecht Institute for Developmental Biology and Stem Cell Research, Netherlands).
6.15. Modified HEK-293T R-spondin secreting cell line
6.16. Dulbecco's Modified Eagle Medium (DMEM) # 12634-010 (Thermo Fisher Scientific)
6.17. Fetal bovine serum # SI2450H (FBS, Atlanta Biologicals)
6.18. OPTIMEM # 51985-034(Thermo Fisher Scientific)
6.19. Zeocin # R25001 (Thermo Fisher)
6.20. Matrigel™ # CB40230C (Corning)
6.21. Ca2+/Mg2+ -free Dulbecco's Phosphate Buffered Saline # 14190-144 (DPBS, Fisher Scientific)
6.22. Collagenase P # 11 249 002 001 (Sigma-Aldrich)
6.23. 5ml Round bottom polystyrene tubes # 14956-3C (Fisher Scientific)
6.24. HBSS # 14175095 (Thermo Fisher Scientific)
6.25. 70micron filter # 22363548 (Fisher Scientific)
6.26. TZV (THIAZOVIVIN) # SML1045 (SIGMA)
6.27. CHIR99021 # SML1046 (SIGMA)
6.28. A8301 # A2939 (Tocris)
6.29. DMEM+ Glutamax-1 # 10569-010 (Thermo Fisher)
6.30. Optimem + Glutamax 1 # 51985-034 (Thermo Fisher)
6.31. Zeocin (Thermo Fisher # R25001)
6.32. T-175 Tissue culture flask # 431038 (Fisher Corning)
6.33. 0.25% Trypsin/EDTA # 25200056 (Thermo Fisher)
6.34. Freezing media # 12648-010 (Thermofisher)
6.35. 150 mm Tissue culture Petri dish # 430599 (Fisher)
6.36. Bottle-Top Filters with 0.22µm Membrane # 430513 (Fisher)
6.37. 500 mL Sterile Collection bottles # 430282 (Fisher Sci)
6.38. 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 1% Penicillin/Streptomycin, 0.25 mg/mL Amphotericin B /10 mg/mL Gentamicin, 1% Kanamycin, 1x B27, 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% pancreatic 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: pancreatic 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 TUMOR-DERIVED PANCREATIC 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 Add tissue to 5mL pancreatic wash buffer with 5mg collagenase P (1mg collagenase P/mL)
(Composition of wash buffer: HBSS supplemented with 1% Penicillin/Streptomycin, 0.25 mg/mL Amphotericin B /10 mg/mL Gentamicin, 1% Kanamycin, 5% FCS).
10.3 Vortex and Incubate in 37°C shaker for 15-45 mins
10.4 Check for small clusters of cells under a microscope every 15 min.
10.5 Add 5ml pancreatic wash buffer to tissue. (1:1 v/v)
10.6 Run through 70-micron sterile filter.
10.7 Rinse filter with pancreatic wash buffer.
10.8 Centrifuge at 400g for 5mins
10.9 Resuspend pellet in PBS+ Antibiotic and transfer cells to a 5mL round bottom tube
10.10 Centrifuge @ 40 x g, 5min
10.11 Carefully remove the supernatant.
10.12 Resuspend the pellet with the desired amount of Matrigel™
10.13 Plate 50μL Matrigel™ per well of a 12 well tissue culture plate
10.14 Add pre-warmed thawing media in each well

(Composition of pancreatic growth media: advanced DMEM/F12, 1X B27 , 284 μM ascorbic acid, 20 μg/μL Insulin, 0.25 μg/μL hydrocortisone, 100 ng/mL fibroblast growth factor-basic (FGF2), 100 nM all trans Retinoic Acid (ATRA), 10 μM Y27632, 100 ng/mL fibroblast growth factor 10 (FGF10), 1% Penicillin/Streptomycin, 0.1% Gentamicin/ Amphotericin B, 2 mM glutamax and 56 μg/mL bovine pituitary extract, 10% R-Spondin, and 50% Wnt conditioned media)

10.15 Maintain the 3D organoid cultures at 37°C in 5% CO_2. Replace with fresh medium every 3-4 days depending on the organoid growth.

10.16 Passage organoids once every 7-10 days in 1:2 or 1:3 ratio, based on the organoid density.

10.17 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.
(Pancreatic growth media contains

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 Rspondin 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), 23mL/dish
15.21 Let them grow for 1 week
15.22 Collect Rspondin condition media, filter, aliquot, label and store at -80°C
15.23 Dispose all waste and the pipet tip in the biohazard container.