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 tissy 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. K2 EDTA Vacutainer tubes, lavender top, plastic, 6 mls, BD367815 (VWR).
6.2. Serum Vacutainer tubes, red top, plastic, 6 ml tubes: BD367863 (VWR).
6.3. 2.0ml Round Bottom, self-standing, cryogenic vials # 03-337-7D (Fisher).
6.4. P-1000 Pipettors and sterile P-1000 filter pipette tips.
6.5. Sorvall Legend RT Tabletop refrigerated centrifuge located in Room 0936.
6.6. Lymphoprep # 7851 (STEMCELL Technologies)
6.7. RPMI # 10-040-CV (Thermo Fisher Scientific)
6.8. SepMateTM 50-IVD tube # 85450 (STEMCELL technologies)
6.9. Ficoll-Paque™ density gradient medium # 171440-02 (GE Healthcare)
6.10. AIMV Medium # 12055091 (Thermo Fisher Scientific)
6.11. Human serum AB # 21985023 (HSA, Gemini Bio-Products)
6.12. β-mercaptoethanol # 800-120 (Thermo Fisher Scientific)

6.13. Insulin-Transferrin-Selenium # 41400045 (ITS, Thermo fisher scientific)
6.14. Interleukin 2 # RP-8608 (IL-2, Thermo fisher scientific)
6.15. Interleukin 7 # RP-8645 (IL-7, Thermo fisher scientific)
6.16. Transforming growth factor beta 1 # 7754-BH-005/CF (TGF-β1 Thermo Fisher Scientific)
6.17. Vascular endothelial growth factor # RVEGFI (VEGF Thermo Fisher Scientific)
6.18. Prostaglandin E2 # P0409 (Sigma Aldrich)
6.19. Granulocyte-macrophage colony-stimulating factor # PHC6025 (GM-CSF, Thermo Fisher Scientific)
6.20. Interleukin 4 # RIL4I (IL-4, Thermo Fisher Scientific)
6.21. Tumor necrosis factor α # PHC3015 (TNF-α, Thermo Fisher Scientific)
6.22. Interleukin 1β # RIL1BI (IL-1β, Thermo Fisher Scientific)
6.23. Interleukin 6 # RIL6I (IL-6, Thermo Fisher Scientific)
6.24. EasySep buffer # 20144 (Stem Cell Technologies)
6.25. EasySep™ Human CD8+ T Cell Enrichment Kit # 19053 (Stemcell Technologies)
6.26. DMSO # D4540 (Sigma Aldrich)
6.27. Human serum AB # 21985023 (HSA, Gemini Bio-Products)
6.28. Biohazard containers

7. BLOOD 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 two 6ml tubes of blood from consented study participants, K2 EDTA lavender top tube and a red top serum tube.
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 one red top tube, one lavender top tube and a Tissue Bank index card with the Biorepository phone number (626-7319) on it.
7.5. When called, Biorepository personnel proceeds to Pre-op and retrieve the blood. Pre-op puts the blood in a basket 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 blood collection as well as the date and time of processing.

8. PLASMA PROCESSING

8.1 Centrifuge the remaining blood in the EDTA tube at 3000 RPM, 4°C, for 15 minutes. The Sovall Legend RT table top refrigerated centrifuge is located in room 0936.
8.2 With a marking pen, label two cryovials with sequential collection ID numbers designated in the collection kit. The collection number suffix for EDTA plasma is 01, and 02. For example, R160XXX 01, R160XXX 02.
8.3 Carefully remove the cap from the Vacutainer tube and using a P-1000 pipet with a filter tip, transfer 1000ul of plasma into each pre labeled cryovial. Depending on the amount of plasma in the tube, aliquots may be less than 1000ul.
8.4 Place the cap back on the Vacutainer tube and store the tube on ice for further processing of PBMCs.

9. SERUM PROCESSING

9.1 After red top Vacutainer has been allowed to clot for 30 minutes, centrifuge it at 3000 RPM, 4°C, for 15 minutes. The Sovall Legend RT tabletop refrigerated centrifuge is located in room 3957. Tubes should be placed in biosafety aerosol canister and each canister balanced prior to spinning.
9.2 With a marking pen, label two cryovials with the sequential collection ID numbers designated in the collection kit. The collection number suffix for serum is 04 and 05. For example, R160XXX 04, R160XXX 05.
9.3 Carefully remove the cap from the Vacutainer tube and using a P-1000 pipet with a filter tip, transfer 1000ul of serum into pre labeled cryovial. Depending on the amount of serum in the tube, aliquots may be less than 1000ul.
9.4 Place the cap back on the Vacut4ainer tube and dispose of it and the pipet tip in the biohazard container.

10. STORAGE

10.1 Filled cryovials be transfered to their permanent box in the -80°C freezer.
10.2 Record the box and slot number of each vial on the collection event form.

11. PBMC Processing using Ficoll-Paque™ density gradient medium

11.1 Dilute the blood from step 9.4 with an equal volume of DPBS.
11.2 Add 3-5 ml of Ficoll-Paque™ density gradient medium into a 15 ml Falcon® tube.
11.3 The recommended ratio is 3ml of Ficoll-Paque™ to 4 ml of the diluted blood sample.
11.4 Carefully layer the diluted blood sample onto the Ficoll-Paque™.
11.5 Centrifuge at 400 × g for an hour without brake.
11.6 Carefully transfer the mononuclear cells at the interface (buffy coat) into a new 15 ml Falcon® tube.
11.7 Dilute the collected mononuclear cells with 3 volumes of DPBS.
11.8 Centrifuge at 400 × g for 10 minutes. Discard supernatant. Repeat the centrifugation once more.
11.9 Resuspend pelleted cells in an appropriate culture medium for downstream applications or cryopreserve as frozen stocks.

12. PBMC Processing using Ficoll-Paque™ density gradient medium

12.1 Dilute the blood from step 9.4 with an equal volume of DPBS, supplemented with 1% fetal calf serum and 1% penicillin/streptomycin.
12.2 Add 15mL of LymphoprepTM to SepMateTM 50mL tube directly through the central hole.
12.3 Add the diluted blood slowly to the side of the SepMate tube, avoiding the central hole.
12.4 Centrifuge at 1200 x g for 10 minutes at 4oC.
12.5 Carefully and quickly pour the top layer into a separate 50mL conical and dilute 50% v/v with blood wash.
12.6 Centrifuge the supernatant enriched for leukocytes at 300 x g for 8 minutes at 4oC.
12.7 Discard the supernatant and re-suspend the pellet in 5mL Blood wash. At this point count the total number of PBMCs.
12.8 Centrifuge the leukocyte-enriched solution at 120 x g for 10 minutes at 4oC in order or separate leukocytes from platelets.
12.9 Resuspend the pellet in freezing media or proceed to culture.

13. Freezing PBMCs

13.1 Mix equal volume of stock Human Serum Albumin (HSA, 25%) and RPMI-1640 medium.
13.2 Prepare 2x Freezing Medium enough to resuspend 500µl/ cryovial. To prepare 2X freezing Media, mix equal volume of 25% HSA stock with sterile RPMI-1640 medium. Add 500µl DMSO per 1 ml of HSA/RPMI mixture.

13.3 Re-suspend PBMCs in 500uL of the 12.5% HSA. solution and pipet up and down to mix. Transfer PBMCs into a 2ml cryopreservation tube.
13.4 Add 500uL12.5% HSA solution to the into the cryopreservation tube.
13.5 Gently swirl tube while adding 500uL of 2x Freezing medium.
13.6 Immediately place the tube on ice. Do not mix any further.
13.7 Place in –80°C freezer. Do not store at Liquid Nitrogen.

14. CULTURE OF DENDRITIC CELLS (DCS)

14.1 Resuspend the pelleted PBMCs in AIMV basal medium.
14.2 Plate them in a 24 well tissue culture plate for 2 hours at 37°C in 5% CO2.
Composition of AIMV Basal Medium: AIM V medium containing 10% human serum AB, 1% Penicillin/Streptomycin and 50 µM β-mercaptoethanol.
14.3 Gently tap the culture plate and discard the culture medium containing non-adherent cells.
14.4 Slowly add AIMV DC culture medium to the adherent cells through the side of the plate.
14.5 Maintain cultures for 3 days at 37oC in 5% CO2. Replace with fresh culture medium on alternate days.
Composition of DC Culture Media: AIMV medium supplemented with 10% human serum AB, 1% Penicillin/Streptomycin, 50 µM β-mercaptoethanol, 800 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 500 U/ml interleukin 4 (IL-4).
14.6 On day 3, discard the culture media and add fresh DC maturation medium.
14.7 Continue culturing the DCs for 24 hours at 37°C in 5% CO2.
Composition of DC Maturation Medium: DC culture medium additionally supplemented with 1% Penicillin/Streptomycin, 5 ng/ml tumor necrosis factor α (TNF-α), 5 ng/ml interleukin 1β (IL-1β), 150 ng/ml interleukin 6 (IL-6) and 1 µg/ml prostaglandin E2 (PGE2).

15. CULTURE OF CYTOTOXIC T-LYMPHOCYTES (CTLs)

15.1 Quickly thaw 1 vial of frozen PBMCs at 370C leaving one ice crystal. Add 1ml warm cRPMI slowly dropwise (over 30 seconds).
Composition of cRPMI: RPMI medium supplemented with 10% FCS and 1 % Penicillin/Streptomycin.
15.2 Transfer cells to a 15 ml conical tube.
15.3 Top up the tube with 3 ml warm cRPMI to cells and centrifuge at 300 x g for 5 minutes.
15.4 Resuspend PBMCs in 1ml EasySep buffer and transfer 1 ml of PBMCs to a 5 ml polystyrene round-bottom tube.
15.5 Add 50 µl of Enrichment Cocktail to the PBMCs. Incubate for 10 minutes at room temperature.
15.6 Add 150 µl of Magnetic Particles to the sample. Incubate for 5 minutes at room temperature.
15.7 Top up to 2.5 ml with EasySep™ Buffer. Place the polystyrene round-bottom tube into a magnet for 5 minutes to allow cell separation.
15.8 Pour off enriched cell suspension into a new 15 ml Falcon® tube and centrifuge at 300 × g for 5 minutes.
15.9 Discard the supernatant and resuspend the cells in CTL culture medium.
15.10 Seed the cells in a 24 well cell culture plate and continue culturing CTLs for 24 hours at 37° C in 5% CO2.
Composition of CTL Culture Media: RPMI 1640 medium containing 10% human serum AB, 1% Penicillin/Streptomycin, 50 µM β-mercaptoethanol, 1 × Insulin-Transferrin-Selenium, 0.15 µg/ml interleukin 2 (IL-2) and 0.1 µg/ml interleukin 7 (IL-7)2 hours at 37°C in 5% CO2.

16. CULTURE OF DENDRITIC CELLS (DCS)

16.1 Quickly thaw 1 vial of frozen PBMCs at 370C leaving one ice crystal. Add 1ml warm cRPMI slowly dropwise (over 30 seconds).
Composition of cRPMI: RPMI medium supplemented with 10% FCS and 1 % Penicillin/Streptomycin.

16.2 Transfer cells to a 15 ml conical tube.
16.3 Top up the tube with 3 ml warm cRPMI to cells and centrifuge at 300 x g for 5 minutes.
16.4 Resuspend PBMCs in 1ml EasySep buffer and transfer 1 ml of PBMCs to a 5 ml polystyrene round-bottom tube.
16.5 Add 50 µl of Enrichment Cocktail to the PBMCs. Incubate for 10 minutes at room temperature.
16.6 Add 150 µl of Magnetic Particles to the sample. Incubate for 5 minutes at room temperature.
16.7 Top up to 2.5 ml with EasySep™ Buffer. Place the polystyrene round-bottom tube into a magnet for 5 minutes to allow cell separation.
16.8 Pour off enriched cell suspension into a new 15 ml Falcon® tube and centrifuge at 300 × g for 5 minutes.
16.9 Discard the supernatant and resuspend the cells in CTL culture medium.
16.10 Seed the cells in a 24 well cell culture plate and continue culturing CTLs for 24 hours at 37o C in 5% CO2.
Composition of CTL Culture Media: RPMI 1640 medium containing 10% human serum AB, 1% Penicillin/Streptomycin, 50 µM β-mercaptoethanol, 1 × Insulin-Transferrin-Selenium, 0.15 µg/ml interleukin 2 (IL-2) and 0.1 µg/ml interleukin 7 (IL-7).

17. CULTURE OF MYELOID DERIVED SUPPRESSOR CELLS (MDSCs)

17.1 Culture PBMCs in AIM V MDSC culture medium for 7 days at 37oC in 5% CO2 to enrich for MDSCs.
17.2 Replace with fresh culture medium every alternate day and continue culturing MDSCs until used in organoid – immune cell co-culture.
Composition of MDSC Culture Media: AIMV medium containing 50% conditioned medium collected from organoid cultures, and supplemented with 1% Penicillin/Streptomycin, 10 ng/ml IL-1β, 10 ng/ml IL-6, 1 µg/ml PGE2, 2 ng/ml transforming growth factor beta 1 (TGF-β1), 10 ng/ml TNF-α, 10 ng/ml vascular endothelial growth factor (VEGF) and 10 ng/ml GM-CSF.

18. ORGANOID IMMUNE CELL CO-CULTURE

Procedure: DCs and CTL Co-Culture

18.1 Collect conditioned medium from organoid cultures. The media should be conditioned by organoids for 7 days. Conditioned media can be stored at -20°C until used for cultures (avoid freeze thawing).
18.2 Gently remove 50% of the culture media from the DCs and replace with the organoid conditioned media.
18.3 Incubate DCs with conditioned media for 2h at 37°C in 5% CO2.
18.4 Collect loosely adherent DCs with a pipette and centrifuge at 300X g for 5min at 4°C.
18.5 Remove supernatant and save pellet on ice to resuspend with CTLs (next step).
18.6 Harvest the CTLs and transfer them to the matured DCs. Continue DC-CTL co-culture for 72 hours at 37oC in 5% CO2.
Composition of RPMI Co-Culture Media: RPMI 1640 medium supplemented with 10% human serum AB and 1% Penicillin/Streptomycin.

Procedure: Organoid- Immune Cell Co-culture

18.7 Isolate the CTLs from DC-CTL co-cultures using the EasySep™ Human CD8+T Cell Enrichment Kit.
18.8 Incubate CTLs with 1mL 5µM carboxyfluorescein diacetate succinimidyl ester (CFSE) at 37°C for 20min.
18.9 Quench the staining by adding five times DPBS-FCS medium. Centrifuge the CTLs at 300X g for 5min at 4°C.
Composition of DPBS-FCS media: DPBS supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.
18.10 Resuspend the CTLs in 1mL 3D gastric organoid medium and incubate for a further 10min at 37°C.
18.11 Harvest organoids in cold DPBS and centrifuge at 400 g for 5min at 4°C.
18.12 Mix the organoids with CFSE-labeled CTLs. Resuspend in an appropriate volume of thawed Matrigel® on ice. The volumes are determined based on the experimental design required for the studies.

18.13 When MDSCs are required within the co-culture, a 1:4 (CTL:MDSC) and 1:1 (CTL:organoids) is used for co-culture.

18.14 Seed as 30–50µL Matrigel® droplets containing organoids plus CTLs in 48 or 24 well tissue culture plates.

18.15 Incubate plates at 37°C for 15min to allow the cell-Matrigel® droplets to be solidified.

18.16 Overlay cell-Matrigel® droplets with pre-warmed 3D gastric organoid culture medium. When seeding into 48 or 24 well plates use 250 µL or 500 µL of medium respectively.

18.17 Maintain organoid/immune cell co-cultures at 37°C in 5% CO2 for 24h.

18.18 After 24h, treat the co-culture with appropriate drugs or inhibitors and maintain the culture at 37°C in 5% CO_2 for 48–72h based on experimental needs.
NOTE: CFSE is a blue laser dye that can be used for analyzing CTL proliferation using flow cytometry. For Experimental conditions requiring MDSCs, mix MDSCs together with the organoids and CFSE-labeled CTLs before seeding them in the culture plates.