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CHANGE HISTORY

Revision	Description
8/25/2017, v003	Internal SOP used by PDMR In Vitro Laboratory
2/13/2018	Standardize SOP for posting to PDMR SharePoint site for use by designated NCI intramural laboratories
2/20/2019	Updated reference SOPs.

RELATED SOPS

SOP30101: Recipes for Complete Media for Patient-Derived In Vitro and Organoid Cultures
SOP40102: Thawing and Initial Culture of Patient-Derived Organoid (PDOrg) Cultures
SOP40103: Passaging and Sub-culture of Patient-Derived Organoid (PDOrg) Cultures

1.0 PURPOSE/SCOPE

This Standing Operating Procedure (SOP) describes the procedures for passaging of Patient-Derived organoid cultures (PDOrg) under BSL-2 safety criteria. Optimal tissue culture media for use with specific PDOrg lines will be provided with the individual **Certificate of Analysis**. This SOP is used/performed by the Biological Testing Branch (BTB) at NCI-Frederick, Frederick National Laboratory for Cancer Research.

2.0 SAFETY

BTB treats all patient-derived material under Biosafety Level 2 (BSL2) conditions even when PCR-based screening has not detected the presence of a known set of human pathogens. All work is conducted in a biological safety cabinet (BSC) using personal protective equipment and avoiding the use of sharps where possible. All materials potentially exposed to human-derived material are disinfected by exposure to a 10% bleach solution for a minimum of 10 minutes, double bagging for autoclaving or incineration. Consult with your facility safety professionals regarding the safe handling of BSL2 studies.

3.0 CLEAN-UP

3.1 All materials coming into contact with human-derived material are treated as a potential health threat (BSL-2 precautions) since the human-derived materials could retain human pathogenic agents even if they do not replicate in mouse cells (e.g., EBV, HPV, etc).

3.2 Flush/soak any items (e.g., tubes, syringes, petri dishes, lab mats, etc) that were in contact with human-derived material with disinfectant (e.g., 10% bleach, commercial hydrogen peroxide disinfectant, 2% Virkon®) for a minimum of 10 minutes before disposal in biohazard waste or sharps containers (follow institutional guidelines and manufacturer's recommendations).

3.3 For items that can't be rinsed (e.g., micropipettors), wipe down thoroughly with bleach-soaked gauze or other appropriate disinfectants.

4.0 REAGENTS AND EQUIPMENT

4.1 Certificate of Analysis for PDOrg culture

4.2 Wash Media:

Item	Catalog	Volume
Basic Media	*see SOP30101	100 mL
FBS	HyClone Laboratories, Cat#: SH30071.03HI	2.5 mL

4.2.1 Sterile filter with 0.22 µm filter. Prepare fresh weekly and warm to room temperature 2-3 hours before use.

4.3 Reagents, Material & Equipment

4.3.1 Dispase II (Thermo Fisher Scientific, Cat#: 17105-041)

4.3.2 Advanced DMEM/F12 (1X) (Invitrogen, Cat#: 12634-028)

4.3.3 TrypLE™ Express Enzyme (1X), phenol red (Thermo Fisher Scientific, Cat#: 12605010)

4.3.4 Fetal Bovine Serum (FBS; HyClone Laboratories, Cat#: SH30071.03HI)

4.3.5 Hydrocortisone (Sigma, Cat#: H4001-1G)

4.3.6 Ethanol, 200 Proof, 99.98% (e.g., Pharmco-Aaper, Cat#: 111000200CSPP)

4.3.7 EGF Recombinant Human Protein (hEGF; Invitrogen, Cat#: PHG0311 or R&D Systems, Cat#: AFL236)

4.3.8 DPBS, no calcium, no magnesium (Thermo Fisher Scientific, Cat#: 14190250)

4.3.9 Adenine (Sigma, Cat#: A2786)

4.3.10 Hydrochloric acid, HCl (e.g., Sigma-Aldrich, Cat#: 320331-500mL)

4.3.11 Penicillin-Streptomycin (10,000 U/mL, Thermo Fisher Scientific, Cat#: 15140163)

4.3.12 L-Glutamine (200mM, Thermo Fischer Scientific, Cat#: 25030-081)

4.3.13 DMSO, HPLC-grade, >99.5% pure (Honeywell Research Chemicals/Burdick & Jackson, Cat#: 081-1L)

4.3.14 Sterile, 0.22 µm Filter Units (e.g., Millipore-Sigma, Cat#: SCGPU05RE)

4.3.15 P1000, P200 and P20 Pipetman (or equivalent) and tips

4.3.16 50-mL sterile conical tubes, sterile

4.3.17 5, 10, 25 mL pipettes and automatic pipettor

4.3.18 Aspirator pipettes (e.g., Fisher Scientific, Cat#: 357501)

4.3.19 Cryovials, screw-capped, sterile, 1.8-2.0 mL capacity (Nunc, Cat#: 368632)

4.3.20 Ice bucket with ice

4.3.21 Benchtop Centrifuge equipped with sealed buckets

4.3.22 Biosafety Level 2 certified biosafety cabinet

4.3.23 37°C humidified, 5% CO2 tissue culture incubator

4.3.24 37°C shaker-incubator

5.0 PROCEDURE

5.1 Guidance on Cryopreservation Timing

5.1.1 Organoids are ready to be cryopreserved based on 2 criteria: **density and size**.

5.1.1.1 **High-density Organoid Cultures**: **Figure 1** and **Figure 2**: Left panels: Example of PDOrg cultures at high density when cryopreservation can occur.

5.1.1.2 **Low-density Cultures: Figure 1** and **Figure 2**: Right panels: Example of PDOrg cultures at low density when feeding is all that is required. As described in **SOP40103: Passaging and Sub-culture of Patient-Derived Organoid (PDOrg) Cultures** SOP Step 5.1.1.2, low density cultures should not be cryopreserved. Follow recommendations in that SOP for passaging to increase the density of the culture.

5.2 Prepare Reagents

5.2.1 Before starting, prepare Splitting Media: 1.5 mg/mL Dispase II in Wash Media, sterile filter with 0.22 µm filter, and store on ice. Should be prepared fresh each week, store at 4C.

5.2.2 Prepare Freeze Media

5.2.2.1 Prepare the following solution and sterile filter using a 0.22 µm filter.

Item	Stock Concentration	Volume
Advanced DMEM/F12		500mL
FBS		100 mL
Hydrocortisone	1 mg/mL in 20% EtOH	200 µL
hEGF	50 µg/mL in DPBS	5 µL
Adenine	2.4mg/mL in 0.05M HCL	5 mL
Penicillin-Streptomycin	10,000 U/mL	5 mL
L-Glutamine	200 mM	5 mL

5.2.2.2 Add 68.5 mL DMSO to filtered solution to make Freeze Media. Keep Freeze Media on ice.

5.2.2.3 Prepare Freeze Media fresh each week and store at 4°C.

5.2.3 Have Cryovials labeled with the model name, passage number, and freeze date.

5.2.4 Perform all procedures under sterile conditions in a Biosafety Level 2 certified biosafety cabinet.

5.3 Passaging Organoid Cultures

The NCI PDMR generally cryopreserves 7-9 dense 24-well plates.

While this varies model-to-model, an average of 30 cryopreserved vials at 1-6x10^6 cells per vial can be prepared from this.

5.3.1 Add a volume of Splitting Media equal to the volume of Feeding Media present in each well to be cryopreserved. This provides a final concentration of Dispase II at 0.75 mg/mL. For a single well in a 24-well plate, this would be 750 µL Splitting Media per well to the already present 750 µL Feeding Media.

5.3.2 Place the plate with the Splitting Media in a 37°C tissue culture incubator and incubate for 1.5 to 2 hours.

5.3.3 Using a P1000 Pipetman, gently pipette up and down the Splitting Media in each well to dissociate any residual BME dome.

5.3.4 Transfer the dissociated BME2 domes, organoids, and Splitting Media into a sterile 50-mL conical tube and add an equal volume Wash Media to dilute and neutralize the Dispase II.

5.3.5 Centrifuge the organoid solution at 200xg for 5 min.

5.3.6 With a 10-mL pipette, remove as much Splitting/Wash Media as possible.

5.3.7 Add Wash Media. The volume will depend on the size of the pellet. For example, if the pellet size is approximately 2 mL, add 50 mL Wash Media. Resuspend the organoids by mildly pipetting up and down. The goal is to keep the organoids intact in the suspension.

5.3.8 Count the viable cells present in the organoid suspension as follows

5.3.8.1 Gently mix the organoid suspension to be sure everything is evenly mixed, then pipette a 50-100 µL aliquot of organoid suspension to an Eppendorf tube and add the same volume of TrypLE Express. Keep the organoid suspension on ice.

5.3.8.2 Counting should be done using dissociated single cells.

- Disassociate to single cells by placing the aliquot of organoids/TrypLE Express in a 37°C shaker-incubator for 5 minutes.
- After 5 min at 37°C, pipette the suspension up and down to fully suspend to single cells.

5.3.8.3 Follow manufacturer's instructions for counting viable cells and determine the viable cell number in the original organoid suspension.

5.3.8.4 Once the cell number has been determined discard the tube. **DO NOT** add the dissociated cells back to the tube containing the intact organoids.

5.3.9 Centrifuge the intact organoid suspension at 200xg for 5 min.

5.4 Prepare Cryo Aliquots

5.4.1 With a pipette, carefully remove as much Wash Media as possible from the pellet.

5.4.2 Add chilled Freeze Media to the tube so that the final cell concentration is **1-6x10^6 viable cells/mL**. Gently pipette up and down. The goal is to keep the organoids intact in the suspension.

5.4.3 Keep organoid/Freezing Media suspension 50-mL tube on ice.

5.4.4 Have labeled cryovials in a tube rack with caps loosened for aliquoting.

5.4.5 Using a 5-mL pipette, aliquot three 1-mL aliquots of organoid/Freezing Media suspension into the pre-labeled cryovials.

- **IMPORTANT**: Prior to each aliquot, gently mix the organoid/Freezing Media suspension as the organoids settle quickly to the bottom of the tube.
- We find use of a 5-mL pipette for mixing and preparing three 1-mL aliquots results in a more uniform number of organoids/cryovial and reduces the number of organoids adhering to the pipet wall.

5.4.6 Apply the cap to the cryovials, seal well. Wipe the exterior with disinfectant then place directly into isopropanol-based slow-rate freezing container (e.g., Mr. Frosty) and place into -80°C immediately. Additional details below.

6.0 CRYOPRESERVATION OF PATIENT-DERIVED ORGANOIDS

6.1 We strongly recommend cryopreserving one model at a time and placing the aliquots into a slow-rate freezing container and then -80°C as quickly as possible.

6.2 Slow-rate freezing (isopropanol-based using a cryo 1°C cell-freezing container such as Mr. Frosty Freeze Container [Sigma-Aldrich, Cat#: C1562])

6.2.1 Follow the manufacturer's instructions as provided for the specific cryopreservation device.

6.2.1.1 The base of the cryo-container is filled with room temperature isopropanol per the manufacturer's recommendation and the tube holder is placed on top.

6.2.1.2 Transfer the cryovials filled with organoids/freeze media into the tube holder of the cryo-container, screw the lid securely onto the cryo-container, and place at -80°C overnight.

6.2.1.3 Vials should be transferred to the vapor phase of a liquid nitrogen tank as soon as practical after the 4-hr freeze step, typically the following morning. In no case, should the vials be held longer than 3 days at -80°C before transfer into the vapor phase of a liquid nitrogen storage tank.

7.0 RECOMMENDED QUALITY CONTROL

7.1 Maintain a record of reagents used to prepare media.

7.2 Document vendors and lot numbers of all media components.

7.3 At change-over, parallel new reagents with existing lots prior to placing a new lot into service.

8.0 REFERENCES

- Sato, T., et al., *Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium.* Gastroenterology, 2011. **141**(5): p. 1762-72. https://www.ncbi.nlm.nih.gov/pubmed/21889923
- Tuveson Laboratory Protocols, Cold Spring Harbor Laboratory. *Murine and Human Organoid Protocols* (Version: 4/27/2016). Link to protocol: http://tuvesonlab.labsites.cshl.edu/wp-content/uploads/sites/49/2017/01/20160427-TuvesonOrganoidProtocols.pdf

APPENDIX 1: FIGURES

Figure 1: Example #1 - High (left) and low (right) density PDOrg cultures

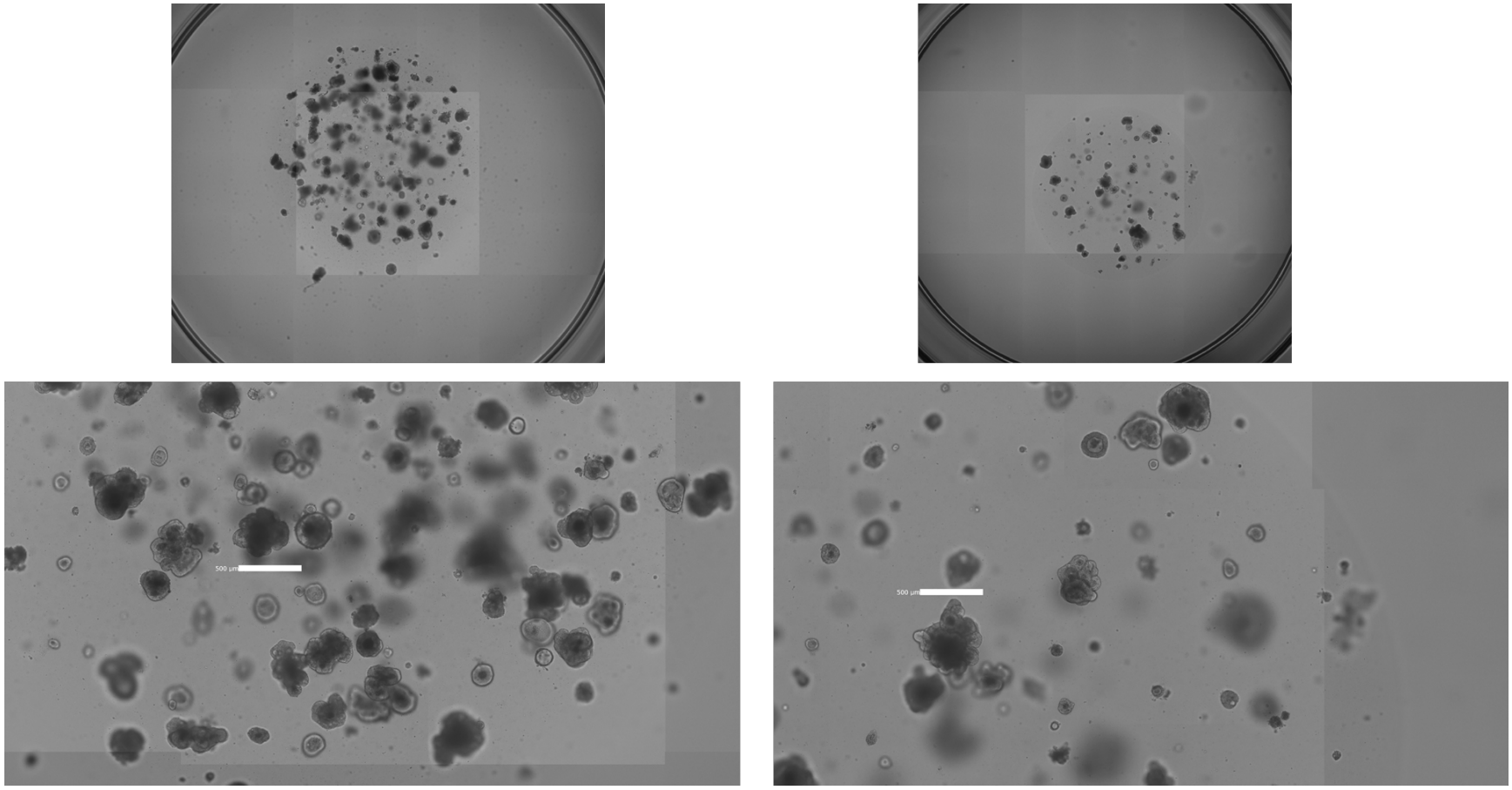

Figure 2: Example #2 - High (left) and low (right) density PDOrg cultures