

SOP40102: Thawing and Initial Culture of Patient-Derived Organoid (PDOrg) Cultures		
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## CHANGE HISTORY

Revision	Description
8/25/2017, v002	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	Added pictogram workflow from patient-derived organoid culture receipt to master cell stock (MCS) preparation. Added details on establishment of MCS. Updated reference SOPs. Added details for cell counting for cryopreservation of MCS. Updated vendor information for BME2.

## RELATED SOPS

SOP30101: Recipes for Complete Media for Patient-Derived In Vitro and Organoid Cultures
SOP40103: Passaging and Sub-culture of Patient-Derived Organoid (PDOrg) Cultures
SOP40104: Cryopreservation of Patient-Derived Organoid (PDOrg) Cultures

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## 1.0 PURPOSE/SCOPE

This Standing Operating Procedure (SOP) describes the procedures for thawing of Patient-Derived Organoid (PDOrg) cultures 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 EQUIPMENT

**4.1 Certificate of Analysis** for PDOrg culture to be grown.

**4.2 Media**

**4.2.1 Complete Media:**

4.2.1.1 Prepare the recommended Complete Media per the PDOrg-specific Certificate of Analysis (COA).

4.2.1.2 Prepare fresh weekly and warm to room temperature 2-3 hours before use.

**4.2.2 Wash Media:**

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

4.2.2.1 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** Cultrex PathClear Reduced Growth Factor BME, Type 2, (R&D Systems, Cat#: 3533-005-02); Request High-protein Content

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

**4.3.3** P1000, P200 and P20 Pipetman (or equivalent) and tips

**4.3.4** 15-mL sterile conical tubes, sterile

**4.3.5** 5, 10, 25 mL pipettes and automatic pipettor

**4.3.6** 24-well tissue culture plate, sterile (e.g., Costar, Cat#: 3524)

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

**4.3.8** Sterile screw cap tubes (e.g., Sarstedt, Cat#: 72692.005)

**4.3.9** Ice bucket with ice

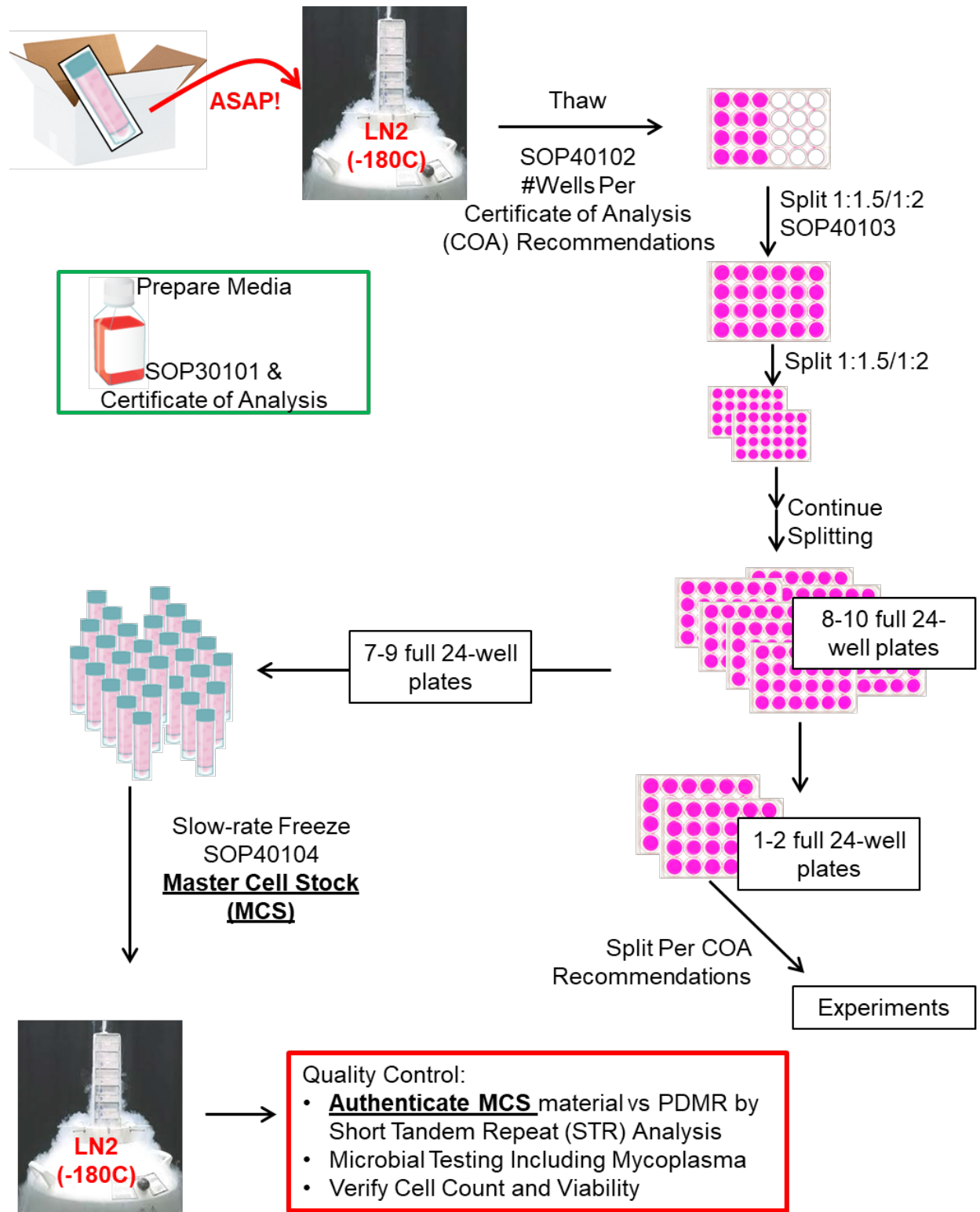
**4.3.10** Benchtop Centrifuge equipped with sealed buckets

**4.3.11** Biosafety Level 2 certified biosafety cabinet

**4.3.12** 37°C humidified, 5% CO2 tissue culture incubator

**4.3.13** Micro-centrifuge (e.g., Corning Model 6765)

## 5.0 GENERAL WORKFLOW



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## 6.0 RECOVERY FROM CRYOPRESERVATION

### 6.1 Prepare Reagents

- 6.1.1 Before starting, pre-warm the Complete Media and Wash Media to room temperature for 2-3 hours. Addition of cold or cool media to the BME2 dome will cause the BME dome to dissipate.
- 6.1.2 Thaw BME2 on ice before use (approx. 5 hrs) or, buried in ice in a 4°C refrigerator overnight.
  - 6.1.2.1 Keep on ice at all times until noted; BME2 polymerizes as it warms
- 6.1.3 Perform procedure under sterile conditions in a Biosafety Level 2 certified biosafety cabinet

### 6.2 Thaw PDOrg stock vial and Prepare BME2 Domes

- 6.2.1 Add 9 mL Wash Media to a 15-mL conical tube.
- 6.2.2 Quickly thaw the frozen PDOrgs by gently agitating the vial in a 37°C water bath. As soon as the solution starts to thaw, place the vial into the hood.
- 6.2.3 Transfer the Freeze Media/PDOrg solution to the prepared 15-mL conical tube.
- 6.2.4 Gently invert the tube and centrifuge 200xg for 5 min.
- 6.2.5 Carefully remove the Wash Media, add 10 mL fresh Wash Media and repeat the centrifugation step.
- 6.2.6 Carefully pipet off all but 1 mL of Wash Media.
- 6.2.7 Resuspend the PDOrg pellet in the remaining 1 mL Wash Media and transfer to a sterile 1.5-mL screw cap conical tube.
- 6.2.8 Using a mini-centrifuge, short-pulse (~1 sec/pulse) spin the tube 3 times. Be careful as prolonged mini-centrifugation can damage the cells.
- 6.2.9 With a P1000 Pipetman, carefully remove as much Wash Media as possible from the pellet.
- 6.2.10 Repeat short-pulse (~1 sec/pulse) spins and media removal using a P200 and then a P20 Pipetman to remove ALL excess media (see [Figure 1](#), appearance of final pellet in tube) as any excess media will dilute the BME2 causing a “wobbly” dome. Try to limit spinning so the cells are not damaged.
- 6.2.11 Place the tube on ice and add the recommended volume of BME2 based on the recommendations from **Section 2.3 of the Certificate of Analysis: 35 µL BME2** times the number of recommended wells. Be careful and **avoid** creating bubbles.

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**6.2.12** Keeping the PDOrg/BME2 mixture on ice, plate 35  $\mu$ L droplets into the center of the wells of a 24-well plate sitting at room temperature (see [Figure 2](#)). Again, be careful and **avoid** creating bubbles.

- Hint: turning the plate diagonally with a corner pointing toward you while plating the BME2 will increase the visibility in the plate and make placing the droplets in the center of the well a bit easier.

**6.2.13** Quickly and smoothly, turn plate upside-down and gently place into a 37°C incubator for 20 min so the BME2 polymerizes into a dome (see [Figure 3](#)).

**Important:** The In Vivo/In Vitro Development and Evaluation Laboratory supporting the PDMR has noticed there can be lot-to-lot variations in the surface coating of the 24-well plate that can result in flattened/spreading domes that is not related to the protein content of the BME2. A new lot of plates should be used.

### **6.3** Add specified media to BME2/PDOrg Domes

**6.3.1** Remove plate from incubator, return to the hood, place plate right-side up, and add 750  $\mu$ L Complete Media per well. Be careful not to disrupt the BME2 domes

**6.3.2** Place plate right-side up in a 37°C humidified, 5% CO<sub>2</sub> tissue culture incubator to culture the organoids.

**6.3.3** Check BME2/PDOrg domes 1-2 times/week and follow recommendations in **SOP40103: Passaging and Sub-culture of Patient-Derived Organoid (PDOrg) Cultures** for passaging or feeding of PDOrg cultures. Images included in the Appendix of that SOP provide guidance for identifying PDOrg cultures that are ready for passaging versus those that just need feeding.

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## 7.0 EXPANSION FOR ESTABLISHING A MASTER CELL STOCK

See Workflow Section 5.0

### 7.1 Splitting

**7.1.1** Follow “SOP40103: Passaging and Sub-culture of Patient-Derived Organoid (PDOrg) Cultures” to expand the initial wells of BME2/PDOrg domes.

**7.1.2** To ensure successful expansion and banking of cells for future use, continue to use the recommended Complete Media (per the PDOrg-specific COA).

**7.2** Repeat splitting the PDOrg cultures 1:1.5/1:2 (or per guidelines in SOP40103) until a total of 8-10 24-well plates of BME2/PDOrg domes have been established.

**7.1** Once the 8-10 24-well plates of BME2/PDOrg domes are ready to be split, 7-9 24-well plates (or ~150-200 wells) should be used to establish a Master Cell Stock (MCS). The MCS will be used for re-establishment of culture material for future experimental work.

**7.2** Follow procedures for digestion of the BME2/PDOrg domes in SOP40103, Section 5.3

**7.3** Follow procedures for cryopreservation in SOP40104.

**7.4** From the original 7-9 24-well plates (or ~150-200 wells) a total of  $\geq 35$  vials of approximately  $1-6 \times 10^6$  cells/vial MCS material (depending on the culture) should be cryopreserved.

**7.4.1** Count the viable cells present in the organoid suspension as follows

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

**7.4.1.2** 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.

**7.4.1.3** Follow manufacturer’s instructions for counting viable cells and determine the viable cell number in the original organoid suspension.

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

**7.4.2** MCS material should be authenticated by Short Tandem Repeat (STR) analysis and compared to the PDMR reported STR profile, undergo microbial/sterility testing, and should be tested for regrowth from freeze to verify viability from frozen stock.

**7.5** The remaining one to two 24-well plates can be split using the recommended split ratio in the PDOrg-specific COA to pursue experimental questions.

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**Note:** Seed Stocks and Working Stocks can be established using the same methodology to increase the amount of banked material ready for experimental use.

## 8.0 RECOMMENDED QUALITY CONTROL

- 8.1 Maintain a record of reagents used to prepare media.
- 8.2 Document vendors and lot numbers of all media components.
- 8.3 At change-over, parallel new reagents with existing lots prior to placing a new lot into service.

## 9.0 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>



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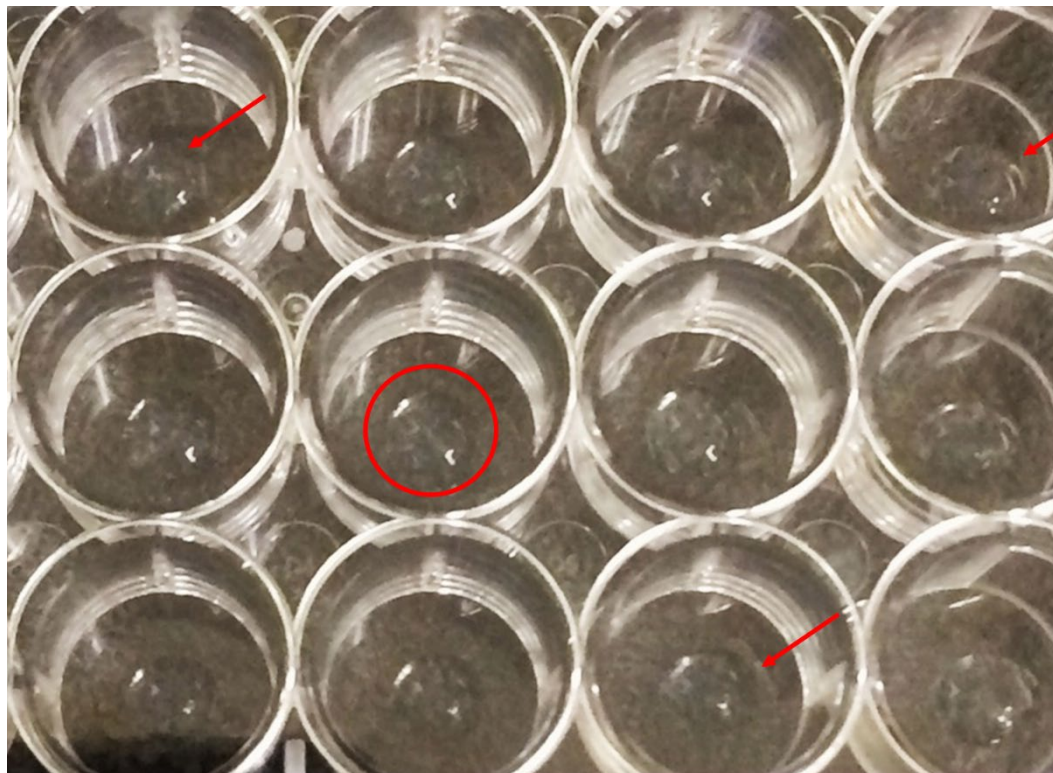
## FIGURES

**Figure 1:** Appearance of pelleted PDOrgs just prior to BME addition.



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**Figure 2:** Appearance of non-polymerized BME2 droplets, freshly pipetted onto bottom of a sterile, 24-well plate.



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**Figure 3:** Appearance of polymerized BME2 domes, ready for addition of Complete Media.

