SOP30104: Initial Culture, Sub-culture, and Cryopreservation of Suspension Patient-Derived Tumor Cultures (PDCs)

Effective Date: 1/19/2019

Please check for revision status of the SOP at

https://pdmr.cancer.gov/sops/

PDMR NCI Patient-Derived Models Repository
An NCI Precision Oncology Initiative℠ Resource

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VERSION INFORMATION

1. Change History

Revision	Description
	Internal SOP used by PDMR In Vitro Laboratory
5/14/2018	Standardize SOP for posting to PDMR internal site for use by designated NCI intramural laboratories
6/28/2018	Added pictogram workflow from cell culture receipt to master cell stock (MCS) preparation. Updated reference SOPs and Purpose/Scope section. Added Appendix 1 providing guidance on changing cell culture conditions. Appendix 2 added with representative PDC Images.
1/16/2019	Updated split recommendations for making master cell stock (MCS) and Freeze Medium recipe

2. Related SOPs

SOP30101: Recipes for Complete Media for Patient-Derived In Vitro and Organoid Cultures

1.0 PURPOSE/SCOPE

This Standing Operating Procedure (SOP) describes common tissue culture media used for growth of Suspension Patient-Derived Tumor Cultures (PDCs) under BSL-2 safety criteria. Early-passage patient-derived in vitro cultures require different growth conditions, have different growth characteristics, and visually appear different than traditional cell cultures; see Appendix 2. The recommended tissue culture media for **each specific** culture are provided as part of the Certificate of Analysis for the culture. **Not all cultures will use the same media**.

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 in vitro cell cultures 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 the cell cultures 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 in contact with patient tissue, as well as the mice carrying patient tumor samples and cultures derived from patient tumor samples, are treated as a potential health threat (BSL-2 precautions) since the human tissues 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 tissue 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 Reagents

4.1.1 Complete Media **without** Y-compound: sufficient volume for thawing steps

4.1.2 Complete Media (including Y-compound)

4.1.3 Fetal Bovine Serum (Hyclone, Cat#: SH30070.03 – HI)

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

4.2 Material & Equipment

4.2.1 50-mL, 25-mL, 10-mL, 5-mL pipettes, sterile

4.2.2 15 and 50-mL polypropylene tubes, sterile

4.2.3 2.0 mL screw-capped cryovials ((Nunc, Cat#: 368632)

4.2.4 Tissue Culture flasks, sterile, vented

4.2.5 Pipetman and sterile tips

4.2.6 Waste container Bleach (Clorox, 5.25% Hypochlorite) diluted 1:10, 2% Virkon®, or similar disinfectant

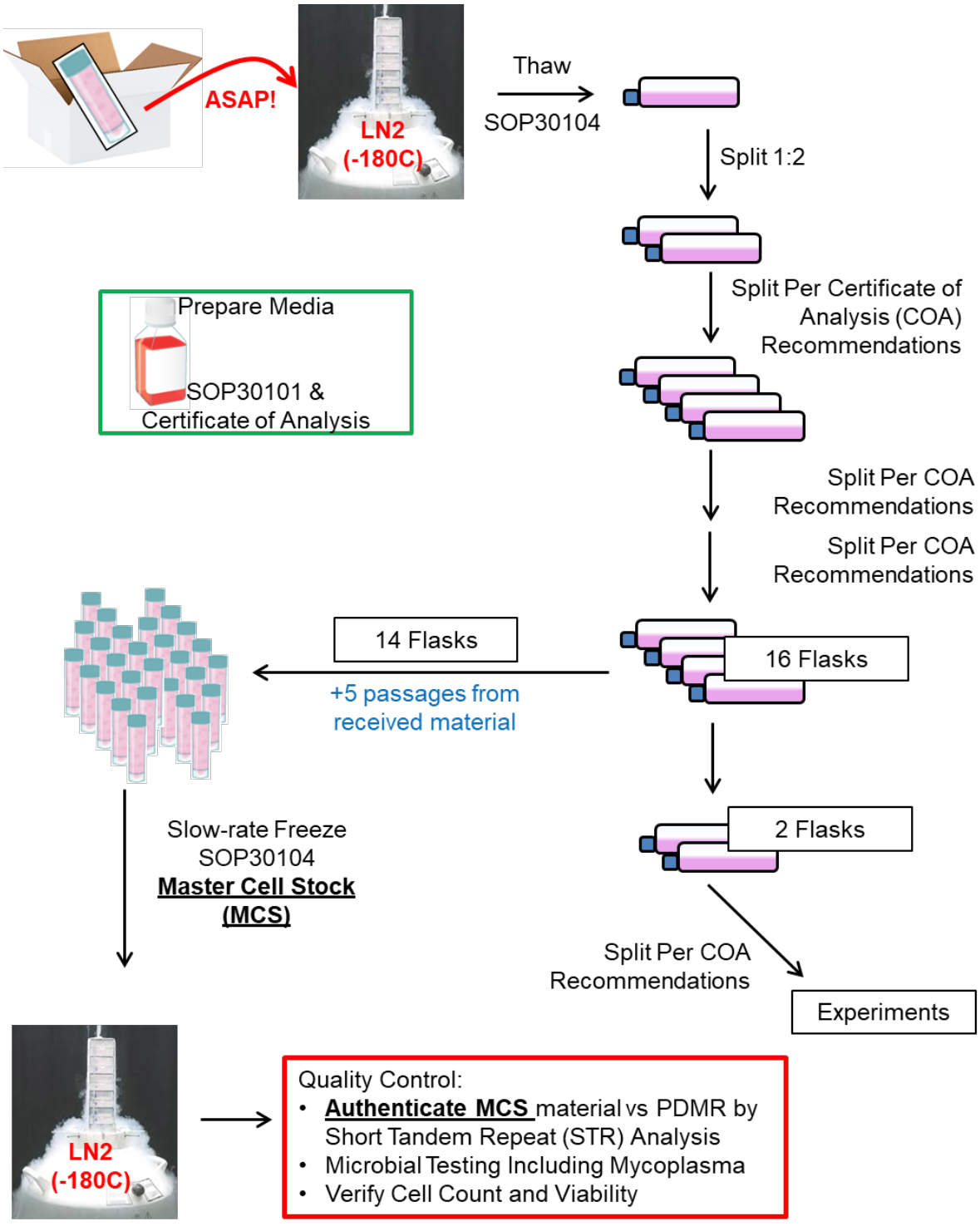
4.2.7 Refrigerator (4°C) and freezer (–20°C)

4.2.8 37°C Incubator (5% CO_2, humidified)

4.2.9 Biological Safety Cabinet (BSC) meeting biosafety level 2 (BSL2) standards

4.2.10 Personal Protective Equipment (PPE) at a minimum laboratory coat, with fitted sleeves, latex or nitrile gloves and safety glasses

5.0 GENERAL WORKFLOW



6.0 RECOVERY FROM CRYO-PRESERVATION

6.1 In a sterile 15-mL conical tube, add 9-mL of the appropriate Complete Media (per the PDC-specific Certificate of Analysis [COA]) **without** Y-compound for the cells being thawed.

6.2 Quickly thaw the frozen cells by gently shaking the vial in a 37°C water bath. As soon as the cells start to thaw (should not be completely thawed), move to the BSC and clean the exterior of the tube with Virkon (or similar disinfectant).

6.3 Transfer the cells to the media-containing 15-mL conical tube.

6.4 Gently invert the tube several times to ensure the cells mix with the fresh Complete Media **without** Y-compound then centrifuge 200xg for 5 min.

6.5 Carefully pipette off the media, add 10-mL fresh Complete Media **without** Y-compound. Invert gently several times and repeat the centrifugation step.

6.6 Carefully pipette off the media and add 10-mL of fresh Complete Media **containing** 10 µM Y-compound. Gently resuspend the cells.

6.7 Gently resuspend the cells using care not to break the cell clusters into single cells.

6.8 Add the resuspended cells to an uncoated T25 flask.

6.9 Incubate flask at 37°C in a 5% CO_2 humidified incubator.

7.0 EXPANSION FOR ESTABLISHING A MASTER SEED STOCK

- Splitting:
  - Split cultures when cells/cell clusters become heavy. Do not allow media to turn bright yellow.

7.1 To ensure successful expansion and banking of cells for future use, expand as follows and continue to use the recommended Complete Media (per the PDC-specific COA).

7.2 Split the initial T25 flask 1:2 as follows:

7.2.1 Transfer media/cell mixture into a 50-mL conical tube.

7.2.2 Centrifuge cells for 5-6 min at 200xg.

7.2.3 Gently decant media from 50-mL conical, being careful not to dislodge the cells. Discard the media in an appropriate waste container.

7.2.4 Add 20-mL Complete Media to the 50-mL conical tube. Triturate gently to break the large clusters into smaller clusters.

7.2.4.1 Be aware: Breaking clusters down to single cells may result in loss of the cell culture.

7.2.5 Add 10 mL of the Complete Media/cell mixture into two new uncoated T25 flasks.

7.2.6 Place flasks in a humidified 5% CO_2 37°C incubator.

7.3 Monitor cells visually at least twice each week.

7.4 Repeat splitting culture using the recommended split ratio in the PDC-specific COA until a total of 16 flasks of cells have been established

7.5 Once the 16 flasks are ready to be split, 14 flasks should be used to establish a Master Cell Stock (MCS). The MCS will be used for re-establishment of culture material for experimental work.

7.5.1 Follow procedures for cryopreservation in SOP Step 9.0.

7.5.2 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 to verify cell count and viability.

7.6 The remaining two (2) T25 flasks should be split using the recommended split ratio in the PDC-specific COA to pursue experimental questions.

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 SUB-CULTURE CONDITIONS

8.1 General Notes

- Splitting:
  - Split cultures when cells/cell clusters become heavy. Do not allow media to turn bright yellow.
  - The recommended split ratio for actively growing cultures is included in the PDC-specific COA provided with the cell line. Primary cell cultures are sensitive to cell density; follow the provided recommendations.
- Change media every 7 days. Change more frequently if the media turns yellow
- Y-compound is included in most Complete Media recipes. See Appendix 1 for recommendations on testing removal of Y-compound or trying different media conditions.

8.2 Sub-culture Method

8.2.1 Transfer media/cell mixture into a 50-mL conical tube.

8.2.2 Centrifuge cells for 5-6 min at 200xg.

8.2.3 Gently decant media from 50-mL conical, being careful not to dislodge the cells. Be sure to discard the media in appropriate waste container.

8.2.4 Add 20-mL Complete Media to the 50-mL conical tube. Triturate gently to break the large clusters into smaller clusters.

8.2.4.1 Be aware: Breaking clusters down to single cells may result in loss of the cell culture.

8.2.5 Following the Split Ratio recommendations in the COA, add additional Complete Media and the media/cell mixture into the needed number of new uncoated T25 flasks.

8.2.6 Place flasks in a humidified 5% CO_2 37°C incubator.

8.2.7 Monitor cells visually at least twice each week.

9.0 CRYOPRESERVATION PROCEDURE

9.1 Prepare Freeze Media

Item	Final Percent/Volume
Complete Media (per COA); which already contains 5% FBS	80%
Fetal Bovine Serum*	15%
DMSO	10%

*Note Fetal Bovine Serum final concentration will be approximately 20% from Complete Media + additional FBS added in Freeze Media Recipe.

9.2 Cryopreservation

9.2.1 Transfer media/cell mixture into a 50-mL conical tube.

9.2.2 Centrifuge cells for 5-6 min at 200xg.

9.2.3 Gently decant media from 50-mL conical, being careful not to dislodge the cells. Be sure to discard the media in appropriate waste container.

9.2.4 Resuspend cell pellet in a small volume of Freeze Media. For example, for MCS establishment from 14 flasks of cells, resuspend in 3 mL.

9.2.4.1 Remove a small aliquot of cells for cell counting (e.g., 25 µL).

9.2.4.2 Triturate to prepare a single cell suspension or as close to a single cell suspension as is possible. Note: Do NOT add these cells back to the tube containing the intact cell clusters

9.2.4.3 Count viable cells by trypan blue exclusion, or similar.

9.2.5 Add additional Freeze Media to the cell/Freeze Media suspension to reach a target of 1 x 10^6 viable cells/mL in Freeze Media.

9.2.6 Aliquot 1-mL of 1 x 10^6 viable cells/mL cell suspension into 2.0 mL screw-capped cryovials. Apply the cap to the cryovials, seal well.

9.2.7 Wipe the exterior with disinfectant then place into wet ice until ready to begin stepped-rate freezing (cooling rate of -1°C/minute).

9.3 Stepped -Rate Cryopreservation Procedure

9.3.1 Mechanically controlled stepped-rate freeze cryopreservation is recommended, when available, as it is believed to result in a lower loss of cell viability due to the decreased formation of ice crystals.

9.3.2 Always follow the manufacturer's guidelines for operation. General stepped-rate cryopreservation parameters used at by the PDMR are:

- Decrease 1°C/minute down to -4°C
- Decrease 25°C/minute down to -40°C
- Increase 15°C/minute up to -12°C
- Decrease 1°C/minute down to -40°C
- Decrease 10°C/minute down to -90°C

9.3.3 Vials should be transferred to the vapor phase of a liquid nitrogen tank as soon as practical after the vials reach minimum temperature. Preferably within 4-6 hours. In no case, should the vials be held longer than 24-hours before transfer into the vapor phase of a liquid nitrogen storage tank.

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

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

9.4.2 Material should be held on wet ice in cryovial tubes until ready for placement into the slow-rate freeze container.

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

9.4.4 Transfer the cryovials filled with cells/freeze media from the ice-bucket into the tube holder of the cryo-container, screw the lid securely onto the cryo-container, and place at -80°C for a minimum of 4 hours, but most commonly for overnight.

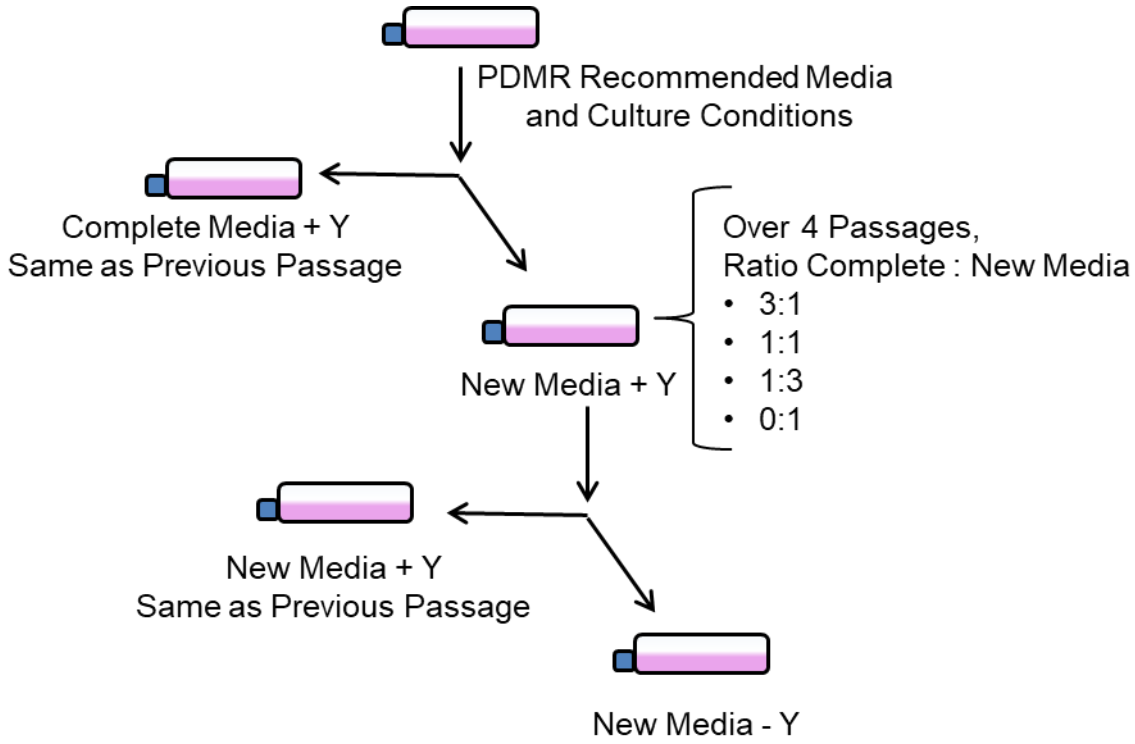
9.4.5 Vials should be transferred to the vapor phase of a liquid nitrogen tank as soon as practical after the 4-hour freeze step. Preferably within 4-6 hours. In no case, should the vials be held longer than 24-hours before transfer into the vapor phase of a liquid nitrogen storage tank.

9.5 Slow-rate freezing (non-isopropanol based such as CoolCell)

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

APPENDIX 1: ALTERNATE CULTURE CONDITIONS

- Changes to culture conditions should not be attempted until the Master Cell Stock (MCS) has been banked. Any time cells are thawed from the MCS, the recommended Complete Media + Y compound per the PDC-specific Certificate of Analysis (COA) should be used.
- Changes to culture conditions should only be attempted on well-established cultures, at least 3-4 passages after thawing.
- Changing culture conditions may result in changes to the cell culture composition, SNP allele frequency, RNA and protein expression levels, etc. Any publications should note all changes to culture conditions relative to the provided PDMR material.
- To minimize shock to the patient-derived cells, changes should be performed in a step-wise controlled manner. Below is a recommended workflow for changing culture conditions.



APPENDIX 2: PHASE CONTRAST IMAGES OF PDCS

Representative images (40x) of PDC cultures from multiple histologies to demonstrate the range of phenotypes that can be expected in adherent and suspension/suspension cluster (yellow boxes) growth types.