Table of Contents

RELATED SOPS 1

1.0 PURPOSE/SCOPE 2

2.0 SAFETY 2

3.0 CLEAN-UP 2

4.0 EQUIPMENT 3

5.0 GENERAL WORKFLOW 4

6.0 RECOVERY FROM CRYO-PRESERVATION 5

7.0 SUB-CULTURE CONDITIONS 5

8.0 CRYOPRESERVATION PROCEDURE 6

APPENDIX 1: PHASE CONTRAST IMAGES OF CAF CULTURES 9

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 with representative CAF images.
1/16/2019	Updated Freeze Medium recipe
9/1/2020	Corrected error in Trypsin concentration from 0.25% to 0.05%; catalog number remains the same

RELATED SOPS

SOP30101: Recipes for Complete Media for Patient-Derived In Vitro and Organoid Cultures
SOP30102: Preparation of Coated Flasks for Adherent Patient-Derived In Vitro Cultures

1.0 PURPOSE/SCOPE

This Standing Operating Procedure (SOP) describes common tissue culture media used for growth of Patient-Derived Tumor Cancer-Associated Fibroblasts (CAFs) 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 (e.g., HeLa). 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 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 Matrigel®- or BME-coated T25 flask (SOP30102)

4.1.4 DPBS (Invitrogen, Cat#: 14190250) or HBSS (Invitrogen, Cat#: 14175-079)

4.1.5 0.05% (w/v) Trypsin-EDTA (Invitrogen, Cat#: 25300120)

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

4.1.7 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

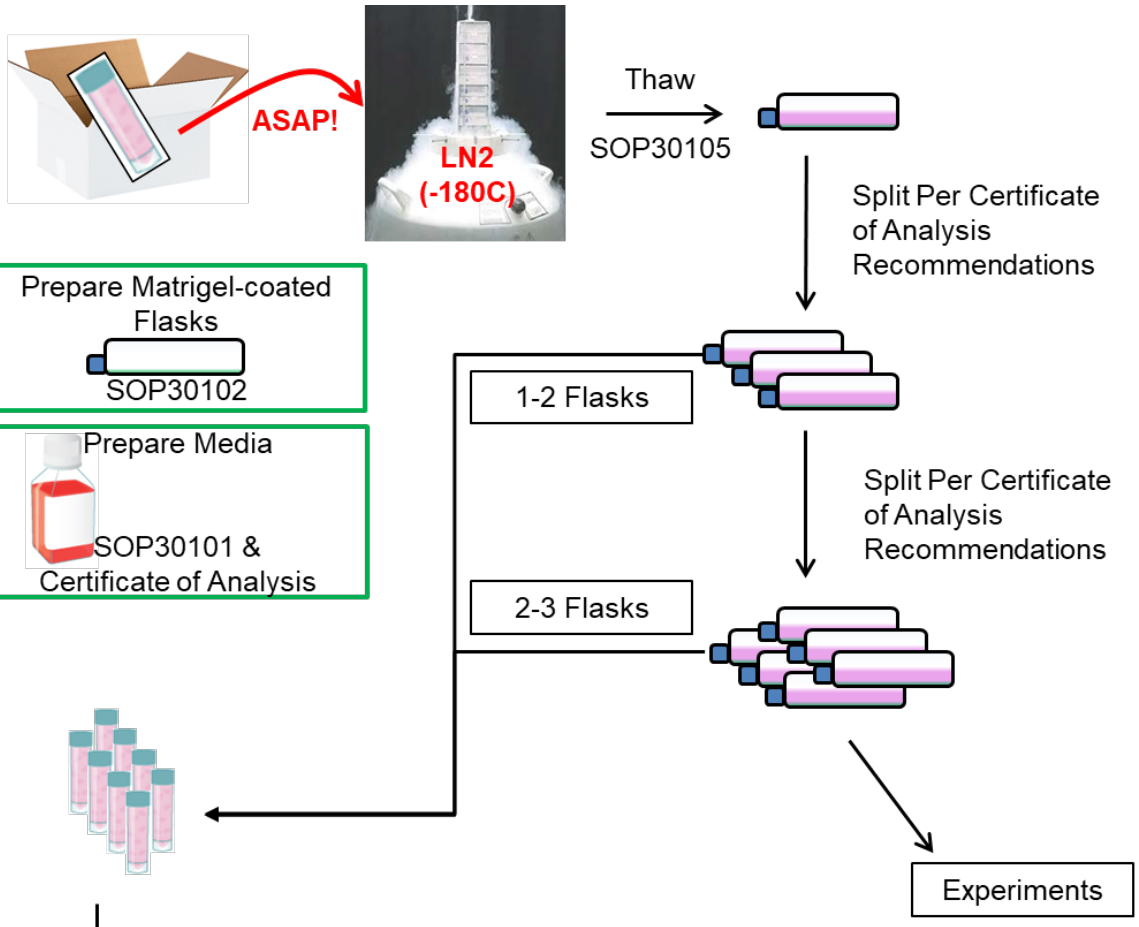
4.2.7 Refrigerator (4°C) and freezer (–20°C)

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



Important

- Cancer Associated Fibroblasts (CAFs) have a finite lifespan in vitro.
- CAFs are guaranteed for experimental use for up to 3 passages when maintained on the recommended Complete Media + Y compound
- Additional population doublings and subcultures are possible, but overall fitness of culture may deteriorate with subsequent passages
- Cryopreservation may compromise CAF cultures especially if the overall fitness is deteriorating.
- Cryopreservation at each split is recommended as long as the culture fitness is healthy.

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 CAF-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 Carefully remove excess media from the Matrigel®- or BME-coated T25 flask and discard taking care to not dislodge the coating.

6.8 Gently add the resuspended cells to the Matrigel®- or BME-coated T25 flask being careful to not dislodge the coating.

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

7.0 SUB-CULTURE CONDITIONS

Important

- Cancer Associated Fibroblasts (CAFs) have a finite lifespan in vitro.
- CAFs are guaranteed for experimental use for up to 3 passages when maintained on the recommended Complete Media + Y compound
- Additional population doublings and subcultures are possible, but overall fitness of culture may deteriorate with subsequent passages

7.1 General Notes

- Splitting:
  - Split cultures at approximately 70%-80% confluence. **Do not** allow cells to reach 100% confluence.
  - The recommended split ratio for actively growing cultures is included in the CAF-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

7.2 Sub-culture Method

7.2.1 Aspirate and discard the media into an appropriate waste container.

7.2.2 Gently, rinse cells with 3-mL sterile DPBS or HBSS.

7.2.3 Add 2-3 mL 0.05% (w/v) trypsin to the T25 flask and return flask to 37°C incubator for 3-5 min.

7.2.4 Gently tap/rock flask to dislodge cells

7.2.5 Add at least twice the volume of Complete Media (per COA) to the flask and mix with gentle pipetting. Transfer media and cells to a sterile 50-mL conical tube.

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

7.2.7 Discard culture media in appropriate waste container.

7.2.8 Resuspend cell pellet in the appropriate volume of Complete Media (per COA) to accommodate recommended split ratio (per COA).

7.2.9 Add cells to flasks and place flasks in a humidified 5% CO_2 37°C incubator.

7.2.10 Monitor cells microscopically at least twice each week.

8.0 CRYOPRESERVATION PROCEDURE

Important

- CAFs have a finite lifespan in vitro.
- Cryopreservation may compromise CAF cultures especially if the overall fitness is deteriorating prior to cryopreservation.
- Cryopreservation at each split is recommended if the culture fitness is healthy.

8.1 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.

8.2 Cryopreservation

8.2.1 Aspirate and discard culture medium from flask into an appropriate waste container.

8.2.2 Rinse cells in flask with 3 mL sterile DPBS or HBSS.

8.2.3 Add 2-3 mL 0.05% (w/v) trypsin to cells in a T25 flask and return flask to 37°C incubator for 3-5 min.

8.2.4 Gently tap flask/rock plate to dislodge cells.

8.2.5 Add at least twice the volume of Complete Media to the flask and mix with gentle pipetting. Transfer media and cells to a sterile 50-mL conical tube(s).

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

8.2.7 Discard culture media in appropriate waste container.

8.2.8 Resuspend cell pellet in a small volume of Freeze Media.

8.2.8.1 Remove a small aliquot of cells for cell counting (e.g., 10-25 µL).

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

8.2.9 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.

8.2.10 Aliquot 1-mL of cell suspension into 2.0 mL screw-capped cryovials. Apply the cap to the cryovials, seal well.

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

8.3 Stepped -Rate Cryopreservation Procedure

8.3.1 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.

8.3.2 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

8.3.3 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.

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

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

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

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

8.4.4 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.

8.4.5 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.

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

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

APPENDIX 1: PHASE CONTRAST IMAGES OF CAF CULTURES

Representative images (40x) of CAF cultures isolated from multiple tumor histologies to demonstrate the range of phenotypes that can be expected in culture.