

Protocol

Cell Culture for Patient-Derived Melanoma Tumor Cell Lines

The basics of cell culture for patient-derived melanoma cell lines share certain similarities, however, cell culture conditions vary typically for each melanoma cell line. Deviating from the culture conditions required for a particular melanoma cell line can result in different phenotypes being expressed. We recommend that you acquaint yourself with each cell line of interest and carefully follow the cell line specific instructions provided with each product.

I. Basic Equipment

1. Biosafety cabinet or laminar-flow hood
2. Incubator (humidity controlled CO₂ incubator maintained at 36°C recommended*)
3. Water bath at 37°C
4. Centrifuge
5. Refrigerator and freezers (–20°C and –80°C)
6. Cell counter
7. Inverted microscope
8. Liquid nitrogen (N₂) freezer or cryo-storage container
9. Cryo-freezing chamber
10. Sterilizer/autoclave

Note: We recommend maintaining CO₂ incubator at 36°C as the cells will die at temperatures slightly over 37°C. These cells will grow better at 36°C than at 37.2°C. To avoid possible errors in the incubator calibration, we suggest maintaining incubators at 36°C.

II. Additional Supplies

1. Sterile cell culture vessels (e.g., T-25 flasks, Petri dishes, multi-well plates, conical tubes)
2. Liquid nitrogen storage Dewar and cryo-vials
3. Pipettes and tips
4. 70% ethanol
5. Syringes and needles
6. Waste containers
7. Media, sera, and reagents (for appropriate media components for desired cell lines, see cell line specification sheet)

III. Procedure

Thawing Frozen Cells

The thawing process is stressful to frozen cells, and using a good technique and working quickly should ensure that a high percentage of cells survive the procedure. Remove vials containing viable cells from liquid nitrogen storage tank and immediately place on dry ice.

1. Before starting the procedure, label and prepare a 15 mL sterile conical tube containing 10 mL of DMEM supplemented with 10% Fetal Bovine Serum (#FBS-01-0100) for each vial being thawed.
2. To thaw a vial, place vial into a 37°C water bath until the frozen cells begin to thaw.
3. Wipe the outside of the vial with 70% ethanol (alternatively, isopropanol may be used) before placing it in the cell culture hood.
4. Immediately transfer the cell suspension into 15 mL conical tube containing 10 mL of DMEM/10% FBS and then centrifuge at 1500 rpm (500 x g) for 5 minutes at room temperature.
5. Discard the supernatant and resuspend cell pellet in 5 mL of Tumor Specialized Media with FBS (heat inactivated).
6. Transfer the suspended cells into a T-25 flask. Place the flask in a humidified incubator (5% CO₂) at 36°C overnight.
7. Check the flask after 24 hours for attachment of cells to the flask. If cells are attached, remove the media and add fresh media to the flask.

Culturing Adherent Cells

1. All solutions and equipment for cell culture must be sterile. All work should be performed in a biological safety cabinet or laminar flow hood to provide aseptic conditions and to prevent contamination of the cells.
2. Tumor-specialized media with 2% FBS is recommended for the majority of melanoma cell lines. The composition of Tumor Specialized media is shown in Table 1.
Note: For information on exceptions to tumor-specialized media with 2% FBS, refer to cell line specific instructions. While not preferred, DMEM with 5% FBS or RPMI with 5% FBS may be used in lieu of Tumor Specialized Media.
3. Cells should be maintained between 30-95% confluence in Tumor Specialized media with 2% FBS (heat inactivated) unless otherwise noted in cell line specific instructions.
4. Media should be changed every 3-4 days. Cells should be split in a ratio according to the cell line specifications.

Table 1. Preparation of Tumor Specialized Media¹

Composition	Final Concentration	Volume (510 mL)
MCDB153	80%	400 mL
Leibovitz's L-15	20%	100 mL
Fetal Bovine Serum	2% ²	10 mL
CaCl ₂	1.68mM	0.42 mL

¹For media preparation and media component ordering instructions, see table 3.

²For information on exceptions to tumor-specialized media with 2% FBS, refer to cell line-specific instructions.

Splitting or Harvesting Cells

1. Detach adherent cells using a dissociation reagent 0.25% trypsin/EDTA solution. The specific volumes of dissociation reagent, quench media, and Tumor Specialized media by flask size are shown in Table 2. The incubation time with 0.25% trypsin/EDTA solution may vary; check for dissociation every 30 seconds. Observe the cells under the microscope for detachment, and when $\geq 90\%$ of the cells have detached, extract the cells from the flask with a serological pipette.
2. Transfer the cells to a 15 mL conical tube, add quench medium (10% DMEM) to the cells according to Table 2 below, and centrifuge at 1500 rpm for 5 minutes at room temperature. Resuspend the cell pellet in Tumor Specialized media with FBS and remove a sample for counting.
3. Determine the total number of cells and percent viability using a hemacytometer. Dilute cell suspension to the seeding density recommended for the cell line. Pipette the appropriate volume of cell suspension into new cell culture flask, and add required volume of Tumor Specialized media with FBS according to flask size as noted in Table 2. Return the cells to the incubator.

Table 2. Specific volumes required by flask size

Flask Size	0.25% Trypsin/EDTA (mL)	Quench Medium (10% DMEM) (mL)	Tumor Specialized Medium with FBS (mL)
T-25	1.0	5	3-5
T-75	3.0	10	10-12
T-150	5.0	15	20-22

Freezing and Storage of Cells

The best method for cryopreserving cells is to store cultures in liquid nitrogen in complete media in the presence of a cryoprotective agent such as dimethyl sulfoxide (DMSO). Cryoprotective agents reduce the freezing point of the media and allow a slower cooling rate, greatly reducing the risk of ice crystal formation, which can damage cells and cause cell death.

To freeze the cells, follow the process for dislodging the cells using trypsin/EDTA solution as described above. Freezing media is composed of 90% FBS and 10% DMSO.

1. Prepare freezing media and store at 2°C to 8°C until use.
2. Gently detach the cells from tissue culture flask following the trypsinization procedure as described above.
3. Centrifuge cell suspension at 1500 rpm for 5 minutes at room temperature. Aseptically decant supernatant without disturbing the cell pellet.
4. Resuspend the cell pellet in cold freezing media (90% FBS/10% DMSO solution) at a density of 1×10^6 cells/mL. Immediately aliquot the cell suspension into cryogenic storage vials.
5. Freeze the cells slowly by decreasing the temperature at approximately 1°C per minute using a controlled-rate cryofreezer or a cryo-freezing container or equivalent.
6. Place the cryo-freezing container at -80°C overnight.
7. Transfer cryogenic vials containing the frozen cells to liquid nitrogen and store them in gas phase above the liquid nitrogen.

IV. Special Considerations

- It is important to keep track of cell line passage number as this is a crucial variable that will likely affect experimental data.
- Cell lines that are at a passage number less than 20, must be maintained at greater than 60% confluence.
- Cell lines that adhere loosely form spheres or tend to float can be made more adherent with the addition of serum up to 5-10%. *See the subculturing conditions in cell line specifications.*
- Cell lines should be tested for mycoplasma contamination and Short Tandem Repeat (STR) profiling every 10 passages or each time a frozen seed stock is made. *Please contact Rockland for Information regarding STR profiling analysis.*

Table 3. Preparation of Tumor Specialized Media¹

Component	Vendor	Item No.
MCDB153	Sigma-Aldrich	M-7403
Leibovitz's L-15	Sigma-Aldrich	L1518
FBS (heat inactivated)	Rockland	FBS-01-0100
Calcium chloride (CaCl ₂)	Sigma-Aldrich	C5670
Sodium Bicarbonate	Sigma-Aldrich	S5761

MCDB-153 Preparation

1. Resuspend MCDB powder in approximately 900 ml of double distilled water.
2. Add 1.2 g cell culture grade sodium bicarbonate and adjust pH to 7.6 with 5N sodium hydroxide using a pH meter.
3. Add double distilled water to have a final volume of 1L and filter sterilize using a 0.22 µm filter.
4. Store at 4°C in dark (up to three weeks).
5. Use 400 ml/511 ml of total media for a final concentration of 80%.

Leibovitz's L-15 Preparation

1. Add 100 mL/511 mL of total media for a final concentration of 20%.

Fetal Bovine Serum (heat inactivated) Preparation

1. Add 10 mL/511 mL of total media for a final concentration of 2%

2M Calcium Chloride (CaCl₂) Preparation

1. Dissolve 44.4 g of CaCl₂ in 200 mL double distilled water and filter sterilize using 0.2 µm filter.
2. Aliquot into 1 mL aliquots and store at room temperature. Use 420 µL/511 mL of total media for a final concentration of 1.68 mM.

Note: Combine all the components together under a cell culture hood. Sterilize using 0.22 µm filter.

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