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OncoPro™ Tumoroid Culture Medium Kit

USER GUIDE

Catalog Number A5701201

Publication Number MAN0029372

Revision 1.0

ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: MAN0029372 1.0 (English)

Revision	Date	Description
1.0	16 May 2023	New document for OncoPro™ Tumoroid Culture Medium Kit.

The information in this guide is subject to change without notice.

Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the “Safety” appendix in this document.

Product description

Gibco™ OncoPro™ Tumoroid Culture Medium Kit is an easy-to-use culture system optimized for expansion of patient-derived tumoroid lines. Currently, the kit enables expansion of colorectal, lung, pancreatic, and head & neck tumoroid lines.

Key features of the medium:

- OPTIMIZED formulation for patient-derived tumoroid culture
- STREAMLINED media preparation, culture methods, and support
- ADAPTABLE use for multiple cancer indications and downstream assays

Procedure overview

In this procedure, tumoroid lines are seeded into suspension culture in complete OncoPro™ Tumoroid Culture Medium plus the ROCK inhibitor Y-27632. Cells are fed every 2–3 days and passaged after approximately 7 days when the average tumoroid diameter is 200-300 µm (depending on the tumoroid line, cultures may be ready to passage anywhere between 4–14 days after seeding). It is recommended that cells are cultured for at least three passages post-thaw prior to use in downstream studies.

For more information, go to: **www.thermofisher.com/oncopro-elearn**

Contents and storage

Table 1 OncoPro™ Tumoroid Culture Medium Kit Contents (Cat. No. A5701201)

Component	Cat. No.[1]	Amount	Storage	Shelf life[2]
OncoPro™ Basal Medium (1X)	A5568501	500 mL	2°C to 8°C; Protect from light.	12 months
OncoPro™ Supplement (50X)	A5568601	10 mL	-5°C to -20°C [3]; Protect from light.	
OncoPro™ BSA	A5568701	40 mL	2°C to 8°C; Protect from light.	
B-27™ Supplement (50X)	17504044	10 mL	-5°C to -20°C[3]; Protect from light.	

[1] OncoPro™ Tumoroid Culture Medium Kit is sold as a complete kit; individual components are not sold separately.
[2] Shelf-life duration is determined from Date of Manufacture.
[3] Store in a non-frost-free freezer.

Culture conditions

Medium: OncoPro™ Tumoroid Culture Medium Kit

Cells: Patient-derived tumoroid lines established from colorectal, lung, pancreatic, or head & neck cancer

Culture type:

- Recommended – Suspension culture
- Optional - Embedded in basement membrane extract (BME) domes (see page 27 for guidance on when to consider embedded culture method)

Non-treated vessels for suspension culture workflow:

- 6-well plate
- T-25 or T-75 flask

Incubator temperature range: 36°C to 38°C

Incubator atmosphere: Humidified atmosphere of 5% CO_2. Ensure that proper gas exchange is achieved in culture vessels.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

Table 2 Required materials not supplied

Item	Source
Equipment	
37°C, 5% CO_2 incubator	**MLS**
EVOS™ XL Core Imaging System	AMEX1000
37°C water bath	**MLS**
Refrigerated centrifuge	**MLS**
Tubes, plates, and other consumables	
Serological pipettetes	**MLS**
Pipette tips	2749-HR (20 µL) 2769-HR (200 µL) 2789-HR (1200 µL)
15 mL conical tubes	339651
50 mL conical tubes	339653
Nunc™ non-treated culture vessels	150239 (6-well) 169900 (T-25) 156800 (T-75)
Reagents	
DMEM/F12 + GlutaMAX™-I (1X)	10565018
Penicillin-Streptomycin (10,000 U/mL)	15140122
InvivoGen Primocin™[1]	Fisher Scientific™; NC9392943
Y-27632 2HCl	Fisher Scientific™; 50-863-6
Distilled water	15230170
Bovine Albumin Fraction V (7.5% solution)	15260037
Basement membrane extract	**MLS**

Table 2 Required materials not supplied *(continued)*

Item	Source
DPBS, no calcium, no magnesium	14190144
StemPro™ Accutase™ Cell Dissociation Reagent	A1110501

[1] Primocin™ is a trademark of InvivoGen.

Table 3 Additional cancer-specific recommended materials

Item	Source
For lung tumoroids:	
Heat Stable FGF-10	PHG0372
For head & neck tumoroids:	
Heat Stable FGF-10	PHG0372
For pancreatic tumoroids:	
Heat Stable FGF-10	PHG0372
Gastrin	Fisher Scientific™; 30-061

Workflow

Example tumoroid suspension culture workflow

Workflow	
Day 0	**Seed tumoroid cultures in suspension** Seed from active tumoroid line cultures or directly from cryopreserved tumoroids. **Note:** For best results, see "Guidelines for initiating tumoroid cultures from either cryopreserved or active cultures" on page 12.
Day 2 and 5	**Feed tumoroid cultures** Feed tumoroid cultures by complete medium exchange. **Note:** For best results, replate into a new culture vessel with each feed.
Day 7	**Passage the suspension tumoroid cultures** Passage the tumoroids using StemPro™ Accutase™ Cell Dissociation Reagent.
Repeat	**Repeat Day 0 to Day 7 for the desired number of passages** Repeat passages can be used to scale-up suspension cultures into larger vessel formats.
Harvest	**Harvest suspension tumoroid cultures for downstream studies** Harvested tumoroids can be used in a wide range of downstream applications.

2 Methods

Guidelines for tumoroid suspension culture

IMPORTANT!

- Perform all procedures inside a laminar flow biosafety cabinet, using aseptic technique to prevent contamination.
- To minimize cross-contamination of tumoroid lines, it is recommended that users wipe down the shaft of all micropipettes with 70% ethanol or isopropanol before and after handling an individual tumoroid line.

Preparing media and reagents

- The volumes provided in this guide are sufficient to seed and passage a single T-25 flask. To seed and passage multiple or different size culture vessels, scale the volumes proportionally based on the size and number of vessels.
- Use non-treated culture vessels for tumoroid suspension cultures.
- StemPro™ Accutase™ Cell Dissociation Reagent should be aliquoted and a fresh aliquot thawed each time cells are passaged.
- Frozen aliquots of 10 mM Y-27632 should be thawed and added to aliquoted OncoPro™ complete medium immediately before each feed and/or passage.
- Basement membrane extract (BME) used in these workflows must have a concentration of > 10 mg/mL prior to dilution. Some ready-to-use formulations may not be suitable for these applications due to their low protein concentration.
- It is recommended to use Heat Stable FGF-10 for cancer indications requiring supplementation with FGF-10. This allows for the use of lower concentrations of growth factor and is more cost-effective than using native FGF-10. If native FGF-10 is used, concentrations should be increased to 100 ng/mL in the complete medium throughout protocol.

Handling cells

- It is recommended to use a serological pipette or micropipette tip instead of a vacuum aspirator setup to remove media during feeding and passaging steps. This reduces the risk of accidental cell loss.
- Pre-wetting serological pipettes and micropipette tips with complete medium can reduce the risk of tumoroids adhering to the pipette or tip and can minimize tumoroid loss. If pre-wetting, it is recommended that you aliquot the complete medium into a secondary container to use for pre-wetting; this will avoid risk of contamination from triturating in the primary container.

Culturing cells

- Tumoroid cultures should be fed every 2–3 days.
- It is recommended that tumoroid lines are transferred to a fresh culture vessel after being fed with a full medium exchange.
- Tumoroid cultures should be passaged when tumoroid size is on average 200–300 µm in diameter, approximately every 7 days. Ensure tumoroids are passaged before the average diameter grows larger than approximately 400 µm.
- Banks of tumoroid cells can be generated from initial cell stocks. We recommend plating into non-treated 6-well plates and/or a non-treated T-25 flask at thaw and increasing flask size or using multiple flasks per tumoroid line as cell number increases over the course of passaging.
- Tumoroids can be cryopreserved in Gibco™ Recovery™ Cell Culture Freezing Medium at time of passage. We recommend freezing at 2×10^6 viable dissociated cells in 1 mL of freezing medium in each cryovial.
- Heated bead baths should not be used during tumoroid dissociation and can lead to culture failures. We recommend using water baths or incubators equipped with orbital shakers to maintain the temperature at 37°C during dissociation.
- The suspension protocol requires the addition of 2% volume/volume diluted BME during initial cell seeding. This promotes the formation and stability of 3D tumoroids. Tumoroids are still free floating at this concentration, as it is much lower than the concentration used for embedded cultures. BME is also added at a 1% volume/volume concentration when feeding suspension cultures.
- A more gentle centrifuge speed (200 × *g*) is recommended when recovering cryopreserved cells than when centrifuging cells from ongoing cultures for passaging (400 × *g*).

Observing cultures

- Complete tumoroid culture media may appear yellow when it is first prepared. This is normal and does not indicate any issues with buffering or pH. The yellow color will dissipate somewhat after refrigeration and/or incubation.
- During suspension culture, tumoroids may be visible in multiple focal planes under microscopic visualization.
- We recommend recording images of representative tumoroids immediately before each passage for monitoring line-specific morphologies and troubleshooting, using the EVOS™ XL Core Imaging System or equivalent imaging system.
- Aggregates of diluted BME may be visible in the tumoroid medium when viewing cultures under a microscope. They may appear as fibrous semi-transparent raft-like structures floating in the culture medium and surrounding tumoroids. This is normal and not cause for concern.

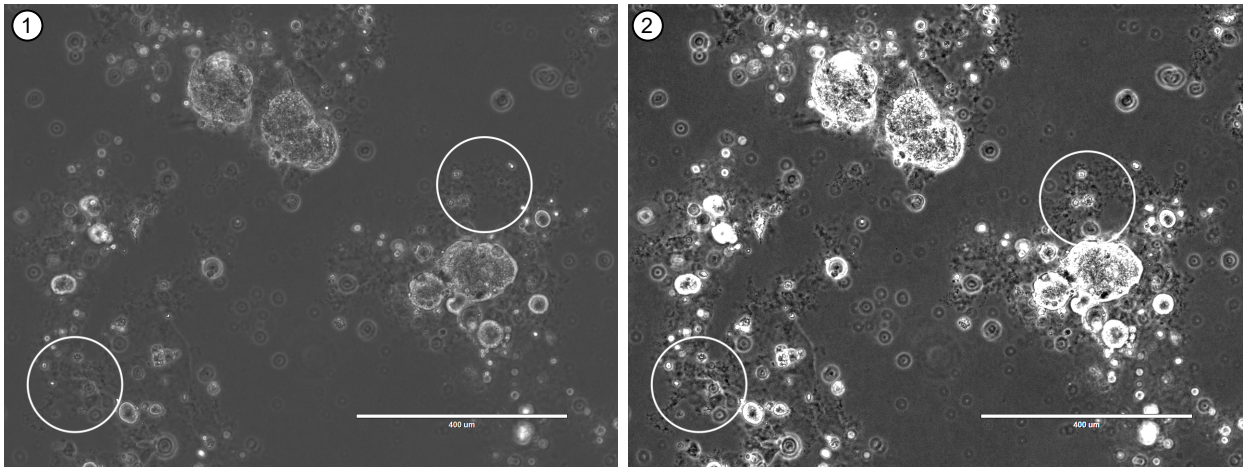

Figure 1 Diluted BME "rafts" in tumoroid culture.

The 2% v/v diluted BME (circled) appears as dark, fibrous, raft-like structures, often surrounding the tumoroids. The same image is shown at low (1) and high (2) brightness and contrast to better demonstrate the appearance of both the BME rafts and the tumoroids.

Guidelines for initiating tumoroid cultures from either cryopreserved or active cultures

The OncoPro™ suspension workflow is compatible with tumoroid cultures maintained in other media systems as well as cultures previously grown in embedded conditions. For more sensitive lines we recommend a stepwise transition to suspension culture in OncoPro™ medium. For best results, we recommend starting with at least 250,000 viable cells during recovery from cryopreservation or transition of cultures between media systems or culture formats. See the following table for additional guidance.

Recommendations for culturing tumoroid lines using OncoPro™ suspension workflow:

Starting cells	Cells previously cultured in	Previous culture format	Start here	Proceed with
Cryopreserved cultures	OncoPro™ medium	Suspension culture	Follow steps in "Recover and seed cryopreserved tumoroid cultures" on page 16. Use OncoPro™ medium.	Proceed with standard suspension workflow in OncoPro™ medium.
		Embedded culture		
	Other media systems	Suspension culture	Follow steps in "Recover and seed cryopreserved tumoroid cultures" on page 16. Use other media systems. Maintain for at least 1 passage with other media system.	
		Embedded culture	• Follow steps in "Recover and seed cryopreserved tumoroid cultures into basement membrane extract domes" on page 28. Use other media systems. • Maintain for at least 1 passage in embedded culture with other media system. • Transition to embedded culture in OncoPro™ medium and maintain for at least 3 passages.	
Active cultures	OncoPro™ medium	Suspension culture	Follow steps in "Passage the suspension tumoroid cultures" on page 20. Use OncoPro™ medium.	
		Embedded culture	Follow steps in "Passage the suspension tumoroid cultures" on page 20. Use OncoPro™ medium.	
	Other media systems	Suspension culture	Follow steps in "Passage the suspension tumoroid cultures" on page 20. Use OncoPro™ medium.	
		Embedded culture	• Follow steps in "Passage the embedded tumoroid cultures" on page 32. Use OncoPro™ medium. • Maintain for at least 3 passages in embedded culture using OncoPro™ medium.	

Before you begin

Prepare reagents

For all tumoroid lines

- Prepare ROCK inhibitor Y-27632
  - Prepare 10 mM solution by reconstituting 10 mg in 3.1225 mL sterile water.
  - Avoid additional freeze-thaw cycles by aliquotting and storing aliquots at -20°C.
- Thaw BME
  - Thaw on ice or at 4°C.
  - Store at 4°C for up to one week, or in working aliquots at -80°C to -20°C for long-term storage.
  - Avoid freeze-thaw cycles.
  - Always keep on ice during use; product will polymerize if not kept at 4°C or on ice.

For lung, head & neck, and pancreatic tumoroid lines

- Prepare Heat Stable FGF-10
  - Prepare 0.1% BSA in DPBS (no calcium, no magnesium) (-/-) by adding 0.2 mL Bovine Albumin Fraction V (7.5% solution) to 14.8 mL DPBS (-/-).
  - Prepare 10 µg/mL Heat Stable FGF-10 stock solution by reconstituting 50 µg lyophilized Heat Stable FGF-10 in 5 mL of 0.1% BSA in DPBS (-/-).
  - Aliquot and store at -20°C.
  - Avoid freeze-thaw cycles.

For pancreatic tumoroid lines

- Prepare gastrin
  - Reconstitute 1 mg in 4.8 mL of DPBS (-/-) to prepare 100 µM stock solution.
  - Aliquot and store at –20°C. Avoid freeze-thaw cycles.

Thaw reagents

- Thaw B-27™ Supplement at room temperature.
  - Mix gently to ensure homogeneity prior to use in complete medium and preparation of aliquots.
  - Prepare single use aliquots and store at -20°C.
  - Avoid additional freeze-thaw cycles.
- Thaw OncoPro™ Supplement (50X) in 37°C water bath until only a small ice crystal remains.
  - Mix gently to ensure homogeneity prior to use in complete medium and preparation of aliquots.
  - Prepare single use aliquots.
  - Flash freeze aliquots overnight at -80°C, and store aliquots for long term storage at -20°C.
  - Avoid additional freeze-thaw cycles.

- Thaw penicillin-streptomycin at room temperature.
  - Mix gently to ensure homogeneity prior to use in complete medium and preparation of aliquots.
  - Prepare single use aliquots and store at -20°C.
  - Avoid additional freeze-thaw cycles.
- Thaw 50 mg/mL Primocin™ at room temperature.
  - Mix gently to ensure homogeneity prior to use in complete medium and preparation of aliquots.
  - Prepare single use aliquots.
  - Store at 4ºC for up to 3-months, or at -20ºC for long-term storage.
  - Avoid additional freeze-thaw cycles.
- Thaw a 10 mM ROCK inhibitor (Y-27632) aliquot at room temperature.
- Thaw additional aliquots of growth factors as recommended for your tumoroid line of interest.

Prepare complete medium

- Prepare appropriate complete medium for your tumoroid line of interest.
- Complete medium may be stored at 4ºC for up to one week. The volumes indicated below are for 50 mL of complete medium. Volumes can be scaled proportionally as needed.

Component	Colorectal	Lung	Head & neck	Pancreatic
	Volume (mL)	Volume (mL)	Volume (mL)	Volume (mL)
OncoPro™ Basal Medium (1X)	43.795	43.745	43.745	43.740
OncoPro™ BSA	3.105	3.105	3.105	3.105
OncoPro™ Supplement (50X)	1	1	1	1
B-27™ Supplement (50X)	1	1	1	1
Penicillin-Streptomycin (10,000 U/mL – 10,000 µg/mL)	1	1	1	1
InvivoGen Primocin™- Antimicrobial agent (50 mg/mL)	0.1	0.1	0.1	0.1
Heat Stable FGF-10 (10 µg/mL)	—	0.05	0.05	0.05
Gastrin (100 µM)	—	—	—	0.005
Total volume (mL)	**50**	**50**	**50**	**50**

Recover and seed cryopreserved tumoroid cultures

The following procedure provides guidance for recovering and seeding cryopreserved tumoroid cultures in suspension. For seeding tumoroid cultures from active cultures please see “Passage the suspension tumoroid cultures” on page 20.

Note: Lower viabilities and growth rates may be observed at the first passage post-thaw. Higher viabilities and more robust tumoroids should be evident by the time of the second passage.

The media volumes in the instructions provided below are sufficient to seed a single T-25 flask. To seed multiple or different size culture vessels, scale the volumes proportionally as needed.

The recommended final volumes post-seed and seeding densities for each type of culture vessel are as follows:

	6-well plate	T-25 flask	T-75 flask
Volume	2 mL per well	5 mL	15 mL
Number of viable cells	2.5×10^5 per well	6.5×10^5 –1×10^6	2×10^6 –3×10^6

Prepare OncoPro™ complete medium + 10 µM Y-27632 (OncoPro + Y)

An aliquoted volume of OncoPro™ complete medium must be supplemented with Y-27632 fresh the day of use.

1. Prepare the appropriate volume of OncoPro + Y per the following table:

   Note: Medium is used during cell recovery from cryopreservation, for cell resuspension and seeding. Volumes listed in the following table account for all medium used in this process. See the previous table for final medium volumes recommended in various culture vessels.

Reagent	Volume		
	Two wells of a 6-well plate	T-25 flask	T-75 flask
OncoPro™ complete medium	25 mL	30 mL	50 mL
10 mM Y-27632	25 µL	30 µL	50 µL

2. Triturate or invert the conical tube 10–20 times to mix, then keep on wet ice in the biosafety cabinet until use.

Prepare the suspension culture vessel with medium

1. Transfer the appropriate volume of OncoPro + Y to each vessel, according to the following table:

	Volume		
Reagent	**Two wells of a 6-well plate**	**T-25 flask**	**T-75 flask**
OncoPro + Y	1 mL per well	4 mL	14 mL

2. Store the prepared vessel in a 37ºC, 5% CO_2 incubator until use.

3. Reserve the remaining OncoPro + Y for preparing and plating the cells.

Prepare the tumoroid cells for seeding into suspension

1. Thaw cells in 37°C water bath until only a small ice crystal remains.

2. Transfer cells from cryovial to 15 mL conical tube.

3. Wash cryovial with 1 mL OncoPro + Y, then slowly drip the wash medium into the 15 mL conical tube containing the cells and mix.

4. Slowly drip additional OncoPro + Y into the 15 mL conical tube containing the cells, swirling the tube as you go, until the total volume reaches 12 mL.

5. Centrifuge cell solution for 5 min at 200 × *g* at 4°C.

6. Use a serological pipette to aspirate the majority of the supernatant, leaving cell pellet.

7. Use a micropipette to aspirate the rest of the supernatant, removing as much media as possible without disturbing the cell pellet.

8. Using a P1000 micropipette, add 1000 µL OncoPro + Y to the cell pellet and triturate 10 times to resuspend.

9. Use a serological pipette to add 4 mL OncoPro + Y to the cells and triturate 10 times to mix.

Count and plate the tumoroid cells

1. If counting cells, proceed to count the number of viable cells according to your method of choice. Alternatively, prepare cells for seeding based on number of tumoroid cells vialed.

2. Depending on the cell count, either dilute the prepared cell solution further with OncoPro + Y to a concentration of 6.5×10^5 viable cells per mL, or centrifuge at 200 × *g*, 4°C for 5 min and resuspend cells in OncoPro + Y at a concentration of 6.5×10^5 viable cells per mL.

3. Retrieve the prepared T-25 flask containing the 4 mL of OncoPro + Y from the incubator.

4. Thoroughly mix cell suspension by inversion, then draw up 1000 µL with a micropipette.

5. Add that 1000 µL cell suspension to the prepared T-25 flask.
6. Gently move the flask back-and-forth several times to ensure the cells are evenly distributed.
7. Drip ice cold BME into the cell solution in the flask to obtain a final concentration of 2% (volume/volume) BME in the medium (e.g. 100 µL BME for 5 mL total media volume).
8. Gently move the flask back-and-forth several times to ensure the cells and BME are evenly distributed.
9. Place flask in a 37°C, 5% CO_2 incubator, then incubate culture for 2–3 days before feeding.

Feed the suspension tumoroid cultures

Tumoroids are fed either every 2 or 3 days. Day 2 and Day 5 are provided as suggested feeding timepoints in the example workflow, however this can be adjusted as needed. It is not recommended to go more than 3 days between feeds. Continue feeding every 2 or 3 days until tumoroids reach the appropriate size for passaging, approximately 200–300 µm in diameter on average. See "Passage the suspension tumoroid cultures" on page 20.

Prepare OncoPro™ complete medium + 10 µM Y-27632 (OncoPro + Y)

Prepare the appropriate volume of OncoPro + Y for your suspension culture format as per the following table. The volumes provided in the instructions below are sufficient to feed a single T-25 flask. For multiple culture vessels, scale volumes proportionally as needed. Please note that an aliquoted volume of OncoPro™ complete medium must be supplemented with Y-27632 fresh on the day of use.

Note: Medium volumes include overage to accommodate for pipetting error. See "Recover and seed cryopreserved tumoroid cultures" on page 16 for recommendations on final medium volume for various cell culture vessels.

Reagent	Volume		
	Two wells of a 6-well plate	T-25 flask	T-75 flask
OncoPro™ complete medium	6 mL	7 mL	17 mL
10 mM Y-27632	6 µL	7 µL	17 µL

1. Combine OncoPro™ complete medium and 10 mM Y-27632 in an appropriately-sized conical tube.
2. Triturate or invert the conical tube 10–20 times to mix, then keep at room temperature until use.

Prepare the suspension culture vessel

Note: For optimal results it is recommended to use a new culture vessel with every feed.

1. Prepare a new culture vessel by transferring the appropriate volume of OncoPro + Y to each well or vessel, according to the following table:

Reagent	Volume		
	Two wells of a 6-well plate	T-25 flask	T-75 flask
OncoPro + Y	1 mL per well	4 mL	14 mL

2. Store the prepared vessel in a 37ºC, 5% CO_2 incubator while preparing cells.

   Reserve the remaining OncoPro + Y for feeding the tumoroids.

Feed the tumoroids

1. Use a serological pipette to transfer all of the cells and medium from the T-25 flask to a 15 mL conical tube.
2. Using a new serological pipette, draw up 5 mL of cold DMEM/F12 + GlutaMAX™-I (1X) and wash the bottom surface of the T-25 flask, then add the wash medium to the 15 mL conical tube.
3. Centrifuge for 5 min at 400 × *g* at 4°C.
4. Use a new serological pipette to aspirate the supernatant without disturbing the cell pellet.
5. Retrieve the prepared T-25 flask containing the 4 mL of OncoPro + Y from the incubator.
6. Using a micropipette, add 1 mL of OncoPro + Y to the 15 mL conical containing the tumoroid cells, triturate ≥10 times to resuspend the cell pellet, then transfer all the cells to the prepared T-25 flask.

   Note: It is important to transfer all of the cell solution into the prepared flask, even if it is more than 1 mL. There should be no cell solution left in the 15 mL conical tube.

7. Gently move the vessel back-and-forth several times to ensure the cells are evenly distributed.
8. Drip ice cold BME into the cell suspension in the culture vessel to add back 1% (volume/volume) BME in the medium (e.g. 50 µL BME for 5 mL total media volume).
9. Gently move the vessel back-and-forth several times to ensure the cells and BME are evenly distributed.
10. Place flask in a 37°C, 5% CO_2 incubator, then incubate culture for 2–3 days.

Passage the suspension tumoroid cultures

Cultures should be passaged when the average tumoroid diameter is approximately 200–300 µm. Day 7 has been provided as a reference point for passaging in the example workflow. However, depending on the tumoroid line, this can range from 4 to 14 days post-seeding. It is not recommended to passage tumoroids that are on average <100 µm or >400 µm in diameter.

The volumes provided in the instructions are sufficient to passage a single T-25 flask. To passage multiple or different size culture vessels, scale the volumes proportionally as needed.

Before you begin

- Thaw StemPro™ Accutase™ Cell Dissociation Reagent at room temperature.
- Any additional StemPro™ Accutase™ Cell Dissociation Reagent not used at the time of passaging should be aliquoted and stored at -20°C for long-term storage. Avoid additional freeze-thaw cycles.
- Thaw 10 mM ROCK inhibitor (Y-27632) aliquot at room temperature.
- Thaw BME on wet ice or at 4°C overnight (if not already thawed).

Prepare OncoPro™ complete medium + 10 µM Y-27632 (OncoPro + Y)

Prepare the appropriate volume of OncoPro + Y for your suspension culture format as per the following table. For multiple culture vessels, scale volumes proportionally as needed.

Note: An aliquoted volume of OncoPro™ complete medium must be supplemented with Y-27632 fresh the day of use.

Reagent	Volume		
	Two wells of a 6-well plate	T-25 flask	T-75 flask
OncoPro™ complete medium	25 mL	30 mL	45 mL
10 mM Y-27632	25 µL	30 µL	45 µL

Note: Media volumes account for volumes required to resuspend cells post-dissociation, prepare new culture vessels, and reseed cells. For recommendations on final medium volume for various cell culture vessels, see “Recover and seed cryopreserved tumoroid cultures” on page 16.

1. Combine OncoPro™ complete medium and 10 mM Y-27632 in an appropriately-sized conical tube.
2. Triturate or invert the conical tube 10–20 times to mix, then keep at room temperature until use.

Prepare the suspension culture vessel

1. Transfer the appropriate volume of OncoPro + Y to each well or vessel, according to the following table:

Reagent	Volume		
	Two wells of a 6-well plate	T-25 flask	T-75 flask
OncoPro + Y	1 mL per well	4 mL	14 mL

2. Store the prepared vessel in a 37ºC, 5% CO_2 incubator until use.

3. Reserve the remaining OncoPro + Y for passaging the tumoroids.

Collect tumoroids from suspension cultures

1. Use a serological pipette to transfer all of the tumoroids and medium from the T-25 flask to a 15 mL conical tube.

2. Using a new serological pipette, draw up 3.5 mL of cold DMEM/F12 + GlutaMAX™-I (1X) and wash the bottom surface of the T-25 flask, then add the wash medium to the 15 mL conical tube.

3. Repeat the previous step with a second wash.

4. Centrifuge for 5 min at 400 × *g* at 4°C.

5. Use a new serological pipette to aspirate the supernatant without disturbing the cell pellet.

6. Use a new serological pipette to add 12 mL of ice cold DPBS (-/-) to the 15 mL conical tube on top of the cell pellet and triturate ≥10 times to fully resuspend the cells.

   Be careful not to overflow the 15 mL conical tube or draw solution into pipet filler.

7. Centrifuge for 5 min at 400 × *g* at 4°C.

Dissociate the tumoroid cells

1. Prepare an appropriate volume of StemPro™ Accutase™ Cell Dissociation Reagent + 10 µM Y-27632 solution (StemPro Accutase + Y) by supplementing StemPro™ Accutase™ Cell Dissociation Reagent 1:1000 with 10 mM Y-27632. 2 mL of StemPro Accutase + Y are needed to passage a T-25 flask. Triturate or invert to mix thoroughly and set aside for use in Step 4.

2. Use a new serological pipette to aspirate the supernatant, without disturbing the cell pellet.

3. Use a micropipette to aspirate the rest of the supernatant, removing as much of the DPBS (-/-) as possible without disturbing the cell pellet.

4. Add 2 mL of StemPro Accutase + Y solution to the conical tube on top of the cell pellet.

5. Use a micropipette set at 800 µL to triturate ≥10 times to fully resuspend the cells.

6. Place cells in water bath at 37°C for 10 minutes. Do not use a bead bath.
7. Every ~2 minutes, swirl tube vigorously in order to resuspend cells in solution.
8. After the 10 minute incubation, use a micropipette set at 800 µL, and triturate the cells in the StemPro Accutase + Y.

Perform cell count and resuspend cells for suspension culture

Seeding densities can be based on either cell count or split ratios. Individual tumoroid lines will exhibit different growth kinetics and will require different seeding densities. If the seeding density for a tumoroid line is unknown, it is recommended to seed based on cell numbers and plate 6.5×10^5 cells per T-25 flask. Cell numbers can be adjusted for future passages as needed, anywhere from 6.5×10^5 to 1×10^6 per T-25 flask. If plating based on split ratios, we recommend starting with a ratio of 1:3 and adjusting as needed from 1:2 to 1:8 depending on individual tumoroid line growth kinetics.

1. If counting cells, aggressively swirl the tube containing the 2 mL cell suspension for ten seconds to mix, then remove an aliquot to perform a cell count.
2. Add 8 mL of OncoPro + Y to the remaining cell suspension in the 15 mL conical tube to dilute the StemPro Accutase + Y.
3. If counting cells, proceed to count the number of viable cells according to your method of choice.
4. When ready to seed, centrifuge the conical tube containing the bulk of your cell solution for 5 min at 400 × *g* at 4°C.
5. Use a new serological pipette to aspirate supernatant, without disturbing the cell pellet.
6. Proceed as appropriate for your workflow:
   - If passaging based on cell count, resuspend cells in OncoPro + Y at a concentration of 6.5×10^5 viable cells per mL. If scaling up, scale seeding density proportionally to the growth area of the culture vessel (e.g. 2×10^6 per T-75 flask).
   - If passaging by split ratio, resuspend cells in volume appropriate for your tumoroid line of interest. For example, for a 1:3 split, resuspend cell pellet in 3 mL of OncoPro + Y.
7. Retrieve the prepared T-25 flask containing 4 mL of OncoPro + Y from the incubator.
8. Thoroughly mix cell suspension by inversion, then draw up 1000 µL with a micropipette.
9. Add that 1000 µL cell suspension to the prepared T-25 flask.
10. Gently move the vessel back-and-forth several times to ensure the cells are evenly distributed.
11. Drip ice cold BME into the cell solution in the culture vessel to obtain a final concentration of 2% (v/v) BME in the medium (100 µL BME for 5 mL total medium volume).

12. Gently move the vessel back-and-forth several times to ensure the cells and basement membrane extract are evenly distributed.

13. Place flask in a 37°C, 5% CO_2 incubator then incubate culture for 2–3 days before feeding.

Harvest suspension tumoroid cultures for downstream studies

To harvest suspension tumoroid cultures for downstream studies, see “Passage the suspension tumoroid cultures” on page 20. Follow the steps and resuspend cells in an appropriate volume of OncoPro + Y. It is recommended that cells are cultured for three or more passages prior to harvesting. As with passaging, the average tumoroid diameter should be approximately 200–300 µm at time of harvest.

Representative images of morphology

Healthy tumoroids can have a variety of morphologies that can range from grape-like to solid to cystic. These morphologies are line-specific and some may be more common for certain cancer indications. It is recommended that you record images of representative tumoroids immediately before each passage, using the EVOS™ XL Core Imaging System or equivalent imaging system to monitor for changes in morphology. These could be indicative of culture drift or cross-contamination with another cell line.

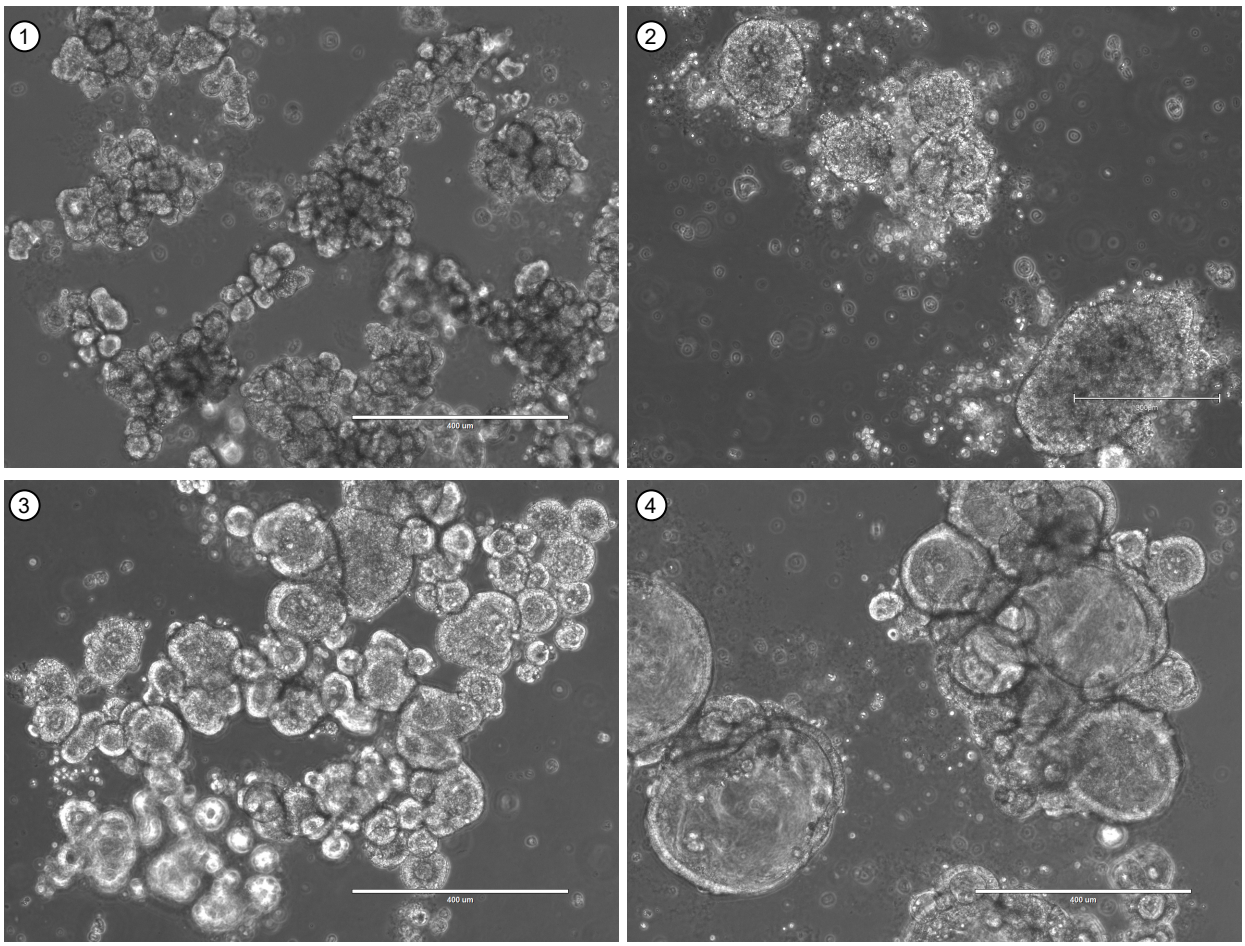

Figure 2 Representative images of tumoroids of various morphologies, imaged immediately prior to passaging.

Tumoroid line morphologies are specific to the patient tissue from which they were derived. Images illustrate examples of (1,2) solid, (3) mixed, and (4) cystic morphologies. Monitor tumoroid cultures closely to ensure line-specific morphologies are being maintained. Imaged with a 10X objective. Scale bars = 400 µm for images 1,3 and 4; scale bar = 300 µm for image 2.

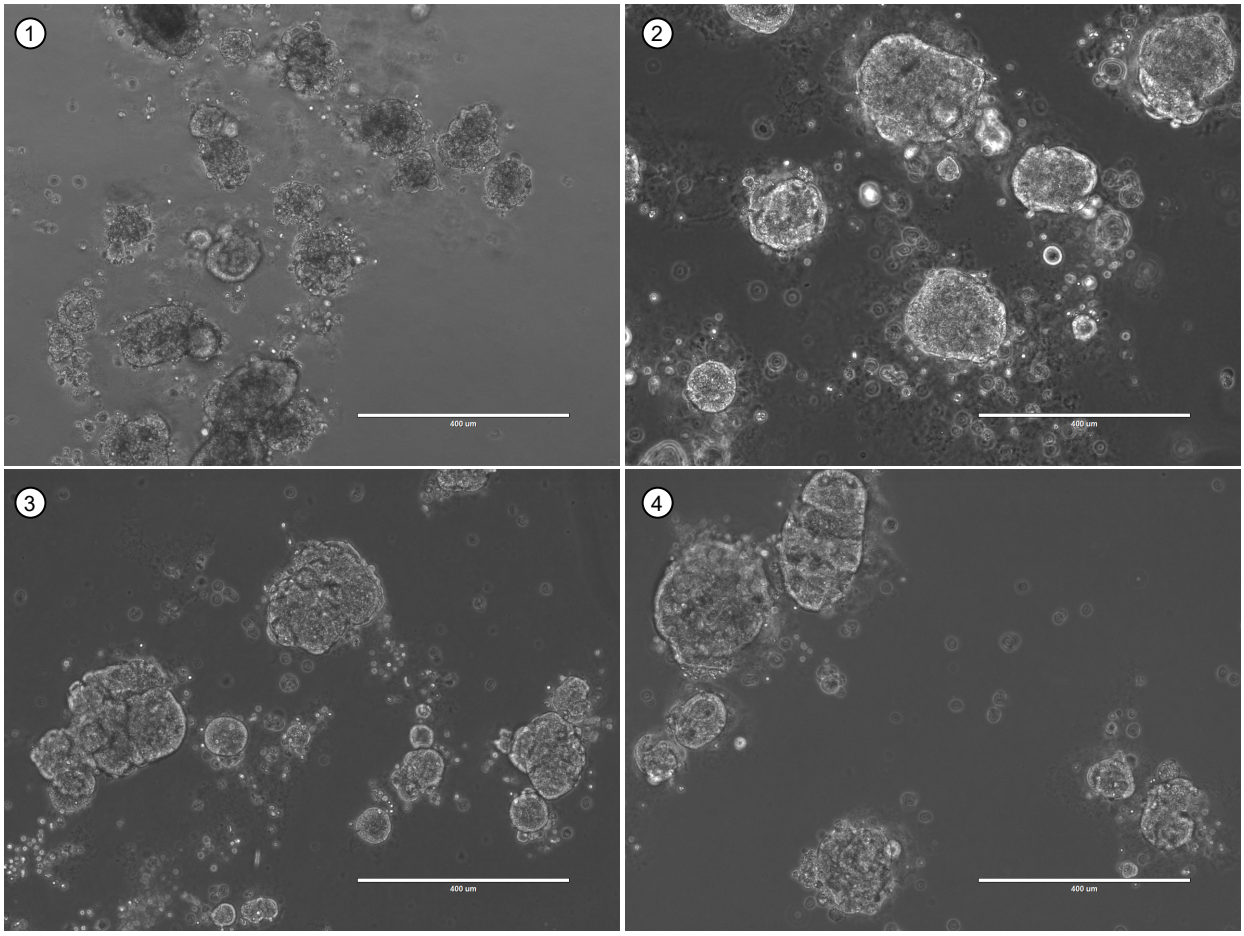

Figure 3 Representative images of tumoroids of various cancer indications, imaged immediately prior to passaging.

The following established tumoroid models were obtained from the NCI Patient-Derived Models Repository (PDMR) and transitioned to suspension culture in OncoPro™ medium: (1) 919269-233-R3-V2-organoid – colorectal (2) K86415-001-R-V1-organoid – lung (3) 549293-155-R-V1-organoid – head & neck (4) 699152-130-R-V1-organoid – pancreatic. Imaged with a 10× objective. Scale bars = 400 µm. We thank the PDMR and their contributing institutions for their contributions to this work.

A

Troubleshooting

Observation	Possible cause	Recommended action
Tumoroids are sticking to the inside of pipette tips or serological pipettes.	Tumoroids are large and/or innately sticky (line-specific).	Before pipetting the cell solution, first pre-wet the inside of the pipette tip or serological pipette by triturating with OncoPro + Y several times to minimize cell loss.
Tumoroids are sticking to the non-treated culture vessel substrate.	Wrong cell culture vessel	Confirm that your cell culture vessel is non-treated.
	Tumoroids are innately sticky (line-specific).	Incubate prepared culture vessel with OncoPro + Y for a longer amount of time before seeding.
Poor dissociation of tumoroids	Tumoroids are innately difficult to dissociate (line-specific).	If poor dissociation is observed after 10 minutes of dissociation, continue to incubate for additional 2 minutes increments for a total incubation time of up to 20 minutes. If poor dissociation is not discovered until after tumoroids have been reseeded, wait 1–3 days before passaging again to further break up the tumoroids.
	Tumoroids are too large.	Continue to dissociate past the recommended 10 minutes in 2 minutes increments up to 20 minutes total.
		If tumoroids are still not dissociated, a 1:1 mix of StemPro™ Accutase™ Cell Dissociation Reagent and TrypLE™ Express Enzyme can be used. Closely monitor minute by minute to minimize risk of over-dissociation.
	There is too much BME present during dissociation.	Keep all buffers and solutions cold on wet ice for passaging workflows. You may also perform an additional wash of the cell solution with cold DMEM/F12 + GlutaMAX™-I (1X) or DPBS (-/-) to further dilute the BME and separate the cells from the BME. Be sure to remove as much of the supernatant as possible without disturbing the cell pellet after the DMEM/F12 + GlutaMAX™-I (1X) or DPBS (-/-) washes.

B Embedded culture workflow

Culturing tumoroids in suspension culture is recommended as this eliminates the manual step of embedding cells in BME domes. As a result, suspension cultures are less labor intensive, more cost efficient, more scalable, and automation compatible when compared to embedded cultures.

However, when adapting tumoroid lines previously cultured as embedded domes in other media systems to OncoPro™ medium it may be beneficial to include a stepwise transition to minimize stress for sensitive lines. Please see "Guidelines for initiating tumoroid cultures from either cryopreserved or active cultures" on page 12 for more details regarding such transitions. Embedded culture conditions may also improve success rates when first deriving new tumoroid lines from patient tissue. In those cases we recommend culturing new lines in embedded conditions for 3–4 passages, or until at least 1×10^6 viable cells have been obtained, before transitioning them to suspension culture. Some users may also choose to expand further prior to transitioning so that cryopreserved banks can be established from the embedded cultures.

Tumoroids can also be maintained long term in suspension or embedded culture conditions in OncoPro™ medium, if desired. We have not found any significant differences in morphology, mutational profiles, or gene expression signatures when comparing tumoroids cultured in parallel in suspension and embedded culture conditions.

Before you begin

- Prepare reagents (e.g. reconstitute and aliquot growth factors) as described in "Before you begin" on page 14.
- Prepare OncoPro™ complete medium for your tumoroid line of interest as for suspension workflow (see "Prepare complete medium" on page 15).
- Thaw 10 mM Y-27632 aliquot at room temperature.
- Thaw BME on ice or overnight at 4°C (if not already thawed).
- Find the protein concentration for your lot of BME (typically found on the Certificate of Analysis). This will be used to calculate the volumes required to resuspend your cells in BME solution wherein the final protein concentration for the cell seeding solution is 10 mg/mL.

Recover and seed cryopreserved tumoroid cultures into basement membrane extract domes

The following procedure provides guidance for recovering and seeding cryopreserved tumoroid cultures in BME domes. For seeding tumoroid cultures from active cultures please see “Passage the embedded tumoroid cultures” on page 32.

Note: Lower viabilities and growth rates may be observed prior to the first passage post-thaw. Higher viabilities and more robust tumoroids should be evident by the time of the second passage.

For embedded culture, cells are resuspended at 1×10^6 cells/mL in a BME solution and plated as 50 µL BME domes (i.e. 5×10^4 cells per dome). The volumes provided in the instructions that follow are sufficient to seed ten 50 µL domes across 3 wells of a 6-well plate (i.e. 5×10^5 cells total). We typically recommend seeding at least three wells of 6-well plate per tumoroid line. The recommended final volumes post-seed and seeding densities for each type of culture vessel are as follows:

	6-well plate	60 mm Petri dish	100 mm Petri dish
Volume of medium	3 mL per well	6 mL	20 mL
Number of domes	3–4	8–12	20–30
Total number of viable cells	1.5×10^5 – 2×10^5 per well	4×10^5 – 6×10^5	1×10^6 – 1.5×10^6

Prepare OncoPro™ complete medium + 10 µM Y-27632 (OncoPro + Y)

Prepare the required volume of OncoPro + Y based on your embedded culture format. The volumes provided in the following instructions are sufficient to seed ten 50 µL basement membrane extract domes seeded into three wells of a 6-well plate. To seed multiple or different size culture vessels, scale the volumes proportionally as needed. Volumes account for medium use during cell recovery from cryopreservation and for cell resuspension, seeding, and medium overlay. See prior table for recommendations on final medium volume used in various culture vessels.

Note: An aliquoted volume of OncoPro™ complete medium must be supplemented with Y-27632 fresh the day of use.

1. Prepare the appropriate volume of OncoPro + Y per the following table:

Reagent	Volume		
	Three wells of a 6-well plate	60 mm petri dish	100 mm petri dish
OncoPro™ complete medium	25 mL	35 mL	45 mL
10 mM Y-27632	25 µL	35 µL	45 µL

2. Triturate or invert the conical tube 10–20 times to mix, then keep on wet ice in the biosafety cabinet until use. Prepared medium should be kept cold until instructed otherwise to avoid premature polymerization of BME.

Prepare the tumoroid cells for seeding into basement membrane extract domes

1. Thaw cells in 37°C water bath until only a small ice crystal remains.
2. Transfer cells from the cryovial to a 15 mL conical tube.
3. Wash cryovial with 1 mL OncoPro + Y, then slowly drip the wash medium into the 15 mL conical tube containing the cells and mix.
4. Slowly drip additional OncoPro + Y into the 15 mL conical tube containing the cells, swirling as you go, until the total volume reaches 12 mL.
5. Centrifuge for 5 min at 200 × *g* at 4°C.
6. Use a serological pipette to aspirate the majority of supernatant, leaving the cell pellet.
7. Use a micropipette to aspirate the rest of the supernatant, removing as much of the medium as possible without disturbing the cell pellet.
8. Use a P1000 pipette to add 1000 µL OncoPro + Y to the cell pellet and triturate 10 times to resuspend.
9. Use a serological pipette to add 4 mL OncoPro + Y to the cells and triturate 10 times to mix.

Embed the tumoroid cells in basement membrane extract domes

1. If counting cells, proceed to count the number of viable cells according to your method of choice. Alternately, prepare cells for seeding according to a split ratio (e.g. 1:3) or number of tumoroid cells vialed.

2. Depending on the cell count, dilute the cell solution or centrifuge and resuspend cells at a concentration of 5×10^5 viable cells per mL in OncoPro + Y.

For seeding, prepare BME solution in OncoPro + Y containing 1×10^6 cells/mL

1. First determine the number of BME domes required for the number of viable cells you would like to seed. For example, since each dome contains 5×10^4 cells per dome, you would require 10 domes to seed 5×10^5 cells.

2. Calculate the volume of the 5×10^5 cells/mL cell suspension prepared above that will be required to obtain the desired number of cells.

   If you are seeding 10 domes (i.e. 3 wells of a 6-well plate) you will require 1000 µL of the 5×10^5 cells/mL cell suspension. This is your "volume of cell suspension".

3. Calculate the volume of BME/cell suspension that will be required to seed the desired number of domes. Each dome is 50 µL, therefore 500 µL of BME/cell suspension would be needed to seed 10 domes. This is your “total volume of BME/cell suspension”.

4. Look up the stock concentration of your lot of BME (you must use a lot with a protein concentration >10 mg/mL). Calculate the volume of stock BME needed to obtain a total volume of BME/cell suspension at a final concentration of 10 mg/mL BME. This is your "volume of stock BME".

5. Calculate the volume of OncoPro + Y that will be needed to bring the volume of stock BME up to the total volume of BME/cell suspension. This is your "volume of OncoPro + Y".

   Volume of OncoPro + Y = Total volume of BME/cell suspension - volume of stock BME

Prepare the BME cell suspension

1. Aliquot the appropriate “volume of cell suspension” into a new 15 mL conical tube (e.g. 1 mL for 10 domes).

2. Centrifuge for 5 min at 200 × *g* at 4°C.

3. Use a serological pipette and/or micropipette to aspirate the supernatant, removing as much of the medium as possible.

4. Move the 15 mL conical tube to wet ice and resuspend the cells in the appropriate "volume of OncoPro + Y".

5. Still working on wet ice, add the appropriate “volume of stock BME”. Triturate to mix cell solution well without making bubbles. You should now have a 10 mg/mL BME solution in OncoPro + Y containing 1×10^6 cells/mL.

6. Use a P1000 pipette to pipet 50 µL drops of cell/BME solution onto a 6-well plate, with 3–4 drops per well.
7. Invert the well plate to form hanging drops of cell/BME solution and incubate at 37°C, 5% CO_2 upside-down for 30 minutes to allow BME to polymerize.
8. During the 30 minutes, allow the OncoPro + Y to come up to room temperature for overlaying the polymerized domes.
9. After 30 minutes, invert the plate back to its normal orientation and carefully overlay domes with 3 mL per well of OncoPro + Y.
10. Add 3 mL sterile distilled water or sterile DPBS (-/-) to the remaining wells that do not contain cells in order to maintain humidity within the plate.
11. Place plate in a 37°C, 5% CO_2, then incubate culture for 2–3 days.

Feed the embedded tumoroid cultures

Tumoroids can be fed either every 2 or 3 days. Day 2 and Day 5 are provided as suggested feeding timepoints in the example workflow. However, this can be adjusted as needed. We do not recommend going more than 3 days in between feeds. Continue feeding every 2 or 3 days until tumoroids reach the appropriate size for passaging, approximately 200–300 µm on average (See “Passage the embedded tumoroid cultures” on page 32).

The volumes provided in the following instructions are sufficient to feed three wells of a 6-well plate. To feed multiple or different size culture vessels, scale the volumes proportionally based on the size and number of vessels.

Prepare OncoPro™ complete medium + 10 µM Y-27632 (OncoPro + Y)

Prepare the appropriate volume of OncoPro + Y for your embedded culture format as per the following table.

Note: An aliquoted volume of OncoPro™ complete medium must be supplemented with Y-27632 fresh the day of use.

Reagent	Volume		
	Three wells of a 6-well plate	60 mm petri dish	100 mm petri dish
OncoPro™ complete medium	10 mL	33 mL	41 mL
10 mM Y-27632	10 µL	33 µL	41 µL

Note: Medium volumes include overage to accommodate for pipetting error. See “Recover and seed cryopreserved tumoroid cultures into basement membrane extract domes” on page 28, for recommendations on final medium volume for various cell culture vessels.

1. Combine OncoPro + Y in an appropriately-sized conical tube.

2. Triturate or invert the conical tube 10–20 times to mix.

IMPORTANT! Use room temperature medium for feeding cells so as to not affect the integrity of the BME domes.

Feed the tumoroids

1. Aspirate the medium from the well containing embedded tumoroids; be careful not to break up domes during media aspiration.

2. Add 3 mL/well OncoPro + Y to the side of each well; be careful not to disrupt domes when adding the medium.

3. Place plate in a 37°C, 5% CO_2 incubator then incubate culture for 2–3 days.

Passage the embedded tumoroid cultures

Cultures should be passaged when the average tumoroid diameter is approximately 200–300 µm. Day 7 has been provided as a reference point for passaging in the example workflow. However, depending on the tumoroid line, this can range from 4 to 14 days post seeding. It is not recommended to passage cells that are on average <100 µm or >400 µm in diameter.

The volumes provided in the following instructions are sufficient to passage ten 50 µL BME domes seeded into three wells of a 6-well plate. To passage multiple or different size culture vessels, scale the volumes proportionally as needed.

Before you begin

- Thaw StemPro™ Accutase™ Cell Dissociation Reagent at room temperature.
- Any additional StemPro™ Accutase™ Cell Dissociation Reagent not used at the time of passaging should be aliquoted and stored at -20°C for long-term storage. Avoid additional freeze-thaw cycles.
- Thaw 10 mM ROCK inhibitor (Y-27632) aliquot at room temperature.
- Thaw BME on wet ice or overnight at 4°C (if not already thawed).

Prepare OncoPro™ complete medium + 10 µM Y-27632 (OncoPro + Y)

Prepare the appropriate volume of OncoPro + Y for your embedded culture format as per the following table:

Note: An aliquoted volume of OncoPro™ complete medium must be supplemented with Y-27632 fresh the day of use.

Reagent	Volume		
	Three wells of a 6-well plate	60 mm petri dish	100 mm petri dish
OncoPro™ complete medium	25 mL	35 mL	45 mL
10 mM Y-27632	25 µL	35 µL	45 µL

Note: Medium volumes account for material required to resuspend cells post-dissociation, reseed cells, and add medium overlay. See page "Recover and seed cryopreserved tumoroid cultures into basement membrane extract domes" on page 28 for recommendations on final medium volume for various cell culture vessels.

1. Combine OncoPro™ complete medium and 10 mM Y-27632 in an appropriately-sized conical tube.
2. Triturate or invert the conical tube 10–20 times to mix, then keep on wet ice in the biosafety cabinet until use. Prepared medium should be kept cold to avoid premature polymerization of BME.

Collect tumoroids from embedded cultures

1. Aspirate medium from wells containing embedded tumoroids. Be careful not to break up domes during media aspiration.
2. Add 1 mL of cold DMEM/F12 + GlutaMAX™-I (1X) to each of the wells containing BME domes.
3. Use a micropipette to draw up 1 mL of the cold DMEM/F12 + GlutaMAX™-I (1X) in one well and triturate repeatedly over the domes to break them up.
4. Transfer the 1 mL of DMEM/F12 + GlutaMAX™-I (1X) and cells to a 15 mL conical tube.
5. Use a micropipette to draw up a fresh 1 mL of cold DMEM/F12 + GlutaMAX™-I (1X) and wash the well from which you transferred the cell solution, then transfer the wash to the 15 mL conical tube containing the cells.
6. Repeat the wash step with an additional 1 mL of cold DMEM/F12 + GlutaMAX™-I (1X).
7. Repeat breaking up the domes, transferring the cells, and wash steps for all other wells.
8. Centrifuge the 15 mL conical for 5 minutes at 400 × *g*, 4°C.
9. Use a serological pipette to aspirate the supernatant; leave the cell and BME layers at the bottom of the conical tube undisturbed.

10. Use a new serological pipette to add 12 mL cold DPBS (-/-) on top of the cell pellet and triturate 10 times to fully resuspend the cells.

    Be careful not to overflow the 15 mL conical tube or draw solution into pipet filler.

11. Centrifuge the 15 mL conical for 5 minutes at 400 × *g*, 4°C.

Dissociate the tumoroid cells

1. Prepare an appropriate volume of StemPro™ Accutase™ Cell Dissociation Reagent + 10 µM Y-27632 solution (StemPro Accutase + Y) by supplementing StemPro™ Accutase™ Cell Dissociation Reagent 1:1000 with 10 mM Y-27632. For example, 2 mL of StemPro Accutase + Y are needed to passage a 10 domes. Triturate or invert to mix thoroughly and set aside for use in Step 4.

2. Using a serological pipette, aspirate the supernatant, leaving the cell and BME layers at the bottom of the conical tube undisturbed.

3. Use micropipette to aspirate the rest of the supernatant, removing as much of the DPBS (-/-) and BME as possible without disturbing the cell pellet.

4. Add 2 mL of freshly prepared StemPro Accutase + Y to the conical tube on top of cell pellet.

5. Use a micropipette set at 800 µL to triturate ≥10 times to fully resuspend the cells.

6. Place cells in water bath at 37°C for 10 minutes. Every ~2 minutes, swirl tube vigorously in order to resuspend cells in solution. Do not use a bead bath.

7. After the 10 minute incubation, use a micropipette set at 800 µL to triturate the cells in the StemPro Accutase + Y solution ≥20 times.

Perform cell count and resuspend cells for embedded culture

Seeding densities can be based on either cell count or split ratios. It is recommended to seed based on cell numbers and to plate 1.5 × 10^5 cells (3 domes) per one well of a 6-well plate. If plating based on split ratios, we recommend starting with a ratio of 1:3 based on the previous number of domes seeded and adjusting as needed from 1:2 to 1:8 depending on individual tumoroid line growth kinetics.

1. If counting cells, aggressively swirl the tube containing the 2 mL cell suspension for ten seconds to mix, then remove an aliquot to perform a cell count.

2. Add 8 mL of OncoPro + Y to the remaining cell suspension in the 15 mL conical tube to dilute the StemPro Accutase + Y.

Resuspend cells in OncoPro + Y

1. If counting cells, proceed to count the number of viable cells according to your method of choice. Alternately, prepare cells for reseeding according to a split ratio (e.g. 1:3) or number of tumoroid cells vialed.

2. Centrifuge the cell solution at 400 × *g*, 4°C for 5 minutes, then resuspend the cells at a concentration of 5×10^5 viable cells per mL in OncoPro + Y.

Prepare 10 mg/mL BME solution in OncoPro + Y

For seeding, prepare 10 mg/mL BME solution in OncoPro + Y containing 1×10^6 cells/mL

1. First determine the number of BME domes required for the number of viable cells you would like to seed. For example, since each dome contains 5×10^4 cells per dome, you would require 10 domes to seed 5×10^5 cells.

2. Calculate the volume of the 5×10^5 cells/mL cell suspension prepared above that will be required to obtain the desired number of cells. If you are seeding 10 domes (i.e. 3 wells of a 6-well plate) you will require 1000 µL of the 5×10^5 cells/mL cell suspension. This is your "volume of cell suspension".

3. Calculate the volume of BME/cell suspension that will be required to seed the desired number of domes. Each dome is 50 uL therefore 500 µL of BME/cell suspension would be needed to seed 10 domes. This is your “total volume of BME/cell suspension”.

4. Look up the stock concentration of your lot of BME (you must use a lot with a protein concentration >10 mg/mL). Calculate the volume of stock BME needed to obtain a total volume of BME/cell suspension at a final concentration of 10 mg/ml BME. This is your "volume of stock BME".

5. Calculate the volume of OncoPro + Y that will be needed to bring the volume of stock BME up to the total volume of BME/cell suspension. This is your “volume of OncoPro + Y”.

   Volume of OncoPro + Y = Total volume of BME/cell suspension - volume of stock BME

Prepare the cell/BME suspension

1. Aliquot the appropriate “volume of cell suspension” into a new 15 mL conical tube (e.g. 1 mL for 10 domes).

2. Centrifuge for 5 min at 400 × *g* at 4°C.

3. Use a serological pipette and/or micropipette to aspirate the supernatant, removing as much of the medium as possible.

4. Move the 15 mL conical tube to wet ice and resuspend the cells in the appropriate “volume of OncoPro + Y”.

5. Still working on wet ice, add the appropriate “volume of stock BME”. Triturate to mix cell solution well without making bubbles You should now have a 10 mg/mL BME solution in OncoPro + Y containing 1×10^6 cells/mL.

6. Use a P1000 pipette to pipet 50 µL drops of cell/BME solution onto a 6-well plate, with 3–4 drops per well.

7. Invert the well plate to form hanging drops of cell/BME solution and incubate at 37°C, 5% CO_2 upside-down for 30 minutes to allow BME to polymerize.

8. During the 30 minutes, allow the OncoPro + Y to come up to room temperature for overlaying the polymerized domes.

9. After 30 minutes, invert the plate back to its normal orientation and carefully overlay domes with 3 mL per well of OncoPro + Y.

10. Add 3 mL sterile distilled water or sterile DPBS (-/-) to the remaining wells that do not contain cells in order to maintain humidity within the plate.

11. Place plate in a 37°C, 5% CO_2 incubate culture for 2–3 days.

Harvest embedded tumoroid cultures for downstream studies

To harvest embedded tumoroid cultures for downstream studies, see steps in “Passage the embedded tumoroid cultures” on page 32 and resuspend cells in an appropriate volume of OncoPro + Y. It is recommended that cells are cultured for three or more passages prior to harvesting. As with passaging, the average tumoroid diameter should be approximately 200–300 µm at time of harvest.

C

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

D Documentation and support

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