Title:	**WiCell Embryonal Carcinoma Cell Line GCT27C4 Protocols**
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1. Preface

This booklet of protocols is intended to serve as a primer for culturing GCT27C4, an embryonal carcinoma (EC) cell line that requires no feeder layer or extracellular matrix. These protocols are representative of how the cells were cultured and banked. WiCell recommends that GCT27C4 should be first thawed and established in the conditions in which they were initially frozen prior to transfer to alternate culture platforms. We recommend that you read through these protocols prior to thawing your cells, and follow them until you have established your own cell bank of frozen vials. As you thaw and expand your initial cell vials, we strongly encourage you to establish your own bank of frozen vials as soon as possible. Once the culture is established, freeze back a portion of the material, and freeze again when you have enough cells for 20 vials. This will ensure you have an adequate stock of material to expand from as you conduct experiments.

Because this bank will be the basis of all future work with this cell line, WiCell recommends that it be screened for STR post thaw to ensure that the identity of the banked material is as expected. This and other tests are available from WiCell's full-service Characterization Laboratory; to learn more about WiCell Characterization's offerings and how to submit samples visit www.wicell.org/characterization.

If you have any additional questions, please contact us through technical support on the WiCell website at www.wicell.org. © 2022 WiCell ®

NOTE: Culture protocols for alternate embryonal carcinoma (EC) cell lines, such as GCT27DC1, are very different from protocols detailed here and must be adhered to for success.

2. Protocols

Section 1: Thawing Embryonal Carcinoma Cell Line GCT27C4

Note: Before thawing, check the certificate of analysis included in the shipping packet insert to acquire the recommended number of wells one vial should be thawed into (this can vary among different lots). In general, ROCK inhibitor is not required for most cell lines. Please consult the Certificate of Analysis to see if ROCK inhibitor is recommended. If ROCK inhibitor is not recommended, it will not be mentioned on the Certificate of Analysis.

Required Equipment

1. Biosafety cabinet
2. 37°C/5% CO_2 incubator
3. 37°C water bath
4. Centrifuge
5. Microscope

Required Supplies

1. Forceps
2. Cryogenic handling gloves and eye protection
3. 5ml and/or 10ml sterile serological pipettes (Fisher, 13-678-27E, 13-67827F) or equivalent
4. 95% Ethanol
5. 15ml conical tubes (Corning, 430052)
6. 250ml Nalgene filter (FisherScientific, 09-741-04)
7. 6-well plates (Falcon, 08-772-1B or Nunc, 140675) [surface area of 1 well = 9.6cm ^{2}]

Required Reagents

1. Fetal bovine serum (FBS) (Peak Serum, PS-FB1) or equivalent, not heat-inactivated
2. GlutaMAX supplement (ThermoFisher, 35050061)
3. Penicillin-Streptomycin (ThermoFisher, 15140-122) or equivalent, optional
4. DMEM (liquid) (ThermoFisher, 11965092) or equivalent
5. **Embryonal Carcinoma (EC) Culture Medium** (may also be referred to as EC culture medium or CS medium) (250ml)
    1. EC Culture medium is also referenced in Sections 2, 3 and 4.
    2. To make EC Culture Medium combine following components, filter sterilize, store at 4ºC for up to 14 days. Scale up or down to suit your needs.
    3. 225ml DMEM
    4. 25ml FBS, not heat-inactivated
    5. 2.5ml GlutaMAX
    6. 2.5ml Penicillin-Streptomycin, optional

Remove Vial and Thaw

1. Prior to removing vial from storage, acquire the thaw recommendation (number of wells one vial should be thawed into) found in the certificate of analysis included in the shipping packet insert.
2. Wear eye protection as vials stored in liquid nitrogen may accidentally explode when warmed.
3. Wear ultra-low temperature cryo gloves. Remove the cell vial from the liquid nitrogen storage tank using forceps.
4. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
5. When only a small ice crystal remains, remove the vial from the water bath.
6. Ensure the vial cap is tightly closed and immerse the vial into a 95% ethanol bath to sterilize the outside of the tube. Briefly (15-30 seconds) air-dry the vial in the sterile biosafety cabinet.

Removal of Cryoprotectant and Re-suspension of Pluripotent EC Cells

1. Transfer the cells gently into a sterile 15ml conical tube using a 1ml or 5ml glass pipette. Do not allow cells to flow down the side of the tube, as this will cause shearing and reduce attachment.
2. Slowly, add 10ml of warmed EC culture medium drop-wise to cells in the conical tube. While adding the medium, gently move the tube back and forth to mix the pluripotent stem cells. This reduces osmotic shock to the cells.
3. Centrifuge the cells at 200 x g for 5 minutes.
4. Aspirate and discard the supernatant with a sterilized Pasteur Pipette.
5. Re-suspend the cell pellet in 2ml EC culture medium for every well that will receive cells (number of wells receiving cells is based on the thaw recommendation found in the certificate of analysis which is included in the shipping packet insert). For example: When the thaw recommendation is to thaw 1 vial into 1 well, re-suspend the pellet in 2ml.
6. Gently pipette cells up and down in the tube a few times.

Plate EC Cells

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1. Label a 6-well plate with the passage number from the vial, the date and your initials. No treatment or cell culture treated plates is required.
2. Slowly add the 2ml cell suspension into the well(s).
3. Place plate into the incubator and gently move the plate back and forth and side to side to evenly distribute the cells—avoid circular motions to prevent pooling in the center of the well.
4. The next day, remove the spent medium and gently add 2ml/well of EC culture medium.
5. Feed every other day until ready to passage or freeze.

Section 2: Feeding Embryonal Carcinoma Cell Line GCT27C4

Equipment

1. Biosafety cabinet
2. 37°C/5% CO_2 incubator
3. Microscope

Required Supplies

1. 5ml and/or 10ml sterile serological pipettes (Fisher: 13-678-27E, 13-67827F) or equivalent

Required Reagents

1. EC Culture Medium as in section 1.

Feeding EC Cells

1. Observe the EC cells using a microscope. If they require passaging, follow the passaging protocol below.
2. Warm enough medium to feed 2ml for each well that will be fed.
3. Aspirate the spent medium with a sterilized Pasteur pipette. If feeding more than one plate, use a different pipette for each plate to reduce risk of contamination.
4. Add 2ml/well. After pipettes are used once, they must be disposed to reduce the contamination potential. Do not reinsert a used pipette into sterile medium for any reason.
5. Return the cells to the 37°C incubator.
6. Feed every other day until ready to passage or freeze.

Section 3: Passaging Embryonal Carcinoma Cell Line GCT27C4

Required Equipment

1. Biosafety cabinet
2. 37°C/5% CO_2 incubator
3. centrifuge
4. 37°C water bath

Required Supplies

1. 5ml and/or 10ml sterile serological pipettes (Fisher, 13-678-27E, 13-67827F), or equivalent
2. 15ml conical tubes (Corning, 430052)
3. 6-well plate with inactivated MEF cells (3-6 wells of MEFs)

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4. 50ml Nalgene filter (Fisher Scientific, 09-741-88)
5. 6-well plates (Falcon, 08-772-1B or Nunc, 140675) [surface area of 1 well = 9.6cm2]

Required Reagents

1. Embryonal Carcinoma (EC) Culture Medium, as seen in section 1.
2. DPBS without Calcium and Magnesium (PBS) (Thermo Fisher, 14-190-144), or equivalent
3. 0.05% Trypsin-EDTA (Thermo Fisher, 25300-054)

Passaging Cells

1. Determine split ratio.
    1. The split ratio is variable, though generally between 1:10 and 1:20. Occasionally cells will grow at a different rate and the split ratio will need to be adjusted.
    2. A general rule is to observe the last split ratio and adjust the ratio according to the appearance and density of the cells. If the cells look healthy and colonies have enough space, split using the same ratio; if they are overly dense and crowding, increase the ratio; and if the cells are sparse, decrease the ratio.
2. Aspirate EC culture medium from wells that will be passaged forward. At least one well of cells should be fed separately and left unpassaged to be used as a backup to protect against problems with the split that would otherwise jeopardize the culture (contamination, etc.).
3. Rinse each well with 1ml of room temperature PBS to rinse away the EC culture medium.
4. Aspirate the PBS and add 1ml/well room temperature trypsin.
5. Place in incubator for 5mins or until cells detach from plate with agitation.
6. Add 2-3ml/well of EC culture medium to de-activate the trypsin.
7. Use a serological pipet to break up the clumps further by gently pipetting up and down 2-3 times. You may move between wells with the same medium to remove cells. Do not work with more than 3ml/well (overfilling wells may lead to contamination).
8. After the cells are removed from the surface of the well, pool the contents of the scraped wells into a sterile conical tube.
9. Centrifuge for 5 minutes at 200 x g.
10. While spinning, prepare the new plates to receive the newly split cells.
    1. No treatment or matrix is needed of the 6-well plates.
    2. Add 1.5-2ml per well of EC culture medium.
    3. Label plate appropriately (WiCell recommends at minimum the cell line name, passage number on the vial, date and technician initials), set aside in the Biosafety cabinet.
11. Aspirate supernatant and suspend pellet in 2-3ml EC culture medium for every well that was harvested.
12. Calculate the volume of cells to add to each new well by dividing total volume of cell suspension by the number of possible wells based on the split ratio.

    EXAMPLE 1: 1 well collected and suspended in a total of 3ml at a split ratio of 1:20
    → 3ml ÷ 20 wells = 0.150ml/well = 150ul cells per well.

    EXAMPLE 2: 4 wells collected and suspended in a total of 6ml at a split ratio of 1:15 → 6ml ÷ 60 wells = 0.1ml/well = 100ul cells per well.

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13. Gently re-suspend the cells using a 5ml pipette.
14. Add calculated volume of cell suspension to each well of the prepared plate.
15. Place plate gently into the incubator and gently move the plate back and forth and side to side to evenly distribute the cells—avoid circular motions to prevent cells from pooling in the center of the well.
16. **Note:** While cells are attaching, try to limit opening and closing the incubator doors, and if you need to access the incubator, open and close the doors carefully. This will prevent disturbing the even distribution of cells to the surface of the well.
17. Incubate cells overnight to allow cells to attach.
18. If passage was successful and free of contamination, the backup well can be discarded the day following passage.
19. Feed 2ml/well EC culture medium every other day until culture is ready to passage or freeze.

Section 4: Freezing Embryonal Carcinoma Cell Line GCT27C4

Required Equipment:

1. Biosafety cabinet
2. 37°C/5% CO_2 incubator
3. centrifuge

Required Supplies:

1. 5ml and/or 10ml sterile serological pipettes (Fisher, 13-678-27E, 13-67827F), or equivalent
2. 15ml conical tubes (Corning, 430052)
3. Isopropanol freezing containers, and isopropanol
4. Ice bucket and ice
5. Cryovials (Fisher Scientific, 03-337-7Y) or equivalent
6. Cryovial rack
7. Cryogenic handling gloves and eye protection
8. Metal forceps
9. Embryonal Carcinoma (EC) Culture Medium, as seen in section 1.
10. DPBS without Calcium and Magnesium (PBS) (Thermo Fisher, 14-190-144), or equivalent
11. 0.05% Trypsin-EDTA (Thermo Fisher, 25300-054)
12. Dimethyl Sulfoxide (DMSO) 10ml ampoules (Sigma Aldrich, D2438), or equivalent
13. Cells are to be frozen at 1-2.5 million cells per vial, with a final total volume of 1ml/vial.
14. Label cryovials with the cell line, passage number (increase the passage number on the plate by 1 to label the vial so that the passage number on the vial is reflective of the passage number at thaw), the freeze date, and your initials. Use an alcohol proof pen or labels that resist liquid nitrogen and ethanol. Place in biosafety cabinet.
15. Sterilize the biosafety cabinet (with the labeled vials in it) for 20 minutes with UV light. Turn on the blower and open the sash. Spray down the whole surface with ethanol and allow it to evaporate for 20 minutes prior to initiating cryopreservation.

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16. Obtain a recharged, room-temperature isopropanol freezing container. The isopropanol must be replaced every 5 uses.

Harvest, Count and Freeze Cells

1. Aspirate EC culture medium from wells that will be frozen.
2. Rinse each well with 1ml of room temperature PBS to rinse away the EC culture medium.
3. Aspirate the PBS and add 1ml/well room temperature trypsin.
4. Place in incubator for 5mins or until cells detach from plate with agitation.
5. Add 2-3ml/well of EC culture medium to de-activate the trypsin.
6. Use a serological pipet to break up the clumps further by gently pipetting up and down 2-3 times. You may move between wells with the same medium to remove cells. Do not work with more than 3ml/well (overfilling wells may lead to contamination).
7. After the cells are removed from the surface of the well, pool the contents of the harvested wells into a sterile conical tube.
8. Repeat harvest for any remaining plates. Continue to pool all cells into the same conical tube to create a uniform lot.
9. Count the cells (excluding dead cells with trypan blue) using a hemacytometer or cell counter.
10. Multiply cell count by volume of remaining cell pool to determine total number of cells available to freeze.
11. Spin cells down for 5 minutes at 200 x g.
12. Aspirate supernatant and suspend with enough EC culture medium to create a cell suspension of about 2 million cells/ml.
13. Use the same 5ml or 10ml serological pipet to very gently pipet up and down to evenly mix the cell suspension.
14. Put cells on ice.
15. Freeze cells by working in batches of 18 vials (or fewer) because 18 vials is the capacity of one isopropanol freezing container.
16. Use a 10ml serological pipet to very gently pipet up and down to evenly mix the cell suspension. Transfer 16.2ml of cells to a new conical tube (0.9ml of cells per vial when 18 vials are being frozen).
17. Calculate the required volume of DMSO, which is 0.1ml/vial (10% of final volume). See Table 1 for DMSO calculation examples.

Number of Vials	ml of DMSO to use (0.1ml/vial)
6	0.6
12	1.2
18	1.8

Table 1.

18. While slowly and gently turning/mixing the conical tube, slowly add calculated DMSO volume down the side of the tube drop by drop.

19. Once all DMSO has been added, use the same 5ml or 10ml serological pipet to very gently pipet up and down to evenly mix the cell suspension.
20. With the same pipette, distribute 1ml of cell suspension to each prepared vial. Mix cell pool every 6-10 vials for even distribution.
21. Ensure all vial caps are tightened and quickly place cryovials into an isopropanol containing freezing container. Place the freezing containers in the -80°C freezer overnight.
22. Repeat freezing in batches until all cells are frozen.
23. Transfer cell vials to liquid nitrogen storage the following day.

3. Version History

Version	Version History	Effective Date
A	CC01011, Document Initiation.	25Feb20
1.0	CC-333, Transitioned SOP-SH-017 to Qualio as SH-17.	19Aug20
2.0	CC-408, updated formatting/numbering of sections.	21Oct20
3.0	CC-1067, Added that FBS should not be heat-inactivated. Added that Pen/Strep is optional.	See Qualio or Controlled Copy

4. Attachments

No attachments.