Wood Breast Cell Model (CB-0401)

Passaging Protocol

A. Materials

Product
500 mL RETM basal medium (CM-0001-A1)
15 mL RETM supplement (15 mL, thawed briefly in a 37°C water bath)(CM-0001-P1)
125 µL Cholera Toxin (100 µg/mL stock solution in cell culture grade water)
25 mL heat-inactivated Fetal Bovine Serum (FBS)(FBS-01-0100)
Tryple Express
Quench solution: 20% FBS, 80% DMEM/F12
Pen/strep (if desired)
(1X) Hank's Balanced Salt Solution (HBSS)

B. Culture Conditions

1. Incubation in a multigas humidified 37°C incubator with 5% CO_2 in ambient air
2. Use of standard tissue culture-treated T-25 vent-cap flasks
3. Using a plating density of 300,000 cells/25 cm^2 should be used when passaging the cells. For initiating a culture from a frozen vial, the Cell Growth Data (Table 1) for the recommended plating density.
4. Use of Renaissance Essential Tumor Medium (RETM) containing 5% heat-inactivated FBS
5. Recommended serum and Cholera Toxin
6. Dissociating cells in Tryple Express
7. HBSS (1X) Hanks' Balanced Salt Solution

C. RETM Complete Medium (5% FBS)

Combine the following to make RETM:

1. 500 mL RETM basal medium
2. 15 mL RETM supplement (15 mL, thawed briefly in a 37°C water bath)
3. 125 µL Cholera Toxin (100 µg/mL stock solution in cell culture grade water)
4. 25 mL heat-inactivated FBS
5. Pen/strep or other antibiotics (if desired)

Initiating a Culture from a Frozen Vial of Wood Cells

I. Procedure

1. Complete RETM Complete Medium (5% FBS) by preparing it according to the instructions above (section C).
2. Prepare the reagent by transfering the growth medium from the refrigerator into a 37°C water bath until warmed. Thoroughly spray all components with 70% alcohol and wipe dry before placing in hood. In the hood, ensure a cryovial rack, a loosely capped 15 mL conical tube, and a loosely capped media bottle are aligned for convenient and sterile work flow.
3. Remove the frozen cell stock from the liquid nitrogen refrigerator by placing the frozen vial in a bucket of dry ice.
   Note: If dry ice is not present, wait until all reagent and hood preparations are compete before removing frozen cell sample. Frozen cell sample must remain completely frozen until ready for thawing.
4. Thaw the frozen cell suspension by removing the sample from the dry ice bucket. Hold frozen cell sample in the 37°C water bath until no less than 1/3 of cryovial contents are frozen.
   Note: Do not let water in bath contact the cap.
   Dry cryovial with paper towel. Spray cryovial with 70% alcohol, wipe dry and place in cryovial rack under the hood.
5. Immediately dilute the thawed cells in media by first uncapping the media bottle, 15 mL conical tube, and partially thawed cell sample in cryovial. Then, with a 10 mL sterile serological pipette, take up a volume of 10 mL media and immediately take up

all liquid from frozen cell suspension cryovial.
6. Dispense 5 mL of serological pipette contents into the 15 mL conical tube.
7. With remaining serological pipette contents, rinse cryovial once and re-collect liquid. Dispense all serological pipette contents into the 15 mL conical tube. Tightly cap and invert to mix while carrying to centrifuge.
8. Centrifuge conical tube at 500 x g for 5 minutes. Observe proper pellet formation.
   Note: Pellets should be opaque with a defined boundary and can have peaks but should be few with well-defined edges. If a pellet appears translucent and poorly defined, check centrifuge settings and centrifuge further.
9. Aspirate the supernatant. Begin by aspirating supernatant and then inverting the tube on a downward angle and aspirating fluid draining away from the pellet.
10. Resuspend pellet in 10 mL RETM Complete Medium (5% FBS).
11. To plate the wood cells, consult the Cell Growth Data (Table 1) to find the recommended number of flasks to be plated. Label the appropriate number of new tissue culture-treated T-25 flask/flasks with information such as "Cell line; Growth Surface; Plating date; Passage number; Growth Media Name." Plating frozen cells adds one passage to the number on the vial.
12. Plate the 10 mL split appropriately among the recommended number of flasks. If multiple flasks are plated, bring the media volume up to 10 mL per T-25 flask.
13. Immediately store newly cultured flask/flasks directly on the metal incubator shelf.
14. Assess the wood cells 24 hours after plating by examining the cells under a microscope and reference the growth curve on the product page. Expect a confluent flask based on the timeline shown in Graph 1. Check degree of confluence and don't allow the flask to become more than 80% confluent.
15. If ready to split, move on to next procedure I-A. If not, move on to next step.
16. To feed the wood cells, aspirate the media, add 5 mL HBSS(+), and rock the flask slowly to wash all sides of the flask.
17. Aspirate HBSS(+) and add 10 mL fresh RETM to the flask and return to incubator.

II. Notes

Change media following step 17–18 above and repeat every 2–3 days thereafter.
Note: Media that appears yellow or yellow-orange must be changed. Media should never be allowed to become yellow.
When a near-confluent flask is reached, passage the cells per the procedure I-A.

Passaging Wood Cultures in RETM Complete Medium (5% FBS)

Passaging is performed when culture flasks exhibit roughly 80% confluency. This protocol outlines the procedure for passaging Wood cells in RETM Complete Medium (5% FBS) prepared according to the instructions above.

It is the responsibility of the Laboratory Manager and Safety Officer to ensure all laboratory personnel are properly trained in and follow this protocol. RETM Complete Medium (5% FBS) contains Cholera Toxin. Use 10% bleach to clean up any spills then dispose of materials as biohazardardous waste. Lab coat and gloves are required.

I-A. Procedure

1. Place Tryple Express 0.25% and RETM Complete Medium (5% FBS) (instructions for RETM shown in section C) in 37°C water bath until warmed. Keep Quench solution at 4°C until ready to use. Thoroughly spray all bottles with 70% alcohol and wipe dry before placing in hood.
2. To rinse the monolayer of cultured flasks with HBSS (-), aspirate the medium in culture flask, add 5 mL HBSS (-), and rinse the inside surfaces of the flask by rocking.
3. Dissociate the wood monolayer by aspirating HBSS (-) and adding 2 mL Tryple Express to the flask.
4. Incubate flask at 37°C /5% CO_2 for no more than 10 minutes, checking after 5 minutes.
5. While flask is incubating, spray cold Quench Solution with alcohol and wipe dry before placing in the hood.
6. Check Wood flask with microscope after incubation period. Ensure all cells have a bright, rounded appearance.
7. Knock flask on bench vigorously to release all cells from the surface. Check if cells are suspended by rapidly moving the flask across the microscope stage. Suspended cells will appear to keep moving once the flask has stopped.
   Note: Do not keep cells in contact with Tryple for over 5 minutes.
   If all cells appear to be suspended, then place flask in the hood for quenching.
8. Quench the Tryple by adding 8 mL of Quench Solution to flask and rinse flask sides, mix by pipetting, and then add to 15 mL conical tube.
9. Transfer ~300 µL to a microcentrifuge tube for counting. Cap the 15 mL conical tube and place in centrifuge at 500 x g for 5 minutes. Count the cells using a hemocytometer, or counting method of choice.
10. Label flask with information such as "cell line name; growth surface; plating date; passage number; # cells seeded; growth media name."
11. Calculate the volume needed to plate 300,000 cells per T-25 flask.
12. Mix cells using a 5 mL pipette immediately before plating.
13. Add the cell suspension by measuring the calculated amount carefully with the appropriate size pipette.
14. Add RETM Complete Medium (5% FBS) to reach a 10 mL total flask volume.
15. Immediately store newly cultured flask directly on the metal incubator shelf.
16. Assess the wood cells 24 hours after plating by examining the cells under the microscope and reference the information shown

below (Table 1). Check degree of confluence, and don't allow the flask become more than 80% confluent.

17. If ready to split, repeat procedure I-A. If not, move on to next step.
18. To feed the wood cells, aspirate the media, add 5 mL HBSS(+), and rock the flask slowly to wash all sides of the flask.
19. Aspirate HBSS(+), add 10 mL fresh RETM Complete Medium (5% FBS) to the flask, and return to incubator.

II-A. Notes

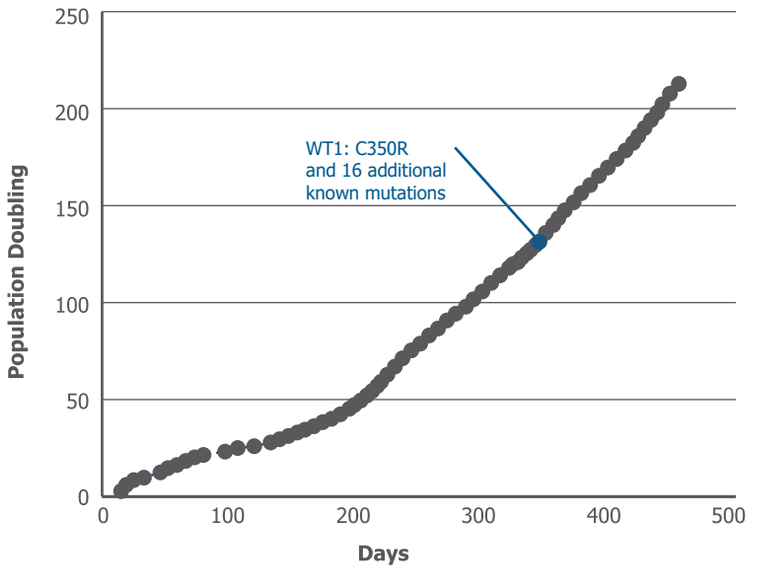
Change media following steps 19–20 and repeat every 2–3 days thereafter.
Note: Media that appears yellow or yellow-orange must be changed. Media should never be allowed to become yellow.
When a near-confluent flask is reached, passage the cells per procedure I-A.

Table 1. Cell Growth Data

Product	Number Seeded	Number Harvested	Days Elapsed
1	1,000,000	2,560,000	2
2	300,000	3,366,000	4
3	300,000	3,820,000	3

Note: One vial was thawed and plated in a T-25 flask. This culture was passaged three times in RETM per teh SOP. If followed, similar growth rates may be achieved.

Graph 1. Growth Curve



Doubling Time: 1.08 days
This growth curve demonstrates consistent growth and indicates a lack of cell crisis. It also demonstrates a continuous cell line accomplished without genetic engineering.