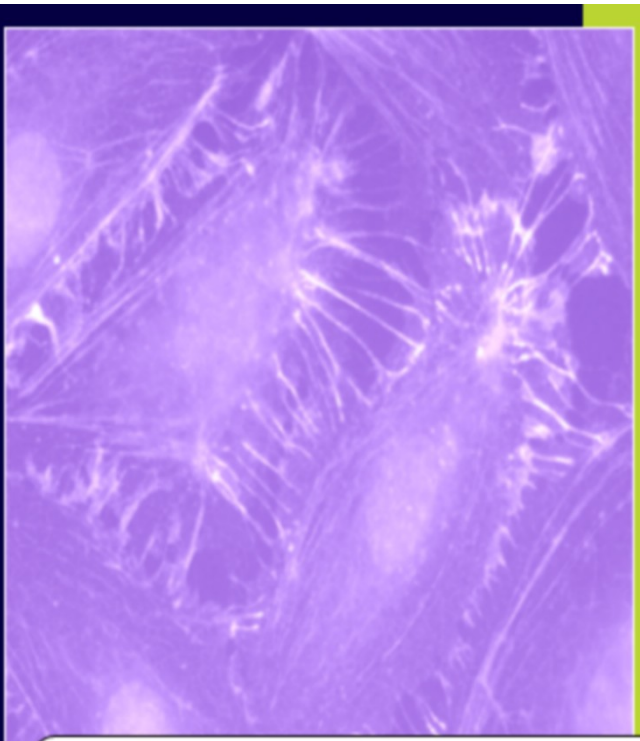

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American Type Culture Collection
10801 University Blvd
Manassas, VA 20110

Thawing, Propagating, and Cryopreserving Protocol

MCF10A-JSB

Breast adenocarcinoma

NCI-PBCF-1000

September 1, 2012; Version 1.0

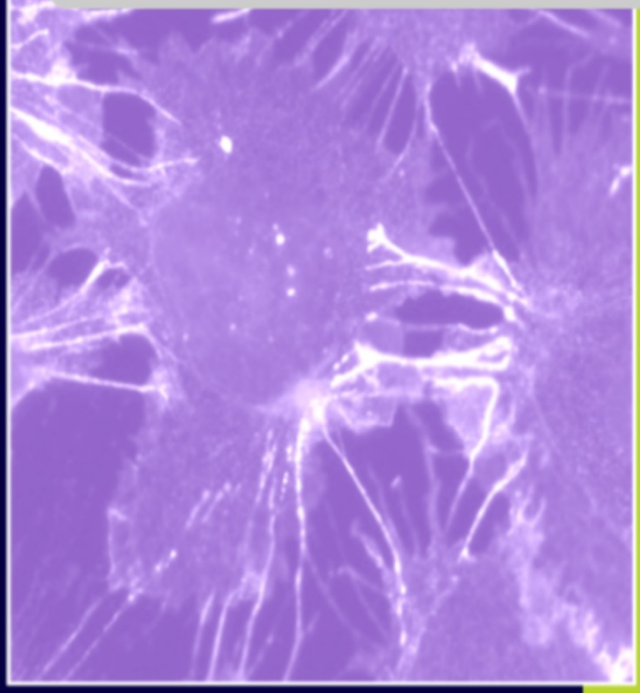

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Table of Contents

1. BACKGROUND INFORMATION ON MCF10A-JSB CELL LINE 3

2. GENERAL INFORMATION FOR THE THAWING, PROPAGATING AND CRYOPRESERVING OF NCI-PBCF-1000 (MCF10A-JSB) CELLS 3

3. REAGENTS 5

A. PREPARATION OF COMPLETE GROWTH MEDIUM 6

4. THAWING AND PROPAGATION OF CELLS 7

A. THAWING CELLS 7
B. PROPAGATING CELLS 8
C. SUBCULTURING CELLS 9

5. HARVESTING OF CELLS FOR CRYOPRESERVATION 11

6. CRYOPRESERVATION OF CELLS 13

A. CRYOPRESERVATION USING A RATE-CONTROLLED PROGRAMMABLE FREEZER 13
i. Using the Cryomed 13
B. CRYOPRESERVATION USING "MR. FROSTY" 14

7. STORAGE 15

APPENDIX 1: PHOTOMICROGRAPHS OF NCI-PBCF-1000 (MCF10A-JSB) CELLS 16

APPENDIX 2: GROWTH PROFILE OF NCI-PBCF-1000 (MCF10A-JSB) CELLS 19

APPENDIX 3: CYTOGENETIC ANALYSIS OF NCI-PBCF-1000 (MCF10A-JSB) CELLS 20

APPENDIX 4: GLOSSARY OF TERMS 23

APPENDIX 5: REFERENCE 24

APPENDIX 6: REAGENT LOT TRACEABILITY AND CELL EXPANSION TABLES 25

APPENDIX 7: CALCULATION OF POPULATION DOUBLING LEVEL (PDL) 27

APPENDIX 8: SAFETY PRECAUTIONS 27

Protocol for Thawing, Propagation and Cryopreservation of NCI-PBCF-1000 (MCF10A-JSB) cells
Mammary gland adenocarcinoma

1. Background Information on MCF10A-JSB cell line

Designations:	MCF10A-JSB	
Biosafety Level:	1	
Shipped:	Frozen (in dry ice)	
Growth Properties:	Adherent **(see Appendix 1)**	
Organism:	*Homo sapiens* (human)	
Source:	**Organ**	Mammary gland; breast
	Disease	Fibrocystic disease

2. General Information for the thawing, propagating and cryopreserving of NCI-PBCF-1000 (MCF10A-JSB) cells

Culture Initiation	• The cryoprotectant (DMSO) should be removed by centrifugation. • The seeding density to use with a vial of MCF10A-JSB cells is about 2.5 x 10^4 viable cells/cm^2 or 1 vial into one T-75 flask containing 10 mL of complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone).
Complete growth medium	• The complete growth medium used to expand MCF10A-JSB cells is DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone. • Complete growth medium should be pre-warmed before use by placing into a water bath set at 37 °C ± 1 °C for 15 min to 30 min. • After 30 min, the complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone) should be moved to room temperature until used. Complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone) should be stored at 2 °C to 8 °C when not in use.
Cell Growth Environment	• The growth temperature for MCF10A-JSB is 37 ºC ± 1 ºC • A 5 % ± 1 % CO_2 in air atmosphere is recommended.
Cell growth properties	• Population Doubling time (PDT) is approximately 16 hours (*see* ***Appendix 2***).

Special Growth Requirements	• Subculture MCF10A-JSB cells at 75 % to 85 % confluence or when cell density reaches an average of 2.5 x 10^5 viable cells/cm^2.
Subculture Medium	• 0.05 % (w/v) trypsin-0.02% EDTA (ATCC cat no. PCS-999-003). • Subculturing reagents should be pre-warmed before use by placing into a water bath set at 37 ºC ± 1 ºC for 15 min to 30 min. • After 30 min, the subculturing medium should be moved to room temperature until used. Subculturing reagents should be stored at 2 ºC to 8 ºC when not in use.
Subculture Method	• The attached MCF10A-JSB cells are subcultured using 0.05 % (w/v) trypsin-0.02% EDTA (ATCC cat no. PCS-999-003). • The enzymatic action of the trypsin-EDTA is stopped by adding complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone) to the detached cells. • A split ratio is about 1:10 or a seeding density of approximately 2.5 x 10^4 viable cells/cm^2 is used when subculturing MCF10A-JSB cells.
Viable Cells/mL/Cryovial	• The target number of viable cells/mL/cryovial is: 2.0 x 10^6 (acceptable range: 1.5 x 10^6 viable cells/mL to 2.5 x 10^6 viable cells/mL).
Cryopreservation Medium	• The cryopreservation medium for MCF10A-JSB cells is complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone) containing 10 % (v/v) DMSO (ATCC cat no. 4-X).

General Procedure to be applied throughout the SOP

Aseptic Technique	• Use good aseptic techniques. Any materials that are contaminated, as well as any materials with which they may have come into contact, must be disposed of immediately.
Traceability of material/reagents	• Record the manufacturer, catalog number, lot number, date received, date expired and any other pertinent information for all materials and reagents used. Record information in the Reagent Lot Traceability Table 4 (***Appendix 6***).
Expansion of cell line	• Record the subculture and growth expansion activities, such as passage number, % confluence, % viability, cell morphology (see Figures 1, 2, 3 - ***Appendix 1***) and population doubling levels (PDLs), in the table for Cell Expansion (Table 5, ***Appendix 6***). Calculate PDLs using the equation in ***Appendix 7***.
Medium volumes	• Medium volumes are based on the flask size as outlined in Table 1.
Glossary of Terms	• Refer to Glossary of Terms used throughout the document (see ***Appendix 4***).
Safety Precaution	• Refer to Safety Precautions pertaining to the propagation and cryopreservation of MCF10A-JSB (See ***Appendix 8***).

Table 1: Medium Volumes

Flask Size	Medium Volume Range
12.5 cm^2 (T-12.5)	3 mL to 6 mL
25 cm^2 (T-25)	5 mL to 13 mL
75 cm^2 (T-75)	10 mL to 38 mL
150 cm^2 (T-150)	30 mL to 75 mL
175 cm^2 (T-175)	35 mL to 88 mL
225 cm^2 (T-225)	45 mL to 113 mL

3. Reagents

Follow Product Information Sheet storage and/or thawing instructions. Below is a list of reagents for the propagation, subcultivation and cryopreservation of MCF10A-JSB cells.

Table 2: Reagents for Expansion, Subculturing and Cryopreservation of MCF10A-JSB cells

Complete growth medium reagents	Subculturing reagents	Cryopreservation medium reagents
DMEM: F-12 Medium (ATCC cat no. 30-2006)	Trypsin-EDTA (0.05% % (w/v) Trypsin/0.02% EDTA) (ATCC cat no. PCS-999-003)	DMEM: F-12 Medium (ATCC cat no. 30-2006)
5 % (v/v) Horse serum (ATCC cat no. 30-2041)	Dulbecco's Phosphate Buffered Saline (DPBS); modified without calcium chloride and without magnesium chloride (ATCC cat no.30-2200)	5 % (v/v) Horse serum (ATCC cat no. 30-2041)
10 µg/mL Recombinant Human Insulin (Sigma cat no. I9278 or equivalent)		10 µg/mL Recombinant Human Insulin (Sigma cat no. I9278 or equivalent)
20ng/mL Recombinant Human Epidermal Growth Factor (hEGF) (Invitrogen cat no. PHG0311L or equivalent)		20ng/mL Recombinant Human Epidermal Growth Factor (hEGF) (Invitrogen cat no. PHG0311L or equivalent)
100ng/mL Cholera toxin (Sigma cat no C8052 or equivalent)		100ng/mL Cholera toxin (Sigma cat no C8052 or equivalent)
0.5 µg/mL Hydrocortisone (Sigma cat no. H0135 or equivalent)		0.5 µg/mL Hydrocortisone (Sigma cat no. H0135 or equivalent)
		10 % (v/v) Dimethyl Sulfoxide (DMSO), (ATCC cat no.4-X)

a. Preparation of complete growth medium

(DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone)

The complete growth medium is prepared by aseptically combining,

1. 25 mL Horse Serum (ATCC cat no. 30-2020) and 0.5 mL human insulin (10 mg/mL stock) and 10 µL hEGF (1 µg/µL stock) and 50 µL Cholera toxin (1 µg/µL stock) and 5 mL Hydrocortisone (50 µg/µL stock) to 469.5 mL of basal medium, DMEM: F-12 (ATCC cat no. 30-2006).
2. Mix gently, by swirling.

4. Thawing and Propagation of Cells

Reagents and Material:

- Complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone)
- Water bath
- T-75 cm^2 polystyrene flask
- 15 mL polypropylene conical centrifuge tubes
- Plastic pipettes (1 mL,10 mL, 25 mL)

a. Thawing cells

Method:

1. Place complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone) in a 37 °C ± 1 ^{o}C water bath.
2. Label T-75 flask to be used with the (a) name of cell line, (b) passage number, (c) date, (d) initials of technician.
3. Wearing a full face shield, retrieve a vial of frozen cells from liquid nitrogen freezer.
4. Thaw the vial by gentle agitation in a 37 °C ± 1 °C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water.

   Note: Thawing should be rapid (approximately 2 min to 3 min, just long enough for most of the ice to melt).
5. Remove vial from the water bath and process immediately.
6. Remove excess water from the vial by wiping with sterile gauze saturated with 70 % ethanol.
7. Transfer the vial to a BSL-2 laminar-flow hood.

b. Propagating cells

Method:

1. Add 9 mL of complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone) to a 15-mL conical centrifuge tube.
2. Again wipe the outer surface of the vial with sterile gauze wetted with 70 % ethanol.
3. Using sterile gauze, carefully remove the cap from the vial.
4. With a 1 mL pipette transfer the content of the vial (1 mL cell suspension) to the 15-mL conical centrifuge tube containing 9 mL complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone). Gently resuspend cells by pipetting up and down.
5. Centrifuge at 125 xg, at room temperature, for 8 min to 10 min.
6. Carefully aspirate (discard) the medium, leaving the pellet undisturbed.
7. Using a 10 mL pipette, add 10 mL of complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone).
8. Resuspend pellet by gentle pipetting up and down.
9. Using a 1 mL pipette, remove 1 mL of cell suspension for cell count and viability. Cell counts are performed using either an automated counter (such as Innovatis Cedex System; Beckman-Coulter ViCell system) or a hemocytometer.
10. Record total cell count and viability. When an automated system is used, attach copies of the printed results to the record.
11. Plate cells in pre-labeled T-75 cm^2 flask at about 2.5×10^4 viable cells/cm^2.
12. Transfer flask to a 37 °C ± 1 °C in 5 % CO_2 incubator if using flasks with vented caps (for non-vented caps stream 5 % CO_2 in the headspace of flask).
13. Observe culture daily by eye and under an inverted microscope to ensure culture is free of contamination and culture has not reached confluence. Monitor, visually, the pH of the medium daily. If the medium goes from red through orange to yellow, change the medium.
14. ***Note: In most cases, cultures at a high cell density exhaust the medium faster than those at low cell density as is evident from the change in pH. A drop in pH is usually accompanied by an increase in cell density, which is an indicator to subculture the cells. Cells may stop growing when the pH is between pH 7 to pH 6 and loose viability between pH 6.5 and pH 6.***

15. If fluid renewal is needed, aseptically aspirate the complete growth medium from the flask and discard. Add an equivalent volume of fresh complete growth medium to the flask. Alternatively, perform a fluid addition by adding fresh complete growth medium to the flask without removing the existing medium. Record fluid change or fluid addition on the Cell Line Expansion Table **(see Table 5 in Appendix 6).**

16. If subculturing cells is needed, continue to Subculturing cells.

    Note: Subculture when cells are 75-85 % confluent (see photomicrographs in Appendix 1). Within 2 days after seeding at 2.5 x 10^4 viable cells/cm^2, the MCF10A-JSB cells form well-attached islands that spread and merge as the cells divide. If the cells are allowed to grow past confluency, they will become compacted and may begin to grow three-dimensionally.

c. Subculturing cells

Reagents and Material:

- 0.05 % (w/v) Trypsin-0.02% EDTA
- DPBS
- Complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone)
- 50 mL or 250 mL conical centrifuge tube
- Plastic pipettes (1 mL, 10 mL, 25 mL)
- T-75 cm^2, T-225 cm^2 polystyrene flasks

Method:

1. Remove and discard culture medium.

2. To the attached cells remaining in the flask, add appropriate volumes of sterile Ca^{2+}- and Mg^{2+}-free DPBS to the side of the flask opposite the cells so as to avoid dislodging the cells (see Table 3).

3. Rinse the cells with DPBS (using a gently rocking motion) and discard.

4. Add appropriate volume of 0.05 % (w/v) Trypsin-0.02% EDTA solution to the flask (see Table 3).

   Note: When subculturing MCF10A-JSB cells it is recommended to use a dissociation solution containing low concentrations of Trypsin (0.05 % (w/v)) and avoid dissociation solutions with higher concentrations of Trypsin.

5. Incubate the flask at 37 °C ± 1 °C until the cells round up. Observe cells under an inverted microscope every 5 min. When the flask is tilted, the attached cells should slide down the surface. This usually occurs after 15-20 min of incubation.

   Note: Do not leave trypsin-EDTA on the cells any longer than necessary as clumping may result.

6. Neutralize the trypsin-EDTA cell suspension by adding an equal volume of complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone) to each flask. Disperse the cells by pipetting, gently, over the surface of the monolayer. Pipette the cell suspension up and down with the tip of the pipette resting on the bottom corner or edge until a single cell suspension is obtained. Care should be taken to avoid the creation of foam.

7. Transfer floating cells collected in Step 1 and again resuspend pooled cells.

8. Using a 1 mL pipette, remove 1 mL of cell suspension for total cell count and viability.

9. Record total cell count and viability.

10. Spin cells at approximately 125 xg for 5 min to 10 min at room temperature. Carefully aspirate and discard the medium, leaving the pellet undisturbed.

11. Resuspend pellet in complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone) and transfer cell suspension (for volume see Table 1) into new pre-labeled flasks at a seeding density of about 2.5 x 10^4 viable cells/cm^2 or a split ratio of about 1:10.

12. Label all new flasks with the (a) name of cell line, (b) passage number, (c) date, (d) initials of technician.

Table 3 - Volume of Rinse Buffer and Trypsin

Flask Type	Flask Size	DPBS Rinse Buffer	Trypsin-EDTA
T-flask	12.5 cm^2 (T-12.5)	1 mL to 3 mL	1 mL to 2 mL
	25 cm^2 (T-25)	1 mL to 5 mL	1 mL to 3 mL
	75 cm^2 (T-75)	4 mL to 15 mL	2 mL to 8 mL
	150 cm^2 (T-150)	8 mL to 30 mL	4 mL to 15 mL
	175 cm^2 (T-175)	9 mL to 35 mL	5 mL to 20 mL
	225 cm^2 (T-225)	10 mL to 45 mL	5 mL to 25 mL

5. Harvesting of Cells for Cryopreservation

Reagents and Material:

- 0.05 % (w/v)Trypsin-0.02% EDTA
- DPBS
- Complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone)
- 50 mL or 250 mL conical centrifuge tube
- Plastic pipettes (1 mL, 10 mL, 25 mL)
- Sterile DMSO
- 1 mL to 1.8 mL cryovials
- Ice bucket with ice

Method:

1. Label cryovials to include information on the (a) name of cell line, (b) passage number (c) date.
2. Prepare cryopreservation medium by adding DMSO to cold complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone) at a final concentration of 10 % (v/v) DMSO. Place cryopreservation medium on ice until ready to use.
3. If most cells are attached and many cells are floating in medium, transfer the floating cells to a centrifuge tube.
4. To the attached cells remaining in the flask, add appropriate volumes of sterile Ca^{2+}- and Mg^{2+}-free DPBS to the side of the flask so as to avoid dislodging the cells (see Table 3).
5. Rinse the cells with DPBS (using a gently rocking motion) and discard.
6. Add appropriate volume of 0.05 % (w/v) Trypsin-0.02% EDTA solution to the flask (see Table 3).

   Note: When harvesting MCF10A-JSB cells it is recommended to use a dissociation solution containing low concentrations of Trypsin (0.05 % (w/v)) and avoid dissociation solutions with higher concentrations of Trypsin.

7. Incubate the flask at 37 °C ± 1 °C until the cells round up. Observe cells under an inverted microscope every 5 min. When the flask is tilted, the attached cells should slide down the surface. This usually occurs after about 15-20 min of incubation.

   Note: Do not leave trypsin-EDTA on the cells any longer than necessary as clumping may result.

8. Neutralize the trypsin-EDTA/cell suspension by adding an equal volume of complete growth medium (DMEM: F-12 + 5 % (v/v) horse serum + 10 µg/mL human insulin + 20ng/mL hEGF + 100ng/mL Cholera toxin + 0.5 µg/mL Hydrocortisone) to each flask. Disperse the cells by pipetting, gently, over the surface of the monolayer.

9. Pipette the cell suspension up and down with the tip of the pipette resting on the bottom corner or edge until a single cell suspension is obtained. Care should be taken to avoid the creation of foam.

10. Transfer floating cells collected in Step 3 to flask and again resuspend cells.

11. Transfer the pooled cell suspension to appropriate conical centrifuge tube (50 mL or 250 mL).

12. Using a 1 mL pipette, remove 1 mL of cell suspension for total cell count and viability.

13. Record total cell count and viability.

14. Spin cells at approximately 125 xg for 5 min to 10 min at room temperature; carefully aspirate and discard the medium, leaving the pellet undisturbed.

15. Calculate volume of cryopreservation medium needed based on the cell count performed at step 13 and resuspend pellet in cold cryopreservation medium at a viable cell density of 2.0×10^6 viable cells/mL (acceptable range: 1.5×10^6 viable cells/mL to 2.5×10^6 viable cells/mL) by gentle pipetting up and down.

16. Dispense 1 mL of cell suspension, using a 5 mL or 10 mL pipette, into each 1 mL cryovial.

17. Place filled cryovials at 2 °C to 8 °C until ready to cryopreserve. A minimum equilibration time of 10 min but no longer than 45 min is necessary to allow DMSO to penetrate the cells.

    Note: DMSO is toxic to the cells. Long exposure in DMSO may affect viability.

6. Cryopreservation of Cells

Material:

- Liquid nitrogen freezer
- Cryomed Programmable freezer (Forma Scientific, catalog no. 1010) **or**
- Mr. Frosty (Nalgene, catalog no. 5100)
- Isopropanol
- Cryovial rack

a. Cryopreservation using a rate-controlled programmable freezer

Method:

A slow and reproducible cooling rate is very important to ensure good recovery of cultures. A decrease of 1 °C per min to -40 °C followed by rapid freeze at about 15 °C to 30 °C per min drop to -90 °C usually work for most animal cell cultures. The best way to control the cooling process is to use a programmable electronic freezer unit. Refer to the manufacturer's handbook for detailed procedure.

i. Using the Cryomed

Starting the Cryopreservation Process

1. Check that the liquid nitrogen valve that supplies the Cryomed is open.

2. Check the gauge to ensure that there is enough liquid nitrogen in the open tank to complete the freeze.

3. Install the thermocouple probe so that the tip is immersed midway into the control fluid

   Note: Be sure that the thermocouple is centered in the vial and the vial is placed centered in the rack. The probe should be changed after three uses or if it turns yellow to ensure accurate readings by the controller during the freezing process. Old medium may have different freezing characteristics.

4. Close and latch Cryomed door.

5. Turn on microcomputer, computer and monitor.

6. Double click the "Cryomed" icon. The machine may need to be pre-programmed for specific cell type and medium.

7. From the top of the screen, select MENU → RUN FUNCTIONS → START RUN.

8. Fill out the box which appears on the screen. Cell line ID; TYPE OF SAMPLE; MEDIA; NUMBER OF SAMPLES.

9. Hit the ESCAPE key and the Cryomed will cool to 4 °C.

10. Once Cryomed chamber has cooled to 4 °C, load cryovials onto racks and close the door.

11. When the Cryomed's chamber temperature and the sample temperature have reached approximately 4 °C; press the space bar to initiate the rate controlled cryopreservation process.

Completing the Cryopreservation Process

1. When samples have reached -90 °C, an alarm will sound. To silence this, select ALARM from the options at the top of the screen.

2. Select MENU →RUN FUNCTIONS→ STOP. Hit the ESCAPE key to return to the main menu and select EXIT.

3. Immediately transfer vials to liquid nitrogen freezer.

4. Shut down the microcomputer and then turn off the monitor.

b. Cryopreservation using "Mr. Frosty"

1. One day before freezing cells, add 250 mL isopropanol to the bottom of the container and place at 2 °C to 8 °C.

2. On the day of the freeze, prepare cells for cryopreservation as described above.

3. Insert cryovials with the cell suspension in appropriate slots in the container.

4. Transfer the container to a -70 °C to -90 °C freezer and store overnight.

5. Next day, transfer cryovials to the vapor phase of liquid nitrogen freezer.

Note: Each container has 18 slots which can accommodate 18 cryovials.

Important information when using the rate-controlled programmable freezer or a manual method (Mr. Frosty) for cryopreservation of mammalian cells.

- Regardless which cooling method is used, it is important that the transfer to the final storage location (between -130 °C and -196 °C) be done quickly and efficiently. If the transfer cannot be done immediately, the vials can be placed on dry ice for a short time. This will avoid damage to cultures by inadvertent temporary warming during the transfer process. Warming during this transfer process is a major cause of variation in culture viability upon thawing.

- Always keep the storage temperature below -130 °C for optimum survival. Cells may survive storage at higher temperatures but viability will usually decrease over time. The ideal storage container is a liquid nitrogen freezer where the cultures are stored in the vapor phase above the liquid nitrogen.

Note: ATCC does not have experience in the cryopreservation of the MCF10A-JSB cells by any other method than the Cryomed programmable freezer.

7. Storage

- Store cryopreserved cells in the vapor-phase of liquid nitrogen freezer (below -130 °C) for optimum long-term survival.

Note: Experiments on long-term storage of animal cell lines at different temperature levels indicate that a -70 °C storage temperature is not adequate except for very short period of time. A -90 °C storage may be adequate for longer periods depending upon the cell line preserved. The efficiency of recovery, however, is not as great as when the cells are stored in vapor phase of the liquid nitrogen freezer.

APPENDIX 1: PHOTOMICROGRAPHS OF NCI-PBCF-1000 (MCF10A-JSB) CELLS

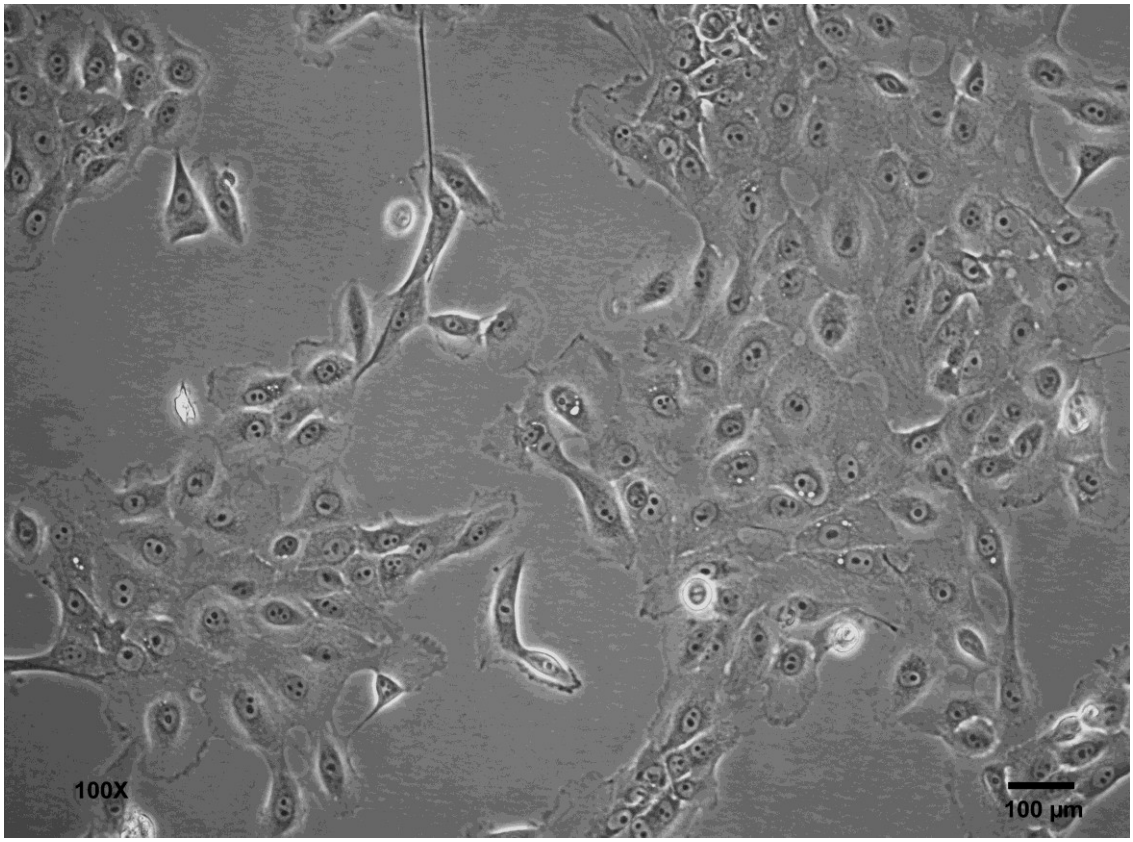


Figure 1: Photomicrograph of MCF10A-JSB cells after one day, post-freeze recovery. Cells were plated at 2.0 x 10^4 viable cells/cm^2.

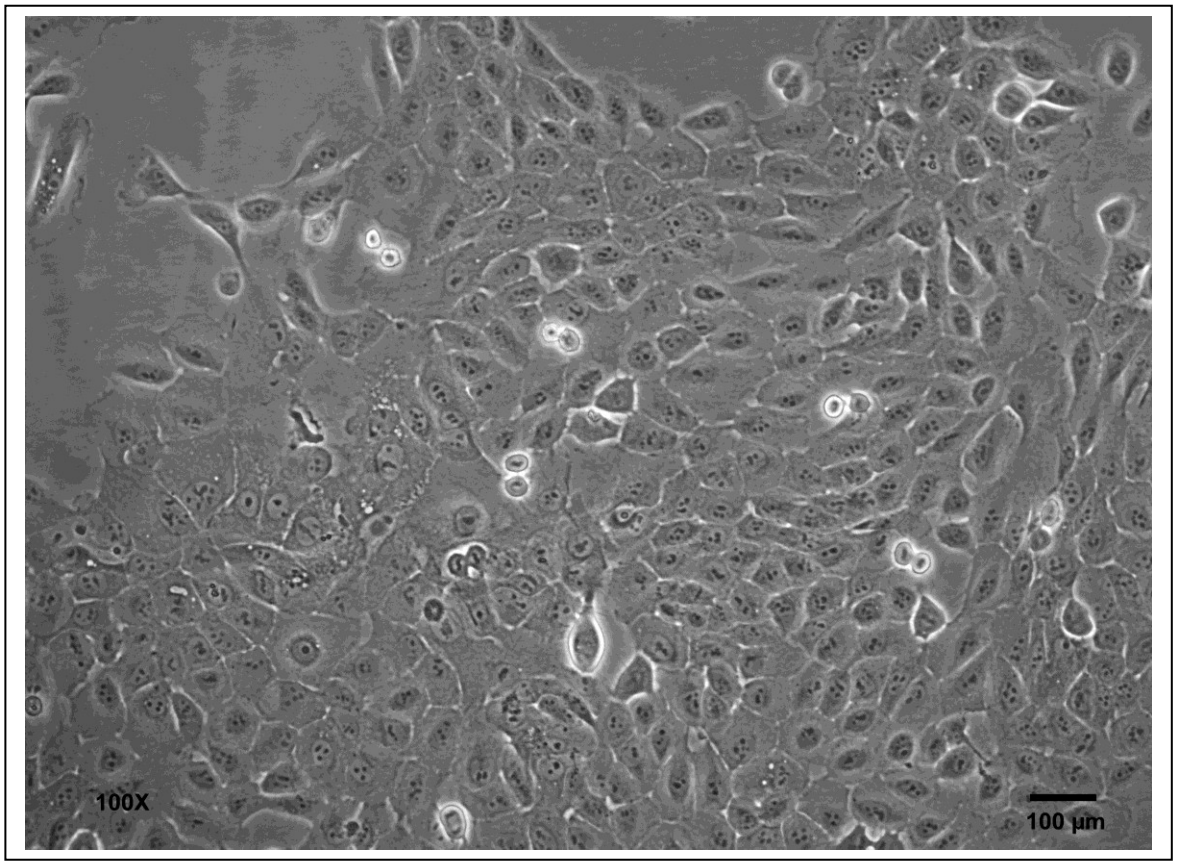


Figure 2: Photomicrograph of MCF10A-JSB cells after two days, post-freeze recovery. Cells were plated at 2.0 x 10^4 viable cells/cm^2.

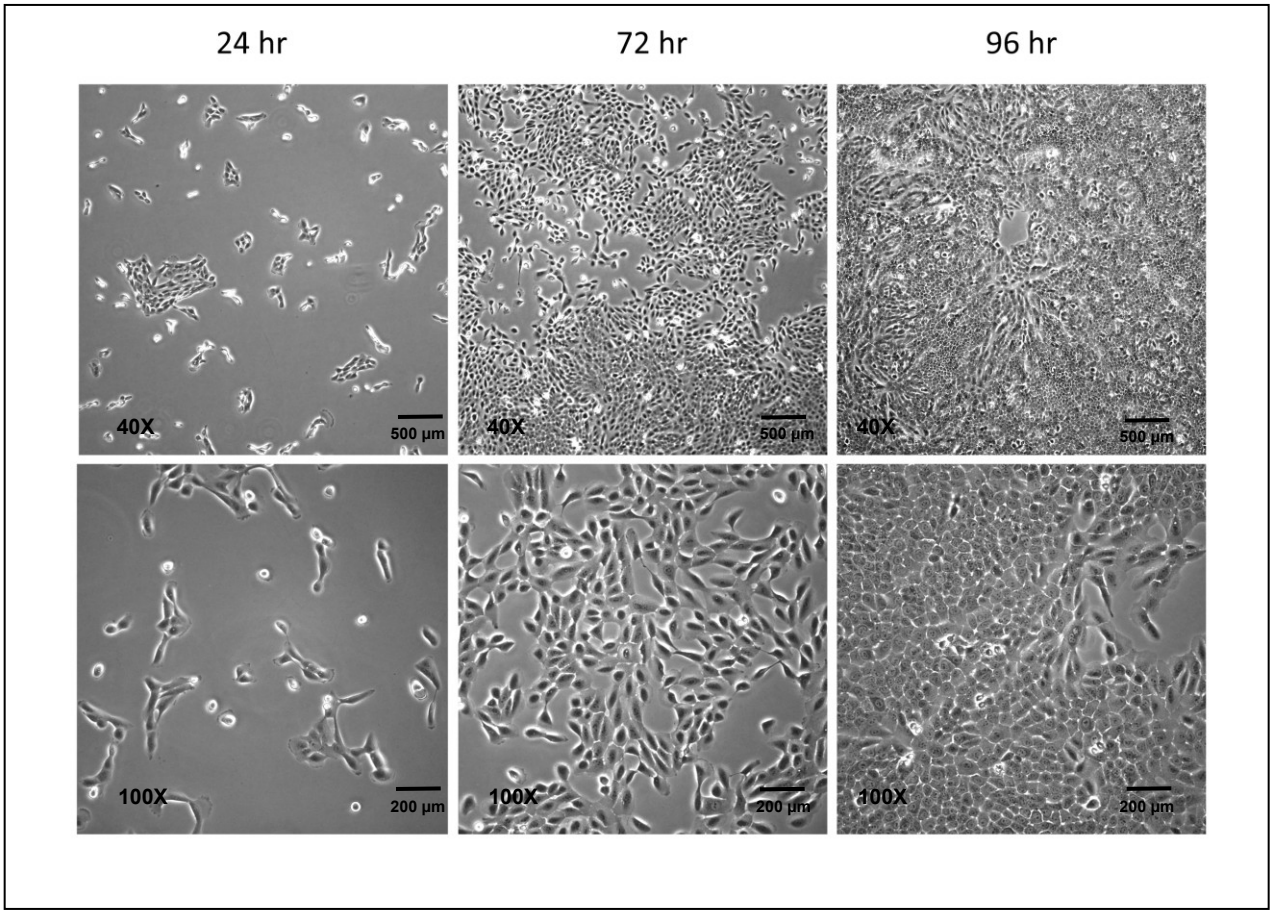


Figure 3: Photomicrographs of MCF10A-JSB cells at various time points after seeding at a cell density of 5 x 10^3 viable cells/cm^2.

APPENDIX 2: GROWTH PROFILE OF NCI-PBCF-1000 (MCF10A-JSB) CELLS

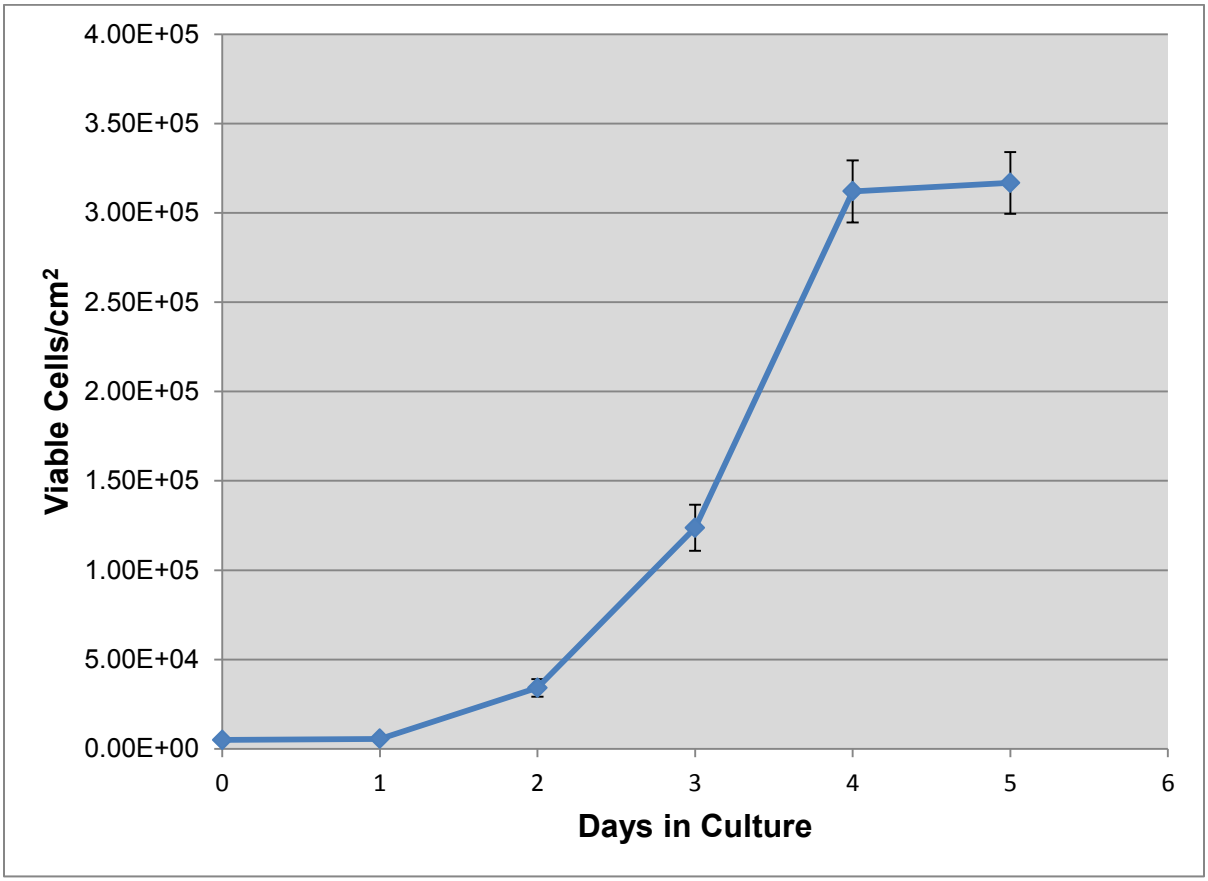
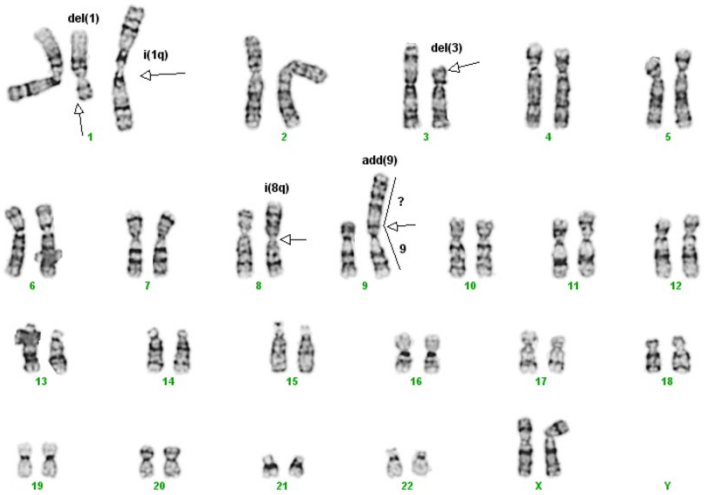


Figure 4: Growth curve for MCF10A-JSB cells; cells were plated at 5 x 10^3 viable cells/cm^2; population doubling time (PDT) is approximately 16 h.

APPENDIX 3: CYTOGENETIC ANALYSIS OF NCI-PBCF-1000 (MCF10A-JSB) CELLS

NCI-PBCF-1000 Karyotype Results	
Metaphase Spread	 **Karyotype**

Number of metaphase spreads counted	10
Band level	300-400
Number of metaphase spreads karyotyped	10
Chromosome range	47
Sex	Female
Comments	Aneuploid*

Karyotype:

47,XX,del(1)(q11q32.1),i(1)(q10),+1,del(3)(p12.3),i(8)(q10),add(9)(p22)[10]

(ISCN nomenclature written based on a diploid karyotype).

*Human diploid karyotype (2N): 46,XX (female) or 46,XY (male)

Karyotype Summary:

In the karyotype image, arrows indicate regions of abnormality. It should be noted that the karyotype description includes the observed abnormalities from all examined metaphase spreads, but due to heterogeneity, not all of the karyotyped cells will contain every abnormality.

This is a human cell line of female origin containing 47 chromosomes per metaphase spread (hyperdiploid). Structural abnormalities include rearrangements to chromosomes 1, 3, 8 and 9.

The rearrangements include:

- Addition of unknown material to the short arm (designated by p) of chromosome 9;
- Deletion of material from the short arm of chromosome 3;
- An interstitial deletion of material from the long arm (designated by q) of chromosome 1 between bands q11 and q32.1.
- Isochromosomes 1 and 8, both which consists of two copies of the q-arms attached at the centromeres [i(1)(q10) and i(8)(q10)].

Numerical changes are based on a diploid karyotype which would contain two copies of each chromosome (2N). *(ISCN 2009: An International System for Human Cytogenetic Nomenclature (2009), Editors: Lisa G. Shaffer, Marilyn L. Slovak, Lynda J. Campbell)*

Karyotype Procedure:

- **Cell Harvest:** Cells were allowed to grow to 80-90% confluence. Mitotic division was arrested by treating the cells with KaryoMax® colcemid for 20 minutes to 2 hours at 37°C. Cells were harvested using 0.05% Trypsin-EDTA, treated with 0.075M KCL hypotonic solution, and then fixed in three changes of a 3:1 ratio of methanol;glacial acetic acid.

- **Slide Preparation:** Slides were prepared by dropping the cell suspension onto wet glass slides and allowing them to dry under controlled conditions.

- **G-banding:** Slides were baked one hour at 90°C, trypsinized using 10X trypsin-EDTA, and then stained with Leishman's stain.

- **Microscopy:** Slides were scanned using a 10X objective and metaphase spreads were analyzed using a 100X plan apochromat objective on an Olympus BX-41 microscope. Imaging and karyotyping were performed using Cytovision® software.

- **Analysis:** Twenty metaphase cells were counted and analyzed, and representative metaphase cells were karyotyped depending on the complexity of the study.

Summary of Karyotyping Procedure:

G-band karyotyping analysis is performed using GTL banding technique: **G** bands produced with **t**rypsin and **L**eishman. Slides prepared with metaphase spreads are treated with trypsin and stained with Leishman's. This method produces a series of light and dark bands that allow for the positive identification of each chromosome.

MCF10A-JSB karyotyping was carried out by Cell Line Genetics, Inc. (Madison, WI 53719)

APPENDIX 4: GLOSSARY OF TERMS

Confluent monolayer: adherent cell culture in which all cells are in contact with other cells all around their periphery and no available substrate is left uncovered.

Split ratio: the divisor of the dilution ration of a cell culture to subculture (e.g., one flask divided into four, or 100 mL up to 400 mL, would be split ratio of 1:4).

Subculture (or passage): the transfer or transplantation of cells, with or without dilution, from one culture vessel to another.

Passage No: the total number of times the cells in the culture have been subcultured or passaged (with each subculture the passage number increases by 1).

Population doubling level (PDL): the total number of population doublings of a cell line since its initiation in vitro (with each subculture the population doubling increases in relationship to the split ratio at which the cells are plated). See Appendix 7.

Population doubling time (doubling time): the time interval, calculated during the logarithmic phase of growth in which cells double in number.

Seeding density: recommended number of cells per cm^2 of substrate when inoculating a new flask.

Epithelial-like: adherent cells of a polygonal shape with clear, sharp boundaries between them.

Fibroblast-like: adherent cells of a spindle or stellate shape.

APPENDIX 5: REFERENCE

1. Hay, R. J., Caputo, J. L., and Macy, M. L., Eds. (1992), ATCC Quality Control Methods for Cell Lines. 2nd edition, Published by ATCC.

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3. Fleming, D.O., Richardson, J. H., Tulis, J.J. and Vesley, D., (1995) Laboratory Safety: Principles and Practice. Second edition, ASM press, Washington, DC.

4. Culture of Animal Cells: A Manual of Basic Technique by R. Ian Freshney, 6th edition, published by Wiley-Liss, N.Y. 2010.

5. J. Debnath, Muthuswamy, S., and Brugge, J.. Morphogenesis and Oncogenesis of MCF-10A Mammary Epithelial Acini Grown In Three-Dimensional Basement Membrane Cultures. Methods. 30: 256-268. 2003.

6. J. Debnath, Mills, K., Collins, N., Reginato, M., Muthuswamy, S., and Brugge, J.. The Role Of Apoptosis In Creating and Maintaining Luminal Space Within Normal and Oncogene-Expressing Mammary Acini. Cell. 111:29-40. 2002.

APPENDIX 6: REAGENT LOT TRACEABILITY AND CELL EXPANSION TABLES

Table 4: Reagent Lot Traceability

Reagent	Vendor	Catalog #	Lot #	Expiration Date

Table 5: Cell Expansion

	From			Fluid Change		Observation under microscope	Cell Count			To		
By / Date	**Flask qty; size**	**Pass #**	**% Confluence**	**Add/ Replace**	**Volume (in mL)**		**Viable cells/mL**	**Total viable cells**	**% Viability**	**Split Ratio**	**Flask qty; size**	**Pass # PDL#**
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								
				Add / Replace								

APPENDIX 7: CALCULATION OF POPULATION DOUBLING LEVEL (PDL)

Calculate the PDL of the current passage using the following equation:

$$PDL = X + 3.322 (\log Y - \log I)$$

Where: X = initial PDL

I = cell inoculum (number of cells plated in the flask)

Y = final cell yield (number of cells at the end of the growth period)

APPENDIX 8: SAFETY PRECAUTIONS

- Use at least approved Biological Safety Level 2 (BSL-2) facilities and procedures.
- Wear appropriate Personal Protective Equipment (PPE), such as isolation gown, lab coat with sleeve protectors, face shield and gloves.
- Use safety precautions for working with liquid nitrogen, nitrogen vapor and cryogenically cooled fixtures.
- Use liquid nitrogen freezers and liquid nitrogen tanks only in areas with adequate ventilation. Liquid nitrogen reduces the concentration of oxygen and can cause suffocation.
- Wear latex gloves over insulating gloves to prevent liquid nitrogen from soaking in and being held next to the skin. Liquid nitrogen is extremely cold and will cause burns and frostbite. Metal inventory racks, tank components, and liquid nitrogen transfer hoses exposed to liquid nitrogen or nitrogen vapor quickly cool to cryogenic temperatures and can cause burns and frostbite.
- Wear a full face mask when thawing and retrieving vials from liquid nitrogen freezer. Danger to the technician derives mainly from the possibility that liquid nitrogen can penetrate the cryovial during storage. On warming, rapid evaporation of the nitrogen within the confines of such cryovial can cause an aerosol or explosion of the cryovial and contents.