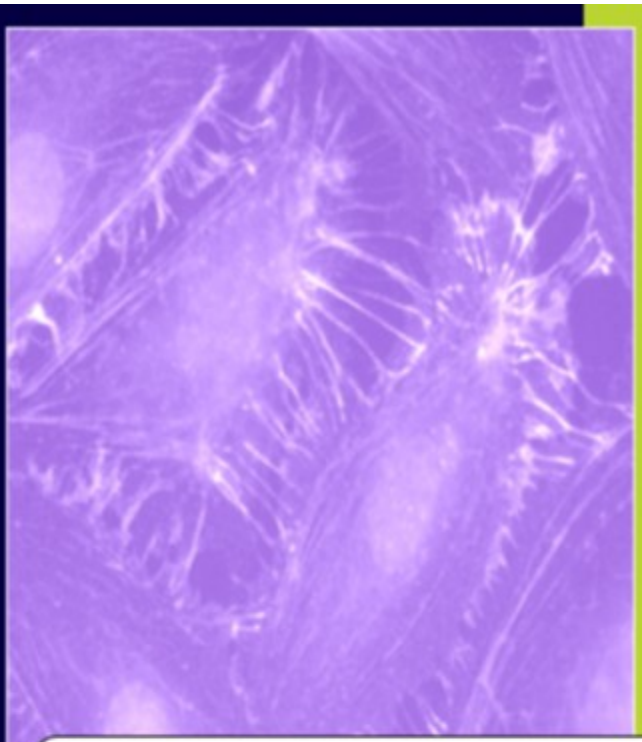


ATCC™

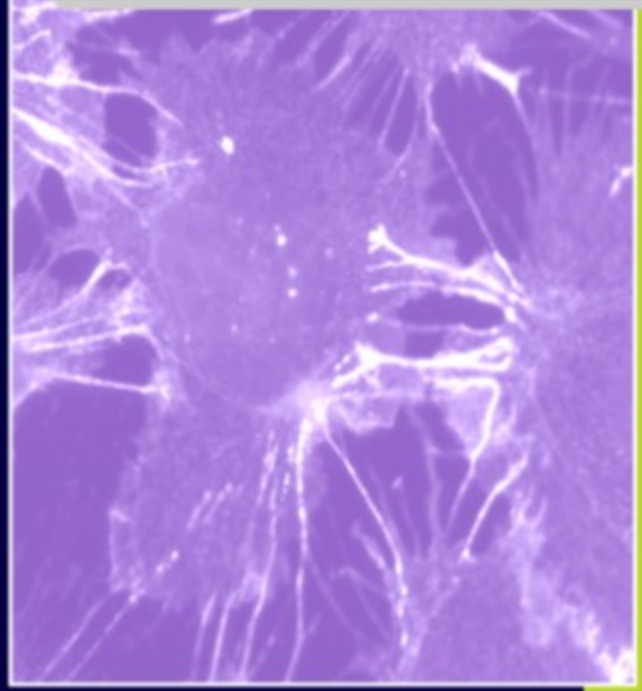
American Type Culture Collection
10801 University Blvd
Manassas, VA 20110

Thawing, Propagating, and Cryopreserving Protocol

NCI-PBCF-CCL256.1 (NCI-BL2126)
B lymphoblast, EBV-transformed

(ATCC® CCL-256.1™)

August 20, 2012; Version 1.0



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Protocol for Thawing, Propagation and Cryopreservation of NCI-PBCF-CCL256.1 (NCI-BL2126) (ATCC® CCL-256.1™) B lymphoblast, EBV transformed

1. Background Information on NCI-PBCF-CCL256.1 (NCI-BL2126) cell line

Designations:	NCI-BL2126 [BL2126]	
Biosafety Level:	2	
Shipped:	Frozen (in dry ice)	
Growth Properties:	Suspension, multicell aggregates (see Appendix 1)	
Organism:	*Homo sapiens*	
Source:	**Organ**	Peripheral blood
	Cell Type:	B lymphoblast; Epstein-Barr virus (EBV) transformed

For more information visit the ATCC webpage:
http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=CCL-256.1&Template=cellBiology

2. General Information for the thawing, propagating and cryopreserving of NCI-PBCF-CCL256.1 (NCI-BL2126)

Culture Initiation	• The cryoprotectant (DMSO) should be removed by centrifugation. • The seeding density to use with a vial of NCI-BL2126 cells is 1 vial into one T-75 flask with 20 mL medium or about 1 x 10^5 viable cells/mL.
Complete growth medium	• The complete growth medium used to expand NCI-BL2126 cells is RPMI 1640 + 10% (v/v) FBS. • Complete growth medium (RPMI 1640 + 10% (v/v) FBS) should be pre-warmed before use by placing into a water bath set at 35 ºC to 37 ºC for 15 min to 30 min. • After 30 min, the complete growth medium (RPMI 1640 + 10% (v/v) FBS) should be moved to room temperature until used. Complete growth medium should be stored at 2 ºC to 8 ºC when not in use.
Cell Growth	• The growth temperature for NCI-BL2126 is 37 ºC ± 1 ºC • A 5 % ± 1 % CO_2 in air atmosphere is recommended.
Growth Properties	• Population doubling time (PDT) is approximately 66h (see Appendix 2).

Special Growth Requirements	• Subculture NCI-BL2126 cells when cell density reaches an average of 3.0 x 10^5 viable cells/mL.
Subculture Method	• The floating NCI-BL2126 cells are subcultured by addition of fresh medium or replacement of medium. • A split ratio of 1:3 or a seeding density of 1 x 10^5 viable cells/mL to is used when subculturing NCI-BL2126 cells.
Viable Cells/mL/Cryovial	• The target number of viable cells/mL/cryovial is: 1 x 10^6 (acceptable range: 1 x 10^6 viable cells/mL to 2 x 10^6 viable cells/mL).
Cryopreservation Medium	• The cryopreservation medium for NCI-BL2126 cells is complete growth medium (RPMI 1640 + 10% (v/v) FBS) containing 5 % (v/v) DMSO (ATCC cat no. 4-X).

General Procedure to be applied throughout the SOP

Aseptic Technique	• Use of good aseptic technique is critical. 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 1-3, ***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 thawing, propagating and cryopreserving of NCI-BL2126 (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 NCI-BL2126 cells.

Table 2: Reagents for Expansion, Subculturing and Cryopreservation of NCI-BL2126 Cells

Complete growth medium reagents	Subculturing reagents	Cryopreservation medium reagents
RPMI 1640 Medium (ATCC cat no. 30-2001)	RPMI 1640 Medium (ATCC cat no. 30-2001)	RPMI 1640 Medium (ATCC cat no. 30-2001)
10 % (v/v) Fetal Bovine Serum (FBS) (ATCC cat no. 30-2020)	10 % (v/v) FBS (ATCC cat no. 30-2020)	10 % (v/v) Fetal Bovine Serum (FBS) (ATCC cat no. 30-2020)
		5 % (v/v) Dimethyl Sulfoxide (DMSO) (ATCC cat no. 4-X)

a. Preparation of complete growth medium

The complete growth medium is prepared by aseptically combining,

1. 56 mL FBS (ATCC cat no. 30-2020) to a 500 mL bottle of basal medium, RPMI 1640 (ATCC cat no. 30-2001).
2. Mix gently, by swirling.

4. Thawing and Propagation of Cells

Reagents and Material:

- Complete growth medium (RPMI 1640 + 10 % (v/v) FBS)
- 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 (RPMI 1640 + 10 % (v/v) FBS) in a water bath set at 35 ^{o}C to 37 ^{o}C.
2. Label T-25 flask to be used with the (a) name of cell line, (b) passage number, (c) date, (d) initials of technician.
3. Wearing full face shield, retrieve a vial of frozen cells from liquid nitrogen freezer.
4. Thaw the vial by gentle agitation in a water bath set at 35 ^{o}C to 37 ^{o}C. 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 the 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 (RPMI 1640 + 10 % (v/v) FBS) 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, slowly, the content of the vial (1 mL cell suspension) to the 15-mL conical centrifuge tube containing 9 mL complete growth medium (RPMI 1640 + 10 % (v/v) FBS). 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 (RPMI 1640 + 10 % (v/v) FBS).
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 a pre-labeled T-75 cm^2 flask at about 1 x 10^5 viable cells/mL.
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.

    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.

14. Three times weekly, using a 1 mL pipette, remove 1 mL of cell suspension for total cell count and viability.
15. Record total cell count and viability.
16. If subculturing of cells is needed, continue to ‘Subculturing cells’.

c. Subculturing cells

Reagents and Material:

- Complete growth medium (RPMI 1640 (ATCC cat no. 30-2001) + 10 % (v/v) FBS (ATCC cat no. 30-2020))
- Plastic pipettes (1 mL, 10 mL, 25 mL)
- T-75 cm^2, T-225 cm^2 polystyrene flasks

Method:

1. Add fresh complete growth medium (RPMI 1640 + 10 % (v/v) FBS) to the flask without removing the existing medium. Record fluid addition on the Cell Line Expansion Table (see Table 4 in Appendix 5). Transfer some cells to new pre-labeled flasks if total volume per flask exceeds maximum recommended (see Table 1).
2. 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:

- Complete growth medium (RPMI 1640 (ATCC cat no. 30-2001) + 10 % (v/v) FBS (ATCC cat no. 30-2020))
- 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 (RPMI 1640 + 10 % (v/v) FBS) at a final concentration of 5 % (v/v) DMSO. Place cryopreservation medium on ice until ready to use.
3. Using a 1 mL pipette, remove 1 mL of cell suspension for total cell count and viability.
4. Record total cell count and viability.
5. Spin cells at approximately 125 xg for 5 min to 10 min at room temperature.
6. Carefully aspirate and discard the medium, leaving the pellet undisturbed.
7. Calculate volume of cryopreservation medium based on the count performed at step 3 and resuspend pellet in cold cryopreservation medium at a viable cell density of 1×10^6 viable cells/mL (acceptable range: 1×10^6 viable cells/mL to 2×10^6 viable cells/mL) by gentle pipetting up and down.
8. Dispense 1 mL of cell suspension, using a 5 mL or 10 mL pipette, into each 1.0 mL cryovial.
9. Place filled cryovials at 2 ^{o}C to 8 ^{o}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, cat no. 1010) **or**
- Mr. Frosty (Nalgene, cat 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 -80 °C followed by rapid freeze at about 15 °C to 30 °C per min drop to -150 °C will usually work for most animal cell cultures. The best way to control the cooling process is to use a programmable, rate-controlled, 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 –80°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 in one freeze.

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 NCI-BL2126 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-CCL256.1 (NCI-BL2126) CELLS

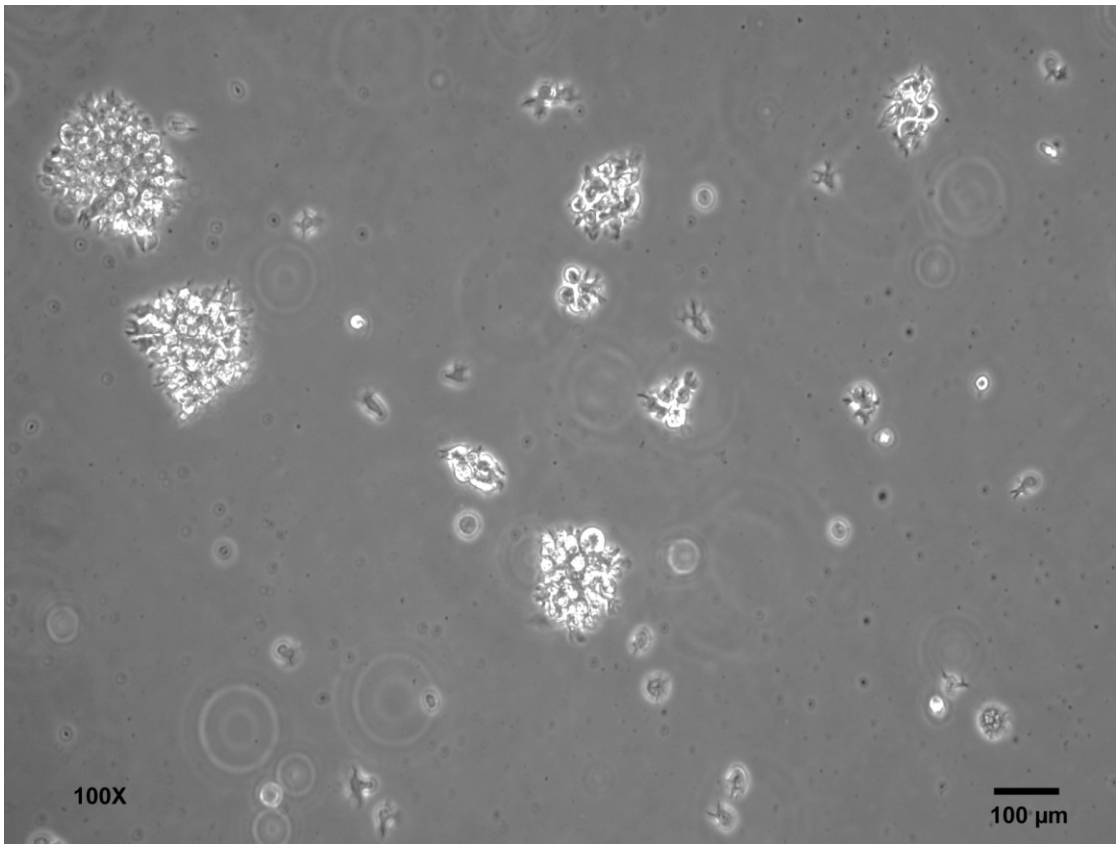


Figure 1: Photomicrograph of NCI-BL2126 cells after one day, post-freeze recovery. Cells were plated at 2.0 x 10^5 viable cells/mL.

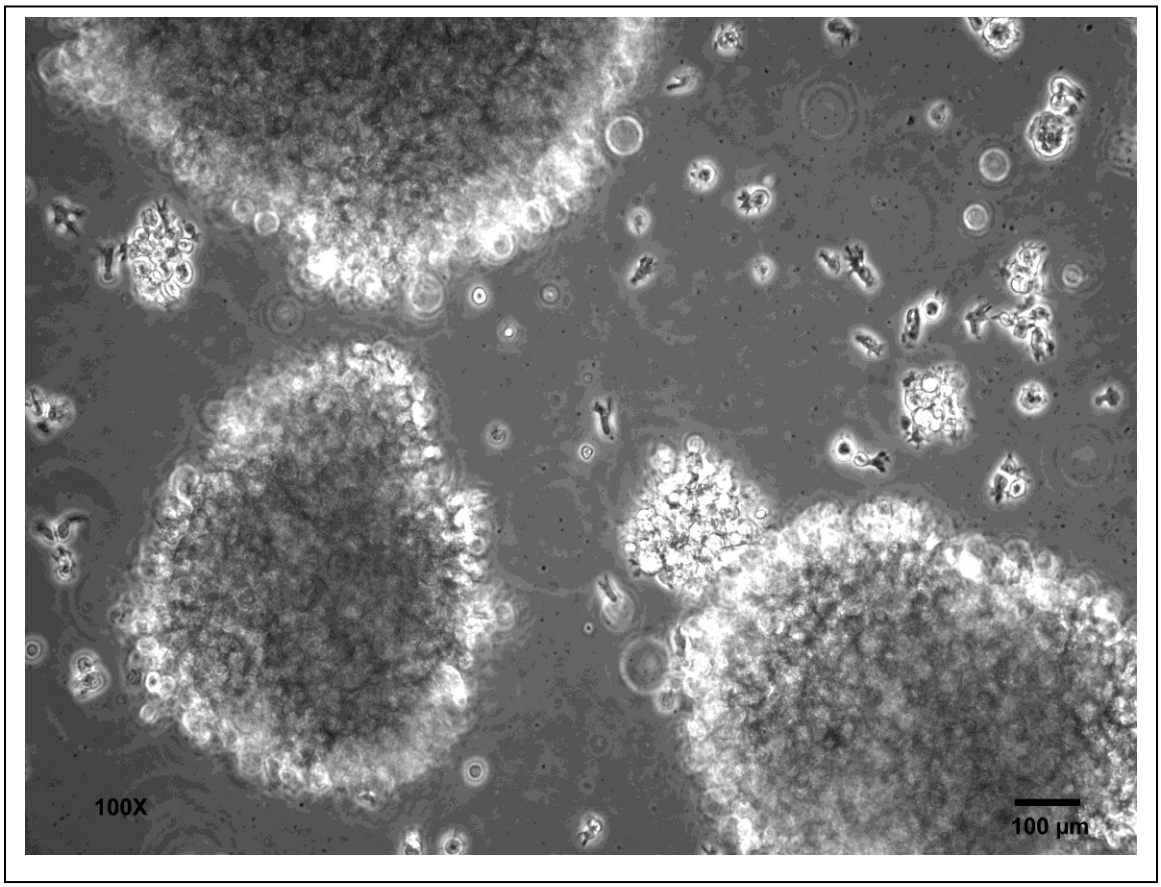


Figure 2: Photomicrograph of NCI-BL2126 cells after five days, post-freeze recovery. Cells were plated at 2.0 x 10^5 viable cells/mL.

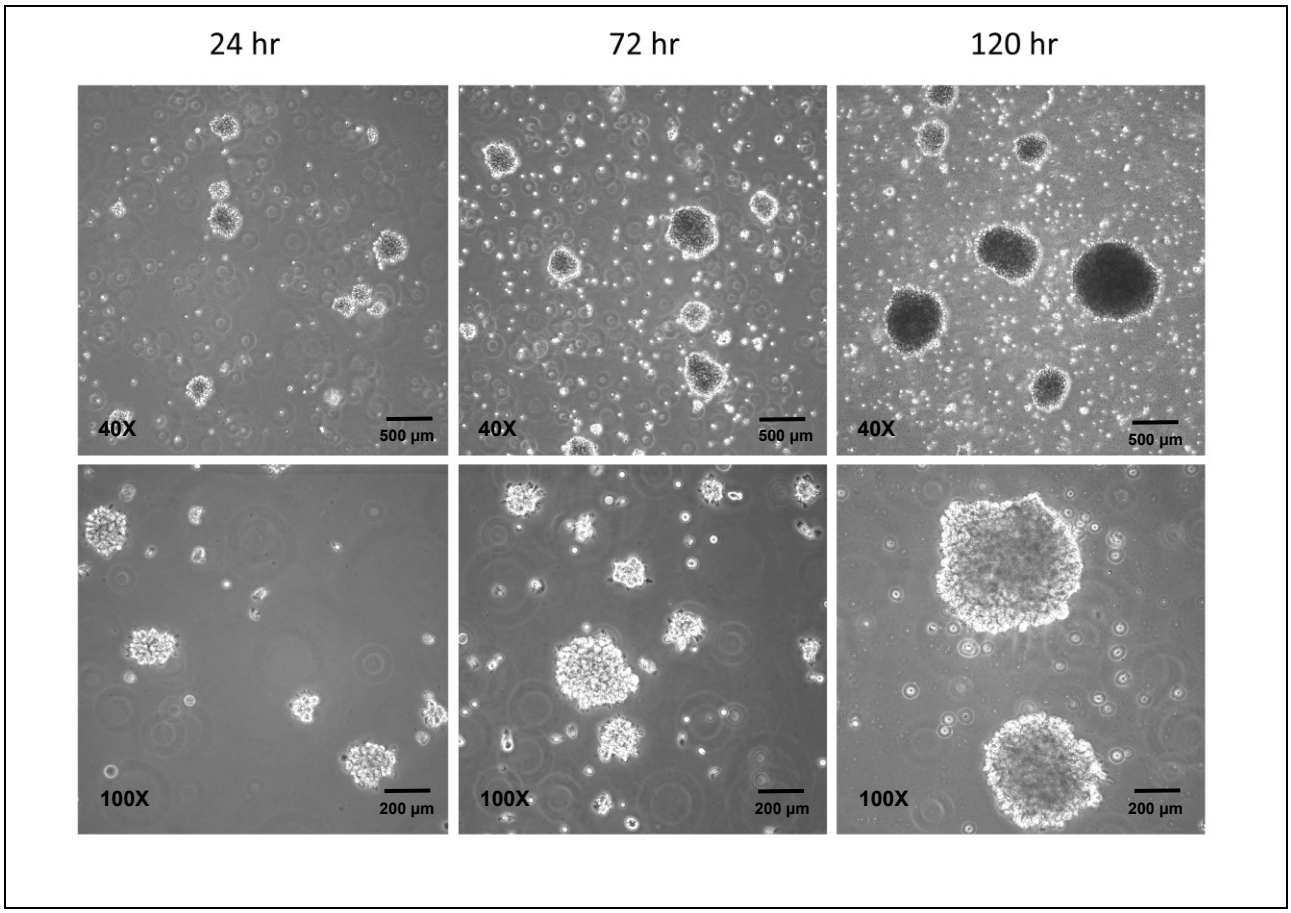


Figure 3: Photomicrographs of NCI-BL2126 cells at various time points after seeding at a cell density of 3.0 x 10^4 viable cells/mL.

APPENDIX 2: GROWTH PROFILE OF NCI-PBCF-CCL256.1 (NCI-BL2126) CELLS

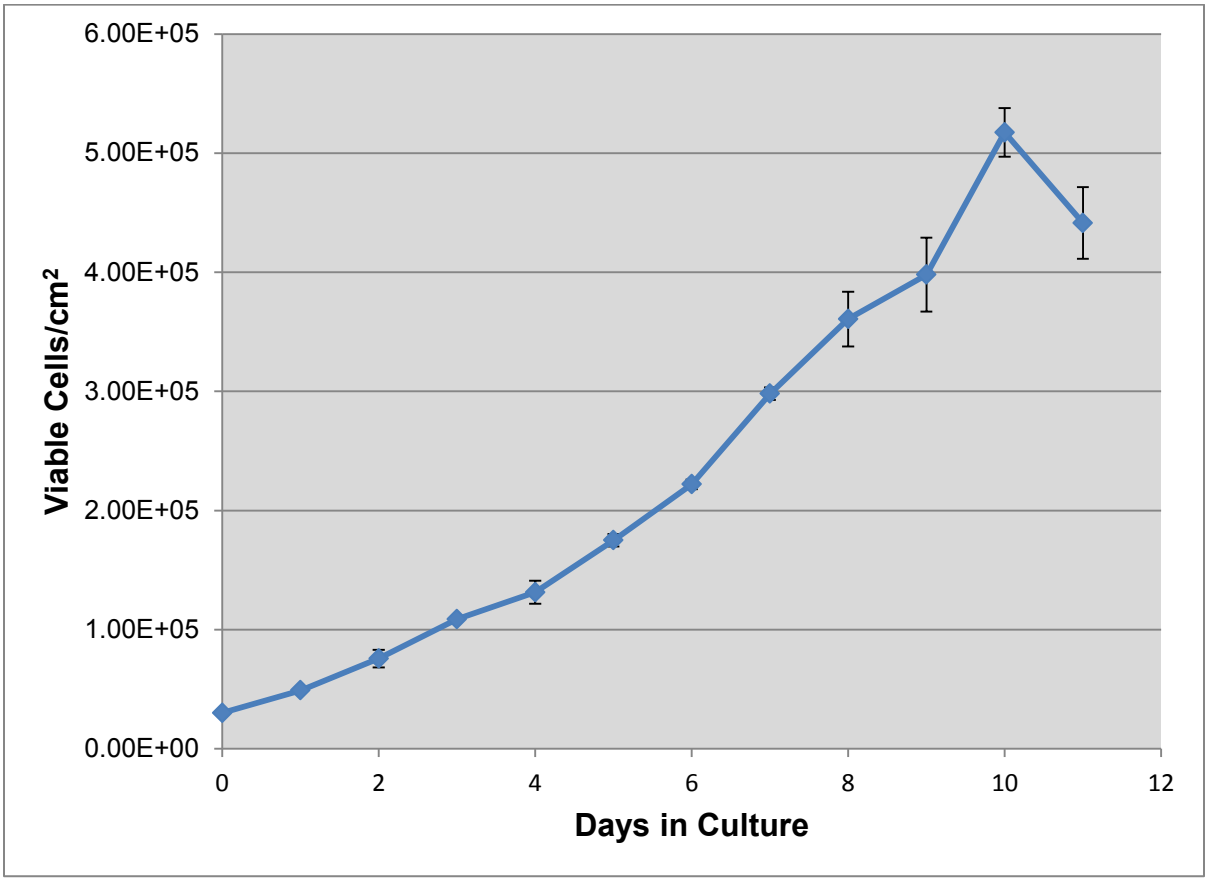
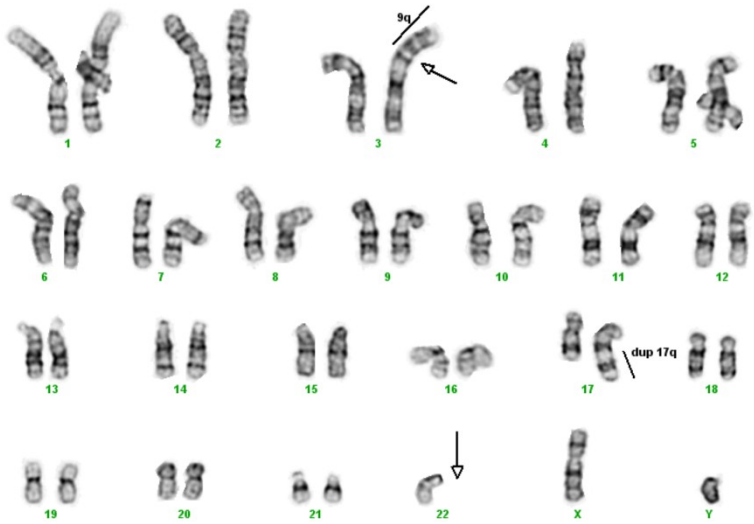


Figure 4: Growth curve for NCI-BL2126 cells; cells were plated at 3.0 x 10^5 viable cells/mL; population doubling time (PDT) is approximately 66 h.

APPENDIX 3: CYTOGENETIC ANALYSIS OF NCI-PBCF-CCL256.1 (NCI-BL2126) CELLS

NCI-PBCF-CCL256.1 Karyotype Results	
Metaphase Spread	 **Karyotype**

Number of metaphase spreads counted	10
Band level	300-400
Number of metaphase spreads karyotyped	10
Chromosome range	43-54
Sex	Male
Comments	Aneuploid*

Karyotype:

45~47,XY,der(3)t(3;9)(p25;q13),-22[cp4] , 46,XY[cp3] and three non-clonal cells (43-54 chromosomes).

(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 male origin containing 45 to 47 chromosomes per metaphase spread (near-diploid) for the clonal populations and 43-54 chromosomes per metaphase spread for the non-clonal cells examined (near-diploid). Approximately 30% of the examined cells were 46,XY (diploid male), 40% had a consistent translocation of chromosomes 3 and 9 and the other 30% of the examined cells had non-clonal aberrations, indicating instability of the cell line karyology.

The rearrangements include:

- A translocation involving chromosomes 3 and 9 [der(3)t(3;9)(p25;q13)];
- Non-clonal rearrangements include: an addition of unknown material to the long arm (q-arm) of chromosome 17 [add(17)(q25)], an isochromosome 8 [i(8)(q10)], a dicentric derivative chromosome involving a translocation between chromosomes 7 and 9 [der(7)t(7;9)(p22;q34.3)dup(7)(p11.2p22)] and a duplicated q-arm of chromosome 17 [dup(17q)].

Numerical changes are based on a diploid karyotype which would contain two copies of each chromosome (2N). Therefore, a karyotype designation such as -22 indicates one copy of a structurally normal chromosome 22 is present per metaphase spread and +2 indicates three copies of structurally normal chromosome 2 per metaphase. *(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.

NCI-BL2126 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. Culture Of Animal Cells: A Manual of Basic Technique by R. Ian Freshney, 6th edition, published by Wiley-Liss, N.Y., 2010.
2. NCI-Navy Medical Oncology Branch Cell Line Supplement. J. Cell. Biochem. suppl. 24: 1996

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 when 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.