**UW Medicine - Pathology**

400-02-01-07

Preservation of Cells in Liquid Nitrogen - Freezing Cells

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| Adopted Date: 09/04/91Review Date: 06/12/09Revision Date: 05/10/11 |

PURPOSE

This procedure allows for storage of living cells in cryogenic suspension for prolonged periods of time (years) in liquid nitrogen (at about ‑190°C).

PROCEDURE

### Material and Equipment

* + - 1. 22 µg Millipore filter
			2. Incubator (37°C)
			3. Inverted microscope
			4. Hemocytometer
			5. Counter
			6. Pasteur pipettes (sterile) or sterile disposable 1 ml pipettes
			7. 15 ml centrifuge tubes
			8. Plastic screw-cap vials (NUNC #3-66524 or Corning #25703)
			9. Diamond pencil. Sharpie or fine tip lab marker
			10. Isopropyl alcohol freezing container
			11. So low freezer (-70 to -80°C)
			12. Liquid nitrogen storage refrigerator
			13. 70% ethanol for wiping outside of vial.

### Reagents and Solutions

* + - 1. Trypsin ‑ EDTA 10X (Gibco Cat. #610-5405)
			2. Versene (Gibco Cat. #15040-066)
			3. Double distilled (DD) H20
			4. Dimethyl sulfoxide (DMSO) (Sigma D2650)
			5. Complete media alpha MEM or Amniomax, according to specimen type to be frozen (fibroblasts, amniotic cells, etc).
			6. Freezing media (complete media + 10% DMSO ‑ made fresh and tested for sterility) to complete bottle of media alpha MEM or Amniomax, add 10% of DMSO and filter it using a .22 µg Millipore filter. Incubate a 1 ml aliquot of medium + DMSO at 37°C overnight to check the sterility.
			7. 1X Trypsin in PBS. Rehydrate the trypsin with 20 ml of DDH20. Take 2 bottles of 90 ml PBS and add 10 ml of trypsin to each bottle.

### Procedure

***Note:*** No mouth pipetting. Adhere to sterile techniques. Use tissue culture hood [Biosafety Hood (BioGard)]. Use gloves.

* + - 1. Trypsinize the cells in the usual way. Four T75 flasks, confluent will be sufficient to prepare 6 vials of 1.2 ml each. Transfer the cell suspension to a 15 or 50 ml centrifuge tube and add an equal volume of complete alpha MEM medium with 10% fetal calf serum and antibiotics (various media work; could use higher serum concentration and other sera; use Amniomax medium for amniotic fluid cells).
			2. Count the number of cells per ml. Spin the cells for 5 min at 1,000 rpm. Decant the supernatant and re-suspend the cells with media containing DMSO to yield 2 x 106 cells per ml. The medium should be cold (DMSO is toxic above 4°C)
			3. Pre-label freezing vials with diamond pencil: name or number of strain, passage number and date. Label vial again with a Sharpie (same information). Set the vials on ice.
			4. With a Pasteur pipette or 1 ml sterile plastic pipette, dispense approximately 1.0 - 2.0 ml of cell suspension in medium + DMSO into freezing vials. Screw cap tightly.

Place the vials rapidly in isopropyl alcohol freezing container in the -80°C freezer for at least 2 hours. Transfer the tubes to the liquid nitrogen tanks within 2 weeks. Currently, cells are kept in liquid nitrogen freezer in K-050. Record in the logbook of the tank the appropriate numbers for date, case number, vials, and location. Record in the logbook of frozen cell lines in AA108 and log information in the computer or book in K-050. Cells can be maintained in liquid nitrogen for many years.

### Thawing

* + - 1. Thaw cells in warm H2O (37°C) holding the vial so that no H2O touches the seal. Thawing should take less than 1 min. When the medium has just thawed (don't let warm up) rinse outer surface of vial in 70% EtOH. Record in logs that vials have been thawed: date and # of vials.
			2. Transfer cells immediately with a Pasteur pipette to a culture flask T25 or T75 containing medium (20 ml). One can also dilute the cells in medium in a centrifuge tube containing 10 ml of medium, spin them down (1,000 g x 5 min) and resuspend them in fresh medium before inoculating a flask.
			3. After cell attachment (overnight) feed with fresh medium.

### Freezing

* + - 1. Freeze 2-6 vials/cell line, depending on cells available. Keep part of culture not frozen. Thaw 1 vial to test freezing. If cells reattached and are not contaminated, discard back-up culture, if problem, freezing should be repeated.
			2. If total seal of the plastic vials is necessary (e.g., if freezing a virus-containing cell line), use the plastic shields from NUNC.

***Note****:* All vessels that come in contact with specimens or cultures from specimens are biohazardous and should be discarded in appropriate containers following the guidelines for disposal**.**

REFERENCES

1. Shannon JE, Macy ML. Freezing, storage and recovery of cell strains. Chapter 8 in: Tissue Culture Methods and Applications. Eds. Kruse PF, Patterson MK, 1973.
2. Barch MT, Knutsen T, Spurbick JL The AGT Cytogenetics Laboratory Manual. Raven Press, 3rd edition, 1997
3. Freshney RI. Culture of Animal Cells, Ch 19, Cryopreservation. Wiley-Liss, 4th edition, 2000.

Written By: Director Approval:

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