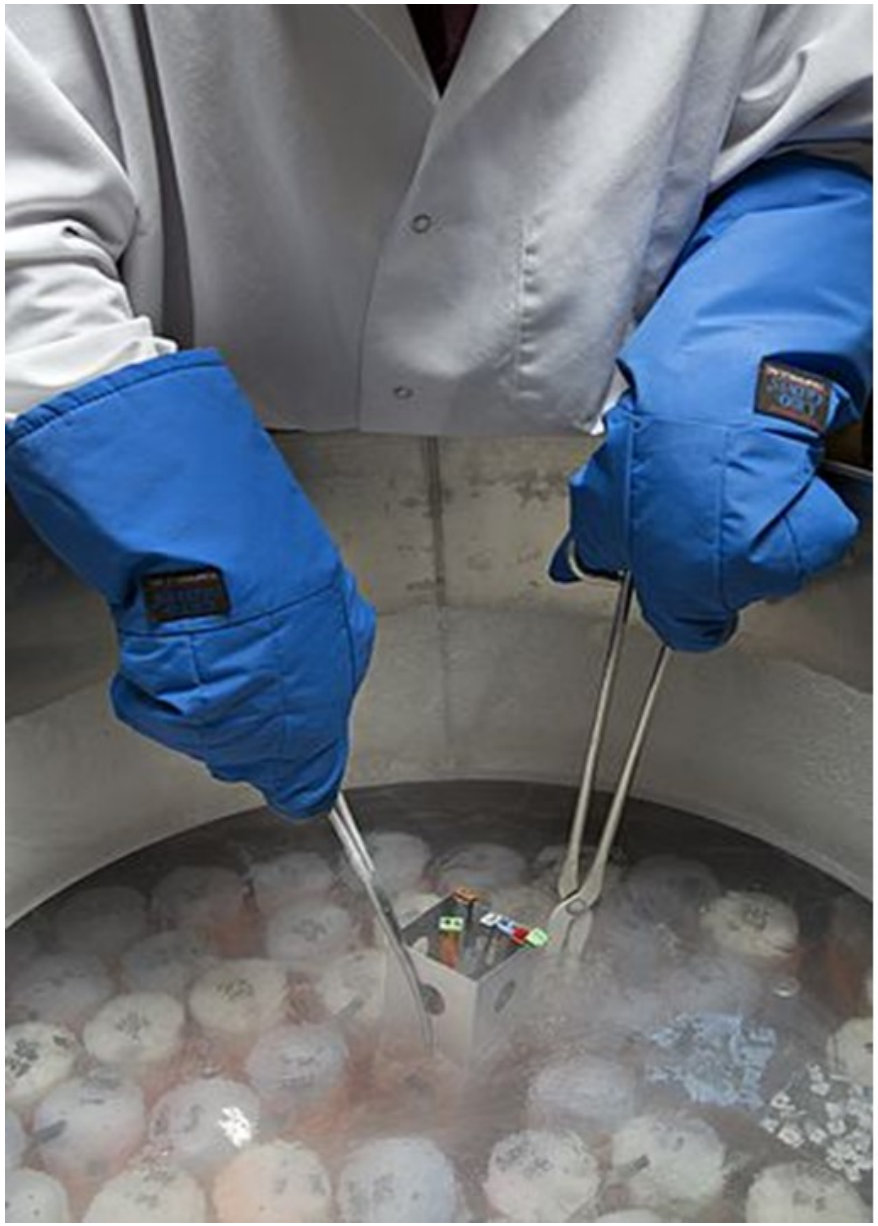

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POST GRADUATE (P. G.) COURSE, MONSOON SEMESTER, 2020

VMC 609: TECHNIQUES IN MICROBIOLOGY AND IMMUNOLOGY

Topic: Reconstitution of preserved cell lines

Dr Manoj Kumar

Assistant Professor, Department of Veterinary Microbiology, Bihar Veterinary College, Patna.

RECONSTITUTION OF PRESERVED CELL LINES

- Unlike the freezing process, rapid thawing of frozen cells is necessary to maintain viability.
- Cryopreserved cells are fragile and require gentle handling.
- Cryopreserved cells are thawed quickly and plated directly into complete growth medium.
- If cells are particularly sensitive to cryopreservative (DMSO or glycerol), they are centrifuged to remove cryopreservative and then plated into complete growth medium.
- The following are suggested procedures for thawing cryopreserved cells.

METHODS OF RECONSTITUTION

1. Direct Plating Method
2. Centrifugation Method

DIRECT PLATING METHOD:

Remove cells from storage and thaw quickly in a 37°C water bath.

Plate cells directly with complete growth medium.

Use 10 to 20 ml of complete growth medium per 1 ml of frozen cells. Perform a viable cell count.	Cell inoculum should be at least 3 × 105 viable cells/ml.

Culture cells for 12 to 24 h. Replace medium with fresh complete growth medium to remove the cryopreservative.

CENTRIFUGATION METHOD

- Remove cells from storage and thaw quickly in a 37°C water bath.
- Place 1 to 2 ml of frozen cells in ~25 ml of complete growth medium. Mix very gently.
- Centrifuge the cells at ~80 × g for 2 to 3 min.
- Discard supernatant.
- Gently resuspend the cells in complete growth medium and perform a viable cell count.
- Plate the cells. Cell inoculum should be at least 3 ×105 viable cells/ml.

PROTOCOL OF RECONSTITUTION (THAWING)

- Remove vials from the cryogenic freezer.
- Directly after removal from storage, vials should be thawed with agitation in a 37°C water bath. with constant gentle shaking until completely thawed (<1 minute).
- As the last ice crystals are melting, the vial is removed from the water.
- Wipe, spray, or wash the vial with 70% ethanol soaked gauge, before opening it in a biosafety hood. .
- Open the cryogenic tube or snap the ampoule aseptically.

- Immediately transfer the contents of the vial into pre-warmed fresh growth medium (Plate cells) following thawing to minimize exposure to the cryoprotective agent.
- For most cultures, the entire contents of the vial may be placed into fresh media.
- For cell lines further dilution may be necessary. It is better that the suspension be centrifuged at 100 x g for 10 minutes after initial dilution, the supernate be removed, and the cells resuspended into fresh growth medium to remove residual cryoprotective agents
- After attachment of monolayer cells, change the medium to remove the cryoprotectant.
- If the cells are non-adherent, allow a sufficient recovery time (about 6 hours), then gently pellet (5 minutes at 400 X g) and resuspend in fresh medium.

DETERMINATION OF RECOVERED CELLS

- Assaying the cells after they have been back in culture for a while, ideally testing not just for viability but functionality as well
- Methods used to estimate the number of viable cells recovered following freezing depend on the type of material preserved.
- Staining and dye exclusion may be effective in determining the presence of viable cells
- Most mammalian cells do not indicate an ability to establish the cell population.

CELL QUANTIFICATION - PROTOCOL

- Equipment
  - Personal protective equipment (sterile gloves, laboratory coat, safety visor)
  - Waterbath set to appropriate temperature
  - Microbiological safety cabinet at appropriate containment level
  - Centrifuge
  - CO_2 incubator
  - Haemocytometer
  - Inverted phase contrast microscope
  - Pre-labelled flasks

- Materials
- Media– pre-warmed to appropriate temperature (refer to the ECACC Cell
- Line Data Sheet for the correct medium and temperature)
- • 70% (v/v) isopropanol in sterile water
- • 0.4% Trypan Blue Solution
- • Trypsin/EDTA

PROCEDURE

1) Transfer cells into suspension using trypsin/EDTA and resuspend in a volume of fresh medium equivalent to the volume of trypsin.
2) Centrifuge cell clumps and resuspend in a small volume and gently pipette to break up clumps.
3) Under sterile conditions remove 100-200µl of cell suspension.
4) Add an equal volume of Trypan Blue (dilution factor =2) and mix by gentle pipetting.
5) Clean the haemocytometer.

- Moisten the coverslip with water.
- Slide the coverslip over the chamber back and forth using slight pressure until Newton's refraction rings appear
- Fill both sides of the chamber with cell suspension (approximately 5-10µl) and view under an inverted phase contrast microscope using x20 magnification.
- Count the number of viable (bright cells) and non-viable cells (stained blue)..
- Calculate the concentration of viable and non-viable cells and the percentage of viable cells using the equations below.

FORMULA

- *Viable Cell Count (live cells per millilitre)* $= \frac{Viable\ Cell\ Count\ (live\ cells\ per\ millilitre)}{Number\ of\ large\ corner\ Squares\ counted} \times Dilution\ factor$

- *Non-viable Cell Count (dead cells per millilitre)* $= \frac{Number\ Dead\ Cells\ Counted}{Number\ of\ large\ corner\ Squares\ counted} \times Dilution\ factor$

- *Non-viable Cell Count (dead cells per millilitre)* $= \frac{No\ of\ Viable\ Cells}{Total\ No.of\ Cells} \times 100$

SAFETY PRECAUTIONS

- Certain precautions should be exercised when thawing cells.
- If cells are stored in liquid nitrogen, use tongs and insulated gloves
- Pressure might build up inside the vials as the nitrogen expands into a gas.
- The improperly sealed glass ampoules/vials may explode or shatter, thus wear insulated gloves and long-sleeved laboratory coats and face shield to protect the skin from exposure.
- Carefully observe whether the glass vials have cracked during freezing or thawing.
- Care must be taken not to vigorously agitate vials containing fragile cells such as mammalian cells while warming in water bath.
- Always thaw and open vials containing hazardous material inside a biological safety cabinet.
- Be prepared for exploding and leaking ampoules/vials.

PRECAUTIONS

- Vials stored in liquid nitrogen, especially screw capped tubes, often fill with liquid nitrogen while submersed.
- When these tubes are removed from the tank, the tubes may pressurize and burst.
- As the last ice crystals are melting, the vial is removed from the water.
- Wipe, spray, or submerse the vial with 70% ethanol before opening it in a biosafety hood.

THANKS

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