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BIOL 4140-Fall2021

Protocol
Cryopreservation

Cryopreservation is the freezing of cells for long-term storage and to provide a secure cell stock to protect against contamination, accidental loss, and genetic or phenotypic instability. We have discussed that importance of proper freezing media and technique for successful cryopreservation. In this experiment, we will vary these factors to experimentally determine the success of each freezing approach.

Materials

Sterile

- 2 T25 of cell cultures -approx 50-80% confluent
- Trypsin at 4°C
- D10 complete media
- Freezing Media at 4°C
- Cryovials (4 per group)
- 15ml conical centrifuge vials (2 per group)

Non Sterile

- Ice bucket

Procedure (Freezing)

1. Examine the culture carefully, **both grossly (naked eye)** and **under the microscope**, and characterize the cells – **focusing on cell health, media condition, contamination, and cell density.** Based on your observations, determine if **freezing is appropriate (flat, attached, red pink clear media, 50-80 confluent)** and choose the most confluent cells (flask) for the procedure, if not enough confluent, mix. Remember to **change the media in your remaining flask.**

2. Prepare the hood for aseptic technique and bring all the reagents and materials to the hood. Place materials needed for trypsinization into the hood. Keep the **ice bucked outside of the hood** and store the tube of freezing media in the ice bucket until it is needed. **Note the 2 media you will freeze cells in are D10 alone and Freezing Media.** The media components are listed below.
    a. **D10 which has no DMSO or high serum concentration (D10)**
    b. **Freezing media with 80% BCS, 10%D10, and 10%DMSO (FM)**
3. From your **2 T25 cell cultures, aspirate the medium using a sterile Pasteur pipette.** You can use the same pipette for both flasks as long as you do not bump anything outside of the flasks with the tip of the pipette.
4. Trypsinize cells in each of the 2 flasks (see protocol "subculturing"). *Remember to rinse with 1.5ml trypsin, incubate in a second 1.5ml of trypsin*

and then inactivate trypsin by adding 1.5ml of D10 to each flask after the cells have rounded up.

TRYPSINIZE (both flasks side by side)

a. RINSE

- Add ~1.5 ml trypsin onto the TOP face of the flask (opposite the cells) so the stream doesn't knock cells off.

- Lay flat, rock gently ~5 sec.

- Aspirate it off.

b. SECOND TRYPSIN

- Add ~1.5 ml fresh trypsin.

- Just enough to cover the cells, no more.

c. INCUBATE

- Keep flask FLAT, 2-5 min (longer on bench).

- Check under the scope often: ready cells look round and bright.

- Tilt test: the cell sheet slides down.

- Firm tap on the side frees the last cells.

d. INACTIVATE

- Add 1.5 ml D10 (its serum stops trypsin).

- Pipette over the growth surface a few times to wash cells off and break clumps.

- Avoid foam.

- Each flask now = 3 ml

(1.5 trypsin + 1.5 D10). Two flasks = 6 ml.

5. Combine the cell suspensions by **moving the volume from one flask to the other and mix gently by pipetting up and down**. At this point, there should be **6ml of solution combined.**
6. Divide the cell suspension equally between **two 15ml falcon tubes**. There should be **3ml in each tube.**
7. **Centrifuge the cells at 1000 RPM for 2 minutes to separate them** from the **D10 and Trypsin mixture.**
8. **Aspirate the media**, while taking care not to disturb the cell pellet. Then resuspend 1 cell pellet in your freezing media and the other cell pellet in D10 Media(See step 3, the two media, **D10 and FM**). **Add all 3ml of your respective media to each tube**. Pipette up and down to disrupt the pellet

and mix the cells well. Make sure that you are using different pipettes for each tube.

9. Once the cells are suspended in a homogenous solution **(even mix),** distribute **1.5ml each to 2 cryovials ; so 4 cryovials in total.**
10. Incubate the cryovials according your experimental plan. (Table 1) At the end of the **30minute cold or warm incubation**. I or our TA will place the vials at -80˚C until needed.

Table 1: Experimental Plan for Cryo Lab

Media	Cryovial-1 Slow Cool  *Room temp or ice for 20-30min. Then -80 freezer*	Cryovial-2 Fast Cool  *-20˚C freezer for 20-30min. Then -80 freezer.*
D10	Label: 1-D10 1ml suspension	Label: 2-D10 1ml suspension
FM	Label: 1-FM 1ml suspension	Label2: -FM 1ml suspension

11. Properly clean and shut down the laminar hood.

Procedure (Thawing): The four frozen vials will be thawed before the following Tuesday. In this lab we are preparing a 6-well plate to observe the effects of freezing and preparing a cluster to be used in our Staining lab, next week. This will be done for you, but for your reference, the procedure if provided below.

Sterile

- 6 well cluster (kept in the incubator)
- D10 Complete Media

1. Prepare the hood for aseptic technique and bring the reagents and materials to the hood.
2. Place **2ml of warm D10 media** into the remaining 4 wells of your cluster.
3. Safely remove the cryopreserved aliquots from the -20° freezer (Your vials will be transferred from -80 less than 30min before you come to lab). Warm to room temperature by holding in a warm water bath. This will be a fast thaw so be ready to transfer to your flasks and cluster.
4. Once thawed, **transfer 250µl of each vial to its respective well and 750µl into the respective flask.** (See Table 2)
5. Properly clean and shut down the laminar hood.
6. 24 hours later we will observe under the microscope. Ask yourself the following questions: Does freezing decrease viability? How does DMSO alter viability with or without freezing?

Table 2: Cryo lab-6 well cluster template for cultured and thawed cells

	Directly Seeded	Slow Cool	Rapid Cool
D10	Do Not Disturb	250µl to 2ml	250µl to 2ml
FM	Do Not Disturb	250µl to 2ml	250µl to 2ml