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Freezing Cells Protocol

August 2021 - Capers Zimmerman

Materials:

Cells (in this particular example, Jurkat cells)
Isopropanol (FisherScientific, Cat No. A4511)
DMSO (FisherScientific, Cat. No. D139-1)
HI-FBS (ThermoFisher, Cat No. A3840202)
Sterile 2 ml Cryogenic Vials (Corning, Cat. No. 430661)
Mr. Frosty Freezing Container (Nalgene, Cat. No. 5100-0001)
Drummond Original Pipet-Aid Pipette Controller (Cat. No. 4-000-220)
Sterile 5 ml serological pipettes (VWR, Cat. No. 89130-896)
Sterile 10 ml serological pipettes (VWR, Cat. No. 89130-898)
Sterile 25 ml serological pipettes (VWR, Cat. No. 89130-900)
Sterile P20 Pipette Tips, unfiltered (Corning, Cat. No. 4121)
Sterile Microcentrifuge tubes, 1.5 ml (VWR, Cat. No. 10025-728)
Sterile 50 ml Falcon tubes (VWR, Cat. No. 89039-656)
Nunclon Delta Surface Sterile 96-well Plate (ThermoFisher, Cat. No. 163320)
P20 micropipette (SCILOGEX, Cat. No. 713121209999)
TC20 Auto Cell Counter (Bio-Rad, Cat. No. 1450102)
Cell Counting Slides (Bio-Rad. Cat. No. 1450011)
Trypan Blue Dye (Bio-Rad, Cat. No. 1450013)
Plastic waste container, tri-corner beaker (VWR, Cat. No. BTC0800)
BioBox Plastic Storage for Cryogenic Vials (ThermoFisher, Cat. No. 22-010-1136)
DMi1 Inverted Microscope for Cell Culture (Leica Microsystems, Cat. No. 11526220)
ZOE Fluorescent Cell Imager (Bio-Rad, Cat. No. 1450031)

Experimental Setup:

- If you have not already prepared freezing media for this protocol, you will need to remember to thaw a 50 ml FBS aliquot from the -80ºC freezer ahead of time in a water bath heated to 37ºC (autoclaved, sterile water)
- Grab a sterile 50 ml Falcon tube, a rack, a sterile 25 ml serological pipette, a plastic waste container, the FBS, DMSO, and your cells

    - Make sure to spray all of your reagents - especially the FBS thawing in the water bath - down with 70% ethanol (EtOH) before bringing them inside the tissue culture (TC) hood to make your freezing media
    - Set up your materials from left → right as follows: cells, FBS, 50 ml Falcon tube/rack, DMSO
    - Loosen the caps to all of your reagents/materials before opening your sterile pipette and remember to keep them covered as much as possible when pipetting
    - Using a sterile 25 ml serological pipette, add 45 ml of FBS against the wall of your sterile 50 ml Falcon tube. Discard your pipette into the plastic waste container
    - Using a sterile 5 ml serological pipette, add 5 ml of *DMSO against the wall of the 50 ml Falcon tube (*DMSO is viscous so mix well by pipetting up and down). Discard your pipette
    - Label the top and side of your freezing media as "10% DMSO in FBS" along with the *date (*freezing media will be good for 1-2 months if kept in the fridge)
    - Put your newly made freezing media into the fridge, as it should be kept cold until right before you are ready to use it
    - You can keep the remaining 5 ml of FBS either in the fridge if you plan to use it soon (1-2 months) or discard it
    - DMSO is stored at room temperature and protected from light
- In this case, our Jurkat cells were previously split (see Ferreira Lab **Suspension Cell Culture Protocol**) and cultured with the intention of expanding them to make liquid nitrogen frozen stocks
    - Before beginning the protocol, examine your cells under a tissue culture microscope to ensure they are confluent and healthy (you never want to freeze cells down in the lag phase of cell division since when thawed, they will come out already 'tired' - always freeze cells down in the log phase of cell division)
    - Take 2 representative images of your current cell population either with a phone through the lens of your tissue culture microscope or using the Zoe Fluorescent Cell Imager (for more information on how to take quality pictures of your cells, see Ferreira Lab **Suspension Cell Culture Protocol**, Experimental Setup)
    - Label your pictures with the date (MM/DD/YY), cell type, magnification, and your initials - this will be helpful when thawing the cells so you know exactly what you started with before they were frozen

***Procedures:**

1. *This protocol is written with respect to suspension cell culture for the sake of simplicity. However, the following procedures are also applicable to adherent cell culture, with the only differences being the additional steps that must be taken to initially harvest adherent

cells and the use of plates rather than flasks in most cases. For step-by-step instructions on how to harvest adherent cells, see Ferreira Lab **Adherent Cell Culture Protocol**

2. Spray down the hood with 70% EtOH
3. Resuspend and transfer your cells from their flask or plate to a sterile 15 or 50 ml Falcon tube(s) in order to count the cells
    a. Our Jurkat cells have already been split once before according to the Ferreira Lab **Suspension Cell Culture Protocol**. As such, they are now growing in a T75 flask, from which the total volume of the cell suspension (100 ml) will be transferred to *two sterile 50 ml Falcon tubes (*having multiple 50 ml Falcon tubes will not always be necessary, depends on the volume of the cell suspension in your flask)
    b. Gather two sterile 50 ml Falcon tubes, a rack, and a sterile 25 ml serological pipette. Label your tubes.
    c. Loosen the cap of your flask of cells and the two Falcon tubes prior to unwrapping your sterile 25 ml serological pipette
    d. Making sure you only have your flask of cells in front of you, use your 25 ml serological pipette to resuspend the cells in the flask (do not pipette all the way up or down —> avoid air bubbles). Also wash the surface of the flask to collect any remaining cells
    e. After you've thoroughly resuspended the cells, *pipette 50 ml of the cell suspension in two successive 25 ml additions to the wall of one of the Falcon tubes (*only one pipette is necessary since the Falcon tubes are sterile). Immediately re-cap Falcon tube 1. Repeat this process using the same pipette, except this time adding to the wall of Falcon tube 2. Re-cap tube 2 and discard the pipette into your plastic waste container
4. Now that you've transferred your cells to two Falcon tubes, you need to count them (if you have more than 1 Falcon tube of your cell suspension, be sure to count each individual tube as described in the procedures below and take the <u>average</u> cell number)
    a. You <u>must</u> count the cells whenever freezing them, since it will give you information about their viability and number prior to freezing (helps you determine the number of cryogenic vials you will need/how many stocks you can make)
    b. Successfully resuspending the cells in your Falcon tube in **Step 3d** is <u>key</u>, because we want to be able to trust the cell count. If the distribution of the cells is not equal throughout the entire volume of the Falcon tube(s), the count will not be representative, hence not a useful data point for extrapolation later on
    c. Gather the Trypan Blue dye, a plastic counting slide, a P20 micropipette, sterile P20 pipette tips, and a sterile 1.5 ml microcentrifuge tube *under the hood (alternatively, to reduce waste, use a sterile 96-well plate to create the mixture

each time you count your cells - 1 well/count - keep the plate in the TC hood and covered in between uses)

   i. *we keep these materials inside the TC hood at all times

d. *Invert the closed Falcon tube a couple of times slowly so as to not introduce bubbles (*necessary, because cells begin to deposit in the bottom of the tube the second they are left standing still, but we want to see an equal distribution of the cells throughout the volume of the Falcon tube)
   i. If you leave your cells standing for an extended period of time (more than 5 mins), you will need to fully resuspend them again using a pipette rather than just inverting the tube
e. Then, make a 1:1 mixture of Trypan Blue dye and an aliquot of your cell suspension
   i. Grab a 1.5 ml sterile microcentrifuge tube, taking care to only touch the tube you will use (or use a 96-well plate)
   ii. Loosen the cap of the Trypan Blue you set aside earlier. Then, using a P20 micropipette with a sterile plastic tip, add 10 µL of Trypan Blue to the microcentrifuge tube/well
      1. Make sure you've re-capped the Trypan Blue vial before adding the 10 µL to the microcentrifuge tube/well to avoid spilling the tube
   iii. Discard that tip into your plastic waste container, replace it with a new one, and pipette a 10 µL aliquot of your well-resuspended cells from the 50 ml Falcon tube into the microcentrifuge tube/well
      1. Before adding the 10 µL aliquot of cells to the microcentrifuge tube/well, make sure you've re-capped the Falcon tube containing the remainder of your cells (remember we want to leave the cells uncovered for as little time as possible to ensure sterility)
   iv. Now, pipette up and down in the microcentrifuge tube/well to mix
f. The cell counting chamber accepts 10 µL of volume
   i. After mixing thoroughly, pipette 10 µL of this 1:1 dilution, touch the tip of your pipette to the loading area of one of the chambers on the plastic counting *slide, and slowly release the volume into the chamber (*slides are good for 2 counts - since there are 2 chambers, discard after 2 uses)
   ii. This process works by capillarity - as you slowly pipette down, the Trypan Blue cell suspension mix will fill the chamber on its own. After the chamber fills, discard your tip

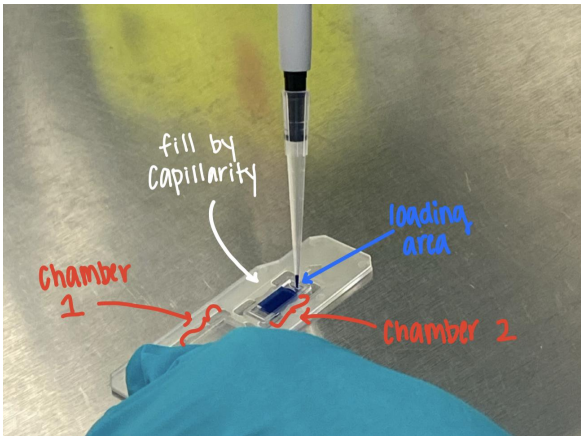

Figure 1. Dual-chamber Cell Counting Slide

iii. Following the directional arrows on the plastic counting slide, insert the slide into the TC20 Auto Cell Counter (use of alternate cell counter ok, but the following procedures will pertain particularly to the aforementioned model)

g. Make note of the *live count and the percent live value (for more detailed information on the significance of each data point reported by the cell counter, see Ferreira Lab **Adherent Cell Culture Protocol**, section **9f**)

i. *The live count is reported in the form of number of cells/ml. Multiply this value by the volume of the cell suspension you have in your Falcon tube to get the total live cell count

ii. It can be easy to forget to multiply the reported live cell count by your total volume so be careful not to incorrectly assume your total live count! **This is why resuspension is extremely important - so that you can extrapolate the number of live cells/ml reported in the 10 µL read by the cell counter to the total number of live cells in the entire volume you have

iii. We do <u>not</u> use cells for experiments that are less than 90% alive

iv. A total cell count is available and can be documented, but it includes both dead and live cells, so we don't typically use this data point

v. The TC20 Automated Cell Counter automatically detects the presence of Trypan Blue in the sample and accounts for the 1:1 Trypan Blue to cells dilution factor. If you ever use a different ratio, make sure to include that in your calculations

h. Repeat **Steps 4c-4g** for the second 50 ml Falcon tube and any remaining tubes you may have. After *measuring the total live count in your Falcon tube(s), determine how many cryogenic vials you can make if aliquoting 5 million cells/vial (*don't forget to take the <u>average</u> cell number if working with multiple Falcon tubes)

i. Here, the total live count was measured to be ~ 50 million cells. Thus, 10 aliquots can be made
j. Before aliquoting the cells for freezing, create a maintenance flask to keep a minimal number of your cells in culture (see Ferreira Lab **Suspension Cell Culture Protocol**). Then, any other leftover cells should be killed with 10% bleach, dumped down the sink, and any empty plastics discarded into a biohazard bin

5. Spin down your 50 ml Falcon tube(s) at 500 G for 5 mins to pellet the cells
    a. While you wait for the centrifugation, grab the number of cryogenic vials you need and a rack. Label the vials with the cell line name, cell number, your initials, and date
    b. Prepare a Mr. Frosty freezing container by adding 250 ml of isopropanol to the bottom of the container. This is where your cells will be stored overnight in the -80ºC freezer before being transferred to liquid nitrogen. The Mr. Frosty allows the cells to cool down slowly, at a rate of 1ºC per minute. Each time you use the Mr. Frosty, add a tally to the sticker on the top of the container
        i. If the container was previously in use and already contains isopropanol you may not need to complete **Step 5b**. However, make sure to look at the number of uses indicated by tallies on top of the container since the isopropanol needs to be changed every 5 uses
        ii. When it does need to be changed, dump the used isopropanol down the sink and add 250 ml of fresh isopropanol (kept at room temperature)
6. After centrifugation, return the tube(s) to the TC hood in order to aspirate the exhausted media
    a. Turn the vacuum on by moving the white lever inside the hood from its initial vertical position to a horizontal position
    b. Loosen the cap of your Falcon tube(s)
    c. Cap the sterile glass pipette attached to the vacuum with a sterile 10 µL unfiltered plastic pipette tip and aspirate the media from each tube (if you have more than one Falcon tube, only one sterile plastic pipette tip is necessary since it is the same cell line in each tube)
        i. After you aspirate the media from one tube, immediately replace the cap before moving on to the next tube. Once you've aspirated the media from all of your tubes, screw the cap all the way back on
7. Grab a sterile 10 ml serological pipette and the freezing media from the fridge
8. Resuspend the cell pellet by scraping the tube(s) across the grate of the TC hood (**Fig. 2**)
    a. *cells tend to clump together when the FBS/DMSO freezing medium is added and it's very important to freeze single cell suspensions!

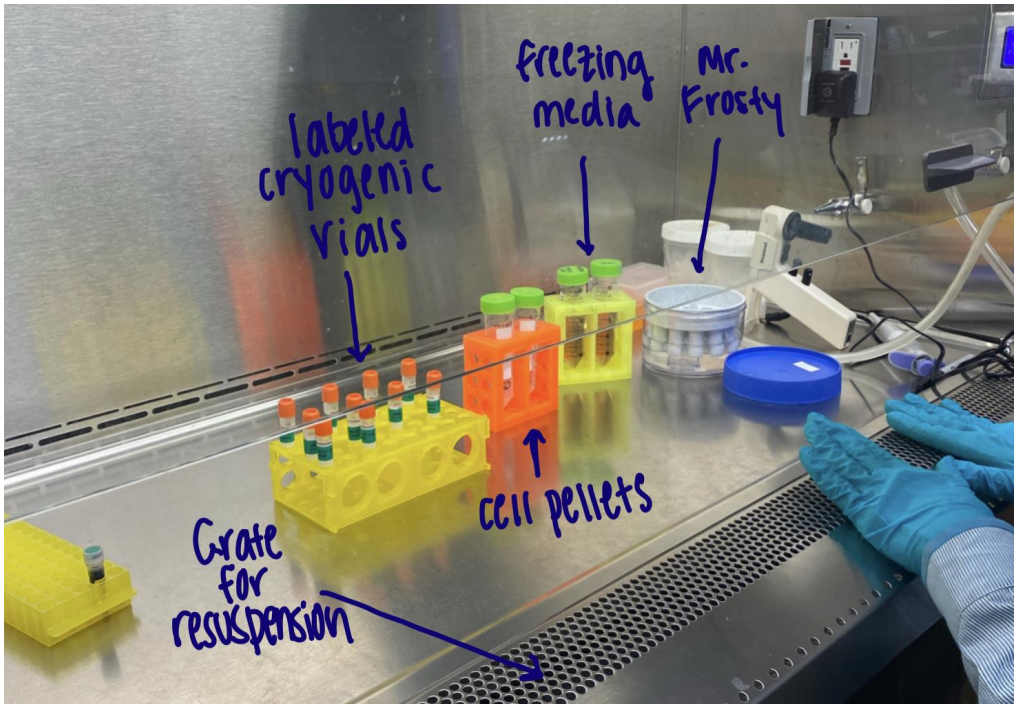

Figure 2. Organizing the Workspace in the Tissue Culture Hood to Freeze Down Cells

9. Remove the caps to all of the cryogenic vials and set them aside facing down (it's ok that the vials are exposed because otherwise the process would not be *quick enough)
    a. This step is important because it will help streamline the process of adding the cell suspension to each cryogenic vial (*needs to be done quickly since DMSO is ideal for freezing cells, as it prevents ice crystal formation, but toxic to cells at room temperature —> need to get them in the -80ºC fast)
10. Double-check that all of the necessary materials are present and that all of the caps to your cryovials have been removed. Then, *resuspend the pellet(s) once more by scraping your Falcon tube(s) across the grate of your hood
    a. *It's important to resuspend a second time since the cells will be prone to clumping once combined with the freezing media in the next step
    b. Loosen the caps to your freezing media and Falcon tube(s) containing the cells
11. Use a sterile 10 ml serological pipette to resuspend the cells in 10 ml of freezing media (be thorough and quick to avoid clumps)
    a. Use your 10 ml serological pipette to take up 10 ml (1 ml x # of cryovials) of freezing media and add the volume to one of your Falcon tubes (without pipetting all the way down/up —> resuspend the pellet in freezing media in your Falcon tube)
        i. Do NOT create bubbles while resuspending. We do not want to freeze bubbles!
        ii. Remember to pipette against the wall of the tube and re-cap the freezing media tube after obtaining the necessary volume

b. If you have more than one Falcon tube, fully resuspend the cells in tube 1, pipette all the way up, and transfer the entire volume of the cell suspension into tube 2. Do not pipette all the way down as you're transferring the volume to tube 2 - instead, resuspend the pellet in tube 2 such that all of your cells are now suspended in 10 ml of freezing media in tube 2 (this step is exactly like the single cell suspensions detailed in the Ferreira Lab cell culture protocols, except now with the cells in FBS/DMSO freezing medium instead)
    i. Avoid bubbles!
c. After you've resuspended both pellets in 10 ml of freezing media in tube 2, take up the entire volume (10 ml) and successively add 1 ml of the cell suspension to each cryovial (pipette against the wall of the vials)
    i. Be careful with your volume so that when you reach the last cryovial you're not pipetting bubbles

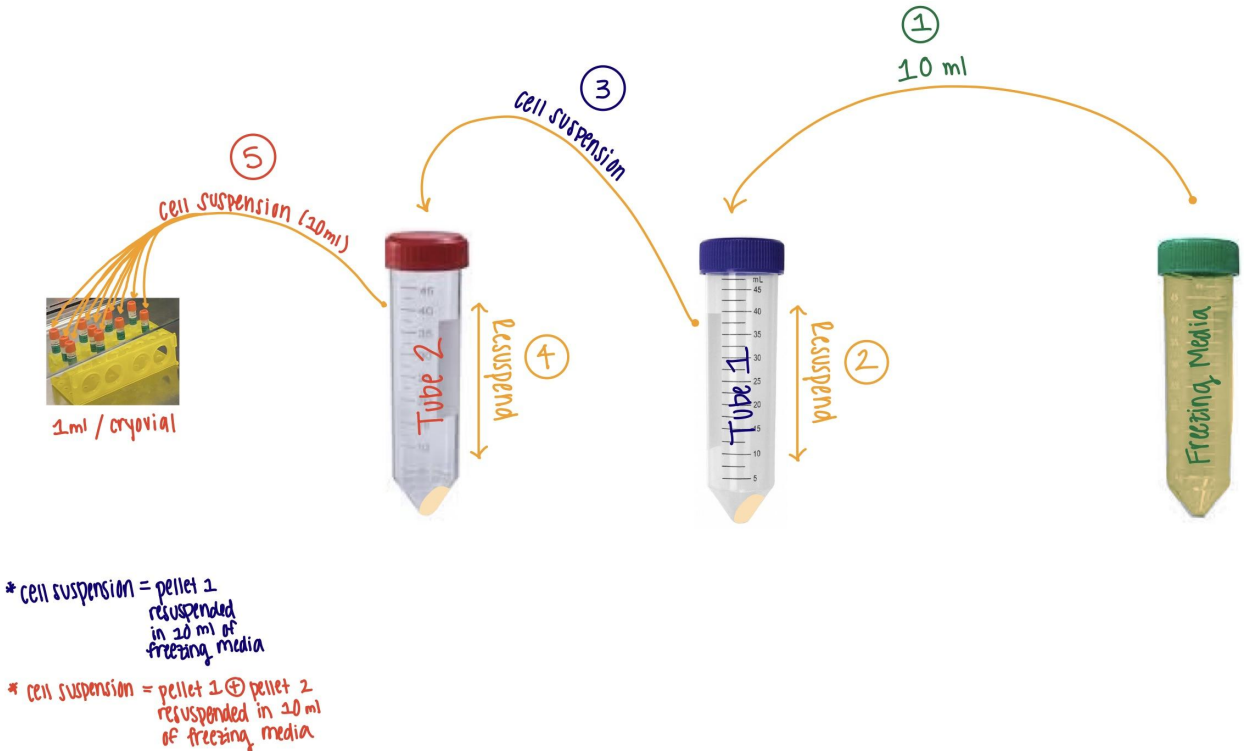

Figure 3. Workflow to Freeze Down a Large Number of Cells (in Two 50 ml Falcon Tubes)

d. If only working with one cell pellet in one 15 or 50 ml Falcon tube, add 10 ml of freezing media to the wall of the Falcon tube and resuspend the pellet. After resuspension, take up the entire volume of the Falcon tube and distribute 1 ml/cryovial
e. Discard your pipette and close all of the cryovials tightly
f. Immediately place all of the cryovials in the Mr. Frosty freezing container, close the lid, and place the container in the -80ºC freezer overnight
    i. After 24 hours, take the cryovials out of the freezing container, put them into a cryogenic plastic storage box, and then into your liquid nitrogen

tank (make sure you use protective gloves when working with liquid nitrogen)

ii. After you are done using the Mr. Frosty freezing container, you can set it aside and leave it at room temperature until the next use

g. If you have leftover freezing media, store it in the fridge (should be good for reuse for 1-2 months)

Supplemental Information:

- The Mr. Frosty freezing container with isopropanol gradually brings the cells down in temperature at 1ºC per minute, all the way down to -80ºC. This is why the cells must be left in this container in the freezer overnight
- Each Mr. Frosty has 18 spots for 18 cryovials
- For some cell lines, passage number is very important. Hence, if we were making HEK293T frozen stocks, we'd want to make as many cryovial stocks in one go as possible. HEK293T cells are necessary for viral production; the lower their passage number, the better the viral production. Thus, the more stocks we have at one time, the more we can avoid using higher passage number HEK293T cells
- When adding 1 ml of the cell suspension (cells + freezing media) to each of the cryovials, we technically could have used a micropipette. However, we opt to use a serological pipette due to ease/speed, but also because it is entirely sterile. Since a micropipette is not disposable, only the tip, the entire instrument is never fully sterile. Thus, we avoid micropipettes whenever possible in cell culture
- Although the number of cells in your entire volume of cell suspension will vary, it is common to aliquot ~5 million cells/vial. However, when freezing cells, be careful not to freeze too few of them or they will not survive (needs to be > 100,000 cells). Freezing too many cells in one vial is not ideal either because as soon as the cells are thawed, they will not fit in the flask/amount of medium - keep these parameters in mind when determining how many cells you aliquot per cryovial