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Suspension Cell Culture Protocol

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Materials:

Complete RPMI medium components:

Product	Amount Used	Supplier	Cat. No.
RPMI1640	500 ml	ThermoFisher	21870092
FBS	50 ml	ThermoFisher	A3840202
HEPES	5 ml	ThermoFisher	15630130
Pen-strep (P/S)	5 ml	ThermoFisher	15140163
Nonessential amino acids (NEAA)	5 ml	ThermoFisher	11140050
GlutaMAX (gMAX)	5 ml	ThermoFisher	35050079
Sodium pyruvate (Pyr)	5 ml	ThermoFisher	11360070

Cells (in this particular example, Jurkat cells)
RPMI Media (see above and Ferreira Lab **Making Media/Sterile Technique Protocol** using RPMI1640 as the basal medium, rather than DMEM)
Millipore Stericup 500 ml Durapore 0.45µm PVDF (Cat. No. SCHVU05RE)
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. 89130-888)
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 15 ml Falcon tubes (VWR, Cat. No. 62406-200)
Sterile 50 ml Falcon tubes (VWR, Cat. No. 89039-656)
Sterile T25 Tissue Culture Flask, .2µm vented cap (Corning, Cat. No. 430639)

Sterile T75 Tissue Culture Flask, vented cap (CytoOne, Cat. No. CC7682-4875)
Nunclon Delta Surface Sterile 96-well Plate (ThermoFisher, Cat. No. 163320)
P20 micropipette (SCILOGEX, Cat. No. 713121209999)
TC20 Automated 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)
DMi1 Inverted Microscope for Cell Culture (Leica Microsystems, Cat. No. 11526220)
ZOE Fluorescent Cell Imager (Bio-Rad, Cat. No. 1450031)

Experimental Setup:

- If you need to make fresh RPMI media for this protocol, remember to thaw a 50 ml FBS aliquot from the -80ºC freezer and a 5 ml Penicillin-Streptomycin (P/S) aliquot from the -20ºC freezer ahead of time in a sterile water bath heated to 37ºC (autoclaved water)
    - Make sure to spray all of your reagents - especially the FBS and P/S aliquot that were in the water bath - down with 70% ethanol (EtOH) before bringing them under the tissue culture (TC) hood to make your media
    - Label the top and side of your media with its components, the date, and your initials
    - For step-by-step directions, see Ferreira Lab **Making Media/Sterile Technique Protocol**
- In this case, our Jurkat cells were already growing in culture in a T25 flask and it is now time to split them
    - These cells need to be split 2x/week (the Ferreira Lab opts to split these cells every Monday/Thursday, leaving a 3.5 day incubation period in between each split)

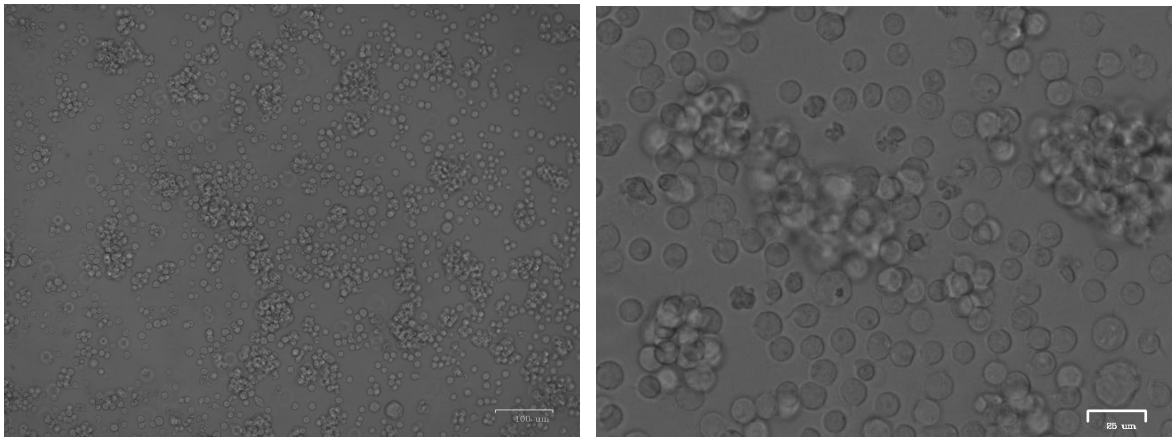

Figure 1. Healthy Jurkat Cells Ready to be Split (magnification left to right: 4x, 16x)

- Before starting the protocol, always examine your cells under a tissue culture microscope and photograph the current condition of the cells (can be done by capturing a picture with your phone through one of the microscope lenses or using the ZOE Fluorescent Cell Imager)

    - Aim to capture two pictures that are representative of your entire population of cells. For example, if there are areas with lots of clumps, but also areas where the cells are nicely spread out, be sure to capture photos of both conditions so that you know exactly what you have in that cell population
        - Label your pictures with the date (MM/DD/YY), cell type, magnification, and your initials
    - The first step is to find the cells. If you can't find any cells whatsoever, resuspend them inside the TC hood and try to view them under the microscope again
    - If the cells are clearly there, but not easily visible, try using a phase contrast slider (for our specific microscope, we've found that the cells are best visualized using the 5F0 setting of the phase contrast slider)
        - If your pictures look dark, increase the LED intensity or gain to brighten them
- Make sure you are using RPMI as your basal medium rather than DMEM since Jurkat cells prefer RPMI media
- If you're expanding your cells transferring them from a T25 flask (25 cm^2 area, holds 20 ml maximum volume, standing upright) to a T75 flask (75 cm^2 area, holds 200 ml maximum volume, standing upright): prepare a rack, a sterile 10 ml serological pipette, a plastic waste container, a sterile T75 flask, and a sterile *50 ml Falcon tube to transfer your cells to (*a 15 ml Falcon tube will not be sufficient in this case since we have 20 ml of cells pre-existing in the T25 flask)

Procedures:

1. If your suspension cells are frozen, first see Ferreira Lab **Thawing Cells Protocol**. In any case, begin by spraying down the hood with 70% EtOH
2. We will be transferring the cells from a T25 flask to a T75 flask - this will give the cells more room to grow
3. Set up your materials and begin by loosening the caps to each of your reagents so you are ready to pipette easily from each one (make sure your cells are the only thing directly in front of you —> maintain sterile technique)
    a. Make sure the cap of your sterile 50 ml Falcon tube is loosened before you open your sterile 10 ml serological pipette, as well as the cap of the T25 flask
    b. Also, make sure the scale of the pipette is turned towards you before opening its packaging since it cannot be adjusted later —> maintain sterile technique
4. Use a sterile 10 ml serological pipette to transfer the *20 ml of cell suspension in the T25 flask to the sterile 50 ml Falcon tube. Then, resuspend the cells
    a. First, tilt your flask at a vertical angle so that all of your volume is collected in a bottom corner of the flask. Take up part of this volume and use it to wash the inside surface of the flask, collecting any cells left behind in the bottom corner of the flask. During this process, remember not to pipette all the way up or down so you don't introduce any bubbles (make sure the tip of the pipet is submerged in liquid at all times)

b. After you've thoroughly washed the surface of the flask, pipette up and down to resuspend the cells. Then, pipette all the way up and transfer everything to the 50 ml Falcon tube (pipetting slowly against the wall). Again, do not pipette all the way down in the tube - instead, pipette almost all of the volume and then pipette up and down, without introducing any bubbles, to *resuspend the cells
    i. *Resuspension is very important here since the next step will be to count your cells (ideally, every time you split your cells you want to count them to ensure we are re-seeding the cells at the same density; for Jurkat cells, a seeding density of 1-2 x 10^5 /ml is recommended)
    ii. To avoid making bubbles, make sure the tip of your pipette remains fully submerged in the liquid as you pipette up and down
    iii. Once you've resuspended the cells, re-cap the 50 ml Falcon tube and discard your 10 ml serological pipette into the plastic waste container inside the TC hood
c. *Because your tip cannot come in contact with air, there will always be some dead volume left in the flask. Hence, some loss in total volume of the cell suspension is to be expected

5. Count the cells
    a. Successfully resuspending the cells in your Falcon tube in **Step 4b** is key, 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, the count will not be representative, hence not a useful data point for extrapolation later on
    b. 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
    c. *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
    d. 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

e. 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

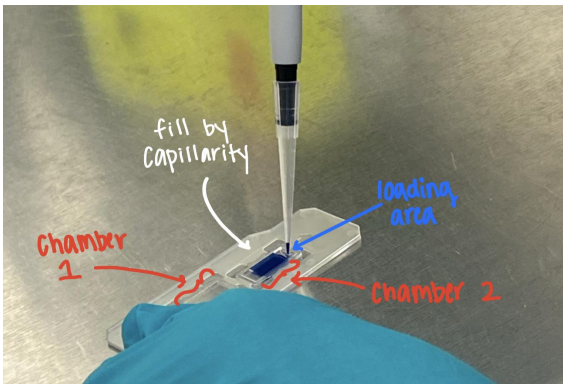

Figure 2. Dual-chamber Cell Counting Slide

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)

f. 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

6. *Spin the 50 ml Falcon tube containing 20 ml of cell suspension down at 500 G (also called "RCF" in some centrifuges) for 5 mins (be sure to include a 20 ml water balance)
    a. This will force your suspension cells into a pellet, so that the exhausted media the cells are currently in can be aspirated
    b. This step is required to make liquid nitrogen frozen stocks of your cells. For this reason, we will be using all of the cells contained in the volume of the Falcon tube. However, if you are only trying to split your Jurkat cells (e.g. 1:10), there is no need to keep the entire volume of the cell suspension. In that case, **Step 6** can be skipped altogether, since you will not need to pellet the cells (the exhausted media your aliquot of cells are in does not need to be aspirated, as once the new medium is added, the 1:10 dilution with fresh medium will render the effect of any metabolites/dead cells negligible)
    c. After centrifugation, it's important to aspirate as much of the residual medium as possible, because it contains dead cells and toxic metabolic by-products, such as lactate, that the cells produce as they are actively growing
        i. This is what makes the media yellow when the cells have been growing for a few days (RPMI contains a pH indicator, phenol red, which changes color from red to yellow when the medium becomes too acidic from excess lactic acid - it is a sign that the media needs to be changed)
7. Once the pellet has formed, turn the vacuum on (make sure the collecting flask is not full), and *aspirate the exhausted media from the 50 ml Falcon tube using a glass pipette capped with a sterile 20 µL plastic pipette tip
    a. *When aspirating, always use the vacuum. Its collecting flask contains bleach that will prevent anything from growing in it, causing contamination. Theoretically, you could just pour out the supernatant, but in practice it is safer and more convenient to use the vacuum
    b. Aspiration in this step does not have to be particularly precise, since we are going to resuspend the pellet in the same type of media
    c. When you are done aspirating, discard the plastic pipette tip into your waste container and immediately re-cap the 50 ml Falcon tube (keep the cells covered as much as possible)

8. Grab a sterile 25 ml serological pipette and a sterile T75 flask - this will be the new, bigger flask we will transfer our cells to so that they have more room to grow. Loosen the caps to the T75 flask and the media bottle
9. Resuspend the pellet of cells in your 50 ml Falcon tube in *100 ml of fresh RPMI media
    a. *Because 100 ml is too much to pipette in the Falcon tube or the T75 flask, we will split the volume between the Falcon tube and the flask
    b. After we add the fresh media, we resuspend the cells. Whenever we mix cells in a tube, to pipette safely, the tube should be no more than ⅔ of the way full. Thus, we will only add 30 ml of fresh media to the 50 ml Falcon tube and 70 ml to the new T75 flask.
    c. Use your sterile 25 ml serological pipette to first add 35 ml (up to "-10" on the pipette graduation) of fresh RPMI media to your vertically-oriented sterile T75 flask. Repeat this procedure for a second time, resulting in a final volume of 70 ml of fresh media added to the flask
        i. Only one pipette is necessary since both the media and the flask are sterile
        ii. Make sure you're covering the media bottle after obtaining the necessary volume each time (this also applies to the flask, replace the cap after each addition of fresh media)
    d. Using the same 25 ml serological pipette, *add 30 ml (up to "-5" on the pipette graduation) of fresh media to the 50 ml Falcon tube and resuspend the cell pellet
        i. *Slowly add the volume to the side of the Falcon tube, but do not pipette all the way down or all the way up. Mix the cells without introducing any bubbles
        ii. Once resuspension is complete, transfer the entire volume of the cell suspension to the new T75 flask containing 70 ml of fresh medium. Pipette slowly and against the wall of the flask to prevent bubbles
        iii. This resuspension may take longer than others because we are using a 25 ml serological pipette - this is for 2 reasons:
            1. It's a bigger volume
            2. The bigger the size of the opening of the pipette, the more cells will happily pass through in clumps (smaller pipettes force cells through a smaller opening so they are less able to remain clumped together)
    e. Make sure you've labeled your new flask with the cell type, cell number, the date, the dilution, your initials, and passage number
    f. It is not necessary to 'Do the Cross', unlike with adherent cell culture (see Ferreira Lab **Adherent Cell Culture Protocol**), since suspension cells will not unevenly attach to the surface of the flask. Simply place the flask in the incubator and this gentle movement will be enough to spread the media and the cells around
        i. Placing the flask in the incubator horizontally will increase the rate of growth of Jurkat cells (due to increased surface area)

ii. Placing the flask in the incubator vertically will decrease the rate of growth of Jurkat cells (this is a trick to use when you want to slow down their growth)

10. Return the media to the fridge and turn the vacuum off if you have not already done so
11. Spray the hood down with 70% EtOH and turn on the UV light for ~30 seconds
12. We will now let the Jurkat cells expand in the T75 flask for 24-48 hours. Then, we will harvest, count, and freeze most of them down to make liquid nitrogen stocks (see Ferreira Lab **Freezing Cells Protocol**). However, depending on the experiment, you can also *keep your suspension cells in culture (*be sure to continuously split them according to this protocol at least 2x/week)

Creating a Maintenance Flask:

- Because Jurkat cells grow so quickly, often when culturing and splitting the cells there will be too many to use all of them. Most of them will be discarded each time, but you can also use a small portion of the cells to create a maintenance flask, in which you will keep the bare minimum amount of cells in culture
- Jurkat cells like to grow at 1-2 x 10^5 cells/ml so this is the standard dilution we will use when making a maintenance flask

1. First, resuspend the cells and count them, as described above in **Step 5**
    a. Counting is optional for maintenance flasks - you can split your cells 1:5, 1:10, or 1:20 (depending on approximately how many cells you have, how many you will need, and the timeline of your experiment)
2. When making a maintenance flask, we will only add the bare minimum number of cells to the flask, since Jurkat cells already grow so quickly and we do not plan on using these cells immediately
    a. After having counted your cells, they should be resuspended in RPMI medium (complete with its added components) in a 15 ml Falcon tube (before this you should have vertically oriented the flask they were originally in and resuspended the cells)
    b. Grab a sterile T25 flask with a vented cap, RPMI media from the fridge, a plastic waste container, and two sterile 5 ml serological pipettes
    c. Label the T25 flask with the cell line name, the date, the cell number, your initials, and the passage number
    d. If your cell suspension has been left standing for a few minutes, invert the Falcon tube slowly a couple of times before beginning this process
    e. Loosen the caps to your media, flask, and Falcon tube
    f. Use a sterile 5 ml serological pipette to add 4.5 ml of RPMI medium to the T25 flask
        i. Remember to replace the cap to the media before adding to the flask and subsequently to replace the cap to the flask after adding the media
        ii. Discard your pipette into the plastic waste container

g. Grab a new sterile 5 ml serological pipette and briefly resuspend the cells in the Falcon tube. Then, add 0.5 ml of your cell suspension to the T25 flask (slowly, against the wall —> no bubbles)
    i. Remember to re-cap the Falcon tube before adding the volume to the flask and subsequently to recap the flask after adding your cells
    ii. Discard your pipette into the plastic waste container
h. Tightly close the vented cap on the T25 flask and place it in the incubator, vertically-oriented (slows their growth)
i. Close the media and return it to the fridge. Put any other remaining materials away if you are only making a maintenance flask (rather than continuing on to freeze the remainder of your cells in suspension in the Falcon tube)
j. Empty your solid plastic waste container into the biohazard bin outside the hood and discard any unused cells by first adding 10% bleach, emptying the contents in the sink, and discarding the empty plastics also in the biohazard bin
k. This maintenance flask will need to be split twice a week so that it does not overgrow (Ferreira Lab has opted to split on Mondays and Thursdays, leaving a 3.5 day incubation period in between each split)

Supplemental Information:

- Suspension cells are more simple to culture than adherent cells because they don't grow as monolayers
    - It's easy to tell what these cells need, e.g. if the media is yellow, then we know the cells need to be split
    - They are also easier to maintain as they can be resuspended directly - there is no need to first wash them with PBS, detach them with trypsin, and then seed them carefully to ensure a uniform distribution on the surface of the new plate or flask
- When resuspending, it is helpful to use certain graduations on your serological pipette as guidelines to keep liquid at the tip of your pipette at all times. For example, when using a 10ml serological pipette in cell culture, staying between 1 and 5 ml reduces the risk of creating air bubbles and ensures you never pipette too far up or down
- The suspension cell culture protocol is significantly easier and less time consuming than the adherent cell culture protocol since we do not have to do much to harvest the cells (basically only have to resuspend the cells and transfer them into a new flask). In contrast, adherent cells must first be washed with PBS and then trypsinized in order to harvest them. These beginning steps to get the cells to work with in the first place are what adds many extra steps to the adherent cell culture protocol
- However, it is also hard to distinguish alive versus dead suspension cells because they don't just peel off the plate like adherent cells. They're suspended so we must rely on looking at certain characteristics like cell shape
- Suspension cells are thawed in a T25 flask in 5 ml of media. The flask can then be placed in the incubator horizontally

- Jurkat cells are pretty sturdy, resilient cells so even if they aren't looking great one day, you could change the media and it's possible they would fully recover
    - Keep them in culture until they are actively expanding and healthy - only freeze cells down when they are healthy. Never freeze cells in the lag phase - only in the log phase of cell division (the cells will thaw out and already be 'tired' if they are frozen down in the lag phase)
- The RPMI media we use has no glutamine, since it degrades and creates toxic ammonia
- When making the 1:1 mixture of Trypan Blue and an aliquot of your cells, we use a P20 micropipette because the fastest/best cell suspension is done using a micropipette
    - Micropipette tips are the smallest openings the cells can be forced through so they are more efficient at resuspending the cells - this is why we use micropipettes when making this mixture since it needs to be very well mixed in order to yield an accurate cell count
    - To verify that the 10 µL sample you inserted into the chamber of the cell counter was representative of the entire volume of cells in your Falcon tube, look at the images produced by the cell counter
        - If you see lots of clumps, that means you did not properly resuspend the cells and the count you are getting is not accurate.You should attempt to resuspend the cells again and then re-do the count