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General protocol for the culture of adherent mammalian cell lines

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Clark Fritsch[1]

[1]University of Pennsylvania

Clark Fritsch
University of Pennsylvania

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ABSTRACT

The purpose of this protocol is to give a general overview of the various methods associated with the culture of adherent mammalian cells. These methods include the use of aseptic technique, maintaining mammalian cell lines, passaging mammalian cell lines and freezing / thawing mammalian cell lines.

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Maintenance of Aseptic Technique and Setup of Work Environment

1 Mammalian cell lines are extremely vulnerable to bacterial and fungal contamination from the environment, as they do not have immune systems of their own. Because of this, extra care must be taken to avoid contamination. This section will cover the various steps that you must take to maintain a sterile working environment for mammalian cell culture, beginning from the moment you enter the cell culture room. Note that aseptic technique must be maintained at all times during cell culture and is perhaps the most time-consuming step of the process.

1.1 Before entering the cell culture room, gather all of the materials that you will need in a container. Once you have entered the cell culture room it is best to minimize the number of times that you leave to get materials, as the constant travel between the cell culture room and the outside environment increases the chances of contamination. Because of this, it is best to maintain separate stocks of materials such as pipets, pipette tips, serological pipettes, and ethanol stocks in the cell culture room. Because of the high cost of pipets, this is not always realistic. If this is the case for you, simply make sure to thoroughly wipe off your pipets with 70% ethanol prior to entering the cell culture room.

1.2 Upon entering the cell culture room there should be a sticky mat in front of the door to remove dirt and other potential contaminants from your shoes.

1.3 After ensuring that your feet are clean, it is important to put on your lab coat. Some labs also require that you place covers on top of your shoes. Lab coats and other personal protective equipment used for cell culture should be housed exclusively in the cell culture room and should ideally never be taken out of the cell culture room. However, this is often unrealistic (often ignored), as you may need to leave the cell culture room while wearing your cell culture lab coat to get something.

1.4 After putting on your lab coat, put on your gloves. You should always wear gloves when handling anything in the cell culture room. It is common to wear two pairs of gloves at a time. This way, if you ever get your topmost pair of gloves dirty, you can simply take them off, dispose of them, and put on a clear pair on top of the first layer of gloves.

1.5 After you have put on your gloves, it is advised to place any cell culture medium that you have in a hot water bath or a bead bath set to 37^{o}C. This includes not only Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS), but also reagents such as Trypsin, PBS and Versene.

- Note that anything that comes into contact with your cells should be warmed up to 37^{o}C to prevent cold-shocking your cells.

- Note that, if using a hot water bath, it is necessary to use an anti-fungal agent to keep the water bath clean. Water baths are potential sources of contamination and must be checked regularly for signs of bacterial or fungal growth.

- Because of potential contamination issues, some labs have switched to using bead baths, which contain metal beads rather than water. These bead baths, while being largely sterile, tend to not

be as good as water for warming up reagents (the temperature of the beads is not always uniform throughout the bath, warming up tends to take longer, etc.).

1.6 Depending on the volume of your reagents, it may take some time to warm up your reagents. In the case of large aliquots of FBS, it is not uncommon to take upwards of an hour to thaw the FBS and then allow it to warm up fully.

1.7 Before leaving your reagents to warm up, however, go to the cell culture biosafety cabinet, turn off the UV light, spray the inside of the cabinet with 70% ethanol, wipe it down with either your clean gloves or paper towels and turn on the blower for the cabinet. Let the blower go for at least 30 minutes before using the hood. It is not uncommon for this to be the first thing people do in the morning. Note that once you turn on the blower, you should keep it on all day (or until you and everyone else who is doing cell culture is done).

1.8 Once you have placed your reagents in the hot water bath or bead bath and turn on the blower in the biosafety cabinet, you will need to wait for a while. When exiting the cell culture room, make sure that you dispose of your gloves in a trashcan meant for biohazardous waste (must have a lid on it that can be opened with a pedal at the base) and hang your lab coat.

- Note that most labs have at least two trashcans in their cell culture rooms. The first trashcan is for normal trash

such as paper towels and serological pipette wrappers (anything that has not come into contact with your cells or
cell culture reagents). The second trashcan is typically for biohazardous waste (anything that has come into
contact with your cells or reagents used for cell culture). This biohazardous waste container must be able to
handle sharps (such as pipette tips and serological pipettes). As stated above, it is also important that you can
open the container with your foot, as you don't want to be opening it with your hands. Both trashcans should be
placed in close proximity to the biosafety cabinet, so that you have the option to dispose of waste materials as
necessary (more on this later).

1.9 After your reagents have warmed up to 37^{o}C and the biosafety cabinet has aired out for at least 30 minutes, you will want to repeat Steps 2 – 4.

1.10 After repeating Steps 2 – 4, you will want to begin transferring materials from the cell culture room to the biosafety cabinet. Note that from this point onwards, extra care must be taken to preserve aseptic technique. To ensure that everything that goes into the cabinet is free of bacterial or fungal contamination, the first thing that you need to do is wipe your gloves down with 70% ethanol. Although this is the first thing you want to do before moving materials into the biosafety cabinet, you will need to wipe your gloves down again anytime you touch something outside of the cabinet. For example, if you are working in the cabinet and open a drawer to get something, you must not only assume that whatever you got from the drawer is dirty, but also that your gloves are dirty. Because of this, it is not uncommon to use to large amounts of 70% ethanol per session of cell culture. Be careful not to slip on the floor.

- Note that it is nice to have the 70% ethanol in a spray bottle that can dispense the ethanol in a mist rather than a

focused stream.

1.11 After washing your gloves with 70% ethanol, you will want to move all materials that you will need that are not already in the cabinet into the cabinet. As stated above, you will want to wipe off all of these materials with 70% ethanol before placing them in the biosafety cabinet. Do not, for example, wipe something off with ethanol and then put it back on the counter. Pick up the material, wipe it off, and put it directly into the cabinet.

1.12 In the case of reagents that have been in the water bath, first wipe the water off with a paper towel, and then wipe them off with 70% ethanol.

1.13 It is useful to have a list of materials that you will need for your cell culture session hung up near the biosafety cabinet. Ideally, once you start working in the cabinet, you will not exit the cabinet until you are done. A list of materials might look as follows:

- P1000 pipette tips, P200 pipette tips
- Serological pipette tips
- Pipets (both manual and automatic)
- DMEM, FBS, PBS, PennStrep, Versene, etc.
- Autoclaved container of glass Pasteur pipets
- Vacuum apparatus for waste disposal
- Pipette rack
- Cell culture flasks and cell culture dishes
- Tape for sealing bags and other containers
- Pens and markers (must be resistant to ethanol)

- Eppendorf tubes, 15 mL conical tubes, and 50 mL conical tubes

- Trays to hold your Eppendorf tubes and other samples

- Freezing vials (must be storable in liquid nitrogen)

- Glass beaker for disposal of pipette tips and other liquids (must contain ethanol)

As stated above, you can dispose of pipette tips and waste wrappers as necessary in the trashcans located outside of the biosafety cabinet. However, some people prefer to wait to dispose of waste materials such as
pipette tips until after they are done working in the cabinet. That way, they don't have to take their hands out of
the sterile environment within the cabinet.

1.14 As can be seen from the above list, the biosafety cabinet can get quite crowded. It is important to organize the environment as best as possible to ensure that you don't get reagents mixed up or confused during your cell culture session. This is especially important with materials such as DMEM and Trypsin, which are the same color (clear red liquids).

- Note that too much clutter in the biosafety cabinet can compromise air flow. Because of this, it is best to try and, at the very least, to not stack things up into high towers or anything like that.

- Note also that some of the materials listed above can (and probably should) be kept I the cabinet permanently. Materials that, once opened, cannot be sealed again should probably stay in the cabinet. In my personal experience, I have kept pipet tip boxes, pipette stands, and bags of 50 mL and 15 mL conical tubes in the biosafety cabinet. You can take the conical tube containers out of the cabinet, but make sure to tape the bags up to prevent the tubes from being exposed to the outside air. This is also true to culture flasks but, due to their large size, I almost never kept them in the cabinet. Instead, I would either take a bag of flasks into the cabinet and make sure to re-seal them with tape once done or take individual flasks into the cabinet after wiping them down with 70% ethanol. Whatever works for you and allows you to comfortably maintain a sterile environment is what you should do.

- Note 70% ethanol is toxic to your cells. Once you have wiped down all of your reagents and materials let them sit in the biosafety cabinet for a couple of minutes so that the ethanol evaporates. This is especially important for your pipettes.

1.15 Now that all necessary reagents and materials have been sanitized and transferred to the biosafety cabinet you are ready to begin working with your cells.

2 For the purpose of this protocol and for overall clarity, I will assume that you are starting from the very beginning with a frozen stock of mammalian cells. If this is not the case, then simply skip this section and begin at the appropriate section.

Mammalian cells are stored in liquid nitrogen so that you don't have to continue maintaining your cells in culture indefinitely and have aliquots of cells as backups should anything go wrong with a culture. The mammalian cells are typically stored in a freezing medium containing 90% FBS and 10% DMSO. This freezing medium prevents the cells from outright dying during the freezing and storage process in liquid nitrogen but is also not particularly healthy for the cells. Because of this you must be fairly gentle with the cells upon thawing and culturing. You also want to work as quickly as possible during this step of the procedure to minimize cell death (you are still going to get a lot of dead cells, though).

2.1 Before taking your cells out of liquid nitrogen and thawing them it is important to have all reagents and samples prepared beforehand so that we have as little work as possible to do once the cells are out of storage.

2.2 For this procedure, I will assume that you have 1 mL stocks of cells in freezing medium, with each stock containing roughly 1 million cells.

2.3 The basic idea of this step in the process is to get the cells out of the freezing medium as quickly as possible and into the normal culture medium so that they can attach to a surface and start recovering and growing.

2.4 First, get a T75 flask and fill it with 6 mL of Maintenance medium (DMEM containing 10% FBS, 1% PennStrep; warmed to 37^{o}C). Tilt the flask so that the medium is distributed evenly on the bottom of the flask. Label the flask with the date, estimated cell number, passage number, cell type, downstream purpose and the time that you seeded the cells (e.g. 12:00 PM). Remember that the marker that you label the flask and all containers with must be resistant to ethanol.

2.5 Then take a 15 mL conical tube and fill it with 9 mL of Maintenance medium. Label the tube "Thawed Cells".

2.6 Warm up a centrifuge that can fit 15 mL conical tubes (ideally one that is specifically for Mammalian cell culture, but this is not absolutely necessary) to room temperature.

2.7 Now that you have prepared what you need to the thawing and seeding of the cells, go to the liquid nitrogen storage container, retrieve your cells, and quickly take them back to the cell culture room. For this step, you

don't need to take on your lab coat (as speed matters), but you will obviously need to wipe your gloves off with 70% ethanol.

2.8 Once you are back to the cell culture room, either place the frozen cells in the 37^{o}C hot water bath to thaw or thaw the cells with your hands. Do not thaw the aliquot completely. Once you see that there is a small clump of frozen cells left, spray your hands with ethanol and wipe off the cell culture vial that the cells are in.

2.9 Then quickly place the cells in the biosafety cabinet and use a P1000 pipet to transfer the cells to the 15 mL conical tube containing 9 mL of Maintenance medium. Get a 10 mL serological pipet and mix the cells gently (very gently).

2.10 Then transfer 1 – 2 mL of this resuspended cell culture back to the freezing vial and wash to collect as many cells as possible in the 15 mL conical tube.

2.11 Then place the 15 mL conical tube in the centrifuge and spin at 500 – 1000 rcf for 5 minutes. This will pellet your cells so that you can separate them from the toxic freezing medium.

2.12 After centrifugation, wipe off the 15 mL conical tube with 70% ethanol and take it back to the biosafety cabinet. You then want to aspirate off the supernatant without disturbing the cell pellet. Note that the pellet might be loose. I typically use a glass Pasteur pipet connected to a vacuum to quickly aspirate off the supernatant. You can also use a serological pipet if you are more comfortable with this.

2.13 Once you have removed most of the supernatant (you don't have to remove all of it), gently add 8 mL of fresh Maintenance medium to the 15 mL conical tube and gently pipet up and down with the serological pipet to resuspend the pellet.

2.14 Then transfer the 8 mL of resuspended cell culture to the T75 flask containing 6 mL of Maintenance medium. Gently tilt the flask in all directions to make sure that the cells are distributed evenly on the surface of the flask. Then transfer back to the 37^{o}C incubator, once again giving the flask a gentle tilt in all directions to evenly distribute the cells on the surface of the flask.

2.15 Allow the cells to remain undisturbed for 1 day. During this period, don't do anything to the cells. Don't even take the flask out of the incubator to look at them. After the 1 day recovery period is over, take the cells out of the incubator and take note of how they look under an inverted microscope (are they spread out evenly?, are they rounded up but attached to the surface?, Are they attached tightly to the surface and spreading out?), how confluent they are, and the color of the Maintenance medium. Also note (very roughly) how many cells are floating around in the medium (dead cells).

2.16 If there are a lot of dead cells floating around, there is a good chance that the cells were excreting apoptotic factors that may kill off more of your presumably healthy cells. To get rid of the dead cells and any factors that may harm your healthy cells, take your flask to the cell culture room, wipe it off with 70% ethanol and transfer it to the biosafety cabinet.

2.17 Tilt the flask so that all of the spent Maintenance medium collects into one corner of the flask and then use a long glass Pasteur pipet connected to a vacuum to quickly remove the spent Maintenance medium that contains dead cells and other crap.

2.18 Then tilt the flask and gently add 12 – 14 mL of PBS (warmed up to 37^{O}C) to one corner of the flask using a serological pipet. When washing the cells, never add PBS or anything else directly to the cells, as this will inevitably detach some of the cells from the surface of the flask.

2.19 Gently rock the flask back and forth so that the PBS covers the bottom of the flask and washes away the spent Maintenance medium. Then, once again, tilt the flask so that all of the PBS collects in one corner of the flask and use a long glass Pasteur pipet to aspirate off the PBS from the cells.

2.20 Finish up by tilting the flask and adding 12 mL of Maintenance medium to one corner of the flask. Then gently rock the flask back and forth so that all of the cells are exposed to the Maintenance medium. You can then transfer the flask back to the incubator and let them grow until they are around 70 – 90% confluent. You will then want to split / passage the cells at least once more before performing experiments on them. The splitting / passaging of cells will be covered in the next section.

Passaging and Maintaining Mammalian Cell Cultures

3 When cells reach a high confluence in culture they will inevitably come into contact with neighboring cells. Once cell-to-cell contacts have been established between neighboring cells and there is no more room to grow, cells will undergo a process known as contact inhibition. Once contact inhibited, cells no longer grow or replicate their DNA and instead enter into a quiescent state. While this quiescent state is desirable for some experiments, it is not desirable for the maintenance of your cell cultures and should be prevented if possible. The good thing is that contact inhibition is easily reversible. All you have to do is break up the cell-to-cell contacts and transfer the cells to another flask at a lower confluence. This basic process is called “passaging” (also referred to as “splitting”) and is an important step in cell culture as it allows you to continue maintaining your cells over time.

Beyond passaging your cells, the process of cell maintenance will vary depending on your specific cell line and downstream application. The growth conditions for your cell line, for example, must be determined empirically. However, for many cell lines a Maintenance medium containing DMEM with 10% FBS and 1% PennStrep is a good starting point. Another source for determining the normal maintenance protocol for your cell line is to refer to the ATCC website. However, the protocols obtained from the ATCC might not apply to your cell line, unless you purchased your cells from them. It is possible that you will need to use different culture mediums depending on whether you are simply maintaining your cells or whether you are growing your cells for a specific downstream application.

3.1 Once your cells have reached a confluence of roughly 70 – 90%, you will want to begin passaging your cells to prevent contact inhibition and maintain the viability of your cells in the future.

Although passaging your cells only takes about 15 – 30 minutes, it can be quite tedious and, depending on

3.2 your cell line and growth conditions, must be repeated several times a week.

3.3 The first part of passaging involves removing the adherent cells from the surface of the cell culture flask. This process is potentially harmful to your cells and will vary depending on the type of cell line that you are using. I will describe several potential methods for removing adherent cells in this section.

3.4 Once you have your cell culture flask containing your cells in the biosafety cabinet, you can begin passaging the cells by first tilting your flask so that all of the spent Maintenance medium collects into one corner of the flask.

3.5 Then use a long glass Pasteur pipet attached to a vacuum apparatus to aspirate off the medium. As noted previously, this should be done as quickly as possible. Once you remove the medium from your cells, they no longer have their food. Try not to keep them out of their medium for more than 5 minutes or so.

3.6 **PBS Wash and Trypsin Digestion Method –**

i. The most basic way to proceed from this point is to gently pipet 10 mL of PBS into one corner of the flask and
then gently rock the flask back and forth to wash away any remaining Maintenance medium.

ii. Once the remaining Maintenance medium has been washed into the PBS, gently tilt the flask again so that the
PBS collects into one corner of the flask. Then use a long glass Pasteur pipet to aspirate off the PBS.

iii. Then add 500 uL of 0.5% Trypsin to the center of the flask and rock the flask back and forth to expose the entire
surface to the Trypsin. Then you ideally want to transfer your flask back to the 37^{o}C incubator for 2 – 5 minutes
to allow the Trypsin to digest the extracellular matrix of your cells, thereby detaching them from the surface of
the flask.

Note that Trypsin, while being very good at detaching cells from the surface of the flask, is also very good at
digesting cell membranes, protein, RNA and DNA (essentially everything). Because of this, the incubation period
should be kept to a minimum. For especially sensitive cell lines, Trypsin is not used simply for this reason.

Note also that Trypsin is inhibited by FBS. This is why is it necessary to wash away any remaining Maintenance
medium from the cells prior to Trypsin digestion. If you were to add Trypsin before washing away residual
Maintenance medium, it would likely be either less effective or completely ineffective.

iv. After the digestion period is over, take your flask out of the incubator and tilt it side to side. If your cells are especially confluent, you will see a film of cells floating around in the flask. Ideally, however, you want your cells to not be clumped together and instead be single cells. For some cell lines, this is easy and for others it is quite difficult. You will simply have to see for yourself by looking at your cells using an inverted microscope following Trypsinization.

For some cell lines it is standard to take the flask out of the incubator and then roughly bash your hand against the side of the flask to break up any clumps of cells. While being fairly rough, it is an effective way to get a suspension of single cells rather than sheets of cells or clumps of cells.

For other cells, this bashing technique is unnecessary and gentle washing of the flask in the next step is enough to de-clump the cells.

v. Following Trypsinization, quickly take your cells back to the biosafety cabinet and add 6 mL of Maintenance medium to the flask. Rock the flask back and forth so that the entirety of the surface is exposed to the medium.
Note that because Maintenance medium contains FBS, it will effectively deactivate the Trypsin and prevent further digestion of your cells.

vi. Once you have washed the surface of the flask, get a 10 mL serological pipet and use it to wash the surface of the flask several times (this is easier to show rather than to describe). Once you have washed the surface several times, tilt the flask so that all of the cell suspension collects into one corner of the flask. Then collect the cell suspension in the serological pipet and gently expel the cell suspension against one of the corners of the flask.
This effectively shears any remaining cell clumps and gives you a nice single cell suspension.

Note that you should only do this shearing two or three times. Too much shearing will obviously damage your cells.

vii. Then collect the cell suspension in a 15 mL conical tube for cell counting.

b. **Versene Wash and Trypsin Digestion Method –**

i. This method is nearly identical to the “PBS Wash and Trypsin Digestion Method” except that instead of washing with PBS (a simple buffer) you will wash with Versene (EDTA). Versene is useful because it sequesters

metal ions
necessary for cell surface adhesion and therefore makes it easier to remove cells using Trypsin digestion.
Because of this, after washing, as described above, you will either be able to use less Trypsin or incubate with
Trypsin for a shorter period of time. This helps to minimize the negative impacts that Trypsin can have on your
cells.

Another lesser benefit is that Versene is actually cheaper than PBS.

c. **Versene Washing Method –**

i. This method is by far the gentlest of the three methods that I have described here and is also the least likely to
succeed if you are using tightly adherent cells. Because of this, it is most useful for cells that detach fairly easily
from the flask surface.

ii. To begin this method, simply aspirate off the spent Maintenance medium as described previously and wash once
with Versene.

iii. The aspirate off the Versene and add 6 mL of Versene to your flask. Rock back and forth several times and then
incubate at room temperature for about 5 – 10 minutes. Rock the flask gently every few minutes. As usual, don't
incubate your cells with Versene for too long, as even it can be toxic to your cells.

iv. After the incubation period is complete, look at your cells using an inverted microscope and confirm at least
partial detachment.

v. Then take your cells back to the biosafety cabinet and wash the surface of the flask with the Versene (easier to
show than to describe) and once again shear the cells against a corner of the flask as described previously.

vi. Then, after confirming that you have a single cell suspension, add 6 mL of Maintenance medium to the flask and
collect the entire cell suspension in a 15 mL conical tube. The spin at 500 rcf for 5 minutes to pellet the cells.

vii. Remove supernatant from the cell pellet and then resuspend in 6 mL of Maintenance medium. You can then
proceed to the counting portion of this section.

3.7 Regardless of the detachment method that you used, you should end up with a suspension of single cells in roughly 6 mL of Maintenance medium in a 15 mL conical tube. While it is not strictly necessary to determine how many cells that you have in your 6 mL cell suspension, it is generally a good idea to do so as it allows you to reliably and reproducibly determine the density of cells that you add to each new culture.

Additionally, it allows you to determine whether something went wrong with your cell culture. If, for example, you typically have a concentration of 12 million cells per mL in your cell suspension following collection of your cells and you find that you are getting only 2 million cells per mL at the moment, then you can generally assume that something is wrong with your culture. Further, it gives you the opportunity to determine roughly how many cells in your cell suspension are dead.

3.8 There are many ways to determine the number of cells that you have per mL of cell suspension, but I have personally found that the most reliable method is to use a simple hemocytometer for the counting. Additionally, to determine the number of dead cells in your culture you can use a simple Trypan Blue staining procedure that I will describe below:

a. To begin the Trypan Blue Cell Exclusion Assay, add 50 uL of your cell suspension to an Eppendorf tube and the
add 50 uL of 0.4% Trypan blue dye to the cells and mix gently by pipetting.

b. Incubate the mixture for about a minute and then add about 9 uL of the mixture to a hemocytometer.

i. Note that Trypan blue is toxic to cells and if you incubate your cells with trypan blue for too long, they will die.
This will lead to inaccurate cell counts for both live and dead cells.

ii. Personally, I never really used trypan blue or any other cell death quantification assay. The trypan blue
exclusion assay works because living cells will pump out the trypan blue (exclude it from their cytoplasm).
Dead cells will be unable to pump out the trypan blue and will therefore appear blue under an inverted microscope. However, just because a cell can pump out trypan blue does not mean that the cell is healthy and
will continue to divide later on. Because of this, I always took the live : dead cell staining with a grain of salt.

c. Each hemocytometer has two loading grids on it that are composed of four 4 x 4 counting grids and several other
grids. The images below illustrate the hemocytometer and the counting grids that you are likely to see.

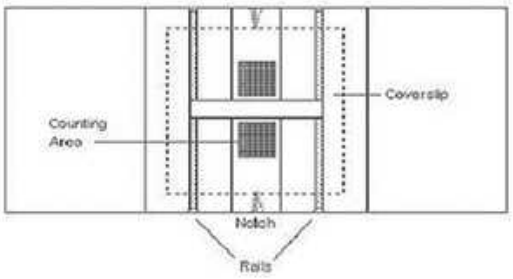

Diagram of a standard hemocytometer

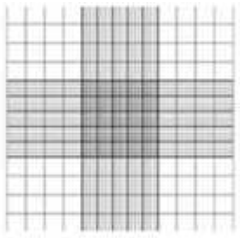

Diagram of a standard counting grid

d. After loading approximately 9 uL of cell suspension / trypan blue mixture onto the hemocytometer, place the hemocytometer on an inverted microscope and focus on the 4 x 4 counting grids. Count all cells contained within each 16 square grid and take note of these cell counts. You should, at the very least, count the cells contained within four of these 4 x 4 grids. If you want more accurate cell counts, you should do at least six 4 x 4 grids. Make sure to take note of how many live cells and dead cells there are per grid.

e. Determine the total number of cells per mL of cell suspension in your 15 mL conical tube by adding the total cell counts from each of the grids together and then dividing by the number of grids that you counted to get the average number of cells per grid. Then multiply this number by the dilution factor (in this case 2) and finally multiply this number by $1x10^4$ to get a measure of how many millions of cells you have per mL of cell suspension.

f. Calculate the percentage of viable cells by dividing the number of viable cells by the number of total cells and multiplying by 100 or % viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100.

g. The below example illustrates how this might work in the end:

i. Grid 1: 105 cells total; 100 living cells, 5 dead cells

ii. Grid 2: 120 cells; 120 living cells, 0 dead cells

iii. Grid 3: 115 cells; 105 living cells, 10 dead cells

iv. Grid 4: 130 cells; 125 living cells, 5 dead cells

v. Grid 5: 110 cells; 110 living cells, 0 dead cells

vi. Grid 6: 112 cells; 110 living cells, 2 dead cells

vii. Calculation for total cell number per mL of cell suspension:

((105 + 120 + 115 + 130 + 110 + 112)) / (6) = 115.3 cells per grid on average

115.3 (2)(1E4) = 2,306,000 cells / mL of cell suspension

2,306,000 cells / mL (6 mL total cell suspension) = 13,836,000 cells total

viii. Calculation of the percentage of viable cells:

((100 + 120 + 105 + 125 + 110 + 110)) / (6) = 111.6 viable cells per grid on average

111.6 (2)(1E4) = 2,232,000 viable cells / mL of cell suspension

((2,232,000 total cells / mL) / (2,306,000 viable cells / mL))(100) = 97% viability

3.9 Now that you know how many cells you have in total, how many viable cells you have, and how many viable cells you have per mL of your 6 mL cell suspension, you can finally add a certain number of cells to each new flask that you have for passaging. Note that by this point the cells in your cell suspension have likely settled to the bottom of the conical tube, so just pipette up and down several times to resuspend them before proceeding.

3.10 Typically, when you are adding new cells to fresh flasks (seeding your cells) you will have a set number of cells that you want to start with so that you have reached 70% - 90% confluence in a given time span. If, for example, I want to have my cells reach 70% confluence in 3 days I might add 500,000 cells to each flask. The number of cells that you need to achieve the desired confluence in the desired number of days must be determined empirically and will vary depending on your growth conditions and the cell line that you are using.

3.11 For the sake of this protocol, however, let's say that I want to seed 500,000 cells into a T75 flask. To do this, I will simply calculate how many milliliters of resuspended cell suspension that I need to add to each flask, add the appropriate volume using a pipette, and then add 12 mL of Maintenance medium to the flask.

3.12 I will then rock the flask back and forth so that the cells are distributed evenly across the surface of the flask and then stick the flask in the 37^{o}C incubator. After putting the cells in the incubator, do not disturb them for at least 24 hours so that they have time to successfully adhere to the surface of the flask.

3.13 You have now successfully passaged your cells. Make sure to note common information on the flask such as the date, time of day, passage number, cell type, and seeding density on the top of the flask and in your notes.

3.14 A few other things to keep in mind is that for some slow growing cells you will need to change the medium several times in between passages. For example, if you have a cell line that takes two weeks to reach confluence, then you may very well have to change out the Maintenance medium three or four times to maintain the health of the cells. You can easily determine when the Maintenance medium needs to be changed by looking at its color. Normally DMEM is bright red in color, but when it is time to change the medium you will see that is starts to turn orange in color. This color change is due to the increasing acidity of the medium as the cells in the flask excrete waste products. If you do need to change your medium in between passages, simply aspirate off the spent medium and gently wash with PBS. Then aspirate off the

PBS and add 12 mL of fresh Maintenance medium (if you are using a T75 flask).

Freezing and Storing Mammalian Cells

4 While you are passaging your cells, you may decide to freeze down several cells stocks for future use. This freezing protocol can be performed right after you finish seeding / passaging your cells. To successfully freeze down and store your mammalian cells, the cells must be suspended in a freezing medium containing a cryo-protectant and a nutrient source. Note that there are many different ways of freezing down mammalian cells, but I will only be going over the way that I have found success with in the past. If you are concerned about the freezing conditions of your specific cell line, it is best to look up the conditions that other have used or to screen different conditions to determine the freezing medium that is optimal for your cell lines.

4.1 Once you have determined how many cells per milliliter you have in your resuspended cell culture and have seeded all of the flasks or dishes that you will be using, you can use the remaining cells in the suspension for freezing cell stocks.

4.2 Each stock should ideally have roughly 1 million cells in it.

4.3 Take your cell suspension that is still in the 15 mL conical tube and centrifuge it at 500 rcf for 5 minutes to pellet the cells. You will then want to aspirate off the supernatant and resuspend the cell pellet in a volume of freezing medium such that the final concentration of cells is roughly 1 million cells per milliliter.

4.4 The freezing medium that I typically use is composed of 90% FBS and 10% DMSO. You will want to make the freezing medium right before you need it. The freezing medium should not be stored and the DMSO that you use should be relatively fresh.

4.5 Then dispense 1 mL of cell suspension in freezing medium into individual cryo-vials that can handle being stored in liquid nitrogen indefinitely. Make sure to write relevant information such as the date, passage number, cell line, freezing medium used, number of cells per aliquot, etc. on the vial.

4.6 Then sandwich the cryo-vials in between two Styrofoam conical tube holders and place them in the -80^{o}C freezer for 24 hours. The point of this is to allow the cells to freeze very slowly so that ice crystals don't form and pierce their cell membranes. Typically, labs that are heavy into cell culture will have special containers for this that prevent the cells from cooling down too rapidly. These, however, are expensive and I have heard that Styrofoam works well enough.

4.7 After the 24-hour cooling period is over, you will then need to transfer the cells to a liquid nitrogen storage container, where they will remain until you need them.

4.8 Because it is difficult to find specific cryo-vials in these liquid nitrogen containers, it is a good idea to keep a cell culture inventory that includes where exactly you stored each vial. This is more important when you are handling multiple different cell lines, passage numbers, etc.

Appendix - Common Mammalian Cell Culture Reagents

5 **Dulbecco's Modified Eagle Medium (DMEM) –**

a. DMEM is a synthetic cell culture medium developed by Harry Eagle in 1955/1959 that can be used to maintain
cells in tissue culture. It contains amino acids, salts (calcium chloride, potassium chloride, magnesium sulfate,
sodium chloride, and monosodium phosphate), glucose, and vitamins (folic acid, nicotinamide, riboflavin, B12).
Many variations of this medium have been developed, mostly adding additional vitamins, amino acids, and/or
other nutrients.

b. DMEM serves as a good cell culture medium for a variety of mammalian cell lines and is often sold with increased levels of glucose and pyruvate. Further, it can be sold with or without certain metal ions such as magnesium that can aid in cell adhesion. DMEM with high glucose and pyruvate sold by ThermoFisher has the
following composition:

A	B	C	D
Components	M.W. (g/mol)	Concentration (mg/l	Concentration
Amino Acids			
Glycine	75.0	30.0	0.40
L-Arginine hydrochloride	211.0	84.0	0.40
L-Cystine 2HCl	313.0	63.0	0.20
L-Glutamine	146.0	584.0	4.00
L-Histidine hydrochloride-H2O	210.0	42.0	0.20
L-Isoleucine	131.0	105.0	0.80
L-Leucine	131.0	105.0	0.80
L-Lysine hydrochloride	183.0	146.0	0.80
L-Methionine	149.0	30.0	0.20
L-Phenylalanine	165.0	66.0	0.40
L-Serine	105.0	42.0	0.40
L-Threonine	119.0	95.0	0.80
L-Tryptophan	204.0	16.0	0.08
L-Tyrosine disodium salt dihydrate	261.0	104.0	0.40

A	B	C	D
L-Valine	117.0	94.0	0.80
Vitamins			
Choline chloride	140.0	4.0	0.03
D-Calcium pantothenate	477.0	4.0	0.008
Folic Acid	441.0	4.0	0.009
Niacinamide	122.0	4.0	0.03
Pyridoxine hydrochloride	206.0	4.0	0.02
Riboflavin	376.0	0.4	0.001
Thiamine hydrochloride	337.0	4.0	0.012
i-Inositol	180.0	7.2	0.04
Inorganic Salts			
Calcium Chloride (CaCl2; anhydrous)	111.0	200.0	1.80
Ferric Nitrate (Fe(NO3)3"9H2O)	404.0	0.1	2.47E-4
Magnesium Sulfate (MgSO4; anhydrous)	120.0	97.67	0.81
Potassium Chloride (KCl)	75.0	400.0	5.33
Sodium Bicarbonate (NaHCO3)	84.0	3700.0	44.04
Sodium Chloride (NaCl)	58.0	6400.0	110.34
Sodium Phosphate monobasic (NaH2PO4-H2	138.0	125.0	0.91
Other Components			
D-Glucose (Dextrose)	180.0	4500.0	25.0
Phenol Red	376.4	15.0	0.04
Sodium Pyruvate	110.0	110.0	1.0

Composition Table for DMEM (Sigma)

c. 500 mL containers of DMEM can be stored at 4^{o}C in a cold room in the dark. The date that the DMEM is opened
should be recorded on the outside of the container using an ethanol resistant marker.

Fetal Bovine Serum (FBS) –

a. Fetal bovine serum (FBS) comes from the blood drawn from a bovine fetus via a closed system of

collection at
the slaughterhouse. Fetal bovine serum is the most widely used serum-supplement for the in vitro cell culture of
eukaryotic cells. This is due to it having a very low level of antibodies and containing more growth factors,
allowing for versatility in many different cell culture applications. The globular protein, bovine serum albumin
(BSA), is a major component of fetal bovine serum. The rich variety of proteins in fetal bovine serum maintains
cultured cells in a medium in which they can survive, grow, and divide. FBS is not a fully defined media component, and as such may vary in composition between batches. As a result, serum free and chemically defined media (CDM) have been developed as a matter of good laboratory practice.

b. Fetal bovine serum is commercially available from many manufacturers, and because cells grown *in vitro* are
highly sensitive, customers usually test specific batches to check for suitability for their specific cell type. When
changing from batch to batch it is usual to adapt the cells to the new batch of material, for example, by mixing
50% of the old serum with 50% of the new serum and allowing the cells to acclimate to the new material. Serum
is stored frozen to preserve the stability of components such as growth factors. When serum is thawed, some
precipitation may be seen. This is a normal phenomenon and it does not compromise the quality of serum in any
way. The precipitate may be removed by transferring the serum to sterile tubes and centrifuging for 5 minutes at
400 × g.

c. Without Fetal bovine serum supplemented to your cell culture medium it is highly likely that your cells will simply
die.

d. Fetal bovine serum should be stored at -20^{o}C at minimum and should be aliquoted to minimize the number of
freeze / thaw cycles that each aliquot experiences. FBS is highly nutritious not only for mammalian cells, but also
bacteria and fungi and is therefore prone to contamination.

Trypsin –

a. Trypsin is a serine protease from the PA clan superfamily, found in the digestive system of many vertebrates,
where it hydrolyzes proteins. Trypsin is formed in the small intestine when its proenzyme form, the trypsinogen
produced by the pancreas, is activated. Trypsin cleaves peptide chains mainly at the carboxyl side of the amino
acids lysine or arginine, except when either is followed by proline. It is used for numerous biotechnological
processes. The process is commonly referred to as trypsin proteolysis or trypsinization, and proteins that have

been digested/treated with trypsin are said to have been trypsinized. Human trypsin has an optimal operating

temperature of about 37^{o}C.

b. Aliquots of trypsin can be stored at -20^{o}C when in storage or at 4^{o}C when you plan on using it.

Appendix - Cell Culture Flasks, Dishes, and their Various Sizes

6 When performing cell culture, you will encounter a wide range of different flasks and cell culture dishes that have different sizes and purposes. Cell culture flasks are typically used for passaging and maintaining cells for extended periods of time due to their large surface area and ability to easily exchange gases through their lid, while at the same time keeping your cells safe from the environment. The most common cell culture flasks that you are likely to encounter are called T-25 flasks, T-75 flasks and T-160 flasks. These flasks have a surface area of 25 cm^2, 75 cm^2, and 160 cm^2, respectively. Typically, I use T-75 flasks for general maintenance and use T-25 flasks to seed cells that have just been thawed (you never really know how many cells will still be alive after freezing, but you definitely don't want to have the cells be too sparse in a flask, as they will grow extremely slowly). T-160 flasks can be used if you need a large number of cells when you are doing things like protein expression. Cell culture dishes also come in various sizes and are typically used when you will need to quickly harvest your cells for an experiment via cell scrapper. Rather than going through the long and potentially destructive process of trypsinization, you can simply harvest clumps of your cells using a cell scrapper. I have done this in the past by adding cell lysis solution to my cell culture dish and then using a cell scrapper to quickly lyse the cells and isolate DNA and RNA. The disadvantage of cell culture dishes is that they are often smaller than flasks and do not exchange gases as well. They also do not protect your cells from the environment as well as flasks do.

To aid in transitioning from one flask type or dish type to another it is a good idea to determine how many cells you need to seed to obtain optimal growing conditions per cm^2. For example, if I know that if I seed 500,000 cells into a T-75 flask my cells with reach confluence in 3 days, I may want to know how many cells I need to seed into a T-25 flask to reach confluence in 3 days. To do this, you simply divide the number of cells that you seed by the surface area of the flask to get cells per cm^2. Therefore, if my optimal seeding density is 500,000 cells per T-75 flask, then my true optimal seeding density is actually 6,666 cells per cm^2. If I want to know how many cells I should optimally seed into a T-25 flask, then I simply multiply (6,666 cells / cm^2)(25 cm^2) to get 166,650 cells per T-25.

When you don't know the optimal seeding conditions for your flask or dish, you may find the following table provided by Invitrogen to be helpful. These numbers serve as good starting points for determining the optimal seeding conditions for your cells:

Useful Numbers for Cell Culture

There are various sizes of dishes and flasks used for cell culture. Some useful numbers such as surface area and volumes of dissociation solutions are given below for various size culture vessels.

	Surface Area (mm^2)	Seeding Density	Cells at Confluency[1]	Versene (ml of 0.53 mM EDTA)	Trypsin (ml of 0.05% trypsin, 0.53 mM EDTA)	Growth Medium (ml)
Dishes						
35 mm	962	0.3×10^6	1.2×10^6	1	1	2
60 mm	2,827	0.8×10^6	3.2×10^6	3	2	3
100 mm	7,854	2.2×10^6	8.8×10^6	5	3	10
150 mm	17,671	5.0×10^6	20.0×10^6	10	8	20
Cluster Plates						
6-well	962	0.3×10^6	1.2×10^6	2	2	3–5
12-well	401	0.1×10^6	0.4×10^6	1	1	1–2
24-well	200	0.05×10^6	0.2×10^6	0.5	0.5	0.5–1.0
Flasks						
T-25	2,500	0.7×10^6	2.8×10^6	3	3	3–5
T-75	7,500	2.1×10^6	8.4×10^6	5	5	8–15
T-160	16,000	4.6×10^6	18.4×10^6	10	10	15–30

[1] The number of cells on a confluent plate, dish, or flask will vary with cell type. For this table, HeLa cells were used.

Appendix - Passage Number and Passage Number Limits

7 You will often see that cell lines have passage numbers associated with them. For example, someone might give you a vial of cells that say Passage 8 or P8 on it. This means that, since the person who froze down the cells received or created the cells, they have been passaged 8 times. In general, the passage number is not always accurate, as it does not necessarily reflect how many times a cell line has been passaged prior to being received. For example, if you purchase a cell culture stock from a company, you may say that these are Passage 1 cells, as you have only thawed and seeded them into a flask. However, you do not know how many times these cells were passaged by the people who originally handled and froze the cells. In general, however, passage number is a good indicator of how many times you yourself have passaged the cells. For experiments, it is generally best to use lower passage number cells. For example, when you have the choice to use Passage 3 cells versus Passage 12 cells for an important experiment, it is probably best to use the Passage 3 cells. This is because cells can only divide a certain number of times before becoming senescent and becoming permanently arrested. This is also why it is a good idea to freeze down a batch of low passage number stocks to use in the future. Some cell lines, referred to as immortal cell lines (such as HeLa cells), can be passaged indefinitely. However, if your cell line is not immortalized it is likely that they will stop dividing after a certain number of divisions. As for how many cell divisions it will take before senescence sets in, you will just have to figure it out over time.

Appendix - Overview of Mammalian Cell Culture Pipeline

8 Aseptic Technique