This section outlines the basic procedures in aseptic technique. This will help minimize the possibility of contamination. Often times, carelessness, but not incompetence, is the cause of contamination. The following is presented as a foundation for aseptic technique, and does apply for research outside of the laminar flow hood as well

Laminar Flow Hood

Although primarily used for cell culture practices, the flow hood is NOT a sterile environment. However, it creates a dead-air space, minimizing the possibility of air-borne dust and microorganisms from entering into the experiment medium, cells, etc.

- Turn off the UV light. You do not want to expose UV light onto your skin. Note that in some models, the "receptacle" button turns the UV light off.
- Turn on the fluorescent light. Note that sometimes the aforementioned procedures may be done with the same switch.
- Open front shield about 7 inches or 8 inches.
- Turn the Laminar Flow Hood on.
- Spray the Laminar Flow Hood liberally with 70% ethanol. Spray deep inside as well as the outer rims of the hood.
- Wait at least 10 minutes before entering the hood. This will give the Laminar Flow Hood sufficient time to create a good "dead-air" space in the hood.

Cardinal Rules

- Spray your hands and arms with 70% ethanol when introducing them into the hood. There are no exceptions to this rule.
- Organize your space. This will minimize the likelihood of contamination.
- Always work in the dead-air space. The hands should be placed deep in the hood, never near the front where the air vent is located (keep elbows off vents).
- Minimize contamination by avoiding contact with bottle/flask necks, caps and openings. If any item is possibly contaminated (through touch, exposure outside hood, etc.), remove from hood, or spray liberally with 70% ethanol.

> Do not pass hands over any open containers. This will result in dust particles falling into the containers.

Media Bottles

- Remove bottle from yellow fluorescence protection bag.

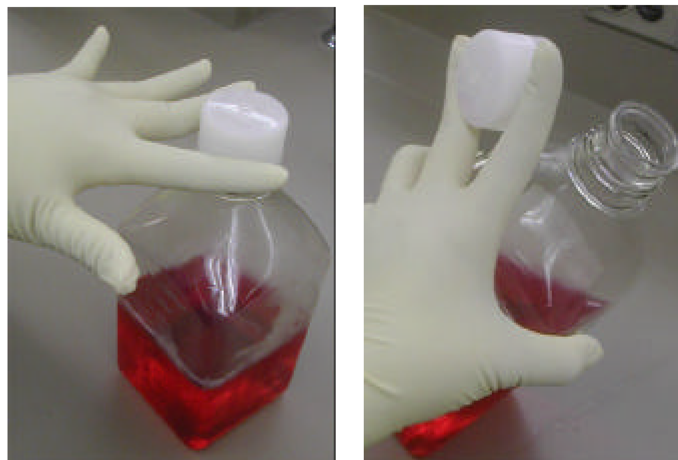
> Never handle the neck of the bottles. Always handle bottles by the base to avoid contamination.

- Warm the media bottle. This is necessary when you are feeding your cells.
- Handle bottles with care. Splashing inside the bottle will create unwanted air bubbles and you do not want any media to touch the neck/cap of the bottle.
- Lightly flame the neck of the bottle with a Bunsen burner. Process should never exceed 2-3 seconds. This will attach the dust particles to the outside of the bottle.
- Spray bottle liberally with 70% ethanol.
- Place the bottle in the hood.
- Unscrew the bottle cap until it is open. Let the lid sit on the bottle mouth, this will give you quick access to the bottle when needed. Never place the bottle cap down.

- Carefully lift the cap and tilt the bottle in one motion. Practice this technique as advised. Tilting of the bottle will minimize contamination by particles falling into the bottle

Method #1

Method #2



- Close the bottle firmly. Remember to close the bottle before removing from the Laminar Flow Hood.

- Remove bottle from the hood. Be careful not to get media in the neck of the bottle while removing.
- Spray with 70% ethanol, then replace in yellow fluorescence protection bag. This will minimize contamination. Pay attention to how you handle the bottle, handling the neck of the bottle is most common in this process.

<u>Flasks</u>

- Handle flasks at the base. The neck of the flask should not be handled under any circumstance. Avoid touching media to the neck of the flask. This will lead to contamination.
- Tighten cap of the flask when removing from incubator. This will minimize contamination, but do not leave flask unattended while sealed, as your cultures will die.
- Lightly flame the neck of the flask with a Bunsen burner. Process should never exceed 2-3 seconds. This will attach the dust particles to the bottle.
- Spray flask with 70% ethanol.
- Introduce into the Laminar Flow Hood.
- Unscrew the flask cap until it is open. Let the lid sit on the flask mouth, this will give you quick access to the bottle when needed.
- Carefully lift the cap and tilt the flask in one motion. Practice this technique as advised. Tilting of the flask will minimize contamination by particles falling into the bottle.

- Close the flask firmly. Remember to do this before removing from the Laminar Flow Hood.

- Remove flask from the hood.
- Unscrew the cap of the flask to allow CO_2 into the flask. The arrows should point upwards and downwards, or cap should be fitted loosely.
- Spray with 70% ethanol, then place back into incubator.

Pipette Canisters

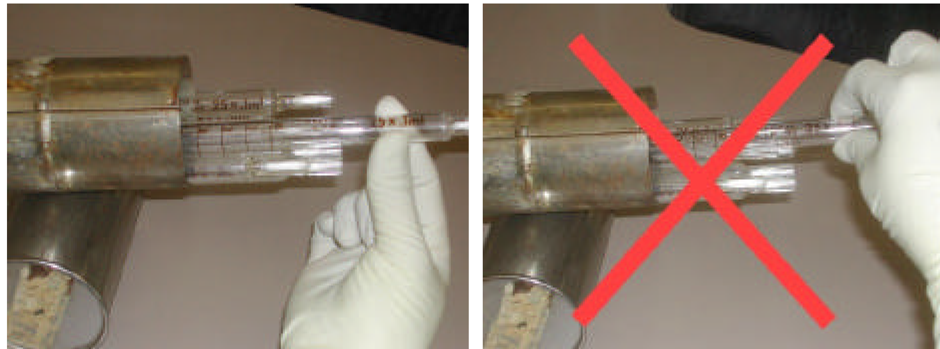
- Handle the bottom of the pipette canister. Do not handle the canister near its opening (neck).
- Lightly flame the neck of the pipette canister with a Bunsen burner. Process should never exceed 2-3 seconds. This will attach the dust particles to the bottle.
- Spray liberally with 70% ethanol.
- Place pipette canister in the hood.
- Shake the pipette canister upside-down gently. This will slide the pipettes inside towards the lid of the canister, making the pipettes more accessible. Note: excessive shaking will crack the pipettes inside the canister.
- Slowly remove the lid of the pipette canister. Be sure that the pipettes inside do not touch the edges of the canister lid.
- Angle the pipette canister upwards. Use the lid of the canister to stabilize. Pipettes should be sticking out of the canister.

When working in the Laminar Flow Hood, keep the pipette canister in an area free of any obstruction. Hitting the pipettes can not only crack them, but also potentially contaminate your cultures.

- When finished, close lid on canister.

Pipettes

- Pipettes should be projecting out from the pipette canister. This will have the pipettes readily accessible.
- Slowly slide one pipette outwards. Grasp the pipette below the notch and above the graduations. Use an underhand grasp, and not an overhand grasp. This is the only area which you should handle these pipettes. Usually, there are brand names, professor names, or department names labeled on the pipette in this area.



- Insert into Pipet-Aid firmly. Some twisting of the pipette might fit the pipette deeper into the Pipet Aid.
- Orient graduations towards yourself. This will insure correct measurement, and minimize contamination.
- Angle the bottle/flask. This will minimize the area for dust particles to potentially fall into the container.
- Pipettes should be dipped into the bottles/flasks without touching anything. Be cautious of pipettes touching the inside walls of the bottles/flasks. This will lead to immediate contamination. If the pipette touches any surface, remove and start with a new pipette.
- Siphon/expel the contents of the pipette. If the pipettes are correctly angled when expelling contents, formation of air bubbles will be minimized. Be cautious to not froth the cell medium. Consult instructor if necessary.
- Remove the pipette from the Pipet-Aid.
- Place pipettes in the clorox soak columns outside the Laminar Flow Hood. Tips of the pipettes should be pointing downwards in the columns.

- Place any unused pipettes in appropriately labeled drawers. This will bypass the washing procedure and the pipettes will go immediately to sterilization.
- Store empty pipette canisters in specified locations.

<u>Pipet-Aid</u>

- Spray liberally with 70% ethanol.
- Introduce into Laminar Flow Hood. Do not let a Pipet Aid rest on its side, the stand is attached for a reason. Do not touch the opening of the Pipet-Aid (where the pipette will be inserted).

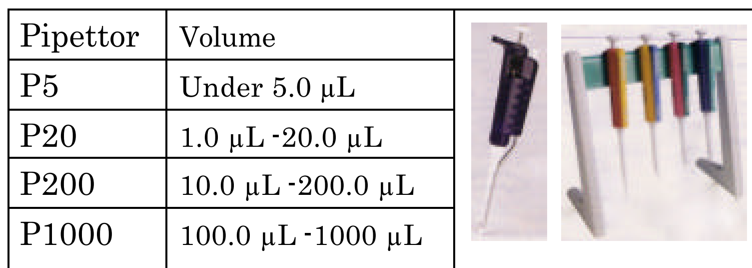
- Adjust the speed of the Pipet-Aid (s, m, f) by turning the analog buttons. The upper button controls siphoning speed, lower button controls expelling speed. Adjust the speed of the siphoning/expelling through depressing the analog buttons. The amount of depression controls the speed of siphoning/expelling.

> DO NOT siphon into the Pipet-Aid. If you do, immediately report it to an instructor

- Remove Pipet-Aid from Laminar Flow Hood. The UV light from the flow hood will damage the Pipet-Aid when left for extensive periods of time.

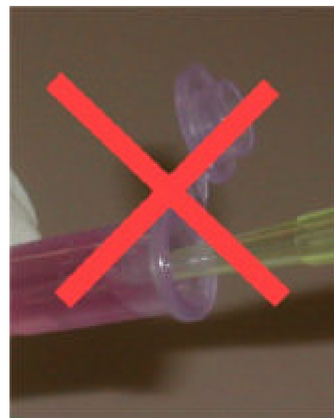
Pipettors

Be sure that you have selected the appropriate pipettor for the amount desired to pipette.



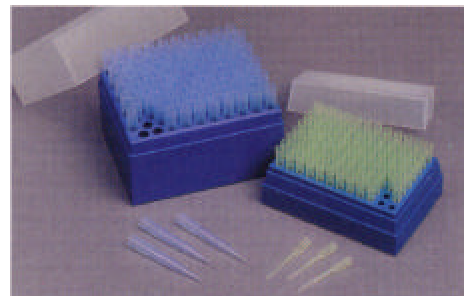
Pipettor	Volume
P5	Under 5.0 μL
P20	1.0 μL -20.0 μL
P200	10.0 μL -200.0 μL
P1000	100.0 μL -1000 μL

- ☐ Spray liberally with 70% ethanol.
- ☐ Introduce into Laminar Flow Hood.
- ☐ Select the appropriate amount to siphon by adjusting the turn knob on the pipettor.
- ☐ Depress pipettor to first point of resistance to siphon, depress past the first point of resistance to expel. Avoid depressing and releasing the button on the pipettor too rapidly; this can result in liquid being siphoned up into the pipettor.
- ☐ Always siphon pipettor straight down. Volumes will slightly change if siphoned at an angle.



Pipette Tips

- All pipette tips must be sterilized in the autoclave prior to work in the Laminar Flow Hood.
- Spray the pipette tip container liberally with 70% ethanol.



- Introduce into the Laminar Flow Hood. Lift the top of the container off carefully without touching the pipette tips inside. Contact with the outside of the container will contaminate your pipette tips.
- Push the pipettor down on the pipette tip. Pressing excessively hard will result in cracked pipettors.

> The pipette tip containers are not meant to keep the tips sterile once they are opened. If in doubt, autoclave the pipette tips again before use. They are usually safe as long as they are kept in the hood.

Epindorph (microcentrifuge) tubes

- Spray the outside of the epindorph tube liberally with 70% ethanol.

- Introduce into the Laminar Flow Hood. Make use of the test tube racks found in the lab to hold the epindorph tubes.

Waste Container

- Spray liberally with 70% ethanol. Keep in mind to spray ALL surfaces exposed to air. This includes both inside and outside surfaces of container.
- Introduce into the Laminar Flow Hood.
- Expel contents down the drain. Do not expel down the drain if it is a radioactive substance or other controlled substance.
- Rinse container with tap water.
- Dry the container and spray lightly with 70% ethanol.

Cleaning Up the Laminar Flow Hood

- Wipe any liquids with a paper towel. Any liquids on the surface of the hood will dry and potentially contaminate the entire Laminar Flow Hood.
- Spray liberally with 70% ethanol. Be sure to spray deep inside hood, as well as the edges and front shield glass.
- Turn off the airflow.
- Lower and close the front shield.
- Turn off fluorescent light and turn on the UV light.

Procedure

Sterilization

- Turn the autoclave on. The knob on the autoclave needs to be turned in a clockwise direction.
- Wait 5 minutes for the autoclave to warm up.
- Place the items that need sterilization into the autoclave. Items that need to be sterilized should be placed in a metal tray. Be sure that nothing touches the walls of the autoclave.
- Close door and seal. Turn the wheel on the door clockwise until the CLOSE light turns on.
- Set sterilization and drying times. Drying time not applicable for liquid items.
- Press either DRY GOODS (dry cycle), or WET GOODS (liquid cycle.) to start sterilization.
- Check to see if STERILIZATION light turns on.

General Autoclave Times			
Item	Time	Dry Time	Cycle
Biohazard Bags	25 min		Liquid
Glassware	45 min	30 min	Dry
Pipette Tips	35 min	30 min	Dry
Canned Pipettes	75 min	45 min	Dry
Media, Liquids	25 min		Liquid
Octapipette	25 min		LIquid

Removing from the autoclave

- Always wear the heat resistant gloves and bring a cart.
- Be sure that the sterilization process is complete. The DONE light should be on.
- Slowly turn the wheel counter clockwise to unseal the autoclave door. This process should be done slowly and carefully, the steam from the chamber can cause serious burns.

- Let the steam gradually come out by opening the autoclave door in small increments. Since the inner chamber has been pressurized, the contents may burst if the autoclave door is opened rapidly.
- After opening the autoclave door, remove the contents with caution and place on metal cart. Carrying the sterilized contents will be dangerous, as items will be at a significantly higher temperature.
- Cool down sterilized items prior to use. Pipettes are usually cooled down in an oven and further dried for 24 hours.

Procedure

Step One: Preparation

- DO NOT ALLOW THE PIPETTES TO DRY. This is a cardinal rule in pipette washing. When the pipettes dry, the contents will attach on to the inner wall of the pipettes, making it very difficult (at times impossible) to clean.
- Remove the pipettes from the columns located next to Laminar Flow Hoods. Removal process should be performed in a sink.
- Remove the cotton from the pipettes. Either use tweezers to remove cotton, or use air to blow cotton out. All cotton is recommended to be removed prior to soaking, or it creates a huge mess.
- Insert the pipettes into a column insert, all tips facing upwards. Pipettes will not be removed from the crates throughout the washing process. The entire crate will move between the following wash steps.

Step Two: Soap Soak

- Insert the pipette column insert into the SOAP SOAK. Slowly insert, as the soap solution will be gradually introduced into the pipettes.
- The pipettes will need to soak for 30-40 minutes. Pipettes can be left in soap soak for extended periods of time if time does not permit for step three.
- Lift the pipette column insert and drain liquids. Once again, slowly drain the liquids from the pipettes as much as possible.

Step Three: Water Wash

- Insert the pipette column insert into the WASH column.
- Turn the water on. Be sure that the water is at a constant flow rate. Turning the water on too fast will result in an overflowing column.
- Rinse the pipettes for 60 minutes.

Step Four: Distilled Water Wash

- Fill the distilled water wash column with distilled water. Fill to 3/4 full.
- Slowly dip the pipette column insert into the distilled water. Wait for all air to escape from the pipettes.
- Lift the pipette column insert to drain distilled water contained in the pipettes. Be sure to drain all remaining water contained in the pipettes.
- Repeat above procedure a minimum of 5 times.
- Immediately drain all water from the wash column after one complete wash.

Step Five: Drying

- Empty pipettes from pipette crate into a steel pan. Remember to put the tips pointing upwards.
- Place in oven to dry for 24 hours. An option would be to air dry as well – however this takes longer.

Step Six: Preparation for Autoclave

- Remove the pipettes from oven and cool.
- Stuff the ends of the pipettes with cotton using the cotton stuffing machine.
- Start filling pipette canisters with pipettes. Fill the same types of pipettes (5L, 10L, 10S etc…) into their own pipette canisters. Never fill the pipette canisters more than half full – one-third is about ideal.
- Label the pipette canisters appropriately with autoclave tape. The tape should label the contents of the pipette canister (5L, 10L, 10S, etc…) and be placed over the opening of the pipette canister. Fold over the tape to make a tab for easy removal of the tape. This will serve as a seal to insure sterility.

Step Seven: Autoclave Sterilization

- Place all full pipette canisters into an autoclave tray.
- Autoclave pipettes. Use autoclave gloves to protect yourself from the heat.
- Remove pipette canisters and cool down before use. Use autoclave gloves to protect yourself from the heat. Pipettes are usually cooled for at least 24 hours prior to use.

Making Media

Complex media will serve as a source of carbohydrates, vitamins, and proteins for the cells in your culture. In addition, it will provide undefined growth factors, a pH buffering system, and a specific salt concentration to match osmolarity. Appropriately calculate amounts of each component to be added to the base medium. Keep note that it may be necessary to take out some media from your base medium to make the appropriate concentrations in the provided containers. All preparations should be done in the Laminar Flow Hood. Be confident with your aseptic techniques, as contamination of the media will lead to a contaminated culture.

Recipe

- Base Medium 500mL or 1000mL
- 10% serum or 20% serum
- 1.5g/L Sodium Bicarbonate ($NaHCO_3$)
- 2.05mM L-Glutamine

Example Calculation for 10% supplemented media

- 435mL RPMI 1640
- 50mL Bovine Calf Serum
- 5mL L-Glutamine (200nM stock solution)
- 10mL Sodium Bicarbonate (7.5% stock solution)

Base Media

There are many different types of base medium available. Usually contained in 500mL bottles, the container will be used to comprise our complex media. All supplements are added to this bottle. Some cell types prefer one type of medium to another, be sure to know all this information ahead of time.

Types of Base Medium

Types of Base Medium
Dulbecco's Modified Eagle's Medium Low Glucoase/High Glucose
Iscove's Modified Dulbecco's Medium
Medium 199 Earle's
Minimum Essential Medium (MEM)
Nutrient Micture F10 (F12) Hams
Roswell Park Memorial Institute 1640 (RPMI1640)
Dulbecco's Phosphate Buffered Saline
Earle's Balanced Salt Solution (EBSS)
Hank's Balanced Salt Solution (HBSS)

- ❑ Select appropriate medium for cell culture. RPMI 1640 is our lab standard. Unopened media is found in the walk-in refrigerator.
- ❑ Yellow fluorescent protection bag is needed during storage. Only take the bottle out of the bag during media use.
- ❑ Remove seal on the media bottle in the hood. Only for first time use.

Serum

Serum is the supernatant of clotted blood. Many undefined growth factors are contained, including many proteins and metal ions. Although Fetal Bovine (Calf) Serum is most commonly used in publications, there are alternatives for economic purposes.

Types of Sera

Serum	Description
Bovine Calf Serum	Standard sera used in our lab, a cost efficient alternative to FBS.
Fetal Calf Serum	Most commonly used sera in literature. However, FBS is more expensive than BCS.
Cosmic Calf Serum	BCS supplemented with extra nutrients, provided exclusively through HyClone.
Equine Serum	Serum from horses. Some select cell types prefer this serum.

- Select appropriate sera. Bovine calf serum and Cosmic Calf Serum are the usual standards for our laboratory. Sera are found in the freezer.
- Thaw in 37°C water bath. Stir occasionally, the sera may form insoluble clusters if you do not pay attention. Let sera thaw slowly in the water bath. Rapid thawing will denature the proteins in the sera.
- Remove seal on the serum bottle in the hood. Only first time use.

Sodium Bicarbonate

This will act as a buffer for pH changes in your culture due to metabolic processes.

- Stock 7.5% $NaHCO_3$ is found in the refrigerator.

L-Glutamine

This will provide the carbohydrates for your culture. A purchased stock solution of L-Glutamine is usually available, but when comprising this from the power form, remember to sterilize the solution before use.

- Stock 205mM L-Glutamine should be stored in –20°C freezer. This will be diluted down to 2.05mM L-Glutamine when added into medium.
- Thaw in 37°C water bath.
- Vortex solution until the precipitate dissolves. Leftover precipitate will lead to an altered concentration.
- Note that the half-life of L-Glutamine is 2-3 weeks. Reconstitute L-Glutamine by adding 0.5% (of remaining media volume) every 2-3 weeks.

Media Test

You will need to perform media test each time you constitute complex media. This will save time and efforts, as contaminated media will lead to contaminated cell cultures.

- Examine your media. Look for any turbidity, or color change in media. You can examine the media under the microscope if desired.
- Incubate your media. Pipette some media into a 15mL conical vial. Loosen the cap and let incubate for 24 hours.
- Agar streak test. You can streak your media onto an agar plate, let sit in incubator for 24 hours and observe for growth.

Suspension Cells

In general terms cultures derived from blood (e.g. lymphocytes) grow in suspension. Cells may grow as single cells or in clumps (e.g. HL-60's grow as single cells, as RAJI's like to cluster). These subcultures are relatively easy to grow, and are well suited for many studies as they are easy to run in assays. For lines that grow in clumps it may be necessary to bring the cells into a single cell suspension by centrifugation and re-suspension by pipetting in a smaller volume before counting.

- View cultures using an inverted phase contrast microscope. Cells growing in exponential growth phase should be bright, round and refractile. Hybridomas may be very sticky and require a gentle knock to the flask to detach the cells. EBV transformed cells can grow in very large clumps that are very difficult to count and the center of the large clumps may be non-viable.
- Do not centrifuge to subculture unless the pH of the medium is acidic (color = yellow) which indicates the cells have overgrown and may not recover. If this is so, centrifuge at 150g for 5 minutes, re-seed at a slightly higher cell density and add pre-warmed media to the flask.
- You may check for cell concentration and viability (see Cell Quantification section).
- Repeat this every 2-3 days.

> NEVER dilute suspension cell concentration to less that 250,000 cells/mL, as cell line may not recover at such low concentrations.

Adherent (monolayer) Cells

Most cells found in the body are not in suspension. Cell Adhesion Molecules (CAMs) and desmosomes adhere some cells to the surfaces of the culture flasks. Since adherent cells grow in one layer (monolayer), cell morphology is easy to observe, and treatments applied to these cultures are homogenous. Unfortunately, growth curves are very difficult to perform, as it is hard to take a sample culture without disrupting the cells.

Adherent cell lines will keep growing until they have covered the surface area available, or until the medium is depleted of nutrients. At this point the cell lines should be sub-cultured in order to prevent the culture from dying. The first step in the subculture of these cells is to bring them into suspension. The degree of adhesion varies from cell line to cell line but in the majority of cases proteases (trypsin), are used to release the cells from the flask. However, this may not be appropriate for some lines where exposure to proteases is harmful or where the enzymes used remove membrane markers/receptors of interest. In these cases cells should be brought into suspension into a small volume of medium mechanically with the aid of cell scrapers.

<u>Trypsinization of Adherent Cells</u>

- Aspirate the medium and discard.
- Wash cells with pre-warmed PBS or HBSS, aspirate and discard. The volume of PBS of HBSS should be approximately the same as the volume of medium used for culturing cells. This will remove the chelating ions from serum that will inhibit trypsin enzymatic activity.
- Repeat above procedure.
- Add enough 1x trypsin-EDTA solution to cover the monolayer and rock the flask 4-5 times to coat the monolayer. Our stock solution is 10x trypsin-EDTA solution. Dilute to 1x concentration using PBS or HBSS.
- Loosen the flask cap and place the flask in the incubator for 1-2 minutes.

Placing the flask directly on the incubator may dislodge the cells faster

- Remove flask from incubator, tighten flask cap and firmly rap the side of the flask with palm of hand to assist detachment. If cells have not dislodged, loosen the flask cap and return the flask to the incubator for a few minutes. Note: do not leave the cells in trypsin for extended periods of time as trypsin will kill the cells. Overly confluent cultures, senescent cells and some cell lines may be resistant. Some cells lines may detach when repeatedly pipetteing the cells up and down in media. This should be done as gently as possible to avoid damaging the cells.
- Once dislodged, resuspend the cells in pre-warmed growth medium containing serum. The magnesium and calcium ions found in the serum will deactivate the trypsin.
- Remove about 100µL of the cell culture and perform a cell count (see Cell Quantification section).
- If your cell concentrations are low or if you are only seeding one flask, centrifuge the cells using a 15mL conical vial at 1000rpms for 3 minutes.
- Transfer the required number of cells to a newly labeled flask containing pre-warmed medium. Refer to online references for recommended seeding densities, such as the ATCC.
- Incubate the cell culture.

Troubleshooting

- Some cultures while growing as attached lines adhere only lightly to the flask, thus it is important to ensure that the culture medium is retained and the flasks are handled with care to prevent the cells detaching prematurely.
- Although most cells will detach in the presence of trypsin alone, the EDTA is added to enhance the activity of the enzyme.
- Trypsin should be neutralized with serum prior to seeding cells into new flasks otherwise cells will not attach.

Trypan Blue Exclusion

In order to find the concentration of cells in our flask we take a representative sample. From that representative sample we are able to calculate cell concentration and viability.

This will give an overview of the trypan blue exclusion method, which is a standard in manual cell counting. A trypan blue solution is added to the cell culture, and dead cells will stain blue. The live cells will take up the dye, then pump it out through the membrane immediately, hence do not stain blue.

Trypan Blue Constitution

- 0.4% Trypan Blue, 0.002% Sodium Azide in double distilled water, or PBS.

- Prepare a 50mL conical vial with a Whatman filter.
- Weigh out 0.125g of Trypan blue and add to top of the filter.
- Weigh out 0.015g of sodium azide and add to top of filter
- Slowly add 50 mL of ddH_2O to the filter.
- Centrifuge at 1300 rpm for 5 minutes when filtration is complete.

Trypan Blue Exclusion Method

- Siphon and expel media in the flask two to three times. This will make your cell culture homogenous throughout. Be careful not to froth the cell media.
- Pipette out less than 0.5 mL of your cell culture into an epindorph tube. This should be performed in a Laminar Flow Hood to insure the flask is protected from contamination.
- Seal the epindorph tube, put culture flask back into the incubator.
- Remove epindorph tube from the Laminar flow hood.
- Cut out a small strip of parafilm.
- Vortex the epindorph tube.
- Using a P20/P200 pipettor, pipette out 20µL of culture and expel on a strip of parafilm. Discard pipette tip into normal trash receptacle.

- Using a P20/P200 pipettor, pipette out 20 µL of Trypan blue and mix with the cell culture on the parafilm. Mix carefully by pipetting up and down without producing any bubbles. Use from the top layers of the stock trypan blue, the bottom 1/4 usually contains a significant amount of garbage.
- Wait 2-3 minutes. This will allow the dead cells to stain, and the live cells to pump out the dye.
- Pipette mixture onto a hemocytometer. Slowly pipette the mixture in, and let the capillary action fill the hemocytometer. Filling in too much will change the volume of your sample, and alter your calculations.

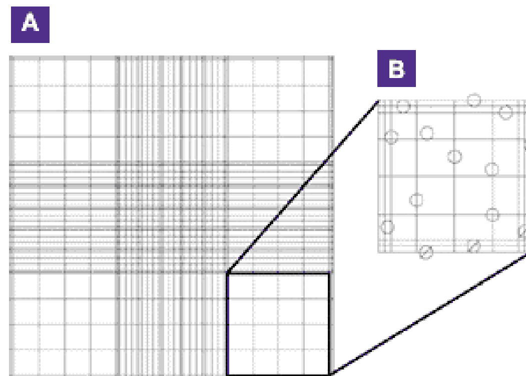


Fig A. The four squares of a hemocytometer.

Fig B. The individual cells that you should be counting on one square

- Count cells manually by using the cell counter. Dead cells will stain blue, while live cells should be translucent.
- Calculate the amount of cells per milliliter. The volume within the four squares of the hemocytometer is $0.4mm^3$ (0.4mL). Also remember to double the actual count, due to the 50/50 dilution of the culture with trypan blue.

Count all the squares and multiply by 5000, and it should give you the number of cells per milliliter.

- When finished, rinse hemocytometer and glass cover slip with distilled water and dry with Kim Wipe. Be careful not to break the cover slips.
- Trypan blue is toxic and is a potential carcinogen. Protective clothing, gloves and face/eye protection should be worn. Do not breathe the vapor.
- The central area of the counting chamber is $1mm^2$. This area is subdivided into 25 smaller squares ($1/25mm^2$). Each of these is surrounded by triple lines and is then further divided into 16 ($1/400mm^2$). The depth of the chamber is 0.1mm.
- There are several sources of inaccuracy:

  - The presence of air bubbles and debris in the chamber.
  - Overfilling the chamber such that sample runs into the channels or the other chamber
  - Incomplete filling of the chamber.
  - Cells not evenly distributed throughout the chamber.
  - Too few cells to count. Centrifuging the cells, resuspending in a smaller volume and recounting can overcome this.
  - Too many cells to count. This can be overcome by using a higher dilution factor in trypan blue e.g. 1:10

Some Useful Calculations

- Total time = end of exponential → start of exponential.
- N = concentration at end of exponential
- N_0 = concentration at beginning of exponential
- X= number of generations
- $N = N_0 2^x$
- $\log N = \log N_0 + x \log 2$
- Generation time = Total time / number of generations

Cell Freezing

Cryopreservation of cultured cell lines is essential in the cancer research laboratory. The aim of cryopreservation is to enable stocks of cells to be stored to prevent the need to have all cell lines in culture at all times. It is invaluable when dealing with cells of limited life span. The other main advantages of cryopreservation are:

- Reduced risk of microbial contamination
- Reduced risk of cross contamination with other cell lines
- Reduced risk of genetic drift and morphological changes
- Work conducted using cells at a consistent passage number
- Reduced costs (consumables and staff time)

There has been a large amount of developmental work undertaken to ensure successful cryopreservation and resuscitation of a wide variety of cell lines of different cell types. The basic principle of successful cryopreservation is a slow freeze and quick thaw.

Standard Freezing Medium

- 92% fetal calf serum and 8% sterilized DMSO

Cell Freezing

- Check that cells are healthy, not contaminated, and have the correct morphological characteristics.
- Change the medium 24 hours prior to freezing the cells. Both monolayers and suspension cells should be at about ~5,000,000 cells per milliliter. Cells must be in exponential phase
- For monolayers, trypsinize cells, resuspend in medium containing serum, pellet the cells by centrifugation at 200G for 5 minutes, and resuspend the cells in freezing medium.
- Transfer 1mL of cells into each cryotube. Label the vials with name of cell line, date, and growth medium.

- Place the freezing vials into a polystyrene box and freeze at the rate of 1~3ºC /min. The box is placed in the refrigerator for 30-40 minutes, and then placed in the –20ºC freezer for 1.5-2 hours. Then place the cells in the –80ºC overnight before transferring them into the liquid nitrogen.

<u>Troubleshooting</u>

- The most commonly used cryoprotectant is dimethyl sulphoxide (DMSO) however, this is not appropriate for all cell lines e.g. HL60 where DMSO is used to induce differentiation. In such cases an alternative such as glycerol should be used. WEAR GLOVES WHENEVER YOU USE DMSO.
- It is essential that cultures are healthy and in the log phase of growth. This can be achieved by using pre-confluent cultures (cultures that are below their maximum cell density) and by changing the culture medium 24 hours before freezing.
- The rate of cooling may vary but as a general guide, a rate of between –1°C and –3ºC per minute will prove suitable for the majority of cell cultures.
- When freezing in the –80ºC freezer it is important to have an allocated section for cell line freezing so that samples are not inadvertently removed. If this happens at a crucial part of the freezing process then viability and recovery rates will be adversely affected.

Cell Thawing

Many cultures obtained from a culture collection, such as ATCC, will arrive frozen and in order to use them the cells must be thawed and put into culture. It is vital to thaw cells correctly in order to maintain the viability of the culture and enable the culture to recover more quickly. Some cryoprotectants, such as DMSO, are toxic above 4ºC therefore it is essential that cultures are thawed quickly and diluted in culture medium to minimize the toxic effects.

- Run tap water to 37°C and place a beaker under the tap.

- Remove a vial of frozen cells from liquid nitrogen, and place in beaker containing 37°C. Wear protective goggles and gloves when thawing vials that have been stored in liquid nitrogen. Vials may explode when removed from liquid nitrogen.
- As soon as the solidified cyrotube begins to liquefy, immediately remove from water bath. Leaving the cells in the freezing medium (containing DMSO) is toxic, and it will kill the already-weakened cells.
- Spray and wipe the outside of the vial with 70% ethanol.
- Prewarm a suitable amount of medium to 37°C in an appropriately sized flask. The size of flask depends on the cell type as well as the desired cell density.
- Slowly pipet the cell suspension into the prewarmed medium. Mix during addition of the cells to the medium. Note: Immediate removal of DMSO may sometimes be necessary, especially for suspension cells. Following the addition of the thawed cell suspension to the prewarmed medium, the cells should be centrifuged (1000rpm 3-4 minutes) and resuspended in fresh, prewarmed medium.
- Incubate cells at 37°C overnight, and then change medium.

Consent Form

I, ____________________________ hereby state that I have read and understood all of the presented material in this manual. I will abide by the safety standards and practices set forth by this manual, and I will be fully responsible for all my actions (under condition that manual information is not out of date).

Signature ____

Date ____