George Lab Cell Culture Training Guide

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Topics Covered:

- Before You Get Started: Important Terminology
- Before You Get Started: General Cell Culture Equipment & Proper Use
- Before You Get Started: Lentiviral Transfections & Transductions
- Before You Get Started: IMPORTANT Chemical, Drug & Cell Line Notifications
- Cell Culture Protocols
- Notes

Before You Get Started: Important Terminology

Aseptic Techniques

Procedures to prevent contaminations and maintain sterility

Basal Media

Growth medium containing salts, amino acids, sugars, and other factors

Buffered Saline Solutions

Maintains required ph (7.2-7.6) for cell cultures; uses CO_2

Biosafety Cabinet/Hood

Continuous laminar flow through a HEPA filter which creates a protective barrier

Cell Type

Refers to all cells with a common phenotype

Confluency

The amount of the growth surface covered with cells expressed as a percentage

Conicals

Centrifuge tubes for volumes ranging between 15ml to 50ml

Continuous Cell Lines

Cells that can propagate in vitro for an indefinite time period

Cryopreservation

Process to store cells for an indefinite time period

Cryoprotectant solution

Solution containing components to help protect cells from damage during cryopreservation

Dewars

Vessel containing liquid nitrogen (-156°C gas phase, -196°C liquid phase) for cryopreservation

DMSO (Dimethyl sulfoxide)

Used in cryopreservation to minimize ice crystals from puncturing cell membranes (preventing membrane permeability)

FBS (Fetal Bovine Serum)

Plasma from fetal calf; contains growth factors

Growth Factors

Typically proteins that promotes cellular growth and proliferation

Incubator

Maintains optimum environment for cell cultures (5% CO_2, 37°C, humidity)—unless otherwise noted

In Vitro

Taking place outside a living organism

In Vivo

Taking place within a living organism

Labware/Lab Supplies

Diposable cell culture items including conicals, serologicals, pipettes, syringes, filters, etc

Microcentrifuge Tubes/Eppitubes

Centrifuge tubes for volumes ranging from 1µl to 2ml

Micropipettor

Pipettes that accurately dispense volumes from 1µl to 1000µl

Media

A medium containing amino acids, glucose, salts, antibiotics, vitamins, proteins

Mr. Frosty

Container filled with isopropyl alcohol used to control the freezing rate of cells (1°C/min)

Passage Number

Refers to the number of times that a cell population has been removed from a growth vessel and undergone a subculture (passage) process

Pasteur Pipette

Tapered glass tubes used for aspirating liquids

Phenol Red

A pH indicator (visual) where yellow indicates a pH < 6.8 and magenta indicates a pH >8.2

Pipette Aid

Mechanical liquid transfer device used with pipettes/serologicals

Pipette

Instrument to transfer measured volumes

Primary Cell Lines

Cells taken from living organisms that propagate in vitro but are **not** immortalized

Reagent

Biological or non-biological substance/mixture

Serological

Graduated pipette to accurately transfer measured volumes

Serum

Plasma containing growth factors; example FBS

Subculture

Culture of daughter cells from passaging a growth vessel

Tissue Culture Flasks/Plates/Dishes

Containers with treated surfaces to promote cell attachment

Trypsin/EDTA

Enzyme/chelator agent used to detach cells from growth surface

Typsin Neutralizing Solution

Inhibits trypsin by reducing the availability of the enzyme

Wash Solution/Buffers (HBSS, DPBS, PBS)

Buffered saline solutions without Ca^{2+} and Mg^{2+} used to remove trypsin neutralizers and divalent cations

Before You Get Started: General Cell Culture Equipment & Proper Use

Microorganisms are constantly present; they can be found on bench tops, objects, clothing, and skin as well as floating in the air. Cell cultures are highly susceptible to contamination by microorganisms such as bacteria, fungi, yeast, and mycoplasma. These microorganisms are easily transferred to cultures by incidental surface touches, simply falling off an object over an exposed culture, or moving along liquid pathways. Aseptic technique is the practice of sterile procedures to eliminate contamination sources. To safe guard cell cultures from unwanted contamination or cross contamination with other cell lines, aseptic techniques must be stringently followed.

• PPE (Personal Protective Equipment)—

* **ALWAYS** wear gloves when performing cell culture work.
* Wear a clean, barrier lab coat (optional: additional disposable sleeves) to eliminate exposure to contaminants residing on skin and clothing.
* **SPRAY YOUR GLOVES WITH 70% ETHANOL EVERY TIME YOUR HANDS ENTER THE HOOD.**

Why do we use 70% Ethanol??
Between 60-80% Ethanol is typically effective in destroying the cell membrane of most microbes/bacteria, thus killing it. Higher concentrations aren't as effective as it will evaporate fairly quickly, minimizing it's efficacy.

• CENTRIFUGES—

* Make sure caps are on tight before placing conicals in rotor buckets.
* Use safety caps on buckets to prevent accidental spills and contaminations.
* ALWAYS use the correct counterbalance(s) to balance across the rotor.

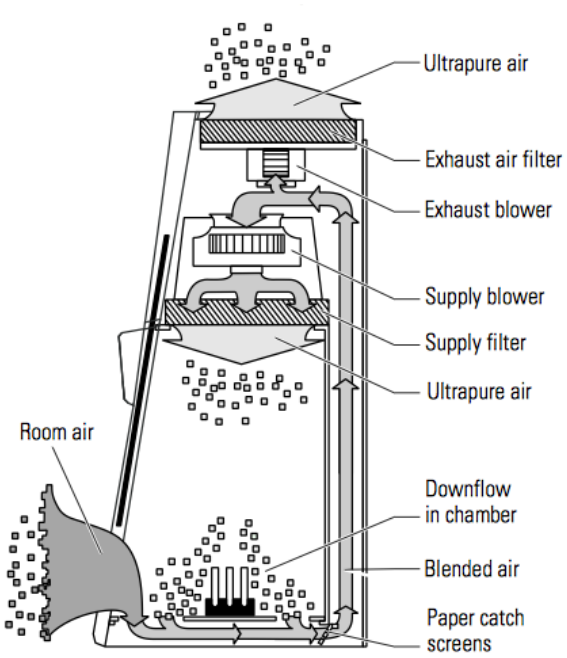


Figure 1-2. Airflow Filtering System

• BIOSAFETY CABINETS/HOODS USE— BEFORE & AFTER CULTURING

BSCs are designed to protect individuals and the environment from potentially infectious aerosols and to maintain a sterile workspace inside the cabinet.

Biosafety cabinets are certified once per year.

* Turn on the fluorescent light, and lift sash to marked location (the hood's alarm will stop beeping).
* Spray the hood down with 70% Ethanol **PRIOR** to working in the hood AND **AFTER** working in the hood (including the pipettes, vacuum knob and aspirator line)—DO NOT SPRAY the ceiling of the biosafety cabinet.
* Spray ALL ITEMS in the hood with 70% Ethanol **PRIOR** to placing in the hood INCLUDING your gloves—DO NOT SPRAY the caps of flask as to prevent wetting the filters.
* Turn the hood OFF when done working in it.
* **DO NOT** cover the front or rear air grilles with paper, absorbent pads, or any other materials.
* When working in the biosafety cabinet/hood, leave gaps between items to allow proper air flow; do not build a "wall" which will block airflow.
* When finished, turn the fluorescent light off and close the sash to marked location (the hood's alarm will then stop beeping).

➢ Pipette Use…

Determine which pipette best suites your needs for the desired volume. **BE SURE YOU DO NOT EXCEED THE MAX VOLUME** as this will offset the calibration, as well as damage the pipette.

Pipettes are calibrated once per year and should be handled with care.

P1000	200µl -1000µl
P200	50µl – 200µl
P100	20µl – 100µl
P20	2µl – 20µl
P10	1µl – 10µl

HOW TO USE THE PIPETTE: There are three plunger positions.

Open Position 1st Stop 2nd Stop

1. Press the plunger to the 1st Stop to void out air.
2. Place tip into solution. **DO NOT submerge it.**
3. Slowly release the plunger to the Open Position. **DO NOT let the plunger spring back.**
4. Place tip into receiving conical/flask/container.
5. Slowly press the plunger to the 1st Stop. Wait a few seconds.
6. Press plunger to 2nd Stop to release any residual volume.
7. Release plunger. Do not let the plunger spring back.

➢ *If tip is immersed in a liquid, retract tip from solution before releasing plunger.*

8. Discard tip into waste receptacle by pressing ejector button.

➢ Handling Liquids w/Serological Pipettes…

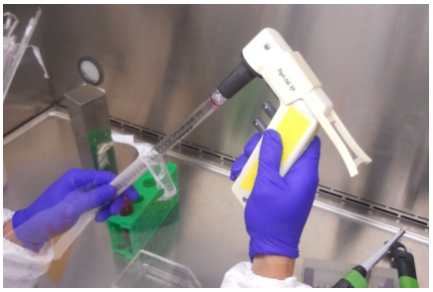
*** ALWAYS** label flasks, dishes, etc appropriately:
Cell Type, Passage Number, Date and Initials

*** DO NOT** leave bottles, conical tubes, flasks, and plates open and exposed.

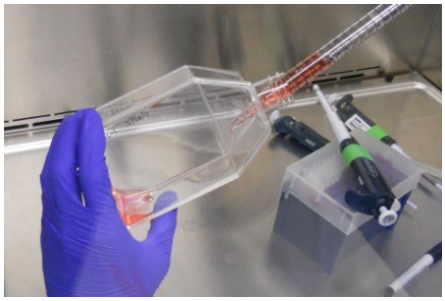
* **DO NOT** cross over open conical tubes, bottles, flasks, or containers.

* DISCARD serological/ tip if it touches any surface to minimize contamination.

* Caps can be placed faced down, faced up, or on its side.



1. Banana peel serological wrapper and push pipette straight into pipette aid.
2. Orientate serological in pipette aid for ease of reading volumes.
3. Carefully remove wrapper ensuring the tip does not contact anything including the wrapper—set aside for disposing of the tip once finished using it.
4. Remove cap from bottle/conical.
5. Place serological in the center of conical/bottle's neck. Avoid contact with the container's neck.
6. Place tip into solution; DO NOT submerge the shaft of the serological.
7. Press the top button on the pipette aid until the desired volume of liquid has been withdrawn. Follow the meniscus while pipetting to avoid drawing air.
8. Carefully remove serological from container without touching the interior.
9. Recap bottle/conical and uncap secondary container.
10. Insert serological in the center of secondary container. Slowly push bottom button on pipette aid to dispense liquid. Avoid blowing bubbles.
    When transferring liquid to a TC flask, angle the flask and insert the serological just pass the neck. Release liquid against a non-growth surface.



11. Remove serological from secondary container and recap.
12. To remove serological from pipette aid, pull straight out and re-sleeve pipette in wrapper and place in waste receptacle.

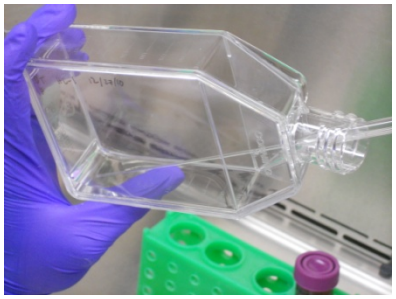
➢ Aspirating Liquids…

Liquids are removed using a:

Micropipettor
Serological pipette
Pasteur pipette
…attached to a vacuum line—DO NOT use the POUR OFF method.

Liquid is removed and disposed of in a waste receptacle containing 10% bleach or 2% Bacdown.

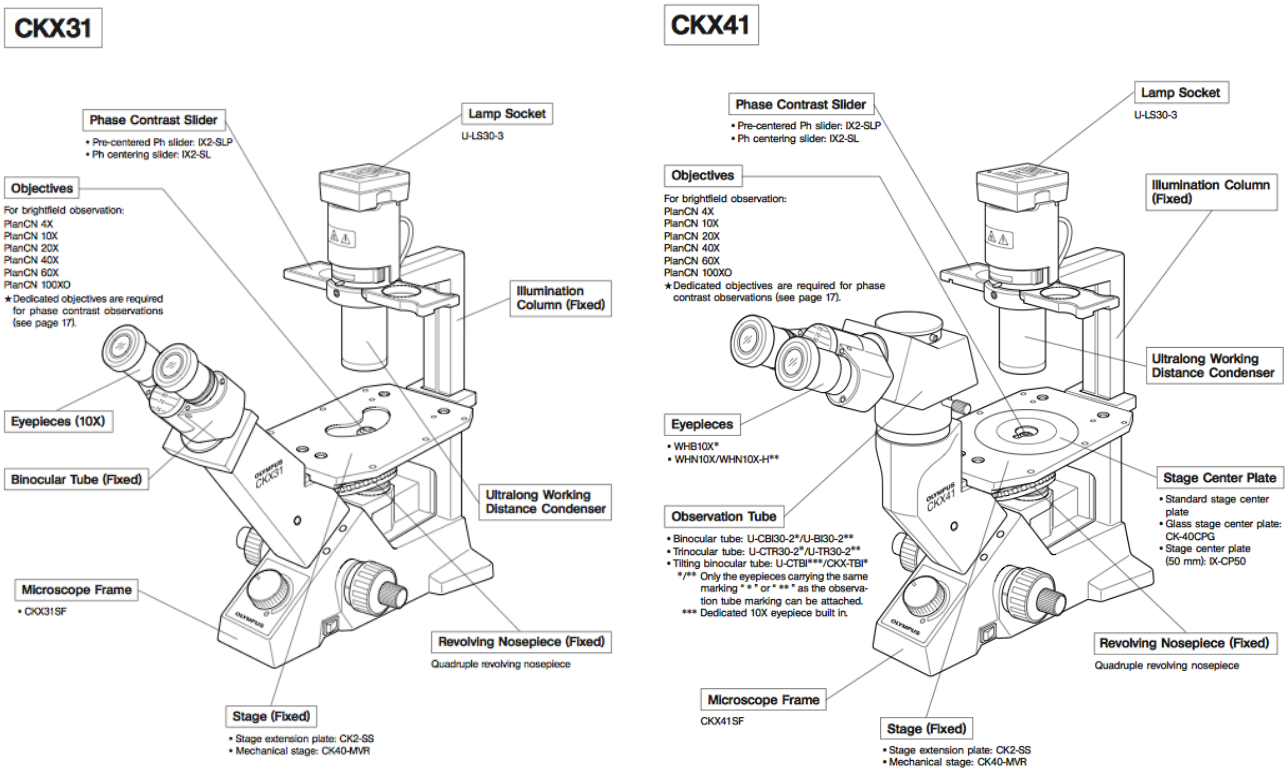
1. Make sure the vacuum line is on prior to attaching the Pasteur pipette.
2. Remove a Pasteur pipette from canister without touching remaining pipettes.
3. Attach the Pasteur pipette to vacuum line by inserting it into the tubing.
4. Be careful not to touch the pipette's shaft while attaching.
5. When aspirating liquid from a TC flask, angle the flask towards you and aspirate from the bottom left front corner.

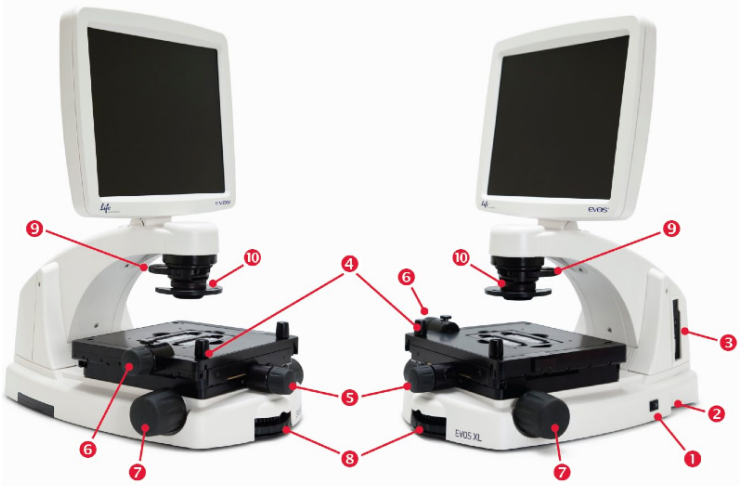


6. When aspirating supernatant from a conical, hold conical at 45˚ and place the tip just above the liquid line. Follow the liquid line down while aspirating.

• INCUBATORS—

* Incubators are non-sterile chambers that must be kept as clean as possible.
* Immediately wipe any spills with a disinfectant.





❶ Power switch
❷ Power input jack
❸ USB and DVI ports
❹ Coarse stage positioning knobs
❺ Stage X-axis knob
❻ Stage Y-axis knob
❼ Focusing knobs
❽ Objective selection wheel
❾ Phase annuli selector
❿ Condenser slider slot

• MICROSCOPES—

* **ALWAYS** use gloves when using the microscopes and the computers connected to them.
* Periodically wipe down the microscopes with a germicidal wipe, alcohol prep pad, or a Kimwipe sprayed with 70% Ethanol.

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* To clean the objective, **only use lens paper** and lens cleaning solution. Everything else will scratch the lens.
* Turn the microscope **OFF** after each use.

• PUMP MAINTENANCE—

Some cell culture experiments will require culturing under conditions where constant pressure or constant flow is necessary for chemcial/drug delivery. Syringe Pumps provide a constant pressure dispensing system for such experiments.

* Be sure to label ALL chemicals connected to the pump for your experiment.
* Pump Maintenance:

  WIPE DOWN the pump, chemical tray and table with a germicidal after **EACH USE** to keep chemical residue, etc from building up and causing damage to the pump.

  If any leakage occurs from the tubing onto the diaper…. change the diaper IMMEDIATELY.

  Try to MINIMIZE the amount of tubing hanging from the pump so that it does not interfere with waste disposal and going into/out of the incubators.

• WATER BATH—

* Maintain 37°C temperature at all times.
* Wipe down all bottles (coming from the water bath) **PRIOR** to placing in the hood AND **AFTER** coming out of the water bath.
* Spray ALL ITEMS **PRIOR** to being placed in the water bath with 70% Ethanol.
* Use ring weights to partially submerge bottles in water.
* Place cryovials and eppitubes in floating holders.

• BIOHAZARD SPILLS—

[**FIRST**—READ the posting in the cell culture room regarding care for spills and clean up and be prepared to follow those WUSTL guidelines regarding spills]

* PLEASE Notify Cristi & your mentor ASAP as soon as a spill occurs, and we will evaluate if security needs to be notified (5-5555) *

• LIQUID WASTE DISPOSAL—

* **IF YOU PLAN TO WORK** with special chemicals/drugs in your cell culture experiment, please notify Cristi so that a Sewer Disposal Form can be filled out and approved **BEFORE** disposing of it down the drains.

IF your experiment needs to start before the approval (for some reason), please use A SEPARATE CONTAINER (see Cristi on what you can use) with the appropriate BLUE EHS STICKER (found in the chemical room), to collect the waste and we can notify EHS for a pickup.

- **ASPIRATOR FLASK WASTE:**
  * Aspirate biological liquid waste with aspirator (waste flask should contain 10% bleach).
  * Most cell culture waste has been approved for sewer disposal once in 10% bleach for 20-30 minutes.

• BIOHAZARD WASTE—

* Place **ANY** supplies (eg. tips, flasks, tubes, etc) in contact with cells in the biohazard trash—if you're not sure, dispose of it in the biohazard!

- **SHARPS:**
  * Sharps (including glass Pasteur pipettes, needles, etc) should go in the red Sharps Biohazard Containers.

Before You Get Started: Lentiviral Transfections & Transductions

→ FOR LENTIVIRAL TRANFECTIONS & TRANSDUCTIONS:

**What is Lentiviral Titer??*

For our purposes, lentiviral vectors (gene delivery vehicles) plus 3 lentiviral "helper" plasmids are transfected into HEK293T cells ('packaging cell line" required for lentivirusreplication; easy to grow and transfect readily) are used to generate.

Once transfected and the supernatant/lentiviral titer harvested, it can be used in a single-round infection (transduction) that results in long-term integration into the genome of both dividing and non-dividing cells using vectors derived from human immunodeficiency virus (HIV).

IF you are using lentiviral titer/transduced cells (Azurite, EGFP or mCherry) for , you are REQUIRED to use a SEPARATE beaker w/10% bleach for ANYTHING in contact with the actual titer.

When actually working with the lentiviral titer (eg. doing transductions), you will need a separate beaker with bleach to directly bleach ANYTHING coming in contact with the actual titer. Simply pour off of the bleach and dump the remaining plastics in the biohazard waste (eg. pipet tips, microcentrifuge tubes, etc)

- Pipet tips should be bleached (pipet up and down a few times and leave in bleach
- Any tubes or other supplies (including the original titer tube), etc should be bleached
- Wipe down the hood and pipets with germicidal wipes really good after you're done and then spray with ethanol

*Until you've changed the media at least 3 times, please CONTINUE to do the separate bleaching to ensure all titer has been removed. Once the media has been changed 3 times, you can then proceed as normal with using the main cell culture waste in the flasks beneath the BSCs and no longer bleaching the pipet tips, etc separately.

**Once you're ready to passage, the original flask or plate with the titer should be bleached for 20 minutes before discarding in the biohazard trash as well—despite any additional media changes.

Before You Get Started: IMPORTANT Chemical, Drug & Cell Line Notifications

• CELL LINE NOTIFICATIONS—

Most cell lines that you will work with will come from stocks stored in the lab dewars. However, some experiments will require purchasing cells from an online vendor or will be obtained from a collaborator.

- *In cases where you obtain cells from collaborators:*

IF you plan to receive cells from a collaborator, please notify Cristi **FIRST** as the appropriate documentation will be necessary for use in the lab.

- *In cases where you obtain cells via online vendor purchases/Cristi:*

No additional action will be necessary as it will be noted once purchased.

• CHEMICAL/DRUG NOTIFICATIONS—

Cell culture experiments will require manipulations via chemicals already in the lab. However, some experiments will require purchasing various chemicals/drugs from an online vendor or will be obtained from a collaborator.

- *In cases where you obtain chemicals/drugs from collaborators:*

IF you plan to receive ANY chemical/drug from a collaborator, please notify Cristi **FIRST** as the appropriate documentation will be necessary for use in the lab.

- *In cases where you obtain cells via online vendor purchases/Cristi:*

No additional action will be necessary as it will be noted once purchased.

- *For ANY chemcials/drugs stored in secondary containers:*

Please label the container appropriately:

Chemical Name
Date
Your initials
*Hazard Class Color Coded Sticker/Tape:

Basic hazard class color code example:

Flammable =	RED
Corrosive =	WHITE
Oxidizer/Reactive =	YELLOW
Toxic/Carcinogenic =	BLUE
Non-hazardous =	GREEN

Note: This color coding scheme can easily be accomplished by using either using colored adhesive dots or colored tape.

George Lab Cell Culture Protocols

• Thawing and Seeding Cells

You'll need:

- Pasteur Pipettes
- Cryovial of Cells
- Flask
- Media (warmed)
- 15ml conical tube
- Serological Pipettes

-->Protocol:

1. Grab and LABEL a flask (eg. T150)
    a. Name/Date/Cell Type/Passage #
2. Dispense 20mL of FGM Media into an empty/labeled T150 flask
    or T25 Flask = 3 ml
    or T75 Flask = 10 ml
3. Place 5 ml media into 15 ml conical.
4. Remove frozen cell vial from liquid nitrogen.
5. Quick thaw of vial in water bath (37˚C)--thaw until a small ice piece remains in cryovial.
    *NOTE: move quickly because cells contain DMSO and when heated/RT for a long time will cause the cells to lyse
6. Transfers contents of vial into the prepared 15ml conical with media.
7. Centrifuge conical at 1000rpm for 5 minutes.
8. Aspirate supernatant leaving cell pellet untouched.
9. Resuspend pellet in 1 ml media. Carefully break up cell pellet with micropipettor.
10. Transfer cell suspension into prepared TC flask.
11. Gently rock TC flask to evenly distribute cells.
12. Using the microscope, check cell morphology and uniform cell distribution. Place in the incubator.

• Feeding a Flask

You'll need:

- Pasteur Pipettes
- Media (warmed)
- Serological Pipettes
- Flask w/cells

* Wipe down all bottles (coming from the water bath) **PRIOR** to placing in the hood
* Spray all items in the hood with 70% Ethanol **PRIOR** to placing in the hood
* Check your media for COLOR and CLARITY
    Golden Orange Media→ possible contamination

Cloudy Media → possible contamination

-->Protocol:

1. Warm media in the waterbath (37˚C)
2. Pull flask from the incubator and under the microscope, check the cell morphology and confluency.
3. Aspirate the media from the flask (tilting flask AWAY from the bound cells in the flask)
4. Add the appropriate volume of media to the flask.
5. Check the flask under the microscope.
6. Place the flask in the incubator.

*At desired confluency freeze cells.

• Passaging Cells

You'll need:

- Flasks w/cells
- Washing Buffer (warmed)
- Trypsin (warmed)
- Media (warmed)
- 10ul and 1000ul pipette tips
- Pasteur Pipettes
- Serological Pipettes
- Microcentrifuge Tubes

-->Protocol:

1. Under the microscope, check TC flask for confluency—passage when cells are 90% confluent.
2. Aspirate Media from the flask
3. Add appropriate amount of wash buffer (HBSS, PBS) and gently rock the flask.
   T25 Flask = 1 ml
   T75 Flask = 3 ml
   T150 Flask = 4 ml
4. Aspirate Wash Buffer
   a. **WASH/ASPIRATE 3x**
5. Add Trypsin (0.025% - .05%) to cut proteins and place in the incubator (37˚C)
   a. MAX Time: 3-5 minutes –you'll begin to see cells floating and lifted off of the flask (tap the flask gently if needed)
      T25 Flask = 1 ml
      T75 Flask = 3 ml
      T150 Flask = 6 ml
6. Neutralize the Trypsin with media (2:1 ratio of media/Trypsin).
7. Spin down the cells in a conical tube at 1000rpm for 5 minutes.

8. Aspirate media off of the cell pellet at the bottom of the conical tube and add media (1mL for low cells—up to 3mL depending upon pellet).
    a. Pipet up and down to break the pellet apart SLOWLY.
9. To **COUNT CELLS**:
    a. Grab a microcentrifuge tube and place 10ul of cells into the tube.
    b. Add 10ul Trypan Blue and pull 10ul TOTAL off and dispense into a slide for the Cell Countess.
10. To **RESEED**:
    a. Grab a NEW flask and LABEL it appropriately.
    b. Add appropriate volume of media and add your cells (volume will need adjusting depending on the cell count).
    c. Place in the incubator.

• Freezing Cells

You'll need:

- FBS (thawed)
- DMSO (in chemical room—light sensitive, so save this for the last steps)
- Conical Tubes
- Cryovials
- Labels for the Cryovials
- Mr. Frosty Container
- Pipette Tips

80% Cells + Media 10% FBS 10% DMSO

1. Count the cells you wish to freeze
   **optimal concentration 1x10^6 cells per cryovial*
2. Label each cryovial appropriately
3. Add the appropriate amounts of Cells + Media, FBS and DMSO to each vial
    a. Determine **how many vials will be frozen**

        Number of Vials = Total number of cells/10^6 cell/vial
        = 9.0 x 10^6 cells/10^6cell/vial
        = 9 vials

    b. Determine total **cryoprotectant volume needed**

        Cryoprotectant Volume = number of vials* 1ml cryoprotectant/vial
        = 9 vials * 1ml/vial
        = 9ml or 9000µl

    c. Determine volumes of **INDIVIDUAL cryoprotectant solution components**.

        →10% DMSO = 0.1*total cryoprotectant volume
        = 0.1*9000 µl

= 900 µl

→10% FBS = 0.1*total cryoprotectant volume
= 0.1*9000 µl
= 900 µl

→80% Media = 0.8*total cryoprotectant volume
= 0.8*9000 µl
= 7200 µl

- ***NOTE: Recall the cells are sitting in a resuspension volume!*** *This volume is part of the Media calculated volume. Subtract out the resuspension volume to determine how much additional Media is required.*

d. Check Math = 10% DMSO + 10% FBS + 80% Media (resuspension + additional)

4. Add the calculated cryoprotectant components to a conical tube…
   Media [to the cell suspension]—FBS—DMSO
   ** Always add DMSO component **LAST** to minimize unfrozen cell exposure to DMSO.*
5. Store vials in Mr. Frosty.
6. Place Mr. Frosty in the -80˚C for a minimum of 6 hours but no more than 48 hours.
7. Enter vial information into Liquid Nitrogen Storage Log Book.

• Cell Counting with the Cell Countess

1. Take 10ul of cell suspension sample and add it to a small microfuge tube that's been sterilized in the hood.
2. Add 10ul Trypan Blue to the sample and mix well.
3. Remove 10ul of the sample and load it into the Countess Chamber Slide.
4. Insert the chamber slide into Cell Countess.
   a. Focus (using the knob) and Zoom in to the cells to ensure it counts accurately
      *__DO NOT__ focus on cell shape but on the light intensity of the cells' center.
   b. OPTIMAL READINGS: 95%+ Viability
5. Press COUNT CELL.
6. **Calculate the Total Number of Cells**:
   Total number of cells = Alive cells/ml *[resuspension volume]
   Alive cells/ml is given by Cell Countess

   Example: = $4.5x10^6$ cells/ml * 2ml resuspension volume
   = $9.0\ x10^6$ total number of cells

***DO NOT LEAVE SLIDES IN THE CELL COUNTER—**The plastic slides must be disposed of in the biohazard waste immediately after use.

- **Sample Protocol for Transducing Endothelial Cells with Azurite Lentiviral Titer in T150 Flasks…**

Day 1
-Seed ECFC-ECs into the T150 flask and wait until 30-40% confluency

Day 2- Transduction
-Aspirate off Media
-Add 5ml of EGM2 media to the flask
-Add Polybrene/ Hexadimethrine bromide (stored in the –20) drop wise to the dish for a final concentration of 8ug/mL. Gently swirl the dish to mix. (20ul should get you to 200ug)
-Add remaining media + viral supernatant (**1:50** dilution—500ul + remaining 20ml EGM2 media) and incubate for ~24hrs at 37C

Day 3
-Image on the inverted microscope to check for fluorescence and continue to change media every other day until cells are 100% transduced

Notes