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*** Starred sections have been borrowed and/or adapted from the "Chan Group Policy Guidelines 2010"**

Wheeler Lab General Rules

In order to maintain a professional and harmonious atmosphere in the laboratory, all individuals under my supervision and all visitors must obey the following rules. There can be no exceptions. New individuals will be instructed in the significance of these rules by the labs Safety Officers (currently Calvin and Roger).

Safety

1. All safety measures must be observed. Special attention must be taken in dealing with biological materials (including culture media and blood) and radioisotopes. See resources under Biosafety Resources for more on that.

2. Eating and drinking is not permitted in the laboratory.

3. Turn off electrical appliances after use.

4. All waste must be labeled (with name, date and type of waste).

5. Keep all passage clear (no bike parking in the lab).

6. Broken glass, used needles, tissue culture waste must be disposed of in the proper containers.

Security

1. Lab doors lock automatically and must be kept closed at all times.

2. Keys are not to be loaned out, except with Aarons permission.

3. All individuals not under Aarons supervision are considered to be visitors and are not allowed to be in the laboratory on their own. Exceptions can be made with Aarons permission. These visitors will need to present themselves to the labs Safety Officers (currently Calvin and Roger) for instruction in these rules.

4. Report the presence of an unauthorized person to the labs to one of the lab captains (currently Sebastian and Richard), Aaron or Lisa .

Common areas

1. Balance, centrifuge, pH meter should be cleaned after use.

2. Do not contaminate deionised/distilled water lines or tanks.

3. Follow instructions for use of all instruments. If you don't know how to do something, ASK!

4. Do not store things in the fume hoods. Do not occupy them unnecessarily.

Tissue Culture Room

1. Keep your personal supplies in your TC storage area.

2. Clean hoods with 70% ethanol and Conflikt before and after each use.

3. Dispose of biological waste in appropriate containers.

4. Clean haemocytometer immediately after use.

5. Shut off microscope when not in use.

6. Clear incubators of unused or unneeded cells promptly.

Professionalism

1. The lab is a workplace and not a common room. Friends are welcome but their presence should not interfere with others.

2. Record all results in your bound lab notebook directly. Do not use separate pieces of paper, etc. If necessary, fasten result into the book at the appropriate locations. Record objectives and conclusions with each experiment. The lab notebook, and all other data, are the property of the lab and remain in the lab after your departure.

3. Do not "borrow" equipment or supplies without permission. If you must, fill the loan book.

4. Label all solutions or preparations with its name, concentration, your name and date.

5. Use the loan book for recording loaned items, no matter how trivial. Record item, name of borrower and their supervisor.

6. To order items, give Lisa a note or e-mail with the catalogue number and supplier information. Lisa will place all orders for the lab.

7. Return items to original locations after use.

8. Confine your experiment to your own work area.

9. Clean up your area as soon as practical after each experiment.

Biosafety Resources

When dealing with biological compounds ensure you are following all of the proper safety precautions. The two biosafety manuals below contain the info necessary to ensure safe handling of biological materials.

University of Toronto Biosafety Manual
(https://www.canada.ca/en/public-health/services/canadian-biosafety-standards-guidelines/second-edition.html)

Canadian Biosafety Standards 2nd edition
(https://www.canada.ca/en/public-health/services/canadian-biosafety-standards-guidelines/second-edition.html)

Managing Your Research*

In order to start doing research on a project, all researchers must provide a project title, project aim, a paragraph describing the study, materials & methods, a timetable of activities (with defined milestones), intended figures, and literature search. This document should not be longer than 3 pages. It is recommended that the researcher spend ~ 2 weeks in this process before the start of the project. Once this document has been submitted, Aaron will go through it and will discuss the proposal with researcher and either approve, modify, or reject the project. The project will be continuously changed and re-written as the project starts and continues.

Use the Research Project Flowchart (Figure 1) and the Research Project Checklist (Figure 2) to help you plan and manage your research. This is a guide taken from other sources and needs adaptation for any specific project. For example, one item that is not clearly stated in these figures and in item 5, are defining the appropriate control groups.

Milestones

Once the project begins, the timetable and milestones will be used to monitor the progress of the project. Milestones are basic bi-weekly reports that describe research progress and activities (Table 1). Whenever you write-up your milestones, you should make 2 copies (one for Aaron, the other to place in your lab notebook.)

Timesheets

You are required to create a schedule of "lab-dedicated" time slots, based on your course timetable and thesis project, during which you will present in the lab on a weekly basis. A minimum of six (6) hours per week is required. Use the Monthly Timesheet (Table 2) to track your hours, and have one of the graduate students initial the sheet weekly to confirm your presence.

Figure 1. Research Project Flow Chart *

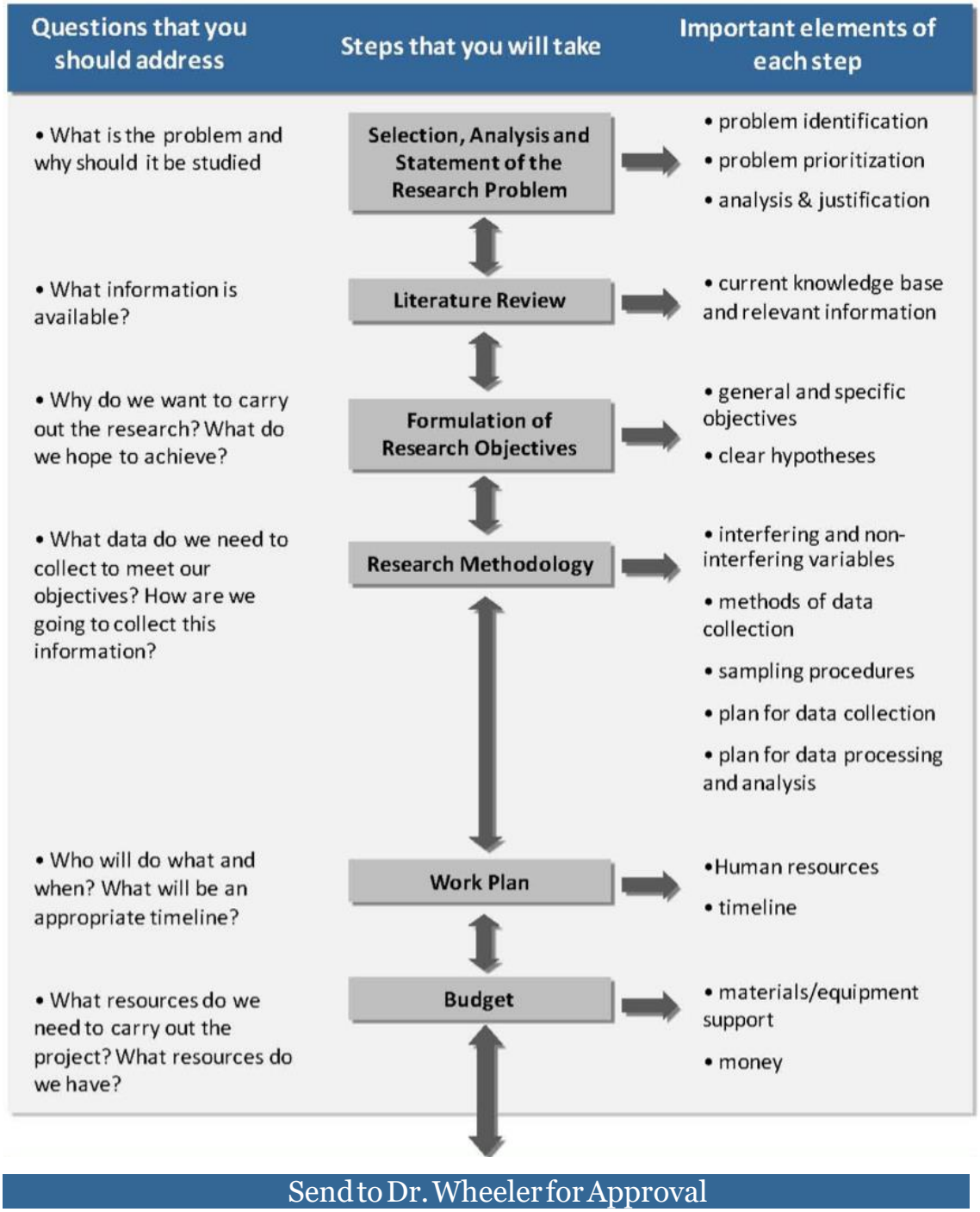


Figure 2. Research Project Checklist *

1. **The Problem**
   - ☐ Identify the problem
     - Present a clear, brief statement of the problem
   - ☐ Describe the significance of the problem to current societal and scientific issues. Refer to one or more of the following considerations:
     - Is it timely?
     - Does it affect a wide/influential/critical population?
     - Does it fill a research gap?
     - Does it permit generalization to broader principles or scientific knowledge?
     - Does it sharpen the definition of an important concept or relationship?
     - Does it have many implications for a wide range of practical problems?
     - Does it have the potential to create or improve an instrument for sampling and analyzing data?
     - Does it provide a possibility for a fruitful exploration with known techniques?

2. **The Research Question**
   - ☐ State the specific question(s) you wish to answer
   - ☐ Highlight proposed answers to the question
     - Describe what answers have been suggested in the literature
     - Add to the above your own suggestions and answers
     - Briefly describe how various answers are related to particular theoretical framework(s)

3. **The Research Proposal**
   - ☐ State a research proposition derived from Section 2 above
     - Elaborate on the theoretical framework(s) that relate to your proposal
     - Define the main concepts and identify Objectives
     - Clarify the nature of the relationships proposed or expected
     - Indicate the significance of this proposal for advancement of research and theory
     - Is the proposed research feasible?

- State the research objectives clearly
    - Define and elaborate on the concepts in your research proposal as outlined in Section 3 above. State a clear hypothesis and use operational terms if necessary
- Identify dependent and independent variables and parameters and any methods or scales that may be used to assess them
- Identify any serious scientific and/or operational limitations in your proposal

5. Research Design

- Describe the ideal experimental design(s) with special attention to
    - Procedures, subjects, experimental methods
    - The control of interfering variables
- State the assumptions in the ideal design
- Specify the limitations and nature of any interfering parameters in the design and describe any relevant measures you will take to control and/or account for them

6. Methodology

- Describe the experiment(s) you will conduct for data collection
    - Specify the phenomena relevant to your experiments
    - Describe the type of equipment and materials you will need
    - Describe general sampling and instrumental procedures
    - Describe the measures of reliable quantitative variables
- Identify any training or collaborative effort(s) that may be required

7. Working Guide and Budgeting Resources

- Prepare a working guide that covers total estimated time and cost of proposed research. Refer to the following:
    - Timelines- estimated time required to complete defined tasks e.g.,
        - Literature review
        - Planning research
        - Conducting experiments
        - Collecting data
        - Processing data
        - Trial plan
        - Potential for revised plans
        - Preparing a final report and/or writing manuscript for publication
- Perform a feasibility analysis of the proposed research by budgeting resources
    - Financial
    - Human
    - Materials and tools required
    - Time

8. Research Progress

- ☐ Summarize conducted experiments
- ☐ Process and analyze data and research goals
    - Look for patterns
    - Organize data
- ☐ Evaluate results
    - Are the results reproducible?
    - Were the results expected? Do the results agree with scientific indications in the literature? Justify
    - Were the results not expected?
        - If so, what might be the possible reasons? What are the probabilities of error?
        - What experiments would be needed to elucidate the cause of unexpected results?
        - Are there any pre-existing references to this unexpected behavior in the scientific literature?
        - Do the experiments need to be repeated?

9. Analysis and Interpretation of Results

- ☐ How do the results relate to the original theoretical framework of the problem?
    - Have the research questions been answered? If not, why not?
    - Are there new and interesting questions that need to be answered before arriving at a conclusion?
    - Do the results validate the theory?
    - How significant are the new results in light of the present knowledge?
- ☐ Arrive at conclusion(s) if any, including revising plans and conducting more experiments if appropriate

10. Projected Future Considerations for Research

- ☐ Describe the anticipated direction of growth for your research and its applicability to the original framework
- ☐ Identify any new, specific research questions that have arisen from your results

Milestones

Milestones are basic bi-weekly reports that describe research progress and activities. These reports will be used to monitor the progress of your project over the course of the year.

Table 1. Bi-Weekly Research Milestones

Date Range:		
Milestone	**Notes**	**Date Competed**

Table 2. Monthly Time Sheet

Name: Month:

Supervising Graduate Student:

Date	Time in	Time out	Hours	Student	Grad. Student
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					
21					
22					
23					
24					
25					
26					
27					
28					
29					
30					
31					
TOTAL					

Your Lab Notebook*

A lab notebook is a crucial document that demonstrates what you worked on. A lab notebook demonstrates the validity of your data if a journal, patent agency, or company requests proof. Therefore, you must keep a neat notebook that contains all of the data and analysis. Your daily planning of activities should be written or taped in your notebook (see Table 2). Any figures should be placed in your notebook, plus any analysis or thoughts should be written in your notebook. State the objectives and conclusions for each experiment. Use the Notebook Up-keeping Checklist (Table 3) for guidelines on maintaining your lab notebook.

Also, the **lab notebook belongs to the lab**. The lab notebook must be kept on the shelf of a researcher's desk or on the Lab bench. When a researcher leaves the lab, all notebooks must be left behind. A researcher may make Xerox copies of the lab notebook to take with them. I will keep the notebooks for five years after a researcher leaves. If there are any patents associated with the research, I will keep the notebook(s) for 26 years. All notebooks should be labeled on the side with your name, year, and book #.

Table 2. Daily To-Do List for your Lab Notebook*

Date:

Task	Notes	Status	
		Complete	Not Complete

Table 3. Notebook Upkeeping Checklist*

1. Did I update the Table of Contents? ☐
2. Did I date each entry? ☐
3. Are all my pages numbered consecutively? ☐
4. Did I use cross-references and continuation notes when necessary? ☐
5. Did I properly void all blank sections? ☐
6. Did I enter all information directly into my notebook? ☐
7. Did I paste and/or reference all graphs and images in my notebook? ☐
8. Did I properly introduce and summarize each experiment? ☐
9. Did I include complete details of all first-time procedures? ☐

Dos for Keeping Lab Notebooks

1. Do use permanently bound books
2. Do sign and date every entry
3. Do keep a Table of Contents
4. Do use permanent ink
5. Do use past tense when describing experiments that were performed
6. Do explain abbreviations and special terms
7. Do paste attachments like graphs and computer printouts into the notebook and sign and date
8. Do outline new experiments with special focus on objectives and rationale
9. Do record relevant lab meeting discussions and the names of people making ideas or suggestions
10. Do provide adequate detail for: test descriptions, operating conditions
11. Do leave you notebook in the lab at your desk space

Don'ts for Keeping Lab Notebooks

1. Don't use loose/leaf or temporary notebooks to record experiments
2. Don't leave any entry without a date
3. Don't leave blank spaces
4. Don't modify prior entries at a later date
5. Don't remove original pages; Null experiments must simply be crossed out in ink with a clear line
6. Don't use pencil
7. Don't take notebooks away from the lab

From Chan Lab Guidelines 2010; adapted from "At the elm" by Kathy Barker

Standard Operating Procedures

Guidelines for using Pubmed

Author: Dean Chamberlain

This is an introduction on how to use Pubmed for a literature search.

Materials and Equipment

Computer with Internet connection

Method

1. Go to Pubmed: http://www.ncbi.nlm.nih.gov/pubmed
2. On the main page, go to the advanced search function.
3. Type what you are searching for into the search builder box.
4. Click on the "all fields" to select the right filter (ie author) or leave at "all fields" if doing a general search.
5. Change the "and, or, not" tab to what you want.
6. Click the "add to search box" button. The search parameter keyword should move to the search box at the top of the window.
7. Add a new key word to the search builder box.
8. Repeat steps 4 through 6.
9. After building your search press the preview button beside the search box. This will open a Query in the search history. From this you can determine the number of papers that your search found. Try for a couple of hundred papers for a general search.
10. Add or remove key words from the search box and press "preview" to refine your search.
11. To look at the list of papers, either push the "search" button or click on the results in the search history.
12. This brings you to the results paper. From the right hand menus, you can select all papers or reviews only. Also on the right, there is a "search details box" which shows all of the keywords that you have used for you search. Some of the single words you enter will bring up multiple words that are searched (ie isolation) and some will not. It is a good idea to check what the key words you are using are.
13. To get more information about a paper click on the title.
14. To get the paper itself, either click on the journal button or look it up through the library e-journals.

Additional Comments

For more information, go to the Pubmed tutorial website: http://www.nlm.nih.gov/bsd/disted/pubmedtutorial/

Waste Disposal

Waste must be properly disposed at the end of every day. Your bench space should be cleaned up, and reagents and glassware returned to their proper location promptly upon the completion of your experiment.

Disposal of chemical waste

Author: Ema Ciucurel

1. Use appropriately labeled waste containers to dispose of your chemical waste.
    - You have to make sure that the container material is compatible with the stored chemical waste. In general, we use empty glass bottles (or sometimes plastic bottles if the chemicals we are disposing off can be stored in plastic containers) to dispose of the chemical liquid waste.
2. Use a Chemical Waste label to identify the content of your waste bottle. Fill in all the required information on the Chemical Waste label (contact information, list of chemicals, % of each chemical in the chemical waste mix etc.).
    - You can find the Chemical Waste labels on the shelves in between the chemical fume hood and the sinks in the lab.
3. Liquid waste containers should not be more than 70-80% full.
4. Do not leave your waste bottles in the fume hood. We temporarily store our liquid chemical waste under the sink and we take all the waste bottles regularly to the Chemical Waste Room (MSB 5376).
5. Do not flush chemicals down the drain.
6. Do not put dry ice or liquid nitrogen in the sink. This can cause serious damage to the plumbing.
7. Do not mix waste chemicals with the general garbage.
8. Do not mix your chemical waste with the biohardous waste.
9. In order to avoid accidents (explosions, fires, spills etc.), make sure you don't mix incompatible chemicals together in the same waste container. A few general rules regarding subtances that should never be mixed together can be found here:

   http://www.ehs.utoronto.ca/services/environmental/chmdisp.htm#segregate

   If you are still not sure if you can mix 2 or more chemicals together, it is always better to use a separate waste container for each one of those chemicals rather that take the risk of mixing two chemicals that are not compatible and causing an accident.

Disposal of Biological Waste

Author: Derek Voice

Liquid Waste

All liquid biological waste (e.g. cell media, blood) should be aspirated into the glass bottles beneath the biosafety cabinets (BSC). MSDS sheets should be consulted for proper disposal of other chemicals (see Chemical Waste section). Chemicals which are not compatible with strong oxidizing agents should not be mixed with bleach.

Glass bottles beneath biosafety cabinets must be emptied at least once throughout a two-week cleaning period. Liquid waste should be poured into empty plastic ethanol or bleach containers using the funnel located beneath the sink in the tissue culture room. Containers should be filled to a maximum of 3/4 total volume. Empty containers are found beneath either lab sink. After emptying glass bottles, pour in fresh bleach to 1/10 of the total volume. A Chemical Waste Sticker with lab contact information must be placed on all waste containers. 'Bleached organic waste' should be written after the 'List of Chemicals'. Be sure to check how many stickers are left and pick up new ones from the waste room if supply is running low. Waste will be transported to the Medical Science Building room 5376 using the lab cart. Keys to this room can be borrowed from Chuen from the Sefton lab. Gloves and lab coat should be brought but only worn when moving waste containers from the cart to the waste room cabinets. NOTE: gloves and lab coats are not to be worn outside of the laboratory.

Solid Waste

Sharps (e.g. needles, razors) are disposed of in the yellow sharps container located between BSCs. Needles attached to syringes are not to be re-capped and should be disposed of in the sharps container.
Serological pipettes and syringes (without needles) are disposed of in the yellow 'Bio Medical Waste' cylindrical containers located between BSCs. When these containers are filed they should be placed outside the tissue culture room with white lids set on top. NOTE: Do not try and seal white lids on top of waste containers.

Pasteur pipettes (glass) and pipette tips are disposed of in 'Terminal Biohazard' cardboard containers hanging from the front of the BSC. When these containers are filled they should be placed in the 'Bio Medical Waste' containers and replaced. NOTE: This is the responsibility of the last user, not the person in charge of weekly clean up.

Tissue culture plates, Petri dishes and centrifuge tubes should be placed in plastic biohazard autoclave bags attached to the front of the BSC. When these bags are filled they should be taped closed and placed outside the tissue culture room within a larger waste bag. NOTE: This is the responsibility of the last user, not the person in charge of weekly clean up. When hanging new bags, be sure to fold the top down to

allow ~2 inches of room for sealing when full. Use 3 pieces of tape to ensure bag is secure.

Yellow 'Bio Medical Waste' containers and biohazard autoclave bags are picked up on Tuesday mornings for disposal. It is the responsibility of the person in charge of weekly clean up to ensure all waste is placed directly outside the tissue culture room with lab information tags attached.

Glass washing

Author: Ema Ciucurel

1. Wash all dirty glassware as soon as you are done using them. Do not leave dirty glassware in the sink – someone else might need it.
2. Use soap and water to wash your glassware.
3. Use distilled water for a final rinse.
4. Dry on the drying racks and return to the proper storage space (The drying rack is NOT a storage space).

Centrifuge

Author: Ema Ciucurel

Note: The most critical concept for centrifuging is ensuring your centrifuge is balanced; otherwise you can damage the centrifuge.

1. Make a counterbalance for each centrifuge tube you want to put in the centrifuge. You have to make sure the masses (not volumes) of the tubes are the same. If the tubes are unbalanced, you may damage the centrifuge.

2. Place the samples in the rotor. The rotor is balanced when each sample is offset by another of equal weight located geometrically opposite. **IMPORTANT NOTE:** Balance the samples using the scale (not a pipette) down to <1mg. If the rotor is not balanced properly, you will destroy the equipment and could cause serious injury.

3. Not all centrifuge buckets have the same weight (you can find the weight of each bucket written on the bucket itself). You have to make sure that the 2 centrifuge buckets that you are planning to use have the same weight. Alternatively, if you have to use 2 buckets of different weight, you can still balance them by placing a tube that weighs less in the bucket that is heavier and a tube that weighs more in the bucket that is lighter, so that the overall weights of each bucket plus the tube that it holds are equal.

4. Enter your settings (centrifugation time, RPM, temperature…) and press the Start button.

   Note: RPM (revolutions per minute) and RCF (relative centrifugal force) are not the same thing. Make sure that you know the difference and that you choose the right settings (RCF = 1.12*R*(RPM/1000)^2, where R is the radius of rotation measured in millimetres). For the Sefton lab large centrifuge, the radius is 160 mm. The microcentrifuge can be programmed in either rpm or rcf.

   If you require a temperature other than room temperature, be sure to turn on the centrifuge early, set the temperature then close the lid and allow the spin chamber to come to the desired temperature. After you are finished, be sure to return the temperature setting to ~20^{o}C and turn off machine.

5. After the centrifuge has stopped spinning, press button for opening the lid (the "STOP" button on the Sefton centrifuge) and remove the tubes carefully (you want to make sure you don't mix the two phases again).

6. Turn off centrifuge after use.

Weighing Samples

Author: Ema Ciucurel

1. Depending on the sample that you need to weigh, select the right balance (i.e. make sure your sample is in the weight range that the balance can measure accurately).
2. Place the weighing boat in the center of the balance.
3. Tare. (This resets the weight to zero, so you're only measuring the weight of the sample you are adding to the container).
4. Transfer your sample to the weighing boat
5. Wait for the display to stabilize and read off the result.
6. Clean up any spills promptly (you will find a brush that you can use to clean up the spills next to the balance).

Proper Use of Pipettes

Authors: Dean Chamberlain and Lindsay Fitzpatrick

Our lab has pipettes from two of the major pipette companies: Gilson and Eppendorf (Figure 1).

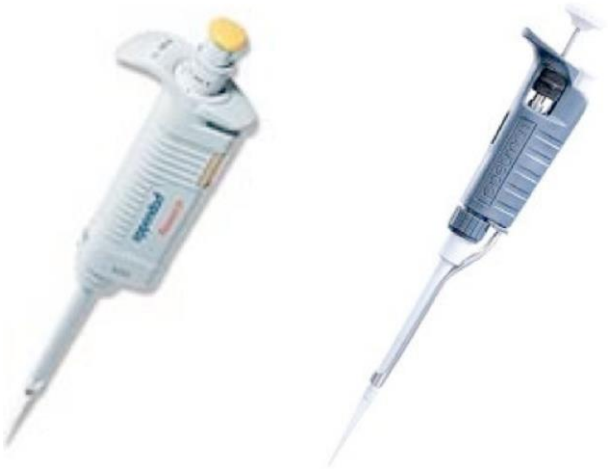


Figure 1. Eppendorf (left) and Gilson (right) pipettes.

Rules for using a pipette

1) Never set a pipette outside the range of the pipette. This will wreck the calibration.
    a. For Eppendorf pipettes the range is written on the pipette
    b. For Gilson ranges are:
       P2 – 0.1 to 2 μL
       P10 – 0.5 to 10 μL
       P20 – 2 to 20 μL
       P100 – 20 to 100 μL
       P200 – 50 to 200 μL
       P1000 – 200 to 1000 μL
2) Each pipette is designed to be used with a specific type of tip. Use the right tip for the pipette or the calibration will be off and you may draw liquid into the pipette.
3) Release the plunger slowly or liquid may be drawn into the pipette.
4) If liquid is drawn into the pipette it needs to be cleaned. Tell someone so that it can be cleaned. It is much harder to clean if the liquid dries.
5) Never set a pipette with liquid in it down.
6) Never tilt a pipette with liquid in it more than 45 degrees.

How to use a pipette (Figure 2)

1) Set the pipette to the volume needed.
2) Put on a tip.

3) Push plunger down to the first stop.
4) Place tip just below the surface of the liquid.
5) Slowly release the plunger.
6) Place the tip into the new container against the wall of the container.
7) Slowly push the plunger to first stop.
8) Slowly push the plunger to second stop.
9) Remove pipette tip from container.
10) Release plunger.
11) Use tip ejector to remove tip into waste container.

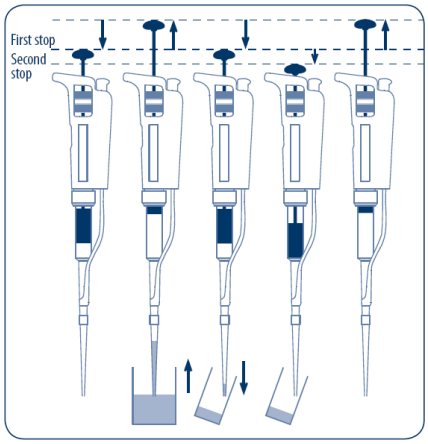


Figure 1. How to use a pipette

NOTE: There is a poster on methods to improve your pipetting skill in the lab. Please look at it.

Pipette Aids

When working with larger volumes of liquid, we have pipette aids that are used with serological (or Mohr) pipette tips (Figure 2).

The serological pipettes fit into the nose of the pipette aid, and liquid can be taken up be pressing the upper button, or dispensed by pressing the lower button. The rate of aspirating or dispensing depends on the how much you depress the button (i.e. if you depress the button to whole way, the pipette will fill or empty at the greater rate than if you only slightly depress the button).
To pipette a particular volume, draw up more liquid than you desire, then slowly drain the pipette until the bottom of the meniscus reaches the appropriate level, as seen in Figure 3.

When using a pipette aid, it is extremely important to be aware of the amount of liquid in your pipette, and to never allow the liquid to reach the cotton baton fitted in the end of each pipette (Figure 2, lower right inset).

Each pipette aid has a self-locking filter in the nosepiece, designed to protect the electronics and to prevent cross-contamination in case of over-pipetting. If you accidentally over fill the serological pipette (don't panic), the filter becomes wet and the pipette aid will cease to work. Please inform one of the pipette people (currently Monika and Harrison) immediately, so we can replace the filter.

We have sterile serological pipettes for use in tissue culture and non-sterile pipettes for general lab use. We routinely stock 1 ml, 5 ml, 10 ml and 25 ml pipette tips.

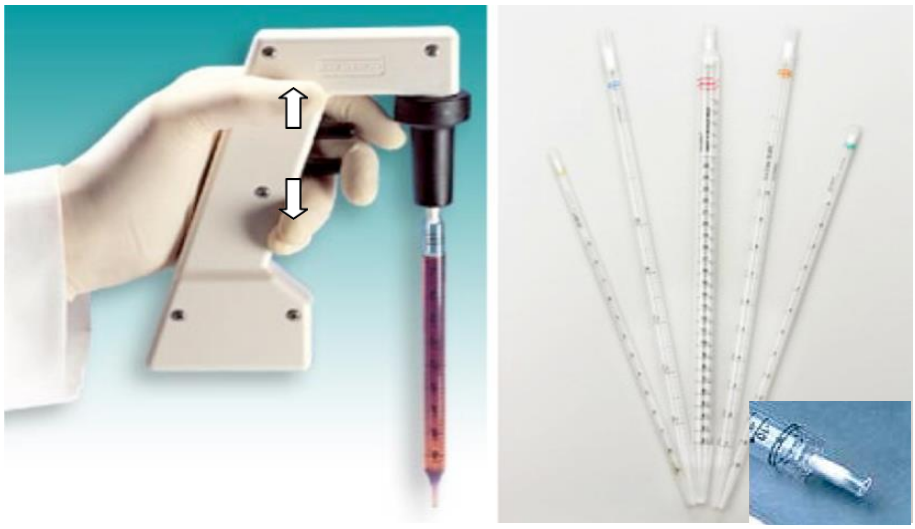


Figure 2. Pipette aid (left) and serological pipettes (right).

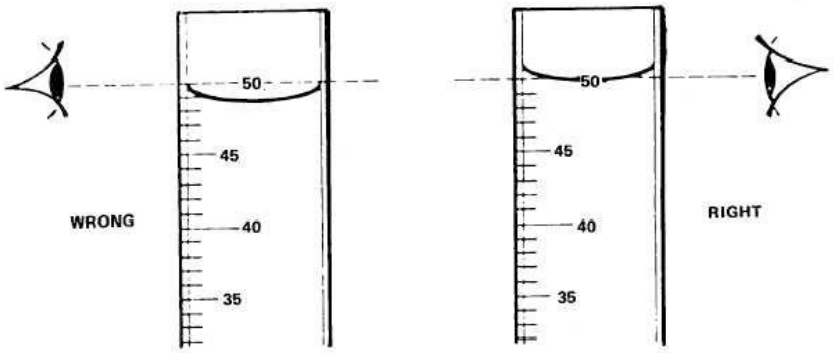


Figure 3. Measure the volume of liquid in the pipette using the bottom of the meniscus.

Basic Cell Culture

Authors: Brendan Leung, Lindsay Fitzpatrick

General Guidelines for working in a biosafety cabinet

General guidelines to ensure sterile environment:

- Always disinfect gloves with 70% ethanol before re-entering into the Biosafety cabinet.
- Use 70% ethanol and wipes to thoroughly clean the pipettes before use
- Make sure the pipette tips have been autoclaved. If there is any doubt, use another box of autoclaved tips.
- When using the pipette aid (gun), make sure fluids to not touch the cotton filter at the top
- Work at least 4 inches inside the cabinet's opening
- Do not clutter the cabinet
- Do not place large bottles directly behind your working area
- Avoid rapid "jerky" movements
- Minimize exit and re-entrance of your arms into the cabinet.

Work space and reagent preparation

1. Make sure the fan on the biosafety cabinet is ON
2. Wipe down the work surface of biosafety cabinet with surface disinfectant
3. Wipe down the work surface of biosafety cabinet with 70% ethanol
4. Warm up cell culture medium in water bath
    a. Make sure you have the correct bottle with your name on it.
5. Set up your workspace with all the supplies you will need (pipette tips, pipettes, Pasteur pipettes, flasks, tubes, serological pipettes, pipette aid, etc). Be sure to wipe everything down with 70% ethanol before bringing it into the biosafety cabinet. You should have your workspace completely set-up before introducing your cells to ensure you minimize entering and exiting the cabinet.

Initiation of adherent and non-adherent cell culture from frozen cell stock

1. Warm up appropriate medium in 37°C in water bath
2. Fill tissue culture flask with the appropriate medium
    a. For a T25, use 5 mL of medium.
3. Retrieve frozen vial from liquid nitrogen storage and place immediately in the water bath while maintaining the seal and cap of the vial above water.
4. As soon as no ice crystal is visible in the vial, wipe the vial down with 70% ethanol and transfer the content into the flask prepared in step 2.

5. Disperse the cells in the flask by gentle shaking, and place the flask in the incubator.
6. Replace medium between 12 hrs and 24 hrs after plating to remove residual DMSO.
    a. For adherent cells, simply aspirate DMSO-containing medium and add fresh culture medium to the flask.
    b. For non-adherent cells, transfer the cell suspension to a centrifuge tube. Centrifuge for 5 minutes at 1000 rpm for 5 minutes. Aspirate supernatant and resuspend pelleted cells in fresh culture medium.

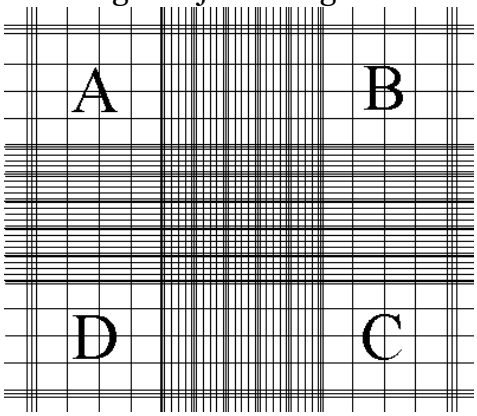
N.B.

- DMSO is a cryo-preservative. It is harmful to cells at 37°C.
- Some cell types are particularly sensitive to DMSO. In these cases, cells should be suspended in 10ml of warm medium in a 15 ml centrifuge tube and centrifuged at 900rpm for 5 minutes, immediately upon being thawed. Aspirate the medium containing DMSO from the centrifuge tube. Then resuspend the pelleted cells in fresh, DMSO-free medium and transfer to a culture flask or dish. Cells are fragile upon thawing, so take care to handle them gently.

Sub-culture of adherent cells

1. Warm up the appropriate medium and Trypsin-EDTA solution in water bath
    a. Aliquot 40 mL of Trypsin-EDTA in a 50 mL Falcon tube and keep using that as your own stock.
2. Remove tissue culture flask (T25) from incubator, aspirate the medium using a glass Pasture pipette.
3. Place 5 mL of 1xPBS into flask and aspirate using a glass Pasture pipette.
4. Place 1 ml of Trypsin-EDTA into flask. Incubate at 37°C for 2 min.
5. Remove the flask from the incubator and examine under light microscope to ensure all cells are detached from the surface.
    a. If some cells remain attached, incubate for another 2 minutes, the repeat step 5
    b. Note that you will not need to detach every single cell; there will always be a few cells left on the flask. Over incubation in trypsin (>10 minutes) is harmful for cells.
    c. Firmly taping flask with the palm of your hand will also dislodge the cells.
6. Place 1 mL of medium (must contain serum) into the flask and transfer the mixture into a 15 ml centrifuge tube.
7. Centrifuge at 1000 rpm for 5 min.
8. Aspirate the supernatant and resuspend cell pellet in medium (1 to 10 mL).
9. Determine cell density using a hemacytometer.

a. Take a 20uL aliquot from the cell suspension in step 9 and mix with 20 uL of Trypan blue.
b. Deliver 10 μL of the cell/Trypan blue mixture into hemacytometer.
c. Count number cells in 2 or more area (A, B, C and D). Some cells inevitably fall on top of the outside gridlines that mark the edges of the chamber. The usual practice is to include cells overlapping the top and left lines, but not those overlapping the bottom or right lines --- this has the advantage of eliminating redundant counting if adjacent regions are counted.



d. Determine cell concentration by using the following formula:

(Total cell #)/(# areas counted) x (Trypan blue dilution factor) x 10,000 = cell conc.(cell/mL)

10. Place the desired amount of cells into a new flask and top up the flask to its working volume with fresh medium.

N.B. Certain cell types are typically subcultured using dilution methods. For this method:

a. Resuspend cells from step 8 in 10 mL.
b. To the new flask add the desired dilution of cells. For example 1 mL for a 1 in 10 dilution or 5 mL for a 1 in 2 dilution.
c. Top up the flask to its working volume with fresh medium.

11. Label flask with the name of the cell line, the passage number and ratio, the date and your name. [Note: Use the same passage ratios for repeat experiments, and note your ratio and passage number in you lab notebook]
12. Place flask in incubator with the cap slightly lose (for solid caps) or tightly closed (for vented caps) and replace medium every 2-3 days.
13. Cells need to be split again when they are at 80% confluent or higher.

Maintaining non-adherent cell cultures

For non-adherent cell cultures, the concentration of cells in the medium (volume), and not the surface area of the flask, determines when the culture must be split.

Non-adherent cell cultures must be maintained within a certain range of concentration (e.g. THP-1 cells should be cultured between 5 x10^5 - 1 x 10^6 cells/ml) and when the culture approaches the upper limit of acceptable range, the culture should be split. The concentration range and splitting ratio will depend on the cell type; therefore it is recommended you consult the literature or the supplier for the appropriate values. When a culture flask is ready to be split:

1. Warm culture medium in water bath.
2. Remove the tissue culture flask from the incubator.
3. Transfer cell suspension to a centrifuge tube.
4. Count cells as described in "adherent cell subculture" to determine the appropriate splitting ratio
5. Divide the cell suspension into the appropriate number of new flasks (i.e. 1:5 dilution → split 1 flask into 5 flasks) and top-up the culture volume with fresh medium.
6. Label flask with the name of the cell line, the passage number and ratio, the date and your name.
7. Place flask in incubator with the cap slightly loose (for solid caps) or tightly closed (for vented caps).
8. Cells should split every 2-3 days to ensure the culture medium is being replenished.

Flasks can be used for up to one month, at which point cells should be transferred to a new flask.

General guidelines and tips

Flasks name	Surface area (cm^2)	Working medium volume (mL)	Trypsin volume (mL)
10 cm plate	56.75	10	1
T-25	25	5	1
T-75	75	15	3

1. Generally speaking, the amount of medium used during culture scales linearly with culture surface area. In other works, the depth of medium is more or less the same regardless of culture surface area.
2. If you need to extend medium change interval by one day, you may overfill the flask/plate by 10-20%. However, cell morphology and viability may suffer.
3. If you see anything other than your cell growing in the flask, or if your culture medium turns yellow or bright pink, please consult a grad student to make sure it is not contaminated.

4. Every time you split your cells the passage number goes up by one. When passaging, you should typically use a splitting ratio of 1/5 to 1/20. Use a consistent splitting ratio, especially when working with primary cells.
5. Confluency is commonly used as a measure of the number of the cells in a flask and refers to the coverage of the flask by the cells. For example, 100% confluency means the flask is completely covered by the cells and there are no more room left for the cells to grow, where as 50% confluency means roughly half of the flask is covered and there is room for cells to grow.
6. In general it takes the average cell type 24 hrs to double in number. Therefore, the confluency doubles every 24 hrs so if you have cells that are 10% confluent they will be 80% confluent in 3 days and need to be split.
7. Do not allow cells to become 100% confluent as this can change the growth characteristics of the cells.