

Before we begin our project for the semester, it's important to set some ground rules. The molecular biology lab is different from a chemistry lab in one fundamental way: rather than fearing contamination OF you by the experiment, we are now most concerned with preventing contamination of the experiment BY you! To prevent such contamination, always follow the guidelines below regarding: 1) Aseptic technique, 2) Safety, and 3) Cleaning up.

## 1) Aseptic Technique

To avoid unwanted and costly contamination in the molecular biological laboratory, it is very important to use aseptic technique. Contamination can include the presence of unwanted organisms growing in a culture (i.e. bacteria) or unwanted DNA or RNA in a molecular reaction such as polymerase chain reaction (PCR). The former type of contamination can destroy a culture and the later type of contamination can lead to false positive results in an experiment.

We must also be concerned with unwanted nucleases, which are enzymes that can rapidly degrade DNA or RNA. Nucleases are found on human skin; therefore, one must avoid direct or indirect contact of fingers with nucleic acids. While most DNases are not very stable, many RNases are highly stable and can adsorb to glass or plastic and remain active (<http://userpages.umbc.edu/~jwolf/method2.htm>).

### Some Steps to Prevent Contamination:

- You do NOT need safety goggles or closed-toe shoes for this lab. However, you should always wear clothing that will not interfere with the activity for the day (i.e., no scarves or dangling threads). If you have long hair, you should always tie it back before beginning lab to prevent your hair from contaminating your samples.
- Bring ONLY relevant lab materials to your bench. These include: the lab protocol, a writing instrument, and any associated documents (background, pre-labs, etc.). You should NOT bring your bookbag or cell phone or computer (unless instructed to do so by your TA). There will be a designated shelf in the lab for your other belongings.
- Always wear clean gloves while working in the molecular biological laboratory. One common source of nuclease contamination is gloves that have become contaminated by touching surfaces in contact with human hands, such as doorknobs (or cell phones!). You may have to fight the urge to touch your face or hair while wearing gloves, but remember that you are the main source of potential contamination for your project!
- Always sterilize your bench area with 70% ethanol before beginning lab procedures, or after a break in the procedure. Use your hands or a chem-wipe/paper towel to spread a small amount of ethanol over the entire surface of your bench.
- Never leave open any bottles of reagents (including sterile water) or tubes and plates with yeast cultures. Likewise, never leave open containers of sterile micropipette tips. Open just before use and close immediately after you are finished.
- Never allow your gloves to touch the inside of any bottles of reagents or tubes and plates with yeast cultures.

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- Never allow the micropipettor to touch the inside of containers with reagents or yeast cultures. Only the pipette tip should touch the sample being removed. Follow the same procedure when adding a reagent or volume of yeast culture to a microcentrifuge tube.
- Always use a fresh, non-aerosol pipette tip when removing cultures or reagents from their containers. Never use a pipette tip twice for this purpose!
- It is essential to carry out proper use of the micropipettors! Otherwise, they will become contaminated or damaged.
- Always be mindful about the sources of contamination and, most importantly, use common sense.

### 2) Safety in the Molecular Biological Laboratory

You must assume that all cultures used in the lab are biologically hazardous, as they might be infected with latent viruses that are not characterized. Also, given the right circumstances (e.g., compromised immune system, or a cut or wound that provides a portal of entry for the microbe), many microorganisms have the potential to be opportunistic pathogens. Some have even reported *Saccharomyces cerevisiae* as an emerging opportunistic pathogen (McCusker 2006). Note that one does not need to have HIV/AIDS to be immunocompromised; other conditions can weaken the immune system, such as the common cold, the flu, poor diet, and stress. Even benign microorganisms may evolve pathogenicity, so treat all microorganisms in the lab as potential pathogens and follow lab safety protocols.

- NO eating or drinking! No food or drink should be out at any point during lab. If you need a drink of water, ask your TA if you can step outside briefly.
- Wash your hands thoroughly after handling cultures and before you leave the lab each lab period.
- Do not remove anything from the lab (e.g., slides or cultures).
- Use proper aseptic technique as explained above.

### 3) Clean-up

One of the most important ways to prevent contamination, as well as being courteous to your classmates and the prep staff, is to clean up your bench before you leave. Before leaving the lab, ALWAYS do the following:

- Put away all items in their proper place (as they were when you first entered the lab). Recap all reagents tightly and securely. Make sure your pipettors are securely in their racks with the dials facing out.
- Dispose of waste items PROPERLY! Proper disposal of all cultures, glassware and other materials is very important for your safety and the safety of your classmates, TAs, and the prep staff. The cultures used in this lab are potential pathogens (see above), so follow lab safety protocols.

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- Contaminated plastics (e.g., Petri plates, pipet tips, etc.) go into the orange biohazard bags. By law, these must be autoclaved before going into the landfill. Do NOT place glass (broken or intact) into biohazard bags. There are two sizes of orange biohazard bags. The small bags are provided on each bench for the disposal of pipet tips and small microcentrifuge tubes ONLY. Chemical solid waste (i.e., pipette tips and microcentrifuge tubes contaminated with non-biological agents) should be disposed of in their own properly labeled receptacles, NOT in the biohazard solid waste containers. Chemical waste and biohazard waste are subject to different EPA disposal guidelines, so you MUST pay attention when discarding solid waste in lab! Do NOT eject **liquid** waste into either the biohazard solid waste bags or the chemical solid waste containers!
  - Do NOT place any gloves or larger contaminated materials (chem-wipes, paper towels) into the small biohazard solid waste containers!! Use only the larger biohazard containers for this purpose.
  - Liquid waste should be disposed of in the proper receptacles, either Biohazard (for cellular materials) or Chemical (for reagents and post-cellular material). These waste bottles will be clearly labeled. Be careful NOT to eject your pipette tips into the liquid waste!
  - Broken glass should be placed in the broken-glass containers (labeled cardboard boxes usually containing a clear plastic bag lining). Your TA will know where this container is located. Do NOT place broken glass anywhere else.
- Make sure the microcentrifuges at the end of your bench are closed, locked and turned off.
  - Clean your bench space with 70% ethanol and paper towels after you have disposed of everything properly. Use the 70% ethanol in the red squirt bottles on your bench tops.
  - Push your chair back under the bench, and wash your hand thoroughly!

### References

Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *New England Journal of Medicine* 348:1967-76.

Emery SL, Erdman DD, Bowen MD, Newton BR, Winchell JM, Meyer RF, Tong S, Cook BT, Holloway BP, McCaustland KA, Rota PA, Bankamp B, Lowe LE, Ksiazek TG, Bellini WJ, and LJ Anderson. 2004. Real-time reverse transcription-polymerase chain reaction assay for SARS-associated coronavirus. *Emerging Infectious Diseases* 10:311-316.

McCusker, John "Saccharomyces cerevisiae: an Emerging and Model Pathogenic Fungus." in *Molecular Principles of Fungal Pathogenesis*. Joseph Heitman, et al., eds. ASM Press, 2006.

Mifflin TE. 1997. Control of contamination associated with PCR and other amplification reactions. Molecular BioProducts, Inc.

This lab will often involve the use of math to impress upon you the extreme scales of the molecular world, from the small (a yeast cell is TINY, but how tiny?) to the large (even a tiny cell has LOTS of molecules, but exactly how many?), so it is important for you to be comfortable manipulating numbers in scientific notation. If you do not use scientific notation on your lab assignments, you will lose valuable points! Below are some basic facts about metric units, conversions, and the micropipettors you will be using all semester to measure volumes of various reagents and samples.

### Part 1: Metric units and conversions

Before we start the lab, it is important that you review metric measurements and the prefixes that are used for describing very small, or large, volumes or lengths. Most of our work in the lab will be with liquid measurements based on the liter, but the same prefixes also apply to measurements of dry weight based on the gram and measurements of length based on the meter. If you are unfamiliar with how to go between orders of magnitude or want a reminder on the importance of keeping track of your units, consult the Helpful Background Knowledge page entitled "A Refresher on Dimensional Analysis".

**Prefixes used for describing objects that are larger or smaller than the base unit.**

Prefix	Symbol	Exponent	Factor
pico-	p	$10^{-12}$	One trillionth
nano-	n	$10^{-9}$	One billionth
micro-	$\mu$	$10^{-6}$	One millionth
milli-	m	$10^{-3}$	One thousandth
centi-	c	$10^{-2}$	One hundredth
kilo-	k	$10^3$	One thousand times
mega-	M	$10^6$	One million times

### Part 2: Using the Micropipettor

The liquid pipetting you will do in the lab this semester involves very small volumes that are less than 1 mL. Your lab benches are supplied with three standard versions of the micropipettor or micropipette: the **P-1000**, **P-200** and the **P-20** (and occasionally the **P-10**). It is essential that you learn the proper use of these instruments so you can ensure accuracy in your lab work and you don't break the micropipettors. These are delicate and very expensive instruments! To introduce you to the micropipette, we have provided written rules below for working with the micropipettor in the lab. We suggest you review the written material first and then watch the YouTube video "Using a Micropipette- University of Leicester."

[http://www.youtube.com/watch?v=uEy\\_NGDfo\\_8](http://www.youtube.com/watch?v=uEy_NGDfo_8)

The maximum volume a micropipettor can dispense is printed at the top of the instrument on the plunger. The units are in microliters ( $1000\ \mu\text{L} = 1\text{mL}$ ). Thus, a P-1000 dispenses up to  $1000\ \mu\text{L}$  ( $=1\text{ mL}$ ), a P-200 up to  $200\ \mu\text{L}$  ( $=0.2\text{ mL}$ ), and a P-20 up to  $20\ \mu\text{L}$  ( $=0.02\text{ mL}$ ). When the instrument is labeled with two numbers, this is the range of volumes that the micropipettor will accurately dispense. Thus a micropipettor labeled 100/1000 should not be used to pipet less than  $100\ \mu\text{L}$  or more than  $1000\ \mu\text{L}$ . Of course, since you will almost always have both a P-1000 AND a P-200, you should use the P-200 for volumes under  $200\ \mu\text{L}$ .

### Exploring the Micropipette:

The plunger can be pushed down to 2 different levels, called stops. The first stop is used to pick up the exact volume on the display. Once you pick up a volume with a pipettor and want to dispense this volume, push the plunger slowly to the first stop, then continue to push the plunger down until the second stop. This second stop allows you to dispense the entire volume contained in the tip. Once you have dispensed the proper volume, the ejector button is used to eject the plastic tip into the proper waste container.

### Rules for Proper Micropipettor Use

1. Do not set the pipettor higher than the maximum volume listed on top of the pipettor or lower than the recommended minimum. If you are not sure how to properly set the pipettor or are unsure of its volume limits, ASK YOUR TA.
2. When setting the volume, never force the pipettor. The proper volume should be easily set. It should not be hard to turn the knob.
3. Do not use the pipettor without a pipette tip. Press firmly to attach the tip. Do NOT “jack-hammer” the tip onto the pipettor or you may not be able to eject it safely!
4. Do not turn a pipettor upside down or sideways when there is liquid in the tip. This can contaminate the pipettor.
5. Use the first stop when drawing up liquids, NEVER the second stop. Using the second stop will result in an inaccurate measurement, and may draw liquid into the pipettor, contaminating it.
6. Always draw up and dispense liquids SLOWLY. Many of the solutions we work with in this lab have low surface tension and will rush into the pipette tip and pull air with them, resulting in an inaccurate volume.
7. Immediately eject your pipette tip into the proper waste receptacle when you are finished using it.
8. When not in use, place the pipettor in a rack (not on the bench!).

For further reading see:

#### **Micropipetting: An Important Laboratory Skill for Molecular Biology**

Jon S Miller; Michell E Sass; Susan J Wong; James Nienhuis *The American Biology Teacher*, Apr 2004; 66, 4; Research Library pg. 291

Credit To Professor Rytas Vilgalys for “Rules for Proper Micropipettor Use”



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Reading a micropipettor's dial takes a little getting used to, but there are a few tricks to make it easier. For a P-1000, the RED digit is the thousands place and should always be either a 0 or a 1. A P-200 does not any red digits, and shows the hundreds, tens, and ones places. A P-20 has a red digit for the TENTHS place.



1
6
5

This is the volume display window of the pipetman.

For a P-1000:

1	← thousands
0	← hundreds
0	← tens

Do not set:  
Above or below

1	0
0	2
0	0

For a P-200:

2	← hundreds
0	← tens
0	← ones

Do not set:  
Above or below

2	0
0	2
0	0

For a P-20:

2	← tens
0	← ones
0	← tenths

Do not set:  
Above or below

2	0
0	2
0	0

**Objective: To learn how to accurately measure and pipette small volumes using micropipettes.**

**Part A: Learning the Ropes**

Micropipettes differ in the volume of liquid they can accurately measure. The number on the top of the micropipette indicates the range of volume it can pipette. A P1000 is used to measure from 200-1000 $\mu$ L; a P200 is used to measure volumes from 20-200 $\mu$ L; a P20 is used to measure volumes from 2-20 $\mu$ L and a P10 is used to measure volumes from 0.5-10 $\mu$ L. In Part 1 of this exercise, you will use the various micropipettes at your station to combine all of the volumes listed on the table in one tube together. Record the pipette you use and the setting in the window.

**Example:**

Color	Volume to add to the tube	Which micropipette was used?	Record the setting as it appears in the window
Blue	150 $\mu$ L	P200	150

Find the white/clear canister on the shelf above your bench and get out ONE small microcentrifuge tube. Add the following amounts to your tube. NOTE: all of the amounts will be added to the same tube!

Color	Volume to add to the tube	Which micropipette was used?	Record the setting as it appears in the window
Blue	400 $\mu$ L		
Yellow	150 $\mu$ L		
Blue	17 $\mu$ L		

a) What is the final volume of your tube in  $\mu$ L? \_\_\_\_\_

b) What is the final volume of your tube in mL? \_\_\_\_\_

**Part B: Small Volume Pipetting**

Gee, Roy and Biv are having problems with their science lab. The question they are trying to answer requires very precise measurements, and the results come out differently each time they attempt the experiment. The teacher suspects pipetting errors, and asks them to complete the following exercises to check their technique. Use the following directions to complete the same task and check your technique.

To begin, find the canister on the shelf above your bench with the clear microcentrifuge tubes (also called “Eppendorf” tubes) and put **six** tubes in a rack on your bench top. Label the caps of the tubes “1-6” with a black Sharpie (also on the shelf above your bench).

**Carefully follow steps 1-12 below putting a check mark to the left of each instruction line upon completion. Be sure to eject the yellow pipet tip into the small biohazard bin after each use.**

- \_\_\_ 1. Put 19 $\mu$ L of red liquid into tube number 1.
- \_\_\_ 2. Put 22  $\mu$ L of yellow liquid into tube number 3.
- \_\_\_ 3. Put 25  $\mu$ L of blue liquid into tube number 5.
- \_\_\_ 4. Take 4  $\mu$ L from tube number 1 and put it into tube number 2.
- \_\_\_ 5. Take 4  $\mu$ L from tube number 1 and put it into tube number 6.
- \_\_\_ 6. Take 4  $\mu$ L from tube number 3 and put it into tube number 4.
- \_\_\_ 7. Take 7  $\mu$ L from tube number 3 and put it into tube number 2.
- \_\_\_ 8. Take 7  $\mu$ L from tube number 5 and put it into tube number 4.
- \_\_\_ 9. Take 7  $\mu$ L from tube number 5 and put it into tube number 6.
- \_\_\_ 10. Calculate the total volume in each tube.
- \_\_\_ 11. Compare the amount of liquid in your tubes with your partners. Are the volumes the same or different? If the amounts are different, try to figure out why. Watch each other micropipette and check each other’s technique.



**Analysis:**

1. What common error in handling a micropipette can account for pipetting **too much** reagent into a tube?
2. What common error in handling a micropipette can account for pipetting **too little** reagent into a tube?
3. Complete the following conversions:

$$1\mu\text{L} = \underline{\hspace{2cm}} \text{ mL}$$

$$10\mu\text{L} = \underline{\hspace{2cm}} \text{ mL}$$

$$100\mu\text{L} = \underline{\hspace{2cm}} \text{ mL}$$

$$\underline{\hspace{2cm}} \mu\text{L} = 1 \text{ mL}$$

# Master Mix Lab

## 1 Labelling

Because most samples and reactions are indistinguishable by eye, it is essential to label tubes in such a way that you can distinguish them. Note that the teaching lab does not allow tubes with writing on top in the PCR machines, so be sure to label the side of any tubes that will go in the PCR machine, not the top.

## 2 Keeping Track

### 2.1 Tube Shuffling

When adding multiple reagents to a reaction it is easy to lose track of what you have already added, so many experienced molecular biologist develop one or more systems for keeping track. Here is one system:

1. Put the "reaction tube", the microcentrifuge tube (MCT) in which you will be preparing the reaction in the first row, first column of the tube rack.
2. Put the MCTs containing reagents in the second row of the tube rack, in the order that you will add that reagent, starting in the third column.
3. When it is time to add reagent A, move its tube to the first row.
4. Set the micropipette to the volume of reagent A that you will need
5. Pick up a tip with the micropipette
6. Open tube A.
7. Draw up reagent A into the pipette tip
8. Dispense reagent A into the reaction tube
9. Close tube A and move it to the last row in the rack
10. Repeat steps 3-9 with the rest of the reagents

### 2.2 Marking Off

Another system is to mark off reagents in the protocol as you add them.

## 2.3 Et cetera

Ask the TAs, instructor, or other experienced molecular biologists in the class what systems they use.

## 3 Enzymes

Enzymes are the workhorses of molecular biology, so we will be using several different enzymes during the RNA-Seq library preparation process. Enzymes are molecular machines, so they can be fragile. Vortexing enzymes can denature (i.e. break) them, so it is considered good practice to avoid vortexing any solution containing enzymes. There are two common methods for safely mixing solutions containing enzymes:

1. Pipette up and down (gently)
2. Flick the tube, then spin down in a microfuge to collect the solution at the bottom (otherwise you might lose any that is on or around the cap).

If you are unsure at a given step whether you are using an enzyme, you can play it safe, or check with a TA or the instructor.

## 4 Master Mixes

### 4.1 Master Mixes Explanation

For many of the steps in the library prep you will need to add multiple reagents to each of your six samples. The naive approach, adding reagents one at a time to each sample tube, is both tedious and error prone. It is error prone in two ways: a) you might forget to add a reagent to some samples, or add the wrong amount b) even if you don't make any mistakes, you might add different amounts to different samples due to inherent pipetting variation. The smart way to solve this problem is to make master mixes. Bonus: You get an extra benefit from master mixes because you will be working with larger volumes, which is always easier and more accurate. For example, if your reaction requires 0.5ul of a reagent and you are making a 10x master mix, you will be pipetting 5ul, instead of a tricky 0.5ul.

Exceptions: Of course there are always exceptions - there are rare cases where reagents should not be pre-mixed, but unless warned otherwise, you can assume that master mixes are safe.

What is a master mix? It is a combination of all the components of a reaction that are the same for all samples, in the correct proportions. Let's say you have the following recipe for a reaction:

Reagent	Volume
Awesome Enzyme	0.3 ul
Reaction Buffer	10 ul
Secret Ingredient	2.7 ul
Sample DNA	2 ul
Total	15 ul

If you have 9 samples, you would make the following master mix

Reagent	1 rxn	10 rxn
Awesome Enzyme	0.3 ul	3 ul
Reaction Buffer	10 ul	10 ul
Secret Ingredient	2.7 ul	27 ul
Total	13 ul	

Once I make the master mix and mix it (see discussion above about enzymes) I will add 13ul of master mix to each reaction tube, then add the 2ul of sample, for a total reaction volume of 15ul.

Margin of error: Why did I make a master mix for 10 reactions if I only have 9 samples?  
To allow for pipetting error. You always need to make a little extra master mix. A good rule of thumb is 5-10% extra, although it is often easiest to round to the nearest 0.5 or 1 reaction.

## 4.2 Master Mix Setup

Now your turn! You have 6 samples to test, here is the recipe for one "reaction". Calculate enough master mix for 6 samples. Have a TA or instructor review your master mix calculations before you continue.

Reagent	Volume
PCJ Indicator	160 ul
Dilution Buffer	10 ul
Thickener	10 ul
Sample	20 ul
Total	200 ul

Fill in the following :

In tube **A** I will put .....

In tube **B** I will put .....

In tube **C** I will put .....

In tube **D** I will put .....

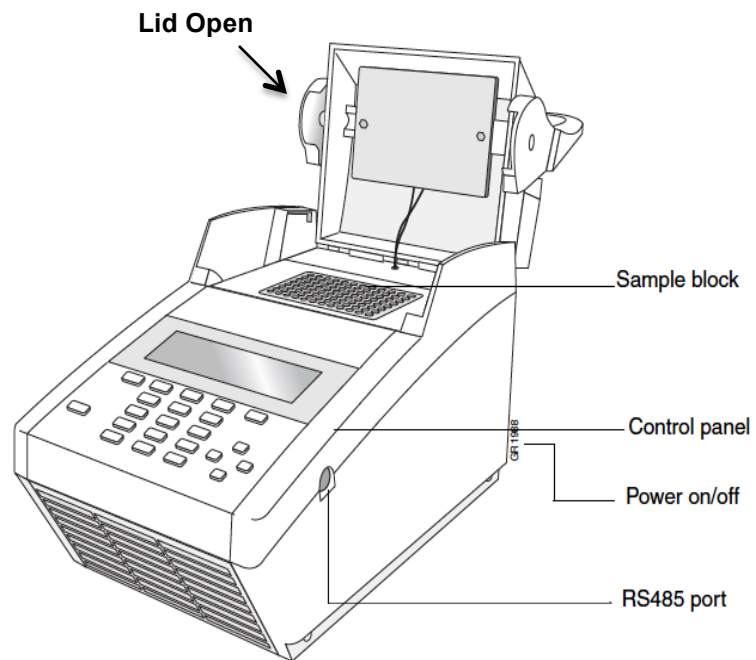
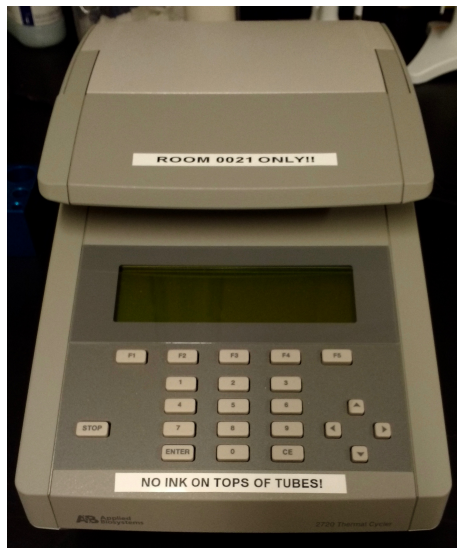
In tube **E** I will put .....

In tube **F** I will put .....

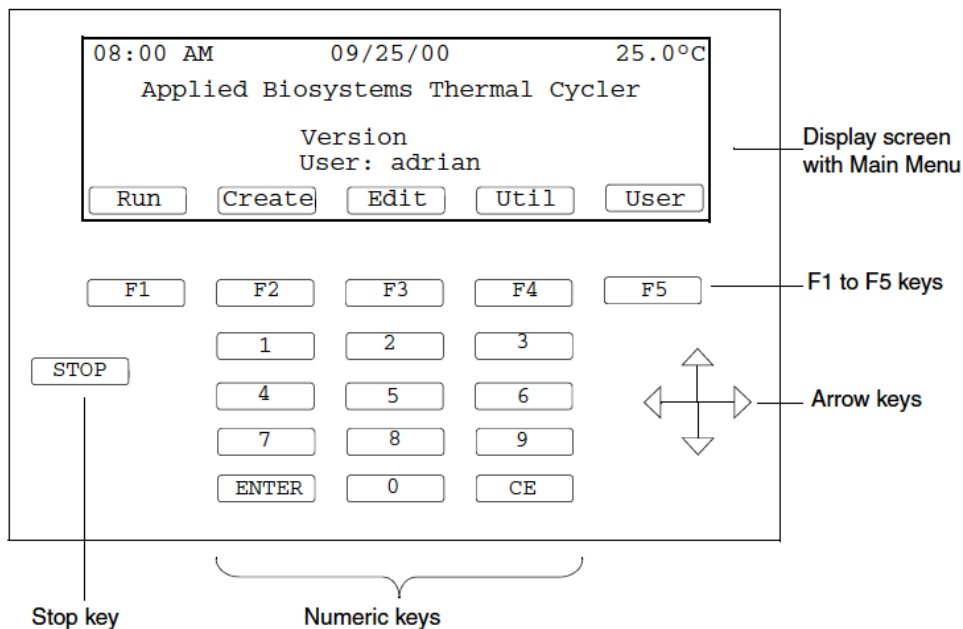
Once the above has been checked, assemble you master mix in a microcentrifuge tube, then set up the individual reactions in 0.2ml PCR tubes and observe the color. Once you have prepared you reactions, run them with the "PCJ" program in one of the thermal cyclers.

# Instructions for Using the ABI 2720 Thermal Cycler

## The ABI 2720:

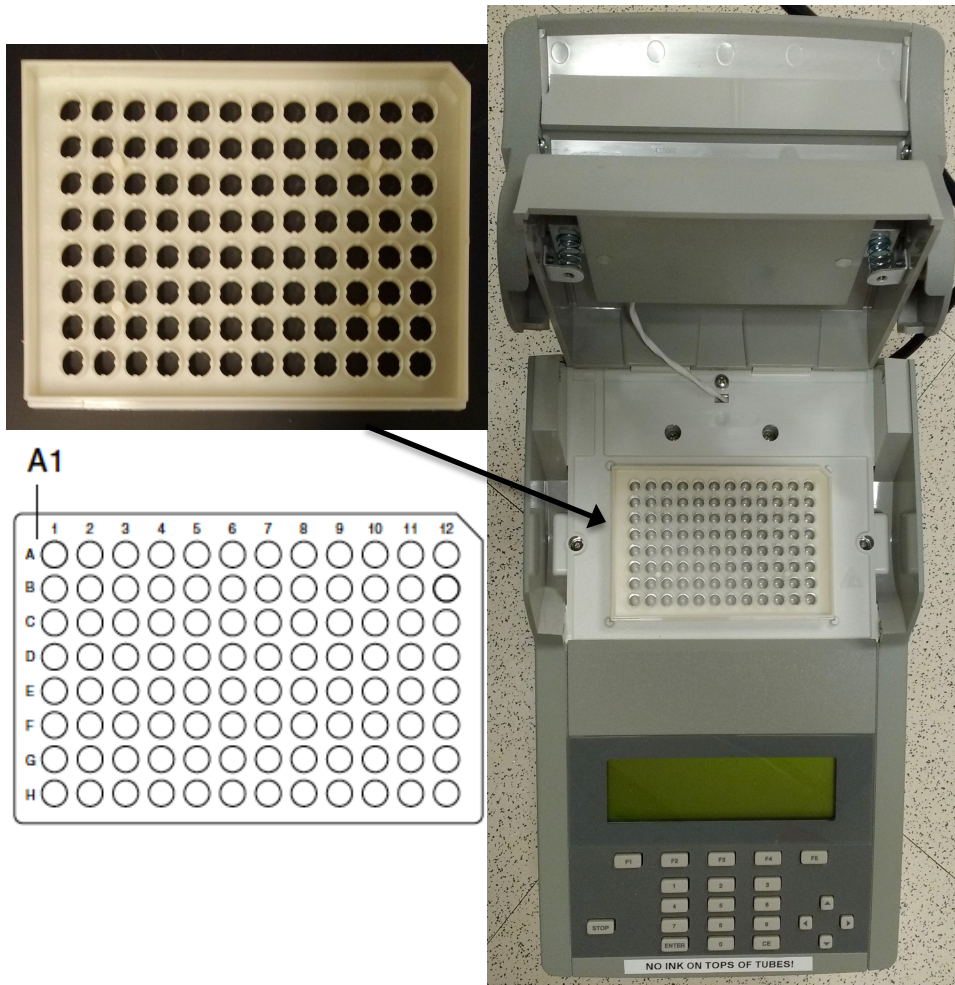


1. Once the instrument is turned on, the Main Menu will appear on the display screen after several seconds:

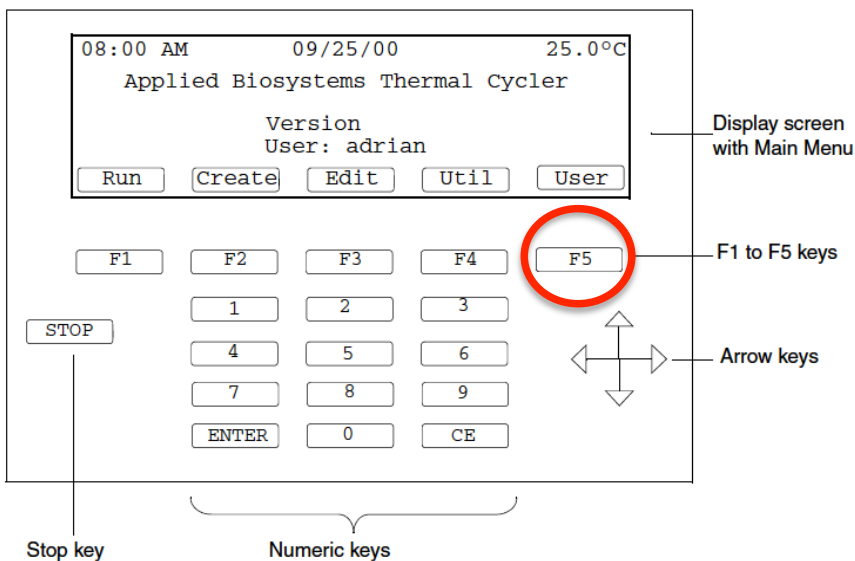


2. Lift up the lever to open the instrument lid. Make sure the MicroAmp 96-well sample tray is in the sample block in proper orientation, with well A1 located in the upper left-hand corner of the tray, and the notched end in the upper right-hand corner:

## Proper Orientation



3. Place the PCR tubes into the wells. Make sure the caps are completely closed, otherwise evaporation during cycling will occur. Only the **sides** of the PCR tubes should be labeled, **NOT** the tops of the tube caps. Carefully pull the lever down to close the instrument lid after all of the tubes have been inserted into the wells.
4. To start a run, first select "User" by pressing key F5:



5. The “Select User Name” screen will appear. Using the Arrow Keys, highlight the user name “**hts.**” (Note: This user name is not shown below.) Press key F1 to accept this user name.

Select User Name

<<ab>>	dave	jenife	murray
adrian	doug	mamood	nayana
anna	faye	mike	ngui

Accept New Edit Delete Cancel

F1 F2 F3 F4 F5

6. The stored “Methods” screen will appear. Using the Arrow Keys, highlight the stored method named “**Test PCR.**” (Note: This method name is not shown below.) Press the key F1 to start.

Methods	User	Size	LastUsed
appl01	adrian	11	03/04/00
exp000	adrian	10	02/22/00
exp001	adrian	12	02/10/00
exp002	adrian	13	02/02/00

Start View User Sort Cancel

F1 F2 F3 F4 F5

7. The “Select Method Options” screen will appear. Enter the volume of your PCR samples and press key F1 to start the thermal cycling method you selected.

Select Method Options

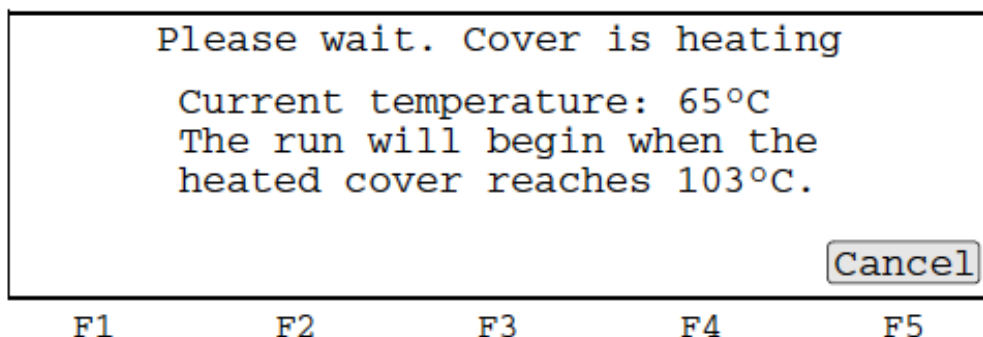
Reaction Volume: 50 µL

Enter a value from 5 to 100 µL

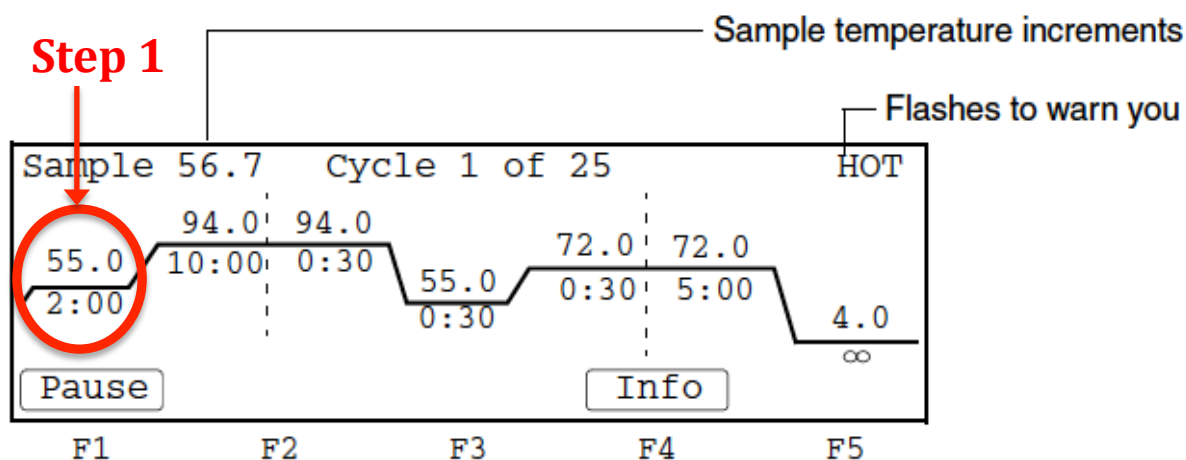
Start Cancel

F1 F2 F3 F4 F5

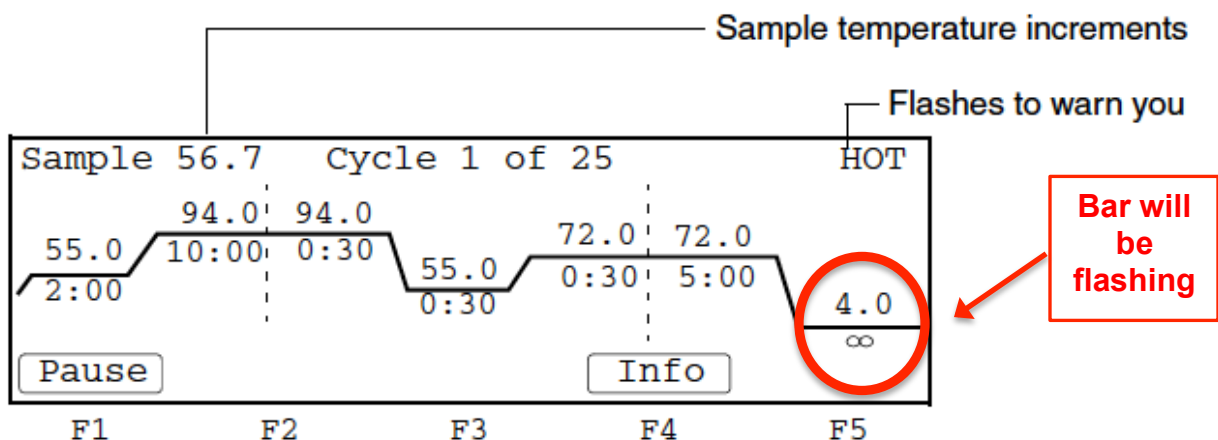
8. If the heated cover is less than 103°C, the following screen will appear:



9. Once the cover reaches 103°C, the "Run Time" screen will appear. The screen will display details of the method you selected and its progression during cycling. At each cycle step in progress, the bar between the temperature and time will be flashing. (For example, when the cycling method begins, the bar between 55.0°C and 2:00 minutes will be flashing.)



10. The run is complete when the last step is completed, which is 4.0°C for infinity (∞). The bar between 4.0 and ∞ will be flashing when the temperature reaches 4.0°C.





11. To stop the run, press the "STOP" key. The "Confirm Stop" screen will appear:

Sample 50.1	Confirm Stop	HOT		
Press STOP to abort. Press Resume to continue.				
<input type="button" value="Resume"/>				
F1	F2	F3	F4	F5

12. Press the "STOP" key again. The "End of Run" screen will appear.:

11:30 AM	End of Run	25.1°C		
Method: General PCR				
Run completed at 11:30:05 AM, 02/01/00.				
Length of run is 01:34:25.				
<input type="button" value="Hist"/>		<input type="button" value="Exit"/>		
F1	F2	F3	F4	F5

13. Press key F5 (Exit) to return to the Main Menu screen:

## Main Menu Screen

08:00 AM	09/25/00	25.0°C		
Applied Biosystems Thermal Cycler				
Version				
User: adrian				
<input type="button" value="Run"/>	<input type="button" value="Create"/>	<input type="button" value="Edit"/>	<input type="button" value="Util"/>	<input type="button" value="User"/>

14. Lift the instrument lid and remove your samples from the thermal cycler. Close the instrument lid when finished.