2022 COSMOS Cluster 3

Biology Laboratory Manual

Overview:

The laboratory skills course will cover basic experimental design/techniques through relevant experimental procedures.

Topics:

- Lab notebook, pipette tutorial, solutions, lab orientation
- Working with bacteria
- Sterile tissue culture techniques
- Sample preparation (RNA isolation and cDNA synthesis)
- QPCR

Schedule:

7/12	7/14	7/19	7/26	7/28	8/2	8/4
Orientation	Bacteria	Bacteria	TC	cDNA synth	QPCR set up	Analysis
Notebook	(Transformation)	(count)	(RNA)	RT		
Pipette	TC					
Solutions	(Plate)					

Instructor:

Emi Embler, PhD (eembler@uci.edu)

TA:

Amina Hussein – 3rd year MCSB student (Advisor: Ken Cho)

Review of Lab Safety Rules:

1) EH&S safety training

2) Wear protective clothing/gear at all times when performing experiments:
    a. Lab coats
    b. Gloves
    c. Eye safety glasses
    d. Closed-toe shoes

3) No eating or drinking in the lab.

4) Follow all directions and instructions carefully. If in any doubt, stop, ask.

5) Be aware of specific safety and environmental hazards.

6) Use designated areas for specific tasks e.g. fume hoods if dispensing any volatile reagent.

7) Disposal of waste: use the appropriate containers provided.

8) Spills: notify your instructor or TA immediately.

9) Emergency procedures:
    a. Evacuation – routes and procedures
    b. Personal exposure
    c. Medical

Lab Notebooks

1) USE PEN
2) Write up your protocol before you start
3) Every experiment should include:
    a. Title
    b. Date
    c. Goal – short description of the purpose of the experiment
    d. Methods – stepwise detailed description of what you did, reagents used, details on incubation times, temperatures etc.
    e. Results – record all your data/observations. Include machine output files/printouts, graphs and calculations.
    f. Conclusions – every experiment should include a brief summary of your conclusions and a brief statement about what experiment will be performed next.

Avoid common mistakes

1) Label everything – clearly with waterproof marker pen, sticky tabs.
2) Use new pipette tips to avoid contamination
3) Don’t rush.
4) Follow your protocol stepwise. Check off completed tasks.
5) Avoid distractions – e.g. engaging in unrelated conversation or texting.

1) Micropipette skills: Determine the accuracy of P20, P200 and P1000 micropipettes.

Materials:

P10, P1000 micropipettes
Appropriate tips for micropipettes
Weigh dish
Water

Procedure:

1) Pipette 10 % of maximum volume (i.e. 100 µL for P1000) of water onto a weighing dish. Record the weight. Repeat the measurement 3 times. (**Create an appropriate data table in your notebook to record your data**).
2) Repeat the experiment by weighing out 90% of the maximum volume of water using each of the micropipettes.

Questions/Things to think about:

1) What are the expected weight of water at 10% and 90% of the maximum volume for each micropipette?
2) Calculate the accuracy (% error) at 10% and 90% of maximum volume for each micropipette.
3) Considering your findings, what is the best way to choose your micropipettes?
4) How precise were you in your measurements?

Micropipette Skills Data Table (sample)

	P10		P1000	
	10%	90%	10%	90%
1				
2				
3				

2) Micropipette skills: Serial dilution

Materials:

P10, P20, P200, P1000 micropipettes
dH_2O
500 uM Bromophenol Blue solution
1.5ml microfuge tubes
Cuvettes

Procedure:

1) Prepare to serially dilute stock Bromophenol Blue solution. Use the table to show your dilution scheme.

µM (final)	50	5	0.5	0.05	0
Total (ul)					1000
Stock (ul)					0
dIH_2O (ul)					1000

2) Get 5 microcentrifuge tubes and label them with each concentration.
3) First add indicated amount of dH_2O to each tube. (**Do you need to change the tip each time?**)
4) Pipet mix or vortex stock Bromophenol Blue solution before using.
5) Add XX µl of 500uM stock solution to the tube marked for 50 µM solution – mix well (pipette mix!).
6) Transfer XX µl of the 50 µM solution to the tube for 5 µM – pipette mix well (**Do you need to change the tip?**)
7) Repeat for the remaining tubes for 0.5 µM and 0.05 µM.
8) Transfer the diluted solutions to the plastic cuvette. Do not hold the cuvette at the flat side (**WHY?**).
9) BLANK the spectrophotometer with **_____?**.
10) For each concentration, read absorbance at **570 nm**.
11) Create a data table in your notebook and record values.

Questions/Things to think about:

1) Draw a A_{570} vs concentration plot for Bromophenol Blue.
2) What does the plot look like?
3) Were you accurate/precise in your measurements?

2) Bacterial growth

Introduction:

In this series of experiments, you will transform bacteria to amplify and select for your plasmid vector.

Subcloning is a technique to put a piece of DNA into a plasmid vector. Subsequently, this plasmid vector carrying your gene of interest can be used to express protein in cells to study its function. Furthermore, you can select a specific vector with different elements to tag your protein or control its expression.

Material:

DH5α competent cells
SOC medium
LB broth
Ampicillin (stock @ 50mg/ml; dilute 1:1000)
LB agar plate (+/- Amp)
Bacteria cell spreader
Pop-top tubes
Ice bucket
H_2O bath @ 42°C

Procedure:

1) Thaw DH5α competent cells on ice. Write your initials on an empty 1.5ml Eppi and place it on ice.
2) Gently mix the DH5α cells with pipette (stir, not up and down or flick the tube) and aliquot 50 ul of cells into the empty 1.5ml Eppi tube on ice.
3) Add 2.5 ul of pU19 vector (250 pg) to the cells and mix gently (stir not up and down).
4) Let sit on ice for 30 min.
5) After 30 min, heat shock cells for 20 seconds in a 42°C water bath (no shaking; just submerge the tube up to where the cells are).
6) After 20 seconds, immediately place the tube back on ice for 2 minutes.
7) Add 950ul of SOC medium to the tube.
8) Let the tubes incubate at 37°C for 1 hour.
9) Get 2 plates: 1 Amp and 1 LB plates. Write your name on the lid. Place them at 37°C to pre-warm.
10) After 1 hour, plate 100ul on each plate.
11) Incubate plates O/N at 37°C.

12) <will be done for you> Pick 2 individual colonies from the AMP plate and grow them up in separate tubes containing 3ml LB broth + Amp (50ug/ml).

13) Spin down the bacteria at 13,000 rpm for 30 seconds to form a “pellet”.
14) Remove the supernatant (spent LB broth).
15) Follow the manufacturer’s protocol for QIAprep Spin Miniprep kit to purify plasmid.

3) Tissue Culture Techniques

Many types of eukaryotic cells have been adapted for tissue culture growth. Typically, primary cells collected from whole organisms or tissues contain a variety of different cell types and may initially grow poorly without the proper stimuli (growth factor signals), grow only for a limited number of divisions or not grow at all. Also, such cell populations may rapidly lose cell type specific characteristics or change over time as cells differentiate.

On the other hand, established tissue culture cell lines represent partially or fully transformed cells and have properties that permit easy passage, expansion and specialized phenotypes. These characteristics include increased and consistent rate of cell proliferation (increase from 24-60+ hrs 20 hrs per cell cycle), reduced requirement for exogenous growth factors, adaptation to fetal bovine serum, high O_2/low CO_2 levels, loss of contact inhibition, increased cloning rate at limiting dilution and foci formation. Fully transformed cells are usually derived from cancers or modified through mutation or introduction of oncogenic genes.

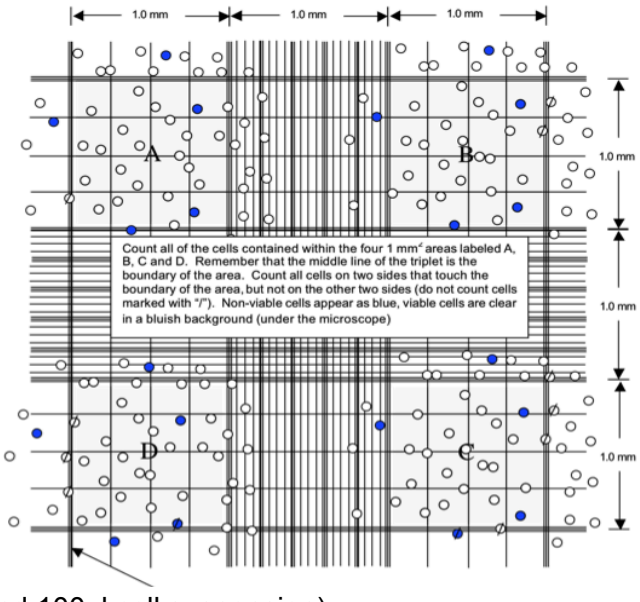
Techniques for culturing cells depend on their properties e.g. adherent or suspension (non-adherent), requirement for substrate coatings and media factors.

Materials:

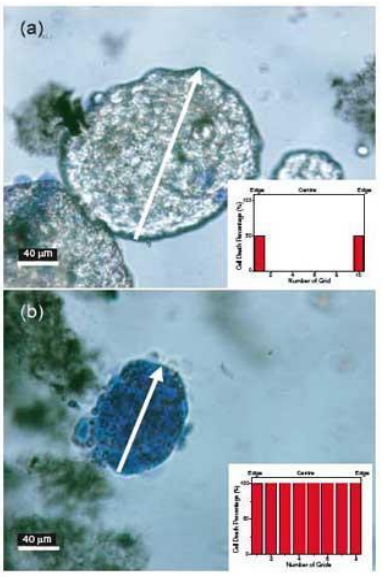
- Complete growth media
- Trypsin solution
- PBS

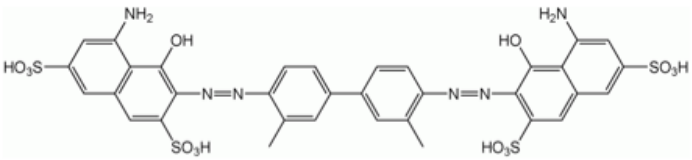
General Protocol for Passaging Mammalian Cells (C2C12)

1) Spray and wipe down the working surface inside of the hood with 70% EtOH. The space is now considered sterile.
2) Spray and wipe down anything else you are brining into the hood with 70% EtOH.
3) If your supply is in a sterile packaging, open it inside of the hood.
4) For adherent cells, aspirate media from a confluent (or near confluent) TC plate.
5) Wash once with 10 mL of sterile phosphate buffered saline (PBS w/o Ca^{2+} or Mg^{2+}). Aspirate.
6) Treat with 1 ml Trypsin/EDTA solution. Place in 37 ºC incubator for 5 minutes. Check that cells are detached and separated under a microscope.
7) Add 9 mL complete media (contains serum to inactivate trypsin), collect the suspension into a 15ml conical tube, spin at low speed and aspirate the supernatant.
8) Triturate with 5-10ml of fresh complete media to make a single cell suspension.
9) Count the number of viable cells and calculate the suitable ratio to split the cells (in a 1.5 ml Eppi, mix 100ul of Trypan Blue and 100ul cell suspension).

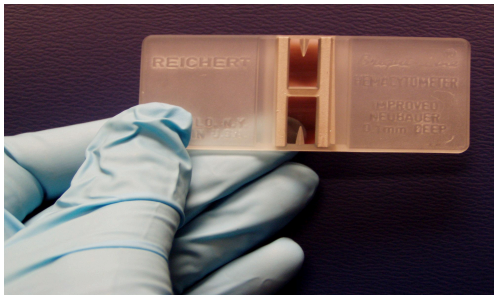


Counting Cells





Trypan Blue Staining



1) Follow steps 2-6 above for adherent cells.
2) To count, mix 1 part cell culture (100 µl) with 1 part Trypan Blue (100 µL).
3) Place a cover slip onto a clean hemocytometer.
4) Gently touch 10-20 ul of the mixed cell suspension to the notch and allow fluid to be drawn under the coverslip by capillary action.
5) Count the number of live cells (bright) and dead cells (blue) on bright field setting on tissue culture microscope.
6) You should try to count at least 100 cells in the quadrants.
7) Establish a consistent rule to counting. For example, count all cells within or touching 2-sides. Do not count cells on or touching the remaining 2 boundaries.
8) Calculate the cell density (cells/ml). Don't forget to multiply by the dilution factor you used with Trypan Blue.

9. To re-plate the cells, on 6-well plate (or 6cm dish), add 2.5 ml of your cell suspension to the fresh plate or dish.
10. Add 2.5 ml fresh media to the cell suspension.
11. Place the plate in 37C incubator.

<u>Tissue Culture Reagents</u>

Growth media for C2C12 cells

DMEM
10% Calf Serum
Pen/strep

6) Molecular biology technique: RT-QPCR

Polymerase chain reaction (PCR) is a commonly used lab technique to amplify specific segment of DNA. Here, you will be using a version of PCR called Reverse Transcription Quantitative PCR (RT-QPCR). Your template will be RNA, which is reverse transcribed into complementary DNA (cDNA) and subsequently amplified. Your target RNA transcripts can be quantified as their template cDNA is amplified in the presence of SYBR Green dye, which binds double-stranded DNA (dsDNA) and fluoresces, and the fluorescence intensity is measured real time during the process.

RNA isolation using TRIZOL

Isolation of total cellular RNA is the first step in many molecular biology protocols. Several methods can be used. The TRIZOL method is convenient because it is effective, fast, efficiently inactivates/removes RNAases and is robust for a wide variety of samples. TRIZOL uses a mix of guanidinium isothiocyanate, phenol and chloroform to disrupt and denature proteins and differentially separate nucleic acids (DNA v RNA). Clean RNA can then be precipitated and pelleted using isopropyl alcohol and centrifugation.

Materials:

Ice-cold PBS
TRIZOL
Chloroform
Isopropanol
70% Ethanol
DEPC-dH_2O
RNase-free microfuge tubes
Phase Lock Gel tubes
4 ^oC centrifuge
Spectrophotometer

Procedure:

1. Aspirate the media and wash once with 5 ml PBS. Aspirate PBS.
2. Add 1 mL TRIZOL reagent to each well.
3. Allow plates to gently agitate for 5 minutes, then tip plate at an angle, dislodge cells with a cell scraper and transfer the TRIZOL mixture to a microfuge tube.
4. Add 0.2 ml chloroform to the microfuge tube. Vortex vigorously for 30 seconds and let the mixture incubate for 3 minutes at room temperature.
5. Centrifuge the mixture for 15 minutes at 13,000 rpm at 4 ^oC to separate the phases.
6. Carefully transfer top (aqueous) layer to a new microfuge tube. (Note - Try not to disturb or carryover the interface or bottom layer. It contains cellular proteins and genomic DNA)

7. To precipitate the RNA, add 0.5 mL isopropanol to the microfuge tube. Vortex briefly (1-3 seconds) to mix.
8. Let the mixture incubate at room temperature for 10 minutes.
9. Centrifuge for 15 minutes at 13,000 rpm at 4 ^{o}C.
10. Carefully aspirate the supernatant. Take care not to disturb or suck up the small gel-like pellet (RNA!!).
11. Rinse the pellet with 0.5 mL 70 % ethanol. Briefly (1-3 seconds) vortex.
12. Centrifuge for 15 minutes at 13,000 rpm at 4 ^{o}C.
13. Aspirate the supernatant and remove all visible droplets of liquid. The pellet might easily dislodge at this point – BE CAREFUL !!
14. Centrifuge for 3 minutes at 13,000 rpm at 4 ^{o}C to ensure removal of all ethanol.
15. Let pellet air-dry for 2 minutes. DO NOT OVER-DRY.
16. Resuspend the pellet in 20 µl DEPC-dH_2O. Gently pipette up and down. Take care to avoid getting the sticky pellet stuck on the inside of the pipette tip.
17. Take an aliquot (1 µl) of your RNA, dilute it 1:40 with dH_2O (add to 39 ul DEPC-dH_2O), and measure the OD at 260nm and 280nm on a UV spectrophotometer to calculate sample concentration and purity. The A_{260}/A_{280} ratio should be above 1.6 for optimal purity. Calculate the RNA concentration by using the convention that OD of 1 at 260 nm = 40ug/ml RNA. Don't forget to account for the dilution factor.
18. Adjust the concentration with DEPC-dH_2O to give a final RNA concentration of 1 µg/µl.
19. Immediately proceed to cDNA synthesis or store at – 80 ^{o}C.

cDNA synthesis

mRNA is reverse transcribed into 1st strand complementary DNA as the template for the subsequent QPCR reaction. Minimally reverse transcriptase requires a template (RNA) and DNA primer complementary to a known 3' location and sequence e.g. polyA tail, (or more generally random hexamer oligonucleotides), a mixture of dexoynucleotide triphosphates (dNTPs), Mg^{2+} cations and appropriate buffer/salts. Lucky for us, there are many commercially available short-cuts to streamline the procedure and minimize source of error.

Materials:

iScript cDNA Synthesis Kit
PCR tubes (strip)
Thermocycler

Procedure:

* When preparing cDNA reactions for multiple RNA samples, make a **MASTER MIX** with common reagents to eliminate inter-sample variability. Especially important if downstream applications require quantitative analyses. *

1) Make a master mix for all of your samples according to the table below (Volunteer to make for the class):

Component	Volume per reaction (ul)
5X iScript Reaction Mix	4
iScript Reverse	1

Transcriptase	
RNA sample (1 ug @ 1ug/ul)	1
Nuclease-free H2O	14
Total reaction volume	20

(What should be in your master mix? How many samples do you have? How much error should you account for?)

6) Aliquot 19ul of the master mix to 6 PCR tubes (labeled 1-6).
7) Put your RNA sample (1ul) in a PCR tube.
8) Add 19 ul of your Master Mix to the tube with your RNA sample and gently pipette mix.
9) Place the sample in a designated thermocycler and run the program called “RT4SC”. The reaction protocol of the program is as follows:

Priming	5 min @ 25 ^{o}C
Reverse transcription	20 min @ 46 ^{o}C
RT inactivation	1 min @ 95 ^{o}C
Storage	Hold @ 4 ^{o}C

10) Save your reaction product for next lab.

Quantitative PCR

You will be measuring the gene expression levels for MyoD (proliferation marker). GAPDH "housekeeping" gene will be used as the control.

Each sample will be run in a triplicate, i.e. 1 samples x 3 = 3 data points per gene. For each gene, a standard curve will also be generated to ensure that the reaction was optimal. QPCR plates have 96 wells. Come up with a plating scheme to minimize the number of runs.

Sample plate map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 5	Std 1	Std 0.2	Std 0.04	Std 0.008	NTD	Std 5	Std 1	Std 0.2	Std 0.04	Std 0.008	NTD
B	Std 5	Std 1	Std 0.2	Std 0.04	Std 0.008	NTD	Std 5	Std 1	Std 0.2	Std 0.04	Std 0.008	NTD
C	Std 5	Std 1	Std 0.2	Std 0.04	Std 0.008	NTD	Std 5	Std 1	Std 0.2	Std 0.04	Std 0.008	NTD
D	1.1	1.2	1.3				1.1	1.2	1.3			
E												
F												
G												
H												

* The green wells = MyoD; white wells = GAPDH

Materials:

iTaq Universal SYBR Green Supermix
Primers @ 10 uM (F & R)
Nuclease-free water
Microfuge tubes
96-well PCR plate & cover film

Procedure:

*** For best results, it is critical that all reagents (buffer components etc.) are equivalent. Even small differences will affect each cycle's PCR efficiency cumulative errors !!

*** Make a master mix in microfuge tubes then dispense aliquots into the 96-well PCR plates.

*** To account for errors, make 30% more than what you actually need.

1. Make a pooled template cDNA for the standard curve (done as class – 1 volunteer).

    a. Set up SIX 0.5ul microfuge tubes and label them as 1, 1/5, 1/25, 1/125, 1/625, 0.

b. In a tube marked “1”, take 1 ul from each of your (and that of your lab-mates) 2 cDNA samples, i.e. reaction product from the RT, and pool them in a microfuge tube (2ul x 6 students = 12ul total).
c. In the tube marked 0, add 10 ul of Nuclease-free water.
d. In the remaining five tubes, perform serial dilution according to the table below. Start by adding the nuclease-free water in applicable tubes first.

Conc.	1	1/5	1/25	1/125	1/625	0
Total vol. (ul)	12	12.5	12.5	12.5	10	
Stock (ul)		2.5	2.5	2.5	2	
H_2O (ul)		10	10	10	8	10

2. Make a master mix for all of your reactions according to the table below:

Component	Volume per reaction (ul)
iTaq Universal SYBR Green Super mix	10
Primer (Forward) @ 10 uM	0.2
Primer (Reverse) @ 10 uM	0.2
DNA template (RT product)	1
Nuclease-free H_2O	8.6
Total reaction volume (ul)	20

(What should be in your master mix? How many reactions do you have? How much error should you account for?)

3. Label 2 microfuge tubes with GAPDH and MyoD. In each tube, make a “smaller-scale” master mix for your triplicate reactions of each sample. (**What should be in this master reaction mix? Room for error?**)
4. Add cDNA template to the tubes containing reaction mix.
5. Put 20 ul of the reaction mixture into the wells of the 96-well PCR plate.
6. Seal the plate with transparent film.
7. Briefly (10 seconds) spin down the plates to make sure all the reaction material is at the bottom of the plate. (Make sure you balance the plate spinner)
8. Place the plate in a designated thermocycler and run the program called “iTaq2step”. The thermal cycling protocol is as follows:

Polymerase activation/DNA denaturation	30 seconds @ 95 °C
Denaturation	5 seconds @ 95 °C
Annealing/Extension/Reading	30 seconds @ 60 °C
Repeat steps 2 & 3	39 times
Melt curve analysis	Ramp up 65-95 °C in 0.5 °C increments @ 5 seconds/step

Analysis:

9. Determine the Ct values for your samples and also compare the melting curves of the product (should be a well-defined single Tm for each gene).

1) Calculate the relative expression levels of the target genes by using the ΔCt method (cf housekeeping gene e.g. GAPDH).

Questions:

How consistent are your triplicate data points?
What kind of information can be deduced from the melting curves?
Identify the most likely sources of variability (error) for this type of experiment.
How would you minimize these errors?

Calculate the relative expression levels of the various genes in response to the cell treatments. What can you conclude about the cell's response to your chosen treatment?