Immunology Lab
Biology 477
Lab Manual

Spring 2016

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SOLUTIONS AND UNITS OF MEASURE IN BIOLOGY

I. Units of Measure

Length

meter = m = 39.37 inches
centimeter = cm = 0.39 inches or 0.01 m
millimeter = mm = 0.04 inches or 0.001 m
micrometer = μm = one-millionth of a meter or 10-6 m
nanometer = nm = one-billionth of a meter or 10-9 m
angstrom = Å = 0.1 nm or 10-10 m

a prokaryotic cell = 1-10 μm in diameter
a eukaryotic cell = 10-100 μm in diameter
large organelles such as nuclei, mitochondria, chloroplasts = 1-5 μm in diameter
small organelles = 0.2-1.0 μm in diameter
ribosome = 25 nm in diameter
membranes = 7-10 nm thick
actin microfilament = 7 nm in diameter
DNA helix = 2 nm in diameter

Since the resolution of the light microscope is about 0.25 μm, only whole cells and large organelles may be observed, even under the best conditions.

Mass

gram = g = 0.002 pounds
milligram = mg = .001 g
microgram = μg = one-millionth of a gram or 10-6 g
nanogram = ng = one-billionth of a gram or 10-9 g
picogram = pg = one-trillionth of a gram or 10-12 g

C. Volume

liter = l = 0.908 quarts; volume of one kilogram of water at 4° C
milliliter = ml = 0.001 l, the volume contained in a cube with sides of 1 cm; one ml of water weights 1 g at 4° C
microliter (μl) = one-millionth of a liter or 10-6 liter; one microliter of water weighs one μg

January 29, 2016

1. Safety Training

2. Introduction to Immunological Techniques

Cell culture

ELISA

Flow cytometry

Histology

Magnetic cell sorting

3. Lab Notebooks

5 points per week for a total of 65 points

Must have in lab notebook: Purpose, Materials/Methods from the lab we are going to do and Results/Data, Discussion of data for the lab we previously did.

4. Lab Reports

There will be three lab reports. Each is due after a key methods section. Reports will be required to have a title, and either: (lab report 1) Methods and Materials used (detailed with WHY each step is taken), (lab report 2) Results (Figures and figure legends will be explained), (lab report 3) Discussion (put the data into context of the field of immunology). All three reports will need a

bibliography with at least 5 primary research articles referenced. You will be required to use your own words in all written sections, plagiarism will not be tolerated.

5. Plagiarism Policy:

Students are responsible for honest completion and representation of their work. Plagiarism will not be tolerated in any of the projects. Any evidence of cheating (including plagiarism, i.e. presenting the words or ideas of others as your own, cutting and pasting phrases from the internet, copying or modifying someone else's work) will result in a failing grade for that assignment and possibly a failing grade for the semester for the course. There will be zero tolerance for infractions. The instructor reserves the right to discipline any student for academic dishonesty and plagiarism, in accordance with the general rules and regulations of the university.

Please see me if you have any questions about what exactly constitutes plagiarism. The writing center is available to help any students struggling with the art of scientific writing. In addition, I am holding a scientific writing group on Wed at noon in the library to give one-on-one advice.

6. Methods Presentations

Worth 85 points (first 15 minutes of class) includes:

1. What we will be doing for this assay (describe the methods with an emphasis on *why*).
2. Scientific principles of the assay (how does it work scientifically?)
3. Uses of the assay in immunology (past and present).
4. An example of current published research that has used this method (show a figure from the paper and explain what the results mean).

Any video content should be less than 30 seconds or created by the group.

7. Pipet aid training

Using a pipet aid can be frustrating at first since it is easy to accidentally suck media up in to the filter at the top of the pipet. This can destroy the pipet aid and thus we will practice performing the measurement of liquid with pipet aids.

1. Hold the pipet at the end (never touch the tip that goes into the liquid), pop the end out of the paper (if this is a sterile, paper-covered pipet).

2. Place the end of the pipet into the pipet aid securely. Push hard enough that it fits tight, but do not overly shove it in.

3. Place the tip of the pipet into the liquid and gently push on the top button with your index finger. Start very gently and then slowly increase pressure until you

reach the desired volume. If you get too much liquid, gently push the bottom button with your middle finger to push liquid out of the pipet.

4. Move the pipet tip to the desired vessel to move the liquid (i.e. another empty tube). Gently push the bottom button to release the liquid from the pipet.

5. Practice measuring liquids with a variety of pipets.

For your purposes in this class:

Use the 5 ml pipet for liquid volumes of 4 ml or less.
The 10 ml pipet for liquid volumes from 9 ml to 4 ml.
The 25 ml pipet for liquids from 24 ml to 9 ml.

1. Pipet 3 ml, 6 ml, 9 ml, 12 ml, 15 ml and 18 ml of liquid. Move the water from a 50 ml tube to a fresh 15 or 50 ml tube and verify the volume you moved. Let the professor know immediately if the filter at the top of the pipet becomes wet due to overpipeting.

2. Practice until you are confident in your ability to pipet the liquid.

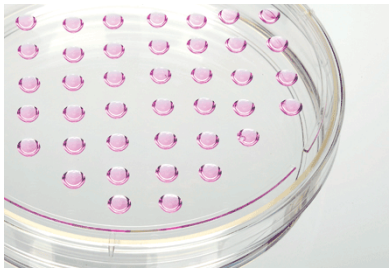
8. Rules in the Tissue Culture Room

9. Design Experiments for Part 1

Key aspects of design (Cell number, Cell type, Read-out, Methods Used)
Lab math
In vivo versus *In vitro* studies
Clinical research

February 5, 2016
1. Introduction to Cell Culture Techniques (Watch Video from link below)

The ability to culture cells outside the natural environment (i.e. from an animal or plant) has been named one of "medicine's ten great discoveries". One of the first biology courses in the US was taught at Yale's Sheffield Scientific School in 1870. Ross Harrison was recruited to start a new zoology department there in 1907. Dr. Harrison had been studying how to culture amphibian embryo neural tube fragments using tissue culture techniques and his data were published over the next three years elucidating the principles of short-term cell culture (Harrison, 1910). His initial breakthrough occurred when he added the cells to fresh frog lymph and used a hanging drop technique for culture (see below).



Primary Cell Cultures: Cells cultured directly from an animal or plant. Many cell types only survive a set number of divisions before undergoing senescence or losing the ability to proliferate.

Cell Lines: When primary cells are subcultured, or transferred in small numbers to a new vessel to continue proliferation, they are called a *cell line*. Some cell lines undergo senescence, while others undergo transformation, either spontaneously or induced, and become immortal (i.e. HeLa, A20 B cell lymphoma).

When subcultured cells are further selected and propagated they are called a *cell strain*, which may have undergone a variety of genetic changes distinguishing it from the primary cell culture.

<u>Growth Characteristics</u>:
Suspension Cells: grow without attaching to the flask or culture vessel.

Adherent Cells: attach to the surface of the flask creating a monolayer.

<u>General Cell Culture Conditions</u>:
Each cell type has unique requirements for culture in vitro, however several variables are generally consistent.

1. Media (solid or liquid)- includes amino acids, vitamins, minerals, carbohydrates for survival
2. Growth Factors
3. Hormones

4. Gases
5. A stable environment with constant physiological temperature, pH, and osmotic pressure
6. Antibiotics

Sterile Technique

Dirt, dust, spores, bacteria live on surfaces and in the air. To keep cell cultures from becoming contaminated it is important to follow rules of sterility.

1. Keep the working space clear. Only have necessary bottles, pipets and containers in the biosafety hood when working with cells.
2. Keep surfaces clean. Wipe everything down with 70% EtOH: bottles (prior to entering biosafety hood), surfaces in the hood, your gloves, and pipets.
3. Only use "sterile" media and reagents. A solution is only considered sterile if it has never been opened outside the hood. Pipets and pipet-tip boxes must only be opened in the biosafety cabinet.
4. Minimize hand motion, especially over any open tubes, bottles, flasks or plates. Place everything in an easy to reach and maneuver spot.
5. Prepare well prior to beginning in order to have everything needed for your cell culture work.
6. If you need to put a sterile cap down, put it down face up.
7. Wear gloves to protect your cells from you and you from your cells!

We will be using a Class II Biosafety Cabinet, which creates a barrier between the sterile environment and the outside air by using HEPA filtered air. Movement of arms in and out of the cabinet breaks the air curtain disturbing the flow. Therefore, *do not block the airflow and work at least 6 inches inside of the hood*.

Signs of Contamination: Watch the media for dramatic changes in color. The pH indicator will turn yellow when acidic or purple for alkaline. If media becomes yellow, the culture is overgrown or contaminated. Purple indicates dead cells or no growth. Similarly cloudy media suggests contamination or overgrowth. If adherent cells come off from the flask or if suspension cells clump up, contamination is also possible. Observe cells at a higher magnification, bacteria will appear in the cytoplasm or media as small moving columnar objects.

Videos:

http://media.invitrogen.com.edgesuite.net/Cell-Culture/videos/CellCultureBasics.html?CID=ccbvid1

References:

http://www.invitrogen.com/site/us/en/home/References/gibco-cell-culture-basics/introduction-to-cell-culture.html

Harrison, R. (1910) The outgrowth of the nerve fiber as a mode of protoplasmic movement. J. Exp. Zool. 9:787-846.

2. MAKE MEDIA

We will prepare two kinds of media for the cells we are growing this semester.
10% FCS in DMEM and
10% FCS in RPMI

1. First squirt all materials with 70% ethanol prior to putting them in the hood. Lightly loosen any caps within the hood of liquids that you will be moving.

2. Using the 50 ml conical tube to hold your media add each of the following to both your 40 ml of DMEM and your 40 ml of RPMI:

4 ml of FCS
.5 ml Pen/Strep/Glu
.5 ml nonessential amino acids
.5 ml sodium pyruvate
1.25 ml Hepes
40ul of β-2me (this is a stinky, and nasty chemical, add quickly and shut the tube, wash the pipet tip in the media you add it to)

3. Label your tubes well!!
How do we label tubes?
With group name, date, and what has been added to the tube.

3. THAW CELLS FOR GROWING (*this will be done prior to class*)

To thaw cells you have to act quickly. The cells have been frozen in DMSO which is toxic to them once they thaw out in the media. The cells will be thawed using a 37C water bath. Once the tube has only one small piece of ice left immediately squirt with ethanol and place it in the biosafety hood.

1. Remove the liquid from the vial and place it in 10 ml of 10% DMEM in a 15 ml conical tube (it is best to already have the 10 ml of 10% DMEM in the tube you are transferring to).

2. Spin the cells down into a pellet using a table top centrifuge at 1250 RPM for 5 minutes (this is a typical "wash" for cells).

3. The cell pellet will be at the bottom of the tube while the supernatant is the liquid above that.

4. Inside the biosafety hood remove and dispose of the supernatant by putting the supernatant in the liquid waste.

5. Add 10 ml of fresh 10% DMEM to the cell pellet, put on the cap and disrupt the pellet using a vortex.

6. After vortexing remove 1 ml of the well mixed cell suspension and put it in a 15 ml conical tube (this will be taken to the lab for counting at your lab bench).

7. Wash the cells in 10ml of 10% DMEM using centrifugation again (as in step 2).

8. Remove supernatant and place the cells in 10 ml of fresh 10% DMEM. Resuspend the cells using the vortex and move the cells and media into a T25 flask with filter.

9. Label the flask well.

4. COUNTING AND SEEDING CELLS

Prepared back at your bench and viewed in microscope. Once the cells are in trypan blue you must count quickly since they will eventually all die and take up the trypan blue.

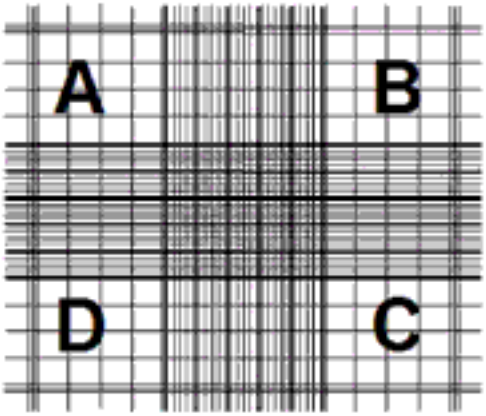
1. Dilute 10ul of your cell sample into 40 ul trypan blue working solution. This is a __:__ dilution.

2. Clean the coverslip and hemocytometer with ethanol. Let it dry. Place the coverslip on the hemocytometer.

3. Take all 50 ul of the cell sample + trypan blue solution into the pipetman and allow about 20 ul to be taken under the slide on each side using capillary action.

4. Immediately place the hemocytometer on the stage of the microscope and locate the grids at low power.

5. Count cells within one large grid ("A" with 16 very small squares). It is easiest to move to one of the corner grids (i.e. A, B, C or D) and increase the magnification of the microscope so the grid takes up the entire space.



6. Count the viable (not blue) cells in the upper left 1-mm square grid ("A" with 16 very small squares). Repeat with each corner grid "B", "C", and "D" so that you have four numbers. Average the numbers.

7. Do the same but only count dead (blue) cells.

8. Calculate the number of viable cells per ml using the formula:
Average # of cells in one grid x 10,000 x dilution factor = # of cells/ml

9. Calculate the percentage of viable cells by dividing the viable cells in one grid by the viable + dead cells in one grid (total cells) and multiply by 100.

10. 80% viability or less suggests cells are not healthy.

11. How many cells do you have per ml?

12. How many ml are left in your cell culture?

13. Thus, how many cells are now in your flask?

February 12, 2016
Harvest Cells, Count Cells, Activate Cells, and Harvest Supernatant

Harvest Cells in Biosafety Cabinet
1. Cells will be removed from the T25 flask by scraping with a cell scraper.
2. Place cells and media in a 15 ml conical tube. Vortex well.
3. Count cells.
4. Place $1x10^5$ cells/well in 6 wells of a 96-well flat bottom plate in 200ul of 10% RPMI.
5. Add LPS (1ug/ml) to the experimental wells (3 wells) and No LPS to the control wells (3 wells).
6. Incubate at 37C for 48-72 hours.
7. Identify students who would like to come and harvest the supernatant and freeze in the -80C.
8. To harvest supernatant, use a p200 pipet to remove the top 100ul of supernatant only (leaving the cells). Place into a 96 well plate (well labeled). Seal plate with parafilm and freeze the plate in the -80C.

February 19, 2016
Perform ELISA on Supernatants from Last Week

TNF-a ELISA
1. (TNF ELISA READY SET GO, eBiosciences) Coat ELISA plate with 100 µL/well of capture antibody in Coating Buffer (dilute as noted on C of A, which is included with the reagent set). Seal the plate and incubate overnight at 4°C.

2. Aspirate wells and wash 3 times with >250 µL/well Wash Buffer*. Allowing time for soaking (~ 1 minute) during each wash step increases the effectiveness of the washes. Blot plate on absorbent paper to remove any residual buffer.

3. Dilute 1 part 5X concentrated Assay Diluent with 4 parts DI water.* Block wells with 200 µL/well of 1X Assay Diluent. Incubate at room temperature for 1 hour.

4. Optional: Aspirate and wash at least once with Wash Buffer.

5. Using 1X Assay Diluent*, dilute standards as noted on the C of A to prepare the top concentration of the standard. Add 100 µL/well of top standard concentration to the appropriate wells. Perform 2-fold serial dilutions of the top standards to make the standard curveor a total of 8 points. Add 100 µL/well of your samples to the appropriate wells. Seal the plate and incubate at room temperature for 2 hours (or overnight at 4°C for maximal sensitivity).

6. Aspirate/wash as in step 2. Repeat for a total of 3-5 washes **.

7. Add 100 µL/well of detection antibody diluted in 1X Assay Diluent* (dilute as noted on C of A). Seal the plate and incubate at room temperature for 1 hour.

8. Aspirate/wash as in step 2. Repeat for a total of 3-5 washes **.

9. Add 100 µL/well of Avidin-HRP* diluted in 1X Assay Diluent (dilute as noted on C of A). Seal the plate and incubate at room temperature for 30 minutes.

10. Aspirate and wash as in step 2. In this wash step, soak wells in Wash Buffer* for 1 to 2 minutes prior to aspiration. Repeat for a total of 5-7 washes **.

11. Add 100 µL/well of Substrate Solution to each well. Incubate plate at room temperature for 15 minutes.

12. Add 50 µL of Stop Solution to each well.

13. Read plate at 450 nm. If wavelength subtraction is available, subtract the values of 570 nm from those of 450 nm and analyze data.

February 26, 2016
Analyze Data from Last Week
See: http://www.abcam.com/protocols/calculating-and-evaluating-elisa-data

AND for help on using Excel:
http://encorbio.com/protocols/ELISA-Excel.htm

March 4, 2016

Making a Single Cell Suspension from Spleen and Thymus

1. Each team will either place a spleen or a thymus in 5 ml 10% DMEM.
2. Mash the organ with the rough end of the sterile slides (use a fresh set of slides for each organ). Remove the remaining organ pieces and throw them away in the biohazard trash.
3. Move cells to a 15 ml conical tube (one for each organ).
4. Underlay the spleen cells with 5 ml of Lympholyte M. Keep the thymocytes on ice.
5. Centrifuge the splenocytes for 10 minutes at 1450 RPM in table top centrifuge with rotor for 15 ml tubes.
6. Remove the splenocytes from the interface between the media and the lympholyte M (it should look cloudy). Place them in a fresh 15 ml tube with 5 mls of 10% DMEM.
7. Spin down thymocytes and splenocytes in centrifuge at 1250 RPM for 5 minutes.
8. Discard supernatant.
9. Vortex cells. Add 10 ml of FACS wash buffer.
10. Count cells.
11. Add $1x10^6$ cells per small 5ml conical tube (FACS tube)
12. Each group will need to prepare the following FACS tubes:
    A. Unstained splenocytes/thymocytes
    B. Splenocytes/thymocytes + anti CD3 Fitc
    C. Splenocytes/thymocytes + anti CD4 APC
    D. Splenocytes/thymocytes + anti CD8 PerCP
    E. Splenocytes/thymocytes + anti B220 PE
    F. Splenocytes/thymocytes + all four antibodies
13. After labeling the tubes, add the correct amount of cells to the correct tubes.
14. Spin down cells in centrifuge at 1250 RPM using table top centrifuge with 5 ml tube insert.
15. Discard supernatant.
16. Each tube will have 100ul of facs wash
17. Add 1ul of antibodies to appropriate tubes. Vortex.
18. Incubate on ice, covered for 30 minutes. Vortex at least once.
19. Add 4 ml facs wash to each tube.

20 Spin down cells in centrifuge at 1250 RPM using table top centrifuge with 5 ml tube insert.

21. Discard supernatant.
22. Vortex cells. Add 300ul of FACS Fixative. Vortex, cap tightly, put in tube rack and cover with foil.

March 11, 2016
Run stained cells on flow cytometer from last week
Read flow cytometry guide on CC before class.

During class review how to do a "Results" section of a lab report.

March 18, 2016

Analyze Flow Cytometry Data

Use the Accuri flow cytometry program to analyze and interpret your data. Save plots for your lab report and make tables that summarize your data.

April 1, 2016

Stain Spleen and Thymus Sections for T cell and B cell zones- Immunofluorescent Microscopy

1. Each group will receive 1 slide with spleen sections and one slide with thymus sections 5um thick.
2. Use pap pen to draw around the sections on the slide
3. Add 4% Formaldehyde using a p1000 to cover all sections (about 300ul). Incubate 1-2 minutes. Make sure the sections do not dry out at any point in the procedure.
4. Add PBS to sections, fully covering them.
5. Block sections with blocking buffer. Incubate for 30 minutes.
6. Add primary antibodies with 2%FCS in PBS. The antibodies should be diluted 1:150 in the 2%FCS in PBS. Antibodies to be used are: CD3 Fitc, B220 PE
7. Incubate for 1 hour at 37C.
8. Dump antibody.
9. Add 2%FCS in PBS for 3-5 minutes to wash the sections. Keep covered.
10. Add mounting media with DAPI and coverslips.

Image the slides at Science Hall 1, microscope room.

April 8, 2016

Analyze Data from Microscopy Experiment

Use photoshop to examine images and make into figures for the report.

April 15, 2016

Done in Biosafety Cabinet: Polarization of T cells into TH1 cells

1. The night before the experiment: Coat a 24-well plate with anti-CD3 at 1 ug/ml and anti-CD28 at 2 ug/ml. Sterile!!!

2. Harvest splenocytes as in March 4, 2016 protocol steps 2-10. Done by instructor.

3. Plate $1x10^6$ cells/ml in 2ml per well in two wells of a coated 24-well plate.

4. ADD 100U/ml IL-2, 10 ng/ml IL-12, and anti-IL4 antibody at 10ug/ml (for TH1 polarization) to one well of the coated plate. Just add 100U/ml of IL-2 to the other well of the coated plate.

5. On Day 3: Pool 2 wells of TH1 cells into a 6 well plate containing 2 more ml of fresh media with IL-2 100U/ml. Do the same with cells from the IL-2 alone control coated plate.

April 22, 2016

Intracellular IFN-γ production by TH1 Cells

1. Stimulate cells with PMA (50 ng/ml)-Ionomycin (1uM) for 5 hours with Bref A (1ug/ml).

2. Harvest cells and place in facs tubes.

3. Each group will need to prepare the following FACS tubes:
   A. Unstained cells
   B. skewed cells + anti CD3 Fitc
   C. skewed cells + anti CD4 APC
   D. skewed cells + anti CD8 PerCP
   E. skewed cells + anti cytokine (either IL-2 or IFN-g or TNF) PE
   F. skewed cells + all four antibodies (B-E)
   G. unskewed cells + all four antibodies (B-E)

3. Wash with facs buffer and follow intracellular cytokine protocol in kit. Details at: http://www.bdbiosciences.com/ds/ab/others/559311%20Book_Website.pdf

April 29, 2016

Run Stained Cells on Flow Cytometer

Re-read flow cytometry guide on CC before class.

During class review how to do a “Discussion” section of a lab report.

May 6, 2016

Analyze Intracellular IFN-γ production by TH1 Cells

Use the Accuri flow cytometry program to analyze and interpret your data. Save plots for your lab notebook and summarize your data and what it means to the field of Immunology for interpretation in the discussion of the lab report.