EUREKA PROGRAM 2017

Estrogens and breast cancer

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TABLE OF CONTENTS

MEASURING THINGS

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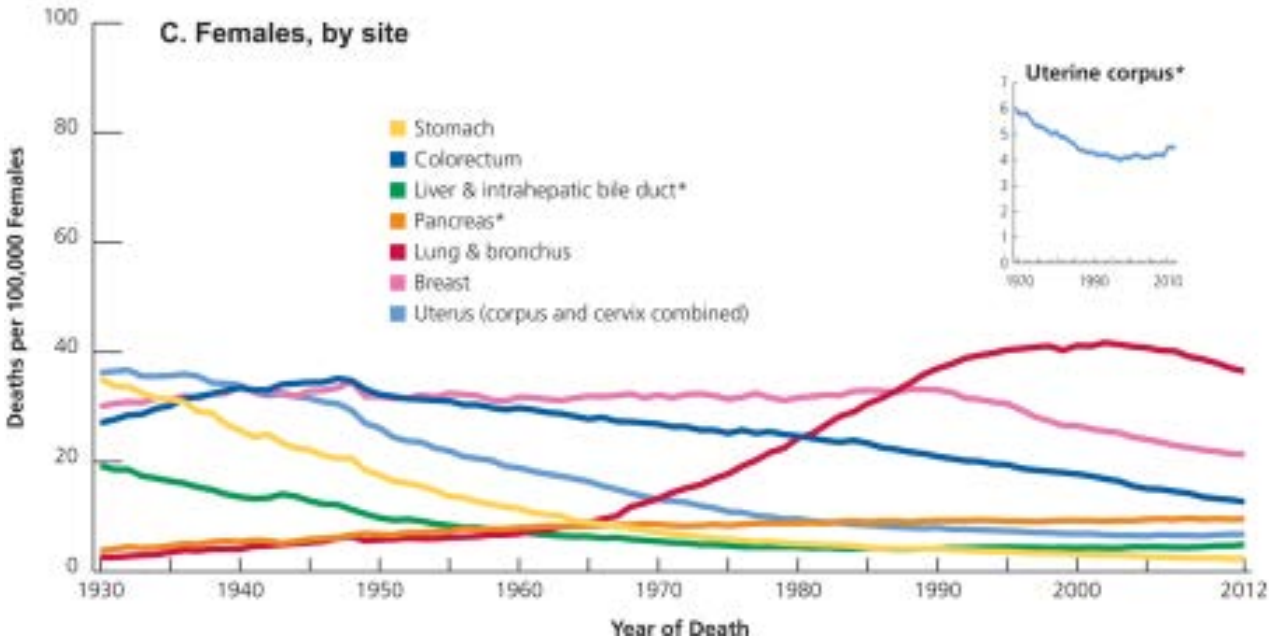


BACKGROUND

What causes breast cancer? How do we investigate this? What do we need to measure? There are many factors that contribute to the risk of developing breast cancer. Different factors may require very different experiments to test whether they play a significant role. For example, it is relatively simple to measure levels of a chemical. We can show that the chemical causes a response in cells grown in an incubator. But does that chemical really cause breast cancer? Often times expose rodents to the chemical to see how they respond. We can measure the chemical in humans and demonstrate associations with the chemical and disease. But does that chemical really cause the disease? Different experiments with different interpretations. But they all start with developing a way to measure a chemical or a response in cells or in humans.

PROCEDURES

Exercise 1 --- Measurements and data reporting

Introduction to Pipettors: One of the most important skills you will need in this course is your ability to use a micropipettor. Micropipettors are used to make accurate measurements of extremely small volumes—from one milliliter down to one microliter (1 mL to 1 µL). Most of what we do in molecular biology involves manipulating volumes of liquid in this range. If you learn to do it accurately now, your experiments will go much more smoothly later.

Equipment and Supplies:

- *Pipettors (one set per student)*
- *Color-coded tips*
- *Weigh boats*
- *Scales*
- *Water in beakers*

1. ***Micropipettors**: These are precision scientific instruments, and must be treated with respect. The pipettor is used to draw liquid up into a cheap disposable tip. The three pipettors you will use take up and deliver liquids in the volume range from ~0.5 µL to 1.0 mL. Your instructor will show you how to use this device. Walk through these points with your instructors to learn how to use them:*

a. ***Holding the pipettor**: For best control, grasp the micropipettor in*

your palm and wrap your fingers around the barrel; work the plunger (piston) with the thumb. Hold the micropipettor almost vertical when filling it.

b. *Adjusting the setting*: *There are three numbers on the display of each pipettor. Look at the top of the pipet to see which one you are holding, then look at the display. The numbers represent volumes as shown below. The color change represents crossing the decimal place or changing units.*

L 20		L 200		L 1000	
1	10µL	**1**	100µL	**1**	1000µL = 1.0 mL
0	1µL	**0**	10µL	**0**	100µL
0	0.1µL = 100 nL	**0**	1µL	**0**	1µL

c. *Using the pipettor*: *smoothly depress the plunger to the first and second stops to become familiar with the feel of the pipettor. Only go to the second stop when expelling liquid (never when sucking liquid into the tip). Never snap the plunger quickly, only use smooth controlled motions.*

d. *Pipetting a sample*: *To withdraw the sample from a reagent tube: Depress the plunger to first stop and hold it in this position. Dip the tip into the solution to be pipetted, and draw fluid into the tip by gradually releasing the plunger. Be sure that the tip remains in the solution while you are releasing the plunger. Slide the pipet tip out along the inside of the reagent tube to dislodge any excess droplets adhering to the outside of the tip.*

- *If you notice air space at the end of the tip or air bubbles within the sample in the tip, carefully expel the sample back into its supply tube and try again with a new tip, making sure it is firmly attached to the pipet.*
- *To expel the sample into a reaction tube: Touch the tip of the pipet to the inside wall near the bottom of the reaction tube into which the sample will be emptied. This creates a capillary effect that helps draw fluid out of the tip.*
- *Slowly depress the plunger to the first stop to expel the sample. Depress to second stop to blow out the last bit of fluid. Hold the plunger in the depressed position.*
- *Slide the pipet out of the reagent tube with the measurement plunger depressed, to avoid sucking any liquid back into the tip.*
- *Use the ejector button (located at the back and different from the plunger) to eject the tip into a waste container.*

e. *Important pipettor don'ts:*

- *Never rotate the volume adjustor beyond the upper or lower range of the pipet.*
- *Never use the micropipettor without the tip in place; this could ruin the piston. Pipettors use disposable plastic tips. Every molecular biology lab circulates its own version of the story of the not-too-bright grad student who did not use a tip. Do not be this student!*
- *Never invert or lay the micropipettor down with a filled tip; fluid will run back into the piston.*
- *Never let the plunger snap back after withdrawing or expelling fluid; smooth motions are the key to success.*
- *Never immerse the barrel of the micropipettor in fluid. Only the disposable tip touches the liquid.*
- *Never reuse a tip when pipetting reagents (it's OK for this exercise since we are just using water). Tips are inexpensive, so when in doubt just change tips. If you do not change tips when pipetting reagents, you will cross-contaminate your reagents and samples, making them both useless. Tips also must be changed if they touch anything except the sample (i.e. the table top/your glove) to avoid contamination.*

2. ***Calibration of*** *pipets --- Instruments vary in their accuracy and precision. Accuracy is the measure of how close a measurement of quantity is to its intended value. If accurate, a pipet set to 0.5 ml should deliver exactly that amount. In fact, instruments have a variation as well as the operator. Precision is the reproducibility/repeatability of a measurement. If a pipet set to 0.5 ml but delivers only 0.44 ml reproducibly, it would be considered precise but not very accurate. This exercise will test the accuracy and precision of a pipet as well as the variation due to the operator.*
    a. *Pipet the following volumes of water and record the weights. (Remember 1 ml = 1 cc^3 = 1 g)*

Volume	Pipet used	Obs#1	Obs#2	Obs#3	Obs#4	Mean	SEM
1.00 ml	**Blue**						
0.80 ml							
0.60 ml							
0.40 ml							
0.20 ml	**Blue**						
200 ul	**Green**						

150 ul							
100 ul							
50 ul	**Green**						
20 ul							
15 ul							
5 ul	**Red**						
1 ul							

b. *Calculate the means for each volume. Calculate the standard error of the mean (SEM). Look up formulas as needed and record the method below. You can use Excel, but you need to calculate at least one by hand showing the work below.*
c. *Mean:*

d. *SEM:*

Exercise 2 --- Measuring protein content

The BCA protein assay depends on a reaction between 4 particular amino acids (cysteine, cysteine, tryptophan, tyrosine) and peptide bonds connecting the amino acids to make a protein. The bicinchonic acid (BCA) changes color in the presence of protein together with the copper in an alkaline solution. The solution absorbs light maximally at a wavelength of 562 nm. The reaction is linear over concentrations of proteins from 20-2,000 ug/ml).

Equipment and Supplies:

- *Plate reader*
- *37C incubator*
- *Microplate (and foil to cover)*
- *Pipettors and tips*
- *Pre-homogenized samples*

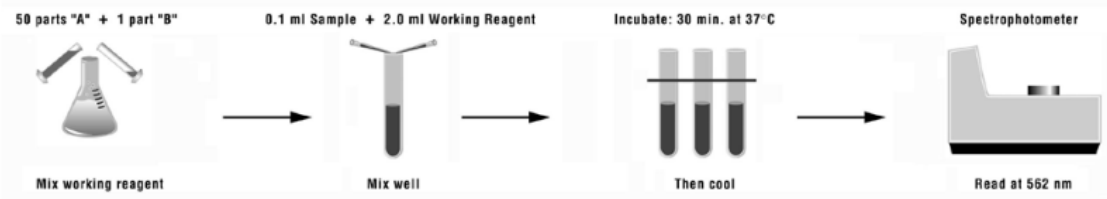
Experimental notes --- Please record notes regarding the procedures and measurements you make.

Preparing the samples:

- *What is your sample?*
- *How much sample?*
    - *Concentrated sample:*
    - *Diluted 1:10 sample:*
- *How much water?*
- *How much did you put into the BCA reaction?*

Preparation of the BCA reagents:

Procedure Summary (Test-tube Procedure, Standard Protocol)



1. Pipet 25ul of each standard and unknown sample into labeled tubes or well of a microplate.
2. Add 200 ul of WR to each tube or well and mix.
3. Cover and incubate at 37 C for 30 min.
4. With the spectrophotometer set to 562nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 minutes.
5. Subtract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm absorbance measurement of all other individual standard and unknown sample replicates.
6. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/mL.
7. Use the standard curve to determine the protein concentration of each unknown sample.

Procedure notes and results:

MEASURING ESTROGENS

BACKGROUND

There are 3 forms of estrogens made in the ovaries of women naturally. The most abundant and most potent form of estrogen is 17β-estradiol which we will refer to as E2. So how can we measure the minute amounts that could be present from various sources? Are there chemicals that can mimic the effects of estrogens? What levels are important to us?

PROCEDURES

Exercise 1 --- Measuring estrogen levels using an ELISA (enzyme-linked immunosorbent assay)

An ELISA is a biochemical technique that uses an “antibody” that is attached to the bottom of a plastic well to detect an “antigen”. An antibody is a Y-shaped protein that recognizes unique molecules. An

antigen is the molecule that the antibody was designed to interact with or detect. We can measure estrogen levels using an ELISA that has an antibody that is specific for detecting estrogen as its antigen. You will *add the specimen* to one of the wells in combination with a solution that contains "E2-Enzyme Conjugate". The E2-enzyme conjugate is simply a molecule of E2 attached to an enzyme. The enzyme is another protein that will cut a substrate in a solution and cause a color change in the solution. The E2 in your specimen and the E2-enzyme conjugate will compete for the antibodies attached to the bottom of the well. If your specimen only has a little E2, more E2-enzyme will bind to the antibodies and the color will be dark. If your specimen has a lot of E2, only a few E2-enzyme conjugate molecules will be able to bind to the antibodies and the color in the wells will be light. We will compare this to a standard – six samples with known concentrations of E2 from 0 pg/ml to 1000 pg/ml.

Equipment and Supplies:

- *ELISA wells*
- *Standards*
- *Samples to measure*
- *Enzyme conjugate*
- *Foil to cover wells/ 4C incubation space*
- *Pipettors and tips*

Procedure Day 1 (Tuesday):

Each experimenter will get one strip of wells. Each strip has 8 little cups (or wells). Mark the flat end using your initials with a sharpie.

Adding the specimen: In the first 6 wells add 25μl of a standard, in order starting at the wells near your initials, (0, 10, 30, 100, 300, 1000). In the last two wells add 25μl of your test sample.

Adding the E2-enzyme conjugate: Now add 100μl of the E2-enzyme conjugate to each well.

We will allow the E2 in the specimens and the E2-enzyme conjugate to compete for the antibody on the bottom of the wells overnight. We will complete the assay and quantify the results tomorrow.

Exercise 2 --- Where do we find estrogens?

Now we know how to measure E2, we will find out where we find estrogens and the amount that is present. Let's find out how much we all know about estrogens and xenoestrogens.

What comes to mind when you first hear the word "estrogen"?

1. *Limits menopause symptoms*
2. *Helps memory*
3. *Causes cancer*
4. *Maintains bone structure*

Finish this sentence - Xenoestrogens are:

1. *Only found in manufactured products*
2. *Harmful to our health*
3. *Mimic estrogens*
4. *Don't know*

Which of these personal care products do you use?

1. *Sunscreen*
2. *Makeup*
3. *Skin/hand lotion*
4. *Nail polish*
5. *More than one of these*
6. *None of these*

When you purchase personal care products, do you commonly read the product label for content/ingredient information?

1. *All the time*
2. *Most of the time*
3. *Infrequently*

4. *Never*

What is the most significant factor that determines your selection of personal care products?

1. *Cost*
2. *Content/ingredients*
3. *Advertisement*
4. *Quality/performance*
5. *Recommendation from peers*

Propylparaben
What It Is: Paraben
Health Risks: Endocrine disrupters that mimic estrogen; linked to breast cancer
Found In: Used as a preservative in lotions and shampoos

Fragrance, DEHP, DBP, Dibutyl Phthalate, Butylbenzyl phthalate
What It Is: Phthalate
Health Risks: infertility, endocrine disruptor
Found In: Used as a plasticizer in nail polish, fragrance carrier in shampoo, deodorant, lotion

Benzophenone-3, BP-3
What It Is: Phenol
Health Risks: Estrogenic activity, endocrine disruptor
Found In: Sunscreen, lip balm, moisturizer to protect products from UV light

Resources to learn more about chemicals in personal care products:

www.ewg.org www.cdc.gov www.safecosmetics.org
www.info.bcerp.org/outreach/ www.thinkdirtyapp.com
www.fda.gov/Cosmetics/Productsingredients/

INTERPRETING DATA

BACKGROUND

We often talk about “high levels” of pollen but what is “high”? We need to know the actual numbers --- we need to quantify the amount of E2. But we also need to interpret that and put it in the context of what is normally present.

PROCEDURES

Exercise 1 --- Continue the ELISA for E2

Complete the ELISA and collect the data. Graph the standard curve, then determine the amount of your unknown. What happens when the unknown is above highest standard or below the lowest standard? How do you interpret that?

Equipment and Supplies:

- *Plate reader*
- *Wash solution aliquots*
- *TMB aliquots*
- *Stop solution aliquots*
- *Pipettors and tips*
- *Computers with excel analysis file*

Procedure Day 2 (Wednesday)

Dump out the solution onto bench paper. We want to dump out any excess E2 or E2-enzyme conjugate that isn't attached to an antibody.

Wash the wells. Add 300μl of wash buffer to each well and then dump it out on the bench paper. Do this 3 times. We want to wash out any excess E2 or E2-enzyme conjugate that is sticking to the plastic.

Add color substrate: Add 100μl of TMB Reagent to each well. The enzyme on the E2-enzyme conjugate needs 30 minutes to work, but you can watch it change the color of the solution from clear to blue.

Add stop solution: Add 50μl of Stop Solution to each well. Mix gently and make sure that all the blue color changes to yellow color.

Read absorbance: The microplate reader will read the absorbance of the yellow color at 450 nm and convert it to a numerical intensity that we can use to quantify the amount of E2 in the specimen.

Graph the Data in Excel

Using the absorbance values from the plate reader make a chart using your standard (sample 1) and the standard of your partner (sample 2).

	A	B	C
1	dose pg/ml	Absorbance	
2		Sample 1	Sample 2
3	0	2.48	2.342
4	10	1.786	1.862
5	30	1.145	1.326
6	100	0.602	0.66
7	300	0.287	0.267
8	1000	0.131	0.128

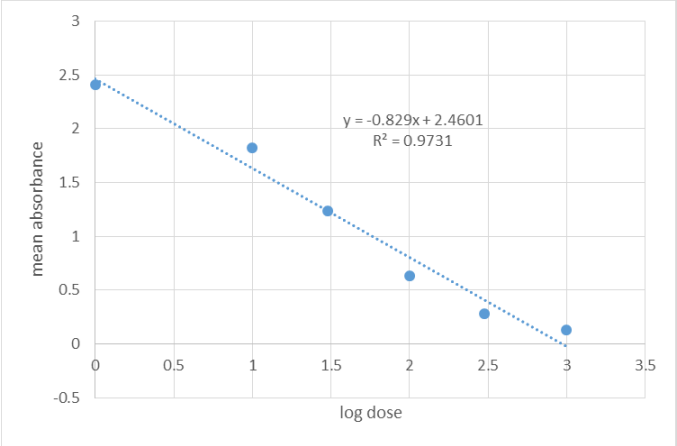
Determine the log dose of the pg/ml. The log dose for the 0 pg/ml is zero. For all other values (10 to 1000) Type =log(select cell or type value)

	A	B	C	E	F
1	dose pg/ml	Absorbance			log dose of pg/ml
2		Sample 1	Sample 2		
3	0	2.48	2.342		0
4	10	1.786	1.862		1
5	30	1.145	1.326		1.477121
6	100	0.602	0.66		2
7	300	0.287	0.267		2.477121
8	1000	0.131	0.128		3

Determine the mean of the absorbance. Type =average(B4:C4, or whatever the correct cells happen to be)

	A	B	C	E	F	G
1	dose pg/ml	Absorbance			log dose of pg/ml	mean absorbance
2		Sample 1	Sample 2			
3	0	2.48	2.342		0	2.411
4	10	1.786	1.862		1	1.824
5	30	1.145	1.326		1.477121	1.2355
6	100	0.602	0.66		2	0.631
7	300	0.287	0.267		2.477121	0.277
8	1000	0.131	0.128		3	0.1295

Graph the log dose vs mean absorbance by selecting the boxed values. Select the table, select insert, choose scatter plot, enter. Then right click on graph, select +, select trendline, more options, and check “display equation on chart” and “display R-square value on chart”. Enter.



Now let’s find out how much E2 is in your unknown samples.

	A	B	C	E	F	G
1	dose pg/ml	Absorbance			log dose of pg/ml	mean absorbance
2		Sample 1	Sample 2			
3	0	2.48	2.342		0	2.411
4	10	1.786	1.862		1	1.824
5	30	1.145	1.326		1.477121	1.2355
6	100	0.602	0.66		2	0.631
7	300	0.287	0.267		2.477121	0.277
8	1000	0.131	0.128		3	0.1295
		unknown sample 1	unkown sample 2			
		0.085	0.346			
		0.08	0.325			
	mean	0.0825	0.3355			
	log dose	2.835378	2.538408	log dose = TREND(log dose for the standard curve, mean absorbance of the standard curve, mean absorbance of the sample)		
	dose in pg/ml	684.5064	345.4679		dose in pg/ml = 10^(log dose)	

In our example we have two values for sample 1 (0.085 & 0.08) an two values for sample 2 (0.346 & 0.325). Calculate the mean for each sample. =average(B13”B14), =average(C13:C14).

Calculate the log dose for each sample by =TREND(select log dose for the standard curve, select mean absorbance of the standard curve, select the mean absorbance of the sample).

Alternatively, you could calculate the log dose using the equation of the line where y is the mean absorbance and you want to solve for x which is the log dose.

X=(y-2.4601)/-0.829. For the first sample you would get 2.868034, which is pretty close to 2.835378 using the first method.

Finally, calculate the dose in pg/ml by =10^(log dose). Our unknown samples in our example had 685 pg/ml and 345 pg/ml of E2.

Exercise 2 --- Dilutions of 17β-estradiol

Equipment and Supplies:

- *Pipettors and tips*
- *Microfuge tubes*
- *Water and colored solution*

The major form of estrogen in humans is 17β-estradiol which we will refer to as E2. If we start with a 1 mM solution of E2, we have to dilute it as follows to get it to levels found in the blood of women.

Take 10 ul of a 1 mM solution and add to 1 ml (1,000 ul) of water. This 1:100 dilution yields a solution that is 10 uM. Now take 10 ul of the 10 uM solution and dilute it in 1 ml of water. This 1:100 dilution yields a solution that is 100 nM. This is still much higher than what is found in humans. We would need to dilute it between 1:10 and 1:100 again to get to levels that are found in women.

Let's do this with a colored solution. When are you no longer able to see the color? This shows you how potent this natural estrogen is.

WHAT DOES ESTROGEN DO IN THE BREAST?

BACKGROUND

During development, the mammary glands of mice undergo dramatic changes. Wholemount slides and thin sections of tissue (4 um thick) allow us to see the magnitude of the changes that the mammary tissues undergo during development. Remember, there changes are in response to the normal range of estrogens that we measured.

The human breast is similar to that of the mouse in its structure. However, with the larger size, a more extensive fibrous network of tissue is required to support the breast cells that are responsible for making milk.

EQUIPMENT AND SUPPLIES

- *Microscopes*
- *Sets of wholemounts*
- *H&Es*

PROCEDURES

Exercise 1 --- A tour of mammary gland development in the mouse

Look at the whole mounts of mouse mammary glands and notice the following landmarks.

Immature 3 wks female --- Find the lymph node. It is the large red circular structure. Can to see the primary duct? It is a single duct with minimal branches.

Pubescent female 6-7 wks old --- The lymph node provides a consistent landmark. Notice how far the ducts have extended toward the lymph node. How do these ducts “grow”? How many ducts are there now?

Mature female 8 wks old --- The fine ductal architecture is now established. Notice that the ducts are well-spaced. How do they know not to grow into each other? What is in the white area between the ducts?

Mature female 12 wks old --- The tissue is now stable, but it remains very active. The ducts show signs of proliferating cells and changes in structure within each ovarian cycle. This is driven by hormones from the ovaries and represents a snippet of what happens in pregnancy when the hormone exposures are prolonged.

Pregnant female --- The ducts have proliferated wildly in only 14 days! The space that was filled by fat cells (adipose) has now been replaced with more ducts and grape-like structures called alveoli. The alveoli are where milk synthesis takes place.

Lactating female --- Even more proliferation has taken place. Now, the ducts are barely visible because the alveoli have filled the entire gland. These

structures are complex, now called lobuloalveolar units, and they are filled with milk.

Postweaning female --- Once the demand for milk decreases, the alveoli and many ducts are no longer needed. Therefore, they receive signals to die. The gland now returns to an architecture that resembles that of the mature female.

You've been provided with five whole mount mammary glands. Use the dissection microscope to view these mammary glands. Work with your partner to draw diagrams that illustrate each of the slides you were provided.

After you have examined all five slides, work together to determine which stages in mammary gland development you were given.

Wholemount mammary gland worksheet

Sample A:

Guess the stage:

Sample B:

Guess the stage:

Sample C:

Guess the stage:

Sample D:

Guess the stage:

Sample E:

Guess the stage:

With so many cells proliferating, the growth during puberty and pregnancy resemble that of cancer. So, why don't you typically see breast cancer during these periods? Instead, the vast majority of breast cancer is in older women.

The average age of women who develop breast cancer in the US is 64 years old.

Exercise 2 --- Looking at the structure of cells on H&E-stained tissue sections

Using the 20X objective, look at the ducts.

Draw simple pictures of what you can see in the slides you were provided.

Does tissue differ among women? Are they all “normal”? If the tissue varies among people, does that have an impact on their risk of breast cancer or sensitivity to environmental estrogens?