

# Using a Comparative Endocrinology Model to Recruit Future Scientists

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Students in an undergraduate research program, **Research PackTrack (RP)**, investigate the structural and functional evolution of the three estrogen receptors (ERs) found in *Micropogonias undulatus*. This neuroendocrine model introduces students to a comparative, evolutionary approach that offers hands-on experience in a working research laboratory. Students use a robust experimental protocol, *in vitro* bacterial expression of the ER and subsequent competitive binding assays, to test their novel hypotheses about the effects of site-directed gene mutations on ER-ligand interactions. This course allows for hypothesis-driven authentic research projects that are loosely based on the laboratory model rather than standardized laboratory exercises.

**Keywords:** estrogen receptors, authentic research, undergraduate research program, studio lab

## Introduction

There is a large, unmet need for undergraduate research opportunities at colleges and universities (AAAS, 2011), especially for underclassmen. Many challenges have to be overcome, because incorporating undergraduates into a research program is time consuming, expensive, and as a result, faculty at research-intensive institutions are hesitant to invest in what is considered to be a service activity that may reduce overall lab productivity. Likewise, faculty at major undergraduate institutions struggle with finding the time or resources to maintain a viable research program. This further limits opportunities for students to experience authentic scientific research. A successful undergraduate research program must benefit the faculty who participate as well as the students. To this end, we created the Research PackTrack Program (RP) to provide a gateway into the world of scientific research for students early in their undergraduate careers and allows participating faculty to gather meaningful data. Research PackTrack is centered on two required courses. After completion of the two-semester series, students transition into research laboratories throughout the university and surrounding scientific community.

In the first course, second-semester freshmen (enrollment of ~30) are introduced to the process of scientific inquiry and discourse in an active, collaborative learning environment. Students participate in local symposia and conferences and attend weekly lab/problem sessions. They produce a scientific primary literature review, a poster representing their semester-long research projects, and a research proposal. They also conduct oral presentations, peer review, and extensive analysis of scientific findings.

In the second course, first semester sophomore students who successfully completed the first course enter the PackTrack Laboratory where they design and execute a research project that will directly contribute to NCSU faculty research programs. We have coined the term *Studio Biology Laboratory* to describe the creative, independent, and open-ended nature of the laboratory experience. Lab times are highly flexible, vary from week to week, and depend on individual progress for each student. The lab is generally open 8:00 a.m.-5:00 p.m. each day and students sign up for times using Google Calendar<sup>®</sup>.

The laboratory is designed around a few, carefully chosen research questions that are currently being pursued by the participating faculty in their own labs. Equipment and supplies are not rotated through the lab as in traditional biology laboratories, reducing labor, equipment costs and storage needs. Instead, students use a core set of techniques and focus on the scientific questions rather than learning many different procedures. This leads to economies of scale and time and allows students to achieve competency and confidence in the laboratory. After initial training, students can work independently with minimal supervision and prep time. Although their proposed research must fall within the scope of faculty research interests, there is much opportunity for students to design creative and innovative projects that speak to them individually. Collaborations with outside faculty are encouraged, and these student-initiated interactions have been extremely successful in expanding the program to include faculty and graduate students in multiple departments at NCSU and beyond.

The three main course outcomes are:

- (1) students will design a project and make novel discoveries,
- (2) students will prepare and present a scientific poster describing their experimental results, and
- (3) students will write a review paper based on their proposed project.

In this paper, we describe the research model currently used in the Research PackTrack lab in order to illustrate the key aspects of the Biology Studio Laboratory. This model can be adapted to address a wide range of research interests while providing exceptional learning opportunities for participating undergraduate students.

## Student Outline

### Overview of Estrogen Receptors

Estrogen is a steroid hormone that is usually thought of as the *female hormone*. However, estrogen has many different functions in both males and females at all developmental stages and ages. Estrogen is found in all vertebrates and has similar functions across species, which makes it possible to use a wide variety of model organisms to investigate the role of estrogen in physiology. Estrogens exert their effects by binding to estrogen receptors (ERs) found inside the cells of target tissues. Estrogen receptors, and all steroid hormone receptors, belong to a large family of signaling molecules called *gene transcription factors*. After estrogen binding, the ligand-receptor complex moves to the chromosomal DNA where it interacts with specific response elements in the promoter regions of estrogen-responsive genes. This interaction promotes or inhibits transcription of the proteins encoded by these target genes.

The binding of estrogen to the estrogen receptor is very specific, and is often thought of as acting like a *lock and key*. However, there are many compounds that can mimic estrogen and bind to the ER. For example, drugs used to treat estrogen-related diseases through binding the estrogen receptor and altering its signaling capabilities. Chemicals that are released into the environment, such as BPA or DDT, can inappropriately bind to ERs and cause adverse effects in organisms. It is important for scientists to understand why these different compounds are able to interact with the estrogen receptor. All estrogen receptors in all organisms share a modular protein structure that includes a Ligand Binding Domain (LBD). The LBD of the estrogen receptor determines the shape of the lock for estrogen and estrogenic compounds. Overall, the amino acid sequences of the LBD are very similar across species, but there are some notable differences. Scientists still do not know a lot about how the amino acids of the ER protein form the specific lock required for ligand binding. Understanding how the amino acid structure of the estrogen receptor affects its function will help us develop more specific drugs to treat estrogen-related disease and also tell us more in general about how estrogen receptors, and all steroid hormone receptors, work.

Through the discovery of a new estrogen receptor in a fish, the Atlantic croaker (*Micropogonias undulates*) investigators (Hawkins et al., 2000) deduced that all teleost fish have three estrogen receptor genes, ER $\alpha$ , ER $\beta$ a, and ER $\beta$ b, which evolved from a single gene that duplicated in fish over 200 million years ago. Since that duplication event, the three ER genes have accumulated changes in their amino acid sequences that are strongly conserved across species (Hawkins et al., 2000). The fact that these specific changes have been conserved for such a long span of evolutionary time and in so many different species suggests they are very important to the different functions of the three ER subtypes (Hawkins et al., 2000). The investigators wanted evidence to support the hypothesis that the three estrogen receptors had different functions in fish. Since mammals, including humans, had recently been discovered to have at least two estrogen receptors, their findings could shed light on the role of multiple ERs in mammals too.

#### *The Teleost Estrogen Receptor Research Model*

Hawkins and Thomas (2004) demonstrate that the three Atlantic croaker estrogen receptors (acERs) have different binding affinities for many compounds and ask, do their different amino acid sequences explain their binding differences? In this paper, the investigators used site-directed mutagenesis to demonstrate that one of the amino acid changes in the fish ERs can explain their binding differences for two estrogenic compounds, DES and tamoxifen. Using the information in Hawkins and Thomas (2004) we can form additional hypotheses about the importance of amino acid changes to the differential binding profiles of the three ERs. We will use the site-directed mutagenesis protocol described in the Methods as a guide for designing our own experiments investigating how and why estrogen receptors can bind to different compounds.

In our experiments, we will test whether a specific amino acid position contributes to the particular binding characteristics of the acERs for a specific compound. To do this, we will mutate one of the diagnostic amino acids (Table 2 in Hawkins and Thomas, 2004), into the equivalent amino acid found in one of the other ER subtypes. This approach is called reciprocal mutagenesis. We will then investigate the binding abilities of our mutant ER to see if altering this amino acid also changed the receptor's binding characteristics.

## Protocol

### Activity 1: Hypothesis Formation, Step by Step

1. Open the Hawkins and Thomas (2004) paper on the computer and have your hard copy handy. In Table 1, do you see an interesting compound that has different binding affinities for at least one of the three ERs? Think “orders of magnitude” rather than smaller changes. List this compound in Table 1 and include the RBA% for each receptor. DES and TAM data from the Hawkins and Thomas (2004) paper are given as examples.

**Table 1.** Compound RBAs.

	<b>ER alpha</b>	<b>ER beta b</b>	<b>ER beta a</b>
DES	4898	96	315
TAM	25	1	4.8

- In your table, circle the value for the compound that you think is *most different*. If there is something mentioned in the Hawkins and Thomas (2004) paper about any of these values being unusual, consider this in your decision or just make notes off to the side. Remember that this is a fluid process! The fastest way to find information about your compound in the paper may be to use the *find function* in the PDF file.
- In the *Discussion* of the Hawkins and Thomas (2004) paper, look for any evidence from previous studies that suggest how particular amino acids in the ligand binding domain may play a role in the differential binding of your compound. You don't have to understand this evidence, just recognize that there is some relationship between your compound and this position. **Hint:** There are four amino acid positions we already know a lot about and are discussed in the paper. We call these positions *The Big Four*, and have provided them in the following table. When we discuss a particular position, we always use the human ER alpha amino acid and number (Column 1, Table 2).

**Table 2.** The Big Four amino acids.

<b>Human ER alpha AA #</b>	<b>Human (tetrapod) ER beta</b>	<b>acER alpha</b>	<b>acER beta a</b>	<b>acER beta b</b>	<b>Compound evidence?</b>
LEU 349					
MET 421	I	M	F	I	
TYR 526					
CYS 530					

- If any of the Big Four are implicated in the binding of your compound, indicate them in Table 2. The Big Four Amino Acids and in your hard copy of the Hawkins and Thomas (2004) paper. For example, on page 2975, there is a discussion of Met 421 and Cys 530 as amino acids that may be important for TAM and DES binding. We would mark these in the compound column of the Big Four table as potential positions to investigate for these compounds. Use Table 2 in the Hawkins and Thomas (2004) paper to help fill in the corresponding Atlantic croaker (ac) amino acids for each position. If any other amino acid positions in addition to the Big Four Table are thought to affect your compound, you can add them to the table if you like. Use the *find function* to search the PDF file for any discussions of your compound.
- Put the information together from the Hawkins and Thomas (2004) tables and the Big Four table to narrow down candidate amino acid positions to investigate. Are there amino acid *patterns* within The Big Four that match your compound's ER binding pattern? (Think *same/different*)? Are any of these positions highlighted on the Big Four table because they are discussed in the paper? If so, you have identified your amino acid position! List any candidates in the space below. If there are no good matches, you may need to look beyond the Big Four amino acids. Go back to Table 2 of the Hawkins and Thomas (2004) paper. Are there any other amino acid positions whose mutation pattern correlates with the binding data? List those candidate amino acid positions.  
  
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- Based on your observations and notes, as well as your personal interests, pick a single amino acid position to investigate and list it here. \_\_\_\_\_
- Once you pick your amino acid position, you must decide on your specific mutation. Will you change the ER $\beta$  amino acid to the ER $\alpha$ ? To the ER $\beta$ b? What do you predict will happen to binding as a result of this mutation? Write a brief pre-proposal with a specific hypothesis.  
  
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## Activity 2: Analyze the Data

### *Data Entry and Analysis for ER Competition Curves*

There are 30 students running assays in our lab each week. To process this amount of data accurately and efficiently, it is **ABSOLUTELY ESSENTIAL** that you follow these guidelines for labeling and arranging your assay tubes and samples. It is also essential that you input your data correctly into your individual data files as well as the group data template files on the lab computer. Failure to follow this procedure may make your data unusable. Please follow these instructions to ensure that you can analyze all your data and include it in your final poster presentation.

#### **1. Data entry.**

In your lab notebook, make a data table for each competition assay. Number all your assays sequentially with your initials as the prefix i.e. BH1, BH2, BH3... All samples should be on the test tube rack in the same arrangement for each assay. For example, your first two tubes will always be non-specific binding control tubes (NSB), the last two will always be total binding control tubes (TB), and your competitor concentrations will always go from most concentrated to least concentrated. The concentrations for your competitors will vary depending on which competitor you are investigating. You must always record these concentrations, even if you do the same ones each time. The range of concentrations for your competitors may change from assay to assay. You will record the scintillation vial numbers and DPMs from the data sheets you will receive after the assays are completed.

#### **2. Set up your assay tubes.**

Label test tubes with the assay number and tube #. Place labeled tubes on a test tube rack in the correct configuration.

#### **3. Add competitors to the assay tubes.**

#### **4. Add the lysate working solution to the assay tubes.**

#### **5. Prepare tubes for <sup>3</sup>H-E2 addition and charcoaling.**

Lab personnel will transport your assays to the radiation lab to add the radioactive tracer. You must submit your assays for tracer addition and data collection by filling out the daily data submission sheet. After placing your assay tubes in the 4°C, fill out the next empty row on the data submission form and record the row ID number in your lab notebook. Write the ID number **prominently** on the parafilm covering your tubes. We need to be able to see this number clearly while standing with the fridge door open! Do not remove another person's tubes from the 4°C without assistance.

#### **6. The next day, collect DPM data from the scintillation counter printout.**

The data sheets will be available approximately 24 hours after submitting them for processing. Timing will depend on the number of vials that need to be processed each day. The scintillation vial numbers on the data submission form will correspond to the SAM NO column on the DPM data sheet. Record the DPMs in your data notebook for each sample. Also record the time of charcoal addition and <sup>3</sup>H-E2 addition and any notes from the data submission sheet that accompanies your data.

#### **7. Input your data into your Excel data template.**

Take care to record all DPM values in the correct cells in your Excel sheet.

#### **8. Input your data into the lab prism file**

If your Excel template is formatted correctly, you should be able to cut and paste your data from Excel to GraphPad Prism (2011) without adding or removing any rows or cells. You will not get credit for assays that are not recorded in the lab PRISM file! This group file allows us to analyze data and share control assays easily.

## Materials

The materials and equipment required will vary depending on the research question chosen. The research question should be based on a single mass protocol with the possibility of many experimental variables that students can choose to test.

### Lab Space and Time Requirements

The Studio Laboratory must be available for students to come and go throughout the day. One lab supervisor in the lab 8-5 is adequate for our estrogen receptor lab. The lab supervisor can often be working on their own projects while students are working.

### Meeting Space Requirements

The students and lab supervisors need a space near the lab where students can meet, work on computers, eat and drink, and study while they are waiting on experiment steps etc. The meeting space is key to establishing a learning community for the students and giving them ownership of the program. It does not need to hold all the students at once.

**Table 3.** General semester outline and plan of work.

Week	Activities
1	<ul style="list-style-type: none"> <li>Pipette calibrations</li> <li>Pre-proposal workbook</li> <li>Scheduling with Google calendar</li> <li>Safety</li> </ul>
2	<ul style="list-style-type: none"> <li>“Dummy Data Run”</li> <li>PRISM/EXCEL workshops</li> <li>Pre-proposal meetings as needed.</li> </ul>
3	<ul style="list-style-type: none"> <li>Competition Assays I: Estradiol (E2) controls</li> <li>ORDER MUTANTS AND COMPOUNDS!</li> <li>Scientific literature search (proposal -specific)</li> <li>Optional: Help with compound dilutions.</li> </ul>
4	<ul style="list-style-type: none"> <li>Assays II: E2 and compound controls</li> <li>Biotech module</li> <li>pET manual worksheet</li> <li>Make LB media and plates</li> <li>Ongoing literature search</li> </ul>
5	<ul style="list-style-type: none"> <li>Assays III: Proposed compound controls</li> <li>Make LB media and plates</li> <li>Data analysis</li> </ul>
6	<ul style="list-style-type: none"> <li>Prepare for mutant expression:</li> <li>Transformations</li> <li>“Streak out” transformed mutants onto plates.</li> <li>Mutant ER expression in bacteria</li> </ul>

7	<ul style="list-style-type: none"> <li>“Streak out” transformed mutants onto plates.</li> <li>Mutant ER expression in bacteria</li> </ul>
8	<ul style="list-style-type: none"> <li>Mutant expression</li> <li>Lysis of bacterial preps</li> <li>Saturation curves begin</li> </ul>
9	<ul style="list-style-type: none"> <li>Lysis of bacterial preps <i>cont.</i></li> <li>Saturation curves <i>cont.</i></li> <li>Data analysis PRISM and EXCEL</li> </ul>
10	<ul style="list-style-type: none"> <li>Mutant competition assays I: E2 and compounds</li> <li><i>NOTE: All mutant assays (Two E2 and three compound) due Tuesday Nov 25.</i></li> </ul>
11	<ul style="list-style-type: none"> <li>Mutant competition assays II: E2 and compounds</li> <li>Data analysis</li> </ul>
12	<ul style="list-style-type: none"> <li>Mutant competition assays III: E2 and compounds</li> <li>Data summary</li> </ul>
13	<ul style="list-style-type: none"> <li>Mutant competition assays IV: E2 and compounds</li> <li>PRISM analyses</li> <li>Data summary EXCEL sheet</li> </ul>
14	<ul style="list-style-type: none"> <li>Data analysis, assay reruns and posters.</li> </ul>
15	<ul style="list-style-type: none"> <li>Poster work, background research, and data analysis</li> <li>Assay reruns with consent of instructor</li> </ul>
Final Exam	<ul style="list-style-type: none"> <li>Poster symposium</li> </ul>

## Notes for the Instructor

Research questions that fulfill the following key criteria are suitable for the Studio Lab model:

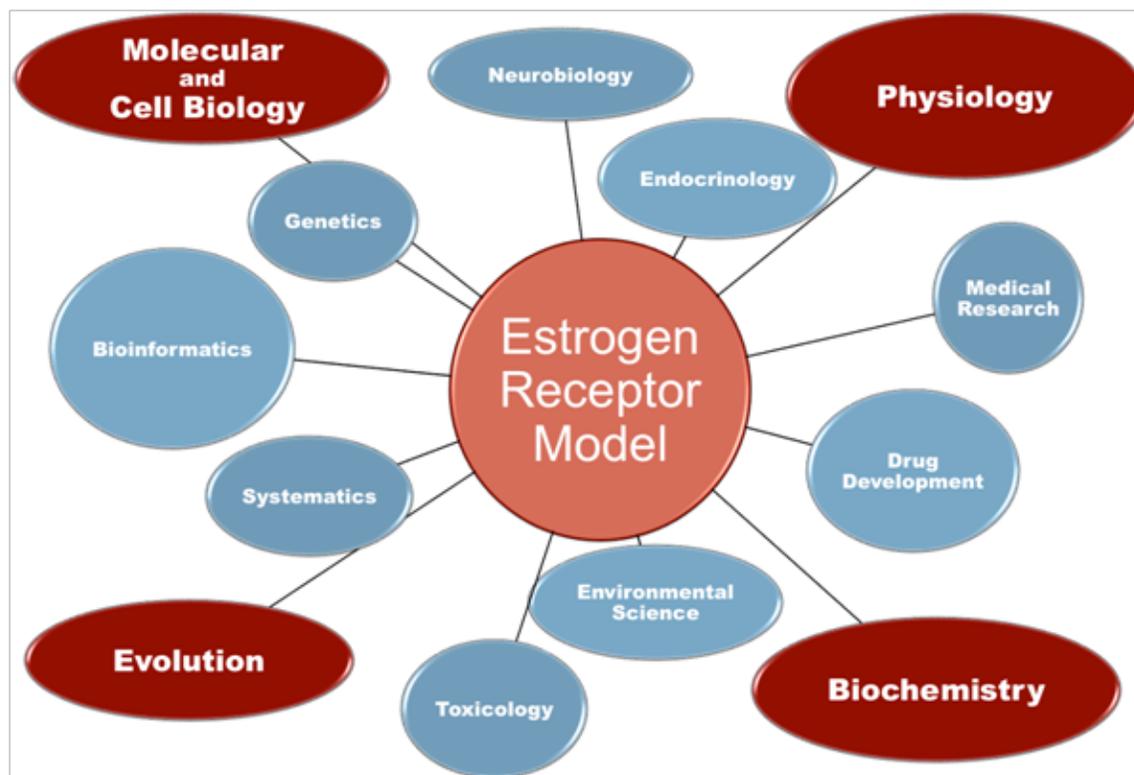
1. Original research worthy of publication and funding.
2. Unifying theme with many possible directions (Fig. 1).
3. Common techniques, equipment, buffers, i.e. a “Mass Protocol” (Fig. 2).
4. Robust protocols that require little troubleshooting.
5. Results achievable in 10 weeks or less.

## Challenges

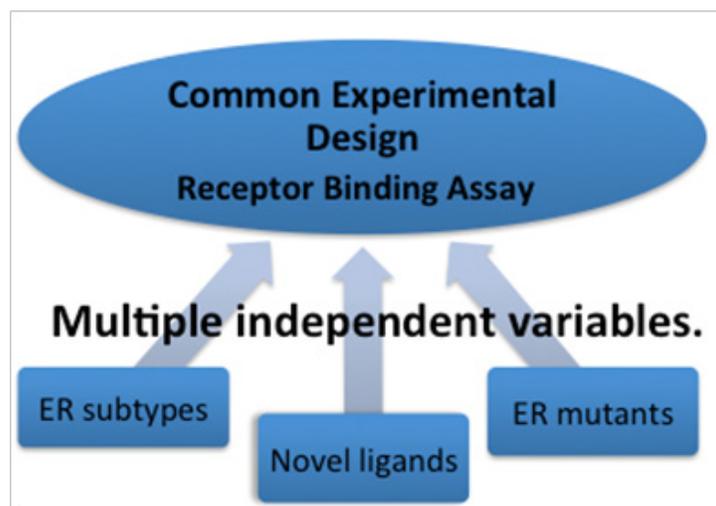
One of the biggest challenges for both instructor and student is the reluctance to start doing the science before it is fully understood. This was illustrated in the ABLE 2013 Workshop by the Pre-proposal Activity (Activity 1). Although additional information was not required, several workshop participants were very uncomfortable forming a

hypothesis without first searching the Internet for more information. Similarly, the novice sophomore students are often reluctant to create a hypothesis because they “don’t know anything about these compounds or how they bind to receptors”.

Time management is difficult for lower classmen who have many 3-hour labs and little flexibility in the planning of their schedule. We solve this problem by discussing the time expectations each week and creating a general plan before the students leave on Monday morning. Students write up their plans on the white board, and we use Google calendar, text messaging and e-mail to communicate. Adding more evening hours for the lab would help to solve this problem.



**Figure 1.** Studies of ER structure and function can lead students in many directions.



**Figure 2.** PackTrack students use the same experimental protocol but choose novel independent variables to investigate.

It is a common misconception that the Studio Lab model requires many more resources per student credit hour than a traditional laboratory. However, Research PackTrack requires fewer FTEs per student credit hour than our large biology laboratories. Also, the considerable infrastructure and weekly preparation of traditional labs is not needed for a Studio Lab. Although the lab is open all day, lab supervisors can often be productive while they are teaching, and the course requires fewer instructors overall. In addition, the value to the students far exceeds that of a traditional biology lab course, and the success of RP students after they complete the program has earned the support of NCSU faculty and administrators.

### Activity 1: Write a Pre-Proposal

In this activity, students will work through the process of forming a hypothesis and writing up a short pre-proposal. Students need a lot of help with putting together the material from scientific papers and forming a hypothesis. We have found that this assignment requires a large time investment by instructors at the beginning of the semester. We exchange emails of rough drafts and schedule small workshops with groups of 4-6 students where we look over the papers together, discuss posters with data that are displayed in the lab, and search the internet for how to link compounds with student interests. For example, a student who is interested in human health will discover that tamoxifen treatment for breast cancer may increase a patient's chances of getting Parkinson's disease. A student who is interested in endocrine disruption will find that genistein found in soy is prevalent throughout the environment and may be causing deleterious effects in organisms. The students submit a pre-proposal early in the semester so that we can produce their mutant while they conduct control assays and work on their research paper. The pre-proposal is a very informal document that is centered on ideas, not style. At this point, students do not understand the ER model system or the experiments, so we use generalizations and analogies to walk them through the process.

### Activity 2: Analyze the Data

Keeping track of the samples and data generated by dozens of undergraduate students is a daunting task, but if you structure this process carefully, you can save enormous amounts of time and feel more confident about the integrity of the experimental results.

When analyzing samples and data created by a large number of students, it is essential to standardize these processes. This is true even if lab supervisors do not perform any portion of the protocol, because it eliminates the considerable time required to properly identify samples or interpret lab notebooks when answering student questions, providing materials for subsequent steps, etc. Standardization of a *mass protocol* also makes it possible to quickly scan the lab for potential problems with student technique. In addition, reading instructions completely and following them precisely is

an important learning objective. A surprising number of students struggle with this seemingly simple task, leading to much frustration and time loss by the lab supervisors. We emphasize to students that in large research laboratories, there are very rigorous protocols, some required by law, for moving and storing samples. We show the students real life examples, such as Chain of Custody forms or online DNA sequencing facility submission forms to emphasize this point. However, despite our repeated written, graphic and spoken instructions, conscientious students will fall short here, so it is important to make your procedures very clear and enforce them consistently.

Likewise, a universal data spreadsheet format that all the students must use allows the lab supervisor/researcher to pool class data sets for analyses with minimal effort. This also reduces data entry mistakes because the data is first typed into the student's personal data sheet where it can be reviewed before it is transferred all at once into the group data spreadsheet.

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## About the Authors

Dr. Mary Beth Hawkins' research focuses on the functional evolution of estrogen receptors and endocrine disruption in teleost fishes. She is also a dedicated educator who is passionate about introducing young people to the creative world of life science research. Her goal is to develop and promote practices that foster a positive relationship between the scientific teaching and research communities. In addition to developing and directing the Research PackTrack Program at North Carolina State University, Dr. Hawkins teaches undergraduate and graduate courses in Physiology. She has received numerous *Thank a Teacher* awards from the student body and is an Outstanding Advisor Award recipient. She is currently President of the Carolinas Regional Chapter of the Society of Environmental Toxicology and Chemistry. Dr. Hawkins received her B.S. degree from The University of Texas at Austin and her Ph.D. from The University of Texas at Austin Marine Science Institute.

Dr. Miriam Ferzli is a Teaching Associate Professor whose approach to teaching focuses on giving students an active role in the learning process. She strives to provide them with the opportunity to help shape learning activities so that they de-

velop a sense of ownership about the learning process. Her mission is to help students attain strong academic skills by helping them understand what it means to be a scholar—how to ask questions, seek answers, solve problems, and work within a community of teachers and learners.

A large majority of Ferzli's research focuses on developing methods that aim to increase student engagement in the authentic practice and discourse of science. Over the past years, Ferzli has taken the lead in issues dealing with teaching effectiveness, earning the title of *Alumni Distinguished Undergraduate Professor*. She has been awarded the National Award for Excellence in Teaching from the Association of Public and Land-grant Universities and is a recipient of both NCSU's University Outstanding Teaching Award and Outstanding Adviser Award. She is also an Education Fellow for the National Academies of Science and a member of the Academy of Outstanding Teachers. Dr. Ferzli received B.S. and B.A. degrees from the University of Miami and her master's and Ph.D. degrees are from North Carolina State University.

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## Appendix

### Pre-Proposal Guidelines

This is a short, informal statement of what you propose to work on this semester. This will allow us to plan your experiments and obtain your compounds and mutations so you can begin your project. You will expand this statement into a complete research document with *Abstract*, *Introduction*, *Methods* and *Discussion* later on in the semester.

### Pre-proposal: Half page or less

Consider these questions while formulating your hypothesis:

1. What is estrogen? Where/when is it released and why?
2. What are estrogen receptors? How do they work? What do they do?
3. What about the estrogen receptors of the Atlantic croaker make it a good model system?
4. How do estrogens and other compounds interact with the ER?
5. What is the impact of your study on future research efforts? (Why is this important?)

Directly address the following questions in your pre-proposal:

1. What is your compound of interest?
2. Why are you investigating your compound of interest?
3. What do you know (if anything) about how your compound interacts with ERs?
4. What amino acid position are you investigating?
5. Why did you choose this amino acid position? Was it proposed as important to the binding of your compound to ERs by previous researchers? Do the amino acid substitutions in the three fish ERs at this position correlate in some way with the binding profiles for your compound?

For example, "Compound A binds to ER alpha and ER beta B in a very similar way, but it binds to ER beta A differently. I propose changing the ER beta A amino acid, V317 to M, because both ER beta b and ER alpha have an M at this position, while ERβa has changed this amino acid to V. I think this change may explain the difference in binding seen for compound A."

Is there another reason for your specific choice?

6. Based on your answers above, what are your hypothesis and predictions?

### Sample Student Abstracts

#### *Abstract 1*

Estrogen receptors (ER) are intracellular proteins that promote gene transcription when activated by binding to estrogen. ERs play critical roles in the development and function of many tissues, including those of the reproductive, cardiovascular, and nervous systems. There are multiple ER subtypes (ER $\alpha$ , ER $\beta$ ) in vertebrates, with differential binding affinities and trans-activation properties for estrogens and estrogenic compounds. Genistein is an estrogenic compound found in soy that binds to ER $\beta$  subtypes with a higher affinity than ER $\alpha$ . It has been proposed that the amino acid change of Met421 in the ligand-binding domain (LBD) of human ER $\beta$  to Ile in ER beta may be responsible for this difference. Fish have three estrogen receptors, ER $\alpha$ , and two ER beta subtypes, ER $\beta$ a and ER $\beta$ b. Like mammalian receptors, fish ER betas have an amino acid substitution at Met421 and a higher binding affinity for genistein. To examine the role of this amino acid position in the binding of genistein to ERs, I will mutate the Ile at the equivalent position in the Atlantic croaker ER $\beta$ b (acER $\beta$ b) to Met and perform competition-binding assays using bacterially expressed ER mutant proteins (Human ER $\beta$  M421). I predict that the mutation of ER $\beta$ b Ile to Met will increase the binding affinity of the receptor for genistein.

#### *Abstract 2*

Raloxifene, a SERM, binds to estrogen receptors and can inhibit estrogenic actions. There are three different subtypes of estrogen receptors in fish (ER $\alpha$ , ER $\beta$ a, and ER $\beta$ b) and they bind differently due to different amino acids in their ligand binding domains. For example, Raloxifene has a higher affinity for ER $\beta$ a than it does for ER $\alpha$ . I propose the mutation of ER $\beta$ a M to C (Human ER $\alpha$  C530), and predict that this will reduce ER $\beta$ a's binding affinity for Raloxifene to be similar to that of ER $\alpha$ .

*Abstract 3*

Estrogenic compounds act as hormones that regulate cell growth and function in several key systems of the body including the reproductive, cardiovascular, and bone maintenance systems. Estrogenic ligands bind to estrogen receptors within target cells, forming complexes that bind to DNA and regulate gene transcription. Teleost fish have three distinct estrogen receptors, ER $\alpha$ , ER $\beta$ a, and ER $\beta$ b, each with its own spectrum of ligand affinities. For example, the antiprogestin RU486 binds to Atlantic croaker (ac)ER $\beta$ a with a higher affinity than to acER $\alpha$  or acER $\beta$ b. These data agree with findings for human ERs where RU486 has a higher affinity for ER $\beta$  than ER $\alpha$ . These differences in binding affinity are attributed in part to amino acid substitutions in the ligand-binding domain of teleost ERs. To better understand the nature of RU486 interactions with ERs, I will mutate the acER $\beta$ a Phe396 amino acid to the corresponding Met found in ER alphas (human ER $\alpha$  Met421) and perform competition binding assays using bacterially-expressed ER $\beta$ a(F-M) fusion proteins. I predict that the relative binding affinity of RU486 for acER $\beta$ aF-M will shift towards that for acER $\alpha$ . This shift in binding affinity after changing a single amino acid would suggest a critical role for this position in the interaction of RU486 with teleost ERs.

**Some Guidelines for Using GraphPad Prism (2011)***1. To Copy And Paste Your Data From Excel.*

Folder: Data with Results > File: Competition Template

Notes about inputting data:

- a. **Be sure that your DPM data is pasting into the correct row in PRISM.** It should be in the row labeled with the nM concentration of competitor you used in your assay. Some people will need to add rows for unusual data points. If you do so, you will also need to add rows to your Excel file so when you copy and paste, the data will be entered properly,
- b. You will need to manually add in names of the assays. It is important for graphing that the names are inputted properly.

*2. Check The Assay Names on Analysis and Results Sheets.*

Folder: Data with Results > File: TB-NSB=SB

Go to this file and check the names. Some may not transfer over from the Competition Template. Enter names as needed. I do not know why this happens, it is a program glitch.

*3. Look at Your Graphs.*

Folder: Graphs

Graphs: the PRISM template file default settings have curves 1-6 on Graph 1, 7-14 on Graph 2, *etc.* You may change the appearance and data on these graphs as needed.

*4. To Change the Data Shown on a Graph.*

- a. Select the graph you want to change so that it is in the visible window.
- b. Go to the pull-down menu at the top of the page labeled Change.
- c. Select Remove/Replace Data sets: The data that is currently on your graph will be listed. To add data, select "Add". All the data sets not on your graph will appear. Select those you want to add.
- d. Note: You will only add data from the following two files!
  - Transform: Final M to Log (M)
  - Results 3 Nonlinear regression (fit)

*5. To Change the Appearance of the Data on a Graph.*

- a. Select the graph you want to change so that it is in the visible window.
- b. Go to the pull-down menu at the top of the page labeled Change.
- c. Select Remove/Replace Data sets: The data that is currently on your graph will be listed. To change the appearance of data, select appearance. All the data sets on your graph will be in the pull-down menu. Select the data you want to change.

Note:

- Final M to Log (M) are the individual data points on the graph. Select “Show Symbols”, “Show Error Bars”, and De-select “Show Connecting Line/ Graph”. Select Show Legend.
- Results 3 Nonlinear regression (fit) is the calculated curve on your graph. Select “show connecting line/curve” only. De-select “Show Legend”.