Laboratory Exercise

Simulated Sandwich Enzyme-Linked Immunosorbent Assay for a Cost-Effective Investigation of Natural and Engineered Cellular Signaling Pathways[S]

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Abstract

The ability to separate, identify, and quantify proteins from complex mixtures are key foundational methods across biochemistry teaching and research. In particular, enzyme-linked immunosorbent assay (ELISA) is an important technique that is used to measure antigen concentrations in both industry and academia. There are four categories of ELISA, direct, indirect, competitive, and sandwich, each with their own applications. Sandwich ELISAs are used to determine antigen concentrations from complex mixtures of protein, such as a cell lysates, and are regularly used as medical diagnostics to diagnose illness and diseases ranging from hepatitis to celiac disease. One major problem with teaching the sandwich ELISA technique to students is the prohibitive cost due to the need to coat a 96-well plate with a capture antibody. One solution to this problem would be to significantly reduce the role of each student in the lab, but this does not adequately prepare students to perform the procedure in a research or industry lab. Instead, this laboratory exercise teaches students the procedural knowledge needed to perform a direct sandwich ELISA, but uses a simulated experience performed within a wet-lab environment. The presented scenario is the analysis of phosphorylated proteins within a synthetic signaling pathway, but because the lab uses simulated samples, it can be tailored to different topics and educational aims. The procedure is 10- to 26-fold less expensive per student to deploy than an authentic sandwich ELISA. Students in the course report that the ELISA lab significantly strengthened the connection between theory and practice. © 2019 International Union of Biochemistry and Molecular Biology, 00(00):1–7, 2019.

Keywords: *ELISA; laboratory exercises; biotechnology education; synthetic biology; signaling pathways*

Introduction

The ability to separate individual proteins from complex mixtures of proteins extracted from cells is a foundational technique in many areas of laboratory research. In particular, the ability to measure protein presence and abundance within cells is critical for investigating the signaling pathways of cells. A suite of methods currently exists to measure protein from cellular samples, with each having a unique set of strengths and weaknesses.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is able to separate proteins out of a complex mixture, but it lacks the resolution to completely isolate each of the ~10,000 different types of proteins in a human cell [1]. To achieve the resolution required in order to measure the presence/absence and quantitative amount of a given protein in a human cell, SDS-PAGE needs to be combined with another technique called Western Blotting. By attaching separated proteins onto a membrane, antibodies can be used to specifically and sensitively pick out a given protein of interest from the separated mixture of protein. A complementary method to Western blotting is the enzyme-linked immunosorbent assay (ELISA). Whereas with Western blotting, the migration distance of the protein of

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The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-activated protein kinase; ERK2, Extracellular signal-regulated kinase 2; TMB, 3,3′,5,5′-tetramethylbenzidine; PBS, phosphate buffered saline.

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interest through the SDS-PAGE gel is used as a proxy for protein size, this size information is lost in the ELISA. Where the ELISA test excels is in the linear range of quantitation (up to six orders of magnitude [2]), whereas Western blotting only has about three to four orders of magnitude dynamic range [3, 4].

Several techniques for measuring protein are also commonly used within the diagnostics and testing industries. For example, the CDC-endorsed Lyme disease testing protocol uses a two-tier approach with an initial ELISA test, which if positive, is followed by a Western blot test for presence of antigens [5, 6]. Lyme diagnosis is positive if both the ELISA and Western Blot are positive. Human immunodeficiency virus (HIV) diagnosis also proceeds through a similar pathway, with serial ELISA and Western blot tests ensuring low false positive/negative rates [7].

This laboratory exercise was designed as part of a suite of labs offered in a third-year undergraduate Cell Biology and Biochemistry course. The ELISA laboratory experiment serves several teaching purposes within a third-year biochemistry and cell biology course. We have designed the lab to explicitly teach the procedural knowledge needed to perform a sandwich ELISA. We have done this because this technique is commonly used in both industry and academic settings. Second, we teach the ELISA laboratory in Week 7 of our course, following several labs on SDS-PAGE and Western Blotting. In this way, we are able to explicitly link the new ELISA material with the prior knowledge of features of protein identification, such as mass and specific interaction with antibodies, from our own course [8]. The ELISA lab allows us to link and discuss the similarities and differences between ELISA and SDS-PAGE/Western Blotting in the context of the uses of protein mass as well as identification using a specific interaction with a known antibody. For example, we teach that ELISA is similar to Western blotting in that we identify an antigen by specific protein–protein interaction with an antibody with known binding-specificity, but unlike Western Blotting, ELISAs do not contain the second piece of information useful in identifying an antigen, protein mass. We also explicitly link the prior knowledge the students gained in the previously performed Western Blotting labs where we observed our antibodies having nonspecific interaction with proteins that did not match the expected mass of the antigen. In this way, we are reinforcing prior learning that multiple pieces of information are useful to identify unknowns and that without this knowledge (in the ELISA) we may miss measuring something important, or our measurements may be skewed by nonspecific effects.

The ELISA laboratory exercise also supports student learning by enabling the creation of richer knowledge organizational hierarchy around protein identification and quantification. The laboratory demonstrates to the students that there are multiple techniques that can be used for protein quantification using antibodies, for instance, and that each has pros and cons and was developed in a different historical problem-solving environment. Whereas the Western blot was developed to detect specific proteins from a complex mixture, with less regard for quantitative measurement of the protein [9], the ELISA was developed specifically to quantify the target protein from a mixture using nonradioactive detection [10, 11].

Practical Issues of the Course

The laboratory skill taught here is a simulation of a sandwich ELISA. Direct and competitive ELISA laboratory exercises have already been detailed previously [12]. The sandwich variant of the ELISA assay is important for students to learn because it is the basis of common research and commercial diagnostic protocols. For example, the Abbott PRISM platform that provides automated Hepatitis B and HIV diagnostic tests, along with the commonly used HIV-1 urine enzyme immunoassay test (Maxim HIV-1 urine enzyme immunoassay), all use the sandwich ELISA method [13]. Unfortunately, the sandwich ELISA is the most expensive of the ELISA variants due to the need to coat the detection plates with antibody rather than antigen, and it is currently prohibitively expensive to offer a real sandwich ELISA experience to undergraduates with current teaching resources. One way around this problem would be to have each student only load one well of a larger sandwich ELISA experiment, but this experience does not adequately prepare the student to perform a sandwich ELISA experiment in a research or industry lab. I therefore designed this laboratory experiment and experience to simulate the actual setup a technician or graduate student would utilize in the "real world." Calculations of relative cost show that the simulated method described in this article is 10-fold less expensive per student than an authentic self-coated sandwich ELISA (not including labor), and 26-fold less expensive than a sandwich ELISA kit (Supporting Information Table S1). The method of simulated ELISA was based on a previously described method [14] with additional modifications for modern reagent availability and for the requirements of our specific experiment, as detailed below. Simulated or simplified experiments have been shown before to be effective learning experiences [15–17].

Overview of the Procedure

In the current form that the laboratory exercise is offered, students are presented with a scenario based on developing an experimental strategy to measure the amount of phosphorylated ERK2 protein produced from a human map kinase pathway that has been transplanted in yeast and is under inducible control (Fig. 1) [18]. This scenario was chosen because it linked several different topics (bioengineering and cellular signaling pathways) presented concurrently in the lecture portion of the course. Because the lab is based

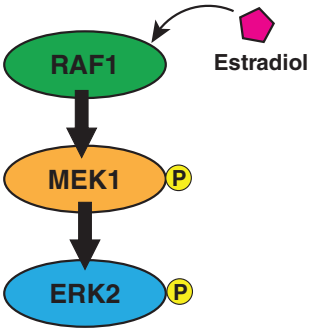

FIG 1 *Synthetic map kinase pathway schematic. The RAF1 enzyme was engineered to turn on kinase activity in the presence of estradiol inducer molecule. Activated RAF1 phosphorylates MEK1. Phosphorylated MEK1 phosphorylates ERK2. [Color figure can be viewed at wileyonlinelibrary.com]*

on a simulation, the choice of scenario is essentially limitless and can be adapted to suit the material currently being presented in a course. The focus for this laboratory exercise was on the process of selecting appropriate samples to measure based on anticipated or hypothesized results and on learning the procedures necessary to successfully perform the sandwich ELISA laboratory technique.

Students worked in groups of two. This choice was mainly done for logistical reasons, since there was a limited amount of time to measure the plates in our lab and we had 64 students and only one ELISA plate reader. The entire lab exercise was performed within one 3-hour block. Assuming it would take ~1-min to measure each plate, we estimated we would have had to spend at least 1-hour of the 3-hour lab measuring plates. Because we estimated it would take the fastest students 2 hours 35 minutes to complete the procedure (Fig. 2), we decided that groups of two would give the best balance between hands-on time for each student and available lab time. Students also generally enjoyed working in groups and we found in the past that a significant amount of student-to-student (peer) teaching was done within groups.

Students prepared for the laboratory by reading background material provided by the instructor in the form of "Student Notes" that included information on the: 1) addition and removal of phosphoryl groups (PO_3^-) from proteins by kinases and phosphatases, 2) the map kinase (MAPK) pathway, and 3) suite of ELISA techniques, with emphasis on the sandwich variety. Students were also presented with figures and descriptions of MAPK pathways under different activation regimes and response dynamics (Fig. 3 and Supporting Information Fig. S1) and asked them to select a limited subset of the samples to measure that would enable them to capture all the dynamics of the system. For example, if one was only able to sample six data points for the examples in Fig. 3, one would need to pick points evenly spread across the region of greatest change (highlighted in green in Fig. S1). This pre-lab exercise was designed to

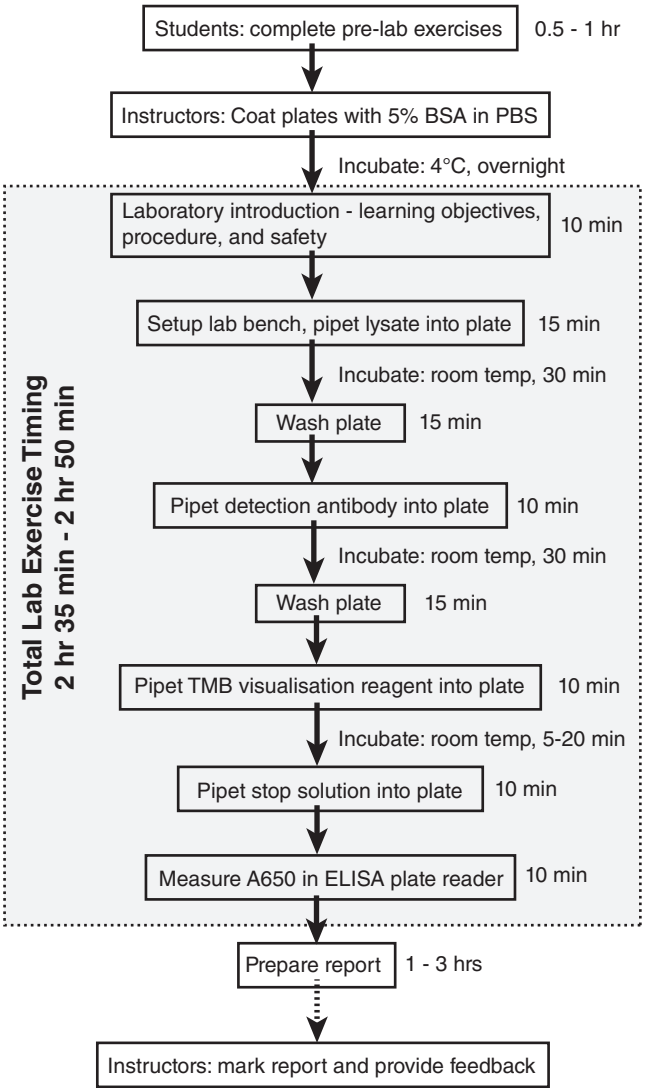

FIG 2 *Flowchart of steps and estimated timing in laboratory exercise.*

prime/prompt students to think about several concepts common in laboratory experiments, including sampling density and its dependence on expected phenomena dynamics, limited time and reagents (i.e. you cannot measure everything), and the possible dynamics of the MAPK pathway.

Prior to the lab class, the 96-well plates were coated with 200 μL of 5% BSA in phosphate buffered saline (PBS), covered with cling wrap, and stored overnight at 4 °C. See Supporting Information for technician notes. Note, to save on reagents and technician time, we only coated the wells that would be used for the laboratory. In our case, we coated columns 1–12 for rows A and B only (24 wells total). The next day the BSA solution was removed and the wells were washed twice by filling the wells with 200 μL PBS and discarding. The last remaining liquid in the wells was removed by gently tapping the plate upside down on some

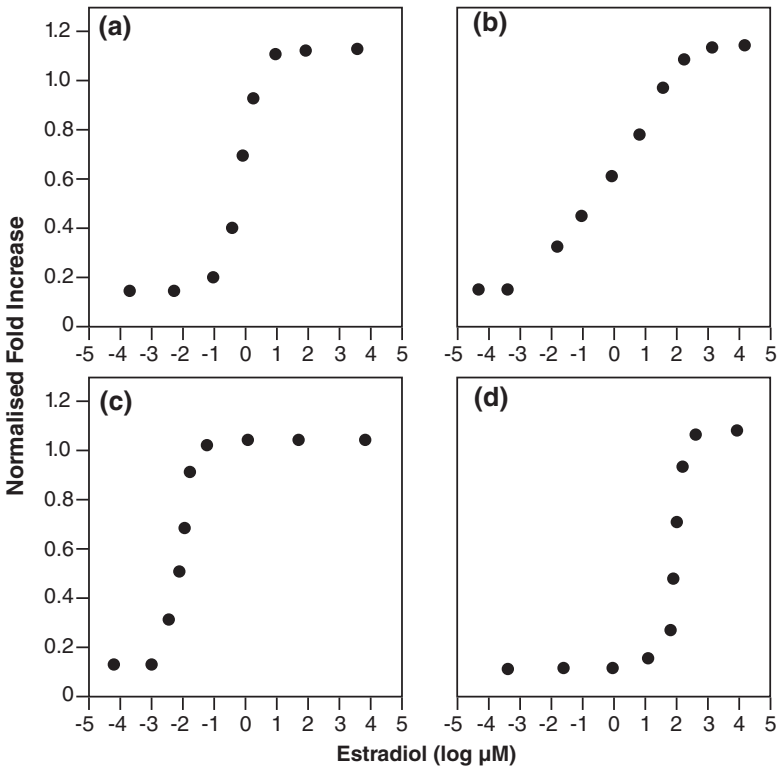

FIG 3 *Measurements of output from example synthetic MAPK pathways under different activation regimes and response dynamics. Within the presented scenario, each point represents the fold-increase of phosphorylated ERK2 due to estradiol-activation of a human MAPK pathway cloned within a yeast cell, based on a previously published pathway [18].*

paper towels. We cling wrapped the plate again and kept at 4 °C until needed. We only kept the plates in the 4 °C for 3 days prior to the lab exercise.

The lab class started with a short presentation outlining the basic idea behind the lab and a short discussion of the experimental steps needed to complete the lab. Additionally, safety and common pitfall advice was discussed. The student groups then selected yeast lysate samples that had been induced with various amounts of estradiol. The simulated estradiol-induced cell lysates in this method, based on prior methods [14, 19], are actually dilutions of an anti-BSA antibody. A range of primary antibody dilutions was made up in PBS prior to the lab exercise to simulate yeast cell lysates from cells that had different concentrations of estradiol added. Hereafter, the primary antibody dilutions will be referred to as the "lysate."

The students selected eight lysate samples based on their pre-lab work estimating which samples would be most informative over the sampling window. They also received one pre-coated ELISA plate, a single-channel micropipette with volume range 10–200 μL, waste container, tip trash, 20 mL PBS plus Tween® 20 (PBS-T) wash solution, and timer. The students planned out how they would load the eight antigen-containing solutions in triplicate into the 24 available wells. One example plate-loading pattern is shown in Supporting Information Fig. S2. Teaching assistants checked and provided feedback to the student well-loading plans before they attempted the experiment.

The students then added 200 μL of each lysate to the appropriate wells of the ELISA plate. The plates were incubated at room temperature for 30 minutes to allow antibody–antigen association. Following incubation, the lysate was removed by dumping the ELISA plate upside down onto a stack of paper towels and tapping gently. The plate was washed by filling each well with 200 μL PBS-T followed by turning the plate upside down onto the same stack of paper towels. This wash was repeated once for a total of two washes.

The next step in a sandwich ELISA is to add the detection antibody coupled to horseradish peroxidase to enable visualization of the capture antibody–antigen-detection antibody "sandwich." The simulated detection antibody in this method is actually an anti-rabbit secondary antibody conjugated to HRP, hereafter referred to as the "detection antibody." Following the lysate incubation and wash, the students added 200-μL detection antibody to all 24 of the wells. The plate was incubated at room temperature for 30 minutes. Following incubation, the detection antibody was removed by dumping the ELISA plate upside down onto a stack of paper towels and tapping gently. The plate was

washed by filling each well with 200 μL PBS-T followed by turning the plate upside down onto the same stack of paper towels. This wash was repeated once for a total of two washes.

To visualize the quantitative binding of the detection antibody to the captured antigen, the students added 100 μL 3,3′,5,5′-tetramethylbenzidine (TMB) substrate to each well. TMB is oxidized to 3,3′,5,5′-tetramethylbenzidine diimine by HRP, producing a blue color that absorbs strongly at 650 nm. Because the students were using single-channel micropipettes, they started timing at the first addition of TMB to the first well and tried to add the TMB to succeeding wells in a consistently timed manner. Although the kinetics of the reaction are not so quick as to change significantly on the second time-scale, longer delays on the minute time-scale could affect the final result. The students incubated the plate at room temperature for 10–20 minutes, watching for blue color development. When the blue color had sufficiently developed, or 20 minutes had passed, whichever was sooner, the students added 100 μL stop solution (0.1% sodium fluoride solution) to each well, starting with the first well and proceeding to the last well. Stop solution addition was meant to occur at the same pace as the addition of TMB to ensure each well was exposed to the TMB substrate for a similar amount of time. After stop solution addition, the blue color was stable for at least 1 hour afterward. The students then took their plate to the microplate reader and the teaching assistants showed them how to measure the plate. A spreadsheet file containing one measurement of absorbance at 650 nm for each well on the plate was returned to the students the following day via an online portal.

Assessment of Student Learning

The ELISA laboratory exercise learning objectives were:

1. Recall from lecture the process and mechanism of protein phosphorylation.
2. Recall from lecture the structure of the MAPK pathway.
3. Define and describe ELISA.
4. Relate different protein separation and identification techniques.
5. Discuss and relate student laboratory exercises to "real-world" commercial and academic uses of ELISA.
6. Demonstrate proficiency with ELISA method.
7. Record experimental procedures performed and identify any deviations from standard protocol.
8. Record data from experiments.
9. Practice using micropipettors.
10. Employ advanced scientific software to graph and analyze data.
11. Construct a Hill-plot comparing relative A650 reading for each sample to estradiol-induction.
12. Calculate EC_{50} and Hill-slope data from a Hill-plot.
13. Report ELISA results within context of experimental objectives.

Student understanding of the major concepts of the experiment was assessed through a written report. Questions included those designed to assess student understanding of the objective of the experiment, recorded details of any variations to the experimental plan, identification of experimental variables held constant and those varied, and extrapolations from the results of this experiment to more general features of the MAPK (or other) signaling pathway.

Some example questions included "Describe the objective of this experiment," "In your experiment, identify: 1) the independent variable, 2) the dependent variable, and 3) one controlled variable," "Did your group have any variations from the written protocol plan? If so, please describe," "We did not use a positive control in this experiment. Identify one positive control that we could have used."

The report also included analysis of the absorbance (650 nm) data collected from the student's ELISA plate. In the simulated scenario, the A650 values represent the amount of phosphorylated ERK2 in each sample. The student's objective in graphing their data was to determine the EC_{50} value and Hill-Slope value for the human MAPK pathway response to RAF1 induction (Fig. 1). In our laboratory exercise the EC_{50} value represents the half-maximal response of ERK2 phosphorylation due to RAF1 induction, and the Hill-Slope represents ultrasensitivity of the reaction.

To perform this analysis, the students created a spreadsheet containing 24 measurements of absorbance (650 nm), one for each well (sample) of the ELISA. The students then graphed the normalized fold-increase of the amount of phosphorylated ERK2 protein versus estradiol/inducer concentration of the culture. To successfully create the graph, the students needed to transform the data to relate the A650 measurements to the different amounts of estradiol inducer added to the system. To setup the y-axis data, averages and standard deviations of biological triplicates were calculated. To setup the x-axis data, the students needed to log-transform the inducer concentration (in μM) and normalize the A650 measurements against the lowest estradiol sample's average A650 measurement. These transformations are standard data analysis and data cleanup procedures that are frequently used in academic research labs. Instructions on how to accomplish the transformations were provided to the students. The students graphed their transformed data using GraphPad Prism software's nonlinear regression (curve fit), sigmoidal dose–response (variable slope, Hill-plot) analysis function. The software then derived an EC_{50} value and Hill-slope value from the fitted line. Other options for similar data analysis through free software exist [20]. Representative student data (Fig. 4) show that the experimental samples can be reliably measured by the students to produce a curve simulating the

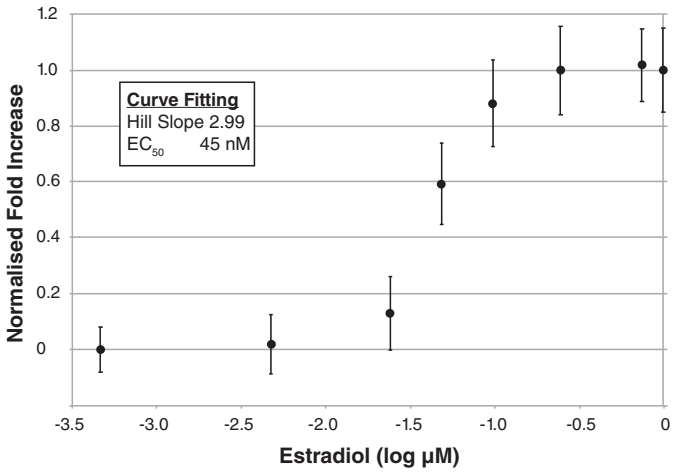

FIG 4

Representative ELISA student data. The samples were simulated to be a yeast strain containing human MAPK pathway that was activatable with estradiol, based on a previously published pathway [18]. Within the presented scenario, each point represents the fold-increase of phosphorylated ERK2 due to estradiol-activation of a human MAPK pathway cloned within a yeast cell. Each point represents the mean of measured absorbance of oxidized TMB substrate at 650 nm for a given sample, normalized against the lowest reading. Error bars represent one standard deviation (n = 27). Curve fitting data generated from student data using Prism 8 (GraphPad) using nonlinear regression (curve fit) analyses with sigmoidal dose–response (variable slope) parameter.

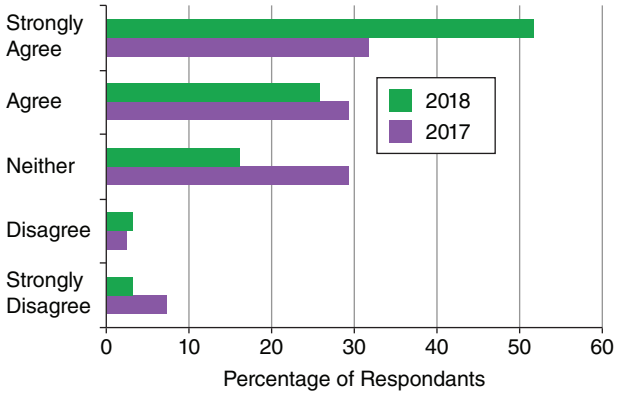

FIG 5

Increased proportion of positive student responses to question "The laboratory sessions helped me to link classroom/textbook theory and practice" before and after implementation of ELISA laboratory exercise. Students were asked question as part of standard anonymous paper survey during course. Number of survey respondents in 2017 (n = 41) and 2018 (n = 31). [Color figure can be viewed at wileyonlinelibrary.com]

desired system response, in this case the dynamics of estradiol-stimulation on phosphorylated ERK2 proteins in a synthetic MAPK pathway.

Some example questions from the analysis part of the report included: "Graph your results using the instructions on the following page. Put an image of your graph below," "What was the EC_{50} value for your experiment?," "What can you say, from your experimental results, about the response of the synthetic MAP kinase pathway to estradiol? Is the amount of phosphorylated ERK2 sensitive to estradiol concentration? Explain why or why not with specific reference to features from your graphed data," "If you were able to do the experiment again, would you try lysates induced with different concentrations of estradiol than you used? If so, what would they be? Why did you choose those values?

Several student pitfalls were observed during the laboratory exercise. The main one seemed to involve either inconsistent pipetting technique, accidentally pipetting the wrong volumes of lysate, or adding the TMB and stop solutions at inconsistent pace. These experimental events that deviated from protocol tended to result in data points that were outside of the expected values and resulted in curve fitting with EC_{50} and Hill-slope values ranging widely from expected. The second type of pitfall involved over-incubating the TMB. If the incubation went any longer than 20 minutes, the differences between the conditions became dampened.

Educational Impact

The positive educational impact of this laboratory exercise was clearly shown by the differences in student feedback to the course after the introduction of the ELISA laboratory exercise. For example, student answers to our course's regular survey question "The laboratory sessions helped me to link classroom/textbook theory and practice" changed from 31.7% to 51.6% "Strongly Agree" when we introduced the ELISA laboratory exercise outlined in this article (Fig. 5).

Aside from positive links formed between theory and practice, the students leave the ELISA laboratory exercise with concrete procedure knowledge and skills on how to perform a foundational lab technique for analyzing and quantifying single proteins of interest from complex mixtures. Furthermore, students are equipped with data analysis skills that can be adapted to future work within academia or industry.

One possible future extension to the educational impact of this experiment could be through the use of both a local lab experience, as outlined in this article, as well as having the students replicate the work in a cloud lab. A cloud lab is an automated lab environment where liquid handling, plate manipulation, mixing, incubation, and measurement are all performed offsite in a central robotics lab. This offsite lab is controlled by software programmed by the user [21]. These programmable cloud labs, exemplified by companies such as Emerald Cloud Lab and Transcriptic, are expected to form important parts of the research ecosystem in the near future and preparing students for this transition with core

competencies will soon become imperative for programs within the biological sciences.

In summary, this work has outlined a flexible and cost-effective simulated sandwich ELISA method that enables delivery of an important laboratory technique, and associated procedural knowledge, to undergraduate students.

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Conflict of Interest

The author declares no conflict of interest related to this work.

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