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Creating a Reliable, Cost-Effective ELISA Simulation

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Abstract

The enzyme-linked immunosorbent assay (ELISA) is a fundamental laboratory technique with direct applications across scientific research and clinical diagnostics as well as everyday life. Unfortunately, many challenges exist that inhibit both its introduction and implementation in the high school biology classroom. We present a reliable yet inexpensive way of effectively simulating this assay, allowing student exposure to several advanced topics, including immunodetection, clinical diagnostics, and qualitative and quantitative colorimetric analysis.

Keywords

ELISA; biomarkers; immunodetection; colorimetric analysis

INTRODUCTION

The enzyme-linked immunosorbent assay, or ELISA, is a fundamental laboratory technique used for detecting immunologically relevant biomarkers (Engvall & Perlmann, 1971). Its footprint on the scientific world is widespread, including use for diagnostics in the clinical laboratory, immunodetection of biologically relevant molecules in the basic science research laboratory, and even in pregnancy tests as part of our daily lives (Tsujii et al., 1998; Lyer et al., 2015; Santaclara et al., 2015; Depuydt et al., 2015). ELISAs are used for diagnosis or detection of allergic responses (insect stings, indoor allergens, food, latex), communicable diseases (HIV, ebola, lyme disease, chagas disease, rocky mountain spotted fever, leishmasiasis), and endocrine disorders (hyper- and hypothyroidism). Specifically, the ELISA refers to an assay immobilizing a ligand or antigen with an antibody coupled to a quantitative indicator (such as an enzyme or fluorophore).

One common variant, the sandwich ELISA, immobilizes the target antigen using a capture antibody, and then uses a detection antibody conjugated to an indicator (Figure 1). Its application of basic biological and immunological principles makes it an incredibly valuable case study in the classroom, and should be used as a point of discussion and laboratory activity in all advanced high school biology courses, such as AP Biology or Anatomy & Physiology. Unfortunately, a number of hurdles make such an experiment incredibly

challenging to complete. First, it comes with a high price tag, with commercial kits costing between \$100 and \$200, and only enough material for 10–12 groups. In addition, these experiments typically take several hours to complete—a luxury not available to most high school biology teachers. Second, kits that don't use actual antigens and antibodies to save on cost and time typically skip several key steps. As a result, students do not fully grasp the entire process. Third, most kits contain materials requiring refrigeration or freezing. This poses a problem for many teachers, both in terms of storage and shipment. Overall, these factors create a challenging environment for experimentation, with many opportunities for error on the part of both student and teacher.

To circumvent some of these issues, while also respecting the importance and integrity of the ELISA in its standard form, we have created a reliable yet inexpensive way of effectively simulating this assay in the high school classroom (Figure 1). In addition, the process described below includes minimal use of sensitive materials and requires no special storage. At the same time, all relative steps of an ELISA (we modeled the sandwich variation) are maintained or modeled, allowing students to experience for themselves how the actual assay might look in a laboratory research setting.

Materials

- 12-well plates (Educational Innovations, LSP-150)
- agarose (Flinn Scientific, A0307)
- egg albumin (Flinn Scientific, A0258)
- distilled water
- graduated pipettes or micro-pipettes (500–1000 μ L)
- test tubes (> 2mL capacity)
- protein assay reagent (multiple choices)
 - Pierce™ BCA Protein reagent (#23227)
 - Pierce™ Coomassie Protein Assay (#23200)
 - Bio-Rad™ Protein Assay Dye Reagent (#5000006)
- colorimeter or spectrophotometers and corresponding cuvettes (optional)
- isopropanol, 70 percent (in spray bottle)

Safety Issues

The teacher should inquire if any students have severe egg allergies (accidental ingestion of egg albumin). Students should wear proper personal safety equipment (lab coats or aprons, gloves, and goggles), as these would be worn in an industrial or collegiate laboratory when working with real patient samples.

Procedure

Teacher Instructions

Material Preparation

- Antigen: 5% egg albumin solution (w/v) equal to 0.50 $\mu\text{g}/\mu\text{L}$, or 5.0 g in 100 mL distilled water. This can be communicated to the students as an antigen for an infectious agent or food allergen, etc. Each student will need 2 mL to prepare their standard curve.
- Preparation of Samples: Each student group will need 1 mL of any of the following:
 1. 5% egg albumin from stock solution
 2. 1% egg albumin: 2 mL 5% albumin in 8 mL distilled water
 3. 0% egg albumin: distilled water
- Blocking Buffer: distilled water in clean test tubes or beakers, 10mL/group
- Capture Antibody (1.5% Agarose): 1.5 g agarose in 100 mL distilled water
 1. Microwave for 30 sec, mix
 2. Microwave 15–30 sec more to bring to a boil
 3. Let it cool a few minutes
- Wash Buffer: distilled water in clean test tubes or beakers, 30 mL/group
- Detection Antibody: distilled water in clean test tubes or beakers, 10 mL/group
- Colorimetric Substrate: multiple options to choose from; prepare per manufacturer's instructions.

Preparing ELISA plates (teacher can choose to make these ahead of time and place in plastic bags in a refrigerator):

1. Distribute the freshly made Capture Antibody (1.5% agarose) solution to students in 15 mL aliquots.
2. Students will place 1 mL in each well of a 12-well plate, making sure to coat the bottom of each well.
3. Allow 5–10 min for agarose to solidify

Colorimetric Analysis Preparation (for optional quantitative analysis):

1. Prepare a colorimeter or spectrophotometer (whichever is available) per manufacturer's instructions.
2. Set the colorimeter at the following wavelengths for the respective protein indicators:

As an alternative to using a colorimeter, images can be captured and quantified using free ImageJ software from the NIH, available at <http://imagej.nih.gov/ij/>.

Indicator	Colorimeter (nm)	Spectrophotometer (nm)
Pierce™ BCA Protein Assay	565 nm	562 nm
Pierce™ Coomassie Protein Assay	635 nm	595 nm
Bio-Rad™ Protein Assay Dye Reagent	635 nm	595 nm

Student Implementation

Prepare six standard curve solutions corresponding to “antigen” albumin concentrations of 0, 0.03, 0.06, 0.12, 0.25, and 0.50 $\mu\text{g}/\mu\text{L}$, respectively, using distilled water for serial dilutions:

1. Obtain six small test tubes (2 mL volume capacity), labeled #1–6.
2. Add 1 mL of distilled water to tubes #2–5.
3. Place 1 mL of the 5% egg albumin stock solution in tubes #5 and #6.
4. Mix tube #5 thoroughly.
5. Take 1 mL from tube #5 and add it to tube #4, mixing thoroughly.
6. Continue the serial dilution process through tube #2.
7. Place 2 mL distilled water in tube #1.

ELISA Experiment

1. 1. *Add blocking buffer* to bind to any antibody sites not taken up with the antigen:
 - a. a. Add 500 μL (0.5 mL) of blocking buffer to each well.
 - b. b. Incubate for 1 min at room temperature.
 - c. c. Fold clean paper towels over the well plate and invert to remove excess blocking buffer.
2. 2. *Add antigen* from standard dilution and patient samples:
 - a. Take 500 μL of solution from tubes #1–6 and add to wells #1–6 on the plate.
 - b. In wells #7–12, place 500 μL of a patient or unknown sample(s). We recommend that unknown samples should be run in duplicate to demonstrate the concept of experimental reproducibility through calculation of means and variation within and between groups. This would allow for 3 samples to be analyzed within each 12-well plate.
 - c. Incubate the plates for 5 min at room temperature.
 - d. Fold clean paper towels over the well plate and invert.
3. *Wash with wash buffer* to remove any unbound antigen, repeating the procedure from step 1.

4. *Add detection antibody* to bind to the captured antigen, repeating the procedure from step 1.
5. *Wash with wash buffer* to remove excess capture antibody, repeating the procedure from step 1.
6. *Add colorimetric substrate* to visualize the detection antibody:
 - a. Add 3 mL of the colorimetric substrate to each well.
 - b. Incubate for 5 min.
 - c. Visually inspect the colors and estimate the concentration of the patient sample, comparing it to the standard dilutions for qualitative analysis (Figure 2A).

Colorimetric Analysis and Determination of Patient Titer (for quantitative analysis)

1. Place 2.5 mL of each well solution into a labeled cuvette.
2. Prepare a negative control by placing 2.5 mL of pure indicator into another labeled cuvette.
3. Place the negative control into the cuvette holder, and zero the colorimeter or spectrophotometer per manufacturer's instructions.
4. Place each of the standard dilutions into the cuvette holder, recording both the absorbance and corresponding concentration.
5. Prepare a standardization curve using a spreadsheet (Figure 2B).
6. Place the patient samples into the colorimeter, and obtain the absorbance and corresponding concentration.
7. Determine the patient titer, or antigen concentration, by using the standardization curve. Calculate means and variation for experimental replicates.

Agarose can be discarded, and colorimetric dye in plates and cuvettes can be cleaned with 70 percent isopropanol for reuse.

Conclusions

This protocol describes a reliable yet inexpensive way of effectively simulating an ELISA experiment in the high school classroom. Although an actual ELISA poses many challenges that are difficult to overcome in a high school setting, including everything from cost to implementation, this activity allows students to experience and appreciate how the assay might be carried out in a research setting. Although this protocol does not recapitulate the biology of an ELISA (Figure 1), alternative protocols relying on antibody-mediated detection (Brokaw & Cobb, 2009) or commercially available kits remain cost-prohibitive and have technical restrictions in the high school classroom. All steps of the assay are modeled (see Table 1), and can be completed in a relatively short period of time, providing a realistic approach to a complex topic. However, the activity maintains great flexibility to be redesigned targeting a wide variety of diagnostic applications with easy replicate sampling,

and can serve as a tactile learning alternative or supplement for a virtual lab (HHMI, 2017). Such characteristics make this ELISA modeling experiment ideal for the advanced high school biology classroom, and provide a manageable approach to introducing students to immunodetection and clinical diagnostics through the application of this fundamental molecular assay.

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ELISA	Coat plate with capture antibody	block protein-binding sites on plate Add blocking buffer	other antigens target antigen Add antigen or patient sample	remove other antigens Add wash buffer	detection Ab Add detection antibody	colorimetric reaction Add colorimetric substrate
Simulated Lab	Add melted agarose	Wash with distilled water	Add "antigen" albumin dilution	Wash with distilled water	Add/wash with distilled water	Add protein assay reagent

Figure 1.
Comparative analysis of an ELISA and the simulated lab activity.

Figure 2. Simulated ELISA results using the Pierce BCA Protein Assay. **(A)** Qualitative and **(B)** quantitative results using standardization tubes #1–6 containing 0.50, 0.25, 0.12, 0.06, 0.03, and 0 $\mu\text{g } \mu\text{L}^{-1}$ egg albumin.

Table 1

Summary of simulated ELISA lab protocol.

ELISA	Actual Chemical	Volume	Time
Coat plate with capture antibody	Melted 1.5% agarose	1 mL	5–10 min
Blocking buffer	Distilled water	500 µL	1 min
Antigen or sample	Egg albumin solutions	500 µL	5 min
Wash buffer	Distilled water	500 µL	1 min
Add detection antibody with conjugated enzyme	Distilled water	500 µL	1 min
Wash buffer	Distilled water	500 µL	1 min
Add colorimetric substrate	Protein assay reagent	3 mL	5 min