Edvo-Kit # 266

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What's In My Lunch? Quantitative Food Allergy ELISA

Experiment Objective:

In this inquiry-based experiment, students will master the concepts and methodology behind the enzyme-linked immunosorbent assay (ELISA). Students will perform an ELISA to detect the presence of whey protein in various food products. A standard curve will be created to quantify the concentration of whey in each sample.

See page 3 for storage instructions.

Version 266.190719

SAMPLE LITERATURE
Please refer to included weblink for correct version

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Experiment Components

	Components	Storage	Check (✓)
A	10x ELISA Wash Buffer	Refrigerator	❑
B	ELISA Dilution Buffer	Refrigerator	❑
C	Whey Antigen (Lyophilized)	Refrigerator	❑
D	Anti-Whey Antibody (Lyophilized)	Refrigerator	❑
E	Secondary Antibody (Lyophilized)	Refrigerator	❑
F	TMB Substrate	Refrigerator	❑
G	Stop Solution	Refrigerator	❑

Experiment #266 contains enough reagents for 10 lab groups.

REAGENTS & SUPPLIES

Store all components below at room temperature.

- Strip tubes (8-well) ❑
- Snap-top microcentrifuge tubes ❑
- Homogenization pestles with tubes ❑
- 15 mL conical tubes ❑
- Transfer pipets ❑

Experiment Requirements *(NOT included in this experiment)*

- Various food samples to be tested
- Distilled or deionized water
- Beakers or flasks
- Paper towels
- Disposable lab gloves
- Safety goggles
- Automatic micropipettes (5-50 µL, 100-1000 µL) and tips
- Digital camera or cell phone with camera
- Computers with Internet, image analysis program, and graphing program

NOTE:
Disposable transfer pipets can be substituted for automatic micropipettes if necessary. See **Cat. #632**.

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals. None of the experiment components are derived from human sources..

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Background Information

UNDERSTANDING ALLERGIES

Allergies are one of the most common diseases of the immune system, occurring in up to 20% of people in developed countries. An allergic response occurs when the immune system overreacts to a foreign material, known as an antigen (short for antibody generator). Common antigens for allergy sufferers include infectious agents, such as bacteria or viruses, chemicals and various environmental materials such as pollen and food. Once an antigen enters the body it triggers white blood cells to produce antibodies, leading to a swift immune response.

Antibodies are specialized proteins that are used by the body to identify and eliminate pathogens. Each antibody is composed of four polypeptide chains, two heavy chains and two light chains. These chains are linked together by disulfide bonds to create a distinct "Y" shape (Figure 1). At each tip of the Y is a highly variable region composed of 110-130 amino acids that gives the antibody its specificity for binding to antigens. Each antibody molecule can bind to two antigen molecules, one at each tip. This binding neutralizes the antigen and forms an insoluble complex through a process known as immunoprecipitation.

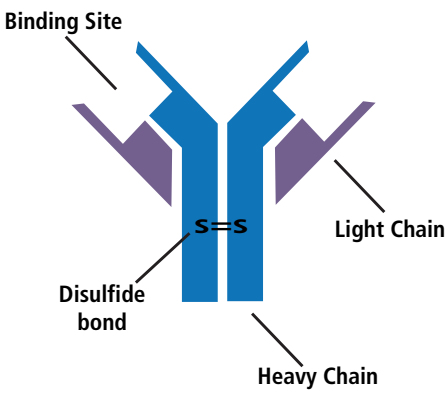

Figure 1: Structure of an antibody.

All allergies begin with sensitization, which starts when a normally non-hazardous antigen enters the body and encounters a lymphocyte cell (Figure 2A). For reasons still being researched, the lymphocyte cell registers this non-harmful particle as threatening, which triggers the production of novel immunoglobulin E (IgE) antibodies (Figure 2B). The highly specific IgE antibodies then attach themselves to immune cells, like mast cells and basophils, which circulate throughout the body (Figure 2C). The sensitization process can take between 6-10 days. After sensitization, IgE antibodies in the bloodstream can rapidly bind to their antigen, triggering immune cells to release mediator compounds such as histamine and proteoglycans into the body (Figure 2D). Once a person has become sensitized, small amounts of the antigen can trigger a full allergic reaction. Symptoms of an allergic reaction are varied, ranging from sneezing and itchy eyes to anaphylaxis (Table 1).

Anaphylaxis is a severe, whole-body reaction to an allergen. French scientists Charles Richet and Paul Portier coined the term in 1902 while studying the toxin produced by the tentacles of the Portuguese Man of War. They isolated the toxin to inject into dogs hoping to obtain protection, or "prophylaxis", against it. However, they were horrified to find that even small doses of the toxin resulted in the rapid onset of breathing difficulty in vaccinated dogs. Richet and Portier rightly concluded that the initial exposure caused the dog's immune system to become hyper-sensitized to the toxin. After the first exposure, re-exposure to the same compound resulted in a severe reaction, regardless of the dosage. They termed this state "anaphylaxis", which means "against protection".

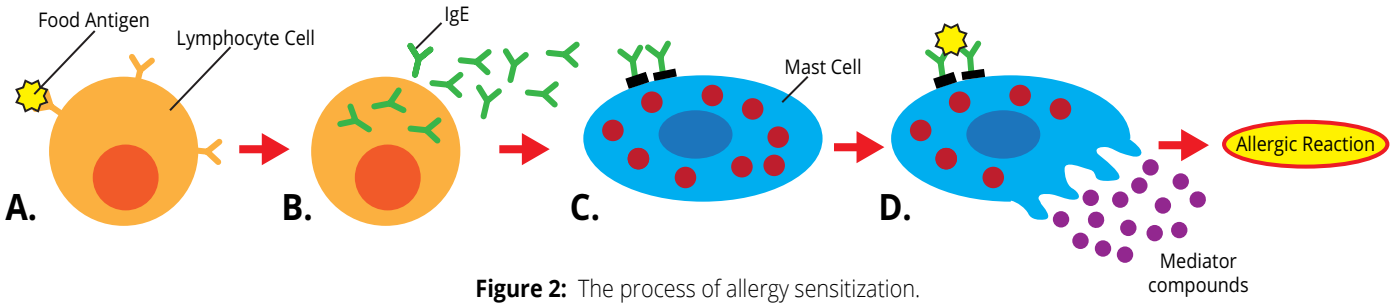

Figure 2: The process of allergy sensitization.

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The leading cause of anaphylaxis in humans is related to the food we eat. Food allergies are a serious and growing health issue that affects around 15 million people in the United States alone. For example, Cow's Milk Protein Allergy (CMPA) is the most common food allergy in children. CMPA results when the immune system mistakenly attacks one or more milk proteins present in food products. The immune response can be immediate (within minutes), delayed (within hours to days), or both. Common symptoms include vomiting, wheezing, and eczema (a detailed list is provided in Table 1).

TABLE 1: POTENTIAL SYMPTOMS OF AN ALLERGIC REACTION

Immediate Reactions	Anaphylaxis, acute rash, wheezing, sneezing, congestion, dry cough, vomiting, acute asthma, swelling of the larynx
Delayed Reactions	Atopic dermatitis, vomiting and diarrhea, constipation, poor growth, inflammation in digestive tract.

Between 2 to 3% of the general population are diagnosed with CMPA, although many (79%) outgrow it by the age of 16. For sufferers, the main treatment for this condition is to eliminate cow's milk protein from their diet. Most milk is around 3% protein, which can be classified into two categories based on the presence or absence of the element phosphorus. Caseins contain phosphorus and will coagulate or precipitate at a pH of 4.6. This coagulation at reduced pH is the basis for cheese curd formation. Most mammal species contain 3 or 4 different casein proteins, which make up approximately 82% of the total protein in milk. All other proteins found in milk lack phosphorus and are grouped together as whey or serum proteins. The major whey proteins in cow milk are beta-lactoglobulin and alpha-lactalbumin. Together, the whey proteins comprise the remaining 18% of protein in milk.

DETECTING FOOD ALLERGENS

In 2005, the FDA began requiring manufacturers to label the presence/absence of eight of the most common allergens (milk, eggs, fish, shellfish, peanuts, wheat, soybeans, and tree nuts) in their food products. This labeling helps consumers more easily identify and avoid potentially dangerous allergens. To detect these allergens in their product, food companies can use PCR, mass spectrometry, or immunochemical assays, although immunochemical assays tend to be the most popular due of their approachability and robustness. Immunochemical assays identify a particular substance by its ability to bind to an antibody. One commonly used immunochemical assay is the Enzyme Linked Immunosorbent Assay (ELISA).

ELISAs can determine the presence and concentration of specific antigens in complex solutions. To accomplish this, ELISAs rely on the ability of an antibody to recognize and bind to specific antigens. Most ELISAs use two different antibodies – a primary antibody that is specific to the antigen of interest and a secondary antibody that recognizes the antigen-antibody complex (Figure 3A). This secondary antibody is coupled to an enzyme that reacts with a substrate to produce a signal. ELISAs can be designed to detect antigens for a large variety of purposes. For example, in medicine ELISAs are often used to determine serum antibody concentrations. This information helps doctors to diagnose viral, bacterial, and parasitic infections. ELISAs can also be used to identify genetically modified organisms, trace drug use, and confirm pregnancy.

Traditional ELISAs are performed in transparent microtiter plates made of polystyrene or polyvinyl chloride. The sample to be tested is deposited into small wells and proteins present in the solution, including the antigen under investigation, will stick to the plate. These proteins/antigens are allowed to bind to the plate during a short incubation period (Figure 3B). After this the wells are washed to remove unabsorbed antigens and a solution that contains the primary antibody is added to the wells. If the antigen is present in the wells then it will bind to the antibody and form a complex.

Following a second wash, a solution containing the enzyme-linked secondary antibody is added to the wells (Figure 3B). This secondary antibody will bind to the antigen and primary antibody complex. After a final washing step, a colorless substrate solution containing 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (also known as ABTS) is added. If there is any secondary antibody bound to the well then the linked enzyme Horseradish Peroxidase (HRP) will catalyze a reaction that produces a colored product from the ABTS (Figure 3B).

Since each secondary antibody can produce many molecules of the converted substrate ELISAs are very sensitive, even at extremely low levels of antigen. ELISAs can be qualitative, in which case they indicate whether or not an antigen is present,

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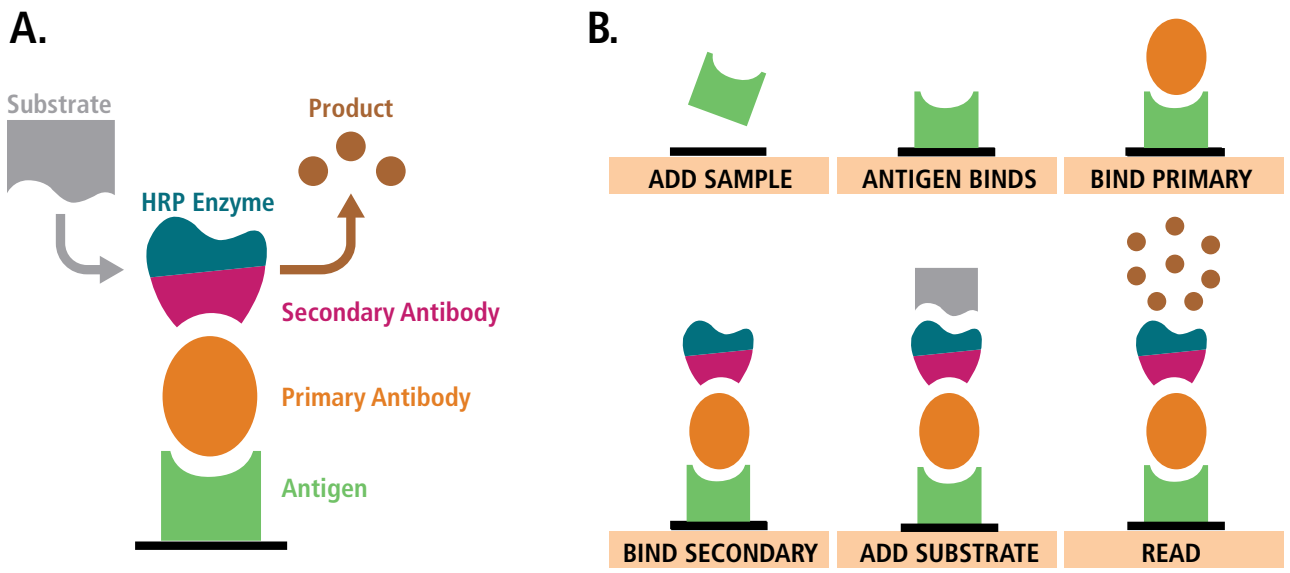

Figure 3: Overview of the ELISA.

or quantitative, in which case they also measure the antigen concentration. A quantitative ELISA requires that several wells be set-aside as standards. Each standard will contain a solution where the concentration of the antigen of interest is already known. The signal intensity in each well is measured and the values of the standards are plotted to create a standard curve. The intensity of the unknown samples can then be compared to the standard curve to determine an approximate protein concentration. Quantitative ELISAs are used in research, medicine, and industry tests.

In this exploration, students will perform an ELISA to examine the presence of whey protein in various food products. Students will also prepare a standard curve containing known amounts of whey protein. This standard curve will be used to estimate the concentration of whey in their food samples. As a STEM activity, students will create a standard curve base on image density measurements. This exercise will allow students to calculate the amount of whey found in a food sample.

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Experiment Overview

EXPERIMENT OBJECTIVE:

In this inquiry-based experiment, students will master the concepts and methodology behind the enzyme-linked immunosorbent assay (ELISA). Students will perform an ELISA to detect the presence of whey protein in various food products. A standard curve will be created to quantify the concentration of whey in each sample.

LABORATORY SAFETY

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Always wash hands thoroughly with soap and water after handling contaminated materials.

Wear gloves and safety goggles

LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Module I: 15 min.
Preparation of Food Samples

↓

Module II: 45 min.
Allergen Detection by ELISA

↓

Module III-A: 15 min.
Qualitative Analysis of Allergen Testing

↓

Module III-B: 45 min.
Quantitative Analysis of Allergen Testing

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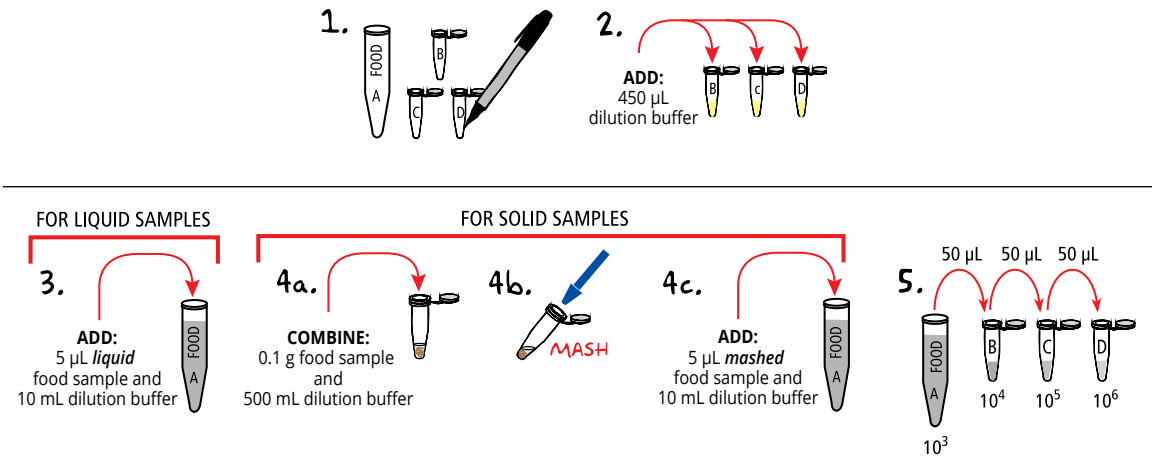
Module I: Preparation of Food Samples

In this module you will prepare and dilute your food sample. Creating a serial dilution ensures that at least one tested food concentration falls inside the concentration range described by the standard curve.



1. **LABEL** one 15 mL tube with the name of your food sample and the letter "A". Also **LABEL** three microcentrifuge tubes "B", "C", and "D" for the food sample dilutions.
2. **ADD** 450 µL of Food Dilution Buffer to each microcentrifuge tube.
3. **FOR LIQUID SAMPLES** (like milk), **ADD** 5 µL of liquid food sample and 10 mL Food Dilution Buffer to the labeled 15 mL tube to create a $1x10^3$ dilution. Mix well. **PROCEED** directly to step 5.
4. **FOR SOLID FOOD SAMPLES** (like cheese):
   a. **COMBINE** 0.1 g of the food with 500 µL of Food Dilution Buffer in a microcentrifuge tube.
   b. Use a pestle to completely **MASH** food.
   c. **ADD** 5 µL of the mashed food sample solution and 10 mL Food Dilution Buffer to the labeled 15 mL tube to create a $1x10^3$ dilution. Mix well.
5. Prepare a serial dilution of your food sample. **ADD** 50 µL of the 10^3 food sample (tube A) to tube B, **MIX** well. **ADD** 50 µL of the 10^4 food sample (tube B) to tube C, **MIX** well. **ADD** 50 µL of the 10^5 food sample (tube C) to tube D, **MIX** well and **PROCEED** to Module II.

Table 2: Food Product Dilutions and Concentrations

Well	A	B	C	D
Concentration	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$
Dilution	Food sample dilution prepared in step 3 or 4	50 µL of $1:10^3$ (A) sample plus 450 µL food dilution buffer	50 µL of $1:10^4$ (B) sample plus 450 µL food dilution buffer	50 µL of $1:10^5$ (C) sample plus 450 µL food dilution buffer

STOP

OPTIONAL STOPPING POINT:
The experiment can be stopped after step 5 by placing the diluted food samples (tubes A-D) into overnight storage at 4° C. The experiment can be resumed the following day by continuing with Module II.

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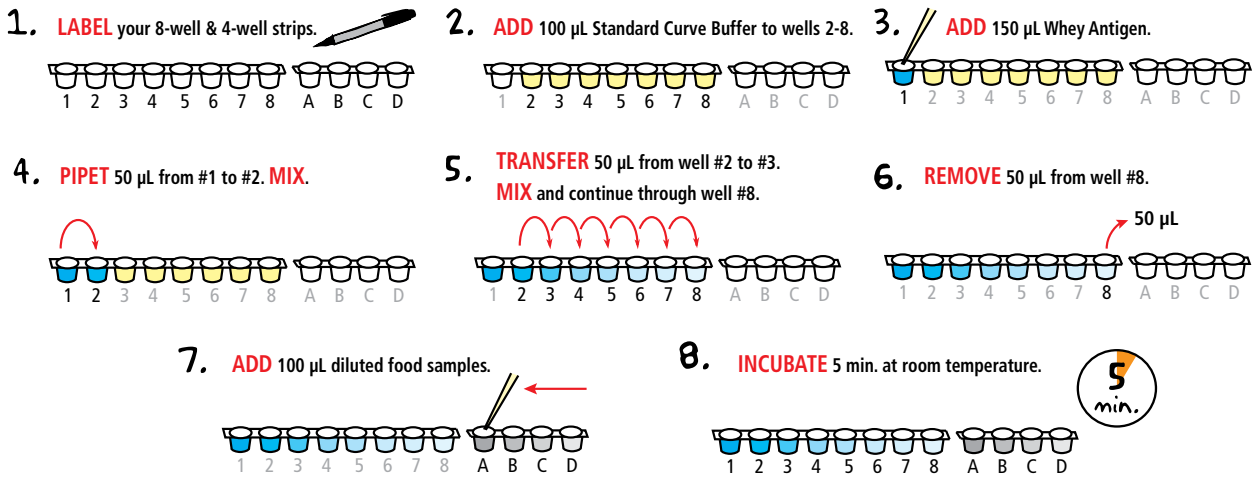
Module II: Allergen Detection by ELISA

In this module you will perform a 1:3 (3-fold) serial dilution of whey protein to buffer, in order to create a standard curve of eight known antigen concentrations. You will then conduct an ELISA on both the diluted food samples and the standard curve samples.



Preparation of the Standard Curve and Loading Diluted Food Samples:

1. Using a fine-tipped marker, **LABEL** the wells of your 8-well strip 1-8 and the wells of your 4-well strip A-D.
2. **ADD** 100 µL Standard Curve Buffer to wells 2-8.
3. **ADD** 150 µL of the whey antigen solution to well #1. The antigen is provided at a concentration of 10 µg/ml.
4. **PIPET** 50 µL from well #1 into well #2 and **MIX** the sample by gently pipetting up and down 5 times.
5. Using the same pipette tip, **TRANSFER** 50 µL from well #2 into well #3 and **MIX**. Continue to serially **DILUTE** the remaining samples through well #8 according to table 3 below.
6. **REMOVE** and **DISCARD** 50 µL of the diluted antigen from well #8.
7. **ADD** 100 µL of the diluted food samples (A, B, C, D) to wells A, B, C, and D. Add the solutions starting with the most dilute sample (well D) and ending with the most concentrated sample (well A) or change tips between each sample.
8. **INCUBATE** the strips for 5 minutes at room temperature. While you are incubating, **CALCULATE** the antigen concentrations for wells 1 through 8 and **RECORD** your answers in Table 3.

Table 3: Standard Dilutions and Concentrations

Well	1	2	3	4	5	6	7	8
Dilution	---	1:3	1:9	1:27	1:81	1:243	1:729	1:2187
Dilution Procedure	150 µL of whey antigen	50 µL of well 1 into 100 µL standard curve buffer	50 µL of well 2 into 100 µL standard curve buffer	50 µL of well 3 into 100 µL standard curve buffer	50 µL of well 4 into 100 µL standard curve buffer	50 µL of well 5 into 100 µL standard curve buffer	50 µL of well 6 into 100 µL standard curve buffer	50 µL of well 7 into 100 µL standard curve buffer
Concentration	10 µg/mL							

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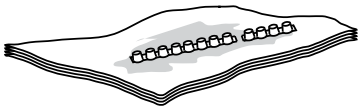
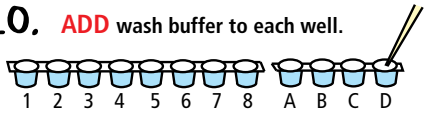
Module II: Allergen Detection by ELISA, continued

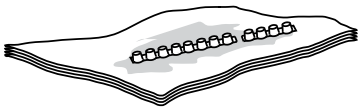
9. **INVERT** onto paper towels.



10. **ADD** wash buffer to each well.



11. **INVERT** onto paper towels.



12. **REPEAT** steps 10 & 11.

Removal of Sample and Washing the Wells:

9. **INVERT** the strips over the sink or a stack of paper towels to remove the samples. Gently **TAP** the strips 4-5 times onto a fresh paper towel. **DISCARD** the wet paper towels.

10. Using a transfer pipet, **ADD** Wash Buffer to each well until they are almost full (~200 µL). DO NOT allow the buffer to spill over into adjacent wells.

11. **INVERT** the strips over the sink or a stack of paper towels to remove the samples. Gently **TAP** the strips 4-5 times onto a fresh paper towel. **DISCARD** the wet paper towels.

12. **REPEAT** steps 10-11 to wash the wells once more.

STOP

OPTIONAL STOPPING POINT:
The experiment can be stopped after step 12 but requires that Wash Buffer be left in all the wells for overnight storage at 4° C. The experiment can be resumed up to 24 hours later. Remove the Wash Buffer and continue with step 13.

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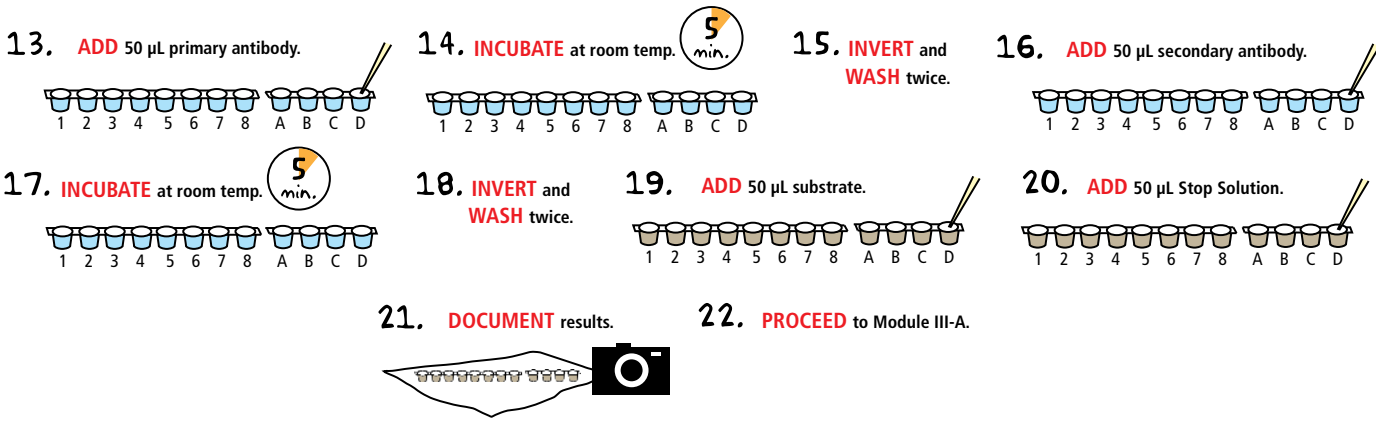
Module II: Allergen Detection by ELISA, continued



Addition and Removal of Antibodies

13. Using a new micropipette tip, **ADD** 50 µL of the primary antibody solution to each well.

14. **INCUBATE** at room temperature for 5 minutes.

15. **INVERT** onto paper towels and **TAP. WASH** the wells twice as in steps 10-12.

16. Using a new micropipette tip, **ADD** 50 µL of the secondary antibody solution to each well.

17. **INCUBATE** at room temperature for 5 minutes.

18. **INVERT** onto paper towels and **TAP. WASH** the wells twice as in steps 10-12.

Addition of Substrate

19. Using a new micropipette tip, **ADD** 50 µL of the TMB substrate to all 12 wells. When the first well is a dark blue (2-5 min), proceed to Step 20.

    NOTE: The enzymatic reaction can quickly saturate in wells with a high antigen concentration. Work quickly but steadily when adding the TMB substrate.

20. Using a new micropipette tip, **ADD** 50 µL of Stop Solution to each well. Gently **TAP** tubes to **MIX**.

21. **DOCUMENT** results by using a digital camera to take a picture. Placing the microtiter strips on a white sheet of paper or a white light box can enhance the contrast between wells. Photograph both strips simultaneously. The best images are taken with the two strips parallel and with the camera directly overhead and 1-2 ft above. When possible, select high resolution.

22. **PROCEED** to Module III-A.

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Module III-A: Qualitative Analysis of Allergen Testing

The color intensity of each well reflects the initial concentration of whey. Color intensity can be estimated by eye and described or ranked on a scale of 0 (clear) to 10 (blue). Because the ELISA solution can quickly evaporate it is important to answer questions 1 - 3 the same day as Module II. Alternatively you can use the picture taken in Module II to answer these questions at a later time.

1. Which standard curve samples are the darkest? Which are the lightest? What does this mean? How does color intensity relate to the concentrations you calculated in Table 3?

2. Observe the food sample dilutions. Did any of them change color?

3. Do any of the food sample dilutions wells (A through D) closely resemble the standard curve wells (1 through 8)?

4. Based on this similarity can you estimate the original concentration of whey in the food sample you tested? Remember that wells A to D represent food that has been diluted.

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Module III-B: Quantitative Analysis of Allergen Testing

The color intensity of each well can be determined using densitometry, the quantitative measurement of light absorption. In this ELISA the initial concentration of whey determines how many molecules of TMB are oxidized. Oxidized TMB turns the solution blue, which leads to more light absorption. Therefore, by measuring the sample color intensity in the eight wells of known concentration, you can establish a relationship between whey concentration and light absorption. This relationship is described by the equation of the standard curve. You can then use the standard curve equation to estimate the original concentration of whey in your chosen food.

1. Calculate the mean gray value and the whey protein concentration for the eight standard curve wells.
   a. Save the digital image of your results as a JPEG on the computer.
   b. Open the ImageJ program on your computer by going to **https://imagej.net/ij/** and following the instructions for either downloading or opening the program in browser.
   c. Go to File > Open and open your image.
   d. Go to Image > Type > 32 bit.
   e. Go to Edit > Invert.
   f. Go to Analyze > Set Measurements and select "mean gray value".

NOTE:
ImageJ can be found at:
https://imagej.net/ij/
You can download the program or run ImageJ in your browser.

NOTE: In digital images each pixel has a luminance – or light intensity – value which ranges from black (zero intensity) to white (full intensity). In ImageJ, this value is called gray value. The mean gray value is calculated by adding all the gray values in a selection and then dividing by the total number of pixels.

   g. Choose the round selection tool and draw a circle that fits within the first well. ***NOTE: This circle will be used to measure all wells. Because some cameras can distort the shape of the edge wells, we suggest making this circle slightly smaller than the actual well.***
   h. Go to Analyze > Measure. A new window titled results should appear. Record the results for "mean gray value" in your lab notebook or in the table below.
   i. Click back to the digital image of your results and use the mouse or arrow keys to move the circle to next well. Repeat step h for all remaining wells.
   j. Complete Table 4 (at right) using the mean gray values and the antigen concentrations from Table 3 (page 9).

Table 4: Calculations for the Standard Curve and Diluted Food Samples

Well	Dilution	Mean Gray Value	Whey Protein Concentration (µg/mL)
1	1:1		10
2	1:3		
3	1:9		
4	1:27		
5	1:81		
6	1:243		
7	1:729		
8	1:2187		
A	$1:10^3$		
B	$1:10^4$		
C	$1:10^5$		
D	$1:10^6$		

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Module III-B: Quantitative Analysis of Allergen Testing, continued

2. Create a standard curve.
   a. Plot the whey protein concentration (x-axis) against the mean gray value (y-axis) for each standard concentration.
   b. Draw a best-fit curve through the points in the graph (for best results we suggest using graphing software).
   c. Record the equation of your curve for use later.

NOTE: We suggest a logarithmic best-fit line. The best-fit line may not pass through every data point.

3. Determine the concentration of target protein in a food sample well.
   a. Find the mean gray values of the sample in wells A to D as in step 1.
   b. Select a sample with an intensity value that falls between the maximum and minimum standard curve values.
   c. Use the equation of the best-fit line to solve for x given the y value. Alternatively, extend a horizontal line from the absorbance value of the sample (y-axis) to the standard curve and read the corresponding concentration (x-axis). See Figure 5.

4. Determine the concentration of whey in the original food sample.
   a. Convert the dilution ratio of the well to a decimal number by dividing the number on left by the number on the right. For example, the $1:10^4$ dilution can be rewritten as 0.0001.
   b. Divide the well whey protein concentration (calculated in step 3c) by this number.

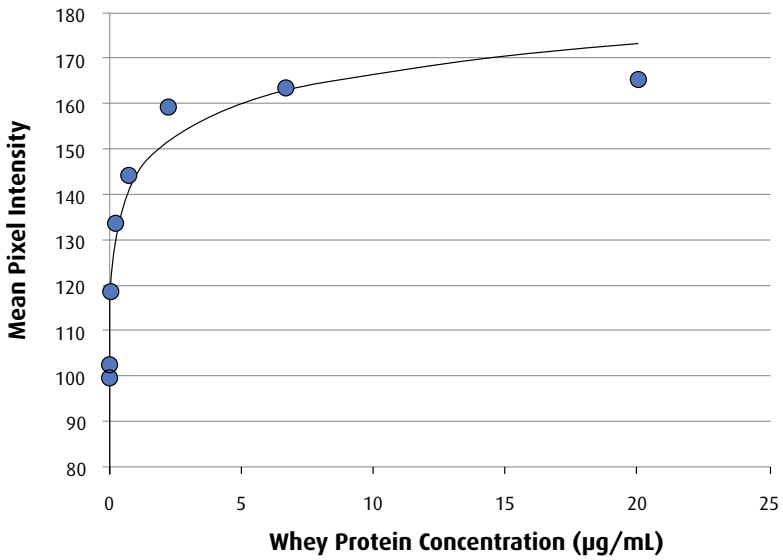

Figure 4: Plotting a Standard Curve.

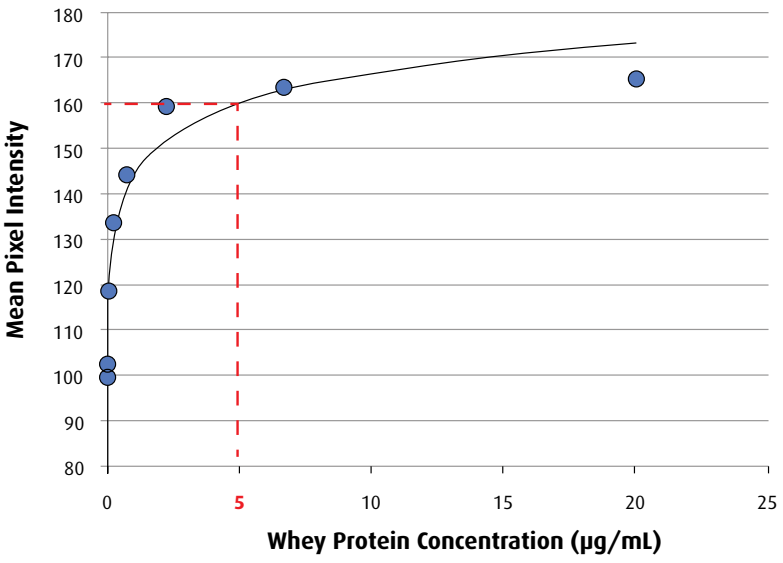

Figure 5: Determining a Protein Concentration.

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Study Questions

1. Describe the structure of an IgE antibody protein. What type of cell produces these proteins?

2. Name and describe two types of milk proteins.

3. Describe an ELISA reaction beginning with the addition of an antigen and ending with the substrate color change.

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Instructor's Guide

OVERVIEW OF INSTRUCTORS PRELAB PREPARATION:

This section outlines the recommended pre-lab preparations and approximate time requirement to complete each pre-lab activity. In addition to preparing and aliquoting the provided components for Module II (table below) you will also need to acquire food samples before Module I and make sure your students have access to digital cameras before Module II and access to computers with image processing and graphing programs before Module III.

Component:	What to do:	When:
Strip Tubes	Divide five strips	Anytime before the experiment.
10X ELISA Wash Buffer (A)	Dilute to 1X solution and aliquot	Anytime before the experiment. Cover and store in the refrigerator.
ELISA Dilution Buffer (B)	Use to rehydrate components C, D, and E and to prepare food and curve buffers.	Anytime before the experiment. Store tubes in the refrigerator.
Whey Antigen (C)	Rehydrate and aliquot	Up to one week before performing the experiment.
Anti-Whey Antigen (D)	Rehydrate and aliquot	Up to one week before performing the experiment.
Secondary Antibody (E)	Rehydrate and aliquot	On the same day as performing the experiment.
TMB Substrate (F)	Aliquot	Up to one week before performing the experiment.
Stop Solution (G)	Aliquot	Up to one week before performing the experiment.

Red = Prepare immediately before module. Yellow = Prepare shortly before module. Green = Flexible / prepare up to a week before the module.

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Pre-Lab Preparations

Acquire Food Samples

A variety of dairy and dairy-free products can be tested. Encourage student groups to bring in their own samples! While results will vary depending on the brand used Table 5 provides a list of suggested products and their general results.

Table 5: Expected Results from Dairy Products

Product Tested	ELISA Color Change
Cow's Milk	Dark
Soy Milk	None
Protein Drink	Light
Dairy Coffee Creamer	Light
Fresh Cream	Light to Dark (depending on brand)
Dairy Cheese	Light
Dairy Yogurt	Light to Dark
Dairy Ice Cream	Light to Dark
Dairy Free Yogurt	None
Dairy Free Ice Cream	Light
Cookies/Cakes	None to Light
Salad Dressing	Light
Butter	None
Candy bar (nougat)	Light
Energy/Protein bar	Dark

Prepare Strip Tubes

Fifteen strip tubes are provided in this kit. For the experiment, each student group will need one full strip (8 wells) for the standard curve and one half strip (4 wells) for the food sample. To create the 4-well strip, divide 5 full strips in half.

Digital Camera

For the purposes of this experiment almost all digital cameras, including those found on cell phones, can be used. Images will need to be uploaded (if using image analysis software in browser) or transfered onto a local computer for image analysis.

Requirements For Module III-B

In order to perform the quantitative analysis, students will need access to image analysis software and a graphing program capable of finding a best-fit curve.

- **Image analysis software**
  A number of programs can be used to compare the intensity of the twelve wells. We recommend **ImageJ**. ImageJ is an image processing program that is in the public domain and can be used freely in browser (smart phone or computer) or downloaded to your local computer. Instructions can be found at **https://imagej.net/ij/**. ImageJ accepts many image formats, including JPEG, PNG, and TIFF. If needed, please consult the appropriate technology support at your institution for assistance.

- **Graphing**
  Numerous graphing programs are available. Alternatively, results can be plotted on semi-logarithmic paper and a linear trend line added by hand.

Removing the Samples and Washing the Wells

As an alternative to inverting the strips to remove the samples during Module II, students can use a pipette to remove the samples and wash buffers. In this case, it is important to change pipettes or pipette tips between wells to prevent cross-contamination. Additional microtransfer pipets can be ordered from Edvotek (**Cat. #632**). To reduce waste, transfer pipets can be labeled and reused for the same well in future steps.

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Pre-Lab Preparations

Preparation of Wash Buffer

1. **ADD** all of the 10x ELISA Wash Buffer (A) to 180 mL of distilled water. **MIX** well.
2. **DISPENSE** 18 mL into small beakers for each of the 10 lab groups. **LABEL** the beakers as "Wash Buffer".

Preparation of Sample Preparation Buffers

1. In a 250 mL beaker/flask, **DILUTE** 7 mL of the ELISA Dilution Buffer (B) in 150 mL of distilled water.
2. **DISPENSE** 13 mL of the dilution from step 1 into ten small beakers. **LABEL** as "Food Dilution Buffer".
3. **DISPENSE** 1 mL of the dilution from step 1 into ten microcentrifuge tubes and **LABEL** as "Standard Curve Buffer".
4. **SAVE** the remaining ELISA Dilution Buffer (B) for subsequent steps.

Preparation of Whey Antigen for Standard Curve

1. **TRANSFER** 7 mL of ELISA Dilution Buffer (B) to a 15 mL conical tube. Label as "Antigen".
2. Carefully **REMOVE** the stopper from the vial of lyophilized Antigen (C) and **TRANSFER** approximately 0.5 mL of the ELISA Dilution Buffer from the tube in step 1. **CLOSE** the stopper and gently shake the vial to **MIX**.
3. **TRANSFER** 0.25 mL (250 µL) of the rehydrated Antigen back to the conical tube from step 1. **MIX** well.
4. **LABEL** ten microcentrifuge tubes as "Whey Antigen" and **DISPENSE** 200 µL of the prepared solution into these tubes.

FOR MODULE I
Each Group should receive:
- 13 mL Food Dilution Buffer
- Food Sample for analysis
- 15 mL conical tube
- 3 microcentrifuge tubes
- 1 tube with pestle (for solid foods)

FOR MODULE II
Each Group should receive:
- One 8-well strip and one 4-well strip
- 18 mL Wash Buffer
- 1 mL Standard Curve Buffer
- 200 µL Whey Antigen
- 700 µL Primary Antibody
- 700 µL Secondary Antibody
- 700 µL Substrate
- 700 µL Stop Solution
- Adjustable micropipette and tips
- 1 large transfer pipet
- Empty beaker for waste
- Paper towels

FOR MODULE III
Each Group will need:
- Access to computer with image processing and graphing programs

Preparation of Primary Antibody

1. **TRANSFER** 7 mL of ELISA Dilution Buffer (B) to a 15 mL conical tube. Label as "Primary Antibody".
2. Carefully **REMOVE** the stopper from the vial of lyophilized Primary Antibody (D) and **TRANSFER** approximately 0.5 mL of the ELISA Dilution Buffer from the tube in step 1. **CLOSE** the stopper and gently shake the vial to MIX.
3. **TRANSFER** the entire volume of the rehydrated Primary Antibody back to the conical tube from step 1. **MIX** well.
4. **LABEL** ten microcentrifuge tubes as "Primary Antibody" and **DISPENSE** 700 µL of the prepared solution into these tubes.

Preparation of Secondary Antibody

Prepare the same day as needed for the experiment.

1. **TRANSFER** 7 mL of ELISA Dilution Buffer (B) to a 15 mL conical tube. Label as "Secondary Antibody".
2. Carefully **REMOVE** the stopper from the vial of lyophilized Secondary Antibody (E) and **TRANSFER** approximately 0.5 mL of the ELISA Dilution Buffer from the tube in step 1. **CLOSE** the stopper and gently shake the vial to **MIX**.
3. **TRANSFER** the entire volume of the rehydrated Secondary Antibody back to the conical tube from step 1. **MIX** well.
4. **LABEL** ten microcentrifuge tubes as "Secondary Antibody" and **DISPENSE** 700 µL of the prepared solution into these tubes.

Preparation of TMB Substrate and Stop Solution

1. Dispense 700 µL of the TMB Substrate (F) into ten microcentrifuge tubes labeled "Substrate".
2. Dispense 700 µL of Stop Solution (G) into ten microcentrigue tubes. Label the tubes as "Stop".

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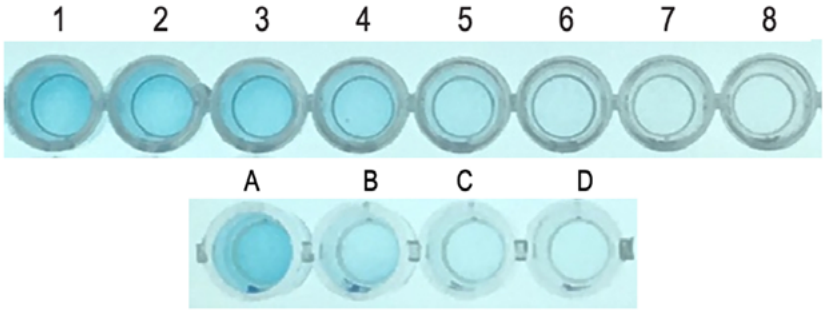
Experiment Results and Analysis

MODULE II

Below are the results for an ELISA we ran using half and half cream. The results in wells A through D will vary depending on what food samples are used. The results in wells 1 through 8 can also vary depending on a number of factors, including incubation times, accuracy of pipetting, and background lighting.



MODULE III-A

1. **Which standard curve samples are the darkest? Which are the lightest? What does this mean? How does color intensity relate to the concentrations you calculated in Table 3?**

   Well 1, which contained the standard curve sample of whey at a 10 µg/mL concentration, is the darkest while well 8, which contained the standard curve sample of whey at 0.0046 µg/mL, is the lightest. This means that the darker the reaction the higher the concentration of whey.

2. **Observe the food sample dilutions. Did any of them change color?**

   All four wells changed color. Well A showed the most color change while well C And D showed only slight color changes.

3. **Do any of the food sample dilutions wells (A through D) closely resemble the standard curve wells (1 through 8)?**

   Well A looks similar in color intensity to wells 1 and 2, well B looks similar to wells 5 and 6, and wells C and D both look similar to wells 7 and 8.

4. **Based on this similarity can you estimate the original concentration of whey in the food sample you tested? Remember that wells A to D represent food that has been diluted.**

   Based on the similarity between well A and wells 1, it would seem that the original food concentration had a whey concentration of 10 µg/mL * 1000 which equals 10,000 µg / mL.

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Experiment Results and Analysis

Module III-B

Below are the image and table generated in step 1.

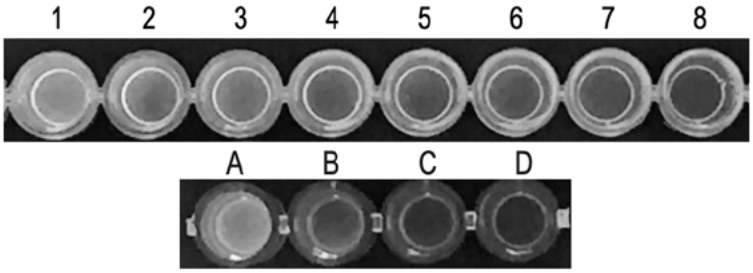

Table 4: Calculations for the Standard Curve and Diluted Food Samples

Well	Dilution	Mean Gray Value	Whey Protein Conc. (µg/mL)
1	1:1	177.84	10.0000
2	1:3	162.645	3.3333
3	1:9	160.823	1.1111
4	1:27	149.56	0.3704
5	1:81	145.953	0.1235
6	1:243	144.029	0.0412
7	1:729	142.504	0.0137
8	1:2187	135.357	0.0046
A	1:103	199.33	Unknown
B	1:104	164.01	Unknown
C	1:105	134.667	Unknown
D	1:106	129.667	Unknown

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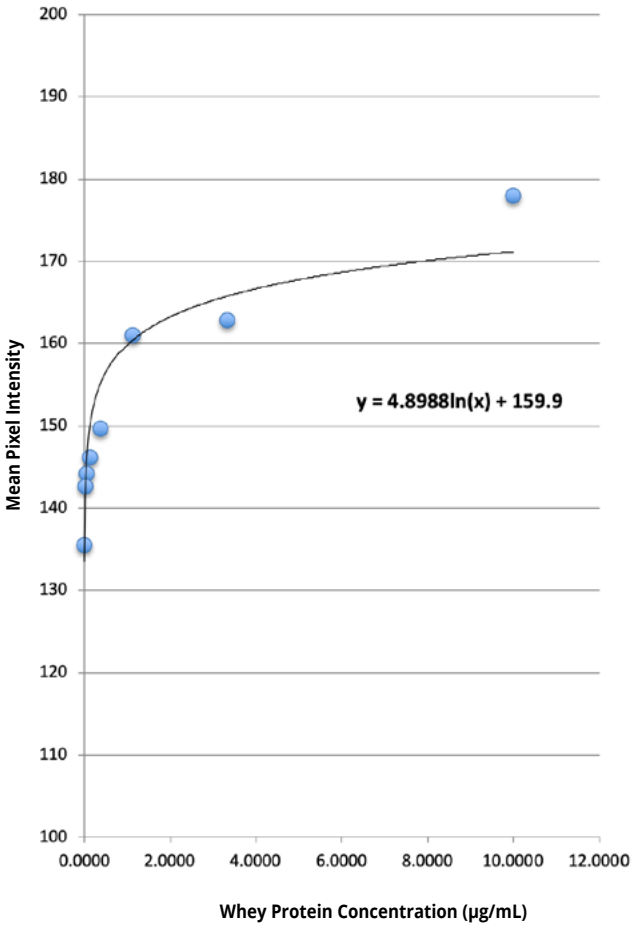
Experiment Results and Analysis

Module III-B, continued

Below is the scatter plot graph and logarithmic best-fit line created in step 2.



For step 3 only the food dilution in well B (mean grey value of 164.01) fell within the range of the standard curve. This was used to calculate the whey concentration using the best-fit line equation.

Equation of the line: $y = 4.8988 \ln(x) + 159.9$
y represents the mean gray scale value
x represents the whey concentration

Solving for x:
$164.01 = 4.8988 \ln(x) + 159.9$
$4.11 = 4.8988 \ln(x)$
$0.8389 = \ln(x)$
$e^{(0.8389)} = e^{\ln(x)}$
$2.3138 = x$

Finally, in step 4, the original dilution factor ($1:10^4$) is converted into a decimal (0.0001). The x value from above (2.314) is then divided by this number to calculate the original concentration of whey in the food sample: 2.3138/0.0001 = 23,138 µg/mL.

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Please refer to the kit insert for the Answers to Study Questions