THE BIOTECHNOLOGY EDUCATION COMPANY®

Edvo-Kit # 266

Edvo-Kit #266

# What's In My Lunch? Quantitative Milk 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.**

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# Experiment Components

| COMPONENTS | | Storage | Check (√) |
|---|---|---|---|
| A | 10x Dilution Buffer | 4°C Refrigerator | ❑ |
| B | Whey Antigen | 4°C Refrigerator | ❑ |
| C | 10x PBST (Wash Buffer) | 4°C Refrigerator | ❑ |
| D | Primary Antibody | 4°C Refrigerator | ❑ |
| E | Secondary Antibody | 4°C Refrigerator | ❑ |
| F | Aminosalicylic Acid (Peroxide Co-Substrate) | 4°C Refrigerator | ❑ |
| G | Hydrogen Peroxide | 4°C Refrigerator | ❑ |

**Experiment #266 is designed for 10 lab groups.**

**REAGENTS & SUPPLIES** Check (√)
**Store all components below at room temperature**

- Microtiter plates ❑
- Snap-top Microcentrifuge tubes ❑
- Homogenization pestles with tubes ❑
- 15 ml conical tubes ❑
- 50 ml conical tube ❑
- Transfer pipets ❑

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.

# Requirements *(not included with this kit)*

- Various food samples to be tested
- Distilled or deionized water
- Beakers or flasks
- Paper towels
- 37°C Incubator
- Disposable lab gloves
- Safety goggles
- Automatic micropipets (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 micro transfer pipets can be substituted for automatic micropipets if necessary. See Cat. #632.



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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.

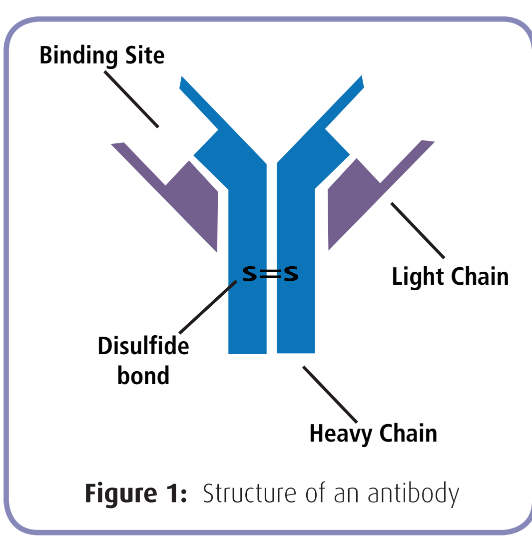


**Figure 1:** Structure of an antibody

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".

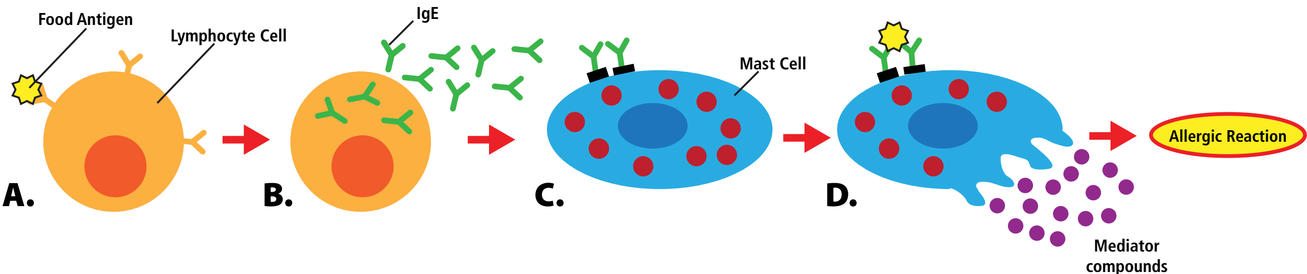


**Figure 2:** The process of allergy sensitization.



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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).

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.

| 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. |

## 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 (hydrogen peroxide and amino salicylate) is added. If there is any secondary antibody bound to the well then the linked enzyme Horseradish Peroxidase (HRP) will convert the hydrogen peroxide to water and oxygen using the amino salicylate as the hydrogen donor. Oxidized salicylate is brown and can be easily observed in wells containing the antigen (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, 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



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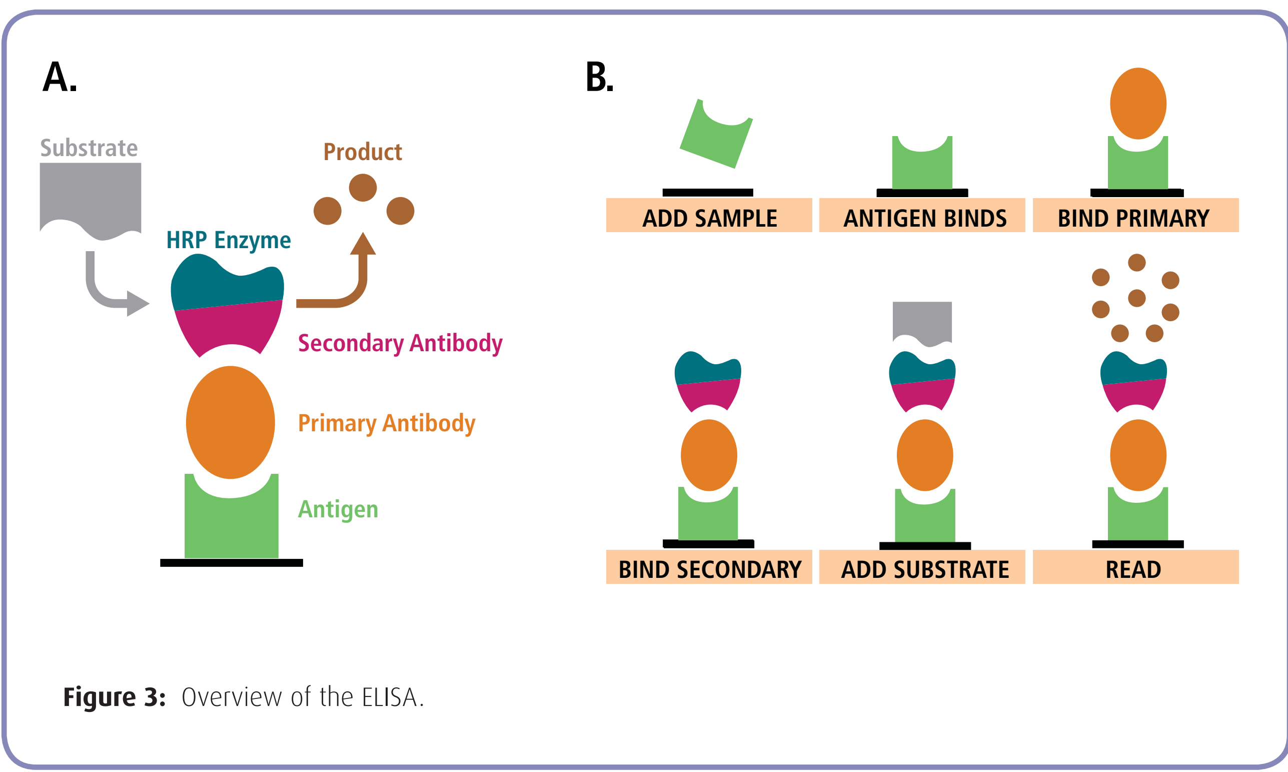


**Figure 3:** Overview of the ELISA.

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. Exercise extreme caution when working with equipment which is used in conjunction with the heating and/or melting of reagents.

3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.

4. 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:

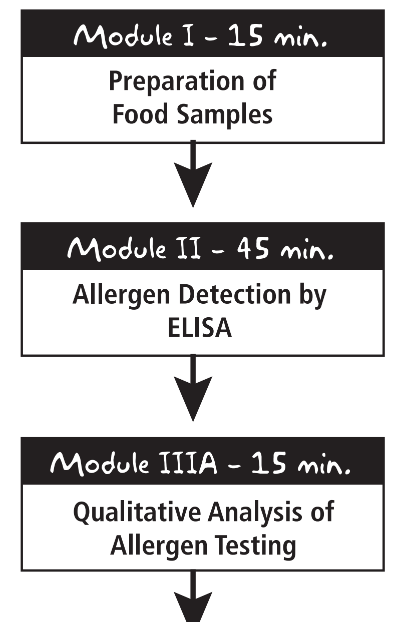
- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

### 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.



Quantitative Analysis of Allergen Testing



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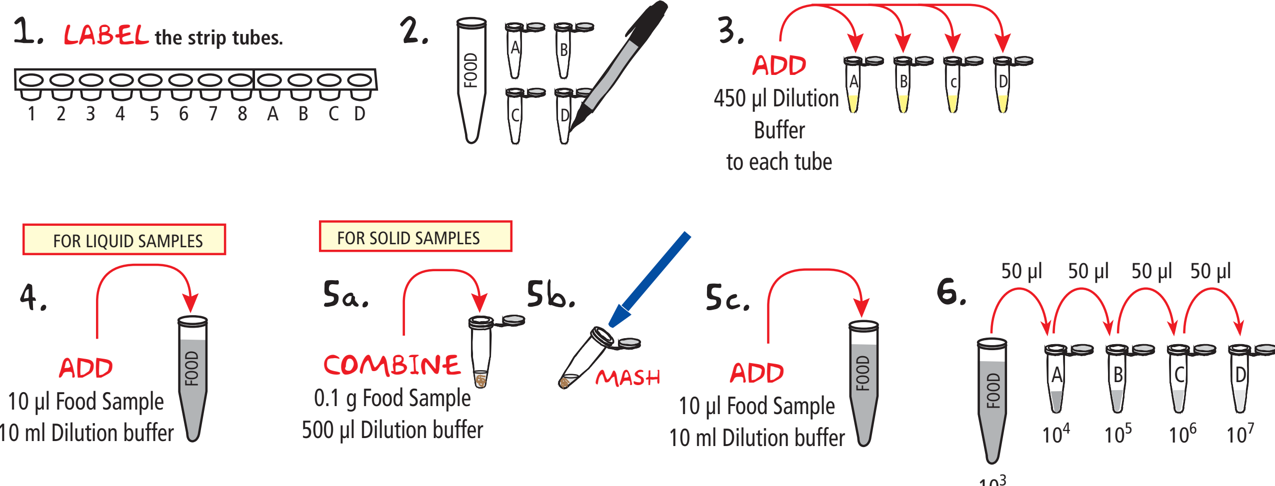
# Module I: Preparation of Food Samples




The food samples must be diluted to ensure that the concentrations will fall inside the range established by the standard curve. Best results are typically obtained using a 1:10 dilution series for the food sample.

1. Using a fine-tipped marker, **DRAW** a box around columns 1-8 and another around columns 9-12. **LABEL** the first eight wells of the microtiter strip with the numbers 1-8 and the last four wells with the letters A, B, C, D.
2. **LABEL** a 15 ml tube with the name of your food sample. Also **LABEL** four microcentrifuge tubes A,B, C, D for the food sample dilutions.
3. **ADD** 450 µl of Dilution Buffer to each microcentrifuge tube.
4. For **LIQUID** samples (like milk):
   a. **ADD** 10 µl of liquid food sample and 10 ml Dilution Buffer to the labeled 15 ml tube to create a $1x10^3$ dilution. Mix well. **PROCEED** directly to step 6.
5. For **SOLID** food samples (like cheese):
   a. **COMBINE** 0.1 g of the food with 500 µl of Dilution Buffer in a microcentrifuge tube.
   b. Use a pestle to completely **MASH** food.
   c. **ADD** 10 µl of the mashed food sample and 10 ml Dilution Buffer to the labeled 15 ml tube to create a $1x10^3$ dilution. Mix well.
6. **ADD** 50 µl of the $10^3$ food sample to tube A, mix well. **ADD** 50 µl of the $10^4$ food sample (tube A) to tube B, mix well. Continue to serially **DILUTE** the remaining samples through tube D.
7. **PROCEED** to Module II.

Table 2: Food Product Dilutions and Concentrations

| Well | A | B | C | D |
|---|---|---|---|---|
| Concentration | $1:10^4$ | $1:10^5$ | $1:10^6$ | $1:10^7$ |
| Dilution | 50 µl of food sample + 450 µl dilution buffer | 50 µl of $1:10^4$ (A) sample + 450 µl dilution buffer | 50 µl of $1:10^5$ (B) sample + 450 µl dilution buffer | 50 µl of $1:10^6$ (C) sample + 450 µl dilution buffer |

**OPTIONAL STOPPING POINT:**
The experiment can be stopped after step 6 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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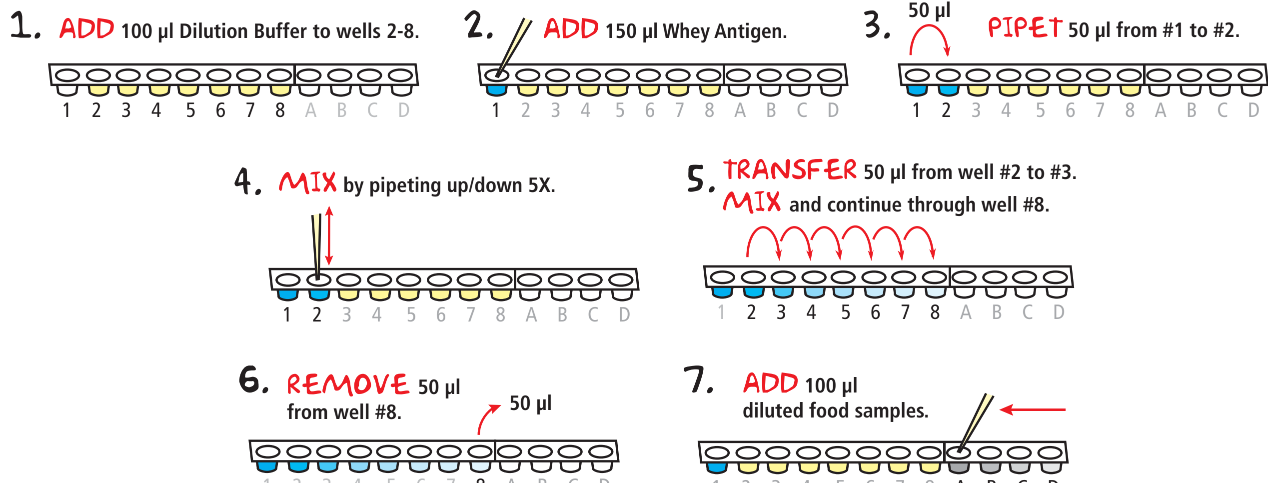
# Module II: Allergen Detection by ELISA




A standard curve allows researchers to determine the relationship between two known quantities. To do this, we will perform a 1:3 (3 fold) serial dilution of whey protein to buffer, creating eight samples of known antigen concentration. After performing the ELISA, these samples are used to create a standard curve that establishes the relationship between whey concentration and sample color intensity.

### Preparation of Dilutions for Standard Curve:

1. **ADD** 100 µl Dilution Buffer to wells 2-8 of the microtiter strip.
2. **ADD** 150 µl of the whey antigen solution to well #1. The antigen is provided at a concentration of 20 µg/ml.
3. **PIPET** 50 µl from well #1 into well #2.
4. Fully **MIX** the sample by gently pipetting up and down 5 times.
5. Using the same pipet tip, **TRANSFER** 50 µl from well #2 into well #3 and **MIX** as in step 4. Continue to serially **DILUTE** the remaining samples through well #8.
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 (well D) and ending with the most concentrated (well A) or change tips between each sample.
8. **CALCULATE** the antigen concentrations for each well 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 dilution buffer | 50 µl of well 2 into 100 µl dilution buffer | 50 µl of well 3 into 100 µl dilution buffer | 50 µl of well 4 into 100 µl dilution buffer | 50 µl of well 5 into 100 µl dilution buffer | 50 µl of well 6 into 100 µl dilution buffer | 50 µl of well 7 into 100 µl dilution buffer |
| Concentration | 20 µg/ml | | | | | | | |



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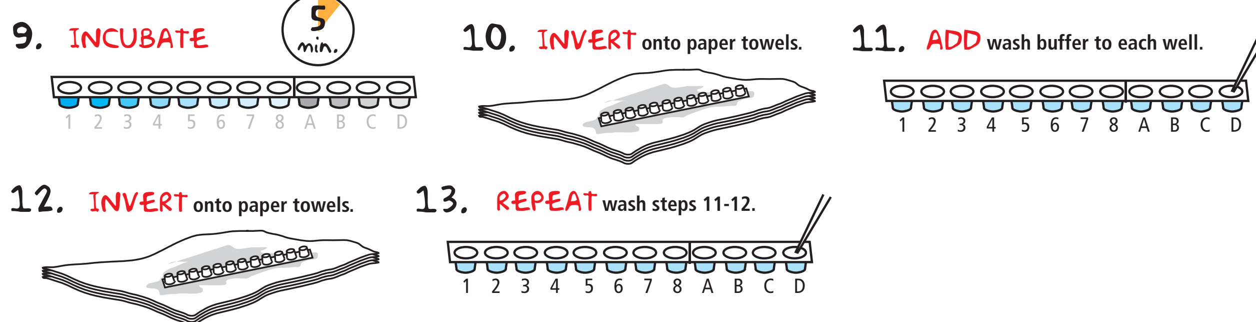
# Module II: Allergen Detection by ELISA, continued




### Removal of Sample and Washing the Wells:

9. **INCUBATE** for 5 minutes at room temperature.
10. **INVERT** the microtiter strip over a stack of paper towels to remove the samples. **TAP** the microtiter strip gently onto a fresh paper towel to remove any remaining sample. **DISCARD** the wet paper towels.
11. Using a transfer pipet, **ADD** wash buffer to fill each well.

**NOTE: To minimize cross-contamination it is important that you avoid spilling buffer into neighboring wells.**

12. **REPEAT** step 10 to **REMOVE** the wash buffer.
13. Using the same transfer pipet, **REPEAT** steps 11 and 12 to wash the wells.

**OPTIONAL STOPPING POINT:**

The experiment can be stopped after step 13 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 14.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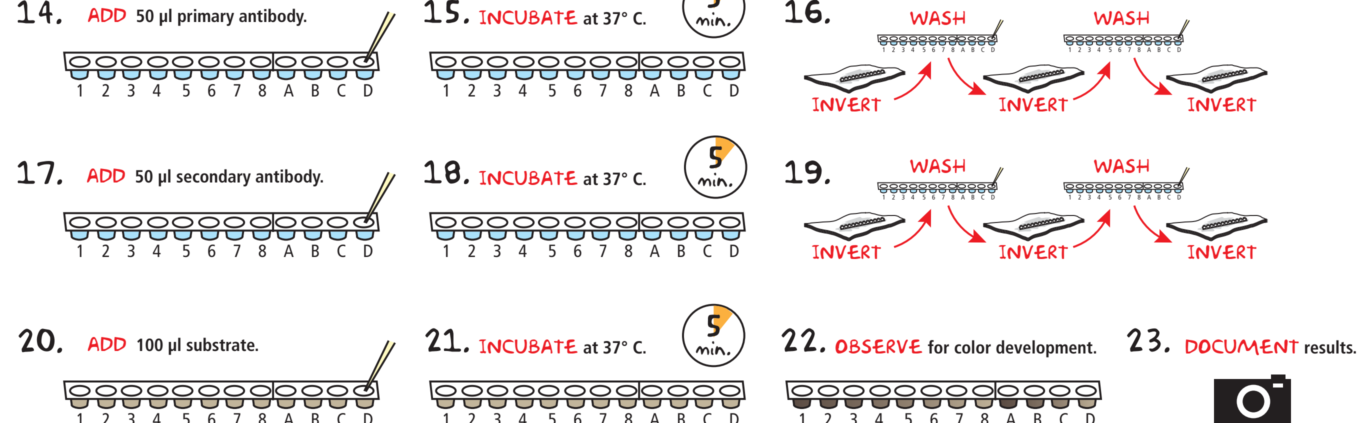
# Module II: Allergen Detection by ELISA, continued




### Addition and Removal of Antibodies

14. **ADD** 50 µl of the primary antibody solution to each well.
15. **INCUBATE** at 37° C for 5 minutes.
16. **WASH** the wells twice as in steps 10 through 13.
17. **ADD** 50 µl of the secondary antibody solution to each well.
18. **INCUBATE** at 37° C for 5 minutes.
19. **WASH** the wells twice as in steps 10 through 13.

### Addition of Substrate

20. **ADD** 100 µl of the substrate to all 12 wells.
21. **INCUBATE** at 37° C for 5 minutes.
22. Periodically **OBSERVE** the plate for color development. If the color is not fully developed in well 1 after 5 minutes, **INCUBATE** for an additional 5 minutes.
23. **DOCUMENT** results by using a digital camera to take a picture. Placing the microtiter strip on a white sheet of paper or a white light box can enhance the contrast between wells.
24. **PROCEED** immediately to Module IIIA and B: Data Analysis.

**NOTE:**
It is important that the incubation is not allowed to proceed for too long as the reaction can saturate.



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# Module IIIA: 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 (dark reddish brown). Because the aminosalicylic acid will continue to oxidize it is important to complete steps 1 through 3 within fifteen minutes of completing Module II. Alternatively a picture can be taken of the microtiter strip and examined later.

1. Which 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 dilutions (wells 1 through 8)? 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 IIIB: 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 aminosalicylic acid are oxidized. Oxidized salicylate turns the solution brown, which leads to more light absorption. Therefore, by measuring the sample color intensity in the eight wells of known concentration, we can establish a relationship between whey concentration and light absorption. This relationship is described by the equation of the standard curve. Using the equation we can then 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.
   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:**
Detailed download instructions for ImageJ can be found at: http://rsb.info.nih.gov/ij/download.html

**NOTE: In digital images each pixel has a luminance – or light intensity – value which ranges from black (zero intensity) to white (full intensity). In Image J 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 around the first 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.

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

| Well | Dilution | Mean Gray Value | Whey Protein Concentration |
|---|---|---|---|
| 1 | 1:1 | | 20 µg/ml |
| 2 | 1:3 | | |
| 3 | 1:9 | | |
| 4 | 1:27 | | |
| 5 | 1:81 | | |
| 6 | 1:243 | | |
| 7 | 1:729 | | |
| 8 | 1:2187 | | |
| A | $1:10^4$ | | |
| B | $1:10^5$ | | |
| C | $1:10^6$ | | |
| D | $1:10^7$ | | |



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## Module IIIB: 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). Record the equation for your curve for use later.

**NOTE:** The best-fit line may not pass through every data point.

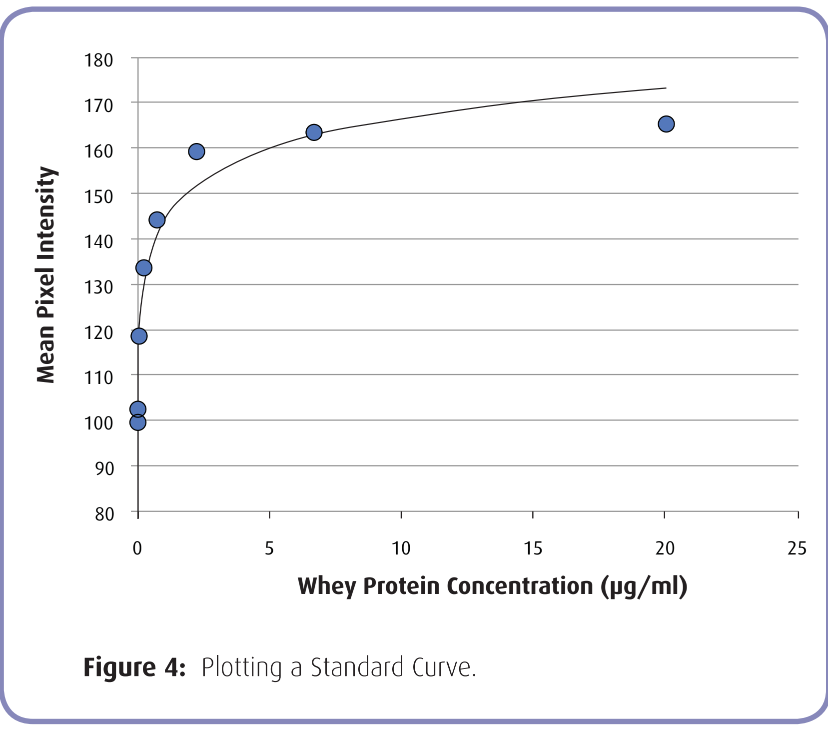


**Figure 4:** Plotting a Standard Curve.

3. Determine the concentration of target protein in the food samples.
    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. From the Y-axis of the standard curve graph, extend a horizontal line from this absorbance value to the standard curve. At the point of intersection extend a vertical line to the X-axis and read the corresponding concentration. Alternatively use the equation of the best-fit curve to solve for x given the y value (see Figure 5).

Based on your calculations, what is the concentration of whey in the tested food samples? Remember that wells A to D represent food that has been diluted.

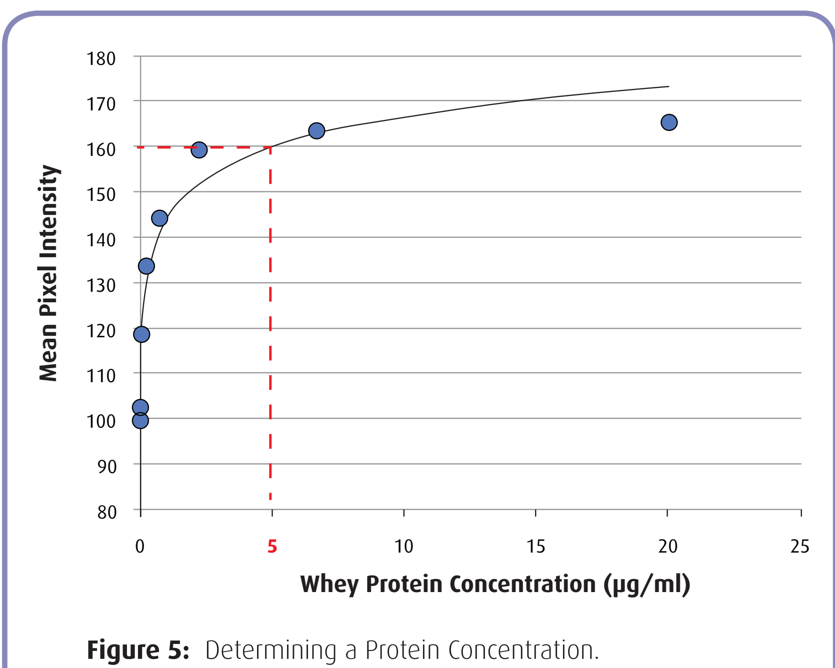


**Figure 5:** Determining a Protein Concentration.

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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.

### Module 1: Preparation of Food Samples

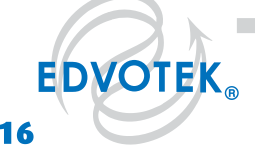
| What to do: | When: | Time Required: |
|---|---|---|
| Acquire Food Samples | Before the lab | ----- |
| Divide Microtiter Plates | Before the lab | 15 minutes |

### Module 2: Allergen Detection by ELISA

| What to do: | When: | Time Required: |
|---|---|---|
| Equilibrate a 37° C Incubator | Day of the lab | 15 minutes |
| Prepare and aliquot reagents | Up to 24 hours before the lab. Store at 4° C. | 20 minutes |
| Prepare peroxidase substrate | During the lab (15-30 min. before last incubation) | 10 minutes |

### Module 3b: Quantitative Analysis of Allergen Testing

| What to do: | When: | Time Required: |
|---|---|---|
| Obtain a digital camera | Before the lab | ----- |
| Obtain access to computers | Before the lab | ----- |
| Install image processing program | Before the lab | ----- |



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## Pre-Lab Preparations

### Acquire Food Samples

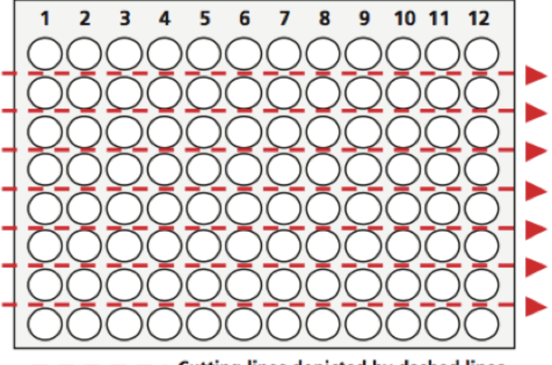
A variety of dairy and dairy free products can be tested and you can encourage student groups to bring in their own samples. While results will vary depending on the brand used Table 5 provides a list of suggested products and their general results.

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 |

### Divide Microtiter Plates

1. **ORIENT** the microtiter plates so that the numbers 1-12 are at the top (see figure, below right).
2. **CUT** each plate on the dotted lines as shown in the figure. Each piece will have 12 wells. Each lab group will receive one strip.




Cutting lines depicted by dashed lines

### Obtain Digital Camera

For the purposes of this experiment almost all digital cameras, including those found on cell phones, can be used. Digital cameras will include a USB cable and/or memory cards for transferring the pictures to the computers. If you are using a cell phone, the images can be sent to an email address. Many image formats can be used including TIFF, GIF, and JPEG.

### Computer Requirements For Module IIIB

In order to perform the quantitative analysis, students will need a computer with image analysis software and a graphing program capable of finding a best-fit curve. A number of programs can be used to compare the intensity of the twelve wells. We recommend ImageJ (version 1.49 or newer). ImageJ is an image processing program developed at the National Institute of Health. It is in the public domain and so can be freely downloaded and installed. Detailed download instructions and the appropriate ImageJ file for your platform can be found at http://rsb.info.nih.gov/ij/download.html.

In order to run ImageJ, you will need to have Java running on the computer. Please consult the appropriate technology support personnel for your institution for assistance. 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 pipet for removing the samples and wash buffers. In this case it is important to change tips or transfer pipets between wells to prevent cross-contamination. To reduce waste, tips or transfer pipets can be labeled and reused for the same well in future steps.



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# Pre-Lab Preparations - Day of the Lab

## MODULES I AND II

### Preparation of Dilution Buffer

1. **ADD** all of the 10x Dilution Buffer (A) to 180 ml of distilled water. Mix well.
2. **DISPENSE** 15 ml into small beakers for each of the 10 lab groups. **LABEL** the beakers as "Dilution Buffer". Reserve the remaining Dilution Buffer for subsequent steps.

### Preparation of Whey Solution for Standard Curve

1. **ADD** 3 ml of diluted Dilution buffer to the bottle of Whey Antigen (B). Cap and invert the bottle to mix. This is the 20 µg/ml whey sample for the standard curve.
2. **LABEL** 10 microcentrifuge tubes "Antigen" and **DISPENSE** 200 µl of the diluted whey for each group.

### Preparation of Wash Buffer

1. **ADD** all of the 10x PBST Wash Buffer (C) to 270 ml of distilled water. **MIX** well.
2. **RESERVE** 30 ml of diluted wash buffer for anti-whey primary antibody and peroxidase substrate preparations.
3. **DISPENSE** 20 ml into small beakers for each of the 10 lab groups. **LABEL** the beakers as "Wash Buffer".

### Preparation of Anti-whey Primary Antibody

1. **DISPENSE** 700 µl of Primary Antibody (D) into 1.5 ml tubes for each of the 10 lab groups. **LABEL** the tubes as "Primary Antibody".

### Preparation of Anti-IgG Peroxidase Conjugate Secondary Antibody

1. **ADD** 0.3 ml of diluted Wash Buffer to the Secondary Antibody (E). **MIX** thoroughly by tapping and inverting the tube.
2. **ADD** 7 ml of Wash Buffer to a 15 ml conical tube.
3. **TRANSFER** the entire contents of tube E prepared in step 1 to the 15 ml tube prepared in step 2. **MIX** well.
4. **DISPENSE** 700 µl into microcentrifuge tubes for each of the 10 lab groups. **LABEL** the tubes as "Secondary Antibody".

**NOTE:**
The sample volume of the secondary antibody is very small - the tube can be centrifuged to collect the sample at the bottom.

**NOTE: Store all reagents at 4° C until needed. Components can be prepared up to 24 hours before needed.**

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## Pre-Lab Preparations - During the Lab

### MODULE II

#### Preparation of Peroxidase Substrate During Lab Experiment

**Prepare 15 – 30 minutes before the last incubation:**

1. **DISPENSE** 13.5 ml of diluted Wash Buffer to the 50 ml tube provided.
2. **ADD** Amino-salicylic acid (F) to the 50 ml tube prepared in step 1. Cap and mix thoroughly by shaking and/or vortexing. It is common to see undissolved material remaining and is okay to proceed to the next step.
3. **ADD** 1.5 ml of Hydrogen peroxide (G). Cap and mix.
4. **DISPENSE** 1.4 ml into microcentrifuge tubes for each of the 10 lab groups. **LABEL** the tubes “Substrate”.

**For Module I, Each Group should receive:**

- 15 ml conical tube
- 4 microcentrifuge tubes
- 15 ml Dilution Buffer
- 1 row of microtiter plate
- Food Sample for analysis
- 1 tube with pestle (for solid foods)

**For Module II, Each Group should receive:**

- 200 µl Whey Solution
- 1 large transfer pipet
- Empty beaker for waste
- 20 ml Wash Buffer
- 700 µl Primary antibody
- 700 µl Secondary antibody
- 1.4 ml Substrate (prepared before the last incubation)
- Paper towels

**QUICK REFERENCE:**

The substrate is prepared for the peroxidase enzyme, which is attached to the anti-IgG peroxidase conjugate (secondary antibody).

**Prepare the substrate 15-30 minutes before students require it for plate development (last incubation).**



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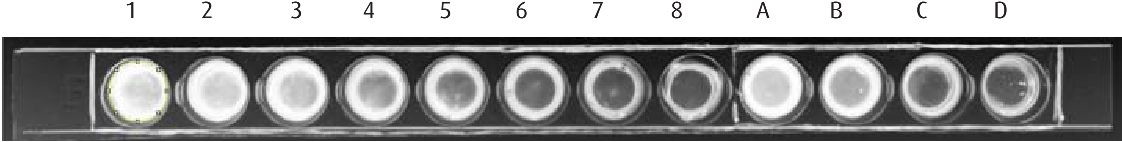
## Experiment Results and Analysis

Below are the results for an ELISA we ran using Vitamin D milk. 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.

1 2 3 4 5 6 7 8 A B C D

Following the directions from Module III B step 1 the image was modified. For reference we have shown the round selection tool around the first well.

1 2 3 4 5 6 7 8 A B C D



The mean gray values for this ELISA are given in our completed Table 4.

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

| Well | Dilution | Mean Gray Value | Whey Protein Concentration |
|---|---|---|---|
| 1 | 1:1 | 165.207 | 20 µg/ml |
| 2 | 1:3 | 163.486 | 6.67 µg/ml |
| 3 | 1:9 | 159.111 | 2.22 µg/ml |
| 4 | 1:27 | 144.114 | 0.74 µg/ml |
| 5 | 1:81 | 133.631 | 0.25 µg/ml |
| 6 | 1:243 | 118.459 | 0.08 µg/ml |
| 7 | 1:729 | 102.436 | 0.03 µg/ml |
| 8 | 1:2187 | 99.515 | 0.01 µg/ml |
| A | 1:$10^4$ | 161.376 | 5.931033753 |
| B | 1:$10^5$ | 139.004 | 0.6080918305 |
| C | 1:$10^6$ | 115.311 | 0.05450055394 |
| D | 1:$10^7$ | 100.051 | 0.01152634047 |



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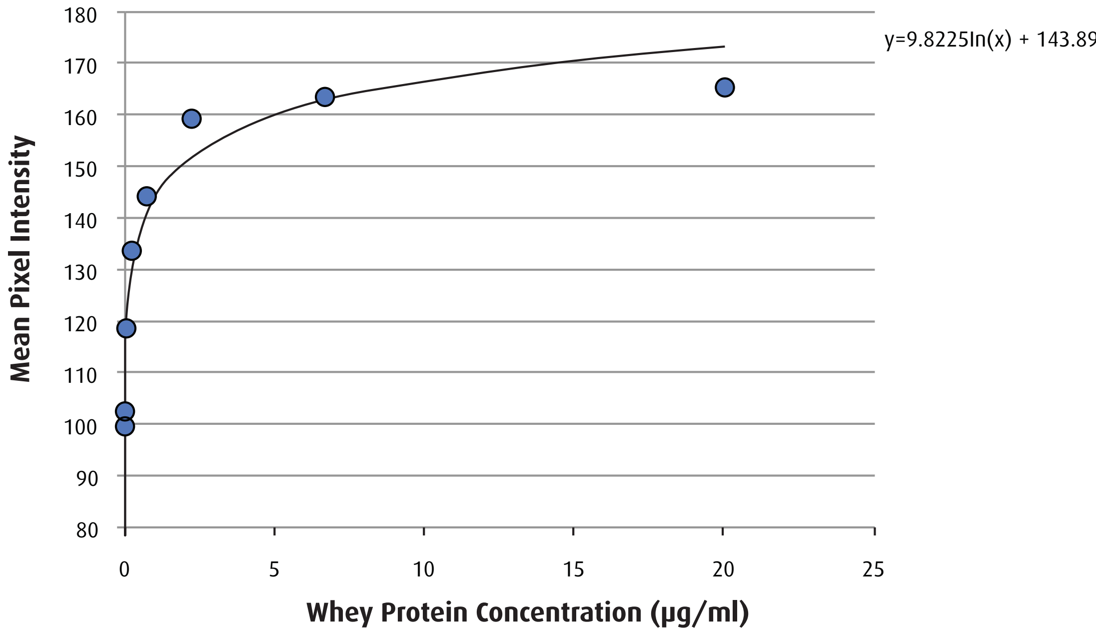
## Experiment Results and Analysis

Creating a standard curve according to the instructions in Module III B step 2 resulted in the below graph.




All four of the food sample dilutions fell within the range of the standard curve so any could be used to calculate the initial whey concentration in the milk. To illustrate step 3 of Module III we will use well A.

Mean gray scale value of well A: 161.376

Equation of the line: $y = 9.8225\ln(x)+143.89$
y represents the mean gray scale value
x represents the whey concentration (μg/ml)

Solving for x:
$9.8225\ln(x)+143.89 = 161.376$
$9.8225\ln(x) = 17.486$
$\ln(x) = 1.78019852$
$e^{\ln(x)} = e^{1.78019852}$
$x = e^{1.78019852}$
$x = 5.93103373052$ which we will round to 5.931

Factoring in the dilution: $5.931 \times 10^4 = 59{,}310$ μg/ml of whey



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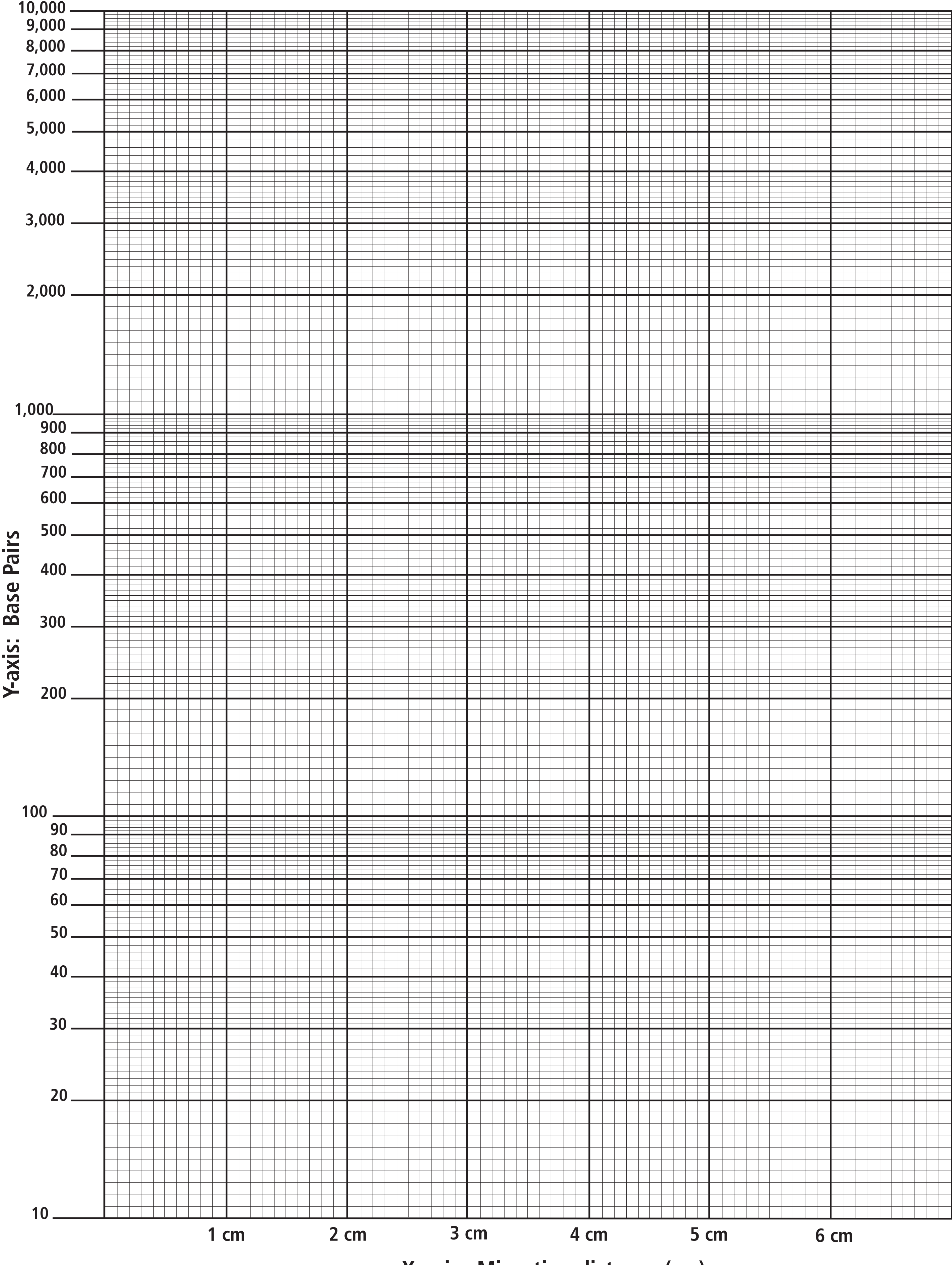
**Please refer to the kit insert for the Answers to Study Questions**


10,000
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8,000
7,000
6,000
5,000
4,000
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2,000
1,000
900
800
700
600
500
400
300
200
100
90
80
70
60
50
40
30
20
10
Y-axis: Base Pairs
1 cm
2 cm
3 cm
4 cm
5 cm
6 cm
X-axis: Migration distance (cm)