Edvo-Kit # 195

Edvo-Kit #195

Forensic Toxicology

Experiment Objective:

In this forensic science experiment, students will use the Enzyme-Linked Immunosorbent Assay (ELISA) to analyze crime scene samples for the presence of drugs.

See page 3 for storage instructions.

Version 195.190618

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Table of Contents

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

	COMPONENTS	Storage	Check (✓)
A	10X ELISA Wash Buffer	Refrigerator	❑
B	ELISA Dilution Buffer	Refrigerator	❑
C	Antigen (lyophilized)	Refrigerator	❑
D	Primary Antibody (lyophilized)	Refrigerator	❑
E	Secondary Antibody (lyophilized)	Refrigerator	❑
F	ABTS (lyophilized)	Refrigerator	❑
G	ABTS Reaction Buffer	Refrigerator	❑

Experiment #195 is designed for 10 groups.

REAGENTS & SUPPLIES

- Microtiter plates ❑
- Snap-top microcentrifuge tubes ❑
- 15 mL conical tubes ❑
- Transfer pipets ❑

Requirements *(NOT included with this kit)*

- Distilled or deionized water
- Beakers
- Disposable lab gloves
- Safety Goggles
- Paper Towels
- Recommended: Automatic micropipets (5-50 µL, 100-1000 µL) and tips

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.

Background Information

THE HISTORY OF FORENSIC TOXICOLOGY

Since antiquity, people have realized that certain chemical compounds are very potent poisons. Archaeological evidence shows ancient man used poisons as early as 4500 BCE to hasten the death of prey in hunting situations. The ancient Greeks used poison extracted from the hemlock plant to execute prisoners, most famously Socrates. Over time, poison became more common as a simple and fast way to remove political opponents. In fact, arsenic became known as "inheritance powder", given that many a frustrated person used the poison to speed up the death of a family member.

While humans have used chemicals for nefarious purposes for many years, tests for these substances did not exist until recent times. At autopsy, the coroner would examine the body for symptoms of the poisoning, but the symptoms often matched natural causes of death. For example, symptoms of arsenic poisoning – nausea, vomiting, and diarrhea – are consistent with cholera, a common disease of the time. Determined to distinguish between cholera and arsenic poisoning, chemist James Marsh devised a test to determine whether samples contained arsenic in the early 1800s (Figure 1). The samples in question are mixed with acid, which converts arsenic to arsine gas. Next, the mixture is heated, and a glass or porcelain plate is held above the sample. The heat reduces the arsine gas to metallic arsenic, which coats the plate with a shiny metallic film. This "arsenic mirror" is considered a positive result for arsenic.

The Marsh test would provide a crucial piece of evidence in a murder trial in the mid-1800s. Marie Capelle was charged with the murder of her husband, Charles Lafarge. During their betrothal, Charles boasted of his palatial estate in France. After the marriage, Marie realized that Lafarge had exaggerated his wealth – the palatial estate was a run-down monastery, and he only married Marie because her dowry would allow him to pay off debts. A few months after they wed, Charles Lafarge repeatedly fell ill with cholera-like symptoms. During this time, Marie tended to her invalid husband and prepared all of his food and beverages. Lafarge passed away from this mysterious ailment, leaving his family wondering whether Marie was responsible for his demise.

In time, the Lafarge family convinced the local authorities to investigate the death of Charles Lafarge as murder. The investigation uncovered that Marie Lafarge purchased arsenic to use as poison for rats. Although the coroner found arsenic in foods prepared by Marie, he could not prove that it caused Charles Lafarge's death. Mathieu Orfila, a renowned toxicologist, used the Marsh test to analyze samples taken from Lafarge's body. In his experiments, the Lafarge samples tested positive for arsenic, leading to a murder conviction for Marie. Because of the use of "modern" science to analyze samples, the Lafarge affair unofficially marks the birth of forensic toxicology.

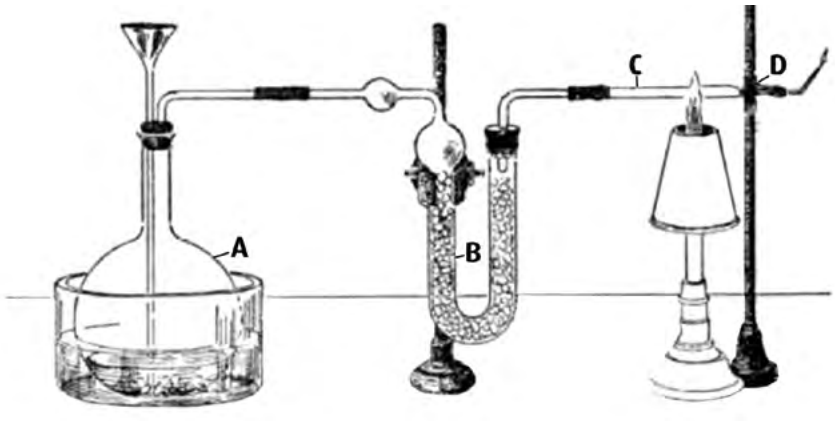


Figure 1: Marsh Test Apparatus. To form the arsenic mirror, a porcelain plate is placed over the valve labeled "D".

TOXICOLOGY IN TODAY'S FORENSIC SCIENCE LABORATORY

In today's forensic science laboratory, toxicologists identify drugs and toxins in tissues and body fluids collected from crime scenes, victims, and potential suspects. If present, the toxicologist also determines whether the drug or toxin

contributed to a person's behavioral changes or death. For example, biological samples from deceased people are analyzed for poisons, drugs and/or alcohol, which can provide evidence as to whether these substances contributed to death. Toxicologists will analyze samples from living people in criminal cases like DUI and drug-assisted sexual assault. These samples are important in establishing motive and intent in criminal investigations. Additionally, forensic toxicologists analyze unknown powders and fluids found at crime scenes to determine their composition.

Before being analyzed in the forensic science laboratory, samples must be carefully collected to prevent contamination or sample degradation. The sample is labeled and sealed in a tamper-proof evidence bag before being removed from the crime scene. A careful record is kept of all people who have handled the evidence as it is transferred from the crime scene to the forensic laboratory to make sure it remains unadulterated. While the samples are being analyzed, forensic toxicologists keep careful records of all tests performed, as their results may be used as evidence in the court of law.

Once in the forensic toxicology lab, scientists use modern biotechnology, pharmacology, and chemistry techniques to analyze crime scene samples. The first series of analysis identifies potential drugs and toxins in the crime scene sample using immunoassays like the **E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay, or ELISA (Table 1). Immunoassays can also detect metabolites (break-down products) of these chemicals to determine how long they have been in the body. For example, cocaine can only be detected for a few hours after use, whereas its metabolites are detectable in bodily fluids for several days after ingestion.

Table 1: Substances Tested For in Forensic Toxicology Labs

Substance	Examples
Illegal drugs	Methamphetamine, cocaine, ecstasy (MDMA)
Over-the-counter drugs	Ibuprofen, acetaminophen
Prescription drugs	Benzodiazepines, opiates, amphetamines, barbiturates
Alcohols	Ethanol, methanol, acetone
Drug Metabolites	Break-down products of drugs (i.e. heroin breaks down into morphine)

ELISAs use antibodies to recognize the antigen of interest in a complex crime scene sample (summarized in Figure 2). The sample of interest is added to the wells of a plastic plate, where its molecules non-specifically adhere to the wells through hydrophobic and electrostatic interactions. After washing away any excess fluid, the wells are "blocked" with a protein-containing buffer, which prevents non-specific interactions between the antibody and the plastic wells. Next, the primary antibody is added to the wells. This "primary" antibody will recognize and bind to the target molecule if present within the sample.

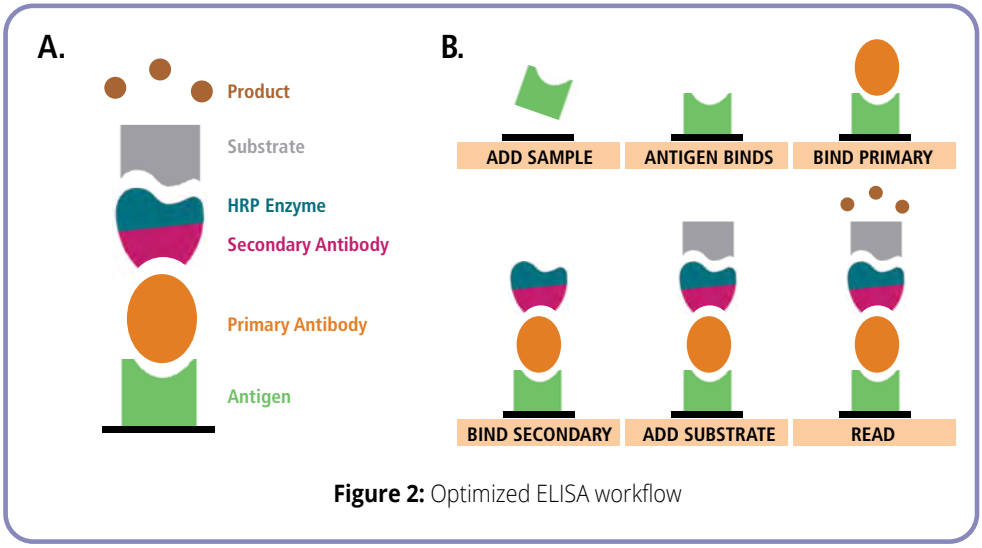


Figure 2: Optimized ELISA workflow

After an incubation period, the wells are washed to remove any primary antibody that did not bind. The secondary antibody is added to the wells where it recognizes and binds to the primary. Excess antibody is removed from the wells by washing several times with buffer. If the secondary antibody has bound to the primary antibody, it will stay in the well.

The secondary antibody is covalently linked to a detection enzyme (in this experiment, Horseradish Peroxidase, or HRP). To detect antibody-antigen interactions, a clear, colorless solution of hydrogen peroxide and 2,2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid), or ABTS, is added to each well. In wells where the enzyme-linked secondary antibody is present, HRP converts hydrogen peroxide (its substrate) to $H_2O + O_2$. This oxidizes the ABTS, turning the clear substrate solution to green.

HRP has a high catalytic activity – its substrate turnover rates exceed 10^6 per second – allowing us to quickly identify any antigen present within the crime scene sample. Since each enzyme breaks down many substrate molecules, the ELISA can detect even the smallest amount of antigen. If a preliminary immunoassay is positive for drugs or toxins, the sample in question is further analyzed using gas chromatography-mass spectroscopy (GC-MS). This analytical technique identifies and quantitates any compounds present in the sample, confirming the earlier ELISA results.

THE CASE OF THE MISSING LAB NOTEBOOK

Late one night, an intrepid scientist worked on an important biotechnology experiment in the laboratory. She was very close to creating a groundbreaking medication that could save many lives. Invigorated by her work, the scientist decided to pull an all-nighter in the lab. The next morning, her labmate found her sound asleep at her desk, with important pages ripped from her lab notebook. Furthermore, security footage showed that someone had stolen some critical reagents from the laboratory. After waking, the scientist insisted a drug had been slipped into her food or drink. Results from a blood test showed that she had ingested a powerful sleeping pill. The detectives believe that the scientist was drugged because the sleeping pill is not something that she is normally prescribed.

Upon investigating the crime scene, the police found the following items at her desk: a bottle of water, a half-eaten sandwich, a cold cup of coffee, a bottle of pain relievers, and a bowl of soup. According to the security footage, a different individual brought each of these items to her. Detectives identified these items as potential vehicles for poisoning and collected them as evidence to be analyzed. As a forensic toxicologist, you will perform the ELISA on these samples. The resulting evidence will be presented as a toxicology report in the court of law.

Experiment Overview

EXPERIMENT OBJECTIVE:

In this forensic science experiment, students will use the Enzyme-Linked Immunosorbent Assay (ELISA) to analyze crime scene samples for the presence of drugs.

LABORATORY SAFETY

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
3. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

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.

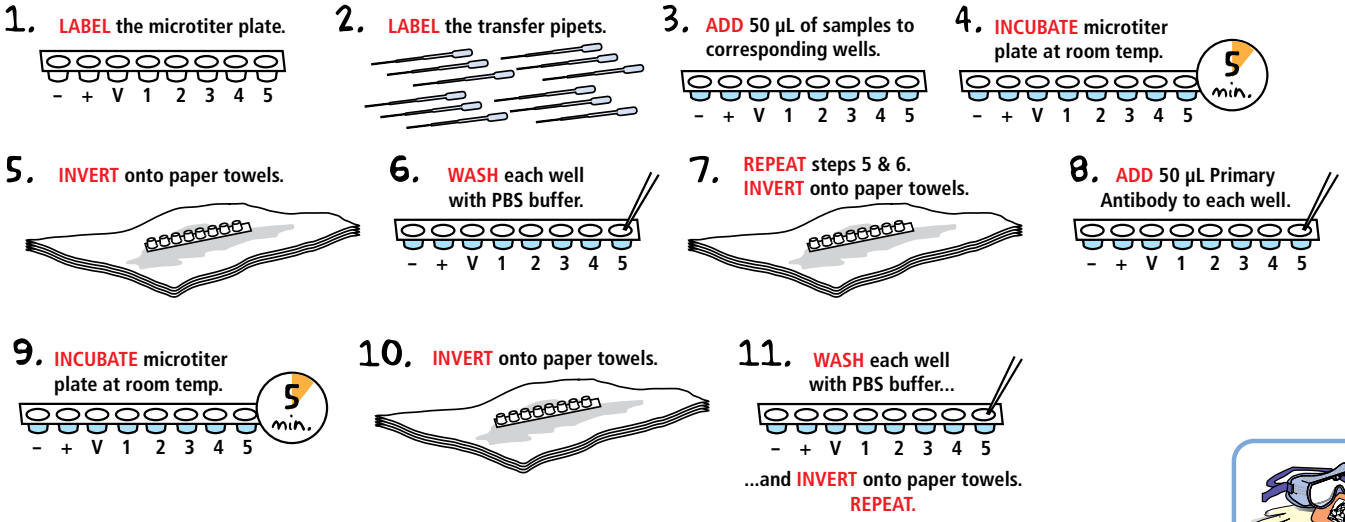
During the Experiment:

- 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.

Testing for Drugs Using the ELISA



Wear gloves and safety goggles

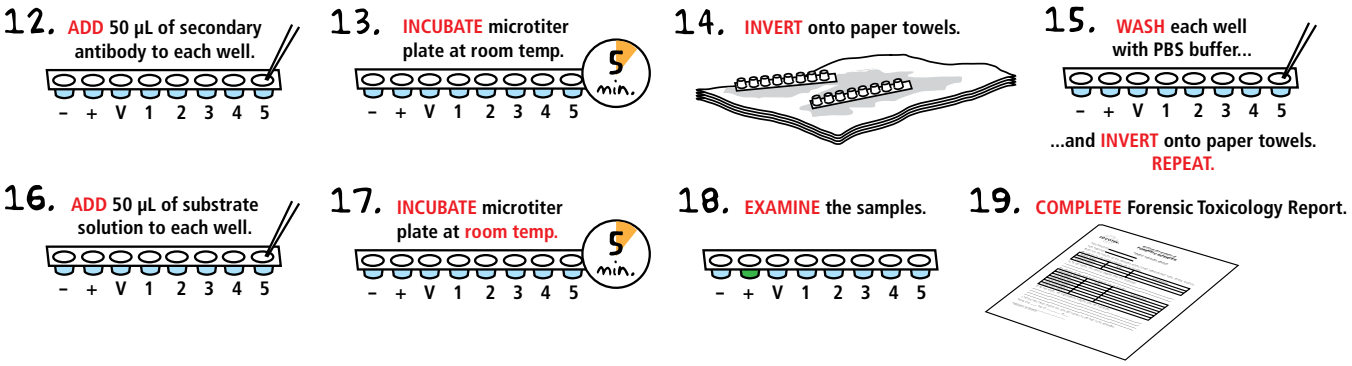
1. **LABEL** the wells of the microtiter plate as shown above.
2. If not using an adjustable volume micropipet, **LABEL** the transfer pipets as outlined in the box below. These 12 pipets will be used to add and remove liquid from the wells.

(–)	Negative Control	(PA)	used to add the primary antibody
(+)	Positive Control	(SA)	used to add the secondary antibody
(V)	Victim Sample	(PBS)	used to add PBS buffer to each well
(1)	Crime Scene Sample 1	(SUB)	used to add substrate to each well
(2)	Crime Scene Sample 2		
(3)	Crime Scene Sample 3		
(4)	Crime Scene Sample 4		
(5)	Crime Scene Sample 5		

3. **ADD** 3 drops (50 µL) of each of the samples to the corresponding well. Be sure to change tips between samples.
4. **INCUBATE** the plate at room temperature for five minutes.
5. **INVERT** the 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.
6. **WASH** each well by adding the PBS buffer to each well until it is almost full (~ 200 µL). Do not allow the buffer to spill over into adjacent wells.
7. **REPEAT** steps 5 and 6 to wash the wells once more. **INVERT** the strip over a stack of paper towels.
8. Using the labeled transfer pipet or a micropipet, **ADD** 3 drops (50 µL) of the primary antibody to each well.
9. **INCUBATE** the plate at room temperature for 5 minutes.
10. Repeat step 5 to **REMOVE** the primary antibody from the wells.
11. **WASH** each well twice with fresh PBS buffer. Between washes, **INVERT** the strip gently onto a fresh paper towel to remove any remaining sample.

OPTIONAL STOPPING POINT: For overnight storage, ADD 200 µL of PBS to each well. Carefully cover the samples and leave the plate at 4° C. The experiment should be resumed during the next lab period. Remove the PBS and continue with step 12.

Testing for Drugs Using the ELISA, continued



12. Using the labeled transfer pipet or a micropipet, **ADD** 3 drops (50 µL) of the secondary antibody solution to each well.
13. **INCUBATE** the plate at room temperature for 5 minutes.
14. Repeat step 5 to **REMOVE** the secondary antibody from the wells.
15. **WASH** each well twice with fresh PBS buffer. Between washes, **INVERT** the strip gently onto a fresh paper towel to remove any remaining sample.
16. Using the labeled transfer pipet or a micropipet, **ADD** 3 drops (50 µL) of the substrate solution (ABTS) to each well.
17. **INCUBATE** the plate at room temperature for 5 minutes or until you see a color change.
18. **EXAMINE** the samples. Negative tests will remain clear and positive tests will appear green in color. **RECORD** your results in Table 2, below.
19. Using the data from your experiment, **COMPLETE** the Forensic Toxicology Report (page 10).

Table 2: Experimental Results

Sample	Color	Interpretation
A. Negative Control Sample		
B. Positive Control Sample		
C. Victim Sample		
D. Crime Scene Sample 1		
E. Crime Scene Sample 2		
F. Crime Scene Sample 3		
G. Crime Scene Sample 4		
H. Crime Scene Sample 5		

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EDVOTEK DEPARTMENT OF
FORENSIC SCIENCES

FORENSIC TOXICOLOGY ANALYSIS

Date Received: _____

Date Reported: _____

Below are the results of the requested enzyme linked immmunosorbent assay (ELISA) analysis.

The results of the control samples are as follows:

Control Sample	Color	Interpretation
Positive Control		
Negative control		

In my own words, the control samples show the following:

_____

_____

The results of the experimental samples are as follows:

Experimental Sample	Color	Interpretation
Victim Sample		
Crime Scene Sample 1		
Crime Scene Sample 2		
Crime Scene Sample 3		
Crime Scene Sample 4		
Crime Scene Sample 5		

In my own words, the experimental samples show the following:

_____

_____

_____

_____

I affirm that these results are true and correct to the best of my knowledge.

Dated this _____ day of _____, 20____

_____

Signature of Forensic Scientist

Study Questions

1. Why was the development of the Marsh test important for forensic science?
2. Why would forensic toxicology be important for criminal cases?
3. What is the ELISA? Describe the purpose of each component used in the ELISA.

Instructor's Guide

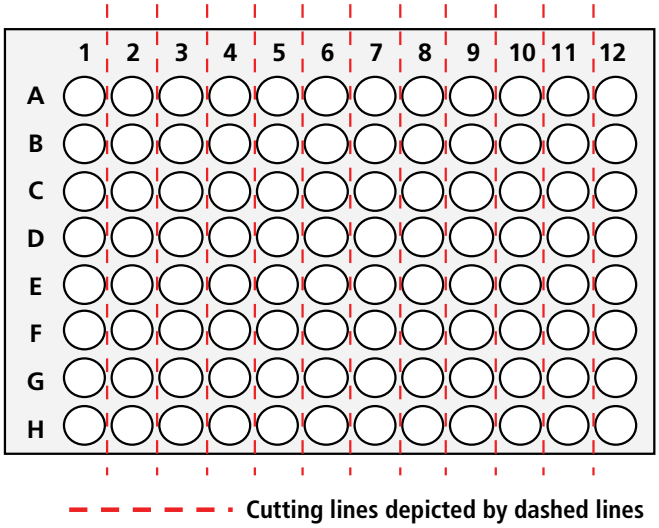
OVERVIEW OF INSTRUCTOR'S PRELAB PREPARATION:

This section outlines the recommended prelab preparations and approximate time requirement to complete each prelab activity.

Preparation for:	What to do:	When:	Time Required:
Testing for Drugs Using the ELISA	Divide microtiter plates	Anytime before the class	5 minutes
	Dilute ELISA Wash Buffer	Anytime before the class	5 minutes
	Prepare Antigen	Up to 1 week before the class	5 minutes
	Prepare Primary Antibody	Up to 1 week before the class	5 minutes
	Prepare Secondary Antibody	Up to 1 day before the class	5 minutes
	Aliquot Dilution Buffer, TMB, and Stop Solution	Up to 1 week before the class	5 minutes

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

Prelab Preparations



Preparing the Microtiter Plate

1. As shown in the figure (right), orient the microtiter plate so that the numbers 1-12 are at the top and the letters A-H are on your left.
2. Cut each plate on the dotted line as show in the figure. Each piece will have eight wells on one axis and one on the other axis. Each lab group will receive one piece.

Preparation of 1X ELISA Wash Buffer

1. Add all of the 10X ELISA Wash Buffer (component A) to 180 mL of distilled water and mix well. Label as "1X wash buffer".
2. Dispense 18 mL into small beakers for each group and label "wash buffer".

Preparation of the Crime Scene and the Control Samples

NOTE: in this simulation, we have designated crime scene sample 3 to be the positive sample, however you may choose whichever crime scene sample fits within your classroom scenario. In the scenario provided in the background, the crime scene samples are: (1) bottle of water, (2) half-eaten sandwich, (3) cup of coffee, (4) bottle of pain relievers, (5) bowl of soup. Positive samples will be reconstituted antigen while negative samples will be dilution buffer.

1. Transfer 7 mL of ELISA Dilution Buffer (component B) into a 15 mL conical tube labeled "Antigen".
2. Carefully remove the stopper from the vial of Antigen (component C). Transfer 0.5 mL from the conical tube "Antigen" to the vial. Close the stopper and shake gently to mix.
3. Transfer the entire contents of the reconstituted Antigen back to the 15 mL conical tube from Step 1. Mix well.
4. Label 80 snap-top microcentrifuge tubes as follows:
   a. 10 – Negative Control (-)
   b. 10 – Positive Control (+)
   c. 10 – Victim Sample (V)
   d. 10 – Crime Scene Sample 1 (CS1)
   e. 10 – Crime Scene Sample 2 (CS2)
   f. 10 – Crime Scene Sample 3 (CS3)
   g. 10 – Crime Scene Sample 4 (CS4)
   h. 10 – Crime Scene Sample 5 (CS5)
5. Dispense 100 µL of ELISA Dilution Buffer (component B) into the tubes labeled "Negative Control", "Crime Scene Sample 1", "Crime Scene Sample 2", "Crime Scene Sample 4", and "Crime Scene Sample 5".
6. Dispense 100 µL from "Antigen" 15 mL conical tube into the tubes labeled "Positive Control", "Victim Sample", and "Crime Scene Sample 3".

Prelab Preparations, continued

Preparation of the Primary Antibody

1. Transfer 7 mL of ELISA Dilution Buffer (Component B) to a 15 mL conical tube. Label the conical tube "1°AB".
2. Carefully remove the stopper from the vial of Primary Antibody (Component D). Transfer 0.5 mL from the conical tube "1°AB" to the vial. Close the stopper and gently sake the vial to mix.
3. Transfer the entire contents of the reconstituted Primary Antibody back to the 15 mL conical tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "1°AB" and dispense 0.5 mL per tube.

Dilution of the Secondary Antibody

(Prepare on the same day as needed for the experiments.)

1. Transfer 7 mL of ELISA Dilution Buffer (component B) to a 15 mL conical tube. Label the conical tube "2°AB".
2. Carefully remove the stopper from the vial of Secondary Antibody (component E). Transfer 0.5 mL from the conical tube "2°AB" to the vial. Close the stopper and gently shake the vial to mix.
3. Transfer the entire contents of the reconstituted Secondary Antibody back to the 15 mL conical tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "2°AB" and dispense 0.5 mL per tube.

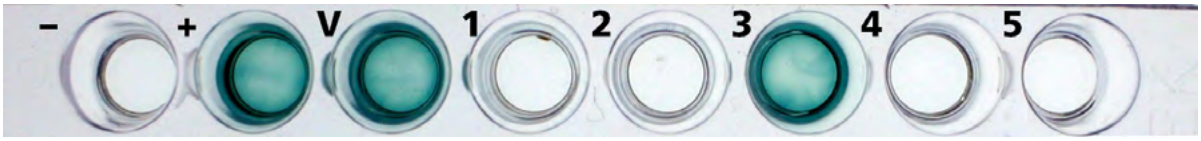
Preparation of ABTS Substrate

1. Transfer 7 mL of ABTS Reaction Buffer (component G) into a 15 mL conical tube labeled "ABTS".
2. Remove 0.5 mL of ABTS Reaction Buffer from the conical tube above and add to vial of ABTS (component F). Close the stopper and gently shake the vial to mix.
3. Transfer the entire contents of the reconstituted ABTS back to the 15 mL conical tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "ABTS" and dispense 0.5 mL per tube.

Each Student Group should receive:

- 1 Beaker containing Wash Buffer
- 8 Snap-top microcentrifuge tubes containing 100 µL of each sample
- 1 Snap-top microcentrifuge tube containing 0.5 mL of the Primary Antibody
- 1 Snap-top microcentrifuge tube containing 0.5 mL of the Secondary Antibody
- 1 Snap-top microcentrifuge tube containing 0.5 mL of ABTS Substrate
- 12 Small transfer pipets or 1 Automatic micropipet and tips
- 1 Stack of paper towels for washes and waste
- 1 8-well microtiter strip

Experiment Results and Analysis



	Component	Sample	Color	Interpretation
A	Negative Control Sample		Clear	No sleeping pill
B	Positive Control Sample		Green	Sleeping pill present
C	Victim Sample		Green	Sleeping pill present
D	Crime Scene Sample 1	Bottle of water	Clear	No sleeping pill
E	Crime Scene Sample 2	Half eaten sandwich	Clear	No sleeping pill
F	Crime Scene Sample 3	Cup of coffee	Green	Sleeping pill present
G	Crime Scene Sample 4	Bottle of pain relievers	Clear	No sleeping pill
H	Crime Scene Sample 5	Bowl of soup	Clear	No sleeping pill

Samples that test positive for the sleeping pill by ELISA will turn green in color within five minutes. The results show that the Crime Scene Sample 3 (cup of coffee) tests positive for the sleeping pills. This evidence suggests that the victim was drugged by the person who brought her the cup of coffee.

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

Appendix A
EDVOTEK® Troubleshooting Guide

PROBLEM:	CAUSE:	ANSWER:
Cross-contamination: Color develops in negative controls	Used wrong transfer pipet.	Be careful to use the correct transfer pipet. Alternatively, use an adjustable-volume micropipette and change tips between samples.
	Too much force was used to wash out wells.	Wash the wells gently and slowly.
	Cross contamination while emptying microtiter plate onto paper towel.	Invert the wells gently and slowly. Alternatively, use transfer pipets to remove liquids from the wells.
Color doesn't develop or is slow to develop.	Incubation time too short.	Incubate ELISA at room temperature for five more minutes.