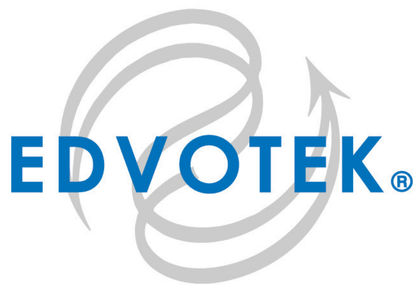

THE **BIOTECHNOLOGY EDUCATION** COMPANY®

REVISED & UPDATED

Edvo-Kit # 280

Edvo-Kit #280

Detecting the Silent Killer: Clinical Diagnosis of Diabetes

Experiment Objective:

In this experiment, students will distinguish between the two main types of diabetes using simulated urinalysis and ELISA tests.

See page 3 for storage instructions.

SAMPLE LITERATURE
Please refer to included weblink for correct version.

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

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Experiment Components

Module II Components	Storage	Check (√)
A Urinalysis Powder 1	Room Temp.	❑
B Urinalysis Powder 2	Room Temp.	❑
C Urinalysis Powder 3	Room Temp.	❑
D Diabetic Urine Powder	Room Temp.	❑
E Yellow Dye	Room Temp.	❑

Module III Components	Storage	Check (√)
F 10X ELISA Wash Buffer	Refrigerator	❑
G ELISA Dilution Buffer	Refrigerator	❑
H Antigen (lyophilized)	Refrigerator	❑
I Primary Antibody (lyophilized)	Refrigerator	❑
J Secondary Antibody (lyophilized)	Refrigerator	❑
K ABTS (lyophilized)	Refrigerator	❑
L ABTS Reaction Buffer	Refrigerator	❑

Experiment #280 contains material for up to 10 lab groups.

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.

REAGENTS & SUPPLIES

Store all components below at room temperature.

Component	Check (√)
• Microtiter plates	❑
• Snap-top Microcentrifuge tubes (1.5 mL)	❑
• Screw-top Microcentrifuge tubes (1.5 mL)	❑
• 15 mL conical tubes	❑
• Transfer pipets	❑

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Requirements *(NOT included in this experiment)*

- Distilled or deionized water
- Beakers
- 37° C Incubator
- 99° C Waterbath
- Pipet pump or bulb
- Lab glassware
- Disposable lab gloves
- Safety goggles
- Recommended: Automatic micropipettes (0-50 μL, 100-1000 μL) and tips

Make sure glassware is clean, dry and free of soap residue.

For convenience, additional disposable transfer pipets can be purchased for liquid removal and washing steps.

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Background Information

Detecting the "Silent Killer": A Simulation of the Clinical Detection of Diabetes

Diabetes mellitus, commonly called the "Silent Killer", is a chronic disease that leads to increased levels of sugar (glucose) in the blood. It is estimated that 8.3% of the United States population is diabetic, of which 7 million are undiagnosed. In addition, this disease affects millions of people around the world, both young and old.

Blood sugar levels are regulated by insulin, a hormone that is synthesized in and secreted by the beta cells in the pancreas. Mature insulin consists of two distinct protein chains that are linked by disulfide bonds. However, insulin is initially synthesized as a single protein chain called preproinsulin, which consists of four domains: a signal peptide, the carboxyl-terminal a chain, the amino-terminal B chain, and the C-peptide that connects the two terminals (Figure 1). The signal peptide at the amino terminal facilitates the transport of preproinsulin into the endoplasmic reticulum to process insulin. Within the endoplasmic reticulum, enzymes known as signal peptidases remove the signal peptide to form what is called, proinsulin (Figure 1A). Subsequently, disulfide bonds form between the A and B chains. The next cleavage occurs by enzymes called endopeptidases that remove the C-peptide from proinsulin (Figure 1B), producing the mature form of insulin (Figure 1C).

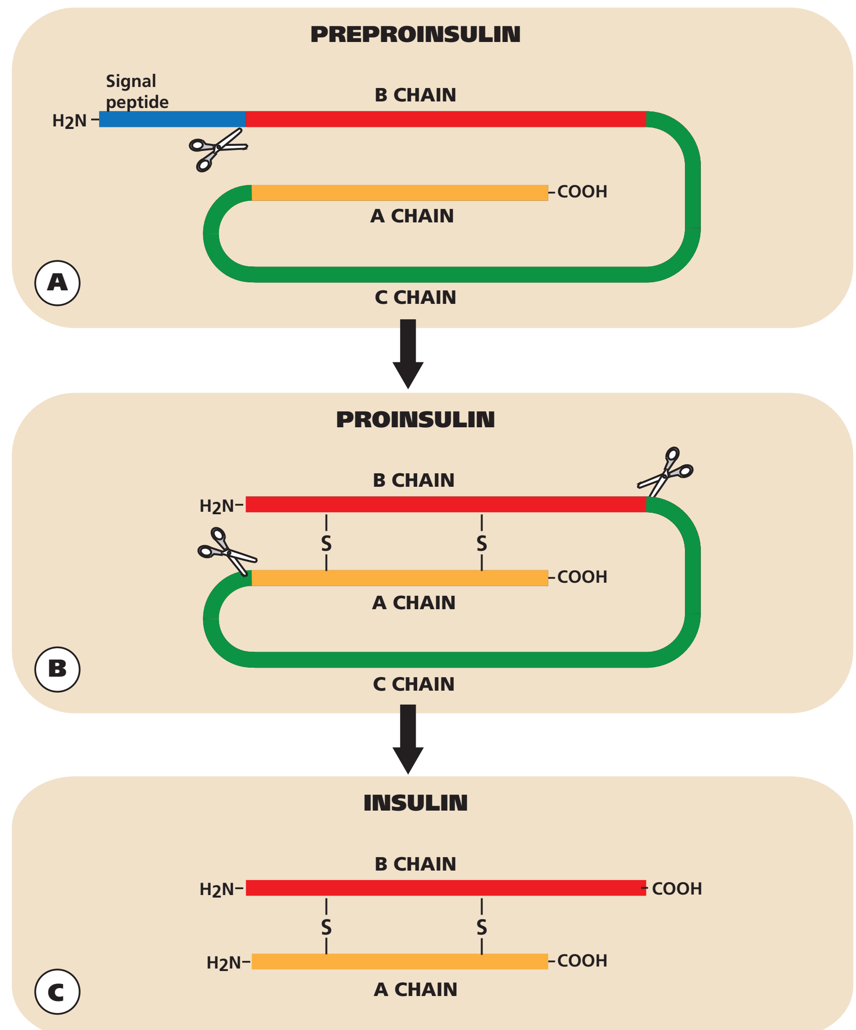

Figure 1: Maturation of Insulin.

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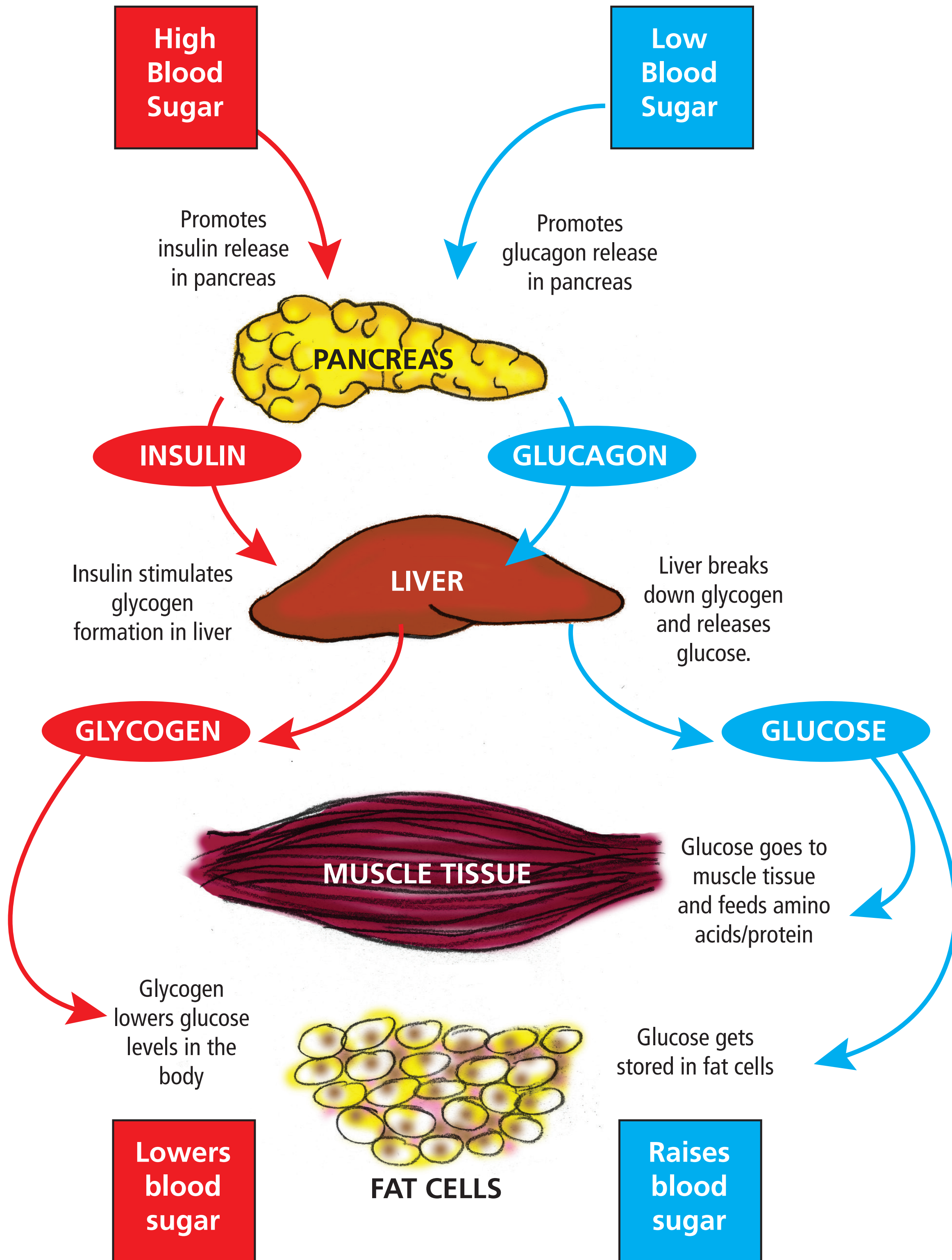

Figure 2: Glucose Homeostasis.

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In healthy individuals, insulin is stored in the pancreas until blood glucose levels begin to rise (e.g. after a meal). At this time, the insulin is secreted (Figure 2). Once in the blood stream, insulin signals the surrounding cells to import glucose (Figure 3A). The cells immediately break down some of glucose to produce energy. Excess glucose is generally stored in one of two forms: glycogen and triacylglycerols. Glucose monomers can assemble into a branched polysaccharide molecule called glycogen, which is stored in liver and muscle tissues. Excess glucose stimulates the conversion of free fatty acids to triacylglycerols, which are stored as fat in adipose tissue. When glucose levels are low, the hormone glucagon stimulates the release of the stored energy from the liver, muscle, and adipose tissues (Figure 2).

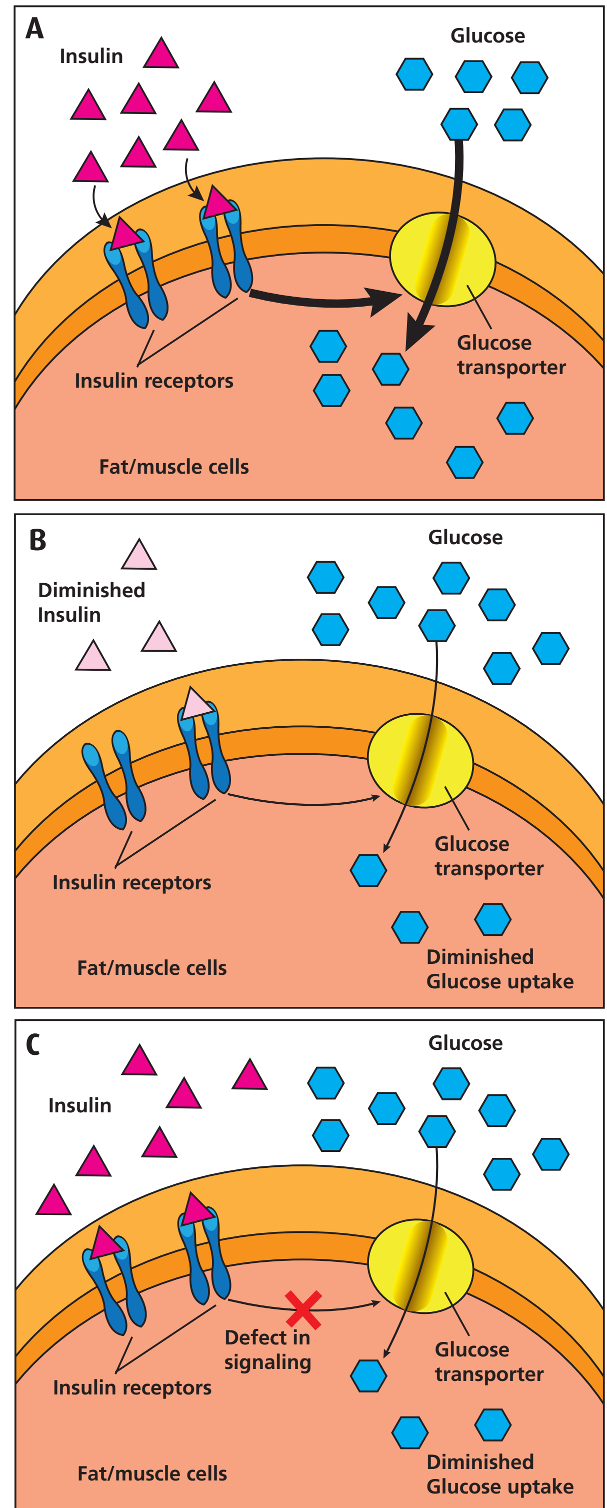

Figure 3: The effect of insulin levels on glucose uptake.

Diabetes occurs when the body becomes unable to regulate the level of glucose in the blood. The most common forms of diabetes are Type 1 and Type 2 diabetes. Type 1 diabetes, also known as juvenile or insulin-dependent diabetes, occurs in childhood and is more severe. In Type 1 diabetes, the beta cells undergo autoimmune destruction progressively and the pancreas releases little or no insulin (Figure 3b). Due to this, the body starts relying on energy derived from the breakdown of fats stored in the adipose cells. Ketones are formed as byproducts of the fat being metabolized. An excess of ketones in the body results in a condition known as ketoacidosis, which can lead to a diabetic coma, or even death. To prevent this, patients with Type 1 diabetes rely on daily injections of insulin for proper glucose metabolism. Some of the symptoms of Type 1 diabetes include excessive thirst (polydipsia), frequent urination (polyuria), high blood sugar (hyperglycemia), fatigue and weight loss.

Type 2 diabetes occurs when the body becomes resistant to insulin, or if the pancreas is not producing enough insulin (Figure 3C). The symptoms of type 2 diabetes are similar to that of Type 1, but they often go unrecognized because of the slow progression of the disease. Individuals affected by Type 2 diabetes may have a genetic predisposition to diabetes, but environmental factors—such as an unhealthy lifestyle— also trigger the development of this life-long disease. Due to sedentary lifestyles and the convenience of fast food restaurants, many overweight adults (>25 BMI) over the age of 40 are at a high risk of developing Type 2 diabetes. Although some patients with Type 2 diabetes can regulate the condition with lifestyle changes, most will rely on daily insulin injections to metabolize glucose.

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Diagnosing Diabetes

If a person experiences symptoms that suggest diabetes, a physician first will determine whether the patient has high blood sugar. Blood glucose levels can be monitored with a simple urine test, as high levels of glucose cannot be reabsorbed by the kidney. As such, excess glucose is excreted into the urine (glucosuria). The patient's urine sample is analyzed using a reagent that undergoes a chemical conversion in the presence of glucose and/or ketones, producing a dramatic color change. The final color of the sample indicates the level of glucose present in the urine (Figure 4). A healthy individual will have a fasting glucose level of around 75-100 milligrams per deciliter (mg/dL). If the urine test indicates an elevated level of sugars and ketones (usually around 125 mg/dL), blood is drawn and sent for further analysis before a formal diagnosis is made.

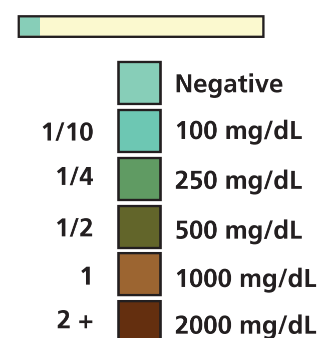

Figure 4: Example of Urinalysis Testing for Diabetes.

The Enzyme-Linked Immunosorbent Assay (or ELISA) uses antibodies to detect the presence of specific biomolecules (i.e. peptides, proteins, antigens and hormones) in a complex sample. This highly sensitive assay can detect the presence of the C-peptide in a patient's blood sample. Since a patient with Type I diabetes will not produce any insulin, the C-peptide is not detected in the blood sample. In contrast, patients with Type II diabetes still produce a low amount of insulin, so the test will detect low amounts of the C-peptide in the blood. In this way, the ELISA can be used to differentiate between Type I and Type II diabetes.

One of the most sensitive ELISA techniques is the sandwich ELISA, in which two separate antibodies are used to detect the antigen – one antibody that is bound to the plate to "capture" the antigen, and one that is used to detect it (Figure 5). First, the capture antibody added to the wells of a transparent plastic microtiter plate. The antibody non-specifically adheres to the plastic through hydrophobic and electrostatic interactions. Any unbound antibody is washed out with a non-reacting buffer. Next, the wells are "blocked" with a protein-containing buffer (commonly casein or bovine serum albumin) to prevent non-specific interactions between the sample and the plastic wells. Following the blocking step, the patient samples are added to the wells. The bound antibody recognizes a specific area of the C-peptide (called an epitope) and non-covalently binds. After the incubation period, the wells are washed to remove excess sample that did not bind.

Next, the purified detection antibody is added and allowed to bind with the antigen. After a short incubation period, any unbound antibody is washed away with buffer. The detection antibody is covalently linked to an enzyme like Horseradish Peroxidase (HRP) that allows for the detection of the antibody-antigen complex. A clear, colorless substrate solution, containing hydrogen peroxide and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), or ABTS, is added to each well. In wells where the secondary antibody is present, the HRP will convert hydrogen peroxide to $H_2O + O_2$. This oxidizes the ABTS, turning the clear substrate solution to green. HRP has a high catalytic activity, with its substrate turnover rates exceeding 10^6 per second, allowing us to quickly detect even the smallest amount of C-peptide.

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The following experiments simulate the medical testing performed by doctors to diagnose Type I or Type II Diabetes. After collecting the patient histories (Module I), students will receive simulated urine samples of patients and use a chemical reagent to distinguish between the diabetic and non-diabetic condition (Module II). In the final part of the experiment (Module III), students will use the ELISA to detect the presence of the C-peptide in patient samples. The data collected in the three modules will allow the students to diagnose the patients as healthy, Type I or Type II Diabetic.

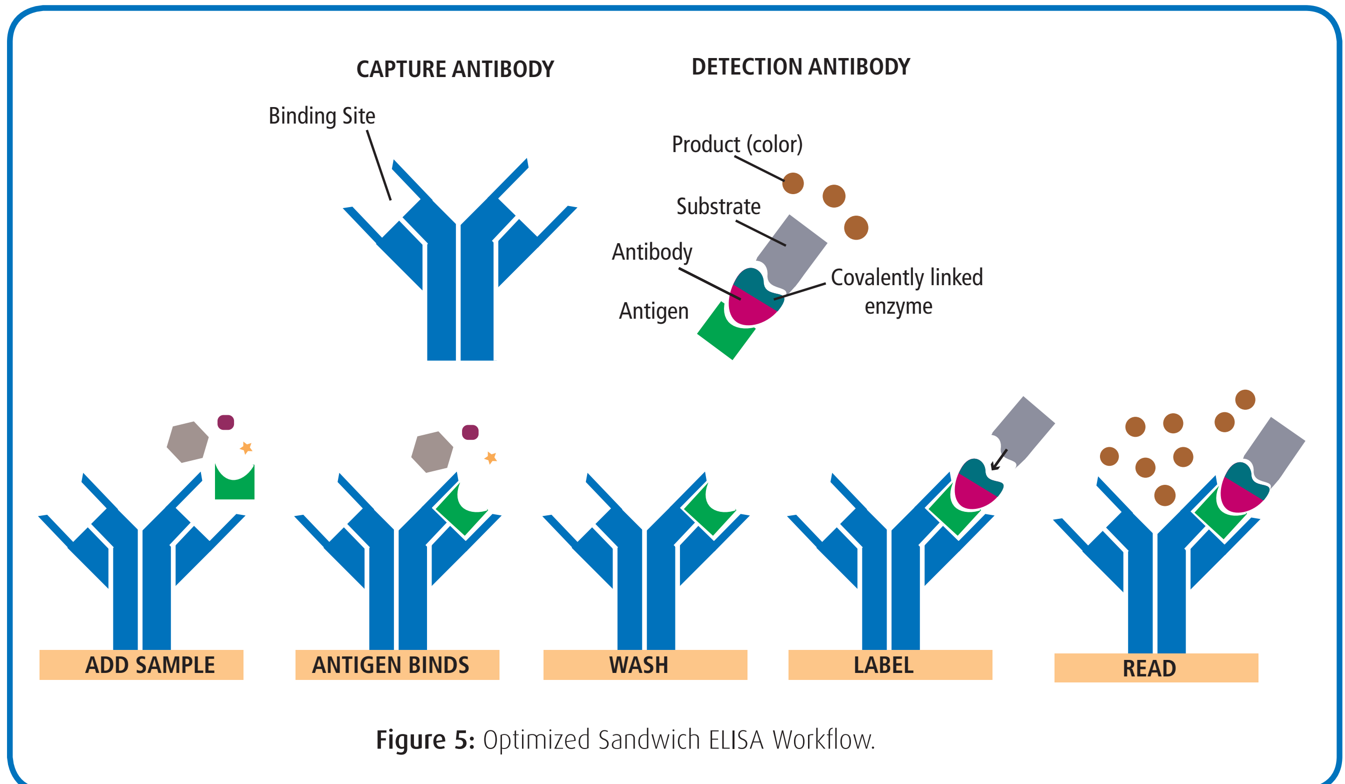

Figure 5: Optimized Sandwich ELISA Workflow.

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Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment, students will distinguish between the two main types of diabetes using simulated urinalysis and ELISA tests.

LABORATORY SAFETY

Wear gloves and safety goggles

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

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.

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Module I: Patient History

REVIEW the Patient History **BEFORE** performing the experiment.

Three patients were examined in the doctor's office, and the following symptoms were identified.

Patient 1 50 year old male, average weight, symptoms include excessive urination. Patient is very active. Last physical indicated high blood pressure.

Patient 2 12 year old female, under weight, symptoms include excessive thirst and dramatic weight loss. Parents report the child often falls asleep in class.

Patient 3 50 year old male, overweight, symptoms include excessive urination. Patient is sedentary. Last physical indicated borderline high blood sugar.

Each patient has several symptoms, some which are indicative of diabetes. Because of these symptoms, the primary care physician has recommended further testing before a diagnosis can be made. **RECORD** any symptoms that suggest diabetes in Table 1 before performing the medical tests.

When the patient arrives for further testing, they will provide a urine sample (Module II) and a blood sample (Module III) for analysis. In practice, the patients are required to fast for 12 hours before testing for diabetes. This allows the clinician to establish a patient's baseline blood sugar level. If the patient were to eat right before the tests were performed, his/her blood sugar levels would be high because of the body's response to food. This would mask the presence of high blood sugar.

TABLE 1: Patient Medical Records

Sample	Symptoms	Urinalysis	ELISA	Diagnosis
Patient 1				
Patient 2				
Patient 3				

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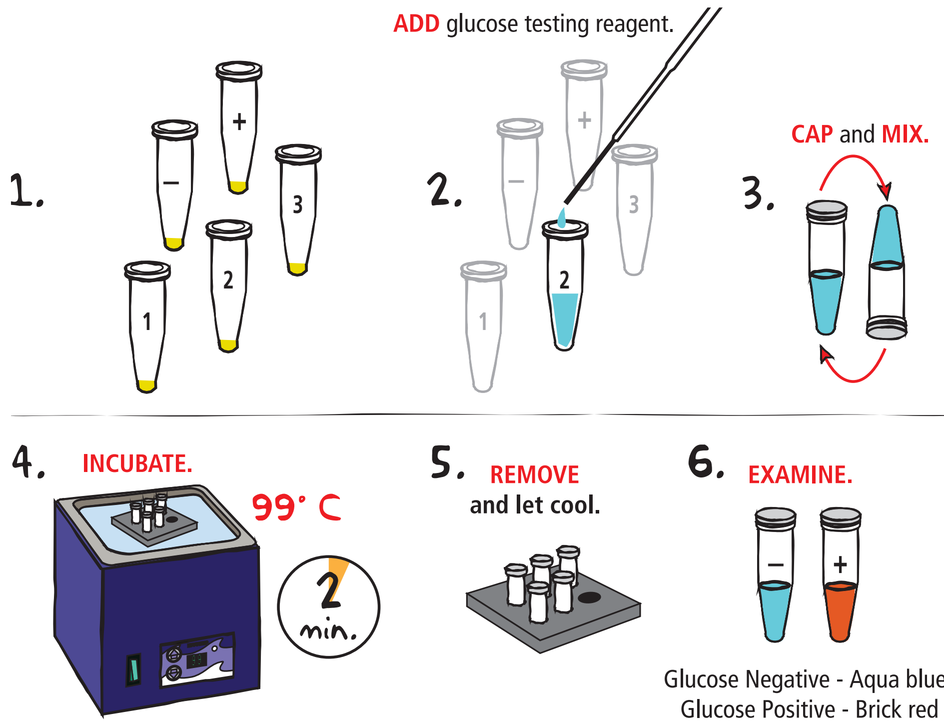
Module II: Urine Glucose Testing



1. **PICK UP** the patient samples from your instructor. They should be labeled as follows:
   (-) Negative Control
   (+) Positive Control
   (1) Patient 1
   (2) Patient 2
   (3) Patient 3
2. **ADD** 750 µL glucose testing reagent to each tube.
3. **SCREW** the lids on tightly. **MIX** the samples by inversion.
4. **INCUBATE** the samples in a 99° C water bath for two minutes.
5. Carefully **REMOVE** the samples from the water bath and place them on your bench to cool.
6. **EXAMINE** the samples. Negative samples will remain blue, while positive samples will turn brick red. **RECORD** your results in Table 1 on page 11.

NOTE: A brownish precipitate may appear in the samples. Mix the samples well and proceed with the data analysis. This will not interfere with the results.

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Module III: C-Peptide Detection by ELISA

1. – + P1 P2 P3
2. CAP WASH SUB DET – + P1 P2 P3
3. ADD CAPTURE ANTIBODY 5 min. INCUBATE
4.
5. WASH
6.
7. REPEAT wash steps 5 & 6.
8. ADD PATIENT SAMPLES 5 min. INCUBATE
9. INVERT then WASH and INVERT two times.

Serum sample	Label	Well
Negative control	-	1
Positive control	+	2
Patient 1	P1	3
Patient 2	P2	4
Patient 3	P3	5

1. **LABEL** the wells of the microtiter plate as shown in diagram.
2. **LABEL** the transfer pipets as follows. These pipets will be used to add and remove liquid from the wells.

(–)	Negative Control	(CAP)	used to add/remove the capture antibody
(+)	Positive Control	(WASH)	used to add wash buffer to each well
(P1)	Patient 1	(SUB)	used to add substrate to each well
(P2)	Patient 2	(DET)	used to add detection antibody to each well
(P3)	Patient 3		

3. **ADD** 50 μL of the capture antibody solution (CAP) to all of the wells. (If using transfer pipets, three drops is approx. 50 μL). **INCUBATE** the plate at room temperature for 5 minutes.
4. **INVERT** the strip over the sink or a stack of paper towels to remove the samples. Gently **TAP** the strip 4-5 times onto a fresh paper towel. **DISCARD** the wet paper towels.
5. Using the WASH transfer pipet, **ADD** wash buffer to fill each well, being careful not to overfill.
   NOTE: To minimize cross-contamination it is important that you avoid spilling buffer into neighboring wells.
6. **REPEAT** step 4 to **REMOVE** the wash buffer.
7. Using the same transfer pipet, **REPEAT** the wash a second time. **INVERT** strip onto fresh paper towels and **TAP.**
8. Using the appropriately labeled transfer pipet, **ADD** 50 μL of each of the controls and patient samples to the appropriate well (see above table). **INCUBATE** the plate for 5 minutes at room temperature.
9. **INVERT** onto paper towels and **TAP**. **WASH** the wells twice as in steps 4-7.

OPTIONAL STOPPING POINT: For overnight storage, **ADD** 200 μL of PBS to each well. Carefully **COVER** the samples and leave the plate at room temperature. The experiment should be resumed during the next lab period. **REMOVE** the PBS before continuing with step 10.

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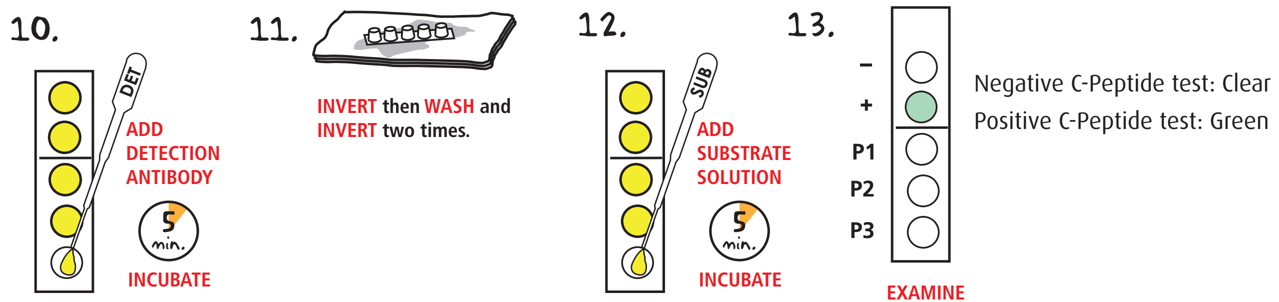
Module III: C-Peptide Detection by ELISA, continued



10. **ADD** 50 μL of the detection antibody solution (DET) to each well. **INCUBATE** the plate for 5 minutes at room temperature.
11. **INVERT** onto paper towels and **TAP**. **WASH** the wells twice as in steps 4-7.
12. **ADD** 50 μL of the substrate solution (SUB) to each well. **INCUBATE** the plate for 5 minutes at room temperature.
13. **EXAMINE** your results. Negative tests will remain clear and positive tests will appear green in color. **RECORD** your results in Table 1 on page 11.

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Study Questions

1. Compare and contrast Types I and II Diabetes.

2. Why do you need both the urine and blood test to diagnose diabetes?

3. Why is the signal peptide important? What would happen if preproinsulin did not have the signal peptide?

4. What is the ELISA test and how is it used in diagnosing diabetes?

5. You have collected a medical history from three different patients and performed the urinalysis and ELISA on samples from them. Knowing their test results and the patient medical history, make a diagnosis for each of the patients.

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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:
Module I: Collecting the Patient Histories	Photocopy/print patient history table.	Before the class period.	5 min.
Module II: Urine Glucose Test	Prepare and Aliquot Reagents.	Up to 1 day before performing the experiment.	30 min.
Module II: ELISA for the C-peptide	Divide microtiter plate.	Before the class period.	15 min.
	Dilute 10X ELISA Wash Buffer to 1X solution and aliquot.	Anytime before the experiment. Cover and store in the refrigerator.	5 min.
	Aliquot ELISA Dilution Buffer for negative control and patient samples.	Anytime before the experiment. Store tubes in the refrigerator.	5 min.
	Rehydrate and aliquot the Antigen.	Up to 1 week before performing the experiment.	10 min.
	Rehydrate and aliquot the Primary Antibody.	Up to 1 week before performing the experiment.	5 min.
	Rehydrate and aliquot the Secondary Antibody.	Up to 1 day before performing the experiment.	5 min.
	Rehydrate and aliquot the ABTS Substrate.	Up to 1 week before performing the experiment.	5 min.

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: Modules I & II

MODULE I: PATIENT HISTORY

- Print or photocopy patient history page.
- Students will record the patient histories in the provided table or a similar table in the student's notebook.

MODULE II: URINE GLUCOSE TEST

Preparation of Glucose Testing Reagent:

1. In a 150 mL beaker, dissolve Components A and B in 50 mL distilled water.
2. In a separate beaker, dissolve Component C in 25 mL distilled water.
3. Slowly add the entire volume of the Component C solution to the beaker containing the Component A/B solution. Mix well.
4. Dispense 6 mL of the Glucose Testing Reagent into 15 mL tubes. Each student group will receive one.

Preparation of Simulated Urine Samples:

1. Label fifty 1.5 mL screw-top microcentrifuge tubes as follows:
   a. 10 – Positive Control (+)
   b. 10 – Negative Control (-)
   c. 10 – Patient 1 (P1)
   d. 10 – Patient 2 (P2)
   e. 10 – Patient 3 (P3)
2. In a small flask or beaker, dissolve Component E in 6 mL distilled water.
3. Dispense 100 μL of the solution into each of the Negative Control and Patient 1 sample tubes.
4. Dissolve Component D in the remaining solution. Mix well.
5. Dispense 100 μL of the solution into each of the Positive Control, Patient 2, and Patient 3 tubes.

FOR MODULE II
Each Student Group should receive:

1 15 mL conical tube containing 6 mL Glucose Testing Reagent
5 1.5 mL screw-top microcentrifuge tubes containing 100 μL of the control and the patient samples (+, -, P1, P2, P3)
1 Small transfer pipet

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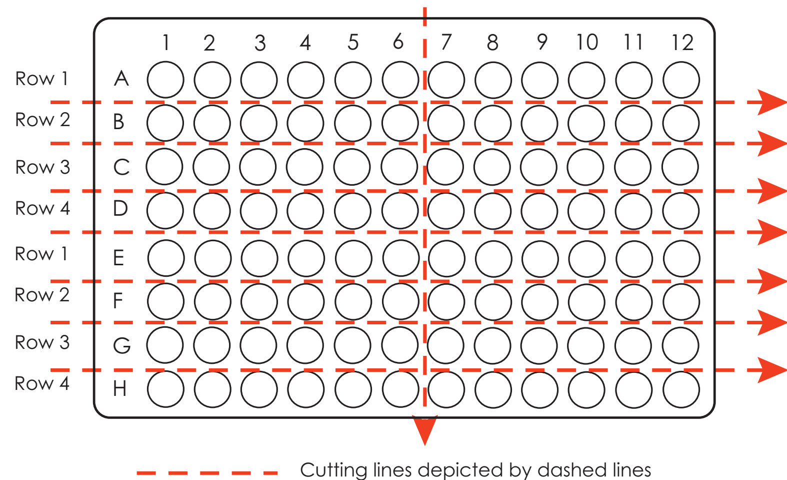
Pre-Lab Preparations: Module III

MODULE III: ELISA

Microtiter Plates

1. As shown in the figure at right, orient the microtiter plates 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 lines as shown in the figure. Each piece will have six wells on one axis and one on the other axis. Each lab group will receive one piece.



Preparation of the Wash Buffer

1. Add all of the 10x ELISA Wash Buffer (F) to 180 mL of distilled water and mix well. Label as "Wash Buffer".
2. Dispense 18 mL into small beakers for each lab group.

Preparation of the "Capture Antibody"

1. Transfer 7 mL of ELISA Dilution Buffer (G) to a 15 mL conical tube. Label the tube "Antigen".
2. Carefully remove the stopper from the vial of lyophilized Antigen (H) 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 contents of reconstituted Antigen back to the 15 mL tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "CAP" and dispense 650 µL into each tube.

Preparation of the Patient and the Control Samples

1. Label fifty 1.5 mL snap-top microcentrifuge tubes as follows:
   a. 10 – Positive Control (+)
   b. 10 – Negative Control (-)
   c. 10 – Patient 1 (P1)
   d. 10 – Patient 2 (P2)
   e. 10 – Patient 3 (P3)
2. Dispense 100 µL ELISA Dilution Buffer (G) into the negative control (-) and patient 2 (P2) tubes.
3. Transfer 7 mL of ELISA Dilution Buffer to a 15 mL conical tube. Label the tube "1°AB".
4. Carefully remove the stopper from the vial of lyophilized Primary Antibody (I) and transfer approximately 0.5 mL of the ELISA Dilution Buffer from the tube in step 3. Close the stopper and gently shake the vial to mix.
5. Transfer the entire contents of reconstituted Primary Antibody back to the 15 mL tube from step 1. Mix well.
6. Dispense 100 µL of the primary antibody into the positive control (+), patient 1 (P1), and patient 3 (P3) tubes.

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Pre-Lab Preparations: Module III

Preparation of Detection Antibody
(Prepare on the same day as needed for the experiment.)

1. Transfer 7 mL of ELISA Dilution Buffer (G) to a 15 mL conical tube. Label the tube “2°AB”.
2. Carefully remove the stopper from the vial of lyophilized Secondary Antibody (J) 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 contents of reconstituted Secondary Antibody back to the 15 mL tube from step 1. Mix well.
4. Label 10 microcentrifuge tubes "DET". Dispense 650 µL per tube.

Preparation of ABTS Substrate

1. Transfer 10 ml ABTS Reaction Buffer (L) into a 15 mL conical tube. Label the tube "ABTS".
2. Carefully remove the stopper from the vial of lyophilized ABTS (K) and transfer approximately 0.5 mL of the ABTS from the tube in step 1. Close the stopper and gently shake the vial to mix.
3. Label 10 microcentrifuge tubes “SUB”. Dispense 650 µL per tube.

FOR MODULE III
Each Student Group should receive:

1 6-well microtiter plate
1 Beaker containing approximately 18 mL wash buffer
5 Snap-top microcentrifuge tubes containing 100 µL of the control and the patient samples (+, -, P1, P2, P3)
1 Snap-top microcentrifuge tube containing 650 µL of the Capture Antibody (CAP)
1 Snap-top microcentrifuge tube containing 650 µL of the Detection Antibody (DET) prepared on the day of the experiment
1 Snap-top microcentrifuge tube containing 650 µL of the ABTS Substrate (SUB)
9 Small transfer pipets
1 Automatic micropipette and tips (optional)
1 Empty beaker or tube labeled “waste”

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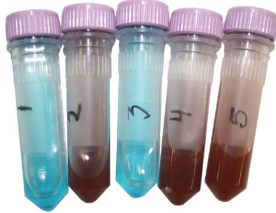
Experiment Results and Analysis

MODULE II:



Sample	Color	Interpretation
Negative Control (-)	Blue	Glucose absent
Positive Control (+)	Brick Red	Glucose present
Patient 1 (P1)	Blue	Glucose absent
Patient 2 (P2)	Brick Red	Glucose present
Patient 3 (P3)	Brick Red	Glucose present

The presence of glucose in the patient's urine, as indicated by the brown color, suggests that the patient has high blood sugar. Further testing to diagnose diabetes is recommended.

MODULE III:

Sample	Color	Interpretation
Negative Control (-)	Clear	C-peptide absent
Positive Control (+)	Green	C-peptide present
Patient 1 (P1)	Green	C-peptide present
Patient 2 (P2)	Clear	C-peptide absent
Patient 3 (P3)	Green	C-peptide present

The presence of the C-peptide in the patient's blood, as indicated by the green color, suggests that the patient is producing insulin. In patients with high blood sugar, the absence of the C-peptide denotes Type I Diabetes, while the presence of the C-peptide denotes Type II Diabetes.

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

Appendix A

EDVOTEK® Troubleshooting Guides

TROUBLESHOOTING GUIDE FOR MODULE II

Problem:	Cause:	Answer:
Brownish precipitate in Urine Glucose Test	Some precipitation is expected due to the chemical reaction.	Thoroughly mix samples before analyzing results.

TROUBLESHOOTING GUIDE FOR MODULE III

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 micropipet and change tips between samples.
	Too much force was used to wash out wells.	Wash the wells gently and slowly.
Color doesn't develop or is slow to develop.	Incubation time too short.	Incubate ELISA at room temperature for five more minutes.

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