Country: _____	Student Code: _____

19th INTERNATIONAL BIOLOGY OLYMPIAD

13th – 20th July, 2008

Mumbai, INDIA

PRACTICAL TEST 3

BIOCHEMISTRY AND CELL BIOLOGY

Total Points: 43

Duration: 60 minutes

Dear Participants,

- In this test, you have been given the following task:

  Task 1: A: Study of β-lactamase activity and its inhibition (35 points)

  B: Correlating β-lactamase expression to resistance (4 points)

  C: Correlating K_i values of pesticides to bacterial growth (4 points)

- **You have to write down your results and answers in the ANSWER SHEET. Answers written in the Question Paper will not be evaluated.**
- Please make sure that you have received all the materials and equipment listed for the task. In case any of these items is missing, please raise the yellow card.
- At the end of the test, put the Answer Sheet and Question Paper in the envelope. The supervisor will collect this envelope.

Good Luck!!

Country: _____

Country Code: _____

First Name: _____

Middle Name: _____

Family Name: _____

Student Code: _____

Task 1

PART A (35 points)

Study of β-lactamase activity and its inhibition

Materials and equipment	Quantity
1. Colorimeter, with a set of seven cuvettes	1
2. Test tubes	8
3. Test tube stand	1
4. Micropipette (10 – 100 µl capacity)	1
5. Micropipette (100 – 1000 µl capacity)	1
6. Micropipette tips (10 – 100 µl capacity)	20
7. Micropipette tips (100 – 1000 µl capacity)	20
8. Photographs of Petri plates	6
9. Permanent marker	1
10. Tissue paper roll	1
11. Wash bottle containing distilled water	1
12. Container for wash and discard	1
13. Graph paper	1

Reagents (please see the next page)

Label	Reagent	Container
A	β – Lactamase enzyme (1.85 mg/ml)	Vial
B	Inhibitor (100 mM)	Vial
C	Penicillin G (0.54 mM)	Blue-stoppered tube
D	Sodium phosphate buffer, pH 7.0 (10 mM)	Blue-stoppered tube
E	$CuSO_4$-Neocuproine reagent	Blue-stoppered tube
F	HCl (2 M)	White-stoppered tube

Handling of micropipette:

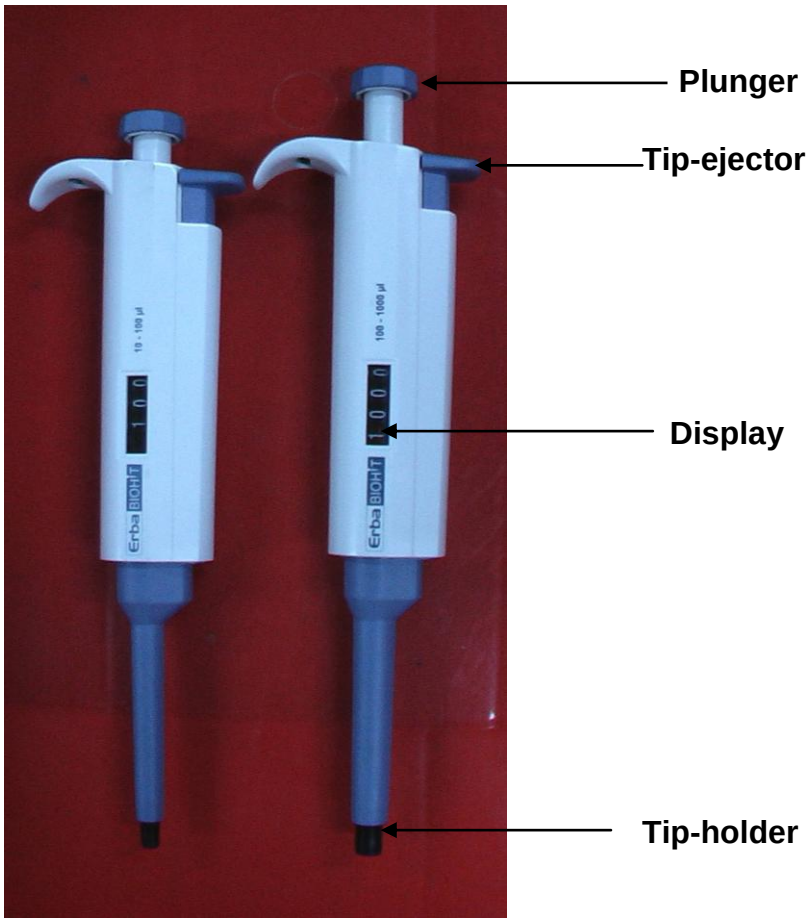


Figure 1

Adjustment method

Turn the plunger (Figure 1) to set the value to the desired volume, which can be seen in the display.

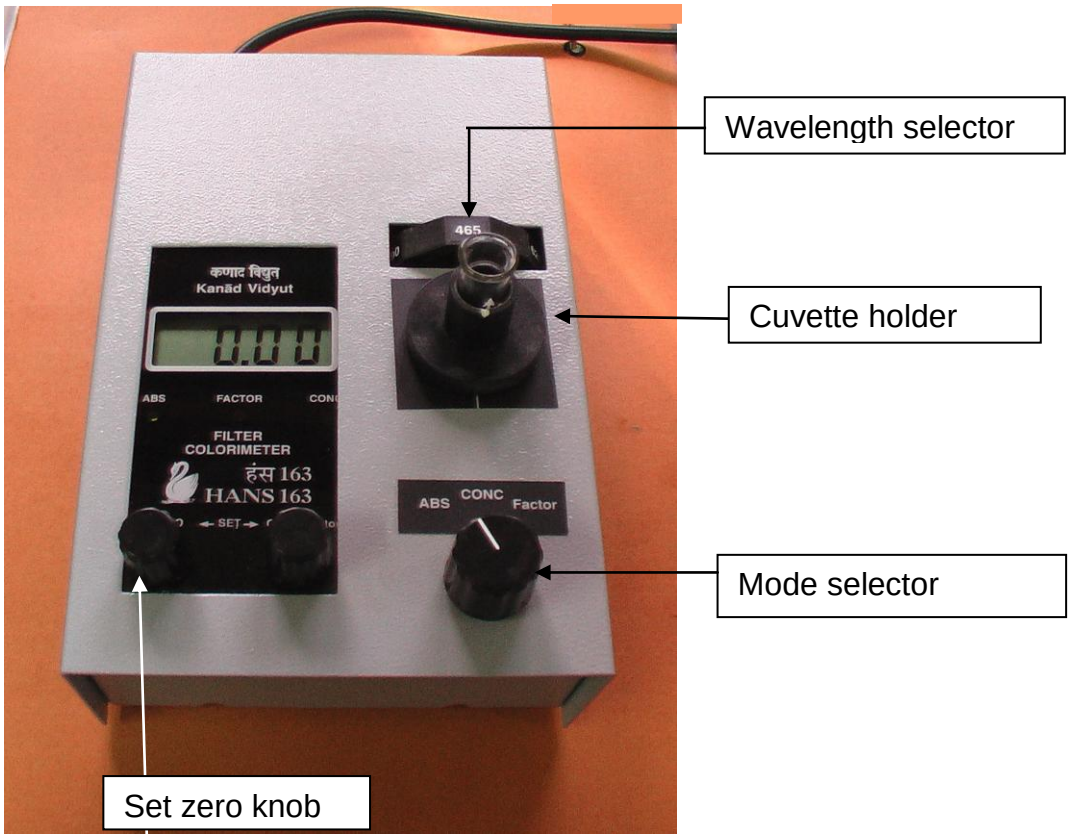
Remember that each micropipette has a fixed range of volumes as indicated on the pipette. DO NOT CROSS THE LIMITS OF THIS RANGE.

Usage method

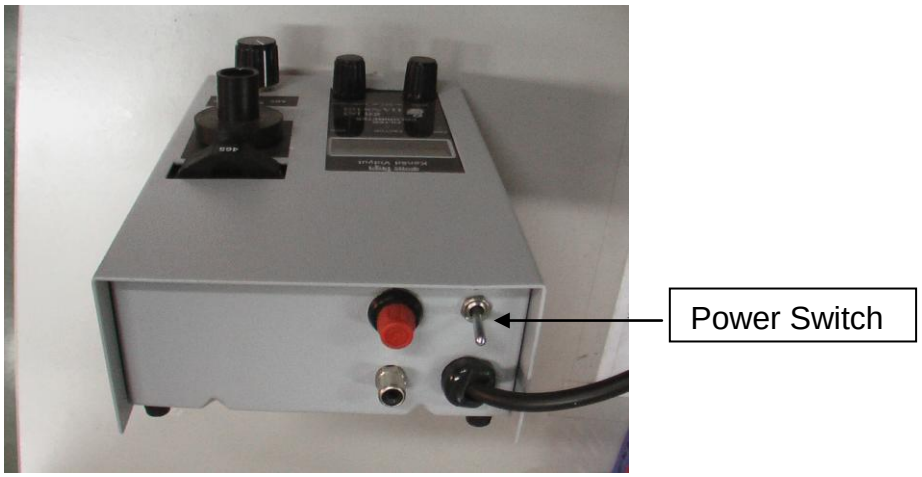
Secure the pipette tip to the tip holder (Figure 1). Gently push down the plunger to the first stop, hold, and dip the tip into the solution vertically to a depth of 2 - 4 mm. Release the plunger slowly and make it return to the original position. Remove the pipette from the liquid and transfer the contents to the desired tube. Make sure that the tip is close to the inner wall of the tube. Push the plunger to the first stop and then push further to discharge the solution completely from the tip. Remove the pipette from the tube. Eject the used tip into the discard container by pressing the tip-ejector.

Operating Instructions for the colorimeter:

Figure 2



Top view of colorimeter



Rear view of colorimeter

1) Turn the power switch (Figure 2) of the colorimeter ON.

2) Set the instrument to Absorbance mode ("ABS") using the mode selector.

3) Set the wavelength to 465 nm using the wavelength selector.

4) Put the blank solution in a cuvette. Clean the outside surface of the cuvette with tissue paper and insert it into the cuvette holder. Gently push the cuvette all the way down.

5) Rotate the 'set zero' knob to set the reading to zero. The instrument is now ready for measuring the absorbance of the test solutions.

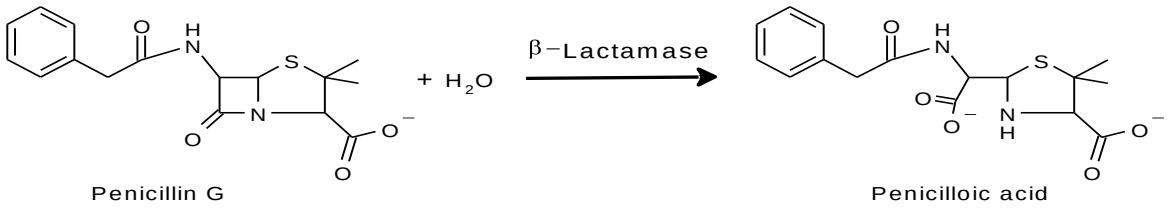
Introduction

Penicillins are antibiotics with a characteristic β-lactam ring in their structure. This antibiotic kills bacteria by inhibiting the cell wall synthesis. However, these molecules are rendered inactive by some bacteria, which synthesize an enzyme called β-lactamase. These bacteria, which produce β-lactamases, are resistant to penicillins. Due to this, penicillin treatment is ineffective in patients infected with such resistant bacteria. One approach to overcome this problem is to develop effective β-lactamase inhibitors.

The effectiveness of a β-lactamase inhibitor can be evaluated by determining its IC_{50} and K_i values. The IC_{50} of an inhibitor is defined as the concentration of the inhibitor required to inhibit the enzyme activity by 50 percent. The K_i of an inhibitor is a measure of its binding affinity for the enzyme.

Principle of β-lactamase assay

β-Lactamase inactivates penicillin by catalyzing the following reaction:



Penicillin G Penicilloic acid

The penicilloic acid generated is complexed with $CuSO_4$ in the presence of neocuproine. The yellow-colored product formed can be monitored by measuring its absorbance at 465 nm using a colorimeter.

In this task, you will:

- determine the IC_{50} value of a given inhibitor by generating a dose-response curve, and
- calculate the K_i value for the inhibitor.

A dose-response curve for the inhibitor is generated by measuring the activity of β-lactamase in the presence of varying concentrations of the inhibitor at a fixed concentration of the substrate.

Q. 1.A.1. (18 points) Follow the protocol given below and enter the absorbance values **in Table 1.A.1. in the Answer Sheet**.

I. Prepare the following reaction mixtures:

Test tube	Sodium phosphate buffer, pH 7.0	Inhibitor (100 mM)	β-lactamase enzyme	Distilled water
1	1.48 ml	-	20 µl	-
2	1.46 ml	20 µl	20 µl	-
3	1.44 ml	40 µl	20 µl	-
4	1.42 ml	60 µl	20 µl	-
5	1.40 ml	80 µl	20 µl	-
6	1.38 ml	100 µl	20 µl	-
Blank	1.43 ml	50 µl	-	20 µl

II. Mix gently and incubate at room temperature for 5 minutes. **You may use the wall clock or your wrist watch to keep track of the incubation time.**

III. Add 1 ml of penicillin G (0.54 mM) to each tube and mix gently. Incubate at room temperature for 10 minutes.

IV. Add 1.5 ml of the $CuSO_4$-neocuproine reagent to each tube and mix gently. Incubate at room temperature for 5 minutes.

V. Stop the color development by adding 100 µl of HCl to each tube and mix gently.

VI. Set the colorimeter to 465 nm.

VII. Use the Blank to set the absorbance to zero.

VIII. Measure the absorbance values of the solutions in Test tubes 1 to 6, and enter these values in the table. **You should get any one absorbance reading countersigned by the supervisor. To call the supervisor, raise the yellow card.**

Table 1.A.1.

Test tube	Absorbance
1	
2	
3	
4	
5	
6	

Data analysis and interpretation

Q. 1.A.2. (6 points)

I. Calculate the concentrations (in mM) of the inhibitor [I] in 2.5 ml of the enzyme reaction in Test tubes 1 to 6 and enter these values **in Table 1.A.2. in the Answer Sheet**.

II. Consider the absorbance values to be the rates of hydrolysis of penicillin G. Now calculate v_i/v_0, where:

v_0 is the rate of hydrolysis of penicillin G by β-lactamase in the absence of the inhibitor, and v_i is the rate of penicillin G hydrolysis in the presence of the inhibitor. Note that for Test tube 1, $v_i = v_0$.

Enter these values (up to two decimals) **in Table 1.A.2. in the Answer Sheet**.

Table 1.A.2.

Test tube	[I] (mM)	v_i/v_0
1		
2		
3		
4		
5		
6		

Q. 1.A.3. (5 points) Plot a graph of v_i/v_0 versus [I] in the **Graph Paper attached to the Answer Sheet**.

Determination of the IC_{50} and K_i value of the inhibitor

Q. 1.A.4. (3 points) Determine the IC_{50} value by interpolation of the data points in the graph. Write the value (up to two decimals) in the box **in the Answer Sheet**.

IC_{50} = _____mM

Q. 1.A.5. (3 points) Calculate the dissociation constant K_i of the inhibitor using the equation:

$$IC_{50} = K_i\left(1 + \frac{[S]}{K_m}\right)$$

where K_m is the Michaelis-Menten constant of β-lactamase for penicillin G and [S] is the initial concentration of substrate (penicillin G) present in the enzyme reaction mixture.

Assume the K_m of β-lactamase for penicillin G to be **0.05 mM**. Write down your answer (up to two decimals) in the box **in the Answer Sheet**.

K_i = _____mM

PART B (4 points)

Correlating β-lactamase expression to resistance

When penicillin-resistant bacteria are grown in liquid culture media, β-lactamase is secreted into the medium. The supernatant of such a medium can be assayed for β-lactamase activity. Culture supernatants from four different organisms (P, Q, R and S), which are suspected to be penicillin-resistant, were obtained and 20 μl of each was assayed for β-lactamase activity. The corresponding absorbance values were measured at 465 nm and are given in the table below.

Organism	Absorbance
P	0.090
Q	0.450
R	0.075
S	0.220

These four organisms were tested for their resistance to penicillin G by the disc diffusion plate assay as follows:

1. Each organism was separately inoculated into warm growth medium and poured into a sterile Petri plate. On cooling, the medium solidified.
2. Filter paper discs impregnated with varying concentrations of penicillin G were then placed on the surface of the medium.
3. The plates were incubated allowing penicillin to diffuse and organisms to grow.

4. Organisms sensitive to penicillin will not be able to grow in the vicinity of the antibiotic disc and hence, a clear zone will be obtained around the disc.

You have been given labeled photographs of six plates I - VI.

Plate I is a control plate showing uniform mat growth of organisms in the absence of penicillin G.

Plate II is also a control plate that contains media without the growth of any organism.

Plates III to VI show the growth of the four organisms in the presence of penicillin G. 2.5, 5, 7.5, 10 and 12.5 are the micrograms of penicillin G present in the respective discs.

Q. 1.B.1. (4 points) Observe these plates and infer which organism is growing in each plate. Write your answers **in Table 1.B.1. in the Answer Sheet**.

Table 1.B.1.

Plate	Organism
III	
IV	
V	
VI	

PART C (4 points)

Correlating K_i values of pesticides to bacterial growth

Four pesticides P1 to P4 are reversible inhibitors of an enzyme E that is essential for the growth of a bacterium B. Their K_i values are given in the table below. Each of these four pesticides is used in four geographically different regions R1 to R4. The residual concentrations of these four pesticides in the respective regions are also shown in the table below:

Region	R1	R2	R3	R4
Pesticide	P1	P2	P3	P4
K_i for the enzyme E	1 nM	5 nM	0.45 μM	0.55 μM
Residual concentration	60 nM	100 pM	30 nM	5.5 μM

Q. 1.C.1. (4 points) Indicate whether the bacterium B would grow or not in each of the four regions by putting tick marks (√) in the appropriate boxes **in the Table 1.C.1. in the Answer Sheet**.

Table 1.C.1.

Region	R1	R2	R3	R4
Bacterium B grows				
Bacterium B does not grow				

********** END OF PRACTICAL TEST 3 **********

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Total Points: 43

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ANSWER SHEET

STUDENT CODE:

Task 1

Study of β-lactamase activity and its inhibition

PART A (35 points)

Q. 1.A.1. (18 points)

Table A.1.

Test tube	Absorbance
1	
2	
3	
4	
5	
6	

Q. 1.A.2. (6 points)

Table 1.A.2.

Test tube	[I] (mM)	v_i/v_0
1		
2		
3		
4		
5		
6		

STUDENT CODE:

Q. 1.A.3. (5 points): Points will be transferred from the Graph Paper.

Q. 1.A.4. (3 points)

IC_{50} = _____mM

Q. 1.A.5. (3 points)

K_i = _____mM

PART B (4 points)

Q. 1.B.1. (4 points)

Table 1.B.1.

Plate	Organism
III	
IV	
V	
VI	

STUDENT CODE:

PART C (4 points)

Q. 1.C.1. (4 points)

Table 1.C.1.

Region	R1	R2	R3	R4
Bacterium B grows				
Bacterium B does not grow				

********** END OF PRACTICAL TEST 3 **********