INTERNATIONAL BIOLOGY OLYMPIAD e. V. IBO

Biochemistry

34th International Biology Olympiad

3-10 July 2023, United Arab Emirates University

Practical Exam

Biochemistry

Total points: 100

Duration: 90 minutes

General Instructions:

You have 90 minutes to complete **ONE task in this practical exam, plus 9 questions.**

Task: Purification, quantitative analysis and kinetic measurements of bacterial diaphorase activity (100 points)

Important information:

Write your answers in the answer sheet. Only answers given in the answer sheet will be evaluated.

Make sure that you have received all the materials and equipment listed, including a graph paper. Check tubes contain solutions (with exception of tubes 1 to 12). If any of these items are missing, please raise your card immediately.

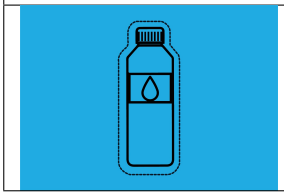
Read the protocol before you start.

During experiments, ensure that you wear gloves, and handle the equipment and samples carefully. Compounds A and B are hazardous.

Any spilled solutions, samples or equipment damaged by you will not be replaced, but the gloves can be replaced.

Your plate results will be scored and you do not need it anymore.

Use the following cards to ask for water/washroom/help.

Drinking water	Washroom	Spectrophotometer measurement	Other queries
			

Stop answering as soon as you hear the whistle at the end of the exam.

No paper, materials or equipment should be taken out of the laboratory.

Good luck!

Required materials with labels and equipment:

1. Eppendorf tubes labelled with the numbers from 1 to 12
2. 1.0 ml of bacterial lysate (BL)
3. Standard BSA solution (BSA)
4. Lysis buffer (LB)
5. Wash buffer (WB)
6. Elution buffer (EB)
7. Bradford buffer (BB)
8. Substrate solution (SUB)
9. Compound A (A)
10. Compound B (B)
11. Assay buffer (AB)
12. Bradford reagent (BR) in a black 15 ml tube in the paper cup
13. 96-well plate
14. Affinity chromatography column with resin, on the column holder
15. 10-100 µl Micropipette
16. 100-1000 µl Micropipette
17. Yellow tips for 10-100 µl Micropipette
18. Blue tips for 100-1000 µl Micropipette
19. Paper cup to discard waste
20. Piece of aluminium foil
21. Piece of parafilm
22. Pair of gloves
23. Paper white cup for discarding liquids under the column

Introduction:

Diaphorase (NADH dehydrogenase) is an enzyme (23.0 kDa) that converts nicotinamide adenine dinucleotide (NAD) from its reduced form (NADH) to its oxidized form (NAD+):

$NADH + H+ + acceptor \rightleftharpoons NAD+ + reduced\ acceptor$

Diaphorase is a flavoprotein that contains iron-sulfur centers. It is used in the electron transport chain for ATP generation.

In this experiment, you will purify a bacterial diaphorase from a crude extract, then measure its activity.

First, you must purify diaphorase from a crude bacterial lysate. *E. coli* has previously been transformed with a plasmid expression vector, into which the gene encoding diaphorase tagged with 6-histidine had been cloned. You will purify diaphorase by affinity chromatography; the 6-histidine tag has affinity for, and binds to, nickel which is attached to the resin in the (Ni-NTA Sepharose) columns.

The bound protein can be eluted from the resin by changes of buffers used in the chromatography protocol. You will collect eluted fractions into two Eppendorf tubes.

After protein elution, you will determine the protein concentration of the two fractions, using a Bradford quantification assay. The Bradford assay is a colorimetric assay during which binding of 'Coomassie brilliant blue' to protein results in increased absorbance of 595 nm wavelength light. You can determine the protein concentration of your fractions by comparing their absorbance to a standard curve derived by assaying bovine serum albumin (BSA) protein solutions of known concentration.

Finally, you will measure the activity of diaphorase in one of the fractions using a colorimetric assay in the absence and presence of some additives. The reduced form of the acceptor (NBT) has a maximum absorbance at the same wavelength of Bradford reagent (595 nm).

Part 1.

Purification of bacterial diaphorase using Ni-NTA affinity chromatography.

Procedure:

In all steps, take care to avoid the resin **drying out.**

The chromatography column is already placed in the hole of the column holder rack. Note that it fits into the hole tightly. Handle the column carefully during the course of the experiment. The column is sealed at the bottom with a stopper and the resin in the column is covered with a small volume of 20% ethanol.

It is important you perform the following steps quickly and carefully to protect the resin.

1. Carefully look at the contents of the column to identify the border between the resin and the overlaying liquid.
2. Break and remove the stopper at the bottom of the column by twisting it clockwise and anticlockwise.
3. Unscrew the lid of the column to allow the liquid to start flowing through the resin. Discard the flow-through into the paper cup placed under the column stand. Collection should continue just until there is no liquid left above the resin. Approximately **5 - 7 drops** will be discarded.
4. Gently add **500 µl** of lysis buffer (LB) to the column, without disrupting the resin, in order to equilibrate the column with lysis buffer. Discard the flow-through into the paper cup placed under the column stand. Collection should continue just until there is no liquid left above the resin.
5. Add **500 µl** of provided bacterial lysate (BL) onto the column and discard the flow-through into the paper cup placed under the column stand. Be sure that all the bacterial lysate has entered the column. Collection should continue just until there is no liquid left above the resin.
6. **Wash Step**: Add 500 µl wash buffer (WB) and discard the flow-through into the paper cup placed under the column stand. As the volume of the buffer above the resin decreases to about 50 µl, add 500 µl additional wash buffer and continue collection of flow-through into the same cup. Add a further 500 µl to wash a third time and continue collecting into the same cup. Collection should continue just until there is no liquid left above the resin.
7. **Elution Step:** Gently transfer the column to the Eppendorf **tube 1**, in the rack or in the column holder. Add **500 µl** elution buffer (EB) to start release of protein (diaphorases) bound to the resin in the column. Start collecting drops into **tube 1**. As the volume of the buffer above the resin decreases to about **50 µl**, add **500 µl** additional elution buffer and continue collection of the eluent into **tube 2**. (Approximately 1000 µl will be collected, into tubes 1 and 2 in total).
8. Seal the bottom of the column with a provided piece of parafilm and place the screw cap on top.

NOTE: Tube 3 will not be used.

Part 2.

Measurement of diaphorase **protein** using a quantitative method

Q.2.1 Complete the protocol below. The Bradford assay will worth 33 points. 33.0pt

1. Make bovine serum albumin (BSA) dilutions as shown in Table 1:

Tube number	4	5	6	7	8	9	10
Standard BSA (1 mg/ml), µl	0	20	40	60	80	100	120
Bradford buffer, µl	200	180	160	140	120	100	80

2. Ensure the contents of each tube (4-10) are well mixed by pipetting up and down.

3. Make dilutions of tubes 1 and 2 in tubes 11 and 12 as shown in Table 2:

	11	12
Eluted protein	50 µl of 1	50 µl of 2
Bradford buffer	50 µl	50 µl

4. Each sample will be assayed in duplicate. For this purpose, add 10 µl of tubes 4-10 into wells of A1-A7, and then consecutively to wells of C1-C7, of your 96 well plate. Subsequently, add 10 µl of tubes 11 into A9 and C9, and 10 µl of tube 12 to wells A11 and C11.

Pause the Bradford assay at this stage, and start Part 3 in the same plate.

Part 3.

Measurement of diaphorase activity using a colorimetric assay

Q.3.1 Complete the protocol below. The enzyme assay will worth 15 points. 15.0pt

Prepare the following solutions in Table 3 into wells E1-E7. Use the enzyme you purified into tube 1 and 2 **without dilution**.

Table 3 - Enzyme assay

Well number	E1	E2	E3	E4	E5	E6	E7
Enzyme (μl) - tube 1	-	20	-	20	20	20	20
Enzyme (μl) - tube 2	20	-	20	-	-	-	-
Compound A (μl)	-	-	-	10	10	-	-
Compound B (μl)	-	-	-	-	-	10	10
Assay buffer (AB), μl	190	10	10	-	-	-	-
Substrate (SUB), μl	-	180	180	180	180	180	180

Immediately continue with the Bradford assay in the same plate.

Bradford Assay (Continued)

5. Add 190 μl of the Bradford reagent (BR) into each of the wells (**only A1-A7, A9, A11 and C1- C7, C9 and C11**) to which samples had been added in the previous stage (do **not** add Bradford reagent to wells E1-E7, used for the enzyme assay). Mix the contents of each well gently with a micropipette tip. Take care not to create bubbles; bubbles could interfere with absorbance measurements.

6. Cover the 96-well plate with aluminium foil to prevent exposure to light. Incubate in the dark for 5 minutes. Keep your time using wall clocks in front of you.

7. After completion of the 5-minute incubation, raise your card (Spectrophotometer image). A scientific volunteer will take your coded plate to a Spectrophotometer station and have the absorbance of all your wells read at a wavelength of 595 nm.

Note: The 96-well plate will <u>not</u> be accepted for reading after the exam is finished.

Part 4

The experiments in the previous sections were previously carried out by other students. You are given the absorbance readings at 595 nm in the table below. Calculate the mean of absorbance and BSA concentration (tubes 4-10) and write your answers to 2 decimal places. Use this data to answer the questions in Part 4.

Q.4.1 4.0pt

BSA (mg/ml)	Well	A (595 nm)	Well	A (595 nm)	Mean
	A1	0.50	C1	0.40	
	A2	0.60	C2	0.50	
	A3	0.74	C3	0.62	
	A4	0.84	C4	0.80	
	A5	0.95	C5	0.91	
	A6	1.00	C6	0.96	
	A7	1.10	C7	0.99	
Tube 11	A9	0.90	C9	0.94	
Tube 12	A11	0.58	C11	0.56	

Q.4.2 Based on the linear part of data, presented in the table above, draw a calibration curve between BSA concentration, mg/ml (A) and mean absorbance at 595 nm (B). You should indicate all of your points in the graph paper. Label the axes of the graph using the A and B letters and indicate corresponding concentration of tubes 11 and 12. 15.0pt

Q.4.3 Using the standard curve and the absorbance at 595 nm of samples from tubes 11 and 12, calculate the protein concentration of the fractions in tubes 1 and 2. Write the protein concentrations of tubes 1 and 2 (rounded to two decimal places) on the answer sheet. Molecular weight of diaphorase is 23.0 kDa. 10.0pt

Tube Number	Mean Absorbance (595 nm) from table at beginning of Q4.1	Dilution Factor	Tube Number	Concentration (mg/ml)	Concentration (μM)
11			1		
12			2		

Kinetic parameters calculation

The same students measured the enzyme activity of 10 µl from **tube 2** in a total volume of 1.0 ml. Absorbance of the solution was measured over a 1-cm path length.

Over 3 minutes, the absorbance at 595 nm increased linearly by a total of 0.6 units.

Assume the extinction coefficient of NBT to be 12.3 $M^{-1}\,cm^{-1}$. What was the specific activity of the enzyme purified by this student? Write the answer to 2 decimal places, in Table Q.4.4

Q.4.4 5.0pt

Specific activity (U/mg) **or µmoles** $\times$ **min**$^{-1}$ $\times$ **mg**$^{-1}$	

As a rule, enzyme kinetics, can be described by the Michaelis-Menten equation:

$V_0 = \frac{V_{max}[S]}{K_m + [S]}$

where,

V_0 is reaction rate,

V_{max} is maximal reaction rate.

S is substrate concentration

K_m is Michaelis-Menten constant.

When the oxidised NBT (substrate) concentration is 20 µM, diaphorase activity is 6500 µmoles $\times$ min^{-1} $\times$ mg^{-1}.

K_m is equal to 10 µM.

$k_{cat} = \frac{V_{\text{max}}}{[E]}$

where,

k_{cat} is enzyme turn over number and [E] is the concentration of the enzyme.

Q.4.5 Calculate the V_{max} and k_{cat} for the diaphorase. Write the answer to 2 decimal places. 10.0pt

V_{max}	
$k_{cat}\ (sec^{-1})$	

Q.5

Conventional purification

To purify two proteins, A (MW: 25 kDa, pI: 4.5) and B (MW: 55 kDa, pI: 8.8) from a crude extract, based on their physical properties, a size exclusion chromatography (using appropriate Sephadex type) and an anion exchange chromatography (DEAE-Sepharose) column were used.

Identify the following statements as True or False by marking a cross (X) in the appropriate cell

Q.5.1 4.0pt

Statement	True	False
Protein A has a higher affinity to DEAE-Sepharose than B at pH 7.0.		
Protein A elutes faster than B from Sephadex.		
Protein A elutes faster than B from a cation exchange resin at pH 4.5.		
Protein A elutes from DEAE-Sepharose at higher salt concentration than protein B at pH 7.0		

Q.5.2

Q.5.2 Bacterial hosts 4.0pt

Different *E. coli* strains are optimized for expressing different kinds of synthetic proteins, as shown in the table below.

Bacterial Code	**Bacterial strain (*E. coli*)**	**Characteristic**
A	BL21- Codon Plus	For rare codon expression
B	Origami	Improve disulfide bond in cytosol.
C	BL21-Codon Plus (DE3)-RP-X	Enhance expression of heterologous proteins.
D	C43 (DE3)	Prevents cell death due to expression of toxic proteins.
E	SoluBL21	Suitable for expression of insoluble protein in soluble form
F	C41 (DE3)	Suitable for expression of membrane proteins

Which *E. coli* strain is most suitable for the expression of the following proteins? Choose only one option for each protein, and write the 'Bacterial code' from the above table:

Protein	**Bacterial code**
Insulin	
N-terminal domain of Gasdermin D with ability to make a membrane pore	
Glut 1, which is responsible for glucose transport	
Proteins with high beta-sheet content	

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A.2.1 (33.0 pt)
Will be scored after reading 96 well plate.

A.3.1 (16.0 pt)
Will be scored after reading 96 well plate.

A.4.1 (4.0 pt)

BSA (mg/ml)	Well	A (595 nm)	Well	A (595 nm)	Mean
	A1	0.50	C1	0.40	
	A2	0.60	C2	0.50	
	A3	0.74	C3	0.62	
	A4	0.84	C4	0.80	
	A5	0.95	C5	0.91	
	A6	1.00	C6	0.96	
	A7	1.10	C7	0.99	
Tube 11	A9	0.90	C9	0.94	
Tube 12	A11	0.58	C11	0.56	

A.4.2 (15.0 pt)
It will be scored on graph paper.

A.4.3 (10.0 pt)

Tube Number	Mean Absorbance (595 nm) from table at beginning of Q4.1	Dilution Factor	Tube Number	Concentration (mg/ml)	Concentration (μM)
11			1		
12			2		

A.4.4 (5.0 pt)

Specific activity (U/mg) or µmoles $\times \text{min}^{-1} \times \text{mg}^{-1}$	

A.4.5 (10.0 pt)

V_{max}	
$K_{cat}\ (sec^{-1})$	

A.5.1 (4.0 pt)

Statement	True	False
Protein A has a higher affinity to DEAE-Sepharose than B at pH 7.0		
Protein A elutes faster than B from Sephadex.		
Protein A elutes faster than B from a cation exchange resin at pH 4.5		
Protein A elutes from DEAE-Sepharose at higher salt concentration than protein B at pH 7.0		

A.5.2 (4.0 pt)

Protein	**Bacterial code**
Insulin	
N-terminal domain of Gasdermin D with ability to make a membrane pore.	
Glut 1 which is responsible for glucose transport.	
Proteins with high beta-sheet content.	

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Answer key and Marking Scheme

Total (100 points):

Q2.1 33 points (will be scored after reading the 96-well plate)

Well #	A (595 nm)	Well #	A (595 nm)
A1	0.45	C1	0.45
A2	0.56	C2	0.56
A3	0.68	C3	0.68
A4	0.82	C4	0.82
A5	0.93	C5	0.93
A6	0.98	C6	0.98
A7	0.99	C7	0.99
A9	0.98	C9	0.98
A11	0.57	C11	0.57

A1-A7 wells and C1-C7 wells, ±10%, each well 1.5 point; ±20%, each well 0.75 point, out of ±20%, each well 0 point.
A9, C9, A11, C11 wells, ±10%, each well 3 points; ±20%, each well 1.5 point, out of ±20%, each well 0 point.

Q3.1 15 points (will be scored after reading the 96-well plate)

Well #	A (595 nm)
E1	0.04
E2	0.5
E3	0.5
E4	0.5
E5	0.5
E6	0.2
E7	0.2

E1-E6 wells, ±10%, each well 2 point; ±20%, each well 1.0 point, out of ±20%, each well 0 point. E6 and E7 wells less than 0.2, 3 points if only E2 is 0.5; higher than 0.2, 0 points.

Please note Q. 2.1 and Q.3.1 in the exam day will be measured again with the scientific volunteers and possible changes will be amended in the answer key.

Q4.1 4 points, each box 0.25 point

BSA (mg/ml)	Well #	A (595 nm)	Well #	A (595 nm)	Mean
0.0	A1	0.50	C1	0.40	0.45
0.1	A2	0.60	C2	0.50	0.55
0.2	A3	0.74	C3	0.62	0.68
0.3	A4	0.84	C4	0.80	0.82
0.4	A5	0.95	C5	0.91	0.93
0.5	A6	1.00	C6	0.96	0.98
0.6	A7	1.10	C7	0.99	1.05
11	A9	0.90	C9	0.94	0.92
12	A11	0.58	C11	0.56	0.57

Q4.2 15 points.

Will be scored on graph paper as indicated in the template graph.

1. Correct label of X and Y axis (as mentioned in the text); X=A and Y=B

   1 point for each label = **2 points; Opposite labelling= 1 points**

2. Scaling of X axis ;**1 points** ;(Partial scaling = 0.5 point)

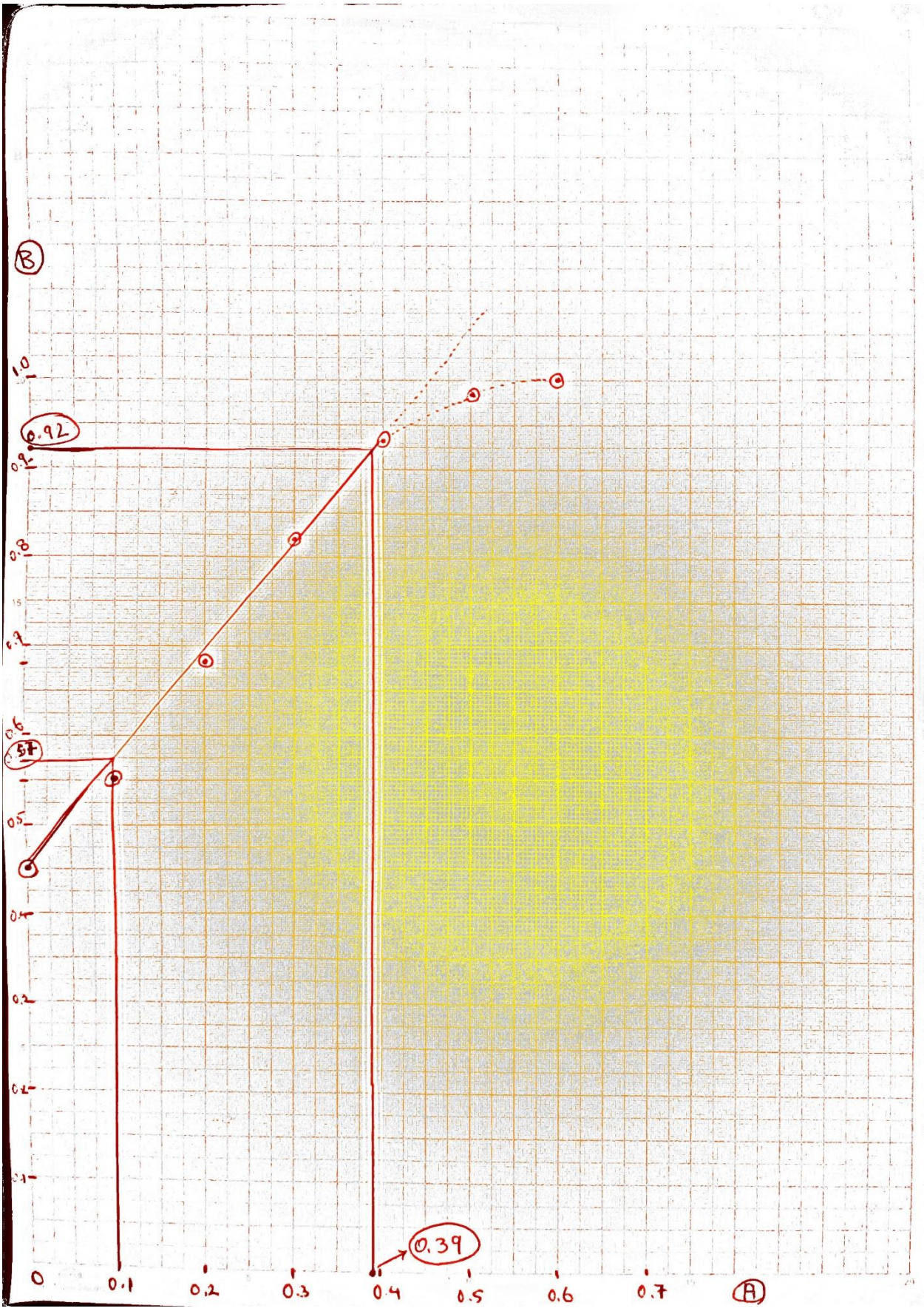
3. Scaling of Y axis; **1 points** ;(Partial scaling: any other scale will be given 0.5 point)

4. Indication of 7 points on graph; 7 points; each 1, 1 point

5. Indication of 2 unknown samples, 2 points

6. Linear graph, using first 5 points (2 Mark), using 6 points (1 mark), other graphs (0 Mark)

Total = 15 points


B
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.2
0.1
0.92
57
0.39
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
A

Q4.3 10 points

tube number	Mean Absorbance (595 nm) from table at beginning of Q4.1	Dilution	Concentration (mg/ml)	Concentration (µM)
#11	0.92	2	0.78	33.91
#12	0.57	2	0.20	8.69

For concentrations; ±10%, each well 2 point; ±20%, each well 1.0 point, out of ±20%, each well 0 point. for other boxes each one 0.5 points.

Q4.4 5 points

Specific activity (U/mg) **or µmoles × min^{-1} × mg^{-1}**	8100.00

±10%, 5 point; ±20%, 2.5 point; out of ±20%, 0 point.

Q4.5 10 points

V_{max}	9750.00
k_{cat} (sec^{-1})	18.70

±10%, 5 point; ±20%, 2.5 point; out of ±20%, 0 point.

Q 5.1. **4 points, 1 point for each correct statement**

1. Protein A has a higher affinity to DEAE-Sepharose than B at pH 7.0. T
2. Protein A elutes faster than B from Sephadex. F
3. Protein A elutes faster than B from a cation exchange resin at pH 4.5. T
4. Protein A elutes from DEAE-Sepharose at higher salt concentration than protein B at pH 7.0. T

Q 5.2. Bacterial hosts. **4 points, each statement 1 point**

Protein	Required *E. coli* code
Insulin	B
N-terminal domain of Gasdermin D with ability to make a membrane pore	D or F
Glut 1 which is responsible for glucose transport	F
Proteins with high beta-sheet content	E