INTERNATIONAL BIOLOGY OLYMPIAD e. V. IBO

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Student name:	Student code:	Country:

28th International Biology Olympiad

July 23-30, 2017
University of Warwick
United Kingdom



Practical Exam 2

BIOCHEMISTRY

The exam will start and end with a whistle.

Total points: 78
Duration: 120 minutes

GENERAL INSTRUCTIONS

In this practical test you have **TWO hours** to do **THREE Questions**.

You should perform the tasks in the order given here:

Question 1: Analysis of blood markers (11 marks). This section should take approximately 15 minutes to complete. You will be provided with some data to analyse.

Question 2: Practical determination of kinetic parameters (60 marks). This section should take approximately 90 minutes to complete. You will be generating your own data.

Question 3: Analysis of genetic markers (7 marks). This section should take approximately 5 minutes to complete. You will be provided with some data.

In this exam you will analyse a patient history through blood marker characteristics, enzyme kinetics and family inheritance of a genetic disorder.
Good luck!

Important Information:

- Please remember to write your name, your student code and your country in the given boxes.
- Write your answers in this question booklet. Only the answers given in this question booklet will be evaluated.
- Make sure that you have received all the materials and equipment listed. If any of these items are missing, please raise your Green card immediately.
- During experiments, ensure to handle equipment properly. Any spilled solutions or equipment damaged by you will not be replenished.
- Stop answering and put down your pen immediately when the whistle sounds at the end of the exam.
- Leave the question booklet on your desk at the end of the exam.
- No paper, materials or equipment should be taken out of the laboratory.
- **An English translation of this paper is available upon request.**

MATERIALS

- Ice Box
- Solution A: 2 x Concentration Reaction Master Mix containing: 50 mM potassium phosphate buffer, pH 7.6; 14 mM $MgSO_4$; 2.64 mM ADP; 40 U Lactate Dehydrogenase; 0.4 mM NADH (10 ml) (in ice box)
- Solution B: H_2O (10 ml)
- Solution C: 10 mM phosphoenol pyruvate (PEP) in 0.1 M potassium phosphate buffer, pH 7.6 (1.5 ml) (in ice box)
- Diluted plasma samples F, M and D (0.5 mL each) (in ice box)
- A 20-200 µl pipette
- A 100-1000 µl pipette or 200-1000 µl pipette
- Pipette tips, to suit pipettes
- 1 ml plastic cuvettes (1 cm path length) x 20
- Waste tip and cuvette disposal vessel
- A visible light spectrophotometer - check the Spectrophotometer Guide sheet, included as a separate document, for instructions on using you spectrophotometer
  - a cuvette will be placed in sample chamber to indicate the correct orientation.
- A Cuvette stand
- Parafilm® squares x 30
- Digital Timer
- 30 cm ruler
- Scientific Calculator
- Pen
- Pencils

Important Background Information
Pyruvate kinase (PK), a 58 kiloDalton (kDa) protein, functions as a homotetramer and has a pivotal role in the glycolytic pathway (Fig. 1), converting its substrate phosphoenol pyruvate (PEP) into pyruvate in a reaction that also generates ATP.

PK deficiency (PKD) is the commonest cause of hereditary non-spherocytic haemolytic anaemia, a group of genetically inherited diseases associated with a net loss of red blood cells. In the PKD disorder, red blood cells are broken down (undergo haemolysis) prematurely, resulting in a shortage of red blood cells (anaemia). In hereditary non-spherocytic haemolytic anaemia, the red blood cells do not assume a spherical shape as they do in some other forms of haemolytic anaemia. Blood analysis can also be helpful in understanding the PKD disease. In addition, the disease is often associated with reticulocytosis; an increase in number of reticulocytes (immature red blood cells).

PKD is inherited in an autosomal recessive pattern. The parents of an individual with an autosomal recessive condition may, or may not, show signs and symptoms of the condition depending on the alleles that they possess.

Given equal subunit expression and random association, PK in either simple heterozygotes or compound heterozygotes (heterozygotes where there are two different recessive alleles) is a spectrum of heterotetrameric isozymes (A4, A3B, A2B2, B3A, B4) in a theoretic ratio of 1:4:6:4:1. If B is a variant and A is normal, some 94% of all PK in PKD patients contains one or more mutant subunits; in vivo, non-assembled monomers are prone to protease digestion. It is important to note that specific alleles code for PK proteins that form tetramers, whilst other alleles code for PK proteins that remain as monomers.

Clinical symptoms usually observed in simple heterozygotes, compound heterozygotes and homozygotes are variable, ranging from neo-natal jaundice requiring blood transfusions, to haemolytic anaemias that are self-regulated and show minimal clinical signs. Many of the mutant PKs have been identified on the basis of the biochemical characteristics of the defective enzyme.

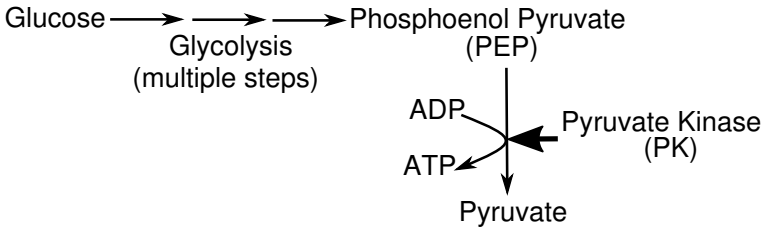


Figure 1: *The role of pyruvate kinase (PK)*

A clinical scenario:
Various family members have been diagnosed with non-spherocytic haemolytic anaemia, perhaps associated with PK deficiency. Numerous tests can be conducted including blood work to determine the parameters of the blood composition (haemogram) and an enzyme analysis of plasma specifically for PK activity. Additionally, a genetic analysis including identification of PK proteins from individual members and an analysis of a family pedigree to confirm the likely inheritance pattern can be undertaken.

You will analyse and collect data from the 3 family members: Father (F), Mother (M) and Daughter (D), in order to determine the family pedigree.

QUESTION 1

Blood Analysis:
Whole blood samples were collected from the Father (F), Mother (M) and their Daughter (D). After blood is taken and analysed the following partial results were obtained (Table 1):

Table 1

	Patient F (Father)	Patient M (Mother)	Patient D (Daughter)
Red Blood Cells (RBCs) count	5.35×10^{12}/l	3.65×10^{12}/l	3.01×10^{12}/l
White Cell count	7.5×10^{9}/l	4.2×10^{9}/l	3.4×10^{9}/l
Haemoglobin concentration	14.4 g/dl	10.5 g/dl	8.5 g/dl
Reticulocytes (% of RBCs)	1.67 %	2.63 %	10.46 %

Various analyses can be undertaken, including the determination of the haematocrit, the packed red blood cell volume, given as a percentage of total blood volume. The haematocrit is produced by taking up a sample of blood into a micro-capillary tube and centrifuging the sample so that the red blood cells become packed. The percentage of red blood cells is calculated by measuring the packed volume as a function of the total volume in the capillary tube.

Task 1a
Determine the haematocrit (% packed red blood cells by volume) shown in Figure 2, for each individual. Include these data in the first row of Table 3. This is expressed as a percentage to one decimal place (d.p.).



Figure 2 Haematocrit diagrams from the Father, Mother and Daughter. *The figure shows diagrammatic representations of the haematocrit, depicting a capillary tube of total sample containing red blood cells (left hand side) white blood cells (buffy coat) (between RBC and plasma) and plasma (right hand side).*

Table 2: *Normal Values for Haemogram of healthy people*

	Male	Female
Haemoglobin (g/dl)	13.5 - 17.5	11.5 - 15.5
Haematocrit (%)	40.0 – 52.0	36.0 – 48.0
Red blood cell Count ($x10^{12}$/l)	4.50 - 6.50	3.90 - 5.60
Mean Cell Haemoglobin (MCH) (pg)	26.0 - 34.0	26.0 - 34.0
Mean Cell Volume (MCV) (fl)	78.0 – 95.0	78.0 – 95.0
Mean Cell Haemoglobin Concentration (MCHC) (g/dl)	30.0 - 35.0	30.0 - 35.0
White Blood Cell count ($x10^{9}$/l)	4.0 – 11.0	4.0 – 11.0
Platelet Count ($x10^{9}$/l)	105 – 450	105 – 450
Reticulocyte Count (%)	0.5 – 1.5	0.5 – 1.5
PK activity (U/g Haemoglobin)	11.8 – 18.6	11.8 – 18.6

Task 1b
Using the packed cell volume from Figure 2, and the patient data from Table 1, calculate the following parameters for each patient (F, M and D):

The red blood cell volume, known as the **Mean Cell Volume** (**MCV**),
The mass of haemoglobin per red blood cell, known as the **Mean Cell Haemoglobin** (**MCH**),
The concentration of haemoglobin per red blood cell, known as the **Mean Cell Haemoglobin Concentration** (**MCHC**).

The MCV is given in femtolitres (fl) (fl is $1x10^{-15}$ l) to one d.p. MCH is expressed in picograms (pg) (pg is $1x10^{-12}$ g) to one d.p. MCHC is the total concentration of haemoglobin in the RBC fraction. This is expressed as g/dl (deci litre) ($1x10^{-1}$ l) to one d.p

Write the numbers in Table 3 for the Father (F), Mother (M) and Daughter (D).

Show your calculations for each parameter, in Box 1 for the Father (F).

Box 1: Calculations for patient F (Father) ***(4 marks)***

Element	Show your working here
Haematocrit	
MCV	
MCH	
MCHC	

Table 3 ***(4 marks)***

	Sample F (Father)	Sample M (Mother)	Sample D (Daughter)
Haematocrit (%)			
MCV (fl)			
MCH (pg)			
MCHC (g/dl)			

Further analysis
Anaemias are classified, according to the size of the red blood cell, as being either normocytic (normal MCV), macrocytic (increased MCV) or microcytic (decreased MCV). Microcytic anaemias are also often described as being hypochromic based on peripheral whole blood smear examination. The optical properties of the small, thin microcytes make them appear hypochromic on the blood smear, while the haemoglobin concentration remains in the normal range (microcytic; hypochromic anaemia). Normochromic blood smears show no change in optical properties on the blood smear with the haemoglobin concentration remaining in the normal range.

Table 2 gives the normal ranges for MCV, MCH and MCHC.

Task 1d
Using the values you have obtained (included in Table 3.), establish the classification in terms of size of blood cells and levels of haemoglobin of the blood of sample F, M and D.

You should answer from one of the following options for each patient (N.B. you can use any of the options more than once):

A. Macrocytic, normochromic
B. Microcytic, hypochromic
C. Normocytic, normochromic

(3 marks)

Patient	Classification (A, B or C)
Father (F)	
Mother (M)	
Daughter (D)	

QUESTION 2

Assay of pyruvate kinase activity
Biochemistry can be utilised to determine properties of well characterised enzymes by studying the variation in their kinetics. Enzyme activity is usually determined by monitoring the disappearance of a substrate or the appearance of a product, often using changes in spectrophotometric properties of the reaction mixture.

PK is conveniently assayed by a **coupled assay** in which the product of the reaction, pyruvate (Equation 1), is used as substrate for the NADH-linked enzyme lactate dehydrogenase (LD) (Equation 2), which is added to the reaction mixture in sufficient amounts to convert all of the pyruvate produced during the reaction to lactate. The reaction can be monitored by following the decrease in absorbance at 340 nm over time. The equations for the coupled reaction is shown (Fig. 3).

(equation 1) Phosphoenol pyruvate (PEP) + ADP $\xrightarrow{PK}$ ATP + pyruvate

(equation 2) Pyruvate (from equation 1) + NADH $\xrightarrow{LD}$ NAD^+ + lactate

Figure 3. *Pyruvate kinase and Lactate dehydrogenase-catalysed breakdown of Phosphenol pyruvate (PEP) (Equation 1) and Pyruvate (Equation 2)*

An enzyme's kinetic properties can be determined by varying the PEP substrate concentration (Equation 1).

Aims of the experiment
To determine the functionality of PK from the three patient plasma samples, Father (F), Mother (M) and the Daughter (D).

- Determination of activity of PK in patient plasma samples through the use of the coupled assay.
- Calculation of the kinetic properties of PK in absolute units.

Methods: pyruvate kinase assay
Before starting the assay, please note the following:

1. Solution A, C and patient samples F (father), M (mother) and D (daughter) should be kept on ice, whilst Solution B (H_2O) should be kept at room temperature.
2. The spectrophotometer should be blanked against water (provided) at 340 nm.
3. Make up the reaction mix by pipetting the reagents shown in Table 4 directly into a plastic 1 ml cuvette. You should add all the components *except* the plasma sample, *then* add the plasma sample last when you are ready to start the reaction.

Table 4. Pyruvate kinase assay components

You are provided with the following reagents	
Solution A	2x stock concentration Reaction Master Mix (10 ml) (the working concentration for the Reaction Master Mix is 1x)
Solution B	H_2O (10 ml)
Solution C	10 mM phosphoenol pyruvate (PEP) (1.5 ml)
Diluted patient plasma labelled F (Father), M (mother), or D (Daughter)	Diluted plasma from each patient (0.5 ml each)

Task 2a
Calculate the reaction volumes for FIVE concentrations of PEP (Solution C) (chose FIVE concentrations ranging from 0.2 mM to 1.5 mM). Show an example of your calculations for the highest concentration in Box 2.

PEP will drive the reaction and allow the determination the Pyruvate Kinase activity in each plasma sample (from Father (F), Mother (M) and Daughter (D)).

Box 2 ***(1 mark)***

Show an example of your workings for the calculation of volumes to be used of the highest PEP concentration.

Task 2b
Write the volumes used to make the reaction mixes into the blank spaces in Table 5; note that these will be the same for each plasma sample (F, M and D).

Table 5, Reaction Volumes. You should write the substrate concentrations, PEP [S], you have decided to use in the first row at the top of each column, and the volumes of each solution in the respective boxes. ***(3 marks)***

Concentration	**1**	**2**	**3**	**4**	**5**
PEP concentration					
Solution A (µl)					
Solution B (µl)					
Solution C (µl)					
Plasma	50 µl	50 µl	50 µl	50 µl	50 µl
Final volume	**1000 µl**	**1000 µl**	**1000 µl**	**1000 µl**	**1000 µl**

The PK activity Assay
Mix contents by inverting of the cuvette (making sure there is a piece of parafilm held in place over the opening using your thumb or forefinger), take the A_{340} reading at time zero, then record the A_{340} at 30 second intervals for 90 seconds. Record your absorbance values to the number of decimal places as displayed on your spectrophotometer.

For each sample you need to determine the initial rate of reaction $\Delta A/\Delta t$ (time expressed in minutes) for the various concentrations of PEP ([S]) you used, using your time zero value and another value of your choice. Highlight the time points which you have used to calculate the rate by circling the values.

Task 2c
Perform the experiment as described and record the absorbance readings using the tables provided (Tables 6.1, 6.2 and 6.3).

Table 6.1 Sample F (6 marks)

Concentration	**1**	**2**	**3**	**4**	**5**
[S]					
Absorbance at 0s					
Absorbance at 30s					
Absorbance at 60s					
Absorbance at 90s					
Rate ($\Delta A/\Delta t$) (min^{-1})					

Table 6.2 Sample M (6 marks)

Concentration	1	2	3	4	5
[S]					
Absorbance at 0 s					
Absorbance at 30 s					
Absorbance at 60 s					
Absorbance at 90 s					
Rate (ΔA/Δt) (min^{-1})					

Table 6.3 Sample D (6 marks)

Concentration	1	2	3	4	5
[S]					
Absorbance at 0 s					
Absorbance at 30 s					
Absorbance at 60 s					
Absorbance at 90 s					
Rate (ΔA/Δt) (min^{-1})					

Calculation of Kinetic Parameters

It is possible to express a change in absorbance over time as a change in product/substrate concentration. For each initial rate, ΔA/Δt, calculate the initial velocity (v_0) (ΔConc/Δt) of the reactions for each PEP substrate concentration ([S]). The absolute units for velocity should be given as µmol min^{-1} using the Beer-Lambert law. The Beer-Lambert law (or Beer's law) is the linear relationship between absorbance and concentration of an absorbing species.

The equation is: $A = \varepsilon lc$

- A = Absorbance
- ε = molar extinction coefficient
- l = path length of light
- c = concentration

The reaction in this experiment measures the conversion of NADH, which has a molar extinction coefficient of 6220 L mol^{-1} cm^{-1}, to NAD^+ (which has a negligible absorbance at 340 nm), and therefore the conversion of PEP to lactate. As there is a 1:1 molar ratio of components the conversion of NADH is therefore equal to the conversion of PEP. The cuvette has a path length of 1 cm.

Task 2d

Calculate the initial velocity (v_0) and write your answers in the tables below (Table 7.1, 7.2 and 7.3) for each patient (Father (F), Mother (M) and Daughter (D)). Your values should be given to the nearest 3 decimal places (d.p.).

Table 7.1 Patient F (1 mark)

[S]	v_0

Table 7.2 Patient M (1 mark)

[S]	v_0

Table 7.3 Patient D (1 mark)

[S]	v_0

Calculation of enzyme parameters K_M and V_{max}

By using the different rates of reaction for differing substrate concentrations two enzymatic parameters can be determined: K_M and V_{max}. These two parameters can be estimated by plotting the data in one of two ways: a Michaelis-Menten plot (Figure 4 A) or a Hanes-Woolf plot (Figure 4 B).

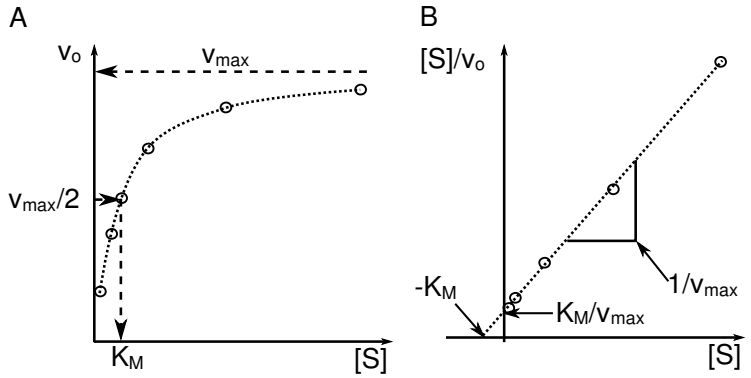
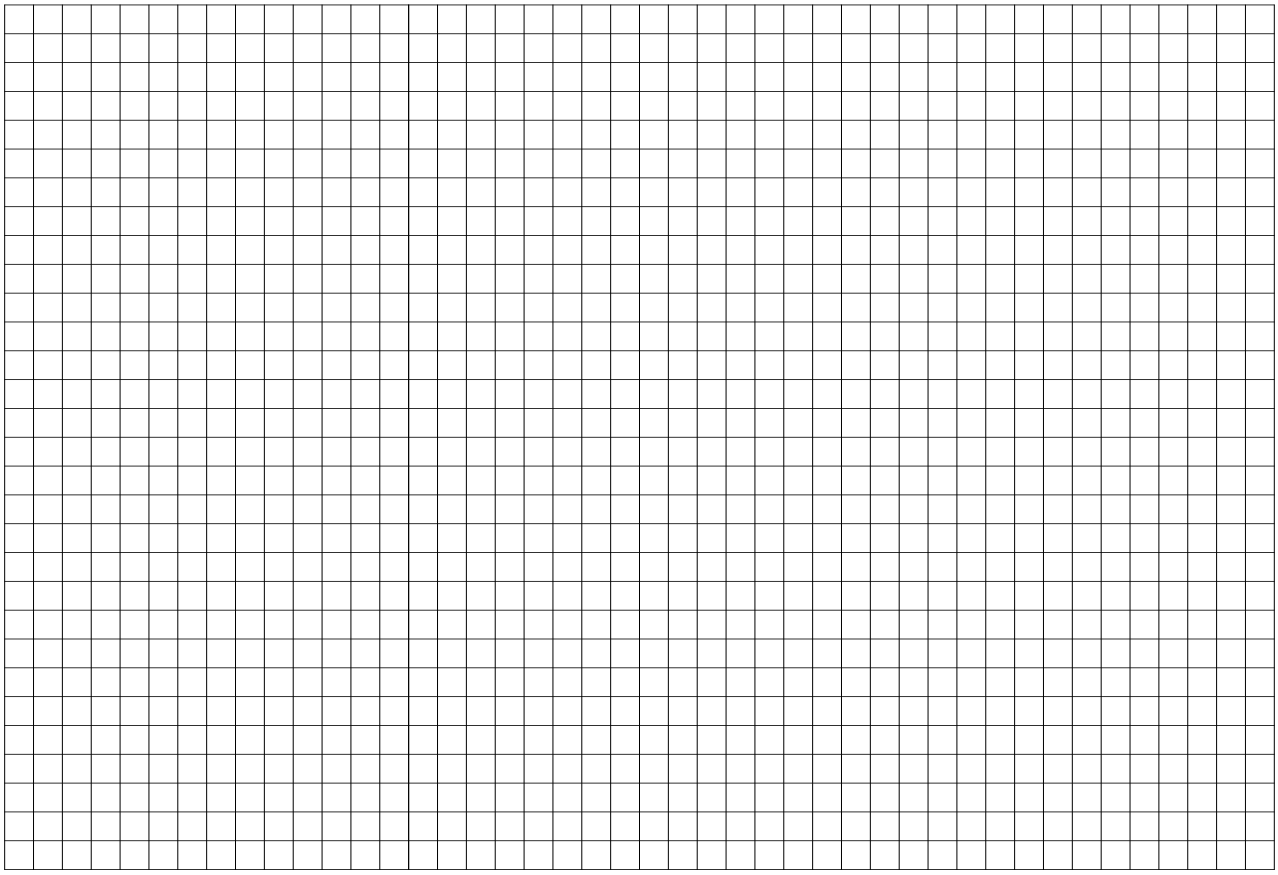


Figure 4. Plotting kinetic data. A. *Michaelis-Menten plot of initial reaction rate (v_0) over substrate concentration [S]. K_M is the substrate concentration that gives half maximal reaction rate. V_{max} is the maximal rate where [S] is saturating.* ***B.*** *Hanes–Woolf plot of the ratio of the substrate concentration and initial rate ([S]/v_0) over the substrate concentration. K_M can be determined from the x-axis intercept of the best linear fit to the data points, and V_{max} can be determined from the slope.*

Task 2e

Use your data to construct a Michaelis-Menten plot of v_0 over [S] for each patient (F, M and D) on the graph area below. You should draw one graph with three data plots; one each for Father (F), Mother (M) and Daughter (D). Use a cross (X) to mark data from the Father (F), a circle (O) to mark data from the Mother (M) and a triangle (Δ) to mark data from the Daughter (D).



Michaelis-Menten plot ***(10 marks)***

Task 2f

Using the three data plots, estimate V_{max} and K_M; write the estimates in the table below using the correct units and to the nearest 2 decimal places (d.p.), for each sample from the graph you have drawn.

(6 marks)

Patient	**V_{max}**	**K_M**
Father (F)		
Mother (M)		
Daughter (D)		

Task 2g (3 marks: one mark for each completed table)

Determine $[S]/v_0$ for each substrate concentration and each sample and write your answers in the tables below for each patient (Father (F), Mother (M) and Daughter (D)). Your values should be given to to the nearest 1 decimal places (d.p.).

Patient F (1 mark)

[S]	[S]/*v*0

Patient M (1 mark)

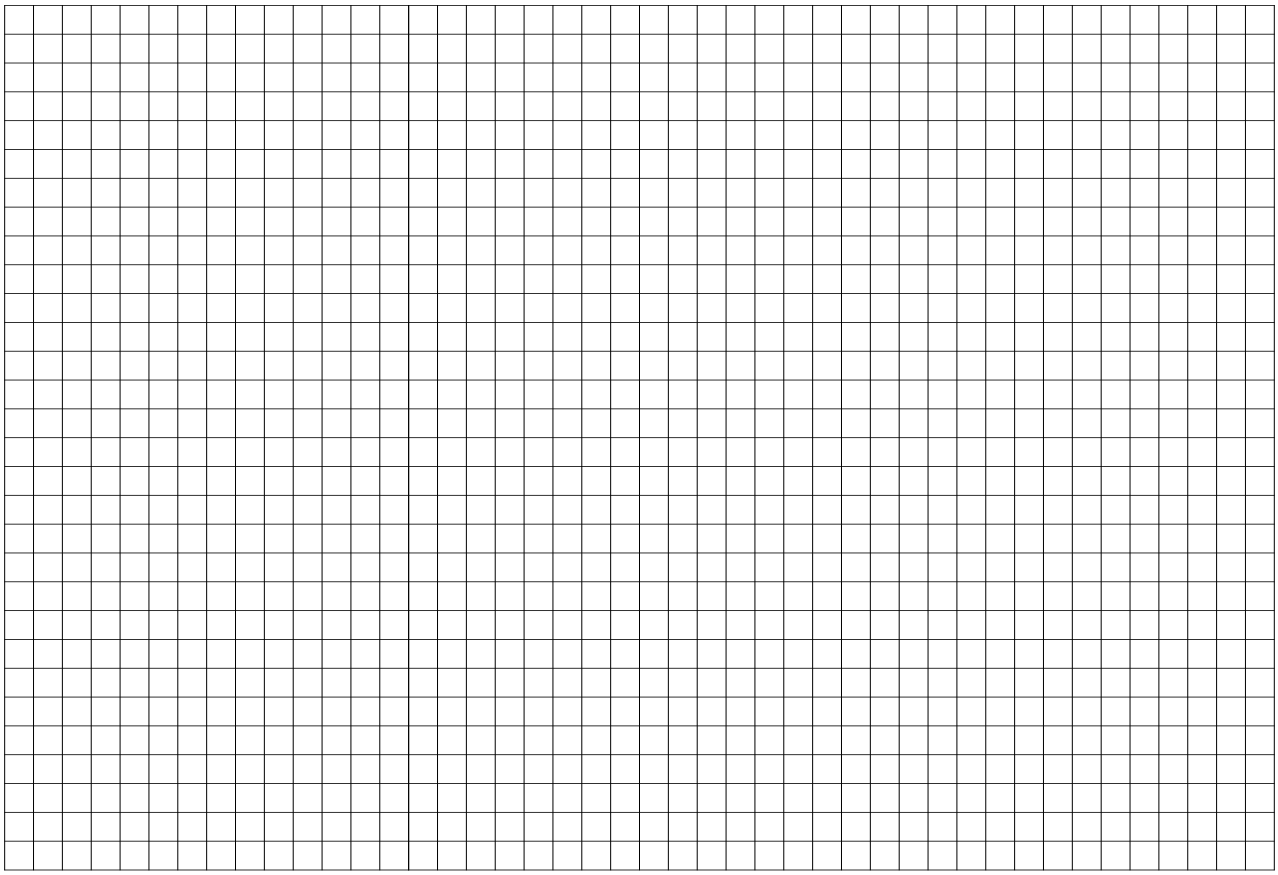
[S]	[S]/*v*0

Patient D (1 mark)

[S]	[S]/*v*0

Task 2h

Plot [S]/v_0 over [S] for each patient (F, M and D) on the graph area below. You should draw one graph with three data plots; one each for Father (F), Mother (M) and Daughter (D). Add a line of best fit for each data plot. Use a cross (X) to mark data from the Father (F), a circle (O) to mark data from the Mother (M) and a triangle (Δ) to mark data from the Daughter (D).



Hanes-Woolf plot ***(10 marks)***

Task 2i

Calculate V_{max} and K_M for each patient and include in the table below using the correct units to two decimal places (d.p.).

(6 marks)

Patient	**V_{max}**	**K_M**
Father (F)		
Mother (M)		
Daughter (D)		

QUESTION 3

Genetic Analysis and Diagnosis

Blood samples from the father, mother and daughter were sent to a clinical testing laboratory for further analysis. Proteins were extracted from whole blood, before separation on a polyacrylamide denaturing electrophoresis gel. The samples were visualised, after transferring the proteins to a nitrocellulose membrane (western blot), by detection using enzyme-conjugated anti-PK antibodies and subsequent chemiluminescence. The resulting visualisation of the PK protein content is shown in the image in Figure 5. Please refer to the Important Background Information to help interpret the figure.

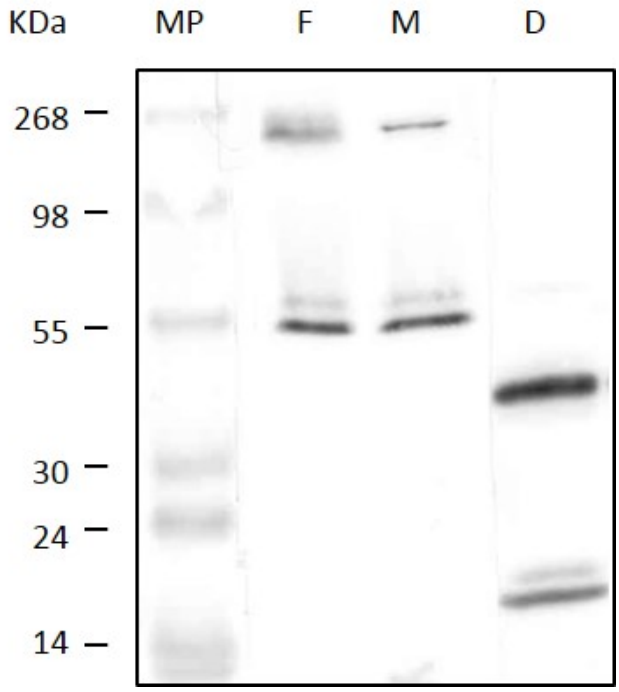


Figure 5. Immunoblot of PK variants in the affected family. *Red blood cell lysates from the father (F), the mother (M) and the daughter (D) were separated by Polyacrylamide gel electrophoresis (PAGE), transferred to membrane (western blot),and probed with anti-PK antibodies (immunoblotted). Sizes and migration of marker proteins (MP) are indicated on the left.*

Task 3a
Complete the table below, indicating which size protein bands are visible for each patient (F, M or D), as either ‘present’ or ‘not present’. Use the letter P to mark present, and NP to mark not present.

Task 3b
Indicate on the table whether any of the three patients (F, M or D) contain a PK tetramer by marking as either ‘present’ or ‘not present’. Use the letter P to mark present, and NP to mark not present.

(5 marks)

Molecular weight	Patient F	Patient M	Patient D
232 kDa			
58 kDa			
40 kDa			
18 kDa			
PK tetramer			

The clinical testing laboratory also received samples from siblings and other family members and analysed the PK activity of these individuals. In the family, the daughter has two further brothers (Table 8).

The PK K_M data for other family members is also shown in Table 8.

Task 3c
Write your values for the K_M, derived from the Hanes-Woolf calculation, for the Father, Mother and Daughter in the table for your convenience (**zero marks**).

***Table 8**. K_M [PEP] for subjects in the extended family; the Proband is a person serving as the starting point for the genetic study of a family.*

Subject	K_M [PEP]
Daughter (IV-3) (Proband)	
Aunt (III-6)	0.45
Grandfather (II-2)	0.40
Great Grandmother (I-1)	0.40
Mother (III-3)	
Brother (IV-4)	0.48
Brother (IV-5)	0.35
Father (III-4)	
Grandmother (II-1)	0.20
Aunt (III-2)	0.15
Uncle (III-5)	Unknown/Unavailable

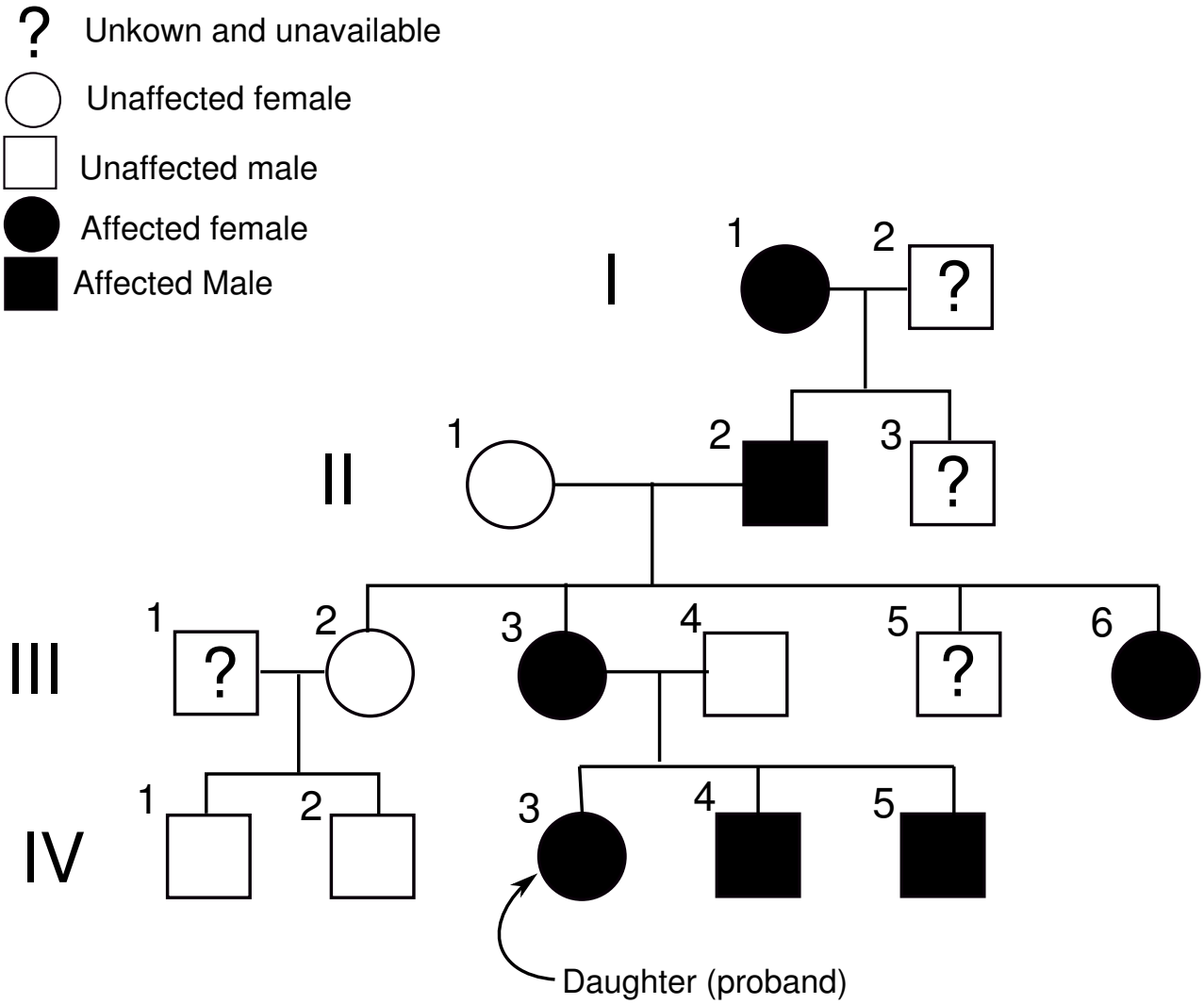


Figure 6. Family Pedigree.

Task 3d (1 mark for the correct answer; you cannot gain a mark for Task 3e unless you have answered Task 3d correctly).

Indicate, by drawing a circle around your choice of either A, B or C, father III-4's genetic PK gene arrangement in terms of:

A. Simple Heterozygote (Aa_1, Aa_2, Aa_3)
B. Compound Heterozygote (a_1a_2; a_1a_3; a_2a_3)
C. Homozygous (AA)

Task 3e (1 mark for the correct answer; you can only receive a mark if you have answered Task 3d correctly).
Indicate, by drawing a circle around your choice of either A or B, brother IV-5's genetic PK gene arrangement in terms of:

A. Simple Heterozygote
B. Compound Heterozygote

END OF EXAM