INTERNATIONAL BIOLOGY OLYMPIAD e. V. IBO

27th International Biology Olympiad

July 17-23, 2016
Hanoi, Vietnam

Practical Exam 1

PLANT ANATOMY AND PHYSIOLOGY

Total points: 91
Duration: 90 minutes

DEAR PARTICIPANTS,

This exam consists of three experiments:

EXPERIMENT 1. LEAF PIGMENT ASSAY (30 points)

Task 1. Pigment quantitative determination by spectrophotometer

Task 2. Pigment qualitative determination by TLC

EXPERIMENT 2. PLANT ANATOMY (31 points)

Task 3. Observe the anatomical characters of four samples

Task 4. Make the data matrix and identify the position of each sample in a given phylogenetic tree

Task 5. Draw the detailed structures of that vascular bundle

EXPERIMENT 3. IDENTIFICATION OF PLANT SPECIES AND MAKE THE DATA MATRIX (30 points)

Task 6. Identify morphological and anatomical characters of five given floral samples

Task 7. Name samples PG-PK using a given dichotomous key

Task 8.Make the data matrix

A. Please remember to write your **Country** and **Student code** in the given box.
B. Write your answers in the separate **Answer Sheet**. Only the answers given in the **Answer Sheet will be evaluated**.
C. Make sure that you have received all the materials and equipment listed. If any of these items are missing, please raise the **Red card** within 10 minutes immediately.
D. During experiments, ensure to handle equipment properly. Any spilled solutions or broken equipment will not be replenished.
E. Stop answering and put down your pen immediately when the bell rings at the end of the exam. Enclose the **Answer Sheet**, **Question Paper**, and **Data printout** in the provided envelope.
F. No paper, materials or equipment should be taken out of the laboratory
G. Ensure to obtain spectrophotometer readings in Task 1 and to answer the questions that follow.

CAUTIONS: This experiment deals with materials that are fragile and sharp. Exercise care when handling these materials. Do not let them get in contact with your skin or clothes. Wear safety goggles to protect your eyes from splashes.

Good luck!!!

EQUIPMENT AND MATERIALS FOR 3 EXPERIMENTS

Experiment 1. Leaf pigment assay

Name	Quantity
Soybean leaf samples (sample A and sample B)	2 microcentrifuge tubes
Positive control	1 microcentrifuge tube
TLC plate with student code in a plastic bag	1 piece
Cuvettes	2 pieces
95% ethanol	40 mL in falcon tube
Ethanol for washing pipette	20 mL in falcon tube
Chromatography solvent (n-hexane : acetone = 7:3 in volume)	25 mL in TLC bottle
Mortars and pestles	2 pieces
Falcon rack	2 pieces
Funnels	2 pieces
Filter papers	2 pieces
Forceps	1 pairs
1 mL glass pipette	2 pieces
5 mL glass pipette	1 piece
Pipetting ball	1 piece
1.5 mL microcentrifuge tube	2 pieces
Microcentrifuge rack	1 piece
15 mL Falcon tube	4 pieces
Cuvette rack	1 piece
Capillary tube	2 pieces
Calculator	1 piece
Scratch paper for calculating	1 Set
Gloves	3 pairs
Tissue papers	5 pieces
Pencil and sharpener	1 piece
Ruler	1 piece
Marker pen	1 piece
Mask	1 piece
Plastic goggle	1 pairs
Waste container	1 piece

Experiment 2. Plant anatomy

Name	Quantity
4 stems of different plant species labelled as SC, SD, SE and SF. Each species include 2 samples	8 pieces
Microscope	1 piece
Lanceolate needle	1 piece
Glass Slide	10 pieces
Glass cover slip	10 pieces
Filter paper	20 pieces
Razor blade	2 pieces
12% bleach solution	20 mL in bottle
3% HCl solution	20 mL in bottle
7.5% carmine solution	20 mL in bottle
1.5% green methyl solution	20 mL in bottle
Distilled water	20 mL in bottle
Timer	1 piece
Marker pen	1 piece
Carrot slice (serves as a cutting board)	1 piece

Experiment 3. Identification of plant species and make the data matrix

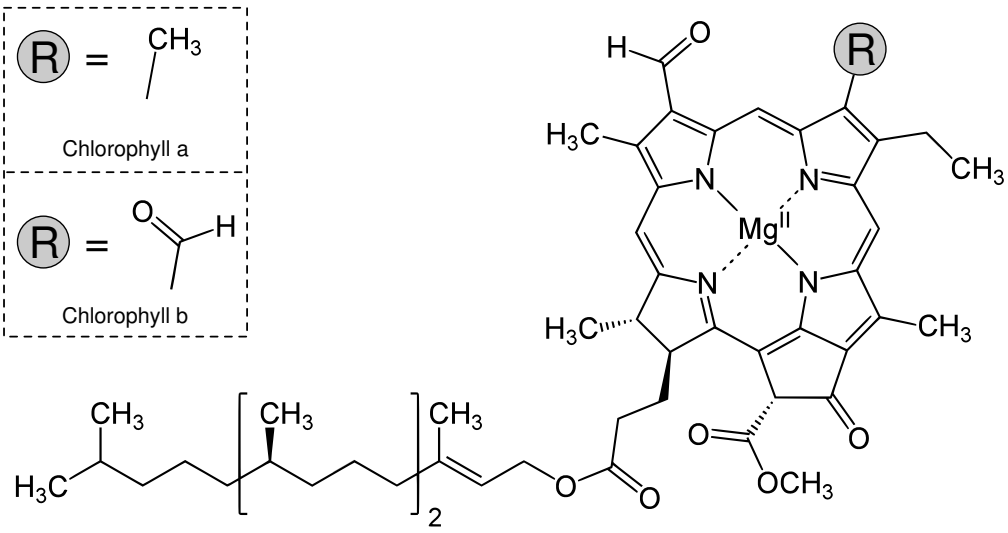
Names	Quantity
5 floral specimen in 70% ethanol labelled as sample PG, PH, PI, PJ, PK. Two flowers for each specimen	5 tubes
Microscope	1 piece
Carrot slice (serves as a cutting board)	1 piece
Magnifier glass	1 piece
Pointed needle	1 piece
Lanceolate needle	1 piece
Glass slide	5 pieces
Cover slip	5 pieces
Forceps	1 piece
Razor blade	2 pieces
Filter paper	5 pieces
Mask	1 piece
Marker pen	1 piece
Distilled water	1 bottle

EXPERIMENT 1. LEAF PIGMENT ASSAY (30 POINTS)

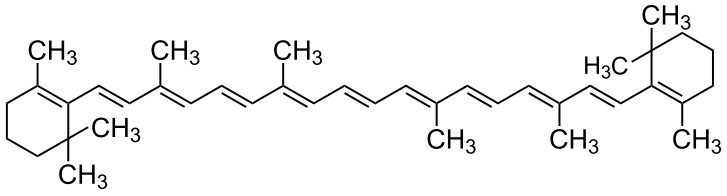
Introduction

Acclimatization to different light intensities involves changes in several physiological characteristics. Leaves exposed directly to sunlight (sun leaves) show differences in leaf structure and their pigment compositions compared with leaves grown under canopy condition (shade leaves). This acclimatization of leaves can be recognized by qualitative and quantitative identification of leaf pigments.

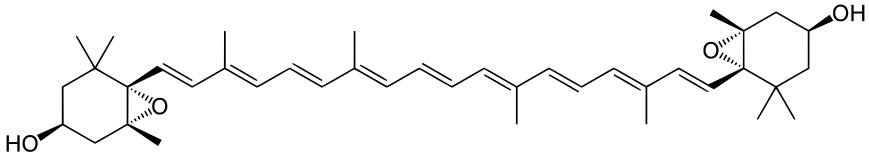
Thin layer chromatography (TLC) is a technique for separating and analyzing the different pigments in the mixture. Leaf pigments including chlorophyll a, chlorophyll b, carotenoids and xanthophylls (violaxanthin, neoxanthin) (see formulas below) can be visualized on TLC plates. To determine the amount of each pigment, leaf extract is quantified by using spectrophotometry at different wavelengths.



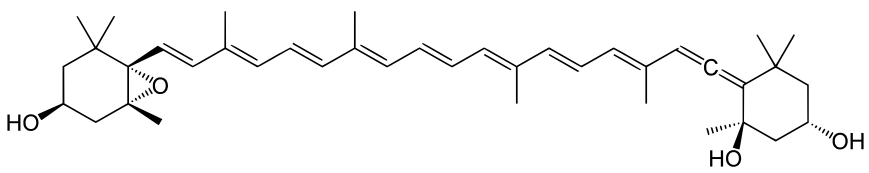
Chlorophyll a and b



b- carotene

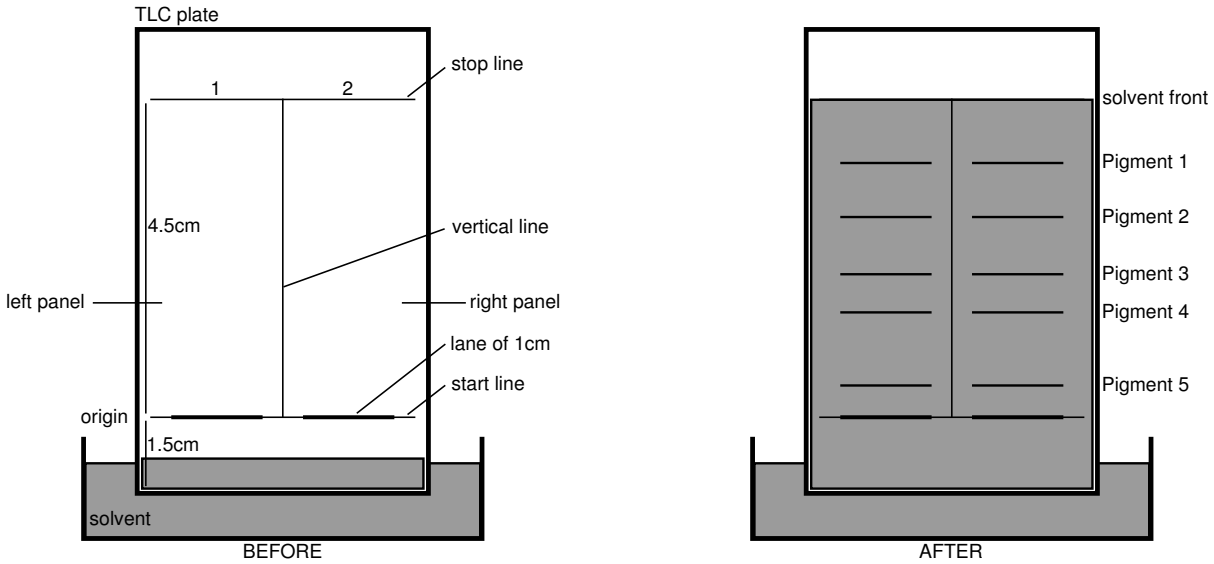


Violaxanthin



Neoxanthin

In this experiment, soybean plants are grown under either sunlight condition or canopy condition to collect leaf samples for qualitative and quantitative analyses of leaf pigments.



Thin layer chromatography of the plant extract

Spectrophotometer

Experiment procedure

1. Grind each leaf sample using a separate mortar and its pestle with 2 mL 95% ethanol into a fine mixture. Add a further 5 mL of 95% ethanol to the mixture, and continuously grind to a homogeneous mixture. Transfer the mixtures onto separate filter papers placed into the funnel. Collect the extract into a labeled 15 mL Falcon tube up to 5 mL resulting in extract A and extract B.
2. Transfer 0.5 mL of each extract into newly labeled 15 mL Falcon tubes.
3. Dilute each extract to 5 mL with 95% ethanol and mix the solution gently.
4. Transfer the diluted extracts A and B to the labeled cuvette A and B, respectively. Measure absorbance at 649 and 664 nm for both cuvette A and B. (*Raise your green card when your cuvettes are ready. The assistants will take your samples to measure and give absorbance values back to you. While waiting for the measurement, you should continue with the next steps of the experiment*).
5. Using a ruler and pencil, lightly draw across the TLC plate 1.5 cm from its bottom edge to make a start line. Place a mark 4.5 cm from the start line to determine the stop line . Lightly draw a vertical line to divide the TLC plate into 2 panels: left panel and right panel. Very lightly draw a 1cm line in the center of the left panel as well as right panel at the start line for indicating loading point.
6. Transfer approximately 0.5 mL of extract A (without dilution) into a 1.5 mL centrifuge tube. Use a glass capillary tube to take extract A from centrifuge tube and load the extract A along the 1 cm line of the left panel. Allow the solvent to dry slightly and apply the pigment again up to 10 times. Similarly load the positive control 10 times onto the lane along start line of right panel of TLC plate.
7. Let the plate dry for approximately 1 minute at room temperature. Put the plate into the TLC bottle containing a shallow pool of chromatography solvent and close the lid (the pigment area on the plate must not be in contact with the chromatography solvent). As the eluent reaches the stop line, remove the TLC plate immediately from the TLC bottle. (*Raise your* **GREEN card***, the assistant will take a photograph of your TLC plate result for grading*). **6 points** will be graded to your photo of TLC plate.

Answer following questions in the <u>Answer sheet</u>

Q.1.1 (10 POINTS)

Record the absorbance values into a table in **Answer sheet**. Calculate concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Total Chl) according to the following formulae (Lichtenthaler, 1987). Calculate the ratios of chlorophyll a to b for extract A, extract B (to 2 decimal points).

$$\text{Chl a (mg/L)} = -5.19 * (A_{649}) + 13.36 * (A_{664})$$
$$\text{Chl b (mg/L)} = 27.43 * (A_{649}) - 8.12 * (A_{664})$$
$$\text{Total Chl (mg/L)} = 22.24 * (A_{649}) + 5.24 * (A_{664})$$

Q.1.2. (2 POINTS)

Indicate in the **Answer sheet** if each of the following statements is True or False by using this mark ✓

A Leaves used for extract A are generally thicker than those of extract B
B Leaves used for extract A are more sensitive to photoinhibition
C Extract A is derived from shaded leaves
D Leaves for extract A have lower light compensation point than that of leaves for extract B

Q.1.3. (6 POINTS)

After taking the photograph of your TLC plate, place it back into the small plastic bag. Seal the top and staple it to your **Answer sheet.**

Q.1.4. (10 POINTS)

Calculate the Rf values according to the following formula and determine the name of each pigment. The ***distance travelled by pigment*** is measured from the start line to the horizontal and vertical centre of the pigment band. The **distance travelled by solvent** is measured from the start line to the solvent front.

$$Rf = \frac{\text{Distance travelled by pigment}}{\text{Distance travelled by solvent}}$$

*no band = no score

Q.1.5. (2 POINTS)

Indicate in the **Answer sheet** if each of the following statements is True or False by using this mark ✓

A Chlorophyll a and chlorophyll b show different Rf values due to their molecular weight.
B Rf value of chlorophyll and b-carotene are different owing to their different polarity
C Speed of pigment movement mainly depends on interaction with stationary phase on TLC plate.
D In the chromatography experiment, n-hexane and acetone are combined as chromatography solvents. These two solvents are used in order to enhance the solubility of different pigments.

EXPERIMENT 2. PLANT ANATOMY (31 POINTS)

Land plants evolved from algae nearly 500 million years ago. Many new features facilitating survival and reproduction on dry land emerged after land plants diverged from their algal relatives. In land plants, the stem is one of the most important organs that supports leaves and reproductive organs by means of mechanical strengthening and transportation of water, minerals and organic compounds. These functions are carried out by the vascular system, including xylem and phloem, which are present in certain plants. The inner structure (anatomy) of plant stem sections can be observed through microscope.
In this task, you will perform stem sectioning of four plants and observe anatomical traits. Based on characteristics of vascular system, a phylogenetic tree representing the evolutionary trend of vascular system and the relationship between given plant taxa, can be generated.

Experimental procedure

1. Slice samples using a razor blade. Make cross-sections as thin as possible.
2. Transfer sections onto a glass slide. Add drops of bleach solution to fully cover the sections and let stand for 2 minutes. Use filter paper to remove excess bleach solution from the sections.
3. Add drops of HCl solution to fully cover the sections and let stand for 30 seconds. After that, use filter paper to remove excess HCl solution from the sections.
4. Add drops of water to wash the sections. After that, use filter paper to remove water from the sections.
5. Add drops of carmine solution to stain the sections for 3 minutes. Use filter paper to remove carmine solution.
6. Add drops of green methyl solution to fully cover the sections and stain for 30 seconds. After that, use filter paper to remove excess green methyl solution.
7. Add drops of water on the sections, cover the sections with a glass cover slip, use filter paper to remove excessive water, and then observe under a microscope.

Answer these following questions into the Answer Sheet:

Q.2.1. (8.0 POINTS)

Which of these following tissues are present in each plant sample?

Mark ✓ for tissues present and × for tissues absent in observed samples in the table in the **Answer sheet**

Q.2.2. (6.4 POINTS)

Use the plant sections to observe 4 stem anatomical characters and state whether they are absent (0) or present (1). Write 0 or 1 of each character to the data matrix in the **Answer sheet** below. The data of outgroup taxon SH are already given.

A phylogenetic tree (shown in Fig. below) of four experimental plant species and one given species (SH) is generated using parsimony method, based on the above data matrix. The primitive character state (state 0) is hypothesized to be the same as the state in the taxon SH.

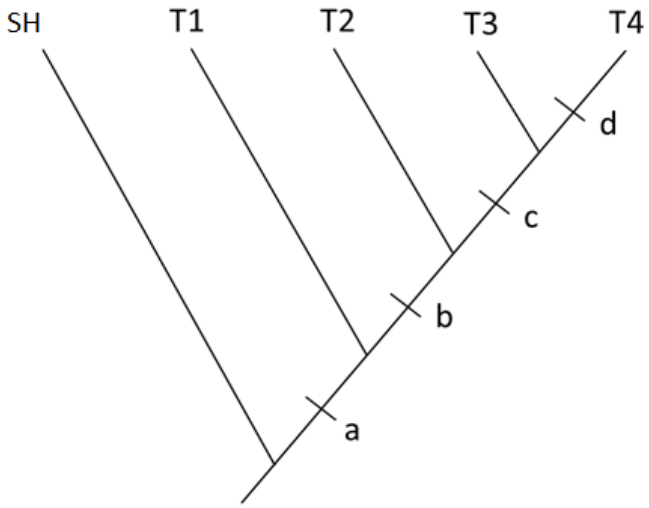


Fig. Phylogenetic tree of 5 species
(Taxon: T)

Q.2.3. (8.0 POINTS)

Using the result from Q.2.2, determine the stem character (C1 to C4) corresponding to the character (a to d) in the phylogenetic tree, and define the position of each experimental plant species (SC to SF) corresponding to its taxon (T1 to T4) in the phylogenetic tree.

- Write down the stem character (C1 to C4) corresponding to the character (a to d) in the phylogenetic tree:

Q.2.4. (6.0 POINTS)

- Write down the name of plant species (SC to SF) corresponding to its taxon (T1 to T4) in the phylogenetic tree:

Q.2.5. (1.0 POINT, 0.25 POINTS EACH)

Refer to the diagram of one vascular bundle on the**Answer Sheet** and label the metaxylem (1), phloem (2), protoxylem (3) and sclerenchyma (4) into the open boxes

Q.2.6. (1.6 POINTS)

Indicate in the **Answer sheet** if each of the following statements is True or False by using this mark ✓

A The stem of plant SC could not transport water as efficiently as plants SD and SE

B The abundance of sclereid in plant SD makes the stem hard.

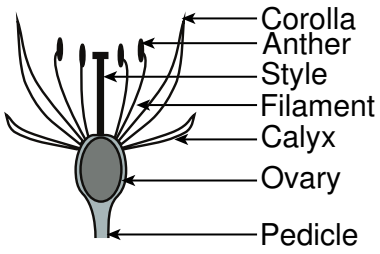
C The stem diameter of plant SE does not increase continuously during plant development because sclerenchyma restricts the development of vascular bundles.

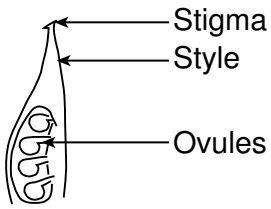
D The sclerenchyma ring below the epidermis strengthens the stem of plant SF.

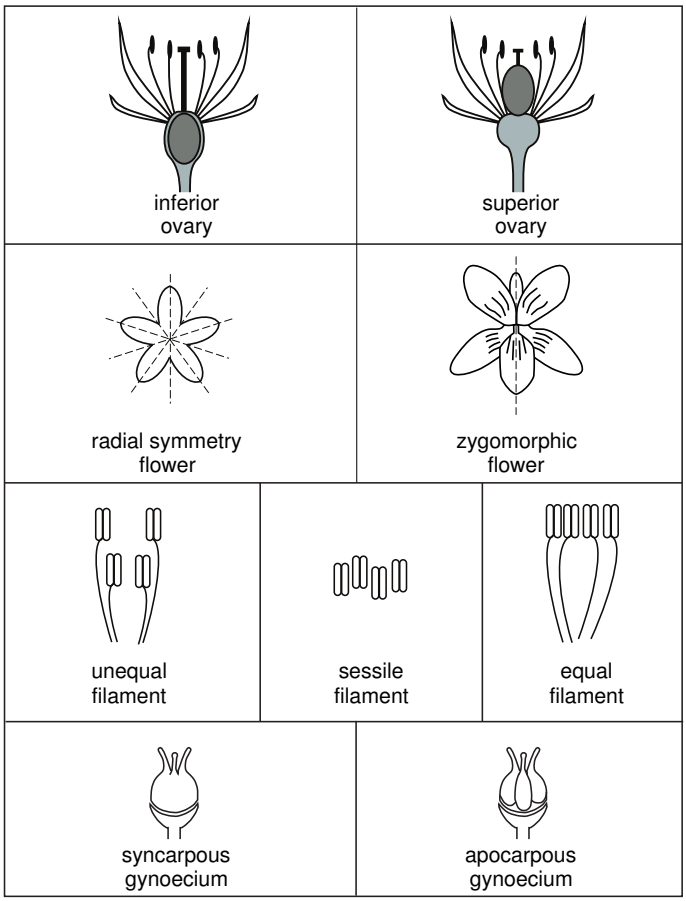
EXPERIMENT 3. IDENTIFICATION OF PLANT SPECIES AND MAKE DATA MATRIX (30POINTS)

Introduction

Flower structures are exceedingly varied but they are useful for plant species identification.
In this task, you will identify morphological and anatomical traits of five given floral samples (sample PG-PK) and answer the questions in the **Answer sheet**. Based on the given dichotomous key and using observed morphological and anatomical characteristics you have to name samples PG-PK. Make data matrix for given samples.
To help understand the terminology used below figures:







Experimental procedure

3.1. Flower symmetry

- Use the forceps to take each sample out of the falcon tube onto a glass slide. Close the tube to avoid ethanol vapor in the room. Use filter paper to remove excess ethanol.
- Handle the flower specimens carefully, as you need the given plant material for all your observations.

Q.3.1. (2.5 POINTS)

Distinguish flower symmetry in each sample and fill "✓" in the table if the flower is radial symmetrical or zygomorphic in the **Answer sheet**

3.2. Number and characteristics of floral parts

- Use the pointed needle, lanceolate needle, razor blade and magnifier glass to analyze in turn the calyx, corolla, androecium and gynoecium for all samples provided.
- Observe all samples carefully and complete Q.3.2 and Q.3.3.

Q.3.2. (4.5 POINTS)

Determine the number of calyx or calyx lobes, corolla lobes, stamens in each sample and put these numbers in table in the **Answer sheet**

Q.3.3. (9.0 POINTS, 0.3 POINTS PER BOX)

Determine the characteristics of calyx, corolla, filament, and gynoecium in each sample and fill "✓" in the table if with characteristic, "x" if without characteristics in the **Answer sheet**.

- Put the gynoecium on the carrot piece, use razor blade to make cross sections of the ovary as thin as possible on one flower and cut along the ovary on the other flower of the same sample PG, PI, PJ (sample PH, PK are already given). Put the sections on the glass slide, add one drop of water on the section, cover with a cover slip. Observe the sections under the microscope.

Q.3.4. (3.0 POINTS)

Determine the number of locules and ovules per locule in each sample and put these numbers in the table in the **Answer sheet**.

3.3. Identify plant species

Dichotomous key to the species: the key was determined by the presence or absence of characteristic. Read the dichotomous key carefully, if you can't find the characteristic in the first line, please move to the second line in the same number.

1	Filaments **equal or sessile**	Go to 2
	Filaments **unequal**	Go to 6
2	Ovary **more than 2**-loculed	Species **ta**
	Ovary **2**-loculed	Go to 3
3	Number of ovule per locule as **1**	Go to 4
	Number of ovule per locule **more than 1**	Go to 5
4	Corolla **with** hair in abaxial	Species **tb**
	Corolla **without** hair in abaxial	Species **tc**
5	Corolla **with** hair in abaxial, **syncarpous** gynoecium	Species **td**
	Corolla **without** hair in abaxial, **apocarpous** gynoecium	Species **te**
6	Corolla **4-5** lobed	Go to 7
	Corolla **more than 5** lobed	Species **tm**
7	Number of ovule per locule only **1**	Go to 8
	Number of ovule per locule **more than 1**	Go to 9
8	Calyx without hair in abaxial	Species **tf**
	Calyx with hair in abaxial	Species **tg**
9	Corolla **with** hair in abaxial; ovary **inferior**	Species **th**
	Corolla **without** hair in abaxial; ovary **superior**	Species **tk**

Q.3.5. (6.0 POINTS)

Using the dichotomous key, identify the name of the species for samples PG-PK, choose and fill the name of species (ta, tb, tc, td, te, tf, tg, th, tk, tm) in the table in the **Answer sheet.**

3.4. MAKE DATA MATRIX

Q.3.6. (5.0 POINTS)

Write down the correct character state of each character to the data matrix in the table in the **Answer sheet.**

End of practical Exam 1

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Practical Exam 2

ANIMAL SYSTEMATICS AND ANATOMY

Total points: 100
Duration: 90 minutes

DEAR PARTICIPANTS,

In this practical test, do the following two parts:

EXPERIMENT 1. CLASSIFICATION OF BUTTERFLIES (65 points)

- Task 1. Identify all the butterfly specimens provided
- Task 2. Compile the character matrix
- Task 3. Calculate the distance matrix based on the characteristic matrix provided
- Task 4. Resolve the phylogenetic relationship of all the specimens
- Task 5. Draw a phylogenetic tree (dendrogram)

EXPERIMENT 2. ANATOMY OF EARTHWORM *Amynthas aspergillum* (35 points)

- Task 6. Identify the external structures of *Amynthas aspergillum*
- Task 7. Dissect and identify the internal structures of *Amynthas aspergillum*

Important Information:

- Please remember to write your **Country** and **Student code** in the given box.
- Write your answers in the separate **Answer Sheet**. Only the answers given in the **Answer Sheet will be evaluated**.
- Make sure that you have received all the materials and equipment listed. If any of these items are missing, please raise the **Red card** immediately.
- During experiments, ensure to handle equipment properly. Any spilled solutions or broken equipment will not be replenished.
- Stop answering and put down your pen immediately when the bell rings at the end of the exam. Enclose the **Question Paper** and **Answer Sheet** in the provided envelope.
- No paper, materials or equipment should be taken out of the laboratory.

Good luck!!!

Materials and Equipment

Experiment 1. Classification of Butterflies

Name	**Quantity**
Box containing 8 butterfly specimens	1 box
Mask	1 piece
Forceps	1 pair
Magnifier glass	1 piece
Ruler	1 piece
Pen	1 piece
Calculator	1 piece
Scratch papers for calculating	1 set
Gloves	2 pairs
Tissue papers	1 box

Name	Quantity
Alcohol pre-treated earthworm	1 specimen
Stereomicroscope	1 piece
Tray	1 piece
Forceps	1 pair
Mounted needle	1 piece
Scissors	1 piece
Knife	1 piece
Petri disc	1 piece
Glass slide	1 piece
Pipette	1 piece
Plate with pins	1 plate
Plate with 5 colour-headed pins	1 plate
Magnifier glass	1 piece
Gloves	2 pairs
Mask	1 piece
Student Code Sheet	1 piece
Pen	1 piece
Tissue papers	1 box

* Participants carefully check the materials and equipment. If any of these items is missing or damaged or unable to distinguish the colour-headed pins, please raise the RED card immediately.

EXPERIMENT 1. CLASSIFICATION OF BUTTERFLIES (65 POINTS)

Introduction

Vietnam has rich and diverse fauna and flora. There are more than 1,200 species of butterflies. However, due to habitat disturbance and destruction, some species are threatened and endangered, thus learning about butterflies may contribute to preserving their diversity. The purpose of this practical task is to identify some butterfly species in Vietnam and compile the phylogenetic relationship of these species based on their morphological characters.

Note: Butterfly box will be a gift for the participants after completing all the practical (*Please, write your name in the label on the box*).

Task1. Identify all the butterfly specimens provided

Identify all butterfly specimens (A to H) using the following identification keys. Consult the figures (1, 2, 3) below to identify the required morphological characters.

Morphological characters

The following figures describe the required morphological characters.

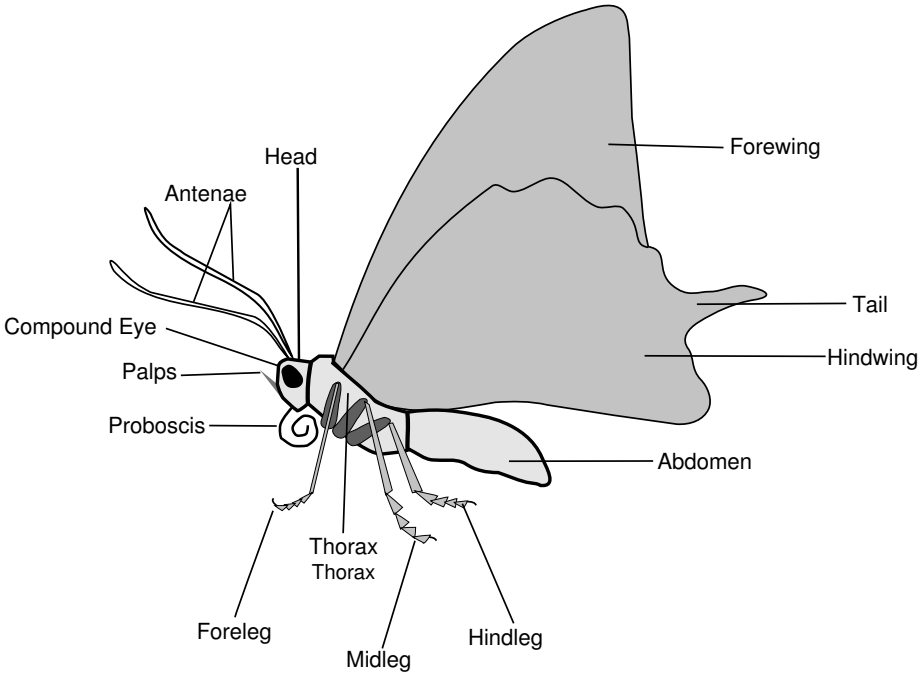

Figure 1. External structure of butterfly.

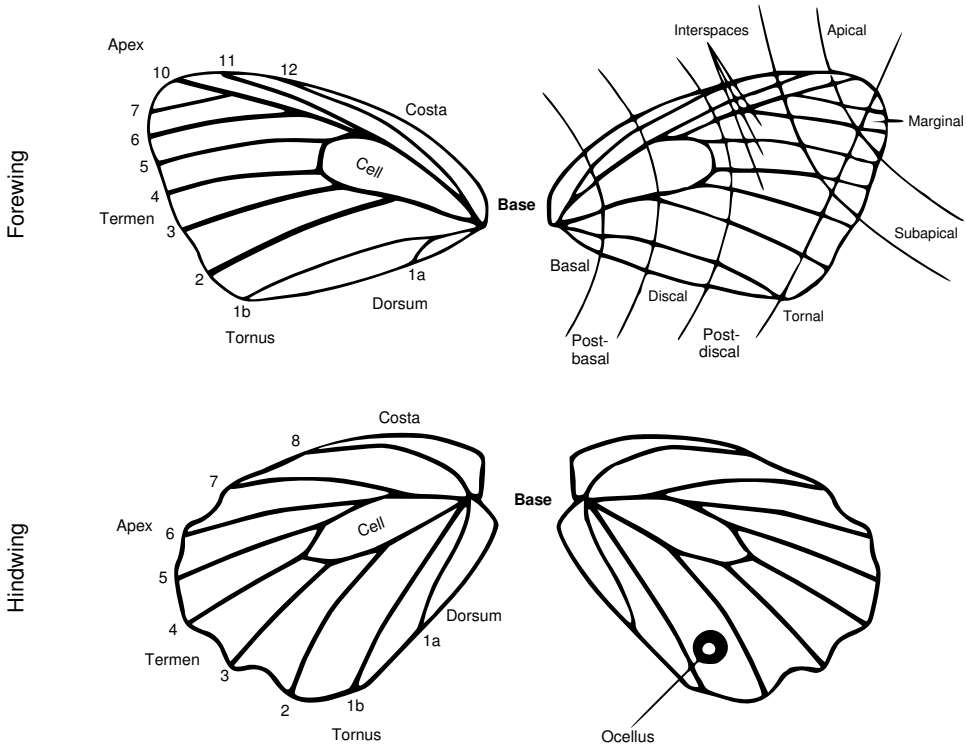

Figure 2. Butterfly wing terms.

The wing is divided into several areas and interspaces. The wing vein are numbered. The forewing veins are numbered from 1 (1a, 1b) to 12; the hindwing veins are numbered from 1 (1a, 1b) to 8.

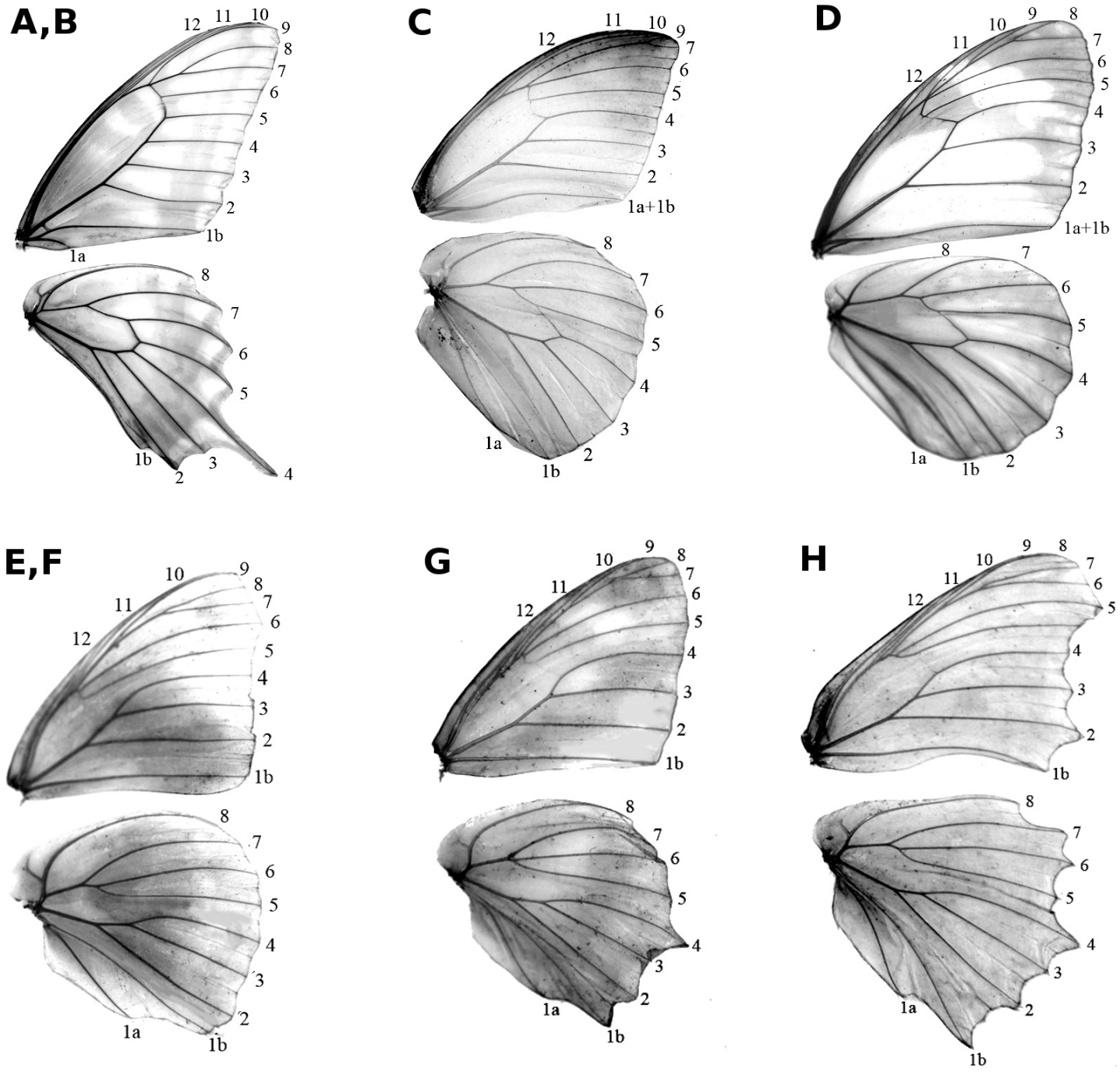

Figure 3.

Specimens A, B) With Veins 1a, 1b in the forewing; without Vein 1a on the hindwing; with long tail on the hindwing,

Specimen C) With Veins 1a, 1b in the forewing intersected; Veins 8 and 9 in the forewing overlapped.

Specimen D) With Vein 1a, 1b in the hindwing; Vein 1a and 1b on the forewing intersected.

Specimens E, F) With Vein 1a, 1b in the hindwing, without Vein 1a on the forewing, open wing cells ; apex of forewing cut

Specimen G), H) Without Vein 1a in the forewing, with Veins 1a and 1b in the hindwing, knob tail on the Vein 4 in the hindwing; open wing cells.

Specimen H) Apex of forewing cut.

Identification Key for Butterflies

1.	Hindwing **with** long tail	Continue at 2.
	Hindwing **without** long tail	Continue at 6.
2.	A white **patch** on the wing	Continue at 3.
	No white patch on the wing	Continue at 4.
3.	Forewing **with** white spots in interspace between 1a and 1b (Figure 2)	***Papilio noblei***
	Forewing **without** white spots in interspace between 1a and 1b	***Papilio helenus***
4.	Upper side of wings **with** a pale yellowish green macular band from the apex to the mid of dorsum of the forewing	***Papilio demolion***
	Upper side of wings **without** a pale yellowish green macular band from the apex to the mid of dorsum of the forewing	Continue at 5.
5.	Upper side of hindwings tornus with a **red spot** but **no black dot** inside	***Papilio machaon***
	Upper side of hindwings tornus with an **orange or pale yellow spot** and a **black dot** inside	***Papilio xuthus***
6.	White or yellow wings	Continue at 7.
	No white or yellow wings	Continue at 8.
7.	**Yellow** wings with a wide orange band in the forewings	***Ixias pyrene***
	White wings with a big red-orange patch on half of forewing	***Hebomoia glaucippe***
8.	Apex of forewing **rounded or pointed**	Continue at 9.
	Apex of forewing **cut**	Continue at 11.
9.	Hindwing with knob tail; upper side with orange bands on brown wings	***Symbrenthia lilaea***
	Hindwing **without** knob tail	Continue at 10.
10.	Wing veins **brown**; upper side of hindwings orange **without** black spots	***Danaus genutia***
	Wing veins **not brown**; upper side of hindwings orange **with** black spots	***Danaus chrysippus***
11.	**Ocelli** on wings	Continue at 12.
	No ocelli on wings	Continue at 13.
12.	**Blue** hindwing; **upper** side of hindwing with 2 ocelli	***Junonia orythia***
	No blue hindwing; **lower** side with darker brown transverse bands	***Junonia iphita***
13.	Black wings with white macular bands and spots	***Athyma asura***
	Orange wings with black spots	***Polygonia c–aureum***

Q.1.1. CLASSIFICATION OF BUTTERFLIES (16 POINTS)

Mark the correct species name for each **Specimen A-H** with a "✓" in the **ANSWER SHEET**.

Task 2. Compile the character matrix

Consider the following characters:

a. Long tail on hindwing
b. Knob tail on hindwing
c. Vein 1a on forewing
d. Vein 1a on hindwing
e. Wing vein 8 and 9 in forewing: overlapped = 1, not overlapped = 0
f. Wing vein 1a: "stretches the dorsum near the base of forewing " = 1, "other cases" = 0
g. Wing vein 1a and 1b in forewing: intersected = 1, other cases = 0
h. Wing cells: open = 1, close = 0
i. Orange hindwings with brown wing veins
j. A big red-orange patch on half of forewing
k. Upper side with orange bands on brown wings
l. A series of white spots on the marginal area of wings
m. White patch on hindwing
n. Upper side hindwing with an orange or a pale yellow tornal spot with a black dot inside
o. Ocelli on wings
p. Apex of forewing: cut = 1, rounded or pointed = 0

Q.2.1 CHARACTER MATRIX (25.6 POINTS)

Compile the character matrix for the character listed above in the **ANSWER SHEET.** Use “1” for present and “0” for absent.

Task 3. Calculate the distance matrix based on the characteristic matrix provided

For the remaining part of this experiment use the character matrix provided Table 1. This matrix is not related to Tasks 1 and 2.

Characters	A	B	C	D	E	F	G	H
1	1	1	0	0	0	0	0	0
2	1	0	0	0	0	0	0	0
3	0	1	0	0	0	0	0	0
4	0	0	1	0	0	0	0	0
5	0	0	0	1	0	0	0	0
6	0	0	1	0	0	0	0	0
7	0	0	0	0	1	0	0	0
8	1	1	1	0	0	0	0	0
9	1	1	0	0	0	0	0	0
10	0	0	0	0	0	0	1	1
11	0	0	1	0	0	0	0	0
12	0	0	1	1	0	0	0	0
13	0	0	0	1	1	1	1	1
14	1	1	0	0	0	0	0	0
15	0	0	0	0	1	1	1	1
16	0	0	0	1	0	0	0	0
17	0	0	0	0	1	1	0	0
18	0	0	0	1	1	1	1	1
19	1	0	0	0	0	0	0	0
20	0	0	0	0	1	1	1	1
21	0	0	0	0	1	1	0	1
22	0	0	0	0	0	0	0	1
23	0	0	0	1	0	0	0	0
24	0	1	0	0	0	0	0	0
25	0	0	0	0	0	1	0	0
26	0	0	0	0	0	0	1	0

Q.3.1. CALCULATE DISTANCE MATRIX

Calculate the distance matrix based on the character matrix provided in Table 1. The distance between two specimens is defined as the number of characters at which the two specimens show different character states (present: “1”; absent: “0”). Write the numerical results in the **ANSWER SHEET (8.4 points).**

Reconstructing Phylogenetic Realtionship using UPGMA

UPGMA (Unweighted Pair Group Method with Arithmetic Mean) is considered the simplest method for reconstructing phylogenetic trees with the assumption that the data provided have constant rates of evolution. In the method, the pair of clusters with the shortest distance is combined into a cluster of higher level at each iteration. To illustrate this concept, consider the numbers of character differences between the taxa (specimens) M, N, O, P, and Q.

Taxa	M	N	O	P	Q
M	0				
N	2	0			
O	6	6	0		
P	4	5	7	0	
Q	7	8	9	7	0

Iteration 1: The pair of clusters with the smallest distance is the pair M and N, which is thus combined into a higher–level cluster (M,N). The relative age of newly formed cluster is computed as half the distance between two original clusters. In this case, the relative age of the cluster is 1. Next, a new matrix of all distance is generated by computing the distance between clusters as the average distance between all taxa from one cluster to all taxa of the other cluster. The distance between Cluster (M,N) and Cluster (P), for instance, is computed as the average between d(M,P) and d(N,P) as (4+5)/2, where d(x,y) is a notation to indicate the distance between Clusters x and y. The result is presented as the table below:

Taxa	(M,N)			
(M,N)	0.0	O		
O	6	0.0	P	
P	4.5	7	0.0	Q
Q	7.5	9	7	0.0

Iteration 2: The pair of clusters with the smallest distance is now the pair of MN and P, which is thus combined into a higher–level cluster ((M,N),P) with a relative age of 2.25. Again, a new matrix is constructed by calculating all distances as indicated above. The distance between Cluster ((M,N),P) and Cluster (O), for instance, is computed as the average between d(M,0), d(N,O), and d(P,O) as (6+6+7)/3 = 6.33. The result is presented as the table below:

Taxa	((M,N),P)		
((M,N),P)	0.0	O	
O	6.33	0.0	Q
Q	7.33	9	0.0

*Iteration 3***:** The pair of clusters with the smallest distance is now the pair of MNP and O, which is thus combined into a higher–level cluster **(((M,N),P),O)** with a relative age of 3.17. Again, a new matrix is constructed by calculating all distances as indicated above. The result is presented as the table below:

Taxa	(((M,N),P),O)	
(((M,N),P),O)	0.0	Q
Q	7.75	0.0

Iteration 4: In the last cluster, the two remaining taxa are combined into the new cluster (**(((M,N),P),O),Q)** with a relative age of 3.88.

Task 4. Resolve the phylogenetic relationship of all the specimens

Resolve the phylogenetic relationship of all specimens (A–H), showed at the Table 1, iteratively using the UPGMA method and based on the distance matrix you compiled above (Task 3). Make sure to report the names of the clusters using the Specimen codes A to H. Write the numerical results in the **ANSWER SHEET (10.75 points**).

Q.4.1. UPGMA ITERATION 1 (3 POINTS)

Q.4.2. UPGMA ITERATION 2 (2.5 POINTS)

Q.4.3. UPGMA ITERATION 3 (2 POINTS)

Q.4.4. UPGMA ITERATION 4 (1.5 POINTS)

Q.4.5. UPGMA ITERATION 5 (1 POINT)

Q.4.6. UPGMA ITERATION 6 (0.5 POINTS)

Q.4.7. UPGMA ITERATION 7 (0.25 POINTS)

Task 5. Draw a phylogenetic tree (dendrogram)

Q.5. PHYLOGENETIC TREE (4.25 POINTS)

Draw a phylogenetic tree (dendrogram) based on the UPGMA result in the **ANSWER SHEET**. Indicate the relative length of each branch by writing the correct numbers next to it.

EXPERIMENT 2. ANATOMY OF EARTHWORM (*AMYNTHAS ASPERGILLUM*) (35 POINTS)

Introduction

The earthworm *Amynthas aspergillum* belongs to the Family Megascolecidae, Phylum Annelida and is a common species in Vietnam. It is rich in protein and suitable food for fish, poultry, and cattle. The species is experimentally raised and used for improving the quality of soil in several areas in Vietnam. This practical test is for you to dissect and identify the external and internal structures of *Amythas aspergillum*.

Task 6. Identify the external structure of *Amynthas aspergillum*.

Use a magnifier glass or stereomicroscope to observe the dorsal pores, clitellum, and chaetae (setae) of *Amynthas aspergillum*. Then, answer the following three questions the **ANSWER SHEET.**

Q.6.1. (3 POINTS)

Indicate in the Answer sheet with a "✓" which of the following statements is True.
Location of the clitellum is from (The segment number is counted from the position behind the labium)

Q. 6.2. (3 POINTS)

Indicate in the Answer sheet with a "✓" which of the following statements is True.
The chaetae distribution in each segment is

Q.6.3. (3 POINTS)

Indicate in the Answer sheet with a "✓" which of the following statements is True.
The number of dorsal pore on each segment just behind the clitellum is

Task 7. Dissecting and identifying the internal structure of *Amynthas aspergillum*

- Place the specimen in the dissecting tray, dorsal side up (Figure 4A).
- Locate the clitellum and insert the tip of the scissors about 3 cm from the clitellum posteriorly (Figure 4B).

A **B**

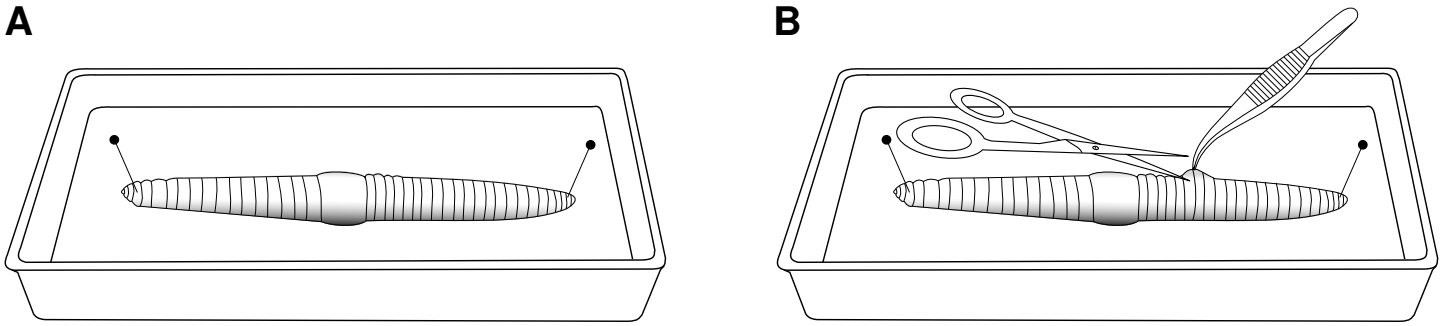

Figure 4.

- Cut skin carefully in both ways up to the head and to the anus. Try to keep the scissors pointed up, and only cut through the skin.
- Spread the skin of the worm out, use a knife to cut the septa (avoid damaging the internal organs).
- Place pins in the skin to hold it apart, angle the pins out so that they are not in your way.
- Pour water into the tray until the earthworm is submerged.

Q.7.1. (3 POINTS)

How many pairs of spermatheca are in *Amynthas aspergillum*? Write the correct number of spermatheca pair in the box in the **ANSWER SHEET**.

Q.7.2 (6 POINTS)

Observe the inside of the body wall and determine the presence/absence of septa between the following segments.

Indicate in the Answer sheet with a "✓" if septa are present or absent in the **ANSWER SHEET.**

Q.7.3. (10 POINTS)

- Identify the following organs by using the appropriate colour–headed pins.

Colour-headed pin	**Organ**
Blue	Stomach
White	Caecum
Red	Seminal vesicle
Yellow	Prostate gland
Purple	Nerve ganglion chain

- Write your student code on the "Student Code Sheet" and place it besides the tray.
- ***Raise the green card to inform the supervisor to take photographs and confirm the results on the "Dissecting result confirmation sheet".***

Q.7.4. (4 POINTS)

- Using the knife to make a cross section (about 0.5 – 1 mm) of the intestine at around segments 30th to 40th. Put this cross section into Petri dish containing water and gently move it in water to remove all remained food. Put the section on the glass slide, add a drop of water, observe the section under the stereomicroscope.
- ***Raise the green card to inform the supervisor to take photographs and confirm the results on the "Dissecting result confirmation sheet".***

Q.7.5 (3 POINTS)

Which of the following best describes the intestinal typhlosole observed in the cross section?

A. Intestinal typhlosole ≥ radius of intestine (Figure 5A)
B. Intestinal typhlosole branched (Figure 5B)
C. Intestinal typhlosole < 1/2 radius of intestine (Figure 5C)
D. No intestinal typhlosole (Figure 5D)

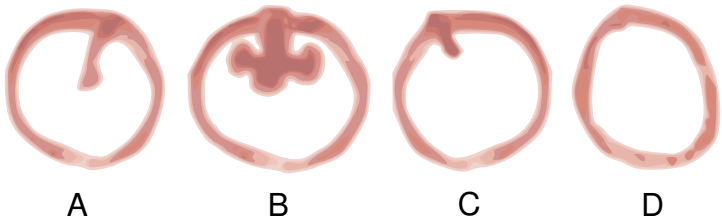

Figure 5.

Indicate in the Answer sheet with a "✓" the cross section that is observed.

End of Practical Exam 2

INTERNATIONAL BIOLOGY OLYMPIAD e. V. IBO

27th International Biology Olympiad

July 17-23, 2016
Hanoi, Vietnam

Practical Exam 3

BIOCHEMISTRY and MICROBIOLOGY

Total points: 100
Duration: 90 minutes

DEAR PARTICIPANTS,

This exam consists of three experiments:

- **Experiment 1: Expression, purification and characterization of proteins (40 points)**
- **Experiment 2: Antioxidant activity of coffee extract (30 points)**
- **Experiment 3: Lactic acid fermentation (30 points)**

Please note the following:

- Please remember to write your **Country** and **Student code** in the given box.
- Write your answers in the separate **Answer Sheet**. Only the answers given in the **Answer Sheet will be evaluated**.
- Make sure that you have received all the materials and equipment listed. If any of these items are missing, please raise the **Red card** immediately.
- During experiments, ensure to handle equipment properly. Any spilled solutions or broken equipment will not be replenished.
- Gel electrophoresis in Experiment 1 must not be performed in the last 30 minutes. You are recommended to do Experiment 1 first.
- Ensure to obtain spectrophotometer readings to answer the questions in Experiment 2.
- Stop answering and put down your pen immediately when the bell rings at the end of the exam. Enclose the **Question Paper**, **Answer Sheet**, and **Data printout** in the provided envelope.
- No paper, materials or equipment should be taken out of the laboratory.

Materials & Equipment

Equipment and Materials for 3 experiments

Name	**Quantity**
Micropipette P1000 (100-1000 µl)	1 piece
Micropipette P200 (20 – 200 µl)	1 piece
Micropipette P20 (2 – 20 µl)	1 piece
Pipette tips for micropipette P1000	1 box
Pipette tips for micropipette P20 and P200	1 box
Deionized water (**dH_2O**)	1 bottle
Microcentrifuge rack	1 piece
Round plastic container for liquid waste (**Liquid waste**)	1 piece
Square plastic container for solid waste (**Solid waste**)	1 piece
Timer	1 piece
Gloves	1 pair
Tissue paper	1 box
Glue	1 tube
Labels of student code	5 pieces
Red card	1 piece
Green card	1 piece
Calculator	1 piece
Marker	1 piece
Goggles	1 piece

For Experiment 1

Name	**Quantity**
SDS-PAGE electrophoresis gel tank and power supply	1 set
Gel comb	1 piece
Gel container (with student code)	1 piece
Microcentrifuge tubes 1.5mL	10 piece
Polyacrylamide gel cassette assembled in the electrophoresis gel tank	1 piece
Magenta microcentrifuge tube with 2X SDS-PAGE loading buffer (**Buffer**)	1 piece
Yellow microcentrifuge tube with 8 µl of protein marker (**M**)	1 piece
Microcentrifuge tube with 30 µl of cells without IPTG (**NO_IPTG**)	1 piece
Microcentrifuge tube with 30 µl of cells with IPTG (**IPTG**)	1 piece
Microcentrifuge tube with 30 µl containing pellet of cell extract from cells with IPTG (**Pellet)**	1 piece
Microcentrifuge tube with 30 µl of supernatant of cell extract from cells with IPTG (**Super**)	1 piece
Microcentrifuge tube with 30 µl of purified proteins (**Puri-P**)	1 piece
Falcon tube (green cap) with 40 mL of SDS-PAGE staining solution (**STAIN**)	1 piece

For Experiment 2

Name	Quantity
96-well microplate with student code (**do not touch the bottom of the plate**)	1 piece
Blue microcentrifuge tube with 300 µl of 1 mg/mL ascorbic acid solution (**AA**)	1 piece
Blue microcentrifuge tube with 300 µl of 5 mg/mL coffee extract (**CC**)	1 piece
Brown bottle with 15 mL of 0.2 mM DPPH solution (**DPPH**)	1 piece

For Experiment 3

Name	Quantity
Burette containing 25 mL of 0.1M NaOH solution with a stand and a clamp	1 set
Magnetic stirrer	1 piece
Stirring bar	1 piece
Forceps	1 piece
Measuring cylinder (10 mL)	1 piece
Measuring cylinder (25 mL)	1 piece
Beakers (100 mL)	8 piece
Hanna portable pH meter and a screw driver	1 piece
Rinsing water bottle (**H_2O**)	1 piece
Bottle with pH 4.01 buffer (**pH 4.01**)	1 piece
Bottle with pH 7.01 buffer (**pH 7.01**)	1 piece
Falcon tubes with 15-30 mL supernatant of culture broth (samples **A0, A2, A3 and A5**)	4 piece

EXPERIMENT 1. EXPRESSION, PURIFICATION AND CHARACTERIZATION OF PROTEINS (40 POINTS).

Introduction

H and B proteins are two important proteins of *Aeromonas hydrophilas.* To study them, a scientist wanted to co-express them in *E. coli*. For this, gene *b* was cloned into multiple cloning site 1 (MCS-1) and gene *h* in MCS-2 of expression vector p1 (**Fig.1.1**). The obtained p1-*b*-*h* vector was transformed into *E. coli* and protein expression was induced by IPTG (isopropyl *β*-D-1-thiogalactopyranoside). The proteins were then purified by affinity chromatography, in which a protein containing 6xHis-tag binds to the nickel column. Expression and purification were finally evaluated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), a method for separation of proteins based on their molecular weight. Note that H is smaller than B.

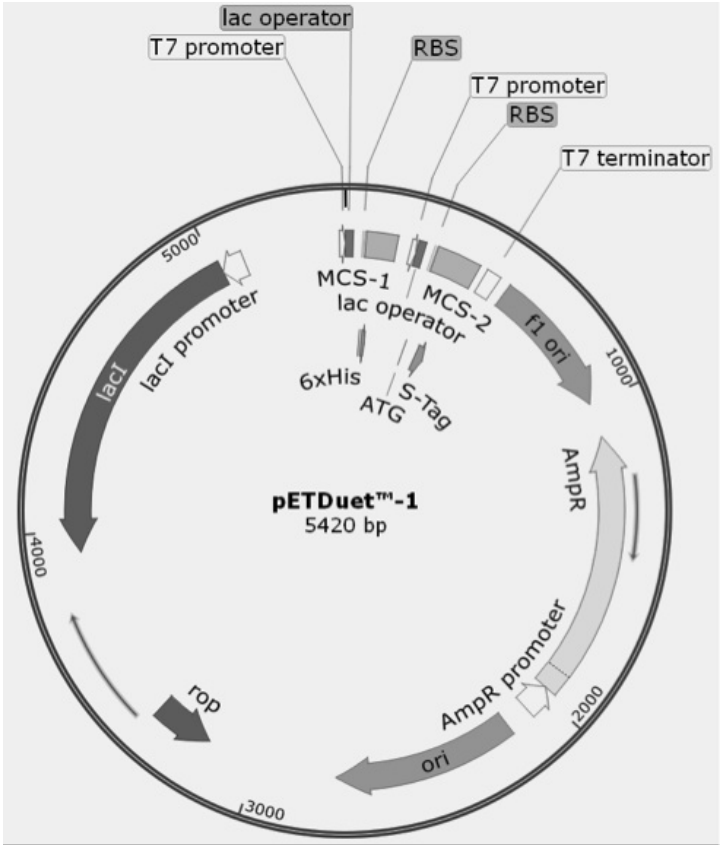

Fig.1.1. Overview map of plasmid p1 expression vector
(ori: origin, rop: repressor of primer, AmpR: ampicillin resistant)

A single colony of *E. coli* containing p1-*b*-*h* vector was cultured in 50 mL of LB medium. The culture was grown at 37ºC until the OD600 reached 0.6. In order to analyze the expression and purification of the recombinant proteins, the scientist has collected the following cell and protein samples:

- **NO_IPTG**. One mililiter of the culture was transferred to a – tube, grown at 2θC for 16 hours (OD_{600} = 2.4) and then centrifuged. The supernatant was discarded and the cell pellet was resuspended in 50 µl H_2O and then mixed with 50 µl 2X SDS-PAGE loading buffer to yield 100 µl sample.

In the remaining 49 mL culture, protein expression was induced by adding IPTG. The culture was further grown at 20oC for 16 hours.

- **IPTG**: One milliliter of the culture with IPTG (OD_{600} = 1.4) was centrifuged. The supernatant was discarded and the cell pellet was resuspended in 50 µl H_2O and then mixed with 50 µl of 2X SDS-PAGE loading buffer to yield 100 µl sample.

The remaining 48 ml culture was centrifuged, the supernatant was discarded and the cell pellet resuspended into 2 mL of nickel binding buffer. The cell suspension was lysed and subsequently centrifuged. Both pellet and supernatant were collected.

- **Pellet**: The pellet obtained from the IPTG-cell lysate was resuspended into 2 mL buffer and then mixed with 2 mL of 2X SDS-PAGE loading buffer (pellet stock solution).
- **Super**: 10 µl of supernatant obtained from IPTG-cell lysate was mixed with 10 µl of 2X SDS-PAGE loading buffer
- **Puri-P:** The rest of the supernatant was loaded onto the nickel column for protein purification. The purified proteins were eluted from the column using 2 mL elution buffer. 10 µl of purified protein was mixed with 10 µl of 2X SDS-PAGE loading buffer.

All samples for SDS-PAGE analysis were boiled at 100ºC for 5 min.

Design your SDS-PAGE experiment to analyze protein expression.

The standard final concentration of total protein for SDS-PAGE analysis must be equal to $5x10^6$ cells/µl. At first, calculate the concentration of cells in each sample, knowing that OD_{600} value of 1 corresponds to $8x10^8$ cells/mL and take into consideration the dilution of each sample during the procedure.

Q.1.1 (6 POINTS)

Calculate and fill the volume (µl) of samples in the table in the **Answer Sheet**. Use one decimal place.

Procedures

1. Based on the table above, prepare all SDS-PAGE samples in empty microcentrifuge tubes provided. Mix each sample by pipetting up and down 4-5 times.

   After completing this step please raise the Green card. An assistant will guide you to the loading area and help to stick your student code to the gel tank.
2. Load 20 µl of each sample on SDS-PAGE gel. Samples must be loaded in order from tube 1 to 6. To load a sample, use the P20 micropipette with tip to withdraw 20 µl of a sample, and carefully place the tip on the top of the well (**Fig.1.2**).

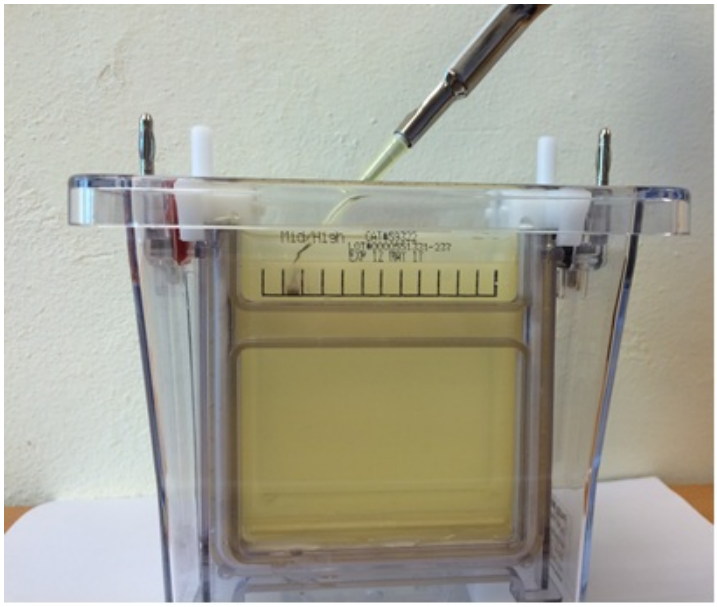

Fig.1.2. Sample loading on SDS gel

3. The assistant will run the SDS-PAGE for 20 min and tell you to set your timer for 20 min.

   You can do another experiment while running SDS-PAGE. After 20 min, please raise the Green card to inform an assistant to return the SDS-PAGE gel to you.

4. Remove the SDS-PAGE gel from the plastic cassette using the gel comb as in the diagram below (**Fig.1.3.)** and put the gel into the gel container.

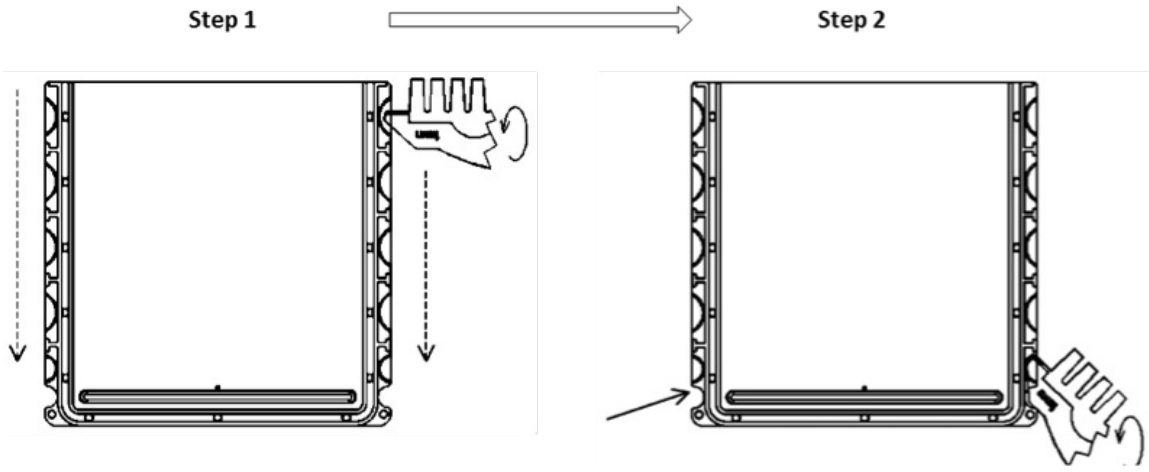

Fig.1.3. Remove the SDS-PAGE gel from the plastic cassette

Step 1. Crack open cassette sides by inserting the comb's slanted edge into each of the notches around the cassette and twisting firmly. Starting with the notches at the top, move down each side of the cassette.

Step 2. After the sides are open, place the comb's slanted edge at a 45-degree angle between the plates at each bottom corner and twist firmly.

Step 3. Gently separate the two pieces of the cassettes.

5. Add 40 mL of staining solution (**STAIN**) into the gel container and rock on the rocker for 10 min.
6. Discard the staining solution from the gel container and rinse the gel 3 times using deionized water.

When finished, raise the Green card to ask an assistant to take photo of the gel.

Q.1.2. SDS-PAGE RESULT (10 POINTS)

After obtaining the photo of SDS-PAGE gel, stick it to the place given in the **Answer Sheet**.

Q.1.3 (4 POINTS)

Based on the information provided in **Fig.1.4A** below, plot the molecular weight of at least five marker proteins versus their relative migration-Rf values on the graph paper provided in the **Answer Sheet**. (Rf: distance migrated by protein/distance migrated by dye front)

Q.1.4. (4 POINTS)

Use the graph from **Q.1.3** and the SDS-PAGE gel to estimate the molecular mass of H and B proteins.

Q.1.5 (4 POINTS)

Based on the SDS-PAGE result, indicate if each of the following statements is true or false. Mark "√" for True or False statements in the **Answer Sheet**.

A H protein is over expressed in LB media with IPTG.
B B protein is completely soluble in the nickel binding buffer.
C H and B protein interact with each other.
D Majority of recombinant proteins was bound to the nickel column.

Q.1.6. (4 POINTS)

Based on detailed restriction map of the p1 expression vector **Fig.1.5**), indicate if each of the following statements is True or False. Mark "√" for True or False statements in the **Answer Sheet**.

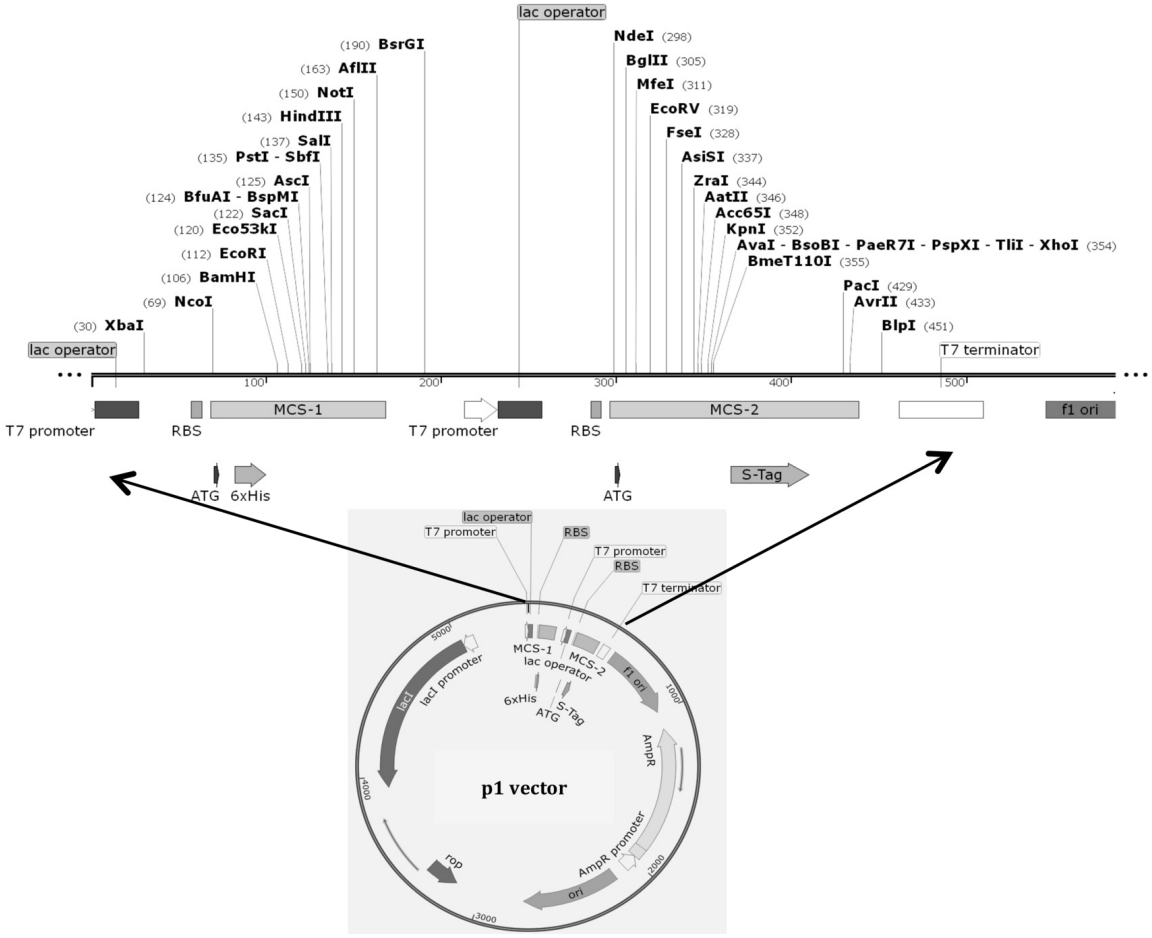

Fig.1.5. Detailed restriction map of p1 expression vector(RBS: ribosome binding site)

A *Sal*I and *Bam*HI can be used to insert *b* gene into MCS-1.
B Gene *h* and *b* should be cloned in the same orientation to be expressed simultaneously.
C Gene *h* and *b* should be in the same reading frame to be expressed simultaneously.
D To maintain the plasmid, ampicillin should be added to the culture medium.

In order to characterize the oligomeric states of H and B proteins, 3 protein samples were prepared: (1) H protein; (2) H and B proteins obtained from the above experiment; (3) B protein. Samples 1 and 2 were transparent but in sample 3 most of the protein was precipitated. Samples 1 and 2 were then loaded on a gel-filtration column. The obtained profiles are shown in **Fig.1.6A**. The sizes of reference molecule on a gel-filtration column are shown in **Fig.1.6B**.

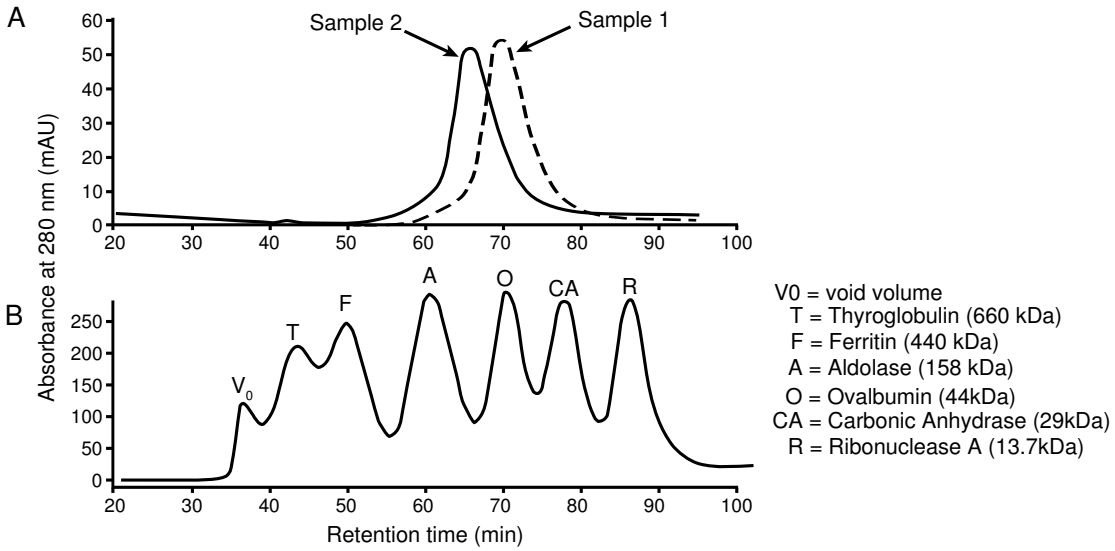

Fig.1.6. Gel filtration analysis of H and B proteins
(A) Chromatogram of sample 1 (dashed line) and 2 (solid line)
(B) Chromatogram of reference molecules

Q.1.7. (4 POINTS)

Calculate and report the relative size of the proteins corresponding to gel filtration peaks from sample 1 and 2 on the table in the **Answer Sheet**.

Q.1.8. (4 POINTS)

Indicate if each of the following statements is True or False. Mark "√" for True or False statements in the **Answer Sheet**.

A H protein exists as monomer
B H and B probably exist as heterodimer
C H protein helps to stabilize B protein.
D In native gel-filtration column analysis, retention time of a protein is proportional to their molecular weight.

EXPERIMENT 2. ANTIOXIDANT ACTIVITY OF COFFEE EXTRACT (30 POINTS)

Introduction

Biological oxidation produces reactive oxygen radicals that can cause serious damage to cells. Antioxidants are molecules that can scavenge radicals and thus inhibit oxidative reactions. Antioxidants include reducing agents such as thiol compounds, ascorbic acid and phenolics. Coffee, prepared from roasted coffee beans, is a potential source of antioxidants.

In this experiment, a 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, in which DPPH is reduced and looses its purple color, is performed. SC_{50} value (scavenging capacity) is commonly used for evaluation of antioxidant activity. This value represents the concentration of sample which scavenge 50% of DPPH radicals. Absorbance of DPPH will be measured at the wavelength of 517 nm. Absorbance of blank is assumed negligible. Absorbance of control (without scavenger, Ac) and sample (As) will be used to calculate scavenging percentage (SC%) for each concentration of samples as:

$SC\% = (Ac - As) \times 100/Ac$

A plot will be created based on the logarithm of concentration series of samples and corresponding scavenging percentage, from which SC_{50} value will be calculated.

In this experiment, beans of a Vietnamese coffee variety (*Coffea canephora*) will be investigated for antioxidant activity. Coffee bean powder (1g) was suspended in deionized water at 80°C for 30 min, then filtered and water was added to a final volume of 200 mL of extract solution.

Procedure and questions

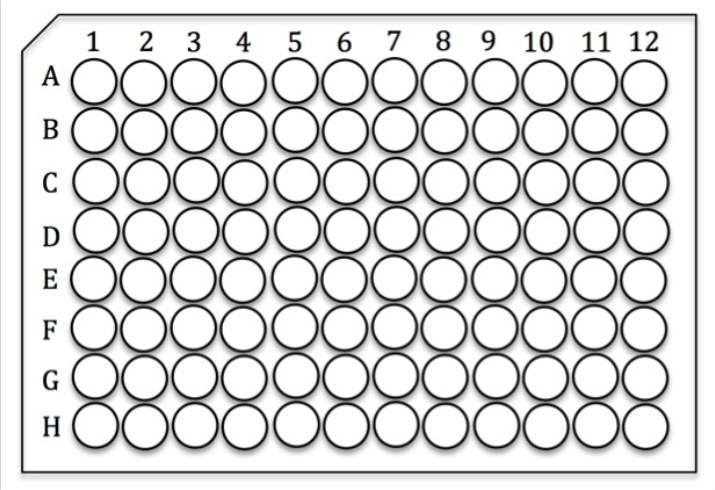

Fig 2.1 96-well microplate

The 96- well microplate above can be used to perform a serial dilution. Positions on this plate are indicated by a letter (A – H) and a number (1 – 12) specifying rows and columns respectively.

1. Use your micropipette to prepare 4 solutions of ascobic acid (AA1-AA4 in wells A1 – A4 in the 96-well microplate) and 4 solutions of coffee extract (CC1-CC4 in wells A6 – A9 in the 96-well microplate) by **serial dilution** with a dilution factor of 2, to achieve a lowest concentration of 0.025 mg/mL and 0.625 mg/mL, respectively. The volume of each solution produced should be 200 uL before further dilution is performed.
   Note: If an error is made in loading any of these wells use wells H1-H4 for ascobic acid solutions AA1 to AA4 and/or wells H6 – H9 for coffee extract solutions CC1 – CC4.

Q.2.1 (4 POINTS)

Fill in the table in the **Answer Sheet** what you calculated for preparing ascorbic acid and coffee extract dilutions.

2. Pipette 20 uL of ascorbic acid solution and/or coffee extract solution from each well in row A to the corresponding wells in rows B, C and D. If an error is made during this step, the procedure may be repeated using the corresponding wells in rows E, F and G.

3. Pipette an aliquot of 20 µl H_2O into wells B11, C11 and D11.
4. Pipette 180 µl of DPPH solution into all wells prepared in steps 2 and 3.
5. Cover the plate with the lid and incubate at room temperature for 10 min and set the timer.

After completing this step, raise the Green card for the assistant to help you in measuring absorbance on a microplate reader and return your data printout.

Q.2.2 (5 POINTS)

Calculate the logarithm (log_{10}) of ascorbic acid and coffee extract concentration and fill in the table in the **Answer Sheet** (all numbers are rounded to 2 decimal places). You can use your calculator to calculate common logarithm value following steps below:

- Press the **ON** key to turn on the your calculator
- Press the four keys **SHIFT, CLR, 2,** and **=** to return to calculation mode.
- Press the **log** key
- Enter the number
- Press the **=** key

Calculate the mean absorbance for each dilution, the scavenging percentage for each sample and fill in the table in the **Answer Sheet**.

Q.2.3. (5 POINTS)

Use the calculated value to plot a linear curve on scavenging percentage versus logarithm (log_0) of ascorbic acid concentration in the grid lines given in the **Answer Sheet**.

Q.2.4. (5 POINTS)

Calculate SC_{50} value of ascorbic acid and coffee extract and fill in the table in the **Answer Sheet** (You can plot a linear curve for coffee extract in the grid lines given in Q.2.3 but this curve will not be scored).

Q.2.5. (3 POINTS)

Using the same protocol, SC_{50} value of extracts of some coffee varieties were collected as follows:

Coffee extract	SC_{50}
X	3.8 mg/mL
Y	2.6 mg/mL

Compare antioxidant activity of different coffee bean types including the one in this experiment (Z) and arrange them in order from the strongest to the weakest and fill in the given space in the **Answer Sheet**.

Q.2.6. (4 POINTS)

Assume that in your experiment the absorbance of all the mixtures of different diluted coffee extracts and DPPH were similar and negligible. Indicate if each of the following statements is true or false.

Mark "√" for True or False statements in the **Answer Sheet**.

A Antioxidant activities of the diluted coffee extracts are negligible.

B To obtain more accurate determination of antioxidant activities, another experiment with higher concentration samples needs to be carried out.

C Activity of antioxidant enzymes in coffee extract resulted in the above result.

D If NADH is added in the wells, there will be no change in the assumed absorbance value.

Q2.7. (4 POINTS)

A student measured antioxidant activity of a sample using the same protocol as you have done (Protocol A) and a different protocol (Protocol A*) which was modified from Protocol A. The result is shown in the following table.

	Protocol A	**Protocol A***
SC_{50} (mg/mL)	1.95	3.9

Which of the following changes could result in a higher value of SC_{50}?

Indicate if each of the following statements is true or false. Mark "√" for True or False statements in the **Answer Sheet**.

A The student has used 0.1 mM DPPH for Protocol A*.

B The student has loaded 10 µl of sample in each well for Protocol A*.

C After adding DPPH, the student has incubated the 96-well plate for a shorter time than in Protocol A.

D The student has used better solvent for antioxidants.

EXPERIMENT 3. LACTIC ACID FERMENTATION (30 POINTS)

Introduction

Recently, a scientist isolated a homolactic acid *Lactobacillus* strain (*Lactobacillus* sp. VN156) from traditionally fermented mustard in Vietnam. In this experiment, *Lactobacillus* sp. VN156 was grown in MRS medium. The initial pH of the culture medium was 5.6. Samples were taken at different times during the cultivation for measuring the optical density (OD) of bacterial cells at 600 nm (**Fig.3.1**). An OD_{600} value of 1 corresponds to $2x10^8$ cells/mL. The samples A0, A2, A3 and A5 are supernatants of collected samples which will be used for analysis of lactic acid production.

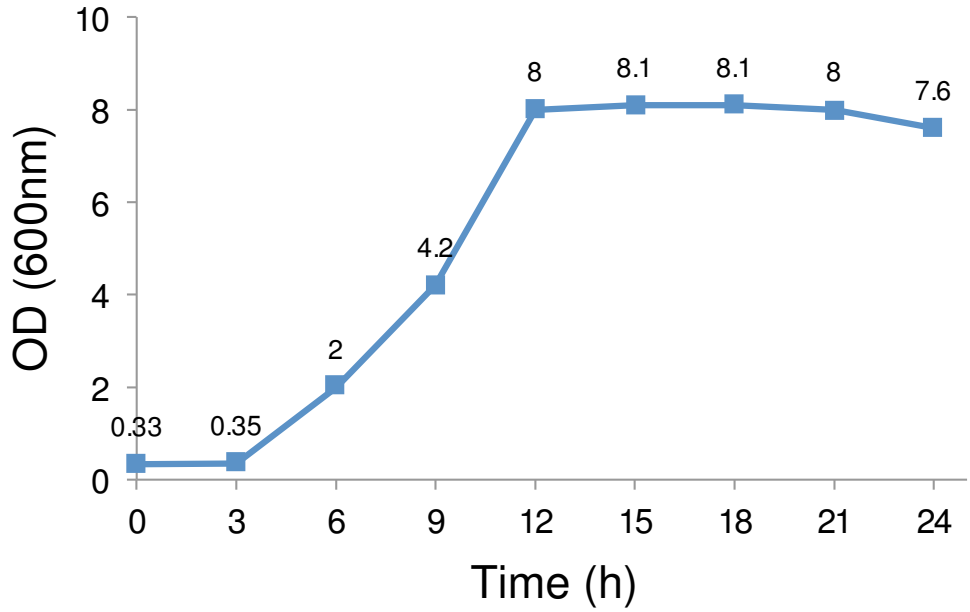

Fig.3.1. Growth curve of *Lactobacillus* sp. VN156

Q.3.1. (3 POINTS)

Assume that the **Fig 3.1** represents the real course of the growth. Calculate the generation time (h) of *Lactobacillus* sp. VN156 during exponential phase and record the value in the **Answer Sheet**.

Q.3.2. (3 POINTS)

If 1 mL of the culture at 9 h is diluted into fresh MRS medium, calculate the number of bacterial cells after 6 h of cultivation and record the value in the **Answer Sheet**.

Calibration of the pH meter

Use Hanna portable pH meter **(Fig 3.2)** for measuring the pH

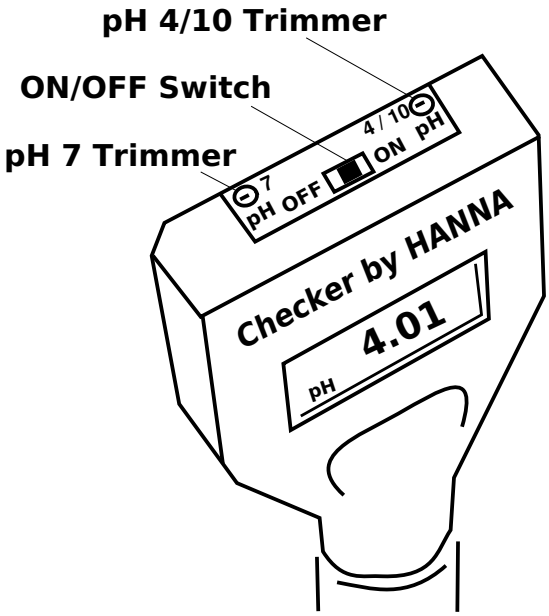

Fig 3.2.Hanna portable pH meter

Calibrate the pH meter according to the following procedure**Fig 3.3.**

- Turn the pH meter on by pressing ON/OFF button.
- Remove the protective cap and rinse the tip of the electrode with water, gently wipe with tissue paper.
- Dip the tip of the electrode in pH 7.01 buffer solution. Ensure the electrode tip and junction are fully immersed in the solution (about 2 cm of the tip is in the solution). Allow the reading to stabilize.
- Use a screwdriver to adjust the pH 7 trimmer until the display reads pH**7.01**
- Rinse the pH electrode with water, gently wipe with tissue paper.
- Dip the tip of the electrode in pH 4.01 buffer solution. Allow the reading to stabilize.
- Use the screwdriver to adjust the pH 4 trimmer until the display reads pH**4.01**.
- *Calibration is completed.*
- **Note:** if you switch off the pH meter you should calibrate it again

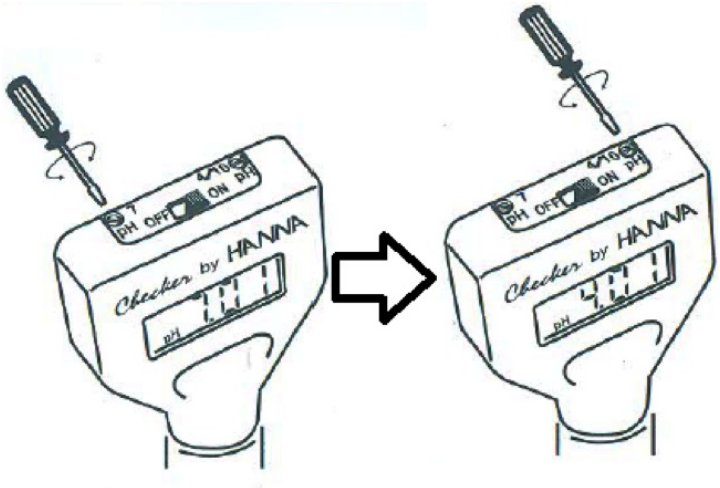

Fig 3.3. pH Calibration

Lactic acid titration

Titration is an analytical technique, which allows the quantitative determination of a specific substance (analyte) dissolved in a solution. It is based on a complete chemical reaction between the analyte and a reagent (titrant) of known concentration, which is added to the solution. In this part, titration method will be used to determine lactic acid concentration in the samples by titration with 0.1M sodium hydroxide solution as shown in **Fig.3.4**.

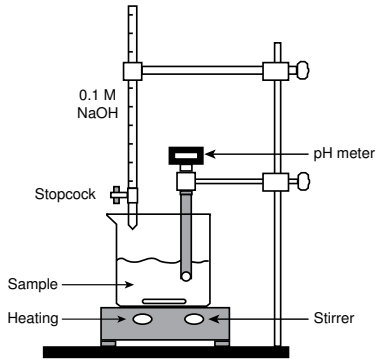

Fig.3.4. Titration setup

Q.3.3. (2 POINTS)

Calculate the volume of sample (mL) and water (mL) needed to be used according to the table in **Q.3.2** and record the values in the table provided in the**Answer Sheet**.

1. Based on your calculation, prepare sample dilutions for each sample in 100 mL beakers. Prepare two replicates for each sample using 25 mL measuring cylinder for deionized water, micropipette and 10 mL measuring cylinder for samples.
2. Carefully place a magnetic stirring bar into the diluted sample solution. Clamp the pH meter and position the pH electrode deep in the solution (about 2 cm of the tip is in the solution) so that the stirring bar will not hit the pH electrode while stirring. Begin stirring slowly and record the starting volume of 0.1M NaOH solution.
3. Open the stopcock of the burette to allow the NaOH solution from the burette to slowly run into the sample in the beaker. Stop adding NaOH when the pH of sample changes to neutral (6.95-7.05). Record the final volume of 0.1M NaOH solution.
4. After each titration, carefully remove the pH electrode from the solution, rinse it with water. Remove the stir bar by using a pair of forceps and rinse it with water.
5. Repeat steps 2–4 with for each sample.

Q.3.4. (9 POINTS)

Record the volume of 0.1 M NaOH used to titrate each sample in the table provided in the **Answer Sheet**.

Q.3.5. (10 POINTS)

Calculate the mean volume of 0.1 M NaOH needed to be used to titrate 30 mL of each stock sample and the concentration of lactic acid in each sample based on the final volume of base titrated. Record all values in the table provided in the **Answer Sheet**.

Note: NaOH (Mw=40) and lactic acid -$C_3H_6O_3$ (Mw=90)

Q.3.6. (3 POINTS)

Based on **Fig 3.1**, assuming that the OD_{600} of 1.0 equals to the densitiy of 2×10^8 cells/mL. The concentration of lactic acid will be increased 1g/L if the number of bacterial cells increases 2×10^8 cells/mL. If at 11 hours of cultivation the lactic acid concentration is 6 g/L, calculate the number of bacterial cells (cells/mL) and record the value in the **Answer Sheet**.

Record the value in the **Answer Sheet**.

END OF PRACTICAL 3

INTERNATIONAL BIOLOGY OLYMPIAD e. V. IBO

27th International Biology Olympiad

July 17-23, 2016
Hanoi, Vietnam

Practical Exam 4

MOLECULAR BIOLOGY

Total points: 100
Duration: 90 minutes

DEAR PARTICIPANTS,

In this exam, you are going to perform a combined PCR-RFLP experiment for two purposes simultaneously:

1. **Genotyping drug metabolising enzyme (NAT2) to determine relevant oral drug dosage in treatment of tuberculosis (TB) patients.**
2. **Forensic identification of unidentified biopsy specimens**

The experiment consists of five tasks:

- Task 1: Design of RFLP experiment (17 points)
- Task 2: Performance of RFLP experiment (44 points)
  (*Notice:* Electrophoresis must start not later than **75 minutes** after the exam begins. After this, you will NOT be allowed to run the gel).
- Task 3: Forensic identification of unidentified biopsy specimens (9 points)
- Task 4: Interpretation of patients' genotypes (12 points)
- Task 5: Determination of drug dosage relevant to patients' genotypes (18 points)

Please take note of the following:

- Please remember to write your **Country** and **Student code** in the given box.
- Write your answers in the separate **Answer Sheets** (using pencil and eraser). Only answers given in the **Answer Sheets will be evaluated**.
- Make sure that you have received all the materials and equipment listed at the beginning of the exam. If any of these items are missing, please raise the **Red card** immediately to notify the lab assistants.
- During the experiment, ensure to handle the equipment properly. Any spilled chemicals or broken equipment will **NOT** be replenished. However, if any equipment appears to malfunction, please raise the **Red card**. A lab assistant will come to help and if necessary replace the equipment.
- Stop answering and put down your pen immediately when the bell rings at the end of the exam. Enclose the **Answer Sheets**, **Question papers**, and **Data printout** in the provided envelope.
- No paper or materials should be taken out of the laboratory.

Good luck!!!

MATERIALS AND EQUIPMENT

Materials and equipment	**Quantity**
FlashGel™ horizontal electrophoresis chamber with lighting switch	1 piece
FlashGel™ (precast) agarose gel cassette (in sealed bag)	1 piece
Electrophoresis power supply (one for 2 students; operated by lab assistants)	1 piece
Water-bath 37ºC (one for 4 students; located behind your seat)	1 set
Heat block 80ºC (one for 4 students; located behind your seat)	1 set
Micro-centrifuge (spin-down) with adapters for 0.2 / 1.5 mL tubes	1 set
Micropipette P200	1 piece
Micropipette P20	1 piece
Sterile micropipette tips in box for p20	1 box
Sterile micropipette tips in box for P200	1 box
Ice box filled with flaked ice (with cover)	1 box
1.5 mL microfuge tube rack	1 piece
0.2 mL microfuge tube rack	1 piece
1.5 mL microtubes	5 pieces
0.2 mL microtubes (PCR tubes)	15 pieces
Stopwatch	1 piece
Foam floating rack (15 holes for 0.2 mL microtubes)	1 piece
Green card to signal assistant(s) for proceeding experiment	1 piece
Red card to signal assistant(s) for technical problem(s)/supports	1 piece
A tip disposal container (plastic beaker with lid)	1 piece
Polygloves (disposable gloves)	3 pairs
Twin marker pen (permanent ink)	1 piece
Student code sticker (to attach to your worked-out image)	1 piece
Kimwipe paper for blotting excess liquid on precast gel cassette	1 box
Tissue (Pussy®) paper for cleansing bench/equipment (if needed)	1 box
Safety goggle	1 piece
Squirt bottle containing deionized water (500 mL)	1 bottle
Scissor (to unpack the bag containing precast gel cassette)	1 piece

Other tools, including handy calculator, pencil (2B Type), eraser (for pencil) and ruler you are provided for commonly using in all the labs.

Reagents	Quantity
PCR products of *NAT2* gene derived from three patients (green caps labeled P1, P2 and P3)	3 tubes x 10 µL
PCR products of *NAT2* gene derived from unidentified biopsy specimens (red caps labeled X, Y and Z)	3 tubes x 10 µL
Restriction enzyme *Kpn*I, labeled RE1 (green label)	1 tube x 10 µL
Restriction enzyme *Bam*HI, labeled RE2 (blue label)	1 tube x 10 µL
10x Restriction buffer, labeled BF (purple tube)	1 tube x 50 µL
MiliQ water tube, labeled W (white label on blue tube)	1 tube x 200 µL
DNA staining dye, labeled D (red label on red tube)	1 tube x 50 µL
100 bp DNA ladder, labeled M (orange label on yellow tube)	1 tube x 10 µL

TASK 1. DESIGN OF PCR-RFLP EXPERIMENT (17 POINTS)

Introduction

Isoniazid (INH) is a pivotal agent in first-line anti-tuberculosis (TB) treatment. Despite the rather successful therapeutic effects of this regimen, there are still treatment failures (ineffective treatments) and unmanageable side effects (most commonly liver injury and occasionally mortality). INH acetylation was found to be the major contributor to drug-induced hepatotoxicity. **Figure 1** presents the major pathway for INH acetylation catalysed by non-inducible hepatic enzyme arylamine N-acetyltransferase type 2 (NAT2). The rate of acetylation is constant in an individual but varies between patients. The human population can be divided into three different phenotypic groups according to acetylation rate: slow, intermediate and rapid acetylators. It is well known that INH-induced hepatotoxicity develops more frequently in NAT2 slow-acetylators. In contrast, treatment failure is likely to occur in rapid-acetylators. Most commonly, rapid-acetylators are those whose genotypes are homozygous for the wild-type SNP allele at all the three positions, thus NAT2*4 (C481, G590, G857). Intermediate-acetylators are heterozygous for a mutant SNP allele at a single position, ie one out of the 3 positions NAT2*5 (C481T), NAT2*6 (G590A) or NAT2*7 (G857A) (**Figures 2a** and **2b**). Slow-acetylators are those who have more than one mutant alleles (either two alleles at one position or multiple positions).

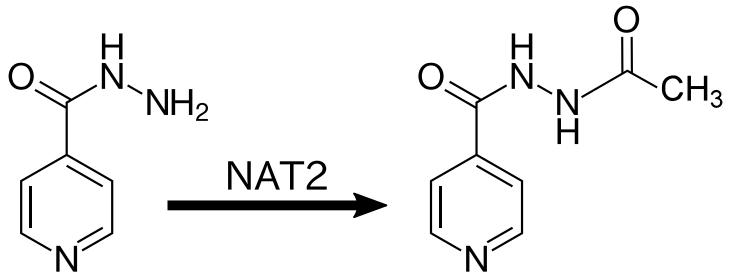

Figure 1. Metabolism of isoniazide catalysed by NAT2 (N-acetyltransferase)

The NAT2 genotype can be determined by using an allele-specific polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Analysis of INH concentrations in the blood of patients of different NAT2 genotypes receiving the same doses of INH revealed that the serum concentration of INH was 2-to 7-fold higher among slow-acetylators compared to rapid- and intermediate-acetylators. Thus, genotyping NAT2 enables personalization of INH doses.

In this experiment, you will receive PCR products of *NAT2* gene derived from total genomic DNA of three TB patients P1, P2 and P3, and PCR products of 3 unidentified biopsy specimens from these 3 patients labelled X, Y and Z. You are to design and perform a combined PCR-RFLP experiment to determine *NAT2* genotype for each patient and identify their biopsy specimens. To determine the genotype, appropriate restriction enzymes (RE) are used on the PCR products. With the data obtained, identify biopsies X, Y and Z. Finally determine the appropriate dose of INH for patients P1, P2 and P3.

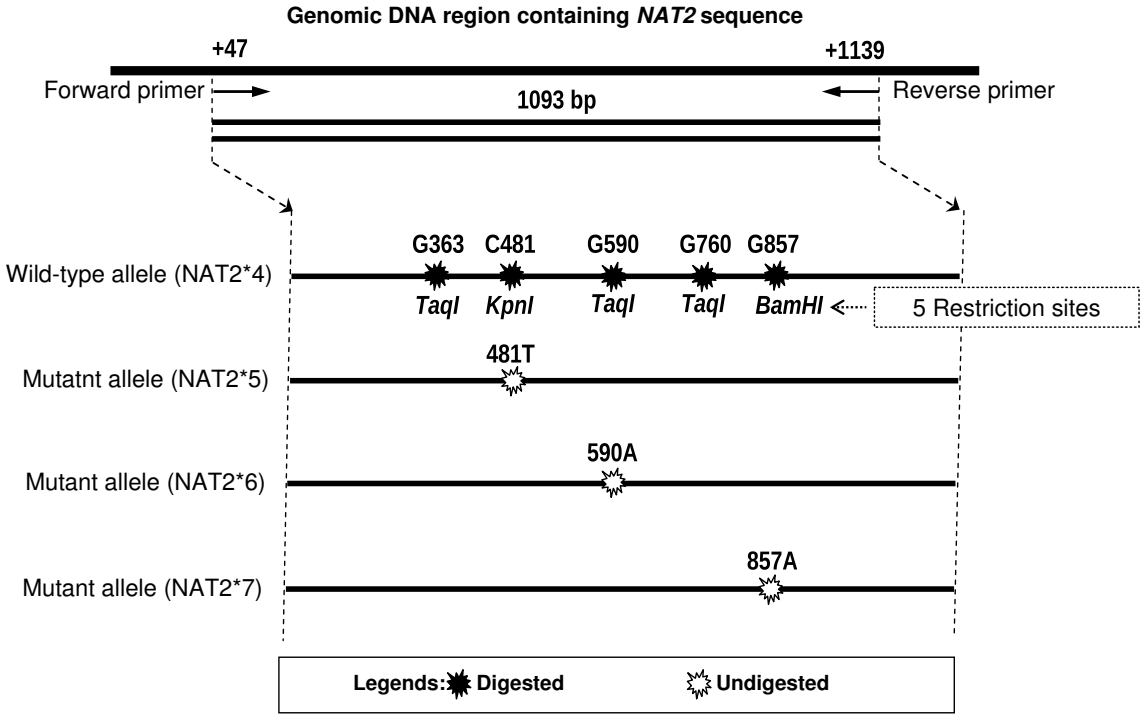

Figure 2(a). Restriction sites of the three restriction enzymes (REs)*Kpn*I, *Taq*I, and *Bam*HI in the gene coding for N-acetyl transferase type 2 (NAT2). These REs are used to generate PCR-RFLP fingerprints for detecting mutant alleles NAT2*5, NAT2*6 and NAT2*7 as distinguished to the wild-type allele NAT2*4. Forward and reverse PCR primers anneal correspondingly to the +47 and +1139 from the start codon of *NAT2* gene.

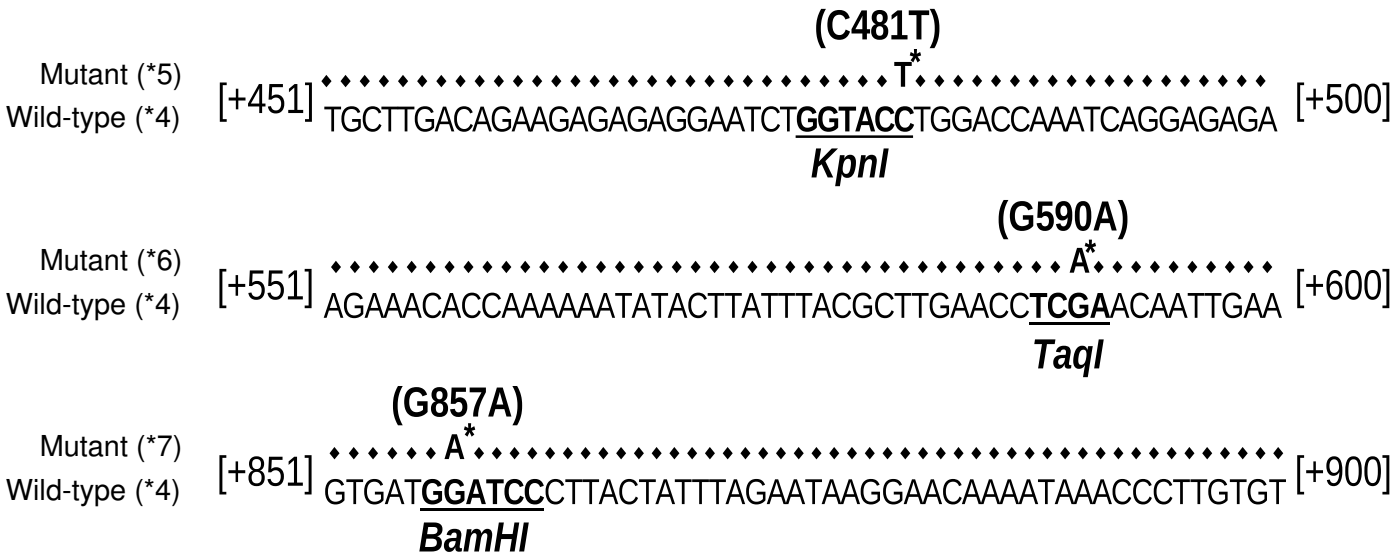

Figure 2(b). Truncated sequence of PCR products of wild-type and mutant NAT2 alleles. Numbers in brackets at the beginning and the end of each row indicate the first and the last base of the presented sequence of the wild-type allele (NAT2*4; represented in A/T/G/C) and of the corresponding mutant alleles (NAT2*5, NAT2*6, and NAT2*7) where dot marks (♦) reveal the nucleotides identical to the wild-type allele. A*/T* presents the SNP mutants.

Q.1.1 (12 POINTS)

Complete the expected RFLP patterns in the figure provided on the**Answer Sheet** by drawing in pencil the expected bands of completely RE digested PCR products of the four *NAT2* alleles: NAT2*4 (wild-type), NAT2*5 (481T), NAT2*6 (590A) and NAT2*7 (857A). Examples are already given for heterozygotes.

For this task, you are required to perform RFLP reactions in a total of 12 tubes for genotyping the wild-type and the two alleles NAT2*5 (labelled with a) and NAT2*7 (labelled with b) for each patient (P1, P2 and P3) and their biopsy specimens (X, Y and Z). Always use 7.0 µg DNA, restriction buffer and where appropriate use 1.0 µL RE, per 0.2 mL microtube (PCR tube).

Q1.2 (5 POINTS)

Design your restriction digests or *NAT2* genotyping of patients (P1 – P3) and specimens (X - Z) in a total volume of 10 µL by completing the table provided in the **Answer Sheet**.

TASK 2. PERFORMANCE OF RFLP EXPERIMENT (44 POINTS)

Notes:

1. For electrophoresis, you are handling two parts of the FlashGel® System, 12+1-well Cassette and Dock (**Figure 3**), while the Power Supply and Camera are operated by Lab assistants. For best results, flood the wells with deionized water prior to sample loading. To observe the bands, turn on the light (using the knob on the Dock), and wear the safety goggles.
2. You may request for a second Cassette (precast gel) but there will be a penalty of 20 points.
3. Spin down all reagents in microtubes before directly pipetting (be sure to balance the micro-centrifuge by placing the microtubes opposite each other. If there is only one tube, balance with an empty tube).

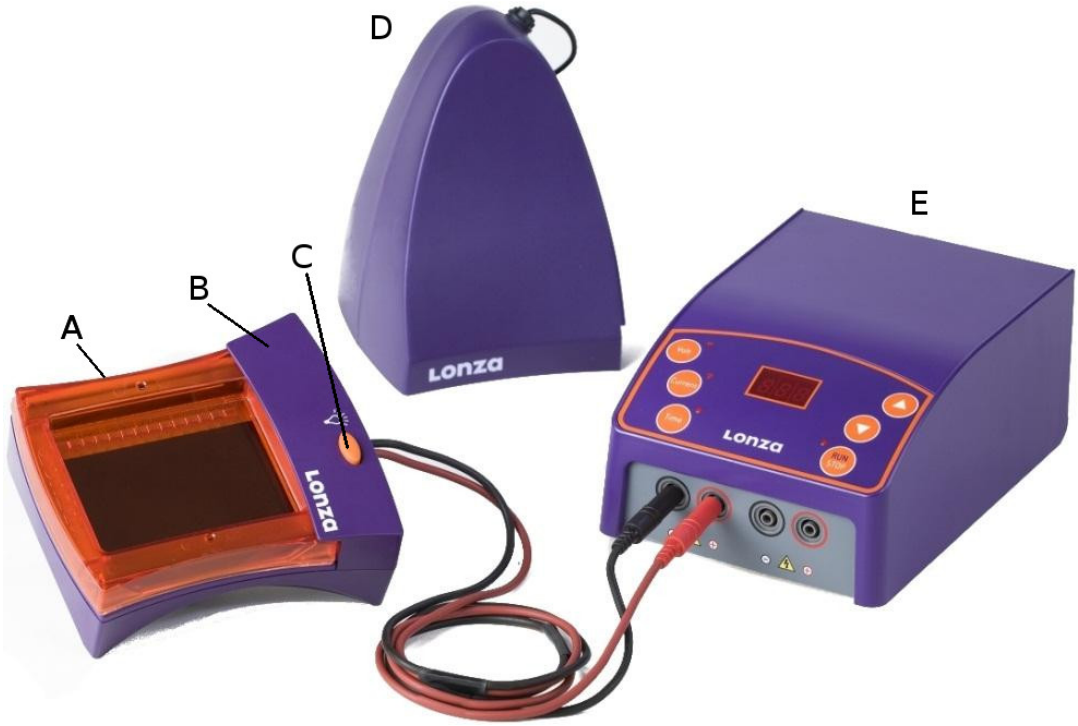

Figure 3. FlashGel® Horizontal Electrophoretic System. **(A)** Cassette. **(B)** Dock. **(C)** Knob for lighting. **(D)** Camera. **(E)** Power supply.

This protocol consists of two stages: PCR-RFLP digestion and electrophoresis:

Step 1 (*Preparation of microtubes for RE's digestion reaction*)**:** Label 12 microtubes with the fine tipped marker pen as P1a, P1b, P2a, P2b, P3a, P3b, Xa, Xb, Ya, Yb, Za and Zb to correspond to PCR products of genomic DNA of the three patients (P1, P2 and P3) and the three biopsy specimens (X, Y and Z), digested either with *Kpn*I or *Bam*HI.

Step 2 (*Preparation of the restriction digestion mixtures*): According to your setting-up of restriction digestion reactions (**Tables 1.2** in your **Answer Sheet)**, prepare the restriction digestion mixtures relevant to each microtube you labelled in Step 1. Gently mix the reagents by pipetting them up and down in each tube or finger-tapping the base of the microtubes. Do not contaminate one sample with another when preparing the mixture (use a new pipette tip for each operation). Spin down the mixture in the micro-centrifuge by using appropriate adapters (please balance the tubes before the centrifugation). During preparation and after spinning, always keep the tubes on ice.

Step 3 (*Incubation of digestion reaction and preparation of precast gel*)**.** After all the tubes have been prepared, remove them from ice and place them into your color-coded foam floating rack and incubate for 5 minutes at 37ºC in the water bath assigned for you (located behind your seat). Make sure to retrieve your own samples after 5 minutes of incubation.

During 37ºC incubation, you can prepare the Cassette (precast gel) as you were instructed when visiting the Lab the day before, with the steps as follows:

1. Use scissor to cut off a side of the bag and carefully take out the Cassette.
2. Remove white seals from the Cassette (but do not remove the clear side vent seals).
3. Use a squirt bottle to flood the sample wells with deionized water (please be sure to flush all the wells), then tilt the Cassette to drain excess liquid, blot off with Kimwipe paper (do not blot wells directly).
4. Insert the Cassette into the Dock (raise your Red Card if you need assistance) and now your gel cassette is ready for sample loading.

Step 4 (*Stopping digestion reaction by deactivating REs*): When the 5 minutes of restriction digestion duration is up, retrieve your own tubes and move them into a nearby 80^{o}C heat block (*use **tissue paper** to blot excess liquids from outside of the microtubes if necessary*) and incubate for another 5 minutes.

Step 5 (*Staining DNA/PCR-RFLP products*): After 80^{o}C incubation period, spin down the tubes for cooling off and collecting all reagents to the base of the tubes. Add 2.5 µL of DNA staining dye solution (labeled D) into each microtube. Mix them well, then spin down any residual liquid using micro-centrifuge again.

Step 6. (*Loading samples for electrophoresis*): Load 5 µL of each of the 12 samples (P1a to Zb) and 100 bp ladder solution (labelled M) into the wells (*Notice: do not exceed 5 µL per lane as it is the maximum limit of the well volume*). Make sure you position the pipette tips carefully on top of the wells and gently load the mixtures into the wells without spilling them. Add your samples according to the following scheme of lanes (from the left side and the wells are away from you).

P1a	P1b	P2a	P2b	P3a	P3b	M	Xa	Xb	Ya	Yb	Za	Zb

Step 7 (*Running electrophoresis*): When you have finished loading the gel, raise your GREEN CARD. The lab assistant will start the electrophoresis run. The voltage of the power supply should be set up at 200V. After about 7 or 8 minutes (when the fastest band of the ladder M has migrated beyond two third of the gel), raise your GREEN CARD again to notify the assistant to disconnect (turn off) the power supply.

Step 8 (*Documenting your gel*)**.** The assistant will remove the Dock with Cassette from the power supply. Plug in the Dock to electric socket and turn on the light. Observe the gel (with the safety goggles) and draw in pencil the bands of each lane you observed into **Figure Q.2.1** in the **Answer Sheet** (Notice the scale of the preprinted molecular ladder (M), and any lanes or bands drawn but not matching the gel photograph in next step will not be scored).

Step 9 (*Photographing your gel*). After finishing the drawing, label the sticker with your **Student ID** and affix onto the frame (the red part) of your gel cassette. Raise your GREEN CARD to hand over your whole gel Dock with Cassette to a Lab assistant. The gel will be photographed and its image will be attached onto **Figure Q.2.2** in your **Answer Sheet** by the assistants afterwards (Notice lanes appearing in a wrong position as compared to those described in Step 6 will not be scored, but they can be used in solving next questions of this exam).

Q.2.1. DRAWING OF GEL (18 POINTS)

Q.2.2 PHOTO (26 POINTS)

TASK 3: FORENSIC IDENTIFICATION OF BIOPSY SAMPLES (9 POINTS)

Q.3.1. (9 POINTS)

Based on PCR-RFLP profiling of samples derived from the three patients (P1 – P3) and the three biopsy specimens in Task 2, match X, Y and Z to the patients by filling in the table on the **Answer Sheet**.

NOTE!

For solving questions in Tasks 4 and 5, if you did not succeed in genotyping any patient specimens (P1, P2 and P3) in Task 2, you might deduce from profiling their biopsy specimens (X, Y and Z). In those cases, write down X/Y/Z into the column “Patients”. However, there will be a penalty of 1.5 points for each substitution.

TASK 4: INTERPRETATION OF PATIENTS' GENOTYPES (12 POINTS)

Q.4.1 (9 POINTS)

Indicate the genotypes of the three patients based on the PCR-RFLP profile you obtained from your own digestion with *Kpn*I (NAT2*5 or C481T) and *Bam*HI (NAT2*7 or G857A) by completing the table in the **Answer Sheet**. The genotype of the locus NAT2*6 (G590A) based on the *Taq*I digestion is already given for the three patients.

Q.4.2 (3 POINTS)

Indicate the acetylator phenotype of the three patients based on their genotypes you determined in this task (Question 4.1) by ticking (√) in relevant boxes of the table in the **Answer Sheet**.

A Patient P1
B Patient P2
C Patient P3

TASK 5. DETERMINATION OF DRUG DOSAGE RELEVANT TO PATIENTS' GENOTYPES (18 POINTS)

Introduction

In 2015, a study performed by Jung AJ and co-workers (*Journal of Drug Design, Development and Therapy; 9: 5433-8*) on 206 patients with TB who received INH at the dose of the standard regimen (5 mg/kg body weight, usually 300 mg INH daily) indicated that 2-hour post-dose serum concentrations of INH were significantly lower in the rapid-acetylators than in the slow-acetylators. A multivariate stepwise linear regression analysis that included the variables of age, sex, body weight, and NAT2 genotype revealed that NAT2 and body weight independently affected INH concentrations ($P < 0.001$), while other variables did not alter INH concentration ($P > 0.05$). According to the regression analysis, the equation that best predicts INH concentration is as follows:

Serum INH concentration (mg/L) = 13.821 - 0.1 × (body weight, kg) - 2.273 × (number of high activity alleles of NAT2; 0, 1, 2) **(Equation 1)**

In this equation, number of high activity alleles of NAT2 (0, 1 or 2) corresponds to the three phenotypes slow-, intermediate- and rapid-acetylators, respectively.

The most effective anti-TB therapy of INH was found when its 2-hour post-dose serum concentration fall within the range of 3.0 – 6.0 mg/L. Based on the concentration of INH shown on the drug label in **Figure 4**, you are to determine the appropriate prescription for patients P1, P2 and P3. Work according to the next two steps (**Questions 5.1** and **5.2**) and assume all three patients are 70 kg in weight each.

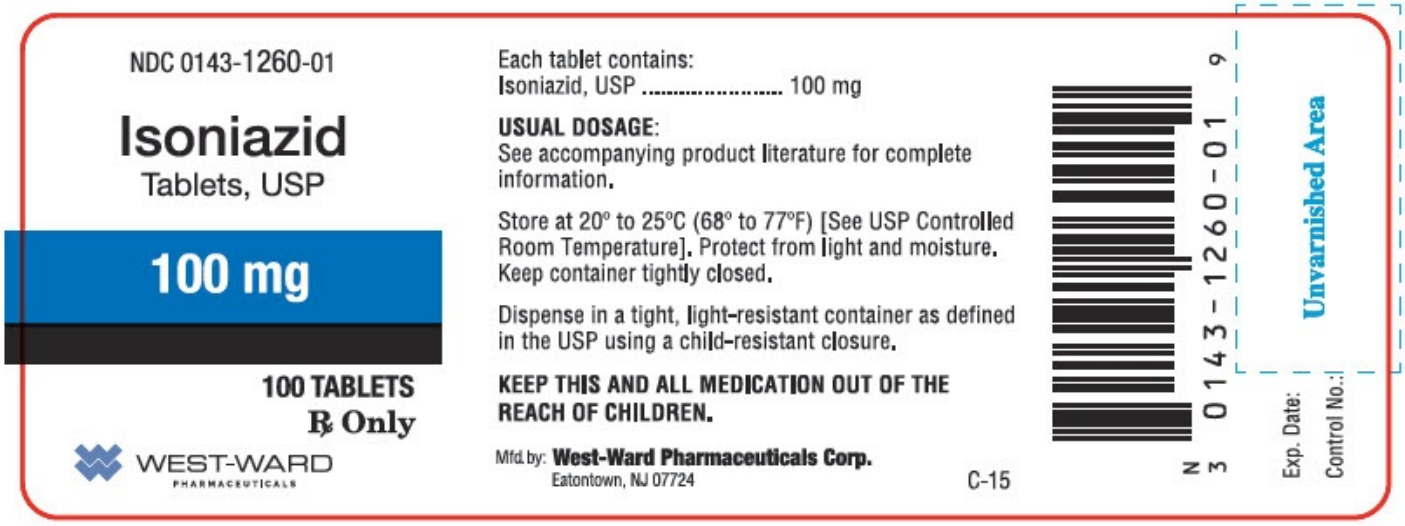

Figure 4. Isoniazid (INH) Drug Label

Figure 4. Isoniazid (INH) Drug Label

Q.5.1 (6 POINTS)

Assume that each patient takes a daily dose of 300 mg INH, estimate the 2-hour post-dose serum concentration of INH in P1, P2 and P3 based on Equation 1 and their genotypes you identified by completing the table in the **Answer Sheet** (numbers are presented to 3 decimal digits).

Q.5.2 (12 POINTS)

Based on the patients' corresponding *NAT2* genotype, determine the least number of drug tablets (shown in **Figure 4)** each patient should be administrated as daily dose to achieve an anti-TB therapy within the most effective range and fill in the table in the **Answer Sheet (**numbers are presented in integer).

END OF PRACTICAL EXAM 4!