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).

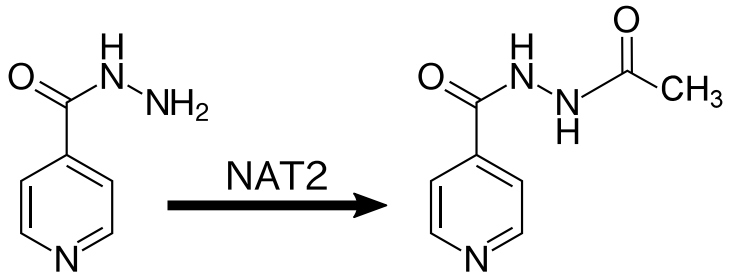

Figure 1. Metabolism of isoniazide catalysed by NAT2 (N-acetyltransferase)

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.

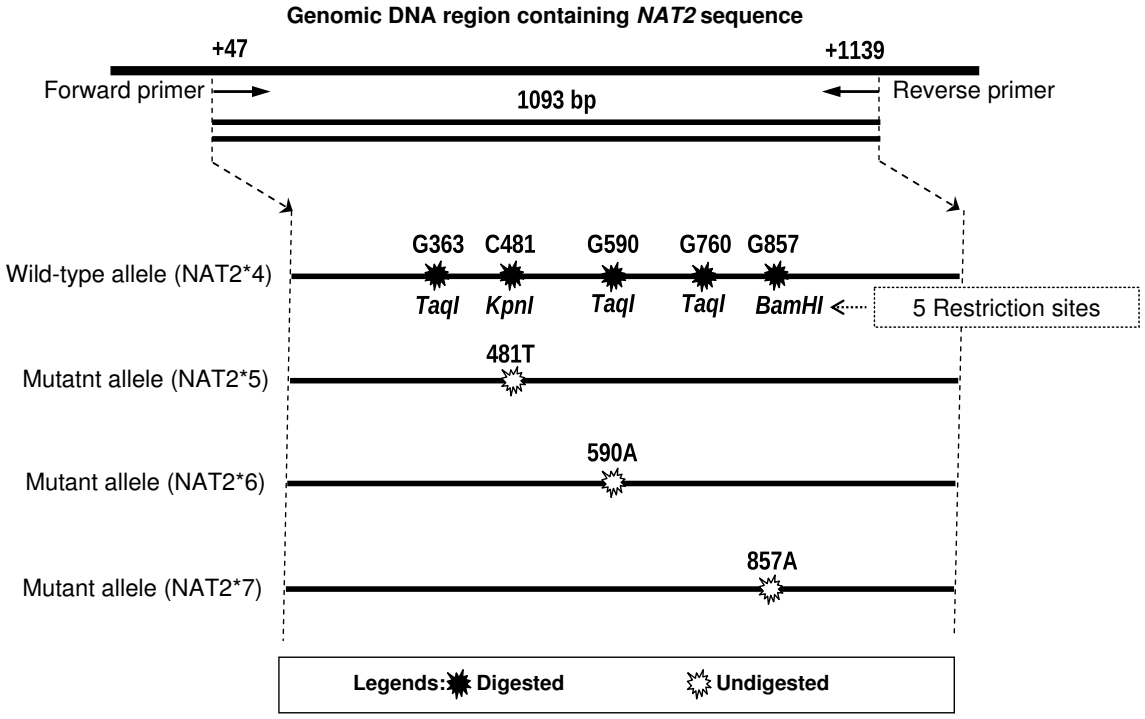

Figure 2(a). Restriction sites of the three restriction enzymes (REs)*Kpn*I, *Taq*I, and *Bam*HI in the gene coding for N-acetyl transferase type 2 (NAT2). These REs are used to generate PCR-RFLP fingerprints for detecting mutant alleles NAT2*5, NAT2*6 and NAT2*7 as distinguished to the wild-type allele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

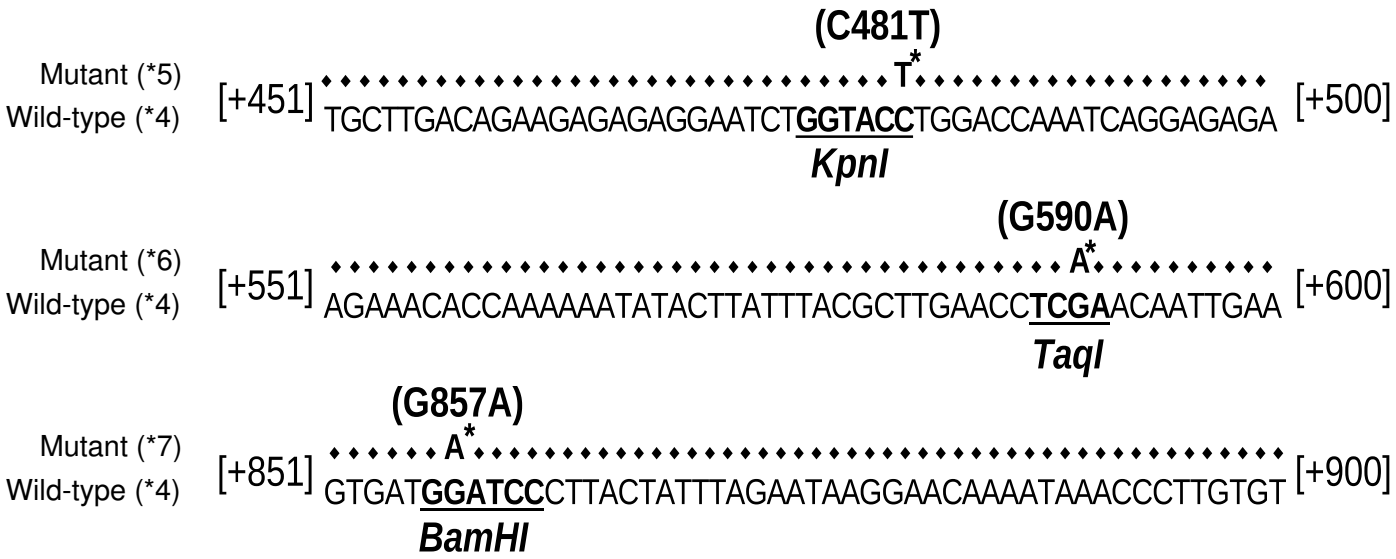

Figure 2(b). Truncated sequence of PCR products of wild-type and mutant NAT2 alleles. Numbers in brackets at the beginning and the end of each row indicate the first and the last base of the presented sequence of the wild-type allele (NAT2*4; represented in A/T/G/C) and of the corresponding mutant alleles (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).

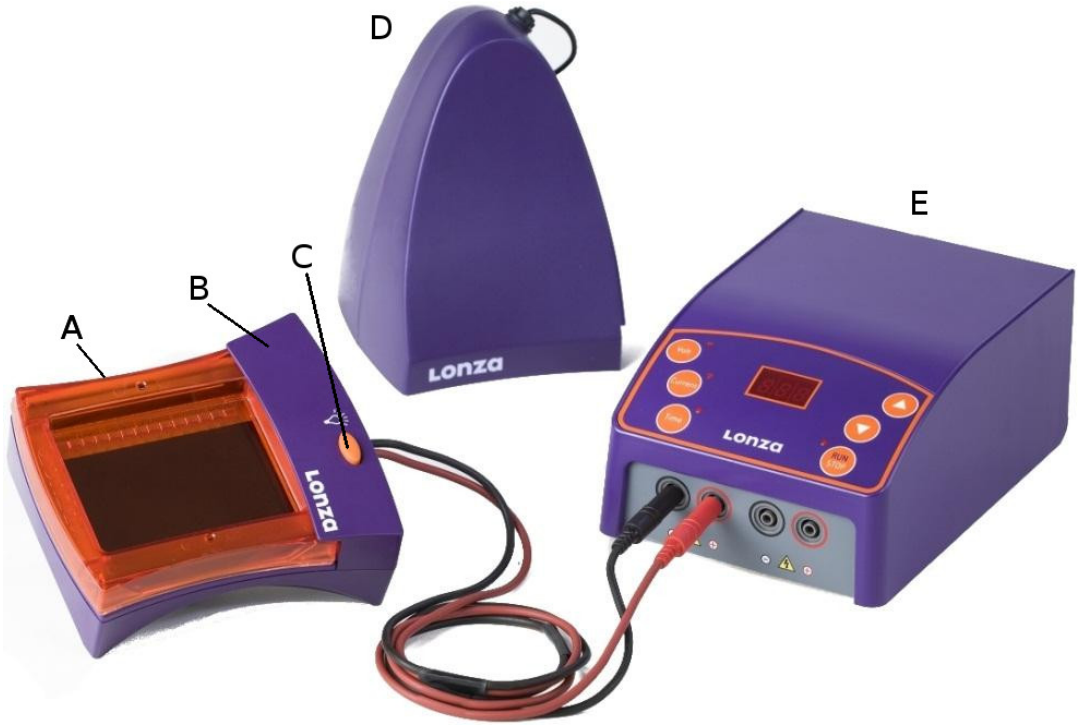

Figure 3. FlashGel® Horizontal Electrophoretic System. **(A)** Cassette. **(B)** Dock. **(C)** Knob for lighting. **(D)** Camera. **(E)** Power supply.

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.

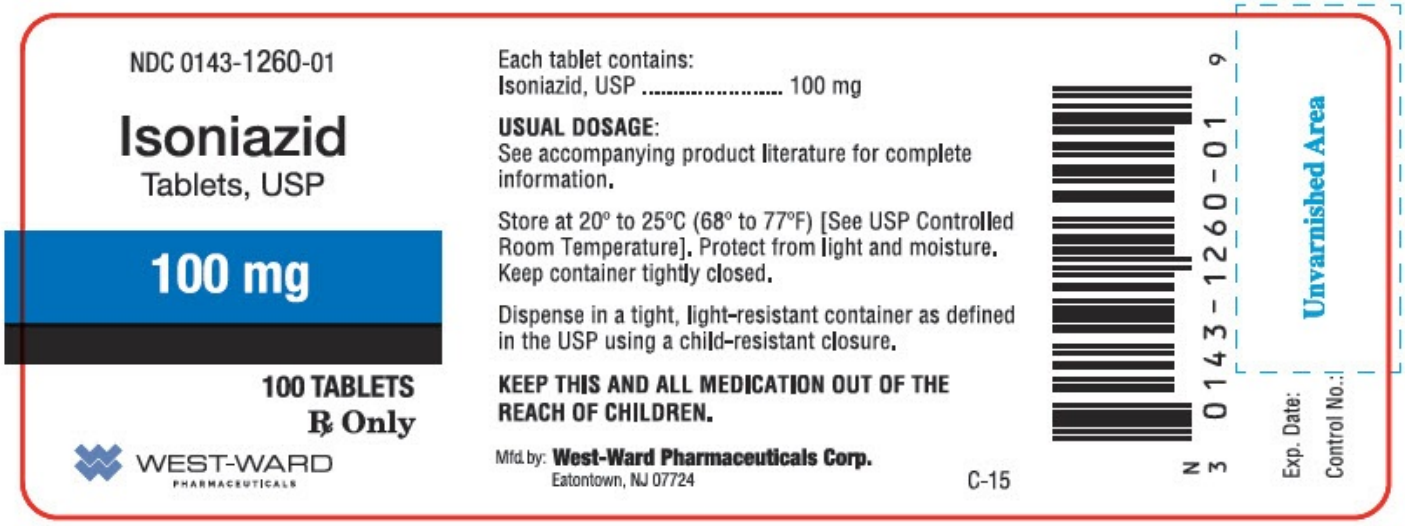

Figure 4. Isoniazid (INH) Drug Label

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!