Date …… Name …… Group ……

Instructions for the practical lesson on biochemistry

Topic: Examination of liver and pancreas

Task 1: Estimation of aspartate aminotransferase (AST) in serum

Reagents:

Commercial kit BioLaTest AST-UV L 500, made by PLIVA-Lachema Diagnostika, is used.

1. **Working solution:** malate dehydrogenase ≥ 12.5 μkat/l, lactate dehydrogenase ≥ 12.5 μkat/l, Tris buffer (pH 7.8) 100.0 mmol/l, L-aspartate 300.0 mmol/l, pyridoxal-5'-phosphate 120.0 μmol/l.
2. **Starter:** 2-oxoglutarate 60.0 mmol/l NADH 900.0 μmol/l
3. **Serum** – unknown sample

Procedure:

The starter solution, as well as the cuvette for the measurement, must be pre-warmed for at least 5 minutes at 37 ˚C.

Measure in ml directly to the cuvette:	
Working solution	1.000
Serum	0.100
Mix and pre-incubate for 5 – 10 minutes at 37 ˚C	
Starter	0.250
Mix and incubate for 1 minute at 37 ˚C	

During 1 minute incubation prepare photometer for measurement: set the wavelength to 340 nm, and zero absorbance using a cuvette filled with distilled water. After the 1 minute incubation place the cuvette with reaction mixture to the spectrophotometer and immediately read the absorbance. Then keep reading the absorbance in (exactly) one-minute intervals for 3 minutes.

Important!!!: once the starter is added, the reaction is running and the measurement must be really started immediately after the 1 minute incubation. In case of any unexpected delay do the experiment again from the beginning.

Calculation:

First calculate the average change of absorbance per minute (ΔA):

Time:		ΔA_{340}
0	A_0 ……	
1 minute	A_1 ……	$A_0 - A_1 \rightarrow \Delta A_1$ ……
2 minutes	A_2 ……	$A_1 - A_2 \rightarrow \Delta A_2$ ……
3 minutes	A_3 ……	$A_2 - A_3 \rightarrow \Delta A_3$ ……

$$\Delta A_{340} = \frac{\Delta A_1 + \Delta A_2 + \Delta A_3}{3}$$

Next, calculate catalytic AST concentration using the equation:

$$\text{S-AST (μkat/l)} = \frac{\Delta A_{340} \times 100 \times 1.35 \times 10^{-3} \times 10^{6}}{622 \times 1000 \times 0.1 \times 10^{-3} \times 60} = \Delta A_{340} \times 36.2$$

- ΔA_{340} is change in absorbance per minute
- number 100 converts 1 cm light-pass (cuvette thickness) to 1 m
- number 1.35×10^{-3} is total volume of reaction mixture
- 10^6 converts result in katals to microkatals
- 622 is molar absorption coefficient NADH at 340 nm ($m^2\ mol^{-1}$)
- number 1000 converts liters to m^3
- 0.1×10^{-3} is volume of serum sample
- number 60 converts one minute measuring interval to seconds

Task 2: Estimation of alanine aminotransferase (ALT) in serum

Reagents:

Commercial kit BIOLATEST® ALT-UV Liquid, made by Erba-Lachema a.s., is used.

1. **Working solution:** lactate dehydrogenase ≥ 26.6 μkat/l, Tris buffer (pH 7.5) 110 mmol/l, L-alanine 567 mmol/l, pyridoxal-5'-phosphate 100.0 μmol/l, 2-oxoglutarate 17.0 mmol/l, NADH 0.21 mmol/l.
2. **Serum** – unknown sample

Procedure:

Cuvettes for the measurement should be pre-warmed for at least 5 minutes at 37 ˚C before use.

Measure in ml directly to the cuvette:	
Working solution	1.00
Serum	0.1
Mix and incubate for 1 minute at 37 ˚C	

During 1 minute incubation prepare photometer for measurement: set the wavelength to 340 nm, and zero absorbance using a cuvette filled with distilled water. After the 1 minute incubation place the reaction mixture in the cuvette to spectrophotometer and immediately read the absorbance. Then keep reading the absorbance in (exactly) one-minute intervals for 5 minutes.

Again, once the working solution and serum are mixed, the reaction is running and the measurement must be really started immediately after the 1 minute incubation. In case of any unexpected delay do the experiment again from the beginning.

Calculation:

First calculate the average change of absorbance per minute (ΔA):

Time:		ΔA_{340}
0	A_0	
1 minute	A_1	$A_0 - A_1 \rightarrow \Delta A_1$ .....
2 minutes	A_2	$A_1 - A_2 \rightarrow \Delta A_2$ .....
3 minutes	A_3	$A_2 - A_3 \rightarrow \Delta A_3$ .....
4 minutes	A_4	$A_3 - A_4 \rightarrow \Delta A_4$ .....
5 minutes	A_5	$A_4 - A_5 \rightarrow \Delta A_5$ .....

$$\Delta A_{340} = \frac{\Delta A_1 + \Delta A_2 + \Delta A_3 + \Delta A_4 + \Delta A_5}{5}$$

Next, calculate catalytic ALT concentration using the equation:

$$\text{S-ALT (µkat/l)} = \frac{\Delta A_{340} \times 100 \times 1.1 \times 10^{-3} \times 10^{6}}{622 \times 1000 \times 0.1 \times 10^{-3} \times 60} = \Delta A_{340} \times 29.5$$

- ΔA_{340} is change in absorbance per minute
- number 100 converts 1 cm light-pass (cuvette thickness) to 1 m
- number 1.1×10^{-3} is total volume of reaction mixture
- 10^6 converts result in katals to microkatals
- 622 is molar absorption coefficient NADH at 340 nm ($m^2\ mol^{-1}$)
- number 1000 converts liters to m^3
- 0.1×10^{-3} is volume of serum sample
- number 60 converts one minute measuring interval to seconds

Task 3: Estimation of γ-glutamyl transferase (GGT) in serum

Reagents:

Commercial kit Gamma-Glutamyltransferase made by BioSystems S.A. Barcelona is used.

1. **Working solution:**  L-γ-glutamyl-3-carboxy-4-nitroanilide 6.5 mmol/l in buffer consisting of glycyl-glycine 165 mmol/l and sodium hydroxide 104 mmol/l pH 7.9
2. **Serum** – unknown sample

Procedure:

The working solution and the cuvette for the measurement, must be pre-warmed for at least 5 minutes at 37 ˚C. Then proceed according to the table:

Measure in ml directly to the cuvette:	
Working solution	1.0
Serum	0.1

Mix, incubate for about 30 sec. at 37 °C, and immediately measure the initial absorbance (A_0) at 405 nm against **solution of potassium dichromate**. Then keep reading the absorbance in (exactly) one-minute intervals for 3 minutes.

Again, once the serum is added, the reaction is running and the measurement must be really started immediately after the 30 sec. incubation. In case of any unexpected delay do the experiment again from the beginning.

Calculation:

First calculate the average change of absorbance per minute (ΔA):

Time:		ΔA_{405}
0	A_0	
1 minute	A_1	$A_1 - A_0 \rightarrow \Delta A_1$ .....
2 minutes	A_2	$A_2 - A_1 \rightarrow \Delta A_2$ .....
3 minutes	A_3	$A_3 - A_2 \rightarrow \Delta A_3$ .....

$$\Delta A_{405}/\text{min.} = \frac{\Delta A_1 + \Delta A_2 + \Delta A_3}{3}$$

Next, catalytic activity of GGT is calculated using the molar absorption coefficient for the colored product 5-amino-2-nitrobenzoic acid:

$$\text{GGT } (\mu\text{kat/l}) = \Delta A_{405}/\text{min.} \times 18.52$$

Task 4: Estimation of serum amylase (AMS)

Reagents:

Commercial kit α-amylase direct made by BioSystems S.A. Barcelona is used.

1. **Working solution:** 2-chloro-4-nitrophenyl-malto-trioside 2.25 mmol/l, MES buffer 50 mmol/l pH 6.1, calcium chloride 5 mmol/l, sodium chloride 300 mmol/l, sodium thiocyanate 450 mmol/l.
2. **Serum** – unknown sample

Procedure:

1. Measure 1.00 ml of working solution directly to a spectrophotometric cuvette and allow to pre-warm in thermo block set to 37°C for 5-10 minutes.
2. During this time, prepare photometer for measurement at 405 nm and set absorbance to zero using a cuvette with distilled water.
3. Add 20 µl of serum to the cuvette, cover with parafilm, and mix quickly by turning upside down. Immediately insert the cuvette to the spectrophotometer, read the initial absorbance at 405 nm and start the stopwatch.
4. Keep reading the absorbance in exactly one-minute intervals for 3 minutes.

N.B.: If the absorbance at 405 nm rises more than 0.4 per minute, dilute the serum 1:5 with distilled water and repeat the measurement. Take the dilution into account in the subsequent calculation.

Calculation:

Calculate the average change of absorbance per minute (ΔA):

Time:		ΔA_{405}
0	A_0 ……	
1 minute	A_1 ……	$A_1 - A_0 \rightarrow \Delta A_1$ ……
2 minutes	A_2 ……	$A_2 - A_1 \rightarrow \Delta A_2$ ……
3 minutes	A_3 ……	$A_3 - A_2 \rightarrow \Delta A_3$ ……

$$\Delta A_{405} = \frac{\Delta A_1 + \Delta A_2 + \Delta A_3}{3}$$

Next, catalytic activity of AMS is calculated using the molar absorption coefficient for the colored product 2-chloro-4-nitrophenol:

S-AMS (µkat/l) = ΔA_{405}/min. × 54.9