BIOCHEMISTRY AND CLINICAL BIOCHEMISTRY INTEGRATION: PRACTICAL SESSIONS

Degree in Medicine. 2nd Year

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CLINICAL ENZYMOLOGY: MEASUREMENT OF ENZYME ACTIVITIES IN SERUM

I. PLASMA ENZYMES AND THEIR DIAGNOSTIC UTILITY

Clinical enzymology measures enzyme activity in serum (and other biological samples) as a tool for the diagnosis and monitoring of diseases.

The blood contains plasma-specific enzymes that perform their function in the plasma itself (e.g. coagulation enzymes) and intracellular enzymes that pass into the blood during cell turnover. The number of intracellular enzymes that appear in serum depend on their rate of exit from cells and the rate of their elimination from plasma. In the absence of pathology, the serum concentration of these enzymes is very low in relation to their concentration in the cells or tissues of origin, but any alteration or tissue injury (necrosis, altered membrane permeability) can accelerate their release and significantly increase their presence in serum. Thus, an increase in the plasma activity of an enzyme may indicate an alteration or pathology in the tissue of origin.

Cells contain many enzymes needed for their metabolic functions. Clinical interest is focused on those enzymes whose variations are indicative of certain diseases or functional alterations and which therefore have diagnostic value (see Table 1). The fact that certain enzymes are known to originate from tissues can help us to identify the organ of origin. Although enzymes are not usually exclusive to a particular organ, the alteration of the serum concentration of a particular enzyme can provide an indication of the tissue (or tissues) most likely to be affected.

Table 1. Diagnostically useful enzymes and what they may indicate

Enzyme	**Tissue where it predominates**	**Clinical utility**
Aldolase	Skeletal muscle Heart	Muscular disease
Amylase	Salivary glands Pancreas	Pancreatic disease
Alanine aminotransferase (ALT)	Liver Skeletal muscle Heart	Liver disease
Aspartate aminotransferase (AST)	Liver Skeletal muscle Heart	Liver disease
Creatine kinase (CK)	Skeletal muscle Heart	Muscular disease Myocardial infarction
Acid phosphatase (ACP)	Prostate	Prostate cancer
Alkaline phosphatase (ALP)	Liver Bone Intestine	Liver disease Bone disease
Gamma-glutamyl transferase (GT, GGT)	Liver Kidney	Hepatobiliary disease
Lactate dehydrogenase (LDH)		Liver diseases Myocardial infarction Haemolysis
Lipase	Pancreas	Pancreatitis
5'-Nucleotidase	Liver	Hepatobiliary disease
Pseudocholinesterase	Liver Pancreas Heart	Organophosphate poisoning

Enzymes can be located in different cellular compartments and are progressively released into the extracellular environment, depending on the degree of damage to the cell. Thus, small disturbances of the cell may result in the release of cytosolic enzymes, whereas high levels of mitochondrial enzymes in serum are an indicator of severe cellular damage.

Some enzymes have molecular variants, known as isoenzymes or isozymes, that are expressed differently in different tissues. Isoenzymes have different physicochemical, catalytic or antigenic characteristics, enabling them to be identified in the laboratory by electrophoretic techniques, the use of selective substrates or inhibitors, or immunochemical methods. Isoenzyme analysis often provides additional information on the causes of an alteration of the levels of a particular enzyme. In other words, if an isoenzyme is known to predominate in a particular tissue, its specific identification and measurement in serum can help us to determine the source of the alteration. Isoenzyme analysis of creatine kinase, alkaline phosphatase or lactate dehydrogenase are some examples of tests with diagnostic utility.

II. MEASUREMENT OF ENZYME ACTIVITIES

The rate of an enzyme reaction depends on the concentration of the enzyme and other factors, such as the concentration of substrates and cofactors, temperature, pH and the presence of inhibitors. Therefore, the measurement of enzyme activities in the clinical enzymology laboratory should be performed using standardized methods under defined (pH, temperature) and controlled conditions.

Enzyme activity is usually measured using spectrophotometric methods that allow the analysis of the disappearance of the substrate from the reaction, the appearance of a product, or the variation in a cofactor as a function of reaction time. For the activity measurement to be valid, it must be performed during the linear phase of the reaction in which **the only rate-limiting factor is the concentration of the enzyme** in the reaction medium.

It is important that the biological samples (usually serum) have been properly handled and stored prior to activity measurement. Some precautions to note are to obtain the serum (after clot separation) as soon as possible, not to use haemolysed samples, to keep samples cold (preferably not frozen, unless storage is to be prolonged) and not to use samples that have been frozen/thawed several times.

In addition, the presence of certain drugs can influence the study of enzyme activity by interfering with analytical techniques, modifying physiological levels through enzyme induction or triggering toxic effects (mainly hepatotoxicity). Therefore, when interpreting test results, it is important to have a precise knowledge of the drugs administered prior to sample collection.

Serum enzyme activities are usually expressed in **International Units per unit volume** (U/L, U/dL, etc.). The International Unit is defined as the enzyme activity that, under standard conditions, converts one µmol of substrate per minute.

III. CASE STUDY: MEASUREMENT OF ENZYME ACTIVITIES IN SERUM

Alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities will be determined in serum samples (control and test samples) using standardised kinetic methods. The assays will be performed directly in spectrophotometer cuvettes by incubating the samples in a suitable reaction medium and measuring absorbance (A) at regular time intervals. The corresponding A/min value (absorbance variation per minute) will then be obtained and the appropriate calculations performed to express the enzyme activity in serum in U/L. Finally, the activity values obtained in the test samples will be

analysed (by comparison with control serum and reference values) and a possible clinical interpretation of the result obtained will be suggested.

Objectives

1) To understand the kinetic methods used for the measurement of enzyme activities in biological samples.

2) To understand the importance of the use of standardized procedures and controlling test conditions.

3) To draw clinical conclusions from the results and to assess possible sources of error.

III.A) Measurement of alanine aminotransferase (ALT)

Aminotransferases or transaminases are transferase enzymes that catalyse a reversible reaction of the transfer of an α-amino group from an amino acid to an α-keto acid. Aminotransferase enzymes are involved in the intermediary metabolism of amino acids and the direction of the reaction they catalyse is determined by the concentration of substrates and products in the cell.

Among the different transaminases that can be found in cells, aspartate aminotransferase (AST; formerly GOT or SGOT) and alanine aminotransferase (ALT; formerly GPT or SGPT) are the most clinically important.

ALT catalyses the transfer reaction of an amino group from alanine to α-ketoglutarate with the formation of pyruvate and glutamate.

It is a cytosolic enzyme that predominates in the liver, although it is also abundant in the heart, skeletal muscle, pancreas, spleen, lung and erythrocytes. Its activity in serum increases when it is released from damaged or necrotic cells and it is usually measured together with AST.

In inflammatory liver disease, caused by viral infection or alcoholism, there is a marked elevation of ALT and AST (x 10 the reference value). The elevation of ALT is usually higher and longer lasting, due to the longer half-life of the enzyme. If the ALT elevation lasts longer than 6 months, this indicates that the process has become chronic.

In cases of liver cirrhosis or hepatocellular carcinoma, the increase in both transaminases is moderate, with AST activity being higher than ALT.

Extrahepatic cholestasis also causes an increase in aminotransferases, and this increases further as the lesion becomes chronic. This increase is accompanied by an even greater increase in ALP activity.

Table 2. Serum reference values for ALT activity

	25ºC	30ºC	37ºC
Women	≤ 18 U/L	≤ 22 U/L	≤ 32 U/L
Men	≤ 22 U/L	≤ 29 U/L	≤ 40 U/L

Higher reference values than those for adults (up to x 2-fold) have been reported in normal newborns due to hepatic immaturity. These values normalise after three months.

The ALT values shown in Table 2 are typical values only and it is recommended that each laboratory establishes its own reference values.

III.A.1. ALT activity measurement

ALT activity is measured using spectrophotometry to determine the rate of NADH disappearance according to the following coupled reactions:

$$\text{L-alanine} + \text{-ketoglutarate} \xrightarrow{\textit{ALT}} \text{pyruvate} + \text{L-glutamate}$$

$$\text{pyruvate} + \textbf{NADH} + \text{H+} \xrightarrow{\textit{LDH}} \text{lactate} + \text{NAD+}$$

NADH (reduced form) shows absorbance at 340-365 nm, which does not appear in the oxidised form (NAD+).

III.A.2. Material

- Automatic pipettes
- Disposable yellow and blue tips
- Eppendorf tubes
- Tube racks
- Spectrophotometer
- Spectrophotometer cuvettes
- Parafilm

III.A.3. Reagents

ALT titration reagent:

The working reagent is ready for use. No additional preparation is required by the student.

The reagent has been prepared as follows:

Reagent 1 (R.1): Tris buffer pH 7.8, 100 mM + L-alanine 500 mM Reagent

2 (R.2): NADH 0.18 mM + LDH 1200 U/L +α-ketoglutarate 15 mM

Dissolve one R.2 tablet in 50 mL of R.1 buffer. The mixture is stable for 72 h at 15-25°C or 21 days at 2-8°C.

III.A.4. Sample

Serum or plasma. Commonly used anticoagulants (heparin, EDTA, oxaloacetate, fluoride) do not affect the results. Due to the presence of ALT in erythrocytes, haemolysis increases the activity of the sample. Some drugs may interfere with the ALT measurement.

III.A.5. Practical procedure

ALT activity will be measured in 2 different samples:

a) control serum (Ctrl)

b) problem serum (PS)

The enzyme activity in each of the samples will be measured separately according to the following procedure (**see Figure 1**):

1) Set the wavelength of the spectrophotometer to 340 nm and set the absorbance value to 0 (with an empty spectrophotometer, without a cuvette).
2) Pipette directly into a spectrophotometer cuvette:

   1 mL of the working reagent and

   100 uL of the corresponding sample (water, control serum or test serum)
3) Mix carefully by inversion (with the help of a piece of *parafilm* placed at the mouth of the cuvette), clean the exterior of the cuvette, place the cuvette in the spectrophotometer, note the absorbance reading. This will be the absorbance value at zero time (**A_0**). Record the reading in the data table.
4) Do not touch the cuvette (it must remain inside the spectrophotometer throughout the test). Exactly one minute after the first reading, a new absorbance value is recorded: **A_1**.
5) Note two more absorbance readings taken at one-minute intervals: **A_2** and **A_3**.
6) Remove the cuvette from the spectrophotometer and repeat the procedure with the next sample.

Important*: The reaction mixture (working reagent + sample) must be prepared in each cuvette immediately before starting to take absorbance readings.*

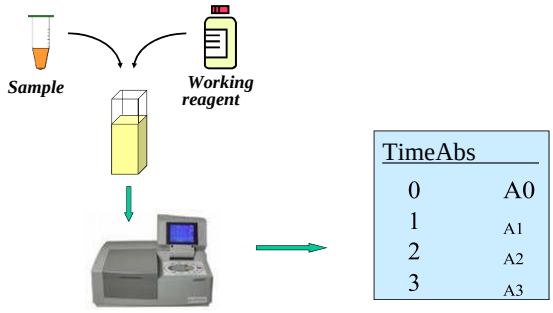


Figure 1. Schematic diagram of the enzyme activity measurement procedure.

Table for ALT activity measurements

	A_0	A_1	A_2	A_3	ΔA / min	ΔU / L
Ctrl						
PS						

III.A.6. Calculations

Use the absorbance data for each time and calculate the absorbance variation rate per minute (ΔA/min) for each sample.

(*Hint: take the difference between each interval (each ΔA) and then calculate de mean value*).

The A/min value obtained for the blank cuvette will be subtracted from that calculated for the serum samples.

The ALT activity value in each sample will be expressed in U/L according to the following expression: (A/min) x 3240 = U/L.

Note: The A/min value is to be considered as an absolute value.

III.B) Measurement of alkaline phosphatase (ALP) activity

Phosphatase enzymes hydrolyse ester bonds of phosphoric acid, their natural substrates being various organic phosphates present in cells. As products of the reaction, inorganic phosphate and the corresponding organic residue with which it was esterified are generated. Among the numerous phosphatases in the body, only the serum levels of two groups of phosphatases are of clinical interest:

- Acid phosphatases (ACP): with an optimum pH around 5 and located mainly in the prostate, red blood cells and platelets.
- Alkaline phosphatases (ALP): with an optimum pH around 9 and essentially of bone and liver origin.

ALP enzymes hydrolyse numerous types of phosphate esters in alkaline media and use Mg^{2+}.

ALP is present in numerous tissues of the body and particularly in the sinusoids and bile canaliculi of the liver, osteoblasts (bone) and the intestinal epithelium. It is also abundant in the placenta, spleen and kidney.

The increase in serum ALP activity is primarily due to:

- Increased osteoblastic activity: hyperparathyroidism, vitamin D deficiency (rickets, osteomalacia), Paget's disease, fracture healing and bone metastases.
- Disorders of the biliary tree and intra- or extrahepatic cholestasis.

Decreased plasma ALP levels also have clinical significance (e.g., cretinism).

Table 3. Serum reference values for ALP activity

	25ºC	30ºC	37ºC
Children	< 400 U/L	< 480 U/L	< 645 U/L
Adults	60-170 U/L	73-207 U/L	98-279 U/L

ALP levels in children are higher than in adults (2-5 times) due to the bone remodelling that occurs during the growth stages.

Values increase (2-3 fold) during pregnancy due to placental ALP release. An alteration of values can also be observed as a consequence of exercise.

The ALP values in Table 3 are typical values only. It is recommended that each laboratory establish its own reference values.

ALP isoenzymes

There are different isoenzymes of ALP (encoded by different genes), varying according to their tissue of origin. The main isoenzymes detected in serum are of hepatic, bone, placental and intestinal origin.

If ALP activity in serum is found to be elevated, identification of the isoenzyme responsible for the increase in ALP activity will help to determine the cause or origin of the alteration.

The particular characteristics of the ALPs from each tissue allow them to be differentiated relatively easily by electrophoretic techniques combined with chemical inhibition or heat inactivation for some of them.

With regard to thermal inactivation, placental ALP is the most thermostable, followed by intestinal, hepatic and bone (Table 4) ALP.

Table 4. Inactivation of ALP activity after treatment of a serum sample at 56°C for 15 min.

Tissue of origin	Inactivation (%)
Liver	50-70
Bone	90-100
Intestine	50-60
Placenta	0

III.B.1. ALP activity measurement

For activity titration, sodium p-nitrophenyl phosphate is used as substrate which, in a basic medium, is hydrolysed by ALP, releasing p-nitrophenol and phosphate.

$$\text{p-nitrophenyl phosphate} \xrightarrow{ALP/Mg2+} \textbf{p-nitrophenol} + \text{phosphate}$$

p-nitrophenol has a strong yellow colouring in an alkaline medium and is quantifiable spectrophotometrically at 405 nm.

III.B.2. Material

See material list in III.A.2.

III.B.3. Reagents

ALP titration reagent:

The working reagent is ready for use. No additional preparation is required by the student.

The reagent has been prepared as follows:

Reagent 1 (R.1): Diethanolamine buffer 1 mM pH 10.4 + magnesium chloride 0.5 mM
Reagent 2 (R.2) (tablets/powder): p-nitrophenyl phosphate 10 mM

Dissolve the contents of vial R.2 in 50 mL of buffer R.1. The mixture is stable for 5 days at 18-20°C or 21 days at 2-8°C.

III.B.4. Sample

Heparinised serum or plasma. Do not use calcium chelators (oxaloacetate, citrate, EDTA, fluoride) as an anticoagulant (ALP uses Mg2+) or haemolysed samples (ALP concentration in erythrocytes is higher than in plasma). Some drugs may interfere with the measurement.

III.B.5. Practical procedure

ALP activity will be measured in 5 different samples:

a) control serum (Ctrl)

b) problem serum (PS)

c) control serum (Ctrl) pre-incubated 15 min at 56ºC

d) test serum (PS) pre-incubated 15 min at 56 °C

The enzyme activity in each of the samples will be measured separately according to the following procedure (see Figure 1):

1) Set the wavelength of the spectrophotometer to 405 nm and set the absorbance value to 0 (with an empty spectrophotometer, without a cuvette).
2) Pipette directly into a spectrophotometer cuvette:

   1.2 mL of working reagent and

   20 uL sample (water, control serum, test serum, control serum 56°C or test serum 56°C).
3) Mix carefully by inversion (with the help of a piece of *parafilm* placed at the mouth of the cuvette), clean the exterior of the cuvette, place the cuvette in the spectrophotometer, wait for about 1 minute and note the absorbance reading. This will be the absorbance value at zero time (A_0). Record this and susbequent readings in the data table.
4) Exactly one minute after the first reading, record a new absorbance value, A1.
5) Record two more absorbance readings taken at one-minute intervals: A2 and A3.
6) Remove the cuvette from the spectrophotometer and repeat the procedure with the next sample.

Important*: The reaction mixture (working reagent + sample) must be prepared in each cuvette immediately before starting to take absorbance readings.*

Table for ALP activity measurements

	A_0	A_1	A_2	A_3	ΔA / min	U / L	
Sample SN							
Sample SP							
Sample SN 56 ºC							
Sample SP 56 ºC							

III.B.6. Calculations

IV Use the absorbance data for each time and calculate the absorbance variation rate per minute (ΔA/min) for each sample.

(*Hint: take the difference between each interval (each ΔA) and then calculate the mean value*).If the A/min value obtained for any of the samples is > 0.250, the measurement will be repeated with the sample diluted in saline.

If the A/min value for the blank cuvette is > 0, this value shall be subtracted from the value obtained for the serum samples.

The ALP activity value in each of the samples shall be expressed in U/L according to the following expression: (A/min) x 3300 = U/L

I. QUESTIONS FOR PRACTICAL SESSION 1

1) Calculate the ALT activity in the test serum and interpret the result.

2) Calculate the ALP activity in the test serum and in the thermally inactivated test serum. Interpret the result.

3) To express the serum activity of the enzymes tested, the value of the increase in absorbance per minute (A/min) is multiplied by a certain factor. What does the value of this factor depend on?

4) Explain why haemolysis can interfere with the measurement of enzyme activity.

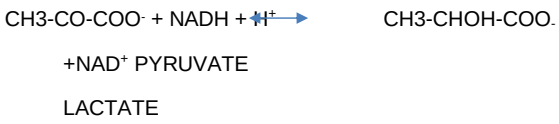
2

STUDY OF LDH ISOENZYMES AS MARKERS OF DIFFERENT PATHOLOGIES

PRACTICAL SESSION 2
LDH ISOENZYME ANALYSIS

I. INTRODUCTION

Lactate dehydrogenase (LDH) is an enzyme widely distributed in animal and plant tissues and microorganisms. It is involved in anaerobic metabolism by catalysing the redox reaction in which pyruvate is reduced to lactate by the oxidation of NADH. In humans, it is especially abundant in heart, liver, kidney, muscle, red blood cells, brain, and lungs.



LDH is a cytoplasmic enzyme and so its appearance in the extracellular medium is a marker of cell damage or cell death. Due to its wide distribution in the human body, its appearance in serum is associated with various pathological conditions such as fasting, ischaemia, dehydration, exposure to bacterial toxins or drugs. For this reason, its detection in serum, despite being a very sensitive method that indicates the appearance of damage, is a very non-specific test. To optimise its diagnostic value, we need to identify specific LDH isoenzymes.

LDH (140 kDa) is made up of 4 subunits, each about 35 kDa.

Three types of subunits are known: H (LDHB), M (LDHA) and X (LDHC), which combine to form tetramers giving rise to **five isoenzymes** (isoforms of the enzyme), each of which tends to be found in certain tissues and can be identified by electrophoresis.

- LDH-1 (H4): in the heart and erythrocytes.
- LDH-2 (H3M): in leukocytes. Abundant in the heart, but less so than LDH-1.
- LDH-3 (H2M2): in the lungs.
- LDH-4 (HM3): in the kidneys, placenta and pancreas.
- LDH-5 (M4): in the liver and skeletal muscle.

Type X is found in mammalian and avian spermatozoa.

Normal values

Although the reference values for normal conditions vary according to the detection method of each laboratory, in general, they are in the following ranges

- LDH-1: 17-27%
- LDH-2: 27-37%
- LDH-3: 18-25%
- LDH-4: 8-16%
- LDH-5: 6-16%.

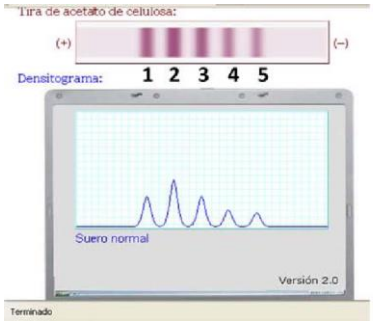
Normal values: < 120-240 U/L with the assay used in this practical session.

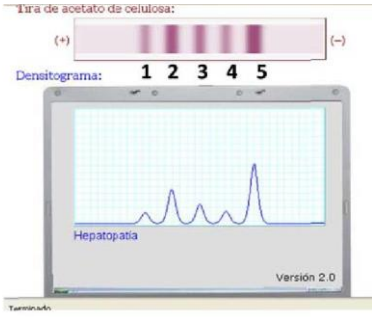
Abnormal values

Elevated **LDH-1** values are indicative of myocardial infarction, red blood cell diseases such as haemolytic anaemia, kidney disease and testicular tumours. Elevated **LDH-2** values are associated with lymphomas. Elevated **LDH3** values are indicative of lung disease. Elevated **LDH-4** may indicate pancreatitis. Elevated **LDH-5** levels are found in liver disease and muscular dystrophy.

II. LDH ISOENZYME ANALYSIS

The levels of the different isoenzymes can be determined via ***electrophoresis using cellulose acetate***, polyacrylamide or agarose because their different composition leads to their migration at different speeds on these types of supports under native conditions.





In this practical session, we are going to take advantage of another of the differences that LDH isoenzymes present due to their different protein composition and that will allow us to easily differentiate between groups of isoenzymes. This characteristic ***is thermal stability***. The different isoenzymes differ in their thermal stability, enabling us to establish two groups of isoenzymes: those which are heat-resistant (or thermoresistant) and those which are thermolabile.

LDH-1 and LDH-3 are **heat-resistant** (they remain active after heating to 60°C), while LDH-2, -4 and -5 are **thermolabile**.

Thus, if we compare the activity of **normal serum** before and after heating to 60°C, the heat-resistant fraction in normal serum is between 30 and 60%. In cases of liver disease, the heat-resistant fraction decreases below 30%, whereas in myocardial infarction, the heat treatment causes an increase in the heat-resistant fraction above 60%. The LDH level rises within 24 to 72 hours after infarction, peaks in 3 to 4 days and normalises within about 14 days.

The measurement of the heat-resistant fraction therefore increases the diagnostic value of LDH measurement.

Preparation

This test requires a blood sample and serum collection. The patient does not need to fast for the test to be carried out.

Precautions

- Intense exercise can increase levels of total LDH, specifically the isoenzymes LDH-1, LDH-2, and LDH-5.
- Alcohol, anaesthetics, aspirin, narcotics, procainamide and fluorides can also increase LDH levels.
- Ascorbic acid (vitamin C) can lower LDH levels.

HOW THE METHOD WORKS

The rate of decrease of the NADH concentration in the medium measured spectrophotometrically is proportional to the catalytic concentration of LDH in the sample tested.

$$\text{Pyruvate} + \text{NADH} + H^+ \rightarrow \text{Lactate} + NAD^+$$

NADH (reduced form) shows an absorbance maximum at 340 nm, which is not the case for the oxidised form (NAD+). Thus, the absorbance value in the sample will be proportional to the amount of NADH present in the solution that has not been transformed into NAD+ in the reaction catalysed by the LDH present in the serum.

Material

- Automatic pipettes
- Disposable yellow and blue tips
- Eppendorf tubes
- Tube racks
- Spectrophotometer
- Spectrophotometer cuvettes
- Thermo-block
- Parafilm

REAGENTS

***Reagent 1:** Phosphate Buffer pH 7.8 [80mM]
Pyruvate [0.6mM].
NADH [0.18mM].

***Phosphate buffer pH 7.4:** 80 mM
***Pyruvate stock solution:** 120 mM
***NADH solution:** 36 mM

Mix 25µL of 36mM NADH + 25µL of 120 mM pyruvate + 4.95 mL 80mM phosphate buffer.

SAMPLE

Serum separated as soon as possible from red blood cells. Due to the presence of LDH in erythrocytes, haemolysis increases the activity of the sample. Stability: 2 days at 2-8 ºC.

PRACTICAL PROCEDURE

LDH activity will be determined in 4 different samples:

a) Control serum (Ctrl).

b) Problem serum (PS1 or PS2).

c) Control serum inactivated at 60°C (Ctrl-60).

d) Test serum inactivated at 60°C (PS1-60 or PS2-60).

The enzyme activity in each of the samples will be measured separately according to the following procedure (see Figure 1):

1) Set the wavelength of the spectrophotometer to 340 nm and set the absorbance value to 0 (versus air).
2) Pipette directly into a spectrophotometer cuvette:

   1 mL of working reagent and

   35 uL of the corresponding sample (Ctrl, PS, Ctrl-60 and PS1-60 / PS2-60)

3) Mix carefully by inversion (with the help of a piece of *parafilm* placed at the mouth of the cuvette), clean the exterior of the cuvette, place the cuvette in the spectrophotometer, wait for about 1 minute and note the absorbance reading. This will be the absorbance value at zero time ($\mathbf{A_0}$). Record the reading in the data table.
4) Do not touch the cuvette (it must remain inside the spectrophotometer throughout the test). Exactly one minute after the first reading, record another absorbance value: $\mathbf{A_1}$.
5) Record two more absorbance readings taken at one-minute intervals: $\mathbf{A_2}$ and $\mathbf{A_3}$.
6) Remove the cuvette from the spectrophotometer and repeat the procedure with the next sample.

Important*: The reaction mixture (working reagent + sample) must be prepared in each cuvette <u>immediately</u> before starting to take absorbance readings.*

Table for LDH activity measurement

	A_0	A_1	A_2	A_3	ΔA / min	IU / L
Ctrl						
PS1/PS2						
Ctrl-60						
PS1-60/PS2-60						

Calculations

Use the absorbance data for each time and calculate the absorbance variation rate per minute (ΔA/min) for each sample.

(*Hint: take the difference between each interval (each DA) and then calculate de mean value*).If the A/min value obtained for any of the samples is > 0.250, the measurement will be repeated with the sample diluted in saline.

The LDH activity value in each of the samples will be expressed in IU/L according to the following expression: **(A/min) x 9131 = IU/L**

PRACTICAL QUESTIONS

1.- Calculate the enzymatic activity in I.U./L of the two test tubes (Table I)

2. Calculate the % of LDH resistant at 60ºC.

3.- Interpret the results

3

SERUM LIPID PROFILE

PRACTICAL SESSION 3:
LIPID METABOLISM

1. OBJECTIVE:

To understand the use of enzyme activity assays in biological fluids and tissues for diagnostic and disease monitoring purposes.

2. INTRODUCTION:

A lipid profile performed in the clinical biochemistry laboratory provides essential information to determine risk factors related to cardiovascular diseases.

3. METHOD OF TOTAL CHOLESTEROL QUANTIFICATION

3.1. Rationale:

In the presence of oxygen, cholesterol oxidase oxidises free cholesterol to form cholestenone and hydrogen peroxide, which can be detected by a chromogenic oxygen acceptor in the presence of peroxidase. The quinone formed is proportional to the concentration of cholesterol in the sample analysed.

3.2. Reagents:

Reagent 1:

- PIPES pH 6.9 (90 mmol/L); Phenol (26 mmol/L)
- Enzymes (Peroxidase 1250U/L; Cholesterol esterase 300U/L; Cholesterol oxidase 300U/L; 4-Aminoantipyrine 0.4mmol/L)

Standard: Cholesterol Solution (200 mg/dL)

3.3. Samples:

- Normal serum (use directly)
- Hyperlipidaemic serum (use directly)

3.4. Procedure:

	Blank	**Standard**	**Normal Serum**	**Hyperlipidaemic Serum**
Reagent 1	1 ml	1 ml	1 ml	1 ml
Water	10 ul	--	--	--
Standard	--	10 ul	--	--
Normal Serum	--	--	10 ul	--
Hyperlipidaemic Serum	--	--	--	10 ul

- Mix and wait for 10 minutes at room temperature.
- Set the device to zero with the blank.
- At 505 nm, record the absorbance of the standard and the samples.

3.5. Calculation:

$$\frac{\text{Sample optical density}}{\text{Standard optical density}} \times \text{Standard Conc. (200mg/dL)} = \text{Sample Concentration (mg/dL)}$$

Conversion factor: mg / dL x 0.0258 = mmol / L

4. METHOD OF HDL-CHOLESTEROL QUANTIFICATION

4.1. Rationale:

The addition of phosphotungstic acid and Mg ions^{2+} to the sample causes the precipitation of chylomicrons, VLDL and LDL. The supernatant after centrifugation contains HDL, whose cholesterol concentration can be determined enzymatically.

4.2. Reagents:

Precipitating Reagent: Phosphotungstic acid (14 nmol/L); Magnesium chloride (2mmol/L)

Reagent 1:

- PIPES pH 6.9 (90 mmol/L); Phenol (26 mmol/L)
- Enzymes (Peroxidase 1250U/L; Cholesterol esterase 300U/L; Cholesterol oxidase 300U/L;
- 4-Aminoantipyrine 0.4mmol/L)

4.3. Samples:

- Normal serum (prepare as indicated in 4.4)
- Hyperlipidaemic serum (prepare as indicated in 4.4)

4.4. Preparation of serum samples:

Use one Eppendorf tube for the normal serum and one Eppendorf tube for the hyperlipidaemic serum.

	Normal Serum	**Hyperlipidaemic Serum**
Normal Serum	100 ul	**--**
Hyperlipidaemic Serum	--	100 ul
Precipitating Reagent	20 ul	20 ul

- Mix, leave to stand for 10 minutes at room temperature, then centrifuge for 20 minutes at 4000 rpm.
- Transfer the supernatant to the Eppendorf tubes.

  Supernatant from the normal serum

  Supernatant from the hyperlipidaemic serum
- Determine the amount of cholesterol in the supernatant.

4.5. Procedure:

	Blank	**Normal Serum (supernatant)**	**Hyperlipidaemic Serum (supernatant)**
Reagent 1	1 ml	1 ml	1 ml
Water	20 ul	--	--
Normal Serum	--	20 ul	--
Hyperlipidaemic Serum	--	--	20 ul

- Mix and wait 10 minutes at room temperature.
- Set the device to zero with the blank

- At 505 nm, record the optical density (absorbance) of the standard and the samples.

4.6. Calculation:

Absorbance X 320 = mg/dL HDL-cholesterol

Conversion factor: mg / dL x 0.0258 = mmol / L

5. TRIGLYCERIDE (TG) QUANTIFICATION METHOD:

5.1. Rationale:

Triglycerides incubated with a lipase are hydrolysed to glycerol and fatty acids. Glycerol is converted to glycerol phosphate in the presence of glycerol kinase and ATP. Glycerol phosphate is oxidised to dihydroxyacetone phosphate by the action of glycerol phosphate oxidase and the hydrogen peroxide generated can be detected by a chromogenic oxygen acceptor in the presence of peroxidase. The quinone formed is proportional to the concentration of triglycerides in the sample analysed.

5.2. Reagents

Reagent TG:

- Good's buffer pH 7.5 (50mmol/L); p-chlorophenol (2mmol/L)
- Enzymes (Lipase 150000U/L; Glycerol Kinase 500U/L; Glycerol-P-oxidase 2500U/L; Peroxidase 440U/L; 4-Aminophenazone 0.1mmol/L; ATP 0.1mmol/L)

Standard: Triglyceride Solution (200 mg/dL)

5.3. Samples:

- Normal serum (use directly)
- Hyperlipidaemic serum (use directly)

5.4. Procedure:

	Blank	**Standard**	**Normal Serum**	**Hyperlipidaemic Serum**
TG reagent	1 ml	1 ml	1 ml	1 ml
Water	10 ul	--	--	--
Standard	--	10 ul	--	--
Normal serum	--	--	10 ul	--
Hyperlipidaemic serum	--	--	--	10 ul

- Mix and wait 10 minutes at room temperature.
- Set the device to zero with the blank.
- At 505 nm, record the optical density (absorbance) of the standard and the samples.

5.5. Calculation:

$$\frac{\text{Sample Absorbance}}{\text{Standard Absorbance}} \text{ X Standard Conc. (200 mg/dL) = Sample Concentration (mg/dL)}$$

Conversion factor: mg / dL x 0.0113 = mmol / L

The **Framingham Heart Study**, a community-wide prospective study that has confirmed the relationship between various risk factors and the likelihood of developing cardiovascular disease. It is a longitudinal study of three generations (1948, 1971 and 2002). The **risk factors with a direct relationship** are:

- **Family history** of coronary artery disease
- **Age:** In general, the older you are, the higher the risk
- **Sex:** Women of childbearing age have a lower cardiovascular risk than men of the same age. After the menopause, the risk is equalised as the protective factor of oestrogens on the vascular endothelium disappears.
- **Total cholesterol**: The higher the total cholesterol, the higher the risk.
- **LDLc** (low-density lipoprotein cholesterol)**:** There is a causal relationship between the **LDLc value, the length of time** that this cholesterol is elevated, and the risk of suffering cardiovascular disease**.** For **the general population LDLc** values of **50-70mg/dL** are recommended.
- **HDLc:** The lower the HDL cholesterol, the higher the risk. HDL < 40mg/dl in men and < 50mg/dl in women is considered low.
- **Arterial hypertension**
- **Diabetes mellitus**
- **Smoking**
- **Being overweight**
- **Sedentary lifestyle**
- **Diets** high in cholesterol, saturated fats, trans-saturated fats and salt.
- **Other risk factors:**

    - Race**:** higher risk in African-Americans and Hispanics
    - Hyperhomocysteinaemia
    - High levels of apolipoprotein B: Its value provides more complete information on cardiovascular risk than LDLc as it includes other apolipoproteins with atherogenic potential such as VLDLc. In fact, **its measurement** is **recommended instead of LDLc in** patients with diabetes mellitus, metabolic syndrome, obesity, very low levels of LDLc and high levels of TG (TG>300mg/dL).
      (refs: Mach et al, "2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk", Eur. Heart J., 2020, 41, 111-188;
      Sniderman AD et al, "Apolipoprotein B vs Low-Density Lipoprotein Cholesterol and Non-High-Density Lipoprotein Cholesterol as the Primary Measure of Apolipoprotein B Lipoprotein-Related Risk", JAMA Cardiol. 2021, Nov 13)

    - Calcium score in arteries determined by CT scan
    - Presence of atherosclerotic plaques as determined by imaging tests such as

carotid Doppler ultrasound.

- Presence of other concomitant diseases such as: chronic kidney disease, autoimmune diseases, obstructive sleep apnoea syndrome, **psychiatric disease, atrial fibrillation, left ventricular hypertrophy, lipoprotein (a) >50mg/dL** and in general diseases with a chronic state of mild inflammation (**ultra-sensitive CRP> 2).**

CALCULATIONS AND REFERENCE VALUES

How to calculate cholesterol: LDLc: Total Cholesterol – HDLc -TG/5

This formula is valid as long as LDLc is not < 50mg/dL and/or TG is not > 400mg/dL.

Desirable values

- ✓ Total Cholesterol: < 170mg/dL
- ✓ **LDLc: Desirable values vary depending on the patient's risk. Optimal values are 50-70mg/dL but values should be lower in high and very high-risk patients (established CVD, familial hypercholesterolaemia, chronic kidney disease, etc.).**
- ✓ HDLc: >40mg/dL in men and >50mg/dL in women
- ✓ TC/HDLc < 3.5 in people with risk factors.
- ✓ TC/HDL< 4.5 in people with no risk factors.
- ✓ TG<150mg/dl in people with and without risk factors.
- ✓ **ApoB values <80mg/dL are recommended for the general population but, as is the case for LDLc, lower values are recommended in high and very high-risk patients.**

Recommended bibliography:

D'Agostino RB et al. "General cardiovascular risk profile for use in primary care: The Framingham Heart Study. Circulation. 2008, 117:743-753.

Fruchart JC et al. "The Residual Risk Reduction Initiative: a call to action to reduce residual vascular risk in patients with dyslipidemia". Am J Cardiol 2008,102(10 Suppl).

Voight BF, et al. "Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study". Lancet 2012, 380:572-580.

Boekholdt SM et al. "Association of LDL cholesterol, non-HDL cholesterol, and apolipoprotein B levels with risk of cardiovascular events among patients treated with statins". JAMA 2012, 307:1302 - 1309.

Piepoli MF et al. "Update on cardiovascular prevention in clinical practice: A position paper of the European Association of Preventive Cardiology of the European Society of Cardiology". Eur. J. Prev. Cardiol. 2020, (2):181-205

Visseren et al, "2021 ESC Guidelines on cardiovascular disease prevention in clinical practice", Eur. Heart J., 2021, 42, 3227-3337

4

NITROGEN METABOLISM

PRACTICAL SESSION 4:

NITROGEN METABOLISM

1. MEASUREMENT OF URIC ACID:

$$\text{Uric ac.} + 2H_2O + O_2 \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + 2H_2O_2$$

$$2H_2O_2 + 4AF + DCPSQ \xrightarrow{\text{POD}} \text{Quinone} + 4H_2O$$

1.1. Reagents:

- **Reagent**: Phosphate buffer/ 2-4 DCPS/ Ascorbate oxidase/ Uricase/ Peroxidase/ 4-Aminophenazone
- **Uric Acid Standard**: 6.0 mg/dl

1.2. Problem samples:

- Normal serum (Spintrol) Ctrl: to use directly
- Pathological serum (Spintrol) PS: to use directly

1.3. Procedure:

	Blank	Normal serum (Ctrl)	Pathological serum (PS)	Uric acid standard
Reagent	1 ml	1 ml	1 ml	1 ml
Water	25 ul	-	-	-
Normal serum SN	-	25 ul	-	-
Pathological serum SP	-	-	25 ul	-
Uric A. standard	-	-	-	25 ul

- Shake the tubes and wait 10 minutes at room temperature.
- Set to zero with the blank
- At 520 nm record the optical density (absorbance) of the samples and of the standard

1.4. Calculations:

$$\frac{\text{Sample Optical Density}}{\text{Standard Optical Density}} \times \text{Concentration of the Standard} = \text{mg/dl}$$

NOTE: Uric acid should be measured in blood and 24-hour urine samples.

2. DETERMINATION OF UREA:

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + CO3^{2-}$$

$$2NH_4^+ + \text{Salicylate} + \text{Hypochorite} \longrightarrow \text{Indophenolic derivative}$$

2.1. Reagents:

- **Reagent A**: Phosphate buffer/Urease/Sodium salicylate/ Sodium nitroprusside/ EDTA-Na_2
- **Reagent B**: Sodium hypochlorite / Sodium hydroxide
- **Urea standard**: 40 mg/dl

2.2. Problem samples:

- Normal serum (Spintrol) Ctrl : use directly
- Pathological serum (Spintrol) PS: use directly

2.3. Procedure:

	Blank	Normal Serum (Ctrl)	Pathological Serum (PS)	Urea Standard
Reagent A	1 ml	1 ml	1 ml	1 ml
Water	10 ul	-	-	-
Normal Serum SN	-	10 ul	-	-
Pathological Serum SP	-	-	10 ul	-
Urea Standard	-	-	-	10 ul

- Shake the tubes and wait 5 minutes at room temperature,

Reagent B	1 ml	1 ml	1 ml	1 ml

- Shake the tubes again and wait 5 minutes at room temperature.
- Set to zero with the blank
- At 600 nm, record the optical density (absorbance) of the samples and of the standard.

2.4. Calculations:

$$\frac{\text{Sample Absorbance}}{\text{Standard Absorbance}} \times \text{Concentration of the Standard} = \text{mg /dl}$$

3. CREATININE MEASUREMENT:

3.1 Reagents:

- **Reagent mixture** : picric acid / sodium hydroxide
- **Creatinine standard**: 2 mg/dl

3.2. Problem samples:

- Normal serum (Spintrol) Normal Ctrl: use directly
- Pathological serum (Spintrol) PS: use directly

3.3. Procedure:

	Normal serum (Ctrl)	Pathological Serum (PS)	Creatinine Standard
Reagent Mixture	1 ml	1 ml	1 ml
Normal Serum Ctrl	100 ul	-	-
Pathological Serum PS	-	100 ul	-
Creatinine Standard	-	-	100 ul

- Shake the tubes and start the stopwatch.
- Record the Absorbance at 30 seconds (A1) and at 90 seconds (A2).
- At 492 nm, record the optical density of the samples and of the standard.

3.4. Calculations:

$$\frac{(A2 - A1)\ \text{sample}}{(A2 - A1)\ \text{standard}} \times \text{concentration of the standard} = \text{mg dl}$$

BIOCHEMISTRY PRACTICALS: QUESTIONNAIRE FOR

NAME..... PRACTICAL GROUP.....

Calculate the concentration values of the samples studied:

1. URIC ACID:

Control serum:	rng/dl
Problem serum:	rng/dl

2. UREA:

Control serum:	rng/dl
Problem serum:	rng/dl

3. CREATININE:

Control serum:	rng/dl
Problem serum:	rng/dl

4. Importance in Clinical Biochemistry

4.1 Recommended equations for the estimation of glomerular filtration rate based on plasma creatinine concentration.

1. CKD-EPI (Chronic kidney disease epidemiology collaboration)
2. MDRD (Modification of diet in renal disease study)
3. Cockcroft-Gault equation (1976)

Recommended bibliography

Jameson, Fauci, Kasper, Hauser, London and Loscalzo. "Harrison, Principles of Internal Medicine", Vols 1 and 2. Ed, J Larry

Florkowski CM and Chew-Harris HSC, "Methods of Estimating GFR - Different Equations Including CKD-EPI", Clin. Biochem. Rev. 2011, 32: 75-78

Caliceti C et al, "Fructose Intake, Serum Uric Acid, and Cardiometabolic Disorders: A Critical Review", Nutrients, 2017, 9:325.

Kashani K et al, "Creatinine: From physiology to clinical application", Eur. J. Med. 2020, 72:9-14.

Zou F et al (2021), "A review on the fruit components affecting uric acid level and their underlying mechanisms", J Food Biochem, 2021 Oct;45(10):e13911.

5

METABOLIC ADAPTATION TO FASTING: QUANTIFICATION OF KETONE BODIES

Practical session 5: Adaptation to fasting: Quantification of ketone bodies.

1. Objective and introduction

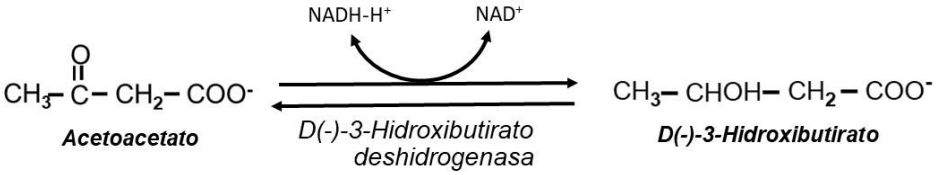
To understand the importance of ketone bodies in adaptation to fasting.

Ketone bodies are produced in the liver. They are water-soluble compounds that are oxidised in the brain, skeletal muscle, heart, kidney and gut. They therefore provide energy and save glucose, which helps to maintain blood glucose levels in times of fasting.

The liver cannot utilise them because it does not produce 3-oxoacid-CoA transferase. Ketone bodies have now also been shown to have functions as regulatory molecules beyond their role as an energy substrate.

2. Method for the quantification of acetoacetate and 3-hydroxybutyrate in plasma using 3-hydroxybutyrate dehydrogenase.

In the presence of NADH, the amount of acetoacetate is proportional to the NADH consumed. In the presence of NAD^+, the amount of 3-hydroxybutyrate is proportional to the NADH formed. The consumption or formation of NADH is measured spectrophotometrically at a wavelength of 340 nm.



2.1. <u>Measurement of acetoacetate</u>

2.1.1. Reagents: 0.1 M phosphate buffer pH 7.0 and 0.05% NADH-H+ 0.05%.

REAGENT 1: 1 part of 0.1M phosphate buffer + 0.05 parts of 0.5% NADH-H+ (in our case we will prepare 4 ml of phosphate buffer and 0.2 ml of 0.5% NADH-H+ solution).

STANDARD: acetoacetate 2.5mM

2.1.2. Procedure

	Blank	Standard	Sample A	Sample B
REAGENT 1 (µL)				
Sample (µL)		10	10	10
Water (µL)		590	590	590

- Shake the cuvettes
- Subsequently, at 340 nm record the absorbance of the standard and the samples (A1).
- Add 5 µL of 3-hydroxybutyrate dehydrogenase
- Shake the cuvettes
- After 30' and at 340nm, record the absorbance of the standard and the samples (A2).

Comentado [GDH1]: No?

2.1.3. Calculations

- Use the absorbance differences (A1-A2). The molar extinction coefficient () of NADH is 6.22 mM-1.cm-1.
- Calculations must be expressed in mM.

Comentado [GDH2]: Falta un símbolo

$$\frac{\Delta \text{Abs}}{\varepsilon} \times Vf \times \frac{1}{Vm} = \mu\text{mol/mL}$$

where: Abs = absorbance difference (E1-E2)

ε = molar extinction coefficient 6.22 mM-1.cm-1

Vf = final volume of the cuvette.

Vm= volume of sample in the cuvette

2.2. Measurement of 3-hydroxybutyrate

2.2.1. **Reagents**: Tris-hydrazine buffer pH 9.5 and NAD+ 1%.

REAGENT 1: Prepare 4.5ml Tris-hydrazine buffer pH 9.5 + 0.5ml NAD+ 1%.

STANDARD: 3-hydroxybutyrate 2.5mM

2.2.2. Procedure

	Blank	Standard	Sample 1	Sample 2
REAGENT 1 (µL)			500	
Sample (µL)		25	25	25
Water (µL)		475	475	475

- Shake the cuvettes
- Subsequently, record the optical density of the standard and the samples (E1) in the spectrophotometer at 340 nm.
- Add 10 µL of 3-hydroxybutyrate dehydrogenase
- Shake the cuvettes
- After 40' and at 340nm, record the optical density of the standard and the samples (E2).

2.2.3. Calculations

- Use the absorbance differences (E2-E1). The molar extinction coefficient (E) of NADH is 6.22 mM-1.cm-1.
- Calculations must be expressed in mM.

$$\frac{\Delta \text{Abs}}{\varepsilon} \times Vf \times \frac{1}{Vm} = \mu\text{mol/mL}$$

where: Abs = absorbance difference (E2-E1)

ε = molar extinction coefficient 6.22 mM-1.cm-1.

Vf = final volume of the cuvetteGood
Vm= volume of sample in the cuvette

Substrates present in the adult blood circulation

	Overnight fasting	4 days of fasting
	mol /mL	mol /mL
LGA	0.42	1.15
TG	1.0	1.0
Glucose	4.7	3.6
Lactate	0.5	0.5
Ketone bodies	0.03	3.0

Plasma concentration of ketone bodies in humans

		mM
Good diet	.....	0.1
Fasting 12-24h	.....	Up to 0.3
Fasting 48-72h	.....	2 - 3
Post-exercise	.....	Up to 2
Late Pregnancy	.....	Up to 1
End of pregnancy + Fasting 48h	.....	4 - 6
Neonate	.....	0.5 - 1
Ketogenic diet.....		Up to 3
Uncontrolled type 1 diabetes (Ketoacidotic coma)		Up to 25

Recommended bibliography

Cahill GF Jr, "Fuel Metabolism in Starvation", Annu Rev. Nutr. 2006, 26:1-22.

Puchalska P and Crawford PA, "Multi-dimensional Roles of Ketone Bodies in Fuel Metabolism, Signaling, and Therapeutics", Cell Metabolism, 2017 Feb 7;25(2):262-284.

6

GENE EXPRESSION ANALYSIS USING PCR

1. INTRODUCTION

1.1 Polymerase Chain Reaction (PCR)

PCR is a technique used to amplify a segment of DNA that lies between two regions of a known sequence. DNA amplification allows the simple detection of such small amounts of DNA that would otherwise be undetectable. Kary Mullis developed this technique in 1985 after observing that the DNA polymerase of a micro-organism living in hot springs (discovered near the Yellowstone geysers), *Thermus aquaticus* or Taq, is capable of synthesising multiple copies of DNA from an initial minimum amount of the order of nanograms. The product of this amplification can then be detected by standard laboratory techniques. The particularity of this enzyme is the fact that it is a thermostable enzyme capable of withstanding the large temperature changes required by the technique without affecting its activity.

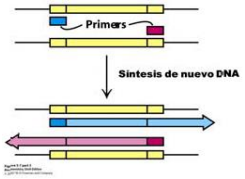
In addition to the DNA polymerase Taq, a PCR reaction requires the presence of several components:

- DNA template or templates of the known sequence in which the region to be amplified or amplicon is located.

- **Buffer**: this provides the appropriate salinity ($ClMg_2$) and pH conditions under which the reaction can proceed.

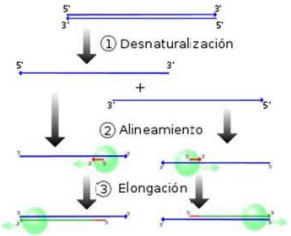
- **dNTPs** (deoxyribonucleotide triphosphates): dATP, dCTP, dGTP, dTTP.

- Primers: from which the polymerase synthesises new strands. Each primer is an oligonucleotide of 20-22 nucleotides whose sequence is complementary to regions flanking the area to be amplified in the template DNA.



During the PCR process, successive PCR cycles will be repeated:

(1) Denaturation - The template DNA is denatured by heating in the presence of a molar excess of the two primers and dNTPs.



(2) Annealing - The reaction mixture is cooled to the alignment, binding or *annealing* temperature, which allows the primers to be matched to their complementary sequences in the template DNA.

(3) Elongation - Starting from the ends of each template first, going in the 5'-3' sense polymerase (Taq) copies the amplicon in the DNA.

The cycle of denaturation, pairing and DNA synthesis is repeated successively, yielding a product that corresponds to a double-stranded DNA segment whose 5' ends are flanked by the primers and whose length is defined by the distance between them.

1.2 Variable reaction parameters

- The appropriate **annealing temperature** for each reaction is determined from the theoretical melting temperature (Tm) calculated for each primer. The Tm of each primer is estimated using the following equation (Itakura et al., 1984):

  Tm (ºC) = **2(A+T) + 4(G+C)**

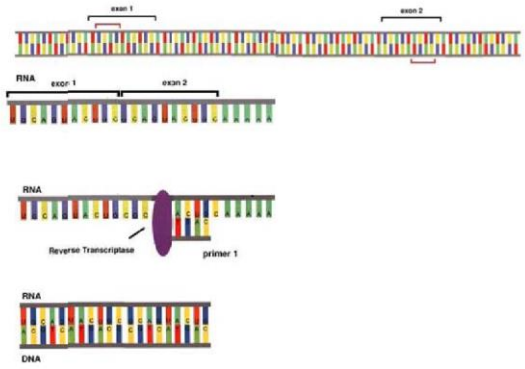
  Where **(A+T)** is the number of nucleotides containing A or T as a nitrogenous base and **(G+C)** is the number of nucleotides containing G or C.
  For oligonucleotides of the same size, the higher the G/C content, the higher the Tm. The reason for this is the number of hydrogen bonds that stabilise this pairing: three, whereas the A/T pairing has only two bonds.

- The **extension time** varies according to the size of the DNA fragment to be amplified (approximately 1 minute per 1 kb).

- The **number of cycles** of the amplification process varies according to the estimated number of copies of template DNA. The more templates, the fewer cycles are required.

1.3 Gene expression analysis using RT-PCR

RT-PCR is used to amplify copies of coding DNA (cDNA) obtained from RNA. It is a very sensitive and versatile technique that can be applied to amplify cDNA as a rapid alternative to cloning, to detect mutations and polymorphisms in transcribed sequences and above all to analyse the expression levels of specific mRNA in different tissues, physiological processes, developmental stages, etc.



- In a first step, the enzyme transcriptase (RT) catalyses the synthesis of a cDNA strand using the mRNA as the starting mole and starting the synthesis at at the site determined by the primer used, one option is to use a poly-T that matches the 3' end of most eukaryotic mRNAs, although it is more efficient to use random to use random banding with a mixture of degenerate hexanucleotides.

- Next, the cDNA of interest is amplified by PCR using primers specific for the sequence to be amplified. The primers chosen to determine the expression levels of a gene must be located in contiguous exons, to avoid possible artefacts due to amplification of contaminated (genomic) DNA in the sample.

1.4 Detection and analysis of RT-PCR products by electrophoresis using horizontal agarose gels

Electrophoresis has become the most widely used technique for the separation and characterisation of nucleic acid molecules. For the separation of DNA fragments from a few hundred to approximately 20,000 base pairs, the most commonly used medium is agarose gel. To prepare the gels, solid agarose is melted in a saline buffer by heating to 80°C and allowed the mixture to gel at temperatures below 45°C. The size of a given fragment (or amplicon) conditions the speed with which it can migrate through the agarose gel: its size can be determined by including a mixture of DNA fragments of known size in the gel and subjecting them to electrophoresis together with the amplification mixture. In order to visualise the amplified DNA, the gel or sample is then stained with a particular intercalating dye, which fluoresces under ultraviolet light.

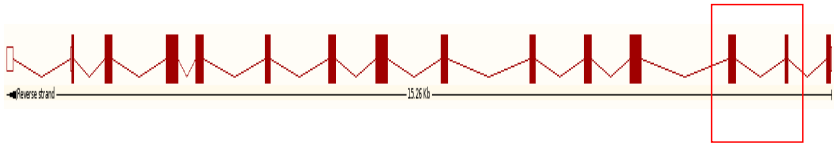
2. PRACTICAL SESSION

In this practical, the expression of two genes subject to tissue-dependent differential regulation mechanisms, *smooth muscle albumin* and *smooth muscle actin,* will be analysed using RT-PCR performed on a liver sample. Although all cells in the body contain the same genes, under physiological conditions, tissue-dependent transcriptional regulation mechanisms allow the exclusive expression of those genes required for the performance of tissue-specific functions. The *albumin* gene is expressed constitutively and abundantly in the liver, whereas expression of the *actin* gene under physiological conditions is restricted to a few tissues. In many cases, a particular pathology causes an alteration in the expression pattern of these genes. In liver cirrhosis, *albumin* expression levels are not altered, whereas *actin* expression is increased as a consequence of the proliferation of fibroblasts and hepatic stellate cells involved in fibrosis.

To measure the expression levels of both genes by RT-PCR, RNA must be extracted from the liver sample and the corresponding reverse transcription undertaken to obtain the cDNA, following the steps described on the previous page. Due to time constraints, in this practical, the student will be provided with the cDNA already obtained after reverse transcription of the RNA sample.

The selected region will then be amplified by PCR. For this purpose, a pair of primers has been designed for each gene whose sequence is underlined over the sequence of the corresponding gene: the introns appear in grey letters and the exons in black letters. In addition, the exonic/intronic structure of the genes is schematically illustrated and the region whose sequence is shown is outlined in boxes. The exons are shown as solid rectangles and introns as the lines connecting them.

RAT ALBUMIN GENE



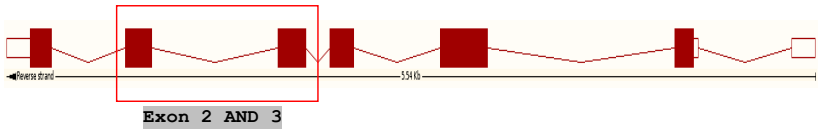
Exon 12 and 13

Exon/Intron

19130994 AATTGGTGCATGCTTTCTATTAAATCATCTCTCTCTTTTCACCACTTCCTTTGAGCTTGT 19130935
GTCA
19130934 CAATGCTGGTATGTGTGTACGTTTGTGTGACAGAGTCTATTATTTAAAGCAGCAGCATGG 19130875
AGTACATTAA
19130874 TACCATCGGGCTAGAGAAACAATACTTCCTTGTGATTCTAGTACAGATTTAAGACTAAGA 19130815
19130814 TCTTACGCTCCAAGTAGTAGTTTTTTTGCATCTACTTGGTTTCTTAGGACAAGGTCTTCC 19130755
TCCAG
19130754 TACTATGCTATCACTCCAGAAGTAAAGATACTTTGATTAGTGGGGGGGAAAAAAATCTTA 19130695
AT
19130694 TGGTTTTTGGGCGAATCTTCTTCTTGAAATATATTACATCAGGCCAGTTTTCTGTCTGGA 19130635
TTAAA
19130634 CCATGTAAGTTATTGTAAGTAATAACTTTTTGTTACACACAGCRTTHTGRTTCGATACAC 19130575
CC
19130574 **AGAAAGCACYTCARGTGTGTCGACCCCAACTYTCGTGGAGGCAGCAAGAAACCTGRGAAR** 19130515
AG
19130514 **TGGGCACCACCAAGTGTTGTAYCCTTCCTGAASCTCARAGACTGCCCTGTGTGTGRAARA** 19130455
CTATG
19130454 TGAGTCTTTTAAACAACATCAAAGTTAACAGGGGACAGGCTGTTGTTGTCCTCAGCAGAC 19130395
CTTGAT
19130394 AAATCTAACTTTCAGGAGCAAGGGGGGGTTCTAATATGTGTGCTTACGTGTGTGTATATA 19130335
GTATCCGG
19130334 GTCATACACCCTCTCTCTCTCCCATATCTAACTAAGATATTATTGAAACAGTTTTTTTAA 19130275
TAAGTCTCACA
19130274 TGTGCAGTTTCAAAGATATATATAAATTACCAAACTGGGCAAGGCTCGCTTCATAGTGGC 19130215
TA
19130214 GTCCAACATGACAGTTTTTTTAGTGCACCGGAGAGCGGATGTTTGTGTGGAATGCTTGTG 19130155
TGACCT
19130154 TCCCTCTCTCCGCTTGTCTTCTTCTTCTTTAKCTGTCTGTCTGCCATCCTRAACCGYCTG 19130095
TGTGTGTGTGCTGC
19130094 **ATRARAAGACCYCAGTRAGCGARAARRTCACCAAGTGCYGTAGTGRRTCCCTGGTRGAAA** 19130035
19130034 **GACGGCCATGTTTCTCTGCTGCTCTCTGACAGTTGACRARACAYATGTCCCCAAARAGTT** 19129975
TAAAG
19129974 **CTRAGACCTTYACCTTCCACTCTRATCTGCACACTCCCADACARRRARAAGCARATAA** 19129915
19129914 **ARAAGCAAACGTGAGGATATATATATTATTCTTTCGCATCTCTCTCTGTTTTTTTTCATA** 19129855
TTGCATATGTATT
19129854 GACAGTGAGAACCGTCAACTGGACAATTTTTCTTTTTTGATGCCGTGGTTAGAGTCCTTC 19129795
19129794 AAGAGAAGTGTTTAGATGCTGGCATCACTAAGTTGAAGAAGAAGTGTGTCAAAGTTCTTT 19129735
TGTCT
19129734 ACGTGAAAATTTTTTTTTTTTGTATTAAAATCTTTCATTCAACTTATTTTTATCACCGTGG 19129675
GTAG
19129674 AAACCACACAGTAGAATACAATTCTAGGGGGGAGGCACAATAGAAAAACCGAAACACTCG 19129615
TTCA
19129614 TGGAAATAGGTAGAAATATATGCTGGTCACGGAAACCATAGGAGTATTTATTTCAAGGTA 19129555
TG
19129554 AGAAACTCACTGAAAATGTTCCACGAAGAAGCCACGCCAGGAGGCTGGACAAGATCGTGG 19129495
G

Forward primer: 5'-TCGATACACACCCAGAAAGC-3' -5'-TCGATACACCCAGAAAGC-3'.
Reverse primer: 5'-GTCAACTGTCAGAGCAGA-3'.

SMOOTH MUSCLE ALPHA ACTIN ALPHA GENE



Exon 2 AND 3

Exon/Intron

99906078 TGGTGAAAATGTGGAGAGAGAGCTTAAAGGAGCTGAAAGGGCCGGATTTCCGTGGTTAGG 99906019
ACCA
99906018 TGGCTCCTTGCGCGTGGCGGCGGGAGGGGGTGTGTGTGTGTGTGTGAGCCTTGGCTAGAA 99905959
GAGAGCAAGGG
99905958 TGGGGGGTCTCACCACCAGCCTTCTTCTCTCACCACCCACACACAGTCAGTCAGTCTCCC 99905899
TGATTCTTCCAAGGCTT
99905898 TTTTGTTTAGGTTTGGTTTTTTTATCTTGAGCATAAGGGAGAGAGTACAGCTGTCTCCTT 99905839
ACTC
99905838 TCTACAATCCAATAACCCCACTGGGCCCTTCAGAAGGAGGCCAGCTCTGCAAGCACTGAG 99905779
99905778 **CATGGACCACTCTTCTTTCCTTAGCCTACAGATCCCACAGAAGCTGTACCAGGATGTGTG** **99905719**
TG
99905718 ACGACGAGGAGAGACCACAGCTTTGGTGTGCGACAATGGCTCCGGACTGGTGAAGGCTGG **99905659**
CT
99905658 TTGCGGGTGATGATGATGCTGCTCCCAGAGAGCTGTCTGTCTTCTTCCCATCCATCCATC **99905599**
GTGGGTCGCCCACGCCACC
99905598 AGGTAACACTCTCCGCTCCTCCTCCTAGCCTGCTCCCTCCCTGTCCATCATTCTAGACCC 99905539
CTTCTGGT
99905538 CACCCTCTCTCAGTCCAGCAATCTGGGAAGTGACTGTCTTGACCCAACTGTTCAGAGAGA 99905479
TCCT
99905478 TTAGGTCATACATGTGAAGCAGAGAGTGAGTTTCAAATCCAAGCCATCCCACGAAACTCC 99905419
CT
99905418 GCGAACCTCCAGATTCCAGGAAGGAAGTCCACTGCAGTGGCTCACCTGTTGATGGGTCAG 99905359
CTCTCT
99905358 CTTGTGAAAGRCCACCAGCTTCTTCTCATTCTGAACCTGTGGGTTTTTTCAACTGCAGTG 99905299
TGTCTTT
99905298 TGTGTCTCTGAAACATGAATTTCTTCTTCTTTTTCCCCTAGGCTCTTTGTCTGTTCAATT 99905239
TAGTT
99905238 TCAACAATACACAGCTAACTATTAGTAATGTAGCTACTTGGAAATAACCAGTACCCAATC 99905179
TA
99905178 CATTCTTCCCCCCCTTGTTCACAGCCAGTTTTCAGAACTCCCCAAAGCTGTAATGGACAC 99905119
TC
99905118 CTTCCTGGGTGCTGCATTTTTTTAGAAGTCAGATGTGTGACTAGAGCAACAAGTGCCACA 99905059
GTGA
99905058 TAGTGGGGCATACCACCACCTTGTTTATTATATTAAAAGAAATGCCACGCTCTCTCTCTG 99904999
GGTTTGTCGCATGC
99904998 TTGGCCAGACAGGCTGCCAGAGAGCACAGAGAGCTGGAGTCCTCAGGCTTGGCCAGTGAA 99904939
GGCT
99904938 ACTCCAGGATAGTAGTCTGAGTGGTTTGACTTTTCAGCTGAGTGATAGCTGCCTATCAGC 99904879
CTA
99904878 TGTACAGTGGATGAGTGTGTGTCAAAACCTTTTCTTAAGGGGACTTAATTAATTAATTCA 99904819
GTTGTGCC
99904818 AAAAGATATGGTCATCTATTTCTTCTTAGGTGACATCAGTGATAAGCTTAGTAATTTAAT 99904759
GAAGT
99904758 CATTATGCTATTATTTCAAGAACAAGATAAGGCTCTGCTTCTTCTTCAGGGAAATCCCTT 99904699
TATTCT
99904698 CTCATCCACAGTTATAATCAAATTAATGCACACACACTTCCTGCTGCTGCGGAGATGAAG 99904639
TATAT
99904638 AACATGAGTCTAAGTAATTCGTTAAAATTAGGATTATGTCTTCACTCCTGGAGGCGGGTC 99904579
99904578 TCACACTATGGCCCAGGCTGGCCTGGCCTGGAACTCGGCTCTACCGACCAGGCTAAATGG 99904519
AAGCT
99904518 GTGACCTGTTCCAGCCTTTTTTGAATGCTGGTTTTTTTTATATAGGCGTGAACCAGCATC 99904459
CCTAACTT
99904458 TCAAATTCTTTTTTTTTACTTTTTTTTCCTCCTAGTATTTTACAGCTCTCTTCACTACCT 99904399
TGAGGATTG
99904398 GATATATATAGCTTAACAAGGCCAGGACTGTTCTTTTTTTTTATTTTTTTTTTTTTGCCT 99904339

TGTTGAGAACTTGA
99904338 TGTTCTCTCATCTTCTTCTCCTTTTTACAGGGTGTGTCATGGTAGGTATGGGGGGCAGAA 99904279
AGACTCCTA
99904278 **TGTAGGTGACGAGGCTCAGAGAGCAAGCGAGGCATCCTGACTCTCTGAAGTACCCCATAG** 99904219
AGAGCA
99904218 **CGGCATTATCACCAACTGGGACGACGACGACATGGAGAAGATCTGGCACCACCACACCTT** 99904159
CTACAATGA
99904158 **GCTCCGTGTGGCCCCCCTGAGGAGCACCCGACCCTGCTGCTCACTGAGGGCCCCACTGAA** 99904099
CCCCAA
99904098 **GGCCAACCGTGAGAAGATGACACACACAGATCATGTTTGAGACCTTCAATGTGCCTGCCA** 99904039
TGTA
99904038 **TGTGGCCATCCAGGCGGTGCTGCTGTCCCTGTACGCTTCTTCTGGGAGAACCACAGGTTT** 99903979
GTTTGTTTGG
99903978 GCTGGGAACAGTCACTGGTTAATCAGTCTCCACTCACTGACTTWGCTGTGAACCCGAATT 99903919
99903918 CTCTCCCGACACACAGAAAAGGTCCTCTCTCCCTAACTCTCTCTCTAGCAAGGTATCTAT 99903859
CTATGCTGAG
99903858 AGAAAGGACTAGCAAATCAGTTTCAGGAACATTTACTGTAAAATCCGATAACCCGTGGAG 99903799
99903798 TTAGATCTTTCCCCTATTATTCAAGATATATGTGAGATGCTACATTCACACAGCCTCAGA 99903739
CATATTC

Forward primer: 5'-TACAGATCCCACAGAAGAAGC-3'
Reverse primer: 5'-TAATGCCGTGCTCTCTATGG-3'.

2.1 Procedure for carrying out PCR

- **Reaction mix:**2xPCR TaqNova-RED (dNTPs mix; MgCl2; TaqNova DNA Polymerase; Loading buffer with dye)

- Prepare the PCR reaction for each gene. Label two small Eppendorf tubes (0.2 ml) and add the following reagents in the following order:

Reagents (x 3)	**PCR *alb***	**PCR *act***
Reaction mixture	25 l	25 l
Forward alb	1 l	----------
Reverse alb	1 l	----------
Forward actin	----------	1 l
Reverse actin	----------	1 l
cDNA	2λ	2λ
H_2O	21λ	21λ
Final volume	50λ	50λ

- Once the tubes have been prepared, place them in a thermal cycler programmed as shown in the table below:

	Temperature	**Time**	**No. Cycles**
Initial Denaturation	95 ºC	2 min	1×
Denaturation **Annealing** **Elongation**	95 ºC 55ºC 72 ºC	30 s 30 s 1 min	25×
Final elongation	72 ºC	7 min	1×

2.2 Analysis of PCR products by agarose gel electrophoresis

- Development of electrophoresis.

  - Remove the comb from the agarose gel and place it in the electrophoresis cuvette by immersing the gel in the buffer.
  - Load the molecular weight marker (8λ) and each sample (15λ) into the wells.
  - Once the loading on the agarose gel is completed, connect the electrophoresis system to a power supply providing a 120 V potential difference.
  - When the front visualised by the migration of bromophenol blue (a component of the loading buffer) runs through two thirds of the gel, interrupt the electrical current by switching off the power supply.
  - Finally, visualise the result and photograph the gel in a UV transilluminator.

3. PRACTICAL QUESTIONS

1. Determine the expected size of the amplicon for the albumin and actin according to the primers that have been selected in this exercise.

2. Determine the Tm for each direction used. Why does the G/C content increase the Tm more than the A/T content?

3. Does the sequence detailed in this booklet for each gene correspond to its cDNA? Explain the answer.

4. Why have primers been chosen in contiguous exons and not in the same exon?

5. Assess the RT-PCR result and interpret it.