Laboratory Exercises in Chemistry and Biochemistry

1st year, Dentistry

SUMMER SEMESTER

Department of Medical Chemistry and Biochemistry

Faculty of Medicine in Pilsen
Charles University

STUDENT´S LABORATORY

A lab coat is required to be worn over your clothes, when working in a lab!

After the work is done, clean your working place so that it is the same as it was in the beginning!

Before you leave the lab, ask laboratory assistant for check of your working place!

Rules of occupational safety

1. Only practising students, specified by the timetable, are right of entry at the practical classes. No admittance of any visitors. Authorized personnel only.
2. Students are required to familiarize with their task. Laboratory coats and working instructions are obligatory. Long hair must be adapted for working with a burner without any risk of injury. Overgarments and bags must be put on the given place.
3. Any leaving is allowed just with a lecturer´s permission.
4. Only prescribed activities are allowed in laboratories. No eating, no drinking, no smoking and no storing food in laboratories. Laboratory equipment is not allowed to use for any other purposes.
5. If there is a leakage of harmful chemicals possible, the extraction must be ensured. Working with fuming substances, substances irritating to the respiratory, toxic gases and vapours, as well as annealing and combustion is allowed to do just in a fume chamber.
6. Students must be careful during the manipulation with a safety bulb pipette filler. Pieces of broken glass must be put in a specific container, label "GLASS".
7. It is possible to pour out only the solvents perfectly miscible with water into the sink. They must be sufficiently diluted (at least 1:10), maximum of 0.5 litre. Aqueous solutions of acids and alkalis must be diluted at least 1:30. Solvents immiscible with water, poisons, acids and alkalis over the given concentration and substances loosing toxic gases and gases irritating to the respiratory must be disposed into the special waste container.
8. An acid is pouring into the water during the dilution of acids, never vice versa.
9. It is forbidden to suck in solution into a pipette per mouth. A safety bulb pipette filler must be used.
10. Spilt acids must be washed by water immediately, if need be neutralized by sodium carbonate. Spilt alkalis must be just washed by water.
11. All burners and electrical current must be switched off due to spilling of flammable liquids and it is necessary to clear the air. Pouring liquids must be absorbed by suitable porous material and it is liquidate in the appropriate way.
12. During the heating of a liquid in a boiling flask superheating must be prevented by using a boiling chip.
13. It is necessary to check all devices before the start of working. Possible faults and defects must be reported to a lecturer or a laboratory technician.
14. Intentional handling with electrical device and substances is forbidden. To switch on a device and to light a burner is allowed by the approval of a lecturer or a laboratory technician.
15. All centrifugation procedures must be controlled by a lecturer or a laboratory technician. Vessels for the centrifugation must be well balanced and the top of the centrifuge must be closed safely during the operation.
16. The gas intake and electrical current must be switch off and clear the air if there is a leakage of gaseous fuels.
17. A lighted burner without supervision is not permited. If there are any problems with a turner, it is necessary to switch off the gas intake and the burner must be regulated.
18. Students are obliged to inform a lecturer of any accident, injury, or in case of ingestion chemicals.
19. Serious breach of rules because of a lack of discipline or ignorance is the reason of leaving the practical classes as an unexcused absence.
20. Students must be informed about classification of toxic, carcinogenic, mutagenic and damaging fertility substances. Safety sheets of particular substances are available in laboratories.
21. Students must be informed about rules of occupational safety with highly toxic substances (label T+) using in laboratories (e.g. mercury, potassium cyanide, ethidium bromide, mercury (II) nitrate).

Program

Name: *Date:*
Group:
Co-worker(s):

Lab 1: Examination of blood I (proteins)

a) Estimation of total protein in blood serum by biuret reaction

Proteins and peptides, similarly to biuret, react with cupric ions in alkaline solutions to form a violet complex suitable for the photometric determination.

What is biuret? How is it formed (draw the equation)?

Task: Determine the concentration of total protein in the blood serum

Pipette into three test tubes:

	sample (sa)	**standard (st)**	**blank (0)**
sample (mL)	0.1	-	-
standard (mL)	-	0.1	-
physiol. solution (mL)	-	-	0.1
biuret reagent (mL)	5.0	5.0	5.0

Mix properly and allow to stand for ***30 min*** at room temperature.

Measure the absorbances of the sample and the standard at ***546 nm*** against the blank.

A_{sample}	
$A_{standard}$	

Calculate the total protein concentration:

$$\text{Total protein concentration (g/L)} = \frac{\mathbf{A\ sample}}{\mathbf{A\ standard}} \times \mathbf{c\ standard}\ (70\ \text{g/L})$$

	Result
	g/L

Reference value of total proteins in the blood:

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

b) Estimation of albumin

Sulphonphthalein dyes as bromocresol purple or bromocresol green yield with albumin in the presence of detergents in a blue-green complex suitable for the photometric determination.

Task: Determine the concentration of albumin in the blood serum

Pipette into three test tubes:

	sample (sa)	**standard (st)**	**blank (0)**
sample (mL)	0.02	-	-
standard (mL)	-	0.02	-
distilled water (mL)	-	-	0.02
reagent (mL)	2.0	2.0	2.0

Mix properly and allow to stand for ***10 min*** at room temperature.

Measure the absorbances of the sample and the standard at ***600 nm*** against the blank.

A_{sample}	
$A_{standard}$	

Calculate the albumin concentration:

Albumin concentration (g/L) = $\frac{\mathbf{A\ sample}}{\mathbf{A\ standard}} \times \mathbf{c\ standard}$ (40 g/L)

	Result
	c_{sample} = g/L

Reference value of albumin in the blood:

Calculate the A/G ratio (albumin/globulin ratio):

$$\mathbf{A/G} = \frac{\mathbf{c\ albumin}}{\mathbf{c\ total\ protein} - \mathbf{c\ albumin}}$$

	Result
	A/G =

*The approximate A/G range is **1.3 – 2.0***

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

c) Estimation of C-reactive protein (CRP) in blood serum

CRP is the classic acute phase protein, one of the first to be recognized. An increase in serum or plasma almost invariably indicates the presence of *inflammation*, most markedly *bacterial infections*. In addition, increased CRP concentrations accompany *tissue necrosis* and *malignancies*, reflecting severity of the disease and the mass of affected tissue. In an acute event, plasma CRP is elevated after 6 h, reach a peak at 48 h and decline with a half time of about 48 h.

CRP is composed of five polypeptide subunits each of 206 amino acid residues, which places CRP in the family of pentraxins, proteins with immune defence properties found in all vertebrates and most invertebrates.CRP is synthesised rapidly in the liver following induction and at the peak of an acute phase response its synthesis may account for as much as 20% of the liver protein synthetic capacity.

The biological functions of CRP are its ability to bind a wide range of endogenous and exogenous substances and then to facilitate their removal from blood and tissues by opsonization (ie. by enhancing the process of phagocytosis or killing by specific lymphocytes). CRP binding to host cells only occurs when the normal structure of the lipid bilayer has been disrupted. On the contrary, the binding to the cell wall in bacteria and other parasites will occur to live, intact organisms. CRP binding may even crosslink some ligands to precipitate them and localize in the tissues.

CRP is determined mostly by an immunoturbidimetric method. The specimen (serum plasma) is incubated in the presence of specific antibodies against human CRP (antiserum, monoclonal antibodies) and the extent of immunoprecipitation is quantified as turbidity at 700 nm.

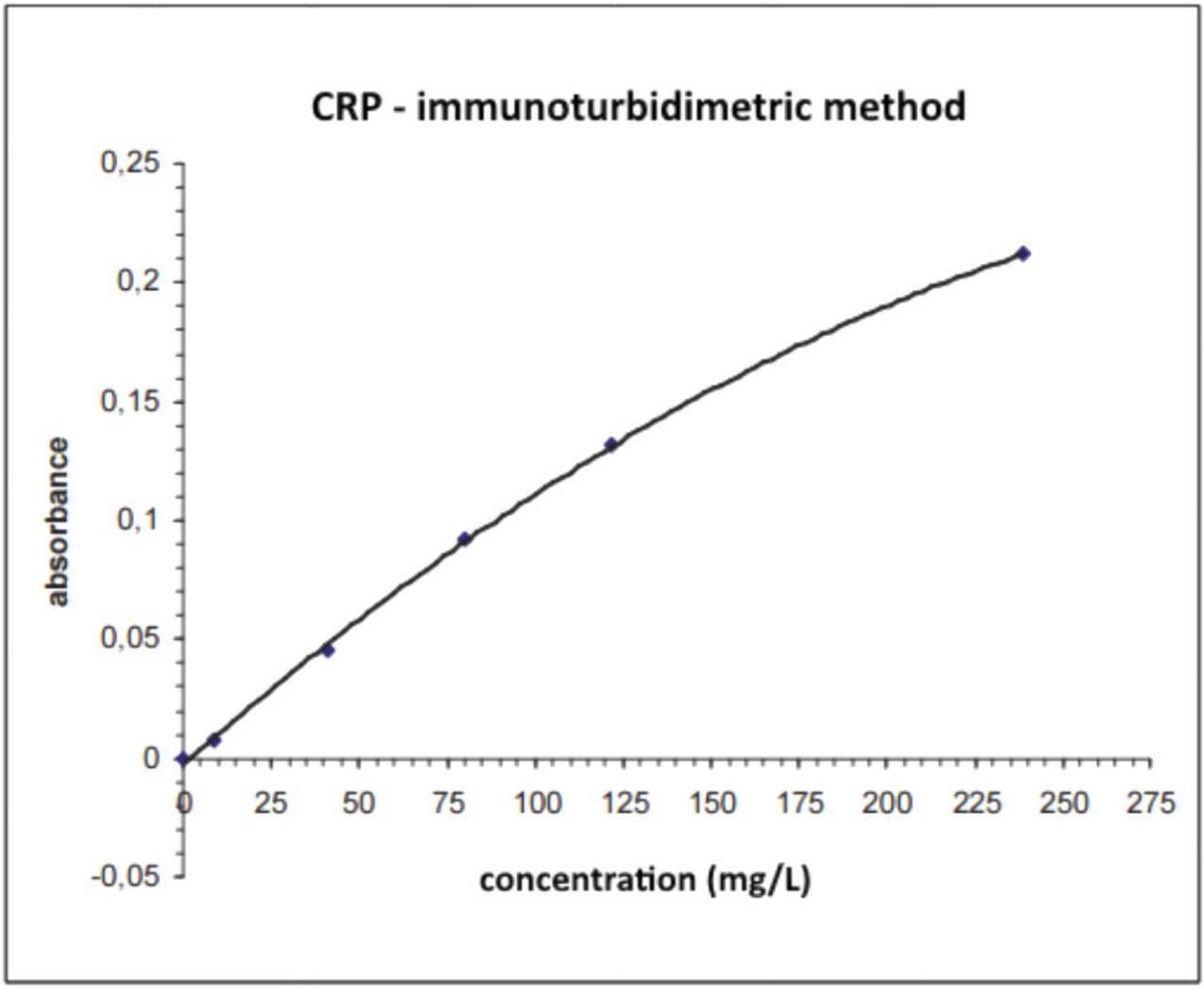
Task: Determine the level of CRP protein in the blood serum

To ***0.05 mL*** of serum add ***2.0 mL*** of antibody solution, mix and allow to stand ***at 37^{o} C for 10 min***.
Read absorbance of a turbid solution against water at ***700 nm***.

A $_{sample}$	

Plot the absorbance value onto the calibration graph and read the concentration of CRP in mg/L. Record the measured CRP value in mg/L and interpret the result.

CRP = mg/L



Reference value of CRP in the blood:

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

Name: *Date:*
Group:
Co-worker(s):

Lab 2: Examination of blood II (glucose,lipids)

a) Estimation of glucose in blood serum

Enzymatic estimation of glucose uses a reagent containing two enzymes and a chromogen. *Glucose oxidase* catalyses the oxidation of glucose to gluconolactone with the formation of hydrogen peroxide as a side product. Hydrogen peroxide produced is determined by oxidative copulation of a substituted phenol with 4-aminophenazone, catalyzed by *peroxidase*, yielding a coloured compound, the amount of which can be measured photometrically.

Draw D-glucose using following types of formulas:

linear form (Fischer projection)	cyclic form (Tollens projection)	cyclic form (Haworth)
D-glucose	*α-D-glucopyranose*	*α-D-glukopyranose*

Task: Determine the concentration of glucose in the blood serum

Pipette into three test tubes:

	sample (sa)	**standard (st)**	**blank (0)**
sample (mL)	0.02	-	-
standard (mL)	-	0.02	-
distilled water (mL)	-	-	0.02
reagent (mL)	2.0	2.0	2.0

Mix properly and incubate ***for 15 min at 37°C***.

Measure the absorbances of the sample and the standard at ***498 nm*** against the blank.

A_{sample}	
$A_{standard}$	

Calculate the concentration:

$$\text{Glucose (mmol/L)} = \frac{\textbf{A sample}}{\textbf{A standard}} \times \mathbf{c_{standard}}\ (10\ \text{mmol/L})$$

	Result
	c_{sample} = mmol/L

Reference value of glucose in the serum:

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

b) Estimation of glucose in capillary blood using glucometer

The Optimum Xceed glucometer uses the electrochemical principle to measure glucose. It is a combination of glucose oxidase reaction and amperometry. There is a narrow capillary on the test strip through which blood is sucked in. Here, glucose oxidation takes place to form hydrogen peroxide. The more glucose in the blood, the more hydrogen peroxide molecules are produced. Hydrogen peroxide is electrolytically decomposed into positive hydrogen cations and negative oxygen anions in the glucometer. Oxygen anions travel to the registration electrode. This creates a stream of negatively charged particles, which can be measured by the glucometer as an electric current. The amount of the current corresponds to the resulting glycemia.

Task: Determine the capillary blood glucose level with a glucometer

Remove the test strip from the foil pouch before sampling. Insert the end of the three black strips into the port. Insert the test strip as far as it will go. The glucometer will turn on automatically. The drop symbol appears on the display. Disinfect and dry your fingertip. Inject with the lancet. Place a drop of blood on the white spot at the end of the test strip. A blood drop is drawn into the test strip. After a few seconds, the display shows the glucose reading. Removing the test strip from the port will turn off the glucometer.

Record the measured glucose value in mmol/L and interpret the result.

Glucose = mmol/L

Reference value of glucose in the whole blood:

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

c) Estimation of total cholesterol

Enzymatic estimation of cholesterol uses a reagent containing three enzymes and a chromogen. Cholesterol esters are hydrolyzed to free cholesterol by *cholesterol esterase*. The free cholesterol produced is oxidized by *cholesterol oxidase* to cholestenone with the simultaneous production of hydrogen peroxide, which in the presence of *peroxidase* allows oxidative copulation of 4-aminoantipyrine with phenol to yield a coloured compound suitable for the photometric determination.

Draw the equation of the cholesterol esterase-catalyzed reaction in the structural formulas.

Task: Determine the concentration of total cholesterol in the blood serum

Pipette into three test tubes:

	sample (sa)	**standard (st)**	**blank (0)**
sample (mL)	0.02	-	-
standard (mL)	-	0.02	-
distilled water (mL)	-	-	0.02
reagent (mL)	2.0	2.0	2.0

Mix properly and incubate for ***20 min at 37°C***.

Measure the absorbances of the sample and the standard at ***498 nm*** against the blank.

A_{sample}	
$A_{standard}$	

Calculate the concentration:

$$\text{Total cholesterol (mmol/L)} = \frac{\textbf{A sample}}{\textbf{A standard}} \times \mathbf{c_{standard}}\ (5.17\ \text{mmol/L})$$

	Result
	c_{sample} = mmol/L

Reference value of total cholesterol in the blood:

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

d) Estimation of triglycerides in blood serum

Enzymatic estimation of triglycerides (triacylglycerols) uses a reagent containing four enzymes and a chromogen. Triglycerides are hydrolyzed to fatty acids and glycerol by a *lipase*. The glycerol produced is phosphorylated by *glycerol kinase* to glycerol-3-phosphate, which is then oxidized by *glycerol-3-phosphate oxidase* to dihydroxyacetone phosphate with the simultaneous production of hydrogen peroxide, which in the presence of *peroxidase* allows oxidative copulation of some chromogens to yield a coloured compound suitable for the photometric determination.

Draw the equation of the lipase-catalyzed reaction in the structural formulas.

Task: Determine the concentration of triglycerides in the blood serum

Pipette into three Eppendorf tubes:

	sample (sa)	**standard (st)**	**blank (0)**
sample (mL)	0.01	-	-
standard (mL)	-	0.01	-
distilled water (mL)	-	-	0.01
reagent (mL)	1.0	1.0	1.0

Mix properly and incubate in the thermoblock ***for 10 min at 37°C***.

Measure the absorbances of the sample and the standard at 546 nm against the blank.

The total volume of the reaction mixtures is rather small, transfer by pipetting all the content from the Eppendorf tube into a special cuvette for low volume samples and make the measurements with cooperation of lab assistant using spectrophotometer suitable for this purpose.

A_{sample}	
$A_{standard}$	

Calculate the concentration:

$$\text{Triglycerides concentration (mmol/L)} = \frac{\mathbf{A\ sample}}{\mathbf{A\ standard}} \times \mathbf{c_{standard}}\ (2.26\ \text{mmol/L})$$

	Result
	c_{sample} = mmol/L

Reference value of triglycerides in the blood:

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

Calculation of LDL cholesterol

Calculation of LDL cholesterol concentration can be done using the Friedewald formula, based on the known values of total cholesterol, HDL cholesterol and triglycerides:

$$\text{LDL cholesterol} = \textit{total cholesterol} - \textit{HDL cholesterol} - \frac{triglycerides}{2.2}$$

Calculate LDL cholesterol level in your sample (use measured levels during practice, you need also to know the level of HDL cholesterol, count with **0.9 mmol/L**).

	Result
	c_{sample} = mmol/L

Reference value of LDL cholesterol in the blood:

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

Atherosclerosis risk assessment

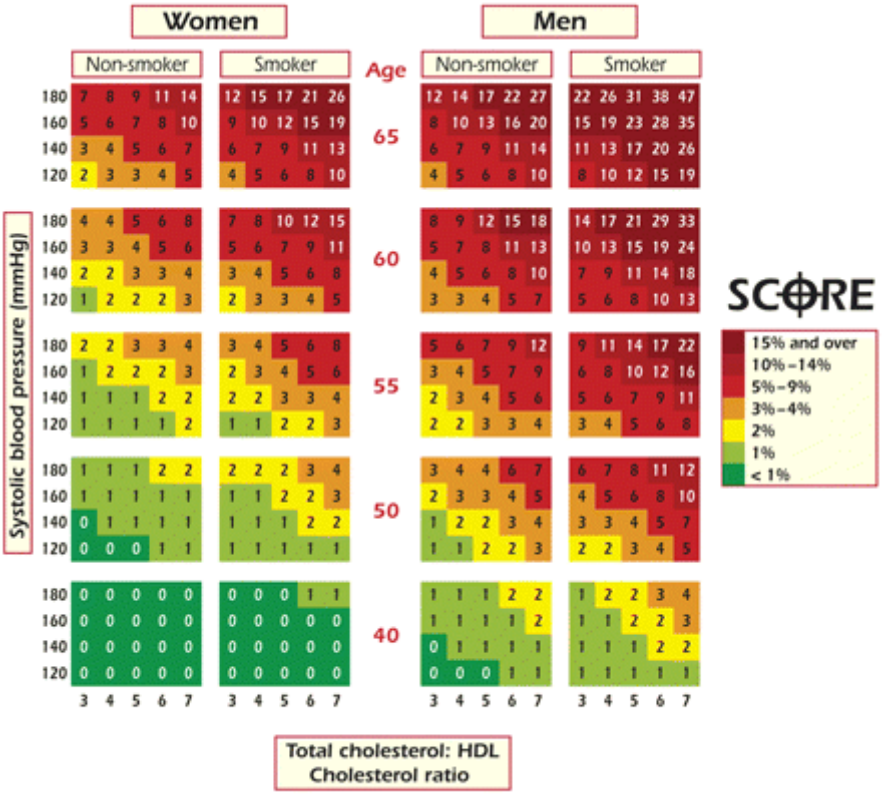
Cardiovascular diseases are a serious concern, accounting for approximately one-third to one-half of all deaths. Parameters of lipid metabolism can be used to calculate some indexes predicting the risc:

Atherogenic index (AI) = $\frac{total\ cholesterol}{HDL\ cholesterol}$

Atherogenic index of plasma (AIP) = log $\frac{triglycerides}{HDL\ cholesterol}$

	Results
	AI **=**
	AIP **=**

There are many other risc factors of atherosclerosis (age, gender, systolic blood pressure, smoking status). In clinical practice, overall risc can be simply predicted using the charts originating in the SCORE project (Systematic COronary Risk Evaluation). The charts show 10-year risk of fatal cardiovascular disease event.



SCORE risc chart (European Society of Cardiology)

Evaluate the risk of atherosclerosis for a model patient (write the data about the patient in the table below). Use cholesterol levels measured during laboratory exercises.

Patient number:	
Gender: male / female	Smoking status: YES / NO
Age years	Blood pressure: mm Hg

Conclusion:

Name: *Date:*
Group:
Co-worker(s):

Lab 3: Examination of blood III (nitrogen compounds)

a) Estimation of urea

Urea is synthesized in the liver from the ammonia produced mostly by the catabolism of amino acids. Kinetic enzymatic estimation of urea uses these reactions:

$$\text{urea} + 2\ H_2O \xrightarrow{\text{urease}} 2\ NH_4^+ + CO_3^{2-}$$

$$\text{2-oxoglutarate} + NH_4^+ + \text{NADH} \xrightarrow{\text{glutamate dehydrogenase}} \text{L-glutamate} + NAD^+ + H_2O$$

Urease hydrolyses urea to ammonia. *Glutamate dehydrogenase* combines the ammonia with 2-oxoglutarate to form glutamate. In this reaction, the NADH is oxidized to NAD^+ and this change is detected photometrically as a decrease in absorbance at 340 nm (Warburg´s optical test).

structural formula of urea

Task: Determine the concentration of urea in the blood serum

Switch the photometer on and let it to heat up for ***10 minutes at 37°C***.
Set up the wavelength to ***340 nm*** and use distilled water to make the blanking.
All the absorbances described further are read against distilled water as a blank.

1) Measuring of blank

There are 3 cuvettes available – one for the blank reaction, one for the standard reaction and one for the sample (blood serum) reaction. First the blank reaction will be performed: Pipette **0.02 mL of distilled water** into the cuvette and **2 mL of the reagent** (working solution). Press the stop-watch at this moment. Pipette the solution inside the cuvette once again up and down to mix it properly. Immediately transfer the cuvette into the heated photometer. Measure the initial absorbance A_1 exactly 30 seconds after the pipetting of the working solution and the second measurement of the absorbance A_2 make after additional 1 minute.

$A_{1\ \text{blank}}$	
$A_{2\ \text{blank}}$	

2) Measuring of standard

Pipette **0.02 ml of the standard solution** into the cuvette and **2 mL of the reagent** (working solution). Press the stop-watch in this moment. Pipette the solution inside the cuvette once again up and down to mix it properly. Immediately transfer the cuvette into the heated photometer.

Measure the initial absorbance A_1 exactly 30 seconds after the pipetting of the "working solution" and the second measurement of the absorbance A_2 make after additional 1 minute.

$A_{1\ standard}$	
$A_{2\ standard}$	

3) Measuring of sample

Pipette **0.02 mL of the serum sample** into the cuvette and **2 mL of the reagent** (working solution). Measure A_1 and A_2 analogically to the previous measurements.

$A_{1\ sample}$	
$A_{2\ sample}$	

Calculation:

Calculate the differences in absorbances:

$\Delta A_{\ blank} = A_{1\ blank} - A_{2\ blank}$	
$\Delta A_{\ standard} = A_{1\ standard} - A_{2\ standard}$	
$\Delta A_{\ sample} = A_{1\ sample} - A_{2\ sample}$	

Calculate the concentration:

$$\text{urea (mmol/L)} = \frac{\mathbf{\Delta A\ sample - \Delta A\ blank}}{\mathbf{\Delta A\ standard - \Delta A\ blank}} \times \mathbf{c_{standard}}\ (15\ \text{mmol/L})$$

	Result
	c_{sample} = mmol/L

Reference value of urea in the blood:

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

b) Estimation of uric acid

Enzymatic estimation of uric uses a reagent containing two enzymes and a chromogen. Uric acid is oxidized by *uricase* to allantoin and hydrogen peroxide.

$$\text{uric acid} + O_2 + 2\ H_2O \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2$$

Hydrogen peroxide in the presence of *peroxidase* allows oxidative copulation of chromogens to yield a coloured compound suitable for the photometric determination.

structural formula of uric acid

Task: Determine the concentration of uric acid in the blood serum

Pipette into three ***Eppendorf tubes***:

	sample (sa)	**standard (st)**	**blank (0)**
reagent (mL)	1.00	1.00	1.00
sample (mL)	0.02	-	-
standard (mL)	-	0.02	-
distilled water (mL)	-	-	0.02

Mix properly and incubate in the thermoblock ***for 2 min at 37°C.***

Measure the absorbances of the sample and the standard at ***550 nm*** against the blank.

The total volume of the reaction mixtures is rather small, transfer by pouring all the content from the Eppendorf tube into a special cuvette for low volume samples and make the measurements with cooperation of lab assistant using spectrophotometer suitable for this purpose.

A_{sample}	
$A_{standard}$	

Calculate the concentration:

$$\text{uric acid } (\mu\text{mol/L}) = \frac{\textbf{A sample}}{\textbf{A standard}} \times \mathbf{c_{standard}}\ (357\ \mu\text{mol/L})$$

	Result
	c_{sample} = μmol/L

Reference value of uric acid in the blood:

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

c) Determination of the total and conjugated bilirubin

Bilirubin forms with diazotized sulfanilic acid an azo-dye suitable for the photometric determination. The total bilirubin (conjugated and unconjugated) reacts in the presence of the accelerator, the direct (conjugated) bilirubin reacts without the accelerator.

structural formula of bilirubin

Task: Determine the concentration the total and conjugated bilirubin in the blood serum

TOTAL BILIRUBIN:

Pipette into test tubes:

	sample (sa)	**blank (0)**
reagent - sulfanilic acid (mL)	0.2	0.2
sodium nitrite ($NaNO_2$)	1 drop	1 drop
accelerator (mL)	1.0	1.0
serum (mL)	0.2	-
physiol. solution (mL)	-	0.2

Mix properly and ***after 10 min*** add into both test tubes **1.0 mL of the buffer solution**.

Measure the absorbance of the sample against the blank at ***580 nm***.

A_{sample}	

Read the total bilirubin concentration from a calibration graph.

$c_{total\ bilirubin}$ = μmol/L

CONJUGATED BILIRUBIN:

Pipette into test tubes:

	sample (sa)	**blank (0)**
reagent - sulfanilic acid (mL)	0.2	0.2
sodium nitrite ($NaNO_2$)	1 drop	1 drop
serum (mL)	0.2	-
physiol. solution (mL)	1.0	1.2

Mix properly, allow to stand ***for 5 min*** and then measure the absorbance of the sample against the blank at ***510 nm***.

A_{sample}	

Read the conjugated bilirubin concentration from a calibrattion graph.

$c_{conjugated\ bilirubin}$ =	μmol/L

Reference value of total bilirubin in the blood:
Reference value of conjugated bilirubin in the blood:
Reference value of unconjugated bilirubin in the blood:

Interpret the results *(compare the results with the reference values above; in case of non-physiological values state possible reasons)*. What type of hyperbilirubinaemia is this?

Conclusion:

Name: *Date:*
Group:
Co-worker(s):

Lab 4: Examination of urine I

a) Physical examination of urine

Task: Perform a physical examination of your own urine

Collect urine into a clean vessel provided. Record colour and odour.

Urine sample description:

Measure the density (specific gravity) with urinometer.
Pour the urine into the cylinder and carefully immerse the urometer so that it does not break on the bottom of the cylinder. The urometer must not touch the bottom or the walls of the cylinder!

Urine density (specific gravity):

Conclusion:

b) Basic chemical examination of urine

(proteins, glucose, ketone bodies, blood and hemoglobin, bilirubin, urobilinogen)

I. Classic test tube tests

To see the positive reaction, please use as "a sample being tested" the imitation of urine positive for the presence of the analyte tested (container labeled with the name of the analyte). Of course, you may try to do the tests with a sample of your own urine. However, being healthy, results must be negative.

Task 1: Perform a basic chemical examination of your own urine by classic test tube tests

Use the *included dropper to measure samples and reagents, prescribed volumes are approximate. Do not confuse droppers to avoid contamination of solutions!*

1. **Proteins**

What is the common principle of all performed tests for proteins in urine?
What is proteinuria?
What is Bence-Jones protein?
What is microalbuminuria?

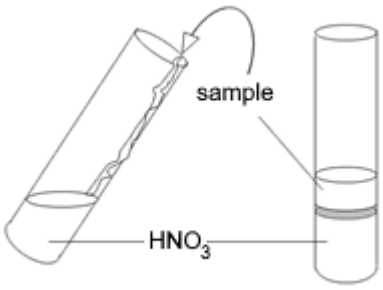
a) Sulfosalicylic acid test

Place about 1 mL of the sample being tested into a test tube and add 5-10 drops of 20% sulfosalicylic acid. A white precipitate is produced if protein is present. This is a very sensitive test.

structural formula of sulfosalicylic acid:

sample being tested	**your own urine**

b) Heller's test *Johann Florian Heller (1813-1871)*



Place carefully about 1 mL of concentrated nitric acid (warning: corrosive !) in a test tube. Incline the tube and slowly pour down the side of the tube in a manner to produce a stratification (two separated layers) about 1 mL of the sample being tested. A white ring appears between the two layers if the test is positive.

sample being tested	**your own urine**

c) Heat coagulation

Place about 1 mL of the sample being tested into a test tube, add about 0.2 mL of acetate buffer (pH=4.6) and heat it in the water bath (75 °C) for 5 minutes. If protein is present, white turbidity appears.

sample being tested	**your own urine**

2. Glucose

What is the common principle of all performed tests for glucose in urine?
What is glycosuria?

a) Fehlings's test *Hermann von Fehling (1812-1885)*

Take a clean test tube and prepare Fehlings's reagent by mixing equal volumes (about 1 mL) of Fehling I (copper(II) sulfate) and Fehling II (NaOH, NaK – tartarate). The reagent prepared is dark blue in colour, without any precipitate inside. Take another test tube and put there about 1 mL of the sample being tested. Add equal volume of Fehlings's reagent prepared in the previous step. Heat the test tube in the water bath (75 °C) for 5 minutes. If the test is positive, reddish brown (orange, olive-green) precipitate is formed.

sample being tested	**your own urine**

b) Benedict's test *Stanley Rossiter Benedict (1884-1936)*

Place about 1 mL of Benedict's reagent in a test tube. Add 4-5 drops of the sample being tested and heat the test tube in the water bath (75 °C) for 5 minutes. If the test is positive, reddish brown (orange, olive-green) precipitate is formed. *(In principle, Benedict's reagent is only a modification of Fehlings's reagent.)*

sample being tested	**your own urine**

c) Nylander's test *Claus Wilhelm Gabriel Nylander (1835-1907)*

Place about 1 mL of the sample being tested into a test tube, add about 1 mL of Nylander's reagent and heat the test tube in the water bath (75 °C) for 5 minutes. If the test is positive, the solution turns grayish-yellow due to formation of black precipitate of metallic bismuth.

sample being tested	**your own urine**

3. Ketone bodies

The term "ketone bodies" refers to 3 compounds. Write their names and draw their structural formulas:

One of the ketone bodies is not a ketone (no KETO group present). Which one?

Is this compound detectable by the tests described below? *YES* *NO*

Ketone bodies are formed if excessive fat metabolism occurs in conjunction with inadequate metabolism of carbohydrates (sugars).
Give an example of disorder when this situation occurs:

a) Lestradet's test *Henri Lestradet (1921-1997)*

Take a small round filter paper and place it unfolded on the white tile. Use a little spoon (it is inside the plastic box with the reagent) to put Lestradet's reagent onto the center of filter paper. Moisten the reagent on the filter paper with a drop of the sample being tested. If purple colour develops within 1 minute, the test is positive.

sample being tested	**your own urine**

b) Legal's test *Emmo Legal (1859-1922)*

Take a clean test tube and disolve few grains of solid sodium nitroprusside in about 1 mL of water. Take another test tube and put there about 5 mL of the sample being tested, add 5 drops of sodium nitroprusside solution prepared in the previous step and 5 drops of 10% NaOH. Red colour appears due to the presence of creatinine. Add few drops of concentrated acetic acid. If ketone bodies are present, the coloration turns to deeper colour.

sample being tested	**your own urine**

4. Blood and hemoglobin

Heitz - Boyer's test
In a test tube combine about 1 mL of the sample being tested with equal volume of the Heitz-Boyer reagent. Carefully overlay with hydrogen peroxide. In the presence of hemoglobin (blood) a red-violet ring appears at the interface of two layers.

sample being tested	your own urine

5. Bilirubin

Naumann's test *Hans Norbert Naumann (1901-1985)*

In a test tube, mix about 5 mL of the sample being tested with talc powder. Prepare what you need for filtration (little funnel, filtrate paper) and filter the mixture to separate talc with bilirubin adsorbed. After the filtration, put a drop of Fouchet's reagent (a solution of $FeCl_3$ and trichloroacetic acid) on the talc on filtration paper. A blue colour indicates that bilirubin is present. This test is more sensitive than the other tests.

sample being tested	your own urine

6. Urobilinogen

Ehrlich's aldehyde test *Paul Ehrlich (1884-1915)*

Place about 1 mL of the sample being tested into a test tube. Add few drops of Ehrlich's aldehyde reagent. A red colour suggests that "Ehrlich positive substances" are present. This is a very sensitive test.

sample being tested	your own urine

Conclusion:

Task 2: Analyze the unknown sample

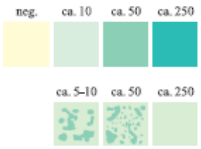
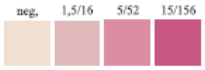
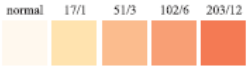
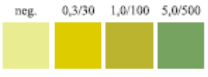
Test the sample for the presence of proteins, glucose, ketone bodies, blood and urobilinogen using the tests listed.

Sample number:	
Substance being tested	**Result**
proteins (Sulfosalicylic acid test)	
glucose (Fehling´s test)	
ketone bodies (Lestradet´s test)	
Blood (Heitz-Boyer´s test)	
Urobilinogen (Ehrlich´s aldehyde test)	
Conclusion:	

II. Diagnostic strips

The principal benefits of the diagnostic strips are simplicity and speed of the testing procedure (results obtained within 1-2 minutes) as well as a high level of diagnostic specificity and sensitivity. The strips are to be used mainly by general practitioners and specialized physicians.

HeptaPHAN

Parameter	Principle of the test	Colour scale
Haemoglobin	oxidation of chromogene by organic hydroperoxide in the presence of the haemoglobine	
Erytrocytes		
Ketones	sodium nitropruside in alkaline buffer (Legal's test)	
Bilirubin	reaction of diazonium salt in acidic surroundings	
Urobilinogen	reaction of diazonium salt in acidic surroundings	
Glucose	enzymatic reaction - glucoseoxidase, peroxidase, chromogene	
Protein	protein error of pH indicator - mixed acido-basic indicator changes colour in the presence of proteins	
pH	mixed acido-basic indicator	

The result can be read by visual comparison with the color scale on the box or by an objective evaluation using an analyzer. The LAURA® Smart system is available for reading the "PHAN" strips. Evaluation of urine analysis with LAURA® Smart eliminates subjective interpretation of the colour response of the diagnostic zones.

Task: Test your own urine with the diagnostic strip

Procedure:

1. Remove the strip from the tube. Do not touch the indication zones on the strips.
2. Close the tube carefully.
3. Immerse the strip in urine (2-3 s) so that all zones are wetted.
4. Remove the strip and the excess of urine remove by wiping the edge about the edge of the container.
5. Dry the edge of the strip (do not wipe the test zones).
6. Evaluate the result – by means of *Laura*® *Smart* analyzer (objective evaluation) or by comparing the tests pads to the corresponding colour scale on the label after approx. 1-2 minutes (visual evaluation).

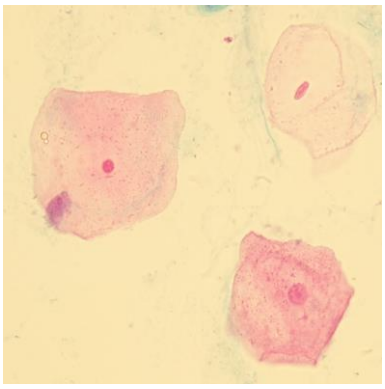
Test your own urine with the heptaPHAN diagnostic strip.
(Laura® Smart can be used for reading the PHAN strips.)

c) Microscopic examination of urinary sediment

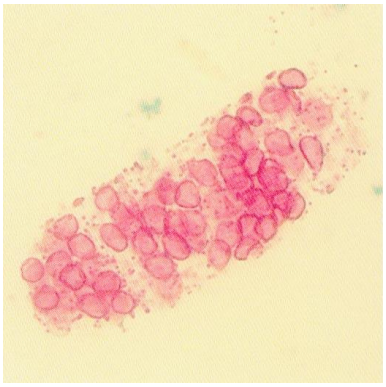
Urinary sediment is prepared by centrifugation of the fresch morning urine. It can be prepared either as native sediment or as stained sediment. The most convenient is the Sternheimer´s supravital cytodiagnostic staining method for urinary sediment.

This staining provides specific colours to cellular elements. In epithelial cells and in leukocytes the nucleus appears dark blue and the cytoplasm pink. Red blood cells appear pink or grey-pink. Hyaline casts appear blue.

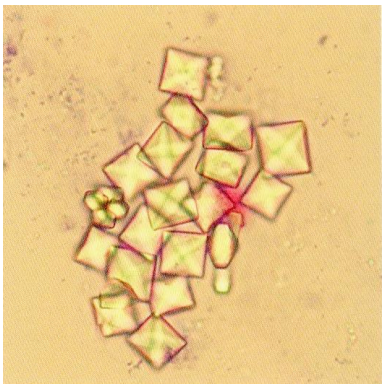
Cells



Casts



Crystals



Task: Perform a microscopic examination of your own urine

Collect your own urine into a clean jar. Mark conical centrifugation tube. Mix the urine and transfer 5 mL into centrifugation tube. The lab.asistent will place the tube into the centrifuge and will perform the centrifugation at 2000 rpm for 5 minutes.

Immediately after centrifugation pour out carefully the content of tube leaving in about 0.5 mL of supernatant. Add 1 drop of working solution from the staining kit and shake thoroughly to mix fully the sediment and the staining solution. Leave 5 – 10 minutes to complete the staining.

Lab.asistent will transfer one drop into the plastic chambre and observe under microscope.

Finding and evaluation:

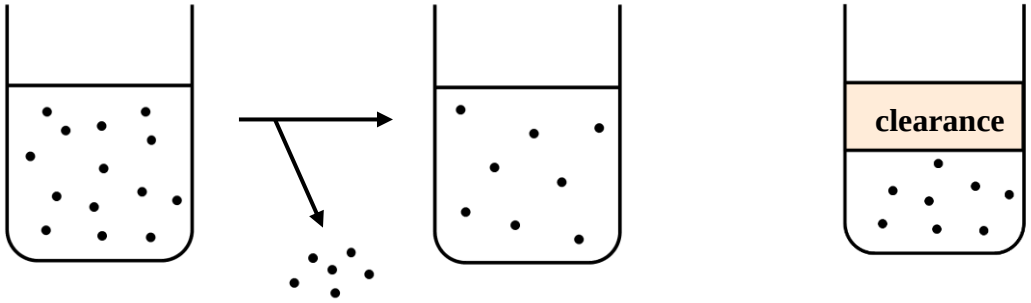
Name: *Date:*
Group:
Co-worker(s):

Lab 5: Examination of urine II

a) Estimation of glomerular filtration rate as a creatinine clearance

Renal function tests allow to assess whether kidney function is physiological or reduced. The basic methods include the determination of *glomerular filtration rate* (GFR). Glomerular filtration is one of the basic processes taking place in the nephron. It can be measured by the excretion of a substance that passes freely into the glomerular filtrate (i.e. at the same concentration as in the blood plasma) and is neither resorbed nor secreted in the tubules.

Clearance indicates the volume of blood plasma that has been completely cleared of a given substance per unit time.



In fact, probably nothing will be completely purified from the substance, only the concentration of the substance will be reduced. The picture on the right shows how to look at it. Assume that the original concentration remains in the part and the part is completely purified. It is this completely purified part that represents the clearance.

Understanding the term clearance is necessary mainly in pharmacology, "cleansing" blood plasma is possible by the action of several organs:

kidneys - excretion of the substance in the urine

liver - removal of the substance by its metabolism

lungs - exhalation

skin - elimination of the substance in sweat

Next, we will deal only with renal clearance. Renal clearance refers to the volume of blood plasma that has been completely cleared of the substance per unit time of renal activity.

glomerular filtration rate = clearance of a substance that passes freely into the glomerular filtrate and is neither resorbed nor secreted in the tubules.

Inulin is such a theoretical ideal substance.

What is inulin?

Inulin is not normally found in the human body, examination using inulin would be very complicated. In practice, it is possible to measure glomerular filtration using the clearance of the endogenous filtration marker - creatinine. Even so, the method is quite complex, the main limiting factor is the correct collection of urine.

structural formula of creatinine

Traditionally, creatinine clearance is calculated from 24-h urine collection, although shorter collection times may be appropriate in certain clinical situations.
For the clearance calculation. Plasma and urine creatinine concentrations, and volume of urine per sec should be determined. The chemical estimation is based on Jaffe´s test, *i.e*. on photometric measurement of the orange coloured reaction product of creatinine with alkaline picrate.

Task: Determine creatinine clearance

Collect your own urine into a clean jar.
Dilute urine sample 100×.
Pipette 1 mL of urine into volumetric flask of a volume 100 mL and add distilled water to the final volume of 100 mL.

Why do we dilute urine?

Substances that are freely filtered in the glomerulus and are subsequently not subject to either tubular resorption or tubular secretion (we assume that creatinine behaves this way) have a concentration in the definitive urine roughly 100 times greater than in the glomerular filtrate (the concentration in the glomerular filtrate is close to in blood plasma).

In photometric determination, when we process the sample and the standard in parallel, it is necessary that the concentration of the sample is not too far from the concentration of the standard. Most often, a standard with a concentration comparable to the expected concentration of the sample is used. Another possibility is diluting the sample so that after dilution we reach the concentration of the standard. From the measured concentration of the diluted sample and the known dilution, we can easily calculate the concentration of the original (undiluted) sample we are interested in.

In our case, we will use blood serum with a known concentration of creatinine (177 μmol/l) as a standard. To determine creatinine clearance, we must measure its concentration both in the blood plasma (serum) sample of the examined patient and in the average sample for 24-hour urine collection. The expected concentration of creatinine in serum is comparable to the concentration of the standard, but this is not the case for urine, the expected concentration in urine is 100 times higher, so we have to dilute the urine sample before measurement.

Pipette according to the following scheme:

- **serum sample** and **standard** into **centrifuge tubes**
- urine sample and blank sample into ordinary thin-walled tubes

Blood serum and urine are completely different biological matrices, so they will be processed in a different way – it is necessary to remove proteins from serum by precipitation with trichloroacetic acid. Without this step, proteins would interfere with the creatinine determination.

	centrifuge test tubes		ordinary test tubes	
	serum	**creatinin standard**	**urine**	**blank**
serum (mL)	0.5	-	-	-
creatinine standard (mL)	-	0.5	-	-
diluted urine (mL)	-	-	0.5	-
distilled water (mL)	1.0	1.0	0.25	0.75
trichloracetic acid (mL)	0.5	0.5	0.25	0.25
Mix properly each test tube and allow to stand for 5 min, centrifuge 10 min at 3000 rpm, take supernetant fluid.			-	-
Supernatant (mL)	1.0	1.0	-	-
Picric acid (mL)	0.5	0.5	0.5	0.5
NaOH (mL)	0.5	0.5	0.5	0.5
Mix properly, allow to stand for 20 min, then measure against the blank at 505 nm.				

A_{serum}	
$A_{standard}$	
$A_{diluted\ urine}$	

Serum creatinine: c_{st} = 177 µmol/L

$$S_{creatinine} = c_{standard} \times \frac{A_{serum}}{A_{standard}}$$

Diluted urine creatinine:

$$U_{diluted\ urine} = c_{standard} \times \frac{A_{diluted\ urine}}{A_{standard}}$$

Non diluted urine creatinine:

$$U_{creatinine} = 50 \times U_{diluted\ urine}$$

Note: The urine was diluted 100 times before the determination of creatinine, but tubes 1 and 2 (where the matrix was serum) were processed differently than the tube with diluted urine, after centrifugation only half of the initial amount was further processed!

Creatinine clearance: *V = 1.78 L/day*

$$GF \approx C_{creatinine} = \frac{U_{creatinine}}{S_{creatinine}} \times V$$

Reference value of creatinine in the blood:	
Reference value of creatinine in the first morning urine:	♂ 3.5 – 24.6 mmol/l ♀ 2.6 – 20.0 mmol/l
Reference value of creatinine clearance rate:	

Conclusion *(compare the results with the reference values above; in case of non-physiological values state possible reasons)*:

Performing in practical exercises does not correspond to the examination of one given patient, even if you work with your own urine, the calculated glomerular filtration says nothing about the function of your kidneys !!!

Think about how you proceeded:

$U_{creatinine}$	own urine – single random specimen
S_{kr}	fetal bovine serum
V	24-hour urine collection was not performed, the value for the calculation is given in advance

Instructions for performing the test (cited by: Department of Clinical Biochemistry and Hematology, University Hospital Pilsen):

3 days before and during the test omit meat, meat products, drugs - if clinically possible. Avoid physical exertion.

On the day of the test, receive the average amount of fluids; the patient must not drink too much, but also must not be thirsty. Do not administer substances with a diuretic effect (diuretics, coffee, tea).

We provide 24-hour urine collection. Urine does not need to be preserved, any preservation does not matter. Send 5 mL of an average urine sample to the estimation of creatinine level ($U_{creatinine}$) to the laboratory. On order form, mark the volume of urine/24 h measured in a collecting vessel to the nearest 100 mL. At the same time in the morning, at the end of the collection period, to take fasting venous blood to determine the level of creatinine ($S_{creatinine}$).

The calculation refers to an ideal body surface area of 1.73 m^2; the patient's weight and height must be reported to calculate the actual body surface area.

The biggest source of error is urine collection!

Therefore, in current practice, glomerular filtration is preferred to be estimated from parameters whose determination does not require urine collection – *estimated glomerular filtration rate* (***eGFR***). There are many formulas for calculation of eGFR:

Historical:

Cockcroft and Gault (1973)

MDRD (2005)

CKD-EPI Creatinine (2009)

CKD-EPI Cystatin C (2012)

Currently reccomended:

CKD-EPI Creatinine (2021)

CKD-EPI Creatinine-Cystatin C (2021)

b) Amino acids and their metabolites in urine

Amino acids are low molecular weight substances (i.e. they have a low relative molecular weight), so they are freely filtered in the glomeruli, but like glucose they are actively resorbed in the proximal tubule. Therefore, they are not normally present in urine at all, physiological excretion is minimal. The excretion of amino acids in the urine is called ***aminoaciduria***.

Renal aminoaciduria results from the involvement of the renal tubules, which are unable to resorb all amino acids from the glomerular filtrate even at their normal level in the blood. There are also genetic defects in specific reabsorption systems.

In ***overflow aminoaciduria***, the supply of one or more amino acids exceeds the resorption capacity of the renal tubules, i.e. the kidneys workinfg well, the problem is in the increased level of some amino acid in the blood (same principle as in the case of glucose in the urine of diabetics).

Task: Perform amino acid testing in your own urine

Use a model urine sample to determine amino acids in urine. It contains the given amino acid, so the result will be positive. Use your own urine as a negative control.

1. Cystine

Cystine is reduced by alkaline cyanide to cysteine which gives a magenta colour with nitroprusside.

Elevated urinary cystine levels are typical of **cystinuria**. Give a brief description of this disease:	*structural formula of cystine*

Place a small amount of thr powder reagent *(sodium nitroprusside , ammonium sulphate, sodium carbonate, sodium cyanide – Caution, poisson!)* onto a glass or porcelainebase. Add a drop of urine. The colour reaction of cystine appears immediatelly.

sample being tested	**your own urine**

2. Phenylpyruvate

Urine in phenylketonuria containing phenylpyruvate gives with ferric chloride in acid a well-marked green colour.

Elevated urinary phenylpyruvate levels are typical of **phenylketonuria**. Give a brief description of this disease:	*structural formula of phenylpyruvate*

To about 2 mL of urine add 2 drops of 10% HCl and several drops of $FeCl_3$. In the presence of phenylpyruvate a deep green colour appears.

sample being tested	**your own urine**

3. Tyrosine

Elevated urinary tyrosine levels are typical of **tyrosinemia** (there are several types). Give a brief description of this disease:	*structural formula of tyrosine*

Tyrosin react with Millon´s reagent (mercury in fuming HNO_3) upon red colour formation.

sample being tested	**your own urine**

Conclusion:

Name: *Date:*
Group:
Co-worker(s):

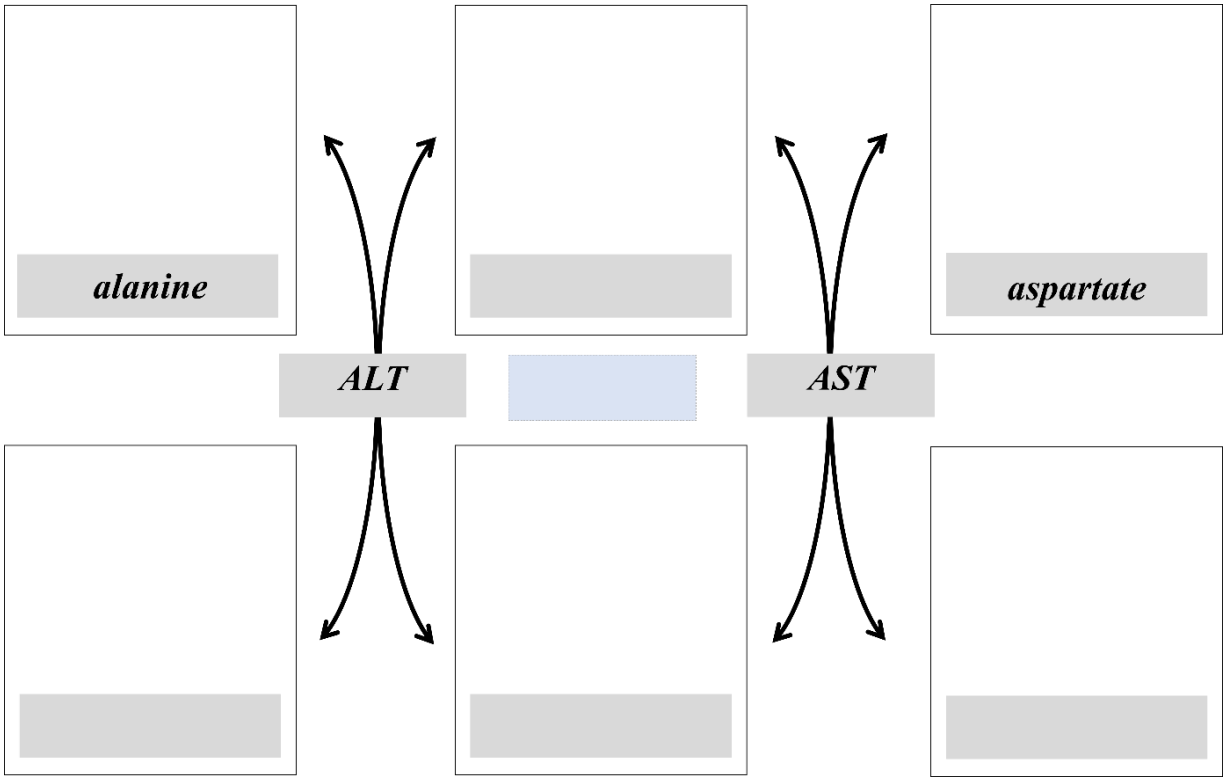
Lab 6: Clinical enzymology

a) Estimation of ALT and AST activity in liver

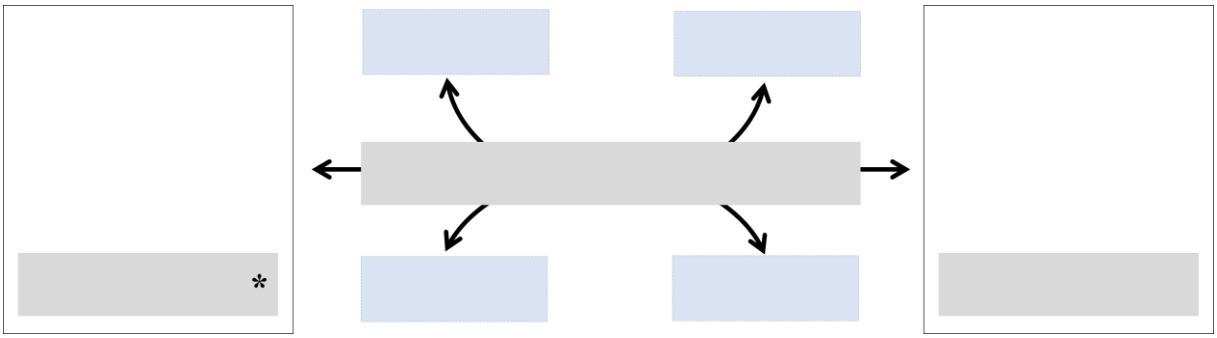
ALT and AST are enzymes that catalyze transamination reactions, they are among the most determined enzymes in clinical practice. In our experiment, we will not measure their catalytic concentration in blood serum, we will only demonstrate their presence in liver tissue (hepatocytes).

ALT =
AST =

Write the equations of the reactions in the structural formulas:



By this mechanism (indirect deamination = transamination), most amino acids are removed from the nitrogen during degradation. Only one single amino acid is freed of nitrogen directly in the form of ammonia (direct deamination = oxidative deamination). It is precisely this that arises from "nitrogen deposition" during transamination (marked * in the diagrams).



Both of these enzymes are intracellular and appear in the blood serum only ín very low concentrations.

ALT is found mostly in the liver, it is localized only in the cytoplasm. The determination of ALT in blood serum is a sensitive and relatively specific test for liver damage. The activity in the serum increases even with a small damage of hepatocytes, only the disruption of the permeability of their cell membrane is enough.

AST occurs in a number of organs - in the **liver, myocardium, skeletal muscle**, but also in erythrocytes. AST is present in mitochondria (70%) and in the cytoplasm (30%). The mitochondrial fraction is released only in serious damage of the cell (cell necrosis). A marked increase in serum AST activity is therefore a sign of more severe damage. AST is not only specific for liver tissue, its increase can also be caused by damage to skeletal muscle or myocardium.

Reference value of ALT in the blood serum:
Reference value of AST in the blood serum:
What is the De Ritis ratio used for?

The estimation is based on a different light absorption 2,4-dinitrophenylhydrazine of 2-oxoglutarate and pyruvate acid that originates directly for reaction of ALT, after spontaneous decarboxylation of oxalacetate for reaction of AST. The activity of aminotransferases is established from a calibration curve.

Task: Demonstrate the presence of aminotransferases in liver cells

Pippete solutions into ***4 test tubes*** according to the table:

Test tubes	**1**	**2**	**3**	**4**
	ALT		**AST**	
	sample	*blank*	*sample*	*blank*
substrate for ALT (mL)	0.50	0.50	-	-
substrate for AST (mL)	-	-	0.50	0.50

Put the test tubes into ***37°C*** water bath for minimally ***10 minutes***.

Preparing of the cell extract:

Crush approximately 1 cm^3 of liver tissue in a mortar, add ***5 mL of phosphate buffer*** and continue in homogenisation. All homogenate transfere to a centrifugation tube, centrifugate ***5 min/2,000 rpm*** (must do the lab technician). The supernatant is our enzyme solution.

Add enzyme solution into the ***sample tubes***:

enzyme solution (mL)	0.10	-	0.10	-

Put all 4 test tubes into the water bath at ***37°C*** for ***30 minutes*** and then add the solution of dinitrophenylhydrazine:

Dinitrophenylhydrazine (mL)	0.50	0.50	0.50	0.50

Mix the tubes and let them for ***15 minutes*** at room temperature.

Add the solution of sodium hydroxide ***NaOH*** into ***all test tubes***:

NaOH (mL)	5.00	5.00	5.00	5.00

Add enzyme solution into the ***blank tubes***:

enzyme solution (mL)	-	0.10	-	0.10

Mix the tubes and wait for ***5 minutes***. Measure absorbance of test tube No. 1 against No. 2 (it corresponds to the activity of ALT) and absorbance of test tube No. 3 against No. 4 (it corresponds to the activity of AST) at ***506nm***.

A_{ALT}	
A_{AST}	

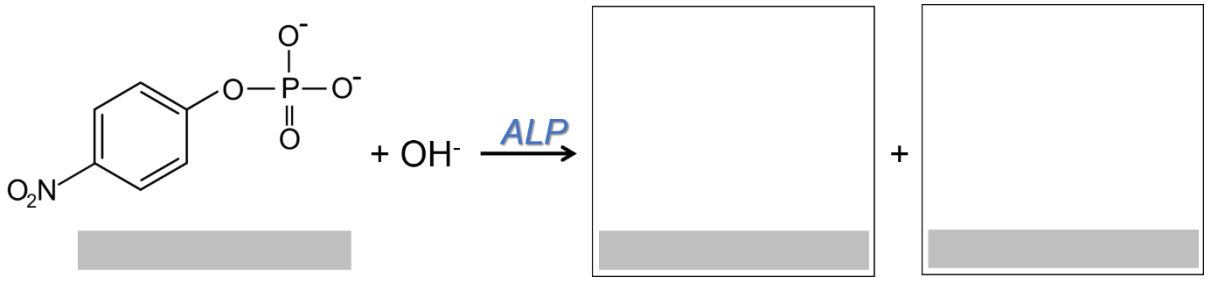
Evaluation, conclusion:

b) Estimation of alkaline phosphatase (ALP) in blood serum

Alkaline phosphatase (ALP) is an enzyme that catalyzes the hydrolysis of phosphate esters in an alkaline environment (pH optimum 9.5–10.5). Alkaline phosphatase is located in the cytoplasmic membranes of bile duct epithelial cells, liver, bones, intestines, placenta. Increased ALP is characteristic for the liver diseases connected with cholestasis and bone diseases. Higher levels of this enzyme are physiologically found in children.

The determination of ALP activity is based on the cleavage of a synthetic substrate into a colour product, which allows direct photometric measurement.

Add structural formulas and names of reactants to the reaction scheme!



Task: Determine the activity of alkaline phosphatase in the blood serum

Pipette solutions into ***two test tubes*** according to the table:

- It is necessary to work very attentively. The serum must be pipetted to the bottom of the tube, it must not stay on the wall of the tube!

	Test tube No. 1 ***sample***	**Test tube No. 2** ***blank***
buffer (mL)	1.00	1.00
serum (mL)	0.02	-

Mix and put both into a water bath at ***37ºC*** for ***5 minutes:***

Add substrate to both tubes:

substrate (mL)	0.20	0.20

Put tubes into a water bath at ***37ºC*** for ***10 minutes*** exactly and then stop the reaction by adding the inhibition solution:

inhibitor (mL)	0.50	0.50

Add just to the tube ***No. 2*** (blank) ***0.02 mL of serum***:

serum (mL)	-	0.02

- Both tubes contain the same solutions. The serum was added to the tube No. 2 (blank) after adding the inhibition solution. The reaction can run only in the tube No. 1. The more enzyme is presented in the serum the more substrate is cleaved and the more colored product can be obtained.

Mix the tubes and measure the absorbance (A) of the sample (tube No. 1) against the **blank** (tube No. 2) at ***420nm***.

A	

Calculation:

Calculation of the catalytic concentration of ALP in an examinated serum.

ALP (μkat/L) = A × 10.236

ALP	μkat/l

Reference value of ALP in the blood serum:

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

c) Estimation of lactate dehydrogenase activity in blood serum

Lactate dehydrogenase (LD) catalyses the reversible interconversion of pyruvate to lactate with oxidation of NADH + H^+ to NAD^+. The rate of absorbance decreses of reduced form of nicotinamide adenine dinucleotide coenzyme at 340 nm is used for kinetic estimation of enzyme activity.

Draw the lactate dehydrogenase reaction equation in the structural formulas. Mark the reactant that absorbance is measured.

Task: Determine the activity of lactate dehydrogenase in the blood serum

Switch on photometer (heating at 37˚C) and adjust the wavelength ***340 nm*** *(according to the enclosed instructions).*

Put a glass cuvette for measuring ***LD*** into the photometer.

Pipette ***1000 µL*** of ***destilled water (blank)*** and measure ***A = 0.000.***

Empty a glass cuvette and put it back into the photometer.

Estimation of standard activity:

Pipette ***20 µL of standard*** and ***1000 µL of „working solution“,*** start up stopwatch, mix the solution and insert it into the cuvette immediately. Measure absorbance at the end of ***2nd***, ***3rd***, ***4th*** and ***5th minute***. Then wash the cuvette with distilled water.

Estimation of sample activity:

Pipette ***20 µL of sample*** and ***1000 µL of „working solution“,*** start up stopwatch, mix the solution and insert it into the cuvette immediately. Measure absorbance at the end of ***2nd, 3rd, 4th*** and ***5th minute***. Then wash the cuvette with distilled water.

	standard	sample
A_2		
A_3		
A_4		
A_5		

Enter the obtained values into a table in the computer *(LD.xls*).

Insert the obtained graph here.

LD	μkat/L

Reference value of LD in the blood serum:

Conclusion *(compare the result with the reference value above; in case of non-physiological value state possible reasons)*:

Name: *Date:*
Group:
Co-worker(s):

Lab 7: Molecular biology I

Task: Examine the presence of the Leiden mutation in a sample of your own DNA

The block of laboratory exercises in molecular biology deal with the detection of the Leiden mutation.

What is the Leiden mutation *(what type of mutation is it, what will it cause)*?

a) DNA isolation *(from buccal swabs)*

You must first obtain the own DNA needed to detect the Leiden mutation.

Students who provide a sample for DNA isolation should not drink hot beverages and alcohol before collection, and should avoid using mouthwash. This significantly reduces the DNA yield.

MACHEREY-NAGEL isolation kit
Nucleic acids can be isolated from any biological material containing cells with conserved nuclei. If material for DNA isolation in a non-invasive manner is to be obtained, buccal mucosa sampling is used.
In laboratory exercise, we use the column method, which works on the principle of ion exchange chromatography. DNA molecules carry a negative charge. In the presence of a high concentration of so-called chaotropic salts, the nucleic acids from the cell lysate bind to the silicate, while most of the contaminants flow through the column. After washing the column, the pure DNA is eluted in a suitable buffer.

Procedure:

1. Gently rub and rotate swab along the inside of the cheek (both left and right side), ensuring that the entire swab tip has made contact with the cheek, **approx. 2-3 min**. During removing the swab from mouth, be careful not to touch swab tip against teeth, lips, or other surface.
2. Put the swab tip into **1.5 mL eppendorf tube** and break the wooden stem so that the part with cotton stays in the eppendorf tube.
3. Add **100 µL PBS**, **15 µL proteinase K**, **100 µL Buffer B3**. Vortex vigorously for **60 s**.
4. Put the tube into thermoblock and incubate at **56°C** for **10 min**. Vortex for **30 s**.
5. Put the tube into thermoblock and incubate at **70°C** for **5 min**. Vortex for **30 s**.
6. Add **100 µL 96% ethanol** and vortex for **10s**. Spin down shortly.
7. Load the **lysate** (without cotton part of the forensic swab) onto the **NucleoSpin® Column**. Centrifuge **1 min** at **12,000 RPM**. Discard Collection Tube with flow-through.
8. Place the **NucleoSpin® Column** into a new Collection Tube and add **400 µL Buffer BW**. Centrifuge **1 min** at **12,000 RPM**. Discard Collection Tube with flow-through.
9. Place the **NucleoSpin® Column** into a new Collection Tube and add **400 µL Buffer B5.** Centrifuge **1 min** at **12,000 RPM**. Discard the flow-through and reuse Collection Tube.
10. Place the **NucleoSpin® Column** back into the Collection Tube and centrifuge **3 min** at **12,000 RPM**.
11. Place the **NucleoSpin® Column** in a new 1.5 mL eppendorf tube and add carefully **50 µL preheated Buffer BE** (**70°C**). Dispense the buffer directly onto the silica membrane! Incubate at room temperature for **1 min**.
12. Centrifuge **1 min** at **12,000 RPM**.

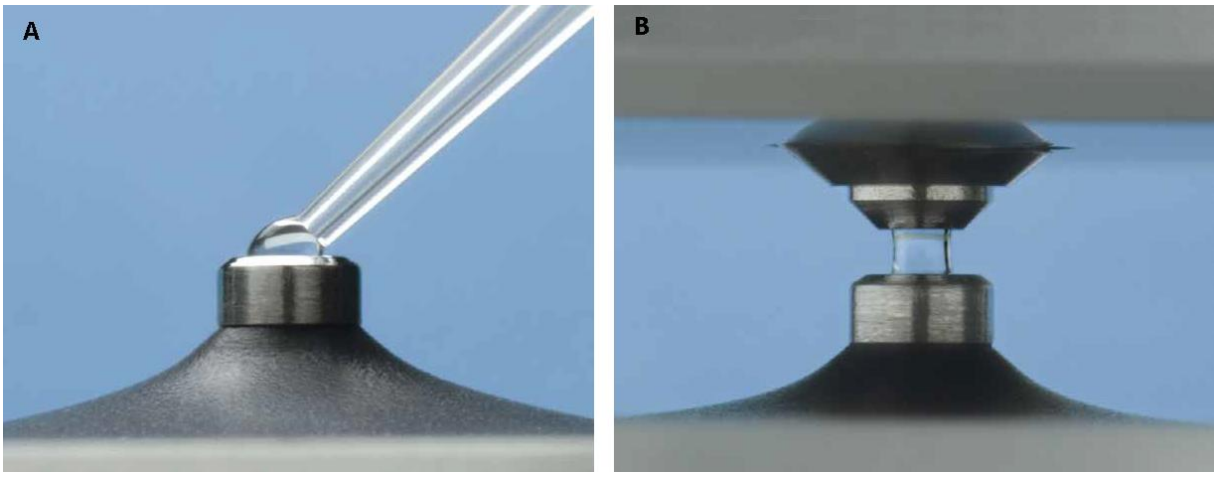
b) Assessment of DNA concentration and purity

There are several methods to determine the concentration of nucleic acids as well as their purity - spectrophotometry (absorbance measurement), agarose gel electrophoresis, fluorometry using fluorescent DNA-binding dyes.
In our practical classes, we will use spectrophotometric estimation. Nucleic acids absorb ultraviolet light with an absorption maximum at 260 nm, proteins at 280 nm. Low molecular weight substances (e.g. phenol, chloroform, EDTA, polysaccharides ...) have its absorption maximum at 230 nm. Absorbance at 320 nm indicates the presence of undissolved solid particles or a contaminated cuvette.
The nucleic acid concentration is calculated from the absorbance measured at 260 nm.
A_{260} = 1 corresponds to:

- double stranded DNA (dsDNA) at concentration 50 µg/mL
- single stranded DNA (ssDNA) at concentration 37 µg/mL
- RNA at concentration 40 µg/mL

The ratios A_{260}/A_{280} a A_{260}/A_{230} is used to assess the purity of nucleic acids. The ratio A_{260}/A_{280} should be for pure DNA around 1.8, for pure RNA around 2. Lower A_{260}/A_{280} values may indicate protein contamination. The ratio A_{260}/A_{230} should be for pure DNA higher than 2.0. Lower A_{260}/A_{230} values indicate contamination with salts or solvents, such as phenol. Residual chemical contamination from nucleic acids extraction procedures may result an overestimation of the nucleic acid concentration and/or negatively influence downstream analysis. If the required purity is not met, reprecipitation of the sample is required, resulting in a significant reduction in the impurity content.

Procedure:
DeNovix DS-11 microvolume Spectrometer - apply 1 µL of sample.



Pipetting sample solution onto pedestal *Measurement*

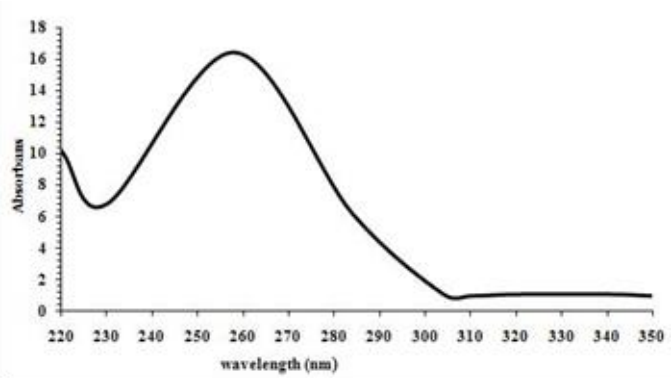
1. Select the program: "dsDNA" on the spectrophotometer.
2. Set the Spectrophotometer against the blank solution ("blank") - in our case BE Buffer:

Ensure both top and bottom sample surfaces are clean. Pipette 1 µL of BE Buffer onto the lower sample surface, lower the top arm and press the BLANK button. Then open the lid, remove the solution from both sample surfaces using a clean, dry lab wipe.

3. Measure the absorption spectrum of the sample within the range 220 nm – 350 nm:

Pipette 1 µL of sample onto the lower sample surface, lower the top arm and press the MEASURE button. Then open the lid, remove the solution from both sample surfaces using a clean, dry lab wipe.

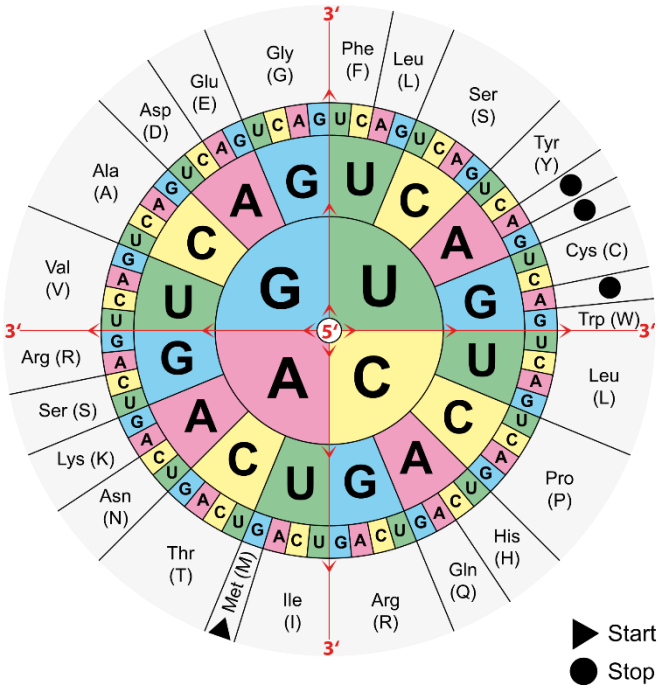
4. The device displays the measured spectrum, DNA concentration, and the A_{260}/A_{280} and A_{260}/A_{230} ratios that give us information about the purity of the solution.



Parameter	Value
A_{260}	
concentration	
A_{260} / A_{230}	
A_{260} / A_{280}	

Lab 8: Molecular biology II

„Translate“ the nucleotide sequence of the region of the gene encoding hemocoagulation factor V, where the Leiden mutation is found, into the amino acid sequence (use the three-letter amino acid abbreviations, example of sequence entry: Gly-Asp-Leu). The image shows the genetic code.



FV wild type

DNA sequence: 1684 GAC AGG CGA GGA ATA CAG

Amino acid sequence: 504 ……

Write the same segment of the factor V gene sequence carrying the mutation:

FV Leiden G 1691 A *(= at position 1691, nucleotide G was replaced by A)*

DNA sequence: 1684 ……

Amino acid sequence: 504 ……

By convention, DNA sequences are always written in the 5′-3′ direction. Numbering - the first nucleotide or amino acid in a given sequence corresponds to the position indicated by the corresponding number.

a) Polymerase chain reaction

In this laboratory, we will amplify a region of the Factor V gene (the region which contains SNP of our interest) using Polymerase Chain Reaction (PCR).

Explain the principle of PCR:
(How is the region for amplification selected? What is the composition of the reaction mixture? How is the amplification taking place?)

Wear gloves (powder-free)!
Fill a prepared box with ice from the icemaker. Keep the reagents on ice always when possible.

Master Mix Preparation *(one common for the study group)*

The total volume for a single PCR reaction is very small (20 μL). It would be uncomfortable to pipette separately all the components needed for each PCR reaction. The strategy is to prepare so called "Master Mix" which contains all the common components for a set of reactions. It improves consistency among the reactions and reduces pipetting error. Today, companies offer pre-prepared master mixes (containing Taq polymerase in a suitable buffer, dNTPs and Mg^{2+}), to which only primers and DNA need to be added.

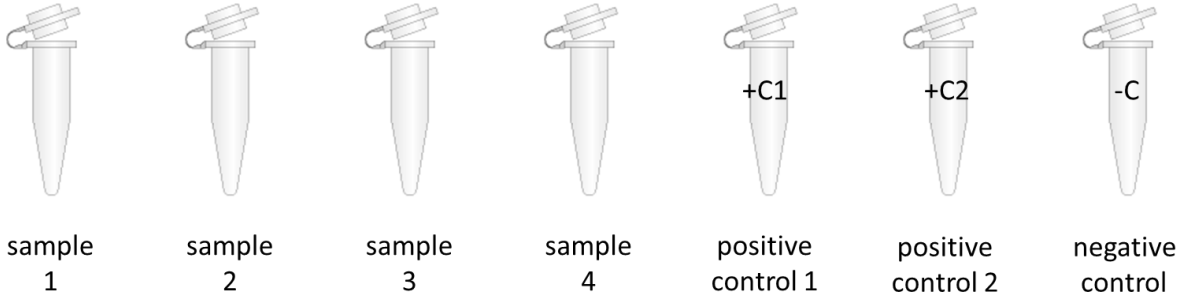
Take a new **1.5 mL eppendorf tube** and prepare the Master Mix for 7 PCR reactions *(you will amplify 3 samples, two positive controls and a negative control, the "excess" reaction is intended for losses during pipetting)*, each of the total volume 20 μL (18 μL Master Mix + 2 μL DNA sample).

	1 reaction **µL**	7 reactions **µl**
water	7.6	53.2
gb Basic PCR Master Mix (2×)	10	70
forward primer (F)	0.2	1.4
reverse primer (R)	0.2	1.4
DNA sample	2	DNA sample will be added later into individual PCR test tubes
Total volume:	20	

Vortex the eppendorf tube with Master Mix for 10 s. Spin down shortly. Keep on ice.

Setting up PCR reactions

Prepare two PCR test tubes in the plate. Dispense **18 µL** of the **Master Mix** into each tube. Each working group add **2 µL** of the DNA sample (DNA isolated during last practices). In addition, the first working group pipette the first positive control - 2 µL of DNA solution from a tube marked **+ C1**, the second working group pipette the second positive control - 2 µL of DNA solution from a tube marked **+ C2**, and the last working group pipette a negative control - instead of DNA 2 µL of water.



Put the PCR tubes into the block of thermocycler and start up the predefined program.

PCR Parameters:

Step		***Temperature***	***Time***
initial denaturation		95°C	2 min
35 cycles:	denaturation	95°C	30 s
	annealing	60°C	30 s
	extension	72°C	30 s
final extension		72°C	5 min
soak		4°C	indefinitely

In the following sequence (part of the sequence of the gene encoding coagulation factor V), mark the sites where the primers will bind during PCR amplification.

Primer sequence:

FVL F	5’- GGA ACA ACA CCA TGA TCA GAG CA -3’	23 mer
FVL R	5’- TAG CCA GGA GAC CTA ACA TGT TC -3’	23 mer

```
1274    GGTGCAGCACACCAACATGACACATGTATACATATGTAACAAACCTGCACGTTGTGCACA
1334    TGTACCCTAGAACTTAAAGTATAATTTAAAAAAAATAAAAATAAAAGAATTCCTTTTGCA
1394    ATATTAATTGGTTCCAGCGAAAGCTTATTTATTTATTTATTATCATGAAATAACTTTGCA
1454    AATGAAAACAATTTTGAATATATTTTCTTTCAGGCAGGAACAACACCATGATCAGAGCAG
1514    TTCAACCAGGGGAAACCTATACTTATAAGTGGAACATCTTAGAGTTTGATGAACCCACAG
1574    AAAATGATGCCCAGTGCTTAACAAGACCATACTACAGTGACGTGGACATCATGAGAGACA
1634    TCGCCTCTGGGCTAATAGGACTACTTCTAATCTGTAAGAGCAGATCCCTGGACAGGCGAG
1694    GAATACAGGTATTTTGTCCTTGAAGTAACCTTTCAGAAATTCTGAGAATTTCTTCTGGCT
1754    AGAACATGTTAGGTCTCCTGGCTAAATAATGGGGCATTTCCTTCAAGAGAACAGTAATTG
1814    TCAAGTAGTCCTTTTTAGCACCAGTGTGATAACATTTATTCTTTTTTTTTTTTGTCTTG
1874    TCTATTTTTATCAGTACCATCACTGCCGAAGGCAAGTCTAGAGTGTGATAACATATTTTG
```

Sequence – normal allele, position marked in yellow where the FV Leiden mutation is located.

Determine how long the product will be amplified with the given primers:

Lab 9: Molecular biology III

a) Restriction cleavage analysis

Explain what restriction endonucleases are and how they work:

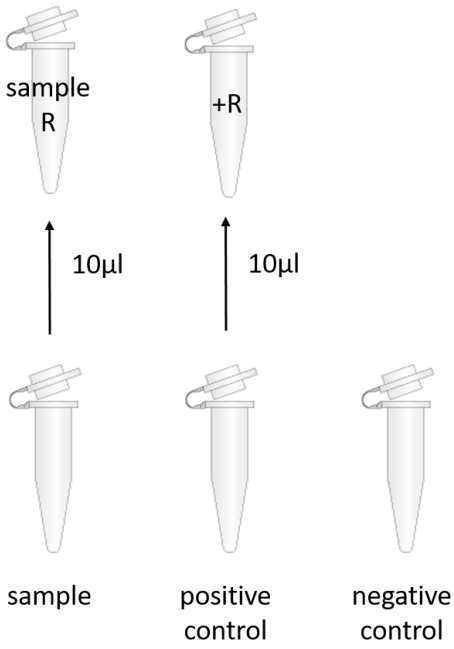
In this laboratory exercise, you will use the restriction endonuclease MnlI for analysis of the DNA fragment amplified by PCR method last time.

Wear gloves (powder-free)!

You will receive ***2 PCR test tubes*** processed last time.
There is 20 μL of PCR reaction mixture (hopefully with the PCR product) in each tube.

- your DNA sample — *test tube marked with your initials*
- positive control (factor V Leiden, homozygote) — *test tube marked "+C1"*
- positive control (factor V Leiden, heterozygote) — *test tube marked "+C2"*
- negative control — *test tube marked "–C"*

To perform restriction enzyme analysis, take **two new PCR test tubes** (or only one new PCR test tube, the working group that processed the negative control) and transfer exactly **10 μL** of the PCR product from the test tube with your DNA sample and positive control into the new test tubes.
Mark the new test tubes with symbol "R", i.e. "*your initials R*", "*+R*".



Restriction analysis

PCR products

The total volume of restriction analysis reaction will be 20 µL. Now there is 10 µL of the PCR product.

1. Add the following components in the order indicated:

PCR product (DNA)	10 µL
water	**7 µL**
10x FastDigest Green Buffer	**2 µL**
FastDigest enzyme MnlI	**1 µL**
	----------
	20 µL

2. Mix gently and spin down.

3. Incubate at **37° C** in a heat block for **10 min**.

b) Electrophoresis, interpretation of the results

Gel electrophoresis

Explain the principle on which electrophoresis is based:

Gel preparation

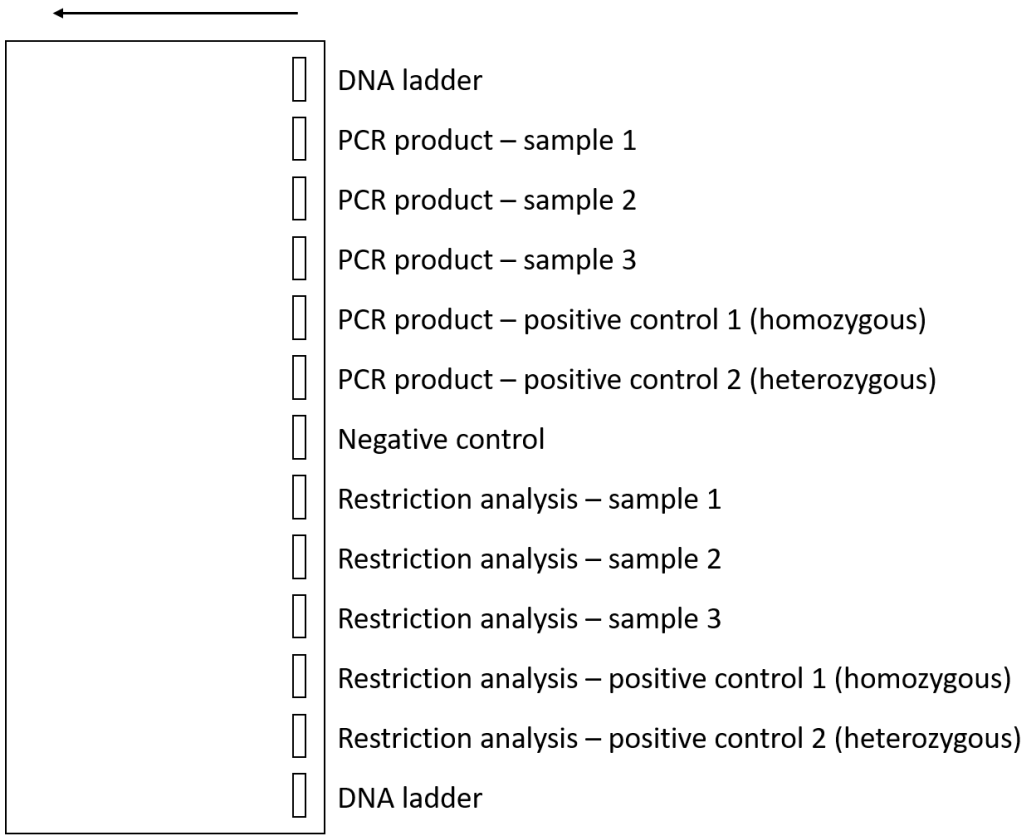
T*he gel must be prepared before starting the restriction cleavage - it must solidify!*

Use a 3% agarose gel in TAE buffer - boil 1.5 g of agarose in 50 mL of TAE buffer in the microwave, after cooling to about 50 ° C, add 2.5 μL of ethidium bromide solution (stock solution - 10 g/L, ethidium bromide in gel - 0.5 μg/mL), mix and pour into a pre-prepared tray (equipped with combs and blanking plugs on the sides). After the gel solidifies, remove the combs and plugs, place the tray in an electrophoretic bath with TAE buffer. Thus, the gel is ready for pipetting samples

Samples preparation

Add **2 μL of loading dye** into the test tubes with PCR products. The test tubes where the restriction digestion took place already contain the loading dye.

Load **10 μL** of the reaction mixtures on a gel as demonstrated in the figure. There are 13 well in the gel we use, all samples fit on one gel.

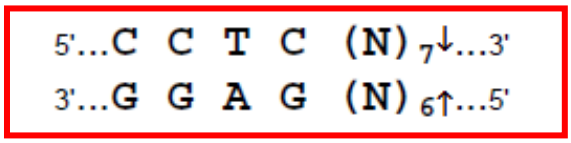


We maintain the electrophoresis at a voltage of 85 V for about 50 minutes (until the blue reaches 2/3 of the gel). Then we examine the gel under UV and take a photo.

Insert a photo, redraw or paste an electrophoreogram here.
Describe which fragment sizes the individual strips correspond to.

Interpretation of the results

The restriction endonuclease MnI I used in the task recognizes this restriction site:



In the sequence of the amplified PCR product (part of the sequence of the gene encoding coagulation factor V - normal allele, marked in red the position where the FV Leiden mutation is located) find the sites where the DNA is cleaved by restriction endonuclease Mnl I

```
  1 GGAACAACACCATGATCAGAGCAGTTCAACCAGGGGAAACCTATACTTATAAGTGGAACA
 61 TCTTAGAGTTTGATGAACCCACAGAAAATGATGCCCAGTGCTTAACAAGACCATACTACA
121 GTGACGTGGACATCATGAGAGACATCGCCTCTGGGCTAATAGGACTACTTCTAATCTGTA
181 AGAGCAGATCCCTGGACAGGCGAGGAATACAGGTATTTTGTCCTTGAAGTAACCTTTCAG
241 AAATTCTGAGAATTTCTTCTGGCTAGAACATGTTAGGTCTCCTGGCTA
```

Determine how many fragments are formed by restriction digestion of the PCR product amplified from normal allele:

In the sequence of the amplified PCR product (part of the sequence of the gene encoding coagulation factor V - Leiden allele, marked in red the position where the FV Leiden mutation is located) find the sites where the DNA is cleaved by restriction endonuclease Mnl I.

```
  1 GGAACAACACCATGATCAGAGCAGTTCAACCAGGGGAAACCTATACTTATAAGTGGAACA
 61 TCTTAGAGTTTGATGAACCCACAGAAAATGATGCCCAGTGCTTAACAAGACCATACTACA
121 GTGACGTGGACATCATGAGAGACATCGCCTCTGGGCTAATAGGACTACTTCTAATCTGTA
181 AGAGCAGATCCCTGGACAGGCGAAGAATACAGGTATTTTGTCCTTGAAGTAACCTTTCAG
241 AAATTCTGAGAATTTCTTCTGGCTAGAACATGTTAGGTCTCCTGGCTA
```

Determine how many fragments are formed by restriction digestion of the PCR product amplified from Leiden allele:

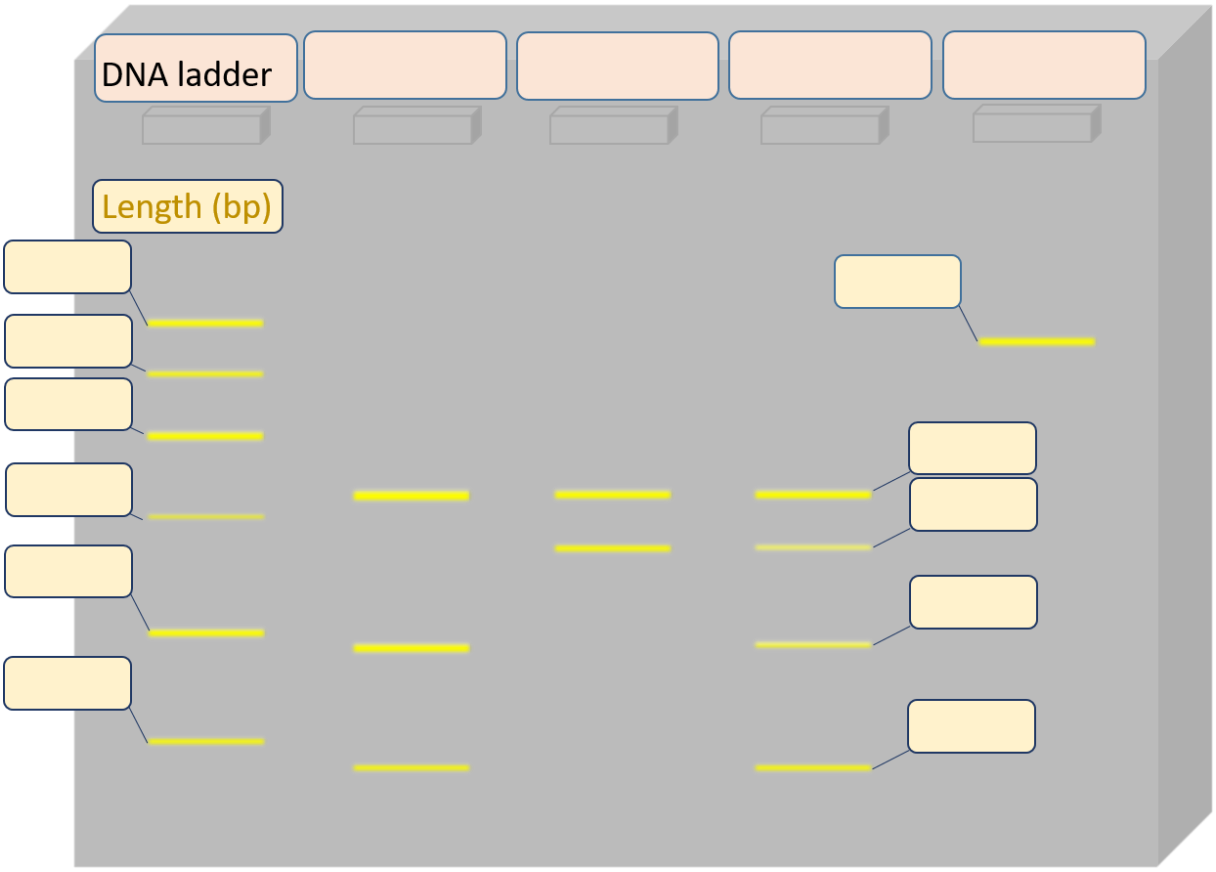
Complete the following table:

Length of PCR product:	**bp**
Restriction digestion:	
genotype	**the length of the fragments (bp)**
homozygous wild type:	
factor V Leiden heterozygous:	
factor V Leiden homozygous:	

The samples listed in the previous table were electrophoresed in the same manner as in laboratory exercise. Complete the scheme of obtained electrophoreogram.

Fill in the top line with a description of the individual lanes, ie in which lane which type of sample was - cleaved/uncleaved PCR product, if it is possible to determine, which genotype.

Add in the columns which fragment sizes each strip corresponds to, if you know that the DNA ladder contained fragments of 50, 100, 150, 200, 250 and 300 bp.



Evaluate and interpret the results:

Conclusion:

Table of Reference Values for Clinical Testing *update 26. 1. 2024*

A result of a laboratory investigation is a measured value that may be physiological, increased or decreased. For classification of lab results as physiologic or pathologic we need to compare measured values with a physiological (reference) range of values. The reference range is obtained by measuring the reference (means healthy) population. Obtained values are arranged in ascending order and then a definite percentage (usually 2.5%) of the extreme minimal and the extreme maximal values are discarded. Then the lowest and the highest remaining values signify the lower and upper reference limits. In this case 95% of healthy people belong to the reference range. But on the other hand, there are about 5% of healthy people, whose values do not belong to the reference range. However, significant deviations of values are almost always associated with pathology.

For some substances any significant deviations from the physiological range are associated with pathologies (*e.g.* glucose). There is a danger only if the level of value of some substances exceeds a certain limit or if the value declines under the limit. For such substances is used only the upper or lower reference value (*e.g.* lowering of cholesterol does not signify a clinical danger).

Reference population, laboratory reagents or procedure of measurement, could be different among particular laboratories in some detail. That is the reason of different reference values used in different labs. The students should be familiarized with the most important reference values used in University Hospital Pilsen in present in the enclosing list. Some reference ranges even include for example a patient's age or a gender. In such cases for study purposes we tried to simplify these ranges. But it should still provide at least a general idea of the average values for healthy adults.

The same metabolites may be found in various body fluids in a different concentration. Therefore, it is necessary to mark an analyzed metabolite with an abbreviation of an investigated material. In common the special term is used for a concentration of certain substances in the specific body fluids. For example, glycaemia refers to blood glucose, the term glycorrhachia relates to cerebrospinal fluid and glycosuria means glucose in urine. If the blood glucose is under the physiological limit we talk about hypoglycaemia. High level of blood glucose is called hyperglycemia.

E.g.:

B_glucose - means glucose concentration in full blood

S_glucose - means glucose concentration in serum

P_glucose - means glucose concentration in blood plasma

Csf_glucose - means glucose concentration in cerebrospinal fluid

U_glucose - means glucose concentration in urine

Common Lab Values to Remember

Lab Investigation	Normal Range
Blod Gas Analysis (Arterial Blood Gas)	
pH	7.36 - 7.44
pCO_2	4.8 - 5.9 kPa
pO_2	9.6 kPa and higher
HCO_3^-	22 - 26 mmol/L
Base Excess (BE)	± 2.5 mmol/L
Anion Gap (AG)	14 - 18 mmol/L
Blood Tests	
Plasma Osmolality	275 - 295 mmol/kg H_2O
Sodium	136 - 144 mmol/L
Potassium	3.8 - 5.2 mmol/L
Total Calcium	2,2 - 2,6 mmol/L
Ionized Calcium	1.15 - 1.30 mmol/L
Magnesium	0.7 - 0.9 mmol/L
Chloride	98 - 109 mmol/L
Phosphorus	0.7 - 1.7 mmol/L
Serum Iron	6 - 35 µmol/L
Lactic Acid	less then 2.2 mmol/L
B/S Glucose	3.6 - 5.6 mmol/L
Glycated Hemoglobin (HbA1c)	20 - 42 mmol/mol
Results of Glucose Tolerance Test	
Normal Glucose Tolerance	less than 7.8 mmol/L
Impaired Glucose Tolerance	7.8 - 11.0 mmol/L
Diabetes Mellitus	11.1 mmol/L and higher

Total Bilirubin	less than 25 µmol/L
Bilirubin - Conjugated ("direct")	less than 8 µmol/L
Total Cholesterol	less than 5 mmol/L
Triglycerides	less than 1.7 mmol/L
LDL Cholesterol	less than 3 mmol/L
HDL Cholesterol	♂ 1 mmol/L and higher
	♀ 1.2 mmol/L and higher
Urea	3 - 8 mmol/L
Uric Acid	♂ 210 - 450 µmol/L
	♀ 140 - 360 µmol/L
Creatinine	♂ 60 -100 µmol/L
	♀ 50 - 90 µmol/L
Ammonia	less than 50 µmol/L
Alpha-amylase (AMS)	less than 2.0 µkat/L
Aspartate Transaminase (AST)	♂ less than 0.8 µkat/L
	♀ less than 0.6 µkat/L
Alanine Transaminase (ALT)	♂ less than 1.2 µkat/L
	♀ less than 0.8 µkat/L
Alkaline Phosphatase (ALP)	0.7 - 2.2 µkat/L
Lactate Dehydrogenase (LD)	less than 4.0 µkat/L
Gamma-glutamyltransferase (GGT)	♂ less than 1.2 µkat/L
	♀ less than 0.7 µkat/L
Lipase (LPS)	less than 1.0 µkat/L
C-reactive Protein (CRP)	less than 5 mg/L
Serum Total Protein	63 - 80 g/L
Albumin	37 - 52 g/L
Serum Protein Electrophoresis Fractions	
Albumin	0.530 (53%) - 0.650 (65%)
Alpha-1 Zone	0.020 (2%) - 0.040 (4%)
Alpha-2 Zone	0.080 (8%) - 0.130 (13%)
Beta Zone	0.090 (9%) - 0.160 (16%)
Gamma Zone	0.115 (11.5%) - 0.19 (19%)

Cerebrospinal Fluid Tests	
Protein	less than 0.5 g/L
Glucose	2.7 - 4.5 mmol/L
Lactic Acid	1.2 - 2.2 mmol/L
Urine Tests	
Density	1010 - 1030 g/L
pH	5 - 6
Glucose	0
Protein	0
Alpha-amylase (AMS)	less than 8.0 µkat/L
Creatinine Clearance Rate	1.6 - 2.6 mL/s