Date ..... **Name** ..... **Group** .....

Instructions for the practical lesson on biochemistry

Topic: Selected immunochemical methods

Task 1: Immunoprecipitation curve of human albumin and estimation of albumin concentration by means of immunoturbidimetry

Reagents:

a) Diluted ram antiserum against human albumin (**Antiserum Task 1**)
b) Phosphate buffer 0.01 mol/l with 0.9 % NaCl, pH 7.2 (**Phosphate Buffer Task 1**)
c) Unknown sample of albumin (**Unknown Sample Task 1**)

Procedure:

Wear gloves during the whole experiment**.**

a) Dilution of the unknown sample of albumin

Proceed according to the table:

Tube No.	Buffer (ml)	Albumin solution (ml)
1	-	0.4 (from stock solution)
2	0.2	0.2 (from tube 1)

b) Immunoprecipitation reaction

Both the diluted and undiluted samples are pipetted to new test tubes 1 – 2 (the tube numbers correspond to the ones in the previous table). The tube no. 3 serves as the blank sample. Then the antiserum against human albumin is given to all the tubes, according to the table:

Measure in ml	**Tube 1** Unknown undiluted	**Tube 2** Unknown diluted	**Tube 3** Blank
Sample	0.1	0.1	–
Buffer	–	–	0.1
Antiserum	1.0	1.0	1.0

The tubes are mixed and allowed to incubate 20 minutes at room temperature. After the incubation the absorbances of all solutions are measured against the blank in 1 cm cuvette at wavelength 400 nm.

Evaluation:

a) For construction of a calibration curve, the absorbances of standard albumin solutions measured by the same method are provided. Plot these values of absorbances against the corresponding standard albumin concentrations, construct the precipitation curve and describe it.

b) Read the concentration of albumin in the unknown sample from the linear ascendent part of the curve. The value for the diluted sample must be multiplied with two. Compare the results obtained for the diluted and undiluted sample and explain the difference.

Task 2: Evaluation of single radial immunodiffusion for estimation of IgG or IgM

Procedure and evaluation:

a) Construction of calibration curve

Measure diameters of the precipitation rings for the standard solutions marked $S_1 - S_8$ by means of the special ruler and read the second power – d^2. Fill the d^2 of standards into the table in your report.

Plot the obtained squared diameters of the precipitation rings against the corresponding standard concentration values on a graph paper; d^2 on the y-axis and concentration on the x-axis. Draw a straight line that best connects all the points.

b) Estimation of IgG or IgM concentration in unknown samples

Measure diameters of the rings of 5 unknown samples, read the second powers and then the concentrations from the calibration curve.

Task 3: Estimation of antibodies in unknown samples by means of ELISA test

Reagents:

a) ELISA strip (8 wells) with bound antigen (strip is inserted to frame)
b) Positive control (**Positive Control Task 3**)
c) Negative control (**Negative Control Task 3**)
d) Cut-off control (**Cut-off Control Task 3**)
e) Unknown sample no. 1 (infectious material) (**Unknown Sample 1 Task 3**)
f) Unknown sample no. 2 (infectious material) (**Unknown Sample 2 Task 3**)
g) Diluting solution for samples **(Diluting Solution Task 3)**
h) Washing solution (**Washing SolutionTask 3**)
i) Conjugate anti-Ig/labeled with peroxidase **(Conjugate Task 3)**

j) Substrate solution: 3´,3´´,5´,5´´ tetramethylbenzidine (TMB) and hydrogen peroxide **(Substrate Task 3)**

k) Stopping solution (sulfuric acid 0.2 mol/l) **Stop Solution Task 3**)

Procedure:

Wear gloves during the whole experiment.

1. Dilution of unknown samples:

Each sample will be diluted before the assay: 1 part of sample + 100 parts of diluting solution. The dilution is performed in microtubes:

	Microtube 1	**Microtube 2**
Diluting solution	1.0 ml	1.0 ml
Unknown sample 1	0.010 ml	–
Unknown sample 2	–	0.010 ml

Mix contents of the microtubes with vortex.

2. ELISA procedure:

Well:	**Layout**
A	Blank (DS)
B	Negative control
C	Positive control
D	Cut-off control
E	Sample 1
F	Sample 1
G	Sample 2
H	Sample 2

a) Application of samples and controls

According to the provided layout measure 0.1 ml of the controls or samples to the wells. Pipette carefully to the bottom of the wells. The first well will serve as a blank – pipette the diluting solution (DS) instead of the samples. Each unknown sample is added to two wells (in a doublet).

Close the strip with the provided lid and allow to incubate for ***1 hour at 37 °C***.

During this incubation the antibodies present in the sample react with the antigen immobilized to the strip wells.

b) Removal of unbound serum components

After the incubation turn the strip upside down above the vessel with chloramine and with a moderate swipe empty its contents to the vessel. Then pipette 0.3 ml of ***washing solution*** to each well. Gently shake the strip and remove the contents by emptying to the vessel with chloramine as above. Repeat this washing step 3-times in total. Finally dry the strip by turning upside down and pressing onto a layer of tissue.

c) Addition of conjugate

Add 0.1 ml of ***conjugate (anti-Ig/Px)*** to each well and incubate the strip ***30 minutes at room temperature***.

During this incubation the second antibody against human immunoglobulin labeled with peroxidase binds onto the immunocomplexes formed in the wells during the previous steps.

d) Removal of unbound molecules of conjugate

After the incubation wash the wells as described above in the step **b)**. This time a redundant unbound conjugate is removed.

e) Addition of substrate

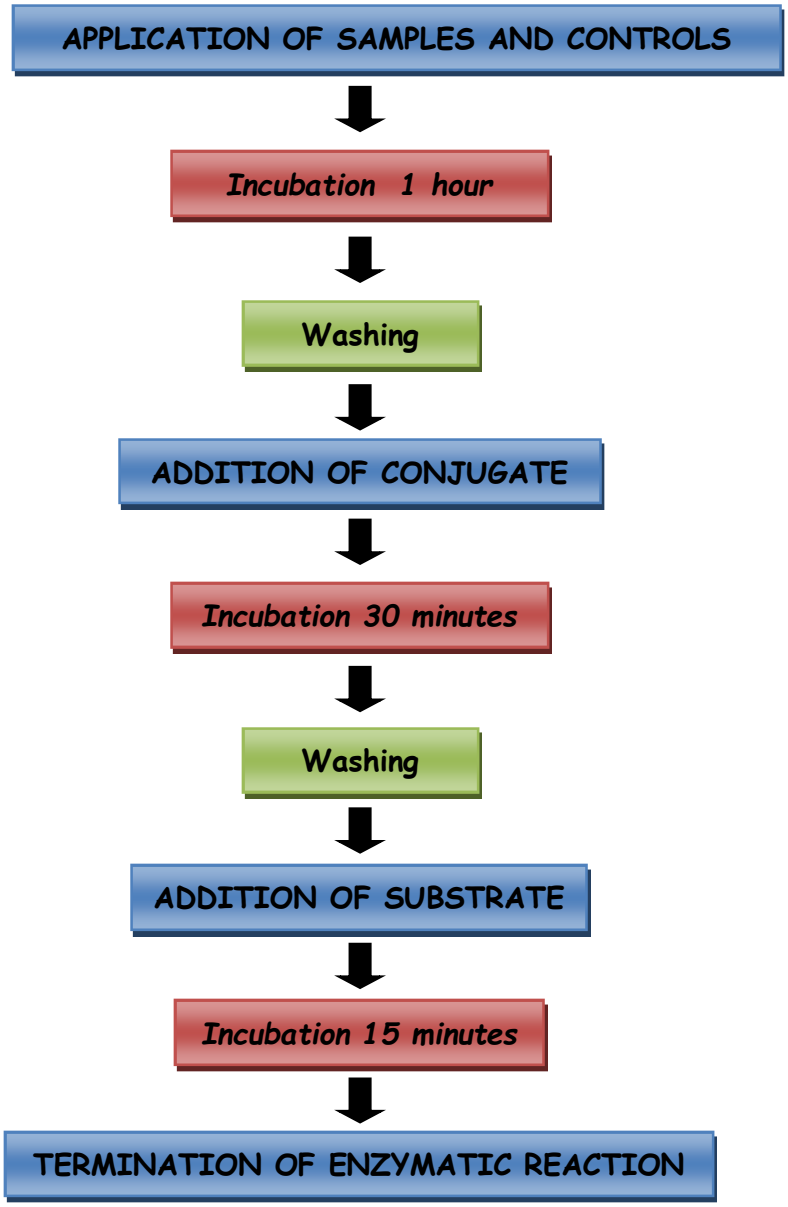
Add 0.1 ml of the ***substrate solution*** to each well, cover the strip with the lid and incubate about ***15 minutes at room temperature in dark***.

In this step the substrate is converted to a product by the action of peroxidase present in the conjugate. The wells with samples containing antibodies against the immobilized antigen now turn blue.

f) Termination of the enzymatic reaction

To each well add 0.1 ml of the ***stopping solution*** that terminates the enzymatic reaction. The blue color in the wells changes to yellow.

Overview of the ELISA procedure



g) Measurement and data processing

Reading of absorbances in the wells is performed by means of the special spectrophotometer with vertical light beam at wavelength 450 nm.

The blank absorbance is to be subtracted from the absorbance values obtained for samples and controls. After the subtraction write all the corrected absorbance values to the table in your report and calculate the arithmetic means for the two unknown samples measured in doublets.

Calculation and interpretation of results

Compare the values of the averaged absorbances of the unknown samples with the value of the cut-off control sample, which sets the limit of positivity.

- If the averaged absorbance of the sample is at least 10 % higher that the absorbance of cut-off control, the sample is considered ***positive***.
- If the averaged absorbance of the sample is at least 10 % lower that the absorbance of cut-off control, the sample is considered ***negative***.
- Sample whose absorbance is within the range ± 10% of the cut-off control absorbance is considered neither positive nor negative – the borderline zone. It is recommended to repeat the examination using a new sample taken 2-4 weeks later.

In the conclusion decide whether the tested specific antibodies in each of the unknown samples were present (positive) or absent (negative).

Task 4: Estimation of concentration of C-reactive protein in serum by means of turbidimetric POCT test (demonstration)

Tools and reagents:

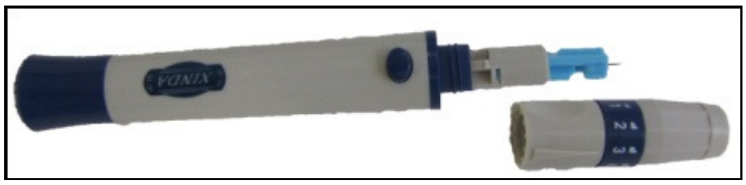
a) QuikRead instrument
b) Program card
c) Reagent (contains latex particles with antibodies against CRP; stabilizer: sodium azide )
d) Sample collection capillaries and pistons
e) Cuvettes filled with buffer
f) Pen and sterile needles for collection of capillary blood
g) Disinfection and tissue
h) Vessels for biologic waste

Procedure:

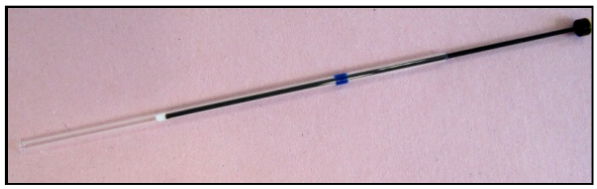
1. Switch on the turbidimeter QuikRead. Start and the automatic test of the instrument lasts about 2 minutes, then the display shows message “Nactete kartu” (read card). Draw the magnetic card through the instrument slit (with the magnetic strip down and facing forward, whichever direction). The display now shows “Pripraven k mereni – CRP” (ready to measure CRP).

Collection of capillary blood:

2. Insert one sterile needle to the collection pen. Turn around the needle cap and remove it. Put the pen cover on and set the puncture depth (usually no. 3). Wind the pen up by pulling the rear part until you hear a click.



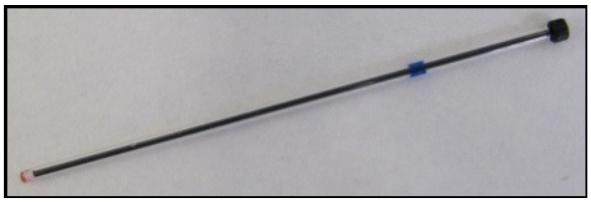
3. Disinfect the fingertip. A right-handed person usually gives the blood from sides of the third or fourth finger of his left hand.
4. Prepare the sample capillary: Insert the black piston through the blue-marked end of the capillary down to the white hydrophobic plug.



5. Perform the puncture with the pen. Wipe out the first blood drop, and then collect the blood to the capillary up to the white plug. The blood fills the capillary by capillary force; the piston should not be moved. Facilitate the blood collection by pressing onto the fingertip if needed.
6. Stop further bleeding with a piece of tissue pre-wet with a disinfectant.

Preparation of sample for measurement:

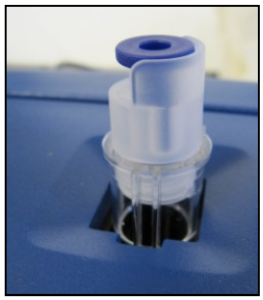
7. Remove the cover foil from the cuvette. Dip the capillary to the one ml of buffer in the cuvette and press the piston into the capillary as far as you can. The blood is released to the cuvette while the white plug stays in the capillary.



8. Cap the cuvette with the reagent-containing stopper. AVOID pressing the blue part of the stopper at this stage.

9. Gently shake the buffer with blood. Avoid turning the cuvette upside down. Wait until the sample becomes transparent – hemolysis has taken place.
10. Insert the cuvette to the turbidimeter. The cuvette has projections that must snap into the grooves in the sample space. The display now reads “Mereni blanku” and a time countdown starts (40 s).

Measurement:



11. When a message “Pridejte cinidlo” (add reagent) appears on the display, press the blue part of the cuvette cap to release the reagent.
12. Immediately remove the cuvette from the instrument, turn it several times upside down and shake the contents vigorously.
13. As soon as the instrument shows message “Vlozte kyvetu” (insert cuvette), return the cuvette to the instrument. Delay in this step results in abortion of the measurement!
14. The display shows “Probiha mereni” (measurement in progress). After two minutes the instrument displays the CRP concentration in mg/l.
15. The whole cuvette with the cap as well as the used sample capillary with the piston is to be discarded to the container designated for biological waste. The needle from the collection pen should be discarded to another vessel for sharp biological waste.

In the conclusion, compare the measured CRP concentration with the reference range.