ÚSTAV LÉKAŘSKÉ BIOCHEMIE A LABORATORNÍ DIAGNOSTIKY 1. LF UK

Selected immunochemical methods

Practical Lesson on Medical Biochemistry

General Medicine

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(translated and edited by Jan Pláteník)

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Task 1: Estimation of circulating immune complexes

Reagents:

a) Solution A (buffer without PEG):

550 ml 1.24% boric acid
450 ml 1.9% borax
1000 ml purified water

b) Solution B (buffer with PEG):

41.66 g PEG 6000
1000 ml solution A

c) Unknown serum sample No. 1 – infectious material

Procedure:

Wear gloves during the whole experiment.

Mix serum on vortex before use.

To 4 test tubes prepare the following reaction mixtures:

Measure in ml:	**Test tube 1** Serum (buffer without PEG)	**Test tube 2** Serum (buffer with PEG)	**Test tube 3** Blank (buffer without PEG)	**Test tube 4** Blank (buffer with PEG)
Serum	0.03	0.03		
Solution A	1.0		1.0	
Solution B		1.0		1.0

Vortex the reaction mixtures and allow to stand 60 minutes at the room temperature (21–25 °C). Then mix again on vortex and then measure absorbance at 450 nm always against the corresponding blank (mixture in tube 1 against solution in tube 3, and mixture in tube 2 against solution in tube 4).

Task 2: Immunoprecipitation curve of human albumin and estimation of albumin concentration by means of immunoturbidimetry

In this experiment you will only evaluate the data that are in your report form. The following experimental procedure is included only for better understanding.

Reagents:

a) Stock solution of human albumin 1000 mg/l
b) Diluted ram antiserum against human albumin
c) Phosphate buffer 0.01 mol/l with 0.9 % NaCl, pH 7.2
d) Unknown sample of albumin

Procedure:

a) Dilution of the unknown sample of albumin

The stock solution of human albumin 1000 mg/l is diluted geometrically with phosphate buffer 0.01 mol/l according to the table 1:

Test tube No.	Buffer (ml)	Albumin solution (ml)	Final concentration of albumin (mg/l)
1	-	0.4 (from stock)	1000
2	0.2	0.2 (from tube 1)	500
3	0.2	0.2 (from tube 2)	250
4	0.2	0.2 (from tube 3)	125
5	0.2	0.2 (from tube 4)	62.5
6	0.2	0.2 (from tube 5)	31.25
7	0.2	0.2 (from tube 5)	15.63

b) Actual immunoprecipitation reaction

Solutions of diluted albumin are pipetted to new test tubes 1–7 (numbers correspond to the test tubes numbers in the table 1 above). To other two test tubes 8–9 the unknown sample undiluted or diluted 1 : 1 with phosphate buffer (0.1 ml undiluted sample + 0.1 ml buffer) is measured, respectively. The last tube 10 serves as the blank. Finally, a diluted antiserum against human albumin is added to all tubes, according to the table 2:

Measure in ml	**Tube 1** Albumin 1000 mg/l	**Tube 2** Albumin 500 mg/l	**Tube 3** Albumin 250 mg/l	**Tube 4** Albumin 125 mg/l	**Tube 5** Albumin 62.5 mg/l	**Tube 6** Albumin 31.25 mg/l	**Tube 7** Albumin 15.63 mg/l	**Tube 8** Sample undiluted	**Tube 9** Sample diluted	**Tube 10** Blank
Albumin diluted	0.1	0.1	0.1	0.1	0.1	0.1	0.1	–	–	–
Sample	–	–	–	–	–	–	–	0.1	0.1	–
Buffer	–	–	–	–	–	–	–	–	–	0.1
Antiserum	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Contents of the tubes is mixed and allowed to stand 20 minutes at room temperature. Then absorbances of all dilutions of albumin and sample are measured against the blank in 1 cm cuvette at the wavelength 400 nm.

Evaluation:

a) Plot the provided values of standard absorbances against the corresponding standard albumin concentrations, construct the precipitation curve and describe it.

b) Use the linear ascendent part of the precipitation curve as the calibration curve and read the concentration of albumin in the unknown sample, both undiluted and diluted. 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 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
c) Negative control
d) Cut-off control
e) Unknown sample no. 1 (infectious material)
f) Unknown sample no. 2 (infectious material)
g) Diluting solution for samples
h) Washing solution
i) Conjugate anti-Ig/labeled with peroxidase

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

k) Stopping solution (sulfuric acid 0.2 mol/l) 

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.

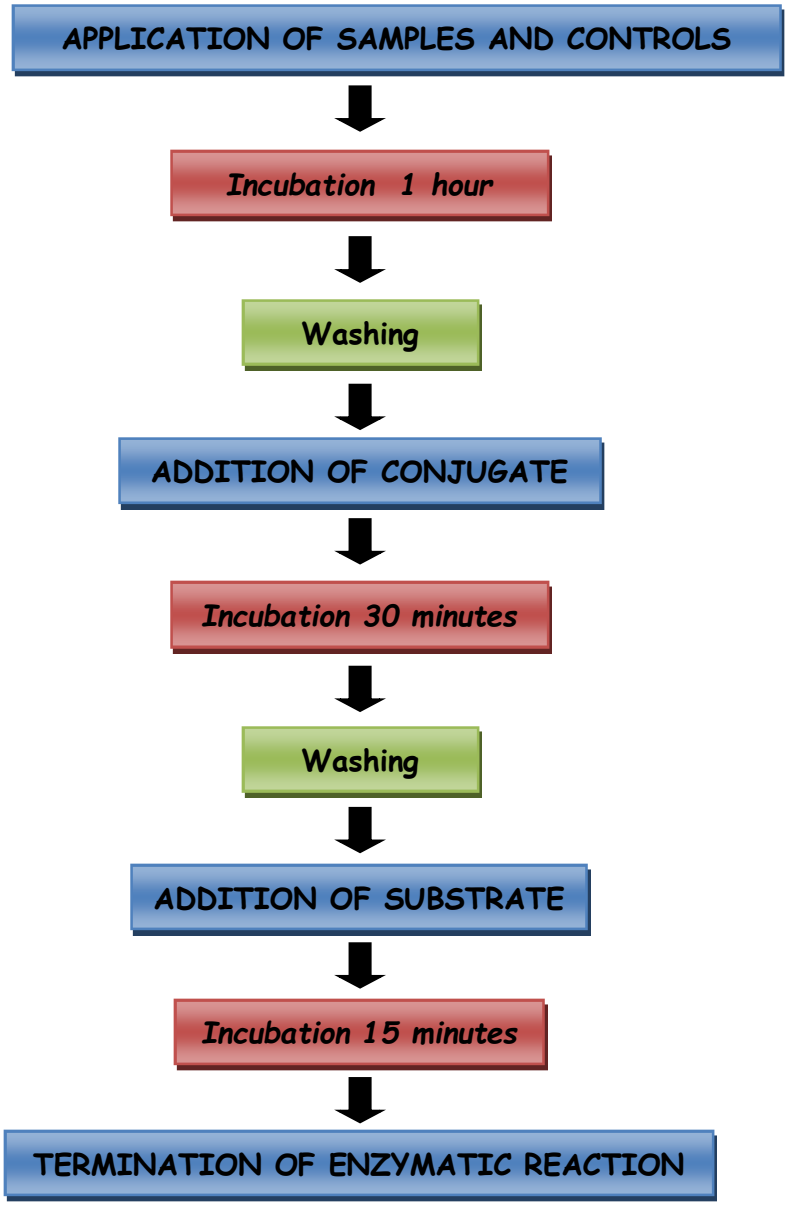
e) Addition of substrate
Add 0.1 ml of the ***substrate solution*** to each well, cover the strip with the lid and incubate about ***15 minutes at room temperature in dark***.
In this step the substrate is converted to a product by the action of peroxidase present in the conjugate. The wells with samples 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.

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.

Overview of the ELISA procedure



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: 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 5: Determination of blood group by means of hemagglutination test

Procedure:

a) Disinfect the fingertip from which the sample will be taken. Typically, capillary blood is drawn from the fingertip of the 3rd or 4th finger, from the left hand in right-handed individuals.

b) Using a sterile needle, make a puncture in the fingertip and apply 2 drops of blood near the center of a glass slide.

c) On the sides of the slide, place a drop of anti-A antibody on the left and anti-B on the right.

d) Mix the blood with each of the antibodies using the pipette tip, and after about 1 minute, read the result.

Task 6: 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

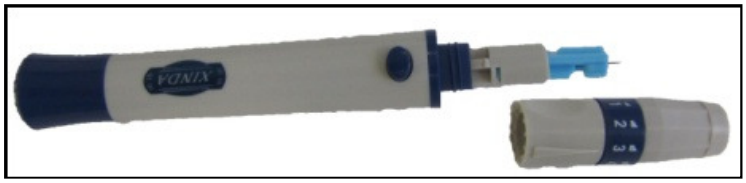
h) Vessels for biologic waste

Procedure:

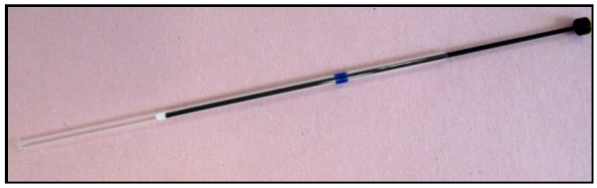
1. Switch on the turbidimeter QuikRead. Start and the automatic test of the instrument lasts about two minutes, then the display shows message “Nactete kartu” (read card). Draw the magnetic card through the instrument slit (with the magnetic strip down and facing forward, whichever direction). The display 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.



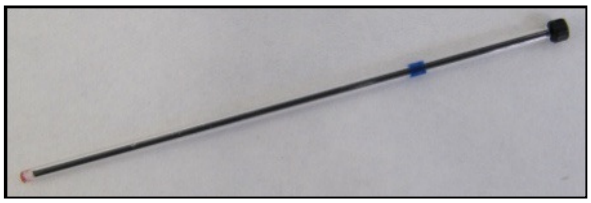
3. Disinfect the fingertip. A right-handed person usually gives the blood from sides of the third or fourth finger of his left hand.
4. Prepare the sample capillary: Insert the black piston through the blue-marked end of the capillary down to the white hydrophobic plug.



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.

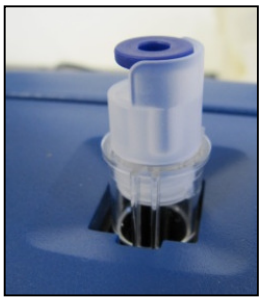
Preparation of sample for measurement:

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.