Topic J11: Molecular-biological methods

To study: PCR, ELISA

Task No. 1: DNA isolation of causative agent of Lyme Borreliosis from clinical material

From the submitted clinical material the DNA of *Borrelia burgdorferi* s.l was isolated by the help of commercial set. Concrete process depends on the type of used set.
Observe the videoclip and read the following text and write to each chemical its function.

DNA isolation is a routine procedure to collect DNA for subsequent molecular or forensic analysis. There are three basic steps in a DNA extraction: Breaking the cells open, commonly referred to as cell disruption, to expose the DNA within, such as by grinding or sonicating the sample. Removing membrane lipids by adding a detergent. Precipitating the DNA with an alcohol — usually ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a pellet upon centrifugation. This step also removes alcohol-soluble salt. Refinements of the technique include adding a chelating agent to sequester divalent cations such as Mg2+ and Ca2+. This stops dnase enzymes from degrading the DNA. Cellular and histone proteins bound to the DNA can be removed either by adding a protease or by having precipitated the proteins with sodium or ammonium acetate, or extracted them with a phenol-chloroform mixture prior to the DNA-precipitation. If desired, the DNA can be resolubilized in a slightly alkaline buffer. *(http://en.wikipedia.org/wiki/DNA_extraction)*	
detergent	
ethanol or isopropanol	
chelating agent	

Task No. 2: Amplification of the specific sections DNA of the causative agent of Lyme Borreliosis by PCR method

Watch the videoclip illustrates the process of polymerase chain reaction (PCR). Read the following text and explain role of given terms.

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. With PCR it is possible to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations. Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*. This DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary to physically separate the strands (at high temperatures) in a DNA double helix (DNA melting) used as the template during DNA synthesis (at lower temperatures) by the DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions. *(http://en.wikipedia.org/wiki/Polymerase_chain_reaction)*	
primer	
Taq polymerase	
thermal cycling	

Task No. 3: The detection of PCR products by gel electrophoresis

B. burgdorferi s.l. nucleic acid from clinical material was isolated. The specific part of this microbial DNA was multiple amplificated with help of PCR in next task. We prove the presence of these specific DNA parts by their separation in 1.5% agarose gel containing ethidiumbromid. This dye bounds on DNA and fluoresces in UV-light. At PCR product detection, gel with wells for pile up of examinating sample is prepared in electrophoretic bath. Gel is flooded with tris-boric buffer solution. 10 μl of positive control are pipetted into the 1st well of this gel. 100 pb ladder are pipetted into following well. The ladder enables sizing of PCR products. 10 μl of mixture of PCR products is piled of with gel loading buffer into remaining wells. Electrophoretic bath is connected into electric source. Correct linkage of electrodes should be checked up. Wells with specimens must be on the side of cathode so that parts of DNA were able to migrate towards anode. The instrument gets started for one hour (100 MV). The gel is placed on UV-transiluminator after finish of electrophoresis. Protective board is covered up and the result is surveyed. Protective board is used against UV-radiation and rubber gloves at work.
Draw in the result of the reaction and interprete it (in negative reactions, make a „minus“ from a „plus“

Drawing	Patient No.	Proper reaction	Internal contol	Conclusion
	1	+	+	
	2	+	+	
	3	+	+	
	4	+	+	

Task No. 4: Comparsion of results obtained by two methods: specific DNA sequence detection and specific antibody detection

4a) PCR of nucleid acid of Lyme borreliosis

Write down, that patient is positive in the PCR. Remark positive and negative controls. Positive is No. ___

4b) Detection of antibodies against ethiological agent of Lyme borreliosis

We detected the specific DNA of the causative agent of lyme borreliosis In previous task. Investigate now the serum from the same patients to presence antibody against these bacteria. The results of detection of antibodies IgG and IgM obtained by ELISA are available in microtiter plate. There are results of spectrophotometry measurment of optical density of particular wells in enclosed paper. Evaluate and write down the results of the reaction of the separate patients according to lecturer's instruction.

IgM	Cut off = (+)/2 =	Controls OK? yes - no	Positive are patients No.
IgG	Cut off = (+)/2 =	Controls OK? yes – no	Positive are patients No.

4c) Compare the results of specific DNA sequence detection and specific antibodies detection of ethiological agent of lyme borreliosis.

Patient	PCR result	ELISA – IgM	ELISA – IgG	Interpretation
1				
2				
3				

Task No. 5: Detection of *Mycobacterium tuberculosis* DNA by means of PCR with detection of the products by ELISA

ELISA is used for detection of PCR products, so – called amplicons, next to gel electrophoresis. PCR proceeds with biotinylyzing primers in this case. Amplicons are immobilized on the wall of wells of microtitre plate with using of specific capture probes. The avidin with enzyme binds hard to biotin on 5' end of amplicon. After addition of substrate the reaction is vizualizated and it can be read on spectrophotometer.
Using of measurement of optical density of each wells by spectrophotometer evaluate the results of the reaction in the patients.
Attention, ELISA technique is exploited here, but otherwise it is very different from ELISA reaction for antigen/antibody detection (J10, task 4a of today's practicle).

Cut off: ______________________________________

Patient No.	Product of reaction	Internal control	Conclusion
1	positive – negative	positive – negative	
2	positive – negative	positive – negative	
3	positive – negative	positive – negative	
4	positive – negative	positive – negative	

Check-up questions:

1. Which microorganism is suitable to detect with PCR?

2. How to prevent contamination of PCR?

3. You (as a doctor) have got a report from PCR laboratory: „PCR test result of patient XY for Lyme boreliosis: inhibition of reaction". How would you interprete such result?

4. What are advantages and disadvantages of nucleic acids specific fragments detection by means of PCR in comparison to methods of classical diagnostics?

5. What other methods of detection of nucleic acids of microorganisms do you know?