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Realizováno v rámci dotačního programu
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Analysis of the *PTPN22* gene via PCR-RFLP

Theoretical part:

Genomic studies revealed hundreds of risk variants in signaling proteins, which lead to the development of autoimmune diseases. Interactions between antigens and receptors of T- and B-cells activate a Src family of protein tyrosine kinases, for example **Lck kinase**. Inactivation of Lck requires both dephosphorylation of tyrosine Y394 (important for autoactivation) and phosphorylation of inhibitory tyrosine Y505 (maintains inactive conformation). This negative regulation of Lck activation is catalyzed by two proteins – lymphoid tyrosine phosphatase (LYP) and Csk kinase. Csk kinase phosphorylates the inhibitory C-terminal tyrosine Y505 of Lck, and behaves as a strong negative regulator of T cell receptor signaling. LYP, the product of the *PTPN22* gene, destabilizes the kinase domain of Lck by dephosphorylating tyrosine Y394. In response to the combined action of Csk and LYP, Lck is closed in its inactivated conformation. The mutation Arg620Trp (R620W; c.1858C>T) in tyrosine phosphatase LYP disturbs the interaction between LYP and SH3 domain of Csk. This R620W mutation increases the risk to many autoimmune diseases. Polymorphisms in the *PTPN22* gene are associated with type 1 diabetes, LADA diabetes (late-onset autoimmune diabetes in adults), autoimmune disorders of the thyroid gland (e.g., Graves' disease and Hashimoto's thyroiditis), rheumatoid arthritis, systemic lupus erythematosus, vitiligo, etc.

The ***PTPN22* gene** (protein tyrosine phosphatase nonreceptor type 22) encodes phosphatase LYP, which is primarily expressed in lymphoid tissues, including mature and immature T- and Blymphocytes. The binding of antigen to TCR leads to activation of the CD4/CD8-associated Src kinase Lck, which gains the active conformation by autophosphorylation of Y394. The activated Lck phosphorylates tyrosines at immunoreceptor tyrosine-based activation motifs (ITAMs) of CD3 and ζchains associated with TCR. Phosphorylated ITAMs serve as docking sites for the Src homology 2 (SH2) domains of ζ-associated protein of 70 kDa (ZAP70). ZAP70, due to its tyrosine kinase activity, mediates signals towards downstream responses, and stimulates activation of transcription factors (NFAT (nuclear factor of ... proliferation and production of cytokines.

... cilitate their termination. Downregulation ... eceptor, Csk-mediated phosphorylation of Lck at Y505, dephosphorylation of Lck at Y394 and/or dephosphorylation of ITAMs on CD3 and ζchains by variety of protein tyrosine phosphatases (PTPs), including LYP, SHP-1, and PTPH1.

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Manjarrez-Orduño, N., *et al*. (2012) CSK regulatory polymorphism is associated with systemic lupus erythematosus and influence B cell signaling and activation. *Nat. Genet.* **44**:1227–1230.

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PCR (Polymerase Chain Reaction)

PCR is a method enabling amplification of DNA sequences. For amplification of DNA sequences, specific short complementary single-stranded oligonucleotides (termed **primers**) must be designed and match the beginning and end of the DNA target. For DNA amplification, thermostable **DNA polymerase** is used; the first one was isolated from the bacterium living in hot springs, named *Thermus aquaticus* (referred as Taq polymerase). PCR proceeds in a **thermocycler**, which can rapidly increase or decrease temperature in the PCR tube by tens of Celsius degrees within just several seconds. The PCR reaction results in a huge amount of original DNA copies. This method is so sensitive that can reveal even a single molecule of DNA.

PCR program comprises of several steps:

1/ **Denaturation** – DNA is warmed up to 94 – 98°C for 20 – 30s. At this temperature, hydrogen bonds in DNA are disrupted. Thus, DNA looses its double-helix conformation, the DNA helix separate and form a single-stranded DNA. Then primers can bind to complementary single-stranded DNA sequences.

2/ **Annealing** – temperature is decreased to 45 – 70°C, thereby enabling the binding of primers to complementary DNA sites. DNA polymerase binds to the DNA-primer complex.

3/ **DNA synthesis** – exact temperature used for the DNA synthesis depends on DNA polymerase used, but is usually within the 68 – 72°C range. The new DNA strand complementary to the original molecule of DNA is synthesized in the 5'→3' direction.

These steps need to be repeated in 30 – 35 cycles. The very first denaturation step and the very last DNA synthesis step are usually prolonged to ensure that everything denatured at the beginning and that all the products were synthetized to the desired length at the end.

In 1993, an American biochemist Kary Banks Mullis and a Canadian biochemist Michael Smith shared Nobel Prize in Chemistry for the invention of PCR and site-directed mutagenesis.

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RFLP (Restriction Fragment Length Polymorphism)

RFLP allows to differentiate between DNA molecules from two or more donors by means of the analysis of the length of DNA fragments. These DNA fragments are prepared by cleavage of DNA samples from individuals in order to analyze variations in homologous DNA (polymorphisms). This cleavage is mediated by restriction endonucleases, thereby distinguishing inherited changes in DNA sequences. If DNA sequence contains a restriction site, site-specific restriction endonuclease can cleave it. In the case of a nucleotide change (a polymorphism) in the restriction site, DNA is not cleaved by specific restriction endonuclease. The fragments are visualized by agarose gel electrophoresis.

Practical part:

A/ Leukocytes isolation from unclotted blood

1/ Put freshly obtained unclotted blood into the centrifuge cooled down to 4°C and centrifuge it at 1500 ×*g*, 10 min.

2/ Remove plasma (the upper part) into the labeled tube and freeze it for further analyses (e.g., antibody assay).

3/ Take the middle part, referred as **buffy coat** (leukocytes, thrombocytes), into a 50 ml-tube and add **10 ml of 1× Ery** (the solution used for lysis of erythrocytes).

4/ Incubate the 50 ml-tube for 10 – 20 min on ice, until the solution becomes transparent.

5/ Centrifuge the solution at 240 ×*g*, 5 min, 4°C.

6/ Pour out the supernatant (the solution above the sediment=pellet) and add **5 ml of 1× Ery**, mix the pellet gently.

7/ Centrifuge the solution at 240 ×*g*, 5 min, 4°C.

8/ Pour out the supernatant, wash the pellet with **10 ml of PBS, pH 7.2** (phosphate-buffered saline – commonly used buffer).

9/ Centrifuge the solution at 240 ×*g*, 5 min, 4°C.

10/ Pour out the supernatant, wash the pellet with **5 ml of PBS, pH 7.2.**

11/ Centrifuge the solution at 240 ×*g*, 5 min, 4°C.

12/ Remove the supernatant carefully and completely.

13/ Resuspend the pellet containing leukocytes in **600 µl of PBS, pH 7.2**; keep **200 µl separately in the new 1.5-ml tube** (use it later for DNA isolation).

14/ Centrifuge the solution at 240 ×*g* , 5 min, 4°C and resuspend the pellet in **300 µl of RNAlater** (the solution for RNA storage for further analyses) and keep it in the fridge.

B/ DNA isolation from leukocytes

For total DNA isolation (genomic, mitochondrial), you will use **QIAamp DNA Blood Mini kit**. The purified DNA is suitable for PCR. The principle of DNA purification by this kit is DNA adsorption on the silica membrane under specific conditions (salts, pH), thereby removing other components of the lysate (such as proteins and lipids), which do not bind to the silica membrane.

1/ Mix gently **200 µl of leukocytes in PBS** prepared in the part A with **20 µl of QIAGEN Protease**.

2/ Add **200 µl of Buffer AL** (lysis buffer) and vortex for 15 s.

3/ Incubate at 56°C, 10 min.

4/ Centrifuge shortly and gently in order to remove drops from the cap.

5/ Add **200 µl of 96% ethanol** and vortex for 15 s, again remove drops from the cap by centrifuging.

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6/ Pipette the mixture carefully into the column (**QIAamp Mini spin column**) placed in 2 ml-tube (**collection tube**), close the column and centrifuge at 6000 ×*g*, 1 min.

7/ Place the column into the new 2-ml tube, trash the previous tube with the filtrate.

8/ Open the column carefully and add **500 µl of Buffer AW1** (DNA wash; removal of contaminants); close the column and centrifuge at 6000 ×*g*, 1 min.

9/ Place the column into the new 2-ml tube, trash the previous tube with the filtrate.

10/ Open the column carefully and add **500 µl of Buffer AW2** (DNA wash; removal of contaminants); close the column and centrifuge at 20 000 ×*g*, 3 min.

11/ Place the column into the new 1,5-ml tube; open the column carefully and add **200 µl of distilled water**.

12/ Incubate at room temperature for 5 min and then centrifuge at 6000 ×*g*, 1 min.

13/ Measure DNA concentration using the spectrophotometer NanoDrop 1000.

14/ Use isolated DNA in the following step (PCR).

C/ PCR

You are going to prepare PCR from all your DNA samples in order to amplify *PTPN22* sequence containing the polymorphism c.1858C>T.

1/ Pipette every reaction keeping the following order:

13.25 µl PCR water

5 µl Q5 buffer (5× concentrated)

0.5 µl dNTP (10 mM)

2.5 µl forward primer (10 µM)

2.5 µl reverse primer (10 µM)

0.25 µl Q5 DNA polymerase (2 U/µl)

1 µl DNA

2/ Mix and centrifuge shortly.

3/ Set the following program on the thermocycler:

	Temperature	**Duration**	**Number of cycles**
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	
Annealing	55°C	10 s	35
Extension	72°C	5 s	
Final extension	72°C	2 min	1

Cooling	4°C	Hold	

D/ Purification of PCR products

For purification of PCR products, you will use **Wizard SV Gel and PCR Clean-Up System (Promega).**

1/ Add the equal volume of **Membrane Binding Solution** to the PCR amplification.

2/ Insert the column (**SV Minicolumn**) into the 2-ml tube (**Collection tube**); pipette the mixture (step 1) into the column.

3/ Centrifuge at 16 000 ×*g*, 1 min.

4/ Add **700 µl of Membrane Wash Solution** and centrifuge at 16 000 ×*g*, 1 min.

5/ Pour out the supernatant (going through the column), and place the column into the same 2-ml tube. Add **500 µl of Membrane Wash Solution** and centrifuge at 16 000 ×*g*, 5 min.

6/ Pour out the supernatant again, and place the column into the same 2-ml tube; centrifuge at 16 000 ×*g*, 1 min.

7/ Insert the column into the new 1.5-ml tube and add **50 µl of Nuclease-Free Water**. Keep for 1 min at room temperature and then centrifuge at 16 000 ×*g*, 1 min.

8/ Trash the column. Use the purified PCR products for RFLP analysis.

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E/ RFLP

For RFLP analysis, you are going to use the **XcmI** restriction endonuclease.

1/ Pipette the reaction for every PCR product **keeping the following order**:

9	**µl**	**Purified PCR product**
1.1	**µl**	**NEB 10× CutSmart buffer**
1	**µl**	**XcmI**

2/ Incubate the restriction reaction for 3 hours at 37°C.

3/ Mix **4 µl of cleaved and non-cleaved PCR** with **1 µl of 6× loading dye** (a dye enabling visualization of the electrophoresis run and due to its high viscosity a sample can sink on the bottom of the well) and **1 µl od GelRed** (a fluorescent dye for UV visualization). Pipette the mixture into wells of 2% agarose gel.

4/ Pipette **3 µl of DNA marker** mixed with **0.5 µl of GelRed** into the outer well.