ÚSTAV LÉKAŘSKÉ BIOCHEMIE A LABORATORNÍ DIAGNOSTIKY 1. LF UK A VFN

Restriction fragment length polymorphism (RFLP)

Practical lesson on medical biochemistry

General Medicine

Martin Vejražka

Edited by Jan Plášteník

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Restriction fragment length polymorphism

Reagents:

PCR product for the DNA sample from the previous practical lesson

KpnI (endonuclease KpnI 0.3 U/µl, Tris-HCl 10 mmol/l pH 7.5, $MgCl_2$ 10 mmol/l, Triton X-100 0.02 %, albumin 0.3 mg/ml)

TaiI (endonuclease TaiI 0.3 U/µl, Tris-HCl 10 mmol/l pH 8.5, $MgCl_2$ 10 mmol/l, KCl 0.1 mol/l, albumin 0.3 mg/ml)

Control sample - fragment of λ-DNA

Loading dye 6× (e.g. bromophenol blue 0.1 %, glycerol 30 %, EDTA 100 mmol/l)

Procedure

You receive a micro tube with 25 µl of the PCR product. Take two more clean 200 µl micro tubes and measure 8 µl of the PCR product into each. Thus, you will work with three micro tubes in the following procedure: 8 µl of the PCR product will be used for restriction with endonuclease KpnI (mark "K"), another 8 µl for endonuclease TaiI (mark ("T"), and remaining 9 µl will be used as untreated control (mark "–").

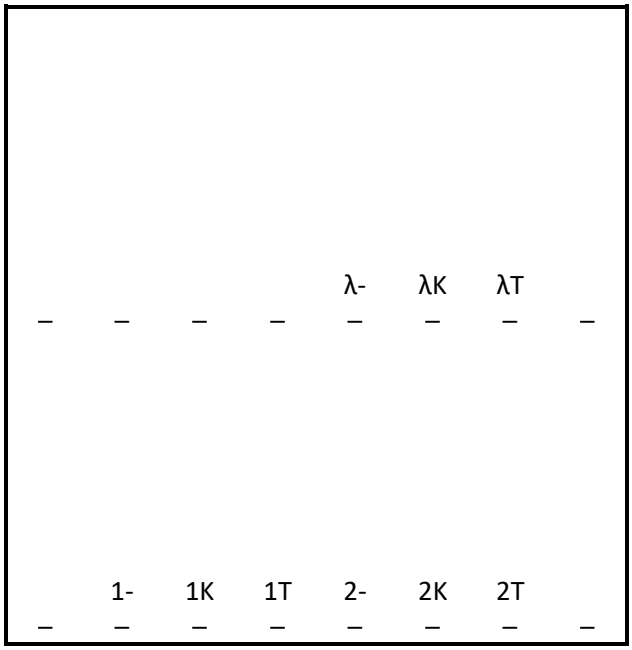
A positive control with a fragment of λ-DNA will be used to make sure that the restriction takes place. The fragment of λ-phage DNA is about the same length as our PCR product, and contains the recognition sequences for both endonucleases. The control will be performed once for several groups of students.

	Restriction of PCR product			**Positive control with λ-phage DNA** (set once for several groups)		
Label of micro tube	**K**	**T**	**–**	**λK**	**λT**	**λ–**
	Restriction KpnI	Restriction TaiI	Untreated product	Control restriction KpnI	Control restriction TaiI	Untreated λ-DNA
PCR product	8 µl	8 µl	remaining 9 µl	8 µl	8 µl	8 µl
KpnI	8 µl			8 µl		
TaiI		8 µl			8 µl	
	Spin on centrifuge (5 s)					
Incubation 30 min	37 °C block	65 °C cycler	4 °C block	37 °C block	65 °C cycler	4 °C block
Loading dye	3 µl	3 µl	3 µl	3 µl	3 µl	3 µl
	Spin on centrifuge (5 s)					

Evaluation of restriction by electrophoresis

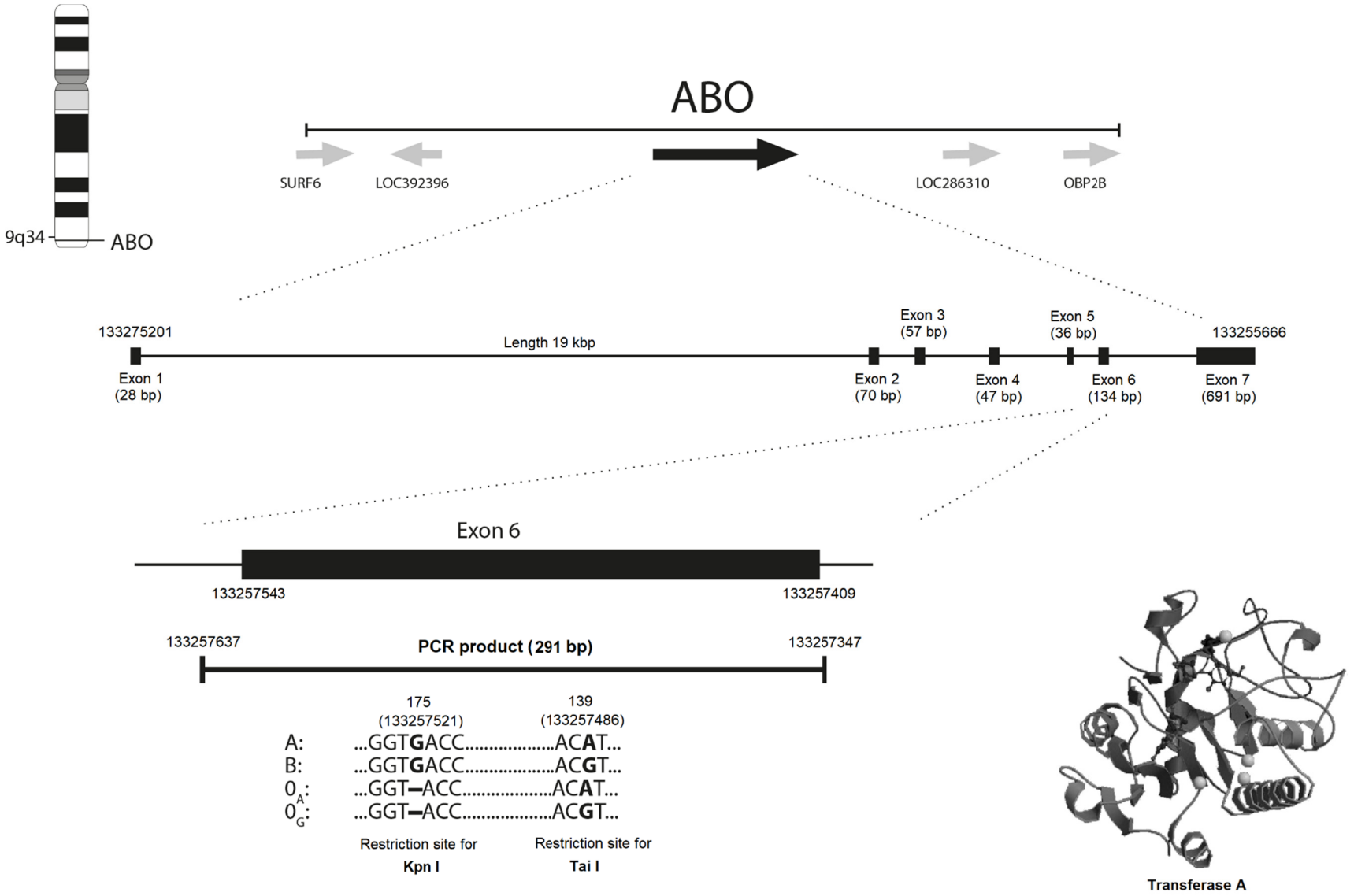
Restriction fragments will be separated by electrophoresis in 2% agarose gel (see the separate instructions below). One gel will be used by two groups of students. Samples from all three micro tubes ("K", "T" and "–") which contain the same PCR product, should be applied to neighboring wells in order to compare position of bands easily; the same applies to all three micro tubes with λ-DNA.

An example of arrangement of samples in the gel is provided in the figure. In this case, the gel was used for evaluation of restriction of two PCR products (marked 1 and 2) and for the positive control with λ-DNA. Some wells remained unused.



The length of our untreated PCR product is 291 bp. KpnI cleaves the sequence GGTACˆC that is present in alleles O_A and O_G. TaiI cleaves ACGTˆ that is found in B and O_G. If the corresponding restriction sites are present, both endonucleases will cleave the PCR product approximately in the middle. In both cases the lengths of the resulting fragments are so similar that in electrophoresis may not be separated from each other, and appear as a single band. On the other hand, the sizes of the cleaved fragments are markedly different from the length of the original PCR product, so that it is possible to distinguish whether cleavage has occurred or not.

If the PCR product treated with endonuclease yields a band in the same position as untreated product, DNA was not cleaved because it does not contain the recognition sequence for given endonuclease. Such result is recorded as KpnI –/–, or TaiI –/–. In case the band of treated DNA is farther from the start than the untreated one, DNA was fully cleaved by endonuclease. Such result is recorded as KpnI +/+ or TaiI +/+. In case the PCR product contains both DNA with and without the recognition sequence (i.e., it is heterozygous) two bands will appear: one in the position of uncleaved DNA, and another one farther from the start. Such result is recorded as KpnI +/– or TaiI +/–.

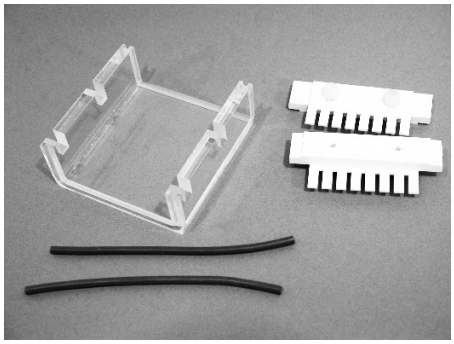

9q34
ABO
ABO
SURF6
LOC392396
LOC286310
OBP2B
133275201
Length 19 kbp
133255666
Exon 1
(28 bp)
Exon 2
(70 bp)
Exon 3
(57 bp)
Exon 4
(47 bp)
Exon 5
(36 bp)
Exon 6
(134 bp)
Exon 7
(691 bp)
Exon 6
133257543
133257409
133257637
PCR product (291 bp)
133257347
175
(133257521)
139
(133257486)
A: ...GGTGACC.....ACAT...
B: ...GGTGACC.....ACGT...
0A: ...GGT–ACC.....ACAT...
0G: ...GGT–ACC.....ACGT...
Restriction site for
Kpn I
Restriction site for
Tai I
Transferase A

From the results of both restrictions the genotype ABO can be determined:

	KpnI +/+	**KpnI +/–**	**KpnI –/–**
TaiI +/+	$O_G O_G$	B O_G	B B
TaiI +/–	$O_A O_G$	A O_G or B O_A	A B
TaiI –/–	$O_A O_A$	A O_A	A A

For combination KpnI +/–, TaiI +/– the genotype cannot be determined using this simple experiment only (it can be genotype A O_G corresponding to phenotype of blood group A, as well as genotype B O_A associated with blood group B). It is necessary to know phenotype or to employ other diagnostic methods (e.g. double digestion with both enzymes simultaneously).

Electrophoresis of DNA in 2% agarose gel



Reagents and tools:
TBE buffer 0.5× (Tris-HCl 44.5 mmol/l, boric acid 44.5 mmol/l, EDTA 1.25 mmol/l, pH 8.4)
Agarose
GelRed® 10,000×
Equipment for horizontal electrophoresis and accessories, Erlenmeyer flasks

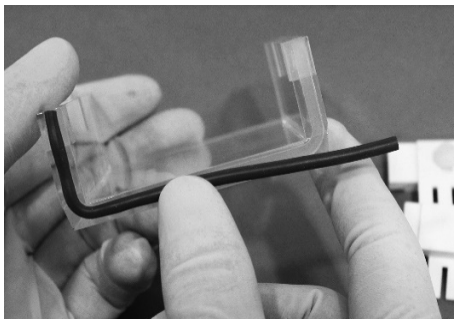
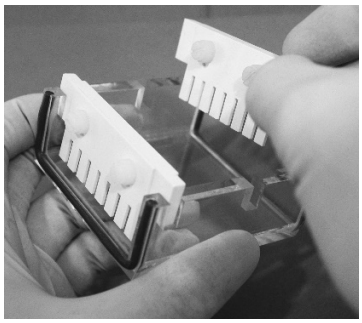
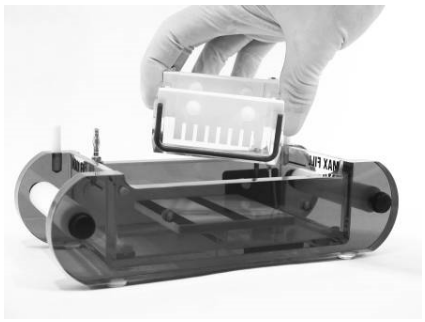
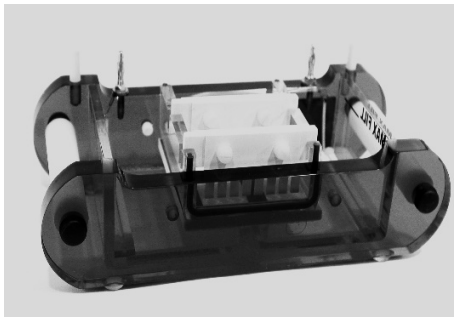
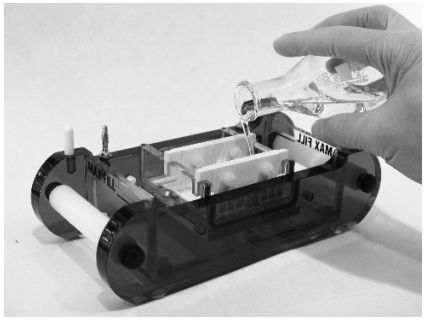
Preparing the gel

2% agarose gel containing fluorescent dye GelRed will be used. Agarose solution is always prepared for casting two gels.

1. In Erlenmeyer flask, mix 1 g of agarose (already pre-weighed) and 50 ml of the buffer.
2. Heat to boiling in the microwave owen. The mixture needs to get boiling, but the heating must be switched quickly after that in order to prevent overflow of the hot gel.
3. Mix the hot gel by whirling the flask. The gel must be homogenous: if undissolved grains of agarose are still apparent, repeat the heating step.
4. Add 2 µl of GelRed dye. Mix gently. Now, agarose is ready to be poured. Solution must get cooled down below 60 °C (it is possible to hold the flask in a hand), otherwise a damage to the electrophoretic chamber can occur.

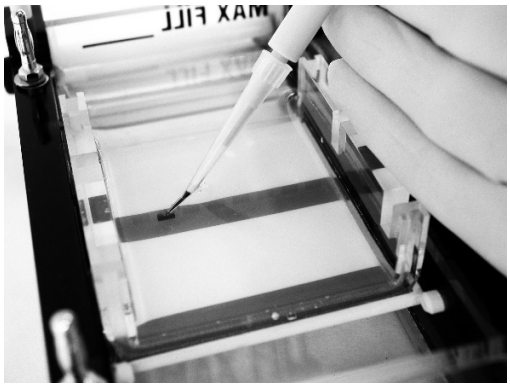
Pouring the gel

1. Set up the tray for casting the gel: silicone sealing is placed on both sides of the tray. Then, insert one or two combs; the first comb is placed so that the wells are close to the edge of the tray. Place the tray to the electrophoretic unit so that the sealing adheres to the wall of the unit.
2. Check that the unit is in horizontal position. Pour the solution of agarose with GelRed.
3. Allow the gel to solidify for about 20 minutes. Do not move or disturb the unit during this time.

Preparing the gel for electrophoresis and application of samples

1. Remove the tray from the unit and take out the sealings. Rotate the tray by 90° and reinsert it to the unit in such a way that the wells are above the colored strips on the support.
2. Pour 250 ml of the buffer to the unit.
3. Careful remove the combs. Pay attention not to damage the wells.
4. Apply samples to the wells (10 µl per well). Record the order of samples in the gel.



Electrophoresis and evaluation

1. Close the lid of the unit. Connect it to the power supply. Set the voltage to 90 V.
2. Electrophoresis should run about 40 minutes. The dye should move to approximately 1 cm from the end of the lane. Then, switch off the power, disconnect the cables, and remove the tray with gel.
3. Carefully remove the gel from the tray and place it on the screen of the transilluminator. Observe the position of bands and take pictures. It is necessary to work quickly because the bands will fade during several minutes of UV illumination.