MOLECULAR BIOLOGY PRACTICAL LESSONS – SS 2025

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Name:_____ Group:_____

Day:_____

1	*Excercise 1*. Introductory week (seminar)	DPD Toxicity Tools: PubMed, Gene Sequence (c.DNA/g.DNA). Who does not want to analyze himself, follow instructions
2	*Excercise 2.2.* DNA isolation (theory) *Excercise 3.2.1.* Measuring of DNA and dilution	*Theory*: DNA isolation from tissues
3	*Excercise 3.2.2* DNA electrophoresis *Excercise 4.2.1.* PCR	*Theory*: PCR
4	*Excercise 4.2.2.* Elfo PCR *Excercise 5.2.1.* Restriction rxn PCR	*Theory*: RE/RFLP
5	*Excercise 5.2.2.* Elfo RE *Excercise 6.2.1.*, 6.2.2. Sequencing reaction	*Theory*: sequencing - Sanger SW Finch, examples of mutations
6	*Excercise 6.2.2.* Precipitation and Seq Run	*Theory:* Cloning, CRISPR.
7	*Excercise* 7. Evaluation	Test, Evaluation, Case reports

Content

1 Introduction

The study of genetic information has a special position in medical diagnosis. Unlike most biochemical examinations that reflect current events in the body, genetic material brings information about the unchanging characteristics of individuals or entire families. Analysis of the genetic material of a particular individual often contains sensitive personal data, which must be handled appropriately. In the light of these aspects, we can divide the genetic analysis into several groups:

1. **Analysis of hereditary genetic information** – DNA isolated from any nuclear body cells (most commonly from peripheral blood leukocytes or epithelial cells of the buccal mucous membrane) can be used, to which apply the notes above. The analysis of the hereditary information and the interpretation of the results of these examinations is the domain of medical genetics**.**

2. **Analysis of somatic genetic information** that comes from changes in the genome in specific tissues during the life period of the person under investigation. The identification of somatic changes in the genome is an important diagnostic tool for the characterization of tumor diseases. In this type of examination, it is required to take the genetic material from the tumor cells derived from the tumor.

Somatic DNA alterations can also be identified in circulating tumor cells (eg. for the diagnosis of minimal residual disease) or circulating DNA released from tumor cells into the blood stream (cfDNA).

The analysis of somatic DNA alterations (and possible epigenetic modifications of DNA) lies, in terms of interpretation, on the edge between the analysis of inherited changes (which can be captured as a "secondary finding" in the examination of somatic DNA mutations) to classical biochemical examinations. Pathology is a dominant field for analysis of somatic changes of genetic and epigenetic information of tumor diseases.

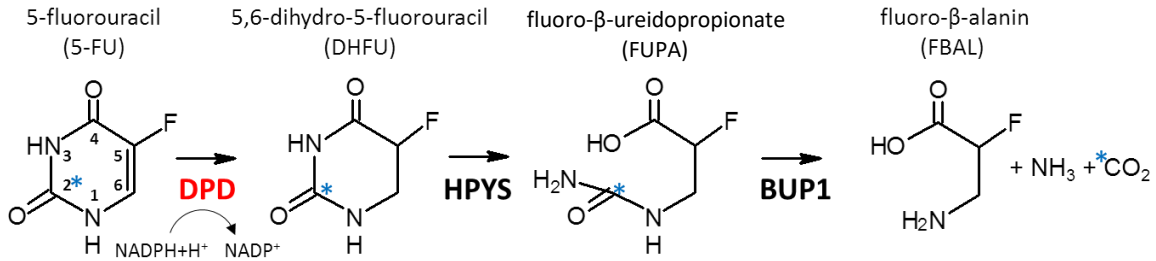
3. **Analysis of allogeneic genetic information** identifies and characterizes the presence of a wide range of foreign DNA in the organism. This foreign DNA may be genetic information of pathogens (eg. bacteria, viruses, yeasts, parasites) causing human diseases. However, the presence of fetal cfDNA in mother circulation can also be characterized by molecular biology methods. Specialized molecular biological assays analyzing foreign DNA, the results of which can be interpreted similarly to classical biochemical examinations, are performed by a number of disciplines, including pathology, infectious, hygienic, acute, forensic or reproductive medicine**.**

The aim of the practical lessons is to demonstrate basic molecular biological approaches and techniques. As a modeling problem we will be able to analyze genetic variants of the *DPYD* gene that affect the activity of the enzyme dihydropyrimidine dehydrogenase (DPD) in the degradation of fluoropyrimidines.

Fluoropyrimidines (eg. **5-fluorouracil**; **5-FU** or capecitabine) are one of the primary cytotoxic agents in the treatment of cancer, including colorectal cancer. In addition to standard therapeutic regimens, they are also used in the form of adjuvant treatment in patients with a high chance of long-term survival without relapse of cancer. Fluoropyrimidines represent effective and economically advantageous anticancer drugs. However, their administration is associated with a number of serious and approximately 1% life-threatening side effects,

including primarily blood vessel cell depletion (including bone marrow depression) and mucosal epithelial defects (stomatitis/mucositis) that cause serious diarrhea when the intestinal epithelium is affected. Approximately 25% of treated patients may have side effects so severe that intensive and costly treatment, including hospitalization, is necessary.
The mechanism of antitumoral action of 5-FU is based on the production of fluorinated nucleotide analogues (FdUMP, FUTP, FdUTP) directly causing DNA and RNA synthesis disorders. Fluorinated nucleotide analogues also inhibit thymidylate synthase which uses uracil (or dUMP) substrate for thymine (or dTMP) formation.
The inherited mutations affecting genes encoding catabolic enzymes of 5-fluorouracil (5-FU) degradation are the major **cause of the occurrence of severe toxicity following fluoropyrimidine** administration. The key enzyme of 5-FU catabolism is the initial reduction of 5-FU to DHFU catalysed by **dihydropyrimidine dehydrogenase (DPD**, EC 1.3.1.2, Figure 1). DPD provides degradation of uracil and thymine under normal conditions. This enzyme is encoded by the *DPYD* gene.

Fig. 1. 5-FU degradation pathway



Carriers of *DPYD* variants on one allele [eg. c.1905+1G>A (IVS14+1G>A)], which reduce the catalytic activity of DPD, are normally quite capable of degrading naturally occurring pyrimidines. The reduced catalytic capacity of the enzyme only occurs when high levels of pyrimidines are used, as is the case with 5-FU. For 5-FU mutation carriers, the 5-FU systemic concentration increase due to decreased degradation. Extending exposure to elevated doses of fluoropyrimidines beyond the toxic uptake limit causes mitotic high-activity tissue cell growth disruptions, which result in undesirable effects of the treatment.
***DPYD* gene** has a size over 800 kbp (843 317 bp; sequence https://www.ncbi.nlm.nih.gov/gene/?term=DPYD) and is located on the 1st chromosome (1p21.3; http://omim.org/entry/612779).

In practical lessons, we will examine three areas of the *DPYD* gene that contain known variants leading to the synthesis of enzyme isoforms that cause a reduction in the catalytic activity of DPD. We will discuss the results of genetic analyzes. Examination will be performed on a sample of DNA isolated from the students´ own buccal mucosal cells. For analysis, we will also use samples from patients who have been treated with fluoropyrimidines and who have developed serious side effects during this therapy.

For each practical lesson you will bring:

- This printed manual
- Marking indelible thin fix
- Disposable gloves

2 DNA isolation from buccal mucous membrane

2.1 THEORY

The isolation of DNA or RNA is the basic step of all molecular-biology analyses that examine the genetic material. Isolation of genomic DNA can be performed from any material containing nuclear cells (blood, tissue, urinary sediment) or their fragments or from samples containing free DNA (e.g. serum). Generally, genomic DNA is isolated from peripheral incoagulable blood from the leukocytes. Buccal mucous membranes are a non-invasive source of nuclear cells for genomic DNA isolation.
DNA isolation from buccal mucosal epithelial cells requires disruption of the cell‘s intercellular mass to release individual cells (by proteinase K)[1]. This step is not necessary for DNA isolation from dispersion tissues (blood or bone marrow) or cell suspensions. Subsequently, DNA has to be released by disrupting the cytoplasmic and nuclear membrane and releasing DNA from nucleoprotein complexes (by precipitating proteins). Released DNA is then precipitated by non-polar solvents (isopropanol or ethanol) from the aqueous solution. The DNA precipitate, after being purified from the contaminants (nonpolar solvent) and carefully dried, is dissolved in redistilled water. The resultant solution of dissolved DNA is necessary to characterize – to determine the concentration of DNA and its fragmentation.
We use the modified Promega Wizard Genomic DNA Purification Kit protocol to isolate genomic DNA; A1125; manual). Isolation takes place in several steps:

1. Disruption of tissue (proteinase K), lysis of the cells and nuclei (Nuclei Lysis Solution)
2. Removal of proteins by precipitation (salting out with salt)
3. DNA precipitation and desalting with isopropanol
4. Dissolving DNA in water

Working with genetic material puts high demands on the precision and purity of laboratory procedures. The basis for most nucleic acid analyzes is the subsequent amplification of the target DNA segment. Amplification means amplification of the selected target sequence in the order 10^5 – 10^7. For this reason, it is necessary to maintain strictly sterile conditions (gloves, sterile solutions, sterile tubes, and sterile pipette tips) so that the analyzed genetic material is not contaminated with foreign DNA (e.g. another sample) or DNA from "external environment".

2.2 WORKFLOW

1. Write down the **number of your samples** assigned by your assistant:_____.
2. Mark the **2 ml** tube with the assigned number and pipette into it:
   a. 300 µl PBS,
   b. 300 µl Nuclei Lysis Solution,
   c. 15 µl Proteinase K.
3. Obtain samples of buccal mucous membranes by **scraping off buccal mucous cells by circular motion** (approx. 1 min) using a special spatula. CAUTION: *Wash out your mouth with water before sampling. The recommended time between sampling and last food intake is at least 10 minutes.*
4. **Insert the spatula into the tube** prepared in step 1 and break the handle.
5. Close the tube, **vortex it** (20 s) and **incubate it** in a dry block for **30 min** at 55°C.
6. **Centrifuge 1 min at 13 000 rpm** (maximum speed). CAUTION: *Place the test tubes in all centrifuges so that the lid points towards the center of the centrifuge.*
7. **Transfer the supernatant (approx. 750 µl)** with a pipette to a **new 1.5 ml tube** marked with your number. Discard the tube with the remainder of the spatula.
8. **Add 200 µl** Protein Precipitation Solution to the supernatant and **immediately** vortex for 20 s. NOTE: *The solution should be cloudy*.
9. **Centrifuge 4 min at 13 000 rpm** (maximum speed). CAUTION: *Place the test tubes in all centrifuges so that the lid points towards the center of the centrifuge.*
10. Protein pellet is placed on the bottom of the tube. **Carefully** transfer **supernatant containing DNA** with the pipette set to 700 µl **into a clean 1.5 ml tube** labeled with your number. Discard the tube with protein pellet.
11. Add **800 µl isopropanol** to the supernatant and invert the tube **carefully** until you see a forming DNA clot. CAUTION: *The DNA precipitate will be (poorly) visible as a miniature fibers or flakes*.
12. **Centrifuge 4 min at maximum speed**. CAUTION: *Place the test tubes in all centrifuges so that the lid points towards the center of the centrifuge.*
13. DNA pellet is (poorly) visible on the wall of the tube. **Carefully spill** the supernatant (do not pipette!) into the waste beaker. **Pellet should stay on the wall**. Dry the tube mouth with the filter paper.
14. Pipette **300 µl of 70% ethanol** into the pellet. Invert the closed tube several times **gently** to wash the pellet.
15. **Centrifuge 2 min at maximum speed**. CAUTION: *Place the test tubes in all centrifuges so that the lid points towards the center of the centrifuge.*
16. **Carefully spill the supernatant** into the waste beaker**. Carefully** dry the inside of the tube with filter paper rolled with a tweezer. **Do not touch the DNA pellet.**
17. Let the open tube stand on the table until ethanol residue evaporates (about 10 minutes). CAUTION: *Be carefull not to overdry the DNA, otherwise it will be poorly soluble.*
18. Pipette **50 µl of rehydration solution** to the DNA pellet. Close the tube and vortex gently, let the DNA dissolve in a dry block at 50°C. Store the isolated DNA at 4°C for further analysis.

3 Electrophoresis and DNA quantification

3.1 THEORY

For further analysis, it is necessary to determine the concentration of isolated nucleic acid, to verify its purity and quality – integrity.

The nucleic acid concentration is determined spectrophotometrically. Nucleic acids absorb due to the presence of nitrogen bases in the ultraviolet region of the spectrum with an absorbtion maximum at 260 nm (Figure 2).

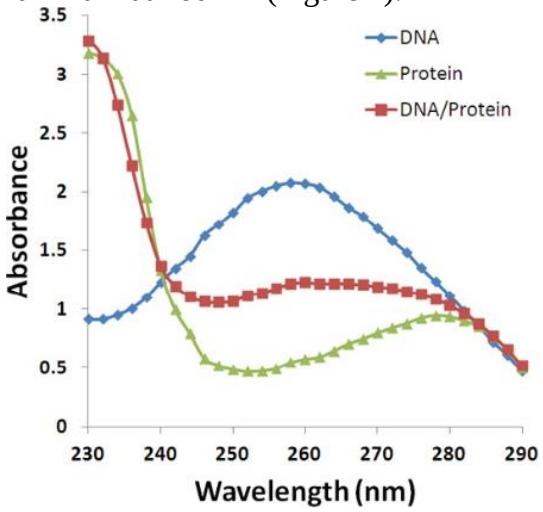


Fig. 2. Absorption graph of DNA, protein and DNA/protein mixtures (1:10), depending on the wavelength in the ultraviolet spectrum 230-290 nm (source).

To determine the **concentration** of isolated **DNA**, we measure the absorption of the solution in a 1 cm cuvette, where the unit optical density (OD) is equivalent to a 50 µg/ml of double stranded DNA solution, i.e. 1 $OD_{260\ nm}$ dsDNA = 50 µg/ml.

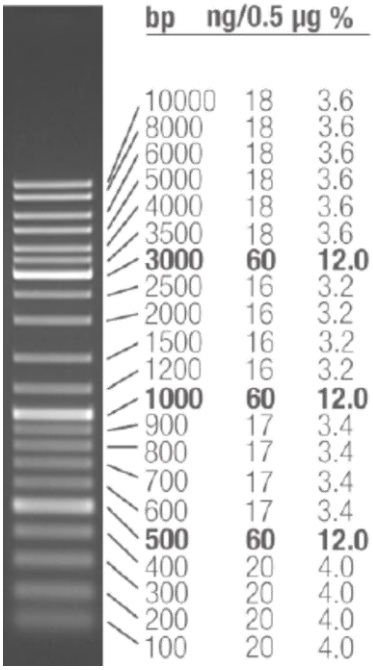
To determine the **purity** of **DNA** we need to know the protein contamination. The proteins reach the absorption maximum at 280 nm (Figure 2), therefore we also perform the evaluation of the ratio of nucleic acids $OD_{260\ nm/280\ nm}$. This ratio should be about 1.8 in pure DNA solution.

Gel electrophoresis is used to verify nucleic acid integrity by separating DNA fragments according to their length in a DC (direct current) electric field onto a gel matrix (agarose or polyacrylamide gel). The selection of the gel matrix depends on the length of the analyzed nucleic acid. In practice, we will only perform agarose electrophoresis capable of separating DNA fragments from 10 bp to 20 kbp. The rate of DNA migration (~ 7 V/cm gel length) is inversely proportional to the length of the DNA fragments (shorter traveling faster).

DNA applied to electrophoresis must be first mixed with sample buffer to increase the density of the sample. As a result, the solution of the analyzed DNA falls down on the bottom of the well due to increased density. The added colors (bromophenol blue and xylene cyanol) enable the visual inspection of the electrophoresis process (they travel together with DNA to the anode). Bromophenol blue migrates in 1% agarose gel (in 1x TBE buffer) with ~ 300 bp dsDNA, xylene cyanol with ~ 4 kb dsDNA.

Size determination of the fragments of analyzed DNA is performed by comparison with the DNA size standard containing fragments of known length (Figure 3).

A.
For genome DNA and PCR



B.
For restriction

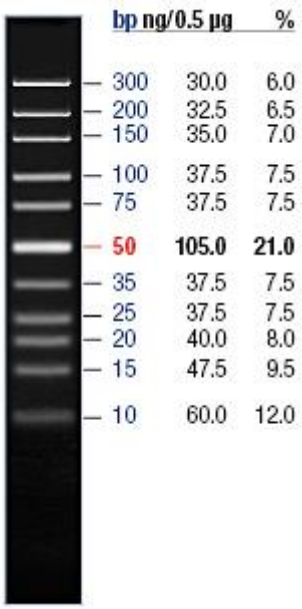


Fig. 3. DNA size standards for DNA length evaluation by gel electrophoresis used in the practical lessons. We use appropriate size standards for genomic DNA analysis:

A. For genome DNA and PCR products analysis: O'GeneRuler DNA Ladder Mix (100 bp – 10 kbp; ThermoFisher Scientific; #SM1173). 1% agarose gel

B. For analysis of products of restriction reaction: GeneRuler Ultra Low Range DNA ladder (10 bp – 300bp; ThermoFisher Scientific; #SM1213). 5% agarose gel

Ethidium bromide is used for visualization of double-stranded nucleic acids. It is an **intercalating agent** that is bound between planar-oriented DNA/RNA base pairs linked by hydrogen bonding (Figure 4). After illumination of the gel with UV light, ethidium bromide emits bright orange light at its high concentration sites, which means at the sites where DNA is located.

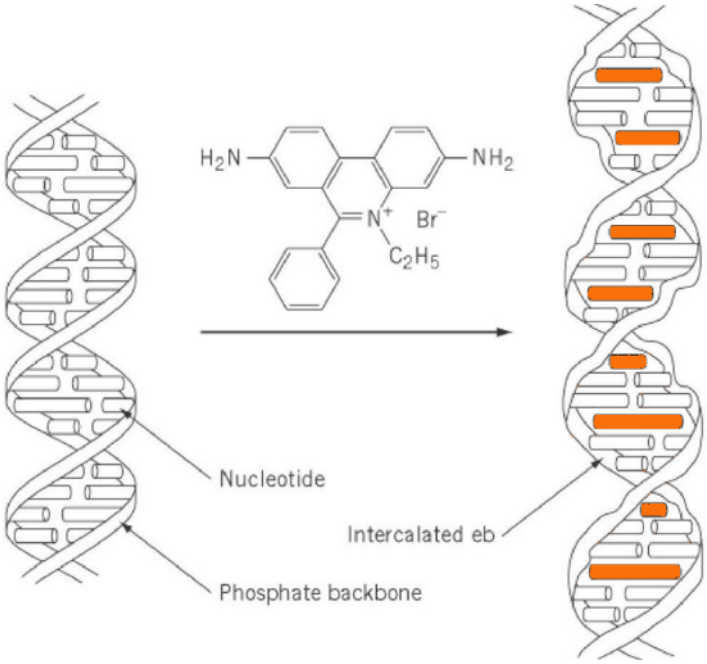


Fig. 4. The ethidium bromide molecule (eb) is intercalated between the DNA base pairs (right). http://what-when-how.com/molecular-biology/ethidium-bromide-molecular-biology/.

For the visualization of DNA (or RNA), the non-toxic GelRed fluorescence dye can be used instead of the mutagenic ethidium bromide as performed in the task.

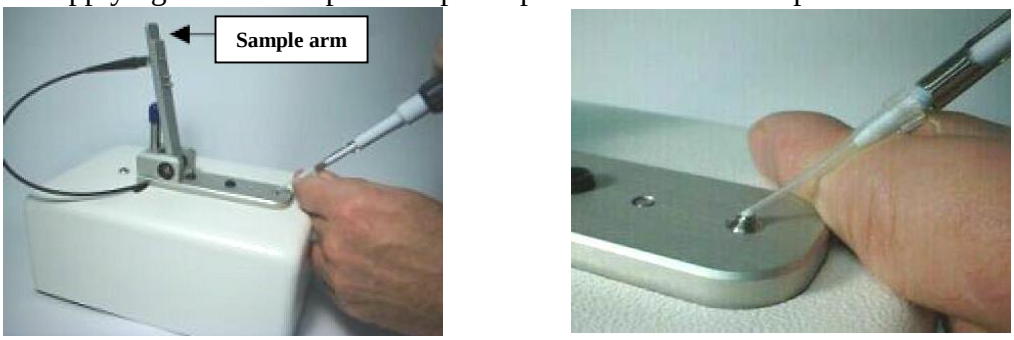
3.2 WORKFLOW

3.2.1 Spectrophotometric determination of DNA concentration

Quantification of isolated DNA is performed using a UV/VIS spectrophotometer NanoDrop 1000 (Thermo Scientific), which enable quantification of samples in a micro volume of 1 µl.

1. Lift the "sample arm" of the instrument and (0-2 µl) **apply 1 µl of DNA sample to the lower measuring area** by pipette (see Fig. 5):

Fig. 5. Applying a DNA sample to a spectrophotometer NanoDrop 1000



2. **Close** the sample arm, click on "MEASURE" and write down the sample DNA concentration and its 260/280 nm purity on the computer screen.
3. Clean both measuring surfaces with a square of pulp before further measuring.
4. Write the parameters of your isolated DNA into the table below:

sample no.	**$OD_{260\ nm}$**	**$OD_{280\ nm}$**	**µg/ml**	**$OD_{260\ nm/280\ nm}$**

5. **Dilute the DNA** concentration to **50 ng / µl**. Count dilution:

Your DNA sample (volume)_____µl
ddH_2O (volume) _____µl

3.2.2 Control of DNA integrity by agarose gel electrophoresis

Reagents:

- sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol)
- TBE buffer (Tris-borate 0.04 mol/l, EDTA 0.001 mol/l)
- 1.0% agarose gel in 1xTBE containing GelRed Nucleic Acid Gel Stain (Biotium)
- ddH2O

The sample mixed with the sample buffer is applied to the wells in an agarose gel placed in an electrophoresis bath containing 1xTBE buffer.

1. **Mark** the sterile 0.2 ml micro-tube on the cap with your DNA number: _____ (number).
2. Pipette **2 µl of blue sample buffer** and **3 µl of your DNA** into the labeled tube.
3. **Mix** on the vortex and **briefly centrifuge** at 13 000 rpm.
4. Apply **the entire sample volume (5 µl) to the bottom of the well** in agarose gel placed in an electrophoresis bath containing 1xTBE buffer. DO NOT APPLY THE SAMPLE TO THE FIRST WELL ON THE LEFT.

5. **Write down the position** of your DNA sample in gel:
   (from the left):_____, row (from top):_____.
6. Connect the electrophoresis tub to an electrical current source (80-100 V).
7. Stop electrophoresis when bromophenol blue reaches the end of the gel.
8. Remove the gel and **check it under the UV** light (on the transducer in the photoroom).
9. Print the electrophoresis image of your DNA (posted on MS Teams study group folder) and paste it into the protocol:

Electrophoresis of DNA. Date_____.

10. **Mark your sample with the arrow, mark the sizes of the size standard fragments** (in bp as shown in Fig. 3A).
11. **Briefly write down the result** of your DNA isolation (method, yield, purity, integrity, size):

4 PCR amplification of selected gene segments *DPYD*

4.1 THEORY

Polymerase Chain Reaction (PCR) is the enzymatic synthesis of DNA used to amplify the selected DNA fragmnet *in vitro*. Currently, PCR is quite routine and probably the most widespread molecular biology technique, its primary task is to amplify a defined DNA segment for its further analysis.

The target segment of amplified DNA (usually dsDNA) is defined by a pair of oligonucleotides (**primers**) that hybridize (annealing) to complementary **DNA template strands** after thermal denaturation of the DNA. Primers are artificially prepared (custom-synthesized) ssDNA oligonucleotides complementary to the target DNA segment. The primer length is 18-30 b (a logical condition for their synthesis is knowledge of the DNA sequence studied). The primers indicate sites for attachment of **a thermostable DNA-dependent DNA polymerase** which synthesizes complementary DNA strand from the 3'end of the primer. The polymerization proceeds in the 5' to 3' direction by linking the nucleotides (as in vivo replication). The DNA polymerase substrate is deoxynucleotide triphosphates (**dNTPs**, Fig. 6) incorporated into the growing polynucleotide.

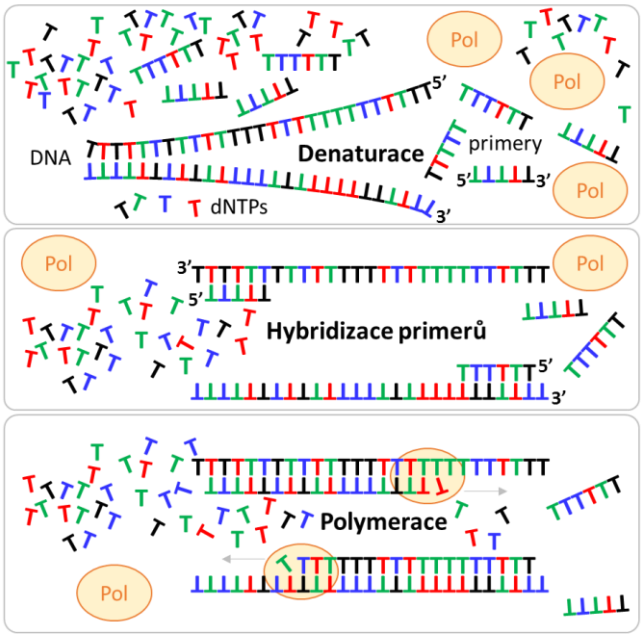


Fig. 6. PCR scheme. Necessary **PCR components** are template DNA, primers (forward and reverse), thermostable polymerase (Pol), dNTPs. In addition, the PCR contains Mg^{2+} ions (Pol cofactor) and buffer to maintain optimal pH.

PCR begins with **dsDNA denaturation**. The dissociated ssDNA subsequently **hybridize with primers** that serve to mount a thermostable polymerase that provides the synthesis (**polymerization**) of the complementary DNA strand. The exponential amplification of the target DNA segment occurs by repeating the individual steps of the PCR cycle – denaturation, primer hybridization (annealing), polymerization.

The introduction of PCR allowed the discovery of thermostable DNA polymerase, **Taq DNA polymerase**, isolated from thermophilic bacteria ***Thermus aquaticus***. The Taq polymerase polymerization rate is 2 - 4 kbp/min.

Amplification of the target fragments of the DNA, defined by primers, is accomplished by multiple repeating of **the PCR cycles.** Since the products of each PCR cycle serve as templates for subsequent cycles, the number of copies of the amplified region increases exponentially (2^n). The PCR cycle consists of three sections with different incubation temperatures:

1. thermal **denaturation** of target dsDNA (dissociation dsDNA to ssDNA at 95°C)
2. **hybridization** of primers (annealing) to ssDNA (at 50°C – 72°C)
3. **polymerization** using thermostable DNA-polymerase (at 72°C).

Automatic repeating of individual cycles allows **PCR-thermocyclers**. The length of PCR-amplified regions is several tens of bases up to 10 kb.

In practical lessons we perform PCR amplification of three segments (fragments) of the *DPYD* gene. Variants known for causing formation of isoforms of protein DPD with decreased catalytical activity of DPD are located in these fragments.
These fragments are (see sequence of *DPYD* page 15):

intron 10 (**i10**) with variant c.1129-5923 C>G/hapB3 (position in gene 341167)
exon 13 (**E13**) with variant c.1679T>G (position in gene 405273)
exon 14 (**E14**) with variant c.1905+1G>A (IVS14+1G>A)

For amplification we use following primers:

Amplification of i10:

i10f: 5'-CACTCAGCATCAGCCACATATC-3'
i10r: 5'-TGAGGGACAACTGGTTTATCAAGC-3'

Amplification of E13:

E13f: 5'-AGATGTAATATGAAACCAAGTATTGG-3'
E13r: 5'-TTAATGTGTAATGATAGGTCTTGTC-3'

Amplification of E14:

E14f: 5'-CTGCAAAAATGTGAGAAGGGACC-3'
E14r: 5'-TCACCAACTTATGCCAATTCTCTTG-3'

On the next page there is a section of genomic DNA coding selected *DPYD* fragments (the entire gene (also translated) is in its original form on the NCBI/Gene). The nucleotide sequence is shown in **`black`** (numbers refer to nucleotides in the *DPYD*), the protein sequence is in **`blue`** (numbers refer to amino acids in the DPD protein).

In the sequence on page 15, <u>find and mark</u>:

1. **PCR primers** (forward and reverse) for amplification individual fragments
2. **Write down the borders of amplicons** (amplified segments) and **count their presumed lengths**:

   i10 (_____-_____) i.e. _____bp

   E13 (_____-_____) i.e. _____bp

   E14 (_____-_____) i.e. _____bp

3. Find and **mark consensus splicing sites.**
4. Find and **mark variant nucleotides**, where the desired variants of *DPYD* are found.

```
LOCUS       NC_000001              843317 bp    DNA     linear   CON 06-JUN-2016
DEFINITION  Homo sapiens chromosome 1, GRCh38.p7 Primary Assembly.
ACCESSION   NC_000001 REGION: complement(97077743..97921059) GPC_000001293
VERSION     NC_000001.11
DBLINK      BioProject: PRJNA168
            Assembly: GCF_000001405.33
KEYWORDS    RefSeq.
SOURCE      Homo sapiens (human)
```

```
341041 aatcatcgcattacataaagttaatcattaaaatgatgatcattattgcaagtttatagc i10

341101 atgctatttttatttcactcagcatcagccacatatctttcctgaatatggaggtgaaaa

341161 tcaaagctgagaatttttttaactttcccactactgtgtaaaaagaaagtgacaacctg

341221 atttgtcaaatgtaaaaaatggacccttatagctgcttactactttattggtggagacta

341281 ggaaaagagaggttggcatgtttgcttgataaaccagttgtccctcataaagacatgctc
```

```
   509                                                           S  E13
405061 atattatatggacaatttagatgtaatatgaaaccaagtattggtttgtattttgcagTC

   510  Q  Y  G  A  S  V  S  A  K  P  E  L  P  L  F  Y  T  P  I  D
405121 ACAATATGGAGCTTCCGTTTCTGCCAAGCCTGAACTACCCCTCTTTTACACTCCTATTGA

   530  L  V  D  I  S  V  E  M  A  G  L  K  F  I  N  P  F  G  L  A
405181 TCTGGTGGACATTAGTGTAGAAATGGCCGGATTGAAGTTTATAAATCCTTTTGGTCTTGC

   550  S  A  T  P  A  T  S  T  S  M  I  R  R  A  F  E  A  G  W  G
405241 TAGCGCAACTCCAGCCACCAGCACATCAATGATTCGAAGAGCTTTTGAAGCTGGATGGGG

   570  F  A  L  T  K  T  F  S  L  D  K
405301 TTTTGCCCTCACCAAAACTTTCTCTCTTGATAAGgtaagaaaatattattgaagtcatat

405361 agaaatgtctatcatatattttaattaaggtataaacattataatggatttttttacta

405421 agaaatactacttattaaactatttgacaagacctatcattacacattaatctttagttt
```

```
470701 aatgtatattaaaaattcctctgcaaaaatgtgagaagggacctcataaaatattgtcat
470761 atggaaatgagcagataataaagattatagcttttctttgtcaaaaggagactcaatatc

   581                 D  I  V  T  N  V  S  P  R  I  I  R  G  T  T  E14
470821 tttactctttcatcagGACATTGTGACAAATGTTTCCCCCAGAATCATCCGGGGAACCAC

   596  S  G  P  M  Y  G  P  G  Q  S  S  F  L  N  I  E  L  I  S  E
470881 CTCTGGCCCCATGTATGGCCCTGGACAAAGCTCCTTTCTGAATATTGAGCTCATCAGTGA

   616  K  T  A  A  Y  W  C  Q  S  V  T  E  L  K  A  D  F  P  D  N
470941 GAAAACGGCTGCATATTGGTGTCAAAGTGTCACTGAACTAAAGGCTGACTTTCCAGACAA

   636
471001 Cgtaagtgtgatttaacatctaaaacaagagaattggcataagttggtgaatgtttattt
```

4.2 WORKFLOW

4.2.1 PCR amplification of three fragments of the DPYD gene

Reagents:

- 5x PCR mastermix (5x HOT FIREPol® EvaGreen® qPCR Supermix; Solis BioDyne):
  - HOT FIREPol (Taq) DNA Polymerase
  - 5x PCR buffer (s 12,5 mM $MgCl_2$)
  - 5x dNTPs (2,5 mM of each dNTP)
  - EvaGreen dye, No ROX dye

1. Prepare **three sterile 0.2 m**l PCR micro-tubes.
2. **Mark the cap by your number (#)** and by the appropriate PCR number**: # -i10 | # -E13 | # -E14.**
3. **Calculate the composition of your PCR reaction**:

PCR reaction mixture	**_i10**	**_E13**	**_E14**
5x PCR mastermix	2,4 µl	2,4 µl	2,4 µl
1,8 pmol primer Forward (1 µM)	**i10f**: _____µl	**E13f**: _____µl	**E14f**: _____µl
1,8 pmol primer Reverse (1 µM)	**i10r**: _____µl	**E13r**: _____µl	**E14r**: _____µl
50 ng DNA (50 µg/ml)*	_____µl	_____µl	_____µl
ddH_20 ad 12 µl	_____µl	_____µl	_____µl
Final volume	12 µl	12 µl	12 µl

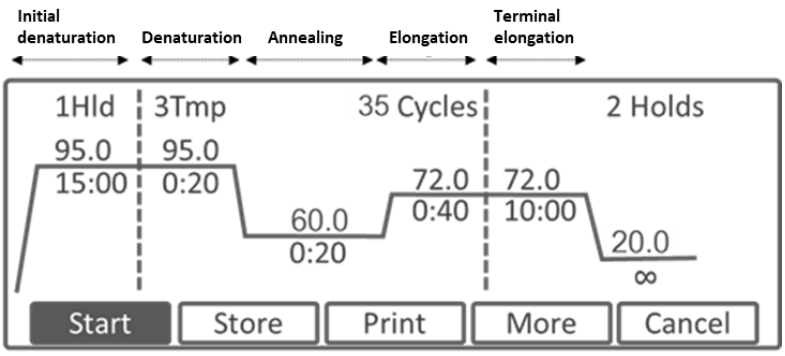
* see page 9. If the concentration of your DNA is too high to add <2 µl, dilute your DNA as needed (eg. 2 - 50x – consult with the assistant).

4. **Pipette** the reaction mixture (a pipette of 1-10 µl volume) **into the tubes according to the table in the following order**:
   1: ddH_20
   2. primers (2 pcs)
   3. PCR mastermix,
   4. 50 ng of your DNA

Perform for all three segments of the *DPYD* gene.

5. Mix the reaction mixture on vortex and briefly centrifuge.
6. Insert the tube into the GeneAmp PCR System 2700 Cycle Block and activate "DPYD_PCR" (Fig. 7).

Fig. 7. The protocol scheme of PCR cycler GeneAmp PCR System 2700



4.2.2 Electrophoresis of PCR products

Reagents:

- Sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol)
- TBE buffer (Tris-borate 0,04 mol/l, EDTA 0,001 mol/l)
- 1.5% agarose gel in 1xTBE containing GelRed Nucleic Acid Gel Stain (Biotium)
- ddH_2O

Workflow:

1. **Mark** three sterile 0,2 ml microtubes on cap with your number (#) and with the appropriate PCR number: **#-i10** | **#-E13**| **#-E14**.
2. **Pipette 2 µl of blue sample buffer** and **4 µl of your PCR product into the labeled tube**.
3. Mix on vortex and briefly centrifuge.
4. Apply **the entire sample volume (6 µl) to the bottom of the well** in agarose gel placed in an electrophoresis bath containing 1xTBE buffer. DO NOT APPLY THE SAMPLE TO THE FIRST WELL ON THE LEFT.
5. **Write down** the position of your DNA sample in the gel (fill in the number of your DNA).

   ____-i10 (position from the left):_____, row (from top)_____.

   ____-E13 (position from the left):_____, row (from top)_____.

   ____-E14 (position from the left):_____, row (from top)_____.

6. Connect the electrophoresis tub to an electrical current source (80 - 100 V).
7. Stop electrophoresis when bromphenol blue reaches the end of the gel.
8. Remove the gel and **check it under the UV** light (on the transducer in the photoroom).
9. Print the electrophoresis image of your PCR product (posted on MS Teams study group folder) amd paste it into the protocol:

Elektrophoresis of PCR products. Date_____.

10. **Mark by numbers your samples, mark the sizes of the size standard fragments** (in bp; according to Fig. 3B).
11. **Briefly enter the result** of your individual PCR (method, yield, size):

5 Restriction analysis of the PCR products of *DPYD* gene – intron 10, exon 14

5.1 INTRODUCTION

For subsequent detection of known varinats, sequencing and **restriction analysis**, i.e. fragmentation of the PCR fragment by **restriction endonuclease**, are used. **Restriction endonucleases are bacterial enzymes** that bacteria use to protect against viral (bacteriophage) infections, these enzymes are able to **recognize a particular DNA sequence at a so-called recognition site and then digest this DNA.** Recognition sites often have the character of genetic **palindromes**. Restriction enzymes cleave both DNA strands at the site of the phosphodiester bond in the polynucleotide.
Restriction analysis, i.e. restriction reaction is chosen to target the site of polymorphic sequence, change in this sequence leads to formation or destruction of a restriction site. Restriction reaction products are then separated on an agarose (or polyacrylamide) gel.
By restriction, we analyze the presence of *DPYD* variants in intron 10 (c.1129-5923C>G; IVS10-5923C>G) and exon 14 (c.1905+1G>A; IVS14+1G>A).

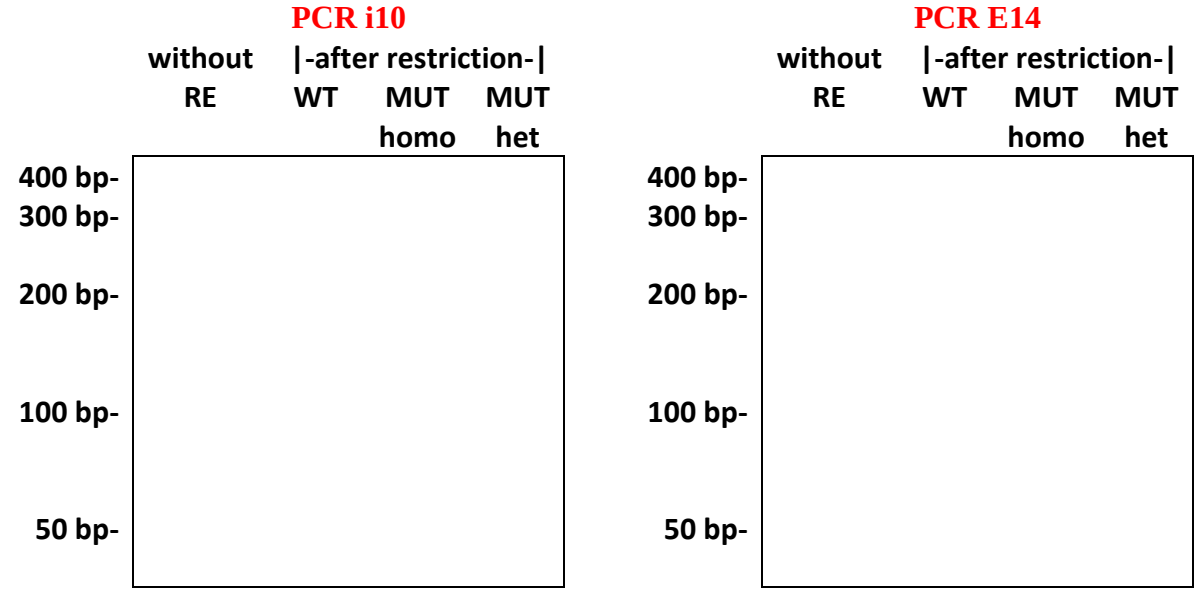
For restriction of PCR fragment i10 we use restriction enzyme AluI:
Endonuclease AluI recognizes sequence (cleavage site of DNA strand is represented by |):

5′...A G|C T...3′
3′...T C|G A...5′

For restriction of PCR fragment E14 we use restriction enzyme HpyCH4IV
Endonuclease HpyCH4IV recognizes sequence (cleavage site of DNA strand is represented by |):

5′...A|C G T...3′
3′...T G C|A...5′

Find and mark AluI resp. HpyCH4IV restriction site(s) of PCR products in a *DPYD* sequence (page 13). **Calculate** the lenght of products of restriction reaction considering in all three situations: **homozygous** carriers of both wt and mutant sequence (c.1129-5923C resp. IVS14+1G) and **heterozygous** carriers of mutant variant (c.1129-5923G resp. IVS14+1A). **Mark** expected PCR fragments after restriction cleavage and before (without) restriction (RE) and mark the lenght of fragment (in bp) **into the scheme of the electrophoresis:**



5.2 PROTOCOL

Reagents:

- 2x restriction mix AluI (R0137S; NEB):
  - 0.2 µl AluI (10 U/µl)
  - 1.0 µl 10X CutSmart® Buffer (1X: 50 mM Potassium acetate, 20 mM Tris Acetate, 10 mM Magnesium acetate, 100 µg/ml BSA, pH 7.9 @ 25°C)
  - 3.8 µl ddH_2O
- 2x restriction mix HpyCH4IV (R0619S; NEB):
  - 0.0125 ul HpyCH4IV (10 U/µl)
  - 1.0 µl 10X CutSmart® Buffer (see above)
  - 3.9875 µl ddH_2O

5.2.1 Preparation and incubation of restriction reaction

1. **Write** your number (#) and number of corresponding PCR **(#-i10R | #-E14R)** on the top of two sterile 0.2ml microtubes.
2. **Pipette 5 µl <u>of the CORRECT</u> restriction mix** into the tubes:
   For restriction of PCR fragment i10 use restriction enzyme AluI
   For restriction of PCR fragment E14 use restriction enzyme HpyCH4IV
3. Add by pipetting **5 µl of your <u>CORRECT</u> PCR products**. (For i10 use restriction mix with AluI, for E14 mix with enzyme HpyCH4IV).
4. **Vortex** and **centrifuge** briefly.
5. Place the tube into **cycler block** GeneAmp PCR System 2700.
6. Activate program "restriction" (Incubation in 37°C over night).

5.2.2 Electrophoresis of PCR products after restriction

Reagents:

- sample buffer (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol)
- TBE buffer (Tris-borate 0.04 mol/l, EDTA 0.001 mol/l)
- 2.5% agarose gel in 1xTBE containing GelRed Nucleic Acid Gel Stain (Biotium)
- ddH_2O

1. **Into each tube containing restriction reaction add 4 µl of blue sample buffer**.
2. Vortex both tubes and spin briefly.
3. **Apply the entire volume** of samples (14 µl) to the bottom of two adjacent wells in an agarose gel in electrophoretic apparatus with 1xTBE buffer. DO NOT APPLY ANY SAMPLE INTO THE FIRST WELL ON THE LEFT SIDE AND LAST TWO ONES ON THE RIGHT.
4. **Note** the position of the wells (fill in the number of your DNA).

   ____-i10R (position from the left):_____, line (from the top)_____.

   ____-E14R (position from the left):_____, line (from the top)_____.

5. Connect the electrophoretic apparatus to the source (80 - 100 V).
6. Stop the electrophoresis when the bromphenol blue reaches the end of the gel.
7. Remove the gel and check it in the UV light (transilluminator in the dark room).
8. Picture of your PCR products restriction electrophoresis will be available at MS Teams study group folder, print it and paste it into your protocol sheet:

Electrophoresis of PCR product restriction. Date:

9. **In the picture, mark your samples with numbers, mark the lenght (size) of restriction fragments (last two wells in the row) and marker (size standard) in bp** (according to pic. 3B).
10. **Shortly and simply write down your result** of restriction of your PCR products (result of restriction cleavage, found variants):

6 Sequencing analysis of PCR product of exon 13 of *DPYD* gene

6.1 INTRODUCTION

DNA sequencing is a method used to determine the exact order of individual nucleotides in the DNA polynucleotide chain, i.e. **the primary DNA structure**. For the analysis of individual DNA fragments (amplified by PCR), **Sanger sequencing** is used. The principle of Sanger sequencing is a combination of two methods: **1) sequencing reaction** using labeled **2',3'-dideoxynucleotide triphosphates** (**ddNTPs; terminators**) which cause disruption of replication process when incorporated into *in vitro* replicating strands. **2) high resolution electrophoresis**, which allows separation of ssDNA fragments according to their size that differ by only 1 base.

In the sequencing reaction the concentration of terminators (ddNTPs) is lower than of dNTPs and the ddNTPs are randomly incorporated into synthetized DNA strand. After their incorporation into synthetized polynucleotide, synthesis of DNA strand is stopped because **ddNTPs lack 3'-OH group** neccessary for phosphodiester bond formation (**Fig.8**).

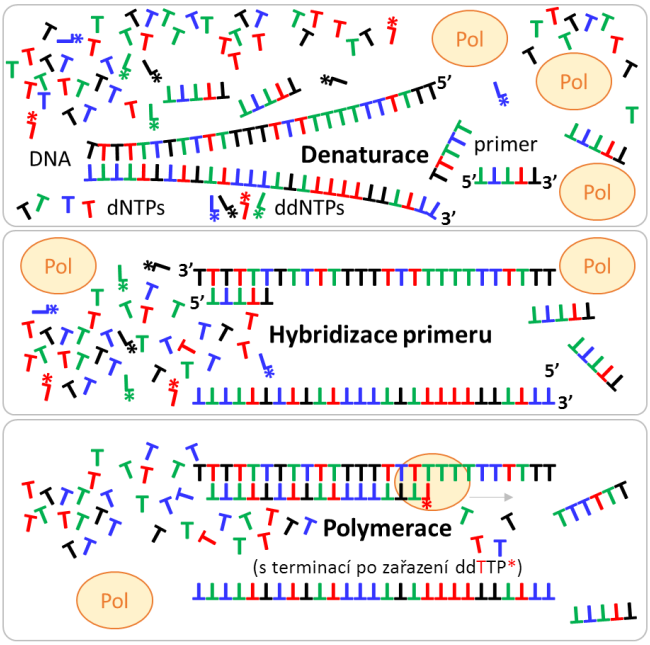


Fig. 8. Scheme of sequencing reaction by Sanger method.

Composition of reaction resembles one of PCR (Fig. 6). The difference is the addition of only one primer and labeled ddNTPs.

Similarly to PCR, sequencing reaction starts with **denaturation of dsDNA. Sequencing primer then hybridizes** to one of ssDNA strands, that serve to bind DNA polymerase providing a complementary strand synthesis. This happens until the terminator ddNTP is randomly incorporated into the sythetized strand. By repeating the individual steps of the sequencing reaction target DNA sequence is linearly amplified.

During sequencing it is neccessary to use **only one sequencing primer** that specifically binds to a template DNA site. There must be sufficient concentration of the template DNA (millions of copies of the target sequence); for this reason sequencing requires the use of amplified DNA (PCR product, plasmid, etc.; Fig. 9).

Note:

Prior to the sequencing reaction itself, resulting directly from PCR, it is necessary to remove the remaining unincorporated PCR primers and dNTPs. This can be done by precipitating the PCR reaction (similar to DNA isolation when short oligonucleotides of primers and dNTPs are dissolved in the polar alcohol solution and separated by decantation fom the DNA precipitate of PCR products, or enzymatically as will be done it this practical course. In case of enzymatic degradation, PCR products are incubated with a mixture of thermolabile enzymes – 1) DNA exonuclease (Exo) cleaving ssDNA (which means primers but not dsDNA = PCR product) to dMNP and 2) alkaline phosphatase (shrimp alkaline phosphatase; SAP) cleaving 5'-phosphate groups from dNTPs.

DNA template	Quantity
PCR product:	
• 100–200 bp	1–3 ng
• 200–500 bp	3–10 ng
• 500–1000 bp	5–20 ng
• 1000–2000 bp	10–40 ng
• >2000 bp	20–50 ng
Single-stranded DNA	25–50 ng
Double-stranded DNA	150–300 ng
Cosmid, BAC	0.5–1.0 µg
Bacterial genomic DNA	2–3 µg

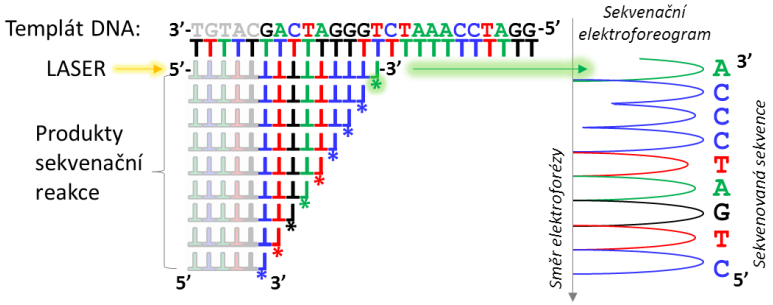
Fig. 9. Recommended amounts of DNA for sequencing with BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher).
Calculate how much of target sequence DNA of the lenght of 300 bp is in your isolated DNA (in ng) :

Total yield of DNA_____ng

Target sequence 300 bp_____ng

By repeating the individual steps of the sequencing reaction, only linear amplification of the target DNA segment occurs. **The result of the sequencing reaction is a mixture of variously long ssDNAs (differing in length by 1 base), all of them end with the labeled terminator** (*, Fig. 10).
From the resulting sequencing reaction, it is necessary to **purify** pure DNA (removal of admixtures from sequencing reaction, such as DNA polymerase protein molecules, unincorporated dNTPs and ddNTPs, ions). After purification, the resulting DNA pellet is dissolved in formamide and denatured.
In this form, the sample is ready for **analysis on the automatic sequencer**. The sequencing analyzer performs **capillary electrophoresis** in which samples of ssDNA ending with a fluorescent tag (fluoroform) migrate through a thin capillary having a glass window irradiated with a laser beam. Each terminator (ddGTP, ddATP, ddTTP, and ddCTP) contains a unique **fluorophore** that emits light of different wavelength after laser irradiation. Emitted radiation is detected by a detector that translates it into an analog signal of the **sequencing electropherogram.** The electropherogram curve is software editted to evaluate the sequenced DNA sequence (Fig. 10).

Fig. 10. Scheme of capillary electrophoresis with detection and sequencing analysis. In sequencing capillary electrophoresis individual ssDNA fragments labeled with fluorophore (*) labeled terminator (ddNTP) migrating according to their size through thin capilary filled with a gel. In the capillary detection window, fragments are irradiated with a laser beam. Detected radiation of the individual fluorophores is transferred to a sequencing chromatogram allowing the reconstruction of the primary sequence of the read DNA segment.



6.2 PROTOCOL

We will analyze the presence of variant c.1679T>G (p._____; fill in); see page 13 "sequences") in exon 13 of the *DPYD* gene by sequencing the PCR product. In addition to this variant there may be several other variants in exon 13. To perform sequencing reaction use your PCR product **_____-E13** from task 4.2.1.

Sequencing reaction is performed with reverse primer E13r.

6.2.1 Removal of unincorporated primers and dNTPs from PCR product

Reagents:

- ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFisher); contains SAP and Exo:

1. Label 0.2 ml eppendorf tube with the number of your PCR product:_____.
2. To this labeled eppendorf tube pipette **1 µl ExoSAP-IT** and add **1 µl of your PCR product E13**.
3. **Vortex** the tube and **spin** briefly.
4. Put the tube in the cycler GeneAmp PCR System 2700 **block** and activate the program – **"exosap"**.

6.2.2 Preparation of sequencing reaction

Reagents:

- Primer E13r (5 µM)
- BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher):
  - 0.4 ul BigDye® Terminator v3.1 Ready Reaction Mix (DNA Pol. dNTPs; ddNTPs*; buffer)
  - 1.0 µl 5X Sequencing Buffer
  - 0.6 µl ddH_2O

1. Into 0.2 ml eppendorf tube with purified PCR product, pipette **2 µl of Sequencing Master Mix**, and 1.0 µl of primer_____ .
2. **Vortex** the tube and **spin** briefly.
3. Put the tube into the GeneAmp PCR System 2700 cycler **block** and activate the sequencing reaction – **program "BD"**.

6.2.3 Precipitation of sequencing reaction and analysis of sequencinf data

Protocol:

1. **Add 1.3 µl of EDTA**, **1.3 µl of sodium acetate** and **30 µl of 100% ethanol** to sequencing reaction.
2. **Vortex** and **centrifuge** for **15 minutes at 14000 rpm**.
3. **Discard** the supernatant, **dry** the edges of the tube with filter paper and add **60 µl of 70% ethanol**.
4. **Vortex** and **centrifuge for 10 minutes at 14000 rpm**.
5. **Discard** the supernatant, **dry** the edges of the tube with filter paper.

6. Let the **OPEN** tube **dry for 5 minutes při 90°C** in the open cycler.
7. Into dried tube **pipette 12 µl of formamide**.
8. Vortex and spin briefly.
9. Denaturate at **95°C for 5 minutes** in the cycler; cool **on ice**.
10. The processed sample is now prepared for DNA analysis in ABI3130 sequencer.
11. Pipette your sample into 96-well sequencing plate. Note:

    Sample position in the plate:_____

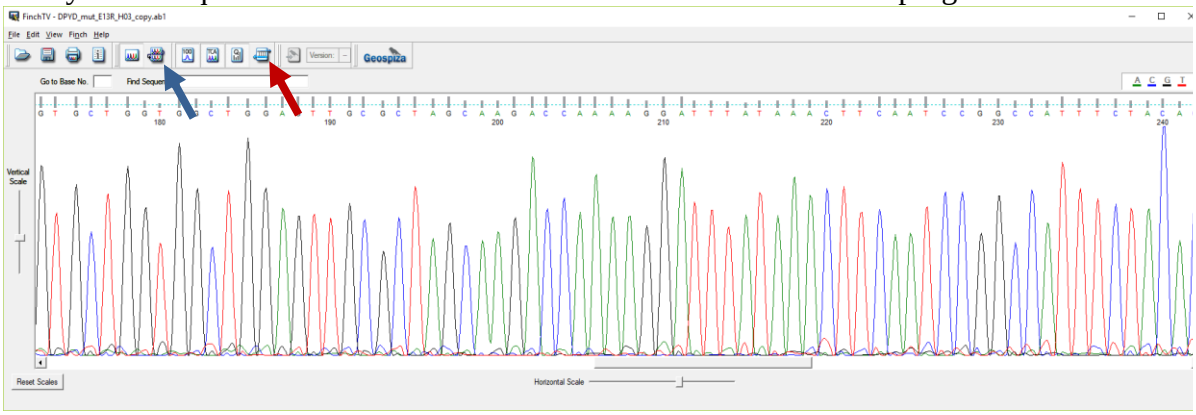
    Sample name in the sample sheat:_____

12. Create sample name:
    #group_#DNA_DPYD_E13r
13. The final file from the sequencer will be named:
    #group_#DNA_DPYD_E13r_position in the plate.ab1

6.2.4 Analysis of sequencing chromatogram

The output from the ABI3130 Automatic Sequencer (Applied Biosystems) is a data file where each file contains analysis of one sequenced DNA fragment. The files are in *.ab1 format. To read these files, use the FinchTV freeware program (to download on the site http://www.softpedia.com/get/Science-CAD/FinchTV.shtml):

Fig. 11. Program FinchTV 1.4.0 for sequenced data analysis.
Analyze the sequence available on the Institute website in a FinchTV program.



1. From the Institute website, download the **file *.ab1 with your sequence**.
2. **Open the sequence in FinchTV** (Fig. 11). Look at the **forward** sequence properly. By clicking „Wrapped view“ (blue arrow) whole sequence will be displayed in several lines; by clicking „Reverse complement“ (red arrow) or keyboard shortcut CTRL+R, the sequence will be diplayed in forward sense.
3. **Identify** possible variants.
4. **Mark** identified variants into the sequence on page 13.
5. Print your sequence (File>Print…) on one page (File>Print Setup…; Choose Fit to one page). Attach printed sequence to your protocol sheet and bring it with you to the last practical lesson of Molecular Biology Course.

7 Conclusion

Evaluate the results of the analysis of selected region of the *DPYD* gene (analyzed gene regions, used methods, found variants, cennection of genotype of possible toxicity of fluoropyrimidine treatment). Name the identified variants and interprete their clinical significance according to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/).

Analyses conlusion:

8 Abbreviations:

agarose		linear polysaccharide composed of units of D-galactose and 3,6-anhydro-L-galactose
bp		base pairs
BUP1		β-ureidopropionase
cDNA		complementary single-stranded DNA synthesized by reverse transcription of mRNA
ddH_2O		redistilled sterile water
DPD		dihydropyrimidine dehydrogenase (enzyme)
DPYD		dihydropyrimidine dehydrogenase (gene)
DMSO		dimethyl sulfoxide
dNTPs		deoxynucleotide triphosphates (dATP, dGTP, dCTP, dTTP)
dsDNA		double-stranded DNA
EDTA		ethylenediaminetetraacetic acid
HPYS		hydropyrimidinase
IVS		intervening sequence (= intron)
kbp		kilo base pairs
OD_{260nm}		optic density at 260 nm
TBE		TRIS – borate – EDTA
RT		room temperature
ssDNA		single-stranded DNA
PCR		polymerase chain reaction
wt		wild type (normal allele)

Genetic Code