EasyPCR XY

determination of human sex using PCR
(with reagents for DNA isolation)

Storage conditions pg. 5
The purpose of the exercise is to carry out a genomic DNA isolation and a PCR reaction in order to determine the human sex. The procedure is based on an amplification of the human amelogenin gene fragments, which are 90% homologous on both chromosomes and differ by 189 bp on the Y chromosome as compared to the X chromosome. The expected PCR products are 977 bp and 788 bp for males, or only 977 bp for females.
### 1. Kit contents and storage

**1. Kit contents**

<table>
<thead>
<tr>
<th></th>
<th>EasyPCR XY 1 exercise (DY10)</th>
<th>EasyPCR XY 5 exercises (DY105)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSL Lysis Buffer</td>
<td>6 tubes (4 rxns per tube)</td>
<td>6 bottles (20 rxns per bottle)</td>
</tr>
<tr>
<td>Proteinase K (lyophilisate)</td>
<td>1 tube (24 rxns)</td>
<td>5 tubes (24 rxns per tube)</td>
</tr>
<tr>
<td>Proteinase Buffer</td>
<td>1 tube</td>
<td>1 tube</td>
</tr>
<tr>
<td>SSB Binding Buffer</td>
<td>6 tubes (4 rxns per tube)</td>
<td>6 bottles (20 rxns per bottle)</td>
</tr>
<tr>
<td>DNA Purification Columns</td>
<td>6 sets (4 pcs per set)</td>
<td>5 sets (24 pcs per set)</td>
</tr>
<tr>
<td>Collection Tubes</td>
<td>6 sets (4 pcs per set)</td>
<td>5 sets (24 pcs per set)</td>
</tr>
<tr>
<td>SSW1 Wash Buffer</td>
<td>6 bottles (4 rxns per bottle)</td>
<td>6 bottles (20 rxns per bottle)</td>
</tr>
<tr>
<td>SSW2 Wash Buffer</td>
<td>6 tubes (4 rxns per tube)</td>
<td>6 bottles (20 rxns per bottle)</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>6 tubes (4 rxns per tube)</td>
<td>6 tubes (20 rxns per tube)</td>
</tr>
<tr>
<td>96% ethanol</td>
<td>6 tubes (4 rxns per tube)</td>
<td>6 bottles (20 rxns per bottle)</td>
</tr>
<tr>
<td>10× reaction buffer</td>
<td>6 tubes (10 rxns per tube)</td>
<td>6 tubes (50 rxns per tube)</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>6 tubes (10 rxns per tube)</td>
<td>6 tubes (50 rxns per tube)</td>
</tr>
<tr>
<td>dNTPs mixture</td>
<td>6 tubes (10 rxns per tube)</td>
<td>6 tubes (50 rxns per tube)</td>
</tr>
<tr>
<td>FORWARD primer</td>
<td>6 tubes (10 rxns per tube)</td>
<td>6 tubes (50 rxns per tube)</td>
</tr>
<tr>
<td>REVERSE primer</td>
<td>6 tubes (10 rxns per tube)</td>
<td>6 tubes (50 rxns per tube)</td>
</tr>
<tr>
<td>PCR-grade water</td>
<td>6 tubes (10 rxns per tube)</td>
<td>6 tubes (50 rxns per tube)</td>
</tr>
<tr>
<td><em>TaqNova</em> DNA polymerase</td>
<td>6 tubes (10 rxns per tube)</td>
<td>6 tubes (50 rxns per tube)</td>
</tr>
<tr>
<td>Positive controls with MALE and FEMALE genomic DNA</td>
<td>12 tubes (5 rxns per tube)</td>
<td>12 tubes (25 rxns per tube)</td>
</tr>
<tr>
<td>Agarose</td>
<td>5 g</td>
<td>25 g</td>
</tr>
<tr>
<td>50× TAE buffer for agarose electrophoresis</td>
<td>50 ml</td>
<td>100 ml</td>
</tr>
<tr>
<td>M100-1000 ready-to-use DNA Ladder</td>
<td>1 tube (10 electrophoretic lanes)</td>
<td>1 tube (50 electrophoretic lanes)</td>
</tr>
<tr>
<td>6× GREEN DNA Gel Loading Buffer</td>
<td>0.5 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Student’s laboratory protocols</td>
<td>6 pcs</td>
<td>6 pcs</td>
</tr>
<tr>
<td>Instructor’s laboratory protocol</td>
<td>1 pcs</td>
<td>1 pcs</td>
</tr>
</tbody>
</table>
2. Storage conditions

- Frequent freezing and thawing of all reagents should be avoided.
- The Proteinase K solution should be stored at -20°C
- All the other reagents for DNA isolation should be stored at room temperature. In order to avoid evaporation, ensure that tubes and bottles are tightly closed before storing.
- The dNTPs solutions should be stored at -20°C and thawed only before use.
- The TaqNova DNA polymerase should be stored at -20°C.
- DNA templates should be stored at -20°C for long-term. After thawing, samples can be kept at +4°C.
- The Molecular Weight DNA Ladder may be stored at room temperature or +4°C. If a longer storage period is required, a temperature of -20°C is recommended.
- The DNA Gel Loading Buffer (6x GREEN) may be stored at either room temperature or +4°C for up to 12 months.

3. Shipping conditions

- Reagents for DNA isolation are shipped at room temperature.
- Reagents for PCR set up are shipped on dry or blue ice.
- DNA templates are shipped on dry or blue ice.
- DNA Ladder is shipped on dry or blue ice.
- Other reagents for electrophoresis are shipped at room temperature.
II. Notes for the instructor

1. Additional equipment and materials required (Not included in the kit)

- Sterile swab sticks
- Agarose electrophoresis equipment (apparatus, power supply)
- PCR tubes (0.2 ml)
- 1.5 ml Eppendorf tubes
- Microcentrifuge
- UV transilluminator or lamp
- Thermal cycler
- Automatic pipettes with appropriate tips
- Thermoblock, incubator or thermostated water bath
- Microwave oven or laboratory burner
- Ethidium bromide solution or other stain for DNA visualization
- Nitrile gloves

2. Additional notes for the instructor

- We suggest conducting the laboratory exercise with twelve students working in pairs.
- One laboratory protocol per pair is provided.
- The reagents are divided into 6 sets containing the reagents necessary to carry out the DNA isolation and PCR reactions.
- The purpose of the exercise is to carry out a genomic DNA isolation from a buccal swab and conduct PCR reactions with the isolated templates in order to determine human sex.
- Each pair of students should prepare 5 reaction mixtures for the amplification reaction of the amelogenin gene fragment:
  1. Negative control
  2. for the isolated DNA (person 1)
  3. for the isolated DNA (person 2)
  4. Positive control – female DNA
  5. Positive control – male DNA

The reactions can be prepared either separately or by preparing a “Master Mix”. Table 1 in the laboratory protocols provides the fields for the relevant calculations.
IMPORTANT! Students should be clearly informed that the results they obtain are not reliable, since the analyses will not have been made by a laboratory specialist in a diagnostic laboratory. The results are purely educational in nature.

3. Theoretical introduction

Molecular diagnostics based on a genetic material analysis (including the PCR technique) can be applied in various branches of medicine, such as diagnostics of genetic and infectious diseases, early detection of cancer (determination of the risk of developing a disease), sanitary diagnostics and forensics (determination of paternity, determination of the origin of traces of body fluids stains etc.). Human sex determination is used in forensics for the analysis of traces of bloodstains. It is also used in preimplantation diagnostics for embryo testing, where the estimation of the risk of diseases related to sex is important. For these applications, FISH (Fluorescent In-situ Hybridization) and PCR techniques are used. Fluorescent probes specific to X and Y chromosomes are employed in the FISH technique. They are hybridized specifically to repetitive sequences (pHY10), which are present in approximately 3000 copies in the Y chromosome. With the PCR technique, various regions can be amplified. These sequences are specific to X and Y chromosomes or are characterized by polymorphism, depending on the chromosome they are localized on, such as repetitive regions on the Y chromosome, zfx and zfy genes (PCR/RFLP analysis), and sry gene on the Y chromosome.

**Figure 1. Amelogenin gene fragment amplification scheme.**

- **PCR product from X chromosome – 977 bp**
  - FORWARD
  - REVERSE

- **PCR product from Y chromosome – 788 bp**
  - FORWARD
  - REVERSE
  - Region of 189 bp deletion
The purpose of this exercise is to carry out a genomic DNA isolation and a PCR reaction in order to determine human sex. The detection is based on the amplification of a human amelogenin gene fragment, which is characterized by polymorphism depending on the chromosome it is localized on. The amelogenin is a main extracellular matrix protein of tooth premordium. The coding sequences are localized in the shorter arm of the X chromosome and near the centromere on the Y chromosome. The amelogenin gene fragments are 90% homologous on both chromosomes and differ by 189 bp on the Y chromosome as compared to the X chromosome. The expected PCR products are 977 bp for male, or 788 bp for female.

The PCR technique was invented by Kary Mullis and his collaborators in 1983, an achievement for which they were awarded the Nobel Prize in Chemistry in 1993. The technique is based on carrying out a DNA polymerase chain reaction which allows the replication of any DNA fragment of a specific nucleotide sequence. This is possible thanks to the use of the thermostable enzyme (DNA polymerase) isolated from, for example, thermophilic bacteria *Thermus aquaticus*, and it allows multiple heating of the mixture to temperatures as high as 95°C without loss of activity. The PCR allows particular DNA sequences to be amplified from the complex mixture. It is possible to design primers which enable amplification of a single locus from an entire DNA genome. If they bind to more than one locus, then more than one product may be created as a result of the amplification. More than one billion copies can be obtained per one template molecule. Adequate precautions, as described in the protocol, should thus be taken in order to avoid contamination of the sample with other DNA or previously obtained amplicons.

The following components, which have an effect on the sensitivity, specificity and efficiency of the amplification, are required for the preparation of the reaction mixture:

→ DNA template containing the target sequence;
→ thermostable DNA polymerase;
→ buffer for the PCR and magnesium salts, which ensure adequate buffer conditions for polymerase activity;
→ an equimolar mixture of deoxyribonucleotide triphosphates (dNTP: dATP, dCTP, dTTP, dGTP);
→ primers; in other words, short oligonucleotide segments which enable amplification of the locus in question.
Literature:


III. Laboratory exercises

IMPORTANT!!!

⚠️ Remember to centrifugate the samples with the reagents after thawing so that the entire mixture is located at the bottom of the tube!!!

⚠️ Before the PCR reaction preparation, hands should be washed and the workstation equipped with tips, tubes and gloves. 3% hydrogen peroxide solution or other decontamination solutions should be used to disinfect the work surface and small items of laboratory equipment such as pipettes and so forth.

⚠️ The system’s extreme sensitivity means that it is highly susceptible to contamination. Disposable gloves should therefore be worn at all times.

⚠️ It is recommended that each of three stages of the determination, namely, the isolation of human DNA, the preparation of the PCR and the electrophoresis of the PCR products is carried out in a separate room. Special attention should be paid to PCR products from previous reactions since they represent the greatest danger of contamination.

⚠️ The use of autoclavable pipettes is the best solution for preparing the PCR. The pipettes should be autoclaved from time to time.

⚠️ Using separate pipettes is recommended for each stage of the determination, in other words, for preparing and portioning the master mix, adding the template and loading the PCR products onto the gel.

⚠️ The rule that only one tube may be open at any particular moment should be observed as this reduces the risk of contamination.

⚠️ Touching the edges of the tubes should be avoided.
1. Isolation of human DNA from the swabs

The DNA purification procedure consists of four stages and utilises spin minicolumns with membranes which efficiently and selectively bind the nucleic acids. The swab sample is subjected to enzymatic lysis by Lysis Buffer and Proteinase K. The cell membranes and proteins are degraded during this step. After the addition of chaotrope salts, the lysate is applied to the purification column membrane and the DNA is bound. The two-step washing stage effectively removes impurities or/enzyme inhibitors. The purified DNA is eluted using either the Elution Buffer or a low ionic strength buffer.

A. Before starting the isolation

1. Mix well each buffer supplied with the kit.
2. Prepare the Proteinase K solution by reconstituting the lyophilisate in an appropriate quantity of the Proteinase Buffer.  
   ☢️ *After reconstitution, store Proteinase K at −20°C.*
3. Examine the buffers. If a sediment has occurred in any of the buffers, incubate it at 37°C (both Wash Buffers) or at 50 – 60°C (other buffers), mixing occasionally until the sediment has dissolved. Cool to room temperature.
4. Set a dry block heater or water bath to 56°C.
5. Heat a sufficient quantity of the Elution Buffer to 70°C.
6. Unless otherwise stated, conduct all the isolation steps at room temperature.

B. Sample preparation

To collect a buccal swab, first ensure that the person providing the sample has not consumed any food or drink during the 30 minutes prior to the sample collection. Scrape a sterile swab stick firmly against the inside of the cheek at least 10 times. Place the swab holding the tissue cells in a 1.5 – 2 ml Eppendorf tube and cut off the excess from the end of the shaft. It is not necessary to dry the swab.
C. DNA isolation protocol (according to EXTRACTME DNA SWAB & SEMEN)

1. Place the buccal swab holding the material in a 1.5 ml Eppendorf tube and cut off the excess from the end of the shaft so that the tube lid can be closed without difficulty.
2. Add 350 μl SSL Buffer and 6 μl Proteinase K, vortex for 3 s.
3. Incubate at 56°C for 30 min. ▲ Mix by inverting the tube at several-minute intervals during the incubation.
4. Add 350 μl SSB Buffer and vortex for 3 s.
5. Incubate for 6 min at 70°C.
6. Press the swab firmly against one side of the tube in order to retrieve the maximum possible volume of the lysate. Discard the swab. ▲ The lysate can be transferred to the purification column without retrieving it from the swab. However, the isolation efficiency will be reduced.
7. Add 200 μl 96% ethanol and mix well by inverting the tube several times.
8. Transfer the 700 μl of the lysate onto a purification column placed in a collection tube.
9. Centrifuge for 1 min at 11 – 15k x g.
10. Discard the flow-through and reuse the collection tube.
11. Transfer all of the remaining lysate onto the purification column.
12. Centrifuge for 1 min at 11 – 15k x g.
13. Transfer the purification column to a new collection tube (2 ml).
14. Add 600 μl SSW1 Buffer and centrifuge for 30 s at 11 – 15k x g.
15. Discard the filtrate and reuse the collection tube.
16. Add 400 μl SSW2 Buffer and centrifuge for 30 s at 11 – 15k x g.
17. Discard the flow-through and reuse the collection tube.
18. Centrifuge for 1 – 2 min at 15 – 21k x g. ▲ The wash buffer contains alcohol, which may interfere with some enzymatic reactions and also decrease the elution efficiency. It is therefore vital to remove the alcohol completely from the column before elution.
19. Discard the collection tube and flow-through and carefully transfer the purification column to a sterile, 1.5 ml Eppendorf microcentrifuge tube.
20. Add 60 μl Elution Buffer, pre-heated to 70°C, directly onto the purification column membrane.
21. Incubate the column at room temperature for 2 min.
22. Centrifuge at 11 – 15k x g for 1 min.
23. Remove the column. The isolated DNA is ready for use in downstream applications or for either short-term storage at +4°C or long-term storage at -20°C.
2. **Agarose gel electrophoresis of human DNA**

### A. Exemplary agarose gel preparation

- The electrophoretic separation should be conducted in 1% agarose gel.
- Prepare 1x TAE buffer working solution. In order to obtain a working solution, dilute one part of the 50x TAE buffer stock solution in 49 parts of distilled water; for example, add 980 ml of distilled water for every 20 ml of the stock solution.
- Dissolve 0.5 g of agarose in 50 ml of 1x TAE buffer by heating the suspension in a microwave. Use caution when handling, as the temperature of the solution can be extremely high. Cool the solution to approximately 60°C.

⚠️ **CAUTION!** From this point on, nitrile gloves, which provide protection from ethidium bromide, a carcinogenic agent, must be worn at all times during all subsequent procedures.

- Add 5 μl of 1 mg/ml ethidium bromide solution (or other nucleic acid stain).
- Mix well and carefully pour into a gel casting tray equipped with the appropriately placed comb(s).
- Take care not to leave any bubbles in the gel. Bubbles already in the gel prior to pouring may be indicative of the gel’s being too cool. If this is the case, re-heat the gel.
- After gel solidification, remove the combs carefully, ensuring that wells stay intact. Place the gel in an agarose gel electrophoresis apparatus and add 1x TAE buffer to a level above the surface of the gel.

### B. Gel electrophoresis

- 10 μl of each DNA sample should be pre-mixed thoroughly with 2 μl of 6x GREEN DNA Loading Buffer before applying in a gel well.
- The electrophoretic separation should be conducted for 30–60 min, with a voltage of 10–12 V/cm.
- Once the electrophoresis has been completed, the gel should be analyzed in the UV light of the transilluminator.
3. Interpretation of the results

Following the electrophoresis, bands of various intensity should be visible in the lanes in which the separation of the human DNA was performed. This results from the varying efficiency of the DNA isolation, which in turn, may be connected with the quantity of material collected or the reliability of the performance of the subsequent stages of the isolation procedure.

The absence of any signal in the lane in which the separation of human DNA was performed may be indicative of a very low DNA isolation efficiency. However, owing to the high sensitivity of the amplification reaction, this does not exclude the performance of an analysis using PCR.

4. PCR reaction with the isolated human DNA

A. Preparation of the reaction mixture for a single sample.

The mixture components should be added to a 0.2 ml PCR tube in the order presented in the table below. Each pair should analyze the human DNA that they have isolated.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume [μl]</th>
<th>MMix for ... tests</th>
<th>Sum [μl]</th>
<th>To be portioned into amounts of [μl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR – grade water</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10x reaction buffer</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl₂ [50 mM]</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dNTPs [8 mM]</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FORWARD primer [10 μM]</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REVERSE primer [10 μM]</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TagNova DNA polymerase [2 U/μl]</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA template – ANALYZED SAMPLE</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final volume</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Composition of PCR reaction mixture for the analyzed sample.
B. Preparation of the reaction mixture for the Positive control.

For each series of analyses, a Positive control should be performed, the purpose of which is to verify the quality of the reagents being used. The composition of the reaction mixture for the preparation of the Positive control – FEMALE DNA is presented in Table 2 and, for the Positive control – MALE DNA, in Table 3. The composition of the reaction mixture is the same as the one for the analyzed sample, but here provided female and male DNA are used as the template.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume [µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR – grade water</td>
<td>29</td>
</tr>
<tr>
<td>10x reaction buffer</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂ [50 mM]</td>
<td>3</td>
</tr>
<tr>
<td>dNTPs [8 mM]</td>
<td>5</td>
</tr>
<tr>
<td>FORWARD primer [10 µM]</td>
<td>2</td>
</tr>
<tr>
<td>REVERSE primer [10 µM]</td>
<td>2</td>
</tr>
<tr>
<td>TaqNova DNA polymerase [2 U/µl]</td>
<td>2</td>
</tr>
<tr>
<td>Positive control – FEMALE DNA</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 2. Composition of PCR reaction mixture for the Positive control of female DNA.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume [µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR – grade water</td>
<td>29</td>
</tr>
<tr>
<td>10x reaction buffer</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂ [50 mM]</td>
<td>3</td>
</tr>
<tr>
<td>dNTPs [8 mM]</td>
<td>5</td>
</tr>
<tr>
<td>FORWARD primer [10 µM]</td>
<td>2</td>
</tr>
<tr>
<td>REVERSE primer [10 µM]</td>
<td>2</td>
</tr>
<tr>
<td>TaqNova DNA polymerase [2 U/µl]</td>
<td>2</td>
</tr>
<tr>
<td>Positive control – MALE DNA</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 3. Composition of PCR reaction mixture for the Positive control of male DNA.**
For each series of analyses, a **Negative control** should be performed in order to verify the correctness of work and the purity of the reagents for the purpose of excluding possible false positive results. The composition of the mixture is presented in Table 4.

### Table 4. Composition of PCR mixture for the Negative control.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume [μl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR – grade water</td>
<td>29</td>
</tr>
<tr>
<td>10x reaction buffer</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂ [50 mM]</td>
<td>3</td>
</tr>
<tr>
<td>dNTPs [8 mM]</td>
<td>5</td>
</tr>
<tr>
<td>FORWARD primer [10 μM]</td>
<td>2</td>
</tr>
<tr>
<td>REVERSE primer [10 μM]</td>
<td>2</td>
</tr>
<tr>
<td>TaqNova DNA polymerase [2 U/μl]</td>
<td>2</td>
</tr>
<tr>
<td>PCR – grade water</td>
<td>2</td>
</tr>
<tr>
<td><strong>Final volume</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

The 5 reaction mixtures listed above (2 samples + Negative control + 2 Positive controls) may be prepared simultaneously. In such a case, the Master Mix (MMix) should be prepared; the quantities of reagents used for one sample should be multiplied by 6 (the number of the samples + 1). Table 1 provides the fields for the relevant calculations to be made. The value obtained for the total volume of MMix should be divided by the number of the samples for which the MMix is calculated; in other words, for 6. The value obtained is the volume of the reaction mixture, which should be portioned into the specific tubes. It should be consistent with the sum of components calculated for the reaction mixture for 1 sample. Before portioning, the prepared MMix should be mixed by pipetting or vortexing. The tubes containing the prepared reaction mixtures should be placed in the thermal cycler and the temperature and time profile presented in Table 5 should be set.

### Table 5. PCR cycling conditions.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature [°C]</th>
<th>Time [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial denaturation</td>
<td>94</td>
<td>120</td>
</tr>
<tr>
<td>denaturation</td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>primer annealing</td>
<td>61.5</td>
<td>60</td>
</tr>
<tr>
<td>elongation</td>
<td>72</td>
<td>90</td>
</tr>
<tr>
<td>final elongation</td>
<td>72</td>
<td>120</td>
</tr>
<tr>
<td>cooling</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

40 cycles
5. Detection of the PCR products

A. Exemplary agarose gel preparation

→ The electrophoretic separation should be conducted in 2% agarose gel.
→ Prepare 1x TAE buffer working solution. In order to obtain a working solution, dilute one part of the 50x TAE buffer stock solution in 49 parts of distilled water; for example, add 980 ml of distilled water for every 20 ml of the stock solution.
→ Dissolve 1 g of agarose in 50 ml of 1x TAE buffer by heating the suspension in a microwave. Use caution when handling, as the temperature of the solution can be extremely high. Cool the solution to approximately 60°C.

⚠️ CAUTION! From this point on, nitrile gloves, which provide protection from ethidium bromide, a carcinogenic agent, must be worn at all times during all subsequent procedures.

→ Add 5 μl of 1 mg/ml ethidium bromide solution (or other nucleic acid stain).
→ Mix well and carefully pour into a gel casting tray equipped with the appropriately placed comb(s).
→ Take care not to leave any bubbles in the gel. Bubbles already in the gel prior to pouring may be indicative of the gel's being too cool. If this is the case, re-heat the gel.
→ After gel solidification, remove the combs carefully, ensuring that wells stay intact. Place the gel in an agarose gel electrophoresis apparatus and add 1x TAE buffer to a level above the surface of the gel.

B. Gel electrophoresis

→ The PCR products obtained should be applied to the gel wells with pipettes, as follows:
  1. 10 μl of M100-1000 ready-to-use DNA Ladder
  2. 2 μl of 6x GREEN DNA Loading Buffer and 10 μl of the PCR mixture of the **analyzed sample (person 1)**
  3. 2 μl of 6x GREEN DNA Loading Buffer and 10 μl of the PCR mixture of the **analyzed sample (person 2)**
  4. 2 μl of 6x GREEN DNA Loading Buffer and 10 μl of the PCR mixture of the **Positive control – FEMALE DNA**
Instructor’s Protocol

5. 2 μl of 6x GREEN DNA Loading Buffer and 10 μl of the PCR mixture of the **Positive control – MALE DNA**
6. 2 μl of 6x GREEN DNA Loading Buffer and 10 μl of the PCR mixture of the **Negative control**

⚠️ **CAUTION!** Special care should be taken while applying the samples to the gel in order to avoid an overflow of the samples from well to well, which could produce false results.

→ The electrophoretic separation should be conducted for 30 – 60 min, with a voltage of 10 – 12 V/cm.
→ Once the electrophoresis has been completed, the gel should be analyzed in the UV light of the transilluminator.

6. **Interpretation of the PCR results**

**Analysis of electropherogram obtained**

→ As the result of the agarose electrophoresis in the lane in which the separation of the DNA size marker (M100-1000) is performed, an electrophoretic profile consisting of 10 bands (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp) should be obtained (*Photo 1. Lane M*).
→ No signals should be obtained as the result of the electrophoresis in the lane in which the separation of the PCR reaction mixture of the **Negative control** is performed. This is indicative of the correct preparation of the mixture and a good reagents quality (*Photo 1. Lane K*-).
→ As the result of electrophoresis in the lane in which the separation of the PCR reaction mixture of the **Positive control – FEMALE DNA** is performed, a product with a size of 977 bp should be obtained. This is indicative of a PCR reaction having occurred correctly (*Photo 1. Lane XX*).
→ As the result of electrophoresis in the lane in which the separation of the PCR reaction mixture of the **Positive control – MALE DNA** is performed, two products with sizes of 977 bp and 788 bp should be obtained. This is indicative of a PCR reaction having occurred correctly (*Lane XY*).
→ If there is one PCR product with a size of 977 bp present in the lane in which the separation of the reaction mixture of the **tested sample** is performed, this means that we are dealing with a female DNA (*Photo 1. Lane XX*).
If there are two PCR products with sizes of 977 bp and 788 bp present in the lane in which the separation of the reaction mixture of the tested sample is performed, this means that we are dealing with a male DNA (Photo 1. Lane XY).

The presence of any product in the lane in which the separation of the reaction mixture of the Negative control is performed is indicative of contamination of the reagents. The PCR should be prepared again and the analyses should be carried out in line with the instructions given above.

The absence of the 977 bp and/or 788 bp product in the lanes in which the separation of reaction mixture of the analyzed sample is performed, with the correct results obtained in the lanes in which the separation of the products for Positive controls is performed, may be indicative of a low quality template DNA (low concentration or PCR inhibitors). The DNA should be isolated again and the analysis should be repeated.

If there are no products with a size of 977 bp and 788 bp in the lanes in which the separation of reaction mixture of the analyzed sample and the Positive controls are performed, incorrect procedure or incorrect action on the part of the reagents being used may be suspected. The reaction should be carried out again and the analyses should be repeated in line with the instructions given above. A repeated lack of the signals for the positive control is indicative of the need to replace the reagents.

When the presence of bands of sizes other than 977 bp and 788 bp on the path in which the separation of reaction mixture of the analyzed sample is performed, those bands should not be taken into consideration. They are the result of non-specific amplification and may be caused by contamination of the reaction mixture.

Photo 1. Example of an electropherogram with PCR results.
WARRANTY
The kit components will remain stable for 12 months from the date of purchase providing it is stored properly. The electrophoresis buffers will remain stable for 6 months.