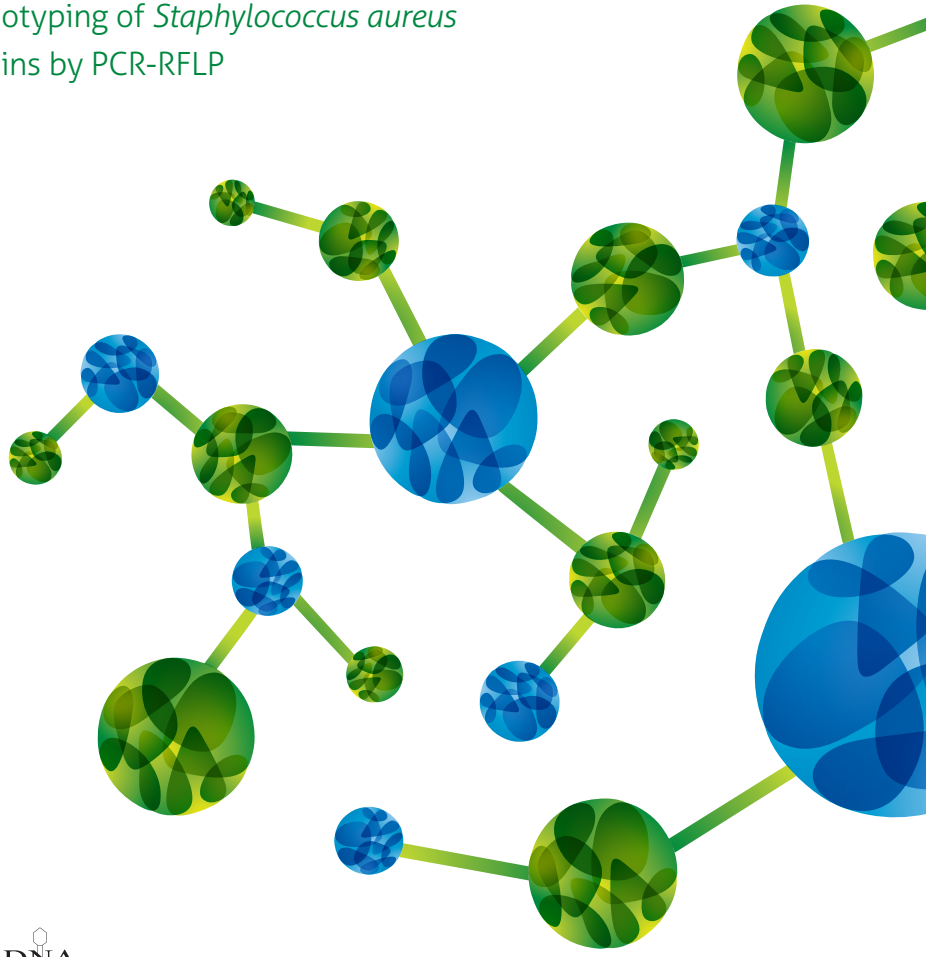
Educational Kit
Instructor's Laboratory Protocol

Cat. No.
DY87 / DY875

*Easy*Genotyping PCR-RFLP – *S. aureus*

genotyping of *Staphylococcus aureus* strains by PCR-RFLP



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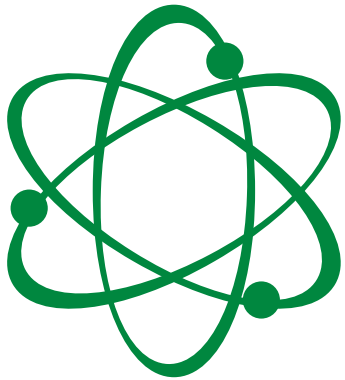
⚠ Storage conditions pg. 5

The purpose of the exercise is to carry out the genotyping of the clinical strains of *Staphylococcus aureus* (supplied as DNA) and assign them to individual genotyping groups or genotypes.



I. Kit contents and storage

1. Kit contents	*Easy*Genotyping 1 exercise (DY87)	*Easy*Genotyping 5 exercises (DY875)
10x reaction buffer	6 tubes (10 reactions per tube)	6 tubes (50 reactions per tube)
50 mM $MgCl_2$	6 tubes (10 reactions per tube)	6 tubes (50 reactions per tube)
dNTPs mixture	6 tubes (10 reactions per tube)	6 tubes (50 reactions per tube)
FORWARD primer	6 tubes (10 reactions per tube)	6 tubes (50 reactions per tube)
REVERSE primer	6 tubes (10 reactions per tube)	6 tubes (50 reactions per tube)
PCR-grade water	6 tubes (10 reactions per tube)	6 tubes (50 reactions per tube)
***TaqNova* DNA polymerase**	6 tubes (10 reactions per tube)	6 tubes (50 reactions per tube)
DNA isolated from 12 *S.aureus* strains	12 tubes (5 reactions per tube)	12 tubes (25 reactions per tube)
10x restriction buffer	6 tubes (10 reactions per tube)	6 tubes (50 reactions per tube)
Restriction enzyme (*Csp*6I)	1 tube (60 reactions)	1 tube (300 reactions)
Agarose	5 g	25 g
50xTAE buffer for agarose electrophoresis	50 ml	100 ml
Ready-to-use **M100-1000 DNA Ladder**	1 tube (10 electrophoretic lanes)	1 tube (50 electrophoretic lanes)
6xGREEN DNA Gel Loading Buffer	0.5 ml	1 ml
Student's laboratory protocols	6 pcs	6 pcs
Instructor's laboratory protocol	1 pcs	1 pcs

2. Storage conditions

- Frequent freezing and thawing of all reagents should be avoided.
- The **dNTPs** solutions should be stored at -20°C and thawed only before use.
- The ***TaqNova* polymerase** and **Restriction enzyme** should be stored at -20°C.
- **Template DNA** 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 (**6xGREEN**) may be stored at either room temperature or +4°C for up to 12 months.

3. Shipping conditions

- Restriction enzyme is shipped on dry or blue ice.
- 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)

- Agarose electrophoresis equipment (apparatus, power supply);
- PCR tubes (0.2 ml)
- 1.5 ml Eppendorf tubes
- Microcentrifuge
- UV transilluminator or lamp
- Thermoblock (dry bath incubator)
- Thermal cycler
- Automatic pipettes with appropriate tips
- 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 is provided per pair.
- The reagents are divided into 6 sets containing the reagents and tubes necessary to carry out PCR and restriction reactions.
- The purpose of the exercise is to carry out the genotyping of the 12 strains of *S. aureus* and assign them to individual genotyping groups or genotypes.
- Each pair of students should carry out an analysis of 2 selected strains and prepare 3 reaction mixtures:
  - 1. and 2. for the DNA samples analyzed
  - 3. Negative control

  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.
- After verification of the PCR reaction results by gel electrophoresis, PCR products should be subjected to enzymatic restriction.

3. Theoretical introduction

The PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) method is based on the analysis of particular genome regions which are first amplified and then subjected to restriction analysis. The method thus consists of two steps; PCR amplification , followed by enzymatic restriction. The genome region to be analyzed is selected in line with the purpose of the particular analysis. It can be a species-specific sequence, which may allow for simultaneous genus and species identification, or a region-enabling phylogenetic analysis. The PCR-RFLP method is also used for detecting single nucleotide mutations which occur or disappear in the analyzed sequence. The region selected as a molecular target should include a variable sequence, which enables the differentiation of the analyzed strains. Primer sequences are designed at conserved sequences flanking the variable region. The restriction enzymes for subsequent digestion reaction usually comprise a frequent-cutting enzyme which recognizes 4-nucleotide sequences. The appropriate enzyme is selected on the basis of the analyzed sequence, or experimentally if the sequence of the product is unknown.

The PCR-RFLP method is usually characterized by a lower differentiation potential than methods such as REA-PFGE (Restriction Endonuclease Analysis combined with Pulsed Field Gel Electrophoresis), which are based on whole genome analysis. In order to increase the differentiation potential, a multiplex PCR may be performed with simultaneous amplification of several targets. The mixture of the products thus obtained should be subjected to digestion with one or more restriction enzymes. The advantage of the PCR-RFLP method is that it enables simultaneous species identification and strain genotyping. The method is widely utilized in epidemiological studies on account of its high reproducibility, straight forward analysis and ease of implementation.

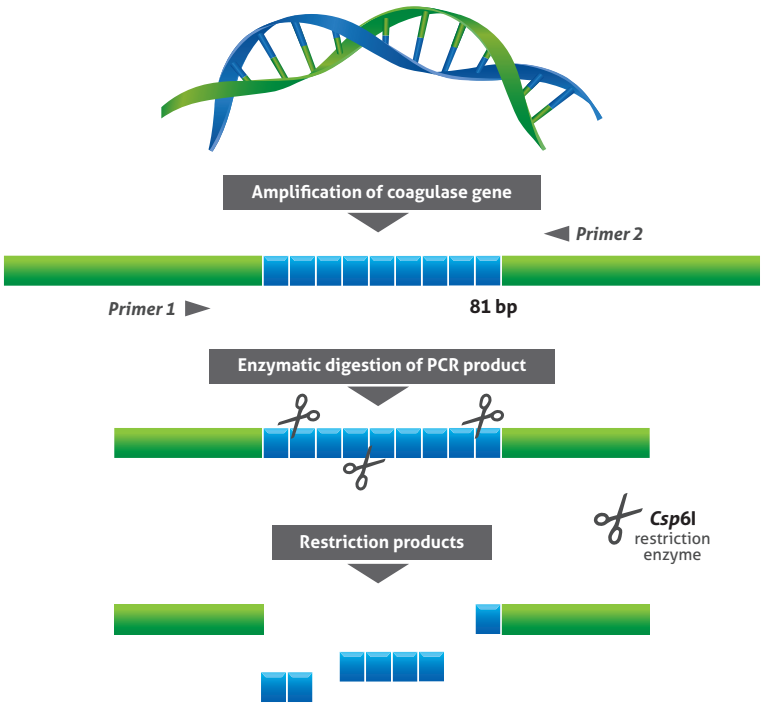


Figure 1. The diagram of coa-RFLP method.

The method has several variants:

- → ARDRA (Amplified Ribosomal DNA Restriction Analysis) – analysis of the variable region of the ribosomal operon;
- → *rec*A-RFLP – for identification of *Acinetobacter* genomic species;
- → *ure*C-RFLP – for *Helicobacter pylori* strains genotyping;
- → *coa*-RFLP – for innerspecie differentiation of *Staphylococcus aureus* strains.

The purpose of the exercise is to use the *coa*-RFLP method to differentiate *S. aureus* strains. Coagulase is one of the major targets for identification of *Staphylococcus aureus* strains used in clinical microbiology. The coagulase is an adhesin, which binds to host's inactive prothrombin, forming an active stphylothrombin that facilitates clot formation by converting fibrinogen into fibrin. The bacterial cell covers its surface with fibrin and thus protects itself

from phagocytosis and immunological response. Most *S. aureus* strains shows coagulase activity (CPSA – coagulase positive *S. aureus*); however strains without this activity are also known (CNSA – coagulase negative *S. aureus*).

There may be more than one version of *coa* gene within one genome. The gene sequence analysis showed that the variable region is at the 3'-end of the gene. This region consists of 81 bp repeating sequences. The number of these repeats differ from strain to strain. Although this sequence is conserved, there are small differences conferring the presence or absence of restriction recognition sites for the *Csp*6I restriction enzyme. After enzymatic digestion of the amplification products of the *coa* gene, restriction profiles are generated, enabling differentiation of *S. aureus* strains within species. Owing to the presence of several versions of the *coa* gene within one genome, more than one amplification product might be obtained.

Literature:

1. Krawczyk B , Lewandowski K, Kur J. *Comparative studies of* Acinetobacter *genus and the species identification metod based on the* rec*A sequences.* Mol Cell Probes 2002, 16; 1-11.
2. Goh SH, Byrne SK, Zhang JL, Chow AW. *Molecular typing of* Staphylococcus aureus *on the basis of cocagulase gene polymorphism.* J Clin Microbiol 1992, 30; 1642-1645.
3. Piechowicz L, Garbacz K, Wisniewska K, Dajnowska-Stanczewa A, Galiński J. *Wytwarzanie białka A prze izolaty* Staphylococcus aureus *nie wytwarzające koagulazy lub czynnika zlepnego (CF).* Med Dosw Mikrobiol 57; 253-261.
4. Moore RA, Kureishi A, Wong S, Bryan LE. *Categorization of clinical isolates of* Helicobacter pylori *on the basis of restriction digest analyses of polymerase chain reaction-amplified* ure*C genes.* J Clin Microbiol 1993, 31; 1334–1335.
5. Kumar R, Barbacid M. *Oncogene detection at the single cell level.* Oncogene 1988, 3; 647-651.
6. Deng G. *A sensitive non-radioactive PCR-RFLP analysis for detecting point mutations at 12th codon of oncogene c-Ha-*ras *in DNAs of gastric cancer.* Nucl Acids Res 1988, 16; 6231.

III. Laboratory exercises

IMPORTANT!!!

- Remember to centrifuge the samples with the reagents after thawing so that the entire mixture is located at the bottom of the tube!!!
- Before beginning to prepare the PCR, the 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 two stages of the determination, namely, 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. PCR reaction of *coa* gene

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 two *S. aureus* strains.

Reagent	Volume [µl]	MMix for ... tests	Sum [µl]	To be portioned into amounts of [µl]:
PCR - grade water	31			
10x reaction buffer	5			
$MgCl_2$ [50 mM]	2			
dNTPs [8 mM]	5			
FORWARD primer [10 µM]	2			
REVERSE primer [10 µM]	2			
TaqNova DNA polymerase [2 U/µl]	1			
DNA template – ANALYZED SAMPLE	2			
Final volume	50			

Table 1. Composition of PCR reaction mixture for the analyzed sample.

B. Preparation of the reaction mixture for the Negative control.

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 2.

Reagent	Volume [µl]
PCR - grade water	31
10x reaction buffer	5
$MgCl_2$ [50 mM]	2
dNTPs [8 mM]	5
FORWARD primer [10 µM]	2
REVERSE primer [10 µM]	2
TaqNova DNA polymerase [2 U/µl]	1
PCR-grade water	2
Final volume	50

Table 2. Composition of PCR mixture for the Negative control.

The 3 reaction mixtures listed above (2 samples + negative control) 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 4 (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 4. 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 3 should be set.

Stage	Temperature [°C]	Time [s]	
initial denaturation	94	120	
denaturation	94	30	35 cycles
primer annealing	62	30	
elongation	72	30	
final elongation	72	120	
cooling	10	∞	

Table 3. PCR cycling conditions.

2. 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. Agarose gel electrophoresis

- 10 µl of each DNA sample should be pre-mixed thoroughly with 2 µl of 6xGREEN DNA Loading Buffer before applying in a gel well.
- The PCR products obtained should be applied to the gel wells with pipettes, as follows:
  1. 2 µl of 6xGREEN DNA Loading Buffer and 10 µl M100-1000 DNA Ladder
  2. 2 µl of 6xGREEN DNA Loading Buffer and 10 µl of the PCR mixture of the **analyzed sample**
  3. 2 µl of 6xGREEN 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 separation should be conducted for 30–60 min, with a voltage of ca. 5 – 10 V/cm of gel length.
- Once the electrophoresis has been completed, the gel should be analyzed in the UV light of the transilluminator.

3. Interpretation of the PCR results

Analysis of electropherogram obtained

- The agarose electrophoresis in the lane in which the separation of the DNA size marker (M100-1000) is performed should produce an electrophoretic profile consisting of 10 bands (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp; *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 good reagent quality. Products below 100 bp, which may occur in the lane, are derived from primer interactions and are not taken under analysis. The presence of products above 100 bp indicates reagent contamination. The reaction should be set up again and analyzed as before (*Photo 1. Lane K-*).

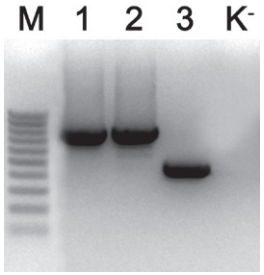


Photo 1. Example of an electropherogram with PCR-coa results.

In the lane containing the PCR product of **analyzed sample**, there should be one or more product between **500** and **900 bp**, depending on the number of versions of the *coa* gene within the *S. aureus* genome (*Photo 1. Lanes 1–3*). On the basis of the obtained results, the students should assign the analyzed strains to individual genotyping groups by completing Table 5. Strains should be assigned to one genotyping group if the amplification products are the same size (have the same product profile). Strains should be assigned to separate genotyping groups if the obtained amplification products are of different sizes (have a different product profile).

- If there are no PCR products in the lane of the analyzed samples, this may have happened for several reasons: incorrect handling during the reaction set up, either reagents or equipment malfunction.

4. Enzymatic digestion – *coa* / RFLP

The mixture components should be added to a new 0.2 ml PCR tube in the order presented in the table below. Each pair should analyze two *S. aureus* strains.

Reagent	Volume [µl]	MMix for ... tests	Sum [µl]	To be portioned into amounts of [µl]:
10x restriction buffer	2.5			
*Csp*6I enzyme [10 U/µl]	0.5			
PCR products	22			
Final volume	25			

Table 4. Composition of restriction reaction mixture for the analyzed sample.

The two reaction mixtures may be prepared simultaneously. In such a case, the Master Mix (MMix) should be prepared following instructions for setting up a PCR-*coa* reaction (section 1A). Reaction mixtures should be placed in a thermoblock (dry bath incubator) or thermal cycler and incubated at 37°C for 1 h.

5. Detection of the *coa* / RFLP products

Follow the instructions in step 2.

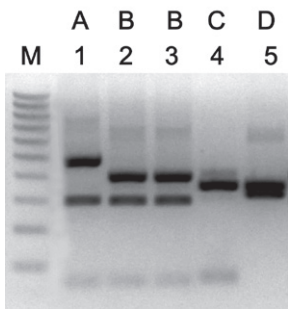


Photo 2. Example of an electropherogram with coa*-RFLP results.*

6. Interpretation of the PCR results

- The agarose electrophoresis in the lane in which the separation of the DNA size marker (M100-1000) is performed should produce an electrophoretic profile consisting of 10 bands (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp; *Photo 2. Lane M*).
- In the lane containing the **analyzed samples**, there should be an electrophoretic product profile characteristic of a particular *S. aureus* strain (*Photo 2. Lanes 1–5*). Classification of the strains to individual genotyping groups is carried out by completing Table 5 on the basis of the number and sizes of the restriction products. Strains should be assigned to one genotyping group if the amplification products are the same size (have the same product profile). Strains should be assigned to separate genotyping groups if the amplification products obtained are of different sizes (have a different product profile or profiles).
- Student should compare the results obtained after each stage of PCR/RFLP and assess whether conducting the digestion step after amplification has the capability of increasing the method's differentiation potential. If the same, intact PCR product is still present in any lane after digestion, then the digestion reaction did not occur and should be repeated.

GENOTYPE		
S. aureus strain no.	Stage I. PCR-*coa* amplification	Stage II. *coa*-RFLP digestion
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		

Table 5. Results of PCR-RFLP genotyping – classification of S. aureus strains to individual genotyping groups (genotypes).

NOTES

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.

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