











AmpliKit HPA-3

Key to symbols used

	List Number		Store at -20°C
	For Research Use Only		Caution!
	Lot Number		Version
	Expiration Date		Consult instructions for use
	Contains reagents		Manufacturer

INTENDED USE

For detection of the glycoprotein IIb (GpIIb) Ile843Ser polymorphism. This Test-Kit is intended for the analysis of 50 genomic DNA samples.

PRINCIPLE

The AMPLIKIT-HPA-3 Test-Kit is based on the PCR mediated amplification of human genomic DNA region surrounding the position 843 of the glycoprotein IIb, and subsequent restriction fragments length polymorphism analysis (PCR-RFLP). Electrophoresis in 3% agarose gel should be performed to resolve the restriction fragments specific for each polymorphic allele.


MATERIALS PROVIDED

1. Double distilled water (H₂O), 300 µl – 1 tube
2. Master-mix, 55 µl – **5 tubes**
3. 10xPCR buffer, 100 µl – 1 tube
4. 25 mM MgCl₂, 40 µl – 1 tube
5. Taq-polymerase (5 U/µl), 15 µl – 1 tube
6. Dilution buffer for Taq-polymerase, 80 µl – 1 tube
7. Mineral oil, 2.5 ml – 1 tube
8. Control DNA “HPA-3 (-/-)” (Ile/Ile, or 3A/3A genotype), 25 µl– 1 tube
9. Control DNA “HPA-3 (+/-)” (Ile/Ser, or 3A/3B genotype), 25 µl– 1 tube
10. Control DNA “HPA-3 (+/+)” (Ser/Ser, or 3B/3B genotype), 25 µl – 1 tube
11. Restriction buffer, 300 µl – 1 tube
12. Restriction enzyme, 35 µl – 1 tube
13. Bromphenol blue buffer, 300 µl – 1 tube

MATERIALS REQUIRED BUT NOT PROVIDED

- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g); Eppendorf 5415D or equivalent
- 60°C ± 5°C dry heat block
- Vortex mixer
- Biohazard waste container
- Refrigerator
- Freezer
- Thermalcycler
- Workstation
- Pipettors (capacity 0,5-10 µl; 5-40 µl) with aerosol barrier
- Tube racks

Reagents non provided

- Detection agarose kit (Precast gel 2-3%  G1-1)
- DNA extraction kit

STORAGE CONDITIONS

The Test-Kit should be stored before use at -20°C . After opening the Test-Kit, all the components should be placed at an appropriate temperature conditions. Reagents 2, 5, 6, and 12 should be stored at -20°C . Reagents 7 and 13 should be stored at room temperature. Other components of the Test-Kit should be thawed at room temperature for 15-20 min, then placed at $60-65^{\circ}\text{C}$ for 15-20 min and subsequently stored at $+4^{\circ}\text{C}$. An enzyme activities of the Reagents 5 and 12 could decrease slightly before expiration date. We recommend to increase the amounts of these Reagents given per each analysis up to 50% over their initial values, when the time to expiration date is less than 2 months.

WARNINGS AND PRECAUTIONS

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Do not pipette by mouth.
3. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
4. Do not use a kit after its expiration date.
5. Heparin has been shown to inhibit reaction. Use of heparinized specimens is not recommended.
6. Dispose of all specimens and unused reagents in accordance with local regulations.
7. Specimens should be considered potentially infectious and handled in biological cabinet in accordance with Biosafety Level 2 or other appropriate biosafety practices.
8. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant.
9. Avoid contact of specimens and reagents with the skin, eyes and mucose membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
10. Material Safety Data Sheets (MSDS) are available on request.
11. Use of this product should be limited to personnel trained in the techniques of DNA amplification.
12. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where you performed previous step.

GENERAL REMARKS:

1. It is recommended to create the protocol for PCR and subsequent restriction analysis each time before the assay performance.
2. This Test-Kit does not contain the reagents for DNA extraction. DNA samples with concentration in range from 30 to 150 ng/ μl could be analyzed.
3. It is recommended to include the Reagents 8, 9 and 10 in your PCR protocol each time when the assay is performed.
4. To avoid microbial contamination during storage, the Reagents 1, 3, 4, 8, 9, 10 and 11 should be placed at 65°C for 20 min after each month of being in use.
5. To protect the Reagent 2 from damage due to frequent thawing-freezing procedures, it has been divided into 5 equal tubes (each tube contains the master-mix sufficient for 11 PCR reactions).
6. This Test-Kit does not contain the reagents for nucleic acid detection by gel electrophoresis.

PREPARATION OF THE REAGENT 5 WORKING SOLUTION:

Before the assay performance, working solution of the Reagent 5 should be prepared by its dilution with the Reagent 6 as described below:

1. In the sterile PCR tube add consequently 10 μl of the Reagent 6 and 2 μl of the Reagent 5 (these quantities will be enough for 10 PCR reactions).
2. Vortex the mix carefully, then concentrate it by quick spin on microfuge.
3. This working solution of the Reagent 5 should be stored at -20°C .

Please note: Reagent 5 working solution should be stored no more than 3 weeks.

ASSAY PROCEDURE:

Amplification

1. Thaw enough quantity of the Reagent 2 at room temperature for 10-15 min.
2. Vortex the Reagent 2 carefully, then concentrate it by quick spin on microfuge.
3. In the sterile tube, prepare the total for all DNA samples tested mix from the following Reagents (**here, the quantities are given for one DNA sample**):

Reagent 1 - 5,0 μ l
Reagent 2 - 5,0 μ l
Reagent 3 - 1,5 μ l
Reagent 4 - 0,6 μ l
Reagent 5 working solution - 1,0 μ l

4. Vortex the mix carefully, then concentrate it by quick spin on microfuge.
5. Aliquot the mix per 13 μ l into each PCR tube.
6. Add 2 μ l of each DNA sample tested into respective PCR tube.
7. Add one drop (30-40 μ l) of the Reagent 7 into each PCR tube.
8. Close the PCR tubes tightly and place into the PCR machine.
9. PCR should be run in the following regimen: (95°C – 3 min, 55°C – 50 sec, 72°C – 50 sec) – 1 cycle; (95°C – 50 sec, 55°C – 50 sec, 72°C – 50 sec) – 39 cycles; (72°C – 2 min) – 1 cycle.

ASSESSMENT OF PRODUCT BY GEL ELECTROPHORESIS:

Please note: If you are sure, that both quantity and quality of the analyzed DNA samples are sufficient enough for PCR analysis, this Section could be omitted.

The PCR product could be easily detected in 2-3% agarose gel with the run distance at least 1 cm. Please, prepare the respective gel beforehand.

To assess the quantity and quality of the obtained PCR product:

1. Aliquot 3 μ l of the PCR product and mix it with 1 μ l of the Reagent 13.
2. Analyze the PCR product aliquot by electrophoresis in 2-3% agarose gel with the run distance 1-2 cm.

Please note: It is forbidden to add the Reagent 13 into PCR tube(s) before the restriction analysis will be done.

RESTRICTION ANALYSIS:

1. In sterile tube, prepare the total for all DNA samples tested stock from the following Reagents (**here, the quantities are given for one sample**):

Reagent 11 - 5,0 μ l
Reagent 12 - 0,3 μ l

2. Vortex this mix carefully, then concentrate it by quick spin on microfuge.
3. Aliquot the mix per 5 μ l into tubes with PCR products (over the mineral oil).
4. Spin the PCR tubes on microfuge for 15-20 sec, and then incubate at 37°C for 2-24 hours.

ANALYSIS OF RESTRICTION FRAGMENTS BY ELECTROPHORESIS IN AGAROSE GEL:

Please note: The restriction fragments could be resolved by electrophoresis in 3% agarose gel with the run distance 5 cm. Please, prepare the respective gel beforehand.

1. When the restriction analysis of PCR product(s) is over, add 5 μ l of the Reagent 13 to each PCR tube (over the mineral oil).
2. Spin the PCR tubes on microfuge for 10-15 sec.
3. Mix the PCR product with the Reagent 13 and then apply 10 μ l of this mix to the gel well.
4. Perform electrophoresis in standard conditions until the run distance of the Reagent 13 will achieve 4-5 cm.
5. Analyze the results on UV-transilluminator.

INTERPRETATION OF THE RESULTS

The size of amplified PCR product from the glycoprotein IIb gene equals to 250 base pairs (bp). In the presence of GpIIb HPA-3A allele in the tested DNA sample, the 250 bp product is digested to 150 bp and 100 bp fragments. Therefore, the detection of 100 bp and 150 bp fragments (complete digestion) after electrophoresis corresponds to the HPA 3A/3A genotype of the GpIIb. This two bands must always be seen, when the control DNA “HPA-3 (-/-)” (Reagent 8) is included in analysis. Identification of 250 bp, 150 bp and 100 bp fragments in the gel corresponds to the heterozygous GpIIb genotype (HPA-3A/3B). This set of restriction fragments must always be seen, when the control DNA “HPA-3 (+/-)” (Reagent 9) is included in analysis. When the DNA sample contains the 843 Ser encoding allele of GpIIb in the homozygous form (843 Ser/Ser, or HPA-3B/3B genotype), only the native 250 bp PCR product should be present in the gel. This pattern must always be seen, when the control DNA “HPA-3 (+/+)” (Reagent 10) is included in analysis.