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EliGene® Spondylitis HLA-B27 RT

REF 90060-RT (for 50 samples)

Kit components:

5 x 200 µl **HLA-B27 Mix**
2 x 50 µl **PC DNA HLA-B27**
Instruction for Use

Storage and shelf life after first opening:

All components of the kit must be transported and stored at -20°C. Kit and remaining MasterMixes must be stored at -20°C in a dark.

Intended use

EliGene® Spondylitis HLA-B27 RT is intended for the genotypization of HLA-B27 allele (subtypes B*2701-2759) from isolated DNA.

Principle of the method

This diagnostic kit is based on RealTime PCR method. In this kit primers and labeled probes (FAM and HEX) for the detection of HLA-B27 alleles and for the detection of internal control are used.

Introduction

Human Leukocyte Antigen (HLA) B27 (subtypes B*2701-2759) is an HLA class I surface antigen that is encoded in the B locus in the major histocompatibility complex (MHC) on the short arm of chromosome 6. In HLA-B27 antigen a strong association with ankylosing spondylitis (AS) and spondyloarthropathies (SpA) was found. Moreover the increased incidence of this antigen in other diseases as Reiter´s syndrome or uveitis was found. Association studies found an association between HLA-B27 antigen and AS in all ethnic and racial groups worldwide however the prevalence of HLA-B27 antigen and the strength of its association with AS does vary. For example, the prevalence of HLA-B27 antigen is about 8% in Caucasians, 4% in North Africans, 2-9% in Chinese, and 0.1-0.5% in Japanese. Further, among northern Europeans only 8% of the general population possesses HLA-B27, but more than 90% of the patients with AS possess this gene. In contrast, among African Americans 2% to 4% of the general population and only 50% to 60% of patients with AS possess this gene.

From this reasons HLA-B27 test cannot be used to screen an asymptomatic population to detect AS but the test provides a statement of increased probability of the existence of AS in the symptomatic patient. Also, the presence of HLA-B27 antigen influences the clinical manifestations of AS disesase, because HLA-B27–positive patients have a significantly younger age at onset of their disease and a higher prevalence of episodes of eye inflammation (acute anterior uveitis) and hip joint involvement.

EliGene® Spondylitis HLA-B27 RT kit detects HLA-B27 allele (subtypes B*2701-2759). As an internal control the gene SYPL2 (synaptophysin-like 2) is used.

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Primary sample collection, handling and storage

Clinical material:	Recommended DNA isolation procedure:
Blood	Manual: EliGene Urine Isolation Kit (ELISABETH PHARMACON) Automatic: **ZEPHYRUS Magneto** (ELISABETH PHARMACON)
Swabs	Manual: EliGene Urine Isolation Kit (ELISABETH PHARMACON) Automatic: **ZEPHYRUS Magneto** (ELISABETH PHARMACON)

Blood:

Manual isolation:

Add 10 µl of Proteinase K to the sample and then continue according to the standard protocol of EliGene Urine Isolation Kit (ELISABETH PHARMACON) for DNA isolation from blood. Isolated DNA use immediately for the detection or store it hours to one week at 4°C or freeze DNA at -20°C for longer period than one week.

Automatic isolation:

Isolate DNA from the sample by using MAGNETO ***BodyFluid DNA/RNA isolation kit*** according to protocol for Blood samples with elution to 50 µl of Elution buffer.

Buccal swabs:

These specimens should be collected according to standard protocol in collection tubes. Specimens should be transported to the laboratory and elaborated in 3 days after collecting. For longer storage, swabs should be dried at room temperature and than it can be stored at room temperature for few weeks.

Manual isolation:

1. Into 2.0 ml tube pipette 400 µl of MI3 solution and 20 µl of Proteinase K.
2. Put the swab into the 2.0 ml tube and with sterile scissor cut the swab – cut about 0.5 cm above the swab. Close the tube.
3. Incubate tube 20 minutes at 56°C in thermo shaker at 1000 rpm. Consequently shortly spin the tube.
4. By sterile pincers remove the swab and add 330 µl of solution MI4 to lysate. Vortex and shortly spin.
5. Continue according to the standard protocol of EliGene Urine Isolation Kit. Isolated DNA use immediately for the detection or store it hours to one week at 4°C or freeze DNA at -20°C for longer period than one week.

Automatic isolation:

1. Into 2.0 ml tube pipette 450 µl of Lysis buffer, 200 µl of PCR water and 10 µl of Proteinase K.
2. Put the swab into the 2.0 ml tube and with sterile scissor cut the swab – cut about 0.5 cm above the swab. Close the tube.
3. Incubate tube 20 minutes at 56°C in thermo shaker at 1000 rpm. Consequently shortly spin the tube.
4. By sterile pincers remove the swab, vortex and shortly spin.
5. Pipette all volume of sample to position H at Deep well plate from MAGNETO ***BodyFluid DNA/RNA isolation kit***.
6. Isolate DNA from the sample by using MAGNETO ***BodyFluid DNA/RNA isolation kit*** according to protocol for plasma samples with elution to 50 µl of Elution buffer.

Recommended concentration of analyzed DNA is 1-10 ng/µl. It is not recommended to test samples at lower concentration than 1 ng/µl.

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Additional required equipment

- Automatic pipette 5-20 µl and sterile tips with filter DNA RNA free, DNase-, RNase- free (we recommended plastic with CE certificate for diagnostic purposes).
- Sterile stand DNA-, RNA- free, DNase-, RNase- free.
- Equipment for RealTime PCR – the kit is designed for RealTime Systems ABI 7000, 7300, 7500 (Applied Biosystems), LightCycler 480 and LightCycler Nano (Roche), RotorGene 6000 or RotorGene Q (Qiagen), Smart cycler.
- Sterile plastic (strips, plates, tubes) DNase-, RNase- free compatible with given RealTime PCR system.
- Lab safety gloves.

Configuration of Real Time instrument

- For HLA-B27 allele the probe labeled with FAM are used (exc. 494nm – em. 518 nm)
- For gene SYPL2 (internal control) detection the probe labeled with HEX is used (exc. 520nm – em. 548nm)
- Reaction Mix includes passive reference control dye ROX for signal normalization

RealTime Systems ABI 7000, 7300, 7500FAST (Applied Biosystems):

Use the program module for absolute quantification (Plate Type "Absolute Quantification" for ABI 7300, "Quantitation-Standard Curve" experiment for ABI 7500FAST). In case of ABI7500FAST use "7500 (96wels)" instrument type.

Set up the following temperature profile:

Holding stage

95°C 3 min

Cycling stage – 40 cycles

95°C 10 s

62°C 30 s Data collection ON

Collect emission signal at the second step – 60 °C.

The complete temperature profile can be up-loaded from Run Template "HLAB27_RT_ABI7300_v03.sdt" or "HLAB27_RT_ABI7500_v03.edt". The Run Template can be copied from the CD included in the kit.

LightCycler® 480 (Roche):

For reaction use white plates only. The usage of natural plates can lead to decreased sensitivity of the kit. Do not reuse plates; the contamination of your laboratory could occur during the manipulation with plates.

In option Detection format choose "Dual Color Hydrolysis probe".

Set up the following temperature profile:

Step 1 - Analysis mode "None", 1 Cycle

95°C	3 min	Ramp rate (4.4°C/s)	Acquisition mode "None"

Step 2 - Analysis mode "Quantification", 40 Cycles

95°C	10 s	Ramp rate (4.4°C/s)	Acquisition mode "None"
62°C	30 s	Ramp rate (2.2°C/s)	Acquisition mode "Single"

Step 3 - Analysis mode "None", 1 Cycle

40°C	1 min	Ramp rate (2.2°C/s)	Acquisition mode "None"

The complete temperature profile can be up-loaded from Run Template "HLAB27_RT_LC480_v03.ixo". The Run Template can be imported to the software in menu "Navigator" by clicking to icon "Import" from the CD included in the kit.

LightCycler Nano (Roche):

For reaction use clear strips only.

In "Run Settings" menu choose "Hydrolysis Probes" option and "High Quality" option.

In "Profile menu" set up the following temperature profile:

Step 1 - Hold

95°C	3 min	Ramp rate (5°C/s)

Step 2 – 2-Step Amplification, 40 cycles

95°C	10 s	Ramp rate (5°C/s)	
62°C	30 s	Ramp rate (4°C/s)	"Acquire" signal

Step 3 - Hold

40°C	1 min	Ramp rate (4°C/s)

In "Samples" menu click in window "Targets" (upper right window) on icon "+ " and choose FAM dye as "Target 1". Then click once again on icon "+ " and choose HEX dye as "Target 2". In window "Samples" (upper left window) click on icon "+ " and add your samples. Then assign the samples with positions and Targets FAM and HEX as an Unknown sample (Samples).

The complete temperature profile can be up-loaded from Run Template "HLAB27_RT_LCNANO_v03.ppf". The Run Template can be copied from the CD included in the kit.

RotorGene 6000 or Q (Qiagen):

In the "New Run" window choose "Two Step" run

Choose the appropriate "Rotor Type" and click "Next".

Set up the following temperature profile:

Holding stage

95°C	3 min

Cycling stage – 40 cycles

95°C	10 s	
62°C	30 s	Acquiring in channels "Green" and "Yellow"

The complete temperature profile can be up-loaded from Run Template "HLAB27_RT_RG_v03.ret". The Run Template can be copied from the CD included in the kit.

SmartCycler® (Cepheid):

In the Main menu window click “Define protocols” icon

Set up the following Protocol:

Stage 1 - Repeat 1 - Hold

95°C	180 sec	Deg/Sec (NA)	Optics “OFF”

Stage 2 - Repeat 40 -2- temperature cycle

95°C	10 s	Deg/Sec (NA)	Optics “OFF”
62°C	30 s	Deg/Sec (NA)	Optics “ON”

To run the protocol select FCTC25 dye set and in Define graphs option **set up the Graphic configuration as follows**:

Graph type	Optics
Channel(s)	Ch 1 (FAM) and Ch 2 (Cy3)
Show	Primary curve, Threshold (Horizontal), Threshold Crossing (Vertical)
Axes	Fluorescence vs. Cycle

Reagent preparation

- To avoid the contamination keep all tubes closed and follow the instructions.
- Before the usage, all reagents must be completely thawed, briefly mix on vortex and shortly spin.
- Isolate DNA according to standard protocol.

Preparation of Reaction Mix

WARNING: The contamination in laboratory space is also possible. Use separate pipette for MasterMixes, separate pipette for positive controls and separate pipette for samples. Follow all recommendations for laboratories of DNA analyses.

1. Detection: Take one microtube with HLA-B27 Mix and after the thawing pipette 20 µl of each mix to separate amplification microtube. Thereafter add 5.0 µl of isolated DNA to each microtube. During the pipetting of samples be careful to avoid cross-contamination of samples. If you do not use all the volume of MasterMix, freeze it and store at -20 °C in a dark. Do not freeze tubes with HLA-B27 Mix repeatedly. Under these conditions it is stable at least for 14 days.
2. Positive control: Take one microtube with HLA-B27 Mix and after the thawing pipette 20 µl of each mix to amplification microtube and add 5.0 µl of Positive Control PC DNA HLA-B27.

Insert the microtubes or plate with samples to the RealTime PCR instrument and run the program according to chapter “Configuration of Real Time instrument” above.

Result reading

RealTime Systems ABI 7000, 7300, 7500FAST (Applied Biosystems):

In “Analysis Settings” choose “Automatic Treshold” and “Automatic Baseline” option and analyze results.

Positive result for *HLA-B27 allele*: The positive result is characterized by amplification and growth of signal in FAM channel (530) of HLA-B27 Mix reaction. In a case of negative results the amplification will not occur.

The Internal Control is amplified every time. The Internal Control amplification is characterized by amplification and growth of signal in HEX channel (560) of HLA-B27 Mix reaction.

The values of Qty correspond to the quantity of positive result, "result undet." means negative result. Positive result is characterized by increasing of fluorescence signal in given channel.

LightCycler 480 (Roche):

In menu "Analysis" choose "Abs Quant/2nd Derivative Max" option.

In Analysis window click to "Color Comp" icon and choose Universal CC FAM (510)-HEX (580) calibration. Analyze results by clicking to icon "Calculate".

Positive result for *HLA-B27 allele*: The positive results are characterized by amplification and growths of signal in FAM channel (510-528) of HLA-B27 Mix reaction. In a case of negative results the amplification will not occur.

The Internal Control is amplified every time. The Internal Control amplification is characterized by amplification and growth of signal in HEX channel (533-580) of HLA-B27 Mix reaction.

The values of Concentration correspond to the quantity of positive result; "Negative" means negative result. Positive result is characterized by increasing of fluorescence signal in selected channel.

LightCycler Nano (Roche):

In "Analysis" menu click in window "Select Analysis" on icon "+ " and choose "Automatic Quantification".

Positive result for *HLA-B27 allele*: The positive results are characterized by amplification and growths of signal in FAM channel (510-528) of HLA-B27 Mix reaction. In a case of negative results the amplification will not occur.

The Internal Control is amplified every time. The Internal Control amplification is characterized by amplification and growth of signal in HEX channel (530-548) of HLA-B27 Mix reaction.

The values of Concentration correspond to the quantity of positive result; "Negative" means negative result. Positive result is characterized by increasing of fluorescence signal in selected channel.

RotorGene 6000 or Q (Qiagen)- version 1.7 and higher:

Click to "Analysis" icon in the menu and choose Analysis option "Quantitation". In "Quantitation Analysis" window choose "Dynamic Tube" and "Slope Correct" option. In menu "Quantitative settings" set up for the FAM channel the parameter NTC threshold of 30%.

Positive result for *HLA-B27 allele*: The positive results are characterized by amplification and growth of signal in FAM channel (Green) of HLA-B27 Mix reaction. In a case of negative results the amplification will not occur.

The Internal Control is amplified every time. The Internal Control amplification is characterized by amplification and growth of signal in HEX channel (Yellow) of HLA-B27 Mix reaction.

The values of "Calc. conc." correspond to the quantity of positive result; "Negative" means negative result. Positive result is characterized by increasing of fluorescence signal in given channel.

SmartCycler® (Cepheid):

To analyze the Ct values data select option "Results Table" in the "View Results" menu.

Positive result for *HLA-B27 allele*: The positive result is characterized by amplification and growth of signal in FAM channel (Ch1) of HLA-B27 Mix reaction. In a case of negative results the amplification will not occur.

The Internal Control is amplified every time. The Internal Control amplification is characterized by amplification and growth of signal in Cy3 channel (Ch 2) of HLA-B27 Mix reaction.

The values of "Calc. conc." correspond to the quantity of positive result; "Negative" means negative result. Positive result is characterized by increasing of fluorescence signal in given channel.

Interpretation of results

Negative result:

If the increasing of amplification signal in FAM channel of HLA-B27 Mix does not appear before cycle number 35, the result of test should be interpreted as HLA-B27 negative. The signal for Internal Control must be positive – see article Quality control.

Positive result:

Amplification signal in FAM channel of HLA-B27 Mix appears before cycle number 35. HLA-B27 allele was detected in the sample. The sample is HLA-B27 allele positive.

WARNING: The contamination in laboratory space is also possible. Use separate pipette for MasterMixes, separate pipette for positive controls and separate pipette for samples. Follow all recommendations for laboratories of DNA analyses.

Inhibited sample:

If in both channels, FAM and HEX, the amplification signal of HLA-B27 Mix is not observed. In this case it is necessary to repeat the analysis. The best is to use DNA samples prepared by new DNA isolation.

Control procedure

EliGene® Spondylitis HLA-B27 RT kit detects as an internal isolation control the human gene *SYPL2* (synaptophysin-like 2) in HLA-B27 in HEX channel. *SYPL2* gene is present in each human DNA sample, so it is not necessary to add internal control to the sample. Internal isolation control follows the quality of DNA isolation and detects mistakes in the isolation process. It detects the occurrence of an inhibition of amplification process. In the case that the sample is HLA-B27 negative, the Ct of internal control must be Ct < 35.

Reference material:

To monitor the all examination process covering DNA isolation and RealTime PCR detection is possible to use reference material positive for HLA-B27 allele. The commercial positive material is not available.

Troubleshooting:

1. If there is no amplification of Internal Control, there is some problem in the isolation of DNA or the kit is after the expiration date or there is RealTime instrument breakdown.
2. If there is no amplification of Positive Control, the kit is after the expiration date or there is RealTime instrument breakdown.

Performance characteristics

Analytical performance characteristics:

EliGene® Spondylitis HLA-B27 RT kit is specific for detection of HLA-B27 allele. The kit detects human genomic DNA of concentration higher than 1 ng/µl added to the amplification mix.
Analytical sensitivity is 1 ng of DNA in reaction mix.

Analytical specificity of method is 100%. Analytical specificity of the method was validated by searching in the DNA databases.

Clinical specificity EliGene® Spondylitis HLA-B27 kit specificity was tested on 202 samples of human DNA with genotypes determined by DNA sequencing.

Diagnostic performance characteristics:

EliGene® Spondylititis HLA-B27 RT kit specificity was tested on 100 samples of human DNA with genotypes determined by DNA sequencing. Totally 202 samples were right determined by EliGene® Spondylititis HLA-B27 kit.
The clinical specificity of EliGene® Spondylititis HLA-B27 RT kit is 100%.

Measuring interval

The kit enables the detection of ≥1 ng DNA molecules in reaction mix.

Internal control of quality

As an internal control of quality the Internal Control for checking the process of DNA isolation together with Positive Control for functional control of MasterMix and as a reference sample is used.

Limitation of the examination procedure

The sensitivity of kit depends on handling with specimen (isolation of DNA). It is strictly recommended to use isolation kits and procedures mentioned above.

Biological reference intervals

Not applicable information for this kit.

Warning

Unused content of the tube with MasterMix is stable for 2 weeks at -20 °C. Do not freeze tubes with MasterMix repeatedly! Do not mix components of the kits of different lots.

Warnings and general precautions

- Handle and dispose of all biological samples as if they were capable of transmitting infective agents. Avoid direct contact with the biological samples. Avoid splashing or spraying. The materials that come into contact with biological samples must be treated with 3% sodium hypochlorite for at least 30 minutes or autoclaved at 121 °C for one hour before disposal.
- Handle and dispose of all reagents and all assay materials as if they were capable of transmitting infective agents. Avoid direct contact with the reagents. Avoid splashing or spraying. Waste must be treated and disposed of in compliance with the appropriate safety standards. Disposable combustible materials must be incinerated. Liquid waste containing acids or bases must be neutralized before disposal.
- Wear suitable protective clothing and gloves and protect eyes/face.
- Never pipette solutions by mouth.
- Do not eat, drink, smoke or apply cosmetic products in the work areas.
- Wash hands carefully after handling samples and reagents.
- Dispose of leftover reagents and waste in compliance with regulations in force.
- Read all the instructions provided with the kit before running the assay.
- Follow the instructions provided with the kit while running the assay.

- Do not use the kit after the expiry date.
- Only use the reagents provided in the kit and those recommended by the manufacturer.
- Do not mix reagents from different batches.
- Do not use reagents from other manufacturer´s kit.

Warnings and precautions for molecular biology

- Molecular biology procedures, such as extraction, reverse transcription, amplification and detection of nucleic acids, require qualified staff to prevent the risk of erroneous results, especially due to degradation of the nucleic acids contained in the samples or due to sample contamination by amplification products.
- It is necessary to have separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions.
- It is necessary to have lab coats, gloves and tools which are exclusively employed in the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never transfer lab coats, gloves or tools from the area designed for the amplification/detection of amplification products to the area designed for the extraction/preparation of the amplification reactions.
- The samples must be exclusively employed for this type of analysis. Samples must be handled under a laminar safety box. Tubes containing different samples must be never opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Reagents must be handled under PCR box or laminar flow box. The reagents required for amplification must be prepared in such a way that they can be used in a single session. The pipettes employed to handle the reagents must be used exclusively for this purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Amplification products must be handled in such way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be employed exclusively for this specific purpose.

Warnings and precautions specific to components of the kit

- The tubes containing mix (HLA-B27 Mix) are disposable and therefore must be used once only in the preparation of the reaction mixture.
- These mixes carry the following safety warnings (P):

P280 Wear protective gloves/protective clothing/eye protection/face protection.

P281 Use personal protective equipment as required.

Literature

Khan MA. Remarkable Polymorphism of HLA-B27: An Ongoing Saga. Curr Rheumatol Report. 2010; 12: 337-41

Robinson PC, Brown MA. 2012. The genetics of ankylosing spondylitis and axial spondyloarthritis. Rheum Dis Clin North Am. 38(3):539-53

Thomas GP, Brown MA. Genetics and Genomics of Ankylosing Spondylitis. Immunol Rev. 2010; 233:162-180.

Symbols

	Catalog number
	Upper limit of temperature
	Batch code
	Use by (last day of month)
	*in vitro* diagnostic medical device
	Fulfilling the requirements of European Directive 98\79\EC for *in vitro* diagnostic medical device.
	Contains sufficient for "N" tests
	Attention, consult instructions for use
	Manufacturer

Manufacturer

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