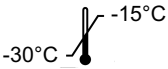
ViennaLab®

HLA-B27
RealFast™ Assay

Instructions For Use

REF	Σ
7-620	100 reactions
7-623	32 reactions
7-620-TRIAL	20 reactions



IVD

CE 0123

Version: rev 1.0 / English
eIFU and other languages available at
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REVISION HISTORY:

version	date	description
rev 1.0	2022-11	Initial version

Summary of Safety and Performance (SSP) of the RealFast™ Assay is retrievable from the European Database on Medical Devices (EUDAMED): https://ec.europa.eu/tools/eudamed or from the manufacturer.

I. INTENDED PURPOSE

The HLA-B27 RealFast™ Assay is a non-automated real-time PCR test for the detection of *HLA-B*27* alleles, specific variants of the *human leukocyte antigen B (HLA-B)* gene. *HLA-B*27* alleles are strongly associated with seronegative spondyloarthropathies. The assay is an aid to diagnosis to confirm suspected ankylosing spondylitis, reactive arthritis, juvenile rheumatoid arthritis, or anterior uveitis. The qualitative assay discriminates the presence or absence of *HLA-B*27* alleles in a human genomic DNA extract from blood and detects the majority of disease-relevant *HLA-B*27* subtypes.

For human *in vitro* diagnostics.

II. BACKGROUND

HLA-B molecules are cell surface proteins that play an important role in immunity. The frequency of the *HLA-B*27* variant is about 8% in the general Caucasian population, whereas it is found in over 90% of patients suffering from ankylosing spondylitis (AS). The disease, predominantly striking men between the age of 20 and 40 years, is characterized by inflammation of the sacroiliac joint and progressing stiffness of the spine. Due to the high correlation with the disease, *HLA-B*27* genotyping is suitable for the differential diagnosis of AS. Other subsets of spondyloarthritis are also associated with *HLA-B*27*, although to a lesser degree.

III. METHODOLOGY

The HLA-B27 RealFast™ Assay is based on the fluorogenic 5' nuclease assay, also known as TaqMan® assay. Each reaction contains gene-specific primers, which amplify a 202 bp fragment of the *HLA-B* gene. Further components are two dual-labeled, gene-specific hydrolysis probes which hybridize to the target sequence of the corresponding fragment. The proximity of the 5'-fluorescent reporter and 3'-quencher dye on intact probes prevents the reporter from fluorescing. During the extension phase of PCR, the 5' – 3' exonuclease activity of the Taq DNA polymerase cleaves the 5'-fluorescent reporter from the hybridized probe. The physical separation of the fluorophore from the quencher dye generates a fluorescent signal in real-time, which is proportional to the accumulated PCR product. In samples positive for *HLA-B*27* both, the **FAM-labeled *HLA-B*27*** probe as well as the **HEX-labeled PCR control** probe bind to the PCR fragment. A strong fluorescence signal is detected in the FAM channel (520nm) and the HEX channel (556nm). In samples negative for *HLA-B*27* only the HEX-labeled PCR control probe hybridizes to the complementary strand of the gene fragment. A strong fluorescence signal is detected in the HEX channel and no or only a baseline signal in the FAM channel.

The HLA-B27 RealFast™ Assay is designed to be capable of detecting the majority of known *HLA-B*27* alleles and subtypes (Table 1).

Table 1: *HLA-B*27* alleles detected by the HLA-B27 RealFast™ Assay

***HLA-B*27* alleles**	**supposedly abrogated or reduced detection**
*B*27:01* to *B*27:05:57*	*B*27:05:09, B*27:05:23, B*27:05:51*
*B*27:06*	
*B*27:07:01* to *B*27:08*	*B*27:07:06*
*B*27:09*	
*B*27:10* to *B*27:15*	*B*27:12:01:01* to *B*27:12:01:03*
*B*27:16*	*B*27:16*
*B*27:17* to *B*27:19*	*B*27:18*
*B*27:20* to *B*27:21*	
*B*27:23* to *B*27:25*	*B*27:23*
*B*27:26* to *B*27:27*	*B*27:26*
*B*27:28*	
*B*27:29* to *B*27:48*	*B*27:29, B*27:31*
*B*27:49*	
*B*27:50:01* to *B*27:256*	*B*27:59N, B*27:77, B*27:85, B*27:91, B*27:92, B*27:101, B*27:109, B*27:119, B*27:129, B*27:140, B*27:153, B*27:157, B*27:204, B*27:239, B*27:242, B*27:246N*

Reference Sequence (RefSeq):

NG_023187.1 (*HLA-B*)

Note: The HLA-B27 RealFast™ Assay detects the *HLA-B* alleles *B*27:03, B*27:05:12, B*27:06:02, B*27:07:03, B*27:17, B*27:65N, B*27:94N, B*27:111, B*27:139, B*27:151, B*27:170, B*27:182, B*27:237, B*27:243N* and *B*27:250*, but the corresponding HEX-labelled TaqMan Probe binding to the control fragment may fail in homozygous samples.

IV. KIT COMPONENTS

				REF 7-620	7-623	7-620-TRIAL
1.	RealFast™ 2x **Genotyping Mix**	1 vial	white cap	1000 µl	320 µl	200 µl
2.	HLA-B27 **Assay Mix**	1 vial	purple cap	550 µl	550 µl	550 µl
3.	HLA-B27 **Positive Control**	1 vial	green cap	75 µl	75 µl	75 µl
4.	HLA-B27 **Negative Control**	1 vial	red cap	75 µl	75 µl	75 µl
5.	**Instructions for Use**			1	1	1

The kit contains reagents for 100 / 32 reactions in a final volume of 20 µl each. The RealFast™ 2x Genotyping Mix comprises hot-start Taq DNA polymerase and dNTPs in an optimized buffer system. The HLA-B27 Assay Mix consists of gene-specific primers and dual-labeled hydrolysis probes for *HLA-B*27* and a control gene. A positive and a negative control for *HLA-B*27* are supplied with the kit.

Note: HLA-B27 RealFast™ Assay is shipped on cooling blocks. On arrival, store the kit at -30°C to -15°C. Alternatively, it can be stored at 2°C to 8°C for short-term use within one month. The kit withstands up to 20 freeze/thaw cycles with no loss of activity. Avoid exposure to intense light.

V. MATERIALS REQUIRED BUT NOT SUPPLIED

In addition to standard molecular biology laboratory equipment, the following is needed:

- real-time PCR instrument with FAM (520 nm) and HEX (556 nm) filters
- instrument-compatible reaction vessels
- disposable powder-free gloves
- vortex mixer
- mini-centrifuge for 2.0 ml tubes
- tube racks
- set of calibrated micropipettes (0.5 – 1000 µl)
- sterile tips with aerosol-barrier filter
- molecular grade water
- DNA extraction system. GENXTRACT™ Blood DNA Extraction System (REF 2-014, ViennaLab) or Spin Micro DNA Extraction Kit (REF 2-020, ViennaLab) recommended
- biohazard waste container

VI. ASSAY PROCEDURE

1. Sample Preparation

Specimen: Use fresh or frozen blood with EDTA anticoagulant. Blood containing heparin or citrate has not been tested. Do not store blood for more than 3 days at ambient temperature or more than 1 week at 2°C to 8°C before use. Blood that has been kept frozen for more than one year, or gone through more than three freeze-thaw cycles shall not be used. For specimen collection and transportation follow the instructions for use of the EDTA-blood collection tube and general recommendations for blood sampling.

DNA Extraction: DNA extraction reagents are not supplied with the kit. DNA isolated from whole peripheral blood can be used. Ensure extracted DNA is suitable for amplification in terms of concentration, purity, and integrity. For accurate genotype calling, DNA should be within a range of 2 to 10 ng/µl and an $OD_{A260/280}$ ratio of 1.7 to 2.0. Higher DNA concentrations have to be diluted to the recommended range prior to PCR input.

Extracted DNA shall be stored at 2°C to 8°C (up to one week) or at -30°C to -15°C (for long term) until analysis is carried out.

2. PCR Control Templates

- **Always** include a **no-template control** (NTC) in each experiment to confirm the absence of potential contamination. It is advisable to run the NTC (use PCR-grade water instead of DNA) in duplicates.
- **Always** include the HLA-B27 **Positive Control** as positive reference signal.
- **Always** include the HLA-B27 **Negative Control** as negative reference signal for threshold setting in the FAM channel.

Note: The controls are potential sources for cross-contamination. Make sure to handle them carefully.

3. Preparation of HLA-B27 RealFast™ Master Mix

- Gently vortex and briefly centrifuge all solutions after thawing.
- Set up PCR at room temperature.
- Prepare sufficient **Master Mix** for all your reactions (N samples + positive control + negative control + NTC) plus at least one additional reaction to compensate for pipetting inaccuracies:

component	per reaction	e.g. 24+1 reactions
RealFast™ 2x Genotyping Mix	10 µl	250 µl
HLA-B27 Assay Mix	5 µl	125 µl
Master Mix	**15 µl**	375 µl

Note: The kit is supplied without ROX. For use with real-time PCR instruments requiring high ROX for normalization of data (e.g. Applied Biosystems® instruments: StepOne™, 7300, 7900/7900HT) add ROX at a final concentration of 1 µM to the 2 x Genotyping Mix.

- Dispense **15 µl Master Mix** into each well.
- Add **5 µl** purified **DNA** or **control** template to reach a final reaction volume of 20 µl. To minimize the risk of contamination, always pipette templates in the following order: first NTC, then samples, last controls. Immediately close reaction vessels.

Note: Avoid creating bubbles in the final reaction mix and avoid touching the optical surface of the cap or sealing film without gloves. Both may interfere with fluorescence measurements. Centrifuge briefly if needed.

4. PCR Program

Program the real-time PCR instrument according to the manufacturer's instructions for quantitation experiments with two targets/reporter dyes. Place the samples into the thermal cycler and run the following program:

cycles	temp	time	steps
1	95°C	3 min	initial denaturation
40	95°C	15 sec	denaturation
	60°C	1 min	annealing/extension **data acquisition** on FAM and HEX channel

The HLA-B27 RealFast™ Assay is validated for the AB 7500 Fast, StepOne™, CFX96™, LightCycler® 480, Mx3005P, MIC qPCR Cycler, and Rotor-Gene® 6000 instruments.

Important!
An adapted program is required for **Rotor-Gene® 6000** instruments using a **36-well rotor**:

cycles	temp	time	steps
1	95°C	3 min	initial denaturation
40	95°C	15 sec	denaturation
	56°C	1 min	annealing/extension **data acquisition** on green and yellow channel

Note: For MIC qPCR Cycler and Rotor-Gene® 6000 instruments, terminology used for FAM and HEX channels is green and yellow channel, respectively.

VII. DATA ANALYSIS AND INTERPRETATION OF RESULTS

The presence or absence of the *HLA-B*27* allele is defined by whether there is a signal in the **FAM channel** or not. Successful PCR is verified by the amplification of PCR products detected in the **HEX channel**. Thus, genomic DNA samples positive for *HLA-B*27* as well as the HLA-B27 Positive Control show amplification in both the HEX and the FAM channel. *HLA-B*27* negative samples as well as the HLA-B27 Negative Control show amplification in the HEX channel only. Fluorescent levels and corresponding amplification curves are automatically displayed in amplification plots in the real-time PCR software.

sample type	amplification in **FAM** channel (520 nm)	amplification in **HEX** channel (556 nm)
*HLA-B*27* positive	**YES**	**YES**
*HLA-B*27* negative	NO	**YES**
HLA-B27 Positive Control	**YES**	**YES**
HLA-B27 Negative Control	NO	**YES**
NTC	NO	NO

Note: Some instrument software may need manual threshold settings for accurate analysis.

Recommendations for threshold settings (Cq): Set the threshold value for the FAM channel just above the background fluorescent signal generated by the HLA-B27 Negative Control (Fig. 1). Samples crossing the threshold line beyond Cq 37 give invalid results and must be repeated. To analyze the acquired data, please follow your instrument software instructions.

See examples of RealFast™ results on page 13 (Fig. 2).

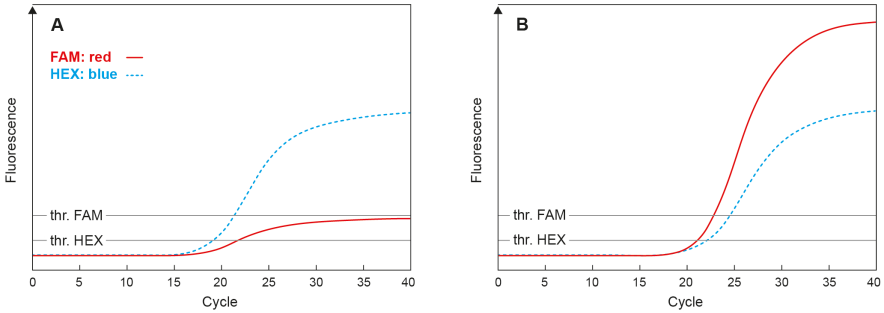

Fig. 1: Correct threshold setting based on the provided PCR Negative Control sample.

A. HLA-B27 Negative Control
B. HLA-B27 Positive Control

VIII. PERFORMANCE EVALUATION

Accuracy of the HLA-B27 RealFast™ Assay was determined by analyzing 198 pretyped genomic DNA samples. Results were concordant with the reference methods that included sequence-specific primer PCR and a commercially available *HLA-B*27* real-time-PCR kit. The assay correctly detected 66 *HLA-B*27* positives (= 100% Positive Percent Agreement) and 132 *HLA-B*27* negatives (= 100% Negative Percent Agreement).

Precision of the HLA-B27 RealFast™ Assay was assessed as variability between replicates, operators, days, and real-time PCR thermocyclers. Tests further included different RealFast™ 2x Genotyping Mix lots and HLA-B27 Assay Mix lots and DNA samples obtained with different extraction methods. A total of 296 tests were carried out under the investigated parameters. All 296 tests showed the expected genotyping results. The HLA-B27 RealFast™ Assay was validated on the ABI 7500 FAST, ABI StepOne, BioRad CFX96, Mx3005Pro, LightCycler 480 II, RotorGene 6000 (72-well rotor and 36-well rotor), and MIC cycler. These instruments have a heating rate range of 2.0 to 5.9°C/sec and a cooling rate in the range of 2.4 to 3.9°C/sec. Manual baseline and threshold setting is required for some instruments. As a consequence, Cq values may differ between cyclers. Use of other thermocyclers must be verified by the user.

Limit of Detection (LoD): LoD is reached at 0.2 ng DNA input per reaction. The recommended DNA concentration is 2 to 10 ng/µl genomic DNA.

Analytical Specificity is ensured by the thorough selection of primers and probes for the detection of *HLA-B*27* alleles, as well as the selection of stringent reaction conditions. All oligonucleotides were checked by sequence comparison analysis against publicly available sequences at the IPD-IMGT/HLA Database (www.ebi.ac.uk/ipd/imgt/hla; release 3.47.0, 2022-01) to ensure that all *HLA-B*27* variants as listed in Table 1 and the internal control will be detected. *In-silico* analysis of selected PCR primers and probes was performed with regard to interfering single nucleotide polymorphisms (SNPs) in their binding sites, and to evaluate the risk of false-positive or false-negative results in conjunction with the *HLA-B* locus. In most cases, experimental confirmation is pending due to limited accessibility of suitable sample materials. Based on *in-silico* analysis, the non-*HLA-B*27* alleles *B*07:428*, *B*14:57*, *B*15:594*, *B*37:60*, *B*44:97*, and *B*55:121* are likely to confer cross-reactivity. Cross-reactivity of *B*73:01:01:01* to *B*73:03* alleles in heterozygous samples is unlikely, but very late Cq values for the FAM-channel in homozygous *B*73:01:01:01* to *B*73:03* samples are possible. Table 1 in section III summarizes the predicted coverage of known *HLA-B*27* alleles based on published sequences.

Clinical Performance

Assessment of the clinical performance of the HLA-B27 RealFast™ Assay to support clinical evidence was established by a systematic literature search. A cross-sectional study was identified as pertaining to the safety and performance of the HLA-B27 RealFast™ Assay. The clinical utility of the HLA-B27 RealFast™ Assay has been demonstrated by analysing 83 ankylosing spondylitis (AS) patients. HLA-B*27 was detected in 66.3% of AS patients, which was comparable to other studies from the region. The study showed that the HLA-B27 RealFast™ Assay has been used in a clinical setting. No adverse events or deviations have been identified. In summary, the clinical performance, the benefits, and the safety of the HLA-B27 RealFast™ Assay are confirmed when the device is used as intended.

IX. INTERFERING SUBSTANCES

Five interfering substances (hemoglobin, immunoglobin G, traces of blood, ethanol, and EDTA) potentially present in EDTA-blood-derived DNA preparations have been tested. Their effects on PCR were evaluated in three purified DNA samples spiked with various concentrations of substances and compared to controls without addition of any interfering substances. All samples were analyzed in triplicates.

A final concentration of <0.2 µM hemoglobin, 0.1 µM immunoglobulin G, <0.02% peripheral blood, 2% ethanol, or 0.1 mM EDTA in the reaction did not interfere with RealFast™ Assay performance.

X. LIMITATIONS OF THE ASSAY

The HLA-B27 RealFast™ Assay is exclusively designed for the detection of *HLA-B*27* alleles as listed in Table 1, section III. Other genetic variants that may be present in a patient's sample, cannot be detected by the assay. The presence of a rare or private polymorphism located within the sequence spanned by primers or probes may result in loss of amplification products and/or fluorescence signals.

The HLA-B27 RealFast™ Assay is intended for laboratory professional use only.

XI. QUALITY CONSIDERATIONS

- A thorough understanding of the procedure outlined here, as well as standard laboratory techniques and appropriate equipment are required to obtain reliable results.
- Do not use RealFast™ components beyond the expiration date.
- After first opening of the primary container, RealFast™ Assay reagents are stable until the expiry date printed on the outer label of the kit when stored properly at -30°C to -15°C or alternatively at 2°C to 8°C for short-term use within one month.
- Avoid exposure to intense light.
- Perform reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- Use sterile disposable pipette tips with filters to avoid microbial contamination and cross-contamination of reagents or samples. Do not interchange tube caps.
- Use instrument-compatible reaction vessels with optically clear caps or sealers.
- Do not mix reagents from different lots.

XII. SAFETY

- Do not drink, eat, smoke, or apply cosmetics in designated work areas. Wear laboratory coats and disposable gloves when handling specimens and kit reagents. Wash hands thoroughly afterwards.
- Handle specimens as if capable of transmitting infectious agents. Thoroughly clean and disinfect all materials and surfaces that have been in contact with specimens. Discard all waste associated with clinical specimens in a biohazard waste container.
- The ingredients of the components of the RealFast™ Assays are not dangerous or their concentrations do not exceed the limits specified in the regulation 1272/2008/EC.
- Adhere to all local and federal safety and environmental regulations which may apply.

XIII. TECHNICAL SUPPORT

Technical support may be obtained by:

- the local ViennaLab Diagnostics distributor (www.viennalab.com/distribution)
- QuickGuides (www.viennalab.com/support)
- contacting techhelp@viennalab.com

XIV. REFERENCES

- OMIM Online Mendelian Inheritance in Man (www.omim.org)
- Allele Frequency Net Database (www.allelefrequencies.net)
- IPD-IMGT/HLA Database (www.ebi.ac.uk/ipd/imgt/hla)
- Khan MA. An Update on the Genetic Polymorphism of *HLA-B*27* With 213 Alleles Encompassing 160 Subtypes (and Still Counting). Curr Rheumatol Rep. 2017;19(2):9. DOI: 10.1007/s11926-017-0640-1

XV. FEEDBACK TO THE MANUFACTURER

Any serious incident that has occurred in relation to the RealFast™ Assay must be reported to the competent authority of the country and to the manufacturer.

XVI. SYMBOLS

REF	Catalog number
LOT	Batch code
IVD	*In vitro* diagnostic medical device
CE 0123	Compliant with European IVD Regulation 2017/746 Identification number of notified body
Σ	Sufficient for <n> tests
	Storage temperature limits
	Use by
	Manufacturer
	Date of manufacture
	Consult Instructions For Use

XVII. EXAMPLES OF TEST RESULTS

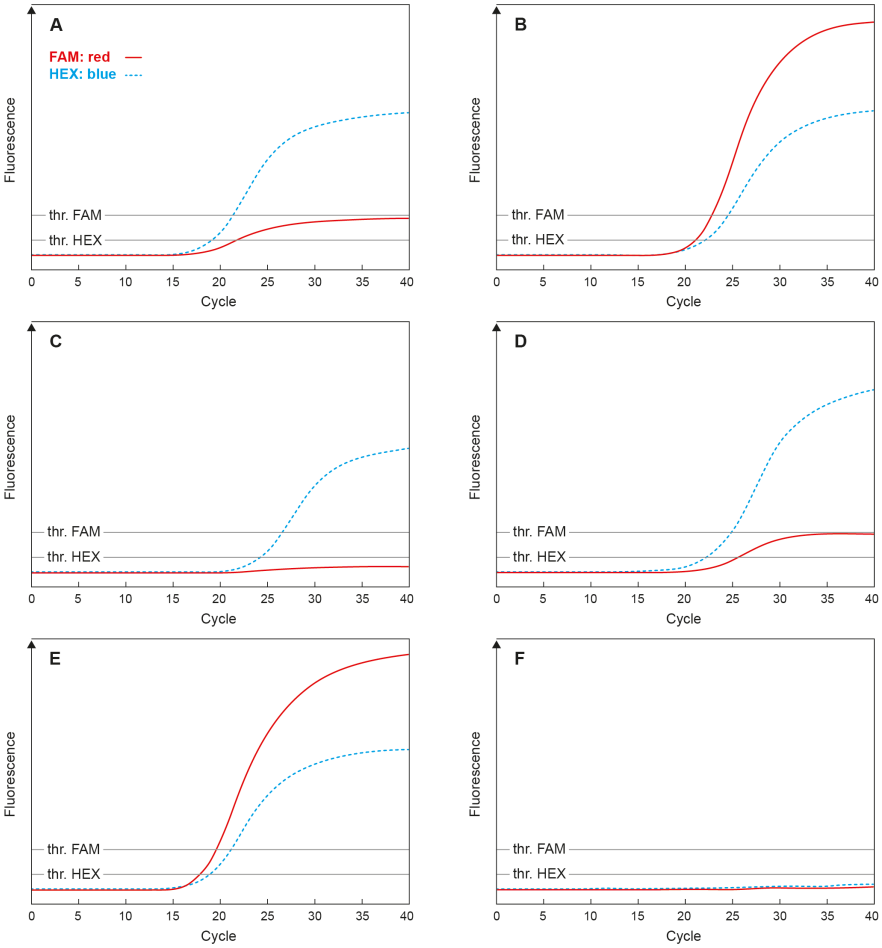

Fig. 2: Amplification plots of controls and samples.

A. HLA-B27 Negative Control. It is recommended to set the threshold value for the FAM channel just above the background fluorescent signal generated by the HLA-B27 Negative Control.
B. HLA-B27 Positive Control
C. HLA-B27 negative sample
D. HLA-B27 negative sample
E. HLA-B27 positive sample
F. No template control (NTC)

Abbreviations: thr. = threshold

NOTES

NOTES

XVIII. RELATED PRODUCTS

REF		Σ
2-014	GENXTRACT™ Blood DNA Extraction System	100 extractions
2-020	Spin Micro DNA Extraction Kit	20 extractions
7-150	LCT -13910C>T RealFast™ Assay	100 reactions
7-153	LCT -13910C>T RealFast™ Assay	32 reactions
7-630	HLA-B1502 RealFast™ Assay	100 reactions
7-633	HLA-B1502 RealFast™ Assay	32 reactions
7-640	HLA-A3101 RealFast™ Assay	100 reactions
7-643	HLA-A3101 RealFast™ Assay	32 reactions

Distributor:

Manufacturer:

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