LightMix® Kit

HLA-B27

Cat.-No.: 40-0606-64

Detection of the *Human Leukocyte Antigen B27 alleles*

for use with the

Roche Diagnostics LightCycler® Instruments

SimpleProbe® format

Reagents for 64 reactions

Upon arrival:

Store Premixed PCR reagents and Controls protected from light at room temperature (do not freeze)

Store FastStart DNA Master HybProbe reagents frozen (-20℃) (if included)

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1. Product Information

1.1 Contents: LightMix® Kit HLA-B27

Lyophilized premixed PCR reagents

⚠ **Store at room temperature in the dark**

	Cap color	Label	Description content	Reaction / Tube status	Total
1 x	**Red**	**PSR**	Parameter Specific Reagents (PSR) containing premixed and lyophilized primers and probes for 64 reactions. <0,01pg unlabeled oligonucleotides (HLA-B27 primers, Globin primers); <0,01pg SimpleProbe 519 labelled HLA-B27 and Globin probes	64 reactions / lyophilized	64 rxs

Standards (Control DNA)

⚠ **Store at room temperature**

	Cap color	Label	Description content	Reaction Tube status	Total
1 x	**Yellow**	**NEG**	HLA Negative Control (Globin Gene) <0,01pg plasmid target (synthetic) [about 10E4 genome equivalents]	24 reactions / lyophilized	24 rxs
1 x	**Yellow**	**POS**	Positive Control (HLA-B27 and Globin) <0,01pg plasmid target (synthetic) [about 10E4 genome equivalents]	24 reactions / lyophilized	24 rxs
1 x	**Yellow**	**REF**	Reference (HLA-B27 only) <0,01pg plasmid target (synthetic) [about 10E4 genome equivalents]	32 reactions / lyophilized	32 rxs

Polymerase Mix: LightCycler® FastStart DNA Master HybProbe

⚠ **Store at -20℃ upon arrival**

FastStart DNA Master HybProbe is included in kits supplied directly by TIB MOLBIOL for customers in Central Europe only[1].
The FastStart DNA Master HybProbe is not included in HLA-B27 kits supplied through Roche Diagnostics or its local distributor.

	Cap color	Label	Description content	Reaction Tube storage	Total
1 x	**Red**	**1a**	LightCycler® FastStart Enzyme	64 reactions / frozen	64 rxs
1 x	**White**	**1b**	LightCycler® FastStart Reaction Mix HybProbe	64 reactions / frozen	64 rxs
1 x	**Color-less**	**Water**	H_2O PCR grade	frozen	
1 x	**Blue**	**$MgCl_2$**	$MgCl_2$, 25 mM	frozen	

1) FastStart enzyme is shipped by TIB MOLBIOL at ambient temperature.

Note : Probe Tm values changed compared to the previous kit version.

1.2 Intended Use

This kit allows to detect the most prevalent HLA-B27 alleles (see below) from genomic human DNA extracted from peripheral blood.

The presence of the HLA-B27 allele is associated with spondyloarthopathie (Morbus Bechterew) and other autoimmune type diseases, but the presence of the HLA-B27 allele is not an indication for the disease.

This product is indicated to help the clinician to analyze the genetic background of patients showing :

- Spondylitis ankylosans (Morbus Bechterew)
- Morbus Reiter
- Psoriatic arthritis
- Juvenile idiopathic arthritis with enthesitis
- Rheumatoid arthritis
- Acute uveitis, Inflammation of the eyes

The primers used in this kit are similar to those published by Tiemann et al.,[2] and are expected to share to miss the following HLA B27 alleles: B*27:04:03, B*27:07:01, B*27:07:02, B*27:07:03, B*27:07:04, B*27:102, B*27:11, B*27:125, B*27:14, B*27:19, B*27:20, B*27:21, B*27:24, B*27:30, B*27:32, B*27:33, B*27:34, B*27:36, B*27:43, B*27:70, B*27:81, B*27:90:01, B*27:90:02.

Note: The performance of the assay can be guaranteed only when used with LightCycler® Instruments (see 1.3.2 for details).

1.3 Specifications

The *LightMix® Kit HLA-B27* allows to test for the presence of this HLA variant.

1.3.1 Clinical Samples

The test requires 2 µl of purified genomic DNA in aqueous solution extracted from clinical specimen, containing from 5 to 100 ng/µl of DNA (10 ng – 200 ng total amount), as determined by UV spectrophotometry (1 OD = 50 µg DNA/ml).

1.3.2 Instruments, Software and Productivity

One kit contains reagents for 64 reactions performed in a 10 µl volume.
Each run requires including one standard and one negative control.
The table below summarizes some features of the kit :

LightCycler® Instrument	Software Version (or higher)	Run Time (approx.)	Max Samples per run [2]	Maximum Productivity of the kit [3]	Minimum Productivity of the kit [4]
LC 1.2	4.10 [1]	60 min	31+ 1 ctrl.	60	31
LC 1.5	4.10 [1]	60 min	31+ 1 ctrl.	60	31
LC 2.0	4.05	60 min	31+ 1 ctrl.	60	31
LC480 (96 wells)	1.5	100 min	63 + 1 ctrl.	61	31
LC480 (384 wells)	1.5	100 min	383 [5] + 1 ctrl.	61	31
Z480 (open channel)	1.5	100 min	63 + 1 ctrl.	61	31
LC96	1.6 [6]	100 min	63 + 1 ctrl.	61	31
Nano	1.0 [6]	60 min	31+ 1 ctrl.	62	31

1. Running the test with the LightCycler® 1.2 or 1.5 Instruments with the software version 3.5 yields comparable results. Instruction for programming, data analysis and interpretation of results are not described in this manual. **Upgrade to version 4.10 or higher when possible.**
   LightCycler® software 3.5.3 does not contain the automatic genotyping module; equivalent results can be obtained by trained personnel which must analyze each sample manually.
2. Each run must include the **Ref** control; when using **NTC** and **POS,** controls becomes two and the number of samples is reduced accordingly (see 3.4 Use of Controls and Reference tubes).
3. The first run of the kit requires to include 3 controls. The maximum number of samples that can be processed is reduced accordingly.
   Depending on local regulations, all 3 controls may have to be included in each run, reducing the total number of patient's samples that can be analyzed.
4. Calculated considering one single clinical sample analyzed in each run.
5. It requires using more than one kit.
6. Nano LightCycler® software 1.0 and LC96 software 1.6 do not contain the automatic genotyping module therefore it is not necessary to add the **NEG** and **POS** controls; equivalent results can be obtained by trained personnel which must analyze each sample manually

1.4 Storage and Stability

Note the **different storage conditions** for reagents and polymerase mix !

Storage Conditions

Reagents and Controls:
Store the lyophilized reagents (PSR and Standards) protected from light and at room temperature (18℃ - 25℃).
Do not freeze these dried reagents. Expiration date is printed on the kit label.

Polymerase mix :
Store the LightCycler® FastStart DNA Master HybProbe at -15℃ to -25℃.
See expiration date on the polymerase tube label.

Shipping:
Products are shipped at ambient temperature. Transport stability of reagents and enzyme components have been tested under shipping conditions.

2. Additional Devices and Reagents

2.1 Required

LightCycler® 2.0 Instrument	**Roche Diagnostics**
LightCycler® 2.0 Instrument	Cat.-No. 12 011 468 001
LightCycler® Software Version 4.05 or	Discontinued
LightCycler® Software Version 4.10 or higher	Cat.-No. 04 779 584 001
LightCycler® Capillaries (20 µl)	Cat.-No. 11 909 339 001
Or	
LightCycler® 480 Instruments	**Roche Diagnostics**
LightCycler® 480 Instrument (model I)	Discontinued
LightCycler® 480 II Instrument	Cat.-No. 05 015 278 001
Cobas® 4800 System (Z480 Instrument)	Cat.-No. 05 200 881 001
LightCycler® Software Version 1.5 or higher	Cat.-No. 04 994 884 001
LightCycler® 480 Multiwell Plate 96 or	Cat.-No. 04 729 692 001
LightCycler® 480 Multiwell Plate 384 white	Cat.-No. 04 729 749 001
Or	
LightCycler® 96 Instrument	**Roche Diagnostics**
LightCycler® 96 Instrument	05 815 916 001
LightCycler® Software Version 1.0 or higher	Included with Instrument
LightCycler® 480 Multiwell Plate 96	Cat.-No. 04 729 692 001
LightCycler® 8 tube strips (white)	Cat.-No. 06 612 601 001
Or	
LightCycler® Nano Instrument	**Roche Diagnostics**
LightCycler® Nano Instrument	Cat.-No. 06 407 773 001
LightCycler® Software Version 1.0 or higher	Included with Instrument
LightCycler® Nano tubes	Cat.-No. 06 327 672 001
Or	
LightCycler® 1.x Instruments	**Roche Diagnostics**
LightCycler® 1.2 and 1.5 Instruments	Discontinued
LightCycler® Software Version 4.10	Cat.-No. 04 779 584 001
LightCycler® Capillaries (20 µl)	Cat.-No. 11 909 339 001

2.2 Optional

Instruments:	**Roche Diagnostics**
LC Carousel Centrifuge 2.0 (230 Volt)	Cat.-No. 03 709 582 001
Capping Tool	Cat.-No. 03 357 317 001

2.3 Sample Preparation

Manual Sample Preparation:	**Roche Diagnostics**
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
Nuclease-free PCR grade water	any supplier
Ethanol p.a.	any supplier
Isopropanol p.a.	any supplier

Automatic Sample Preparation:	**Roche Diagnostics**
MagNA Pure Instrument	Discontinued
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure 2.0 Instrument	Cat.-No. 05 197 686 001
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure Compact Instrument	Cat.-No. 03 731 146 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	Cat.-No. 03 730 964 001
MagNA Pure 96 Instrument	Cat.-No. 05 195 322 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 05 467 497 001

3. Background Information

3.1 Medical Background

The Human Leukocyte Antigen (HLA) system is the major histocompatibility complex (MHC) in humans, consisting of many genes for cell-surface antigen-presenting proteins located on chromosome 6.

HLA-A, B and C (MHC class I) define small peptides made inside the cell while HLA-DP, DM, DOA, DOB, DQ and DR (MHC class II) are antigens presented outside of the cells to stimulate T-helper cells.

The HLA haplotypes are defined by a stretch of genes located on one singular chromosome recombine in children from each one haplotype of the parents.

The HLA type is most important for transplantations and must be as similar as possible to reduce the risk of transplant rejections.
Some HLA types are associated with certain diseases or the response to viral infections; testing for these HLA types allows to predict the disease risk.

HLA-B27 is encoded by the B locus, including subtypes B*27:01 to *27:59 (might be meantime extended) and is associated with ankylosing spondylitis (Morbus Bechterew) and related inflammatory diseases referred to as "spondyloarthritis"; in particular reactive arthritis (Reiter's Syndrome), some eye disorders like acute anterior uveitis and iritis, psoriatic arthritis and ulcerative colitis associated spondyloarthritis.

The prevalence of HLA-B27 varies regionally. The frequence in Causasians is 8%, in Northern Scandinavia up to 25% and less frequent in Southern Europe, North Africa as well as in China (2-8%), and very rare in Japan (< 0.5%).

Information: The following alleles will be detected according to alignments :

*27:01, *27:02:01, *27:02:02, *27:03, *27:04:01, *27:04:02, *27:04:04, *27:05:02, *27:05:03, *27:05:04, *27:05:05, *27:05:06, *27:05:07, *27:05:08, *27:05:09, *27:05:10, *27:05:11, *27:05:12, *27:05:13, *27:05:14, *27:05:15, *27:05:16, *27:05:17, *27:05:18, *27:05:19, *27:05:20, *27:05:21, *27:05:22, *27:05:23, *27:05:24, *27:05:25, *27:05:26, *27:05:27, *27:05:28, *27:06, *27:08, *27:09, *27:10, *27:12, *27:13, *27:15, *27:16, *27:17, *27:18, *27:23, *27:25, *27:26, *27:27, *27:28, *27:29, *27:31, *27:35, *27:37, *27:38, *27:39, *27:40, *27:41, *27:42, *27:44, *27:45, *27:46, *27:47, *27:48, *27:49, *27:50, *27:51, *27:52, *27:53, *27:54, *27:55, *27:56, *27:57, *27:58, *27:59N, *27:60, *27:61, *27:62, *27:63, *27:64N, *27:65N, *27:66N, *27:67, *27:68, *27:69, *27:71, *27:72, *27:73, *27:74, *27:75, *27:76, *27:77, *27:78, *27:79, *27:80, *27:82, *27:83, *27:84, *27:85, *27:86, *27:87, *27:88, *27:89, *27:91, *27:92, *27:93, *27:94N, *27:96:01, *27:96:02, *27:97, *27:98, *27:99, *27:95, *27:100, *27:101, *27:103, *27:104, *27:105, *27:106, *27:107, *27:108, *27:109, *27:110, *27:111, *27:112, *27:113, *27:114, *27:115, *27:116, *27:117, *27:118, *27:119, *27:120, *27:121, *27:122, *27:123, *27:124.

3.2 Methodology and Assay Principle

Using PCR methodology, a fragment of the HLA-B27 gene is amplified with specific primers. A second fragment from the beta globin gene is amplified as internal control. Both fragments are detected with a specific detection probe internally labeled with SimpleProbe® 519 reagent.

SimpleProbes® are fluorescent only when bound to complementary DNA.

The probe binds to a part of the amplified fragment. During the melting curve analysis the temperature is slowly increased. The probe melts off at specific melting temperatures causing the fluorescence to decrease.

The HLA-B27 probe yields a 8℃ higher melting peak than the globin control. HLA-B27 negative samples display only the globin low melting peak, while HLA-B27 positive samples exhibit two melting peaks; sometime the globin amplification is not visible in HLA-B27 positive samples because the assay is devised with a preferential amplification for HLA-B27 target, thus only the high melting peak will be visible.
Reading of the genotype results is based on the melting temperatures compared to the supplied standards. If permitted by the instrument software reading of the genotype results can be achieved by the automated genotyping (instrument-dependent: software module 'Melt Curve Genotyping'); in case that automated genotyping fails to report the genotype results, they must be deducted from the melting temperatures following the criteria described in chapter 7.

The supplied control DNA standards allow a comparison with clinical samples.

3. 3 Performance Characteristics

Analytical Specificity

The specificity to the target gene and the suitability of the PCR amplification employed in the present test for the detection of the allele variant was demonstrated by direct sequencing of the generated 133 bp amplicon.

Analytical Sensitivity

Detection of serial dilutions of several heterozygous human genomic DNA has revealed that the limit of detection of the present kit is 350 copies (2 ng).

Diagnostic Specificity and Sensitivity

A total number of 20 genomic DNA samples from Caucasian individuals were analyzed with this kit and a published SYBR Green based assay[1].

Study results: results for both analytical methods were in 100% concordance.

In particular 1 sample was HLA-B27 positive and 19 samples were negative.

3.4 Use of Controls and Reference tubes

Two controls are included in the kit: **POS** representing HLA-B27 positive patients, containing the HLA-B27 and the globin target, and **NEG** representing HLA-B27 negative patients, containing the globin target only.

The procedure described throughout the manual requires the use of the **POS** control and a **NTC** (**N**o **T**arget **C**ontrol) in each run. The **NEG** control is used only in the first analysis; laboratory or local procedure might require to include the **NEG** control in all runs.

To increase the productivity of the kit a different procedure can be implemented and the **NTC** control, the **POS** control and the **NEG** control can be substituted with the third control tube present in the kit: **REF** Reference.
The blue line on the right of the text indicates the instruction for the utilization of the **REF** HLA Reference tube).
The **REF** contains only the HLA-B27 target. Please note that this approach can be used only when analyzing more than five patients not deriving from the same family. In case that all sample from one run are HLA-B27 positive the run has to be repeated with **POS** and **NEG** controls included.

Follow this interpretation scheme when using the **REF** approach:
1) Biological samples generate a low melting curve, proving the functionality of the Globin reagents and substituting the **NEG** control.

2) **REF** contains HLA B-27 gene, but misses the Globin target: a condition that does not exist in nature.
The **REF** must produce only the higher melting peak, thus ensuring the functionality of the HLA-B27 reagents and representing a substitute of the **POS** standard. The concomitant presence of the low melting peak is indication of contamination with the Globin target and therefore with genomic DNA.
Statistically less than 20% of biological samples from not blood related patients are HLA-B27 positive; the presence of the high melting peak in all, or almost all samples, is a strong suggestion of contamination with HLA-B27 target. In this case the run has to be repeated with **NTC**, **POS** and **NEG** controls included.

3) The absence of the high melting peak in the majority of biological samples together with the absence of the low melting peak in the **REF** substitute the **NTC**.

4. Precautions and Warnings

Handling Requirements

The present product is an *in-vitro* device and therefore must be used by qualified personnel only.

General precautions for the handling of generic laboratory materials are required.

The laboratory work-flow must conform to standard practices. Due to the risk of contamination, PCR preparation and PCR amplification must be performed in physically separated areas.

Do not mix reagents from different lots.

Do not use the reagents after the expiration date.
Use the manual version which is delivered with the kit (see kit label).

Laboratory Procedures

All materials of human origin and related waste must be considered potentially infectious. Thoroughly clean and treat all work surfaces with disinfectants approved by local authorities.

Do not eat, drink or smoke in the laboratory working area.

Do not pipet by mouth.

Wear disposable protective gloves, laboratory coats and adequate eye protection during the handling of samples and set components.

Avoid microbial or nuclease contamination of the reagents while pipetting the aliquots. The use of disposable sterile tips is essential.

Thoroughly wash your hands after handling the samples and the sets components.

Sample Preparation

Regarding proper handling and disposal refer to the safety instructions enclosed in the package insert of the product employed (see chapter 2.3).

Amplification and Detection

Before using this product, please read the LightCycler® Operator's Manual.

Please save a sample file to identify each position for correct sample identification.

Check LightCycler® Instrument settings and make sure that they match those reported in the following section "PCR protocol" specific for your Instrument.

Do not touch the capillary surface or plate cover without gloves.

Please refer to all the operative and safety instructions of the LightCycler® Instrument.

Handling of Waste Materials

Dispose of the unused reagents and waste materials according to the current laws.

5. Programming

5.1 Color Compensation

No Color Compensation is required for the use of the *LightMix® Kit HLA-B27.* Reading data with 'Color Compensation' activated will not change the readout of the results.

5.2 Capillary Based LightCycler® Instruments

For details see the LightCycler® Operator's Manual.

Programming:
The protocol consists of four program steps (Tab.1):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	**None**	**Quantification mode**			**Melting Curves mode**			**None**
Cycles	1	45			1			1
Target [℃]	95	95	60	72	95	43	75	40
Hold [hh:mm:ss]	**00:10:00**	**00:00:05**	**00:00:10**	**00:00:15**	**00:00:20**	**00:00:20**	**00:00:00**	**00:00:30**
Ramp Rate [℃/s]*	**20**	**20**	**20**	**20**	**20**	**20**	**0.2**	**20**
Sec Target [℃]	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Step Size [℃]	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Step Delay [cycles]	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Acquisition Mode	None	None	Single	None	None	None	Cont.	None

* For LightCycler 1.x Instruments using software version 3.5.3 read 'Temperature Transition Rate' [℃/s] instead of Ramp Rate.

Tab. 1: Programming of capillary based Instruments for using *LightMix® Kit HLA-B27*

Note:
While programming maintain default software values: channel = 530, max. samples = 32, seek temperature = 30℃ and capillary size = 20 µl. Do not change the capillary size value to 100 µl.
Store the program and the default values as '**RUN Template'** which can be loaded to start every HLA-B27 LightCycler® run.

Just before starting the run, modify max. samples (default = 32) to the number of samples plus controls included in the run to prevent stopping of the instrument due to missing capillaries.
For instructions see the Operator's Manual.

5.3 Cycler® 480 Instruments

For details see the LightCycler® Operator's Manual.

Detection Format: SimpleProbe

Note:
This kit can be run in combination with LightMix®_40-0340-32 CE_HFE63-65-282, following the instruction for Detection Format and Programming described in the HFE manual.

Reaction Volume: 10 µl

Programming:

The protocol consists of four program steps (Tab.2):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	**None**	**Quantification mode**			**Melting Curves mode**			**None**
Cycles	1	45			1			1
Target [°C]	95	95	60	72	95	43	75	40
Acquisition Mode	**None**	**None**	**Single**	**None**	**None**	**None**	**Cont.**	**None**
Hold [hh:mm:ss]	**00:10:00**	**00:00:05**	**00:00:10**	**00:00:15**	**00:00:30**	**00:02:00**	**00:00:00**	**00:00:30**
Ramp Rate [C°/ s] **96**	**4.4**	**4.4**	**2.2**	**4.4**	**4.4**	**1.5**	**0.29**	**1.5**
Ramp Rate [C°/ s] **384**	**4.6**	**4.6**	**2.4**	**4.6**	**4.6**	**2.0**	**0.29**	**2.0**
Acquisitions [per °C]	**-**	**-**	**-**	**-**	**-**	**-**	**2**	**-**
Sec Target [°C]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Size [°C]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Delay [cycles]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**

Tab. 2: Programming of multiwell based instrument (96 wells and 384 wells formats) and Z480 Instrument for using the LightMix® Kit *HLA-B27*

Note:

a) Store the program and the default values as '**RUN Template'** which can be loaded to start every HLA-B27 LightCycler® run.
b) Ensure to program **2 acquisitions per second** instead the default value 5; more acquisitions reduce the slope of the melting curve, increase experiment time and cause kit malfunction.

5.4 LightCycler® 96 Instrument

For details see the LightCycler® Operator's Manual.

Measurement:

Detection Format: 470/514 FAM			**General**
Quant Factor	Melt Factor	Integration Time (S)	Volumes (µl)
10.00	1.20	dynamic	10

Profile:

The protocol consists of four program steps (Tab.3):

1. **Preincubation** of sample and activation of the enzyme
2. **Step Amplification** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Cycles	**1**	**45**			**1**			**1**
Ramp [°C/ s]	**4.4**	**4.4**	**2.2**	**4.4**	**4.4**	**1.5**	**0.20**	**1.5**
Duration [s]	**600**	**5**	**10**	**15**	**30**	**120**	**1**	**30**
Target [°C]	**95**	**95**	**60**	**72**	**95**	**43**	**75**	**40**
Mode		Standard	Standard	Standard				
Acquisition Mode	**None**	**None**	**Single**	**None**	**None**	**None**	**Cont.**	**None**
Readings /°C							**5**	

Tab. 3: Programming of LightCycler® 96 Instrument for using the LightMix® Kit *HLA 27B*

Note: Store the program and the default values as **'Experiment file'** which can be loaded to start every LightCycler® run.

5.5 LightCycler® Nano Instrument

For details see the LightCycler® Operator's Manual.

Run Setting / Optical setting

Intercalating Dyes
Normal Quality

Profile:

The protocol consists of four program steps (Tab.3):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Denaturation** of amplified PCR product.
4. **Melting** Identification of PCR amplified DNA sequence

Step:	1	2			3	4	
Parameter							
Name	Hold	3 Step Amplification			Hold	Melting Stage Initial Stage	Melting Stage Final Stage
Cycles		45					
Temp [℃]	95	95	60	72	95	43	75
Ramp (℃/s)	5	5	4	5	5	4	0.2
Hold (s)	600	10	15	20	30	120	1
Acquire			√				

Tab. 4: Programming of LightCycler Nano® Instrument for using the *LightMix® Kit HLA-B27*

Note:

Store the program and the default values as **'Experiment file'** which can be loaded to start every HLA B27 LightCycler® run.

6. Experimental Protocol

Program the Instrument before preparing the solutions (see 5. Programming and read the LightCycler® Operator's Manual for details).
The described performance of the assay can be guaranteed only when used with Roche Diagnostics LightCycler® systems.

6.1 Sample Preparation

For preparation of genomic DNA use human peripheral blood (EDTA, citrate). The use of heparin is strongly discouraged since this anticoagulant might interfere with the PCR.
Perform nucleic acid purification using the High Pure PCR Template Preparation Kit or the MagNA Pure LC Instruments using the extraction kit appropriate to the MagNA Pure Instrument used (see 2. Additional Devices and Reagents) as described in the respective protocols.

In the depicted assays (7.5. Reading of the Results) the DNA was manually extracted from 200 µl of blood using the High Pure PCR Template Preparation Kit following the manufacturer's instructions; 100 µl of elution buffer were used for the final elution of the purified DNA from the column.

6.2 Reagents Preparation

6.2.1 Preparation of the LightCycler® FastStart DNA Master

1	Keep LightCycler® FastStart Enzyme **1a** cold.
2	Thaw the LightCycler® FastStart Reaction Mix **1b** by warming up the tube at 30°- 35°C for 3 - 5 minutes.
3	Quickly spin tubes to collect drops.
4	The solution must be free of particles.
5	Add 60 µl of **1b** to the vial **1a**.
6 ⚠	Mix the solution carefully with a pipette. Do not vortex! Avoid the production of bubbles.
7	Spin the tubes to collect drops.
8	Use reagent to prepare the Reaction Mix (6.3).
9	Store left over reagent at 4°C.

6.2.2 Preparation of Parameter-Specific Reagents (PSR)

►	Each **PSR** reagent tube is sufficient for 64 reactions
1	Spin the premixed **PSR** tube at 10,000 RPM for 1 minute
2	Check that the pellet is located at the bottom.
3	To each **PSR** tube add **66 µl** of PCR-grade **Water**
4	Incubate for 20 sec at room temperature
5	Vortex for 10 sec
6	Spin the tubes to collect drops

► Use 1 µl of **PSR** reagent for a 10 µl PCR reaction.

6.2.3 Preparation of HLA-B27 Controls

The LightCycler® software 4.05 and later (capillary based instruments) and software 1.5 (plate based instruments) can be calibrated with reference standards to perform an automated genotyping of unknown clinical samples.

►	**HLA-B27 Controls** tubes are sufficient for 24 reactions
1	Spin the **NEG** and **POS** tubes at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the bottom of the tube.
3	Dissolve pellet by adding 50 µl PCR-grade **Water**.
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use 2 µl of **NEG Control** and **POS Control** for a 10 µl PCR reaction
Please note: Opening the vials may cause contaminations of the work-space (aerosol).

6.2.4 Preparation of REF Reference

►	**REF** tube is sufficient for 32 reactions
1	Spin the **REF** tube at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the bottom of the tube.
3	Dissolve pellet by adding 66 µl PCR-grade **Water**.
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use **2 µl** of **REF** for a 10 µl PCR reaction.
Please note: Opening the vials may cause contaminations of the work-space (aerosol).

The rational for the use of **REF** is described in chapter 3.4

6.3 Preparation of the Reaction Mix

6.3.1 Preparation of 64 LightCycler® Reaction Mix

We recommend preparing 64 reactions to prevent storage of dissolved or activated reagents in varying volumes (6.2). Pre-mixed reagents can be stored at 4°C but must be used within 30 days. For the pre paration of reaction mix for less reactions, please go to step 6.3.2 "Reaction mix for single reaction".

Prepare the reaction mix in the PSR reagent tube (cooled):

Components	**64 reactions**
To the **PSR** tube (red cap) already containing	66.0 µl
Add:	
H_2O, PCR-grade (colorless cap)	343.2 µl
Mg^{2+} solution 25 mM (blue cap)	52.8 µl
LightCycler® FastStart DNA Master HybProbe (red cap), see **6.2.1**	66.0 µl
⚠ Substitute of the "long neck cap" of the **PSR** tube with the red cap from FastStart	
Total Volume	**528.0 µl**

Tab. 5: Volumes of components for preparing 64 reaction mixture

6.3.2 Preparation of the Single LightCycler® Reaction Mix

Prepare the reaction mix by multiplying each volume (Tab. 5) by the number of biological samples to be analyzed plus three reactions **NTC**, **NEG** and **POS** and one excess.

When the HLA **REF** is used, the number of reactions to be prepared is equal to the number of all biological samples plus two reactions. See chapter 3.4 for details.

Prepare the reaction mix in a cooled vial:

Components	Single reaction
H_2O, PCR-grade (colorless cap)	5.2 µl
Mg^{2+} solution 25 mM (blue cap)	0.8 µl
PSR (red cap), see **6.2.2**	1.0 µl
LightCycler® FastStart DNA Master HybProbe (red cap), see **6.2.1**	1.0 µl
Volume of reaction mix	**8.0 µl**

Tab. 6: Volumes of components for preparing a single reaction mixture

Gently pipette up and down the reaction mix.
A high percentage of experimental failure is due
to a non homogeneous reaction mix!

6.3.3 Capillary / Well Loading Procedure

Each run must include one Negative Control (**NTC**) to demonstrate the absence of contaminations with genomic DNA or HLA-B27 PCR product and a **POS** Control to identify run specific melting temperatures. Regulatory agencies or local laboratory rules might require including also the **NEG** Control

Capillary / Well Loading Procedure

▶	Remember to include the controls when setting up the run.
1	Mix gently, spin down and check for the absence of air bubbles in the reaction mix vial.
2	Dispense **8 µl** per capillary/well of reaction mix
3	**Mandatory:** Add **2 µl** of PCR-grade **H_2O** as **Negative Control** (**NTC**) in position 1 (A1). Add **2 µl** of **POS** Control in position 2 (A2).
	Optional*: Add **2 µl** of **NEG** Control in position 3 (A3)
4	Add **2 µl** of **Sample** in the remaining capillaries / wells.
5 ⚠	Close the capillary/plate and centrifuge. Check that no air bubbles are present.
6	Place the rotor/plate into the LightCycler® Instrument.
7	Capillary-based users only: input number of samples.
8	Start the run.
9	Input experiment's name when instructed.
10	Save sample data in the samples' window.

* See section **6.5** for the Sample loading and Typing calibration.

REF procedure does not allow Typing calibration.
Substitute **NTC** and **POS** with **REF** in position 1 (A1) See chapter 3.4 for details

6.4 Storage and Stability of Diluted Components

Reaction Mix

The complete reaction mix containing Parameter-Specific Reagents (**PSR**), LightCycler® FastStart DNA Master HybProbe and $MgCl_2$ can be stored refrigerated (4℃ - 8℃) for 30 days.

Avoid prolonged exposure to light.

Parameter Specific Reagents (PSR)

Once diluted, store PSR refrigerated at 4℃ - 8℃ f or up to 30 days.
Avoid prolonged exposure to light.

LightCycler® FastStart DNA Master HybProbe

The combined FastStart DNA Master HybProbe master mix (1a+1b) can be stored refrigerated (4℃ - 8℃) for 30 days.

NEG, POS Controls and REF

The dissolved Controls are stable for 30 days when stored refrigerated (4℃ - 8℃).

6.5 Loading of Controls

Samples in positions 1 to 2 (A1 to A2) must be filled in each run as described in the tables below.

Genotype results are based on melting temperatures.
The use of the automated genotyping module present in the LightCycler® 2.0 and LightCycler® 480 software is optional.

Sample in position 3 (plate: A3) is required for teaching of Genotyping Standards only in the first run of the kit.
For routine runs **NTC** and **POS** controls can be substitute with **REF** Reference control in position 1 (A1). See chapter 3.4 for details
Refer to LightCycler® Operator's Manual for details.

6.5.1 Capillary Based Instruments

In "Samples data - Capillary View", input Sample Name as described in the second column.
Select "Analysis Type – Genotyping". Select Channel 530 and deselect all others. From the pull down menu select "Sample Type" and copy the "Genotype" description

Pos	Sample Name	Channel	Target Name	Sample Type	Genotype
1	**NTC**	530	Target 1	Negative Control	
2	**POS**	530	Target 1	Melting Standard	HLA-B27 positive
3	**NEG**	530	Target 1	Melting Standard	HLA-B27 negative

6.5.2 LightCycler® 480 Instruments

In the "Sample Editor" window, in "Step1: Select Workflow" section, select "Melt Geno". Select filter combination 465-510 and deselect all others. Input the description of controls as follows:

Pos	Sample Name	Melt Geno Sample Type	Melt Geno Genotype
1	**NTC**	Negative Control	
2	**POS**	Melting Standard	HLA-B27 positive
3	**NEG**	Melting Standard	HLA-B27 negative

6.5.3 LightCycler® 96 Instrument

In the "Sample Editor" window input, as described below, the description of Controls in:

Table View

Color	Position	Sample Name	Sample Type	Dye
	A1	**NTC**	Unknown	FAM
	A2	**POS**	Unknown	FAM
	A3	**NEG**	Unknown	FAM

Leave empty all other not described cells.

6.5.4 LightCycler® Nano Instrument

Samples:

Input, as described below, the description of **Controls** into the "Samples" window; input name and select Dye into the "Target" window.

Samples:

Color	Name	Note
	NTC	
	POS	
	NEG	

Target:

Color	Name	Dye	Reference
	channel 530	FAM	

Well as table

Pos	#	Note	Sample	FAM	Type
A1	1		**NTC**	channel 530	U
A2	2		**POS**	channel 530	U
A3	3		**NEG**	channel 530	U

7. Data Analysis and Interpretation

7.1 Limits and Interferences

The present assay is specific for the HLA-B27 DNA. The alleles B*27:04:03, B*27:07:01, B*27:07:02, B*27:07:03, B*27:07:04, B*27:102, B*27:11, B*27:125, B*27:14, B*27:19, B*27:20, B*27:21, B*27:24, B*27:30, B*27:32, B*27:33, B*27:34, B*27:36, B*27:43, B*27:70, B*27:81, B*27:90:01, and B*27:90:02 are not detected as predicted by the assay design.
No other interferences for this assay are known.

7.2 Calibration

Calibration has to be performed following the procedure described in 6.2.3 and 6.3.3, 6.5, 7.3.2 and 7.3.3.

7.3 Quality Control – Acceptance Criteria

In order to perform a reliable typing analysis, it is essential that at least **NTC** and **POS** control are included in each run.
For the alternative use of the **REF** see 7.3.4
NOTE: The PCR is performed at an annealing temperature of 60ºC; at this temperature the HLA-B27 probe will not bind the amplicon very tightly and the amplification might appear absent. For this reason, the acceptance criteria for the analysis results are based only on the definition of the melting-curve patterns as described below.

7.3.1 NTC No Target Control (position 1)

NTC Negative Control (position 1).
Melting-curve analysis of the Negative Control must provide a negative result: no assay-specific melting peaks (see 7.3.2) must be detected.

In case that the **NTC** should report one or both specific peaks (compare signal with sample results to avoid that the software enlarges background noise to window size suggesting the presence of melting peaks), a contamination or a pipetting error has occurred; the session is not valid and the procedure has to be repeated. If the problem sustains, change water and/or reagents and re-peat. Feel free to ask for assistance at service@tib-molbiol.de.

In case a peak is detected at an unspecific temperature (see paragraph 7.3.4), the software might incorrectly identify it as positive, causing automatic genotyping impossible (LightCycler® 480 software 1.5 reports: "*Sample NTC in position A1 is a negative control not in the negative group*").
In this case - to enable the automatic genotyping - the NTC sample must be set as "Unknown" instead of "Negative Control" (see 6.5 Sample loading and calibration of Genotyping Standards); alternatively, results must be read from the melting temperatures (7.3.4 Samples; 7.6 Interpretation of the Results).

7.3.2 POS Control DNA (position 2)

Melting-curve analysis should show two melting peaks at 54-56℃+1.5℃ and 62-64℃+1.5℃ (depending on the type of instrument used).
The temperature difference should be about 8℃+1.5℃.
POS Positive Control mimics an HLA positive clinical sample (see **7.5**).

7.3.3 NEG Control DNA (position 3)

Melting-curve analysis should display a single melting peak at 54-56℃+1.5℃.
HLA NEG is mimicking an HLA-negative clinical samples (see **7.5**).

7.3.4 REF Reference

Melting-curve analysis of **REF** must show one single melting peak at 62-64℃+1.5℃ demonstrating the performance of the PCR.
The HLA Reference works also as No-Target-Control (NTC) for the globin gene: the presence of a second melting peak at 54-56℃+1.5℃ is an indication of genomic or PCR product contamination: the session is not valid and the whole procedure has to be repeated (sample preparation, amplification and detection).

7.3.5 Samples

The result of the present assay must always show one or two melting peaks at 54-56℃+1.5℃ (low) and/or 62-64℃+_1.5℃ (high melting).
HLA B27 **negative** clinical samples must show only the low-melting peak at 54-56℃ representing the amplification of the Globi n gene.
HLA B27 **positive** clinical samples show the high-melting peak at 62-64℃ while the presence of the low-melting at 54-56℃ (G lobin) is not relevant.
⚠ No more than two peaks per sample are expected.
The melting peak profiles must be conformable to the acceptance criteria described in the present chapter. Otherwise, the result is not valid and the procedure has to be repeated (sample preparation, amplification, detection).
Samples functions also as No Target Control for the HLA-B27 gene: the presence of a second melting peak at 62-64℃+1.5℃ in **all** samples is an indication of genomic or PCR product contamination: the run is not valid and the procedure must repeated (sample preparation, amplification and detection).
See chapter 7.6.

7.3.6 Abnormal Melting Curves

If an abnormal melting curve persists, it can be due to a defect in the product or can be caused by other variations (mutations) in the probe binding region. In the latter case any other method must be used for comparison of the results and verification of the sequence.
Submit the PCR fragment for DNA sequencing to confirm the sequence or identify any unknown mutations. Report deviations to service@tib-molbiol.de

7.4 Saving External Genotyping Standards

Not applicable for LC1.x software versions below 4.0 and for LightCycler® Nano Instruments.
After the genotyping analysis, if samples 1 to 3 comply with the acceptance criteria (see **7.3 Quality Control – Acceptance Criteria**), save the Genotyping Standards as follows and use External Standard in all successive runs.

7.4.1 Capillary Based Instruments

In the Melting Curve analysis - Genotyping window open the "Standard (Int)" menu and select "Save standards as External".

7.4.2 LightCycler® 480 Instruments

In the Melt Curve Genotyping analysis window open the "Standards (In-run)" menu and select "Save as ext."

7.5 Reading the Results

Perform data analysis as described in the LightCycler® Operator's Manuals.

7.5.1 Typical Data for Amplification

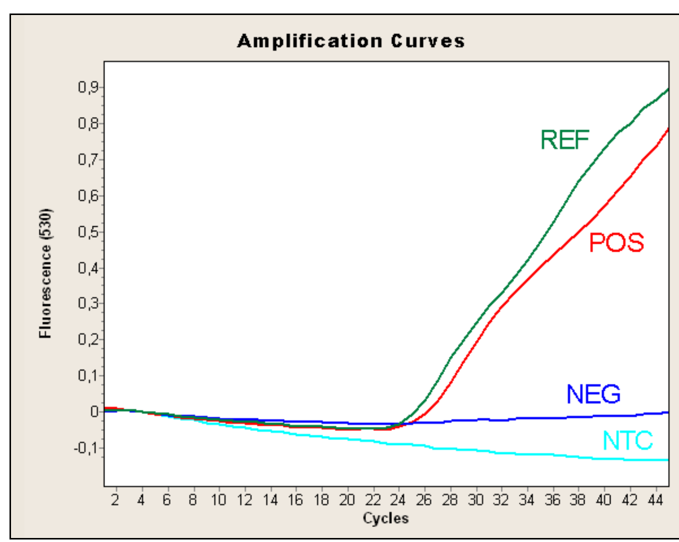
The amplification curves do not contain any analytical information (see section **7.3 Quality Control – Acceptance Criteria**), but, nevertheless, an example from LightCycler® 2.0 is depicted below (Fig. 1).
The PCR has been performed with approximately 50 ng of genomic DNA from samples previously sequenced, one Negative Control **NTC**, **Controls**, and **REF**.

View data for amplification as follows:

LC 2.0 Instrument (or LC1.x with software versions 4.1):
View amplification in channel 530, "Absolute Quantification" analysis mode.

LC 480 Instruments:
View amplification data in channel 483-533 for use with LightCycler® 480 Instrument and in channel 465-510 for use with LightCycler® 480 II Instrument, "Abs Quant/2nd Derivative Max" analysis mode.

LC1.x, software versions 3.5:
View amplification in fluorescence channel F1 "Quantification – Second Derivative Maximum" mode.



Data from channel 530.

NTC No Target Control (H_2O - light blue line) must show no amplification signal.

POS Control (HLA-B27 and Globin - red line), and all HLA-B27 positive samples (not depicted) show an amplification signal.

NEG Control (Globin - blue line) and HLA-B27 negative samples (not depicted) show no or very weak amplification signal.

REF Reference (green line) shows an amplification signal.

Fig. 1: Amplification plot.
Sample data for *HLA-B27*

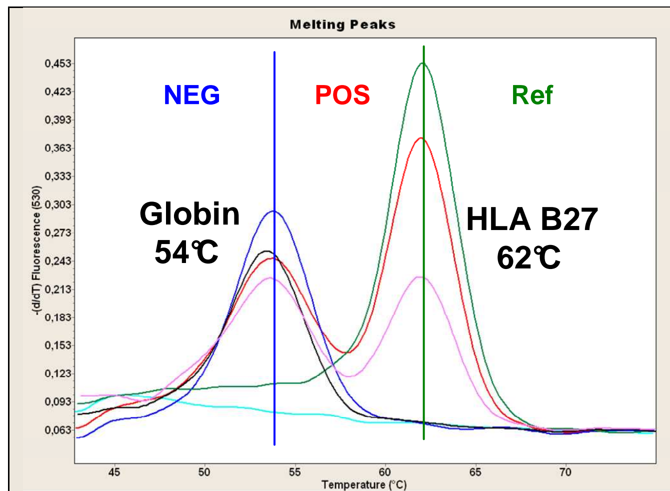
7.5.2 Melting Analysis: Capillary Based Instruments

The melting-curve peaks (Fig. 2) discriminate between the HLA-B27 positive and HLA-B27 negative samples..

View *HLA-B27* data for Melting as follows:

LC 2.0 Instrument (or LC1.x with software versions 4.1):
View Melting data in channel 530. Analysis Type "Melting Curve Analysis – Genotyping" mode.

LC1.x, software version 3.5.3
View Melting data in channel F1 instead of channel 530. "Melting Curve" mode.



Channel 530

NTC No Target Control (H_2O - light blue line) no assay-specific melting peaks must be detected.
POS Control (HLA-B27 and Globin - red line) and positive sample (fuchsia) shows melting peaks at 54°C and 62°C.
NEG HLA Negative Control Standard (Globin - blue line) and HLA-B27 negative sample (black line) show a melting peak at 54°C.

REF Reference (green line) shows a melting peak at 62°C; a second peak at 54°C would be an indication of a contamination.

Fig. 2: Melting sample data for HLA-B27

Interpretation of the Results

Genotype:	HLA-B27 negative	HLA-B27 positive	
Number of melting peaks	1	2	1
Melting temperature of peaks	54°C	54°C and 62°C	62°C
Temperature difference between peaks	---	8°C	---
Phenotype	**HLA-B27 negative**	**Increased Risk**	**Increased Risk**

Tab. 7: Typical analysis results

Note:

The values of the melting temperatures (Tm) may vary ±2.5°C between different experiments.
The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.

In case of variations see instructions: **7.3.6 Abnormal Melting Curves.**

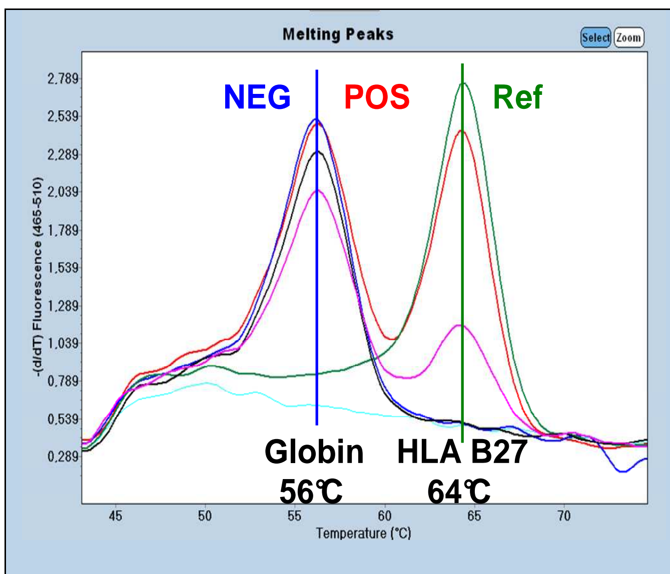
In case of automatic genotype module failure (score <0.6 or res<0.4), switch to manual identification of melting curve (Tm calling) and compare results with table above or use table 11 in chapter **7.6. Interpretation of the Results**.

7.5.3 Melting Analysis: LightCycler® 480 Instruments

The melting-curve peaks (Fig. 3) discriminate between HLA-B27 positive and HLA-B27 negative samples.

View data for Melting as follows:

LC 480 Instruments:
For use in LightCycler® 480 Instrument view Melting data in channel 483-533.
For use in LightCycler® 480 II Instrument view Melting data in channel 465-510.
For use in Cobas® Z480 Instruments view Melting data in channel 465-510.
“Melt Curve Genotyping” mode.



Channel 465-510

NTC No Target Control (H_2O - light blue line) no assay-specific melting peaks must be detected.

POS Control (HLA-B27 and Globin - red line) and positive sample (fuchsia) show melting peaks at 64℃ and 56℃.

NEG HLA Negative Control Standard (Globin - blue line) and HLA-B27 negative sample (black line) show a melting peak at 56℃.

REF Reference (green line) shows a melting peak at 64℃; a second peak at 56℃ is indication of contamination.

Fig. 3: Melting sample data for HLA-B27.

Interpretation of the Results

Genotype:	HLA-B27 negative	HLA-B27 positive	
Number of melting peaks	1	2	1
Melting temperature of peaks	56℃	56℃ and 64℃	64℃
Temperature difference between peaks	---	8℃	---
Phenotype	**HLA-B27 negative**	**Increased Risk**	**Increased Risk**

Tab. 8: Typical analysis results

Note:

The values of the melting temperatures (Tm) may vary ±2.5℃ between different experiments.
The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5℃.

In case of variations see instructions: **7.3.6 Abnormal Melting Curves.**

In case of automatic genotype module failure (score <0.6 or res<0.4), switch to manual identification of melting curve (Tm calling) and compare results with table above or use table 11 in chapter **7.6. Interpretation of the Results**.

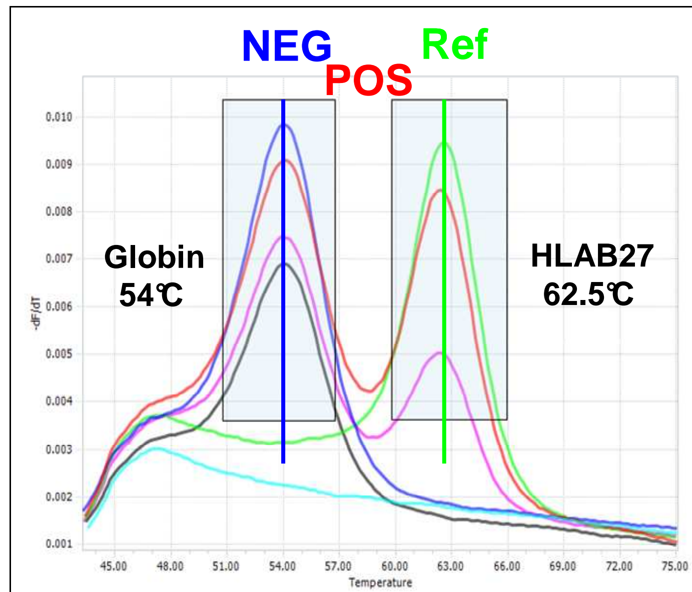
7.5.4 Melting Analysis: LightCycler® 96 Instrument

The melting-curve peaks (Fig. 4) discriminate between HLA-B27 positive and HLA-B27 negative samples.

Add Analysis: **Tm Calling**
View data in: **Melting peak**
Select peaks by using the: **Area marker tool**

Note : The LightCycler® 96 Instrument needs a manual input to mark the region which shall be used for determining the melting peak; if the peak is not contained in the box (marker tool) the software will not report a Tm value.

Manually compare the melting curve of each patient with the melting curve of Standards and use table 9 below or table 11 in chapter **7.7. Interpretation of the Results** for comparison.



Channel FAM

NTC No Target Control (H_2O - light blue line) no assay-specific melting peaks must be detected.

POS Control (HLA-B27 and Globin - red line) and positive sample (fuchsia) show melting peaks at 62.5℃ and 54℃.

NEG HLA Negative Control Standard (Globin - blue line) and HLA-B27 negative sample (black line) show a melting peak at 54℃ .

REF Reference (green line) shows a melting peak at 62.5℃; a second peak at 54℃ would be an indication for a contamination.

Fig. 4: Melting sample data for HLA B27.

Interpretation of the Results

Genotype:	HLA-B27 negative	HLA-B27 positive	
Number of melting peaks	1	2	1
Melting temperature of peaks	54℃	54℃ and 62.5℃	62.5℃
Temperature difference between peaks	---	8.5℃	---
Phenotype	**HLA-B27 negative**	**Increased Risk**	**Increased Risk**

Tab. 9: Typical analysis results

Note:

The values of the melting temperatures (Tm) may vary ±2.5℃ between different experiments. The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5℃.

In case of variations see instructions: **7.3.6 Abnormal Melting Curves.**

7.5.5 Melting Analysis: LightCycler® Nano Instrument

The melting-curve peaks (Fig. 4) discriminate between HLA-B27 positive and HLA-B27 negative samples.

.

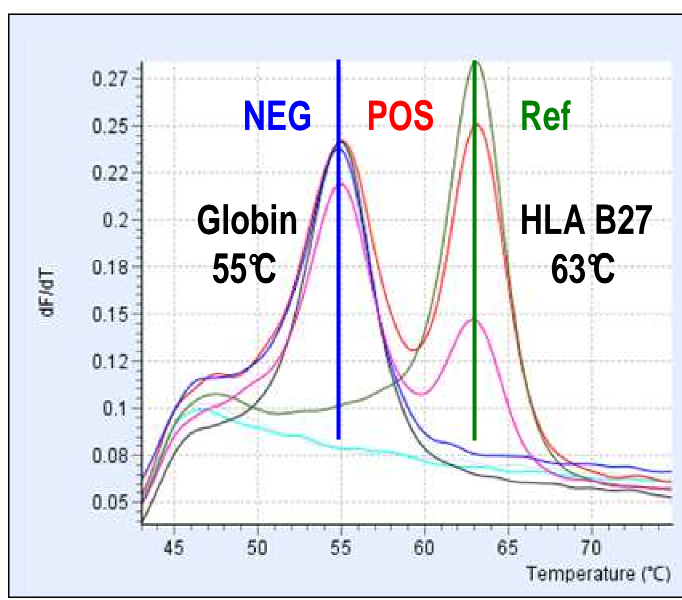
View data for Melting as follows:

In window: **Select Analysis** — Select: Tm Calling

In window: **Setting** — Select: Use negative Derivative "Yes"
Select: Noise Reduction Range (℃) = 1
Select: Target: Channel 530

Melt Peaks

Manually compare the melting curve of each patient with the melting curve of Standards and use table 10 below or table 11 in chapter **7.7. Interpretation of the Results** for comparison.



Channel 530

NTC No Target Control (H_2O - light blue line) no assay-specific melting peaks must be detected.

POS Control (HLA-B27 and Globin - red line) and positive sample (fuchsia) show melting peaks at 63℃ and 55℃.

NEG HLA Negative Control Standard (Globin - blue line) and HLA-B27 negative sample (black line) show a melting peak at 55℃ .

REF Reference (green line) shows a melting peak at 63℃ a second peak at 55℃ is indication of contamination.

Fig. 5: Melting sample data for HLA-B27.

Interpretation of the Results

Genotype:	HLA-B27 negative	HLA-B27 positive	
Number of melting peaks	1	2	1
Melting temperature of peaks	55℃	55℃ and 63℃	63℃
Temperature difference between peaks	---	8℃	---
Phenotype	**HLA-B27 negative**	**Increased Risk**	**Increased Risk**

Tab. 10: Typical analysis results

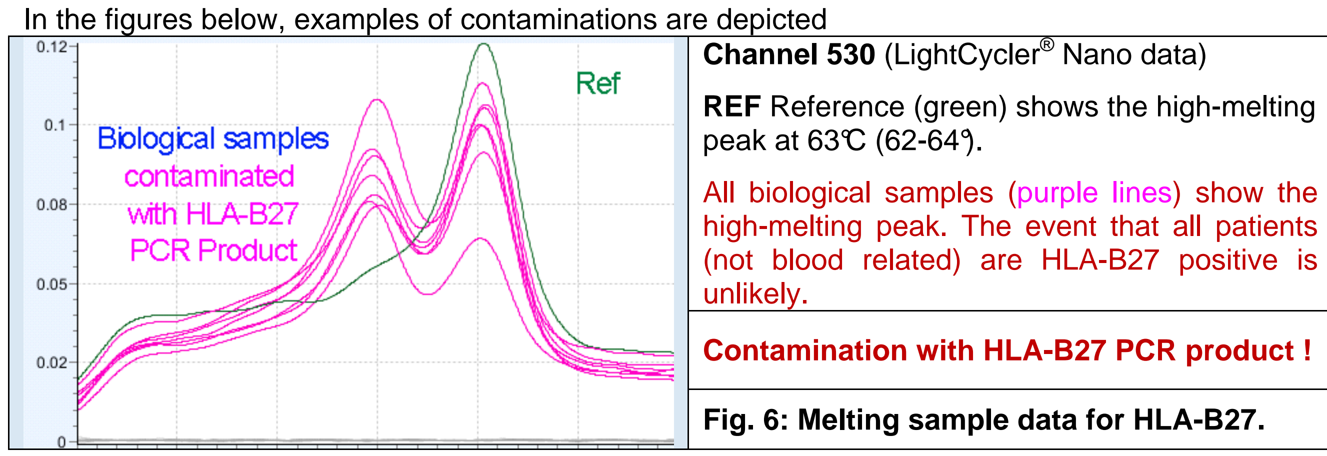
Note:

The values of the melting temperatures (Tm) may vary ±2.5℃ between different experiments. The ∆T between the melting peaks for heterozygous genotypes may vary ±1.5℃.

In case of variations see instructions: **7.3.6 Abnormal Melting Curves.**

7.6 Sample Results in case of Contaminations

In the figures below, examples of contaminations are depicted



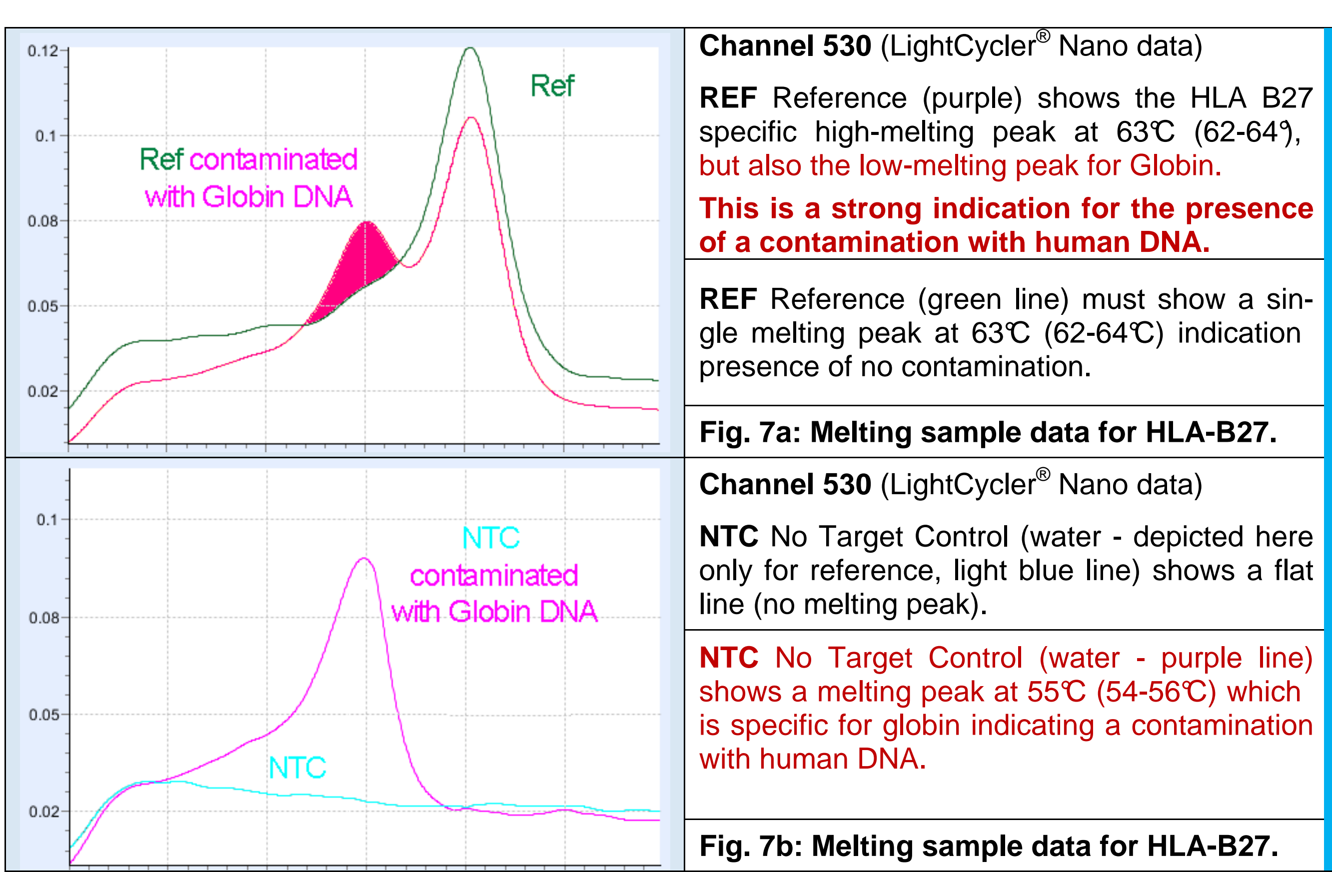
Channel 530 (LightCycler® Nano data)

REF Reference (green) shows the high-melting peak at 63°C (62-64°).

All biological samples (purple lines) show the high-melting peak. The event that all patients (not blood related) are HLA-B27 positive is unlikely.

Contamination with HLA-B27 PCR product !

Fig. 6: Melting sample data for HLA-B27.



Channel 530 (LightCycler® Nano data)

REF Reference (purple) shows the HLA B27 specific high-melting peak at 63°C (62-64°), but also the low-melting peak for Globin.

This is a strong indication for the presence of a contamination with human DNA.

REF Reference (green line) must show a single melting peak at 63°C (62-64°C) indication presence of no contamination.

Fig. 7a: Melting sample data for HLA-B27.

Channel 530 (LightCycler® Nano data)

NTC No Target Control (water - depicted here only for reference, light blue line) shows a flat line (no melting peak).

NTC No Target Control (water - purple line) shows a melting peak at 55°C (54-56°C) which is specific for globin indicating a contamination with human DNA.

Fig. 7b: Melting sample data for HLA-B27.

Figure 7a and 7b are equivalent.

7.7 Interpretation of the Results - Overview

In case of automatic genotype module failure, switch to manual identification of melting curve (Tm calling) and compare results.

HLA-B27 Channel 530 Melting peak(s)		HLA-B27 Genotypes	Metabolizers Phenotype
Globin	HLA-B27		
Melting Peaks 530 Temperature (°C)		HLA-B27 negative	Normal risk
54-56	-		
Melting Peaks 530 Temperature (°C)		HLA-B27 positive	Increased Risk
54-56	62-64		
Melting Peaks 530 Temperature (°C)		HLA-B27 positive	Increased Risk
-	62-64		
ΔTm 8°C			

Tab. 11: Typical analysis results

Compare results obtained with biological samples with published frequencies.

Note: The values of the melting temperatures (T_M) may vary ±2.5°C between different runs. The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.

In case of variations see instructions: **7.3.4 Samples: Abnormal Melting Curves**

8. Troubleshooting

Instrument specific codes:	Capillary based instruments	LightCycler® 480 instruments
	LightCycler® Nano	LightCycler® 96 instrument
Event	**Possible Reason**	**Solution**
No sample detected	No centrifugation	Centrifuge capillary
All negative PCRs	Incorrect selection of detection channel	Set correct channel before analyzing
	Incorrect amplification protocol	Check instrument program
Inconsistent baseline among various samples	Incorrect pipetting	Ensure homogeneity of mix quantity in each sample
	Non homogenous reaction mix	Pipette reaction mix up and down 10 x with a clean 200µl tip before distribution in reaction vessel
	Multiwell plate badly sealed	Ensure that multiwell plate is properly sealed
Baseline “Saw teeth like”	Bubble in the well	Centrifuge plate before run
	Bubble in the well	Centrifuge plate before run
	Incorrect positioning of capillary in the carousel	Firmly press capillary in the carousel
No signal in the Positive Control	Error while setting the instrument	Check the position settings of the Positive Control
	Incorrect PSR / $MgCl_2$ concentration	Repeat assay
	Positive Control or standard degradation	Use a new aliquot of Positive Control or standard
Positive signal in **NTC** Negative Control	Error while setting the instrument	Check the position settings of the Negative Control
	Dispensing error	Comply with the work sheet when dispensing samples, Negative Controls, Positive Controls and standards
	Dispensing error	Always change tips among samples
	Dispensing error	Avoid spilling the contents of the sample test tube
	Contamination of the PCR- grade water.	Use a new aliquot of PCR- grade water
	Contamination of the reaction mix	Use new aliquots of reagents to prepare the reaction mix
	Contamination of the extraction/preparation area for amplification reactions	Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use
	Contamination of the extraction/preparation area for amplification reactions	Add LightCycler® Uracil-DNA Glycosylase (Cat.-No.03 539 806 001) to the reaction mix according to instructions
No signal in samples	Low DNA amount	Check DNA concentration
	Sample inhibition	Dilute sample and repeat PCR, or repeat extraction and PCR, or add Positive Control and repeat
Melting curve outside the expected temperature range	With peaks TM **concordant** with Positive Control: Incorrect reagent concentration	Manually assign results accordingly to Positive Control
	With peaks TM **discordant** with Positive Control: Possible extraction inhibitor	Repeat assay diluting the DNA 1:3
	With peaks TM **discordant** with Positive Control: Possible different mutation	Repeat assay by sequencing and report unexpected variant to service@tib-molbiol.de

9. References

1) Bon, M., van Oeveren-Dybicz, A. and van den Berg, F.
Genotyping of HLA-B27 by Real-Time PCR without Hybridization Probes
Clinical Chemistry 46: 1000-1002, 2000

2) Tiemann C. et al.,
Rapid DNA Typing of HLA-B27 Allele by Real-Time-PCR Using LightCycler technology.
Clin.Lab. 47 (2001) 131-134

3) Seipp. M.T., Erali, M., Wies, R.L and Wittwert, C
HLA-B27 Typing: Evaluation of an Allele-Specific PCR Melting Assay and Two Flow Cytometric Antigen Assays. *Clin.Cytometry (2005) 63B 10-15*

4) Faner R, Casamitjana N, Colobran R, Ribera A, Pujol-Borrell R, Palou E, Juan M.
HLA-B27 genotyping by Fluorescent Resonance Emission Transfer (FRET) probes in real-time PCR. *Hum Immunol. 2004 Aug;65 826-38*

1) Bon, M., van Oeveren-Dybicz, A. and van den Berg, F.
Genotyping of HLA-B27 by Real-Time PCR without Hybridization Probes
Clinical Chemistry 46: 1000-1002, 2000

Classification / Reference

Reference	**Classification**
EDMA	16 01 04 01
CPV	33694000-1
EAN	4260159332261
Roche SAP No.	06896537001

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According to OSHA 29CFR1910.1200, Commonwealth of Australia [NOHSC:1005, 1008 (1999)] and the European Union Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

Version History

Notes in red mark events that require to change laboratory procedures
Notes in blue mark improvements and changes in composition

Version	Event	Date
V120512	Release Version	12-05-2012
V130301	Reference numbers and classification completed	02-04-2013
V130506	Exact temperatures for high and low melting peaks replaced by temperature range (3.2 and 7.3). LightCycler® 96 Instrument included	25-04-2013
V131001	Detection probes for HLA and Globin have been substituted by lower melting probes, shifting the melting peaks to lower temperatures and an increased ΔTm in order to improve the performance of the automated genotyping.	01-10-2013
V150101	Instrument setting LC 96 corrected. List of detected alleles included.	07-01-2015

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