

# READY TO USE, PREALIQUOTED

Instructions for use

## HISTO TYPE B27

CE 0123

Test kit for typing of B\*27 alleles  
on a molecular genetic basis

IVD

48 Typing tests / REF 7070

96 Typing tests / REF 7071

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# 1. Introduction

## 1.1 Clinical significance of B27

Associations between certain HLA types and certain diseases have been recognised for more than 40 different combinations. The most significant is the association of HLA-B27 with the disease picture of seronegative arthritis (Bechterew's disease, Reiter's disease, reactive arthritis). A positive HLA-B27 result is associated with a very high disease risk (see Table 1) [1, 2]. Most notably, a confirmed HLA-B27 diagnostics result makes an important contribution to the therapy of the patient in unclear cases of suspected Bechterew's disease.

Disease	B27 Frequency in patients	Relative risk
Ankylosing spondylitis (Bechterew's disease)	90.2 %	91
Reiter's disease	78.8 %	37.6
Post-infection reactive arthritis	70.2 %	

Table 1: HLA-B27 Frequencies and risks.

## 1.2 Principle of the test

The use of the polymerase chain reaction (PCR) in HLA typing has now become routine. The sequence determination of all HLA alleles [3] makes possible a specific and positive typing at the DNA level with a high resolution and provides decisive advantages compared with conventional serological methods. The starting material for typing with the **HISTO TYPE B27** test is purified leukocyte DNA. The test is based on a **Sequence Specific Primer (SSP)** PCR (see Fig. 1). This method takes advantage of the fact that for a successful reaction to take place, the two primers must exhibit no mismatch [4], particularly at the 3' end. In this way, only complete agreement of the primers with the target sequence will lead to the production of an amplificate, which is then rendered visible by subsequent gel electrophoresis.

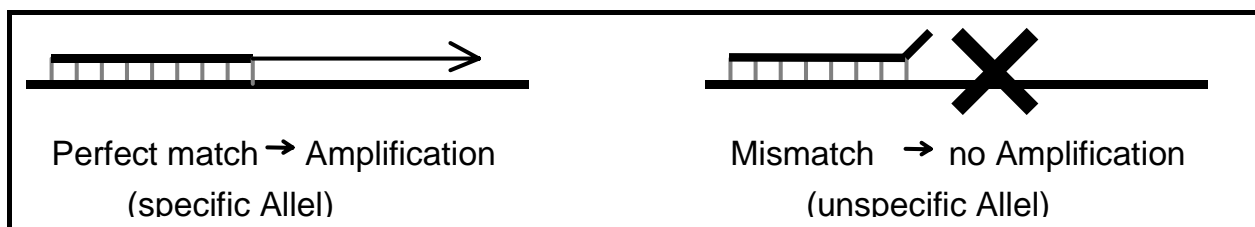


Fig. 2: Principle of the SSP-PCR

The specific primers have been selected such that the most frequent subtypes (\*2701-17, 19-21, 24-28, 30-32, 34-45) are recognized. In addition, each reaction mixture contains a control primer pair which must always form an amplificate.

If this band is missing, either a pipetting error has been made, or the DNA contained inhibitory substances (see Troubleshooting). In this case, the test cannot be evaluated! For each test, a reaction well with **prealiquoted** and **dried** reagents is required. The final volume is 10  $\mu$ l.

## 2. Materials

### 2.1 Contents of the HISTO TYPE B27 Kit

- ◆ 6 (12) HISTO TYPE strips (each containing 8 thin-walled PCR reaction wells) sufficient for 48 (96) HLA-B27 typing tests. The prealiquoted and dried reagents consist of allele-specific primers, internal control primers (specific for the human G3PDH gene) and nucleotides.
- ◆ 1.1 ml 10 x PCR buffer
- ◆ Instructions for use

### 2.2 Requirements and supplementary material

#### 2.2.1 Reagents

- ◆ **BAG EXTRA-GENE** Kit (optional) for DNA extraction from blood / lymphocytes / leukocytes or material for other DNA extraction methods
- ◆ Taq polymerase (5 U/ $\mu$ l) , (e.g. : Qiagen)
- ◆ DNA agarose
- ◆ 0.5 x TBE buffer (45 mM tris, 45 mM boric acid, 0.5 mM EDTA )
- ◆ DNA-length standard (Cat.-No.: 7097)
- ◆ Ethidium bromide (EtBr)

#### 2.2.2 Equipment

- ◆ DNA cycler (e.g. PTC 200-96V with "Hot Bonnet"; BioRad)
- ◆ submarine electrophoresis unit with combs and power supply (200 - 300 V, 200 mA)
- ◆ UV transilluminator (220 - 310 nm)
- ◆ DNA-length standard (Cat.-No.: 7097)
- ◆ Photographic equipment (e.g. Polaroid system) with films (Polaroid Type 667)
- ◆ Piston pipettes (0.5 - 250  $\mu$ l) and sterile tips with filter

## 2.3 Storage and stability

The kit is delivered unfrozen. After receipt, store all kit reagents at -20...-80°C protected from light. The expiry date printed on the outer label applies to all reagents contained in the kit and is also valid after first use. Thaw the 10x PCR buffer shortly before use.

## 3. Data of performance

Analytical Sensitivity: A reliable typing is guaranteed by using 40-80 ng DNA per reaction mix

Diagnostical Specificity: The composition of the primer mixture guarantees a reliable identification of the B27 subtypes (based on the latest sequence data) indicated in the principle of the test (Chapter 1). Updates will be done regular.

For every lot the specificity of the primer mix was verified with DNA from reference samples.

A study of performance was done for the HISTO TYPE B27 kit with at least 50 DNA samples. The comparison of the test results with other typings, done with SSP kits of another supplier, showed no discrepancy.

## 4. Test procedure

### 4.1 Safety conditions and special remarks

The PCR reaction is an extremely sensitive test method and should be performed by well trained personnel, experienced in molecular genetic techniques and histocompatibility testing. Transplantation guidelines as well as EFI standards should be followed in order to minimize the risk of false typings, in the particular case of discrepancies in serological and molecular genetic method. Special safety conditions must be noted in order to avoid contamination and thus false reactions:

- ◆ always wear protective gloves (preferably powder-free)
- ◆ use new tips for every pipetting step (with filter insert or integrated wad)
- ◆ work in two separate work areas for the **pre-amplification** (DNA isolation, preparations for the reactions) and the **post-amplification** (amplification, gel electrophoresis, documentation); if possible work in two separate rooms
- ◆ Use equipment and other materials only at their assigned work areas and do not interchange them

## 4.2 DNA isolation

About 50 ng of leukocyte DNA are needed for typing one patient. E.g. the **BAG EXTRA-GENE** Kit is most suitable for isolation since pure DNA can be obtained from whole blood in the minimum of time without the use of toxic chemicals or solvents. Other suitable methods for the isolation of DNA of adequate purity include those described in the literature [5], such as the chloroform-triethylammonium bromide (CTAB) method or phenol-chloroform purification. Heparin can under certain circumstances inhibit the PCR reaction [6]. It is therefore recommended to use EDTA or citrate blood for typing. The DNA should have a purity index (extinction ratio  $OD_{260}/OD_{280}$ ) between 1.5 and 2.0.

## 4.3 Amplification

All prealiquoted and dried reaction mixtures already contain the allele-specific primers, nucleotides and the primers for the internal amplification control. The amplification parameters are optimised for a final volume of 10  $\mu$ l.

## 4.4 Test procedure

Remove the required number of HISTO TYPE B27 strips from the freezer and thaw the 10x PCR buffer.

<b>I. Taq predilution:</b>	<b>0.08 <math>\mu</math>l</b>	Taq polymerase (5 U/ $\mu$ l) <i>x no. determinations + 1</i> (0.5 U per reaction)
	<b>1.0 <math>\mu</math>l</b>	10 x PCR buffer <i>x no. determinations + 1</i> mix thoroughly

<b>II. Sample mixture:</b>	<b>1.1 <math>\mu</math>l</b>	Taq predilution
	<b>1.0 <math>\mu</math>l</b>	DNA solution (approx. 50 ng)
	<b>7.9 <math>\mu</math>l</b>	sterile distilled water (to 10 $\mu$ l) ; mix well

**III. Distribution :** Pipette **10  $\mu$ l** of the sample mixture into a reaction well, carefully close the well with the lid. The reaction solution must be in the bottom of the well !There is no need to cover the reaction mixture with mineral oil when a heated cover is used!

**IV. Place the reaction wells into the cycler (check that they are firmly seated!)**

## V. Start the PCR program

### Amplification protocol

Programme-Step	Temp.	Time	No. of Cycles
First Denaturation	96°C	5 Min	1 Cycle
Denaturation	96°C	20 Sec	5 Cycles
Annealing+Extension	68°C	1 Min	
Denaturation	96°C	20 Sec	10 Cycles
Annealing	64°C	50 Sec	
Extension	72°C	45 Sec	
Denaturation	96°C	20 Sec	15 Cycles
Annealing	61°C	50 Sec	
Extension	72°C	45 Sec	
Final Extension	72°C	5 Min	1 Cycle

#### Cycler types:

PTC 100 / 200  
(MJ Research/ BioRad),  
GeneAmp PCR-System  
9600 / 9700 (use heating  
rate of 9600 please) (ABI)  
and Mastercycler  
epGradient S (Eppendorf)

**By using thermal cyclers with a very fast heat- and coolingrate, it's recommended to use a slower heat- and coolingrate.**

**Since cyclers of different manufacturers perform differently and even individual machines of one type may be calibrated differently, it may be necessary to optimize the amplification parameters.**

To optimize your machine use the following guide:

With **false positive** reactions (unspecific bands, additional types): Increase of the annealing temperature in 1°C steps.

With **false negative** reactions (bands missing): Decrease of the annealing temperature in 1°C steps and/or increase of the annealing periods in 5 second steps and/or increase of the denaturation periods in 5 second steps.

**It's recommended to use only regularly calibrated cyclers. For this the BAG-Cycler Check kit is very suitable (Cat.-No.: 7104).**

**The quality control tests were done on a PTC-200 resp. 100 (MJ Research), 9700 (ABI) and Mastercycler epGradient S (Eppendorf)**

### 4.5 Gel electrophoresis

Separation and evaluation of the amplification products is performed using electrophoresis on a (horizontal) agarose gel. The recommended separation buffer is a 0.5 x TBE (45 mM tris, 45 mM boric acid, 0.5 mM EDTA) buffer. The gel concentration should be 2.0 - 2.5 % agarose. The gel should be allowed to polymerise for at least 30 minutes before applying the sample. After completion of the amplification, the samples are taken out of the cycler

and the complete reaction mixture carefully pipetted into a slot of the gel. The addition of sample buffer is not necessary. One further slot should be loaded with a DNA length standard for size comparison. The electrophoretic separation is carried out at 10 - 12 V/cm (for 20 cm gel length approx. 200 - 240 V) for 20 - 40 minutes.

When the run is finished, stain the entire gel for 30 - 45 minutes in a solution of ethidium bromide (EtBr - approx. 0.5 µg/ml EtBr in H<sub>2</sub>O or TBE buffer). Alternatively, EtBr (0.5 µg/ml) can also be added to the electrophoresis buffer or to the agarose gel. If necessary, the gel can be decolorised for approx. 20 - 30 minutes in H<sub>2</sub>O.

#### **4.6 Documentation and evaluation**

For documentation, the gel is placed on a UV transilluminator (220 - 310 nm) and photographed with a suitable camera (e.g. Polaroid, film type 667). The exposure time and aperture should be selected such that the bands are sharply delineated and contrast well with the dark background (approximates aperture 11, exposure time 1 second).

### **5. Interpretation**

Only bands which possess the correct length of **420 bp and/or 85 bp** with reference to the DNA length standard are to be considered positive. In all tracks which show no specific amplification, the internal control must appear at **1070 bp** in all cases. In the samples which show a specific reaction, the internal control can appear weaker or can disappear completely! For results which cannot be evaluated, see 7. Troubleshooting.

### **6. Warnings and Precautions**

Ethidiumbromide is a powerful mutagen. Wear gloves when handling gels or solutions containing EtBr! Note the instructions for use and warnings and precautions of the manufacturer! The transilluminator radiates very short-wave UV light which may cause burns of the skin and the retina. Use a UV face safety mask!

All for extraction of DNA used biological material, e.g. blood or human tissue, should be handled as potentially infectious. When handling biological material appropriate safety precautions are recommended (do not pipet by mouth; wear disposable gloves while handling biological material and performing the test; disinfect hands when finished the test).

Biological material should be inactivated before disposal (e.g. in an autoclave). Disposables should be autoclaved or incinerated after use.

Spillage of potentially infectious materials should be removed immediately with absorbent paper tissue and the contaminated areas swabbed with a suitable standard disinfectant or 70% alcohol. Material used to clean spills, including gloves, should be inactivated before disposal (e.g. in an autoclave).

## 7. Troubleshooting






Problem	Possible Reason	Solution
no amplification, length standard visible	DNA contaminated with PCR-inhibitors	repeat DNA isolation, try different methods
	DNA concentration too high/too low	alter DNA concentration, repeat DNA isolation
	enzyme is missing or concentration too low	repeat typing, alter enzyme concentration
	DNA from heparinized blood	repeat typing with EDTA- or citrate-blood
	wrong amplification parameters	optimize the amplification parameters (see 4.4) ☆
repeated failure in single lanes (no amplification-control)	leak in reaction tubes; water loss and change in concentration during PCR	close tubes tight with caps; use other reaction tubes
unspecific amplification, additional bands, (additional bands of the wrong size must be neglected)	contamination with amplification products	repeat typing, ensure exact working
	DNA contaminated with salts	repeat DNA isolation, try different methods
	DNA concentration too high	use less DNA
	enzyme concentration too high	use less enzyme
	wrong amplification parameters	optimize the amplification parameters (see 4.4) ☆
no or only very weak bands visible, length standard invisible	EtBr staining too weak	repeat staining
gel background shines too bright	staining was too long, EtBr concentration too high	soak gel in H <sub>2</sub> O or TBE lower EtBr concentration
blurred band	electrophoresis buffer too hot or too old wrong electrophoresis buffer gel not polymerized completely	Lower the voltage use (fresh) 0,5x TBE buffer allow gel 30 min. to polymerize

☆ When using the equipment and materials listed, optimisation of the amplification parameters should be looked upon as a last resort. In most cases, it is possible to evaluate the test by eliminating the additional bands caused by size discrepancies.

## 8. References

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A Laboratory Manual. New York: Cold Spring Harbour Laboratory
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## 9. Explanation of symbols used on Labelling

	For in vitro diagnostic use
	Storage temperature
	Batch code
	Use by
REF	Catalogue number
	Consult instructions for use



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