

READY TO USE, PREALIQUOTED

Instructions for use

HISTO TYPE Null Kits

REF 70862: Null A*2409N

REF 70872: Null B*5111N

REF 70882: Null Cw*0409N

CE 0123

Test kit for typing of HLA class I Null alleles
on a molecular genetic basis



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1. Introduction

1.1 NMDP Policy for high resolution HLA typing

The NMDP (National Marrow Donor Program, USA) requires a high resolution HLA-A, B, C and DRB1 typing of the recipient and the potential donors before a stemcell transplantation can be carried out. The definition of an acceptable result and the common and well-documented (CWD) alleles that need to be clearly identified in order to meet the criteria for “high resolution” are described in the 2007 manuscript by Cano et al [1]. Generally, it is not required to resolve CWD alleles that are identical in nucleotide sequence in the exons encoding the antigen recognition site. However, NMDP requires to test for the CWD Null alleles A*2409N, B*5111N and Cw*0409N when evidence exists for the presence of alleles or haplotypes that are associated with a specific Null allele [2].

Null Allele	Alternative Common Allele	Associated Alleles in Haplotype	Location of Polymorphism
A*2409N	A*24020101	B*40 or B*27	Exon 4
B*5111N	B*510101	A*02 or DRB1*04 or Cw*15 (A*0201, B*5111N, DRB1*0402, Cw*15BJ)	Exon 4
Cw*0409N	Cw*04010101	B*4403	Exon 7

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 a specific typing at the DNA level with a high resolution possible and provides decisive advantages compared with conventional serological methods. The basis for typing with the **HISTO TYPE Null Kits** 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 the two primers must fit exactly to the target sequence [4], particularly at the 3' end. Therefore, only completely matched primers will lead to the formation of an amplificate, which is then rendered visible by subsequent gel electrophoresis.

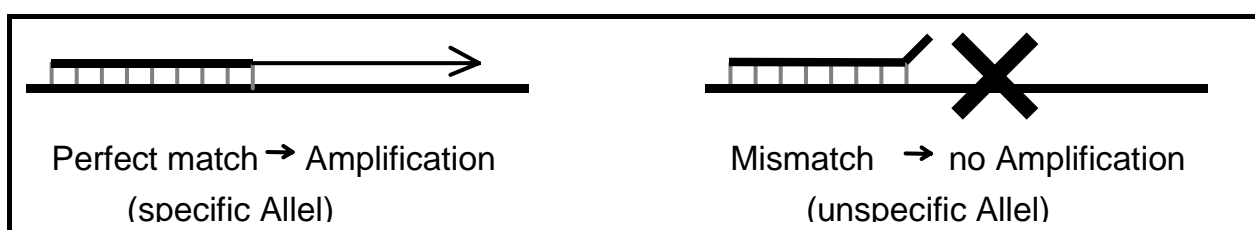


Fig. 1: Principle of the SSP-PCR

The specific primers have been selected in order to recognize only the respective Null allele (A*2409N, B*5111N or Cw*0409N). In addition, each reaction mixture contains internal control primers that must always amplify.

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, one well with **prealiquoted** and **dried** reagents is required. The final reaction volume is 10 µl.

2. Materials

2.1 Contents of the HISTO TYPE Null Kits

- ◆ 3 HISTO TYPE strips (each containing 8 thin-walled PCR reaction wells) sufficient for 24 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/µl) , (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)
- ◆ Photographic equipment (e.g. Polaroid system) with films (Polaroid Type 667)
- ◆ Piston pipettes (0.5 - 250 µl) 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 alleles A*2409N, B*5111N, Cw*0409N respectively (based on the latest sequence data).

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

A study of performance was done for the HISTO TYPE Null Kits with at least 50 DNA samples. The comparison of the test results with previous results achieved with another typing method 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. For example, the **BAG EXTRA-GENE** Kit is very 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 μ l.

4.4 Test procedure

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

I. Taq predilution:	0.08 μl	Taq polymerase (5 U/ μ l) x <i>no. determinations</i> + 1 (0.5 U per reaction)
	1.0 μl	10 x PCR buffer x <i>no. determinations</i> + 1 mix thoroughly
II. Sample mixture:	1.1 μl	Taq predilution
	1.0 μl	DNA solution (approx. 50 ng)
	7.9 μl	sterile distilled water (to 10 μ l) ; mix well

III. Distribution : Pipette **10 μ 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	60 Sec	
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 H₂O 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 H₂O.

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 that have the correct length with reference to the DNA length standard are to be considered positive (see Table 1). In all tracks which show no specific amplification, the internal control must appear. In 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.

Table 1: Size of amplicons

HISTO TYPE Null Kit	Size of specific band	Size of control band
A*2409N	315 bp	1070 bp
B*5111N	670 bp	410 bp
Cw*0409N	700 bp	410 bp

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 biological material used for extraction of DNA, e.g. blood or human tissue, should be considered 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 ₂ 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

- 1 Cano P, et al., 2007, Human Immunology 68:392-417
- 2 http://bioinformatics.nmdp.org/POLICIES/policies_idx.html
- 3 Bodmer, J. et al., 1997. Tissue Antigens 49:297-321
- 4 Newton CR, 1989. Nucleic Acids Res. **17**:2503-2516
- 5 Maniatis et al., 1989. Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbour Laboratory
- 6 Beutler, E. et al., 1990. BioTechniques **9**:166

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