

Instructions for use
HISTO TYPE Narcolepsy

CE 0123

Test kit for tissue typing of HLA alleles
(Class II: HLA-DR, DQA, DQB)
on a molecular genetic basis

IVD

20 Typings
ready to use prealiquoted

REF 70716: HISTO TYPE Narcolepsy

(8 mixes, red)

Contents

| | |
|---|----|
| 1. Introduction | 2 |
| 1.1 Background | 2 |
| 1.2 Product description | 3 |
| 2. Material | 4 |
| 2.1 Contents of the HISTO TYPE Narcolepsy Kit | 4 |
| 2.2 Requirements and supplementary material | 4 |
| 2.3 Storage and stability | 5 |
| 3. Data of performance | 5 |
| 4. Test procedure | 6 |
| 4.1 Safety conditions and special remarks | 6 |
| 4.2 DNA isolation | 6 |
| 4.3 Amplification | 6 |
| 4.4 Gel electrophoresis | 9 |
| 4.5 Documentation and interpretation | 9 |
| 5. Warnings and precautions | 10 |
| 6. Troubleshooting | 11 |
| 7. References | 12 |
| 8. Explanation of symbols used on Labelling | 12 |

Version 01/2010

1. Introduction

1.1 Background

Narcolepsy belongs to the group of sleep disorders (dyssomnia). It is a disabling-sleep disorder that affects 0.02 to 0.18% of the general population in various countries and ethnic groups. Characteristic symptoms of the disorder are excessive daytime somnolence, abnormal sleep, sleep paralysis, cataplexy and hypnagogic hallucinations. Narcolepsy is caused by a selective loss of hypocretin producing neurons in the hypothalamus.

Its close association to the HLA system has been known for a long time period. About 85 – 95% of the narcoleptic patients are carrying the HLA haplotype DRB1*1501 -DQA1*0102 -DQB1*0602, compared to 30% of the normal population. Highest association to narcolepsy has been reported for the subtype DQB1*0602, which is the most specific marker for narcolepsy across all ethnic groups. About 98% of narcoleptic patients has been found to be carrier of the DQB1*0602 subtype. Association to DRB1*1501, DQA1*0102 are reported in less extend.

Based on these informations, the detection of the DRB1*1501, DQA1*0102, and DQB1*0602 genotypes are helpful tools in the diagnostic of narcolepsy. In the case of a negative DQB1*0602 test result, narcolepsy can be excluded most likely. On the other hand, in the case of a positive DQB1*0602 test result, existence of narcolepsy is **not** mandatory, because the frequency of DQB1*0602 is about 11-22% in general populations.

1.2 Product description

With the use of Polymerase Chain Reaction (PCR) in HLA typing much progress has been made recently. Sequencing of HLA alleles has facilitated clear typing at the DNA level with a high resolution and has many advantages over the traditionally used serologic methods. The basic material for typing with the **HISTO TYPE Narcolepsy kit** is purified DNA. The test procedure is done by using the **Sequence Specific Primers (SSP)** -PCR (see Fig. 1). This method is based on the fact that primer extension, and hence successful PCR relies on an exact match at the 3'-end of both primers. Therefore, only if the primers entirely match the target sequence is amplification obtained which is subsequently visualised by agarose gel electrophoresis.

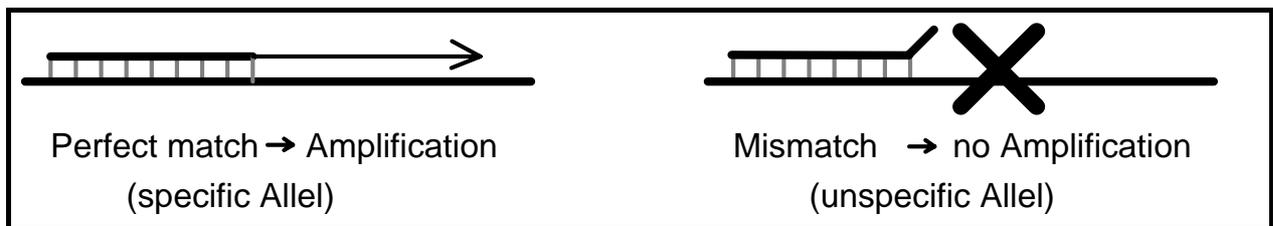


Fig. 1: Principle of SSP-PCR

The composition of the individual primer mixtures makes clear identification of the HLA types indicated in the respective evaluation diagram possible. With each typing a certain number (8) **prealiquoted** and **dried** reaction mixes including internal amplification control with a final volume of 10 µl are used.

2. Material

2.1 Contents of the HISTO TYPE Narcolepsy kit

- ◆ 20 HISTO TYPE stripes sufficient for 20 Narcolepsy typings. The prealiquoted and dried reaction mixtures consist of allele specific primers, internal control primers (specific for the human G3PDH gene) and nucleotides. The first reaction mix is marked with the print of the lot number on each stripe.
- ◆ PCR reaction mixes contamination control with internal control primers and amplificate specific primers
- ◆ 10 x PCR-buffer sufficient for 20 typings
- ◆ 8er strip-caps à 12 sufficient for 20 typings
- ◆ instructions for use, specificity table, evaluation diagram and worksheet

2.2 Requirements and supplementary material

- ◆ Taq Polymerase (5 U/μl)
- ◆ **BAG EXTRA-GENE** (optional) Kit for DNA extraction from blood / lymphocytes / leucocytes or material for other DNA extraction methods
- ◆ piston pipettes (0,5-250 μl)
- ◆ sterile tips with integrated filter
- ◆ DNA Cycler (e.g. PTC 200 with a heated and adjusted cover, MJ Research/BioRad)

Devices and material for gel electrophoresis

- ◆ DNA agarose
- ◆ 0.5 x TBE buffer (45 mM of Tris, 45 mM of boric acid, 0,5 mM of EDTA)
- ◆ Ethidium bromide (EtBr)
- ◆ submarine electrophoresis unit
- ◆ DNA-length standard (Cat.-No.: 7097)
- ◆ power supply (200-300 V, 200 mA)

Devices for interpretation and documentation

- ◆ UV source (220-310 nm)
- ◆ camera (e.g. Polaroid system) with films (Polaroid type 667)

2.3. Storage and stability

The kit is delivered unfrozen. Store all reagents at -20...-80°C in the dark. The expiry date is indicated on the label of each reagent and is also valid for opened reagents. The expiry date indicated on the outer label refers to the reagent with the shortest stability contained in the kit. Thaw the 10 x PCR-buffer shortly before use.

3. Data of performance

Analytical Sensitivity: A reliable typing is guaranteed by using 50 - 80 ng DNA per reaction mix

Diagnostical Specificity: The composition of the primer mixture guarantees a reliable identification of the HLA-Types (based on the latest sequence data) indicated in the evaluation diagram. Updates will be done regularly.

For every lot the specificity of each primer mix was verified with DNA from reference samples: each PCR reaction has been positively tested at least once. Alleles, which are not included and cause of their rareness not tested respectively, are indicated on the evaluation diagram or specificity table.

A study of performance was done for the HISTO TYPE Narcolepsy 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 is a particularly sensitive 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 observed in order to avoid contamination and thus false reactions:

- ◆ Wear gloves during work (powder-free, if possible).
- ◆ Use new tips with each pipetting step (with integrated filter).
- ◆ Use separate working areas for pre-amplification (DNA isolation and preparation of the reactions) and post-amplification (gel electrophoresis, documentation). Preferably use two separate rooms.
- ◆ Use devices and other materials only at the respective places and do not exchange them.

4.2 DNA isolation

For a HLA-SSP typing of one patient at least 1 µg of DNA (corresponding to approximately 0,1 - 0.5 ml of blood) is required. E.g. the **BAG EXTRA-GENE** kit is most suitable for isolation since pure DNA can be obtained from whole blood in a short time without the use of toxic chemicals or solvents. Also, other methods described in literature such as the chloroform-triethyl-ammonium-bromide (CTAB) method or phenolchloro-form purification are suitable to provide DNA of sufficient purity. The presence of heparin potentially inhibits PCR. Therefore EDTA or Citrate Blood is recommended for typing. 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 allele and control-specific primers and nucleotides. These are supplied dried down in the reaction vessel. Amplification parameters are optimized to a final volume of 10 µl.

- 1.: Remove the required number of HISTO TYPE stripes from - 20°C and thaw the 10 x PCR-buffer at room temperature.

- 2.: Pipet the Master-Mix consisting of 10 x PCR-buffer, DNA solution, Taq-Polymerase and Aqua dest. and mix well. The HISTO TYPE Narcolepsy Kit works with the same Master-Mix as other BAG HISTO TYPE SSP Kits do and can therefore be combined. The composition of the Master-Mix is given in Table 1 (p.7).
 If the **contamination control** should be performed, produce the Master-Mix without the DNA solution first and pipet 10 µl of this mix in the contamination control. Afterwards add the DNA solution and distribute the Master-Mix on the predropped reaction mixes.

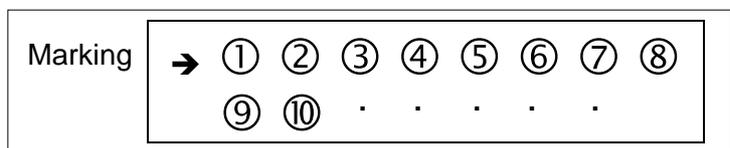
Table 1: Composition of the Master-Mix:

| no. of mixes | Aqua dest. | 10 x PCR-buffer | DNA-sol. (25 - 40 ng/µl) | Taq-Polymerase (5 U/µl) | whole volume | |
|--------------|------------|-----------------|--------------------------|-------------------------|--------------|----|
| 8 | 69 | 10 | 20 | 0,8 | 100 | µl |

⇒ The quantity of DNA should be 50 – 80 ng per mix. According to DNA-concentration, the amount of DNA and water must be varied.

(e.g.: 20 µl DNA solution (25 ng/µl) and 69 µl Aqua dest.; 10 µl DNA solution (50 ng/µl) and 79 µl Aqua dest.).

- 3.: After vortexing add **10 µl** of this mixture immediately to the predropped and dried reaction mixtures. Change the tip after each pipetting step. Tightly close the tubes with the



respective caps. Ensure that you do not touch the inner side of the caps and the upper edges of the tubes with the fingers so that contamination is avoided. If cyclers with tightly closable lid are used, it is also possible to use reusable PCR mats.

Slightly shake the plate downwards to dissolve the blue pellet at the bottom of the plate. All PCR solution should settle on the bottom.

4.: Place the reaction tubes into the thermocycler and tighten lid so that the reaction vessels do not warp in heating. Start the PCR programme. Overlaying of the reaction mixtures with mineral oil is **not** required if a heated and adjusted cover is used!

Amplification parameters:

| Programme-Step | Temp. | Time | No. of Cycles |
|---------------------|-------|--------|---------------|
| First Denaturation | 96°C | 5 Min | 1 Cycle |
| Denaturation | 96°C | 20 Sek | 5 Cycles |
| Annealing+Extension | 68°C | 60 Sek | |
| Denaturation | 96°C | 20 Sek | 10 Cycles |
| Annealing | 64°C | 50 Sek | |
| Extension | 72°C | 45 Sek | |
| Denaturation | 96°C | 20 Sek | 15 Cycles |
| Annealing | 61°C | 50 Sek | |
| Extension | 72°C | 45 Sek | |
| 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).

4.4 Gel electrophoresis

Separation of the amplification products is done by electrophoresis via a (horizontal) agarose gel. As electrophoresis buffer, 0.5 x TBE (45 mM of tris, 45 mM of boric acid, 0.5 mM of EDTA) buffer is recommended. The gel concentration should be 2.0 - 2.5% of agarose. Allow the gel to polymerize at least 30 minutes before sample loading. After amplification has been finished, take the samples out of the thermocycler and load the complete reaction mixtures carefully in each slot of the gel. In addition, apply 10 µl of the DNA length standard for size comparison. Electrophoretic separation is done at 10-12 V/cm (with 20 cm distance between the electrodes approx. 200-240 V), for 20-40 min.. After the run has been completed, the complete gel is stained in an ethidiumbromide (EtBr) solution (approx. 0.5 µg/ml of EtBr in H₂O or TBE buffer) for 30-45 min.. As alternative, EtBr (0.5 µg/ml) can also be added to the electrophoresis buffer or the agarose gel. If required, excess of EtBr can be removed by soaking the gel in H₂O or 0.5 x TBE buffer for 20-30 minutes.

4.5 Documentation and interpretation

For documentation, visualize the PCR amplification using an UV transilluminator (220-310 nm) and photograph it with a suitable camera, film and filters (e.g. polaroid, film type 667). Choose exposure time and aperture such that the bands are drawn sharp and stand out against the dark background (approximates aperture 11, exposure time 1 second).

For interpretation use the specificity table and evaluation diagram (see extra sheet); only bands that have the correct size compared to the DNA length standard should be considered positive. The correct sizes are given in the table resp. diagram. In all lanes without allele-specific amplification, the **1070 bp** internal control should be clearly visible. In most cases where there is allele-specific amplification the internal control is weaker or completely disappears! For improper results see troubleshooting (6.).

No band should be visible in the **contamination control**. If there is a contamination with genomic DNA there will be a band at 282 bp. Additional bands may occur at 78 bp, 104 bp, 176 bp and around 580 bp. If there is a contamination with amplicates bands will occur at 78 bp and/or 104 bp and/or 282 bp.

5. 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).

6. 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 blood |
| | wrong amplification parameters | optimize the amplification parameters (see 4.3) ☆ |
| 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 4.3) ☆ |
| evaluation shows more than 2 specificities | carry-over contamination (amplification products!) new allele | check typing mixtures (no DNA added) ensure exact working |
| 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 wrong electrophoresis buffer | lower the voltage use 0,5x TBE buffer |

☆ 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.

7. References

- Nishino, S. et al., 2000. Sleep Medicine Reviews **4**:75-99
- Mignot, E. et al., 2001. Am. J. Hum. Genet. **68**:686-699
- Overeem, S. et al. 2008. Sleep Medicine Reviews **12**:95-107
- Bodmer, J. et al., 1997. Tissue Antigens **49**:297-321
- Olerup, O., Zetterquist H., 1992. Tissue Antigens **39**:225-235
- Olerup, O., Zetterquist H., 1993. Tissue Antigens **41**:55-56
- Maniatis et al., 1989. Molecular Cloning: A Laboratory Manual.
New York: Cold Spring Harbour Laboratory
- Beutler, E. et al., 1990. BioTechniques **9**:166

8. Explanation of symbols used on Labelling

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

Instructions for Use in other languages see:

<http://www.bag-healthcare.com/en/Diagnostika/Downloads/>



BAG Health Care GmbH
Amtsgerichtsstraße 1-5
35423 Lich/Germany
Tel.: +49 (0) 6404 / 925-0
Fax: +49 (0) 6404 / 925-250
www.bag-healthcare.com
info@bag-healthcare.com

Auftragsannahme/Ordering:
Tel.: +49 (0) 6404 / 925-450
Fax: +49 (0) 6404 / 925-460
verkauf@bag-healthcare.com

Customer Service:
Tel.: +49 (0) 6404 / 925-125
Fax: +49 (0) 6404 / 925-421
service@bag-healthcare.com