

# Lymphotype HLA

For the detection of human HLA antigens

## Package size

REF <sup>(4)</sup>	823 020	HLA-ABC 72
REF <sup>(4)</sup>	823 023	HLA-AB 72 AN
REF <sup>(4)</sup>	823 028	HLA-AB 72 oriental
REF <sup>(4)</sup>	823 030	HLA-ABC 120
REF <sup>(4)</sup>	823 031	HLA-AB 120 special
REF <sup>(4)</sup>	823 045	HLA-ABC 144 Italia
REF <sup>(4)</sup>	823 070	HLA-AB 144
REF <sup>(4)</sup>	823 100	HLA-DR/DQ 60
REF <sup>(4)</sup>	823 102	HLA-DR/DQ 72
REF <sup>(4)</sup>	823 110	HLA-B27 special
REF <sup>(4)</sup>	823 111	HLA-B27 common
REF <sup>(4)</sup>	823 115	HLA-B5

MP	Microplate
COMP	Complement
IVD	For In Vitro Diagnostic Use

## Intended Use

Biotest Lymphotype HLA is used for the detection of human HLA antigens in a complement-dependent microlymphocytotoxicity test (1,2,3,4,6,9). Biotest Lymphotype HLA consists of ready-to-use microplates containing pre-loaded anti-HLA reagents. The anti-HLA reagents can be monoclonal HLA antibodies or human polyclonal HLA antisera (5). Lymphotype HLA-B27 is used to detect HLA-B27 antigen. Since there is found to be a strong association worldwide between HLA-B27 and ankylosing spondylitis, it is one of the tools used to diagnose this disease (7). Lymphotype HLA-B5 is used to detect HLA-B5 antigens.

The order and specificity of the pre-loaded anti-HLA reagents can be obtained from the worksheet enclosed with every kit. Antibodies with identical specificity but different ID numbers are from different donors and serve the purpose of providing greater confidence in the results.

## Summary and Explanation

HLA antigens are glycoproteins present on the cell membrane. They are divided into two classes. Class I antigens (HLA-ABC) can be found on almost all nucleated cells. Class II antigens (HLA-DR/DQ) are to be found on the surface of only a few cell populations. All circulating lymphocytes in the peripheral blood contain HLA-ABC antigens. B-lymphocytes possess DR/DQ antigens in addition. For HLA-ABC typing, either a whole lymphocyte population can be used or a T-lymphocyte population. For DR/DQ typing, a sample rich in B-lymphocytes must be produced, as these account for only about 10 - 15 % of the whole lymphocyte population.

## Principle of the Microlymphocytotoxicity Test

For the determination of HLA antigens, HLA antibodies with known specificity must be incubated with a lymphocyte suspension of the samples in the presence of complement.

After the addition of lymphocytes to Lymphotype HLA, the lymphocytes will be lysed in the presence of the corresponding antibody and complement. This is made visible using a stain (e.g. eosin). The assessment of lysed and non-lysed lymphocytes is carried out using an inverse phase contrast microscope.

## Reagent Description

Biotest Lymphotype HLA contains human, polyclonal HLA antisera or monoclonal HLA antibodies of stated specificity for the determination of HLA class I or HLA class II antigens. The monoclonal antibodies can be of murine or human origin. Each well contains 1 µl of anti-HLA reagent and 6 - 8 µl of covering oil to prevent evaporation. Every Lymphotype HLA-ABC contains a negative and a positive control. Every Lymphotype HLA-DR/DQ contains in addition an anti-B-lymphocyte and an anti-T-lymphocyte control. The arrangement of the individual anti-HLA reagents can be found in the accompanying worksheet. For macroscopic checking purposes and for controlling of pH value the anti-HLA reagents are coloured with phenol red. 0.095% sodium azide is added to each anti-HLA reagent as a preservative.

## Statement of Precautions

The test must be performed by well-trained and authorised laboratory technicians.

All materials of human origin used in this product have been tested and found to be non-reactive for HBsAg, anti-HCV and anti-HIV-1/-2. However, all products of human origin should be considered to be potential transmitters of hepatitis, HIV or other infectious agents. Appropriate safety measures are recommended.

All used microplates [MP] should be treated as potentially infectious and should be destroyed according to the valid national guidelines.

## Storage

The microplates [MP] and the lyophilised complement [COMP] supplied are stable up to the date stated on their individual labels.

Lymphotype HLA must be stored at -20 °C or below and used before the expiration date. The package supplied in dry ice must be transferred **immediately** after receipt to storage at -20 °C or below. Opened Lymphotype HLA packages must not be stored together with dry ice.

Thawed microplates [MP] should be used for testing within 60 minutes. **Do not refreeze already thawed plates!**

## Indications of deteriorations

A yellow colouration of the anti-HLA reagents which still remains after thawing may indicate a change of the pH value. Those microplates [MP] should **not** be used for the test.

## Specimen Collection and Preparation

### Isolation of the lymphocytes (density gradient centrifugation)

- Mix approx. 5 ml (10 - 20 ml for HLA-DR/DQ typing) of a fresh, heparinised blood sample (approx. 10 units of heparin per ml of blood) with the same volume of Hanks Solution.  
It is possible to store the blood sample at room temperature (20...24 °C) for up to 24 hours.
- Place a quantity of Lymphoflot (20...24°C) into a centrifuge tube and cover it with an equal quantity of diluted blood such that blood and Lymphoflot do not mix.
- Centrifuge for 20 minutes at 1000 x g without braking. The lymphocytes will settle as a white ring at the boundary between the plasma and the Lymphoflot. Using a pipette, carefully transfer the ring to a fresh centrifuge tube, fill the tube with Hanks Solution and mix.
- Centrifuge for 10 minutes at 230 x g, pour off the supernatant, resuspend the sediment, fill the tube with Hanks Solution and mix.
- Centrifuge again for 10 minutes at 110 x g. Pour off the supernatant.
- For HLA-ABC typing, resuspend the lymphocyte sediment in Lymphostabil. The lymphocyte suspension should contain approximately 2000 - 3000 lymphocytes/µl.

**Note:** The lymphocytes can also be isolated using immunomagnetic beads. A description of the lymphocyte separation method and the differing incubation times can be found in the Beads package insert.

For performing the HLA-ABC typing test, continue at Section "Microlymphocytotoxicity test". For performing the HLA-DR/DQ typing test, continue at Section "Separation in T- and B-lymphocytes".

## Separation in T- and B-lymphocytes

- Loosely fill a 2 ml disposable syringe or similar with approx. 0.15 g of nylon wool (= wool column). Wash out the column using approx. 20 ml of Hanks Solution and approx. 10 ml of medium (medium = 10 % (V/V) FCS in Lymphostabil), in both cases warmed to 37°C.
- Seal the wool column at both ends using Parafilm and incubate for 30 min. at 37 °C in an incubator or water bath. Prepared in this way, wool columns can be stored at -20°C or less until they are needed. After thawing and washing with medium at 37°C, the wool columns are ready for use again.
- Resuspend the lymphocyte sediment from step 5 of specimen preparation in 1 ml of medium warmed to 37°C and place onto the top of the wool column. Seal the wool column with Parafilm and incubate for 30 minutes at 37 °C.
- Place a cannula on the wool column to reduce the flow rate.
- Wash out the T-lymphocytes, which do not adhere, using 10 - 20 ml of Hanks Solution warmed to 37°C and collect them in a suitably labelled centrifuge tube.
- After removing the cannula, wash out the B-lymphocytes into a second centrifuge tube using approx. 2 ml of medium warmed to 37°C. Using tweezers, carefully squeeze out the nylon wool and loosen it again. Repeat this procedure 4 - 5 times.
- Centrifuge the T- and B-lymphocytes for 10 minutes at 230 x g. Pour off the supernatant.
- Resuspend the T- and B-lymphocytes using Lymphostabil and adjust to a concentration of 2000 - 3000 lymphocytes/µl.

Use the tube containing the T-lymphocytes for HLA-ABC typing and the tube containing the B-lymphocytes for HLA-DR/DQ typing.

## Procedure

### Materials Provided

- Each Lymphotype HLA consists of 6 microplates [MP] and two bottles of complement [COMP].
  - Lymphotype HLA-ABC 72 (6 microplates [MP] with 72 anti-HLA reagents for 6 HLA-ABC typing tests)
  - Lymphotype HLA-AB 72 (6 microplates [MP] with 72 anti-HLA reagents for 6 HLA-AB typing tests)
  - Lymphotype HLA-ABC 120 or 144 (3 x 2 microplates [MP] with 120 or 144 anti-HLA reagents for 3 HLA-ABC typing tests)
  - Lymphotype HLA-AB 120 or 144 (3 x 2 microplates [MP] with 120 or 144 anti-HLA reagents for 3 HLA-AB typing tests)
  - Lymphotype HLA-B27 common (6 microplates [MP] for 30 HLA-B27 determinations)
  - Lymphotype HLA-B27 special (6 microplates [MP] for 18 HLA-B27 determinations)
  - Lymphotype HLA-B5 (6 microplates [MP] for 36 HLA-B5 determinations)
  - Lymphotype HLA-DR/DQ 60 or 72 (6 microplates [MP] with 60 or 72 anti-HLA reagents for 6 HLA-DR/DQ typing tests)
- 2 x 2 ml lyophilized complement [COMP] (rabbit) in each Lymphotype HLA package, non-toxic to human lymphocytes
- Worksheets for each typing (enclosed)
- 1 reaction pattern chart (enclosed)

### Additional materials required

1. Hanks Solution (free from Ca<sup>++</sup> and Mg<sup>++</sup>)
2. Lymphoflot (density gradient for the isolation of lymphocytes, density 1.077 g/ml)
3. Lymphostabil (McCoy's Medium 5A, modified according to Park and Terasaki, free from Ca<sup>++</sup> and Mg<sup>++</sup>, for storage of lymphocytes)
4. Fetal calf serum (FCS), heat inactivated, e.g. Sigma
5. Nylon wool
6. Eosin Y (dissolved 5 % in distilled water and filtered), e.g. Merck
7. Formaldehyde for histology, 37 %, acid-free (filtered and adjusted to pH 7.2 ± 0.2 with 0.1 N sodium hydroxide solution), e.g., Merck
8. 2 ml disposable syringe or similar
9. Covering oil
10. Cover slides, 50 x 75 mm
11. Microlitre syringes, e.g., Hamilton (0.05 ml syringe)
12. Volume dispenser, e.g., Hamilton No. PB-600-1
13. Terasaki dispenser, e.g., Hamilton (6x 0.250 ml syringe)
14. Microdispenser for microtest plates, e.g. Greiner
15. Inverse phase contrast microscope

### Microlymphocytotoxicity Test Procedure

1. Thaw the microplates [MP] at room temperature (20...24°C) immediately before use. Do not allow to stand for longer than 60 minutes at room temperature. DO NOT REFREEZE!
2. Carefully add 1 µl of the adjusted lymphocyte suspension to the HLA reagent into each well just under the oil (antigen-antibody reaction) and incubate for  
HLA-ABC typing 30 minutes and  
HLA-DR/DQ 60 minutes  
at room temperature (20...24 °C).
3. 15 minutes before completion of the incubation time, reconstitute the complement [COMP] enclosed with the kit through gentle swirling using the quantity of water stated on the label. DO NOT REFREEZE! DISCARD ANY RESIDUAL COMPLEMENT!
4. Add 5 - 6 µl of complement [COMP] to each well (lysis of the lymphocytes) and incubate for  
HLA-ABC typing 60 minutes and  
HLA-DR/DQ 120 minutes  
at room temperature (20...24 °C).
5. Add 3 - 4 µl of 5% Eosin to each well. Incubate for approx. 3 minutes.
6. Fix the reaction by adding 6 - 7 µl of buffered formaldehyde to each well.
7. If necessary, oil should be added to the microplates [MP].
8. Cover the completed microplates [MP] with a cover slip and read the results at least 30 minutes after completion of the test. Since lysed lymphocytes sediment more slowly than viable lymphocytes we recommend to reread the microplates [MP] after 12-24 hours.  
If stored at 2 ...8 °C, the test result can still be assessed for up to 3 days afterwards. Read the tray in the following serpentine pattern which corresponds to the worksheet. 1A through 1F; 2F through 2A; 3A through 3F; etc.

### Quality control

**Negative control:** The negative control is used to test the viability of the lymphocyte suspension used. Other reactions in the tray are scored by evaluating the viability compared to this negative control, which should be negative. If the negative control is weakly positive, it must be taken into account during evaluation.

**Positive control:** The positive control is used to check the complement activity. The positive control should produce 81 - 100 % lysed lymphocytes.

**Anti-B-lymphocyte control (only in Lymphotype HLA-DR/DQ):** The anti-B-lymphocyte control is in well 1C. This control is used to check the purity of the B-lymphocyte suspension.

**Anti-T-lymphocyte control (only in Lymphotype HLA-DR/DQ):** The anti-T-lymphocyte control is in well 1D. This control is used to check the separation of the isolated T- and B-lymphocytes.

The anti-B-lymphocyte control should produce at least 81-100 % lysed lymphocytes and the anti-T-lymphocyte control should produce less than 20 % lysed cells. If these values are not achieved, the separation of T- and B-lymphocytes was incomplete. The test should be repeated.

### Results

The reactions are read under an inverse phase contrast microscope. Vital lymphocytes appear bright and luminous (= negative reaction), lysed lymphocytes appear somewhat larger and stained dark (= positive reaction). The number of lysed lymphocytes compared with the total number of lymphocytes is quoted as a score value in each well.

% lysed cells		Evaluation
0 - 10 %	=	Score 1 negative
11 - 20 %	=	Score 2 doubtful negative
21 - 50 %	=	Score 4 weak positive
51 - 80 %	=	Score 6 positive
81 - 100 %	=	Score 8 strong positive
	=	Score 0 not readable

Following recording of the results, identify the antigens which are present on the lymphocyte preparation being tested. Positive reactions occur where antigens correspond to antibody present in the antisera.

### Limitations

#### Causes of false negative or weak reactions:

- Erythrocyte contamination can make microscopic evaluation difficult because of visual confusion with negative lymphocytes.
- Platelet contamination can deplete antibody and complement.
- Lymphocyte count is too high. Cell concentration is important since the test is standardized using a certain antigen-antibody-ratio.
- Yellow colour of the anti-HLA reagents is an indication of bacterial contamination or change of pH value (dry ice!).
- Opened packages or individual plates have been stored together with dry ice.
- Microplates [MP] have been thawed and refrozen.
- Reconstituted complement [COMP] kept too long at room temperature (20...24 °C) before use.
- Residual complement [COMP] was frozen and thawed.
- Incubation times were reduced.
- Incubation temperature was too low. The microlymphocytotoxicity test is temperature dependent.

#### Causes of false positive reactions:

- Because of similar molecular structures, HLA antigens can react with anti-HLA reagents of other specificities (cross-reaction).
- Incubation temperature was too high.
- Incubation times were too long.
- Prior damage to the lymphocytes = negative control is positive.
- Failure to add fixative.

### Expected Values

The phenotype frequencies for HLA Class I and Class II will vary among different populations (8).

### Specific Performance Characteristics

#### Specificity and reaction strength of the anti-HLA reagents

All anti-HLA reagents selected for Biotest Lymphotype HLA have a defined specificity. They have been tested for specificity and reaction strength against several panels of freshly isolated or frozen HLA-typed lymphocytes.

The selected anti-HLA reagents have shown positive reactions (Score 4-8) with lymphocytes which have been demonstrated to carry the relevant antigen. Multispecific antibodies are used only if no monospecific ones are available for a particular specificity.

The selected anti-HLA reagents have shown negative reactions (Score 1-2) with lymphocytes which have been demonstrated not to carry the relevant antigen.

Every batch of Lymphotype HLA is tested with an adequate number of well-defined lymphocyte suspensions. The proportion of false positive or false negative reactions must not be greater than 10% in relation to all possible reactions. Typing tests performed using Lymphotype HLA provided unambiguous results.

The **negative control** consists of serum of a healthy male donor with blood group AB and showed no cytotoxic reactions in the microlymphocytotoxicity test with randomly selected donor lymphocytes.

The **positive control** is an anti-lymphocyte serum from rabbit which reacts cytotoxicity with all human lymphocytes.

The **anti-B-lymphocyte control** is a monoclonal antibody (clone Tü 35, isotype IgG2a) which reacts cytotoxicity with B-lymphocytes. It does not react with T-lymphocytes, granulocytes, platelets, monocytes or erythrocytes.

The **anti-T-lymphocyte control** is a monoclonal antibody (clone 12F6, isotype IgG2a) which reacts cytotoxicity with T-lymphocytes. It is directed against the pan-T-cell antigen CD3 which is expressed exclusively on all T-lymphocytes and not on B-lymphocytes, granulocytes, macrophages or erythrocytes.

### Literatur

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