

Lymphotype HLA (Reagent Block)

Package Size

REF	823 021	Lymphotype HLA-ABC 72 (Reagent Block)
REF	823 024	Lymphotype HLA-AB 72 AN (Reagent Block)
REF	823 033	Lymphotype HLA-ABC 120 (Reagent Block)
REF	823 037	Lymphotype HLA-AB 120 special (Reagent Block)
REF	823 046	Lymphotype HLA-ABC 144 Italia (Reagent Block)
REF	823 071	Lymphotype HLA-AB 144 (Reagent Block)
REF	823 101	Lymphotype HLA-DR/DQ 60 (Reagent Block)
REF	823 103	Lymphotype HLA-DR/DQ 72 (Reagent Block)

Intended Use

Lymphotype HLA (Reagent Block) consists of pre-loaded anti-HLA reagents in bulk blocks. The anti-HLA reagents can be monoclonal HLA antibodies or human polyclonal HLA antisera. Lymphotype HLA (Reagent Block) is made for final production of trays used for the detection of human HLA antigens in a complement-dependent microlymphocytotoxicity test.

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 the lymphocytes of a sample to the typing tray, 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

Lymphotype HLA (Reagent Block) 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 approx. 200 µl of anti-HLA reagent. Every Lymphotype HLA-ABC (Reagent Block) contains a negative and a positive control. Every Lymphotype HLA-DR/DQ (Reagent block) 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. Antibodies with identical specificity but different ID numbers are from different donors and serve the purpose of providing greater confidence in the results.

For filling level control 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

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 using FDA licensed test kits. 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.

Storage

Lymphotype HLA (Reagent Block) and the separately delivered, lyophilised complement supplied are stable up to the date stated on their individual labels.

Lymphotype HLA (Reagent Block) 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. Open Lymphotype HLA (Reagent Block) packages must not be stored together with dry ice.

The blocks have been taken out of the aluminium pouch immediately after taking them out of the -20°C freezer. The condensation water which will appear immediately on the connections between the wells has to be removed carefully using a pulp swab before the HLA reagents are thawed.

Thawed Lymphotype HLA (Reagent Block) should be stored at 2...8°C and should be used within 8 hours. **Do not refreeze remaining HLA reagents!**

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 reagents should **not** be used for the production of typing trays.

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. For a description of the lymphocyte separation method and the differing incubation times please refer to the conditions mentioned 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

- Lymphotype HLA ABC -72 (Reagent Block)-
+ 50 x 2 ml complement
(1 reagent block with 72 anti-HLA reagents for HLA-ABC typings)

Lymphotype HLA -AB 72 (Reagent Block)
+ 50 x 2 ml complement
(1 reagent block with 72 anti-HLA reagents for HLA-AB typings)

Lymphotype HLA -ABC 120 or 144 (Reagent Block)
+ 100 x 2 ml complement
(1 x 2 reagent blocks with 120 or 144 anti-HLA reagents for HLA-ABC typings)

Lymphotype HLA -AB 120 or 144 (Reagent Block)
+ 100 x 2 ml complement
(1 x 2 reagent blocks with 120 or 144 anti-HLA reagents for HLA-AB typings)

Lymphotype HLA -DR/DQ 60 or 72 (Reagent Block)
+ 50 x 2 ml complement
(1 reagent block with 60 or 72 anti-HLA reagents for HLA-DR/DQ typings)

- To each Lymphotype HLA (Reagent Block) package will be delivered the proper quantity of rabbit complement. (á 2 ml, lyophilized, non-toxic to human lymphocytes)
- Worksheets (enclosed)
- 1 reaction pattern chart (enclosed)

Additional materials required

- Microtest plates with 60 or 72 wells for HLA typing (Biotest art.no. 830 031/ 830 032)
- Hanks Solution (free from Ca⁺⁺ and Mg⁺⁺) (Biotest art.no. 824 035)
- Lymphoflot (density gradient for the isolation of lymphocytes, density 1.077 g/ml) (Biotest art.no. 824 012)
- Lymphostabil (McCoy's Medium 5A, modified according to Park and Terasaki, free from Ca⁺⁺ and Mg⁺⁺, for storage of lymphocytes) (Biotest art.no. 824 020)
- Fetal calf serum (FCS), heat inactivated, e.g. Sigma
- Nylon wool
- Eosin Y (dissolved 5 % in distilled water and filtered), e.g. Merck
- 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
- 2 ml disposable syringe or similar
- Covering oil (Biotest art.no. 824 040)
- Cover slides, 50 x 75 mm (Biotest art.no. 830 033)

12. Microlitre syringes, e.g., Hamilton (0.05 ml syringe)
13. Volume dispenser, e.g., Hamilton No. PB-600-1
14. Terasaki dispenser, e.g., Hamilton (6x 0.250 ml syringe)
15. Microdispenser for microtest plates, e.g. Greiner
16. Inverse phase contrast microscope

Production of Typing Plates

1. Fill the wells of an empty microtest plate with approx. 7 µl of cover oil.
2. Thaw Lymphotype HLA (Reagent Block) at room temperature (20...24°C) immediately before use.
Do not refreeze any residual anti-HLA reagents!
Mix the anti-HLA reagents in each well before pipetting.
3. Fill each 1 µl anti-HLA reagent from the Lymphotype HLA (Reagent Block) in the corresponding positions of the microtest plate under the oil. This procedure should be done within 8 hours. The anti-HLA reagents should be placed in the middle of each well.
4. Produced typing plates have to be stored at -20°C or below.

Microlymphocytotoxicity Test Procedure

1. Thaw the typing plates 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 form 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 through gentle swirling using the quantity of distilled water stated on the label. **Do not refreeze! Discard any residual complement!**
4. Add 5 - 6 µl of complement 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 microtest plates.
8. Cover the completed microtest plate with a cover slip and read the results at least 30 minutes after completion of the test. Since lysed lymphocytes sediment more slowly than vital lymphocytes we recommend to reread 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 (Reagent Block)): 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 (Reagent Block)): 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.
- Lymphotype HLA (Reagent Block) has been thawed and refrozen.

- Lymphotype HLA (Reagent Block) has been stored too long at room temperature.
- Microtest plates have been thawed and refrozen.
- Reconstituted complement kept too long at room temperature (20...24 C) before use.
- Residual complement 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.

Specific Performance Characteristics

Specificity and reaction strength of the anti-HLA reagents

All anti-HLA reagents selected for Lymphotype HLA (Reagent Block) 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.

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 equine anti-lymphocyte serum 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.