Instruction Manual

Mycobacterium tuberculosis
IgG ELISA sensitive

Enzyme immunoassay based on microtiter plate for the detection and quantitative determination of human IgG antibodies against Mycobacterium tuberculosis in serum and plasma

Cat. No.: ILE-TUS01
Storage: 2-8°C
For in-vitro diagnostic use only

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**Contents**

1. Intended Use ........................................... 3
2. General Information ................................. 3
3. Principle of the Tests ................................. 3
4. Limitations, Precautions and General Comments 4
5. Reagents Provided .................................... 4
6. Materials Required but not Provided .......... 6
7. Specimen Collection and Handling ........... 6
8. Assay Procedure ...................................... 6
9. Evaluation ............................................. 7
10. Assay Characteristics .................. ........... 9
11. References ............................................ 9
12. Short Instruction ................................. 10

**Symbole und Übersetzungen / Symbols and Translations**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>English</th>
<th>French</th>
<th>German</th>
<th>Italian</th>
<th>Spanish</th>
<th>Greek</th>
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<td>CAL</td>
<td>Calibrator</td>
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<td>CONJ</td>
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<td>Coniugato</td>
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<td>Concentré</td>
<td>Konzentrat</td>
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<td></td>
<td>(&lt;n&gt;-fold)</td>
<td>(&lt;n&gt; fois)</td>
<td>(&lt;n&gt;-fach)</td>
<td>(&lt;n&gt;-volte)</td>
<td>(&lt;n&gt;-veces)</td>
<td>η (&lt;n&gt; φορές)</td>
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<tr>
<td>SAMP</td>
<td>Sample</td>
<td>Diluant</td>
<td>Probenverdünner</td>
<td>Diluente del</td>
<td>Diluyente de</td>
<td>Αραίωσης</td>
</tr>
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<td>DIL</td>
<td>Diluent</td>
<td>échantillon</td>
<td>verdünnner</td>
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<td>Αραίωσης</td>
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<td>STOP</td>
<td>Stop</td>
<td>Solution</td>
<td>Stopp-Lösung</td>
<td>Soluzione d’arresto</td>
<td>Solución de</td>
<td>Αναστολής</td>
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<td>SUBS</td>
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<td>Sustrato</td>
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<td></td>
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<td></td>
<td>ματος</td>
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<tr>
<td>MT PLATE</td>
<td>Microtiter</td>
<td>Microplaque</td>
<td>Mikrötiterplatte</td>
<td>Piastre</td>
<td>Placa microtiter</td>
<td>Μικρόπλακα</td>
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<tr>
<td>WASH BUF</td>
<td>Wash buffer</td>
<td>Tampon de lavage</td>
<td>Waschpuffer</td>
<td>Soluzione di lavaggio</td>
<td>Tampón de lavado</td>
<td>Πλυστικό Διάλυμα</td>
</tr>
</tbody>
</table>
1. Intended Use

The IMMUNOLAB Mycobacterium tuberculosis IgG antibody ELISA kit has been designed for the detection and the quantitative determination of specific IgG antibodies against Mycobacterium tuberculosis in serum and plasma. Further applications in other body fluids are possible and can be requested from the Technical Service of IMMUNOLAB.

Laboratory results can never be the only base of a medical report. The patient history and further tests have additionally to be taken into account.

2. General Information

Mycobacterioses (tuberculosis, leprosy, atypical mycobacterioses, paratuberculosis, and perhaps Crohn’s Disease) are the infectious diseases of men and animals with the largest diffusion on earth. The infectious agents of tuberculosis are acid-resistant rod-like formed bacteria of the family Mycobacteriaceae, genus Mycobacterium. The germ was detected by Robert Koch in 1882. Owing to the very high infectious power of pathogenic mycobacteria, early diagnosis is essential to prevent spreading of the disease. Convergence of various approaches are necessary to control the mycobacterioses, immune reactions and bacterial shedding being variable during the diseases. However, usual diagnostic procedures were up to now unsatisfactory and did not allow to distinguish among different mycobacterial species. The illness is normally transferred by droplets of saliva from infected persons. The target of the infection are mostly the lungs, but also other organs like the brain, intestinal tract, bones, lymph nodes and kidneys can be afflicted. Tuberculosis is not only found in developing countries with 8 million of new infections yearly, but also in industrialized civilizations, as an actual disease with some thousands of cases yearly. Without treatment, the disease leads in 50% of the cases to death within less than two years. Clinical symptoms are fatigue, loss of weight, lack of appetite, light fever, nocturnal sweat and pain in the chest. Especially patients with HIV are threatened by tuberculosis due to their impaired immune system. A vaccination with living attenuated bacteria is possible (BCG = Bacille Calmette Guérin). This is mostly done with newborn or young children. With older patients, before the vaccination there is normally performed the tuberculin test (Pirquet or Mantoux), where a small amount of tuberculin is injected under the skin. In a positive case, there exist antibodies against Mycobacteria, and a vaccination is not necessary. Up to recently, there have not existed any serological methods to detect tuberculosis antibodies in serum. The only available procedure was besides the skin tuberculin test the direct microscopical identification of the dyed bacteria in sputum. Meanwhile specific antigens have been prepared either by purification of natural material or by recombinant methods. This ELISA test kit for the determination of IgG antibodies uses purified PPD proteins in order to determine an immune response against the bacteria in human serum. A fresh or chronically active infection can be diagnosed by IgA and IgM tests, which are also available.

3. Principle of the Tests

The IMMUNOLAB Mycobacterium tuberculosis IgG antibody test kit is based on the principle of the enzyme immunoassay (EIA). Mycobacterium tuberculosis antigen is bound on the surface of the microtiter strips. Diluted patient serum or ready-to-use standards are pipetted into the wells of the microtiter plate. A binding between the IgG antibodies of the serum and the immobilized Mycobacterium tuberculosis antigen takes place. After a one hour incubation at room temperature, the plate is rinsed with diluted wash solution, in order to remove unbound material. Then ready-to-use anti-human-IgG peroxidase conjugate is added and incubated for 30 minutes. After a further washing step, the substrate (TMB) solution is pipetted and incubated for 20 minutes, inducing the development of a blue dye in the wells. The color development is terminated by the addition of a stop solution, which changes the color from blue to yellow. The resulting dye is measured spectrophotometrically at the wavelength of 450 nm. The concentration of IgG antibodies is directly proportional to the intensity of the color.
4. Limitations, Precautions and General Comments

- Only for in-vitro use! Do not ingest or swallow! The usual laboratory safety precautions as well as the prohibition of eating, drinking and smoking in the lab have to be followed.
- All sera and plasma or buffers based upon, have been tested respective to HBsAg, HIV and HCV with recognized methods and were found negative. Nevertheless precautions like the use of latex gloves have to be taken.
- Serum and reagent spills have to be wiped off with a disinfecting solution (e.g. sodium hypochlorite, 5%) and have to be disposed of properly.
- All reagents have to be brought to room temperature (18 to 25 °C) before performing the test.
- Before pipetting all reagents should be mixed thoroughly by gentle tilting or swinging. Vigorous shaking with formation of foam should be avoided.
- It is important to pipet with constant intervals, so that all the wells of the microtiter plate have the same conditions.
- When removing reagents out of the bottles, care has to be taken that the stoppers are not contaminated. Further a possible mix-up has to be avoided. The content of the bottles is usually sensitive to oxidation, so that they should be opened only for a short time.
- In order to avoid a carry-over or a cross-contamination, separate disposable pipet tips have to be used.
- All reagents have to be used within the expiry period.
- In accordance with a Good Laboratory Practice (GLP) or following ISO9001 all laboratory devices employed should be regularly checked regarding the accuracy and precision. This refers amongst others to microliter pipets and washing or reading (ELISA-Reader) instrumentation.
- The contact of certain reagents, above all the stopping solution and the substrate with skin, eye and mucosa has to be avoided, because possible irritations and acid burns could arise, and there exists a danger of intoxication.

5. Reagents Provided

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume / Qty.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobacterium tuberculosis antigen coated microtiter strips</td>
<td>12</td>
</tr>
<tr>
<td>Calibrator A (Negative Control)</td>
<td>2 mL</td>
</tr>
<tr>
<td>Calibrator B (Cut-Off Standard)</td>
<td>2 mL</td>
</tr>
<tr>
<td>Calibrator C (Weak Positive Control)</td>
<td>2 mL</td>
</tr>
<tr>
<td>Calibrator D (Positive Control)</td>
<td>2 mL</td>
</tr>
<tr>
<td>Enzyme Conjugate</td>
<td>15 mL</td>
</tr>
<tr>
<td>Substrate</td>
<td>15 mL</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>15 mL</td>
</tr>
<tr>
<td>Sample Diluent</td>
<td>60 mL</td>
</tr>
<tr>
<td>Washing Buffer (10×)</td>
<td>60 mL</td>
</tr>
<tr>
<td>Plastic foils</td>
<td>2</td>
</tr>
<tr>
<td>Plastic bag</td>
<td>1</td>
</tr>
</tbody>
</table>

Storage and Stability (refer to the expiry date on the outer box label)
Store kit components at 2-8°C. After use, the plate should be resealed, the bottle caps replaced and tightened and the kit stored at 2-8°C. The opened kit should be used within three months.
Universal Reagents
Washing buffer, substrate and stop solution are identical for all infectious disease test kits from IMMUNOLAB with Peroxidase as detecting enzyme and may be interchanged between products and lots. All other reagents are assigned to a special kit lot and must not be mixed.

5.1. Mikrotiter Strips
12 strips with 8 breakable wells each, coated with M. tuberculosis antigen (purified PPD extract of Mycobacterium tuberculosis). Ready-to-use.

5.2. Calibrator A (Negative Control)
2 mL, protein solution diluted with PBS, contains no IgG antibodies against Mycobacterium tuberculosis. Addition of 0.01 % methylisothiazolone and 0.01 % bromonitrodioxane. Ready-to-use.

5.3. Calibrator B (Cut-Off Standard)
2 mL human serum diluted with PBS, contains a low concentration of IgG antibodies against Mycobacterium tuberculosis. Addition of 0.01 % methylisothiazolone and 0.01 % bromonitrodioxane. Ready-to-use.

5.4. Calibrator C (Weak Positive Control)
2 mL, human serum diluted with PBS, contains a medium concentration of IgG antibodies against Mycobacterium tuberculosis. Addition of 0.01 % methylisothiazolone and 0.01 % bromonitrodioxane. Ready-to-use.

5.5. Calibrator D (Positive Control)
2 mL, human serum diluted with PBS, contains a high concentration of IgG antibodies against Mycobacterium tuberculosis. Addition of 0.01 % methylisothiazolone and 0.01 % bromonitrodioxane. Ready-to-use.

5.6. Enzyme Conjugate
15 mL, anti-human-IgG-HRP (rabbit), in protein-containing buffer solution. Addition of 0.01 % methylisothiazolone and 0.01 % bromonitrodioxane and 5 mg/L Proclin™. Ready-to-use.

5.7. Substrate
15 mL, TMB (tetramethylbenzidine). Ready-to-use.

5.8. Stop Solution
15 mL, 0.5 M sulfuric acid. Ready-to-use.

5.9. Sample Diluent
60 mL, PBS/BSA buffer. Addition of 0.095 % sodium azide. Ready-to-use.

5.10. Washing Buffer
60 mL, PBS + Tween 20, 10x concentrate. Final concentration: dilute 1+9 with deionized water. If during the cold storage crystals precipitate, the concentrate should be warmed up at 37°C for 15 minutes.

5.11. Plastic Foils
2 pieces to cover the microtiter strips during the incubation.

5.12. Plastic Bag
Resealable, for the dry storage of non-used strips.
6. Materials Required but not Provided

- 5 µL-, 100 µL- and 500 µL micro- and multichannel pipets
- Microtiter Plate Reader (450 nm)
- Microtiter Plate Washer
- Reagent tubes for the serum dilution
- Deionized water

7. Specimen Collection and Handling

Principally serum or plasma (EDTA, heparin) can be used for the determination. Serum is separated from the blood, which is aseptically drawn by venipuncture, after clotting and centrifugation. The serum or plasma samples can be stored refrigerated (2-8°C) for up to 7 days. For a longer storage they should be kept at -20°C. The samples should not be frozen and thawed repeatedly. Lipemic, hemolytic or bacterially contaminated samples can cause false positive or false negative results.

For the performance of the test the samples (not the standards) have to be diluted 1:101 with ready-to-use sample diluent (e.g. 5 µL serum + 500 µL sample diluent).

8. Assay Procedure

8.1. Preparation of Reagents

Washing Solution: dilute before use 1+9 with deionized water. If during the cold storage crystals precipitate, the concentrate should be warmed up at 37°C for 15 minutes.

- Strict adherence to the protocol is advised for reliable performance. Any changes or modifications are the responsibility of the user.
- All reagents and samples must be brought to room temperature before use, but should not be left at this temperature longer than necessary.
- A standard curve should be established with each assay.
- Return the unused microtiter strips to the plastic bag and store them dry at 2-8°C.

8.2. Assay Steps

1. Prepare a sufficient amount of microtiter wells for the standards, controls and samples as well as for a substrate blank.
2. Pipet 100 µL each of the diluted (1:101) samples and the ready-to-use standards and controls respectively into the wells. Leave one well empty for the substrate blank.
3. Cover plate with the enclosed foil and incubate at room temperature for 60 minutes.
4. Empty the wells of the plate (dump or aspirate) and add 300 µL of diluted washing solution. This procedure is repeated totally three times. Rests of the washing buffer are afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.
5. Pipet 100 µL each of ready-to-use conjugate into the wells. Leave one well empty for the substrate blank.
6. Cover plate with the enclosed foil and incubate at room temperature for 30 minutes.
7. Empty the wells of the plate (dump or aspirate) and add 300 µL of diluted washing solution. This procedure is repeated totally three times. Rests of the washing buffer are afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.
8. Pipet 100 µL each of the ready-to-use substrate into the wells. This time also the substrate blank is pipetted.
9. Cover plate with the enclosed foil and incubate at room temperature for 20 minutes in the dark (e.g. drawer).
10. To terminate the substrate reaction, pipet 100 µL each of the ready-to-use stop solution into the wells. Pipet also the substrate blank.
11. After thorough mixing and wiping the bottom of the plate, perform the reading of the absorption at 450 nm (optionally reference wavelength of 620 nm). The color is stable for at least 60 minutes.

9. Evaluation
The mean values for the measured absorptions are calculated after subtraction of the substrate blank value. The difference between the single values should not exceed 10 %.

Example:

The above diagram contains only an example, which was achieved under arbitrary temperature and environmental conditions. The described data constitute consequently no reference values which have to be found in other laboratories in the same way.

9.1. Qualitative Evaluation
The calculated absorptions for the patient sera, as mentioned above, are compared with the value for the cut-off standard. If the value of the sample is higher, there is a positive result. For a value below the cut-off standard, there is a negative result. It seems reasonable to define a range of +/- 20 % around the value of the cut-off as a grey zone. In such a case the repetition of the test with the same serum or with a new sample of the same patient, taken after 2-4 weeks, is recommended. Both samples should be measured in parallel in the same run.

The positive control must show at least the double absorption compared with the cut-off standard.

9.2. Quantitative Evaluation
The ready-to-use standards and controls of the Mycobacterium tuberculosis IgG antibody kit are defined and expressed in arbitrary units (U/mL). This results in an exact and reproducible quantitative evaluation. Consequently for a given patient follow-up controls become possible. The values for controls and standards in units are printed on the labels of the vials.
For a quantitative evaluation the absorptions of the standards and controls are graphically drawn point-to-point against their concentrations. From the resulting reference curve the concentration values for each patient sample can then be extracted in relation to their absorptions. It is also possible to use automatic computer programs. As curve fit point-to-point has to be chosen.

Calibrator B with its concentration of 10 U/mL serves as cut-off standard. Analogous to the qualitative evaluation a range of +/-20% around the cut-off is defined as a grey zone. Thus results between 8 and 12 U/mL are reported as borderline. Consequently, quantitative results should be interpreted as follows:

**Interpretation of quantitative results:**

<table>
<thead>
<tr>
<th>Absorption Range</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 8 U/mL</td>
<td>negative</td>
</tr>
<tr>
<td>8-12 U/mL</td>
<td>borderline</td>
</tr>
<tr>
<td>12-40 U/mL</td>
<td>weakly positive</td>
</tr>
<tr>
<td>40-150 U/mL</td>
<td>positive</td>
</tr>
<tr>
<td>&gt; 150 U/mL</td>
<td>strongly positive</td>
</tr>
</tbody>
</table>

In case of borderline results it is recommended to repeat the test with the same serum or with a new sample of the same patient, taken after 2-4 weeks. Both samples should be measured in parallel in the same run.

Since the cut-off limit may be elevated in regions of high incidence it is recommended to adapt them by the laboratory/clinician, if required. In general, the cut-off limit can be adjusted in any region by testing a minimum of 40 non-infected healthy individuals.

### 9.3. Interpretation

Serological data provide information about the humoral response of a patient i.e. presence of specific antibodies. In contrast to the tuberculin test evaluating the cellular immune status of a patient, the antibody production depends most likely on the antigen load. Further, three immunoglobulin isotypes IgA, IgG and IgM can be detected providing important information about the infection status and disease history.

Although antibody responses may occur in healthy persons in rare cases, normally they indicate a colonization with Mycobacterium tuberculosis. Positive IgM results are related to the early stage of an infection. During its course a seroconversion towards IgG antibodies takes place. The simultaneous presence of IgG and IgM antibodies denote an infection at its early stage or a reactivation in chronic infections. The sole occurrence of IgG is a sign for a completed immunological response. IgA antibodies occur after the initial activation of the immunoreaction as indicated by the presence of IgM and are associated to a high inflammatory potential. IgA results can further increase specificity and sensitivity of the overall diagnosis. Since they are not as affected by anergy effects as IgG antibodies, they are a useful marker for patients which show a reduced IgG response due a pre-existing immune depression.

Briefly the humoral immune response can be summarized as follows:

- **IgM (-), IgG (-), IgA (-):** no infection
- **IgM (+), IgG (-), IgA (-):** infection at a very early stage, often found among children living under unhealthy conditions
- **IgM (-), IgG (+), IgA (+/-):** completed infection
- **IgM (+), IgG (+), IgA (+/-):** infection at an early stage or re-infection
- **IgM (-), IgG (-), IgA (+):** completed infection in patients suffering from IgG reducing effects

**Abnormal cases:**

- The absence of IgG and IgA antibodies in patients showing symptoms of a TB infection:
  - should not be considered as a false-negative result but can be caused by anergy at an advanced state of the infection.
- The detection of high IgG and IgA antibody titers in healthy individuals indicates:
  - contacts with infected individuals who need to be identified and monitored.
• The detection of IgM antibody titers in healthy individuals indicates:
  o the initial infection phase. The affected individuals need to be monitored in order to check if they develop clinical symptoms or
  o the application of a tuberculin skin test (IgM may occur 7 to 10 days after the test) or
  o a BCG vaccination which may induce the formation of IgM antibodies.
• A reduced antibody production is expected in children and immunocompromised patients (cancer, chemotherapy, HIV infection etc.). Indeed, the disease appears often in the presence of co-factors like cancer, HIV, malnutrition promoting the infection. The immunological weakness caused by these co-factors isenhanced by the immunological anergy caused by the bacteria itself.
• Infections with Nocardia and Corynebacterium which are closely related to Mycobacterium may cause ‘false-positive’ serological results. These infections can be identified by their clinical symptoms.

10. Assay Characteristics

<table>
<thead>
<tr>
<th>Mycobacterium sensitive ELISA</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-Assay-Precision</td>
<td>6.4 %</td>
<td>8.3 %</td>
<td>6.4 %</td>
</tr>
<tr>
<td>Inter-Assay-Precision</td>
<td>6.9 %</td>
<td>8.1 %</td>
<td>1.7 %</td>
</tr>
<tr>
<td>Inter-Lot-Precision</td>
<td>2.2 %</td>
<td>3.3 %</td>
<td>7.7 %</td>
</tr>
<tr>
<td>Analytical Sensitivity</td>
<td>1.15 U/mL</td>
<td>1.15 U/ml</td>
<td>1.05 U/mL</td>
</tr>
<tr>
<td>Recovery</td>
<td>98 – 106 %</td>
<td>102 – 108 %</td>
<td>88 – 94 %</td>
</tr>
<tr>
<td>Linearity</td>
<td>77 – 108 %</td>
<td>76 – 113 %</td>
<td>83 – 125 %</td>
</tr>
<tr>
<td>Cross-Reactivity</td>
<td>No cross-reactivity to Helicobacter pylori and Bordetella pertussis.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interferences</td>
<td>No interferences to bilirubin up to 0.3 mg/mL, hemoglobin up to 8.0 mg/mL and triglycerides up to 5.0 mg/mL</td>
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<tr>
<td>Clinical Specificity</td>
<td>93 %</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>Clinical Sensitivity</td>
<td>100 %</td>
<td>100 %</td>
<td>100 %</td>
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</table>

11. References
12. Short Instruction

<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction:</th>
<th>Volume</th>
<th>Description</th>
</tr>
</thead>
</table>
| 1.   | Serum     | 100 µL | Standards/Controls (ready-to-use)  
                  Patient serum (diluted 1:101)  
                  60 min ↓ at room temperature  
                  Wash 3x, 300 µl washing buffer (diluted 1:10) |
| 2.   | Conjugate | 100 µL | Conjugate (ready-to-use)  
                  30 min ↓ at room temperature  
                  Wash 3x, 300 µl washing buffer (diluted 1:10) |
| 3.   | Substrate | 100 µL | Substrate (TMB, ready-to-use)  
                  20 min ↓ at room temperature  
                  100 µL Stop Solution (0.5M sulfuric acid, ready-to-use)  
                  ↓  
                  Read at 450 (620-630) nm |