



Instruction Manual

Diphtheria Toxoid IgG ELISA

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



Cat. No.: ILE-DIP01
Storage: 2-8°C
For in-vitro diagnostic use only

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

Symbole und Übersetzungen / Symbols and Translations

Symbol	English	French	German	Italian	Spanish	Greek
CAL	Calibrator	Etalon	Kalibrator	Calibratore	Calibrador	Πρότυπο Διάλυμα
CONJ	Conjugate	Conjugué	Konjugat	Coniugato	Conjugado	Διάλυμα Συμπλόκου
CONC	Concentrate (<n>-fold)	Concentré (<n> fois)	Konzentrat (<n>-fach)	Concentrato (<n>-volte)	Concentrado (<n>-veces)	Συμπύκνωση (<n> φορές)
SAMP DIL	Sample Diluent	Diluant échantillon	Proben- verdünner	Diluyente del campione	Diluyente de muestra	Διάλυμα Αραίωσης Δειγμάτων
STOP	Stop Solution	Solution d'arrêt	Stopp-Lösung	Soluzione d'arresto	Solución de parada	Διάλυμα Αναστολής
SUBS	Substrate	Substrat	Substrat	Substrato	Sustrato	Διάλυμα Υποστρώ ματος
MT PLATE	Microtiter plate	Microplaque	Mikrotiterplatte	Piastre	Placa microtiter	Μικρόπλακα
WASH BUF	Wash buffer	Tampon de lavage	Waschpuffer	Soluzione di lavaggio	Tampón de lavado	Πλυστικό Διάλυμα

1. Intended Use

The IMMUNOLAB Diphtheria Toxoid IgG Antibody ELISA Test Kit has been designed for the detection and the quantitative determination of specific IgG antibodies against Diphtheria Toxoid in serum and plasma. Further applications in other body fluids are possible and can be requested from the Technical Service of IMMUNOLAB.

This assay is intended for in-vitro diagnostic use only.

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

Diphtheria is a bacterial infectious disease which appears predominantly during the childhood. The disease leads particularly to an inflammation of the pharynx, larynx and nasal mucosa. Additionally, bacterial toxins cause via long-distance effect damages of the heart, circulation and CNS. Only the toxigenic strains are pathogenic.

The etiologic agent is the *Corynebacterium diphtheriae*. These gram-positive bacteria prefer a microaerophil to anaerobe environment. Its pathogenicity is based on the secretion of an exotoxin that is circulating in the blood and effecting the heart muscle, kidneys and CNS. The Diphtheria toxoid will be produced by lysogenic strains.

Depending on the stage of disease, the three types 'slight, middle and serious' can be distinguished. The natural source of infection is the sick individual, whereas a carrier not absolutely shows symptoms. The infection is spread both through the aerial-droplet route and rarely by milk or smear infection. The appearance of Diphtheria shows a seasonal prevalence with the greatest incidence in winter. Especially non-vaccinated children will be infected. The incubation time is depending on the number of invasive germs.

The place of infection is the mucosa of the respiratory tract, where an acute local infection is developing. The secreted toxin leads to a superficial inflammation of the mucosa associated with the formation of a brown film (pseudo-membrane) upon it, consisting of bacteria, necrotic epithelial cells, fibrin, red and white cells. From this local inflammation, the toxin reaches other organs by using the blood and lymphatic circulation. Here it may cause severe damages. The grade of disease depends on the immunostate of the child. Usually, a limited Diphtheria arises, whereas in case of an immunosuppression, a severe Diphtheria is observed. As a result of this disease course, patients may die.

In most cases children will be vaccinated (e.g. DTP = Diphtheria-Tetanus-Pertussis) after the third month of life. The state of immunity can be monitored by determining the antitoxin IgG.

3. Principle of the Test

The IMMUNOLAB Diphtheria Toxoid IgG antibody test kit is based on the principle of the enzyme immunoassay (EIA). Diphtheria Toxoid 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 Diphtheria Toxoid 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 the 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.
- No reagents from different kit lots have to be used, and they should not be mixed with one another.
- 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

Store kit components at 2-8°C and do not use after the expiry date on the box outer label. Before use, all components should be allowed to warm up to ambient temperature (18-25°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.

Components	Volume / Qty.
Diphtheria Toxoid antigen coated microtiter strips	12
Standards with 0, 0.01, 0.1, 0.5 and 1 IU/mL	5 x 2 mL
Enzyme Conjugate	15 mL
Substrate	15 mL
Stop Solution	15 mL
Sample Diluent	60 mL
Washing Buffer (10×)	60 mL
Plastic foils	2
Plastic bag	1
Instruction Manual	1

5.1. Microtiter Strips

12 strips with 8 breakable wells each, coated with a Diphtheria Toxoid antigen. Ready-to-use.

5.2. Standards

5 x 2 mL, human serum diluted with PBS, with 0, 0.01, 0.1, 0.5 and 1 IU/mL of IgG antibodies against Diphtheria Toxoid. Addition of 0.01 % methylisothiazolone and 0.01 % bromonitrodioxane. Ready-to-use.

5.3. 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 ProclinTM. Ready-to-use.

5.4. Substrate

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

5.5. Stop Solution

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

5.6. Sample Diluent

60 mL, PBS/BSA buffer. Addition of 0.1 % sodium azide. Ready-to-use.

5.7. Washing Buffer

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

5.8. Plastic Foils

2 pieces to cover the microtiter strips during the incubation.

5.9. 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
- Bidistilled 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 (4-8°C) for up to 48 hours, 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 distilled 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.
- Standards and samples should be assayed in duplicates.
- A standard curve should be established with each assay.
- Return the unused microtiter strips to the plastic bag and store them dry at 4-8°C.

8.2. Assay Steps

1. Prepare a sufficient amount of microtiter wells for the standards, controls and samples in duplicate 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

	OD Value	corrected OD	Mean OD Value
Substrate Blank	0.016		
Standard 1 (0 IU/mL)	0.037 / 0.045	0.021 / 0.029	0.025
Standard 2 (0.01 IU/mL)	0.072 / 0.063	0.056 / 0.047	0.052
Standard 3 (0.1 IU/mL)	0.376 / 0.386	0.360 / 0.370	0.365
Standard 4 (0.5 IU/mL)	1.480 / 1.461	1.464 / 1.445	1.455
Standard 5 (1 IU/mL)	2.149 / 2.126	2.133 / 2.110	2.122

The above table 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. Quantitative Evaluation

The ready-to-use standards of the Diphtheria Toxoid antibody kit are defined and expressed in International Units (IU/mL). This results in an exact and reproducible quantitative evaluation. Consequently for a given patient follow-up controls become possible. The values for the standards in International Units are printed on the labels of the vials.

For a quantitative evaluation the absorptions of the standards are graphically drawn 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.

The dilution factor of the samples (1:101) has already been reconsidered in the concentration given for the standards.

9.2. Interpretation

The results of each patient sample can be assessed as follows:

< 0.01 IU/mL	basic immunisation recommended
0.01 – 0.1 IU/mL	booster vaccination recommended
> 0,1 IU/mL	good immunity

10. Assay Characteristics

Diphtheria Toxoid ELISA	IgG
Intra-Assay-Precision	7.5 %
Inter-Assay-Precision	4.9 %
Inter-Lot-Precision	2.3 – 7.4 %
Analytical Sensitivity	0.004 U/mL
Recovery	96 – 102 %
Linearity	78 – 133 %
Cross-Reactivity	No cross-reactivity to Clostridium tetani
Interferences	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
Clinical Specificity	94 %
Clinical Sensitivity	94 %

11. References

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