

# **Diphtheria ELISA IgG Testkit**

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**FOR IN VITRO DIAGNOSIS ONLY**

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## 1. Intended Use

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The Diphtheria ELISA is intended for the quantitative detection of IgG antibodies against the Diphtheria toxin to follow up the success of vaccinations and to determine the immunisation status.

## 2. Diagnostic Relevance

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Diphtheria is caused by *Corynebacterium (C.) diphtheriae*, an aerobic, non-sporulating rod bacterium. The virulence of *C. diphtheriae* derives from the diphtheria toxin. Humans are the only reservoir for the bacterium. It is transmitted by droplet infection. Within a few days, there is a general feeling of illness with fever, headache, abdominal pain and pains in the limbs. The infectious disease occurs worldwide but has been greatly pushed back by active immunisation. There are locally limited outbreaks again and again because of gaps in immunisation and it is associated with high mortality. In Germany only one clinical case of diphtheria was recorded in 1998 and again in 1999 and there was no case recorded in 2000 (1,4).

Active immunisation is the method of choice for the prevention of diphtheria. The immunity produced by the antitoxin largely prevents the illness but not the infection so that there can also be carriers among those who have been vaccinated (1). Diphtheria immunisation in infants and preschool children is as good as that against tetanus in Central Europe. According to investigations by the RKI [Robert Koch Institute], approx. 95% of all children are vaccinated regularly against diphtheria (4). However, the recommended boosters are often no longer given so that deficits in vaccine immunity occur in adolescents and younger adults (1, 3, 4). It is estimated that about half of them are not vaccinated as recommended for their age (4). In Germany at present, only about one third of adults have reliably protective antibodies (1, 4, 6). Similar figures are also found for other parts of Europe and the USA (5, 7).

The neutralisation test is the method of choice for detecting specific antibodies to the toxin. However this is very expensive and time-consuming. By means of this ELISA, the IgG antibody level can be measured so that conclusions can be drawn about the vaccination status. Furthermore, it can be used to examine the need for vaccination and to check immunity after vaccination has taken place.

## 3. Test Principle

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The antibody searched for in the human serum forms an immune complex with the antigen coated on the microtiter-plate. Unbound immunoglobulins are removed by washing processes. The enzyme conjugate attaches to this complex. Unbound conjugate is again removed by washing processes. After adding the substrate solution (TMB), a blue dye is produced by the bound enzyme (peroxidase). The color changes to yellow when the stopping solution is added.

## 4. Package Contents (IgG Testkit)

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1. **1 Microtiter-Plate** consisting of 96 with antigen coated, breakable single wells, lyophilised
2. **PBS-Dilution Buffer (blue, ready to use) 2x50ml**, pH 7,2, with preservative and Tween 20
3. **PBS-Washing Solution (20x concentrated) 50ml**, pH 7,2, with preservative and Tween 20
4. **IgG-Ab-standard sera** for drawing a standard curve, 6 vials à 2ml, ready to use, human serum with preservative, 0,001IU/ml, 0,002IU/ml, 0,005IU/ml, 0,01IU/ml, 0,02IU/ml, 0,05IU/ml (IU=international units)
5. **IgG high positive Control, 2 ml**, human serum with preservative, ready to use
6. **IgG low positive Control, 2 ml**, human serum with preservative, ready to use
7. **IgG-Conjugate (anti-human), 11 ml**, (sheep or goat)-horseradish-peroxidase-conjugate with protein-stabilizer and preservative in Tris-Buffer, ready to use
8. **Tetramethylbenzidine substrate solution (3,3',5,5'-TMB), 11 ml**, ready to use
9. **Citrate-Stopping Solution, 6ml**, contains an acid mixture

## 5. Storage and Shelflife of the Testkit and the ready to use reagents

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Store the testkit at 2-8°C. The shelf life of all components is shown on each respective label; for the kit shelf life please see Quality Control Certificate.

1. Microtiter strips/single wells are to be resealed in package after taking out single wells and stored with desiccant at 2-8°C. Reagents should immediately be returned to storage at 2-8°C after usage.

- The ready to use conjugate and the TMB-substrate solution are sensitive to light and have to be stored in dark. Should there be a color reaction of the substrate dilution due to incidence of light, it is not useable anymore.
- Take out only the amount of ready to use conjugate or TMB needed for the test insertion. Additional conjugate or TMB taken out may not be returned but must be dismissed.

Material	Status	Storage	Shelflife
Test Samples	Diluted	+2 to +8°C	max. 6h
	Undiluted	+2 to +8°C	1 week
Controls	After Opening	+2 to +8°C	3 months
Microtitreplate	After Opening	+2 to +8° (storage in the provided bag with desiccant bag)	3 months
Rheumatoid factor - Absorbent	Undiluted, After Opening	+2 to +8°C	3 months
	Diluted	+2 to +8°C	1 week
Conjugate	After Opening	+2 to +8°C (protect from light)	3 months
Tetramethylbenzidine	After Opening	+2 to +8°C (protect from light)	3 months
Stop Solution	After Opening	+2 to +8°C	3 months
Washing Solution	After Opening	+2 to +8°C	3 months
	Final Dilution (ready-to-use)	+2 to +25°C	4 weeks

## 6. Precautions and Warnings

- Only sera which have been tested and found to be negative for HIV -1 antibodies, HIV-2 antibodies, HCV antibodies and Hepatitis-B surface-antigen are used as control sera and standards. Nevertheless, samples, diluted samples, standards, controls, conjugates and microtiter strips should be treated as potentially infectious material. Please handle products in accordance with laboratory directions.
- Those components that contain preservatives, the Citrate Stopping Solution and the TMB have an irritating effect to skin, eyes and mucous. If body parts are contacted, immediately wash them under flowing water and possibly consult a doctor.
- The disposal of the used materials has to be done according to the country-specific guidelines.

## 7. Material required but not supplied

- Aqua dest./demin.
- Eight-channel pipette 50µl, 100µl
- Micropipettes: 10µl, 100µl, 1000µl
- Test tubes
- Paper towels or absorbent paper
- Cover for ELISA-plates
- Disposal box for infectious material
- ELISA hand washer or automated EIA plate washing device
- ELISA plate spectrophotometer, wavelength = 450nm, reference length = 620nm (Reference Wavelength 620-690nm)
- Incubator

## 8. Test Procedure

Working exactly referring to the Sekisui Virotech user manual is the prerequisite for obtaining correct results.

### 8.1 Examination Material

Either serum or plasma can be used as test material, even if only serum is mentioned in the instructions. Any type of anticoagulant can be used for plasma.

Always prepare patient-dilution freshly.

For a longer storage the sera must be frozen. Repeated defrosting should be avoided.

- Only fresh non-inactivated sera should be used.
- Hyperlipaemic, haemolytic, microbially contaminated and turbid sera should not to be used (false positive/negative results).

## 8.2 Preparation of Reagents

The Sekisui Virotech System Diagnostica offers a high degree of flexibility regarding the possibility to use the dilution buffer, washing solution, TMB, citrate stopping solution as well as the conjugate for all parameters and for all different lots. The standard sera, high positive control and low positive control are only intended for this testkit. Do not use in other lots.

1. Set incubator to 37°C and check proper temperature setting before start of incubation.
2. Bring all reagents to room temperature before opening package of microtiter strips.
3. Shake all liquid components well before use.
4. Make up the washing solution concentrate to 1 L with distilled or demineralised water. If crystals have formed in the concentrate, please bring the concentrate to room temperature before use and shake well before use.

## 8.3 Virotech ELISA Test Procedure

1. For each test run, pipette 100µl each of ready to use dilution buffer (blank), standard- and control sera as well as diluted patient sera. We propose a double insertion (blank, standards, controls and patient sera).  
Working dilution of patient sera: 1+100; e.g. 10µl serum + 1ml dilution buffer.
2. After pipetting start incubation for 30 min. at 37°C (with cover).
3. End incubation period by washing microtiter strips 4 times with 350 – 400µl washing solution per well. Do not leave any washing solution in the wells. Remove residues on a cellulose pad.
4. Pipette 100µl of ready to use conjugate into each well.
5. Incubation of conjugate: 30 min. at 37°C (with cover).
6. Stop conjugate incubation by washing 4 times (pls. refer to point 3 above).
7. Pipette 100µl of ready to use TMB into each well.
8. Incubation of substrate solution: 30 min. at 37°C (with cover, keep in dark).
9. Stopping of substrate reaction: pipette 50µl of citrate stopping solution into each well. Shake plate carefully and thoroughly until liquid is completely mixed and a homogeneous yellow color is visible.
10. Measure extinction (OD) at 450/620nm (Reference Wavelength 620-690nm). Set your photometer in such a way that the blank value is deducted from all other extinctions. Extinctions should be measured within 1 hour after adding the stopping solution!

Pls. refer to last page for Test Procedure Scheme

## 8.4 Usage of ELISA processors

All Sekisui Virotech ELISAs can be used on ELISA processors. The user is bound to proceed a validation of the devices (processors) on a regular basis.

Sekisui Virotech recommends the following procedure:

1. Sekisui Virotech recommends to proceed the validation of device referring to the instructions of the device manufacturer during the implementation of the ELISA processor respectively after bigger reparations.
2. It is recommended to check the ELISA-processor with the Validationkit (EC250.00) afterwards. A regular check using the Validationkit shall be proceeded minimum once a quarter to test the accuracy of the processor.
3. The release criteria of the Quality Control Certificate of the product must be fulfilled for each testrun.

With this procedure, your ELISA processor will function properly and this will support quality assurance in your laboratory.

## 9. Test Evaluation

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### 9.1 Test function control

#### a) OD-values

The OD-value of the blank should be < 0.15.

The OD-values of the lowest standard (0,001 IU/ml) shall be >0,05 OD and the OD-values of the highest standard (0,050 IU/ml) shall be < 2,800 OD.

#### b) The concentration of the low and high positive control have to be within the ranges (IU/ml) stated in the Quality Control Certificate.

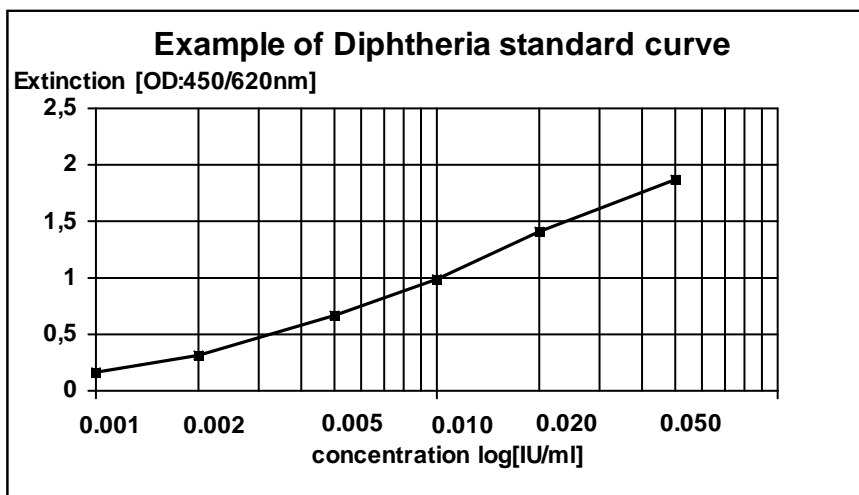
## 9.2 Evaluation

By using the standards, a standard curve is plotted on the semilogarithm paper included in the kit in order to determine the Diphtheria-antitoxin-IgG-antibody level in the serum. The mean values of the extinctions are plotted on the ordinate and the concentrations (IU/ml of the ready to use standards) of the standard sera on the abscissa. You have to be aware that the patient sera intended for the test procedure have been diluted 1:100. This is why the result read from the diagram must be multiplied by the factor of 100. For preparing the standard curve, either a point-to-point procedure as well as a 4-parameter-calculation can be used.

### Please note:

Samples found to have a concentration of under 0.1IU/ml can be retested with a 1:10 dilution. The difference in the dilution must be considered in the evaluation.

Samples with an extinction above the value of the 0,05IU/ml standard have to be used in a higher dilution in the test, e.g. 1:200, 1:400 etc. **At OD values above 2.00, the measuring precision decreases with increasing optical density. It is therefore recommended that sera which attain OD values above 2.00 at a dilution of 1:100 be used in higher dilution, e.g. 1:200, 1:400 etc., in the test.** These dilutions have to be considered during the test evaluation.



## 9.3 Interpretation

The Diphtheria antitoxin concentrations are expressed in International Units (IU/ml) following the WHO Standard.

According to references in literature a sufficient immune protection can be expected above 0.1IU/ml (2,8,9,11). But this figure has to be considered as recommendation only.

We would like to draw your attention to vaccination recommendation mentioned below, that was taken from the publication Pietsch, M. (10).

IU/ml	recommended vaccination
<0,1	basic immunisation immediately
0,1 - 1,0	booster vaccination immediately
1,0 - 1,4	booster vaccination after 5 years
1,4 - 2,0	booster vaccination after 7 years
>2,0	booster vaccination after 10 years

The detection of the Diphtheria-antitoxin-IgG-antibody level can be a decisive help for determining the necessity of vaccination and for checking the success of vaccination. In addition information on the different reactions of the individual immune system can be obtained.

## 9.4 Limits of the Test

1. The interpretation of serological results shall always include the clinical picture, epidemiological data and all further available laboratory results.
2. The Virotech Diphtheria ELISA is not suitable for laboratory diagnosis of an infection.
3. Please refer also to vaccination certificate or information about last Diphtheria vaccination for interpretation of the antitoxin titer.
4. An interpretation of antibody titers below 0,1 IU/ml is not recommendable, as they are below the technically reproducible sensitivity limit when using ELISA test systems. The vaccination anamnesis should be therefore considered in the individual case to decide if a basic immunisation or a booster vaccination should be performed.

## 10. IgG test evaluation with the 4-parameter method

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By means of the Virotech Diphtheria IgG ELISA, it is possible to carry out a quantitative assay with the 4-parameter method. For this purpose the 0.01 IU/mL standard is used for calibration control. The calibration control compensates for the fluctuations caused by performance of the test. Mean values of the OD readings are used for the calculation.

### 10.1 Test function control

#### a) OD-values

The OD-value of the blank should be < 0.15.

The OD-value of the calibration control must lie within the reference range indicated in the quality control certificate.

#### b) IU/mL

The anti-diphtheria IgG concentrations (IU/mL) of the weakly positive control and of the strongly positive control must lie within the ranges indicated in the quality control certificate.

If the requirements (OD readings, IU/mL) are not satisfied, the test is to be repeated.

### 10.2 Conversion of the quantitative results to international units per milliliter (IU/mL)

The extinction of the blank value (450/620 nm) must be subtracted from all extinctions.

The patient sera are quantified by expressing them in international units. The standard curve is determined by non-linear regression on the basis of extensive tests and is described mathematically by the following formula (12):

$$\text{IU/mL} = e^{(C - \ln((D - A) / (OD \text{ corr} - A) - 1) / B)}$$

Where

- A: expected OD at an anti-diphtheria IgG concentration of 0  
B: slope factor  
C: inflection point  
D: expected OD at an infinitely high anti-diphtheria IgG concentration  
OD corr: corrected OD of the patient serum

To allow for fluctuations within the course of the tests, the measured OD of the patient serum is corrected on the basis of a calibration control:

$$\text{ODcorr} = \text{OD patient serum} * \frac{\text{OD calibration control specified}}{\text{OD calibration control measured}}$$

See the certificate for the values of the parameters A, B, C and D as well as the specified OD of the calibration control. In the case of evaluation software not compatible with this calculation method, 6 standard value pairs that also describe the standard curve are additionally defined in the certificate.

The quantifiable range is between 0.03 IU/mL and 10 IU/mL.

#### Determination of the IU/mL

The IU/mL can be determined using software available for purchase from Virotech. Alternatively, an evaluation template for common tabular estimates can be provided.

## 11. Performance Data

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### 11.1 Sensitivity and Specificity

It is not possible to determine diagnostic sensitivity and specificity because this ELISA is a quantitative test, which is not intended to differentiate between positive and negative results.

### 11.2 Recovery rate

The standard curve of the VIROTECH Diphtheria ELISA has been checked with the Diphtheria Antitoxin Human Serum (00/496) of the Institute for Biological Standards and Control, WHO International Laboratory for Biological Standards in Great Britain.

### 11.3 Prevalence (Expected Values)

117 blood bank sera were tested and the IU/ml concentrations were calculated:

Concentration	Number of sera found
< 0,1 IU/ml	67 %
0,1 – 1,0 IU/ml	33 %
1,0 – 1,4 IU/ml	0 %
1,4 – 2 IU/ml	0 %
> 2 IU/ml	0 %

The sera distribution corresponds approximately to the prevalence rate described in some literature (1,2,3,4,6). The data can only partly be compared as no information about the age of the blood donors is available.

### 11.4 Intra-assay-Coefficient of Variation (Repeatability)

In one assay, strips of different plates of one batch have been tested with the same serum sample. The obtained coefficient of variation is lower than 9%.

### 11.5 Inter-assay-Coefficient of Variation (Reproducibility)

Three sera were tested in 10 independent test runs by different persons in different laboratories. The obtained variation coefficient values are lower than 15%.

### 11.6 Proficiency

Within the period from November 2005 to April 2010, 20 sera from interlaboratory comparisons and of known concentrations were measured in the Virotech ELISA. All 20 declared sera were correctly identified.

### 11.7 Detection Limit

Tests within the company have found a lower reproducible limit of detection of 0.04 IU/ml with a coefficient of variation of 2.6%.

## 12. Literature

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## Preparation of Patient Samples and Washing Solution

▼ **Washing Solution:** Fill up concentrate to 1 liter with aqua dest./demin.

### **IgG-Samples – Dilution**

**1:101**

e.g.:

10 µl serum/plasma + 1000 µl Dilution Buffer  
(Serum Dilution Buffer is ready to use)

## Testprocedure

Samples Incubation	30 minutes at 37°C	<b>100 µl Patient Samples</b> blank value (Dilution Buffer) standards and controls
↓		
Wash 4times		<b>400 µl Washing Solution</b> Remove Residues on a Cellulose Pad
↓		
Conjugate Incubation	30 minutes at 37°C	<b>100 µl Conjugate</b> IgG
↓		
Wash 4times		<b>400 µl Washing Solution</b> Remove Residues on a Cellulose Pad
↓		
Substrate Incubation	30 minutes at 37°C	<b>100 µl Substrate</b>
↓		
Stopping		<b>50 µl Stopping Solution</b> shake carefully
↓		
Measure Extinctions		<b>Photometer at 450/620nm</b> (Reference Wavelength 620- 690nm)