VIROTECH Tetanus IgG ELISA
(Tetanus IgG ELISA)

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FOR IN VITRO DIAGNOSIS ONLY

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Contents

1. Intended Use

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

2. Diagnostic Relevance

Tetanus is caused by Clostridium (C.) tetani, an obligate anaerobic spore-forming bacterium. Its spores are ubiquitous in soil and are extremely resistant to heat and disinfectants. The vegetative form of C. tetani produces two exotoxins, tetanolysin and tetanospasmin, of which the tetanospasmin causes the typical clinical symptoms such as increased muscle tone and spasms. The clinical forms of tetanus that can be distinguished are generalised, local and neonatal disease (1).

The bacteria can get into the human body through contaminated wounds which thus represent an infection risk. The detection of specific antibodies is unimportant in diagnosing the infection. The serological measurement of the IgG antibodies is more important and therefore more suitable for obtaining evidence of tetanus immunity (1). Up-to-date vaccination is an absolute necessity for everyone, especially for elderly people (as these are often inadequately immunised) (4,5). Overall, tetanus vaccination rates are higher than those of other vaccinations, such as diphtheria (5, 8). After basic immunisation, periodic boosters should be given at intervals of 10 years. However, the booster is often neglected so that the immunisation is often inadequate (1).

The IgG antibody level can be measured by means of this ELISA so that conclusions can be drawn about immunisation status. Furthermore, it can be used to determine the need for vaccination and to check immunity after vaccination has taken place.

3. Test Principle

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)

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), 11ml**, (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), 11ml**, 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

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.
2. 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.
3. 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	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

1. 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.
2. 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.
3. The disposal of the used materials has to be done according to the country-specific guidelines.

7. Material required but not supplied

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

8. Test Procedure

Working exactly referring to the VIROTECH Diagnostics 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.

The patient samples can be stored for 1 week at 2-8°C.

Always prepare patient-dilution freshly. Maximum shelf life 6h at 2-8°C

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

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

8.2 Preparation of Reagents

The VIROTECH Diagnostics 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).
   If the standard 0.01 IU/ml is used as calibration control for evaluation of the test using the 4-parameter method, double insertion is a mandatory requirement. 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 VIROTECH Diagnostics ELISAs can be used on ELISA processors. The user is bound to proceed a validation of the devices (processors) on a regular basis.

VIROTECH Diagnostics recommends the following procedure:

1. VIROTECH Diagnostics 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

9.1 Test function control

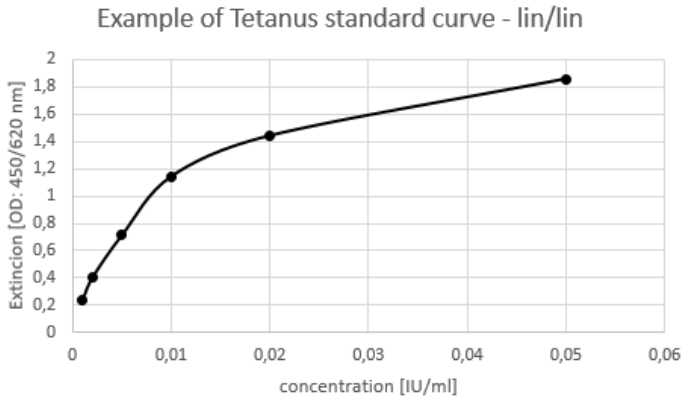
a) OD-values
   The OD-value of the blank should be < 0.15.
   The OD values of the lowest standard (0.001 IU/ml) should exceed the OD value specified in the quality certificate and the OD values of the highest standard (0.050 IU/ml) should not exceed the OD value specified in the quality certificate.
b) The concentration of the low and high positive control have to be within the ranges (IU/ml) stated in the Quality Control Certificate.
c) If the requirements (OD / IU/ml) are not met, the test must be repeated.

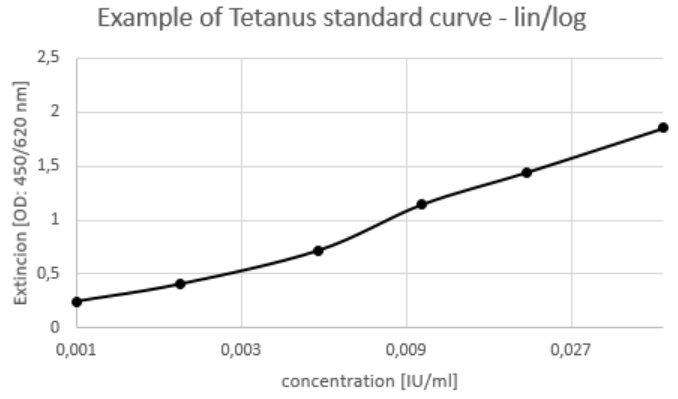
9.2 Evaluation

By using the standards, a standard curve is plotted on the semilogarithm paper included in the kit in order to determine the Tetanus-antitoxoid-IgG-antibody level in the serum. The mean values of the extinctions are plotted on the ordinate and the concentrations (lU/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-paramter-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 Tetanus antitoxoid concentrations are expressed in International Units (IU/ml) following the WHO Standards. A Tetanus-antitoxoid-IgG-antibody concentration of >0,1 IU/ml is indicated as immune protection (2,11) or safe immune protection (9,10). Booster vaccinations are not indicated where antibody concentrations exceed 0.5 IU/ml (10). In the information below, we would like to point out the following vaccination recommendations. These have been drafted based on the recommendations of the working group for immunoprophylaxis (13):

IU/ml	**Interpretation and next steps**
< 0.01	- No vaccine protection

	- Depending on medical history, initial vaccination or booster vaccination required - Serological test after 4 to 8 weeks
0.01 – 0.1	- Vaccine protection uncertain - Booster vaccination required - Serological test after 4 to 8 weeks
0.11 – 0.5	- Vaccine protection still ensured for a short time - Booster vaccination recommended - Booster vaccination will provide long-term vaccine protection
0.51 – 1.0	- Vaccine protection provided - Booster vaccination or serological test recommended after 3 years - **Note: Vaccination in the case of antibody concentrations > 0.5 IU/ml could lead to undesired reactions to the vaccine**
> 1.0 – 5.0	- Long-term vaccine protection provided - Booster vaccination or serological test recommended at the earliest after 5 years
> 5.0 – 10.0	- Long-term vaccine protection provided - Booster vaccination or serological test recommended at the earliest after 8 years
> 10	- Long-term vaccine protection provided - Booster vaccination or serological test recommended at the earliest after 10 years

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 Tetanus ELISA is not suitable for laboratory diagnosis of an infection.
3. Please refer also to vaccination certificate or information about last Tetanus vaccination for interpretation of the antitoxoid titer (7).
4. An interpretation of antitoxoid 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 therefore be considered in the individual case to decide if a basic immunisation or a booster vaccination shall be performed (=> 10.2).

10. IgG test evaluation with the 4-parameter method

By means of the VIROTECH Tetanus 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 indiciated in the quality control certificate.

b) IU/mL

The anti-tetanus 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):

IU/ml = exp(-(ln((D-A)/((OD corr)-A)-1)-B)/C)

Where

A:	expected OD at an anti-tetanus IgG concentration of 0
B:	slope factor
C:	inflection point
D:	expected OD at an infinitely high anti-tetanus 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:

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

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 0.01 IU/mL and 15 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. The calculated concentrations always indicate the actual concentrations of the undiluted serum where this has been diluted for test purposes using a 1/100 dilution. If a different dilution was used during testing, the concentration values must be adjusted accordingly.

11. Performance Data

11.1 Sensitivity and Specificity

It is not possible to determine diagnostical sensitivity and specificity because this ELISA is a quantitave test, which is not intended to differentiate between positve and negative results.

11.2 Detection Limit

In internal tests a lower reproduceable determination limit of 0,06 IU/ml at a coefficient of variation of 3,9% could be determined.

11.3 Proficiency

Within the period from March 2002 to November 2009, 32 sera from interlaboratory comparisons and of known concentrations were measured in the VIROTECH ELISA. 30 sera corresponded to the declared value. Two sera did not meet the specifications.

11.4 Recovery rate

The international standard of the WHO for Tetanus Immunglobuline (human) TE-3 was tested on the VIROTECH ELISA to determine the recovery rate of the Tetanus ELISA. By using the standard curve the concentration in IU/ml was calculated considering the respective dilution and then compared with the expected initial value. The results show that the starting concentration of 120 IU/ml could exactly be recovered in the diagnostic relevant lower area of the standard curve (0,1 to 0,5IU/ml).

11.5 Prevalence (Expected Values)

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

Concentration	Serum values

< 0.01 IU/ml	0 %
0.01 – 0.1 IU/ml	7 %
0.11 – 0.5 IU/ml	15 %
0.51 – 1.0 IU/ml	26 %
> 1.0 – 5.0 IU/ml	51 %
> 5.0 – 10.0 IU/ml	1 %
> 10.0 IU/ml	0 %

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

11.6 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 then 9%.

11.7 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%.

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