TestLine
Clinical
Diagnostics

Instruction for use

EIA Toxoplasma IgG

REF TgG096

Σ 96

Kit for professional use

IVD CE 1023

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CONTENT

Enzyme immunoassay for the detection of IgG antibodies to *Toxoplasma gondii* and IgG avidity in human serum or plasma

1 Introduction

Toxoplasmosis is a widespread parasitic disease caused by protozoan *Toxoplasma gondii* – a parasite with a complicated life cycle consisting of several morphologically different stadia. Primary hosts are members of the feline family. Humans and most warm-blooded animals can be infected by either primarily infected food (insufficiently heat-treated meat) or by ingestion of oocysts (secondary contaminated food or contaminated fingers, objects, etc.).

Acquired toxoplasmosis in immunocompetent individuals is usually asymptomatic or can manifest itself with flu-like symptoms (subfebrility, fatigue, lymphadenopathy, muscle aches), but without lasting ill effects. Severe life-threatening infections (encephalitis, hepatitis, chorioretinitis, myocarditis, generalized form of the disease) may develop in immunocompromised patients usually because of a reactivation of a latent infection.

Congenital toxoplasmosis is caused by transmission of infection from mother to foetus. The mother has been primary infected with toxoplasmosis shortly before becoming pregnant or during pregnancy. Congenital toxoplasmosis might result in severe damages of the foetus (brain calcification, hydrocephalus, vision disorders, mental affections), still birth or abortion.

Diagnosis of the disease is based on epidemiological anamnesis, clinical manifestation and laboratory tests. Direct detection of the parasite is not available for routine diagnostics. Serology is the most important tool for laboratory diagnostics of toxoplasmosis. Screening consists in determination of total antibodies by complement fixation test (CFT). Determination of specific IgA, IgE, IgM, IgG antibodies and IgG avidity is performed by ELISA and confirmation of results by immunoblot method.

IgA, IgE and IgM antibodies are significant markers of acute toxoplasmosis. IgM antibodies are a highly sensitive marker of acute infection and they can persist more than 1 year. IgA antibodies can persist for 6-9 months from the beginning of infection. IgE antibodies as a highly specific marker of acute infection can persist up to 6 months from the beginning of infection.

IgG antibodies reach maximum level in serum after 6 months from the beginning of infection and they can be detected for many years after past infection.

2 Test Principle

The kit is intended for detection of specific IgG antibodies in a sample by means of a sandwich type of the EIA method (i.e. a solid phase coated with specific antigen – antibody from the analysed sample – labelled antibody). The labelled antibody (conjugate) is an animal immunoglobulin fraction to human IgG conjugated with horseradish peroxidase. Peroxidase activity is determined in the test by a substrate containing TMB. Positivity is indicated when blue colour appears; after stopping solution has been added, blue changes to yellow. The yellow colour intensity is measured by a photometer at 450 nm, and it is proportional to the concentration of specific IgG antibodies in the sample.

Antigen Used

Purified and inactivated antigen of *T. gondii* (RH strain)

3 Materials Provided

MICROPLATE	**Microtitre Plate** coated with antigen, 12 x 8 wells in bag with desiccant	1 pc
CONTROL - CAL1	**Negative Control (Calibrator 1) 0.1 IU/ml** Solution containing no specific human antibodies, ready to use	1 × 2 ml
CUTOFF CAL2	**CUT-OFF (Calibrator 2) 6 IU/ml** Solution containing specific human antibodies in cut-off concentration, ready to use	1 × 3 ml
CONTROL + CAL3	**Positive Control (Calibrator 3) 60 IU/ml** Solution containing specific human antibodies, ready to use	1 × 2 ml
CAL4	**Calibrator 4 (240 IU/ml)** Solution containing specific human antibodies, ready to use	1 × 2 ml
CONJUGATE	**Conjugate** Solution containing peroxidase labelled animal immunoglobulin to human IgG, ready to use	1 × 15 ml
DILUENT 5	**Sample Diluent 5** Buffer with protein stabilisers, ready to use	1 × 105 ml
SUBSTRATE 2	**TMB-Complete 2** Chromogenic substrate solution containing TMB/H_2O_2, ready to use	1 × 15 ml
WASH 20x	**Wash Solution** 20× concentrated buffer	1 × 75 ml
STOP	**Stop Solution** Acid solution, ready to use	1 × 15 ml
AVIDITY 1	**Avidity Solution 1** Stabilised urea solution	1 × 7 ml
	Instructions for use	1 pc

4 Other Material Required for Manual Test Performance

Single and multichannel pipettes

Disposable tips

Microplate washer

Timer

Incubator (37°C)

Microplate reader

5 Storage and Stability

Store the kit at +2°C to +8°C. Do not freeze. If the kit is stored as described, the labelled expiration date is valid. The expiration date is indicated on the package. The opened kit should be used within three months.

Sample Preparation and Storage

The following human body liquids can be used for testing: serum and citrate plasma. Anticoagulants in the plasma (except for citrate) as well as bacterially contaminated, haemolytic or chylous samples can affect the test results.

Samples can be stored at +2°C to +8°C for one week. For a longer period, store samples at -20°C. Diluted samples should be used as soon as possible.

6 Preparation of Reagents

Dilute the Wash Solution 1:20 (1 part of solution and 19 parts of distilled water); e.g. 75 ml of the concentrated Wash Solution + 1425 ml of distilled water.

Salt crystals might develop in the bottle with the concentrated Wash Solution. Prior to use, it is necessary to dissolve the crystals by warming the bottle in a water bath. The diluted Wash Solution is stable at +2°C to +8°C for one week.

The Controls and the Calibrators are supplied ready to use, do not dilute further!

The Conjugate is supplied ready to use, do not dilute further!

TMB-Complete is a one-component chromogenic substrate solution ready to use, do not dilute further!

Interchangeability of reagents

The Sample Diluent, TMB-Complete and the Avidity Solution are interchangeable in EIA kits of TestLine Clinical Diagnostics s.r.o., provided they have the identical numeric marking (e.g. Sample Diluent 2, Sample Diluent 3, etc.). The Stop Solution and the Wash Solution are universal in all kits.

7 Preparation of Samples

Mix gently the Sample Diluent prior to use.

Dilution of sera and plasma samples

Dilute well mixed samples 1:101 with the Sample Diluent:

E.g.: 10 µl of sample + 1 ml of the Sample Diluent

Mix well.

Dilute foetal and neonatal sera 1:101 with the Sample Diluent:

E.g.: 10 µl of serum + 1 ml of the Sample Diluent

Mix well.

8 Assay Procedure

Allow all reagents to come to room temperature and mix well. If you do not use a whole microplate, return unnecessary strips into the bag with desiccant. Seal the bag tightly and store at +2°C to +8°C. Keep dry!

1. Dispense the controls (calibrators) and the diluted samples according to the working schedule.

Semiquantitative evaluation in Index of Positivity (IP)

- Leave A1 well empty (blank).
- Pipette 100 µl of the Negative Control (Calibrator 1) into 1 well.
- Pipette 100 µl of CUT-OFF (Calibrator 2) into 2 wells.
- Pipette 100 µl of the Positive Control (Calibrator 3) into 1 well.
- Pipette 100 µl of the diluted samples (see Chapter Preparation of Samples) into the other wells.

Quantitative evaluation in Units IU/ml

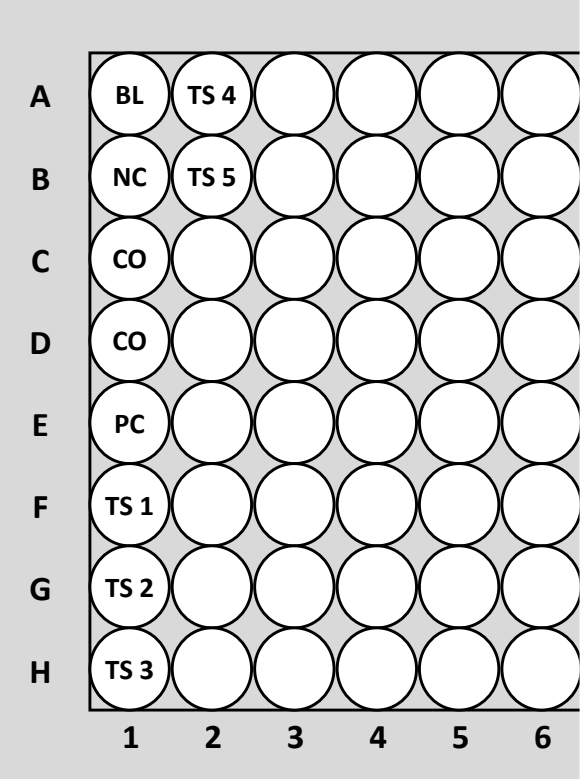
- Leave A1 well empty (blank).
- Pipette 100 µl of the Negative Control (Calibrator 1) into 1 well.
- Pipette 100 µl of CUT-OFF (Calibrator 2) into 2 wells.
- Pipette 100 µl of the Positive Control (Calibrator 3) into 2 wells.
- Pipette 100 µl of the Calibrator 4 into 2 wells.
- Pipette 100 µl of the diluted samples (see Chapter Preparation of Samples) into the other wells.

2. Cover the microplate with the lid and incubate at 37°C for 60 minutes.
3. Aspirate the content of the wells and wash 5× with the working strength Wash Solution. Fill the wells up to the edge. Finally, tap the inverted microplate thoroughly on an absorbent paper to remove solution remnants.

4. Pipette 100 µl of the Conjugate into all wells except A1 well.
5. Cover the microplate with the lid and incubate at 37°C for 60 minutes.
6. Aspirate the content of the wells and wash 5× with the working strength Wash Solution. Fill the wells up to the edge. Finally, tap the inverted microplate thoroughly on an absorbent paper to remove solution remnants.
7. Pipette 100 µl of TMB-Complete into all wells. Avoid contamination – see Chapter Procedural Notes.
8. Cover the microplate with the lid and incubate at 37°C for 20 minutes. Keep out of light.
9. Stop the reaction by adding 100 µl of the Stop Solution in the same order and intervals as the substrate was added.
10. Read the colour intensity in wells against blank (A1 well) using photometer set to 450 nm. The absorbance should be read within 30 minutes after stopping the reaction.

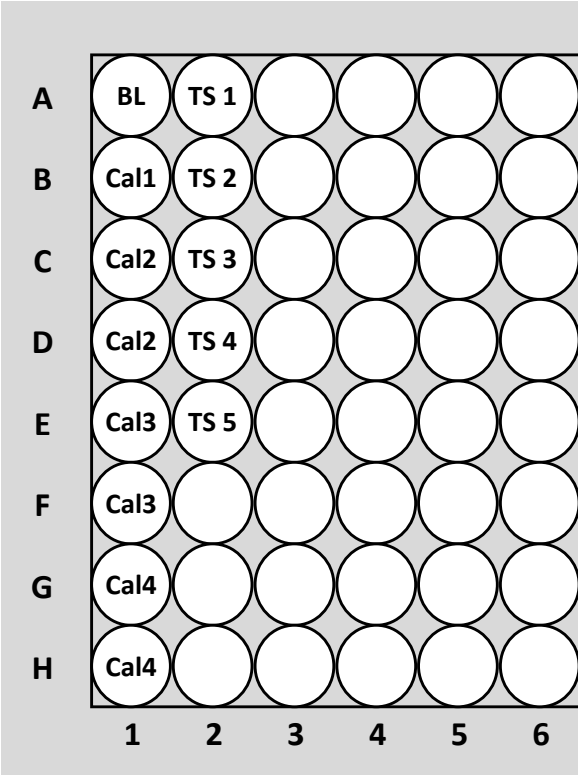
9 Working Schedule

Semiquantitative evaluation
Index of Positivity (IP)



BL	Blank (empty well)	
NC	100 µl	CONTROL - CAL1
CO	100 µl	CUTOFF CAL2
PC	100 µl	CONTROL + CAL3
TS 1-x	100 µl	diluted tested sample

Quantitative evaluation
Units IU/ml



BL	Blank (empty well)	
Cal1	100 µl	CONTROL - CAL1
Cal2	100 µl	CUTOFF CAL2
Cal3	100 µl	CONTROL + CAL3
Cal4	100 µl	CAL4
TS 1-x	100 µl	diluted tested sample

10 Quality Control

The test is valid if:

The absorbance of blank is lower than 0.150.

BLANK < 0.150

The absorbance of the Negative Control (Calibrator 1) is lower than half of the mean absorbance of CUT–OFF (Calibrator 2).

CONTROL - CAL1 < 0.5 × CUTOFF CAL2

The mean absorbance of CUT-OFF (Calibrator 2) is within a range of 0.200 – 0.800.

0.200 < CUTOFF CAL2 < 0.800

The absorbance of the Positive Control (Calibrator 3) is 1.5-fold higher than the mean absorbance of CUT–OFF (Calibrator 2).

CONTROL + CAL3 > 1.5 × CUTOFF CAL2

The absorbance of the Calibrator 4 is higher than the absorbance of the Positive Control (Calibrator 3).

CAL4 > CONTROL + CAL3

11 Results Interpretation

Calculation of Index of Positivity (IP)

Divide the absorbance of a tested sample by the mean absorbance of CUT-OFF measured in the same test run:

$$IP = \frac{\text{Absorbance of sample}}{\text{Mean absorbance of CUT-OFF}}$$

Interpretation of the test results is described in Table 1.

Table 1 Interpretation of test results

Index of Positivity (IP)	Evaluation
lower than 0.9	negative
0.9 to 1.1	borderline
higher than 1.1	positive

Examination of borderline samples, i.e. samples with Index of Positivity from 0.9 to 1.1, should be repeated from a new sample collected after 2 to 6 weeks regarding to the disease specifics.

Quantitative evaluation in International Units (IU/ml)

Construct a calibration curve by plotting the concentration (X) of the calibrators in IU/ml against the corresponding absorbance (Y). Construct the calibration curve by single point cross connection. Read the values of antibody level (IU/ml) in samples from the calibration curve.

If the level of antibodies in a sample is higher than 240 IU/ml, dilute the sample in two steps and repeat the test (final dilution 1:1010).

Step 1. Dilute the sample 1:101 with the Sample Diluent: 10 µl of the sample + 1 ml of the Sample Diluent.

Step 2. Dilute 100 µl of the sample diluted in Step 1 with 900 µl of the Sample Diluent.

Multiply the concentrations of antibodies read from the calibration curve by 10.

Interpretation of the quantitative test results is described in Table 2.

Table 2 Quantitative interpretation in International Units (IU/ml)

Antibody level (IU/ml)	Evaluation
lower than 5.4	negative
5.4 to 6.6	borderline
higher than 6.6	positive

Examination of borderline samples should be repeated from a new sample collected after 2 to 6 weeks regarding to the disease specifics.

Serological finding can be interpreted only in the context of results of other laboratory tests and patient clinical picture.

12 Safety Precautions

The kit is intended for in vitro diagnostic use only.

The sera used for controls were tested and found to be negative for HIV 1 and HIV 2, HBsAg, HCV, TPHA. In spite of this fact, they still need to be handled as potentially infectious materials.

Some reagents contain sodium azide, which is a toxic compound. Avoid contact with skin.

The Stop Solution contains diluted acid solution. Avoid contact with eyes and skin.

It is necessary to observe the local safety rules and regulations.

First aid

In case of contact with eyes, flush with copious amount of water and seek medical assistance. In case of contact with skin and clothing, remove all the contaminated clothes. Wash the skin with soap and plenty of running water. In case of contact with solutions containing plasma or clinical samples, disinfect the skin. In case of accidental ingestion, flush the mouth with drinking water and seek medical assistance.

Remnants disposal

All the materials used for performing the test must be treated as potentially infectious due to the contact with biological materials. Therefore they need to be disposed together with biological waste.

Expired kit disposal

Disassemble the kit and dispose the components as biological material. Discard the packaging material as required by local regulations.

13 Procedural Notes

In order to obtain reliable results, it is necessary to **strictly follow the Instructions for Use**. Always use clean preferably disposable tips and glassware.

Microtitre Plate – in order to prevent water condensation on the surface of the microplate, always allow the bag with the microplate to warm up to room temperature before opening.

Wash Solution – use high quality distilled water for preparing the working strength Wash Solution.

Washing procedure – keep to the prescribed number of wash cycles and fill the wells to the upper edge. The soak time (i.e. interval between two different wash cycles during which the wells stay filled up with the Wash Solution) should be approx. 30-60 seconds.

TMB-Complete – the vessel used for multichannel pipetting should not be used for other reagents. Do not return the surplus TMB-Complete from the pipetting vessel into the vial.

Non-reproducible results might be caused by improper methodology as following:

- insufficient mixing of reagents and samples before use
- improper replacement of vial caps
- using the same tip for pipetting different reagents
- reagent exposure to excessive temperature; bacterial or chemical contamination
- insufficient washing or filling of the wells (the wells should be filled to the upper edge), improper aspiration of Wash Solution remnants
- contamination of the well edges with Conjugate or samples
- using reagents from different kit lots
- contact of reagents with oxidants, heavy metals and their salts

The kit might be used for sequential examinations. When preparing working strength solutions, use only the amount of reagents needed for the analysis.

The kit might be used in all types of automatic EIA analysers.

If necessary, TestLine Clinical Diagnostics s.r.o. can offer a certified modification of the Instructions for Use for the specific type of analyser.

The producer cannot guarantee that the kit will function properly if the assay procedure instructions are not strictly adhered to.

14 Index of Avidity

14.1 Introduction

Antibody avidity expresses the strength of bond between antigen and antibody. Low avidity antibodies are produced in the early stages of a primary infection. As the infection progresses, immune response of organism matures and avidity of antibodies increases. Antibodies show high avidity in the latent phase of the disease. High avidity IgG antibodies are produced by memory B-cells from the beginning of a secondary infection or reactivation.

Determination of IgG antibodies avidity enables differentiation of various stages of infection and is a useful addition to serological diagnostics.

14.2 Test Principle

Avidity determination is based on dissociation of antigen-antibody bond by means of Avidity Solution (urea solution). After Avidity Solution treatment, low avidity antibodies are released and washed out while high avidity antibodies remain bound to the antigen. The binding strength is expressed by the Index of Avidity (IAv). IAv determines the portion of IgG antibodies that remains bound to antigen after incubation with Avidity Solution. IAv assay procedure is a modification of the standard ELISA procedure using Avidity Solution. The IgG avidity is determined only in IgG positive samples.

Result interpretation of 90% of serum samples will not be influenced by different serum dilution, i.e. whether a sample has been diluted 1:101 or more. However, IAv analysis of 10% of sera diluted 1:101 leads to high-avidity results while further dilution of such sera may provide a low-avidity outcome. Optimal results are obtained for antibody concentration of approx. 60 IU/ml. Therefore, one- or three-point analysis can be used for the test.

One-point analysis

Determine quantitatively antibody level in serum (in IU/ml) using the usual procedure (see Chapter Assay Procedure). Then dilute the analysed sample with Sample diluent to 60 IU/ml and determine the IAv in a subsequent ELISA test.

Three-point analysis

The IAv is directly determined without any previous analysis. In this procedure, three different serum concentrations are simultaneously analysed - the serum sample is diluted 1:101 (basic dilution) and then further 5× and 25× diluted. This procedure is especially useful in time-critical situations when rapid results are required (STAT examinations).

14.3 Preparation of Reagents

Allow all reagents including the Avidity Solution to come to room temperature and mix well. Crystals might develop in the vial with the Avidity Solution. Prior to use, it is necessary to dissolve the crystals by short-time warming up. The functionality of the Avidity Solution is indicated by yellow colour. The solution is thermolabile. The solution is deteriorated, if it changes its colour from yellow to red. A red-coloured Avidity Solution cannot be used further.

14.4 One-Point Analysis

14.4.1Dilution of samples

Determination of antibody concentration in IU/ml

Determine antibody concentration (IU/ml) by EIA Toxoplasma IgG test as described (see Chapter Assay Procedure).

Predilution to 60 IU/ml

1. If antibody concentration is lower than 60 IU/ml, do not dilute the sample.
2. If antibody concentration ranges from 60 to 240 IU/ml, dilute the sample with the Sample Diluent to 60 IU/ml (e.g. dilute the sample three times if the content of antibodies is 180 IU/ml).
3. If antibody concentration is higher than 240 IU/ml, dilute the sample 1:1010, repeat the analysis and multiply the result by 10. Then, dilute the sample with the Sample Diluent to 60 IU/ml according to the obtained antibody concentration.

Dilution of Samples for IAv analysis

Dilute a prediluted serum sample (60 IU/ml) 1:101 with the Sample Diluent (10 µl of sample + 1 ml of the Sample Diluent). Mix well.

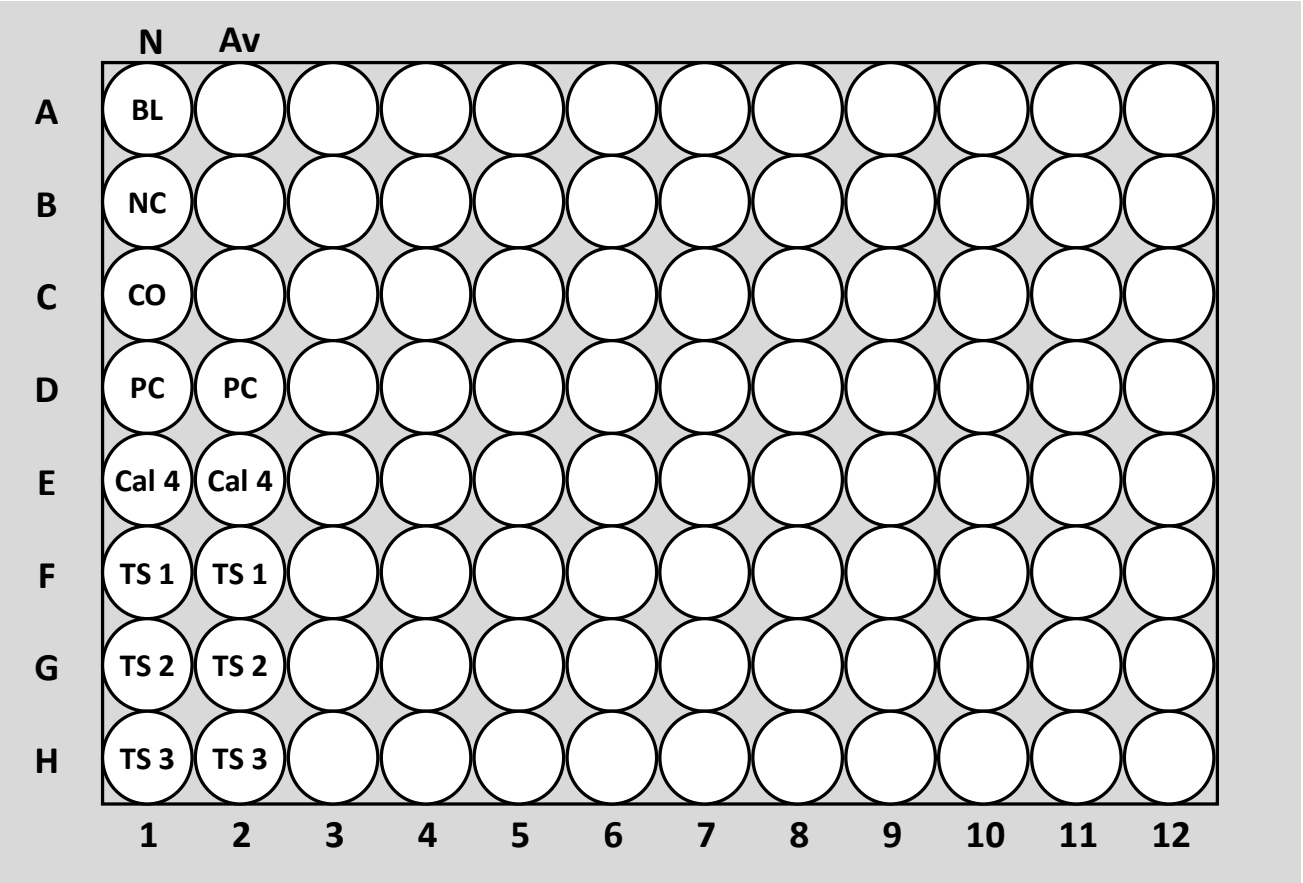
14.4.2Assay Procedure

1. Dispense the controls and the diluted samples according to the working schedule.

- Leave A1 well empty (blank).
- Pipette 100 µl of the Negative Control (Calibrator 1) 0.1 IU/ml into 1 well of N strip.
- Pipette 100 µl of CUT-OFF (Calibrator 2) 6 IU/ml into 1 well of N strip.
- Pipette 100 µl of the Positive Control (Calibrator 3) 60 IU/ml into two adjacent wells of N and Av strips.
- Pipette 100 µl of the Calibrator 4 (240 IU/ml) into two adjacent wells of N and Av strips.

- Pipette 100 µl of the diluted samples into two adjacent wells of N and Av strips.

Working Schedule of One-Point Analysis



N	strip for EIA (without the Avidity Solution)	
Av	strip for avidity test (incubation with the Avidity Solution)	
BL	Blank (empty well)	
NC	100 µl	CONTROL - CAL1
CO	100 µl	CUTOFF CAL2
PC	100 µl	CONTROL + CAL3
Cal 4	100 µl	CAL4
TS 1-x	100 µl	diluted tested samples

14.5 Three-point analysis

14.5.1Dilution of samples

Dilute each sample in 3 subsequent steps and determine the IAv of all three dilution.

Step 1. Dilute serum 1:101 with the Sample Diluent:
10 µl + 1 ml of the Sample Diluent (final dilution 1:101)

Step 2. Dilute serum prepared in Step 1with Sample diluent:
100 µl + 400 µl of the Sample Diluent (final dilution 1:505)

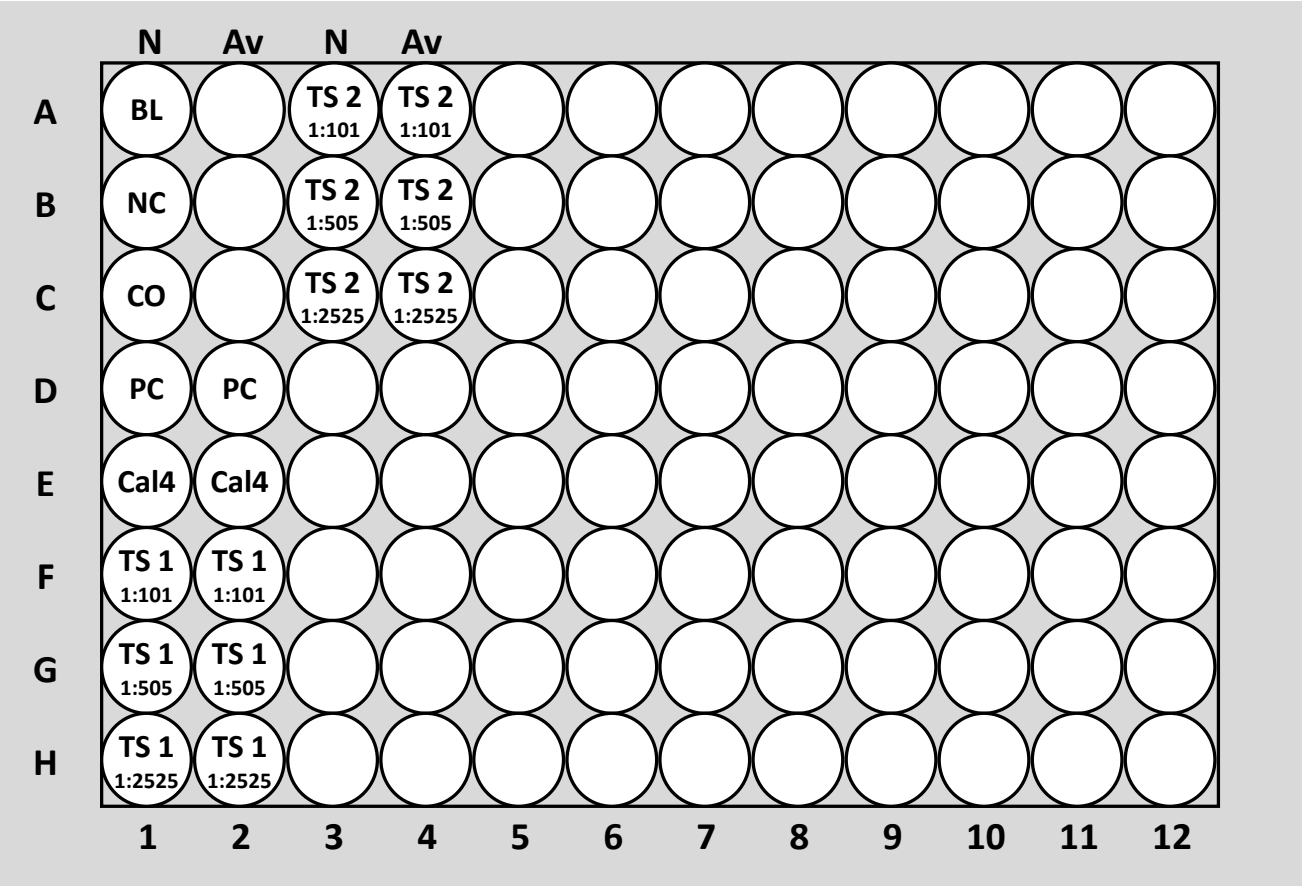
Step 3. Dilute serum prepared in Step 2 with the Sample Diluent:
100 µl + 400 µl of the Sample Diluent (final dilution 1:2525)

14.5.2 Assay Procedure

1. Dispense controls and diluted samples according to the working schedule.

- Leave A1 well empty (blank).
- Pipette 100 µl of the Negative Control (Calibrator 1) 0.1 IU/ml into 1 well of N strip.
- Pipette 100 µl of CUT-OFF (Calibrator 2) 6 IU/ml into 1 well of N strip.
- Pipette 100 µl of the Positive Control (Calibrator 3) 60 IU/ml into two adjacent wells of N and Av strips.
- Pipette 100 µl of the Calibrator 4 (240 IU/ml) into two adjacent wells of N and Av strips.
- Pipette 100 µl of samples diluted 1:101 in Step 1. into two adjacent wells of N and Av strips.
- Pipette 100 µl of samples diluted 1:505 in Step 2. into two adjacent wells of N and Av strips.
- Pipette 100 µl of samples diluted 1:2525 in Step 3. into two adjacent wells of N and Av strips.

14.6 Working Schedule of Three-Point analysis



N	strip for ELISA (without Avidity Solution)	
Av	strip for avidity test (incubation with Avidity Solution)	
BL	Blank (empty well)	
NC	100 µl	CONTROL - CAL1
CO	100 µl	CUTOFF CAL2
PC	100 µl	CONTROL + CAL3
Cal 4	100 µl	CAL4
TS 1-x	100 µl	diluted tested sample

Follow-up procedure is identical for both one- and three-point analysis.

1. Cover the microplate with the lid and incubate it at 37°C for 60 minutes.
2. Aspirate the content of the wells and wash 5× with the working strength Wash Solution. Fill the wells up to the edge. Finally, tap the inverted microplate thoroughly on an absorbent paper to remove solution remnants.
3. Pipette 100 µl of Avidity Solution into all wells of Av strips.
4. Pipette 100 µl of working strength Wash Solution into all wells of N strip.
5. Cover the microplate with the lid and incubate it at 37°C for 10 minutes.
6. Aspirate the content of the wells and wash 5× with the working strength Wash Solution. Fill the wells up to the edge. Finally, tap the inverted microplate thoroughly on an absorbent paper to remove solution remnants.
7. Pipette 100 µl of Conjugate into all wells except A1 well.
8. Cover the microplate with the lid and incubate it at 37°C for 60 minutes.
9. Aspirate the content of the wells and wash 5× with the working strength Wash Solution. Fill the wells up to the edge. Finally, tap the inverted microplate thoroughly on an absorbent paper to remove solution remnants.
10. Pipette 100 µl of TMB – Complete into all wells. Cover the microplate with the lid and incubate it at 37°C for 20 minutes. Keep out of light.
11. Stop the reaction by adding 100 µl of Stop Solution in the same order and intervals as the substrate was added.
12. Read the colour intensity in wells against blank (A1 well) using photometer set to 450 nm. The absorbance should be read within 30 minutes after stopping the reaction.

14.7 Quality Control

Quality control of the test is performed in N strips (see Chapter Quality Control).

The test is valid if:

Index of Avidity of the Positive Control (Cal 3) is lower than 30 %.

IAv | CONTROL + CAL3 | < 30 %

Index of Avidity of the Calibrator 4 is higher than 60 %.

IAv | CAL4 | > 60%

14.8 Results Interpretation

Calculation of Index of Avidity (IAv)

Divide the absorbance of a tested sample in Av strip by the absorbance of the tested sample in N strip measured in the same test run:

$$IAv = \frac{\text{Absorbance of sample in Av strip}}{\text{Absorbance of sample in N strip}} \times 100\ [\%]$$

Interpretation of the test results is described in Table 3.

If the three-point analysis is used, calculate the IAv for 1:101, 1:505 and 1:2525 sample dilutions. Determine the sample dilution with the antibody level closest to 60 IU/ml. The IAv of this sample dilution represents the relevant IAv result.

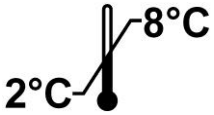
Table 3 Interpretation of test results

Index of Avidity (IAv) in %	Evaluation of avidity	Interpretation of results
< 30	low	acute toxoplasmosis <4 months after infection
30 – 35	borderline	repeat examination after 3 – 4 weeks
35 – 100	high	> 4 months after infection

Notes to interpretation

- Less than 5% of patients, who overcame toxoplasmosis and their disease, are not acute, show low avidity even after 4 month after the infection. The Index of Avidity remains lower than or equal to 30. It typically occurs in sera with low Anti-toxoplasma IgG concentration. Samples with specific IgG concentration lower than 25 IU/ml, especially when diluted (for three-point analysis), can give inadequately low IAv values.
- Three-point analysis cannot be evaluated if IgG concentration in a sample is higher than 5000 IU/ml.
- Examination of borderline samples should be repeated with a new sample collected after 3-4 weeks.
- Low as well as high levels of specific IgM and IgA antibodies can persist in some patients who have high IAv values.
- Results of IgG avidity examination should be interpreted only in the context of results of other tests, especially CFT, NIFR titres and IgG, IgM and IgA levels.

15 IFU Symbols

	Temperature limitation
	Keep dry
	Expiry date
	Lot number
	Manufactured by
	Consult instructions
	Catalogue number
	Number of tests
	In vitro diagnostic medical device

Notes

Notes

Summary of EIA Toxoplasma IgG Protocol

Step No.	Symbol	Test steps
1		Dilute samples serum/plasma 1:101 (10 µl + 1 ml) foetal and neonatal serum/plasma 1:101 (10 µl + 1 ml)
2		Pipette Controls and diluted samples – 100 µl Blank = empty well
3		Incubate at 37°C for 60 min
4		Aspirate and wash the wells 5×
5		Pipette Conjugate – 100 µl Blank = empty well
6		Incubate at 37°C for 60 min
7		Aspirate and wash the wells 5×
8		Pipette Substrate (TMB-Complete) – 100 µl Including blank
9		Incubate at 37°C for 20 min
10		Pipette Stop Solution – 100 µl Including blank
11		Read colour intensity at 450 nm