Chagatest

ELISA lisado

Enzyme-linked Immunosorbent assay (ELISA) for the detection of antibodies anti-*Trypanosoma cruzi*

SUMMARY

Chagas' disease is a parasitic infection caused by *Trypanosoma cruzi*. The laboratory diagnosis depends on the stage of the disease. During the acute phase, the diagnosis is done by identification of parasite in blood or through immunological methods that detect IgM. During the chronic phase, immunological methods may be used, such as hemagglutination, immunofluorescence, enzyme immunoassay or western blot.

PRINCIPLE

Chagatest ELISA lisado is an "in vitro" enzyme immunoassay for the qualitative detection of antibodies anti-*T. cruzi* in human serum or plasma samples.

The sample is diluted in the wells in which *T.cruzi* antigens are immobilized, corresponding to highly preserved zones among different strains. If the sample contains specific antibodies, they will bind to the antigens and remain bound to the solid phase. Unbound fractions are eliminated by washing, after which conjugate is added (monoclonal antibodies anti-human IgG, conjugated to peroxidase), which specifically reacts with immunocaptured antibodies anti-*T.cruzi*. The unbound conjugate is removed by washing. The presence of peroxidase bound to the complex is recognized by the addition of a chromogenic substrate, tetramethylbenzidine. The reactive samples develop a light blue color. The enzymatic reaction is stopped by the addition of sulphuric acid, producing a light blue to yellow color change. The optical density is measured bichromatically at 450/620-650 nm or at 450 nm.

PROVIDED REAGENTS

Coated microtitration plate: microtitration plate with removable strips and 96 wells containing *Trypanosoma cruzi* antigens.
Sample Diluent: saline buffer with surfactant. Violet color.
Concentrated Conjugate: monoclonal antibody anti-Human IgG, conjugated to peroxidase (10x). Red color.
Conjugate Diluent: saline buffer with proteins.
Substrate: tetramethylbenzidine and hydrogen peroxide solution.
Stopper: 2 N sulfuric acid.
Concentrated Wash Buffer: saline buffer with surfactant (25x). Green color.
Positive Control: inactivated human serum containing antibodies anti-*Trypanosoma cruzi.* Orange color.
Negative Control: inactivated human serum, non-reactive. Yellow color.

NON-PROVIDED REAGENTS

Distilled or deionized water

REQUIRED MATERIAL (non-provided)

- Micropipettes for measuring stated volumes
- Disposable tips
- Volumetric material to prepare stated dilutions
- 37ºC incubator
- Absorbent paper
- Disposable gloves
- Timer or stopwatch
- Sodium hypochlorite
- Microtitration plate wash system (manual or automatic)
- Spectrophotometer for microtitration plate reading

WARNING

- Reagents are for "in vitro" diagnostic use.
- All patient samples should be handled as capable of transmitting infection.
- The control sera have been tested for hHepatitis B surface antigen (HBsAg) and antibodies against hepatitis C virus (HCV) and human immunodeficiency virus (HIV) and found to be negative. However, because no test method can offer complete assurance that infectious agents are absent, they should be handled as infectious material.
- To ensure the inactivation of pathogenic agents, all materials used to perform the test must be decontaminated before disposal. The recommended method is autoclaving one hour at 121ºC. Liquid waste may be disinfected with sodium hypochlorite (5% final concentration) for at least 60 minutes.
- Do not exchange reagents from different lots.
- Do not use reagents from other origin.
- Avoid touching the walls of the wells with the tips.
- Avoid using metal objects that may be in contact with the reagents.
- The microtitration plates should be placed in incubator. Do not open the incubator during this process. Do not use water bath.
- Avoid contact of strips with hypochlorite fumes from biohazards disposal containers or other sources, since hypochlorite affects the reaction.
- Avoid contact of the sulfuric acid (Stopper) with the skin and eyes. If this occurs, rinse the affected area with copious quantities of water. R36/38: irritates eyes and skin, R34 causes burns, S24/25: avoid contact with the eyes and skin. S26: if splashing onto skin occurs, rinse the affected area with copious quantities of water and seek medical attention, S28: after contact with skin, wash immediately with plenty of water, S37/39: wear suitable gloves and eye/face protection.
- Avoid the spill of liquids and the formation of sprays.

- Do not pipette by mouth.
- All reagents and samples should be discarded according to current regulations.

REAGENT PREPARATION

All the material used for reagent preparation should be clean and free from detergent and hypochlorite.

- Wash Buffer: constituents of the concentrated reagent may precipitate at low temperature. In such case, bring the solution to 37ºC until complete dissolution. To obtain a ready-to-use wash buffer, dilute 1 part Concentrated Wash Buffer (25x) with 24 parts distilled or deionized water. Example: for one microtitration plate, 20 ml with 480 ml.

Conjugate: dilute 1 part Concentrated Conjugate (10x) with 9 parts Conjugate Diluent. Example: see table with concentrated Conjugate and Conjugate Diluent required volumes.

Nº of wells	Concentrated Conjugate	Conjugate Diluent
8	100 ul	0.9 ml
16	200 ul	1.8 ml
24	300 ul	2.7 ml
32	400 ul	3.6 ml
96	1200 ul	10.8 ml

Coaed microtitration plate, Sample Diluent, Conjugate Diluent, Substrate, Stopper, Negative and Positive Controls: ready to use.

STABILITY AND STORAGE INSTRUCTIONS

Provided Reagents are stable at 2-10ºC until expiration date stated on the box. Do not freeze.

Concentrated Wash Buffer and Stopper: they may be stored at 2-25ºC.

Wash Buffer (1x): once diluted is stable for up to 3 months at 2-25ºC.

Conjugate: once diluted is stable for up to 6 hours at 2-25ºC.

Sensitized microtitration plate: do not open the pouch until performing the test and until it has reached room temperature. Otherwise, the contents could get moistened. Unused well strips should be kept in the pouch with the desiccant and stored at 2-10ºC. Test strips stored in this manner are stable for 4 months if it does not exceed the date printed on the pouch label.

SAMPLE

Serum or plasma

a) Sample collection: obtain in the usual way.

b) Additives: not required for serum. Employ plasma collected in EDTA, heparin or citrate based anticoagulants.

c) Known interfering substances: no interference has been observed with bilirubin up to 21 mg/dl, ascorbic acid up to 50 mg/dl, triglycerides up to 1500 mg/dl or hemoglobin up to 300 mg/dl. Samples containing particles should be clarified by centrifugation.

d) Stability and storage instructions: sample should be stored at 2-10ºC. If the test is not performed within 72 hours, sample should be frozen at -20ºC. Samples should not be repeatedly frozen and thawed. This may lead to erroneous results. In case of using frozen samples, they should be homogenized and centrifuged before use. Heat inactivation may affect the result. Do not use samples with microbial contamination. If samples are to be transported, they should be packaged according to local regulations in force for biohazards materials shipment.

TEST PROCEDURE

1- Bring reagents and samples to room temperature before use.

2- Prepare the necessary volume of diluted wash buffer (1x)

3- Place the number of required wells in the strip holder for the quantity of determinations to be used, including 2 wells for Positive Control (PC) and 3 for Negative Control (NC).

4- Dispense Sample Diluent, then sample (S) and controls according to the following scheme:

	S	PC	NC
Sample Diluent	100 ul	100 ul	100 ul
Positive Control	-	20 ul	-
Negative Control	-	-	20 ul
Sample	20 ul	-	-

Homogenize mixing 2-3 times by loading and unloading the micropipette. When sample is added, Sample Diluent will change color, according to the following:

Sample type	Without sample	Serum or plasma	Positive Control	Negative Control
Color	Violet	Light blue	Dark orange	Green

Control or sample dispensing to the wells may be inspected visually or by spectrophotometric reading (at 610/650 nm).

Warning: turbid, hemolyzed or jaundice samples may change the final color without affecting the results. Color change may depend on the added sample volume and its composition. A less intense color change may be due to a lower sample volume dispensed, sample not being in the appropriate conditions or a low protein level.

5- To avoid evaporation, cover the plate with the provided adhesive tape and incubate for 30 ± 2 minutes at 37ºC ± 1ºC. At the same time, prepare the diluted conjugate (see table in REAGENTS PREPARATION).

6- After incubation, completely remove the liquid from each well. Wash 5 times according to washing instruction (see Washing Procedure).

7- Add Conjugate:

Conjugate	100 ul	100 ul	100 ul

To avoid evaporation cover the microplate with adhesive tape.

8- Incubate for 30 ± 2 minutes at 37ºC ± 1ºC.
9- Wash 5 times according to the washing instructions.
10- Dispense the Substrate. Transfer to a clean recipient only the required Substrate volume. Do not transfer the remaining Substrate to the original bottle. Avoid reagent contact with oxidizing agents.

Substrate	100 ul	100 ul	100 ul

11- Incubate for 30 ± 2 minutes at room temperature (18-25ºC), protecting from light.
12- Add the Stopper:

Stopper	100 ul	100 ul	100 ul

13- Measure absorbance in spectrophotometer bichromatically at 450/620-650 nm or at 450 nm.
Note: bichromatic reading is always recommended. If reading is monochromatic, perform a reagent blank which, in turn, must be subtracted from all sample values.

STABILITY OF THE FINAL REACTION
Reaction color is stable for 10 minutes. Thus, results should be read within this period.

WASHING PROCEDURE
Remove the liquid from the wells by aspiration or inversion. The wells are washed with 300 ml diluted wash buffer. When filling the wells make sure not to spill. The wash solution should be in contact with the wells for 30 to 60 seconds. Make sure no residual liquid remains after the final washing step. Perform double aspiration to remove excess buffer. If after such procedure, it still persists, invert the plate onto absorbent paper and tap it several times. Otherwise, erroneous results may be obtained.
Note: the washing procedure is crucial for the test result. If excess wash buffer remains in the wells, or if the wells are not completely filled, erroneous results may be obtained. Do not let the wells air dry during the procedure. Automatic washers should be rinsed with distilled or deionized water at the end of the day to avoid obstructions due to the presence of salt in the wash buffer.

SUMMARY OF THE PROCEDURE

STAGE	PROCEDURE	WARNINGS/OBSERVATIONS
Dilution	Prepare Wash solution (1x)	Dissolve salt crystals
Sample Diluent	Add 100 ul Sample Diluent in each well	
Samples	Add 20 ul S, PC and NC	Color change is observed when adding the sample and controls
Incubation	Cover the wells and incubate for 30 ± 2 minutes at 37 ± 1ºC	In incubator
Washing step	Wash each well with 300 ul diluted Wash Buffer (5 times)	Time of contact of the Wash solution from 30 to 60 seconds. Completely remove the residual liquid from the wells
Dilution	Conjugate preparation (1x)	During incubation with the sample, dilute the Concentrated Conjugate (10x)
Conjugate	Add 100 ul diluted Conjugate	
Incubation	Cover the wells and incubate for 30 ± 2 minutes at 37 ± 1ºC	In incubator
Washing step	The same as above	
Substrate	Add 100 ul Substrate	Transfer the required Substrate volume to be used. Do not pipette from the original bottle. Discard the remaining reagent. Avoid contact with oxidizing agents. Do not expose to light
Incubation	30 ± 2 minutos at 18-25ºC	Keep the wells protected from light
Stop	Add 100 ul Stopper	
Reading	Read in spectrophotometer	Read within 10 minutes

ASSAY VALIDATION CRITERIA

The assay is considered valid if the following conditions are simultaneously met:

1- The optical density (O.D) average of the Negative Controls should be less than or equal to 0.100.
Example:
Reading 1 = 0.034, Reading 2 = 0.028, Reading 3 = 0.029
Average = (0.034+0.028+0.029) / 3= 0.030

2- Remove any Negative Control with O.D. greater than 0.100.

3- If any Negative Control has been removed, recalculate the Negative Control average. An assay is valid when at least two of the Negative Controls are accepted.

4- The Positive Controls O.D average should be greater or equal to 1.300.
Example:
Reading 1 = 1.697, Reading 2 = 1.774
Mean = (1.697+1.774) / 2 = 1.736

5- The difference between the O.D average of the Positive and Negative Controls should be greater or equal to 1.200.

In case one of the above conditions is not met, repeat the assay.
Remember that the obtained readings will depend on the sensitivity of the instrument used.

INTERPRETATION OF RESULTS

a) With optical instruments

The presence or absence of antibodies anti-*T.cruzi* is determined, associating the sample absorbance with the Cut-off value.

Cut-off = NC + 0.200

NC: Negative Control O.D average

Example: 0.030 + 0.200 = 0.230

Non-reactive samples: samples with absorbance value lower than the Cut-off value.
Reactive samples: samples with absorbance value greater or equal to the Cut-off value.

b) Visual interpretation

If this type of interpretation is selected, every sample not presenting more color than the Negative Controls should be considered non-reactive. On the contrary, an evidently yellow sample is considered Reactive.

All samples initially reactive should be repeated by duplicate. If one or both repetitions are positive, it should be considered reactive.
A sample initially reactive may be non-reactive in both repetitions. This may be due to:
- Cross contamination of a non-reactive well with a reactive sample.
- Sample contamination during dispensation, lack of precision in sample, conjugate and/or Substrate dispensation into the well.
- Tip reutilization.
- Well contamination with hypochlorite or other oxidizing agents.

In certain cases a non-reactive sample may produce a falsely reactive reaction, both in the initial analysis as in its repetitions. Some probable causes of this effect may be:
- Sample contamination during collection, processing or storage.
- Presence of interfering substances, such as autoantibodies, drugs, etc.
- Ineffective dispensation and/or aspiration of the wash solution (obstructed system).

PROCEDURE LIMITATIONS

See Known interfering substances under SAMPLE.
Do not use pooled samples.
Do not use other body fluids such as saliva, cerebrospinal fluid or urine.
Any reactive result must be verified by another technique. Remember the criteria recommended by the Fatala Chabén Institute, whereby immunodiagnosis of infection must be performed with 2 of the following methods at a minimum: indirect immunofluorescence, indirect hemagglutination and ELISA, duly validated by the National Reference Center.

SPECIFIC PERFORMANCE FEATURES

a) Sensitivity

Clinical Sensitivity in Performance Panels

In a study performed on different international commercial panels, the following results were obtained:
PMT 201 (Anti-*T.cruzi* Performance Panel, BBI, USA): 14 out of 14 reactive samples were detected.
PMT 202 (Anti-*T.cruzi* Performance Panel, BBI, USA): 14 out of 14 reactive samples were detected.
PP 0409 (Performance Panel for Chagas, Q Panel, Brazil): 16 out of 16 reactive samples were detected.
PP 0508 (Performance Panel for Chagas, Q Panel, Brazil): 16 out of 16 reactive samples were detected.

Clinical Sensitivity in Panels of reactive anti-T.cruzi samples

In a study performed on 85 samples from children from endemic regions with *T. cruzi* infection, confirmed by different methods, all of the samples were found reactive with the **Chagatest ELISA lisado** kit.
In a study of 89 reactive samples from a hospital institution, all the samples were detected.

b) Specificity

In a study performed on 739 sera and plasma samples from blood banks, the obtained specificity was 99.29%.
In other study performed on 251 sera and plasma samples from blood blank, the specificity obtained was 99.60%.
A possible cross-reactivity was evaluated, assaying samples from 341 individuals with different clinical conditions that may be the cause of unspecific reactions for the **Chagatest ELISA lisado** test. These conditions include pregnant wo-men, hemodialyzed patients, patients with autoimmune diseases or infectious diseases other than Chagas (HIV, HTLV, hepatitis C, hepatitis B, syphilis, others). For this population the specificity was 99.04%.

c) Precision

The test precision was evaluated following EP5A protocol

recommended by the NCCLS. The assays were performed using samples having different reactivity levels and controls. One daily assay was performed testing each sample by quadruplicate during 5 days.

	Mean (O.D.)	Intra-assay		Total	
		S.D.	C.V.	S.D.	C.V.
Sample 1	0.590	0.021	3.54%	0.041	6.92%
Sample 2	0.993	0.043	4.34%	0.067	6.72%
(+)Control	2.255	0.062	2.76%	0.087	3.86%
(-)Control	0.025	0.001	4.88%	0.002	7.38%

n = 20

WIENER LAB. PROVIDES

- 96 tests (Cat. Nº 1293096)
- 192 tests (Cat. Nº 1293192)

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- User Demostration of Performance for Precision and Accuracy - Approved Guideline EP15-A (2001).

SYMBOLS EXPLANATION

Policubeta | Sensib.
Coated microtitration plate

Diluyente | Muestra
Sample Diluent

Conjugado | Conc.
Concentrated Conjugate

Conjugado | Diluy.
Conjugate Diluent

Revelador
Substrate

Buf. Lavado | Conc.
Concentrated Wash Buffer

Control | +
Positive Control

Control | -
Negative Control

Stopper
Stopper

The following symbols are used in packaging for Wiener lab. diagnostic reagent kits.

This product fulfills the requirements of the European Directive 98/79 EC for "in vitro" diagnostic medical devices

EC REP Authorized representative in the European Community

IVD "In vitro" diagnostic medical diagnostic device

Contains sufficient for <n> tests

Use by

Temperature limitation (store at)

Do not freeze

Biological risks

Volume after reconstitution

Cont. Contents

LOT Batch code

Manufactured by:

Xn Harmful

Corrosive / Caustic

Xi Irritant

Consult instructions for use

Calibr. Calibrator

CONTROL Control

CONTROL + Positive Control

CONTROL - Negative Control

REF Catalog number

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