Vitrotest®

Vitrotest Borrelia-IgG

ELISA test-kit for the detection of IgG class antibodies to *Borrelia burgdorferi*

Instruction for use

IVD	For in vitro diagnostic use
REF	TK084

ELISA test for the diagnosis of infectious diseases TS (ТУ.У 24.4 – 36555928 – 001:2011)

«Vitrotest Borrelia-IgG»
ELISA test-kit for the detection of IgG class antibodies to *Borrelia burgdorferi*

1. Intended use

ELISA test-kit «Vitrotest Borrelia-IgG» is intended for the detection of IgG class antibodies to *Borrelia burgdorferi* in human serum or plasma.

The test kit may be used both for the ELISA using automatic pipettes and standard equipment and for setting with the open-system immunoenzymatic automated analyzer.

2. Clinical value

Lyme disease (tick borreliosis) is the most common disease in the northern hemisphere, which is transmitted by ticks. The name of the disease comes from the town of Old Lyme (USA), where in the mid-1970s has been described a number of cases of arthritis after tick bites. The causative agent of Lyme disease – spirochete from group *Borrelia burgdorferi* – was first identified in 1982 by the american microbiologist Willie Burgdorferi.

Signs and symptoms of Lyme borreliosis, as well as other diseases caused by spirochetes, occur in stages and related with lesions of various tissues and organs, including skin, joints, heart and nervous system. Early disease (stage 1) is characterized by primary migrating erythema - ringlike skin rash (diameter 10-20 cm), which begins in a few days or weeks after the tick bite; It occurs in 60-80% of patients. Other manifestations of the disease are nonspecific: chills, fever, headache, aching muscles, severe weakness and fatigue. Stage 2 begins in a few weeks or months after the localized stage. It is caused by hematogenous spread of spirochetes, which can lead to multiple skin lesions (secondary erythema migrans) and meningitis, neuritis, arthritis and myocarditis. Chronic infection (stage 3) can arise from several months to several years after the primary lesion and cause acrodermatitis chronica atrophicans (ACA), varying degrees of encephalopathy and encephalomyelitis, and persistent arthritis.

Thus, Lyme disease is characterized by very diverse clinical picture, which may complicate its timely diagnosis. Early diagnosis is based on clinical and epidemiological data. The diagnosis is confirmed by laboratory, mainly serological methods - detection of specific antibodies to *Borrelia burgdorferi* in the blood. Serological methods have high sensitivity and specificity, unlike such as crop, pathogen antigen detection and histological examination of the skin, which additionally is invasive.

Specific IgM class antibodies appear within the first few weeks after infection, in high titers they are usually in patients with clinical signs of early infection. IgG antibodies begin to appear in 4-6 weeks after infection, the maximum titer of IgG-antibodies is synthesized in 2-3 months after the onset of early symptoms. IgG antibodies titer slightly reduced after 2-4 months after successful antimicrobial therapy, but continue to occur and circulate in the blood for a long time (from several months to several years). However, IgG antibodies often are not detected at an early, localized stage of the disease or after early designated antimicrobial therapy. By tick-borne borreliosis is not produced projective immunity - people who recover from it may have re-infection in a few years.

3. Principle of the test

Principle of the test of «Vitrotest Borrelia-IgG» kit is based on an indirect enzyme immunoassay technique.

The solid phase is made of strip microplate coated with antigens of *Borrelia burgdorferi*. During incubation of samples in wells of ELISA plate specific to *Borrelia burgdorferi* antibodies are bound to the antigen on the solid phase. After washing out unbound components anti-specific conjugate of anti-IgG monoclonal antibodies with horseradish peroxidase added to the wells, it binds to immune complexes in the solid phase. Unbound components are washed out. The immune complex formed in the wells are visualized by adding chromogen solution of 3,3',5,5'- tetramethylbenzidine (TMB). As a result of the reaction solution in wells where immune complexes were formed would be painted in blue. The reaction is stopped by adding stop reagent, blue colored wells become yellow. The result of the analysis is determined by spectrophotometric reading at 450/620 nm.

4. Materials and equipment

4.1 Contents of the kit

ELISA plate – 12 strips of 8 wells (with the possibility of separation of the wells) with immobilized recombinant antigens of *Borrelia burgdorferi*.

Positive control – 1 vial containing 0,3 ml solution of human antibodies specific to *Borrelia burgdorferi* (pink).

Negative control – 1 vial containing 0,5 ml negative human serum (yellow).

Sample diluent – 1 bottle containing 12 ml buffer with skim milk extract, detergent and preservatives (violet).

Conjugate solution – 1 bottle containing 12 ml buffer solution of monoclonal antibodies to human IgG conjugated with horseradish peroxidase, with stabilizers and preservatives (green). Ready to use.

TMB Solution – 1 bottle containing 12 ml of TMB solution and hydrogen peroxide, with stabilizers and preservatives (colourless).

Washing solution Tw20 (20x) – 1 bottle containing 50 ml 20-fold concentrated phosphate buffer with Tween-20 (colourless).

Stop-reagent – 1 vial containing 12 ml of 0,5M sulphuric acid solution (colourless).

Adhesive film – 2 sheets of adhesive film for covering the plates during incubation.
Sera identification plan – 1 sheet of paper for noting the schemes of samples distribution.
Instruction for use – one copy of user manual.

4.2 Additional reagents, materials and equipment

In order to conduct the analysis, the following additional reagents, materials and equipment are required:

– deionized or distilled water;
– filter paper;
– graduated cylinders of 10, 200 and 1000 ml capacity;
– disposable gloves;
– hydrogen peroxide solution 6%;
– disposable glassware for preparing the reagents (bottles and trough);
– timer;
– mono- and multi-channel automatic adjustable pipettes capable of delivering volumes of 20, 200 and 1000 microliters and tips for them;
– thermostat for 37 °C;
– container for solid waste;
– container for liquid waste;
– [1]automatic or semi-automatic washer;
– [2]mono- or multi-channel reader for microplates at 450/620-695 nm.

[1,2] Please, consult us about the adaptation of your equipment.

5. Reservations and safety precautions

5.1. Reservations:

– do not use expired reagents;
– do not use in the analysis and do not mix components of different lots and components of test kits with different nosology;
– do not use reagents of other manufacturers in composition with the Vitrotest® sets;

- Note: possible to use washing solution Tw20 (20X), TMB solution and Stop-reagent with other series that are different from those attached to the test kit. These reagents are used in other test systems of Ramintek IPC. Please consult us for details.

– close reagent vials after use only with their appropriate caps;
– strictly follow to the washing procedure requirements described in this instruction;
– control the filling and full aspiration of the solution in the wells;
– use a new distribution tip for each serum and reagent;
– protect kit reagents from straight sun rays;
– TMB solution must be colourless or light blue before its use. If solution is dark blue or yellow, it cannot be used. Avoid any contact of the TMB solution with metals or metal's ions. Use glassware thoroughly washed and rinsed with deionized water.
– use only disposable pipette tips for adding TMB-substrate into plate's wells;
– never use the same glassware for conjugate solution and chromogen.

5.2. Safety precautions:

– all reagents included in the kit are intended for "in vitro" diagnostic use;
– wear disposable gloves when perform analysis;
– do not pipette by mouth;
– the controls of «Vitrotest Borrelia-IgG» are negative for anti-HCV, anti-HIV1/2, anti-*T.pallidum* antibodies and HBsAg. Nevertheless, all controls and sera should still be regarded and handled as potentially infectious;
– the liquid waste must be inactivated, for example, with the hydrogen peroxide solution at the final concentration of 6% for 3 hours at room temperature, or with the sodium hypochlorite at the final concentration of 5% for 30 minutes, or with other disinfectant agents;
– the solid waste must be inactivated with autoclaving at 121°C for 1 hour;
– do not autoclave the solutions that contain sodium azide or sodium hypochlorite;
– avoid spilling of TMB-solution and Stop-reagent and any contact of these solutions with the skin or mucosa;
– in case of spilling of solutions, that do not contain acid, e.g. sera, rinse the surface with hydrogen 6% solution, then dry with filter paper.

6. Storage and stability

Reagents of the kit are stable up to the expiry date on the label, when stored at 2-8 °C.

Transport the test-kit at 2-8 ºC. Disposable transportation at temperature not higher than 20ºC during two days is allowed.

7. Specimen collection

The serum or plasma samples should be stored at 2-8 ºC not more then 3 days after collection. It is possible to store them longer, but frozen (-20 to-70 ºC). Before use frozen samples, wait for 30 minutes for the reagents to stabilize at room temperature. Mix thawed samples well to homogeneity. Avoid repeated freezing/thawing. Samples containing aggregates must be clarified by centrifugation. Do not use samples with contaminated, hyperlipeamic and hyperhaemolysed sera.

8. Reagent preparation

It is very important to bring all reagents of the «Vitrotest Borrelia-IgG» kit to room temperature (18-25°C) for 30 minutes before use.

8.1. ELISA plate preparation

Before opening the ELISA plate, allow it to stabilize at room temperature for 30 minutes to avoid water condensation inside the wells. Open the vacuum bag and remove necessary amount of wells. Immediately after removal of wells, the remaining strips should be **resealed with zip-lock and stored at 2-8 °C**. Microplate in thus packed bag is stable for 3 month.

8.2. Washing solution

The vial contains 50 ml of a concentrated 20X buffer with detergent. Dilute the washing solution 1:20 (1+19) with distilled or deionised water, then mix. For example: for 4 ml of concentrate – 76 ml of distilled water is enough for one strip. Crystals in the solution disappear by warming up to 37°C for 15-20 min.

The diluted washing solution can be stored at 2-8°C not more than 7 days.

9. Assay procedure

9.1. Take out from the protective packing the support frame and the necessary number of wells (the number of investigated samples and four wells for controls). Wells with the controls must be included in each analysis.

9.2. Complete the sera identification plan.

9.3. Prepare washing solution (refer to point 8.2).

9.4. Dispense 90 µl of sample diluent in each well.

9.5. Dispense 10 µl of controls and unknown samples into the wells in the following order: A1 – positive control, B1, C1 and D1 – negative control, E1 and rest wells – unknown samples. Gently pipette the mix in wells, avoiding foaming. After addition of serum color of the solution in wells changes from violet to blue**.**

9.6. Cover strips with an adhesive film and incubate for 30 min at 37 °C.

9.7. After completing the incubation remove the adhesive film carefully and wash the wells five times using the automatic washer or 8-channel pipette in the following order:

– aspirate the content of wells strips into a liquid waste container;

– fill the strip wells with a minimum of 300 microliters of washing solution for each well (respect the soak time of a minimum of 30 seconds);

– aspirate the solution of all wells, the residual volume of solution after aspiration must be lower than 5 microliters;

– repeat the washing step four more times;

– after the last aspiration blot the microplate by turning it upside down on absorbent paper.

9.8. Dispense 100 µl of conjugate solution per well. Cover strips with an adhesive film, incubate for 30 min at 37°C.

9.9. After completing the incubation remove the adhesive film carefully and wash the wells five times as described above (refer to point 9.7).

9.10. TMB-solution is ready to use TMB-substrate solution with hydrogen peroxide. TMB-solution should be colorless, protect TMB-substrate solution from straight sun rays. To add TMB-solution only new tips must be used: carefully select a TMB-solution from the vial and without touching the bottom and walls of the hole plate, add 100 µl TMB solution per well.

9.11. Incubate the strips for 30 minutes at room temperature of 18-25°C in the dark. Do not use adhesive film in this incubation.

9.12. Add 100 µl of stop-reagent in each well. Respect the same distribution sequence than for the TMB-substrate solution.

9.13. Read the optical density of every strip well in dual wavelength reading at 450/620 nm, within the 5 minutes of stopping the reaction. Pay attention to the cleanness of the wells bottom outside.

Measurement in the single-wave procedure at 450 nm is possible. Reserve blank well to adjust spectrophotometer in such analysis. Only TMB-substrate and stop-reagent must be added in blank well.

10. Calculation and interpretation of the results

10.1. Test validity conditions:

Calculate the mean optical density (OD) of negative control

$$OD\ NC_{mean} = (OD\ NC1 + OD\ NC2 + OD\ NC3)/3.$$

In order for an assay to be considered valid, the following criteria must be met:

– OD of positive control is not lower than 1,2 optical unit (OU),

– OD of negative controls should be lower or equal to 0,15 OU,

– OD of every negative control differs no more than twice from the mean value of negative control that is

$$OD\ NC_{mean} \times 0,5 \leq OD\ NCn \leq OD\ NC_{mean} \times 2,0.$$

If one of negative controls does not respect this norm, disregard and recalculate the mean using remaining values.

10.2. Calculation of the results.

Calculate cut-off by adding value 0,30 to the mean NC, that is

$$Cut\ off = OD\ NC_{mean} + 0,30.$$

Calculate the index of positivity (IP) for each sample

$$IP = \frac{ODsample}{Cut\ off}$$

10.3. Interpretation of the results

The samples with IP above 1,1 are considered **positive (IP > 1,1)**.

The samples with IP below 0,9 are considered **negative (IP < 0,9**).

The samples with IP **within 0,9-1,1** are considered **indeterminate (0,9≤ IP ≤1,1)**. It is recommended to retest the appropriate samples in duplicate. If the results are again within indeterminate, it is necessary to test sera obtained after 2-4 weeks. If you get undefined results assume that the serum does not contain specific antibodies to *Borrelia burgdorferi*.

11. Performance characteristics

11.1. Specificity and sensitivity

Diagnostic characteristics of the test «Vitrotest Borrelia-IgG» was determined in comparative studies with other two commercial test kits, one of which has a CE marking. Among 82 negative and 219 positive for anti-Borrelia IgG antibodies sera was observed 99% coincidence of positive and 98% coincidence of negative results with both commercial sets.

11.2. Accuracy

Intra assay reproducibility

Coefficient of variation (CV) for two sera with different levels of specific antibodies was calculated in 48 repetitions on two series of test kits.

Serum №	IP	CV_1, %	CV_2, %
57D	1,73	7,1	6,8
0363	5,26	5,6	5,2

Inter assay reproducibility

Coefficient of variation (CV) for two sera with different levels of specific antibodies was calculated for three days in four ELISA performances, in four repetitions for each analysis.

Serum №	IP	CV, %
57D	1,85	7,0
0363	4,95	5,3

12. Limits of the test

A positive result in the test «Vitrotest Borrelia-IgG» is an evidence of presence in patient of IgG class antibodies specific to *Borrelia burgdorferi*.

A negative result in the test «Vitrotest Borrelia-IgG» indicates the absence of antibodies to *Borrelia burgdorferi* in studied human serum, or the concentration of specific antibodies is below the sensitivity of analysis.

We cannot completely exclude false-positive results that may be due to the presence in blood of specific antibodies for diseases caused by spirochete (syphilis, relapsing fever).

The final diagnosis cannot be established only on the basis of serological test. At diagnosis should take into account the complex of laboratory and instrumental studies and clinical manifestations of disease.

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Legend

Interpretation of notation conventions:

IVD	For in vitro diagnostic use
LOT	Batch code
REF	Catalogue number
	Production date
	Expiry date
	Storage temperature limitation
	Meant for <n> tests
	Protect from direct solar radiation
	Attention! Consult instruction for use
	Manufacturer and its address
	Consult instructions for use

For questions and suggestions regarding the kit, contact the manufacturer:

Ramintek Innovation-Production Company
03039 Ukraine, Kiev, 7 October 40th Anniversary Av., of. 227 (registered address)
07300 Vishgorod, Kiev region, 19 Sholudenko Str. (factual address)
Tel. +380 44 222-76-72

Scheme of the assay «Vitrotest Borrelia-IgG»

Keep reagents at room temperature (18-25°C) during 30 minutes

Prepare washing solution, dilute 20x concentrate washing solution *Tw20* with distilled water 1:20 (1+19). For example, 4 ml of solution + 76 ml of water

Complete the sera identification plan

Dispense 90 µl of sample diluents into the wells

Dispense 10 µl of controls and patient samples into the wells:
A1 – positive control,
B1, C1, D1 – negative control,
E1 and other wells – patient samples
After dispensing of serum the color in well switches from violet to blue

Cover wells with adhesive film, incubate for 30 min at 37°C

Wash wells five times

Dispense 100 µl of conjugate solution (green) into the wells

Cover wells with adhesive film, incubate for 30 min at 37°C

Wash wells five times

Dispense 100 µl of TMB substrate solution into the wells

Incubate the plate for 30 min in the dark at room temperature (18-25°C)

Add 100 µl of stopping solution in each well

Read optical density at 450/620 nm

Calculate the cut-off of the assay «Vitrotest Borrelia-IgG»:

Cut-off = OD NC mean + 0,3

Calculate the index of positivity (IP) for patient samples: $IP = \frac{OD\ of\ patient\ sample}{cut\ off}$

Interpret the results according to the table:

IP value	Result
IP_{sample} >1,1	**positive**
0,9≤ IP_{sample} ≤1,1	**indeterminate**
IP_{sample} <0,9	**negative**