CE

Declaration of Conformity

EUROIMMUN Medizinische Labordiagnostika AG
Seekamp 31, D-23560 Lübeck, Germany

declare under our sole responsibility that the WESTERBLOT products

Anti-Borrelia burgdorferi-WESTERNBLOT (IgG)	*DY 2132-#### G*
Anti-Borrelia burgdorferi-WESTERNBLOT (IgM)	*DY 2132-#### M*

(product name, order no)

meet the demands of

Directive 98/79/EC on in vitro diagnostic medical devices of 27 October 1998 and its transpositions in national laws which apply to it.

Conformity assessment procedure: Annex III

Digitally signed by Ceaicovschi Tudor
Date: 2024.03.12 15:51:12 EET
Reason: MoldSign Signature
Location: Moldova

Lübeck, 04.10.2016
(Place and date of issue)

Dr. Wolfgang Schlumberger
- Member of the Board -

Susanne Aleksandrowicz
- Member of the Board -

Anti-Borrelia burgdorferi-WESTERNBLOT (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
DY 2132-3001 G DY 2132-24001 G	Borrelia burgdorferi (complete antigens)	IgG	Antigen coated membrane strips	30 x 01 (30) 240 x 01 (240)

Indication: The Westernblot test kit provides a qualitative in vitro assay for human autoantibodies of the immunoglobulin class IgG against Borrelia in serum or plasma for the diagnosis of Lyme borreliosis and associated diseases (Erythema chronicum migrans, lymphadenosis cutis benigna, acrodermatitis chronica atrophicans, arthritis, carditis, lymphocytic meningoradiculoneuritis and neuroborreliosis).

Principles of the test: The test kit contains test strips with electrophoretically separated antigen extracts of Borrelia burgdorferi. The blot strips will be blocked and incubated in the first reaction step with diluted patient samples. In the case of positive samples, specific antibodies of the class IgG (and IgA, IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (enzyme conjugate) catalysing a colour reaction.

Contents of the test kit:

Component	Format	Format	Symbol
1. Test strips Single strips with electrophoretically separated Borrelia burgdorferi antigens	30 x 1	240 x 1	STRIPS
2. Evaluation matrix with control strip Test strip incubated with a positive control serum	1 pattern	1 pattern per test strip lot	---
3. Enzyme conjugate Alkaline phosphatase-labelled anti-human IgG (goat), 10x concentrate	2 x 3 ml	16 x 3 ml	CONJUGATE 10x
4. Universal buffer 10x concentrate	1 x 100 ml	8 x 100 ml	BUFFER 10x
5. Substrate solution Nitroblue tetrazolium chloride/5-Bromo-4-chloro-3-indolylphosphate (NBT/BCIP), ready for use	1 x 50 ml	8 x 50 ml	SUBSTRATE
6. Adhesive foil	---	8 sheets	---
7. Test instruction	1 booklet	1 booklet	---

LOT Lot description
IVD In vitro diagnostic medical device
CE
Storage temperature
Unopened usable until

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C, do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Undiluted patient samples and incubated blot strips should be handled as infectious waste. Other reagents do not need to be collected separately, unless stated otherwise in official regulations.

The following components are not provided in the test kits but can be ordered at EUROIMMUN under the respective order numbers.Performance of the test requires an **incubation tray**:
ZD 9895-0130 Incubation tray with 30 channels (black)
ZD 9898-0144 Incubation tray with 44 channels (black, for the EUROBlotOne and EUROBlotCamera system)
For the creation of work protocols and the evaluation of incubated test strips using **EUROLineScan** green paper and adhesive foil are required:
ZD 9880-0101 Green paper (1 sheet)
ZD 9885-0116 Adhesive foil for approx. 16 test strips
ZD 9885-0130 Adhesive foil for approx. 30 test strips
If a **visual evaluation** is to be performed in individual cases, the required evaluation protocol can be ordered under: ZD 2132-0101 Evaluation protocol visual Anti-Borrelia burgdorferi-WESTERNBLOT.

Preparation and stability of the reagents

Note: The bag containing the blot strips is printed with a number in addition to the test kit lot number. This number refers to the strip batch and is also printed on the corresponding evaluation template. These two numbers must match to ensure correct evaluation of test results.
All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. Unopened, reagents are stable until the indicated expiry date when stored at +2°C to +8°C. After initial opening, reagents are stable for 12 months or until the expiry date, if earlier, unless stated otherwise in the instructions. Opened reagents must also be stored at +2°C to +8°C and protected from contamination.

- **Coated test strips:** Ready for use. Open the packing with the test strips only when the strips have reached room temperature to prevent condensation on the strips. After removal of the strips the packing should be sealed tightly and stored at +2°C to +8°C. To ensure correct evaluation of results, the lot number on the bag must match the lot number on the strips as well as on the evaluation matrix.

- **Enzyme conjugate:** The enzyme conjugate is supplied as a 10x concentrate. For the preparation of the ready for use enzyme conjugate the amount required should be removed from the bottle using a clean pipette and diluted 1:10 with ready for use diluted universal buffer. For 1 test strip dilute 0.15 ml anti-human IgG concentrate with 1.35 ml ready for use diluted universal buffer. The ready for use diluted enzyme conjugate should be used at the same working day.

- **Universal buffer:** The universal buffer is supplied as a 10x concentrate. For the preparation of the ready for use universal buffer the amount required should be removed from the bottle using a clean pipette and diluted 1:10 with deionised or distilled water. For the incubation of 1 test strip 1.5 ml buffer concentrate should be diluted with 13.5 ml deionised or distilled water. The ready for use diluted universal buffer should be used at the same working day.

- **Substrate solution:** Ready for use. Close bottle immediately after use, as the contents are sensitive to light ✳.

Warning: The control of human origin has tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the agent sodium azide in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the serum or plasma samples

Sample material: Human serum or EDTA, heparin or citrate plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

Sample dilution: The **patient samples** for analysis are diluted **1:51** in ready for use diluted universal buffer. For example, add 30 µl of sample to 1.5 ml ready for use diluted universal buffer and mix well by vortexing. Sample pipettes are not suitable for mixing.

Incubation

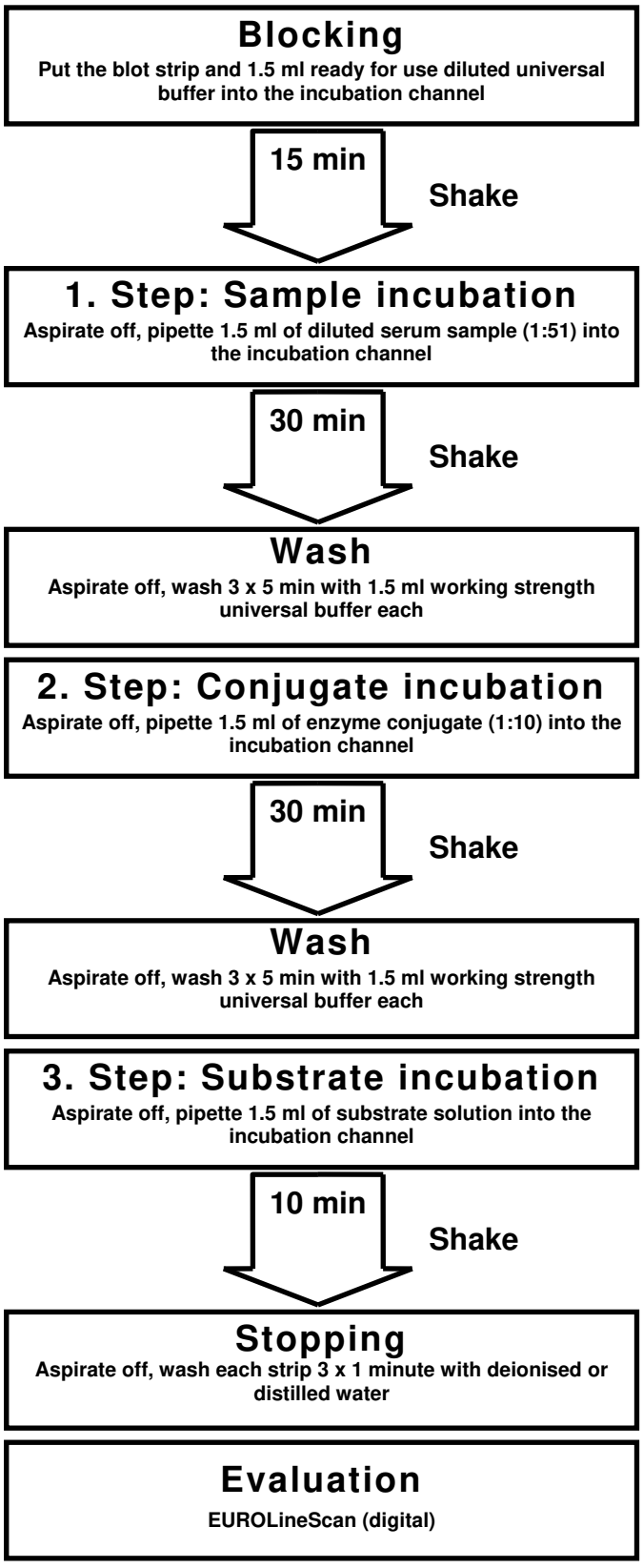
Blocking:	According to the number of serum samples to be tested fill each channel of the incubation tray with 1.5 ml ready for use diluted universal buffer and a blot strip. Remove the required amount of blot strips from the packing using a pair of tweezers. The number on the test strip should be visible. Incubate for **15 minutes** at room temperature (+18°C to +25°C) on a rocking shaker. Afterwards aspirate off all the liquid.
Sample incubation: (1st step)	Fill each channel with 1.5 ml of the diluted serum samples. Incubate at room temperature (+18°C to +25°C) for **30 minutes** on a rocking shaker.
Wash:	Aspirate off the liquid from each channel and wash **3 x 5 minutes** each with 1.5 ml working strength universal buffer on a rocking shaker.
Conjugate incubation: (2nd step)	Pipette 1.5 ml diluted enzyme conjugate (alkaline phosphatase-conjugated anti-human IgG) into each channel. Incubate for **30 minutes** at room temperature (+18°C to +25°C) on a rocking shaker.
Wash:	Aspirate off the liquid from each channel. Wash as described above.
Substrate incubation: (3rd step)	Pipette 1.5 ml substrate solution into the channels of the incubation tray. Incubate for **10 minutes** at room temperature (+18°C to +25°C) on a rocking shaker.
Stopping:	Aspirate off the liquid from each channel and wash each strip **3 x 1 minute** with deionised or distilled water.

For automated incubation with the EUROBlotMaster select the program **Euro02 Inf WB30**.

For automated incubation with the EUROBlotOne select the program **Euro 01/02**.

Anti-Borrelia burgdorferi-WESTERNBLOT (IgG)

Incubation protocol



Anti-Borrelia burgdorferi-WESTERNBLOT (IgG)
Evaluation and interpretation

Handling: For evaluation of incubated test strips we generally recommend using the **EUROLineScan** software. After stopping the reaction using deionised or distilled water, place the incubated test strips onto the adhesive foil of the green work protocol using a pair of tweezers. The position of the test strips can be corrected while they are wet. As soon as all test strips have been placed onto the protocol, they should be pressed hard using filter paper and left to air-dry. After they have dried, the test strips will be stuck to the adhesive foil. The dry test strips are then scanned using a flatbed scanner (EUROIMMUN AG) and evaluated with **EUROLineScan**. Alternatively, imaging and evaluation is possible directly from the incubation trays (EUROBlotCamera and EUROBlotOne). For general information about the EUROLineScan program please refer to the EUROLineScan user manual (EUROIMMUN AG). The code for entering the **Test** in EUROLineScan is **B.b._WB_IgG**.

If a visual evaluation must be performed in exceptional cases, hold the evaluation matrix next to the stuck-on blot strips and position it so that the black band above the number on the blot strips lines up with the alignment bar of the evaluation matrix. **The lot number on the evaluation matrix must match the lot number on the blot strips.** Clearly recognisable bands on the blot strips which concur with the labelled bands on the evaluation matrix are noted in the evaluation protocol.

Antigens: The antigen source for the EUROIMMUN Anti-Borrelia burgdorferi-WESTERNBLOT is provided by a particularly suitable Borrelia burgdorferi strain. The cultured Borrelia have been solubilised using sodium dodecyl sulphate followed by a separation of the solubilised protein using discontinuous polyacrylamide gel electrophoresis according to molecular mass and transfer of the separated proteins to nitrocellulose. From each test kit, 2 control test strips have been removed and incubated with a reference serum. One of these stained strips is included in the kit, the other remains with EUROIMMUN for documentation purposes. Diagnostically relevant antigens have been characterised with monoconal reference antibodies from the German National Reference Laboratory for Borreliae.

Specificity of the antigens on the test strips:

Band	Antigen	Specificity
83 kDa	Membrane-vesical protein, p83	Degradation product of p100, **high specificity**
75 kDa	Heat shock protein, p75	Unspecific
62 kDa	Heat shock protein, p62	Unspecific
57/59 kDa	p57 and p59	Unspecific
50 kDa	p50	Unspecific
47 kDa	p47	Probably genus specific
43 kDa	p43	Unspecific
41 kDa	Flagellin, p41	Genus specific, cross reactivity to other spirochaetaceae and bacteria having flagella
39 kDa	Bmp A, p39	**High specificity**
34 kDa	Osp B, p34	Outer surface protein B, **high specificity**
32 kDa	p32	Unspecific
31 kDa	Osp A, p31	Outer surface protein A, **high specificity**
29 kDa	p29	Probably specific, poorly investigated
28 kDa	p28	Unspecific
25 kDa	Osp C, p25	Outer surface protein C, **high specificity**
21/22 kDa	p21/22	**High specificity**
18 kDa	p18	**Probably specific**
17 kDa	p17	Poorly investigated

In the lower part of the test strip there is a conjugate control membrane chip (IgA, IgG and IgM). Below the conjugate control, there is a membrane chip with a control band (Control).

Attention: A correctly performed determination of antibodies of class IgG against the antigens described above is indicated by a positive reaction of the control band and a positive reaction of the IgG band.

If one of these bands only shows a very weak reaction or none at all, the result is not valid.

Specificity of the antigens: Borrelia burgdorferi antigens can generally be divided into three categories.

Category	Antigens
1	Cross-reacting and undefined antigens with the molecular mass 17 kDa, 28 kDa, 32 kDa, 43 kDa, 47 kDa, 50 kDa, 57 kDa, 59 kDa, 62 kDa and 75 kDa.
2	A genus-specific antigen with a molecular mass of 41 kDa (flagellin).
3	Species-specific and highly specific antigens with the molecular mass 18 kDa, 21/22 kDa, 25 kDa, 31 kDa, 34 kDa, 39 kDa and 83 kDa.

IgG class antibodies against Borrelia burgdorferi

Interpretation of results: In order to evaluate the signals, the band positions and intensity of staining must be taken into consideration, as negative sera sometimes produce weak signals in individual bands. Based on experience, the results of the Borrelia burgdorferi WESTERNBLOT test can be divided into negative, borderline and positive results.

Result	Characteristics
Negative	No bands, or weak intensities of some antigens from categories 1 and 2.
Borderline	A distinctive band from category 3 (antigens in category 3 are shaded grey in the evaluation protocol!) **and** several distinctive signals from categories 1 and 2. It is recommended that a new sample be taken and the test repeated after a few weeks.
Positive	More than one distinctive band from category 3 (antigens in category 3 are shaded grey in the evaluation protocol!). In addition, and particularly in the case of patients in the late stage of the disease, numerous bands from categories 1 and 2 can be observed.

IgM class antibodies against Borrelia burgdorferi

Interpretation of results: In order to evaluate the signals, the band positions and intensity of staining must be taken into consideration. The results of the Borrelia burgdorferi WESTERNBLOT test can be divided into negative, borderline and positive results.

In the early phase of a Borrelia infection IgM antibodies are typically directed against Osp C (p25). IgM antibodies against other specific Borrelia antigens are not considered definitive indicators of a fresh Borrelia infection.

IgM antibodies against flagellin (p41) can represent the initial response of the body to Borrelia burgdorferi. However, an unspecific reaction cannot be excluded, since it is known that antibodies directed against other microorganisms cross react with Borrelia burgdorferi flagellin (p41). For this reason, a single band at the position of flagellin (p41) in IgM detection should not be considered proof of a fresh infection with Borrelia burgdorferi. If only the flagellin band (p41) reacts positively, the test should be repeated several weeks later with a fresh blood sample.

In the serological investigation of a Borrelia infection the determination of antibodies of class IgM often yields unclear results. IgM antibodies can sometimes be found in serum years after an infection or following antibiotic treatment. Therefore, the detection of IgM antibodies does not necessarily indicate a fresh infection. A negative IgM results does not exclude a fresh infection. With a second infection, only antibodies of class IgG and not IgM can be formed.

In the late stage of borreliosis a positive IgM result does not provide any additional information, due to the antibody persistence mentioned above. The cause of such false-positive IgM results often remains unclear. They are observed, for example, in infectious mononucleosis, herpes virus infections and various autoimmune diseases.

For the diagnosis of a fresh Borrelia infection, a positive IgM result should be confirmed with a positive IgG result using a fresh blood sample 3 to 6 weeks later.

Result	Characteristics
Negative	No antigen bands recognisable or weak intensities of some bands of category 1
Borderline	One antigen band of category 2 (flagellin, p41) or a weak band of category 3. It is recommended that a fresh sample be taken and the test repeated after a few weeks. Antibodies against Osp C are characteristic for a fresh infection.
Positiv	At least one distinctive band from category 3 (antigens in category 3 are shaded grey in the evaluation protocol!). Antibodies against Osp C are characteristic for a fresh infection.

For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Measurement range: The Westernblot is a qualitative method. No measurement range is provided.

Inter- and intra-assay variation: The inter-assay variation was determined by multiple analyses of characterised samples over several days. The intra-assay variation was determined by multiple analyses of characterised samples on one day. In every case, the intensity of the bands was within the specified range. This Westernblot displays excellent inter- and intra-assay reproducibility.

Interference: Haemolytic, lipaemic and icteric sera showed no effect on the analytical results.

Prevalence: Sera from 156 clinically characterised patients and 517 healthy blood donors were investigated with the EUROIMMUN Anti-Borrelia burgdorferi-Westernblot.

		Prevalence		
Clinically characterised sera	**n**	**IgG**	**IgM**	**IgM/IgG**
Erythema migrans	108	64%	67%	84%
Neuroborreliosis	32	85%	44%	88%
Arthritis	10	100%	30%	100%
Acrodermatitis	6	83%	50%	100%

		Prevalence	
Serum samples	**n**	**IgG**	**IgM**
Healthy blood donors *	517	5%	4%

*Medical University of Luebeck

The prevalence of anti-Borrelia antibodies from the healthy blood donor samples agrees with the values from literature [14].

Cross reactivity: The quality of the antigen used (whole antigen, SDS extract) and the antigen source (Borrelia burgdorferi sensu stricto) ensure high specificity of the Westernblot. The determination of cross reactivity is not necessary with the Westernblot, since specific reactions and unspecific or cross reactions can be differentiated directly with this test system.

Clinical significance

The history of Lyme disease, a contagious condition caused by Borrelia burgdorferi and transmitted to humans by ticks, offers infectiologists a formidable lesson on how medicine progresses. Clinical description started in Europe at the turn of the $19^{th}/20^{th}$ century with Pick's description of what was then labelled chronic atrophic acrodermatitis. Fifty years later, Hauser noted the affection was transmitted by ticks. Independently, Afzelius, then Lipschutz, described erythema migrans and its relationship with tick bites. Neurological involvement was also described with the skin signs. These early dermatological descriptions suddenly came into the limelight in 1975 when an epidemia of arthritis occurred in children in Lyme, Connecticut, USA. Many of the affected children had erythema migrans. Based on these observations and an epidemiological analysis of the epidemia, Steele and co-workers defined "Lyme disease" as a rheumatological disorder commonly associated with erythema migrans and sometimes with multiple organ involvement. In 1982 Burgdorfer suggested that ticks transmitted "treponema-like spirochaetes", which were later authentified as the causal agent: Borrelia burgdorferi.

Borrelia burgdorferi as a species of bacteria is belonging to the spirochaetaceae family. This family includes five genera: Borrelia, spirochetes, cristispira, treponema and leptospira. Among about 30 tick species feeding on humans, Ixodes ricinus is the most frequent tick species biting humans in Europe. It is the vector of Borrelia burgdorferi, which causes Lyme disease, of anaplasma phagocytophilum and the tick-borne encephalitis virus. I. ricinus ticks pass through three developmental stages: larvae, nymphs and adults (females and males). The density of this tick species can be very high, reaching in some places more than 300 ticks/100 m^2. Lyme borreliosis is the most frequent tick-transmitted disease in the northern hemisphere. In Europe, especially in Central Europe and Scandinavia there are up to 155 cases per 100,000 individuals caused by the species B. burgdorferi sensu stricto, B. afzelii, and B. garinii. The human seroprevalence rate of antibodies against Borrelia burgdorferi in the normal population of Germany and other Central European countries is about 8% (for IgG); in highly endemic areas even considerably higher with ranges from 18% to 52%. In East Asia, e.g. in an endemic area in China, the seroprevalence amounts to 26%. Persons working in the area of forestry display anti-Borrelia antibodies in about 40%, hunters in more than 50% of cases.

A Borrelia burgdorferi infection can manifest itself in the areas of dermatology, neurology, ophthalmology, rheumatology and internal medicine. The clinical expression of borreliosis can be divided into three stages:

Stage I: The typical primary manifestation of a Borrelia burgdorferi infection is erythema migrans (80%), a reddening of the skin which appears around the area of the tick bite and spreads in a circular manner. The erythema is accompanied by influenza-like general symptoms with fever, shaking chill, headaches and vomiting. Lymphadenopathies are observed in a few cases (lymphadenosis cutis benigna). The leading clinical types of the erythema-free borreliosis (20%) are neurological, arthromyalgic, influenza-like, cardiovascular borreliosis and borreliosis with hepatitis, with regional lymphadenitis and mixtures of these. Stage I can result in spontaneous healing or can develop into a generalised borreliosis. The transition phase is generally symptom-free. IgM antibodies against Borrelia burgdorferi can be detected serologically in 50% to 90% of patients during stage I. Humans produce highly specific antibodies against the outer surface protein C (OspC) shortly after infection with Borrelia burgdorferi. The main antigen VlsE, which is exclusively expressed in vivo, is the most sensitive antigen for IgG antibody detection, whereas OspC is the most sensitive antigen for IgM antibody detection.

Stage II: A variety of symptoms can develop several weeks to some months after receiving the tick bite. In the foreground of these are neurological manifestations: meningitis, encephalitis, asymmetric polyneuritis, cranial nerve pareses, Bannwarth's lymphocytic meningoradiculoneuritis. One of the most frequent neurological symptoms of borreliosis in children is the peripheral facial palsy. Usually the paresis of the facial nerve appears at one side and after several weeks on the opposite side. Also arthritis, particularly of the knee joints, and non-localised pains in the bones, joints and muscles are frequently found. Less frequently are cardiological manifestations such as myocarditis and pericarditis. Antibodies against Borrelia burgdorferi can be detected in 50% to 90% of patients in stage II. In the early phase of this stage mainly IgM antibodies are found but in the late phase often only IgG antibodies occur. However, IgM antibodies can persist for a long time. By additionally determining antibodies against VlsE the serological hit rate can be increased by 20% to 30%. VlsE displays the highest sensitivity of all antigens tested for IgG detection.

Stage III: The typical manifestations of a Borrelia burgdorferi infection in stage III are chronic-relapsing erosive arthritis, acrodermatitis chronica atrophicans and progressive encephalomyelitis, which can proceed in a similar way to multiple sclerosis. Without treatment, the tertiary stage can develop over a period of years to decades after the original infection. In this stage, IgG antibody titers are significantly increased in 90% to 100% of patients, while IgM antibodies are only rarely detectable.

For the diagnosis of neuroborreliosis, the determination of antibodies in cerebrospinal fluid is of decisive importance and far superior to serological testing. An antibody index (AI) discriminates between a blood-derived and a pathological, brain-derived specific antibody fraction in cerebrospinal fluid (CSF) and takes into account individual changes in the blood/CSF barrier function.

Various techniques come into question for the detection of antibodies against Borrelia burgdorferi: ELISA, indirect immunofluorescence (IIFT), passive haemagglutination and immunoblot. Generally, ELISA or IIFT are used as screening test for preliminary characterisation of the serum sample. According to guidelines in the USA and in Germany, serological diagnosis should follow the principle of a two-step procedure: ELISA or indirect immunofluorescence (IIFT) as a first step, which, if reactive, is followed by an immunoblot. In fresh infections it is recommended performing ELISA/IIFT and immunoblot in parallel, since some weak reactions become detectable earlier in the blot than in the screening test. VlsE, the most sensitive antigen for IgG antibody detection, and OspC for IgM antibody detection should be included in the test. By additionally determining antibodies against VlsE the serological hit rate can be increased by 20% compared to whole extract Westernblots. Of all recombinant antigens tested, VlsE possesses the highest sensitivity for the detection of a Borrelia infection.

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Anti-Borrelia burgdorferi-WESTERNBLOT (IgM)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
DY 2132-3001 M DY 2132-24001 M	Borrelia burgdorferi (complete antigens)	IgM	Antigen coated membrane strips	30 x 01 (30) 240 x 01 (240)

Indication: The Westernblot test kit provides a qualitative in vitro assay for human autoantibodies of the immunoglobulin class IgM against Borrelia in serum or plasma for the diagnosis of Lyme borreliosis. Associated diseases: Erythema chronicum migrans, lymphadenosis cutis benigna, acrodermatitis chronica atrophicans, arthritis, carditis, lymphocytic meningoradiculoneuritis and neuroborreliosis.

Principles of the test: The test kit contains test strips with electrophoretically separated antigen extracts of Borrelia burgdorferi. The blot strips will be blocked and incubated in the first reaction step with diluted patient samples. In the case of positive samples, specific antibodies of the class IgM (and IgA, IgG) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgM (enzyme conjugate) catalysing a colour reaction.

Contents of the test kit:

Component	Format	Format	Symbol
1. Test strips Single strips with electrophoretically separated Borrelia burgdorferi antigens	30 x 1	240 x 1	STRIPS
2. Evaluation matrix with control strip Test strip incubated with a positive control serum	1 pattern	1 pattern per test strip lot	---
3. Enzyme conjugate Alkaline phosphatase-labelled anti-human IgM (goat), 10x concentrate	2 x 3 ml	16 x 3 ml	CONJUGATE 10x
4. Universal buffer 10x concentrate	1 x 100 ml	8 x 100 ml	BUFFER 10x
5. Substrate solution Nitroblue tetrazolium chloride/5-Bromo-4-chloro-3-indolylphosphate (NBT/BCIP), ready for use	1 x 50 ml	8 x 50 ml	SUBSTRATE
6. Adhesive foil	---	8 sheets	---
7. Test instruction	1 booklet	1 booklet	---

LOT Lot description
IVD In vitro diagnostic medical device
CE
Storage temperature
Unopened usable until

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C, do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Undiluted patient samples and incubated blot strips should be handled as infectious waste. Other reagents do not need to be collected separately, unless stated otherwise in official regulations.

The following components are not provided in the test kits but can be ordered at EUROIMMUN under the respective order numbers.
Performance of the test requires an **incubation tray**:
ZD 9895-0130 Incubation tray with 30 channels (black)
ZD 9898-0144 Incubation tray with 44 channels (black, for the EUROBlotOne and EUROBlotCamera system)
For the creation of work protocols and the evaluation of incubated test strips using **EUROLineScan** green paper and adhesive foil are required:
ZD 9880-0101 Green paper (1 sheet)
ZD 9885-0116 Adhesive foil for approx. 16 test strips
ZD 9885-0130 Adhesive foil for approx. 30 test strips
If a **visual evaluation** is to be performed in individual cases, the required evaluation protocol can be ordered under: ZD 2132-0101 Evaluation protocol visual Anti-Borrelia-burgdorferi-WESTERNBLOT

Preparation and stability of the reagents

Note: The bag containing the blot strips is printed with a number in addition to the test kit lot number. This number refers to the strip batch and is also printed on the corresponding evaluation template. These two numbers must match to ensure correct evaluation of test results.
All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. Unopened, reagents are stable until the indicated expiry date when stored at +2°C to +8°C. After initial opening, reagents are stable for 12 months or until the expiry date, if earlier, unless stated otherwise in the instructions. Opened reagents must also be stored at +2°C to +8°C and protected from contamination.

- **Coated test strips:** Ready for use. Open the packing with the test strips only when the strips have reached room temperature to prevent condensation on the strips. After removal of the strips the packing should be sealed tightly and stored at +2°C to +8°C. To ensure correct evaluation of results, the lot number on the bag must match the lot number on the strips as well as on the evaluation matrix.

- **Enzyme conjugate:** The enzyme conjugate is supplied as a 10x concentrate. For the preparation of the ready for use enzyme conjugate the amount required should be removed from the bottle using a clean pipette and diluted 1:10 with ready for use diluted universal buffer. For 1 test strip dilute 0.15 ml anti-human IgG concentrate with 1.35 ml ready for use diluted universal buffer. The ready for use diluted enzyme conjugate should be used at the same working day.

- **Universal buffer:** The universal buffer is supplied as a 10x concentrate. For the preparation of the ready for use universal buffer the amount required should be removed from the bottle using a clean pipette and diluted 1:10 with deionised or distilled water. For the incubation of 1 test strip 1.5 ml buffer concentrate should be diluted with 13.5 ml deionised or distilled water. The ready for use diluted universal buffer should be used at the same working day.

- **Substrate solution:** Ready for use. Close bottle immediately after use, as the contents are sensitive to light.

Warning: The control of human origin has tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the agent sodium azide in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the serum or plasma samples

Sample material: Human serum or EDTA, heparin or citrate plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

Sample dilution: The **patient samples** for analysis are diluted **1:51** in ready for use diluted universal buffer. For example, add 30 µl of sample to 1.5 ml ready for use diluted universal buffer and mix well by vortexing. Sample pipettes are not suitable for mixing.

Incubation

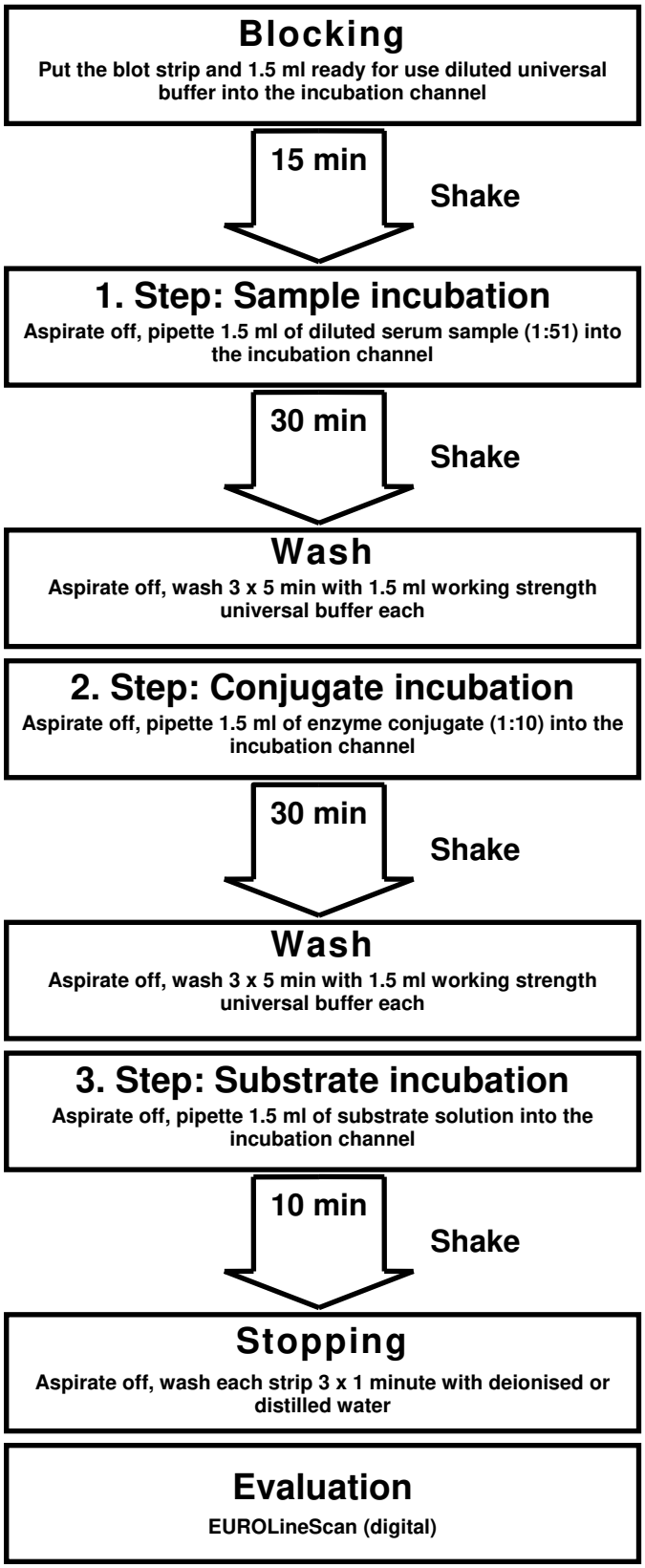
Blocking:	According to the number of serum samples to be tested fill each channel of the incubation tray with 1.5 ml ready for use diluted universal buffer and a blot strip. Remove the required amount of blot strips from the packing using a pair of tweezers. The number on the test strip should be visible. Incubate for **15 minutes** at room temperature (+18°C to +25°C) on a rocking shaker. Afterwards aspirate off all the liquid.
Sample incubation: (1st step)	Fill each channel with 1.5 ml of the diluted serum samples and incubate at room temperature (+18°C to +25°C) for **30 minutes** on a rocking shaker.
Wash:	Aspirate off the liquid from each channel and wash **3 x 5 minutes** each with 1.5 ml working strength universal buffer on a rocking shaker.
Conjugate incubation: (2nd step)	Pipette 1.5 ml diluted enzyme conjugate (alkaline phosphatase-conjugated anti-human IgM) into each channel and incubate for **30 minutes** at room temperature (+18°C to +25°C) on a rocking shaker.
Wash:	Aspirate off the liquid from each channel. Wash as described above.
Substrate incubation: (3rd step)	Pipette 1.5 ml substrate solution into the channels of the incubation tray. Incubate for **10 minutes** at room temperature (+18°C to +25°C) on a rocking shaker.
Stopping:	Aspirate off the liquid from each channel and wash each strip **3 x 1 minute** with deionised or distilled water.

For automated incubation with the EUROBlotMaster select the program **Euro02 Inf WB30**.

For automated incubation with the EUROBlotOne select the program **Euro 01/02**.

Anti-Borrelia-burgdorferi WESTERNBLOT (IgM)

Incubation protocol



Evaluation and Interpretation of the results of the Anti-Borrelia-burgdorferi WESTERNBLOT (IgM)

Handling: For evaluation of incubated test strips we generally recommend using the **EUROLineScan** software. After stopping the reaction using deionised or distilled water, place the incubated test strips onto the adhesive foil of the green work protocol using a pair of tweezers. The position of the test strips can be corrected while they are wet. As soon as all test strips have been placed onto the protocol, they should be pressed hard using filter paper and left to air-dry. After they have dried, the test strips will be stuck to the adhesive foil. The dry test strips are then scanned using a flatbed scanner (EUROIMMUN AG) and evaluated with **EUROLineScan**. Alternatively, imaging and evaluation is possible directly from the incubation trays (EUROBlotCamera and EUROBlotOne). For general information about the EUROLineScan program please refer to the EUROLineScan user manual (EUROIMMUN AG). The code for entering the **Test** in EUROLineScan is **B.b._WB_IgM**.

If a visual evaluation must be performed in exceptional cases, hold the evaluation matrix next to the stuck-on blot strips and position it so that the black band above the number on the blot strips lines up with the alignment bar of the evaluation matrix. **The lot number on the evaluation matrix must match the lot number on the blot strips.** Clearly recognisable bands on the blot strips which concur with the labelled bands on the evaluation matrix are noted in the evaluation protocol.

Antigens: The antigen source for the EUROIMMUN Anti-Borrelia-burgdorferi WESTERNBLOT is provided by a particularly suitable Borrelia burgdorferi strain [2, 3]. The cultured borrelia have been solubilised using sodium dodecyl sulphate followed by a separation of the solubilised protein using discontinuous polyacrylamide gel electrophoresis according to molecular mass and transfer of the separated proteins to nitrocellulose. From each test kit, 2 control test strips have been removed and incubated with a reference serum. One of these stained strips is included in the kit, the other remains with EUROIMMUN for documentation purposes. Diagnostically relevant antigens have been characterised with monoconal reference antibodies from the German National Reference Laboratory for Borreliae [39].

Specificity of the antigens on the test strips: [41, 42, 43]

Band	Antigen	Specificity
83 kDa	Membrane-vesical protein, p 83	Degradation product of p 100, **high specificity**.
75 kDa	Heat shock protein, p 75	Unspecific.
62 kDa	Heat shock protein, p 62	Unspecific.
57/59 kDa	p 57 and p 59	Unspecific.
50 kDa	p 50	Unspecific.
47 kDa	p 47	Probably genus specific.
43 kDa	p 43	Unspecific.
41 kDa	Flagellin, p 41	Genus specific, cross reactivity to other spirochaetaceae and bacteria having flagella.
39 kDa	Bmp A, p 39	**High specificity**.
34 kDa	Osp B, p 34	Outer surface protein B, **high specificity**.
32 kDa	p 32	Unspecific.
31 kDa	Osp A, p 31	Outer surface protein A, **high specificity**.
29 kDa	p 29	Probably specific, poorly investigated.
28 kDa	p 28	Unspecific.
25 kDa	Osp C, p 25	Outer surface protein C, **high specificity**.
21/22 kDa	p 21/22	**High specificity**.
18 kDa	p 18	**Probably specific**.
17 kDa	p 17	Poorly investigated.

In the lower part of the test strip there is a conjugate control membrane chip (IgA, IgG and IgM). Below the conjugate control, there is a membrane chip with a control band (Control).

Attention: A correctly performed determination of antibodies of class IgM against the antigens described above is indicated by a positive reaction of the control band and a positive reaction of the IgM band.

If one of these bands only shows a very weak reaction or none at all, the result is not valid.

Specificity of the antigens: Borrelia burgdorferi antigens can generally be divided into three categories [40].

Category	Antigens
1	Cross-reacting and undefined antigens with the molecular mass 17 kDa, 28 kDa, 32 kDa, 43 kDa, 47 kDa, 50 kDa, 57 kDa, 59 kDa, 62 kDa and 75 kDa.
2	A genus-specific antigen with a molecular mass of 41 kDa (flagellin).
3	Species-specific and highly specific antigens with the molecular mass 18 kDa, 21/22 kDa, 25 kDa, 31 kDa, 34 kDa, 39 kDa and 83 kDa.

IgG class antibodies against Borrelia burgdorferi

Interpretation of results: In order to evaluate the signals, the band positions and intensity of staining must be taken into consideration, as negative sera sometimes produce weak signals in individual bands. Based on experience, the results of the Borrelia burgdorferi WESTERNBLOT test can be divided into negative, borderline and positive results.

Result	Characteristics
Negative	No bands, or weak intensities of some antigens from categories 1 and 2.
Borderline	A distinctive band from category 3 (antigens in category 3 are shaded grey in the evaluation protocol!) and several distinctive signals from categories 1 and 2. It is recommended that a new sample be taken and the test repeated after a few weeks.
Positive	More than one distinctive band from category 3 (antigens in category 3 are shaded grey in the evaluation protocol!). In addition, and particularly in the case of patients in the late stage of the disease, numerous bands from categories 1 and 2 can be observed.

IgM class antibodies against Borrelia burgdorferi

Interpretation of results: In order to evaluate the signals, the band positions and intensity of staining must be taken into consideration. The results of the Borrelia burgdorferi WESTERNBLOT test can be divided into negative, borderline and positive results.

In the early phase of a Borrelia infection IgM antibodies are typically directed against Osp C (p 25). IgM antibodies against other specific Borrelia antigens are not considered definitive indicators of a fresh Borrelia infection.

IgM antibodies against flagellin (p 41) can represent the initial response of the body to Borrelia burgdorferi. However, an unspecific reaction cannot be excluded, since it is known that antibodies directed against other microorganisms cross react with Borrelia burgdorferi flagellin (p 41). For this reason, a single band at the position of flagellin (p 41) in IgM detection should not be considered proof of a fresh infection with Borrelia burgdorferi. If only the flagellin band (p 41) reacts positively, the test should be repeated several weeks later with a fresh blood sample.

In the serological investigation of a Borrelia infection the determination of antibodies of class IgM often yields unclear results. IgM antibodies can sometimes be found in serum years after an infection or following antibiotic treatment. Therefore, the detection of IgM antibodies does not necessarily indicate a fresh infection. A negative IgM result does not exclude a fresh infection. With a second infection, only antibodies of class IgG and not IgM can be formed.

In the late stage of borreliosis a positive IgM result does not provide any additional information, due to the antibody persistence mentioned above. The cause of such false-positive IgM results often remains unclear. They are observed, for example, in infectious mononucleosis, herpes virus infections and various autoimmune diseases [39].

For the diagnosis of a fresh Borrelia infection, a positive IgM result should be confirmed with a positive IgG result using a fresh blood sample 3-6 weeks later.

Result	Characteristics
Negative	No antigen bands recognisable or weak intensities of some bands of category 1
Borderline	One antigen band of category 2 (flagellin, p 41) or a weak band of category 3. It is recommended that a fresh sample be taken and the test repeated after a few weeks. Antibodies against Osp C are characteristic for a fresh infection.
Positive	At least one distinctive band from category 3 (antigens in category 3 are shaded grey in the evaluation protocol!). Antibodies against Osp C are characteristic for a fresh infection.

For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Measurement range: The Westernblot is a qualitative method. No measurement range is provided.

Inter- and intra-assay variation: The inter-assay variation was determined by multiple analyses of characterised samples over several days. The intra-assay variation was determined by multiple analyses of characterised samples on one day. In every case, the intensity of the bands was within the specified range. This Westernblot displays excellent inter- and intra-assay reproducibility.

Interference: Haemolytic, lipaemic and icteric sera showed no effect on the analytical results.

Prevalence: Sera from 156 clinically characterised patients and 517 healthy blood donors were investigated with the EUROIMMUN Anti-Borrelia burgdorferi-WESTERNBLOT.

		Prevalence		
Clinically characterised sera	**n**	**IgG**	**IgM**	**IgM/IgG**
Erythema migrans	108	64%	67%	84%
Neuroborreliosis	32	85%	44%	88%
Arthritis	10	100%	30%	100%
Acrodermatitis	6	83%	50%	100%

		Prevalence	
Serum samples	**n**	**IgG**	**IgM**
Healthy blood donors *	517	5%	4%

*Medical University of Luebeck

The prevalence of anti-Borrelia antibodies from the healthy blood donor samples agrees with the values from literature [7].

Cross reactivity: The quality of the antigen used (whole antigen, SDS extract) and the antigen source (Borrelia burgdorferi sensu stricto) ensure high specificity of the Westernblot. The determination of cross reactivity is not necessary with the Westernblot, since specific reactions and unspecific or cross reactions can be differentiated directly with this test system.

Clinical significance

The history of Lyme disease, a contagious condition, caused by Borrelia burgdorferi, transmitted to humans by ticks, offers infectiologists a formidable lesson on how medicine progresses. Clinical description started in Europe at the turn of the $19^{th}/20^{th}$ century with Pick's description of what was then labelled chronic atrophic acrodermatitis [1]. Fifty years later Hauser noted the affection was transmitted by ticks [1]. Independently, Afzelius, then Lipschutz, described erythema migrans and its relationship with tick bites. Neurological involvement was also described with the skin signs. These early dermatological descriptions suddenly came into the limelight in 1975 when an epidemic of arthritis occurred in children in Lyme, Connecticut, USA [1]. Many of the affected children had erythema migrans. Based on these observations and an epidemiological analysis of the epidemic, Steele and co-workers defined "Lyme disease" as a rheumatological disorder commonly associated with erythema migrans and sometimes with multiple organ involvement. In 1982 Burgdorfer suggested that tick bites transmitted a Spirochaeta which was later authentified as the causal agent: Borrelia burgdorferi [1, 2, 3].

Borrelia burgdorferi as a species of bacteria is belonging to the spirochaetaceae family. This family includes five genera: borrelia, spirochaetes, cristispira, treponema and leptospira. Among about 30 tick species feeding on humans, Ixodes ricinus is the most frequent tick species biting humans in Europe. It is the vector of Borrelia burgdorferi which causes Lyme disease, and of the tick-borne encephalitis virus [4, 5, 6, 7]. I. ricinus ticks pass through three developmental stages: larvae, nymphs and adults (females and males) [8]. The density of this tick species may be very high, reaching in some places more than 300 ticks/100 m^2 [8]. Lyme borreliosis is the most frequent tick-transmitted disease in the northern hemisphere [9, 10, 11]. In Europe, especially in Central Europe and Scandinavia there are up to 155 cases per 100,000 individuals caused by the species B. burgdorferi sensu stricto, B. afzelii, and B. garinii [4, 6, 10, 11, 12]. The human seroprevalence rates of antibodies against Borrelia burgdorferi in the normal population of Germany and other Central European countries ranges from 18% to 52% [13]. In East Asia, e.g. in China, there are 26% on the average [14]. Persons working in the area of forestry display anti-borrelia antibodies in about 40%, hunters in more than 50% of cases [7, 15].

A Borrelia burgdorferi infection can manifest itself in the areas of dermatology, neurology, ophthalmology, rheumatology and internal medicine [7, 16]. The clinical expression of borreliosis can be divided into three stages:

Stage I: The typical primary manifestation of a Borrelia burgdorferi infection is erythema migrans (80%), a reddening of the skin which appears around the area of the tick bite and spreads in a circular manner [17]. The erythema is accompanied by influenza-like general symptoms with fever, shaking chill, headaches and vomiting [18]. Lymphadenopathies are observed in a few cases (lymphadenosis cutis benigna). The leading clinical types of the erythema-free borreliosis (20%) are neurological, arthromyalgic, influenza-like, cardiovascular, hepatitis, regional lymphadenitis, and mixed [19, 20, 21]. Stage I can result in spontaneous healing or can develop into a generalised borreliosis. The transition phase is generally symptom-free. IgM antibodies against Borrelia burgdorferi can be detected serologically in 50% to 90% of patients in Stage I. The prevalence of specific IgG antibodies is considerably lower [22, 23]. However, serological tests often provide negative results in this stage of the illness [3, 19]. In order to overcome this problem a new test was created including the newly identified VlsE (**v**ariable major protein-**l**ike **s**equence, **e**xpressed), which can be considered as the major antigen for Borrelia serology. Over 85% of IgG-positive sera can be identified at a glance by assessing the VlsE band in incubated Westernblot strips. VlsE allows detection of antibodies against all Borrelia species. The risk of a false negative reaction due to species differences is ten times lower [24]. Humans also produce highly specific borreliacidal antibodies against outer surface protein C (OspC) shortly after infection with Borrelia burgdorferi sensu stricto: immunoglobulin M (IgM) OspC and immunoglobulin G (IgG) OspC borreliacidal antibodies [25]. VlsE is the most sensitive antigen for IgG antibody detection, OspC for IgM antibody detection [3, 26, 27].

Stage II: A variety of symptoms can develop several weeks to some months after receiving the tick bite. In the foreground of these are neurological manifestations: meningitis, encephalitis, asymmetric polyneuritis, cranial nerve pareses, Bannwarth´s lymphocytic meningoradiculoneuritis [20, 28, 29]. One of the most frequent neurological symptoms of borreliosis in children is the peripheral facial palsy. Usually the paresis of the facial nerve appears at one side and after several weeks on the opposite side [20]. Also arthritis, particularly of the knee joints, and non-localised pains in the bones, joints and muscles are frequently found [30]. Rarer are cardiological manifestations, such as myocarditis and pericarditis. Antibodies against Borrelia burgdorferi can be detected in 50% to 90% of patients in stage II [24]. In the early phase of this stage, mainly IgM antibodies are found, but in the late phase, often only IgG antibodies occur. However, IgM antibodies can persist for a long time [3, 30]. By additionally determining antibodies against VlsE the serological hit rate can be increased by 20%-30% [31]. VlsE displays the highest sensitivity of all antigens tested for IgG detection [26].

Stage III: The typical manifestations of a Borrelia burgdorferi infection in stage III are chronic-relapsing erosive arthritis, acrodermatitis chronica atrophicans and progressive encephalomyelitis, which can proceed in a similar way to multiple sclerosis. Without treatment, the tertiary stage can develop over a period of years to decades after the original infection. In this stage, IgG antibodies are significantly increased in 90% to 100% of patients, while IgM antibodies are only rarely detectable [3, 24].

For the diagnosis of neuroborreliosis, the determination of antibodies in cerebrospinal fluid is of decisive importance and far superior to serological testing [21, 29, 32]. An Antibody Index (AI) discriminates between a blood-derived and a pathological, brain-derived specific antibody fraction in cerebrospinal fluid (CSF) and takes into account individual changes in blood/CSF barrier function [33].

Various techniques come into question for the detection of antibodies against Borrelia burgdorferi [34, 35, 36, 37, 38, 39]: ELISA, indirect immunofluorescence, passive haemagglutination and immunoblot [26]. Many investigators employ an ELISA test in parallel with an indirect immunofluorescence test for preliminary characterisation of the serum sample [26, 34]. According to guidelines of the USA and Germany, serological diagnosis should follow the principle of a two-step procedure: ELISA or indirect immunofluorescence (IIFT) as first step: if reactive, followed by Westernblot [3, 24, 40]. In fresh infections it is recommended performing ELISA/IIFT and Westernblot in parallel, since some weak reactions become detectable earlier in Westernblot than in the screening tests [12, 24]. VlsE, the most sensitive antigen for IgG antibody detection, and OspC for IgM antibody detection, should be included in the test [3]. By additionally determining antibodies against VlsE the serological hit rate can be increased by 20% compared to whole extract Westernblots and by 30% compared to recombinant antigen Westernblots [24, 27, 31]. Of all recombinant antigens tested, VlsE possesses the highest sensitivity for the detection of a Borrelia infection [31, 32, 33].

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Declaration of Conformity

EUROIMMUN Medizinische Labordiagnostika AG
Seekamp 31, D-23560 Lübeck, Germany

declare under our sole responsibility that the ELISA-products

Anti-Borrelia plus VlsE ELISA (IgG)	*EI 2132-9601-2 G*
CSF: Anti-Borrelia plus VlsE ELISA (IgG)	*EI 2132-9601-L G*
CSQ pair of controls Anti-Borrelia (IgG)	*EI 2132-0208-8 L G*

(product name, order no)

in combination with automated analyzer for ELISA

EUROIMMUN Analyzer I

meet the demands of

Directive 98/79/EC on in vitro diagnostic medical devices of 27 October 1998

Lübeck, 26.05.2010

(Place and date of issue)

Wolfgang Schlumberger, PhD
- Member of the Board -

Susanne Aleksandrowicz
- Member of the Board -

Declaration of Conformity

EUROIMMUN Medizinische Labordiagnostika AG
Seekamp 31, D-23560 Lübeck, Germany

declare under our sole responsibility that the ELISA products

Anti-Borrelia ELISA (IgG)	*EI 2132-9601 G*
Anti-Borrelia ELISA (IgM)	*EI 2132-9601 M*
CSF: Anti-Borrelia ELISA (IgM)	*EI 2132-9601-L M*

(product name, order no)

meet the demands of

Directive 98/79/EC on in vitro diagnostic medical devices of 27 October 1998

Lübeck, 25.04.2008
(Place and date of issue)

Wolfgang Schlumberger, PhD
- Member of the Board -

Susanne Aleksandrowicz
- Member of the Board -

Anti-Borrelia plus VlsE ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2132-9601-2 G	Borrelia burgdorferi, Borrelia afzelii, Borrelia garinii, Borrelia VlsE Antigen	IgG	Ag-coated microplate wells	96 x 01 (96)

Indications: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human antibodies of the IgG class against Borrelia antigens in serum or plasma for the diagnosis of infection with Borrelia and associated diseases: Erythema chronicum migrans, lymphadenosis cutis benigna, acrodermatitis chronica atrophicans, arthritis, carditis, lymphocytic meningoradiculitis and neuro-borreliosis.

Application: Clinical diagnosis of borreliosis can be achieved by determination of Borrelia-specific antibodies of classes IgG and IgM. The Anti-Borrelia plus VlsE ELISA (IgG) is based on an optimised lysate mixture of the most releveant human pathogenic Borrelia strains and also contains recombinant VlsE. Due to its complete antigen spectrum, the ELISA offers a very high sensitivity and is therefore ideally suited for antibody screening. Samples with positive or borderline ELISA results should always be further investigated using immunoblot.

Principle of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with a mix of whole antigen extracts of Borrelia burgdorferi sensu stricto, Borrelia afzelii, Borrelia garinii and recombinant VlsE of Borrelia burgdorferi. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgG antibodies will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (enzyme conjugate) catalysing a colour reaction.

Contents of the test kit:

Component	Colour	Format	Symbol
1. Microplate wells coated with antigens 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	12 x 8	STRIPS
2. Calibrator 1 200 RU/ml (IgG, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2 20 RU/ml (IgG, human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 2 RU/ml (IgG, human), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
8. Sample buffer ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
9. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
10. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
11. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
12. Test instruction	---	1 booklet	
13. Quality control certificate	---	1 protocol	

LOT Lot description
IVD In vitro diagnostic medical device
CE
Storage temperature
Unopened usable until

Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bags).
  Once the protective wrapping has been opened for the first time, the wells coated with antigens can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.

- **Calibrators and controls:** Ready for use. The reagents must be mixed thoroughly before use.

- **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.

- **Sample buffer:** Ready for use.

- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallisation occurs in the concentrated buffer, warm it to 37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionised or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working strength wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.

- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.

- **Stop solution:** Ready for use.

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Warning: The calibrators and controls of human origin have tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the agent sodium azide in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the patient samples

Samples: Human serum or EDTA, heparin or citrate plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples must be incubated within one working day.

Sample dilution: Patient samples are diluted **1:101** with sample buffer.
Example: Add 10 µl of sample to 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: The calibrators and controls are prediluted and ready for use, do not dilute them.

Incubation

For **semiquantative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1, 2 and 3** along with the positive and negative controls and patient samples.

(Partly) manual test performance

Sample incubation: (1st step)
Transfer 100 µl of the calibrators, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol.
Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Manual: Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash.
Automatic: Wash the reagent wells 3 times with 450 µl of working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Modus").

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

Note: Residual liquid (> 10 µl) remaining in the reagent wells after washing can interfere with the substrate and lead to false low extinction values. Insufficient washing (e.g., less than 3 wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction values.
Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

Conjugate incubation: (2nd step)
Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgG) into each of the microplate wells.
Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Empty the wells. Wash as described above.

Substrate incubation: (3rd step)
Pipette 100 µl of chromogen/substrate solution into each of the microplate wells.
Incubate for **15 minutes** at room temperature (+18°C to +25°C), protect from direct sunlight.

Stopping:
Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:
Photometric measurement of the colour intensity should be made at a **wavelength of 450 nm** and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution**. Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

Test performance using fully automated analysis devices

Sample dilution and test performance are carried out fully automatically using an analysis device. The incubation conditions programmed in the respective software authorised by EUROIMMUN may deviate slightly from the specifications given in the ELISA test instruction. However, these conditions were validated in respect of the combination of the EUROIMMUN Analyzer I, Analyzer I-2P or the DSX from Dynex and this EUROIMMUN ELISA. Validation documents are available on enquiry.
Automated test performance using other fully automated, open system analysis devices is possible. However, the combination should be validated by the user.

Pipetting protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	**C 2**	**P 6**	**P 14**	**P 22**			**C 1**	**P 4**	**P 12**	**P 20**		
B	**pos.**	**P 7**	**P 15**	**P 23**			**C 2**	**P 5**	**P 13**	**P 21**		
C	**neg.**	**P 8**	**P 16**	**P 24**			**C 3**	**P 6**	**P 14**	**P 22**		
D	**P 1**	**P 9**	**P 17**				**pos.**	**P 7**	**P 15**	**P 23**		
E	**P 2**	**P 10**	**P 18**				**neg.**	**P 8**	**P 16**	**P 24**		
F	**P 3**	**P 11**	**P 19**				**P 1**	**P 9**	**P 17**			
G	**P 4**	**P 12**	**P 20**				**P 2**	**P 10**	**P 18**			
H	**P 5**	**P 13**	**P 21**				**P 3**	**P 11**	**P 19**			

The pipetting protocol for microtiter strips 1-4 is an example for the **semiquantitative analysis** of 24 patient sample (P 1 to P 24).
The pipetting protocol for microtiter strips 7-10 is an example for the **quantitative analysis** of 24 patient sample (P 1 to P 24).

The calibrators (C 1 to C 3), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample.
The wells can be broken off individually from the strips. Therefore, the number of tests performed can be matched to the number of samples, minimising reagent wastage.
Both positive and negative controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

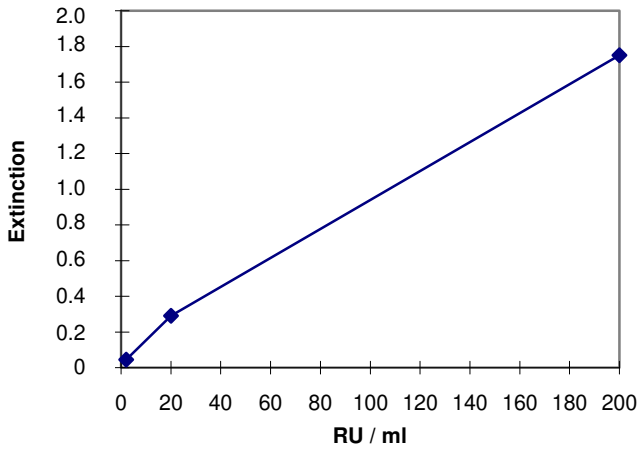
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2. Calculate the ratio according to the following formula:

$$\frac{\textbf{Extinction of the control or patient sample}}{\textbf{Extinction of calibrator 2}} = \textbf{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <0.8:	**negative**
Ratio ≥0.8 to <1.1:	**borderline**
Ratio ≥1.1:	**positive**

Quantitative: The standard curve from which the concentration of antibodies in the patient samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 3 calibration sera against the corresponding units (linear/linear). Use “point-to-point” plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.



If the extinction for a patient sample lies above the value of calibrator 1 (200 RU/ml), the result should be reported as “>200 RU/ml“. It is recommended that the sample be retested at a dilution of e.g. 1:400. The result in RU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4. The upper limit of the normal range of non-infected persons (cut-off value) recommended by EUROIMMUN is 20 relative units (RU)/ml. EUROIMMUN recommends interpreting results as follows:

<16 RU/ml:	**negative**
≥16 to <22 RU/ml:	**borderline**
≥22 RU/ml:	**positive**

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another, EUROIMMUN recommends to retest the samples.

A negative serological result does not exclude an infection. Particularly in the early phase of an infection, antibodies may not yet be present or are only present in such small quantities that they are not detectable. In case of a borderline result, a secure evaluation is not possible. If there is a clinical suspicion and a negative test result, we recommend clarification by means of other diagnostic methods and/or the serological investigation of a follow-up sample. A positive result indicates that there has been contact with the pathogen. In the determination of pathogen-specific IgM antibodies, polyclonal stimulation of the immune system or antibody persistence may affect the diagnostic relevance of positive findings. Significant titer increases (exceeding factor 2) and/or seroconversion in a follow-up sample taken after 7 to 10 days can indicate an acute infection. To investigate titer changes, sample and follow-up sample should be incubated in adjacent wells of the ELISA microplate within the same test run. For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Calibration: As no international reference serum exists for antibodies against Borrelia, the calibration is performed in relative units (RU)/ml.

For every group of tests performed, the extinction values of the calibrators and the relative units and/or ratio determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

The binding activity of the antibodies and the activity of the enzyme used are temperature-dependent. It is therefore recommended using a thermostat in all three incubation steps. The higher the room temperature (+18°C to +25°C) during the incubation steps, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The antigen source is provided by particularly suitable Borrelia strains (Borrelia burgdorferi sensu stricto, Borrelia afzelii, Borrelia garinii) as well as recombinant VlsE (**v**arible major protein-**l**ike **s**equence, **e**xpressed) of Borrelia burgdorferi. VlsE is a newly characterised surface protein of Borrelia which is expressed exclusively in vivo and which contains conserved and highly immunogenic epitopes.The cultured borrelia have been solubilised by using sodium dodecyl sulphate. The used antigen mixture contains all relevant proteins.

Linearity: The linearity of the Anti-Borrelia plus VlsE ELISA (IgG) was determined by assaying 4 serial dilutions of different patient samples. The coefficient of determination R^2 for all sera was > 0.95. The Anti-Borrelia plus VlsE ELISA (IgG) is linear at least in the tested concentration range (8 RU/ml to 124 RU/ml).

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable antibody titer. The lower detection limit of the Anti-Borrelia plus VlsE ELISA (IgG) is 0.7 RU/ml.

Cross reactivity: Cross reactivity of the Anti-Borrelia plus VlsE ELISA (IgG) was evaluated in a study performed on 256 patient sera with antibodies against Treponema pallidum and 16 patient sera with antibodies against Leptospira. 95 sera of the Anti-Treponema pallidum-positive sera and 1 serum of the Anti-Leptospira-positive sera presented reactivity. Therefore, the cross reactivity amounts to 37.1% and. 6.3%, respectively. Cross reactions with antibodies against other spirochaetes cannot be excluded. The Anti-Borrelia plus VlsE ELISA is designed as a screening test which should be followed by a confirmatory test (Western- or line blot). Further sera from patients with different possible influencing factors were investigated. An overview of these results can be found in the following table.

Possible influencing factors	n	Anti-Borrelia plus VlsE ELISA (IgG) positive
Acute EBV infection	33	0%
Anti-HSV 1	12	0%
Anti-CMV	12	0%
Anti-VZV	12	0%
Anti-Adenovirus	12	0%
Anti-RSV	12	0%
Anti-Parainfluenza	12	0%
Anti-Influenza-A virus	12	0%
Anti-Influenza-B virus	12	0%
Anti-Mycoplasma pneumoniae	12	0%
Anti-Measles virus	12	0%
Anti-Mumps virus	12	0%
Anti-Rubella virus	12	0%
Anti-Toxoplasma gondii	11	0%
Anti-Chlamydia pneumoniae	11	0%
Anti-Helicobacter pylori	12	0%
ANA + DNS (AAb)	36	2.8%
Rheuma factor	37	0%
Neurological diseases	54	3.7%

Interference: Haemolytic, lipaemic and icteric samples showed no influence on the result up to a concentration of 10 mg/ml for haemoglobin, 20 mg/ml for triglycerides and 0.4 mg/ml for bilirubin in this ELISA.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 3 sera. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed in 6 different test runs.

Intra-assay variation, n = 20		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	52	3.5
2	97	3.5
3	128	3.6

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	55	3.6
2	103	3.7
3	138	3.8

Specificity and sensitivity: Sera of 165 patients with suspected borreliosis were analysed using the EUROIMMUN Anti-Borrelia plus VlsE ELISA (IgG) and the EUROIMMUN Anti-Borrelia EUROLINE-Westernblot (IgG) as a reference method. The test showed a specificity of 90.2% and a sensitivity of 100%.

n = 165		**EUROIMMUN EUROLINE-Westernblot**		
		positive	borderline	negative
ELISA	positive	60	11	9
	borderline	0	1	1
	negative	0	0	83

Borderline results are not included by the calculation of specificity and sensitivity.

Clinical study: 138 patients with clinically characterised borreliosis in different disease stages were screened with the Anti-Borrelia plus VlsE-ELISA (IgG) and Anti-Borrelia-ELISA (IgM). Sensitivities between 89 to 96% were found.

n = 138		**EUROIMMUN Anti-Borrelia plus VlsE-ELISA (IgG), EUROIMMUN Anti-Borrelia-ELISA (IgM)**		
		IgG positive	IgM positive	IgG and/or IgM positive
Clinic	erythema migrans, n = 97	60	68	86 (88.7%)
	mono- or poly- arthritis, n = 26	22	5	25 (96.2%)
	neuroborreliosis, n = 15	13	4	14 (93.3%)

All ELISA negative results were confirmed as negative by an Anti-Borrelia Westernblot.

Reference range: The levels of the anti-Borrelia antibodies (IgG) were analysed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors. With a cut-off of 20 RU/ml, 5% of the blood donors were anti-Borrelia positive (IgG), wich reflects the known percentage of infections in adults.

Clinical significance

The history of Lyme disease, a contagious condition caused by Borrelia burgdorferi and transmitted to man by ticks, offers infectiologists a formidable lesson on how medicine progresses. Clinical description started in Europe at the turn of the 19th/20th century with Pick's description of what was then labelled chronic atrophic acrodermatitis. Fifty years later, Hauser noted the affection was transmitted by ticks. Independently, Afzelius, then Lipschutz, described erythema migrans and its relationship with tick bites. Neurological involvement was also described with the skin signs. These early dermatological descriptions suddenly came into the limelight in 1975 when an epidemia of arthritis occurred in children in Lyme, Connecticut, USA. Many of the affected children had erythema migrans. Based on these observations and an epidemiological analysis of the epidemia, Steele and co-workers defined "Lyme disease" as a rheumatological disorder commonly associated with erythema migrans and sometimes with multiple organ involvement. In 1982 Burgdorfer suggested that ticks transmitted "treponema-like spirochaetes", which were later authentified as the causal agent: Borrelia burgdorferi.

Borrelia burgdorferi as a species of bacteria is belonging to the spirochaetaceae family. This family includes five genera: borrelia, spirochaetes, cristispira, treponema and leptospira. Among about 30 tick species feeding on humans, Ixodes ricinus is the most frequent tick species biting humans in Europe. It is the vector of Borrelia burgdorferi, which causes Lyme disease, of anaplasma phagocytophilum and the tick-borne encephalitis virus. I. ricinus ticks pass through three developmental stages: larvae, nymphs and adults (females and males). The density of this tick species can be very high, reaching in some places more than 300 ticks/100 m^2. Lyme borreliosis is the most frequent tick-transmitted disease in the northern hemisphere. In Europe, especially in Central Europe and Scandinavia there are up to 155 cases per 100,000 individuals caused by the species B. burgdorferi sensu stricto, B. afzelii, and B. garinii (in rare cases also by B. spielmanii and B. bavariensis). The human seroprevalence rate of antibodies against Borrelia burgdorferi in the normal popu-lation of Germany and other Central European countries is about 8% (for IgG); in highly endemic areas even considerably higher with ranges from 18% to 52%. In East Asia, e.g. in an endemic area in China, the seroprevalence amounts to 26%. Persons working in the area of forestry display anti-Borrelia antibodies in about 40%, hunters in more than 50% of cases.

A Borrelia burgdorferi infection can manifest itself in the areas of dermatology, neurology, ophthalmology, rheumatology and internal medicine. The clinical expression of borreliosis can be divided into three stages:

Stage I: The typical primary manifestation of a Borrelia burgdorferi infection is erythema migrans (80%), a reddening of the skin which appears around the area of the tick bite and spreads in a circular manner. The erythema is accompanied by influenza-like general symptoms with fever, shaking chill, headaches and vomiting. Lymphadenopathies are observed in a few cases (lymphadenosis cutis benigna). The leading clinical types of the erythema-free borreliosis (20%) are neurological, arthromyalgic, influenza-like, cardiovascular borreliosis and borreliosis with hepatitis, with regional lymphadenitis and mixtures of these. Stage I can result in spontaneous healing or can develop into a generalised borreliosis. The transition phase is generally symptom-free. IgM antibodies against Borrelia burgdorferi can be detected serologically in 50% to 90% of patients during stage I. Humans produce highly specific antibodies against the outer surface protein C (OspC) shortly after infection with Borrelia burgdorferi. The main antigen VlsE, which is exclusively expressed in vivo, is the most sensitive antigen for IgG antibody detection, whereas OspC is the most sensitive antigen for IgM antibody detection.

Stage II: A variety of symptoms can develop several weeks to some months after receiving the tick bite. In the foreground of these are neurological manifestations: meningitis, encephalitis, asymmetric polyneuritis, cranial nerve pareses, Bannwarth's lymphocytic meningoradiculoneuritis. One of the most frequent neurological symptoms of borreliosis in children is the peripheral facial palsy. Usually the paresis of the facial nerve appears on one side and after several weeks on the opposite side. Also arthritis, particularly of the knee joints, and non-localised pains in the bones, joints and muscles are frequently found. Less frequently are cardiological manifestations such as myocarditis and pericarditis. Antibodies against Borrelia burgdorferi can be detected in 50% to 90% of patients in stage II. In the early phase of this stage mainly IgM antibodies are found but in the late phase often only IgG antibodies occur. However, IgM antibodies can persist for a long time. By additionally determining antibodies against VlsE the serological hit rate can be increased by 20% to 30%. VlsE displays the highest sensitivity of all antigens tested for IgG detection.

Stage III: The typical manifestations of a Borrelia burgdorferi infection in stage III are chronic-relapsing erosive arthritis, acrodermatitis chronica atrophicans and progressive encephalomyelitis, which can proceed in a similar way to multiple sclerosis. Without treatment, the tertiary stage can develop over a period of years to decades after the original infection. In this stage, IgG antibody titers are significantly increased in 90% to 100% of patients, while IgM antibodies are only rarely detectable.

For the diagnosis of neuroborreliosis, the determination of antibodies in cerebrospinal fluid is of decisive importance and far superior to serological testing. An antibody index (AI) discriminates between a blood-derived and a pathological, brain-derived specific antibody fraction in cerebrospinal fluid (CSF) and takes into account individual changes in the blood/CSF barrier function.

Various techniques come into question for the detection of antibodies against Borrelia burgdorferi: ELISA, indirect immunofluorescence (IIFT), passive haemagglutination and immunoblot. Generally, ELISA or IIFT are used as screening test for preliminary characterisation of the serum sample. According to guidelines in the USA and in Germany, serological diagnosis should follow the principle of a two-step procedure: ELISA or indirect immunofluorescence (IIFT) as a first step, which, if reactive, is followed by an immunoblot. In fresh infections it is recommended performing ELISA/IIFT and immuno-blot in parallel, since some weak reactions become detectable earlier in the blot than in the screening test. VlsE, the most sensitive antigen for IgG antibody detection, and OspC for IgM antibody detection should be included in the test. By additionally determining antibodies against VlsE the serological hit rate can be increased by 20% compared to whole extract Westernblots. Of all recombinant antigens tested, VlsE possesses the highest sensitivity for the detection of a Borrelia infection.

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Anti-Borrelia ELISA (IgM)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2132-9601 M	Borrelia burgdorferi, Borrelia afzelii, Borrelia garinii	IgM	Ag-coated microplate wells	96 x 01 (96)

Indications: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human antibodies of the IgM class against Borrelia antigens in serum or plasma for the diagnosis of Infection with Borrelia. Associated diseases: Erythema chronicum migrans, lymphadenosis cutis benigna, acrodermatitis chronica atrophicans, arthritis, carditis, lymphocytic meningoradiculitis, neuroborreliosis.

Application: Clinical diagnosis of borreliosis can be achieved by determination of Borrelia-specific antibodies of classes IgG and IgM. The Anti-Borrelia ELISA (IgM) is based on an optimised lysate mixture of the most releveant human pathogenic Borrelia strains. Specific cultivation methods help to ensure a large proportion of OspC in the test. Due to its complete antigen spectrum, the ELISA offers a very high sensitivity and is therefore ideally suited for antibody screening. Samples with positive or borderline ELISA results should always be further investigated using immunoblot.

Principle of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with antigen extracts of Borrelia burgdorferi sensu stricto, Borrelia afzelii and Borrelia garinii. In the first reaction step, diluted patient samples are incubated with the wells. In the case of positive samples, specific IgM antibodies (also IgA and IgG) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgM (enzyme conjugate) catalysing a colour reaction.

Contents of the test kit:

Component	Colour	Format	Symbol
1. Microplate wells coated with antigens 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	12 x 8	STRIPS
2. Calibrator 1, 200 RU/ml (IgM, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2, 20 RU/ml (IgM, human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3, 2 RU/ml (IgM, human), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control, (IgM, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control, (IgM, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgM (goat), ready for use	red	1 x 12 ml	CONJUGATE
8. Sample buffer containing IgG/RF absorbent (anti-human IgG antibody preparation obtained from goat), ready for use	green	1 x 100 ml	SAMPLE BUFFER
9. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
10. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
11. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
12. Test instruction	---	1 booklet	
13. Quality control certificate	---	1 protocol	

LOT	Lot description	CE	Storage temperature
IVD	In vitro diagnostic medical device		Unopened usable until

Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature (+18°C to +25°C) to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bags).
  Once the protective wrapping has been opened for the first time, the wells coated with antigens can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.

- **Calibrators and controls:** Ready for use. The reagents must be mixed thoroughly before use.

- **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.

- **Sample buffer:** Ready for use. The green coloured sample buffer contains IgG/RF absorbent. Serum or plasma samples diluted with this sample buffer are only to be used for the determination of IgM antibodies.

- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallisation occurs in the concentrated buffer, warm it to 37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionised or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working strength wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.

- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.

- **Stop solution:** Ready for use.

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Warning: The calibrators and controls of human origin have tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the agent sodium azide in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the patient samples

Samples: Human serum or EDTA, heparin or citrate plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

Introduction: Before the determination of specific antibodies of class IgM, antibodies of class IgG should be removed from the patient sample. This procedure must be carried out in order to prevent any rheumatoid factors from reacting with specifically bound IgG, which would lead to false positive IgM test results, and to prevent that specific IgG displace IgM from the antigen, which would lead to false IgM negative test results.

Functional principle: The sample buffer (green coloured!) contains an anti-human antibody preparation from goat. IgG of a serum or plasma sample is bound with high specificity by these antibodies and precipitated. If the sample also contains rheumatoid factors, these will be absorbed by the IgG/anti-human IgG complex.

Separation properties:
- All IgG subclasses are bound and precipitated by the anti-human IgG antibodies.
- Human serum IgG in concentrations of up to 15 mg per ml are removed (average serum IgG concentration in adults: 12 mg per ml).
- Rheumatoid factors are also removed.
- The recovery rate of the IgM fraction is almost 100%.

Performance: The **patient samples** for analysis are diluted **1:101** with green coloured sample buffer. For example, add 10 µl sample to 1.0 ml sample buffer and mix well by vortexing. Sample pipettes are not suitable for mixing Incubate the mixture for at least **10 minutes** at room temperature (+18°C to +25°C). Subsequently, it can be pipetted into the microplate wells according to the pipetting protocol.

Notes:
- Antibodies of the class IgG should not be analysed with this mixture.
- It is possible to check the efficacy of the IgG/RF absorbent for an individual patient sample by performing an IgG test in parallel to the IgM test using the mixture. If the IgG test is negative, the IgM result can be considered as reliable.
- The calibrators and controls are ready for use, do not dilute them.

Incubation

For **semiquantative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1, 2 and 3** along with the positive and negative controls and patient samples.

(Partly) manual test performance

Sample incubation: (1st step)
Transfer 100 µl of the calibrators, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol. Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Manual: Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash.
Automatic: Wash the reagent wells 3 times with 450 µl of working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Modus").

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

Note: Residual liquid (> 10 µl) remaining in the reagent wells after washing can interfere with the substrate and lead to false low extinction values.
Insufficient washing (e.g. less than 3 wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction values.
Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

Conjugate incubation: (2nd step)
Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgM) into each of the microplate wells. Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Empty the wells. Wash as described above.

Substrate incubation: (3rd step)
Pipette 100 µl of chromogen/substrate solution into each of the microplate wells. Incubate for **15 minutes** at room temperature (+18°C to +25°C), protect from direct sunlight.

Stopping:
Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:
Photometric measurement of the colour intensity should be made at a **wavelength of 450 nm** and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution.** Prior to measuring, carefully shake the microplate to ensure a homogeneous distribution of the solution.

Test performance using fully automated analysis devices

Sample dilution and test performance are carried out fully automatically using an analysis device. The incubation conditions programmed in the respective software authorised by EUROIMMUN may deviate slightly from the specifications given in the ELISA test instruction. However, these conditions were validated in respect of the combination of the EUROIMMUN Analyzer I, Analyzer I-2P or the DSX from Dynex and this EUROIMMUN ELISA. Validation documents are available on enquiry.
Automated test performance using other fully automated, open system analysis devices is possible. However, the combination should be validated by the user.

Pipetting protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	**C 2**	**P 6**	**P 14**	**P 22**			**C 1**	**P 4**	**P 12**	**P 20**		
B	**pos.**	**P 7**	**P 15**	**P 23**			**C 2**	**P 5**	**P 13**	**P 21**		
C	**neg.**	**P 8**	**P 16**	**P 24**			**C 3**	**P 6**	**P 14**	**P 22**		
D	**P 1**	**P 9**	**P 17**				**pos.**	**P 7**	**P 15**	**P 23**		
E	**P 2**	**P 10**	**P 18**				**neg.**	**P 8**	**P 16**	**P 24**		
F	**P 3**	**P 11**	**P 19**				**P 1**	**P 9**	**P 17**			
G	**P 4**	**P 12**	**P 20**				**P 2**	**P 10**	**P 18**			
H	**P 5**	**P 13**	**P 21**				**P 3**	**P 11**	**P 19**			

The pipetting protocol for microtiter strips 1-4 is an example of the **semiquantitative determination** of antibodies in 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7-10 is an example of the **quantitative determination** of antibodies in 24 patient samples (P 1 to P 24).

Calibrators (C 1 to C 3), positive (pos.) and negative (neg.) control as well as the patient samples have been incubated in one well each. The reliability of the ELISA test can be improved by duplicate determinations of each sample.
The wells can be broken off individually from the strips. This makes it possible to adjust the number of test substrates used to the number of samples to be examined and minimises reagent wastage.
Both positive and negative controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

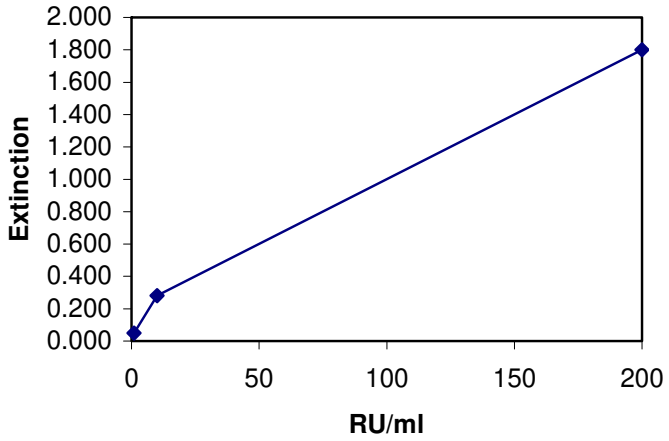
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of calibrator 2. Calculate the ratio according to the following formula:

$$\frac{\textbf{Extinction of the control or patient sample}}{\textbf{Extinction of calibrator 2}} = \textbf{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <0.8:	**negative**
Ratio ≥0.8 to <1.1:	**borderline**
Ratio ≥1.1:	**positive**

Quantitative: The standard curve from which the concentration of antibodies in the patient samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 3 calibrators against the corresponding units (linear/linear). Use “point-to-point” plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.



If the extinction for a patient sample lies above the value of calibrator 1 (200 RU/ml), the result should be reported as “>200 RU/ml“. It is recommended that the sample be retested at a dilution of e.g. 1:400. The result in RU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4. The upper limit of the reference range of non-infected persons (cut-off value) recommended by EUROIMMUN is 20 relative units RU/ml. EUROIMMUN recommends interpreting results as follows:

<16 RU/ml:	**negative**
≥16 to <22 RU/ml:	**borderline**
≥22 RU/ml:	**positive**

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another, EUROIMMUN recommends to retest the samples.

A negative serological result does not exclude an infection. Particularly in the early phase of an infection, antibodies may not yet be present or are only present in such small quantities that they are not detectable. In case of a borderline result, a secure evaluation is not possible. If there is a clinical suspicion and a negative test result, we recommend clarification by means of other diagnostic methods and/or the serological investigation of a follow-up sample. A positive result indicates that there has been contact with the pathogen. In the determination of pathogen-specific IgM antibodies, polyclonal stimulation of the immune system or antibody persistence may affect the diagnostic relevance of positive findings. Significant titer increases (exceeding factor 2) and/or seroconversion in a follow-up sample taken after 7-10 days can indicate an acute infection. To investigate titer changes, sample and follow-up sample should be incubated in adjacent wells of the ELISA microplate within the same test run. For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Calibration: As no international reference serum exists for antibodies against Borrelia, the calibration is performed in relative units (RU)/ml.

For every group of tests performed, the extinction values of the calibrators and the relative units and/or ratio values determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

The binding activity of the antibodies and the activity of the enzyme used are temperature-dependent. It is therefore recommended using a thermostat in all three incubation steps. The higher the room temperature (+18°C to +25°C) during the incubation steps, the greater will be the extinction values. Corresponding variations apply also to the incubation times.
However, the calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The antigen source is provided by particularly suitable Borrelia strains (Borrelia burgdorferi sensu stricto, Borrelia afzelii and Borrelia garinii). The cultured bacteria were solubilised using sodium dodecyl sulphate. The used antigen mixture contains all relevant proteins.

Linearity: The linearity of the Anti-Borrelia ELISA (IgM) was determined by assaying 4 serial dilutions of different patient samples. The coefficient of determination R^2 for all sera was > 0.95. The Anti-Borrelia ELISA (IgM) is linear at least in the tested concentration range (3 RU/ml to 177 RU/ml).

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable antibody titer. The lower detection limit of the Anti-Borrelia ELISA (IgM) is 1.3 RU/ml.

Cross reactivity: Cross reactivity of the Anti-Borrelia ELISA (IgM) was evaluated in a study performed on 263 patient sera with antibodies against Treponema pallidum and 18 patient sera with antibodies against Leptospira. 26 sera of the Anti-Treponema pallidum-positive sera and 3 sera of the Anti-Leptospira-positive sera presented reactivity. Therefore, the cross reactivity amounts to 9.9% and 16.7%, respectively. Cross reactions with antibodies against other spirochaetes cannot be excluded. The Anti-Borrelia ELISA is designed as a screening test which should be followed by a confirmatory test (Western- or line blot). Further sera from patients with different possible influencing factors were investigated. An overview of these results can be found in the following table.

Possible influencing factors	n	Anti-Borrelia ELISA (IgM) positive
Acute EBV infection	27	0%
Anti-CMV	19	0%
Anti-Measles virus	13	0%
Anti-Mumps virus	12	0%
Anti-Toxoplasma gondii	14	0%
Anti-VZV	15	0%
Anti-Rubella virus	10	0%
Anti-HSV	5	0%
Anti-HAV	7	0%
Anti-HBV	8	0%
Anti-HAMA	4	0%
ANA + DNS (AAb)	45	0%
Rheuma factor	39	0%
Neurological diseases	54	1.9%

Interference: Haemolytic, lipaemic and icteric samples showed no influence on the result up to a concentration of 10 mg/ml for haemoglobin, 20 mg/ml for triglycerides and 0.4 mg/ml for bilirubin in this ELISA.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 3 sera. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed in 6 different test runs.

Intra-assay variation, n = 20		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	75	4.3
2	103	2.9
3	115	3.4

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	78	6.9
2	109	6.2
3	122	4.7

Specificity and sensitivity: Sera from 150 patients with suspected borreliosis were analysed using the EUROIMMUN Anti-Borrelia ELISA (IgM) and the EUROIMMUN Anti-Borrelia burgdorferi Westernblot (IgM) as a reference method. The test showed a specificity of 96.4% and a sensitivity of 100%.

n = 150		**EUROIMMUN Westernblot**		
		positive	borderline	negative
ELISA	positive	34	5	4
	negative	0	1	106

Clinical study: 138 patients with clinically characterised borreliosis in different disease stages were screened with the Anti-Borrelia plus VlsE-ELISA (IgG) and Anti-Borrelia-ELISA (IgM). Sensitivities between 89-96% were found.

n = 138		**EUROIMMUN Anti-Borrelia plus VlsE-ELISA (IgG), EUROIMMUN Anti-Borrelia-ELISA (IgM)**		
		IgG positive	IgM positive	IgG und/oder IgM positive
Clinic	erythema migrans, n = 97	60	68	86 (88.7%)
	mono- or poly- arthritis, n = 26	22	5	25 (96.2%)
	neuroborreliosis, n = 15	13	4	14 (93.3%)

All ELISA negative results were confirmed as negative by an Anti-Borrelia Westernblot.

Reference range: The levels of the anti-Borrelia antibodies (IgM) were analysed with this EUROIMMUN ELISA in 500 healthy blood donors. With a cut-off of 20 RU/ml, 1.6% of the blood donors were anti-Borrelia positive (IgM).

Clinical significance

The history of Lyme disease, a contagious condition caused by Borrelia burgdorferi and transmitted to man by ticks, offers infectiologists a formidable lesson on how medicine progresses. Clinical description started in Europe at the turn of the 19^{th}/20^{th} century with Pick's description of what was then labelled chronic atrophic acrodermatitis. Fifty years later, Hauser noted the affection was transmitted by ticks. Independently, Afzelius, then Lipschutz, described erythema migrans and its relationship with tick bites. Neurological involvement was also described with the skin signs. These early dermatological descriptions suddenly came into the limelight in 1975 when an epidemia of arthritis occurred in children in Lyme, Connecticut, USA. Many of the affected children had erythema migrans. Based on these observations and an epidemiological analysis of the epidemia, Steele and co-workers defined "Lyme disease" as a rheumatological disorder commonly associated with erythema migrans and sometimes with multiple organ involvement. In 1982 Burgdorfer suggested that ticks transmitted "Treponema-like spirochaetes", which were later authentified as the causal agent: Borrelia burgdorferi.

Borrelia burgdorferi as a species of bacteria is belonging to the spirochaetaceae family. This family includes five genera: borrelia, spirochaetes, cristispira, treponema and leptospira. Among about 30 tick species feeding on humans, Ixodes ricinus is the most frequent tick species biting humans in Europe. It is the vector of Borrelia burgdorferi, which causes Lyme disease, of anaplasma phagocytophilum and the tick-borne encephalitis virus. I. ricinus ticks pass through three developmental stages: larvae, nymphs and adults (females and males). The density of this tick species can be very high, reaching in some places more than 300 ticks/100 m^2. Lyme borreliosis is the most frequent tick-transmitted disease in the northern hemisphere. In Europe, especially in Central Europe and Scandinavia there are up to 155 cases per 100,000 individuals caused by the species B. burgdorferi sensu stricto, B. afzelii, and B. garinii (in rare cases also by B. spielmanii and B. bavariensis). The human seroprevalence rate of antibodies against Borrelia burgdorferi in the normal popu-lation of Germany and other Central European countries is about 8% (for IgG); in highly endemic areas even considerably higher with ranges from 18% to 52%. In East Asia, e.g. in an endemic area in China, the seroprevalence amounts to 26%. Persons working in the area of forestry display anti-Borrelia antibodies in about 40%, hunters in more than 50% of cases.

A Borrelia burgdorferi infection can manifest itself in the areas of dermatology, neurology, ophthal-mology, rheumatology and internal medicine. The clinical expression of borreliosis can be divided into three stages:

Stage I: The typical primary manifestation of a Borrelia burgdorferi infection is erythema migrans (80%), a reddening of the skin which appears around the area of the tick bite and spreads in a circular manner. The erythema is accompanied by influenza-like general symptoms with fever, shaking chill, headaches and vomiting. Lymphadenopathies are observed in a few cases (lymphadenosis cutis benigna). The leading clinical types of the erythema-free borreliosis (20%) are neurological, arthromyalgic, influenza-like, cardiovascular borreliosis and borreliosis with hepatitis, with regional lymphadenitis and mixtures of these. Stage I can result in spontaneous healing or can develop into a generalised borreliosis. The transition phase is generally symptom-free. IgM antibodies against Borrelia burgdorferi can be detected serologically in 50% to 90% of patients during stage I. Humans produce highly specific antibodies against the outer surface protein C (OspC) shortly after infection with Borrelia burgdorferi. The main antigen VlsE, which is exclusively expressed in vivo, is the most sensitive antigen for IgG antibody detection, whereas OspC is the most sensitive antigen for IgM antibody detection.

Stage II: A variety of symptoms can develop several weeks to some months after receiving the tick bite. In the foreground of these are neurological manifestations: meningitis, encephalitis, asymmetric poly-neuritis, cranial nerve pareses, Bannwarth's lymphocytic meningoradiculoneuritis. One of the most frequent neurological symptoms of borreliosis in children is the peripheral facial palsy. Usually the paresis of the facial nerve appears on one side and after several weeks on the opposite side. Also arthritis, particularly of the knee joints, and non-localised pains in the bones, joints and muscles are frequently found. Less frequently are cardiological manifestations such as myocarditis and pericarditis. Antibodies against Borrelia burgdorferi can be detected in 50% to 90% of patients in stage II. In the early phase of this stage mainly IgM antibodies are found but in the late phase often only IgG antibodies occur. However, IgM antibodies can persist for a long time. By additionally determining antibodies against VlsE the serological hit rate can be increased by 20% to 30%. VlsE displays the highest sensi-tivity of all antigens tested for IgG detection.

Stage III: The typical manifestations of a Borrelia burgdorferi infection in stage III are chronic-relapsing erosive arthritis, acrodermatitis chronica atrophicans and progressive encephalomyelitis, which can proceed in a similar way to multiple sclerosis. Without treatment, the tertiary stage can develop over a period of years to decades after the original infection. In this stage, IgG antibody titers are significantly increased in 90% to 100% of patients, while IgM antibodies are only rarely detectable.

For the diagnosis of neuroborreliosis, the determination of antibodies in cerebrospinal fluid is of decisive importance and far superior to serological testing. An antibody index (AI) discriminates between a blood-derived and a pathological, brain-derived specific antibody fraction in cerebrospinal fluid (CSF) and takes into account individual changes in the blood/CSF barrier function.

Various techniques come into question for the detection of antibodies against Borrelia burgdorferi: ELISA, indirect immunofluorescence (IIFT), passive haemagglutination and immunoblot. Generally, ELISA or IIFT are used as screening test for preliminary characterisation of the serum sample. According to guidelines in the USA and in Germany, serological diagnosis should follow the principle of a two-step procedure: ELISA or indirect immunofluorescence (IIFT) as a first step, which, if reactive, is followed by an immunoblot. In fresh infections it is recommended performing ELISA/IIFT and immuno-blot in parallel, since some weak reactions become detectable earlier in the blot than in the screening test. VlsE, the most sensitive antigen for IgG antibody detection, and OspC for IgM antibody detection should be included in the test. By additionally determining antibodies against VlsE the serological hit rate can be increased by 20% compared to whole extract Westernblots. Of all recombinant antigens tested, VlsE possesses the highest sensitivity for the detection of a Borrelia infection.

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