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Article

Assessing the Need for Multiplex and Multifunctional Tick-Borne Disease Test in Routine Clinical Laboratory Samples from Lyme Disease and Febrile Patients with a History of a Tick Bite

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Abstract: Human polymicrobial infections in tick-borne disease (TBD) patients is an emerging public health theme. However, the requirement for holistic TBD tests in routine clinical laboratories is ambiguous. TICKPLEX[®] PLUS is a holistic TBD test utilized herein to assess the need for multiplex and multifunctional diagnostic tools in a routine clinical laboratory. The study involved 150 specimens categorized into Lyme disease (LD)-positive ($n = 48$), LD-negative ($n = 30$), and febrile patients from whom borrelia serology was requested ($n = 72$, later “febrile patients”) based on reference test results from United Medix, Finland. Reference tests from DiaSorin, Immunetics, and Mikrogen Diagnostik followed the two-tier LD testing system. A comparison between the reference tests and TICKPLEX[®] PLUS produced 86%, 88%, and 87% positive, negative, and overall agreement, respectively. Additionally, up to 15% of LD and 11% of febrile patients responded to TBD related coinfections and opportunistic microbes. The results demonstrated that one (TICKPLEX[®] PLUS) test can aid in a LD diagnosis instead of four tests. Moreover, TBD is not limited to just LD, as the specimens produced immune responses to several TBD microbes. Lastly, the study indicated that the screening of febrile patients for TBDs could be a missed opportunity at reducing unreported patient cases.

Keywords: Lyme disease; tick-borne disease; zoonoses; spirochetes; polymicrobial; summer flu; misdiagnosis; persister; Borrelia; Lyme diagnostic



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1. Introduction

Lyme disease (LD) is a tick-borne disease (TBD) caused by bacteria from the *Borrelia burgdorferi sensu lato* group that can cause arthritic, dermatitis, or neurological manifestations [1–4]. Other common TBDs also include Babesiosis, Ehrlichiosis, Anaplasmosis, Encephalitis, and more [5–8]. Currently, TBDs are present in over 80 countries and may affect 35% of the world's population by 2050 [9]. In the meantime, the number of ticks that carry pathogens and can cause TBDs are ever-increasing [10–13]. Over the years, the reported TBD cases have spiked in various countries around the world [14–16]. Healthcare authorities like the Centers for Disease Control and Prevention (CDC) in the USA recognize that the real frequency of TBD cases in humans is much higher than the reported cases [17]. In 2018, the European Commission made headway by adding Lyme Neuroborreliosis to the list of diseases under the European Union's epidemiological surveillance [18]. Additionally, the European Parliament resolution recognized that the current TBD diagnostic tools are inaccurate, as they test for only one microbe at a time [19].

Globally, the CDC two-tier testing algorithm for LD stands undisputed by regulatory and healthcare authorities [20]. The literature is rife with evidence concerning the effectiveness of the CDC two-tier system for diagnosing LD [1,21,22]. The CDC recently revised its

LD testing algorithm by endorsing the use of two enzyme-linked immunosorbent assays (ELISAs) in both tiers [23]. However, the testing recommendations for other TBDs in LD patients is not clear, despite the growing evidence of coinfections in such patients [24]. An estimated 85% of LD patients can produce an immune response to TBD-related coinfections or opportunistic microbes [25]. Yet, 83% of all commercial TBD tests—for example, in the USA—are solely prescribed for LD [26]. The most putative diagnostic test manufacturers have popularized the use of a single test for a single disease following the Germ Theory [21,22,27]. As a result, the role, relevance, and requirements for a multiplex and multifunctional tool in the diagnosis of a complex disease like TBD are unclear for routine use in clinical laboratories.

Internationally, the research community has confirmed the likelihood of immune dysfunction in LD patients due to pathogenesis by *Borrelia* [28–33]. A TBD patient may experience an increase in disease severity, as *Borrelia* can sabotage, undermine, or trick the host immune system by evasion [32–34]. For example, *Borrelia* can repress the antigen-induced proliferation of lymphocyte cells or anti-*Borrelia* antibody response in immunocompromised patients [28,33]. Additionally, *Borrelia* can meddle with the kinetics and quality of B-cell and T-cell responses [34,35]. Hence, LD patients can present seronegative, delayed, or persistent antibody responses to *Borrelia*, indicating the complex nature of TBDs and a possible reason for misdiagnosed or undiagnosed cases [35–38]. Additionally, the regular discovery of novel and emerging TBD pathogens such as *Rickettsia monacensis*, Powassan virus, Omsk hemorrhagic virus, and others further complicates treatment for TBD patients without a holistic diagnostic tool [39].

A holistic diagnostic test may also help realize the need to institute a differential diagnosis in TBD testing recommendations. Patients with common symptoms like fever, headache, cough, and chills in the absence of laboratory evidence for LD could be misdiagnosed or remain undiagnosed for other conditions [40–42]. The prevalence of well-known TBD-related coinfections and opportunistic microbes are evident in individuals suffering from myalgia, fatigue, arthritis, and more [43]. For example, infection with *Bartonella* species can cause patients to complain about myalgia and fatigue [44]. Similarly, patients with fibromyalgia and chronic fatigue syndrome demonstrate an immune response to *Mycoplasma pneumoniae* or *Mycoplasma fermentans* [45,46]. While TBD is complicated to diagnose according to the literature mentioned above, will the use of comprehensive diagnostic tests prove practical to help reduce unrecognized patient cases? The goal of this study was to assess the need for a multiplex and multifunctional TBD immunoassay in routine clinical laboratory samples from Lyme disease and febrile patients with a (suspected) history of a tick bite.

2. Materials and Methods

2.1. Index Test and Interpretation

TICKPLEX® PLUS (herein, TICKPLEX®) is an ELISA index test used in this study that is a CE-IVD registered product (i.e., European In-Vitro Diagnostic Devices Directive (98/79/EC) compliant) manufactured in an ISO 13485:2016 accredited facility at Tezted Ltd, Jyväskylä, Finland. TICKPLEX® can measure the immunoglobulin M (IgM) and immunoglobulin G (IgG) immune responses in human serum samples against *Borrelia burgdorferi sensu lato* species in spirochete and persistent forms, coinfections, and opportunistic microbes. Mainly, TICKPLEX® includes *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, and *Borrelia garinii* in spirochete and persistent form, *Babesia microti*, *Bartonella henselae*, *Ehrlichia chaffeensis*, *Rickettsia akari*, Coxsackievirus, Epstein–Barr virus, Human parvovirus B19, *Mycoplasma fermentans*, and *Mycoplasma pneumoniae* [25]. The clinical relevance for all TICKPLEX® microbes in TBD patients has been previously demonstrated [25]. The 150 human serums were tested blindly with the index test at Tezted Ltd. Normalized optical density values at 450 nm lower than 0.90, between 0.91 to 0.99, and higher than 1.00 were negative, borderline, and positive immune responses for all microbes, respectively.

2.2. Ethics Statement

United Medix Laboratories (Finland) provided anonymized and leftover human sera samples for research purposes. Sera sample included reference test results for LD, age, and gender for all patients. Following the General Data Protection Regulation (GDPR) [47], researchers at Tezted Ltd. did not have access to any private information (i.e., name, profession, or ethnicity) from the specimens that could be linked back to the patients. Hence, following the Declaration of Helsinki embodied in Common Rule set forth by the Code of Federal Regulations, USA, informed consent was not collected, as the present study was not considered as human subject research [48,49]. In Finland, the medical research act (488/1999) and the law on the medical usage of human organs, tissues, and cells (2.2.2001/101; section 20 (30.11.2012/689)) supports the use of leftover and deidentified human serum samples with consent from the collection unit [50,51]. United Medix Laboratories (Finland) was the collection unit for this study that contributed the deidentified human serum specimens according to their International Organization for Standardization (ISO) 15189 section 5.9.1. quality management system [52].

2.3. Reference Tests and Interpretation

Healthcare providers in Finland follow the CDC two-tier guidelines for LD diagnosis. Thus, Diasorin LIAISON[®] Borrelia chemiluminescence immunoassay (CLIA), Immunetics[®] C6 Lyme ELISA[™] (C6 ELISA), and Mikrogen Diagnostik *recom*Bead Borrelia IgG 2.0 (IgG Blot) were used to confirm LD in human specimens. The CLIA test separately measures human IgM and IgG immune responses to *Borrelia burgdorferi sensu lato*. In contrast, the C6 ELISA measures human IgM and IgG combined immune reactions to the C6 synthetic peptide derived from the VlsE protein conserved in *Borrelia burgdorferi sensu stricto* or *Borrelia afzelii* and *Borrelia garinii*. For LD confirmation purposes, IgG Blot measured the human IgG immune response against *Borrelia burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, *B. bavariensis*, and *B. spielmanii*.

For the CLIA IgM test, arbitrary units per milliliter (AU/ml) less than 18, between 18 to 22, and more than 22 were considered negative, borderline, and positive immune responses, respectively. Similarly, for the CLIA IgG test, AU/ml less than 10, between 10 to 15, and more than 15 were considered negative, borderline, and positive immune responses, respectively. Like AU/ml, the C6 ELISA test utilized the Lyme Index (LI) with a normalized optical density value at 450 nm and a reference wavelength at 650 nm. As a result, LI less than 0.9, between 0.91 to 1.09, and more than 1.10 were considered negative, borderline, and positive immune responses, respectively. In the case of the IgG Blot test, normalized fluorescence intensities below 0.67, between 0.67 to 1.00, and above 1.00 were considered negative, borderline, and positive immune responses, respectively.

2.4. Patient Categorization

According to the CDC two-tier algorithm [24] for LD diagnosis and related test interpretation criteria, as mentioned above, the 150 human serum samples were organized in three different categories. LD-positive category ($n = 48$) included specimens with positive IgM or IgG immune responses to one ($n = 7$), two ($n = 17$), three ($n = 9$), or all four ($n = 15$) diagnostic tests. Category two included LD-negative ($n = 30$) serum samples with a negative immune response to all four tests ($n = 15$) and a positive immune response limited to the CLIA IgM or IgG test ($n = 15$). The last category included serum samples from patients with fever and a known or suspected history of a tick bite, i.e., from whom borrelia serology was requested (later, the febrile patient group) ($n = 72$). For the febrile patient group, the test results from the C6 ELISA and IgG Blot tests were not available.

2.5. Index Test and Interpretation

TICKPLEX[®] PLUS (herein, TICKPLEX[®]) is an ELISA index test used in this study that is a CE-IVD registered product manufactured in an ISO 13485:2016 accredited facility at Tezted Ltd. TICKPLEX[®] can measure IgM and IgG immune responses in human serum

samples against *Borrelia burgdorferi sensu lato* species in spirochete and persistent forms, coinfections, and opportunistic microbes. Mainly, TICKPLEX[®] includes *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, and *Borrelia garinii* in spirochete and persistent form, *Babesia microti*, *Bartonella henselae*, *Ehrlichia chaffeensis*, *Rickettsia akari*, Coxsackievirus, Epstein–Barr virus, Human parvovirus B19, *Mycoplasma fermentans*, and *Mycoplasma pneumoniae* [25]. The clinical relevance for all TICKPLEX[®] microbes in TBD patients has been previously demonstrated [25]. The 150 human serums were tested blindly with the index test at Tezted Ltd. Normalized optical density values at 450 nm lower than 0.90, between 0.91 to 0.99, and higher than 1.00 were negative, borderline, and positive immune responses for all microbes, respectively.

2.6. Statistical Analysis

For quality control purposes, an inter-plate and inter-operator precision analysis was conducted by assessing the coefficient of variance [53] (CV %) on the optical density values for IgM/IgG plate controls and all microbial antigens on TICKPLEX[®]. To assess the CV % for index test microbial antigens, the negative serum control (TEZ1) in the kit was repeatedly performed in each plate by each operator. Equations (1)–(3) were utilized to calculate the proportion of positive (PA), negative (NA), and overall (OA) agreement, respectively, among the reference tests and between the reference tests versus (vs.) index test [54]. The PA, NA, and OA agreements among the reference tests and between reference tests with the index test were combined for the IgM and IgG immune responses. In Equations (1)–(3), the letters a, b, c, and d stand for true positives, false positives, false negatives, and true negatives, respectively. Further, the reliability for each PA and NA comparison was evaluated by calculating Cohen’s kappa (k) with a 95% confidence interval [54,55].

$$PA = \frac{2a}{2a + b + c} \quad (1)$$

$$NA = \frac{2d}{2d + b + c} \quad (2)$$

$$OA = \frac{a + d}{a + b + c + d} \quad (3)$$

Cohen’s k ranges from -1 to $+1$, wherein k values ≤ 0 indicates no agreement, 0.01–0.20 as none to a slight agreement, 0.21–0.40 as fair agreement, 0.41–0.60 as moderate agreement, 0.61–0.80 as substantial agreement, and 0.81–1.00 as almost perfect agreement [55]. Proportionate positive and negative agreements, along with Cohen’s k , were calculated using the EPITOOLS diagnostic test evaluation and comparison calculator. The inter-rater reliability and proportional agreement analysis between various tests were carried out using just LD-positive and -negative patient groups. Further, Fisher’s exact test was used to assess the statistical differences in IgM or IgG immune responses between the LD (positive and negative) and febrile patient groups. The two-tailed p -values for the Fisher’s exact test were calculated using GraphPad (<https://www.graphpad.com/quickcalcs/contingency1/>) (accessed on 28 May 2019). Fisher’s exact test results with p -values < 0.05 were considered statistically associated or dependent [56].

3. Results

The United Medix Laboratories in Finland collected specimens from LD-positive ($n = 48$) and LD-negative ($n = 30$) patients and from febrile patients from whom borrelia serology was requested ($n = 72$). The samples were collected amid routine clinical diagnostic services (convenience sampling) in the summer of 2018, beginning from late-May to mid-September. On average, patients were 42, 39, and 36 years old in the LD-positive, LD-negative, and febrile groups, respectively. The LD-positive patient group included 27 male and 21 female human serum samples. Likewise, the LD-negative group included specimens from 15 male and 15 female patients. Lastly, specimens from the febrile patients consisted of 31 male and 41 female human specimens. Overall, the average age for 73 male and

77 female serum samples was 39 years. Further, the inter-plate and inter-operator CV % for IgM and IgG on the index test were 6.280% and 4.692%, respectively. Additionally, the CV % for the internal negative control (TEZ1) was observed to be $\leq 15\%$ for all microbial antigens on the index test.

Figure 1 illustrates the PA, NA, OA, and Cohen's k among the reference tests and between the reference tests with the index test. The PA for the individual reference or index test ranged between 53% for IgG Blot vs. TICKPLEX[®] to 72% for CLIA IgM/IgG vs. C6 ELISA. Similarly, the lowest NA was observed for CLIA IgM/IgG vs. TICKPLEX[®] (49%) and the highest between IgG Blot vs. TICLPLEX (76%). The OA ranged from 55% for CLIA IgM/IgG vs. TICKPLEX[®] to 73% between C6 ELISA vs. IgG Blot. Except for a moderate Cohen's k agreement between C6 ELISA vs. IgG Blot ($k = 0.45$), all the other individual test combinations displayed fair Cohen's k agreements ($k = 0.12$ to 0.31). Among the different test comparisons individually, the average PA, NA, and OA were 63.5%, 62.33%, and 64%, respectively. As mentioned earlier, four Lyme disease tests (i.e., reference tests) were used to confirm *Borrelia* infection according to the CDC two-tier criteria. A substantial Cohen's k agreement was observed between the commercial two-tiered tests vs. TICKPLEX[®] ($k = 0.74$). The PA, NA, and OA for comparisons between all reference tests and TICKPLEX[®] were 86%, 88%, and 87%, respectively (Figure 1).

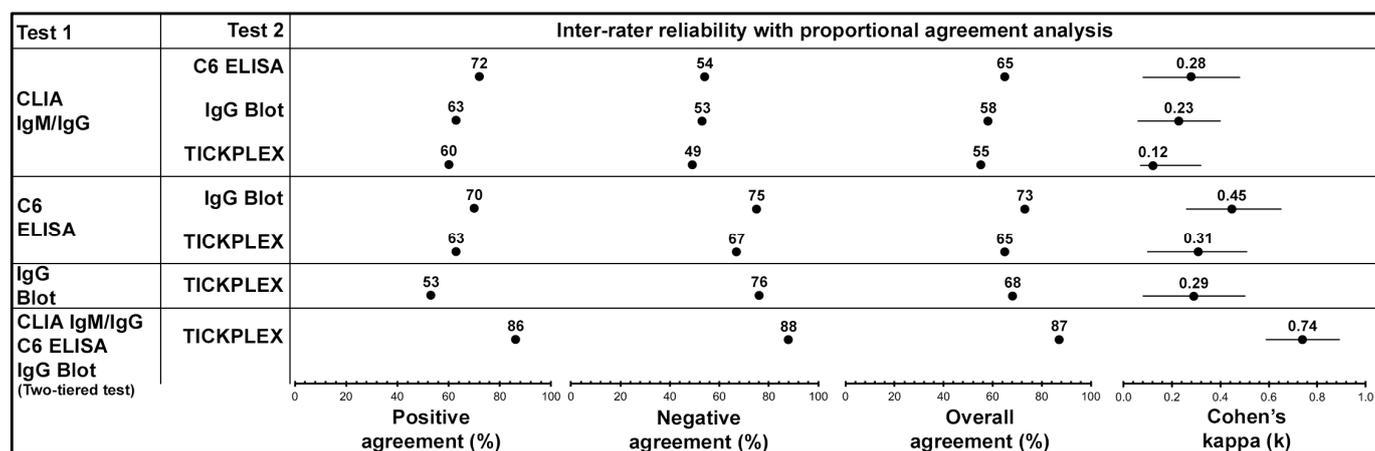


Figure 1. TICKPLEX[®] can aid replace the need for four Lyme disease diagnostic tests, as the index test clinical performance substantially agrees with the Centers for Disease Control and Prevention (CDC) two-tier system. The collective immunoglobulin M/immunoglobulin G (IgM/IgG) inter-rater reliability (i.e., Cohen's k) and proportional agreement analysis (i.e., positive, negative, and overall agreement) among reference tests and between the reference tests with the index test. Herein, reference tests refer to Diasorin LIAISON[®]Borrelia chemiluminescence immunoassay (CLIA), Immunetics[®]C6 Lyme ELISATM (C6 ELISA), and Mikrogen Diagnostik *recom*Bead Borrelia IgG 2.0 (IgG Blot). Similarly, the index test refers to TICKPLEX[®] PLUS (TICKPLEX[®]). Further, Cohen's k ranges from -1 to $+1$, wherein k values ≤ 0 indicates no agreement, 0.01 – 0.20 as none to a slight agreement, 0.21 – 0.40 as fair agreement, 0.41 – 0.60 as moderate agreement, 0.61 – 0.80 as substantial agreement, and 0.81 – 1.00 as almost perfect agreement. The present figure uses the reference and index test results from Lyme disease-positive ($n = 48$) and -negative ($n = 30$) groups.

In addition to Lyme disease, the LD-positive, LD-negative, and febrile patient groups were also tested against TBD related coinfections and opportunistic microbes using TICKPLEX[®]. Figure 2 is a cooccurrence heat map indicating the percentage of IgM or IgG immune responses by LD (positive and negative) and febrile patient groups to TICKPLEX[®] antigens. *Borrelia* spirochete species and persistent forms witnessed the most significant percentage of IgM and IgG immune responses in both patient groups. Apart from *Borrelia*, an average 2% for IgM and 8% for IgG immune responses were noted by LD specimens to coinfections and opportunistic microbes related to TBD (herein other microbes). Likewise, on average, 4% for IgM and 6% for IgG immune responses were observed for febrile patient samples against other TBD-related microbes. Overall, a statistical association or dependence was observed

between LD and the febrile patient group's IgM and IgG responses to the Epstein–Barr virus and *Borrelia* spirochete species, respectively (Figure S1). No association in IgM or IgG immune responses with the remaining TICKPLEX® antigens were noted between the LD and febrile patient groups (Figure S1).

		<i>Borrelia</i> spirochete species	<i>Borrelia</i> persistent forms	<i>Babesia microti</i>	<i>Bartonella henselae</i>	<i>Ehrlichia chaffeensis</i>	<i>Rickettsia akari</i>	Coxsackievirus	Epstein-Barr virus	Human parvovirus B19	<i>Mycoplasma</i> species	
IgM	Lyme disease	6	5	4	4	0	5	0	0	1	3	
	Febrile	11	4	3	3	3	6	3	6	6	3	
IgG	Lyme disease	44	15	9	10	5	12	6	9	6	4	
	Febrile	25	11	7	6	6	8	7	6	6	7	
Co-occurrence scale (%)		0	10	20	30	40	50	60	70	80	90	100

Figure 2. Screening of febrile patients for tick-borne diseases could be a missed opportunity at reducing misdiagnosed and undiagnosed patient cases, as their positive IgM and IgG immune response percentages are similar to the Lyme disease group. *Borrelia* spirochete species and *Borrelia* persistent forms refer to *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, and *Borrelia garinii* in spirochete and persistent forms, respectively. Similarly, *Mycoplasma* species refers to *Mycoplasma fermentans* and *Mycoplasma pneumoniae*.

The IgM and IgG immune responses by the LD and febrile patient groups to *Borrelia* alone, *Borrelia* and other microbes, and just other microbes in the index test were further analyzed (Figure 3). For IgM, 1%, 6%, and 0% LD patients responded to only *Borrelia*, *Borrelia* and other microbes, and only other microbes, respectively (Figure 3A). Similarly, 4%, 7%, and 4% of the febrile patient specimens produced an IgM response against only *Borrelia*, *Borrelia* and other microbes, and only other microbes, respectively (Figure 3A). In the case of IgG immune responses by LD patients, 28%, 15%, and 4% of the patients responded to only *Borrelia*, *Borrelia* and other microbes, and only other microbes, respectively (Figure 3B). Likewise, 14%, 11%, and 0% of the febrile patient specimens produced IgG response against only *Borrelia*, *Borrelia* and other microbes, and only other microbes, respectively (Figure 3B). A statistical association was observed between the LD and febrile patient groups' IgG responses to only *Borrelia* (Figure 3).

Figure S2 demonstrates the percentage of LD or febrile patient IgM and IgG immune responses to the number of other microbes along with *Borrelia*. The IgM or IgG immune responses to *Borrelia* and one other microbe was the most significant percentage of the reaction seen in both the LD and febrile patient groups. In the case of the LD patient group, 3% for IgM and 6% for IgG responded to *Borrelia* and one other microbe, respectively. Similarly, 4% and 3% of the febrile patients produced IgM and IgG responses to *Borrelia* and one other microbe, respectively. Not more than 1% of the LD or febrile patient specimens in IgM or IgG responded to *Borrelia* and two other microbes to seven other microbes.

Remarkably, the second most significant percentage of IgM or IgG immune responses was seen in both the LD and febrile patient specimens for *Borrelia* and eight other microbes. Approximately 4% IgM or IgG immune responses were noted from the LD and febrile

patient groups against *Borrelia* and eight other microbes. An IgM or IgG immune response to *Borrelia* and eight other microbes primarily responded to all ten TICKPLEX[®] antigens. At random, a serum sample with IgM and IgG immune response to *Borrelia* and eight other microbes was selected and serially diluted on TICKPLEX[®]. As a result, a clear dose-dependent response was observed (Figure S3).

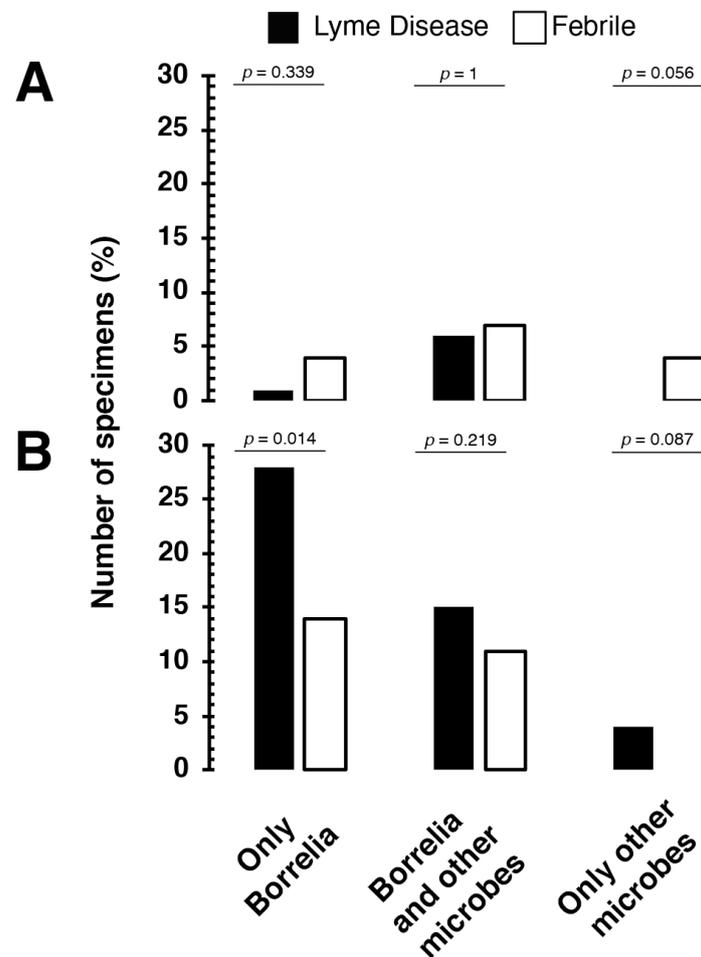


Figure 3. Lyme disease and febrile patients produce (A) IgM and (B) IgG immune responses to *Borrelia* and multiple coinfections and opportunistic microbes related to tick-borne diseases. Other microbes refer to *Babesia microti*, *Bartonella henselae*, *Ehrlichia chaffeensis*, *Rickettsia akari*, Coxsackievirus, Epstein–Barr virus, Human parvovirus B19, *Mycoplasma fermentans*, and *Mycoplasma pneumoniae* in the index test. The *p*-value originates from the Fisher’s exact test that was used to assess the statistical differences in IgM or IgG immune responses between Lyme disease (LD) (positive and negative) and febrile patient groups. The two-tailed *p*-values for the Fisher’s exact test were calculated using GraphPad (<https://www.graphpad.com/quickcalcs/contingency1/> (accessed on 28 May 2019)). The Fisher’s exact test results with *p*-values < 0.05 were considered statistically associated or dependent.

4. Discussion

To evaluate the use for a multiplex and multifunctional TBD immunoassay in a routine clinical laboratory, LD-positive ($n = 48$), LD-negative ($n = 30$), and febrile ($n = 72$) patient specimens were tested against TICKPLEX[®] microbial antigens for their IgM and IgG immune responses. The clinical performance of TICKPLEX[®] (index test) for testing LD was compared to four reference tests (CLIA IgM and IgG, C6 ELISA, and IgG Blot) used at the United Medix Laboratories in Finland following the CDC two-tier criteria. Individual comparisons among the reference tests and between the reference with the index tests resulted in an average PA, NA, and OA of 63.5%, 62.33%, and 64%, respectively

(Figure 1). A substantial Cohen's k agreement ($k = 0.74$) was mainly observed when the clinical outcome from all four reference tests was compared with the TICKPLEX[®] results (Figure 1). A comparison between the commercial CDC two-tiered LD testing system with TICKPLEX[®] produced 86% PA, 88% NA, and 87% OA (Figure 1).

Variations in the PA, NA, or OA among the LD diagnostic tests is a rule rather than the exception, because several in vitro diagnostic test manufacturers utilize different *Borrelia* proteins [21,22,27,57]. For example, the positivity rate for LD patients with an Erythema Migrans rash can range from 18% to 53% for whole-cell antigen LD tests vs. 31% to 50% for recombinant antigen LD tests [57]. Generally, diagnostic test sensitivities improve from the early to late LD stages [21,22,57]. In later LD stages like neuroborreliosis, the positivity rate can vary from 41% to 86% for whole-cell antigen LD tests and 49% to 81% for recombinant antigen LD tests [57]. Additionally, with regards to the CDC two-tier testing system, a PA among commercial LD tests can vary from 5% to 98.5%, and a NA can range from 28.6% to 100% [21]. Overall, at any given LD stage, the average accuracy for LD diagnostic tests is 62.3% [21,27]. Similar accuracy averages in this study were observed among the reference tests and between the reference and index tests (Figure 1).

While the accuracy averages for LD diagnostic tests between this study and the literature are comparable, the study findings herein also indicated that TICKPLEX[®] is a suitable replacement for the CLIA IgM/IgG, C6 ELISA, and IgG Blot reference tests. A dramatic increase in correlations between the commercial CDC two-tiered LD tests and TICKPLEX[®] is connected to a consistent PA (60% to 63%) with CLIA IgM/IgG and C6 ELISA plus a high NA (76%) with the IgG Blot test (Figure 1). The C6 ELISA demonstrates a similarly dramatic change in a PA and NA when compared with either an individual LD test or a CDC two-tiered testing system [21]. A previous comparison between the C6 ELISA and CLIA IgM/IgG tests yielded 70% PA and 99.1% NA [21]. However, the current study demonstrated 72% PA and only 54% NA between the C6 ELISA and CLIA IgM/IgG tests (Figure 1). Nevertheless, a 98.5% PA and 49% NA was evident between the C6 ELISA and CDC two-tiered tests, which included the Wampole Bb (IgG/IgM) ELISA test system, MarDx Lyme Disease (IgG and IgM), and Marblot strip test system [21]. As a result, the PA, NA, and OA of LD tests strongly depend on the type of reference test used for comparison [21,22,27,57].

The TICKPLEX[®] results also indicated that 6% to 15% of the LD individuals responded to TBD-related coinfections and opportunistic microbes (Figure 2). Traditionally, a TBD-linked opportunistic infection in a LD patient could be the result of a vulnerable immune system due to a prolonged TBD infection [32,33,58]. Immune responses by LD patients to multiple other TBD microbes with or without *Borrelia* demonstrate that TBD is not limited to just LD in Finland (Figures 2 and 3). In several other countries, like Germany, Sweden, the Netherlands, and more, 4% to 60% of LD patients can suffer from LD and TBD-related coinfections [59–61]. Multiple TBD-associated infections in LD patients primarily originate from ticks that can carry over 120 distinct bacterial and other microbial species [62]. In various regions of Finland, the cooccurrence percentage for multiple pathogens in ticks ranges from 1.02% to 28.3% [11–13,63,64]. In 2004, a Finnish LD patient suffered from fatal Babesiosis [65]. Therein, no research articles on PubMed elucidated the relevance of TBD-related coinfections or opportunistic microbes in Finland.

Furthermore, the IgG immune responses were statistically correlated between the LD and febrile patient groups (Figure S1 and Figure 3). Moreover, 7% to 11% of the febrile patients reacted to other TBD-related microbes (Figures 2 and 3). The current study demonstrated that individuals with fever and a putative history of a tick bite can respond to TBD microbes similar to LD patients (Figures 2 and 3). A misdiagnosis of early LD as summer flu is an understudied topic in the field of TBDs [41]. Not all LD patients demonstrate an Erythema Migrans (EM) rash or produce detectable antibodies in the first two to four weeks. A misdiagnosis is probable for nearly 16% of LD cases that do not display an EM rash [66]. Additionally, 60% of early-stage LD individuals receive a negative LD diagnostic test result, as they do not develop a detectable level of antibodies and are

therefore susceptible to misdiagnosis [67]. A TBD infection can cause nonspecific febrile illness wherein individuals may suffer from LD (11%), human granulocytic ehrlichiosis (13%), or coinfections (3%) [68].

Lastly, an IgM or IgG immune response to all TICKPLEX[®] antigens by 4% of the LD and febrile patient groups is an unexpected finding in this study (Figure S2). The unspecific binding of human specimens to recombinant proteins or blocking agents on an ELISA test is a plausible interpretation [69,70]. However, all TICKPLEX[®] antigens comprise of either whole-cell lysates or synthetic peptides and not recombinant proteins. Secondly, a sera sample from 4% of the LD and febrile patients at random was serially diluted to correlate the declining antibody concentration with optical density values. In the presence of an unspecific reaction, a serial dilution of sera specimen will not make any difference on the resulting optical density value. Figure S3 indicates no unspecific binding on TICKPLEX[®] for IgM and IgG. Immune evasion and host immune response suppression, modulation, or subversion by *Borrelia* in LD patients is a common finding [28,30,32,33,71–73]. For example, *Borrelia* can trick the host immune system into producing a strong yet inadequate response while it evades the lymph nodes [34]. We postulate that a universally positive IgM or IgG immune response in TBD patients could be the result of a B-cell-related immune dysfunction, such as unspecific B-cell activation [29,34,74].

A noticeable improvement to the current study would be to increase the overall sample size and improve the statistical confidence in the findings. In the future, the study design could also include a comparison between TICKPLEX[®] non-*Borrelia* antigens and related reference tests in a routine lab clinical setting. Additionally, a multicenter prospective study approach with several TBD disease patient groups would aid in a health economic assessment and awareness for TBD diagnosis with TICKPLEX[®]. Furthermore, a systematic investigation is required to assess the significance and prevalence of TBD patients with an IgM or IgG-positive immune response to every microbial protein (universally positive).

In conclusion, the present study makes evident that the clinical performance of *Borrelia* spirochete species and *Borrelia* persistent forms on TICKPLEX[®] is in-line with the industry standard PA, NA, and OA. Additionally, the unique *Borrelia* protein combination in TICKPLEX[®] can reduce the need from four tests for a LD diagnosis to just one test. Furthermore, in a routine clinical lab, a multiplex and multifunctional test can help detect TBD-related coinfections and opportunistic microbes in LD patients. Moreover, the screening of febrile or summer flu patients for TBDs could be a missed opportunity at reducing misdiagnosed and undiagnosed patient cases.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2414-6366/6/1/38/s1>, Figure S1. Statistical association or dependence was observed between Lyme disease and febrile patient groups' IgM and IgG responses to the Epstein–Barr virus and *Borrelia* spirochete species, respectively. *Borrelia* spirochete species and *Borrelia* persistent forms refer to *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, and *Borrelia garinii* in spirochete and persistent forms, respectively. Similarly, *Mycoplasma* species refers to *Mycoplasma fermentans* and *Mycoplasma pneumoniae*. The *p*-value originates from the Fisher's exact test that was used to assess the statistical differences in the IgM or IgG immune responses between the LD (positive and negative) and febrile patient groups. The two-tailed *p* values for the Fisher's exact test was calculated using GraphPad (<https://www.graphpad.com/quickcalcs/contingency1/> (accessed on 28 May 2019)). Fisher's exact test results with *p*-values < 0.05 were considered statistically associated or dependent. Figure S2. Lyme disease and febrile patient specimens demonstrated (A) IgM and (B) IgG immune responses for up to eight other microbes with *Borrelia* using the TICKPLEX[®] test. In the present figure, the other microbes refer to *Babesia microti*, *Bartonella henselae*, *Ehrlichia chaffeensis*, *Rickettsia akari*, Coxsackievirus, Epstein–Barr virus, Human parvovirus B19, *Mycoplasma fermentans*, and *Mycoplasma pneumoniae* in the index test. Figure S3. No (A) IgM or (B) IgG unspecific binding is observed in the TICKPLEX[®] test. *Borrelia* spirochete species and *Borrelia* persistent forms refer to *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, and *Borrelia garinii* in spirochete and persistent forms, respectively. Similarly, *Mycoplasma* species refers to *Mycoplasma fermentans* and *Mycoplasma pneumoniae*. Table S1. Normalized IgM optical density values for Lyme disease-positive (sera ID 1-48), -negative (sera ID

49-78), and febrile patients (sera ID 79-150) from the index test. Table S2. Normalized IgG optical density values for Lyme disease-positive (sera ID 1-48), -negative (sera ID 49-78), and febrile patients (sera ID 79-150) from the index test.

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TICKPLEX® PLUS INSTRUCTIONS FOR USE

1. TITLE: TICKPLEX® PLUS. A complete diagnostic kit for tick-borne disease pathogens.

2. INTENDED USE

The TICKPLEX® PLUS test provides a quantitative and qualitative *in vitro* assay for human IgM and IgG antibodies against multiple microbial antigens in human serum or plasma. The kit tests for *Borrelia afzelii*, *Borrelia burgdorferi*, and *Borrelia garinii* infections and the tests include persistent antigens of the different *Borrelia* species. In addition, the kit tests for co-infections (*Babesia microti*, *Bartonella henselae*, *Ehrlichia chaffeensis*, *Rickettsia akari*), and opportunistic infections (Coxsackievirus, Epstein-Barr virus, Human parvovirus B19, *Mycoplasma fermentans* and *Mycoplasma pneumoniae*) associated with tick-borne diseases. This kit is intended to aid in diagnosis of tick-borne pathogen infections. The test kit and TICKPEX® PLUS analyze excel sheet are intended to aid in the diagnosis of diseases associated with a tick bite. The test kit and TICKPEX® PLUS analyze excel sheet are for professional use only in clinical laboratory environment and are not to be used for self-testing.

3. TEST PRINCIPLE

TICKPLEX® PLUS is an indirect enzyme linked immunosorbent assay (ELISA). Micro wells on TICKPLEX® PLUS ELISA plates have been coated with inactivated whole cell lysate or peptide antigens. Antigen (A1 to A10) coating order for TICKPLEX® PLUS can be noted from TICKPLEX® PLUS plate layout in section 7. Antibodies that are specific against antigens being tested in TICKPLEX® PLUS will bind to the immobilized antigen(s) on the plate. Antibodies that do not bind with antigens are washed away. However, antibodies that form complexes with the antigen(s) can be recognized by anti-human IgM and anti-human IgG antibodies. Anti-human IgM/IgG are conjugated with horseradish peroxidase (HRP). Presence of conjugated anti-human IgM/IgG can be seen by an enzymatic reaction with 3,3',5,5'-tetramethylbenzidine (chromogenic substrate). Human antibodies that is negative against the antigens being tested in TICKPLEX® PLUS will not form complexes with immobilized antigens. A mild change in the chromogenic substrate color due to a reaction with a negative human antibody can be differentiated from a reaction with a borderline, and positive human antibody binding.

4. TICKPLEX® PLUS ELISA KIT COMPONENTS AND STORAGE

Table 1. List of TICKPLEX® PLUS ELISA kit components, quantities provided in the kit, and storage conditions.

ID	TICKPLEX® PLUS ELISA kit components	Quantity	Storage upon receipt
A	ELISA microplate coated with inactivated microbial antigens	10 plates	+2°C to +8°C
B	Wash buffer concentrate (25X phosphate buffer saline)	2 x 125 ml	+2°C to +8°C
C	Sample buffer concentrate (2% bovine serum albumin)	150 ml	+2°C to +8°C
D	100 % IgM serum diluent concentrate	50 ml	+2°C to +8°C
E	Enzyme substrate solution [3,3',5,5'-tetramethylbenzidine (TMB)]	125 ml	+2°C to +8°C
F	Stop solution [2 M Sulphuric acid (H ₂ SO ₄)]	100 ml	+2°C to +8°C
G	Anti-human IgM conjugated with HRP	60 ml	+2°C to +8°C
H	Anti-human IgG conjugated with HRP	60 ml	+2°C to +8°C
I	Negative serum control (TEZ1) with SDS document	25 ml	+2°C to +8°C
J	TICKPLEX PLUS ANALYZE (sent via email)	1	N/A
K	TICKPLEX PLUS Quality control certificate	1 document	N/A

Note: Please inspect that all kit components are available in the kit and packed as specified.

Note: Please check that you have received Analyze Excel Sheet with matching LOT information marked on Containers 1 and 2.

Note: Please check that Analyze Excel Sheet opens up in your computer. Instructions how to use software for test result calculations are included as part of program.

5. MATERIALS REQUIRED BUT NOT PROVIDED WITH THE KIT

- i. Distilled/deionized water for dilution of wash buffer concentrate.
- ii. Appropriate equipment for pipetting, liquid dispensing, and washing ELISA microplate.
- iii. Spectrophotometer/colorimeter/microplate reader. Absorbance wavelength required = 450 nm with bottom reading.
- iv. Sixty (60) 2 ml Eppendorf tubes.
- v. Waste for safe disposal of potentially infectious material (example; human serum sample).

6. REAGENT AND SERUM SAMPLE PREPARATIONS FOR PERFORMING ONE PLATE

A TICKPLEX® PLUS ELISA microplate can test negative serum control (TEZ1) and three patients. The following kit components have been provided in ready to use format: the ELISA microplates coated with inactivated microbial antigens, enzyme substrate solution [3,3',5,5'-tetramethylbenzidine (TMB)], and stop solution [2 M Sulphuric acid (H₂SO₄)].

Follow instructions below to dilute the remaining kit components to perform one (1) TICKPLEX® PLUS microplate:

i. Diluting buffer solution

- **Wash buffer concentrate dilution** → Add 300 ml of distilled / deionized water to 12.5 ml wash buffer concentrate (kit component B) to obtain **1X wash buffer**.
- **Sample buffer concentrate dilution** → Add 15 ml of 1X wash buffer to 15 ml sample buffer concentrate (kit component C) to obtain **1 % sample buffer**.
- **100 % IgM serum diluent concentrate dilution** → Add 2.5 ml of 1 % sample buffer to 2.5 ml of 100 % IgM serum diluent (kit component D) to obtain **50 % IgM serum diluent**.

Storage after dilution:

Store 1X wash buffer and 1 % sample buffer at +2°C to +8°C for maximum 6 weeks.

50 % IgM serum diluent should be used immediately after preparation.

CAUTION: Negative serum control is a human serum sample which is classified as non-hazardous substance. This human serum has been verified as negative for the most relevant infections, but it is not a complete assurance that infectious agents are absent. Thus, the user should handle this product as if capable of transmitting infection.

ii. Diluting patient serum sample

A TICKPLEX® PLUS ELISA microplate can test three (3) patients. To test one patient at 1:200 dilution, 1 ml of diluted sera for IgM testing and 1 ml of diluted sera for IgG testing are needed.

- **For IgM testing** → add 5 µl of patient sera sample to 995 µl of **50 % IgM serum diluent**.

- For IgG testing → add 5 µl of patient sera sample to 995 µl of 1 % sample buffer

7. DIRECTIONS TO PERFORM THE TICKPLEX® PLUS ELISA

To perform TICKPLEX® PLUS, the plate layout provided below and the instructions in this section must be followed. Read the instructions carefully before performing the test.

TICKPLEX® PLUS PLATE LAYOUT

Legends to illustration presented below in a 96-microwell plate format.											
A1 = <i>Borrelia burgdorferi</i> , <i>Borrelia afzelii</i> , and <i>Borrelia garinii</i>											
A2 = <i>Borrelia burgdorferi</i> , <i>Borrelia afzelii</i> , and <i>Borrelia garinii</i> persistent form											
A3 = <i>Babesia microti</i> A4 = <i>Bartonella henselae</i> A5 = <i>Ehrlichia chaffeensis</i> A6 = <i>Rickettsia akari</i>											
A7 = <i>Coxsackievirus</i> A8 = Epstein-Barr virus A9 = Human parvovirus B19 A10 = <i>Mycoplasma pneumoniae</i> and <i>Mycoplasma fermentans</i>											
P = Positive Control N = Negative Control C1, C2, and C3 = Calibrator Control											

	1	2	3	4	5	6	7	8	9	10	11	12	
TEZ1 control	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	P	C1	Test for IgM Antibody type from A1 to D12
Patient 1	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	N	C2	
Patient 2	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10		C3	
Patient 3	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10			
TEZ 1 control	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	P	C1	Test for IgG Antibody type from E1 to H12
Patient 1	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	N	C2	
Patient 2	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10		C3	
Patient 3	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10			

i. Negative serum control (TEZ1) and patient sample addition and incubation

- Place the reagents and plate at room temperature (+15°C to +25°C) for 30 min before performing the test.
- Unwrap the TICKPLEX® PLUS ELISA microplate from its vacuum packing.
- Pipetting order for **IgM** test is as follows:
 - Pipette 100 µl of negative serum control (TEZ1) in wells A1 to A10.
 - Pipette 100 µl of patient 1 serum sample freshly diluted in 50 % IgM serum diluent in wells B1 to B10 each.
 - Pipette 100 µl of patient 2 serum sample freshly diluted in 50 % IgM serum diluent in wells C1 to C10 each.
 - Pipette 100 µl of patient 3 serum sample freshly diluted in 50 % IgM serum diluent in wells D1 to D10 each.
- Pipetting order for **IgG** test is as follows:
 - Pipette 100 µl of negative serum control (TEZ1) in wells E1 to E10 each.
 - Pipette 100 µl of patient 1 serum sample freshly diluted in 1 % sample buffer in wells F1 to F10 each.
 - Pipette 100 µl of patient 2 serum sample freshly diluted in 1 % sample buffer in wells G1 to G10 each.
 - Pipette 100 µl of patient 3 serum sample freshly diluted in 1 % sample buffer in wells H1 to H10 each.

- Pipette 100 µl of 1 % sample buffer to IgM and IgG positive control (P), negative control (N), and calibrator (C1, C2, and C3) wells.
- Cover the plate with its lid and incubate for 1 hour at room temperature.

ii. Anti-human IgM and IgG HRP conjugated antibodies addition and incubation

- **Wash step** → Dispense and aspirate 200 µl of 1X wash buffer five times in all wells.
- After washing, thoroughly dispose of all liquid from the microplate by tapping it on an absorbent paper with the openings facing downwards to remove all residual wash buffer.

IMPORTANT: Anti-human IgM and IgG conjugated with HRP dilution should be gently mixed before use (example; 1 ml single-channel pipette).

- In each well, pipette 100 µl of anti-human **IgM** conjugated with HRP in rows A, B, C, and D.
- In each well, pipette 100 µl of anti-human **IgG** conjugated with HRP in rows E, F, G, and H.
- Cover the plate and incubate for 1 hour at room temperature.

iii. Enzyme substrate solution addition and incubation

- **Wash step** → Dispense and aspirate 200 µl of 1X wash buffer five times in all wells.
- After washing, thoroughly dispose of all liquid from the microplate by tapping it on an absorbent paper with the openings facing downwards to remove all residual wash buffer.

IMPORTANT: Enzyme substrate solution (TMB) must reach room temperature before use. The enzyme substrate solution must be clear to use. DO NOT USE if the solution is colored.

- Add 100 µl of enzyme substrate solution (TMB) in all wells.
- Cover the plate and incubate in the dark (example, covering with aluminum foil) at room temperature.
- Incubate plate for a total of **30 min** at room temperature.

iv. Stop solution addition and plate reading at 450 nm

- Add **100 µl** of stop solution (H₂SO₄) in all well.
- Read the optical absorbance immediately at 450 nm (bottom reading).
- Resulting optical density (OD) values will be used to calculate patient 1, 2, and 3 responses to TICKPLEX® PLUS antigens.

8. CALCULATION OF RESULTS

For manual calculations, the quality control certificate provided with the test kit and the plate layout provided above must be followed along with the instructions in this section.

i. Optical density index (ODI) calculations

- **Evaluate validity of the test performed for ODI analysis**

The test performed is valid if;

- The OD values for IgM (well A11) and IgG (well E11) **positive controls are**
≥ 1.0

- The OD values for IgM (well B11) and IgG (well F11) **negative controls are ≤ 0.5**
- **Calculate individual cut-off values for antigens A1 to A10**
 - **For IgM test result** → Insert optical density values from wells A1 to A10 in table 2 (last row named “Tested results”) provided in the quality control certificate.
 - **For IgG test result** → Insert optical density values from wells E1 to E10 in table 3 (last row named “Tested results”) provided in the quality control certificate.
 - For each antigen in tables 2 and 3 (in quality control certificate), calculate the mean.
 - Multiply each IgM and IgG mean with the correction factor provided in table 4 of the quality control certificate to obtain cut-off values.
- **Calculate the ODI**
 - For each antigen (A1 to A10) and patient (patient 1, 2, and 3), divide the OD value over its cut off value calculated above.
For IgM and IgG, calculate the ODI according to the following formula,

$$\frac{\text{OD value of a patient for antigen A\#}}{\text{Cut off value of antigen A\#}} = \text{ODI}$$

where, A# = Antigen A1, A2, A3, A4, A5, A6, A7, A8, A9, or A10.

Interpret results as follows,

ODI < 0.9	NEGATIVE
ODI ≥ 0.9 to < 1.0	BORDERLINE
ODI ≥ 1	POSITIVE

ii. **Antibody titer calculations**

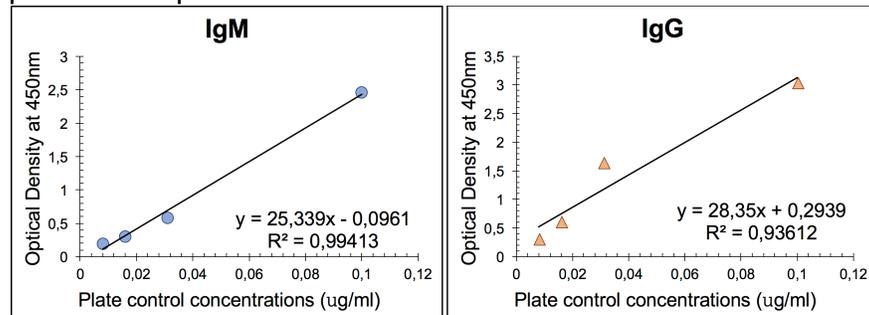
- **Create a standard curve for IgM and IgG**
 - Use a computer to create a scatter plot with OD-values of positive, calibrators 1, 2, and 3 controls on y-axis and their corresponding concentrations on x-axis in the same order as presented below in Table 2.

Table 2. Positive, calibrators 1, 2, and 3 controls concentrations (in µg) for the standard curve.

Controls required for creating a standard curve	Reference well number		Concentration (µg/ml)
	IgM	IgG	
Calibrator 3	C12	G12	0.008
Calibrator 2	B12	F12	0.016
Calibrator 1	A12	E12	0.031
Positive control	A11	E11	0.1

- Add a linear trend line with straight-line equation and R-squared (R²) value to the scatter plot.

The following plots are an example of a typical standard curve. DO NOT use the standard curves below to determine antibody concentrations in patient samples.



- **Evaluate validity of the test performed for antibody titer analysis**

The test is valid if,

- The **R² value is ≥ 0.75** for IgM and IgG.

- **Calculate antibody concentrations in patient samples**

Use the straight-line equation ($y = mx \pm c$) from IgM and IgG standard curves.

- In place of "y", insert OD value of an antigen for a patient and solve for "x". Resulting value for "x" is the antibody concentration in µg/ml.

iii. Test result interpretation

It is recommended that both ODI and corresponding antibody titer values are taken into clinical consideration. A negative immune response on TICKPLEX® PLUS does not preclude the possibility of a positive immune response to microbes associated with tick-borne disease: false negative immune responses can be due to stage of infection (e.g., specimen obtained prior to the development of cellular immune response), co-morbid conditions which affect immune functions, incorrect handling of the blood collection tubes following venipuncture, incorrect performance of the assay, or other immunological variables. Patients in early stages of infection may not produce detectable levels of antibody. Further, early antibiotic therapy after ECM (*erythema chronicum migrans*) may diminish or abrogate good antibody response). Immunocompromised patients may never generate detectable antibody levels.

A positive immune response on TICKPLEX® PLUS result should not be the sole or definitive basis for determining infection with tick-borne pathogen. Incorrect performance of the assay may cause false-positive responses. A positive immune response on TICKPLEX® PLUS should be followed by further medical evaluation and diagnostic evaluation for an active tick-borne disease. Sera/plasma from patients with other spirochetal diseases (syphilis, yaws, pinta, leptospirosis and relapsing fever), infectious mononucleosis or systemic lupus erythematosus may give false positive results.

The IgM antibody test can be used for asymptomatic patients a minimum of 2 weeks after confirmed tick bite.

The IgG antibody test can be used for asymptomatic patients a minimum of 4 weeks after confirmed tick bite.

The IgM antibody test can be used for tick-borne diseases symptomatic patients a minimum of 2 weeks after confirmed tick bite.

The IgG antibody test can be used for tick-borne diseases symptomatic patients a minimum of 4 weeks after confirmed tick bite.

9. CHARACTERISTICS OF THE TEST

i. Validity of the test

The test is intended for determination of specific IgM and IgG antibody responses in human serum or plasma. Te?ted Oy does not guarantee kit operation with EDTA plasma, heparin plasma, citrate plasma, or hemolytic serum/plasma. The test can be performed on minimum 4 ml of whole drawn blood, on samples stored generally at +4°C for up to 14 days, on diluted samples within one working day, and on samples stored at -20°C for up to one year.

ii. Clinical studies

Figure 1 demonstrates the clinical relevance of TICKPLEX® PLUS antigens using the IgM and IgG immune responses from several patient categories. Following guidelines from the Centers for Disease Control and Prevention (CDC) two-tier criteria and the Infectious Disease Society of America (IDSA), we categorized specimens into CDC acute, CDC late, CDC negative, and Post-Treatment Lyme Disease Syndrome (PTLDS). Further, the study included immunocompromised patients who suffered from flu-like symptoms or late Lyme disease symptoms, low CD57 cell count and tested negative by lymphocyte cells against *Borrelia* antigens. Likewise, the unspecific patient category included individuals with flu-like signs or late Lyme disease symptoms but no laboratory tests for tick-borne disease. Lastly, the healthy group comprised specimens without flu-like signs or late Lyme disease symptoms, negative IgM or IgG serology utilizing the CDC two-tier LD diagnosis criteria, and healthy donors.

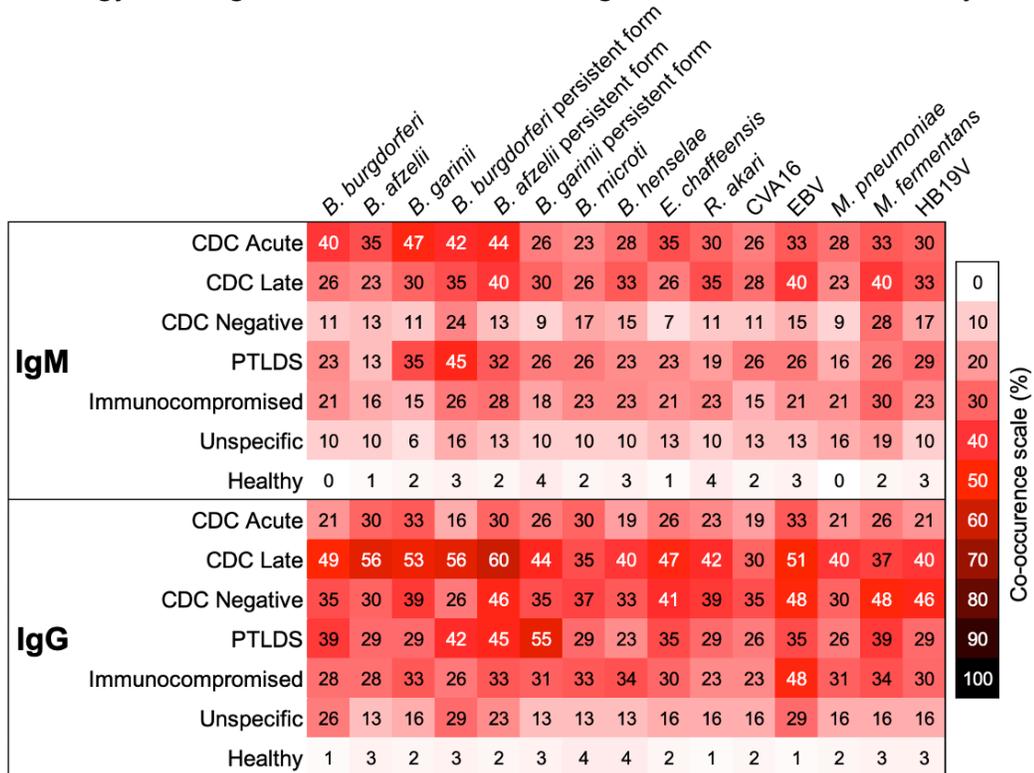


Figure 1. IgM and IgG immune responses by individual patient categories to TICKPLEX® PLUS antigens. Patient categories refer to individuals from the Centers for Disease Control and Prevention (CDC) acute, CDC late, CDC negative, Post-Treatment Lyme Disease Syndrome (PTLDS), immunocompromised, and unspecific. Microbes include *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, *Borrelia garinii*, *Borrelia burgdorferi sensu stricto* persistent form, *Borrelia afzelii* persistent form, *Borrelia garinii* persistent form, *Babesia microti*, *Bartonella henselae*, *Ehrlichia*

chaffeensis, *Rickettsia akari*, Coxsackievirus A16 (CVA16), Epstein-Barr virus (EBV), *Mycoplasma pneumoniae*, *Mycoplasma fermentans*, and Human parvovirus B19 (HB19V).

iii. Diagnostic sensitivity and specificity

Table 3. The diagnostic sensitivity, specificity, and their evaluation has been tabulated.

Antigen	IgM/IgG Sensitivity	IgM/IgG Specificity	Evaluation criteria
Borrelia species and persistent Borrelia forms	95%	98%	Previous clinical diagnostic results for 331 patients with Lyme disease were compared with test results from the Tickplex study performed at the Jyväskylä University.
<i>Babesia microti</i>	100%	80%	Previous clinical diagnostic results for 200 patients with <i>Babesia microti</i> were compared with test results from the Tickplex study performed at the Jyväskylä University.
<i>Bartonella henselae</i>	80%	100%	Previous clinical diagnostic results for 206 patients with <i>Bartonella henselae</i> were compared with test results from the Tickplex study performed at the Jyväskylä University.
<i>Ehrlichia chaffeensis</i>	94%	90%	Previous clinical diagnostic results for 204 patients with <i>Ehrlichia chaffeensis</i> were compared with test results from the Tickplex study performed at the Jyväskylä University.
<i>Rickettsia akari</i>	100%	100%	Previous clinical diagnostic results for 187 patients with Lyme disease were tested in the Tickplex study performed at Jyväskylä University. Results from Tickplex study were compared with test results from the verification study at Te?ted Oy.
Coxsackievirus	90%	100%	Previous clinical diagnostic results for 182 patients with Coxsackievirus were compared with test results from the Tickplex study performed at the Jyväskylä University.
Epstein-Barr virus	100%	100%	Previous clinical diagnostic results for 188 patients with Epstein-Barr virus were compared with test results from the Tickplex study performed at the Jyväskylä University.
Human parvovirus B19	86%	100%	Previous clinical diagnostic results for 178 patients with Human parvovirus B19 were compared with test results from the Tickplex study performed at the Jyväskylä University.
<i>Mycoplasma pneumoniae</i> and <i>Mycoplasma fermentans</i>	100%	100%	Previous clinical diagnostic results for 187 patients with Lyme disease were tested in the Tickplex study performed at Jyväskylä University. Results from Tickplex study were compared with test results from the verification study at Te?ted Oy.

10. SAFETY PRECAUTIONS

- i. For *in vitro* diagnostic use only.
- ii. When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. Do not smoke, drink, or eat while performing or preparing for the assay. Do not pipette by mouth. Wear disposable gloves while handling reagents or samples and wash your hands thoroughly afterwards. Avoid spilling or producing aerosols.
- iii. Do not use kit if any reagent bottle or the microplate shows signs of damage or leakage prior to use. Do not use blood collection tubes or ELISA kit after the expiration date.

- iv. Ensure that laboratory equipment such as plate washers and readers have been calibrated / validated before use.
- v. Handle stop solution (H₂SO₄) with care. Avoid contact with skin or mucous membranes. In case of contact with skin, rinse immediately with plenty of water and seek for medical advice. Liquid waste containing acid [stop solution (H₂SO₄)] should be neutralized in 4% sodium bicarbonate solution.
- vi. Handle human blood as if potentially infectious. Observe relevant blood handling guidelines. While preparing and perform the assay, all material that encountered human serum should be considered as contagious and therefore should be handled / disposed according to appropriate regulations.
- vii. If not using the complete solutions at one time, use in sterile conditions to prevent contamination.

11. HANDLING PRECAUTIONS

- i. Follow assay procedure indicated above.
- ii. Avoid microbial contamination of serum samples and kit reagents.
- iii. If not using the complete solutions at one time, use sterile conditions to prevent contamination.
- iv. Avoid cross-contamination of reagents.
- v. Avoid contact of enzyme substrate solution (TMB) with oxidizing agents, metal surfaces, and light.
- vi. Enzyme substrate solution (TMB) must be clear on use; do not use if the solution is colored.
- vii. Store reagents in conditions mentioned above when not in use.
- viii. Do not re-use diluted human antibodies or IgM/IgG conjugated with HRP samples in 1% sample buffer. Always prepare fresh.
- ix. Do not use reagents after the expiry date printed on the label.
- x. Variations in the test results are usually due to:
 - Insufficient mixing of reagents and samples
 - Inaccurate pipetting and inadequate incubation times
 - Poor washing techniques or spilling the rim of well with sample or IgM/IgG conjugated with HRP
 - Use of identical pipette tips for different solutions
 - Use of contaminated pipette tips
 - The ELISA microplate and assay reagents such as enzyme substrate solution (TMB) did not reach room temperature +15°C to +25°.

Technical Assistance: info@teztet.com More information: www.teztet.com
UDI-DI product identifier: UDI-DI: 111745456742 (Tickplex Plus) Classification B.

TICKPLEX® BASIC INSTRUCTIONS FOR USE



- 1. TITLE: TICKPLEX® BASIC. A complete diagnostic kit for tick-borne disease pathogens.**
- 2. INTENDED USE**

The TICKPLEX® BASIC test provides a quantitative and qualitative *in vitro* assay for human IgM and IgG antibodies against multiple microbial antigens in human serum or plasma. The kit tests for *Borrelia afzelii*, *Borrelia burgdorferi*, and *Borrelia garinii* infections and the tests include persistent antigens of the different borrelia species. The test kit and TICKPEX® BASIC analyze excel sheet are intended to aid in the diagnosis of tick-borne diseases. The test kit and TICKPEX® BASIC analyze excel sheet are for professional use only in clinical laboratory environment and are not to be used for self-testing.

3. TEST PRINCIPLE

TICKPLEX® BASIC is an indirect enzyme linked immunosorbent assay (ELISA). Micro wells on TICKPLEX® BASIC ELISA plates have been coated with inactivated whole cell lysate or peptide antigens. Antigens (A1 and A2) coating order for TICKPLEX® BASIC can be noted from TICKPLEX® BASIC plate layout in section 7. Antibodies that are specific against antigens being tested in TICKPLEX® BASIC will bind to the immobilized antigen(s) on the plate. Antibodies that do not bind with antigens are washed away. However, antibodies that form complexes with the antigen(s) can be recognized by anti-human IgM and anti-human IgG antibodies. Anti-human IgM/IgG are conjugated with horseradish peroxidase (HRP). Presence of conjugated anti-human IgM/IgG can be seen by an enzymatic reaction with 3,3',5,5'-tetramethylbenzidine (chromogenic substrate). Human antibodies that is negative against the antigens being tested in TICKPLEX® BASIC will not form complexes with immobilized antigens. A mild change in the chromogenic substrate color due to a reaction with a negative human antibody can be differentiated from a reaction with a borderline, and positive human antibody binding.

4. TICKPLEX® BASIC ELISA KIT COMPONENTS AND STORAGE

Table 1. List of TICKPLEX® BASIC ELISA kit components, quantities provided in the kit, and storage conditions.

ID	TICKPLEX® BASIC ELISA kit components	Quantity	Storage upon receipt
A	ELISA microplate coated with inactivated microbial antigens	10 plates	+2°C to +8°C
B	Wash buffer concentrate (25X phosphate buffer saline)	2 x 125 ml	+2°C to +8°C
C	Sample buffer concentrate (2% bovine serum albumin)	150 ml	+2°C to +8°C
D	100 % IgM serum diluent concentrate	50 ml	+2°C to +8°C
E	Enzyme substrate solution [3,3',5,5'-tetramethylbenzidine (TMB)]	125 ml	+2°C to +8°C
F	Stop solution [2 M Sulphuric acid (H ₂ SO ₄)]	100 ml	+2°C to +8°C
G	Anti-human IgM conjugated with HRP	60 ml	+2°C to +8°C
H	Anti-human IgG conjugated with HRP	60 ml	+2°C to +8°C
I	Negative serum control (TEZ1) with SDS document	25 ml	+2°C to +8°C
J	TICKPLEX BASIC ANALYZE (sent via email)	1	N/A
K	TICKPLEX BASIC Quality control certificate	1 document	N/A

Note: Please inspect that all kit components are available in the kit and packed as specified.

5. MATERIALS REQUIRED BUT NOT PROVIDED WITH THE KIT

- i. Distilled/deionized water for dilution of wash buffer concentrate and sample buffer concentrate.
- ii. Appropriate equipment for pipetting, liquid dispensing, and washing ELISA microplates.
- iii. Spectrophotometer/colorimeter/microplate reader. Absorbance wavelength required = 450 nm.
- iv. Five hundred (500) 2 ml Eppendorf tubes.
- v. Waste for safe disposal of potentially infectious material (example; human serum sample).

6. REAGENT AND SERUM SAMPLE PREPARATIONS REQUIRED

A TICKPLEX® BASIC ELISA microplate can test negative serum control (TEZ1) and twenty patients. The following kit components have been provided in ready to use format: the ELISA microplates coated with inactivated microbial antigens, enzyme substrate solution [3,3',5,5'-tetramethylbenzidine (TMB)], and stop solution [2 M Sulphuric acid (H₂SO₄)].

Follow instructions below to dilute the remaining kit components to perform one (1) TICKPLEX® BASIC microplate:

i. Diluting buffer solution

- **Wash buffer concentrate dilution** → Add 300 ml of distilled / deionized water to 12.5 ml wash buffer concentrate (kit component B) to obtain **1X wash buffer**.
- **Sample buffer concentrate dilution** → Add 15 ml of 1X wash buffer to 15 ml sample buffer concentrate (kit component C) to obtain **1 % sample buffer**.
- **100 % IgM serum diluent concentrate dilution** → Add 2.5 ml of 1 % sample buffer to 2.5 ml of 100 % IgM serum diluent (kit component D) to obtain **50 % IgM serum diluent**.

Storage after dilution:

Store 1X wash buffer and 1 % sample buffer at +2°C to +8°C for maximum 6 weeks.

50 IgM serum diluent should be used immediately after preparation.

CAUTION: Negative serum control is a human serum sample which is classified as non-hazardous substance. This human serum has been verified as negative for the most relevant infections but it is not a complete assurance that infectious agents are absent. Thus, the user should handle this product as if capable of transmitting infection.

ii. Diluting patient serum sample

A TICKPLEX® BASIC ELISA microplate can test twenty (20) patients. To test one patient at 1:200 dilution, 200 µl of diluted sera for IgM testing and 200 µl of diluted sera for IgG testing are needed.

- **For IgM testing** → add 1 µl of patient sera sample to 199 µl of **50 % IgM serum diluent**
- **For IgG testing** → add 1 µl of patient sera sample to 199 µl of **1 % sample buffer**

IMPORTANT: Anti-human IgM and IgG conjugated with HRP antibodies should be **thoroughly mixed before use** (example; vortex-mixer).

7. DIRECTIONS TO PERFORM THE TICKPLEX® BASIC ELISA

To perform TICKPLEX® BASIC, the plate layout provided below and the instructions in this section must be followed. Read the instructions carefully before performing the test.

TICKPLEX® BASIC PLATE LAYOUT

		Patient number layout presented in "P #" format for IgM and IgG test													
		1	2	3	4	5	6	7	8	9	10	11	12		
Antigen order	Antigen A1	A	P #1	P #3	P #5	P #7	P #9	P #11	P #13	P #15	P #17	P #19	TEZ1	Positive	IgM
	Antigen A2	B	P #1	P #3	P #5	P #7	P #9	P #11	P #13	P #15	P #17	P #19	TEZ1	Negative	
	Antigen A1	C	P #2	P #4	P #6	P #8	P #10	P #12	P #14	P #16	P #18	P #20	TEZ1	EMPTY	
	Antigen A2	D	P #2	P #4	P #6	P #8	P #10	P #12	P #14	P #16	P #18	P #20	TEZ1	CAL 1	
	Antigen A1	E	P #1	P #3	P #5	P #7	P #9	P #11	P #13	P #15	P #17	P #19	TEZ1	Positive	IgG
	Antigen A2	F	P #1	P #3	P #5	P #7	P #9	P #11	P #13	P #15	P #17	P #19	TEZ1	Negative	
	Antigen A1	G	P #2	P #4	P #6	P #8	P #10	P #12	P #14	P #16	P #18	P #20	TEZ1	EMPTY	
	Antigen A2	H	P #2	P #4	P #6	P #8	P #10	P #12	P #14	P #16	P #18	P #20	TEZ1	CAL 1	

NOTE: Antigen A1 includes *Borrelia burgdorferi*, *Borrelia afzelii*, and *Borrelia garinii*; antigen A2 includes *Borrelia burgdorferi*, *Borrelia afzelii*, and *Borrelia garinii* in persisted form

i. Negative serum control (TEZ1) and patient sample addition and incubation

- Place the reagents and plate at room temperature (+15°C to +25°C) for 30 min before performing the test.
- Unwrap the TICKPLEX® BASIC ELISA microplate from its vacuum packing.
- Pipetting order of **twenty patients and TEZ1 serum samples freshly diluted in 50 % IgM serum diluent for IgM test** is as follows:
 1. **Column 1**, pipette 100 µl of serum sample of patient number 1 (**P #1**) to wells A1 and B1, and patient number 2 (**P #2**) to wells C1 and D1.
 2. **Column 2**, pipette 100 µl of serum sample of patient number 3 (**P #3**) to wells A2 and B2, and patient number 4 (**P #4**) to wells C2 and D2.
 3. **Column 3**, pipette 100 µl of serum sample of patient number 5 (**P #5**) to wells A3 and B3, and patient number 6 (**P #6**) to wells C3 and D3.
 4. **Column 4**, pipette 100 µl of serum sample of patient number 7 (**P #7**) to wells A4 and B4, and patient number 8 (**P #8**) to wells C4 and D4.
 5. **Column 5**, pipette 100 µl of serum sample of patient number 9 (**P #9**) to wells A5 and B5, and patient number 10 (**P #10**) to wells C5 and D5.
 6. **Column 6**, pipette 100 µl of serum sample of patient number 11 (**P #11**) to wells A6 and B6, and patient number 12 (**P #12**) to wells C6 and D6.
 7. **Column 7**, pipette 100 µl of serum sample of patient number 13 (**P #13**) to wells A7 and B7, and patient number 14 (**P #14**) to wells C7 and D7.
 8. **Column 8**, pipette 100 µl of serum sample of patient number 15 (**P #15**) to wells A8 and B8, and patient number 16 (**P #16**) to wells C8 and D8.
 9. **Column 9**, pipette 100 µl of serum sample of patient number 17 (**P #17**) to wells A9 and B9, and patient number 18 (**P #18**) to wells C9 and D9.
 10. **Column 10**, pipette 100 µl of serum sample of patient number 19 (**P #19**) to wells A10 and B10, and patient number 20 (**P #20**) to wells C10 and D10.
 11. **Column 11**, pipette 100 µl of negative serum control (**TEZ1**) to wells A11, B11, C11, and D11.
- Pipetting order of **twenty patients and TEZ1 serum samples for IgG test** is as follows:

1. **Column 1**, pipette 100 μl of serum sample of patient number 1 (**P #1**) to wells E1 and F1, and patient number 2 (**P #2**) to wells G1 and H1.
2. **Column 2**, pipette 100 μl of serum sample of patient number 3 (**P #3**) to wells E2 and F2, and patient number 4 (**P #4**) to wells G2 and H2.
3. **Column 3**, pipette 100 μl of serum sample of patient number 5 (**P #5**) to wells E3 and F3, and patient number 6 (**P #6**) to wells G3 and H3.
4. **Column 4**, pipette 100 μl of serum sample of patient number 7 (**P #7**) to wells E4 and F4, and patient number 8 (**P #8**) to wells G4 and H4.
5. **Column 5**, pipette 100 μl of serum sample of patient number 9 (**P #9**) to wells E5 and F5, and patient number 10 (**P #10**) to wells G5 and H5.
6. **Column 6**, pipette 100 μl of serum sample of patient number 11 (**P #11**) to wells E6 and F6, and patient number 12 (**P #12**) to wells G6 and H6.
7. **Column 7**, pipette 100 μl of serum sample of patient number 13 (**P #13**) to wells E7 and F7, and patient number 14 (**P #14**) to wells G7 and H7.
8. **Column 8**, pipette 100 μl of serum sample of patient number 15 (**P #15**) to wells E8 and F8, and patient number 16 (**P #16**) to wells G8 and H8.
9. **Column 9**, pipette 100 μl of serum sample of patient number 17 (**P #17**) to wells E9 and F9, and patient number 18 (**P #18**) to wells G9 and H9.
10. **Column 10**, pipette 100 μl of serum sample of patient number 19 (**P #19**) to wells E10 and F10, and patient number 20 (**P #20**) to wells G10 and H10.
11. **Column 11**, pipette 100 μl of negative serum control (**TEZ1**) to wells E11, F11, G11, and H11.

- Pipette 100 μl of 1 % sample buffer to IgM and IgG positive control (Positive), negative control (Negative), blank (EMPTY), and calibrator 1 (CAL 1) wells.
- Cover the plate with its lid and incubate for 1 hour at room temperature.

ii. Anti-human IgM and IgG HRP conjugated antibodies addition and incubation

- **Wash step** → Dispense and aspirate 200 μl of 1X wash buffer five times in all wells.
- After washing, thoroughly dispose of all liquid from the microplate by tapping it on an absorbent paper with the openings facing downwards to remove all residual wash buffer.
- In each well, pipette 100 μl of anti-human **IgM** conjugated with HRP in rows A, B, C, and D.
- In each well, pipette 100 μl of anti-human **IgG** conjugated with HRP in rows E, F, G, and H.
- Cover the plate and incubate for 1 hour at room temperature.

iii. Enzyme substrate solution addition and incubation

- **Wash step** → Dispense and aspirate 200 μl of 1X wash buffer five times in all wells.
- After washing, thoroughly dispose of all liquid from the microplate by tapping it on an absorbent paper with the openings facing downwards to remove all residual wash buffer.

IMPORTANT: Enzyme substrate solution (TMB) must reach room temperature before use. The enzyme substrate solution must be clear to use. DO NOT USE if the solution is colored.

- Add **100 μl** of enzyme substrate solution (TMB) in all wells.

- Cover the plate and incubate in the dark (example; covering with aluminum foil) at room temperature.
- Incubate plate for a total of **30 min** at room temperature.

iv. Stop solution addition and plate reading at 450 nm

- Add **100 µl** of stop solution (H₂SO₄) in all well.
- Read the optical absorbance immediately at 450 nm (bottom reading)..
- Resulting optical density (OD) values will be used to calculate patient (number 1 to 20) responses to TICKPLEX® BASIC antigens.

8. CALCULATION OF RESULTS

For manual calculations, the quality control certificate provided with the test kit and the plate layout provided above must be followed along with the instructions in this section.

i. Optical density index (ODI) calculations

- **Evaluate validity of the test performed for ODI analysis**

The test performed is valid if;

1. The OD values for IgM (well A12) and IgG (well E12) **positive controls are ≥ 1.0**
2. The OD values for IgM (well B12) and IgG (well F12) **negative controls are ≤ 0.5**

- **Calculate individual cut-off values for antigens A1 and A2**

1. **For IgM test result** → Insert optical density values from wells A11 and C11 for antigen A1, and wells B11 and D11 for antigen A2 in table 2 (last row named “Tested results reads 5 and 6”) provided in the quality control certificate.
2. **For IgG test result** → Insert optical density values from wells E11 and G11 for antigen A1, and wells F11 and H11 for antigen A2 in Table 3 (last row named “Tested results reads 5 and 6”) provided in the quality control certificate.
3. For each antigen in tables 2 and 3 (in quality control certificate), calculate the mean.
4. Multiply each IgM and IgG mean with the correction factor provided in table 4 of the quality control certificate to obtain cut-off values.

- **Calculate the ODI**

1. For each antigen (A1 and A2) and patient (patient 1 to 20), divide the OD value over its cut off value calculated above.

For IgM and IgG, calculate the ODI according to the following formula,

$$\frac{\text{OD value of a patient for antigen A\#}}{\text{Cut off value of antigen A\#}} = \text{ODI}$$

where, A# = Antigen A1 or A2

Interpret results as follows,

ODI < 0.9

ODI ≥ 0.9 to < 1.0

ODI ≥ 1

NEGATIVE

BORDERLINE

POSITIVE

ii. Antibody titer calculations

- **Create a standard curve for IgM and IgG**

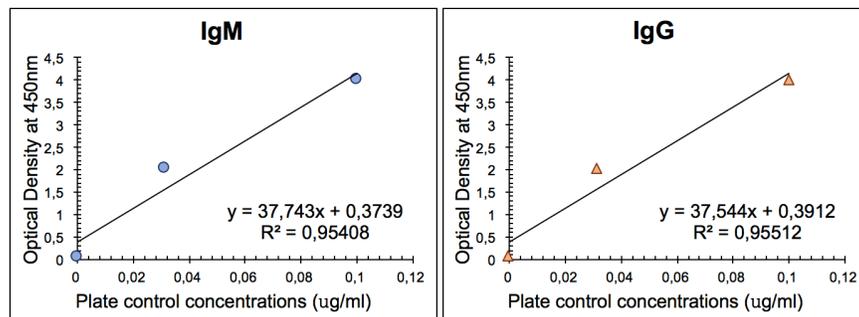
1. Use a computer to create a scatter plot with OD-values of negative control, calibrator 1, and positive control on y-axis and their corresponding concentrations on x-axis in the same order as presented below in Table 2.

Table 2. Negative control, Calibrators 1, and Positive control concentrations (in µg/ml) for the standard curve.

Controls required for creating a standard curve	Reference well number		Concentration (µg/ml)
	IgM	IgG	
Negative control	B12	F12	0.000
Calibrator 1	D12	H12	0.031
Positive control	A12	E12	0.1

2. Add a linear trend line with straight-line equation and R-squared (R^2) value to the scatter plot.

The following plots are an example of a typical standard curve. DO NOT use the standard curves below to determine antibody concentrations in patient samples.



- **Evaluate validity of the test performed for antibody titer analysis**

The test is valid if,

1. The **R^2 value is ≥ 0.75** for IgM and IgG.

- **Calculate antibody concentrations in patient samples**

Use the straight-line equation ($y = mx \pm c$) from IgM and IgG standard curves.

1. In place of "y", insert OD value of an antigen for a patient and solve for "x". Resulting value for "x" is the antibody concentration in µg/ml.

iii. Test result interpretation

It is recommended that both ODI and corresponding antibody titer values are taken into clinical consideration. A negative immune response on TICKPLEX[®] BASIC does not preclude the possibility of a positive immune response to microbes associated with tick-borne disease: false negative immune responses can be due to stage of infection (e.g., specimen obtained prior to the development of cellular immune response), co-morbid conditions which affect immune functions, incorrect handling of the blood collection tubes following venipuncture, incorrect performance of the assay, or other immunological variables. Patients in early stages of infection may not produce detectable levels of antibody. Further, early antibiotic therapy after ECM (*erythema chronicum migrans*) may diminish or abrogate good antibody response. Immunocompromised patients may never generate detectable antibody levels. A positive immune response on TICKPLEX[®] BASIC result should not be the sole or definitive basis for determining infection with tick-borne pathogen. Incorrect performance of the assay may cause false-positive responses. A positive immune response on TICKPLEX[®] BASIC should be followed by further medical evaluation and diagnostic evaluation for an active tick-borne disease. Sera/plasma from patients with other spirochetal diseases (syphilis, yaws, pinta, leptospirosis and relapsing fever), infectious mononucleosis or systemic lupus erythematosus may give false positive results.

The IgM antibody test can be used for asymptomatic patients a minimum of 2 weeks after confirmed tick bite.

The IgG antibody test can be used for asymptomatic patients a minimum of 4 weeks after confirmed tick bite.

The IgM antibody test can be used for tick-borne diseases symptomatic patients a minimum of 2 weeks after confirmed tick bite.

The IgG antibody test can be used for tick-borne diseases symptomatic patients a minimum of 4 weeks after confirmed tick bite.

9. CHARACTERISTICS OF THE TEST

i. Validity of the test

The test is intended for determination of specific IgM and IgG antibody responses in human serum or plasma. Te?ted Oy does not guarantee kit operation with EDTA plasma, heparin plasma, citrate plasma, or hemolytic serum/plasma. The test can be performed on minimum 4 ml of whole drawn blood, on samples stored generally at +4°C for up to 14 days, on diluted samples within one working day, and on samples stored at -20°C for up to one year.

ii. Precision of test and coefficient of variation

Coefficient of variation was assessed by calculating intra- and inter-assay variation. Intra-assay variation was determined by a duplicate high titer, and low titer measurement from the same plate. For inter-assay, variation was determined by measuring three high titer samples and three low titer samples from different plates that were performed on three different days. Coefficient of intra-assay variation is max 4.25%, and the coefficient of inter-assay variation is max 4.29%.

iii. Clinical studies

Please refer to peer-reviewed article Garg, K et al. *Trop. Med. Infect. Dis.* **2021**, 6, 38.

iv. Diagnostic sensitivity and specificity

Table 2. The diagnostic sensitivity, specificity, and their evaluation has been tabulated.

Antigen	IgM/IgG Sensitivity	IgM/IgG Specificity	Evaluation criteria
Borrelia species and persistent Borrelia forms	95%	98%	Previous clinical diagnostic results for 331 patients with Lyme disease were compared with test results from the Tickplex study performed at the JYU University.

10. SAFETY PRECAUTIONS

- For *in vitro* diagnostic use only.
- When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. Do not smoke, drink, or eat while performing or preparing for the assay. Do not pipette by mouth. Wear disposable gloves while handling reagents or samples and wash your hands thoroughly afterwards. Avoid spilling or producing aerosols.
- Do not use kit if any reagent bottle or the microplate shows signs of damage or leakage prior to use. Do not use blood collection tubes or ELISA kit after the expiration date.
- Ensure that laboratory equipment such as plate washers and readers have been calibrated / validated before use.
- Handle stop solution (H₂SO₄) with care. Avoid contact with skin or mucous membranes. In case of contact with skin, rinse immediately with plenty of water and seek for medical advice. Liquid waste containing acid [stop solution (H₂SO₄)] should be neutralized in 4% sodium bicarbonate solution.
- Handle human blood as if potentially infectious. Observe relevant blood handling guidelines. While preparing and perform the assay, all material that encountered human serum should be considered as contagious and therefore should be handled / disposed according to appropriate regulations.
- If not using the complete solutions at one time, use in sterile conditions to prevent contamination.

11. HANDLING PRECAUTIONS

- Follow assay procedure indicated above.
- Avoid microbial contamination of serum samples and kit reagents.
- If not using the complete solutions at one time, use sterile conditions to prevent contamination.
- Avoid cross-contamination of reagents.
- Avoid contact of enzyme substrate solution (TMB) with oxidizing agents, metal surfaces, and light.
- Enzyme substrate solution (TMB) must be clear on use; do not use if the solution is colored.
- Store reagents in conditions mentioned above when not in use.
- Do not re-use diluted human antibodies or IgM/IgG conjugated with HRP samples in 1% sample buffer. Always prepare fresh.
- Do not use reagents after the expiry date printed on the label.
- Variations in the test results are usually due to:
 - Insufficient mixing of reagents and samples
 - Inaccurate pipetting and inadequate incubation times
 - Poor washing techniques or spilling the rim of well with sample or IgM/IgG conjugated with HRP
 - Use of identical pipette tips for different solutions
 - The ELISA microplate and assay reagents such as enzyme substrate solution (TMB) did not reach room temperature +15°C to +25°C

A negative immune response on TICKPLEX® BASIC does not preclude the possibility of a positive immune response to microbes associated with tick-borne disease: false negative immune responses can be due to stage of infection (e.g., specimen obtained prior to the development of cellular immune response), co-morbid conditions which affect immune functions, incorrect handling of the blood collection tubes following venipuncture, incorrect performance of the assay, or other immunological variables. Patients in early stages of infection may not produce detectable levels of antibody. Further, early antibiotic therapy after ECM (*erythema chronicum migrans*) may diminish or abrogate good antibody response). Immunocompromised patients may never generate detectable antibody levels.

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Technical Assistance: info@tezted.com

More information: www.tezted.com

UDI-DI: 111745457308 (Tickplex Basic)