# Supplementary Information: Rapid single-tier serodiagnosis of Lyme disease

Rajesh Ghosh, Hyou-Arm Joung, Artem Goncharov, Barath Palanisamy, Kevin Ngo, Katarina Pejcinovic, Nicole Krockenberger, Elizabeth J. Horn, Omai B. Garner, Ezdehar Ghazal, Andrew O'Kula8, Paul M. Arnaboldi, Raymond J. Dattwyler, Aydogan Ozcan* and Dino Di Carlo*

*Corresponding Authors: ozcan@ucla.edu ; dicarlo@ucla.edu

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**Supplementary Table 1.** Anti-human secondary IgM and IgG antibodies screened for binding against anti-*Borrelia* antibodies for the detection of LD infection.

| Code | Vendor | Host | Type | CAT# | Antibody |
|---|---|---|---|---|---|
| G1 | abcam | Mouse | Monoclonal | ab99770 | Mouse monoclonal [H2] Anti-Human IgG Fc |
| G2 | SouthernBiotech | Mouse | Monoclonal | 9040-01 | Mouse Anti-Human IgG Fc-UNLB (JDC-10) |
| G3 | | Mouse | Monoclonal | 9042-01 | Mouse Anti-Human IgG Fc-UNLB (H2) |
| G4 | | Goat | Polyclonal | 2014-01 | Goat Anti-Human IgG Fc, Multi-Species SP ads-UNLB |
| M1 | abcam | Mouse | Monoclonal | ab99741 | Mouse monoclonal UHB Anti-Human IgM mu chain |
| M2 | SouthernBiotech | Mouse | Monoclonal | 9020-01 | Mouse Anti-Human IgM-UNLB (SA-DA4) |
| M3 | | Mouse | Monoclonal | 9022-01 | Mouse Anti-Human IgM-UNLB (UHB) |
| M4 | | Goat | Polyclonal | 2023-01 | Goat Anti-Human IgM, Mouse/Bovine/Horse SP ads-UNLB |

**Supplementary Table 2.** List of all synthetic peptide antigens and their amino acid sequences used in this study.

| Synthetic peptide name | Amino acid sequence | Strain | Molecular weight |
|---|---|---|---|
| OspC | MTLFLFISCNNSGKDGNTSAC | B31 | 2223.53 |
| OppA | CYGQNWTSPENMVTSGPFKLKERIPNEKYVFEKNNK | B31 | 4277.88 |
| p35 | CDTGSERSIRYRRRVY | B31 | 2017.17 |
| ErpP | CKIEFSKFTVKIKNKD | B31 | 1928.33 |
| DbpA | CTILVNLLISCGLTGA | B31 | 1590.96 |
| DbpB | CKDLKNKILKIKKEATGKGVLFEAFTGLKTG | B31 | 3380.12 |
| BBK32 | CKKPMNKKGKGKIARKKGKSKVSRKEPYIHS | B31 | 3541.36 |
| BBA64 | CNESKKHKKEKRKGKV | B31 | 1927.31 |
| BBA65-94 | CTIKKISGGIRIQQGFEVA | B31 | 2048.45 |
| BBA65-101-02 | CKNTLEEIYNLIVDLTLIKKEW | B31 | 2679.19 |
| RecA164 | CIMFINQIRMRIGVMFGNPETTT | B31 | 2673.25 |
| RecA168-69 | CGGNALKFYSSLRLEVRKIEQVTR | B31 | 2768.25 |
| LA-7 | CIPSKENAKLIVYFYDNVYAG | B31 | 2407.78 |
| FlilB4 | CVSRKGGLLPDIIIKI | B31 | 1725.18 |
| modVlsE-FlaB | CVQEGVQQ EGAQQPGGGMKKNDQ IVAAIALRGVA | B31 | 3451.95 |
| Var2FlaB | CMKKTDNIAAAIVIRGVAKDGQFALKGGGVQEGVQQEGAQQP | B31 | 4312.97 |
| DbpA4-B6 | TILVNLLISCGLTGAGGGGGKDLKNKILKIKKEATGKGVLFEAFTGLKTGC | B31 | 5135.19 |
| BBA73-196-99 | MKRNKIWKTLKLFQITLLFSCSFYSKSNNTC | B31 | 3743.75 |

**Supplementary Table 3.** Clinical samples and corresponding ground truth data from Cohort 3 of the Lyme Disease Biobank used to train the single-tier xVFA.

| | *Clinical presentations* | | *Reference tests* | | | | *First-tier tests* | | | *Second-tier tests* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample #** | ***EM/ Annular Rash*** | ***EM > 5 cm at Enrollment*** | ***B. burgdorferi PCR*** | ***B. miyamotoi PCR*** | ***Anaplasma PCR*** | ***B. Microti PCR*** | ***Whole Cell Lysate ELISA*** | ***C-6 Peptide ELISA*** | ***VlsE/PepC 10 ELISA*** | ***Western Blot IgM**** | ***Western Blot IgG#*** | ***Two-tier Positive*** | ***xVFA prediction (triplicate)*** |
| LDB01 | YES | NO | NEG | NEG | NEG | NEG | POS | POS | NA | POS | EQV | YES | |
| LDB04 | YES | NO | NEG | NEG | NEG | NEG | NEG | POS | NA | POS | EQV | YES | |
| LDB05 | NO | NA | NEG | NEG | NEG | NEG | POS | POS | NA | POS | EQV | YES | |
| LDB07 | YES | YES | NEG | NEG | NEG | NEG | NEG | POS | NA | POS | POS | YES | |
| LDB08 | NO | NA | NEG | NEG | NEG | NEG | NEG | POS | NA | POS | EQV | YES | |
| LDB10 | YES | NO | NEG | NEG | NEG | NEG | EQV | POS | NA | POS | EQV | YES | |
| LDB11 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB12 | NO | NA | NEG | NEG | NEG | NEG | NA | EQV | POS | POS | NEG | YES | |
| LDB13 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | EQV | POS | NEG | YES | |
| LDB14 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB15 | NO | NA | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB16 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | NA | NEG | POS | YES | |
| LDB17 | YES | NO | NEG | NEG | NEG | NEG | NA | NEG | POS | POS | NEG | YES | |
| LDB19 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | POS | POS | NEG | YES | |
| LDB20 | NO | NA | NEG | NEG | NEG | NEG | NA | POS | POS | POS | POS | YES | |
| LDB21 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB22 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | POS | YES | |
| LDB23 | NO | NA | NEG | NEG | NEG | NEG | NA | POS | POS | POS | POS | YES | |
| LDB24 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB25 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | NA | POS | NEG | YES | |
| LDB36 | NO | NA | NEG | NEG | NEG | NEG | EQV | NEG | NA | EQV | EQV | NO | |
| LDB37 | NO | NA | NEG | NEG | NEG | NEG | NEG | NEG | NA | NEG | EQV | NO | |
| LDB38 | NO | NA | NEG | NEG | NEG | NEG | NEG | NEG | NA | NEG | NEG | NO | |
| LDB40 | NO | NA | NEG | NEG | NEG | NEG | NEG | NEG | NA | EQV | EQV | NO | |
| LDB41 | NO | NA | NEG | NEG | NEG | NEG | NEG | NEG | NA | EQV | EQV | NO | |
| LDB42 | NO | NA | NEG | NEG | NEG | NEG | NEG | NEG | NA | EQV | EQV | NO | |
| LDB43 | NO | NA | NEG | NEG | NEG | NEG | NEG | NEG | NA | NEG | EQV | NO | |
| LDB44 | NO | NA | NEG | NEG | NEG | NEG | NEG | NEG | NA | EQV | EQV | NO | |
| LDB46 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB47 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | EQV | NEG | NEG | NO | |
| LDB48 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB49 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB50 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB51 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NA | NEG | NEG | NO | |
| LDB52 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NA | NEG | NEG | NO | |
| LDB54 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB55 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB56 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB58 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB59 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |

*Minimum of 2 of the 3 CDC specific bands (23,39,41 Kda) must be present.

#Minimum of 5 of the 10 CDC specific bands (18,23,28,30,39,41,45,58,66,93 Kda) must be present.

¶Blue indicates positive and grey indicates negative outcomes. Each sample was tested in triplicate, and all replicates are shown.

**Supplementary Table 4.** Clinical samples and corresponding ground truth data from Cohort 3 of the Lyme Disease Biobank used to validate the single-tier xVFA.

| | *Clinical presentations* | | *Reference tests* | | | | *First-tier tests* | | | *Second-tier tests* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample #** | ***EM/ Annular Rash*** | ***EM > 5 cm at Enrollment*** | ***B. burgdorferi PCR*** | ***B. miyamotoi PCR*** | ***Anaplasma PCR*** | ***B. Microti PCR*** | ***Whole Cell Lysate ELISA*** | ***C-6 Peptide ELISA*** | ***VlsE/PepC 10 ELISA*** | ***Western Blot IgM**** | ***Western Blot IgG#*** | ***Two-tier Positive*** | ***xVFA prediction (triplicate testing)¶*** |
| LDB02 | YES | YES | NEG | NEG | NEG | NEG | NEG | POS | NA | POS | EQV | YES | |
| LDB03 | YES | YES | NEG | NEG | NEG | NEG | NEG | POS | NA | POS | EQV | YES | |
| LDB06 | YES | YES | NEG | NEG | NEG | NEG | RE | POS | NA | POS | EQV | YES | |
| LDB09 | YES | YES | NEG | NEG | NEG | NEG | EQV | POS | NA | POS | EQV | YES | |
| LDB18 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | POS | YES | |
| LDB26 | NO | NA | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB27 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB28 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | POS | YES | |
| LDB29 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB30 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB31 | NO | NA | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB32 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | POS | YES | |
| LDB33 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB34 | YES | YES | NEG | NEG | NEG | NEG | NA | POS | POS | POS | NEG | YES | |
| LDB35 | YES | YES | NEG | NEG | NEG | NEG | NEG | POS | POS | POS | NEG | YES | |
| LDB39 | NO | NA | NEG | NEG | NEG | NEG | NEG | NEG | NA | NEG | NEG | NO | |
| LDB45 | NO | NA | NEG | NEG | NEG | NEG | NEG | NEG | NA | EQV | EQV | NO | |
| LDB53 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB57 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB60 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB61 | NO | NA | NEG | NEG | NEG | NEG | NEG | NEG | NA | EQV | EQV | NO | |
| LDB62 | NO | NA | NEG | NEG | NEG | NEG | NEG | NEG | NA | EQV | NEG | NO | |
| LDB63 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NA | NEG | NEG | NO | |
| LDB64 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB65 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB66 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB67 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB68 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB69 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |
| LDB70 | NO | NA | NEG | NEG | NEG | NEG | NA | NEG | NEG | NEG | NEG | NO | |

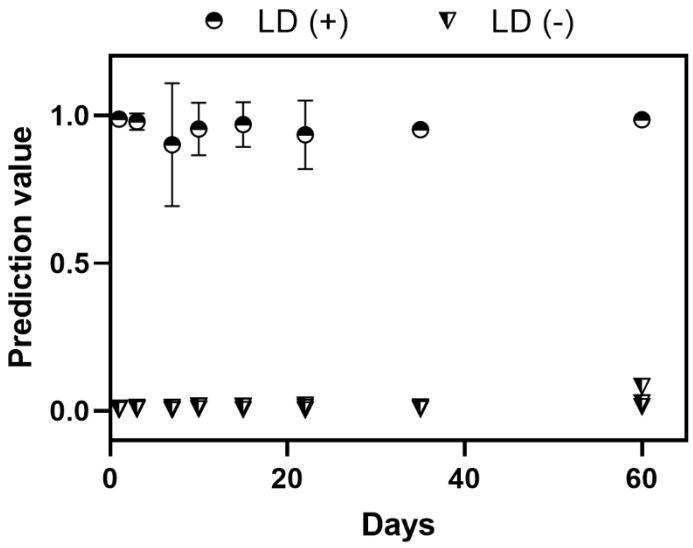
*Minimum of 2 of the 3 CDC specific bands (23,39,41 Kda) must be present.

#Minimum of 5 of the 10 CDC specific bands (18,23,28,30,39,41,45,58,66,93 Kda) must be present.

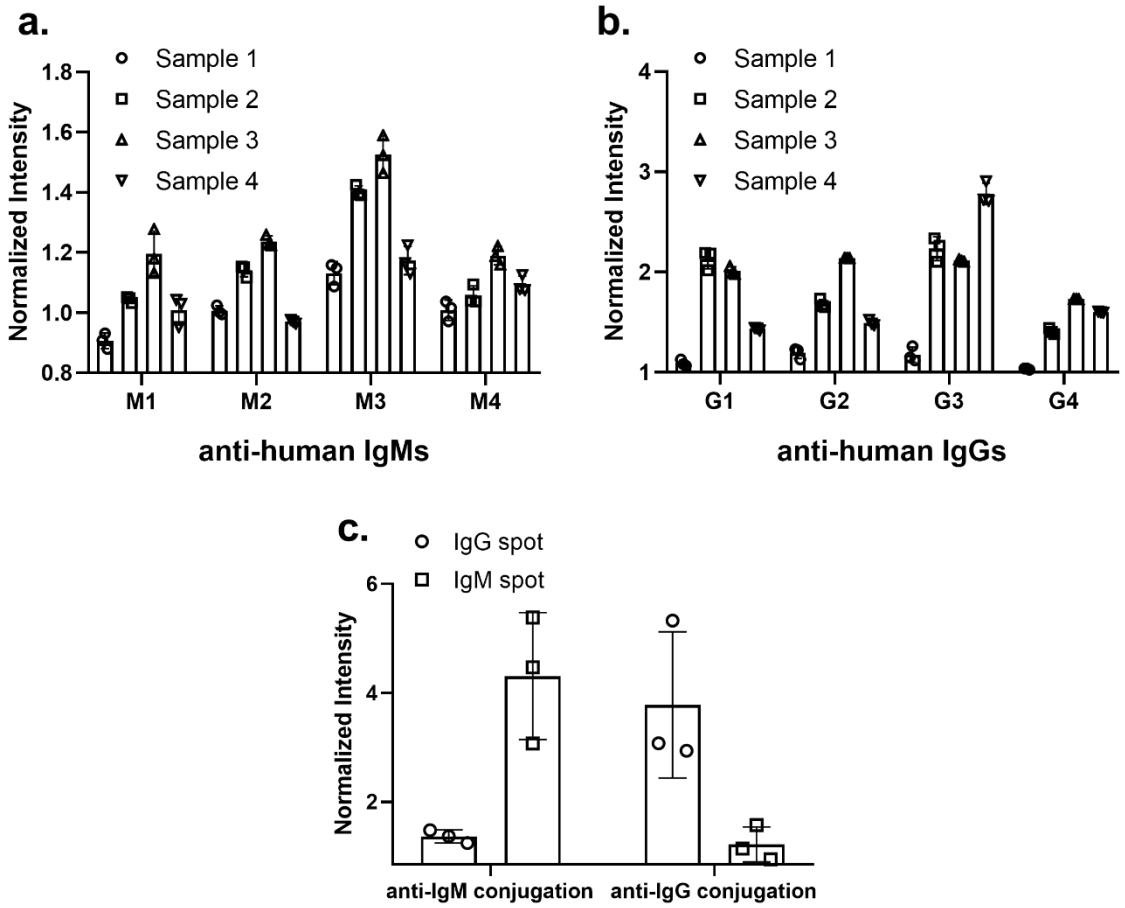
¶Blue indicates positive and grey indicates negative outcomes. Each sample was tested in triplicate, and all replicates are shown.

**Supplementary Table 5.** Cost breakdown of xVFA test using recombinant proteins and peptide-based antigen panel.

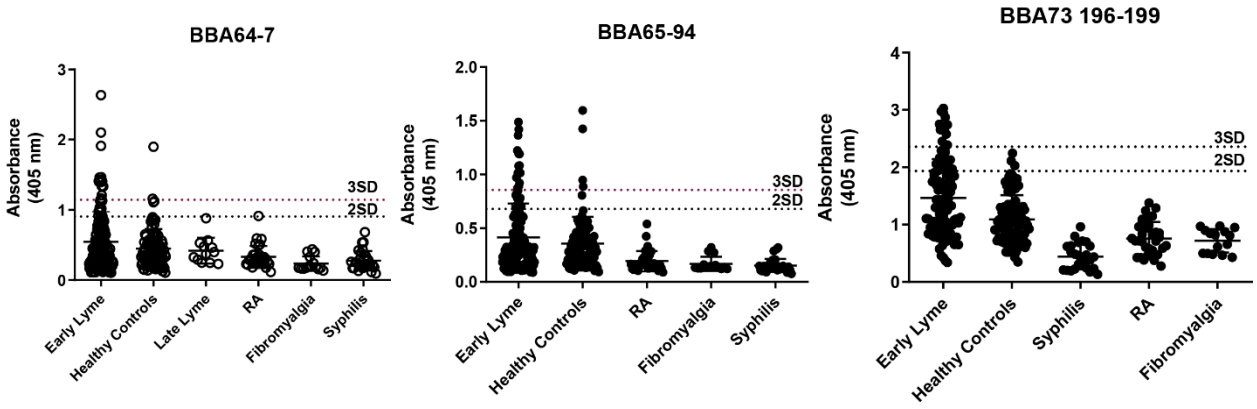
| Types | Name | Cost/test ($) | |
|---|---|---|---|
| | | Antigen assay | Peptide assay |
| Paper materials | Asymmetric membrane | 0.38 | |
| | Interpad | 0.07 | |
| | NC membrane | 0.17 | |
| Catridge (3D printed) | | 1.00 | |
| Bio/chemical resources | Gold nanoparticles | 0.20 | |
| | Anti-Human IgG | 0.17 | |
| | Anti-Human IgM | 0.17 | |
| | Anti-Mouse IgG | 0.002 | |
| | BSA | 0.03 | |
| | Others | 0.40 | |
| | Antigens | **10.4** | |
| | Peptides | | **0.32** |
| | **Total cost per test** | **12.99** | **2.59** |



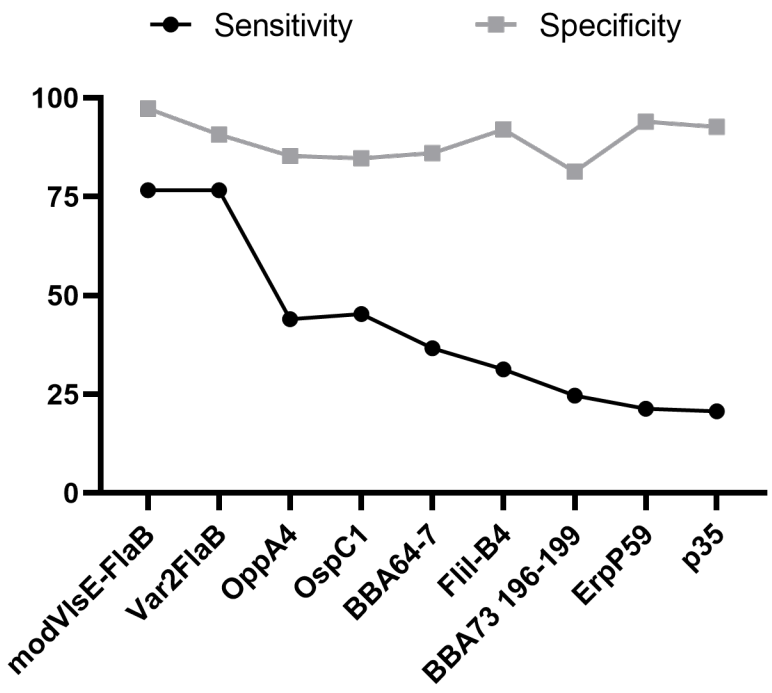

**Supplementary Figure 1.** The stability of the combined synthetic peptide panel was assessed over a period of 60 days, using both LD-positive and healthy control samples as benchmarks. The resulting plot illustrates the mean predictive values generated by the trained machine-learning diagnostic model for each of the xVFA assays throughout the testing duration (N=3). Data are presented as mean values ± SD.



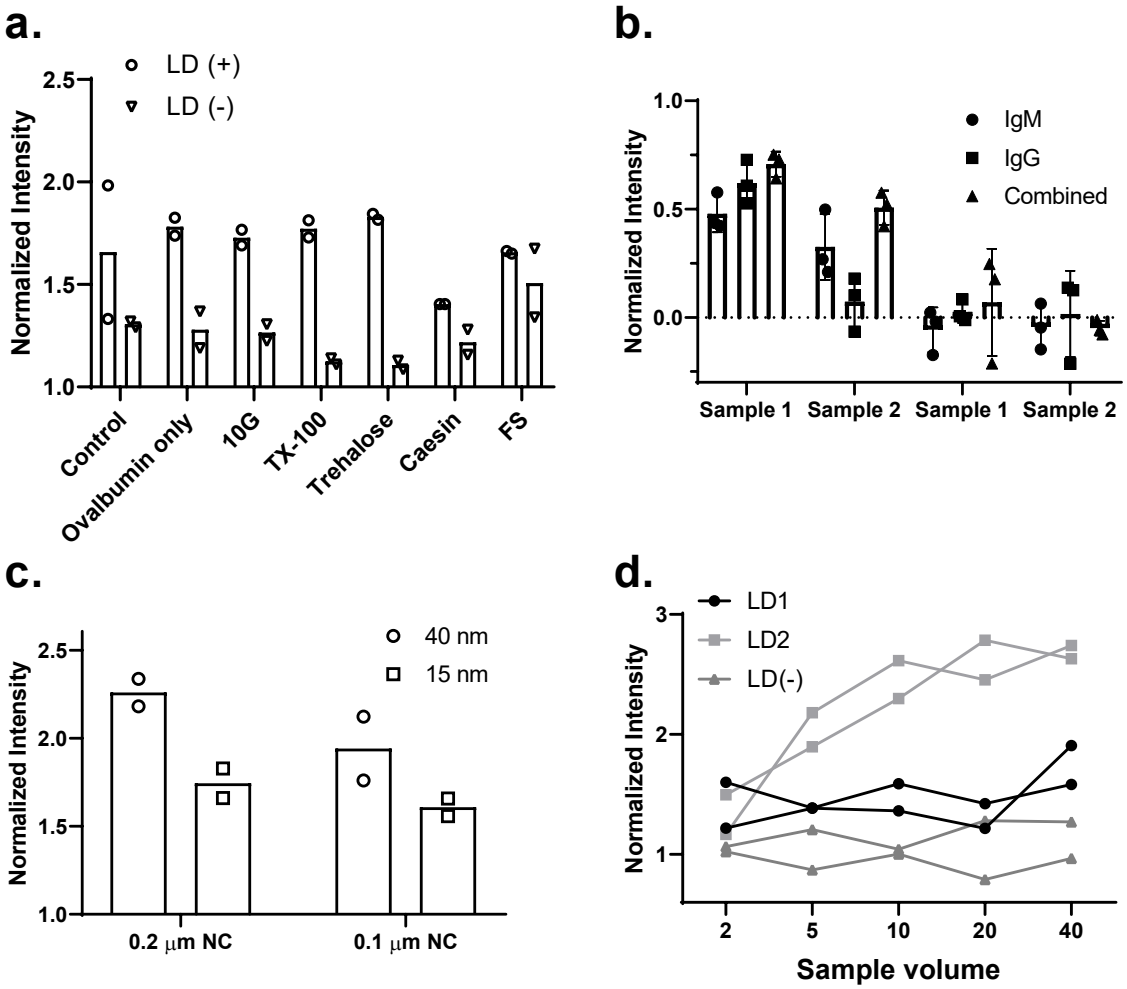

**Supplementary Figure 2.** Screening of anti-human antibodies for binding to Lyme disease **a** IgM and **b** IgG antibodies. **c** Cross-reactivity evaluation of the IgM and IgG antibodies selected in the detection of a control LD patient sample containing anti-*Borrelia* IgM and IgG antibodies. Data are presented as mean values ± SD, with three replicates for each (N=3).




**Supplementary Figure 3.** Screening of BBA64-7, BBA65-94, BBA73 196-199 peptides using ELISA to measure interaction of synthesized peptides with anti-*Borrelia* IgM antibodies tested against sample groups containing early Lyme disease patient samples, late Lyme disease, healthy control groups and cross-reactive diseases such as rheumatoid arthritis, fibromyalgia and syphilis. The cut-off for positivity was defined as three times the standard deviation of the healthy controls and an equivocal result was defined as a signal above two times the standard deviation. If the absorbance recorded for a healthy control sample was above three times the standard deviation, it was not considered for the cut-off determination.




**Supplementary Figure 4.** Sensitivity and specificity of individual synthetic peptides selected for diagnosing LD using xVFA without combining the signal intensities from reaction spots using a neural network model.




**Supplementary Figure 5.** Optimization of the xVFA operation and assay reagents. **a** Evaluation of different running buffer condition where control represents a buffer containing 2% (w/v) ovalbumin, and 3% (w/v) Tween-20. Other compositions tested along with the control buffer were ovalbumin without the Tween-20 surfactant, 3% (w/v) 10G surfactant, 1% (w/v) Triton X-100, 2% (w/v) trehalose, 2% (w/v) casein and 10x dilution of Thermofisher Scientific superblock® buffer. Buffer containing 2% (w/v) ovalbumin, 3% (w/v) Tween-20 and 2% (w/v) trehalose was found optimal. **b** Testing of control Lyme disease patient samples with gold nanoparticles labelled with IgM only, IgG only and combined IgM and IgG secondary antibodies. Data are presented as mean values ± SD (N=3). **c** Screening of deposition of 15 nm and 40 nm gold nanoparticles on 0.2 μm and 0.1 μm nitrocellulose membrane. 40 nm gold nanoparticles deposited on 0.2 μm nitrocellulose membrane was found optimal. **d** Optimization of the sample volume added during the xVFA operation using two control Lyme disease patient samples and one control healthy sample. The signal was almost saturated at 10 μL sample volume and 20 μL was chosen as the volume of sample added for all clinical tests carried out.