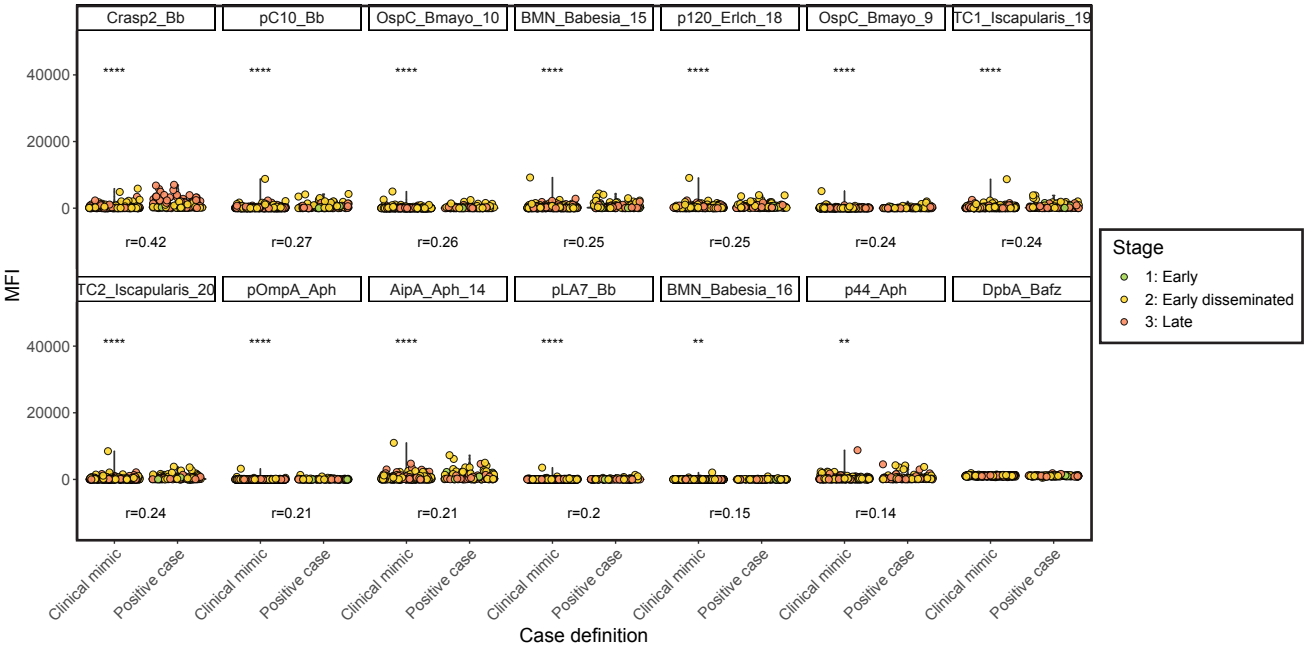

Figure S1 | Expression of uninformative antigens.
Here, antigens that were excluded from subsequent analysis due to low variance are presented in the same way as in **Figure 2**.

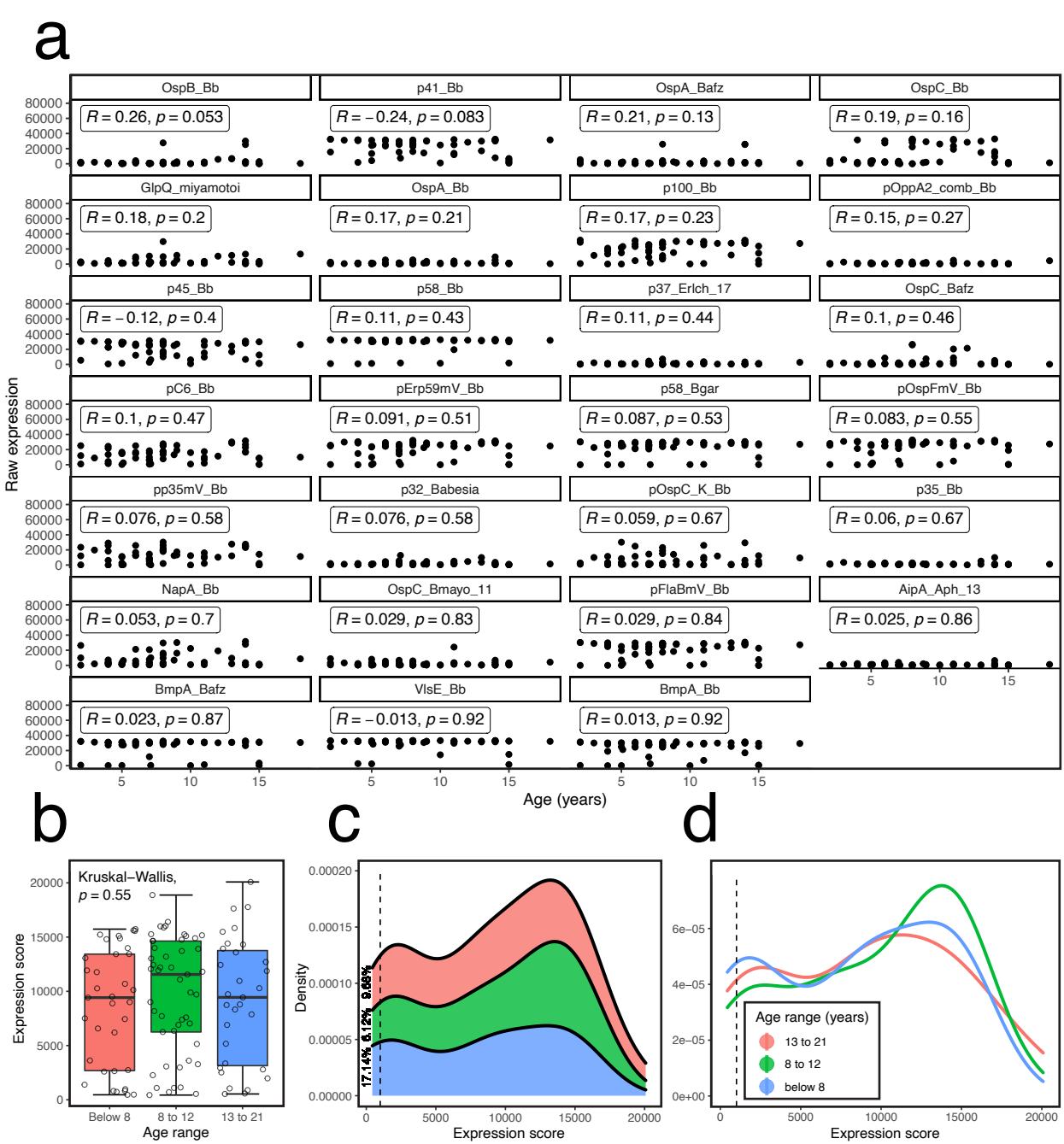

Figure S2 | Serologic reactivity to *Borrelia* antigens by age.
Uninformative antigens were filtered as stated in the Methods section. Children were filtered for positivity by case definition. (a) Antigen expression of the remaining 115 children was plotted versus age. *R* indicates Pearson's *R*. In all cases, the null hypothesis ($R = 0$) was not rejected. (b)-(d), Shared legend. Children were classified into 3 categories by age (n=35 below 8, n=49 between 8 and 12, n=31 between 13 and 21). Expression score was calculated as mean antigen expression over all antigens. (b) Expression score by Age range. (c)-(d) Stacked (c) and unstacked (d) density plots. Dashed line indicates overall 10th percentile. Percentage in (c) indicates fraction of scores in in 10th percentile by age range.

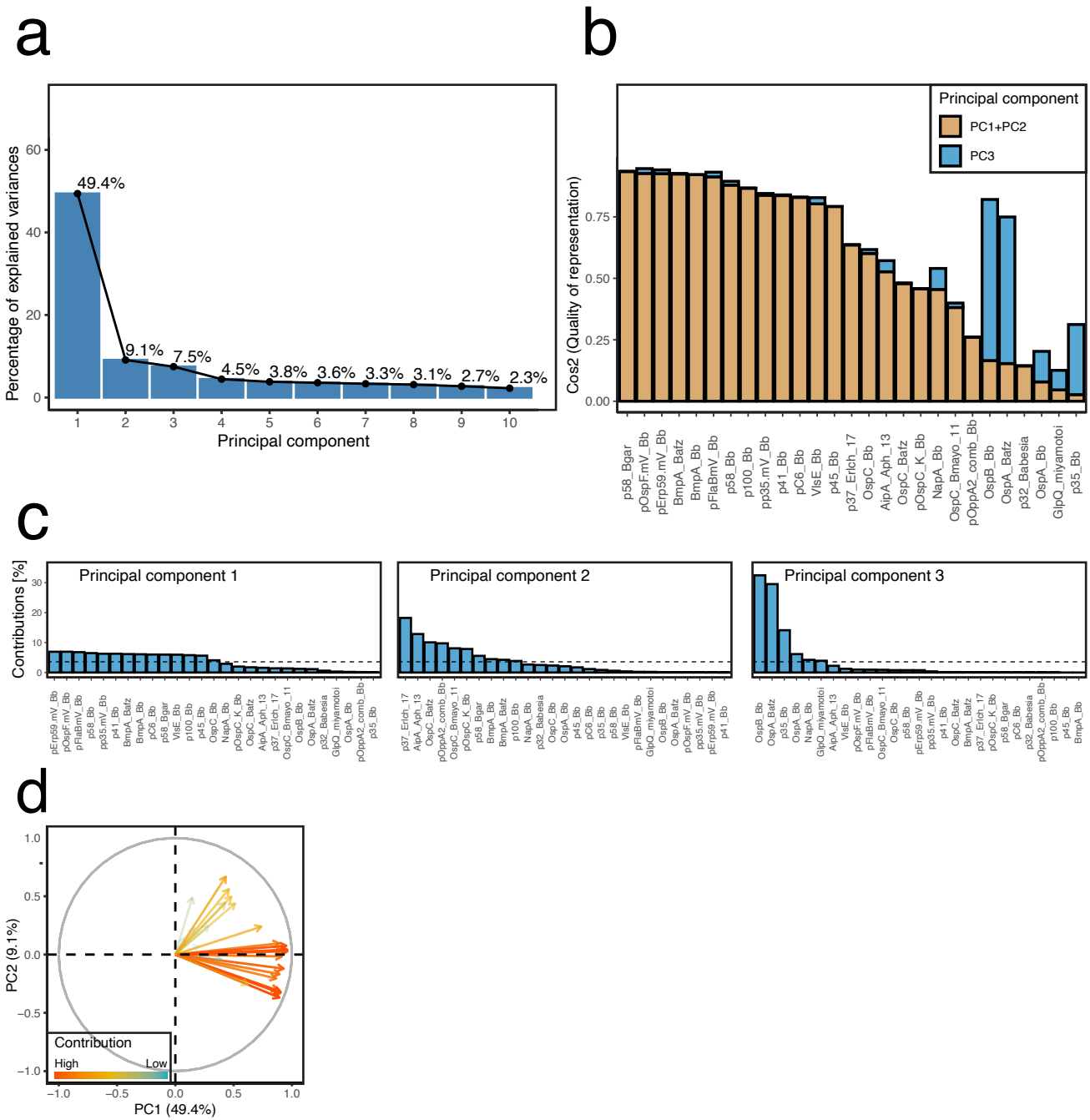

Figure S3 | Antigen contribution to principal components.
(a) Scree plot indicating the amount of variance explained by the first ten principal components used in Figure 5. (b) Bar plot indicating the quality of representation in a 2-dimensional visualization and the increase of quality of representation in a 3-dimensional visualization of the principal components. (c) Shared y-axis. Relative contribution of antigens to the first three principal components per principal component. Dashed line indicates expected contribution in a uniform variable contribution scenario. (d) Correlation circle of MFI per antigen. Color and transparency indicate contribution of the variables to the first two principal components. Variable contributions were obtained as stated in the Methods section.

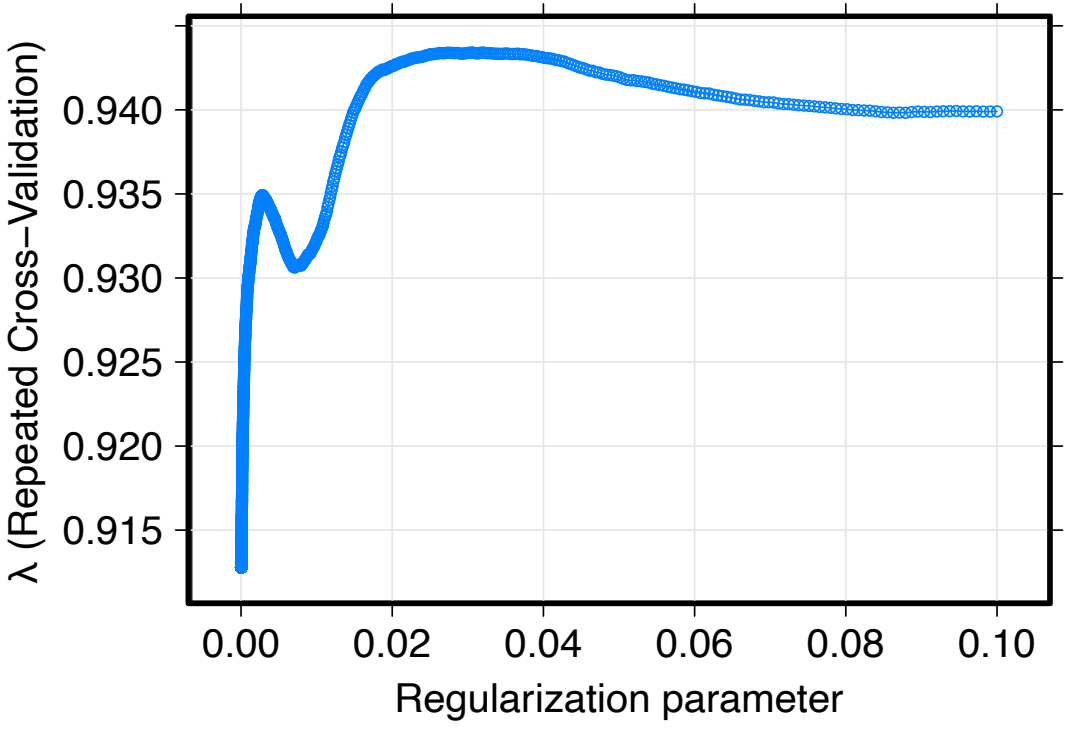

Figure S4 | LASSO feature selection and generalization for logistic regression modelling.
The logistic model's performance across the regularization parameter λ for a 10-fold cross-validation of a LASSO logistic model, repeated 100 times, using ROC (receiver operating characteristic) as the target metric.

Table S1: Multiplex antigen panel.

#	Short Name	Type	Species	Category	Sequence	Source/Notes
1	VlsE_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Abcam; ab138339
2	pFlaBmV_Bb	peptide	B. burgdorferi	B. burgdorferi	CVQEGVQQEGAQQPGGGMKKNDQIVAAIALRGVA	From Lahey et al., 2015, doi: 10.1128/JCM.02111-15
3	p100_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Diarect; 40100
4	GlpQ_miyamotoi	recombinant protein	B. miyamotoi	Other Borrelia	--	Diarect; 42400
5	Crasp2_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Rockland; 000-001-C19
6	p41_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Diarect; 40200
7	DpbA_Bafz	recombinant protein	B. afzelii	Other Borrelia	--	Diarect; 40901
8	OspC_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Diarect; 40300
9	BmpA_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Diarect; 40500
10	p58_Bgar	recombinant protein	B. garinii	Other Borrelia	--	Diarect; 40700
11	p58_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Diarect; 40700
12	pOppA2_comb_Bb	peptide	B. burgdorferi	B. burgdorferi	YGQNWTSPENMVTSGPFKLKERIPNEKYVFEKNNKGGGKK	From Lahey et al., 2015, doi: 10.1128/JCM.02111-15; two linear epitopes linked by triglycine
13	OspB_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Rockland; 000-001-C15
14	p32_Babesia	recombinant protein	Babesia microti	co-infection	--	Diarect; 44100
15	BmpA_Bafz	recombinant protein	B. afzelii	Other Borrelia	--	Diarect; 41100
16	OspA_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Meridian Life Science; R8A131 or Diarect 41200
17	NapA_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Diarect; 41300
18	p45_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Diarect; 41500
19	p66_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Diarect; 41700
20	OspA_Bafz	recombinant protein	B. afzelii	Other Borrelia	--	Diarect; 41000
21	OspC_Bafz	recombinant protein	B. afzelii	Other Borrelia	--	Diarect; 41800
22	pOspC_K_Bb	peptide	B. burgdorferi	B. burgdorferi	AKKAILITDAAKDKGGGGMTLFLFISCNNSGKDGNTSA	From Lahey et al., 2015, doi: 10.1128/JCM.02111-15
23	pErp59mV_Bb	peptide	B. burgdorferi	B. burgdorferi	KIEFSKFTVKIKNKDGGGMKKNDQIVAAIALRGVA	From Lahey et al., 2015, doi: 10.1128/JCM.02111-15
24	pp35mV_Bb	peptide	B. burgdorferi	B. burgdorferi	DTGSERSIRYRRRVYGGGMKKNDQIVAAIALRGVA	From Lahey et al., 2015, doi: 10.1128/JCM.02111-15
25	pOspFmV_Bb	peptide	B. burgdorferi	B. burgdorferi	INKLEAKKTSLKTYSEYEEQGGGMKKNDQIVAAIALRGVA	From Lahey et al., 2015, doi: 10.1128/JCM.02111-15
26	pLA7_Bb	peptide	B. burgdorferi	B. burgdorferi	IPSKENAKLIVYFYDNVYAG	From Lahey et al., 2015, doi: 10.1128/JCM.02111-15
27	pC6_Bb	peptide	B. burgdorferi	B. burgdorferi	CMKKDDQIAAAMVLRGMAKDGQFALK	Equivalent to commercial C6 assay
28	ISB	control	--	--	--	control bead
29	pC10_Bb	peptide	B. burgdorferi	B. burgdorferi	PVVAESPKKP	Commercial 'ZEUS' C10 assay
30	OspC_Bmayo_9	peptide	B. mayonii	Other Borrelia	GKKIQQNNGLGNEAGKNGSLLSGIY	Bmayo OspC AA70-94, designed peptide to be unique to mayonii species
31	OspC_Bmayo_10	peptide	B. mayonii	Other Borrelia	AKKAILRTNGDKTKGAE	Bmayo OspC AA147-163, designed peptide to be unique to mayonii species
32	OspC_Bmayo_11	peptide	B. mayonii	Other Borrelia	AKKAILRTNGDKTKGGGGMTLFLFISCNNSGKDGNASN	B. mayonii homolog of Dattwyler et al. OspC K combined peptide
33	pOmpA_Aph	peptide	A. phagocytophilum	co-infection	TTQSRGKAEPEVLVYSTDAQ	OmpA AA124-123. Peptide seq has some degree of homology with A. marginale and Erlichia spp.
34	AipA_Aph_13	peptide	A. phagocytophilum	co-infection	KGVDSDRKHDAEKTEDKKHGLGSL	AipA AA178-201
35	AipA_Aph_14	peptide	A. phagocytophilum	co-infection	KLSLDPTQGSHTAENILATA	AipA AA3-22
36	p35_Bb	recombinant protein	B. burgdorferi	B. burgdorferi	--	Rockland; 000-001-C12
37	BMN_Babesia_15	peptide	Babesia microti	co-infection	DTQTTQEISEESEEGHDKINKNKS	BMN1-17, AA149-172, 157 & 160 C > S (internal cysteines replaced with serine); partial overlap with immunetics peptide
38	BMN_Babesia_16	peptide	Babesia microti	co-infection	GGPSGTVGPSEAGGPSEAGGPSE	BMN1-2, AA37-59 with added C-term E
39	p44_Aph	recombinant protein	A. phagocytophilum	co-infection	--	Bioclone.us; #PP-0050
40	p37_Erlch_17	peptide	Ehrlichia chaffeensis	co-infection	KYGEFTTALIDDIQKTEHGN	
41	p120_Erlch_18	peptide	Erlichia chaffeensis	co-infection	SHQKEDEIVSQSSSEPFVAE	
42	TC1_Iscapularis_19	peptide	I. scapularis	Borrelia vector	EEAPAGGDAEEEHKHDHE	Tick calreticulin
43	TC2_Iscapularis_20	peptide	I. scapularis	Borrelia vector	EDFEEEDDEDKKEDEEAP	Tick calreticulin

Table S2: Clinical and laboratory features of patients with negative two-tier serology with positive three-antigen panel.
Two children also had multiplex antigens that clustered with early and three with late Lyme disease.

Age (years)	*Sex*	*Race*	*Ethnicity*	*Month*	*Clinical symptoms*	*Days*	*Previous Lyme disease*	*C6 EIA*	*Clinical EIA*	*IgG bands*	*IgM bands*	*ESR*	*CRP*
1	F	White	Non-Hispanic	Apr	Unilateral knee swelling	6	N	0.22	0.93[b]	ND	ND	42	8.74
3	M	White	Non-Hispanic	Jun	Fever	22	N	5.99	4.29[a]	41,45	none	40	0.04
5	F	Black	Non-Hispanic	Dec	Fever and arthritis	4	Y	1.41	1.11	none	none	50	6.89
8	M	White	Non-Hispanic	June	Fever and headache	1	N	0.63	neg	ND	ND	32	1.9
9	F	White	Non-Hispanic	Jul	Facial palsy	2	N	4.67	2.53	none	none	ND	ND
10	M	White	Non-Hispanic	Jul	Fever and headache	1	N	1.10	1.32	18,39, 41	ND	ND	ND
13	M	White	Non-Hispanic	Jul	Fever and headache	1	N	0.17	0.40	ND	ND	ND	ND
13	M	White	Hispanic	Jun	Fever and headache	6	N	1.49	0.48	41	none	ND	ND
14	F	White	Non-Hispanic	Jan	Headache	14	Y	0.21	1.6	none	41,23	6	0.07
14	M	Black	Non-Hispanic	Sep	Headache	2	N	0.18	0.19	ND	ND	ND	ND
17	M	White	Non-Hispanic	Jul	Fever, headache and neck pain	2	N	4.72	1.22	none	none	ND	ND
18	F	White	Non-Hispanic	Jul	Fever and headache	5	N	0.23	0.13	ND	ND	ND	ND
Cluster with early disseminated Lyme cases													
5	M	White	Non-Hispanic	Aug	Fever and headache	4	N	0.36	0.26[b]	ND	ND	65	0.38
6	M	White	Non-Hispanic	Jul	Unilateral knee swelling	4	N	0.79	0.72[b]	ND	ND	ND	ND
Cluster with late Lyme cases													
12	F	White	Non-Hispanic	Oct	Unilateral knee swelling	3	N	0.25	0.35[b]	ND	ND	26	0.21
14	F	Black	Non-Hispanic	Aug	Fever, headache and neck pain	3	N	0.50	0.37[b]	ND	ND	ND	ND
6	M	White	Non-Hispanic	Sep	Unilateral knee swelling	2	N	0.64	0.01[b]	ND	ND	32	1.32

ND: Not Done

[a]Whole Cell Sonicate EIA (negative < 1.0)

[b]Diasorin VlSE (negative < 1.0)

Table S3: Clinical characteristics of children excluded from the analysis.

	Lyme disease N=12 n (%)	Not Lyme disease N=47 n (%)
Median age, years (interquartile range)	14 (11, 15)	10 (7, 14)
Male gender	6/12 (50%)	25/47 (53%)
Female gender	6/12 (50%)	22/47 (47%)
Race		
White	12/12 (100%)	38/47 (81%)
Black	*n/a*	5/47 (11%)
Asian	*n/a*	2/47 (4%)
Other	*n/a*	2/47 (4%)
Hispanic ethnicity	*n/a*	9/47 (19%)
Presentation during peak Lyme season[a]	9/12 (75%)	41/47 (87%)
Early (single EM lesion)	*n/a*	*n/a*
Early disseminated	8/12 (67%)	17/47 (36%)
Multiple EM lesions	1	*n/a*
Facial palsy	4	2
Meningitis	3	2
Carditis	1	2
Late (arthritis)	4/12 (33%)	9/47 (19%)
Non-specific symptoms	*n/a*	21/47 (45%)

[a]Peak Lyme season defined as June to October
n/a = not applicable

VIDEO LEGENDS

Video S1 | Stage dependency of serological response in 3D PCA.
3D principal component plot, colored by stage, based on the same data as **Figure 4a**. Cases have been colored as stated in legend of **Figure 4a**. Gray indicates clinical mimics, green indicates early stage, yellow indicates early disseminated stage and orange indicates late stage.

Video S2 | Correct classifications and false positives in 3D PCA.
3D principal component plot based on **Figure 5d**. Green spheres indicate correct classifications, yellow tetrahedrons indicate false positives based on the case definition.