SUPPLEMENTAL MATERIALS

A Diverse Set of Single-Domain Antibodies (VHHs) Against the Anthrax Toxin Lethal and Edema Factors Provides a Basis for Construction of a Bispecific Agent That Protects Against Anthrax Infection

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SUPPL. FIGURE LEGENDS

FIGURE S1. **Alignment of amino acid sequences of EF- and LF-binding VHHs**. Multiple sequence alignment was performed with the Clustal Omega algorithm (Suppl. Ref. 1, Suppl. Ref. 2). Symbols below the alignment are as follows: asterisk, a fully conserved amino acid residue; colon, strongly similar amino acid properties (>0.5 in the Gonnet PAM 250 matrix); period, weakly similar amino acid properties (<0.5 in the Gonnet PAM 250 matrix).

FIGURE S2. Alignment of EF and LF amino acid sequences. Full-length LF and EF were aligned using Clustal Omega (Suppl. Ref. 1, Suppl. Ref. 2), with sequence similarities indicated by BOXSHADE. Black boxes indicate amino acid identity between the two proteins, and grey boxes indicate amino acid biochemical similarity. Blue bars below the alignment indicate representative regions of high similarity between EF and LF in the N-terminal domain of each protein. Note that the sequences used here include the N-terminal signal peptides (first 33 residues) in the numbering, and the C-terminal tail is omitted.

FIGURE S3. Schematic of sandwich ELISA method for determination of epitope groups. Schematically depicts the experimental procedure used to collect data for Fig. 2. Pre-binding of a molar excess of the blocking VHH with HRP-labeled EF or LF was followed by application of the complexes to a plate coated with the test VHH. A low signal signifies competition for epitope binding between the two VHHs.

FIGURE S4. Modified sandwich ELISA data for additional LF competition group mapping. (A). Schematic of the modified sandwich method for assessing competition. Immulon plates were coated with 10 µg/ml of the blocking VHH, followed by blocking (1% gelatin or 3% BSA) and a 1 h incubation with 5 µg/ml LF. After washing, wells were incubated with a second, HRP-labeled test VHH, using a dilution that produced approximately 50% of the peak binding signal in LF binding curves. Positive controls for direct binding of the HRP-labeled VHHs to LF were included on the plate, and HRP-VHH signal in the absence of LF was a negative control. **(B).** Table of LF ELISA results for the modified sandwich ELISA, representing data from three experiments. Plus symbols indicate pairs of VHHs that demonstrated competition, defined as a >10-fold lower signal than that observed for HRP-VHH binding of LF directly coated on the plate. VHHs are arranged and boxed by epitope competition group. The table depicts repeat pairs, via reversal of the test vs. blocking VHH for each pair. JMO-C10 is a relatively weak binder of LF, and since signals were low when JMO-C10 was coated on the plate as the blocking VHH (but not when used as a test VHH), the JMO-C10 blocking VHH values are omitted.

FIGURE S5. Additional ELISA assays for epitope mapping. **(A).** Assessment of binding of two additional members of the EF1/LF1 competition group (JMN-D10, JMO-B3) as compared to JMO-B9 in the LF2 competition group. Experiment was conducted essentially as in Fig. 4C-D. Closed symbols, LF binding; Open symbols, LF_N binding. Error bars indicate ± SEM of 3 technical replicates; the experiment was also independently repeated. **(B-C).** Standard binding ELISAs were conducted as described in Methods, probing LF(Δ1-36) coated on the plate with JMN-D10 (B), JMO-B3 (B), or JMO-G1 (C). Closed symbols, LF binding; Open symbols, LF(Δ1-36) binding. Results were repeated in three independent experiments.

FIGURE S6. VHH neutralization of MEK cleavage by LF under cellular conditions. RAW264.7 cells were treated with or without LT pre-incubated with vehicle or VHHs. LT was used at 250 ng/mL

for 3 h or 1 μg/mL for 1 h, and VHHs were used at 1.75 μg/mL, a 5:1 or 20:1 VHH to LF molar ratio. Western blotting of cell lysates was performed to assess cleavage of MEK2 and -3 by LF. Actin is a control for cell viability.

FIGURE S7. LF neutralization assays under "high toxin" conditions. Representative LF neutralization experiments using three different doses of toxin (250, 750 and 1500 ng/ml). JKH-C7 is a PA neutralizing VHH that was used as a control.

SUPPL. REFERENCES

1. Sivers, F., Wilm, A., Dineen, D. G., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J. D., and Higgins, D. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **7**, 539

2. Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J., and Lopez, R. (2010) A new bioinformatics analysis tools framework at EMBL-EBI. *Nucleic Acids Res*. **38 (suppl 2),** W695-W699

SUPPL TABLE 1. LF_N linear peptides for epitope mapping.

LF Peptide	LF_N Residue Numbers	Peptide Sequence (N → C)
LF1	97-106	LSEDKKKIKD
LF2	135-143	EDYVENTEK
LF3	178-187	KNASDSDGQD
LF4	226-234	EPQHRDVLQ
LF5	230-239	RDVLQLYAPE

Fig. S1

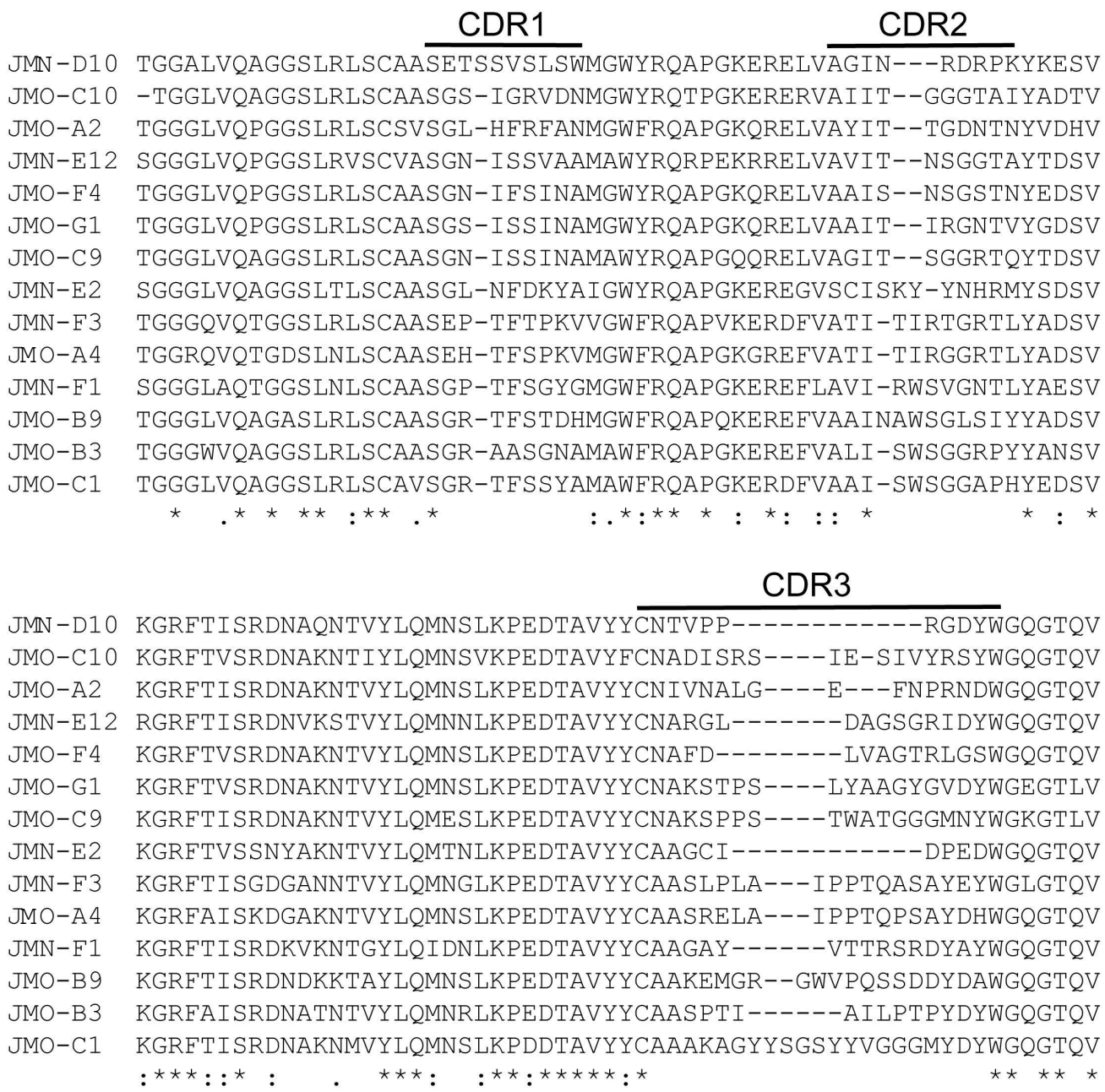

CDR1
CDR2
CDR3
JMN-D10 TGGALVQAGGSLRLSCAASETSSVSLSWMGWYRQAPGKERELVAGIN---RDRPKYKESV
JMO-C10 -TGGLVQAGGSLRLSCAASGS-IGRVDNMGWYRQTPGKERERVAIIT--GGGTAIYADTV
JMO-A2 TGGGLVQPGGSLRLSCSVSGL-HFRFANMGWFRQAPGKQRELVAYIT--TGDNTNYVDHV
JMN-E12 SGGGLVQPGGSLRVSCVASGN-ISSVAAMAWYRQRPEKRRELVAVIT--NSGGTAYTDSV
JMO-F4 TGGGLVQPGGSLRLSCAASGN-IFSINAMGWYRQAPGKQRELVAAIS--NSGSTNYEDSV
JMO-G1 TGGGLVQPGGSLRLSCAASGS-ISSINAMGWYRQAPGKQRELVAAIT--IRGNTVYGDSV
JMO-C9 TGGGLVQAGGSLRLSCAASGN-ISSINAMAWYRQAPGQQRELVAGIT--SGGRTQYTDSV
JMN-E2 SGGGLVQAGGSLTLSCAASGL-NFDKYAIGWYRQAPGKEREGVSCISKY-YNHRMYSDSV
JMN-F3 TGGGQVQTGGSLRLSCAASEP-TFTPKVVGWFRQAPVKERDFVATI-TIRTGRTLYADSV
JMO-A4 TGGRQVQTGDSLNLSCAASEH-TFSPKVMGWFRQAPGKGREFVATI-TIRGGRTLYADSV
JMN-F1 SGGGLAQTGGSLNLSCAASGP-TFSGYGMGWFRQAPGKEREFLAVI-RWSVGNTLYAESV
JMO-B9 TGGGLVQAGASLRLSCAASGR-TFSTDHMGWFRQAPQKEREFVAAINAWSGLSIYYADSV
JMO-B3 TGGGWVQAGGSLRLSCAASGR-AASGNAMAWFRQAPGKEREFVALI-SWSGGRPYYANSV
JMO-C1 TGGGLVQAGGSLRLSCAVSGR-TFSSYAMAWFRQAPGKERDFVAAI-SWSGGAPHYEDSV
* .* * ** :** .* :.*:** * : *: :: * * : *
JMN-D10 KGRFTISRDNAQNTVYLQMNSLKPEDTAVYYCNTVPP----------RGDYWGQGTQV
JMO-C10 KGRFTVSRDNAKNTIYLQMNSVKPEDTAVYFCNADISRS----IE-SIVYRSYWGQGTQV
JMO-A2 KGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNIVNALG----E---FNPRNDWGQGTQV
JMN-E12 RGRFTISRDNVKSTVYLQMNNLKPEDTAVYYCNARGL-------DAGSGRIDYWGQGTQV
JMO-F4 KGRFTVSRDNAKNTVYLQMNSLKPEDTAVYYCNAFD--------LVAGTRLGSWGQGTQV
JMO-G1 KGRFTVSRDNAKNTVYLQMNSLKPEDTAVYYCNAKSTPS----LYAAGYGVDYWGEGTLV
JMO-C9 KGRFTISRDNAKNTVYLQMESLKPEDTAVYYCNAKSPPS----TWATGGGMNYWGKGTLV
JMN-E2 KGRFTVSSNYAKNTVYLQMTNLKPEDTAVYYCAAGCI----------DPEDWGQGTQV
JMN-F3 KGRFTISGDGANNTVYLQMNGLKPEDTAVYYCAASLPLA---IPPTQASAYEYWGLGTQV
JMO-A4 KGRFAISKDGAKNTVYLQMNSLKPEDTAVYYCAASRELA---IPPTQPSAYDHWGQGTQV
JMN-F1 KGRFTISRDKVKNTGYLQIDNLKPEDTAVYYCAAGAY------VTTRSRDYAYWGQGTQV
JMO-B9 KGRFTISRDNDKKTAYLQMNSLKPEDTAVYYCAAKEMGR--GWVPQSSDDYDAWGQGTQV
JMO-B3 KGRFAISRDNATNTVYLQMNRLKPEDTAVYYCAASPTI------AILPTPYDYWGQGTQV
JMO-C1 KGRFTISRDNAKNMVYLQMNSLKPDDTAVYYCAAAKAGYYSGSYYVGGGMYDYWGQGTQV
:***::* : . ***: :**:*****:* ** ** *

Fig. S2

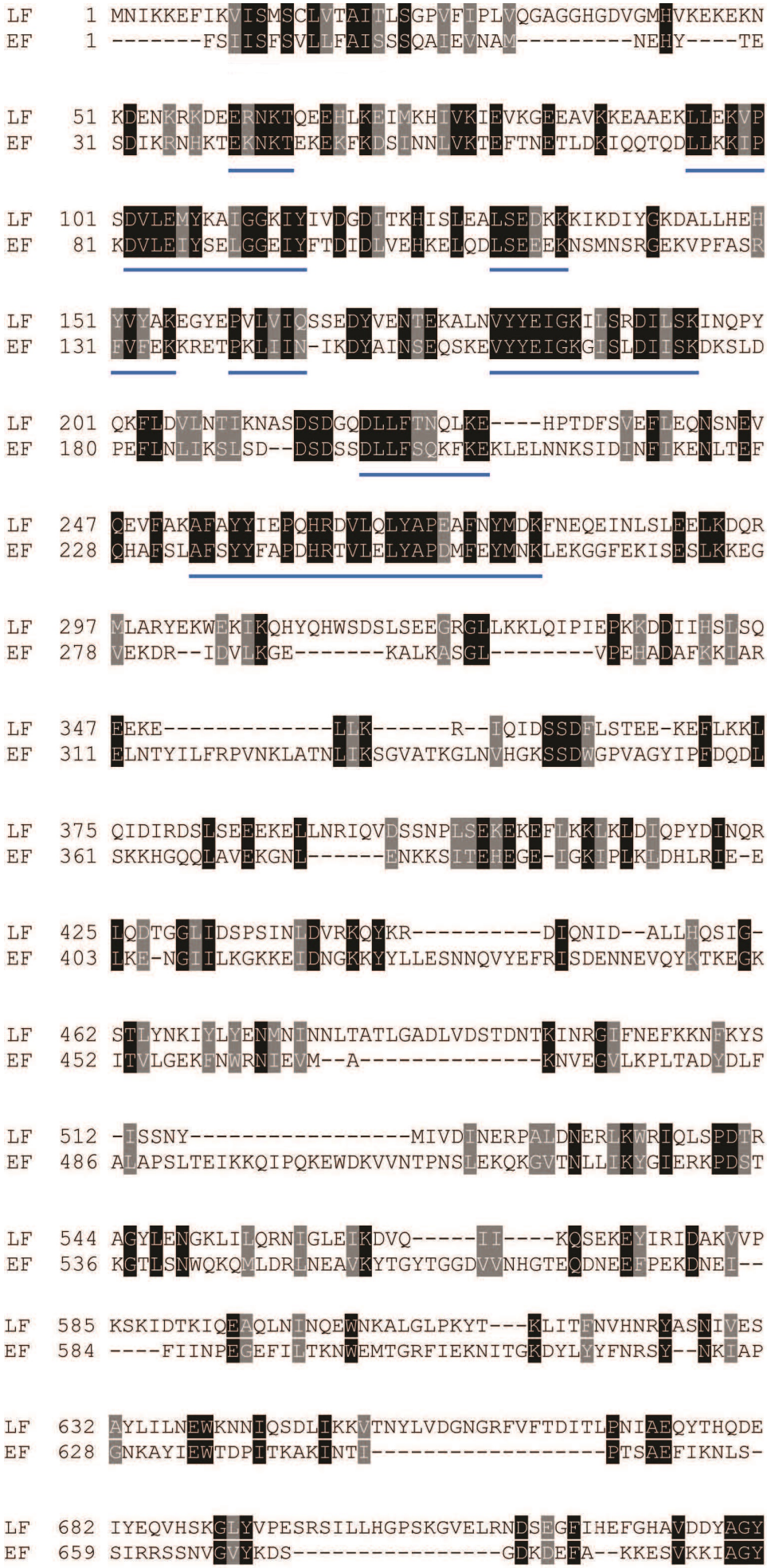

LF 1 MNIKKEFIKVISMSCLVTAITLSGPVFIPLVQGAGGHGDVGMHVKEKEKN
EF 1 -------FSIIISFSVLIFAISSSQAIEVNAM---------NEHY----TE
LF 51 KDENKRKDEERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAEKLLEKVP
EF 31 SDIKRNHKTEKNKTEKEKFKDSINNLVKTEFTNETLDKIQQTQDLLKKIP
LF 101 SDVLEMYKAIGGKIYIVDGDITKHISLEALSEDKKKIKDIYGKDALLHEH
EF 81 KDVLEIYSELGGEIYFTDIDLVEHKELQDLSEEEKNSMNSRGEKVPFASR
LF 151 YVYAKEGYEPVLVIQSSEDYVENTEKALNVYYEIGKILSRDILSKINQPY
EF 131 FVFEKKRETPKLIIN-IKDYAINSEQSKEVYYEIGKGISLDIISKDKSLD
LF 201 QKFLDVLNTIKNASDSDGQDLLFTNQLKE----HPTDFSVEFLEQNSNEV
EF 180 PEFLNLIKSLSD--DSDSSDLLFSQKFKEKLELNNKSIDINFIKENLTEF
LF 247 QEVFAKAFAYYIEPQHRDVLQLYAPEAFNYMDKFNEQEINLSLEELKDQR
EF 228 QHAFSLAFSYYFAPDHRTVLELYAPDMFEYMNKLEKGGFEKISESLKKEG
LF 297 MLARYEKWEKIKQHYQHWSDSLSEEGRGLLKKLQIPIEPKKDDIIHSLSQ
EF 278 VEKDR--IDVLKGE-------KALKASGL--------VPEHADAFKKIAR
LF 347 EEKE-------------LLK------R--IQIDSSDFLSTEE-KEFLKKL
EF 311 ELNTYILFRPVNKLATNLIKSGVATKGLNVHGKSSDWGPVAGYIPFDQDL
LF 375 QIDIRDSLSEEEKELLNRIQVDSSNPLSEKEKEFLKKLKLDIQPYDINQR
EF 361 SKKHGQQLAVEKGNL------ENKKSITEHEGE-IGKIPLKLDHLRIE-E
LF 425 LQDTGGLIDSPSINLDVRKQYKR----------DIQNID--ALLHQSIG-
EF 403 LKE-NGIILKGKKEIDNGKKYYLLESNNQVYEFRISDENNEVQYKTKEGK
LF 462 STLYNKIYLYENMNINNLTATLGADLVDSTDNTKINRGIFNEFKKNFKYS
EF 452 ITVLGEKFNWRNIEVM--A----------KNVEGVLKPLTADYDLF
LF 512 -ISSNY----------MIVDINERPALDNERLKWRIQLSPDTR
EF 486 ALAPSLTEIKKQIPQKEWDKVVNTPNSLEKQKGVTNLLIKYGIERKPDST
LF 544 AGYLENGKLILQRNIGLEIKDVQ-----II----KQSEKEYIRIDAKVVP
EF 536 KGTLSNWQKQMLDRLNEAVKYTGYTGGDVVNHGTEQDNEEFPEKDNEI--
LF 585 KSKIDTKIQEAQLNINQEWNKALGLPKYT---KLITFNVHNRYASNIVES
EF 584 ----FIINPEGEFILTKNWEMTGRFIEKNITGKDYLYYFNRSY--NKIAP
LF 632 AYLILNEWKNNIQSDLIKKVTNYLVDGNGRFVFTDITLPNIAEQYTHQDE
EF 628 GNKAYIEWTDPITKAKINTI----------PTSAEFIKNLS-
LF 682 IYEQVHSKGLYVPESRSILLHGPSKGVELRNDSEGFIHEFGHAVDDYAGY
EF 659 SIRRSSNVGVYKDS----------GDKDEFA--KKESVKKIAGY

Fig. S3

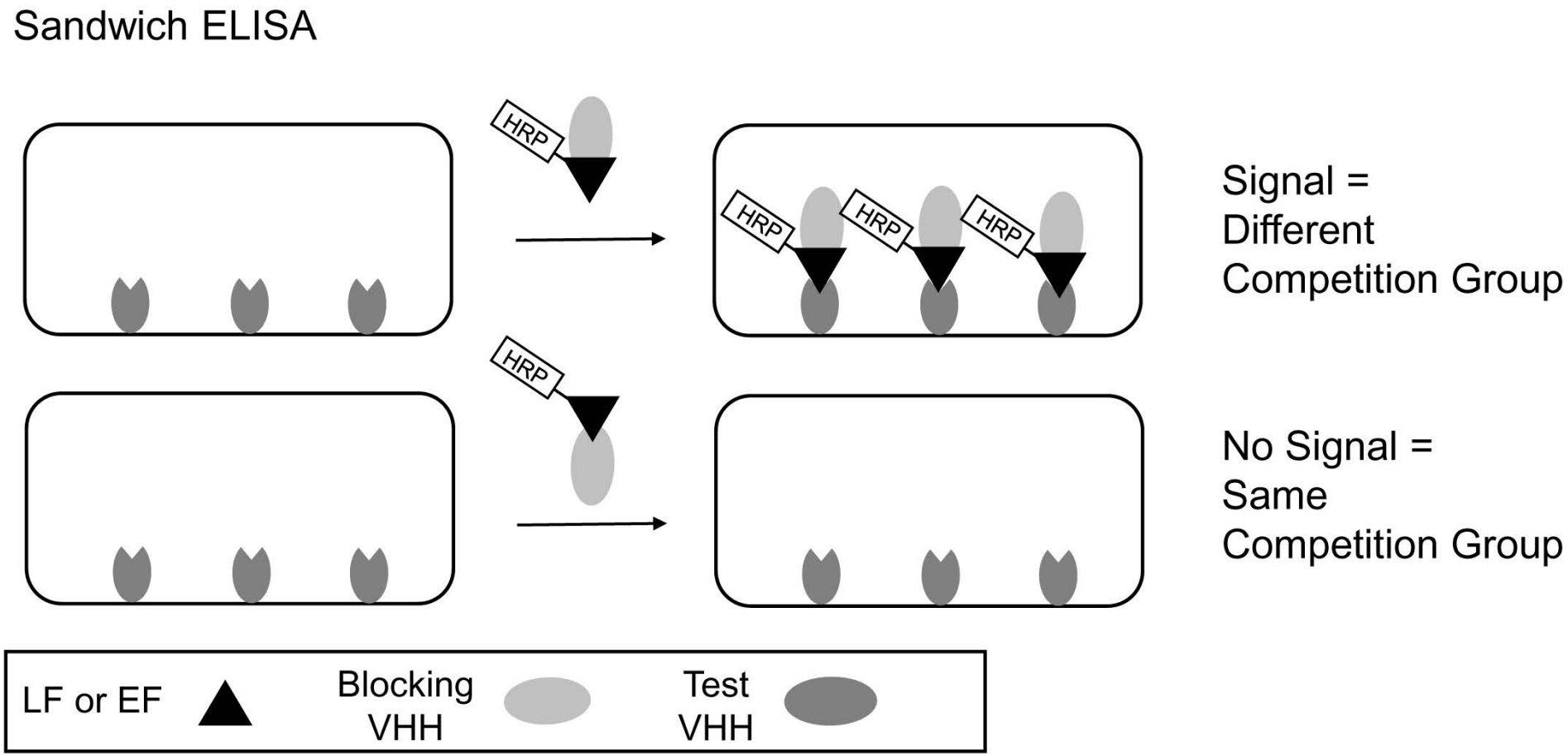

Sandwich ELISA
HRP
HRP
HRP
HRP
Signal =
Different
Competition Group
HRP
No Signal =
Same
Competition Group
LF or EF
Blocking
VHH
Test
VHH

Fig. S4

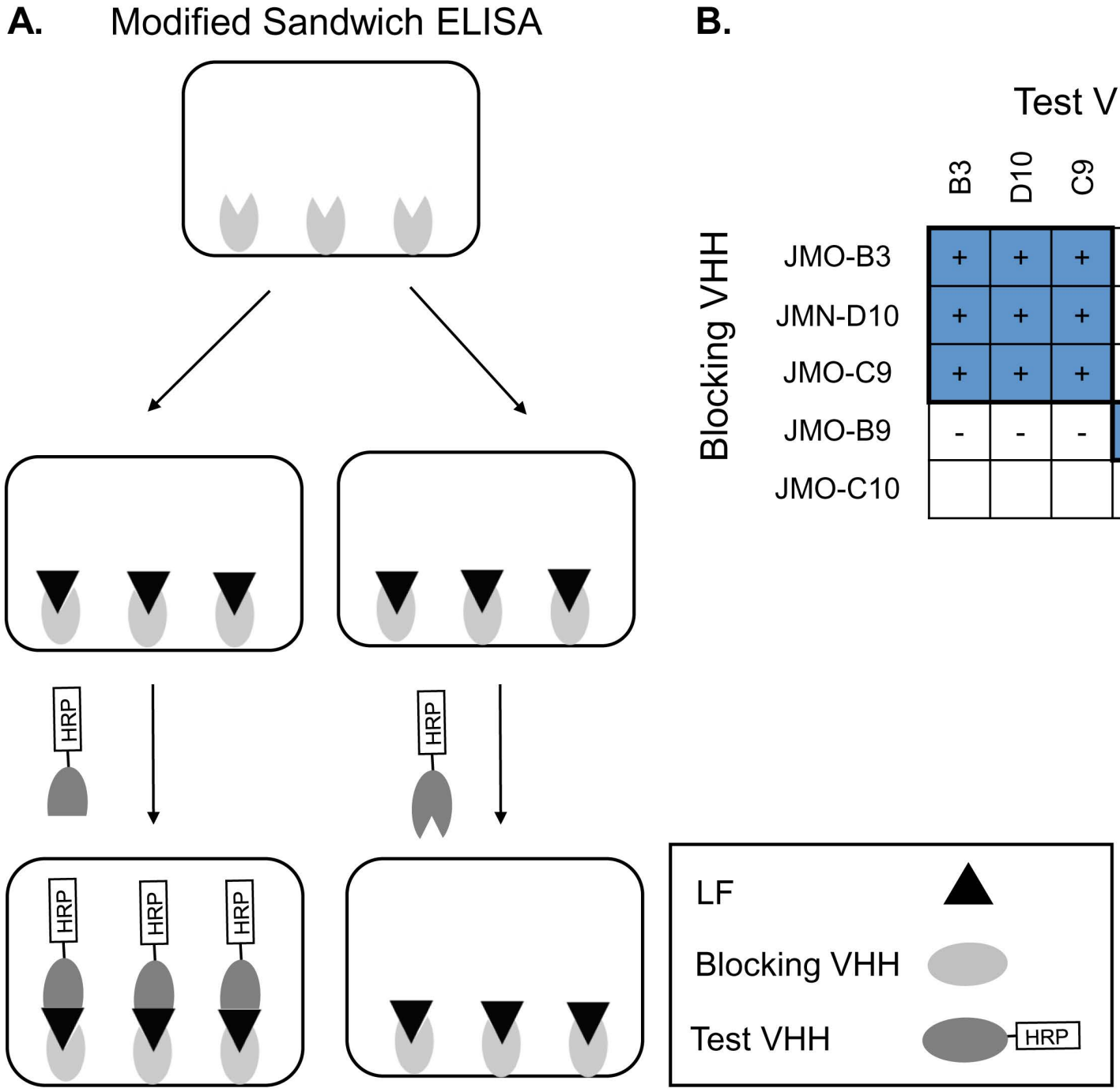

A.
Modified Sandwich ELISA
HRP
HRP
HRP
HRP
HRP
B.
Test VHH
B3
D10
C9
B9
C10
Blocking VHH
JMO-B3
JMN-D10
JMO-C9
JMO-B9
JMO-C10
LF
Blocking VHH
Test VHH
HRP

Fig. S5

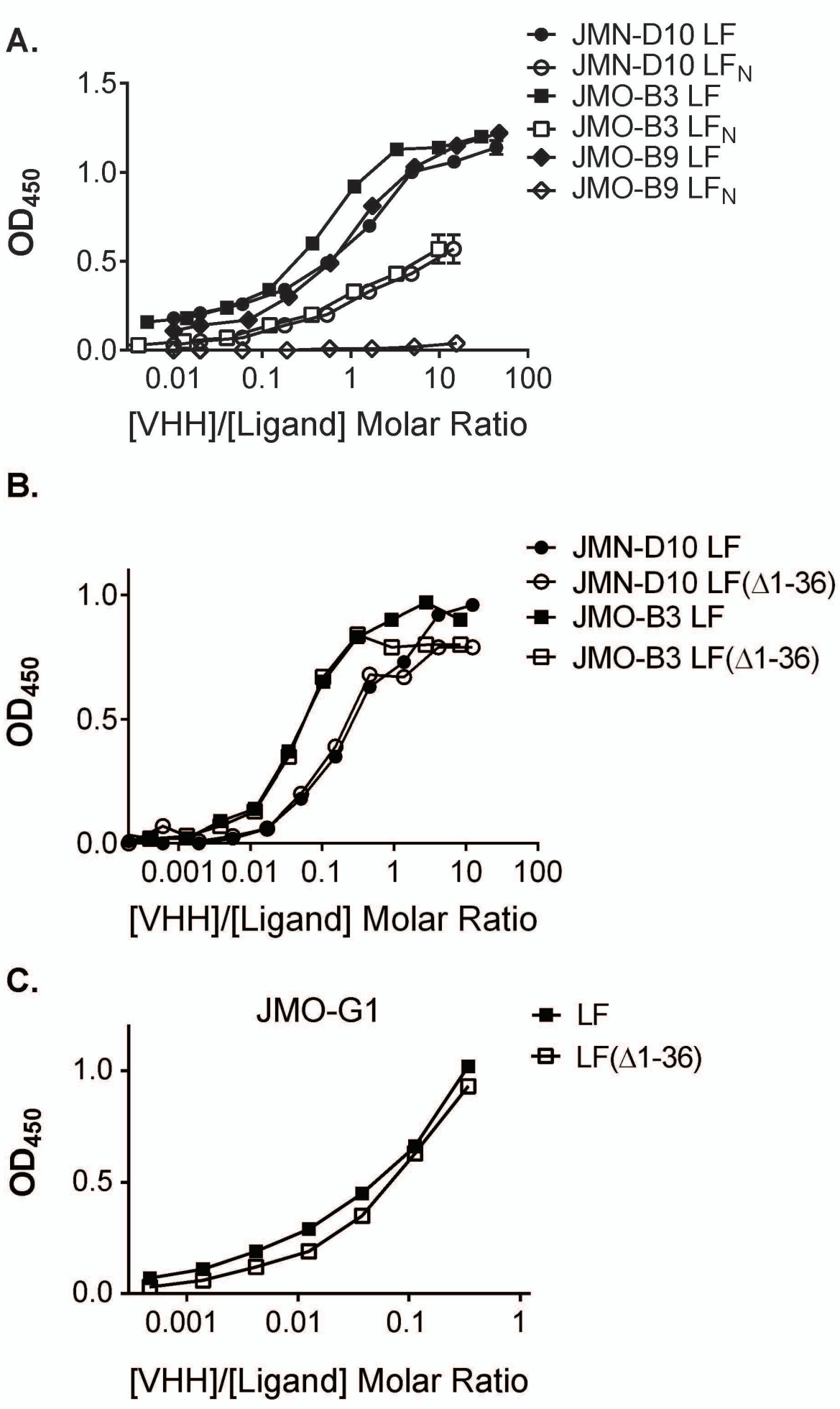

A.
JMN-D10 LF
JMN-D10 LF$_N$
JMO-B3 LF
JMO-B3 LF$_N$
JMO-B9 LF
JMO-B9 LF$_N$
OD$_{450}$
1.5
1.0
0.5
0.0
0.01
0.1
1
10
100
[VHH]/[Ligand] Molar Ratio
B.
JMN-D10 LF
JMN-D10 LF(Δ1-36)
JMO-B3 LF
JMO-B3 LF(Δ1-36)
OD$_{450}$
1.0
0.5
0.0
0.001
0.01
0.1
1
10
100
[VHH]/[Ligand] Molar Ratio
C.
JMO-G1
LF
LF(Δ1-36)
OD$_{450}$
1.0
0.5
0.0
0.001
0.01
0.1
1
[VHH]/[Ligand] Molar Ratio

Fig. S6

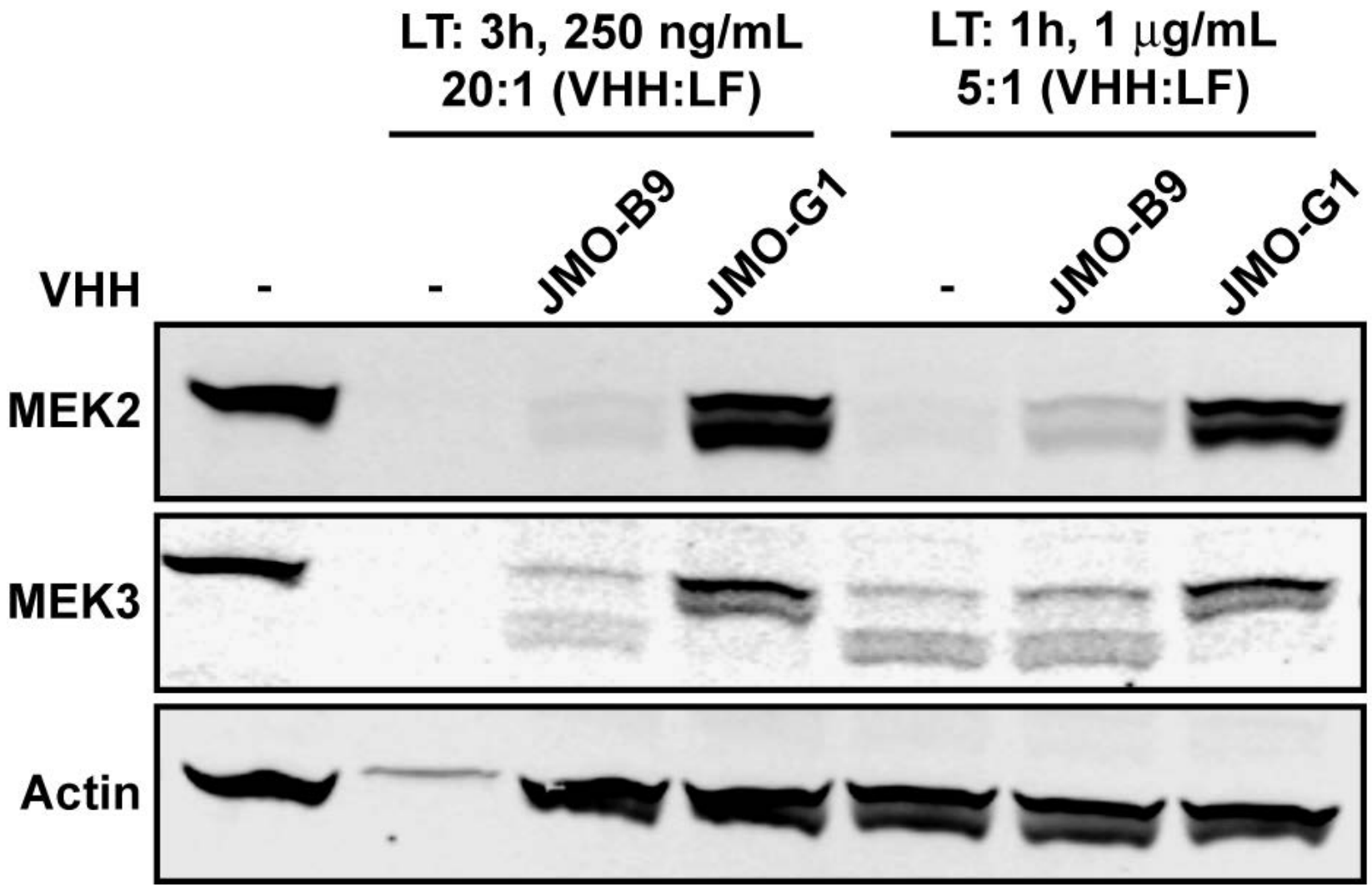
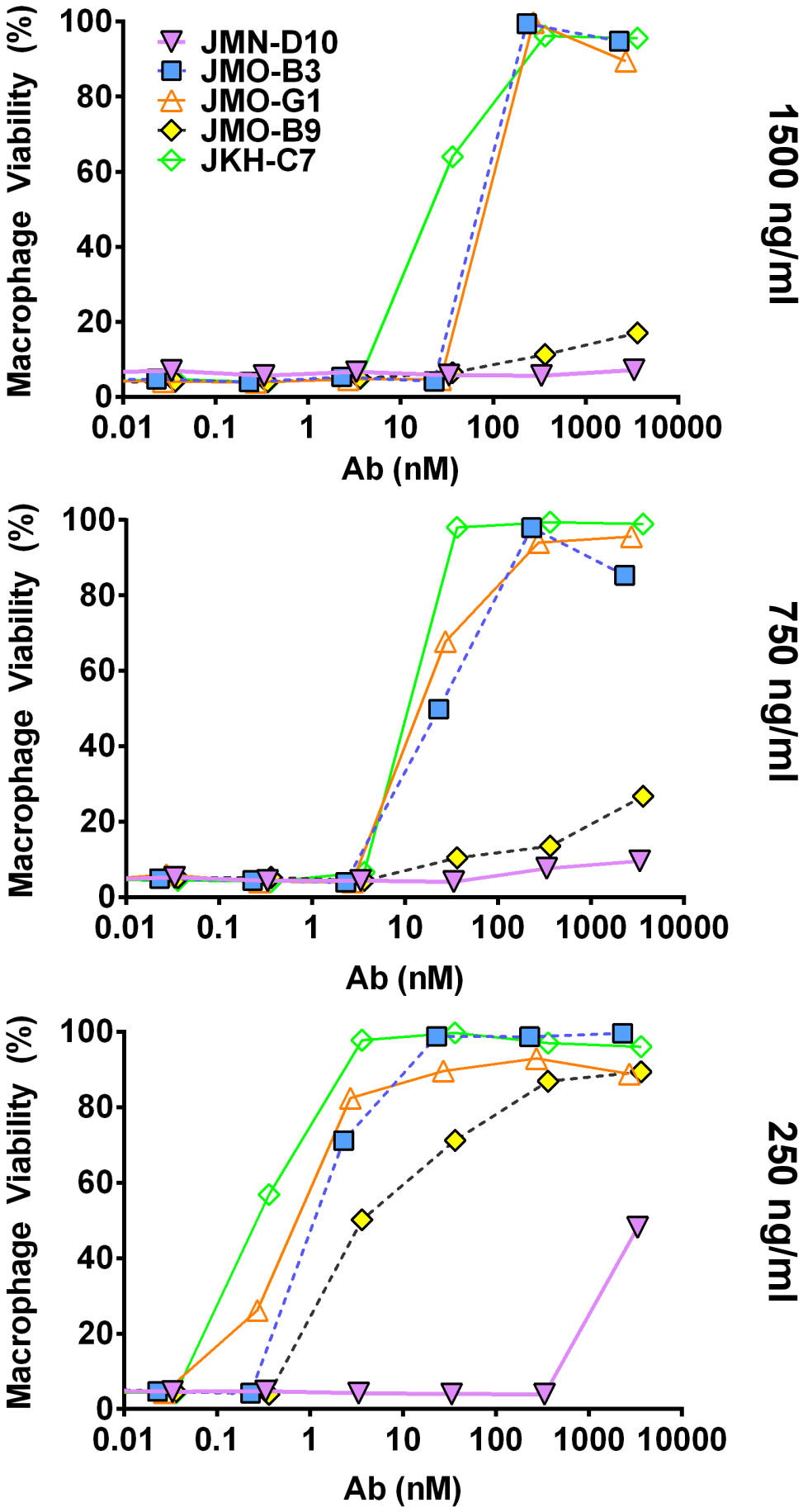

LT: 3h, 250 ng/mL
20:1 (VHH:LF)
LT: 1h, 1 μg/mL
5:1 (VHH:LF)
VHH
-
-
JMO-B9
JMO-G1
-
JMO-B9
JMO-G1
MEK2
MEK3
Actin

Fig. S7


JMN-D10
JMO-B3
JMO-G1
JMO-B9
JKH-C7
Macrophage Viability (%)
Ab (nM)
0.01
0.1
1
10
100
1000
10000
0
20
40
60
80
100
1500 ng/ml
750 ng/ml
250 ng/ml