

Supplemental Information

**Viral Fitness Landscapes in Diverse Host
Species Reveal Multiple Evolutionary Lines
for the NS1 Gene of Influenza A Viruses**

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FIGURE S1

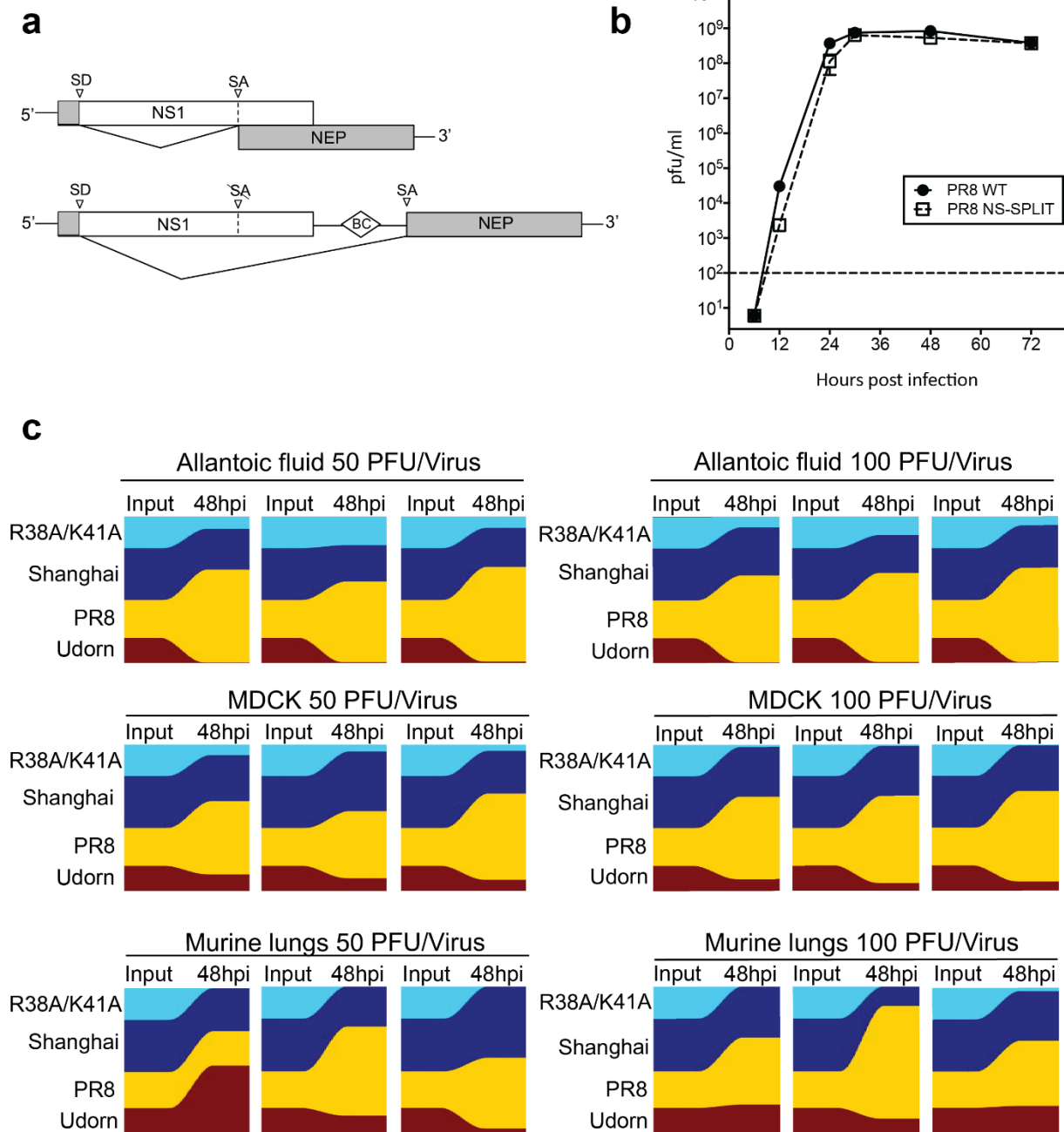


Figure S1, related to Figure 1. Reproducible barcode-based abundance is achieved after infecting *in vitro*, *in ovo* and *in vivo* systems with a small NS1 recombinant library. (a) Schematic representation of WT NS segment (top) and split NS segment (bottom) with modified splicing mechanism that allows the expression of NS1 and NEP in different open reading frames, flanking a short neutral barcode. **(b)** Replication comparison of a WT PR8 virus and a recombinant NS-split PR8 virus. MDCK cells were infected at a MOI of 0.001 pfu/cell. Error bars depict the standard deviation (SD) of three independent experiments. **(c)** Viral progression diagrams (Matlab) depicting the relative viral abundance present in a “proof-of-principle” library relative to the total number of barcode reads. Each color represents the relative proportion of a specific barcode within the viral population. “Input” indicates the proportion within the initial inoculum at the time of infection. 10-day-old chicken embryonated eggs (upper panels), MDCK cells (middle panels) and 8-week-old WT C57BL/6 mice (lower panels) were infected in triplicates with the viral library by adding either 50 pfu/virus or 100 pfu/virus and samples were collected 48 hours post infection.

FIGURE S2

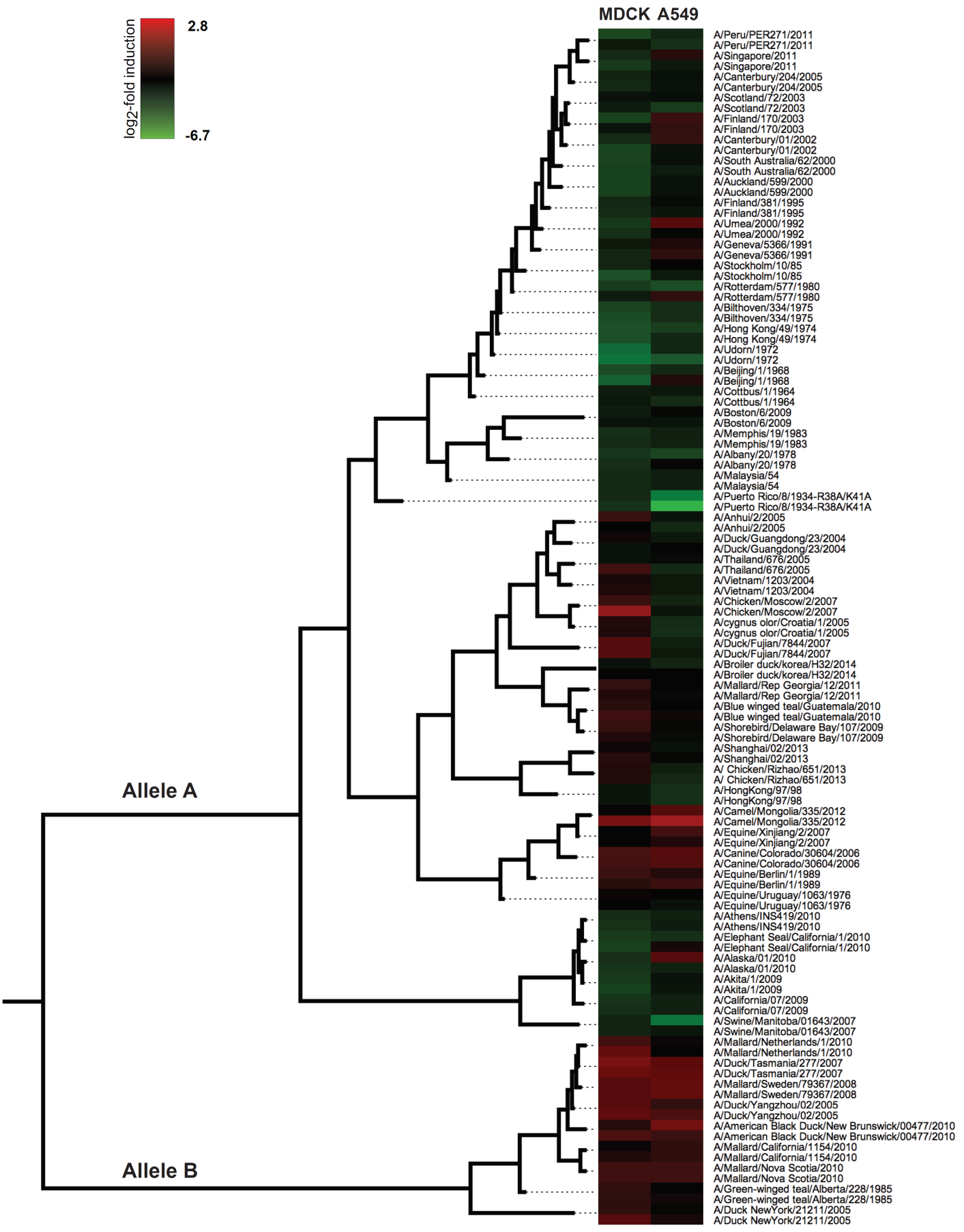


Figure S2, related to Figure 2. Different NS1 selection profiles upon MDCK or A549 cells library infection. Phylogenetic tree containing selected NS1 sequences within the library was assembled following Bayesian analysis (BEAST). Heat map displaying the relative abundance of barcode reads for each recombinant virus within the library upon infection of MDCK or A549 cells. Viral RNA samples were isolated at 48 hours post infection and further analyzed. Average of the triplicates are listed in columns and expressed as the \log_2 -fold induction over the initial relative proportion of barcode reads found in the initial viral mix (input). Red and green colors indicate high or low barcode representation versus the input, respectively. Additional information of each specific NS1 is available in **Table 1**.

FIGURE S3

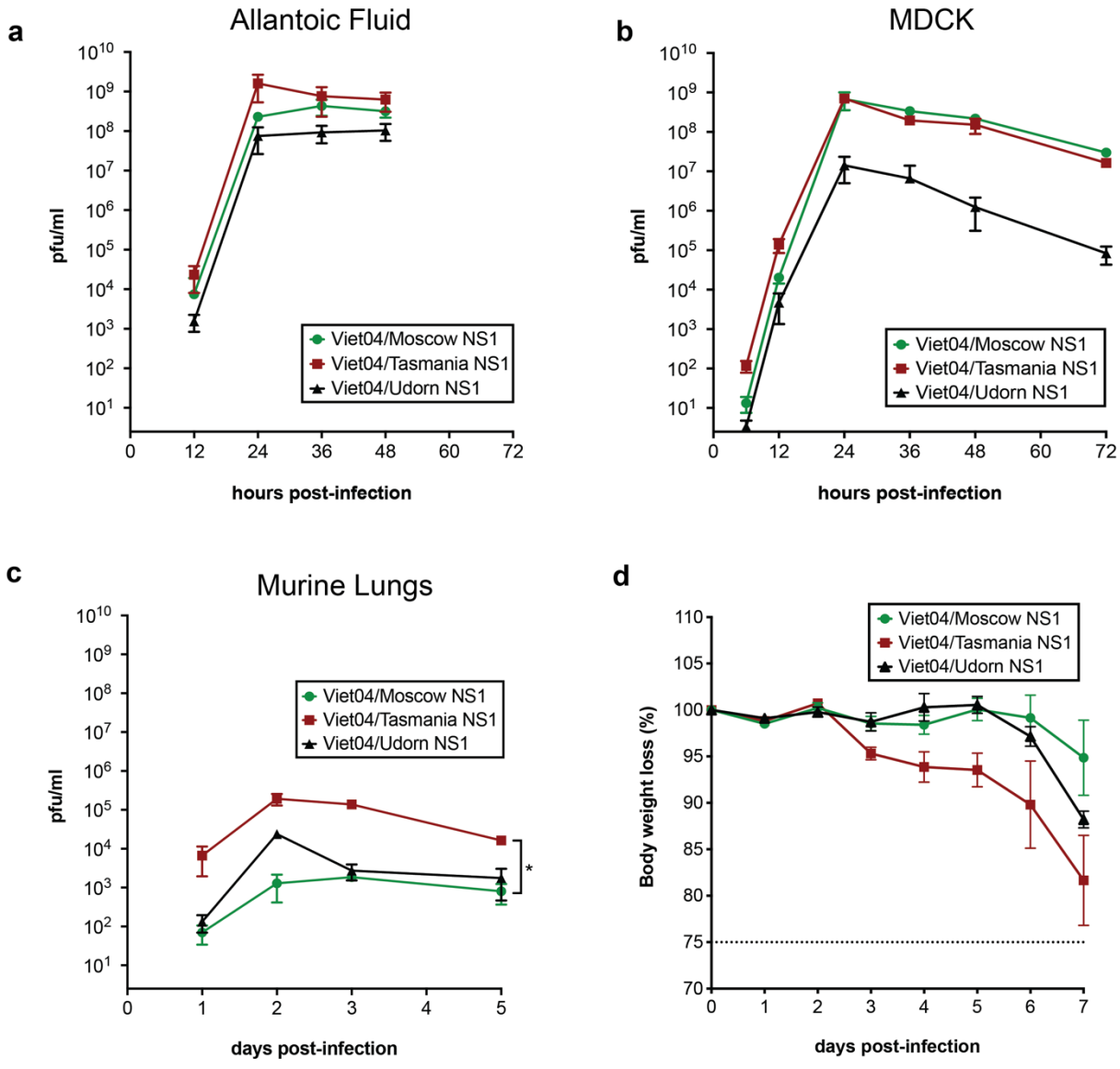


Figure S3, related to Figure 3. Viral library profile dynamics can be reproduced in single-virus experiments in A/Vietnam/1203/04 (H5N1 HALo) background. Same NS1 variants as in Figure 3 were rescued in a H5N1 HALo background and single-virus infections were conducted in triplicates using 10-day-old chicken embryonated eggs (a) MDCK cells (b) and 8-week-old C57BL/6 mice (c). Viral replication was quantified at different time points post infection. Body weight loss of infected mice was daily monitored (d). Error bars depict the standard deviation (SD). *, $p < 0.05$.

FIGURE S4

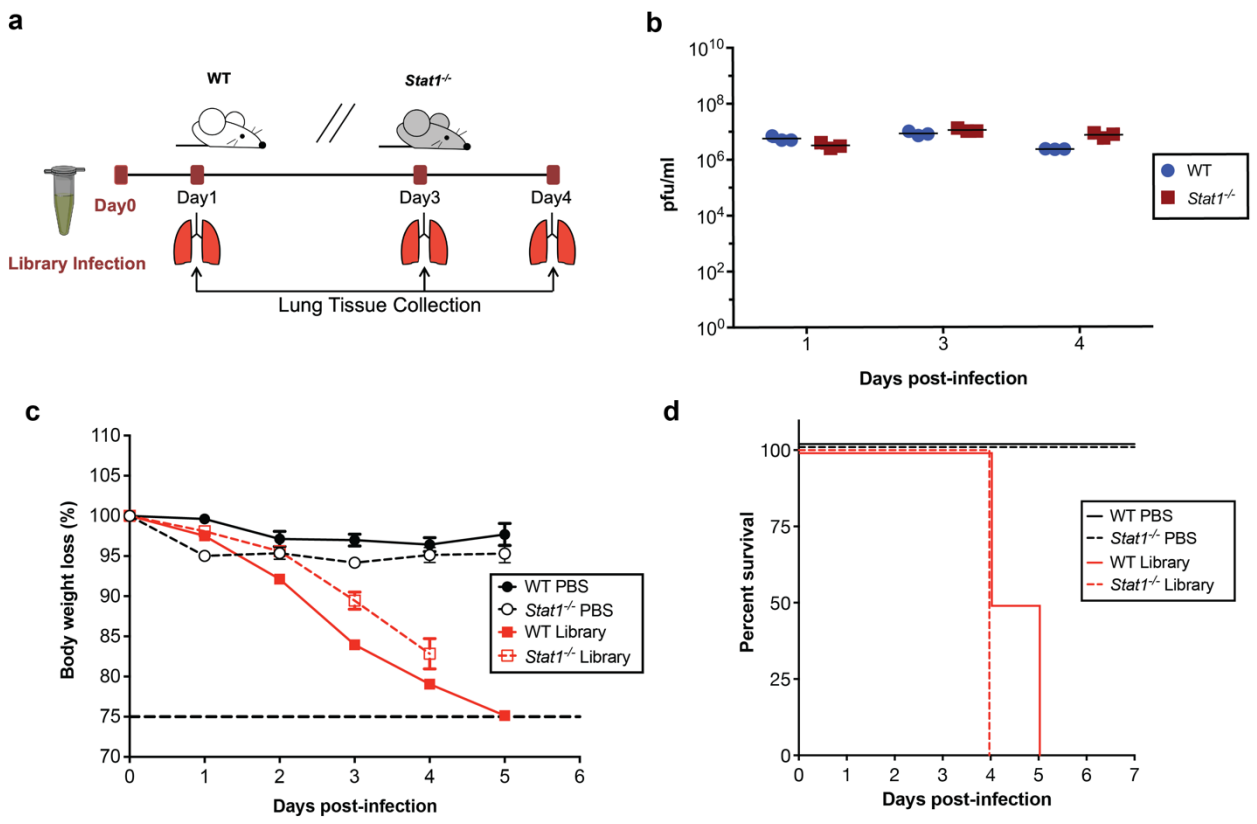


Figure S4, related to Figure 5. NS1 library selection profile depends on the type-I IFN response. (a) Experimental layout of library infection in 129S wild type (WT) and 129S *Stat1*^{-/-} mice (n=4). (b) Mouse lung viral titers were determined by plaque assay on MDCK cells at days 1, 3 and 4 post-infection. (c) Body weight loss and (d) survival rates were daily monitored during the infection. Error bars depict the standard deviation (SD).

FIGURE S5

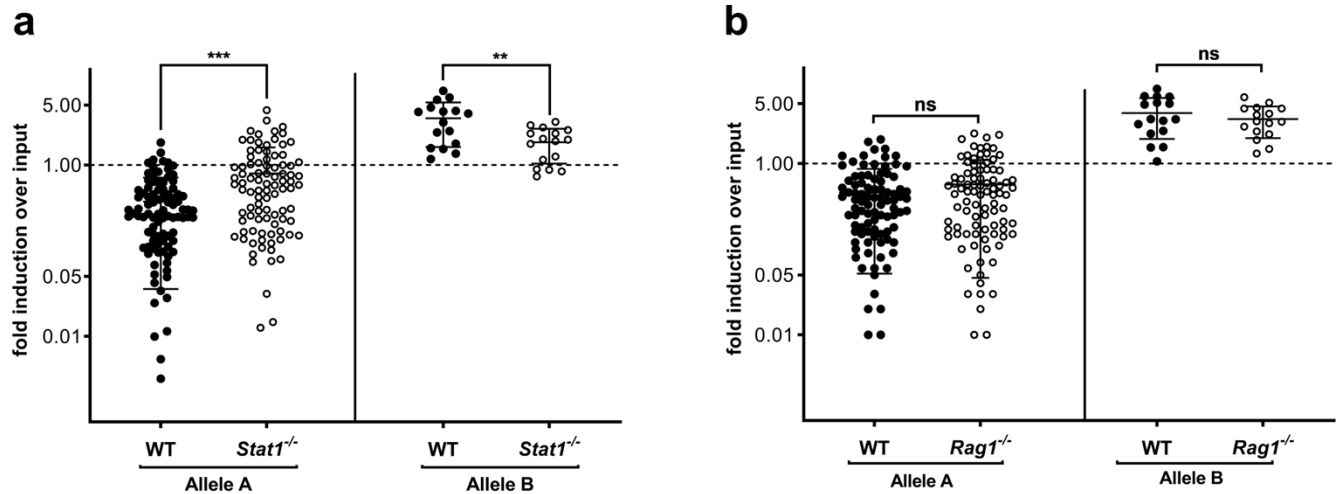


Figure S5, related to Figure 5. Differences in fitness between allele A and allele B NS1. Scatter plot graphs depicting the abundance profiles of viruses containing allele A or allele B NS1 within the library. Average of the triplicates are represented as the fold induction over the input (initial relative proportion of barcode reads found in the viral mix) and represented in a log₂ scale. Profiles from *Stat1*^{-/-} at 3 days post-infection (a) and *Rag1*^{-/-} mice at 4 days post infection (b) are shown. Error bars depict the standard deviation (SD). *, P<0.05; **, P<0.01; ***, P<0.001.

FIGURE S6

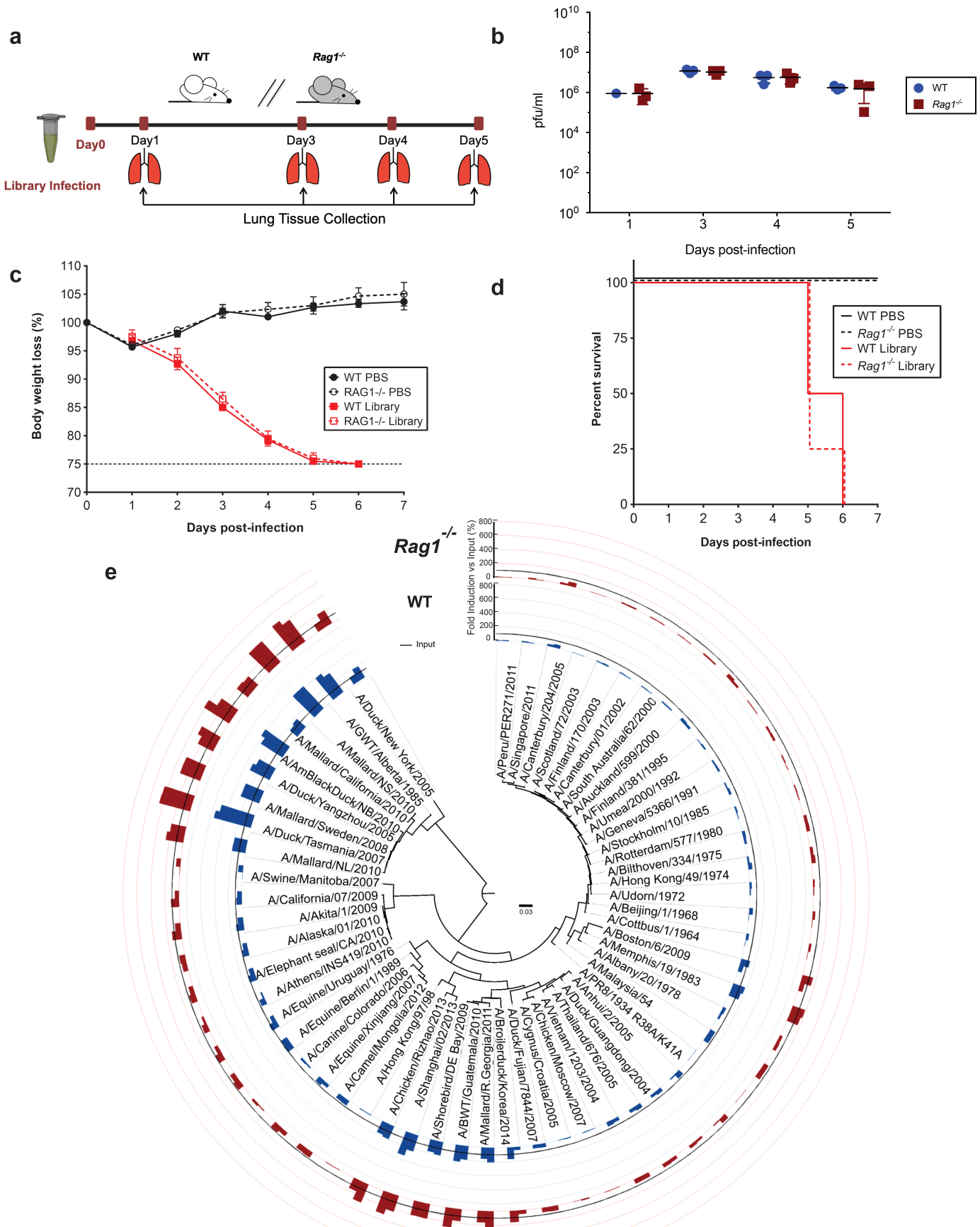
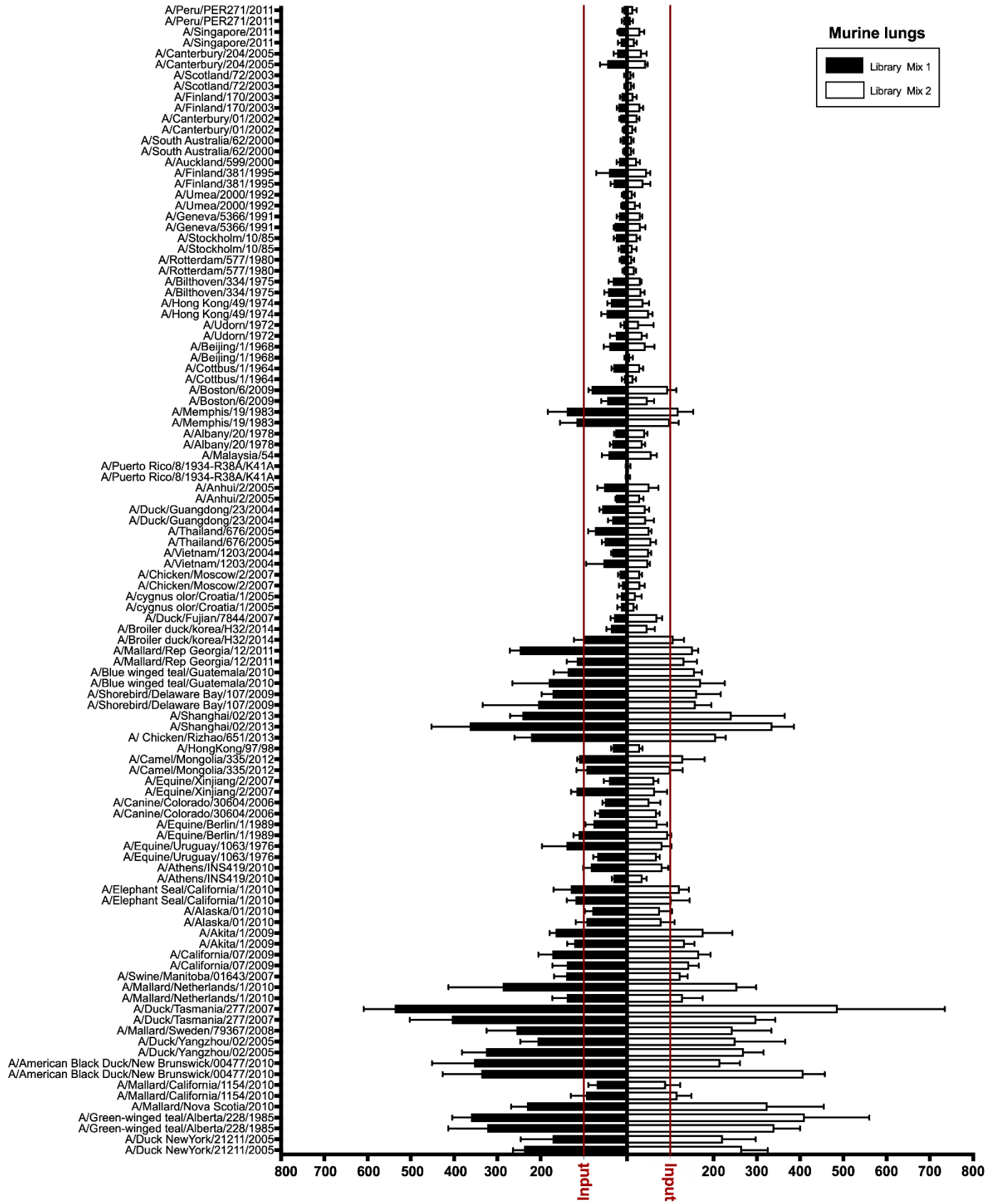


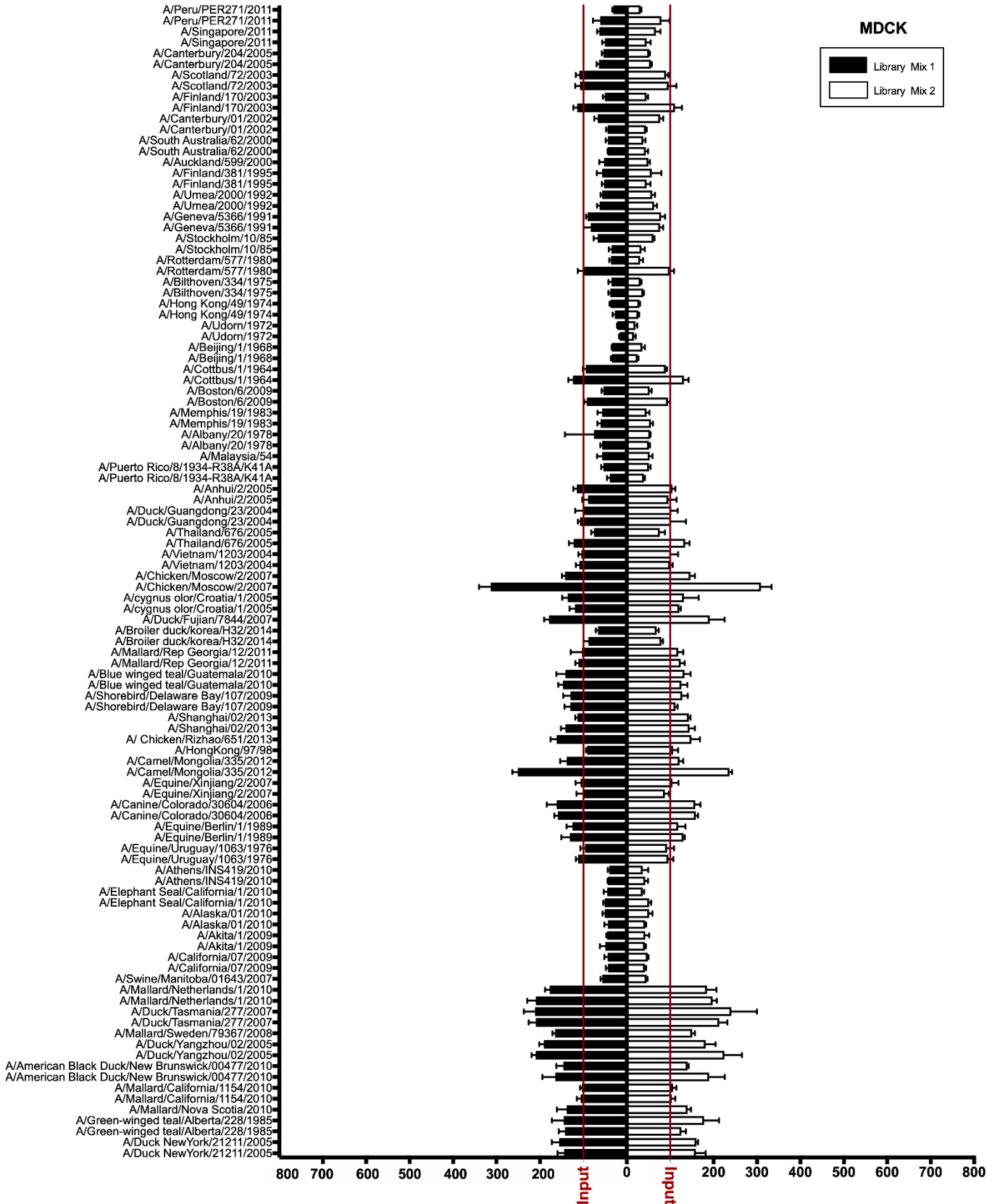
Figure S6, related to Figure 5. NS1 library selection profile is early host adaptive immune response independent. (a) Experimental layout of library infection in C57BL/6 wild type (WT) and C57BL/6 *Rag1*^{-/-} mice. (b) Lung tissue from infected mice (n=3) were collected and homogenized at the indicated timepoints. Viral titers were determined by plaque assay on MDCK cells. Additionally, weight loss (c) and survival rates (d) were daily monitored during the infection. (e) Circular bar graph comparing results obtained from infecting WT or *Rag1*^{-/-} at day 4 post infection and expressed as the relative barcode-fold increase percentage over the input. Error bars depict the standard deviation (SD).

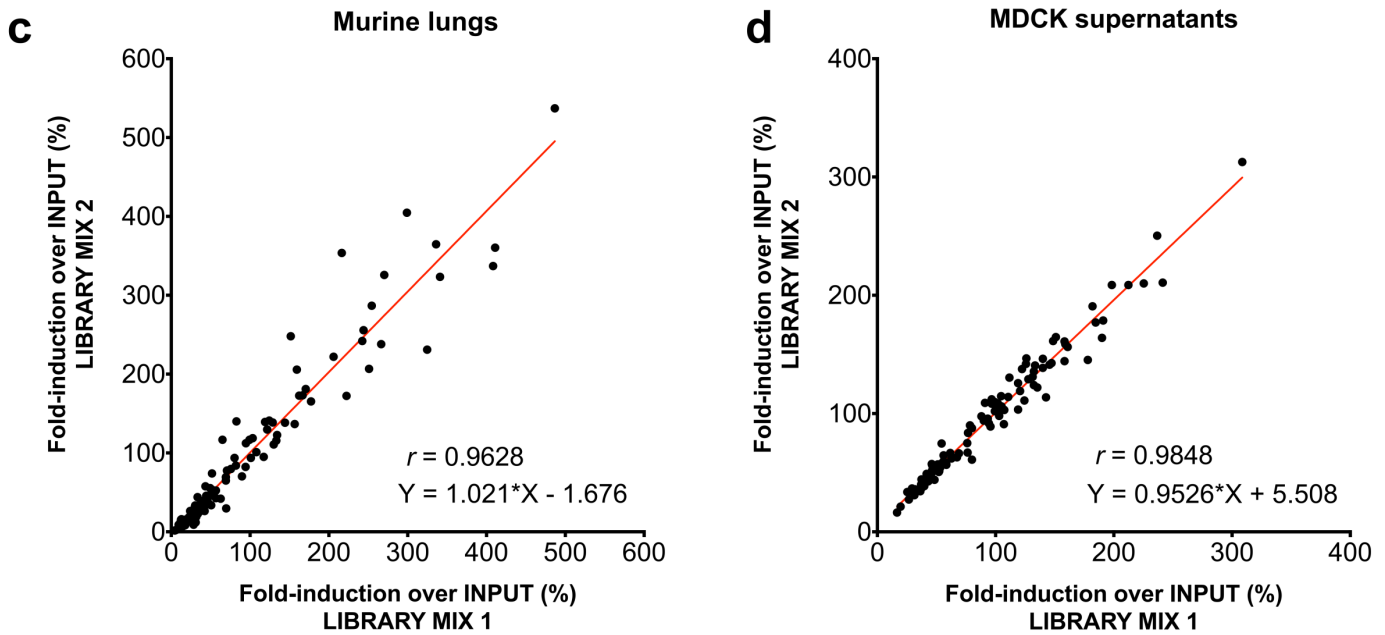
DATA S1

a



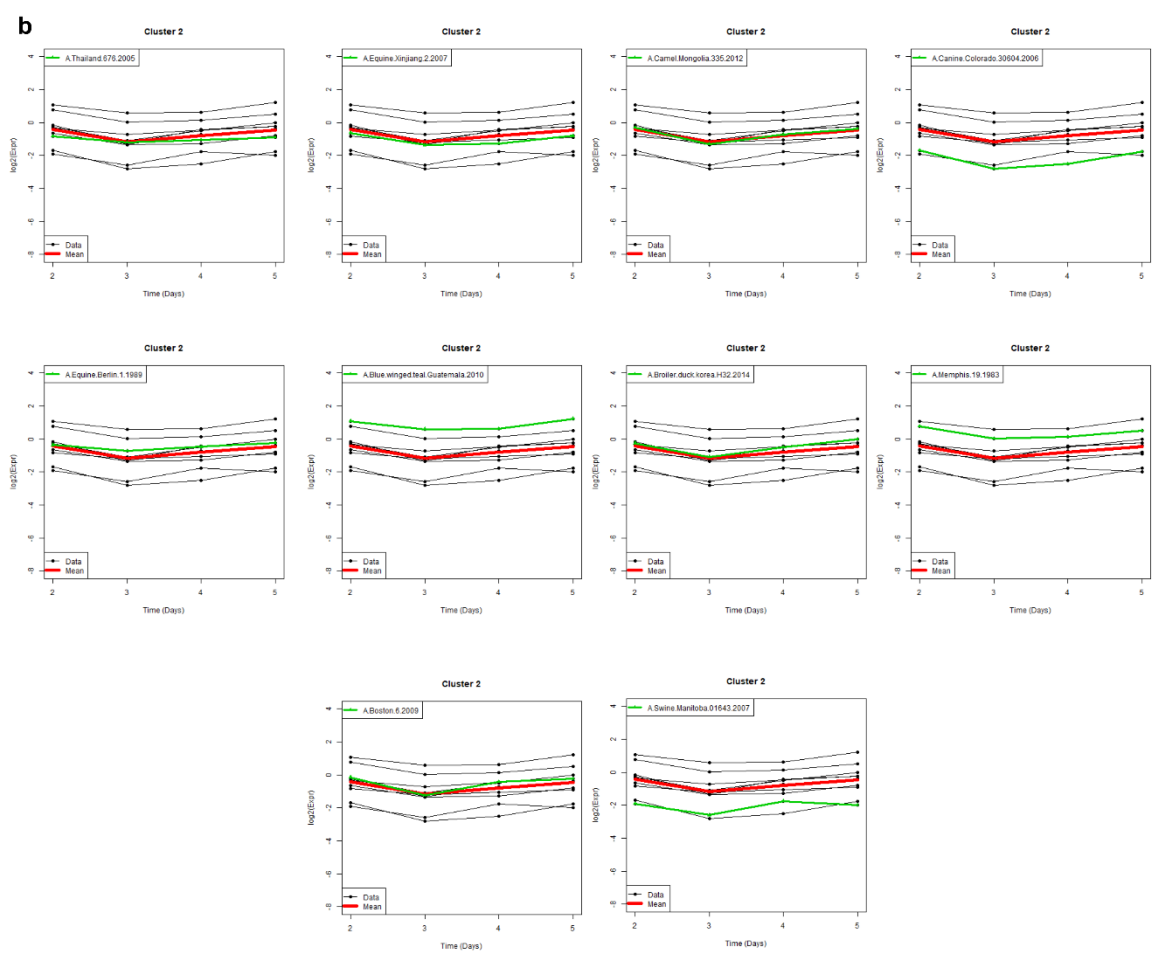
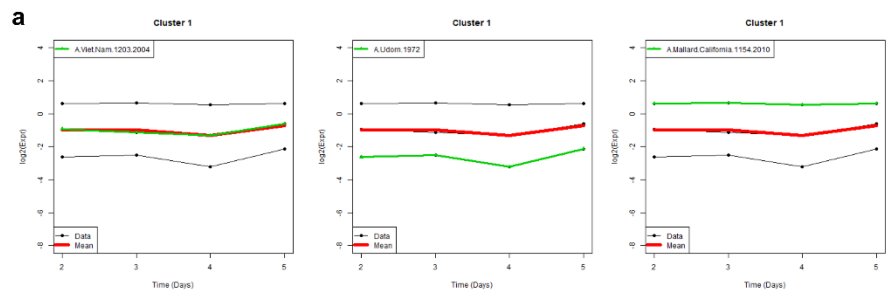
b

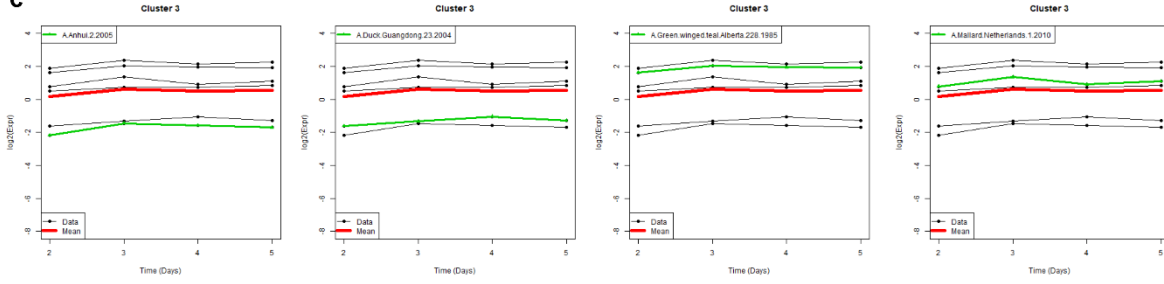
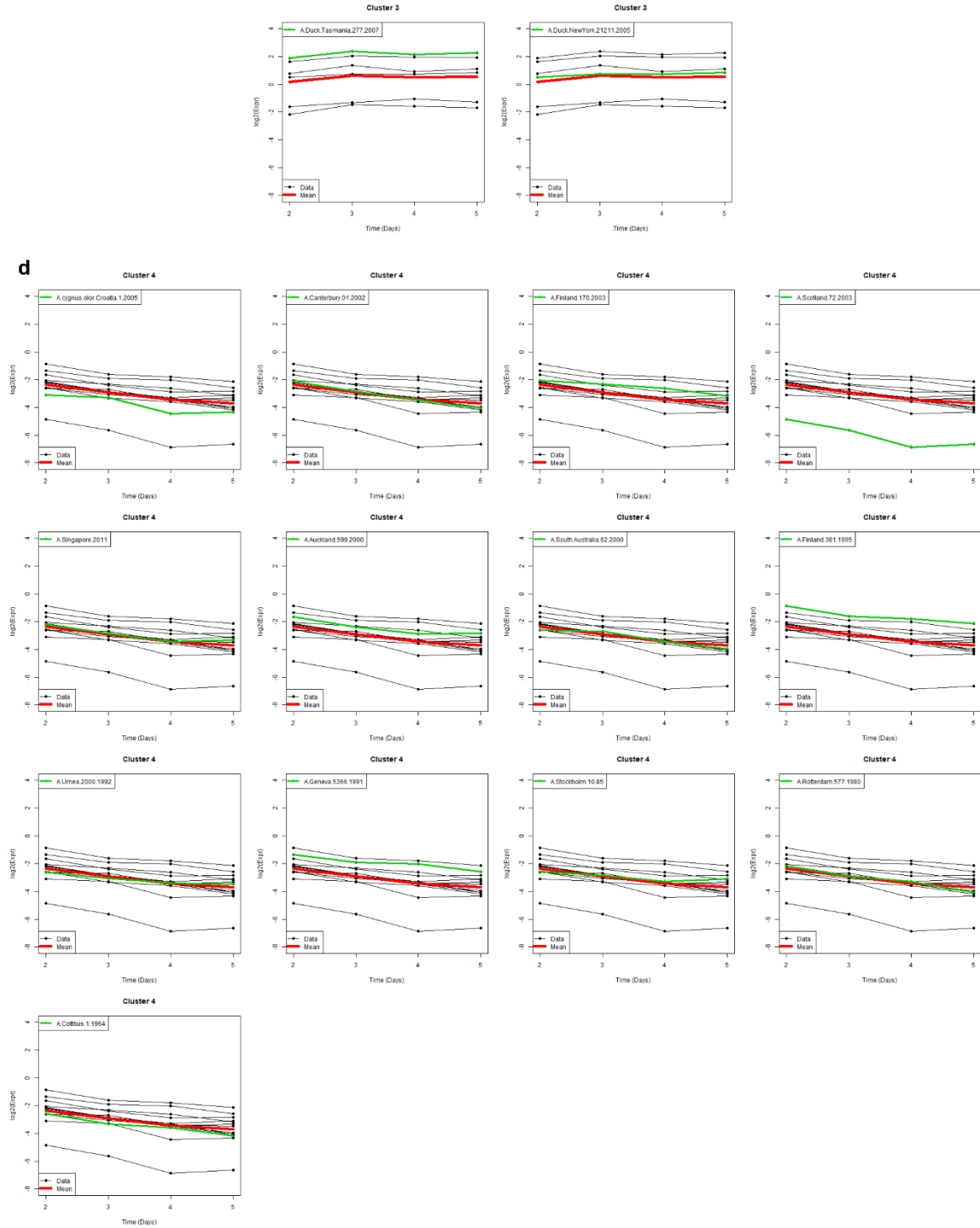


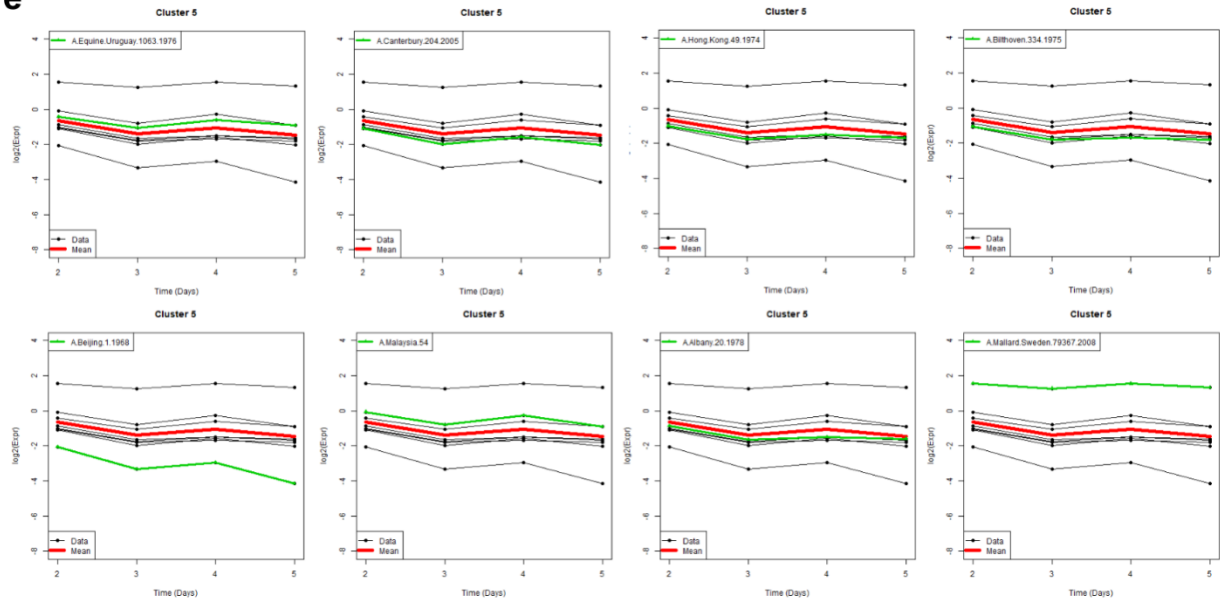
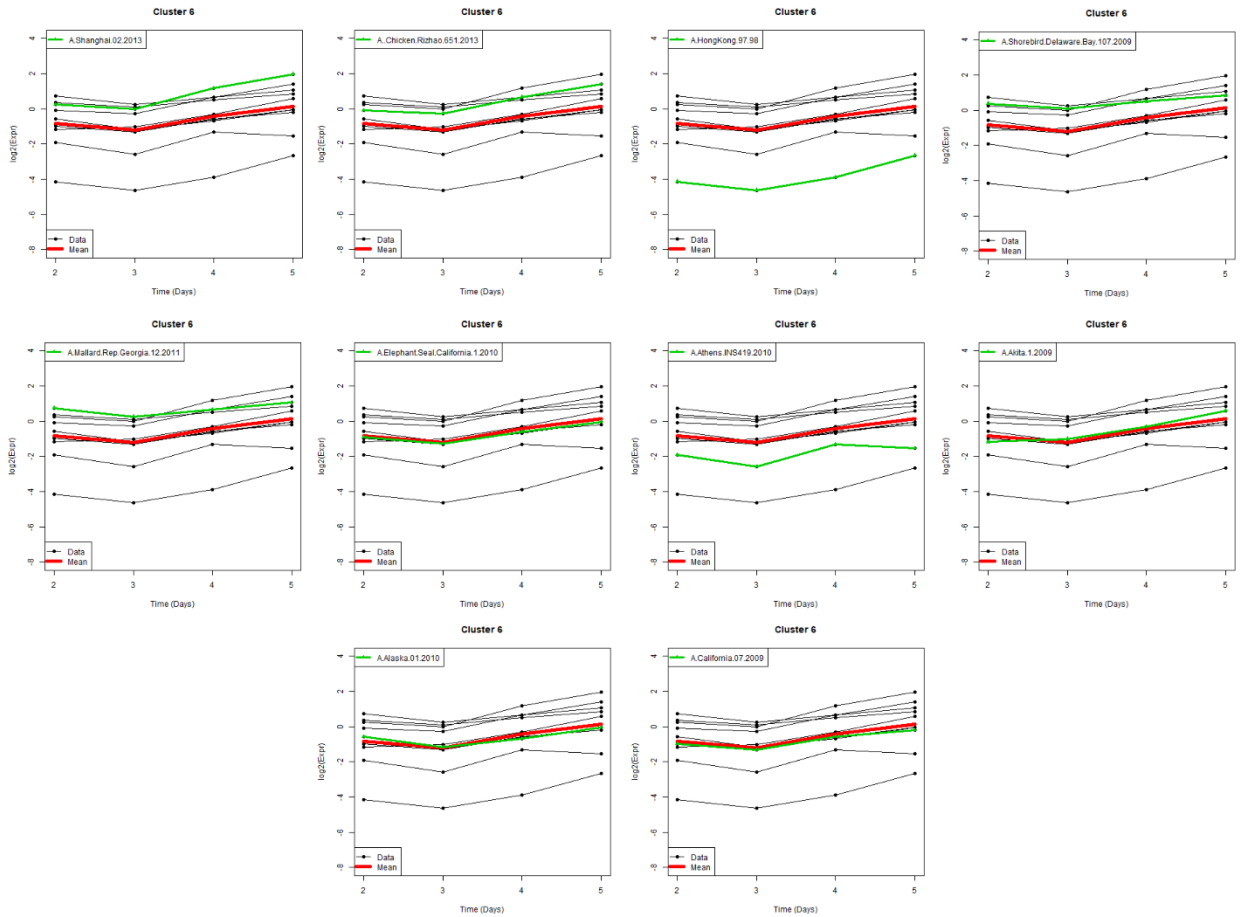


Data S1, related to Figure 2. Validating the reproducibility of our NS1 recombinant viral library *in vitro* and *in vivo*. Two independent viral library sets (100 pfu/virus) were used to infect 8-week-old female BALB/c mice (a) and MDCK cells (b) in triplicates. Relative barcode abundance was analyzed at 48 hours post-infection. Results are expressed as the fold-induction percentage over the initial relative proportion of barcode reads found in the initial viral mix (input, red dotted line). Scatter plots expressing the fold induction (%) over the input after infecting BALB/c mice (c) and MDCK cells (d) in triplicates with two different pools of library (Library Mix 1 or 2). Error bars depict the standard deviation (SD). Two-tailed paired t tests between independent library mixes were used to obtain Pearson correlation coefficients (r), with linear regression analysis.

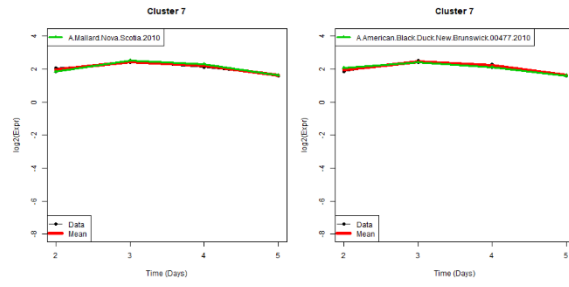
DATA S2



C**d**

e**f**

g



Data S2, related to Figure 6. Time course analysis of clusters in Figure 6. Barcode reads for each recombinant virus within clusters 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), 6 (f) and 7 (g) at days 2, 3, 4 and 5 post-infection were plotted (black arrows). Each panel highlights a data set of a particular virus (green) and the average values of the whole cluster (red).

TABLE S1

List of barcode sequences used in the viral library		
Barcode #	Sequence	NS1 strain
BC1	ATTTATATAAGCAGCTAACTGT	A/Udorn/1972
BC2	ATATTGATTAACAACTAGCCA	A/Puerto Rico/8/1934
BC3	ATAGCTTACCGGACTCGGTGCC	A/Puerto Rico/8/1934 R38A/K41A
BC4	TAAAATTGCGATTCACTGGCCT	A/Shanghai/02/2013
BC5	TACAGTTAGCATAAGATGTGAG	A/Udorn/1972
BC6	TTTGTTTATATTTAACAGTGTG	A/Puerto Rico/8/1934
BC7	TTTCAGCATGAGAATCTCCTTC	A/Puerto Rico/8/1934 R38A/K41A
BC8	CTGCTCTTTGGCTGAATGGGCC	A/Shanghai/02/2013
BC9	AATTAATTTCACTGACTTCGGC	Akita/1/2009
BC10	ATAACCTGAAAGTACATATGCT	Akita/1/2009
BC11	ATAATGTCCCTCAATGTCGGCC	A/Alaska/01/2010
BC12	ATTTGGGCTGCCATGTCCAGGA	A/Alaska/01/2010
BC13	TATACAAATTATATTATGTCCG	A/Albany/20/1978
BC14	TTCTGTTAGAAGTGCAGCATAT	A/Albany/20/1978
BC15	TTCAATCAATATCCAATTCCTC	A/Anhui/2/2005
BC16	CTTCAAAGTCTTCTAAGATCCG	A/Anhui/2/2005
BC17	AATAATCTCCACAGCCATCCAT	A/Athens/2010
BC18	TAAATCCATTATGTACTTGGTC	A/Athens/2010
BC19	TTTAACCCATTAATAATATGCA	A/Auckland/2000
BC21	TTTGGTGATGAATGACGCTGGC	A/Beijing/1/1968
BC22	ATTGATTTATTCAGTAACCGGA	A/Beijing/1/1968
BC23	AATATCAACAAGTAGTCCCGCT	A/Bilthoven/334/1975
BC24	TTAAACTTTCTGGGAGATGGCC	A/Bilthoven/334/1975
BC25	TTAAGCCAGCTAATAAAGAGTC	A/Blue winged teal/Guatemala/2010
BC26	TTATATAAACTCAGGACCGCCA	A/Blue winged teal/Guatemala/2010
BC27	TTACACCACTACATTGAAGGAT	A/Boston/6/2009
BC28	TTTGGTTTGCCTGGAGATGCCA	A/Boston/6/2009
BC31	TATATCTGTGTTTGTCCGAGAC	A/Broiler_duck/korea/2014
BC32	TTAAATTCTCCACTCAGGTGGA	A/Broiler_duck/korea/2014
BC33	ACAATTCCTTCTGCAAGCGGT	A/California/07/2009
BC34	TTCATCAAAGATATTCTTGGTG	A/California/07/2009
BC35	ATTTCAAATAACAGATTAAGTT	A/Camel/Mongolia/2012
BC36	TTCATATGGCCCGTGCTTAGCC	A/Camel/Mongolia/2012
BC37	TATAGTGATGCCGTTGTCAAGT	A/Canine/Colorado/2006
BC38	ATAACCCCTTATGTTCAAATGGA	A/Canine/Colorado/2006
BC39	TTAACTTCTCTTCTTGTAAGGC	A/Canterbury/01/2002
BC40	TTCTGTAATAATATTCCAGGCC	A/Canterbury/01/2002
BC41	AATGTTGAACACAAGTTGCTC	A/Canterbury/204/2005
BC42	TTCTTAAATAAGAATAAGAGCC	A/Canterbury/204/2005
BC43	GTCTAATACGATAATAAGCCGG	A/Moscow/2007
BC44	TTTCTCGTGATACTTCAATGGG	A/Moscow/2007
BC47	TTTGGCGATGATAATTGTAGGG	A/ Rizhao/2013
BC48	ATAACAAGAATTAGATATCTT	A/ Rizhao/2013
BC49	TTAATGGAGAGCTGGCTGGCCT	A/Cottbus/1964
BC50	TAGCTCATAGATGTAGTGTCTG	A/Cottbus/1964
BC51	TATATTTCAAGCATGACAGACCA	A/Cygnus olor/2005
BC52	TTTCTGGATGGATCACTGGGTG	A/Cygnus olor/2005

BC53	ATTCATTAATCTTTACAGTGCG	A/Fujian/2007
BC54	TATTGTGTTAATTATGGATGAA	A/Fujian/2007
BC55	TATACCTCTGAGGTTTCTTCCA	A/Guangdong/2004
BC56	TTAACGTAGACTTTCAGCTGCA	A/Guangdong/2004
BC57	ATCATCTGACTCATGATAGGTC	A/Equine/Berlin/1989
BC58	TTACTTCATTCACTTGTGTGTT	A/Equine/Berlin/1989
BC59	ATTCGTCATAATTCATGATCTT	A/Equine/Uruguay/1976
BC60	TTATTATCTTGGATAAGGAGGC	A/Equine/Uruguay/1976
BC61	TAACAGTTACTTATCTAATCCC	A/Equine/Xinjiang/2007
BC62	TTTCTGGTAATGACGAAGGGTC	A/Equine/Xinjiang/2007
BC63	TACAATCTCGATCTTACTGCGA	A/Finland/2003
BC64	CATTCATCTCCATTGCATTGGA	A/Finland/2003
BC65	TAGTGAAGCCACAGATGTA	A/Finland/95
BC66	ATATTACATAAATTTATCATGC	A/Finland/95
BC69	TAATTGTGGATGGATTGGAGAT	A/Geneva/1991
BC70	TTTCCGATCATCCTGAAGAGGC	A/Geneva/1991
BC71	TTAATTATCTTTCAAACGAGGA	A/Hong Kong/1974
BC72	ATAATCTTCTGCGAATGTGGG	A/Hong Kong/1974
BC75	TTGCTTTAAATCTCTCCTTGGA	A/Malaysia/54
BC77	TTTCTAAGTAACCAACATAGCC	A/Rep Georgia/2011
BC78	ATCTGATATGGCAATCTTTCCT	A/Rep Georgia/2011
BC79	TAAGTCCAAACTCCTTAACAGC	A/Memphis/1983
BC80	TTAATATGTCAAATCCATATTG	A/Memphis/1983
BC81	ATAACTCCTTACATTTACCCAA	A/Peru/2011
BC82	ATATGTAAACTCTAGGACGTTG	A/Peru/2011
BC83	ATAAGGTGACACAGGAACATAC	A/Rotterdam/1980
BC84	ATATCTGGAGTAAGAAACAGAG	A/Rotterdam/1980
BC85	ATTAAGCATCATAATCAGGA	A/Scotland/2003
BC86	AAGATGAGACATCTTGTAAGCA	A/Scotland/2003
BC89	TTTCCAGTCACTTTTGTCTGCT	A/Shorebird/Delaware/2009
BC90	TTAGAGTAATCTCCAATCAGCT	A/Shorebird/Delaware/2009
BC91	TTGAAATTTCTGTAGGAGATT	A/Singapore/2011
BC92	TTAGCGCTGGAGCCCAGGTGAC	A/Singapore/2011
BC93	TTACTTAAAGCCGGTGGTGCCG	A/South Australia/2000
BC94	TTCCAGTTTAGTTATCTGAGCG	A/South Australia/2000
BC95	TAATAGCTAAGTTATTACAGGC	A/Stockholm/85
BC96	TTTCAAAGCAAATTAATACAG	A/Stockholm/85
BC97	TTGCCATGGATAAGTAATTGGT	A/Manitoba/2007
BC98	TAATGCTAACAACAACAACCA	A/Manitoba/2007
BC101	ATGGAAATTACAAGTAGGAGCC	A/Thailand/2005
BC102	TTAGTTCCTGCCTGCTTGAGGT	A/Thailand/2005
BC103	TTAATAATTCATTTAGGAGCCG	A/Umea/92
BC104	AATTGTATACATCCTCCGAGGG	A/Umea/92
BC105	ATTACTTTGAGAACTTAAAGCA	A/Black Duck/New Brunswick/2010
BC106	ATAAGTTTTCGTCATAACTCGCC	A/Black Duck/New Brunswick/2010
BC107	TTCCAGGTTACATACGACTGCG	A/Duck New York/21211/2005
BC108	TAATGCCAGGTGAAATTCTCTC	A/Duck New York/21211/2005
BC109	TTGTGCCTACTGCCTCGGAATT	A/Tasmania/2007
BC110	TTATCTTCTCAGCCAGATCCGT	A/Tasmania/2007
BC111	TTCTCTTAAAGGGCAGTGTCAG	A/Duck/Yangzhou/02/2005
BC112	TATGAGAATAAGGATGAGAGGT	A/Duck/Yangzhou/02/2005
BC113	TACAATGATAAGAGAAGCACCA	A/Mallard/California/1154/2010
BC114	ATATTACAAGGCTTGAAAGGT	A/Mallard/California/1154/2010

BC115	AAGTCGCCCTACGGCGGGTGCC	A/green-winged teal/1985
BC116	TTTCATATATGGGTTCTCACAG	A/green-winged teal/1985
BC117	AATAGTCCAGAATTTCACTGGC	A/Mallard/Netherlands/2010
BC118	TTAATAGCATCGTATTTGTCCT	A/Mallard/Netherlands/2010
BC120	TTACCACAAGAAATAAGACCA	A/Mallard/Nova Scotia/2010
BC121	TTAAGATAAACAGGATTCAGCC	A/Mallard/Sweden/2008
BC123	ATAGAGGTCAGAGAGTCGTCCC	A/Elephant Seal/California/1/2010
BC124	GACATTCATAACAGCAAATGGC	A/Elephant Seal/California/1/2010
BC125	TATTAGTCATACATCTTCCTTG	A/Hong Kong/97/98
BC127	TTTCTCAAACAGCTGGTAACGC	A/Vietnam/1203/2004
BC128	AATATAAGTATCTTTAGCGGCG	A/Vietnam/1203/2004

Table S1, related to STAR Methods. List of barcode sequences used in the viral library.

TABLE S2**List of primers used for the deep-sequencing analysis**

Primer Name	Sequence
3-Ambi-NS	GATCGCTCTTCTGGGAGCaAAAGCAGGgtgac
5-SAP-NS	CATCGCTCTTCTATTAGTAGAAACAAGGgtggtt
Illumina_mir30_F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGTGAAGCCACAGATGTA
Illumina_mir30_R_1	CAAGCAGAAGACGGCATAACGAGATaaaatcGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_2	CAAGCAGAAGACGGCATAACGAGATaccgccGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_3	CAAGCAGAAGACGGCATAACGAGATacgtaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_4	CAAGCAGAAGACGGCATAACGAGATagagagGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_5	CAAGCAGAAGACGGCATAACGAGATagtagaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_6	CAAGCAGAAGACGGCATAACGAGATatgggtGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_7	CAAGCAGAAGACGGCATAACGAGATcaatggGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_8	CAAGCAGAAGACGGCATAACGAGATcataatGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_9	CAAGCAGAAGACGGCATAACGAGATcgctaaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_10	CAAGCAGAAGACGGCATAACGAGATcggcgcGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_11	CAAGCAGAAGACGGCATAACGAGATctgacgGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_12	CAAGCAGAAGACGGCATAACGAGATgattcaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_13	CAAGCAGAAGACGGCATAACGAGATgcctgtGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_14	CAAGCAGAAGACGGCATAACGAGATgcccagGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_15	CAAGCAGAAGACGGCATAACGAGATgtacgaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_16	CAAGCAGAAGACGGCATAACGAGATgtcaacGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_17	CAAGCAGAAGACGGCATAACGAGATtaccttGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_18	CAAGCAGAAGACGGCATAACGAGATtaggacGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_19	CAAGCAGAAGACGGCATAACGAGATtcaacaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA
Illumina_mir30_R_20	CAAGCAGAAGACGGCATAACGAGATtctgtgGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCAAAGTGATTTAATTTATACCATTTTA

Table S2, related to STAR Methods. List of primers used for the deep-sequencing analysis.