

# **Incorporation of the influenza A virus NA segment into virions does not require cognate non-coding sequences.**

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## Supplementary figure legends

### Supplementary Figure S1. Incorporation of the different X-NA-X virus segments. (a)

Incorporation of the eight segments at passage 10. (b) For **PB1-NA-PB1** and **HA-NA-HA** viruses, incorporation of the eight segments at passage 20. For (a) and (b), viral RNAs were extracted from the supernatants collected at day three post infection and the level of each segment was evaluated by specific two-step RT-qPCR<sup>19</sup>. Results were expressed as relative amounts calculated using the  $2^{-\Delta\Delta C_t}$  method with **NA-NA-NA** as virus reference and segment M as segment reference, except for segment M quantification where segment NP was used as segment reference. Results for segment NA are already presented in Fig. 2.

### Supplementary Figure S2. Effects of mutations identified in PB1-NA-PB1 virus on virus

**fitness and segment 6 incorporation.** (a-b) Growth kinetics. Infections were performed in MDCK cells at a low m.o.i. ( $10^{-5}$  pfu/cell). Titres were determined by standard plaque assays at indicated time points. Results correspond to the mean of at least two independent experiments. (c) Incorporation of segment 6. Viral RNAs were extracted from the supernatants collected at three days post infection of the growth kinetics and the NA segment vRNA level was evaluated by specific two-step RT-qPCR<sup>19</sup>. Results were expressed as relative amounts calculated using the  $2^{-\Delta\Delta C_t}$  method with **NA-NA-NA** as virus reference and segment M as segment reference. Each bar represents the range (minimum - maximum) of the values obtained from the growth kinetics. Symbols used in the growth kinetics graphs are presented next to the name of each virus in panel c. (d) Infections in A549 cells at a low m.o.i. ( $10^{-4}$  pfu/cell). Supernatants were collected at three days p.i. Left panel: viral titres. Right panel: segment 6 incorporation.

### Supplementary Figure S3. Effects of additional mutations identified in PB2-NA-PB2 virus

**on virus fitness and segment 6 incorporation.** (a-b) Growth kinetics. Infections were performed

in MDCK cells at a low m.o.i. ( $10^{-5}$  pfu/cell). Titres were determined by standard plaque assays at indicated time points. Results correspond to the mean of at least two independent experiments. (c) Incorporation of segment 6. Viral RNAs were extracted from the supernatants collected at three days post infection of the growth kinetics and the NA segment vRNA level was evaluated by specific two-step RT-qPCR <sup>19</sup>. Results were expressed as relative amounts calculated using the  $2^{-\Delta\Delta C_t}$  method with NA-NA-NA as virus reference and segment M as segment reference. Each bar represents the range (minimum - maximum) of the values obtained from the growth kinetics. Symbols used in the growth kinetics graphs are presented next to the name of each virus in panel c. (d) Infections in A549 cells at a low m.o.i. ( $10^{-4}$  pfu/cell). Supernatants were collected at three days p.i. Left panel: viral titres. Right panel: segment 6 incorporation.

**Supplementary Figure S4. Effects of additional mutations identified in PA-NA-PA virus on virus fitness and segment 6 incorporation.** (a-b) Growth kinetics. Infections were performed in MDCK cells at a low m.o.i. ( $10^{-5}$  pfu/cell). Titres were determined by standard plaque assays at indicated time points. Results correspond to the mean of at least two independent experiments. (c) Incorporation of segment 6. Viral RNAs were extracted from the supernatants collected at three days post infection of the growth kinetics and the NA segment vRNA level was evaluated by specific two-step RT-qPCR <sup>19</sup>. Results were expressed as relative amounts calculated using the  $2^{-\Delta\Delta C_t}$  method with NA-NA-NA as virus reference and segment M as segment reference. Each bar represents the range (minimum - maximum) of the values obtained from the growth kinetics. Symbols used in the growth kinetics graphs are presented next to the name of each virus in panel c. (d) Infections in A549 cells at a low m.o.i. ( $10^{-4}$  pfu/cell). Supernatants were collected at three days p.i. Left panel: viral titres. Right panel: segment 6 incorporation.

**Supplementary Figure S5. Effects of additional mutations identified in NS-NA-NS virus on virus fitness and segment 6 incorporation.** (a) Growth kinetics. Infections were performed in MDCK cells at a low m.o.i. ( $10^{-5}$  pfu/cell). Titres were determined by standard plaque assays at indicated time points. Results correspond to the mean of at least two independent experiments. (b) Incorporation of segment 6. Viral RNAs were extracted from the supernatants collected at three days post infection of the growth kinetics and the NA segment vRNA level was evaluated by specific two-step RT-qPCR <sup>19</sup>. Results were expressed as relative amounts calculated using the  $2^{-\Delta\Delta C_t}$  method with NA-NA-NA as virus reference and segment M as segment reference. Each bar represents the range (minimum - maximum) of the values obtained from the growth kinetics. Symbols used in panel a are presented next to the name of each virus in panel b. (c) Infections in A549 cells at a low m.o.i. ( $10^{-4}$  pfu/cell). Supernatants were collected at three days p.i. Left panel: viral titres. Right panel: segment 6 incorporation.

**Supplementary Figure S6. Effects of additional mutations identified in M-NA-M virus on virus fitness and segment 6 incorporation.** (a) Growth kinetics. Infections were performed in MDCK cells at a low m.o.i. ( $10^{-5}$  pfu/cell). Titres were determined by standard plaque assays at indicated time points. Results correspond to the mean of at least two independent experiments. (b) Incorporation of segment 6. Viral RNAs were extracted from the supernatants collected at three days post infection of the growth kinetics and the NA segment vRNA level was evaluated by specific two-step RT-qPCR <sup>19</sup>. Results were expressed as relative amounts calculated using the  $2^{-\Delta\Delta C_t}$  method with NA-NA-NA as virus reference and segment M as segment reference. Each bar represents the range (minimum - maximum) of the values obtained from the growth kinetics. Symbols used in the growth kinetics graphs are presented next to the name of each virus in panel b. (c) Infections in A549 cells at a low m.o.i. ( $10^{-4}$  pfu/cell). Supernatants were collected at three days p.i. Left panel: viral titres. Right panel: segment 6 incorporation.

**Supplementary Figure S7. Effects of additional mutations identified in NP-NA-NP virus on virus fitness and segment 6 incorporation.** (a-b) Growth kinetics. Infections were performed in MDCK cells at a low m.o.i. ( $10^{-5}$  pfu/cell). Titres were determined by standard plaque assays at indicated time points. Results correspond to the mean of at least two independent experiments. (c) Incorporation of segment 6. Viral RNAs were extracted from the supernatants collected at three days post infection of the growth kinetics and the NA segment vRNA level was evaluated by specific two-step RT-qPCR <sup>19</sup>. Results were expressed as relative amounts calculated using the  $2^{-\Delta\Delta Ct}$  method with **NA-NA-NA** as virus reference and segment M as segment reference. Each bar represents the range (minimum - maximum) of the values obtained from the growth kinetics. Symbols used in the growth kinetics graphs are presented next to the name of each virus in panel c. (d) Infections in A549 cells at a low m.o.i. ( $10^{-4}$  pfu/cell). Supernatants were collected at three days p.i. Left panel: viral titres. Right panel: segment 6 incorporation.

**Supplementary Figure S8. NA protein expression level evaluated by Western-Blot using chemiluminescence acquisition with a G. Box (Syngene).** (a) Original and additional mutant constructs tested by functional vRNP transient reconstitution assays. Twenty-four hours after transfection, 293T cell lysates were analysed for viral NA protein and for GAPDH as cellular control. (b) MDCK infections were performed with viruses rescued by reverse genetics. Upper part of b: **PB2-NA-PB2** and **PA-NA-PA** constructs, as well as **NA-NA-NA**, were tested at high m.o.i. (5 pfu/cell) and cell lysates were analysed at eight hours after infection for viral NA and NS1 proteins and for  $\beta$ -actin as cellular control. Lower part of b: **M-NA-M**, **NP-NA-NP**, and **NA-NA-NA** constructs were tested at low m.o.i. ( $10^{-2}$  pfu/cell) and cell lysates were analysed at 24 hours after infection for viral NA and NS1 proteins and for  $\beta$ -actin as cellular control.

**Supplementary Figure S9. Effects of a shorter 5'NC region in NA-NA-NA virus on virus**

**fitness and segment 6 incorporation.** (a) Growth kinetics. Infections were performed in MDCK

cells at a low m.o.i. ( $10^{-5}$  pfu/cell). Titres were determined by standard plaque assays at indicated

time points. Results correspond to the mean of at least two independent experiments. (b)

Incorporation of segment 6. Viral RNAs were extracted from the supernatants collected at three

days post infection of the growth kinetics and the NA segment vRNA level was evaluated by

specific two-step RT-qPCR<sup>19</sup>. Results were expressed as relative amounts calculated using the  $2^{-\Delta\Delta C_t}$

method with NA-NA-NA as virus reference and segment M as segment reference. Each bar

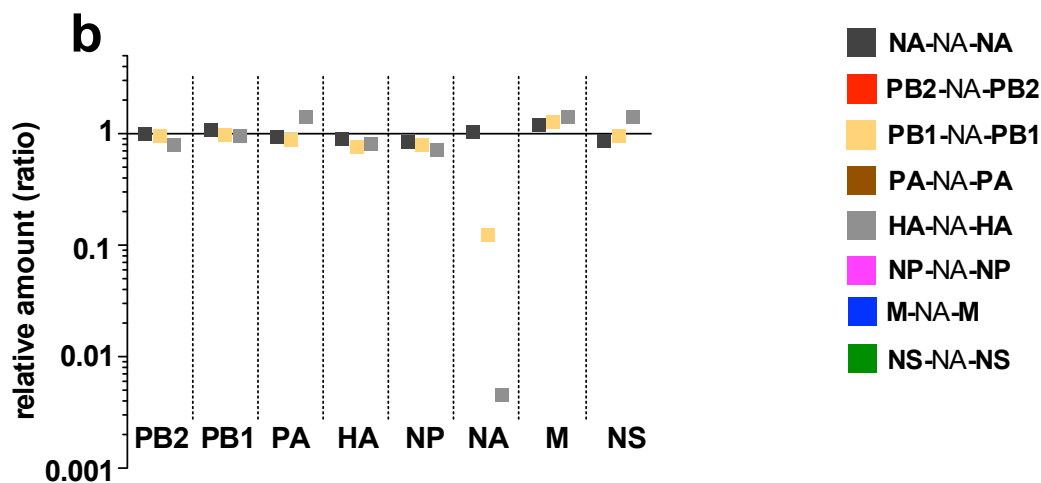
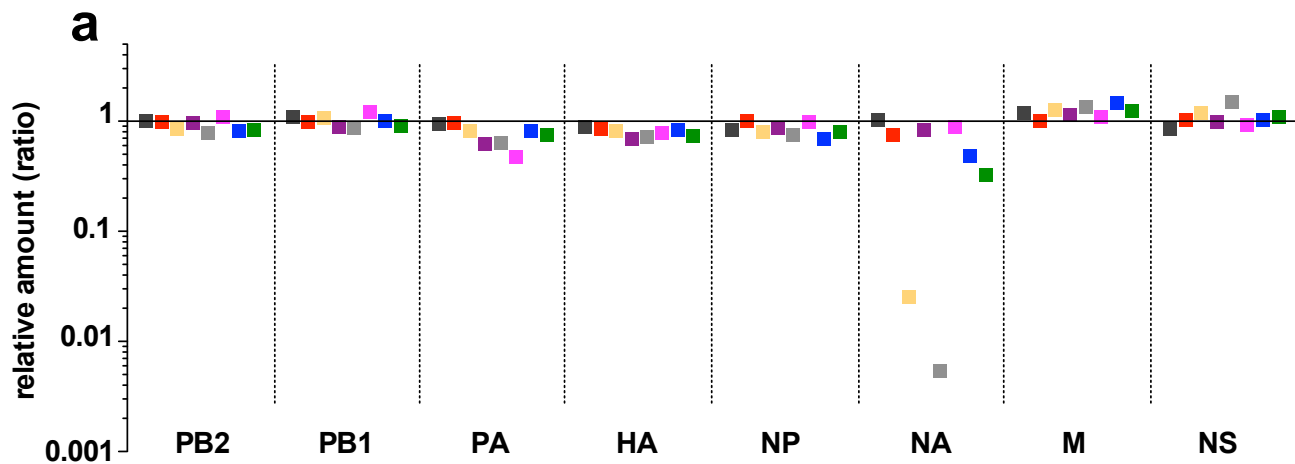
represents the range (minimum - maximum) of the values obtained from the growth kinetics.

Symbols used in the growth kinetics graphs are presented next to the name of each virus in panel

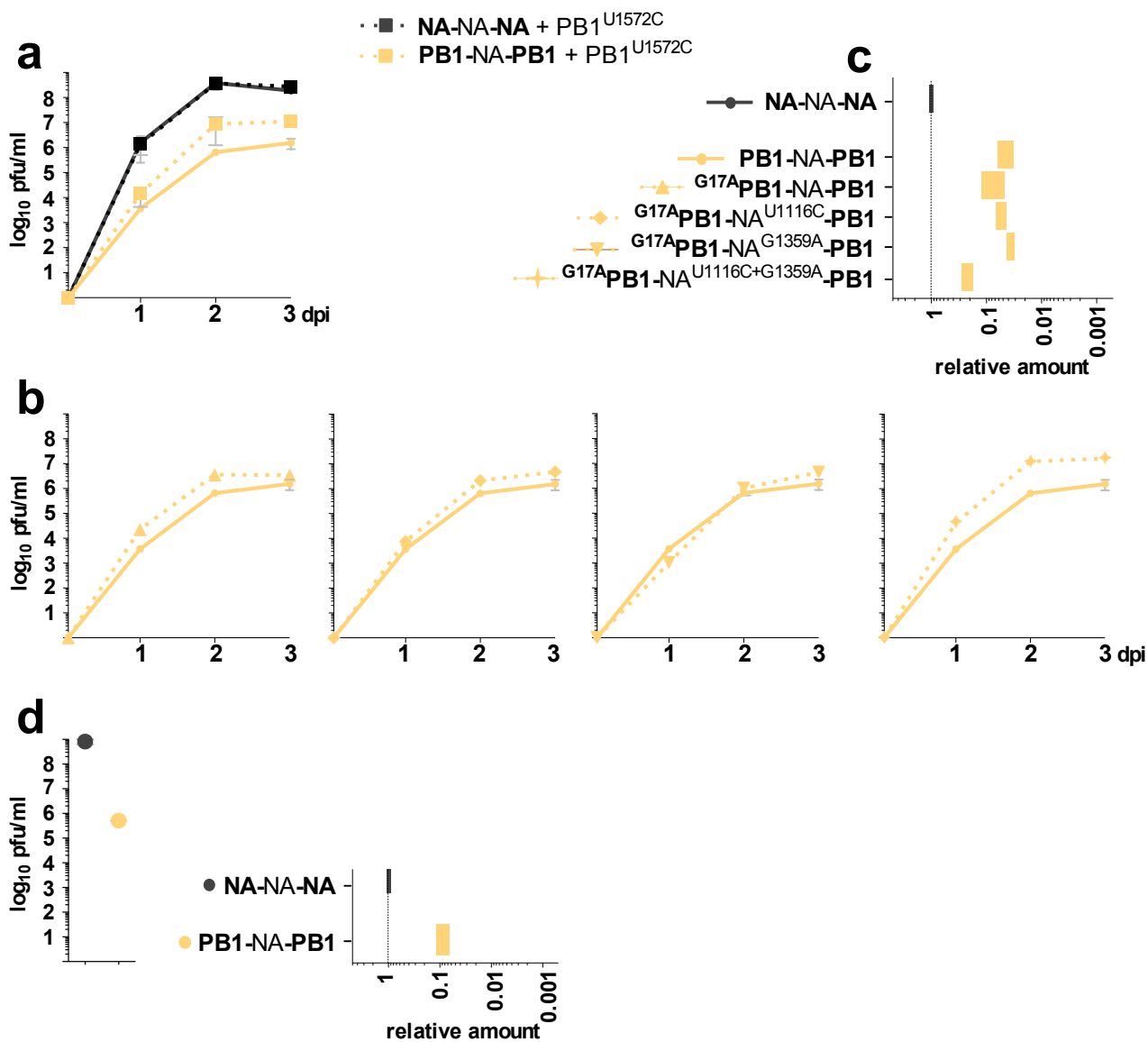
b. The stop codon of the NA-NA-NA<sup>20nt</sup> was modified to respect the polyU sequence (see

Supplementary Table S1). (c) Infections in A549 cells at a low m.o.i. ( $10^{-4}$  pfu/cell). Supernatants

were collected at three days p.i. Left panel: viral titres. Right panel: segment 6 incorporation.

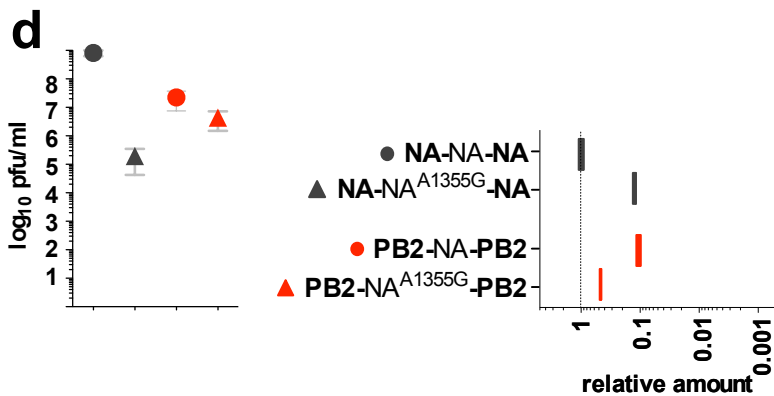
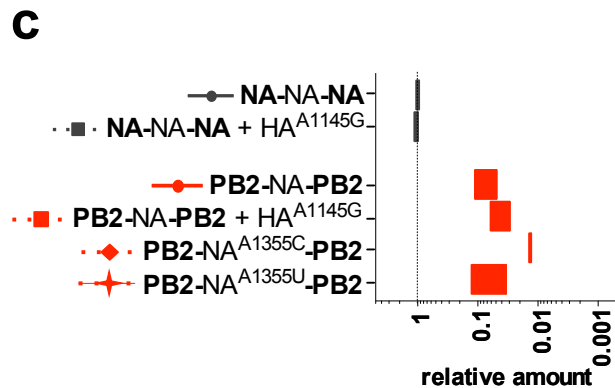
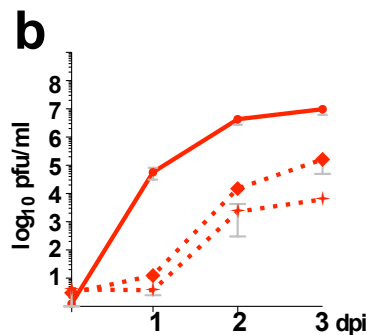
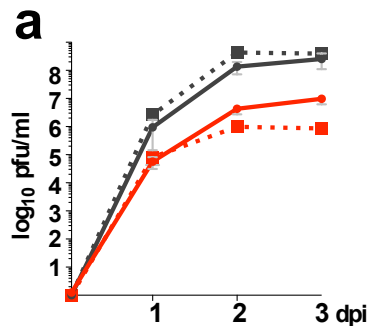


Supplementary Figure S1.

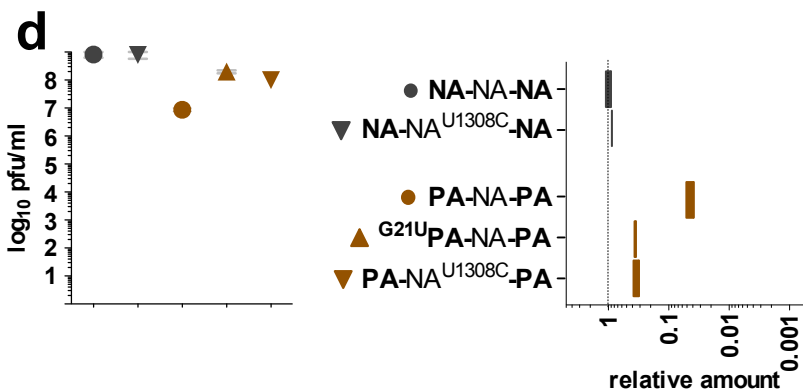
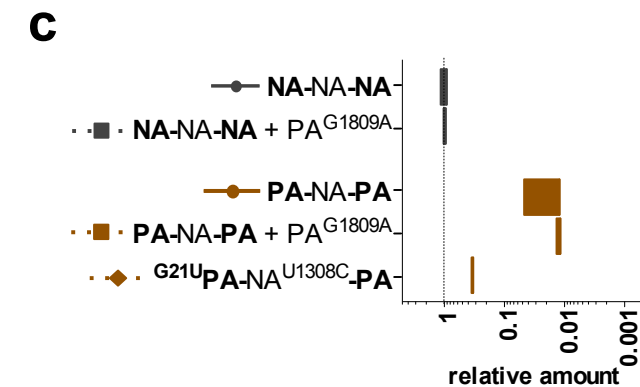
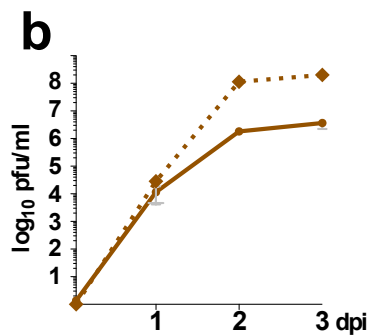
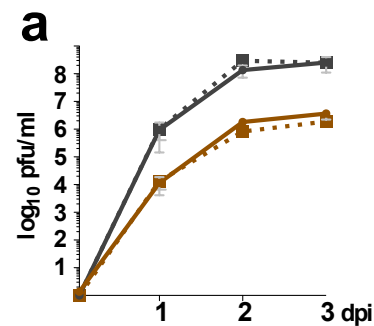


Supplementary Figure S2.

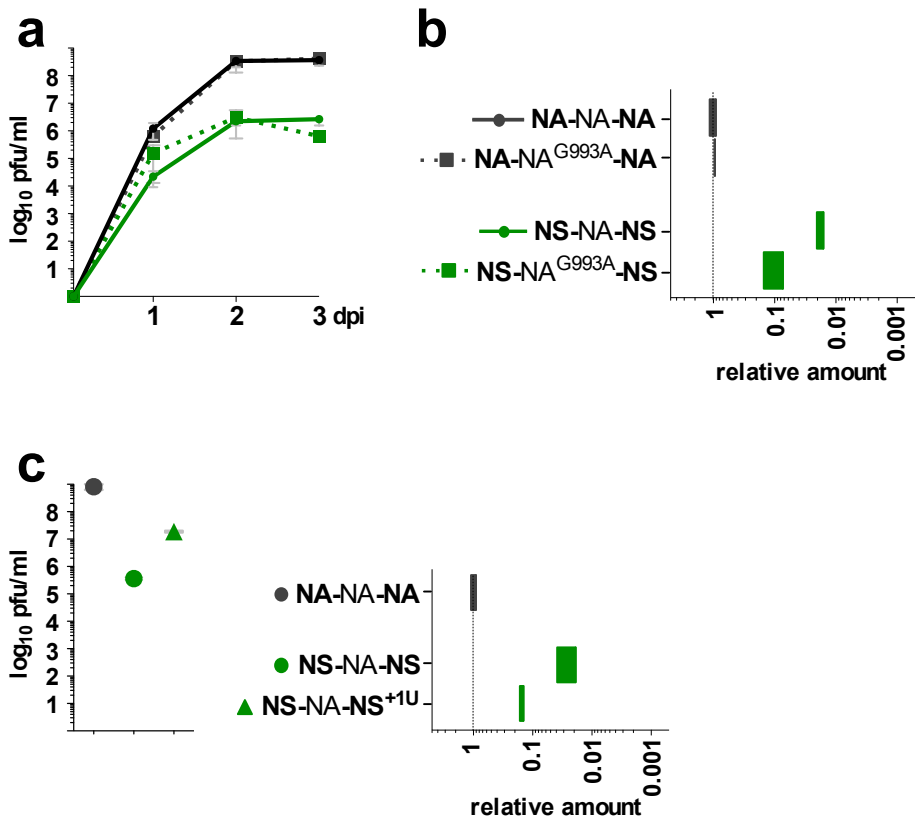




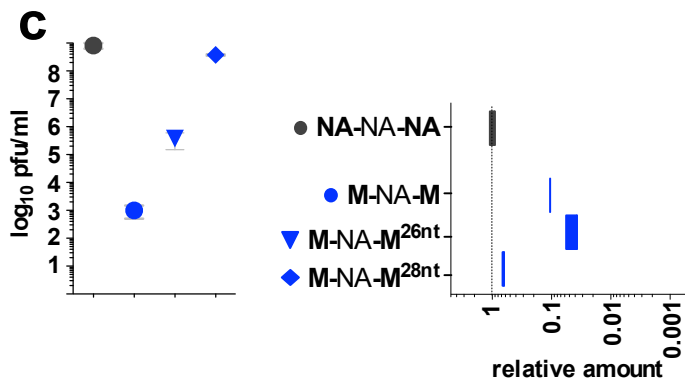
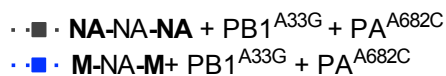
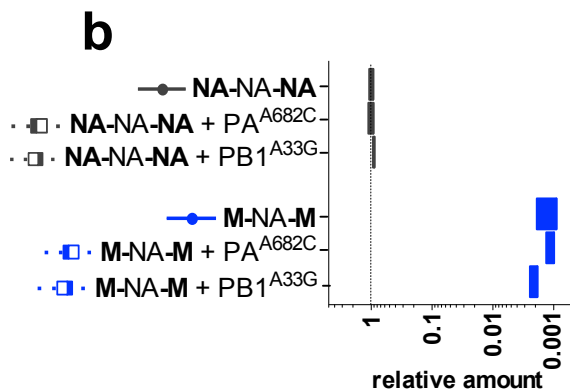
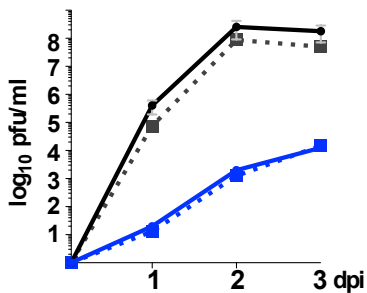
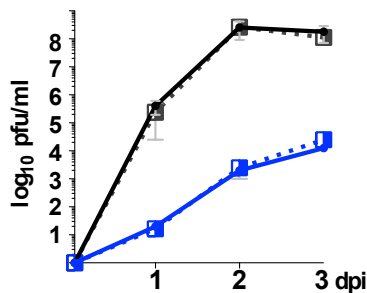
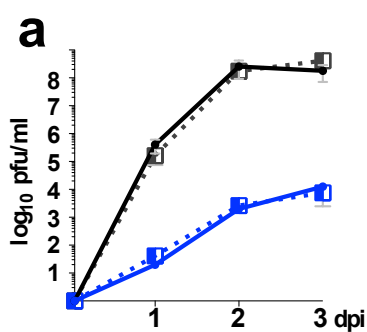
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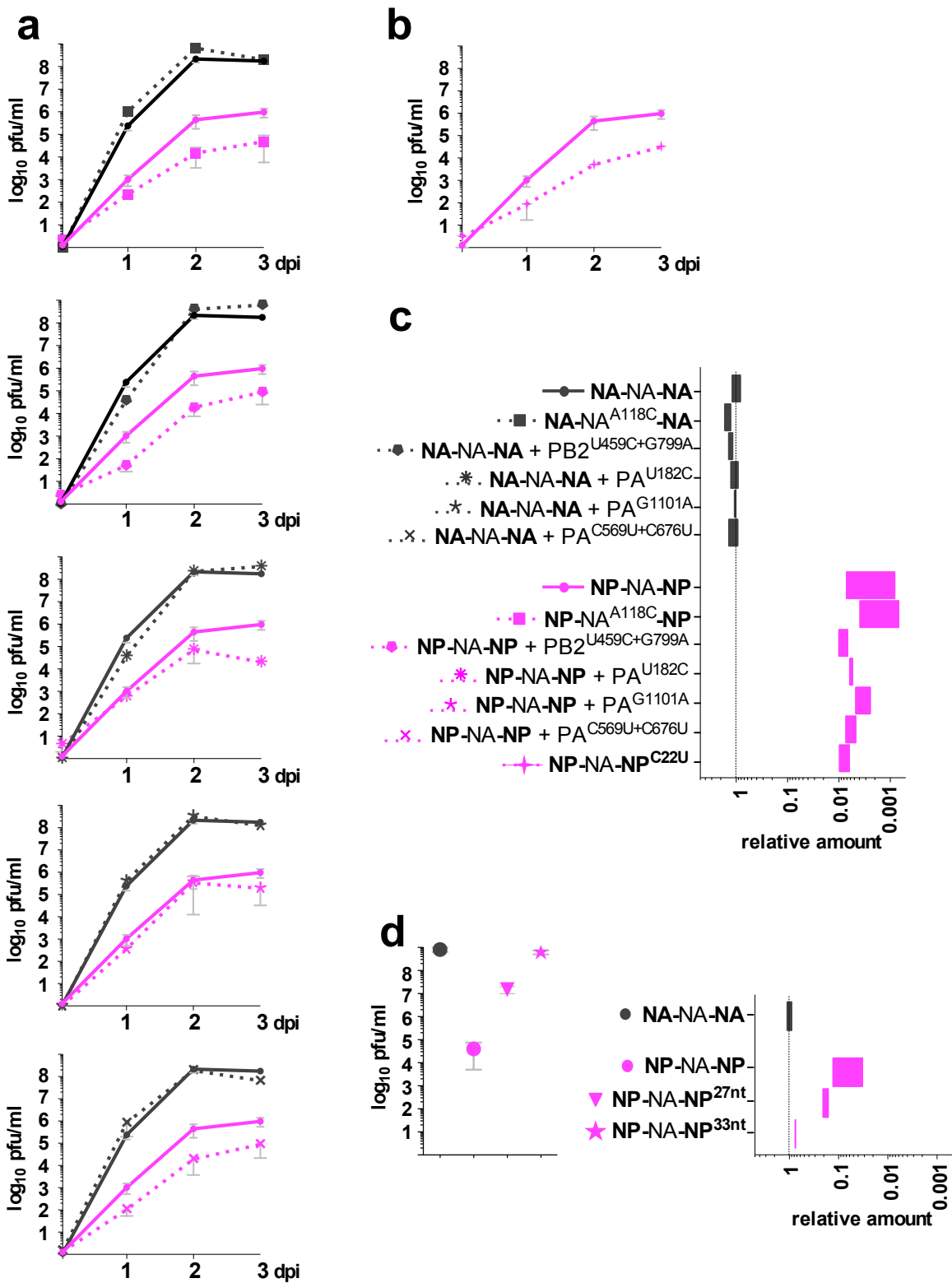
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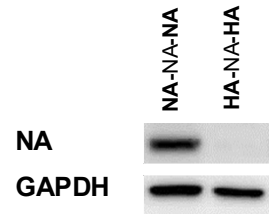
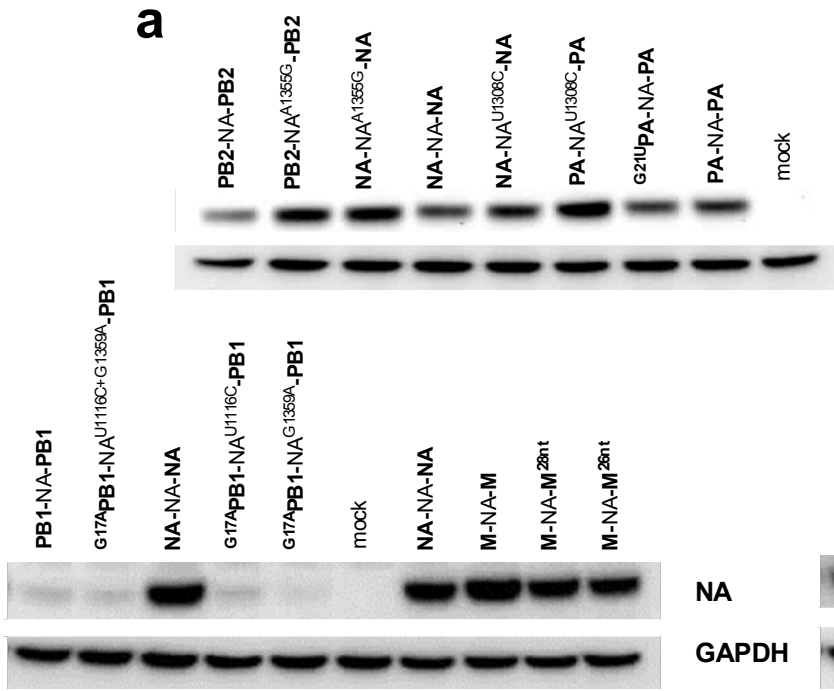
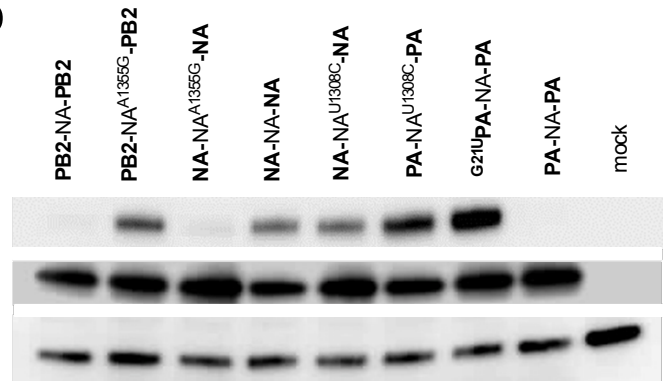
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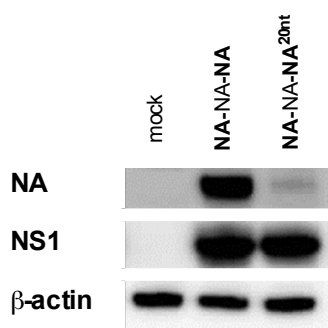
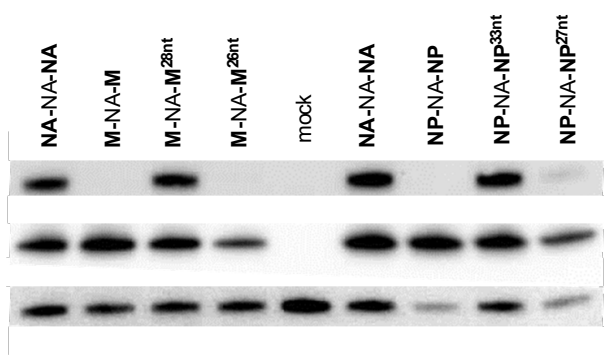
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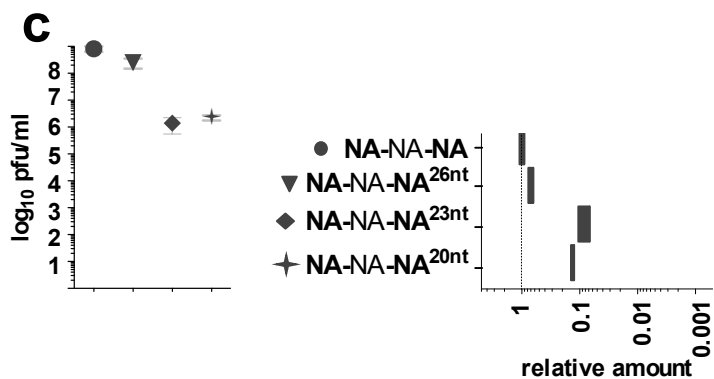
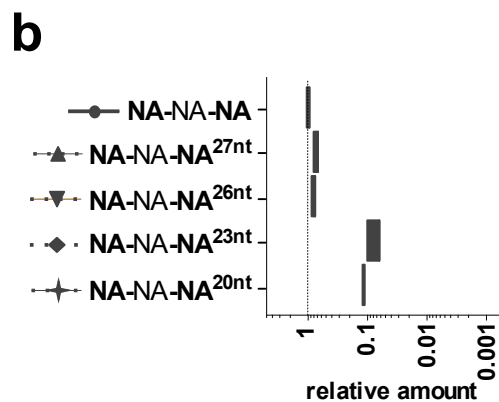
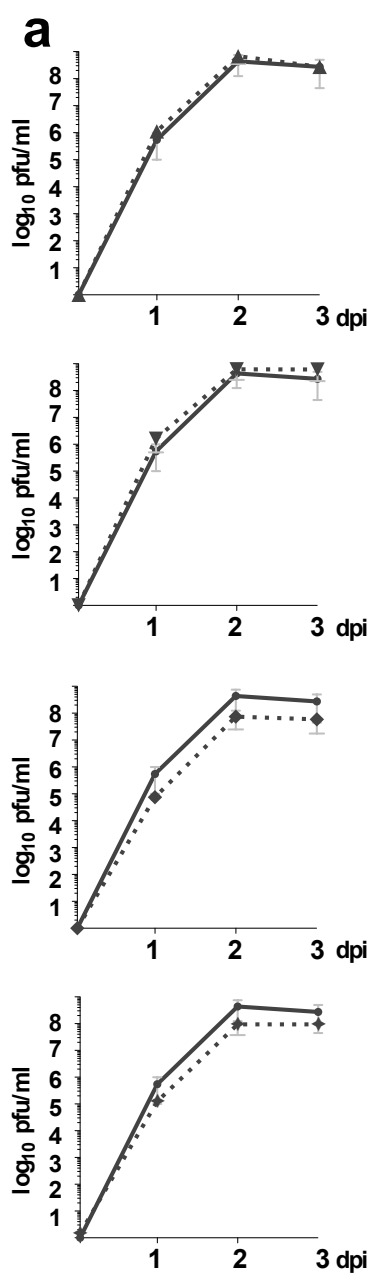
Supplementary Figure S7.

**a****b**

NA  
NS1  
beta-actin



Supplementary Figure S8.



Supplementary Figure S9.

**Supplementary Table S1: Genomic vRNA sequences of the 5' and 3' NC regions and mutations introduced in the X-NA-X viruses produced by reverse genetics**

3'X-NA-XS <sup>a</sup> reverse genetics viruses	vRNA 5'NC length (nt)	vRNA 5'NC sequence <sup>b</sup>	NA coding sequence <sup>c</sup> nt (aa)	vRNA 3'NC sequence <sup>b</sup>	vRNA 3'NC length (nt)	coding sequence <sup>d</sup> segment-nt (aa)
PB2-NA-PB2	34	AGUGAAACAAGGUCUUUUUAAACUAUUCGACACUA		CAUUAUGAAUAUAUUGACUCGCUUUUCGCU	27	
PB2-NA-PB2	34	AGUGAAACAAGGUCUUUUUAAACUAUUCGACACUA		CAUUAUGAAUAUAUUGACUCGCUUUUCGCU	27	HA-A1145G (K382R)
PB2-NA <sup>A1359G</sup> -PB2	34	AGUGAAACAAGGUCUUUUUAAACUAUUCGACACUA	NA-A1355G (D452G)	CAUUAUGAAUAUAUUGACUCGCUUUUCGCU	27	
PB2-NA <sup>A1359C</sup> -PB2	34	AGUGAAACAAGGUCUUUUUAAACUAUUCGACACUA	NA-A1355C (D452A)	CAUUAUGAAUAUAUUGACUCGCUUUUCGCU	27	
PB2-NA <sup>A1359U</sup> -PB2	34	AGUGAAACAAGGUCUUUUUAAACUAUUCGACACUA	NA-A1355U (D452U)	CAUUAUGAAUAUAUUGACUCGCUUUUCGCU	27	
NA-NA <sup>A1355G</sup> -NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA	NA-A1355G (D452G)	CAUUUAAACUCUCGCUUUUCGCU	19	
NA-NA-NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	HA-A1145G (K382R)
PB1-NA-PB1	43	AGUGAAACAAGGCACUUUUUUCACUAGAGGACAAGCUAAAUUCAUA		CAUUCAAAGGCUUUGCUCGCUUUUCGCU	24	
PB1-NA-PB1	43	AGUGAAACAAGGCACUUUUUUCACUAGAGGACAAGCUAAAUUCAUA		CAUUCAAAGGCUUUGCUCGCUUUUCGCU	24	PB1-U1572C (-)
G1179-PB1-NA-PB1	43	AGUGAAACAAGGCACUUUUUUCACUAGAGGACAAGCUAAAUUCAUA		CAUUCAAAGGCUUUGCUCGCUUUUCGCU	24	
G1179-PB1-NA <sup>G1194C</sup> -PB1	43	AGUGAAACAAGGCACUUUUUUCACUAGAGGACAAGCUAAAUUCAUA	NA-U1116C (-)	CAUUCAAAGGCUUUGCUCGCUUUUCGCU	24	
G1179-PB1-NA <sup>G1198A</sup> -PB1	43	AGUGAAACAAGGCACUUUUUUCACUAGAGGACAAGCUAAAUUCAUA	NA-G1359A (-)	CAUUCAAAGGCUUUGCUCGCUUUUCGCU	24	
G1179-PB1-NA <sup>G1198C-G1359A</sup> -PB1	43	AGUGAAACAAGGCACUUUUUUCACUAGAGGACAAGCUAAAUUCAUA	NA-U1116C (-) + NA-G1359A (-)	CAUUCAAAGGCUUUGCUCGCUUUUCGCU	24	
NA-NA-NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	PB1-U1572C (-)
PA-NA-PA	58	AGUGAAACAAGGUACUUUUUUGGACAGUUGGAUAGCAAUAUGAGCAUUGCCACAAACUA		CAUUUUUGAAACAGUACCGUCUUUCGCU	24	
PA-NA-PA	58	AGUGAAACAAGGUACUUUUUUGGACAGUUGGAUAGCAAUAUGAGCAUUGCCACAAACUA		CAUUUUUGAAACAGUACCGUCUUUCGCU	24	PA-G1809A (-)
G2120-PA-NA-PA	58	AGUGAAACAAGGUACUUUUUUGGACAGUUGGAUAGCAAUAUGAGCAUUGCCACAAACUA		CAUUUUUGAAACAGUACCGUCUUUCGCU	24	
PA-NA <sup>A1308R</sup> -PA	58	AGUGAAACAAGGUACUUUUUUGGACAGUUGGAUAGCAAUAUGAGCAUUGCCACAAACUA	NA-U1308C (-)	CAUUUUUGAAACAGUACCGUCUUUCGCU	24	
G2120-PA-NA <sup>A1308R</sup> -PA	58	AGUGAAACAAGGUACUUUUUUGGACAGUUGGAUAGCAAUAUGAGCAUUGCCACAAACUA	NA-U1308C (-)	CAUUUUUGAAACAGUACCGUCUUUCGCU	24	
PA-NA <sup>A1308R</sup> -NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA	NA-U1308C (-)	CAUUUAAACUCUCGCUUUUCGCU	19	
NA-NA-NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	PA-G1809A (-)
HA-NA-HA	45	AGUGAAACAAGGGUGUUUUUCCUUAUAUUCUGAAAUCCUAUUCUA		CAUUUUUGGUUUUUUAUUUUCUCCCGCUUUUCGCU	32	
NP-NA-NP	23	AGUGAAACAAGGUAUUUUUUCUA		CADGAUUUCGAUGUCACUCUGUGAGUGAUAUAUACUACCCGCUUUUCGCU	45	
NP-NA-NP	23	AGUGAAACAAGGUAUUUUUUCUA		CADGAUUUCGAUGUCACUCUGUGAGUGAUAUAUACUACCCGCUUUUCGCU	45	PB2-U459C (-) + G799A (V267I)
NP-NA-NP	23	AGUGAAACAAGGUAUUUUUUCUA		CADGAUUUCGAUGUCACUCUGUGAGUGAUAUAUACUACCCGCUUUUCGCU	45	PA-U182C (I61T)
NP-NA-NP	23	AGUGAAACAAGGUAUUUUUUCUA		CADGAUUUCGAUGUCACUCUGUGAGUGAUAUAUACUACCCGCUUUUCGCU	45	PA-G1101A (-)
NP-NA-NP <sup>C22U</sup>	23	AGUGAAACAAGGUAUUUUUUCUA		CADGAUUUCGAUGUCACUCUGUGAGUGAUAUAUACUACCCGCUUUUCGCU	45	PA-C569U (S190F) + C676U (L226F)
NP-NA-NP <sup>C22U</sup>	23	AGUGAAACAAGGUAUUUUUUCUA		CADGAUUUCGAUGUCACUCUGUGAGUGAUAUAUACUACCCGCUUUUCGCU	45	
NP-NA <sup>A118C</sup> -NP	23	AGUGAAACAAGGUAUUUUUUCUA	NA-A118C (T40P)	CADGAUUUCGAUGUCACUCUGUGAGUGAUAUAUACUACCCGCUUUUCGCU	45	
NP-NA-NP <sup>24nt</sup>	24	AGUGAAACAAGGUAUUUUUUCUUCUA		CADGAUUUCGAUGUCACUCUGUGAGUGAUAUAUACUACCCGCUUUUCGCU	45	
NP-NA-NP <sup>27nt</sup>	27	AGUGAAACAAGGUAUUUUUUCUUCUCUA		CADGAUUUCGAUGUCACUCUGUGAGUGAUAUAUACUACCCGCUUUUCGCU	45	
NP-NA-NP <sup>28nt</sup>	28	AGUGAAACAAGGUAUUUUUUCUUCUUCUA		CADGAUUUCGAUGUCACUCUGUGAGUGAUAUAUACUACCCGCUUUUCGCU	45	
NP-NA-NP <sup>33nt</sup>	33	AGUGAAACAAGGUAUUUUUUCUUCUUCUUCUA		CADGAUUUCGAUGUCACUCUGUGAGUGAUAUAUACUACCCGCUUUUCGCU	45	
NA-NA <sup>A118C</sup> -NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA	NA-A118C (T40P)	CAUUUAAACUCUCGCUUUUCGCU	19	
NA-NA-NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	PB2-U459C (-) + G799A (V267I)
NA-NA-NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	PA-U182C (I61T)
NA-NA-NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	PA-G1101A (-)
NA-NA-NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	PA-C569U (S190F) + C676U (L226F)
NA-NA-NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	
NA-NA-NA <sup>27nt</sup>	27	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	
NA-NA-NA <sup>28nt</sup>	26	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	
NA-NA-NA <sup>28nt</sup>	23	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	
NA-NA-NA <sup>28nt</sup>	20	AGUGAAACAAGGAGUUUUUCUA		CAUUUAAACUCUCGCUUUUCGCU	19	
M-NA-MI	20	AGUGAAACAAGGUAUUUUUCUA		CAUCUUUCAAUAUUAUACCGUCUUUCGCU	25	
M-NA-M	20	AGUGAAACAAGGUAUUUUUCUA		CAUCUUUCAAUAUUAUACCGUCUUUCGCU	25	
M-NA-M	20	AGUGAAACAAGGUAUUUUUCUA		CAUCUUUCAAUAUUAUACCGUCUUUCGCU	25	PB1-A33G (-)
M-NA-M	20	AGUGAAACAAGGUAUUUUUCUA		CAUCUUUCAAUAUUAUACCGUCUUUCGCU	25	PA-A682C (N228H)
M-NA-M	20	AGUGAAACAAGGUAUUUUUCUA		CAUCUUUCAAUAUUAUACCGUCUUUCGCU	25	
M-NA-M <sup>24nt</sup>	24	AGUGAAACAAGGUAUUUUUUAUUUA		CAUCUUUCAAUAUUAUACCGUCUUUCGCU	25	PB1-A33G (-) + PA-A682C (N228H)
M-NA-M <sup>24nt</sup>	26	AGUGAAACAAGGUAUUUUUUAUUUUUA		CAUCUUUCAAUAUUAUACCGUCUUUCGCU	25	
M-NA-M <sup>28nt</sup>	28	AGUGAAACAAGGUAUUUUUUAUUUUUUUA		CAUCUUUCAAUAUUAUACCGUCUUUCGCU	25	
NA-NA-NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	PB1-A33G (-)
NA-NA-NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	PA-A682C (N228H)
NA-NA-NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA		CAUUUAAACUCUCGCUUUUCGCU	19	PB1-A33G (-) + PA-A682C (N228H)
NS-NA-NS	26	AGUGAAACAAGGGUGUUUUUUAUUUAUA		CAUUAUGCUUUUGUACUCCGUCUUUCGCU	26	
NS-NA-NS <sup>21U</sup>	27	AGUGAAACAAGGGUGUUUUUUAUUUAUAUA		CAUUAUGCUUUUGUACUCCGUCUUUCGCU	26	
NS-NA <sup>G993A</sup> -NS	26	AGUGAAACAAGGGUGUUUUUUAUUUAUA	NA-G993A (-)	CAUUAUGCUUUUGUACUCCGUCUUUCGCU	26	
NA-NA <sup>G993A</sup> -NA	28	AGUGAAACAAGGAGUUUUUUGAACAAACUA	NA-G993A (-)	CAUUUAAACUCUCGCUUUUCGCU	19	

a: name of virus produced by reverse genetics

b: nucleotide substitutions or insertions are in bold; start and stop codons are in italics

c: mutations in the NA coding region, in brackets amino acid substitution; (-) indicates a synonymous mutation

d: mutations in the coding region of the indicated segments; in brackets amino acid substitution; (-) indicates a synonymous mutation