Supplementary information file

Mini viral RNAs act as innate immune agonists during influenza virus infection

Aartjan J.W. te Velthuis[1,2,*,§], Joshua C. Long[1,*], David L.V. Bauer[1,*], Rebecca L.Y. Fan[3], Hui-Ling Yen[3], Jane Sharps[1], Jurre Y. Siegers[4], Marian J. Killip[1,5], Hollie French[2], Maria José Oliva-Martín[1], Richard E. Randall[5], Emmie de Wit[6], Debby van Riel[4], Leo L.M. Poon[3], Ervin Fodor[1,§]

[1] Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, United Kingdom.
[2] Division of Virology, Department of Pathology, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0QQ, United Kingdom.
[3] School of Public Health, University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong SAR, China.
[4] Department of Viroscience, Erasmus Medical Centre, 3015 CN, Rotterdam, The Netherlands.
[5] Biomedical Sciences Research Complex, University of St Andrews, North Haugh, St Andrews, Fife KY16 9ST, United Kingdom.
[6] Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA

[*] These authors contributed equally to this work.
[§] Corresponding authors

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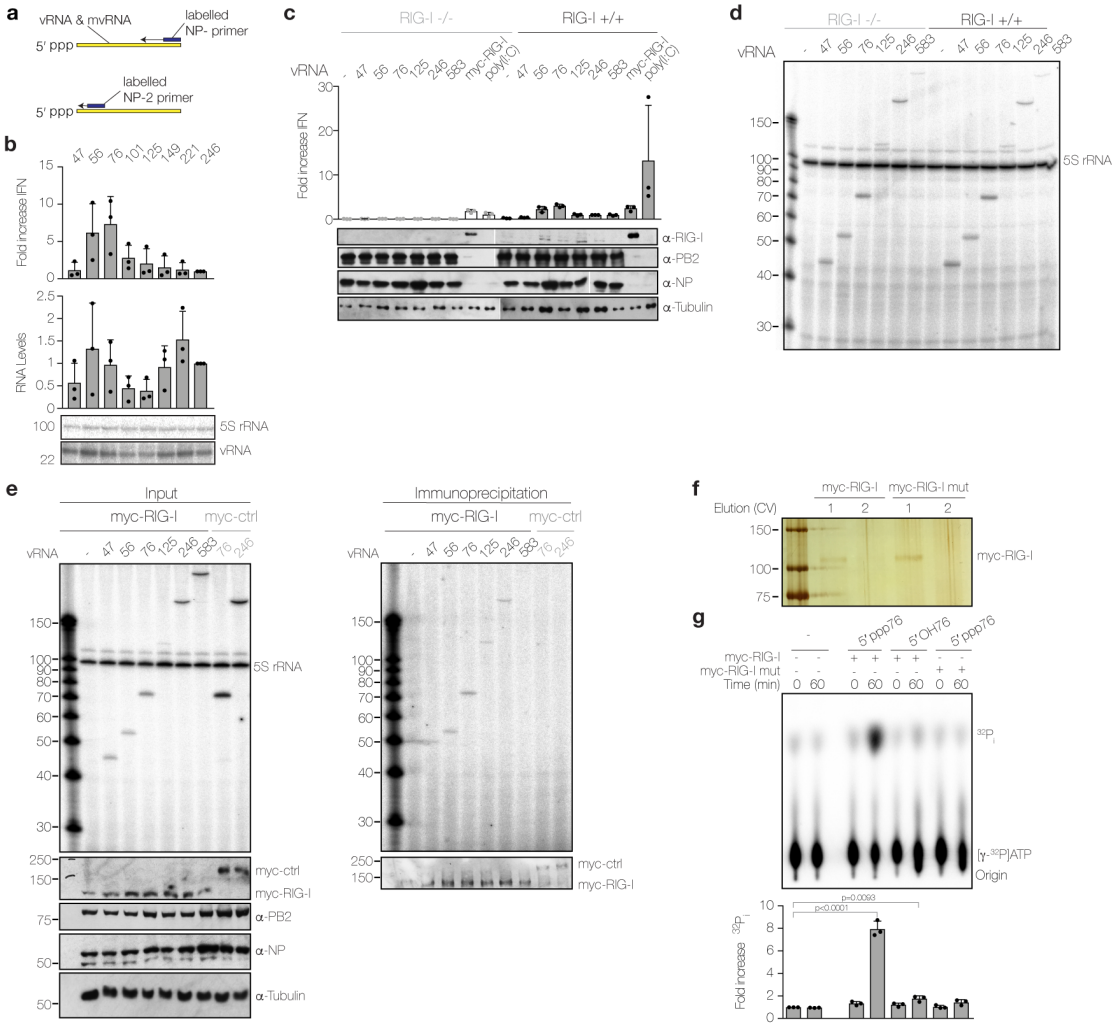
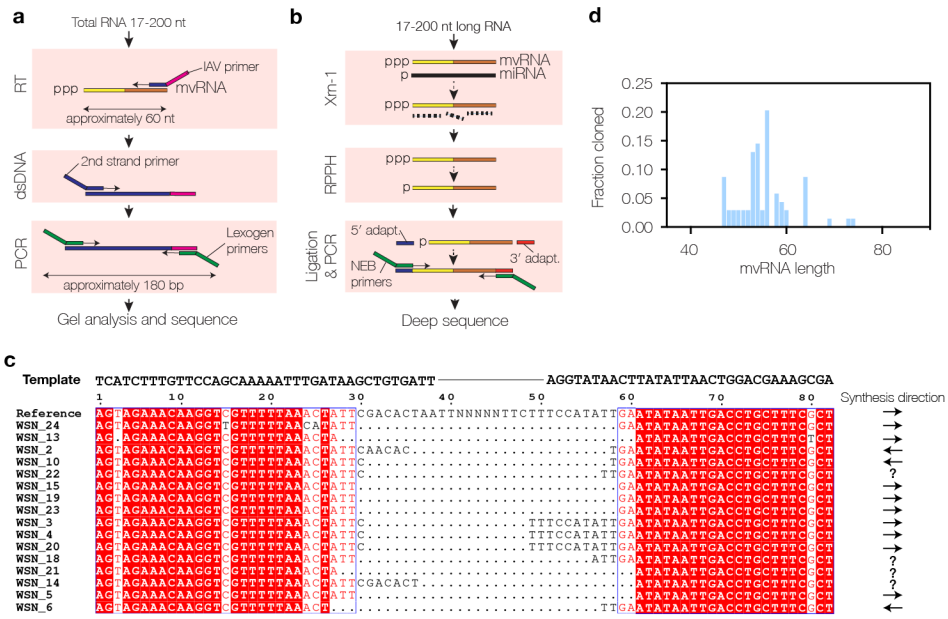
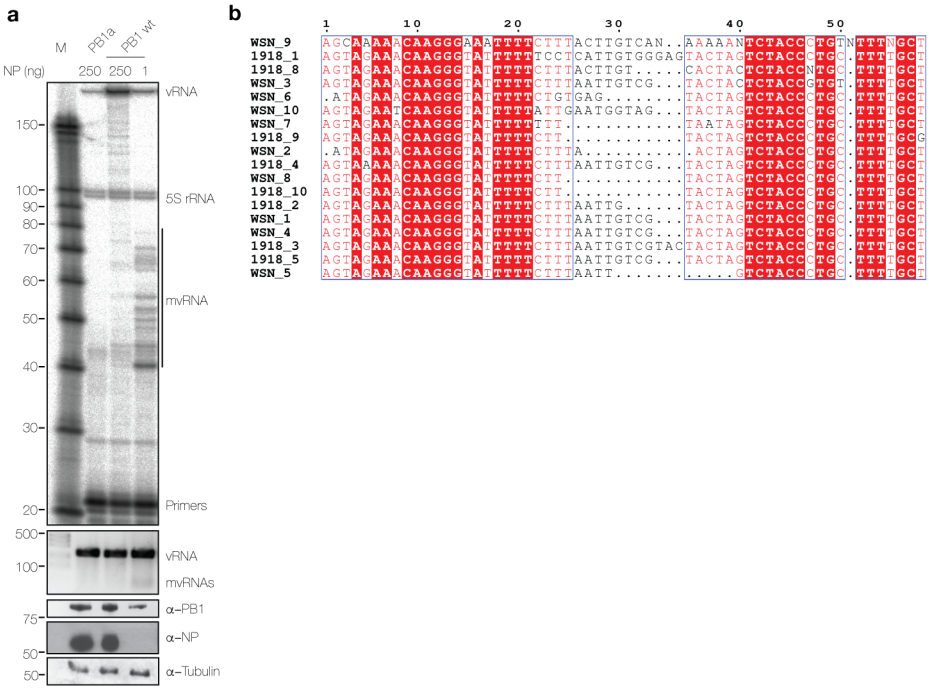


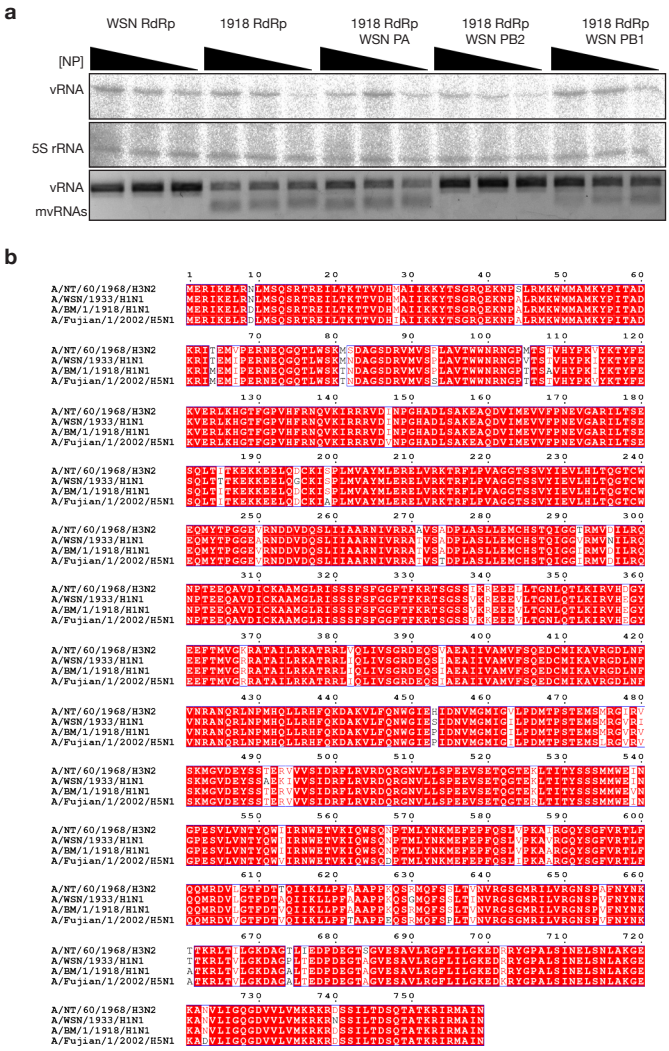
Supplementary Figure 1 I Induction of IFN-β promoter activity by replication of mvRNAs and binding of mvRNAs to RIG-I. (a) Schematic of primer extension analysis using primers binding at the 3′ end of vRNAs (NP-) or to an internal sequence (NP-2). (b) IFN-β promoter activity induced by the replication of segment 5-based vRNA templates before normalisation to RNA levels measured by primer extension with primer NP-2. Normalised data is shown in Fig. 1c. (c) INF-β promoter activity induced by the replication of segment 5-based vRNA templates in wild-type (RIG-I +/+) or RIG knockout (RIG-I -/-) HEK 293T cells expressing luciferase from an IFN-β promoter. (d) Primer extension performed with primer NP- on samples from Supplementary Fig. 1c and analysed by denaturing PAGE. Figure is a representative result of three independent experiments. (e) Detection of segment 5-derived vRNAs with the NP- primer before and after myc-RIG-I or myc-EGF immunoprecipitation. Primer extension analysis was resolved by denaturing PAGE (top panel). Protein expression was analysed by western blot (other panels). Quantified RNA levels of three independent experiments are shown in Fig. 1e. (f) SDS-PAGE and silverstain analysis of myc-RIG-I elutions per column volume (CV). Figure is representative of three independent purifications. (g) ATPase assay of wildtype myc-RIG-I or mutant (mut) in the presence of a triphosphorylated 76 nt mvRNA (5′ ppp76) or a dephosphorylated mvRNA (5′ OH76). Gel is a representative result of three independent experiments. P-values were determined using ANOVA relative to the 0 min buffer control. All graphs show standard deviation and mean of three biologically independent experiments.



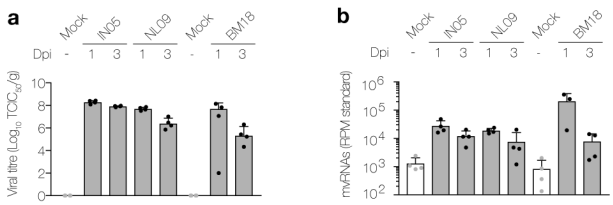
Supplementary Figure 2 | Deep sequencing schematic and alignment of cloned mvRNAs. (**a**) Schematic of deep sequencing protocol using universal primers for the influenza A virus (IAV) promoter. (**b**) Deep sequencing via adapter ligation was performed on the total small RNA fraction (RNAs 17-200 nt in length) after treatment with XRN-1 and RppH. (**c**) Alignment and (**d**) size distribution of segment 1-derived mvRNAs generated during infection with influenza A/WSN/33 (H1N1) virus in the presence of overexpressed viral polymerase. Sequences were produced after gel extraction and Sanger sequencing. The possible direction of synthesis (vRNA to mcRNA or cRNA to mvRNA) via the intramolecular copy-choice model is indicated. Unknown directions of synthesis are indicated with '?'.



Supplementary Figure 3 | mvRNAs synthesis in replication assays with WSN polymerase in the presence of limiting NP and alignment of cloned mvRNAs. (**a**) Analysis of steady state RNA levels in replication assays with an active site mutant WSN PB1 (PB1a) or WSN PB1 wild-type (PB1 wt) and a 246-nt vRNA that was derived from segment 5 of the influenza A virus genome, in the presence of limiting NP. The NP- primer was used for primer-extension analysis of vRNA levels (top panel) and 5S rRNA was used a loading control. RT products were resolved by denaturing PAGE. vRNA and mvRNA levels were also analysed by RT-PCR and agarose gel electrophoresis (2nd panel). Viral and cellular protein levels were analysed by western blot (3rd, 4th and 5th panel). The top panel is representative of two independent experiments, while the other panels are representative of three independent experiments. (**b**) Alignment of mvRNAs generated in replication assays with the WSN and BM18 polymerases from segment 5-derived 246 nt vRNA template.



Supplementary Figure 4 | mvRNA synthesis by reassorted WSN and BM18 polymerases and sequence alignments of the PB2 subunit. (**a**) Analysis of steady state RNA levels in replication assays with WSN and BM18 polymerases and their reassortants and a 246-nt vRNA that was derived from segment 5 of the influenza A virus genome, in the presence of decreasing NP. 5S rRNA and vRNA signals were detected with radiolabeled primers and RT products were resolved by denaturing PAGE (top and middle panel). The vRNA and mvRNA signals were analysed by RT-PCR and agarose gel electrophoresis (bottom panel). The top and middle panel are representative of two independent experiments. The bottom panel is representative of four independent experiments. (**b**) Amino acid sequence alignment of the PB2 subunits of A/WSN/33 (H1N1) (accession number ACF54608), A/Brevig Mission/1/18 (H1N1) (accession number ABA55038), A/Northern Territory/60/68 (H3N2) (accession number AAA43613), and A/duck/Fujian/1/2002 (H5N1) (accession number AY585504) influenza viruses.



Supplementary Figure 5 | **Viral titre and mvRNA levels in ferret lungs.** (**a**) Published viral titres of ferret lung tissue samples[29,30]. (**b**) mvRNA levels in lungs of ferrets one and three days after infection with IN05, NL09 or BM18 as measured using deep sequencing. Graphs show standard deviation and mean of data from four (n=4) biologically independent animal samples. A day 1 BM18 ferret sample with low titre was excluded from the analysis.

Supplementary Table 1 | Primers for mutagenesis

Mutant	Fw/rv	Sequence (5′ to 3′)
PB1 V43I	Fw	GGAACAGGATACACCATGGATACTATTAACAGGACACATCAGTACTCAG
	Rv	CTGAGTACTGATGTGTCCTGTTAATAGTATCCATGGTGTATCCTGTTCC
PB2 E627K	Fw	CATTTGCAGCAGCCCCACCGAAGCAGAGCAGAATGCAGTTTTCTTC
	Rv	GAAGAAAACTGCATTCTGCTCTGCTTCGGTGGGGCTGCTGCAAATG
PB2 T64M	Fw	CCAATTACAGCAGACAAGAGGATAATGGAAATGATTCCTGAGAGAAATGAGC
	Rv	GCTCATTTCTCTCAGGAATCATTTCCATTATCCTCTTGTCTGCTGTAATTGG
PB2 M81T	Fw	CAGGGACAAACTTTATGGAGTAAAACGAATGACGCCGGATCAGAC
	Rv	GTCTGATCCGGCGTCATTCGTTTTACTCCATAAAGTTTGTCCCTG
Myc-RIG-I mut	Fw	AAGTTTTGAAGCAAGAGCAGCGATATTCTGTGCCCGACAGAACTGCAGC
	Rv	GAAAACTGCGCTGGCTTGGGATGTGGTCTACTCACAAAGCATTCC

Sequences of DNA primers used to generate influenza A virus polymerase subunit and RIG-I mutants. Forward (fw) and reverse (rv) primers are listed.

Supplementary Table 2 | RNA templates

Template	Sequence (5′ to 3′)
NP47	AGUAGAAACAAGGGUAUUUUUCUUUCUCGAGCGUACUAGUCUACCCUGCUUUUGCU
NP76	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUGAUUUCGAUGUCACUCUGUGAGUGAUUAUCUACCCUGCUUUUGCU
NP101	AGUAGAAACAAGGGUAUUUUUCUUUAAUUGUCGUACUCACUAGUUUUGGUCGCCAUGAUUUCGAUGUCACUCUGUGAGUGAUUAUCUACCCUGCUUUUGCU
NP125	AGUAGAAACAAGGGUAUUUUUCUUUAAUUGUCGUACUCCUCUGCAUUGUCACUAGUUCGUUUGGUGCCUUUGGUCGCCAUGAUUUCGAUGUCACUCUGUGAGUACUAGUCUACCCUGCUUUUGCU
NP149	AGUAGAAACAAGGGUAUUUUUCUUUAAUUGUCGUACUCCUCUGCAUUGUCUCCGAAGAAAUAACUAGUCUGUUCGUAAGAUCGUUUGGUGCCUUUGGUCGCCAUGAUUUCGAUGUCACUCUGUGAGUGAUUAUCUACCCUGCUUUUGCU
NP246	AGUAGAAACAAGGGUAUUUUUCUUUAAUUGUCGUACUCCUCUGCAUUGUCUCCGAAGAAAUAAGAUCCUUCAUUACUCAUGUCAAAGGAGGGCACGAUCGGGCUCGUUGCCUUUUCGUCCGAGAGCUCGAAGACUCCCCGCCCCUGGAAAGACACUAGUCUCCAUCUGUUCGUAAGAUCGUUUGGUGCCUUUGGUCGCCAUGAUUUCGAUGUCACUCUGUACUAGUCUACCCUGCUUUUGCU
NP583	AGUAGAAACAAGGGUAUUUUUCUUUAAUUGUCGUACUCCUCUGCAUUGUCUCCGAAGAAAUAAGAUCCUUCAUUACUCAUGUCAAAGGAGGGCACGAUCGGGCUCGUUGCCUUUUCGUCCGAGAGCUCGAAGACUCCCCGCCCCUGGAAAGACACAUCUUCUGGUCUUGCACUUUCCAUCAGCCUUAUGAUUUCGGUUCUCAUGUCAGAUGUUCUCCCCUCUGUAUUCCCAGUGAAUGCUGCCAUAAUGGUUGGUCUGUCAAAAGGGAGAUUUCUCUGUACUGAGAACGUAGGUUGUAUGCUGAUUUGGCCCGAGGAAGCCCUCUGUUGAUUGGUGUUCCCUCCACUUCUGGUCCUUAUGGCCCAGUAUCUGCUUCUCAGUUCAAGGGUACUAGUUUAAGUUCGGUGCACAUUUGGAUGUAGAAUCGUCCAAUUCCAUCAAUCAUUUUUCCGACAGAUGCUCUGAUUUCAGUGGCAUUCUGGCGUUCUCCAUCAGUCUCCAUCUGUUCGUAAGAUCGUUUGGUGCCUUUGGUCGCCAUGAUUUCGAUGUCACUCUGUGAGUGAUUAUCUACCCUGCUUUUGCU
HA77	AGUAGAAACAAGGGUGUUUUUCCUUAUAUUUCUGAAAUCCUUCAUUUUGGUUGUUUUUAUUUUCCCCUGCUUUUGCU
HA245	AGUAGAAACAAGGGUGUUUUUCCUUAUAUUUCUGAAAUCCUAAUCUCAGAUGCAUAUUCUGCACUGCAAAGACCCAUUAGAACACAUCCAGAAACUGAUUGCCCCCAGGGAGACCAAAAGCACCAGCCUCCUCGCUGGCGCCGGCUGGGCAACAUUCCGAGGGGACCGUCCCCUCGGUAAUGGCGAAUGGGACUCUAGUACAAAAGCCUUCAUUUUGGUUGUUUUUUAUUUUCCCCUGCUUUUGCU
NA76	AGUAGAAACAAGGAGUUUUUUGAACAAACUACUUGUCAAUUUCUGGUUUGGAUUCAUUUAAACUCCUGCUUUUGCU
NA244	AGUAGAAACAAGGAGUUUUUUGAACAAACUACUUGUCAAUGGUGAACGGGAGCUCAGCACCGUCUGGCCAAGACCAAUCUACAGUAUCACCAUUCACACCACAAAAAGAAAUGAUGCUCCCACAAUCCAUAUUGAGAUUAUAUUUCCUAUUUGCAAUAUUAGGCUAAUUAUUCCGACUACCAUACAGAUCGAUCCAAUGGUUAUUAUUUUCUGGUUUGGAUUCAUUUAAACUCCUGCUUUUGCU
49-vRNA spike	AGUAGAAACAAGGGUGUUUUUUCAGAUCUCGAGUCACCCUGCUUUUGCU
37-cRNA spike	AGCAAAAGCAGGGUAGACUAGUACCCUUGUUUCUACU

Sequences of RNA templates used to perform RNP reconstitution and IFN induction assays. The NP, HA, and NA templates were expressed from plasmid DNAs containing an RNA polymerase I (Pol I) promoter. The spike RNAs were purchased as synthetic oligonucleotides.

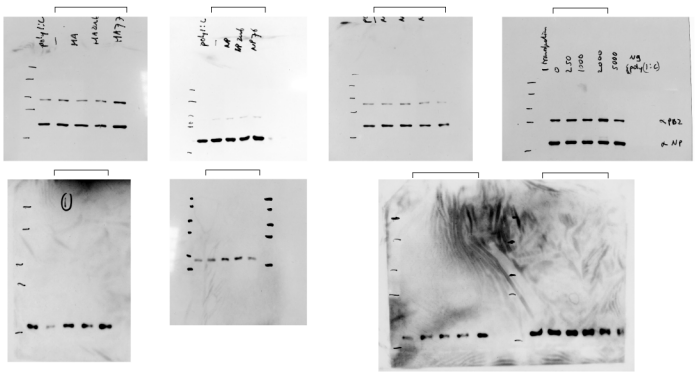
Supplementary Table 3 | Primer-extension and RT-PCR primers

Primer name	Sequence (5′ to 3′)	Target
NP-	AGCAAAAGCAGGGTAGACTAGT	NP negative strand (3′ terminus)
NP-2	AAAGAAAAATACCCTTGTTTC	NP negative strand
5S100	TCCCAGGCGGTCTCCCATCC	5S rRNA (100 nt product)
NA-	GTTCAAAAAACTCCTTGTTTC	NA negative strand
HA-	GGAAAAACACCCTTGTTTCT	HA negative strand
NP 5'	AGTAGAAACAAGGGTATTTTTCT	NP positive strand (5′ terminus)
NP 3'	AGCAAAAGCAGGGTAGATAATC	NP positive strand (3′ terminus)
PB2v	AGCGAAAGCAGGTCAATTATAT	PB2 negative strand (3′ terminus)
PB2c	AGTAGAAACAAGGTCGTTTTTAAAC	PB2 positive strand (5′ terminus)
Lv3ga	GTTCAGACGTGTGCTCTTCCGATCTAGCG+AAAGCAGG	vRNA RT primer (contains LNA)
Lv3aa	GTTCAGACGTGTGCTCTTCCGATCTAGC+A+AAAGCAGG	vRNA RT primer (contains LNA)
Lv5	CACGACGCTCTTCCGATCTHNNNNNNNAGTAGAA+A+CAAGG	vRNA forward primer (contains LNA)
Lc3	GTTCAGACGTGTGCTCTTCCGATCTAGTAGAA+A+CAAGG	cRNA RT primer (contains LNA)
Lc5a	CACGACGCTCTTCCGATCTHNNNNNNNAGC+AAAAGCAGG	cRNA forward primer (contains LNA)
Lc5g	CACGACGCTCTTCCGATCTHNNNNNNNAGCGAAAGCAGG	cRNA forward primer
P5	AATGATACGGCGACCACCGAGATCTACACTCTTTC-CCTACACGACGCTCTTCCGATCT	vDNA and cDNA forward
i7003	CAAGCAGAAGACGGCATACGAGATACTGGTGTGA-CTGGAGTTCAGACGTGTGCTCTTCCGATCT	vDNA and cDNA reverse
Actin human fw	AGAGCTACGAGCTGCCTGAC	Actin B mRNA (forward)
Actin human rv	AGCACTGTGTTGGCGTACAG	Actin B mRNA (reverse)
Actin ferret fw	GATATTACCAGATATCTTATCAAGCTGCTGC	Actin B mRNA (forward)
Actin ferret rv	GCGGCCATCTGGGAGTGTGTAAG	Actin B mRNA (reverse)
H5N1_VN NA fw	CAATTTGGACTAGTGGGAGCAGC	NA H5N1 VN qRT-PCR (forward)
H5N1 VN NA rv	TTGAACAAACTACTTGTCAATGGTGAATG	NA H5N1 VN qRT-PCR (reverse)
NA rv	GCAACTCAGCACCGTCTGGCC	NA qRT-PCR (reverse)
NA 1918 fw	TGCTTCTGGGTTGAATTAATCAGGG	NA BM 1918 qRT-PCR (forward)
NA NL fw	GCTTCTGGGTTGAACTAATCAGAGGG	NA NL 2009 qRT-PCR (forward)
NA IND fw	GTTTCTGGGTTGAGTTGATCAGAGGG	NA Ind H5N1

Sequences of DNA primers used to perform primer extension reactions and RT-PCR reactions. Targets of the primers are listed. Some RT primers contain LNA bases. The location of these LNA bases in the primers is indicated with +N, where N represents the base.

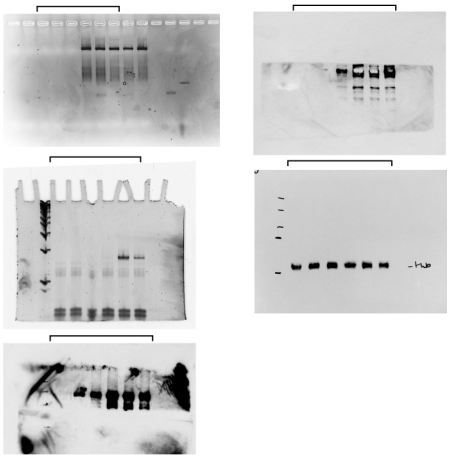
Raw images Figure 1

Figure 1b



Raw images Figure 2

Figure 2a



Raw images Figure 3

Figure 3a

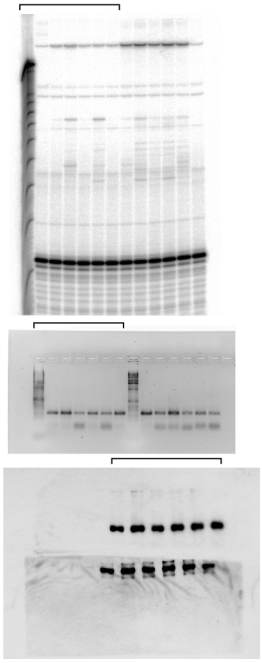
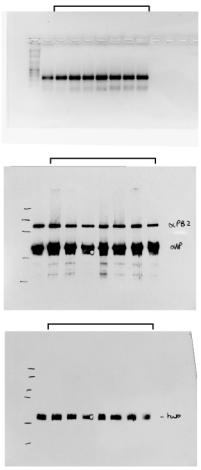
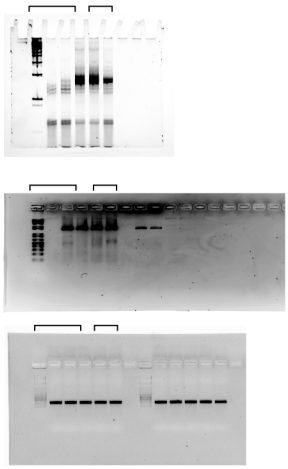


Figure 3c



Raw images Figure 4

Figure 4a



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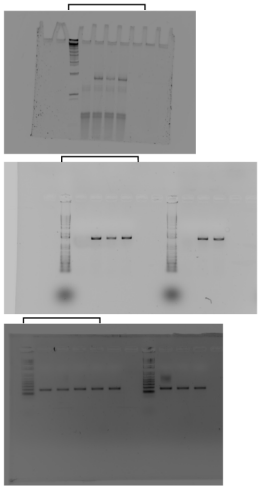
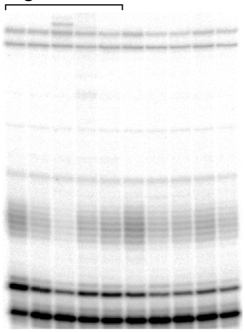


Raw images Supplementary Figure 1

Figure S1b



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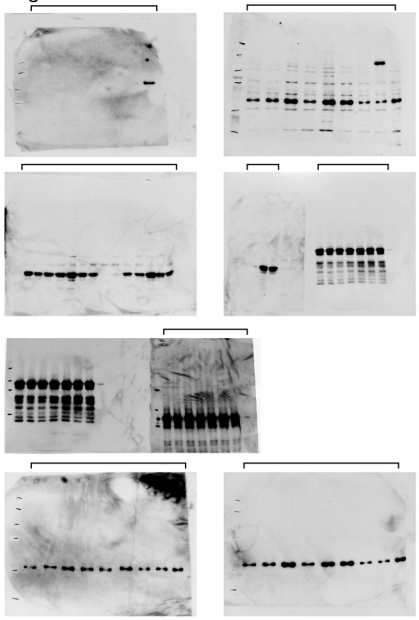
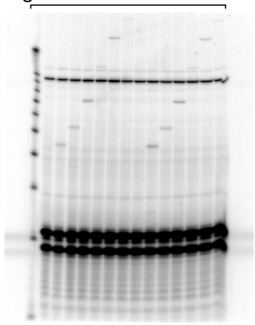


Figure S1d



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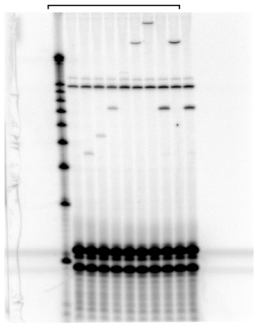


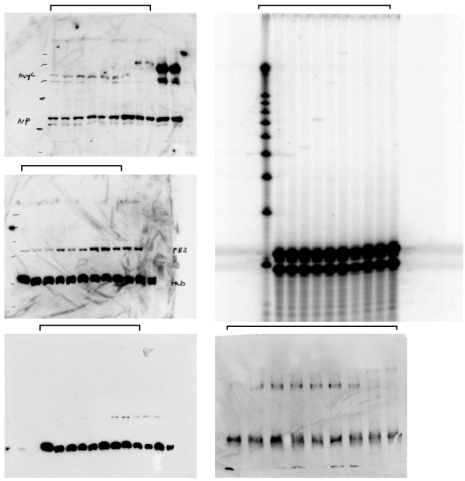


Figure S1f

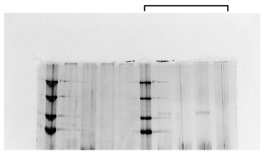
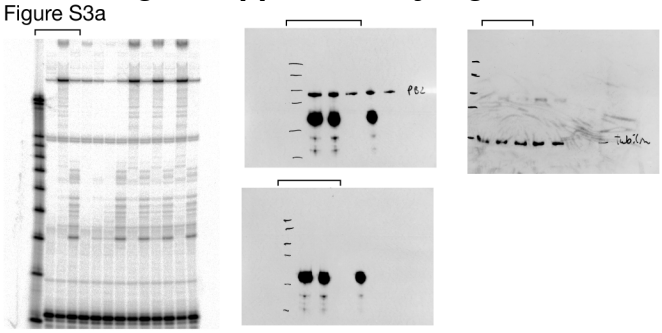


Figure S1g

Raw images Supplementary Figure 3

Figure S3a



Raw images Supplementary Figure 4

Figure S4