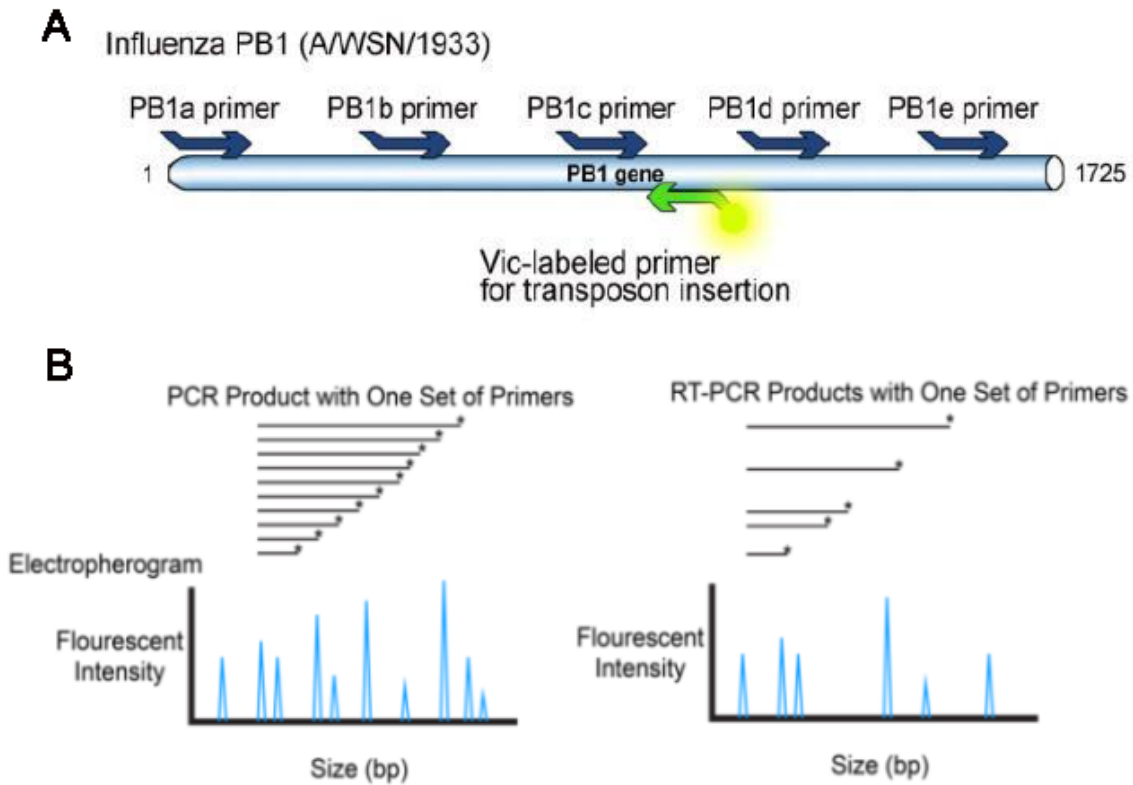
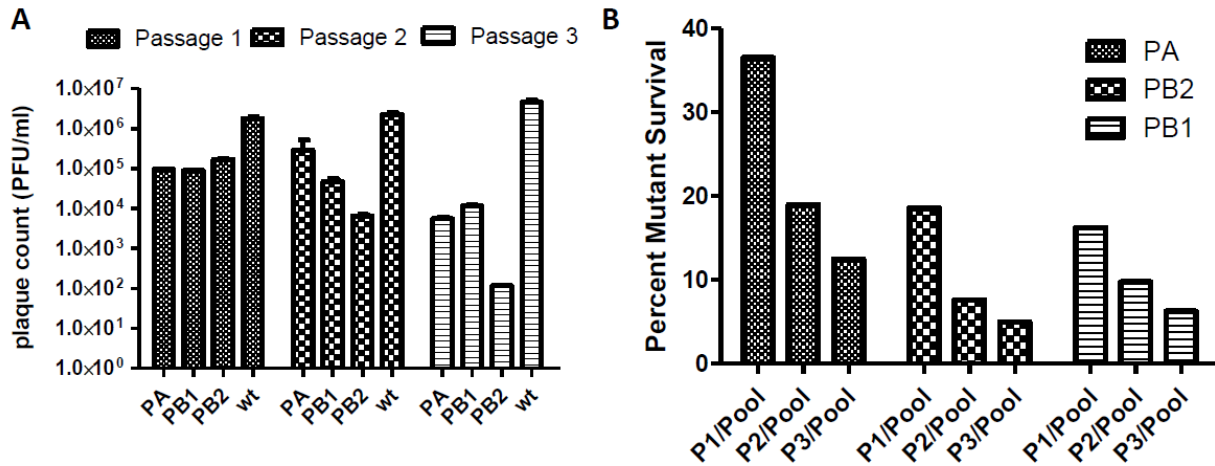


1 **Supplementary Figures**
2 **Figure S1.**



3
4 **Figure S1. Genome-wide virus mutagenesis and data collection.** (A) Individual
5 mutations are genotyped by PCR amplification with a gene-specific primer and a Vic-
6 labeled primer against the 15nt insertion sequence. (B) The mutant pool library can be
7 put through selection such as growth *in vitro* or *in vivo*. The selected pool is collected
8 and genotyped. Essential and non-essential regions in the genome required for growth
9 can be determined by comparing genotyping data from the unselected pool (total
10 mutant library) and selected pool.

1 **Figure S2.**

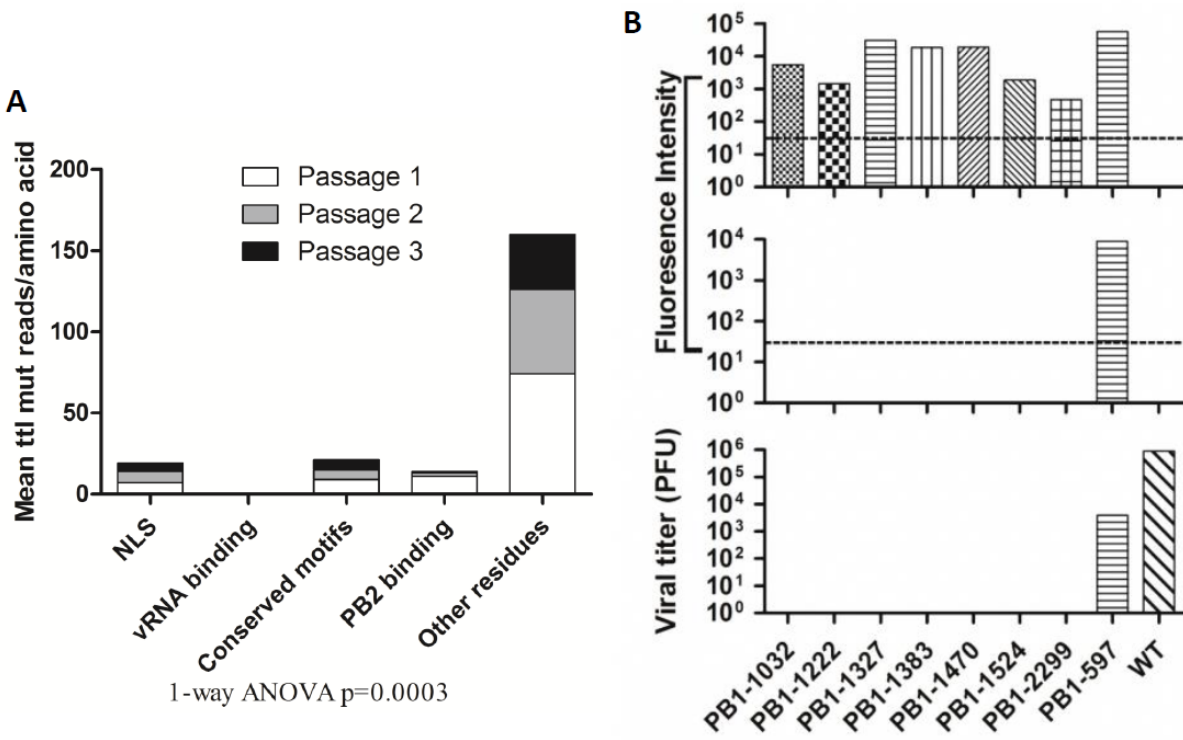


2

3 **Figure S2. Characterization of virus pool from each passage.** (A) The viral titer of
4 WT and mutant pools at the indicated passage was measured by plaque assay using
5 MDCK cells. (B) Total numbers of mutants detected in the initial PB1 mutant DNA library
6 and recovered from the first three passages in MDCK cells after reconstitution of the
7 influenza mutant pool.

8

1 **Figure S3.**

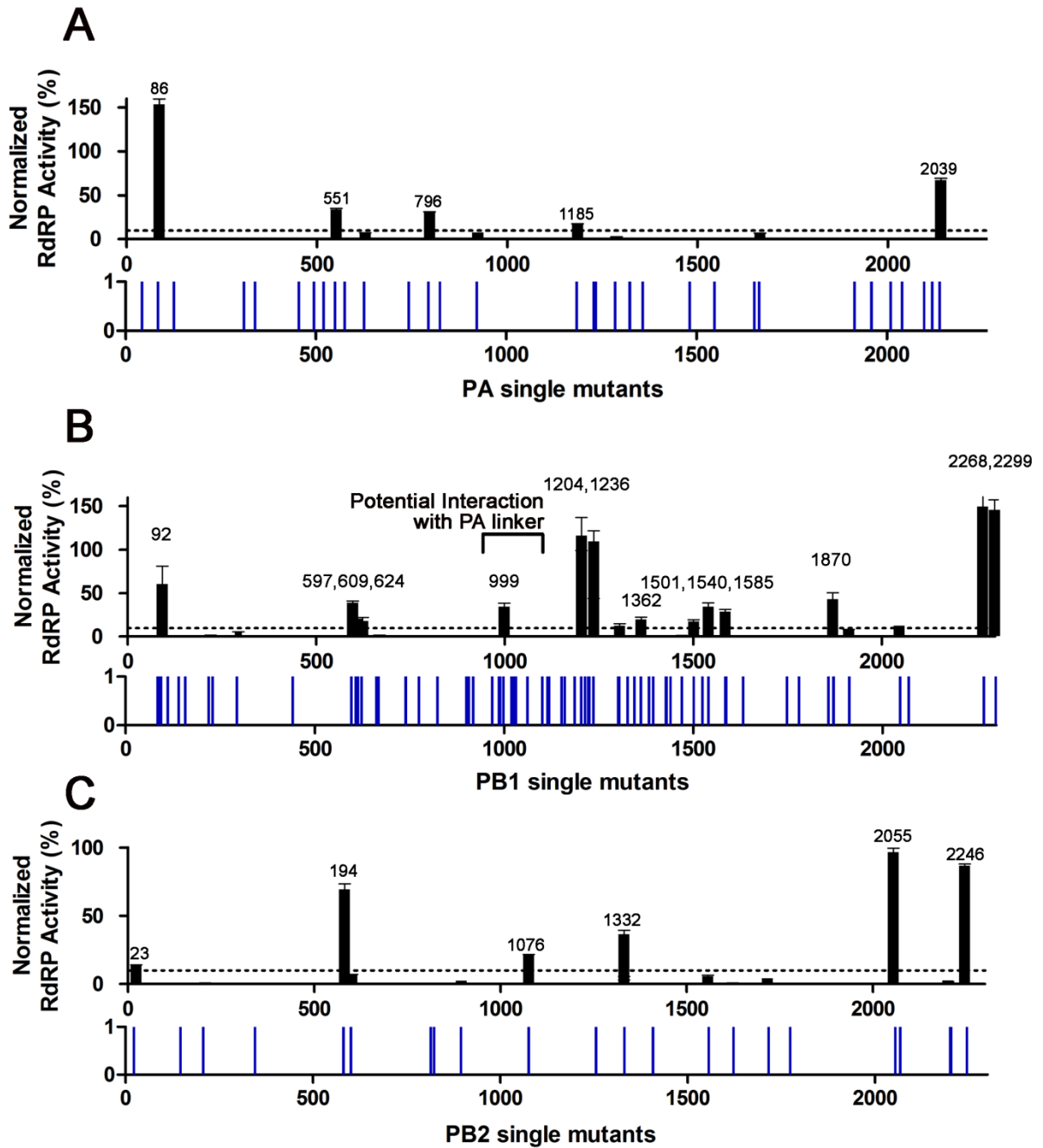


2

3 **Figure S3. Validation of genotyping data using isolated single clones.** (A) The
 4 location of significant viable mutants at functional regions of the PB1 gene by amino
 5 acid position. NLS, Nuclear Localization Signal. (B) To verify our mutagenesis, the
 6 mutant PB1 plasmid library was co-transfected with 7 WT plasmids in 293T cells to
 7 recover virus in passage 1. Eight randomly selected single clone mutants were isolated
 8 from the plasmid pool and were co-transfected independently with 7 WT plasmids. The
 9 graph indicates where single clone mutants and viruses from passage 1 were unable to
 10 grow.

11

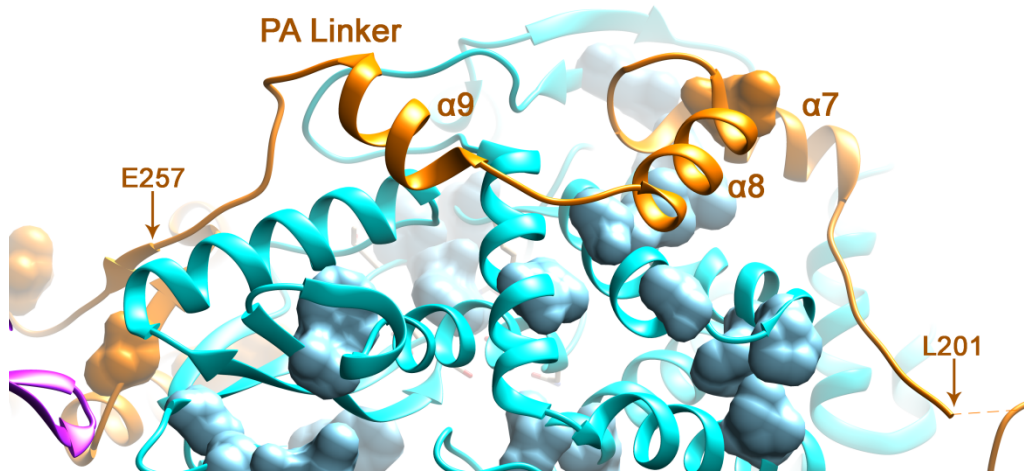
1 **Figure S4.**



2

3 **Figure S4. Location and RdRP activity of all isolated single clones from PA, PB1**
4 **and PB1 library.** The coverage and location of all single mutants from the PA (A), PB1
5 (B) and PB2 (C) libraries shown in blue bars, whereas the relative polymerase activities
6 are labeled in black bars.

1 **Figure S5.**

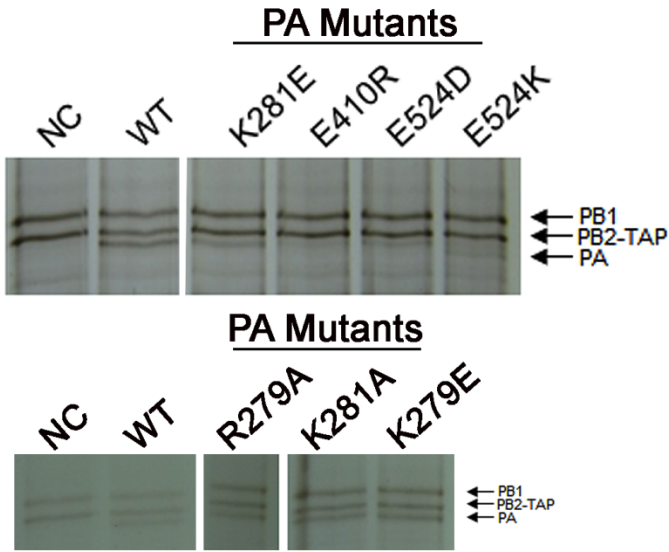


2

3 **Figure S5. Structure of the PA linker.** The PA linker that interacts with PB1 subunit is
4 labeled in orange, whereas the PB1 interface is labeled in cyan. PB2 subunit is labeled
5 in purple. Site 264 (green) was highlighted on the PA linker (blue). A charged amino
6 acid tunnel surrounding the linker on PAc is shown in purple. PB1c and PAc are shown
7 as transparent grey and orange surfaces, respectively.

8

1 **Figure S6.**



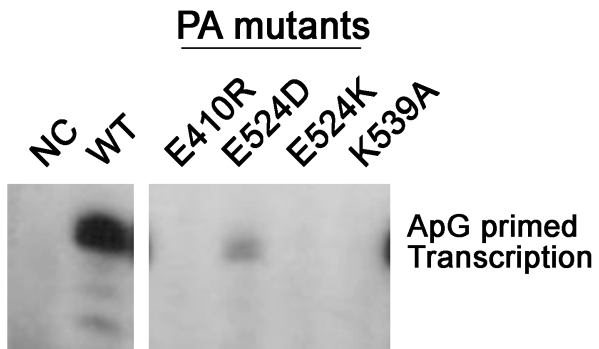
2

3 **Figure S6. TAP pull-down of influenza virus polymerase complex with PA mutants.**

4 PB2-TAP tagged heterotrimer polymerase complex was purified in 293T cells at 40
5 hours after transfection. The positions of PA, PB-TAP and PB1 are indicated.

6

1 **Figure S7.**



2

3 **Figure S7. ApG primed transcription assay.** *In vitro* ApG primed transcription
4 assay of influenza virus polymerase with WT and indicated mutations on PA. The
5 positions of transcription products are indicated.

Table S1. Primers, nucleotide locations, and sequences.

PA	Primer Positions & Sequence
13	ATTTGAATGGATGTCAATCC
456	ACACAATAGAAGTGTTTCAG
847	GATAAGGAAGATGATGACC
1276	AATCTTGGACAAAAGAGAC
1687	ATACAACATTAGAAATCTCC
PB1	Primer Positions & Sequence
1	GAAGATTTTGTGCGACAATGC
439	ATTCAGTGGGGAGGAAATGG
861	TCCAAATTCCTGCTGATGG
1278	ATAGAGCTCGATGAGATTGG
1799	CCTATGTTCTTGTATGTGAGG
PB2	Primer Positions & Sequence
85	ATGTCGCAGTCTCGCACTC
383	AACCTTTGGCCCTGTCCATTTTA G
739	GAGGGGAGGCGAGGAATG
1094	GGTTGGGAGAAGAGCAACAGC
1449	GGGGTAGATGAGTATTCCAGCG
1876	CAAAGCAAAGTGGAAATGCAGTT CTC

Table S2. Single mutants isolated from the PB1 pool.

	PB1	Nuc Pos	RdRP activity^a	Std Dev	Viral titer	Plaque size	Plaque Characteristic	AA Pos
1	PB1-wt	0	100	19.147	4.67E+06	Normal	Clear	0
2	PB1-240	85	0.01	0.01	None	ND	ND	28
3	PB1-241	85	0.01	0.01	None	ND	ND	28
4	PB1-242	92	60.391	20.682	1.23E+05	Normal	Clear	31
5	PB1-140	94	0	0.01	None	ND	ND	31
6	PB1-272	112	0	0.01	None	ND	ND	37
7	PB1-262	141	0.01	0.01	None	ND	ND	47
8	PB1-3	158	0	0.01	None	ND	ND	53
9	PB1-258	220	1.2042	0.5247	None	ND	ND	73
10	PB1-222	230	0.1719	0.0385	None	ND	ND	77
11	PB1-214	294	5.35	0.09	None	ND	ND	98
12	PB1-8	442	0.0257	0.0111	None	ND	ND	147
13	PB1-16	597	38.972	2.1601	1.10E+03	Small	Clear	199
14	PB1-125	609	19.575	0.4957	None	ND	ND	203
15	PB1-213	614	2.83	0.02	None	ND	ND	205
16	PB1-157	624	17.98	3.9762	None	ND	ND	208
17	PB1-209	663	0.01	0.01	None	ND	ND	221

18	PB1-27	668	1.2335	0.3948	None	ND	ND	223
19	PB1-56	740	0.16	0.01	None	ND	ND	247
20	PB1-34	740	0.13	0.00	None	ND	ND	247
21	PB1-6	741	0	0.01	None	ND	ND	247
22	PB1-275	775	0	0.00	None	ND	ND	258
23	PB1-221	824	0.02	0.00	None	ND	ND	275
24	PB1-25	901	0	0.01	None	ND	ND	300
25	PB1-235	907	0.81	0.00	None	ND	ND	302
26	PB1-38	919	0.22	0.00	None	ND	ND	306
27	PB1-154	969	0.01	0.00	None	ND	ND	323
28	PB1-236	987	0.01	0.01	None	ND	ND	329
29	PB1-263	987	0.01	0.00	None	ND	ND	329
30	PB1-233	990	0.02	0.01	None	ND	ND	330
31	PB1-232	999	34.26	4.28	8.65E+04	Normal	Clear	333
32	PB1-17	1020	0.11	0.01	None	ND	ND	340
33	PB1-135	1026	0.076	0.0171	None	ND	ND	342
34	PB1-144	1031	0.4445	0.1403	None	ND	ND	344
35	PB1-269	1031	0.02	0.01	None	ND	ND	344
36	PB1-101	1031	0.01	0.02	None	ND	ND	344
37	PB1-43	1032	0.15	0.00	None	ND	ND	344
38	PB1-238	1032	0.02	0.00	None	ND	ND	344
39	PB1-111	1062	0.18	0.03	None	ND	ND	354
40	PB1-279	1101	0.02	0.00	None	ND	ND	367
41	PB1-200	1115	0.01	0.01	None	ND	ND	372
42	PB1-250	1119	1.25	0.01	None	ND	ND	373
43	PB1-160	1152	0.12	0.00	None	ND	ND	384
44	PB1-100	1152	0.03	0.02	None	ND	ND	384
45	PB1-48	1160	0.1115	0.0155	None	ND	ND	387
46	PB1-252	1186	0.03	0.00	None	ND	ND	395
47	PB1-47	1204	116.04	20.999	5.10E+03	Normal	Clear	401
48	PB1-103	1204	92.04	6.79	8.70E+04	Normal	Clear	401
49	PB1-270	1214	0	0.01	None	ND	ND	405
50	PB1-168	1222	0.01	0.01	None	ND	ND	407
51	PB1-15	1225	0.13	0.02	None	ND	ND	408
52	PB1-113	1236	109.56	12.096	7.50E+05	Normal	Clear	412
53	PB1-205	1236	39.74	4.28	2.22E+05	Normal	Clear	412
54	PB1-248	1236	0	0.01	None	ND	ND	412
55	PB1-57	1302	0.23	0.01	None	ND	ND	434
56	PB1-152	1305	12.69	2.28	1.70E+03	Small	Turbid	435
57	PB1-259	1327	0.01	0.01	None	ND	ND	442
58	PB1-261	1345	0.01	0.01	None	ND	ND	448
59	PB1-153	1362	19.61	2.7679	None	ND	ND	454
60	PB1-245	1383	0	0.01	None	ND	ND	461
61	PB1-150	1394	0.0507	0.0056	None	ND	ND	465
62	PB1-11	1428	0.24	0.02	None	ND	ND	476
63	PB1-266	1428	0	0.00	None	ND	ND	476
64	PB1-20	1429	0	0.00	None	ND	ND	476
65	PB1-134	1440	0.19	0.02	None	ND	ND	480
66	PB1-122	1440	0.04	0.02	None	ND	ND	480
67	PB1-230	1470	1.99	0.01	None	ND	ND	490

68	PB1-120	1470	0.01	0.01	None	ND	ND	490
69	PB1-161	1470	0	0.00	None	ND	ND	490
70	PB1-202	1501	17.57	1.67	9.69E+03	Small	Turbid	500
71	PB1-104	1524	0.13	0.02	None	ND	ND	508
72	PB1-215	1540	34.46	4.50	1.12E+05	Normal	Clear	513
73	PB1-247	1585	28.94	2.29	1.90E+03	Normal	Clear	528
74	PB1-142	1587	0.12	0.00	None	ND	ND	529
75	PB1-41	1631	0.15	0.01	None	ND	ND	544
76	PB1-163	1631	0.01	0.00	None	ND	ND	544
77	PB1-21	1632	0.09	0.00	None	ND	ND	544
78	PB1-278	1748	0	0.00	None	ND	ND	583
79	PB1-166	1779	0.01	0.00	None	ND	ND	593
80	PB1-121	1857	0.01	0.01	None	ND	ND	619
81	PB1-9	1870	43.06	7.3892	9.80E+04	Normal	Clear	623
82	PB1-223	1871	0.01	0.01	None	ND	ND	624
83	PB1-237	1871	0.01	0.01	None	ND	ND	624
84	PB1-264	1912	7.3321	1.0362	None	ND	ND	637
85	PB1-139	2046	12.58	0.02	9.31E+02	Small	Turbid	682
86	PB1-159	2069	0.38	0.01	None	ND	ND	690
87	PB1-148	2268	163.45	30.068	None	ND	ND	756
88	PB1-137	2299	145.75	11.779	None*	Small	Turbid	766

Nuc Pos is abbreviated for the location of the insertion in nucleotide position. Std Dev is abbreviated for standard deviation of the RdRp activity. AA pos is abbreviated for the location of the insertion in amino acid position. ^aRelative polymerase activity, which was normalized against WT.

Table S3. Single mutants isolated from the PB2 pool.

	PB2	Nuc Pos	Std Dev	RdRP activity ^a	AA pos
1	PB2-wt	0	1.00	100.00	0
2	PB2-17	23	0.34	13.86	8
3	PB2-145	147	0.05	1.04	49
4	PB2-2	147	0.05	0.76	49
5	PB2-112	208	0.06	0.83	69
6	PB2-66	346	0.01	0.67	115
7	PB2-125	582	4.13	69.43	194
8	PB2-59	602	0.11	6.85	201
9	PB2-79	815	0.02	0.32	272
10	PB2-95	824	0.05	0.55	275
11	PB2-52	824	0.09	0.40	275
12	PB2-84	824	0.01	0.28	275
13	PB2-157	896	0.11	2.59	299
14	PB2-4	1076	0.43	21.32	359
15	PB2-173	1200	0.00	0.01	400
16	PB2-22	1256	0.06	1.05	419
17	PB2-87	1332	3.08	36.43	444
18	PB2-158	1332	0.35	5.20	444

19	PB2-123	1332	0.29	2.60	444
20	PB2-72	1408	0.01	0.37	469
21	PB2-65	1557	0.47	5.99	519
22	PB2-47	1557	0.00	0.44	519
23	PB2-122	1623	0.18	0.68	541
24	PB2-40	1717	0.03	4.28	572
25	PB2-32	1774	0.00	0.02	591
26	PB2-70	2055	2.85	96.71	685
27	PB2-42	2068	0.00	0.24	689
28	PB2-131	2201	0.02	2.82	734
29	PB2-46	2204	0.02	2.27	735
30	PB2-15	2246	1.12	86.85	749

Nuc Pos is abbreviated for the location of the insertion in nucleotide positions. Std Dev is abbreviated for standard deviation of the RdRp activity. AA pos is abbreviated for the location of the insertion in amino acid positions.¹Relative polymerase activity, which was normalized against WT.

Table S4. Single mutants isolated from the PA pool.

	PA	Nuc Pos	Std Dev	RdRP activity^a	AA pos
1	PA-wt	0	1	100	0
2	PA-84	44	0.00	0.01	15
3	PA-83	86	5.90	168.06	29
4	PA-49	128	0.02	0.52	43
5	PA-76	311	0.00	0.06	104
6	PA-100	340	0.00	0.01	113
7	PA-89	456	0.00	0.02	152
8	PA-2	495	0.00	0.02	165
9	PA-12	521	0.00	0.02	174
10	PA-7	551	0.88	34.10	184
11	PA-51	576	0.07	0.77	192
12	PA-88	627	0.39	6.79	209
13	PA-35	796	0.68	30.49	265
14	PA-103	923	0.11	7.35	308
16	PA-31	1185	0.87	16.84	395
17	PA-92	1230	0.00	0.01	410
18	PA-94	1235	0.00	0.02	412
19	PA-26	1287	0.21	2.28	429
20	PA-75	1325	0.01	0.02	442
21	PA-72	1359	0.00	0.01	453
22	PA-17	1482	0.00	0.04	494
23	PA-24	1482	0.04	0.84	494
24	PA-68	1547	0.00	0.01	516
25	PA-87	1651	0.11	1.67	550
26	PA-96	1664	0.31	6.54	555
27	PA-52	1915	0.00	0.02	638
28	PA-20	1959	0.00	0.01	653

29	PA-50	1960	0.01	0.06	653
30	PA-48	2010	0.00	0.03	670
31	PA-65	2040	0.00	0.01	680
32	PA-54	2098	0.00	0.01	699
33	PA-59	2119	0.00	0.02	706
34	PA-23	2139	1.86	67.47	713

Nuc Pos is abbreviated for the location of the insertion in nucleotide positions. Std Dev is abbreviated for standard deviation of the RdRp activity. AA pos is abbreviated for the location of the insertion in amino acid positions. ^aRelative polymerase activity, which was normalized against WT.

Table S5. Viable PB1, PB2 and PA mutant viruses in 3 passages.

PB1			PA			PB2		
Passag e 1	Passag e 2	Passag e 3	Passag e 1	Passag e 2	Passag e 3	Passag e 1	Passag e 2	Passag e 3
82	82	82	89	100	100	155	162	158
89	89	89	100	101	101	161	164	162
157	157	194	101	119	119	189	182	165
164	190	277	119	126	126	259	183	169
169	194	334	126	135	135	358	185	180
190	277	534	135	145	145	363	186	181
194	334	542	143	146	146	364	188	189
199	534	569	144	149	161	414	194	460
200	542	588	146	161	180	415	219	463
277	550	599	149	242	235	446	220	487
317	569	602	161	299	240	456	222	488
334	588	611	175	345	269	459	291	489
341	597	618	177	349	298	463	293	602
349	599	629	180	351	335	479	359	676
359	602	669	181	359	346	480	361	759
382	609	672	240	361	358	487	362	768
534	611	693	241	393	360	488	397	818
542	618	781	242	415	361	510	452	823
550	622	789	265	513	390	512	453	824
569	626	875	298	549	415	603	457	937
588	629	880	299	552	448	676	459	975
599	669	897	304	569	453	721	486	1198
602	672	925	312	595	513	762	487	1311
609	693	927	315	646	552	810	510	1425
611	771	934	335	647	595	818	512	1528

618	781	958	345	648	647	826	602	1552
622	789	961	356	649	792	839	818	1553
626	875	980	358	695	914	844	818	1607
629	880	989	359	789	918	846	826	1608
659	897	1026	361	794	966	859	843	1669
669	925	1037	368	914	971	866	877	1742
672	927	1050	393	917	980	869	935	1743
693	934	1117	395	918	1031	875	962	1979
771	948	1123	415	951	1032	877	1002	1980
781	958	1134	416	959	1067	879	1053	1993
782	961	1171	417	966	1073	889	1060	2093
789	980	1179	447	971	1075	894	1061	
875	984	1313	475	980	1075	898	1080	
880	989	1472	476	1030	1077	906	1085	
897	993	1476	513	1031	1077	917	1135	
925	999	1637	527	1032	1084	925	1172	
927	1026	1708	529	1035	1085	928	1240	
934	1037	1752	575	1046	1086	935	1272	
948	1050	1864	595	1067	1088	946	1339	
958	1076	1931	640	1072	1181	950	1528	
961	1084	2066	642	1074	1183	962	1552	
980	1102		646	1074	1306	978	1669	
984	1108		647	1075	1402	1002	1846	
989	1117		648	1077	1442	1019	1979	
993	1123		649	1077	1500	1020	1983	
1026	1134		675	1083	1503	1050	1993	
1037	1168		683	1085	1512	1076	1994	
1050	1171		683	1085	1514	1090	2059	
1076	1179		686	1086	1610	1096	2069	
1084	1189		687	1087	1649	1105	2097	
1102	1313		694	1088	1650	1122		
1108	1472		695	1109	1753	1144		
1117	1476		697	1110	1757	1185		
1123	1510		715	1160	1759	1207		
1134	1637		737	1166	1763			
1168	1708		771	1273	1791	1216		
1171	1726		773	1306	1822	1225		
1179	1752		781	1312	1825	1227		
1189	1864		822	1347	1845	1228		
1313	1931		886	1379	1873	1232		
1332	2036		899	1390	1913	1237		

1353	2066	914	1394	1916	1243
1357		917	1400	1920	1247
1472		918	1402	1924	1267
1476		926	1403	1926	1284
1481		953	1404	1927	1291
1486		955	1408	1929	1299
1507		965	1442	1929	1312
1510		966	1443	1978	1314
1636		970	1460	2020	1323
1637		971	1462	2070	1329
1708		973	1494		1333
1726		980	1495		1351
1728		986	1503		1358
1752		1007	1503		1360
1830		1028	1506		1362
1851		1028	1514		1367
1864		1030	1515		1383
1874		1031	1517		1384
1894		1032	1524		1392
1915		1045	1548		1395
1927		1046	1565		1404
1931		1047	1566		1421
1938		1056	1609		1428
2021		1057	1622		1431
2036		1063	1632		1457
2041		1064	1633		1462
2058		1066	1644		1463
2066		1066	1647		1477
2077		1070	1648		1493
2119		1071	1649		1496
2143		1072	1650		1498
2209		1073	1669		1502
2230		1074	1670		1505
2232		1075	1753		1515
2265		1076	1773		1524
		1077	1791		1525
		1081	1792		1527
		1082	1804		1552
		1083	1822		1567
		1084	1825		1635
		1085	1830		1657

1086	1842	1657
1087	1844	1668
1088	1845	1675
1110	1853	1676
1110	1855	1678
1160	1928	1702
1166	1932	1720
1181	1961	1720
1205	1978	1728
1208	1985	1811
1273	1998	1916
1287	2006	1918
1306	2009	1919
1311	2070	1923
1362		1946
1364		1967
1364		1971
1376		1980
1378		1994
1378		2050
1380		2058
1395		2059
1400		2096
1401		2128
1402		2135
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