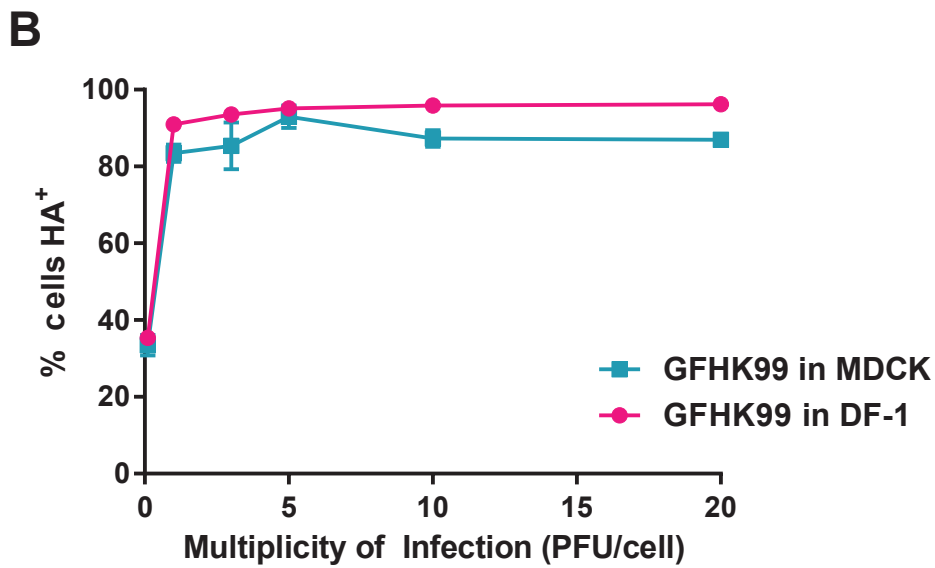
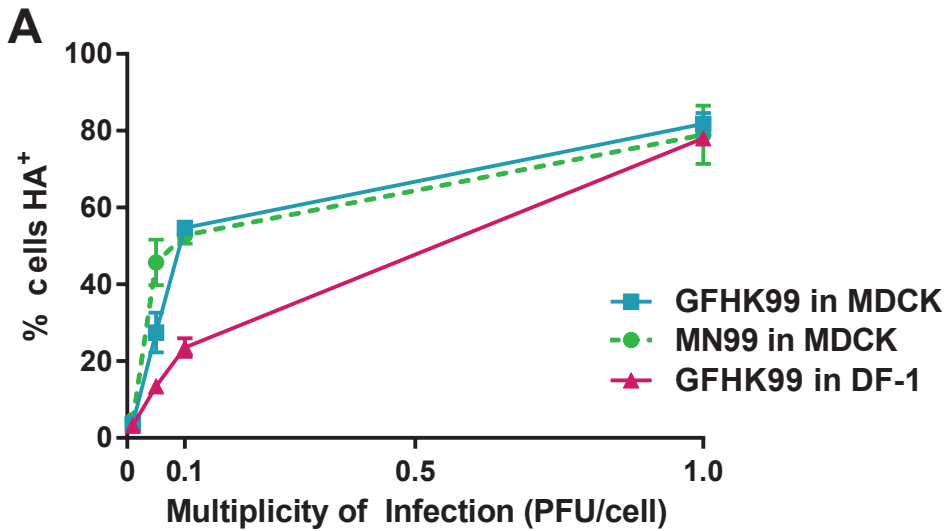
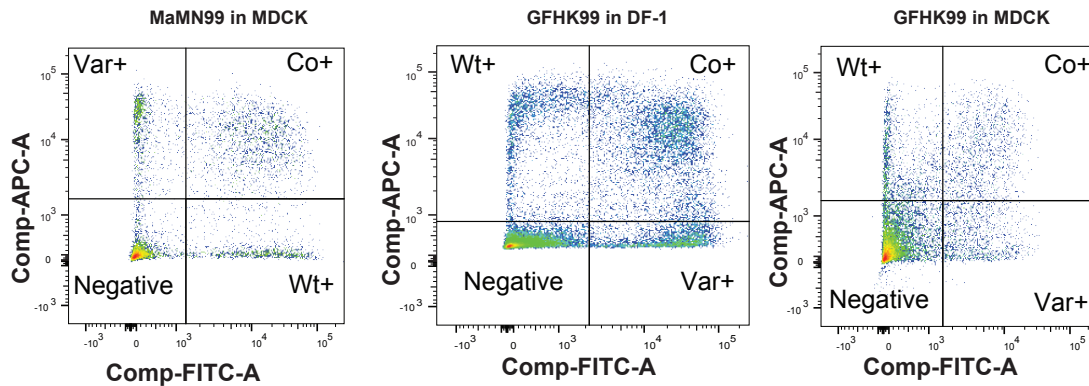


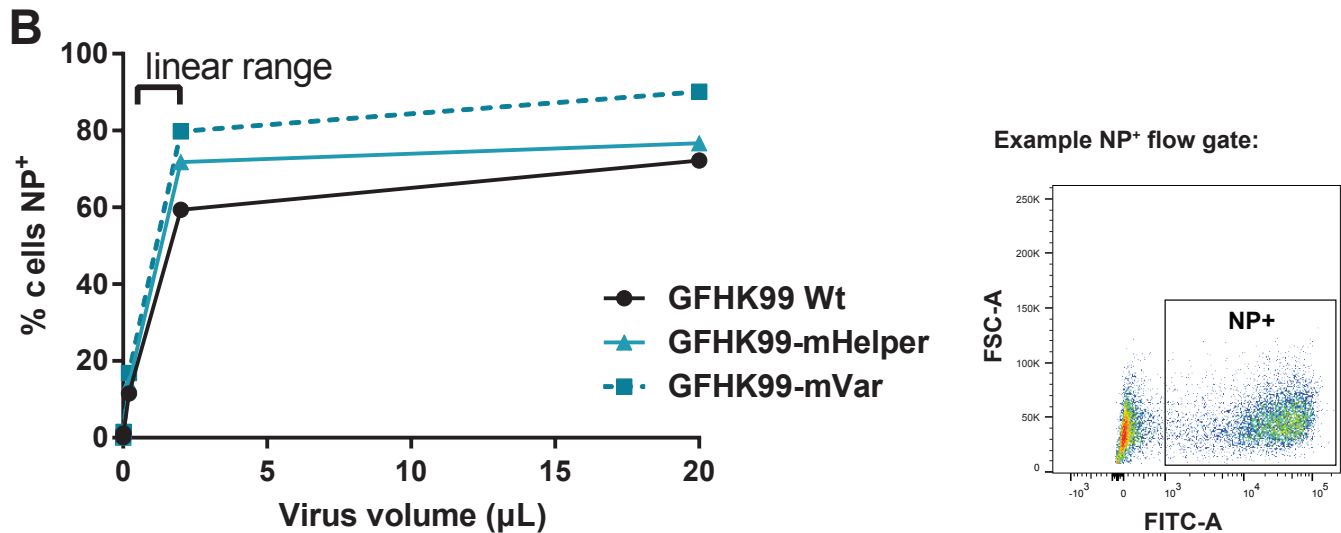
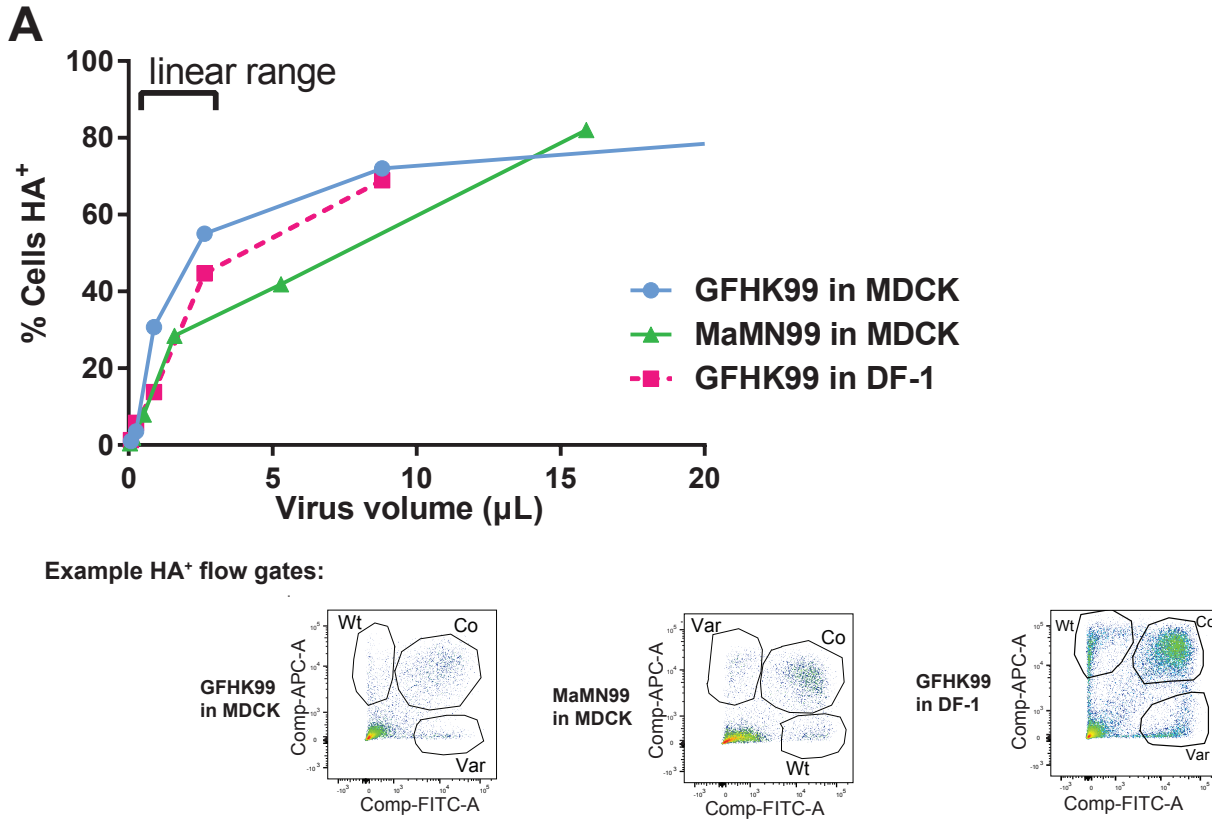
**Supplementary Figure 1 | Co-infection and reassortment of GFHK99 viruses in a human cell line.** Synchronized, single cycle co-infections were performed for a 1:1 mixture of GFHK99 virus in A549 cells. A) HA expression and B) reassortment was measured as described in Figure 1.



Example HA<sup>+</sup> flow gates:



**Supplementary Figure 2 | Flow cytometry of HA expression represents infection level across MOIs for single cycle growth assays.** Triplicate or duplicate wells of cells were harvested 24 h post infection and stained to detect surface expression of HA and HIS epitope tags. Panel A) corresponds to Figure 2 A-C and Panel B) corresponds to Figure 2 D-E. Flow gating was performed by excluding cell debris and multiplet cells. Quadrant gates were used to quantify each population.



**Supplementary Figure 3 | Titration of virus stocks for HA expressing units and NP expressing units by flow cytometry.** A) The doses to be used in RNA kinetics studies shown in Figure 6 were determined via flow titration of HA expressing units in relevant cell lines. GFHK99 and MaMN99 virus mixtures were titrated in MDCK and DF-1 cell lines to calculate HA expressing units/mL in each virus-cell line combination. Serial dilutions of virus were used to infect cells under synchronized, single cycle conditions. Cells were harvested at 24 h post infection and stained for epitope tags. Data points of percent cells positive within the linear range were used to calculate the viral titer. B) GFHK99 viruses used in mRNA sequencing experiments were titered in DF-1 cells. Previous experiments indicated DF-1 cells were more permissive to infection and thus should give a more sensitive measurement of virus present. As the virus strains used did not contain epitope tags, virus detection was accomplished through cell permeabilization and detection of the viral NP protein. Data points within the linear range were used to calculate viral titers. Examples of flow cytometry gates show selection of HA epitope tag or internal NP expressing singlets following gating to exclude cell debris and doublets.

**Table 1. Genotypes of viruses used in this study.**

	<b>PB2</b>	<b>PB1</b>	<b>PA</b>	<b>HA</b>	<b>NP</b>	<b>NA</b>	<b>M</b>	<b>NS</b>
<b>MaMN99 Var</b>	G399A	G573A	G402A	A344G	A414G	G548A	A433G	A458G
<b>GFHK99 Var<sub>1</sub></b>	A285G	A420G	A426G	T341C	T327C	T295C	A349G	T329C
<b>GFHK99 Var<sub>2</sub></b>	300G, 303T, 306C, 459C, 461A, 467T	282C, 285C, 288G, 420G, 426C, 432T	351G, 354T, 357T, 501G, 504T, 507T	338G, 351C, 344C, 432G, 435A, 438T	345G, 351A, 354G, 485C, 488A, 494A	424G, 430A, 433A, 583G, 586C, 589C	340A, 343G, 349G, 439A, 442T, 445G	386T, 389A, 392G, 479G, 482C, 488G
<b>GFHK99 mVar<sub>1</sub></b>	A2151G, C2164T	A2193G, A2185C	C2064A, A2061G	T1574C, G1589A	G1442A, T1411C	C1315T, G1300A	A818C, G815A	A694C, G690A
<b>GFHK99 mVar<sub>2</sub></b>	A2127G, A2124G	C2175T, T2184C	C2017T, A2019G	G1553A, G1556A	G1383A, A1374G	A1255C, A1240C	C809T, T806C	A681G, C678T

**Table 2. Primers for the differentiation of Wt and Var<sub>1</sub> by HRM.**

<b>MaMN99<sup>1</sup> Primers</b>	
MN99 PB2 337 F	CCGACAACAAGCACAGTTCA
MN99 PB2 420 R	GCCAAAGGTCCCATGTTTTA
MN99 PB1 522 F	CCTCAAGGACGTGATGGAAT
MN99 PB1 622 R	CCATTTTCTTGGTCATGTTGTC
MN99 PA 379 F	GAAATTGGAGTGACACGGAGA
MN99 PA 461 R	TGAATGTGTGTCTTCTCGGATT
MN99 HA 322 F	AAACCTGGGACCTTTATGTGG
MN99 HA 402 R	TGAGCGATGCATAGTCTGGT
MN99 NP 378 F	CGACAAAGAAGAGATCAGAAGGA
MN99 NP 457 R	TCATCAAATGGGTGAGACCA
MN99 NA 522 F	TACCAGGCAAGGTTTGAAGC
MN99 NA 605 R	GCCCGTTACTCCAATTGTCA
MN99 M 404 F	TGCATGGGCCTCATATACAA
MN99 M 493 R	ATCAGCAATCTGCTCACACG
MN99 NS 389 F	GGCCATTATGGACAAGAGGA
MN99 NS 483 R	CGTCTGTGAAAGCCCTCAGT
<b>GFHK99<sup>2</sup> Primers</b>	
WF10 PB2 240 F	TGAGCAAGGCCAAACTCTTT
WF10 PB2 320 R	CACGTTACAGCCAGAGGTGA
WF10 PB1 362 F	TTGTCCAGCAAACGAGAGTG
WF10 PB1 441 R	AGCCGGCTGGTTTCTATTC
WF10 PA 386 F	GTGTGACACGGAGGGAAGTT
WF10 PA 461 R	TGGATATGTGTTTTCTCGGATTT
WF10 HA 278 F	CCCTTCTTGTGACCTGCTGT
WF10 HA 364 R	CCAGGGTAACACGTTCCATT
WF10 NP 279 F	CCTAGAGGAACATCCCAGTGC
WF10 NP 369 R	CAGCTCTCTCACCCATTTCC
WF10 NA 270 F	ATTGGTCAAACCGCAATGT
WF10 NA 346 R	GCCTGCAGAAAGCCTAATTG
WF10 M 291 F	ACCCAACAACATGGACAGG
WF10 M 373 R	TGCAACTTCCTTTGCTCCAT
WF10 NS 265 F	CTATCGCTTCAATGCCTGCT
WF10 NS 357 R	CTTTCTGCTTGGGAATGAGC

<sup>1</sup>A/mallard/Minnesota/199106/99 [H3N8], also referred to as “MN99”

<sup>2</sup>A/guinea fowl/Hong Kong/WF10/99 [H9N2], also referred to as “WF10”

**Table 3. Primers for the differentiation of Wt and Var<sub>2</sub> by PCR.**

<b>GFHK99 WT Virus Primers</b>	
WF10wt PB2 286F	GACAGGGTAATGGTATCACCT
WF10wt PB2 480R	GGCCAGGGTTCATGTCAACCCT
WF10wt PB1 266F	GGTATGCACAAACAGATTGTGTAT
WF10wt PB1 440R	CCGGCTGGTTTCTATTCAAT
WF10wt PA 337F	TCTTCCGGACCTATACGACTA
WF10wt PA 521R	CTTCATCAAGGGTGTAGTCAG
WF10wt NP 336F	GAAGGAGAGACGGGAAATG
WF10wt NP 505R	GGCTCTTGTCTCTGGTATG
WF10wt HA 323F	CGTCGAAAGATCATCAGCTGTA
WF10wt HA 451R	CAGGTTGTGTCTGGGAAGATT
WF10wt NA 413F	CTTGGGCAGGGAACCACTTTG
WF10wt NA 601R	CCCAGTGACACAAACATGTAAC
WF10wt M 328F	GAAGCTGAAGAGGGAAATGACA
WF10wt M 457R	AAGAGCCACTTCTGTGGTC
WF10wt NS 374F	CATTAGAGTGGACCAGGCA
WF10wt NS 499R	CCCACTATTGCTCCTTCATCT
<b>GFHK99 VAR<sub>2</sub> Virus Primers</b>	
WF10help PB2 286F	GACAGGGTAATGGTgTcTCCc
WF10help PB2 480R	GGCCAGGGTTCATaTCAAcTcG
WF10help PB1 266F	GGTATGCACAAACAGAcTGcGTgT
WF10help PB1 440R	CCGGCTGaTTTCTgTTCAAc
WF10help M 331F	GCTGAAGAGAGAGATGACG
WF10help M 459R	CAAGAGCCACTTCCGTAGTTA
WF10help NS 373F	GCATTAGAGTGGATCAAGCG
WF10help NS 496R	ACTATTGCCCTTCGTCC

**Table 4. Primers for identification of strand specific RNA species.**

<b>MaMN99 Reverse Transcription and PCR Primers</b>	
MN99 NS 552F	GGCCGTCATGGTGGCGAAT AATGCAATTGGAATCCTCAT
MN99 NS mRNAtag_dTR 13	CCAGATCGTTCGAGTCGT TTT TTT TTT TTT TTT AGTACTAAATAAG
MN99 NS cRNAtag_dTR 25	GCTAGCTTCAGCTAGGCATC AGTAGAAACAAGGGTGTTTTTTAG
MN99 NS 795F	CTTGCAGGCATTGCAAC
MN99 NS 643R	CGGACTCCCCAAGCGAATCTC
<b>GFHK99 Reverse Transcription and PCR Primers</b>	
WF10 vRNA NS 520F	GGCCGTCATGGTGGCGAAT CCCTTCCAGGACATACTGAC
WF10 NS mRNAtag_dTR 13	CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTTATCATTAAATAAG
WF10 NS cRNAtag_dTR 25	GCTAGCTTCAGCTAGGCATC AGTAGAAACAAGGGTGTTTTTTATC
WF10 NS 592R	TCATTCCATTCAAGTCCTCCGATGAG
WF10 NS 791F	CCTTTATGCAAGCCTTACAAC
<b>MaMN99 and GFHK99 Tagged PCR Primers</b>	
vRNA	GGCCGTCATGGTGGCGAAT
cRNA	GCTAGCTTCAGCTAGGCATC
mRNA	CCAGATCGTTCGAGTCGT