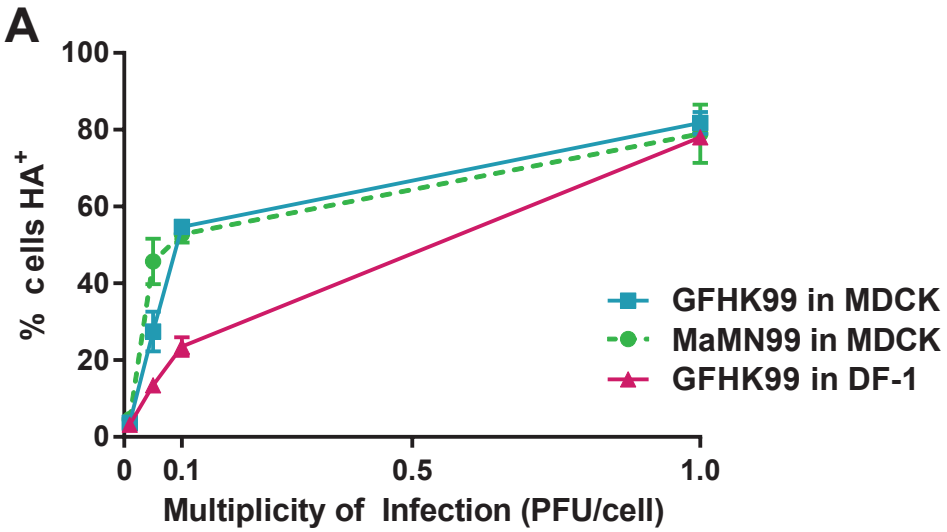
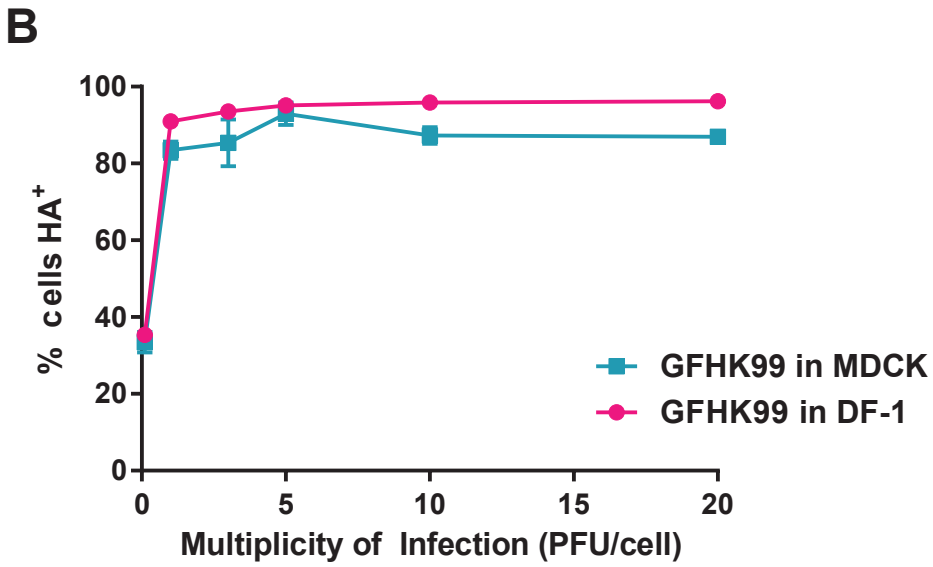
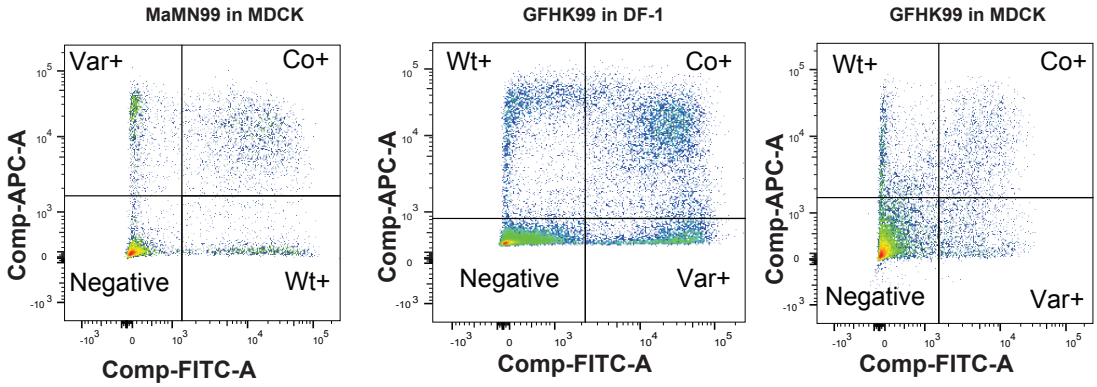
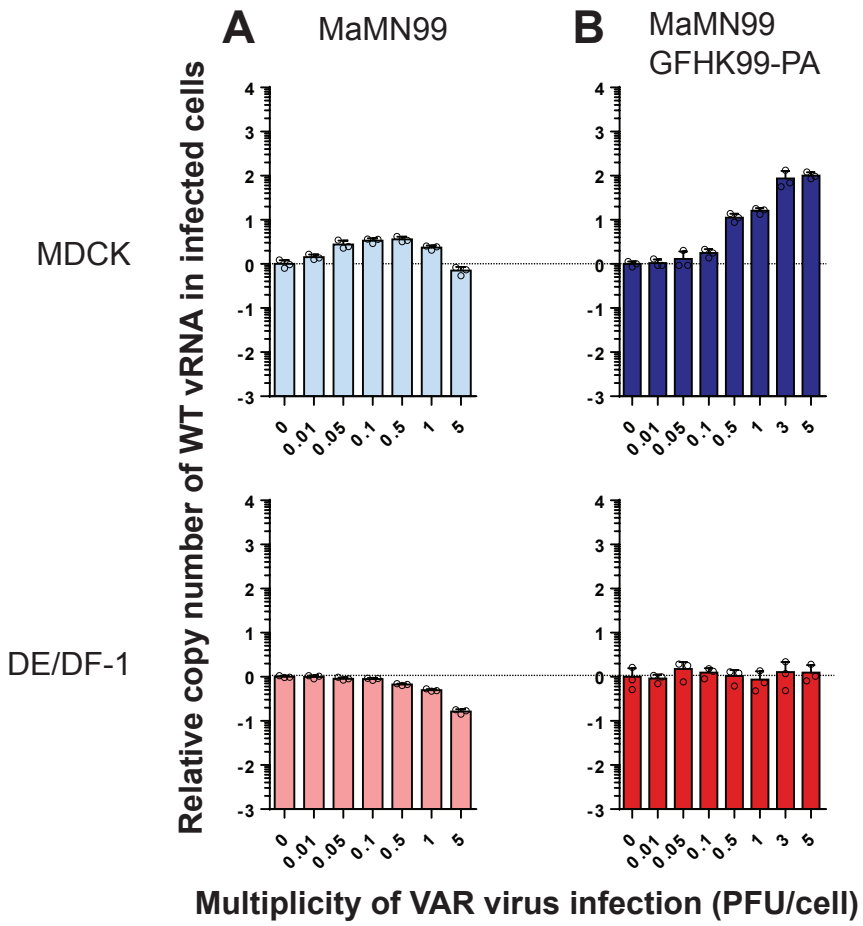




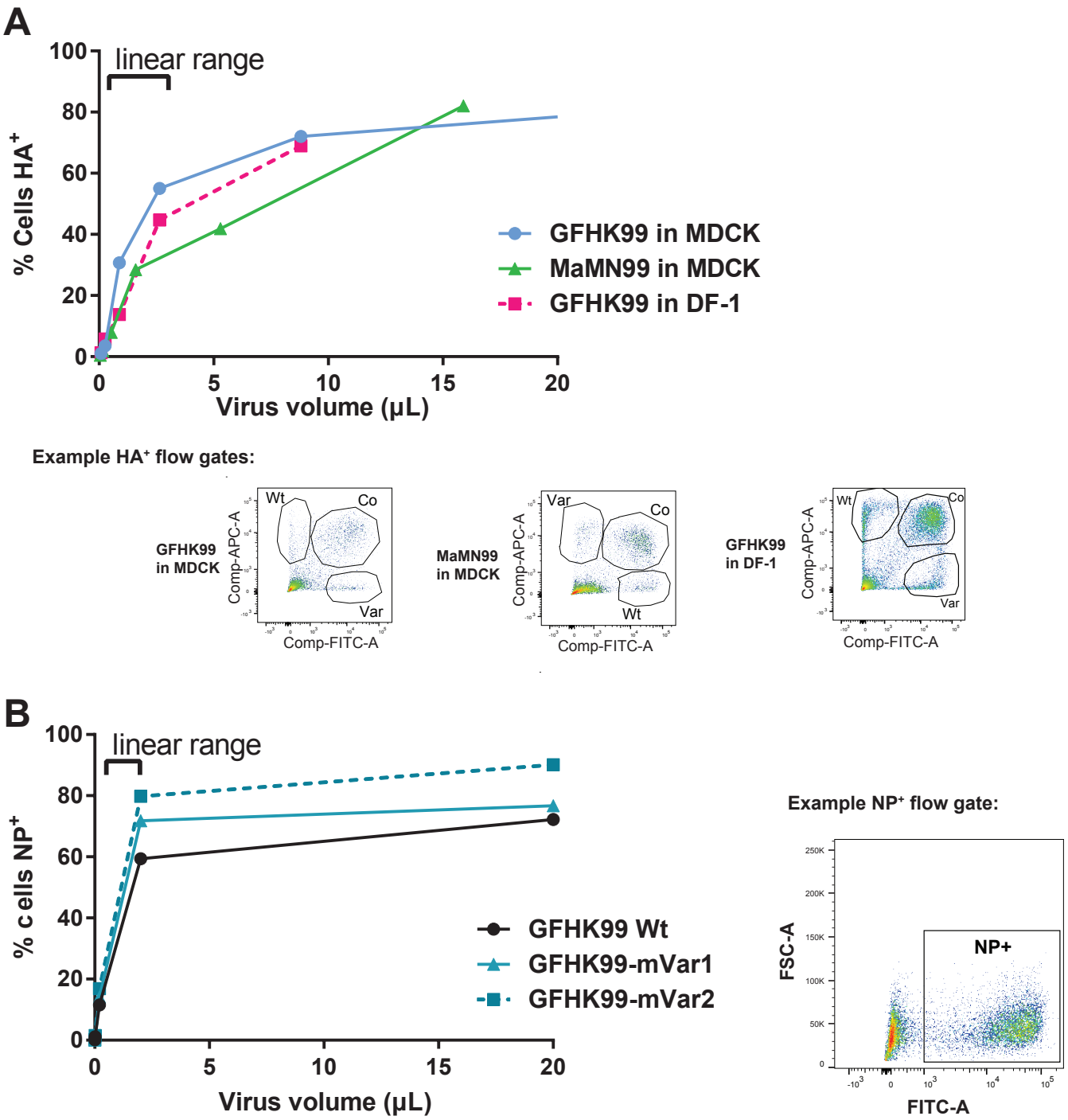
Example HA+ flow gates:



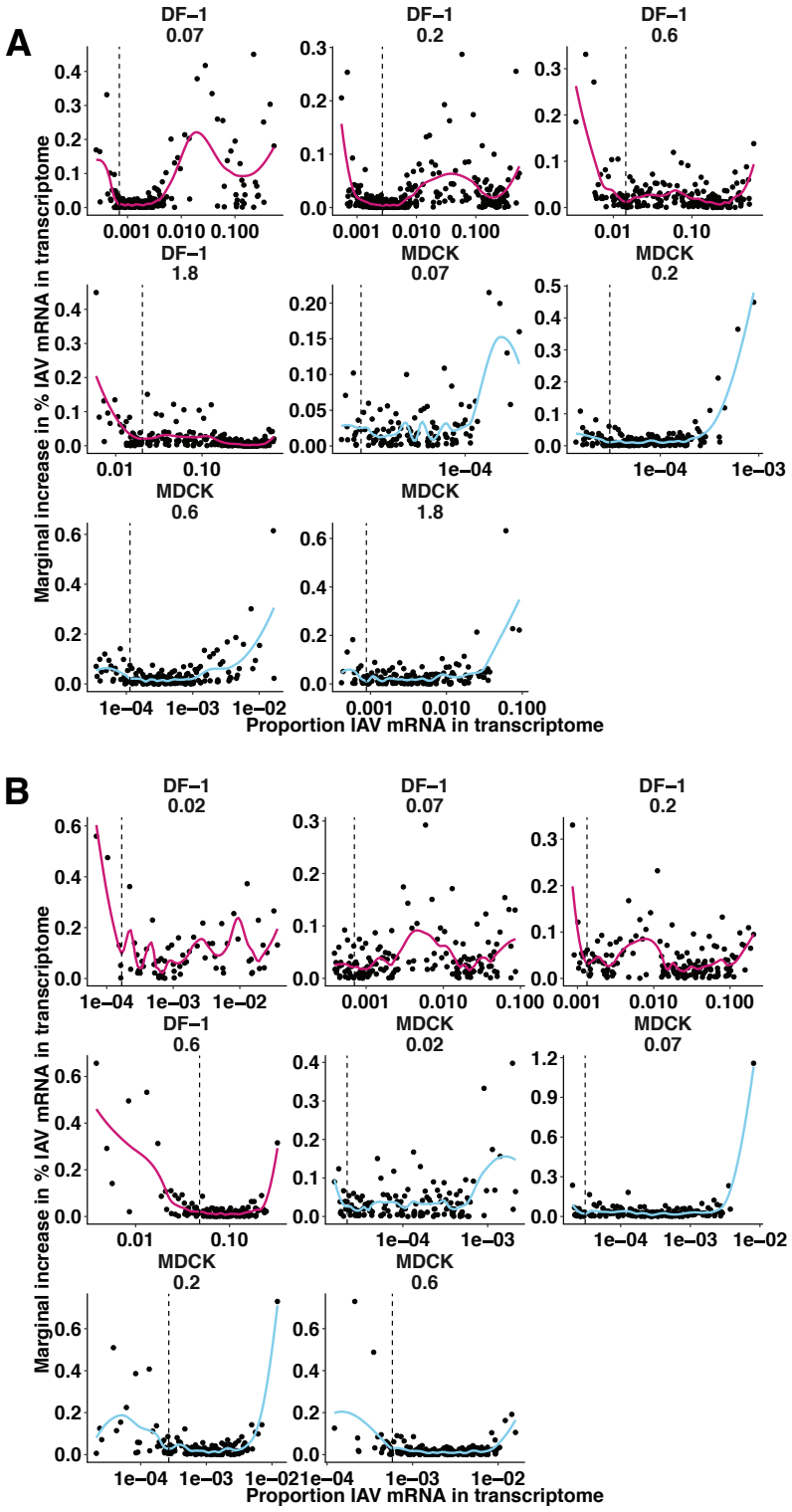
Supplementary Figure 1 | HA positive cells detected by flow cytometry indicate levels of infection achieved across MOIs for the single cycle growth assays. (Relates to Figure 2) Triplicate or duplicate wells of cells were harvested 24 h post infection and stained to detect surface expression of HA and HIS epitope tags. Panel A) corresponds to Figure 2 A-C and Panel B) corresponds to Figure 2 E-F. Flow gating was performed by excluding cell debris and multiplet cells. Quadrant gates were used to quantify each population.



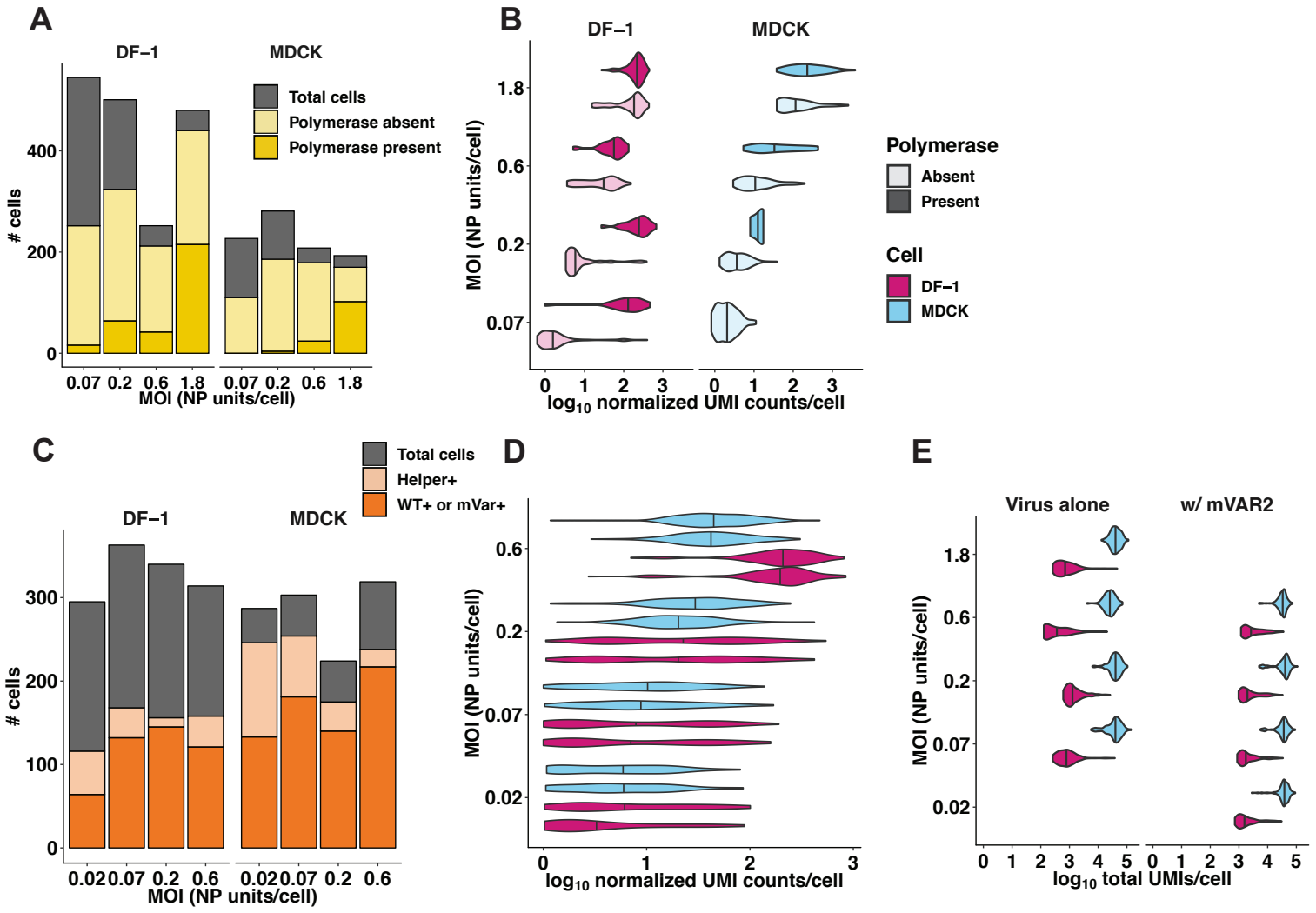
Supplementary Figure 2 | Introduction of PA gene segment from GFHK99 virus into MaMN99 virus confers increased dependence on multiple infection for vRNA synthesis. (Relates to Figures 3 and 4) Cells were coinfected with WT virus and increasing doses of VAR virus. WT virus MOI was 0.005 PFU per cell. The fold change in WT vRNA copy number, relative to that detected in the absence of VAR virus, is plotted for MaMN99 virus (A) and MaMN99-GFHK99-PA virus (B). Data shown in panel (A) are also shown in Figure 3. MaMN99 virus was tested in MDCK and DE cells; MaMN99 GFHK99-PA virus was tested in MDCK and DF-1 cells.



Supplementary Figure 3 | Titration of virus stocks for HA expressing units and NP expressing units by flow cytometry. (Relates to Figures 5 and 6) A) The doses to be used in RNA kinetics studies shown in Figure 5 were determined via flow titration of HA expressing units in the 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 shown in Figure 6 were titered in DF-1 cells. DF-1 cells are more permissive to infection and thus give more sensitive detection of infectious virus compared to MDCK cells. 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. Representative flow plots show gates used following exclusion of cell debris and doublets.



Supplementary Figure 4 | Preliminary analysis of single-cell mRNA sequencing data to exclude cells with viral mRNA that are likely uninfected. (Relates to Figure 6) A) Within each infection, cells in which viral RNA was detected were rank ordered by the proportion of their transcriptome that was comprised of viral RNA (% viral RNA), and the relative gain in % viral RNA from one cell to the next was plotted against the proportion of viral RNA in each cell. Local regression was performed separately for each infection, and the first local minimum of the resulting functions (indicated by dashed lines) indicated the point at which the marginal gain in % viral RNA was more consistent and less sensitive to the % viral RNA of the prior cell. Cells with % viral RNA values below this threshold were deemed falsely positive and considered uninfected for the analyses shown in **Figure 6** and **Supplementary Figure 5**. Facets indicate individual infections, with lines colored by cell type (DF-1 = pink, MDCK = blue). B) The same analysis in panel A) was applied to the data from the second experiment, in which cells were co-inoculated with a 1:1 mixture of WT and $mVAR_1$ viruses, as well as $mVAR_2$ virus at an MOI of 0.1 PFU/cell in DF-1 cells, or 1.0 PFU/cell in MDCK cells. Only cells containing all eight $mVAR_2$ segments were analyzed in this manner.

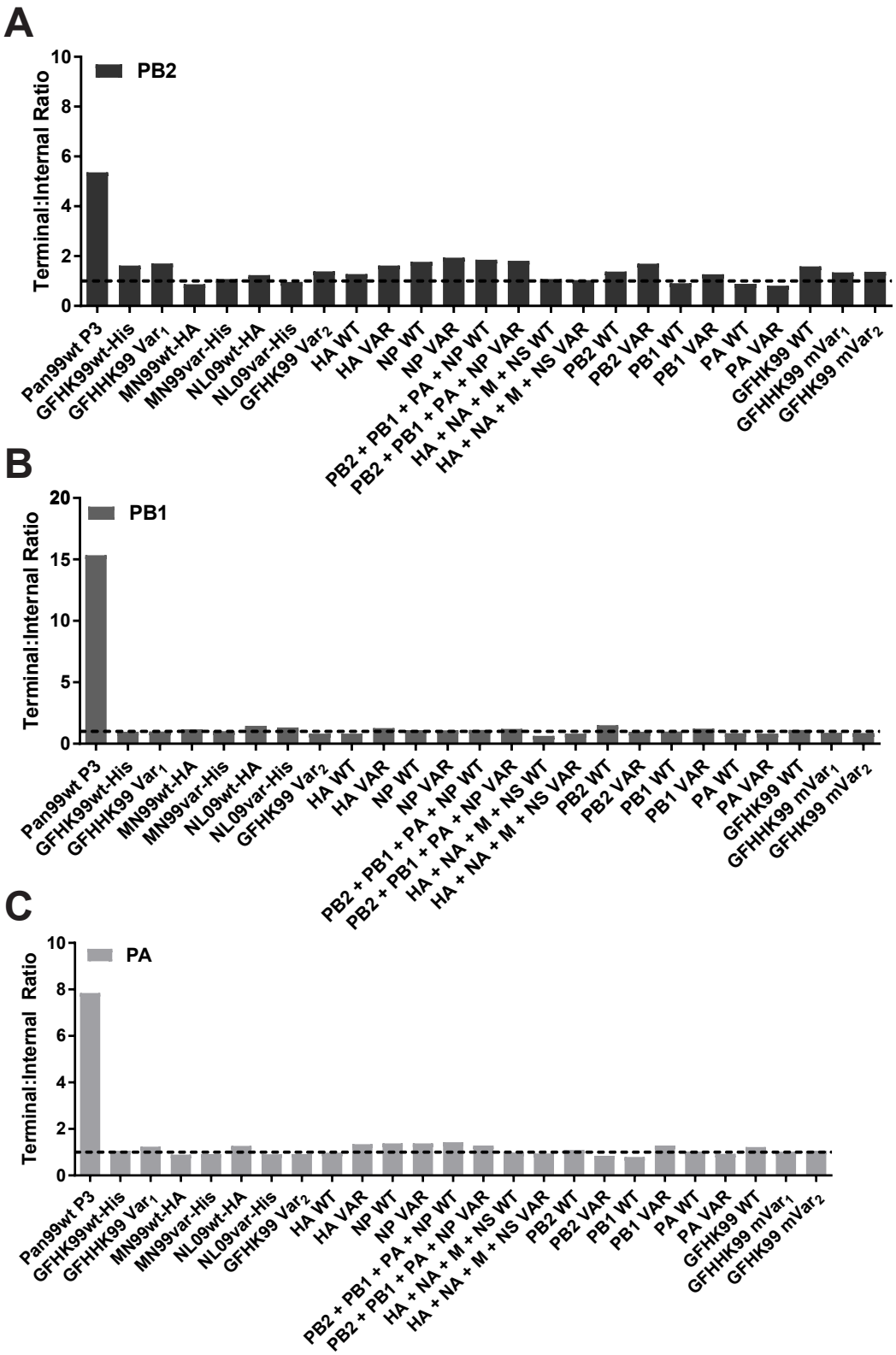


Supplementary Figure 5 | Validation of single-cell mRNA sequencing data. (Relates to Figure 6) A) The total number of cells sequenced, infected, and containing PB2, PB1, PA, and NP segments are represented by the cumulative heights of the gray, light yellow, and dark yellow bars, respectively. Cells that were excluded by the analysis shown in **Supplementary Figure 4** are contained within the gray bar. B) DF-1 or MDCK cells were infected with GFHK99 WT virus at four different MOIs (0.07, 0.2, 0.6, 1.8 NP units per cell), and the transcriptomes of 1,873 individual infected cells were sequenced using the 10x Genomics Chromium platform. Violin plots show distributions of $\log_{10}$-transformed viral mRNA abundance, for all eight viral transcripts combined, in individual infected cells. The data are stratified by cell type (MDCK cells in blue, DF-1 cells in pink), MOI, and the presence of polymerase complex (light shading = cells missing PB2, PB1, PA, or NP; dark shading = cells in which PB2, PB1, and PA are all detected). The absence of a dark shaded distribution for MDCK cells at the lowest MOI is due to the absence of any cells in which all four of these segments were detected. C) The total number of cells sequenced, containing all eight $mVAR_2$ genome segments, and infected with either WT or $mVAR_1$ virus are represented by the cumulative heights of the gray, light orange, and dark orange bars, respectively. As in panel A), cells that were deemed falsely positive are contained within the gray bar. D) Distributions of viral UMIs per cell are shown separately for WT (bottom of each cell-MOI pair) and $mVar_1$ (top of each cell-MOI pair). Vertical lines represent the median of each distribution. E) The distributions of UMIs detected per cell are shown for each cell type, MOI, and infection type. Vertical lines represent the median of each distribution.

Supplementary Figure 6 | Alignment of MaMN99 and GFHK99 virus PA and PA-X amino acid sequences. Sequences and functional domains of the PA protein are displayed in panel (A), and those of the PA-X protein are shown in panel (B). N-ter = the N-terminal endonuclease domain[1]; C-ter = C-terminal domain[1]; X-ORF = the 61 aa region of PA-X encoded in frame 2 of the PA gene[2]; Active site = the active site of the endonuclease[3]; Dim. Loop = dimerization loop important for formation of polymerase dimers[4]; Site 1 and Site 2 = sites mediating the interaction of PA with cellular Pol II C-terminal domain[5].

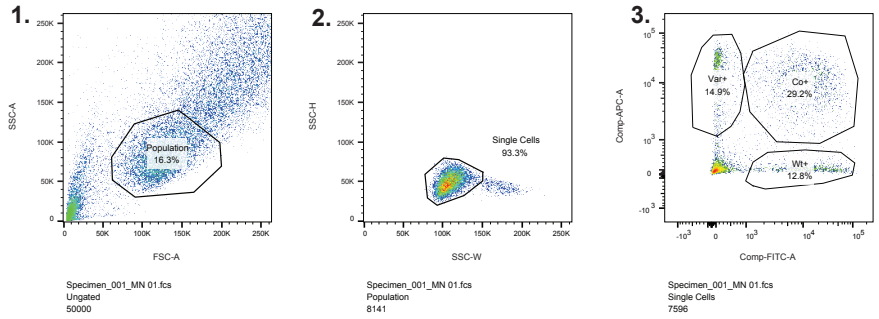
References:

1. Pflug, A., Guilligay, D., Reich, S. *et al.* Structure of influenza A polymerase bound to the viral RNA promoter. *Nature* **516,** 355–360 (2014) doi:10.1038/nature14008
2. Jagger, B. W., et al. "An Overlapping Protein-Coding Region in Influenza A Virus Segment 3 Modulates the Host Response." *Science*, vol. 337, no. 6091, 2012, pp. 199–204., doi:10.1126/science.1222213
3. Dias, A., Bouvier, D., Crépin, T. *et al.* The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. *Nature* **458,** 914–918 (2009) doi:10.1038/nature07745
4. Fan, H., Walker, A.P., Carrique, L. *et al.* Structures of influenza A virus RNA polymerase offer insight into viral genome replication. *Nature* **573,** 287–290 (2019) doi:10.1038/s41586-019-1530-7
5. Lukarska, M., Fournier, G., Pflug, A. *et al.* Structural basis of an essential interaction between influenza polymerase and Pol II CTD. *Nature* **541,** 117–121 (2017) doi:10.1038/nature20594

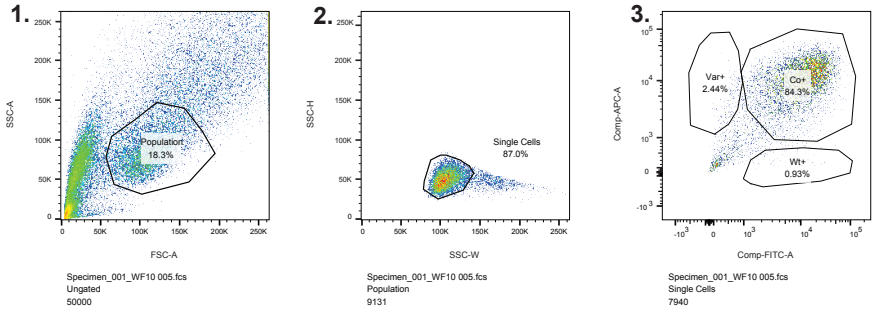


Supplementary Figure 7 | Quanitification of virus stock defective interfering RNA content by ddPCR. Defective RNA content for A) PB2, B) PB1, and C) PA segments was determined using primer pairs targeting terminal and internal portions of each polymerase gene segment to determine their absolute copy number and produce a ratio of terminal:internal copies. All virus stocks used in this study contained low DI content (terminal:internal ratio less than or equal to 2). A DI-rich control virus, Pan99wt P3 (A/ Panama/2007/99 [H3N2]), is included for comparison. This virus stock was passaged three times in MDCK cells at high MOI. For the MaMN99-GFHK99 chimeric viruses, the segments derived from GFHK99 virus are listed in place of the full strain names.

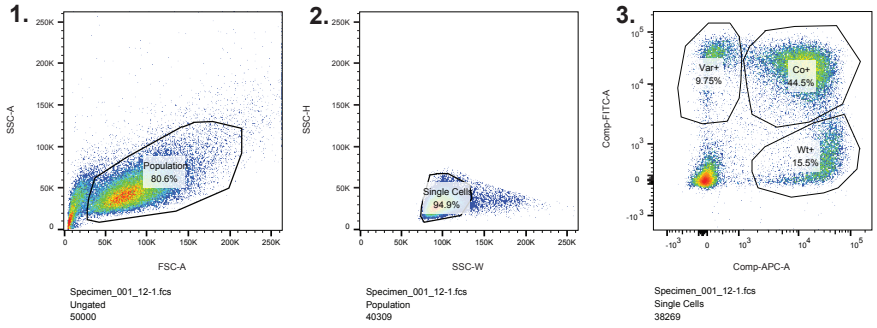
HA expression of MaMN99 in MDCK cells:



HA expression of GFHK99 in MDCK cells:



HA expression of GFHK99 in DF-1 cells:



Supplementary Figure 8 | Example gating for flow cytometry to evaluate HA positive cell numbers. (Relates to Figure 1) Following staining for HA expression 1) a population of cells was first selected by gating out cell debris by SSC-A vs FSC-A. 2) Multiplets were excluded by gating for single cells in SSC-H vs SSC-W. In 3) populations of infected cells were gated by populations expressing the appropriate epitope tag.

Supplementary Table 1. Genotypes of variant viruses

	PB2	PB1	PA	HA	NP	NA	M	NS
MaMN99[1] VAR	G399A	G573A	G402A	A344G	A414G	G548A	A433G	A458G
GFHK99[2] VAR_1	A285G	A420G	A426G	T341C	T327C	T295C	A349G	T329C
GFHK99-VAR_2	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
GFHK99-$mVAR_1$	A2151G, C2164T	A2193G, A2185C	C2064A, A2061G	T1574C, G1589A	G1442A, T1411C	C1315T, G1300A	A818C, G815A	A694C, G690A
GFHK99-$mVAR_2$	A2127G, A2124G	C2175T, T2184C	C2017T, A2019G	G1553A, G1556A	G1383A, A1374G	A1255C, A1240C	C809T, T806C	A681G, C678T
NL09[3] VAR	C273T	T288C	C360T	C305T	A351G	G336A	G295A	C341T

[1]A/mallard/Minnesota/199106/99 (H3N8), also referred to as “MN99”

[2]A/guinea fowl/Hong Kong/WF10/99 (H9N2), also referred to as “WF10”

[3]A/Netherlands/602/2009 (H1N1)

Supplementary Table 2. Primers for the differentiation of WT and VAR by HRM

MaMN99 Primers	
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
GFHK99[1] Primers	
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	ATTGGTCAAAACCGCAATGT
WF10 NA 346 R	GCCTGCAGAAAGCCTAATTG
WF10 M 291 F	ACCCAAACAACATGGACAGG
WF10 M 373 R	TGCAACTTCCTTTGCTCCAT
WF10 NS 265 F	CTATCGCTTCAATGCCTGCT
WF10 NS 357 R	CTTTCTGCTTGGGAATGAGC

[1]These primers were used for differentiation of GFHK99 WT and VAR_1 viruses.

Supplementary Table 3. Primers and Probes for the differentiation of WT and VAR in ddPCR

GFHK99 WT Virus Primers	
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	GGCTCTTGTTCTCTGGTATG
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
GFHK99 VAR_2 Virus Primers	
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	ACTATTGCCCCTTCGTCC
MaMN99 Virus Primers and Probes	
MaMN99 NP 378 F	CGACAAAGAAGAGATCAGAAGGA
MaMN99 NP 457 R	TCATCAAATGGGTGAGACCA
MaMN99wt NP Probe	FAM-CGT(+C)[a]AA(+G)(+C)(+A)AA(+T) A(+A)TGG-IBFQ
MaMN99var NP Probe	HEX-CGT(+C)AA(+G)(+C)(+G)AA(+T)AATGG-IBFQ
NL09 Virus Primers and Probes	
NL NP 309 F	CCCTAAGAAAACAGGAGGACCC
NL NP 411 R	TTGGCGCCAAACTCTCCTTA
NLwt NP Probe	FAM-AGAC(+G)(+G)(+A)AA(+G)T(+G)GATGA-IBFQ
NLvar NP Probe	HEX-AGACG(+G)(+G)(+A)AGTGGATGA-IBFQ
dkHK78 Virus Primers and Probes	
dkHK78 NP 467 F	CCAACTTGAATGATGCCACA
dkHK78 NP 552 R	TCCTTGCATCAGAGAGCACA

dkHK78wt NP probe	FAM-TGC GTA CTG +G+G+A TGG AC-IBFQ
dkHK78var NP probe	HEX-TGC GTA +CTG +G+A+A TGG AC-IBFQ
QaHK88 Virus Primers and Probes	
QaHK88 NP 313 F	AAGAAAACTGGAGGCCCAAT
QaHK88 NP 400 R	TCCTCCTGATCTCCTCCTTG
QaHK88wt NP Probe	FAM-AGG A+GA +GA+T +G+GA AAA TG-IBFQ
QaHK88var NP Probe	FAM-AGG A+GA +GA+C +G+GA AAA TG-IBFQ

[a] + notation indicates Locked nucleic acid (LNA) bases

Supplementary Table 4. Primers for quantification of viral mRNA and vRNA by ddPCR

MaMN99 Reverse Transcription and PCR Primers	
MaMN99 NS 552F	GGCCGTCATGGTGGCGAAT AATGCAATTGGAATCCTCAT
MaMN99 NS mRNAtag_dTR 13	CCAGATCGTTCGAGTCGT TTT TTT TTT TTT TTT AGTACTAAATAAG
MaMN99 NS 795F	CTTGCAGGCATTGCAAC
MaMN99 NS 643R	CGGACTCCCCAAGCGAATCTC
GFHK99 Reverse Transcription and PCR Primers	
GFHK99 vRNA NS 520F	GGCCGTCATGGTGGCGAAT CCCTTCCAGGACATACTGAC
GFHK99 NS mRNAtag_dTR 13	CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTATCATTAAATAAG
GFHK99 NS 592R	TCATTCCATTCAAGTCCTCCGATGAG
GFHK99 NS 791F	CCTTTATGCAAGCCTTACAAC
MaMN99 and GFHK99 Tagged PCR Primers	
vRNA	GGCCGTCATGGTGGCGAAT
mRNA	CCAGATCGTTCGAGTCGT