

SUPPLEMENTARY MATERIAL for “Assessing baloxavir susceptibility of influenza viruses circulating in the United States during the 2016/17 and 2017/18 seasons”

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Supplementary document containing information on (1) polymerase acidic amino acid substitutions of concern with no evident effect on baloxavir susceptibility, (2) the workflow of the high-content imaging neutralization test (HINT) and (3) pyrosequencing results showing nucleotide changes associated with reduced susceptibility to baloxavir.

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Supplemental Table S1. Viruses containing polymerase acidic amino acid substitutions that did not affect baloxavir susceptibility, tested by HINT, United States, 2016/17 and 2017/18 influenza seasons

Type/subtype	Virus name	Codon ^a	PA AA (% substitution) ^a	EC ₅₀ , nM Mean ± SD ^c	Fold change to control ^d	Fold change to median ^e
A(H3N2)	A/Maine/17/2017	GCA	A37	0.79 ± 0.13	1	1
	A/Maine/15/2017	G/ <u>A</u> CA	A37T/A (44)	0.63 ± 0.10	1	1
	A/Hawaii/26/2017	ATA	I38	0.36 ± 0.12	1	1
	A/Hawaii/28/2017	AT/ <u>A</u> A	I38K/I (27)	0.54 ± 0.12	2	1
	A/West Virginia/06/2018	GAA	E119	0.30 ± 0.09	1	1 ^f
	A/California/148/2017	G/ <u>A</u> AA	E119K/E (40)	0.50 ± 0.16	2	1
	A/Hawaii/67/2016	ATA	I38	1.17 ± 0.20	1	1
	A/Hawaii/89/2016	<u>G</u> TA	I38V (100)	0.51 ± 0.13	1 ^g	1
A(H1N1)pdm09	A/Connecticut/28/2016	ATA	I38	1.90 ± 0.52	1	1
	A/California/153/2016	<u>G</u> TA	I38V (100)	4.09 ± 1.73	2	3
B Yamagata	B/Texas/98/2017	ATC	I38	9.17 ± 4.30	1	2
	B/Oregon/07/2018	<u>G</u> TC	I38V (100)	9.40 ± 1.28	1	2

AA: amino acid; PA: polymerase acidic; SD: standard deviation.

^a Underlined base indicates the nt change.

^b Number in parentheses indicate % containing substitution determined by next generation sequencing (20-25% as threshold)

^c Mean and SD of ≥ three independent tests.

^d Fold change to EC₅₀ of test virus compared to respective sequence-matched control virus. A/California/153/2016 and B/Oregon/07/2018 also contained H144Y and K605R in PA, respectively.

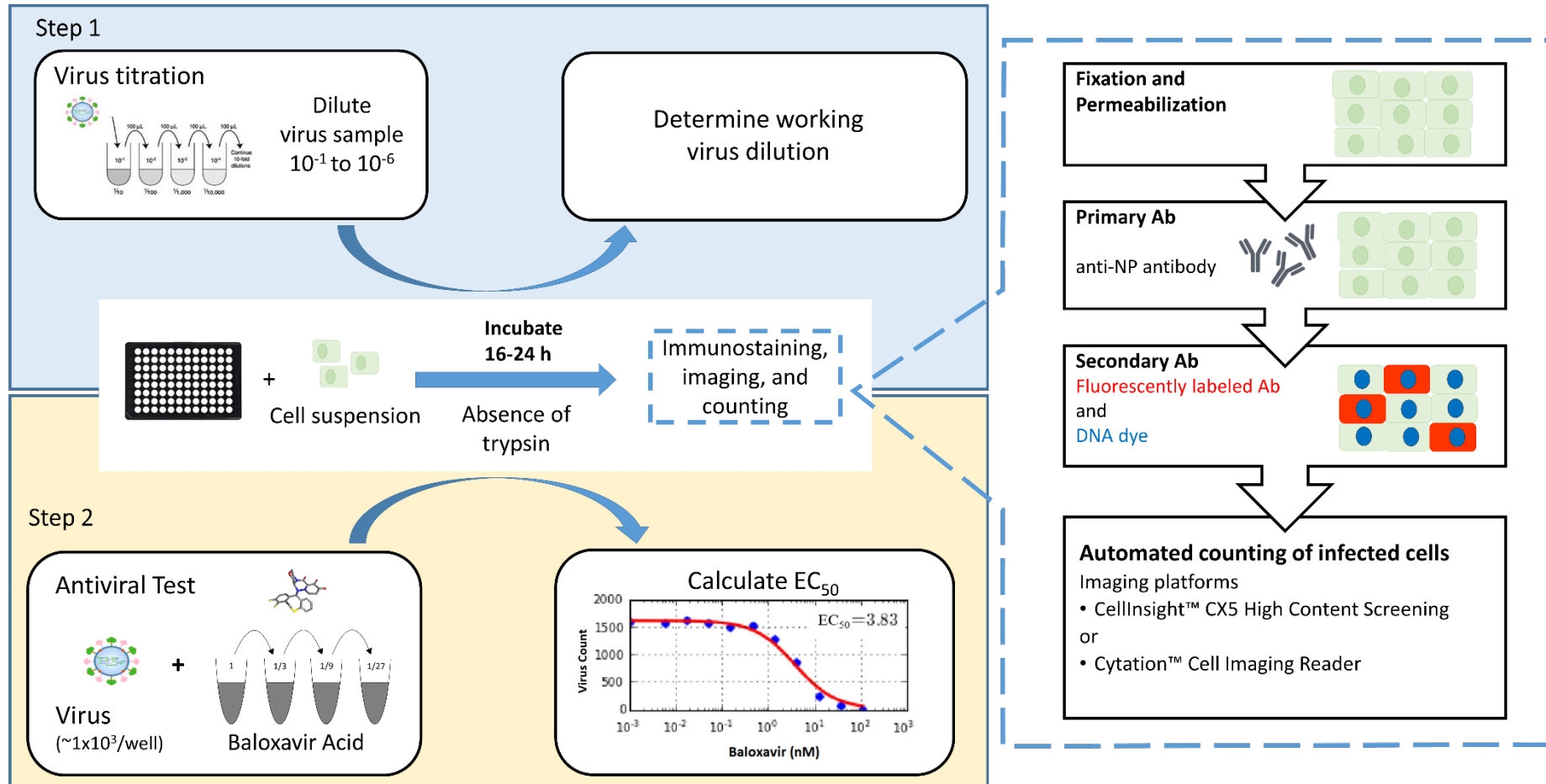
^e Fold change to EC₅₀ of test virus compared with baseline median values: 0.80 for A(H3N2), 1.57 for A(H1N1)pdm09 and 4.92 for B Yamagata (Table 4).

^f Fold change < 0.5 is shown as 1.

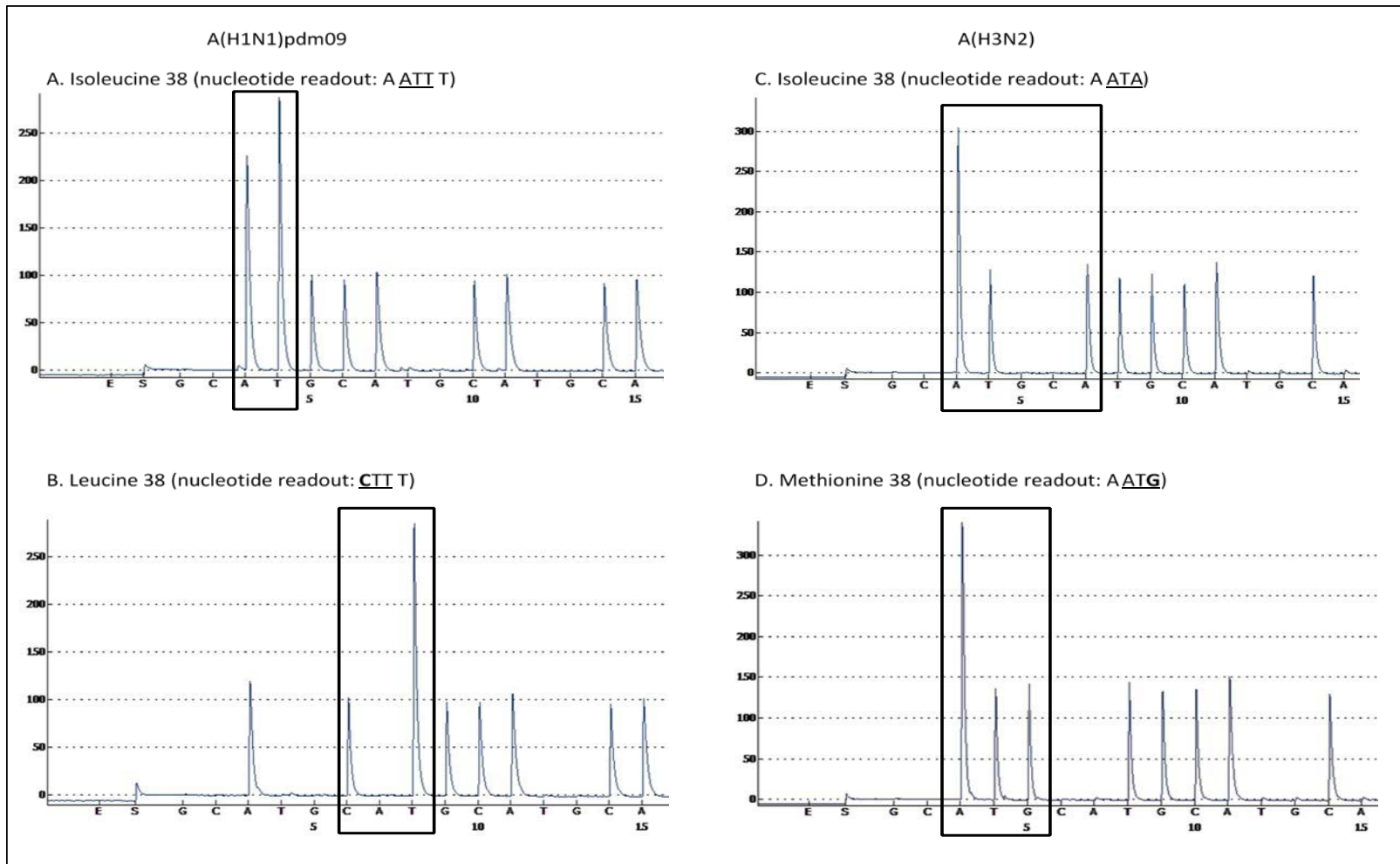
^g Fold change < 0.5 is shown as 1.

PA amino acid substitutions causing ≤ three-fold change in EC₅₀ are not considered as affecting baloxavir susceptibility.

Supplemental Figure S1. High-content imaging neutralization test (HINT) workflow



Supplemental FIGURE S2. Pyrosequencing readouts of polymerase acidic amino acid residue 38 for influenza A(H1N1)pdm09 and A(H3N2) viruses



Pyrosequencing assay conducted as previously described [reference 15].

Forward RT-PCR primer nt58, 5'- GCA ATG AAA GAR TAT GGG G-3'.

Reverse RT-PCR primer nt280, Biotin-5'-TAC TGT TYA CYA CTG TCC AGG CCA-3'.

Sequencing primers: A(H1N1)pdm09 nt91, 5'-GAA ACT AAT AAG TTT GCT GC-3'; A(H3N2) nt95, 5'-CC AAC AAA TTT GCA GC-3'.

Black box designates nt readout containing the PA-38 codon. Sequence readouts: A) A ATT TGC ACA CA; B) A CTT TGC ACA; C) A ATA TGC AC; D) A ATG TGC AC. Underline designates nt triplet encoding amino acid at residue 38 of PA protein. Bold indicates nt change.