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Supplementary Materials for

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The spike receptor-binding motif G496S substitution determines the replication

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fitness of SARS-CoV-2 Omicron sublineage

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The file includes:

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Supplementary Table 1

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Supplementary Figures 1 to 3

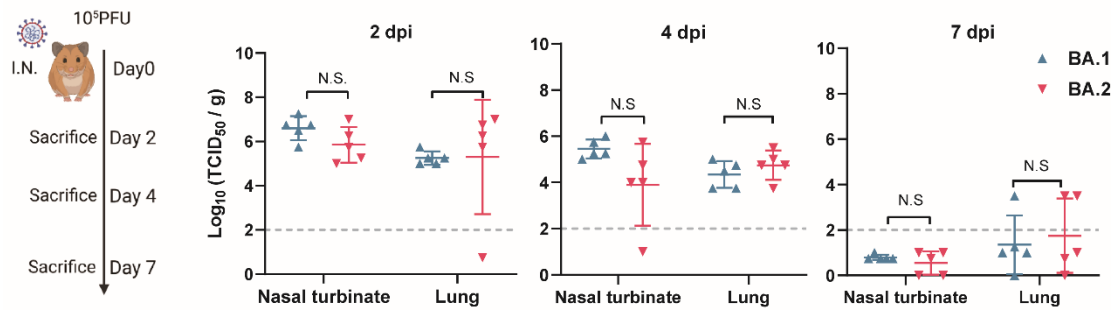
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14 **Supplementary Material**15 **Supplementary Table 1**

Primer	Sequence 5' – 3'
galK-F'	GCACACCTTGTAATGGTGTGAAGGTTTTAATTGTTAC TTTCCTTTACAACCTGTTGACAATTAATCATCGGCA
galK-R'	GTAGAAGTTCAAAGAAAGTACTACTACTCTGTATGG TTGGTAACCAACATCAGCACTGTCCTGCTCCTT
S496-F'	GCACACCTTGTAATGGTGTGAAGGTTTTAATTGTTAC TTTCCTTTACAATCATAT <u>AGTTTCCA</u> ACCCACTAATGG TGTTGGTTACCAACCATAACAGAGTAGTAGTACTTTCTT TTGAACTTCTAC
S496-R'	GTAGAAGTTCAAAGAAAGTACTACTACTCTGTATGG TTGGTAACCAACACCATTAGTGGGTTGGAA <u>ACT</u> ATAT GATTGTAAAGGAAAGTAACAATTAACCTTCAACAC CATTACAAGGTGTGC
G496 _{R498} -F'	GCACACCTTGTAATGGTGTGAAGGTTTTAATTGTTAC TTTCCTTTACAATCATATGGTTT <u>CCG</u> ACCCACTAATGG TGTTGGTTACCAACCATAACAGAGTAGTAGTACTTTCTT TTGAACTTCTAC
G496 _{R498} -R'	GTAGAAGTTCAAAGAAAGTACTACTACTCTGTATGG TTGGTAACCAACACCATTAGTGGG <u>TCCG</u> GAAACCATAT GATTGTAAAGGAAAGTAACAATTAACCTTCAACAC CATTACAAGGTGTGC
S496 _{R498} -F'	GCACACCTTGTAATGGTGTGAAGGTTTTAATTGTTAC TTTCCTTTACAATCATAT <u>AGTTTCCG</u> ACCCACTAATGG TGTTGGTTACCAACCATAACAGAGTAGTAGTACTTTCTT TTGAACTTCTAC
S496 _{R498} -R'	CCCACTAATGGTGTGGTTACCAACCATAACAGAGTAG TAGTACTTTCTTTTGA <u>ACTTCTACTCGGAACT</u> ATATGATTGTAAAGGAAAGTAACAATTAACCTTCA ACACCATTACAAGGTGTGC

16 * Targeted amino acid sequences are underlined.



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18 **Supplementary Figure 1. Replication kinetics of BA.1 and BA.2 in hamsters.** Nasal
 19 turbinate and lung tissues were serially collected from the Omicron BA.1-infected and
 20 Omicron BA.2-infected male Syrian hamsters (n = 5 per group) at the indicated time-
 21 points for virus titration by TCID₅₀ assays. Data represent mean ± standard deviations.
 22 N.S., not significantly different by Student's t-test.

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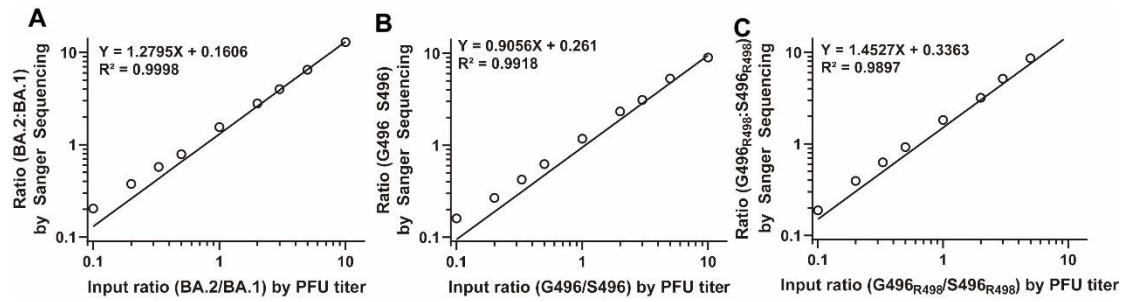
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41 **Supplementary Figure 2. Validation of the competition assay.** The correlation
 42 between input PFU ratios and output RT-PCR amplicon ratios determined by Sanger
 43 sequencing. The BA.1 and BA.2 variants were mixed at PFU ratios of 10:1, 5:1, 3:1,
 44 1:1, 1:3, 1:5, or 1:10. Total RNA of the mixture was extracted and amplified by RT-
 45 PCR. Ratio was calculated by the peak heights of Sanger sequencing. **(A)** Ratios of
 46 BA.2 to BA.1. **(B)** Ratios of G496 mutant virus to S496 virus. **(C)** Ratios of G496_{R498}
 47 single mutant virus to S496_{R498} double mutant virus.

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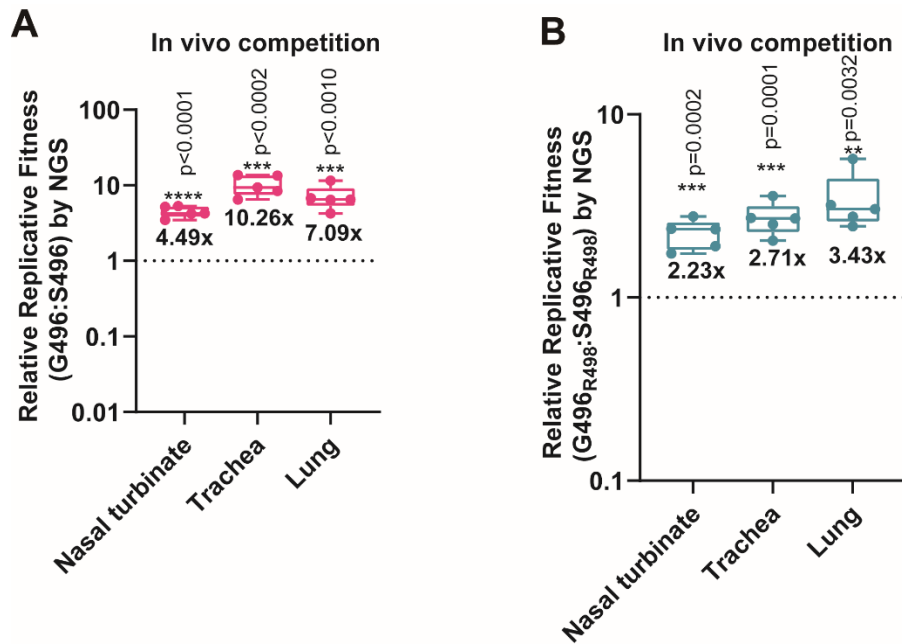
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60 **Supplementary Figure 3. (A)** *In vivo* virus competition assay measuring the wildtype
 61 (G496) to S496 viral-load ratios in the nasal turbinates, trachea, and lung of male
 62 hamsters (n=5 hamsters) at 2 days post-infection (2dpi) by NGS. **(B)** *In vivo* virus
 63 competition assay measuring the viral-load ratios of G496_{R498} single mutant to the
 64 S496_{R498} double mutant were performed in male hamsters (n=5 hamsters) at 2dpi by
 65 NGS. All data are indicated as mean ± SD. The ratios between the comparative variants
 66 were calculated based on the ratio of reads containing the 2 spike mutations, G496S
 67 and G498R. P values are calculated for the group (strain) coefficient for each linear
 68 regression analysis of indicated sample ratios versus baseline ratio (1:1). **P < 0.01;
 69 ***P < 0.001; ****P < 0.0001.

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