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Supplementary appendix

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Supplement to: Uraki R, Ito M, Kiso M, et al. Antiviral and bivalent vaccine efficacy against an omicron XBB.1.5 isolate. *Lancet Infect Dis* 2023; published online Feb 8. https://doi.org/10.1016/S1473-3099(23)00070-1.

Antiviral and Bivalent Vaccine Efficacy against an Omicron XBB.1.5 isolate

Supplementary Appendix

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Supplementary Materials

Cells.

Vero E6-TMPRSS2-T2A-ACE2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Calf Serum (FCS), 10 mM HEPES pH 7.3, 100 U/mL penicillin–streptomycin, and 10 μg/mL puromycin. VeroE6/TMPRSS2 (JCRB 1819) cells were propagated in the presence of 1 mg/ml geneticin (G418; Invivogen) and 5 μg/ml plasmocin prophylactic (Invivogen) in DMEM containing 10% FCS. Vero E6-TMPRSS2-T2A-ACE2 and VeroE6/TMPRSS2 cells were maintained at 37 °C with 5% CO_2. Chinese hamster ovary (CHO) cells were maintained in DMEM containing 10% FCS and antibiotics at 37 °C with 5% CO_2. Expi293F cells (Thermo Fisher Scientific) were maintained in Expi293 expression medium (Thermo Fisher Scientific) at 37 °C under 8% CO_2. The cells were regularly tested for mycoplasma contamination by using PCR, and confirmed to be mycoplasma-free.

Viruses.

hCoV-19/USA/MD-HP40900-PIDYSWHNUB/2022 (Omicron XBB.1.5), hCoV-19/Japan/TY41-795/2022 (Omicron XBB)[1], hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron BA.2)[2], and SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo (ancestral strain) were propagated in VeroE6/TMPRSS2 cells.

All experiments with SARS-CoV-2 were performed in enhanced biosafety level 3 (BSL3) containment laboratories at the University of Tokyo and the National Institute of Infectious Diseases, Japan, which are approved for such use by the Ministry of Agriculture, Forestry, and Fisheries, Japan.

Clinical specimens.

After informed consent was obtained, plasma specimens were collected from COVID-19 convalescent individuals and vaccinees. The research protocol was approved by the Research Ethics Review Committee of the Institute of Medical Science of the University of Tokyo (approval numbers: 2019–71–0201 and 2020-740226).

Antibodies.

Amino acid sequences for the variable region of the heavy and light chains of the following human monoclonal antibodies against the S protein were used for gene synthesis: clones tixagevimab (COV2-2196/AZD8895; GenBank accession numbers QLI33947 and QLI33948), casirivimab (REGN10933; PDB accession numbers 6XDG_B and 6XDG_D), cilgavimab (COV2-2130/AZD1061; GenBank accession numbers QKY76296 and QKY75909), imdevimab (REGN10987; PDB accession numbers 6XDG_A and 6XDG_A), S309 (PDB accession numbers 6WS6_A and 6WS6_F), and bebtelovimab (LYCoV1404; PDB accession numbers 7MMO_D and 7MMO_E). An artificial signal sequence and the constant gamma heavy (IgG1, UniProtKB/Swiss-Prot accession number P01857) and kappa (UniProtKB/Swiss-Prot accession number P01834) or lambda (UniProtKB/Swiss-Prot accession number P0DOY2) light chain coding sequences were added before and after each variable region. Codon usage was optimized for expression in CHO cells. The synthesized genes were cloned into a plasmid for protein expression and transfected into CHO cells. Cell culture media were harvested after incubation for 10–14 days at 37 °C. Monoclonal antibodies were purified by using MabSelect SuRe LX (Cytiva) or a protein A column. Purity was confirmed by SDS-PAGE and/or HPLC before use. The reactivities of these antibodies against SARS-CoV-2, including the Alpha, Beta, Delta, Gamma, and Omicron variants, have been tested previously[3].

Antiviral compounds.

Active components of remdesivir and molnupiravir (i.e., GS-441524 and EIDD-1931), and nirmatrelvir (PF-07321332) were purchased from MedChemExpress. Ensitrelvir (S-217622) was kindly provided by Shionogi & Co., Ltd. All compounds were dissolved in dimethyl sulfoxide.

Supplementary Methods

Whole genome sequencing

Viral RNA was extracted by using a QIAamp Viral RNA Mini Kit (QIAGEN). The whole genome of SARS-CoV-2 was amplified by using a modified ARTIC network protocol in which some primers were replaced or added. Briefly, viral cDNA was synthesized from the extracted RNA by using a LunarScript RT SuperMix Kit (New England BioLabs). The DNA was amplified by performing a multiplexed PCR in two pools using the ARTIC-N5 primers and the Q5 Hot Start DNA polymerase (New England BioLabs). The DNA libraries for Illumina NGS were prepared from pooled amplicons by using a QIAseq FX DNA Library Kit (QIAGEN) and were then analyzed by using the iSeq 100 System (Illumina). To determine the virus sequences, the reads were assembled by CLC Genomics Workbench (version 22, Qiagen) with the Wuhan/Hu-1/2019 sequence (GenBank accession no. MN908947) as a reference. The sequence of XBB.1.5 (hCoV-19/USA/MD-HP40900-PIDYSWHNUB/2022) was deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database with Accession ID: EPI_ISL_16543623.

Focus reduction neutralisation test (FRNT).

Neutralisation activities of monoclonal antibodies and human plasma were determined by using a focus reduction neutralisation test as previously described.[4] Serially diluted antibodies (starting concentration, 50,000 ng/ml) were mixed with 100–400 focus-forming units (FFU) of virus/well and incubated for 1 h at 37 ℃. The antibody-virus mixture (50 μl) was then inoculated onto Vero E6-TMPRSS2-T2A-ACE2 cells in 96-well plates in triplicate. After a 1-h incubation at 37 °C, 100 μl of 1.5% Methyl Cellulose 400 (FUJIFILM Wako Pure Chemical Corporation, Japan) in culture medium was added to each well. The cells were incubated for 14–18 h at 37 ℃ and then fixed with formalin. For human plasma, the samples were first incubated at 56 °C for 1 h. Then, the treated plasma samples were serially diluted five-fold with DMEM containing 2% FCS in 96-well plates and mixed with 100–400 FFU of virus/well, followed by incubation at 37 °C for 1 h. The plasma-virus mixture was inoculated onto Vero E6-TMPRSS2-T2A-ACE2 cells in 96-well plates in duplicate and incubated for 1 h at 37 °C. An equal volume of 1.5% Methyl Cellulose 400 (FUJIFILM Wako Pure Chemical Corporation) in culture medium was then added to each well. The cells were incubated for 14–16 h at 37 °C and then fixed with formalin.

After the formalin was removed, the cells were immunostained with a mouse monoclonal antibody against SARS-CoV-2 nucleoprotein [N45 (TAUNS Laboratories, Inc., Japan)], followed by a horseradish peroxidase-labeled goat anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories Inc.). The infected cells were stained with TrueBlue Substrate (SeraCare Life Sciences) and then washed with distilled water. After cell drying, the focus numbers were quantified by using an ImmunoSpot S6 Analyzer, ImmunoCapture software, and BioSpot software (Cellular Technology). The results are expressed as the 50% focus reduction neutralisation titre ($FRNT_{50}$). The $FRNT_{50}$ values were calculated by using GraphPad Prism (GraphPad Software). Samples under the detection limit (<10-fold dilution) were assigned an $FRNT_{50}$ of 10.

Inhibitory effect of compounds against SARS-CoV-2 *in vitro*.

Antiviral susceptibilities of SARS-CoV-2 were determined by applying a focus reduction assay as previously described[1,5]. Vero E6-TMPRSS2-T2A-ACE2 cells in 96-well plates were infected with 100–400 FFU of virus/well. Virus adsorption was carried out for 1 h at 37 ℃ and then the inoculum was removed and 1% Methyl Cellulose 400 (FUJIFILM Wako Pure Chemical Corporation) in culture medium containing serial dilutions of antiviral compounds was added to each well in triplicate. The cells were incubated for 18 h at 37 ℃ and then fixed with formalin. After the formalin was removed, immunostaining was performed as described for the FRNT. The results are expressed as the 50% inhibitory concentration (IC_{50}). The IC_{50} values were calculated by using GraphPad Prism (GraphPad Software).

Acknowledgements
We thank Susan Watson for scientific editing. We also thank Mashiho Yanagi, Kyoko Yokota, Naoko Mizutani, Rie Onoue and Madoka Yoshikawa for technical assistance. Vero E6-TMPRSS2-T2A-ACE2 cells were provided by Dr. Barney Graham, NIAID Vaccine Research Center.

Author Contributions
R.U.: conceptualization, formal analysis, validation, visualization, and writing the first draft. M. Ito, M. Kiso: data curation, formal analysis, and methodology. S. Yamayoshi: conceptualization and methodology. K.I-H.: resources and validation. Y.F.: data curation. Y.S-T., M. Imai, M. Koga, S. Yamamoto, E.A., M.S., T.T, A.O., T.K., H.Y., PJ.H., A.P.: resources. Y.K.: conceptualization, supervision, writing (review and editing), and funding acquisition. R.U., M. Ito, and M. Kiso contributed equally.

Declaration of Interests
Y.K. has received unrelated funding support from Daiichi Sankyo Pharmaceutical, Toyama Chemical, Tauns Laboratories, Inc., Shionogi & Co. LTD, Otsuka Pharmaceutical, KM Biologics, Kyoritsu Seiyaku, Shinya Corporation, and Fuji Rebio. T.K.is employed by Nihon Sumo Kyokai. The remaining authors declare that they have no competing interests.

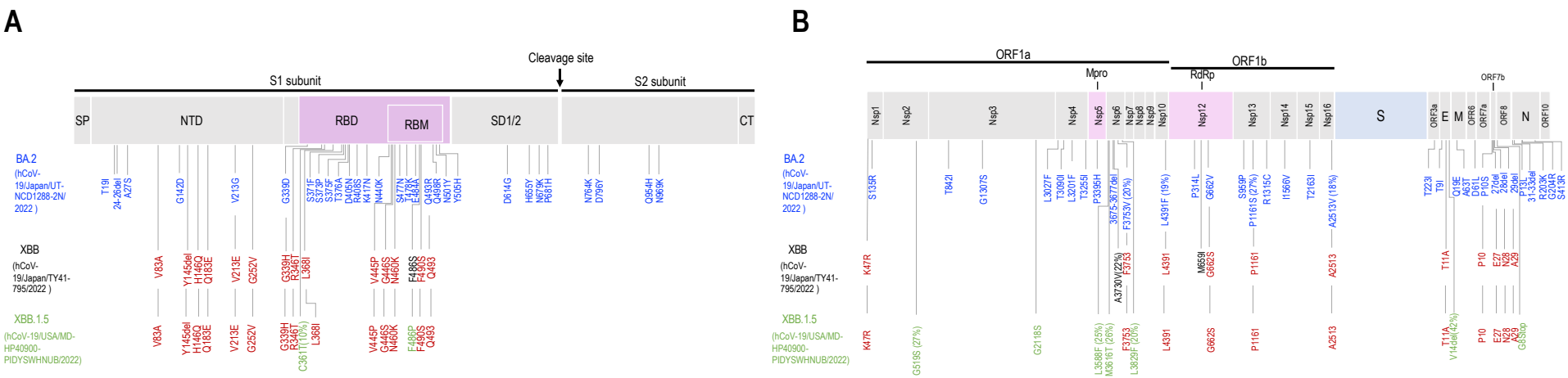

Figure S1. Mutations of Omicron subvariants.

Panels A. Spike (S) protein substitutions in the XBB.1.5 clinical isolate used in this study. The BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022) isolate possesses 31 amino acid changes in its S protein relative to the reference strain Wuhan/Hu-1/2019. Compared with BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022), substitutions are shown in black for XBB (hCoV-19/Japan/TY41-795/2022) and green for XBB.1.5 (hCoV-19/USA/MD-HP40900-PIDYSWHNUB/2022). The conserved substitutions between XBB and XBB.1.5 are shown in red. The S protein comprises two subunits, S1 and S2. The arrow indicates the S1/S2 proteolytic cleavage site. SP, signal peptide; NTD, N-terminal domain; RBD, receptor-binding domain; RBM, receptor-binding motif; SD1/2, subdomain 1 and 2; and CT, cytoplasmic tail. Panel B. Non-spike protein substitutions in the XBB.1.5 clinical isolate used in this study. Compared with the reference strain Wuhan/Hu-1/2019, the BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022) isolate possesses 37 amino acid changes in regions other than the S protein. Compared with BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022), substitutions are shown in black for XBB (hCoV-19/Japan/TY41-795/2022) and green for XBB.1.5 (hCoV-19/USA/MD-HP40900-PIDYSWHNUB/2022). The conserved substitutions between XBB and XBB.1.5 are shown in red. ORF, open reading frame; Mpro, main protease; RdRp, RNA-dependent RNA polymerase; S, Spike; E, Envelope; M, Membrane; and N, Nucleocapsid.

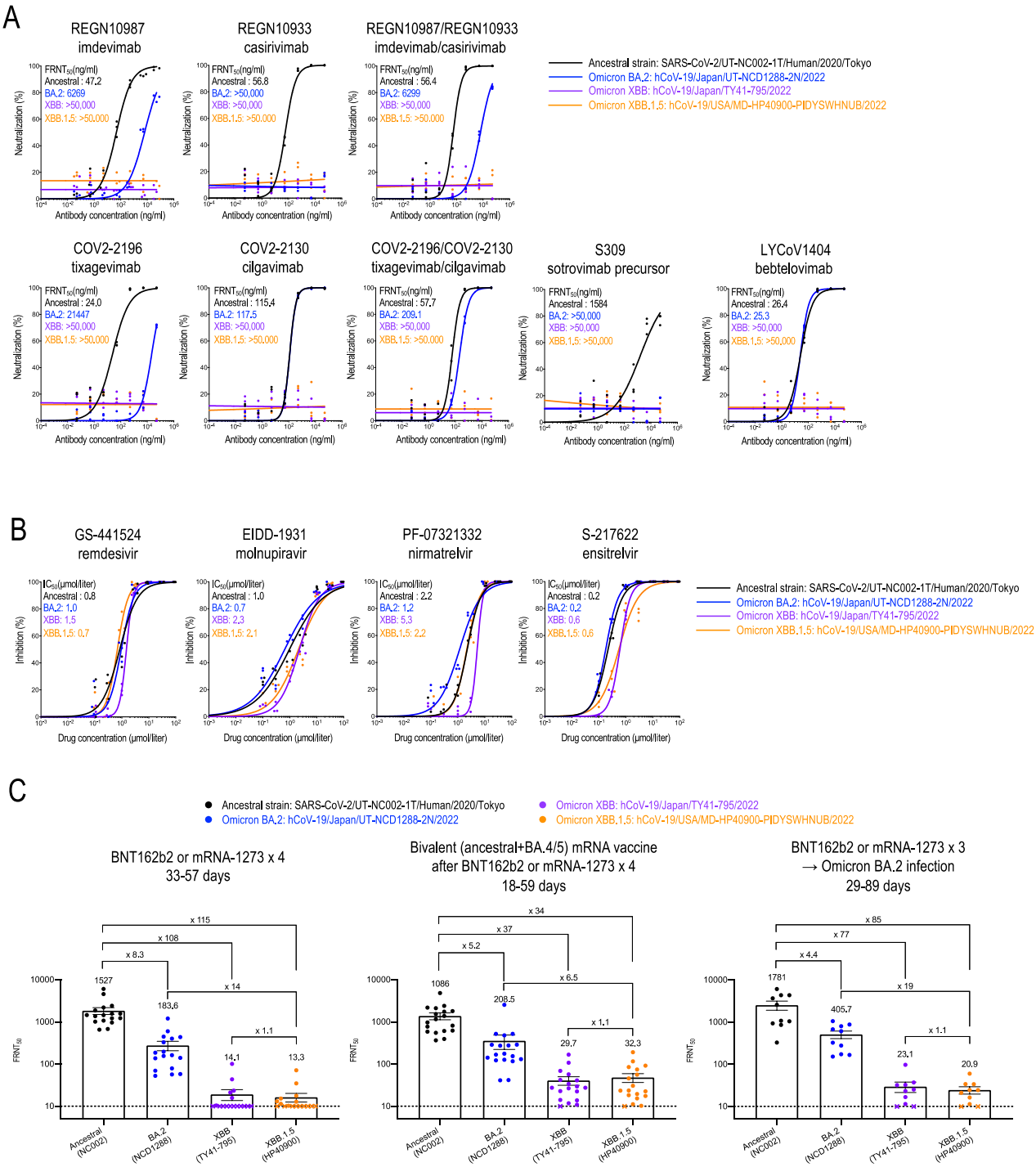

Figure S2. *In vitro* antiviral efficacy and neutralizing activity of plasma against Omicron subvariants.
A. The neutralizing activity of therapeutic monoclonal antibodies. Tested antibodies were produced in the authors' laboratories and are not identical to the commercially available products. The 50% focus reduction neutralization test titres ($FRNT_{50}$) were determined in Vero E6-TMPRSS2-T2A-ACE2 cells.
B. The inhibitory activity of antiviral drugs. The *in vitro* 50% inhibitory concentration (IC_{50}) values were determined in Vero E6-TMPRSS2-T2A-ACE2 cells. GS-441524 (the main metabolite of remdesivir) and EIDD-1931 (the active form of molnupiravir) are RNA-dependent RNA polymerase inhibitors. PF-07321332 (nirmatrelvir) and S-217622 (ensitrelvir) are inhibitors of Mpro (also called 3CLpro). (Panel A and B) Data are the mean values for triplicate experiments. Statistical analysis of the data was not performed.
C. The neutralizing titres of plasma obtained from individuals who had received four doses of BNT162b2 or mRNA-1273 vaccine (left), individuals immunized with the bivalent (ancestral and BA.4/5) vaccine as a fifth dose (middle), and patients who were infected with the omicron BA.2 subvariant after receiving either the BNT162b2 or mRNA-1273 vaccine (right). Detailed information about the participants is provided in

Tables S1, S2, and S3. $FRNT_{50}$ were determined in Vero E6-TMPRSS2-T2A-ACE2 cells. The lower limit of detection (value=10) is indicated by the horizontal dashed line. Samples under the detection limit (<10-fold dilution) were assigned an $FRNT_{50}$ of 10 and are represented by X. Geometric mean titres are shown.

Table S1. Neutralising antibody titres of human plasma obtained from individuals who received four doses of COVID-19 vaccines

Sample ID	Age	Gender	Plasma collection day post-final vaccination	Vaccine type	FRNT50: 50% focus reduction neutralisation titre			
					SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo (A)	hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron BA.2)	hCoV-19/Japan/TY41-795/2022 (Omicron XBB)	hCoV-19/USA/MD-HP40900-PIDYSWHNUB/2022 (Omicron XBB.1.5)
HP(H)-032	39	M	53	BNT162b2x 3, mRNA-1273 x 1	963.0	69.4	<10	10.2
HP(H)-058	58	F	48	BNT162b2x 3, mRNA-1273 x 1	6036.7	1199.1	101.1	71.3
HP(H)-088	46	F	43	BNT162b2x 3, mRNA-1273 x 1	1456.0	186.0	21.2	15.2
HP(H)-113	62	M	41	BNT162b2x 3, mRNA-1273 x 1	1564.2	137.0	<10	12.8
HP(H)-158	29	F	41	BNT162b2x 3, mRNA-1273 x 1	678.4	52.6	<10	<10
HP(H)-182	52	F	54	BNT162b2x 4	1867.1	327.4	15.8	17.7
HP(H)-183	47	F	44	BNT162b2x 3, mRNA-1273 x 1	1568.3	196.4	<10	<10
HP(H)-185	48	F	50	BNT162b2x 3, mRNA-1273 x 1	2411.9	447.0	23.4	11.4
HP(H)-189	57	F	33	BNT162b2x 4	1225.0	436.4	<10	13.1
HP(H)-198	33	F	45	BNT162b2x 3, mRNA-1273 x 1	1011.9	79.0	<10	<10
HP(H)-220	34	M	44	BNT162b2x 3, mRNA-1273 x 1	1071.0	56.6	<10	<10
HP(H)-228	49	F	51	BNT162b2x 3, mRNA-1273 x 1	1365.7	400.8	<10	<10
HP(H)-241	62	F	57	BNT162b2x 4	1447.7	140.0	10.4	<10
HP(H)-250	35	F	49	BNT162b2x 3, mRNA-1273 x 1	4585.8	597.7	43.6	34.9
HP(H)-255	51	F	42	BNT162b2x 3, mRNA-1273 x 1	2191.2	155.2	<10	<10
HP(H)-264	43	M	41	BNT162b2x 3, mRNA-1273 x 1	1218.9	57.8	<10	<10
HP(H)-282	43	M	48	BNT162b2x 3, mRNA-1273 x 1	652.9	162.1	<10	<10

Table S2. Neutralising antibody titres of human plasma obtained from individuals who received the bivalent (ancestral and BA.4/5) mRNA vaccine after four doses of COVID-19 vaccines

Sample ID	Age	Gender	Plasma collection day post-final vaccination	Vaccine type	FRNT50: 50% focus reduction neutralisation titre			
					SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo (A)	hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron BA.2)	hCoV-19/Japan/TY41-795/2022 (Omicron XBB)	hCoV-19/USA/MD-HP40900-PIDYSWHNUB/2022 (Omicron XBB.1.5)
HP(H)-172	53	F	19	BNT162b2x 3, mRNA-1273 x 1, bivalent (Moderna)	552.2	117.0	23.0	18.3
HP(H)-173	40	M	59	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	1743.1	481.1	27.5	23.6
HP(H)-179	52	F	21	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	610.6	127.2	22.4	22.7
HP(H)-185	49	F	21	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	4826.5	2538.3	168.0	191.9
HP(H)-189	58	F	22	BNT162b2x 4, bivalent (Pfizer/BioNTech)	966.0	167.2	26.8	19.5
HP(H)-215	49	M	21	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	1378.9	521.6	68.9	74.3
HP(H)-228	49	F	18	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	2183.2	276.2	34.0	56.5
HP(H)-230	35	M	18	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	2397.4	123.2	14.2	15.4
HP(H)-235	43	F	42	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	393.5	41.9	13.9	10.2
HP(H)-241	62	F	18	BNT162b2x 4, bivalent (Pfizer/BioNTech)	1534.5	272.3	57.0	85.2
HP(H)-247	49	F	28	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	588.8	142.2	27.5	23.5
HP(H)-248	60	F	18	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	769.6	154.2	11.8	17.5
HP(H)-252	49	M	21	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	620.6	130.7	41.1	45.7
HP(H)-282	44	M	21	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	815.4	176.4	10.9	11.2
HP(H)-297	57	M	18	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	2117.1	519.9	107.5	116.1
HP(H)-299	54	F	30	BNT162b2x 3, mRNA-1273 x 1, bivalent (Pfizer/BioNTech)	364.2	41.3	<10	<10
HP(H)-300	72	F	21	BNT162b2x 4, bivalent (Pfizer/BioNTech)	1154.5	288.3	42.1	92.0
HP(H)-305	71	M	34	BNT162b2x 4, bivalent (Pfizer/BioNTech)	1798.5	287.6	29.6	30.7

Table S3. Neutralising antibody titres of human plasma obtained from individuals who were infected with the Omicron BA.2 variant after three doses of COVID-19 vaccines

Sample ID	Age	Gender	Plasma collection day post-onset	Vaccine type	FRNT50: 50% focus reduction neutralisation titre			
					SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo (A)	hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron BA.2)	hCoV-19/Japan/TY41-795/2022 (Omicron XBB)	hCoV-19/USA/MD-HP40900-PIDYSWHNUB/2022 (Omicron XBB.1.5)
HPCo-383	56	F	29	mRNA-1273 x 2, BNT162b2 x 1	327.6	152.5	<10	<10
HP-S(H)0377	29	M	44	BNT162b2 x 3	933.8	325.7	25.2	21.1
HP-S(H)0380	22	M	42	BNT162b2 x 3	2571.8	278.6	11.4	16.3
HP-S(H)0381	22	M	44	BNT162b2 x 3	859.4	167.0	<10	<10
HP-S(H)0382	21	M	44	BNT162b2 x 3	6077.2	1049.9	32.2	33.8
HP-S(H)0383	18	M	42	BNT162b2 x 3	1044.1	610.3	23.3	24.4
HP-S(H)0882	23	M	89	BNT162b2 x 3	1076.8	172.5	16.7	11.4
HP-S(H)0883	24	M	84	BNT162b2 x 3	4355.7	673.7	37.2	24.7
HP-S(H)0888	21	M	89	BNT162b2 x 3	3675.2	800.2	32.6	33.9
HP-S(H)1056	22	M	42	BNT162b2 x 3	4343.2	877.2	97.4	60.1

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