# Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Uriu K, Kimura I, Shirakawa K, et al. Neutralization of the SARS-CoV-2 mu variant by convalescent and vaccine serum. N Engl J Med. DOI: 10.1056/NEJMc2114706

**Supplementary Appendix**

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## Materials and Methods

### Ethics statement

For the use of human specimens, all protocols involving human subjects recruited at Kyoto University and Chiba University were reviewed and approved by the Institutional Review Boards of Kyoto University (approval number G0697) and Chiba University (approval number HS202103-03). All human subjects provided written informed consent.

### Human sera

Peripheral blood was collected from three COVID-19 convalescents and the sera were isolated (**Table S6**). Moreover, peripheral blood was collected four weeks after the second vaccination with BNT162b2 (Pfizer-BioNTech), and the sera of fourteen vaccinated-individuals were isolated (**Table S7**). Sera were inactivated at 56°C for 30 min and stored at –80°C until use. Ten additional COVID-19 convalescent sera were purchased from RayBiotech (**Table S6**).

### Epidemic data

The data of daily COVID-19 cases in Colombia (until August 31, 2021) were downloaded from Our World in Data (https://ourworldindata.org/covid-cases)[1] on September 2, 2021.

### Viral genome sequences

SARS-CoV-2 annotation information used in this study was downloaded from the GISAID database (https://www.gisaid.org) as of August 30, 2021 (3,059,698 genomes). Based on the annotation by Phylogenetic Assignment of Named Global Outbreak (PANGO), we determined the daily frequency of SARS-CoV-2 Gamma (P.1), Delta (B.1.617.2, AY.4, AY.5, AY.12), Lambda (C.37), and Mu (B.1.621) variants isolated in Colombia. We also examined amino acid replacements of the spike protein of each SARS-CoV-2 variant by comparing it to the sequence of the Wuhan-Hu-1 strain (GISAID ID: EPI_ISL_1532199).

### Cell culture

HEK293T cells (a human embryonic kidney cell line; ATCC CRL-3216) and HOS-ACE2/TMPRSS2 cells,[2,3] a derivative of HOS cells (a human osteosarcoma cell line; ATCC CRL-1543) stably expressing human ACE2 and TMPRSS2, were maintained in Dulbecco's modified Eagle's medium (high glucose) (Wako, Cat# 044-29765) containing 10% fetal calf serum, 100 units penicillin and 100 ug/ml streptomycin.

### Plasmid construction

Plasmids expressing the SARS-CoV-2 spike proteins of the parental D614G (B.1), Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), Epsilon (B.1.427), and Lambda (C.37) variants

were prepared in our previous studies.[3-5] A plasmid expressing the spike protein of the Mu (B.1.621) variant was generated by site-directed overlap extension PCR using pC-SARS2-S D614G[3] as the template and the primers listed in **Table S8**. The resulting PCR fragment was digested with KpnI and NotI and inserted into the corresponding site of the pCAGGS vector.[6] Nucleotide sequences were determined by DNA sequencing services (Eurofins), and the sequence data were analyzed by Sequencher v5.1 software (Gene Codes Corporation).

**Neutralization assay**

Pseudoviruses were prepared as previously described.[3-5,7] Briefly, lentivirus (HIV-1)-based, luciferase-expressing reporter viruses were pseudotyped with the SARS-CoV-2 spikes. HEK293T cells ($1 \times 10^6$ cells) were cotransfected with 1 μg psPAX2-IN/HiBiT,[8] 1 μg pWPI-Luc2,[8] and 500 ng plasmids expressing parental S or its derivatives using PEI Max (Polysciences, Cat# 24765-1) according to the manufacturer's protocol. Two days post transfection, the culture supernatants were harvested and centrifuged. The pseudoviruses were stored at –80°C until use.

Neutralization assays were performed as previously described.[4,7] Briefly, the SARS-CoV-2 spike pseudoviruses (counting ~20,000 relative light units) were incubated with serially diluted (40-, 120-, 360-, 1,080-, 3,240-, 9,720-, and 29,160-fold dilution at the final concentration) heat-inactivated human sera at 37°C for 1 h. Pseudoviruses without sera were included as controls. Then, an 80 μl mixture of pseudovirus and serum was added to HOS-ACE2/TMPRSS2 cells (10,000 cells/50 μl) in a 96-well white plate. Two days post infection, the infected cells were lysed with a One-Glo luciferase assay system (Promega, Cat# E6130), and the luminescent signal was measured using a GloMax explorer multimode microplate reader 3500 (Promega). The assay of each serum was performed in triplicate, and the 50% neutralization titer was calculated using Prism 9 (GraphPad Software).

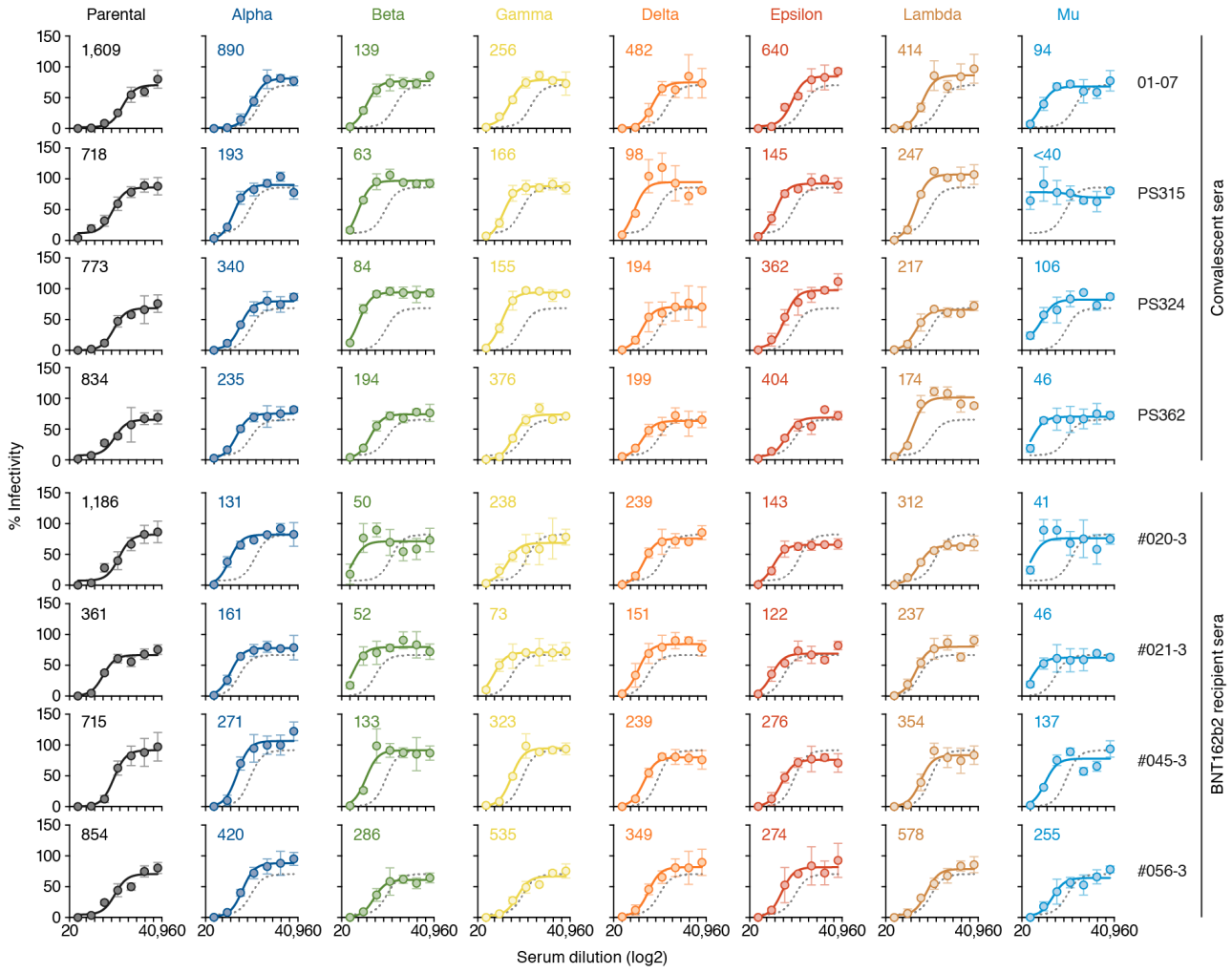


**Figure S1. Representative neutralization curves.** Representative results of virus neutralization assays using four convalescent sera (donors 01-07, PS315, PS324 and PS362; top panel) and four BNT162b2-vaccinated sera (donors #020-3, #021-3, #045-3 and #056-3; bottom panel) are shown. The percentages of pseudovirus infectivity compared to that without sera are shown. Assays were performed in triplicate, and data are average with standard deviation. The number in each panel indicates the 50% neutralization titers of the respective serum. The fitting curve of parental virus of respective serum is shown as broken black lines in all panels. The 50% neutralization titers are summarized in **Tables S6 and S7**.

**Table S1. Number of Mu variant sequences isolated in each country.**

| Country | B.1.621 (Mu) | B.1.621.1 |
|---|---|---|
| USA | 1,492 | 505 |
| Colombia | 844 | 8 |
| Spain | 344 | 122 |
| Mexico | 338 | 6 |
| Ecuador | 168 | 0 |
| Aruba | 74 | 5 |
| Chile | 70 | 2 |
| Netherlands | 68 | 2 |
| Italy | 63 | 14 |
| United Kingdom | 48 | 35 |
| Costa Rica | 46 | 0 |
| Canada | 43 | 4 |
| Switzerland | 23 | 25 |
| Belgium | 22 | 12 |
| Portugal | 21 | 3 |
| Curacao | 14 | 5 |
| France | 14 | 1 |
| Brazil | 11 | 0 |
| Denmark | 7 | 0 |
| Germany | 7 | 7 |
| Peru | 6 | 0 |
| Poland | 6 | 0 |
| Bonaire | 5 | 1 |
| Venezuela | 5 | 0 |
| Finland | 3 | 0 |
| Sweden | 3 | 0 |
| Dominican Republic | 2 | 41 |
| Hong Kong | 2 | 0 |
| Ireland | 2 | 2 |
| Japan | 2 | 0 |
| Slovakia | 2 | 1 |
| Turkey | 2 | 0 |
| Austria | 1 | 48 |
| Romania | 1 | 0 |
| Sint Maarten | 1 | 1 |
| Liechtenstein | 1 | 0 |
| Turks and Caicos Islands | 1 | 0 |
| Luxembourg | 0 | 1 |
| Malta | 0 | 1 |

**Table S2. Weekly new cases and numbers of different VOCs/VOIs in Colombia.**

| Week number | Date from (MM/DD) | Weekly new cases | Number | | | | | Percentage | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Gamma | Delta | Lambda | Mu | Others | Gamma | Delta | Lambda | Mu | Others |
| 1 | 01/04 | 111,080 | 4 | 0 | 0 | 0 | 41 | 8.9 | 0 | 0 | 0 | 91.1 |
| 2 | 01/11 | 121,513 | 1 | 0 | 0 | 1 | 44 | 2.2 | 0 | 0 | 2.2 | 95.7 |
| 3 | 01/18 | 107,072 | 8 | 0 | 0 | 0 | 29 | 21.6 | 0 | 0 | 0 | 78.4 |
| 4 | 01/25 | 79,399 | 2 | 0 | 0 | 0 | 16 | 11.1 | 0 | 0 | 0 | 88.9 |
| 5 | 02/01 | 62,332 | 5 | 0 | 0 | 0 | 6 | 45.5 | 0 | 0 | 0 | 54.5 |
| 6 | 02/08 | 37,823 | 2 | 0 | 0 | 0 | 8 | 20.0 | 0 | 0 | 0 | 80.0 |
| 7 | 02/15 | 31,223 | 5 | 0 | 0 | 1 | 6 | 41.7 | 0 | 0 | 8.3 | 50.0 |
| 8 | 02/22 | 25,428 | 0 | 0 | 0 | 5 | 16 | 0 | 0 | 0 | 23.8 | 76.2 |
| 9 | 03/01 | 24,966 | 4 | 0 | 0 | 8 | 36 | 8.3 | 0 | 0 | 16.7 | 75.0 |
| 10 | 03/08 | 26,488 | 8 | 0 | 0 | 13 | 22 | 18.6 | 0 | 0 | 30.2 | 51.2 |
| 11 | 03/15 | 34,086 | 8 | 0 | 0 | 7 | 19 | 23.5 | 0 | 0 | 20.6 | 55.9 |
| 12 | 03/22 | 45,500 | 5 | 0 | 0 | 11 | 30 | 10.9 | 0 | 0 | 23.9 | 65.2 |
| 13 | 03/29 | 63,489 | 16 | 0 | 1 | 23 | 31 | 22.5 | 0 | 1.4 | 32.4 | 43.7 |
| 14 | 04/05 | 89,979 | 111 | 0 | 8 | 85 | 152 | 31.2 | 0 | 2.2 | 23.9 | 42.7 |
| 15 | 04/12 | 116,749 | 95 | 0 | 6 | 62 | 85 | 38.3 | 0 | 2.4 | 25.0 | 34.3 |
| 16 | 04/19 | 121,517 | 36 | 0 | 3 | 54 | 35 | 28.1 | 0 | 2.3 | 42.2 | 27.3 |
| 17 | 04/26 | 119,191 | 50 | 0 | 5 | 47 | 50 | 32.9 | 0 | 3.3 | 30.9 | 32.9 |
| 18 | 05/03 | 109,103 | 40 | 0 | 5 | 78 | 42 | 24.2 | 0 | 3.0 | 47.3 | 25.5 |
| 19 | 05/10 | 115,668 | 37 | 0 | 5 | 56 | 29 | 29.1 | 0 | 3.9 | 44.1 | 22.8 |
| 20 | 05/17 | 114,030 | 32 | 0 | 0 | 51 | 12 | 33.7 | 0 | 0 | 53.7 | 12.6 |
| 21 | 05/24 | 150,823 | 10 | 0 | 1 | 18 | 3 | 31.3 | 0 | 3.1 | 56.3 | 9.4 |
| 22 | 05/31 | 187,788 | 3 | 0 | 0 | 15 | 6 | 12.5 | 0 | 0 | 62.5 | 25.0 |
| 23 | 06/07 | 182,157 | 10 | 0 | 3 | 33 | 7 | 18.9 | 0 | 5.7 | 62.3 | 13.2 |
| 24 | 06/14 | 191,942 | 6 | 0 | 0 | 22 | 2 | 20.0 | 0 | 0 | 73.3 | 6.7 |
| 25 | 06/21 | 213,550 | 7 | 0 | 1 | 20 | 1 | 24.1 | 0 | 3.4 | 69.0 | 3.4 |
| 26 | 06/28 | 191,779 | 4 | 1 | 4 | 49 | 5 | 6.3 | 1.6 | 6.3 | 77.8 | 7.9 |
| 27 | 07/05 | 161,465 | 2 | 2 | 1 | 30 | 5 | 5.0 | 5.0 | 2.5 | 75.0 | 12.5 |
| 28 | 07/12 | 127,506 | 2 | 5 | 1 | 39 | 5 | 3.8 | 9.6 | 1.9 | 75.0 | 9.6 |
| 29 | 07/19 | 88,380 | 0 | 3 | 1 | 26 | 0 | 0 | 10.0 | 3.3 | 86.7 | 0 |
| 30 | 07/26 | 66,568 | 0 | 3 | 0 | 3 | 0 | 0 | 50.0 | 0 | 50.0 | 0 |
| 31 | 08/02 | 44,570 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 100.0 | 0 |

**Table S3. Haplotypes of the spike protein of Mu variant.**

| Haplotype | Sequence number | Percentage |
|---|---|---|
| T95I\|YY144-145TSN\|R346K\|E484K\|N501Y\|D614G\|P681H\|D950N | 1488 | 39.6 |
| T95I\|YY144-145TSN\|R346K\|K417N\|E484K\|N501Y\|D614G\|P681H\|D950N | 189 | 5.0 |
| T95I\|YY144-145TSN\|R346K\|E484K\|N501Y\|D614G\|P681H | 129 | 3.4 |
| T95I\|YY144-145TSN\|R346K\|E484K\|N501Y\|D614G\|P681H\|D950N\|M1229I | 128 | 3.4 |
| T95I\|YY144-145SN\|R346K\|E484K\|N501Y\|D614G\|P681H\|D950N | 111 | 3.0 |
| T95I\|V143VT\|Y145N\|R346K\|E484K\|N501Y\|D614G\|P681H\|D950N | 108 | 2.9 |
| T95I\|R346K\|E484K\|N501Y\|D614G\|P681H\|D950N | 76 | 2.0 |
| T95I\|YY144-145TSN\|R346K\|E484K\|N501Y\|T572I\|D614G\|P681H\|D950N | 58 | 1.5 |
| L5F\|T95I\|YY144-145TSN\|R346K\|E484K\|N501Y\|D614G\|P681H\|D950N | 45 | 1.2 |
| T95I\|YY144-145TSN\|R346K\|D614G\|P681H\|D950N | 43 | 1.1 |

**Table S4. Mutations in the spike protein of Mu variant.**

| Mutation | Sequence number | Percentage |
|---|---|---|
| D614G | 3,755 | 99.8 |
| P681H | 3,743 | 99.5 |
| R346K | 3,684 | 97.9 |
| N501Y | 3,607 | 95.9 |
| T95I | 3,604 | 95.8 |
| E484K | 3,591 | 95.5 |
| D950N | 3,385 | 90.0 |
| Y145N | 3,264 | 86.8 |
| Y144S | 3,053 | 81.2 |
| V143VT | 2,952 | 78.5 |
| K417N | 302 | 8.0 |
| M1229I | 192 | 5.1 |
| Y144T | 97 | 2.6 |
| T572I | 96 | 2.6 |
| Y145SN | 71 | 1.9 |
| M1237I | 64 | 1.7 |
| L5F | 64 | 1.7 |
| E583D | 41 | 1.1 |
| E1258D | 39 | 1.0 |

**Table S5. Mutations in the spike proteins of SARS-CoV-2 variants used in this study.**

| Classification | Parental | Alpha | Beta | Gamma | Delta | Epsilon | Lambda | Mu |
|---|---|---|---|---|---|---|---|---|
| PANGO lineage | B.1 | B.1.1.7 | B.1.351 | P.1 | B.1.617.2 | B.1.427 | C.37 | B.1.621 |
| Mutations | D614G | HV69-70del | L18F | L18F | T19R | S13I | G75V | T95I |
| | | Y144del | D80A | T20N | G142D | W152C | T76I | YY144-145TSN |
| | | N501Y | D215G | P26S | EFR156-158G | L452R | RSYLTPGD246-253N | R346K |
| | | A570D | R246I | D138Y | L452R | D614G | L452Q | E484K |
| | | D614G | K417N | R190S | T478K | | F490S | N501Y |
| | | P681H | E484K | K417T | D614G | | D614G | D614G |
| | | T716I | N501Y | E484K | P681R | | T859N | P681H |
| | | S982A | D614G | N501Y | D950N | | | D950N |
| | | D1118H | A701V | D614G | | | | |
| | | | | H655Y | | | | |
| | | | | T1027I | | | | |

**Table S6. Summary of COVID-19 convalescent sera used in this study.**

| Donor ID | Sex | Age | Severity | Date of test (MM/DD/YY) | Date of sampling (MM/DD/YY) | 50% neutralization titer | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Parental | Alpha | Beta | Gamma | Delta | Epsilon | Lambda | Mu |
| 01-07 | Male | 57 | Severe | 09/01/20 | 11/04/20 | 1,609 | 890 | 139 | 256 | 482 | 640 | 414 | 94 |
| 03-03 | Male | 58 | Severe | 09/30/20 | 12/04/20 | 500 | 158 | <40 | 131 | 92 | 200 | 163 | <40 |
| 12-01 | Male | 86 | Moderate | 08/19/20 | 09/18/20 | 293 | 370 | 117 | 95 | 222 | 260 | 159 | 44 |
| PS315 [a] | Female | 61 | NA [b] | 04/01/20 | 05/03/20 | 718 | 193 | 63 | 166 | 98 | 145 | 247 | <40 |
| PS324 [a] | Female | 76 | NA [b] | 04/01/20 | 05/03/20 | 773 | 340 | 84 | 155 | 194 | 362 | 217 | 106 |
| PS329 [a] | Female | 92 | NA [b] | 04/02/20 | 05/03/20 | 7,834 | 2,801 | 1,365 | 2,697 | 5,039 | 3,613 | 4,442 | 844 |
| PS362 [a] | Female | 52 | NA [b] | 04/17/20 | 05/17/20 | 834 | 235 | 194 | 376 | 199 | 404 | 174 | 46 |
| PS364 [a] | Female | 74 | NA [b] | 04/17/20 | 05/17/20 | 2,579 | 893 | 320 | 537 | 503 | 624 | 489 | 260 |
| PS313 [a] | Female | 69 | NA [b] | 04/01/20 | 05/03/20 | 327 | 147 | 48 | 225 | 88 | 278 | 76 | 47 |
| PS314 [a] | Female | 64 | NA [b] | 04/02/20 | 05/03/20 | 318 | 132 | 172 | 377 | 129 | 877 | 129 | <40 |
| PS330 [a] | Female | 60 | NA [b] | 04/02/20 | 05/03/20 | 430 | 194 | 42 | 420 | 106 | 260 | 84 | <40 |
| PS340 [a] | Female | 73 | NA [b] | 04/02/20 | 05/03/20 | 349 | 210 | 68 | 542 | 117 | 371 | 214 | 48 |
| PS354 [a] | Male | 71 | NA [b] | 04/17/20 | 05/17/20 | 1,762 | 2,054 | 973 | 1,777 | 501 | 1,502 | 569 | 265 |
| | | | | | Geometric mean | 806 | 378 | 153 | 357 | 235 | 471 | 257 | 111 |

[a] Purchased from RayBiotech.

[b] Not applicable.

**Table S7. Summary of BNT162b2-vaccinated sera used in this study.**

| Donor ID | Sex | Age | Date of 2nd vaccination (MM/DD/YY) | Date of sampling (MM/DD/YY) | 50% neutralization titer | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Parental | Alpha | Beta | Gamma | Delta | Epsilon | Lambda | Mu |
| #005-3 | Female | 34 | 04/30/21 | 05/27/21 | 555 | 440 | 64 | 168 | 84 | 225 | 228 | <40 |
| #006-3 | Female | 41 | 04/30/21 | 05/27/21 | 2,417 | 880 | 248 | 1,171 | 623 | 641 | 1,062 | 259 |
| #007-3 | Female | 35 | 04/26/21 | 05/24/21 | 613 | 415 | 128 | 202 | 260 | 138 | 219 | <40 |
| #020-3 | Female | 43 | 04/30/21 | 05/28/21 | 1,186 | 131 | 50 | 238 | 239 | 143 | 312 | 41 |
| #021-3 | Female | 47 | 04/27/21 | 05/25/21 | 361 | 161 | 52 | 73 | 151 | 122 | 237 | 46 |
| #026-3 | Female | 28 | 04/26/21 | 05/25/21 | 206 | 179 | 58 | 289 | 149 | 581 | 127 | 69 |
| #027-3 | Male | 41 | 04/27/21 | 05/25/21 | 368 | 227 | 205 | 300 | 96 | 89 | 195 | 52 |
| #028-3 | Female | 42 | 04/28/21 | 05/26/21 | 595 | 597 | <40 | 112 | 520 | 463 | 434 | 112 |
| #030-3 | Female | 29 | 04/27/21 | 05/25/21 | 256 | 81 | 97 | 112 | 224 | 319 | 272 | 59 |
| #036-3 | Male | 34 | 04/27/21 | 05/27/21 | 448 | 192 | 54 | 334 | 218 | 179 | 358 | 70 |
| #045-3 | Female | 34 | 04/30/21 | 05/24/21 | 715 | 271 | 133 | 323 | 239 | 276 | 354 | 137 |
| #056-3 | Male | 38 | 04/30/21 | 05/26/21 | 854 | 420 | 286 | 535 | 349 | 274 | 578 | 255 |
| #078-3 | Female | 32 | 04/22/21 | 05/06/21 | 1,260 | 691 | 141 | 291 | 265 | 347 | 579 | 121 |
| #103-3 | Male | 35 | 04/07/21 | 05/07/21 | 313 | 1,407 | 66 | 112 | 511 | 203 | 406 | 44 |
| | | | | Geometric mean | 575 | 324 | 101 | 233 | 239 | 243 | 332 | 85 |

**Table S8. Primers used for the construction of Mu spike expression plasmid.**

| Primer name | Sequence (5'-to-3') |
|---|---|
| T95I Fwd | TCTACTTTGCCAGCAttGAGAAGAGCAACATC |
| T95I Rev | GATGTTGCTCTTCTCaaTGCTGGCAAAGTAGA |
| YY144-145TSN Fwd | CCATTCCTGGGAGTCacctccaacCACAAGAACAACAAG |
| YY144-145TSN Rev | CTTGTTGTTCTTGTGgttggaggtGACTCCCAGGAATGG |
| R346K Fwd | GTTCAATGCCACCAaGTTTGCCTCTGTCT |
| R346K Rev | AGACAGAGGCAAACtTGGTGGCATTGAAC |
| E484K Fwd | CCATGTAATGGAGTGaAGGGCTTCAACTGTT |
| E484K Rev | AACAGTTGAAGCCCTtCACTCCATTACATGG |
| N501Y Fwd | TGGCTTCCAACCAACCtATGGAGTGGGCTA |
| N501Y Rev | TAGCCCACTCCATaGGTTGGTTGGAAGCCA |
| P681H Fwd | CCCAGACCAACAGCCatAGGAGGGCAAGGTCT |
| P681H Rev | AGACCTTGCCCTCCTatGGCTGTTGGTCTGGG |
| D950N Fwd | CTGGGCAAACTCCAAaATGTGGTGAACCAG |
| D950N Rev | CTGGTTCACCACATtTTGGAGTTTGCCCAG |

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