			← CDR1→	-CDR2
Nb-007	OLOLVESGGGLV	OAGGSMRLSCAAS	. ISFSSFPMGWHROAPGK	ORELVAKIG, IGGTAYDDSV
Nb-001	EVOLVESGGGLV	ÕA <mark>G</mark> GSLRLSCAAS	GSIFRLYTIDWYROAPGK	ORELVATISGTSPTNYADSV
Nb-002	EVOLVESGGALVO	OPGGSLRLSCAAS	GFTLDAAAIG <mark>W</mark> FROAPGK	EREW <mark>V</mark> SCINRVGGGFYSD S A
Nb-003	EVOVVESGGGLV	ÕAGGSLRVSCAAS	GSTSSRYIMAWY <mark>RÕ</mark> A PG K	ORELVASITLVGATIYKDSV
Nb-004	OLOLVESGGGLV	OAGGSLGLSCAAS	GITLGDYRMGWYROAPGK	ORELVARFTNAGAILYGDS V
Nb-005	EVOLVESGGGLV	OAGGSLRLSCAAS	GSAFNDYAMGWYROAPGK	ORELVARFTNVGATLSSNS V
Nb-006	OVOLVESGGGLV	OAGESLRLSCAAS	GSTVSPYTMGWYROAPGK	OREWVATIRSDRSTNYADSV
Nb-008	EVOVVESGGGLV	OAGGSLRLSCAAS	GSTSNLRVTGWYROTPGK	ORELVAAISNAGATVYPDSV
Nb-009	OVOLVESGGGLV	OAGGSLKLSCAAS	GITESSYDMAWYROAPGK	OREWVATVRSDGITNYRDSV
Nb-010	EVOVVESGGGLV	OAGGSLRLSCTAS	GSTETRYPMGWYROAPGK	ORELVAGETVVGATIYADSV
				3
Nt- 007				
Nb-007	KGRFTISRDNTKI	NTVYLQMNSLKVE	dTAVYYCWGWR	3
Nb-007 Nb-001	KG <mark>R</mark> FTISRDNTK KGRATISRDNAK	NTVYLOMNSLKVE NTVYLOINSLKPE	DTAVYYCWGWR DTAVYYCHTVS	3
Nb-007 Nb-001 Nb-002	KG <mark>R</mark> FTI <mark>S</mark> RDNTKI KG <mark>RATIS</mark> RDNAKI KGRFTISRDNDKI	NTVYLQMNSLKVE NTVYLQINSLKPE NTVYLQMNSLKFE	DTAVYYCWGWR DTAVYYCHTVS DTAVYYCAADSDSPRFGC	3 MNDYWGQGTQVTVSS GNTWGQGTQVTVSS SDSRARYEYWGQGTQVTASS
Nb-007 Nb-001 Nb-002 Nb-003	KG <mark>R</mark> FTISRDNTK KG <mark>R</mark> ATISRDNAK KGRFTISRDNDK KGRFTISGDNAK	NTVYLOMNSLKVE NTVYLOINSLKPE NTVYLOMNSLKFE KTVYLOMNNLKPE	DTAVYYCWGWR DTAVYYCHTVS DTAVYYCAADSDSPRFGC DTAVYYCAADSDSPRFGC	3 MNDYWGQGTQVTVSS GNTWGQGTQVTVSS SDSRARYEYWGQGTQVTASS GGPGYWGQGIQVTVSS
Nb-007 Nb-001 Nb-002 Nb-003 Nb-004	KGRFTISRDNTK KGRATISRDNAK KGRFTISRDNDK KGRFTISGDNAK KGRFTISMNNAK	NTVYLQMNSIKVE NTVYLQINSIKPE NTVYLQMNSIKFE KTVYLQMNNIKPE NTVYLQINSIKPE	DTAVYYCWGWR DTAVYYCHTVS DTAVYYCAADSDSPRFGC DTAVYYCNQIDPLI DTAVYYCNA.RTT.	3 MNDYWGQGTQVTVSS GNTWGQGTQVTVSS SDSRARYEYWGQGTQVTASS GGPGYWGQGIQVTVSS RGIDNWGQGTQVTVSS
Nb-007 Nb-001 Nb-002 Nb-003 Nb-004 Nb-005	KGRFTISRDNTK KGRATISRDNAK KGRFTISRDNDK KGRFTISGDNAK KGRFTISMNNAK KGRFTISRDNAK	NTVYLQMNSLKVE NTVYLQINSLKPE NTVYLQMNSLKFE KTVYLQMNNLKPE NTVYLQINSLKPE NTVFLQMNSLKPE	CDTAVYYCWGWR. DTAVYYCHTVS DTAVYYCAADSDSPRFGC DTAVYYCNQIDPLI DTAVYYCNA.RTT DTAVYYCNA.RTT	3 GNTWGQGTQVTVSS SDSRARYEYWGQGTQVTASS GGPGYWGQGTQVTASS RGIDNWGQGTQVTVSS LGREYWGQGTQVTVSS
Nb-007 Nb-001 Nb-002 Nb-003 Nb-004 Nb-005 Nb-006	KGRFTISRDNTK KGRATISRDNAK KGRFTISRDNDK KGRFTISGDNAK KGRFTISNNAK KGRFTISRDNAK LGRFTISRDNAK	NTVYLQMNSLKVE NTVYLQINSLKPE KTVYLQMNNLKFE KTVYLQMNNLKPE NTVYLQINSLKPE NTVYLQMNSLKPE NTVYLQMNSLKPE	DTAVYYCWGWR DTAVYYCHTVS DTAVYYCAADSDSPRFGC DTAVYYCNQIDPLI DTAVYYCNA.RTT DTAVYYCNA.RTT DTAVYYCHAGHRIL	3 MNDYWGQGTQVTVSS GNTWGQGTQVTVSS SSDSRARYEYWGQGTQVTVSS GGPGYWGQGIQVTVSS RGIDNWGQGTQVTVSS LGREYWGQGTQVTVSS GGLLLGQGTQVTVSS
Nb-007 Nb-001 Nb-002 Nb-003 Nb-004 Nb-005 Nb-006 Nb-008	KGRFTISRDNTK KGRATISRDNAK KGRFTISRDNDK KGRFTISGDNAK KGRFTISRDNAK KGRFTISRDNAK LGRFTISRDNAK AGRFTISRDNAK	NTVYLQMNSLKVE NTVYLQINSLKPE NTVYLQMNSLKFE NTVYLQINSLKPE NTVFLQINSLKPE NTVFLQMNSLKPE NTVYLQINSLKPE NTVFLQINSLKPE	CDT DTAVYYCWGWR DTAVYYCHTVS DTAVYYCAADSDSPRFGC DTAVYYCNQIDPLI DTAVYYCNA.RTT DTAVYYCHAGHRIL DTAVYYCHAGHRIL DTAVYYCHAGHRI	3 GNTWGQGTQVTVSS GNTWGQGTQVTVSS SDSRAYEYWGQGTQVTASS GGPGYWGQGIQVTVSS GGLDNWGQGTQVTVSS GGLLLGQGTQVTVSS GGLLLGQGTQVTVSS FGRDYWGQGTQVTVSS
Nb-007 Nb-001 Nb-002 Nb-003 Nb-004 Nb-005 Nb-006 Nb-008 Nb-008 Nb-009	KG <mark>R</mark> FTISRDNTKI KGRATISRDNAK KGRFTISRDNDK KGRFTISGDNAK KGRFTISRDNAK LGRFTISRDNAK AGRFTISRDNAK KDRFTISRDNAK	NTVYLQMNSLKVE NTVYLQINSLKFE NTVYLQMNSLKFE NTVYLQINSLKPE NTVFLQMNSLKPE NTVFLQMNSLKPE NTVFLQMNSLKPE NTVYLQINSLKPE NTVYLQ	CDT DTAVYYCWGWR DTAVYYCHTVS DTAVYYCAADSDSPRFGC DTAVYYCNQIDPLI DTAVYYCNA.RTT DTAVYYCHAGHRIL DTAVYQCYALQ DTAVYYCNARR DTAVYYCNARR	3 GNTWGQGTQVTVSS GNTWGQGTQVTVSS SDSRARYEYWGQGTQVTASS GGPGYWGQGTQVTVSS RGIDNWGQGTQVTVSS GGLLLGQCTQVTVSS FGRDYWGQGTQVTVSS FGRDYWGQGTQVTVSS WAADYWGQGTQVTVSS

Supplementary Figure 1. Multiple sequence alignment of the ten nanobodies with

unique sequences identified in this study. The CDR regions are marked.



Supplementary Figure 2. Purification and gel-filtration characteristics of the indicated nanobodies. Solution behaviors of the 9 non-neutralizing nanobodies identified in the study on a SuperdexTM 75 10/300 GL column are shown. The inset figure shows the SDS-PAGE analyses of the pooled samples.



Supplementary Figure 3. Binding of the indicated nanobodies to SARS-CoV-2 S-RBD verified by single-concentration ELISA-binding assay. The experiments were performed with 200 ng SARS-CoV-2 S-RBD immobilized on the plate and 0.2 μ g nanobodies (green) or ACE2 (magenta) as the analyte. The emission OD450 was plotted as histograms. The error bar shows the mean \pm SD from three independent experiments.



Supplementary Figure 4. Initial screenings for the potential virus-entry inhibition by the indicated nanobodies based on the S-mediated syncytium-formation assay. SARS-CoV-2 S-mediated cell–cell fusion experiments were performed in the presence of 10 μ M nanobody. The formed syncytia are marked with white arrows. Scale bar equals 100 μ m.



Supplementary Figure 5. Binding capacity of ACE2 to SARS-CoV-2 S-RBD detected via SPR. The recorded binding profiles and calculated kinetic parameters are shown.



Supplementary Figure 6. Solution behavior and pseudovirus neutralization of Nb20. (A) Purification and gel-fitration characterization of Nb20. The inset figure shows the SDS-PAGE analyses of the pooled samples. (B) Neutralization of SARS-CoV-2 pseudovirus (original strain) by Nb20. The error bar stands for the mean \pm SD from triplicate experiences.



Supplementary Figure 7. The currently-defined antigenic sites on S-RBD of SARS-CoV-2. For easy comparison, the S-RBD molecules are oriented similarly and rendered as molecular surface in cyan. The antigenic sites are depicted as follows: RBS-A epitope in magenta, RBS-B epitope in yellow, CR3022 site in gray, and S309 site in orange. The epitope of RBS-C is shown in main-text figure 4.



Supplementary Figure 8. Comparison of the epitope of Nb-007 with those of the indicated antibodies and nanobodies. (**A**) Superimposition of the structures of S-RBD (gray) in complex with Nb-007 (magenta) and antibodies CB6 (lemon, PDB code: 7C01), CV07-250 (blue, PDB code: 6XKQ), CV07-270 (light-pink, PDB code: 6XKP), P2B-2F6 (cyan, PDB code: 7BWJ), CR3022 (salmon, PDB code: 6W41), S309 (yellow, PDB code: 6WPS) and A23-58.1 (orange, PDB code: 7LRS). (**B**) Superimposition of the structures of S-RBD (gray) in complex with nanobodies Ty1 (cyan, PDB code: 6ZXN), Nb20 (blue, PDB code: 7JVB), Nb12 (light-blue, PDB

code: 7MY3), Nb30 (lemon, PDB code: 7MY2), VHHE (green, PDB code: 7B14), Re5D06 (yellow, PDB code: 7OLZ), Re9F06 (orange, PDB code: 7OLZ), and Nb-007 (magenta). Antibodies and nanobodies are shown in cartoon and RBD is shown in surface representation.



Supplementary Figure 9. Solution behaviors and binding capacity of Nb-007 mutants. **(A-E)** Characterization of the solution behavior of Nb-007/I26D (A), Nb-007/I26S (B), Nb-007/S27D (C), Nb-007/R97D (D), Nb-007/R97E (E) by gel filtration chromatography (upper panel) and the affinity calculation of the binding of individual mutants to SARS-CoV-2 Beta variant S-RBD using SPR (lower panel).



Supplementary Figure 10. Binding capacity and neutralizing activity of Nb-007-Fc against SARS-CoV-2 variants. (**A**, **B**) Affinity analysis of the binding of Nb-007-Fc to SARS-CoV-2 Beta variant S-RBD (A) and Delta variant S-RBD (B) using SPR. The real-time binding kinetics are shown. (**C**) Inhibition of the pseudovirus entry by Nb-007-Fc at the indicated concentrations for the SARS-CoV-2 Beta variant. Error bar represents the mean \pm SD of triplicate.

SARS-CoV-2 S-RBD with Nb-007					
(PDB code: 7W1S)					
Data collection					
Space group	$P4_{1}2_{1}2$				
Cell dimensions					
<i>a</i> , <i>b</i> , <i>c</i> (Å)	56.80, 56.80, 219.77				
α, β, γ (°)	90.00, 90.00, 90.00				
Wavelength (Å)	0.97915				
Resolution (Å)	50.00-2.00 (2.07-2.00)				
Unique reflections	25547 (2451)				
R _{merge}	0.182 (0.739)				
CC _{1/2}	0.999 (0.900)				
I/sigI	16.50 (2.00)				
Completeness (%)	99.9 (99.2)				
Redundancy	17.4 (11.4)				
Refinement					
Resolution (Å)	29.65-2.00				
No. reflections	25292				
$R_{ m work}/R_{ m free}$	0.199/0.223				
No. atoms					
Protein	2388				
Ligand/ion	0				
Water	268				
B-factors					
Protein	28.5				
Ligand/ion	-				
Water	39.1				
R.m.s. deviations					
Bond lengths (Å)	0.004				
Bond angles (°)	0.567				
Ramachandran plot (%)					
Favored region	98.01				
Allowed region	1.99				
Outlier region	0				

Supplementary Table 1. Data collection and structure refinement statistics.

A single crystal was used to collect the data.

Values in parentheses are for the highest-resolution shell.