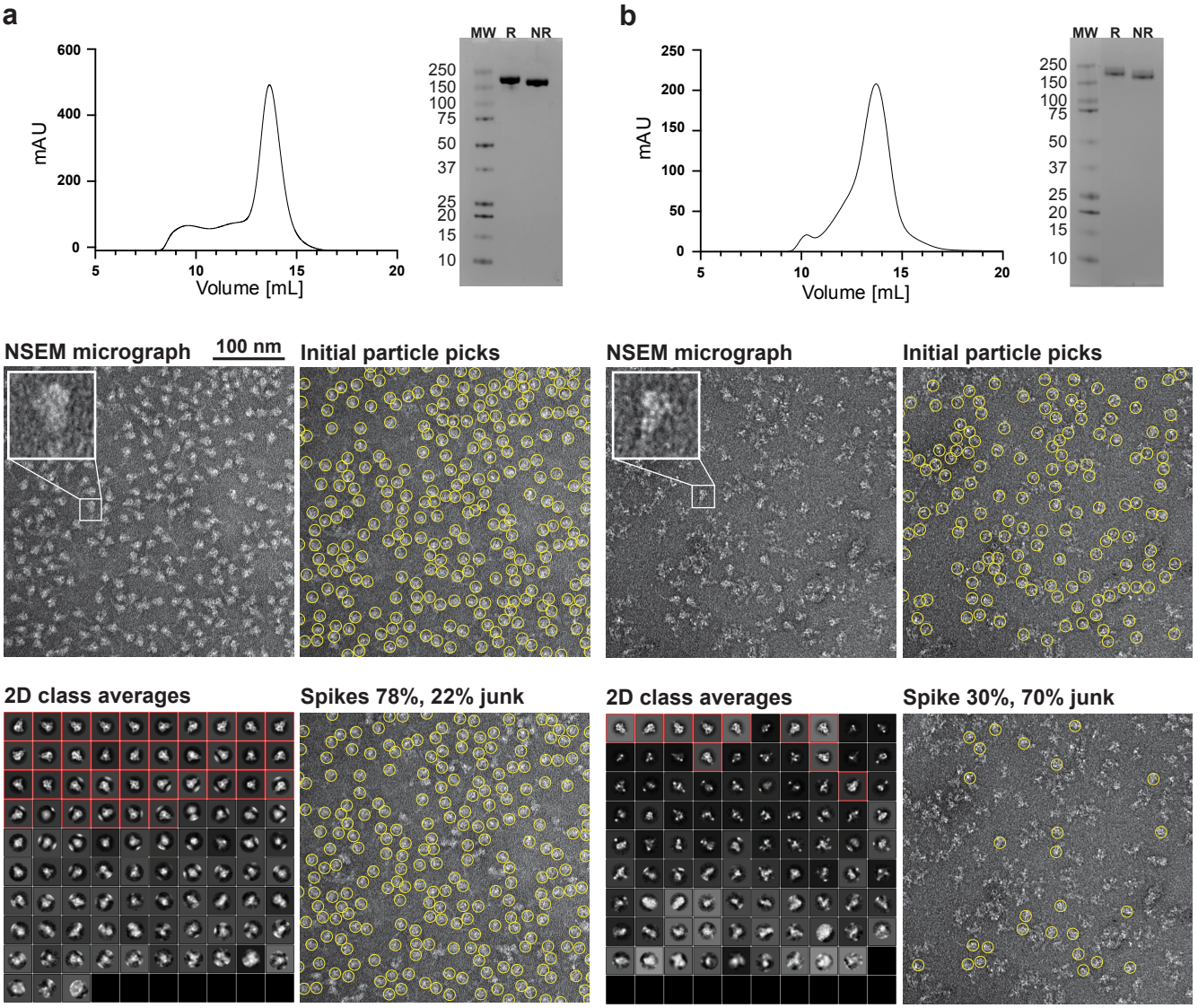
Supplementary Table 1. Thermostability of the SARS-CoV-2 2P S ectodomain stored at different temperatures measured by DSF. Inflection temperatures from DSF plots shown in Figure 1F, expressed as averages ± standard deviation, N=5.

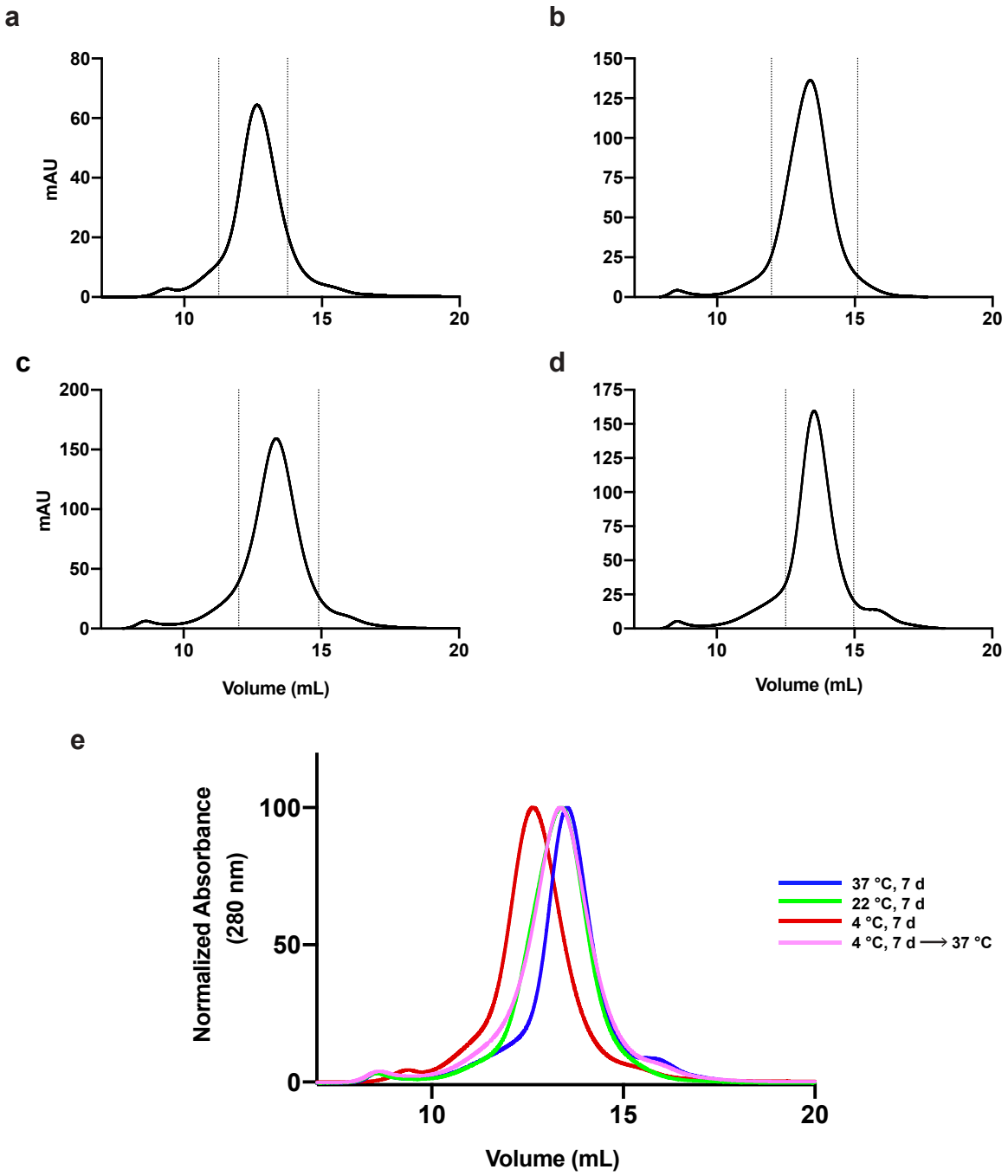
Storage Temp.	T^i_1(°C)	T^i_2 (°C)	T^i_3 (°C)
37 °C	53.7 ± 1.1	65.0 ± 0.2	74.8 ± 0.2
22 °C	49.9 ± 0.2	58.6 ± 0.3	74.2 ± 0.2
4 °C	49.9 ± 0.4	57.5 ± 0.3	71.9 ± 0.2
4 → 37 °C Recovery	52.2 ± 0.5	58.8 ± 0.5	74.1 ± 0.3

Supplementary Table 2. Thermostability of the SARS-CoV-2 2P S ectodomain stored at different temperatures measured by DSC. Table of melting temperatures, T_m, with values expressed as mean ± range, N=2.

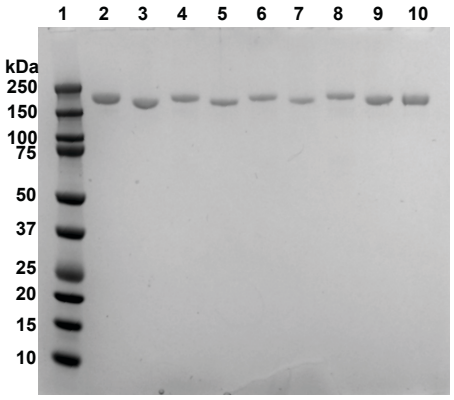
	Transition Peak 1	Transition Peak 2
Storage Temp.	**T_m (°C)**	**T_m (°C)**
37 °C	N/A	65.5 ± 0.2
22 °C	48.2 ± 0.1	65.8 ± 0.1
4 °C	48.41 ± 0.01	65.1 ± 0.3
4 → 37 °C Recovery	49.2 ± 0.1	65.95 ± 0.02



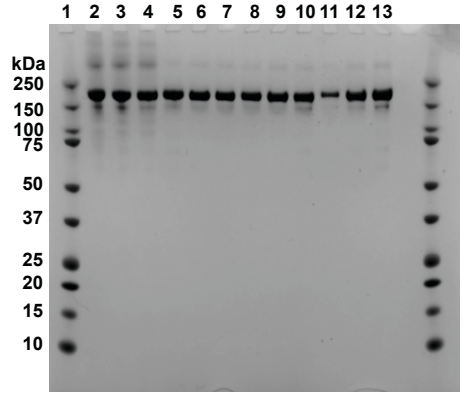
Supplementary Figure 1. Purification and quality control of the SARS-CoV-2 2P S ectodomain. (a and b) Two representative spike preparations that together highlight the role of NSEM in discriminating between good **(a)** and bad **(b)** spike preparations that otherwise appear to be of similar quality by SEC (top, left) and SDS-PAGE (top, right). The SEC and SDS-PAGE runs were performed with spike samples that were produced in 293F cells and purified from the cell culture supernatant using a Strep-Tactin column (see methods). Representative micrographs from each prep are shown, middle left. Protein appears as white blobs on gray background. Insets show a single kite-shaped spike particle enlarged. At the middle right, automatic particle picking is shown as yellow circles superimposed on the micrograph. Sets of ~20,000 initial particles picks are subjected to automated 2D classification to group together and average particles with similar features into discrete classes, bottom left. In the 2D class averages for each sample, the classes that contain the SARS-Cov-2 S ectodomain (shown within red boxes) can be clearly distinguished from classes that contain junk. The final spike picks come from the particles contained within the indicated classes and their total number provide an estimate of the ratio between the SARS-CoV-2 2P S ectodomain and junk seen in the NSEM sample.



Supplementary Figure 2. Size Exclusion Chromatography of SARS-CoV-2 2P S ectodomain incubated at different temperatures. SEC profiles run on a Superose 6 increase 10/300 column for SARS-CoV-2 2P S ectodomain samples that were incubated at **(a)** 4 °C for one week, **(b)** 22 °C for one week. **(c)** 37 °C for one week, and **(d)** 4 °C for one week and moved to 37 °C for 3 hours prior to the experiment. **(e)** Overlay of SEC plots normalized to allow better visualization of peak shifts. The spike preparations were purified by SEC before the start of the temperature incubations. Only the peak corresponding to the 2P S was used and any shoulder and aggregates were discarded.

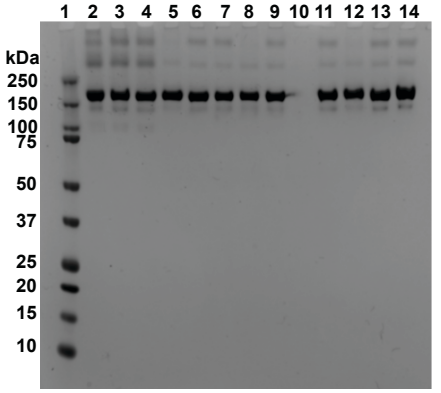


1. Molecular weight marker
2. 2P 097VS 4 °C reduced
3. 2P 097VS 4 °C non-reduced
4. 2P 097VS 22 °C reduced
5. 2P 097VS 22 °C non-reduced
6. 2P 097VS 37 °C reduced
7. 2P 097VS 37 °C non-reduced
8. 2P 097VS 4 °C Rebound reduced
9. 2P 097VS 4 °C Rebound non-reduced
10. 2P thawed from frozen reduced



Reduced

1. Molecular weight marker
2. 2P-N165A 095VS 37 °C
3. 2P-N234A 096VS 37 °C Repeat 1
4. 2P-N234A 096VS 37 °C Repeat 2
5. 2P-N165A 095VS 4 °C
6. 2P-N234A 096VS 4 °C Repeat 1
7. 2P-N234A 096VS 4 °C Repeat 2
8.2P-N165A 095VS 22 °C
9. 2P-N234A 096VS 22 °C Repeat 1
10. 2P-N234A 096VS 22 °C Repeat 2
11. 2P-N165A 095VS 4 °C Rebound
12. 2P-N234A 096VS 4 °C Rebound Repeat 1
13. 2P-N234A 096VS 4 °C Rebound Repeat 2



Non-Reduced

1. Molecular weight marker
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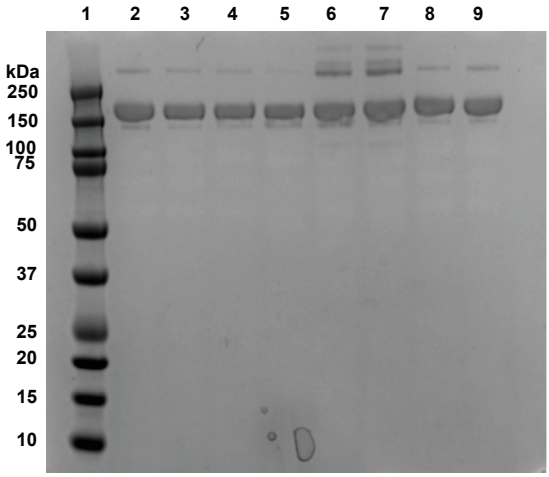
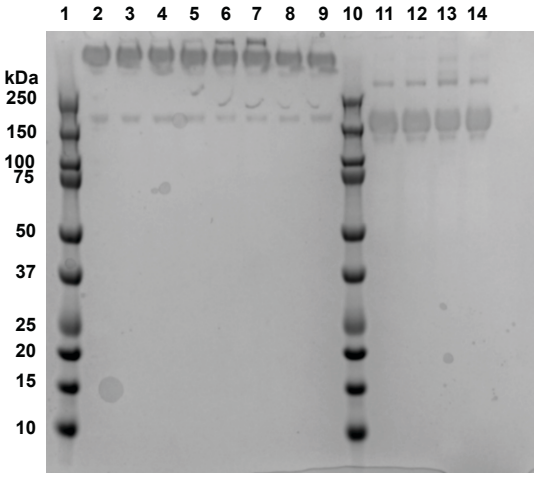
3. 2P-N234A 096VS 37 °C Repeat 1
4. 2P-N234A 096VS 37 °C Repeat 2
5. 2P-N165A 095VS 22 °C
6. 2P-N234A 096VS 4 °C Repeat 1
7. 2P-N234A 096VS 4 °C Repeat 2
8. 2P-N165A 095VS 22 °C
9. 2P-N234A 096VS 22 °C Repeat 1
10. Intentionally left blank
11. 2P-N234A 096VS 22 °C Repeat 2
12. 2P-N165A 095VS 4 °C Rebound
13. 2P-N234A 096VS 4 °C Rebound Repeat 1
14. 2P-N234A 096VS 4 °C Rebound Repeat 2

Supplementary Figure 3 . SDS-PAGE analysis of spike ectodomains incubated at different temperatures. Top. SDS-PAGE of 2P S under reducing and non-reducing conditions. Bottom. SDS-PAGE of the 2P-N165A and 2P-N234A spikes under reducing (left) and non-reducing (right) conditions. The legend below each gel lists the sample loaded in each lane. The sample description follows this order- construct name (e.g. 2P-N165A), lot # (e.g. 095VS), temperature at which sample was incubated for 1 week (e.g. 4 °C). Samples that were incubated first for 1 week at 4 °C, then moved to 37 °C hours are labeled as “Rebound”. 3 µg sample was loaded in each well.

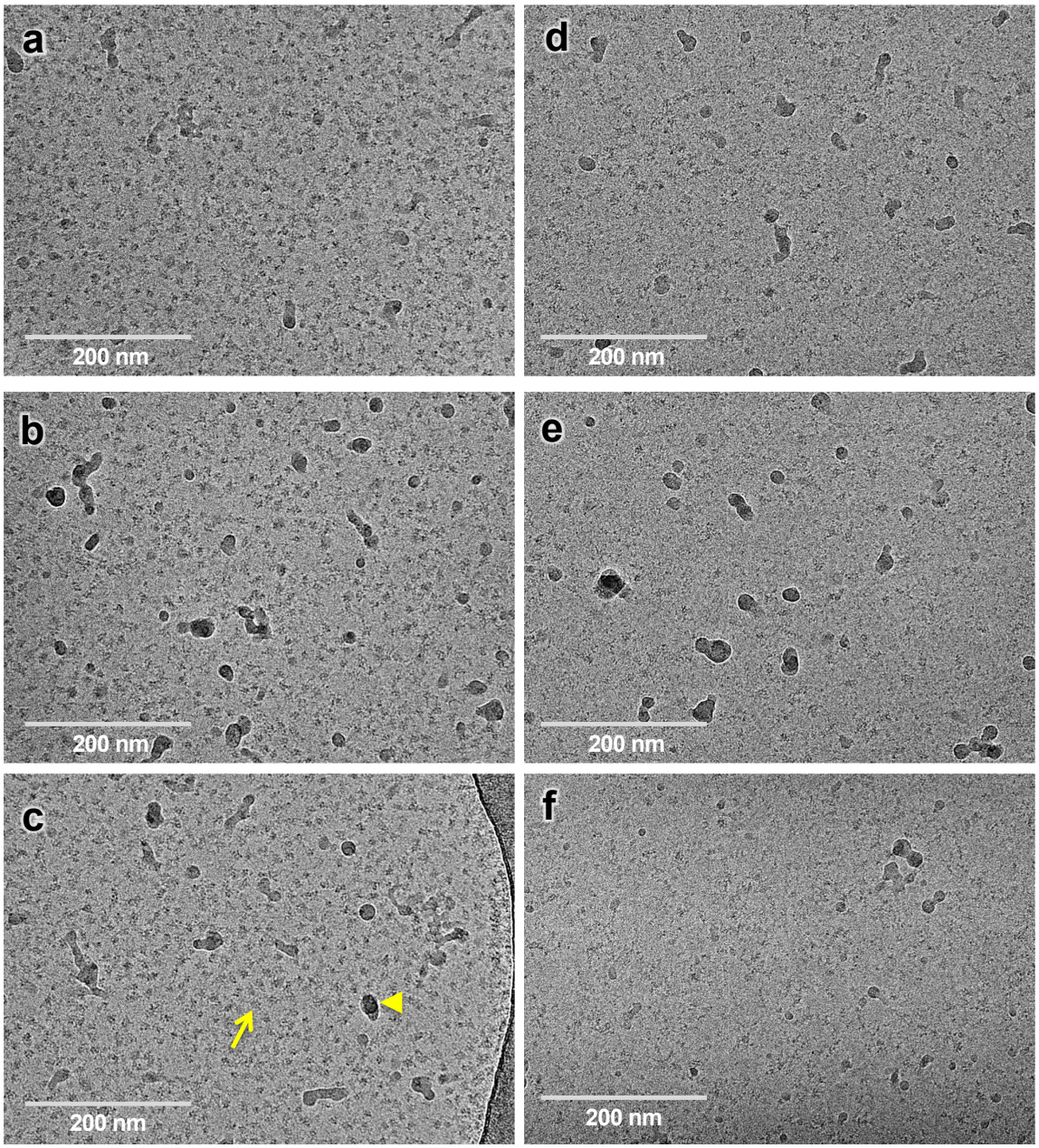


1. Molecular weight marker
2. Hexapro111KJ 4 °C Repeat 1
3. Hexapro111KJ 4 °C Repeat 2
4. Hexapro111KJ 22 °C Repeat 1
5. Hexapro111KJ 22 °C Repeat 2
6. Hexapro111KJ 37 °C Repeat 1
7. Hexapro111KJ 37 °C Repeat 2
8. Hexapro111KJ 4 °C Rebound Repeat 1
9. Hexapro111KJ 4 °C Rebound Repeat 2

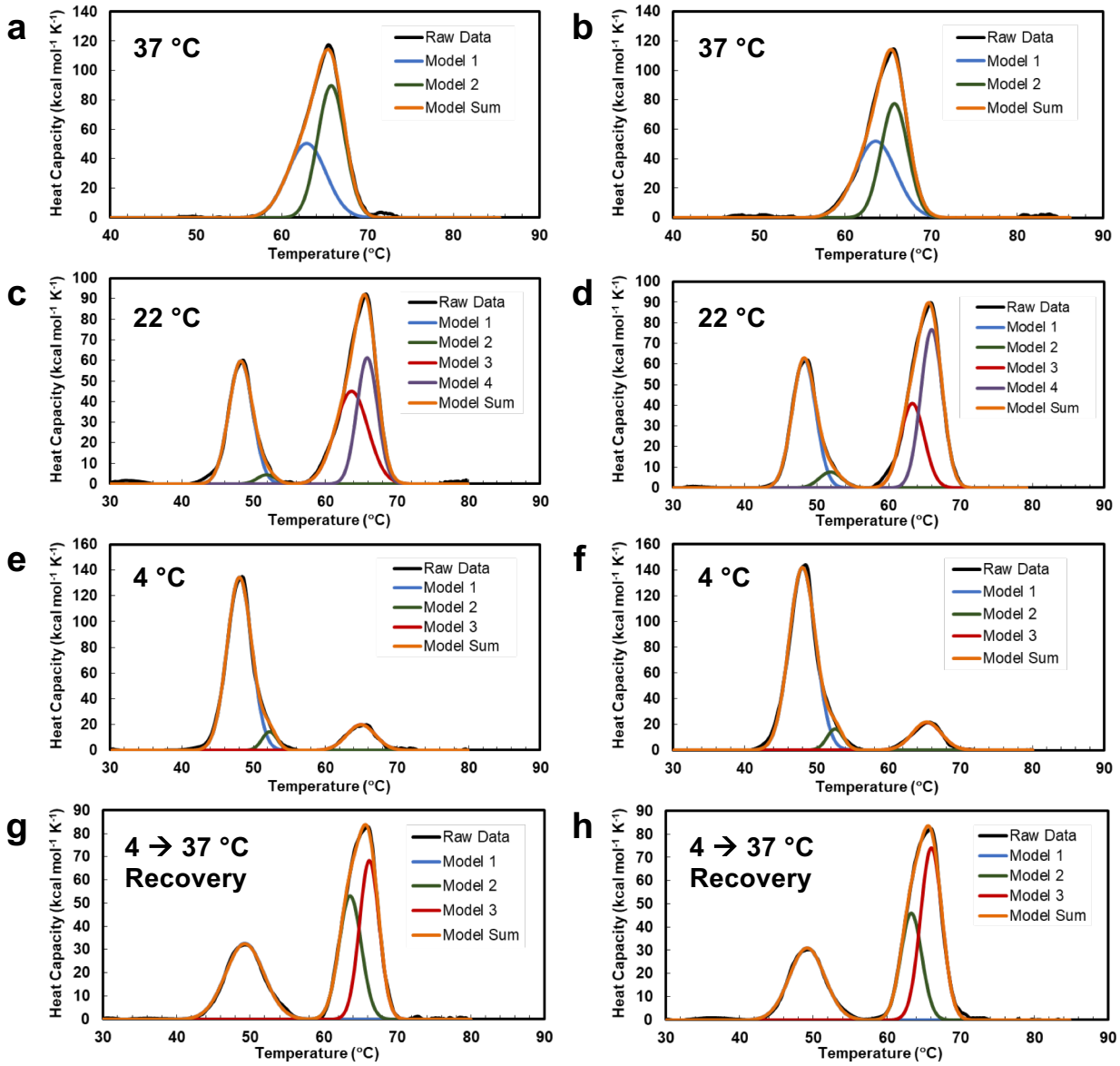


1. Molecular weight marker
2. rS2d-Hexapro 126KJ 4 °C Repeat 1
3. rS2d-Hexapro 126KJ 4 °C Repeat 2
4. rS2d-Hexapro 126KJ 22 °C Repeat 1
5. rS2d-Hexapro 126KJ 22 °C Repeat 2
6. rS2d-Hexapro 126KJ 37 °C Repeat 1
7. rS2d-Hexapro 126KJ 37 °C Repeat 1
8. rS2d-Hexapro 126KJ 4 °C Rebound Repeat 1
9. rS2d-Hexapro 126KJ 4 °C Rebound Repeat 2
10. Molecular weight marker
11. 2P 025MFK 4 °C
12. 2P 025MFK 22 °C
13. 2P 025MFK 37 °C
14. 2P 025MFK 4 °C Rebound

Supplementary Figure 3 (contd). SDS-PAGE analysis of spike ectodomains incubated at different temperatures. SDS-PAGE under non-reducing conditions. The legend below each gel lists the sample loaded in each lane. The sample description follows this order- construct name (e.g. HexaPro), lot # (e.g. 111KJ), temperature at which sample was incubated for 1 week (e.g. 4 °C). Samples that were incubated first for 1 week at 4 °C, then moved to 37 °C are labeled as “Rebound”. 10 µg sample was loaded in each well.



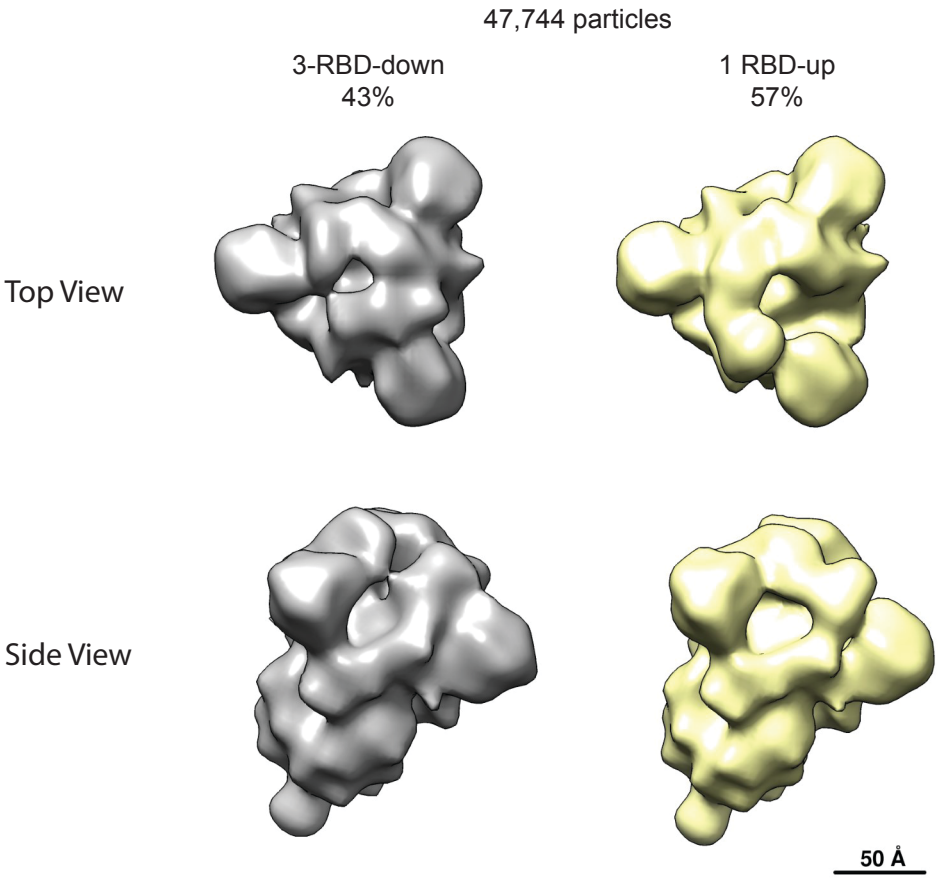
Supplementary Figure 4. Effect of SARS-CoV-2 2P S ectodomain sample storage on cryo-EM specimen preparation. (**a-c**) Representative cryo-EM micrographs of a SARS-CoV-2 2P S ectodomain sample that was flash frozen immediately after purification and stored in -80 °C, then thawed rapidly and incubated for ~ 5 min at 37 °C immediately prior to grid preparation. Cryo-EM images are low contrast, and the desired spike particles appear as medium gray spots (*e.g.* arrow) on a light gray background. Dark gray or black spots are slight ice contamination (*e.g* arrowhead). These panels on the left show an excellent distribution of discrete spike particles. **(d-f)** Representative cryo-EM micrographs of SARS-CoV-2 2P S ectodomain samples that were stored for ~1 week at 4 °C prior to grid preparation. Compared to the panels of the left, these panels on the right show a sparse field-of-view with very few intact spike particles visible. A similar spike concentration (~1 mg/ml) was used to freeze all the samples. Micrographs were collected on a Titan Krios microscope with a Gatan K3 camera.



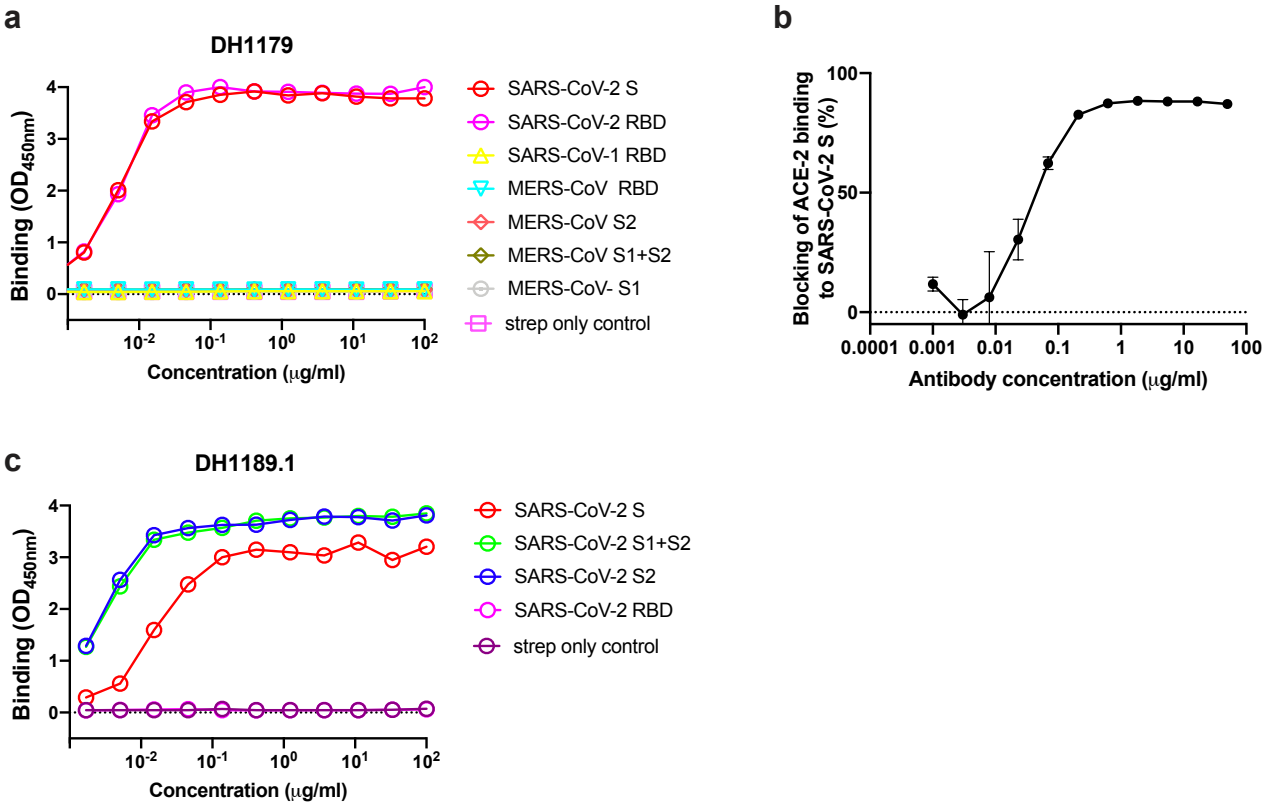
i

	Transition Peak 1		Transition Peak 2		DSC Model Fits			
	T_{onset} (°C)	T_m (°C)	T_{onset} (°C)	T_m (°C)	$T_{m,1}$ (°C)	$T_{m,2}$ (°C)	$T_{m,3}$ (°C)	$T_{m,4}$ (°C)
37 °C	N/A	**N/A**	57.5 ± 0.4	**65.5 ± 0.2**	63.2 ± 0.7	65.74 ± 0.02	N/A	N/A
22 °C	43.64 ± 0.02	**48.2 ± 0.1**	59 ± 1	**65.8 ± 0.1**	48.2 ± 0.1	51.87 ± 0.03	63.5 ± 0.5	65.9 ± 0.1
4 °C	43.0 ± 0.4	**48.41 ± 0.01**	61.1 ± 0.2	**65.1 ± 0.3**	48.02 ± 0.03	52.4 ± 0.3	65.1 ± 0.3	N/A
4 → 37 °C Recovery	42.5 ± 0.5	**49.2 ± 0.1**	59.59 ± 0.04	**65.95 ± 0.02**	49.2 ± 0.1	63.4 ± 0.3	66.1 ± 0.2	N/A

Supplementary Figure 5. Thermostability of the SARS-CoV-2 2P S ectodomain stored at different temperatures. Representative thermal denaturation profiles of the SARS-CoV-2 2P S ectodomain after 1 week incubations at (**a,b**) 37 °C , (**c,d**) 22 °C, (**e,f**) 4 °C, and (**g,h**) 4 °C followed by 37 °C for 3 hours. Profiles and transition parameters (**I**) were obtained by DSC and analyzed as described in Methods. Raw data (black) was best fit with two or more Gaussian transition models ($T_{m,1\text{-}4}$) (blue, green, red, purple). The peak observed at ~65 °C was best fit with two Gaussian transition models suggesting a complex unfolding mechanism. Data shown are the mean and range from two experimental replicates.



Supplementary Figure 6. 3D classification and reconstructions from NSEM data on SARS-CoV-2 2P S that had been incubated for 1 week at 4 °C followed by recovery for 3 hours at 37 °C .



d

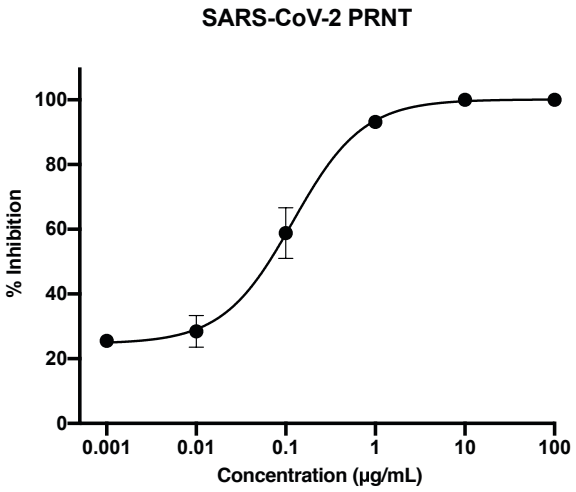
	SARS-CoV-2 Spike/2p	SARS-CoV-2 RBD	SARS-CoV-2 S2
DH1189.1	11.59	0	16.11
DH1179	16.705	17.048	0

Supplementary Figure 7. Characterization of antibodies isolated from COVID-19 convalescent donors by ELISA (**a**) Epitope mapping of antibody DH1179 shows that the antibody binds the SARS-CoV-2 RBD region, (**b**) Blocking of ACE-2 binding to SARS-CoV-2 S ectodomain by DH1179, **(c)** Epitope mapping of antibody DH1189.1 shows that the antibody binds the SARS-CoV-2 S2 region **(d)** Summary of epitope mapping from ELISA showing log of the area under the curves.

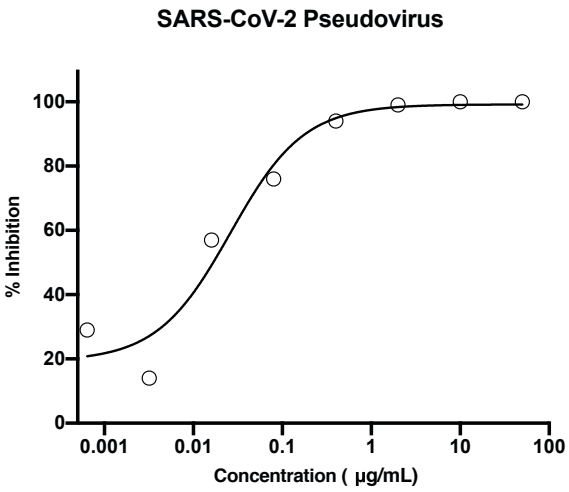
a

	Microneutralization assay	Pseudovirus assay		PRNT		
	Geometric Mean Effective conc. (µg/ml)	IC50 (µg/ml)	IC80 (µg/ml)	IC50 (µg/ml)	IC80 (µg/ml)	IC90 (µg/ml)
DH1189.1	>100	NA	NA	NA	NA	NA
DH1179	0.55	0.012	0.11	0.316	0.120	0.863

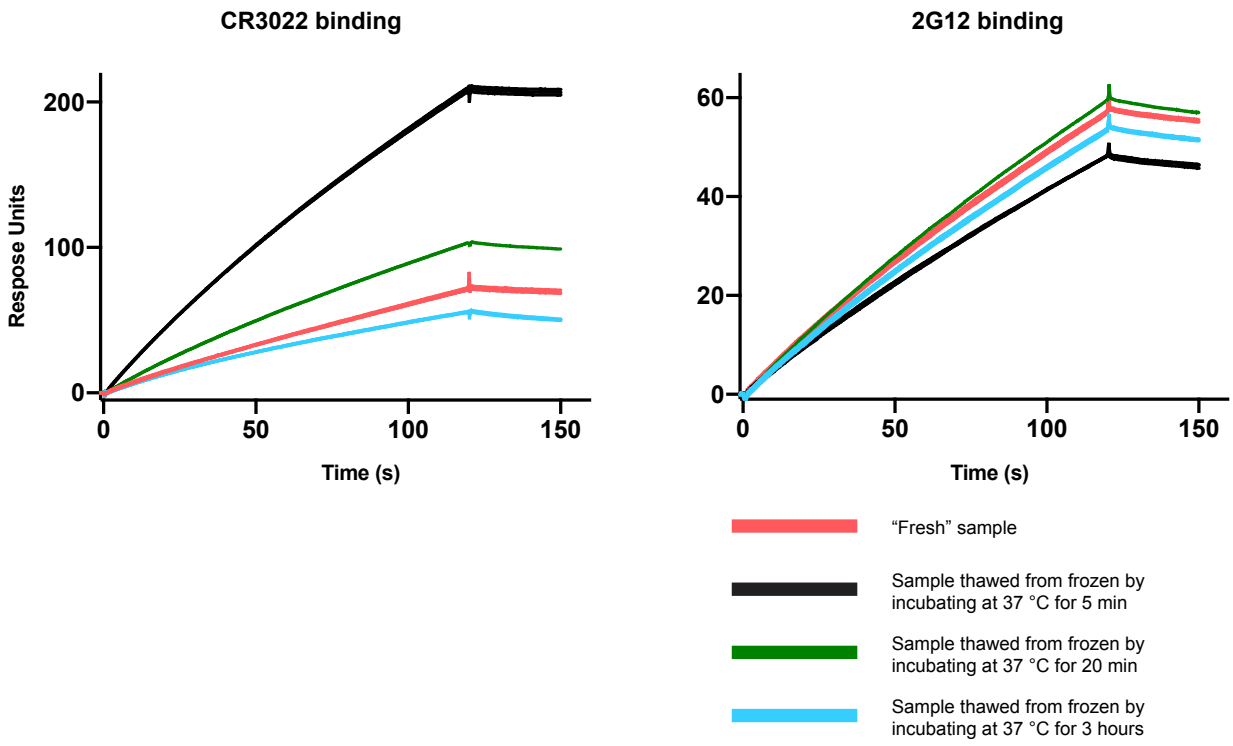
b



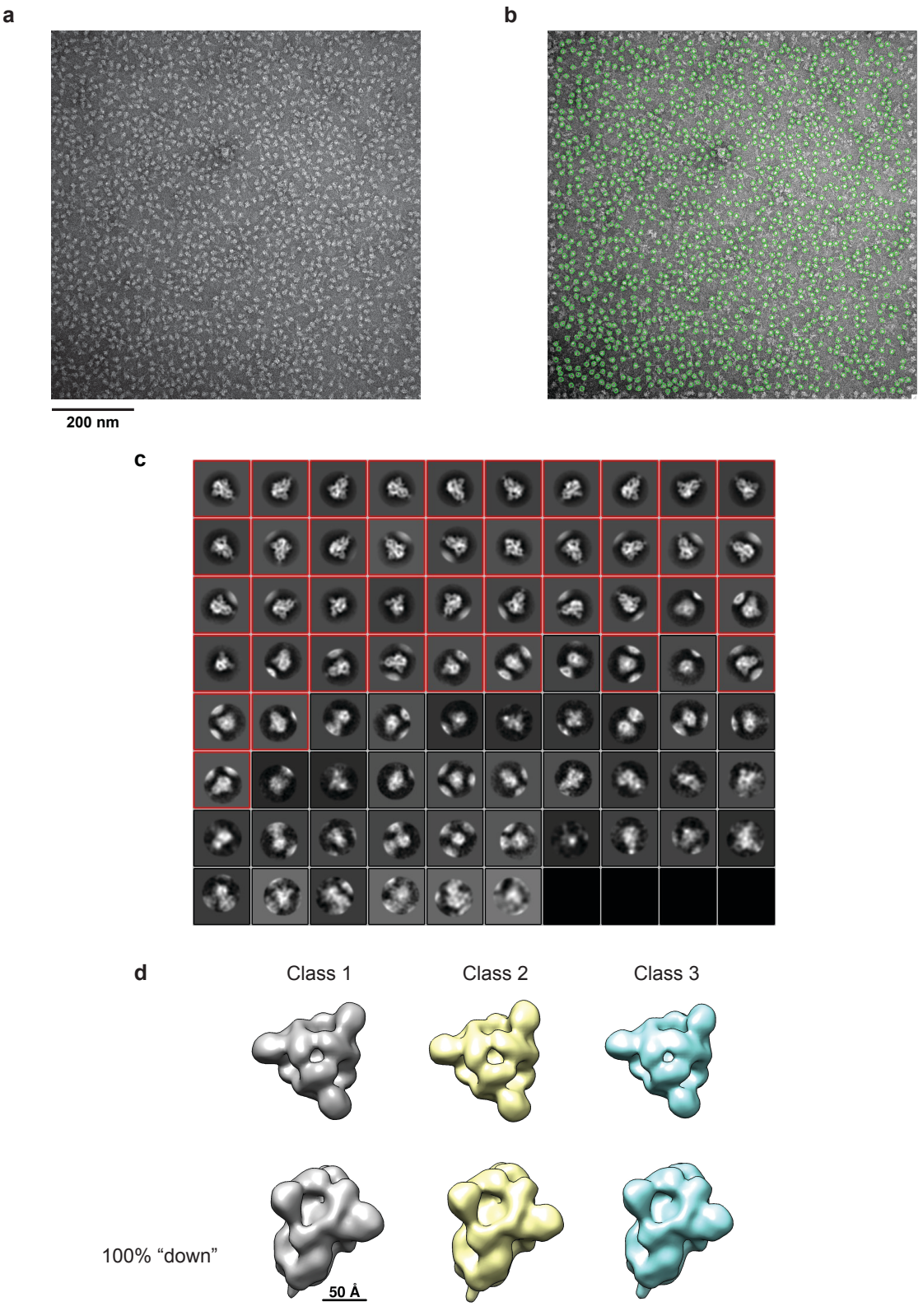
c



Supplementary Figure 8. Characterization antibodies isolated from COVID-19 convalescent donors in neutralization assays. (**a**) Summary of neutralization profiles of antibodies DH1179 and DH1189.1 by three different assays. (**b**) Neutralization of SASR-CoV-2 by DH1179 measured using plaque reduction neutralization test. **(c)** Neutralization of SASR-CoV-2 by DH1179 measured using a pseudovirus assay.



Supplementary Figure 9. CR3022 and 2G12 binding to freshly purified spike and spike that has been flash frozen then thawed. Antibody CR3022 IgG (left) and 2G12 IgG (right) binding to spike that was either freshly purified or flash-frozen in liquid N2, then thawed by incubating at 37 °C for different periods of time.


a
b
200 nm
c
d
Class 1
Class 2
Class 3
100% “down”
50 Å

Supplementary Figure 10. NSEM workflow for rS2d-HexaPro. (a) Representative NSEM micrograph **(b)** Representative NSEM micrograph showing particle picks in green **(c)** 2D class averages; the particles in the classes marked with a red box were taken forward to the next steps on the analysis **(d)** 3D classes showing top views in the top row and side views in the bottom row. 30,000 particles were used for 3D classification. These were separated into three 3D classes that were reconstructed using C1 symmetry. Only all-RBD-down classes were observed. Also see Supplementary Movie S1 that shows the residual movement in the RBDs despite being locked down by a RBD-to-S2 disulfide.