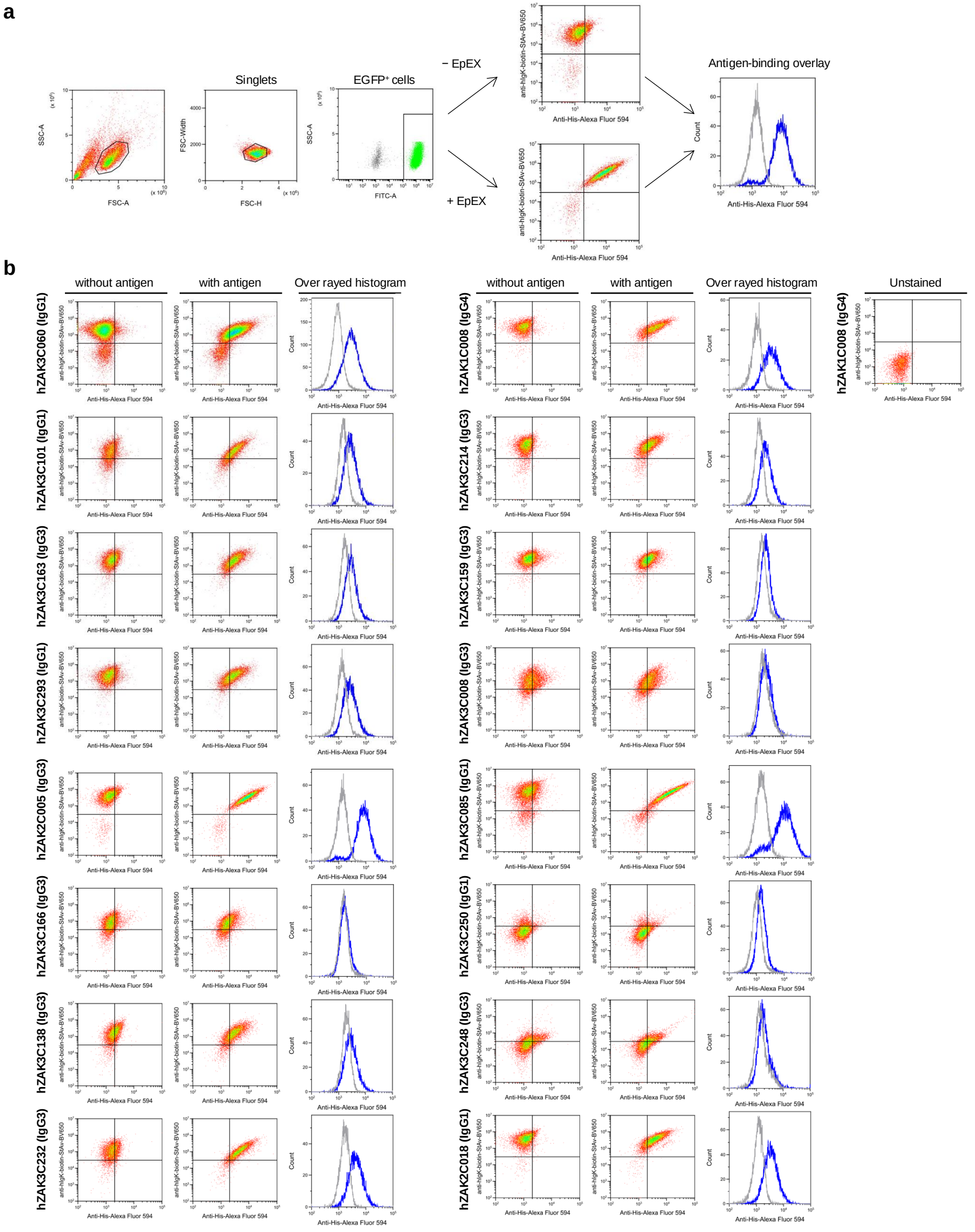
Supplementary Information

Developing a workflow for the isolation of hybridoma cells producing fully human antigen-specific antibodies using a surface IgG detection method

Satofuka and Wang et al.



Supplementary Figure 1. Flow cytometry to detect hybridoma cells expressing antigen-specific monoclonal antibodies.
(a) Gating strategy for the antigen (EpCAM) reactivity analysis of the hybridoma cell surface. Hybridoma cells were generated from TC-mAb mice as previously described[7]. The preparation of the EpCAM extracellular domain recombinant protein (EpEX with a His-tag at the C-terminus) was also reported previously[8]. The hybridoma cells were stained with EpEX (left) or left unstained (right), before being treated with anti-His antibody conjugated to Alexa Fluor 594. **(b)** Analysis of the reaction between surface IgG and antigen. The overlaid histograms correspond to the data presented in the dot plots, where the presence or absence of EpEX protein is indicated in blue and gray, respectively. The names of the hybridoma clone and their subclasses are indicated on the left of each set of graphs. An unstained hZAK1C008 (IgG4) control sample is shown in the upper right-hand corner of the figure.

a

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Mutated hIgG1  PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
               |||.|||||||||||||  |.||||||||||||||||||||||||||||||||||||||
P01857         PKSSDKTHTCPPCPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN

Mutated hIgG1  WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
               |||||||||||||||||||||||||||||||||||||||||||||| ||||||||.|||||
P01857         WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPASIEKTI

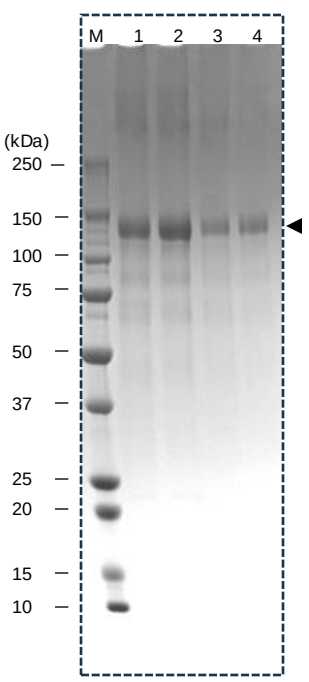
Mutated hIgG1  SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
               ||||||||||||||||||.|.||||||||||||||||||||||||||||||||||||||
P01857         SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP

Mutated hIgG1  VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
               ||||||||||||||||||||||||||||||||||||||||||||||||
P01857         VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
```

b

MYRMQLLSCIALSLALVTNSQSTIEEQAKTFLDKFNHEAEDLFYQSSLASWNYNTNITEENVQNMNNA
GDKWSAFLKEQSTLAQMYPLQEIQNLTVKLQLQALQQNGSSVLSEDKSKRLNTILNTMSTIYSTGKVC
NPDNPQECLLLEPGLNEIMANSLDYNERLWAWESWRSEVGKQLRPLYEEYVVLKNEMARANHYEDY
GDYWRGDYEVNGVDGYDYSRGQLIEDVEHTFEEIKPLYEHLHAYVRAKLMNAYPSYISPIGCLPAHLL
GDMWGRFWTNLYSLTVPFGQKPNIDVTDAMVDQAWDAQRIFKEAEKFFVSVGLPNMTQGFWENSM
LTDPGNVQKAVCHPTAWDLGKGDFRILMCTKVTMDDFLTAHHEMGHIQYDMAYAAQPFLLRNGANE
GFHEAVGEIMSLSAATPKHLKSIGLLSPDFQEDNETEINFLLKQALTIVGTLPFTYMLEKWRWMVFKGEI
PKDQWMKKWWEMKREIVGVVEPVPHDETYCDPASLFHVSNDYSFIRYYTRTLYQFQFQEALCQAAK
HEGPLHKCDISNSTEAGQKLFNMLRLGKSEPWTLALENVVGAKNMNVRPLLNYFEPLFTWLKDQNKN
SFVGWSTDWSPYADQSIKVRISLKSALGDKAYEWNDNEMYLFRSSVAYAMRQYFLKVKNQMILFGEE
DVRVANLKPRISFNFFVTAPKNVSDIIPRTEVEKAIRMSRSRINDAFRLNDNSLEFLGIQPTLGPPNQPP
VSGGGGSTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG
VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY
TLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

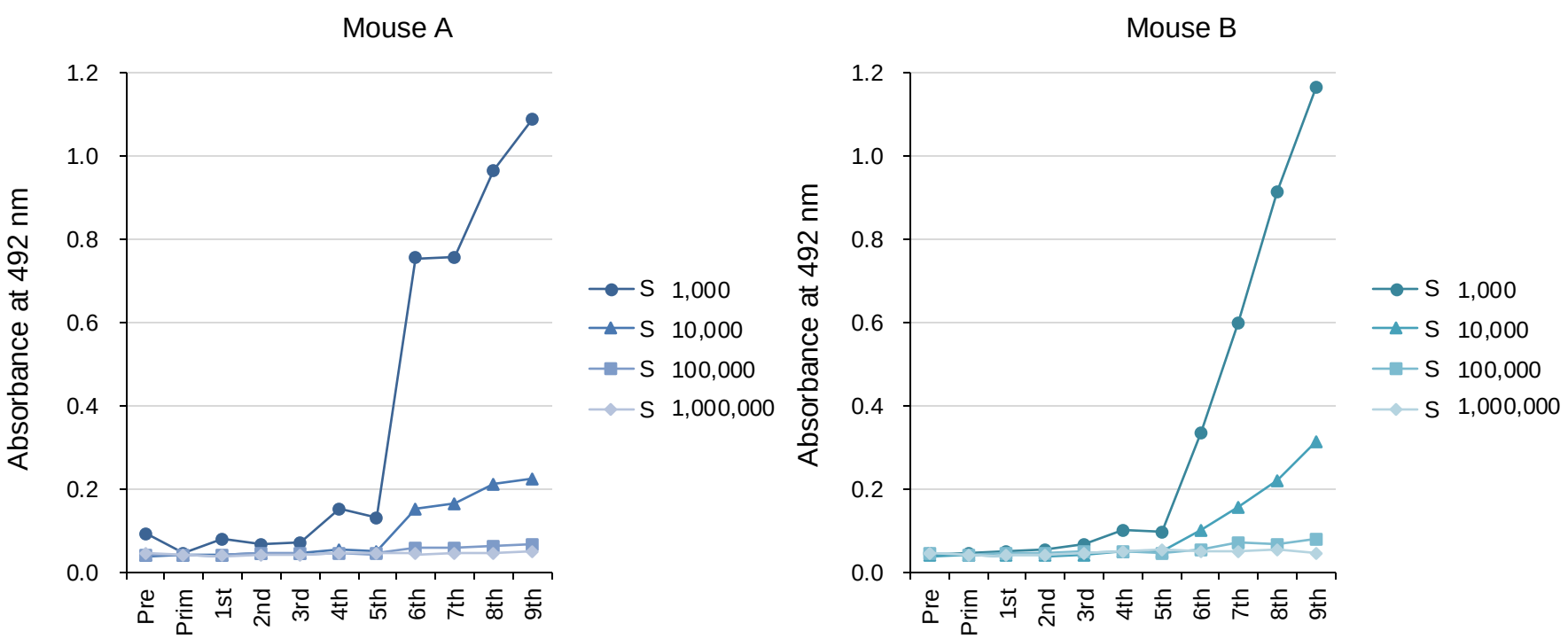
Supplementary Figure 2. Production of human IgG1-fused antigen for the immunization of TC-mAb mice.
(**a**) Sequence differences between human IgG1 and mutated IgG1. The amino acid sequence of human IgG1 (P01857) was obtained from the UniProt database (https://www.uniprot.org/) as an immunoglobulin heavy constant gamma 1 sequence (*Homo sapiens*). The amino acids highlighted in red represent mutated residues. **(b)** Amino acid sequence of the hACE2-Fc fusion protein. The IL2 signal sequence (sky blue), hACE2 extracellular domain (red), linker (green), and mutated hIgG1 (blue) are indicated.



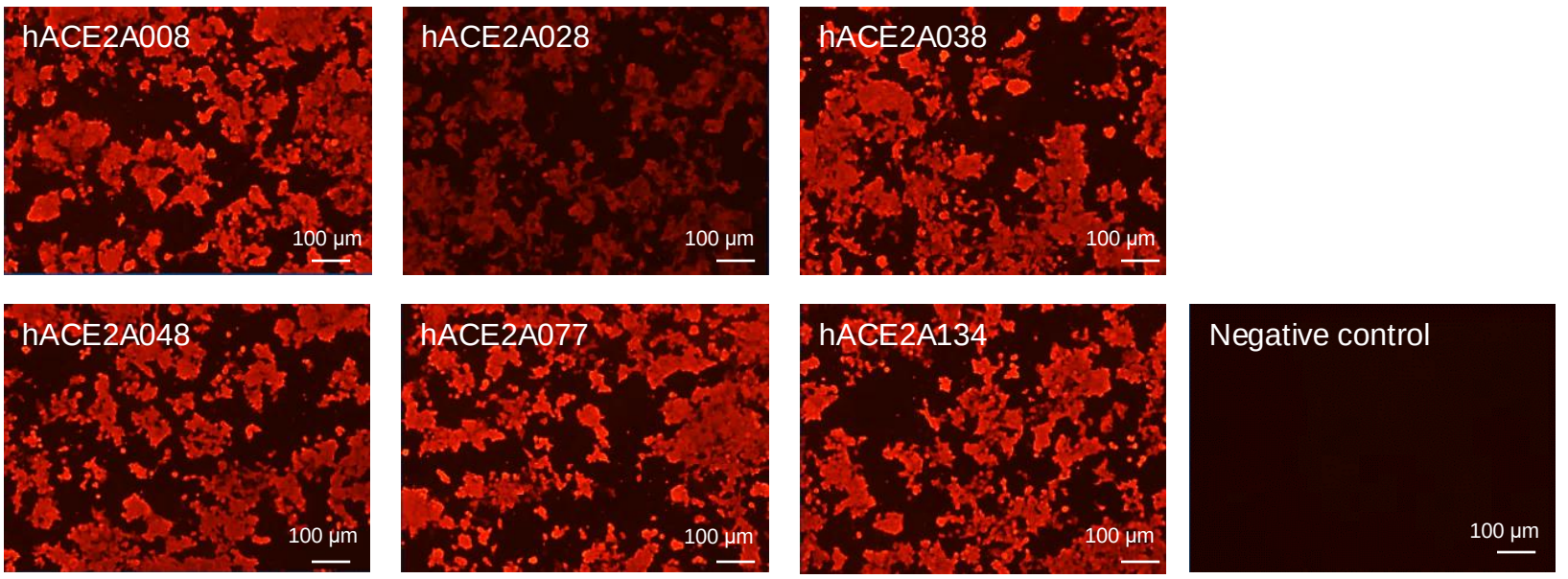
M: Marker
1 : Purified, PEI-1, 5 µg
2 : Purified, PEI-2, 5 µg
3 : Purified, PEI-1, 2.5 µg
4 : Purified, PEI-2, 2.5 µg

Estimated molecular weight of hACE2-Fc: 109.143 kDa

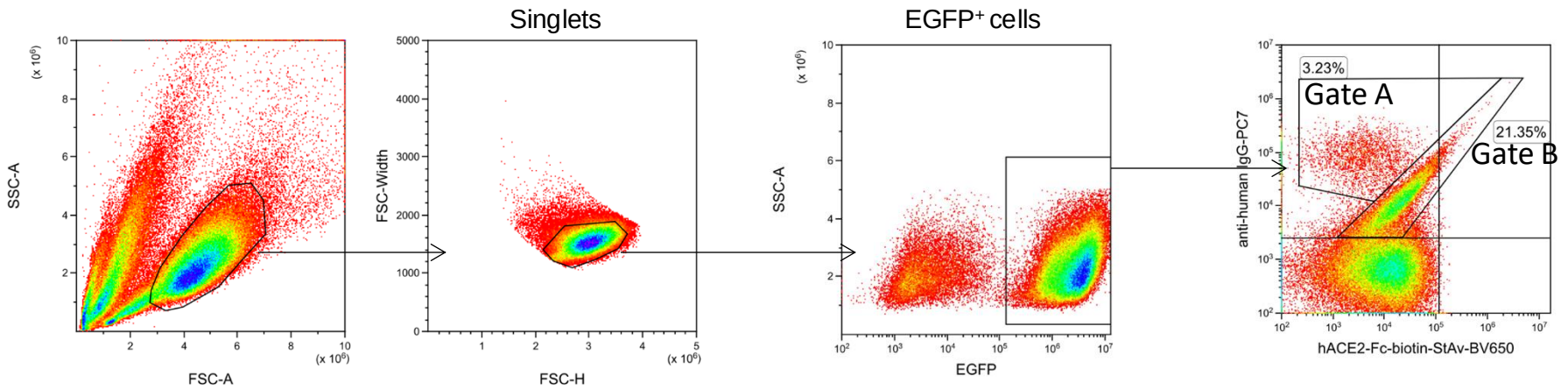
Supplementary Figure 3. SDS-PAGE analysis of the purified hACE2-Fc fusion protein.
After protein G column purification, the purified hACE2-Fc fusion protein was analyzed by SDS-PAGE using a 5%–20% gradient gel. The black arrowhead indicates the main signal originating from hACE2-Fc. The molecular weight of hACE2-Fc was estimated from amino acid sequence to be 109.143 kDa, but a signal with a higher molecular weight was observed, probably due to glycosylation. The uncropped gel is shown in Supplementary Figure 8.



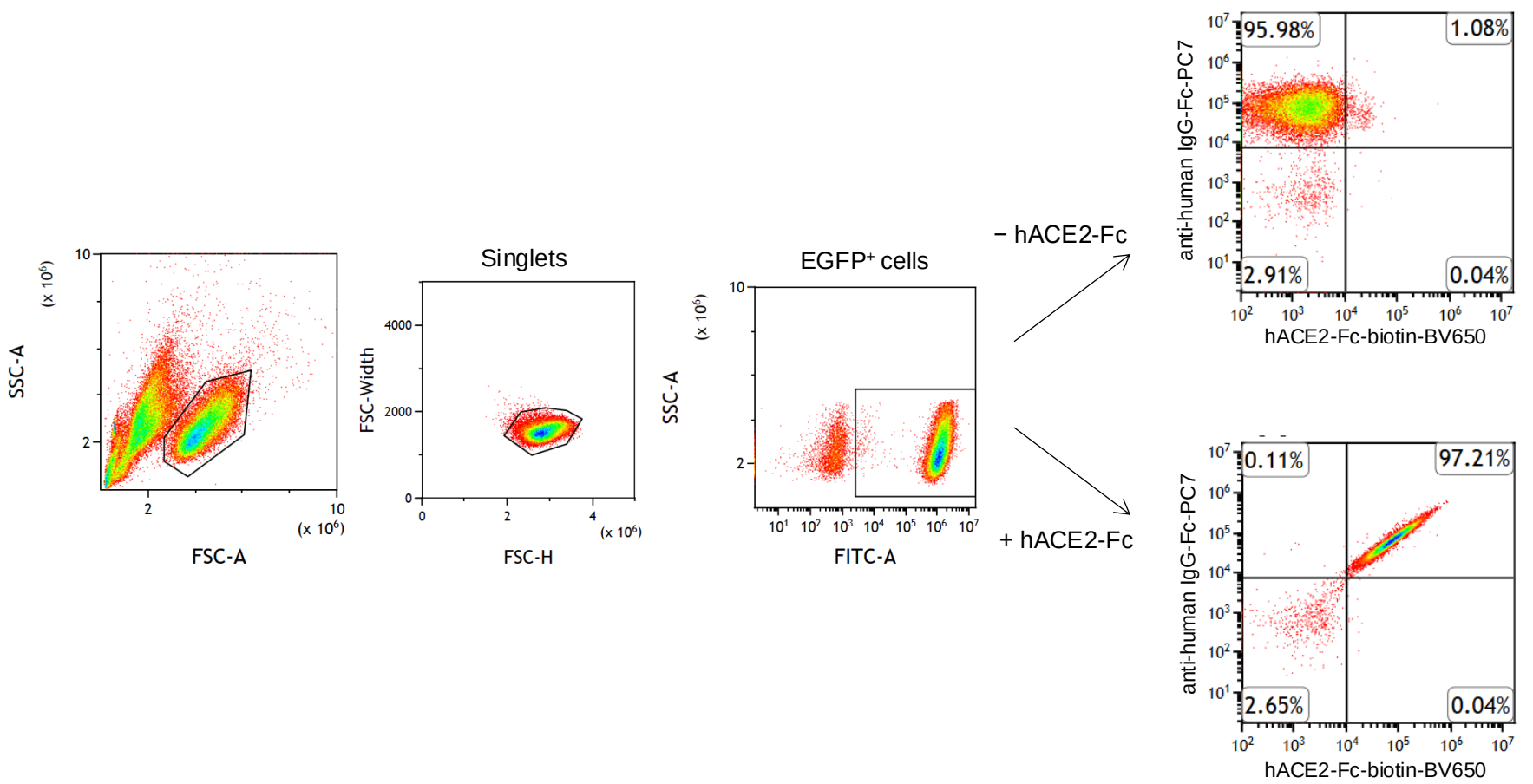
Supplementary Figure 4. Analysis of anti-serum titers in TC-mAb mice immunized with the hACE2-Fc protein. The titers of anti-serum were analyzed using the fusion protein, hACE2-Fc; four different dilution factors were used as indicated in the legend. Because the increase in Ab titer to hACE2-Fc was weak, nine immunizations were performed to achieve sufficiently high Ab titers.



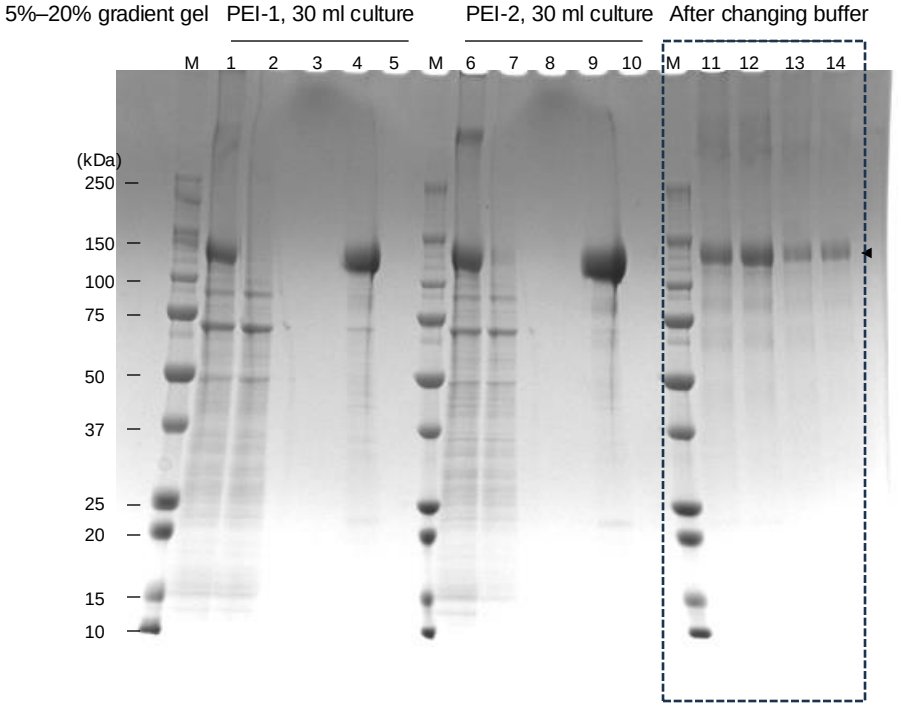
Supplementary Figure 5. Immunocytochemistry analysis of the monoclonal antibodies obtained.
HEK293-hACE2 cells were incubated with the hybridoma cell culture supernatants or medium only (negative control). The name of each monoclonal antibody analyzed is indicated in the upper left corner of each image. The images were obtained using an IncuCyte S3 instrument (Sartorius, Gettingen, Germany) at ×20 magnification.



Supplementary Figure 6. Flow cytometry gating strategy for detecting hybridoma cells expressing antigen-specific monoclonal antibodies.
Because anti-human IgG-Fc-PE/Cyanine7 can react with both surface IgG and hACE2-Fc-biotin, hybridoma cells expressing antigen-specific antibodies were identified within Gate B, while IgG-expressing cells which did not bind antigen are shown in Gate A.



Supplementary Figure 7. Flow cytometric detection of hACE2-specific sIgG-expressing hybridoma cells.
Gating strategy for antigen (EpCAM) reactivity analysis of hybridoma cells. The hybridoma cells were stained with biotinylated hACE2-Fc or left unstained, followed by staining with a Streptavidin-Alexa Fluor 594 conjugate.

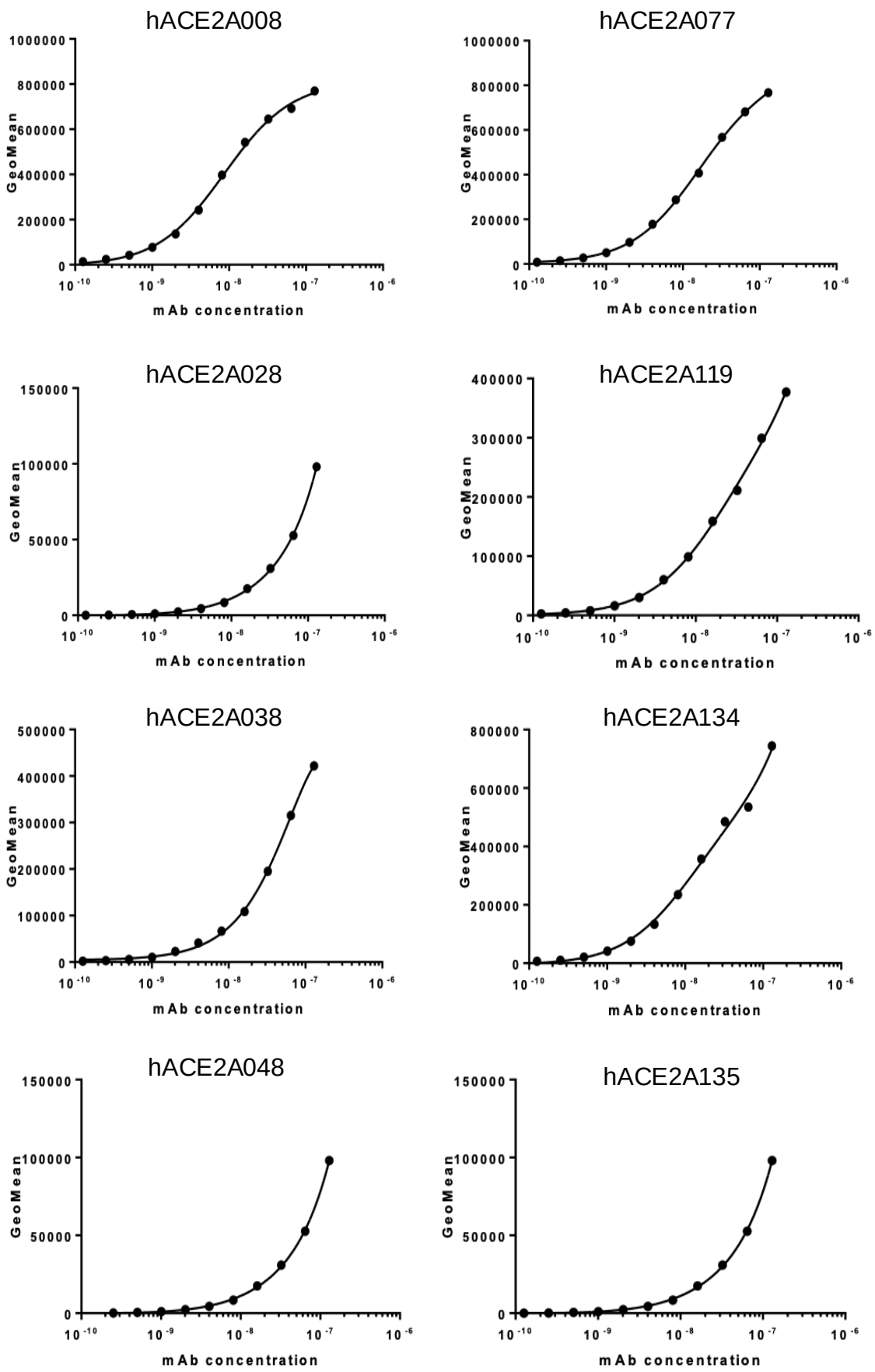


M: Marker
1 : Expi293, Supernatant, PEI-1
2 : Flowthrough
3 : Wash
4 : Elution 1
5 : Elution 2
6 : Expi293, Supernatant, PEI-2
7 : Flowthrough
8 : Wash
9 : Elution 1
10 : Elution 2
11 : Purified, PEI-1, 5 µg
12 : Purified, PEI-2, 5 µg
13 : Purified, PEI-1, 2.5 µg
14 : Purified, PEI-2, 2.5 µg

Estimated molecular weight of hACE2-Fc: 109.143 kDa

Supplementary Figure 8. SDS-PAGE analysis of the purified hACE2-Fc fusion protein (uncropped).
The hACE2-Fc protein was expressed by using the Expi293 Expression System (Thermo Scientific) in combination with PEI MAX - Transfection Grade Linear Polyethylenimine Hydrochloride (Polysciences, Inc., Warrington, PA, USA). The hACE2-Fc protein was purified using Protein G Sepharose FF column. This figure is related to Supplementary Figure 3.

(a)



(b)

	hACE2 A008	hACE2 A028	hACE2 A038	hACE2 A048	hACE2 A077	hACE2 A119	hACE2 A134	hACE2 A135
KD ($\times 10^{-8}$ M)	0.86	2.08	0.66	15.5	1.59	1.86	1.07	5.16
R^2	0.998	0.999	0.998	0.999	0.999	0.999	0.995	0.999

Supplementary Figure 9. KD value of hACE2 antibodies.
The data were collected using CytoFLEX S and analyzed using Kaluza software (a), and the KD values (b) were estimated by fitting binding isotherms to built-in one-side binding models of GraphPad Prism 8 (GraphPad Software, Inc. La Jolla, CA, USA).

(a) hACE2A038-H chain

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                                                <---------------------------------FR1-IMGT--------------------------------><-------CDR1-IM
                                                 Q  V  Q  L  V  Q  S  G  A  E  V  K  K  P  G  S  S  V  K  V  S  C  K  A  S  G  Y  T  F  T
                      Query_1      1            CAGGTTCAACTGGTGCAGTCTGGAGCTGAGGTGAAGAAGCCTGGGTCCTCAGTCAAGGTCTCCTGCAAGGCTTCTGGTTACACCTTTACC  90
V  96.6% (284/294)    IGHV1-18*01  1            ........G....................................G.......G....................................  90
                                                 Q  V  Q  L  V  Q  S  G  A  E  V  K  K  P  G  A  S  V  K  V  S  C  K  A  S  G  Y  T  F  T
V  96.3% (283/294)    IGHV1-18*03  1            ........G....................................G.......G....................................  90
V  96.3% (283/294)    IGHV1-18*04  1            ........G....................................G.......G....................................  90

                                                GT------><-----------------FR2-IMGT------------------------><-------CDR2-IMGT------><-----
                                                 S  C  G  I  S  W  V  R  Q  A  P  G  Q  G  L  E  W  M  G  W  I  S  V  Y  N  D  N  T  N  Y
                      Query_1      91           AGCTGTGGTATCAGCTGGGTCCGACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGATGGATCAGCGTTTACAATGATAACACAAACTAT  180
V  96.6% (284/294)    IGHV1-18*01  91           ....A...............G..............................................C........G.............  180
                                                 S  Y  G  I  S  W  V  R  Q  A  P  G  Q  G  L  E  W  M  G  W  I  S  A  Y  N  G  N  T  N  Y
V  96.3% (283/294)    IGHV1-18*03  91           ....A...............G..............................................C........G.............  180
V  96.3% (283/294)    IGHV1-18*04  91           ....AC..............G..............................................C........G.............  180

                                                -----------------------------------------FR3-IMGT-----------------------------------------
                                                 A  Q  K  F  Q  G  R  V  T  M  T  T  D  T  S  T  S  T  A  Y  M  E  L  R  S  L  R  S  D  D
                      Query_1      181          GCACAGAAGTTCCAGGGCAGAGTCACCATGACCACAGATACATCCACGAGCACAGCCTACATGGAACTGAGGAGCCTGAGATCTGACGAC  270
V  96.6% (284/294)    IGHV1-18*01  181          .........C............................C..........................G........................  270
                                                 A  Q  K  L  Q  G  R  V  T  M  T  T  D  T  S  T  S  T  A  Y  M  E  L  R  S  L  R  S  D  D
V  96.3% (283/294)    IGHV1-18*03  181          .........C............................C..........................G........................  270
V  96.3% (283/294)    IGHV1-18*04  181          .........C............................C..........................G........................  270

                                                -----------------><---------------------CDR3-IMGT----------------------><-----------FR4-IM
                                                 T  A  V  Y  Y  C  A  R  S  G  C  I  T  M  I  R  G  V  M  D  W  F  D  P  W  G  Q  G  T  L
                      Query_1      271          ACGGCCGTGTATTACTGTGCGAGATCTGGGTGTATTACTATGATTCGGGGAGTTATGGACTGGTTCGACCCCTGGGGCCAGGGAACCCTG  360
V  96.6% (284/294)    IGHV1-18*01  271          ........................------------------------------------------------------------------  294
                                                 T  A  V  Y  Y  C  A  R
V  96.3% (283/294)    IGHV1-18*03  271          .T......................------------------------------------------------------------------  294
V  96.3% (283/294)    IGHV1-18*04  271          ........................------------------------------------------------------------------  294
D  96.0% (24/25)      IGHD3-10*01  1            -------------------------------...........G.............----------------------------------  25
D  92.0% (23/25)      IGHD3-10*03  1            -------------------------------...........G...A.........----------------------------------  25
D  96.0% (24/25)      IGHD3-10*02  1            -------------------------------...........-.............----------------------------------  24
J  100.0% (47/47)     IGHJ5*02     4            ----------------------------------------------------------................................  35
J  95.7% (45/47)      IGHJ5*01     4            ----------------------------------------------------------...........T..........A.........  35
J  100.0% (34/34)     IGHJ4*02     14           -----------------------------------------------------------------------...................  32

                                                GT------------>
                                                 V  T  V  S  S
                      Query_1      361          GTCACCGTCTCCTCA  375
J  100.0% (47/47)     IGHJ5*02     36           ...............  50
J  95.7% (45/47)      IGHJ5*01     36           ...............  50
J  100.0% (34/34)     IGHJ4*02     33           ...............  47
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(b) hACE2A038-L chain

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                                                <----------------------------------FR1-IMGT----------------------------------><----CDR1-IM
                                                 D  I  Q  M  T  Q  S  P  S  A  M  S  A  S  V  G  D  R  V  T  I  T  C  R  A  S  Q  G  I  S
                      Query_1      1            GACATCCAGATGACCCAGTCTCCATCTGCCATGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGTCGGGCGAGTCAGGGCATTAGC  90
V  97.9% (278/284)    IGKV1-17*03  1            ..........................................................................................  90
                                                 D  I  Q  M  T  Q  S  P  S  A  M  S  A  S  V  G  D  R  V  T  I  T  C  R  A  S  Q  G  I  S
V  97.2% (275/283)    IGKV1D-17*01 2            -............................................................................G............  90
V  94.4% (268/284)    IGKV1-17*01  1            ..........................CT..C.....................................C.....A..............A  90

                                                GT---><------------------FR2-IMGT-----------------------><CDR2-IM><-----------------------
                                                 N  Y  L  A  W  F  Q  Q  K  P  G  K  V  P  K  R  L  I  Y  A  A  S  S  L  Q  S  G  V  P  S
                      Query_1      91           AATTATTTAGCCTGGTTTCAGCAGAAACCAGGGAAAGTCCCTAAGCGCCTGATCTATGCTGCCTCCAGTTTGCAAAGTGGGGTCCCATCA  180
V  97.9% (278/284)    IGKV1-17*03  91           ..............................................................A...........................  180
                                                 N  Y  L  A  W  F  Q  Q  K  P  G  K  V  P  K  R  L  I  Y  A  A  S  S  L  Q  S  G  V  P  S
V  97.2% (275/283)    IGKV1D-17*01 91           ..............................................A...............A...........................  180
V  94.4% (268/284)    IGKV1-17*01  91           ...G......G....A....................C........................A...........................  180

                                                ------------------------FR3-IMGT---------------------------------------------------><-----
                                                 R  F  S  G  N  G  S  G  T  E  F  T  L  T  I  S  S  L  Q  P  E  D  F  A  T  Y  F  C  L  Q
                      Query_1      181          AGGTTCAGCGGCAATGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCTGAAGATTTTGCAACTTATTTCTGTCTACAG  270
V  97.9% (278/284)    IGKV1-17*03  181          .............G.................................................................A..........  270
                                                 R  F  S  G  S  G  S  G  T  E  F  T  L  T  I  S  S  L  Q  P  E  D  F  A  T  Y  Y  C  L  Q
V  97.2% (275/283)    IGKV1D-17*01 181          .............G.................................................................A..........  270
V  94.4% (268/284)    IGKV1-17*01  181          .............G.................................................................A..........  270

                                                ---CDR3-IMGT--------><----------FR4-IMGT---------->
                                                 H  N  Y  F  P  I  T  F  G  Q  G  T  R  L  E  I  K
                      Query_1      271          CATAATTATTTCCCGATCACCTTCGGCCAAGGGACACGACTGGAGATTAAA  321
V  97.9% (278/284)    IGKV1-17*03  271          ......AG..A...-------------------------------------  284
                                                 H  N  S  Y  P
V  97.2% (275/283)    IGKV1D-17*01 271          ......AG..A...-------------------------------------  284
V  94.4% (268/284)    IGKV1-17*01  271          ......AG..A...-------------------------------------  284
J  100.0% (37/37)     IGKJ5*01     1            --------------.....................................  37
J  100.0% (14/14)     IGKJ1*01     8            ---------------------..............----------------  21
J  76.5% (26/34)      IGKJ2*01     5            -----------------...T..T.....G.....CAAG........C...  38

Lambda      K        H
    1.10    0.333    0.549

Gapped
Lambda      K        H
    1.08    0.280    0.540

Effective search space used: 49421775
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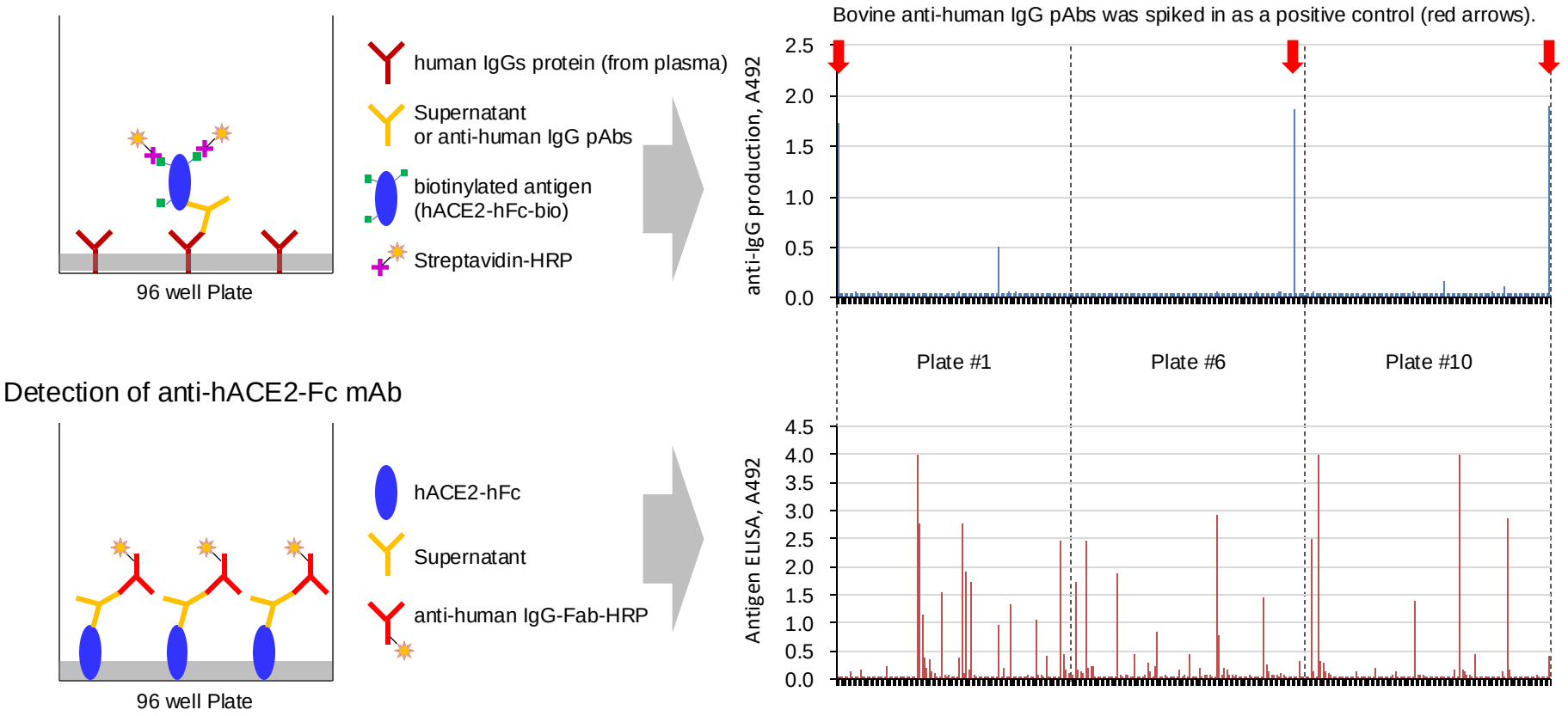
Supplementary Figure 10. Somatic hypermutation analysis of the hACE2A038 mAb.

The nucleotide sequences of the H (a) and L (b) chains of hACE2A038 were analyzed with the IgBlast tool (https://www.ncbi.nlm.nih.gov/igblast/). The results showed that there were 10 nucleotide mutations in the H chain and 6 nucleotide mutations in the L chain, including amino acid mutations. We previously reported that the average somatic hypermutations in the H and L chains of TC-mAb mice were 0.90 and 1.47, respectively (Satofuka et al., *Nature Communications*, 2022), indicating that hACE2A038 has an above-average number of mutations.



Supplementary Figure 11. ELISA analysis of magnetic-bead-enriched hybridoma cells.
Three plates were randomly selected from plates seeded with cell fractions enriched by beads (plate numbers 1, 6, and 10), and 288 wells of hybridoma cell culture supernatants were analyzed to determine whether mAbs reacted with the human Fc region (a) or hACE2 (b). Red arrows indicate positive controls when antibodies against human IgG were produced by spiking with 2 μg/mL of bovine anti-human IgG polyclonal antibodies (Bethyl Laboratories, Montgomery, TX, USA). The results of the experiment showed that few antibodies that reacted to human antibodies were induced.
To detect the induction of anti-human IgG Abs, plates were coated with human IgGs protein and blocked with 5% skim milk for 30 minutes at room temperature. After washing, supernatant or anti-human IgG polyclonal Abs (pAbs) was added to the wells and incubated for 60 minutes. After washing, biotinylated hACE2-Fc protein was added and incubated for 60 minutes. When anti-human IgG mAbs are produced in the supernatant, they interact with immobilized human IgG and the human Fc of biotinylated hACE2-Fc. The binding of biotinylated hACE2-Fc was detected by developing with streptavidin-HRP conjugate for 10 minutes.

Supplementary Table 1. Determination of yield using the bead separation method

TC-mAb mouse identifier	#6292	#6978
Bead treatment	Anti-human IgG-biotin-StAv-beads	
Number of splenocytes ($\times 10^8$)	3.0	4.0
Number of cultured cells ($\times 10^8$)*	1.9	3.6
Number of separated cells ($\times 10^8$)	0.9	2.0
Yield from **precleared cells	47.4%	55.6%

*After cell fusion, cells were cultured for 2 days and precleared using magnetic beads.
**Precleared cells are a pre-washed cell fraction of cells that bind nonspecifically to the beads by treatment with antigen-free beads.