1 # Title:

2 Development of a new genotype–phenotype linked antibody screening system

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22 Impact Statement:

23 A Golden Gate-based dual-expression vector enables rapid screening, facilitating efficient

24 isolation of high-affinity cross-reactive antibodies for therapeutic or diagnostic use, offering

25 a crucial advance for pandemic preparedness.

26 Keywords:

27 Membrane-bound Ig expression, NGS-based Ig-seq, Golden Gate Cloning, influenza

28 broadly-reactive

29

Abstract:

Antibodies are powerful tools for the therapy and diagnosis of various diseases. In addition to conventional hybridoma-based screening, recombinant antibody-based screening has become a common choice; however, its application is hampered by two factors: 1) screening starts only after Ig gene cloning and recombinant antibody production, and 2) the antibody is composed of paired chains, heavy and light, commonly expressed from two independent expression vectors. Here, we introduce a method for the rapid screening of recombinant monoclonal antibodies by establishing a Golden Gate-based dual-expression vector and *in vivo* expression of membrane-bound antibodies. Using this system, we demonstrated the efficient isolation of influenza cross-reactive antibodies with high affinity from mouse germinal center B cells over 4 days. This system is particularly useful for isolating therapeutic or diagnostic antibodies (e.g., during foreseen pandemics).

43 # Introduction:

44 Technical improvements in the rapid isolation of monoclonal antibodies (mAbs) are critical
45 for their diagnostic and therapeutic development. This need became apparent to the
46 general public during the COVID-19 pandemic. Antibodies isolated from convalescent
47 COVID-19 patients are valuable resources for the development of mAb therapeutics and
48 rapid diagnostic tools. Human antibodies can be administered to other patients without the
49 need to humanize in an emergency and with a slight modification of Fc receptor binding
50 (1) to increase the half-life or reduce the risk of antibody-dependent enhancement.
51 Although production costs remain high, the efficiency of generating recombinant
52 antibodies has dramatically improved with the use of mammalian cell lines, and the
53 application of therapeutic antibodies is likely to expand in the near future (2).

54 The gold standard method for isolating mAbs is the hybridoma technology developed
55 by Kohler and Milstein in 1975 (3), in which antibody-producing B cells are fused with
56 immortal B cells, called myelomas, to produce long-lasting antibody-producing B cells. In
57 addition to hybridoma technology, new methods have been developed to increase mAb
58 screening efficiency. These include direct immortalization of B cells by gene
59 reprogramming using the Epstein–Barr virus (4) or retrovirus-mediated gene transfer (5),
60 cloning of variable region-encoding genes by single-cell PCR (6,7), single-cell culture

61 screening (8), and *in vitro* screening of recombinant antibody libraries (9–13). Some of
62 these methods successfully yield high-affinity antibodies in various formats, including
63 single variable domain on a heavy chain or single-chain fragment variable antibodies.
64 Although hybridoma technology is highly reliable for the isolation of valuable antibodies
65 from animal models, such as mice, rats, and hamsters, it is time-consuming and requires
66 technical skill. The development of a new fusion partner cell line, SPYMEG, has enabled the
67 production of human hybridomas and opened a new direction for the isolation of human
68 mAbs (14). Although many of these new technologies have increased the throughput of
69 mAb isolation, the procedure still requires significant resources and time.

70 To dramatically enhance the efficiency of mAb isolation, we used next-generation
71 sequencing (NGS) technology, which has changed the method for sequencing
72 immunoglobulin (Ig) variable-region genes. For instance, tens of thousands of Ig genes
73 specific to certain antigens can be identified by combining droplet-based single-cell
74 isolation with DNA barcode antigen technology, followed by NGS (15). Although Ig genes
75 can be sequenced at a high throughput, there is no method for screening antibodies in a
76 high-throughput format that is compatible with NGS technology.

77 In this study, we developed a new functional screening method that is compatible
78 with NGS to rapidly identify antigen-specific clones. We first generated an Ig dual-

79 expression vector using Golden Gate Cloning (16), which enabled the linkage of heavy-
80 chain variable and light-chain variable DNA fragments obtained from a single-sorted B cell,
81 followed by the expression of membrane-bound Ig. This single-step procedure enabled the
82 enrichment of antigen-specific, high-affinity Igs by flow cytometry, which was significantly
83 faster than conventional cloning-based methods that require sequential steps. To
84 demonstrate the efficiency of our new method, we screened for potent broadly reactive
85 antibodies (17–19) against the influenza virus in an experimental mouse model using our
86 technology. Broad reactivity allows for influenza vaccines that work across seasonal
87 variations in influenza strains, which can only be accomplished by screening a large
88 number of candidate antibodies. We first raised cross-reactive B cells against various
89 hemagglutinin (HA) antigens from the influenza virus by sequential immunization with
90 heterotypic HA antigens from group 1 influenza. Using this technology, we obtained
91 several mAbs that bind to other group 1 HA antigens and even to group 2 HA antigens of
92 the influenza virus. Our technology can also be applied to human antibody screening and
93 represents a new line of mAb screening that accelerates the isolation of therapeutic and
94 diagnostic mAbs.

95 # Results:

96 ## Rapid membrane-bound dual Ig expression screening system

97 Cloning-based Ig screening involves several steps: cloning two Ig gene fragments
98 encoding heavy and light chains independently from a single cell, co-expressing these Ig
99 chains, and purifying individual recombinant antibodies. These steps are labor intensive.
100 We established a new antibody screening system for the isolation of valuable mAbs
101 (details in the method). Eventually, the antigen-binding Ig transformants were collected
102 by sorting in a bulk fashion, and the unique CDR3 region and clones of interest were
103 identified using the Ig-seq database (Fig. 1A).

104 We generated a dual-expression vector to express both Ig heavy and kappa/lambda
105 in a single-expression vector (Fig. 1B). These plasmids were individually transfected
106 transiently to the floating human FreeStyle 293 cell line. Igs were expressed on the cell
107 surface in 2 days; their expression could be confirmed and normalized by *Venus*
108 expression, since the Ig heavy chain is fused with *Venus* at the cytoplasmic domain tail
109 (Fig. 1C). The advantage of this system is the rapid enrichment of clones of interest
110 through bulk screening. In the current single-cell-based cloning/screening, plasmid DNA
111 extracted from the collected antigen-binding bulk transformants was sequenced for the
112 heavy chain CDR3 region. The CDR3 region is unique and can be used for the identification

113 of a clone (20,21). This membrane-bound Ig expression system links antigen-binding

114 features and genetic information of the Ig repertoire.

115 Building the Ig-seq database and analysis

116 As a model experiment, we obtained broadly reactive antibodies against influenza viruses

117 using multiple HA probes. We prepared two HA proteins as probes: A/Puerto Rico/8/1934

118 (H1N1), designated as PR8, and A/Okuda/1957 (H2N2), designated as H2. A total of 380

119 IgG1^{+} germinal center B cells of either PR8^{+} (204 cells), H2^{+} (99 cells), or PR8^{+}H2^{+} (77

120 cells) were collected in a single-cell fashion. We obtained sequences of 284 independent

121 clones. The success rate of cloning the paired Ig fragments was 75.9%. An overview of

122 heavy chain V-D-J and light chain V-J usage and repertoire clonality is shown in

123 Supplementary Fig. 1A and B. Mutation rates and CDR3 lengths of the heavy chains of the

124 three cell populations (PR8^{+}, H2^{+}, and PR8^{+}H2^{+}) were comparable (Supplementary Fig. 1C

125 and D). These results indicate that broadly reactive antibodies do not require unique

126 genetic traces to obtain breadth in our experimental setup.

127 Isolation of H2- and H1-reactive B cells

128 On day 2 after transfection, 287 transformants (Ig-expressing transformant libraries) were

129 stained with HA probes (Fig. 2A). As shown in Fig. 2B, we selected three prominent

130 populations, H1^{+}H2^{+} (cross), H2^{+} (H2), and H1^{+} (H1), and collected them in bulk. In

131 contrast to bacterial transfection, mammalian cell lines, such as FreeStyle 293 cells, can

132 contain multiple plasmids in a cell and ectopically express multiple proteins. Therefore, we
133 decided to mix the transformants instead of plasmids. We sorted 2,981 cells from these
134 three populations and performed sequencing. Bulk Ig-seq data are referred to as Ig-seq
135 data, as described above. All the transformants were examined individually. A total of 90
136 clones from the three populations were verified for their sequences and HA binding by flow
137 cytometry analysis, as shown in Fig. 2. Among these, 110 “H1^{+},” 67 “H2^{+},” and 13 “cross”
138 clones were successfully isolated. Then, 138 transformants were verified for their binding
139 to HAs by flow cytometry. Of these, 81 "H1^{+}," 48 "H2^{+}," and nine "cross" clones were
140 successfully isolated. Finally, 8 of the 13 "cross" clones obtained in bulk were included in
141 this group; the binding of one clone (D11p4) was so weak that it was not included in the
142 gates sorted in bulk. Thus, we established a high-throughput method for sorting "cross"
143 clones.

144 Characterization of “cross” clones for broad reactivity against the influenza virus

145 By generating a secretory form of mAbs for these nine cross clones, including C10p2, we
146 characterized their affinity and broadness using six different HA antigens from strains
147 A/Okuda/1957 (H2N2) named for H2, A/Puerto Rico/8/1934 (H1N1) for PR8,
148 A/California/2009 (X-179A) [H1N1] Pdm09 for Cal, A/Texas/50/2012 (X-223) [H3N2] for
149 H3, A/Egypt/N03072/2010(H5N1) for H5, and A/Brisbane/59/2007 [H1N1] (19) for Stem.
150 We determined the affinities (K_d) of the antibodies for the six HA antigens using surface

151 plasmon resonance analysis (Table 1 and Supplementary Fig. 2), which ranged from 500

152 to 100 nM. With the highest affinity among them, the C10p2 antibody bound to Cal at K_d

153 $\simeq 5.66 \times 10^{-10}$ (M). Surprisingly, the antibodies showed a broader spectrum of HA binding

154 among the influenza strains. Seven of the nine mAbs bound to HA from the highly

155 pathogenic avian influenza strain H5N1. Furthermore, the six antibodies that bound to H3

156 were categorized into group 2 (22). In addition, we observed that two of the broadly

157 reactive antibodies recognized the stem region of the HA of A/Brisbane/59/2007. A

158 possible scenario for other stem cell-negative antibodies is that they may either bind to

159 the stem region of other strains or to the head region of HA. We tested whether some of

160 these antibodies shared features with classic broadly neutralizing antibodies. C179, the

161 first broadly neutralizing mouse antibody isolated in 1993 (17), reacts with the stem

162 region of HAs from group 1 influenza virus strains (22). C179 binds to HAs from group 1

163 influenza viruses, including H1N1, H2N2, and H5N1. A competition assay for the binding of

164 H2 and PR8 revealed that A6p4 competes with C179 (Fig. 3). In addition, our two-

165 dimensional phylogeny map indicated that A6p4 was unique to all clones (Supplementary

166 Fig. 3). Note that we used NSP2 as a negative control antibody, which binds specifically to

167 the HA from A/California/2009 (X-179A) [H1N1] Pdm09 (23). These results demonstrate

168 that we were able to isolate a broadly reactive mAb with an affinity comparable to that of

169 a classic broadly reactive mAb (C179) using our technology.

170 # Discussion:

171 The discovery of therapeutic antibodies is accumulating, and their demand is increasing
172 (24). Efficiently isolating one biologically significant antibody from the 10^{13} antibody
173 repertoire is challenging. The hybridoma method has long been used for this purpose and
174 remains reliable. However, the fusion rate of B and myeloma cells is < 0.001% in this
175 method; therefore, the number of hybridomas obtained from one individual B cell is
176 limited to several hundred clones, limiting its use in the production of mAbs. As an
177 alternative choice, cloning-based antibody screening has gained popularity, especially for
178 human antibody discovery, because of the lack of a human myeloma cell line similar to
179 that used for murine cell fusion partners. The SPYMEG cell line can generate antigen-
180 specific antibody-producing cells by fusing the murine SP2/0 myeloma cell line and human
181 megakaryoblastic leukemia cell line MEG-01 with human PBMCs (14, 25). In addition to
182 gene expression profiling, NGS facilitates antibody repertoire analysis. However, despite
183 breakthroughs in sequencing technology, the process of linking antibody function and its
184 genes is still long and labor intensive. To overcome this issue, we developed a new
185 antibody screening system to directly link antigen–antibody binding (as a function) with a
186 gene encoding the antibody by expressing its membrane-bound form. Among conventional
187 cloning-based mAb isolation methods, this system demonstrates several advantages. 1)

188 Membrane Ig expression can link the antigen-binding feature of membrane-expressed Ig,
189 which can be linked to Ig DNA sequence information using our plasmid construct. Similar
190 to the panning procedure used in phage display (26), antigen-binding cells carry the
191 plasmid encoding the Ig genes through surface Ig so that the selection process in bulk
192 format enriches the relevant plasmids for use in further experiments. 2) The dual Ig
193 expression vector links heavy- and light-chain genes, which reduces the plasmid
194 preparation time and stock by half. 3) Highly reliable Golden Gate Cloning technology
195 using type IIs restriction enzymes can readily generate plasmid clones. This reduced the
196 time required to generate an Ig plasmid library. 4) The population profile, defined by the
197 fluorescence intensity during flow cytometry, directly reflected the affinity of a clone
198 (Supplementary Fig. 4). Collectively, our technology has streamlined the isolation of mAbs
199 for therapy and diagnosis.

200 Conclusion:

201 Our findings indicate that the developed antibody presentation system facilitates antibody
202 functional analysis and is well suited for the discovery of antibodies important for
203 infectious diseases when combined with conventional NGS-based antibody repertoire
204 analysis. Compared with droplet-based experimental systems, well-based systems are
205 limited in the number of cells they can process. Furthermore, experiments involving

206 infectious bacteria and viruses have imposed limitations on human experimentation. To
207 solve these problems, the automation of experiments will become important in the future.
208 By combining our screening system with robotic automation of experiments, it will be
209 possible to obtain useful mAbs for various diseases quickly and in large quantities, which
210 has broad implications for the development of vaccines against various diseases.

211 Materials and Methods:

212 Protein purification of influenza HAs (H1 and H2) as antigens

213 A mixture of 25.7 μg of HA, comprising H1 strain A/Puerto Rico/8/1934 (PR8), H1 strain
214 A/California/7/2009 (X-179A), H2 strain A/Okuda/1957 (H2), H3 strain A/Texas/50/2012
215 (X-223), or H5 strain A/Egypt/N03072/2010], 1.3 μg of NA (Strain-matched), and 3 μg of
216 BirA-expressing plasmids were transfected into Expi293 cells using the Expifectamine 293
217 transfection kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the
218 manufacturer's instructions. The cells were then cultured in Expi293 Expression Medium
219 (Thermo Fisher Scientific) supplemented with D-biotin at a final concentration of 100 μM
220 for *in vivo* BirA biotinylation in a humidified incubator containing 8% CO_2 at 37°C and 125
221 rpm. If biotinylation was not necessary, transfection was performed without BirA, and cell
222 culture was carried out without D-biotin. Culture supernatants were harvested at 3 days

223 post-transfection and filtered through a 0.45 μm filter (Merck Millipore, Burlington, MA,
224 USA). All proteins were purified with a Talon metal affinity resin (Takara Bio USA,
225 Mountain View, CA, USA) and dialyzed against pyrogen-free PBS. The oligomeric state and
226 purity were determined at 25°C by size-exclusion chromatography on a Superdex 200
227 10/300GL column (GE Healthcare Technologies, Chicago, IL).

228 HA (H1 and H2) protein immunization

229 Two individual BALB/c mice were intraperitoneally immunized sequentially, 2 weeks apart,
230 with 15 μg of H1 (PR8) HA followed by 15 μg of H2 HA protein as an antigen and
231 supplemented with AddaVax adjuvant (InvivoGen, San Diego, CA, USA). BALB/c mice
232 were purchased from CLEA Japan (CLEA Japan, Inc., Tokyo, Japan) All animal experiments
233 were performed using protocols approved by the Institutional Animal Care and Use
234 Committee (IACUC) of the RIKEN Yokohama Branch.

235 Fluorescence-activated cell sorting

236 CD43-negative B cells were collected using AutoMACS (Miltenyi Biotec, Inc., Bergisch
237 Gladbach, North Rhine-Westphalia, Germany) and stained with IgG1 (Bv510), non-
238 biotinylated His-tagged purified recombinant H1 (PR8) protein, and biotinylated purified
239 recombinant H2 protein on ice for 30 min. After washing three times, the cell suspension
240 was incubated with Alexa Fluor 488-labeled anti-His antibodies on ice for 30 min. After

241 washing three times, the cell suspension was incubated with Brilliant Violet 421-
242 conjugated streptavidin on ice for 3 min. Next, the B cells were subjected to single-cell
243 sorting using a BD FACSAria III (BD Biosciences, Franklin Lakes, NJ, USA) and collected into
244 96-well plates pre-loaded with lysis buffer. The single cells in the lysis buffer were
245 immediately snap-frozen on powdered dry ice and stored at −80°C.

246 Amplification of a paired B cell repertoire amplicon from a single cell

247 Single cells were dropped into pre-loaded plates containing 4 μL/well of lysis buffer (2
248 U/μL RNase inhibitor, 0.5× PBS, 10 mM DTT) and 1 μL of 10 μM Oligo(dT)18 Primer. Cell
249 lysates were incubated at 70°C for 90 s, followed by 35°C for 15 s, and then placed on ice
250 for 2 min. For first-strand cDNA synthesis, 5 μL of RT mix (2× PCR buffer, 5 mM DTT, 2
251 mM dNTP Mix, 10 U RNase inhibitor, 100 U SuperScript III Reverse Transcriptase) was
252 added to the cell lysate and incubated consecutively at 35°C for 5 min, 45°C for 20 min,
253 70°C for 10 min, and 4°C for chilling. For primer digestion, 5 μL of the primer digestion
254 mix (1× ExoI Buffer, 7.5 U Exonuclease I) was prepared and incubated at 37°C for 30
255 min, 80°C for 20 min, and 4°C for chilling. For the poly-A tailing reaction, 5 μL of the poly-
256 A tailing reaction mix (4× PCR buffer, 5 mM dATP, 0.1 U RNase H, 60 U Terminal
257 transferase) was prepared and incubated at 37°C for 50 s, 65°C for 10 min, and 4°C for
258 chilling. For the 2nd strand cDNA synthesis, 25 μL of the amplification mix (1× KAPA HiFi

259 HotStart ReadyMix, 400 nM SMART_dT primer, 10 μL of poly-A tailing product, 1.5 μL of
260 nuclease-free H_2O) was prepared and incubated at 95°C for 3 min, 40°C for 1 min, 65°C
261 for 10 min, and 72°C for 5 min and chilled. For the cDNA amplification using an Ig-specific
262 outer primer set, 20 μL of the outer amplification mix (1× KOD FX Neo buffer, 400 μM
263 dNTPs, 1× UPM, 300 nM outer primer, 3.5 μL of the SMART product, 0.5 U KOD FX Neo
264 polymerase) was prepared and incubated at 94°C for 2 min, followed by 25 cycles of 98°C
265 for 10 s, 60°C for 30 s, and 68°C for 45 s and then chilled. For cDNA amplification with an
266 Ig-specific inner primer set, 20 μL of the inner amplification mix (1× KOD FX Neo buffer,
267 400 μM dNTPs, 500 nM 5′ matrix primer mix, 500 nM 3′ matrix primer mix, 3.5 μL of the
268 outer cDNA amplified product, 0.5 U KOD FX Neo polymerase) was prepared and
269 incubated at 94°C for 2 min, followed by 25 cycles of 98°C for 10 s, 60°C for 30 s, and
270 68°C for 45 s and then chilled. Finally, 3 μL of the amplified inner cDNA product was used
271 for further plate indexing by amplification. Then, 20 μL of the plate-indexing amplification
272 mix (1× KOD FX Neo buffer, 400 μM dNTPs; 500 nM i5-Fwd or i7-Fwd, 500 nM i7-Rev or
273 i5-Rev; 2 μL of the inner cDNA amplified product; 0.5 U KOD FX Neo polymerase) was
274 prepared and incubated at 94°C for 2 min, followed by 25 cycles at 98°C for 10 s, 60°C
275 for 30 s, and 68°C for 45 s and then chilled. All primers used are listed in Supplementary
276 Table 1 and Supplementary Table 2.

277 ## Paired-end Illumina sequencing strategy and analysis

278 The libraries were sequenced on a MiSeq instrument (Illumina, San Diego, CA, USA)
279 following the manufacturer's protocol. As the sequencing length was limited to 2 × 300
280 bp, we used the following strategy. Amplicons in both orientations were generated using
281 an Illumina adapter sequence. Then, asymmetric 400+100 nt paired-end sequencing was
282 performed to obtain high-quality 400 + 400 nt paired-end sequences of the original cDNA
283 molecule. This approach provided a sufficient sequencing length to capture extra-long Ig
284 variants. We followed Turchaninova's method (27). Only reads that met the following four
285 conditions were used as the resulting Ig sequences: 1) reads with a length of 260 nt or
286 more; 2) cumulative clustered reads representing the top 5% of the entire reads; 3) 5'-
287 and 3'-reads overlapping a length of 20 bases or more; and 4) a total length of 460 ± 50
288 bp for heavy chain or 390 ± 40 bp for light chain. All resulting sequence data were
289 analyzed using IMGT/High V-QUEST (http://www.imgt.org/IMGT_vquest/vquest) for
290 annotation (28). Using the sequences and annotations, Ig database construction and
291 visualization of the Ig repertoire were performed using the in-house BONSCI software.

292 ## Construction of a mouse IgG/IgK expression plasmid

293 The BsaI restriction sites were inserted into the paired B cell repertoire amplicon from a
294 single cell using PCR. The destination vector contained the IL6 signal peptide, EF1a

295 promoter, ccdB gene, and mouse IgK Fc region. The donor vector contained the mouse
296 IgG1 Fc region, the *Venus* gene, and the EF1a promoter. The paired B cell repertoire
297 amplicons, destination vector, and donor vector containing BsaI restriction sites were
298 assembled using our assembly method. For this step, 10 μL of the assembly mix (1× T4
299 DNA ligase buffer, 1× BSA, 1 U BsaI restriction enzyme, 40 U T4 DNA ligase, 100 ng
300 heavy chain amplicon, 100 ng light chain amplicon, 100 ng destination vector, and 100 ng
301 donor vector) was prepared and incubated for 25 cycles at 37°C for 3 min, 16°C for 4 min,
302 50°C for 5 min, and 80°C for 5 min, and then chilled. The antibody sequence that entered
303 the construct was fused to the *Venus* sequence and expressed in membrane form.

304 Antibody display on FreeStyle 293 cultured cells

305 A mixture of 1 μg of antibody-expressing plasmid was transfected into FreeStyle 293 cells
306 using the 293fectin Transfection Reagent (Thermo Fisher Scientific) according to the
307 manufacturer's instructions and cultured in FreeStyle 293 Expression Medium (Thermo
308 Fisher Scientific) in a humidified incubator with 8% CO_2 at 37°C and 125 rpm. Antibodies
309 were displayed on the surface of cultured cells, which enabled the determination of
310 antigen specificity. Furthermore, the antibody-display cells were tested for binding activity
311 with Alexa647-labeled H1 and Alexa568-labeled H2 using a BD FACSAria III (BD
312 Biosciences).

313 ## Antibody production

314 To obtain a large amount of secretory antibodies, PCR fragments from the antibody-
315 expressing plasmid were cloned into the pcDNA3.4-mIgG1 or pcDNA3.4-kappa vectors. A
316 mixture of 15 μg of pcDNA3.4-V-gene-mIgG1 vector and 15 μg of pcDNA3.4-V-gene-
317 kappa vector were transfected into Expi293 cells using the ExpiFectamine 293 Transfection
318 Kit (Thermo Fisher Scientific), according to the manufacturer's instructions, and cultured in
319 Expi293 Expression Medium (Thermo Fisher Scientific) in a humidified 8% CO_2 incubator at
320 37°C and 125 rpm. Then, 5 days post-transfection, the culture supernatants were
321 harvested, and all proteins were purified with PureSpeed IMAC resin (Mettler Toledo,
322 Columbus, OH, USA), according to the manufacturer's instructions.

323 ## Surface plasmon resonance

324 Kinetic analyses were performed at 25°C using a BIAcore 3000 machine (GE Healthcare
325 Technologies). A2p1, B10p2, C10p2, E11p2, G6p2, A6p4, D4p4, D11p4, or G12p4
326 antibodies were immobilized on a CM5 sensor chip (GE Healthcare Technologies) with an
327 amine-coupling kit according to the manufacturer's instructions. HA probes were serially
328 diluted at five different concentrations and injected at a flow rate of 30 μL/min for 3 min
329 with a dissociation time of 7 min in HBS–EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl,

330 3.4 mM EDTA, and 0.005% Surfactant P20). The chip was regenerated using 10 mM

331 glycine (pH 2.5) as an HA probe.

332

333 Data access:

334 All raw and processed sequencing data generated in this study have been submitted to the

335 NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) under

336 accession number GSE140720.

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Conflict of interest:

None declared.

Author contributions:

T.W. and H.F. designed the study, the main conceptual ideas, and the proof outline. H.H., Y.M., F.Y., T.H., and N.K. collected the data. O.O. and T.K. supervised the project. T.W. wrote the manuscript with support from H.F. All authors discussed the results and commented on the manuscript.

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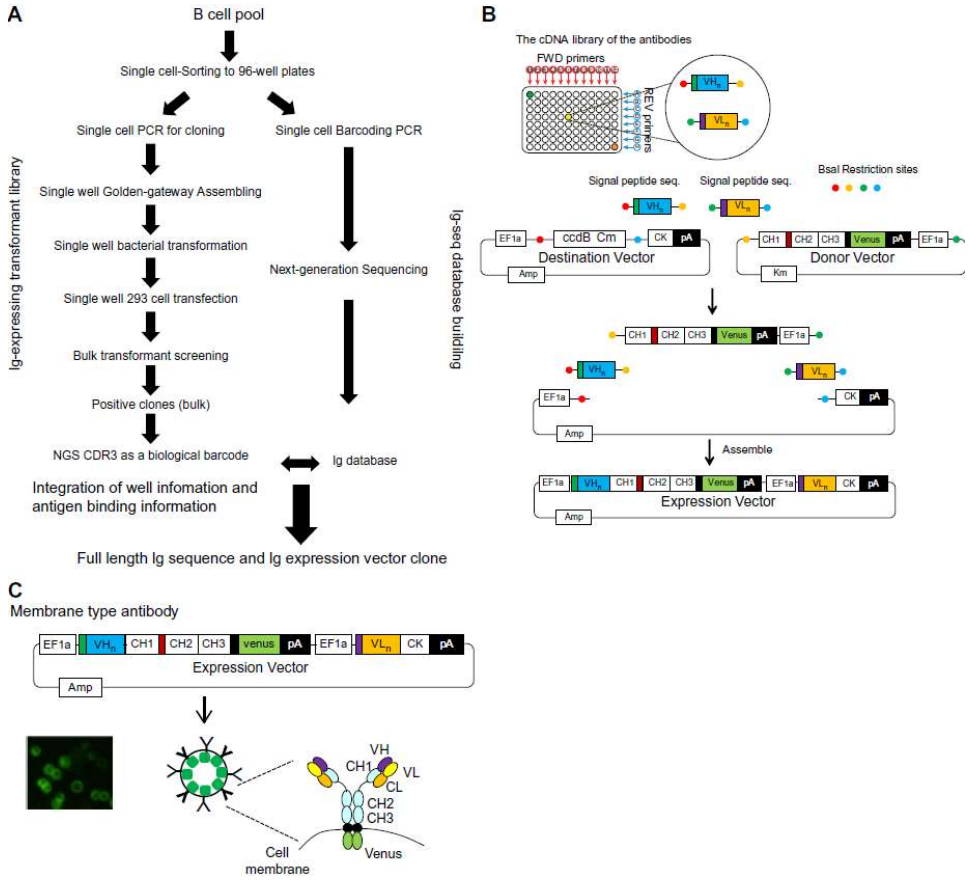
422

423 # Figures:



424

425

426 **Figure 1.** System to link the antigen-binding feature with the Ig repertoire genetic

427 information. **A.** Single cell-sorted B cells were subjected to two processes: Ig-seq database

428 building and Ig-expressing transformant library preparation. Antigen-binding Ig

429 transformants were collected by sorting in bulk and the sequences of the unique CDR3

430 regions and clones of interest were identified by referring to the Ig-seq database. **B.** To

431 express both Ig heavy and kappa/lambda chains in a single-expression vector, we

432 generated a dual-expression vector. Four gene fragments were assembled by the Golden
433 Gate method using the BsaI restriction enzyme. They included: 1) the destination vector
434 containing the Ig kappa constant gene and the ccdB Chloramphenicol cassette for negative
435 selection, 2) fragments containing the Ig heavy constant gene fused to *Venus* derived
436 from the donor vector, 3) the Ig heavy variable gene fragment, and 4) the Ig light variable
437 gene fragment. **C.** Individually purified plasmids were transiently transfected to the
438 floating human FreeStyle 293 cell line. Igs were expressed on the cell surface in 2 days,
439 and expression levels were confirmed and normalized by *Venus* expression, since the Ig
440 heavy chain was fused to *Venus* at the cytoplasmic domain tail.
441

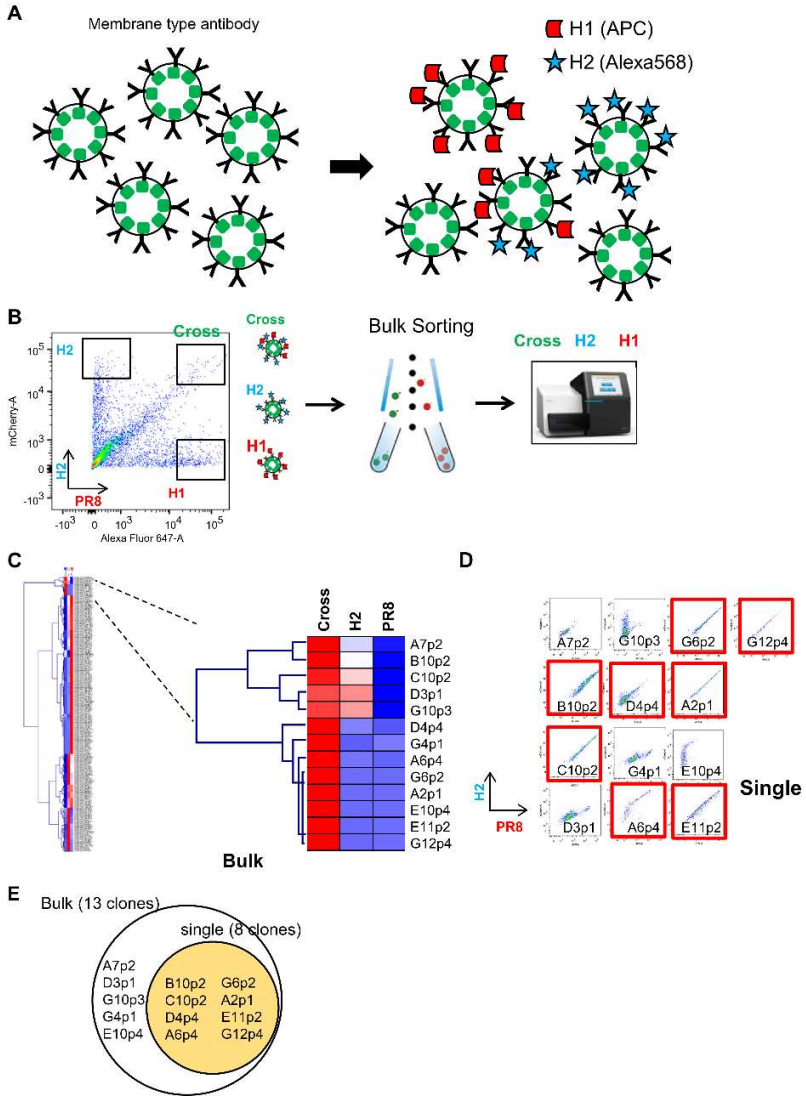


Figure 2. Isolation of H2 and H1-reactive B cells. **A.** Mixture of Ig-expressing transformant libraries stained with HA probes. **B.** Three prominent populations, $H1^{+}H2^{+}$ (cross), $H2^{+}$ (H2), and $H1^{+}$ (H1), were selected and collected in a bulk fashion. Then, the three prominent populations were sorted and sequenced. **C.** The bulk Ig-seq data were referred to as the single-cell Ig-seq data. Wells containing crossed antibodies were determined by comparing the sequence of the heavy chains of the populations included in $H1^{+}H2^{+}$ (cross)

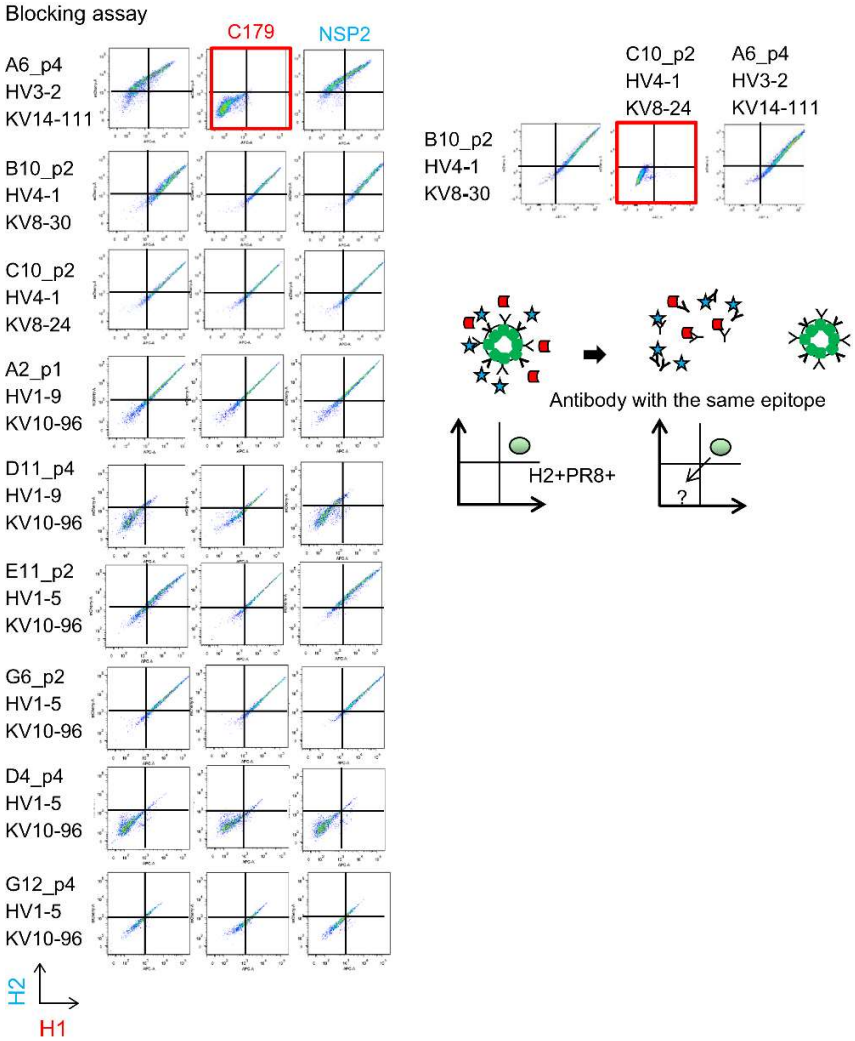
449 with the Ig-seq data of single cells. **D.** Cross antibodies obtained by bulk sorting were

450 examined by single-cell presentation. **E.** A summary of the results to check whether a

451 candidate mAb was cross-reactive.

452

453



454

455 **Figure 3.** Competition assay using various mAbs for binding to H2 and PR8 was

456 performed, including two previously reported antibodies, C179, a broadly-reactive mAb

457 binding to the Stem region of HA, and NSP2 (Narita strain-specific mAb), a strain-specific

458 mAb that binds to the head region of HA.

459

460 # Table 1

461 Surface plasmon resonance (Biacore) KD (M)

Group	1	1	1	2	1	1
	H2	**PR8**	**Cal**	**H3**	**H5**	**Stem**
A6p4	4.51E-08	1.41E-06	1.14E-05		2.91E-05	1.59E-08
B10p2	6.75E-09	2.3E-09	3.29E-09			
C10p2	1.66E-09	1.29E-09	5.66E-10			4.26E-08
A2p1	2.49E-06	1.42E-07	1.14E-05	1.25E-04	1.91E-05	
D11p4	7.93E-07	1.72E-06	1.04E-06	1.36E-05	1.05E-11*	
E11p2	4.4E-08	1.86E-08	1.67E-06	2.92E-07	2.37E-07	
G6p2	3.51E-08	3.53E-08	1.44E-08	1.78E-05	3.05E-08	
D4p4	1.1E-05	1.78E-05	6.4E-05	4.38E-05	2.47E-05	
G12p4	1.45E-07	1.52E-07	5.7E-07	9.44E-07	1.58E-07	

462

High
Middle
Low

463
464
465
466 **Table 1.** Affinities (K_d) of the antibodies were determined to 6 HA antigens from strains

467 A/Okuda/1957(H2N2) named for H2, A/Puerto Rico/8/1934(H1N1) for PR8,

468 A/California/2009 (X-179A) [H1N1] Pdm09 for Cal, A/Texas/50/2012 (X-223) [H3N2] for

469 H3, A/Egypt/N03072/2010(H5N1) for H5, and A/Brisbane/59/2007 (H1N1) for Stem using

470 surface plasmon resonance analysis. H2, PR8, Cal, and H5 belong to group 1, and H3

471 belongs to group 2.

472 * The affinity of D11p4 for H5 was low and inaccurate (Supplementary Figure 2).
473

Supplementary Tables

Supplementary Table 1. Oligonucleotides

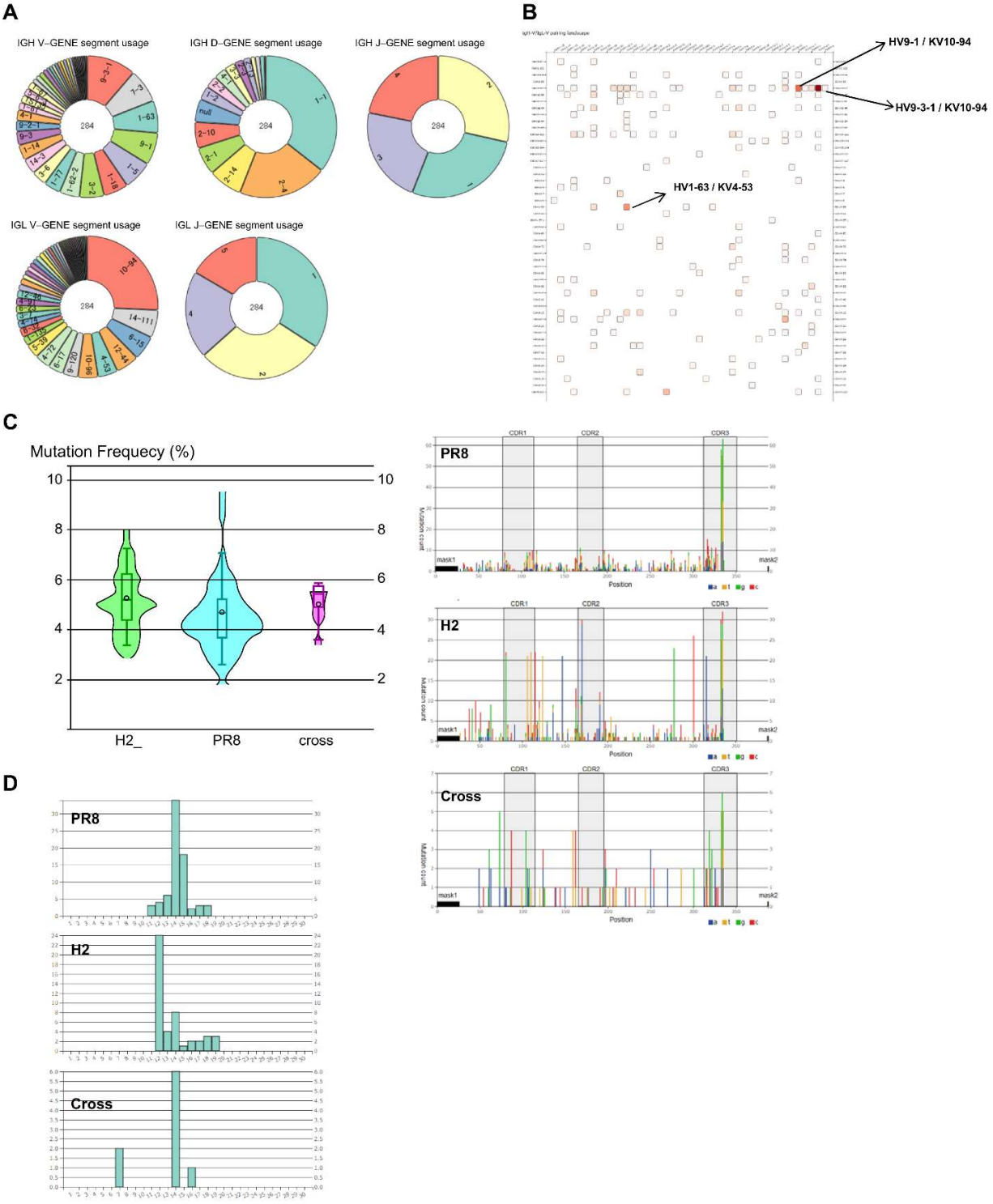
Primer	Application	Sequence
First-strand cDNA synthesis		
Oligo(dT)18	Primer for cDNA synthesis	TTTTTTTTTTTTTTTTTT
Second-strand cDNA synthesis		
SMART_dT	TdT oligo	AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN
Mouse IGH/IGK primer		
TdT_L_BsaI_heavy	mouse IgG heavy-chain cDNA	CTAGGGTCTCAAGCAGTGGTATCAACGCAGAGTAC
msVHE		GAGGTGCAGCTGCAGGAGTCTGG
W-gamma-outer		AGAAGGTGTGCACACCGCTGGAC
mC_G new2		ATCTCCACACACAGGGGCCAGTGGATAGAC
BsaI_IL6sp_L		CTAGGGTCTCAAGCAGATGAACTCCTTCTCCACAAGCG
mC_G_new2_BsaI		TCCTAGGTCTCCCACACACAGGGGCCAGTGGATAGAC
TdT_L_BsaI_light	mouse IgK light-chain cDNA	CTAGGGTCTCAGTGGTATCAACGCAGAGTAC
mvkappa		GAYATTGTGMTSACMCARWCTMCA
mCkappa-outer		ACTGAGGCACCTCCAGATGTT
mC_K_new		AGGCACCTCCAGATGTTAACTGCTCACTGG
BsaI_IL6sp_kappa_L		CTAGGGTCTCAGTGGTATGAACTCCTTCTCCACAAGCG
mC_K_new_BsaI		TCCTAGGTCTCCTCCAGATGTTAACTGCTCACTGG

478 **Supplementary Table 2. Oligonucleotides**

Primer	Application	Sequence
Next Generation sequencing		
M13fwd_i5_mC_G_new2[a]	Well index	TGTAAAACGACGGCCxxxxxxxxATCTCCACACACAGGGGCCAGTGGATAGAC
M13fwd_i5_mC_K[a]		TGTAAAACGACGGCCxxxxxxxxGCACCTCCAGATGTTAACTG
M13fwd_i5_mC_L[a]		TGTAAAACGACGGCCxxxxxxxxAGCTCCTCAGRGGAAGGTG
M13rev_i7_msVHE[b]		GGAAACAGCTATGACyyyyyyyyGAGGTGCAGCTGCAGGAGTCTGG
M13rev_i7_mvkappa[b]		GGAAACAGCTATGACyyyyyyyyGAYATTGTGMTSACMCARWCTMCA
i5-Fwd[c]	Plate index	AATGATACGGCGACCACCGAGATCTACACxxxxxxxxACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTAAAACGACGGCC
i7-Rev[d]		CAAGCAGAAGACGGCATACGAGATyyyyyyyyGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGAAACAGCTATGAC
i5-Rev[c]		AATGATACGGCGACCACCGAGATCTACACxxxxxxxxACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAAACAGCTATGAC
i7-Fwd[d]		CAAGCAGAAGACGGCATACGAGATyyyyyyyyGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGTAAAACGACGGCC

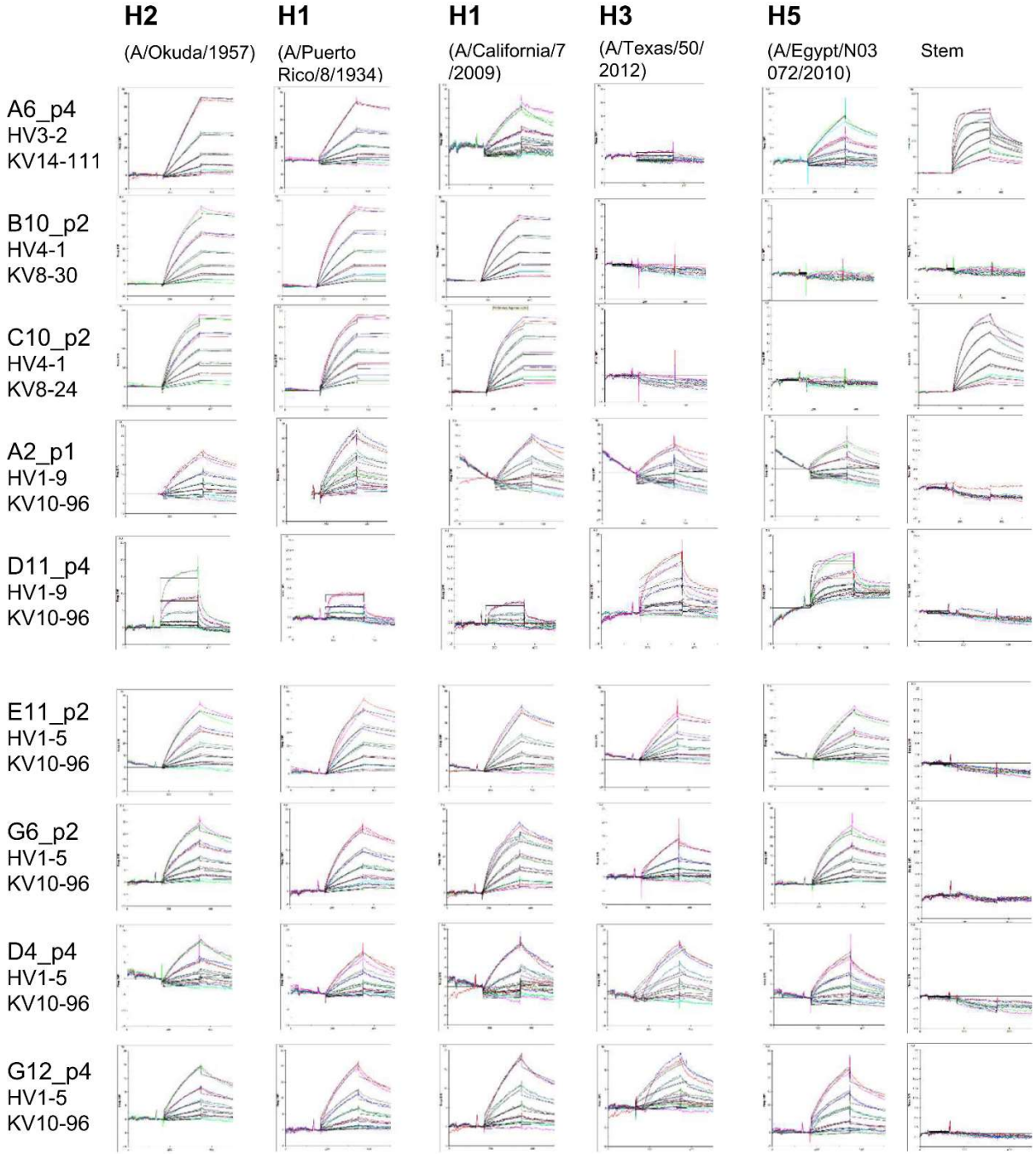
479 [a]xxxxxxxx: sequence showing the well index from D501 to D508. [b]yyyyyyyy: sequence
480 showing the well index from D701 to D712. [c]xxxxxxxx: sequence showing the plate index
481 from D501 to D508. [d]yyyyyyyy: sequence showing the plate index from D701 to D712.
482

Supplementary Figures



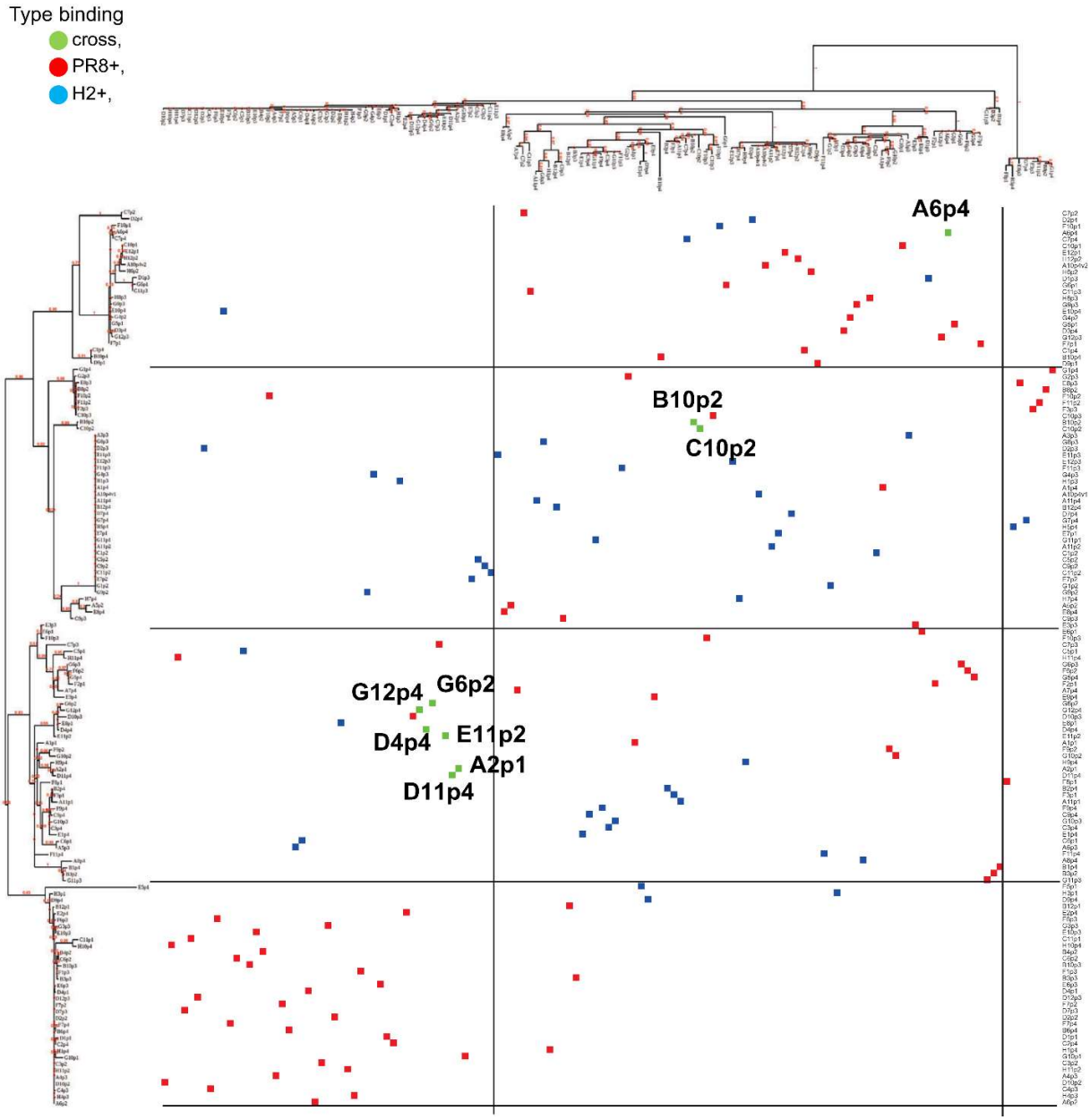
Supplementary Figure 1.

The Ig repertoires were visualized using the in-house software. **A,** Overview of heavy (V-D-J) and light (V-J) chain usages. **B,** the repertoire clonality from single cells. **C,** the mutation rates of heavy chains in three cell populations (PR8+, H2+, PR8+H2+) were compared. **D,** the lengths of CDR3 of heavy chains of three cell populations (PR8+, H2+, PR8+H2+) were compared.



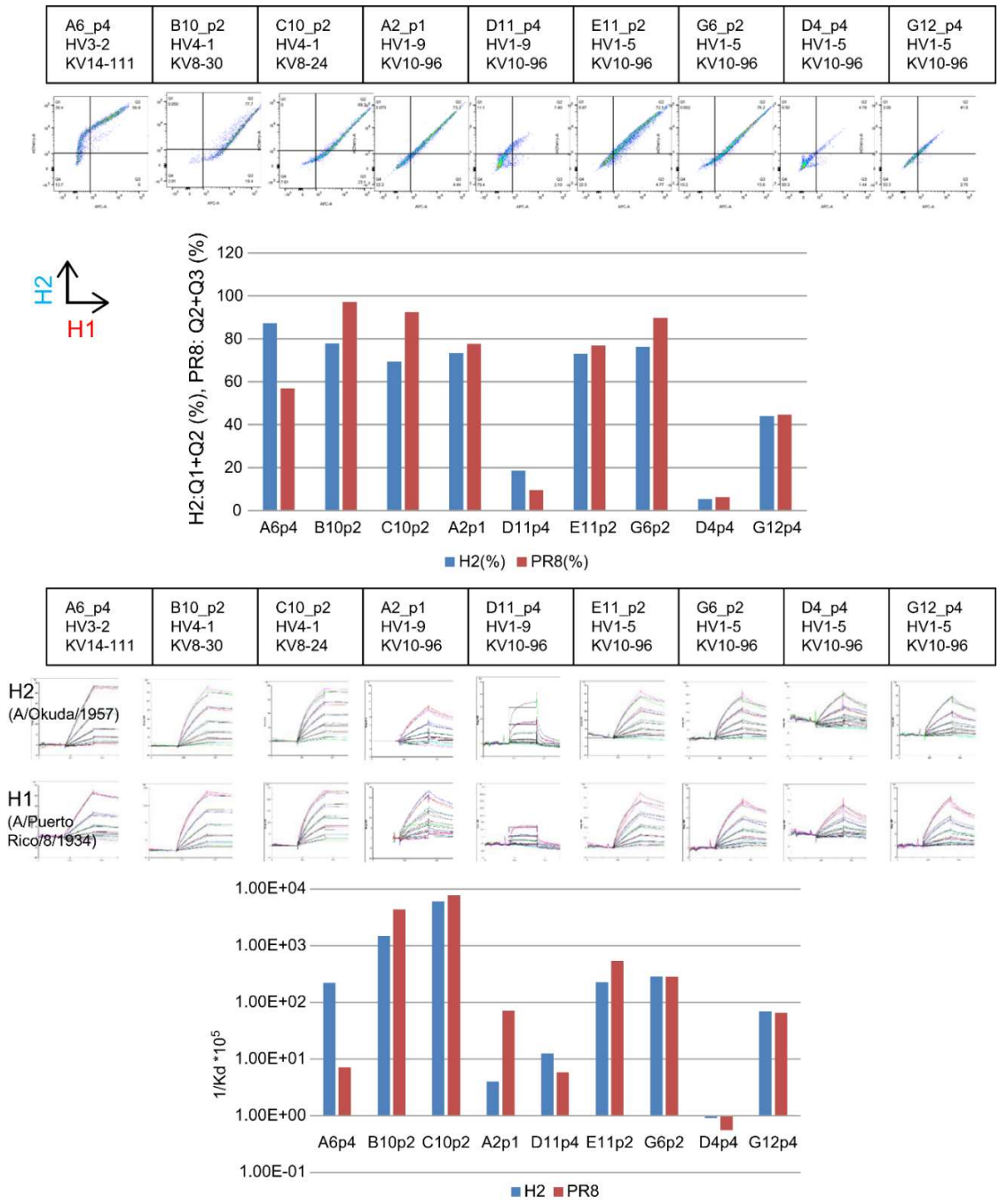
Supplementary Figure 2.

Kinetics analysis was performed using a BIAcore 3000 machine (GE Healthcare). A6p4, B10p2, C10p2, A2p1, D11p4, E11p2, G6p2, D4p4 or G12p4 antibodies were immobilized on a CM5 sensor chip (GE Healthcare) using an amine-coupling kit according to the supplier's instructions. HA probes were serially diluted at five different concentrations in HBS-EP buffer.



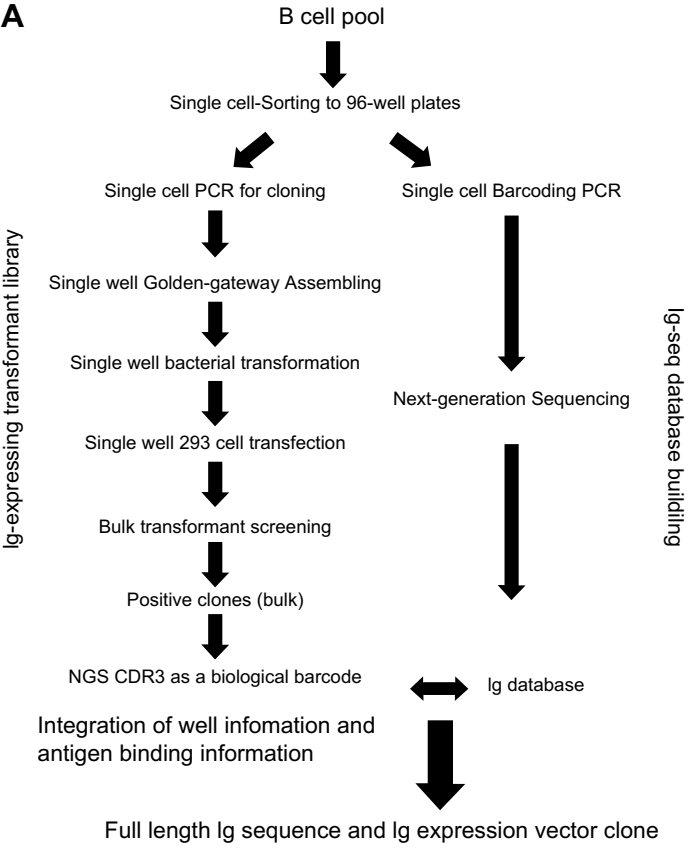
Supplementary Figure 3.

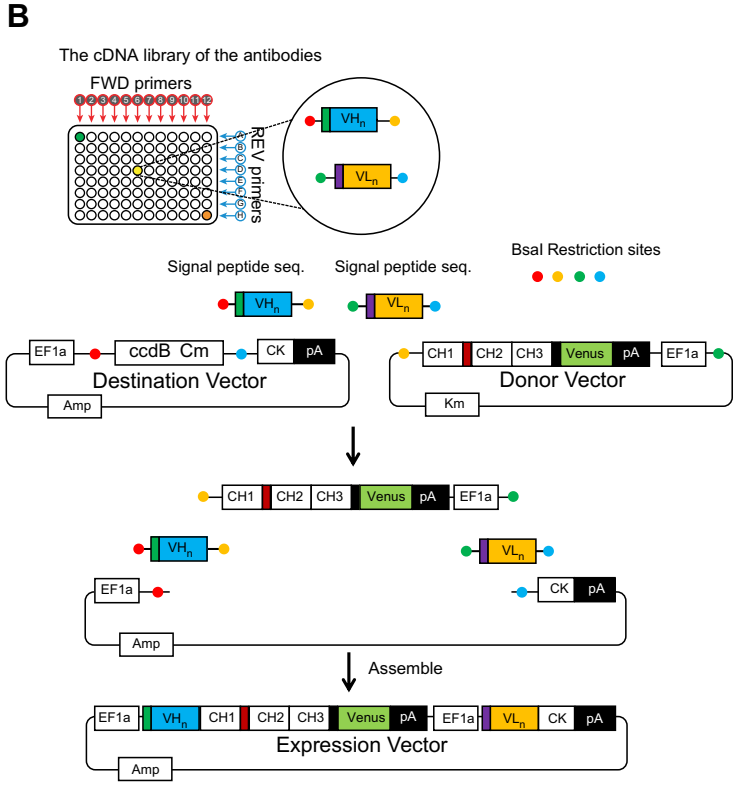
Two hundred and eighty-four clones are plotted in a two-dimensional map of phylogeny trees for heavy and light chain genes. Nine mAb clones are overlaid in green. Six “cross” clones are located in a major cluster. B10p2 and C10p2 are co-localized and distant from the cluster. A6p4 is uniquely located in the map.

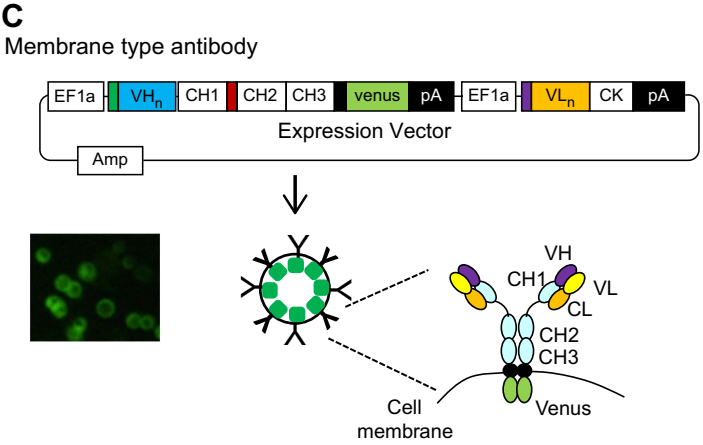


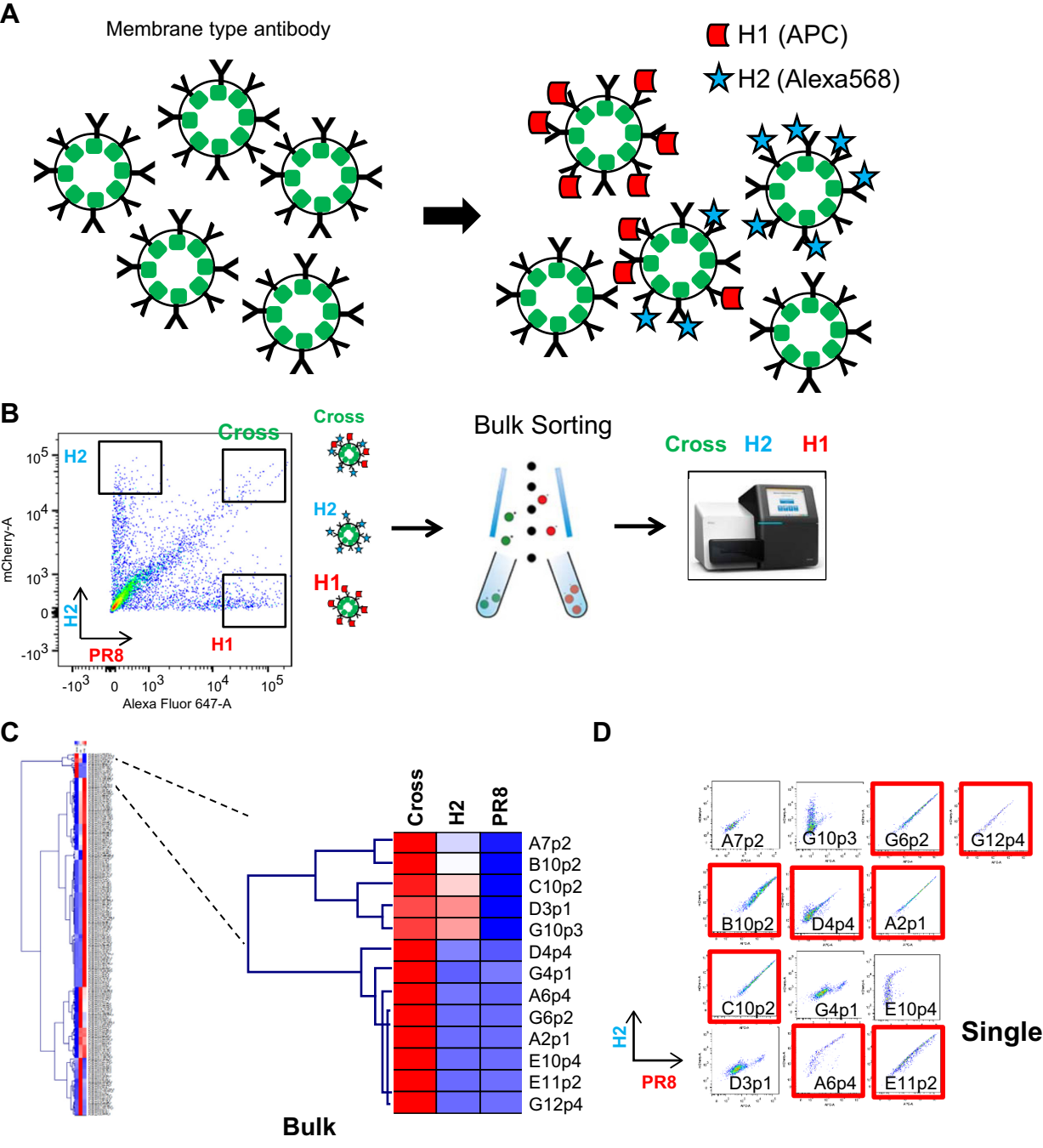
Supplementary Figure 4.

The affinity of antigen-antibody binding (in this case, probe and membrane-bound antibody expressing cell) was inferred from the fluorescence intensity (flow cytometry analysis).

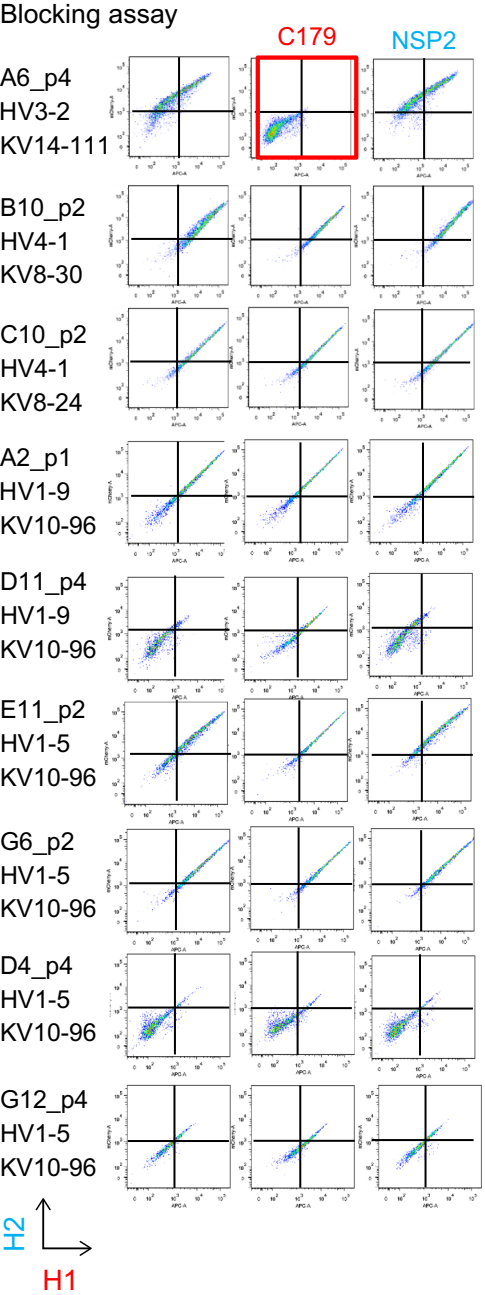

A
B cell pool
Single cell-Sorting to 96-well plates
Single cell PCR for cloning
Single cell Barcoding PCR
Single well Golden-gateway Assembling
Single well bacterial transformation
Single well 293 cell transfection
Bulk transformant screening
Positive clones (bulk)
NGS CDR3 as a biological barcode
Next-generation Sequencing
Ig database
Integration of well infomation and antigen binding information
Full length Ig sequence and Ig expression vector clone
Ig-expressing transformant library
Ig-seq database building

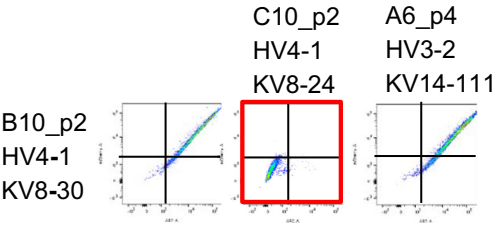

B
The cDNA library of the antibodies
FWD primers
REV primers
VHn
VLn
Signal peptide seq.
Signal peptide seq.
BsaI Restriction sites
EF1a
ccdB Cm
CK
pA
Destination Vector
Amp
CH1
CH2
CH3
Venus
pA
EF1a
Donor Vector
Km
Assemble
Expression Vector

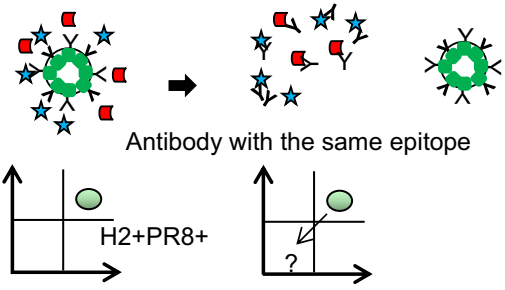

C
Membrane type antibody
EF1a
VHn
CH1
CH2
CH3
venus
pA
EF1a
VLn
CK
pA
Expression Vector
Amp
VH
CH1
VL
CL
CH2
CH3
Cell membrane
Venus

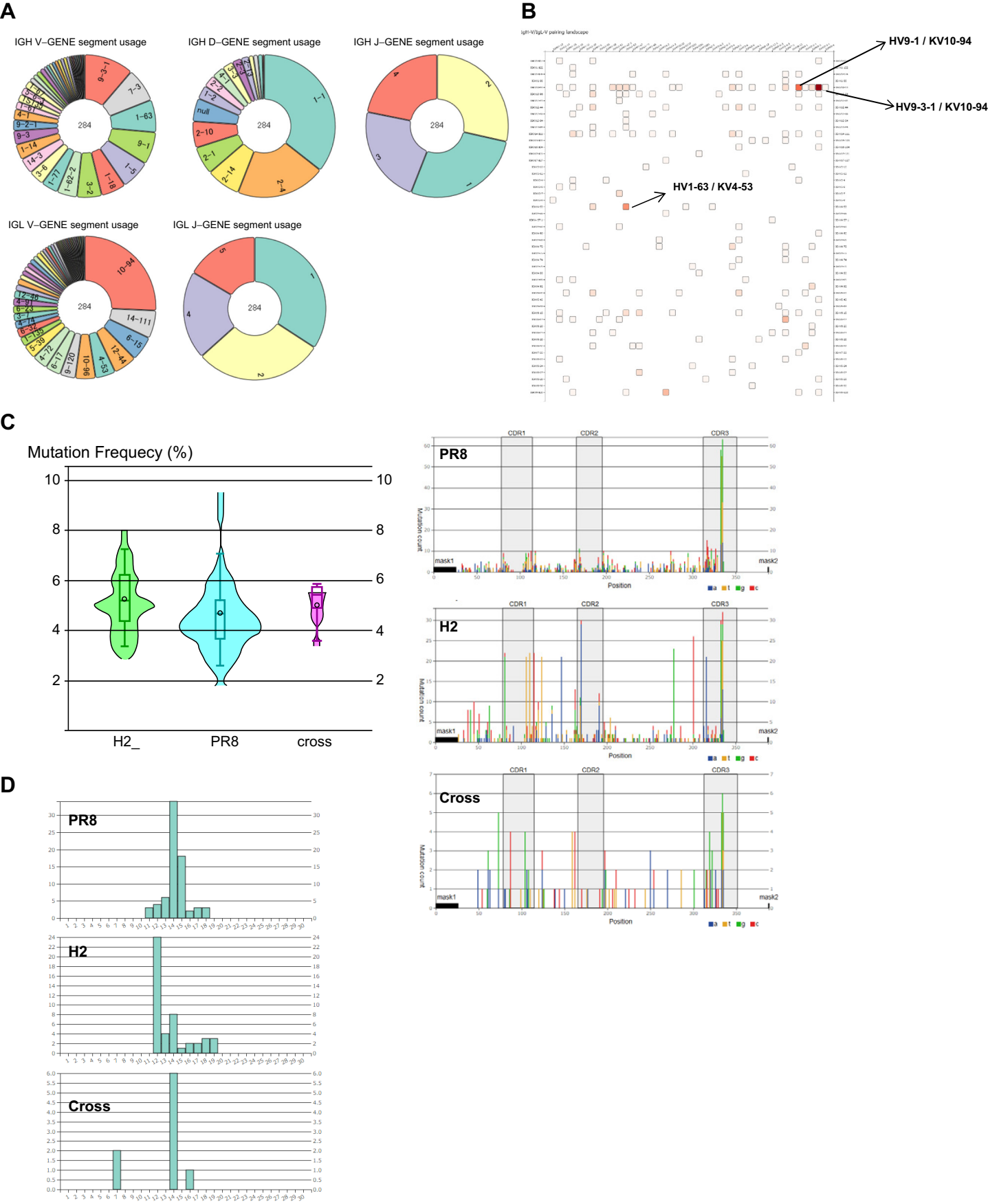

A
Membrane type antibody
H1 (APC)
H2 (Alexa568)
B
Cross
H2
H1
PR8
mCherry-A
Alexa Fluor 647-A
Bulk Sorting
C
Cross
H2
PR8
A7p2
B10p2
C10p2
D3p1
G10p3
D4p4
G4p1
A6p4
G6p2
A2p1
E10p4
E11p2
G12p4
Bulk
D
A7p2
G10p3
G6p2
G12p4
B10p2
D4p4
A2p1
C10p2
G4p1
E10p4
D3p1
A6p4
E11p2
Single
E
Bulk (13 clones)
single (8 clones)
A7p2
D3p1
G10p3
G4p1
E10p4
B10p2
C10p2
D4p4
A6p4
G6p2
A2p1
E11p2
G12p4

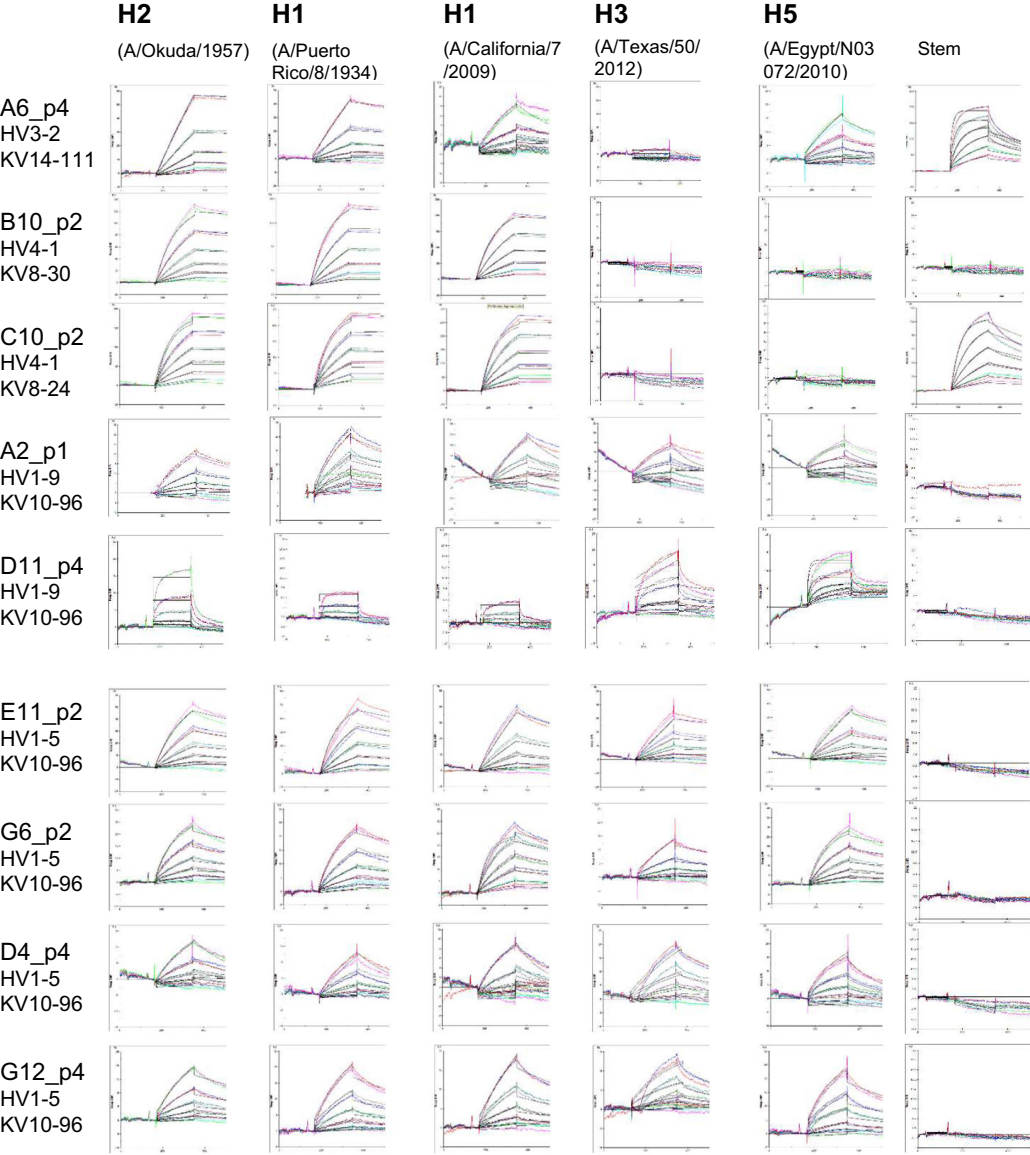
Blocking assay








A
IGH V–GENE segment usage
IGH D–GENE segment usage
IGH J–GENE segment usage
IGL V–GENE segment usage
IGL J–GENE segment usage
284
B
igh1-V/igL-V pairing landscape
HV9-1 / KV10-94
HV9-3-1 / KV10-94
HV1-63 / KV4-53
C
Mutation Frequecy (%)
H2_
PR8
cross
CDR1
CDR2
CDR3
mask1
mask2
Mutation count
Position
a
t
g
c
D
PR8
H2
Cross


H2
(A/Okuda/1957)
H1
(A/Puerto Rico/8/1934)
H1
(A/California/7/2009)
H3
(A/Texas/50/2012)
H5
(A/Egypt/N03072/2010)
Stem
A6_p4
HV3-2
KV14-111
B10_p2
HV4-1
KV8-30
C10_p2
HV4-1
KV8-24
A2_p1
HV1-9
KV10-96
D11_p4
HV1-9
KV10-96
E11_p2
HV1-5
KV10-96
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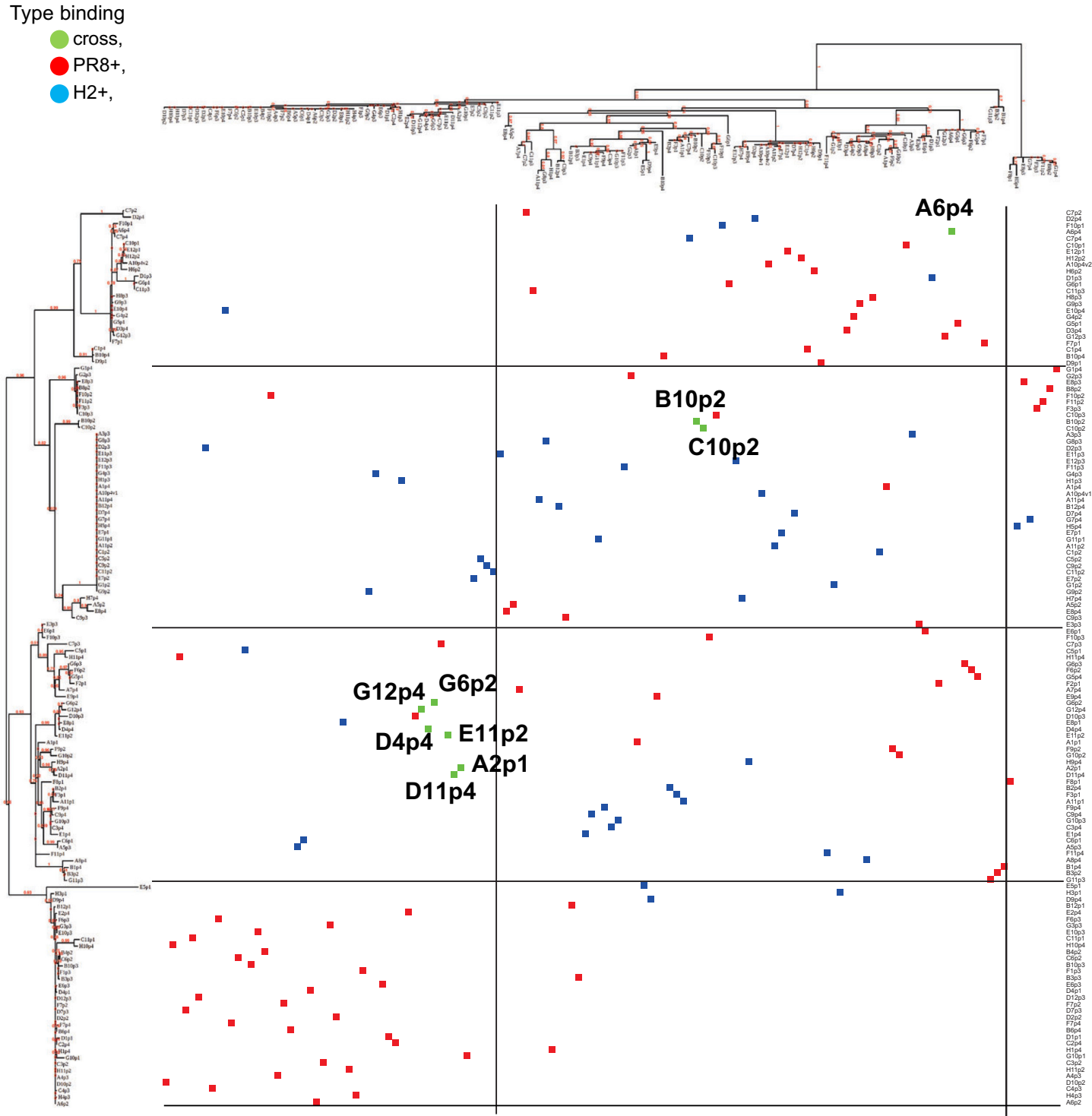
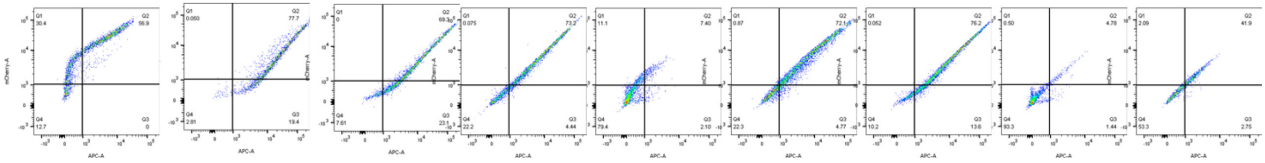
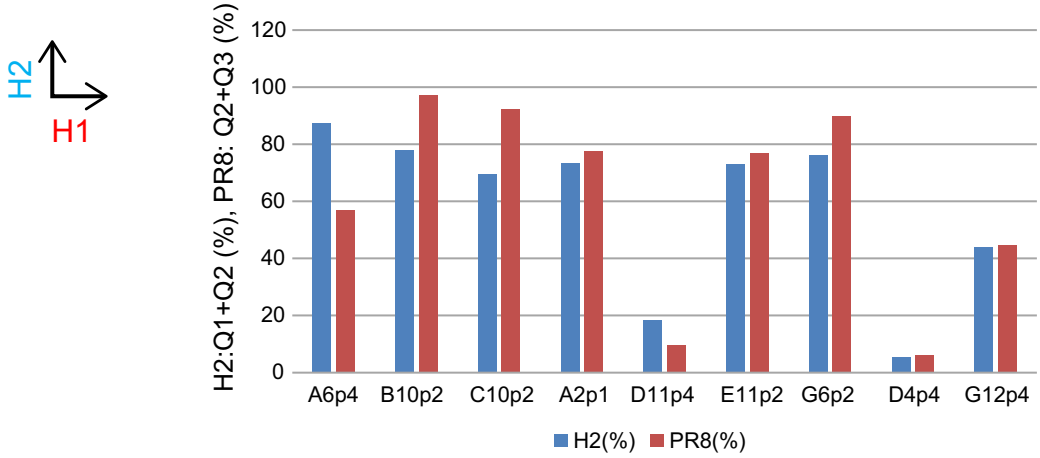
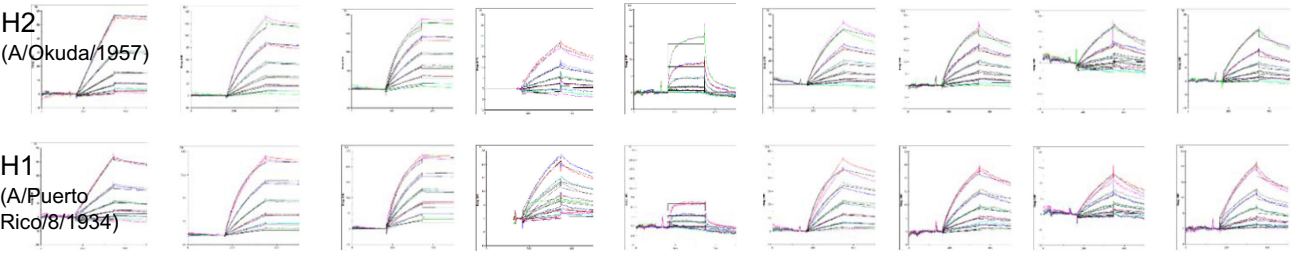
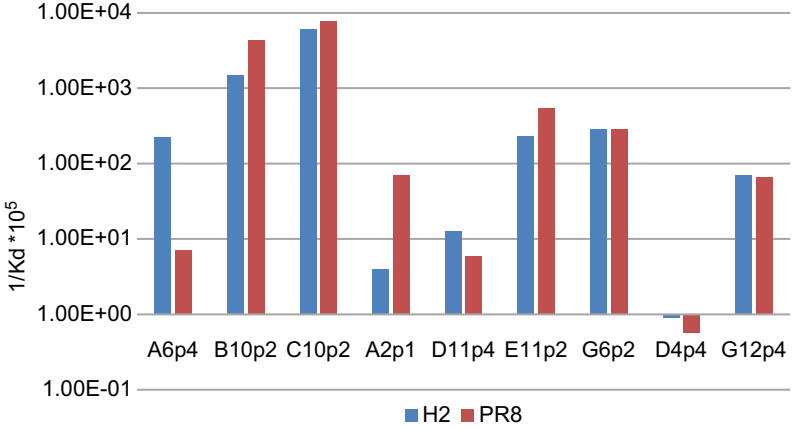
HV1-5
KV10-96


Type binding
cross,
PR8+,
H2+,
A6p4
B10p2
C10p2
G12p4
G6p2
D4p4
E11p2
A2p1
D11p4

A6_p4 HV3-2 KV14-111	B10_p2 HV4-1 KV8-30	C10_p2 HV4-1 KV8-24	A2_p1 HV1-9 KV10-96	D11_p4 HV1-9 KV10-96	E11_p2 HV1-5 KV10-96	G6_p2 HV1-5 KV10-96	D4_p4 HV1-5 KV10-96	G12_p4 HV1-5 KV10-96





A6_p4 HV3-2 KV14-111	B10_p2 HV4-1 KV8-30	C10_p2 HV4-1 KV8-24	A2_p1 HV1-9 KV10-96	D11_p4 HV1-9 KV10-96	E11_p2 HV1-5 KV10-96	G6_p2 HV1-5 KV10-96	D4_p4 HV1-5 KV10-96	G12_p4 HV1-5 KV10-96