

1 **Development of whole-porcine monoclonal antibodies with potent neutralization**
2 **activity against classical swine fever virus (CSFV) from single B cells**

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7 Running Head: Isolation of monoclonal antibodies against CSFV from single B cells

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16

17 **Abstract (max 250 words, currently 219)**

18 Classical swine fever (CSF) is a highly contagious swine disease found worldwide that has
19 caused devastating economic losses. However, there are few efficacious mAbs against the
20 CSF virus (CSFV) that can be used for treatment because most mAbs against CSFV are
21 derived from mouse hybridoma cells and these murine mAbs have disadvantages of
22 inefficient effector functions elicitation and high immunogenicity *in vivo*. Accordingly, we
23 characterized whole-porcine anti-CSFV neutralizing mAbs (NAbs) isolated directly from
24 single B cells sorted from a CSFV-vaccinated pig using the fluoresceinated conserved linear
25 neutralizing epitope of the CSFV E2 protein and fluorophore conjugated goat anti-pig IgG.
26 Immunoglobulin (Ig) genes were isolated via nested PCR, and two porcine mAbs termed
27 HK24 and HK44 were produced. We determined that these mAbs can bind to E2 protein and
28 recognize sites within this major antigenic epitope. In addition, we found that mAbs HK24
29 and HK44 exhibit potent neutralizing activity against CSFV, and they can protect PK-15 cells
30 from infections *in vitro* with potent IC₅₀ values of 9.3 µg/ml and 0.62 µg/ml, respectively.
31 Notably, we demonstrated that these two mAbs can be used as novel reagents for detecting
32 virus infection. These data suggest that our results not only provide a method for efficiently
33 obtaining mAbs against CSFV but also offer promising mAb candidates for development of
34 antibody-based diagnostic and antiviral agents.

35 **Keywords:** Classical swine fever; epitope of E2 protein; whole-porcine anti-CSFV
36 neutralizing mAbs; single B cells;

38 **Importance (max 150 words, currently 123)**

39 Neutralizing monoclonal antibodies (NAbs) can prevent and may slow the spread of virus
40 infection. The discovery of NAbs that recognize classical swine fever virus (CSFV)
41 necessitates new technologies because the NAbs produced by immunization and hybridoma
42 technology could not be transferred to *in vivo* research. Multiple full-length human
43 therapeutic antibodies have been produced via single-cell polymerase chain reactions but
44 whole-porcine NAbs for CSFV have not been generated. In this study, two whole-porcine
45 mAbs, named HK24 and HK44, were isolated from epitope-specific single B cells. We
46 demonstrate that these two mAbs have potent neutralizing activity against CSFV and can
47 protect cells against viral infection. Therefore, they may facilitate the development of
48 vaccines or antiviral drugs that offer the advantages of stability and low immunogenicity.

50 Introduction

51 Classical swine fever (CSF) caused by classical swine fever virus (CSFV) is a highly
52 contagious and fatal viral swine disease that remains a serious problem for the pork industry
53 (1-3). CSFV is an enveloped virus with positive-sense RNA and belongs to the genus
54 *Pestivirus* of the family *Flaviviridae* (4). The mature CSFV virion contains twelve viral
55 proteins, N^{pro}, C, E^{ms}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (5). E2 is a
56 protein with multiple functions that can form a dimer with E1 and mediate virion entry into
57 target cells; additionally, it is the major antigen for the production of neutralizing antibodies
58 that protect the body from the virus. The ability of an anti-E2 antibody to neutralize CSFV has
59 previously been characterized in detail (6, 7). The E2 protein consists of four antigen domains,
60 A, B, C, and D, at the N-terminus with a total of 177 amino acids, ranging from amino acids
61 690 to 866. These domains contain some conformational epitopes and linear epitopes that
62 play critical roles in inducing neutralizing antibodies. Comparison of the different epitopes of
63 the E2 protein has demonstrated that the linear epitope CTAVSPTTLRTEVVK, found
64 between amino acids 828 and 842, a region recognized by the monoclonal antibody WH303,
65 is strongly conserved in different CSFV strains but is highly divergent among bovine virus
66 diarrhoea virus (BVDV) and border disease virus (BDV) strains (8, 9).

67 NAbs that are capable of directly neutralizing most strains of a given highly antigenic
68 variable pathogen have attracted considerable attention because they can be used to treat some of
69 the difficult infectious diseases encountered in modern medicine and have potential for
70 development of passive immunotherapy treatment or vaccine reagents (10). In addition, given
71 their potential antiviral effects, NAbs also play an essential role in studying the
72 structure-function properties of infectious agents and related pathogenesis. However, mAbs of
73 mouse origin that are produced by hybridoma technology cannot be applied in *in vivo*
74 research because they can induce the production of anti-mouse antibodies *in vivo*, leading to a
75 short half-life (11-13). Other traditional methods of generating mAbs, such as Epstein-Barr
76 virus-transformation and phage-display libraries, are limited by being unstable, time
77 consuming or inefficient (14, 15). With the advent of single-cell reverse transcription PCR
78 (RT-PCR) technologies, the amplification of full-length immunoglobulin gene (Ig) fragments
79 from single B cells by utilizing nested PCR has allowed the bypass of many of these
80 limitations and this technology has been shown to provide a versatile tool for generation of
81 new mAbs; this is an important advancement in mAbs production (16, 17). Prior to the
82 amplification of Ig-encoding genes, antigen-specific memory B cells must be stained with
83 fluorescently-labelled antigen baits and then sorted. Several human mAbs against HIV, ZIKV
84 and Ebola virus have been obtained through this technology (18-20). However, the production
85 of whole-porcine mAbs against the E2 protein of CSFV using linear specific-epitopes has not
86 been reported. Whole-porcine neutralizing antibodies usually exhibit the lowest
87 immunogenicity, longer half-lives and have a great potential for superior applications in *in*
88 *vivo* (21, 22).

89 In this study, we report the acquisition of the whole-porcine mAbs HK24 and HK44.
90 These mAbs were isolated from single B cells of a vaccinated pig using the fluoresceinated
91 epitope CTAVSPTTLRTEVVK and fluorescein isothiocyanate (FITC)-labelled goat anti-pig
92 IgG via fluorescent-activated cell sorting (FACS) (23). Their ability to significantly neutralize
93 CSFV was identified through a panel of assays. We also confirmed that these mAbs

94 demonstrate significant value for the serological diagnosis of CSFV infection. Together, the
95 porcine mAbs HK24 and HK44 show potential as candidates for immunotherapy or
96 diagnostic reagents.

97

98 **Results**

99 **Isolation of single B cells from a vaccinated pig and mAb generation**

100 Individual pigs were immunized with an attenuated vaccine strain of CSFV to generate a
101 spectrum of antibody responses ranging from low to high levels of blocking, which was
102 determined using the Classical Swine Fever Virus Antibody Test Kit (IDEXX, Switzerland);
103 unvaccinated pigs served as negative controls (**Fig. 1A**). As shown in **Fig. 1B**, pigs #3748 and
104 #3757 exhibited a high blocking rate; therefore, blood samples from pig #3748 were used to
105 isolate single B cells that were stained with FITC-conjugated anti-pig IgG and
106 5-TAMRA-conjugated epitope-76 via FACS. Epitope-76 is strongly conserved in different
107 CSFV strains but is highly divergent among the BVDV and BDV strains, as shown in **Fig. 1C**.
108 As shown in **Fig. D**, epitope-specific IgG⁺ B cells constituted approximately 0.39% of the cell
109 population. Full-length heavy- and light-chain antibody genes were amplified from the cDNA
110 of single purified B cells via nested PCR and were sequenced. All nine antibodies were
111 obtained from one hundred single epitope-specific IgG⁺ B cells. Nucleotide sequence analysis
112 of these genes demonstrated that they could be divided into four different groups. Some of
113 these antibodies shared the same *IgH* and *IgL* genes, and most clones were somatic variants of
114 the IGHV1-4*02 and IGKV1-11*01/IGKV2-10*02 germline genes (**Fig. 1E**). This result was
115 similar to that obtained in a study of ZIKV (20).

116 In summary, we deduced that individuals with high serologic neutralizing titres against
117 the same epitopes of CSFV may express isotype antibodies. We also found that the mAbs
118 HK24 and HK44 each had a long complementarity determining 3 region of antibody
119 heavy-chain (CDR H3) loop composed of 17 and 21 amino acids, respectively. The antibody
120 gene pairs HK24 *IgH*, *IgK* and HK44 *IgH*, *IgK* were selected to produce mAbs, which were
121 identified via SDS-PAGE. Polyclonal antibody IgGs were isolated from porcine serum by
122 protein A were served as a positive control (**Fig. 1F**).

123

124 **The mAbs HK24 and HK44 can bind to specific epitopes of CSFV**

125 To map the epitopes of the mAbs HK24 and HK44, we first checked whether these
126 mAbs react with epitope-76. For this purpose, HEK293T cells were used for the transient
127 expression of mAb HK24 or HK44, and the binding activity of the mAbs HK24 and HK44 to
128 FITC-conjugated epitope-76 (20 µg/ml) was detected and analysed through microscopy. As
129 shown in **Fig. 2A**, there was a high level of green fluorescence in cells transfected with the
130 expression vector for either HK24 or HK44, and cells without expression vectors showed no
131 green fluorescence. This result indicated that HK24 and HK44 could bind to the conserved
132 epitope-76 with a higher affinity. This distinctive feature of the mAbs HK24 and HK44 was
133 further confirmed by flow cytometric analysis, as shown in **Fig. 2B**. FACS results indicated
134 that a higher percentage of HEK293T cells expressing HK24 or HK44 was stained by
135 FITC-conjugated epitope-76 (60.7% and 40.8%, respectively) than HEK293T cells that did
136 not express HK24 and HK44 (0.128%).

137 We also performed a direct fluorescence experiment to ascertain whether the mAbs

138 HK24 or HK44 possess binding specificity to epitope-76. As shown in **Fig. 2C**, the mAbs
139 HK24 and HK44, as well as positive IgGs purified from pig #3748, were recognized
140 efficiently by epitope-76, while HK24 and HK44 did not bind to the unrelated epitope-75
141 which is a linear epitope on the E2 protein of CSFV. Meanwhile, the positive IgGs interacted
142 efficiently with both epitope-76 and epitope-75.

143 In summary, these data demonstrated that the mAbs HK24 and HK44 can be specifically
144 recognized by epitope-76 with a high affinity.

145

146 **The mAbs HK24 and HK44 exhibit high specificity and sensitivity to the CSFV E2** 147 **protein**

148 The reactivity between the mAbs and the CSFV E2 protein was deciphered via Western
149 blotting. The results showed that the 55-kDa E2 protein band from lysates of CSFV-infected
150 cells was clearly detected by the mAbs HK24 and HK44 with high specificity, unlike the
151 lysates of uninfected cells (**Fig. 3A and 3B**).

152 To further verify the reactivity of the mAbs HK24 and HK44 with the CSFV E2 protein,
153 recombinant E2 protein was subsequently produced and used to detect antibody affinity via
154 Western blotting. As expected, the mAbs HK24 and HK44 reacted with recombinant E2 with
155 high specificity (**Fig. 3C**). Indirect ELISA also showed that the mAbs HK24 and HK44
156 exhibited excellent affinity for the recombinant E2 protein, and binding occurred in a
157 dose-dependent manner, whereas negative IgGs (isolated from CSFV-negative swine serum
158 by protein A) did not show significant reactivity (**Fig. 3D**). Together, these results
159 demonstrated that the mAbs HK24 and HK44 possess specific reactivity with either CSFV E2
160 protein in cells or purified recombinant E2 protein.

161 Additionally, to investigate whether the mAbs HK24 and HK44 could be applied for
162 CSFV detection, these antibodies were used to detect CSFV in PK-15 cells via an
163 immunofluorescence assay. As shown in **Fig. 3E**, the mAbs HK24 and HK44 showed strong
164 fluorescence at 30 nM, as observed for CSFV-positive serum, unlike CSFV-negative serum.
165 This result suggested that both mAbs isolated in the present study could be used to detect
166 CSFV. Hence, our results suggested that the mAbs HK24 and HK44 exhibited high sensitivity
167 towards E2 of CSFV and can be used for the serological detection of CSFV infection or for
168 research purposes.

169

170 **Evaluation of the binding affinity of mAbs HK44 and HK24 to E2 protein by SPR**

171 SPR is a powerful technique that provides affinity and kinetic information for
172 protein-protein interactions (24, 25). Thus, SPR was used to assess the ability of the mAbs
173 HK44 and HK24 to bind the recombinant protein E2. For this purpose, the mAbs HK44 and
174 HK24 were immobilized on CM5 sensor chips with a general 1:1 interaction; the results are
175 shown in **Fig. 4**. As shown in **Fig. 4A and 4B**, SPR dose-dependent binding assays verified
176 that the mAbs HK44 and HK24 bound the E2 protein with high affinity at E2 protein
177 concentrations ranging from 125 nM to 2000 nM. The SPR data indicated that the association
178 rate constant (k_a) of the mAbs HK44 and HK24 with the recombinant E2 protein was $1.70 \times$
179 $10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $5.01 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, indicating a fast association constant between mAbs
180 HK44 or HK24 and E2 protein and the equilibrium dissociation constant (KD) of 1.85×10^{-7}
181 M and 3.67×10^{-8} M respectively. These results suggest that mAbs HK44 and HK24 exhibit a

182 high binding affinity to the E2 protein. The responses of E2 protein at each concentration to
183 mAb HK44 or HK24 are depicted as a sigmoidal dose response curve in **Fig. 4 C and 4D**.
184 These results clearly illustrate the higher specific affinity of the mAbs HK44 and HK24 for
185 the E2 protein.

186

187 **Identification of the binding of the mAbs HK24 and HK44 to a conserved epitope on the** 188 **CSFV E2 protein based on a 3D structural model**

189 To obtain detailed structural information on E2-mAbs interactions, protein-protein
190 docking was performed. Before docking, the best protein modelling result was selected based
191 on the probability density function (PDF) total energy and discrete optimized protein energy
192 (DOPE). The PDF total energy and DOPE of HK24, HK44 and the E2 protein were
193 2296.5857, -45243.882812; 2390.8101, -46726.902344; and 1949.7089, -28135.984375,
194 respectively. Furthermore, there were fewer irrational amino acid residues in the
195 Ramachandran Plot, suggesting that the models we built may exist. Thus, protein-protein
196 docking was performed by setting the Fab as the receptor protein and the E2 as the ligand
197 protein. The docking results of E2-HK24 and E2-HK44 are shown in (**Fig. 5A and 5B**). More
198 precisely, we found that the epitope Cys141-Lys155 of the E2 motif in both cases was buried
199 in the middle of V_L-V_H interface of the E2-HK24 or E2-HK44 protein complex, and this
200 epitope contacted the Fab CDR H3, CDR L3 and CDR L1 loops in both structures in addition
201 to CDR H2 in the HK44-E2 complex (**Fig. 5C and 5D**). In addition, the mAb HK44 also
202 directly bind to Thr88 and Pro90 (a linear epitope on E2 protein spanning Leu84-Pro90
203 residues that has been reported) (26, 27) by means of three residues (Val111A, Ser111B and
204 Tyr111C) on the tip of the loop which is a relatively long CDR H3 loop (21 amino acids) (**Fig.**
205 **5C**). As shown in **Fig. 5D**, similar to E2-HK44 protein complex, the long CDR H3 of mAb
206 HK24 (17 amino acids) not only binds to the epitope-76, but also binds to the linear epitope
207 on E2 protein spanning Leu84-Pro90, via some hydrogen bonds and hydrophobic interaction.
208 Detailed interactions between antigen and antibodies are shown in the **Table 2 and 3**.

209 To further confirm the interaction between mAb HK44 or HK24 and epitope-76, Virtual
210 Alanine Mutation Scanning (VAMS) was employed to calculate mutation energy of
211 individual amino acid residues changes using Discovery Studio 2017. In the case of E2-HK44
212 complex, when the residues of epitope-76 of E2 protein were individually mutated to alanine,
213 seven residues on epitope-76, namely, Val144, Ser145, Pro146, Thr147, Arg150, Glu152,
214 Val154 generated the mutation energy higher than 0.5 kcal/mol, indicating an unstable protein
215 complex (**Fig. 5E**). The mutations of epitope-76 in the E2-HK24 complex procedure also
216 destabilize the protein complex (**Fig. 5F**). When we mutate all of the epitopes to alanine, the
217 mutation energy is 3.63 kcal/mol for E2-HK24 protein complex and 7.28 kcal/mol for
218 E2-HK44 protein complex, indicating that the protein complex is an unstable state. In
219 summary, these results suggested that the mAb HK24 or HK44 binds to the E2 protein by the
220 recognition of the epitope-76 (CTAVSPTTLRTEVV).

221

222 **MAbs HK24 and HK44 can significantly neutralize CSFV *in vitro***

223 We next evaluated the protective efficacy of the mAbs HK24 and K44 against CSFV
224 infection in PK-15 cells. A neutralization test was first performed via indirect
225 immunofluorescence assay (IFA). As shown in **Fig. 6A**, the mAbs HK24 and HK44 displayed

226 high neutralizing activity against CSFV, as no fluorescence was observed at the concentration
227 of 75 µg/ml, and only weak fluorescence signals were detected at the concentration of 1.1
228 µg/ml, which was same as that observed for CSFV-positive serum. In contrast, strong
229 fluorescence signals were detected in cells treated with CSFV-negative serum, indicating no
230 inhibition of CSFV infection. The range of 50% inhibitory concentrations (IC₅₀) to the
231 non-neutralizing concentration for mAbs HK24 and HK44 is presented in **Fig. 6B**. These
232 results indicated that the mAbs HK24 and HK44 exhibited neutralizing activity against CSFV,
233 and they can prevent CSFV infections in cells.

234 To further directly detect whether the mAbs HK24 and HK44 block CSFV infection, the
235 copy numbers of CSFV RNA in PK-15 cells were analysed via qRT-PCR with specific
236 primers. The results suggested that treating PK-15 cells with the HK44-CSFV or
237 HK24-CSFV complex at concentrations of 75 µg/ml to 1.1 µg/ml resulted in obviously lower
238 numbers of virus copies in the cells than those observed with negative control treatment; at
239 the same time, the mAb HK44 showed better neutralization activity than the mAb HK24 (**Fig.**
240 **6C and 6D**).

241 In summary, these data indicated that the mAbs HK24 and HK44 significantly
242 neutralized CSFV and played an essential role in preventing CSFV from infecting target
243 cells.

244

245 **Discussion**

246 CSF is a disease that threatens the pork industry and has caused tremendous economic
247 losses. As an alternative, development of effective vaccines and therapeutics agents may be a
248 direct and effective approach to minimizing these losses. With the technological advances,
249 mAbs now represent an important class of biotherapeutics, which can be used to treat
250 autoimmune disorders diseases, cancer and viral or bacterial infections (28). Thus, it is
251 necessary to establish a simple, highly efficient, low-cost method to produce anti-CSFV
252 NAbs.

253 For generation of human mAbs, there have been previous reports of approaches
254 involving the sorting of single B cells via FACS using fluorescently-labelled specific
255 membrane immunoglobulins as antigen bait; However, this method may be limited by the
256 number of antigenic baiting reagents (29-31). Here, we describe a method for efficient
257 production of mAbs from immunized swine. We used a fluoresceinated linear neutralizing
258 epitope CTAVSPTTLRTEVVK and FITC-labelled goat anti-pig IgG as bait for flow
259 cytometry to screen B cells from a pig that had been pre-screened for high-level
260 CSFV-specific antibody response in the blood. The cognate immunoglobulin heavy- and
261 light-chain genes were then isolated from epitope-specific single B cells using single-cell
262 RT-PCR technology. The key features of this method are the (a) sorting of B cells using a
263 specific linear epitope by FACS, which may avoid the limitation of the availability of multiple
264 antigenic baiting reagents; (b) direct isolation of natively matched full-length heavy- and
265 light-chain pairs from a single B cell, thus, producing natural mAbs with a lower the risk of
266 immunogenicity *in vivo* and greater suitability for *in vivo* applications; (c) rescue of mAbs
267 that recognize the specific linear epitope. In contrast to traditional methods for generating
268 mAbs, such as hybridoma technology, Epstein-Barr virus-transformation and phage-display
269 libraries, this method helps to improve the recovery efficiency of mAbs as 78% of the mAbs

270 produced showed neutralizing activity against CSFV. Taken together, this method is more
271 convenient and efficient for obtaining epitope-specific NAbs against CSFV.

272 In this study, we amplified full-length heavy- and light-chain genes from one hundred
273 single B cells and obtained nine mAbs, among which three were identical to HK24 and four
274 to HK44. Thus, two mAbs HK24 and HK44 were selected to detect the strong binding
275 reactivity against CSFV via Western blotting. To further define their binding paratopes, we
276 tested the reactivity of the mAbs HK24 and HK44 with recombinant E2 via indirect ELISA
277 and SPR. The results showed that the mAbs HK24 and HK44 exhibited comparable reactivity
278 to the E2 protein. The binding sites within the linear epitope CTAVSPTTLRTEVVK on the
279 E2 protein surface were predicted through a 3D structural model. Notably, these results are
280 consistent with reports that the linear epitope CTAVSPTTLRTEVVK on the E2 protein is
281 specifically recognized by CSFV antibodies (23, 32). Because the linear epitope is conserved
282 in diverse CSFV strains and is divergent in the BVDV and BDV strains, we deduced that the
283 mAbs HK44 and HK24 might be applied for the development of diagnostic reagents for
284 CSFV.

285 We also confirmed that the mAbs HK24 and HK44 exhibited a powerful neutralizing
286 response against the CSFV Shimen strain *in vitro*; in particular, the mAb HK44 showed an
287 $IC_{50} \leq 1.1 \mu\text{g/ml}$ (**Fig. 6B**). This result indicated that these mAbs are capable of blocking virus
288 infection and provided implications for therapeutic designs. Moreover, neutralization assays
289 showed that in this work, the mAb HK44 exhibited more excellent neutralizing activity than
290 the mAb HK24. We deduced that this may be due to a longer CDR H3 (21 amino acids)
291 because the amino acid lengths of the predominant CDR H3 are conserved in most vertebrates,
292 averaging 12-16 amino acids (33). Indeed, some longer CDR H3 loops usually play an
293 important role in the adaptive immune response such as virus neutralization as described
294 previously (34-36). Elongated CDR H3 can better overcome the structural barriers presented
295 by antigens to confer protective functions.

296 In conclusion, this is the first report to describe the isolation of whole-porcine NAbs
297 against the E2 protein of CSFV from single B cells of a vaccinated pig using a specific linear
298 epitope. In this study, we characterized the functions of the mAbs HK24 and HK44 through a
299 panel of assays. The results demonstrated that HK24 and HK44 displayed high sensitivity to
300 CSFV, indicating that these mAbs have great potential for the detection and treatment of viral
301 infections. In addition, we established a simple and rapid method for the isolation of specific
302 B cells. The mAbs that we generated were derived directly from porcine B cells and are
303 therefore safer and more efficient than mAbs produced in mice, rabbits or other species.

304

305 **Materials and methods**

306 **Cell lines and virus**

307 PK-15 cells (porcine kidney cell line, ATCC, CCL-33) and HEK293T cells (human
308 embryonic kidney cell line, ATCC, Manassas, VA) were cultured in Dulbecco's minimal
309 essential medium (DMEM, Gibco, America) supplemented with 10% heat-inactivated foetal
310 bovine serum (FBS, Gibco, America), at 37 °C in 5% CO₂. CSFV (strain Shimen) was gifts
311 from Dr. Changchun Tu (Academy of Military Medical Sciences, Changchun, China).

312

313 **Single B cell sorting**

314 Blood samples were isolated from a swine with a high-level CSFV-specific antibody
315 response that received primary immunization with an attenuated vaccine strain of CSFV and
316 subsequent boosting two times at one-week intervals with the same vaccine. Peripheral blood
317 mononuclear cells (PBMCs) were isolated and suspended in phosphate-buffered saline (PBS).
318 cells were stained for 30 min at 4 °C using FITC-labelled goat anti-pig IgG (Sigma-Aldrich,
319 USA) and 5-TAMRA-conjugated epitope-76, which is a conserved epitope
320 (CTAVSPTTLRTEVVK) on the E2 protein of CSFV (synthesized by China Peptides Co., Ltd.,
321 China) (37). All buffers used for staining contained 2% FBS (vol/vol) to block non-specific
322 binding. Single cells were sorted via flow cytometry into 0.2-ml thin-walled PCR tubes
323 containing 20 µl of cell lysis buffer supplemented with 1 µl of RNase inhibitor, 19 µl of 0.2%
324 (vol/vol) Triton X-100 (Sigma-Aldrich, USA), 1 µl of oligo-dT primer and 1 µl of dNTP mix.
325 The cells were stored at -80 °C until use (38, 39).

326

327 **Isolation and expression of Ig gene**

328 PCR was performed to amplify the full-length immunoglobulin heavy- and light-chain
329 genes (*IgH* and *IgL*) from single B cells as described previously (40, 41). Briefly, to obtain Ig
330 genes, we performed a reverse transcription reaction using SuperScript II reverse transcriptase
331 (38, 42). Next, the *IgH*, *Igλ* and *Igκ* genes were amplified separately via nested PCR reactions
332 using a mixture of primers that were specific for heavy chain, kappa light chain and lambda
333 light chain; all oligonucleotide primers are listed in **Table 1**. The PCR products of the
334 full-length heavy- and light-chain genes were confirmed via sequencing and were separately
335 cloned into the pEGFP-C1 expression vector with the EF1α promoter and WPRE element
336 (**Fig. S1**). To produce mAbs, plasmids encoding immunoglobulin heavy- and light-chains
337 were transiently co-transfected into HEK293T cells using PowerTrans293 Transfection
338 Reagent (Throne Science, Shanghai, China) following the manufacturer's instructions. After
339 culturing for four days at 37 °C under 5% CO₂ in 6-well plates, supernatants were harvested
340 via centrifugation, and antibodies were purified using Protein A (GenScript, USA Inc.) and
341 quantified using a NanoDrop 2000.

342

343 **SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis**

344 SDS-PAGE was performed as follows. Briefly, antibody samples were separated in 12%
345 (reducing conditions) resolving gels and 4% stacking gels, and protein bands were stained
346 using Coomassie brilliant blue R-250 (43). For Western blotting analysis, we collected
347 CSFV-infected cells and uninfected cells separately in cold PBS and lysed them using a lysis
348 buffer (Beyotime, China) that contained 1 mM phenylmethylsulfonyl fluoride (PMSF) for 5
349 min on ice. After centrifugation at 10,000 × g at 4 °C for 10 min, the supernatants were
350 harvested and transferred into new 1.5-ml centrifuge tubes (44). Protein concentrations were
351 determined with an enhanced-bicinchoninic acid (BCA) protein assay kit (Beyotime, China).

352 Western blotting was performed according to a previously described procedure (45).
353 Briefly, equal amounts of proteins were loaded on a 12% SDS-PAGE gel. Next, the separated
354 proteins were transferred onto nitrocellulose membranes, which were blocked with 5%
355 (wt/vol) non-fat milk in Tris-buffered saline that contained 0.2-0.4% Tween-20 (TBS-T) for 2
356 h at room temperature. The membranes were probed with 4 µg/ml mAb or porcine IgGs
357 (hyperimmune serum). Next, the bound antibody was stained using 2 µg/ml horseradish

358 peroxidase (HRP)-conjugated rabbit anti-pig antibodies (Beijing Biosynthesis Biotechnology
359 Co. Ltd, China). Immunoreactive bands were visualized using the ECL detection reagent
360 (Beyotime, China) and the Azure c600 Western blot imaging system.

361

362 **Immunofluorescence to detect the binding specificity of mAbs HK44 and HK24 to** 363 **epitope-76**

364 The binding of epitope-76 to mAbs HK44 and HK24 were determined via
365 immunofluorescence. In this procedure, enzyme-linked immunosorbent assay (ELISA) plates
366 were coated with 100 μ l (2 μ g/ml) of a positive polyclonal antibody, mAbs HK24 and HK44
367 diluted in carbonate bicarbonate buffer (pH 9.6) and were incubated at 4 $^{\circ}$ C overnight (46).
368 The next day, the plates were washed thrice with PBS containing 0.05% Tween-20 (PBS-T),
369 and 100 μ l of blocking buffer (5% non-fat dry milk in PBS) was added per well. The plates
370 were incubated at 37 $^{\circ}$ C for 1 h. After blocking, 100 μ l of FITC-conjugated epitope-76 or
371 unrelated FITC-conjugated epitope-75 was added into each well, and the plates were
372 incubated at 37 $^{\circ}$ C for 2 h. Finally, fluorescence intensity was measured using a Tecan
373 Microplate reader.

374

375 **Expression of the E2 protein**

376 The partial sequence of E2 gene (GenBank: AY775178.2) was amplified from
377 CSFV-infected cell mRNA via PCR and cloned into an expression vector (47). The vector was
378 transformed into *E. coli* Rosetta (DE3) cells, and the transformants were selected and cultured
379 in LB. The overnight cultures were subcultured at 1:100 and grown to an OD₆₀₀ of 0.6 at 37 $^{\circ}$ C.
380 Next, 0.1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) was added to induce the
381 expression of the protein at 25 $^{\circ}$ C for 12 h. The induced culture was harvested via
382 centrifugation at 6,500 \times g for 15 min at 4 $^{\circ}$ C and sonicated on ice. Subsequently, the inclusion
383 bodies were separated from the crude cell lysate via centrifugation at 10,000 \times g for 10 min
384 and dissolved in 6 M urea at room temperature for 6 h. Finally, soluble E2 protein was
385 purified through Ni-NTA resin as described previously (48, 49).

386

387 **Detecting the affinity of mAbs HK24 and HK44 for the CSFV E2 protein via indirect** 388 **ELISA**

389 An indirect ELISA was used to determine the affinity of the mAbs for the CSFV E2
390 protein. Purified E2 protein (2 μ g/ml) was used to coat ELISA plates at 4 $^{\circ}$ C overnight. The
391 next day, the plates were washed thrice with PBS-T, and 100 μ l of blocking buffer (5% nonfat
392 dry milk in PBS) was added per well. The plates were incubated at 37 $^{\circ}$ C for 1 h. After
393 blocking, 100 μ l of antibody samples were added per well, and the plates were incubated at
394 37 $^{\circ}$ C for 2 h. Thereafter, an HRP-conjugated rabbit anti-pig antibody (Beijing Biosynthesis
395 Biotechnology Co. Ltd, China) was added into each well at 37 $^{\circ}$ C for 1 h. Finally, the wells
396 were washed and incubated with 100 μ l of TMB/H₂O₂ substrate per well at room temperature
397 for 10 min. The reaction was stopped using 50 μ l of 2 M H₂SO₄ per well, and the absorbance
398 was read at 450 nm.

399

400 **Modelling of the E2 protein and the antigen-binding (Fab) fragment of the mAbs HK24** 401 **and HK44**

402 The interactions of mAbs HK24 and HK44 with the CSFV E2 protein were simulated
403 using Discovery Studio 2017. To obtain a three-dimensional model of the mAb HK24, we
404 searched for a similar sequence in the Protein Data Bank (PDB) database. We found that the
405 sequence homology between an anti-G-quadruplex-containing RNA antibody crystal (PDB
406 4KZD) and Fab of HK24 consisted of 84.1% similarity and 72.7% identity. Therefore, we
407 used PDB 4KZD as a template for modelling the mAb HK24 Fab. Considering the quality of
408 mAb HK44 model, we selected a chimeric template for modelling. For this purpose,
409 humanized antibody 4B12 Fab (PDB 4LKX), an anti-TDRD3 FAB (PDB 3PNW) and an
410 anti-CMV Fab Fragment (PDB 4LRI) were used as templates for modelling the light chain
411 (LC), heavy chain (HC) and the interface of the mAb HK44 Fab; the sequence homologies
412 (similarity and identity) between each template and the mAb HK44 Fab were 87.7% and 74.9%
413 for V_L, 80.4% and 70.9% for V_H and 82.8% and 69.8% for the entire Fab, respectively. E2
414 protein modelling was performed using the template of the crystal of the BVDV1 envelope
415 glycoprotein E2 (PDB 2YQ2, 60% sequence identity with E2) (50).

416

417 **Docking and analysis of protein complexes**

418 Before docking, models were typed using the CHARMM Polar H forcefield. We set E2
419 as the ligand protein and the Fab of HK24/HK44 as the receptor protein. ZDOCK, a
420 rigid-body protein-protein docking algorithm based on the Fast Fourier Transform Correlation
421 technique, was used with a 6° angular step size to generate 54,000 poses, of which the top
422 2000 were re-ranked by ZRANK (51). These poses were then processed with RDOCK, and
423 only the top 10 clusters with the highest density of poses were further considered. Finally, we
424 used the “Analyze Protein Interface” protocol for analysis. All docking calculations were
425 performed using Discovery Studio 2017.

426

427 **Surface plasmon resonance (SPR) analysis**

428 To analyse the affinity of the mAbs for the CSFV E2 protein, we performed SPR analysis
429 on a Biacore 8K system (GE Healthcare) using CM5 sensor chips (GE Healthcare) and PBS-T
430 as the running buffer during immobilization and binding analysis at a constant temperature of
431 25 °C (52). Initially, the surface was activated with
432 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS),
433 and the mAb HK44 or HK24 was diluted in 10 mM sodium acetate buffer (pH 4.0) and
434 immobilized on the CM5 sensor chip using Amine Coupling Kit (GE Healthcare). Next, for
435 binding analysis, five different concentrations of the recombinant E2 protein diluted in the
436 running buffer (2000 nM, 1000 nM, 500 nM, 250 nM, and 125 nM) were allowed to flow
437 over the chip surface. After sample injection was completed, the running buffer was allowed
438 to flow over the surface to perform dissociation. At the end of dissociation, the sensor surface
439 was regenerated with a glycine-HCl solution (53). Finally, all experimental data were
440 analysed using Biacore 8K evaluation software (GE Healthcare).

441

442 **Neutralization assays**

443 The neutralization activity of the mAbs HK24 and HK44 against CSFV was tested via an
444 immunofluorescence assay as described previously (54). Briefly, PK-15 cells were grown to
445 30-40% confluence in complete DMEM containing 10% FBS on 96-well plates at 37 °C in 5%

446 CO₂. Antibodies (150 µg/ml) were diluted two-fold in DMEM in a series and mixed 1:1 with
447 100 TCID₅₀ CSFV, and the mixtures were incubated at 37 °C for 1 h. After incubation, 100 µl
448 of the antibody-virus mixture was added into the wells of the 96-well plates to infect the
449 PK-15 cells for 2 h. Uninfected cells and virus-infected cells acted as positive and negative
450 controls, respectively. After 2 h of incubation, the supernatants were aspirated, and the cells
451 were washed three times with PBS. Next, 200 µl of fresh medium was added, and the cells
452 were incubated at 37 °C for 72 h. Subsequently, the cells were fixed with 80% cold acetone at
453 -20 °C overnight. The next day, the cells were washed and incubated with porcine
454 CSFV-positive serum (1:100 dilution in PBS containing 10% FBS) at 37 °C for 2 h. After
455 being washed three times with PBS, FITC-labelled goat anti-pig IgG (Sigma-Aldrich,
456 America) was added into each well, and the plates were incubated at 37 °C for 0.5 h (55).
457 Images were obtained using fluorescence microscopy (Olympus BX51).

458

459 **Quantification of CSFV RNA**

460 Quantitative RT-PCR (qRT-PCR) was performed to examine CSFV in PK-15 cells. Total
461 RNA was extracted from the uninfected and virus-infected cells using TRIzol (Tiangen,
462 Beijing, China), according to the manufacturer's instructions. Reverse transcription reactions
463 were performed using the FastKing RT Kit (Tiangen, Beijing, China) to synthesize cDNA, and
464 qRT-PCR was performed on a Bio-RadiQ5 instrument (BioRad, USA) with SuperReal
465 PreMix Plus (Tiangen, Beijing, China). A standard curve was simultaneously created to
466 calculate the viral load in each sample.

467

468 **Statistical analysis**

469 All data presented in the figures are expressed as the mean ± SD from at least three
470 independent experiments. When the data from the neutralization assays were analysed, the
471 antibody concentrations were transformed to log₁₀, and the IC₅₀ was calculated using
472 GraphPad Prism software 7.0 (La Jolla, CA, America) with the equation for dose response
473 (variable slope). P < 0.05 was considered statistically significant.

474

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483

484 **Conflict of Interest**

485 The authors declare that they have no competing interests.

486

487 **Ethical Approval**

488 Not applicable.

489

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654

655

656 **Figure legends**

657

658 **Figure 1. Isolation of epitope-specific antibodies.**

659 (A and B) Detection of CSFV antibodies was performed using a CSFV antibody test kit
660 (positive blocking rate $\geq 40\%$, negative blocking rate $\leq 30\%$). (A) Blocking rate of serum
661 samples isolated from unvaccinated pigs. (B) Blocking rate of serum samples isolated from
662 individuals immunized with attenuated vaccine strain of CSFV. (C) Sequence alignment
663 between E2 from four different CSFV strains, one BVDV strain and one BDV strain. The
664 conserved amino acid residues are highlighted in red, and the somatically mutated residues
665 are highlighted in green (BVDV stains) and blue (BDV strains). (D) Frequency of
666 epitope-specific, IgG⁺ memory B cells in the peripheral blood of pig #3748. An unvaccinated
667 pig, #3741, was used as the negative control. Flow cytometry plots display the percentage of
668 IgG⁺ B cells that bound to FITC-conjugated epitope-76 bait. (E) List of V (D) J segments of
669 antibody genes that we isolated and germline (GL) assignment which were derived using the
670 international ImMunoGeneTics information system (IMGT). (F) Analysis of the expression of
671 the mAbs HK24 and HK44 via SDS-PAGE. Samples were resolved by reducing 12%
672 SDS-PAGE, followed by staining with Coomassie blue. Lane M, marker protein; lane 1,
673 porcine IgGs 8 μg ; lane 2, porcine IgGs 20 μg ; lane 3, mAb HK24; lane 4, mAb HK44; lane 5,
674 supernatant of control HEK293T cells. The black arrows on the right indicate the antibody
675 heavy chains (HC) and light chains (LC).

676

677 **Figure 2. Specific reactions of the mAbs HK24 and HK44 with epitope-76.**

678 (A) Fluorescence microscopic images of cells. Green fluorescence was only observed on cells
679 transfected with the HK24 or HK44 expression vectors. Cells transfected with empty vectors
680 served as a negative control. Scale bar, 200 μm . (B) Analysis of the affinity of HK24 and
681 HK44 for epitope-76. Affinity was analysed using FACS. NC is cells transfected with empty
682 vectors. (C) Antibody specificity against epitope-76 was measured via a fluorescence assay.
683 The mAbs HK24 and HK44 and positive IgGs isolated from pig #3748 were immobilized on
684 an ELISA plate. Fluoresceinated epitopes were used to assess the binding activity of these
685 antibodies. Epitope-75 is an unrelated epitope. The results from at least three biological
686 replicates (mean \pm SD) and analysed using t tests with GraphPad Prism software. * $p < 0.05$,
687 ** $p < 0.005$, and *** $p < 0.0001$.

688

689 **Figure 3. The mAbs HK24 and HK44 bound to the E2 protein.**

690 (A and B) The mAbs HK24 and HK44 bound to the CSFV E2 protein. Protein samples were
691 obtained from PK-15 cells infected with CSFV. Uninfected PK-15 cells served as negative
692 controls. All samples were resolved via 12% reducing SDS-PAGE and were identified using
693 the mAbs HK24 (A) and HK44 (B). β -Actin was used as an internal control. (C) Recombinant
694 CSFV E2 protein (690-866 aa) expressed in a bacterial system was recognized by the mAbs
695 HK24 and HK44 and determined via Western blotting. (D) Detection of the binding of the
696 mAbs HK24 and HK44 to the recombinant E2 protein using ELISA. Recombinant E2 protein
697 (2 $\mu\text{g}/\text{ml}$) was used to coat the ELISA plates. The mAbs HK24 or HK44 (200 $\mu\text{g}/\text{ml}$), which
698 were serially diluted in PBS, were added to each well. Negative IgGs were purified from
699 CSFV-negative serum and used as a negative control. Results were obtained from at least

700 three biological replicates (mean \pm SD). (E) Detection of CSFV by the mAbs HK24 and
701 HK44. PK-15 cells were infected with CSFV and identified via IFA using HK24, HK44,
702 anti-CSFV swine serum and negative swine serum. Green fluorescence was observed on cells
703 treated with HK24, HK44 and anti-CSFV swine serum. However, cells treated with negative
704 serum showed no fluorescence. Scale bar, 200 μ m.

705

706 **Figure 4. SPR binding assays.**

707 MAbs HK44 and HK24 bound to recombinant E2 protein. (A and B) Binding of E2 protein at
708 concentrations of 2000, 1000, 500, 250 and 125 nM to mAbs (A) MAb HK44, (B) MAb
709 HK24. The SPR data for each binding step were calculated and are shown in the table at the
710 bottom. (C and D) Dose–response curve of the E2 binding signal. (C) The mAb HK44
711 binding response against each concentration of E2 protein. (D) The mAb HK24 binding
712 response against each concentration of E2 protein.

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714 **Figure 5. 3D modelling of the combination of the Fab fragments of mAbs HK24 and** 715 **HK44 with the CSFV E2 protein.**

716 (A and B) Cartoon representation of E2-HK44 and E2-HK24 protein complex. The best
717 docking results are displayed by the surface. Light and heavy chains of the antibody are
718 shown in slateblue and salmon, except for the CDR loops, which are shown in cyan and violet.
719 The E2 protein is shown in pale green, except for the epitope, which is shown in green. (A)
720 E2-HK44 protein complex. (B) E2-HK24 protein complex. (C and D) Bonds in the interface
721 of the protein complex. Conventional hydrogen bond is colored in green, carbon hydrogen
722 bond is colored in cyan, electrostatic (attractive charges and Pi-Cation) is colored in orange,
723 Alkyl Hydrophobic is colored in pink, Pi-Sigma Hydrophobic is colored in purple. (C)
724 E2-HK44 protein complex. (D) E2-HK24 protein complex. (E and F) The value of mutation
725 energy during Virtual Alanine Mutation on the epitope-76 in the protein-protein complex. (E)
726 E2-HK44 protein complex. (F) E2-HK24 protein complex.

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728 **Figure 6. The mAbs HK24 and HK44 neutralize CSFV.**

729 (A) The mAbs HK24 and HK44 were tested for neutralization reactivity with CSFV via the
730 IFA neutralization assay. CSFV-positive serum isolated from pig #3748 served as the positive
731 control, and CSFV-negative serum isolated from pig #3741 was used as the negative control.
732 (B) The percentage of CSFV inhibition. The dashed line represents 50% inhibition of the
733 virus compared to the virus-only control. (C and D) Neutralization activity of the mAbs HK24
734 and HK44 was measured via quantitative qRT-PCR. (C) The number of CSFV copies in cells
735 treated with the mAb HK24. (D) The number of CSFV copies in cells treated with the mAb
736 HK44. Results were obtained from at least three biological replicates (mean \pm SD) and
737 analyzed using t tests with GraphPad Prism software. * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0001$
738 (versus the positive serum).

739

740 **Figure S1. Physical maps of the expression vectors of the mAbs HK24 and HK44.**

741 Plasmid pEGFP-C1 was selected as the backbone, and the CMV promoter was substituted by
742 the EF1 α promoter. (A) P24H is the expression vector of the kappa light chains of HK24. (B)
743 P24K is the expression vector of the heavy chains of HK24. (C) P44H is the expression vector

744 of the kappa light chains of HK44. (D) P44K is the expression vector of the heavy chains of
745 HK44.

747 **TABLE 1.** Oligoes used in this study.

Oligo name	Sequences (5'→3')	748
E2-F	CATG <u>CCATGG</u> CTCGGCTAGCCTGCAAGG	749
E2-R	CCG <u>CTCGAG</u> TAGATCTTCATTTTCCACTGTGGTGGTC	750
IGH F1	GTGTCCAGGGTGAGGAGAAGCTG	751
IGH F2	GTGTCCAGGGTCAGGAGAAGCTG	752
IGH F3	GTGTCCAGGGTGAGGTGAAGCTG	753
IGH R	CGGTGATGCACGAGGCTCTGCACAACCA	754
IGK F1	GCCATCCAGCTGACCCAGTCTCCAGCCTCC	755
IGK F2	GCCATYGTGCTGACCCAGASTCCACTCTC	756
IGK F3	GAAATTGTGCTGACCCAGTCTGCAGCC	757
IGK F4	GAAACAACAGTCACTCAATCTCCAGCAT	758
IGK R	AGCCTCACACTCGTTCCTGYTGAAGCTTTTG	759
IGL F1	TCCTATGAGGTGACTCAGCCACCCT	760
IGL F2	TCCTATGAGCTGACCCAGCCGTCTT	761
IGL F3	TCTTCTAAGCTGACTCAGCCCCCG	762
IGL F4	CAGTCTGCCCTGACTCAGCCCCCCT	763
IGL F5	TCTCAGACTGTGATCCAGGAGCCGG	764
IGL F6	TCCCTCTCCCAGGCTGTGCTGA	765
IGL F7	TCCCAGATGGTGACTCAGGAA	766
IGL F8	CCAAGCTGTGTGACTCAGGAACC	767
IGL R	GGCGCACTCGGAGGGCGTCACTGTCTT	768
IS	AAGCAGTGGTATCAACGCAGAGT	769
Oligo-dT ₃₀ N	AAGCAGTGGTATCAACGCAGAGTACT ₃₀ VN	770
TSO	AAGCAGTGGTATCAACGCAGAGTACATrGrG+G	771
CSFV F	CTAGCCATGCCACAGTAGGA	772
CSFV R	CTCCATGTGCCATGTACAGCA	773

774 Underlining indicates NcoI and XhoI sequences.

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785 **TABLE 2.** Intermolecular force affecting the interaction of mAb HK44 and E2 proteins.

Intermolecular force	Amino acid
Conventional Hydrogen Bond	Arg64 (HC):Glu152 (E2)
	Ser145 (E2):Asp111Z (HC)
	Ala143 (E2):Asp34 (LC)
	Ser145 (E2):Asp111Z (HC)
	Pro146 (E2):Asp111Z (HC)
Carbon Hydrogen Bond	Thr147 (E2):Leu108 (LC)
	Thr147 (E2):Tyr116 (LC)
	Ser111B (HC):Thr88 (E2)
	Pro90 (E2):Tyr111 (HC)
Electrostatic (Attractive Charges)	Arg64 (HC): Glu152 (E2)
Electrostatic (Pi-Cation)	Arg150 (E2):Phe111X (HC)
Alkyl Hydrophobic	Pro146 (E2):Leu108 (LC)
	Val111A (HC):Pro90 (E2)
	His31(LC):Pro146(E2)
Pi-Sigma Hydrophobic	Tyr38(LC):Pro146(E2)
	Tyr110(HC):Ala143(E2)

786 HC: Heavy Chain; LC: Light Chain; E2: E2 protein.

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809 **TABLE 3.** Intermolecular force affecting the interaction of mAb HK24 and E2 proteins.

Intermolecular force	Amino acid
Conventional Hydrogen Bond	Ser36 (LC):Val144 (E2)
	Ser109 (LC):Thr147 (E2)
	Thr147 (E2):Leu114 (LC)
	Asn111A (HC):Gly87 (E2)
	Asn111A (HC):Asp86 (E2)
	Ser36 (LC):Val144 (E2)
Carbon Hydrogen Bond	Pro146 (E2):Ser109 (LC)
	Pro146 (E2):Ser28 (LC)
	Thr147 (E2):Leu114 (LC)
Alkyl Hydrophobic	Asn111A (HC):Gly87 (E2)
	Ala143 (E2):Cys111Y (HC)
	Pro146 (E2):Ile29 (LC)

810 HC: Heavy Chain; LC: Light Chain; E2: E2 protein.

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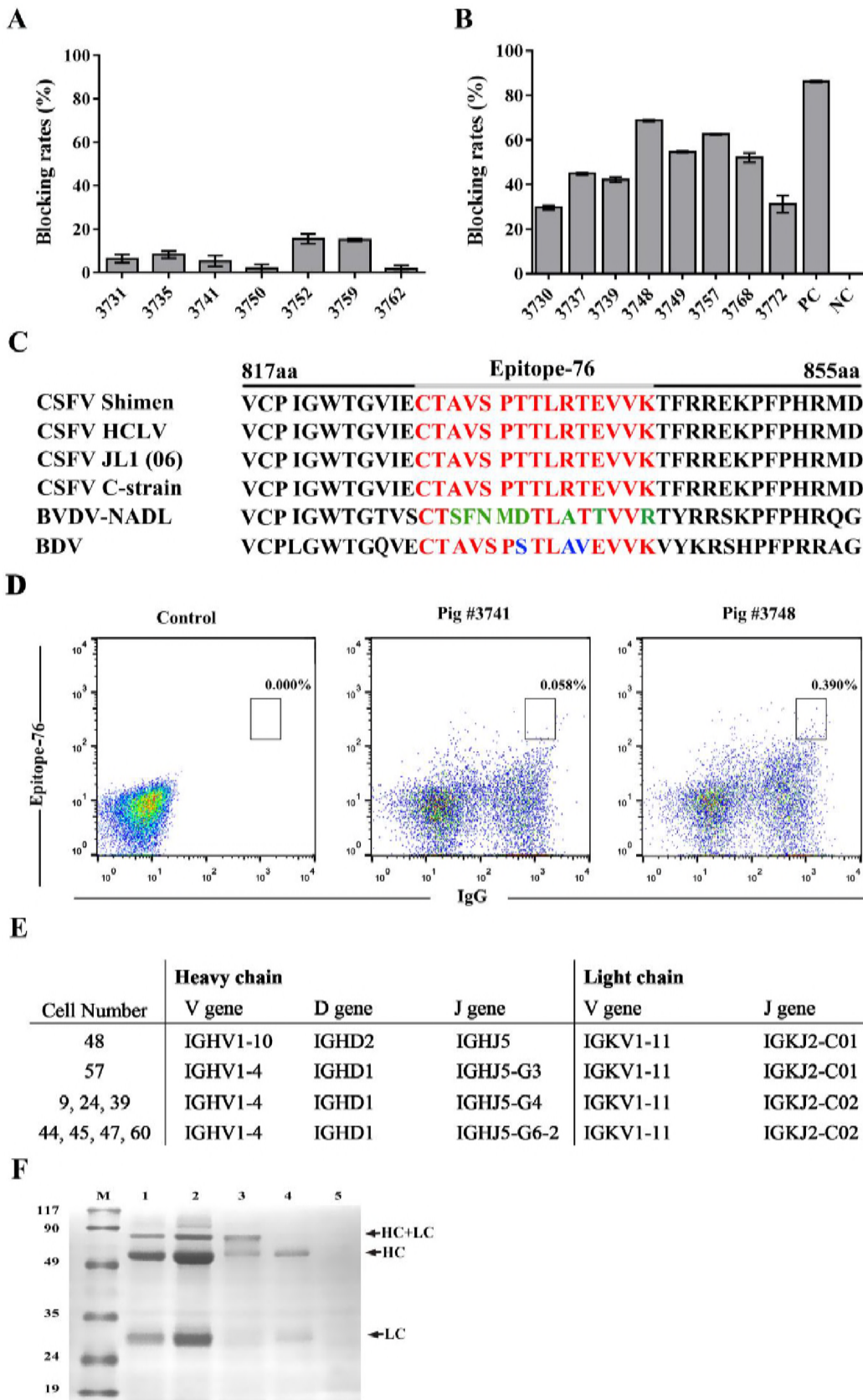
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837 Fig. 1



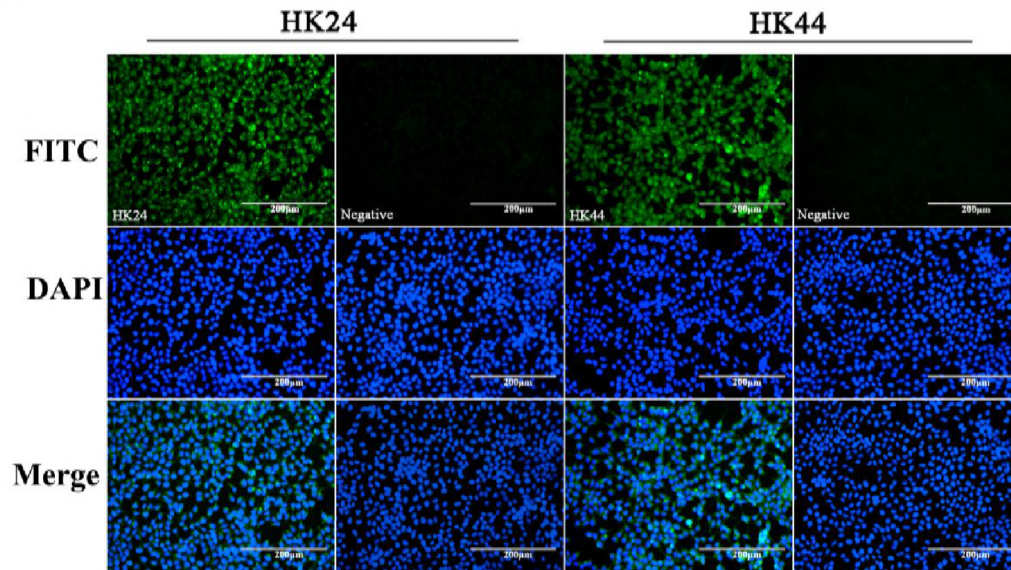
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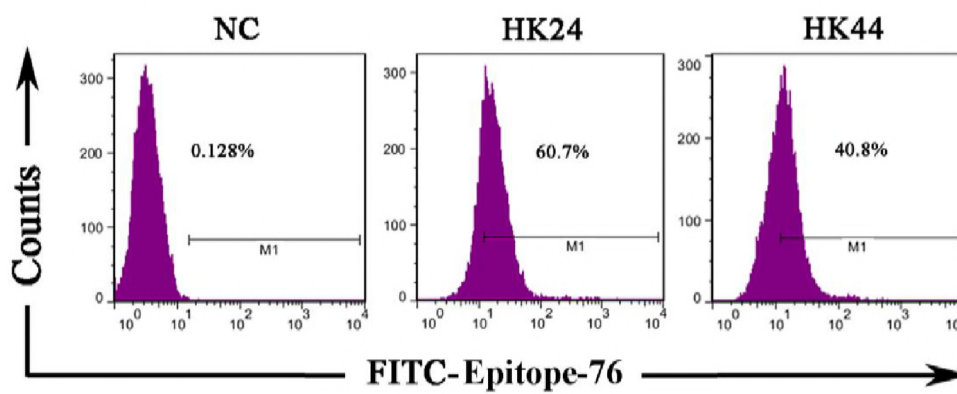
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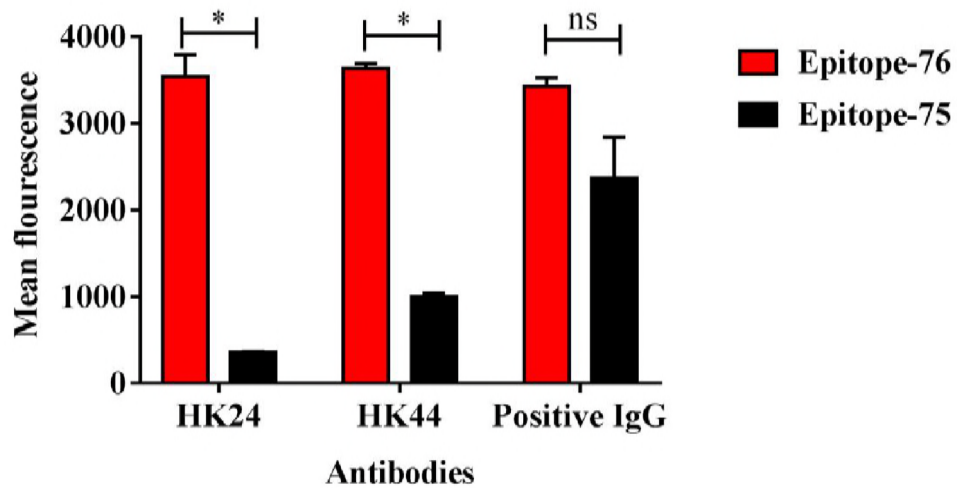
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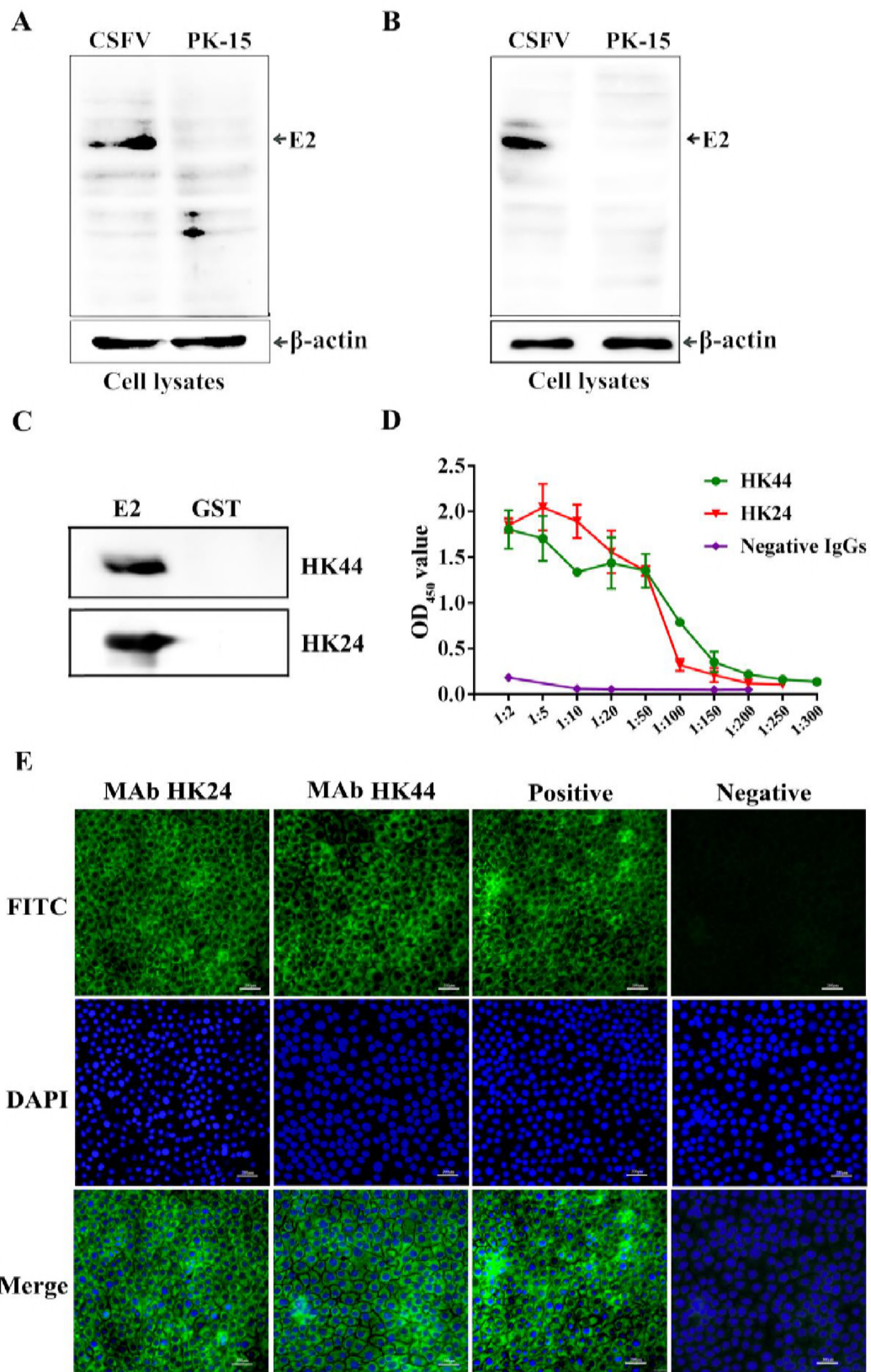
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847 Fig. 3



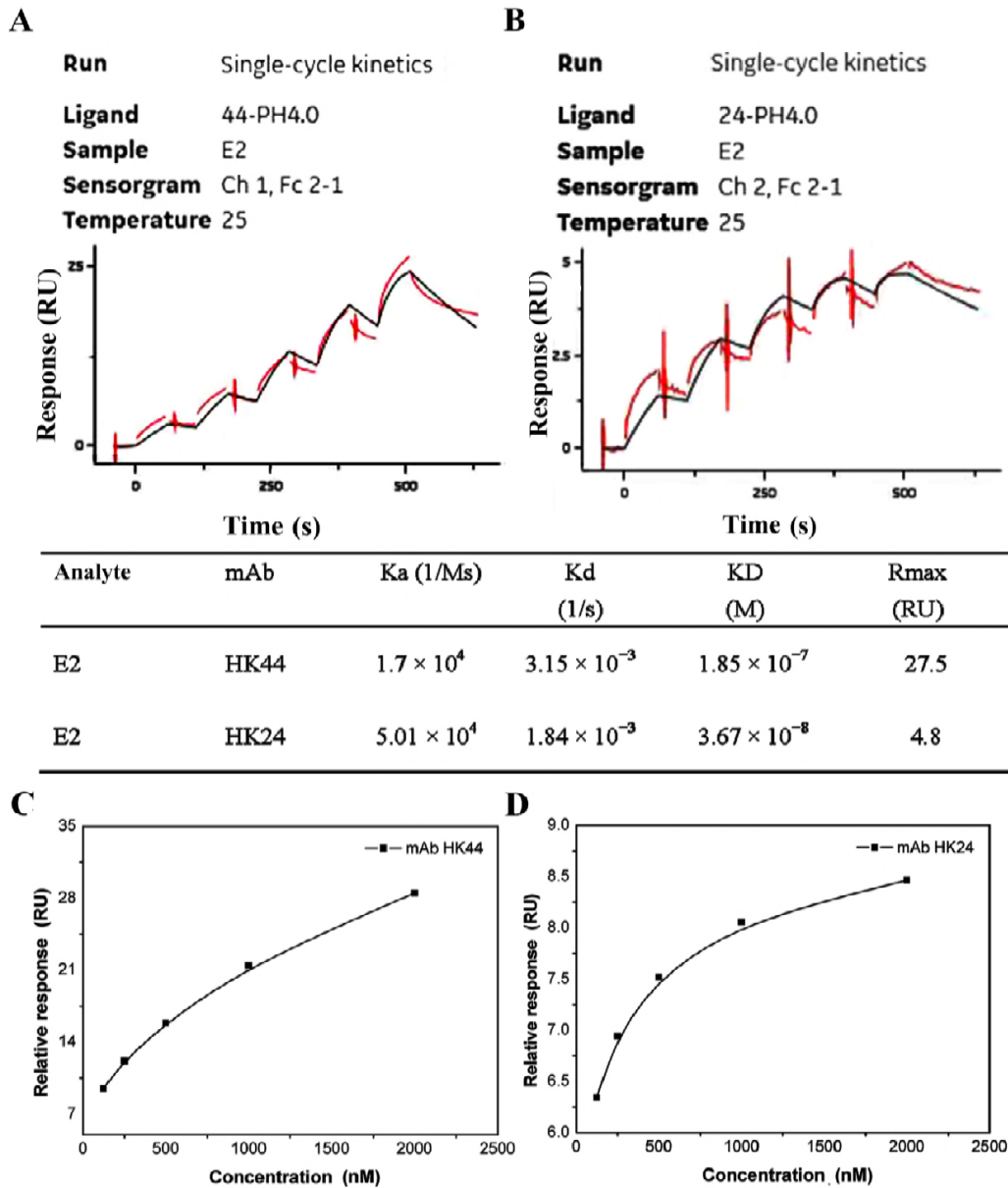
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852 Fig. 4



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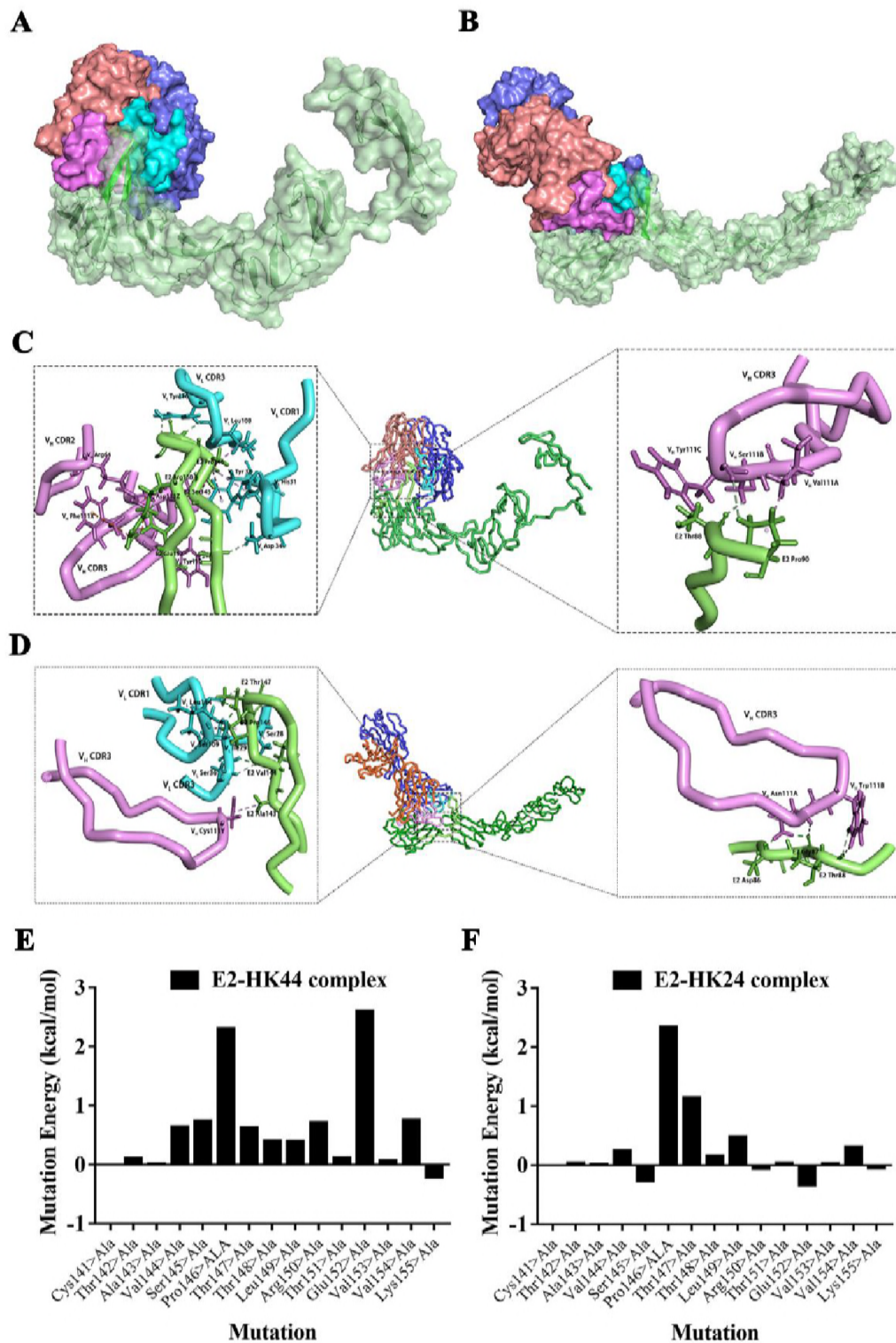
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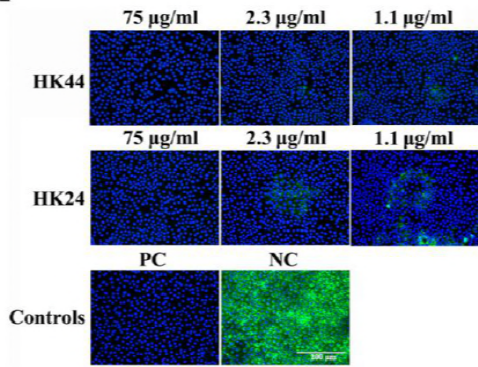
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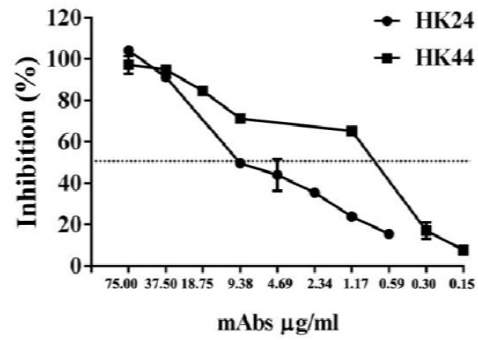
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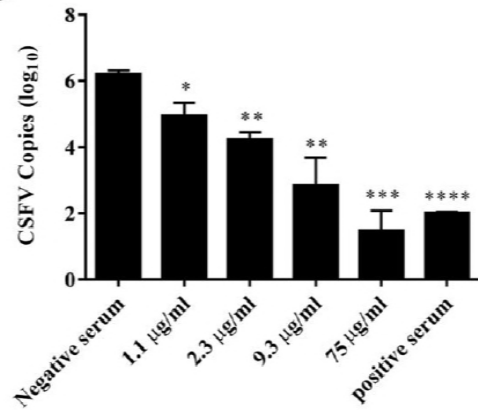
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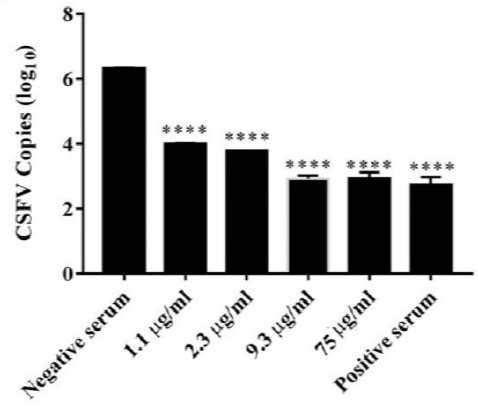
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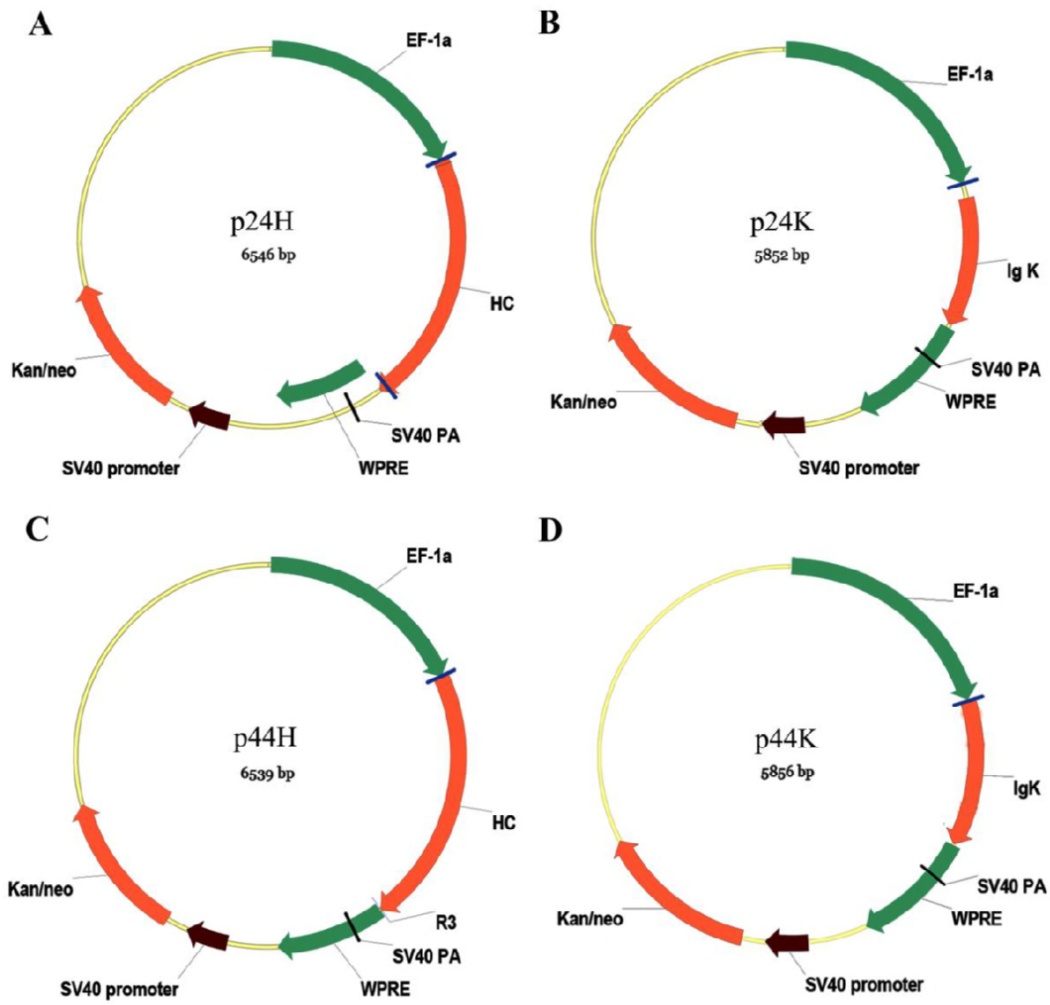
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918 Fig. S1



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