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## Selection of antigenically advanced variants of seasonal influenza viruses

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### Accession numbers

The nucleotide sequences of the HA and NA genes of A/Norway/3568/2009 (H1N1), A/Norway/3858/2009 (H1N1) and A/Yokohama/UT-K1205T/2009 (H1N1) determined in this study were deposited in GenBank (accession numbers CY189537-CY189542).

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## Abstract

Influenza viruses mutate frequently, necessitating constant updates of vaccine viruses. To establish experimental approaches that may complement the current vaccine strain selection process, we selected antigenic variants from human H1N1 and H3N2 influenza virus libraries possessing random mutations in the globular head of the haemagglutinin protein (which includes the antigenic sites) by incubating them with human and/or ferret convalescent sera to human H1N1 and H3N2 viruses. Further, we selected antigenic escape variants from human viruses treated with convalescent sera and from mice that had been previously immunized against human influenza viruses. Our pilot studies with past influenza viruses identified escape mutants that were antigenically similar to variants that emerged in nature, establishing the feasibility of our approach. Our studies with contemporary human influenza viruses identified escape mutants before they caused an epidemic in 2014–2015. This approach may aid in the prediction of potential antigenic escape variants and the selection of future vaccine candidates before they become widespread in nature.

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The haemagglutinin (HA) protein is the major influenza viral antigen<sup>1</sup> and the primary target of infection-neutralizing antibodies. During influenza virus circulation in humans, the antigenicity of the virus gradually changes due to mutations in the globular head of HA, necessitating frequent updates of the influenza vaccine<sup>2,3</sup>. Selection of antigenic variants of influenza viruses in humans is believed to be driven by neutralizing antibodies<sup>4</sup>. Currently, seasonal influenza vaccine strains are selected based on the antigenicity of clinical isolates and cross-reactive immunity in human populations in combination with genetic and epidemiologic data<sup>5</sup>. This decision has to be made more than six months prior to the onset of the influenza season to allow sufficient time for vaccine manufacture. At the time of decision making, novel antigenic clusters may not have emerged or may not yet be recognized, occasionally resulting in the selection of vaccine strains that differ antigenically from the viruses circulating during the subsequent influenza season, leading to low vaccine effectiveness<sup>6,7</sup>. Presently, no experimental or computational methods exist to predict future antigenic variants before they emerge in nature. We, therefore, developed an experimental strategy to predict the antigenic evolution of human influenza A viruses.

In our approach (Fig. 1), mutant virus libraries with random mutations in the antigenic region of HA are screened *in vitro* with antisera against circulating viruses or in mice immunized against the circulating viruses. The antigenic properties of the escape mutants are analyzed by using haemagglutination inhibition (HI) assays. Antigenic cartography (an established method to visualize and analyze HI data<sup>8</sup>) is then used to identify novel antigenic

clusters that may necessitate the update of an influenza vaccine. This experimental approach could identify novel antigenic clusters before they emerge in nature and complement the current vaccine selection process.

## Antigenic evolution of A(H1N1)pdm09 viruses

First, we performed a proof-of-concept study to identify and characterize antigenic escape variants to pandemic (H1N1) 2009 influenza A (A(H1N1)pdm09) viruses. Eight convalescent sera collected from A(H1N1)pdm09-infected individuals in Japan (Supplementary Table 1) were mixed with two A(H1N1)pdm09 clinical isolates and escape viruses were isolated using plaque assays in Madin-Darby canine kidney (MDCK) cells. Escape mutants possessed the K153E/N239K, D127E, or K163E mutations (H1 numbering; Supplementary Table 2). HI assays with ferret antisera against the escape variants confirmed that they were antigenically distinct from their parental viruses (Supplementary Table 3).

To further explore the antigenic landscape, we generated a virus library with random mutations in the HA globular head (encompassing amino acids 54–253; Supplementary Fig. 1 and Supplementary Table 4). This region spans the four antigenic domains including antigenic site Sa (positions 124, 125, 153–157, and 159–164)<sup>9–12</sup> to which two of the selected mutations (K153E and K163E) map. For library generation, we chose the A(H1N1)pdm09 A/Norway/3858/2009 HA whose globular head sequence closely matches the consensus sequence of A(H1N1)pdm09 viruses. The HA mutant library was screened for escape mutants with the 14 human convalescent sera indicated in Supplementary Table 1. We sequenced the HA gene of more than 500 plaque-purified viruses and identified 92 HA mutant clones (Supplementary Table 5), including viruses with the mutations D127E and K163E detected in our preliminary experiments. Importantly, no mutations were found in the HA gene of wild-type virus after three consecutive passages in MDCK cells, indicating that the identified mutations were not selected during virus replication in these cells. HI assays confirmed that single amino acid changes at four positions in the Sa antigenic site (153, 154, 155, 156) caused 4–8-fold reductions in HI titres; Supplementary Table 5). In addition, 2–4-fold reduction in HI titres were caused by mutations at other positions in antigenic site Sa (125, 127, 129 and 163) as well as one position adjacent to site Sb (183). Collectively, these data demonstrate the importance of antigenic site Sa for A(H1N1)pdm09 antigenicity.

Previously, Koel *et al.* demonstrated that amino acid mutations at seven positions (142, 152, 153, 155, 156, 186, and 190; H1 numbering) of seasonal H3N2 influenza viruses altered antigenicity<sup>13</sup>. Although the primary amino acid sequences of H1 and H3 HAs differ by ~57%, their antigenic sites substantially overlap<sup>9,10,14</sup>. We, therefore, included these positions in our analysis of A(H1N1)pdm09 viruses. On the basis of sequence comparisons of seasonal H1N1 vaccine virus strains from 1977 to 2007, we also selected position 141 (identified in our preliminary escape mutant selection) and position 187 (located close to key antigenic positions 186 and 190) for further analysis.

Next, we generated A/Norway/3858/2009 HA libraries that represent all 20 amino acids at each of the 15 selected positions (i.e., HA positions 125, 127, 129, 141, 142, 152, 153, 154, 155, 156, 163, 183, 186, 187, and 190) (Supplementary Fig. 1 and Supplementary Table 6).

These HA mutant libraries were individually screened with ferret antisera as shown in Supplementary Table 7. Libraries for which we did not have escape mutant-specific antisera (HA positions 141, 142, 152, 186, 187, and 190) were screened with antiserum to wild-type virus; libraries for which we isolated antigenic escape mutants in our earlier experiments (namely, HA positions 125, 127, 129, 153, 154, 155, 156, 163, and 183) were screened with antisera to the N125D, D127E, N129D, K153E, K154N, G155E, N156D, K163E, or S183P escape mutants, respectively, to obtain additional mutants at the respective positions. For each screen, at least 11 plaque-purified viruses were characterized for their HA sequence and antigenicity (Supplementary Table 8). Mutations at positions 153–156 reduced HI titres up to 16-fold, suggesting that these HA positions play a major role in A(H1N1)pdm09 HA antigenicity. We did not identify antigenic escape mutants at positions 141, 163, or 187.

Our data suggested a key role for amino acid positions 153–156 in A(H1N1)pdm09 antigenicity; therefore, we generated HA mutant libraries with random mutations at two, or all four, of these positions (Supplementary Table 6). Random mutations at positions 153–156 were also combined with the D127E mutation in HA. This mutation is located near the Sa antigenic site, was selected in our screens (Supplementary Tables 2 and 5), and has been detected in several natural A(H1N1)pdm09 isolates. Virus libraries possessing mutations at two sites were screened with 50:50 mixtures of antisera against escape mutants at the respective positions (Supplementary Table 7); libraries possessing mutations at four or five sites were screened with a human serum (A163) obtained from an individual vaccinated with a monovalent, inactivated split-virus vaccine against A/California/07/2009 (A(H1N1)pdm09) (Supplementary Table 7). More than 320 escape mutants were isolated that exhibited reduced reactivity with antiserum against the wild-type virus (Supplementary Tables 9–11). Among those, certain amino acid changes were found at higher frequency (e.g., the N156G mutation). Sequence analysis of mutagenized plasmids used for virus library generation did not show sequence biases (Supplementary Tables 12 and 13); therefore, more frequently detected variants were most likely selected due to immune pressure and/or structural changes that conferred a fitness advantage.

We also carried out antigenic selection studies in which A/California/04/2009 virus-infected mice (Supplementary Table 14) were challenged with the mutant HA virus library possessing random amino acid changes at positions 153–156 together with the D127E mutation. We sequenced the HA gene of 53 escape viruses and detected several amino acid combinations that had also been selected in our *in vitro* antigenic selection experiments (Supplementary Table 15). These findings suggest that our *in vitro* selection experiments with human sera in part reflect *in vivo* immune evasion mechanisms.

To obtain a comprehensive picture of the antigenic properties of selected escape variants, 469 viruses (including 447 HA escape variants selected in this study) were characterized in HI assays with a panel of ferret antisera against H1N1 influenza viruses (Supplementary Table 16). The HI data were then analyzed using antigenic cartography<sup>8,13,15,16</sup>. Many of the escape mutants form a single cluster approximately four antigenic units (representing a 16-fold reduction in HI titre) away from wild-type A/Norway/3858/2009 virus (Fig. 2a). Thus, although our *in vivo* selection studies resulted in the isolation of 28 different combinations of amino acid changes (Supplementary Table 15), they all localized to the same novel

antigenic cluster. Mutations at positions 153–156 had the greatest impact on antigenicity (Fig. 3). Importantly, single amino acid changes are sufficient to produce antigenic variants with >16-fold difference in HI titre (Fig. 2b and Supplementary Fig. 2).

To exclude the possibility that the antisera did not adequately resolve the antigenicity of the escape mutants in the antigenic cluster we identified, we raised additional ferret antisera against 10 antigenic variants that lie in the new cluster or were outliers of the original cluster (Supplementary Fig. 3; Supplementary Table 17). HI assays with these antisera did not result in appreciable changes to overall relationships or placement of any of the viruses (Supplementary Fig. 3b). Thus, the identification of only one new antigenic cluster is a valid finding and not an artifact of poor resolution of antigenic differences by the antisera used in this study. Further analysis also revealed that the accumulation of mutations at positions tested here resulted in viruses that are increasingly distinct from the parental virus (Supplementary Fig. 4); however, the effect of individual mutations cannot be summed to predict the effect of combinations of mutations (Supplementary Fig. 5).

Since our study identified several potential A(H1N1)pdm09 escape variants, we analyzed the antigenicity of 2,555 recently circulating A(H1N1)pdm09 strains derived from routine WHO surveillance from 2009–2013. Isolates from each season were analyzed separately due to the lack of common antigens and antisera between the datasets (Fig. 2c, Supplementary Fig. 6a–e). Over 6% of the strains were antigenically different (i.e., more than 4-fold difference in HI titre) from the vaccine strain, A/California/07/2009. The antigenic variation seen among these viruses was representative of that seen in A(H1N1)pdm09 viruses currently circulating worldwide<sup>17</sup>. The antigenic differences found were identical to those identified with experimentally selected antigenic variants in this study in terms of distance and direction from the main antigenic cluster (typified by the vaccine strain) as well as the formation of a single antigenically distinct cluster. No mutations at any of the other sites identified by Koel *et al.* were observed<sup>13</sup>. All but two of the antigenic variants identified among circulating strains contained mutations shown in this study to cause antigenic change [K153E, K154E, G155E or N156D]. The exceptions were two isolates of the same virus: egg-passaged A/Iraq/8529/2009 (E3) and MDCK cell-passaged A/Iraq/8529/2009 (MDCK1). To directly compare the two datasets, we tested 20 field viruses against a subset of ferret antisera used in this study and generated a new antigenic map that merged the datasets from both experimentally generated mutants (Fig. 2b) and field strains (Fig. 2c) (Fig. 2d; a zoomable version is provided in Supplementary Fig. 6f). Isolates that were antigenically advanced, but had no sequence information, were subsequently sequenced. The antigenic properties of these viruses were consistent between the two datasets and demonstrated that a laboratory-based antigenic selection procedure can produce variants seen in surveillance samples.

Our experimental data demonstrate that the amino acids at positions 153–156 of HA are critical for antigenicity. This finding is consistent with several studies showing that a small proportion of A(H1N1)pdm09 clinical strains with amino acid changes in HA positions 153–157 showed antigenic drift compared with the A/California/07/2009-like reference viruses<sup>18–20</sup>. Moreover, mutations at positions 153–157 of HA have been detected after *in vitro* passage of A(H1N1)pdm09 viruses in the presence of monoclonal antibodies<sup>21,22</sup> or after A(H1N1)pdm09 passage in suboptimally vaccinated ferrets<sup>20</sup>. The HA positions 153–

156 are located at the edge of the receptor-binding pocket; hence, the amino acids at these positions may affect receptor-binding avidity and specificity<sup>20,23</sup>, in addition to antigenicity.

To determine whether the HA mutants selected in our study and the field strains from the new antigenic cluster could indeed evade immunity to current A(H1N1)pdm09 strains, mice were immunized by infection or vaccination with an A(H1N1)pdm09 virus (Supplementary Figs 7 and 8). Four weeks later, the mice were challenged with the viruses indicated in Supplementary Figs 9 and 10 (see also Supplementary Tables 18 and 19). No virus was detected on day 4 post-challenge in the lungs of immunized mice challenged with A/California/04/2009 or A/Norway/3858/2009 wild-type viruses (Supplementary Figs 9 and 10). By contrast, 12 of the 13 antigenic variants tested replicated in the lungs of immunized mice. Similarly, several viruses representing antigenic variants isolated in our study and found in field strains also replicated in mice immunized against current A(H1N1)pdm09 viruses (Supplementary Fig. 10).

Next, we tested whether the selected antigenic variants could evade immunity to the current A(H1N1)pdm09 viruses in ferrets, a commonly used animal model in the evaluation of influenza vaccines. Ferrets were immunized by infection with A/California/04/2009 virus. Pre-challenge serum HI titres were between 1:160 and 1:640 (Supplementary Fig. 11). Twelve months later, ferrets were challenged with either A/California/04/2009 control virus, three different mutant viruses that replicated in mice immunized with wild-type virus (Fig. 4), or with an A/Norway/3568/2009 reassortant virus possessing HA changes identified in a field strain (this mutant virus replicated in mice immunized with the wild-type A/California/04/2009 virus or with the A/California/07/2009 split vaccine) (Fig. 4). No virus was detected from the nasal washes collected from the immunized ferrets challenged with A/California/04/2009 virus. By contrast, the three antigenic mutants identified in our *in vivo* screens and the antigenic mutant with HA changes found in the field strain replicated efficiently in immunized ferrets. Thus, viruses possessing these mutations in HA may have the potential to cause epidemics in the future.

As influenza viruses evolve and amino acid substitutions become fixed within an antigenic cluster, these changes in genetic background may affect the future evolution of the viruses. If so, antigenic evolution studies should be updated repeatedly with contemporary viruses.

## Retrospective antigenic evolution of H3N2 viruses

To further test the potential of our experimental approach to identify future antigenic drift of influenza viruses, we carried out a retrospective study with human H3N2 viruses, whose antigenic evolution is well characterized<sup>8,13,24,25</sup>. The antigenic transition from the Sydney/1997 (SY97) cluster to the Fujian/2002 (FU02) cluster required two amino acid changes: HA-H155T and HA-Q156H<sup>13</sup>. To assess whether this cluster transition could be recapitulated experimentally, we generated a virus library with random mutations in the HA globular head of A/Hong Kong/CUHK5250/2002 (CUHK5250, H3N2), a representative of the SY97 cluster that encodes HA-155H-156Q (the virus library was generated with the NA gene of CUHK5250 virus and the internal genes of A/Puerto Rico/8/34, H1N1). Our sequence analysis of human H3N2 viruses also revealed several natural isolates (for



example, A/Kwangju/219/2002; Kwangju/219), that belong to the SY97 cluster, but encode the FU02-like residue HA-155T. This finding suggests that the SY97-to-FU02 transition may have occurred in two steps: from 155H-156Q to 155T-156Q to 155T-156H. Since evolutionary ‘advanced’ viruses (i.e., those with an ‘intermediate’ amino acid sequence) may be the founders of the next antigenic cluster, we also generated a virus library for Kwangju/219 (since the NA proteins of Kwangju/219 and CUHK5250 differ by only two amino acids, the CUHK5250 NA gene was used for Kwangju/219 library generation). *In vitro* screens for escape mutants were carried out individually with 9 human sera collected in Japan in 1999 (for the CUHK5250 library; Supplementary Table 20), or with ferret antiserum to CUHK5250 (for the Kwangju/219 library; Supplementary Table 21) to select escape mutants from the SY97 cluster; from these screens, we isolated 61 and 110 escape variants, respectively. The antigenic screen of the CUHK5250 mutant library did not result in the isolation of the cluster-transitioning H155T-Q156H mutations. By contrast, nine of the 110 variants isolated from the Kwangju/219 mutant library acquired the cluster-defining HA-Q156H mutation which, together with the HA-155T residue encoded by Kwangju/219, created the FU02 cluster motif of HA-H155T-Q156H.

Next, HI assays were performed for 20 reference strains and for 32 and 56 mutant viruses isolated from the CUHK5250 and Kwangju/219 screens, respectively (we focused on viruses with mutations in amino acid positions known to affect antigenicity; Supplementary Tables 20 and 21). All Kwangju/219 mutants that inherently encoded HA-155T and acquired the HA-Q156H mutation localized to the edge of the FU02 cluster (Fig. 5, red triangles). Most CUHK5250 mutants did not shift to the FU02 cluster, as expected by the absence of the HA-H155T-Q156H mutations. However, one CUHK5250 mutant possessing HA-Q156H-H183L mutations mapped to the outer edge of the FU02 cluster (Fig. 5, red rhombus). This variant is located in the antigenic map close to the cluster of Kwangju/219 mutants encoding HA-155T-156H (Fig. 5, red triangles). Hence, our *in vitro* antigenic selection identified the same antigenic drift mutants that occurred in nature (i.e., HA-H155T-Q156H) and were critical for the transition from the SY97 cluster to the FU02 cluster.

## Antigenic evolution of contemporary H3N2 viruses

In February 2014, the WHO recommended an A/Texas/50/2012(H3N2)-like virus (TX/50) as a vaccine strain for the 2014–2015 influenza season in the Northern hemisphere. From March to September 2014, an increasing percentage of antigenically drifted H3N2 variants belonging to clades 3C.3a or 3C.2a was reported<sup>26</sup>. The antigenic difference between TX/50 and the novel clade 3C.3a and 3C.2a viruses was most likely correlated with HA-F159S and HA-F159Y mutations, respectively<sup>27</sup>. The novel antigenic clusters were dominant in most parts of the world by late 2014 and were responsible for the low vaccine effectiveness of the H3N2 component of the 2014–2015 influenza vaccine<sup>28</sup>. In February 2015, the WHO therefore recommended replacing TX/50 with a clade 3C.3a vaccine virus<sup>28</sup>. During the first half of 2015, viruses of clade 3C.2a became dominant over those of clade 3C.3a<sup>29</sup>. In September 2015, the WHO therefore recommended A/Hong Kong/4801/2014 (HK/4801; clade 3C.2a, encoding HA-159Y) as a novel vaccine virus<sup>29</sup>.

To identify antigenically advanced variants of contemporary human H3N2 viruses, we generated a virus library possessing random amino acid changes at HA positions 63–252 (H3 numbering) of TX/50, the WHO-recommended vaccine virus at that time. The library was screened with 10 human antisera collected in December 2013–January 2014, and with three mixtures of these antisera based on the age of the donor (Supplementary Table 22). We identified 139 different potential escape mutants (Supplementary Tables 23 and 24); the most frequently mutated amino acid position was HA-159, consistent with the role of this amino acid position in recent cluster transitions. For antigenic analysis, we chose a subset of 24 antigenic mutants that encoded amino acid changes frequently detected in these screens and/or amino acid changes known to affect antigenicity (indicated by HI titres in Supplementary Tables 23 and 24). HI assays were conducted in the presence of 20 nM oseltamivir with 19 reference antisera, and with 19 reference strains. The resulting HI data were merged with data from the Centers for Disease Control and Prevention (CDC) (Fig. 6). The TX/50 antigenic cluster (encoding HA-159F; blue) and the minor 3C.3b cluster (also encoding HA-159F, yellow) are antigenically closely related, whereas the 3C.3a cluster (characterized by HA-159S; green) and the now dominant 3C.2a cluster (characterized by HA-159Y; red) are antigenically distinguishable from TX/50. Among the experimentally derived escape variants (shown in numbered mid-sized circles), the majority of antigenically ‘advanced’ mutants [i.e., those that shifted from the vaccine virus by more than two grid units (i.e., four HI units)] moved towards the currently dominant 3C.2a cluster, represented by the A/Hong Kong/4801/2014 vaccine virus (#31); some of these possessed the HA-159Y mutation. However, not all of the antigenic escape variants that shifted towards the current cluster encode a mutation at HA-159. In fact, escape mutant #27 (closest to the vaccine strain, #31) encodes mutations HA-K189E and -N225D, which are known to affect antigenic and receptor-binding specificity, respectively<sup>13,30,31</sup>. The HA-N225D mutation is present in most viruses belonging to clade 3C.2a; however, the HA-K189E mutation has not been detected in human H3N2 influenza viruses collected from January 1, 2014 to February 20, 2016, based on sequence searches in GISAID (<http://platform.gisaid.org>) and the Influenza Research Database ([www.fludb.org](http://www.fludb.org)). Collectively, these findings indicate that although our approach may not always identify the exact sequence of the next epidemic strains, it can predict the antigenic properties of future epidemic strains.

## Discussion

With the current vaccine selection process (based on comparisons of sequence and antigenic data available at the time of the WHO consultations on vaccine strain selection), uncertainty remains as to whether the recommended vaccine virus will provide protective immunity against the next epidemic strain. Here, we provide a methodology that identified H1N1 and H3N2 antigenic clusters similar to those observed in nature. These data can be generated before the variants are detected in nature; in this regard, our approach is conceptually different from methods that predict which circulating variants may become dominant<sup>32–35</sup>. Our method may therefore improve the current WHO influenza vaccine selection process. These *in vitro* selection studies are highly predictive of the antigenic evolution of H1N1 and H3N2 viruses in human populations. Hence, a limited number of experimental antigenic screens may be sufficient to identify potential future clusters. We found that genetic



background can be important for antigenic evolution, as with the SY97-FU02 antigenic cluster transition. To put methods like ours into practice, it will likely be necessary to keep the predictions up to date by reapplying the methodology as new clades emerge. We cannot predict the HA sequence of the next epidemic strain, and (compensatory) mutations in NA may also affect antigenicity; however, our methodology may be sufficient to predict the next antigenic cluster for proactive vaccine development.

## Methods

### Viruses and cells

293T human embryonic kidney cells were maintained in DMEM supplemented with 10% fetal bovine serum. MDCK cells were grown in MEM containing 5% newborn calf serum. After infection with influenza viruses, MDCK cells were maintained in MEM containing 0.3% BSA and 0.5 µg/ml TPCK-trypsin. A/Norway/3206-3/2009 (H1N1), A/Norway/3858/2009 (H1N1), and A/Yokohama/UT-K1205T/2009 (H1N1) were amplified in MDCK cells to generate stock viruses. A/Norway/3568/2009 (H1N1) and A/California/04/2009 (H1N1) were generated using reverse genetics techniques as described<sup>36</sup>. To generate A/Hong Kong/CUHK5250/2002 (CUHK5250, H3N2), and A/Texas/50/2012 (TX/50, H3N2; cell culture-grown; see #1 in Fig. 6) virus libraries, the HA and NA genes, or just the HA gene, of these viruses were combined with the remaining six or seven genes of A/Puerto Rico/8/34 (H1N1) virus. To generate A/Kwangju/219/2002 (Kwangju/219, H3N2) virus libraries, we combined the Kwangju/219 HA gene, the CUHK5250 NA gene (note that the NA proteins of Kwangju/219 and CUHK5250 differ by only two amino acids), and the remaining genes of A/Puerto Rico/8/34 virus. The cell lines used here were maintained in our laboratory and were negative for mycoplasma contamination; they are not listed in the database of commonly misidentified cell lines.

### Generation of virus libraries with random mutations in the HA globular head region

The cDNAs for the HA gene of A/Norway/3858/2009, CUHK5250, Kwangju/219, and TX/50 viruses were randomly mutagenized using error-prone PCR with the GeneMorph II kit (Stratagene, La Jolla, California, USA) (note that the sequence of our CUHK5250 HA gene differed by two silent nucleotide changes from the sequence reported in the database). The randomized coding regions for amino acid positions 54–253 (for the H1 viruses; H1 numbering) or 63–252 (for the H3 viruses) of HA were replaced with the corresponding region of the respective parental HA cDNA plasmid using a PCR-based cloning technique. Unbiased library composition and the targeted mutation rate (1–2 amino acid substitutions per molecule) were confirmed by sequence analysis. The sizes of our *E. coli* libraries were  $9 \times 10^6$ ,  $1.3 \times 10^7$ ,  $1.4 \times 10^7$ , and  $1.5 \times 10^6$  colony-forming units for A/Norway/3858/2009, CUHK5250, Kwangju/219, and TX/50, respectively. The randomized A/Norway/3858/2009 HA plasmid library was then used to generate a virus library in the genetic background of the closely related A/Norway/3568/2009 virus. The CUHK5250 and Kwangju/219 HA plasmid libraries were used to generate virus libraries in the genetic background of the CUHK5250 NA gene and the remaining A/Puerto Rico/8/34 virus genes. The TX/50 HA plasmid library was used to generate a virus library in the genetic background of the 7 remaining A/Puerto Rico/8/34 virus genes.

### **Generation of A(H1N1)pdm09 virus libraries representing all amino acids at a defined position**

For random mutagenesis at a defined amino acid position, the HA plasmid was amplified with degenerate PCR primers representing mixtures of all four nucleotides at the three positions of the respective codon. The PCR products were self-ligated and transformed into *E. coli*, resulting in  $10^4$ – $10^6$  colony-forming units per library. Virus libraries were generated as described above. The titres of the virus libraries were  $3.4 \times 10^4$ – $7.0 \times 10^7$  PFU/ml (Supplementary Table 6).

### **Generation of reassortant viruses possessing HA and NA genes derived from A(H1N1)pdm09 field strains**

Plasmids for the expression of the HA and NA genes of field strains were generated by site-directed PCR mutagenesis using cDNAs for the HA and NA genes of A/Norway/3858/2009, A/Norway/3568/2009, or A/California/04/2009 as templates (for A/Qingdao/1610/2009, whose NA gene sequence is not publicly available, the NA gene of A/Norway/3568/2009 was used; for A/Miyazaki/40/2011 and A/Okinawa/41/2010, whose HA2 sequences are not publicly available, chimeric HA genes with the HA2 sequence of A/Norway/3568/2009 were constructed and used). The resulting plasmids were combined with plasmids encoding the remaining six genes of A/Norway/3568/2009 virus; viruses were generated as described above.

### ***In vitro* selection of A(H1N1)pdm09 antigenic escape mutants from clinical isolates**

Clinical virus isolates (A/Norway/3206-3/2009 or A/Yokohama/UT-K1205T/2009) were mixed with 2-fold serial dilutions of human convalescent sera and incubated at 4°C overnight. After virus neutralization, virus-serum mixtures were inoculated onto MDCK cells and incubated at 37°C for 1 h. After removal of the inoculum, cells were cultured for 2–4 days. Then, culture supernatants were collected from cells with the highest concentration of antiserum at which a cytopathic effect was observed. Viruses in the supernatants were plaque-purified in MDCK cells and the HA genes of the purified viruses were sequenced.

### ***In vitro* selection of antigenic escape mutants from A(H1N1)pdm09 or H3N2 virus libraries**

Virus libraries were mixed with undiluted or 2-fold serial dilutions of the respective ferret or human sera, with the exception of the H3N2 TX/50 virus library, which was mixed with individual or mixed human sera, and incubated at 4°C overnight. The virus-serum mixtures were diluted and subjected to plaque assays in MDCK cells grown in 6-well plates. Antigenic escape variants were isolated from wells that showed inhibition of virus infection (<10 plaques per well), and the HA genes of the isolated viruses were sequenced.

### **Animal experiments**

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-Madison. The facilities where this research was conducted are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The animal experiments described in this

study were not designed to generate data sets for statistical analysis; hence, the sample size was small and randomization and blinding were not carried out.

### ***In vivo* selection of A(H1N1)pdm09 antigenic escape mutants from virus libraries**

Six-week-old female BALB/c (Jackson Laboratory, Bar Harbor, Maine, USA) and C57BL6/J (Jackson Laboratory) mice were used in this study. Three mice per group were immunized by intranasal infection with 10 PFU (BALB/c mice) or  $10^{1.5}$  PFU (C57BL6/J mice) (50  $\mu$ l) of A/California/04/09 virus. Four weeks later, we determined the HI titres of mouse sera against A/California/04/09 virus. Then, animals were challenged by intranasal infection with  $10^6$  PFU (50  $\mu$ l) of either A/California/04/09 virus (control) or the virus library possessing the D127E mutation in HA and random mutations at amino acid position 153–156 of HA. On day 4 post-challenge, virus titres in mouse lungs were determined using plaque assays in MDCK cells. Viruses recovered from the lungs of successfully immunized (HI antibody titres  $\geq 40$ ) and virus library-challenged mice were plaque purified prior to HA sequence analysis.

### **Challenge experiments in mice**

Six-week-old female C57BL6/J mice were immunized by intranasal infection with  $10^{1.5}$  PFU (50  $\mu$ l) of A/California/04/2009 virus or by two vaccinations (with a two-week interval) of a commercial A/California/07/2009 HA split vaccine (CSL, Parkville, Australia, 1.5  $\mu$ g in 50  $\mu$ l). Four weeks later, serum HI titres were determined. Three-to-four mice per group with HI antibody titres  $\geq 40$  (for mice immunized by virus infection) or  $\geq 160$  (for mice immunized by vaccination) were challenged by intranasal infection with  $10^6$  PFU (50  $\mu$ l) of the oseltamivir-sensitive A/California/04/2009 or A/Norway/3858/2009 control viruses, representative antigenic escape mutants, or A/Norway/3568/2009-based reassortant viruses possessing changes in HA detected in field strains. On day 4 post-challenge, virus titres in mouse lungs were determined using plaque assays in MDCK cells

### **Challenge experiments in ferrets**

Six- to ten-month-old female ferrets (Triple F Farms, Sayre, Pennsylvania, USA) that were serologically negative by haemagglutination inhibition (HI) assay for currently circulating human influenza viruses were infected by intranasal inoculation with 500 PFU (500  $\mu$ l) of A/California/04/2009 virus. Twelve months later, serum HI titres were determined. Three or four ferrets per group with HI antibody titres between 160 and 640 were challenged by intranasal infection with  $10^7$  PFU (500  $\mu$ l) of A/California/04/2009, three antigenic escape mutants selected in the *in vivo* screens, or an A/Norway/3568/2009 reassortant virus with HA changes identified in a field strain (Fig. 4 and Supplementary Fig. 11). The nasal washes of the challenged ferrets were collected daily from day 1 to day 8 post-challenge. Virus titres in the nasal washes were determined using plaque assay in MDCK cells.

### **Generation of ferret antisera**

We used 6–10-month-old female ferrets (Triple F Farms, Sayre, Pennsylvania, USA) that were serologically negative by haemagglutination inhibition (HI) assay for currently circulating human influenza viruses. One or two ferrets per group were anaesthetized

intramuscularly with ketamine and xylazine (5–30 mg and 0.2–6 mg/kg of body weight, respectively) and inoculated intranasally with  $10^7$  PFU (500  $\mu$ l) of wild-type or mutant A(H1N1)pdm09, seasonal H1N1, swine H1N1, or human H3N2 virus. Three-to-four weeks later, ferrets were euthanized and sera were collected for subsequent use in HI assays or the selection of escape mutants.

### Antiserum treatment

Human or ferret serum (100  $\mu$ l) was treated with 300  $\mu$ l of receptor-destroying enzyme (RDE; Denka Seiken Co., Ltd., Tokyo, Japan) at 37°C for 18–24 h. To denature the RDE solution, the serum-RDE mixture was incubated at 56°C for 30–60 min. After denaturation, 600  $\mu$ l of sterile PBS and 100  $\mu$ l of pelleted turkey red blood cells (TRBCs) were added and the mixture was incubated at room temperature for 60 min; during that time, the suspension was mixed every 10 min. The mixture was then centrifuged at 3000 rpm at 4°C for 5 min. Supernatants were aliquoted and stored at –20°C for use in HI assays. To confirm that all non-specific binding had been removed, 25  $\mu$ l of PBS and 25  $\mu$ l of treated serum were mixed with 50  $\mu$ l of 0.5% TRBCs and incubated at room temperature for 45 min. If no hemagglutination was detected, the serum could be used for HI assays; otherwise, the antiserum treatment was repeated.

### HI assays

HI titrations were performed in 96-well U bottom microtiter plates (Thermo Scientific, Rochester, New York, USA). Sera were serially diluted 2-fold with PBS and then mixed with the amount of virus equivalent to 8 haemagglutination units, followed by incubation at room temperature for 30 min. Finally, 50  $\mu$ l of 0.5% TRBCs were added. To screen TX/50 variants, sera were serially diluted 2-fold with PBS containing a final concentration of 20 nM oseltamivir, mixed with the amount of virus equivalent to 4 haemagglutination units, and incubated at room temperature for 30 min; finally, 50  $\mu$ l of 0.75% guinea pig red blood cells with 20 nM oseltamivir was added. Serum, virus, and erythrocytes were gently mixed and incubated at room temperature for 45 min. Titres were recorded as the inverse of the highest antibody dilution that inhibited 8 or 4 agglutinating units of virus, respectively.

### Antigenic cartography of escape mutants

Antigenic maps were constructed using the antigenic cartography method<sup>8</sup>. The HI titres were mathematically transformed to create a table of antigenic distances, using the equation  $D_{ij}=b-\log_2(H_{ij})$ , where  $H_{ij}$  is the titre of antigen  $i$  against serum  $j$ , and  $b$  is the logarithm of the maximum titre against serum  $j$ . The error function  $(D_{ij}-d_{ij})^2$  was minimized where  $d_{ij}$  is the Euclidean distance between two points on the map.

### Sequence analysis

Viral RNA was extracted from MDCK cell supernatant or purified plaques using the QIAmp viral RNA mini kit (Qiagen, Valencia, California, USA) or the MagMAX™-96 Viral RNA Isolation Kit (Ambion, Austin, Texas, USA). cDNAs were synthesized from vRNAs by reverse transcription with Uni12 primer<sup>37</sup>, and amplified by PCR with gene-specific primers.

Sequencing was performed on an Applied Biosystems DNA analyzer at the Biotechnology Center of the University of Wisconsin-Madison.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NOTE: The methods used in this manuscript could potentially select for variants of A(H1N1)pdm09 viruses that could escape vaccine-based immunity; therefore, our manuscript was reviewed by the CDC's Institutional Biosecurity Board which concluded this study does not constitute Dual Use Research of Concern (DURC). The selection of antigenic escape variants was completed before the US Government issued a Research Funding Pause on October 17, 2014, on selected gain-of-function research on influenza, MERS, and SARS viruses.

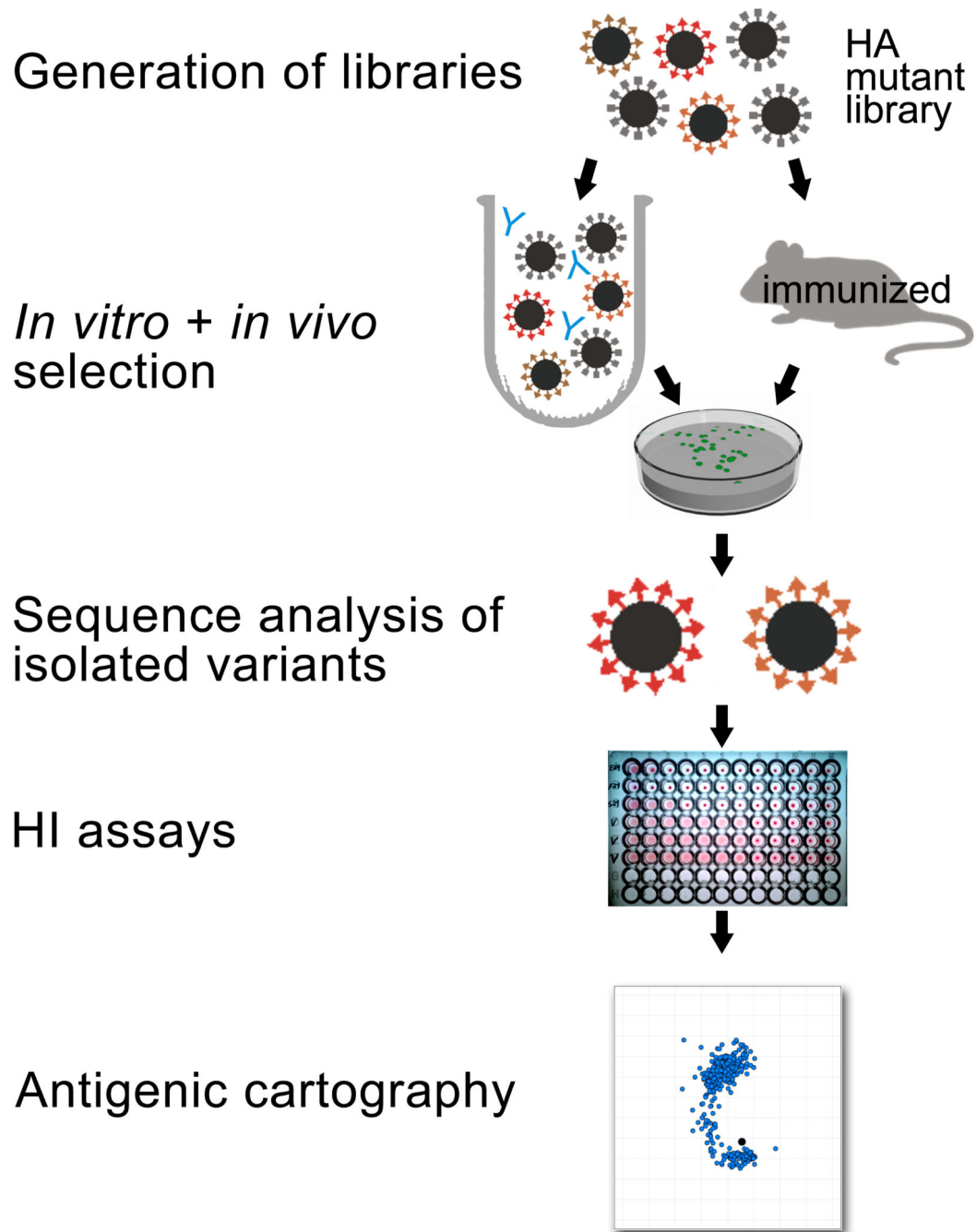
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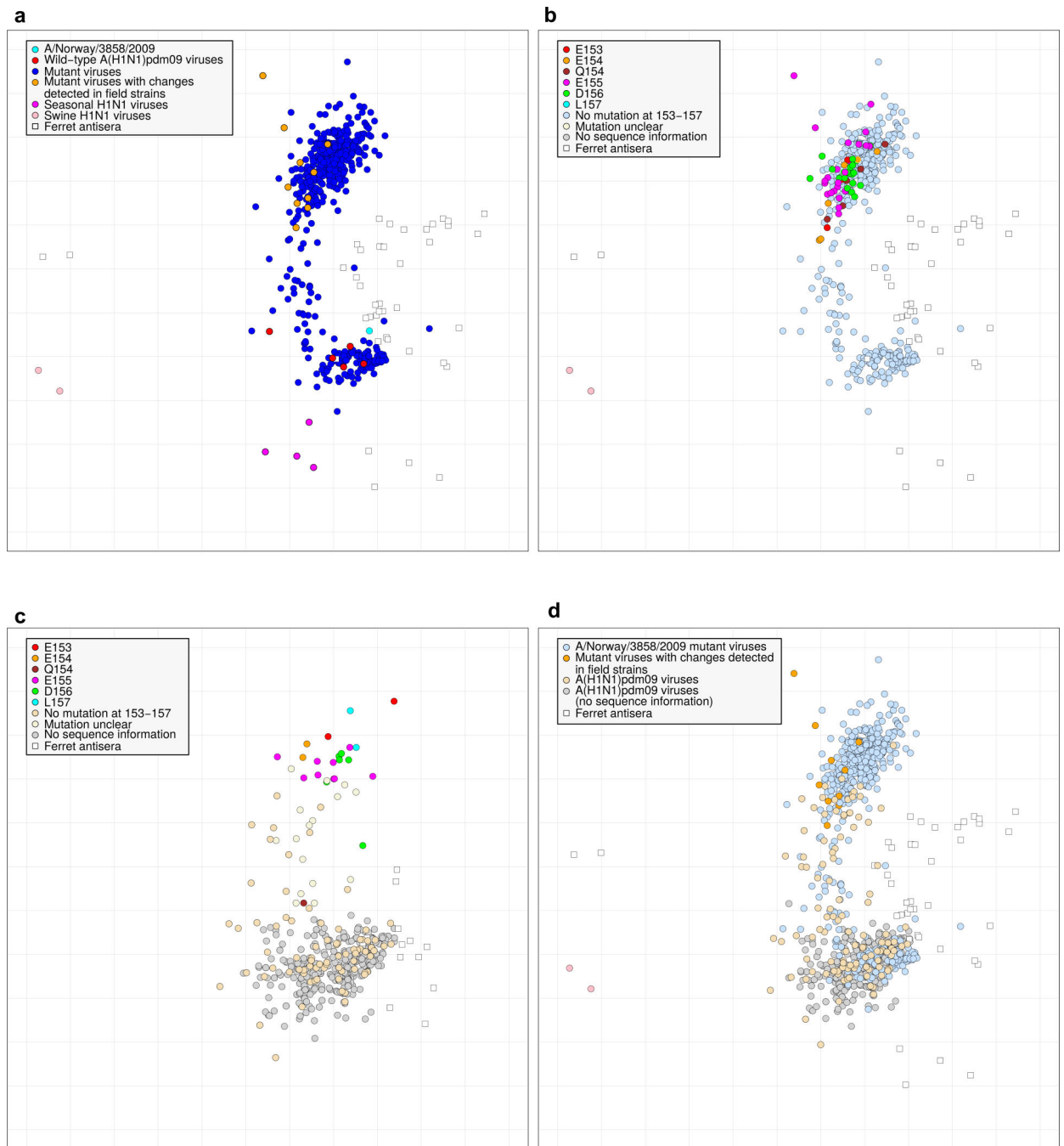
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**Figure 1. Schematic overview of our experimental approach to predict the antigenic evolution of human influenza A viruses**

Virus libraries possessing random or targeted mutations in the antigenic region of HA are screened *in vitro* with antisera against human 2009 H1N1 viruses or *in vivo* in mice immunized with a human 2009 H1N1 virus. Mutant viruses are analyzed by using haemagglutination inhibition (HI) assays, and antigenic cartography is then used to identify viruses that differ antigenically from the parent cluster.

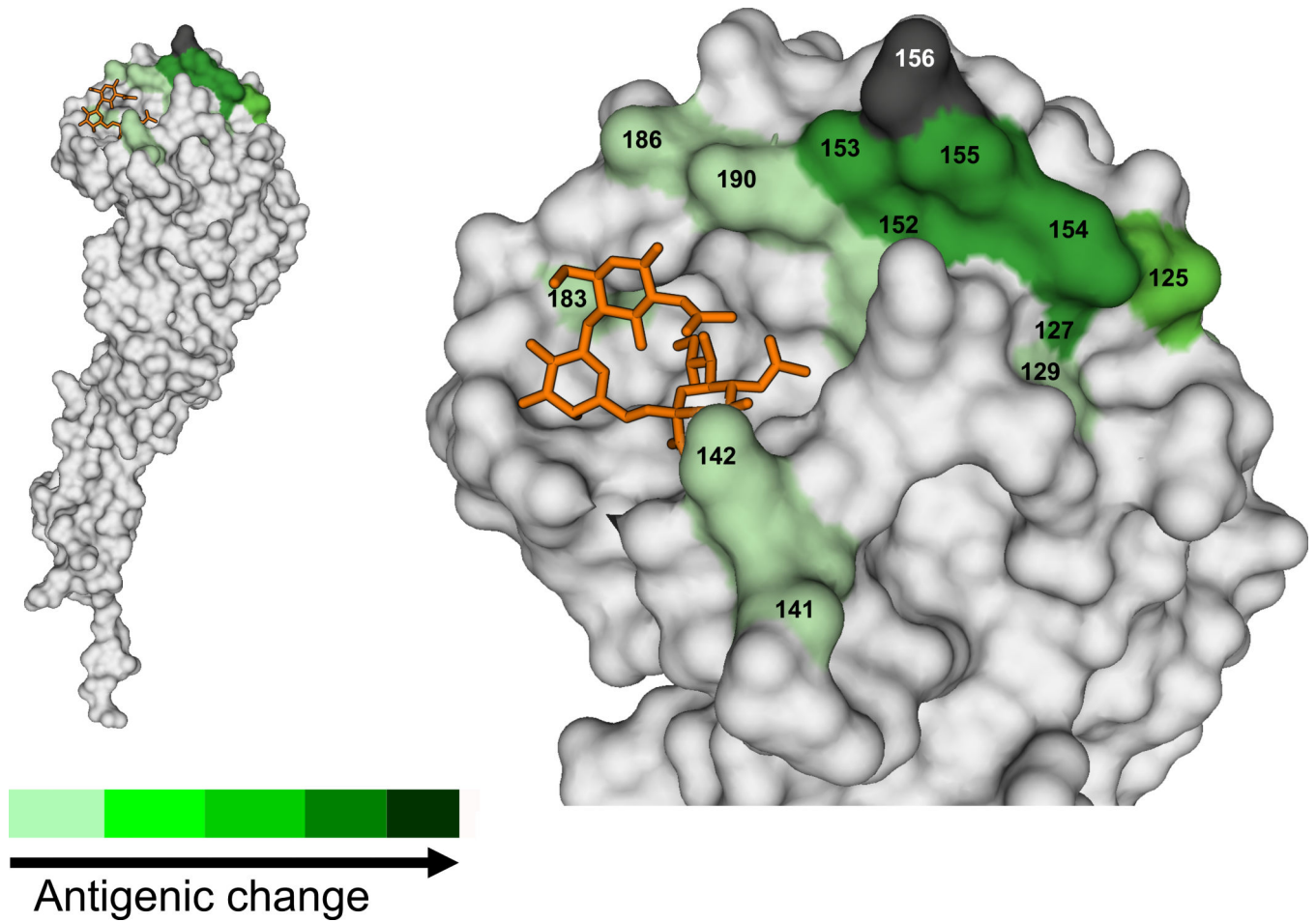


### Figure 2. HA antigenic cartography

An antigenic map, a geometrical representation of the HI assay data. If two viruses have similar HI titres against a panel of sera, then they are close to each other on the map. Conversely, if two viruses differ in their HI titres against a panel of sera, then they will be far apart on the map. Each grid square corresponds to a 2-fold change in HI titre (the x and y axes represent antigenic distances). (a) Map for the 447 mutant viruses (blue circles) and 42 ferret antisera (squares) together with A/Norway/3858/2009 (cyan circle), 5 wild-type A(H1N1)pdm09 viruses (red circles), 10 A(H1N1)pdm09-based reassortant viruses that

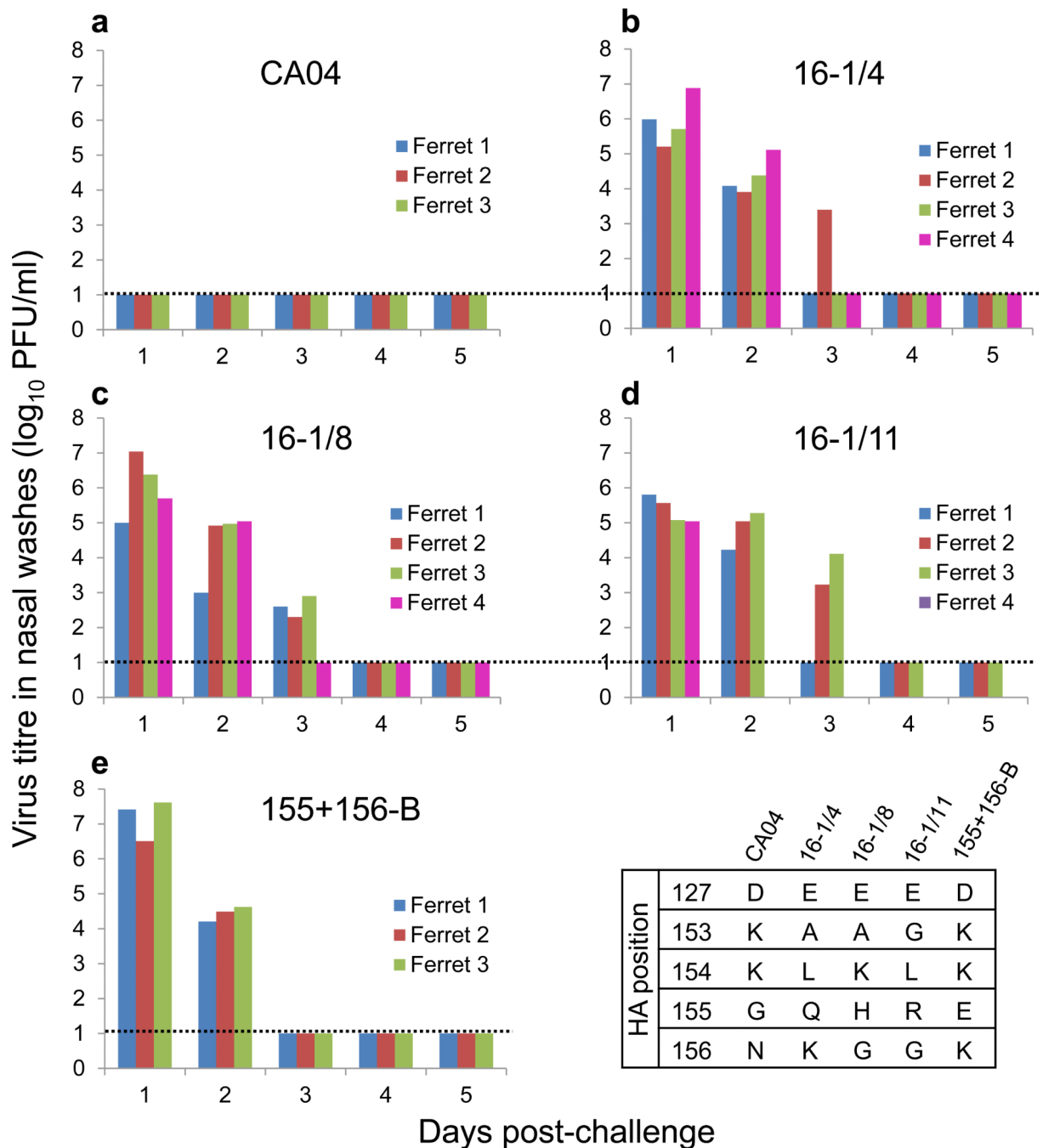
possess changes in HA at positions 153–156 detected in field strains (orange circles), 4 seasonal H1N1 viruses (magenta circles), and two H1N1 swine viruses (pink circles). **(b)** Map focused on A(H1N1)pdm09 viruses (light blue circles) and ferret antisera (squares) from this study. Viruses with mutations at positions 153–157 in HA are colored by amino acid type at that position (note: viruses may have more than one mutation; this can result in a change in position [see Supplementary Figs 2 and 5]). **(c)** Antigenic map of 862 A(H1N1)pdm09 circulating strains from WHO surveillance in 2009 and 2010 (See key in **b**). For some circulating viruses, the identity of one or more amino acids at positions 153–157 was unclear in sequence data (pale yellow circles). Circulating viruses for which the HA sequence has not been determined are shown as light grey circles. Fig. 2c is available as a labeled, zoomable pdf in the Supplementary figures – see Supplementary Fig. 6a. **(d)** Antigenic map of the combined HI data from Fig. 2b,c. This map includes A(H1N1)pdm09 viruses from the mutant screens in this study (light blue circles); A(H1N1)pdm09-based reassortant viruses that possess changes in HA at positions 153–156 detected in field strains (orange circles); and circulating viruses from WHO surveillance studies with HA sequence data (pale orange circles) or without HA sequence data (light grey circles). Circulating A(H1N1)pdm09 viruses tested with a subset of sera from this study are shown as red circles. Fig. 2d is available as a labeled, zoomable pdf in the Supplementary figures – see Supplementary Fig. 6f.





**Figure 3. Structural basis of antigenic changes**

Structure of the HA of A/California/04/2009; PDB 3UBN<sup>38</sup>. Amino acid positions at which single mutations result in appreciable antigenic change are shown as increasingly darker shades of green. The human-type receptor analog, 6'-SLN (6'-sialyl-N-acetylactosamine), is shown in orange.



**Figure 4. Immune evasion in ferrets by HA mutations identified in this study or found in a field strain**

Ferrets were infected intranasally by inoculation with 500 PFU of A/California/04/2009 virus. Twelve months later, serum HI titres against A/California/04/2009 were determined (Supplementary Fig. 11) and ferrets were challenged with  $10^7$  PFU of A/California/04/2009 (a), with representative antigenic escape mutants selected in the *in vivo* screens (b, c, d), or with a A/Norway/3568/2009-based reassortant virus possessing changes in HA at positions 155 and 156 detected in a field strain (e) (see Supplementary Tables 18 and 19). Shown are nasal wash virus titers collected from day 1 to day 5 post-challenge (all nasal wash samples

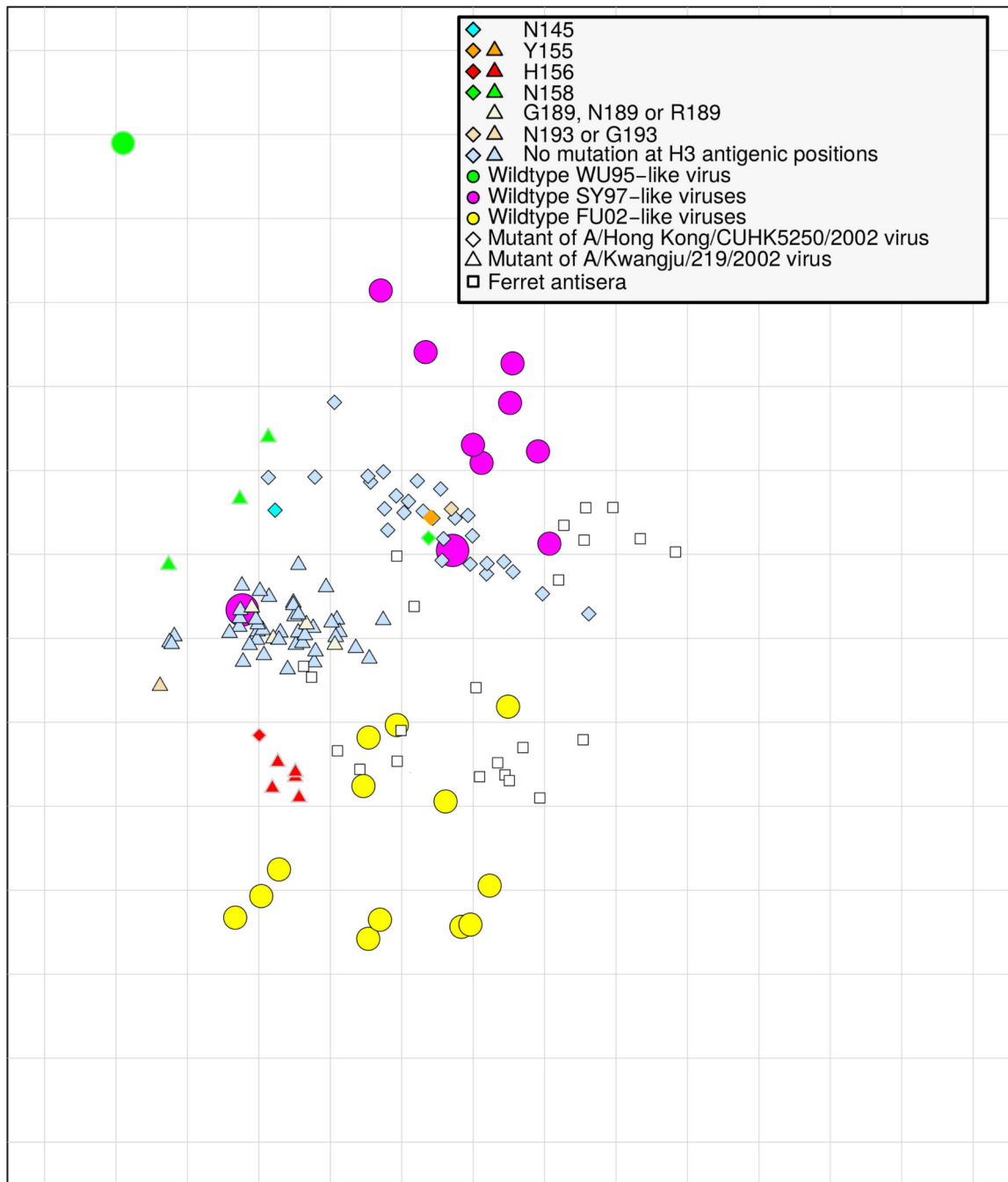
collected on days 6–8 post-challenge were negative for virus). Dashed lines, virus detection limit ( $1 \log_{10}$  PFU/ml).

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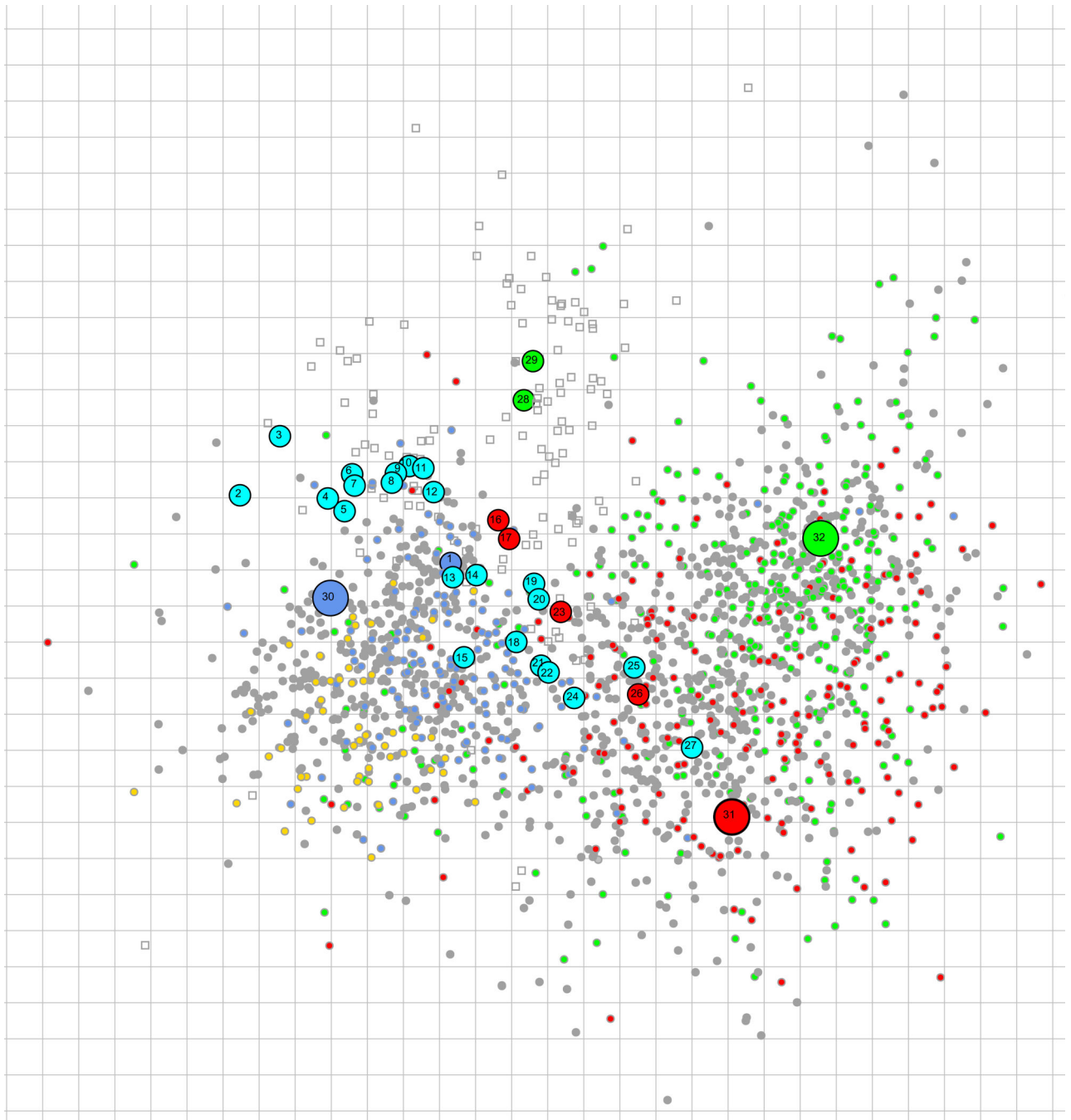
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### Figure 5. Antigenic map of CUHK5250 and Kwangju/219 H3N2 escape mutants

The antigenic map was generated as described in the legend to Fig. 2. Wild-type strains belonging to the Wuhan 1995, Sydney 1997, or Fujian 2002 clusters are shown in green, magenta, or yellow, respectively. Escape mutants selected from the CUHK5250 and Kwangju/219 libraries are shown as diamonds and triangles, respectively, and colored by the presence of a mutation at position 145 (cyan), 155 (orange), 156 (red), 158 (green), 189 (cream), or 193 (wheat), which were recently found to be antigenically important for human H3N2 virus evolution<sup>13</sup>.



**Figure 6. Antigenic map of TX/50 H3N2 escape mutants**

The antigenic map was generated as described in the legend to Fig. 2. Surveillance samples are shown in small circles; experimentally derived antigenic variants are shown in mid-sized circles; vaccine viruses are shown in large circles. Surveillance samples characterized by HI assays, but not sequenced, are indicated in gray. Shown in yellow are viruses belonging to the minor cluster 3C.3b. The TX/50 cluster is indicated in blue. Antigenic escape mutations encoding HA-159F (as found for TX/50) are shown in cyan. The 3C.3a and 3C.2a clusters (characterized by HA-159S and HA-159Y, respectively) are indicated in green and red,



respectively. Viruses are numbered as follows: #1, TX/50 MK1/MDCK2 (used for virus library generation); #2, TX/50 HA-E172G+F193S; #3, TX/50 HA-F193S+T203A; #4, TX/50 HA-S219F; #5, TX/50 HA-I217T+N225K; #6, TX/50 HA-Q75L+N144I+I192T; #7, TX/50 HA-F174Y; #8, TX/50 HA-Y94F+N225D; #9, TX/50 HA-R220G+N225D; #10, TX/50 HA-N158D; #11, TX/50 HA-Q197R; #12, TX/50 HA-K207N+N225S; #13, TX/50 HA-W127L+N225D; #14, TX/50 HA-Y94H+S107T+N225D; #15, TX/50 HA-V88I+K189N; #16, TX/50 HA-F159Y+K160E+I192V+I242T; #17, TX/50 HA-F159Y; #18, TX/50 HA-L157F+N225D; #19, TX/50 HA-N144I+N158D+F193Y; #20, TX/50 HA-N225D; #21, TX/50 HA-K189E; #22, TX/50 HA-K189E+I242M; #23, TX/50 HA-N122S+F159Y; #24, TX/50 HA-E172D+K189E; #25, TX/50 HA-K189E+F193S; #26, TX/50 HA-N128D+F159Y+N225D; #27, TX/50 HA-K189E+N225D; #28, TX/50 HA-H156R+F159S+N246H/WT-NA (propagation and re-generation of viruses possessing HA-F159S+N225D failed; when we attempted to generate a PR8 virus with the NA gene of TX/50 and the TX/50 HA-F159S gene, the virus also acquired HA-H156R and N246H mutations); #29, TX/50 HA-F159S+N246H (propagation and re-generation of viruses possessing HA-F159S+N225D failed; when we attempted to generate a PR8 virus with the TX/50 HA-F159S gene, the virus also acquired a N246H mutation); #30, A/Texas/50/2012 (E5; vaccine strain); #31, A/Hong Kong/4801/2014 (NYMC-263A; vaccine strain); and #32, A/Switzerland/9715293/2013-NIB-88 (E4/E5/E2; vaccine strain).