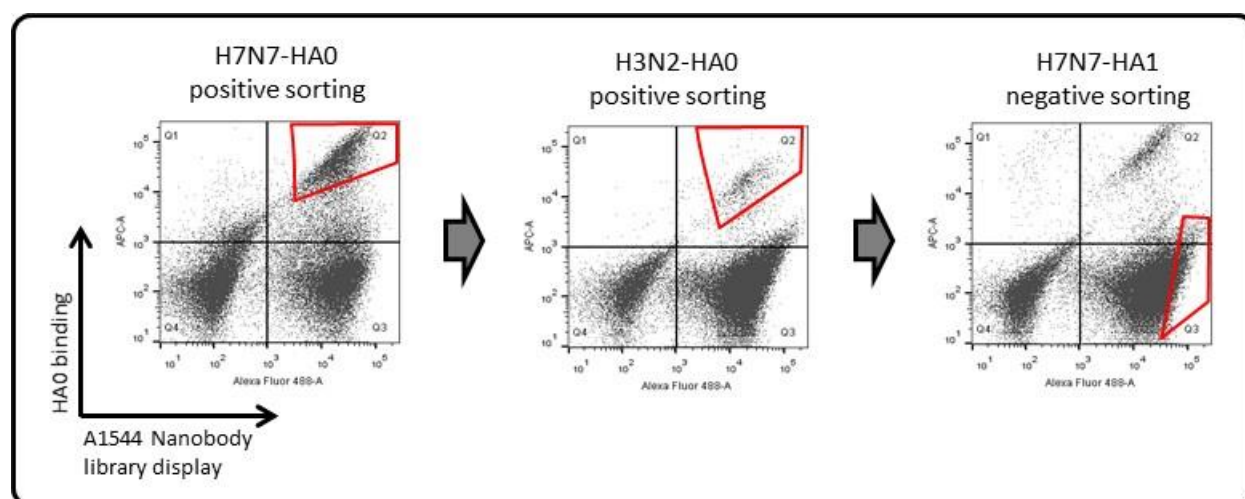# Supplementary Information

# Nanobodies mapped to cross-reactive and divergent epitopes on A(H7N9) influenza hemagglutinin using yeast display.

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**Fig S1. Guided yeast library cell sorting for stem specific binding nanobodies.**
FACS plots (FlowJo 10.4 software) showing the initial positive sorting of yeast Nb library using recombinant H7N7 HA0 (A/chicken/Netherlands/1/2003), followed by a second round of positive sorting using recombinant H3N2 HA0 (A/Brisbane/10/2007) and by a third round of negative sorting using recombinant H7N7 HA1 (A/chicken/Netherlands/1/2003). For each round the gated population for cell sorting is shown in red.
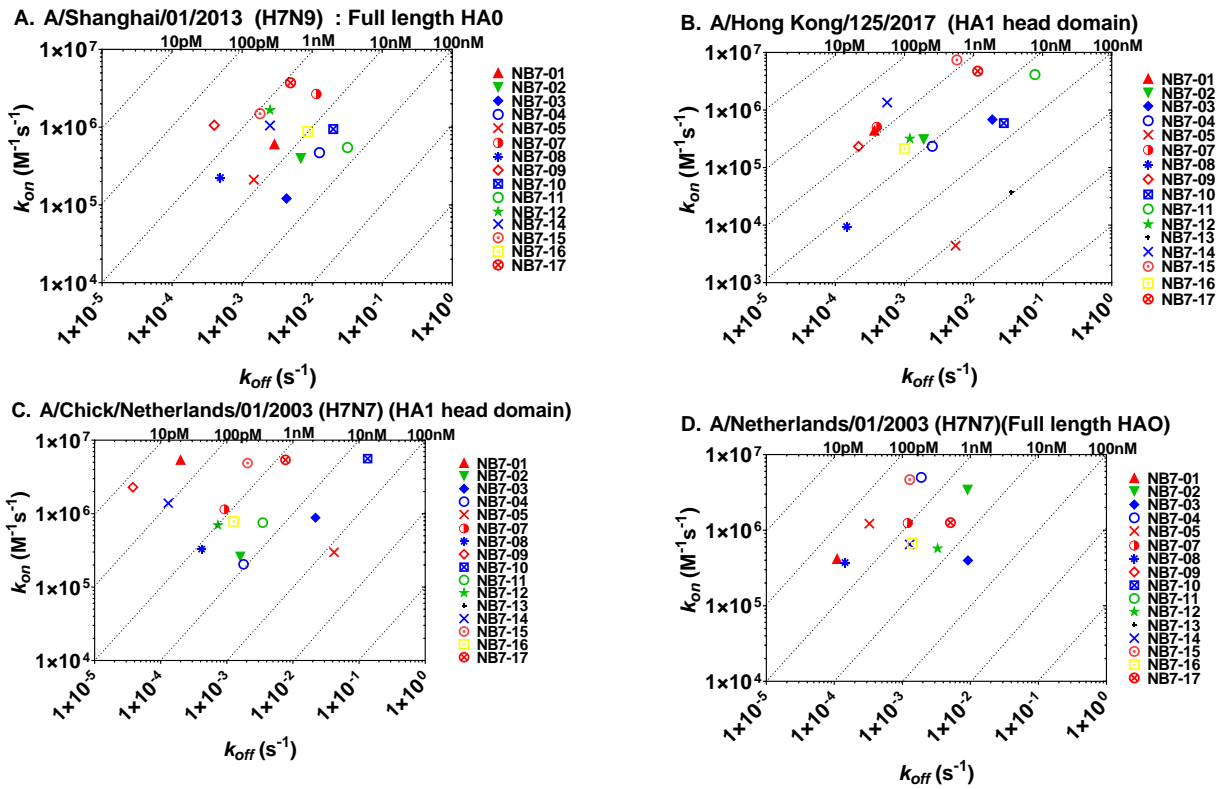
**Fig S2. Binding constants ($k_{on}$ and $k_{off}$) on recombinant HA0 and HA1**. Single cycle kinetic data are presented as rate plots with iso-affinity diagonals (RAPID) where the diagonals (dotted lines) are connecting the points of equal dissociation constant ($K_D$). Affinity on (A) recombinant HA0 from A/Shanghai/01/2013 (H7N9), (B) recombinant HA1 head domain from A/Hong Kong/125/2017 (H7N9) (C) recombinant HA1 head domain from A/Chick/Netherlands/01/2003 (H7N7)(D) recombinant HA0 from A/Netherlands/219/2003 (H7N7). Fitting was with a 1:1 Langmuir fitting model using Biacore T200 evaluation software 3.1 software. Equilibrium dissociation constants ($K_D$) are given in Table 2.
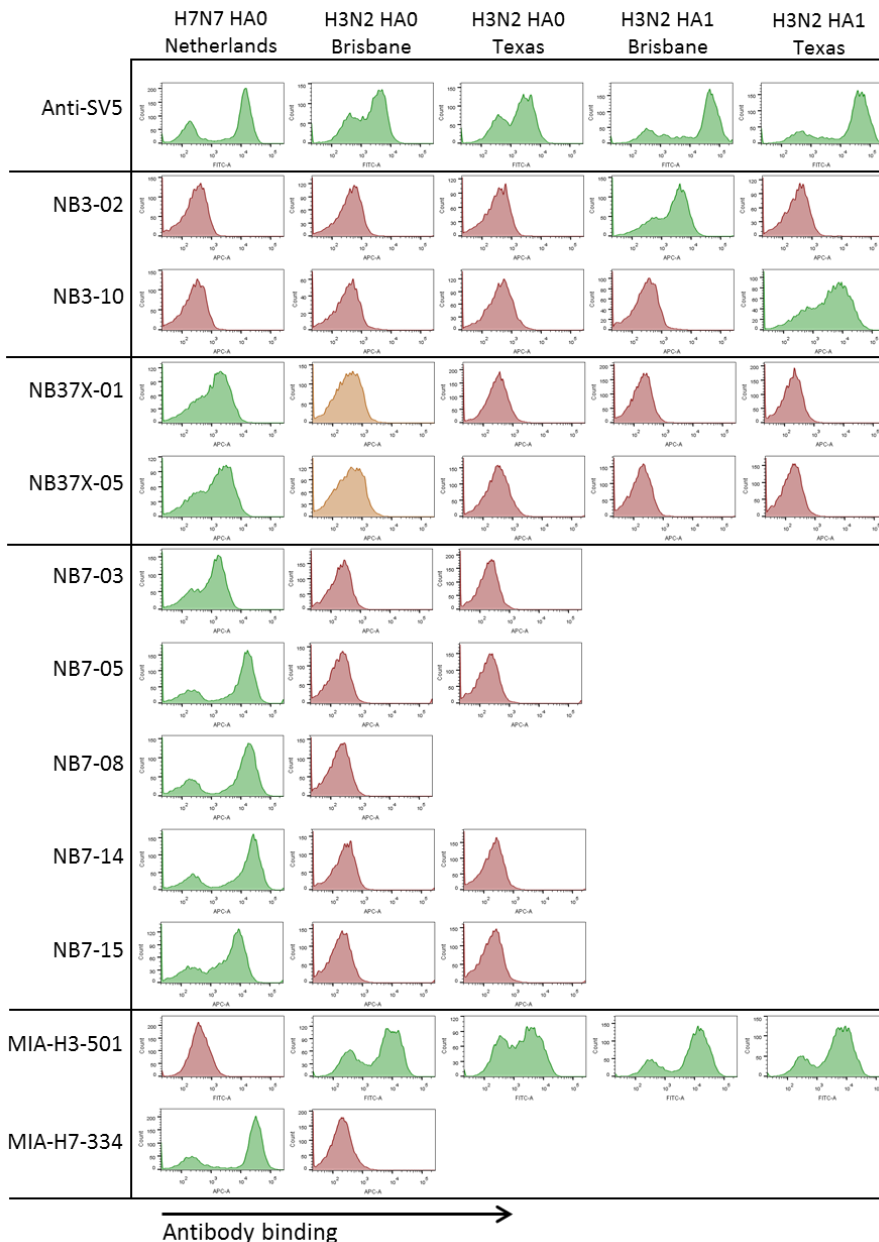
**Fig S3. Nb binding to yeast displayed wild type HA (full-length HA0 or head domain HA1).** Anti SV5 measures HA display via epitope tag. NB3-02 and NB3-10 are nanobodies against H3-HA1 domain as controls to confirm display of H3N2-HA1 on yeast. NB37X-01 and NB37X-05 are cross reactive nanobodies. NB7-03, NB7-05, NB7-08, NB7-14, NB7-15 are nanobodies specific to H7-HA. MIA-H3-501 is control mAb specific for H3N2-HA0. MIA-H7-334 is control mAb specific for H7N7-HA0. HA0 precursor gene of A/Brisbane/10/2007 (H3N2) (Q1-D487 mature protein numbering). HA1 head domain of A/Brisbane/10/2007 (H3N2) (Q1-R329). HA0 precursor gene of A/Netherlands/219/2003 (H7N7) (D1-V508 mature protein numbering). HA0 precursor gene of A/Texas/50/2012 (H3N2) (Q1-D512 mature protein numbering). HA1 head domain of A/Texas/50/2012 (H3N2) (Q1-R328). Green histograms indicate binding, red is no binding and amber is partial binding.
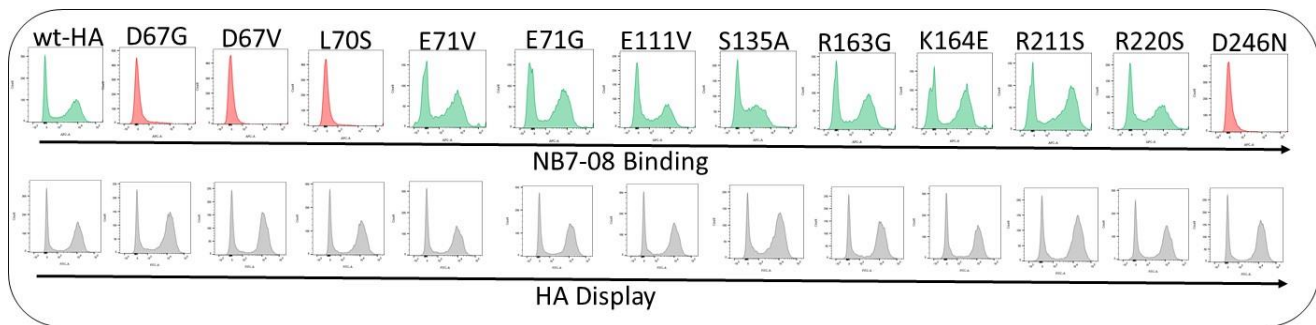
**FIG S4. Example flow cytometry histograms showing NB7-08 binding to yeast displayed wild-type HA and complete panel of HA mutants.** Green indicates mutation has no effect on NB7-08 binding, red indicate NB7-08 binding is lost. Grey histograms indicate HA display and show mutations do not affect the level of HA display.

**Table S1. H7N9 amino acid variability at the 5 distinct nanobody binding epitopes.**

| Ag site* | E | | | A | - | A | - | | D | - | Stem | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gp1a/1b/1c/1d/ Gp2 | | | | | Gp3 | | | Gp4 | | Gp5 | | | | |
| aa** | D67 | L70 | E71 | S135 | D246 | E111 | R163 | K164 | R211 | R220 | M102 | E103 | E114 | M115 | Y119 |
| A | | | | | | | | | | | | | | | |
| C | | | | | | | | | | | | | | | 1 |
| D | 324 | | | | 312 | | | 4 | | | | | | | |
| E | | | 313 | | 11 | 324 | | 3[a] | | | | 319 | 324 | | |
| F | | | | | | | | | | | | | | | |
| G | | | | | | | | | | | | | | | |
| H | | | | | | | | | | | | | | | |
| I | | | | | | | | | | 7 | | | | | |
| K | | | 11 | | | | 2 | 286 | | | | | | | |
| L | | 324 | | | | | | | | | | | | | |
| M | | | | | | | | | | | 323 | | | 324 | |
| N | | | | | | | | 26[b] | | | | | | | |
| P | | | | | | | 3 | | | | | | | | |
| Q | | | | | | | | | | | | | | | |
| R | | | | | | | 317 | | 324 | 317 | | | | | |
| S | | | | 324 | | | | 7 | | | | | | | |
| T | | | | | | | 2 | | | | 1 | | | | |
| V | | | | | 1 | | | | | | | 5 | | | |
| W | | | | | | | | | | | | | | | |
| Y | | | | | | | | | | | | | | | 323 |
| X | | | | | | | | | | | | | | | |

\* Antigenic site A/B/C/D/E (H3 numbering) [1]

\*\*H7 amino acids numbering is from the first residue of the mature protein with DKIC

A total of 324 complete protein sequences corresponding to H7N9 strains were retrieved from NCBI influenza database.

a. K164E substitution present in A/Guangdong/17SF003/2017 (H7N9).

b. K164N substitution present in A/NewYork/107/2003 (H7N2)

1.    Yang, H., et al., *Structural and Molecular Characterization of the Hemagglutinin from the Fifth-Epidemic-Wave A(H7N9) Influenza Viruses.* J Virol, 2018. **92**(16).

2.    Wiley, D.C., I.A. Wilson, and J.J. Skehel, *Structural identification of the antibody binding sites of Hong Kong in influenza haemagglutinin and their involvment in antigenic variation*. Nature, 1981. **289**(5796): p.373-8