**SUPPLEMENTARY MATERIAL**

## Material and Methods

### Analysis of the amount of HA incorporated into virions.

Wildtype and glycosylation mutant viruses in the background of strain A/Netherlands/602/2009 were grown in MDCK cells in the presence of TPCK-trypsin. The supernatants were harvested, cleared by low-speed centrifugation and loaded onto 20% sucrose cushions for ultracentrifugation. The resulting virus pellets were resuspended in PBS and subjected to PNGaseF (New England Biolabs) treatment according to the manufacturer's protocol. The deglycosylated virus preparations were analysed by SDS-Page and Coomassie staining of the gel. The intensities of the HA1 and NP bands were quantified by Multigauge software and plotted as ratio of HA/NP.

### Oseltamivir resistance tests.

***a. NA star assay.*** Viruses were grown in MDCK cells, supernatants were harvested and cleared by low-speed centrifugation. The NA star assay (Invitrogen) was performed using 1:10 dilutions of the virus-containing supernatants according to the manufacturer's instructions.

***b. Inhibition of virus replication in tissue culture.*** MDCK were infected with the different viruses at an MOI of 0.1 in the presence of different concentrations of Oseltamivir (0/10/100 nM). Supernatants containing virus were harvested at 24h post infection and virus titers were determined on MDCK cells.

### Neutralization Assay.

Two-fold serial dilutions of heat (56°C) inactivated mouse sera were mixed and pre-incubated with 100 times the 50% tissue culture infectious dose (TCID50) of each indicated virus per well of a 96-well plate in a final volume of 100 µl. The plates were then incubated for 1hr at 37°C. The serum-

25 virus mixture was then added onto monolayers of MDCK cells that had been seeded at a density of
26 $2X10^4$ cells/well/100µl 18hrs earlier. Infection was allowed to proceed for 18-20hrs. The media was
27 removed and the cells were washed with PBS and then fixed with 80% ice-cold acetone/PBS.
28 Infections were detected by incubation with a rabbit polyclonal anti-NP antibody. Positive wells
29 were visualized by utilizing a secondary antibody labeled with peroxidase and the plates read in a
30 plate reader. Neutralizing titers were determined as the highest dilution that displayed neutralization
31 activity.
32

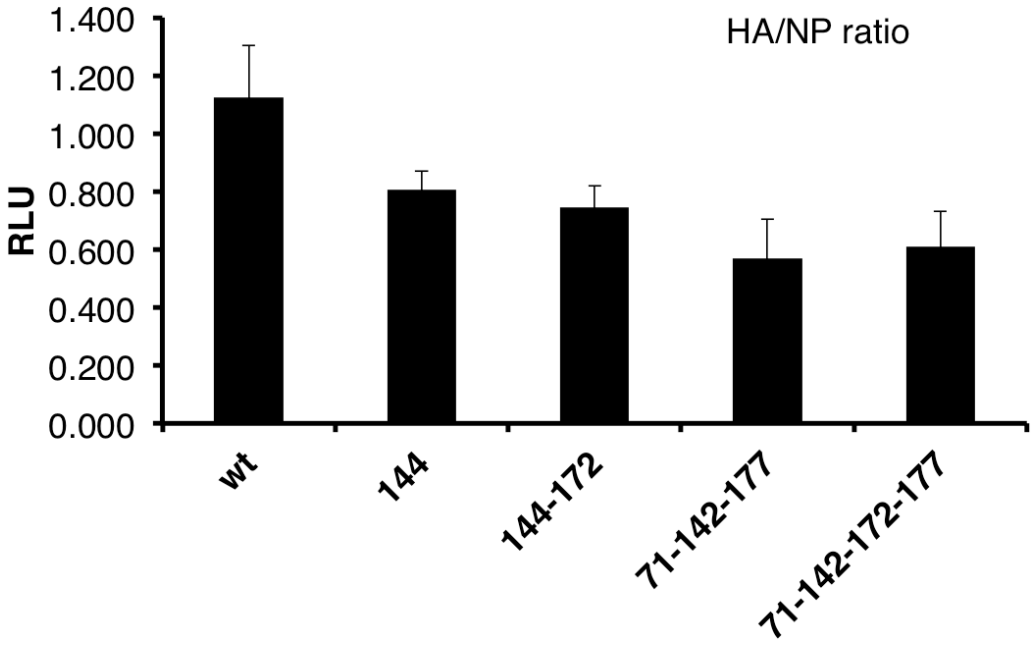



**Figure S1. HA/NP ratio in virions of glycosylation mutant viruses.** Viruses (WT, 144, 144-172, 71-142-177, 71-142-172-177) were grown in MDCK cells and purified by ultracentrifugation through a 20% sucrose cushion. The viruses were deglycosylated by PNGaseF treatment and analysed by SDS-Page. Coomassie stained gels were used to quantify the HA1 band in comparison to the NP band for each virus. The average ratio from three independent gels is graphed.

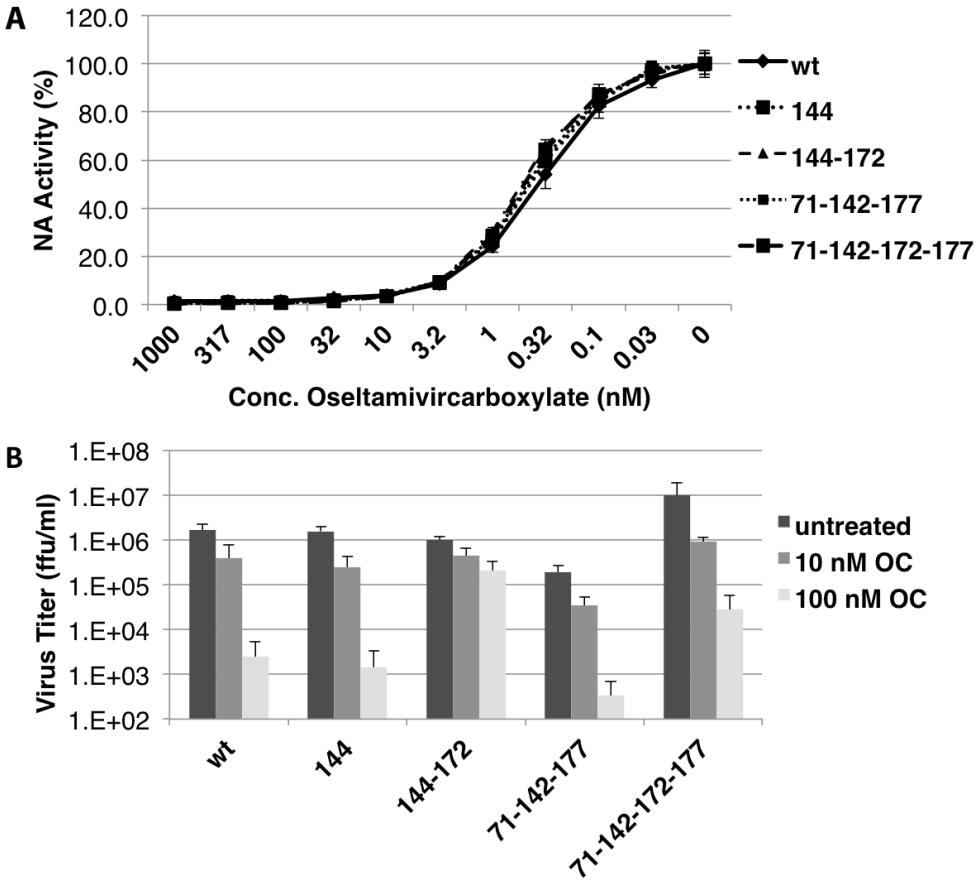
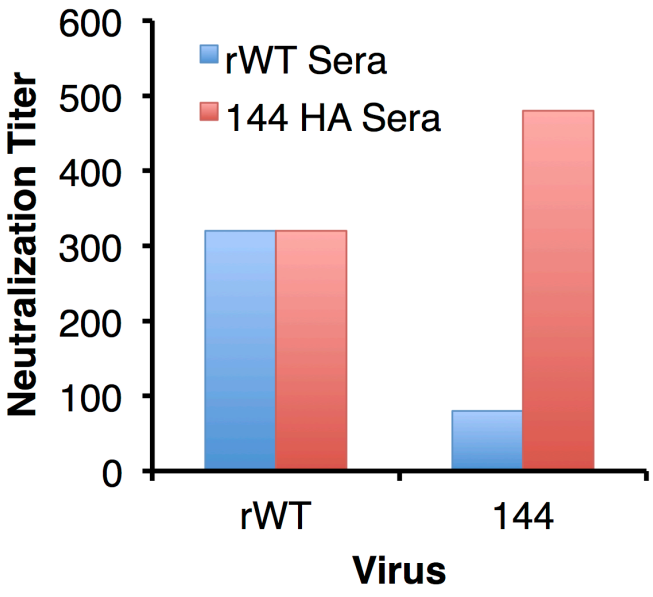



**Figure S2. Sensitivity of the glycosylation mutant viruses to the Oseltamivir antiviral.** A. Neth/09 glycosylation mutant viruses do not differ from WT in their sensitivity to Oseltamivir in an *in vitro* assay. Log dose inhibition curves for the WT Neth/09 and the 144, 144-172, 71-142-177 and 71-142-172-177 viruses were determined in an NA Star chemiluminescent substrate cleavage assay. Data points represent the average number of relative fluorescent units (RLU) at each drug concentration (0-1000nM in 3-fold dilutions), expressed as a percentage of the RLUs obtained in the absence of drug; error bars indicate standard deviations. 2B. Glycosylation mutant viruses show minor differences in their sensitivity to Oseltamivir in a tissue culture replication assay. MDCK were infected with the different viruses at an MOI of 0.1 in the presence of different concentrations of Oseltamivir (0, 10 and 100 nM). Supernatants containing virus were harvested at 24h post infection and virus titers were determined on MDCK cells by standard plaque assay.




| | Sera | |
|---|---|---|
| **Virus** | **rWT** | **144** |
| **rWT** | 320 | 320 |
| **144** | 80 | 480 |

**Figure S3: Neutralization activity of sera from mice infected with rWT and 144 glycosylation mutant viruses.** Sera from mice infected with either rWT or a virus containing glycosylation 144, obtained at day 28 p.i. were analyzed by micro-neutralization assays. Titers represent the geometrical mean of the highest dilution that displayed neutralization activity.

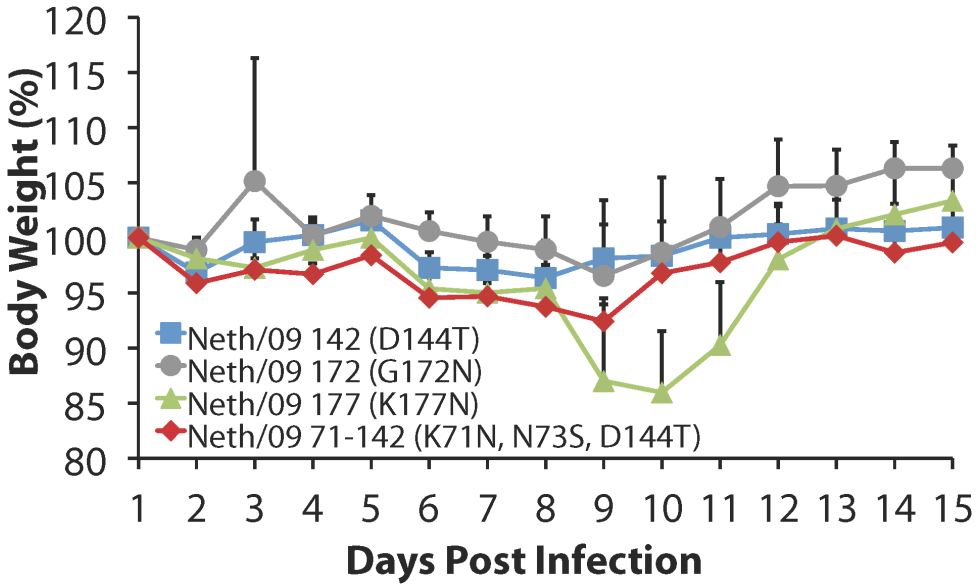



**Figure S4: Pathogenesis of Neth/09 viruses containing single and double glycosylations.** Additional viruses containing single or double glycosylations were generated to evaluate their individual contribution to virulence and cross-reactivity (or lack of) seen with the viruses containing the multiple glycsylations. 8-week-old C57B/6 female mice were infected with $1X10^4$ pfu of each of the indicated viruses and the average body weight of mice n=5 per group was determined for 15 days. Error bars denote the s.d. for each time point. Mice were allowed to seroconvert for 29 days, and convalescent sera were used to test their HI activity against the virus containing the naturally occurring glycosylations. Results are shown in Table 2.

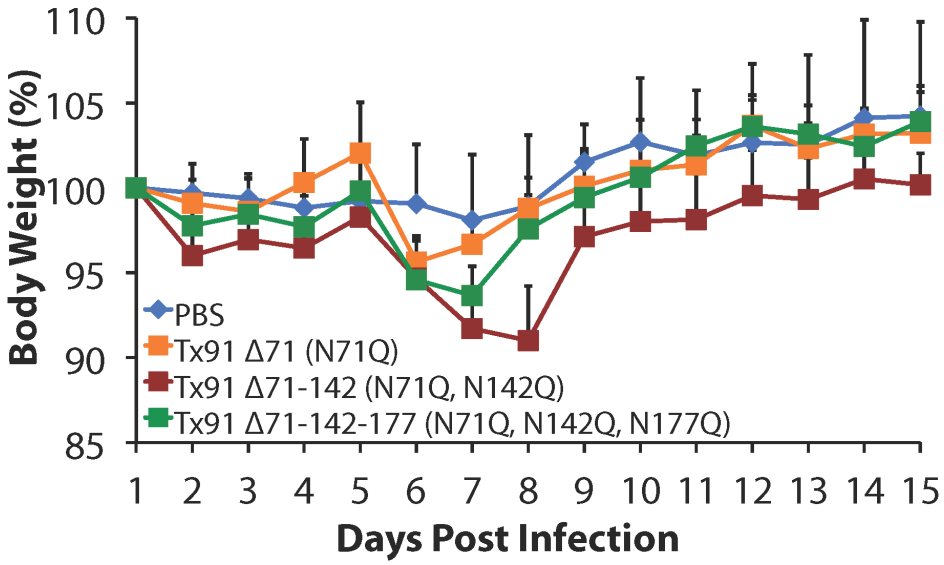



**Figure S5: Pathogenesis of Tx91 viruses containing glycosylation deletions by alternative mutations.** To fully evaluate the contribution of glycosylations in cross reactivity, we generated Tx91 glycosylation mutants that removed the glycosylation motif and introduced a non-relevant amino acid within or close to the antigenic site (amino acid changes are shown). 8-week-old C57B/6 female mice were infected with $1X10^4$ pfu of each of the indicated viruses and average body weights of mice n=5 per group were obtained for 15 days. Error bars denote the s.d. for each time point. Mice were allowed to seroconvert and were then challenged at 29 days p.i with 100LD50 of WT Neth/09. Results are shown in Fig 6.

**Table S1. HAI titers of adult and pediatric human sera after vaccination with 2009 pH1N1 inactivated vaccine.**

| Sera | Age (Years) | Virus | | | | | | | | | | | | | | Vaccine | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | rWT | | 144 | | 144-172 | | 71-142-177 | | 71-142-172-177 | | Bris/59 | | Bris/10 | | 2009 H1N1 | 2009 TIV |
| | | Pre | Post | Pre | Post | Pre | Post | Pre | Post | Pre | Post | Pre | Post | Pre | Post | | |
| 1[a] | 1 | <10 | 20 | <10 | 10 | <10 | 10 | <10 | 20 | <10 | 40 | 20 | 20 | 20 | 20 | Yes | No |
| 2[b] | 5 | <10 | 20 | <10 | 10 | <10 | <10 | <10 | 10 | <10 | 20 | 40 | 40 | <10 | <10 | Yes | No |
| 3[a] | 8 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | 10 | <10 | 10 | 80 | 80 | <10 | <10 | Yes | No |
| 4[b] | 9 | <10 | 20 | <10 | 10 | <10 | <10 | <10 | 10 | <10 | 20 | 10 | 10 | 40 | 160 | Yes | No |
| 5[b] | 10 | <10 | 80 | <10 | 20 | <10 | 40 | <10 | 80 | <10 | 320 | 80 | 1280 | 40 | 1280 | Yes | No |
| 6[a] | 12 | 10 | 80 | 10 | 80 | 10 | 40 | 10 | 160 | 10 | 160 | 10 | 10 | <10 | 10 | Yes | No |
| 7[a] | 13 | <10 | 80 | <10 | 40 | <10 | 40 | <10 | 160 | <10 | 320 | <10 | 10 | 10 | 10 | Yes | No |
| 8[a] | 14 | <10 | 10 | <10 | <10 | <10 | <10 | <10 | 10 | <10 | 20 | 80 | 80 | 10 | 10 | Yes | No |
| 9[c] | 14 | <10 | 160 | <10 | 160 | <10 | 80 | 10 | 640 | 20 | 640 | <10 | 40 | 10 | 40 | Yes | Yes |
| 10[a] | 16 | <10 | 10 | <10 | <10 | <10 | <10 | <10 | 20 | <10 | 10 | 160 | 160 | 20 | 20 | Yes | No |
| 11[c] | 16 | <10 | 20 | <10 | 10 | <10 | <10 | <10 | 20 | <10 | 40 | 10 | 80 | 10 | 80 | Yes | Yes |
| 12[a] | 17 | <10 | 40 | <10 | 10 | <10 | 10 | 20 | 160 | 20 | 160 | 10 | 20 | <10 | <10 | Yes | No |
| 13[c] | 17 | <10 | 160 | <10 | 40 | <10 | 40 | <10 | 640 | <10 | 640 | 20 | 20 | <10 | <10 | Yes | Yes |
| 14[b] | 17 | <10 | 80 | <10 | 20 | <10 | 20 | 10 | 160 | 40 | 320 | <10 | <10 | 10 | 320 | Yes | No |
| 15[a] | 18 | <10 | 80 | <10 | <10 | <10 | 10 | <10 | 80 | 10 | 320 | 80 | 80 | 10 | 10 | Yes | No |
| 16[b] | 19 | 20 | 160 | <10 | 40 | <10 | 20 | 20 | 160 | 20 | 160 | <10 | 80 | <10 | 320 | Yes | Yes |
| 17[a] | 20 | <10 | 1280 | <10 | 320 | <10 | 160 | 10 | 1280 | 20 | 2560 | 40 | 80 | 160 | 160 | Yes | No |
| 18[b] | 21 | 10 | 640 | 10 | 320 | <10 | 160 | 20 | 1280 | 20 | 1280 | 80 | 80 | 80 | 160 | Yes | Yes |
| 19[a] | 31 | <10 | 40 | <10 | 20 | <10 | 40 | <10 | 40 | 10 | 160 | <10 | <10 | <10 | <10 | Yes | No |
| 20[b] | 33 | <10 | 80 | <10 | 20 | <10 | 40 | <10 | 20 | <10 | 80 | 20 | 20 | 10 | 20 | Yes | No |
| 21[b] | 34 | <10 | 2560 | <10 | 1280 | <10 | 1280 | <10 | 80 | <10 | 640 | <10 | 20 | <10 | 10 | Yes | Yes |
| 22[b] | 34 | 10 | 160 | 10 | <10 | 10 | <10 | 20 | 320 | 10 | 640 | <10 | 160 | 40 | 1280 | Yes | Yes |
| 23[a] | 36 | <10 | 160 | <10 | 80 | <10 | 80 | <10 | 160 | <10 | 320 | <10 | <10 | <10 | <10 | Yes | No |
| 24[a] | 40 | <10 | 160 | <10 | 80 | <10 | 80 | <10 | 160 | 10 | 320 | <10 | 10 | <10 | <10 | Yes | No |
| 25[a] | 42 | <10 | 40 | <10 | 20 | <10 | 20 | <10 | 40 | <10 | 160 | 20 | 20 | <10 | <10 | Yes | No |
| 26[a] | 42 | <10 | 640 | <10 | 640 | 10 | 640 | <10 | 80 | 80 | 640 | 10 | 20 | 10 | 10 | Yes | No |
| 27[b] | 42 | 20 | 640 | 10 | 320 | 10 | 160 | 20 | 320 | 20 | 640 | 10 | 20 | 20 | 40 | Yes | Yes |
| 28[b] | 43 | 10 | 640 | 10 | 320 | 10 | 160 | <10 | 640 | 10 | 640 | 10 | 20 | 80 | 40 | Yes | Yes |
| 29[a] | 44 | <10 | 40 | <10 | 20 | <10 | 40 | <10 | 10 | <10 | 80 | <10 | 10 | 160 | 160 | Yes | No |
| 30[a] | 46 | <10 | 1280 | 10 | 1280 | <10 | 1280 | <10 | 160 | 40 | 160 | <10 | <10 | 20 | 20 | Yes | No |
| 31[a] | 46 | <10 | 20 | <10 | <10 | <10 | <10 | <10 | 20 | <10 | 80 | 80 | 80 | 10 | 10 | Yes | No |
| 32[a] | 47 | <10 | 160 | <10 | 160 | <10 | 160 | <10 | 80 | <10 | 80 | <10 | <10 | <10 | <10 | Yes | No |
| 33[a] | 48 | <10 | 40 | <10 | 10 | <10 | 40 | <10 | 40 | <10 | 160 | <10 | 10 | <10 | <10 | Yes | No |
| 34[b] | 49 | <10 | 40 | <10 | 20 | <10 | 20 | <10 | 40 | <10 | 160 | <10 | 10 | <10 | 20 | Yes | Yes |
| 35[a] | 52 | <10 | 640 | <10 | 320 | <10 | 320 | <10 | 160 | <10 | 320 | 80 | 80 | <10 | <10 | Yes | No |
| 36[a] | 54 | <10 | 20 | <10 | <10 | <10 | <10 | <10 | 40 | <10 | 80 | 10 | 10 | 20 | 20 | Yes | No |
| 37[b] | 55 | <10 | 640 | <10 | 320 | <10 | 640 | <10 | 320 | <10 | 640 | <10 | 80 | <10 | <10 | Yes | Yes |
| 38[a] | 57 | <10 | 20 | <10 | 10 | <10 | <10 | <10 | 20 | <10 | 40 | <10 | <10 | <10 | <10 | Yes | No |
| 39[a] | 59 | 40 | 160 | 40 | 80 | 40 | 80 | 40 | 80 | 40 | 160 | 10 | 10 | 20 | 20 | Yes | No |
| 40[d] | 60 | 20 | 1280 | 10 | 320 | 20 | 320 | 20 | 320 | 20 | 160 | <10 | <10 | <10 | 10 | Yes | Yes |
| 41[a] | 63 | 10 | 40 | <10 | 20 | <10 | 20 | <10 | 40 | 10 | 40 | <10 | <10 | <10 | <10 | Yes | No |
| 42[a] | 65 | <10 | 20 | <10 | <10 | <10 | <10 | <10 | 10 | <10 | 40 | <10 | 20 | <10 | 80 | Yes | No |
| 43[a] | 66 | 20 | 160 | 10 | 160 | 10 | 80 | 20 | 80 | 40 | 160 | 40 | 40 | 80 | 80 | Yes | No |
| 44[a] | 67 | <10 | 40 | <10 | 10 | <10 | <10 | <10 | 20 | <10 | 20 | <10 | <10 | 10 | 10 | Yes | No |
| 45[d] | 67 | 10 | 1280 | <10 | 640 | <10 | 320 | 20 | 640 | 10 | 320 | 20 | 40 | 40 | 80 | Yes | Yes |
| 46[d] | 67 | 20 | 640 | <10 | 40 | 20 | 320 | 20 | 640 | 20 | 2560 | 10 | 10 | 40 | 320 | Yes | Yes |
| 47[a] | 68 | 10 | 160 | 10 | 40 | 10 | 40 | 10 | 160 | 40 | 320 | 40 | 40 | 20 | 20 | Yes | No |
| 48[a] | 73 | <10 | 80 | <10 | 20 | <10 | 40 | 10 | 80 | 20 | 320 | <10 | <10 | <10 | <10 | Yes | No |
| 49[a] | 75 | 10 | 320 | 10 | 320 | <10 | 160 | 10 | 160 | 10 | 160 | 10 | 20 | 10 | 10 | Yes | No |
| 50[d] | 82 | 40 | 2560 | 40 | 2560 | 40 | 160 | 40 | 1280 | 40 | 160 | 10 | 20 | 40 | 160 | Yes | Yes |
| 51[d] | 51 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | 40 | 20 | <10 | 40 | No | Yes |
| 52[d] | 74 | <10 | 10 | <10 | <10 | <10 | 10 | <10 | <10 | <10 | 10 | <10 | 320 | 20 | 80 | No | Yes |

[a] Human serum samples obtained on days 0 (Pre) and 42 (Post) after vaccination, respectively. Subjects received the 2009 H1N1 inactivated vaccine on days 0 and 21.

[b] Human serum samples obtained on days 0 (Pre) and 21 (Post) after vaccination, respectively. Subjects received the 2009 H1N1 inactivated vaccine on day 0 only.

[c] Human serum samples obtained on days 0 (Pre) and 42 (Post) after vaccination, respectively. The 2009 TIV: trivalent inactivated influenza virus vaccine which included strains A/Brisbane/57/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B/Brisbane/60/2008, was given on day 0 and the 2009 H1N1 vaccine was given on day 21.

[d] Human serum samples obtained on days 0 (Pre) and 63 (Post) after vaccination, respectively. 2009 H1N1 inactivated vaccine was given on days 0 and 21 and the 2009 TIV: trivalent inactivated influenza virus vaccine, which included strains A/Brisbane/57/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B/Brisbane/60/2008, was given on day 42.