Supplementary Materials and Methods

Transmission electron microscopy. Negative stain TEM was performed in conjunction with the Electron Microscopy Research Core at the University of Rochester. Recombinant HA proteins were adjusted to a concentration of 0.01µg protein/µl of PBS. 10µl of each rHA preparation were placed into a 200nm mesh copper grid coated with formvar/carbon. The rHA suspension was air dried for 2 minutes and 10µl of 2% phosphotungstic acid, pH 6.5, were added to the copper grid. The grid was air dried for 2 additional minutes prior to imaging.

Gel electrophoresis and Silver staining. Recombinant HA proteins were run under reducing conditions on a 4-15% gradient polyacrylamide Tris-HCL "Ready Gel" (BioRad Cat#161-1154). The protein bands were visualized using the silver stain plus kit from BioRad (Cat# 161-0449) according to manufacturer's recommendations.

rHA deglycosylation. Carbohydrate removal from rHA proteins was achieved by treating 50µg of rHA protein with 1000 units of Peptide N-Glycosidase F (New England BioLabs Cat# P070S) for 20 hours. The enzymatic reaction was carried out according to manufacturer's recommendations. It is worth noting that PNGase F treatment does not result in the removal of α1,3 core fucosylated structures present in some insect and plant derived proteins. The enzymatic reaction buffer was exchanged for PBS using Zeba desalting spin columns, with a 40kDa exclusion range (Thermo Scientific Cat# 87764), according to manufacturer's recommendations. The concentration of recovered proteins was determined by bicinchoninic acid assay (Thermo Scientific Cat# 23235).

Supplementary Figure legends

Supplementary Figure 1. Amino acid sequence alignment of the rHA proteins from A/Brisbane/59/07. Amino acid sequences from the rHA proteins were obtained from each supplier and aligned using ClustalW2. The Sf9-derived rHA is expressed as a full-length protein. Tni-, HEK-, Nb-, and Sf9-HA_T-derived proteins contain various truncations and engineered modifications, which are highlighted as follows: Histidine purification tag (Yellow), Thrombin Cleavage Sequence (Red), T4 Fibritin Trimerization domain (Blue), and the Endoplasmic Reticulum retention signal KDEL (Green). Amino acids derived from plasmid sequences are shown in underlined and italicized text.

Supplementary Figure 2. Amino acid sequence alignment of the rHA proteins from B/Florida/04/06. Amino acid sequences from the rHA proteins were obtained from each supplier and aligned using ClustalW2. The Sf9-derived rHA is expressed as a full-length protein. Tni-, HEK-, Nb-, and Sf9-HA_T-derived proteins contain various truncations and engineered modifications, which are highlighted as follows: Histidine purification tag (Yellow), Thrombin Cleavage Sequence (Red), T4 Fibritin Trimerization domain (Blue), and the Endoplasmic Reticulum retention signal KDEL (Green). Amino acids derived from plasmid sequences are shown in underlined and italicized text. The sequence from the Tni-derived HA was unavailable, but strategy utilized to generate this recombinant protein is identical to that used for A/Brisbane/59/07 (i.e. HA ectodomain contained the C-terminal Thrombin Cleavage site, the T4 Fibritin Trimerization domain, and the Histidine purification tag.

Supplementary Figure 3. Influenza recombinant hemagglutinin proteins produced in various protein expression systems differ on the structural organization of the HA oligomeric species. TEM of the hemagglutinin protein from A/Brisbane/59/07 produced in (A) HEK, (B) Sf9, (C) Nb, (D) Tni, and (E) Sf9 HA_T revealed that the presence of rosette-like oligomeric structures varied from each protein preparation. The white arrows highlight defined rosette structures containing 4-8 HA trimers.

Supplementary Figure 4. The bulk of the recombinant hemagglutinin protein in the preparations is in an uncleaved state. Recombinant hemagglutinin proteins were run under reducing conditions on a 4-15% gradient polyacrylamide Tris-HCL "Ready Gel". The protein bands were visualized by silver staining. SDS-PAGE analysis of the rHA preparations from (A) A/Brisbane/59/07 and (B) B/Florida/04/06 shows that the bulk of the hemagglutinin protein contained in the preparations is in its HA0/uncleaved state.

Supplementary Figure 5. The extent of recombinant HA protein glycosylation is dependent on the protein expression system. Recombinant HA proteins were treated with PNGase F to remove N-glycans. Alpha 1-3 fucosylated structures present in some rHAs (*) cannot be removed by PNGase F treatment. Deglycosylated HA proteins were run under reducing conditions on a 4-15% gradient polyacrylamide Tris-HCL "Ready Gel". The protein bands were visualized by silver staining. SDS-PAGE analysis of the rHA preparations from A/Brisbane/59/07 shows a shift in size following the removal of N-glycans. As expected, the greatest size shift was observed in the highly glycosylated HEK-derived HA.

Supplementary Figure 6. The induction of antibodies with HAI activity is affected by the source of the rHA protein. C57BL/6 female mice were immunized intramuscularly (left flank) with 5ug of rHA adjuvanted with CpG. 14 days after priming, the mice received a booster immunization consisting of the same rHA preparation (5ug rHA and CpG). Serum from immunized mice was collected 21 days after boost. Serum was diluted and incubated with (A) A/Brisbane/59/07 or (B) B/Florida/04/06. HAI titer is defined as the highest dilution in which RBC agglutination was observed. Mean HAI titer and standard deviation are plotted on the graphs. Dashed lines represent the mean HAI titer obtained from serum generated following viral infection with the corresponding influenza A or B virus. Mann-Whitney (t-test) statistical analysis performed with n=8 (A) and n=10 (B). Numbers at the top of each bar represent the fraction of mice that antibodies with HAI activity greater that 1:40.