

Additional file 3: Development and optimization of H5 EB-ELISA.

An epitope-blocking (EB) ELISA was developed using the purified recombinant H5 HA protein (aa 17–530, Δ RRRKRR, 6x His) produced in a baculovirus-expression vector system (BEVS; Oxford Expression Technologies Ltd.). Details of the protein, referred to as rH5-BEVS, are provided in Additional file 1: Table S1. During the assay development, the optimal type of ELISA plate and preliminary testing conditions were established. Further optimization of H5 EB-ELISA was performed in three steps.

Plate selection

The PolySorp, MediSorp, MaxiSorp and MultiSorp plates (Nunc, Roskilde, Denmark) were coated by overnight incubation at 2–8 °C with 50 μ L/well of rH5-BEVS at a concentration of 1.0 μ g/mL in PBS. The coated plates were washed three times with 300 μ L/well of PBS containing 0.05% Tween 20 (PBST; pH 7.4) and then incubated with 200 μ L/well of 1% BSA in PBS for 60 min at room temperature (23 ± 2 °C). After blocking, the plates were washed two times with 350 μ L/well of PBST. Next, 50 μ L/well of G-7-27-18 mAb, two-fold serially diluted from 8,000 ng/mL to 0.977 ng/mL in 1% BSA in PBS, was applied to the plates, which were then incubated for 30 min at 37 °C and washed three times with 300 μ L/well of PBST afterwards.

Antigen-bound mAbs were detected using HRP-labeled, anti-mouse IgG (γ -chain specific) antibodies (cat. no. A3673, lot no. 051M6270, Sigma-Aldrich). The plates were incubated with 50 μ L/well of anti-mouse antibodies, diluted 1:1,000 in 1% BSA in PBS, for 30 min at 37 °C and then washed three times with 300 μ L/well of PBST. The reactions were developed with 50 μ L/well of TMB (Sigma-Aldrich) at room temperature (23 ± 2 °C) in the dark for 10 min and subsequently stopped by adding 50 μ L/well of 0.5 M H_2SO_4 . The optical density (OD) was measured at 450 nm (OD_{450}) using a Synergy 2 multidetection microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

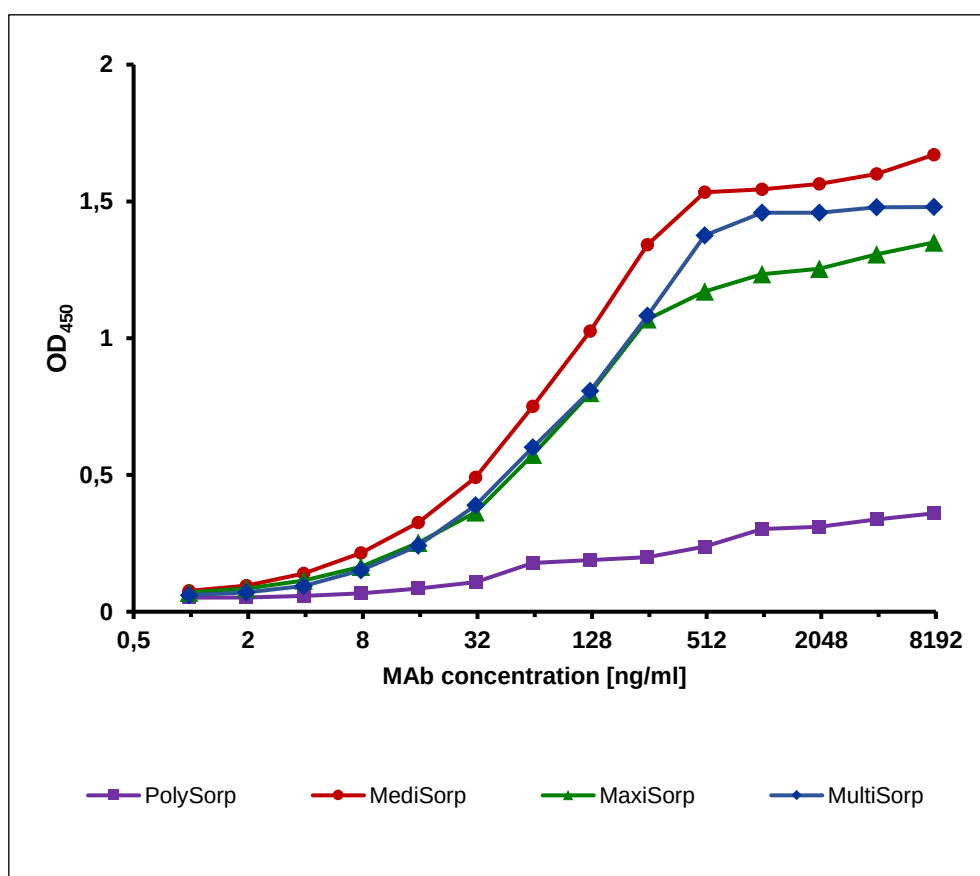


Fig. S1. The ELISA titration curves of G-7-27-18 mAb against rH5-BEVS coated on the well surfaces with varied hydrophilicity.

Titration curves were denoted according to the plate type.

Preliminary assay conditions

The MediSorp plates (Nunc, Roskilde, Denmark) were coated by overnight incubation at 2–8 °C with 50 µL/well of rH5-BEVS at concentrations of 1.0 µg/mL or 0.5 µg/mL in PBS. The coated plates were washed three times with 300 µL/well of PBS containing 0.05% Tween 20 (PBST; pH 7.4) and then incubated with 200 µL/well of 1% BSA in PBS for 60 min at room temperature (23 ± 2 °C). After blocking, the plates were washed two times with 350 µL/well of PBST. Next, 50 µL/well of G-7-27-18 mAb, two-fold serially diluted from 2,000 ng/mL to 15.625 ng/mL in 1% BSA in PBS, was applied to the plates, which were then incubated for 60 min or 30 min at 37 °C and washed three times with 300 µL/well of PBST afterwards.

Antigen-bound mAbs were detected using HRP-labeled, anti-mouse IgG (γ-chain specific) antibodies (cat. no. A3673, lot no. 051M6270, Sigma-Aldrich). The plates were incubated with 50 µL/well of anti-mouse antibodies, diluted 1:1,000 in 1% BSA in PBS, for 60 min or 30 min at 37 °C and then washed three times with 300 µL/well of PBST. The reactions were developed with 50 µL/well of TMB (Sigma-Aldrich) at room temperature (23 ± 2 °C) in the dark for 15 min and/or 10 min and subsequently stopped by adding 50 µL/well of 0.5 M H₂SO₄. The optical density (OD) was measured at 450 nm (OD₄₅₀) using a Synergy 2 multidetection microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

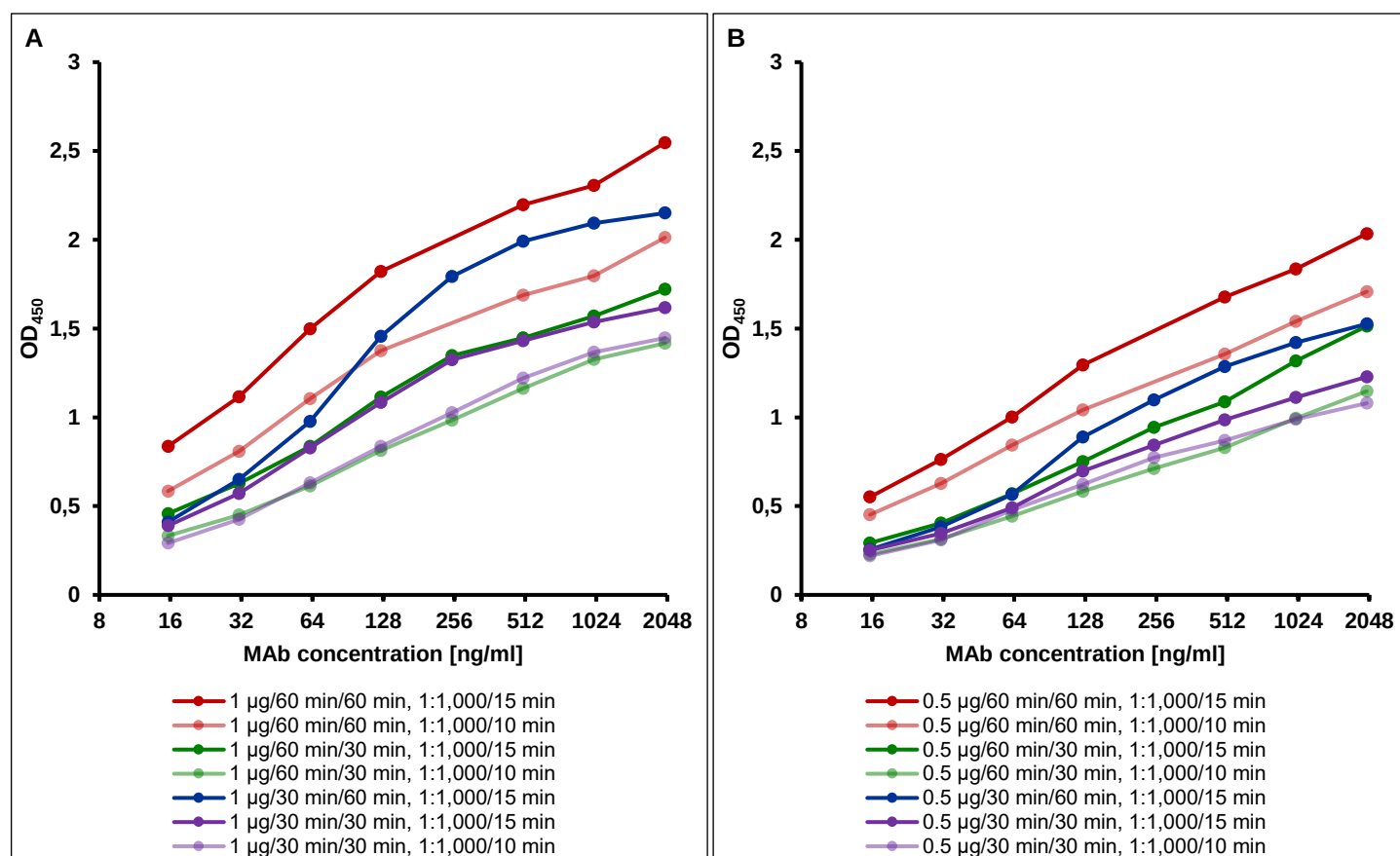


Fig. S2. The ELISA titration curves of G-7-27-18 mAb against rH5-BEVS under preliminary assay conditions.

rH5-BEVS was coated at (A) 1.0 µg/mL or (B) 0.5 µg/mL on MediSorp plates. Titration curves were denoted according to the coating concentration of rH5-BEVS in µg per mL (1 µg or 0.5 µg) and then the time of plate incubation with G-7-27-18 mAb (60 min or 30 min), anti-mouse antibodies (60 min or 30 min) at the indicated dilution (1:1,000) and TMB (15 min or 10 min).

Optimization - step 1

The MediSorp plates (Nunc, Roskilde, Denmark) were coated by overnight incubation at 2–8 °C with 50 µL/well of rH5-BEVS at a concentration of 0.5 µg/mL in PBS. The coated plates were washed three times with 300 µL/well of PBS containing 0.05% Tween 20 (PBST; pH 7.4) and then incubated with 200 µL/well of Protein-Free T20 (PBS) Blocking Buffer (Pierce/Thermo Fisher Scientific) for 60 min at room temperature (23 ± 2 °C). After blocking, the plates were washed two times with 350 µL/well of PBST and then incubated with 100 µL/well of 1% BSA in PBS for 60 min at 37 °C with shaking at 150 rpm and subsequently washed three times with 300 µL/well of PBST. Next, 50 µL/well of G-7-27-18 mAb, two-fold serially diluted from 4,000 ng/mL to 3.906 ng/mL in 1% BSA in PBS, was applied to the plates, which were then incubated for 60 min or 30 min at 37 °C with shaking at 150 rpm and washed three times with 300 µL/well of PBST afterwards.

Antigen-bound mAbs were detected using HRP-labeled, anti-mouse IgG (γ-chain specific) antibodies (cat. no. A3673, lot no. 051M6270, Sigma-Aldrich). The plates were incubated with 50 µL/well of anti-mouse antibodies, diluted 1:1,000, 1:1,500, 1:2,000 or 1:2,500 in 1% BSA in PBS, for 60 min at 37 °C with shaking at 150 rpm and then washed three times with 300 µL/well of PBST. The reactions were developed with 50 µL/well of TMB (Sigma-Aldrich) at room temperature (23 ± 2 °C) in the dark for 15 min and subsequently stopped by adding 50 µL/well of 0.5 M H₂SO₄. The optical density (OD) was measured at 450 nm (OD₄₅₀) using a Synergy 2 multidetection microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

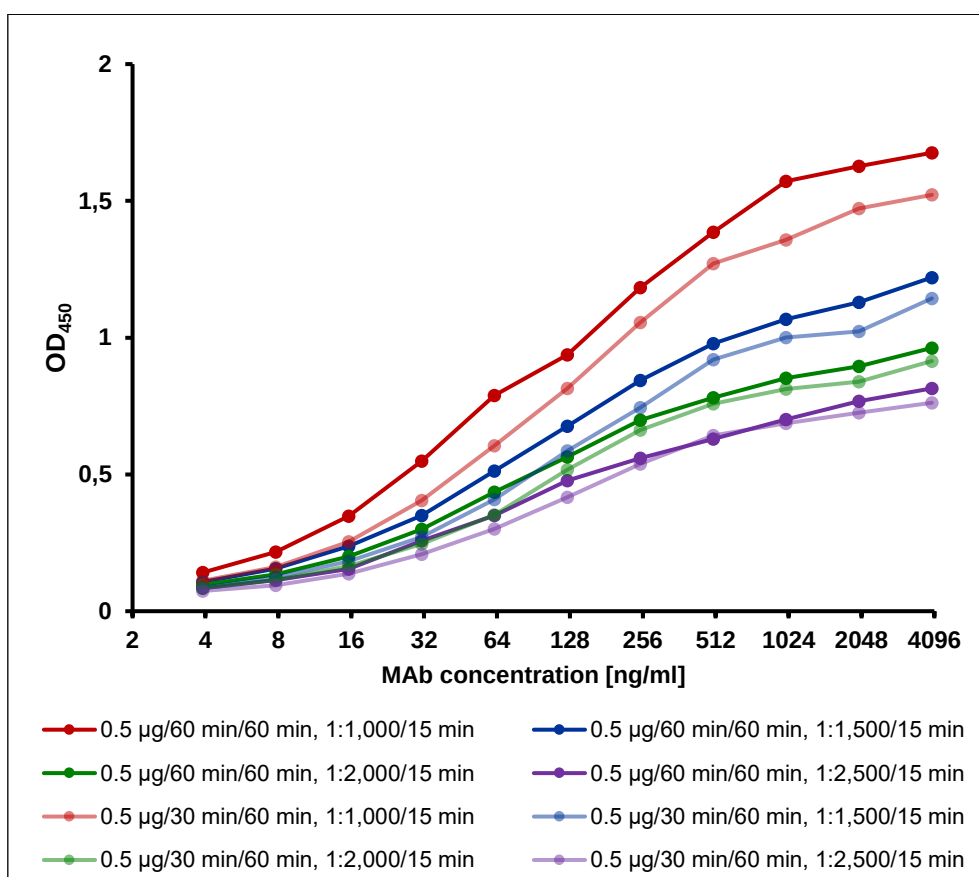


Fig. S3. The ELISA titration curves of G-7-27-18 mAb against rH5-BEVS under assay conditions optimized in step 1.

Titration curves were denoted according to the coating concentration of rH5-BEVS in µg per mL (0.5 µg) and then the time of plate incubation with G-7-27-18 mAb (60 min or 30 min), anti-mouse antibodies (60 min) at the indicated dilutions (1:1,000, 1:1,500, 1:2,000 or 1:2,500) and TMB (15 min).

Optimization - step 2

The MediSorp plates (Nunc, Roskilde, Denmark) were coated by overnight incubation at 2–8 °C with 50 µL/well of rH5-BEVS at a concentration of 0.5 µg/mL in PBS. The coated plates were washed three times with 300 µL/well of PBS containing 0.05% Tween 20 (PBST; pH 7.4) and then incubated with 200 µL/well of Protein-Free T20 (PBS) Blocking Buffer (Pierce/Thermo Fisher Scientific) for 60 min at room temperature (23 ± 2 °C). After blocking, the plates were washed two times with 350 µL/well of PBST and then incubated with 100 µL/well of 1% BSA in PBS for 60 min at 37 °C with shaking at 150 rpm and subsequently washed three times with 300 µL/well of PBST. Next, 50 µL/well of G-7-27-18 mAb, two-fold serially diluted from 4,000 ng/mL to 3.906 ng/mL in 1% BSA in PBS, was applied to the plates, which were then incubated for 60 min or 30 min at 37 °C with shaking at 150 rpm and washed three times with 300 µL/well of PBST afterwards.

Antigen-bound mAbs were detected using HRP-labeled, anti-mouse IgG (γ-chain specific) antibodies (cat. no. A3673, lot no. SLBH0520, Sigma-Aldrich). The plates were incubated with 50 µL/well of anti-mouse antibodies, diluted 1:1,500, 1:2,000, 1:3,000 or 1:4,000 in HRP-Protector (CANDOR Bioscience GmbH), for 60 min at 37 °C with shaking at 150 rpm and then washed three times with 300 µL/well of PBST. The reactions were developed with 50 µL/well of TMB (Sigma-Aldrich) at room temperature (23 ± 2 °C) in the dark for 15 min and subsequently stopped by adding 50 µL/well of 0.5 M H₂SO₄. The optical density (OD) was measured at 450 nm (OD₄₅₀) using a Synergy 2 multidetection microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

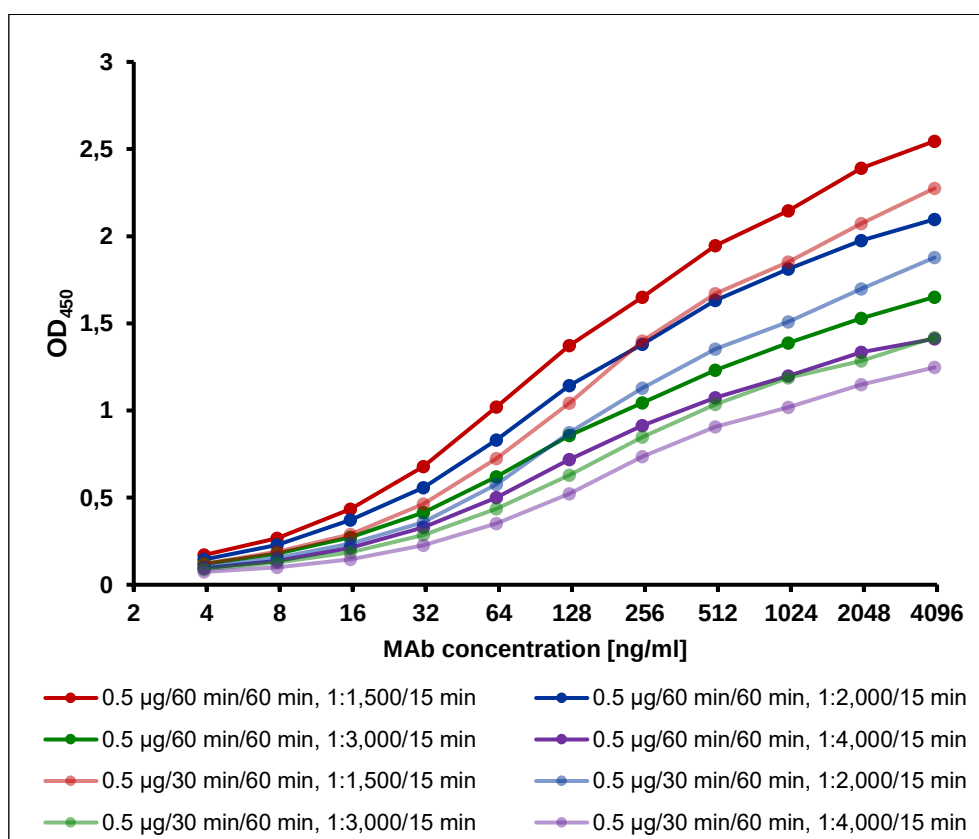


Fig. S4. The ELISA titration curves of G-7-27-18 mAb against rH5-BEVS under assay conditions optimized in step 2.

Titration curves were denoted according to the coating concentration of rH5-BEVS in µg per mL (0.5 µg) and then the time of plate incubation with G-7-27-18 mAb (60 min or 30 min), anti-mouse antibodies (60 min) at the indicated dilutions (1:1,500, 1:2,000, 1:3,000 or 1:4,000) and TMB (15 min).

Optimization - step 3

The MediSorp plates (Nunc, Roskilde, Denmark) were coated by overnight incubation at 2–8 °C with 50 µL/well of rH5-BEVS at a concentration of 0.5 µg/mL in PBS. The coated plates were washed three times with 300 µL/well of PBS containing 0.05% Tween 20 (PBST; pH 7.4) and then incubated with 200 µL/well of Protein-Free T20 (PBS) Blocking Buffer (Pierce/Thermo Fisher Scientific) for 60 min at room temperature (23 ± 2 °C). After blocking, the plates were washed two times with 350 µL/well of PBST and then incubated with 100 µL/well of 1% BSA in PBS for 60 min at 37 °C with shaking at 150 rpm and subsequently washed three times with 300 µL/well of PBST. Next, 50 µL/well of G-7-27-18 mAb, two-fold serially diluted from 4,000 ng/mL to 3.906 ng/mL in Antibody Stabilizer PBS (CANDOR Bioscience GmbH, Wangen, Germany), was applied to the plates, which were then incubated for 60 min at 37 °C with shaking at 150 rpm and washed three times with 300 µL/well of PBST afterwards.

Antigen-bound mAbs were detected using HRP-labeled, anti-mouse IgG (γ-chain specific) antibodies (cat. no. A3673, lot no. SLBH0520, Sigma-Aldrich). The plates were incubated with 50 µL/well of anti-mouse antibodies, diluted 1:2,500, 1:3,000, 1:3,500 or 1:4,000 in HRP-Protector (CANDOR Bioscience GmbH), for 60 min at 37 °C with shaking at 150 rpm and then washed three times with 300 µL/well of PBST. The reactions were developed with 50 µL/well of TMB (Sigma-Aldrich) at room temperature (25 ± 0.1 °C) in the dark for 15 min and subsequently stopped by adding 50 µL/well of 0.5 M H₂SO₄. The optical density (OD) was measured at 450 nm (OD₄₅₀) using a Synergy 2 multidetection microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

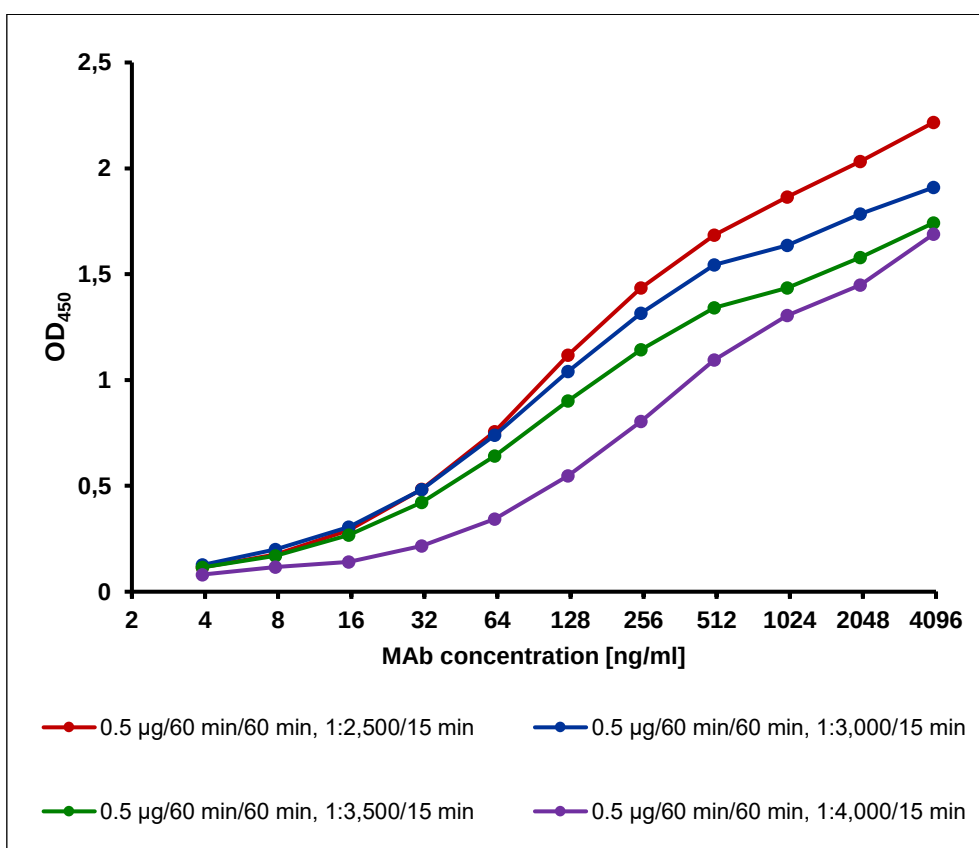


Fig. S5. The ELISA titration curves of G-7-27-18 mAb against rH5-BEVS under assay conditions optimized in step 3.

Titration curves were denoted according to the coating concentration of rH5-BEVS in µg per mL (0.5 µg) and then the time of plate incubation with G-7-27-18 mAb (60 min), anti-mouse antibodies (60 min) at the indicated dilutions (1:2,500, 1:3,000, 1:3,500 or 1:4,000) and TMB (15 min).