Day 1

1. Coat 96-well high affinity plates overnight at 4 ◦C with 5 µg/mL OVA in PBS (100 / 25 µL per well).

Day 2

2. Wash with 0.05% v/v Tween 20 in PBS (abbreviated PBST). Perform 4X.

3. Block plates with 200 / 50 µL of 1% w/v Casein in PBS for 2 hours at room temperature (RT).

4. Repeat step 2.

5. Dilute serum in 1% Casein in PBS in standard 96-well plate, then transfer to ELISA plate.
   a. Samples should be diluted beginning at 1:10 or 1:20 in the first column for the groups you expect to perform the most poorly (e.g., PBS, unadjuvanted antigen).

   b. Higher dilutions (begin at 1:100 or even 1:200) are suggested for adjuvanted groups, especially at later timepoints. Perform serial dilutions across the next 10 columns.

   c. The last column should be buffer only.

6. Incubate 100 µL of diluted serum samples for 2 hours at RT (dilutions in 0.05% v/v Tween 20 in PBS) OR OVERNIGHT – just be consistent

Day 3

7. Repeat step 2. Also warm TMB bottle up to room temperature.

8. Incubate 100 µL of 1:6,000 dilution of HRP-conjugated anti-IgG secondary antibody for 1 hr at RT (dilute in 0.05% v/v Tween 20 in PBS).

9. Repeat step 2 but perform 5 to 7 washes. It is important to get rid of all of the secondary antibody.

10. Add 100 µL TMB solution.

11. After 10 minutes, add 100 µL of 2 N sulfuric acid (recipe = 5.6 mL neat sulfuric acid + 94.4 mL DI water). However, 10 minutes may be too long. Monitor the plates to make sure the buffer only column isn't getting darker.

12. Read at 450 nm and 570 nm (background).

13. The antibody titer is the last sample dilution that is greater than or equal to 3X higher than the buffer only well. Buffer only should be ~0.05 OD (may sometimes be higher). Make sure the signal has gone down as you proceed down the serial dilutions too.