Sample Preparation

1. Collect an appropriate volume of blood from the animal (depending on the species it could be 0.5 -2.5 mL).
2. Let the sample stand for 15-30 min at room temperature or in ice if room temperature exceeds 26°C (80°F).
3. Spin the blood at 2,000 – 3,000 rpm (1,000 – 2,000 x g) for 10 minutes and collect the serum (top layer) in a new clean tube (sterile tube is preferred to avoid serum contamination). Store the serum samples at 4°C, for short term storage or at -20 °C to -80 °C for long term storage.

Recommended Procedure

1. Prepare a 1X solution from the 10X concentrated Wash by making a 1:10 dilution with DiH2O.
   Example: 1 mL of 10X Wash Solution to 9 mL of DiH2O per plate.
2. Wash plate by adding 100 µL per well of 1X Wash Solution; discard the wash buffer and blot on a paper towel. Repeat this step three times.
3. Prepare the Milk Diluent working solution by adding one vial of Milk Powder (0.5 g) into the 10 mL Milk Diluent bottle. Wash the remaining powder in the vial by adding 1-2 mL of working Milk Diluent; pipette up and down and transfer back to Milk Diluent bottle. Mix until the powder is completely dissolved.
4. If necessary, dilute Positive Control to the optimal dilution according to the appropriate Lot number (Indicated in each Kit). If the positive control is provided as "ready to use", skip this step. For the dilution use milk diluent prepared in step 3.
5. Dilute Negative Control antibody to 1:2 in Milk diluent. For each well pipette 50 µL of the negative control (provided in the kit) to appropriate negative control antigen wells and adjacent control antigen well. Next, pipette 50 µL milk diluent to each of these wells to bring to final dilution.
6. Prepare 1:50 dilution of test sample in Milk Diluent. Prepare enough sample for both antigen and control antigen wells. Add 100 µL of diluted samples to appropriate antigen wells and adjacent control antigen wells.
7. Seal the plate to avoid evaporation and incubate at 37°C for 60-65 minutes. You can use disposable adhesive film covers, or silicone sealing mats.
8. Wash plate three times with 100 µL of 1X Wash Solution and blot dry on a paper towel.
9. Add 100 µL per well of species-specific conjugate antibody (what dilution??).
10. Reseal the plate and incubate at 37°C for 60-65 minutes.
11. Wash plate three times with 100 µL of 1X Wash Solution and blot dry on a paper towel.
12. While incubation on step 10; bring to room temperature the substrate solution. After the last wash in step 11, add 100 µL per well of the peroxidase substrate. (any reason why you use ABTS instead of TMB?)
13. Reseal the plate and incubate at 37°C for 30-35 minutes.
14. While the plate is incubating, prepare 1X Stop Solution by a 1:5 dilution with DiH2O.
    For example, add 2 mL of 5X Stop solution (provided) to 8 mL DiH2O for 1 plate.
15. Add 100 µL of 1X Stop Solution to each well immediately after completion of the final incubation.

16. Read the plate at 405 nm within 15 minutes (why recommend a longer period of time?) of adding the stop solution to each well.

Evaluating Results (are these values pre-determined based in preliminary test?)
Net OD= Antigen well – Control Antigen well

- Positive control: Net O.D value should be ≥0.8 OD units.
- Negative control: Net O.D value should be <0.150 OD.
- Samples with net O.D values ≥0.150 are considered reactive.
- Samples with net O.D values <0.150 are considered negative.