Test Validation
• The mean of the Negative Control O.D.s must be >0.200 and <2.000.
• The mean of the Positive Control O.D.s must be ≥ 35%.

Interpreting the Results
• If a test sample produces ≥ 35% inhibition, it is positive.
• If a test sample produces < 35% inhibition, it is negative.
A positive test indicates the presence of antibody specific for EAV as a result of either infection by EAV or vaccination.

Precautions
All potentially infectious components used in this test kit tested negative for adventitious viruses, bacteria, fungi, and mycoplasmas according to 9 CFR 113.52. However, kit components should be handled and disposed of as potentially hazardous. Do not eat, drink, or smoke where serum samples and kit reagents are handled. Do not pipette by mouth. Some reagents may be harmful if ingested. If ingested, seek medical attention. Do not use expired or contaminated reagents, or reagents from other kits or serials. Do not mix reagents from different serials of the same product.
Component B, Positive Control, contains sodium azide as a preservative.
Component C, Negative Control, contains sodium azide as a preservative.
Component D, 10X Primary Antibody, contains sodium azide, ProClin 300, methylisothiazolone, and bromonitrodioxane as a preservative.
Component E, 100X Secondary Antibody-Peroxidase Conjugate, contains ProClin 300, methylisothiazolone, bromonitrodioxane, and thimerosal as preservatives.
Component F, Antibody Diluting Buffer, contains ProClin 300, methylisothiazolone and bromonitrodioxane as preservatives.

Materials Required But Not Included in the Test Kit
Single and multichannel adjustable-volume pipettors and disposable plastic tips, test tubes or non-antigen-coated transfer plate(s), ELISA microplate absorbance spectrophotometer with 450 nm filter, deionized or distilled water, paper towels, graduated cylinder, timer, multichannel pipettor reservoirs, wash bottle, manual multichannel washing device or automatic plate washer, and Parafilm® or equivalent
Storage and Stability
Store all reagents at 2-7°C. Do not freeze. Unopened reagents will remain stable until the expiration date when stored as instructed. Do not use test kit past the expiration date printed on the box.

Preparation
a. Warm reagents: Bring the serum samples, reagents and plate(s) to room temperature (23 ± 2°C) prior to starting the test.
b. Prepare controls and samples: Positive and Negative Controls are provided ready to use. Load Positive Control (B) in duplicate and Negative Control (C) in triplicate, regardless of the number of serum samples to be tested. When whole plates are used, it is best to put the controls in wells on different areas of the plate. Controls must be loaded on every plate. Serum samples are run UNDILUTED.
c. Prepare plates: Remove the plate(s) from the foil pouch(es) (A). If applicable: Return any unused strips to the pouch and securely seal it. Extra pouches and sealer are available from VMRD. Place strips to be used in the frame and number the top of each strip to maintain orientation. Always mark the strips in case they dislodge from the frame during washing.
d. Prepare primary antibody: Prepare 1X Primary Antibody by diluting 1 part of the 10X Primary Antibody (D) with 9 parts of Antibody Diluting Buffer (F). Example: For 96 wells, mix 600 μl of 10X Primary Antibody (D) with 5.4 ml of Antibody Diluting Buffer (F) to yield 6 ml of 1X Primary Antibody. Fifty microliters (50 μl) are needed per well.
e. Prepare secondary antibody-peroxidase conjugate: Prepare 1X Secondary Antibody-Peroxidase Conjugate by diluting 1 part of the 100X Secondary Antibody-Peroxidase Conjugate (E) with 99 parts of Antibody Diluting Buffer (F). Example: For 96 wells, mix 60 μl of 100X Secondary Antibody-Peroxidase Conjugate (E) with 5.940 ml of Antibody Diluting Buffer (F) to yield 6 ml of 1X Secondary Antibody-Peroxidase Conjugate. Fifty microliters (50 μl) are needed per well.
f. Prepare wash solution: Prepare 1X Wash Solution by diluting 1 part of the 10X Wash Solution Concentrate (G) with 9 parts of deionized or distilled water. Approximately 2.25 ml are needed per well. Allow extra quantity for reservoirs, tubing, pipetting, etc.

test Procedure
1. Load controls and serum samples: Using a pipettor set at 50 μl, load controls and serum samples into the Antigen-Coated Plate (A). Serum samples and controls should be loaded into the Antigen-Coated Plate (A) as quickly as possible. When running more than two strips, we recommend that the serum samples and controls be first loaded into a transfer plate and then transferred to the Antigen-Coated Plate (A) using multi-channel pipetting equipment. The sample volume in the transfer plate must be in excess of 50 μl in order to transfer 50 μl from it. Tap the side of the loaded assay plate several times to make sure the samples coat the bottom of the wells. Use care not to spill samples from well to well. Cover the top of the plate with Parafilm® or equivalent to prevent evaporation and incubate the plate 2 hours at room temperature (23 ± 2°C).
2. Wash wells: After the 2-hour incubation, wash the plate 3 times: If an automatic washer is used, place the plate on the washing apparatus and wash plate 3 times, filling the wells each time with 1X Wash Solution. If manual washing is used, dump well contents and remove remaining sera and controls by sharply striking the inverted plate 4 times on a clean paper towel, striking a clean area each time. Immediately fill each well with 1X Wash Solution using a multichannel filling device or a wash bottle. Empty the wash solution from the plate and strike the inverted plate sharply on a clean paper towel as above. Fill and empty the plate by the same method 2 additional times for a total of 3 washes.
3. Add primary antibody: Add 50 μl of diluted (1X) Primary Antibody to each well. Tap the side of the loaded assay plate several times to make sure the primary antibody coats the bottom of the wells. Incubate for 30 minutes at room temperature (23 ± 2°C).
4. Wash wells: After the 30-minute incubation, wash the plate 3 times as in Step 2.
5. Add secondary antibody-peroxidase conjugate: Add 50 μl of diluted (1X) Secondary Antibody-Peroxidase Conjugate to each well. Tap the side of the loaded assay plate several times to make sure the conjugate coats the bottom of the wells. Incubate for 30 minutes at room temperature (23 ± 2°C).
6. Wash wells: After the second 30-minute incubation, wash the plate 3 times as in Step 2.
7. Add substrate solution: Add 50 μl of Substrate Solution (H) to each well. Tap the side of the loaded assay plate several times to make sure the substrate coats the bottom of the wells. Incubate 15 minutes at room temperature (23 ± 2°C). Avoid leaving the plate in direct sunlight. Do not empty wells.
8. Add stop solution: Add 50 μl of Stop Solution (I) to each well. When Stop Solution is added, the color will turn from blue to yellow. Tap the side of the loaded assay plate several times to mix the Substrate Solution and the Stop Solution. Do not empty wells.
9. Read and record the test results: Immediately after adding the Stop Solution, the plate should be read on a microplate absorbance spectrophotometer. Set the optical density (OD) reading wavelength to 450 nm. Blank the instrument on air and read plate(s). Some readers require an empty well on the plate for blanking. In this case, no reagents should be added to this well.
10. Return all remaining kit reagents to 2-7°C for storage.

Calculation of % Inhibition (% I):

% I = 100 [1-(Sample O.D. ÷ NC O.D.)]