For a test to be valid, the reference lines (between Positive Reference Serum (R) in alternating peripheral wells, leaving three empty wells do not break down when agar plugs are removed. (Hardness of the agar may be increased by placing the plates (with the lids replaced) in a refrigerator for 24 and 48 hours, sometimes the results can be determined before the haze obscures the reaction. However, in some cases a determination cannot be made if the samples are positive or negative. Sample serum may produce a specific BTV line as well as a non-specific line). Care must be taken to be certain a specific reaction is not obscured by a non-BTV line. Retesting such samples and observing the reactions at frequent intervals may facilitate making determinations if the samples are positive or negative. A sample serum may produce a specific BTV line as well as a non-specific line). Care must be taken to avoid separating the agar from the plastic when removing the agar plugs. If moisture is observed in the wells prior to introduction of reagents or samples, it should be removed by suction. Agar plates should be used the same day they are cut.

Filling the Wells and Incubation of the Agar Plates
Place 20 μl of Bluetongue Virus Antigen (A) in the center well and 20 μl of Positive Reference Serum (R) in alternating peripheral wells, leaving three empty wells for samples (Figure 1). Place 20 μl of each sample into empty alternating wells. This arrangement provides a positive control line on each side of the test serum, thus facilitating accurate determination of lines of identity. Fill wells level with the agar surface, leaving no meniscus. Serum or antigen must not run on top of the agar. Allow the plates to set a few minutes before moving to reduce the possibility of spillage. Place the plates in a closed humid chamber and incubate at 23 ± 2°C.

Test Validation
For a test to be valid, the reference lines (between Positive Reference Serum wells and the center Bluetongue Virus Antigen well) must be easily visualized. These lines should be clearly visible right to the edge of negative sample wells. If not, the assay cannot be accurately interpreted.

Interpreting the Results
The test may be read at 24 hours of incubation and can be reread at 48 hours to confirm the results, especially when weak positive reactions are observed. The precipitin lines are most easily visualized using an intense narrow beam of light. It is also very helpful to read the plates against a dark background in reduced lighting conditions.

The following types of reactions are observed:
1. Negative: The reference lines continue straight into the test sample well without bending (Figure 1, S1, negative sample).
2. Positive: Control lines join with and form a continuous line with the line between the test serum and antigen (A) (Figure 1, S2, positive sample).
3. Weak Positive: The reference positive control lines bend slightly toward the antigen well (A) and away from the Positive Reference Serum wells (R) but do not form a complete line between antigen (A) and test serum (Figure 1, S3, weak positive sample). These reactions require careful observation and can be easily overlooked. All weak positive samples should be retested before reporting the results.
4. Very Strong Positive: The reference positive control lines turn toward the antigen well before they reach the well containing the test serum and there is a broad, hazy line between the test serum and antigen (A) (Figure 2, S1, strong positive sample). This line is situated very near the antigen well (A), especially if the plate is observed at 24 hours.
5. Non-Specific Lines: These lines are observed between the antigen and test serum well. However, the reference positive control lines will pass through the non-specific line and continue on into the test serum well of negative sera (not shown). The non-specific line does not form a continuous line with the reference positive control lines. The reference positive control lines will form more acute angles with a non-specific line than with a BTV-specific line of identity. The non-specific lines are formed by sample-antibody reactions with antigens other than BTV. A sample serum may produce a specific BTV line as well as a non-specific line (Figure 2, S2, positive sample with non-specific line). Care must be taken to avoid separating the agar from the plastic when removing the agar plugs. If moisture is observed in the wells prior to introduction of reagents or samples, it should be removed by suction. Agar plates should be used the same day they are cut.

Precautions
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 this same product.