INTERNAL VALIDATION REPORT

ID SCREEN® BLUETONGUE COMPETITION

COMPETITIVE ELISA FOR THE DETECTION OF ANTIBODIES AGAINST THE BTV VP7 PROTEIN

METHOD	**Competitive ELISA**
TARGET	Antibodies directed against Bluetongue Virus
SAMPLE TYPES	• Serum • Plasma
VALIDATED SPECIES	• Bovine (cattle and buffalo) • Ovine • Caprine
PRODUCT CODE	BTC

With you at every step

TABLE OF CONTENTS

INTRODUCTION **3**

DESCRIPTION AND PRINCIPLE OF THE TEST **3**

SPECIFICITY **4**

SENSITIVITY **5**

FIELD SAMPLES 5

SEROTYPE SENSITIVITY (INCLUSIVITY/EXCLUSIVITY) 6

CORRELATION WITH PCR 6

ANALYTICAL SENSITIVITY **9**

CUT-OFF DETERMINATION AND ROC CURVE **10**

REPEATABILITY **11**

REPRODUCIBILITY **12**

ROBUSTNESS **13**

STABILITY **14**

CONCLUSION **15**

ADDITIONAL DATA (EXTERNAL STUDIES) **15**

REFERENCES **15**

RELATED PRODUCTS **15**

HISTORY OF REVISIONS **16**

INTRODUCTION

Bluetongue (BT) is an infectious, vector-borne viral disease that affects wild and domestic ruminants, such as sheep. The disease is caused by the Bluetongue virus (BTV), of which 27 serotypes exist (1).

Laboratory detection of BTV may be achieved by detecting antibodies directed against the VP7 protein. The VP7 is a major core protein possessing the serogroup-specific antigens common to the 27 serotypes.

The ID Screen® Bluetongue Competition ELISA allows for detecting anti-VP7 antibodies in serum and plasma from multiple species.

This document summarizes the validation data obtained for this test.

DESCRIPTION AND PRINCIPLE OF THE TEST

Microwells are coated with VP7 recombinant protein. Samples to be tested and controls are added to the microwells. The anti-VP7 antibodies, if present, form an antibody-antigen complex which masks the VP7 epitopes.

After washing, an anti-VP7-peroxidase (HRP) conjugate is added to the microwells. It binds the remaining free VP7 epitopes, forming an antigen-conjugate-peroxidase complex.

After elimination of the excessive amount of the conjugate by washing, the substrate solution (TMB) is added. The resulting coloration is proportional to the amount of specific antibodies present in the sample. In the absence of antibodies, a blue coloration appears, which becomes yellow after addition of the stop solution. In the presence of antibodies, no coloration appears.

The microplate is read at 450 nm.

Note: This kit does not contain infectious material.

For each sample, the S/N % is calculated:

$$\mathbf{S/N\%} = \frac{\mathbf{OD_{samples} - OD_{PC}}}{\mathbf{OD_{NC} - OD_{PC}}} \times \mathbf{100}$$

Samples are then classified as positive or negative depending on their S/N % result, as indicated in the table:

RESULT	STATUS
S/N % < 40%	**Positive**
S/N % ≥ 40%	**Negative**

SPECIFICITY

1500 ovine sera, 650 bovine sera, and 311 caprine sera from disease-free regions in Southern France (Avéyron and Hérault) were tested with the ID Screen® Bluetongue Competition ELISA. These samples were collected before the BTV outbreak in France that occurred in 2006.

150 buffalo sera from a disease-free regions in North Italy and Germany were also tested.

The results are shown in Figure 1 and Table 1.

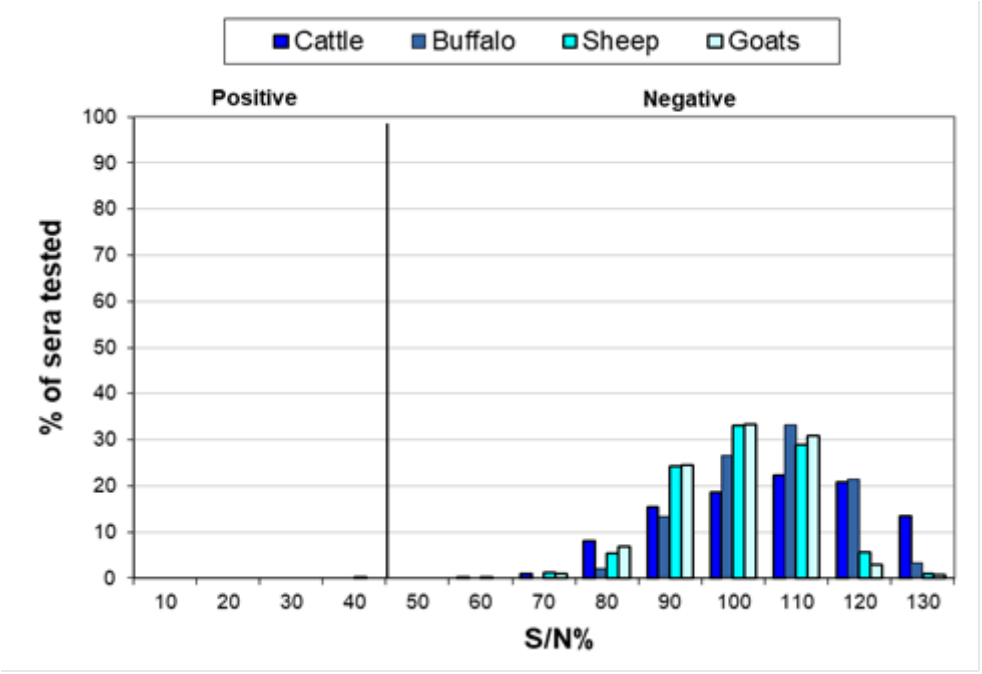

Figure 1: S/N% distribution of negative sera, n=2611

SPECIES	NUMBER OF SAMPLES TESTED	MEASURED SPECIFICITY (%)	95% CI
Cattle	650	100	[99.43, 100]
Buffalo	150	100	[97.57, 100]
Ovine	1500	99.93	[99.63, 100]
Caprine	311	100	[98.82, 100]
TOTAL	**2611**	**99.96**	**[99.79, 100]**

Table 1: Measured specificities according to each species, n=2611

RESULTS (Table 1 and Figure 1):

- Out of the 2611 sera tested, all bovine, buffalo and caprine sera were found negative. One ovine serum was found positive.
- **Measured specificity = 99.96%** (95% CI [99.78, 99.99], n=2611).

SENSITIVITY

FIELD SAMPLES

The following samples from infected herds were tested:

- 300 sera from BTV-8 infected / vaccinated cattle (Germany, Belgium and France, 2007 and 2008).
- 300 sera from BTV-8 infected / vaccinated sheep (Germany, Belgium and France, 2007 and 2008).
- 84 sera from BTV-8 infected / vaccinated goats (Germany, Belgium and France, 2007 and 2008).
- 37 sera from BTV-1 infected cattle (France and Spain, 2008).
- 33 sera from BTV-1 infected sheep (France, 2008)
- 30 sera from BTV-infected buffalo (Italy (Rome, Salerno), 2014-2018).

Results are summarized in Figure 2.

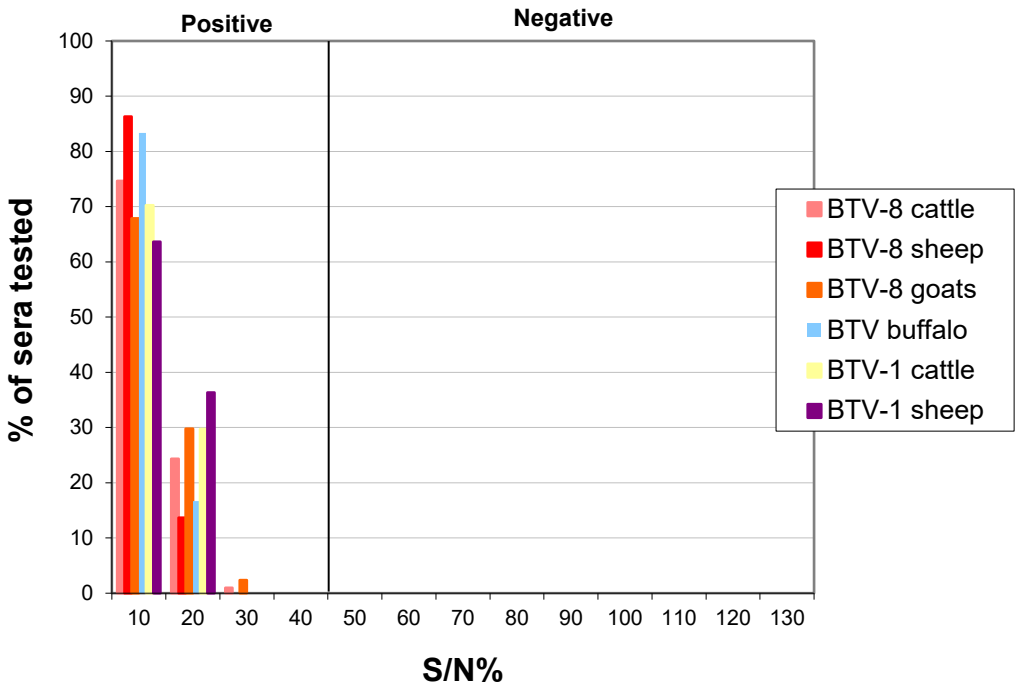

Figure 2: S/N% distribution of positive sera

RESULTS (Figure 2):

- All sera were found positive.
- **Measured sensitivity = 100%** (95%CI [99.51, 100], n=784).

SEROTYPE SENSITIVITY (INCLUSIVITY/EXCLUSIVITY)

The following samples were tested using the ID Screen® ELISA:

- antisera to the 26 BTV serotypes;
- serial dilutions of BTV-16 positive samples (4 samples diluted 1:25, 1:50, 1:100 and 1:200);
- 3 BTV-negative sera;
- sera from animals infected by the 5 Epizootic Hemorrhagic Disease Virus (EHDV) serotypes.

RESULTS:

- **All 26 serotypes were detected by the ID Screen® ELISA.**
- The BTV-16 positive sera were found positive diluted 1:25.
- T**he BTV-negative samples and EHDV-positive samples were correctly identified as negative.**

CORRELATION WITH PCR

Field sera samples from 304 animals from the 2006 Spring-Summer primo-infection in Belgium were first tested by PCR, then tested 3 weeks later with the ID Screen® ELISA.
Results are summarized in Table 2.

		BTV-PCR		
		POSITIVE	**NEGATIVE**	**TOTAL**
ID Screen® ELISA	**POSITIVE**	36	1	37
	NEGATIVE	1	266	267
	TOTAL	37	267	304

Table 2: Seroconversion kinetics on time-course sera tested with the ID Screen® ELISA

RESULTS (Table 2):

- **Correlation between the PCR and ID Screen® ELISA results is excellent with 99.34% agreement** (Kappa=0.969).
- The sample not detected by ELISA probably corresponds to an animal infected and tested before seroconversion.
- The sample not detected by PCR probably corresponds to an animal after seroconversion and after elimination of the virus.
- In the months following these analyses, the percentage of PCR-positive samples decreased, while the number of serology-positive samples remained constant.

EXPERIMENTALLY INFECTED ANIMALS

12 sheep, experimentally infected with BTV-4, were bled 0, 4, 7, 14, 21, 28, 35, and 42 days post-infection. Serum samples were tested with the ID Screen® Bluetongue Competition ELISA. Results are summarized in Table 3.

SAMPLE	DAY 0	DAY 4	DAY 7	DAY 14	DAY 21	DAY 28	DAY 35	DAY 42
1	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
2	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE
3	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
4	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
5	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
6	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
7	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
8	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
9	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
10	NEGATIVE	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
11	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
12	NEGATIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE

Table 3: Seroconversion kinetics for experimentally-infected sheep

RESULTS (Table 3):

- Seroconversion was detected as of **7 days post-infection.**

VACCINATED ANIMALS

The following animals were tested with the ID Screen® Bluetongue Competition ELISA:

- 1 sheep, vaccinated with a South African BTV-2 live vaccine, and bled 0, 7, 14, 21, 28, 35, 42 and 49 days post-vaccination (dpv). The results are shown in Table 4.
- 5 sheep, vaccinated with Merial 2,4 vaccine, and bled 0, 7, 38, 74, and 108 dpv. The results are shown in Table 5.

DAYS POST VACCINATION	S/N% cut-off: <40%	STATUS
0	119	NEGATIVE
7	91	NEGATIVE
14	46	NEGATIVE
21	59	NEGATIVE
28	40	NEGATIVE
35	31	POSITIVE
42	15	POSITIVE
49	8	POSITIVE

Table 4: Seroconversion kinetics of a BTV-2 vaccinated serum

DAYS POST VACCINATION (DPV)	S/N % / (STATUS*)									
	SERUM 1		SERUM 2		SERUM 3		SERUM 4		SERUM5	
0	139	(-)	125	(-)	139	(-)	129	(-)	125	(-)
7	77	(-)	47	(-)	114	(-)	62	(-)	58	(-)
38	42	(-)	53	(-)	50	(-)	90	(-)	62	(-)
74	38	(+)	7	(+)	7	(+)	7	(+)	9	(+)
108	7	(+)	8	(+)	7	(+)	8	(+)	8	(+)

*: (-) negative (+) positive

Table 5: Seroconversion kinetics of 5 vaccinated sheep

RESULTS (Tables 4 and 5):

- For the BTV-2 vaccinated sheep, the kit detected seroconversion as of 35 dpv.
- For sheep vaccinated with the Merial 2,4 vaccine, the kit detected seroconversion between 38 and 74 dpv.

ANALYTICAL SENSITIVITY

As there is no international standard for BTV serology, analytical sensitivity was compared to another commercial ELISA (kit A) through analysis of different dilutions of two BT-positive sera:

- a BTV-4 positive serum, from a Southern Italian slaughterhouse;
- a BTV-8 positive serum, kindly provided by Dr Toussaint and Dr. Clercq, from the CODA CERVA laboratory in Belgium.

Results are summarized in Table 6.

	BTV-4		BTV-8	
DILUTION	**Innovative Diagnostics**	**KIT A**	**Innovative Diagnostics**	KIT A
PURE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
1:2	POSITIVE	POSITIVE	POSITIVE	POSITIVE
1:4	POSITIVE	POSITIVE	POSITIVE	POSITIVE
1:16	POSITIVE	POSITIVE	POSITIVE	POSITIVE
1:32	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE
1: 64	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE

Table 6: Analytical sensitivity comparison between ID Screen® and kit A

RESULTS (Table 6):

- Both kits detected the BTV-4 diluted to 1:16.
- Innovative Diagnostics kit detected BTV-8 at a higher dilution level compared to Kit A.

CUT-OFF DETERMINATION AND ROC CURVE

611 negative samples (1500 ovine, 650 bovine, 311 goat, and 150 buffalo sera) and 784 positive samples (333 ovine, 337 bovine, 84 goat and 30 buffalo sera) were tested for diagnostic specificity and sensitivity, respectively.

Diagnostic specificity and sensitivity were calculated, with the 95% confidence interval (lower and upper limits), for different cut-off values.

The results are presented in the Table 7 below.

THRESHOLD VALUE (S/N%)	SPECIFICITY (%)			SENSITIVITY (%)		
	SPECIFICITY	LOWER LIMIT	UPPER LIMIT	SENSITIVITY	LOWER LIMIT	UPPER LIMIT
10	100	99,9	100	78,1	75	80,9
20	100	99,9	100	99,4	98,5	99,8
30	100	99,9	100	100	99,5	100
40	100	99,8	100	100	99,5	100
50	100	99,8	100	100	99,5	100
60	99,7	99,4	99,8	100	99,5	100
70	98,6	98,1	99,0	100	99,5	100
80	92,6	91,6	93,6	100	99,5	100
90	71,2	69,5	72,9	100	99,5	100
100	42	40,1	43,9	100	99,5	100
110	14,3	13	15,7	100	99,5	100
120	4,2	3,5	5,1	100	99,5	100

Table 7: Specificity and sensitivity values obtained for different threshold values

The data acquired was also used to plot the Receiver Operating Curve shown in Figure 3.

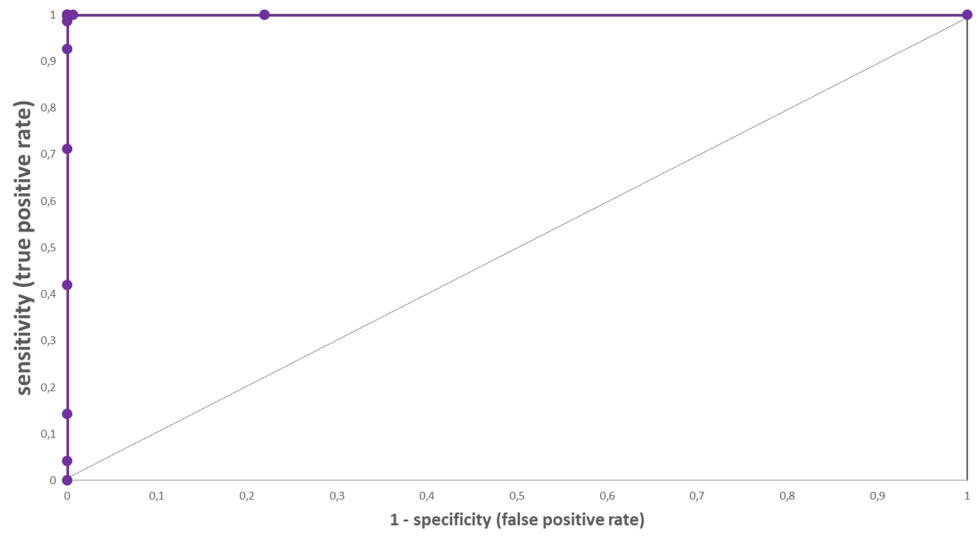

Figure 3: ROC curve obtained for the ID Screen® Bluetongue Competition ELISA (calculation of AUC)

RESULTS (Table 7 and Figure 3):

- The **cut-off at a S/N% value of 40 %** was chosen by Innovative Diagnostics to ensure **the best possible sensitivity and specificity.**
- As illustrated by **an Area Under Curve (AUC) of 1,** the obtained ROC curve confirms **the excellent diagnostic and discrimination capacity of the ID Screen® BlueTongue Competition ELISA.**

REPEATABILITY

Intra-plate repeatability was evaluated by measuring the coefficient of variation (CV%) for 36 repetitions of a negative sample, and 60 repetitions of a weak positive sample.

Results are considered compliant if the CV% is less than 15%.

OD results are shown in Table 8 below.

OD AT 450 NM											
0.901	0.930	0.920	0.929	0.826	1.894	1.768	0.887	0.856	0.949	0.869	0.889
0.989	1.015	1.006	1.021	0.113	1.801	1.707	0.902	0.896	0.958	0.931	0.985
O.888	0.960	0.873	0.980	0.888	1.771	1.833	0.967	0.928	0.073	O.918	0.868
1.916	1.882	1.765	1.730	1.785	1.824	1.713	1.732	1.718	1.859	1.872	1.877
1.942	1.838	1.785	1.764	1.780	1.755	1.847	1.854	1.836	1.864	1.822	1.871
1.032	0.994	0.991	1.016	1.006	1.797	1.854	0.934	0.946	1.0720	0.936	0.953
1.067	1.000	0.874	0.918	0.907	1.929	1.828	0.959	0.920	0.972	0.907	0.959
1.022	1.029	0.938	0.950	1.013	1.881	2.013	0.943	0.920	0.990	0.928	0.955

	AVERAGE OD	STANDARD DEVIATION	MINIMUM	MAXIMUM	CV%
WEAK POSITIVE SAMPLE	0.949	0.054	0.826	1.072	**6**
NEGATIVE SAMPLE	1.825	0.070	1.707	2.013	**4**

Table 8: Repeatability study for the ID Screen® Bluetongue Competition ELISA

RESULTS (Table 8):

- The CV% obtained was 6% for the weak positive sample and 4% for the negative sample, demonstrating **excellent test repeatability**.

REPRODUCIBILITY

A positive serum was diluted in a negative serum pool in order to generate a weak positive serum. This threshold dilution was tested in 9 independent runs by different operators and on different days. Results are considered compliant if the values are within ± 2 standard deviations around the mean and the CV% is less than 15%. Results are shown in Figure 4.

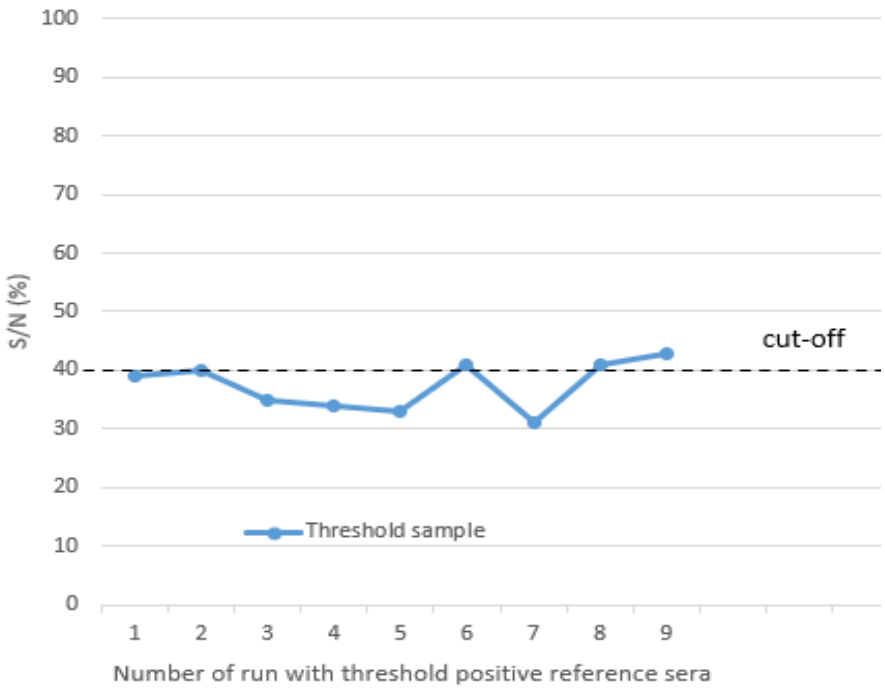

Figure 4: S/N % for a positive serum diluted in a negative serum pool and tested in 9 independent runs

RESULTS (Figure 4):

- All values are within a range of 2 standard deviations around the mean, with a CV of 11%.
- These results illustrate the **high reproducibility** of the ID Screen® Bluetongue Competition ELISA test.

ROBUSTNESS

Test robustness was evaluated by 3 operators in 3 independent runs.

Robustness was evaluated by testing the maximum and minimum conditions of time and temperature of incubation as defined in the instructions for use:

- Samples incubation: 45 minutes ± 4 minutes at 21°C (± 5°C);
- Conjugate incubation: 30 minutes ± 3 minutes at 21°C (± 5°C);
- Substrate Solution incubation: 15 minutes ± 2 minutes at 21°C (± 5°C).

For each condition. the test is validated if:

- The mean value of the negative control OD (OD_{NC}) is greater than 0.7 ($OD_{NC} > 0.7$).
- The ratio of the mean values of the positive and negative controls (OD_{PC} and OD_{NC}) is greater than or equal to 0.3 ($OD_{PC} / OD_{NC} < 0.3$)

Optical densities at 450nm obtained in each condition for both negative and positive controls and the S/N% values obtained for 3 dilutions of a positive reference serum and 2 negative samples are detailed in Table 9 below.

SAMPLES/CONJUGATE/SUBSTRATE INCUBATION TIME		**45 MIN / 30 MIN / 15 MIN**			**41 MIN / 27 MIN / 13 MIN**	**49 MIN / 33 MIN / 17 MIN**	
TEMPERATURE OF INCUBATION		**16°C**	**21°C**	**26°C**	**16°C**	**26°C**	
Positive control		0.076	0.081	0.069	0.070	0.085	OD 450 NM
		0.073	0.075	0.073	0.070	0.070	
Negative Control		1.049	1.433	1.532	0.905	1.828	
		0.983	1.406	1.575	0.892	1.722	
$OD_{NC} > 0.7$		√	√	√	√	√	
$OD_{PC} / OD_{NC} < 0.3$		√	√	√	√	√	
Positive reference serum	diluted 1 :5	16%	11%	10%	16%	8%	S/N%
	diluted 1 :10	36%	34%	28%	39%	26%	
	diluted 1 :20	66%	66%	63%	71%	57%	
Negative sample	1	109%	104%	117%	106%	105%	
	2	104%	102%	113%	107%	102%	

Table 9: Robustness study for the ID Screen® Bluetongue Competition ELISA

RESULTS (Table 9):

- **For each time and temperature condition**, **the test validation criteria** for both positive and negative controls **were obtained, the S/N% values obtained were similar**, and **analytical sensitivity was constant,** thereby demonstrating the **excellent robustness of the ID Screen ELISA.**

STABILITY

The shelf-life of the products is evaluated by the technique of accelerated ageing .

The stability of the plates, the negative control, and the conjugate was tested by evaluating the residual activity of individual components after storage at 37°C ± 2°C, with respect to storage at 5°C ± 3°C. The measured residual activity at 37°C ± 2°C should be greater than 75% after two months. Results are shown in the Figure 5 below.

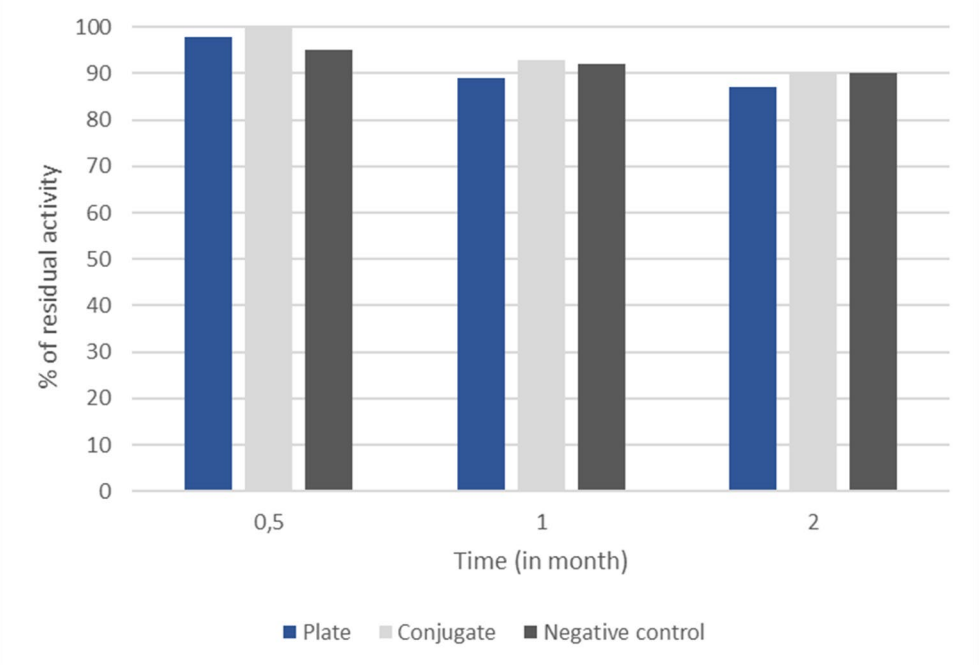

Figure 5: Percentage of residual activity of the plates, negative control and conjugate after stability testing at 37°C

RESULTS (Figure 5):

- After **2 months at 37°C**, the **plates, the conjugate and the negative control** showed **residual activity of 87 %, 90%, 90 %** respectively, thus indicating **high component stability**.

CONCLUSION

The **ID Screen® Bluetongue Competition** ELISA:

- demonstrates excellent specificity and sensitivity (with detection of all BTV serotypes and no cross-reactivity with EHDV).
- can detect Bluetongue antibodies in bovine (cattle and buffalo), ovine, caprine, or other susceptible species.
- detects seroconversion at least 7 days post infection.
- excellent stability, repeatabilty, robustness and reproducibility

The kit is an easy-to-use and reliable tool for Bluetongue disease surveillance.

ADDITIONAL DATA (external studies)

The ID Screen® Bluetongue Competition ELISA test is already widely used in the field for seroepidemiological studies for cattle, sheep, and goats. Literature provides many publications on the use of the kit on these species.

It has also been used on buffalo sera (Ferrara G. *et al.*, 2024[(2)], Puri et al., 2022[(3)]; Douangngeun B. *et al.*, 2016[(4)]) and camelids sera (Shabbir M. Z. *et al*., 2020[(5)], Schulz C. *et al*., 2012[(6)]).

For more information, please contact info@innovative-diagnostics.com.

REFERENCES

(1) WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, thirteenth edition 2021; CHAPTER 3 .1.3. BLUETONGUE (INFECTION WITH BLUETONGUE VIRUS).

(2) Ferrara G. *et al.* (2024). Bluetongue virus seroprevalence and risk factor analysis in cattle and water buffalo in southern Italy (Campania region). Veterinary Research Communications, 48(1), 579-584.

(3) Puri B. *et al*. (2022). Seroprevalence of bluetongue disease among domestic ruminants raised in International Border Areas of Nepal. Sarhad Journal of Agriculture, 38(2), 555-562.

(4) Douangngeun B. *et al.* (2016). Seroprevalence of Q fever, brucellosis, and bluetongue in selected provinces in Lao People's Democratic Republic. The American journal of tropical medicine and hygiene, 95(3), 558.

(5) Shabbir M. Z. *et al.* (2020). Sentinel surveillance of selected veterinary and public health pathogens in camel population originating from Southern Punjab province, Pakistan. Acta tropica, 205, 105435.

(6) Schulz C. *et a*l. (2012). Experimental infection of South American camelids with bluetongue virus serotype 8. Veterinary Microbiology, 154(3-4), 257-265.

RELATED PRODUCTS

For associated products, please consult the Innovative Diagnostics website: www.innovative-diagnostics.com.

HISTORY OF REVISIONS

VERSION	EDIT DATE	REFERENCE	TYPE OF REVISION	REVISION MADE
	10/2019	doc831	First edition	NA
1217	10/2024	doc1405	Update: Addition/Edition of validation data	• Updated introduction. • Updated data for diagnostic specificity and sensitivity on buffaloe samples. • Addition of new chapters with their associated data: - Cut-off determination - Reproducibility - Robustness - Stability - Additional data (External studies). • Updated references and conclusion.

info@innovative-diagnostics.com | www.innovative-diagnostics.com| + 33 (0)4 67 41 49 33