Internal validation report

ID Screen® Influenza A Antibody Competition Multi-species

Competitive ELISA for the detection of antibodies against the nucleoprotein of the Influenza A virus in avian, porcine, or equine serum, plasma or porcine oral fluid.

- **Highly sensitive and specific**
- **Convenient multi-species test** applicable to birds, horses, swine and other species
- **Cost-effective** for the analysis of large sample size, particularly in comparison with Haemagglutination Inhibition (HI) assay
- **Easy-to-use,** with results available under 2 hours
- **New improved protocol for samples from different avian species**

Introduction

The influenza viruses A, B and C, of the family *Orthomyxoviridae*, are responsible for influenza diseases affecting humans and a variety of animals hosts. The viral types A, B, or C are defined by the nature of the internal nucleocapsid antigen. The A type infects humans as well as avian, porcine and equine species.

Type A influenza viruses are further divided into subtypes based on their Haemagglutinin (H) and Neuraminidase (N) antigens. Eighteen H antigens (H1 to H18) and eleven N antigens (N1 to N11) have been isolated. Some subtypes containing H5 and H7 are associated with highly pathogenic forms of the disease.

Given the need for rapid and reliable detection of antibodies directed against Influenza A virus nucleoprotein in different animal species, IDvet has developed the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA kit. This competitive ELISA provides results faster than other diagnostic techniques (AGID, HI). It can be applied to a range of species (horses, pigs and birds), since it is not species dependant.

Kit principle

Samples to be tested and controls are added to the microwells coated with Influenza A nucleoprotein (NP). Anti-NP antibodies, if present, form an antibody-antigen complex which masks the NP epitopes.

An anti-NP-peroxidase (HRP) conjugate is added to the microwells. It binds to the remaining free epitopes, forming an antigen-conjugate-HRP complex. After washing in order to eliminate the excessive amount of conjugate, the substrate solution (TMB) is added.

The resulting coloration depends on the quantity of specific antibodies present in the specimen to be tested. In the absence of antibodies, a blue solution 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.

Result interpretation:

For each sample, the S/N percentage (S/N%) is calculated: $[OD_{sample} / OD_{NC}] \times 100$.

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

RESULT	STATUS
S/N % ≤ 45 %	POSITIVE
45 % < S/N % < 50 %	DOUBTFUL
S/N % ≥ 50 %	NEGATIVE

Avian

Analytical sensitivity

Serial dilutions of an AGID (Agar Gel Immunodiffusion) positive control were tested. This serum becomes negative with the AGID method when diluted from 1:8 to 1:12 in negative sera.

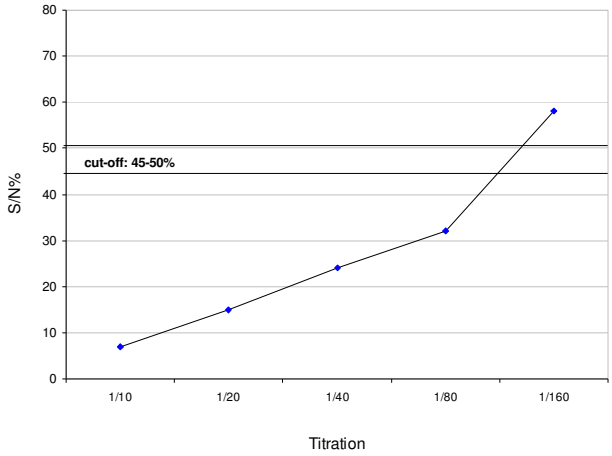


Figure 1: Titration of an AGID-positive serum control using the ID Screen® ELISA.

Results (Figure 1):

- The serum was found positive up to the dilution of 1:80 (included) using the **ID Screen®** ELISA.
- The analytical sensitivity of the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA was 10 times higher than the one of the AGID method.

Specificity

The following sera from disease-free flocks, all negative by hemagglutination inhibition (HI) assay, were tested with the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA:

- 200 chicken sera from France (before 2014)
- 200 chicken sera from Belgium (SPF chickens),
- 100 turkey sera from northern Italy,
- 100 samples from other bird species (duck, ostrich) from China.

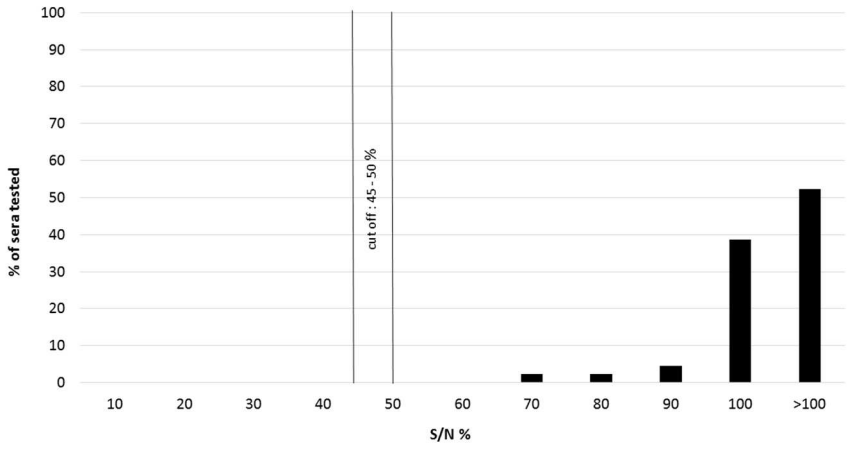


Figure 2: S/N% Distribution for the samples tested (n=600).

Results (Figure 2):

- All samples for all species tested with the **ID Screen®** ELISA were found negative.

  Measured specificity = 100% ($CI_{95\%}$: 99.36% - 100%), n = 600.

Sensitivity

Correlation with HI

The following sera from vaccinated chickens and turkeys were tested in parallel using the Haemagglutination Inhibition (HI) assay (with homologous strain) and the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA:

Strain	Samples	Origin	Titer HI (log_2)
H7N1	8 chickens	SPF vaccine trial	11
H7N4	8 chickens	SPF vaccine trial	11
H5N9	12 chickens	SPF vaccine trial	10
H5N9 / H7N1	50 chickens	northern Italy	2-11
H5N9 / H7N1	50 turkeys	northern Italy	1-6

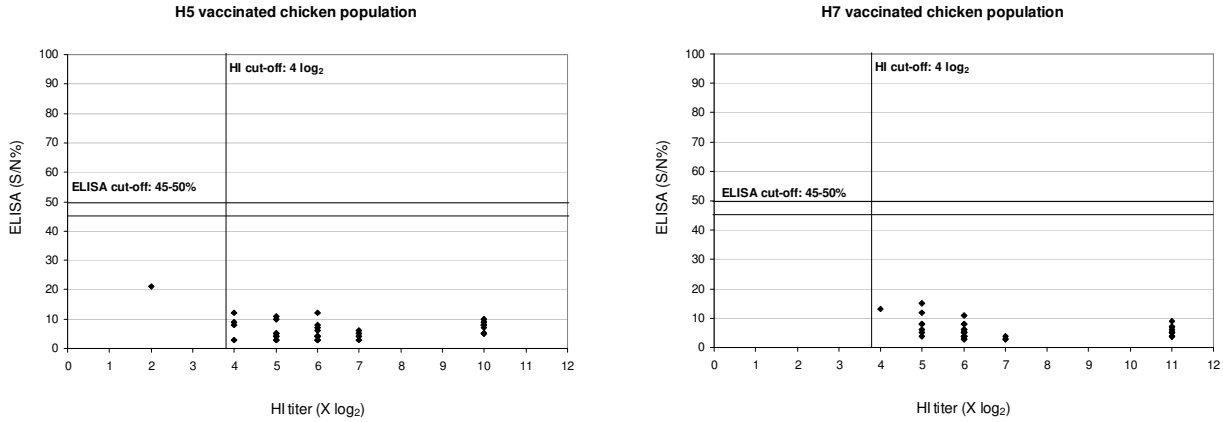


Figure 3: Correlation between the ID Screen® ELISA and HI assay, for chicken sera.

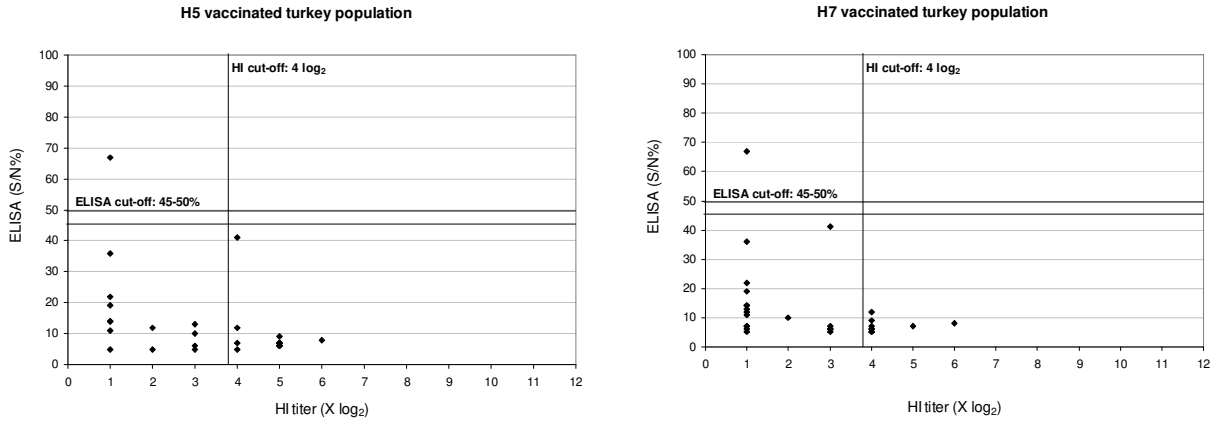


Figure 4: Correlation between the ID Screen® ELISA and HI assay, for turkey sera.

Results (Figure 3 & 4):

- With the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA, as generally observed with other techniques such as HI assay, chickens presented higher antibody titres than turkeys.
- OIE considers that HI titres may be regarded as positive if there is inhibition at a serum dilution of 1:16 ($4log_2$). While all vaccinated chickens were detected positive by HI assay, only 32% and 50% of vaccinated turkeys were detected positive by HI assay for H5 and H7 respectively. The **ID Screen® Influenza A Antibody Competition Multi-species** ELISA, however, detected all vaccinated animals as positive (except one turkey with a titre of 1:2 or $1log_2$ in HI). The **ID Screen® Influenza A Antibody Competition Multi-species** ELISA is more sensitive than the classical HI assay for the detection of anti-Influenza A antibodies; it is therefore, better suited for vaccinated population surveillance (since some vaccinated samples considered as HI-negative will give positive ELISA results).
- The **ID Screen® Influenza A Antibody Competition Multi-species** ELISA is able to detect both low and high levels of anti-Influenza A virus antibodies (up to 11 log_2).

Naturally-infected animals

The following studies where carried out using the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA.

Bird serum samples

15 Influenza A strains from naturally-infected animals were tested:

- H5N2
- H5N6
- H5N9
- H3N8
- H4N6
- H6N1
- H6N2
- H7N1
- H8N4
- H9N2
- H9N7
- H10N7
- H10N8
- H13N6
- H14N5

20 naturally-infected chickens from Belgium, confirmed as positive by H7 and H5 HI, were also tested.

Results:

- All Influenza A samples and strains were detected positive.
- All 20 naturally-infected animals were also found positive.

Duck serum samples:

13 duck sera infected with H5N8 (from the southwest France, 2016) and 24 duck sera infected with H5 (from South Korea) were tested.

Results:

- 36 / 37 duck sera were found positive
  Measured sensitivity for duck sera = 97.3% ($CI_{95\%}$: 86.18% - 99.52%), n = 37

Vaccinated animals

Serum samples from hyperimmunized chickens:

3 sera samples, obtained by a four-fold immunization of chickens with AIV H5 (strains H5N2, H5N3 and H5N8), were tested.

Results:

- All H5 strains samples were detected positive using the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA.

Turkey serum samples:

10 sera from turkeys immunized with a specific H9 vaccine (from Germany) were tested.

Results:

- All the sera from turkeys immunized with a specific H9 vaccine were detected positive using the ID Screen® ELISA.

Swine

Analytical sensitivity

Serial dilutions of three sera hyper-immunized (obtained by a four-fold immunization of pigs) were tested in parallel using the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA and 2 other commercial iELISAs.

	ID Screen® Influenza A Antibody cELISA			H1N1 iELISA		H3N2 iELISA	
Serum HI Titer	**H1N2 640**	**H3N2 2560**	**H1N1 1280**	**H1N2 640**	**H1N1 1240**	**H3N2 2560**	**H1N2 640**
Pure	5 (+)	5 (+)	6 (+)	114 (+)	167 (+)	110 (+)	26 (-)
1/4	6 (+)	4 (+)	6 (+)	57 (+)	107 (+)	72 (+)	12 (-)
1/16	33 (+)	8 (+)	25 (+)	18 (-)	47 (+)	26 (-)	3 (-)
1/64	84 (-)	57 (-)	81 (-)	3 (-)	14 (-)	5 (-)	1 (-)
1/256	98 (-)	84 (-)	96 (-)	1 (-)	2 (-)	1 (-)	3 (-)
1/1024	108 (-)	100 (-)	98 (-)	2 (-)	1 (-)	3 (-)	1 (-)
1/4096	101 (-)	93 (-)	95 (-)	1 (-)	1 (-)	4 (-)	3 (-)
1/16384	102 (-)	94 (-)	91 (-)	1 (-)	2 (-)	4 (-)	4 (-)

Table 1: Titration of three sera hyper-immunized (H1N1, H1N2, H3N2).

Results (Table 1):

- The **ID Screen® Influenza A Antibody Competition Multi-species ELISA** shows a slightly higher analytical sensitivity than the ones of the other commercial iELISAs tested.

Specificity

The following sera from disease-free herds were tested:

- 88 sera from Norway,
- 75 SPF sera.

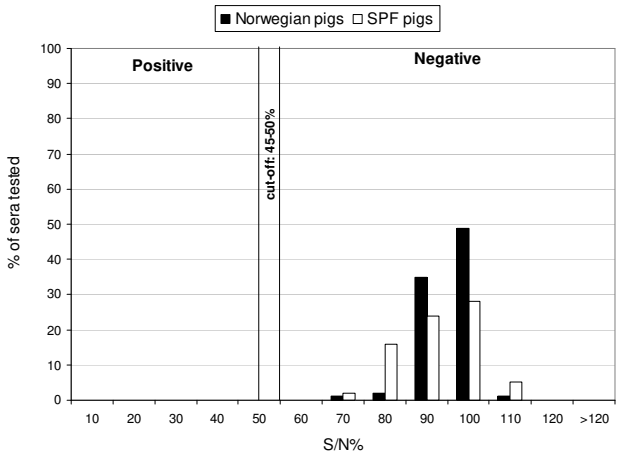


Figure 5: S/N% distribution in a disease-free population.

Results (Figure 5):

- All samples gave negative results.

  Measured specificity = 100% ($CI_{95\%}$: 97.7% - 100%), n = 163.

Correlation with HI assay and Virus Neutralization test (VNT)

The following sera were tested:

- 120 swine sera from herds without any clinical signs but of unknown disease status,
- 25 sera from vaccinated pigs.

These sera were tested in parallel using:

- the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA (ID Screen® cELISA),
- two indirect commercial ELISAs: H1N1 and H3N2,
- Haemagglutination Inhibition (HI): subtype Bakum/909/93 (H3N2), Bakum/1832/00 (H1N2), Potsdam/1/81 and Bakum/3543/98 (H1N1),
- Virus neutralisation test (VNT).

	Number of positive results among the samples tested in Herd A								
	Indirect commercial ELISA		**ID Screen® cELISA**	**HI**			**VNT**		
	H1N1	H3N2		H1N1	H3N2	H1N2	H1N1	H3N2	H1N2
20 young sows	5/20	0/20	8/20	-	-	-	3/8	0/8	0/8
20 male pigs (40-50 Kg)	0/20	0/20	1/20 (+) 1/20 (+ / -)	-	-	-	1/2	0/2	0/2
20 Piglets (20Kg)	1/20	0/20	1/20	-	-	-	1/1	0/1	0/1

	Number of positive results among the samples tested in Herd B								
	Indirect commercial ELISA		**ID Screen® cELISA**	**HI**			**VNT**		
	H1N1	H3N2		H1N1	H3N2	H1N2	H1N1	H3N2	H1N2
20 young sows	2/20	0/20	9/20	-	-	-	3/9	0/9	0/9
40 male pigs	4/40	0/40	9/40 (+) 3/40 (+ / -)	-	-	-	5/9 1/3	0/12	0/12

Table 2: Samples from 2 different herds without clinical signs.

	Indirect commercial ELISA		**ID Screen® cELISA**	**HI**		
	H1N1	N3N2		H1N1	H3N2	H1N2
25	21/25	20/25	21/25	13/25	13/25	0/25

Table 3: Samples from vaccinated pigs.

Results (Tables 2 & 3):

- All HI results were negative, however VNT and ELISAs results indicated that there has been viral exposure in the past.
- The difference in antibody detection may be due to the antigens used for the HI or to the nature of the antibodies detected.
- The capacity of the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA to detect older viral exposure was superior to the one of HI.

Porcine oral fluid samples

Monitoring of Influenza A virus in pig herds can be performed by testing pen-based oral fluids using ELISA technique. A trial study, led by the IVD GmbH, Seelze (near Hannover) Germany, was conducted in 2 successive batches of finishers, in a commercial herd in northwest Germany. The results were published and kindly shared by Dr. Katrin Strutzberg-Minder, IVD GmbH, Seelze, Germany [ref. 1].

Each batch consisted of approximately 100 pigs housed in one room, with 4 pens, with approximatively 25 pigs per pen. Oral fluids were collected once a week from each of the 4 pens, starting when the pigs were 12 weeks old and continuing until they were 24 weeks old for Batch 1 and 22 weeks old for Batch 2. In order to collect the samples, one rope was placed, in the morning prior to feeding, in each pen, and it was left for chewing for 10 to 15 minutes. The samples were chilled and shipped on ice within 24 hours to the IVD GmbH laboratory, Seelze, Germany. Serum samples were also collected from four pigs, randomly selected, for each pen at 12, 16 and 20 weeks for both batches and at 24 weeks for Batch 1 only.

IAV infection was monitored using conventional reverse transcription PCR (RT-PCR) for oral fluid testing. In each batch, 1 pen-based oral fluid sample was found positive for IAV when the pigs were 15 weeks old.

In order to evaluate the suitability of pen-based oral fluids for the monitoring of Influenza A virus (IAV) infection, the samples were tested in comparison with the usual monitoring method, i.e. testing of serum samples.

All the pen-based oral fluids and individual serum samples were tested for both batches, starting at 12 weeks of age, using the ID Screen® Influenza A Antibody Competition Multi-Species to detect antibodies against IAV.

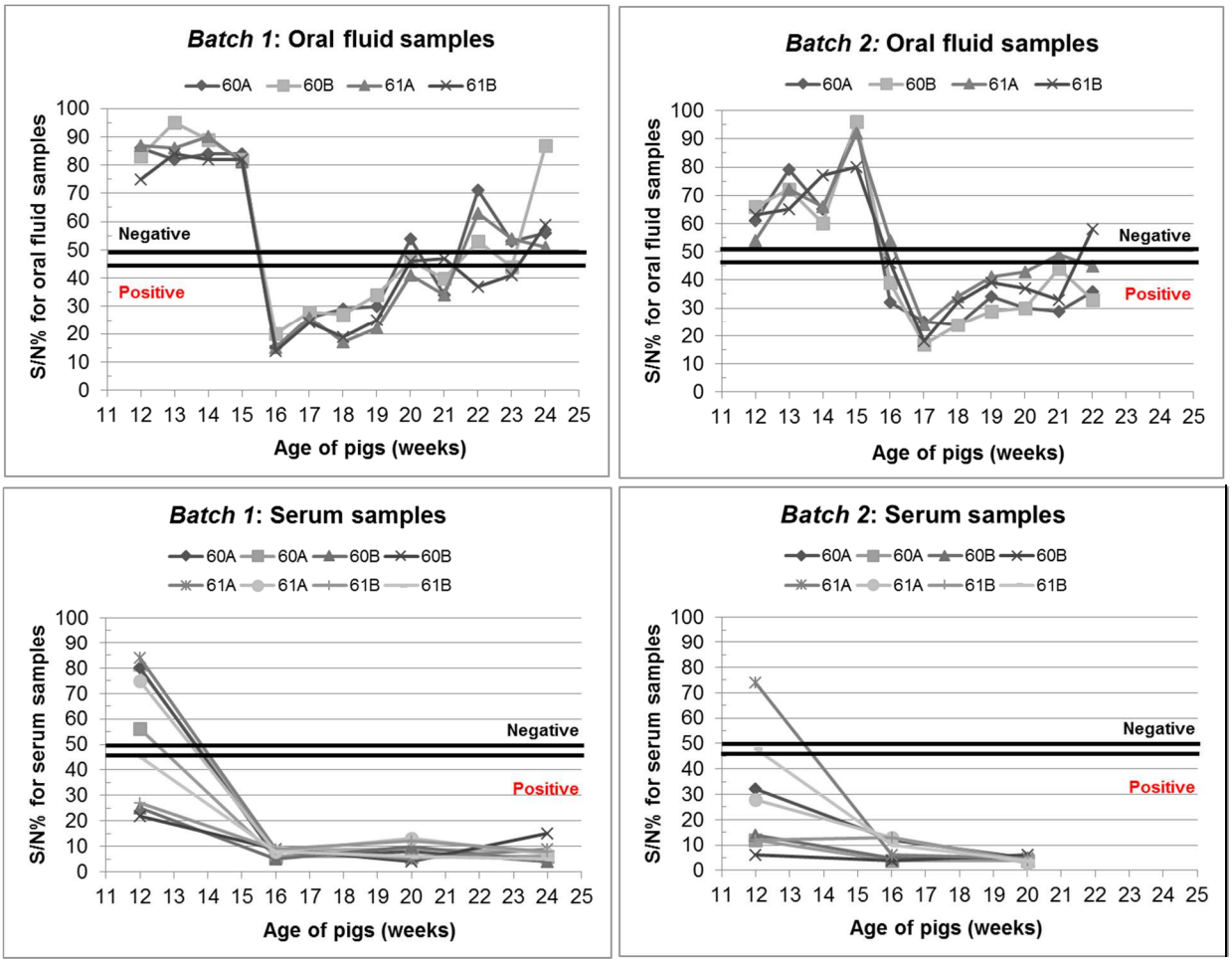
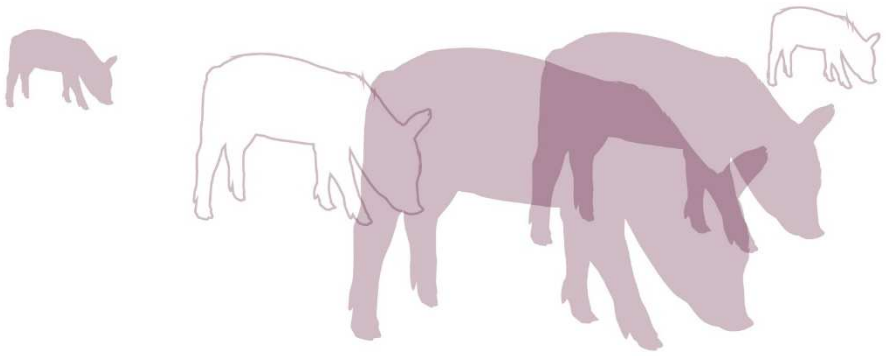


Figure 6: Results obtained for oral-fluids and serum samples using the ID Screen® ELISA

Results (Figure 6):

- At 12 weeks of age, 4 or 6 of the 8 individual serum samples from pigs of Batch 1 or Batch 2, respectively, were found positive by the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA while the pen-based oral fluids samples were all negative.
- With the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA, the oral fluid samples continued to test positive for IAV antibodies for up to 7 weeks, after the initial detection of IAV RNA by RT-PCR, while the serum samples remained positive.
- Oral fluid samples can be used for the monitoring of **recent** IAV infections in pig pens using the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA.



Equine

This chapter summarizes results obtained from a study performed by the Investigation and Diagnostic Centre Wallaceville (New Zealand) (ref. 2) using the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA, 2 other commercial ELISAs and 1 in-house ELISA.

Specificity

365 influenza-naïve samples from horses within New Zealand, unexposed to vaccination or natural disease, were tested. These samples are considered as disease-free because Equine Influenza Virus (EIV) is not present in the country.

Samples were collected between November 2008 and March 2009.

Sensitivity

99 serum samples from vaccinated Australian horses that had been infected during the 2007 EI (Equine Influenza) outbreak were tested.
These samples were confirmed as positive by real time RT-PCR test.

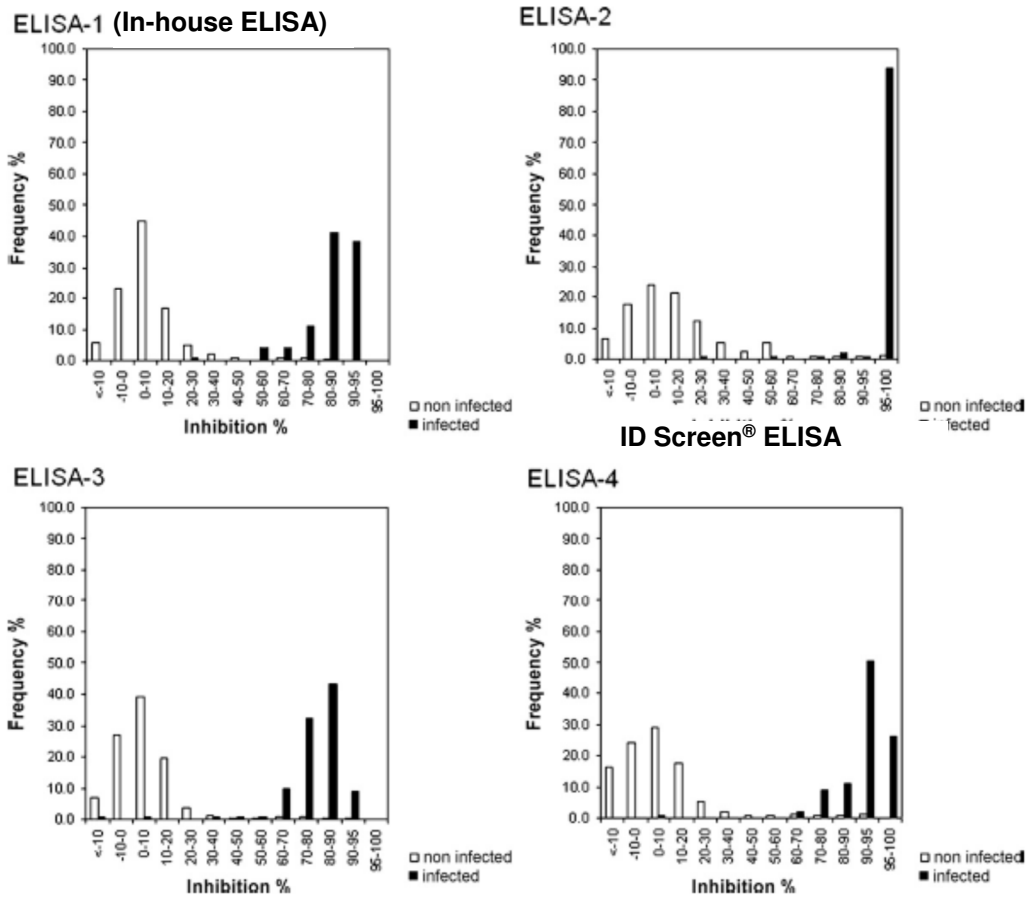


Figure 7: PI% for infected horses from Australia (n = 99) and non-infected horses from New Zealand (n = 365).

Results (Figure 7):

- The **ID Screen® Influenza A Antibody Competition Multi-species** ELISA showed the clearest separation between positive and negative samples with respect to the 3 other commercial ELISAs.
- **Measured specificity = 95.3% ($CI_{95\%}$: 92.7 – 97.3%), n = 365.**
- **Measured sensitivity = 99.0% ($CI_{95\%}$: 94.5 – 99.9%), n = 99.**

Seroconversion kinetics

3 horses were experimentally-infected with the EIV and were bled at 0, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 17 and 20 days post-infection (dpi).
In addition to the ELISA testing, these samples were also tested by Haemaglutination Inhibition (HI).

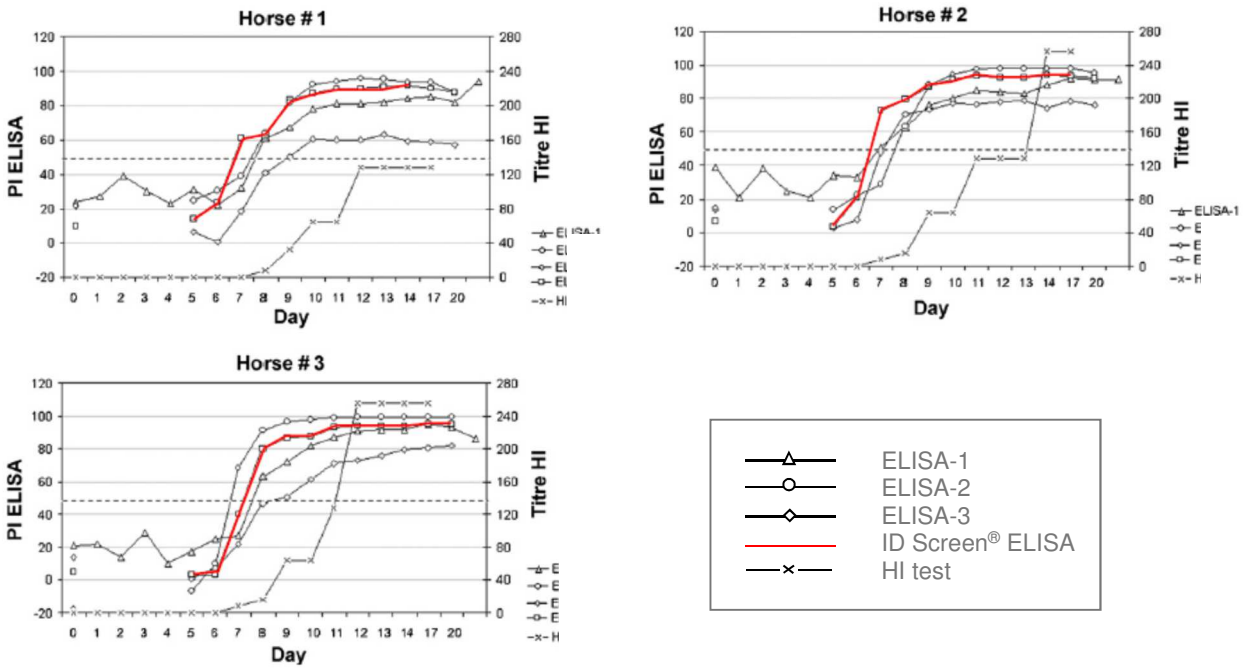
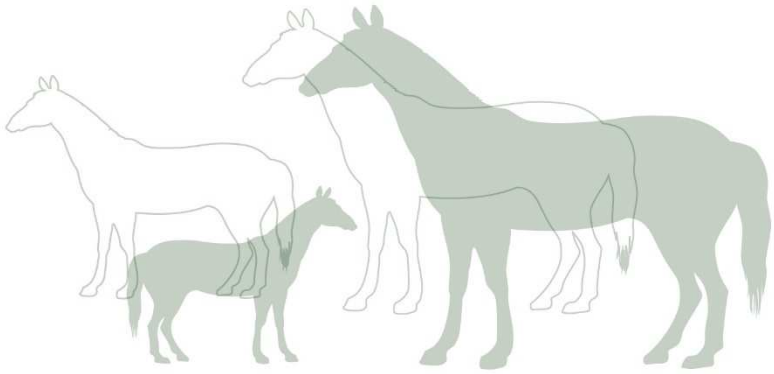


Figure 8: Seroconversion kinetics on experimentally-infected horses.
Note: for the ELISA-2, -3 and the ID Screen® Influenza A Antibody Competition Multi-species ELISA: samples from day 1 to day 4 post-infection were not available.

Results (Figure 8):

- The **ID Screen® Influenza A Antibody Competition Multi-species** ELISA detected seroconversion at 7 days post-infection.
- The **ID Screen® Influenza A Antibody Competition Multi-species** ELISA detected seroconversion earlier than the 3 other commercial tests: ELISAs and HI.



Conclusion

The ID Screen® Influenza A Antibody Competition Multi-species ELISA demonstrates:

- excellent specificity and sensitivity,
- a superior analytical sensitivity than the HI and AGID methods and slightly higher than indirect ELISAs,
- a more effective detection of vaccinated animals than HI assay,
- an earlier detection of seroconversion than other commercial ELISAs and HI assay.
- a good detection of infections by the H5N8 virus (new clade 2.3.4.4).

The **ID Screen® Influenza A Antibody Competition Multi-species** ELISA kit is an excellent, efficient tool for the detection of anti-Influenza A specific antibodies in avian, swine and equine serum and plasma samples.

The kit can be used on pen-based oral fluids to monitor IAV infection dynamics, notably in commercial pig populations.

As it is a competitive test, the **ID Screen® Influenza A Antibody Competition Multi-species** ELISA can be applied to a large variety of species.

Reference

(1) Strutzberg-Minder K., Boehmer J., Fischer S., Homuth M., Gomez-Duran O., Finger G., Genzow M. Monitoring influenza A virus infection in pigs by using a competitive enzyme-linked immunosorbent assay to detect virus antibodies in pen-based oral fluid specimens. Journal of Swine Health and Production, 2015, Volume 23, Issue 3, p. 126-131.

(2) Kittelberger R., McFadden A.M.J., Jenner J., Bueno R., Vait J., Kirkland P.D., Delbridge G., Heine H.G., Selleck P.W., Pearce T.W., Pigott C.J., O'Keefe J.S. Comparative evaluation of four competitive/blocking ELISAs for the detection of influenza A antibodies in horses. Veterinary Microbiology, 2011, Volume 148, Issues 2-4, p. 377-383.

Associated products

ID Screen® ELISAs:

- **ID Screen® Influenza A Nucleoprotein Indirect (product code: FLUNPS):** Indirect ELISA for the detection of antibodies against the Influenza A virus nucleoprotein in chicken or turkey serum or plasma.
- **ID Screen® Influenza H9 Indirect (product code: FLUH9S):** Indirect ELISA for the detection of antibodies against the hemagglutinin H9 of the Avian Influenza in serum or plasma from chickens or turkeys.
- **ID Screen® Influenza A Antibody Competition Multi-species (product code: FLUACA):** Competitive ELISA for the detection of antibodies against the nucleoprotein of the Influenza A virus in avian, porcine, or equine serum, plasma or porcine oral fluid.
- **ID Screen® Influenza H7 Antibody Competition (product code: FLUACH5):** Competitive ELISA for the detection of antibodies against the hemagglutinin H5 of the Avian Influenza virus in avian serum.
- **ID Screen® Influenza H9 Antibody Competition (product code: FLUACH9):** Competitive ELISA for the detection of antibodies against the hemagglutinin H9 of the Avian Influenza virus in avian serum.
- **ID Screen® Influenza A Antigen Capture (product code: INFLAG):** Sandwich ELISA for the detection of Antigen A of the Influenza virus in bronchopulmonary liquids from pigs, horses and birds, as well as in bird cloacal swab samples and in bird faeces.

Internal reference material:

Internal reference sera to be used for quality control with the ID Screen® ELISA kits.

- **Ready-to-use positive serum FLU-C (product code: MRI-FLUC-RTU):** Ready-to-use pool of positive chicken sera which contains significant and known antibody level against Avian Influenza virus.

  For use with the following ID Screen® indirect ELISAs: FLUACA, FLUACH5, FLUACH7, FLUACH9.
- **Ready-to-use negative SPF chicken serum (product code: MRINEG-BIRD-RTU):** Ready-to-use pool of specific pathogen free (SPF) chicken sera. Does not contain any antibodies against avian pathogens.

  For use with all IDvet kits.

ID Gene® PCR:

- **ID Gene® Influenza A Duplex (product code: IDFLUA):** Real-time RT-PCR assay for the qualitative detection of the Avian Influenza A virus. Suitable samples: avian tracheal, oropharyngeal or cloacal swabs, organs and nucleic acid storing cards (individual samples or pools of up to 5).

History of revisions

Version	Edit date	Reference	Type of revision	Revision made
0917	03/2019	DOC747	Update	Inclusion of Associated products section

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