OIE Procedure for Registration of Diagnostic Kits

Abstract sheet

Name of the diagnostic kit: BOVIGAM® - *Mycobacterium bovis* Gamma interferon test kit for cattle
Manufacturer: Prionics AG
OIE Approval number: 20150110
Date of Registration: May 2015

Disease: Bovine Tuberculosis

Pathogen Agent: *Mycobacterium bovis* and other mycobacteria belonging to the tuberculosis complex (e.g. *M. caprae*)

Type of Assay: Sandwich ELISA

Purpose of Assay: Certified by the OIE fit for the detection of cell mediated immune response to infection with *Mycobacterium bovis* and other mycobacteria belonging to the tuberculosis complex on analysis of whole blood specimens in cattle, buffalo (*Syncerus caffer*), goat and sheep (provisionally) for the following purposes:

1. Historical freedom
2. Re-establishment of freedom after outbreaks
3. Certify freedom from infection or agent in individual animals or products for trade/movement purposes
4. Eradication of infection from defined populations
5. Confirmatory diagnosis of suspect or clinical cases (includes confirmation of positive screening test)
6. Estimate prevalence of infection to facilitate risk analysis (surveys/herd health schemes/disease control)
7. Ancillary test for eradication of Tuberculosis

Species and Specimen: Cattle, Buffalo (*Syncerus caffer*), Goats and Sheep (provisionally) - blood-based in vitro laboratory test

1. Information on the kit

Please refer to the kit insert available on the OIE Registry web page or contact manufacturer at:

Website link: http://www.prionics.com/tuberculosis/

Email address: info@prionics.com

2. Summary of validation studies

Analytical characteristics

Analytical sensitivity

BOVIGAM® is adjusted to detect 80 pg/ml of recombinant bovine IFN-ɣ.

Whole blood stimulation: Analytical sensitivity of the stimulation part cannot be evaluated as the detection limit depends on the bovine Tb status of the tested animal. In principal whole blood samples between 1.5 ml and 250 µl have been tested and were assessed as suitable for the diagnosis of bovine Tb. The effect of lymphocyte count on reliability and detection limit is unknown. Lymphocyte counts may vary from cattle to cattle. The minimum number required for a reliable result has not been established.

Analytical specificity

Recombinant bovine IFN-ɣ, α and β were assayed in BOVIGAM® at biologically active concentrations of 1, 10 and 1000 ng/ml respectively. BOVIGAM® did not detect Interferon-α and –β samples. Reactivity of purified protein derivative from *Mycobatcerium bovis* (PPDB) and purified protein derivative from *Mycobacterium avium* (PPDA) stimulated whole blood samples derived from cattle infected with *M. tuberculosis*, *M. africanum*, *M. microti*, *M. canetti*, *M. pinnipedi*, *M. caprae*, who belong to the tuberculosis complex mycobacteria, lead to true positive results in BOVIGAM® and cannot be interpreted as cross reactive or false positive.

***Repeatability data*:**

Within run repeatability data:

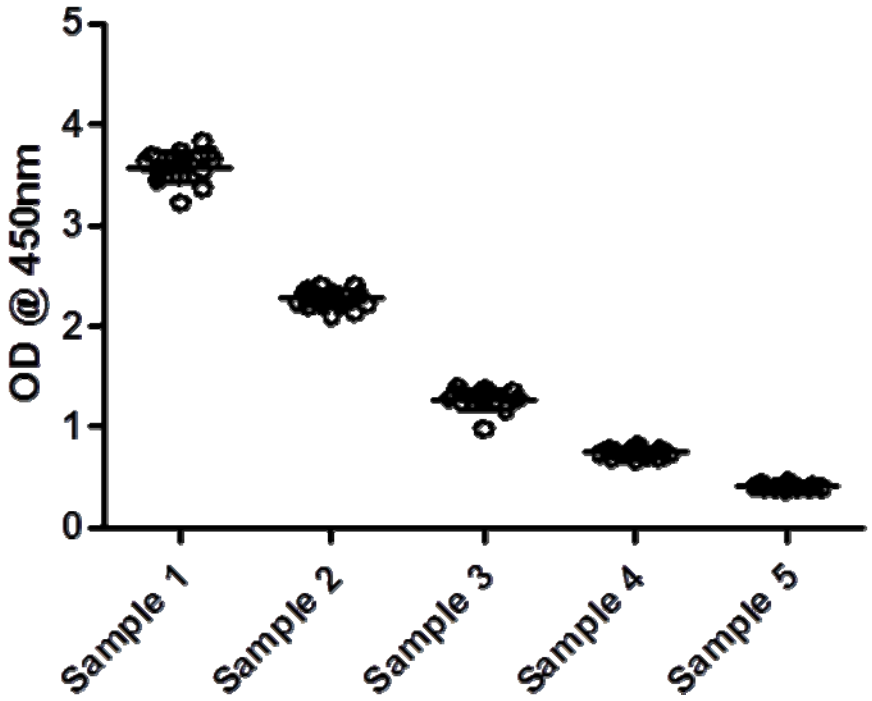
Aim: To demonstrate that the BOVIGAM® ELISA has minimal well-to-well variation.

Methods: The within run repeatability of the BOVIGAM® ELISA was estimated by assaying 5 different concentrations of recombinant bovine IFN-γ in 16 replicates using a single test-kit lot (lot number 633261701). Each IFN-γ sample had an analyte concentration within the operating range of the assay.

Results: Figure 1 shows the optical density readings of the 16 replicates for each of the five concentrations of recombinant bovine IFN-γ. Horizontal lines and error bars represent the mean and standard deviation respectively. As detailed in table 1, the coefficient of variation was less that 10% for all five samples.

Conclusions: The BOVIGAM® ELISA displays excellent interwell repeatability for the detection of bovine IFN-γ at different concentrations across the operating range of the assay.

Figure 1:



Between run repeatability data

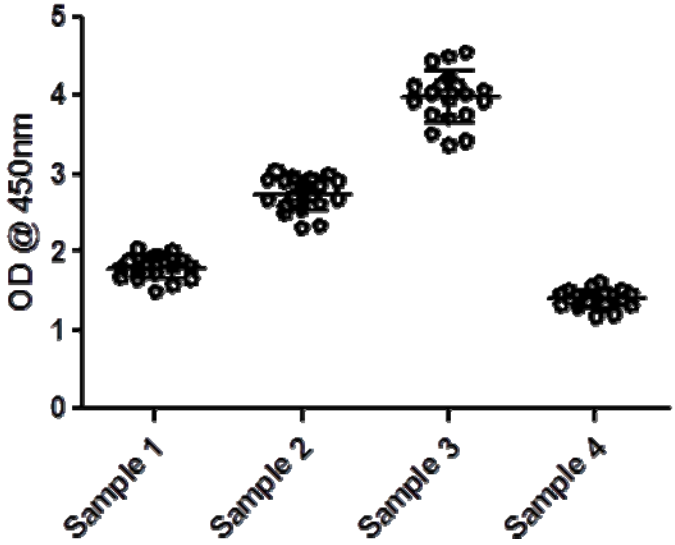
Aim: To demonstrate that the BOVIGAM® ELISA has minimal between run variation.

Methods: Four separate samples of bovine whole blood culture supernatants were aliquoted and stored frozen at -80°C. The antigens used for the stimulation of bovine whole blood generate samples 1, 2, 3 and 4 were avian tuberculin purified protein derivative (PPD-A), staphylococeal Enterotoxin B (SEB), early secreted antigen target 6kD protein (ESAT-6)/ culture filtrate protein 10 kD (CFP-10) peptide cocktail and Rv3615c peptide cocktail respectively. These samples were then used to assess the between run repeatability of the BOVIGAM® ELISA. Each sample was assayed in triplicate in a total of 19 runs, which were performed on 5 separate days by 2 different operators.

Results: Figure 2 shows the optical density readings of the four bovine whole blood culture supernatants run on 19 different occasions. Horizontal lines and error bars represent the mean and standard deviation respectively. As detailed in table 2 the coefficient of variation was less that 10% for all four samples.

Conclusions: The BOVIGAM® ELISA displays excellent between run repeatability for the detection of bovine IFN-γ in supernatants from bovine whole blood assays.

Figure 2: The BOVIGAM® ELISA has minimal between run variation.



Whole blood stimulation on cattle reactor variances of repeatability: Values differ between days less than 20%

Whole blood stimulated samples with pokeweed variances of repeatability: Values differ between days less than 6%

Diagnostic Characteristics

Threshold determination

Each country has to determine its own unique cut-off adopted to the regional cattle TB situation in the country.

Diagnostic sensitivity (DSn) and specificity (DSp) estimates

BOVIGAM®		**Target Species**			
		Cattle	**Buffalo**	**Goats**	**Sheep**
Diagnostic sensitivity*[1] (classical statistics with PPDs)	N DSn CI	8879 84.6% (95%CI = 73.0-95.5%)	2514 81.6-91.9%	472 58-100%	4 100%
Diagnostic specificity*[2] (classical statistics with PPDs)	N DSp CI	10966 97.4% (95%CI = 87.5-99.6%)	608 86.2-99.4%	140 96-100%	3 100%
Diagnostic sensitivity*[3] (Bayesian analysis with PPDs)	N DSn CI	4937 33.9-68.8%[+] n.a.	n.a.	n.a.	n.a.
Diagnostic specificity*[4] (Bayesian analysis with PPDs)	N DSp CI	4937 87.9-99.8%[+] n.a.	n.a.	n.a.	n.a.

Diagnostic sensitivity*[5] (Esat-6/CFP10)	N DSn CI	771 52.2%-85% n.a.[$]	n.a.	n.a.	4 100% n.a.
Diagnostic specificity*[6] (Esat-6/CFP10)	N DSp CI	2039 94%-98.9% n.a.[$]	n.a.	n.a.	3 100% n.a.

* Different cut-offs may apply; [$] Specificity and sensitivity estimates based on several studies thus a 95%CI is not given here; [+]Depending on test assumption

*1-2 cattle → The following different cut-offs have been applied for these studies

Criterion 1: BOD_COD > 0 and BOD_AOD > 0;
Criterion 2: BOD/COD > 1.25 and BOD_AOD > 0;
Criterion 3: BOD/COD > 1.5 and BOD_AOD > 0;
Criterion 4: BOD_COD P0.05 and BOD_AOD > 0;
Criterion 5: If BOD = 0.1, then BOD/COD > 1.5 and BOD_AOD > 0. If BOD > 0.1, then BOD_COD > 0.05 and BOD_AOD > 0;
Criterion 6: BOD_AODP0.1;
Criterion 7: BOD_COD P0.1 and BOD/AODP 1.8;
Criterion 8: BOD_COD P0.1 and BOD/AODP 1.25;
Criterion 9: BOD/AODP1.8;
Criterion 10: BOD_COD P0.05 and BOD/AODP 1.8 (”criterion 4 if BOD/AODP1.0);
Criterion 11: BOVIGAM®: BOD_COD P0.1 and BOD_AOD > 0;
Criterion 12: BOD_COD P2(COD) and BOD_AODP0.05;
Criterion 13: BOD_COD P0.1 and BOD_AODP 0.1;
Criterion 14: BOD_AODP0.04.
BOD: Mean optical density value of the plasma from the bovine PPD-stimulate blood.
AOD: Mean optical density value of the plasma from the avian PPD-stimulated blood.
COD: Mean optical density value of the plasma from blood incubated with phosphate buffered saline (nil antigen control).

*1-2 buffalo → The following different cut-offs have been applied for these studies

Criterion C1= BOD-AOD P0.05 and BOD-AOD > 0;
Criterion C4 = ODbovine readings < 0.385 are interpreted as test negative, and ODbovine readings≥0.385 are interpreted as test positive
Criterion 5= ODbovine – ODavian > 0.20 and if ODfortuitum – ODnil < 0.15, provided that ODnil < 0.25. In cases where ODfortuitum – ODnil > 0.15 the buffalo was classified as multiple reactor (MR).

*1-2 goats → The following different cut-offs have been applied for these studies

Criterion C2= IFN-c assay. Standard interpretation: Goat positive if bovine PPD OD minus no antigen sample ODP0.1 and bovine PPD OD > avian PPD OD. Severe interpretation: Goat positive if bovine PPD OD minus no antigen sample ODP0.05 and bovine PPD OD > avian PPD OD.

*1-2 cattle → The following different cut-offs have been applied for these studies

Criterion C3=OD indices (ODI), that is the ratio of the OD for stimulated cultures compared with the OD for control cultures. An ODI>2 is regarded as positive

*3-4 cattle, baysian analyis → a specific Cut-off did not apply as it is a baysian analysis, details see Using latent class analysis to estimate the test characteristics of the □-interferon test, the single intradermal comparative tuberculin test and a multiplex immunoassay under Irish conditions Tracy A. Clegg, Anthony Duignan, Clare Whelan, Eamonn Gormley, Margaret Good, John Clarke, Nils Toft, Simon J. More Veterinary Microbiology 151 (2011) 68–76

*5-6 cattle, ESAT6/CFP10→ The following different cut-offs have been applied for these studies

Criterion 1: Esat6/CFP10 > 0.1
Criterion 2: PPDB-PPDA > 0.1 And PPDB - Nil > 0.1
Criterion 3: PPDB-PPDA > 0.1 And Esat6/CFP10 > 0.1 (confirmatory)
Criterion 4: bPPD - PBS ≥0.05 and bPPD greater than aPPD
Criterion 5: Prionics PC-EC- Nil > 0.1 (confirmatory)

*5-6 Sheep, ESAT6/CFP10→ The following different cut-offs have been applied for these studies

Criterion C3=OD indices (ODI), that is the ratio of the OD for stimulated cultures compared with the OD for control cultures. An ODI>2 is regarded as

Comparative performance

	Diagnostic sensitivity	Diagnostic specificity
Skin Test - CCT	80%*	96.8%*
Skin Test – CFT/SCT	84%*	99.50%*

Agreement and discrepancies

High agreement between BOVIGAM® and the conventional bio-assay for bovine IFN-ɣ could be observed. BOVIGAM® demonstrates a higher sensitivity than the bioassay. comparative cervical

tuberculin/caudal-fold tuberculin/Single cervical tuberculin Skin tests: Bovine and/or Avian Tuberculin PPDs are administered intradermally and are thus *in vivo* diagnostics. In TB cattle, injection of bovine tuberculin PPD results in an immunological response at the site of injection. This is referred to as the Delayed Type Hypersensitivity (DTH) reaction and is observed as swelling and local inflammation of the skin (lesion). The thickness of the skin is measured with callipers 72 hours following injection. Avian tuberculin PPD is used to control for unspecific reactions. A full set of T-cells can be stimulated. BOVIGAM®: Is an *in vitro* test to stimulate whole blood samples with PPD's or other specific Antigens. A marker concentration, IFN-ɣ is measured. Predominantly, CD4+ cells are stimulated. Proportion of agreement is about 70% as the immune response behind the test system is different and other sub population of TB positive animals can be recognized with each test. In the table below several studies are displayed summarizing the proportion of agreement between skin test applications and BOVIGAM®.

Proportion of agreement between different skin test assays and BOVIGAM®.

Author	Species	Skin test	BOVIGAM®	Proportion of agreement	Kappa (ƙ)
Lopes et al., 2012	Cattle N= 350	CCT	According PI	79.4% to 85.3%	0.546 to 0.663
Antognoli et al., 2010	Cattle N= 900	CCT	According PI	n.a.	0.45 (95%CI 0.28 – 0.62)
Goosen et al., 2013	Buffalo N= 82	SCT	According PI Or South Africa specific for buffalo	63% 64%	n.a. n.a.
Kalis et et, 2003	Cattle N= 1631	SCT	According PI**	85.7%	0.41
Schroeder, 2014	Cattle N=541	CCT	According PI	95.1%	0.501

Reproducibility

Experiment 1:
To investigate the reproducibility of the BOVIGAM® ELISA when performed in different laboratories.

Methods: Given that it is technically impractical to send freshly drawn blood samples to laboratories located in different countries to perform the whole blood stimulations, we have confined the analysis of reproducibility to the detection of IFN-γ using the BOVIGAM® ELISA. Whole blood samples from 21 animals (16 SICCT skin test positive natural field rectors, 3 BCG-vaccinated/*M. bovis* infected and 2 non-vaccinated/non-infected controls) were incubated with PPD-A, PPD-B, ESAT-6/CFP-10 peptide cocktail and Rv3615c peptide cocktail. These stimulations were set up in multiple wells, which allowed for the pooling of replicate samples to create a panel of identical aliquots, which were then subsequently tested in the BOVIGAM® ELISA at the laboratories listed above. A different BOVIGAM® ELISA kit batch was used in each laboratory (VISAVET kit# 6632600201, Luddington kit# 6332601801, Weybridge kit# 6332601701). Each animal was then scored as test positive or negative using three different readout systems: (i) the standard comparative readout of bovine PPD minus avian PPD (B-A), (ii) responses to the ESAT-6/CFP-10 peptide cocktail (E/C), or (iii) responses to the ESAT-6/CFP-10 peptide cocktail and/or the Rv3615c peptide cocktail (E/C ± Rv).

Results: The test results generated by three independent laboratories for 21 animals using either (i) B-A, (ii) E/C, or (iii) E/C ± Rv3615c are shown in table 18.

Table 18: Agreement of test results from three independent laboratories.

	B-A			E/C			E/C and/or Rv3615c		
I.D.	**VISAVET**	**Luddington**	**Weybridge**	**VISAVET**	**Luddington**	**Weybridge**	**VISAVET**	**Luddington**	**Weybridge**
S1	N	N	N	N	N	N	N	N	N
S2	Y	Y	Y	Y	Y	Y	Y	Y	Y
S3	Y	Y	Y	Y	Y	Y	Y	Y	Y
S4	Y	Y	Y	N	N	N	N	N	N
S5	Y	Y	Y	Y	Y	Y	Y	Y	Y
S6	Y	Y	Y	Y	Y	Y	Y	Y	Y
S8	Y	Y	Y	N	N	N	**Y**	**N**	**Y**
S9	Y	Y	Y	Y	Y	Y	Y	Y	Y
S10	Y	Y	Y	Y	Y	Y	Y	Y	Y
S11	Y	Y	Y	Y	Y	Y	Y	Y	Y
S12	Y	Y	Y	Y	Y	Y	Y	Y	Y
S13	Y	Y	Y	N	N	N	Y	Y	Y
S14	Y	Y	Y	Y	Y	Y	Y	Y	Y
S15	Y	Y	Y	Y	Y	Y	Y	Y	Y
S16	Y	Y	Y	Y	Y	Y	Y	Y	Y
S17	Y	Y	Y	Y	Y	Y	Y	Y	Y
S20	N	N	N	N	N	N	N	N	N
S21	N	N	N	N	N	N	N	N	N
S23	N	N	N	N	N	N	N	N	N
S24	Y	Y	Y	N	N	N	N	N	N
S25	Y	Y	Y	N	N	N	N	N	N

Table 18: Y = test positive response, N = test negative response, B-A = the standard comparative readout of bovine PPD minus avian PPD, E/C = responses to the ESAT-6/CFP-10 peptide cocktail, E/C and/or Rv3615c = responses to the ESAT-6/CFP-10 peptide cocktail and/or the Rv3615c peptide cocktail

Complete test agreement (100%) was seen across all three laboratories when using either B-A or E/C as readouts. Furthermore, 100% test agreement was also observed between Weybridge and VISAVET laboratories when using E/C ± Rv3615c as a readout. The only discrepancy in test results occurred when comparing E/C ± Rv3615c results from Luddington laboratory with either Weybridge or VISAVET (highlighted in red), where sample S8 tested negative in the former laboratory but positive in the two latter laboratories. This resulted in a test agreement of 95.24% (kappa value of 0.8966, interpreted as very good agreement) between Luddington and either Weybridge or VISAVET when comparing E/C ± Rv3615c results.

Conclusions:
These results demonstrate the high reproducibility of the BOVIGAM® ELISA when used at different laboratories, with different kit batches and with a variety of different readout systems.

Experiment 2:
To investigate the variability of results obtained at different laboratories using sample tubes from the same animal drawn at the same time.

Methods: 316 blood samples were submitted in parallel to AHVLA Luddington and also to a second laboratory (either AHVLA Weybridge or AHVLA Sutton Bonnington) for blood stimulations and IFN-γ ELISA. These consisted of 285 samples from the IFN-γ Specificity Trial and 31 samples from SICCT skin test positive animals. Each sample was tested for IFN-γ production against a medium (negative) control, PPD-A, PPD-B, and SEB (positive control) according to the relevant SOPs.

Results: All controls were within the ranges specified by the SOP. For the B-A readout, positive results were determined by subtracting the response to avian tuberculin from that to bovine

tuberculin; those of 0.1 or more were considered positive. The results are summarized below, and generate a test agreement for B-A of 96.52%.

Summary of test agreement for the B-A responses.

		Second Laboratory		
		Test negative	Test positive	Total
Luddington	Test negative	275	5	280
	Test positive	6	30	36
	Total	281	35	316

A similar analysis was carried out for responses to the ESAT-6/CFP-10 peptide cocktail, where a total of 287 blood samples were submitted in parallel to AHVLA Luddington and also to AHVLA Weybridge. These consisted of 284 samples from the IFN-γ Specificity Trial and 3 samples from SICCT skin test positive animals. Positive responses were determined by subtracting the response to the negative control from the response to the peptide cocktail; those of 0.1 or more were considered positive. The responses are summarized below, where again good test agreement (94.43%) was seen between the two sites.

Summary of test agreement for ESAT-6/CFP-10 responses.

		Weybridge		
		Test negative	Test positive	Total
Luddington	Test negative	268	6	274
	Test positive	10	3	13
	Total	278	9	287

Experiment 3

In a further trial in France, reproducibility has been tested between laboratories (table below).

	Laboratoire Départemental de l'Hérault, Montpellier, Carmargues		Laboratoire Départemental D`Analyses Agriculture et Vétérinaire; Coulounieix-Chamiers Dordogne	Laboratoire Départemental de la Côte-d'Or, Dijon	
Batch Number	6332603001	6332604201	6332603701	6332602701	6332603401
Mean Ref Material	19.65%	19%	20.43%	22.56%	20.05%
Standard deviation	1.82	2.71	2.69	1.97	1.47
%CV	9.23%	14.56%	13.17%	9.0%	7.0%

These results demonstrate the high reproducibility of the BOVIGAM® ELISA when used at different laboratories, with different kit batches and with a variety of different readout systems at different days.

Application

Some reference laboratories use BOVIGAM® as an ancillary test of animals derived from a skin test positive tested herd but which has been negative tested in skin test (e.g. Ireland, UK). Some reference laboratories use BOVIGAM® as a confirmatory test of animals which has been tested positive in skin test (e.g. Bavaria). Mexican and one Laboratory in France (for bull fighting herds) use BOVIGAM® as primary test for tuberculosis diagnostic in cattle.

BOVIGAM® has been used several million times since its introduction in 1988, mostly in routine laboratories. Typical laboratories have used this test to analyze several hundred samples per day. Minimum turn-around time for the test is 4 hours for the ELISA and 16-24 hours for the stimulation of whole blood samples.

References

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