INTERNAL VALIDATION REPORT

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ID GENE™ NEWCASTLE DISEASE DUPLEX

REAL-TIME RT-PCR ASSAY FOR THE QUALITATIVE DETECTION OF NEWCASTLE DISEASE VIRUS RNA

METHOD	Real time RT-PCR – Duplex – Qualitative
TARGET SEQUENCES	• **Newcastle disease virus RNA** • **Exogenous Non-Target positive control (NTPC-BIRD):** Non-pathogenous micro-organism specific sequence
SAMPLE TYPES	• **Swab** (tracheal, oropharyngeal and cloacal; Individual samples or pools of up to 5) • **Organ** (trachea, lung, spleen, liver, brain and cecal tonsils; Individual samples or pools of up to 5) • **Nucleic acid storage card** (individual samples or pools of up to 5)
VALIDATED SPECIES	Birds
PRODUCT CODE	IDNDV

Table of contents

INTRODUCTION

Newcastle disease (ND) is a widespread avian pathology which may cause severe losses in domestic poultry production. It is a disease subject to official control and should be notified to the OIE. The causative agent is Newcastle disease virus (NDV) which is also called avian paramyxovirus 1 (APMV-1) or Avulavirus.

NDV is an enveloped, negative-sense single-stranded RNA virus, with a nonsegmented genome of approximately 15 kb in size. It encodes six main structural proteins, including the fusion protein (F) and the nucleoprotein (NP). NDV has been shown to be able to infect over 200 species of birds. Morbidity, mortality and symptoms vary widely depending on strain virus, species and age of bird, pre-existing immunity, and concurrent disease. NDVs are classified by virulence into three pathotypes: lentogenic (lowly virulent), mesogenic (moderately virulent), and velogenic (highly virulent; either viscerotropic velogenic or neurotropic velogenic). Furthermore, it has been observed that even APMV-1 strains of low virulence are exacerbated by the presence of other organisms or by adverse environmental conditions.

Innovative Diagnostics has developed a molecular biology detection tool for the Newcastle Disease virus from swabs, organs, and nucleic acid storage cards. This kit is a qualitative Duplex RT-qPCR test. It simultaneously amplifies target RNA as well as an Exogenous Non-Target Positive Control (NTPC-BIRD).

This report summarizes validation data for this test.

TEST PRINCIPLE

The ID Gene™ Newcastle disease Duplex (IDNDV) developed by Innovative Diagnostics is based on the real time PCR method.

Viral RNA is extracted from a given matrix (swab, organ, or nucleic acid storage card) before the amplification step. For the extraction step, Innovative Diagnostics recommends the following extraction kits:

DESCRIPTION	PRODUCT NAME	PRODUCT CODE
Automated extraction system with magnetic beads	ID Gene™ Mag Fast Extraction Kit	MAGFAST384
Manual extraction system with columns	ID Gene™ Spin Universal Extraction Kit	SPIN50/SPIN250

The IDNDV RT-qPCR amplification kit includes:

- a ready-to-use Amplification Reaction Mixture (ARM-NDV) which allows for the amplification and the detection of a target sequence in Newcastle disease virus (NDV) as well as an exogenous non-target positive control. It contains different sets of primers, probes and a master mix including the reverse transcriptase and the polymerase) required to perform the RT-qPCR amplification.
- a NDV Positive Amplification Control (PAC-NDV) which consists of a synthetic nucleic acid target. It allows the validation of the amplification of the NDV specific-target RNA sequence.
- an Exogenous Non-Target Positive Control (NTPC-BIRD) which is the exogenous non-target control. This is non-pathogenic microorganism, acting as a mimic of the target. It is to be added to each sample to test, prior to the nucleic acid extraction step. Provided with the kit in a freeze-dried format. Once

resuspended, its addition allows the validation of the good execution of all the analytical steps of the system (extraction, purification, amplification), for each sample.

Provided separately by Innovative Diagnostics, a freeze-dried NDV Positive Extraction Control (product code: PEC-NDV) can be used as a sentinel for the complete method (nucleic acids extraction and RT-qPCR amplification). The PEC-NDV consists of inactivated NDV strain diluted into tracheal and oropharyngal swabs supernatants from negative birds. If the PEC-NDV, or an internal sentinel sample, is used as positive control for the test runs, the use of the PAC-NDV is not mandatory.

IDNDV is a duplex real time RT-PCR assay allowing the simultaneously amplification of:

- a target sequence within the NDV RNA.
- a non-pathogenous microorganim specific sequence present in the Exogenous Non-Target Positive Control (NTPC-BIRD):
  The non-pathogenous microorganism chosen for the NTPC-BIRD is different from Newcastle disease virus and is not present in the same domain of activity. It acts as a pathogen mimic.

The IDNDV RT-qPCR kit uses fluorescent probes relying on FAM™ and VIC®/HEX™ fluorochromes for detection of the targets, as detailled below:

TARGET	CHANNEL CAPABLE OF READING
Newcastle disease virus specific sequence	FAM™
Non-pathogenous microorganism specific sequence (NTPC-BIRD)	VIC®/HEX™

Two amplification programs are available for the IDNDV kit:

AMPLIFICATION PROGRAMS	RUN LENGTH
Standard	90 minutes
Rapid	60 minutes

ANALYTICAL SPECIFICITY

IN SILICO

The specificity of the IDNDV RT-qPCR assay was evaluated *in silico* by aligning the target PCR systems (primers and probes) with the databases available on the NCBI (National Center for Biotechnology Information).

After alignments, 100% in silico specificity was found for the NDV target. The alignments do not show high sequence homology with pathogens from the same ecological niche.

EXPERIMENTAL

Inclusivity study

Inclusivity was evaluated on a panel of 4 Newcastle disease virus isolates provided by different laboratories.

STRAIN	ORIGIN	IDNDV RESULTS
Ulster C2	GD Deventer	Detected
La Sota	India	Detected
La Sota, VLDIA039	GD Deventer	Detected
Ulster C2	IZSV	Detected

Figure 1: Analytical specificity results obtained with the IDNDV kit on a panel of Newcastle disease virus isolates

RESULTS (Figure 1) :

- The IDNDV kit successfully detected **all the isolates** in this panel.

Exclusivity study

Exclusivity was evaluated on a panel of pathogens with phylogenetic relationship, similarities in target sequence and/or found within the same ecological niche than NDV.

PATHOGEN	SOURCE	IDNDV RESULT
Viruses		
Marek Disease Virus	EA2260-628FRL03-13	Not detected
Paramyxovirus type 2	GD Deventer, VLDIA136	Not detected
Paramyxovirus type 3	GD Deventer, VLDIA239	Not detected
Reovirus	GD Deventer, VLDIA016	Not detected
Adenovirus	GD Deventer, VLDIA010	Not detected
Fowl Pox Virus	GD Deventer, VLDIA011	Not detected
Metapneumovirus Turkey Rhinotracheitis virus	GD Deventer, ref. 021215	Not detected
Egg Drop Syndrome 76 virus	GD Deventer, VLDIA038	Not detected
Reticuloendotheliosis virus	USDA	Not detected
Infectious Laryngotracheitis virus	GD Deventer, VLDIA014	Not detected
Bovine Leucose Virus	Field strain	Not detected
Porcine reproductive and respiratory syndrome virus (US)	Field strain	Not detected
Bluetongue virus serotype 1	Field strain	Not detected
Bluetongue virus serotype 8	Field strain	Not detected
Schmallenberg bovine virus	Field strain	Not detected
Bovine Herpesvirus of type 1	Field strain	Not detected
Infectious Bursal Disease virus	GD Deventer, VLDIA012	Not detected
Infectious Bronchitis Virus D388 (QX)	GD Deventer, VLDIA258	Not detected
Infectious Bronchitis Virus (Beaudette)	GD Deventer, VLDIA013	Not detected
Infectious Bronchitis Virus (Italy02)	GD Deventer, VLDIA259	Not detected
Infectious Bronchitis Virus (D8880)	GD Deventer, VLDIA036	Not detected
Bacteria		
Mycoplasma meleagridis	ATCC 25294	Not detected
Mycoplasma iowae	ATCC 33552	Not detected
Mycoplasma gallinaceum	ATCC 33550	Not detected
Mycoplasma gallinarum	ATCC 19769	Not detected
Mycoplasma gallinarum	ATCC 15319	Not detected
Mycoplasma meleagridis	ATCC 27764	Not detected
Mycoplasma anseri	ATCC 49234	Not detected
Mycoplasma imitans	Strain provided by the IZS Venezie	Not detected
Chlamydophila abortus	Field strain	Not detected
Campylobacter fetus fetus	CIP 5396 T	Not detected
Campylobacter jejuni	NC 13367	Not detected
Campylobacter fetus venerealis	NC 10354	Not detected
Campylobacter coli	CIP 7080	Not detected
Parasites		
Cryptosporidium parvum	Field strain	Not detected
Neospora caninum	Field strain	Not detected
Leishmania infantum	Field strain	Not detected

Figure 2: Evaluation of the analytical specificity on a panel of different pathogens using the IDNDV kit

RESULTS (Figure 2):

- The IDNDV kit is **specific to its target Newcastle Disease virus**.

ANALYTICAL SENSITIVITY – LD_{PCR}

The limit of detection of the PCR (LD_{PCR}) is the smallest number of copies of the target nucleic acid that can be detected, in 95 % of the cases, per unit of volume:

- Number of copies/PCR (total volume per PCR = 13µL)

The limit of detection in PCR-based analyses (LD_{PCR}) was determined by performing serial dilutions (8 replicates) on 3 different sessions using calibrated synthetic RNA containing the Newcastle Disease virus specific sequence.

The amplification step was performed with the IDNDV kit using using the most stringent conditions (meaning using the rapid amplification program).

The results obtained are presented in Figure 3.

NUMBER OF COPIES/PCR	RAPID PROGRAM	
	Nb of positives/Nb of replicates	Detection frequency
100	24/24	100%
50	24/24	100%
20	23/24	96%
15	14/24	58%
10	11/24	46%
5	6/24	25%

Figure 3: Smallest number of copies detected in 95% of cases per PCR

RESULTS (Figure 3) :

- The **LD_{PCR} (95%)** with the rapid amplification program was established around **20 copies / PCR.**

LINEARITY RANGE AND EFFICIENCY

The linearity of a quantitative test is its ability to generate results proportional to the concentration of the target within a given range of values modeled by a linear function. A linear regression exists between the instrumental response (example: Cq) and the decimal logarithm of the amount of the target (copy number of the target per PCR). The determination of the parameters of the regression line (y = a x '+ b) and the validation of the linear equation must allow to:

- determine the equation of the regression line
- determine the efficiency(E)
- determine and validate the performance of the linear regression

In theory, the number of molecules of the target sequence should double during each replication cycle, corresponding to 100% amplification efficiency (E= 100%).

The amplification efficiency is calculated by performing five 10-fold serial dilutions of calibrated synthetic nucleic acids (containing a specific sequence of the target gene) in 4 different runs using the rapid and standard amplification programs. First, the Cq values obtained are ploted in fonction of the corresponding concentrations expressed in a logarithmic scale.

Next, a linear regression curve through the data points is generated to calculate the slope of the regression line to determine the efficiency of the RT-qPCR.

Efficiency is calculated using the equation: $E= -1 + 10^{(-1/slope)}$

In theory, for an efficiency found between 85% and 115%, the slope should be found between -4.115 and 2.839.

RAPID AMPLIFICATION PROGRAM

Efficiency results

RAPID PROGRAM	SLOPE (a)	Y-INTERCEPT (b)	COEFFICIENT R2	EFFICIENCY %
Session 1	-3,439	36,95	0,98	95%
Session 2	-3,551	36,37	1,00	91%
Session 3	-3,369	35,81	1,00	98%
Session 4	-3,45	36,38	0,99	95%
Average	**-3,45**	**36,38**	**0,99**	**95%**

Figure 4 : Efficiency results with the rapid program (R^2: correlation coefficient)

RESULTS (Figure 4) :

- The average **efficiency** is **95%.**
- The average coefficient **R^2** is **0.99**.

DIAGNOSTIC SENSITIVITY AND SPECIFICITY

In order to evaluate diagnostic sensitivity and specificity, several panels of field samples (total of 59 samples) were tested. The status of the different sample types had been previously characterized by other methods.

PANEL DESCRIPTIONS

Panel 1

- 12 outbreak samples obtained from broilers (United Arab Emirates):
  - 2 organ samples (trachea) which were NDV-negative
  - 5 oropharyngeal swab samples: 4 were NDV-positive and 1 NDV-negative
  - 5 nucleic acid storage cards (from organs): 3 were NDV-positive and 2 NDV-negative

  Sample status was determined using another commercially available PCR kit (Kit A)

Panel 2

- 11 organ samples (trachea, kidney, lung, liver) obtained from broilers aged 28 days (Egypt). 3/11 samples were detected positive with Kit A.

Panel 3

- 5 pools of oropharyngeal swabs:from broilers/breeders (Pakistan). They were all detected as NDV-negative with Kit A.

Panel 4

- 31 tracheal swabs samples obtained from chicks, layers and breeders (France). 31/31 samples were detected positive for MG/MS, but negative for NDV by Kit A.

RESULTS

The data obtained for the 59 samples tested are summarised in the Figure 5 below.

SAMPLE TYPE	NUMBER OF SAMPLES TESTED	DIAGNOSTIC SENSITIVITY (SE)	DIAGNOSTIC SPECIFICITY (SP)
		NDV TARGET	
ORGAN	13	3/3 **100%**	10/10 **100%**
OROPHARYNGEAL AND TRACHEAL SWAB	41	4/4 **100%**	37/37 **100%**
NUCLEIC ACID STORAGE CARD	5	2/2 **100%**	3/3 **100%**

Figure 5 : Diagnostic sensitivity and specificity results obtained with the IDNDV RT-qPCR for the different sample types tested.

RESULTS (Figure 5):

- **Diagnostic sensitivity** for **all matrices** is **Se = 100%.**
- **Diagnostic specificity for all matrices** is **Sp = 100%**.

COMMERCIALLY AVAILABLE PCR KIT (KIT A)

A panel of NDV Ring Trial samples from GD Deventer (2018, Netherland) was used to compare the performances of IDNDV RT-qPCR test with another commercially available RT-qPCR kit (Kit A)

Nucleic acid extraction was performed using the Innovative Diagnostics's MAGFAST extraction method. RT-qPCR amplification was done following the manufacturer's instructions for use.

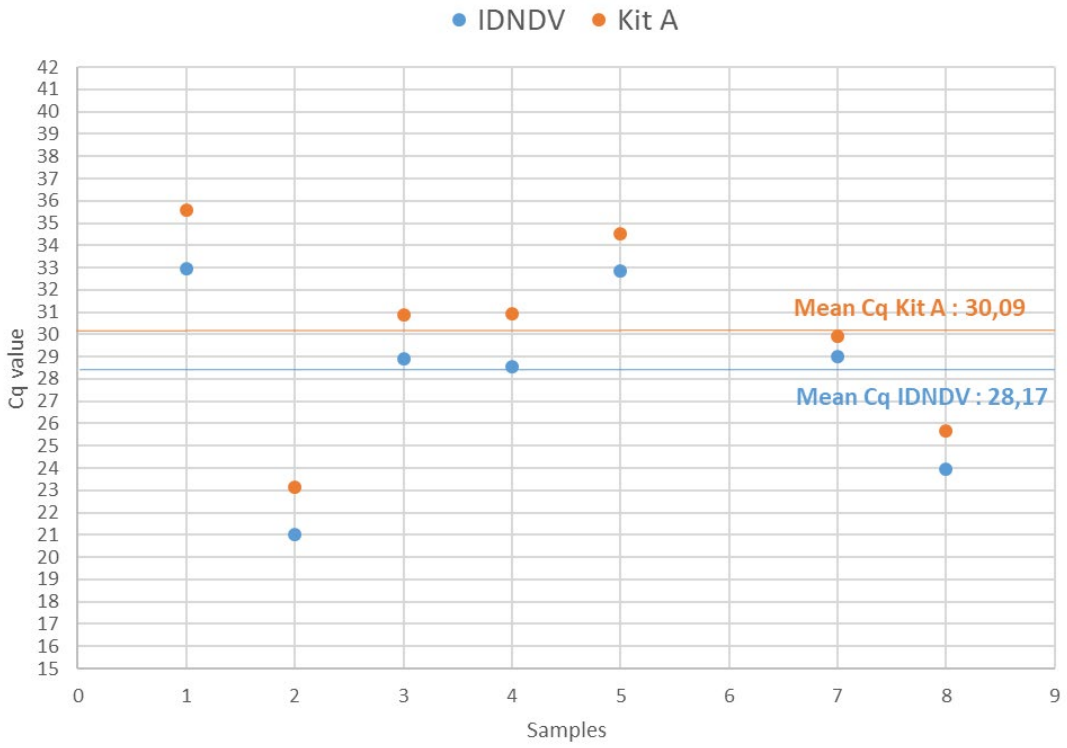

Figure 6 : Comparison of results obtained for NDV Ring Trial samples from GD Deventer using the IDNDV RT-qPCR kit and Kit A.

RESULTS (Figure 6):

- The IDNDV assay gave **earlier Cq values** than the Kit A (ΔCq = -1.9).
- The IDNDV assay has a **slightly higher sensitivity** than Kit A on the tested panel.
- The IDNDV kit shows **good status correlation** with Kit A.

ROBUSTNESS

The IDNDV RT-qPCR kit robustness was evaluated by testing the maximum and minimum conditions of RNA volume and temperatures used, in the different steps of the amplification run, as defined in the instructions for use. In addition, the evaluation was conducted in the limiting conditions described below:

- 3 times the LD_{PCR} using NDV-specific synthetic nucleic acids;
- RNA volume ±10%;
- Reverse transcription: 10 minutes at 45°C ± 1°C;
- Polymerase activation: 2 minutes at 95°C ± 1°C;
- DNA denaturation: 10 seconds at 95°C ± 1°C
- Annealing step: 30 sec at 60°C ± 1°C

RNA samples from La Sota and Ulster strains and from the NDV Positive Extraction Control (PEC-NDV) were tested in these conditions. The amplification reactions were performed in duplicates and the average values are presented in Figures 7 and 8.

		AVERAGE OF 2 CQ VALUES FOR **EACH SAMPLE VOLUME**			
		-10%	**SET VOLUME**	**+10%**	CV%
NEWCASTLE DISEASE VIRUS TARGET	**3 X LD_{PCR}**	31.29	31.57	31.23	**0.6%**
	PEC	27.34	27.26	26.8	**1.1%**
	LA SOTA STRAIN (INDIA)	28.84	28.72	28.86	**0.3%**
	ULSTER STRAIN	23.83	24.35	23.96	**1.1%**
	LA SOTA STRAIN (GD DEVENTER)	24.51	24.47	24.37	**0.3%**

Figure 7: Cq and coefficient of variation obtained in response to changes in RNA volume

		AVERAGE OF 2 CQ VALUES FOR **EACH TEMPERATURE**			
		-1°C	**SET TEMPERATURES**	**+1°C**	CV%
NEWCASTLE DISEASE VIRUS TARGET	**3X LDPCR**	32.32	32.31	32.71	**0.7%**
	PEC	27.31	27.5	27.3	**0.4%**
	LA SOTA STRAIN (INDIA)	29.03	28.67	28.74	**0.7%**
	ULSTER STRAIN	23.98	23.81	23.72	**0.6%**
	LA SOTA STRAIN (GD DEVENTER)	24.64	24.61	24.51	**0.3%**

Figure 8 : Cq and coefficient of variation obtained in response to changes in temperature.

RESULTS (Figures 7 and 8):

- When the **RNA volume** was varied by ± **10%**, the IDNDV kit gave a **CV% of less than 2%**.
- When the **temperatures** was varied by **± 1°C**, the IDNDV kit gave a **CV% of less than 1%.**
- The IDNDV RT-qPCR kit shows **excellent robustness.**

STABILITY

Real time stability studies were conducted at -20°C (recommended storage temperature) using the most stringent conditions of the IDNDV RT-qPCR assay and a control for the complete method as follows:

- 3 times the LD_{PCR} using NDV-specific plasmid
- The NDV Positive Extraction Control (PEC-NDV), after nucleic acid extraction with the MAGFAST system, was tested to ensure the biological integrity and quality of the PEC-NDV over time.
- The PAC-NDV, which consist of a synthetic nucleic acid target
- Rapid amplification program

The proposed shelf-life of the IDNDV kit is of 15 months. Stability results obtained over a period of 18 months are presented below.

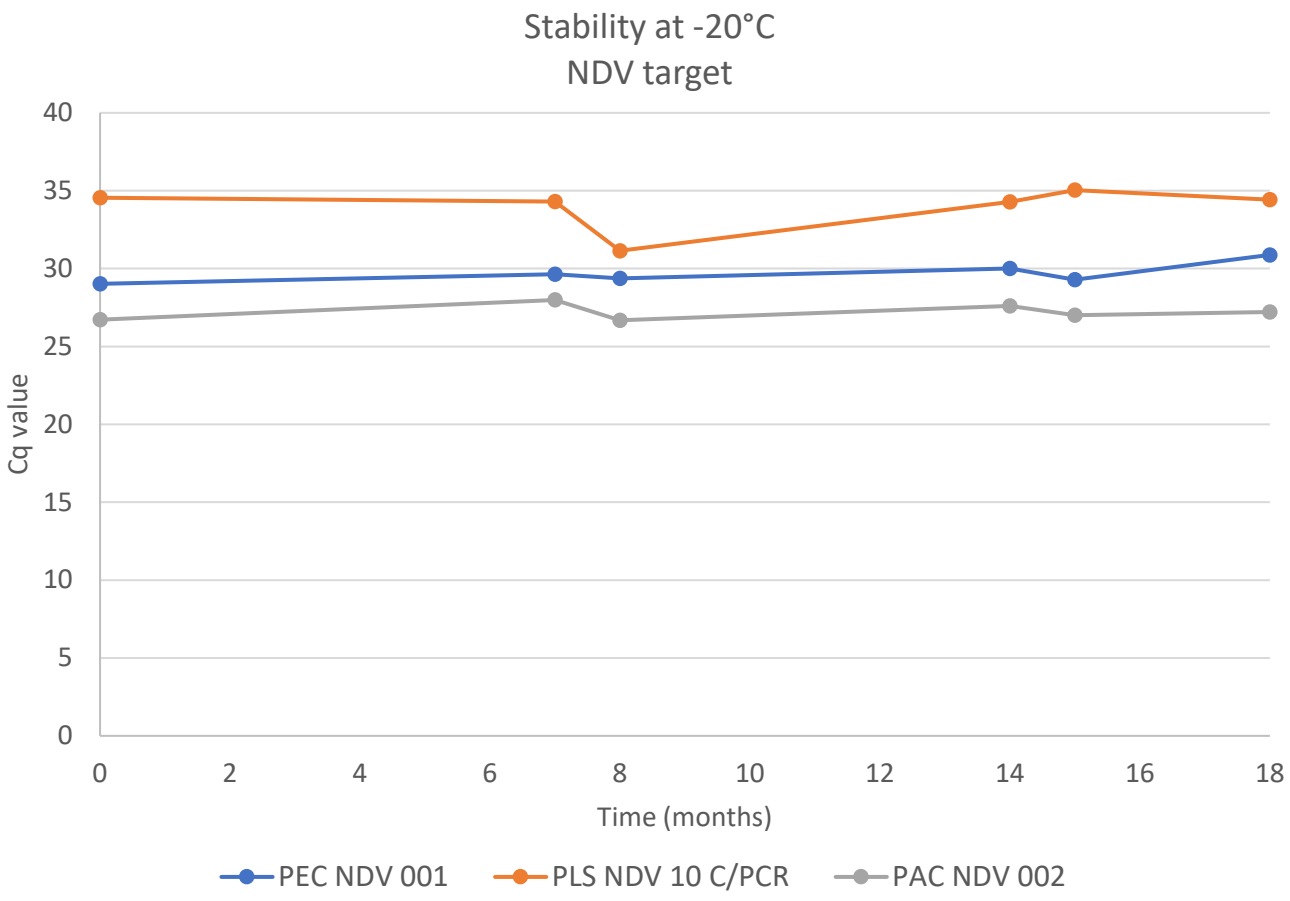

Figure 9. IDNDV real-time stability study at -20°C.

RESULTS (Figure 9):

- The real-time stability study illustrates the **excellent stability** of the IDNDV assay over time.

CONCLUSION

The ID Gene™ Newcastle Disease Duplex RT-qPCR kit:

- **Shows high performance:** LD_{PCR}< 20 copies / PCR and specificity of 100%
- Offers **high specificity** and **sensitivity** on all matrices used for the detection of Newcastle disease virus by real time RT-PCR.
- **Demonstrates excellent correlation with other commercial PCR kit** (Kit A) and **excellent robustness and stability.**

Related products

- **NDV-positive Extraction Control** (product code: PEC-NDV): Freeze dried preparation of an inactivated Newcastle disease virus strain diluted into tracheal and oropharyngal swabs supernatants from negative birds. To be prepared and extracted in the same way as samples. to validate the efficiency of the nucleic acid extraction and the qPCR amplification processes and to monitor variations in analytical sensitivity.
- **ID Screen® Newcastle disease Competition** (product code: NDVC): Competitive ELISA kit for the detection of anti-NDV antibodies in domestic and wild avian species. For diagnosis and control of SPF animals.
- **ID Screen® Newcastle disease Indirect** (product code: NDVS): Indirect ELISA for the detection of anti-NDV antibodies in chicken and turkey sera. For monitoring of recombinant vaccines (rHVT-F vaccines alone).
- **ID Screen® Newcastle disease Indirect Conventional Vaccines** (product code: NDVS-CV): Indirect ELISA for the detection of anti-NDV in chicken and turkey serum or egg yolk. For monitoring of all NDV conventional vaccines (live or inactivated).
- **ID Screen® Newcastle disease Nucleoprotein Indirect** (product code: NDVNP): The only commercial indirect ELISA for the specific detection of anti- NDV Nucleoprotein (NP) antibodies in chicken or turkey serum. Can be used in association with the NDVS kit for DIVA testing (when rHVT-F vaccines are used).
- **Ready-to-use multi-positive serum – ref. A** (product code: MRIPOS-BIRD-RTU-A): Ready-to-use multi-positive chicken serum. Positive notably with the NDVS and NDVS-CV kits. To be used as internal reference material for quality control.

History of revisions

VERSION	EDIT DATE	REFERENCE	TYPE OF REVISION	REVISION MADE
1019	12/2019	DOC0037	Not applicable (first version)	N/A
	10/2021	DOC0076	Correction of anomalies	Correction of the LD_{PCR} from 1 copy/PCR to 10 copies/PCR
0722	07/2022	DOC0095	Technical modification : Update of the document following technical modification of the kit	The PAC-NDV is now provided as a NDV-specific synthetic RNA instead of a synthetic DNA The LD_{PCR} has been updated for this new PAC-NDV from 10 copies/PCR to 20 copies/PCR
			Update	Innovative Diagnostics now mentionned as the kit's manufacturer