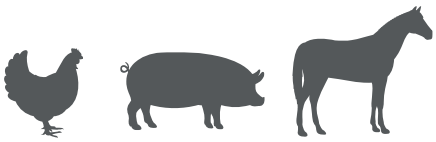
November 2024 | EN

virotype® Influenza A 2.0 RT-PCR Kit

Validation Report

For the detection of RNA from the Influenza A Virus



INDICAL BIOSCIENCE

Contents

1 Introduction

1.1 Intended use

The virotype Influenza A 2.0 RT-PCR Kit is intended for the detection of RNA from Influenza A Virus (IAV) in oropharyngeal, tracheal, and cloacal swabs (individual or pooled), fecal samples, or tissue samples from birds; nasal swabs, bronchoalveolar lavage fluid (BALF), and tissue samples from swine, as well as nasal swabs from equids.

The kit is approved by the Friedrich-Loeffler-Institut and licensed in accordance with § 11 (2) of the German Animal Health Act (FLI-C 116) for use in Germany for veterinary diagnostic procedures.

For veterinary use only.

1.2 General information

The virotype Influenza A 2.0 RT-PCR Kit is a highly sensitive solution for the safe and sensitive detection of RNA from Influenza A Virus in samples from birds, pigs, and equids.

The Influenza A Virus is an enveloped, single-stranded RNA virus and belongs to the family *Orthomyxoviridae*, genus *Alphainfluenzavirus*. Influenza A viruses occur in high genetic diversity and a wide range of virulence. They are grouped into low and highly pathogenic strains. Waterfowl are the natural reservoir of Low-Pathogenic Avian Influenza Viruses (LPAIV). Highly Pathogenic Avian Influenza Viruses (HPAIV) belong to subtypes H5 or H7 and may cause fowl plague in domestic poultry with high economic losses.

The subtypes H1N1, H1N2, and H3N2 of Influenza A Virus can also cause infections of the respiratory tract in swine as can the subtype H3N8 in equids.

1.3 Description of the test principle

Polymerase chain reaction (PCR) is based on the amplification of specific regions of the pathogen genome. In real-time PCR, the amplified product is identified using fluorescent dyes. These are usually linked to oligonucleotide probes that bind specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run (i.e., in real-time) allows detection of the accumulating product without the need to re-open the reaction tubes afterward.

The virotype Influenza A 2.0 RT-PCR Kit contains all of the necessary reagents for the detection of Influenza A Virus RNA, including a Positive and Negative Control. With this kit, both, reverse transcription and PCR are performed in one reaction tube, reducing the risk of contamination.

The virotype Influenza A 2.0 RT-PCR Kit uses two specific primer/probe combinations:

- FAM™ fluorescence for RNA of Influenza A Virus
- HEX™ fluorescence for the endogenous Internal Control (EC; β-actin present within the sample)

A Positive Control serves to verify the functionality of the reaction mix for the amplification of the Influenza A Virus RNA target.

1.4 Kit contents

virotype Influenza A 2.0 RT-PCR Kit	**(100)**
Cat. no.	**VT282625**
Number of reactions	**100**
Master Mix (tube with orange cap), includes enzymes, primers, and probes	2 x 1000 µl
Positive Control (tube with red cap)	1 x 150 µl
Negative Control (tube with blue cap)	1 x 150 µl
Handbook	1

1.5 Storage

The components of the virotype Influenza A 2.0 RT-PCR Kit should be stored at -30°C to -15°C and are stable until the expiration date stated on the label. Avoid repeated thawing and freezing (> 3x), as this may reduce assay sensitivity. Freeze the components in aliquots if they will only be used intermittently.

1.6 Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Pipets
- Nuclease-free, aerosol-resistant pipet tips with filters
- Sterile 1.5 ml Eppendorf® tubes
- Nuclease-free (RNase/DNase-free) consumables. Special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive identification of viral nucleic acids
- Cooling device or ice
- Benchtop centrifuge with rotor for 1.5 ml tubes
- Real-time cycler with appropriate fluorescent channels
- Appropriate software for chosen real-time cycler
- Appropriate strip tubes and caps or 96-well optical microplate with optical sealing film or cover for chosen real-time cycler

1.7 RNA extraction

The virotype Influenza A 2.0 RT-PCR Kit can be used for the detection of Influenza A Virus RNA from the following sample types:

- Birds: oropharyngeal, tracheal, and cloacal swabs (individual or pooled), fecal samples, tissue samples
- Swine: nasal swabs, bronchoalveolar lavage fluid (BALF), tissue samples
- Equids: nasal swabs

Due to the high sensitivity of the test, pools of up to 10 individual swab samples can be tested.

Note: For use in Germany the specifications described in the „Amtliche Methodensammlung“ apply.

Prior to real-time PCR, viral RNA must be extracted from the starting material.

INDICAL offers a range of validated kits for the extraction of RNA from animal samples.

Extraction based on magnetic beads:

- IndiMag® Pathogen Kit (SP947457)
- IndiMag Pathogen Kit w/o plastics (SP947257)
- IndiMag Pathogen IM48 Cartridge (SP947654P608, SP947654P224)
- IndiMag Pathogen KF96 Cartridge (SP947855P196)

Extraction based on spin columns:

- IndiSpin® Pathogen Kit* (SP54104, SP54106)
- IndiSpin QIAcube® HT Pathogen Kit (SP54161)

If real-time RT-PCR is not performed immediately after extraction, store the RNA at -20°C or at -80°C for longer storage.

For further information on automated and manual extraction of Influenza A Virus RNA from different sample types, refer to the respective handbook or contact INDICAL Support at support@indical.com.

1.8 Important notes

General precautions

The user should always pay attention to the following:

- Use nuclease-free pipet tips with filters.
- Store and extract positive materials (specimens, positive controls and amplicons) separately from all other reagents and add them to the reaction mix in a spatially separated facility.
- Thaw all components on ice before starting the assay.
- When thawed, mix the components by inverting and centrifuge briefly.
- Do not use components of the test kit past the expiration date.
- Keep samples and controls on ice or in a cooling block during the setup of reactions.

Negative control

At least one negative control reaction should be included in each PCR run, containing all the components of the reaction except for the pathogen template. This enables assessment of contamination in the reaction.

Positive control

When performing PCR on unknown samples, it is recommended to include a positive control reaction in the PCR run, containing a sample that is known to include the targeted viral RNA. A positive control serves to prove the functionality of the pathogen assay, e.g., the correct setup of the reaction mix. Use 5 µl of the Positive Control provided with the virotype Influenza A 2.0 RT-PCR Kit to test for successful amplification of the target.

Extraction and amplification control

For increased process safety and convenience, one extraction and amplification control assay is included in the test kit.

An endogenous Internal Control (EC) detects the β-actin gene present within the sample. This allows extraction and amplification to be monitored.

2 Procedure

2.1 Important points before starting

- Please read „Important notes“ before starting.
- Include at least one positive control (Positive Control) and one negative control (Negative Control) per PCR run.
- Before beginning the procedure, read through the protocol and ensure that you are familiar with the operation of the chosen real-time PCR cycler.
- RNA is unstable. Perform the protocol without interruption.

2.2 Things to do before starting

- Thaw all reagents on ice and protect from light.
- Before use, spin the reagents briefly.
- Maintain reagents on ice or in a cooling block during PCR setup.

2.3 Test procedure

1. Before use, mix the Master Mix by inverting 5 times or until mixed thoroughly, then centrifuge briefly to collect the fluids.
2. Pipet 20 µl of the Master Mix into each reaction tube. Then add 5 µl of the sample RNA (Table 1).

   Include positive and negative control reactions.

   Positive Control: Use 5 µl of the positive control (Positive Control) instead of sample RNA.

   Negative Control: Use 5 µl of the negative control (Negative Control) instead of sample RNA.

Table 1. Preparation of reaction mix

Component	**Volume**
Master Mix	20 µl
Sample	5 µl
Total volume	**25 µl**

3. Close the reaction tubes or seal the plate and invert 5 times or until mixed thoroughly. Then centrifuge briefly to collect the fluids.

4. Set the filters for the reporter dyes in the software of your thermal cycler according to Table 2.

Table 2. Filter settings for the reporter

Pathogen/ Internal Control	Reporter
Influenza A Virus	FAM
Endogenous Internal Control	HEX™[1]
Passive reference[2]	ROX™

[1] Use the option appropriate for your thermal cycler.

[2] Internal reference for use with Applied Biosystems® ABI PRISM® Sequence Detection Systems

5. Run the real-time PCR protocol according to Table 3.

Table 3. Real-time RT-PCR protocol for Influenza A 2.0.

Step	Temperature	Time	Number of cycles
Reverse Transcription	50°C	10 min	1
Initial Activation	95°C	2 min	1
2-step cycling			
Denaturation	95°C	5 s	40
Annealing/Extension*	60°C	30 s	

* Fluorescence data collection. Approximate run time 67 min (Mx3005P®, Agilent Technologies, Inc.)

3 Data interpretation

Interpretation of results

For the assay to be valid the Positive Control must give a signal in the FAM and HEX channels with a C_T[1] < 35. The Negative Control must give no signal.

The following results are possible if working with unknown samples. The possible sample results are also summarized in Table 4.

The sample is positive for Influenza A Virus, and the assay is valid, if the following criteria are met:

- The sample yields a signal in both, the FAM and the HEX channel.
- The Positive Control yields a signal in both, the FAM and the HEX channel.
- The Negative Control does not yield a signal in the FAM and HEX channels.

Note that very high concentrations of Influenza A Virus RNA in the sample may lead to a reduced HEX signal or no HEX signal due to competition with the Internal Control.

The sample is negative for Influenza A Virus, and the assay is valid, if the following criteria are met:

- The sample yields a signal in the HEX channel but not in the FAM channel.
- The Positive Control yields a signal in both, the FAM and the HEX channel.
- The Negative Control does not yield a signal in the FAM and HEX channels.

A positive HEX signal means that extraction and amplification were successful as the housekeeping gene (β-actin) within the sample is amplified.

The sample results are inconclusive, and the assay is invalid, if the following occurs:

- The sample yields no signal in any of the fluorescence channels.

[1] Threshold cycle (C_T) — cycle at which the amplification plot crosses the threshold, i.e., there is the first clearly detectable increase in fluorescence

If no signal is detected in the FAM (Influenza A Virus) and the HEX (endogenous Internal Control, EC) channel, the result is inconclusive. The absence of a signal for the housekeeping gene indicates strong PCR inhibition and/or other malfunctions, e.g., during extraction.

To check for inhibition, we recommend 1:5 dilution of the sample RNA in nuclease-free water, to repeat the RNA extraction procedure, or repeat the whole test procedure starting with new sample material.

Check that there is a fluorescence signal in all the channels for the positive control reaction (Positive Control). Absence of a signal for the Positive Control indicates an error, which could be due to incorrect setup of the reaction mix or incorrect cycling conditions.

Table 4. Results interpretation table*

Sample result	**FAM (Influenza A Virus)**	**HEX (EC)**
Influenza A Virus positive	X	X
Influenza A Virus strong positive	X	
Influenza A Virus negative		X
Inconclusive		

***** Interpretation of sample results can be determined provided positive and negative control reactions are performed. The Positive Control must yield a signal in the FAM and HEX channels. The Negative Control must yield no signal in any channel. For a complete explanation of possible sample results please refer to “Data analysis and interpretation”.

4 Characteristics of the test

4.1 Analytical sensitivity

4.1.1 Analytical sensitivity using the QuantStudio® 5 instrument

The high analytical sensitivity of the virotype Influenza A 2.0 RT-PCR Kit was verified by a titration series of Influenza A Virus (IAV) *in vitro* RNA [10^6 – 1 copies/well], performed in triplicates of relevant dilutions using the virotype Influenza A 2.0 RT-PCR protocol on the Thermo Fisher Scientific QuantStudio 5 instrument.

Results / Conclusion

The virotype Influenza A 2.0 RT-PCR Kit can detect up to one IAV genome copy per sample (Table 5, Figure 1 and Figure 2). A correlation coefficient of 0.98 with an efficiency of 102.1 % for the *in vitro* RNA was calculated when using the virotype Influenza A 2.0 RT-PCR Kit on the QuantStudio 5 instrument (Figure 2).

Table 5. Individual and mean C_T values of **IAV** (FAM) *in vitro* RNA titration series in triplicates. The test was performed on the QuantStudio 5 instrument using the virotype Influenza A 2.0 real-time RT-PCR protocol.

Type	Copy number	C_T (FAM)	C_T mean	SD	Result
Standard	10^6	19.99			+
Standard	10^6	20.13	20.07	0.06	+
Standard	10^6	20.10			+
Standard	10^5	23.50			+
Standard	10^5	23.48	23.48	0.01	+
Standard	10^5	23.48			+
Standard	10^4	26.90			+
Standard	10^4	26.86	26.92	0.06	+
Standard	10^4	26.99			+
Standard	10^3	30.26			+
Standard	10^3	30.46	30.35	0.08	+
Standard	10^3	30.33			+
Standard	100	33.43			+
Standard	100	33.70	33.69	0.20	+
Standard	100	33.93			+
Standard	10	35.27			+
Standard	10	37.25	36.23	0.81	+
Standard	10	36.19			+
Standard	1	37.73			+
Standard	1	37.80	37.76	0.03	+
Standard	1	-			-

SD = standard deviation, - = no C_T

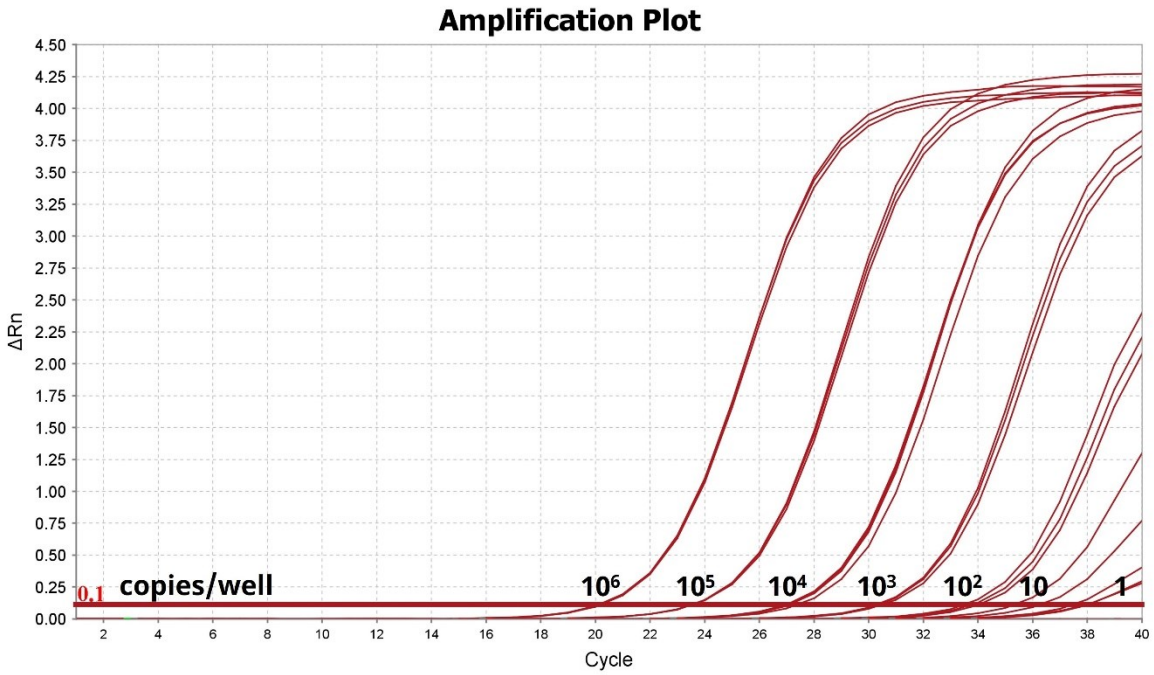


Figure 1. Individual values of a titration series of **IAV** (FAM) *in vitro* RNA in triplicates. The test was performed on the QuantStudio 5 instrument using the virotype Influenza A 2.0 real-time RT-PCR protocol.

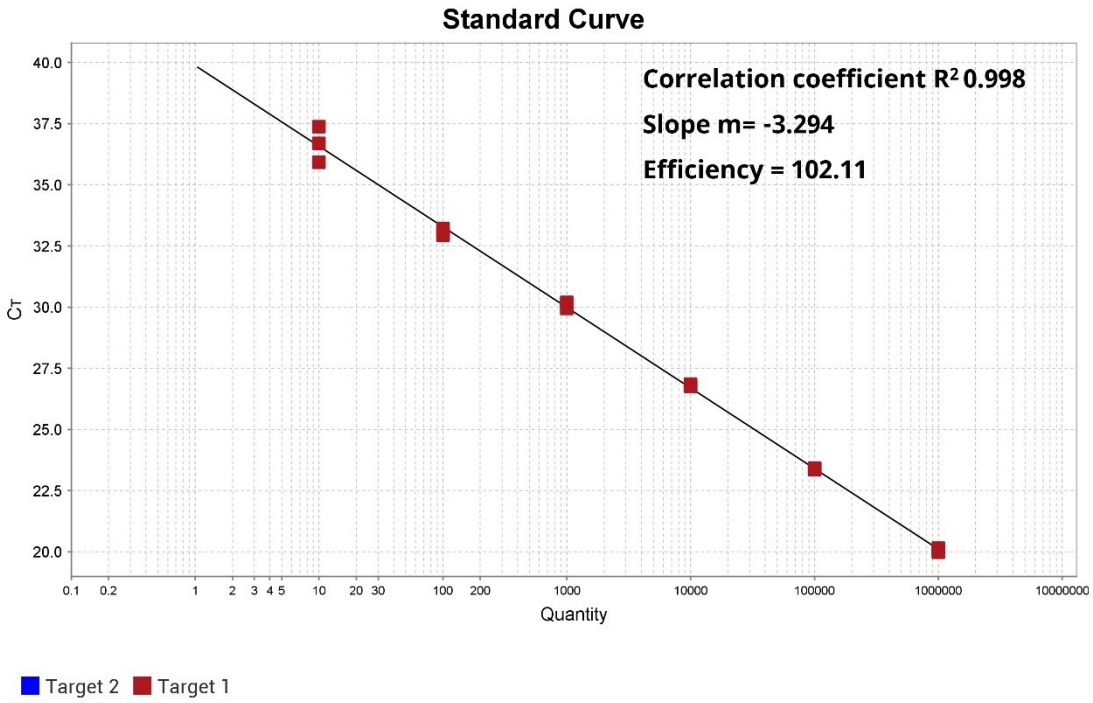


Figure 2. Standard curve of obtained C_T values for a titration series of **IAV** (FAM) *in vitro* RNA. The test was performed on the QuantStudio 5 instrument using the virotype Influenza A 2.0 real-time RT-PCR protocol.

4.1.2 Analytical sensitivity – Limit of detection

The limit of detection (LOD) for the target sequence of the IAV was determined by testing individual titration series of *in vitro* RNA of this sequence in octuplicates. The limit of detection with 95 % confidence interval ($LOD_{95\,\%}$: mean number of copies yielding a probability of detection of 0.95) was determined using the web tool https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory.

Results / Conclusion

Results are summarized in Table 6 and Figure 3. Using the virotype Influenza A 2.0 RT-PCR Kit, a high correlation between RNA copy number and the amount of amplified product was demonstrated for the IAV-targeted sequence. The $LOD_{95\%}$ is 11.3 copies/ reaction with a 95 % confidence interval of [5.911, 21.384] (Figure 3).

Table 6. Limit of detection for **IAV** *in vitro* RNA tested in octuplicates on the QuantStudio 5 instrument using the virotype Influenza A 2.0 real-time RT-PCR protocol.

Copies/test	Total number of replicates	Number of replicates positive	Number of replicates negative
10,000	8	8	0
1,000	8	8	0
100	8	8	0
50	8	8	0
10	8	8	0
5	8	5	3

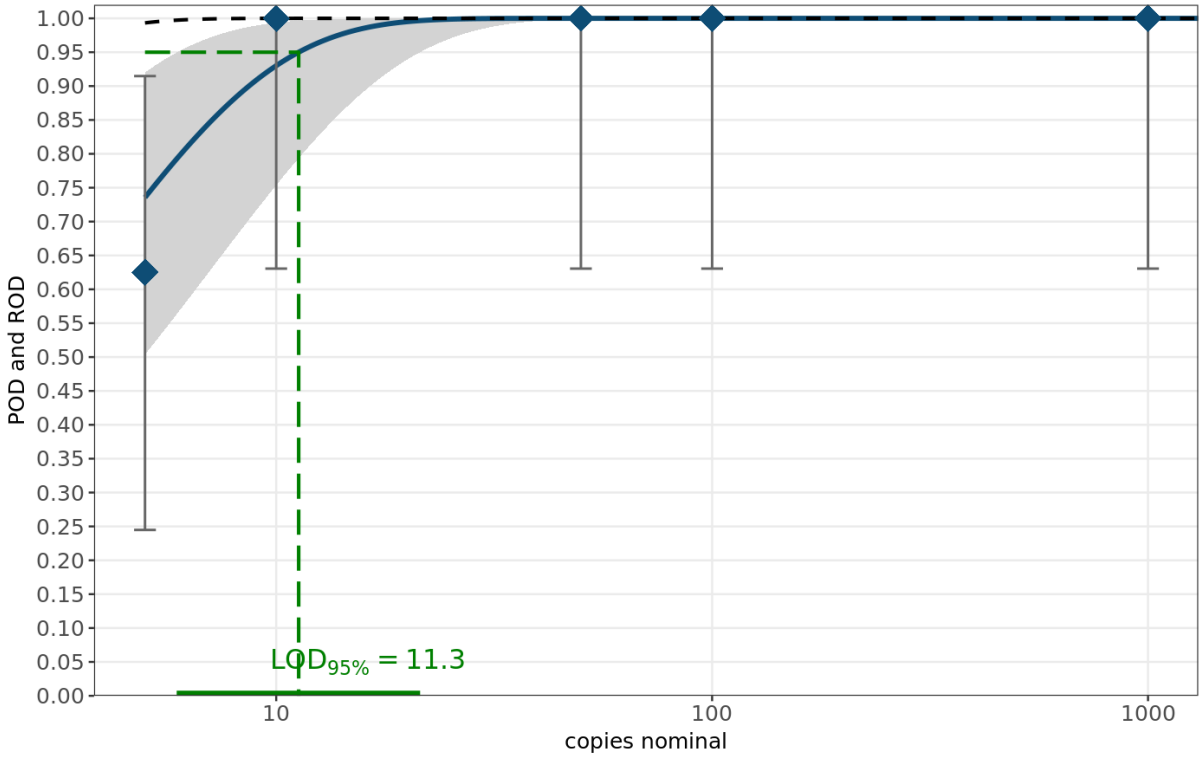


Figure 3. POD (probability of detection) curve and $LOD_{95\,\%}$ for **IAV**. The blue rhombuses characterize the laboratory-specific rates of detection. The blue curve denotes the mean POD curve along with the corresponding 95 % confidence range highlighted as the grey band. The POD curve under ideal conditions is displayed as the black dashed curve.

4.1.3 Analytical sensitivity of pooled samples

Pools were generated by diluting two IAV-positive (H5N3) swab samples (provided by a State Diagnostic Laboratory) in IAV-negative swab samples. Samples were extracted with the QIAamp® Viral RNA Kit (QIAGEN GmbH, Hilden, Germany) following manufacturer´s instructions. The resulting pools were tested with the virotype Influenza A 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 RT-PCR protocol.

Results / Conclusion

The C_T values of IAV (FAM) and the endogenous Internal Control (HEX) are shown in Table 7. Pooled samples up to 20 could be detected with the virotype Influenza A 2.0 RT-PCR Kit.

Table 7. Analysis of **IAV** (FAM) and the endogenous **Internal Control** (HEX) signals for pool samples with the virotype Influenza A 2.0 RT-PCR Kit tested on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 real-time RT-PCR protocol.

Pool size	**Sample**	**Species**	**virotype Influenza A 2.0 RT-PCR Kit**	
			IAV C_T	**Internal Control C_T**
0	19	bird	28.82	34.38
5			32.29	33.52
10			33.91	33.95
15			33.69	33.52
20			34.48	33.79
0	18T	bird	23.88	34.45
5			27.04	34.82
10			28.25	34.71
15			28.93	34.72
20			29.31	34.72

4.2 Specificity

4.2.1 Inclusivity

4.2.1.1 Detection of hemagglutinin (HA) and neuraminidase (NA) subtypes of the Influenza A Virus in birds

Dilutions of RNA samples from the Avian Influenza Virus (AIV) reference panel comprising *n* = 16 HA and 9 NA subtypes of the Influenza A Virus (kindly provided by the OIE, FAO and National reference lab for Avian Influenza at the Friedrich-Loeffler-Institut (FLI), Greifswald, Germany and the European Union Reference Laboratory (EURL) for Avian Influenza, Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Legnaro, Italy) were tested with the virotype Influenza A 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 RT-PCR protocol. The virotype Influenza A RT-PCR Kit served as reference.

Results / Conclusion

All tested Influenza HA and NA subtypes were correctly detected when using the virotype Influenza A 2.0 RT-PCR Kit (Table 8). In comparison the virotype Influenza A 2.0 RT-PCR Kit shows a slightly higher sensitivity (C_T mean 24.57) than the virotype Influenza A RT-PCR Kit (C_T mean 25.51).

Table 8. Analysis of RNA samples from the AIV reference panel tested with the virotype Influenza A 2.0 RT-PCR on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 real-time RT-PCR protocol compared to reference results obtained with the virotype Influenza A RT-PCR Kit.

Influenza A strain (avian origin)	**Subtype**	**virotype Influenza A 2.0 RT-PCR Kit**	**Ref.**
		C_T (IAV)	**C_T (IAV)**
A/wild duck/Germany/R30/06	H1N1	24.19	25.22
A/duck/Potsdam/177/83	H2N2	19.36	20.28
A/duck/Ukraine/1/63	H3N8	18.06	19.16
A/mallard/Germany/1507/07	H4N6	20.79	21.98
A/chicken/Netherlands/SP00153/2014	H5N1	20.34	21.52
A/eurasian wigeon/Italy/20VIR7301-206_feather/2020	H5N1	31.00	32.04
A/chicken/Belgium150/99 soncke99/150 v6	H5N2	20.86	22.25
A/Mallard/Sweden/Eskilstuna/05	H5N3	21.39	23.27
A/duck/Italy/16VIR123/2016	H5N3	27.57	28.68
A/mallard/Italy/20VIR4911-52/2020	H5N3	28.06	29.58
A/peregrine falcon/Denmark/ 13776-1_20VIR7282-13/2020	H5N5	30.94	31.50
A/duck/Nigeria/19VIR8424-2/2019	H5N6	29.82	30.23
A/duck/Nigeria/19VIR8424-20/2019	H5N8	29.44	30.17
A/chicken/Slovakia/14_20VIR205-19/2020	H5N8	29.75	30.17
A/Chicken/Netherlands/SP00213/2017	H5N8	37.71	39.35
A/turkey/Germany/AR2487/2014	H5N8	22.61	23.45
A/Turkey/Germany/R30/99	H6N1	21.48	22.24
A/turkey/Massachussetts/65	H6N2	27.10	28.32
A/turkey/Grub/R41/98	H6N5	19.48	20.33
A/parrot/N. Ireland/vf-73-67/73	H7N1	27.08	28.27
A/chicken/Italy/19VIR5895-21/2019	H7N3	29.80	30.88
A/teal/Italy/21VIR49-39/2021	H7N3	23.32	24.22
A/teal/Italy/16VIR345/2016	H7N7	29.30	30.24

A/turkey/Ontario/6118/68	H8N4	23.63	24.47
A/chicken/Saudi Arabia/SP02525/3AAV2000	H9N2	30.61	30.99
A/chicken/Nigeria/19VIR8424-15/2019	H9N2	22.65	23.52
A/swan/Italy/17VIR1205/2017	H9N2	30.25	30.94
A/mallard/Germany/2075/07	H10N7	22.49	23.23
A/guinea fowl/Hungary/1/69	H10N8	21.10	21.65
A/duck/England/56	H11N6	23.15	24.00
A/mallard/Germany/R2994/07	H11N9	15.59	16.86
A/duck/Alberta/60/76	H12N5	20.11	21.19
A/black headed gull/Germany/ R2622/06	H13N2	22.44	22.76
A/gull/Stralsund/Wv1136-40/03	H13N6	23.82	24.41
A/mallard/Gurev/263/82	H14N5	17.40	18.22
A/shearwater/West Australia/ 2576/79	H15N9	23.65	24.42
A/herring gull/Germany/ R2792/06	H16N2	24.58	25.56
A/herring gull/Germany/ R3309/07	H16N3	22.73	23.79
C_T mean		**24.57**	**25.51**

Ref. = Reference

4.2.1.2 Analysis of equine Influenza A samples using virotype Influenza A 2.0 RT-PCR Kit

Equine Influenza A virus RNA samples of subtype H3N8 isolated from horse nasal swabs were kindly provided by the Institute of Virology at the Free University of Berlin. Samples were tested with the virotype Influenza A 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 RT-PCR protocol. The virotype Influenza A RT-PCR Kit served as reference.

Results / Conclusion

All tested nasal swab samples were correctly detected when using the virotype Influenza A 2.0 RT-PCR Kit (Table 9). In comparison the virotype Influenza A 2.0 RT-PCR Kit shows a slightly higher sensitivity (C_T mean 22.96) than the virotype Influenza A RT-PCR Kit (C_T mean 24.20).

Table 9. RNA samples from horses infected with Influenza A subtype H3N8 analyzed with the virotype Influenza A 2.0 RT-PCR on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 real-time RT-PCR protocol compared to reference results obtained with the virotype Influenza A RT-PCR Kit.

Sample (equine origin)	**virotype Influenza A 2.0 RT-PCR Kit C_T (IAV)**	**Reference C_T (IAV)**
Sample #1 1:100	20.54	21.49
Sample #2 1:10	17.14	18.07
Sample #3 1:10	17.20	18.31
Sample #4 1:100	28.18	29.52
Sample #5 1:10	23.00	24.41
Sample #6 1:10	22.99	24.16
Sample #7 1:100	27.07	28.37
Sample #8 1:100	27.05	28.28
Sample #9 1:10	23.44	24.78
Sample #10 1:10	22.64	24.01
Sample #11 1:10	23.43	24.53
Sample #12 1:10	22.86	24.43
C_T mean	**22.96**	**24.20**

4.2.1.3 Analysis of porcine Influenza A samples using virotype Influenza A 2.0 RT-PCR Kit

Different sample materials from *n* = 56 Influenza A Virus (IAV)-infected pigs were used for nucleic acid extraction and were subsequently tested with the virotype Influenza A 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 RT-PCR protocol. The virotype Influenza A RT-PCR kit served as reference.

Furthermore, *in silico* analyses of different Influenza A virus subtypes occurring in pigs was conducted.

Results / Conclusion

All except one low positive sample were correctly detected when using the virotype Influenza A 2.0 RT-PCR Kit (Table 10). The virotype Influenza A 2.0 RT-PCR Kit shows equal to slightly lower C_T values (C_T mean 29.24) compared to the Reference (virotype Influenza A RT-PCR Kit, C_T mean 30.02).

The detection of different Influenza A virus subtypes occurring in pigs was also confirmed by an *in silico* analysis of sequence data by the Genbank (e.g.: H1N2 (MW362570.1), H1N1 (CY010573.2), H3N2 (EU273781.1).

Table 10. Porcine sample panel derived from Influenza A Virus (IAV)-infected pigs analyzed with the virotype Influenza A 2.0 RT-PCR on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 real-time RT-PCR protocol compared to reference results obtained with the virotype Influenza A RT-PCR Kit.

Sample (porcine origin)	**Sample material**	**virotype Influenza A 2.0 RT-PCR Kit C_T (IAV)**	**Ref. C_T (IAV)**
Pig # 1	Lung	28.26	29.24
Pig # 2	Lung	19.60	20.81
Pig # 3	Lung	25.73	27.00
Pig # 5	Nasal swab	28.79	29.17
Pig # 6	Oral fluid	37.23	37.93
Pig # 7	Nasal swab	32.85	33.49
Pig # 8	Nasal swab	36.88	37.47
Pig # 9	Nasal swab	30.07	30.44
Pig # 10	Lung	21.30	23.47

Pig # 11	Colon	26.82	26.99
Pig # 12	Oral fluid	32.14	33.02
Pig # 13	Swab	31.20	32.06
Pig # 14	Swab	28.21	28.62
Pig # 16	Lung	22.61	23.57
Pig # 17	Swab	-	36.41
Pig # 19	Nasal swab	31.90	32.36
Pig # 20	Nasal swab	24.45	25.04
Pig # 21	Nasal swab	29.97	30.13
Pig # 22	Tracheal swab	34.97	36.68
Pig # 23	Tracheal swab	21.08	21.93
Pig # 24	Oral fluid	31.70	32.74
Pig # 25	Oral fluid	34.93	36.33
Pig # 26	Oral fluid	33.55	34.00
Pig # 29	Oral fluid	31.74	32.65
Pig # 30	Oral fluid	32.37	33.17
Pig # 31	Lung	24.05	25.02
Pig # 32	Lung	25.64	26.28
Pig # 33	Lung	33.57	34.06
Pig # 34	Swab	20.22	20.75
Pig # 35	Swab	35.10	35.01
Pig # 36	Swab	24.96	25.41
Pig # 37	Swab	22.00	22.34
Pig # 38	Swab	30.60	30.71
Pig # 40	Oral fluid	30.50	30.41
Pig # 41	Oral fluid	28.03	28.53
Pig # 42	Oral fluid	30.71	31.73
Pig # 43	Lung	34.57	38.44
Pig # 44	Lung	31.44	32.25
Pig # 45	BALF	32.24	33.28

Pig # 46	BALF	32.27	32.90
Pig # 47	Lung	23.46	25.05
Pig # 48	Lung	28.35	29.50
Pig # 49	Lung	25.34	26.36
Pig # 50	Plural swab	34.20	35.33
Pig # 51	Nasal swab	35.61	38.27
Pig # 52	Nasal swab	28.32	29.08
Pig # 53	Nasal swab	30.62	31.55
Pig # 54	Nasal swab	33.19	33.64
Pig # 55	Nasal swab	31.84	32.77
Pig # 59	Lung	30.31	30.89
Pig # 60	Nasal swab	24.54	25.41
Pig # 61	Nasal swab	22.12	22.75
Pig # 62	Nasal swab	21.46	22.64
Pig # 63	Nasal swab	22.85	23.64
Pig # 64	unknown	25.97	26.61
Pig # 65 (H1N1)	unknown	35.13	35.83
C_T mean		**29.24**	**30.02**

Ref. = Reference, BALF = Bronchoalveolar lavage fluid

4.2.2 Exclusivity (Discrimination of pathogens for differential diagnosis)

The specificity / exclusivity was tested with nucleic acids from samples positive for *Avian Paramyxovirus* (APMV, *n* = 4), *Porcine Reproductive and Respiratory Syndrome Virus* (PRRSV, *n* = 11), *Mycoplasma synoviae* (Ms, *n* = 1)) and *Mycoplasma gallisepticum* (Mg, *n* = 2). The samples were kindly provided by the FLI and State Veterinary Laboratories. Specificity testing was performed on the Bio-Rad CFX96 instrument.

Furthermore, *in silico* analyses of several other respiratory pathogens in horses was conducted.

Results / Conclusion

No cross-reactivity to other relevant avian and porcine viral and bacterial pathogens was detected using the virotype Influenza A 2.0 RT-PCR Kit (Table 11).

An *in silico* analysis of other respiratory pathogens in horses did not reveal any cross-reactivity for *Equid Alphaherpesvirus* 1 (EHV-1, PQ014267), *Equid Alphaherpesvirus* 4 (EHV-4, PP765803), *Equine Arteritis Virus* (EAV, AY349167), *Equine Adenovirus 2* (NC_027705) and *Streptococcus equi* subsp. *equi* (NZ_CP133957).

Table 11. Cross-reactivity of the virotype Influenza A 2.0 RT-PCR Kit to other bird and swine-related pathogens tested on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 real-time RT-PCR protocol.

	Sample	Sample material	virotype Infl. A 2.0 RT-PCR Kit C_T (IAV)	Reference assay* C_T (Pathogen)
***Avian Paramyxovirus* (APMV)**	APMV-1/pigeon/Cyprus/ 20VIR3543-9/2020	-	-	positive
	APMV8 (Virus D)	-	-	positive
	NDV/chicken/Rus/Krasnodar/9.1/19	-	-	positive
	Virus C (NDV Clone 30)	-	-	positive
***Porcine Reproductive and Respiratory Syndrome Virus* (PRRSV) EU**	191 2 (15.12.2010) Routine	Serum	-	27.70
	214 1 (15.12.2010) Routine	Serum	-	25.10
	214 4 (15.12.2010) Routine	Serum	-	23.41
	369 5 (10.01.2011) Routine	Serum	-	29.19
	366 2 (10.01.2011) Routine	Serum	-	27.86
	366 4 (10.01.2011) Routine	Serum	-	31.10
	366 5 (10.01.2011) Routine	Serum	-	27.73
	366 6 (10.01.2011) Routine	Serum	-	34.34
	366 7 (10.01.2011) Routine	Serum	-	25.12
	366 8 (10.01.2011) Routine	Serum	-	36.02
	161 (16-20) (09.12.2010) Routine	Serum	-	29.77
***Mycoplasma synoviae* (Ms), *Mycoplasma gallisepticum* (Mg)**	Ms 1 RV21	-	-	18.46
	Mg/Ms 2 RV21	-	-	21.26, 32.00
	Mg 3 RV21	-	-	14.65

Ref. = Reference, - = no C_T

***** Reference assay were as follows: bactotype Mycoplasma MgMs PCR Kit (Ms/Mg samples), virotype PRRSV RT-PCR Kit (PRRSV samples)

4.3 Diagnostic sensitivity, specificity and efficiency

4.3.1 Definition diagnostic sensitivity

Percentage of positive samples in the new test of a population of true positive samples.
True positive samples giving negative results in the new test are termed false negative.

Calculation: [true positives / (true positives + false negatives)]*100

4.3.2 Definition diagnostic specificity

Percentage of negative samples in the new test of a population of true negative samples.
True negative samples giving positive results in the new test are termed false positive.

Calculation: [true negatives / (false positives + true negatives)]*100

4.3.3 Definition diagnostic efficiency

Diagnostic efficiency refers to the amount of agreement between the results from the new test and those from the reference test. It is expressed as a proportion of correctly identified samples among all samples.

Calculation: [(true positives + true negatives) / (true positives + true negatives + false positives + false negatives)]*100

4.3.4 Validation of the virotype Influenza A 2.0 RT-PCR Kit

The virotype Influenza A 2.0 RT-PCR Kit has been designed as a specific real-time RT-PCR, which means it specifically detects RNA of the Influenza A virus, a single-stranded RNA virus belonging to the family *Orthomyxoviridae*. The probe hybridizes to a highly conserved region coding for the matrix protein of Influenza A virus. High specificity is achieved by the combination of forward and reverse primer specific for swine, bird, and horse.

For validation of the virotype Influenza A 2.0 RT-PCR Kit, *n* = 296 samples (swab (*n* = 61), bronchoalveolar lavage fluid (*n* = 2), oral fluid (*n* = 10), organs (*n* = 43), feces (*n* = 10), serum (*n* = 69), allantoic fluid (*n* = 58), cell culture (*n* = 12), FTA cards (*n* = 7) and unknown (*n* = 24) were tested.

Swab samples comprised of undefined swab samples (*n* = 25), tracheal swab (*n* = 3), nasal swab (*n* = 16), cloacal swab (*n* = 17).

Organ samples comprised of organ pools (*n* = 28), lung (*n* = 14) and colon (*n* = 1).

The samples comprised of following species: bird (*n* = 157), swine (*n* = 127), horse (*n* = 12).

Positive nucleic acid reference samples were among others kindly provided by the FLI and the IZSVe. The IAV positive samples comprised of 16 HA and 9 NA subtypes.

Negative samples from wild birds were among others kindly provided by State Veterinary Laboratories.

Organ samples were processed using the IndiMag® Pathogen Kit (INDICAL Bioscience) on the KingFisher Flex according to the manufacturer's instructions and subsequently tested using the virotype Influenza A 2.0 RT-PCR Kit and the virotype Influenza RT-PCR Kit as reference.

Results/ Conclusion

The summary is shown in Table 12. Individual results are shown in Figure 4.

All but one weak positive IAV sample were detected correctly with the virotype Influenza A 2.0 RT-PCR Kit. Thus, the virotype Influenza A 2.0 RT-PCR Kit demonstrated a diagnostic sensitivity of 99.4 %, a diagnostic specificity of 100 % and a diagnostic efficiency of 99.7 % related to the virotype Influenza A RT-PCR Kit as referenced method. In this study the virotype Influenza A 2.0 RT-PCR Kit mostly demonstrated lower C_T values for IAV-positive samples than the virotype Influenza A RT-PCR Kit.

Table 12. Diagnostic sensitivity, specificity and efficiency of the virotype Influenza A 2.0 RT-PCR Kit.

virotype Influenza A 2.0 RT-PCR Kit (IAV; FAM)		**Comparative data**			
Total	**296**	**Ref. Positive**	**157**	**Ref. negative**	**139**
positive	157	true-positive	156	false-positive	0
negative	139	false-negative	1	true-negative	139

Ref. = Reference

Diagnostic sensitivity:	99.4 %
Diagnostic specificity:	100 %
Diagnostic efficiency:	99.7 %

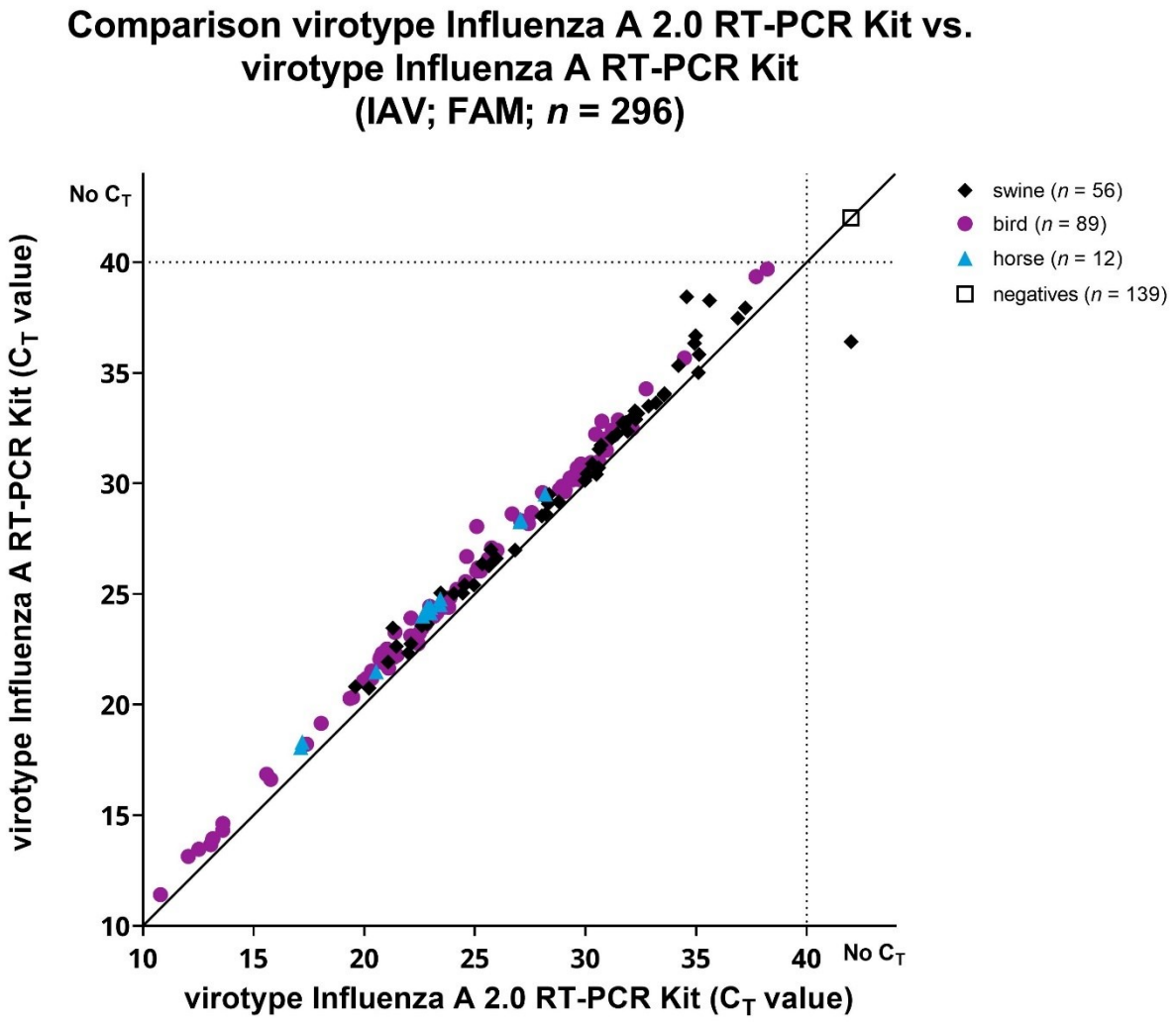


Figure 4. Comparison of C_T values from IAV-positive and IAV-negative samples tested with the virotype Influenza A 2.0 RT-PCR Kit compared to the virotype Influenza A RT-PCR Kit. All samples situated above the black diagonal line showed lower C_T values with the virotype Influenza A 2.0 RT-PCR Kit than tested with the virotype Influenza A RT-PCR Kit.

4.4 Comparative analysis of the virotype Influenza A 2.0 RT-PCR Kit and other commercially available RT-PCR Kits

A sample panel consisting of four IAV-positive RNA samples from different bird species and four IAV-positive RNA samples from swine (Table 13) were tested in a dilution series using the virotype Influenza A 2.0 RT-PCR Kit as well as five RT-PCR-Kits from other suppliers on the Bio-Rad CFX Opus 96 instrument. The material derived from tracheal swabs, nasal swabs, and organ pools and samples were kindly provided by several State Veterinary Laboratories or by routine testing laboratories.

Table 13. Samples for comparative testing of the virotype Influenza A 2.0 RT-PCR Kit and other commercially available RT-PCR Kits

Sample	IAV status	Material	Species
1	positive	swab	bird
2	positive	organ pool	chicken
3	positive	unknown	mallard duck
4	positive	unknown	buzzard
5	positive	nasal swab	swine
6	positive	tracheal swab	swine
7	positive	swab	swine
8	positive	swab	swine

Results / Conclusion

Results obtained with the virotype Influenza A 2.0 RT-PCR Kit compared to the five competitor RT-PCR kits are shown in Table 14. Besides the virotype Influenza A 2.0 RT-PCR Kit, Kits A and B were the only competitor test kits to be registered for testing swine and bird samples as well. All other competitor test kits are either registered for bird samples (Kit C, D) or for swine samples (Kit E) only.

Altogether, the virotype Influenza A 2.0 RT-PCR Kit shows an overall similar to even better sensitivity compared to all used competitor RT-PCR kits.

Table 14. Comparative analysis of the virotype Influenza A 2.0 RT-PCR Kit and competitor kits.

Species	Sample	Dil.	virotype Infl. A 2.0 RT-PCR Kit swine, bird C_T	Kit A swine, bird C_T	Kit B swine, bird C_T	Kit C bird C_T	Kit D bird C_T	Kit E swine C_T
Avian samples	1	1:100	27.57	31.65	31.68	30.14	29.64	
		1: 10^3	30.83	34.09	n.t.	32.41	33.00	
		1: 10^4	36.21	37.03	n.t.	35.74	-	
		1: 10^5	39.21	-	n.t.	-	-	
	2	1:100	23.30	23.85	26.70	23.52	22.17	
		1: 10^3	26.59	27.42	n.t.	26.63	25.06	
		1: 10^4	30.04	30.39	n.t.	29.90	28.16	
		1: 10^5	33.90	33.38	n.t.	33.28	31.35	
		1: 10^6	37.20	39.27	n.t.	37.21	34.26	
		1: 10^7	-	-	n.t.	38.86	-	
	3	1:100	34.17	37.23	37.76	36.11	33.16	
		1: 10^3	35.55	-	n.t.	38.55	-	
	4	1:10	30.60	31.76	35.20	31.85	29.35	
		1: 100	34.23	33.87	n.t.	34.86	32.68	
		1: 10^3	38.66	-	n.t.	38.16	39.98	
Swine samples	5	1:100	27.33	28.78	31.43			29.10
		1: 10^3	30.69	31.92	n.t.			32.06
		1: 10^4	34.12	34.81	n.t.			35.49
		1: 10^5	36.99	-	n.t.			38.14
		1: 10^6	-	-	n.t.			-
	6	1:100	25.55	25.89	n.t.			27.27
		1: 10^3	29.01	29.01	n.t.			30.52
		1: 10^4	32.33	33.20	n.t.			33.90
		1: 10^5	34.96	36.17	n.t.			37.39
		1: 10^6	-	-	n.t.			-

7	1:100	28.07	28.28	30.32		30.00
	1: 10^3	31.24	31.78	n.t.		32.87
	1: 10^4	34.17	35.76	n.t.		35.92
	1: 10^5	38.11	38.13	n.t.		38.82
8	1:100	26.18	26.56	28.79		27.94
	1: 10^3	29.32	29.77	n.t.		31.07
	1: 10^4	32.93	33.72	n.t.		34.58
	1: 10^5	36.91	36.70	n.t.		-

- = no C_T, n.t. = not tested

4.5 Repeatability

The same sample panel comprising one IAV-positive RNA sample from chicken (sample 1), two IAV-positive RNA samples from swine (samples 2 and 3) and one IAV-negative sample from chicken (sample 4), as well as the kit controls (NC, PC) was used for assessment of intra-assay variance, inter-assay variance, batch-to-batch variance, robustness testing, as well as for comparison of test results obtained with the virotype Influenza A 2.0 RT-PCR Kit tested on different real-time PCR thermocyclers (Table 15). Samples were either kindly provided by State Veterinary Laboratories or by routine testing laboratories.

Table 15. Sample panel for assessment of intra-assay variance, inter-assay variance, batch-to-batch variance, stability, and robustness testing for the virotype Influenza A 2.0 RT-PCR Kit.

Sample	IAV status	Material	Species	Dilution
1	positive	organ pool	Chicken	1:200
2	positive	nasal swab	Swine	1:40
3	positive	swab	Swine	1:200
4	negative	organ pool	Chicken	1:20
NC	negative	Negative Control (virotype Influenza A 2.0 RT-PCR Kit)		
PC	positive	Positive Control (virotype Influenza A 2.0 RT-PCR Kit)		

NC = Negative Control, PC = Positive Control

4.5.1 Intra-assay variance

The sample panel listed in Table 15 was tested in a sixfold setup in the same PCR run with the virotype Influenza A 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 RT-PCR protocol.

Results / Conclusion

The intra-assay variance is on average 0.32 % for IAV (FAM) and 0.38 % for the endogenous Internal Control (HEX) (Table 16, Table 17, and Figure 5).

These results show an excellent reproducibility in the same RT-PCR run for the virotype Influenza A 2.0 RT-PCR Kit.

Table 16. Intra-assay variance for **IAV** (FAM) for the virotype Influenza A 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Intra-assay variance for IAV (FAM)										
Sample	**IAV status**	**Reactions (C_T values)**						**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**			
1	pos	23.73	23.92	23.95	23.85	23.92	23.86	23.87	0.08	0.33
2	pos	24.77	24.83	24.70	24.65	24.75	24.72	24.74	0.06	0.25
3	pos	29.66	29.98	29.80	29.69	29.71	29.70	29.76	0.12	0.40
4	neg	-	-	-	-	-	-	-	-	-
NC	neg	-	-	-	-	-	-	-	-	-
PC	pos	26.82	27.00	27.00	26.97	27.05	26.89	26.96	0.08	0.31
Mean										**0.32**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 17. Intra-assay variance for the endogenous **Internal Control** (HEX) for the virotype Influenza A 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Intra-assay variance for the endogenous Internal Control (HEX)										
Sample	**IAV status**	**Reactions (C_T values)**						**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**			
1	pos	33.49	33.43	33.28	33.46	33.08	33.41	33.36	0.15	0.46
2	pos	31.55	31.74	31.64	31.61	31.44	31.65	31.61	0.10	0.32
3	pos	34.60	34.44	34.33	34.37	34.18	34.16	34.35	0.17	0.48
4	neg	28.70	28.85	28.67	28.82	28.78	28.58	28.73	0.10	0.35
NC	neg	-	-	-	-	-	-	-	-	-
PC	pos	26.86	26.96	27.06	27.03	26.97	26.92	26.97	0.07	0.27
Mean										**0.38**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Intra-assay variance virotype Influenza 2.0 RT-PCR Kit

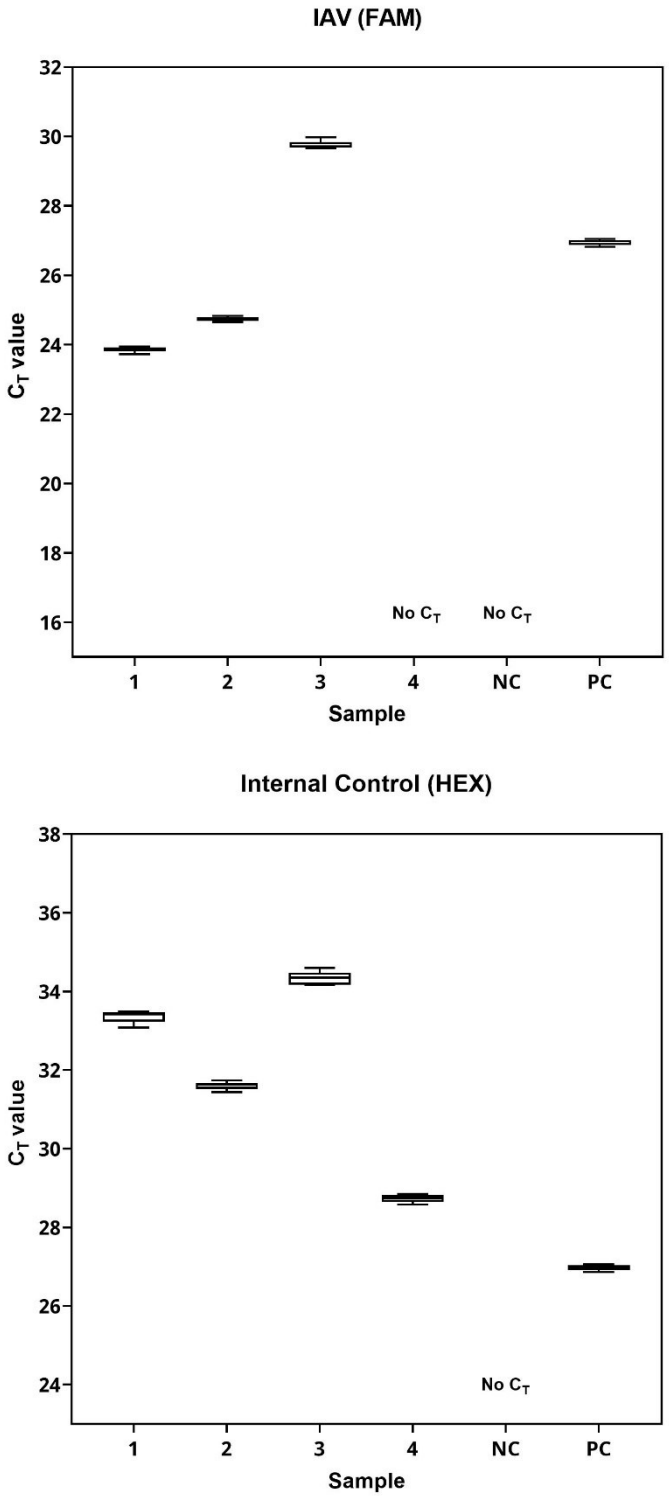


Figure 5. Boxplots of intra-assay variance for **IAV** (FAM) and the **endogenous Internal Control** (HEX) for the virotype Influenza A 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.
NC = Negative Control, PC = Positive Control

4.5.2 Inter-assay variance

The sample panel listed in Table 15 was tested in six independent PCR runs using the virotype Influenza A 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 RT-PCR protocol.

Results / Conclusion

The inter-assay variance is on average 0.42 % for IAV (FAM) and 1.35 % for the endogenous Internal Control (HEX) (Table 18, Table 19, and Figure 6). These results show an excellent reproducibility in different RT-PCR runs for the virotype Influenza A 2.0 RT-PCR Kit.

Table 18. Inter-assay variance for **IAV** (FAM) for the virotype Influenza A 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Inter-assay variance for IAV (FAM)										
Sample	**IAV status**	**RT-PCR runs (C_T values)**						**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**			
1	pos	25.19	25.19	25.33	25.25	25.34	25.35	25.28	0.07	0.30
2	pos	24.94	24.98	25.07	24.74	25.08	25.04	24.98	0.13	0.51
3	pos	29.95	29.78	30.04	29.88	30.14	30.04	29.97	0.13	0.43
4	neg	-	-	-	-	-	-	-	-	-
NC	neg	-	-	-	-	-	-	-	-	-
PC	pos	27.31	27.27	27.38	27.04	27.20	27.18	27.23	0.12	0.43
Mean										**0.42**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 19. Inter-assay variance for the **endogenous Internal Control** (HEX) for the virotype Influenza A 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Inter-assay variance for the endogenous Internal Control (HEX)										
Sample	**IAV status**	**RT-PCR runs (C_T values)**						**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**			
1	pos	37.18	37.05	37.77	34.52	34.94	35.84	36.22	1.32	3.64
2	pos	32.37	31.81	32.38	31.44	31.56	31.58	31.86	0.42	1.32
3	pos	34.66	33.99	34.15	34.10	34.34	34.10	34.22	0.24	0.71
4	neg	28.83	28.97	29.05	28.67	28.78	28.63	28.82	0.16	0.57
NC	neg	-	-	-	-	-	-	-	-	-
PC	pos	27.09	26.76	27.08	26.96	27.09	27.09	27.01	0.13	0.49
Mean										**1.35**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Inter-assay variance virotype Influenza 2.0 RT-PCR Kit

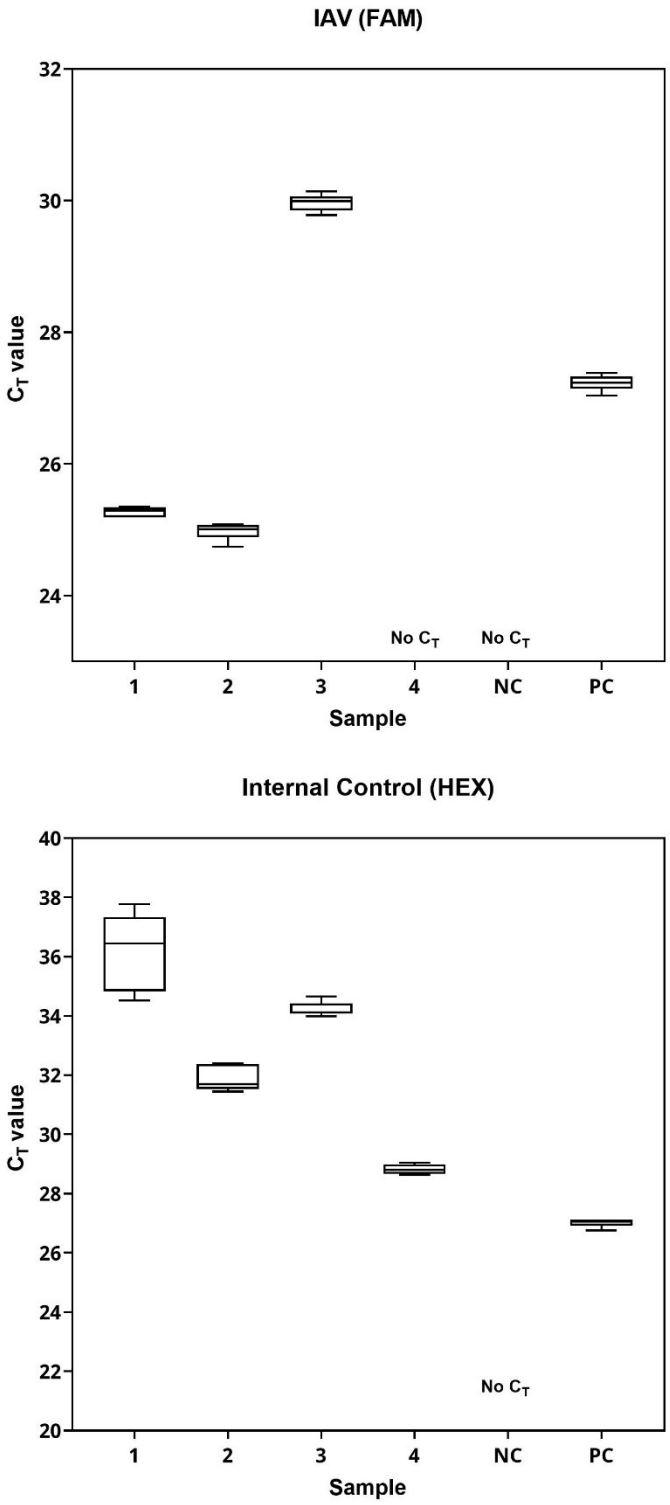


Figure 6. Boxplots of inter-assay variance for **IAV** (FAM) and the **endogenous Internal Control** (HEX) for the virotype Influenza A 2.0 RT-PCR Kit tested using the Bio-Rad CFX96 instrument.
NC = Negative Control, PC = Positive Control

4.5.3 Batch-to-batch comparison

The sample panel listed in Table 15 was tested in in the same PCR run using three different batches of the virotype Influenza A 2.0 RT-PCR Kit (batch 1, 2 and 3) on the Bio-Rad CFX96 instrument using the virotype Influenza A 2.0 RT-PCR protocol.

Results / Conclusion

The batch-to-batch performance showed an average variance of 0.50 % for IAV (FAM) and 0.86 % for the endogenous Internal Control (HEX) (Table 20, Table 21, and Figure 7).

Table 20. Batch-to-batch variance for **IAV** (FAM) for the virotype Influenza A 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Batch-to-batch variance for IAV (FAM)							
Sample	**IAV status**	**Batch number (C_T values)**			**Mean**	**SD**	**CV%**
		1	**2**	**3**			
1	pos	25.23	25.26	25.08	25.19	0.10	0.38
2	pos	24.94	25.18	24.85	24.99	0.17	0.68
3	pos	29.87	30.10	29.94	29.97	0.12	0.39
4	neg	-	-	-	-	-	-
NC	neg	-	-	-	-	-	-
PC	pos	27.06	27.29	27.03	27.13	0.14	0.52
Mean							**0.50**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 21. Batch-to-batch variance for the **endogenous Internal Control** (HEX) for the virotype Influenza A 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Batch-to-batch variance for the endogenous Internal Control (HEX)							
Sample	**IAV status**	**Batch number (C_T values)**			**Mean**	**SD**	**CV%**
		1	**2**	**3**			
1	pos	35.24	35.88	35.58	35.57	0.32	0.90
2	pos	31.86	32.23	31.61	31.90	0.31	0.98
3	pos	34.62	34.96	34.02	34.53	0.48	1.38
4	neg	28.75	29.14	28.92	28.94	0.20	0.68
NC	neg	-	-	-	-	-	-
PC	pos	27.20	27.08	27.27	27.19	0.10	0.35
Mean							**0.86**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Batch-to-batch variance virotype Influenza 2.0 RT-PCR Kit

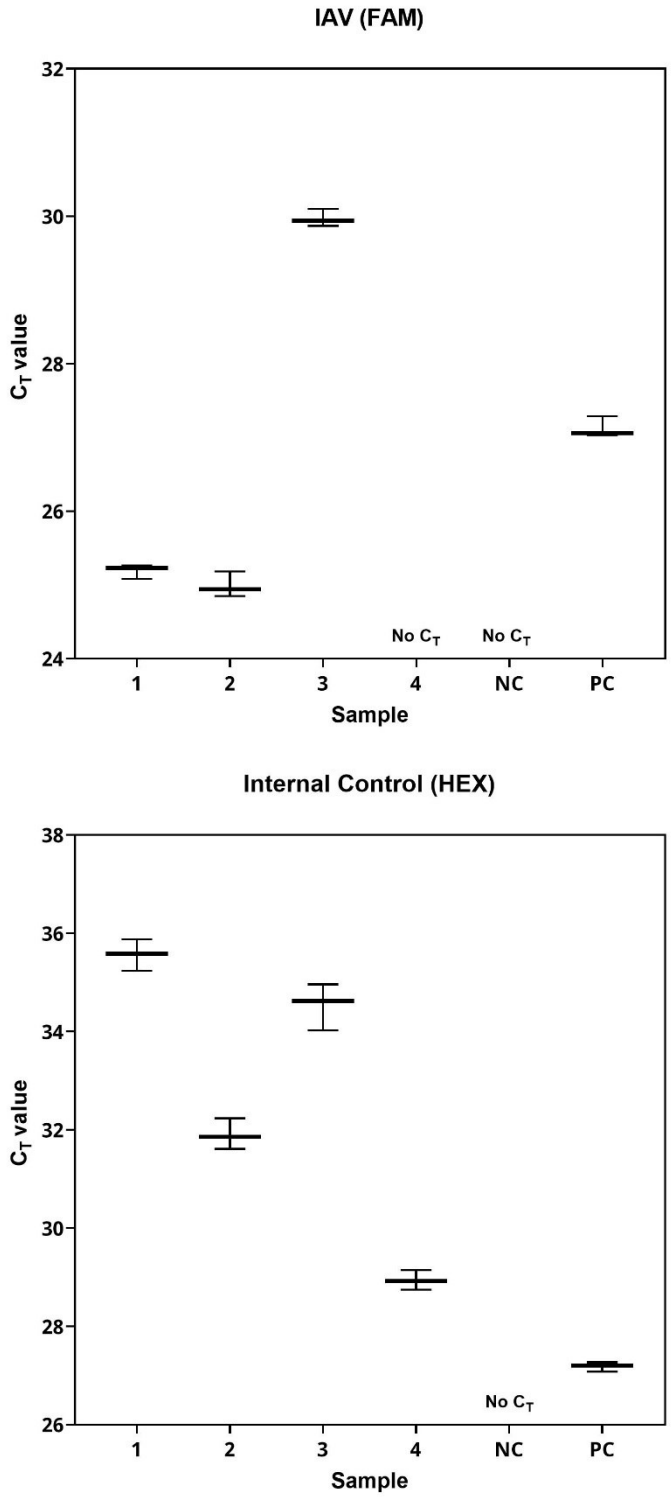


Figure 7. Boxplots of batch-to-batch variance for **IAV** (FAM) and the **endogenous Internal Control** (HEX) for the virotype Influenza A 2.0 RT-PCR Kit tested using the Bio-Rad CFX96 instrument.
NC = Negative Control, PC = Positive Control

4.5.4 Comparison of real-time PCR thermocyclers

The virotype Influenza A 2.0 RT-PCR Kit can be used on different standard real-time PCR cyclers. Table 22 gives an overview of selected PCR cyclers and their approximate run times, using the virotype Influenza A 2.0 RT-PCR protocol.

Note: The use of the RT-PCR Kit is not limited to the mentioned instruments.

Table 22. Selected overview of real-time thermocyclers and their approximate run times for the virotype Influenza A 2.0 RT-PCR protocol.

	Thermocycler			
	Manufacturer	**Model**	**Filters**	**Run time [minutes]**
A	Bio-Rad Laboratories, Inc., Hercules, California, USA	CFX96	FAM, HEX	72
B	Agilent Technologies, Santa Clara, California, USA	AriaMx	FAM, HEX	59
C	Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA	QuantStudio 5	FAM, VIC	65

The sample panel listed in Table 15 was tested with the virotype Influenza A 2.0 RT-PCR Kit on three different real-time PCR thermocycler instruments (Table 22) using the virotype Influenza A 2.0 RT-PCR protocol.

Results / Conclusion

The results are summarized in Table 23 (IAV / FAM channel), and Table 24 (endogenous Internal Control/ HEX channel). All samples tested on different real-time PCR thermocycler instruments showed comparable results.

Table 23. Inter-thermocycler variance for **IAV** (FAM).

Inter-thermocycler variance for IAV (FAM)				
Sample	**IAV status**	**Thermocycler (C_T values)**		
		A	**B**	**C**
1	positive	25.20	25.59	25.06
2	positive	24.91	24.77	25.09
3	positive	29.88	29.70	30.22
4	negative	-	-	-
NC	negative	-	-	-
PC	positive	27.03	26.87	27.25

NC = Negative Control, PC = Positive Control, - = no C_T

Table 24. Inter-thermocycler variance for the **endogenous Internal Control** (HEX).

Inter-thermocycler variance for the endogenous Internal Control (HEX)				
Sample	**IAV status**	**Thermocycler (C_T values)**		
		A	**B**	**C**
1	positive	35.36	33.59	35.33
2	positive	31.65	29.89	31.74
3	positive	34.21	32.89	34.16
4	negative	28.88	28.18	29.18
NC	negative	-	-	-
PC	positive	27.17	26.37	27.24

NC = Negative Control, PC = Positive Control, - = no C_T

4.6 Stability testing

4.6.1 Freeze-thaw-cycles

For stability evaluation, a sample panel consisting of one IAV-positive RNA sample from chicken (sample 1), three IAV-negative samples from chicken (sample 2, 3, and 4) and the kit controls (NC, PC) was tested (Table 25). Samples were kindly provided by State Veterinary Laboratories.

One kit batch was tested at the time of production and after six freeze/thaw cycles. The mean value (Mean), standard deviation (SD) and coefficient of variation (CV) were calculated

Table 25. Sample panel for assessment of stability of the virotype Influenza A 2.0 RT-PCR Kit

Sample	IAV status	Material	Species
1	positive	organ pool	chicken
2	negative	organ pool	chicken
3	negative	organ pool	chicken
4	negative	organ pool	chicken
NC	negative	Negative Control (virotype Influenza A 2.0 RT-PCR Kit)	
PC	positive	Positive Control (virotype Influenza A 2.0 RT-PCR Kit)	

NC = Negative Control, PC = Positive Control

Results / Conclusion

The virotype Influenza A 2.0 RT-PCR Kit shows excellent stability after six freeze/thaw cycles of the virotype Influenza 2.0 Master Mix. The average variance was 1.41 % for IAV (FAM) and 0.41 % for the endogenous Internal Control (HEX) (Table 26, Table 27).

Table 26. Stability testing for **IAV** (FAM) of the virotype Influenza A 2.0 RT-PCR Kit during freeze and thaw cycles. The Bio-Rad CFX96 instrument was used for analysis.

Stability for IAV (FAM)						
Sample	**IAV status**	**Freeze-thaw-cycle (C_T values)**		**Mean**	**SD**	**CV%**
		1	**6**			
1	pos	24.56	24.74	24.65	0.13	0.52
2	neg	-	-			
3	neg	-	-			
4	neg	-	-	-	-	-
5	neg	-	-	-	-	-
PC	pos	27.4	26.52	26.96	0.62	2.31
Mean						**1.41**

PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 27. Stability testing for the **endogenous Internal Control** (HEX) of the virotype Influenza A 2.0 RT-PCR Kit during freeze and thaw cycles. The Bio-Rad CFX96 instrument was used for analysis.

Stability for the endogenous Internal Control (HEX)						
Sample	**IAV status**	**Freeze-thaw-cycle (C_T values)**		**Mean**	**SD**	**CV%**
		1	**6**			
1	pos	28.12	28.1	28.11	0.01	0.05
2	neg	24.19	24.4	24.30	0.15	0.61
3	neg	32.57	32.16	32.37	0.29	0.90
4	neg	30.45	30.31	30.38	0.10	0.33
5	neg	-	-		-	-
PC	pos	27.12	27.05	27.09	0.05	0.18
Mean						**0.41**

PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

4.6.2 Heparin inhibition

To test the stability of the virotype Influenza A 2.0 RT-PCR Kit, sample inhibition was simulated by treating an IAV-positive samples with an increasing concentration of heparin (0.17 – 3.40 U/reaction). The sample was tested in duplicate using the virotype Influenza A 2.0 RT-PCR Kit.

Results / Conclusion

Complete inhibition of the IAV (FAM) signal by heparin was observed at the highest tested heparin concentration of 3.4 U/reaction (Table 28). The inhibition study showed that the endogenous Internal Control signal will be affected first in case of sample inhibition. This will help to identify partially and fully inhibited samples.

Table 28. Stability testing of the virotype Influenza A 2.0 RT-PCR Kit for inhibition by heparin. The Bio-Rad CFX Opus 96 instrument was used for analysis.

	Stability (heparin inhibition)	
Heparin [U/reaction]	**IAV C_T value**	**Internal Control C_T value**
-	24.14	28.40
-	24.20	28.23
0.17	24.54	29.90
0.17	24.70	30.09
0.34	25.04	30.79
0.34	25.15	31.29
1.70	33.49	-
1.70	33.56	-
3.40	-	-
3.40	-	-

- = no C_T

4.6.3 EDTA inhibition

To test the stability of the virotype Influenza A 2.0 RT-PCR Kit, sample inhibition was simulated by treating an IAV-positive sample with an increasing concentration of Ethylenediaminetetraacetic acid (0.5 – 7.0 mM EDTA final concentration in reaction mix). The sample was tested in duplicate using the virotype Influenza A 2.0 RT-PCR Kit.

Results / Conclusion

Complete inhibition of the IAV (FAM) signal occurs at high concentrations only (> 5.0 mM) when using the virotype Influenza A 2.0 RT-PCR Kit (Table 29). The inhibition study using EDTA also showed that the endogenous Internal Control signal will be affected first in case of sample inhibition (EDTA concentration > 4.0 mM). This will help to identify partially and fully inhibited samples.

Table 29. Stability testing of the virotype Influenza A 2.0 RT-PCR Kit for inhibition by EDTA.

Stability (EDTA inhibition)		
EDTA [mM]	**IAV C_T value**	**Internal Control C_T value**
-	24.14	28.40
-	24.20	28.23
0.5	24.40	28.59
0.5	24.38	28.65
1.0	24.69	28.74
1.0	24.54	28.87
2.0	24.62	29.36
2.0	24.50	29.45
3.0	24.35	29.85
3.0	24.32	29.85
4.0	32.21	-
4.0	33.34	-
5.0	37.19	-
5.0	-	-
6.0	-	-
6.0	-	-

- = no C_T

4.6.4 Humic acid inhibition

To test the stability of the virotype Influenza A 2.0 RT-PCR Kit, sample inhibition was simulated by treating an IAV-positive sample with an increasing concentration of humic acid (1 – 250 nm humic acid final concentration in reaction mix). The sample was tested in duplicate using the virotype Influenza A 2.0 RT-PCR Kit.

Results / Conclusion

The inhibition study using humic acid showed that complete sample inhibition (IAV, FAM) occurs at concentration of 100 ng when using the virotype Influenza A 2.0 RT-PCR Kit (Table 30). As seen for EDTA and heparin, inhibition study using humic acid showed that the endogenous Internal Control signal will be affected first in case of sample inhibition (humic acid concentration > 10 ng/ reaction). The increased C_T value will help to identify partially and fully inhibited samples.

Table 30. Stability testing of the virotype Influenza A 2.0 RT-PCR Kit for inhibition by humic acid.

Stability (humic acid inhibition)		
Humic acid [ng / reaction]	**IAV C_T value**	**Internal Control C_T value**
-	24.28	28.27
-	24.33	28.46
1.0	24.33	28.76
1.0	24.25	28.76
10	26.77	31.85
10	26.76	32.19
50	33.17	-
50	32.94	-
100	38.07	-
100	-	-
250	-	-
250	-	-

- = no C_T