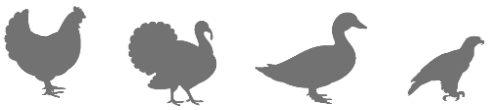
April 2023 | EN

virotype® Influenza A H5/H7/H9 RT-PCR Kit

Validation Report

For simultaneous detection of RNA from Influenza A virus subtypes H5, H7 and H9



INDICAL BIOSCIENCE

Contents

1 Introduction

1.1 Intended use

The virotype Influenza A H5/H7/H9 RT-PCR Kit is intended for the simultaneous detection and differentiation of RNA from Influenza A virus subtypes H5, H7 and H9. The kit allows the detection of RNA in tracheal, and cloacal swabs, as well as in fecal and tissue samples, in cell culture supernatant and filter membranes (e.g., FTA® cards) from birds.

The kit is approved by the Friedrich-Loeffler-Institut and licensed in accordance with § 11 (2) of the German Animal Health Act (FLI-C 072) for use in Germany for veterinary diagnostic procedures.

For veterinary use only.

1.2 General information

The virotype Influenza A H5/H7/H9 RT-PCR Kit is a multiplex PCR assay intended for the simultaneous detection and differentiation of RNA from Influenza A virus subtypes H5, H7 and H9. The kit allows the detection of RNA in tracheal, and cloacal swabs, as well as in fecal and tissue samples, in cell culture supernatant and filter membranes (e.g., FTA® cards) from birds.

Viruses of the genus *Influenzavirus* A belong to the family *Orthomyxoviridae*. They occur in high genetic diversity and a wide range of virulence. Influenza A viruses 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. All infections with subtypes H5 or H7 are notifiable. Infections with subtype H9 often lead to milder symptoms of the respiratory tract and egg production. Co-infections with other pathogens causing respiratory diseases may lead to severe infections with high mortality. Furthermore, avian Influenza A subtype H9 virus can contribute to genetic reassortions due to its wide distribution and co-circulation (esp. in Asia).

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 H5/H7/H9 RT-PCR Kit contains all of the necessary reagents for the detection and differentiation of RNA from Influenza A subtypes H5, H7 and H9, 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.

An internal control excludes the possibility of false-negative results.

The kit uses four specific primer/probe combinations:

- FAM™ fluorescence for RNA from Influenza A subtype H5
- HEX™ fluorescence for RNA from Influenza A subtype H7
- Cy®5 fluorescence for RNA from Influenza A subtype H9
- Texas Red® fluorescence for the internal control

A Positive Control serves to verify the functionality of the reaction mix for the amplification of the pathogen targets.

1.4 Kit contents

virotype Influenza A H5/H7/H9 RT-PCR Kit	**(96)**
Cat. no.	**VT282705**
Number of reactions	**96**
RT-PCR Mix (tube with yellow cap), includes enzymes and the internal control system	2 x 990 µl
Primers/Probes (tube with purple cap)	1 x 210 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 H5/H7/H9 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 (>2x), 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
  Note: Use of Rotor-Gene® Q only in combination with the Q-Rex software
- 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 H5/H7/H9 RT-PCR Kit is intended for the simultaneous detection and differentiation of RNA from Influenza A virus subtypes H5, H7 and H9 in tracheal, and cloacal swabs, as well as in fecal and tissue samples and filter membranes (e.g., FTA cards) from birds.

Prior to real-time RT-PCR, viral RNA must be extracted from the starting material. INDICAL offers a range of validated kits for the extraction of RNA and DNA 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

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

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

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.
- Important: 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. 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 H5/H7/H9 RT-PCR Kit to test for successful amplification of the target.

Internal control

For increased process safety and convenience, an internal control assay is included in the form of an additional primer/probe set in the Master Mix This allows 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. Perform the protocol without interruption.
- **Important**: Ensure to mix the viscous RT-PCR Mix well prior to use.

2.2 Things to do before starting

- Thaw all reagents on ice and protect from light.
- Maintain reagents on ice or in a cooling block during setup of the Master Mix as well as Reaction Mix.
- Before use, spin the reagents briefly.

1. Set up the Master Mix according to Table 1 immediately prior to use. The Master Mix contains all the components that are required for a PCR reaction except the sample. Set up a Master Mix volume that is 10 % greater than is needed for the total amount of PCR reactions. Table 1 lists the required volumes based on the quantity of reactions. Storage of prepared Master Mix is not recommended.

Table 1. Preparation of the Master Mix

	Quantity of reactions		
Component	**1**	**24**	**96**
RT-PCR Mix (yellow cap)	18 µl	432 µl	1896 µl
Primers/Probes (purple cap)	2 µl	48 µl	24 µl
Total volume	**20 µl**	**480 µl**	**1920 µl**

2. Mix well the prepared Master Mix (shortly vortex if possible) and collect fluids by short centrifugation.

3. Pipet 20 µl of the Master Mix into each reaction tube. Then add 5 µl of the sample RNA (Table 2).

   Include Positive and Negative Control reactions.

   Positive Control: Use 5 µl of the Positive Control instead of sample RNA.

   Negative Control: Use 5 µl of the Negative Control instead of sample RNA.

Table 2. Preparation of Reaction Mix

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

4. Close the reaction tubes with the corresponding caps.
5. Set the filters for the reporter dyes in the software of your thermal cycler according to Table 3

Table 3. Filter settings for the reporter

Pathogen/ Internal Control	**Reporter**
Influenza A subtype H5	FAM
Influenza A subtype H7	HEX/ JOE™[1]
Influenza A subtype H9	Cy®5
Internal Control	Texas Red/ROX™[1, 2]

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

[2] ROX as use of internal reference dye **must be deactivated** for use on ABI PRISM® Sequence Detection Systems (Applied Biosystems®)

6. Run the real-time PCR protocol according to Table 4.

Table 4. Real-time RT-PCR protocol for the virotype Influenza A H5/H7/H9 RT-PCR Kit

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 (Agilent Agilent Mx3005P)

3 Data interpretation

Interpretation of results

For the assay to be valid the FAM, HEX, Cy5 and Texas Red fluorescence of the Positive Control must give a signal with a C_T[1] < 35. The Negative Control may only give a Texas Red fluorescence signal.

The following results are possible if working with unknown samples. The possible sample results are also summarized in Table 5.

Note: Please note that, depending on the used thermal cycler, crosstalk between fluorescence channels can occur (e.g., Rotor-Gene® Q, Aria™ Mx). When analyzing the data, please activate „crosstalk correction/ crosstalk compensation“. Further information is given in chapters 3.1 and 3.2, in a product-specific Technical Information or by contacting our INDICAL support under support@indical.com.

The sample is positive for Influenza A virus subtype H5, and the assay is valid, if the following criteria are met:

- The sample yields a signal in both the FAM and the Texas Red channel.
- The Positive Control yields a signal in all channels.
- The Negative Control does not yield a signal in the FAM, HEX and Cy5 channel.

The sample is positive for Influenza A virus subtype H7, and the assay is valid, if the following criteria are met:

- The sample yields a signal in both the HEX and the Texas Red channel.
- The Positive Control yields a signal in all channels.
- The Negative Control does not yield a signal in the FAM, HEX and Cy5 channel.

The sample is positive for Influenza A virus subtype H9, and the assay is valid, if the following criteria are met:

- The sample yields a signal in both the Cy5 and the Texas Red channel.
- The Positive Control yields a signal in all channels.
- The Negative Control does not yield a signal in the FAM, HEX and Cy5 channel.

Important: A sample can be positive for more than one subtype. It will then score positive results in the FAM and/ or HEX and/ or Cy5 channel in addition to the Texas Red fluorescence signal. A detailed interpretation of potential results can be found in Table 5.

[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

The sample is negative for Influenza A virus subtypes H5 and H7 and H9, and the assay is valid, if the following criteria are met:

- The sample yields a signal in only the Texas Red channel.
- The Positive Control yields a signal in all channels.
- The Negative Control does not yield a signal in the FAM, HEX and Cy5 channel.

A positive Texas Red signal excludes the possibility of inhibition, as the internal control was successfully amplified.

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

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

If no signal is detected, including the Texas Red channel (Internal Control), the result is inconclusive. The absence of a signal for the housekeeping gene indicates PCR inhibition and/ or other malfunctions.

To check for inhibition, we recommend 1:5 dilution of the sample RNA in nuclease-free water.

Check that there is a fluorescence signal in the FAM, HEX, Cy5 and Texas Red channels for the 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 5. Results interpretation table*

Pathogen			IC	Sample result
FAM	**HEX**	**Cy5**	**Texas Red/ ROX**	Positive for Influenza A subtype:
X			X	H5
	X		X	H7
		X	X	H9
X	X		X	H5 and H7
X		X	X	H5 and H9
	X	X	X	H7 and H9
X	X	X	X	H5 and H7 and H9
			X	Negative for H5 and H7 and H9
				inconclusive

* Interpretation of sample results can be determined provided positive and negative control reactions are performed. The Positive Control must yield a signal in all channels (FAM, HEX, Cy5 and Texas Red channels. The Negative Control must yield no signal in the FAM, HEX and Cy5 channels.

3.1 Data analysis at Rotor-Gene Q

If Rotor-Gene Q (QIAGEN, Hilden) in combination with Q-Rex Software was used, the crosstalk compensation function has to be activated as follows:

1. Click „Settings…“ in the „Crosstalk compensation“ section in the „Analysis“ tab.
2. Define crosstalk compensation settings in the „Select channels to compensate“ section as follows: select in column „Origin“ „Orange“ and in column „Affected“ „Red“, then click „Add compensation“.
3. Then select in the „Select channels to compensate“ section in column „Origin“ „Green“ and in column „Affected“ „Yellow“, then click „Add compensation“.
4. Select in the „Select methods to calculate compensation factors“ section: „Compensate crosstalk based on“ „all tubes“ and „Crosstalk limit is set“ „automatically“, then click „OK“.
5. Check the „Compensate crosstalk“ checkbox in the „Crosstalk compensation“ section.
6. Then analyze data as usual. If necessary, check the „Ignore first cycles“ checkbox in the „Normalization“ section and enter a number of cycles at the start of the run that are not used to normalize the fluorescence data.

3.2 Data analysis at Aria Mx

If Aria Mx (Agilent Technologies) was used, the crosstalk correction function has to be activated as follows:

1. Select „Adjust“ in „Crosstalk Correction“ section in „Amplification Plots“ tab.
2. In the box for the channel „HEX-JOE“ select the value „1“ for „FAM“.
3. Click „OK“ or „Apply“ and „OK“.
4. Then analyze data as usual.

4 Characteristics of the test

4.1 Analytical sensitivity

The analytical sensitivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit was determined by testing individual titration series of *in vitro* RNA of the Influenza A subtype H5. Tests were performed in duplicates of ten-fold dilutions on different cyclers using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol (50°C 10 min, 95°C 2 min; 40 cycles 95°C 5 s, 60°C 30 s). Results are shown in Table 6 and Table 7 as well as Figure 1 and Figure 2.

Table 6. Analytical sensitivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137): Individual and mean C_T values of an **Influenza A H5** (FAM) titration series in duplicates. The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Copy number	Influenza A H5 (FAM)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
Standard H5_EU	10^5	21.18	21.18	28.90	+
	10^5	21.18		28.91	+
	10^4	24.42	24.47	28.59	+
	10^4	24.52		28.68	+
	10^3	27.96	27.96	28.77	+
	10^3	27.95		28.67	+
	100	31.06	31.07	28.53	+
	100	31.08		28.54	+
	10	34.48	34.43	28.39	+
	10	34.38		28.55	+

IC = Internal Control

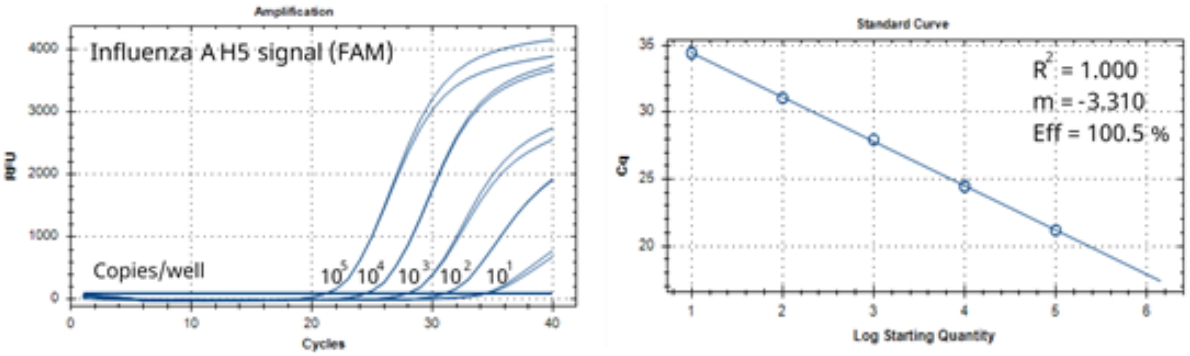


Figure 1. Titration of **Influenza A H5** (FAM) *in vitro* RNA tested with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Bio-Rad CFX96 instrument using the Influenza A H5/H7/H9 real-time RT-PCR protocol. The graph shows the amplification plot (FAM) and standard curve of obtained C_T values for the FAM signal.

Table 7. Analytical sensitivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137): Individual and mean C_T values of an **Influenza A H5** (FAM) titration series in duplicates. The test was performed on the Aria Mx instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Copy number	Influenza A H5 (FAM)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
Standard H5_EU	10^5	20.80	20.54	27.09	+
	10^5	20.28		26.94	+
	10^4	23.81	23.84	27.04	+
	10^4	23.86		26.79	+
	10^3	26.77	26.97	26.92	+
	10^3	27.16		27.03	+
	100	30.28	30.24	26.93	+
	100	30.19		27.11	+
	10	33.55	33.22	27.24	+
	10	32.88		27.16	+

IC = Internal Control

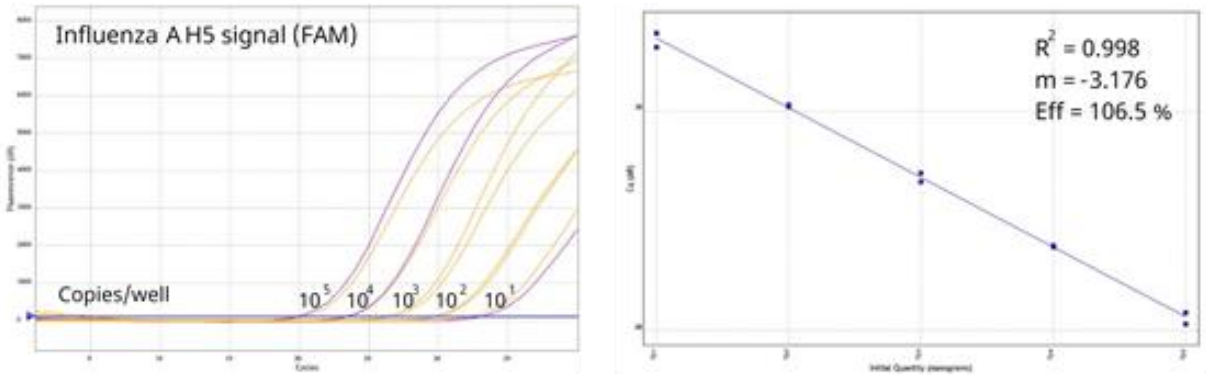


Figure 2. Titration of **Influenza A H5** (FAM) *in vitro* RNA tested with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Aria Mx instrument using the Influenza A H5/H7/H9 real-time RT-PCR protocol. The graph shows the amplification plot (FAM) and standard curve of obtained C_T values for the FAM signal.

The analytical sensitivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit was also determined by testing individual titration series of *in vitro* RNA of Influenza A subtype H7. Tests were performed in duplicates of ten-fold dilutions on different cyclers using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol (50°C 10 min, 95°C 2 min; 40 cycles 95°C 5 s, 60°C 30 s). Results are shown in Table 8 and Table 9 as well as Figure 3 and Figure 4.

Table 8. Analytical sensitivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137): Individual and mean C_T values of an **Influenza A H7** (HEX) titration series in duplicates. The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Copy number	Influenza A H7 (HEX)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
Standard H7_EU	10^5	23.17	23.06	27.74	+
	10^5	22.94		27.55	+
	10^4	26.03	26.00	27.56	+
	10^4	25.96		27.44	+
	10^3	29.10	29.18	27.44	+
	10^3	29.25		27.39	+
	100	32.67	32.49	27.34	+
	100	32.31		27.50	+
	10	35.11	36.40	27.77	+
	10	37.68		27.61	+

IC = Internal Control

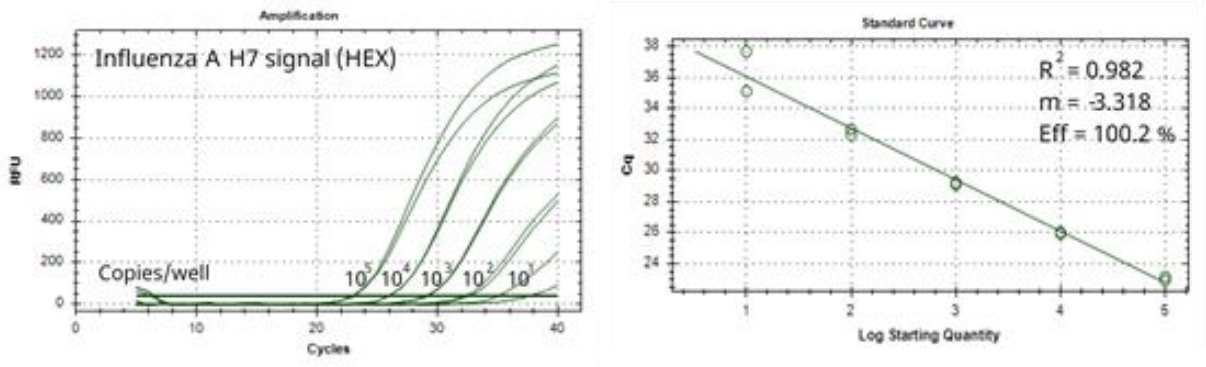


Figure 3. Titration of **Influenza A H7** (HEX) *in vitro* RNA tested with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Bio-Rad CFX96 instrument using the Influenza A H5/H7/H9 real-time RT-PCR protocol. The graph shows the amplification plot (HEX) and standard curve of obtained C_T values for the HEX signal.

Table 9. Analytical sensitivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137): Individual and mean C_T values of an **Influenza A H7** (HEX) titration series in duplicates. The test was performed on the Aria Mx instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Copy number	Influenza A H7 (HEX)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
Standard H7_EU	10^5	22.48	22.42	26.55	+
	10^5	22.35		26.76	+
	10^4	25.63	25.82	27.05	+
	10^4	26.00		27.17	+
	10^3	29.17	29.10	27.53	+
	10^3	29.03		27.05	+
	100	31.94	31.90	27.06	+
	100	31.85		27.11	+
	10	35.89	35.39	26.86	+
	10	34.89		27.06	+

IC = Internal Control

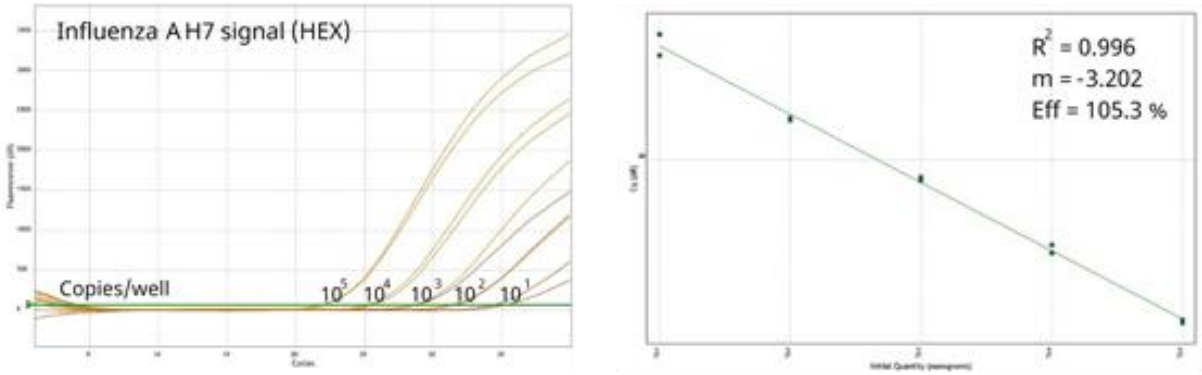


Figure 4. Titration of **Influenza A H7** (HEX) *in vitro* RNA tested with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on Aria Mx instrument using the Influenza A H5/H7/H9 real-time RT-PCR protocol. The graph shows the amplification plot (HEX) and standard curve of obtained C_T values for the HEX signal.

The analytical sensitivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit was furthermore determined by testing individual titration series of *in vitro* RNA of Influenza A subtype H9. Tests were performed in duplicates of ten-fold dilutions on different cyclers using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol (50°C 10 min, 95°C 2 min; 40 cycles 95°C 5 s, 60°C 30 s). Results are shown in Table 10 and Table 11 as well as Figure 5 and Figure 6.

Table 10. Analytical sensitivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137): Individual and mean C_T values of an **Influenza A H9** (Cy5) titration series in duplicates. The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Copy number	Influenza A H9 (Cy5)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
Standard H9_EU/NA	10^5	23.35	23.55	28.31	+
	10^5	23.75		28.37	+
	10^4	27.03	26.82	28.62	+
	10^4	26.60		28.40	+
	10^3	30.07	30.10	28.32	+
	10^3	30.13		28.65	+
	100	33.62	33.55	28.61	+
	100	33.47		28.38	+
	10	37.06	36.81	28.78	+
	10	36.55		28.50	+

IC = Internal Control

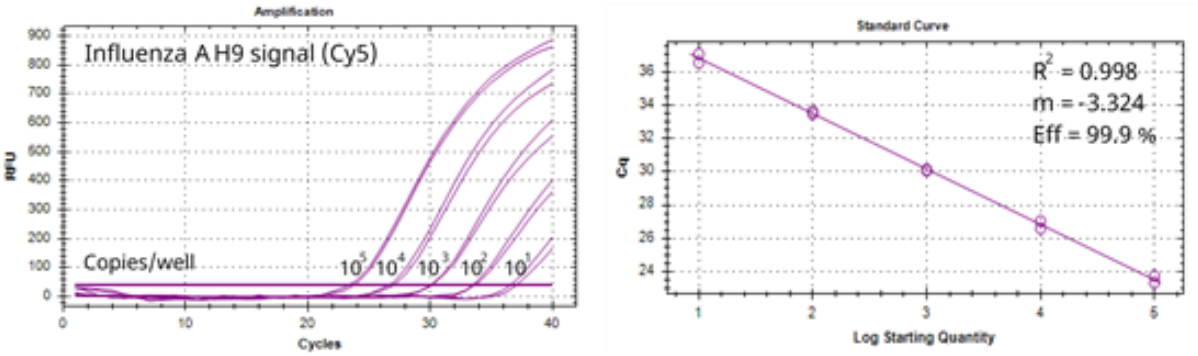


Figure 5. Titration of **Influenza A H9** (Cy5) *in vitro* RNA tested with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Bio-Rad CFX96 instrument using the Influenza A H5/H7/H9 real-time RT-PCR protocol. The graph shows the amplification plot (Cy5) and standard curve of obtained C_T values for the Cy5 signal.

Table 11. Analytical sensitivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). Individual and mean C_T values of an **Influenza A H9** (Cy5) titration series in duplicates. The test was performed on the Aria Mx instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Copy number	Influenza A H9 (Cy5)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
Standard H9_EU/NA	10^5	23.06	23.15	27.08	+
	10^5	23.24		27.19	+
	10^4	26.13	26.07	27.10	+
	10^4	26.01		26.96	+
	10^3	28.68	28.64	26.99	+
	10^3	28.59		26.99	+
	100	31.60	31.76	27.13	+
	100	31.92		27.31	+
	10	34.85	34.69	27.24	+
	10	34.53		27.15	+

IC = Internal Control

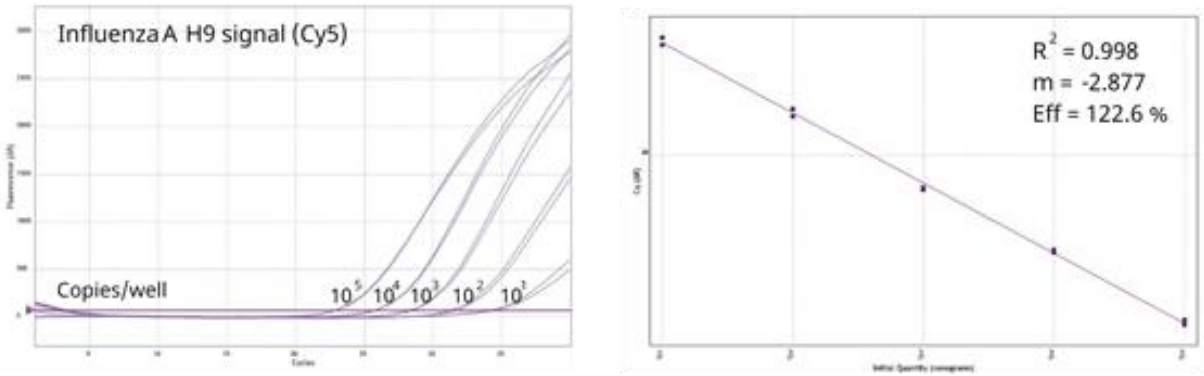


Figure 6. Titration of **Influenza A H9** (Cy5) *in vitro* RNA tested with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Aria Mx instrument using the Influenza A H5/H7/H9 real-time RT-PCR protocol. The graph shows the amplification plot (Cy5) and standard curve of obtained C_T values for the Cy5 signal.

Results/ Conclusion

Using virotype Influenza A H5/H7/H9 RT-PCR Kit, a high correlation between RNA copy number and the amount of amplified product was demonstrated for H5, H7 and H9 RNA. The virotype Influenza A H5/H7/H9 RT-PCR Kit is able to detect at least 10 RNA copies per sample for the subtypes H5 (correlation coefficient of 1.000 with an efficiency of 100.5 % when tested with the Bio-Rad CFX96), H7 (correlation coefficient of 0.982 with an efficiency of 100.2 % when tested with the Bio-Rad CFX96) and H9 (correlation coefficient of 0.998 with an efficiency of 99.9 % when tested with the Bio-Rad CFX96).

4.2 Titration series of Influenza A positive samples

Additionally, the sensitivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit was determined by titration of RNA of H5, H7, and H9 positive samples (kindly provided by Friedrich-Loeffler-Institut, Germany). Extraction of RNA from the samples was performed externally and real-time RT-PCR was run on Bio-Rad CFX96 instrument in both cases.

4.2.1 Titration of an Influenza A H5 positive sample

The sensitivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit (Lot F202000137) for the Influenza A subtype H5 was tested using the H5 strain A/mallard/Germany/R734/08. Results are presented in Table 12 and Figure 7.

Table 12. Results for an RNA titration series of an **Influenza A H5** positive sample (H5N3: A/mallard/Germany/R734/08) tested with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137) in duplicates. Individual and mean C_T values of Influenza H5 amplificates (FAM) and the Internal Control (Texas Red) are shown. The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Dilution	Influenza A H5 (FAM)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
H5N3: A/mallard/Germany/R734/08	$1:10^3$	26.48	26.44	28.29	+
		26.40		28.40	+
	$1:10^4$	29.48	29.33	28.38	+
		29.18		28.08	+
	$1:10^5$	32.61	32.48	27.75	+
		32.35		27.91	+
	$1:10^6$	35.79	35.99	28.08	+
		36.18		28.12	+
	$1:10^7$	-	-	28.45	-
		-		28.16	-

IC = Internal Control, - = no C_T

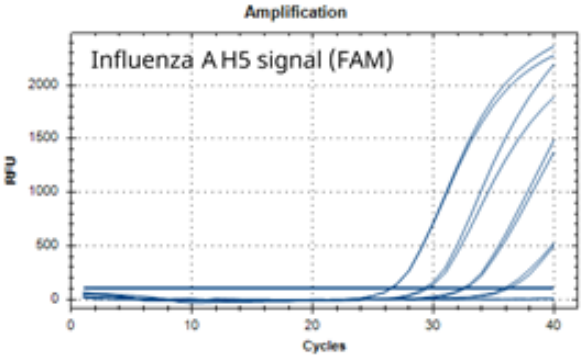


Figure 7. Amplification plot for a titration series of RNA of an **Influenza A H5** positive sample (H5N3: A/mallard/Germany/ R734/08) using the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

4.2.2 Titration of Influenza A H7 positive samples

Sensitivity of the new virotype Influenza A H5/H7/H9 RT-PCR Kit (Lot F202000137) for the Influenza A subtype H7 was furthermore tested using different H7 strains (Table 13). Results are shown in Table 14 - Table 18 and Figure 8 - Figure 12.

Table 13. Influenza A H7 strains tested in a titration series with the virotype Influenza A H5/H7/H9 RT-PCR Kit.

H7 strains	**Subtype**
A/turkey/Germany/R11/01	H7N7
A/chicken/Germany/AR915/2015	H7N7
A/mute swan/Germany/R901/2006	H7N1
A/domestic duck/R1771/2011	H7N7
A/sentinel duck/Germany/SK207R/2007	H7N3

Table 14. Analysis of an RNA titration series of an **Influenza A H7** positive sample (strain H7N7: A/turkey/Germany/R11/01) tested in duplicates with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). Individual and mean C_T values of Influenza H7 amplificates (HEX) and the Internal Control (Texas Red) are shown. The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Dilution	Influenza A H7 (HEX)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
H7N7: A/turkey/Germany/R11/01	$1:10^3$	26.39	26.34	27.96	+
		26.28		28.15	+
	$1:10^4$	29.03	29.02	28.18	+
		29.01		28.09	+
	$1:10^5$	31.40	31.72	28.09	+
		32.04		28.29	+
	$1:10^6$	34.51	34.02	27.85	+
		33.52		28.09	+
	$1:10^7$	-	-	28.03	-
		-		27.88	-

IC = Internal Control, - = no C_T

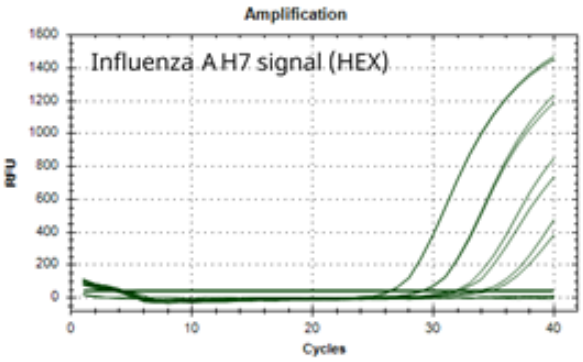


Figure 8. Amplification plot for a titration series of RNA of an **Influenza A H7** positive sample (strain H7N7: A/turkey/Germany/R11/01) tested in duplicates with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Table 15. Analysis of an RNA titration series of an **Influenza A H7** positive sample (strain H7N7: A/chicken/Germany/AR915/2015) tested in duplicates with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). Individual and mean C_T values of Influenza H7 amplificates (HEX) and the Internal Control (Texas Red) are presented. The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Dilution	Influenza A H7 (HEX)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
H7N7: A/chicken/Germany/AR915/2015	$1:10^3$	26.20	26.19	28.28	+
		26.17		28.33	+
	$1:10^4$	29.02	29.04	28.45	+
		29.06		28.22	+
	$1:10^5$	31.34	31.47	28.07	+
		31.60		28.27	+
	$1:10^6$	34.14	34.32	28.03	+
		34.50		28.14	+
	$1:10^7$	-	-	28.25	-
		-		28.47	-

IC = Internal Control, - = no C_T

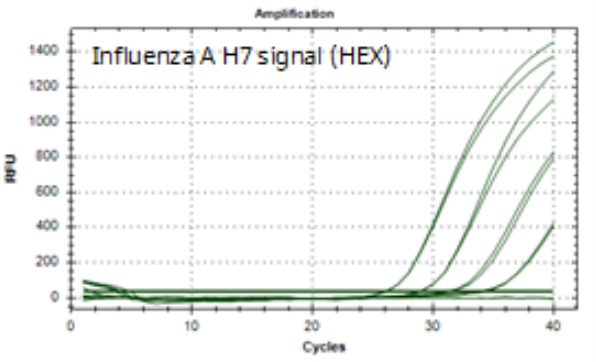


Figure 9. Amplification plot for a titration series of RNA of an **Influenza A H7** positive sample (H7N7: A/chicken/Germany/AR915/2015) using the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Table 16. Analysis of an RNA titration series of an **Influenza A H7** positive sample (strain H7N1: A/mute swan/Germany/R901/2006) tested in duplicates with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). Individual and mean C_T values of Influenza H7 amplificates (HEX) and the Internal Control (Texas Red) are presented. The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Dilution	Influenza A H7 (HEX)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
H7N1: A/mute swan/Germany/R901/2006	$1:10^3$	25.47	25.47	28.20	+
		25.47		27.92	+
	$1:10^4$	28.49	28.43	28.25	+
		28.37		28.02	+
	$1:10^5$	31.48	31.64	27.89	+
		31.79		27.96	+
	$1:10^6$	35.63	35.12	28.33	+
		34.60		28.12	+
	$1:10^7$	37.05	38.25	28.21	+
		39.45		28.41	+

IC = Internal Control

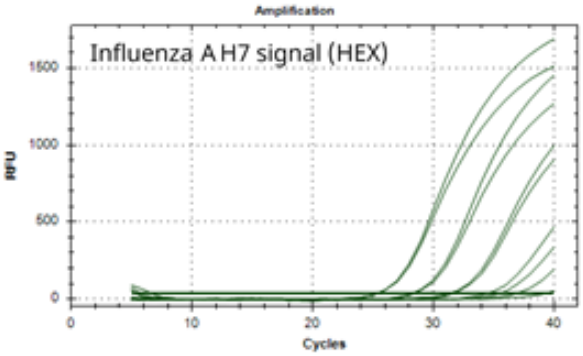


Figure 10. Amplification plot for a titration series of RNA of an **Influenza A H7** positive sample (H7N1: A/mute swan/Germany/R901/2006) using the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Table 17. Analysis of an RNA titration series of an **Influenza A H7** positive sample (strain H7N7: A/domestic duck/R1771/2011) tested in duplicates with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). Individual and mean C_T values of Influenza H7 amplificates (HEX) and the Internal Control (Texas Red) are shown. The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Dilution	Influenza A H7 (HEX)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
H7N7: A/domestic duck/R1771/2011	$1:10^3$	24.15	24.20	28.05	+
		24.24		27.98	+
	$1:10^4$	27.31	27.21	28.27	+
		27.11		28.08	+
	$1:10^5$	30.03	30.01	28.02	+
		29.98		28.31	+
	$1:10^6$	33.41	33.78	28.12	+
		34.15		28.12	+
	$1:10^7$	36.81	37.50	28.31	+
		38.19		28.35	+
	$1:10^8$	37.58	37.58	28.43	+
		-		28.50	-

IC = Internal Control, - = no C_T

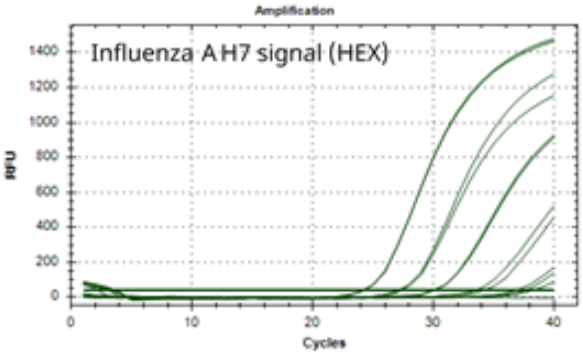


Figure 11. Amplification plot for a titration series of RNA of an **Influenza A H7** positive sample (H7N7: A/domestic duck/R1771/2011) using the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Table 18. Analysis of an RNA titration series of an **Influenza A H7** positive sample (strain H7N3: A/sentinel duck/Germany/SK207R/2007) tested in duplicates with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). Individual and mean C_T values of Influenza H7 amplificates (HEX) and the Internal Control (Texas Red) are presented. The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Dilution	Influenza A H7 (HEX)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
H7N3: A/sentinel duck/Germany/SK207R/2007	$1:10^3$	24.41	24.36	28.01	+
		24.31		28.24	+
	$1:10^4$	27.29	27.24	27.98	+
		27.19		28.43	+
	$1:10^5$	30.34	30.37	28.08	+
		30.39		29.57	+
	$1:10^6$	33.91	33.78	28.93	+
		33.65		32.28	+
	$1:10^7$	36.21	36.21	28.20	+
		-		28.23	-
	$1:10^8$	36.66	36.66	28.01	+
		-		28.32	-

IC = Internal Control, - = no C_T

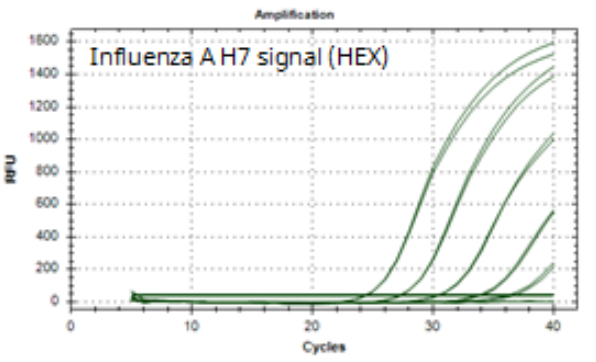


Figure 12. Amplification plot for a titration series of RNA of an **Influenza A H7** positive sample (H7N3: A/sentinel duck/Germany/SK207R/2007) using the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

4.2.3 Titration of an Influenza A H9 positive sample

Sensitivity of the new virotype Influenza A H5/H7/H9 RT-PCR Kit (Lot F202000137) for the Influenza A subtype H9 was furthermore tested using the H9 strain A/Turkey/Germany/R869/12. Results are presented in Table 19 and Figure 13.

Table 19. Results for an RNA titration series of an **Influenza A H9** positive sample (H9N2: A/turkey/Germany/R869/12) tested in duplicates with the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). Individual and mean C_T values of Influenza H9 amplificates (Cy5) and the Internal Control (Texas Red) are presented. The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Type	Dilution	Influenza A H9 (Cy5)		IC (Texas Red)	Result
		C_T	C_T mean	C_T	
H9N2: A/turkey/Germany/R869/12	$1:10^2$	26.12	26.17	27.67	+
		26.21		28.05	+
	$1:10^3$	29.55	29.48	27.96	+
		29.40		28.08	+
	$1:10^4$	33.15	33.03	28.16	+
		32.91		27.89	+
	$1:10^5$	36.40	36.33	28.13	+
		36.26		28.17	+
	$1:10^6$	39.39	39.29	28.23	+
		39.18		28.23	+
	$1:10^7$	-	-	28.02	-
		-		28.00	-

IC = Internal Control, - = no C_T

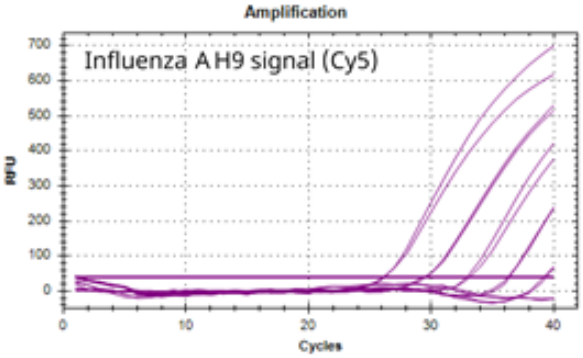


Figure 13. Amplification plot for a titration series of RNA of an **Influenza A H9** positive sample (H9N8: A/turkey/Germany/R869/12) using the virotype Influenza A H5/H7/H9 RT-PCR Kit (batch F202000137). The test was performed on the Bio-Rad CFX96 instrument using the virotype Influenza A H5/H7/H9 real-time RT-PCR protocol.

Conclusion

A titration series of RNA of Influenza A H5, H7 and H9 positive samples revealed a high sensitivity for all three subtypes (Table 12 - Table 19 and Figure 7 - Figure 13).

4.3 Analytical specificity

4.3.1 Cross-reactivity to other Influenza A subtypes

Cross-reactivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit was tested with samples positive for the Influenza A subtypes H1 – H16 (except H5, H7 and H9). Test results with the virotype Influenza H5/H7/H9 RT-PCR Kit as well as those obtained with the M gene-specific virotype Influenza A RT-PCR Kit are shown (Table 20). The samples were kindly provided by the Friedrich-Loeffler-Institut (FLI), by Veterinary State Diagnostic Laboratories in Germany or were obtained as part of ring trials.

Results / Conclusion

No cross-reactivity for other Influenza serotypes was detected.

Table 20. Test for cross-reactivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit with different Influenza A subtypes. C_T values of the **Influenza A H5** (FAM), **H7** (HEX), **H9** (Cy5) and **Internal Control** (Texas Red) signals obtained with the virotype Influenza A H5/H7/H9 RT-PCR Kit as well as C_T values of Influenza A (FAM) signals using the virotype Influenza A RT-PCR Kit are shown. The test was performed on the Bio-Rad CFX96 instrument using according real-time RT-PCR protocols.

Sample	**Species/Strain**	**virotype Influenza A H5/H7/H9 RT-PCR Kit**				**virotype Infl. A RT-PCR Kit**
		C_T H5	**C_T H7**	**C_T H9**	**C_T IC**	**C_T Infl. A**
H1N1 1:100	A/wild duck/Germany/R30/06	-	-	-	27.42	n.d.
H1N1 2009 1:10	Unknown	-	-	-	28.27	31.16
H1N1 SWL 1:10	Unknown	-	-	-	28.76	29.43
H1	Unknown	-	-	-	28.41	13.35
H2N9 1:100	A/wild duck/Germany/R311/07	-	-	-	27.61	n.d.
H2N2 1:100	A/duck/Potsdam/177/83	-	-	-	28.73	19.70
H3N8	A/mallard/Germany/R2619/07	-	-	-	27.81	n.d.

1:100						
H3N8 1:100	A/duck/Ukraine/1/63	-	-	-	28.73	18.72
H3N2 1:100	Unknown	-	-	-	26.60	23.26
RV 2020 ITA #13 (H3N1)	A/chicken/Belgium/3497_0001/2019	-	-	-	26.81	25.71
H4N6 1:100	A/mallard/Germany/R1740/07	-	-	-	27.35	n.d.
H4N6 1:100	A/mallard/Germany/1507/07	-	-	-	28.80	21.50
H6N1 1:100	A/turkey/Germany/R30/99	-	-	-	27.32	n.d.
H6N5 1:100	A/turkey/Grub/R41/98	-	-	-	28.74	19.91
H6N8	Unknown	-	-	-	28.47	13.32
RV 2018 NL #4 (H6N2)	A/turkey/Massachussetts/65	-	-	-	27.44	34.32
H8N4 1:100	A/turkey/Ontario/6118/68	-	-	-	28.52	n.d.
H8N4 1:100	A/turkey/Ontario/6118/68	-	-	-	28.46	21.09
H10N7 1:100	A/mallard/Germany/2075/07	-	-	-	27.35	n.d.
H10N8 1:100	A/guinea fowl/Hungary/1/69	-	-	-	28.41	21.73
RV 2011 FLI #5/11 H10) 1:100	Unknown	-	-	-	26.48	22.21
H11N6 1:100	A/duck/England/56	-	-	-	27.29	n.d.
H11N9 1:100	A/mallard/Germany/R2994/07	-	-	-	28.38	16.59
H12N5	A/duck/Alberta/60/76	-	-	-	27.26	n.d.

1:100						
H12N5 1:100	A/duck/Alberta/60/76	-	-	-	28.36	20.99
H13N2 1:100	A/black headed gull/Germany/ R2622/06	-	-	-	27.36	n.d.
H13N6 1:100	A/gull/Stralsund/Wv1136-40/03	-	-	-	28.46	24.36
H14N5 1:100	A/mallard/Gurjev/263/82	-	-	-	27.37	23.70
H15N9 1:100	A/shearwater/West Australia/2576/79	-	-	-	27.44	23.39
H16N3 1:100	A/herring gull/Germany/ R3309/07	-	-	-	27.49	22.35
H16N2 1:100	A/herring gull/Germany/ R2792/06	-	-	-	28.56	25.39
37/15075 F7	Swine	-	-	-	26.41	26.48
37/15075 H7	Swine	-	-	-	26.54	26.52
37/15075 E5	Swine	-	-	-	26.26	27.16

n.d. = not determined, IC = Internal Control, - = no C_T

4.3.2 Cross-reactivity to other pathogens from birds

Avian Influenza A virus and *Newcastle Disease Virus* (NDV) are two main avian pathogens causing economic problems. Both RNA viruses as well as several other pathogens cause similar symptoms that need to be differentiated from an Influenza A infection.

In order to examine specificity of the virotype Influenza A H5/H7/H9 RT-PCR Kit several relevant pathogens of birds were tested including both, viral and bacterial pathogens: *Newcastle Disease Virus* (NDV; ring trial samples FLI and EURL IZSVe), *Avian Paramyxovirus-8* (APMV-8; ring trial sample FLI), *Mycoplasma gallisepticum* (from a turkey farm; ring trial samples), and *Mycoplasma synoviae* (from ring trial GD Animal Health; University of Vienna; ring trial samples).

Results

The virotype Influenza A H5/H7/H9 RT-PCR Kit showed no cross-reactivity to other avian viruses (NDV, APMV-8) or bacteria (*Mycoplasma gallisepticum* and *Mycoplasma synoviae*) (Table 21).

Table 21. Test for cross-reactivity of the virotype Influenza A H5/H7/H9 RT-PCR Kit with NDV and APMV-8 RNA as well as *Mycoplasma gallisepticum* (Mg) and *Mycoplasma synoviae* (Ms) DNA. C_T values of the Influenza A **H5** (FAM), **H7** (HEX), **H9** (Cy5) and **Internal Control** (Texas Red) signals are shown. The tests were performed on the Bio-Rad CFX96 instrument.

Sample & sample status (sample positive for)		**Species/Strain**	**virotype Influenza A H5/H7/H9 RT-PCR Kit**			
			C_T H5	**C_T H7**	**C_T H9**	**C_T IC**
NDV	NDV #1		-	-	-	26.70
	RV 2011 FLI #10/11 1:100	APMV-1	-	-	-	27.84
	RV 2017 FLI #C	NDV	-	-	-	27.77
	RV 2019 ITA #2	APMV-1/bassette chicken/ Belgium/4096/2018	-	-	-	26.60
	RV 2019 ITA #3	APMV-1/pigeon/Italy/ 96/2019 (PPMV-1)	-	-	-	26.31
	RV 2019 ITA #12 1:5	APMV-1 (V4 like)	-	-	-	27.51
	RV 2020 ITA #3	APMV-1/chicken/ California/18-016505-1/2018	-	-	-	27.87
	RV 2020 ITA #8	APMV-1/pigeon/Italy/ 19Vir8321/2019 (PPMV-1)	-	-	-	29.72
	RV 2020 ITA #9	APMV-1/chicken/ California/18-016505-1/2018	-	-	-	28.10

	RV 2020 ITA #10	NDV La Sota	-	-	-	28.01
APMV-8	RV 2017 FLI # D		-	-	-	28.91
Ms	Ms #1	Chicken	-	-	-	27.71
	Ms #2	Chicken	-	-	-	28.01
	Ms #3 1:50	Chicken	-	-	-	28.03
	Ms #4 1:50	Chicken	-	-	-	27.84
	Ms #5 1:50	Chicken	-	-	-	27.81
Mg	Mg #1	Turkey	-	-	-	28.37
	Mg #2	Turkey	-	-	-	30.47
	Mg #3	Turkey	-	-	-	29.47
	Mg #4	Turkey	-	-	-	30.37
	Mg #5	Turkey	-	-	-	27.58
Mg/ Ms	RV 2016 #4		-	-	-	26.48
	RV 2016 #8		-	-	-	26.49

NDV = *Newcastle Disease Virus*; APMV = *Avian Paramyxovirus*, Ms = *Mycoplasma synoviae*, Mg = *Mycoplasma gallisepticum,* RV = Ringversuch (ring trial), IC = Internal Control, - = no C_T

Conclusion

The virotype Influenza A H5/H7/H9 RT-PCR Kit specifically identifies Influenza A H5, H7 and H9 RNA.

4.4 Diagnostic sensitivity, specificity and efficiency

4.4.1 Definitions

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

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

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

4.4.2 Validation of the virotype Influenza H5/H7/H9 RT-PCR Kit

For validation of the virotype Influenza A H5/H7/H9 RT-PCR Kit a total of *n* = 245 samples with known Influenza A status were tested. Altogether, *n* = 64 out of *n* = 245 samples were Influenza A H5 positive, *n* = 20 samples were Influenza A H7 positive, *n* = 20 samples were Influenza A H9 positive, *n* = 13 samples were double or triple positive for H5 and H9 and/or H7 and *n* = 128 samples were Influenza A H5 and H7 and H9 negative. The samples originated from different outbreaks. RNA samples were kindly provided by FLI (Germany), by veterinary diagnostic labs in Germany, by the Thessaloniki Veterinary Centre (Greece) or were part of ring trials. The results are presented in Table 22.

Table 22. Sensitivity and specificity of the virotype Influenza A H5/H7/H9 RT-PCR Kit.

virotype Influenza A H5/H7/H9 RT-PCR Kit		**Reference status**			
Total	**245**	**Reference positive**	**117**	**Reference negative**	**128**
H5 positive	64	true H5 positive	64	false H5 positive	0
H7 positive	20	true H7 positive	20	false H7 positive	0
H9 positive	20	true H9 positive	20	false H9 positive	0
H5/H7/H9 positive	13	True double/triple positive	13	false double/triple positive	0
H5, H7 and H9 negative	128	false negative	0	true negative	128

Diagnostic sensitivity:	100 %
Diagnostic specificity:	100 %
Diagnostic efficiency:	100 %

Figure 14 shows the origin of the Influenza A H5, H7 and H9 positive samples and Figure 15 the collection date of positive samples (both if known) tested in the internal validation.

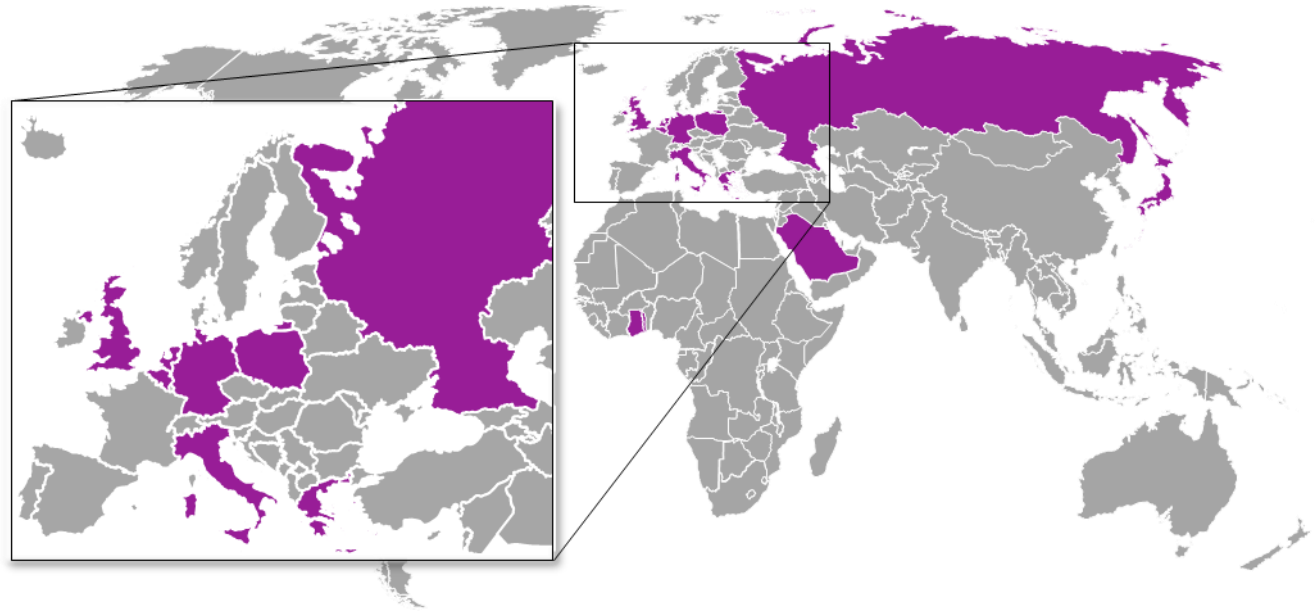


Figure 14. Origin of tested H5, H7 and H9 positive samples (if known) using the virotype Influenza A H5/H7/H9 RT-PCR Kit.

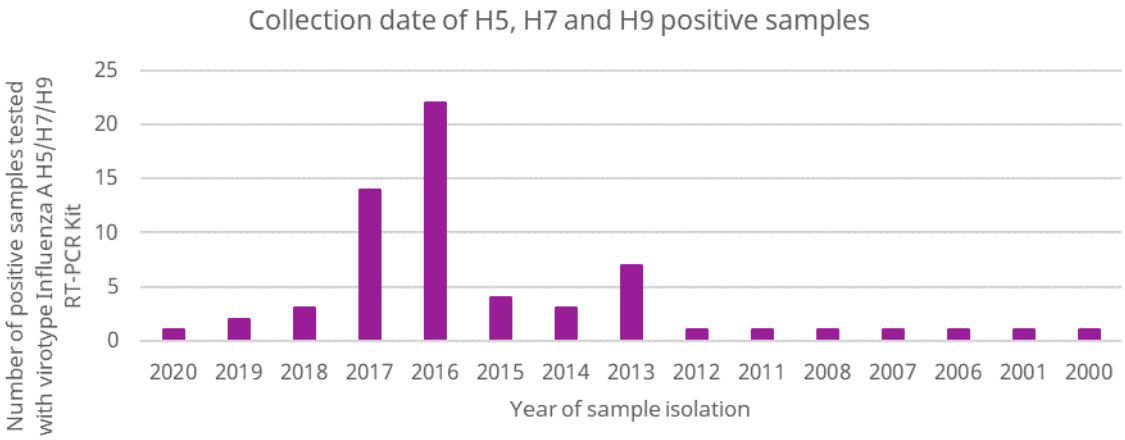


Figure 15. Collection date of tested Influenza A H5, H7 and H9 positive samples (if known) using the virotype Influenza A H5/H7/H9 RT-PCR Kit.

Conclusion

Testing this sample panel resulted in a sensitivity of 100 % and a specificity of 100 % for the virotype Influenza A H5/H7/H9 RT-PCR Kit. In accordance with the results of the testing of synthetically derived RNA (Table 24, chapter 4.4.4), the data show that the virotype Influenza A H5/H7/H9 RT-PCR Kit detects currently circulating strains of the Influenza A subtypes H5, H7 and H9 (Figure 15) as well as Influenza A viruses of the subtypes H5, H7 and H9 originating from different geographical regions (Figure 14).

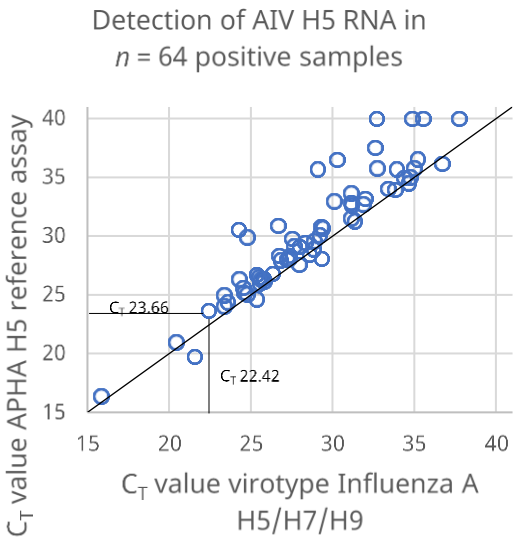
4.4.3 Testing of Influenza A H5, H7 and H9 positive samples in comparison to various reference methods

Several protocols for detection of the Influenza A subtypes H5, H7 and H9 have been described. The virotype Influenza A H5/H7/H9 RT-PCR Kit was tested in comparison to the protocols used by the German National Reference Laboratory for Avian Influenza of the Friedrich Loeffler Institute (FLI) and the Avian Influenza EURL IZSVe (called H5 or H7 APHA assay) as well as the H9 detection assay developed by Monne *et al*., 2008.

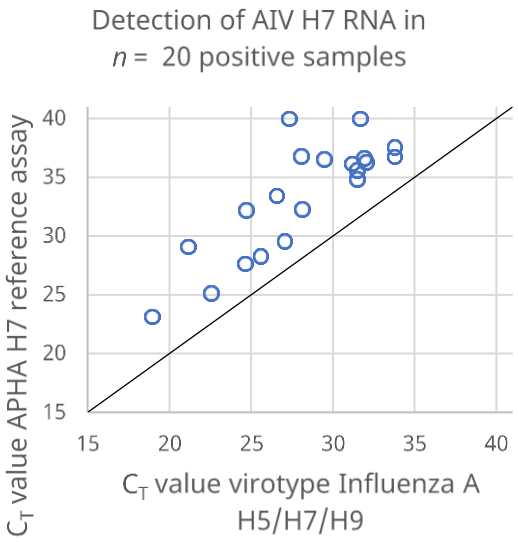
4.4.3.1 Testing of Influenza A H5, H7 and H9 positive samples in comparison to H5 or H7 APHA assays as well as H9 assay by Monne *et al*., 2008

RNA of Influenza A H5, H7 or H9 positive field samples was tested using the virotype Influenza A H5/H7/H9 RT-PCR Kit. The obtained results for H5 and H7 were compared to the results obtained with the protocols used by the Avian Influenza EURL IZSVe, also known as H5 or H7 APHA assays. Results of H9 were compared to the results obtained with the protocol by Monne *et al.*, 2008. The APHA assays detect H5 or H7 RNA in a monoplex RT-qPCR, respectively and the H9 protocol of Monne *et al.,* 2008 was also performed in monoplex – each assay without Internal Control. The results are presented in Figure 16.

A



B



C

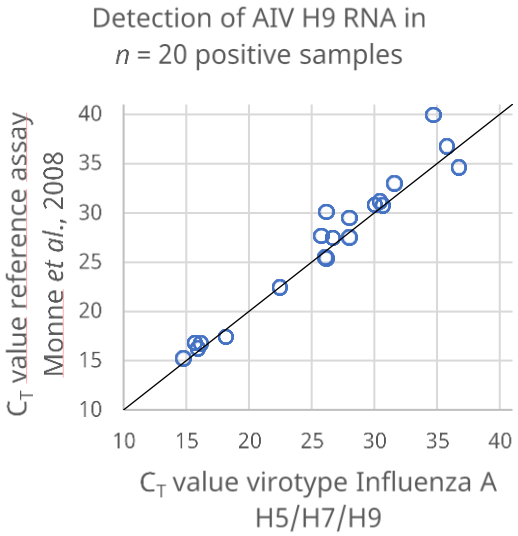


Figure 16. Analysis of RNA of **Influenza A H5**, **H7** and **H9** positive samples using the virotype Influenza A H5/H7/H9 RT-PCR Kit compared to the reference methods of APHA for H5 (**A**) and H7 (**B**) or Monne *et al.*, 2008 for H9 (**C**). The tests were performed on the Agilent Mx3005P or Bio-Rad CFX96 instruments. Black lines in Figure A exemplary depict C_T values for one sample obtained with the virotype Influenza A H5/H7/H9 RT-PCR Kit (C_T 22.42) as well as with the APHA H5 reference assay (C_T 23.66).

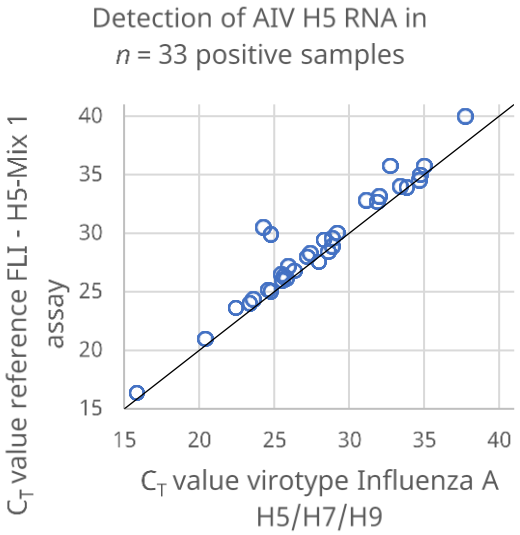
Conclusion

The identification of Influenza A subtypes H5, H7 and H9 using the virotype Influenza A H5/H7/H9 RT-PCR Kit shows comparable or more sensitive results to the reference methods APHA H5, APHA H7 or to the assay of Monne *et al.*, 2008.

4.4.3.2 Testing of Influenza A H5 positive samples compared to the protocol by Hoffmann *et al.*, 2016

RNA of a panel of Influenza A H5 positive field samples was tested using the virotype Influenza A H5/H7/H9 RT-PCR Kit compared to the protocols described by Hoffmann *et al.*, 2016 using the IAV-H5-Mix 1-FAM (FLI – H5-Mix 1 assay) or the IAV-H5a-Mix 1-FAM (FLI – H5a-Mix 1 assay). The FLI assay was performed as indicated, including an Internal Control system (duplex RT-qPCR). The results are depicted in Figure 17.

A



B

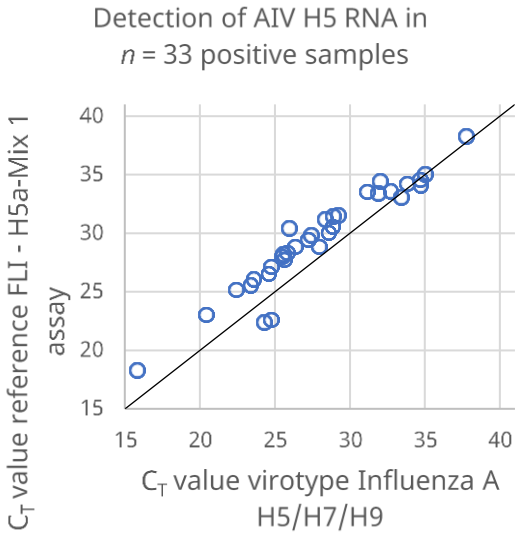


Figure 17. Analysis of RNA of **Influenza A H5** positive samples using the virotype Influenza A H5/H7/H9 RT-PCR Kit compared to the reference protocols described by Hoffmann *et al.*, 2016 using the the IAV-H5-Mix 1-FAM (**A**) and the IAV-H5a-Mix 1-FAM (**B**). The tests were performed on the Agilent Mx3005P instrument.

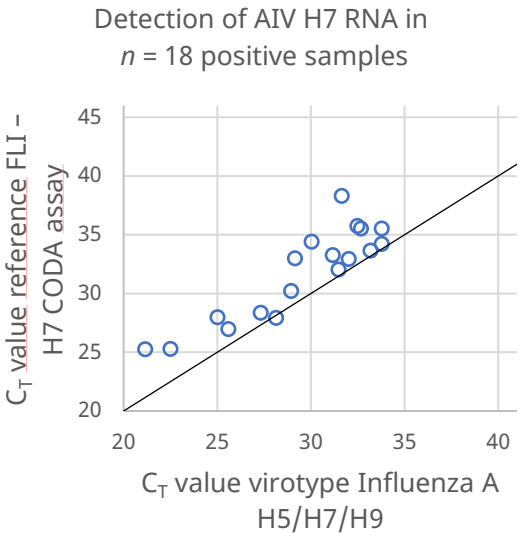
Conclusion

Detection of Influenza A subtype H5 using virotype Influenza A H5/H7/H9 RT-PCR Kit shows comparable or more sensitive results to the Influenza A H5-specific protocols by Hoffmann *et al.*, 2016.

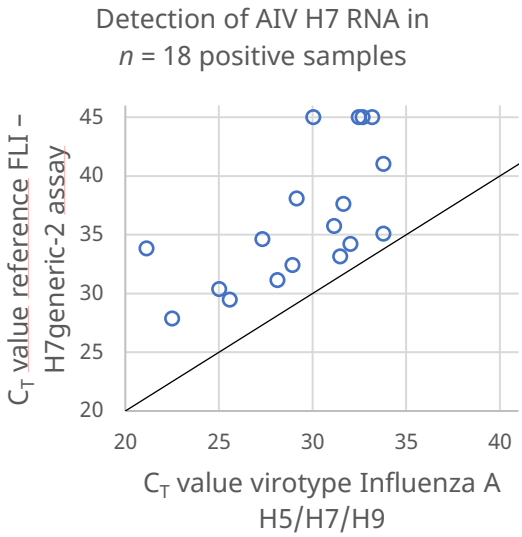
4.4.3.3 Testing of Influenza A H7 positive samples compared to the protocols by Hoffmann *et al.*, 2016

RNA of Influenza A H7 positive field samples was tested using the virotype Influenza A H5/H7/H9 RT-PCR Kit compared to the protocols described by Hoffmann *et al.*, 2016 using the FLI-H7-CODA-Mix, the FLI-H7generic-2-Mix and the IAV-H7-2.2-Mix-FAM. The FLI assays were performed as indicated (except for fluorescence: Cy5 was used instead of FAM), including an Internal Control system (duplex RT-qPCR each). The results are shown in Figure 18.

A



B



C

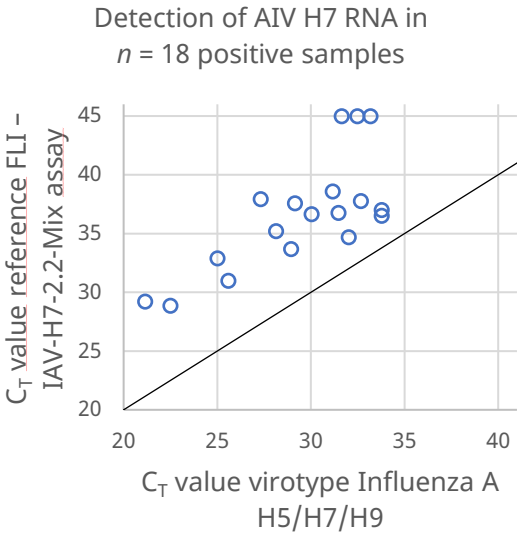


Figure 18. Analysis of RNA of **Influenza A H7** positive samples using the virotype Influenza A H5/H7/H9 RT-PCR Kit compared to the reference protocols described by Hoffmann *et al.*, 2016 using the FLI-H7-CODA-Mix (**A**), FLI-H7 generic-2-Mix **(B**) and the IAV-H7-2.2-Mix (**C**). The tests were performed on the Agilent Mx3005P or Bio-Rad CFX96 instruments.

Conclusion

Detection of Influenza A subtype H7 using the virotype Influenza A H5/H7/H9 RT-PCR Kit shows comparable or more sensitive results compared to the three FLI protocols (using FLI-H7-CODA-Mix, FLI-H7generic-2-Mix or IAV-H7-2.2-Mix) by Hoffmann *et al.*, 2016.

4.4.3.4 Testing of Influenza A H9 positive samples compared to the protocol by Hoffmann *et al.*, 2016

RNA of Influenza A H9 positive field samples was tested using the virotype Influenza A H5/H7/H9 RT-PCR Kit compared to the protocol described by Hoffmann *et al*., 2016 using the IAV-H9-Mix 2-FAM. The FLI assay was performed as indicated, including an Internal Control system (duplex RT-qPCR). Due to the limited number of samples and sample volume, only a small panel of H9 positive samples was tested with both methods.

Run time of the FLI assay is 1 hour and 49 minutes compared to that of the virotype Influenza A H5/H7/H9 RT-PCR Kit of 1 hour and 7 minutes (using the Agilent Mx3005P instrument). The results are shown in Figure 19.

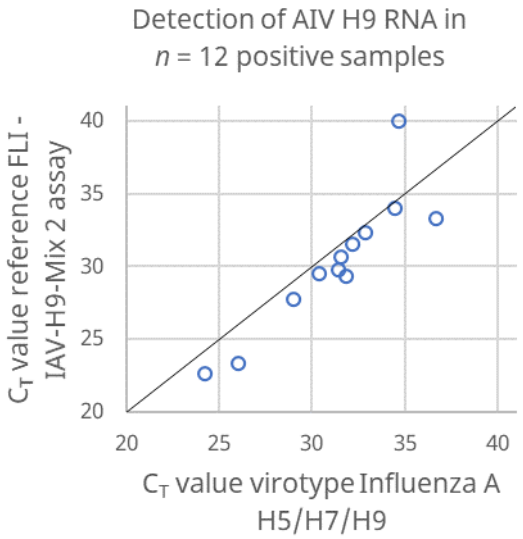


Figure 19. Analysis of RNA of **Influenza A H9** positive samples using the virotype Influenza A H5/H7/H9 RT-PCR Kit compared to the protocol described by Hoffmann *et al.*, 2016 using the IAV-H9-Mix 2-FAM. The tests were performed on the Agilent Mx3005P instrument.

Conclusion

Detection of Influenza A subtype H9 using the virotype Influenza A H5/H7/H9 RT-PCR Kit shows comparable or slightly less sensitive results to the FLI-H9 protocol by Hoffmann *et al*., 2016.

4.4.3.5 Testing of Influenza A H5, H9 and/or H7 double or triple positive samples compared to the FLI protocols

RNA of Influenza A H5, H9 and/or H7 double or triple positive samples was tested using the virotype Influenza A H5/H7/H9 RT-PCR Kit. The results of analysis shown in Table 23 were compared to the methods of FLI. Protocols described by Hoffmann *et al*., 2016 are duplex RT-qPCRs for detection of one Influenza subtype and an Internal control simultaneously.

Table 23. Analysis of RNA of **Influenza A H5, H9 and/or H7** double or triple positive samples using the virotype Influenza A H5/H7/H9 RT-PCR Kit. C_T values of the **H5** (FAM), **H7** (HEX), **H9** (Cy5) and **Internal Control** (Texas Red) signals compared to the protocols performed by FLI are shown. Note that samples were tested undiluted with the FLI reference assays, while samples were diluted as stated in the table for tests with the virotype Influenza A H5/H7/H9 RT-PCR Kit. The tests were performed on the Agilent Mx3005P instruments.

Sample	**Dilution**	**virotype Influenza A H5/H7/H9**				**Reference assay FLI (undiluted samples)**		
		C_T H5	**C_T H7**	**C_T H9**	**C_T IC**	**C_T H5**	**C_T H7**	**C_T H9**
AR 127/18	1:20	22.10	27.33	16.02	26.92	19.40	27.10	14.24
AR 128/18	1:10	18.37	29.37	19.68	26.82	17.45	29.82	19.03
AR 130/18	1:10	32.02	-	31.98	26.98	31.86	-	34.54
AR 131/18	1:200	23.25	21.52	25.24	26.65	17.69	18.91	19.86
AR 520/18	undil.	25.76	-	27.09	27.11	18.90	-	30
AR 528/18	undil.	23.41	-	28.86	27.12	25	-	30
AR 537/18	undil.	37.98	-	16.79	27.39	30	-	14
AR 541/18	undil.	26.57	-	15.07	26.11	29	-	14
AR 544/18	undil.	22.80	-	19.68	26.03	21	-	26
AR 561/18	1:5	38.66	-	35.97	26.51	28	-	27
AR 562/18	undil.	24.45	-	26.95	26.35	25	-	26
AR 593/18	undil.	30.59	-	14.51	26.11	30	-	16
AR 589/18	undil.	27.86	-	16.35	25.94	32	-	17

undil. = undiluted, IC = Internal Control, - = no C_T

Conclusion

Detection of Influenza A subtypes H5, H7 and H9 using the virotype Influenza A H5/H7/H9 RT-PCR Kit is comparable to the FLI methods.

4.4.4 Testing of synthetically derived RNA of different strains or clades of Influenza A viruses

Due to the limited number of Influenza A positive samples, *in vitro* RNA molecules were designed to test the performance of the virotype Influenza A H5/H7/H9 RT-PCR Kit regarding detection of strains from different geographical regions or diverse H5 clades.

Results/ Conclusion

The results of analysis are shown in Table 24. Using the virotype Influenza A H5/H7/H9 RT-PCR Kit, Influenza A positive samples of the subtypes H5, H7 and H9 and negative samples can be identified with high sensitivity and specificity. The virotype Influenza A H5/H7/H9 RT-PCR Kit allows the fast and specific detection of European, North-American and Asian strains of H5, H7 and H9 subtypes as well as the currently circulating H5 clades 2.3.4.4, 2.3.2.1a and 2.3.2.1c.

Table 24. Analysis of *in vitro* RNA molecules of diverse strains or clades of Influenza A by the virotype Influenza A H5/H7/H9 RT-PCR Kit. C_T values of the **H5** (FAM), **H7** (HEX) and **H9** (Cy5) signals obtained with the virotype Influenza A H5/H7/H9 RT-PCR Kit as well as limit of detection (LOD) are shown. Tests were performed on the Agilent Mx3005P instrument.

Subtype	*in-vitro* RNA	Strains/clade	Accession number	GISAID EPI/WSS-number	C_T (10^6 copies/ well)	LOD
H5	H5_EU	EU	EF597267	EPI117978	17.45	1
	H5_NA	NA	MF359882	EPI1061344	17.96	1
	H5_NA2	clade 2.3.4.4 - NA + EU	KP795737	EPI576477	18.72	1
	H5_A1	Asia	JX420142	EPI406344	18.46	1
	H5_EU2	clade 2.3.4.4 - EU	KY621534	EPI964917	17.67	1 - 10
	H5_A2	clade 2.3.4.4 - Asia	KP286563	EPI602028	18.19	1
	H5_A3	clade 2.3.4.4 - Asia	LC316683	EPI1184338	17.67	1 - 10
	H5_A4	clade 2.3.2.1a - Asia	MH135475	EPI1309552	22.75	1 - 10
	H5_A5	clade 2.3.2.1c - Asia	LC364004	EPI1161381	18.10	1
H7	H7_EU	EU	AY999979	EPI26857	18.76	1
	H7_NA	NA	CY099313	EPI336958	19.27	1 - 10
	H7_A1	Asia	MF630173	EPI1090429	19.20	1 - 10
	H7_A2	Asia	n.s.	EPI1319697	16.47	1
	H7_EU2	EU	n.s.	n.s.	16.78	1
H9	H9_EU/NA	EU + NA	CY144763	EPI455027	20.37	1 - 10
	H9_A	Asia	KT157799	EPI623595	19.34	1 - 10

n.s. = not specified, LOD = limit of detection, EU = Europe, NA = North America

4.4.5 Testing of Influenza A H5, H7 and H9 negative samples

RNA of *n* = 128 Influenza A H5, H7 and H9 negative field samples of different origins (tissue, swabs, feces, FTA cards) was tested using the virotype Influenza A H5/H7/H9 RT-PCR Kit. Protocols used by the Avian Influenza EURL IZSVe (called H5 or H7 APHA assay) as well as the H9 detection assay described by Monne *et al*., 2008 served as reference assays.

Results/ Conclusion

All *n* = 128 RNA samples correctly scored negative for Influenza A H5, H7 and H9 when tested with the virotype Influenza A H5/H7/H9 RT-PCR Kit. Therefore, diagnostic specificity was 100 % for the tested field sample panel.

4.5 Reproducibility

4.5.1 Intra-assay variance

Influenza A H5 positive RNA samples (1, 2), H7 positive RNA samples (3, 4), H9 positive RNA samples (5, 6), one negative RNA sample (7), and the Positive Control (PC) as well as the Negative Control (NC) of the kit were tested five times each in the same RT-PCR run with the virotype Influenza A H5/H7/H9 RT-PCR Kit on the Bio-Rad CFX96 instrument.

Results

Test results including calculated mean values, standard deviations (SD), and the coefficients of variation (CV) are given in Table 25 – Table 28. Figure 20 depicts the results for the according positively tested samples per channel.

The intra-assay variance is on average 0.46 % for **Influenza A H5** (FAM), 0.72 % for **H7** (HEX), 1.42 % for **H9** (Cy5), and 0.42 % for the **Internal Control** (Texas Red).

Table 25. Intra-assay variance of C_T values for **Influenza A subtype H5** (FAM). Tests were performed on the Bio-Rad CFX96 instrument.

Samples		**Intra-assay variance for H5 (FAM) Reactions (C_T values)**					**C_T mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**			
1	H5 positive sample	33.72	33.56	33.64	33.46	33.43	33.56	0.12	0.36
2	H5 positive sample	33.12	33.05	33.28	33.18	33.05	33.14	0.10	0.29
3	H7 positive sample	-	-	-	-	-	-	-	-
4	H7 positive sample	-	-	-	-	-	-	-	-
5	H9 positive sample	-	-	-	-	-	-	-	-
6	H9 positive sample	-	-	-	-	-	-	-	-
7	Negative sample	-	-	-	-	-	-	-	-
PC	Positive Control	28.31	28.61	28.52	28.18	28.14	28.35	0.21	0.73
NC	Negative Control	-	-	-	-	-	-	-	-
Mean									**0.46**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 26. Intra-assay variance of C_T values for **Influenza A subtype H7** (HEX). Tests were performed on the Bio-Rad CFX96 instrument.

Samples		Intra-assay variance for H7 (HEX) Reactions (C_T values)					C_T mean	SD	CV%
		1	2	3	4	5			
1	H5 positive sample	-	-	-	-	-	-	-	-
2	H5 positive sample	-	-	-	-	-	-	-	-
3	H7 positive sample	32.78	32.46	32.46	32.93	33.32	32.79	0.36	1.10
4	H7 positive sample	33.29	32.85	33.02	33.06	33.07	33.06	0.16	0.48
5	H9 positive sample	-	-	-	-	-	-	-	-
6	H9 positive sample	-	-	-	-	-	-	-	-
7	Negative sample	-	-	-	-	-	-	-	-
PC	Positive Control	29.04	29.22	28.88	28.76	29.01	28.98	0.17	0.60
NC	Negative Control	-	-	-	-	-	-	-	-
Mean									**0.72**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 27. Intra-assay variance of C_T values for **Influenza A subtype H9** (Cy5). Tests were performed on the Bio-Rad CFX96 instrument.

Samples		Intra-assay variance for H9 (Cy5) Reactions (C_T values)					C_T mean	SD	CV%
		1	2	3	4	5			
1	H5 positive sample	-	-	-	-	-	-	-	-
2	H5 positive sample	-	-	-	-	-	-	-	-
3	H7 positive sample	-	-	-	-	-			
4	H7 positive sample	-	-	-	-	-			
5	H9 positive sample	34.66	34.94	35.02	34.46	34.02	34.62	0.40	1.16
6	H9 positive sample	32.33	32.21	32.52	32.04	31.45	32.11	0.41	1.27
7	Negative sample	-	-	-	-	-	-	-	-
PC	Positive Control	30.04	29.41	29.04	28.91	28.67	29.21	0.53	1.83
NC	Negative Control	-	-	-	-	-	-	-	-
Mean									**1.42**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 28. Intra-assay variance of C_T values for the **Internal Control** (Texas Red). Tests were performed on the Bio-Rad CFX96 instrument.

Samples		Intra-assay variance for the Internal Control (Texas Red) Reactions (C_T values)					C_T mean	SD	CV%
		1	2	3	4	5			
1	H5 positive sample	28.10	28.16	28.20	28.00	28.22	28.14	0.09	0.32
2	H5 positive sample	27.98	27.99	28.15	28.12	28.18	28.08	0.09	0.33
3	H7 positive sample	28.09	28.09	28.05	28.12	27.85	28.04	0.11	0.39
4	H7 positive sample	28.01	28.02	27.96	28.13	27.80	27.98	0.12	0.43
5	H9 positive sample	28.03	28.13	27.95	28.09	27.91	28.02	0.09	0.33
6	H9 positive sample	27.87	27.92	27.88	28.18	27.76	27.92	0.16	0.56
7	Negative sample	28.05	28.09	28.17	28.01	28.24	28.11	0.09	0.33
PC	Positive Control	27.63	28.02	27.96	27.85	27.64	27.82	0.18	0.65
NC	Negative Control	28.12	27.94	28.06	28.09	28.29	28.10	0.13	0.45
Mean									**0.42**

SD = standard deviation; CV = coefficient of variation

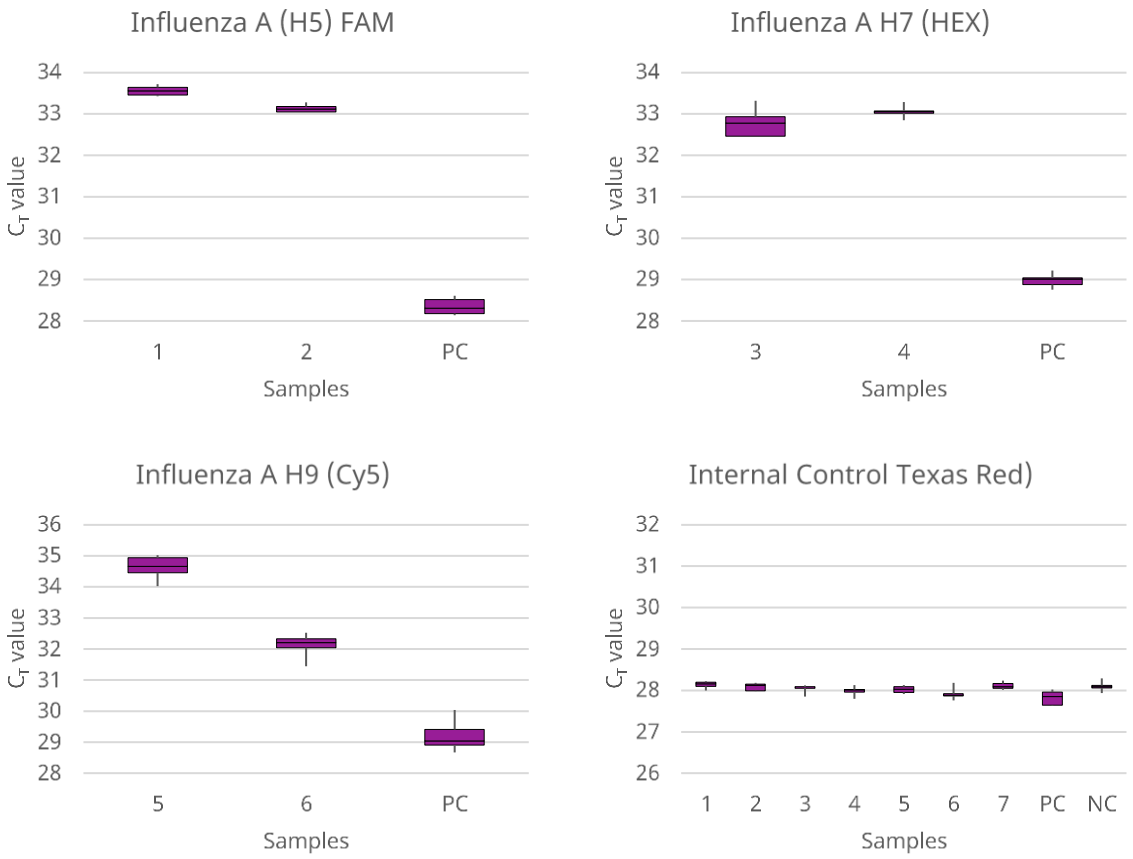


Figure 20. Boxplots of intra-assay variance for **Influenza A H5** (FAM), **H7** (HEX), **H9** (Cy5), and the **Internal Control** (Texas Red) for the virotype Influenza A H5/H7/H9 RT-PCR Kit. Tests were performed on the Bio-Rad CFX96.
NC = Negative Control, PC = Positive Control

Conclusion

The virotype Influenza A H5/H7/H9 RT-PCR Kit shows an excellent repeatability of results with very low intra-assay variance.

4.5.2 Inter-assay variance

Influenza A H5 positive RNA samples (1, 2), H7 positive RNA samples (3, 4), H9 positive RNA samples (5, 6), one negative RNA sample (7), and the Positive Control (PC) as well as the Negative Control (NC) of the kit were tested in five different RT-PCR runs with the virotype Influenza A H5/H7/H9 RT-PCR Kit on the Bio-Rad CFX96 instrument.

Results

Test results including calculated mean values, standard deviations (SD), and the coefficients of variation (CV) are given in Table 29 - Table 32. Figure 21 depicts the results for the according positively tested samples per channel.

The inter-assay variance is on average 0.40 % for **Influenza A H5** (FAM), 0.47 % for **H7** (HEX), 0.66 % for **H9** (Cy5), and 1.21 % for the **Internal Control** (Texas Red).

Table 29. Inter-assay variance of C_T values for **Influenza A subtype H5** (FAM). Tests were performed on the Bio-Rad CFX96 instrument.

Samples		**Inter-assay variance for H5 (FAM) RT-PCR runs (C_T values)**					**C_T mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**			
1	H5 positive sample	33.40	33.50	33.46	33.56	33.74	33.53	0.13	0.39
2	H5 positive sample	33.21	33.11	32.93	33.13	33.30	33.14	0.14	0.41
3	H7 positive sample	-	-	-	-	-	-	-	-
4	H7 positive sample	-	-	-	-	-	-	-	-
5	H9 positive sample	-	-	-	-	-	-	-	-
6	H9 positive sample	-	-	-	-	-	-	-	-
7	Negative sample	-	-	-	-	-	-	-	-
PC	Positive Control	28.39	28.47	28.56	28.35	28.61	28.48	0.11	0.39
NC	Negative Control	-	-	-	-	-	-	-	-
Mean									**0.40**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 30. Inter-assay variance of C_T values for **Influenza A subtype H7** (HEX). Tests were performed on the Bio-Rad CFX96instrument.

Samples		Inter-assay variance for H7 (HEX) RT-PCR runs (C_T values)					C_T mean	SD	CV%
		1	2	3	4	5			
1	H5 positive sample	-	-	-	-	-	-	-	-
2	H5 positive sample	-	-	-	-	-	-	-	-
3	H7 positive sample	32.38	32.67	32.66	32.79	32.94	32.69	0.21	0.63
4	H7 positive sample	32.96	33.21	33.10	33.06	32.98	33.06	0.10	0.30
5	H9 positive sample	-	-	-	-	-	-	-	-
6	H9 positive sample	-	-	-	-	-	-	-	-
7	Negative sample	-	-	-	-	-	-	-	-
PC	Positive Control	29.15	29.10	29.36	28.98	29.17	29.15	0.14	0.47
NC	Negative Control	-	-	-	-	-	-	-	-
Mean									**0.47**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 31. Inter-assay variance of C_T values for **Influenza A subtype H9** (Cy5). Tests were performed on the Bio-Rad CFX96 instrument.

Samples		Inter-assay variance for H9 (Cy5) RT-PCR runs (C_T values)					C_T mean	SD	CV%
		1	2	3	4	5			
1	H5 positive sample	-	-	-	-	-	-	-	-
2	H5 positive sample	-	-	-	-	-	-	-	-
3	H7 positive sample	-	-	-	-	-	-	-	-
4	H7 positive sample	-	-	-	-	-	-	-	-
5	H9 positive sample	34.64	34.78	34.75	34.62	34.42	34.64	0.14	0.41
6	H9 positive sample	32.25	32.41	32.35	32.11	32.70	32.36	0.22	0.68
7	Negative sample	-	-	-	-	-	-	-	-
PC	Positive Control	29.24	29.37	29.48	29.21	29.87	29.43	0.27	0.91
NC	Negative Control	-	-	-	-	-	-	-	-
Mean									**0.66**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 32. Inter-assay variance of C_T values for the **Internal Control** (Texas Red). Tests were performed on the Bio-Rad CFX96.

Samples		Inter-assay variance for the Internal Control (Texas Red) RT-PCR runs (C_T values)					C_T mean	SD	CV%
		1	2	3	4	5			
1	H5 positive sample	28.09	28.47	28.60	28.13	28.84	28.43	0.32	1.12
2	H5 positive sample	28.12	28.46	28.43	28.08	28.92	28.40	0.34	1.19
3	H7 positive sample	28.09	28.33	28.43	28.04	28.87	28.35	0.33	1.17
4	H7 positive sample	27.99	28.19	28.35	27.98	28.52	28.21	0.23	0.83
5	H9 positive sample	27.93	28.17	28.44	28.02	28.85	28.28	0.37	1.31
6	H9 positive sample	27.94	28.43	28.50	27.92	28.60	28.28	0.32	1.14
7	Negative sample	28.09	28.43	28.52	28.11	29.01	28.43	0.37	1.32
PC	Positive Control	27.88	28.38	28.45	27.82	28.87	28.28	0.44	1.54
NC	Negative Control	28.22	28.49	28.27	28.10	29.04	28.42	0.37	1.31
Mean									**1.21**

SD = standard deviation; CV = coefficient of variation

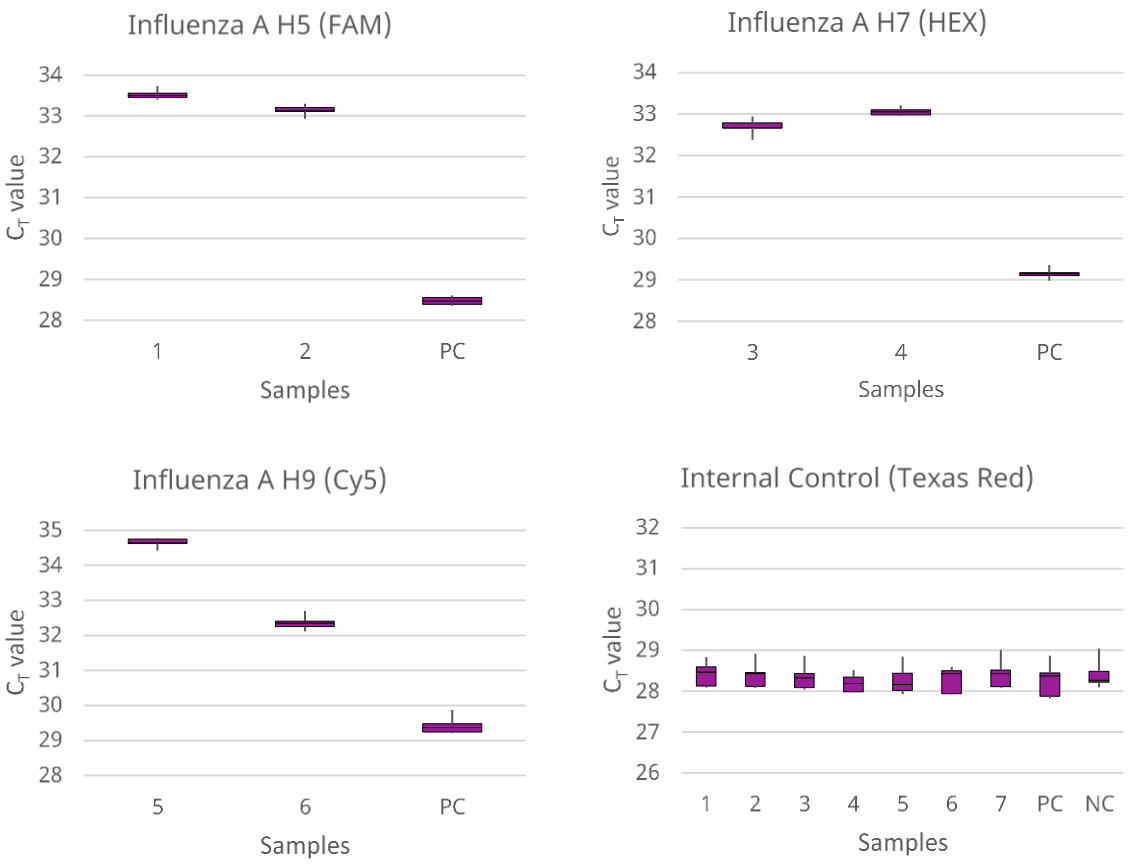


Figure 21. Boxplots of inter-assay variance for **Influenza A H5** (FAM), **H7** (HEX), **H9** (**C**y5), and the **Internal Control** (Texas Red) for the virotype Influenza A H5/H7/H9 RT-PCR Kit. Tests were performed on the Bio-Rad CFX96.
NC = Negative Control, PC = Positive Control

Conclusion

The virotype Influenza A H5/H7/H9 RT-PCR Kit shows an excellent repeatability of results with very low inter-assay variance.

4.6 Stability testing (freeze-thaw-cycles)

To evaluate stability of the virotype Influenza A H5/H7/H9 RT-PCR Kit, the components were frozen and thawed up to five times and subsequently tested using six Influenza A RNA positive (samples 1 – 6) and one Influenza A RNA negative sample (7) as well as the Positive Control (PC) and Negative Control (NC) of the kit. Tests were performed on the Bio-Rad CFX96 instrument.

Results/Conclusion

Results are represented in Table 33 - Table 36. The virotype Influenza A H5/H7/H9 RT-PCR Kit shows excellent stability with a mean C_T difference of -0.12 for **Influenza A H5** (FAM), 0.02 for Influenza A **H7** (HEX), -0.03 for Influenza A **H9** (Cy5), and -0.52 for the Internal Control (Texas Red).

Table 33. Stability testing for **Influenza A H5** (FAM) for the virotype Influenza A H5/H7/H9 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Samples		Stability (Influenza A H5; FAM) Freeze-thaw-cycles (C_T Values)		Δ C_T
		1	5	
1	H5 positive sample	33.74	33.48	-0.26
2	H5 positive sample	33.30	33.23	-0.07
3	H7 positive sample	-	-	-
4	H7 positive sample	-	-	-
5	H9 positive sample	-	-	-
6	H9 positive sample	-	-	-
7	Negative sample	-	-	-
PC	Positive Control	28.61	28.58	-0.03
NC	Negative Control	-	-	-
Mean				**-0.12**

- = no C_T

Table 34. Stability testing for **Influenza A H7** (HEX) for the virotype Influenza A H5/H7/H9 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Samples		Stability (Influenza A H7; HEX) Freeze-thaw-cycles (C_T Values)		Δ C_T
		1	5	
1	H5 positive sample	-	-	-
2	H5 positive sample	-	-	-
3	H7 positive sample	32.94	33.11	0.17
4	H7 positive sample	32.98	32.93	-0.05
5	H9 positive sample	-	-	
6	H9 positive sample	-	-	
7	Negative sample	-	-	-
PC	Positive Control	29.17	29.12	-0.05
NC	Negative Control	-	-	-
Mean				**0.02**

- = no C_T

Table 35. Stability testing for **Influenza A H9** (Cy5) for the virotype Influenza A H5/H7/H9 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Samples		Stability (Influenza A H9; Cy5) Freeze-thaw-cycles (C_T Values)		Δ C_T
		1	5	
1	H5 positive sample	-	-	-
2	H5 positive sample	-	-	-
3	H7 positive sample	-	-	-
4	H7 positive sample	-	-	-
5	H9 positive sample	34.42	34.65	0.23
6	H9 positive sample	32.70	32.35	-0.35
7	Negative sample	-	-	-
PC	Positive Control	29.87	29.91	0.04
NC	Negative Control	-	-	-
Mean				**-0.03**

- = no C_T

Table 36. Stability testing for the **Internal Control** (Texas Red) for the virotype Influenza A H5/H7/H9 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Samples		Stability (Internal Control, Texas Red) Freeze-thaw-cycles (C_T Values)		Δ C_T
		1	5	
1	H5 positive sample	28.84	28.45	-0.39
2	H5 positive sample	28.92	28.07	-0.85
3	H7 positive sample	28.87	28.30	-0.57
4	H7 positive sample	28.52	28.35	-0.17
5	H9 positive sample	28.85	28.29	-0.56
6	H9 positive sample	28.60	28.29	-0.31
7	Negative sample	29.01	28.53	-0.48
PC	Positive Control	28.87	28.11	-0.76
NC	Negative Control	29.04	28.46	-0.58
Mean				**-0.52**

5 Proficiency Test 2021 organized by IZSVe

INDICAL BIOSCIENCE GmbH took part in the 2021 proficiency test for Avian Influenza and Newcastle Disease organized by the AI/ND EURL IZSVe (Instituto Zooprofilattico Sperimentale delle Venezie, Italy). The samples were extracted using the IndiMag Pathogen Kit and the sample panel (Table 37) was tested with the virotype Influenza A H5/H7/H9 RT-PCR Kit as well as several other methods: The APHA assay detects H5 or H7 RNA in a monoplex RT-qPCR and the H9 protocol of Monne *et al*., 2008 was also performed in monoplex – each assay without Internal Control. Protocols described by Hoffmann *et al*., 2016 (FLI) are duplex RT-qPCRs for detection of one Influenza subtype and an Internal control simultaneously.

Results

Test results using the virotype Influenza A H5/H7/H9 RT-PCR Kit are compared to those obtained with the methods of APHA and FLI (Table 38). In contrast to certain reference methods, all samples of the proficiency test panel were successfully identified with the virotype Influenza A H5/H7/H9 RT-PCR Kit.

Table 37. Sample panel of the 2021 proficiency test 2021 for Avian Influenza and Newcastle Disease organized by the AI/ND EURL IZSVe (Instituto Zooprofilattico Sperimentale delle Venezie, Italy).

ID Sample	Isolate	Subtype	Clade/ Lineage	Patho-type
M01	A/duck/Italy/16VIR123/2016	H5N3		LPAI
M02	A/teal/Italy/16VIR345/2016	H7N7		LPAI
M03	APMV-1/pigeon/Cyprus/20VIR3543-9/2020	PPMV-1	XXI.2*	Virulent
M04	A/chicken/Nigeria/19VIR8424-15/2019	H9N2	G1	
M05	A/duck/Nigeria/19VIR8424-20/2019	H5N8	2.3.4.4B*	HPAI
M06	A/teal/Italy/21VIR49-39/2021	H7N3		LPAI
M07	A/chicken/Slovakia/14_20VIR205-19/2020	H5N8	2.3.4.4B*	HPAI
M08	Negative	Negative		
M09	A/mallard/Italy/20VIR4911-52/2020	H5N3		LPAI
M10	A/chicken/Italy/19VIR5895-21/2019	H7N3		LPAI
M11	A/peregrine falcon/Denmark/13776-1_20VIR7282-13/2020	H5N5	2.3.4.4B*	HPAI
M12	A/Eurasian_wigeon/Italy/20VIR7301-206_feather/2020	H5N1	2.3.4.4B*	HPAI
M13	NDV/chicken/Rus/Krasnodar/9.1/19	APMV-1	VII.1.1*	Virulent
M14	A/swan/Italy/17VIR1205/2017	H9N2	G1	
M15	A/duck/Nigeria/19VIR8424-2/2019	H5N6	2.3.4.4B*	HPAI

* According to Terregino *et al*, 2021. Proficiency test 2021 for Avian Influenza and Newcastle Disease organized by ISZVe – EURLAI/ND, Individual report

Table 38. Analysis of RNA of samples of the proficiency test organized by IZSVe in 2021 using virotype Influenza A H5/H7/H9 RT-PCR Kit. C_T values of the virotype **Influenza A H5** (FAM), **H7** (HEX), **H9** (Cy5) signals compared to the reference methods of APHA for H5 and H7 or Monne *et al*., 2008 for H9 and FLI (protocols described by Hoffmann *et al*., 2016 using the IAV-H5a-Mix 1-FAM, FLI-H7-CODA-Mix and IAV-H9-Mix 2 FAM) are shown. Tests were performed on the Agilent Mx3005P instrument.

ID Sample	**Sub-type**	**virotype Influenza A H5/H7/H9**			**APHA**		**Monne *et al.*, 2008**	**FLI**		
		C_T H5	**C_T H7**	**C_T H9**	**C_T H5**	**C_T H7**	**C_T H9**	**C_T H5**	**C_T H7**	**C_T H9**
M01	H5N3	27.68	-	-	30.90	-	-	30.88	-	-
M02	H7N7	-	30.61	-	-	36.81	-	-	30.48	-
M03	PPMV-1	-	-	-	-	-	-	-	-	-
M04	H9N2	-	-	23.86	-	-	25.99	-	-	-
M05	H5N8	30.61	-	-	31.38	-	-	32.27	-	-
M06	H7N3	-	23.81	-	-	30.00	-	-	23.99	-
M07	H5N8	29.84	-	-	31.21	-	-	32.72	-	-
M08	Neg.	-	-	-	-	-	-	-	-	-
M09	H5N3	27.77	-	-	30.51	-	-	28.52	-	-
M10	H7N3	-	28.72	-	-	-	-	-	29.70	-
M11	H5N5	31.55	-	-	32.97	-	-	33.01	-	-
M12	H5N1	30.44	-	-	32.20	-	-	32.57	-	-
M13	APMV-1	-	-	-	-	-	-	-	-	-
M14	H9N2	-	-	30.43	-	-	32.22	-	-	29.97
M15	H5N6	30.36	-	-	32.03	-	-	33.11	-	-

Neg. = Negative, - = no C_T

Conclusion

The virotype Influenza A H5/H7/H9 RT-PCR Kit allows the fast, reliably sensitive and specific detection of various Influenza strains.

6 References

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