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

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

Licensed in accordance with § 11 (2) of the German Animal Health Act
MA No.: FLI-C 072

REF 96 reactions (Cat. no. VT282705)

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INDICAL BIOSCIENCE

Contents

Kit contents

virotype Influenza A H5/H7/H9 RT-PCR Kit **Cat. no.** **Number of reactions**	**(96)** **VT282705** **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
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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.

Symbols

	Legal manufacturer
	Lot number
	Use by date
	Temperature limitations for storage
	Handbook
	Catalog number
	Material number
	Protect from light
	For samples from birds

Quality control

In accordance with INDICAL's ISO-certified Quality Management System, each lot of virotype Influenza A H5/H7/H9 RT-PCR Kit is tested against predetermined specifications to ensure consistent product quality.

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.

Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available from your local sales representative or by Email request under **compliance@indical.com**.

All sample residues and objects that have come into contact with samples must be decontaminated or disposed of as potentially infectious material.

Introduction

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 symptomes 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).

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.

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.

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

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 perform 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.

Protocol: Real-time RT-PCR for detection of RNA from Influenza A virus subtypes H5, H7 and H9

Important points before starting

- Please read „Important notes“ on page 10 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.
- **Important**: Ensure to mix the viscous RT-PCR Mix well prior to use.

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.

Procedure

1. Before use, mix the RT-PCR Mix by inverting 5 times or until mixed thoroughly, then centrifuge briefly to collect the fluids.
2. 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	1728 µl
Primers/Probes (purple cap)	2 µl	48 µl	192 µl
Total volume	**20 µl**	**480 µl**	**1920 µl**

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

4. 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 (Positive Control) instead of sample RNA.

Negative Control: Use 5 µl of the negative control (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**

5. Close the reaction tubes with the corresponding caps and **invert vigorously at least 5 times**. Then centrifuge briefly to collect the fluids.

6. 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	Cy5
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®)

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

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

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 67min (Stratagene Mx3005P)

Data analysis and 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 must give a Texas Red fluorescence signal and must not show a signal in the FAM, HEX and Cy5 channels.

The following results are possible if working with unknown samples. The possible sample results are also summarized in Table 5 on page 19.

Note: Please note that, depending on the used thermal cycler, crosstalk between fluorescence channels can occur (e.g., Rotor-Gene Q, AriaMx). When analyzing the data, please activate „*crosstalk correction/ crosstalk compensation*“. Further information is given 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.

[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 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 detailled interpretation of potential results can be found in Table 5 on page 18.

Note that very high concentrations of Influenza A RNA in the sample may lead to a reduced signal or no signal for the internal control (Texas Red) due to competition.

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 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 5. Results interpretation table*

FAM	HEX	Cy5	Texas Red/ ROX (IC)	Sample result 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 the FAM, HEX, Cy5 and Texas Red channels. The Negative Control must yield a signal in the Texas Red channel and no signal in the FAM, HEX and Cy5 channels. For a complete explanation of possible sample results please refer to “Data analysis and interpretation” on page 16.

INDICAL offers a range of ELISA kits and real-time PCR and real-time RT-PCR kits for the detection of animal pathogens.

Visit **www.indical.com** for more information about afosa, bactotype, cador, cattletype, flocktype, pigtype, Svanovir and virotype products.

For up-to-date licensing information and product-specific disclaimers, see the respective INDICAL kit handbook or user manual.

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Change index

Handbook	Version	Change
HB-2521-EN-005	December 2022	Specification Procedure (mixing of reagents)
HB-2521-EN-004	March 2022	Editorial changes
HB-2521-EN-003	November 2021	Correction Table 1, page 13

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