ViroReal® Typing Kit InfA H5N8

Manual

For veterinary use only

REF **DVET003**

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Index

1. Product description

ViroReal® Typing Kit InfA H5N8 contains two PCR primer sets for the amplification and detection of RNA of avian influenza A H5N8. This test is based on an intercalating dye using one-step reverse-transcription real-time PCR and was developed and validated for the Applied Biosystems® 7500 (Fast) instrument (Thermo Fisher Scientific), but is also suitable for other real-time PCR instruments. This test allows the rapid typing of avian influenza H5N8 from RNA samples.

ViroReal® Typing Kit InfA H5N8 detects the haemagglutinin (H) and neuraminidase (N) genes of avian influenza A virus, subtype H5N8. Amplification curves and specific melting curves for the targets H5 and N8 indicate the amplification of genotype specific RNA. It should be emphasized, however, that the viral genome consists of segmented RNA fragments highly prone to sequence variation. The test is based on currently published viral motives of known virulence, but constant re-arrangement makes it impossible to cover all potential relevant strains.

When using PCR-platforms not validated by ingenetix, an evaluation of the real-time PCR is recommended. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing PCR.

2. Pathogen information

Influenza A virus is a single-stranded, negative-sense RNA virus of the family Orthomyxoviridae. Of the three Influenza genera (A, B, C), only Influenza A is known to infect birds. Avian Influenza is further characterized based on the presence of haemagglutinin/neuroaminidase subtypes. Various strains, especially of H5N1 and H5N8 genotype, are responsible for outbreaks in domestic ducks and migratory birds in East Asia, Northern America, parts of Europe and the Middle East. Further spreading around the globe is possible through migratory birds. Virulence potential of a certain strain can vary from mild to severe due to high sequence variability.

References:

M. Li et al (2017): Highly Pathogenic Avian Influenza A (H5N8) Virus in Wild Migratory Birds, Qinghai Lake, China. Emerging Infectious Diseases 23 (4): 637-641

3. Principle of real-time PCR

When detecting pathogens by reverse transcription real-time PCR, a specific RNA sequence of the pathogen genome is transcribed into cDNA and amplified. The generated PCR-product is detected by an intercalating nucleic acid dye with subsequent melt curve analysis.

4. General Precautions

The user should always pay attention to the following:

- Always include a negative control per PCR-run (water instead of sample).
- Be careful when handling the positive control.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated workspace.
- Periodically decontaminate benches and devices.
- Use sterile pipette tips with filters.
- Thaw all components thoroughly at room temperature before starting an assay. When thawed, mix the components and centrifuge briefly.
- Always keep the SYBR® Green RT-PCR Mix and RT Enzyme Mix on ice.
- Use the RNA immediately after extraction and store at -20°C to -80°C as soon as possible.
- **Caution:** the Positive Controls are stored in RNA stabilizer that contains guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).

5. Contents of the Kit

5.1. ViroReal® Typing Kit InfA H5N8 order no. DVET003

Labelling	Content	Amount DVET003	Storage
Infuenza A H5 Assay Mix (green cap)	Primer for detection of H5 of Influenza A virus	1 x 50 µl	-15 °C to -25 °C
Influenza A N8 Assay Mix (violet cap)	Primer for detection of N8 of Influenza A virus	1 x 50 µl	-15 °C to -25 °C
Influenza A H5 Positive Control (red cap)	Control-RNA	1 x 15 µl	-15 °C to -25 °C
Influenza B N8 Positive Control (red cap)	Control-RNA	1 x 15 µl	-15 °C to -25 °C
2x SYBR® Green RT-PCR Mix (white cap)	One-Step RT-PCR Master Mix	2x 500 µl	-15 °C to -25 °C until first use, then at + 4°C
RT Enzyme Mix	Enzyme mix for reverse transcription	2 x 8 µl	-15 °C to -25 °C
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	-15 °C to -25 °C

The components of ViroReal® Typing Kit InfA H5N8 should be stored at -15°C to -25°C. Components are stable until expiry date stated on the label.

6. Additionally required materials and devices

- Reagents and devices for RNA-extraction
- Nuclease-free water
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material

7. Preparation of real-time PCR

Please make sure that at least one negative control (water, blue cap), as well as one positive control Influenza A H5, and Influenza A N8 (red cap) are included per PCR run.
Ingenetix highly recommends performing PCR analyses in duplicates, which increases the probability of pathogen detection and facilitates interpretation of results.

7.1. Pipetting scheme

Prepare separate Master Mixes for Influenza A H5 and Influenza A N8 for the number of reactions required including a positive control and a negative control (NTC):

		Per sample
Preparation of Master Mix (InfA H5) (mix well)	Nuclease-free water	3.84 µl
	Power SYBR® Green RT-PCR Mix (2x)	10.00 µl
	RT Enzyme Mix	0.16 µl
	Influenza A H5 Assay Mix	1.00 µl
	Total volume Master Mix	**15.00 µl**
Preparation of PCR	Master Mix	15.00 µl
	Sample	5.00 µl
	Total volume	**20.00 µl**

		Per sample
Preparation of Master Mix (InfA N8) (mix well)	Nuclease-free water	3.84 µl
	Power SYBR® Green RT-PCR Mix (2x)	10.00 µl
	RT Enzyme Mix	0.16 µl
	Influenza A N8 Assay Mix	1.00 µl
	Total volume Master Mix	**15.00 µl**
Preparation of PCR	Master Mix	15.00 µl
	Sample	5.00 µl
	Total volume	**20.00 µl**

Positive Control:
Influenza H5 and N8 Positive Controls are *in vitro* synthesized RNAs in RNA-stabilizer stored at -20°C. Before use, freshly dilute positive controls 1:500 with nuclease-free water (approximately 400,000 target copies/µl).

→ Use 1 µl of the freshly 1:500 diluted Influenza A H5 or N8 Positive Control + 4 µl Nuclease-free water, respectively for each of the InfA H5 and InfA N8 Master Mixes.

Caution: The use of more than 1 µl positive control (1:500 diluted) inhibits the RT-PCR reaction.

7.2. Programming of the temperature profile

Please find further information on programming the real-time PCR instrument in the respective operator's manual. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing real-time PCR.

Experiment Properties	Reagents: SYBR® Green Reagents Ramp speed: Standard
Select dyes	Reporter SYBR, Quencher None
Select passive reference	ROX
Sample Volume	20 µl
Temperature Profile:	

Holding Stage Cycles: 1 Analysis: None	**Holding Stage** Cycles: 1 Analysis: None	**Cycling Stage** Cycles: 40 Analysis: Quantification Acquisition at 60°	**Melt Curve Stage** Continuous
48°C 30 min	95°C 10 min	95°C 15 sec 60°C 1 min	95°C 15 sec 60°C 1 min 95°C 30 sec 60°C 15 sec

8. Interpretation of PCR-data

For analysis of PCR results gained with ViroReal® Typing Kit InfA H5N8, check amplification plots and melt curves. The specific amplification and melting curve of a sample needs to be interpreted in context of the Ct/Cp and
Tm [°C] values of the negative (NTC) and positive control.

For a valid interpretation, the following criteria should be fulfilled:

Applied Biosystems® 7500: 515 nm

Target	Positive Control Tm [°C]	Positive Sample Tm [°C]	NTC/ negative sample Tm [°C]
Influenza A H5	75 (+/- 2)	77 (+/- 2)	70 (+/-2)
Influenza A N8	75 (+/- 2)	77 (+/- 2)	70 (+/-2)

Due to sequence variation and other factors (e.g. performance on different PCR instruments), a shift of melting point (Tm) may occur. Therefore, a melting temperature interval is specified.

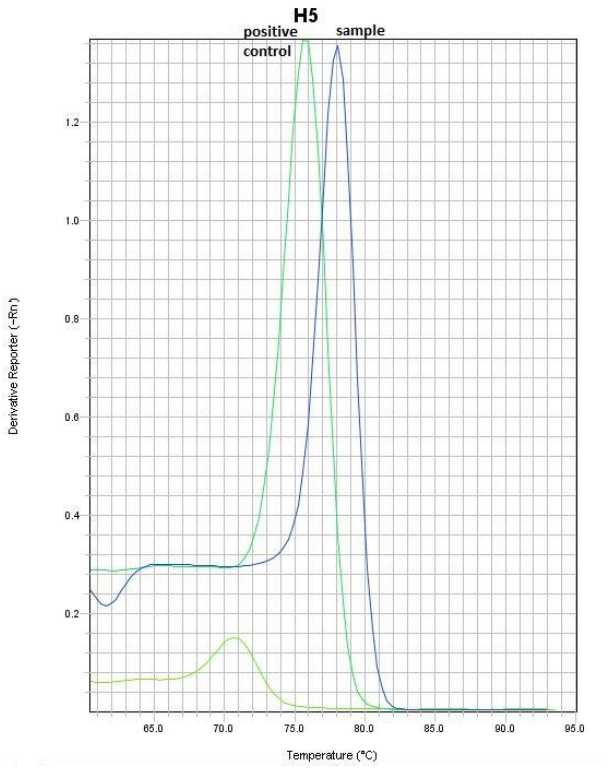
Based on the fact, that the viral genome consists of segmented RNA fragments, variation of InfA H5 and InfA N8 may arise.
Following results are possible:

InfA H5 positive	and	InfA N8 positive
InfA H5 positive	and	InfA N8 negative
InfA H5 negative	and	InfA N8 positive
InfA H5 negative	and	InfA N8 negative

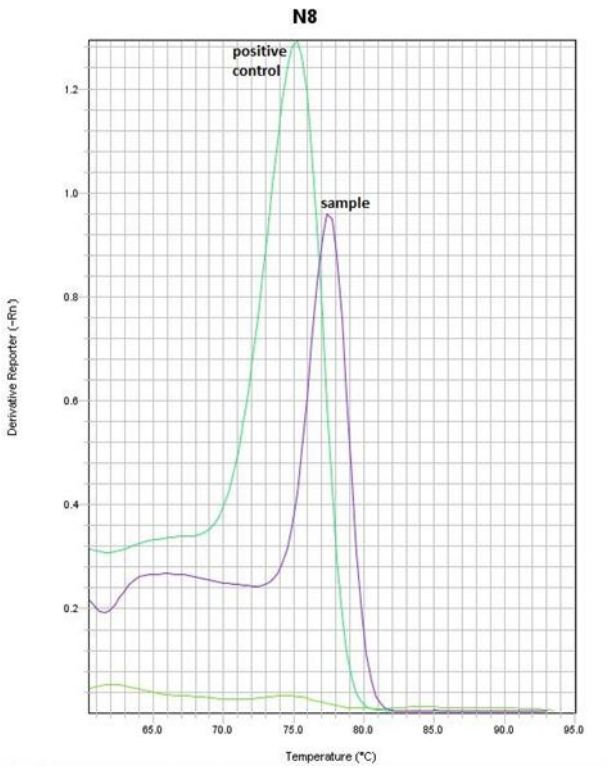
High sequence variation of InfA H5 and N8 may cause cross-reactivity of the assay and can be excluded by comparing sample Ct/Cp values with respect to amplification efficiency.

Before large-scale testing is started, a pretesting of seasonal or local Influenza A strain variants whose virulence has already been confirmed by international agencies is recommended.

Interpretation of melt curve data



Applied Biosystems® 7500: 515 nm
Specific melt curve of Influenza A H5-positive sample and positive control, no primer dimer formation



Applied Biosystems® 7500: 515 nm
Specific melt curve of Influenza A N8-positive sample and positive control, no primer dimer formation

9. Troubleshooting

9.1. No Influenza A H5 or N8 specific signal with positive control

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 7. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 7. Preparation of real-time PCR) and repeat the PCR, if necessary.
- RNA might be degraded.
  → Prepare fresh 1:500 dilutions of the positive controls and repeat the PCR.

9.2. Influenza A H5 or N8 specific signal with negative control

- A contamination occurred during preparation of the PCR.
  → Repeat the PCR with new reagents in replicates.
  → Strictly pipette the positive controls at last.
  → Make sure that work space and instruments are decontaminated at regular intervals.

10. Specifications

ViroReal® Typing Kit InfA H5N8 has been evaluated with Applied Biosystems® 7500 (Fast) instrument (Thermo Fisher Scientific). For further validation data please contact ingenetix.

10.1. Analytical sensitivity and specificity

ViroReal® Typing Kit InfA H5N8 is designed for rapid genotyping purposes of previously tested, strong Influenza A positive samples. The specificity for Influenza A H5N8 is ensured by the selection of highly specific primers. The primers have been checked for possible homologies to currently published sequences by sequence comparison analysis. However, the high sequence variability of influenza A has to be kept in mind. We recommend pre-testing of seasonal or local Influenza A strain variants whose virulence has already been confirmed by international agencies. If those subtypes are tested positive with ViroReal® Typing Kit InfA H5N8, high-throughput screening of field samples is indicated.
Influenza A H5 and N8 assays are not prone to generate primer dimers with the supplied master mix.

11. Annex – symbols

	Batch code		Use by
	Catalogue number		Manufactured by
	Contains sufficient for <n> tests		Store at