ViroReal® Kit Influenza B

Manual

CE

IVD **For *in vitro* diagnostic use**

REF **DHUV01453**

Σ **50 reactions**

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Explanation of symbols

LOT	Batch code		Use by
REF	Catalogue number		Manufactured by
Σ	Contains sufficient for <n> tests		Store at
CE	This product fulfils the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices	IVD	For *in vitro* diagnostic use

Corrosion, GHS05

Exclamation mark, GHS07

1. Intended use

ViroReal® Kit Influenza B is an *in vitro* diagnostic test, based on one-step reverse transcription real-time PCR (RT-PCR), for the detection of influenza B virus. Test material are extracted samples of the respiratory tract.

2. Product description

ViroReal® Kit Influenza B detects RNA of the hemagglutinin gene of influenza B virus. This test allows the rapid and sensitive detection of RNA of influenza B virus from samples purified from the respiratory tract (e.g. with the QIAamp Viral RNA Mini Kit, Qiagen).

A probe-specific amplification-curve in the FAM channel indicates the amplification of influenza B specific RNA. An internal RNA positive control (RNA IPC) is detected in Cy5 channel and is used as RNA extraction as well as RT-PCR inhibition control. The target for the RNA IPC is extracted with the sample.

This test is compatible with real-time PCR instruments which detect and differentiate fluorescence in FAM and Cy5 channel (e.g. ABI® 7500 instrument (Thermo Fisher Scientific), QuantStudio 5, QuantStudio 7 (Thermo Fisher Scientific), Mx3005P® (Agilent), qTOWER^{3}G (Analytik Jena), MIC instrument (bio molecular systems), LightCycler® 480 II (Roche Diagnostics), cobas z 480 Analyzer (Roche)).

When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR is recommended. Keep in mind that some PCR-platforms first have to be calibrated with the corresponding dye before performing multiplex-PCR.

The test is based on one-step reverse transcription real-time PCR (RT-PCR). A specific RNA sequence of the pathogen genome is transcribed into cDNA and amplified in a one-step PCR. The generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows a sequence-specific detection of PCR amplificates.

Ingenetix ViroReal®, BactoReal®, MycoReal, PanReal, ParoReal and SeptiReal Kits have been optimized to run under the same thermal cycling conditions. RNA and DNA material can be analysed in the same run.

3. Pathogen information

Influenza is an acute infectious disease caused by influenza virus A, B or, to a much lesser extent, influenza virus C. Influenza viruses are enveloped viruses with single-stranded, segmented RNA with negative polarity as genome. These viruses can be found worldwide. Epidemics and pandemics are mainly caused by influenza virus A, due to antigenic drift of the hemagglutinin and neuraminidase molecules. Type B and C influenza viruses are isolated almost exclusively from humans, while influenza A viruses infect a wide variety of warm-blooded animals.

4. Contents of the kit, stability and storage

Labelling	Content	Amount	Storage
Influenza B Assay Mix (green cap)	Primer and probe (FAM) for influenza B detection	1 x 50 µl	-15 °C to -25 °C
RNA IPC-3 Assay Mix (yellow cap)	Primer and probe (Cy5) for RNA IPC detection	1 x 50 µl	-15 °C to -25 °C
RNA IPC Target (orange cap)	Target for RNA IPC (internal RNA positive control system)	1 x 100 µl	-15 °C to -25 °C
Influenza B Positive Control (red cap)	RNA positive control (approx. 10^3 copies/µl)	1 x 150 µl	-15 °C to -25 °C
RNA Reaction Mix (white cap)	RNA reaction mix	1 x 250 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® Kit Influenza B are stable until the expiry date stated on the label.

RNA Reaction Mix: The Master Mix provided with the kit has been designed for reliable, high-sensitivity one-step reverse transcription real-time PCR even in the presence of common reaction inhibitors. The Master Mix contains a thermostable MMLV Reverse Transcriptase, an RNase inhibitor, a highly purified Taq Polymerase for rapid hot-start PCR, dNTPs, ROX™ dye (passive reference) and buffer components – additives optimized to handle RT-PCR inhibitors.

5. Additionally required materials and devices

- Reagents and devices for RNA-extraction
- Nuclease-free water
- Disposable powder-free gloves
- Sterile filter pipette tips
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM and Cy5 channel
- Appropriate optical 96-well reaction plates or reaction tubes with optical closing material recommended by the manufacturer of the real-time PCR instrument

6. Precautions and safety information

- For *in vitro* diagnostic use. The use of this kit is limited to qualified personnel instructed in the procedures of real-time PCR and *in vitro* diagnostics.
- The real-time PCR instrument should be serviced and cleaned regularly.
- Clean benches and devices periodically.
- Use sterile filter pipette tips.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective disposable powder-free gloves when handling kit reagents and specimens.
- Use separated workspaces for specimen preparation, reagent preparation and amplification. Supplies and equipment must be dedicated to each of these separate workspaces and ensure workflow in the laboratory from pre- to post-PCR.
- Be careful when handling samples and positive control to avoid cross contamination. Change gloves after handling of samples or positive control. Store positive or potentially positive material separately from reagents.
- Prevent contamination of work equipment and reagents with DNA/RNA, nuclease or amplification products by good laboratory practice.
- Quality of RNA has a profound impact on the test performance. Ensure that the used RNA extraction system is compatible with reverse transcription real-time PCR technology.
- For a valid interpretation of results, a negative control must be included during RNA-extraction (e.g. extraction of water instead of sample material) and tested per PCR-run, in order to exclude false-positive results due to contamination with virus RNA during extraction.
- Optional, also include a negative control per PCR-run (nuclease-free water instead of sample).

- Please note the expiry date of the kit.
- Repeated thawing and freezing of kit components should be avoided. Protect kit components from light. **Caution:** RNA IPC Target is stored in RNA stabilizer which contains DTT/Guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).
- Use established laboratory practices according to your local safety regulations for discarding specimens, reagents and waste disposal.

7. Limitations

- Optimal performance of this test requires appropriate specimen collection, transport and storage, as well as an appropriate RNA extraction procedure. RNA extraction and influenza B virus detection have been validated for nasopharyngeal swabs with this kit. Test performance with other specimen types has not yet been assessed.
- A negative test result does not exclude the possibility of influenza B infection, because test results may be affected by improper specimen collection, technical error, specimen mix-up or viral quantities below the assay sensitivity. The presence of PCR inhibitors may lead to invalid results.
- For this kit highly specific primers and probes have been selected. However, false-negative or less sensitive results might be obtained due to sequence heterogeneity within the target region of not yet described clinical subtypes.
- Results should be interpreted in consideration of clinical and laboratory findings.

8. Preparation of samples and real-time PCR

Swabs can be collected with swab material proper for PCR (e.g. sterile polyester or rayon swabs with aluminium or plastic shaft, not provided) and put into 1 ml isotonic saline solution (NaCl 0.9%, not provided). Do not freeze samples prior to extraction. Extract RNA from 140-200 µl sample (depending on the extraction method) and elute in 50 µl.
Extract samples with an RNA extraction system compatible with reverse transcription real-time PCR technology. A negative control of the RNA extraction must always be included (e.g. extraction of water instead of sample material).
Make sure that at least one extraction negative control, as well as one positive control (red cap) and optional one negative control (water) are included per PCR run.

Just before use, thaw the RNA Reaction Mix on ice, and invert 2 to 3 times to ensure a homogenous solution. The RNA Reaction Mix does not freeze at -20°C, but gelling may occur.
Best use RNA immediately after extraction and keep it on ice. Alternatively, use RNA stored at -20°C to -80°C and avoid prolonged exposure to room temperature, thaw on ice and immediately refreeze the RNA.
If the extracted RNA is stored at -80°C, it should remain stable for 3-6 months, at -20°C for 3-6 weeks, at 4°C for 3-5 hours and at room temperature for 0.5-1 minute.

8.1. Internal RNA Positive Control (RNA IPC)

An Internal RNA Positive Control system containing the RNA IPC assay and the RNA IPC Target excludes false-negative results due to inhibition of reverse transcription real-time PCR.
RNA IPC Target (approx. $6x10^5$ target copies/µl) is stored in RNA stabilizer which contains Guanidinium thiocyanate/Triton X-100. This stabilizer crystallizes by repeated freeze/thaw cycles and can be dissolved again by briefly warming up to approx. 50°C.
→ For control of RNA extraction and reverse transcription real-time PCR (exclusion of PCR inhibition), the RNA IPC Target must be added during extraction. Spike 1 µl of undiluted RNA IPC Target into the sample material after the lysis buffer was added, then continue the extraction procedure. Caution: The RNA IPC Target must not be added directly to the sample material.
→ As alternative (not recommended): For the only control of the reverse transcription real-time PCR, 1 µl of freshly 1:500 diluted RNA IPC Target (approx. 1200 target copies) per reaction has to be added to the master mix. Caution: The RNA IPC Target must not be added undiluted to the master mix.

8.2. Positive Control

Influenza B Positive Control is an *in vitro* synthesized RNA with a concentration of approx. 10^3 copies/µl. It has to be stored at -20°C. Ensure a homogenous solution by gently mixing, do not vortex. To avoid freeze/thaw cycles, it can also be temporarily stored at 4°C if used several times on the same day.
→ As positive control, use 10 µl of the Positive Control.

8.3. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Nuclease-free Water*	3.0 µl
	RNA Reaction Mix	5.0 µl
	Influenza B Assay Mix	1.0 µl
	RNA IPC-3 Assay Mix	1.0 µl
	Total volume Master Mix	**10.0 µl**
Preparation of PCR	Master Mix	10.0 µl
	RNA-Sample*	10.0 µl
	Total volume	**20.0 µl**

*10 µl sample can be used. When using a volume other than 10 µl, the volume of nuclease free water has to be adjusted accordingly.

Not recommended - if RNA IPC Target has not already been added during extraction: add 1 µl freshly 1:500 diluted IPC Target per reaction to the Master Mix. In this case, the IPC monitors the reverse transcription real-time PCR only. **Caution:** undiluted RNA IPC Target inhibits the PCR reaction.

For preparation of RT real-time PCR, dispense 10 µl aliquots of prepared Master Mix into the plate wells and then add 10 µl of RNA sample per well. At last, pipet the Positive Control. Close the plate with appropriate optical closing material.

8.4. Programming of temperature profile

Please find further information on programming the real-time PCR instrument in the respective operator's Further information on programming the real-time PCR instrument can be found in the respective operator's manual. Keep in mind that some PCR-platforms first have to be calibrated with the corresponding dye before performing multiplex-PCR.

Sample Volume: 20 µl

Temperature Profile:

Program 1	**Program 2**	**Program 3**
Cycles: 1 Analysis: None **Reverse Transcription**	Cycles: 1 Analysis: None **Polymerase Activation**	Cycles: 45 Analysis: Quantification Acquisition at 60°
50°C 15 min	95°C 20 sec	95°C 5 sec 60°C 1 min

For ABI® 7500 instrument
Ramp speed: Without "fast cycling" parameter

Note: This temperature profile can be used for all ViroReal®, BactoReal®, MycoReal, PanReal, ParoReal and SeptiReal kits on all real-time PCR instruments.

Detection channels:
FAM-TAMRA: Detection of influenza B virus
Cy5-NONE: Detection of IPC
Passive reference dye, if required: ROX (e.g. for ABI® 7500 instrument)

cobas z 480 Analyzer (Roche):
FAM: Excitation at 465 nm, Emission at 510 nm
Cy5: Excitation at 610 nm, Emission at 670 nm
Detection format: 2 Color Hydrolysis Probe
Passive reference dye: None

LightCycler® 480 II (Roche):
FAM: Excitation at 465, Emission at 510 nm
Cy5: Excitation at 618, Emission at 670 nm
Detection format: 2 Color Hydrolysis Probe
Passive reference dye: None

MIC Instrument (bio molecular systems):
FAM: Green
Cy5: Red
Passive reference dye: None

9. Interpretation of PCR-data

For analysis of PCR results gained with ViroReal® Kit Influenza B, select fluorescence display options FAM channel for the virus target and Cy5 channel for the RNA IPC target. Samples with positive Ct/Cp-values are considered positive.

Important: Please, also check amplification curves, not only Ct-values. Samples should be inspected both in logarithmic and linear scale view and compared with the negative control. Adjust the Threshold, if necessary.

Table 1 shows the criteria for valid controls. Table 2 shows interpretation of data with clinical samples.

Table 1: Criteria for valid controls, IPC Target was added during extraction

	Ct FAM channel Viral target	Ct Cy5 channel RNA IPC target[1]	Interpretation	Action
Positive control	23-25	Negative	Valid	-
Positive control	Negative	Negative	Invalid	See 10.1
Positive control	23-25	Positive	Invalid	See 10.4
Extraction negative control	Negative	25-28	Valid	-
Extraction negative control	Negative	Negative	Invalid	See 10.1
Extraction negative control	Positive	25-28	Invalid	See 10.3
Negative control (optional)	Negative	Negative	Valid	-
Negative control (optional)	Positive	Negative	Invalid	See 10.2
Negative control (optional)	Negative	Positive	Invalid	See 10.4

[1]If the RNA IPC Target has been added directly to the Master Mix, all samples must be positive in Cy5 channel

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. If the controls are not valid, the patient results cannot be interpreted.

Table 2: Interpretation of data with clinical samples

	Ct FAM channel Viral target	Ct Cy5 channel RNA IPC target	Interpretation	Action
Clinical sample	Negative	25-28[1]	Negative	-
Clinical sample	Positive	Positive	Positive	-
Clinical sample	Positive	Negative[2]	Positive	-
Clinical sample	Negative	Negative	Invalid	See 10.5

1)A positive signal excludes PCR inhibition. However, IPC Ct-values should show comparable results among samples. A shift of Ct- values can indicate a partial inhibition of PCR.

2) High pathogen load in the sample can lead to a reduced or absent fluorescence signal of the RNA IPC.

10. Troubleshooting

10.1. No virus specific signal with positive control and with IPC

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 8. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 8. Preparation of real-time PCR) and repeat the PCR, if necessary.
- The RNA IPC Target was added undiluted directly to the mastermix. The PCR reaction is therefore inhibited.
  → Freshly dilute RNA IPC Target and repeat PCR.
- No Positive Control was added.
  → Repeat PCR in case all clinical samples are negative.
- For control of the reverse transcription real-time PCR only, 1 µl of freshly 1:500 diluted RNA IPC Target has to be added to the mastermix. If no RNA IPC Target was added to mastermix:
  → Freshly dilute RNA IPC Target and repeat PCR extraction.
- For control of RNA extraction and PCR inhibition, the RNA IPC Target must be added during extraction. If no RNA IPC Target was added to lysis buffer during extraction:
  → Repeat PCR extraction.

10.2. Virus specific signal (FAM signal) with negative control

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

10.3. Virus specific signal with negative control of extraction (optional)

- A contamination occurred during extraction.
  → Repeat extraction and RT-PCR using new reagents.
  → Make sure that workspace and instruments are decontaminated at regular intervals.
  → See also 10.2

10.4. IPC specific signal with negative control and positive control

- The RNA IPC Target has been added to lysis buffer during extraction, but there is IPC specific signal with negative control and positive control: Contamination with the RNA IPC Target
  → Make sure that workspace and instruments are decontaminated at regular intervals.

10.5. No signal with IPC and no virus specific signal with sample

- PCR reaction was inhibited. No interpretation can be made.
  → Make sure that you use a recommended method for RNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during extractions can be retraced, it is recommended to repeat the PCR with lower amounts of RNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of H_2O).
- Incorrect PCR conditions.
  → Check the RT-PCR conditions and repeat the RT-PCR, if necessary.

10. Troubleshooting

10.1. No virus specific signal with positive control and with IPC

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 8. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 8. Preparation of real-time PCR) and repeat the PCR, if necessary.
- The RNA IPC Target was added undiluted directly to the mastermix. The PCR reaction is therefore inhibited.
  → Freshly dilute RNA IPC Target and repeat PCR.
- No Positive Control was added.
  → Repeat PCR in case all clinical samples are negative.
- For control of the reverse transcription real-time PCR only, 1 µl of freshly 1:500 diluted RNA IPC Target has to be added to the mastermix. If no RNA IPC Target was added to mastermix:
  → Freshly dilute RNA IPC Target and repeat PCR extraction.
- For control of RNA extraction and PCR inhibition, the RNA IPC Target must be added during extraction. If no RNA IPC Target was added to lysis buffer during extraction:
  → Repeat PCR extraction.

10.2. Virus specific signal (FAM signal) with negative control

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

10.3. Virus specific signal with negative control of extraction (optional)

- A contamination occurred during extraction.
  → Repeat extraction and RT-PCR using new reagents.
  → Make sure that workspace and instruments are decontaminated at regular intervals.
  → See also 10.2

10.4. IPC specific signal with negative control and positive control

- The RNA IPC Target has been added to lysis buffer during extraction, but there is IPC specific signal with negative control and positive control: Contamination with the RNA IPC Target
  → Make sure that workspace and instruments are decontaminated at regular intervals.

10.5. No signal with IPC and no virus specific signal with sample

- PCR reaction was inhibited. No interpretation can be made.
  → Make sure that you use a recommended method for RNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during extractions can be retraced, it is recommended to repeat the PCR with lower amounts of RNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of H_2O).
- Incorrect PCR conditions.
  → Check the RT-PCR conditions and repeat the RT-PCR, if necessary.

11. Specifications and performance evaluation

11.1. Kit performance on different real-time PCR instruments

Performance of ViroReal® Kit Influenza B with an Applied Biosystems® 7500 Real-time PCR System (Thermo Fisher Scientific) is shown in Figure 1.

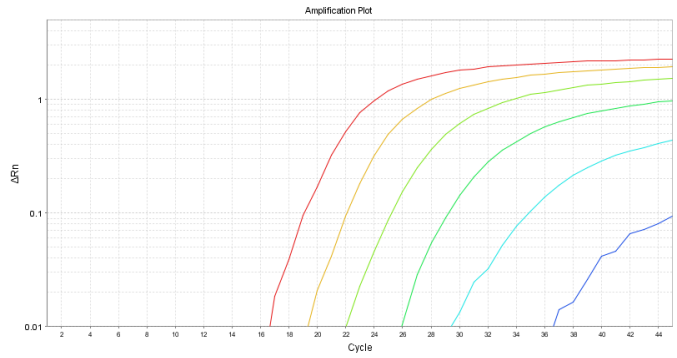
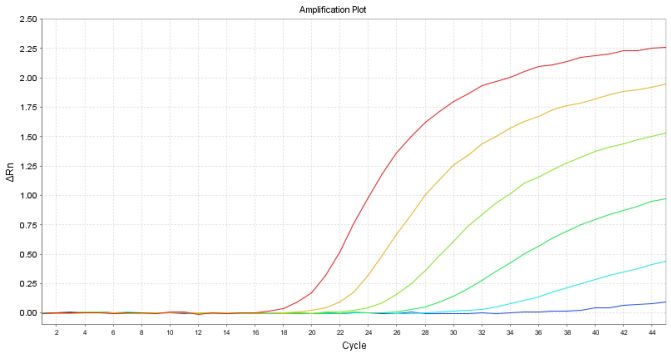
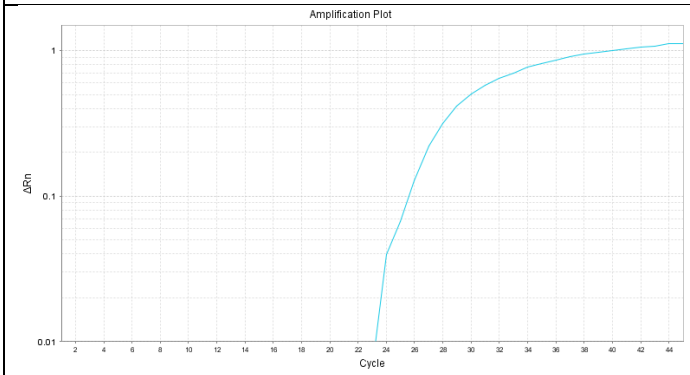
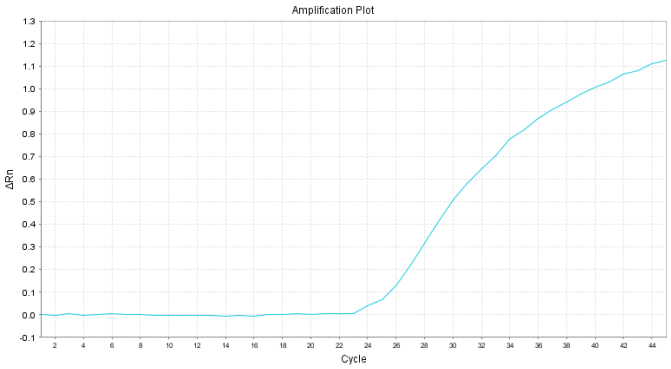
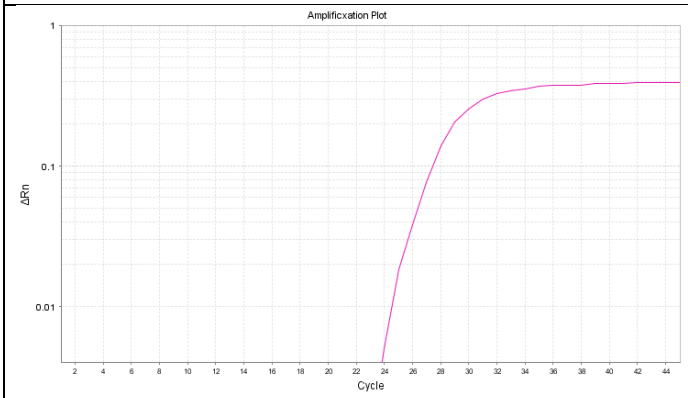
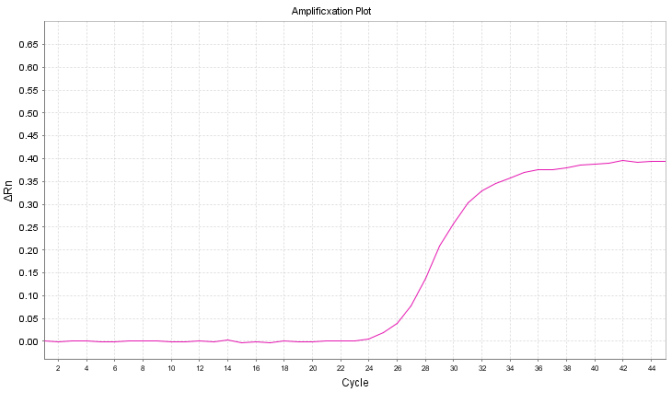
Logarithmic view	Linear view
	
ABI Prism® 7500: FAM channel, 530 nm 1:10 serial dilution of an influenza B RNA target	
	
ABI Prism® 7500: FAM channel, 530 nm Influenza B positive control	
	
ABI Prism® 7500: Cy5 channel, 667 nm Internal RNA positive control	

Figure 1 Performance of ViroReal® Kit Influenza B with ABI® 7500

This test has been validated with the Applied Biosystems® 7500 Real-time PCR System (Thermo Fisher Scientific) and tested with a LightCycler® 480 Instrument II (Roche), Mx3005P® QPCR System (Agilent), MIC instrument (bio molecular systems), QuantStudio™ 7 Pro (Thermo Fisher Scientific) and qTOWER^{3}G (Analytik Jena), but is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM and Cy5 channel.

11.2. Limit of detection and linearity

ViroReal® Kit Influenza B was tested with a 10-fold dilution series of a synthetic RNA representing a fragment of influenza B virus RNA. At least 10 target copies/reaction could be detected.
The **limit of detection** (LoD95 = smallest number of copies of target RNA which can be detected in 95% of cases) is 21.6 target copies/reaction.
The assay shows **linearity** over the range of 100 to 1,000,000 target copies/ with a slope of -3.299 ± 0.043 and a R_2 of 0.99 as shown in Figure 2.

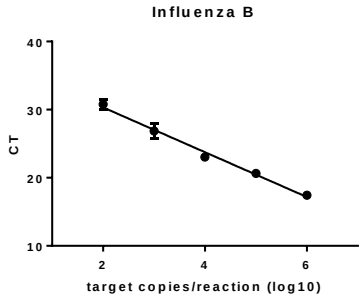


Figure 2 Ten-fold dilution series of influenza B virus RNA plotted against CT

11.3. Precision

Precision within one run (intra-assay), between several runs (inter-assay) and between two lots (intra-lot) was determined. The coefficient of variation is 1.7% on average for the intra-assay, 1.1% on average for the inter-assay and 1.0% on average for the intra-lot comparison.

11.4. Analytical specificity

The selection of highly specific primers and probes ensures analytical specificity. Primers and probes have been checked for possible homologies to currently published sequences by sequence comparison analysis. This validates the detection of so far known influenza B strains.
The analytical specificity was further evaluated by testing genomic DNA or RNA of viruses (adenovirus 3, parainfluenza virus 1 and 3, enterovirus, metapneumovirus A, RSV B, influenza A) and of bacteria (*Bordetella pertussis, Bordetella parapertussis, Streptoccocus pneumoniae, Mycoplasma pneumoniae, Neisseria meningitidis, Haemophilus influenzae*). No cross-reactions were observed.

11.5. Diagnostic evaluation

For diagnostic evaluation, 9 influenza B isolates, 4 nasopharyngeal swabs positive for influenza B and 13 nasopharyngeal swabs negative for influenza B were tested with ViroReal® Kit Influenza B. The presence or absence of influenza B was correctly confirmed in all samples (Table 3, 4).

Table 3 Results of diagnostic evaluation

	Reference			
		pos	neg	Total
ViroReal® Kit Influenza B	pos	13	0	13
	neg	0	30	30
	Total	13	30	43

Table 4 Summary diagnostic evaluation

	Value	95% CI
Sensitivity	100%	75.29% to 100.00%
Specificity	100%	88.43% to 100.00%
NPV	100%	88.43% to 100.00%
PPV	100%	75.29% to 100.00%
Prevalence	30%	

12. References

Taubenberger JK and Morens DM. 2008. The pathology of influenza virus infections. Annu Rev Pathol. 3: 499-522.

13. Revision history

Revision	Date	Description
1.3en	03 March 2021	4. Information about RNA Reaction Mix was added 8.Aadditional information about sample preparation was added 8.2 CAUTION! Positive Control was changed from storage in 15 µl RNA stabilizer (contained Guanidinium thiocyanate/Triton X-100) to storage in 150 µl citratbuffer. It must not be diluted 1:500 any more. 9. Table 1 and 2 were inserted 11.1. qTOWER3G (Analytik Jena) and MIC instrument (bio molecular systems) were added as validated real-time PCR devices 11.3 Added information about precision 11.5 Table 3 and 4 were inserted