

ViroReal[®] Kit Bovine Coronavirus

Manual

For use with the

- ABI PRISM[®] 7500 (Fast)
- LightCycler[®] 480
- Mx3005P[®]



For veterinary use only



DVEV00911, DVEV00913



100



DVEV00951, DVEV00953



50











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Index

1. Product description	3
2. Pathogen information	3
3. Principle of real-time PCR	3
4. General Precautions	3
5. Contents of the Kit.....	4
5.1. ViroReal® Kit Bovine Coronavirus order no. DVEV00911 or DVEV00951	4
5.2. ViroReal® Kit Bovine Coronavirus order no. DVEV00913 or DVEV00953	4
6. Additionally required materials and devices.....	4
7. Preparation of real-time PCR	5
7.1. Internal RNA positive control (RNA IPC):	5
7.2. Positive Control:.....	5
7.3. Pipetting scheme	5
7.4. Programming of the temperature profile	6
8. Interpretation of PCR-data	7
8.1. Signal in FAM channel:.....	7
8.2. No signal in FAM channel but signal of the RNA IPC:.....	7
8.3. No signal in FAM, VIC/HEX or Cy5 channel:.....	7
9. Troubleshooting.....	9
9.1. No BCoV specific signal with positive control:	9
9.2. No signal with the RNA IPC and no BCoV specific signals with the sample:.....	9
9.3. BCoV specific signal with the negative control:	9
9.4. BCoV specific signal with the negative control of RNA-extraction (optional):	9
10. Specifications	9
10.1. Analytical sensitivity	9
10.2. Analytical specificity	9

Explanation of symbols

	Batch code		Use by
	Catalogue number		Manufactured by
	Contains sufficient for <n> tests		Store at
	Corrosion, GHS05		Exclamation mark, GHS07

1. Product description

ViroReal® Kit Bovine Coronavirus is a real-time PCR assay for detection of RNA of the bovine coronavirus (BCoV) using one-step reverse transcription real-time PCR. This test was developed and validated for the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific), LightCycler® 480 (Roche) and Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments. This kit allows the rapid and sensitive detection of RNA of BCoV from nasal swabs or feces (e.g. with the QIAamp Viral RNA Mini Kit, Qiagen).

ViroReal® Kit Bovine Coronavirus detects the nucleocapsid protein gene (N gene) of BCoV. A probe-specific amplification-curve in the FAM channel indicates the amplification of BCoV specific RNA. An internal RNA positive control system for detection in VIC/HEX channel (order no. DVEV00911 or DVEV00951) or in Cy5 channel (order no. DVEV00913 or DVEV00953) allows control of RNA extraction and excludes false-negative interpretation of results caused by inhibition of reverse transcription real-time PCR (see 8. Interpretation of PCR-data).

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

BactoReal®, MycoReal, ParoReal and ViroReal® Kits are optimized to run under the same thermal cycling conditions. RNA and DNA material can be analysed in one run.

2. Pathogen information

Bovine coronavirus (BCoV) is a member of the genus *Coronavirus (Betacoronavirus)*, family *Coronaviridae*. The BCoV virion is enveloped and spherical in shape. The genome is a single-stranded, positive-sense RNA molecule. BCoV causes diarrhea in adult cattle (Winter Dysentery) and wild ruminants, as well as enteric and respiratory diseases in calves. Winter Dysentery is characterized by high morbidity (50-100%) and low mortality (1-2%), the highest incidence is between November and April when cattle are kept in close confinement. In calves morbidity and mortality are high. BCoV has been found in cattle worldwide.

References:

Saif LJ. 2010. Bovine respiratory coronavirus. *Vet Clin North Am Food Anim Pract.* 26: 349-64.8.

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 to cDNA and amplified. The generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows for a sequence-specific detection of PCR amplicates.

4. General Precautions

The user should always pay attention to the following:

- Always include a negative control per PCR-run (Nuclease-free water instead of sample).
- Optional: for valid interpretation of results, a negative control should be included during RNA-extraction (for example extraction of water instead of sample material), in order to exclude false-positive results due to contamination with BCoV RNA during extraction.
- 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 RNA Reaction Mix on ice.
- Use the RNA immediately after extraction and store at -20°C to -80°C as soon as possible.
- **Caution:** the RNA IPC Target is stored in RNA stabilizer that contains guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).

5. Contents of the Kit

5.1. ViroReal® Kit Bovine Coronavirus order no. DVEV00911 or DVEV00951

Labelling	Content	Amount		Storage
		DVEV00911	DVEV00951	
Bovine Coronavirus Assay Mix (green cap)	Primer and probe (FAM) for BCoV detection	2 x 50 µl	1 x 50 µl	-20°C
RNA IPC-1 Assay Mix (yellow cap)	Primer and probe (VIC/HEX) for RNA IPC detection	2 x 50 µl	1 x 50 µl	-20°C
RNA IPC Target (orange cap)	RNA internal positive control	1 x 100 µl	1 x 100 µl	-20°C
Bovine Coronavirus Positive Control (red cap)	RNA positive control	1 x 300 µl	1 x 300 µl	-20°C
RNA Reaction Mix (white cap)	4 x Reaction Mix	2 x 250 µl	1 x 250 µl	-20°C
Nuclease-free water (blue cap)	Nuclease-free water	2 x 1000 µl	1 x 1000 µl	-20°C

5.2. ViroReal® Kit Bovine Coronavirus order no. DVEV00913 or DVEV00953

Labelling	Content	Amount		Storage
		DVEV00913	DVEV00953	
Bovine Coronavirus Assay Mix (green cap)	Primer and probe (FAM) for BCoV detection	2 x 50 µl	1 x 50 µl	-20°C
RNA IPC-3 Assay Mix (yellow cap)	Primer and probe (Cy5) for RNA IPC detection	2 x 50 µl	1 x 50 µl	-20°C
RNA IPC Target (orange cap)	RNA internal positive control	1 x 100 µl	1 x 100 µl	-20°C
Bovine Coronavirus Positive Control (red cap)	RNA positive control	1 x 300 µl	1 x 300 µl	-20°C
RNA Reaction Mix (white cap)	4 x Reaction Mix	2 x 250 µl	1 x 250 µl	-20°C
Nuclease-free water (blue cap)	Nuclease-free water	2 x 1000 µl	1 x 1000 µl	-20°C

The components of ViroReal® Kit Bovine Coronavirus are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

6. Additionally required materials and devices

- Reagents and devices for RNA-extraction
- Nuclease-free water for dilution of RNA IPC Target
- 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 which is able to detect and differentiate fluorescence in FAM and VIC/HEX or Cy5 channel
- 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 (red cap) and one extraction negative control (optional, recommended) are included per PCR run.

Ingenetix highly recommends performing PCR analyses in duplicates, which increases the probability of detection of the pathogen and facilitates interpretation of results.

- Prepare master mix on ice.
- Thaw RNA Reaction Mix on ice, and invert 2 to 3 times to ensure homogenous solution. Do not let it warm to room temperature.
- Use RNA immediately after extraction and store at -20 to -80°C as soon as possible.

7.1. 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 interpretation of results due to inhibition of reverse transcription real-time PCR.

→ Dilute RNA IPC Target freshly 1:500 with nuclease-free water and add to the master mix (use 1 µl/reaction).

→ Alternatively, for control of RNA extraction and PCR inhibition the RNA IPC Target can be added during extraction. Spike 1 µl of undiluted RNA IPC Target into the sample material after the lysis buffer was added.

Caution: Do not add the RNA IPC Target directly to the sample material.

7.2. Positive Control:

The Bovine Coronavirus Positive Control is an *in vitro* synthesized RNA with a concentration of approx. 10³ 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 Positive Control.

7.3. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Nuclease-free Water*	2.0 µl
	RNA Reaction Mix	5.0 µl
	Bovine Coronavirus Assay Mix	1.0 µl
	RNA IPC Assay Mix	1.0 µl
	RNA IPC Target# (freshly diluted 1:500)	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

*1-10 µl of the sample can be used. When using an amount < 10 µl of the sample, the amount of H₂O has to be changed accordingly.

#If RNA IPC Target not already added during extraction.

7.4. 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 multiplex-PCR.

Select dyes: FAM-TAMRA for detection of BCoV
 Cy5-NONE (RNA IPC-3 Assay Mix) **or** VIC/HEX-TAMRA (RNA IPC-1 Assay Mix) for detection of RNA IPC

Select reference dye (passive reference): ROX

Sample Volume: 20 µl

Temperature Profile:

Program 1	Program 2	Program 3
Cycles: 1 Analysis: None	Cycles: 1 Analysis: None	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 PRISM® 7500:
 Ramp speed: Without “fast cycling” parameter

For LightCycler® 480 instrument:
 Detection format: 2 Color Hydrolysis Probe
 (dyes see above)

Note: These instrument parameters can be used for all BactoReal®, MycoReal, ParoReal and ViroReal® kits on all PCR instruments.

8. Interpretation of PCR-data

Examples for interpretation of positive reactions are shown in the amplification plots below.

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

	Ct/Cp (FAM channel) BCoV target	Ct/Cp RNA IPC target	Interpretation
Negative control	Negative	26-29*	Valid
Positive control	<30	26-29*	Valid
Extraction negative control (optional)	Negative	26-29	Valid
Negative sample	Negative	26-29	Valid
Positive sample	Positive	26-29/negative	Valid

*In the case that the RNA IPC target has been added to the master mix

For analysis of PCR data please proceed as follows:

For analysis of PCR results gained with ViroReal® Kit Bovine Coronavirus please select fluorescence display options 530 nm (FAM channel) for the BCoV target and VIC/HEX channel or Cy5 channel for the RNA IPC target. Samples with a positive Ct or Cp-value are considered positive. Please also check the presence of amplification-curves manually.

8.1. Signal in FAM channel:

→ RNA of BCoV was amplified. The sample has to be interpreted as positive.

8.2. No signal in FAM channel but signal of the RNA IPC:

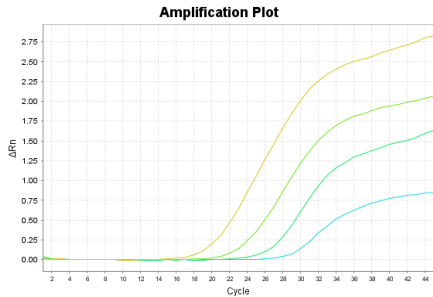
→ No RNA of BCoV is detectable in the sample. The sample has to be interpreted as negative. The positive signal of the internal positive control assay excludes a putative PCR inhibition.

8.3. No signal in FAM, VIC/HEX or Cy5 channel:

→ No interpretation statement can be made.

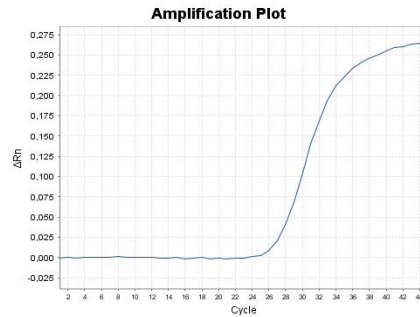
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

Detection of bovine coronavirus



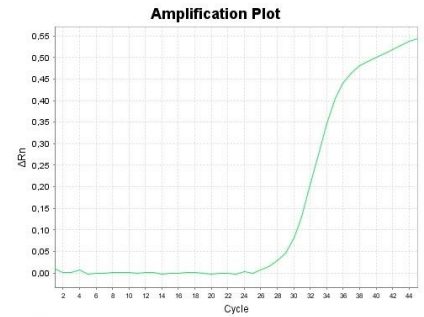
ABI Prism® 7500: FAM channel, 530 nm
1:10 serial dilution of a BCoV RNA positive control

Detection of internal RNA positive control IPC-3

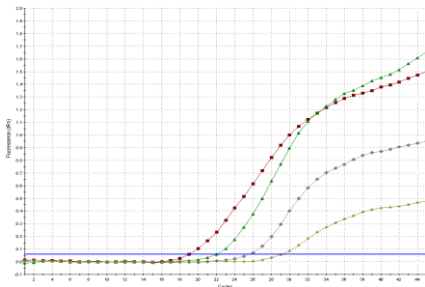


ABI Prism® 7500: Cy5 channel, 667 nm
Detection of internal RNA positive control

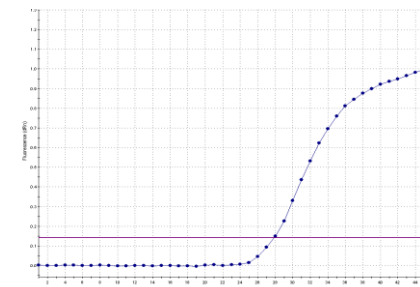
Detection of internal RNA positive control IPC-1



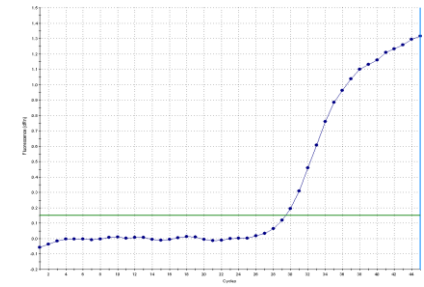
ABI Prism® 7500: VIC channel, 554 nm
Detection of internal RNA positive control



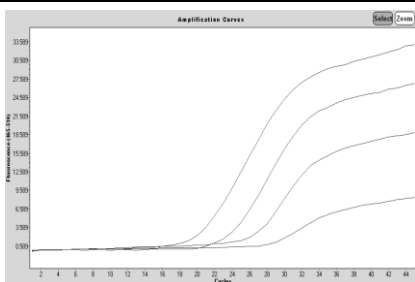
Mx3005P®: FAM channel
1:10 serial dilution of a BCoV RNA positive control



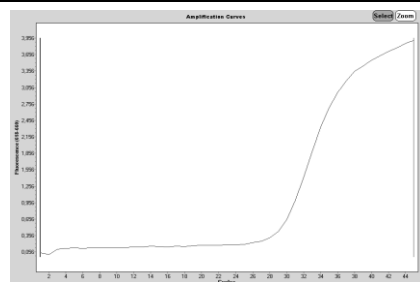
Mx3005P®: CY5 channel
Detection of internal RNA positive control



Mx3005P®: HEX channel
Detection of internal RNA positive control



LightCycler® 480: FAM channel
1:10 serial dilution of a BCoV RNA positive control



LightCycler® 480: Cy5 channel
Detection of internal RNA positive control

9. Troubleshooting

9.1. No BCoV 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.

9.2. No signal with the RNA IPC and no BCoV specific signals with sample

- The 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 amount of H₂O).
- Incorrect PCR conditions.
→ Check the PCR conditions and repeat the PCR, if necessary.

9.3. BCoV specific signal with negative control

- A contamination occurred during preparation of the PCR.
→ Repeat 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.

9.4. BCoV specific signal with negative control of RNA-extraction (optional)

- A contamination occurred during extraction.
→ Repeat the extraction and PCR using new reagents.
→ Make sure that work space and instruments are decontaminated at regular intervals.

10. Specifications

ViroReal® Kit Bovine Coronavirus was evaluated with the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific), with the LightCycler® 480 (Roche) and the Mx3005P® (Agilent). For further validation data please contact ingenetix.

10.1. Analytical sensitivity and linearity

The analytical sensitivity is 10 target copies/PCR reaction. The limit of detection (LoD₉₅ = smallest number of copies of target RNA which can be detected in 95% of cases) of 26 target copies/reaction was determined by several replicates around the detection limit.

The assay shows **linearity** over the range of 100 to 1,000,000 target copies/reaction with a slope of -3.4 and a R₂ of > 0.99 as shown in Figure 1.

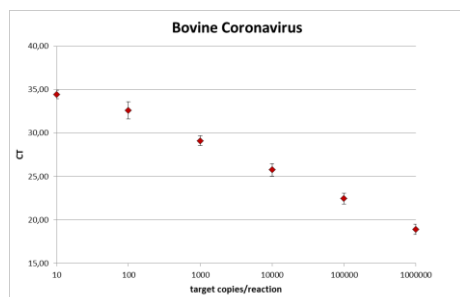


Figure 1 Ten-fold dilution series of a bovine coronavirus RNA standard plotted against CT

10.2. Analytical specificity

The specificity is ensured by the selection of highly specific primers and probes. The primers and probes were checked for possible homologies to currently published sequences by sequence comparison analyses. This also validated the detection of so far known BCoV strains published in the NCBI database.

The kit was tested against RNA isolates of bovine coronavirus, PHEV, TGEV and PEDV. It cross-reacts with PHEV. ViroReal® Kit Bovine Coronavirus detects all BCoV strains published in the NCBI database.