September 2014

# *virotype*® BTV RT-PCR Kit Handbook

24 (catalog no. 280433)*

96 (catalog no. 280435)

480 (catalog no. 280437)*

For detection of RNA from bluetongue virus

Registered in accordance with § 17c of the German Law on Animal Diseases (FLI-B 482)

280433*, 280435, 280437*

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* Available only on request.

Sample & Assay Technologies

# QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

Our mission is to enable you to achieve outstanding success and breakthroughs. For more information, visit www.qiagen.com.

In addition, QIAGEN provides high-quality, easy-to-use, and sensitive molecular solutions to enable veterinary pathogen detection and animal pathogen research. The QIAGEN veterinary portfolio includes a broad range of pathogen-specific PCR-assays and an extensive and growing ELISA portfolio. For more information, visit www.qiagen.com/Animal-and-Veterinary-Testing.

## Contents

## Kit Contents

| *virotype* BTV RT-PCR Kit | (24) | (96) | (480) |
|---|---|---|---|
| **Catalog no.** | **280433*** | **280435** | **280437*** |
| **Number of reactions** | **24** | **96** | **480** |
| PCR Mix (tube with yellow cap) includes primers and probes | 1 x 500 µl | 2 x 1000 µl | 6 x 1650 µl |
| Enzyme Mix (tube with green cap) | 1 x 6.5 µl | 1 x 26 µl | 2 x 65 µl |
| Positive Control (tube with red cap) | 1 x 25 µl | 1 x 70 µl | 2 x 50 µl |
| Negative Control (tube with blue cap) | 1 x 25 µl | 1 x 70 µl | 100 µl |
| Handbook | 1 | 1 | 1 |

* Available only on request.

## Intended Use

The *virotype* BTV RT-PCR Kit is intended for the detection of bluetongue virus RNA in ruminant whole blood (preferred with anticoagulants, for example EDTA-blood) and tissue samples (spleen, lymph nodes) from cattle, sheep, and goats. The *virotype* BTV RT-PCR Kit is for laboratory use only. The kit is approved by the Friedrich-Loeffler-Institut and registered in accordance with § 17c of the German Law on Animal Diseases (FLI-B 482) for use in Germany for veterinary diagnostic procedures. For veterinary use only.

## Symbols

Contains reagents for <N> tests


Legal manufacturer

Lot number


Use by date


Temperature limitations for storage

Handbook

Catalog number

Material number


Protect from light


For cattle, sheep, and goat samples

## Storage

The components of the *virotype* BTV RT-PCR Kit should be stored at –15 to –30°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 online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

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

**24-hour emergency information**
Chemical emergency or accident assistance is available 24 hours a day from:
CHEMTREC
**USA & Canada** ▪ Tel: 1-800-424-9300
**Outside USA & Canada** ▪ Tel: +1-703-527-3887 (collect calls accepted)

## Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of *virotype* BTV RT-PCR Kit is tested against predetermined specifications to ensure consistent product quality.

## Introduction

Bluetongue is an infectious, non-contagious disease of ruminants. The agent is the bluetongue virus (BTV), a double-stranded RNA virus of the genus *Orbivirus* of the family *Reoviridae* which includes at least 25 known serotypes. BTV is widely distributed around the world. Sheep, cattle, and goats are mainly affected by the disease. Clear clinical signs are usually seen only in sheep. In severe cases the tongue may show intense hyperemia and become cyanotic (Bluetongue). BTV serotype 8 is of epidemiological importance in Central Europe and cause of recent major Bluetongue Disease outbreaks. The virus is transmitted by certain midges of the genus *Culicoides*. Furthermore, the virus can be spread by contaminated needles and surgery equipment.

## Principle

Polymerase chain reaction (PCR) is based on the amplification of specific regions of the pathogen genome. In real-time RT-PCR, the amplified product is detected 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 the detection of the accumulating product without the need to re-open the reaction tubes afterward.

The *virotype* BTV RT-PCR Kit contains all of the necessary reagents for the detection of BTV RNA, 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.

The kit uses two specific primer/probe combinations: one for BTV RNA yielding FAM™ fluorescence and one for a housekeeping gene (β-actin mRNA), present within the sample, yielding HEX™ fluorescence.
The Positive Control contains BTV-8 RNA and allows the control of the denaturation step since the successful denaturation of the viral double-stranded RNA is a prerequisite for amplification.

## RNA extraction

*virotype* BTV can be used for the detection of BTV RNA from ruminant whole blood (preferred with anticoagulants, for example EDTA-blood) and tissue samples (spleen, lymph nodes). Due to the high sensitivity of the test, pools of up to 10 individual blood samples may be analyzed. However, the optimum pool size depends on the regional prevalence for BTV.

Prior to real-time RT-PCR, viral RNA must be extracted from the starting material. QIAGEN offers a range of products for RNA extraction from animal samples.

- QIAamp® *cador*® Pathogen Mini Kit
- QIAamp Viral RNA Mini Kit
- RNeasy® Fibrous Tissue Mini Kit for tissue
- RNeasy Mini Kit

If real-time RT-PCR is not performed immediately after extraction, store the RNA at -20°C or at –70°C for longer storage.

RNA extraction using kits based on spin-column technology can be automated using the QIAcube®.

## 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 and ice or liquid nitrogen
- 96-well plate standard PCR cycler
- Benchtop centrifuge with rotor for 1.5 ml tubes
- Rotor-Gene® Q or 96-well plate real-time cycler with appropriate fluorescent channels
- Rotor-Gene Q software version 1.7.94 or higher, or appropriate software for chosen 96-well plate cycler
- Strip Tubes and Caps, 0.1 ml, for use with Rotor-Gene Q (cat. no. 981103 or 981106) or PCR tubes and Caps, 0,2 ml or 96-well optical microplate with optical sealing film or cover for chosen 96-well plate 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 an assay.
- When thawed, mix the components by inverting and centrifuge briefly.
- Do not use components of the test kit past the expiration date.
- 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, for example, the correct setup of the reaction mix. Use 5 µl of the Positive Control provided with the *virotype* BTV RT-PCR Kit to test for successful amplification of the target.

## Extraction and amplification control

For increased process safety and convenience, an extraction and amplification control assay is included in the form of a second primer/probe set that detects a housekeeping gene present within the sample. This allows both extraction and amplification to be monitored.

## Protocol: Real-time RT-PCR using the Rotor-Gene Q

### 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.

### Things to do before starting

- Thaw all reagents on ice and protect from light.
- Maintain reagents on ice during PCR setup.
- Remove the Enzyme Mix from storage at –20°C immediately before use. Keep it on ice. Return it to –20°C immediately after use
- Before use, spin the reagents briefly.

### Procedure

1. Prepare the master mix according to Table 1.
   The master mix contains all of the components needed for PCR except the sample. Prepare a volume of master mix at least 10% greater than that required for the total number of PCR assays to be performed.
   See Table 1 for the volumes per number of reactions for the master mix.

**Table 1. Preparation of master mix**

| Component | 1 | 24 | 96 | 480 |
|---|---|---|---|---|
| PCR Mix (yellow cap) | 19.75 µl | 493.75 µl | 1896 µl | 9480 µl |
| Enzyme Mix (green cap) | 0.25 µl | 6.25 µl | 24 µl | 120 µl |
| **Total volume** | 20 µl | 500 µl | 1920 µl | 9600 µl |

2. **Pipet at least 7 µl of RNA samples or Positive Control into individual 0.2 ml PCR reaction tubes. Cover the reaction tubes (e.g., with PCR sealing film).**
   Include positive control reactions.
   Positive control: Use at least 7 µl of the positive control (Positive Control) instead of sample RNA.
3. **Denature the samples for 5 min at 98°C in a 96-well plate standard cycler with a heated lid.**
4. **Immediately cool down on ice water or liquid nitrogen for at least 20 s. Then store on ice or cooling device.**
5. **Pipet 5 µl of RNA samples, Positive Control, and Negative Control into individual Strip Tubes and Caps, 0.1 ml, for use with Rotor-Gene Q.**
   Use 5 µl of the negative control (Negative Control) instead of sample RNA.
6. **Add 20 µl of the Master Mix into each reaction tube. Thus the final volume is 25 µl (Table 2).**

**Table 2. Preparation of reaction mix**

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

7. Close the reaction tubes with the corresponding caps.
8. Set the filters for the reporter and quencher dyes in the software of your thermal cycler according to Table 3. Select the green and yellow channels on the Rotor-Gene Q (Table 3).

**Table 3. Filter settings for reporter and quencher**

| Pathogen/internal control | Reporter | Quencher |
|---|---|---|
| BTV | **FAM** | TAMRA™ |
| Internal control | **HEX**/JOE®* | TAMRA |

* Use the option appropriate for your thermal cycler.

9. Run the real-time RT-PCR protocol according to Table 4.
   This protocol is also suitable if running other *virotype* assays simultaneously (i.e., *virotype* BTVpan/8, *virotype* BVDV, *virotype* CSFV, *virotype* PRRSV and/or *virotype* SBV).

**Table 4. Real-time RT-PCR protocol**

| Temperature | Time | Number of cycles |
|---|---|---|
| 50°C | 20 min | 1 |
| 95°C | 15 min | 1 |
| 95°C | 30 s | 40 |
| 57°C* | 45 s | |
| 68°C | 45 s | |

* Fluorescence data collection.

## Protocol: Real-time RT-PCR using 96-well plate real-time cycler

Please read "Important Notes", page 10 and "Important points before starting" and "Things to do before starting", page 12.

## Procedure

1. **Prepare the master mix according to Table 5.**
   The master mix contains all of the components needed for PCR except the sample. Prepare a volume of master mix at least 10% greater than that required for the total number of PCR assays to be performed.
   See Table 5 for the volumes per number of reactions for the master mix.

**Table 5. Preparation of master mix**

| Component | 1 | 24 | 96 | 480 |
|---|---|---|---|---|
| PCR Mix (yellow cap) | 19.75 µl | 493.75 µl | 1896 µl | 9480 µl |
| Enzyme Mix (green cap) | 0.25 µl | 6.25 µl | 24 µl | 120 µl |
| **Total volume** | 20 µl | 500 µl | 1920 µl | 9600 µl |

2. **Pipet 5 µl of RNA samples, Positive Control, and Negative Control into individual reaction tubes. Cover the reaction tubes (e.g., with PCR sealing film).**
   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.

3. Denature the samples for 5 min at 98°C in a 96-well plate standard cycler with a heated lid.
4. Immediately cool down on ice water or liquid nitrogen for at least 20 s. Then store on ice or cooling device.
5. Pipet 20 µl of the master mix into each reaction tube. Thus the final volume of a test is 25 µl (Table 6).

**Table 6. Preparation of reaction mix**

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

6. Close the reaction tubes with the corresponding caps.
7. Set the filters for the reporter and quencher dyes in the software of your thermal cycler according to Table 7.

**Table 7. Filter settings for reporter and quencher**

| Pathogen/internal control | Reporter | Quencher |
|---|---|---|
| BTV | FAM | TAMRA™ |
| Internal control | HEX/JOE®* | TAMRA |
| Passive reference† | ROX™ | – |

* Use the option appropriate for your thermal cycler.

† Internal reference for use with the Applied Biosystems® ABI PRISM® Sequence Detection Systems.

8. **Run the real-time RT-PCR protocol according to Table 8.**
   This protocol is also suitable if running other virotype assays simultaneously (i.e., virotype BTVpan/8, virotype BVDV, virotype CSFV, virotype PRRSV and/or virotype SBV).

**Table 8. Real-time RT-PCR protocol**

| Temperature | Time | Number of cycles |
|---|---|---|
| 50°C | 20 min | 1 |
| 95°C | 15 min | 1 |
| 95°C | 30 s | 40 |
| 57°C‡ | 45 s | |
| 68°C | 45 s | |

‡ Fluorescence data collection.

## Data Analysis and Interpretation

### Interpretation of results

For the assay to be valid the Positive Control must give a signal in the FAM and HEX channels with a $C_T$* <35. If no FAM signal, of the Positive Control, is measured the denaturation and cooling steps were insufficient and testing should be repeated. The Negative Control must give no signal.

The following results are possible if working with unknown samples. The possible sample results are also summarised in Table 9 on page 21.

**The sample is positive for BTV, and the assay is valid, if the following criteria are met:**

- The sample yields a signal in both the FAM and HEX† channels
- The Positive Control yields a signal in both the FAM and HEX
- The Negative Control yields no signal

Note that very high concentrations of BTV RNA in the sample may lead to a reduced HEX signal or no HEX signal due to competition with the internal control.

* Threshold cycle ($C_T$) — cycle at which the amplification plot crosses the threshold, i.e., there is the first clearly detectable increase in fluorescence.
† Green and yellow on the Rotor-Gene Q.

**The sample is negative for BTV, and the assay is valid, if the following criteria are met:**

- The sample yields a signal in the HEX channel but not in the FAM channel
- The Positive Control yields a signal in both the FAM and HEX channels
- The Negative Control yields no signal

A positive HEX signal means that extraction and amplification were successful as the housekeeping gene (β-actin mRNA) within the sample is amplified.

**The sample results are inconclusive, and the assay is invalid, if the following occurs:**

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

If no signal is detected in both the FAM (pathogen) and the HEX (Internal Control) channel, the result is inconclusive. The absence of a signal for the housekeeping gene indicates PCR inhibition and/or other malfunctions.

It is recommended to retest the respective individual samples in nuclease free water (e.g., diluted 1:5), to repeat the RNA extraction, or repeat the whole test procedure starting with new sample material.

Check that there is a fluorescence signal in the all 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 RNA denaturation, RNA extraction failure, incorrect setup of the master mix, or incorrect cycling conditions.

Repeat RNA extraction or repeat the whole procedure starting with new sample material.

**Table 9. Results interpretation table***

| | Reporter | |
|---|---|---|
| **Sample result** | **FAM (pathogen)** | **HEX (IC)** |
| BTV positive | X | X |
| BTV positive (strong positive) | X | |
| BTV negative | | X |
| Inconclusive result | | |

* Interpretation of sample results can be determined provided positive and negative control reactions are performed. The positive control must yield a signal in both the FAM and HEX channels. The negative control must yield no signal in the FAM and HEX channels. For a complete explanation of possible sample results please refer to "Data Analysis and Interpretation" on page 19.

## Troubleshooting Guide

The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

## Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| *virotype* BTV RT-PCR Kit (24)* | For 24 reactions: PCR Mix, Enzyme Mix, Positive Control, Negative Control | 280433 |
| *virotype* BTV RT-PCR Kit (96) | For 96 reactions: PCR Mix, Enzyme Mix, Positive Control, Negative Control | 280435 |
| *virotype* BTV RT-PCR Kit (480)* | For 480 reactions: PCR Mix, Enzyme Mix, Positive Control, Negative Control | 280437 |
| **Related products** | | |
| *virotype* BTV pan/8 RT-PCR Kit (96)† | For 96 reactions: Master Mix, Positive Control, Negative Control | 280445 |
| *virotype* PRRSV RT-PCR Kit (96)† | For 96 reactions: Master Mix, Positive Control, Negative Control | 282305 |
| *virotype* BVDV RT-PCR Kit (96)† | For 96 reactions: PCR Mix, Enzyme Mix, Positive Control, Negative Control | 280375 |

* Available only on request.
† Other kit sizes are available; see www.qiagen.com.

| Product | Contents | Cat. no. |
|---|---|---|
| *virotype* CSFV RT-PCR Kit (96)* | For 96 reactions: Master Mix, Positive Control, Negative Control | 281805 |
| *virotype* SBV RT-PCR Kit (96)* | For 96 reactions: Master Mix, Positive Control, Negative Control | 281605 |
| *virotype* Influenza A RT-PCR Kit (96)* | For 96 reactions: Master Mix, Positive Control, Negative Control | 282605 |
| *bactotype* Mycoplasma Mg/Ms PCR Kit (96)* | For 96 reactions: Master Mix, Positive Control, Negative Control | 288105 |
| QIAamp *cador* Pathogen Mini Kit (50)* | For 50 preps: 50 QIAamp Mini Spin Columns, Carrier RNA, Proteinase K, Collection Tubes (2 ml), RNase-free Buffers | 54104 |
| QIAamp Viral RNA Mini Kit (50)* | For 50 RNA preps: 50 QIAamp Mini Spin Columns, carrier RNA, Collection Tubes (2 ml), RNase-free buffers | 52904 |

* Other kit sizes are available; see www.qiagen.com.

| Product | Contents | Cat. no. |
|---|---|---|
| RNeasy Fibrous Tissue Mini Kit (50) | For 50 preps: 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), Proteinase K, RNase-free DNase I, RNase-free Reagents and Buffers | 74704 |
| RNeasy Mini Kit (50)* | For 50 preps: 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers | 74104 |
| Rotor-Gene Q 5plex Platform | Real-time PCR cycler with 5 channels (green, yellow, orange, red, crimson), laptop computer, software, accessories, 1-year warranty on parts and labor | 9001570 |

* Other kit sizes are available; see www.qiagen.com.

QIAGEN offer a range of ELISA kits and real-time PCR and real-time RT-PCR kits for the detection of animal pathogens. Visit www.qiagen.com/Animal-and-Veterinary-Testing for more information about the *bactotype*®, *cador*®, *cattletype*®, *flocktype*®, *pigtype*®, and *virotype* products.

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Limited License Agreement for *virotype* BTV RT-PCR Kit

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