# ViroReal® Kit Rabies Virus

## Manual

### For use with the

- **ABI PRISM® 7500 (Fast)**
- **LightCycler® 480**
- **Mx3005P®**


**For veterinary use only**

REF **DVEV00811, DVEV00813** Σ **100**

REF **DVEV00851, DVEV00853** Σ **50**

**ingenetix GmbH**
Arsenalstraße 11
1030 Vienna, Austria
T +43(0)1 36 198 0 198
F +43(0)1 36 198 0 199
office@ingenetix.com
www.ingenetix.com

# Index



## Explanation of symbols

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

## 1. Product description

ViroReal® Kit Rabies Virus is a real-time PCR assay for detection of RNA of the classical rabies virus (RABV genotype 1, serotype 1, RABV-GT-1) using one-step reverse transcription real-time PCR. This test was developed and validated for the ABI PRISM® 7500 (Fast) instrument (Applied Biosystems), 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 rabies virus from samples purified from tissues (e.g. brain, etc), saliva, urine and cerebrospinal fluid. Serial sampling is recommended. Rabies virus RNA can be recovered efficiently from liquid samples using the QIAamp Viral RNA Mini Kit (Qiagen) or from tissue samples using RNeasy kit (Qiagen) extraction methods, for example.

ViroReal® Kit Rabies Virus detects a region in the nucleoprotein (N) gene of rabies virus (RABV). A probe-specific amplification-curve in the FAM channel indicates the amplification of rabies virus specific RNA. An internal RNA positive control system for detection in VIC/HEX channel (order no. DVEV00811 or DVEV00851) or in Cy5 channel (order no. DVEV00813 or DVEV00853) allows control of RNA extraction and excludes false-negative interpretation of results due to 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

Rabies is caused by a neurotropic virus of the genus *Lyssavirus* of the family *Rhabdoviridae*, and is transmissible to all mammals. As it is transmissible to humans by inoculation or inhalation of infectious virus, all suspected infected material must be handled under the appropriate safety conditions specified by the World Health Organisation (WHO). Previous known lyssavirus genotypes were recently reclassified, and the Lyssavirus genus currently consists of 14 lyssavirus species (Aravan virus, Australian bat lyssavirus (ABLV), Bokeloh bat lyssavirus (BBLV), Duvenhage virus (DUVV), European bat lyssavirus 1 (EBLV-1), European bat lyssavirus 2 (EBLV-2), Ikoma lyssavirus (IKOV), Irkut virus, Khujand virus, Lagos bat virus (LBV), Mokola virus (MOKV), Rabies virus (RABV), Shimoni bat virus (SHIBV) and the West Caucasian bat virus (WCBV). All lyssaviruses cause the severe neurotropic disease known as rabies. RABV (genotype 1, RABV-GT-1) is detected worldwide and is the prototype lyssavirus, the remaining species are known as rabies-related lyssaviruses. Human infections are mainly caused by RABV, DUVV, EBLV-1, EBLV-2 and ABLV.

**References:**

Improved safety for molecular diagnosis of classical rabies viruses by use of a TaqMan real-time reverse transcription-PCR "double check" strategy. 2010. Hoffmann B, Freuling CM, Wakeley PR, Rasmussen TB, Leech S, Fooks AR, Beer M, Müller T.; J Clin Microbiol. 48:3970-8.

## 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 amplificates.

# 4. Contents of the Kit

## 4.1. ViroReal® Kit Rabies Virus order no. DVEV00811 or DVEV00851

| Labelling | Content | Amount | | Storage |
|---|---|---|---|---|
| | | DVEV00811 | DVEV00851 | |
| Rabies Virus Assay Mix (green cap) | Primer and probe (FAM) for rabies virus 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 |
| Rabies Virus Positive Control (red cap) | RNA positive control (approx. 2,000,000 target copies/µl) | 1 x 15 µl | 1 x 15 µl | -20°C |
| RNA Reaction Mix (white cap) | Amplification mix for one-step RT real-time PCR | 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 |

## 4.2. ViroReal® Kit Rabies Virus order no. DVEV00813 or DVEV00853

| Labelling | Content | Amount | | Storage |
|---|---|---|---|---|
| | | DVEV00813 | DVEV00853 | |
| Rabies Virus Assay Mix (green cap) | Primer and probe (FAM) for rabies virus 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 |
| Rabies Virus Positive Control (red cap) | RNA positive control (approx. 2,000,000 target copies/µl) | 1 x 15 µl | 1 x 15 µl | -20°C |
| RNA Reaction Mix (white cap) | Amplification mix for one-step RT real-time PCR | 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 Rabies Virus are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

## 5. 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 rabies virus 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 Positive Control and the RNA IPC Target is stored in RNA stabilizer that contains guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).

## 6. Additionally required materials and devices

- Reagents and devices for RNA-extraction
- Nuclease-free water for dilution of RNA IPC Target and positive control
- 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°C 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 Rabies Virus Positive Control is an *in vitro* synthesized RNA in RNA-stabilizer. It has to be stored at -20°C. Before use it has to be freshly diluted 1:500 with nuclease-free water, which corresponds to approx. 4,000 target copies/µl. Optional: 1:10 dilution of the 1:500 diluted positive controls can be used and defined as second standard value (approx. 400 target copies/µl).

→ As positive control please use 1 µl of the freshly 1:500 diluted Rabies Virus Positive Control + 9 µl nuclease-free water. Caution: The use of more than 1 µl positive control (diluted 1:500) inhibits the RT-PCR reaction.

### 7.3. Pipetting scheme

| | | **Per sample** |
|---|---|---|
| **Preparation of Master Mix** (mix well) | Nuclease-free Water* | 2.0 µl |
| | RNA Reaction Mix | 5.0 µl |
| | Rabies Virus 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 $H_2O$ 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 rabies virus
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<br>Analysis: None | Cycles: 1<br>Analysis: None | Cycles: 45<br>Analysis: Quantification<br>Acquisition at 60° |
| 50°C<br>15 min | 95°C<br>20 sec | 95°C<br>5 sec<br>60°C<br>1 min |

<u>For ABI PRISM® 7500:</u>
Ramp speed: Without "fast cycling" parameter

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

**<u>Note:</u>** 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) Rabies virus target | Ct/Cp RNA IPC target | Interpretation |
|---|---|---|---|
| Negative control | Negative | 26-29* | Valid |
| Positive control (freshly diluted 1:500), approx. 4,000 copies, 1 µl/PCR | 23-25 | 26-29* | Valid |
| Extraction negative control (optional) | Negative | 26-29 | Valid |
| Negative sample | Negative | 26-29 | Valid |
| Positive sample | Positive | 26-29/negative | Valid |

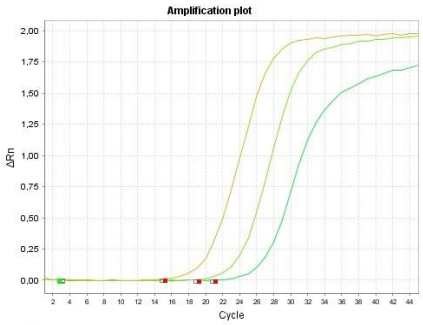
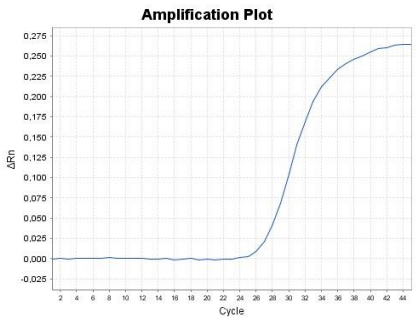
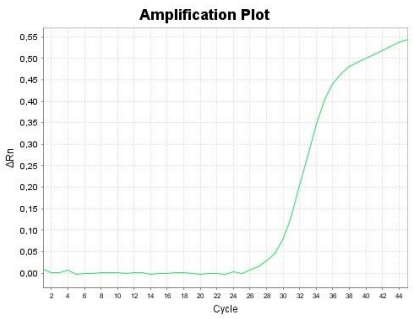
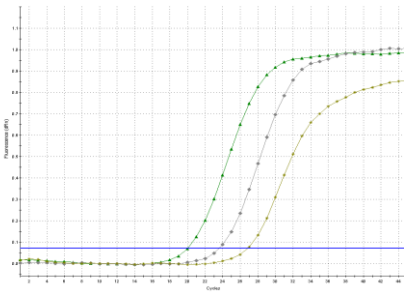
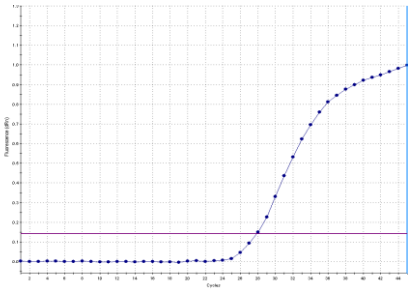
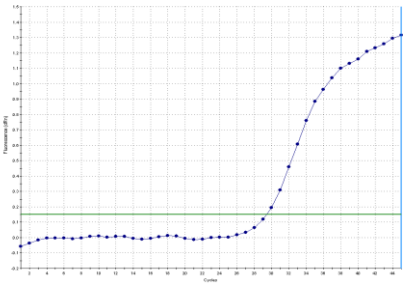
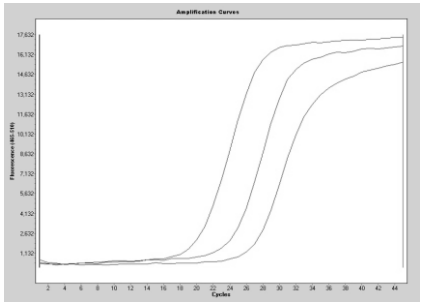
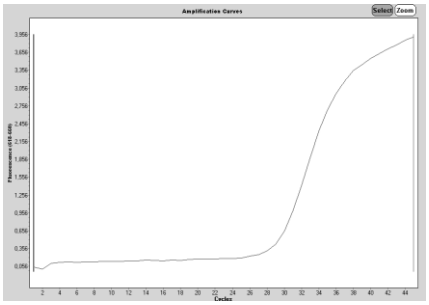
*In the case that the RNA IPC target has been added to the master mix

**For analysis of PCR data please proceed as follows:**
For analysis of PCR results gained with ViroReal® Kit Rabies Virus please select fluorescence display options 530 nm (FAM channel) for the rabies virus 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 rabies virus 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 rabies virus is detectable in the sample. The sample has to be interpreted as negative. However, negative results should not be used to exclude a diagnosis of rabies.
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 **rabies virus (RABV)** | Detection of internal RNA positive control **IPC-3** | Detection of internal RNA positive control **IPC-1** |
|---|---|---|
|  |  |  |
| **ABI Prism® 7500:** FAM channel, 530 nm<br>1:10 serial dilution of a rabies virus positive control | **ABI Prism® 7500:** Cy5 channel, 667 nm<br>Detection of internal RNA positive control | **ABI Prism® 7500:** VIC channel, 554 nm<br>Detection of internal RNA positive control |
|  |  |  |
| **Mx3005P®:** FAM channel<br>1:10 serial dilution of a rabies virus positive control | **Mx3005P®:** CY5 channel<br>Detection of internal RNA positive control | **Mx3005P®:** HEX channel<br>Detection of internal RNA positive control |
|  |  | |
| **LightCycler® 480:** FAM channel<br>1:10 serial dilution of a rabies virus positive control | **LightCycler® 480:** Cy5 channel<br>Detection of internal RNA positive control | |

# 9. Troubleshooting

## 9.1. No rabies virus 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.

## 9.2. No signal with the RNA IPC and no rabies virus specific signals with the 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 $H_2O$).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.

## 9.3. Rabies virus specific signal with the negative control:

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

## 9.4. Rabies virus specific signal with the 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 Rabies Virus was evaluated with the ABI PRISM® 7500 (Fast) instrument, with the LightCycler® 480 and the Mx3005P®. For further validation data please contact ingenetix.

### 10.1. Analytical sensitivity

The analytical sensitivity is 10 template copies/PCR. The sensitivity can vary because of the diversity of rabies virus.

### 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 rabies virus strains. ViroReal® Kit Rabies Virus is specific for the rabies virus.