ViroReal® Kit Monkeypox Virus

Instructions for Use

Research use only

REF DHUV02753

Σ **50 reactions**

ingenetix GmbH
Arsenalstraße 11
1030 Vienna, Austria
T +43(0)1 36 1980 1
office@ingenetix.com
www.ingenetix.com

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Explanation of symbols

	Batch code		Use by date
	Catalogue number		Manufacturer
	Contains sufficient for <n> tests		Temperature limit (Store at)
	Consult instructions for use		Manufacturing date
	Corrosion, GHS05		Exclamation mark, GHS07

1. Intended purpose

ViroReal® Kit Monkeypox Virus is a non-automatic rea-time PCR test (*research use only*) for the qualitative detection of monkeypox virus DNA using real-time polymerase chain reaction. Suitable test materials are DNA extracts from different sample material. The kit detects both clades of Monkeypox Virus, the West African clade and the Congo Basin (Central African) clade. The kit also detects other members of the orthopoxviruses, such as cowpox virus and vaccinia virus, albeit with lower sensitivity.

2. Product description

ViroReal® Kit Monkeypox Virus is a real-time PCR test and detects the B9R gene of the monkeypox virus, which encodes the interferon-γ receptor-like protein. The test was validated with a synthetic oligonucleotide representing this sequence.

A probe-specific amplification-curve in the FAM channel (530 nm) indicates amplification of monkeypox virus-specific DNA. As quality control, the kit contains an internal DNA positive control (DNA IPC), which is detected in the Cy5 channel. The target of the DNA IPC assay (artificial target DNA) can be extracted with the sample and serves as DNA extraction control as well as real-time PCR inhibition control.

This test has been validated with the QuantStudio™ 7 (Thermo Fisher Scientific) but is also suitable for use with other instruments such as ABI® 7500 Real-Time PCR System (Thermo Fisher Scientific), LightCycler® 480 II (Roche Diagnostics), Mx3005P® (Agilent), qTOWER^{3}G (Analytik Jena), and mic instrument (bio molecular systems). Please, keep in mind that some PCR-platforms first have to be calibrated with the corresponding dye before performing multiplex-PCR.

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

3. Pathogen information

Monkeypox is a smallpox-like disease caused by the monkeypox virus. Monkeypox virus belongs to the Orthopoxvirus genus. These viruses have a genome of double-stranded DNA and can replicate in the cytosol. The natural reservoir of monkeypox virus are rodents and can be transmitted from them to other animals and to humans. Further transmission from person to person can occur through contact with skin and bodily secretions.

Monkeypox virus is endemic in Africa. Two genetically different clades have developed, the West African clade and the Congo Basin (Central Africa) clade. In May 2022 there was an increased incidence of monkeypox virus infections outside of Africa, especially in the US, Canada and Europe. This virus belongs to the West African clade.

4. Principle of real-time PCR

The test is based on multiplex real-time PCR technology by 5´-nuclease-assay technology. Specific DNA sequences are amplified and the generated PCR-products are detected by oligonucleotide-probes labelled with fluorescent dyes (FAM und Cy5). This allows a sequence-specific detection of PCR amplificates.

During PCR, primers are extended by *Taq* polymerase and probes hybridized to the target are cleaved by the 5'-exonuclease activity of *Taq* polymerase. According to the accumulation of PCR product, the fluorescence of the probe increases with each PCR cycle. The change in fluorescence of the dyes is recorded cycle by cycle in the real-time PCR instrument in the closed reaction tube at different fluorescence wavelengths.
The Cq value (Quantification cycle, Cq; Cycle threshold, Ct; Crossing point, Cp) describes the cycle at which the fluorescence first rises significantly above the background fluorescence.

5. Contents of the kit, stability and storage

Beschriftung	Inhalt	Menge	Lagerung
Monkeypox Virus Assay Mix (green cap)	Primer + probe for detection of - Monkeypox virus (FAM)	1 x 50 µl	-25 °C to -15 °C
Internal Positive Control Assay (yellow cap)	Primer + probe for detection of - DNA IPC (Cy5)	1 x 50 µl	-25 °C to -15 °C
DNA IPC Target (orange cap)	Target for DNA IPC (internal DNA positive control system)	1 x 200 µl	-25 °C to -15 °C
Positive Control (red cap)	DNA positive control (approx. 10^4 target copies/µl)	1 x 300 µl	-25 °C to -15 °C
DNA Reaction Mix (white cap)	DNA reaction mix	1 x 500 µl	- **25 °C to -15 °C until first use, then at +4 °C**
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	-25 °C bis -15 °C

DNA Reaction Mix
The Master Mix provided with the kit has been designed for reliable, high-sensitivity real-time PCR. The Master Mix contains a highly purified Taq Polymerase for rapid hot-start PCR, dNTPs with dUTP and Uracil-N glycosylase (UNG) to eliminate amplicon carryover, ROX™ dye (passive reference) and buffer components – additives optimized to handle RT-PCR inhibitors.

Delivery and Storage
The kit is delivered at -20 °C to +4°C. The components of the kit are stable at -25 °C to -15 °C until the expiry date stated on the label. After opening and storage at 4°C, the in-use stability of the DNA Reaction Mix is a maximum of 12 months but no longer than the expiry date indicated on the label. Protect components of the kit from light.

Quality Control Release Testing
In accordance with the ISO 13485-certified Quality Management System of ingenetix, each lot is tested against predetermined specifications to ensure consistent product quality. The quality control is carried out with an artificial DNA, which represents parts of the pathogen DNA.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction appropriate for the biological sample material (see 9. Preparation of samples)
- Optional: Nuclease-free water for dilution of DNA IPC Target
- Disposable powder-free gloves
- Pipettes
- Filter pipette tips
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM and Cy5 channel
- Optical 96 well reaction plates or optical reaction tubes with optical closing material Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents.

7. Precautions and safety information

- The use of this kit is limited to qualified personnel instructed in real-time PCR
- Transportation of specimens must comply with local regulations for the transport of etiologic agents.
- Improper collection, transport or storage of specimens may hinder the ability of the assay to detect the target sequences.
- The real-time PCR instrument should be serviced and cleaned regularly.
- Clean benches and devices periodically.
- Use sterile filter pipette tips and disposable powder-free gloves.
- 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 separate areas for specimen preparation, reagent preparation and amplification. Supplies and equipment must be dedicated to each of these separate areas 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.
- Quality of DNA has a profound impact on the test performance. Ensure that the used DNA extraction system is compatible with real-time PCR technology.
- Prevent contamination of work equipment and reagents with DNA/RNA, nucleases or amplification products by good laboratory practice.
- For a valid interpretation of results, a negative control must be included during DNA-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 pathogen DNA during extraction.
- Optionally include a negative control per PCR-run (nuclease-free water instead of sample).
- Reagents from different kits or batches should not be mixed. Please note the expiry date of the kit.
- Repeated freeze/thaw cycles of kit components should be avoided. Protect kit components from light.
- Use established laboratory practices according to your local safety regulations for discarding specimens, reagents and waste disposal.
- **Caution:** DNA IPC Target is stored in RNA/DNA stabilizer which contains DTT/Guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).

8. Limitations

- Optimal performance of this test requires appropriate specimen collection, transport and storage, as well as an appropriate DNA extraction procedure.
- A negative test result does not exclude the presence of monkeypox virus, because test results may be affected by improper specimen collection, technical error, specimen mix-up or pathogen quantities below the assay sensitivity. The presence of PCR inhibitors may lead to invalid results.
- Although highly specific primers and probes were chosen for this kit, any sequence variability in the target region of previously unknown subtypes may lead to false-negative or less sensitive results.

9. Preparation of samples

ViroReal® Kit Monkeypox Virus is suitable for analysis of DNA extracts from different sample material.

Purified DNA should be stored at -25 °C to -15 °C.

Extract samples with a DNA extraction system compatible with real-time PCR technology and appropriate for the biological sample material.

- For manual extraction recommended: QIAamp® DNA Mini Kit (Qiagen)
- For automated extraction recommended: innuPREP AniPath DNA RNA – KFFLX Kit (Analytik Jena) with the KingFisher FLEX instrument (Thermo Fisher Scientific)

Always include an extraction negative control during DNA-extraction (e.g., extraction of water instead of sample material).

Quality control for DNA extraction and PCR inhibition
The DNA IPC system (internal DNA positive control) is used as a control for DNA extraction, identifies possible PCR inhibition and confirms the integrity of kit reagents.
An artificial target DNA (IPC Target, approx. $6x10^5$ copies/µl) is added during extraction and is detected in Cy5 channel.

→ **For control of DNA extraction and real-time PCR:** The undiluted DNA IPC target is added during the extraction. Pipette 1 µl undiluted DNA IPC target (orange cap) per sample directly to the lysis buffer (or add to the sample after the lysis buffer has been pipetted to the sample) and then continue the extraction.

Caution: The undiluted DNA IPC target must not be added directly to the sample material in the absence of lysis buffer as it may degrade. It must be added to the lysis buffer.

→ If the DNA IPC target was not added during the extraction, it can be added to the PCR master mix at a later time as a quality control for the PCR reaction. In this case, freshly dilute the DNA IPC target 1:100 with nuclease-free water and add 1 µl to the dilution/PCR reaction.

Caution: The DNA IPC Target must not be added undiluted to the master mix.

10. Preparation of real-time PCR

- Include one extraction negative control per PCR run, one positive control (red cap) and optionally one PCR negative control (nuclease-free water).
- It is recommended to analyse samples in duplicates, which increases the probability of pathogen detection and facilitates interpretation of results.
- It is best to use the DNA immediately after extraction (always store on ice).
- Thaw DNA samples on ice.
- Kit components must thaw completely at room temperature before preparing the Master Mixes. When thawed, mix components carefully, centrifuge briefly at low speed.
- Gently mix the DNA Reaction Mix to ensure homogeneity of solution.
- **Positive Control**
  → Use 8 µl of Positive Control (red cap). Always, pipette positive control at last.

10.1. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	DNA Reaction Mix	10.0 µl
	Monkeypox Virus Assay Mix	1.0 µl
	Internal Positive Control Assay Mix	1.0 µl
	Total volume Master Mix	**12.0 µl**
Preparation of PCR	Master Mix	12.0 µl
	DNA-Sample*	8.0 µl
	Total volume	**20.0 µl**

*8 µl sample can be used. If a volume other than 8 µl is used, the volume must be adjusted accordingly with nuclease-free water.

→ **If DNA IPC Target was not added during extraction:** Freshly dilute the DNA IPC Target (orange cap) 1:100 with nuclease-free water and add 1 µl per sample directly to the master mix. In this case, the IPC is used for quality control of the real-time PCR.
Caution: The use of more than 1 µl 1:100 diluted DNA IPC Target per reaction causes inhibition of the real-time PCR reaction.

		Per sample
Preparation of Master Mix (mix well)	DNA Reaction Mix	10.0 µl
	Monkeypox Virus Assay Mix	1.0 µl
	Internal Positive Control Assay Mix	1.0 µl
	(optional: IPC Target 1:100)	1.0 µl
	Total volume Master Mix	**13.0 µl**
Preparation of PCR	Master Mix	13.0 µl
	DNA-Sample*	7.0 µl
	Total volume	**20.0 µl**

*7 µl sample can be used. If a volume other than 7 µl is used, the volume must be adjusted accordingly with nuclease-free water.

- Prepare the Master Mix according to the number of samples, calculate an additional volume of approx. 10% to account for pipetting loss.
- Pipette 12 µl or 13 µl (if IPC target is added to the master mix) of the prepared Master Mix per sample into the well of the optical reaction plate.
- Then add 8 µl or 7 µl (if IPC target is added to the master mix) of the extracted sample or controls. Pipette the positive control at last.
- Seal the plate with a suitable optical sealing material.
- Vortex the sealed plate for 1-2 seconds and briefly centrifuge the plate.

10.2. Programming of temperature profile

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 have to be calibrated with the corresponding dye before performing a multiplex-PCR.

Select detection channel: FAM-TAMRA, 530 nm (for Monkeypox virus)
Cy5-NONE, 667 nm (for DNA IPC)
Passive reference dye, if needed (depends on device): ROX (e.g., ABI® 7500, QuantStudio™ 3/5/6/7)
Sample Volume: 20 µl

Temperature Profile:

Program 1 Cycles: 1 Analysis: None	Program 2 Cycles: 1 Analysis: None	Program 3 Cycles: 45 Analysis: Quantification Acquisition at 60 °C
50 °C ***2 min**	95 °C #2 min	95 °C 5 sec 60 °C 1 min

For ABI® 7500, QuantStudio™ 3/5/6/7:
Ramp speed: "Standard", without "fast cycling"

For LightCycler® 480 instrument:
Detection format: 2 Color Hydrolysis Probe

#The previous temperature profile with 20 sec in program 2 can still be used.

***Note:** If viral RNA should be also detected in the same PCR run, program 1 has to be prolonged to 15 min at 50 °C. This temperature profile can be used for all ingenetix ViroReal®, BactoReal®, MycoReal®, PanReal, and ParoReal for the detection of DNA or RNA.

11. Interpretation of PCR-data

For the analysis of the PCR results, select the fluorescence display options FAM channel for the pathogen target and Cy5 channel for the DNA IPC Target in the logarithmic analysis. Please note that some PCR platforms require colour compensation when using a multiplex PCR with FAM and Cy5.
Samples with positive Cq-values (quantification cycle (Cq) = cycle threshold (Ct) = crossing point (Cp)) are considered positive.

IMPORTANT: Please, also check amplification curves, not only Cq-values. Samples should be inspected both in logarithmic (Roche instrument: Abs Quant/Fit Points) and linear scale view and compared with the negative control. Adjust the Threshold (noise band), if necessary. After you have saved the new settings, export the data. For the cobas z 480 Analyzer, export tables per dye.

Table 1 shows the criteria for valid positive and negative controls. Table 2 shows interpretation of data with samples.

11.1. Controls

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

	Cq FAM channel Monkeypox Virus Target	Cq Cy5 channel DNA IPC Target[1]	Interpretation	Action
Positive control	Positive	Negative	Valid	-
Extraction negative control	Negative	Positive	Valid	-
Negative control (optional)	Negative	Negative	Valid	-

[1] If the DNA IPC target was added directly to the master mix, all samples in the Cy5 channel must be positive.

Assessment of sample test results should be performed after the positive and negative controls have been examined and determined to be valid. If results of controls are not valid, no interpretation is possible.

11.2. Samples

Samples with positive Cq values are to be evaluated as positive, see Table 2.

Table 2 Interpretation of data with samples

	Cq FAM channel Monkeypox Virus Target	Cq Cy5 channel DNA IPC Target	Interpretation	Action
Sample	Negative	Positive[1]	Negative	-
Sample	Positive	Positive/Negative[2]	Positive	-
Sample	Negative	Negative	Invalid	see 12.5

[1)] A positive signal of the DNA IPC excludes a possible PCR inhibition. However, IPC Cq-values should show comparable results among samples. A shift of Cq- 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 DNA IPC.

In case of invalid data, analysis has to be repeated with the remaining or newly extracted DNA sample (see 12. Troubleshooting).

12. Troubleshooting

12.1. No signal in FAM channel and Cy5 channel with positive control and sample

- Incorrect programming of the temperature profile or incorrect setting of detection channels on the real-time PCR instrument.

→ Compare temperature profile and setting of detection channels with details specified with the protocol.

- Incorrect configuration of PCR reaction.
  → Check your work steps and repeat PCR, if necessary.
- The DNA IPC Target was added undiluted directly to the master mix. The PCR reaction is therefore inhibited.
  → Freshly dilute DNA IPC Target 1:100 and repeat PCR.
- No Positive Control was added.
  → Repeat PCR in case all samples are negative.
- For control of real-time PCR only, 1 µl of freshly 1:100 diluted DNA IPC Target has to be added to the master mix. If no DNA IPC Target was added to master mix:
  → Freshly dilute DNA IPC Target 1:100 and repeat PCR.
- For control of DNA extraction and real-time PCR inhibition, the undiluted DNA IPC Target must be added to the lysis buffer during extraction. If the DNA IPC target has been forgotten:
  → Repeat DNA extraction.

12.2. Signal in FAM channel with negative control

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

12.3. Signal in FAM channel with extraction negative control

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

12.4. IPC specific signal in Cy5 channel with negative control and positive control

- The DNA 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 DNA IPC Target
  → Make sure that workspace and instruments are decontaminated at regular intervals.

12.5. No virus specific signal in FAM channel and Cy5 channel with sample

- Incorrect assignment of detection channels in sample.
  → Please verify the correct assignment of detection channels.

  Incorrect PCR conditions
  → Check PCR conditions and repeat the PCR if necessary.
- The DNA may be degraded.
- If IPC Target was added during extraction:
  - Inhibition of PCR may have occurred.
  - DNA extraction was unsuccessful.
  - The IPC target was not added to the lysis buffer of the sample.
  - The extracted DNA was not added to the PCR-reaction.

  → No statement is possible. Make sure that you use a recommended method for DNA 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 DNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of nuclease-free water).

13. Specifications and performance evaluation

13.1. Test performance

Performance of ViroReal® Kit Monkeypox Virus with a Quantstudio™ 7 Instrument (Thermo Fisher Scientific) is shown in Figure 1.

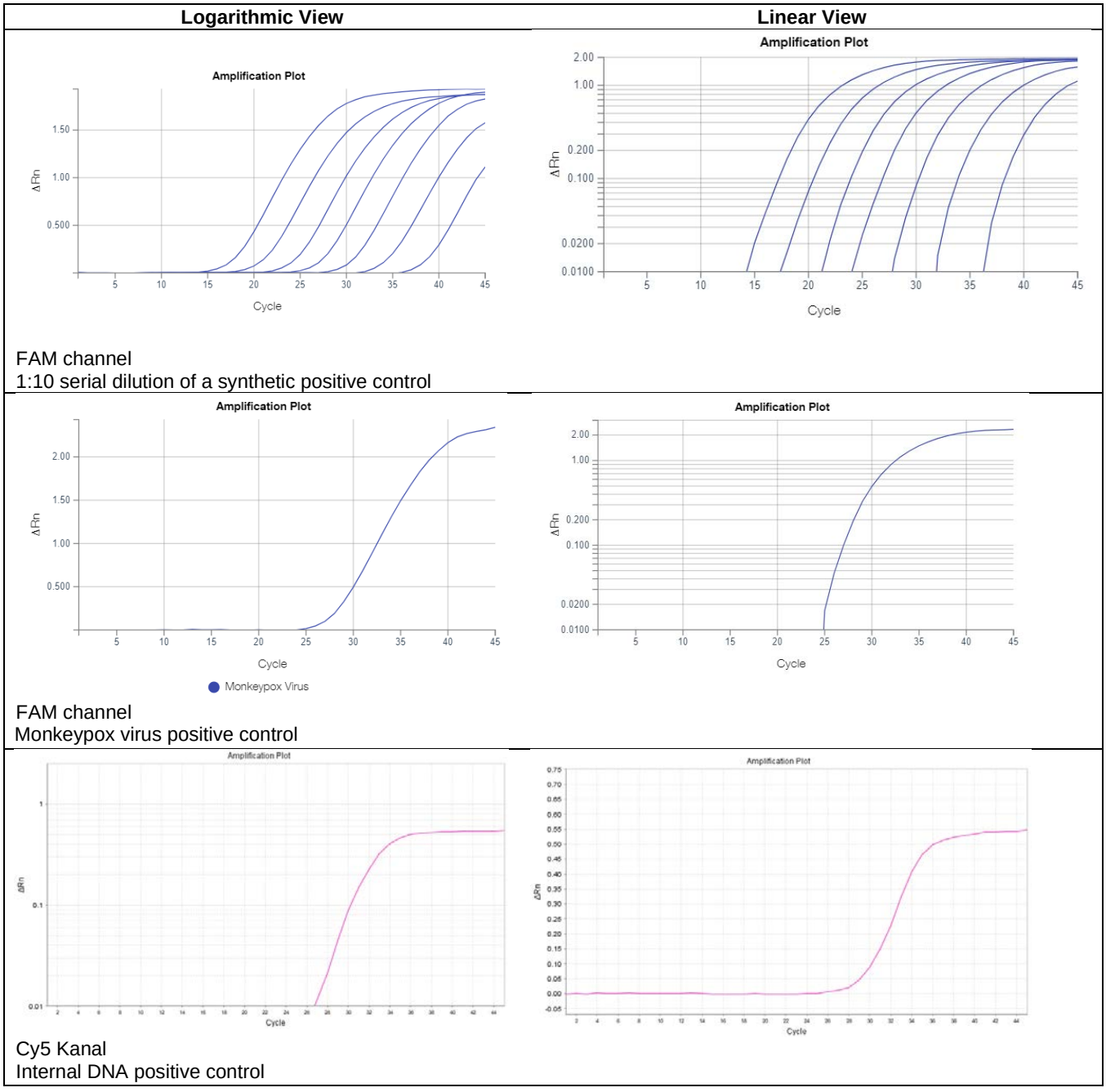

Figure 1 Performance of ViroReal® Kit Monkeypox Virus

13.2. Limit of detection, LoD

Method: ViroReal® Kit Monkeypox Virus was tested with a 10-fold dilution series of a synthetic oligonucleotide representing parts of the Monkeypox virus DNA.
The limit of detection (LoD95: number of target copies detected in 95% of cases) was determined by analysing serial dilutions of the oligonucleotide around the analytical sensitivity using a non-linear curve fitting with the GraphPad Prism Software.

Result: At least 1 target copy/reaction could be detected. The LoD95 is 2.2 target copies/reaction.

13.3. Linearity and dynamic range

Method: Linearity was determined using a 10-fold serial dilution ($6x10^6$ – $6x10^0$ target copies/reaction) of the plasmid. The number of determinations (n) per dilution was nine.

Result: The assay shows linearity over the range of 6 to 6,000,000 target copies/reaction with a slope of -3.36 ± 0.04 and a R^2 of 0.99 as shown in Figure 2.

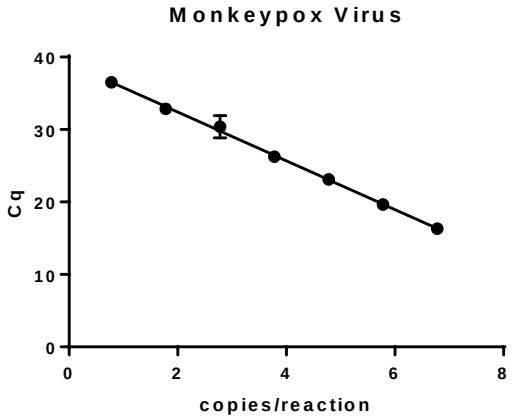

Figure 2 Ten-fold dilution series of a monkeypox virus synthetic DNA standard plotted against Cq

13.4. Precision

Method: Precision within a run (intra-assay), between multiple runs (inter-assay) and between two lots (inter-lot) was determined.

Result: The mean values of the coefficients of variation (CV%) are 0.97% for intra-assay precision, 1.18% for inter-assay precision and 1.08% for inter-lot precision.

13.5. Analytical specificity

Method BLAST analysis: The selection of highly specific primers and probes ensures analytical specificity. *In silico* validation of primers and probes was carried out with the basic local alignment tool (BLAST) against the NCBI database. Primers and probes have been checked for possible homologies to currently published sequences by sequence comparison analysis. This approach validates the detection of so far known Monkeypox virus strains.

Result: During BLAST analysis, no relevant sequence variability was observed in the primer and probe region of monkeypox virus trains.

Method testing exclusivity: There are no cross-reactions with other DNA viruses or with human DNA. Human genomic DNA, and DNA isolates of herpes simplex virus-1, human cytomegalovirus, human varicella zoster virus, human adenovirus type 5 and SV40 polyomavirus were all negative with ViroReal® Kit Monkeypox Virus.

15. Revision history

Revision	Date	Description

Technical support
ingenetix GmbH, Arsenalstr. 11,1030 Vienna, Austria
Telephone: +43 (0)1 36 198 01; **E-Mail:** office@ingenetix.com