AMA AlKhurmah Virus Multiplex qPCR Detection Kit

Product Manual

Version 4.0

This product is intended for research use only.

Contents

Notice to Laboratories

Since AlKhurmah virus is a relatively new virus, chances of mutation through random evolution are considerable. Therefore, two AlKhurmah virus-specific genomic regions are targeted by this kit, so that if any mutations occur in one of them the other serves as a backup; if such case occurs, please contact our support and/or sales team so that the assay targets are re-evaluated and updated accordingly. We have already designed and validated assays for a number of other conserved regions on the viral genome.

Also, since the closely related KFDV virus shares a high degree of homology to AlKhurmah virus, the third, non-specific target (ALKFDV) in this kit enables laboratories to track both viruses.

A. Introduction and Principles of the Test

This is a fourplex RT-qPCR based assay for detecting AlKhurmah virus. Two AlKhurmah-specific viral genomics regions are targeted (Propep and NS4), as well as a non-specific target (spanning NS1/NS2) that facilitates differentiation between AlKhurmah virus and Kyasanur Forest disease virus (KFDV) infections, which we named ALKFDV. The fourth target, the human β2M gene, serves as the internal control (IC) for extraction and PCR reaction conditions. Each sample is evaluated in one well in a 96-well format. So, a full 96 plate can run up to 94 samples (with the positive and negative controls) in nearly 1 hour.

Table1: *The four targets of the kit and their detection channels.*

Target	ALKFDV (NS1/NS2)	AlKhurmah1 (NS4)	AlKhurmah2 (Propep)	IC (β2M)
Detection Channel	FAM	ROX	Cy5	HEX

By targeting the abundantly expressed human β2M gene as the internal control instead of using a synthetic spike-in, our kit provides complete control of all steps of the test, starting from sample collection and transportation and ending with fluorescent detection. This also reduces RNA extraction turnaround time as well as enabling testing of older or archived samples, to which a synthetic IC was not added.

B. Intended Use

This kit is intended for detection of Alkhurma virus and Kyasanur Forest disease virus with extracted RNA as the starting material. The kit does *NOT* include reagents for nucleic acid extraction. See (Specimen Preparation) section for our recommended extraction kits.

It should be noted that this kit is intended for research use only. Please do not use it for human medical diagnostics.

C. Kit Contents and Storage

The kit comes in a package of four tubes, as indicated in the following table. All components must be stored at -20°C. The Oligo Mix should not be exposed to light for extended periods of time.

Table2: *Kit components.*

Component	Volume (uL) PD105-25 (25 reactions)	Volume (uL) PD105 (100 reactions)
One-Step RT-qPCR Master Mix (Green Cap)	250	1,000
AMA Oligo Mix (Purple Cap)	50	200
Positive Control (Red Cap)	50	100
Negative Control (Blue Cap)	50	100

D. Required Materials That Are Not Included in the Kit

- Real-time PCR thermal cycler (see next section: Compatible Instruments)
- PCR plates compatible with your real-time PCR thermal cycler
- Optical adhesive seal and an applicator to seal off the PCR plate OR optical 8-strip caps
- PCR plate microcentrifuge
- Pipettes and filtered pipette tips in the range of 1–1,000µL
- 1.5mL or 2.0mL RNase-free microcentrifuge tubes
- Microcentrifuge with 1.5mL/2.0mL rotor
- (highly recommended) Ice or a cooled PCR stand/rack

E. Compatible Instruments

Our kit is compatible with the following real-time PCR systems:

- QuantStudio™ 5 Real-Time PCR System (ABI, A28574)
- CFX96™ Real-Time PCR Detection System (Bio-Rad, 185-5096)
- CFX Opus 96 Real-Time PCR System (Bio-Rad, 12011319)
- LightCycler® 480 Instrument (Roche Diagnostics, 05015278001)

Please consult us about the compatibility of your thermal cycler by sending an email to support@havensci.com.

F. Good PCR Practices

Contamination is the worst enemy of PCR. Although the positive and negative controls in our kit can help in detecting contamination, dealing with contamination could prove troublesome and costly. Here, we list a few measures that could help you avoid contamination:

- Have physically separate workstation for PCR preparation, sample loading, and nucleic acid extraction. Each workstation should have its own dedicated pipettes and other small instruments, and should preferably be in a separate room.
- Always work under a cabinet (laminar flow for PCR preparation, and safety cabinet for nucleic acid extraction and sample loading).
- Use filtered tips. They are more expensive, but the small extra cost may save you valuable time, effort, and money down the line.
- If you need to analyze the PCR product after PCR cycling has finished, never unseal the plate near your PCR preparation or sample loading workstations. Even the smallest of aerosols could contain tens of millions of PCR templates.

G. Performance Evaluation

Sensitivity

As of September 2022, there are 23 and 78 published full genomic sequences for AlKhurmah virus and AlKhurmah+KFDV, respectively in the NCBI Virus database. Our oligos target all of these sequences with 100% complementarity. Furthermore, 2 independent laboratories used our kit to test >70 samples in total, which were previously diagnosed positive for AlKhurmah Virus. Our kit passed the internal validation parameters in both labs.

Specificity

Wet-lab testing of verified respiratory pathogenic samples purchased from ZeptoMetrix shows that our assay has no false positives in any of the organisms listed in Table3. Furthermore, in silico analysis reveals no cross reactivity with any flavivirus as well as a wide variety of other respiratory viruses (see Table4).

Table3: *List of pathogens that have no cross-reactivity with our kit as revealed wet-lab testing.*

Pathogen Name (strain)		
Adenovirus 3 (N/A)	Chikungunya (R80422)	Dengue Type1 (Hawaii)
Dengue Type2 (New Guinea C)	Dengue Type3 (H87)	Dengue Type4 (H241)
Hepatitis B (NATHBV-0003)	Hepatitis C (NATHCV-0003)	HIV-2 (NIH-Z)
Herpes Type 6A (isolate GS)	Herpes Type 7 (isolate SB)	Herpes Type1 (isolate 11)
Herpes Type2 (isolate 21)	Monkeypox (6C)	Zika (ERCM)

Table4: *List of pathogens that have no cross-reactivity with our kit as revealed by in silico analysis.*

Pathogen Name (NCBI TaxID)		
Flaviviridae (taxid:11050)	Enterovirus EV-A (taxid:138948)	Legionella pneumophila (taxid:446)
SARS-CoV-2 (taxid:2697049)	Enterovirus EV-B (taxid:138949)	Mycobacterium tuberculosis complex (taxid:77643)
Human coronavirus OC43 (taxid:31631)	Enterovirus EV-C (taxid:138950)	Mycobacterium tuberculosis typus humanus (taxid:1773)
Human coronavirus HKU1 (taxid:290028)	Enterovirus EV-D (taxid:138951)	Streptococcus pneumoniae (taxid:1313)
Human coronavirus NL63 (taxid:277944)	Human enterovirus EV68 (taxid:42789)	Streptococcus pyogenes (taxid:1314)
SARS coronavirus (taxid:694009)	Human respiratory syncytial virus (taxid:11250)	Bordetella pertussis (taxid:520)
MERS coronavirus (taxid:1335626)	Human rhinovirus A (taxid:147711)	Bordetella pertussis 18323 (taxid:568706)
Human adenovirus 1 (taxid:10533)	Human rhinovirus B (taxid:147712)	Mycoplasma pneumoniae (taxid:2104)
Human adenovirus 7 (taxid:10519)	Human rhinovirus C (taxid:463676)	Pneumocystis jirovecii (taxid:42068)
Human parainfluenza virus 1 (taxid:12730)	Human rhinovirus C1 (taxid:1219416)	Pneumocystis jirovecii RU7 (taxid:1408657)
Human parainfluenza virus 2 (taxid:1979160)	Human rhinovirus B14 (taxid:12131)	Candida albicans (taxid:5476)
Human parainfluenza virus 3 (taxid:11216)	Human rhinovirus B3 (taxid:44130)	Pseudomonas aeruginosa (taxid:287)
Human parainfluenza virus 4 (taxid:11203)	Human rhinovirus A1 (taxid:573824)	Pseudomonas aeruginosa group (taxid:136841)
Influenza A virus (taxid:11320)	Human rhinovirus NAT001 (taxid:992230)	Staphylococcus epidermis (taxid:1282)
Influenza B virus (taxid:11520)	Chlamydia pneumoniae (taxid:83558)	Streptococcus salivarius (taxid:1304)
Influenza A virus (A/PR 8/34 (H1N1)) (taxid:211044)	Haemophilus influenzae (taxid:727)	Human metapneumovirus (taxid:162145)

Precision

A clinically positive sample of each pathogen and a clinically negative sample were run 10 different times using our kit. The samples were called correctly 100% of the time.

Assay efficiency

A synthetic DNA construct containing each of the 3 assay genomic targets was adjusted for a final concentration of 1 million copies/µL. This construct was serially diluted down to 10 copies/µL and the serial dilutions were run with our assay in triplicates to assess efficiency and linearity (Figure1). All targets had a PCR efficiency of 96-97% with Pearson's Coefficient of Variance (R^2) of ≥0.996 (Table5).

Table5: *Pearson's Coefficient of Variance (R^2) and PCR efficiency of our multiplex assay with serial dilutions from 10^6 down to 10^1 viral equivalents per microliter.*

Component	FAM (ALKFDV)	ROX (AlKhurmah1)	Cy5 (AlKhurmah2)
R^2	0.999	0.997	0.996
PCR Efficiency	96.7%	97.3%	96.2%

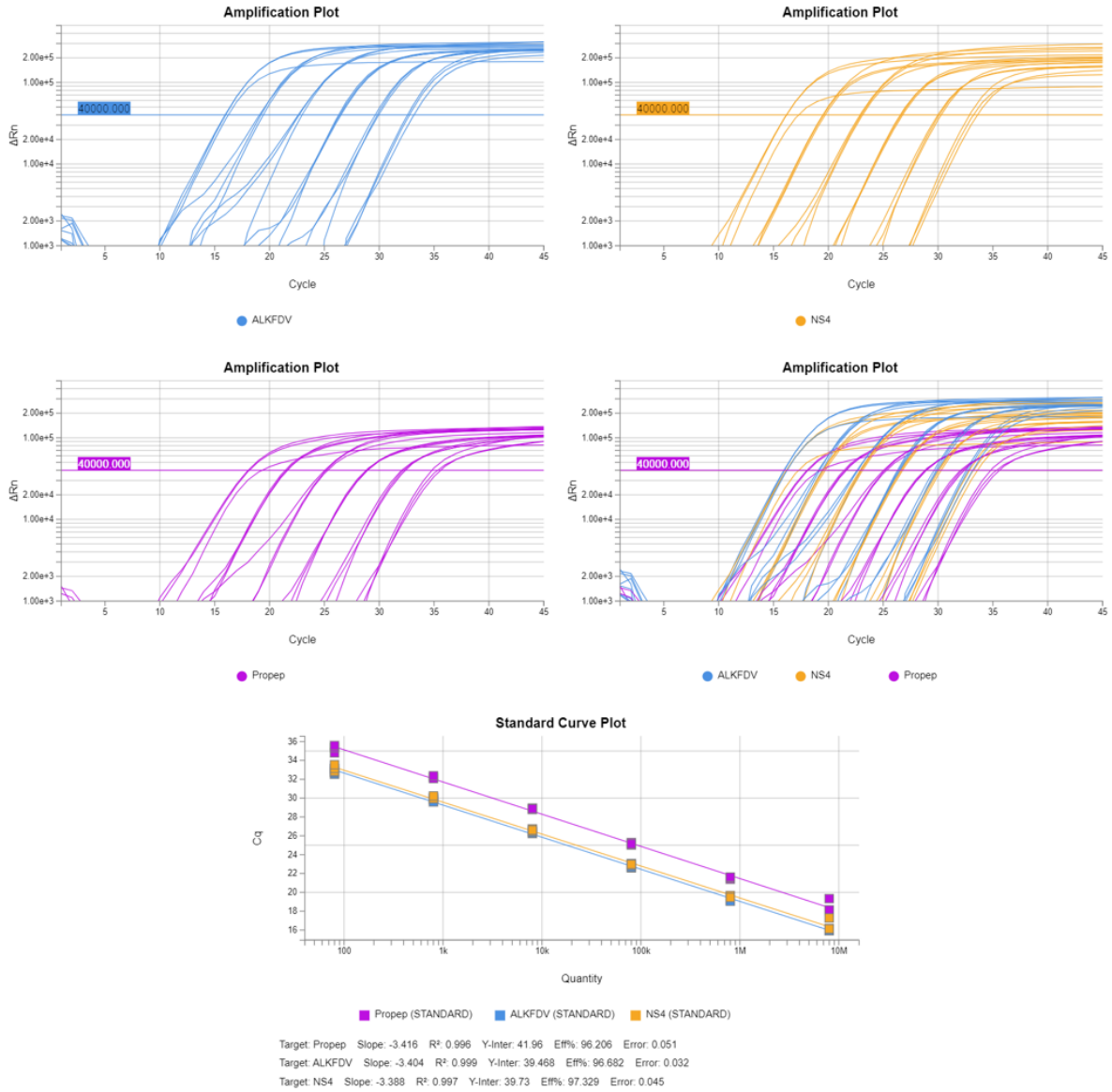

Figure1: *Serially diluted sample containing from 1 million viral equivalent of each of the 3 assay targets per microliter down to 10 copies per microliter, tested by our multiplex. Upper left panel shows the amplification curves for the FAM (ALKFDV) channel selected alone, while the upper right panel shows the ROX (NS4) channel. The middle left and middle right panels show the Cy5 (propep) and all channels selected together, respectively. Lower panel shows the standard curves for all 3 channels.*

H. Specimen Preparation

Make sure to have the extracted RNA/total nucleic acid ready before proceeding to PCR preparation in the next section. We recommend using the following extraction kits depending on the extraction technology used:

Extraction Technology	Extraction Kit
Silica columns	Viral TNAbler, Cat. No. TE98050
Magnetic beads	Mag Viral TNAbler, Cat. No. TE99032 (Optimized on our MagEX 32 automated extraction system, Cat. No. NE032)

I. Protocol

1. Extract RNA or total nucleic acids from each sample using one of our recommended kits in section H of this manual (Specimen Preparation, page 14). Nucleic acid should be used immediately after extraction (within 30 minutes) or stored for up to a week at -20°C if stored immediately after extraction.

2. Thaw all components at room temperature then place them on ice. Mix each tube by gentle flicking followed by a quick spin down to collect the tube's component to the bottom of the tube. ***Do NOT exceed 3 freeze-thaw cycles!***

3. Prepare a reaction master mix by mixing the following components (5% overage should be included to account for pipetting errors):

    a. *10 µL of One-Step RT-qPCR Master Mix / reaction*
    b. *2 µL of AMA Oligo mix / reaction*

Sample calculation

Component	Per 1 reaction	X10 reactions (+5% overage)
One-Step RT-qPCR Master Mix	*10 µL*	*105 µL*
AMA oligo mix	*2 µL*	*21 µL*

NOTE: Don't forget to figure in the positive and negative samples in your calculations!

4. Transfer 12 µL of the reaction master mix (prepared in step3) into each designated well on a 96-well optical qPCR plate

5. Add 8 µL of each sample's extracted nucleic acid to its designated well; add 8 µL of the positive and negative controls to their designated wells

6. Seal the plate with optical cap strips or an optical adhesive seal. Spin with a plate centrifuge.

7. On the Real-Time PCR thermal cycler software, define the detection filters as follows:

Target	ALKFDV	AlKhurmah1	AlKhurmah2	IC
Detection Channel	FAM	ROX	Cy5	HEX

8. On the software, define your Positive and Negative Controls.

9. Run the Real-Time PCR thermal cycler for 45 cycles as follows:

Step1:	*2 minutes - 25°C (the UNG step)*
Step2:	*15 minutes - 50°C (the RT step)*
Step3:	*2 minutes - 95°C (initial polymerase activation)*
Step4:	*10 seconds - 95°C (denaturation)*
Step5:	*30 seconds - 60°C (annealing/extension)* → ***DATA COLLECTION***
Step6:	*go to step4 (X39 times)*

10. Use the next section (Data Interpretation) to call the samples positive or negative

J. Data Interpretation

Follow the 3 steps below sequentially to interpret the qPCR results:

1. Adjustment of software settings
2. Checking qPCR run validity
3. Sample calling

Adjustment of software settings

Adjust the thermal cycler's analysis software settings as follows:

- Quantstudio5:
  - Open the run file using the "Design and Analysis 2" software
  - Go to the "Quality Check" tab on the top of the screen, press on the "Actions" button, select "Primary Analysis Settings…"
  - Stay on the first tab named "General", and, on the "Default Settings" row, uncheck the box under the "Auto Threshold" column, a number should appear under the "Threshold" column, set it at 40,000
  - On the "Default Settings" row, uncheck the box under the "Auto Baseline" column, a number should appear under the "Baseline start" and "Baseline end" columns, set them at 3 and 12, respectively
  - Press the "Save" button on the bottom right, the "Quality Check" tab will reappear, press the "Analyze" button on the top right to apply the new settings
  - Look for qPCR false amplification curves (artifacts) and omit them from analysis; qPCR amplification curves follow a sigmoidal shape (exponential fluorescence increase, Figure2) whereas qPCR artifacts have a linear amplification shape (Figure3)

- CFX Opus96:
  - Open the run file using the "Bio-Rad CFX Maestro 2.3" software, a screen will appear and the "Quantification" tab on top will be selected
  - Just above the well selector, you will find the amplification channels (FAM, HEX, etc.), put a checkmark only on the FAM channel
  - Press the "Settings" button then select "Baseline Threshold…"
  - Under the "Baseline Cycles" option, select "User Defined", select the entire table, set the "Begin" option to 3 and hit Enter, set the "End" option to 12 and hit Enter
  - Under "Single Threshold", select "User Defined", set the value to 150, press OK
  - Repeat the same steps with all other channels
  - Look for qPCR false amplification curves (artifacts) and omit them from analysis; qPCR amplification curves follow a sigmoidal shape (exponential fluorescence increase, Figure2) whereas qPCR artifacts have a linear amplification shape (Figure3)

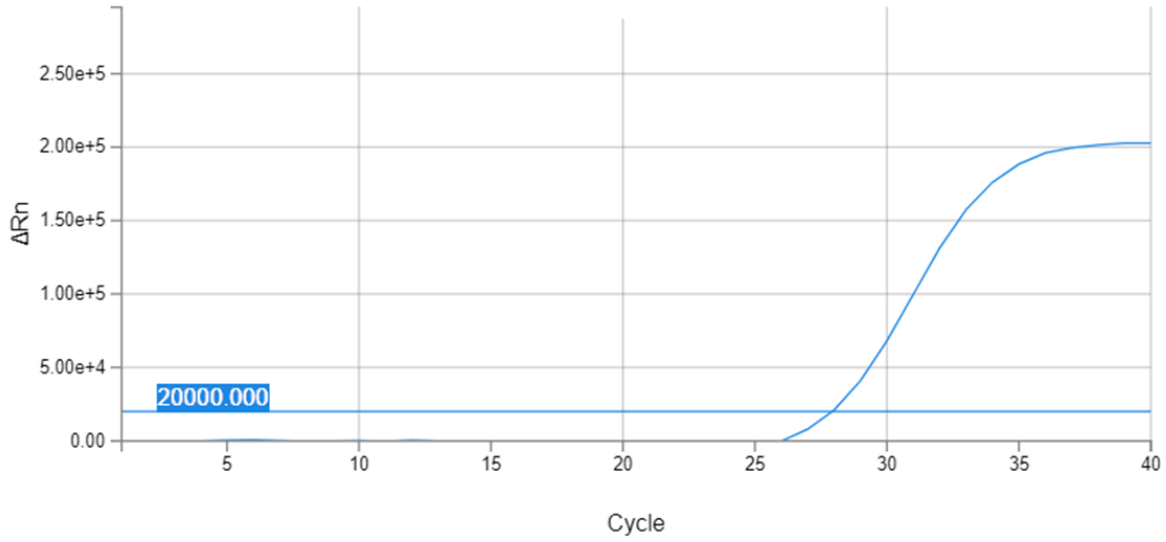

Figure2: *The typical "good" qPCR amplification plot has a sigmoidal shape, in which the fluorescence increases exponentially each subsequent cycle.*

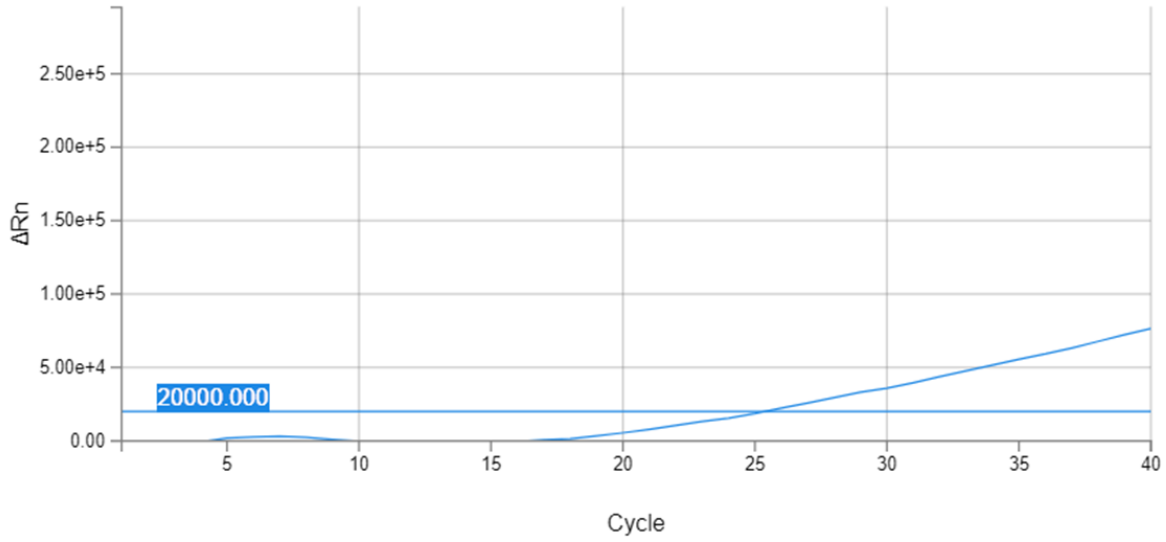

Figure3: *The non-typical "bad" qPCR amplification plot has a linear shape. This is a mere qPCR artifact, not an actual amplification, and it should be omitted from analysis.*

Checking qPCR run validity

Before sample calling, the run must be validated first using the positive and negative controls. For the run to be valid, the Negative Control must return a negative infection result, whereas the Positive Control must return a positive infection result with all targets, including IC, detected. If not, the whole run is considered invalid.

Sample calling

Our assay merely provides Ct values, and interpretation of these values should be in accordance with the international standards. Here, we summarize our recommendations for sample calling in Table6.

It is important to note that acceptable cycle threshold of detection is Ct ≤ 40, which means that Ct > 40 should be neglected.

Table6: *A guide for interpretation of the assay results. A (+) sign indicates a Ct ≤ 40 whereas a (–) sign indicates Ct>40 or no detection*

	ALKFDV (FAM)	ALKHRMH1 (ROX)	ALKHRMH2 (CY5)	IC (HEX)	INTERPRETATION
CASE1	+	+	+	+ or –	**AlKhurmah infection**
CASE 2	–	+	+	+ or –	**AlKhurmah infection**
CASE 3	+	–	+	+ or –	**AlKhurmah infection**
CASE 4	+	+	–	+ or –	**AlKhurmah infection**
CASE 5	–	–	+	+	**AlKhurmah infection**
CASE 6	–	+	–	+	**AlKhurmah infection**
CASE 7	+	–	–	+	**KFDV infection**
CASE 8	–	–	+	–	**Presumptive AlKhurmah, retest**
CASE 9	–	+	–	–	**Presumptive AlKhurmah, retest**
CASE 10	+	–	–	–	**Presumptive KFDV, retest**
CASE 11	–	–	–	+	**No infection**
CASE 12	–	–	–	–	**Sample degraded**

K. Support

For questions, suggestions, or technical support, feel free to contact us by email on:

support@havensci.com

L. Quality Control

Each lot is tested against predetermined parameters to ensure consistent performance between all lots.