



AMA MERS-CoV Multiplex qPCR Detection Assay

Product Manual

Version 1.1

This product is intended for investigation use only.

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A. Introduction and Principles of the Test

This is a fourplex RT-qPCR based assay for detecting the MERS-CoV virus. Three highly conserved viral genomics regions are targeted (nsp3, nsp8, and nsp14), plus the human β 2M gene as the internal control (IC). Each sample is evaluated in one well in a 96-well format. So, a full 96 plate can run up to 94 samples (with 1 positive and 1 negative controls) in a little over 1 hour.

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

Target	nsp8	nsp3	nsp14	IC (β 2M)
Detection Channel	FAM	ROX	Cy5	HEX

By targeting the abundantly expressed human β 2M gene to serve as the internal control, instead of using a synthetic RNA spike-in, our kit provides complete control of all steps of the test, starting from sample acquisition 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 MERS-CoV 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 investigation 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)	
	PD125-25 (25 reactions)	PD125 (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

- CFX Opus 96 Real-time PCR thermal cycler (see next section: Compatible Instruments)
- PCR plates compatible with the 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 validated with the following real-time PCR systems:

- QuantStudio™ 5 Real-Time PCR System (ABI, A28574)
 - The “Design and Analysis 2, Version 2.7.0” is needed
- CFX Opus 96 Real-Time PCR System (Bio-Rad, 12011319)

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 and 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. Each of the 3 MERS-CoV-specific targets in our assay had a positive result only in the MERS-CoV sample, and no false positives were observed in the other samples.

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

Pathogen Name (strain)		
Adenovirus 1 (N/A)	Adenovirus 3 (N/A)	Adenovirus 31 (N/A)
B. parapertussis (A747)	B. pertussis (A639)	C. pneumoniae (IOL-207)
Coronavirus 229E (N/A)	Coronavirus HKU-1 (Recombinant)	Coronavirus NL63 (N/A)
Coronavirus OC43 (N/A)	Influenza A H1N1pdm (A/NY/02/09)	Influenza AH1 (A/New Caledonia/20/99)
influenza AH3 (A/Brisbane/10/07)	Influenza B (B/Florida/02/06)	M. pneumoniae (M129)
Metapneumovirus 8 (Peru6-2003)	Parainfluenza 1 (N/A)	Parainfluenza 2 (N/A)
Parainfluenza 3 (N/A)	Parainfluenza 4 (N/A)	Rhinovirus 1A (N/A)
RSV A (N/A)	RSV B (CH93(18)-18)	SARS-CoV-2 (USA-WA1/2020)
parapertussis (A747)	parapertussis (A747)	

Furthermore, in-silico analysis shows that our assay has no cross-reactivity with any of the organisms listed in table4 below.

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)
Human coronavirus OC43 (taxid:31631)	Enterovirus EV-B (taxid:138949)	Streptococcus pneumoniae (taxid:1313)
Human coronavirus HKU1 (taxid:290028)	Enterovirus EV-C (taxid:138950)	Streptococcus pyogenes (taxid:1314)
Human coronavirus NL63 (taxid:277944)	Enterovirus EV-D (taxid:138951)	Bordetella pertussis (taxid:520)
SARS coronavirus (taxid:694009)	Human enterovirus EV68 (taxid:42789)	Bordetella pertussis 18323 (taxid:568706)
Human adenovirus 1 (taxid:10533)	Human respiratory syncytial virus (taxid:11250)	Mycoplasma pneumoniae (taxid:2104)
Human adenovirus 7 (taxid:10519)	Human rhinovirus C1 (taxid:1219416)	Pneumocystis jirovecii (taxid:42068)
Human parainfluenza virus 1 (taxid:12730)	Human rhinovirus B14 (taxid:12131)	Pneumocystis jirovecii RU7 (taxid:1408657)
Human parainfluenza virus 2 (taxid:1979160)	Human rhinovirus B3 (taxid:44130)	Candida albicans (taxid:5476)
Human parainfluenza virus 3 (taxid:11216)	Human rhinovirus A1 (taxid:573824)	Pseudomonas aeruginosa (taxid:287)
Human parainfluenza virus 4 (taxid:11203)	Human rhinovirus NAT001 (taxid:992230)	Pseudomonas aeruginosa group (taxid:136841)
Human rhinovirus A (taxid:147711)	Chlamydia pneumoniae (taxid:83558)	Staphylococcus epidermis (taxid:1282)
Human rhinovirus B (taxid:147712)	Haemophilus influenzae (taxid:727)	Streptococcus salivarius (taxid:1304)
Human rhinovirus C (taxid:463676)	Mycobacterium tuberculosis complex (taxid:77643)	Human metapneumovirus (taxid:162145)

Precision

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

Limit of detection (LoD)

We made a synthetic DNA construct containing the 3 targeted genome segments of MERS-CoV. Then a standard curve was constructed from which we quantified the MERS-CoV viral RNA in the sample purchased from Zeptomatrix. The viral RNA was diluted down to 10 or 5 copies per reaction. All targets of our assay were able to detect all 21 out of 21 replicates at 10 copies per reaction (table5).

Table5: Detection of MERS-CoV RNA at 10 or 5 viral copies per reaction by our assay. Each viral dilution was run in 21 replicates at each viral concentration.

Component	nsp8	nsp3	Nsp1
10 copies/reaction	21 out 21	21 out 21	21 out 21
5 copies/reaction	21 out 21	21 out 21	18 out 21

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 12). 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 μ L of One-Step RT-qPCR Master Mix / reaction
 - b. 2 μ L of AMA Oligo mix / reaction

Sample calculation

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

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

4. Transfer 12 μ L of the reaction master mix (prepared in step3) into each designated well on a 96-well optical qPCR plate
5. Add 8 μ L of each sample's extracted nucleic acid to its designated well; add 8 μ L 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	nsp8	nsp3	Nsp1	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 40 cycles as follows:

<i>Step1:</i>	<i>2 minutes - 25°C (UNG activation)</i>
<i>Step2:</i>	<i>15 minutes - 50°C (the RT step)</i>
<i>Step3:</i>	<i>2 minutes - 95°C (initial polymerase activation)</i>
<i>Step4:</i>	<i>10 seconds - 95°C (denaturation)</i>
<i>Step5:</i>	<i>30 seconds - 58°C (annealing/extension) → DATA COLLECTION</i>
<i>Step6:</i>	<i>go to step4 (X39 times)</i>
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, figure1) whereas qPCR artifacts have a linear amplification shape (figure2)
- 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 automatically selected
 - Just above the well selector, you will find the amplification channels (FAM, HEX, ROX, 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 7 and hit Enter, set the "End" option to 15 and hit Enter
 - Under "Single Threshold", select "User Defined", set the value to 150, press OK
 - Repeat the same steps with the other channels (HEX, ROX, etc.)
 - Look for qPCR false amplification curves (artifacts) and omit them from analysis; qPCR amplification curves follow a sigmoidal shape (exponential fluorescence increase, figure1) whereas qPCR artifacts have a linear amplification shape (figure2)

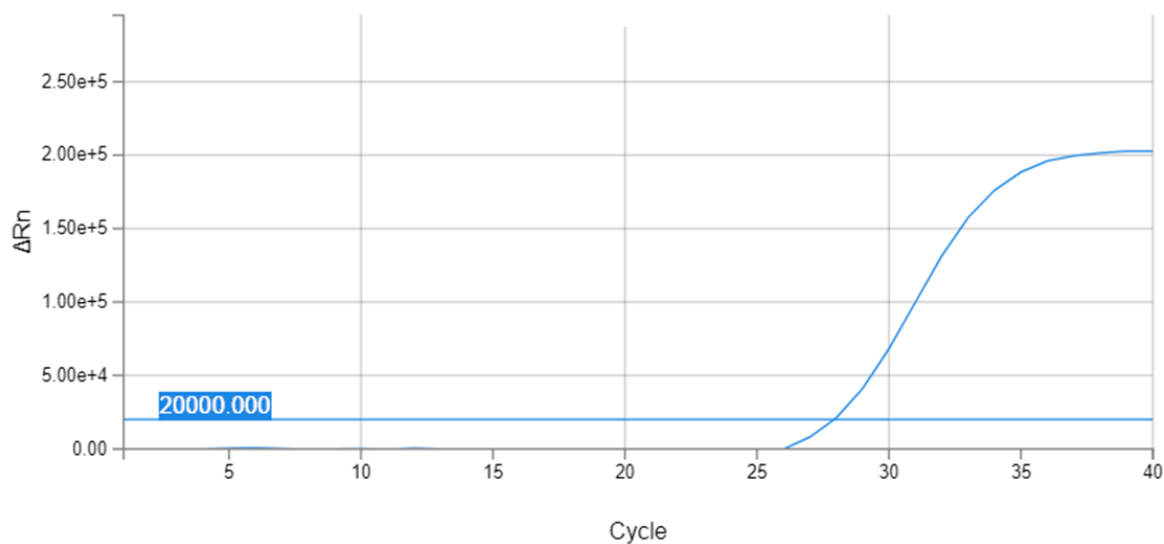


Figure1: The typical “good” qPCR amplification plot has a sigmoidal shape, in which the fluorescence increases exponentially each subsequent cycle.

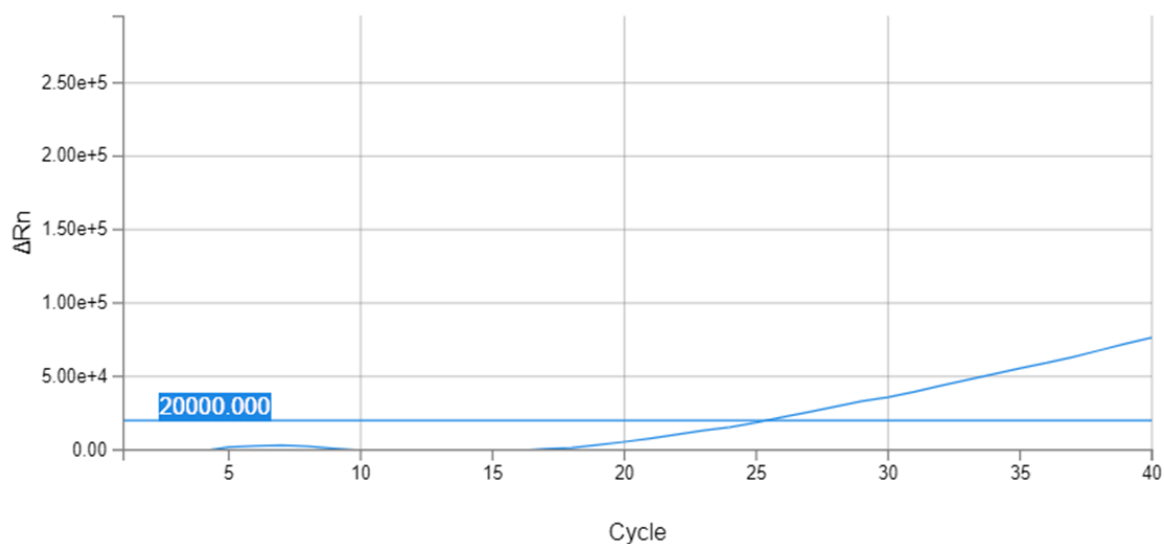


Figure2: The non-typical “bad” qPCR amplification plot has a linear shape. This is a mere qPCR artifact, not an actual amplification, and it should be omitted from analysis.

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 detected (see sample calling below). 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 $Ct \leq 40$, which means that $Ct > 40$ should be neglected.

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

	NSP8 (FAM)	NSP3 (ROX)	NSP14 (CY5)	IC (HEX)	INTERPRETATION
CASE1	+	+	+	+/-	MERS-CoV infection
CASE 2	+	+	–	+/-	MERS-CoV infection
CASE 3	+	–	+	+/-	MERS-CoV infection
CASE 4	–	+	+	+/-	MERS-CoV infection
CASE 5	+	–	–	+	MERS-CoV infection
CASE 6	–	+	–	+	MERS-CoV infection
CASE 7	–	–	+	+	MERS-CoV infection
CASE 8	+	–	–	–	Presumptive MERS-CoV infection, retest
CASE 9	–	+	–	–	Presumptive MERS-CoV infection, retest
CASE 10	–	–	+	–	Presumptive MERS-CoV infection, 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.