AMA SARS – Cov – 2 Multiplex qPCR Detection Assay

This product is intended for research use only (RUO)

Made in
SAUDI ARABIA
www.havensci.com

Table of content

Introduction and Principles of the Test

This is a fourplex RT-qPCR based assay for detecting the SAR-Cov-2 virus. Three viral genomics regions are targeted (M, exonuclease, and nsp6), plus the human β2M 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 less than 2 hours.

Target	M	Exo	Nsp6	IC (β2M)
Detection Channel	FAM	Cy5	Cy5.5	HEX

By targeting the abundantly expressed human β2M 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.

To minimize the chances of carryover contamination by the viral PCR products, especially for labs with high throughput SARS-CoV-2 testing, dT is replaced with dU in the reaction mix and a UNG enzyme is added, which ensures degradation of any PCR product previously amplified by this kit.

Kit Contents and Storage

The kit comes in a package of four tubes, as indicated in Table1. All components must be stored at -20°C. The One-Step RT-qPCR Master Mix and Oligo Mix should not be exposed to light for extended periods of time.

Table1: Kit components

Component	Volume (uL)
One-Step RT-qPCR Master Mix	1,000
AMA Oligo Mix	200
Positive Control	50
Negative Control	50

Sensitivity

Analysis of a total of 174 clinical samples using this kit was performed by two independent labs that routinely perform clinical PCR SARS-CoV-2 testing. The samples included clinically positive SARS-CoV-2 samples of high, medium, or low viral loads, as well as negative samples. The total concordance rate with their internally approved protocols was 97.1%.

Lab A analyzed 78 samples and found a concordance rate of 96%, and Lab B tested 96 samples and reported 98% concordance. Both Lab A and Lab B are certified by the Saudi Public Health Authority to perform diagnostic SARS-CoV-2 testing.

Specificity

Wet-lab testing of clinically positive and/or standardized samples (listed in Table2) showed no false positive results of SARS-CoV-2 by our kit. Furthermore, in-silico analysis shows that our kit has no cross-reactivity with any of the pathogens listed in Table3.

Table2: List of pathogens that have no cross-reactivity with our kit as revealed wet-lab testing.

Pathogen Name (strain)			
Adenovirus 1 (strain N/A)	Adenovirus 3 (strain N/A)	Adenovirus 31 (strain N/A)	Adenovirus 41 (TAK)
B. parapertussis (strain A747)	B. pertussis (strain A639)	C. pneumoniae (strain CWL-029)	Coronavirus 229E (strain N/A)
Coronavirus HKU-1 (recombinant strain)	Coronavirus NL63 (strain N/A)	Coronavirus OC43 (strain N/A)	Influenza A H1N1pdm (strain A/NY/02/09)
Influenza AH1 (strain A/New Caledonia/20/99)	Influenza AH3 (strain A/Brisbane/10/07)	Influenza B (strain B/Florida/02/06)	M. pneumoniae (strain M129)
Parainfluenza 1 (strain N/A)	Parainfluenza 2 (strain N/A)	Parainfluenza 3 (strain N/A)	Parainfluenza 4 (strain N/A)
Metapneumovirus 8 (strain Peru6-20033)	Rhinovirus 1A (strain N/A)	RSV A (strain N/A)	MERS-CoV (clinical sample)
Influenza A (clinical sample)	Influenza B (clinical sample)		

Table2: List of pathogens that have no cross-reactivity with our kit as revealed wet-lab testing.

Pathogen Name (NCBI TaxID)			
Human coronavirus 229E (taxid:11137)	Human parainfluenza virus 1 (taxid:12730)	Human rhinovirus A (taxid:147711)	Bordetella pertussis (taxid:520)
Human coronavirus OC43 (taxid:31631)	Human parainfluenza virus 2 (taxid:1979160)	Human rhinovirus B (taxid:147712)	Bordetella pertussis 18323 (taxid:568706)
Human coronavirus HKU1 (taxid:290028)	Human parainfluenza virus 3 (taxid:11216)	Human rhinovirus C (taxid:463676)	Pneumocystis jirovecii (taxid:42068)
Human coronavirus NL63 (taxid:277944)	Human parainfluenza virus 4 (taxid:11203)	Human rhinovirus C1 (taxid:1219416)	Pneumocystis jirovecii RU7 (taxid:1408657)
SARS coronavirus (taxid:694009)	Influenza A virus (taxid:11320)	Human rhinovirus B14 (taxid:12131)	Candida albicans (taxid:5476)
MERS coronavirus (taxid:1335626)	Influenza B virus (taxid:11520)	Human rhinovirus B3 (taxid:44130)	Pseudomonas aeruginosa (taxid:287)
Human adenovirus 1 (taxid:10533)	Influenza A virus (A/PR 8/34 (H1N1)) (taxid:211044)	Human rhinovirus A1 (taxid:573824)	Pseudomonas aeruginosa group (taxid:136841)
Human adenovirus 7 (taxid:10519)	Enterovirus EV-A (taxid:138948)	Human rhinovirus NAT001 (taxid:992230)	Staphylococcus epidermis (taxid:1282)
Human metapneumovirus (taxid:162145)	Enterovirus EV-B (taxid:138949)	Legionella pneumophila (taxid:446)	Streptococcus salivarius (taxid:1304)
Human respiratory syncytial virus (taxid:11250)	Enterovirus EV-C (taxid:138950)	Mycobacterium tuberculosis complex (taxid:77643)	Streptococcus pneumoniae (taxid:1313)
Chlamydia pneumoniae (taxid:83558)	Enterovirus EV-D (taxid:138951)	Mycobacterium tuberculosis typus humanus (taxid:1773)	Streptococcus pyogenes (taxid:1314)
Haemophilus influenzae (taxid:727)	Human enterovirus EV68 (taxid:42789)	Mycoplasma pneumoniae (taxid:2104)	

Precision

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

Limit of detection (LoD)

An RNA molecule that is identical to our PCR product of the M gene was synthesized by GeneLink (Elmsford NY, United States) and purified by PAGE. Purification by PAGE ensures that artefacts that are common in chemical RNA synthesis are removed. The synthetic-M RNA was used to construct a standard curve (from 1010 to 103 copies) from which the concentration of a clinically positive sample was determined (viral copies/µL). By serially diluting this sample, we determined that the limit of detection of our assay is ~33 copies per reaction.

Singleplex and multiplex PCR efficiency

Each assay of the 3 viral targets and the internal control was analyzed for PCR efficiency as a singleplex in five 10-fold dilutions, from 1 to 10,000 dilution factors of clinically positive SARS-CoV-2 sample (Figure1). All singleplexed assays displayed a linear curve of the plot of log(relative concentration) vs Cq value (R2 ≥ 0.99) and had efficiencies of 100±15% (Table4).

Similarly, to validate that multiplexing did not hinder the assay performance, the assays were analyzed for the same parameters after multiplexing (Figure2). After multiplexing, all assays maintained linearity (R2 ≥ 0.99) and had efficiencies of 100±15% (Table4).

Table4: Assay linearity (R2) and efficiency, as singleplexes and after multiplexing, as determined by testing against five 10-fold dilutions from 1 to 10,000 dilution factors of a clinically positive SARS-CoV-2 sample.

Assay		R2	Slope	PCR Efficiency
M	Singleplex	0.9994	-3.1872	105.95%
	Multiplex	0.9991	-3.1029	110.03%
Nsp6	Singleplex	0.9996	-3.0688	111.77%
	Multiplex	0.9873	-3.0708	111.66%
Exo	Singleplex	0.9993	-3.1291	108.73%
	Multiplex	0.9979	-3.024	114.14%
β2M	Singleplex	0.9987	-3.1312	108.63%
	Multiplex	0.9992	-3.098	110.28%

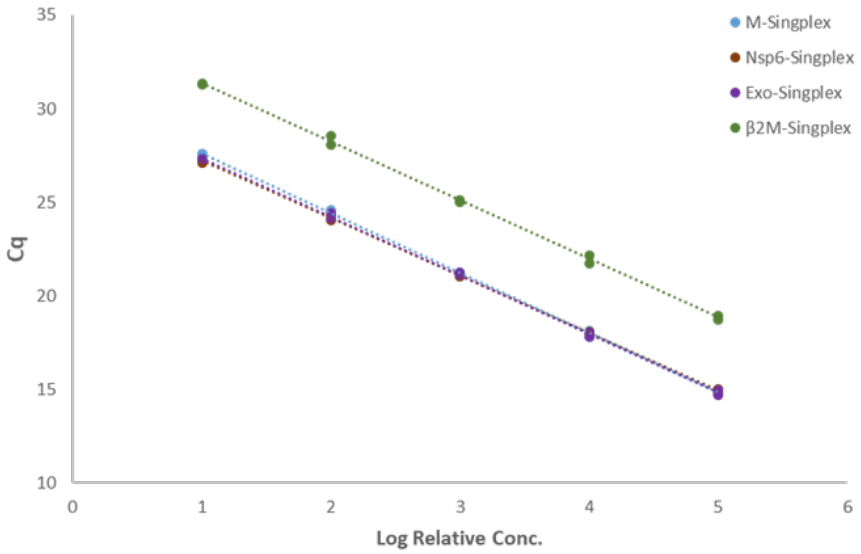

Figure1: Plots of Cq's of our 4 assays in singleplexes against the log of relative concentrations of five 10-fold dilutions of a positive clinical SARS-CoV-2 sample.

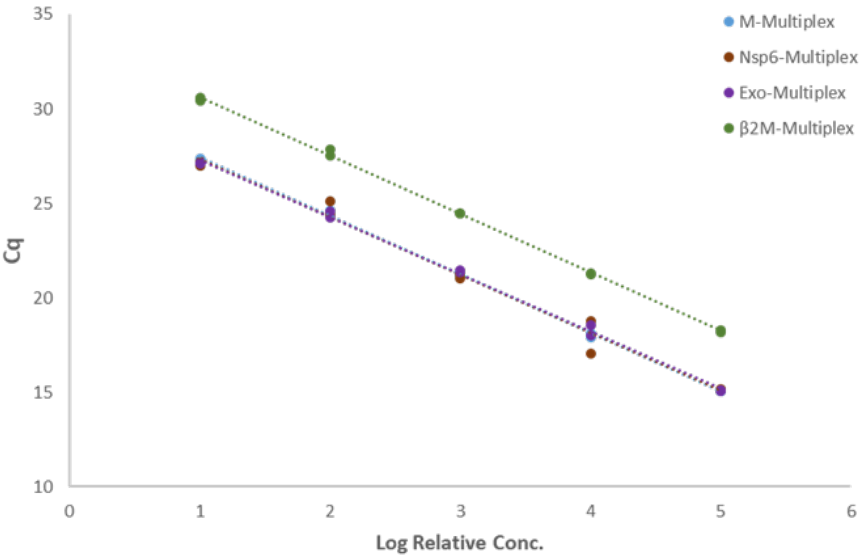

Figure2: Plots of Cq's of our 4 assays after multiplexing against the log of relative concentrations of five 10-fold dilutions of a positive clinical SARS-CoV-2 sample.

Singleplex vs multiplex Cq difference

Comparison of Cq of each assay as a singleplex and after multiplexing throughout the five 10-fold dilutions from 1 to 10,000 dilution factors of a clinically positive SARS-CoV-2 sample showed that multiplexing does not have a significant effect on the Cq (Figure3), as the absolute difference before and after multiplexing was < 0.8 cycles for all assays throughout the 1 to 10,000 dilutions (Table5).

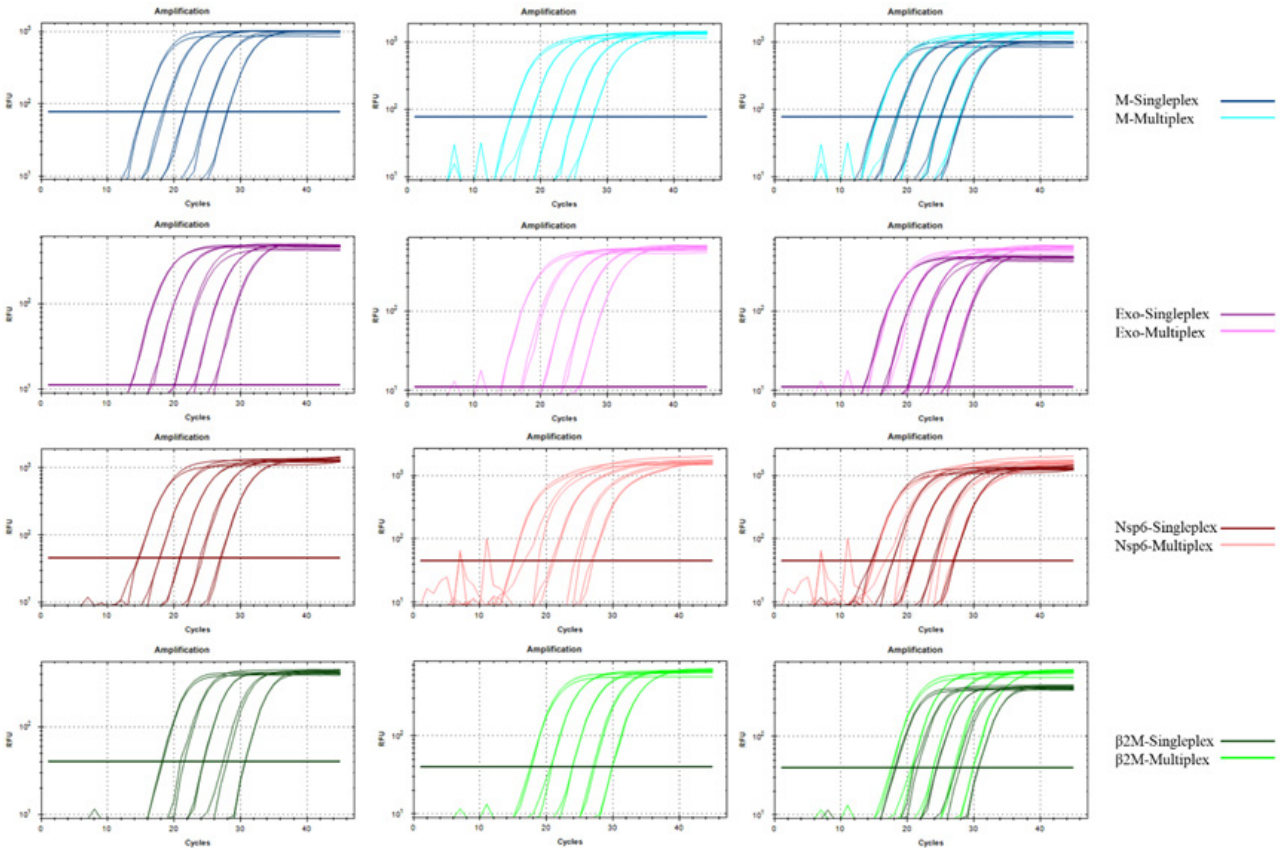

Figure3: Log-scaled amplification plots of the four assays (from top to bottom: M, Exo, Nsp6, β2M) for five 10-fold dilutions, from 1 to 10,000-fold dilutions, of a clinically positive SARS-CoV-2 sample. The left panel shows amplification of singleplexed assays, the middle panel shows amplification after multiplexing, and the right panel is on overlay of singleplex and multiplex amplification plots of each assay. The overlay shows nearly identical amplification plots of the singleplexed and multiplexed assays, indicating no decline in assay performance after multiplexing.

Table5: Cq of each assay before and after multiplexing throughout the five 10-fold dilutions from 1 to 10,000 dilution factors

Assay		Dilution Factor				
		1X	10X	100X	1,000X	10,000X
M	Cq (Singleplex)	14.86	17.98	21.18	24.50	27.54
	Cq (Multiplex)	15.07	18.02	21.23	24.54	27.33
	Cq difference	-0.21	-0.05	-0.05	-0.04	0.21
Nsp6	Cq (Singleplex)	14.91	18.05	21.08	24.19	27.18
	Cq (Multiplex)	15.16	17.93	21.14	24.75	27.11
	Cq difference	-0.25	0.11	-0.06	-0.55	0.08
Exo	Cq (Singleplex)	14.84	17.91	21.21	24.28	27.30
	Cq (Multiplex)	15.08	18.29	21.41	24.42	27.13
	Cq difference	-0.24	-0.37	-0.20	-0.14	0.16
β2M	Cq (Singleplex)	18.84	21.95	25.05	28.31	31.32
	Cq (Multiplex)	18.23	21.28	24.47	27.70	30.51
	Cq difference	0.61	0.67	0.58	0.60	0.81

Cq differences between multiplexed viral targets

Some other commercially available SARS-CoV-2 kits have 2-3 viral targets, but it was noticed that some of their targets are more sensitive than the other targets of the same kit, rendering the other targets somewhat dispensable. In our kit, however, analysis of Cq's of each assay in five 10-fold dilutions of a clinically positive SARS-CoV-2 displayed that, for any given dilution, assays of all of the 3 viral targets were within < 0.4 cycles among each other, whether singleplexed or multiplexed (Table6), indicating that all of the three targets are powerful detectors, and that they all work in tandem to detect the virus.

Table6: Difference of highest and lowest Cq among the three viral targets for each dilution before or after multiplexing. Highlighted in yellow are the highest and lowest Cq's of the viral targets in each given dilution.

		Dilution Factor				
Assay	Cq	1X	10X	100X	1,000X	10,000X
Singleplex	M	14.86	17.98	21.18	24.50	27.54
	Nsp6	14.91	18.05	21.08	24.19	27.18
	Exo	14.84	17.91	21.21	24.28	27.30
	Highest difference	0.07	0.14	0.13	0.31	0.36
Multiplex	M	15.07	18.02	21.23	24.54	27.33
	Nsp6	15.16	17.93	21.14	24.75	27.11
	Exo	15.08	18.29	21.41	24.42	27.13
	Highest difference	0.09	0.36	0.27	0.33	0.22

Nucleic Acid Extraction

This kit has been validated on nucleic acid extracted from nasopharyngeal swabs. Nucleic acid extraction should be performed according to the user's established method of extraction, and according to the standards of country's health authorities.

Protocol

1. Extract RNA or total nucleic acids from each sample, as in your laboratory's approved internal protocol. 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 the 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 the 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.

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

Target	M	Exo	Nsp6	IC
Detection Channel	FAM	Cy5	Cy5.5	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: 10 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 (X44 times)

10. Analyze the results according to your thermal cycler's software. Use your judgement to set/modify the threshold of detection for each filter and/or omit amplification artifacts (e.g. non-sigmoidal amplification curve).

Sample calling

Our assay merely provides Cq values, and interpretation of these values should be in accordance with the international standards. Here, we adopt the CDC guidelines for qPCR results interpretation and we briefly summarize them in Table7.

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

Table7: A guide for interpretation of the assay results. A (+) sign indicates a Cq ≤ 40 whereas a (–) sign indicates Cq>40 or no detection

	NSP6 (CY5.5)	EXO (CY5)	M (FAM)	B2M (HEX)	INTERPRETATION
CASE 1	+	+	+	+ or –	Positive infection
CASE 2	-	+	+	+ or –	Positive infection
CASE 3	+	-	+	+ or –	Positive infection
CASE 4	+	+	-	+ or –	Positive infection
CASE 5	+	-	-	+	Positive infection
CASE 6	-	+	-	+	Positive infection
CASE 7	-	-	+	+	Positive infection
CASE 8	+	-	-	-	Presumptive positive, retest
CASE 9	-	+	-	-	Presumptive positive, retest
CASE 10	-	-	+	-	Presumptive positive, retest
CASE 11	-	-	-	+	No infection
CASE 12	-	-	-	-	Sample degraded

Run validity

This assay contains positive and negative controls. The three targets and internal control must all be detected in the positive control; if not, the whole plate is considered invalid. Similarly, the negative control must show detection of the internal control alone; any other result invalidates the whole plate.

Support

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

Quality control

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

HavenSci

AMA SARS – Cov – 2 Multiplex qPCR Detection Assay

Product Manual (PD115)
Version 2.0
www.havensci.com