



MERS-CoV and H7N9 Influenza Assay Development on NGDX

**MANUEL CABALLERO, CIV
LA'QUITA ARMSTRONG-SPENRATH, CIV**

FINAL REPORT

July 2018

**59th Medical Wing
Office of the Chief Scientist
2520 Ladd Street, BLDG. 3885
JBSA Lackland AFB, TX 78236-7517**

DISTRIBUTION A. Approved for public release; distribution is unlimited.

DECLARATION OF INTEREST

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Air Force, Department of Defense, nor the U.S. Government. This work was funded by Project Code Number AC12EM01. Authors are military service members, employees, or contractors of the US Government. This work was prepared as part of their official duties. Title 17 USC §105 provides that 'copyright protection under this title is not available for any work of the US Government.' Title 17 USC §101 defines a US Government work as a work prepared by a military service member, employee, or contractor of the US Government as part of that person's official duties and that the authors do not endorse any products in this report

NOTICE AND SIGNATURE PAGE

Using Government drawings, specifications, or other data included in this document for any purpose other than Government procurement does not in any way obligate the U.S. Government. The fact that the Government formulated or supplied the drawings, specifications, or other data does not license the holder or any other person or corporation or convey any rights or permission to manufacture, use, or sell any patented invention that may relate to them.

Qualified requestors may obtain copies of this report from the Defense Technical Information Center (DTIC) (<http://www.dtic.mil>).

MERS-CoV AND H7N9 INFLUENZA ASSAY DEVELOPMENT ON NGDX



Jerry R. Spencer, DAF
Medical Modernization Senior Program Analyst
59th Medical Wing-Science & Technology



Carlton C. Brinkley, DAF
Dir, Diagnostics & Therapeutics Research
59th Medical Wing-Science & Technology

This report is published in the interest of scientific and technical information exchange, and its publication does not constitute the Government's approval or disapproval of its ideas or findings.

TABLE OF CONTENTS

Section	Page
1.0 ABSTRACT	6
2.0 INTRODUCTION	7
3.0 MATERIALS AND METHODS	10
3.1 Samples and Reagents	10
3.2 Biomeme two3 TM Instrument	10
3.3 MatrixSeq Kit Assays on ViiA7 Real-time PCR Machine	10
3.4 PCR Assays on the Biomeme two3 TM Instruments	10
3.5 Analysis of Biomeme Panels on ViiA7 Real-time PCR Machine	11
3.6 Biomeme two3 TM Real-time PCR Data	11
4.0 RESULTS	13
4.1 Detection of MERS-CoV N Gene with the BioMatrix MERS-CoV Panel	13
4.2 Detection of MERS-CoV <i>orf1a</i> and upE Targets with Biomeme two3 TM	16
4.3 Detection of Influenza A Virus H7N9 Using the Biomeme H7N9 Panel	23
4.4 Assessment of Specificities of the Biomeme MERS and H7N9 Panels	30
5.0 SUMMARY	33
6.0 REFERENCES	34

MERS-CoV and H7N9 Influenza Assay Development on NGDX

La'Quita Armstrong-Spenrath and Manuel Y. Caballero

Center for Advanced Molecular Detection, Chief Scientist's Office, Science and Technology,
59th Medical Wing, US Air Force, JBSA-Lackland, San Antonio, Texas 78236

Funds for this project were provided by Air Force Medical Support Agency

KEYWORDS

Real-time, polymerase chain reaction, Biomeme two3™, handheld, lightweight, portable

“The views expressed are those of the authors’ and do not reflect the official views or policy of the Department of Defense or its Components”

1.0 ABSTRACT

Deadly infectious diseases pose a prevalent danger to war fighters and warrior medics in remote, hostile areas. Infectious agents inevitably hinder the war fighters' duty performance, even potentially cause mission failures. Therefore a crucial military need is to acquire the capabilities to rapidly detect the threat agents, and to expeditiously devise strategies to counter the threats. The Biomeme two3™ (Biomeme, Inc., Philadelphia, PA) is a light (1.2 lb.), hand-held, field-deployable real-time polymerase chain reaction (PCR) device that could meet these needs. The device is coupled to an iPhone with unique software for data analysis and transmission to intended recipients. This work reports a comparative research testing and evaluation of this system, focusing on detection of the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and Influenza A virus H7N9 for this assessment. Three Biomeme two3™ instruments were purchased for this work. The reagents specifically designed and set in the appropriate format for Biomeme two3™ and the target templates were also purchased from the manufacturer of the instrument. For MERS-CoV, the detection targets were an *orf1a* segment and a segment upstream of gene E (termed upE). For H7N9, the target amplicons were in the H7 and N9 genes. The instrument performance was evaluated for template copy numbers that varied from 50 to 500,000 per reaction.

Our results show that Biomeme two3™ can detect the tested targets at various copy numbers, down to 50 copies per reaction. We also tested the MERS-CoV detection reagents for their capacity to amplify the corresponding genomic segments from two nontarget coronaviruses, OC43 and 229E. These tests yielded no amplicons. Likewise, we tested the H7N9 detection reagents to see whether they would amplify the corresponding gene segments from the nontarget influenza A virus H1N1. These PCR reactions also did not produce any amplicons. Together these results show the specificity of the reagents for detection of MERS-CoV and H7N9 by real-time PCR performed on Biomeme two3™.

For comparison we also employed benchtop real-time PCR instruments. Overall, our findings suggest that the Biomeme two3™ system performance in detecting the targets was similar to those of the benchtop instruments.

2.0 INTRODUCTION

Periodic emergence of highly virulent new and known pathogens poses serious threats to public health. Such pathogens are endemic to certain parts of the World, where they emerge periodically, yet unpredictably. The United States deploys its military personnel in many parts of the World, including those where the periodic deadly outbreaks occur. During these deployments, whether for wartime operations or peacetime efforts, the US military personnel are always at risk of exposure to the deadly disease agents. Given that healthy, effectively functioning forces are vital to the success of any mission, it is crucial that the forces have clear and total situational awareness of the active or likely outbreak areas. To that end, it is crucial that warrior medics have the operational capabilities to expeditiously identify the deadly infectious agents. Therefore, any device and approach that would permit speedy, onsite identification of these organisms would be invaluable to the US Air Force warrior medics. Clearly, in the absence of such preparedness, the exposure of forces to deadly agents could severely hamper missions, and in some cases could altogether cause them to fail.

One example of a deadly pathogen with very high mortality rate is the Ebolavirus, which causes periodic outbreaks largely in the Central and West African countries, but cases in other nations have been reported as well (4, 16). Another highly virulent pathogen is the Zika virus, which has wreaked havoc in outbreak areas of Africa and South America, but cases have been reported in other regions as well (5, 17).

A relatively recent example of a new, deadly infectious agent was the Middle East respiratory syndrome coronavirus (MERS-CoV). The first case of what came to be called the Middle East Respiratory Syndrome was reported in 2012 in Saudi Arabia in a 60 year old man. The causative agent was identified to be a coronavirus, and upon identification it was designated human coronavirus-EMC (hCoV-EMC), after Erasmus Medical Center (EMC), Rotterdam, The Netherlands, where the virus from the Saudi man was identified (18). Soon afterward, another case was identified in a Qatari man who had visited Saudi Arabia, which suggested he contracted the virus while visiting that country. The virus was later identified to be a coronavirus related to hCoV-EMC (1). As more cases of the Syndrome emerged, more viruses were identified and classified (6, 7, and 13). The designation for this virus evolved, and the term MERS-CoV became the accepted norm (9).

From the time of its first emergence, MERS-CoV spread rapidly. Many more cases were reported in the Middle East, an area where the United States routinely deploys military personnel. Cases from other parts of the World were also reported. The World Health Organization (WHO) in its April 2018 assessment revealed that 27 countries had reported a total of 2,206 confirmed cases of MERS-CoV, of which 787 were fatal; a fatality rate of nearly 36 %. Of the Worldwide total, Saudi Arabia had the most; 1,831 confirmed cases, with 713 deaths, translating into a fatality rate of about 39 % (2, 14). MERS-CoV was found to be related to the

Severe Acute Respiratory Syndrome (SARS) virus (1, 18), another coronavirus that had suddenly emerged in China in 2002, and that showed a high mortality rate (8, 11). SARS coronavirus and MERS coronavirus are classified into the same genus, *Betacoronavirus* (9).

Another deadly virus that recently emerged is the Asian influenza A virus H7N9, which first emerged in China in 2013 (10). Poultry in China is the reservoir for H7N9, and thus transmission is from poultry to humans; however, rare human to human transmissions have been reported. Most all cases reported have been in china, but a few were also reported in Hong Kong and Macao in individuals who had traveled to China. Since its emergence in 2013, the H7N9 outbreaks have been in China, a nation currently in its sixth outbreak of the H7N9 flu. In its 2 March 2018 update, WHO reported that since the first case of H7N9 in 2013 in China, 1,567 cases had been reported, of which 615 were fatal, a mortality rate of 39 % (15). In the United States so far, the Asian H7N9 virus has not been detected in people or birds. The CDC risk assessment with Influenza Risk Assessment Tool has classified the H7N9 risk low. But the potential spread of the virus from China to poultry in the neighboring countries remains a serious concern. Further, the potential of H7N9 threat beyond that region would remain. For example, the afore-mentioned CDC assessment also reported that if the virus evolved the ability of sustained human to human transmission, it could pose a serious public health threat, possibly of pandemic nature (3, 15).

Biomeme two3™ is a miniaturized thermocycler with real-time PCR capabilities. It has highly desirable attributes that are potentially of great utility to the US military warrior medics; it is a light (1.2 lbs.), hand-held, battery-operated device, making it highly portable to remote areas; it is mated to an iPhone with the essential software to display and process the real-time PCR data; and the iPhone enables the operators to instantaneously transmit the data in various formats to remote sites, where the decision makers can further analyze the data to make the appropriate decisions (Figure 1). The device therefore has the potential to be very useful for expeditious identification of different infectious agents in far-forward areas of military deployments.

We report here a research assessment of the Biomeme two3™ device in a standard laboratory setup using MERS-CoV and influenza A virus H7N9 detection as examples. For this study, we evaluated three separate Biomeme two3™ instruments, each with its own iPhone (refer to the Materials and Methods section).



<https://shop.biomeme.com/products/two3-real-time-pcr-thermocycler?variant=656490790924>

Figure 1. A Biomeme two3™ hand-held real-time PCR device along with the integrated iPhone (Biomeme, Inc., Philadelphia, PA)

3.0 MATERIALS AND METHODS

3.1 Samples and Reagents

Purified MERS-CoV and influenza A virus H7N9 genomic RNA templates were either purchased from American Type Culture Collection (ATCC, Manassas, VA), or were provided with the real-time PCR assay kits. MatrixSeq kit was purchased from BioMatrix, Inc., (Rancho Santa Fe, CA). The Biomeme MERS and H7N9 freeze-dried 3-tube strip panels were purchased from the manufacturer of the Biomeme two3™ instrument (Biomeme, Inc., Philadelphia, PA). These strips were kept at room temperature until use. The OC43, 229E, and H1N1 genomic RNA preparations were purchased from ATCC (Manassas, VA).

3.2 Biomeme two3™ Instrument

Three of these devices were purchased from Biomeme, Inc. (Philadelphia, PA), the maker of the instrument. The machine is essentially a very small, light thermocycler with real-time PCR capabilities. The cycler has three slots to carry out reactions in three PCR tubes (Figure 1). The machine can detect fluorescence signals from a single probe, or simultaneously from two. Each unit has an integrated iPhone. Including the iPhone, the device weighs only 1.2 lbs., and is thus highly portable. Each iPhone has the essential software to operate the thermocycler, as well as record, display, and process data in different ways, e.g., as sigmoidal graphs for real-time PCR or as melting curve graphs (first derivative plots). The iPhone has the capability to transmit data to various sites.

3.3 MatrixSeq Kit Assays on ViiA7 Real-Time PCR Machine

This MatrixSeq kit contains reagents for detection of MERS-CoV by real-time PCR. The approach targets three separate regions of the viral genome, and it uses HyBeacon probes for real-time detection of the target amplicons. The target regions are *orf1a*, the N gene (detection reagents, Nseq), and a region upstream of the E gene (detection reagents, upE). Each reaction composition was as follows: 1 µL of primer and probe mix, 10 µL of 2X Primerdesign oasis OneStep Mastermix, 4 µL of RNase and DNase free water, and 5 µL of sample or control (20 µL total). As directed (12), reverse transcription and real-time PCR were performed in the same reaction mixtures on a ViiA7 instrument (Life Technologies, Waltham, MA). The thermocycling protocol was as follows: 10 min at 42°C; 2 min at 95°C; and 50 cycles of 15 sec at 95°C, 20 sec at 55°C, and 20 sec at 72°C. Following thermocycling for amplification, the melting curve protocol was done by setting the temperature range of 45-85°C at 0.1°C per second. During the melting curve phase, the fluorescence data was collected through the FAM channel. There is no passive reference dye in the BioMatrix MERS-CoV Assay with this HyBeacon Probe kit to normalize the background fluorescence levels using the ViiA7 real-time PCR instrument.

3.4 PCR Assays on the Biomeme two3™ Instruments

The Biomeme's MERS Panel is a uniplex system that comes as a 3-tube strip (Figure 2). Each of the three tubes contains a freeze-dried mixture of the essential reagents for reverse transcription and real-time PCR (primers, probes, reverse transcriptase, DNA polymerase, dNTPs). The 3-tube

strip layout is as follows: tube 1, *orf1a* assay reagents; tube 2, upE assay reagents; and tube 3, human RNase P gene reagents, which can be used as a general internal positive control (IPC) when needed for the system performance. The *orf1a* and upE probes are FAM-labeled. The freeze-dried contents of tubes 1 and 2 were reconstituted by adding 20 μ L each of the target nucleic acid solution. The tube 3 contents were reconstituted by adding 20 μ L of RT-PCR grade water. Because we added water to tube 3 instead of the RNase P gene template or any other template, the tube 3 reaction essentially functioned as a negative template control (NTC). The total reaction volume in each tube was 20 μ L. The combined reverse transcription and real-time PCR protocol was as follows: 2 min at 48°C, followed by 1 min at 95°C; and 45 cycles of 1 sec at 95°C and 20 sec at 60°C.

Biomeme's H7N9 Panel is a multiplex assay for human influenza A virus H7N9, simultaneously detecting target amplicons from H7 and N9 genes. The H7-specific probe is FAM-labeled and the N9-specific probe Texas Red X-labeled. The 3-tube strip layout was as follows: tube 1, human RNase P gene detection reagents (IPC); tube 2 and 3, combined H7 and N9 detection reagents. However, the experiment to simultaneously detect H7 and N9 was not performed; only one template, either H7 or N9, was added to each tube. The reverse transcription and real-time PCR thermocycling protocol was as for the MERS panel. As described for the MERS Panel, the RNase P gene detection was not used as a positive control; because only water was added to tube 1, the reaction effectively became an NTC.

3.5 Analysis of Biomeme Panels on ViiA7 Real-time PCR Machine

To test Biomeme's MERS and H7N9 Panels on ViiA7 machine, each mixture was reconstituted as described above by adding 20 μ L of either the H7 template or the N9 template to the H7N9 Panel, and either *orf1a* template or upE template to the MERS Panel. The tube contents were then transferred to new PCR tubes for real-time PCR on the ViiA7 instrument. The thermocycling protocol was the same as for Biomeme two3™.

3.6 Biomeme two3™ Real-time PCR Data

The real-time PCR data generated by Biomeme two3™ were stored on the mated iPhone and processed using the especially developed iPhone software for the purpose. The data were also stored in the Biomeme's cloud storage system.

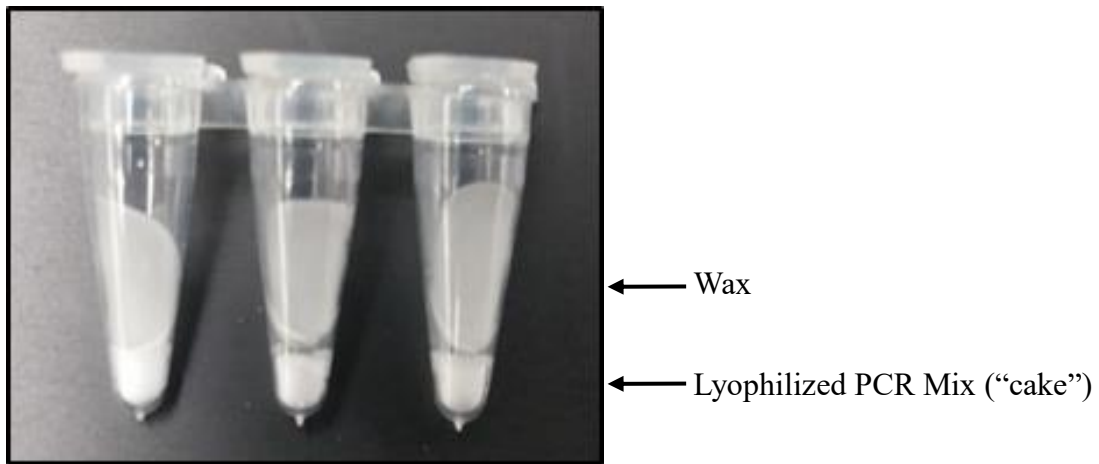


Figure 2. The 3-strip format for real-time PCR in Biomeme two3™

Each tube has lyophilized real-time PCR reagents ("cake") topped with wax to prevent evaporation during thermocycling. Each mixture contains the essential reagents for detection of a target template, or to serve as the positive or negative control.

4.0 RESULTS

4.1 Detection of MERS-CoV N Gene with the BioMatrix MERS-CoV Panel

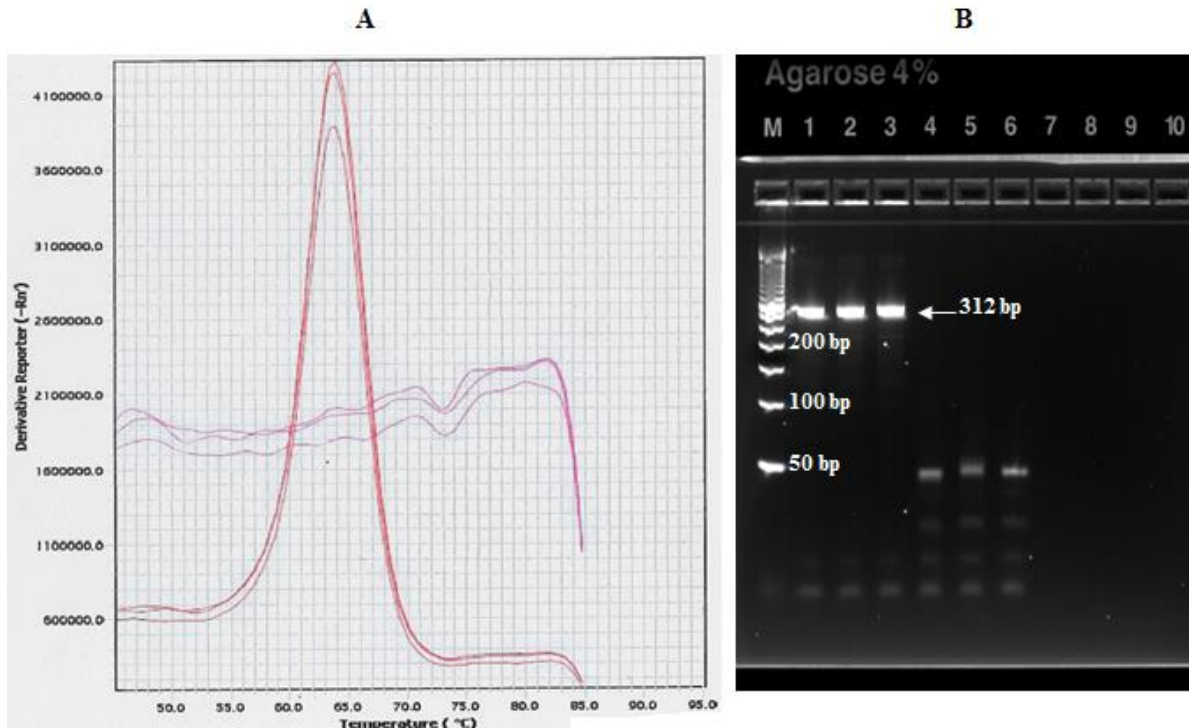


Figure 3. MERS-CoV N gene detection assay (Nseq) with MatrixSeq kit using a ViiA7 real-time PCR machine

The assay was performed in triplicate. **A**, the first derivative plots of real-time PCR results. The triplicate peaks are at ~ 63.8°C (arrow). **B**, agarose gel electrophoresis analysis of the PCR products. The expected N gene amplicon size is 312 bp. M, 50 bp molecular weight marker ladder; the lowest band represents the 50-bp fragment. Lanes 1-3: MERS-CoV Nseq; Lanes: 4-6: NTC. Lanes 7-10, empty.

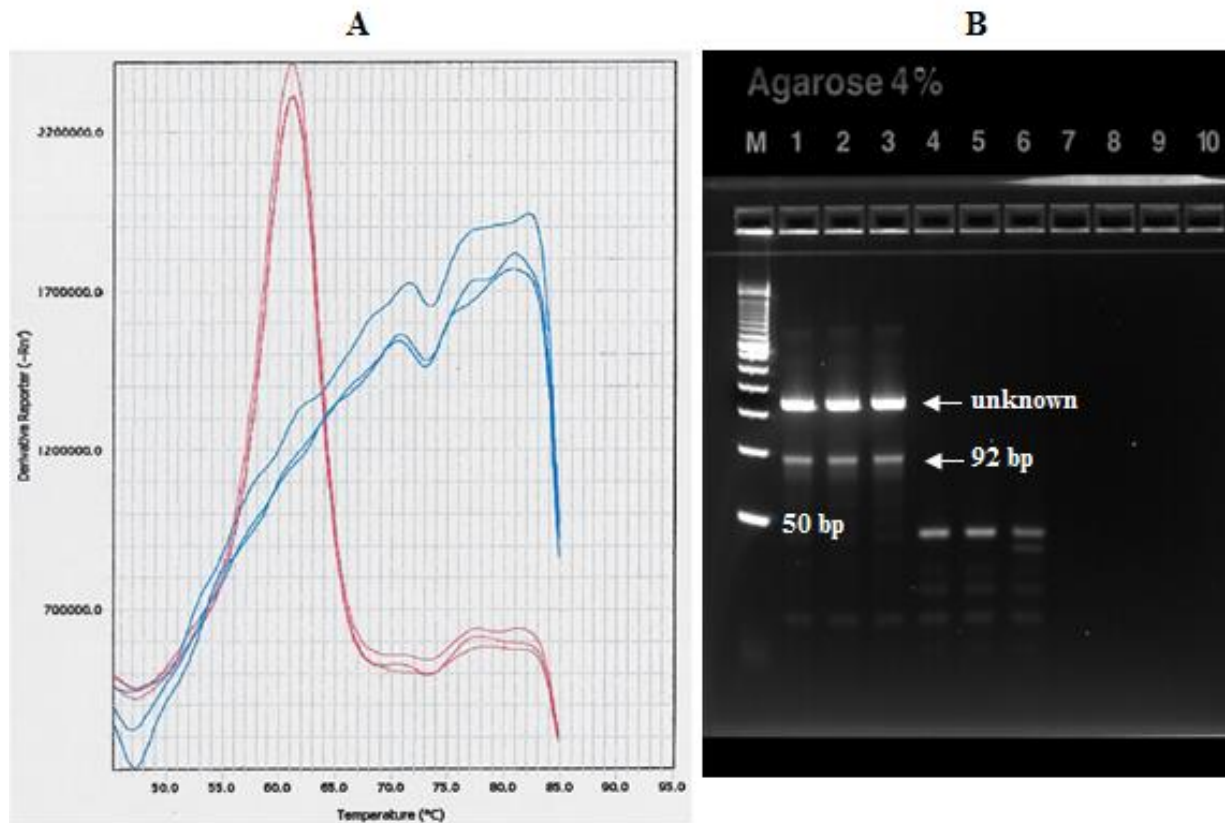


Figure 4. MERS-CoV upE region detection assay with MatrixSeq kit using a ViiA7 real-time PCR machine

The assay was performed in triplicate. **A**, the first derivative plots of real-time PCR results. All three peaks are coincident at 61°C (arrow). **B**, agarose gel electrophoresis analysis of the PCR products. The expected upE region amplicon size is 92 bp. M, 50 bp molecular weight marker ladder; the lowest band represents the 50 bp fragment. Lanes, 4-6, NTC. Lanes 7-10, empty. Lanes 1-3 have an unexpected band (~ 170 bp) in the PCR product. This result was consistent and reproducible when the experiment was performed by two different researchers.

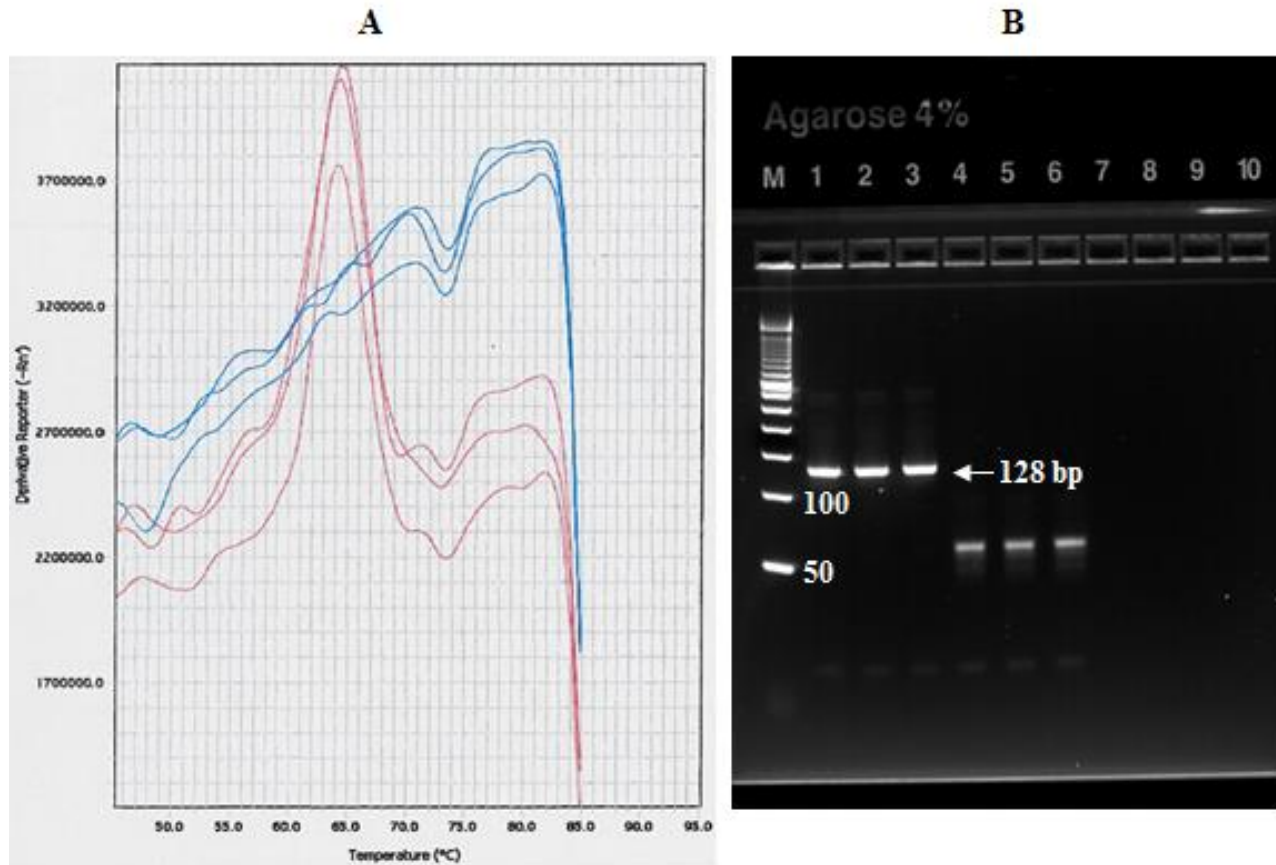


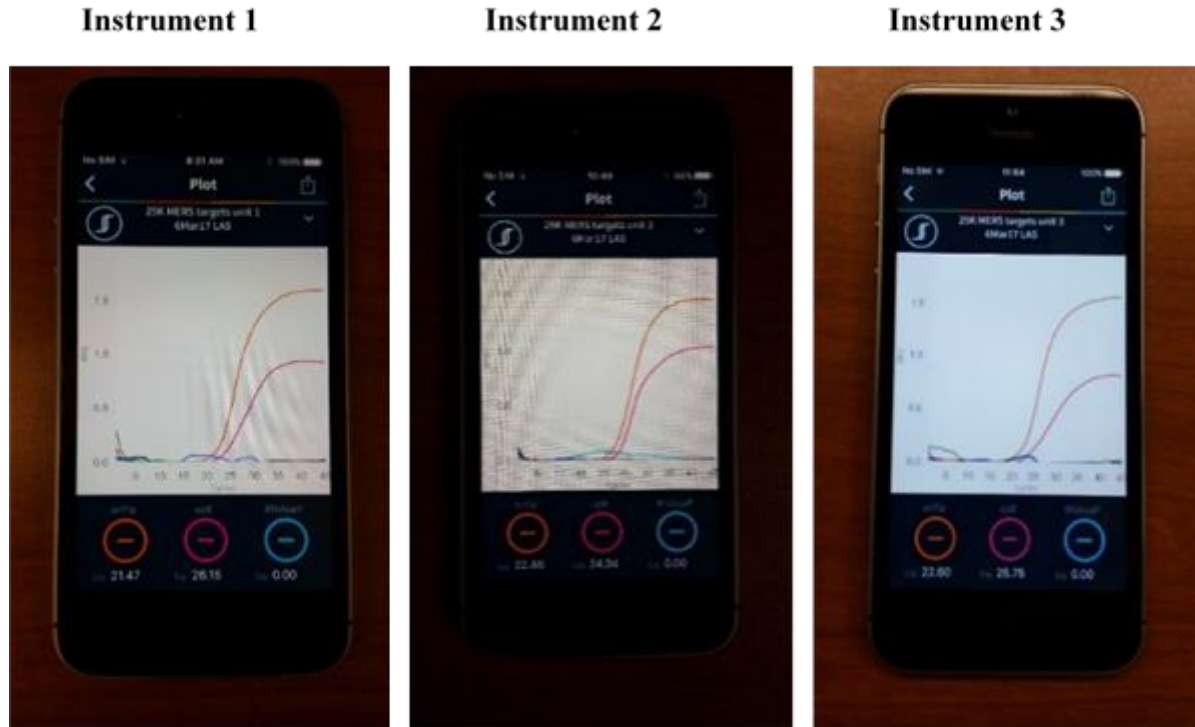
Figure 5. MERS-CoV *orf1a* detection assay with MatrixSeq kit on a ViiA7 real-time PCR machine

The assay was performed in triplicate. **A**, the first derivative plots of real-time PCR results. Approximate average of the triplicate peaks is 64.2°C (arrow). **B**, analysis of the PCR products by agarose gel electrophoresis. M, 50 bp molecular weight marker ladder (the lowest band is 50 bp). Lanes 1-3, *orf1a* detection real-time PCR reactions. Lanes 4-6, NTC. Lanes 7-10, empty. The expected *orf1a* specific amplicon size is 128 bp, clearly present in Lanes 1-3. The NTC lanes have an unexpected band of a size between 50 bp and 75 bp; we did not determine the identity of this band.

4.2 Detection of MERS-CoV *orf1a* and upE targets with Biomeme two3™

The Biomeme MERS Panel was used for real-time PCR on Biomeme two3™ devices. The Panel is specifically designed for use with Biomeme two3™ and for the detection of targets in *orf1a* and upE segments of the MERS-CoV genome. The *orf1a* and upE template stocks provided by Biomeme, Inc., were subjected to 10-fold serial dilutions to obtain concentrations ranging from 25,000 to 2.5 copies/μL. Each original stock concentration was 10,000,000,000 copies/μL (Biomeme, Inc.). The reactions were set up as described in the Materials and Methods section. The final copy numbers of each template per reaction were 500,000, 50,000, 5,000, 500, and 50. The results of these experiments are shown in Figures 6-10. The agarose gel electrophoresis analysis of the real-time PCR products generated by the 50 copy number experiments are shown in Figure 11. Both targets were detectable for all template copy numbers.

For comparison, we also assessed the Biomeme MERS and H7N9 Panels on ViiA7 real-time PCR machine. The procedure was as described in the Materials and Methods section. This approach did not work; the instruments gave “abort run errors,” which may have been due to the very short incubation times at various steps in the thermocycling protocol, which was specifically designed for Biomeme two3™.

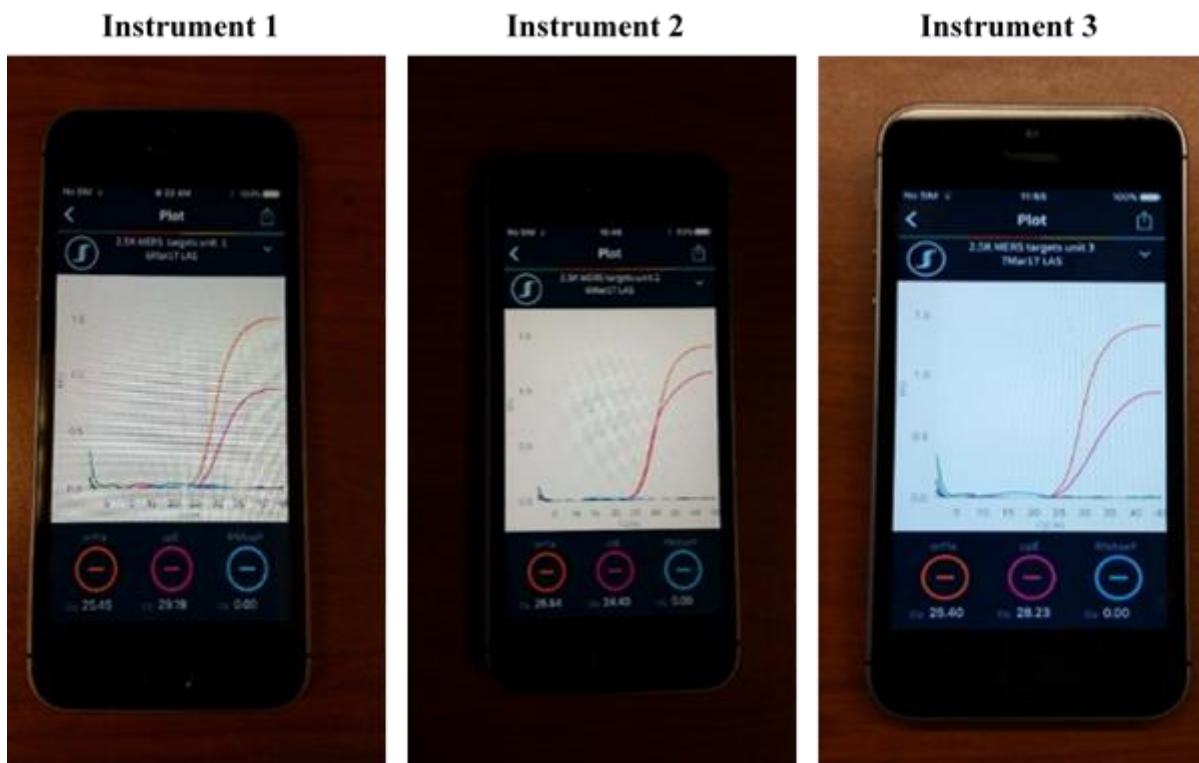


Ct Values (500,000 template copies per reaction)			
Target	Instrument 1	Instrument 2	Instrument 3
<i>orf1a</i>	21.47	22.46	22.60
upE	26.15	24.04	25.75
IPC	None	None	None

Figure 6. Ct values generated by performing real-time PCR on three separate Biomeme instruments.

The assays were independently performed on the three machines. Each reaction mixture contained 500,000 copies of MERS-CoV targets *orf1a* (tube 1) and upE (tube 2). The IPC (tube 3) reaction mixture contained 20 μ L of water. IPC, internal positive control containing human RNase P detection primers and probe. As explained in the Materials and Methods section, the IPC tube became a negative template control because only water was added to it instead of the RNase P gene template.

The results show that the Ct values generated by all three instruments were very similar, reflecting uniformity of instrument and reagent performance in this experiment.

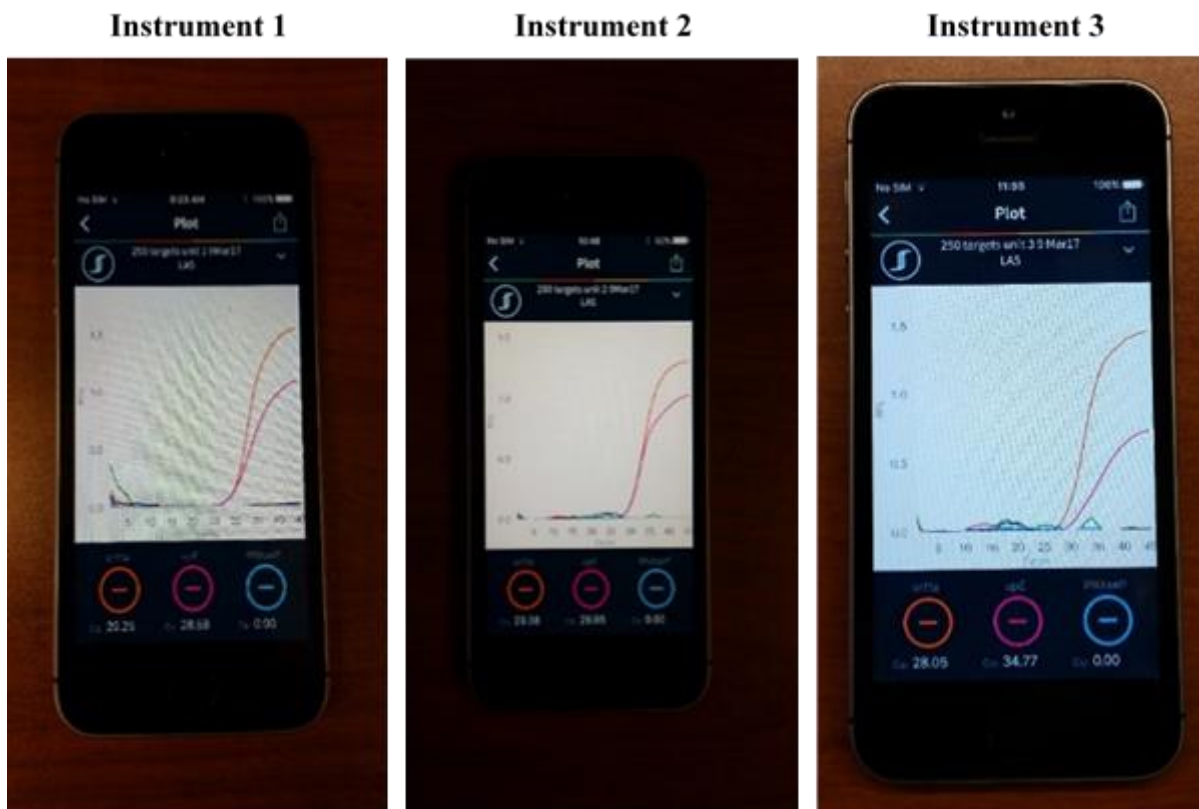


Ct Values (50,000 template copies per reaction)			
Target	Instrument 1	Instrument 2	Instrument 3
<i>orf1a</i>	25.45	25.54	25.40
upE	29.19	24.40	28.23
IPC	None	None	None

Figure 7. Ct values generated by performing real-time PCR on three separate Biomeme instruments

The assays were independently performed on the three machines. Each reaction mixture contained 50,000 copies of MERS-CoV targets *orf1a* (tube 1) and upE (tube 2). The IPC (tube 3) reaction mixture contained 20 μ L of water (see explanations in Materials and Methods).

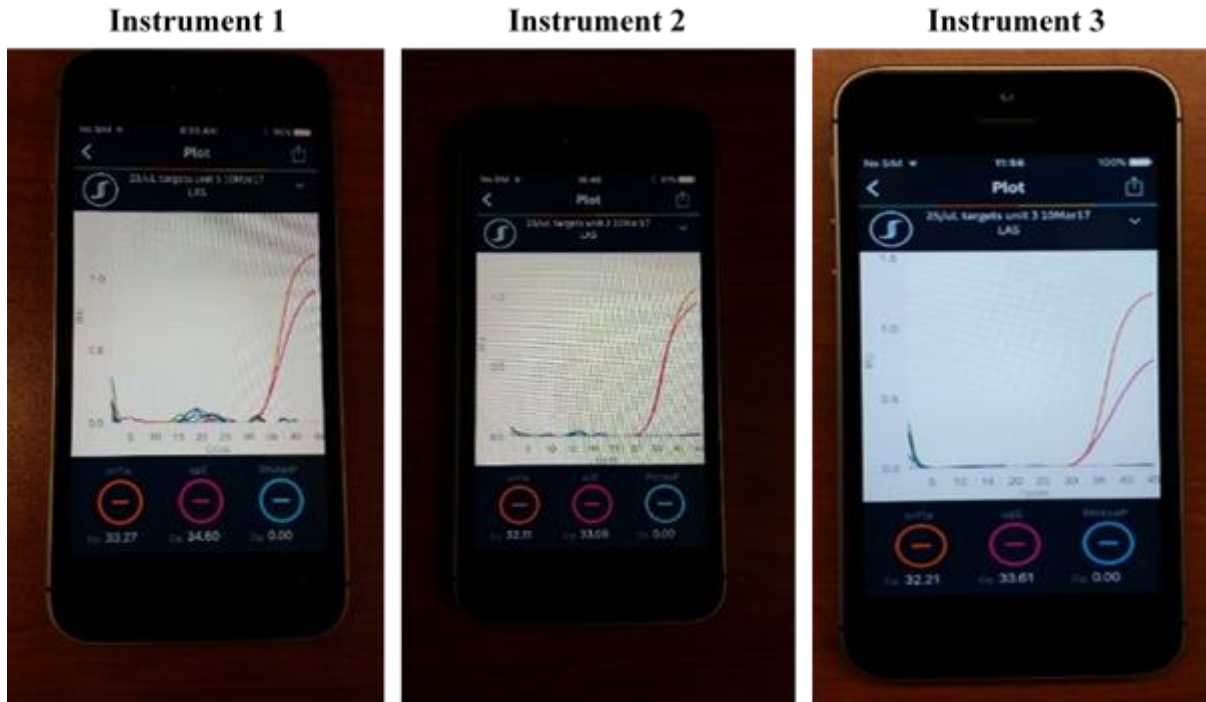
For *orf1a*, the three instruments generated near identical Ct values for 50,000 template copies per reaction, again reflecting precision and uniformity of performance. For upE, however, the Ct value generated by instrument 2 was lower than those by instruments 1 and 3. Further, except for upE on instrument 2, the Ct values were higher than those for the 500,000 copies per reaction, an expected outcome because the template copy number was 50,000 per reaction, much lower than 500,000.



Ct Values (5,000 template copies per reaction)			
Target	Instrument 1	Instrument 2	Instrument 3
<i>orf1a</i>	29.25	29.58	28.05
upE	28.58	29.95	34.77
IPC	None	None	None

Figure 8. Ct values generated by performing real-time PCR on three separate Biomeme instruments

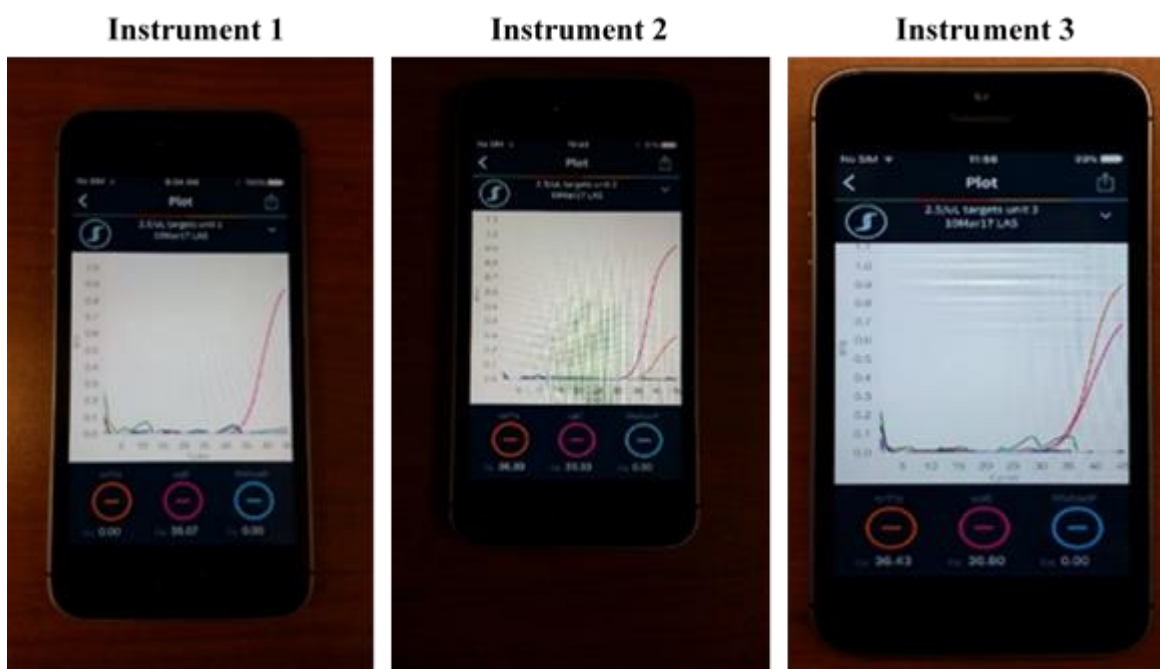
The assays were independently performed on the three machines. Each reaction mixture contained 5,000 copies of MERS-CoV targets *orf1a* (tube 1) and upE (tube 2). The IPC (tube 3) reaction mixture contained 20 μ L of water (see explanations in Materials and Methods).



Ct Values (500 template copies per reaction)			
Target	Instrument 1	Instrument 2	Instrument 3
<i>orf1a</i>	33.27	32.11	32.31
upE	34.60	33.09	33.61
IPC	None	None	None

Figure 9. Ct values generated by performing real-time PCR on three separate Biomeme instruments

The assays were independently performed on the three machines. Each reaction mixture contained 500 copies of MERS-CoV targets *orf1a* (tube 1) and upE (tube 2). The IPC (tube 3) reaction mixture contained 20 μ L of water (see explanations in Materials and Methods).



Ct Values (50 template copies per reaction)			
Target	Instrument 1	Instrument 2	Instrument 3
<i>orf1a</i>	Negative	36.39	36.43
upE	35.07	33.33	36.8
IPC	None	None	None

Figure 10. Ct values generated by performing real-time PCR on three separate Biomeme instruments.

The assays were independently performed on the three machines. Each reaction mixture contained 50 copies of MERS-CoV targets *orf1a* (tube 1) and upE (tube 2). The IPC (tube 3) reaction mixture contained 20 μ L of water (see explanations in Materials and Methods). *Orf1a* PCR reaction on instrument 1 failed to amplify at 50 copies per reaction.

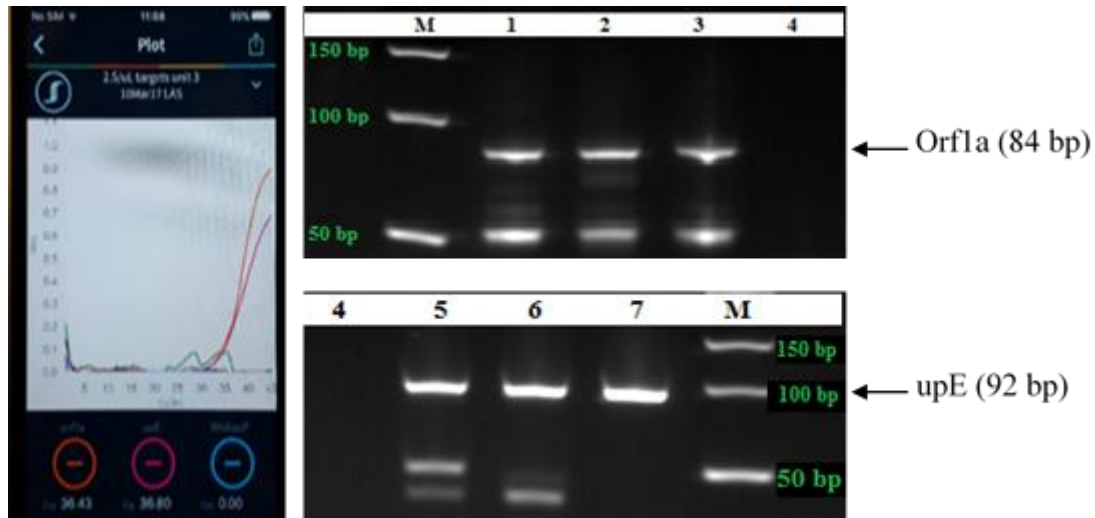


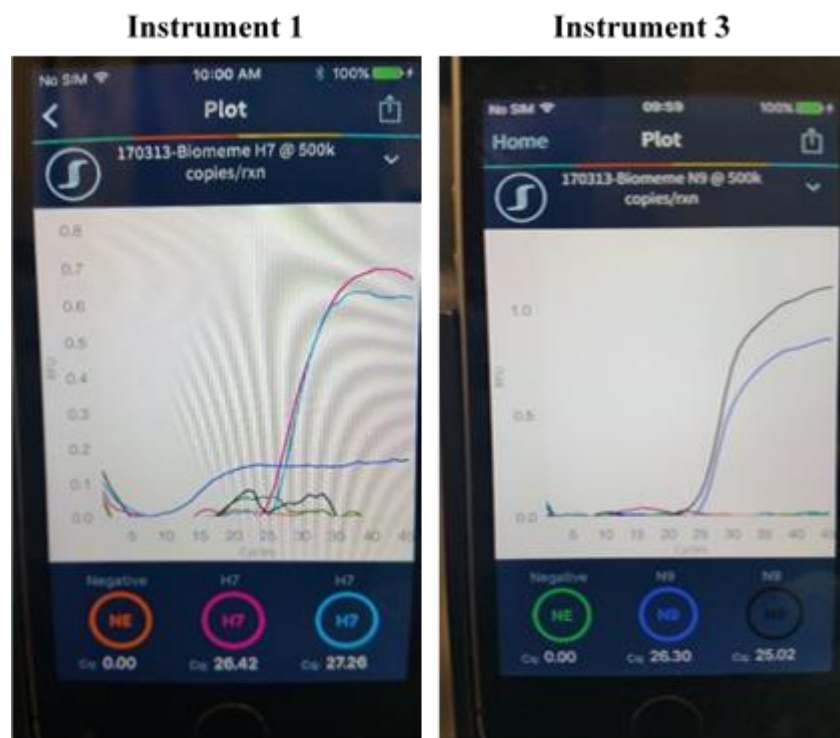
Figure 11. Amplification and detection of MERS-CoV *orf1a* and upE amplicons at 50 copies per reaction using the Biomeme MERS Panel assay.

The assays were independently performed on instruments 1, 2, and 3. Expected *orf1a* band size is 84 bp, and upE is 92 bp. Lanes 1 and 5, Instrument 1. Lanes 2 and 6, Instrument 2. Lanes 3 and 7, Instrument 3. Lane 4 (both gel pictures), NTC.

The lowest template copy number at which Biomeme two3™ afforded target detection was 50 per reaction. As shown in Figure 15, both *orf1a* and upE could be detected. The bands around 50 bp are of unknown identity, likely nonspecific or primer dimers.

4.3 Detection of Influenza A virus H7N9 using the Biomeme H7N9 Panel

The H7 and N9 template dilutions and the experimental protocols were as for the Biomeme MERS Panel and described in the Materials and Methods section. The results are shown in Figures 12-16. Both targets were detectable at all copy numbers of templates. The agarose gel electrophoresis analysis of the real-time PCR products generated by the 50 copy number experiments are shown in Figure 17.



	Ct Values (500,000 template copies per reaction)		
Target	Instrument 1	Instrument 2	Instrument 3
IPC	None	Not tested	None
H7	26.42 & 27.26	Not tested	Not tested
N9	Not tested	Not tested	26.30 & 25.02

Figure 12. Evaluation of the Biomeme instruments 1 and 3 using the Biomeme H7N9 Panel real-time PCR and 500,000 copies of the H7N9 template per reaction

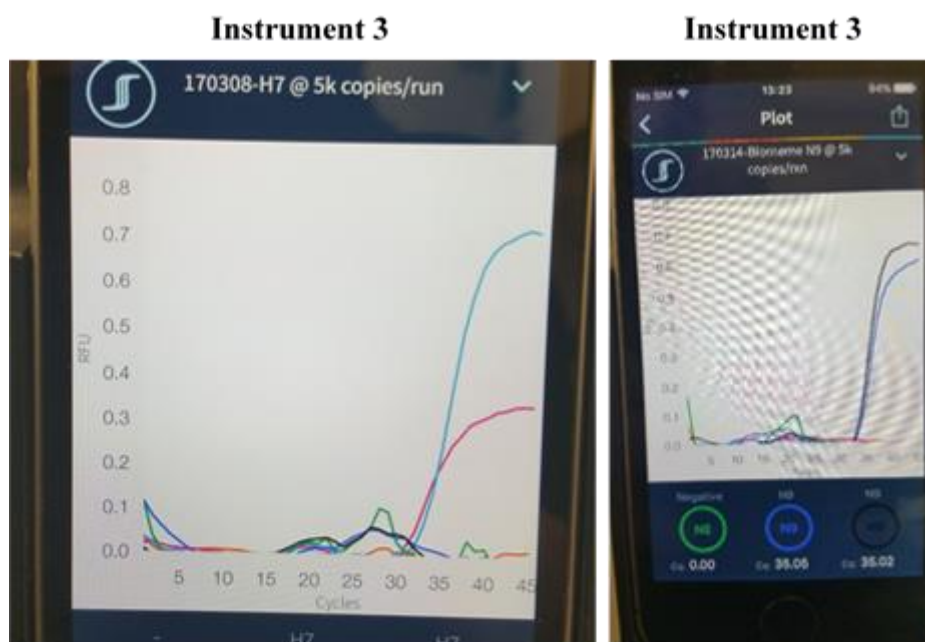
The Table records Ct values generated by each machine. The thermocycling protocol for this experiment was the same as for the Biomeme MERS Panel real-time PCR. The H7N9 template copy number was 500,000 per reaction. However, whereas the MERS Panel experiment was a uniplex experiment, the H7N9 Panel setup is for duplex real-time PCR. The Biomeme 3-tube strip setup was as follows: Tube 1, human RNase P gene detection reagents; only PCR grade water was added to it, making it a negative template control reaction (see details in Materials and Methods). Tubes 2 and 3, H7 and N9 reagents (primers and probes for both genes), but only one template was added to each tube, either H7 or N9, making the experiment uniplex.



	Ct Values (50,000 template copies per reaction)		
Target	Instrument 1	Instrument 2	Instrument 3
IPC	Not tested	None	None
H7	Not tested	27.91 & 27.94	Not tested
N9	Not tested	Not tested	30.11 & 29.31

Figure 13. Evaluation of the Biomeme instruments 2 and 3 using the Biomeme H7N9 Panel real-time PCR and 50,000 copies of the H7N9 template per reaction

Except for the 50,000 H7N9 template copy number per reaction, the experimental procedures were as described for Figure 12. The Ct values are shown in the Table. For IPC, see the explanatory notes in the Materials and Methods section.

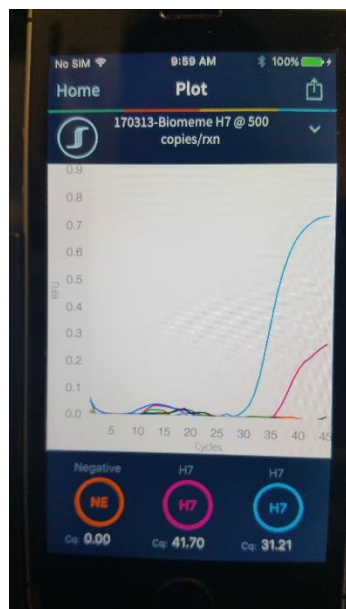


Ct Values (5,000 template copies per reaction)			
Target	Instrument 1	Instrument 2	Instrument 3
IPC	Not tested	Not tested	None
H7	Not tested	Not tested	32.62 & 33.40
N9	Not tested	Not tested	35.05 & 35.02

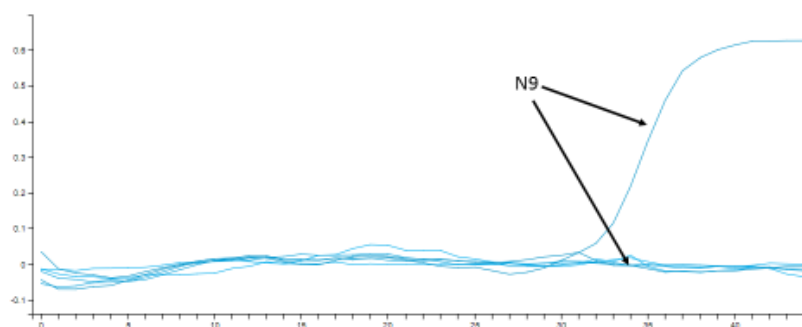
Figure 14. Evaluation of the Biomeme instruments 2 and 3 using the Biomeme H7N9 Panel real-time PCR and 5,000 copies of the H7N9 template per reaction

The H7N9 template copy number per reaction for this experiment was 5,000, and all other experimental parameters were as for the data shown in Figure 12. For IPC, see the explanatory notes in the Materials and Methods section.

Instrument 1



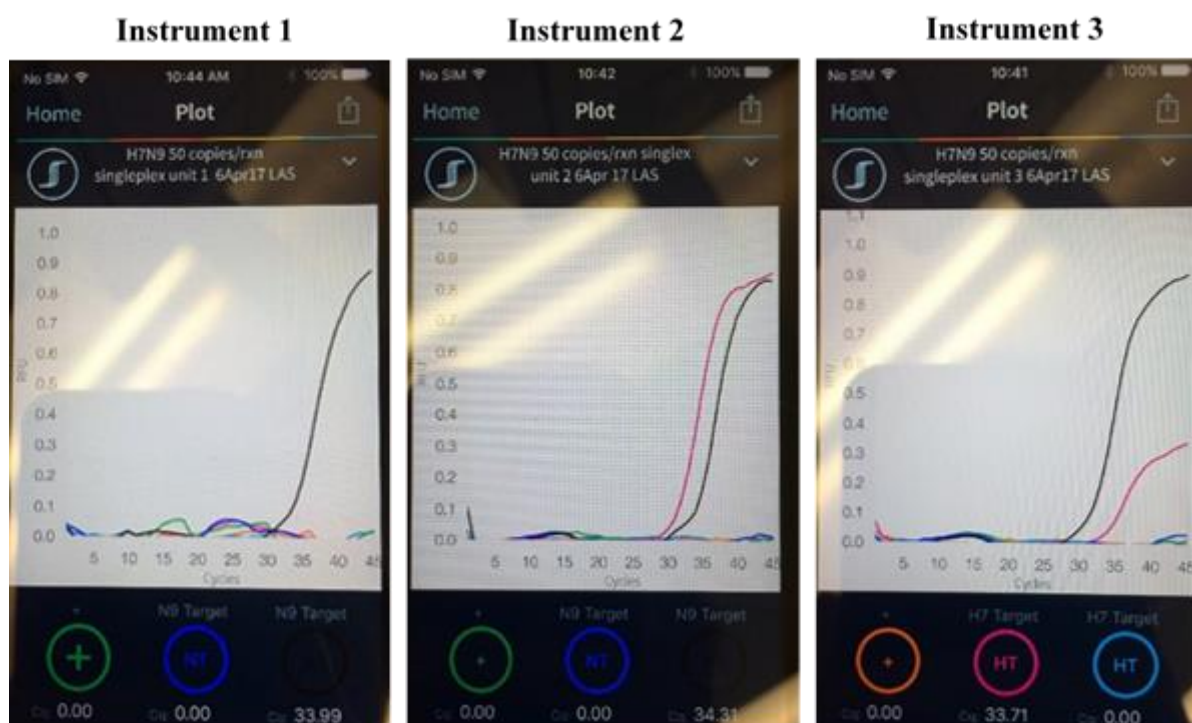
Instrument 2



Ct Values (500 template copies per reaction)			
Target	Instrument 1	Instrument 2	Instrument 3
IPC	None	None	Not tested
H7	41.70 & 31.21	Not tested	Not tested
N9	Not tested	34.66 & Negative	Not tested

Figure 15. Evaluation of the Biomeme instruments 1 and 2 using the Biomeme H7N9 Panel real-time PCR and 5,000 copies of the H7N9 template per reaction

The H7N9 template copy number per reaction for this experiment was 500, and all other experimental parameters were as for the data shown in Figures 12. Instrument 2 gave only one Ct value; the second reaction failed. The screen shot is not available for Instrument 2. For IPC, see the explanatory notes in the Materials and Methods section.



Ct Values (50 template copies per reaction)			
Target	Instrument 1	Instrument 2	Instrument 3
IPC	None	None	None
H7	Negative	30.26	33.71
N9	33.99	34.31	32.10

Figure 16. Evaluation of the Biomeme instruments 1, 2, and 3 using the Biomeme H7N9 Panel real-time PCR and 50 copies of the H7N9 template per reaction

The H7N9 template copy number per reaction for this experiment was 50. The experimental parameters and cycling protocol were as for the experiments shown in Figures 12, except that detection in each tube was uniplex, i.e., either for H7 or N9. For IPC, see the explanatory notes in the Materials and Methods section.

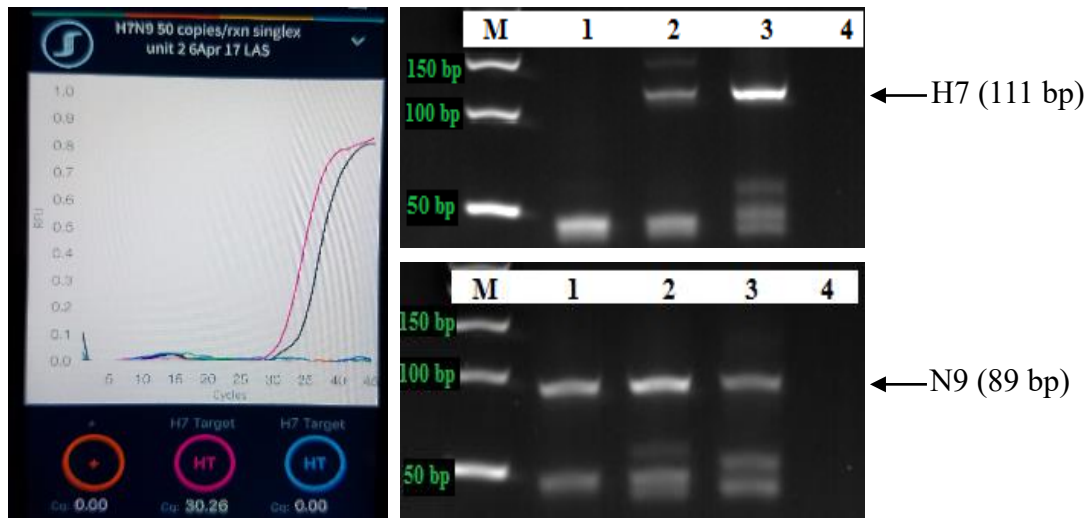


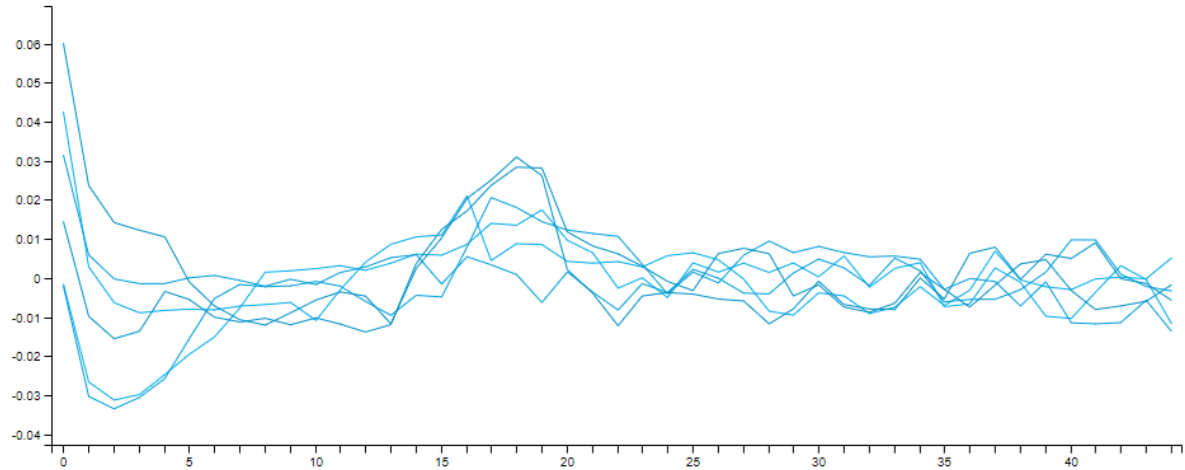
Figure 17. Amplification and detection of H7N9 amplicons at 50 copies per reaction using the Biomeme H7N9 qPCR assay. The expected H7 amplicon size is 111 bp, and N9 is 89 bp. Each gel shows results from all three Biomeme instruments: lane 1, Instrument 1; lane 2, instrument 2; lane 3, instrument 3. Lane 4, negative controls (no template).

Although the Biomeme H7N9 Panel is designed for simultaneous detection of both targets, in this experiment we did not do that. Instead, to a given tube we added either the H7 template or the N9 template, making the experiment uniplex. The results show that at 50 copies of each template per reaction, N9 was detected by all three instruments, while H7 was detected by instruments 2 and 3, not 1. The expected amplicons were 111 bp for H7 and 89 bp for N9.

4.4 Assessment of Specificities of the Biomeme MERS and H7N9 Panels

To evaluate the specificity of the Biomeme MERS Panel for MERS-CoV *orf1a* and upE targets, the Panel was also tested for potential cross-amplification of the corresponding segments of the related coronaviruses OC43 and 229E. Both the MERS-CoV and OC43 coronaviruses are classified in the same genus, *Betacoronavirus*, while the 229E coronavirus is an *Alphacoronavirus*. The OC43 and 229E templates were purchased from ATCC, and the experimental procedures were the same as for the MERS-CoV. The results show that the MERS Panel did not amplify the corresponding *orf1a* and upE segments of OC43 and 229E (Figure 18). Thus, Biomeme MERS Panel proved specific for detection of the intended targets, *orf1a* and upE.

Likewise, to determine the Biomeme H7N9 Panel specificity, we used another influenza A virus template. Again, no amplicon for either target resulted from this real-time PCR was assessed by testing the panel for potential detection of H1 or N1 (Figure 19). These results show that the H7N9 panel is indeed specific for H7N9 detection.



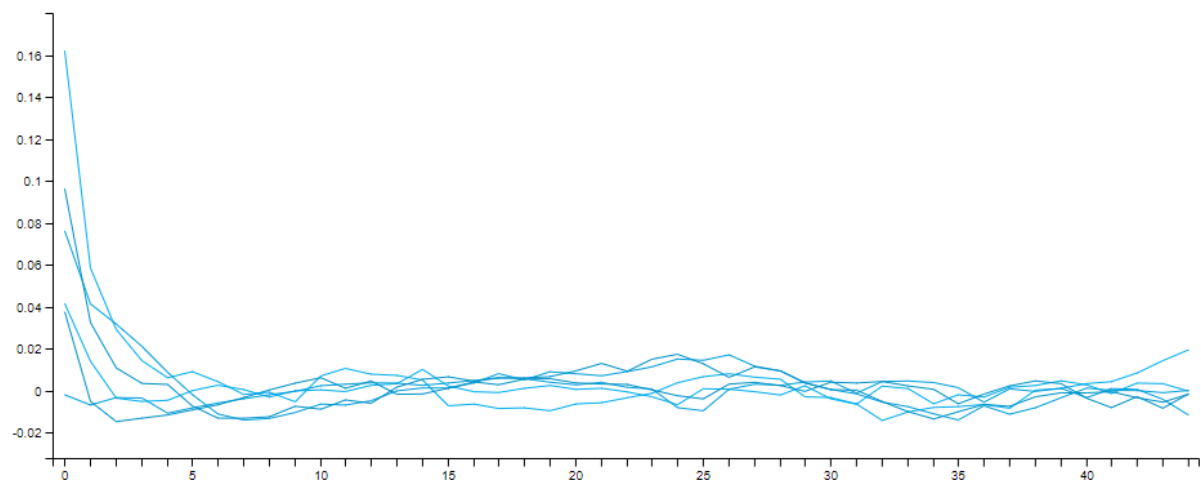
RUN DATA

ID	Near neighbor OC43, 229e 17Feb17 LAS
Date & Time	1/17/2017 3:59 AM
Results	
Location	Lackland A F B, TX, United States
Latitude	29.3992237451939
Longitude	-98.61788208051324
Serial Number	171
Firmware Version	2.0.3
Software Version	133

PROTOCOL DATA

Chemistry	TaqMan	
Start Denature	95	60
Cycling Denature		
Cycling Anneal		
Cycles	45	
Duration		
Reverse Transcription	48	2
Single Threshold	95	60

Figure 18. Evaluation of the Biomeme MERS Panel for cross-amplification of *orf1a* and upE segments of OC43 and 229E



RUN DATA

ID	020217 Biomeme H7N9 Near Neighbor Experiment 1A-NT
Date & Time	1/2/2017 4:57 AM
Results	
Location	Lackland A F B, TX, United States
Latitude	29.39881549469313
Longitude	-98.61711704860066
Serial Number	174
Firmware	2.0.3
Version	
Software	133
Version	

PROTOCOL DATA

Chemistry	TaqMan	
Start Denature	95	60
Cycling Denature		
Cycling Anneal		
Cycles	45	
Duration		
Reverse Transcription	48	2
Single Threshold	95	60

Figure 19. Evaluation of the Biomeme H7N9 Panel for cross-contamination of H7 and N9 genes on Flu A and H1N1

5.0 SUMMARY

1. In this study, we evaluated the overall performance of Biomeme two3™, a light, portable real-time PCR device with coupled iPhone for data processing, storage, and transmission. We used the reagents specifically designed for work on this instrument, and provided by the manufacturer. Three Biomeme two3™ machines were purchased for the work.
2. Initially we employed a proven benchtop real-time PCR machine, the Applied Biosystems ViiA7. The reagents used in this study were procured from BioMatrix. One reason for this work was that Biomeme two3™ was not available at that time. The other was that we wanted to evaluate the performance of the detection reagents, which would have been meant for the upcoming Biomeme instrument. However, that did not turn out to be the case because the Biomeme two3™ machines we bought did not have the melting curve analysis capability. The results of these experiments are shown in Figures 3-5.
3. MERS-CoV and influenza A virus H7N9 (Asian lineage) templates provided by the manufacturer were used for evaluating Biomeme two3™. Specifically, the target amplicons were in the *orf1a* and upE segments of the MERS-CoV genome, and in H7 and N9 segments of the influenza A virus H7N9 genome.
4. Testing was performed using different template copy numbers, in 10-fold serial dilutions ranging from 50 to 500,000 per reaction.
5. Overall, the detection of all four targets was excellent, and the targets could be detected down to 50 copies per reaction.
6. To gauge the specificity of target detection, we performed identical experiments using the near-neighbor templates, the OC43 and 229E coronavirus templates as the MERS-CoV near-neighbors, and H1N1 as the H7N9 near-neighbor. These experiments yielded no amplicons. Thus, the detection reagents (primers, probes) proved highly specific for the detection of their respective targets.
7. Overall, our data suggest that Biomeme two3™ is a very useful device for rapid, specific detection of target pathogens. We note, however, that in as much as specificity in PCR is a function of the specificities of both primers and the probe for each detection, they must be carefully designed to ensure specificity.
8. We also think the instrument and its applicability would greatly benefit from incorporating a greater number of slots for reactions. The instrument we tested has only three slots. Thus, if one is used for the positive control and the other for the negative control, only one test sample reaction can be carried out.
9. Incorporating multiplex detection capacity in each tube would also prove greatly advantageous because it would allow testing of a greater number of samples simultaneously.

6.0 REFERENCES

1. Bermingham A, Chand MA, Brown CS, Aarons E, Tong C, Langrish C, Hoschler K, Brown K, Galiano M, Myers R, Pebody RG, Green HK, Boddington NL, Gopal R, Price N, Newsholme W, Drosten C, Fouchier RA, Zambon M. Severe respiratory illness caused by a novel coronavirus, in a patient transferred to the United Kingdom from the Middle East, September 2012. *Euro Surveill.* 2012 Oct 4;17(40):20290.
2. Centers for Disease Control and Prevention. <https://www.cdc.gov/coronavirus/mers/>
3. Centers for Disease Control and Prevention. <https://www.cdc.gov/flu/avianflu/h7n9-virus.htm>
4. Centers for Disease Control and Prevention. <https://www.cdc.gov/vhf/ebola/index.html>
5. Centers for Disease Control and Prevention. <https://www.cdc.gov/zika/index.html>
6. Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, van Boheemen S, Gopal R, Ballhause M, Bestebroer TM, Muth D, Müller MA, Drexler JF, Zambon M, Osterhaus AD, Fouchier RM, Drosten C. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. *Euro Surveill.* 2012 Sep 27;17(39). pii: 20285.
7. Corman VM, Müller MA, Costabel U, Timm J, Binger T, Meyer B, Kreher P, Lattwein E, Eschbach-Bludau M, Nitsche A, Bleicker T, Landt O, Schweiger B, Drexler JF, Osterhaus AD, Haagmans BL, Dittmer U, Bonin F, Wolff T, Drosten C. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. *Euro Surveill.* 2012 Dec 6;17(49). pii: 20334.
8. Drosten C, Günther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RA, Berger A, Burguière AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Müller S, Rickerts V, Stürmer M, Vieth S, Klenk HD, Osterhaus AD, Schmitz H, Doerr HW. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med.* 2003 May 15;348(20):1967-76. Epub 2003 Apr 10. Reports identification of SARS-CoV.
9. International Committee on Taxonomy of Viruses (ICTV). <https://talk.ictvonline.org/taxonomy/>
10. Kageyama T, Fujisaki S, Takashita E, Xu H, Yamada S, Uchida Y, Neumann G, Saito T, Kawaoka Y, Tashiro M. Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013. *Euro Surveill.* 2013 Apr 11;18(15):20453.

11. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini WJ, Anderson LJ; SARS Working Group. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med*. 2003 May 15;348(20):1953-66. Epub 2003 Apr 10. Reports identification of SARS-CoV.
12. Thermo Fisher Scientific. <https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-instruments/viia-7-real-time-pcr-system.html>
13. van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, Osterhaus AD, Haagmans BL, Gorbalenya AE, Snijder EJ, Fouchier RA. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. *MBio*. 3(6): e00473-12. 2012.
14. World Health Organization Report, April 2018. <http://www.who.int/emergencies/mers-cov/en/>. Checked, 24 May 2018.
15. World Health Organization. http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/
16. World Health Organization. <http://www.who.int/news-room/fact-sheets/detail/ebola-virus-disease>
17. World Health Organization. <http://www.who.int/news-room/fact-sheets/detail/zika-virus>
18. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med*;367:1814–20. 2012.