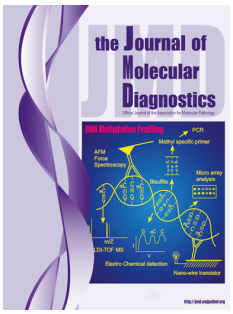
Accepted Manuscript



Evaluation and Clinical Validation of Two Field-deployable Reverse Transcription-Insulated Isothermal PCR Assays for the Detection of the Middle East Respiratory Syndrome Coronavirus

Yun Young Go, Yeon-Sook Kim, Shinhye Cheon, Sangwoo Nam, Keun Bon Ku, Meehyein Kim, Nam Hyuk Cho, Hyun Park, Pei-Yu Alison Lee, Yu-Chun Lin, Yun-Long Tsai, Hwa-Tang Thomas Wang, Udeni B.R. Balasuriya

PII: S1525-1578(17)30188-5

DOI: 10.1016/j.jmoldx.2017.06.007

Reference: JMDI 624

To appear in: *The Journal of Molecular Diagnostics*

Received Date: 25 March 2017

Revised Date: 19 May 2017

Accepted Date: 21 June 2017

Please cite this article as: Go YY, Kim Y-S, Cheon S, Nam S, Ku KB, Kim M, Cho NH, Park H, Alison Lee P-Y, Lin Y-C, Tsai Y-L, Thomas Wang H-T, Balasuriya UBR, Evaluation and Clinical Validation of Two Field-deployable Reverse Transcription-Insulated Isothermal PCR Assays for the Detection of the Middle East Respiratory Syndrome Coronavirus, *The Journal of Molecular Diagnostics* (2017), doi: 10.1016/j.jmoldx.2017.06.007.

Evaluation and Clinical Validation of Two Field-deployable Reverse Transcription-Insulated Isothermal PCR Assays for the Detection of the Middle East Respiratory Syndrome Coronavirus

Yun Young Go[1,2*¶], Yeon-Sook Kim[3¶], Shinhye Cheon[3], Sangwoo Nam[1,2], Keun Bon Ku[1], Meehyein Kim[1,2], Nam Hyuk Cho[4], Hyun Park[5], Pei-Yu Alison Lee[6], Yu-Chun Lin[6], Yun-Long Tsai[6], Hwa-Tang Thomas Wang[6], Udeni B. R. Balasuriya[7*]

[1]Center for Virus Research and Testing, Korea Research Institute of Chemical Technology, Daejeon 34113, Republic of Korea

[2]Department of Medicinal Chemistry and Pharmacology, University of Science and Technology, Daejeon 34113, Republic of Korea

[3]College of Medicine, Chungnam National University, Daejeon, Republic of Korea

[4]Department of Microbiology and Immunology, and Department of Biomedical Science, Seoul National University College of Medicine and Bundang Hospital, Seoul 03080, Republic of Korea

[5]Department of Infection Biology School of Medicine, Wonkwang University, Iksan, 54538 Republic of Korea;

[6]GeneReach USA, Lexington, Massachusetts, USA

[7]Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, College of Agriculture, Food and Environment and Department of Microbiology, Immunology and Molecular Genetics, College of Medicine, University of Kentucky, Lexington, Kentucky, USA

[¶]YYG and YSK contributed equally to this work

Address correspondence to Yun Young Go (yygo@krict.re.kr) or Udeni B. R. Balasuriya (ubalasuriya@uky.edu)

E-mail addresses:

Yun Young Go	yygo@krict.re.kr
Yeon-Sook Kim	idalicekim@gmail.com
Shinhye Cheon	episodes22@cnuh.co.kr
Sangwoo Nam	swnam@krict.re.kr
Keun Bon Ku	kbku@krict.re.kr
Meehyein Kim	mkim@krict.re.kr
Nam Hyuk Cho	chonh@snu.ac.kr
Hyun Park	hyunpk@whu.ac.kr
Pei-Yu Alison Lee	peiyu329@genereachbiotech.com
Yu-Chun Lin	xls-fish@genereachbiotech.com
Yun-Long Tsai	David.tsai@genereachbiotech.com
Hwa-Tang Thomas Wang	thomaswang@genereachbiotech.com
Udeni B. R. Balasuriya	ubalasuriya@uky.edu

Running title: RT-iiPCR assays for MERS-CoV diagnosis

Key words: Middle East Respiratory Syndrome Coronavirus, MERS-CoV, insulated isothermal PCR, RT-iiPCR, POCKIT™, point-of-need diagnosis

Abstract: 219 words, main text: 4,147 words, Tables: 5, Figure: 1

Highlights:

- Development and clinical evaluation of two new reverse transcription-insulated isothermal PCR (RT-iiPCR) assays for the detection of Middle East respiratory syndrome coronavirus (MERS-CoV).
- Both RT-iiPCR assays had a comparable analytical sensitivity to previously described real-time RT-qPCR assays targeting the same genes.
- These assays provide a highly sensitive and specific field-deployable point-of-need method for the diagnosis of MERS.

Conflict of Interest

The authors declare the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: authors PAL, YL, YT, and HTW are employed by GeneReach USA, Lexington, MA. The remaining authors declare no conflicting interests with respect to their authorship or the publication of this article.

Funding

This work was financially supported by intramural funding (KK1603-C00) from Korea

Research Institute of Chemical Technology and Korea Centers for Disease Control and Prevention (KCDC, 2015ER480800), Republic of Korea. YYG, SN, KBK, and MK acknowledge support from the KRICT and YYG, MK, and HP acknowledge support from the KCDC (Grant 2015ER480800 to HP).

Abstract

Middle East respiratory syndrome (MERS) is an emerging zoonotic viral respiratory disease that was first identified in Saudi Arabia in 2012. In 2015, the largest MERS outbreak outside of the Middle East region occurred in the Republic of Korea. The rapid nosocomial transmission of MERS-coronavirus (MERS-CoV) in Korean healthcare settings highlighted the importance and urgent need for a rapid and reliable on-site diagnostic assay to implement effective control and preventive measures. Here, we describe the evaluation and validation of two newly developed reverse transcription-insulated isothermal PCR (RT-iiPCR) methods targeting the *ORF1a* and *upE* genes of MERS-CoV. Compared to World Health Organization-recommended singleplex real-time RT-PCR (reference RT-qPCR) assays, both RT-iiPCR assays had comparable analytical sensitivity for the detection of MERS-CoV RNA in tissue culture fluid and in sputum samples spiked with infectious virus. Furthermore, clinical evaluation was performed with sputum samples collected from subjects with acute and chronic respiratory illnesses including MERS-CoV infected patients. The overall agreement values between the two RT-iiPCR assays and the reference RT-qPCR assays were 98.06% (95% CI, 94.43-100%; $\kappa = 0.96$) and 99.03% (95% CI, 95.88-100%; $\kappa = 0.99$) for *ORF1a* and *upE* assays, respectively. In conclusion, the *ORF1a* and *upE* MERS-CoV RT-iiPCR assays coupled with a field-deployable system provide a platform for a highly sensitive and specific on-site tool for diagnosis of MERS-CoV infections.

Introduction

The Middle East respiratory syndrome coronavirus (MERS-CoV), first identified in Saudi Arabia in September 2012, is an emerging zoonotic pathogen that causes severe acute respiratory illness in humans (1). To date, more than 1,900 laboratory-confirmed MERS-CoV infections and 684 human deaths in 27 countries have been reported with a mortality rate of approximately 36% (World Health Organization, http://www.who.int/emergencies/mers-cov/en/, March 24, 2017). MERS-CoV is a zoonotic virus that has repeatedly moved into the human population via contact with the infected dromedary camels in the Arabian Peninsula (World Health Organization**.** MERS-CoV Global Summary and risk assessment. http://www.who.int/emergencies/mers-cov/mers-summary-2016.pdf. December 3 2016). Recent phylogenetic analysis of viral isolates from humans, camels, and bats revealed that bats may have been the original primary reservoir of the virus, and they may have initially transmitted the virus to camels (2). Thus, transmission of MERS-CoV to humans is suspected to occur by direct or indirect contact with infected camels or camel-related products (e.g. raw camel milk, camel urine) (3, 4). Human-to-human transmission of MERS-CoV requires close contact and can occur among relatives in households and among patients and healthcare workers in healthcare settings (nosocomial infection) (5).

Since its emergence, most of the MERS-CoV infections have occurred in the Arabian Peninsula (Kuwait, Bahrain, Qatar, the United Arab Emirates, Oman, Yemen, and Saudi Arabia), but additional cases have been reported from countries in North Africa, Europe, North America, and Asia due to movement of infected individuals. The outbreak in the Republic of Korea in May 2015 was the largest MERS-CoV outbreak ever recorded outside of Saudi Arabia and resulted in 185 laboratory-confirmed human infections in Korea and one

in China, with 36 deaths (6). The index case was traced back to an individual with a travel history to the Middle East. The MERS outbreaks have been attributed to failures of preventive and control measures in healthcare settings (5). Therefore, early diagnosis, prompt isolation of suspected cases, and timely tracing of case contacts are key strategies to prevent further transmission.

Following the emergence of MERS-CoV, several molecular detection methods and serological assays were developed and deployed internationally through an international collaborative laboratory response (7-10). Currently, real-time reverse transcription PCR (RT-qPCR) is the primary method for laboratory diagnosis of MERS-CoV infection, and it requires at least two different genomic targets for a positive diagnosis according to the case definition announced by the World Health Organization (WHO) as of July 3, 2013 (http://www.who.int/csr/disease/coronavirus_infections/case_definition/en/index.html). The two RT-qPCR assays developed by Corman *et al.* (2012) shortly after the first report of the disease were designated as recommended MERS-CoV molecular diagnostics by the WHO (7, 8). Both assays proved to be highly sensitive and were successfully used for the diagnosis of the majority of the MERS-CoV cases. These assays target genomic regions upstream of the envelope gene (*upE*) and the viral open reading frame 1a (*ORF1a*). The RealStar® MERS-CoV RT-qPCR Kit (Alotona Diagnostics, Hamburg, Germany) has been developed using these WHO-recommended assays (11). However, these assays are costly, demand expensive instrumentation, and require a dedicated laboratory environment with technically skilled personnel. Consequently, simple and rapid methods are required to meet the needs of point-of-need MERS-CoV detection. For this purpose, many isothermal RNA amplification methods were developed for exponential amplification of RNA at low and constant

129 temperatures such as rapid one-step RNA amplification/detection (iROAD) assay (12) and
130 reverse transcriptional loop-mediated isothermal amplification (RT-LAMP) (13, 14). The
131 RT-LAMP assay can be performed in a simple heating block.

132 Recently, fluorescent probe hydrolysis-based insulated isothermal PCR (iiPCR) for
133 amplification and detection of nucleic acid has been described (15). The iiPCR is highly
134 sensitive and specific for the detection of both DNA and RNA and can be performed with a
135 single heating source; thus, it does not require an expensive thermocycler (16, 17). The PCR
136 mix in a capillary tube (R-tube™, GeneReach USA, Lexington, MA, USA) is heated at the
137 bottom. Rayleigh-Bénard convection drives fluid cycling through temperature gradients and
138 the three PCR steps, namely denaturation, annealing, and extension, can be completed
139 sequentially at different zones within the capillary tube. Subsequent integration of hydrolysis
140 probe technology and an optical detection module into the device allow automatic detection
141 and interpretation of iiPCR results (17). Performance of iiPCR assays on a commercially
142 available, field-deployable, and user-friendly iiPCR system, the POCKIT™ Nucleic Acid
143 Analyzer (GeneReach USA), has been demonstrated to be comparable to that of real-time
144 PCR, nested PCR, and/or virus isolation for the detection of various pathogens in different
145 hosts, including dengue virus and malaria in human samples (18-32). Taking advantage of
146 this system, in this study we developed two singleplex RT-iiPCR assays for the detection of
147 MERS-CoV *upE* and *ORF1a* genes separately and determined the ability of the assays for
148 viral nucleic acid detection. The analytical sensitivity, analytical specificity, and
149 reproducibility of the two MERS-CoV-specific RT-iiPCR assays were assessed using viral
150 tissue culture fluid (TCF) and human sputum samples spiked with known amounts of MERS-
151 CoV. The clinical performance of these two assays were further evaluated and validated using
152 RNA extracted from sputum samples of MERS-CoV-infected patients obtained from the

153 recent Korean outbreak and compared to the corresponding reference singleplex real-time
154 RT-qPCR assays recommended by WHO.

155 **Materials and Methods**

156 **Viruses and cells.** A patient-derived MERS-CoV isolate (MERS-
157 CoV/KOR/KNIH/002_05_2015; GenBank accession No. KR029139.1) was kindly provided
158 by the Korea Centers for Disease Control and Prevention (Korea CDC, Osong, Republic of
159 Korea). A working virus stock was prepared by passaging MERS-CoV in a human hepatoma
160 cell line, Huh7 cells (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan).
161 The infectious viral titer of the TCF supernatant, expressed as plaque forming units per ml
162 (PFU/ml) was determined by plaque assay using Vero cells (ATCC® CCL-81™; American
163 Type Culture Collection (ATCC®), Manassas, VA, USA) according to a standard laboratory
164 protocol. All procedures using live MERS-CoV were performed in the biosafety level-3
165 facility at Center for Virus Research and Testing, Korea Research Institute of Chemical
166 Technology, Daejeon, Republic of Korea.

167 Human coronaviruses, hCoV-229E (ATCC® VR-740) and hCoV-OC43 (ATCC® VR-1558)
168 were purchased from ATCC and amplified in human fetal lung fibroblast MRC-5 cells
169 (ATCC® CCL-171). Feline infectious peritonitis coronavirus (FIPV, ATCC® VR-990) and its
170 host cell line Crandall feline kidney were obtained from ATCC and Korean Cell Line Bank
171 (Seoul, Republic of Korea), respectively. Other human viral pathogens included in this study
172 were influenza virus type A (H1N1, A/Puerto Rico/8/34 [ATCC® VR-1469]), and influenza
173 virus type B (B/Panama/45/1990 [Korea CDC]). Influenza viruses (types A and B) were
174 propagated by infection of Madin Darby canine kidney cells.

175 **Ethics statement.** Clinical data and specimens obtained from the MERS-CoV infected
176 patients were used in this study following ethical approval granted by the Institutional
177 Review Board of Chungnam National University Hospital, Daejeon, Republic of Korea. All
178 surviving patients provided written informed consent prior to participating in the study. In
179 fatal cases, an exemption to the patients' consent was obtained from the institutional review
180 board for the retrospective analysis of clinical samples. All experiments were performed
181 according to the approved guidelines.

182 **Clinical specimens.** A total of 55 sequential sputum samples collected from twelve MERS-
183 CoV-infected patients were obtained from the Chungnam National University Hospital. These
184 patients were diagnosed positive for MERS-CoV infection by real-time RT-qPCR assays
185 targeting the *ORF1a* and *upE* sequences at the Korea CDC laboratory between May and June
186 of 2015. Sputum samples collected from patients suffering from other acute and chronic
187 respiratory illnesses (n=48) were included in the study as negative samples. Sputum samples
188 from nine healthy individuals were randomly selected and spiked with serially diluted
189 MERS-CoV TCF working stocks.

190 **Nucleic acid extraction.** For analytical sensitivity analysis, TCF containing MERS-CoV (3.7
191 x 10^6 PFU/ml) was subjected to 10-fold serial dilutions (10^0 to 10^7) in Dulbecco's Modified
192 Eagle Medium (HyClone™, UT, USA) containing 10% fetal bovine serum (HyClone™).
193 Viral RNA was extracted from serial dilutions of MERS-CoV TCF and MERS-CoV-spiked
194 sputum samples (100 µl per sample) by using the taco™ DNA/RNA Extraction Kit
195 (GeneReach USA, Lexington, MA, USA) on a taco™ Nucleic Acid Automatic Extraction
196 System (GeneReach USA), according to the manufacturer's instructions. Viral RNA was
197 eluted in 100 µl of elution buffer. Total RNA from sputum samples collected from MERS-

198 CoV-infected patients and from patients suffering from other acute and chronic respiratory
199 illnesses (controls) was extracted using TRIzol LS reagent (ThermoFisher Scientific,
200 Waltham, MA, USA) according to the manufacturer's instruction in a biosafety level-3
201 facility. The final volume of each extracted sample was 50 µl. All nucleic acid samples were
202 placed at -80^{o}C until further use.

203 ***In vitro* transcribed RNA preparation.** The analytical sensitivity of the singleplex MERS-
204 CoV RT-iiPCR assays was determined by using *in vitro* transcribed (IVT) RNA. Briefly, the
205 sequences of the *ORF1a* and *upE* regions of the MERS-CoV (nt 27361 - 27596 and nt 11137
206 - 11339, respectively; GenBank accession number NC_019843) were synthesized, cloned into
207 the pGEM®-3Z vector (Promega, Madison, WI, USA) downstream of the T7 promoter
208 sequence, and subsequently used as the template in *in vitro* transcription using the
209 mMESSAGE mMACHINE® T7 Transcription Kit (Ambion/Life Technologies, Grand Island,
210 NY, USA). Residual DNA was removed using the Ambion Turbo DNA-free kit (Applied
211 Biosystems, Grand Island, NY, USA). Concentration of RNA was measured by a
212 NanoDrop1000 Spectrophotometer (NanoDrop Technologies, Houston, TX, USA). Single
213 use IVT RNA aliquots were stored at -80^{o}C. The analytical sensitivity of the RT-iiPCR was
214 determined using a dilution series (10^0 to 10^8 molecules/reaction) of the IVT RNA. The
215 concentration of the IVT RNA /µl was calculated as described previously (33).

216 **Reference MERS-CoV *ORF1a* and *upE* real time RT-qPCR tests**. The singleplex *ORF1a*
217 and *upE* real time RT-qPCR assays were carried out according to the WHO-recommended
218 protocol (34) using the S*uper*Script III one step RT-PCR system with Platinum Taq
219 polymerase (Invitrogen, Carlsbad, CA, USA). Primers and probes targeting *ORF1a* and *upE*
220 genes were synthesized according to the previously published sequences by GenoTech Corp.

(Daejeon, Republic of Korea) (7, 8). Thermocycling program was set up as follows: an RT step at 50°C for 30 min, followed by 95°C for 2 min and 40 cycles of 15 sec at 95°C and 1 min at 55°C as described previously (35). Duplicate samples with C_T values >38 were considered negative. Each run included negative controls spiked with water and positive controls with the IVT RNA containing target sequences. All clinical specimens were tested for the human *RNase P* gene by RT-qPCR to monitor nucleic acid extraction efficiency and the presence of PCR inhibitors as described elsewhere (35).

Establishment of reverse transcription-insulated isothermal PCR. The MERS-CoV-specific RT-iiPCR was designed on the basis of the probe hydrolysis-based POCKIT™ method as described previously (17). The primers and probe targeted *ORF1a* or *upE* region of MERS-CoV (GenBank accession number NC_019843). The conserved regions were identified by aligning 253 sequences available in the GenBank database. The RT-iiPCR reaction conditions, such as concentrations of primers and probe, Taq DNA polymerase, and reverse transcriptase, were tested systematically to obtain the highest sensitivity and specificity. Following optimization of the RT-iiPCR assay conditions, the reagents including primers and probe were lyophilized (proprietary) and used in this study. Briefly, the lyophilized premix was reconstituted in 50 µl of Premix Buffer B (GeneReach USA), 5 µl of the test nucleic acid extract was added. A 50 µl volume of the premix/sample mixture was transferred into a labeled R-tube™, which was subsequently sealed with a cap, spun briefly in a microcentrifuge (Cubee™, GeneReach USA), and placed into the POCKIT™ Nucleic Acid Analyzer. The default program, including an RT step at 50°C for 10 min and an iiPCR step at 95°C for about 30 min was completed in less than one hour. Signal-to-noise (S/N) ratios, i.e. light signals collected after iiPCR/fluorescent signals collected before iiPCR (17), were

converted automatically to "+", "-", or "?" according to the default S/N thresholds by the built-in algorithm. The results were shown on the display screen at the end of the program. A "?" indicated that the results were ambiguous and the sample should be tested again.

Statistical analysis. Limit of detection with 95% confidence ($LOD_{95\%}$) was determined by statistical probit analysis (a non-linear regression model) using the commercial software SPSS V.14.0 (SPSS Inc., Chicago, IL, USA). The clinical performance of the assays was calculated based on the analysis of the 55 sputum samples from MERS-CoV infected patients and 48 donors not suspected of MERS-CoV infection. The singleplex *ORF1a* or *upE* RT-qPCR assay recommended by WHO for MERS-CoV diagnosis was used as a reference test (35). The degree of agreement between the two assays was assessed by calculating Cohen's Kappa (k) values. Sensitivity was calculated as (number of true positives)/(number of true positives + number of false negatives), and specificity was calculated as (number of true negatives)/(number of true negatives + number of false positives).

Results

Evaluation of analytical sensitivity and specificity of MERS-CoV *ORF1a* and *upE* RT-iiPCR assays.

(i). Analytical sensitivity. The analytical sensitivities of the singleplex MERS-CoV *ORF1a* and *upE* RT-iiPCR assays were evaluated separately by using RNA extracted from serial 10-fold dilutions of TCF containing MERS-CoV. The detection limit for both *ORF1a* and *upE* RT-iiPCR methods were determined to be approximately 3.7×10^{-1} PFU/ml of MERS-CoV in TCF which is equivalent to a technical $LOD_{95\%}$ of <10 copies of synthetic RNA (Table 1). To obtain an estimate of detection limit in a more clinically relevant setting, viral RNA extracted

266 from sputum samples of healthy individuals spiked with 10-fold serial dilutions of MERS-
267 CoV were used. The limit of detection values for both assays for MERS-CoV in sputum were
268 determined to be approximately 3.7×10^{-1} PFU/ml (Table 2) suggesting that the effect of PCR
269 inhibition is minimal. **(ii). Analytical specificity.** The specificities of the singleplex *ORF1a*
270 and *upE* MERS-CoV RT-iiPCR assays were evaluated with viral nucleic acids extracted from
271 infectious TCF containing human coronavirus 229E (hCoV-229E), hCoV-OC43, FIPV, and
272 influenza virus type A and B strains. All reactions yielded negative results, indicating high
273 analytical specificity with no false-positive test results with either assay **(iii) Reproducibility.**
274 Reproducibility of the singleplex *ORF1a* and *upE* MERS-CoV RT-iiPCR assays was assessed
275 by testing independently (three experimental runs) three replicates of the nucleic acid extract
276 of 10^{-5} dilution (3.7×10^{-1} PFU/ml) of infectious TCF. All nine reactions were detected
277 positive, suggesting excellent intra- and inter-assay reproducibility of the established assays
278 (Table 3).

279 **Evaluation of the MERS-CoV *ORF1a* and *upE* RT-iiPCR assays using clinical samples.**

280 In order to determine clinical sensitivity and specificity of the singleplex MERS-CoV *ORF1a*
281 and *upE* RT-iiPCR assays, we analyzed 55 sputum samples consisting of sequential sample
282 sets taken from twelve patients during the course of acute MERS illness. These samples were
283 confirmed to be positive for MERS-CoV infection by the real-time RT-qPCR assay routinely
284 used at the Korean CDC laboratory during the outbreak in Korea in 2015. To estimate the
285 diagnostic performance of the singleplex RT-iiPCR methods, the assay results were compared
286 with the reference singleplex RT-qPCR assays that was run side-by-side (i.e. the reference
287 RT-qPCR assays were repeated with the newly extracted RNA from sputum samples) (34).
288 The results from the initial laboratory testing together with those from the RT-iiPCR and RT-

qPCR using the clinical specimens are shown in Tables 4. The *ORF1a* RT-iiPCR assay was able to detect MERS-CoV RNA in 54 out of 55 (54/55) positively confirmed samples whereas the reference *ORF1a* RT-qPCR assay positively detected 52 out of 55 (52/55). Meanwhile, the *upE* RT-iiPCR positively detected all 55 samples whereas the reference *upE* RT-qPCR assay confirmed 54 out of 55 positive samples (Table 4). Specifically, the previously positive specimen from patient ID P085 (collection date: 2015-06-14) was tested as negative with *ORF1a* RT-iiPCR and reference singleplex *ORF1a* and *upE* RT-qPCR assays. The *upE* RT-iiPCR was the only assay that identified the P085 specimen as positive. Two positive samples from patients ID P130 (collected on 2015-06-14) and ID P148 (collected on 2015-06-21) were tested as false negatives by the reference *ORF1a* RT-qPCR, resulting in detection of 52 out of 55 positive samples (Table 4). Thus, by comparing the RT-iiPCR results to those from the initial laboratory testing at the time of the outbreak, the sensitivities of the singleplex MERS-CoV *ORF1a* and *upE* RT-iiPCR were 99.03% (54/55; 95% CI, 95.88-100%) and 100% (55/55; 95% CI, 97.43-100%), respectively, while those of the reference RT-qPCR were 97.09% (52/55; 95% CI, 87.43-100%) for *ORF1a* and 99.03% (54/55; 95% CI, 95.88-100%) for *upE*, respectively (Table 4).

All samples from patients suffering from other respiratory illnesses were tested as negative with the corresponding assays indicating high specificity (100%). Lastly, the overall agreement values between the RT-iiPCR and reference RT-qPCR were 98.06% (95% CI, 94.43-100%; $\kappa = 0.96$) for *ORF1a* signature, with two positive samples giving discrepant results; and 99.03% (95% CI, 95.88-100%; $\kappa = 0.99$) for *upE* signature, with one discrepant result from a positive sample between the RT-iiPCR and reference RT-qPCR (Table 5).

Discussion

The 2015 MERS-CoV outbreak in the Republic of Korea revealed that a rapid and reliable diagnostic assay suitable for on-site detection of virus is critical and urgently needed to effectively control the spread of infection among individuals. Unfortunately, the existing RT-qPCR assays are not suitable to be used in clinical settings since they require expensive equipment and laboratory environment staffed with skilled technicians. In this study, we describe the development and evaluation of a rapid and highly sensitive field-deployable system for detection of MERS-CoV that allows mobile detection of the virus directly from clinical materials obtained from patients suspected of infection.

A variety of isothermal amplification methods for nucleic acid detection similar to iiPCR such as LAMP and recombinase polymerase amplification have been developed for use in simple point-of-need systems (36-38). Despite its advantages, most LAMP assays still have some technical limitations, such as propensity to produce false-positive reactions and variations in visual observation of LAMP signals between different observers in particular for weak positive samples (39). Recently, several RT-LAMP assays have been described in the literature for detection of MERS-CoV (13, 14, 40) with advantages of being rapid, simple, accurate, and cost-effective suitable for on-site application. However, further validation of these assays is needed using specimens from infected patients to ensure their clinical performance.

The singleplex *ORF1a* and *upE* MERS-CoV RT-iiPCR assays described in this study are performed in commercially available, simple, and compact instruments, the POCKIT™ Nucleic Acid Analyser series, which can process four to thirty-two samples at a time within an hour. The automated interpretation by the iiPCR machine makes the method easier since it

334 does not require data analysis by the user. Furthermore, the ready-to-use lyophilized
335 amplification reagents that are stable for at least two years at 4°C provide great ease of
336 storage and transportation. Removal of contaminating PCR inhibitors and genomic DNA
337 during nucleic acid extraction is critical to avoid false negative and positive results,
338 respectively (41). Thus, optimal RNA extraction and template preparation is key for obtaining
339 highest sensitivity and specificity of any molecular diagnostic assay including RT-iiPCR.
340 Automatic or semi-automatic nucleic acid extraction methods only require basic laboratory
341 training skills and minimize the hands-on time required for template preparation before
342 assembling the reaction. Furthermore, the clinical samples are directly added to the lysis
343 buffer which immediately inactivates the pathogen and thus, reduces the risk of exposure of
344 the personnel handling the clinical material. However, it is important to use appropriate
345 personal protective wears and follow recommended biosecurity guidelines during handling
346 and testing of the clinical specimens. Utilization of a light-weight, field-deployable automatic
347 nucleic acid extraction device (taco™ mini Nucleic Acid Automatic Extraction System,
348 GeneReach USA) along with the iiPCR/POCKIT™ system greatly reduces the hands-on time
349 from sample collection to results (Figure 1). These instruments also can be connected to a
350 car-battery and are designed as a portable system to be used in clinics, hospitals, farms and
351 public health labs in remote areas without electricity. Taking advantage of this platform, the
352 two MERS-CoV singleplex RT-iiPCR methods targeting either *ORF1a* or *upE* gene were
353 established to aid rapid on-site diagnosis of MERS-CoV infection. Multiple RT-qPCR assays
354 targeting different sequences in the MERS-CoV genome have been recommended to be used
355 as sequential primary and confirmatory tests to help reduce the risks of misidentifying
356 MERS-CoV cases (35). Particularly, the *upE* assay has been recommended for screening

purposes while the *ORF1a* assay for confirmation (8). Both *ORF1a*- and *upE*-specific RT-iiPCR assays described in this study were shown to be analytically sensitive and no cross-reactivity was observed with other respiratory viruses, including human coronaviruses such as the hCoV-229E and hCoV-OC43 strains.

Lower respiratory tract specimens such as sputum and tracheal aspirates are the recommended sample types for accurate diagnosis since they are known to contain high viral RNA loads that persist longer compared to other sample types tested (42). Therefore, we compared the detection limit of singleplex *ORF1a* and *upE* MERS-CoV RT-iiPCR assays to the corresponding WHO-recommended reference singleplex RT-qPCR assays using RNA extracted from infectious TCF or human sputum samples spiked with serially diluted MERS-CoV and assessed the assay performance. The data showed that the singleplex *ORF1a* and *upE* RT-iiPCR and the corresponding singleplex reference RT-qPCR assays were able to detect as low as <10 copies/µl of synthetic RNA and 3.7×10^{-1} PFU/ml of infectious TCF. Similar results were obtained when analytical sensitivities of *ORF1a* and *upE* RT-iiPCR assays were evaluated using sputum samples spiked with a MERS-CoV isolate. Furthermore, the clinical sensitivity and specificity of both singleplex MERS-CoV RT-iiPCR assays were evaluated using archived sputum samples collected from confirmed cases of MERS-CoV infection during the Korean outbreak in 2015. To estimate the diagnostic performance, the singleplex RT-iiPCR assay results were compared side-by-side with the reference singleplex RT-qPCR assay. The data indicated that the sensitivities of the singleplex MERS-CoV *ORF1a* and *upE* RT-iiPCR assays were 99.03% (54/55; 95% CI, 95.88-100%) and 100% (55/55; 95% CI, 97.43-100%), respectively, while those of the reference RT-qPCR assays were 97.09% (52/55; 95% CI, 87.43-100%) for *ORF1a* and 99.03% (54/55; 95% CI, 95.88-100%) for *upE*.

Possible explanation for the discrepancy with initial diagnosis seen in two specimens (P085 [2015-06-14] and P130 [2015-06-14]) could be viral RNA degradation during long-term storage. The viral RNA copy number of false-negative samples was below detection limit in both assays (assessed by reference RT-qPCR <10 copies) while the C_T value of internal amplification control remained moderately low (*Rnase P* C_T 27.3 and 30.2, respectively), indicating the possibility of viral RNA loss during storage and/or the extraction process. Moreover, in a number of specimens tested, the overall estimated viral load detected during our study was lower compared to viral copy number estimated a year earlier (data not shown). These observations suggest that sensitivity of the assay can be adversely affected by collection method and storage of clinical specimens. In the case of the specimen from patient ID P148 (collected on 2015-06-21) that was tested as false negative by the reference *ORF1a* RT-qPCR assay, it could potential be due to the presence of extremely low concentrations of target RNA. The RNA levels of the internal amplification control in this sample (*Rnase P* C_T 38.5) were the lowest compared to the rest of the samples, suggesting relatively low RNA extraction levels. Nevertheless, the data presented in this study demonstrate that the performance of both singleplex *ORF1a* and *upE* MERS-CoV RT-iiPCR tests is equivalent or higher compared to the reference singleplex RT-qPCR tests and provides much faster results (within an hour).

Bats and alpacas are potential reservoirs for MERS-CoV in the wild while dromedary camels may be the only animal host responsible for animal-to-human transmission of MERS-CoV (43, 44). Accordingly, regular screening and isolation of MERS-CoV-infected camels have been recommended to help control MERS-CoV spread (45). Thus, the field-deployable MERS-CoV RT-iiPCR on the POCKIT™ system has potential to be used for timely on-site

403 monitoring of MERS-CoV carriers in camel and alpaca herds and in bat populations in the
404 wild. It would be interesting to see whether these newly developed assays could be used to
405 detect MERS-CoV in samples from dromedary camels, bats, and alpacas in epidemiological
406 investigations.

407 Lastly, we conclude that the two rapid, highly sensitive and specific MERS-CoV RT-
408 iiPCR methods coupled with the field-deployable platform described here can be effectively
409 used as an on-site, point-of-need diagnostic tool to aid diagnosis of MERS-CoV infection in
410 clinics, hospitals, airports, or premises where a large number of people congregate (e.g.
411 religious festivals) as well as in epidemiological investigations including animal reservoirs.

412

413 **Acknowledgements**

414 The authors appreciate Dr. Sung Soon Kim (Korea Centers for Disease Control and
415 Prevention, KCDC) for providing the MERS-CoV strains used in this study.

416

References

1. **Chan JF, Lau SK, To KK, Cheng VC, Woo PC, Yuen KY.** 2015. Middle East respiratory syndrome coronavirus: another zoonotic betacoronavirus causing SARS-like disease. Clin Microbiol Rev **28:**465-522.
2. **Han HJ, Yu H, Yu XJ.** 2016. Evidence for zoonotic origins of Middle East respiratory syndrome coronavirus. J Gen Virol **97:**274-280.
3. **Azhar EI, El-Kafrawy SA, Farraj SA, Hassan AM, Al-Saeed MS, Hashem AM, Madani TA.** 2014. Evidence for camel-to-human transmission of MERS coronavirus. N Engl J Med **370:**2499-2505.
4. **Drosten C, Meyer B, Muller MA, Corman VM, Al-Masri M, Hossain R, Madani H, Sieberg A, Bosch BJ, Lattwein E, Alhakeem RF, Assiri AM, Hajomar W, Albarrak AM, Al-Tawfiq JA, Zumla AI, Memish ZA.** 2014. Transmission of MERS-coronavirus in household contacts. N Engl J Med **371:**828-835.
5. **Oboho IK, Tomczyk SM, Al-Asmari AM, Banjar AA, Al-Mugti H, Aloraini MS, Alkhaldi KZ, Almohammadi EL, Alraddadi BM, Gerber SI, Swerdlow DL, Watson JT, Madani TA.** 2015. 2014 MERS-CoV outbreak in Jeddah--a link to health care facilities. N Engl J Med **372:**846-854.
6. **Korea Centers for Disease C, Prevention.** 2015. Middle East Respiratory Syndrome Coronavirus Outbreak in the Republic of Korea, 2015. Osong Public Health Res Perspect **6:**269-278.
7. **Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, van Boheemen S, Gopal R, Ballhause M, Bestebroer TM, Muth D, Muller MA, Drexler JF, Zambon M, Osterhaus AD, Fouchier RM, Drosten C.** 2012. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill **17**.
8. **Corman VM, Muller 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.** 2012. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro Surveill **17**.
9. **Palm D, Pereyaslov D, Vaz J, Broberg E, Zeller H, Gross D, Brown CS, Struelens MJ, Joint E-WHOROfENCLSp, Points ENMF, Network WHOERE, European Network for Diagnostics of "Imported" Viral D.** 2012. Laboratory capability for molecular detection and confirmation of novel coronavirus in Europe, November 2012. Euro Surveill **17**.
10. **Reusken C, Mou H, Godeke GJ, van der Hoek L, Meyer B, Muller MA, Haagmans B, de Sousa R, Schuurman N, Dittmer U, Rottier P, Osterhaus A, Drosten C, Bosch BJ, Koopmans M.** 2013. Specific serology for emerging human coronaviruses by protein microarray. Euro Surveill **18:**20441.
11. **Corman VM, Olschlager S, Wendtner CM, Drexler JF, Hess M, Drosten C.** 2014. Performance and clinical validation of the RealStar MERS-CoV Kit for detection of Middle East respiratory syndrome coronavirus RNA. J Clin Virol **60:**168-171.
12. **Koo B, Jin CE, Lee TY, Lee JH, Park MK, Sung H, Park SY, Lee HJ, Kim SM, Kim JY, Kim SH, Shin Y.** 2017. An isothermal, label-free, and rapid one-step RNA amplification/detection assay for diagnosis of respiratory viral infections. Biosens

Bioelectron **90:**187-194.

13. **Shirato K, Yano T, Senba S, Akachi S, Kobayashi T, Nishinaka T, Notomi T, Matsuyama S.** 2014. Detection of Middle East respiratory syndrome coronavirus using reverse transcription loop-mediated isothermal amplification (RT-LAMP). Virol J **11:**139.
14. **Lee SH, Baek YH, Kim YH, Choi YK, Song MS, Ahn JY.** 2016. One-Pot Reverse Transcriptional Loop-Mediated Isothermal Amplification (RT-LAMP) for Detecting MERS-CoV. Front Microbiol **7:**2166.
15. **Tsai SM, Liu HJ, Shien JH, Lee LH, Chang PC, Wang CY.** 2012. Rapid and sensitive detection of infectious bursal disease virus by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. J Virol Methods **181:**117-124.
16. **Chang HF, Tsai YL, Tsai CF, Lin CK, Lee PY, Teng PH, Su C, Jeng CC.** 2012. A thermally baffled device for highly stabilized convective PCR. Biotechnol J **7:**662-666.
17. **Tsai YL, Wang HT, Chang HF, Tsai CF, Lin CK, Teng PH, Su C, Jeng CC, Lee PY.** 2012. Development of TaqMan probe-based insulated isothermal PCR (iiPCR) for sensitive and specific on-site pathogen detection. PLoS One **7:**e45278.
18. **Tsen HY, Shih CM, Teng PH, Chen HY, Lin CW, Chiou CS, Wang HT, Chang HF, Chung TY, Lee PY, Chiang YC.** 2013. Detection of *Salmonella* in chicken meat by insulated isothermal PCR. Journal of Food Protection **76:**1322-1329.
19. **Balasuriya UB, Lee PY, Tiwari A, Skillman A, Nam B, Chambers TM, Tsai YL, Ma LJ, Yang PC, Chang HF, Wang HT.** 2014. Rapid detection of equine influenza virus H3N8 subtype by insulated isothermal RT-PCR (iiRT-PCR) assay using the POCKIT Nucleic Acid Analyzer. Journal of Virological Methods **207:**66-72.
20. **Tsai YL, Wang HC, Lo CF, Tang-Nelson K, Lightner D, Ou BR, Hour AL, Tsai CF, Yen CC, Chang HF, Teng PH, Lee PY.** 2014. Validation of a commercial insulated isothermal PCR-based POCKIT test for rapid and easy detection of white spot syndrome virus infection in Litopenaeus vannamei. PLoS One **9:**e90545.
21. **Wilkes RP, Tsai YL, Lee PY, Lee FC, Chang HF, Wang HT.** 2014. Rapid and sensitive detection of canine distemper virus by one-tube reverse transcription-insulated isothermal polymerase chain reaction. BMC Veterinary Research **10:**213.
22. **Ambagala A, Pahari S, Fisher M, Lee PA, Pasick J, Ostlund EN, Johnson DJ, Lung O.** 2015. A rapid field-deployable reverse transcription-insulated isothermal polymerase chain reaction assay for sensitive and specific detection of bluetongue virus. Transboundary and Emerging Diseases **doi: 10.1111/tbed.12388**.
23. **Lung O, Pasick J, Fisher M, Buchanan C, Erickson A, Ambagala A.** 2015. Insulated isothermal reverse transcriptase PCR (iiRT-PCR) for rapid and sensitive detection of classical swine fever virus. Transboundary and Emerging Diseases **doi: 10.1111/tbed.12318.**
24. **Wilkes RP, Kania S, Tsai YL, Lee PY, Chang HH, Ma LJ, Chang HF, Wang HT.** 2015. Rapid and sensitive detection of feline immunodeficiency virus using an insulated isothermal polymerase chain reaction-based assay with a point-of-need PCR detection platform. Journal of Virological Methods **27:**510-515.
25. **Wilkes RP, Lee PY, Tsai YL, Tsai CF, Chang HH, Chang HF, Wang HT.** 2015. An insulated isothermal PCR method on a field-deployable device for rapid and sensitive detection of canine parvovirus type 2 at points of need. Journal of Virological

509 Methods **220:**35-38.
510 26. **Chua KH, Lee PC, Chai HC.** 2016. Development of insulated isothermal PCR for
511 rapid on-site malaria detection. Malaria Journal **15:**134.
512 27. **Go YY, Rajapakse RP, Kularatne SA, Lee PA, Ku KB, Nam S, Chou PH, Tsai YL,**
513 **Liu YL, Chang HG, Wang HT, Balasuriya UB.** 2016. A pan-dengue virus reverse
514 transcription-insulated isothermal polymerase chain reaction (RT-iiPCR) assay
515 intended for point-of-need diagnosis of dengue infection using POCKIT™ Nucleic
516 Acid Analyzer. Journal of Clinical Microbiology **54:**1528-1535.
517 28. **Kuo HC, Lo DY, Chen CL, Tsai YL, Ping JF, Lee CH, Lee PA, Chang HG.** 2016.
518 Rapid and sensitive detection of *Mycoplasma synoviae* by an insulated isothermal
519 polymerase chain reaction-based assay on a field-deployable device. Poultry Science
520 **doi: 10.3382/ps/pew228**.
521 29. **Soltan MA, Tsai YL, Lee PA, Tsai CF, Chang HG, Wang HT, Wilkes RP.** 2016.
522 Comparison of electron microscopy, ELISA, real time RT-PCR and insulated
523 isothermal RT-PCR for the detection of Rotavirus group A (RVA) in feces of different
524 animal species. Journal of Virological Methods **235:**99-104.
525 30. **Zhang J, Tsai YL, Lee PA, Chen Q, Zhang Y, Chiang CJ, Shen YH, Li FC,**
526 **Chang HG, Gauger PC, Harmon KM, Wang HT.** 2016. Evaluation of two
527 singleplex reverse transcription-Insulated isothermal PCR tests and a duplex real-time
528 RT-PCR test for the detection of porcine epidemic diarrhea virus and porcine
529 deltacoronavirus. Journal of Virological Methods **234:**34-42.
530 31. **Carossino M, Lee PY, Nam B, Skillman A, Shuck KM, Timoney PJ, Tsai YL, Ma**
531 **LJ, Chang HF, Wang HT, Balasuriya UB.** 2016. Development and evaluation of a
532 reverse transcription-insulated isothermal polymerase chain reaction (RT-iiPCR) assay
533 for detection of equine arteritis virus in equine semen and tissue samples using the
534 POCKIT system. Journal of Virological Methods **234:**7-15.
535 32. **Go YY, Rajapakse RP, Kularatne SA, Lee PY, Ku KB, Nam S, Chou PH, Tsai YL,**
536 **Liu YL, Chang HF, Wang HT, Balasuriya UB.** 2016. A Pan-Dengue Virus Reverse
537 Transcription-Insulated Isothermal PCR Assay Intended for Point-of-Need Diagnosis
538 of Dengue Virus Infection by Use of the POCKIT Nucleic Acid Analyzer. J Clin
539 Microbiol **54:**1528-1535.
540 33. **Lu Z, Branscum AJ, Shuck KM, Zhang J, Dubovi EJ, Timoney PJ, Balasuriya**
541 **UB.** 2008. Comparison of two real-time reverse transcription polymerase chain
542 reaction assays for the detection of Equine arteritis virus nucleic acid in equine semen
543 and tissue culture fluid. J Vet Diagn Invest **20:**147-155.
544 34. **WHO.** June 2015. Laboratory Testing for Middle East Respiratory Syndrome
545 Coronavirus (MERS-CoV).
546 http://apps.who.int/iris/bitstream/10665/176982/1/WHO_MERS_LAB_15.1_eng.pdf?
547 ua=1. Accessed
548 35. **Lu X, Whitaker B, Sakthivel SK, Kamili S, Rose LE, Lowe L, Mohareb E,**
549 **Elassal EM, Al-sanouri T, Haddadin A, Erdman DD.** 2014. Real-time reverse
550 transcription-PCR assay panel for Middle East respiratory syndrome coronavirus. J
551 Clin Microbiol **52:**67-75.
552 36. **James A, Macdonald J.** 2015. Recombinase polymerase amplification: Emergence as
553 a critical molecular technology for rapid, low-resource diagnostics. Expert Rev Mol
554 Diagn **15:**1475-1489.
555 37. **Parida M, Sannarangaiah S, Dash PK, Rao PV, Morita K.** 2008. Loop mediated

isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Rev Med Virol **18:**407-421.

38. **Mori Y, Notomi T.** 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J Infect Chemother **15:**62-69.
39. **Goto M, Honda E, Ogura A, Nomoto A, Hanaki K.** 2009. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. Biotechniques **46:**167-172.
40. **Bhadra S, Jiang YS, Kumar MR, Johnson RF, Hensley LE, Ellington AD.** 2015. Real-time sequence-validated loop-mediated isothermal amplification assays for detection of Middle East respiratory syndrome coronavirus (MERS-CoV). PLoS One **10:**e0123126.
41. **Schrader C, Schielke A, Ellerbroek L, Johne R.** 2012. PCR inhibitors - occurrence, properties and removal. J Appl Microbiol **113:**1014-1026.
42. **Oh MD, Park WB, Choe PG, Choi SJ, Kim JI, Chae J, Park SS, Kim EC, Oh HS, Kim EJ, Nam EY, Na SH, Kim DK, Lee SM, Song KH, Bang JH, Kim ES, Kim HB, Park SW, Kim NJ.** 2016. Viral Load Kinetics of MERS Coronavirus Infection. N Engl J Med **375:**1303-1305.
43. **Mohd HA, Al-Tawfiq JA, Memish ZA.** 2016. Middle East Respiratory Syndrome Coronavirus (MERS-CoV) origin and animal reservoir. Virol J **13:**87.
44. **Reusken CB, Haagmans BL, Muller MA, Gutierrez C, Godeke GJ, Meyer B, Muth D, Raj VS, Smits-De Vries L, Corman VM, Drexler JF, Smits SL, El Tahir YE, De Sousa R, van Beek J, Nowotny N, van Maanen K, Hidalgo-Hermoso E, Bosch BJ, Rottier P, Osterhaus A, Gortazar-Schmidt C, Drosten C, Koopmans MP.** 2013. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. Lancet Infect Dis **13:**859-866.
45. **Omrani AS, Al-Tawfiq JA, Memish ZA.** 2015. Middle East respiratory syndrome coronavirus (MERS-CoV): animal to human interaction. Pathog Glob Health **109:**354-362.

Figure legend:

Figure 1. POCKIT™ system workflow for point-of-need detection of MERS-CoV RNA.

This system includes a compact automatic nucleic acid extraction device (taco™ mini) and a portable PCR device (POCKIT™). After sample collection, nucleic acids are extracted using a preloaded extraction plate in approximately 30 min and, subsequently, the lyophilized RT-iiPCR reaction is reconstituted and nucleic acids are added. The mixture was transferred to an

595 R-tube™ and tested in a POCKIT™ device. TaqMan® probe hydrolysis-based amplification
596 signals are automatically detected, processed, and interpreted, providing qualitative results on
597 the display screen in 60 min.

598

Table 1. Analytical sensitivity analysis of MERS-CoV *ORF1a* and *upE* RT-iiPCR using TCF containing MERS-CoV

Assay	PFU/ml	MERS-CoV RT-iiPCR	MERS-CoV RT-qPCR
MERS-CoV *ORF1a*	3.7×10^{1}	3/3	3/3
	3.7×10^{0}	3/3	3/3
	3.7×10^{-1}	**3/3**	**3/3**
	3.7×10^{-2}	1/3	1/3
	3.7×10^{-3}	0/3	0/3
	3.7×10^{-4}	0/3	0/3
MERS-CoV *upE*	3.7×10^{1}	3/3	3/3
	3.7×10^{0}	3/3	3/3
	3.7×10^{-1}	**3/3**	**3/3**
	3.7×10^{-2}	1/3	1/3
	3.7×10^{-3}	0/3	0/3
	3.7×10^{-4}	0/3	0/3

599

600

Table 2. Analytical sensitivity analysis of MERS-CoV *ORF1a* and *upE* RT-iiPCR assays using virus-spiked sputum samples

Sample no.	Virus amount (PFU/ml)	*ORF1a*		*upE*	
		RT-iiPCR	RT-qPCR (C_T)	RT-iiPCR	RT-qPCR (C_T)
SP1	3.7×10^{1}	+	+ (28.60)	+	+ (28.28)
	3.7×10^{0}	+	+ (32.31)	+	+ (31.92)
	3.7×10^{-1}	+	+ (35.58)	+	+ (35.31)
	No virus	-	-	-	-
SP2	3.7×10^{1}	+	+ (32.34)	+	+ (31.35)
	3.7×10^{0}	+	+ (36.60)	+	+ (35.30)
	3.7×10^{-1}	+	- (38.93)	+	+ (37.84)
	No virus	-	-	-	-
SP7	3.7×10^{1}	+	+ (31.18)	+	+ (30.85)
	3.7×10^{0}	+	+ (34.87)	+	+ (34.75)
	3.7×10^{-1}	+	+ (36.92)	+	+ (36.49)
	No virus	-	-	-	-
SP8	3.7×10^{1}	+	+ (29.01)	+	+ (28.65)
	3.7×10^{0}	+	+ (32.48)	+	+ (32.14)
	3.7×10^{-1}	+	+ (36.34)	+	+ (35.60)
	No virus	-	-	-	-
SP9	3.7×10^{1}	+	+ (29.54)	+	+ (29.12)
	3.7×10^{0}	+	+ (33.44)	+	+ (32.78)
	3.7×10^{-1}	+	+ (34.61)	+	+ (34.18)
	No virus	-	-	-	-
SP10	3.7×10^{1}	+	+ (31.48)	+	+ (30.94)
	3.7×10^{0}	+	+ (32.80)	+	+ (32.33)
	3.7×10^{-1}	+	+ (36.35)	+	+ (35.77)
	No virus	-	-	-	-
SP11	3.7×10^{1}	+	+ (28.35)	+	+ (28.39)
	3.7×10^{0}	+	+ (31.04)	+	+ (30.80)
	3.7×10^{-1}	+	+ (32.87)	+	+ (32.30)
	No virus	-	-	-	-
SP13	3.7×10^{1}	+	+ (28.04)	+	+ (28.23)
	3.7×10^{0}	+	+ (31.50)	+	+ (31.96)
	3.7×10^{-1}	+	+ (32.62)	+	+ (33.33)
	No virus	-	-	-	-
SP14	3.7×10^{1}	+	+ (28.19)	+	+ (28.50)
	3.7×10^{0}	+	+ (30.93)	+	+ (30.86)
	3.7×10^{-1}	+	+ (32.30)	+	+ (32.94)
	No virus	-	-	-	-

601
602

Table 3. Intra- and inter-assay variability of MERS-CoV *ORF1a* and *upE* RT-iiPCR

MERS-CoV *ORF1a* RT-iiPCR

Assay Run	RT-iiPCR Result* (Positive/Total)	S/N † (Average ± SD)
1	3/3	4.95 ± 0.03
2	3/3	4.92 ± 0.02
3	3/3	4.85 ± 0.11

MERS-CoV *upE* RT-iiPCR

Assay run	RT-iiPCR Result* (Positive/Total)	S/N † (Average ± SD)
1	3/3	4.94 ± 0.05
2	3/3	4.85 ± 0.05
3	3/3	4.93 ± 0.05

* Nucleic acid extract of 10^{-5} dilution (3.7 x 10-1 PFU/ml) of MERS-CoV infectious TCF

†S/N: signal-to-noise ratio; SD: standard deviation

603

Table 4. Detection of MERS-CoV by RT-iiPCR and RT-qPCR in sputum samples collected from MERS-CoV-infected patients

Patient's ID	Age	Gender	Date of onset	Outcome	No. of samples	Collection date*	Initial laboratory testing (RT-PCR)†	MERS-CoV RT-iiPCR		MERS-CoV RT-qPCR		Estimated viral load‡ (Log_{10} [copies])		Internal control (C_T)§
								ORF1a	*upE*	*ORF1a*	*upE*	*ORF1a*	*upE*	*RNase P*
P016	40	M	2015-05-19	Recovered	8	2015-06-09	+	+	+	+	+	3.906	4.195	35.8
						2015-06-10	+	+	+	+	+	2.681	3.111	32.8
						2015-06-10'	+	+	+	+	+	3.507	3.289	32.2
						2015-06-11	+	+	+	+	+	6.024	6.482	30.9
						2015-06-11'	+	+	+	+	+	5.779	6.224	32.1
						2015-06-13	+	+	+	+	+	5.266	5.837	31.9
						2015-06-14	+	+	+	+	+	2.363	2.846	32.3
						2015-06-14'	+	+	+	+	+	2.478	3.128	32.7
P030	60	M	2015-05-30	Recovered	6	2015-06-08	+	+	+	+	+	6.527	7.049	29.7
						2015-06-09	+	+	+	+	+	4.936	5.132	28.4
						2015-06-09'	+	+	+	+	+	4.917	5.243	29.7
						2015-06-11	+	+	+	+	+	6.017	6.406	27.4
						2015-06-11'	+	+	+	+	+	5.986	6.360	28.4
						2015-06-11"	+	+	+	+	+	6.238	6.521	24.9
P031	69	M	2015-05-30	Fatal	5	2015-06-08	+	+	+	+	+	6.821	6.864	27.8
						2015-06-11	+	+	+	+	+	7.878	8.242	28.2
						2015-06-12	+	+	+	+	+	5.996	6.416	34.1
						2015-06-15	+	+	+	+	+	6.478	6.125	25.3
						2015-06-17	+	+	+	+	+	5.322	5.003	24.6
P054	62	F	2015-05-31	Recovered	4	2015-06-12	+	+	+	+	+	6.306	6.447	31.4
						2015-06-14	+	+	+	+	+	6.701	7.025	28.4
						2015-06-15	+	+	+	+	+	5.672	5.700	27.1
						2015-06-16	+	+	+	+	+	5.983	5.812	31.9
P082	82	F	2015-06-04	Fatal	3	2015-06-11	+	+	+	+	+	2.716	4.159	31.0
						2015-06-13	+	+	+	+	+	6.146	6.719	26.3
						2015-06-14	+	+	+	+	+	3.756	4.600	27.9

P085	65	F	2015-06-06	Recovered	5	2015-06-10	+	+	+	+	+	4.347	4.100	26.6
						2015-06-13	+	+	+	+	+	5.049	5.138	35.0
						2015-06-14	+	n.d.	+	n.d.	n.d.	n.d.	n.d.	27.3
						2015-06-15	+	+	+	+	+	4.376	4.590	29.5
						2015-06-16	+	+	+	+	+	3.842	4.241	31.8
P110	57	F	2015-06-02	Recovered	2	2015-06-13	+	+	+	+	+	5.538	5.491	29.7
						2015-06-13'	+	+	+	+	+	5.040	5.411	27.5
P122	55	F	2015-06-06	Recovered	6	2015-06-10	+	+	+	+	+	6.168	6.731	29.2
						2015-06-12	+	+	+	+	+	5.075	5.221	34.5
						2015-06-13	+	+	+	+	+	5.218	5.750	31.7
						2015-06-14	+	+	+	+	+	4.866	5.177	29.5
						2015-06-15	+	+	+	+	+	3.100	3.401	29.8
						2015-06-16	+	+	+	+	+	3.702	3.789	33.2
P130	65	F	2015-06-12	Recovered	2	2015-06-14	+	+	+	n.d.	+	n.d.	2.672	30.2
						2015-06-16	+	+	+	+	+	2.160	2.604	30.2
P148	38	F	2015-06-06	Recovered	4	2015-06-15	+	+	+	+	+	3.564	3.678	35.8
						2015-06-17	+	+	+	+	+	5.781	6.081	33.1
						2015-06-18	+	+	+	+	+	5.516	5.843	31.3
						2015-06-21	+	+	+	n.d.	+	n.d.	2.743	38.5
P172	60	F	2015-06-15	Recovered	10	2015-06-22	+	+	+	+	+	5.359	5.812	29.8
						2015-06-25	+	+	+	+	+	3.936	4.074	28.0
						2015-06-25	+	+	+	+	+	4.614	4.722	28.3
						2015-06-27	+	+	+	+	+	3.133	3.007	28.0
						2015-06-28	+	+	+	+	+	4.949	5.153	26.0
						2015-06-28'	+	+	+	+	+	4.559	4.922	26.4
						2015-06-29	+	+	+	+	+	3.961	4.157	28.2
						2015-06-29'	+	+	+	+	+	2.923	3.328	26.9
						2015-06-29''	+	+	+	+	+	2.710	3.308	25.8
						2015-06-30	+	+	+	+	+	5.497	5.791	27.8

*Dates indicated with (') and ('') indicate sample replicate

†Patients were confirmed positive by real-time RT-qPCR at the Korean Center for Disease Control laboratory during the 2015 outbreak

‡Viral loads (Log_{10} copies) were quantified by using *in vitro* transcribed RNA derived from the amplicon region of each assay

§All specimens were tested for the human *Rnase P* gene to monitor nucleic acid extraction efficiency and the presence of PCR inhibitors as described in Lu et al. (2014).

604 'n.d', not detected.
605

Table 5. Diagnostic performance comparison between MERS-CoV RT-iiPCR and RT-qPCR assays

ORF1a		MERS-CoV RT-qPCR		
		Positive	Negative	Total
MERS-CoV RT-iiPCR	Positive	52	2	54
	Negative	0	49	49
		52	51	103

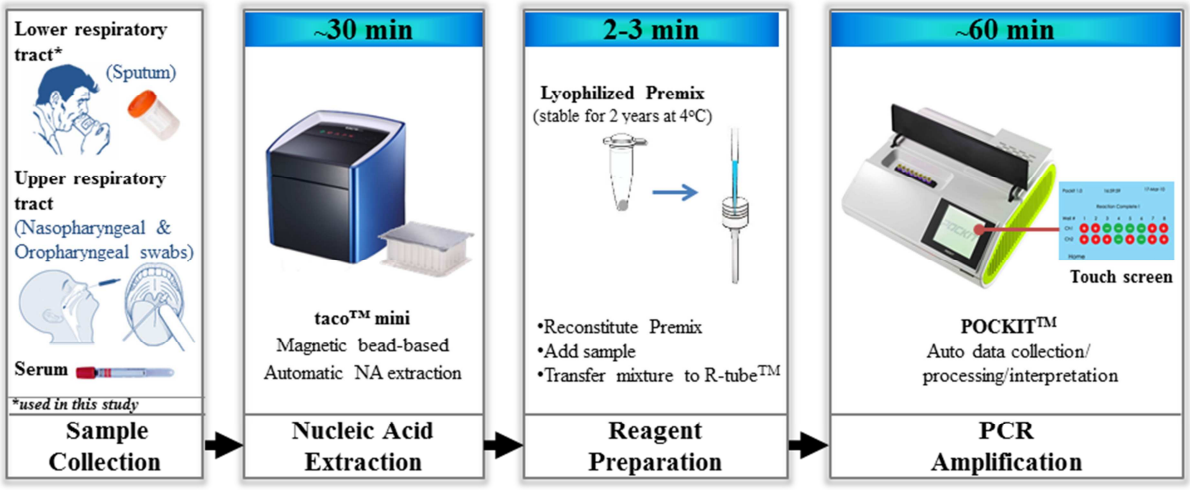
Agreement (95% CI): 98.06% (94.43-100 %); κ = 0.96

upE		MERS-CoV RT-qPCR		
		Positive	Negative	Total
MERS-CoV RT-iiPCR	Positive	54	1	55
	Negative	0	48	48
		54	49	103

Agreement (95% CI): 99.03% (95.88-100 %); κ = 0.99

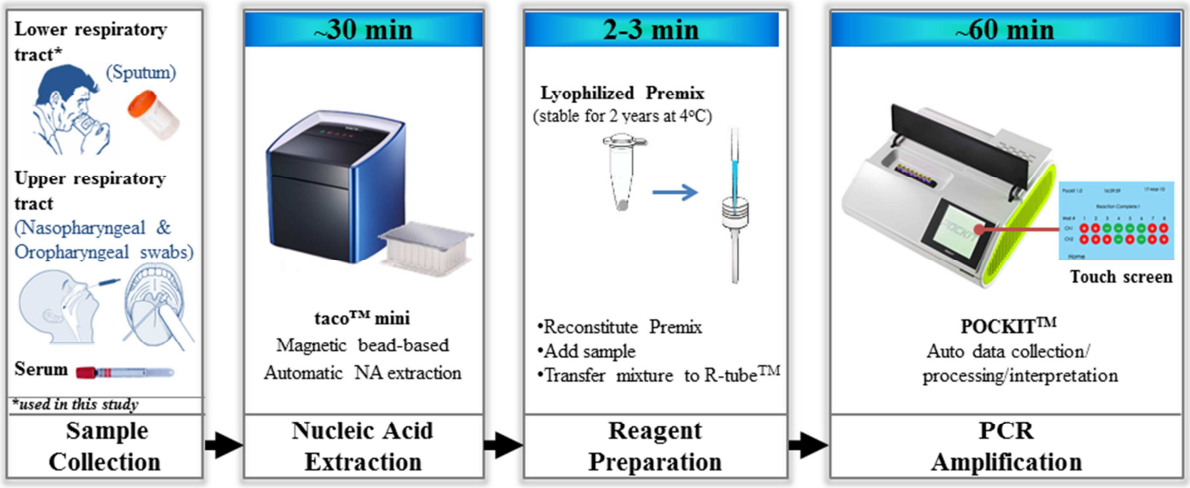
606

607 Figure 1.



POCKIT™ MERS-CoV Detection (~1.5 h)

608



POCKIT™ MERS-CoV Detection (~1.5 h)