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Original Article

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Summary

Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) is usually diagnosed through highly sensitive and specific genetic tests such as real-time reverse transcription polymerase chain reaction (RT-PCR). Currently, two real-time RT-PCR assays targeting the upE and ORF1a regions of the MERS-CoV genome are widely used and are the standard assays recommended by the World Health Organization (WHO). The MERS outbreaks to date suggest that rapid diagnosis and subsequent isolation of infected patients, particularly superspreaders, are critical for containment. However, conventional real-time RT-PCR assays require large laboratory instruments, and amplification takes approximately 2 h. These are disadvantages for rapid diagnosis. Here, an ultra-rapid real-time RT-PCR test was established: a multiplex assay for upE and ORF1a running on the mobile PCR1100 device. As few as five copies of MERS-CoV RNA can be detected within 20 min using the WHO standard assays with similar sensitivity and specificity to those of a conventional real-time PCR instrument such as the LightCyler, enabling timely intervention to control MERS-CoV infection.

Introduction

Middle East respiratory syndrome (MERS) is an emerging respiratory disease caused by

the MERS coronavirus (MERS-CoV). MERS has been endemic mainly in Saudi Arabia since 2012 (1, 2). As of August 26, 2019, a total of 2,458 confirmed cases, with 849 deaths, had been reported from 27 countries [World Health Organization (WHO), https://www.who.int/emergencies/mers-cov/en/]. According to the case definition of the WHO, amplification of at least two different genomic targets is required for positive diagnosis (WHO, http://www.who.int/csr/disease/coronavirus_infections/case_definition/en/index.html). Therefore, the likelihood of positive diagnosis of MERS-CoV increases as the number of sensitive genetic diagnostic methods used increases. Various genetic assays have been developed, such as real-time reverse transcription polymerase chain reaction (RT-PCR) (3, 4), reverse transcription loop-mediated isothermal amplification (RT-LAMP) (5, 6), and reverse transcription recombinase polymerase amplification (RT-RPA) (7). Of these, the real-time RT-PCR assays developed by Corman et al. are widely used (these are the WHO standard); a primer/probe set targeting the upE region of the MERS-CoV genome is used for the first screening test and a set targeting the open reading frame (ORF) 1a region is used for the confirmation test (3, 4). In the Corman assay, positive results in both tests are required for a positive diagnosis of MERS-CoV.

Real-time RT-PCR assays are commonly used to detect the genomes of pathogens; this wide prevalence is one of their advantages. However, conventional laboratory real-time RT-PCR assays require large installed instruments (i.e., a thermal cycler with a fluorometer), and depending on the reagents used, amplification requires approximately 2 h. The tests are thus time-consuming. In 2015, an outbreak of MERS occurred in the Republic of Korea; the 186 cases included 36 fatalities (8). The first case was a returnee from the Middle East who had visited four hospitals prior to MERS diagnosis and had infected 28 patients (9). Further superspreading events occurred in healthcare facilities. Fifteen of the 186 cases transmitted MERS-CoV to others; five were considered superspreaders, two of whom transmitted the virus to 80 and 24 patients, respectively (10, 11). The Korean outbreak emphasizes the importance of rapid diagnosis and isolation of MERS cases, especially superspreaders (10, 11). Therefore, a rapid diagnostic method is essential. The time required for diagnosis via real-time RT-PCR must be dramatically shortened by optimizing the RNA extraction method, the RT approach and real-time RT-PCR master mix, and the real-time RT-PCR instrument; this is the case even for conventional real-time RT-PCR.

Recently, an ultra-rapid mobile PCR device, the PicoGene PCR1100 (Nippon Sheet Glass, Tokyo, Japan), was developed. This device enables ultra-rapid real-time

PCR by moving the reaction mix via air pressure between areas of two different temperatures in the device through shallow grooves in the chips (i.e., there is no requirement to increase or decrease temperature), facilitating rapid execution of a (maximally) three-color multiple real-time RT-PCR assay. Here, we developed an ultra-rapid real-time RT-PCR system featuring the multiplex Corman assay on the PCR1100 for rapid detection of MERS-CoV.

Materials and Methods

Viruses: The MERS-CoV EMC strain was kindly provided by Dr. Ron A. M. Fouchier, Erasmus Medical Center, Rotterdam, The Netherlands. Human orthopneumoviruses [respiratory syncytial virus (RSV), Long, A2, B WV/14617/85 (B1 wild type), and 18537] were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Human metapneumovirus (HMPV; Sendai-H/2404/2003) was obtained from the Virus Research Center, Sendai Medical Center, Japan. Human coronavirus (HCoV)-229E isolates ATCC VR-740, Sendai-H/1121/04, and Niigata/01/08 (12) were used. HCoV-NL63 (Amsterdam I) was supplied by Dr. Lia van der Hoek, University of Amsterdam, the Netherlands. HCoV-OC43 ATCC VR-1558 was used. SARS coronavirus (the Frankfurt strain) was supplied by Dr. J. Ziebuhr, University of

Würzburg, Germany. Human respiroviruses [parainfluenza viruses (PIV) 1 (strain C35) and 3 (strain C243)] were obtained from ATCC. Adenoviruses (ADVs) (serotype 3, strain G.B.; serotype 4, strain RI-67; and serotype 7, strain Gomen) were also obtained from ATCC. Viruses were propagated and titrated using HEp-2, HeLa, RD, Vero, VeroE6, LLC-Mk2, or Vero/TMPRSS2 cells (13); otherwise, copy numbers were calculated by real-time RT-PCR (14). Influenza viruses [Flu; A/California/7/2009 (H1N1pdm), A/Victoria/210/2009 (H3N2), and B/Brisbane/60/2008] were propagated and titrated using MDCK cells. Clinical isolates of HCoV-OC43 (Tokyo/SGH-36/2014, LC315646; Tokyo/SGH-61/2014, LC315647; Tokyo/SGH-06/2015, LC315648), HKU1 (Tokyo/SGH-15/2014, LC315650; Tokyo/SGH-18/2016, LC315651), and NL63 (Tokyo/SGH-15/2017, LC488390; Tokyo/SGH-18/2018, LC488389; Tokyo/SGH-24/2018, LC488388) were isolated and propagated using human bronchial tracheal epithelial cells (Lifeline Cell Technology, Frederick, MD, USA), cultured, and allowed to differentiate at an air-liquid interface, as previously described (15). Copy numbers were calculated via virus-specific real-time RT-PCR (16).

Extraction of nucleic acids from viral stocks: RNA was extracted using TRIzol LS, the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) from viral stocks,

and the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used for extractions from specimens, according to the manufacturers' instructions. Viral DNA was extracted using the SimplePrep Reagent for DNA (TaKaRa Bio Inc., Shiga, Japan), according to the manufacturer's instructions. Nasopharyngeal swab (NPS), nasal swab (NS), sputum, and bronchoalveolar lavage (BAL) specimens were obtained from Discovery Life Sciences (Los Osos, CA, USA); these were used in spike tests with the approval of the Research and Ethical Committee for the Use of Human Subjects of the National Institute of Infectious Diseases, Japan (approval #1003). For spike tests, infectious MERS-CoV was mixed with clinical specimens at various concentrations together with an RNase inhibitor and subjected to RNA extraction. A positive control RNA of ribonuclease (RNase)P-transcribed from T7 promotor-incorporated PCR template was also used in the validation tests.

Real-time RT-PCR: Corman assays (targeting upE and ORF1a regions) were used to detect MERS-CoV (3, 4), using the following primers and probes: upE-forward, 5′-GCAACGCGCGATTCAGTT-3′; upE-reverse, 5′-GCCTCTACACGGGACCCATA-3′; upE-probe, 5′-CTCTTCACATAATCGCCCCGAGCTCG-3′; ORF1a-forward,

5′-CCACTACTCCCATTTCGTCAG-3′; ORF1a-reverse, 5′-CAGTATGTGTAGTGCGCATATAAGCA-3′; and ORF1a-probe, 5′-TTGCAAATTGGCTTGCCCCCACT-3′. To detect single targets, both probes were labeled with fluorescein amidite (FAM) and carboxytetramethylrhodamine (TAMRA). Single-target detection was performed using the AgPath-ID One-Step RT-PCR reagents (Thermo Fisher Scientific) on a LightCycler 480 (Roche, Basel, Switzerland) or LightCycler 96 (Roche) system, following the manufacturer's instructions. The amplification conditions of Corman et al. were utilized (3, 4). For multiplex detection, the upE probe was labeled with FAM and the ORF1a probe with Cy5; Black Hole Quencher (BHQ) was used to quench both reactions. The RNase P gene served as the internal control, using the primer/probe set described previously (17): forward, 5′-AGATTTGGACCTGCGAGCG-3′; reverse 5′-GAGCGGCTGTCTCCACAAGT-3′; and probe HEX-5′-TTCTGACCTGAAGGCTCTGCGCG-3′-BHQ.

An ultra-rapid real-time RT-PCR assay employed the components of the KAPA3G Plant PCR Kit (KAPA Biosystems, Wilmington, MA, USA). FastGene Scriptase II (NIPPON Genetics, Tokyo, Japan) or SMART MMLV Reverse Transcriptase (TaKaRa Bio) served as the reverse transcriptase (RT). The components of the reaction mixture and the primer/probe mixtures are shown in Table 1. The following real-time RT-PCR

conditions were set for the PCR 1100: 55°C for 180 sec; 95°C for 15 sec; and 50 cycles of 95°C for 5 sec and 60°C for 7 sec. The longest reaction time displayed on device screen was 19 min, 17 sec. In the displayed reaction time, the PCR 1100 device estimates the longest required time as 2 min, 15 sec for initial stabilization of block temperatures. The time depends on the environment of the test performed. If the block temperatures are stabilized within a shorter duration than 2 min, 15 sec, the total reaction time also becomes shorter. The assay sensitivity was calculated by the Reed-Muench method.

Results

The PCR1100 is a very small real-time PCR device powered by an alternating current supply or by a portable battery (Fig. 1a). For PCR reactions, specific chips with hollow grooves are required (Fig. 1b). Reagents are applied after peeling the front cover seal (Fig. 1b, i), which is subsequently resealed. The chip is set in place after peeling the back cover seal (Fig. 1b, ii). The device features three heated panels (Fig. 1c). Panel 1 is used for the RT reaction. PCR proceeds via back-and-forth movements between panels 2 and 3. The PCR mixture is moved through the grooves by air pressure; the air is first passed through air filters on the chip (Fig. 1b, iii). PCR is performed under closed

conditions to prevent environmental contamination. Fluorescence is monitored as the mixture moves between panels 2 and 3. The amplifications can be seen in real-time manner on the monitor screen and the cycle thresholds are calculated automatically (Fig. 1d).

First, we evaluated sensitivity (Table 2). The upE and ORF1a primer/probe sets detected MERS-CoV RNA at the level of several copies after singleplex reaction using a general real-time RT-PCR instrument (the LightCycler), as previously described (3, 4). Using ultra-rapid real-time RT-PCR, multiplex assays detecting upE, ORF1a, and RNase P simultaneously detected the internal control and MERS-CoV RNA, and the sensitivity for viral RNA was approximately five copies. We next evaluated specificity (Table 3). As Corman et al. reported, the upE and ORF1a primer/probe sets did not cross-react with any other respiratory pathogens in singleplex reactions (3, 4). After ultra-rapid real-time RT-PCR, the multiplex assays also exhibited no cross-reactions with the genomes of any other respiratory viral pathogens. Thus, the sensitivity and specificity were equivalent to those of the LightCycler singleplex reaction, even when multiplex reactions were concluded within 20 min.

Spike tests. As MERS-CoV-positive clinical specimens are not available in Japan, spike

tests were performed using mixtures of MERS-CoV EMC isolates and clinical specimens. NPS and NS specimens were used to mimic the tests with upper respiratory tract specimens, and sputum and BAL specimens to mimic the tests with lower respiratory tract specimens. As shown in Table 4, the detection kinetics of single- and multi-plex assays were similar, as were their sensitivities. These results suggest that the ultra-rapid real-time RT-PCR assay is capable of detecting MERS-CoV with a sensitivity similar to that of the conventional real-time RT-PCR assay, including in specimens from the upper and lower respiratory tract.

Discussion

We developed an ultra-rapid real-time RT-PCR assay (a multiplex Corman assay) to detect MERS-CoV using a mobile PCR device, the PicoGene PCR1100. The assay detected MERS-CoV RNA with a sensitivity and specificity equivalent to those of conventional real-time PCR instruments, including in clinical specimens. The Corman assay for MERS-CoV detection is the standard WHO assay (https://www.who.int/csr/disease/coronavirus_infections/mers-laboratory-testing/en/). We found that the standard WHO assay for MERS-CoV detection can be performed within 20 min, without compromising quality.

The greatest advantage afforded by the PCR1100 is a reduced total reaction time, which is mediated by moving the reaction mixture between regions at two temperatures, i.e., no change in temperature is needed, reducing the assay duration. The Corman assays use TaqMan probes (3, 4). The KAPA3G DNA polymerase was used for ultra-rapid real-time RT-PCR; this was the only polymerase tested that could drive the TaqMan assay during ultra-rapid real-time RT-PCR on the PCR1100. In the TaqMan assay, probe removal via the 5′ to 3′ exonuclease activity of Taq DNA polymerase is essential (18), but it takes time. We found that the KAPA3G DNA polymerase reduced the extension time; i.e., the probe-disassembly time, to 7 sec. The denaturing step required 5 sec because the upper temperature limit of the PCR1100 was 95°C. Thus, the time required for one TaqMan RT-PCR cycle was 12 sec. To maintain the sensitivity, the number of PCR cycles was increased to 50, but the reduced PCR-cycle time enabled the assay to conclude within 20 min, including 180 sec for the RT reaction.

In this study, RNA purified with an extraction kit was used as a template. The ultra-rapid real-time RT-PCR reduced the reaction time from approximately 2 h to fewer than 20 min; however, RNA extraction remained time-consuming for both ultra-rapid and conventional real-time RT-PCR. Currently, RNA extraction for real-time RT-PCR can be performed with a commercial simple extraction buffer, such as

CellAMP (TaKaRa-Bio), RealTime ready Cell Lysis Kit (Roche), and others. These buffers enable subsequent real-time RT-PCR analysis after a brief mixing procedure. Therefore, the total time for the diagnostic test for MERS-CoV can be shortened in combination with these types of simple extraction buffers, which is worth consideration.

We have developed RT-LAMP assays for the rapid detection of MERS-CoV within 30 min (5, 6). Based on the WHO case definition, detection of at least two distinct genomic targets is required for a positive diagnosis. Therefore, target regions for the two RT-LAMP primer sets differ from those of the two Corman assays; a positive result in at least two of these four tests fulfills the WHO criteria. However, in Japan, the detection method for MERS-CoV is restricted to "PCR" by notification from the Ministry of Health, Labour and Welfare. Therefore, regardless of whether a MERS-CoV positive case fulfills WHO criteria, if the detection method is not PCR, the case results cannot be considered positive in Japan. Importantly, containment measures for a MERS-CoV-positive patient begin when the diagnosis test results fulfill the WHO criteria; however, the result of the Corman assay is required for an official announcement of MERS case. Therefore, acceleration of the Corman assay has been validated in this study as much as possible. We believe that the findings of this study will be helpful for other countries in which Corman assays are the only method

approved by the authorities.

As described above, a rapid diagnostic method is essential for the detection and isolation of superspreaders. The ultra-rapid real-time RT-PCR assay will enable timely intervention by accelerating administrative decisions. The viral titer is higher in the lower than in the upper respiratory tract; thus, specimens from the lower tract are recommended for diagnosis of MERS-CoV (19-21). The ultra-rapid real-time RT-PCR assay detected MERS-CoV RNA in not only upper respiratory tract specimens but also lower respiratory tract specimens. Furthermore, upon detection of MERS-CoV infection, the WHO strongly recommends repeated sampling of multiple bodily compartments (including the upper respiratory tract) for further PCR testing after PCR confirmation (WHO, https://www.who.int/csr/disease/coronavirus_infections/mers-laboratory-testing/en/). It is important to determine the duration of viral shedding when implementing nosocomial infection control. Such testing is also essential to determine when a hospitalized MERS patient can be discharged. Our ultra-rapid real-time RT-PCR assay will aid in the performance of a large number of repeated tests by reducing the effort and valuable time of all staff involved in MERS control measures.

List of Abbreviations

BAL: bronchoalveolar lavage

CoV: coronavirus

EMC: Erasmus Medical Center

HCoV: human coronavirus

MERS: Middle East respiratory syndrome

NPS: nasopharyngeal swab

NS: nasal swab

ORF: open reading flame

PCR: polymerase chain reaction

PIV: parainfluenza virus

RSV: respiratory syncytial virus

RT: reverse transcription

RNase: ribonuclease

SARS: severe acute respiratory syndrome

Competing Interests

The authors declare that they have no competing interests.

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Table 1. Components of the PCR1100 RT-PCR mixture.

Component	Concentration	Amount (µL)	Final concentration
2× buffer (Plant 3G kit)	0.4 mM dNTPs and 1.5 mM $MgCl_2$	10	dNTPs, 0.2mM
$MgCl_2$ (Plant 3G kit)	25 mM	1.35	$MgCl_2$, 3.1875 mM
Primer/Probe mix	See Table 2	0.8	
RT	200 U/µL	1.15	23 U/µL
DNA Polymerase (Plant 3G kit)	2.5 U/µL	1.5	0.375 U/µL
Dithiothreitol (DTT; RT kit)	100 mM	0.2	1 mM
RNA		5	
		Total 20	

Component	Volume of 100 mM stock (µL)	Final concentration
upE Forward	0.085	425 nM
upE Reverse	0.145	725 nM
upE Probe	0.08	400 nM
ORF1a Forward	0.085	425 nM
ORF1a Reverse	0.145	725 nM
ORF1a Probe	0.08	400 nM
RNase P Forward	0.04	200 nM
RNase P Reverse	0.08	400 nM
RNase P Probe	0.06	300 nM
Total	0.8	

Table 2. Sensitivity of ultra-rapid real-time RT-PCR performed using the PCR1100.

Copies/reaction	5,000	500	50	5	0.5	0.05
LightCycler (singleplex)	Positive/number					
upE	6/6	6/6	6/6	3/6	0/6	
ORF1a	6/6	6/6	6/6	5/6	0/6	

Copies/reaction	5,000	500	50	5	0.5	0.05
PCR1100 (multiplex)	Positive/number					
upE	3/3	3/3	3/3	5/6	0/6	0/3
ORF1a	3/3	3/3	3/3	3/6	1/6	0/3
RNase P*	3/3	3/3	3/3	6/6	6/6	3/3

* Each sample contained 45,000 copies of control RNA (encoding RNase P).

Table 3. Specificity of ultra-rapid real-time RT-PCR performed using the PCR1100.

Virus	Strain	Amount / reaction	PCR1100 upE	PCR1100 ORF1a	PCR1100 RNase P*
Coronaviruses					
MERS-CoV	EMC	1×10^5 copies	+	+	+
SARS-CoV	Frankfurt	1×10^4 $TCID_{50}$	-	-	+
HCoV-229E	VR-740	2.5×10^4 PFU	-	-	+
	Sendai-H/1121/04	5×10^4 PFU	-	-	+
	Niigata/01/08	2×10^2 PFU	-	-	+
HCoV-NL63	Amsterdam I	1×10^2 FFU	-	-	+
	Tokyo/SGH-15/2017	6.2×10^5 copies	-	-	+
	Tokyo/SGH-18/2018	3.3×10^6 copies	-	-	+
	Tokyo/SGH-24/2018	5.1×10^5 copies	-	-	+
HCoV-OC43	VR-1558	2.5×10^4 $TCID_{50}$	-	-	+
	Tokyo/SGH-36/2014	2×10^5 copies	-	-	+
	Tokyo/SGH-61/2014	1×10^6 copies	-	-	+
	Tokyo/SGH-06/2015	1×10^5 copies	-	-	+
HCoV-HKU1	Tokyo/SGH-15/2014	5×10^5 copies	-	-	+
	Tokyo/SGH-18/2016	5×10^5 copies	-	-	+
Other respiratory viruses					
Human orthopneumovirus	RSV, Long	5×10^4 copies	-	-	+
	RSV, A2	1×10^6 copies	-	-	+
	RSV, CH/18537	5×10^4 copies	-	-	+
	RSV, B1	1×10^6 copies	-	-	+
Human metapneumovirus	Sendai-H/3404/2003	1.2×10^6 PFU	-	-	+
Human respirovirus 1	PIV1, C-35	1.2×10^3 PFU	-	-	+
Human respirovirus 3	PIV3, C-243	1×10^5 PFU	-	-	+
Human adenovirus 3	G.B.	2×10^6 $TCID_{50}$	-	-	+
Human adenovirus 4	RI-67	2×10^6 $TCID_{50}$	-	-	+
Human adenovirus 7	Gomen	2×10^6 $TCID_{50}$	-	-	+
Influenza viruses					
H1N1pdm	A/California/7/2009	4×10^3 $TCID_{50}$	-	-	+
H3N2	A/Victoria/210/2009	1.25×10^6 $TCID_{50}$	-	-	+
B	B/Brisbane/60/2008	1.25×10^3 $TCID_{50}$	-	-	+

* Each sample contained 50,000 copies of control RNA (encoding RNase P)

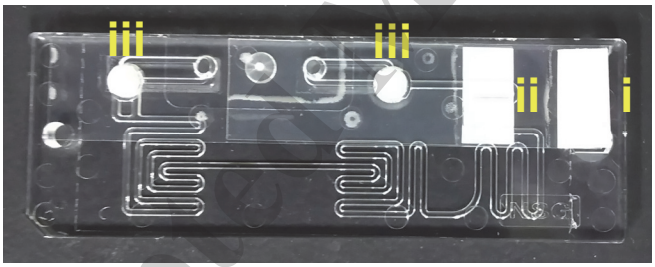
Table 4. Results of spike tests.

NPS	Copies/reaction		100	10	1	
			Positive results/total			Sensitivity (copies)
	Single	upE	4/4	3/4	0/4	5.6
		ORF1a	4/4	2/4	0/4	10.0
	Multiple	upE	6/6	3/6	0/6	10.0
		ORF1a	6/6	5/6	0/6	4.6
		RNase P	6/6	6/6	6/6	
NS	Copies/reaction		100	10	1	
			Positive results/total			Sensitivity (copies)
	Single	upE	4/4	1/4	0/4	17.8
		ORF1a	4/4	0/4	0/4	31.6
	Multiple	upE	6/6	1/6	0/6	21.5
		ORF1a	6/6	1/6	0/6	21.5
		RNase P	6/6	6/6	6/6	
Sputum	Copies/reaction		1000	100	10	
			Positive results/total			Sensitivity (copies)
	Single	upE	4/4	2/4	0/4	100
		ORF1a	4/4	1/4	0/4	178
	Multiple	upE	6/6	2/6	0/6	147
		ORF1a	6/6	0/6	0/6	316
		RNase P	6/6	6/6	6/6	
BAL	Copies / reaction		100	10	1	
			Positive results/total			Sensitivity (copies)
	Single	upE	4/4	0/4	0/4	31.6
		ORF1a	4/4	1/4	0/4	17.8
	Multiple	upE	6/6	3/6	0/6	10.0
		ORF1a	6/6	3/6	0/6	10.0
		RNase P	6/6	6/6	6/6	

Fig.1

a)

b)



c)

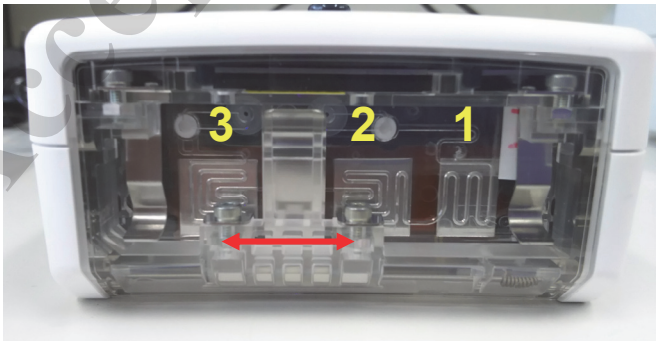


Fig.1

d)

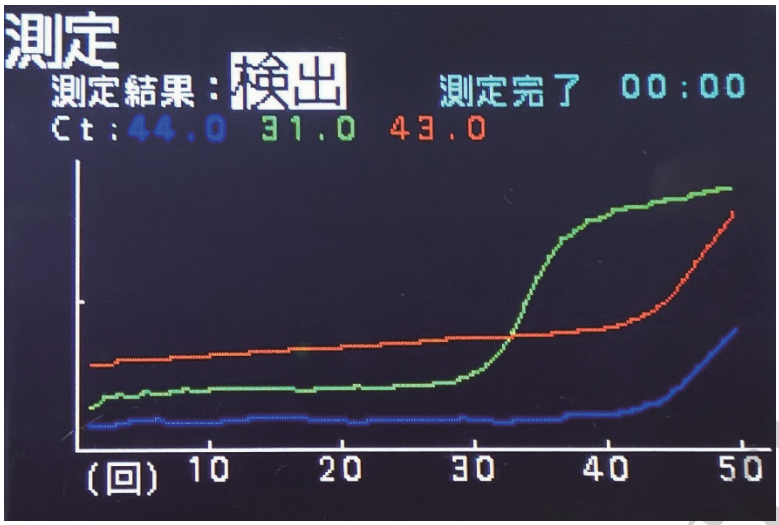


Figure 1. Photographs of the PicoGene PCR1100. a) A photograph of the front. The device is approximately 20 cm in length. The PCR results are shown on the central screen. b) A photograph of a PCR1100 chip; a shallow groove is apparent. The reagent insertion slot is covered by the front seal (i). The vent filters (iii) are covered by the back seal (ii). c) The three heating panels. Panel 1 is used for RT. PCR proceeds via back-and-forth movements between panels 2 and 3. The PCR mixture is moved through the grooves by air pressure. d) The monitor screen image after amplification. The amplifications are plotted in three colors. Blue shows FAM, green shows VIC, and red shows Cy5 fluorescence. Currently, Japanese is the only display language of the PCR1100.