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Evaluation of the commercially available LightMix® Modular E-gene kit using clinical and proficiency testing specimens for SARS-CoV-2 detection

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ABSTRACT

Background: Rapid and sensitive diagnostic assays for SARS-CoV-2 detection are required for prompt patient management and infection control. The analytical and clinical performances of LightMix® Modular SARS and Wuhan CoV E-gene kit, a widely used commercial assay for SARS-CoV-2 detection, have not been well studied.
Objective: To evaluate the performance characteristics of the LightMix® E-gene kit in comparison with well-validated in-house developed COVID-19 RT-PCR assays.
Study design: Serial dilutions of SARS-CoV-2 culture isolate extracts were used for analytical sensitivity evaluation. A total of 289 clinical specimens from 186 patients with suspected COVID-19 and 8 proficiency testing (PT) samples were used to evaluate the diagnostic performance of the LightMix® E-gene kit against in-house developed COVID-19-RdRp/Hel and COVID-19-N RT-PCR assays.
Results: The LightMix® E-gene kit had a limit of detection of 1.8×10^{-1} $TCID_{50}$/mL, which was one $\log_{10}$ lower than those of the two in-house RT-PCR assays. The LightMix® E-gene kit (149/289 [51.6%]) had similar sensitivity as the in-house assays (144/289 [49.8%] for RdRp/Hel and 146/289 [50.5%] for N). All three assays gave correct results for all the PT samples. Cycle threshold (Cp) values of the LightMix® E-gene kit and in-house assays showed excellent correlation. Reproducibility of the Cp values was satisfactory with intra- and inter-assay coefficient of variation values < 5%. Importantly, the LightMix® E-gene kit, when used as a stand-alone assay, was equally sensitive as testing algorithms using multiple COVID-19 RT-PCR assays.
Conclusions: The LightMix® E-gene kit is a rapid and sensitive assay for SARS-CoV-2 detection. It has fewer verification requirements compared to laboratory-developed tests.

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1. Background

In late December 2019, a novel coronavirus, now named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified in patients with pneumonia in Wuhan, China [1]. SARS-CoV-2 is efficiently transmitted from person to person and has rapidly disseminated globally [2,3]. The World Health Organization declared Coronavirus Disease 2019 (COVID-19) as a pandemic in early March 2020. As of 23 May 2020, over 4.9 million COVID-19 cases including more than 327,000 deaths attributable to SARS-CoV-2 have been reported globally (https://www.who.int/emergencies/diseases/novel-coronavirus-2019). Rapid, sensitive and specific diagnostic tests for COVID-19 are of paramount importance to facilitate early identification of cases, contact tracing, and isolation [4,5].

Reverse transcription-polymerase chain reaction (RT-PCR) is the gold standard for laboratory diagnosis of COVID-19 (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance). While a number of in-house and commercial RT-PCR assays for COVID-19 have been developed in the past few months, the clinical performance of some of these assays has not been well studied.

2. Objectives

In the present study, we evaluated the performance of a commercially available LightMix® Modular SARS and Wuhan CoV E-gene kit capable of detecting SARS-CoV-2 RNA against well-validated in-house developed RT-PCR assays targeting the RNA-dependent RNA polymerase (RdRp)/Helicase (Hel) and nucleocapsid (N) regions of SARS-CoV-2 using archived clinical specimens and proficiency testing samples [6].

3. Study design

3.1. Viruses, clinical specimens and proficiency testing samples

SARS-CoV-2 was isolated from the nasopharyngeal aspirate specimen of a patient with COVID-19 in Hong Kong as previously described [7]. SARS-CoV-2 stock (1.8×10^7 $TCID_{50}$/mL) was prepared by one additional passage in VeroE6 cells [8,9]. For analytical sensitivity evaluation, 10-fold serial dilutions of total nucleic acid (TNA) extracted from the SARS-CoV-2 isolate were used. For analytical specificity evaluation, TNA extracted from a clinical specimen positive for human coronavirus HKU1 (HCoV-HKU1) and 17 culture isolates of other human-pathogenic coronaviruses and respiratory viruses were used [6,10]. For clinical evaluation, 289 clinical specimens (236 respiratory tract and 53 non-respiratory tract) from 186 hospitalized patients (male : female = 90 : 96; median age: 37 years; range: 18–97 years) with suspected COVID-19 were selected for SARS-CoV-2 RNA detection. In addition to clinical specimens, 8 proficiency testing samples from Quality Control for Molecular Diagnostics (QCMD) with different concentrations of SARS-CoV-2 RNA or negative for SARS-CoV-2 RNA were also evaluated.

3.2. Nucleic acid extraction

TNA extraction was performed using NucliSENS easyMAG extraction system (BioMerieux, Marcy-l'Étoile, France) according to the manufacturer's instructions and as previously described [11–13]. Briefly, 250 µL of each respiratory tract specimen, rectal swab and stool specimen were subjected to extraction with an elution volume of 55 µL; and 100 µL of each plasma specimen were subjected to extraction with an elution volume of 25 µL. The extracts were stored at −80 °C until use.

3.3. Real-time RT-PCR assays for SARS-CoV-2 RNA detection

LightMix® Modular SARS and Wuhan CoV E-gene kit (TIB Molbiol, Berlin, Germany) with LightCycler Multiplex RNA Virus Master (Roche, Basel, Switzerland) was used according to the manufacturer's instructions. Briefly, each 20 µL reaction mixture contained 5.4 µL of water, 4 µL of Roche Master, 0.5 µL of reagent mix, 0.1 µL of RT Enzyme, and 10 µL of TNA as the template. RT-PCR was performed on a LightCycler 480 II Real-Time PCR System (Roche). The thermal cycling condition was 55 °C for 5 min, 95 °C for 5 min, followed by 45 cycles of 95 °C for 5 s, 60 °C for 15 s and 72 °C for 15 s.

In-house developed COVID-19-RdRp/Hel and COVID-19-N RT-PCR assays were performed using QuantiNova Probe RT-PCR Kit (QIAGEN, Hilden, Germany) on the LightCycler 480 II Real-Time PCR System (Roche) as previously described [6]. Each 20 µL reaction mixture contained 10 µL of 2x QuantiNova Probe RT-PCR Master Mix, 0.2 µL of QN Probe RT-Mix, 1.6 µL of each 10 µM forward and reverse primer, 0.4 µL of 10 µM probe, 1.2 µL of nuclease-free water and 5 µL of TNA as the template. The thermal cycling condition was 45 °C for 10 min, 95 °C for 5 min, followed by 45 cycles of 95 °C for 5 s and 55 °C for 30 s.

3.4. Statistical analysis

Fisher's exact test was used to compare the performance of the assays. Spearman's correlation was used to assess the relation between the Cp values of different assays. The Cp values obtained from the three assays were compared using ANOVA Friedman test with Dunn's multiple comparisons test (a Cp value of 41 was assigned to specimens that tested negative in the real-time RT-PCR assay). Statistical analysis was performed using GraphPad Prism 8. $P < 0.05$ was considered statistically significant.

4. Results

4.1. Analytical sensitivity, analytical specificity and imprecision of the LightMix® E-gene assay

To determine the analytical sensitivity of the LightMix® E-gene assay, the limit of detection (LOD) was evaluated by using TNA extracted from the SARS-CoV-2 isolate. Serial 10-fold dilutions of SARS-CoV-2 TNA extracted from the viral culture isolate were prepared and tested in triplicate for each concentration in two independent runs. The LOD of the E-gene assay was 1.8×10^{-1} $TCID_{50}$/mL (Table 1).

To investigate whether the LightMix® E-gene assay would non-specifically amplify other human-pathogenic coronaviruses and respiratory viruses, we tested TNA extracted from the clinical respiratory specimen with HCoV-HKU1, and TNAs extracted from the 17 culture isolates of SARS-CoV, MERS-CoV, HCoV-OC43, HCoV-NL63, HCoV-229E, influenza A ((H1N1)pdm09 and H3N2) viruses, influenza B virus, influenza C virus, parainfluenza virus types 1–4, respiratory syncytial virus, human metapneumovirus, human rhinovirus and human

Table 1
Test results for determining the limit of detection of the LightMix® Modular SARS and Wuhan CoV E-gene assay with genomic RNA extracted from a SARS-CoV-2 culture isolate.

Virus titer ($TCID_{50}$/mL)	Cp (Intra-run)			Cp (Inter-run)		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
1.8×10^{1}	30.04	30.05	29.91	30.23	30.42	30.44
1.8×10^{0}	33.24	33.61	33.64	33.57	34.01	33.77
1.8×10^{-1}	36.50	36.03	36.64	37.25	37.61	37.05
1.8×10^{-2}	40.00	40.00	–	–	–	38.16
1.8×10^{-3}	37.87	–	–	–	–	–

Abbreviations: -, negative; Cp, cycle number at detection threshold.

Table 2
Imprecision of the LightMix® Modular SARS and Wuhan CoV E-gene assay using the SARS-CoV-2 culture isolate extracts.

Virus titer ($TCID_{50}$/mL)	Number of positive replicates	Intra-assay Mean Cp ± SD (% coefficient of variation)	Inter-assay Mean Cp ± SD (% coefficient of variation)
1.8×10^{1}	3	30.00 ± 0.08 (0.26)	30.18 ± 0.22 (0.72)
1.8×10^{0}	3	33.50 ± 0.22 (0.67)	33.64 ± 0.25 (0.75)
1.8×10^{-1}	3	36.39 ± 0.32 (0.88)	36.85 ± 0.57 (1.54)

adenovirus. The LightMix® E-gene assay did not cross react with these respiratory viruses, except SARS-CoV.

Different concentrations of TNA extracted from the SARS-CoV-2 isolate were used to evaluate intra- and inter-assay variations by the LightMix® E-gene assay. Each concentration was tested in triplicate in two independent runs. The total imprecision (% CV) values ranged from 0.72% to 1.54% (Table 2).

4.2. Comparative performance of the LightMix® E-gene assay and the in-house COVID-19-RdRp/Hel and COVID-19-N assays for the detection of SARS-CoV-2 RNA in clinical specimens and proficiency testing samples

Overall, 289 clinical specimens from 186 patients were evaluated in this study. The LightMix® E-gene kit detected 149/289 [51.6%] specimens, and had similar sensitivity as the in-house assays (144/289 [49.8%] for RdRp/Hel, P = 0.739; and 146/289 [50.5%] for N, P = 0.868). Of these 289 specimens, 195 were initial specimens obtained from the 186 patients with suspected COVID-19. Among these 186 patients, 72 patients were positive for SARS-CoV-2 RNA by at least two of the assays and 114 patients were negative by all three assays in their initial specimens. For the initial specimens of these patients, the positive detection rate of the LightMix® E-gene assay was 71/72 (98.6%), while those of the in-house COVID-19-RdRp/Hel and COVID-19-N assays were 71/72 (98.6%) and 70/72 (97.2%), respectively. The remaining specimens were follow-up specimens of the confirmed cases. Among a total of 94 follow-up specimens obtained from the laboratory-confirmed COVID-19 patients, 11 were negative by all three assays. Seventy-eight (83.0%) were positive by the LightMix® E-gene assay, while 73 (77.7%) and 76 (80.9%) were positive by the in-house COVID-19-RdRp/Hel and COVID-19-N assays, respectively. We then compared the sensitivity of the LightMix® E-gene assay for the follow-up clinical specimens against a testing algorithm involving combination of two of the three or all three assays. We found that adding the in-house assays to the LightMix® E-gene assay did not result in a significant increase in sensitivity (Table 3). There was no significant difference in the detection rate between the LightMix® E-gene assay and our in-house assays. The sensitivity of these assays did not differ significantly for both respiratory and non-respiratory tract specimens (Table 4). For the specimens with discordant results, their mean cycle threshold (Cp) value was 36.7, which represented very low viral RNA load. Among the 8 proficiency testing samples from QCMD, all three assays provided 100% correct results. A good agreement in the performance of the LightMix® E-gene assay compared to the in-house assays was evidenced by a strong correlation (Spearman's $\rho > 0.97$; $P < 0.0001$) (Fig. 1). The Cp values obtained from the 3 different assays were also examined. The median Cp value of the LightMix® E-gene assay (29.3) was significantly lower than those of the COVID-19-RdRp/Hel (30.9; $P < 0.0001$) and COVID-19-N (31.3; $P < 0.0001$) assays (Fig. 2).

Table 3
Comparative performance of the three RT-PCR assays in follow-up specimens of confirmed COVID-19 patients.

Combination of different assays	No. of specimens missed by the assay(s) / total no. of positive follow-up specimens (%)	*P*-value*
LightMix® E-gene	5/83 (6.0)	N/A
COVID-19-RdRp/Hel	10/83 (12.0)	0.279
COVID-19-N	7/83 (8.4)	0.766
LightMix® E-gene + COVID-19-RdRp/Hel	0/83 (0)	0.059
LightMix® E-gene + COVID-19-N	3/83 (3.6)	0.720
COVID-19-RdRp/Hel + COVID-19-N	3/83 (3.6)	0.720
All 3 assays	0/83 (0)	0.059

* *P*-value for comparison between LightMix® E-gene assay and other assay combinations. N/A: not applicable.

5. Discussion

An increasing number of in-house and commercial COVID-19 RT-PCR assays have been described in the past 5 months [14–26]. The commercially available LightMix® Modular SARS and Wuhan CoV E-gene kit is widely used in clinical laboratories, but its performance has not been thoroughly evaluated with clinical specimens. In the present study, we compared the performance of the LightMix® E-gene kit with two previously established and validated in-house COVID-19 RT-PCR assays using a variety of clinical specimens and proficiency testing samples [6].

According to the manufacturer instructions, the LightMix® E-gene assay can detect not only SARS-CoV-2, but other sarbecoviruses including SARS-CoV and bat SARS-related coronaviruses. In our analytical specificity evaluation, the LightMix® E-gene assay detected SARS-CoV but not other common human-pathogenic coronaviruses and respiratory viruses, while our in-house COVID-19-RdRp/Hel and COVID-19-N assays were specific for SARS-CoV-2 without cross-reactivity with SARS-CoV. The LightMix® E-gene assay was likely intentionally designed to cross-react with SARS-CoV because of the scarce information on the genetic diversity of SARS-CoV-2 in human and animals in late December 2019 when it was developed. To avoid under-diagnosis, the primers and probe targeting the viral E gene were designed to detect not only SARS-CoV-2 but also other sarbecoviruses. This strategy was similarly used for designing other RT-PCR assays in the earlier phase of the COVID-19 pandemic [14,15]. It would therefore be a reasonable strategy to use the sensitive LightMix® E-gene assay as the first-line screening assay for suspected COVID-19 cases, followed by confirmation by sequencing or another RT-PCR assay specific to SARS-CoV-2 (https://www.who.int/publications-detail/laboratory-testing-for-2019-novel-coronavirus-in-suspected-human-cases-20200117).

The LightMix® E-gene assay was highly sensitive for SARS-CoV-2 RNA detection, with LOD of 1.8×10^{-1} $TCID_{50}$/mL, which is one $\log_{10}$ $TCID_{50}$/mL lower than our previously described COVID-19-RdRp/Hel and COVID-19-N assays (1.8 $TCID_{50}$/mL) [6]. The median Cp value of the LightMix® E-gene assay was also significantly lower than the in-house assays. This might be due to the higher volume of specimen template used in the LightMix® E-gene assay than the in-house assays. Another possibility is that the LightMix® E-gene assay and our in-house assays were performed using different PCR reagents and thermocycling conditions. Nevertheless, no significant difference in the sensitivity was noted among these three assays for both respiratory and non-respiratory tract specimens. Reproducibility of the Cp values was satisfactory with the intra- and inter-assay coefficient of variation values of < 5% [27–29]. The Cp values of the LightMix® E-gene/COVID-19-RdRp/Hel and E-gene/COVID-19-N assays showed excellent

Table 4
Comparative performance of the three RT-PCR assays in respiratory and non-respiratory tract specimens.

Specimen type[a]	No. of positive test results/no. of specimens (%)			*P* value[b]	*P* value[c]
	LightMix® Modular E-gene	COVID-19-RdRp/Hel	COVID-19-N		
Respiratory tract	117/236 (49.6)	113/236 (47.9)	115/236 (48.7)	0.782	0.927
NPA/NPS/TS	94/202 (46.5)	87/202 (43.1)	90/202 (44.6)	0.548	0.764
Saliva	23/34 (67.6)	26/34 (76.5)	25/34 (73.5)	0.590	0.791
Non-respiratory	32/53 (60.4)	31/53 (58.5)	31/53 (58.5)	1	1
Stool/rectal swabs	23/33 (69.7)	24/33 (72.7)	22/33 (66.7)	1	1
Plasma	9/20 (45.0)	7/20 (35.0)	9/20 (45.0)	0.748	1
Total	149/289 (51.6)	144/289 (49.8)	146/289 (50.5)	0.739	0.868

[a]Abbreviations: NPA, nasopharyngeal aspirate; NPS, nasopharyngeal swab; TS, throat swab [b] *P* value for LightMix® E-gene and COVID-19-RdRp/Hel assays [c] *P* value for LightMix® E-gene and COVID-19-N assays.

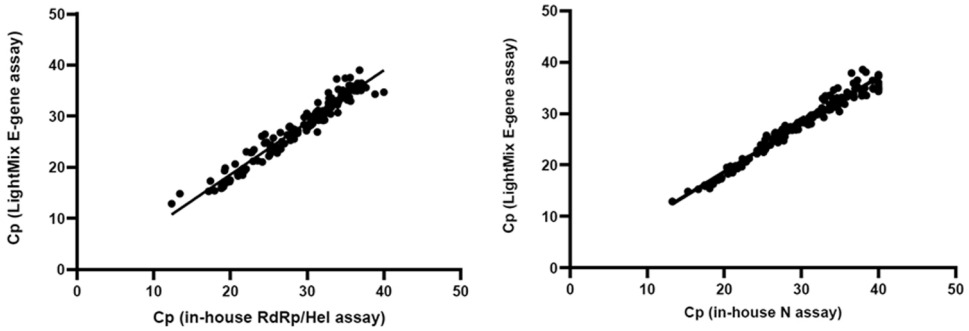

Fig. 1. Correlation of the Cp values of the specimens tested positive for SARS-CoV-2 RNA by the assays. (A) LightMix® E-gene assay vs in-house COVID-19-RdRp/Hel assay and (B) LightMix® E-gene assay vs in-house COVID-19-N assay.

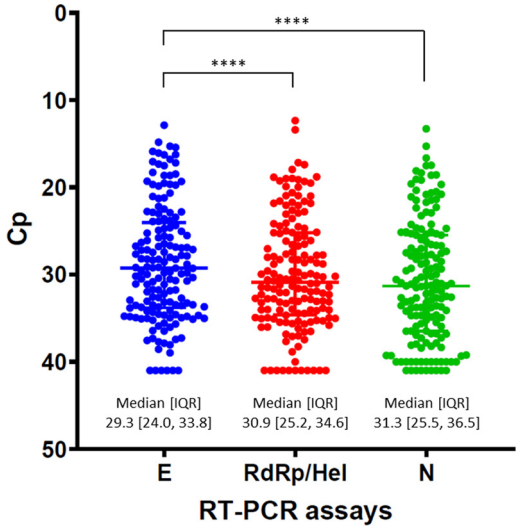

Fig. 2. Comparison of the Cp values of the three RT-PCR assays in this study. **** indicates P < 0.0001.

correlation. All three assays performed well in the proficiency testing samples from QCMD. These findings suggested that the LightMix® E-gene assay and our in-house assays showed excellent diagnostic performance for SARS-CoV-2 RNA detection.

Healthcare facilities including our hospital use RT-PCR negativity as a criterion for hospital discharge. However, the false-negative rate of RT-PCR assays may rise during the convalescent phase of illness as the patient's viral RNA load decreases. Thus, it remains controversial as to how many RT-PCR assays targeting different gene regions should be used to test convalescent phase patients. Our results showed that the LightMix® E-gene assay performed well as a stand-alone test with similar sensitivity as other testing algorithms using multiple tests for follow-up clinical specimens. This feature is reassuring and obviates the need for testing follow-up specimens with multiple assays, especially in areas where diagnostic kits are limited.

In addition to the analytical and clinical performance, the turnaround time and cost are also essential factors affecting the choice of diagnostic assays, especially when there is a large number of clinical specimens from patients with suspected COVID-19 during this pandemic. The sample-to-extract time was the same among the three assays because the same extraction method was used, while the PCR running time of the LightMix® E-gene assay (66 min) was slightly shorter than our in-house COVID-19-RdRp/Hel and COVID-19-N assays (72 min). For the reagent cost including the PCR reagents and primers/probes, our in-house assays (US$2 per reaction) were much lower than the LightMix® E-gene assay (US$10 per reaction). For clinical laboratories without the necessary expertise in the development of in-house assays, the LightMix® E-gene kit may be an alternative commercially available diagnostic option.

In conclusion, the LightMix® Modular SARS and Wuhan CoV E-gene kit is a rapid and highly sensitive assay for screening suspected cases of COVID-19. Further confirmation can be achieved by performing another assay specific to SARS-CoV-2, such as our in-house COVID-19-RdRp/Hel and COVID-19-N assays with lower cost.

Ethics approval and consent to participate

This study was approved by Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Data records were de-identified and completely anonymous, so informed consent was waived.

Availability of data and materials

The datasets used and/or analyzed during the current study are

available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Cyril Chik-Yan Yip: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing - original draft. **Siddharth Sridhar:** Formal analysis, Writing - original draft, Writing - review & editing. **Andrew Kim-Wai Cheng:** Validation, Investigation, Data curation. **Kit-Hang Leung:** Validation, Investigation. **Garnet Kwan-Yue Choi:** Methodology. **Jonathan Hon-Kwan Chen:** Investigation. **Rosana Wing-Shan Poon:** Investigation. **Kwok-Hung Chan:** Investigation. **Alan Ka-Lun Wu:** Investigation. **Helen Shuk-Ying Chan:** Investigation. **Sandy Ka-Yee Chau:** Investigation. **Tom Wai-Hin Chung:** Investigation. **Kelvin Kai-Wang To:** Investigation. **Owen Tak-Yin Tsang:** Investigation. **Ivan Fan-Ngai Hung:** Investigation, Resources. **Vincent Chi-Chung Cheng:** Resources. **Kwok- Yung Yuen:** Writing - review & editing, Supervision. **Jasper Fuk-Woo Chan:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

J.F.-W.C. has received travel grants from Pfizer Corporation Hong Kong and Astellas Pharma Hong Kong Corporation Limited, and was an invited speaker for Gilead Sciences Hong Kong Limited and Luminex Corporation. S.S. has received speaker's honoraria from Abbott Laboratories Limited. The other authors declared no conflict of interests. The funding sources had no role in study design, data collection, analysis or interpretation or writing of the report.

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