Evaluation of 3 Commercial Real-time RT-PCR Assays for Middle East Respiratory Syndrome Coronavirus

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Background

The recent emergence of the Middle East respiratory syndrome coronavirus (MERS-CoV) prompted the US CDC and others to develop real-time RT-PCR (rRT-PCR) assays to support MERS-CoV surveillance. Soon thereafter, several companies introduced commercial MERS-CoV rRT-PCR kits available for purchase. In this study, we compared the performance of 3 commercial rRT-PCR kits with the CDC reference assay.

MERS Update (as of April 21, 2015)

- 1106 laboratory-confirmed cases with 421 deaths (38.1% mortality)
- Onsets between April 2012 and April 2015
- Gender: 66.5% male
- Median age: 50 yrs (0, 99 yrs)
- Most cases hospitalized have underlying conditions
- Recent exportations: Austria, Turkey, Jordan, Philippines, Germany

MERS-CoV rRT-PCR Kits

Fig. 1. CDC Novel Coronavirus 2012 rRT-PCR kit (CDC). Comprised of 3 individual (singleplex) hydrolysis probe rRT-PCR assays (upE, N2 and N3). Primers/probes for each assay come dried within individual tubes. End user must procure rRT-PCR enzyme mix to perform test (Invitrogen SuperScript III one-step). Kits are currently offered through FDA Emergency Use Authorization in the USA for diagnostic use. Shipping conditions of the primers/probes/ positive control are flexible from dry ice to ambient temperatures.

Fig 2. Altona Diagnostics RealStar® MERS-CoV RT-PCR kit (RS). This is a complete testing reagent kit, including 2 multiplex primer/probe (upE/internal control, ORF1a/internal control) pools which contain the enzyme and buffers, positive controls and PCR grade water. Kits must ship on dry ice.

Fig 3. Fast-track Diagnostics® hCoV-EMC kit (FTD). This kit can be purchased as a complete reagent package containing 1 multiplex screening assay primer/probe pool (screening assay/internal control), 1 singleplex confirmation assay and positive controls. FTD offers proprietary enzyme and buffer separately, but can also be requested and shipped with the primer/probe kit. Some 3rd party commercial one-step RT-PCR enzyme mixes are also compatible per manufacturer's specifications. Kits must ship on dry ice.

Fig 4. Primerdesign genesig® Novel Coronavirus hCoV-MERS kit (GS). Primers/probe/controls and lyophilized rRT-PCR enzyme/buffer kits sold separately. Assays are in 1 multiplex primer/probe pool (upE/internal control) and one singleplex primer/probe pool (ORF1a). Kits ship at ambient temperature.

Methods

- Analytical sensitivity was determined using serial 10-fold dilutions (10^{-5} - 10^{-9}) of a quantified MERS-CoV isolate (strain Jordan-N3/2012) spiked in a mock respiratory specimen matrix.
- Clinical sensitivity was evaluated with 10 MERS-CoV positive clinical specimens (Ct range, 27.7 - 40.2) from hospitalized patients previously confirmed positive.
- Assay specificity was assessed using concentrated nucleic acid from 12 other respiratory viruses and 10 archived respiratory specimens containing diverse respiratory pathogens.
- Nucleic acid extracts were simultaneously tested by the CDC assay and each commercial kit following the manufacturer's instructions, using the Applied Biosystems® 7500 fast Dx Real-time PCR system (Thermofisher). Individual assays were performed in triplicate by 2 technicians.

Fig 1. CDC kit

Fig 2. RS kit

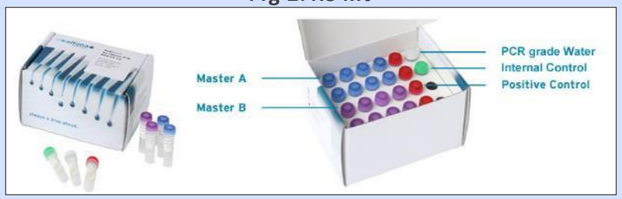


Fig 3. FTD kit

Fig 4. GS kit

Table 1. Comparative LODs of MERS-CoV rRT-PCR assays (Tech #1 /Tech #2).

Dilution	CDC-upE	CDC-upE	CDC-upE	CDC-N2	CDC-N2	CDC-N2	CDC-N3	CDC-N3	CDC-N3
-5	27.8 / 29.3	27.8 / 29.4	27.0 / 29.3	27.0/ 28.9	26.6 / 28.9	26.3 / 28.7	27.5 / 28.1	27.4 / 28.1	27.4 / 28.2
-6	31.8 / 33.3	31.2 / 33.1	31.8 / 32.9	31.1 / 32.6	31.0 / 32.4	30.7 / 32.2	30.8 / 31.8	30.9 / 31.4	30.8 / 31.7
-7	35.0 / 35.6	36.1 / 35.9	35.0 / 37.4	35.0 / 36.5	34.1 / 36.2	34.1 / 37.7	34.0 / 35.3	34.0 / 34.5	34.4 / 34.8
-8	Neg / 38.8	37.3 / Neg	39.2 / 37.5	37.3 / 40.1	37.3 / 38.3	36.6 / Neg	Neg / Neg	Neg / Neg	Neg / Neg
-9	Neg / Neg	37.0 / 38.4	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg

Dilution	RS-upE	RS-upE	RS-upE	RS-ORF1a	RS-ORF1a	RS-ORF1a
-5	28.2 / 27.1	28.2 / 27.0	28.1 / 26.7	28.1 / 26.7	28.0 / 26.8	28.1 / 26.8
-6	31.3 / 29.8	31.3 / 30.2	31.5 / 30.1	31.5 / 30.1	31.2 / 30.2	31.2 / 30.1
-7	34.2 / 32.9	35.5 / 33.3	34.5 / 33.5	34.0 / 33.1	35.1 / 32.7	34.1 / 32.8
-8	38.4 / Neg	Neg / 39.2	42.5 / 40.6	Neg / 36.7	37.8 / 36.7	37.7 / 35.1
-9	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg

Dilution	FTD-screen	FTD-screen	FTD-screen	FTD-confirm	FTD-confirm	FTD-confirm
-5	28.4 / 28.1	28.9 / 27.9	28.5 / 27.7	28.7 / 27.5	29.0 / 27.8	28.7 / 28.3
-6	31.2 / 31.1	32.9 / 30.3	32.4 / 31.2	33.0 / 32.8	32.0 / 31.4	32.2 / 31.0
-7	Neg / 34.6	32.2 / 34.3	34.5 / 33.9	35.1 / Neg	35.4 / Neg	37.3 / 34.6
-8	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / 35.7
-9	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg

Dilution	GS-upE	GS-upE	GS-upE	GS-ORF1a	GS-ORF1a	GS-ORF1a
-5	30.7 / 31.6	30.1 / 31.0	30.2 / 31.8	31.4 / 33.0	31.7 / 30.1	30.9 / 31.1
-6	34.2 / Neg	35.1 / Neg	38.4 / Neg	Neg / 36.8	35.2 / 36.1	Neg / 37.5
-7	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg
-8	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg
-9	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg	Neg / Neg

Fig 5. Assay performance with MERS-CoV positive clinical specimens.

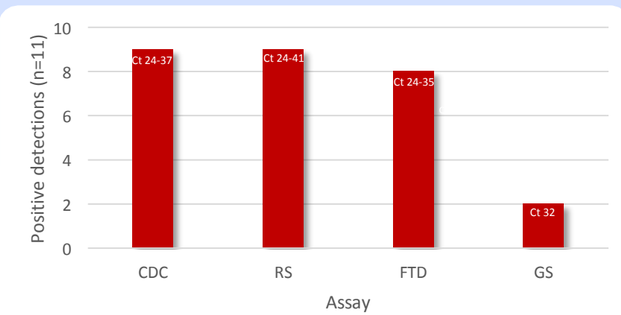
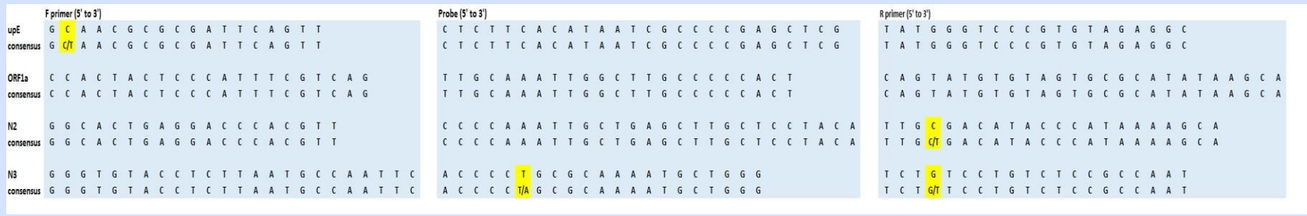


Table 2. Published MERS-CoV rRT-PCR assay signatures: sequence identity with 82 published genome sequences, 2012 –2015.



Results

- CDC, RS, FTD and GS assay limits of detection (LOD) were 10^{-7}, 10^{-7}, 10^{-6} and 10^{-5} dilutions, respectively. Testing was replicated by 2 different technicians (**Table 1**).
- CDC and RS assays correctly identified 9 of 11 (81.8%) positive clinical specimens; 2 samples with low virus loads (Ct 40.2 and 38.9) were judged negative by both assays on repeat testing (**Fig. 5**)
- FTD assays correctly identified 8 of 11 (72.7%) positive clinical specimens (**Fig. 5**)
- GS assays correctly identified 2 of 11 (18.2%) positive clinical specimens (**Fig. 5**)
- Specificity for all assays was 100% with virus isolates and clinical specimens positive for 14 other respiratory viruses, including coronaviruses OC43, 229E, HKU1 and NL63 - data not shown.
- Alignment of upE, N2, N3, and ORF1a primer/probe sequences with a consensus sequence derived from MERS-CoV genomic sequences published in Genbank (2012-2015) revealed some base mismatches between the upE forward primer, N2 reverse primer, and N3 reverse primer and probe (**Table 2**).

Study Limitations

- Number of tests and test replicates were limited by the number of commercial kits that could be purchased for the study and clinical specimens availability. (n=11, updated from n=7 at time of abstract submission)
- MERS-CoV positive specimens were originally identified using CDC assays which may have introduced selection bias in the study.
- LOD determinations were performed with only a single virus strain, Jordan-N3/2012.
- Clinical specimens had been previously tested prior to this study and may have incurred some degradation through transportation and freeze-thaw cycles.

Conclusions and Discussion

The 3 commercial rRT-PCR kits provide all necessary reagents and easy-to-follow instructions for MERS-CoV diagnostic testing. The RS and FTD assays performed comparably to the CDC reference assay, whereas the GS assay lacked sensitivity in both LOD determinations and with authentic MERS-CoV positive specimens. Because test comparisons were limited by kit costs and few available MERS-CoV positive specimens, further kit evaluation is warranted.

The poor performance of the GS kit is likely due to the lyophilized proprietary one-step enzyme/buffer reagents. Experiments have shown that some PCR enzymes, especially reverse transcriptases, are sensitive to the lyophilization process. Because the primer/probe sequences used in the GS kit (upE, ORF1a) are similar to those in other kits, a different enzyme/buffer mix or shipping conditions may improve this products performance. A second GS kit was obtained with no improvement to performance (data not shown).

Upon conducting a review of primer/probe sequences for the upE, N2, N3, and ORF1a assays versus all MERS-CoV sequences published in Genbank (2012-2015), base mismatches were found among the upE, N2, and N3 targets. Internal testing data with sequence confirmation suggest that these mismatches do not have a critical impact on the ability of these primer/probe sequences to detect MERS-CoV in clinical specimens. However it serves as a reminder that assay primer/probe compatibility must be regularly assessed to account for new virus mutations.

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