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# Comparison of fast-track diagnostics respiratory pathogens multiplex real-time RT-PCR assay with in-house singleplex assays for comprehensive detection of human respiratory viruses☆

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Fast-track Diagnostics respiratory pathogens (FTDRP) multiplex real-time RT-PCR assay was compared with in-house singleplex real-time RT-PCR assays for detection of 16 common respiratory viruses. The FTDRP assay correctly identified 26 diverse respiratory virus strains, 35 of 41 (85%) external quality assessment samples spiked with cultured virus and 232 of 263 (88%) archived respiratory specimens that tested positive for respiratory viruses by in-house assays. Of 308 prospectively tested respiratory specimens selected from children hospitalized with acute respiratory illness, 270 (87.7%) and 265 (86%) were positive by FTDRP and in-house assays for one or more viruses, respectively, with combined test results showing good concordance ($K = 0.812$, 95% CI = 0.786–0.838). Individual FTDRP assays for adenovirus, respiratory syncytial virus and rhinovirus showed the lowest comparative sensitivities with in-house assays, with most discrepancies occurring with specimens containing low virus loads and failed to detect some rhinovirus strains, even when abundant. The FTDRP enterovirus and human bocavirus assays appeared to be more sensitive than the in-house assays with some specimens. With the exceptions noted above, most FTDRP assays performed comparably with in-house assays for most viruses while offering enhanced throughput and easy integration by laboratories using conventional real-time PCR instrumentation.
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## 1. Introduction

Respiratory viruses are among the most important causes of human morbidity and mortality worldwide (Nair et al., 2010; Pavia, 2011). Clinically indistinguishable, respiratory virus infections require accurate laboratory diagnosis to guide treatment effectively and prevention decisions. Polymerase chain reaction (PCR) and other molecular assays are now routinely used for diagnosis of respiratory virus infections (Beck and Henrickson, 2010; Kehl and Kumar, 2009), but the large and increasing number of viruses makes laboratory testing with individual (singleplex) virus assays challenging. Conversely, multiplex PCR assays that combine multiple individual assays in a single reaction facilitate more rapid, high throughput and cost-effective testing and are generally preferred in the clinical setting (Elnifro et al., 2000; Jansen et al., 2011).

Numerous laboratory-developed and commercial multiplex PCR assays using different amplification platforms have been described for respiratory viruses and have been generally shown to be superior to traditional diagnostic methods, such as virus culture and antigen detection for sensitive and specific detection of respiratory viruses (Arens et al., 2010; Bibby et al., 2011; Brittain-Long et al., 2010; Caliendo, 2011; Gadsby et al., 2010; Kim et al., 2009; Lamson et al., 2006; Mahony et al., 2007; Raymond et al., 2009). The US Food and Drug Administration (FDA) has cleared recently two commercial assays, the xTAG® RVP Fast (Luminex Molecular Diagnostics, Austin, TX) and FilmArray® Respiratory Panels (Idaho Technology Inc., Salt Lake City, Utah) (Rand et al., 2011), for in-vitro diagnostic use for multiplex detection of respiratory viruses.

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However, many of these assays are costly, require specialized laboratory equipment and use highly multiplexed reactions that may be deficient in individual assay performance and can be difficult to modify without extensive assay reoptimization (Gunson et al., 2008).

The FTD respiratory pathogens (FTDRP) multiplex assay kit (Fast-track Diagnostics, Luxembourg) uses standard commercial one-step reverse transcription (RT)-PCR hydrolysis probe chemistry and common real-time PCR instrumentation. The FTDRP assay consists of 5 discrete primer/probe mixes that together cover 16 common human respiratory viruses. This study reports the results of a comparison of the FTDRP multiplex assay with a panel of validated in-house singleplex real-time RT-PCR assays developed at the Centers for Disease Control and Prevention (CDC).

## 2. Materials and methods

### 2.1. Viruses and specimens

Virus isolates and archived clinical specimens were obtained from CDC collections acquired during routine surveillance and outbreak investigations. These included 26 laboratory reference virus strains and field isolates and 265 geographically (U.S., Central and South America and Africa) and compositionally diverse specimens [nasopharyngeal and oropharyngeal swabs (223), nasal washes and aspirates (21), sputum (1), lung autopsy tissue (1) and unidentified (19)] collected from children and adults with acute respiratory illnesses (ARIs) acquired between 2008 and 2011 and previously testing positive for respiratory viruses by the in-house singleplex assays. All residual samples and extracts were stored at −70 °C. Whenever possible, archived specimens were selected to achieve a proportional representation of viral loads. Forty-six mock human specimens spiked with moderate-to-low concentrations of virus were available from the 2010 Quality Control for Molecular Diagnostics (QCMD, Glasgow, Scotland) external quality assessment (EQA) programs for rhinovirus/coronavirus, adenovirus, parainfluenza viruses, human metapneumovirus/respiratory syncytial virus, and influenza A & B viruses (Wallace, 2003). Pooled nasal wash specimens from 20 consenting healthy new military recruits was kindly provided by Dr. Lisa Lott, Eagle Applied Sciences, L.L.C., San Antonio, TX. Finally, a subset of 308 nasopharyngeal aspirates (NPAs) from an etiologic study of 1162 children < 2 years of age hospitalized with ARI at a tertiary hospital in São Paulo, Brazil, between March 2008 and September 2010, were selected from the seasonal peaks of respiratory virus circulation for each of the study years based on local surveillance data. The NPAs were collected directly into liquid nitrogen, aliquoted and transferred to −70 °C and retained until retrieved for this study. This study was approved by institutional review boards at the University of São Paulo and Santa Casa de Misericórdia de São Paulo Hospital, Brazil, and CDC.

### 2.2. Total nucleic acid extraction

Total nucleic acid (TNA) extracts were prepared from samples using the NucliSENS® easyMAG® (bioMérieux). Because of their multiple study origins and testing histories, residual archived specimen extraction volumes varied from 100 to 300 μL and TNA elution volumes from 55 to 100 μL. RNase-free water was added to a few archived extracts (≤2-fold dilution) to obtain sufficient volume for comparison testing. For prospectively tested nasal aspirate specimens, 300 μL of each sample was extracted and the TNA recovered in 210 μL of elution buffer which was then split into 3 aliquots and frozen at −70 °C until testing. All extracts were subjected to identical freeze-thaw cycles for comparison testing. All extracts were confirmed positive for human RNase P gene by real-time RT-PCR before inclusion in the study.

**Table 1**
Comparison of FTDRP and in-house assays with 26 virus isolates.

| Virus (strain) | In-house (Ct) | FTDRP (Ct)[a] |
|---|---|---|
| AdV C1 (Ad.71) | Pos (13.7) | Pos (16.7) |
| AdV C5 (Ad.75) | Pos (18.4) | Pos (20.1) |
| AdV B7 (SA-104) | Pos (13.8) | Pos (18.8) |
| AdV B14 (deWit) | Pos (20.8) | Pos (23.3) |
| AdV E4 (RI-67) | Pos (15.2) | Pos (18.2) |
| CoV 229E | Pos (10.3) | Pos (13.2) |
| CoV OC43 | Pos (13.0) | Pos (14.9) |
| CoV SARS (Urbani) | Pos (19.2) | n/a[c] |
| EV, echovirus 6[b] | Pos (21.3) | Pos (15.8) |
| EV, echovirus 11[b] | Pos (16.3) | Pos (15.5) |
| EV, enterovirus 68[b] | Pos (21.3) | Pos (24.6) |
| HMPV A (CAN 97-83) | Pos (15.0) | Pos (17.0) |
| HMPV B (CAN 98-75) | Pos (18.3) | Pos (21.2) |
| Inf A H1N1 (A/California/09) | Pos (14.7) | Pos (14.8) |
| Inf A H2N1 (A/Japan/57) | Pos (27.3) | Pos (24.4) |
| Inf B (B/Shanghai/99) | Pos (14.6) | Pos (15.1) |
| PIV 1 (C35) | Pos (16.6) | Pos (19.1) |
| PIV 2 (Greer) | Pos (16.9) | Pos (15.8) |
| PIV 3 (C-43) | Pos (15.2) | Pos (16.3) |
| PIV 4a (M-25) | Pos (16.7) | Pos (19.5) |
| PIV 4b (CH 19503) | Pos (21.5) | Pos (21.1) |
| PeV 1[b] | Pos (16.0) | Pos (16.4) |
| RSV A (Long) | Pos (15.0) | Pos (15.7) |
| RSV B (CH 93-18B) | Pos (15.1) | Pos (16.7) |
| RV A1a | Pos (13.4) | Pos (15.3) |
| RV B14 | Pos (15.7) | Pos (32.0) |

[a] Unless otherwise indicated, all other FTDRP assays were negative.
[b] FTDRP EV/PeV assay does not distinguish between EV and PeV.
[c] FTDRP SARS CoV assay not available (n/a).

### 2.3. FTDRP multiplex assay

The FTDRP multiplex real-time RT-PCR assay (ver.5, cat. no. FTD 2-96/12) consists of 5 separate primer/probe mixes covering 16 human respiratory viruses and brome mosaic virus (BMV), an RNA plant virus that serves as an internal extraction control when spiked into the sample (virus provided); mix #1: influenza A virus (Inf A), influenza B virus (Inf B), BMV; mix #2: coronavirus (CoV) NL63, 229E and OC43 and enterovirus/parechovirus (EV/PeV); mix #3: parainfluenza virus (PIV) 2, 3 and 4; mix #4: PIV 1, human metapneumovirus (HMPV) and human bocavirus (HBoV); mix #5: rhinovirus (RV), respiratory syncytial virus (RSV) and adenovirus (AdV). Individual assays within each pool are distinguished by use of different probe fluorophores, with the exception of the EV and PeV assays, where both probes are ROX-labeled and therefore cannot be distinguished. Each kit also contains a positive plasmid control pool and detailed instructions on test performance. The FTDRP assay was performed following the manufacturer's recommendations. Briefly, 194 μL of 2× RT-PCR buffer was combined with 23.3 μL of each primer/probe pool and 15.5 μL of 25× enzyme mix (AgPath-ID™ One-Step RT-PCR Kit, Applied Biosystems), and 15 μL of each mixture was then added to 14 wells of a PCR plate (12 sample reactions plus one positive and one negative virus control). Ten μL of sample TNA extract or controls were then added to the respective wells of each primer/probe pool. The following cycling conditions were performed on a 7500 Fast Dx Real-Time PCR Instrument (Applied Biosystems): 15 min at 50 °C, 10 min at 95 °C and 40 cycles of 8 s at 95 °C and 34 s at 60 °C. Threshold cycle (Ct) values were determined by manually adjusting the fluorescence baseline to fall within the exponential phase of the amplification curves and above any background signal. A positive test result was considered a well-defined curve that crossed the threshold cycle within 40 cycles. Positive and negative virus plasmid controls provided in the kit were included in all runs to monitor assay performance. The

**Table 2**
Comparison of FTDRP and in-house assays with 46 samples from 5 QCMD EQA programs.

| QCMD EQA[a] | Virus | QCMD Key[b] | In-house (Ct) | FTDRP (Ct) |
|---|---|---|---|---|
| Adenovirus (AdV) | | | | |
| ADV10-02 | AdV F41 | Pos (113) | Neg/Pos (39.6)[c] | Pos (39.4) |
| ADV10-03 | AdV C1 | Pos (64121) | Pos (30.9) | Pos (29.2) |
| ADV10-04 | AdV E4 | Pos (767) | Pos (36.7) | Pos (35.1) |
| ADV10-06 | AdV C1 | Pos (4055) | Pos (34.0) | Pos (32.0) |
| ADV10-08 | AdV B34 | Pos (1225) | Pos (34.0) | Neg/Neg[c] |
| ADV10-07 | No virus | Neg | Neg | Neg |
| Influenza virus (Inf) | | | | |
| INFRNA 09-01 | Inf A subtype H1 | Pos (29.4) | Pos (29.4) | Pos (28.9) |
| INFRNA 09-02 | Inf A subtype H3 | Pos (31.4) | Pos (28.8) | Pos (29.7) |
| INFRNA 09-03 | Inf B | Pos (39.2) | Pos (38.3) | Pos (38.2) |
| INFRNA 09-04 | Inf A subtype H1v[d] | Pos (28.7) | Pos (28.7) | Pos (25.7) |
| INFRNA 09-06 | Inf A subtype H1 | Pos (27.9) | Pos (28.7) | Pos (27.8) |
| INFRNA 09-07 | Inf B | Pos (32.1) | Pos (30.3) | Pos (27.5) |
| INFRNA 09-09 | Inf A subtype H1v[d] | Pos (32.1) | Pos (28.8) | Pos (28.7) |
| INFRNA 09-10 | Inf A subtype H1 | Pos (29.4) | Pos (29.9) | Pos (29.1) |
| INFRNA 09-11 | Inf A subtype H1 | Pos (33.1) | Pos (33.5) | Pos (33.0) |
| INFRNA 09-12 | Inf A subtype H3 | Pos (35.6) | Pos (33.3) | Pos (33.0) |
| INFRNA 09-05 | No virus | Neg | Neg | Neg |
| Parainfluezavirus (PIV) | | | | |
| PINF10-01 | PIV 1 | Pos (33.1) | Pos (32.7) | Pos (38.1) |
| PINF10-02 | PIV 4 | Pos (31.9) | Pos (35.2) | Pos (33.5) |
| PINF10-03 | PIV 1 | Pos (31.0) | Pos (31.1) | Pos (33.8) |
| PINF10-06 | PIV 3 | Pos (34.5) | Pos (25.7) | Pos (23.7) |
| PINF10-07 | PIV 2 | Pos (28.2) | Pos (24.0) | Pos (21.3) |
| PINF10-08 | No virus | Neg | Neg | Neg |
| Respiratory syncytial virus (RSV) & Human metapneumovirus (HMPV) | | | | |
| MPV.RSV10-01 | RSV A | Pos (38.4) | Pos (36.7) | Neg/Pos (36.8)[c] |
| MPV.RSV10-02 | RSV B | Pos (37.1) | Pos (31.7) | Neg/Pos (33.1)[c] |
| MPV.RSV10-04 | RSV A | Pos (33.4) | Pos (31.1) | Pos (30.7) |
| MPV.RSV10-09 | RSV B | Pos (39.9) | Pos (34.8) | Neg/Pos (37.3)[c] |
| MPV.RSV10-10 | RSV B | Pos (32.4) | Pos (24.6) | Pos (25.4) |
| MPV.RSV10-11 | RSV A | Pos (37.3) | Pos (33.7) | Pos (36.5) |
| MPV.RSV10-03 | HMPV B2 | Pos (35.5) | Pos (29.7) | Pos(32.5) |
| MPV.RSV10-05 | HMPV B2 | Pos (38.5) | Pos (32.8) | Pos (34.2) |
| MPV.RSV10-07 | HMPV A1 | Pos (39.3) | Pos (34.9) | Neg/Pos (39)[c] |
| MPV.RSV10-08 | HMPV A1 | Pos (33.2) | Pos (29.1) | Pos (33.2) |
| MPV.RSV10-12 | HMPV B2 | Pos (35.6) | Pos (30.0) | Pos (32.2) |
| MPV.RSV10-06 | No virus | Neg | Neg | Neg |
| Rhinovirus (RV) & Coronavirus (CoV) | | | | |
| RV.CV10-01 | RV B42 | Pos (29.6) | Pos (26.9) | Pos (36.7) |
| RV.CV10-02 | RV A8 | Pos (25.8) | Pos (22.5) | Pos (24.0) |
| RV.CV10-03 | RV B72 | Pos (22.9) | Pos (21.5) | Neg/Neg[c] |
| RV.CV10-05 | RV A90 | Pos (32.6) | Pos (28.7) | Pos (31.6) |
| RV.CV10-07 | RV A16 | Pos (30.5) | Pos (27.5) | Pos (30.3) |
| RV.CV10-09 | RV A16 | Pos (34.1) | Pos (30.9) | Pos (33.3) |
| RV.CV10-04 | CoV 229E | Pos (28.5) | Pos (27.9) | Pos (26.6) |
| RV.CV10-08 | CoV 229E | Pos (35.0) | Pos (34.0) | Pos (32.5) |
| RV.CV10-06 | CoV OC43 | Pos (31.1) | Pos (32.6) | Pos (32.2) |
| RV.CV10-10 | CoV NL63 | Pos (26.9) | Pos (25.7) | Pos (24.1) |
| RV.CV10-11 | EV[e] | Neg | Neg | Neg |

[a] QCMD EQA, 2010 Quality Control for Molecular Diagnostics External Quality Assessment program samples.
[b] QCMD test results; Ct values (RV/CoV, PIV, RSV/HMPV) and genome copies/mL (AdV). QCMD Ct values should not be used for method comparison or as a target for individual laboratory assessment.
[c] Original and repeat result.
[d] Inf A subtype H1v = new variant pandemic H1N1 strain.
[e] QCMD EQA negative RV control sample contained coxsackievirus A1.

BMV internal control was spiked into clinical specimens to monitor sample extraction and reverse transcription. Previously extracted TNA samples were evaluated for RNase P only.

### 2.4. In-house singleplex assays

In-house singleplex real-time RT-PCR assays for RSV, HMPV, PIV1-4, RV, AdV, HBoV and CoVs (229E, OC43, NL63, HKU1, SARS-CoV) as previously described (Dare et al., 2007; Emery et al., 2004; Fry et al., 2010; Heim et al., 2003; Kodani et al., 2011; Lu et al., 2006, 2008; Morgan et al., 2012) were performed on a MX3000P QPCR System (Agilent Technologies) using AgPath-ID™ One-Step RT-PCR reagents (Applied Biosystems) with the following cycling conditions: 45 °C for 10 min, 95 °C for 10 min and 45 cycles of 95 °C for 15 s and 55 °C for 1 min. Primer/probe sequences are available from D.E. on request. The in-house EV and PeV assays as modified from previous reports (Kilpatrick et al., 2009; Nix et al., 2008) were performed on a MX3000P QPCR System using the SuperScript III Platinum® One-Step Quantitative RT-PCR System reagents (Invitrogen) with the following cycling conditions: 50 °C for 30 min, 95 °C for 5 min

and 45 cycles of 95 °C for 15 s, 55 °C (EV) or 58 °C (PeV) for 45 s and 72 °C for 10 s. Universal Inf A and Inf B assays were performed on a 7500 Fast Dx Real-Time PCR Instrument with SDS software ver. 1.4 (Applied Biosystems) using the SuperScript III Platinum® One-Step Quantitative RT-PCR System with the following cycling conditions: 50 °C for 30 min, 95 °C for 2 min and 45 cycles of 95 °C for 15 s and 55 °C for 30 s (Stephen Lindstrom, CDC, personal communication). Following standard operating procedures, all in-house assays were performed in 25 μL final reaction volumes containing 5 μL of sample TNA extract. A positive test result was considered a well-defined curve that crossed the threshold cycle within 40 cycles. Positive and negative virus RNA transcript or whole virus extract controls were included in all runs to monitor assay performance.

### 2.5. Statistics

Percent sensitivity and specificity of the FTDRP assay for prospectively collected specimens were calculated using the in-house assays as the reference standard. Agreement between assays was measured using the Kappa statistic (Cohen, 1960) where 0 indicates no agreement and 1 indicates perfect agreement.

## 3. Results

### 3.1. Virus isolates

The FTDRP assay was first evaluated with undiluted TNA from cultures of 26 respiratory virus strains corresponding to most assays in the multiplex to assess assay specificity and virus strain inclusivity (Table 1). Although no FTDRP assay for SARS-CoV was available, this virus was tested to assess the specificity of the other FTDRP CoV assays. HBoV and CoV NL63 and HKU1 isolates were not available for testing. Positive results were obtained with both assays for all viruses with no cross-reactions detected. FTDRP and in-house assay results were within 3Ct values for 22 (88%) of the viruses tested. Notably, the FTDRP RV assay gave a substantially higher Ct value (Δ 16.3Ct) with one RV isolate (RV-B14). Serial dilutions of RV-B14 TNA showed the FTDRP assay to be >1000-fold less sensitive than the corresponding in-house assay with this virus strain (data not shown).

### 3.2. Pooled human respiratory specimens

The specificity of the FTDRP assay was further evaluated with pooled nasal wash samples from 20 consenting normal healthy adults to represent diverse microbial flora in the human respiratory tract. Positive results were obtained with the in-house assays for RV (Ct 25.0), CoV 229E (Ct 35.1) and AdV (Ct 39.3) which were confirmed by alternate RT-PCR assays and sequencing. The FTDRP assay was positive for RV (Ct 28.3) and CoV 229E (Ct 34.8), but did not detect the AdV on initial or repeat testing. All other in-house and FTDRP assays were negative.

### 3.3. QCMD EQA program samples

Forty-one mock respiratory samples spiked with low to moderate levels of different viruses and 5 negative control samples selected from 2010 QCMD EQA programs for HRV/CoV, AdV, PIV, RSV/HMPV and Inf A/B, were tested to assess assay performance against the reference QCMD assays (Table 2). Overall, expected results were obtained with 40 (98%) and 35 (85%) of positive EQA program samples with the in-house and FTDRP assays, respectively. All program negative control samples were negative by both assays. One sample (AdV10-02), with low concentration AdV-F41, was initially negative by the in-house assay, but positive on repeat testing. The FTDRP assay gave expected results with all PIV (5), CoV (4) Inf

**Table 3**
Comparison of FTDRP and in-house assays with 263 archived respiratory specimens previously positive for respiratory viruses.

| Virus[a] | In-house + | FTDRP + | FTDRP % + | Ct <30[b] | | | | Ct ≥30 to ≤37[b] | | | | Ct >37 to <40[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Total + | FTDRP + | FTDRP − | FTDRP % + | Total + | FTDRP + | FTDRP − | FTDRP % + | Total + | FTDRP + | FTDRP − | FTDRP % + |
| AdV | 25 | 17 | 68% | 8 | 8 | 0 | 100% | 11 | 8 | 3 | 73% | 6 | 1 | 5 | 17% |
| CoV 229E | 5 | 5 | 100% | 3 | 3 | 0 | 100% | 2 | 2 | 0 | 100% | 0 | | | |
| CoV OC43 | 7 | 7 | 100% | 5 | 5 | 0 | 100% | 2 | 2 | 0 | 100% | 0 | | | |
| CoV NL63 | 8 | 8 | 100% | 7 | 7 | 0 | 100% | 1 | 1 | 0 | 100% | 0 | | | |
| EV/PeV[c] | 8 | 8 | 100% | 4 | 4 | 0 | 100% | 4 | 4 | 0 | 100% | 0 | | | |
| HBoV | 2 | 2 | 100% | 2 | 2 | 0 | 100% | 0 | | | | 0 | | | |
| HMPV | 26 | 26 | 100% | 20 | 20 | 0 | 100% | 6 | 6 | 0 | 100% | 0 | | | |
| Inf A | 17 | 17 | 100% | 12 | 12 | 0 | 100% | 5 | 5 | 0 | 100% | 0 | | | |
| Inf B | 11 | 11 | 100% | 10 | 10 | 0 | 100% | 1 | 1 | 0 | 100% | 0 | | | |
| PIV 1 | 20 | 17 | 85% | 9 | 9 | 0 | 100% | 11 | 8 | 3 | 73% | 0 | | | |
| PIV 2 | 13 | 12 | 92% | 6 | 6 | 0 | 100% | 6 | 5 | 1 | 83% | 1 | 1 | 0 | 100% |
| PIV 3 | 31 | 30 | 97% | 15 | 15 | 0 | 100% | 16 | 15 | 1 | 94% | 0 | | | |
| PIV 4 | 12 | 12 | 100% | 9 | 9 | 0 | 100% | 3 | 3 | 0 | 100% | 0 | | | |
| RSV | 31 | 23 | 74% | 12 | 12 | 0 | 100% | 11 | 10 | 1 | 91% | 8 | 1 | 7 | 14% |
| RV | 47 | 37 | 79% | 38 | 33 | 5 | 87% | 9 | 4 | 5 | 44% | 0 | | | |
| Total | 263 | 232 | 88% | 160 | 155 | 5 | 97% | 88 | 74 | 14 | 84% | 15 | 3 | 12 | 20% |

[a] Virus co-detections not included in the analysis.
[b] In-house assay results classified as strong (Ct <30), moderate (Ct ≥30 to ≤37) or weak (Ct >37) positive.
[c] FTDRP EV/PeV assay does not distinguish between EV and PeV. Eight specimens separately tested positive for EV (3Ct <30; 2Ct ≥30 to ≤37) and PeV (1Ct <30; 2Ct ≥30 to ≤37) by in-house assays.

**Table 4**
Comparison of FTDRP and in-house assays with 32 archived respiratory specimens with sequence confirmed rhinovirus (RV) or enterovirus (EV).

| Virus[a] | RV | | EV | PeV | EV/PeV |
|---|---|---|---|---|---|
| | In-house (Ct) | FTDRP (Ct) | In-house (Ct) | In-house (Ct) | FTDRP (Ct)[b] |
| EV, enterovirus 68 | Neg | Neg | Pos (31.1) | Neg | Pos (30.7) |
| EV, enterovirus 68 | Neg | Neg | Pos (28.9) | Neg | Pos (24.4) |
| EV, echovirus 9 | Neg | Neg | Pos (23.5) | Neg | Pos (23.4) |
| EV, coxsackievirus B4 | Neg | Neg | Pos (22.6) | Neg | Pos (21.6) |
| EV, coxsackievirus B5 | Neg | Neg | Pos (31.0) | Neg | Pos (27.1) |
| RV A18 | Pos (17.7) | Pos (23.1) | Neg | Neg | Neg |
| RV A19 | Pos (25.1) | Pos (23.5) | Neg | Neg | Neg |
| RV A22 | Pos (19.1) | Pos (19.6) | Neg | Neg | Neg |
| RV A30 | Pos (16.7) | Pos (16.7) | Neg | Neg | Neg |
| RV A30 | Pos (20.3) | Pos (23.6) | Neg | Neg | Neg |
| RV A33 | Pos (22.3) | Pos (27.7) | Neg | Neg | Neg |
| RV A38 | Pos (18.8) | Pos (22.6) | Neg | Neg | Neg |
| RV A38 | Pos (21.1) | Pos (24.6) | Neg | Neg | Pos (36.1) |
| RV A49 | Pos (19.5) | Pos (18.1) | Neg | Neg | Neg |
| RV A58 | Pos (17.7) | Pos (20.2) | Neg | Neg | Neg |
| RV A76 | Pos (23.2) | Pos (29.6) | Neg | Neg | Neg |
| RV A68 | Pos (22.8) | Pos (22.6) | Neg | Neg | Pos (31.4) |
| RV A96 | Pos (25.9) | Pos (26.3) | Neg | Neg | Neg |
| RV B6 | Pos (12.9) | Pos (24.1) | Neg | Neg | Neg |
| RV B6 | Pos (27.8) | Neg | Neg | Neg | Neg |
| RV B6 | Pos (25.1) | Neg | Neg | Neg | Neg |
| RV B48 | Pos (21.5) | Neg | Neg | Neg | Neg |
| RV B97 + C | Pos (28.3) | Pos (29.5) | Neg | Neg | Neg |
| RV C | Pos (23.2) | Pos (22.9) | Neg | Neg | Neg |
| RV C | Pos (26.8) | Neg | Neg | Neg | Neg |
| RV C | Pos (20.6) | Pos (20.8) | Neg | Neg | Neg |
| RV C | Pos (23.1) | Pos (29.7) | Neg | Neg | Neg |
| RV C | Pos (17.3) | Pos (19.9) | Neg[c] | Neg | Pos (28.3) |
| RV C | Pos (20.0) | Pos (21.5) | Neg | Neg | Neg |
| RV C | Pos (18.7) | Pos (28.7) | Neg | Neg | Neg |
| RV C | Pos (18.6) | Pos (25.5) | Pos (31.6) | Neg | Pos (34.6) |
| RV C | Pos (23.4) | Pos (28.2) | Neg | Neg | Neg |

[a] RV species A, B, C; no serotype-specific determination for RV species C.
[b] FTDRP EV/PeV assay does not distinguish between EV and PeV.
[c] Ct (41.4) above assay cutoff.

A (8) and Inf B (2) positive samples, and 3 of 6 (50%) RSV, 4 of 5 (80%) HMPV, 5 of 6 (83%) RV and 4 of 5 (80%) AdV positive samples. RSV (MPV.RSV10-01, MPV.RSV10-02, MPV.RSV10-09) and HMPV (MPV.RSV10-07) positive samples that were negative by FTDRP assay had generally lower virus loads and were positive on repeat testing. In contrast, EQA samples spiked with RV-B72 (RV.CV10-03) and AdV-B34 (ADV10-08) were consistently negative and RV-B42 (RV.CV10-01) showed substantially higher Ct values ($\Delta$ 9.8Ct) by the FTDRP RV assay.

### 3.4. Archived clinical specimens

Two hundred sixty-five diverse respiratory specimens that previously tested positive for respiratory viruses by in-house assays were selected for comparison with the FTDRP assay. Of these, 263 were positive for at least one of the 16 assays available in the FTDRP multiplex; two specimens positive for CoV HKU1 for which there was no corresponding FTDRP assay were also tested to assess the specificity of the other FTDRP CoV assays (Table 3). Because of limited available sample volume, only FTDRP multiplex mixes containing the virus-specific assay were performed and virus co-detections by the other assays in each multiplex mix were not included in the analysis. All specimens were confirmed positive by in-house singleplex assays on retesting. The FTDRP assay identified all specimens that were positive for HBoV (2), CoV NL63 (8), Inf A (17), Inf B (11), HMPV (26), PIV4 (12) and EV/PeV (5 EV and 3 PeV); >90% for PIV2 (12/13) and PIV3 (30/31); 85% for PIV1 (17/20); 79% for RV (37/47); 74% for RSV (23/31); and 68% for AdV (17/25). Overall, the FTDRP assay identified correctly 88% of the archived specimens positive for respiratory viruses by the in-house assays and 97% of specimens with lower Ct values (<30). Two specimens positive for CoV HKU1 by in-house singleplex assay were negative by the FTDRP CoV 229E, OC43 and NL63 assays.

The FTDRP AdV, RSV and RV assays gave the lowest relative sensitivities with the archived specimens at 68%, 74% and 79%, respectively. With the exception of RV, most discrepancies occurred with samples containing low levels of viral target. For example, most FTDRP AdV false-negatives occurred with moderate to high Ct value specimens (mean Ct 37.3; range 33.0–39.5), but this did not appear to be associated with any particular AdV type. A wide range of sequence-confirmed AdV types were represented among the archived specimens, including species B (types 3, 7 and 50), C (types 2, 5, 6 and untyped) and F (types 40 and 41), suggesting that the FTDRP AdV assay is inclusive for all recognized human AdV types. In contrast, FTDRP RV assay failed to detect 5 RV positive samples with low Ct values by the corresponding in-house assay.

To further assess the FTDRP RV and EV/PeV assays for virus type/strain inclusivity and group exclusivity, 32 archived samples with high RV (27) or EV (5) loads and typed by partial VP1 and/or VP4/2 RT-PCR and sequencing (protocols available from X.L. on request) were retested (Table 4). The FTDRP RV assay gave negative results with 4 samples and was $\geq$10Ct values higher than the in-house assay with 2 others, all species B or C RVs. The FTDRP EV/PeV assay was also positive with 4 sequence-confirmed RV positive specimens of which 1 was also positive by the in-house EV assay; a second sample also gave an exponential fluorescence amplification curve with the in-house EV assay, but with a >40Ct value and was therefore classified as EV-negative based on test cutoff criteria. Although EV was not detected in 2 of these samples by VP4/2 RT-PCR, and PeV was not detected by the in-house assay, the

presence of these viruses could not be ruled out definitively. Nevertheless, the most probable explanation for these results is that the FTDRP and in-house EV assays cross-react with some RV strains. Five sequence-confirmed EV-positive samples were positive by the FTDRP EV/PeV assay with no evidence of cross-reactions with the RV and PeV assays.

### 3.5. Prospectively tested clinical specimens

Three hundred-eight nasopharyngeal aspirates selected from a study of infants and young children hospitalized with acute respiratory infection were tested prospectively by both in-house and FTDRP assays. Of these, 277 (89.9%) were positive for one or more of the 16 viruses by either the in-house singleplex or FTDRP multiplex assays, with 270 (87.7%) positive by the in-house assay and 265 (86%) positive by FTDRP assay alone (Table 5). Overall, the in-house and FTDRP assays showed good concordance ($K = 0.812$, 95% CI = 0.786–0.838) (Table 6). As seen with the archived specimens, however, the FTDRP AdV, RSV and RV assays gave consistently lower detection rates than the corresponding in-house assays, at 43.7%, 72.5% and 75.5%, respectively, and missed some specimens with high virus loads. Coincidently, these three assays are combined in the same reaction mix (mix #5) and had the highest co-detection rate for these viruses by in-house assays at 41.9%; followed by mix #4 (PIV1, HBoV, HMPV) at 23.8%; mix #2 (CoV 229E, CoV OC43, CoV NL63, EV/PeV) at 16.3%; mix #3 (PIV2, PIV3, PIV4) at 7.3%; and mix #1 (Inf A, Inf B) at 2.2%. Simultaneous presence of multiple targets in the same specimen may have led to competitive inhibition of amplification of less abundant targets and may explain some loss of assay sensitivity.

The FTDRP HBoV assay appeared to be more sensitive than the corresponding in-house assay (Lu et al., 2006) with specimens containing low levels of HBoV. To further investigate this finding, limited sequencing studies were performed using a newly developed semi-nested PCR assay specific for the HBoV NS1 gene that amplifies all 4 recognized HBoV types (protocol available from X.L. upon request). Of the 49 specimens positive for HBoV by both in-house and FTDRP assays, 36 of 37 (mean in-house Ct 26.6; range 13.3–37.3) were successfully sequenced (all HBoV type 1). In contrast, only 2 of 4 in-house assay positive (mean Ct 37.7; range Ct 37.3–38.4)/FTDRP negative and none of the 20 FTDRP positive (mean Ct 38.2; range Ct 36.3–39.9)/in-house negative specimens could be confirmed by NS1 PCR and sequencing. Failure to resolve these discrepancies may be due to (i) a higher sensitivity of the FTDRP assay with specimens containing low levels of HBoV DNA, possibly attributable to the larger volume of TNA extract used in the FTDRP assay (10 μL vs. 5 μL), (ii) failure of both assays to detect some variant HBoV strains and/or (iii) non-specific amplification or amplicon contamination in these samples.

The FTDRP EV/PeV assay also appeared to be more sensitive and specific than the corresponding in-house EV assay with some specimens. Of 18 specimens positive by the FTDRP EV/PeV assay (mean Ct 34.6; range 29.9–38.4), and negative by in-house EV and PeV assays, 9 had recoverable VP1 and/or VP4/2 sequences representing 8 different EVs (echovirus 6, 24, 30; enterovirus 68; poliovirus 1; coxsackievirus A4, B1, B4); 3, that were also positive by in-house and FTDRP RV assays (Ct < 30), had sequence-confirmed species A RV of which 2 also had type-indeterminate EV sequences present; 1 gave a fluorescence amplification curve with the in-house PeV assay, but with a Ct value >40 and therefore was classified as PeV negative; and 5 could not be sequenced. Of 9 samples positive by the in-house EV assay and negative by the FTDRP EV/PeV assay, all were strongly positive for RV (Ct < 30) by both in-house and FTDRP RV assays and were confirmed positive for RV species A or C by VP1 and/or VP4/2 sequences. All 12 specimens positive by the in-house

**Table 5**
Comparison of FTDRP and in-house assays with 308 prospectively tested respiratory specimens.

| Virus[a] | In-house + FTDRP + | In-house + FTDRP − | In-house − FTDRP +[c] | In-house − FTDRP − | Ct <30[b] | | | | Ct ≥30 to ≤37[b] | | | | Ct >37 to <40[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Total + | FTDRP + | FTDRP − | FTDRP % + | Total + | FTDRP + | FTDRP − | FTDRP % + | Total + | FTDRP + | FTDRP − | FTDRP % + |
| AdV | 38 | 49 | 0 | 221 | 32 | 29 | 3 | 91% | 47 | 9 | 38 | 19% | 8 | 0 | 8 | 0% |
| CoV 229E | 6 | 0 | 1 | 301 | 4 | 4 | 0 | 100% | 2 | 2 | 0 | 100% | 0 | | | |
| CoV OC43 | 19 | 4 | 0 | 285 | 14 | 14 | 0 | 100% | 6 | 5 | 1 | 83% | 3 | 0 | 3 | 0% |
| CoV NL63 | 19 | 0 | 0 | 289 | 9 | 9 | 0 | 100% | 10 | 9 | 1 | 90% | 0 | | | |
| EV/PeV[d] | 36 | 9 | 18 | 245 | 19 | 16 | 3 | 84% | 23 | 17 | 6 | 74% | 3 | 3 | 0 | 100% |
| HBoV | 49 | 4 | 20 | 235 | 25 | 25 | 0 | 100% | 17 | 17 | 0 | 100% | 11 | 7 | 4 | 64% |
| HMPV | 50 | 17 | 0 | 241 | 39 | 39 | 0 | 100% | 19 | 10 | 9 | 53% | 9 | 1 | 8 | 11% |
| Inf A | 29 | 1 | 0 | 278 | 17 | 17 | 0 | 100% | 10 | 10 | 0 | 100% | 3 | 2 | 1 | 67% |
| Inf B | 14 | 1 | 0 | 293 | 9 | 9 | 0 | 100% | 5 | 5 | 0 | 100% | 1 | 0 | 1 | 0% |
| PIV 1 | 9 | 4 | 0 | 295 | 7 | 7 | 0 | 100% | 5 | 2 | 3 | 40% | 1 | 0 | 1 | 0% |
| PIV 2 | 1 | 0 | 0 | 307 | 1 | 1 | 0 | 100% | 0 | | | | 0 | | | |
| PIV 3 | 51 | 2 | 0 | 255 | 35 | 35 | 0 | 100% | 13 | 12 | 1 | 92% | 3 | 2 | 1 | 67% |
| PIV 4 | 8 | 0 | 2 | 298 | 2 | 2 | 0 | 100% | 3 | 3 | 0 | 100% | 3 | 3 | 0 | 100% |
| RSV | 74 | 28 | 0 | 206 | 80 | 68 | 12 | 85% | 15 | 6 | 9 | 40% | 7 | 0 | 7 | 0% |
| RV | 80 | 26 | 3 | 199 | 90 | 73 | 17 | 81% | 15 | 7 | 8 | 47% | 1 | 0 | 1 | 0% |
| Total | 483 | 145 | 44 | 3948 | 383 | 348 | 35 | 91% | 190 | 114 | 76 | 60% | 53 | 18 | 35 | 34% |

[a] Virus co-detections included in the analysis.
[b] In-house assay results classified as strong (Ct <30), moderate (Ct ≥30 to ≤37) or weak (Ct >37 to <40) positive.
[c] FTDRP assay Ct values, median (range): HBoV, 38.3 (36.3–39.9); CoV 229E, 38.3; PIV 4, 39.4 (38.9, 39.9); RV, 34.8 (34.8–37.0); EV/PeV 34.7 (30.0–38.4).
[d] FTDRP EV/PeV assay does not distinguish between EV and PeV. Twelve samples positive by in-house PeV assay were also positive by FTDRP EV/PeV assay.

**Table 6**
FTDRP and in-house assay sensitivity, specificity and Kappa values with 308 prospectively tested respiratory specimens.

| Virus[a] | FTDRP[b] | | In-house[b] | | Kappa statistic[c] (95% CI) |
|---|---|---|---|---|---|
| | Sensitivity | Specificity | Sensitivity | Specificity | |
| AdV | 43.7 | 100.0 | 100.0 | 82.0 | 0.527 (0.405–0.648) |
| CoV 229E | 100.0 | 99.7 | 85.7 | 100.0 | 0.921 (0.767–1) |
| CoV OC43 | 82.6 | 100.0 | 100.0 | 98.6 | 0.898 (0.798–0.997) |
| CoV NL63 | 100.0 | 100.0 | 100.0 | 100.0 | 1.(1–1) |
| EV/PeV[d] | 80.0 | 93.2 | 66.7 | 96.5 | 0.676 (0.559–0.793) |
| HBoV | 92.5 | 92.2 | 71.0 | 98.3 | 0.756 (0.662–0.85) |
| HMPV | 74.6 | 100.0 | 100.0 | 93.4 | 0.822 (0.739–0.904) |
| Inf A | 96.7 | 100.0 | 100.0 | 99.6 | 0.981 (0.946–1) |
| Inf B | 93.3 | 100.0 | 100.0 | 100.0 | 0.964 (0.893–1) |
| PIV 1 | 69.2 | 100.0 | 100.0 | 98.7 | 0.812 (0.628–0.995) |
| PIV 2 | 100.0 | 100.0 | 100.0 | 100.0 | 1.(1–1) |
| PIV 3 | 96.2 | 100.0 | 100.0 | 99.2 | 0.977 (0.945–1) |
| PIV 4 | 100.0 | 99.3 | 80.0 | 100.0 | 0.886 (0.728–1) |
| RSV | 72.5 | 100.0 | 100.0 | 88.0 | 0.780 (0.702–0.857) |
| RV | 75.5 | 98.5 | 96.4 | 88.4 | 0.780 (0.704–0.856) |
| All assays | 77.0 | 98.8 | 91.3 | 96.5 | 0.812 (0.786–0.838) |

[a] Virus co-detections included in the analysis.
[b] Referenced to FTDRP or in-house assay.
[c] Kappa statistic: <0–0.2 = poor; 0.21–0.4 = fair; 0.41–0.6 = moderate; 0.61–0.8 = good; and 0.81–1 = very good. CI, confidence interval.
[d] FTDRP EV/PeV assay does not distinguish between EV and PeV.

PeV assay (mean Ct 33.8; range 27.7–38.4) were also positive by the FTDRP EV/PeV assay with similar Ct values.

## 4. Discussion

Diagnosis of ARI in both clinical care and public health settings has greatly advanced in recent years with the increased availability of rapid, sensitive and specific molecular tests for the simultaneous detection of multiple respiratory pathogens. Some commercial assays in particular that have received FDA 510(k) clearance have made substantial inroads into the diagnostic laboratory (Rand et al., 2011). However, these assays are often costly, require dedicated laboratory equipment, use highly multiplexed reactions where individual assay performance may be compromised, and can be difficult to modify quickly in response to the emergence of new medically important virus strains, as occurred during the 2009 H1N1 influenza pandemic.

The commercial multiplex FTDRP real-time RT-PCR assay addresses some of these limitations by offering a complete kit with moderate throughput for detection of 16 respiratory viruses that could be easily integrated into the workflow of laboratories using conventional real-time PCR platforms. The FTDRP assay setup and runtime requires approximately 2.5 h for 12 samples and controls (assay reagents are aliquoted in 12 sample test units), excluding sample extraction, and with a kit list price of $27.34/sample (PCR enzyme kit costs not included). By combining assays into 5 multiplex reaction mixes, individual mixes could more easily modified if needed without impacting the other mixes and could allow for more efficient targeted testing based on epidemiologic findings.

In this study, the FTDRP multiplex assay was compared with in-house singleplex assays corresponding to each of the test viruses. Overall, the FTDRP and in-house assays performed comparably for most viruses tested, particularly when the virus was abundant in the sample (low Ct values). With exceptions noted below, most discordant results were seen with samples containing lower concentrations of virus (high Ct values), suggesting that differences in assay sensitivity near their detection limits was responsible for these discrepancies rather than failure of primer/probe hybridization due to critical target sequence mismatches.

FTDRP assays for RSV, RV and AdV in particular showed lower relative sensitivities than the corresponding in-house assays with some clinical specimens. The FTDRP RV assay showed clear evidence of dropouts with some RV strains (see further discussion below), and some prospectively tested specimens were negative for RSV and AdV, even when the viruses were abundant. It is notable that these three FTDRP assays are combined in the same reaction mix and these three viruses showed the highest co-detection rates by singleplex in-house in these specimens. It is possible that competing amplification reactions in some specimens containing multiple virus targets may have reduced the sensitivity of some assays for low abundant targets. This may have had a more noticeable impact on detection of AdV, where a disproportionate number of AdV positive specimens had lower virus loads. This would be expected in a population comprised of infants and young children where persistent low level AdV shedding is common.

Development of real-time RT-PCR assays that can detect all RV and EV strains and distinguish between both groups is challenging due to the extensive sequence diversity within each group and sequence similarity between some EV and RV strains. These data confirmed previous experience with the in-house EV and RV assays: both assays cross-react with some RV and EV strains, particularly if present in high copy number (Lu et al., 2008; Oberste et al., 2010). Although this complicated efforts to evaluate the FTDRP EV/PeV and RV assays, several conclusions can be drawn from these findings. The FTDRP EV/PeV assay appeared to be more sensitive and specific than the in-house assay for detection of some EV strains, including the recently emergent EV68 (CDC, 2011), although cross-reactions with some RV strains identified in the archived sample collection could not be ruled out. Of particular concern, the FTDRP RV assay was insensitive with some sequence confirmed RV species B and C strains. This finding suggests that critical primer/probe mismatches with these viruses substantially diminished target amplification and/or probe hybridization. More extensive testing of culture purified RV and EV strains will be necessary to assess the full extent of this deficiency.

Limited discrepancy testing was conducted and focused primarily on samples with large differences in Ct values between the in-house and FTDRP assays. To resolve all discrepant results for all assays would require extensive confirmatory testing with additional molecular tests possessing equal or greater sensitivity than those evaluated here, which was beyond the scope of this study. Limited specimen volume prevented additional testing in some

cases. The apparent false-positive results by either assay were most likely true positives occurring as the result of the conditions noted above.

Following completion of this work, Fast-track Diagnostics introduced a new version of the FTDRP assay (FTD Respiratory Pathogens 21) that expands testing to include new respiratory pathogens and modifies of some of the existing assays to enhance performance (Miriam Steimer, Fast-track Diagnostics, personal communication). Given these changes, and the promising potential of the FTDRP multiplex assay for diagnosis of respiratory virus infections, further studies with expanded sample collections are warranted.

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