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1 # A novel real-time PCR assay panel for detection of common
2 # respiratory pathogens in a convenient, strip-tube array format

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24 **Abstract**

25 Commercial multiplex assays, built on different chemistries and platforms are widely
26 available for simultaneous detection of pathogens that cause respiratory infections. However,
27 these tests are often difficult to implement in a resource limited setting because of high cost. In
28 this study, we developed and validated a method for simultaneous testing of common respiratory
29 pathogens (Respanel) by real-time PCR in a convenient, strip-tube array format. Primers and
30 probes for sixteen PCR assays were selected from the literature or newly designed. Following
31 optimization of individual PCR assays, strip-tube arrays were prepared by dispensing primer-
32 probe mixes (PPM) into two sets of 8-tube strips. Nucleic acid extracts from specimens were
33 mixed with PCR master mix, and dispensed column-wise into 2X8-wells of a 96-well plate.
34 PPMs from strip-tubes were then added to the wells using a multichannel pipette for real-time
35 PCR. Individual PCR assays were optimized using previously known specimens (n=397) with
36 91%-100% concordance with culture, DFA or PCR results. Respanel was then tested in a routine
37 manner at two different sites using specimens (n=147) previously tested by Qiagen Resplex I&II
38 or Fast-Track Diagnostics Respiratory Pathogens 21 assays. The sensitivity, specificity and
39 accuracy of Respanel were 94%, 95% and 95%, respectively, against Resplex and 88%, 100%
40 and 99%, respectively, against FTDRP21. Respanel detected 48% more pathogens ($p<0.05$) than
41 Resplex but the rate of pathogen detection was not significantly different from FTDRP21.
42 Respanel is a convenient and inexpensive assay that is more sensitive than Resplex and
43 comparable to FTDRP21 for the detection of common respiratory pathogens.

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47 **Introduction:**

48 Acute viral and bacterial respiratory tract infections are among the most common human
49 ailments (1, 2). Symptoms range in severity from mild upper respiratory tract infections (URTI)
50 to serious lower respiratory tract infections (LRTI), many of which are associated with
51 significant morbidity and mortality particularly in children, the elderly and those who are
52 immunocompromised or have underlying comorbidities such as congenital heart defect or
53 chronic respiratory disease (3-9).

54 These infections contribute to an increased burden of Emergency Department visits
55 during the winter season, leading to longer waiting times and higher health care costs, and are a
56 common reason for hospital admission (10-13). They also account for the majority of antibiotic
57 prescriptions, particularly in children, in spite of the fact that most of the infections will resolve
58 on their own without medical intervention. The specific diagnosis of bacterial versus viral
59 infection cannot conclusively be made on clinical examination alone and, as the symptoms
60 overlap significantly, the physician may order diagnostic tests and empirically prescribe
61 antibiotics (10).

62 Traditionally, the laboratory diagnosis of respiratory infections has been a lengthy
63 procedure, using bacterial and viral culture or relatively insensitive techniques such as
64 immunofluorescence (IFA) and rapid enzyme immune assays (EIA) (5, 11, 14-18). These test
65 modalities can identify a limited number of organisms such as respiratory syncytial virus (RSV),
66 influenza and parainfluenza viruses, and bacteria such as *Streptococcus pneumoniae*. With the
67 introduction of rapid molecular tests we now know that the list of common agents known to
68 cause respiratory infections is much longer than previously thought and the laboratory diagnostic
69 options to detect respiratory pathogens have improved significantly (3, 6, 18, 19). Molecular

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70 tests offer many advantages including a rapid response time and the ability to detect organisms
71 that will not grow in culture. In addition these tests allow detection of bacteria in patients where
72 antimicrobial treatment has been initiated prior to collection of the sample. We now have access
73 to accurate and rapid multiplex assays simultaneously detecting both viral and bacterial
74 pathogens within a few hours. The ability to diagnose co-infections is a significant improvement
75 as these can be associated with increased morbidity and mortality (19-21). Rapid molecular tests
76 have been shown to reduce the length of hospital stay and the cost for testing for those with viral
77 respiratory testing and has facilitated a more targeted approach to patients presenting with
78 respiratory infections with respect to treatment regimens, need for admission and infection
79 control concerns (7, 22-24).

80 A wide range of chemistries and platforms are now commercially available for molecular
81 testing of common respiratory pathogens. Examples include BIOFIRE® FILMARRAY®
82 Respiratory Panel (Biomerieux) that enables detection and identification of multiple organisms
83 based on nested PCR and melt-curve analysis (25). A similar test kit, in terms of workflow and
84 pathogen targets, is ePlex® Respiratory Pathogen Panel (GenMark Dx) which makes use of
85 signal probes and capture probes to electrochemically detect target pathogens (26). Both systems
86 fully automate nucleic acid extraction, amplification or probe hybridization and detection. Also,
87 these tests are very easy to perform, provide faster results (<1 hour) and require very little hands
88 on time (~2 minutes). On the other hand, xTAG® Respiratory Viral Panel (Luminex) and
89 Resplex I&II (Qiagen) assays require nucleic acid extraction and amplification of targets prior to
90 probe hybridization, labelling and detection by a flow cytometric method on a Luminex xMAP
91 system (27). Multiplex, real-time PCR based kits are also available such as Fast-Track
92 Diagnostics Respiratory pathogens 21 (FTDRP21) (Siemens Healthineers) is a five tube

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93 multiplex PCR assay to detect a total of 23 pathogen targets (28). The test kit provides reagents
94 only and the users can run the tests using their own nucleic acid extraction platforms and real-
95 time PCR systems.

96 While most commercial assays are rapid and convenient and their performance
97 characteristics meet regulatory requirements, they are invariably expensive, which may be
98 difficult to implement in resource poor settings. Also, commercial test kits are not uniformly
99 available throughout the world. Timely delivery of test kits and reagents are critical for smooth
100 operation of diagnostic laboratories and a delay in shipping may adversely affect the
101 management of patients with respiratory infections. Furthermore, commercial tests are not free of
102 technical limitations. Some tests may be difficult to interpret due to background signals or
103 ambiguous results from multiple targets in the same reaction. It is also difficult to troubleshoot
104 commercial assays because of proprietary test characteristics. In addition, commercial assays
105 cannot be quickly modified when new pathogens or new strains of known pathogens emerge
106 which are missed by the existing assays. Therefore, in this study, we developed an in-house PCR
107 assay panel (Respanel) customized to detect the most common, and clinically significant
108 respiratory pathogens. The assay was designed to use pre-aliquoted assay-specific reagents in
109 strip-tubes for convenience, and to reduce the hands-on time required to perform multiple
110 reactions for each patient specimen. The performance characteristics of Respanel assay were
111 compared with two commercially available assays.

112

113 **Materials and Methods:**

114 *Clinical specimens:* For individual validation of PCR assays for different pathogens, 34
115 reference bacterial and viral strains from American Type Culture Collection (ATCC), a total of

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116 363 nasopharyngeal wash (NPW) and nasopharyngeal flocked swab (NPFS) specimens, 23
117 serum specimens, 4 pleural fluid (PF) specimens, 4 cerebrospinal fluid (CSF) specimens and 68
118 external quality assessment (EQA) specimens were used. These specimens were submitted to the
119 Microbiology and Virology laboratory of BC Children's Hospital, Canada (between January,
120 2012 to January, 2013) for testing either by culture or by molecular methods. For validation of
121 the full PCR panel, 34 NPW or NPFS specimens that tested positive or negative for various
122 pathogens by Resplex I and II (Qiagen) assays were selected from the same site. An additional
123 113 NPW or NPFS specimens were tested at Sidra Medicine, Qatar. These specimens include
124 external quality assessment (EQA) specimens from College of American Pathologists (CAP),
125 validation specimens and clinical specimens collected at Sidra Medicine from May, 2016 to
126 August, 2017, and were previously tested for respiratory pathogens by FTD Respiratory
127 pathogens 21 (FTDRP21) assay (Siemens Healthineers).

128 Resplex and FTDRP21 assays were performed according to laboratory standard operating
129 procedures based on manufacturer's instructions. Specimens or nucleic acid extracts were
130 maintained at -80°C following initial testing. Testing was performed exclusively on
131 retrospective, residual samples. To maintain patient anonymity, each sample was coded and all
132 patient identifiers were removed to ensure that personnel involved in this study were unaware of
133 any patient information. Ethics approval was not sought because studies that involve the
134 secondary use of anonymous human biological materials are exempted from review by the local
135 Research Ethics Board of the University of British Columbia and Sidra Medicine.

136 *Nucleic acid extraction:* At BC Children's Hospital, nucleic acids from 0.35 mL NPW or
137 NPFS specimens were extracted using the QIAsymphony virus/bacteria kit in an automated
138 nucleic acid extraction platform, QIAsymphony SP (Qiagen, USA). At Sidra Medicine, nucleic

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139 acids from 0.5 ml of NPW or NPFS specimens were extracted on a NucliSENS® easyMAG
140 platform (bioMérieux, France) according to the methods described by the manufacturers.
141 *Real-time PCR:* Primers and probes for sixteen PCR assays were either obtained from
142 previously published assays or newly designed, in this study by using the Primer Express
143 software v3.0.1 (Life Technologies) (Table 1). The in silico specificity of the amplicon sequence
144 for the target pathogen was confirmed by nucleotide blast search against the NCBI non-
145 redundant nucleotide database (nr/nt) (29). Each PCR assay was individually tested and validated
146 using reference bacterial or viral strains and clinical samples that were previously tested by a
147 reference method such as culture, direct fluorescent antibody (DFA) tests, Resplex I & II assays
148 (Qiagen) or an alternative PCR. Apart from Resplex assays, standard PCR assays that were
149 available for comparison were a commercial assay for enterovirus (Trimgen Genetic Diagnostics,
150 USA) and previously validated laboratory developed tests (LDTs) for adenovirus, influenza A,
151 human metapneumovirus, *Streptococcus pneumoniae*, *Mycoplasma pneumoniae* and *Bordetella*
152 *pertussis*,
153 For PCR, 5µl of sample extract was mixed with 20µl of a master mix containing 12.5 µl
154 of 2x QuantiTect Probe RT-PCR Master Mix, 0.25 µl of QuantiTect RT Mix (Qiagen, USA) and
155 primers and probes to final concentrations shown in Supplemental Table 1. Thermal cycling was
156 performed in a ABI7500 Fast instrument (Thermofisher Scientific, USA) with 1 cycle of reverse
157 transcription at 50°C for 30 min followed by 1 cycle of denaturation at 95°C for 15 min,
158 followed by 40 amplification cycles each consisting of 94°C-15s and 60°C-60s. DNA extraction
159 and PCR inhibition was monitored by an internal control PCR assay, using the primers and
160 probes shown in Supplemental Table 2. For use as positive control 4 plasmids harboring
161 amplicon sequences of all Respanel targets were custom made (Integrated DNA Technologies),

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162 diluted to 10^6 copies/ml, mixed and aliquoted for use with each PCR runs. The aliquots of
163 positive control were stored at -20°C. For negative controls, 0.2 ml neonatal calf serum (NCS)
164 (Thermofisher Scientific, USA) spiked with 10^5 copies of a plasmid, harboring target amplicon
165 sequence of IC, were extracted along with specimens and used with each PCR runs. The target
166 sequence of IC is a randomly generated sequence with no known homology to any sequences in
167 the nucleotide database (29).

168 *Preparation of strip-tube arrays:* A mixed working stock of primers and probe(s) for
169 each of the assays were prepared to a total volume of 2 ml, according to the working stock
170 concentrations shown in supplemental table 1. Ten µl of primer and probe mix (PPM) for each
171 assay was dispensed into the tubes of MicroAmp Fast 8-Tube Strips (without cap) (Thermofisher
172 Scientific, USA) according to figure 1. Strip-tubes were placed in 96-well PCR tube racks and
173 PPMs were dispensed either with the aid of multichannel, dispensing pipettes (Eppendorf, USA)
174 or in an automated liquid handler (Perkin Elmer, USA) into two sets of strip-tubes labeled as
175 panel I and panel II. The strips were marked at one end with different colors for Panel I and
176 Panel II to maintain proper orientation. Tubes were then covered with MicroAmp Optical
177 Adhesive Film and pressed and sealed using MicroAmp Adhesive Film Applicator
178 (Thermofisher Scientific, USA). Optical adhesive film covers were then cut vertically between
179 the columns of tubes in each rack using a clean scalpel and panel I and II strips were stored in
180 separate boxes at -20°C.

181 *Respanel PCR assay:* For each sample, one panel I strip and one panel II strip are thawed,
182 centrifuged briefly to bring the contents of the tubes at the bottom of the tubes, and placed in a
183 96-well PCR tube rack. A sample mix is then prepared with 88 µl of extracted nucleic acid from
184 the specimen to be analyzed, 220 µl of 2x QuantiTect Probe RT-PCR Master Mix and 4.4 µl of

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185 QuantiTect RT Mix (Qiagen, USA). Ten percent extra volume was added to the required volume
186 for all components, to account for pipetting error. Next, using an electronic dispensing pipette
187 (Eppendorf, USA), 17.5 µl of sample mix was dispensed into the 16-wells of the 96-well PCR
188 plate, in the two adjacent columns containing the primers and probes as described above and in
189 figure 1. Alternatively, 88 µl of nucleic acid extracts were mixed with 88 µl of TaqPath™ 1-Step
190 Multiplex Master Mix (No ROX) (Thermofisher Scientific, USA) and 44 µl of nuclease free
191 water (Thermofisher Scientific, USA). 12.5 µl of sample mix was dispensed in the same way. 7.5
192 µl of each PPM from panel I and panel II strips are then transferred to the respective wells of the
193 PCR plate using a multichannel pipette. The PCR plate was covered with adhesive film and
194 thermal cycling was performed on an ABI7500 Fast instrument as described above
195 (Thermofisher Scientific, USA). Test results did not vary when tests were performed in parallel
196 with two different master mixes in a set of known specimens (data not shown).
197 *Statistical analysis:* Correlation between the results of different assays was determined by
198 Cohen's kappa test. Ninety-five percent CI for sensitivity, specificity and accuracy were
199 calculated by Clopper-Pearson interval or exact method. The significance of differences in
200 pathogen detection rates between Respanel versus Resplex and Respanel versus FTDRP21 were
201 calculated by 2-tailed, paired Student's t-test.
202
203 **Results**
204 Respanel PCR assays included singleplex assays for *Streptococcus pneumoniae*,
205 *Bordetella pertussis*, adenovirus, human metapneumovirus, influenza A, parainfluenza virus 3,
206 respiratory syncytial virus, enterovirus, rhinovirus, and bocavirus and duplex assays for
207 *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*, influenza B and an internal control (IC),

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208 parainfluenza virus 1 and parainfluenza virus 2, coronavirus 229E and coronavirus OC43, and
209 coronavirus NL63 and coronavirus HKU1, respectively. Test comparisons were only made
210 between results for matching pathogens. Resplex assays included all Respanel targets except
211 *Bordetella pertussis* and also incuded *Neisseria meningitidis*, *Haemophilus influenzae* and
212 *Legionella pneumophila* that are not included in Respanel. Similarly, FTDRP21 assays include
213 influenza A(H1N1)pdm09 virus, human parainfluenza virus 4, human parechovirus,
214 *Haemophilus influenzae* B and *Staphylococcus aureus* that are not included in Respanel.
215 PCR conditions for all targets included in Respanel were first optimized using ATCC
216 strains or EQA panels. Primer and probe concentrations were adjusted for maximum analytical
217 sensitivity and specificity (Supplemental table 2). In some cases such as Influenza B and
218 enterovirus, multiple primer and probe sets were tested for superior performance. Because of the
219 cross-reactivity of enterovirus and rhinovirus primers and probes with their respective targets, an
220 additional PCR reaction was added for enterovirus 68 in order to correctly assign results for
221 enteroviruses and rhinoviruses. PCR assays were then tested individually using PCR, DFA or
222 culture confirmed specimens or ATCC reference strains of bacteria and viruses (n=397).
223 Respanel PCR results in 91%-100% agreement with the reference results (Supplemental table 3).
224 Next, a total of 34 nasopharyngeal wash specimens that were previously tested by
225 Resplex I and II were tested by Respanel assay as described in the materials and methods. The
226 overall, observed agreement with Resplex assay was ~95% (*kappa* = 0.734; 95%CI: 0.643-
227 0.826). In total, 71 pathogens were identified in these samples by Respanel, which was 48%
228 higher than that of Resplex assays (Table 1). >90% of pathogens that were undetectable by the
229 Resplex assays had higher C_T values (>30) by real-time PCR. Similarly, 113 NPW or NPFS
230 specimens that were previously tested by FTDRP21 assay were retested by Respanel. The

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231 overall, observed agreement with FTDRP21 assay was ~99.2% (*kappa* = 0.896; 95%CI: 0.845-
232 0.946). In total, 78 pathogens were identified in these samples by Respanel and 82 pathogens
233 were identified by FTDRP21 assay (Table 2). Respanel detected 6 pathogens that were not
234 detected by FTDRP21. On the other hand FTDRP21 assay detected 10 pathogens that were not
235 detected by Respanel. Notably, majority of these specimens had PCR C_T values >35.

236 The sensitivity and specificity of Respanel assay and the accuracy of results obtained
237 by this assay were calculated against both Resplex and FTDRP21 assays. The specificity and
238 accuracy of Respanel results were ≥95% against both commercial assays. Sensitivity of Respanel
239 was 94% and 88% against Resplex and FTDRP21assays, respectively (Table 3). When results
240 for each of the individual pathogens were compared, Respanel assay results were more correlated
241 (>95%) to FTDRP21 assay than Resplex assays (Table 1 and 2).The cost of Respanel is
242 approximately 25% of Resplex assays and requires less than 10 minutes of hands-on time per
243 sample, apart from the initial effort required for producing PCR-ready aliquots of primer/probe
244 mixes in PCR strip-tubes.

245

246 **Discussion**

247 Molecular assays for simultaneous detection of common respiratory pathogens are now
248 widely used, replacing many conventional diagnostic methods such as culture and antigen
249 detection assays. Implementation of these tests have resulted in increased identification of
250 causative pathogens for acute upper and lower respiratory tract infections. In addition, a
251 significant decrease in TAT has assisted institutions in the timely use appropriate isolation
252 precaution measures and reduce inappropriate antibiotic use (30-33). A wide range of multiplex
253 assays are now available commercially which differ in their use of chemistry, optics and the level

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254 of automation. Many test methods have also been described in the literature for potential use as
255 laboratory-developed tests (LDT). To make an appropriate choice, a careful assessment of
256 performance characteristics as well as costs and benefits associated with the test method is
257 necessary. Ideally, when performance characteristics of test methods are comparable and meet
258 regulatory requirements, a test method and a platform that provides rapid TAT and requires
259 minimum technical skill and hands on time would be preferable. However, the costs associated
260 with the platform, such as test kits and service contracts may not be affordable to many
261 laboratories. Furthermore, because the majority of these test platforms are closed systems it is
262 difficult to troubleshoot ambiguous results and test failures in a timely manner without active and
263 continuous support from the vendor. LDTs on the other hand necessitate more labour and
264 responsibilities for the laboratory in order to maintain the quality of the test. Substantial
265 molecular biology expertise and effort are also necessary for validation of LDTs. However,
266 LDTs are less expensive and can be tailored specifically to needs of the laboratory and the
267 patient population it serves. An additional advantage is that LDTs can be updated rapidly to
268 make the test inclusive for a new pathogen of interest or an emerging variant of a known
269 pathogen. Logistically, if stored properly, primers and probes used in PCR based LDTs may
270 have a longer shelf life than the manufacturer recommended shelf life of commercial kits.

271 The purpose of this study was to develop a laboratory-developed, real-time PCR based
272 test panel for selected respiratory pathogens with minimum multiplexing and in a format so that
273 minimum hands-on time is required. Accordingly, we designed our workflow so that assay
274 specific reagents are ready to be used for routine testing (Figure 1). We also minimized repeated
275 pipetting through the use of multichannel pipettes for faster transfer of reagents and to prevent
276 pipetting error. The pre-aliquoted primer and probe mixes in 8-tube strips can be prepared once

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277 in 3 months, 6 months or 1 year according to laboratory needs. Preparation of primer/probe
278 mixes and strip tubes for Panel I and II may take one FTE technologist time for up to 8 hours.
279 We noted that in an automated liquid handler (Perkin Elmer, USA), preparation of 200 test strips
280 takes about 2 hour time.

281 After initial optimization of PCR conditions, Respanel assay was validated in three steps.
282 First, the accuracy of results obtained by each PCR assay was compared with results obtained by
283 bacterial or viral culture, DFA, Resplex and standard PCR assays offered by the Microbiology
284 and Virology laboratories of BC Children's Hospital, Canada. Next test comparisons were made
285 with Resplex and FTDRP21 assays at two different laboratories using nasopharyngeal
286 specimens. Despite multiple test types used as reference methods, Respanel assay demonstrated
287 85%-100% concordance with the reference methods. Respanel detected significantly ($p=0.015$
288 by Student's T-test) more pathogens than Resplex but was not different than the FTDRP21 panel
289 ($p=0.27$ by Student's T-test). This is consistent with the fact that Respanel and FTDRP21 are
290 both real-time PCR based methods, while Resplex assay employed a different chemistry based
291 on highly multiplexed PCR, probe hybridization and flow cytometry.

292 To our knowledge, this is the first report of a strip-tube array based, clinical laboratory
293 developed PCR test panel for respiratory pathogens designed for cost savings, convenience and
294 superior or equivalent performance compared to commercial assays. Furthermore, the laboratory
295 developed, Respanel has the additional advantage of flexibility to update, modify, incorporate or
296 replace pathogen targets as required, and to serve specific patient populations with acute
297 respiratory infections.

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419 **Table 1: Concordance between Respanel and Resplex test results**

Organism	Respanel		Resplex I&II		Concordance (%)
	Positive	Negative	Positive	Negative	
Adenovirus	1	33	3	31	94
Human Metapneumovirus	1	33	1	33	100
Influenza A	8	26	2	32	82
Influenza B	0	34	0	34	100
Parainfluenza Virus 1	1	33	1	33	100
Parainfluenza Virus 2	3	31	2	32	97
Parainfluenza Virus 3	3	31	0	34	91
Respiratory Syncytial Virus	4	30	3	31	97
Mycoplasma pneumoniae	5	29	4	30	97
Chlamydophila pneumoniae	1	33	0	34	97
*Enterovirus/Rhinovirus	17	17	13	21	88
Bocavirus	8	26	6	28	94
Coronavirus 229E	0	34	0	34	100
Coronavirus OC43	2	32	2	32	100
Coronavirus NL63	2	32	1	33	97
Coronavirus HKU1	0	34	0	34	100
Streptococcus pneumoniae	15	19	10	24	85
Total	71	507	48	530	96

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421 *Enterovirus and Rhinovirus results were compared together because Resplex Enterovirus and Rhinovirus assays
422 were reported in aggregate.

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424 **Table 2: Concordance between Respanel and FTD Respiratory pathogens 21 test results**

Organism	Respanel		FTD		Concordance (%)
	Positive	Negative	Positive	Negative	
Adenovirus	7	106	7	106	100
Human Metapneumovirus	2	111	3	110	99
Influenza A	4	109	5	108	99
Influenza B	3	110	3	110	100
Parainfluenza Virus 1	3	110	3	110	100
Parainfluenza Virus 2	2	111	2	111	100
Parainfluenza Virus 3	5	108	6	107	99
Respiratory Syncytial Virus	8	105	8	105	100
Mycoplasma pneumoniae	4	109	4	109	98
Chlamydophila pneumoniae	0	113	0	113	100
Enterovirus	4	109	4	109	98
Rhinovirus	19	94	21	92	96
Bocavirus	4	109	4	109	98
Coronavirus 229E	3	110	4	109	99

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Coronavirus OC43	3	110	3	110	100
Coronavirus NL63	5	108	4	109	99
Coronavirus HKU1	2	111	1	112	99
Total	78	1843	82	1839	99

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426 **Table 3: Diagnostic performance of Respanel compared to Resplex and FTD Respiratory**
427 **pathogens 21 assays**

Statistics	Resplex I&II	FTDRP21
	Value (95% CI)	Value (95% CI)
Sensitivity	94% (83%-99%)	88% (79%-94%)
Specificity	95% (93%-97%)	100% (99%-100%)
Accuracy	95% (93%-97%)	99% (99%-100%)

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430 **Figure legend**

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432 Figure 1: Respanel assay workflow

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Fig 1