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ABSTRACT

We have developed a nucleic acid-based assay that is rapid, sensitive, specific, and can be used for the simultaneous detection of 5 common human respiratory pathogens including influenza A, influenza B, parainfluenza type 1 and 3, respiratory syncytial virus, and adenovirus group B, C, and E. Typically, diagnosis on an un-extracted clinical sample can be provided in less than 3 hours, including sample collection, preparation, and processing, as well as data analysis. Such a multiplexed panel would enable rapid broad-spectrum pathogen testing on nasal swabs, and therefore allow implementation of infection control measures, and timely administration of antiviral therapies. This article presents a summary of the assay performance in terms of sensitivity and specificity. Limits of detection are provided for each targeted respiratory pathogen, and result comparisons are performed on clinical samples, our goal being to compare the sensitivity and specificity of the multiplexed assay to the combination of immunofluorescence and shell vial culture currently implemented at the UCDMC hospital. Overall, the use of the multiplexed RT-PCR assay reduced the rate of false negatives by 4% and reduced the rate of false positives by up to 10%. The assay correctly identified 99.3% of the clinical negatives, 97% of adenovirus, 95% of RSV, 92% of influenza B, and 77% of influenza A without any extraction performed on the clinical samples. The data also showed that extraction will be needed for parainfluenza virus, which was only identified correctly 24% of the time on un-extracted samples.

Each year, between October and March, hospital admissions suddenly increase with patients presenting with influenza or influenza-like symptoms. It is estimated that influenza-associated hospitalizations in the United States range from approximately 54,000 to 430,000 per season (1). Respiratory syncytial virus (RSV) is the most common cause of bronchiolitis and pneumonia among infants and children under one year of age, but most respiratory viruses can trigger severe lower respiratory tract disease at any age, especially among the elderly or among those with compromised cardiac, pulmonary, or immune systems (2). In this context, timely and accurate identification of respiratory viruses is rapidly becoming more relevant as antiviral treatment options increase. Additionally, the resulting improved treatment of patients presenting with respiratory illness will help control infection, prevent nosocomial spread, and reduce patient stay as well as hospital costs.

Although alternative respiratory virus identification techniques such as immunofluorescence and rapid antigen detection tests have been developed to provide rapid diagnostic capabilities, viral culture remains the most prevalent test in use for laboratory identification (3). The main drawback of immunoflorescence and rapid tests kits is their lack of sensitivity. A recent study reported that immunofluorescence assays detect only 19% of respiratory viruses with viral loads below 10^6 copies/mL (4) and rapid test kits have been shown to have typical false negative rates of 30% for influenza (5). While viral culture is both sensitive and specific, it is labor intensive and time consuming. Additionally, because some viral strains grow poorly and/or slowly in cell culture, timely results are not available to impact or inform clinical decisions such as the use of antiviral drug treatment. A recent study undertaken with pediatric patients to determine the impact of rapid diagnosis of influenza (such as the FluOIA test from Biostar Inc. which is only 83-96% sensitive and 64-

76% specific) on physician decision-making and patient management in the ER showed that the use of rapid test kits at point-of-care lead to a reduction of antibiotic prescriptions of 40%, a reduction of laboratory and radiograph charges of 50%, patient discharges occurring one hour more quickly, and an increase in antiviral use by 25% (6). Another study comparing cell culture and immufluorescence focused on the benefits of rapid reporting of respiratory viruses concluded that the mean length of stay for hospital inpatients with respiratory viral isolates was 10.6 days (mean cost of $7,893) when the patients were diagnosed by viral culture and only 5.3 days (mean cost of $2,177) when they were diagnosed using immunofluorescence (7).

To alleviate issues of specificity and sensitivity inherent to the rapid tests as well as the long turnaround times of viral culture, laboratories analyzing clinical samples are progressively moving toward molecular diagnostics as a mean to identify respiratory viruses. Nucleic acid amplification techniques such as PCR followed by gel electrophoresis (8), and quantitative PCR (q-PCR) with corresponding probes (9, 10) have recently been developed for the rapid detection of respiratory pathogens, leading to significant sensitivity and specificity improvements over culture and immunofluorescence techniques. Nevertheless, a limitation of semiquantitative real-time PCR assays is their extremely low level of multiplexing. Multiplexed detection capabilities provide many advantages over conventional detection methodologies. In the event of a respiratory disease outbreak, the use of multiplexed assay panels can provide a cost effective means of handling high volumes (i.e., a surge) of samples. Moreover, custom tailored assay panels designed to respond to genetic mutations and/or new pathogens can be rapidly implemented, and therefore greatly help reduce the impact of infectious disease outbreaks. Additionally, contrary to current q-PCR

assays which require DNA/RNA extraction, the only requirement of our assay is a nasal swab in buffer solution, dramatically reducing processing time and reagent costs.

We have extended the utility of nucleic acid amplification techniques by developing a multiplexed RT-PCR assay that allows timely simultaneous detection of five respiratory viruses. The multiplexed assays (liquid arrays) have been developed on a commercially available flow cytometer (Bioplex, Bio-Rad Inc.). The assay utilizes surface-functionalized polystyrene micro-beads, embedded with precise ratios of red and infrared fluorescent dyes (FIG. 1). There are 100 unique dye ratios, giving rise to 100 unique bead classes. When excited by a 635-nm laser, the two dyes emit light at different wavelengths (658 and 712 nm) and thus each bead class has a unique spectral address. Bead classes can be easily distinguished and therefore they can be combined and up to 100 different analytes can be measured simultaneously within the same sample. Although liquid arrays have been demonstrated in a variety of applications (11) including detection of antigens, antibodies, small molecules, and peptides, in the presently described application, beads are functionalized with a nucleic acid probe approximately 30 bases long, where the probe sequence is complementary to a target amplicon. Nucleic acid from the pathogen of interest is amplified by RT-PCR (FIG. 1), which is conducted using a mixture of all forward and reverse primers for each of the pathogen targets in the multiplexed panel. The amplified product is then introduced to the bead mixture, allowed to hybridize, and subsequently labeled with the fluorescent reporter, streptavidin-phycroerythrin (SAPE). Each optically encoded and fluorescently-labeled micro-bead is interrogated by the Bioplex flow cytometer. A red laser excites the dye molecules inside the bead and classifies it while a green laser quantifies the assay at the bead surface via the median fluorescence intensity (MFI) of the

SAPE reporter. The flow cytometer is capable of reading several hundred beads each second and fluorescence analysis can be completed in as little as 15 seconds.

The current panel (Table 1) includes 16 beads, with assays for influenza A (2 assays) influenza B (2 assays), parainfluenza type 1 (1 assay) and 3 (1 assay), respiratory syncytial virus (1 assay), and adenovirus group B (2 assays), C (2 assays), and E (1 assay). The panel also includes 4 unique internal controls described in the methods section. Typically, results on a clinical sample can be provided in less than 3 hours, including sample collection, preparation, and processing, as well as data analysis.

This article presents a summary of the assay performance in terms of sensitivity and specificity. Limit-of-detection (LOD) values for each targeted respiratory pathogen are presented for the multiplexed panel, and result comparisons are performed on clinical samples collected at the UCDMC (University of California Davis Medical Center, Davis, CA), our goal being to compare the sensitivity and specificity of the multiplexed assay to the currently implemented detection techniques.

MATERIALS AND METHODS

Reagents. Tris-NaCl (0.1 M Tris, 0.2 M NaCl, 0.05 % Triton X-100, pH = 8.0) and TE (10 mM Tris-HCl, 1.0 mM EDTA, pH = 8.0) buffers were purchased from Teknova Inc. (Hollister, CA). Streptavidin-phycoerythrin (SAPE) was purchased from Invitrogen Inc. (Carlsbad, CA) and suspended in Tris-NaCl at a concentration of 3 ng/μL. All primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA) and suspended in TE buffer.

Viruses. Current circulating strains of certified killed respiratory viruses were purchased at a stock concentration of 1 mg/mL. Influenza A viruses (A/H1, New Caledonia strain and A/H3, Shandong strain), RSV, and adenovirus C were purchased from Research Diagnostics Inc. (Flanders, NJ), while Influenza B, Victoria strain, and Parainfluenza type 1 and 3 were purchased from Advanced Immunochemical Inc. (Long Beach, CA). Adenovirus group B, and E were also grown and titered by the method of Reed and Muench (12).

Carbodiimide coupling of amino-substituted probes to carboxylated microbeads. Different sets of carboxylated fluorescent micro-beads were obtained from Luminex Corp. (Austin, TX), and oligonucleotide probes for the respiratory panel were assigned to individual bead sets. Each probe sequence represented the reverse complement to the target region of the forward strand (5'-3') and contained a spacer (18-atom hexa-ethyleneglycol spacer) between the reactive group (Amino Modifier C6, also called phosphoramidite) and the 5' end of the oligonucleotide, to enable optimal hybridization. Phosphoramidite is a primary amine which results in a stable, covalent attachment upon reaction with the ester on the bead coating. Probes for each of the pathogen targets were coupled to the beads using the manufacturer's recommended coupling protocol. Briefly, a 1 mL aliquot of beads (1.25 x 10^7 beads) was re-suspended in 50 µL of 0.1 M 2-[N-morpholino] ethanesulfonic acid (MES) buffer at pH = 4.5 and sonicated. 0.05 mg of 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC) (Pierce Biotechnology, Rockford, IL) was added, along with 10 µL of probe at a concentration of 50 µM. This solution was incubated in the dark at room temperature for 30 minutes. A second aliquot of EDC (0.025 mg) was added and incubated in the conditions described above. The beads were then rinsed in 1 mL phosphate buffered saline (PBS) containing 0.02 % Tween-20 (Sigma, St Louis, MO), centrifuged at

10,000 rpm for 5 min, rinsed in PBS containing 0.1 % sodium dodecyl sulfate (SDS), centrifuged a second time, re-suspended in 250 µL of TE buffer, sonicated, and stored in the dark at 4 °C. A 10X bead set containing all conjugates was then prepared, using 200 µL of each bead in a total volume of 5 mL of Tris-NaCl buffer. A 1X working solution was then prepared from the stock before use, using Tris-NaCl buffer for dilution.

RT-PCR reaction. The All RT-PCR (Reverse Transcription-Polymerase Chain Reaction) reactions were prepared using the end-point Superscript III one step RT-PCR kit from Invitrogen Inc. (Carlsbad, CA). Typically, each 25 µL PCR reaction contained: 12.5 µL of Superscript III Master mix, 0.5 µL of $MgSO_4$ (50 mM), 0.1 µL of each forward and reverse primer (0.4 uM final concentration), 1 µL of reverse transcriptase and Taq DNA polymerase mix, and PCR grade water to complete the volume to 20 µL. 5 µL of un-extracted sample was then added to 20 µL of PCR mix and cycled on a thermocycler using the following parameters: reverse transcription at 50 °C for 30 min, denaturation at 95 °C for 15 min, followed by 35 PCR amplification cycles (denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 15 s).

Microbead hybridization. Following RT-PCR, 5 µL of amplified product was added to 22 µL of bead mix and hybridized to the probe-coated beads using a denaturation step at 95 °C for 2 min, followed by an annealing step at 55 °C for 5 min.

Microbead washing and labeling. The hybridized bead solution was transferred to a 96-well filter plate (Millipore Inc., Bedford, MA) with 1.2 µm pores. The beads were washed 3 times to remove un-bound oligonucleotides, using 100 µL aliquots of Tris-NaCl buffer pipetted in each well and vacuum-aspirated with a vacuum manifold kit (Millipore Inc., Bedford, MA). The washed beads were then incubated with 60 µL of 3 ng/mL SAPE

reporter for 5 min, washed twice using 100 µL aliquots of Tris-NaCl buffer, and transferred into a 96-well microtiter round bottom plate. For each well, 50 µL of solution was analyzed in the Bioplex flow analyzer.

Controls. Controls that convey important diagnostic information regarding reagent addition, quality, and concentration, assay operator performance, and instrument stability were added to the assay. A unique set of four internal controls are built into every sample, monitoring and reporting every step of the protocol. The negative control (NC) is a bead coupled to a Mt7 probe. Mt7 is a nucleic acid sequence obtained from *Maritima maritensis*, an organism found near deep-sea thermal vents. This organism was selected to serve as a NC because its nucleic acid is unlikely to be observed in clinical samples. Thus, Mt7 is not expected to bind exogeneous nucleic acids and consequently, the median fluorescent intensity (MFI) of the NC beads should always be low. High MFIs on the NC beads obtained in the presence of a sample would indicate a lack of specificity. The instrument control (IC) verifies the reporter fluorescence optics of the flow analyzer. The IC is a bead to which a Cy3-labeled Mt7 probe has been coupled. The probe is unlikely to bind other nucleic acids, and the Cy3 dye emits a constant fluorescence (i.e. constant MFI) in all samples when excited by the reporter laser. A change in MFI on the IC bead indicates fluctuations in the reporter laser performance. The fluorescent control (FC) tests for the addition of the fluorescent reporter (SAPE). FC is a bead coupled with biotinylated-Mt7 probe that fluoresces after exposure to SAPE. A bead coupled to an RNAse P probe serves as a positive PCR control, as well as a control for the addition of the clinical sample. Signals are obtained only when PCR product has been generated and bound to the probe, and SAPE has been added; lack of signal on the PCR control bead indicates that either PCR was not performed

properly or that SAPE was not added. The FC control, however, will yield a signal even in the absence of PCR, so these two events can be decoupled. These controls afford high-confidence that the assay is performed correctly by monitoring the addition of sample, confirming PCR was performed, indicating that SAPE was added, checking that the instrument is performing, and verifying that the assay is specific. Every sample is analyzed in the context of the performance of the controls, thereby minimizing the likelihood of false positives.

Limit Of Detection (LOD) data. Each virus was diluted in distilled water starting from a 10^2 ng/μL stock. The concentration range for the LOD study spanned ten orders of magnitude using 2 dilutions per order of magnitude. Each concentration was run in quadruplicate and LOD data sets for each specific virus were run on separate 96-well plates in order to prevent any possible cross-contamination. All experiments were performed on whole virus without nucleic acid extraction. Each plate contained 8 blank wells in which distilled water was added to the RT-PCR mix as negative controls.

Clinical sample collection and handling. From November 2004 through November 2006, over 1,000 nasal swab samples were collected from patients arriving in the emergency room at the UCDMC Emergency Department in Sacramento, CA, which treats 60,000 patients per year including 12,000 children. Nasal swabs were obtained from patients showing respiratory symptoms, as well as from asymptomatic subjects such as accompanying family members.

Nasal swabs were collected in 3 mL of M4 viral transport medium (Remel, Lenexa, KS), which is composed of gelatin, vancomycin, amphotericin B, and colistin. The sample was then de-identified and divided into two tubes. One aliquot was subjected to

immunofluorescence testing and/or viral culture utilizing standard shell vial technique while the other sample aliquots were analyzed with multiplexed assays on the Bioplex platform. According to immunofluorescence and/or viral culture results, the clinical sample inventory contained: 56 RSV samples, 35 influenza A samples, 12 influenza B samples, 46 parainfluenza samples, 30 adenovirus samples, and 828 negative samples.

Extraction. Although extraction was not generally performed on clinical samples, results obtained with parainfluenza virus were suboptimal. In order to asses whether these results derived from poor primer performance or had other roots, viral RNA was purified for 8 parainfluenza samples using the MagMAX™-96 Viral RNA Isolation Kit (Ambion, 1836). During the purification process, the samples were lysed, and magnetic beads were used to bind the nucleic acid. The beads were then washed using two alcohol wash solutions. Following the washes, the nucleic acid was removed from the beads by adding an elution buffer and heating the solution to 65ºC. This eluent, which represents the purified RNA sample, was used for the multiplexed RT-PCR reaction.

RESULTS

Respiratory panel design. An initial set of 24 signatures derived from a variety of sources (Centers for Disease Control, Lawrence Livermore National Laboratory Bioinformatics Group, as well as previously published work (9, 10)) were chosen for their ability to bind and amplify target-specific genes which are phylogenetically conserved and

247 therefore insensitive to strain variations. Each signature typically consisted of two 20 bp
248 primers and a 30 bp probe; typical amplicon length was 90-200 bp.

249 Signatures were first tested in singleplex reactions (only one primer pair present in the
250 primer mix) against their respective targets in order to ascertain the likelihood of identifying
251 target. Four signatures did not identify target except at very high concentrations (100 pg per
252 reaction) and were therefore discarded, leaving twenty signatures for assembly in a
253 multiplexed panel. Starting with a single viral target, individual signatures were added to a
254 growing mixture one at a time, until all target signatures were added and demonstrated to
255 work as effectively in the multiplexed environment as they did in the singleplex format. This
256 viral target signature "block" was then combined with another viral signature block and
257 tested again. After each signature addition, poor performers and/or competing signatures
258 were isolated and removed. Poor performers were typically signatures which provided low
259 but adequate MFI signals in singleplex, but for which the MFI signals further dropped in the
260 presence of other signatures. Competing signatures were comprised of primer sets that
261 amplified overlapping target regions and therefore competed for target amplification. The
262 effect of such a competition is a concomitant drop of the MFI signal for both competing
263 signatures while other signatures keep performing well in the assay.

264 **Assay optimization.** At the end of this iterative process, the multiplexed respiratory
265 panel was composed of 12 signatures and 4 controls. RT-PCR parameters such as the added
266 $MgSO_4$ concentration, the annealing temperature, the extension temperature, and the
267 extension time, were then optimized for this final respiratory panel in order to produce a
268 combination of low backgrounds, high MFI signals, and low cross-reactivity. Four $MgSO_4$
269 concentrations ranging from 1 to 6 mM, three annealing temperatures ranging between 50

and 60 °C, two extension temperatures: 68 and 72 °C, and three extension times ranging from 10 to 20 s were investigated. For each new parameter under study, an LOD curve was built in triplicate for a minimum of three organisms including adenovirus type C, RSV, and influenza A, and the experimental conditions leading to the best combination of background, MFI, and cross-reactivity signals across the range of targets tested was selected (data not shown). All the primer concentrations were maintained at 0.4 µM, except for the RNAse P control primer concentration which was decreased to 0.2 µM to reduce the probability of amplification competition, and the RSV forward primer concentration, which was increased to 0.8 µM due to the fact that two reverse primers are present in the mix, amplifying RSV type A and B respectively. Details of the optimized RT-PCR protocol are provided in the materials and methods section.

Limits of Detection (LODs). The LOD for each target was then determined with the 16-plex respiratory panel, using the protocol described in the materials and methods section. An average of the four MFI values was plotted on the LOD graph for each concentration, as well as the standard deviation. Two examples are provided in FIG. 2: the LOD curves for influenza A are shown on FIG. 2A, for both influenza A signatures when titrating using the New Caledonia strain, which is an A/H1 subtype. The LOD curve for the single signature for parainfluenza 3 is also presented in FIG. 2B. A summary of the LOD values, defined as virus concentrations at which the corresponding average MFI values were above the background by more than three standard deviations, is presented in Table 2. The LOD value obtained for parainfluenza 1 was higher than for the other viruses. As discussed in the clinical evaluation section below, this result was attributed to the remarkable stability of the nucleocapsid which encapsidates the RNA of *paramyxoviruses* (13). All the other LOD

293 values obtained with the multiplexed RT-PCR assay without performing any RNA/DNA
294 extraction step were within one to two orders of magnitude of the LOD values published
295 using both RNA/DNA extraction procedures and significantly lower levels of multiplexing
296 (14-17). The ability to remove the extraction step from the assay protocol may be valuable
297 for point of care applications because it simplifies the handling of clinical samples, lowers
298 the processing costs, shortens the analysis time by up to 30 minutes, and allows for easier
299 assay automation.

300 **Clinical evaluation.** The multiplexed panel was tested on clinical samples collected
301 from patients arriving in the emergency room at the UCDMC. Nasal swabs were collected in
302 viral transport medium and divided into two aliquots. One aliquot was diagnosed using
303 immunofluorescence and/or viral culture while the other aliquot was diagnosed using the
304 multiplexed RT-PCR respiratory panel on the Bioplex platform. For the Bioplex-based
305 assay, 5 μL of nasal swab sample was directly mixed with 20 μL of PCR reagents and the
306 amplification, bead hybridization, washing, labeling, and flow cytometer analysis steps were
307 performed according to the previously described protocol (see materials and methods section
308 for details). A total of 828 negative samples were first analyzed in order to set threshold
309 values for positive identification. Threshold values for each signature were calculated based
310 on the response of the known negative patient samples. First, outliers were removed
311 iteratively using the Grubb's outlier test (18). After the outliers were removed, thresholds
312 were calculated for each signature. The threshold value was chosen such that the MFI values
313 of negative samples that were not determined to be outliers would exceed this value at a rate
314 of 0.005, which corresponds to a set assay specificity of 99.5 %. These thresholds led to a
315 rating scale for which MFI values below the threshold were ruled negative, and MFI values

316 equal to or above the threshold were ruled positive. A summary of the threshold values is
317 provided in Table 3. For viruses for which two signatures were included in the panel, a
318 positive was called when at least one of the signatures had an MFI equal to or above
319 threshold. Out of the 828 samples tested, 791 were confirmed negative by multiplexed RT-
320 PCR (95.5 %) and 37 were identified positive for a respiratory virus. These 37 samples were
321 sent to the Viral and Rickettsial Disease Laboratory (VRDL) at the State of California Health
322 and Human Services Agency (Richmond, CA) for third party confirmatory q-PCR analysis.
323 The positive multiplexed RT-PCR result was validated for 31 samples and invalidated for 6
324 samples, bringing the percentage of correctly identified clinical negatives to 99.3% and
325 reducing the rate of false negatives by 4% compared to the combination of
326 immunofluorescence and/or shell vial culture implemented at the UCDMC.

327 Samples identified positive via viral culture and/or immunofluorescence, including 56
328 RSV samples, 35 influenza A samples, 12 influenza B samples, 46 parainfluenza samples,
329 and 30 adenovirus samples, were then analyzed randomly using 96-well plates and the
330 identification performed using multiplexed RT-PCR was compared to the viral culture and/or
331 immunofluorescence results. A summary of this clinical study is provided in Table 4. For
332 each respiratory virus, the table shows the number of samples identified positive using viral
333 culture and/or immunofluorescence, the number of samples confirmed positive by
334 multiplexed RT-PCR, the number of samples for which the multiplexed RT-PCR result was
335 positive but in disagreement with viral culture and/or immunofluorecence, and the number of
336 samples identified negative by multiplexed RT-PCR. The five samples (1 RSV and 4
337 parainfluenza) for which the positive diagnoses made by viral culture and by multiplexed
338 RT-PCR were in disagreement were cultured a second time and for all 5 samples, the

identification made by multiplexed RT-PCR was confirmed upon re-culture. All the samples identified positive by viral culture and/or immunofluorescence but negative by multiplexed RT-PCR (10 influenza A, 1 influenza B, 8 RSV, and 4 adenovirus) were sent to VRDL for third party confirmatory q-PCR analysis. The separate singleplex semiquantitative assays run on these samples confirmed the negative multiplexed RT-PCR results for 2 influenza samples out of 10, 5 RSV samples out of 8, and 3 adenovirus samples out of 4. Overall, when folding the third party confirmatory results into the study, the multiplexed RT-PCR assay correctly identified 97% of adenovirus, 95% of RSV, 92% of influenza B, and 77% of influenza A without any extraction of the clinical samples (data summarized in the last column of Table 4). Compared to the combination of immunofluorescence and/or viral culture, the use of the multiplexed RT-PCR assay reduced the rate of false positives by up to 10% for adeno virus and RSV.

In order to investigate the poor performance of multiplexed RT-PCR compared to viral culture for parainfluenza (only 24% of correct identification on un-extracted samples), we performed an extraction experiment on a subset of clinical samples diagnosed positive for parainfluenza by viral culture. These samples were extracted using a magnetic bead-based viral RNA isolation kit and 5 μL of the purified and concentrated RNA was tested using the multiplexed RT-PCR protocol. As a control, two samples identified as parainfluenza type 1 and two samples identified as parainfluenza 3 by multiplexed RT-PCR before RNA extraction were extracted and re-analyzed in similar conditions, confirming the initial results. Three randomly selected samples initially identified as parainfluenza by viral culture and immunofluorescence but as negative by multiplexed RT-PCR were then extracted and re-analyzed. All three samples were identified as parainfluenza type 1 or 3 upon extraction,

362 suggesting that an extraction step will be required in order to increase the sensitivity of the
363 parainfluenza assay.

364

365 **DISCUSSION**

366

367 Although immunofluorescence and/or viral culture had initially identified 828 clinical
368 samples as negative, 791 were confirmed negative by multiplexed RT-PCR (95.5 %) while
369 37 were identified positive for a respiratory virus. Confirmatory q-PCR assays performed at
370 the VRDL invalidated the positive diagnostic for 6 samples but validated it for 31 samples.
371 Out of these 31 positive samples missed when using standard detection techniques, 24 were
372 RSV positive, 4 were influenza A positive, 2 were adenovirus positive, and one was
373 parainfluenza positive. This data points out that most of the false negatives (77%) generated
374 by the immunofluorescence/viral culture detection techniques are missed RSV samples. RT-
375 PCR assays enabled improved detection of RSV, which could be particularly important for
376 pediatrics departments since RSV is the most common cause of bronchiolitis and pneumonia
377 among infants and children under one year of age (2).

378 For the analysis of the samples initially identified positive using a combination of
379 immunofluorescence and/or viral culture, all 5 samples for which there was a disagreement
380 on the positive identification were confirmed in favor of the multiplexed RT-PCR result by a
381 second culture. Additionally, 23 samples initially identified positive by viral culture and/or
382 immunofluorescence were identified negative by multiplexed RT-PCR (10 influenza A, 1
383 influenza B, 8 RSV, and 4 adenovirus). Confirmatory q-PCR analysis performed at VRDL
384 on these samples confirmed the negative multiplexed RT-PCR results for 2 influenza, 5 RSV,

385 and 3 adenovirus samples. The detail of the critical PCR threshold (Ct) values obtained with
386 q-PCR for the samples that were missed using RT-PCR (1 influenza B, 1 adenovirus, and 8
387 influenza A) showed that some of these samples had fairly high Ct values after extraction,
388 which is indicative of low levels of viral RNA in the initial sample (Ct of 33.7 for the
389 influenza B sample, and Ct above 31 for 4 of the missed influenza A samples). In addition,
390 most of the missed samples were influenza A (8 out of 10 missed). This can most probably
391 be attributed to the rapid mutation rate of the influenza virus and stresses the necessity of
392 constantly updating viral signatures to adapt the assay to the genetic evolution of the targeted
393 organisms.

394 The weakness of this particular multiplexed assay is its low sensitivity to parainfluenza
395 virus (only 7 samples out of 42 were detected). The LOD data pointed out that the sensitivity
396 to parainfluenza 1 was significantly weaker than the sensitivity to parainfluenza 3. In order
397 to investigate whether the signature design was the cause of the low detection levels observed
398 for parainfluenza, parainfluenza type 1 and 3 clinical samples were extracted and analyzed
399 with the multiplexed assay. Positive identification was obtained in all cases, confirming that
400 the signatures amplify the target RNA. We therefore attribute the weakness of the
401 parainfluenza assay to the lack of available free-floating RNA in un-extracted samples. This
402 hypothesis is supported by the fact that the viral RNA of *Paramyxoviruses* has been reported
403 to be encapsidated with nucleoproteins to form a very stable helical nucleocapside (13). An
404 additional extraction step could be included in the protocol to alleviate this issue when the
405 detection of particularly sturdy viruses is desired.

406 In addition to main advantages such as flexibility, sensitivity, specificity, relative low-
407 cost, and ease-of-use, multiplexed RT-PCR also provides the ability to detect co-infections.

During our clinical study, the multiplexed RT-PCR assay detected influenza A-adenovirus co-infections on three samples. Although not initially detected at the UCDMC, these 3 cases of co-infection were confirmed by a second culture. Despite the fact that only 3 samples showed co-infection, these results stress the unique ability of multiplexed assays to rapidly and concomitantly detect of a broad range of pathogens.

We have demonstrated the ability of the multiplexed respiratory panel to differentiate influenza from pathogens that cause influenza-like illnesses in clinical samples. The current 16-plex RT-PCR panel enables simultaneous detection of influenza A, influenza B, parainfluenza (types 1, and 3), respiratory syncytial virus, and adenovirus (groups B, C, and E) in clinical samples. This panel is being deployed in other laboratories including the State of California Health and Human Services Agency and the Naval Health Research Center for further testing and evaluation with clinical samples. Assay development efforts are underway to expand the capabilities of this assay by including signatures that can differentiate seasonal influenza (e.g., A/H1, A/H3) from A/H5N1 or other potential pandemic strains. We are also in the process of developing an instrument to automate sample analysis. This system is able to process samples, perform multiplexed real-time RT-PCR with the respiratory panel, analyze data, and report results in less than 3 hours. The combination of assay development and automation should ultimately allow the implementation of the assay to perform point-of-care diagnostics as well as disease surveillance.

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Figure captions

FIG. 1. Individual primer pairs (biotinylated forward and standard reverse) that bracket the targeted genomic sequence are included in an RT-PCR master mix. After target amplification by RT-PCR, the amplicons are mixed with beads and target amplicons containing the forward biotinylated primer hybridize to the complementary probe on the appropriate beads. A fluorescent reporter molecule (streptavidin-phycoerythrin) then binds biotin functional groups. The completed assay includes a bead, a probe, and a biotinylated and fluorescently tagged amplicon. The sample is then analyzed using a flow cytometer and a Median Fluorescence Intensity (MFI) value is reported for each bead class, each bead class representing a specific signature.

TABLE 1. Summary table of the 16-plex respiratory panel lay out. The biotinylated forward (*Bio* denotes a biotin placed at the 5' end while *iBiod* denotes an internal biotin) and the reverse primer sequences are provided for each signature. The probe design is also detailed, including the 5' end reactive group (*AmMC6*, Amino Modifier C6, also called phosphoramidite) and the spacer 18 (noted *iSp18*), which is an 18-atom hexa-ethyleneglycol spacer placed between the reactive group and the DNA sequence to allow optimal coupling of the carboxylated bead to the probe.

FIG. 2. LOD determination for A) influenza A (2 signatures), and B) parainfluenza (1 signature). The MFI signals from the other 14 bead classes corresponding to the 14 additional target analytes, as well as the 4 controls have been omitted for clarity.

TABLE 2. Summary table of LOD values in the multiplexed respiratory assay, for each targeted respiratory virus. The LOD value was defined as the virus concentration at which the corresponding average MFI value was above the background by more than three standard deviations.

TABLE 3. Summary table of the MFI thresholds for positive sample identification, determined after removing outliers iteratively using the Grubb's outlier test.

TABLE 4. Summary table of the clinical study performed with the multiplexed RT-PCR respiratory assay. A comparison of the performance of the multiplexed assay against the results initially obtained using viral culture and/or immunofluorescence is presented for both negative and positive samples.

FIG. 1

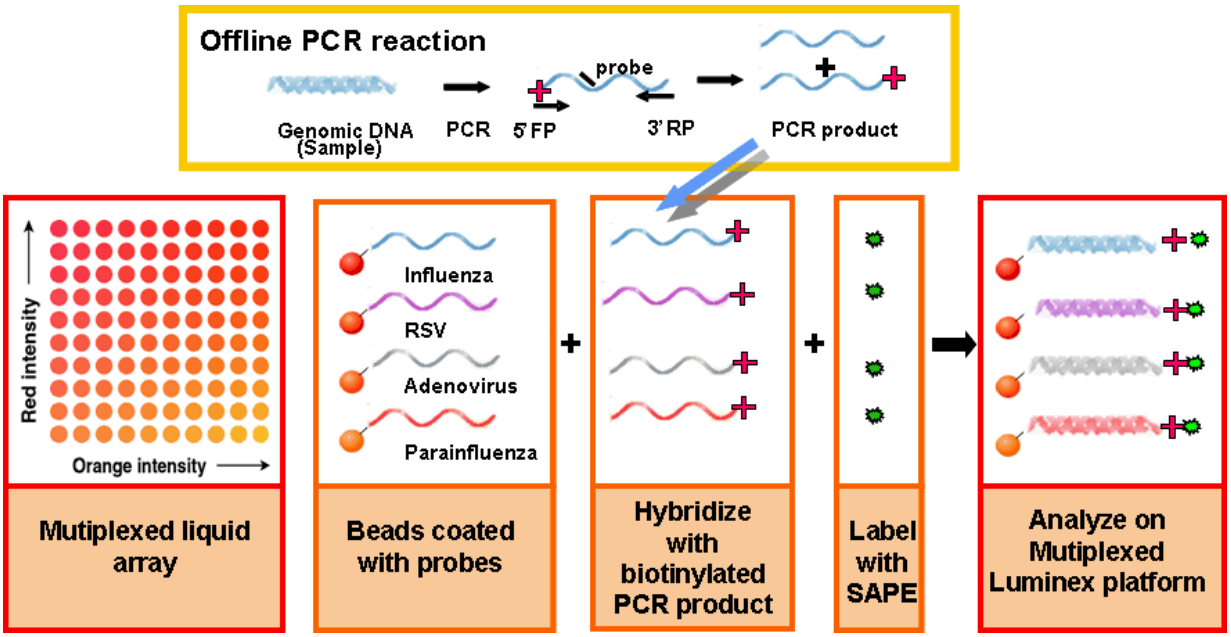
Offline PCR reaction
probe
Genomic DNA (Sample)
PCR
5' FP
3' RP
PCR product
Red intensity
Orange intensity
Influenza
RSV
Adenovirus
Parainfluenza
Mutiplexed liquid array
Beads coated with probes
Hybridize with biotinylated PCR product
Label with SAPE
Analyze on Mutiplexed Luminex platform

TABLE 1

Targets	Signature ID	Biotinylated Forward Primer	Reverse Primer	Probe
Influenza A	Flu A-1	5'/5Bio/GACCRA/iBiodT /CCTGTCACC/iBiodT/C TGAC-3'	5'/AGGGCATTTGGACA AAKCGTCTA-3'	5'/5AmMC6//iSp18/CGTGCCCAGT GAGCGAGGACTGCA-3'
	Flu A-2	5'/5Bio/GGACC/iBiodT/ CCACTTAC/iBiodT/CC AAAACAGAAAC-3'	5'/GTAAGGCTTGCATG AATGTTATTTGCTC-3'	5'/5AmMC6//iSp18/TTGACCTAGTT GTTCTCGCCA-3'
Influenza B	Flu B-1	5'/5Bio/TCC TCAAC/iBiodT/CACTC T/iBiodT/CGAGCG-3'	5'/CGG TGC TCT TGA CCA AAT TGG-3'	5'/5AmMC6//iSp18/CACCGCAGTT TCAGCTGCTCGAATTGG-3'
	Flu B-2	5'/5Bio/GTCCA/iBiodT/C AAGCTCCAG/iBiodT/ttt -3'	5'/TCTTCTTACAGCTTG CTTGC-3'	5'/5AmMC6//iSp18/CCTCCGTCTCC ACCTACTTCGTT-3'
RSV	RSV	5'/5Bio/GGAAACA/iBiod T/ACGTGAACAA GC/iBiodT/TCA-3'	5'/CATCGTCTTTTTCTA AGACATTGTATT GA-3' (RSV a) 5'/TCATCATCTTTTTCT AGAACATTGTAC TGA-3' (RSV b)	5'/5AmMC6//iSp18/TGT GTA TGT GGA GCC TTC GTG AAG CAA G-3'
Para-influenza 1	Para 1	5'/5Bio/ATGCTCC/iBiod T/TGCCCACTG/iBiodT/ GAATG-3'	5'/AATCTTTATCCCACT TCCTACACTTG-3'	5'/5AmMC6//iSp18/TCTATACCTTC ACTCGAGTAATCTG-3'
Para-influenza 3	Para 3	5'/5Bio/ACCAGGAAAC/ iBiodT/ATGC/iBiodT/GC AGAACGGC-3'	5'/GATCCACTGTGTCA CCGCTCAATACC-3'	5'/5AmMC6//iSp18/AGAGCTCCTA AACATGATGGATACC-3'
Adenovirus B	Adeno B-1	5'/5Bio/TCCTGCACCA/i BiodT/TCCCAGA/iBiod T/A-3'	5'/CCTCCGGGACCTGTT TGTAA-3'	5'/5AmMC6//iSp18/CTGACACGAA TAATTCAAGGCTGGAAAGCTG-3'
	Adeno B-2	5'/5Bio/CGCTT/iBiodT/C ACAGTCCAAC/iBiodT/ GC-3'	5'/GCTGCTTGTGGGTTT GATGA-3'	5'/5AmMC6//iSp18/CGTTTTCGGAT TATGATTCCCATCGTTCTTC-3'
Adenovirus C	Adeno C-1	5'/5Bio/AGCGCG/iBiodT /AATATTTGTC/iBiodT/ AGGGC-3'	5'/TCAGCTGACTATAA TAATAAAACGCCA-3'	5'/5AmMC6//iSp18/CGGAACGCGG AAAACACCTGAGAAAA-3'
	Adeno C-2	5'/5Bio/TCGA/iBiodT/CT TACC/iBiodT/GCCACG AG-3'	5'-GCCACAGGTCCTCATA TAGCAA-3'	5'/5AmMC6//iSp18/TGCTCCACAT AATCTAACACAAACTCCTCACC C-3'
Adenovirus E	Adeno E	5'/5Bio/TGCAAT/iBiodT/ TTGTTGGGT/iBiodT/TC G-3'	5'/CCTGGCTGTTATTTT CCACCA-3'	5'/5AmMC6//iSp18/TTAATCATGGT TCTTCCTGTTCTTCCCTCCC-3'
Controls				
RNAse P	RNAse P	5'/5Bio/AGA T/iBiodT/T GGA CC/iBiodT/ GCG AGC G-3'	5'/GAGCGGCTGTCTCC ACAAGT-3'	5'/5AmMC6//iSp18/TTC TGA CCT GAA GGC TCT GCG CG-3'
Mt7	Mt7	n/a		5'/5AmMC6//iSp18/CAAAGTGGGA GACGTCGTTG-3'
Mt7-Cy3	Mt7-Cy3	n/a		5'/5AmMC6//iSp18/CAAAGTGGGA GACGTCGTTG-3'Cy3
Mt7-biotin	Mt7-biotin	n/a		5'/5AmMC6//iSp18/CAAAG/iBiodT/ GGGAGACGTCG/iBiodT/TG-3'

FIG. 2

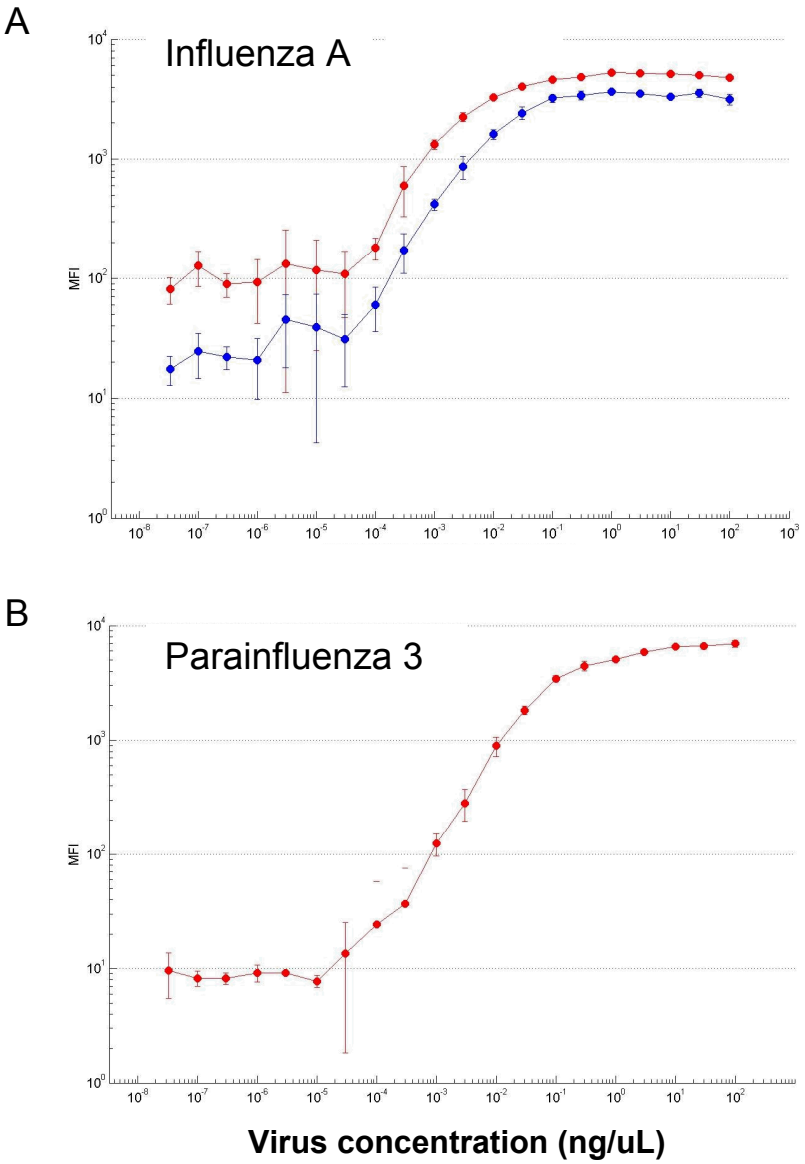
A
Influenza A
MFI
B
Parainfluenza 3
MFI
Virus concentration (ng/uL)

TABLE 2

Signature ID	LOD in multiplexed RT-PCR panel
Flu A	0.005 pg/μL
Flu B	0.01 pg/μL
Para 1	5000 pg/μL
Para 3	0.05 pg/μL
RSV	0.1 pg/μL
Adeno B	0.1 $TCID_{50}$/μL[a]
Adeno C	0.005 pg/uL
Adeno E	0.001 $TCID_{50}$/μL[a]

[a] Only available as live virus.

TABLE 3

Signature	MFI threshold for 'Positive' identification
Flu A-1	112
Flu A-2	40
Flu B-1	63
Flu B-2	119
RSV	181
Para 1	8
Para 3	25.5
Adeno B-1	27
Adeno B-2	47
Adeno C-1	59
Adeno C-2	29
Adeno E	40.5

TABLE 4

Clinical sample analysis	Number of samples (identified by viral culture and /or immunofluorescence)	Number of samples (confirmed by multiplexed RT-PCR)	Other attribution by multiplexed RT-PCR	Negative by multiplexed RT-PCR	% of reconciled multiplexed RT-PCR identifications
Negative for respiratory virus	828	791	37 (31 confirmed at VRDL)[a]	N/A	99.3
Influenza A	35	25	-	10 (2 confirmed at VRDL)[a]	77
Influenza B	12	11	-	1	92
RSV	56	47	1 (confirmed after second culture)[b]	8 (5 confirmed at VRDL)[a]	95
Parainfluenza	46	7	4 (confirmed after second culture) [b]	35	24
Adenovirus	30	26	-	4 (3 confirmed at VRDL)[a]	97

[a] All samples for which there was disagreement between the results obtained using shell vial culture and/or immunofluorescence and multiplexed RT-PCR were sent to the Viral and Rickettsial Disease Laboratory (VRDL) at the State of California Health and Human Services Agency (Richmond, CA) for a third party confirmatory q-PCR analysis.
[b] Samples cultured a second time using standard shell vial procedure.