

**Development of a Respiratory Virus Panel (RVP) Test for the Detection of
Twenty Human Respiratory Viruses using Multiplex PCR and a Fluid
Microbead-based Assay**

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Running title: Detection of human respiratory viruses by multiplex PCR

1 **Abstract**

2 Virology laboratories have historically used DFA and culture to detect six or
3 seven respiratory viruses. Following the discovery of five new human respiratory
4 viruses since 2000 there is an increasing need for diagnostic tests to detect these
5 emerging viruses. We have developed a new test that can detect 20 different
6 respiratory virus types/subtypes in a single five hour test. The assay employs
7 multiplex PCR using 14 virus-specific primer pairs followed by a multiplexed Target
8 Specific Primer Extension (TSPE) reaction using 21 primers for specific respiratory
9 virus types and subtypes. TSPE products were sorted and identified using a fluid
10 microsphere-based array (Universal ArrayTM, TmBioscience) and the Luminex x-
11 MAP system. The assay detected Influenza types A and B, Influenza A subtypes
12 H1, H3, and H5 (including the H5N1 Asian lineage), Parainfluenza types 1, 2, 3, and
13 4, RSV types A and B, Adenovirus, Metapneumovirus, Rhinovirus, Enterovirus, and
14 Coronaviruses OC43, 229E, SARS-CoV, NL63, and HKU1. In a prospective
15 evaluation using 294 NP specimens, DFA/culture detected 119 positives and the
16 RVP test detected 112 for a sensitivity of 97%. The RVP test detected an additional
17 61 positive specimens that were either not detected by DFA/culture or were not
18 tested for by DFA/culture. After resolution of discordants using a second unique
19 PCR assay and using a combined reference standard of positivity, the RVP test
20 detected 180 of 183 true positives for a sensitivity of 98.5% compared to DFA and
21 culture which detected only 126 of 183 for a sensitivity of 68.8%. The RVP test
22 should improve the capability of hospital and public health laboratories for
23 diagnosing viral respiratory tract infections and assist Public Health agencies in
24 identifying etiologic agents in respiratory tract outbreaks.

1 Introduction

2 For diagnosing viral respiratory tract infections clinical virology laboratories
3 have historically used traditional methods such as DFA and culture for the
4 detection of six or seven conventional respiratory viruses. DFA offers a rapid
5 turn around time for results but is labor intensive, subjective, requires trained
6 technologists, and requires specific monoclonal antibodies. With traditional
7 methods such as DFA and culture that use microscopy, turn around times for
8 results are slow especially in laboratories handling large volumes of respiratory
9 specimens. These methods are also limited by the availability of monoclonal
10 antibodies for newly discovered viruses.

11 Over the past 10 years, nucleic acid amplification tests (NAAT) have been
12 developed for a number of respiratory viruses. NAAT assays including PCR and
13 NASBA have shown enhanced sensitivity over DFA and culture (4). Multiplex
14 PCR assays have been used to detect the presence of one or more respiratory
15 viruses in respiratory tract specimens (1,3,5,8,9,11). The emergence of five new
16 respiratory viruses since 2000 including Metapneumovirus, SARS-CoV, Avian
17 influenza H5N1, Coronavirus NL63 and HKU1 and human Bocavirus has
18 presented challenges for the virology laboratory. The absence of commercially
19 available tests often leaves laboratories without the ability to diagnose these
20 important emerging virus infections. There is therefore a need for new and
21 improved diagnostic tests to diagnose both traditional and emerging respiratory
22 virus infections with improved sensitivity. We have developed a multiplex PCR
23 assay called, Respiratory Virus Panel (RVP) test, that can detect 20 different

1 respiratory viruses including the “orphaned” common cold viruses *viz.*
2 Rhinoviruses and Coronaviruses not tested for in most clinical laboratories,
3 seven conventional respiratory viruses detected by most clinical laboratories and
4 emerging viruses such as Metapneumovirus, Coronaviruses SARS-CoV, NL63,
5 HKU1, and H5N1 avian influenza virus not diagnosed by routine clinical
6 laboratories. The RVP test was more sensitive than DFA and culture and
7 detected 43% additional respiratory virus infections not detected by conventional
8 methods used in the clinical virology laboratory.

9

10 **Methods and Materials**

11 1. Specimens – Two hundred and ninety four nasopharyngeal swabs (NP)
12 specimens were collected from hospitalized patients in Hamilton during the
13 winter of 2005/6 under an Ethics Review Board approval (St. Joseph’s
14 Healthcare). Consecutively specimens (2-3 mL Universal Transport Medium,
15 Copan) were collected prospectively and divided into aliquots. One aliquot (1
16 mL) was processed in the routine virology laboratory for DFA and shell vial
17 culture and a second aliquot (0.5 mL) was processed for testing by the multiplex
18 RVP test. DFA was performed using standard methods and slides were stained
19 using virus-specific monoclonal antibodies (Diagnostic Hybrids Inc.) and read by
20 experienced virology technologists. DFA negative specimens were set up in
21 shell vial cultures and stained with a panel of 8 monoclonal antibodies at 48 hr.
22 Shell vial cultures containing R-Mix cells were purchased from DHI.

1 2. Nucleic acid extraction- Total nucleic acid (DNA plus RNA) was extracted from
2 aliquots (0.5 mL) of respiratory tract specimens using the Biomerieux MiniMag
3 extractor according to the manufacturer's instructions. Purified nucleic acid (40
4 μL) was frozen at -80°C in 5 microliter aliquots.

5 3. RT-PCR – For the RVP assay a two step RT-PCR was used. cDNA was
6 synthesized using MMLV reverse transcriptase (Invitrogen) in a 20 μL reaction
7 including 0.5 μM random hexamers, 0.5 μM dNTP, 1x RT buffer, 0.01 M DTT and
8 5 μL nucleic acid for 60 min at 37°C . cDNA (5 μL) was amplified in a multiplex
9 PCR reaction using 14 primer pairs designed to amplify highly conserved regions
10 of individual viral genomes for detection of specific respiratory virus types and
11 subtypes. The multiplex PCR targeted the following viral genes: the matrix of
12 influenza A, the three hemagglutinin genes of H1, H3, and H5 influenza A, the
13 prehemagglutinin of influenza B, the hemagglutinin of Parainfluenza types 1
14 through 3, the phosphoprotein of parainfluenza type 4, the polymerase of RSV A
15 and B, the hexon of Adenovirus, the 5'-UTR of Enterovirus/Rhinovirus, the
16 nucleoprotein of Metapneumovirus, and the polymerase of Coronavirus. The
17 primers were chosen carefully for target specificity and size so that amplicons
18 would be small in size (100-400 bp), to maximize amplification efficiency; the
19 median amplicon size was 204 bp. PCR reactions contained 20 μL PCR Master
20 Mix consisting of 1.5 X TaKara Buffer, 0.15 mM dNTPs, 1 U TaKara Taq
21 polymerase, 14 pairs of primers at optimized concentrations ranging from 0.2-1
22 μM and 5 μL cDNA. The PCR cycling conditions were as follows: 1 cycle of 2
23 min at 95°C , 30 cycles of 30 sec at 95°C , 30 sec at 55°C , 30 sec at 72°C , 1

1 cycle of 2 min at 72° C. Following PCR the remaining dNPTs and primers were
2 removed by incubating the entire 25 µL PCR reaction with 2.5 U shrimp alkaline
3 phosphatase and 10 U Exonuclease (Invitrogen) for 30 min at 37° C followed by
4 30 sec at 99° C.

5 4. Target Specific Primer Extension Reaction (TSPE) – A multiplex TSPE
6 reaction was used to detect specific viral sequences amplified by RT-PCR.
7 Treated PCR products (5 µL) were reacted with a mixture of 21 oligonucleotide
8 primers designed to recognize 19 respiratory virus types and subtypes. TSPE
9 primers were chimeric by design containing both a virus-specific oligonucleotide
10 sequence and a “tag” oligonucleotide that hybridizes to a complimentary anti-tag
11 oligonucleotide bound to 21 spectrofluorometrically-labeled microspheres (see
12 Fig 1). The tag anti-tag oligonucleotides are proprietary sequences and part of
13 the Universal Array from TmBioscience Corporation (Toronto). These are unique
14 3-base, 24-mer oligonucleotide pairs with isothermal hybridization kinetics (2).
15 For the TSPE reaction an aliquot (5 µL) of the treated PCR product was added to
16 15 µL of TSPE Master Mix containing Qiagen PCR buffer, dATP, biotinylated
17 dCTP, dGTP, dTTP, 2.5 U TaKara Hot Start polymerase, TSPE primer mix (2.5
18 µM each) and the reaction incubated under the following cycling conditions: 1
19 cycle for 2 min at 96° C, 35 cycles of 30 sec at 54° C, 30 sec at 72° C.

20 5. Analysis of TSPE products using a fluidic microarray. Biotinylated TSPE
21 products were next hybridized to a fluid microbead-based array (part of the
22 Universal Array, TmBioscience, Toronto, Canada) in wells of a 96-well microtiter
23 plate, and detected using a Streptavidin-Phycoerythrin conjugate (Bortolin et al.

1 2004). The RVP microbead mix consisted of 21 microbeads each containing a
2 different fluorescent dye mix and each containing a unique anti-tag
3 oligonucleotide sequence complimentary to the oligonucleotide tag sequences
4 incorporated into the 21 TSPE primers. An aliquot of the TSPE reaction (3.5
5 μL) was mixed with 20 μL of the microbead mix and incubated for 2 min at 96 ° C
6 followed by 30 min at 37 ° C. Aliquots (0.1 mL) of Streptavidin-Phycoerythrin
7 conjugate (Molecular Probes, Eugene, OR) diluted in 1:100 in 1 X wash buffer
8 were added to the wells and the plates incubated in the dark for 20 min at room
9 temperature. The washed plates were read in a Luminex 100 instrument
10 (Luminex Corporation) after 20 min incubation. TSPE products bound to specific
11 beads were detected by two lasers in the Luminex 100, the first a red laser
12 identifying individual microbeads based on unique spectrophotometric dyes
13 incorporated into the microbeads and the second a green laser detecting
14 phycoerythrin fluorescence bound to each microbead. The signal on each bead
15 is recorded as MFI signal and the output of the instrument determined by TDAS
16 software (TmBioscience Corporation) that analyses the raw data and makes a
17 positive or negative call for each virus type and subtype.

18 6. Confirmatory PCR - Twenty confirmatory RT-PCR assays were developed
19 using a unique pair of primers for each of the 20 virus types/subtypes to confirm
20 positives detected by the RVP assay. Primers were designed in consensus
21 regions of the genome and optimal conditions for amplification were determined
22 for each set of primers. All amplification targets except one were chosen outside
23 the RVP amplicon. For one virus, one primer was located within the amplicon

1 and the other primer was upstream. For the two-step RT-PCR assays, cDNA
2 was synthesized as described above for the RVP assay using random hexamers
3 and PCR was performed under optimal conditions (primer concentration and
4 annealing temperature) derived for each set of primers. Analytical sensitivity for
5 each confirmatory test was similar to the analytical sensitivity of the
6 corresponding target in the RVP test as determined by end point titrations using
7 serial dilutions of *in vitro* generated transcripts (Invitrogen kit) using cloned
8 amplicons.

10 **Results**

11 We designed a multiplex PCR test for the detection and identification of 20
12 different human respiratory virus types and subtypes including conventional
13 respiratory viruses, common cold viruses and newly emerging respiratory
14 viruses. Following nucleic acid extraction, the first step of the assay is a
15 multiplex RT-PCR using 14 consensus primers that amplify consensus regions of
16 the various viral genomes. All PCR reactions gave specific and robust products
17 with predominant bands for most viral targets being amplified and only the
18 occasional minor non-specific bands. The second step of the RVP assay
19 involved detection of specific amplicons using a multiplex TSPE reaction where
20 the TSPE primers annealed to specific amplicons and were extended by Tag
21 polymerase incorporating biotinylated dCTP. The TSPE reaction used chimeric
22 primers containing a virus-specific sequence and a unique “tag” sequence that
23 allowed each product to be addressed to a specific microbead containing a

1 complimentary anti-tag oligonucleotide (Fig 1). TSPE products captured on
2 microbeads were detected with a streptavidin-phycoerythrin conjugate and
3 signals produced for each bead analysed by the Luminex 100 flowcell instrument
4 and expressed as Mean Fluorescence (MFI) Units. A total of 21 microbeads
5 were used in the assay each capturing a specific PCR product (Table 1). The
6 use of consensus PCR primers together with type- and subtype-specific TSPE
7 primers allowed detection of the following viruses: Influenza types A and B,
8 Influenza A subtypes H1, H3 and H5 including the Asian lineage of H5N1,
9 Parainfluenza types 1, 2, 3, and 4, RSV types A and B, Metapneumovirus,
10 Adenovirus, Rhinovirus, Enterovirus, Coronavirus types OC43, 229E, NL63,
11 HKU1 and SARS CoV.

12 The background signals for all 21 beads were low, generally in the range of
13 50-100 MFI compared with positive signals in the 2,000 – 8,000 MFI range giving
14 S/CO ratios in the range of 100-500 for all targets (Fig 2). The low background
15 on all of the remaining beads (excluding the positive bead) indicates the absence
16 of “cross talk” or signal on other beads indicating the high specificity of
17 hybridization of the Universal Array™ Tag and Anti-Tag oligonucleotides used for
18 signal sorting. The excellent specificity afforded by the PCR and TSPE primers
19 together with the excellent S/CO ratios allowed the detection of multiple targets.
20 This is seen in Fig 2 for an influenza A H1N1 virus that has a strong signal on
21 both the matrix gene bead and the H1 gene bead. The high specificity of the
22 assay allowed for the detection of 15 specimens that were positive for two
23 viruses (data not shown).

1 The analytical sensitivity for each viral target was determined by testing
2 serial dilutions of stock virus seeds (TCID₅₀/mL) or *in vitro* generated RNA
3 transcripts from plasmids containing cloned amplicons (genome equivalents).
4 For all 20 virus types and subtypes tested the RVP assay detected between 0.1
5 and 100 TCID₅₀ of virus. RVP had an analytical sensitivity of 0.1 TCID₅₀ for
6 Rhinovirus/Enterovirus, Coronavirus 229E, and Influenza A types H1 and H3, 0.5
7 TCID₅₀ for Influenza B, Parainfluenza type 3, and Metapneumovirus, 1 TCID₅₀ for
8 RSV A and Parainfluenza type 4, 10 TCID₅₀ for Parainfluenza type 2, RSV B,
9 Coronavirus NL63 and OC43, and 100 TCID₅₀ for Adenovirus, Parainfluenza type
10 1 and SARS-CoV. The corresponding analytical sensitivities in genome
11 equivalents were 50-250 for all virus types/subtypes.

12 We evaluated the performance of the RVP assay by testing 294
13 respiratory tract specimens submitted to the clinical virology laboratory for routine
14 investigation of respiratory viruses. Aliquots of each specimen were tested by
15 routine DFA plus culture followed by the RVP test. DFA and culture was
16 performed in the clinical virology laboratory and the RVP was performed in the
17 research laboratory by technologists blinded to previous results obtained for the
18 specimens. Of the 294 specimens there were 228 concordant results including
19 123 positives by DFA/culture and RVP and 105 negative in both tests (Table 2).
20 DFA/culture detected 128 positive specimens and RVP detected 123 of these for
21 an unadjusted sensitivity of 96.1% for the seven conventional respiratory viruses
22 (Influenza A and B, Parainfluenza 1-3, RSV, Adenovirus) routinely detected in
23 most clinical laboratories. The RVP test detected an additional 61 positive

1 specimens, 14 of which were negative by DFA/culture for the seven viruses
2 tested and 47 were positive for viruses not tested by DFA/culture. These 61
3 additional positive specimens included 2 Influenza A, 1 Parainfluenza type 1, 2
4 Parainfluenza type 2, 1 Parainfluenza type 4, 2 RSV, 8 Metapneumovirus, 39
5 Rhinovirus/Enterovirus, 6 OC43 CoV, 2 NL63 CoV, 1 HKU1 CoV and included 3
6 specimens that were positive for two viruses including Metapneumovirus and
7 Rhino/Enterovirus, two OC43 and Rhino/Enterovirus positive specimens. All of
8 the 66 discordant specimens including the 5 DFA/culture positive specimens that
9 were negative by RVP and the 61 specimens that were positive by RVP and
10 negative or not tested by DFA/culture were tested by a second PCR targeting a
11 different area of the viral genome. Table 3 shows the results for the 5
12 DFA/culture positive RVP negative and 14 DFA/culture negative RVP positive
13 discordant specimens. Three of the 5 DFA/culture positive RVP negative
14 specimens (#167, 191, 187) were confirmed positive by PCR indicating two false
15 positives (#286, 62) by DFA/culture. All of the additional 61 RVP positive
16 specimens confirmed as true positives by the second PCR. If a true positive is
17 defined as being positive by two or more tests (DFA, culture, RVP or
18 confirmatory PCR), then there were 183 positives and 111 negatives. To
19 determine how the RVP test performed compared to DFA and culture we
20 eliminated the 47 specimens that were positive for a virus not tested for by DFA
21 and culture (viz. Parainfluenza 4, Metapneumovirus, OC43, 229E, NL63, HKU1,
22 and Entero/Rhinovirus), and used the remaining 247 specimens for analysis. Of
23 these 247 specimens there were 137 positives and 110 negatives. The

1 sensitivity and specificity of DFA/culture was 91.9% (126/137) and 98.2%
2 (108/110) respectively (Table 4). By comparison, the RVP had a sensitivity of
3 97.8% (134/137) and a specificity of 96.4% (107/110). If however, all confirmed
4 respiratory viruses detected by the RVP test are included in the analysis, then
5 the RVP assay detected 180 out of 183 positive specimens and had an overall
6 sensitivity of 98.4% compared with DFA plus culture that detected only 126 out of
7 183 specimens and had a sensitivity of 68.8%.

8 Of particular interest was the finding that 15 out of 294 (5.2%) specimens
9 were positive for two viruses in this group of specimens. The dual infections
10 included the following combinations: 1 Parainfluenza 1+RhinoEnterovirus, 1
11 Parainfluenza 2+RhinoEnterovirus, 2 Parainfluenza 3+RhinoEnterovirus, 3
12 RSV+RhinoEnterovirus, 1 Adenovirus+RhinoEnterovirus, 1 MPV+OC43 CoV, 3
13 MPV+RhinoEnterovirus, 2 OC43 CoV+RhinoEnterovirus, and 1
14 Adenovirus+KHU1 CoV. No specimen was positive for 3 respiratory viruses.
15 Testing additional specimens from a separate study has confirmed a dual
16 positivity rate for NP specimens collected from our combined pediatric and adult
17 population of 5-8% using the RVP assay during the 2005/2006 season.

18

19 **Discussion**

20 The RVP test detects 20 respiratory viruses including conventional respiratory
21 viruses Influenza A and B, Parainfluenza 1-4, RSV, Adenovirus, and
22 Metapneumovirus, common cold viruses such as Rhinovirus, OC43 and 229E
23 Coronavirus, and newly emerging respiratory viruses such as SARS-CoV, Avian

1 influenza H5N1, NL63, and HKU1 CoV not usually tested by clinical laboratories.
2 The assay employs a multiplex PCR using 14 primer pairs followed by a
3 multiplex TSPE reaction using 21 primer pairs to detect and identify 20 different
4 virus types and subtypes in a single test. The test uses a 96-well microtiter plate
5 format and the Luminex 100 flow-cell instrument. Since the current test involves
6 several steps and is unable to produce results as quickly as DFA, a shortened
7 version of the test is currently under development which will cut the run time
8 significantly and allow faster turn around times for results. Following nucleic acid
9 extraction the RVP assay takes about five hours to perform, making it possible
10 for some laboratories to provide same day results. Actual turn around time for
11 results will vary from laboratory to laboratory depending on specimen volumes.
12 One technologist can easily handle two plates and generate results for up to 190
13 specimens plus controls in one work shift recognizing that the extraction may be
14 rate limiting and not the RVP test itself. Laboratories that have two automated
15 extractors would be able to process 192 specimens per day. If routine PCR
16 contamination precautions are followed, the risk of false positives can be
17 minimized and should be no higher than that obtained for any PCR tests. We
18 have not seen PCR contamination issues with the RVP assay in testing over
19 2,000 specimens since both the PCR and TSPE reactions are performed in
20 closed 8-well capped strips and only the hybridization step is performed in open
21 wells of 96-well plates. For this reason we have not used uracil-N-glycosylase in
22 the PCR master mix. For the IUO and subsequent IVD versions of the assay,
23 the manufacturer has added two controls to the test. An aliquot of MS2 RNA

1 phage is added to each specimen prior to nucleic acid extraction. This controls
2 for nucleic acid extraction and also serves as an amplification inhibitor control.
3 The second control is lambda phage DNA which is included in every run as a
4 “run control” to control for amplification and detection.

5 The analytical sensitivity of the RVP for the 20 different viruses ranged
6 from 0.1-1 TCID₅₀ corresponding to approximately 50-250 genome equivalents.
7 The differences in analytical sensitivities obtained for genome equivalents and
8 TCID₅₀ determinations likely reflects the production of viral interfering particles in
9 cell culture and/or the presence of viral transcripts.

10 To determine the clinical performance of the the RVP assay we performed
11 a comparison to DFA/culture using 294 prospectively collectively specimens
12 submitted to the Regional Virology Laboratory for investigation of respiratory
13 viruses. Excluding the 47 specimens that were positive for a virus not tested for
14 by DFA and culture (viz. Parainfluenza 4, Metapneumovirus, OC43, 229E, NL63,
15 HKU1, and Enterovirus/Rhinovirus), the sensitivity of DFA/culture was 92% (126/137)
16 while the sensitivity of RVP was 97.8% (134/137). The major advantage of the
17 RVP test is the ability to identify an additional 8 respiratory viruses not detected
18 by DFA/culture including 5 Coronaviruses, Parainfluenza type 4, Rhinovirus, and
19 Enterovirus and to type Influenza virus subtypes H1, H3, H5 and RSV types A
20 and B. In this study RVP detected 42% more viruses than DFA plus culture (180
21 compared to 126). All of the additional positives were confirmed as true positives
22 by a second PCR assay using different primers. The overall sensitivity for
23 detecting any respiratory virus was 98.5% (180/183) for the RVP test and 68.8%

1 (126/183) for DFA/culture. The specificity of RVP (96.4%) was similar to that of
2 DFA/culture (98.2%). The sensitivities of the RVP assay for detecting individual
3 viruses was 83% (5/6) for influenza B, 92% (11/12) for Parainfluenza 2, 96.6%
4 (28/29) for RSV, 100% for influenza A (10/10), 100% (19/19) for Parainfluenza 1,
5 100% (17/17) for Adenovirus, 100% (22/22) for MPV, and 100% (10/10) for
6 Parainfluenza 3. The numbers of positives for some viruses was quite low
7 however, and the true sensitivity of the RVP assay for individual viruses must
8 await larger studies

9 In resolving the RVP positive-DFA/culture negative specimens, only a
10 single confirmatory PCR was performed to confirm that the additional positives
11 were true positives and not false positives. If these discordants were tested by
12 all 21 confirmatory PCR tests some additional viruses may have been detected
13 and this may have changed the performance characteristics of the RVP assay as
14 well as DFA/culture. The RVP test provides a much broader coverage for
15 respiratory viruses compared with DFA and culture that detects only 7 or 8
16 viruses. Other multiplex PCR assays have been described that detect up to
17 seven viruses in a single test or up to 14 viruses in two multiplex reactions (3).
18 The specificity of the RVP assay and its ability to detect multiple viruses in a
19 single reaction is achieved by the aggregate specificity of primers and
20 oligonucleotides using for the PCR, TSPE, and Universal Array hybridization
21 steps. A commercial version of the RVP test called, ID-TagTM RVP, is presently
22 in clinical trials. This version will include an internal control that is spiked into the
23 specimen to control for extraction of nucleic acid and a run control. The broad

1 coverage of the RVP test should improve the ability of clinical laboratories to
2 diagnose respiratory virus infections in hospitalized patients and assist Public
3 Health Laboratories in identifying viral etiological agents in RTI outbreaks in the
4 community that can be as low as 25-30% (10). The RVP test should increase
5 our understanding of the epidemiology of respiratory viral infections. Much of our
6 knowledge of respiratory virus epidemiology comes from studies performed in the
7 1970s using limited and insensitive laboratory tests. Use of the RVP test in
8 broad population based studies will increase our knowledge of the seasonality
9 and risk factors for respiratory virus infections in different patient populations.
10 For example, the RVP indicated that there were up to 12 respiratory viruses co-
11 circulating in Hamilton in the months of January and February in 2006 compared
12 with 5 viruses detected by DFA and culture that co-circulated in the community
13 the previous winter. We have consistently seen a dual respiratory virus infection
14 rate of 5-8% for symptomatic patients and even some triple virus infections using
15 the RVP assay. In the current study of 294 NP specimens we found positives
16 for RSV and influenza A, RSV and parainfluenza 3, RSV and MPV, combinations
17 which have been reported in the literature plus new combinations of viruses not
18 previously reported including Influenza A with Metapneumovirus, Parainfluenza
19 3 with Rhino/Enterovirus, Metapneumovirus with Rhino/Enterovirus, and
20 Metatpneumovirus with Coronavirus OC43. It is not known whether infections
21 with multiple respiratory viruses are associated with adverse outcomes or
22 increased hospital stays in specific patient populations. Clinical studies are
23 ongoing to determine whether dual respiratory virus infections carry an increased

1 risk for adverse outcomes or increased hospital stays for pediatric and adult
2 patients.

3 The RVP test may also be useful in the global surveillance of emerging or
4 re-emerging respiratory viruses such as SARS-CoV or Avian Influenza Asian
5 lineage H5N1. The RVP was designed to detect all influenza A subtypes but not
6 specifically to identify specific H or N types. An RVP signal on the influenza A
7 matrix bead in the absence of a signal on the H1, H3 or H5 bead would be
8 consistent with a new influenza type and indicate the need for further genotyping
9 of the isolate. The re-emergence of a new influenza A type such as H7N2 or
10 H9N2 into the human population would be detected by the RVP test which
11 detects the matrix gene of all influenza A genotypes H1 through H16 (data not
12 shown). The RVP has detected H1, H3, H5, as well as H7 and H9 (data not
13 shown) and therefore could act as a sentinel test for the global surveillance of
14 newly emerging influenza viruses. Since the assay detects the currently
15 circulating Asian lineage of H5N1, the assay can also be used to signal the
16 “migration” of H5N1 into new areas of the world.

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Table 1. Assignment of virus types and subtypes to individual microbeads for detection by the Luminex X-MAP system.

Virus	Bead number
Influenza A	Bead 1
H1 subtype of Influenza A	Bead 2
H3 subtype of Influenza A	Bead 3
H5 subtype of Influenza A	Bead 4
Influenza B	Bead 5
RSV A	Bead 6 (RSV) and Bead 7 (RSV A)
RSV B	Bead 6 (RSV) and Bead 8 (RSV B)
Metapneumovirus	Bead 9
Rhinovirus	Bead 10
Enterovirus	Bead 11
Parainfluenza 1	Bead 12
Parainfluenza 2	Bead 13
Parainfluenza 3	Bead 14
Parainfluenza 4	Bead 15
Corona SARS	Bead 16
Corona 229E	Bead 17
Corona OC43	Bead 18
Corona NL63	Bead 19
Corona HKU1	Bead 20
Adenovirus	Bead 21

Table 2. Distribution of DFA/Culture and RVP test results for 294 NP specimens.

Number of Specimens	DFA/culture result	RVP result
123	Positive	Positive
105	Negative	Negative
5	Positive	Negative
14	Negative	Positive
47	Not tested	Positive

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Table 3. PCR test results for 5 DFA/culture positive RVP negative and 14 DFA/culture negative RVP positive discordant specimens.

Specimen	DFA/Culture	RVP (MFI reading)	Second PCR
167	Flu B +	Flu B – (42)	Flu B +
191	Para 2 +	Para 2 – (13)	Para 2 +
286	Para 2 +	Para 2 – (29.5)	Para 2 -
62	Para 1 +	Para 1 – (20)	Para 1 -
187	RSV +	RSV – (93)	RSV +
108	Flu A -	Flu A + (298)	Flu A +
53	Para 2 -	Para 2 + (266)	Para 2 +
349	Para 2 -	Para 2 + (6447)	Para 2 +
89	Para 1 -	Para 1 + (215)	Para 1 +
443	RSV -	RSV + (213)	RSV -
58	Flu A -	Flu A + (412)	Flu A -
128	Mpn -	Mpn + (7229)	Mpn +
441	Mpn -	Mpn + (1837)	Mpn +
503	Mpn -	Mpn + (286)	Mpn +
549	Mpn -	Mpn + (656)	Mpn +
566	Mpn -	Mpn + (966)	Mpn +
601	Mpn -	Mpn + (6032)	Mpn +
604	Mpn -	Mpn + (3537)	Mpn +

119	Mpn -	Mpn + (6772)	Mpn +
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*A second confirmatory PCR targeting a unique genomic region was performed to resolve the discordant specimens as described in Methods and Materials.

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Table 4. Sensitivity and specificity of DFA/culture and the RVP test for detection of eight respiratory viruses after resolution of the discordant results.*

	Sensitivity (%)	Specificity (%)
DFA/culture	91.9 (126/137)	98.2 (108/110)
RVP Test	97.8 (134/137)	96.4 (107/110)

* Forty seven specimens that were positive for a virus not tested by DFA/culture were removed from the analysis leaving 247 for comparison. DFA/culture and the RVP discordants (N=66) were tested by confirmatory PCR tests for each target. The sensitivity and specificity was calculated using a combined reference standard of positivity in two or more tests (DFA, culture, RVP, and confirmatory PCR).

Figure Legends

Figure 1. Detection and identification of TSPE reaction products captured onto microsphere beads containing anti-tag oligonucleotides that hybridize to TSPE products containing a complimentary tag oligonucleotide. The microbeads are sorted in the Luminex 100 instrument that identifies spectrophotometrically colored beads with one laser and a phycoerythrin signal on the beads with a second laser.

Figure 2. RVP results obtained for eleven NP specimens showing TSPE signals recorded for all microbeads. The 11 positive specimens are indicated across the bottom with the signals for each of the 21 beads indicated by colored bars. The first sample on the left shows a positive signal for the Influenza A bead (matrix gene positive) and for the H1 hemagglutinin gene. The other beads for this sample are all negative.

Figure 1

Universal Array Sorting

TSPE products containing a Tag oligo are hybridized to specific microbeads containing a complimentary anti-Tag oligo

PCR and TSPE Reaction

TSPE primers bind to specific amplicons and are extended incorporating biotin

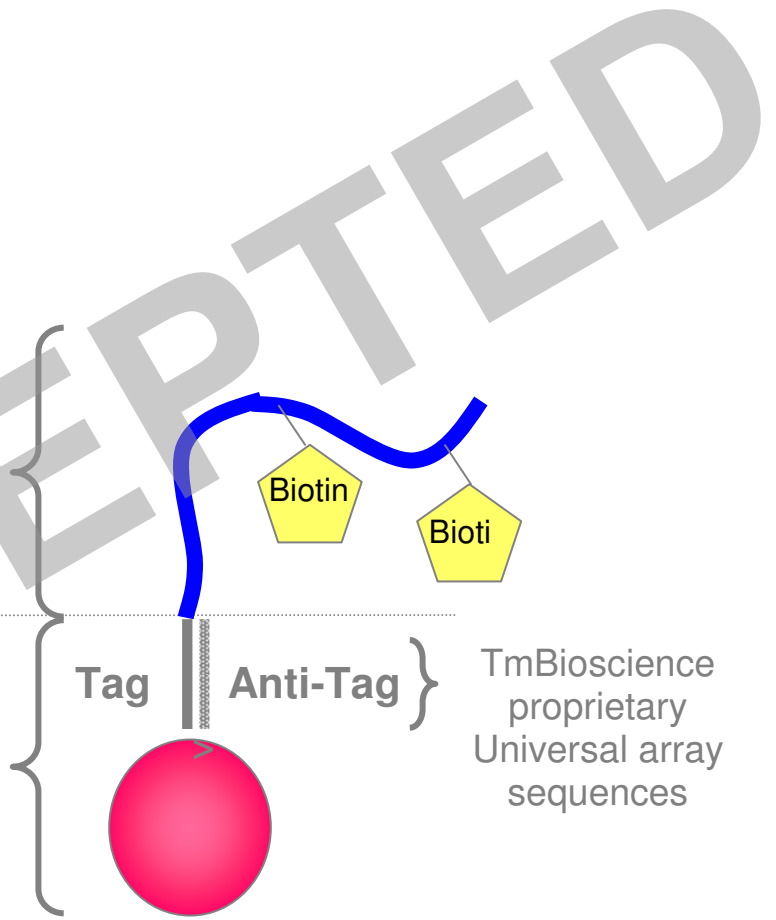
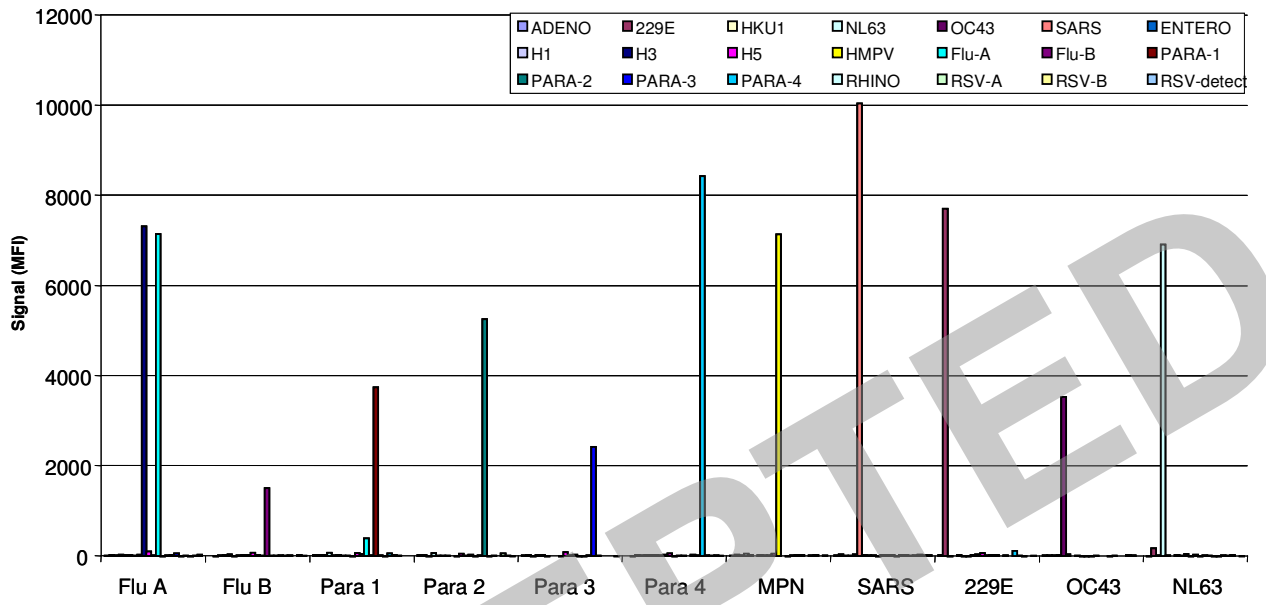


Figure 2



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