

**A High-Throughput, Sensitive and Accurate Multiplex PCR-Microsphere Flow Cytometry
System for Large-Scale Comprehensive Detection of Respiratory Viruses**

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1 ABSTRACT

2 Human respiratory viruses are a diverse group of pathogens composed of hundreds of
 3 virus strains, and this presents a major challenge for diagnostic laboratories. To efficiently detect
 4 numerous viruses in a large epidemiologic study, we developed a fast, multi-target, sensitive and
 5 specific assay, named the Respiratory MultiCode[®]-PLx Assay (RMA). The RMA utilizes
 6 improved multiplex PCR chemistry (EraGen[®] MultiCode[®]-PLx technology) coupled with high-
 7 throughput microsphere flow cytometry (Luminex[®]). Eighteen sets of virus-specific multiplex-
 8 PCR primers were developed based on the conserved sequences of all available respiratory viral
 9 sequences for 8 distinct groups: rhinovirus (HRV), respiratory syncytial virus (RSV),
 10 parainfluenza (PIV), influenza (InfV), metapneumovirus (MPV), adenovirus (AdV), coronavirus
 11 (CoV) and enterovirus (EnV). Each primer set detected 20 cDNA copies of the intended target
 12 per sample and had no reaction with 60,000 copies of human genomic DNA. The accuracy and
 13 sensitivity of RMA for detecting respiratory viruses in human samples was tested with two sets
 14 of clinical specimens. First 101 nasal wash specimens that were positive for HRV, RSV, InfV,
 15 PIV or AdV by traditional techniques were reanalyzed by RMA, and all target viruses were
 16 detected with an overall sensitivity of 94% and specificity of 99%. Secondly, 103 nasal wash
 17 samples from 5-yr old children with asthma and respiratory symptoms were analyzed; RMA
 18 detected viruses in 74 specimens (71.8%) compared to only 24 (23.3%) by traditional culture and
 19 immunofluorescence staining techniques. These results show that RMA is an accurate, sensitive,
 20 and practical test for respiratory viral infections.

1 INTRODUCTION

2 Asthma is a common disease worldwide with high economic burden, particularly in poor
 3 inner cities (4, 22). Multiple epidemiological surveys have been conducted in attempt to
 4 understand risk factors for childhood asthma. Viral respiratory infections, particularly wheezing
 5 illnesses caused by respiratory syncytial viruses (RSV) and human rhinoviruses (HRV), in
 6 infancy have been implicated in these studies as risk factors associated with subsequent asthma
 7 (28, 36-38), although there is little information from urban environments. Moreover, other
 8 studies have suggested that frequent viral infections during infancy could decrease subsequent
 9 rates of wheezing and atopy (19). Therefore, the precise role of viral illnesses in the development
 10 of asthma has yet to be comprehensively evaluated (14, 21, 26).

11 To better understand the contribution of viral respiratory infections in infancy to the
 12 incidence of asthma in urban environments, a large prospective cohort study, the Urban
 13 Environment and Childhood Asthma project (URECA,) was initiated. The URECA study will
 14 enroll more than 500 infants and will include sampling of nasal secretions for viral diagnostics
 15 during periods of health and respiratory illness. Unfortunately, use of standard viral diagnostics
 16 to accomplish this task would be quite costly and time consuming, given the multiple groups of
 17 viruses and hundreds of associated strains. Currently, a typical virology diagnostic laboratory
 18 uses a combination of viral culture, immunofluorescence staining and PCR assays to detect
 19 respiratory viruses. Viral culture is time- and labor-consuming, and is not a sensitive method for
 20 detection of rhinoviruses and other viruses that are labile during transport and have fastidious
 21 growth requirements (17, 39). Immunofluorescence staining is fast, but can be insensitive, often
 22 leading to false-negative results (17, 39). None of the current PCR assays has high-throughput
 23 detection capability to effectively handle large number of samples containing multiple targets (6,
 24 7, 16, 18, 34, 41).

In order to analyze the several thousand nasal lavage specimens expected for URECA, our goal was to develop a new system capable of detecting each of the hundreds of virus strains belonging to at least 8 distinct groups: rhinovirus (HRV), respiratory syncytial virus (RSV), parainfluenza virus (PIV), influenza virus (InfV), metapneumovirus (MPV), enterovirus (EnV), coronavirus (CoV), and adenovirus (AdV). The MultiCode[®]-PLx System (EraGen[®] Biosciences, Madison, WI) is a new multi-target, high-throughput detection platform technology that can be adapted to multiple uses (20, 33, 35). This System integrates multiplexed PCR and microsphere flow cytometry technologies in one operation through the use of an expanded DNA base-pairing of isoguanosine (iG) and isocytidine (iC), allowing reaction products to be labeled and captured without many of the steps required for other methods. The MultiCode-PLx System can simultaneously detect up to 80 targets in one reaction and complete 96 reactions in 4 hours and has been successfully applied to commercial tests for large-scale genetic screening of mutation carrier of cystic fibrosis multi-mutation screening (20), single nucleotide polymorphism analysis of PECAM-1 (33) and human minor histocompatibility antigen genotyping (35).

In this report, we describe the implementation of the MultiCode-PLx System for rapid, sensitive and multiplexed detection of nearly all human respiratory viruses in large number of clinical specimens.

MATERIALS AND METHODS

Viruses, viral RNA and viral DNA clones. Virus stocks were obtained from various sources.

Infected cell lysates of 99 serotypes of HRV (serotype 1A, 1B, 2 to 10, 12 to 100 and Hanks) were from Dr. Fred Hayden (U. Virginia, Charlottesville); HRV11, HRV90, EV69, EV70, EV71, coxsackievirus A22, coxsackievirus B1, PIV4a and PIV4b from ATCC; InfV A (Sydney/05/97-like, H3N2), InfV B (Beijing/184/93-like), and AdV 1, 2, 3, 4, 5, 6, 7, 11, 14, 16, 21, 34, 35 from Wisconsin State Laboratory of Hygiene (WSLH); RSV A (Long strain), RSVB and MPV (Isolate CAN97-83) from Dr. Anne Mosser (U. Wisconsin); CoV 229E from Dr. Holmes (U. Colorado). RNA of CoV NL63 was provided by Drs. van der Hoek and Berkhont (U. Amsterdam). DNA clones of HN genes of PIV1, PIV2 and PIV3 were obtained from Drs. Skiadopoulos and Murphy (National Institutes of Health). A DNA clone of the CoV OC43 N gene was obtained from Drs. Dandekar and Perlman (U. Iowa).

Clinical specimens. To test the clinical sensitivity and specificity of RMA, 101 throat or nasopharyngeal swab specimens from adult patients with respiratory symptoms were obtained from a sample collection of WSLH. These specimens were tested positive for HRV (n= 20), RSV (n=21), InfVA (n=10), InfVB (n=10), PIV1 (n=10), PIV3 (n=10) and AdV (n=20) by traditional viral culture and immunofluorescence staining methods, as described elsewhere (15). Briefly, all specimens were inoculated into various cell cultures for isolation of cultivable respiratory viruses including RSV, InfV A and InfV B, PIV 1-4, HRV, EnV, and AdV (15). Respiratory viruses were initially identified by characteristic cytopathic effect and hemadsorption reaction, and virus type was confirmed by immunofluorescence staining with specific monoclonal antibodies (15). In addition, HRV was identified by acid lability, and EnV was identified by virus neutralization using standard LBM serum typing pools. RSV antigen was detected by immunofluorescence staining of respiratory epithelial cells in the clinical specimens in addition to viral culture (15).

This WSLH sample collection did not have samples tested positive for PIV2, PIV4 and EnV by traditional methods.

To direct compare RMA and traditional methods, we obtained 103 nasal lavage specimens from 5 year-old children with asthma and respiratory symptoms from Dr. Robert Lemanske (U Wisconsin-Madison) (27). These specimens were tested for various respiratory viruses by traditional culture and immunofluorescence staining at WSLH as described above.

Respiratory MutiCode-PLx Assay (RMA). The RMA assay was performed in 96-well PCR plates (BioRad MLL9601). Each assay consists of the following steps that all occur in the same well with reagent additions followed by mixing, sealing with Microseal B film (BioRad MSB1001) and incubation (Figure 1): amplification of viral cDNAs by PCR, labeling of the PCR products with virus-specific tags and site-specific biotins by target-specific extension (TSE) of tagged primers, capture of the tagged TSE products by color-addressed microspheres through the hybridization of each tag to its precise complementary oligonucleotide conjugated to the surface of the microspheres, binding of fluorescent streptavidin-phycoerythrin (SAPE) to the biotins of TSE products, and reading of fluorescent signals on each microsphere using the Luminex LabMap 100 instrument (20).

The PCR step was carried out in 8 μ l reaction mix containing 2 μ l cDNA, 1 μ l MC-PCR buffer (EraGen PN1235) and 0.16 μ l of Taq polymerase (BD Bioscience 639209) and 200 nM PCR forward and and 5'-isoC modified reverse primers using the following conditions: 5 min at 95° C and 28 cycles of (5 sec at 95° C, 10 sec at 55° C, 30 sec at 72° C).

Immediately after the PCR, 2 μ l of TSE mix containing 1 μ l of MC-TSE buffer (EraGen PN1308) and 75 nM TSE primers (EraGen) was added to the PCR product. The TSE reaction was carried out in the following conditions: 30 sec at 95° C and 10 cycles of (5 sec at 95° C, 2 min at 65° C).

After the TSE reaction, 40 μ l of microspheres/hybridization solution (EraGen PN1402/1237) was added to the TSE products. The resulting mixture was incubated (RT, 10 min) in the dark to allow hybridization of TSE products to the tag specific microspheres, and then 40 μ l of sheath fluid (Luminex 40-50000) containing 2 μ g of SAPE (Prozyme PJ31S) was added. Next, the fluorescent signal associated with each microsphere was measured in a Luminex machine (96 well-plate flow cytometer). The signal is expressed as mean fluorescence intensity (MFI). Samples with an average signal >6 standard deviations of average negative control signals (typically 400 to 500 MFI) were regarded as positive.

Identification of detection target sequences for common respiratory viruses. Each of the 8 respiratory virus groups (HRV, RSV, InfV, PIV, MPV, AdV, CoV, and EnV) selected for analysis has multiple strains/serotypes. To be able to detect all members of each virus group, conserved viral genomic regions were needed as the detection targets for designing RMA PCR primers. To locate these conserved genomic regions, we first identified all full-length genome sequences of each virus group (Table1, total 110 sequences) listed in public databases, including NCBI Taxonomy Browser, NCBI nucleotide database, Picornavirus Home Page (<http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/>) and the Influenza Sequence Database (<http://www.flu.lanl.gov/>), and then analyzed them with the alignment program ClustalX (42).

HRV have 101 known serotypes, but only 7 full-length sequences (serotypes 1B, 2, 9, 14, 16, 85 and 89) were found in the databases. Alignment of these 7 sequences showed that the 5' noncoding region (NCR) was the most conserved genomic region, and it was therefore chosen as the detection target. The EnV group consists of polioviruses (3 serotypes), echoviruses (29 serotypes), coxsackieviruses (29 serotypes) and EV68 through EV73. Polioviruses and echoviruses were excluded from our target list because they are not considered as respiratory pathogens. Sixty-two full-length sequences for coxsackieviruses and EV68 through EV73 were

identified. Like HRV, 5' NCR was selected as the detection target. Coronaviruses (CoV) have 3 serotypes that are common respiratory pathogens: OC43, 229E and NL63, although additional serotypes have been recently identified. Alignment of the 2 full-length genome sequences of OC43 and 229E and 3 sequences of NL63 showed very little homology, and separate primer sets were designed for each serotype. The nucleocapsid (N) gene was chosen as the detection target for coronaviruses because it is highly expressed in infected cells.

RSV have 2 serotypes: A and B, and 10 full-length genome sequences (7 RSVA and 3 RSVB) were identified. Although the fusion (F) gene was the most conserved, variability led to synthesis of separate primer sets for each serotype. For metapneumoviruses (MPV), 4 full-length sequences were found, and the polymerase (L) gene was selected as the detection target.

Parainfluenza viruses (PIV) have 5 serotypes: 1, 2, 3, 4a and 4b. Full-length sequences were found for PIV1 (n=2), PIV2 (n=2) and PIV3 (n=2) but not for PIV4a and 4b. Although there was some sequence homology in the hemagglutination-neuraminidase (HN) gene, serotype-specific primer sets were required.

Influenza viruses (InfV) has 3 genera: A, B and C. InfV C was not included in our assay because it is not a common pathogen. Unlike the other virus groups, the genomes of InfV A and B are divided into 8 RNA strands, so we only examined the sequences of the 2 most conserved segments: matrix (M) and nucleoprotein (NP). Analysis of 51 NP (28 A and 23 B) and 59 M (40 A and 19 B) sequences of the recent (after 1990) human isolates showed that M genes were slightly more conserved than NP genes, while M and NP sequences varied between InfV A and B. Therefore, M gene sequences were chosen as the detection target and separate primer sets were selected for InfV A and B.

Adenoviruses (AdV) have more than 50 serotypes that are divided into 6 groups (A to F). Group B, C and E viruses were selected for our assay because they are common respiratory

1 pathogens. Fourteen full-length sequences were found in GenBank: 7 B, 6 C and 1 E. Alignment
 2 of these sequences showed that hexon gene was the most conserved among serotypes within the
 3 same group. Due to variability among serotypes, separate primer sets were needed for each
 4 group.

5 Collectively, based on the alignment analysis of all available complete genome
 6 sequences, 18 conserved viral genomic regions were identified as the detection targets for the 8
 7 respiratory virus groups (Table 1). The sizes of these target viral genomic regions, except for the
 8 L gene of MPV, occupy about 10% or less of the respective genomes (Table1).

9 Many of the detection target genomic regions were better-characterized parts of the
 10 respective viral genomes, and 339 additional partial sequences (Table 1) were identified in public
 11 databases and were included in our primer design process to maximize the sensitivity of the
 12 assay. Altogether, searches of public databases yielded a total of 449 sequences (110 complete
 13 genomes and 339 target genomic regions) for designing RMA primers.

14 **Construction of the cDNA clones of target viral genomic regions.** To generate viral targets
 15 with defined sequences and concentration for testing of RMA primers, we cloned the target
 16 genomic regions of 129 viruses (Table 2): 5'NCR of 101 HRV serotypes, 5'NCR of 6
 17 representative EnV serotypes (EV68, EV69, EV70, EV71, coxsackievirus A22 and
 18 coxsackievirus B1), M-N genes of CoV 229E and NL63, F genes of RSV A and B, L gene of
 19 MPV, HN gene of PIV 4a and 4b, M genes of InfV A and B, and hexon genes of all 13 known
 20 serotypes of AdV B, C and E (types 3, 7, 11, 14, 16, 21, 34, 35, 1, 2, 5, 6 and 4).

21 Total nucleic acids including viral RNA was prepared from 100 μ L of infected cell lysate by
 22 phenol extraction and ethanol precipitation (2). Each target viral genomic region was amplified
 23 using a reverse transcriptase (RT)-PCR mix (Invitrogen 11922-028) and appropriate primers that
 24 annealed to its 5' and 3' ends (online supplement Table 1). PCR products were analyzed by

electrophoresis in a 1% low melting agarose gel. Each PCR fragment band was excised from the gel and purified (2). The resulting DNA was treated with kinase, ligated to a StuI-linearized plasmid vector pMJ3 (24), and then transformed into *E. coli*. Plasmids containing the PCR fragment insertions were identified using the Colony Fast-Screen (Size) Kit (Epicentre FS08250) and agarose gel electrophoresis. Three independent plasmids for each PCR fragment were isolated, amplified and purified. The concentration of each plasmid solution was determined using its OD₂₆₀ nm (50 µg per OD unit) and the size of the plasmid. Each viral DNA fragment was completely sequenced (Automated DNA Sequencing Facility, University of Wisconsin).

This work provided not only purified viral targets with defined sequences and concentration for primer selection but also 129 new target sequences for primer design. In addition, we also obtained DNA clones of N gene of CoV OC43 and HN genes of 3 PIV serotypes (1, 2 and 3) from Dr Dandekar (U. Iowa), and Dr Skiadopoulos (NIH), respectively.

Selection of specific RMA primer sets. Each RMA primer set has 2 PCR primers and 1 TSE primer. Multiple candidate primer sets were generated for each of the 18 detection targets in 2 steps. Stretches (> 60 bases) of conserved sequences in each target genomic region were identified by aligning all the available sequences with program ClustalX. Secondly, candidate primer sets were selected within these conserved sequences with computer software (Visual Oligonucleotide Modeling Platform [OMP], DNA Software, Inc., <http://www.dnasoftware.com>), according to the following criteria: appropriate melting temperature, minimal secondary structure formation, minimal interactions with the other primers in multiplex setting and no interaction with human sequences.

The performance of each candidate primer set was next evaluated in a standard MultiCode-PLx assay by preparing samples containing 20 copies of target cDNA, a target concentration near the lower limit of RMA detection. The candidate primer set that gave the maximal fluorescent signal

for each target was selected. Each of these 18 selected primer sets (Table 3) generated signal (2000 to 10000 MFI) that was markedly higher than the background signal (300 MFI and lower) of the negative control (60000 copies of human DNA per reaction).

Development of a pan-HRV primer set. HRV group has 101 known serotypes that account for >60% of all our target viruses. To design a pan-HRV RMA primer set, we collected and analyzed all available sequences of HRV 5'NCR (146 published and 101 new sequences) that covered all 101 known serotypes. The analysis results revealed that the 5'NCR had 3 stretches (A, B and C) of almost completely conserved sequences, corresponding to nucleotide# 352-368 (A), 442-462 (B) and 535-554 (C) of HRV16. Within these 3 stretches of sequences, 12 PCR forward (A), 9 PCR reverse (C) and 5 TSE (B) candidate primers were designed using the program Visual OMP. The primer set with the best signal/noise ratio (Table 3) was then selected from these candidate primers by testing different combinations of PCR and TSE primers against the DNA clones of the 5' NCR of HRV1A, 2, 17 and 59 in the RMA assay. These 4 serotypes were used because the collective sequences of their primer sites had identity with 99 of the 101 serotypes. This selected primer set detected 10 copies of representative targets per reaction with signals of about 2000 MFI, which was 10-fold higher than the background. The signal strength of the assay increased with target concentration and then reached a plateau (~5000 MFI) at 200 copies of target per reaction.

To determine whether this primer set could sensitively detect all HRV, cDNA clones of the 5'NCR of all 101 serotypes were tested. The results showed that this primer set detected 99 serotypes at 20 copies of target per reaction with a typical signal of about 2000 MFI. However, higher target concentrations were needed for HRV33 (100 copies) and HRV78 (40 copies). Consistent with these results, a single-base mutation was found in the PCR forward primer site of these 2 viruses.

Design and evaluation of respiratory panels. Separate panel assays were used for HRV and EnV detection due to extensive sequence homology at the primer sites and some cross-reactivity. Therefore, we divided the 18 primer sets into 2 detection panels, called A and B. Panel A consists of 11 primer sets for HRV, RSVA, RSVB, MPV, PIV1, PIV2, PIV3, PIV4a, PIV4b, InfVA and InfVB. Panel B consists of 7 primer sets for EnV, CoVOC43, CoV229E, CoVNL63, AdVB, AdVC and AdVE. The performance of each primers set in Panels A and B was evaluated in a standard multiplex MultiCode-PLx assay against the purified DNA clones of detection targets and human genomic DNA (negative control) at 20 and 60,000 copies per reaction respectively.

Preparation of the cDNA from clinical specimens for RMA. To maximize the sensitivity of the RMA, we developed a new protocol by optimizing the RNA extraction conditions of our previous procedures (15), and selecting a reverse transcriptase enzyme (AMV-reverse transcriptase, Promega M510F), with greater cDNA synthesis efficiency at low template concentrations. This procedure applied to not only RNA viruses but also AdVs with DNA genomes because our target was the AdV hexon mRNA in infected cells in the clinical samples.

All RNA preparation steps of the new protocol were carried out in Eppendorf DNA LoBind tubes (Eppendorf 022431021). To 200 µl of specimens (e.g., nasal lavage fluid or swab), 150 µl PBS (phosphate buffered saline), 20 µg of glycogen (Ambion 9510), 15 µg of glycoblue (Ambion 9515), 50 ng of human genomic DNA (BD Bioscience 6550-1) and 750 µl of Trizol LS (Invitrogen 10296) were added. The resulting mixture was vortexed for 10 minutes 25° C, supplied with 230 µl of chloroform, vortexed for 5 minutes and then centrifuged for 5 minutes. 25° C The resulting aqueous phase (~700 µl) was mixed with 600 µl isopropanol in a new tube and then incubated at 25° C for 1 hr to precipitate RNA . The RNA precipitant was pelleted by

centrifugation for 10 minutes 25° C. The RNA pellet was washed once with 700 µl of 75% ethanol, air-dried and dissolved in 20 µl water.

To make cDNA, 16 µl of RNA solution was added to 24 µl of reaction solution containing 6 units of AMV-reverse transcriptase (Promega M510F), 8 µl 5X AMV-RT buffer (Promega M515A), 0.5 µg random primers (Promega C1181), 20 units of RNAsin (Promega N2615), 8 µl 5 mM dNTPs (Promega U1330) and then incubated at 25° C for 5 minutes, 42° C for 10 minutes, 50° C for 20 minutes, and 85° C for 5 minutes.

Comparison the new and old protocols for RNA extraction and cDNA synthesis. Samples containing various amount of purified HRV16 virions: 100, 200, 400, 1000, 4000 and 10000 (25) were processed with the new protocol as described above and the old protocol as described previously (15). Briefly (the old protocol), 100 µl of sample was mixed with 1 ml of Trizol (Invitrogen 15596) in a regular Eppendorf tubes (Eppendorf 022363204) by vortexing (10 min, 25° C). The resulting mixture was supplied with 200 µl of chloroform, vortexed for 5 minutes and then centrifuged for 5 minutes at 25° C. The resulting aqueous phase (~800 µl) was mixed with 5 µl glycogen (20 µg/µl) and 600 µl isopropanol and incubated at -20° C for 10 min to precipitate RNA. The RNA precipitant was pelleted by centrifugation (30 min, 4° C). The RNA pellet was washed once with 70% ethanol, air-dried and dissolved in 20 µl water. cDNA synthesis conditions were similar to those of the new protocol, except Invitrogen (18064) SuperScript II (200 units per reaction) and buffer were used. Each cDNA preparation was then tested by the RMA as described above.

Cloning and sequencing of the 5' NCR of HRV and EnV in nasal specimens that are RMA positive but culture negative. The 5' NCR of HRV and EnV was amplified from the leftover cDNA for the RMA by semi-nested PCR (15) with 3 universal PCR primers for both HRV and EnV. These 3 universal primers (corresponding to nucleotides 163-181, 443-462 and 536-552 of

1 HRV16 were designed according to the conserved regions of 247 HRV and 62 EnV 5'NCR
2 sequences. PCR products of each sample were analyzed in a 1.5% agarose gel for the presence of
3 the predicted 300-base PCR fragment (corresponding to nucleotides 163-462 of HRV16). PCR
4 fragments of each positive sample were purified with 1.2% low melting point agarose gel,
5 inserted into plasmid vector pMJ3 and then transformed into E coli (2). Plasmids with the correct
6 inserts were isolated, amplified and purified. Each insert was completely sequenced as described
7 above.

ACCEPTED

RESULTS

Sensitive and specific detection of target viruses by Panels A and B. The performance of Panels A (HRV, RSVA, RSVB, MPV, PIV1, PIV2, PIV3, PIV4a, PIV4b, InfVA and InfVB) and B (EnV, CoVOC43, CoV229E, CoVNL63, AdVB, AdVC and AdVE) was evaluated in a standard MultiCode-PLx assay against 20 copies of detection target cDNAs and 60,000 copies of human genomic DNA (negative control) per reaction. As shown in Figure 2, each primer set provided a strong target-specific signal (signal/noise ratio of 14-240 with an average of 46) and had no nonspecific reaction with human sequences or the other viral targets. For Panel A primer sets, the target-specific signals were ranged from 2500 to 7900 MFI with an average of 4200 MFI and low background signals (60-270 MFI with an average of 180 MFI) as measured against 60000 copies of human DNA. The PIV4b primer set gave a slightly higher background signal (560 MFI), but it also generated the strongest target-specific signal (7900 MFI). For Panel B primer sets, the target-specific signals ranged from 3600 to 10300 MFI with an average of 5400 MFI and the background signals were between 20 to 180 MFI with an average of 110 MFI.

Improved preparation of cDNA from clinical specimens. To optimize the detection rate of RMA for clinical samples, we refined the RNA extraction and cDNA synthesis procedures of our previous protocol (15) for efficient production of viral cDNA from very small amount of viral targets. The new protocol produced sufficient cDNA from a sample containing only 100 HRV16 virions to generate a strong RMA signal and thus markedly increased the sensitivity of the RMA (Figure 3). Since an infectious unit of HRV16 typically contains 200-400 virions, our detection assay was more sensitive than traditional viral culture assays that have a theoretical sensitivity of ≥ 1 infectious unit.

Sensitivity and specificity of virus detection in clinical specimens. The sensitivity and specificity of both RMA Panels A and B to detect respiratory viruses in human specimens was

evaluated against 101 clinical samples (throat or nasopharyngeal swab) that previously tested positive by traditional culture or immunofluorescence staining methods (15) for HRV (n=20), RSV (n=21), InfVA (n=10), InfVB (n=10), PIV1 (n=10), PIV3 (n=10) and AdV (n=20). After RNA extraction and cDNA synthesis, duplicate samples were then tested by both RMA Panel A and B assays. Samples with an average signal >6 standard deviations of average negative control signals (typically 400 to 500 MFI) were regarded as positive. Positive signals ranged from 1000 to 10000 MFI and background signals from 0 to 300 MFI (Table 4). The RMA detected HRV and AdV in all positive samples, RSV in 95% of RSV samples, and InfVA, InfVB, PIV1 and PIV3 in 90% of respective samples. In AdV samples, all 3 groups, B, C and E, were detected and in RSV samples, both A and B were found. RMA also detected additional HRV (n=3), InfVA (n=3), PIV3 (n=3) and EnV (n=1) in 10 samples that were tested negative for these viruses by traditional methods, and detected MPV (n=1) and OC43 (n=2) that were not tested by WSLH. Therefore, compared to the gold standard traditional methods, the respective sensitivity of RMA to detect HRV, RSV, InfVA, InfVB, PIV1, PIV3 and AdV were 100%, 95%, 90%, 90%, 90%, 90% and 100% and the respective specificity were 96%, 100%, 97%, 100%, 100%, 97% and 100%. The overall sensitivity and specificity of RMA were 94% and 99% respectively.

Detection of respiratory viruses in nasal specimens from children with respiratory

symptoms. The sensitivity of the RMA to detect respiratory viruses in clinical specimens was further assessed by testing 103 additional samples of nasal secretions from 5 year-old children with asthma and acute respiratory symptoms. For comparison, a second aliquot of each sample was tested with traditional culture and immunofluorescence staining (15) at WSLH. By traditional methods, viruses were found in 24 of the 103 samples (Table 5). In contrast, RMA detected respiratory virus in 74 samples (71.8% of the total), including 70 samples with 1 virus and 4 samples with 2 viruses. RMA had improved rates of detection for RSV, AdV, EnV, and

1 especially HRV. RMA detected HRV in 37 samples while traditional methods in only in 6.

2 Detection for InfV A, InfV B, and PIV1, PIV3 and PIV4 were approximately equal for either
3 assay.

4 **Confirmation of positive RMA results by sequence analysis.** To test the possibility that the
5 detection of HRV and EnV by RMA in the 34 culture negative specimens were false-positive
6 results due to a nonspecific reaction or/and a laboratory contaminant, we determine the identities
7 of HRV and EnV in these specimens by sequencing their 5'NCRs amplified from the leftover
8 cDNA for the RMA by semi-nested PCR. Twenty-nine of the 31 HRV samples and all 3 EnV
9 samples produced the predicted 300-base PCR fragments. Each of the 32 PCR fragments was
10 cloned and sequenced. These sequences were compared to the HRV and EnV sequences in
11 Genbank database and our database of the HRV 5' NCR sequences (manuscript in preparation)
12 with computer software (Clustal X and BLAST). The results verified that all 29 HRV samples,
13 and an additional 6 samples that were both culture and RMA positive, had HRV, and all 3 EnV
14 samples had EnV. Therefore, the detection of HRV and EnV by RMA in the culture negative
15 samples was specific. Alignment of these 35 HRV sequences revealed 24 distinct HRV strains
16 (pairwise nucleotide variations between 9-59%): 16 strains were detected once, 5 strains twice,
17 and 3 strains were found in three samples each. None of the sequences identified more than once
18 were from sequential samples, suggesting none of the repeat isolates was due to cross-
19 contamination.

DISCUSSION

In this report we describe a fast, multi-target, sensitive and specific detection assay for many common respiratory viruses (HRV, RSV, InfV, PIV, MPV, AdV, CoV, and EnV) based on the EraGen MultiCode-PLx System. The design of the 18 comprehensive primer sets (Table 3) began with identification and analysis of all available respiratory viral sequences, including more than 2 million bases of public sequences (Table 1) and 129 new sequences recently completed in our laboratory (Table 2). The 18 primer sets targeted conserved regions, and were comprehensively tested against known quantities of sequenced cDNA (Figure 2), followed by clinical specimens that were characterized by traditional viral diagnostics (Tables 4 and 5). The RMA was shown to be both sensitive and specific for a broad array of respiratory viruses. Although the RMA was initially designed to detect virtually all respiratory viruses, new viruses, such as Bocavirus (1) and CoV HKU1 (48), have been discovered since the assay primer set was designed.

Currently, most virology diagnostic laboratories use a combination of viral culture, immunofluorescence staining and PCR assays to detect respiratory viruses. Culture has been and still is considered the gold standard for diagnosis of respiratory viruses because it can be used for a wide spectrum of viruses and can detect as little as 1 infectious unit. However, viral culture often takes 2-3 weeks to complete and has limited ability to detect viruses that have fastidious growth requirements, such as CoV, MPV and many serotypes of HRV and EnV (5, 8, 17, 39, 45). Immunofluorescence staining is fast, providing results within several hours, but assay sensitivity and the availability of antisera can be limiting factors (17, 39).

Since its invention in 1985, PCR assay has become an essential viral diagnostic tool because of superior sensitivity and its ability to detect viruses that cannot be identified by culture or immunofluorescent staining (29, 47). Unfortunately, testing with single PCR assays becomes

time-consuming due to the large number of respiratory virus targets. Using a multiplex assay can help to overcome this limitation. Even so, multiplex protocols utilizing traditional PCR chemistry can be limited by lengthy and labor-intensive detection procedures. Current detection procedures such as gel electrophoresis (6), enzyme hybridization assay (12, 18, 34), enzyme-linked amplicon hybridization assay (40), microwell hybridization assay (16) and reverse-line blot hybridization assay (7) are inefficient for handling large number of samples containing multiple respiratory viruses.

The MultiCode-PLx System offers improvements over current multiplex respiratory virus detection methods in both sample processing and detection system. It utilizes unique chemistry of the specific and avid pairing of non-natural DNA bases isoguanine (iG) and isocytosine (iC) (20). The avidity of this pairing enables extremely specific hybridization, allowing both a simplified sample processing procedure, and high-throughput detection of up to 80 different multiplex PCR products with oligonucleotide-labeled and color-addressed microspheres and flow cytometry (20, 32).

The MultiCode-PLx protocol requires only four simple addition steps (PCR, TSE, microspheres, and SAPE reagents) followed by a final step of multiplex signal detection (Figure 1). All of these steps are carried out in the same 96-well plate, and can be completed in <4 hours. Using this streamlined RMA protocol, analysis of 103 nasal lavage specimens (Table 5) for all common respiratory viruses, from sample preparation to final results, was completed by one technician within a week compared to approximately 4-5 weeks when traditional culture and immunofluorescence methods were used. Moreover, reagent costs for RMA are relatively low, and the fast processing time translates into substantial savings in technician time compared to traditional techniques or single PCR reactions.

The sensitivity and specificity of all multiplex PCR-based assays are dependent on the quality of the primers. As a result of comprehensive analysis of respiratory virus genetic sequences and careful primer design and selection, the RMA primer sets needed as little as 20 copies of purified viral target per reaction to generate a strong target-specific signals with little noise against 60,000 copies of 3 billion-base pair long human genomic DNA and the other viral targets (Figures 2), and detected virus targets in clinical samples specifically and sensitively (Tables 4 and 5). The RMA was more sensitive than culture to detect HRV, EnV and AdV and more sensitive than immunofluorescent antibody techniques to detect RSV (Table 5). These results are consistent with previous reports that culture could not detect many HRV and EnV strains/serotypes with fastidious growth requirements (5, 8, 17, 39, 45), and confirmed that immunofluorescent antibody methods are less sensitive than PCR to detect RSV (5, 8, 17, 39, 45).

A common drawback of highly sensitive PCR assays is the potential to produce false-positive results due to extreme sensitivity. The possibility for cross-contamination was minimized by implementing standardized PCR laboratory procedures (31). This was verified by analysis of the sequences and distribution of 35 HRV detected by RMA in clinical specimens from children with respiratory illnesses, which showed that the specimens were distinct isolates and there was no evidence of cross-contamination.

One of the powerful features of MultiCode-PLx System is its ability to simultaneously detect up to 80 targets in one reaction. Our current RMA uses only a small fraction of this capability and therefore, there is still ample capacity to accommodate additional primer sets for new targets. Some of this excess capacity could be used to safeguard the current assay against false-negative results due to natural mutations at the primer sites of the target viruses. Most of our targets are RNA viruses (HRV, EnV, RSV, InfV, PIV, MPV and CoV) that are notorious for

high mutation rates (10^{-4} to 10^{-5} per nucleotide incorporation) (9). A potential drawback of our current assay is the use of only one primer set for each target, raising the possibility of false-negative results should new mutations occur at critical positions in the primer sites. One approach to circumvent this potential problem would be to add a second independent primer set/microsphere combination for each target. Since the probability of mutating 2 primer sites simultaneously is very low (10^{-8} to 10^{-10} per nucleotide incorporation), the chance of false-negative results due to mutation would be minimized. In addition, this double primer set strategy can also detect mutation in one of the primer sites for updating the primer sequences according to the evolution of the target viruses.

The excess capacity of our RMA also gives us flexibility to respond to the emergence of new respiratory viruses. Our knowledge of respiratory viruses is incomplete, and previously unrecognized respiratory viruses continue to be identified. Two of our current targets, MPV and CoV NL63, were recently discovered (44, 46), and CoV SARS virus emerged in 2002 (10, 23). Two new respiratory viruses, Bocavirus (1) and CoV HKU1 (48), were reported even more recently in 2005. These viruses have been detected worldwide in children and adults (1, 3, 11, 13, 30, 43), and specific primer sets for these new viruses will be added into our panels.

In conclusion, the RMA is a fast, multi-target, sensitive and specific detection assay for hundreds of strains of common respiratory viruses. This high-throughput assay was designed to be practical for use in large clinical studies of respiratory viruses, and with further development may be suitable for routine clinical use in diagnostic virology laboratories.

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REFERENCES

1. Allander, T., M. T. Tammi, M. Eriksson, A. Bjerkner, A. Tiveljung-Lindell, and B. Andersson. 2005. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc. Natl. Acad. Sci. USA* 102:12891-12896.
2. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. *Current protocols in molecular biology*. John Wiley and Sons, New York.
3. Bastien, N., K. Brandt, K. Dust, D. Ward, and Y. Li. 2006. Human Bocavirus infection, Canada. *Emerg. Infect. Dis.* 12:848-50.
4. Beasley, R. 2002. The burden of asthma with specific reference to the United States. *J. Allergy Clinical Immunol.* 109:S482-S489.
5. Chapman, N. M., S. Tracy, C. J. Gauntt, and U. Fortmueller. 1990. Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridization. *J Clin Microbiol* 28:843-50.
6. Coiras, M. T., J. C. Aguilar, M. L. Garcia, I. Casas, and P. Perez-Brena. 2004. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. *J. Med. Virol.* 72:484-495.
7. Coiras, M. T., M. R. Lopez-Huertas, G. Lopez-Campos, J. C. Aguilar, and P. Perez-Brena. 2005. Oligonucleotide array for simultaneous detection of respiratory viruses using a reverse-line blot hybridization assay. *J. Med. Virol.* 76:256-264.
8. Couch, R. B. 2001. Rhinoviruses, p. 777-797. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, and e. al. (ed.), *Fields Virology*, 4th ed, vol. 1. Lippincott Williams and Wilkins, Philadelphia, PA.

- 1 9. Drake, J. W. 1993. Rates of spontaneous mutation among RNA viruses. *Proc. Natl. Acad.*
2 *Sci. USA* 90:4171-4175.
- 3 10. Drosten, C., S. Gunther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau,
4 M. Panning, L. Kolesnikova, R. A. Fouchier, A. Berger, A. M. Burguiere, J. Cinatl, M.
5 Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Muller, V. Rickerts,
6 M. Sturmer, S. Vieth, H. D. Klenk, A. D. Osterhaus, H. Schmitz, and H. W. Doerr. 2003.
7 Identification of a novel coronavirus in patients with severe acute respiratory syndrome.
8 *N. Engl. J. Med.* 348:1967-76.
- 9 11. Esper, F., C. Weibel, D. Ferguson, M. L. Landry, and J. S. Kahn. 2006. Coronavirus
10 HKU1 infection in the United States. *Emerg. Infect. Dis.* 12:775-779.
- 11 12. Fan, J., K. J. Henrickson, and L. L. Savatski. 1998. Rapid simultaneous diagnosis of
12 infections with respiratory syncytial viruses A and B, influenza viruses A and B, and
13 human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse
14 transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex). *Clin.*
15 *Infect. Dis.* 26:1397-1402.
- 16 13. Foulongne, V., M. Rodiere, and M. Segondy. 2006. Human Bocavirus in children. *Emerg.*
17 *Infect. Dis.* 12:862-863.
- 18 14. Gern, J. E., and R. F. J. Lemanske. 2003. Infectious triggers of pediatric asthma. *Pediatr.*
19 *Clin. N. Amer.* 50:555-575.
- 20 15. Gern, J. E., M. S. Martin, K. A. Anklam, K. Shen, K. A. Roberg, K. T. Carlson-Dakes, K.
21 Adler, S. Gilbertson-White, R. Hamilton, P. A. Shult, C. J. Kirk, D. F. Da Silva, S. A.
22 Sund, M. R. Kosorok, and R. F. J. Lemanske. 2002. Relationships among specific viral
23 pathogens, virus-induced interleukin-8, and respiratory symptoms in infancy. *Pediatr.*
24 *Allergy Immunol.* 13:386-393.

- 1 16. Grondahl, B., W. Puppe, A. Hoppe, I. Kuhne, J. A. Weigl, and H. J. Schmitt. 1999. Rapid
2 identification of nine microorganisms causing acute respiratory tract infections by single-
3 tube multiplex reverse transcription-PCR: feasibility study. *J. Clin. Microbiol.* 37:1-7.
- 4 17. Henrickson, K. J. 2004. Advances in the laboratory diagnosis of viral respiratory disease.
5 *Pediatr. Infect. Dis. J.* 23:S6-S10.
- 6 18. Hindiyyeh, M., D. R. Hillyard, and K. C. Carroll. 2001. Evaluation of the Prodesse
7 Hexaplex multiplex PCR assay for direct detection of seven respiratory viruses in clinical
8 specimens. *Am. J. Clin. Path.* 116:218-224.
- 9 19. Illi, S., E. von Mutius, S. Lau, R. Bergmann, B. Niggemann, C. Sommerfeld, U. Wahn,
10 and e. al. 2001. Early childhood infectious diseases and the development of asthma up to
11 school age: a birth cohort study. *Br. Med. J.* 322:390-395.
- 12 20. Johnson, S. C., D. J. Marshall, G. Harms, C. M. Miller, C. B. Sherrill, E. L. Beaty, S. A.
13 Lederer, E. B. Roesch, G. Madsen, G. L. Hoffman, R. H. Laessig, G. J. Kopish, M. W.
14 Baker, S. A. Benner, P. M. Farrell, and J. R. Prudent. 2004. Multiplexed genetic analysis
15 using an expanded genetic alphabet. *Clin. Chem.* 50:2019-2027.
- 16 21. Johnston, S. L. 2005. Overview of virus-induced airway disease. *Proc. Am. Thoracic Soc.*
17 2:150-156.
- 18 22. Keller, M. B., and L. S. R. 2002. Epidemiology of asthma. *Sem. Respir. Crit. Care. Med.*
19 23:317-329.
- 20 23. Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C.
21 Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W.
22 J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J.
23 M. Hughes, J. W. LeDuc, W. J. Bellini, and L. J. Anderson. 2003. A novel coronavirus
24 associated with severe acute respiratory syndrome. *N. Engl. J. Med.* 348:1953-66.

- 1 24. Lee, W. M., S. S. Monroe, and R. R. Rueckert. 1993. Role of maturation cleavage in
2 infectivity of picornaviruses: activation of an infectosome. *J. Virol.* 67:2110-2122.
- 3 25. Lee, W. M., and W. Wang. 2003. Human rhinovirus type 16: mutant V1210A requires
4 capsid-binding drug for assembly of pentamers to form virions during morphogenesis. *J.*
5 *Virol.* 77:6235-44.
- 6 26. Lemanske, R. F. 2004. Viral infections and asthma inception. *J. Allergy Clinical*
7 *Immunol.* 114:1023-1026.
- 8 27. Lemanske, R. F. J. 2002. The childhood origins of asthma (COAST) study. *Pediatr.*
9 *Allergy Immunol.* 13:38-43.
- 10 28. Lemanske, R. F. J., D. J. Jackson, R. E. Gangnon, M. D. Evans, Z. Li, P. A. Shult, C. J.
11 Kirk, E. Reisdorf, K. A. Roberg, E. L. Anderson, K. T. Carlson-Dakes, K. J. Adler, S.
12 Gilbertson-White, T. E. Pappas, D. F. Dasilva, C. J. Tisler, and J. E. Gern. 2005.
13 Rhinovirus illnesses during infancy predict subsequent childhood wheezing. *J. Allergy*
14 *Clinical Immunol.* 116:571-577.
- 15 29. Liolios, L., A. Jenney, D. Spelman, T. Kotsimbos, M. Catton, and S. Wesselingh. 2001.
16 Comparison of a multiplex reverse transcription-PCR-enzyme hybridization assay with
17 conventional viral culture and immunofluorescence techniques for the detection of seven
18 viral respiratory pathogens. *J. Clin. Microbiol.* 39:2779-2783.
- 19 30. Ma, X., R. Endo, N. Ishiguro, T. Ebihara, H. Ishiko, T. Ariga, and H. Kikuta. 2006.
20 Detection of human bocavirus in Japanese children with lower respiratory tract infections.
21 *J. Clin. Microbiol.* 44:1132-1134.
- 22 31. Mifflin, T. E. 2003. Setting up a PCR laboratory, p. 5-14. *In* C. W. D. a. G. S. Dveksler
23 (ed.), *Pcr Primer: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold
24 Spring Harbor.

- 1 32. Nolan, J. P., and F. Mandy. 2006. Multiplexed and microparticle-based analyses:
2 Quantitative tools for the large-scale analysis of biological systems. *Cytometry A* 69:318-
3 325.
- 4 33. Novinska, M. S., B. C. Pietz, T. M. Ellis, D. K. Newman, and P. J. Newman. 2006. The
5 alleles of PECAM-1. *Gene* 376:95-101.
- 6 34. Pehler-Harrington, K., M. Khanna, C. R. Waters, and K. J. Henrickson. 2004. Rapid
7 detection and identification of human adenovirus species by adenoplex, a multiplex PCR-
8 enzyme hybridization assay. *J. Clin. Microbiol.* 42:4072-4076.
- 9 35. Pietz, B. C., M. B. Warden, B. K. DuChateau, and T. M. Ellis. 2005. Multiplex
10 genotyping of human minor histocompatibility antigens. *Hum Immunol* 66:1174-82.
- 11 36. Sigurs, N., R. Bjarnason, F. Sigurbergsson, and B. Kjellman. 2000. Respiratory syncytial
12 virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7.
13 *Am. J. Respir. Crit. Care Med.* 161:1501-1507.
- 14 37. Sigurs, N., P. M. Gustafsson, R. Bjarnason, F. Lundberg, S. Schmidt, F. Sigurbergsson,
15 and B. Kjellman. 2005. Severe respiratory syncytial virus bronchiolitis in infancy and
16 asthma and allergy at age 13. *Am. J. Respir. Crit. Care Med.* 171:137-141.
- 17 38. Stein, R. T., D. Sherrill, W. J. Morgan, C. J. Holberg, M. Halonen, L. M. Taussig, A. L.
18 Wright, and F. D. Martinez. 1999. Respiratory syncytial virus in early life and risk of
19 wheeze and allergy by age 13 years. *Lancet* 354:541-545.
- 20 39. Storch, G. A. 2001. Diagnostic Virology, p. 493-531. *In* D. M. Knipe, P. M. Howley, D.
21 E. Griffin, and e. al. (ed.), *Fields Virology*, 4th ed, vol. 1. Lippincott Williams and
22 Wilkins, Philadelphia, PA.
- 23 40. Syrmis, M. W., D. M. Whiley, M. Thomas, I. M. Mackay, J. Williamson, D. J. Siebert,
24 M. D. Nissen, and T. P. Sloots. 2004. A sensitive, specific, and cost-effective multiplex

- reverse transcriptase-PCR assay for the detection of seven common respiratory viruses in respiratory samples. *J Mol. Diagn.* 6:125-131.
41. Templeton, K. E., S. A. Scheltinga, M. F. Beersma, A. C. Kroes, and E. C. Claas. 2004. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. *J. Clin. Microbiol.* 42:1564-1569.
42. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24:4876-4882.
43. Vabret, A., J. Dina, S. Gouarin, J. Petitjean, S. Corbet, and F. Freymuth. 2006. Detection of the new human coronavirus HKU1: a report of 6 cases. *Clin Infect Dis* 42:634-9.
44. van den Hoogen, B. G., J. C. de Jong, J. Groen, T. Kuiken, R. de Groot, R. A. Fouchier, and A. D. Osterhaus. 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nature medicine* 7:719-724.
45. van den Hoogen, B. G., D. M. Osterhaus, and R. A. Fouchier. 2004. Clinical impact and diagnosis of human metapneumovirus infection. *Pediatr. Infect. Dis. J.* 23:S25-32.
46. van der Hoek, L., K. Pyrc, M. F. Jebbink, W. Vermeulen-Oost, R. J. Berkhout, K. C. Wolthers, P. M. Wertheim-van Dillen, J. Kaandorp, J. Spaargaren, and B. Berkhout. 2004. Identification of a new human coronavirus. *Nature medicine* 10:368-373.
47. van Elden, L. J., M. G. van Kraaij, M. Nijhuis, K. A. Hendriksen, A. W. Dekker, M. Rozenberg-Arska, and A. M. van Loon. 2002. Polymerase chain reaction is more sensitive than viral culture and antigen testing for the detection of respiratory viruses in adults with hematological cancer and pneumonia. *Clin. Infect. Dis.* 34:177-83.

- 1 48. Woo, P. C., S. K. Lau, C. M. Chu, K. H. Chan, H. W. Tsoi, Y. Huang, B. H. Wong, R.
2 W. Poon, J. J. Cai, W. K. Luk, L. L. Poon, S. S. Wong, Y. Guan, J. S. Peiris, and K. Y.
3 Yuen. 2005. Characterization and complete genome sequence of a novel coronavirus,
4 coronavirus HKU1, from patients with pneumonia. *J. Virol.* 79:884-895.
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FIGURE LEGENDS

Figure 1. The RMA consists of 5 main steps that all occur in the same microwell. (1) Target viral cDNA is PCR-amplified with a pair of virus-specific primers (1 hour). One of the primers contains an isocytosine (iC) at its 5' terminus. (2) The PCR product is labeled with an virus-specific EraCode tag (a 7-base oligo composed of both natural bases and iG) and an iG-biotin (biotinylated 2'-deoxy-isoguanosine triphosphate) with a target-specific extension (TSE) primer (30 minutes). Both iG and iC are recognized by natural polymerase. iG and iC pair with each other but not with natural C and G. (3) The tagged TSE product is captured by a color-addressed microsphere through the hybridization of its tag to its precise complementary oligonucleotide conjugated to the surface the microsphere (10 minutes at room temperature). (4) The captured product is labeled with fluorescent streptavidin-phycoerythrin (SAPE) (10 minutes). (5) Fluorescent signal associated with each microsphere is measured in a Luminex LabMap 100 cytometer (1 hour for a 96-well plate).

Figure 2. Detection of target viruses by the RMA is highly sensitive and specific. Each well, containing 20 copies of cDNA of the target genomic regions (Table 2) of the indicated target virus (Panel A: RSV A and B, PIV 1, 2, 3, 4a and 4b, InfV A and B, MPV and HRV1A; and Panel B: AdV3 (group B), AdV1 (group C), AdV4 (group E), CoV 229E, CoV NL63, CoV OC43, EV68, EV69, EV70, EV71, CA22 (Coxsackie A22) and CB1 (Coxsackie B1)), was analyzed with the RMA. Human genomic DNA (60000 copies per well) was used as the negative control (NC). The height of each bar represents the average mean fluorescence intensity (MFI) of triplicate samples.

Figure 3. Improved RNA extraction and cDNA synthesis conditions for the RMA. Samples containing indicated amount of HRV16 virions were processed with both the old and new protocols. Each cDNA preparation was subjected to RMA, and the height of each bar represents

1 the average mean fluorescence intensity (MFI) of triplicate samples.

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Table 1. Sequence information for target respiratory viruses.

Respiratory viruses ^a	Genome Size (Kbs)	No. of complete sequences	Target viral genomic regions ^a	Target Sizes (Kbs)	No. of target sequences for primer design
HRV (101 serotypes)	7	7	5' NCR	0.6	146
EnV (70 serotypes)	8	62	5' NCR	0.7	83
CoV OC43	30	2	N gene	1.3	6
CoV 229E	30	2	N gene	1.2	4
CoV NL63	30	3	N gene	1.1	3
RSV A	15	7	F gene	1.7	27
RSV B	15	3	F gene	1.7	5
MPV	14	4	L gene	6.0	4
PIV1	16	2	HN gene	1.7	32
PIV2	16	2	HN gene	1.7	7
PIV3	16	2	HN gene	1.7	14
PIV4a	- ^b	0	HN gene	1.7	2
PIV4b	- ^b	0	HN gene	1.7	1
InfV A	13	- ^c	M gene	1.0	40
InfV B	13	- ^c	M gene	1.0	19
AdV B (8 serotypes)	37	7	Hexon gene	2.8	35
AdV C (4 serotypes)	37	6	Hexon gene	2.9	16
AdV E (1 serotype)	37	1	Hexon gene	2.6	5

^a) Abbreviations: HRV, human rhinoviruses; EnV, enteroviruses; CoV, coronaviruses; RSV, respiratory syncytial virus; MPV, metapneumoviruses; PIV, parainfluenza viruses; InfV, influenza viruses; AdV, adenoviruses; NCR, noncoding region; N, nucleocapsid; F, fusion; L, polymerase; HN, hemagglutination-neuraminidase; M = matrix.

^b) Unknown.

^c) The genomes InfV A and B are divided into 8 segments. Only the 2 most conserved gene segments (matrix and nucleoproteins) were analyzed.

Table 2. DNA clones of target viral genomic regions.

Target viral genomic regions ^a	Serotypes/Strains/Isolates	Size (kb)
HRV 5'NCR-VP4	HRV1-86, 88-100 and Hanks	0.7
EnV 5'NCR	EV68, 69, 70 & 71, CAV22 and CBV1	0.5
CoV M-N	229E, NL63 and OC43	2.0
RSV F	A/ Long strain and B	2.0
MPV L	CAN97-83	6.0
PIV HN	1, 2 and 3	1.7
	4a and 4b	2.5
InfVA M	A/Sydney/05/97-like (H3N2)	0.9
InfVB M	B/Beijing/184/93-like	1.0
AdVB Hexon	3, 7, 11, 14, 16, 21, 34 and 35	3.0
AdVC Hexon	1, 2, 5, 6	3.1
AdVE Hexon	4	2.8

^a) Abbreviations of viral genomic regions: NCR, noncoding region; VP4, viral capsid protein 4; M-N, membrane glycoprotein-nucleocapsid; F, fusion; L, polymerase; HN, hemagglutination-neuraminidase; M = matrix.

Table 3. Sequences of RMA primers for 18 detection targets.

Viral targets	PCR Primers ^a	TSE primers
HRV 5'NCR	F: AGCCTGCGTGGC R: CGGACACCCAAAGTAGT	CGGCYCCTGAATGYGGCTAA
EnV 5'NCR	F: GGCTGCGYTGGCG R: CGGACACCCAAAGTAGT	ACAWGGTGYGAAGAGTYTATTGAGCTA
CoV OC43 N	F: TGATCAAATTGCTAGTCTTG R: CTTATTCAAATTTCTGTCTG	AGGATGCCACTAAACCTCAGC
CoV 229E N	F: ATTTTCATGCTTTTGTCTT R: ATAAAAAGTCAGCGAAAAC	ACTCTTGCCAGAAAGTTTGAGAAGA
CoV NL63 N	F: TGGCTTTAAAGAACTTAGG R: AAAGAGGCTTATTAGGTTTC	CAGTCGAAGTCACCTAGTTCTTCTG
RSVA F	F: CACCCGTTAGAAAATGT R: TTCAAAAACAGATGTAAGCA	GTTTTGCCATAGCATGACACAATG
RSVB F	F: ATTGCATTTGGTTTCTTTTA R: GTTTTACCAATCGACATGT	CTATTGTTATGACACTGGTATACCAACCTGTT C
MPV L	F: ATGACBACAATGATATGTGC R: CTGGTTTACTKACATCTATTGA	AGACATGCACCACCAGAAAACAAA
PIV1 HN	F: TGAGTGATTAAGTTTGATGA R: ATTATTACCYGGACCAAG	TGCATCACCAATTGATAATGAAGGT
PIV2 HN	F: GGGTTGATTGTGGCCCA R: CTGAGACTTGCTTTCTATTATTATAATGATAC	TGCCCTGTTGTGTTTGAAGAGATATGACT
PIV3 /HN	F: ATGCTTATACCTCRAATCTA R: TRGGATTTAAGTCAGGTACC	ACTCGAGGTTGYCAGGATATAGGAA
PIV4a HN	F: GGGCGATTTCATTTT R: GCAGAGGGTCGATTATATA	CCTCTCTGATAATAAAATATGTTGTTCTCAATG
PIV4b HN	F: TGTGCAGGTGCTTTC R: CCCATAAGGCAAGAAG	CAATGATCTTTTATTTTCGCAATTATGTTTGT T
InfVA M	F: ATTGCCTGCACCATYT R: GTTYTGGCCAGCACT	ACTTGATCCAGCCATTTGCTCCA
InfVB M	F: AAAGAAGATTCATCACAGAG R: TGCTATTTCAAATGCTTCA	CAGGAATGGGAACAACAGCAACAAA
AdVB hexon	F: MAGYACTCTGTTGTCSCC R: GGGTCTGGTGCAGTT	GTCAACGGGCAYRAAGCGCA
AdVC hexon	F: CTGAAGTACGTCTCGG R: GCTACCCCTTCGATG	GGCGTCCTGGCCCGAG
AdVE hexon	F: CATTGGCATAGAGGAAGT R: AGATGCAGGTTCTGAA	AAGGATTGCCTACATGGATTTCATTAGC

^a) F: Forward PCR primer and R: Reverse PCR primer.

PIV3 reverse and AdVB forward PCR primers are degenerate primers.

Table 4. Detection of respiratory viruses by RMA in clinical specimens characterized by traditional methods.

Target viruses	No. of WI State Lab samples	RMA Positive ^a	Additional viruses Detected by RMA
HRV	20	20	
RSV	21	20 ^b	HRV(1), InfVA(2) PIV3(2), OC43(1)
InfVA	10	9	
InfVB	10	9	
PIV1	10	9	
PIV3	10	9	EnV(1)
AdV	20	20 ^c	HRV(2), InfVA(1) PIV3(1), MPV(1), OC43(1)

^a) Samples were called positive when the virus signal was >6 standard deviations of the negative control (~500 MFI in this experiment).

^b) 11 samples were positive for RSVA, 8 samples had RSVB, and 1 sample had both serotypes.

^c) 6 samples had AdVB, 11 samples had AdVC, and 3 samples had both AdVB and AdVE.

Table 5. Detection of respiratory viruses in 103 nasal lavage specimens from 5 year-old children with asthma and respiratory symptoms.

Viruses	No. of virus detected	
	RMA ^a	Traditional methods ^b
HRV	37	6
RSV	13 (7 A and 6 B)	8
InfV A	2	2
InfV B	1	1
MPV	5	Not tested
PIV1	3	3
PIV3	3	2
PIV4	2 ^c	1
AdV	4 (1 B and 3 C)	0
CoV OC43	2	Not tested
CoV NL63	2	Not tested
EnV	4	1
Total	78	24

^a) Samples were called positive when the virus signal was >6 standard deviations of the negative control (~500 MFI in this experiment). A total of 74 samples tested positive for virus; 70 samples had 1 virus, and 4 samples had 2 viruses detected.

^b) A total of 24 samples tested positive for virus by culture and immunofluorescence staining, and each sample had a single virus.

^c) Both samples tested positive for PIV4b.

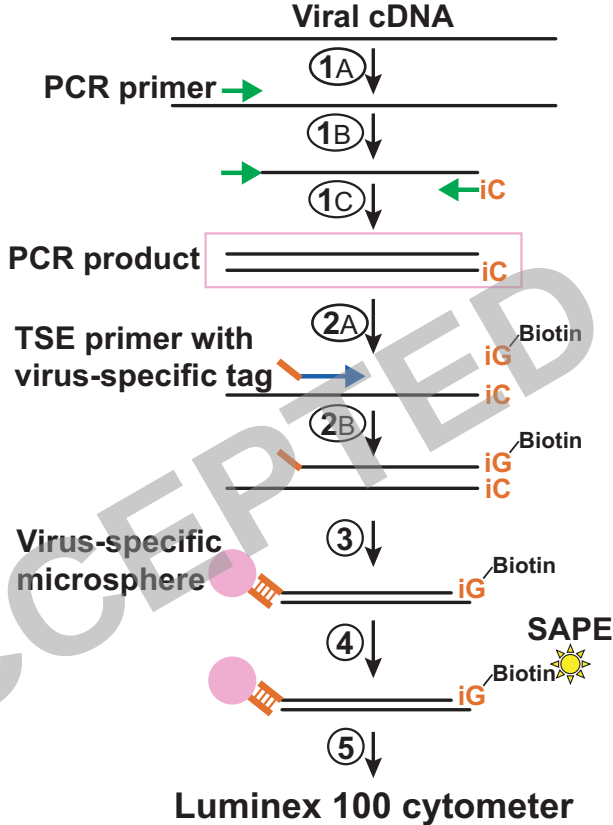


Figure 1, Lee et. al.

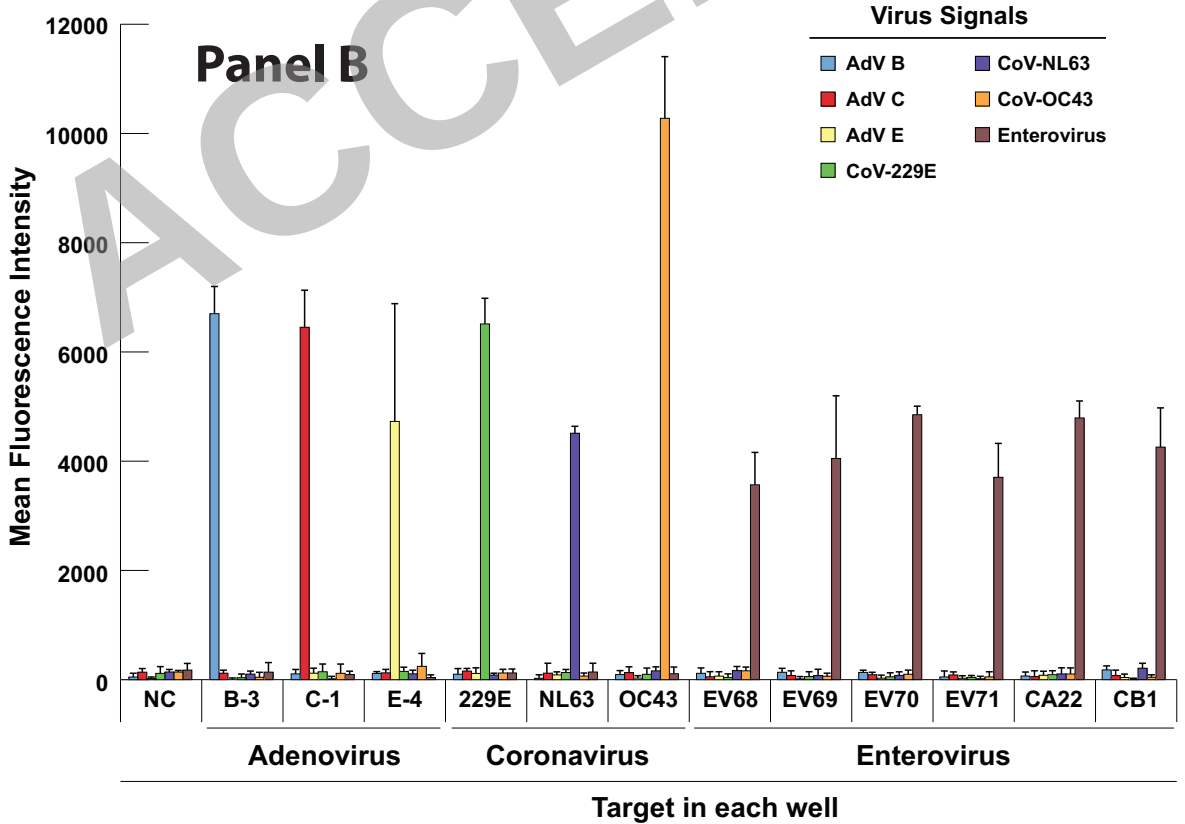
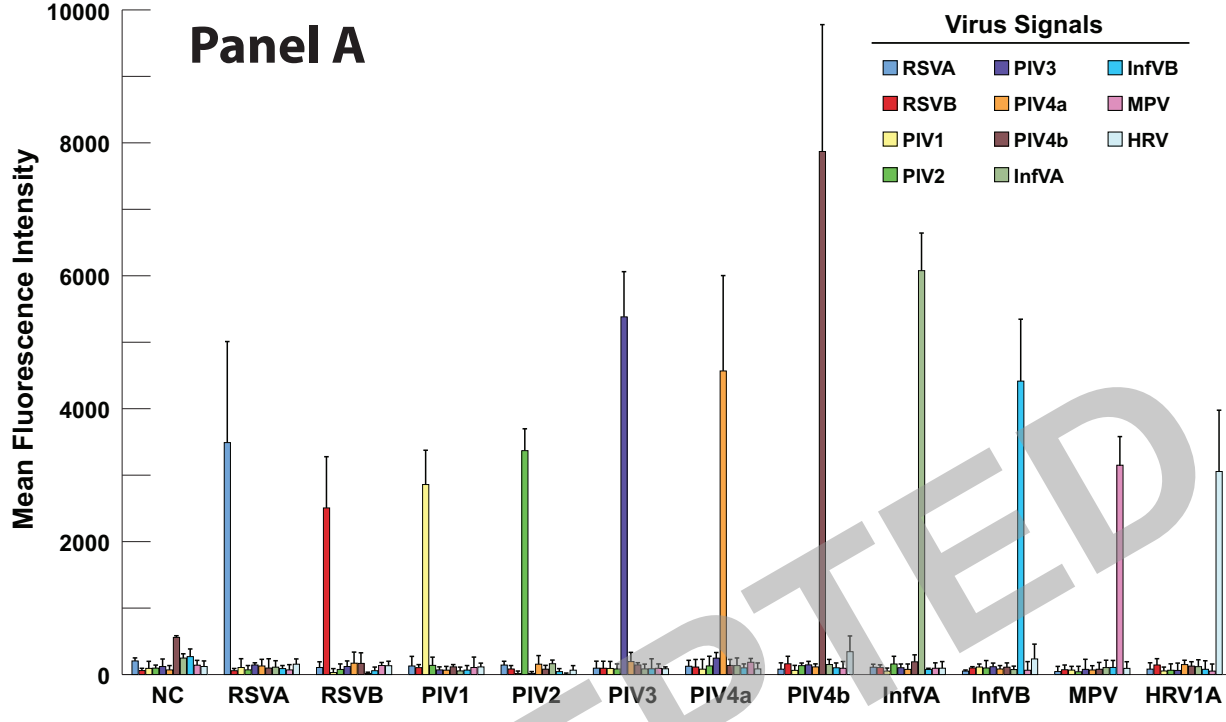


Figure 2, Lee et. al.

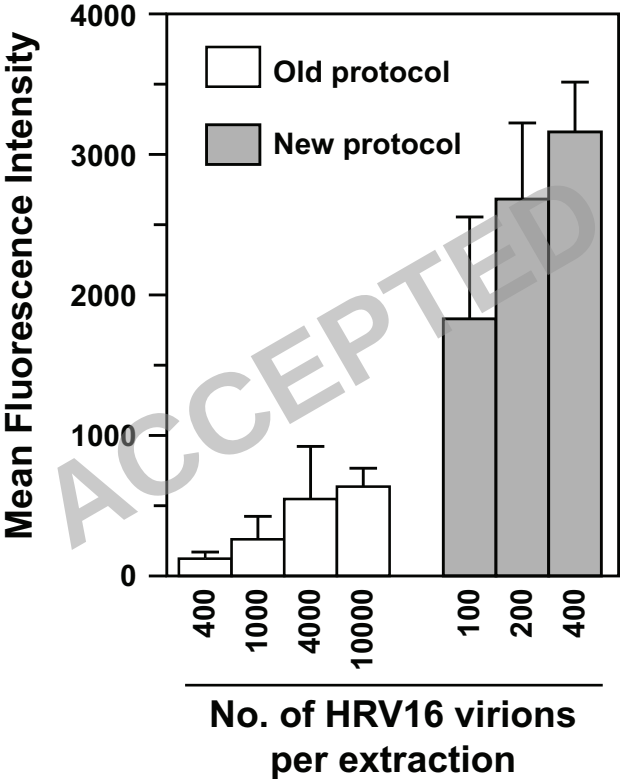


Figure 3, Lee et. al.