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

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

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

To better understand the contribution of viral respiratory infections in infancy to the incidence of asthma in urban environments, a large prospective cohort study, the Urban Environment and Childhood Asthma project (URECA) was initiated. The URECA study will enroll more than 500 infants and will include sampling of nasal secretions for viral diagnostics during periods of health and respiratory illness. Unfortunately, the use of standard viral diagnostics to accomplish this task would be quite costly and time-consuming, given the multiple groups of viruses and hundreds of associated strains. Currently, a typical diagnostic virology laboratory uses a combination of viral culture, immunofluorescent staining, and PCR assays to detect respiratory viruses. Viral culture is time- and labor consuming and is not a sensitive method for detection of rhinoviruses and other viruses that are labile during transport and have fastidious growth requirements (17, 39). Immunofluorescent staining is fast but can be insensitive, often leading to false-negative results (17, 39). None of the current PCR assays has high-throughput detection capability to effectively handle large number of samples containing multiple targets (6, 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 eight distinct groups: HRV, RSV, parainfluenza virus (PIV), influenza virus (InfV), metapneumovirus (MPV), enterovirus (EnV), coronavirus (CoV), and adenovirus (Ad). The Respiratory MultiCode-PLx Assay (RMA) (EraGen Biosciences, Madison, WI) is a new multitarget, 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 isoguanine (iG) and isocytosine (iC), allowing

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reaction products to be labeled and captured without many of the steps required for other methods. The RMA system can simultaneously detect up to 80 targets in one reaction and complete 96 reactions in 4 h and has been successfully applied to commercial tests for large-scale genetic screening of carriers of cystic fibrosis multimutations (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 RMA system for rapid, sensitive, and multiplexed detection of nearly all human respiratory viruses in large numbers 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 (serotypes 1A, 1B, 2 to 10, 12 to 100, and Hanks) were from Fred Hayden (University of Virginia, Charlottesville); HRV type 11 (HRV11), HRV90, EnV serotypes EV69, EV70, and EV71, coxsackievirus A22, coxsackievirus B1, PIV type 4a (PIV4a), and PIV4b were from ATCC; InfV A (Sydney/05/97-like; H3N2), InfV B (Beijing/184/93-like), and Ad type 1 (Ad1), -2, -3, -4, -5, -6, -7, -11, -14, -16, -21, -34, and -35 were from the Wisconsin State Laboratory of Hygiene (WSLH); RSV A (Long strain), RSV B, and MPV (isolate CAN97-83) were from Anne Mosser (University of Wisconsin); and CoV 229E was from K. Holmes (University of Colorado). RNA of CoV NL63 was provided by L. van der Hoek and B. Berkhont (University of Amsterdam). DNA clones of the hemagglutinin-neuraminidase (HN) genes of PIV1, PIV2, and PIV3 were obtained from M. Skiadopoulos and B. Murphy (National Institutes of Health). A DNA clone of the CoV OC43 N gene was obtained from A. Dandekar and S. Perlman (University of 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 from WSLH. These specimens tested positive for HRV ($n = 20$), RSV ($n = 21$), InfV A ($n = 10$), InfV B ($n = 10$), PIV1 ($n = 10$), PIV3 ($n = 10$), and Ad ($n = 20$) by traditional viral culture and immunofluorescent-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, PIV1 to -4, HRV, EnV, and Ad (15). Respiratory viruses were initially identified by characteristic cytopathic effects and hemadsorption reactions, and the virus type was confirmed by immunofluorescent staining with specific monoclonal antibodies (15). In addition, HRV was identified by acid lability and EnV was identified by virus neutralization using standard Lim Benyesh-Melnick serum-typing pools. RSV antigen was detected by immunofluorescent staining of respiratory epithelial cells in the clincal specimens, in addition to viral culture (15). This WSLH sample collection did not have samples that tested positive for PIV2, PIV4, or EnV by traditional methods.

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

RMA. The RMA was performed in 96-well PCR plates (Bio-Rad; 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 (Bio-Rad; MSB1001), and incubation (Fig. 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 surfaces 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 an 8-μl reaction mixture containing 2 μl cDNA, 1 μl MC-PCR buffer (EraGen; PN1235), 0.16 μl of *Taq* polymerase (BD Bioscience; 639209), and 200 nM PCR forward and 5′-iC modified reverse primers under the following conditions: 5 min at 95°C and 28 cycles of 5 s at 95°C, 10 s at 55°C, and 30 s at 72°C.

Immediately after the PCR, 2 μl of TSE mixture containing 1 μl of MC-TSE buffer (EraGen; PN1308) and 75 nM TSE primers (EraGen) were added to the PCR product. The TSE reaction was carried out under the following conditions: 30 s at 95°C and 10 cycles of 5 s at 95°C and 2 min at 65°C.

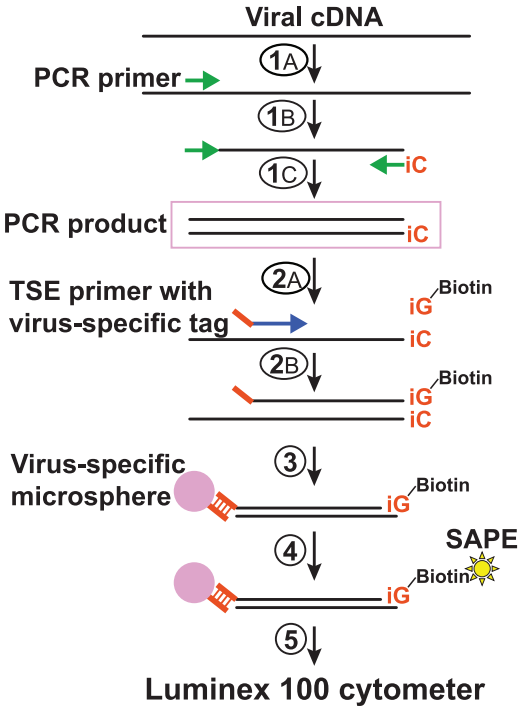

FIG. 1. The RMA consists of five main steps that all occur in the same microwell. (Step 1) Target viral cDNA is PCR amplified with a pair of virus-specific primers (1 h). One of the primers contains an iC at its 5′ terminus. (Step 2) The PCR product is labeled with a virus-specific EraCode tag (an eight-base oligonucleotide composed of both natural bases and iG) and an iG-biotin (biotinylated 2′-deoxy-iG triphosphate) with a TSE primer (30 min). Both iG and iC are recognized by natural polymerase. iG and iC pair with each other, but not with natural C and G. (Step 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 min at room temperature). (Step 4) The captured product is labeled with fluorescent SAPE (10 min). (Step 5) Fluorescent signal associated with each microsphere is measured in a Luminex LabMap 100 cytometer (1 h for a 96-well plate).

After the TSE reaction, 40 μl of microspheres/hybridization solution (EraGen PN1402/1237) was added to the TSE products. The resulting mixture was incubated (room temperature; 10 min) in the dark to allow hybridization of TSE products to the tag-specific microspheres, and then 40 μl of sheath fluid (Luminex; 40-50000) containing 2 μg of SAPE (Prozyme; PJ31S) was added. Next, the fluorescent signal associated with each microsphere was measured in a Luminex machine (a 96-well plate flow cytometer). The signal was expressed as the mean fluorescence intensity (MFI). Samples with an average signal >6 standard deviations from the 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 eight respiratory-virus groups (HRV, RSV, InfV, PIV, MPV, Ad, 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 (Table 1; a total of 110 sequences) listed in public databases, including the NCBI Taxonomy Brower, the NCBI nucleotide database, the 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).

HRVs have 101 known serotypes, but only seven full-length sequences (for serotypes 1B, 2, 9, 14, 16, 85, and 89) were found in the databases. Alignment of

TABLE 1. Sequence information for target respiratory viruses

Respiratory virus	Genome size (kb)	No. of complete sequences	Target viral genomic regions	Target size (kb)	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	Unknown	0	HN gene	1.7	2
PIV4b	Unknown	0	HN gene	1.7	1
InfV A	13	-[a]	M gene	1.0	40
InfV B	13	-	M gene	1.0	19
Ad B (8 serotypes)	37	7	Hexon gene	2.8	35
Ad C (4 serotypes)	37	6	Hexon gene	2.9	16
Ad E (1 serotype)	37	1	Hexon gene	2.6	5

[a] -, the genomes InfV A and B are divided into eight segments. Only the two most conserved gene segments (matrix and nucleoproteins) were analyzed.

these seven 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 to be respiratory pathogens. Sixty-two full-length sequences for coxsackieviruses and EV68 through EV73 were identified. As for HRV, the 5′ NCR was selected as the detection target. CoVs have three serotypes that are common respiratory pathogens, OC43, 229E, and NL63, although additional serotypes have been recently identified. Alignment of the two full-length genome sequences of OC43 and 229E and three 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.

RSVs have two serotypes, A and B, and 10 full-length genome sequences (7 RSV A and 3 RSV B) were identified. Although the fusion (F) gene was the most conserved, variability led to synthesis of separate primer sets for each serotype. For MPVs, four full-length sequences were found, and the polymerase (L) gene was selected as the detection target. PIVs have five 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 HN gene, serotype-specific primer sets were required.

InfV has three 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 eight RNA strands, so we examined only the sequences of the two 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.

Ads have more than 50 serotypes that are divided into six groups (A to F). Group B, C, and E viruses were selected for our assay because they are common respiratory pathogens. Fourteen full-length sequences were found in GenBank: seven B, six C, and one E. Alignment of these sequences showed that the hexon gene was the most conserved among serotypes within the same group. Due to variability among serotypes, a separate primer set was needed for each group.

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

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

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

Total nucleic acids, including viral RNA, were prepared from 100 μl of infected cell lysate by phenol extraction and ethanol precipitation (2). Each target viral genomic region was amplified using a reverse transcriptase PCR mixture (Invitrogen; 11922-028) and appropriate primers that annealed to its 5′ and 3′ ends (see Table S1 in the supplemental material). PCR products were analyzed by electrophoresis in a 1% low-melting-point 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 *Escherichia 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 optical density at 260 nm (50 μg per optical-density unit) and the size of the plasmid. Each viral DNA fragment was

TABLE 2. DNA clones of target viral genomic regions

Target viral genomic region[a]	Serotype/strain/isolate	Size (kb)
HRV 5′ NCR-VP4	HRV1-86, 88-100, Hanks	0.7
EnV 5′ NCR	EV68, -69, -70, -71; CA22; CB1	0.5
CoV M-N	229E, NL63, OC43	2.0
RSV F	A/Long strain, B	2.0
MPV L	CAN97-83	6.0
PIV HN	1, 2, 3	1.7
	4a, 4b	2.5
InfV A M	A/Sydney/05/97-like (H3N2)	0.9
InfV B M	B/Beijing/184/93-like	1.0
Ad B hexon	3, 7, 11, 14, 16, 21, 34, 35	3.0
Ad C hexon	1, 2, 5, 6	3.1
Ad E hexon	4	2.8

[a] VP4, viral capsid protein 4.

TABLE 3. Sequences of RMA primers for 18 detection targets

Viral target	PCR primers[a]	TSE primer
HRV 5′ NCR	F: AGCCTGCGTGGC R: CGGACACCCAAAGTAGT	CGGCYCCTGAATGYGGCTAA[b]
EnV 5′ NCR	F: GGCTGCGYTGGCG R: CGGACACCCAAAGTAGT	ACAWGGTGYGAAGAGTYTATTGAGCTA[b]
CoV OC43 N	F: TGATCAAATTGCTAGTCTTG R: CTTATTCAAAATTTTCTGTCTG	AGGATGCCACTAAACCTCAGC
CoV 229E N	F: ATTTCATGCTTTTGTTCTT R: ATAAAAAGTCAGCGAAAAC	ACTCTTGGCAGAAGTTTGAGAAGA
CoV NL63 N	F: TGGCTTTAAAGAACTTAGG R: AAAGAGGCTTATTAGGTTTC	CAGTCGAAGTCACCTAGTTCTTCTG
RSV A F	F: CACCCGTTAGAAAATGT R: TTCAAAAACAGATGTAAGCA	GTTTTGCCATAGCATGACACAATG
RSV B F	F: ATTGCATTTGGTTTCTTTTA R: GTTTTACCAATCGACATGT	CTATTGTTATGACACTGGTATACCAACCTGTTC
MPV L	F: ATGACBACAATGATATGTGC R: CTGGTTTACTKACATCTATTGA	AGACATGCACCACCAGAAACAAA
PIV1 HN	F: TGAGTGATTAAGTTTGATGA R: ATTATTACCYGGACCAAG	TGCATCACCAATTGATAATGAAGGT
PIV2 HN	F: GGGTTGATTGTGGCCCA R: CTGAGACTTGCTTTCTATTATTATAATGATAC	TGCCCTGTTGTGTTTGGAAGAGATATGACT
PIV3 HN	F: ATGCTTATACCTCRAATCTA R[b]: TRGGATTTAAGTCAGGTACC	ACTCGAGGTTGYCAGGATATAGGAA[b]
PIV4a HN	F: GGGCGATTTCAATTTTT R: GCAGAGGGTCGATTATATA	CCTCTCTGATAATAAAATATGTTGTTCTCAATG
PIV4b HN	F: TGTGCAGGTGCTTTC R: CCCATAAGGCAAGAAG	CAATGATCTTTTATTTTCGCAATTATGTTTGTTT
InfV A M	F: ATTGCCTGCACCATYT R: GTTYTGGCCAGCACT	ACTTGATCCAGCCATTTGCTCCA
InfV B M	F: AAAGAAGATTCATCACAGAG R: TGCTATTTCAAATGCTTCA	CAGGAATGGGAACAACAGCAACAAA
Ad B hexon	F[b]: MAGYACTCTGTTGTCSCC R: GGGTCTGGTGCAGTT	GTCAACGGGCAYRAAGCGCA[b]
Ad C hexon	F: CTGAAGTACGTCTCGG R: GCTACCCCTTCGATG	GGCGTCCTGGCCCGAG
Ad E hexon	F: CATTGGCATAGAGGAAGT R: AGATGCAGGTTCTGAA	AAGGATTGCCTACATGGATTTCATTAGC

[a] F, forward PCR primer; R, reverse PCR primer.
[b] Degenerate primer.

completely sequenced (Automated DNA Sequencing Facility, University of Wisconsin).

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

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

The performance of each candidate primer set was next evaluated in a standard RMA by preparing samples containing 20 copies of the 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 a signal (2,000 to 10,000 MFI) that was markedly higher than the background signal (300 MFI and lower) of the negative control (60,000 copies of human DNA per reaction).

Development of a pan-HRV primer set. The HRV group has 101 known serotypes that accounted 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′ NCRs (146 published and 101 new sequences), which covered all 101 known serotypes. The analysis results revealed that the 5′ NCR had three stretches (A, B, and C) of almost completely conserved sequences, corresponding to nucleotides 352 to 368 (A), 442 to 462 (B), and 535 to 554 (C) of HRV16. Within these three stretches of sequence, 12 PCR forward (A), 9 PCR reverse (C), and 5 TSE (B) candidate primers were designed using the program Visual Oligonucleotide Modeling Platform. 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′ NCRs of HRV1A, -2, -17, and -59 in the RMA assay. These four serotypes were used because the collective sequences of their primer sites had identity with 99 of the 101 serotypes. The selected primer set detected 10 copies of representative targets per reaction, with signals of about 2,000 MFI, which was 10-fold higher than the background. The signal strength of the assay increased with the target concentration and then reached a plateau (~5,000 MFI) at 200 copies of the target per reaction.

To determine whether this primer set could sensitively detect all HRVs, cDNA clones of the 5′ NCRs of all 101 serotypes were tested. The results showed that the primer set detected 99 serotypes at 20 copies of target per reaction with a typical signal of about 2,000 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 sites of these two 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 two detection panels, called A and B. Panel A consisted of 11 primer sets for HRV, RSV A, RSV B, MPV, PIV1, PIV2, PIV3, PIV4a, PIV4b, InfV A, and InfV B. Panel B consisted of seven primer sets for EnV, CoV OC43, CoV 229E, CoV

NL63, Ad B, Ad C, and Ad E. The performance of each primer set in panels A and B was evaluated in a standard multiplex RMA 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 (avian myleoblastosis virus [AMV] reverse transcriptase; Promega; M510F), with greater cDNA synthesis efficiency at low template concentrations. This procedure applied not only to RNA viruses, but also to Ads with DNA genomes, because our target was the Ad 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 specimen (e.g., nasal-lavage fluid or swab), 150 μl 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 min at 25°C, supplied with 230 μl of chloroform, vortexed for 5 min, and then centrifuged for 5 min at 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 h to precipitate RNA. The RNA precipitant was pelleted by centrifugation for 10 min at 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 5× AMV reverse transcriptase buffer (Promega; M515A), 0.5 μg random primers (Promega; C1181), 20 units of RNasin (Promega; N2615), and 8 μl 5 mM deoxynucleoside triphosphates (Promega; U1330) and then incubated at 25°C for 5 min, 42°C for 10 min, 50°C for 20 min, and 85°C for 5 min.

Comparison of the new and old protocols for RNA extraction and cDNA synthesis. Samples containing various numbers of purified HRV16 virions (100, 200, 400, 1,000, 4,000, and 10,000) (25) were processed by the new protocol as described above and the old protocol as described previously (15). Briefly (for the old protocol), 100 μl of sample was mixed with 1 ml of Trizol (Invitrogen; 15596) in regular Eppendorf tubes (Eppendorf; 022363204) by vortexing (10 min; 25°C). The resulting mixture was supplied with 200 μl of chloroform, vortexed for 5 min, and then centrifuged for 5 min 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. The 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′ NCRs of HRV and EnV in nasal specimens that were RMA positive but culture negative. The 5′ NCRs of HRV and EnV were amplified from the leftover cDNA for the RMA by seminested PCR (15) with three universal PCR primers for both HRV and EnV. These three universal primers (corresponding to nucleotides 163 to 181, 443 to 462, and 536 to 552 of HRV16) were designed according to the conserved regions of 247 HRV and 62 EnV 5′ NCR sequences. The PCR products of each sample were analyzed in a 1.5% agarose gel for the presence of the predicted 300-base PCR fragment (corresponding to nucleotides 163 to 462 of HRV16). PCR fragments of each positive sample were purified with a 1.2% low-melting-point agarose gel, inserted into plasmid vector pMJ3, and then transformed into *E. coli* (2). Plasmids with the correct inserts were isolated, amplified, and purified. Each insert was completely sequenced as described above.

RESULTS

Sensitive and specific detection of target viruses by panels A and B. The performance of panels A (HRV, RSV A, RSV B, MPV, PIV1, PIV2, PIV3, PIV4a, PIV4b, InfV A, and InfV B) and B (EnV, CoV OC43, CoV 229E, CoV NL63, Ad B, Ad C, and Ad E) was evaluated in a standard RMA against 20 copies of detection target cDNAs and 60,000 copies of human genomic DNA (negative control) per reaction. As shown in Fig. 2, each primer set provided a strong target-specific signal (a signal/noise ratio of 14 to 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 ranged from 2,500 to 7,900 MFI, with an average of 4,200 MFI and low background signals (60 to 270 MFI, with an average of 180 MFI) as measured against 60,000 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 (7,900 MFI). For panel B primer sets, the target-specific signals ranged from 3,600 to 10,300 MFI, with an average of 5,400 MFI, and the background signals were between 20 and 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 amounts 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 (Fig. 3). Since an infectious unit of HRV16 typically contains 200 to 400 virions, our detection assay was more sensitive than traditional viral culture assays, which 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 were evaluated against 101 clinical samples (throat or nasopharyngeal swab) that previously tested positive by traditional culture or immunofluorescent- staining methods (15) for HRV (n = 20), RSV (n = 21), InfV A (n = 10), InfV B (n = 10), PIV1 (n = 10), PIV3 (n = 10), and Ad (n = 20). After RNA extraction and cDNA synthesis, duplicate samples were tested by both RMA panel A and B assays. Samples with an average signal >6 standard deviations from the average negative control signals (typically 400 to 500 MFI) were regarded as positive. Positive signals ranged from 1,000 to 10,000 MFI and background signals from 0 to 300 MFI (Table 4). The RMA detected HRV and Ad in all positive samples, RSV in 95% of RSV samples, and InfV A, InfV B, PIV1, and PIV3 in 90% of the respective samples. In Ad samples, all three groups, B, C, and E, were detected, and in RSV samples, both A and B were found. RMA also detected additional HRV (n = 3), InfV A (n = 3), PIV3 (n = 3), and EnV (n = 1) in 10 samples that tested negative for these viruses by traditional methods and detected MPV (n = 1) and OC43 (n = 2) that were not tested for by WSLH. Therefore, compared to the gold standard traditional methods, the respective sensitivities of RMA for detecting HRV, RSV, InfV A, InfV B, PIV1, PIV3, and Ad were 100%, 95%, 90%, 90%, 90%, 90%, and 100%, and the respective specificities 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 for detecting 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 by traditional culture and immunofluorescent staining (15) at WSLH. By traditional methods, viruses were found in 24 of the 103 samples (Table 5). In con-

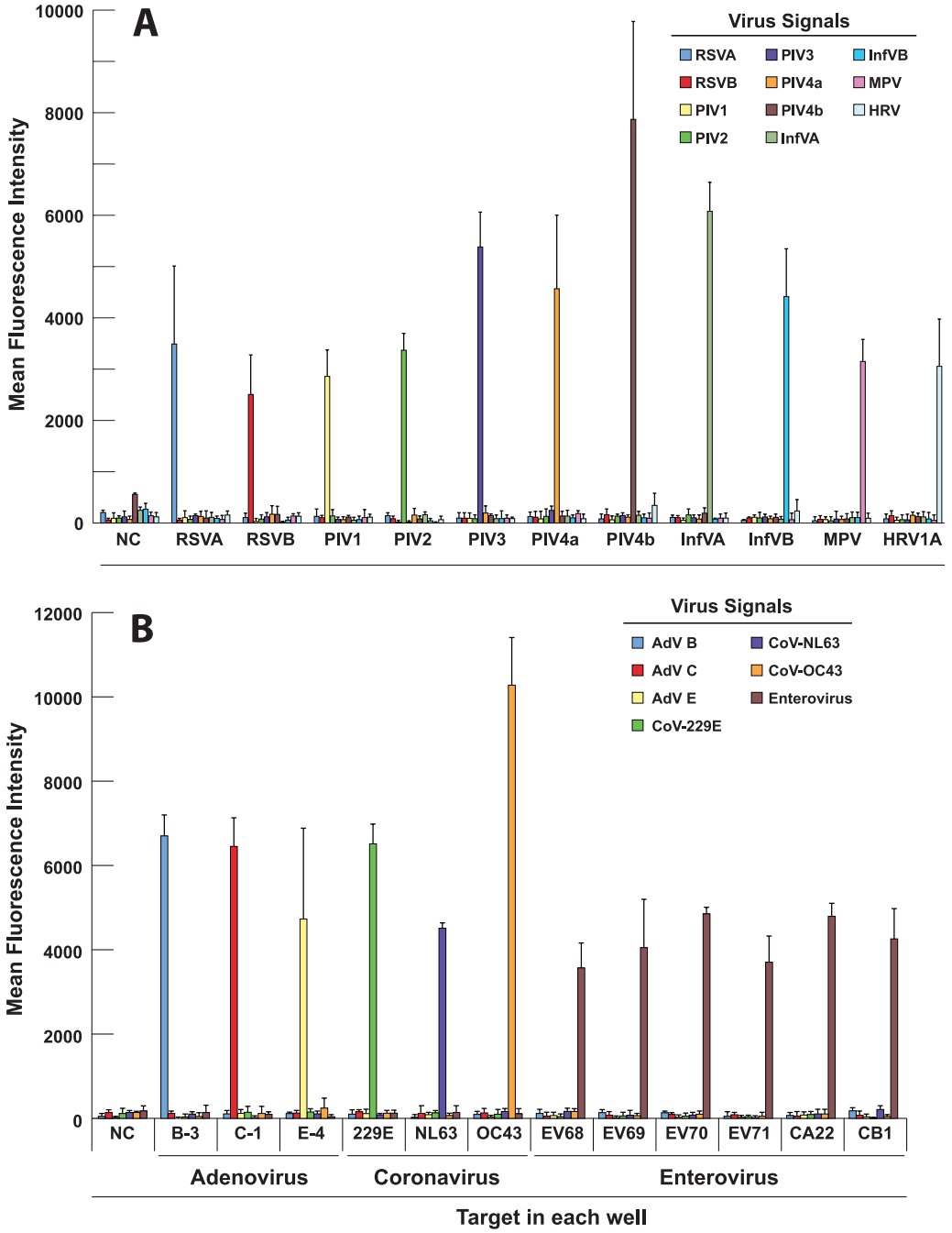

FIG. 2. Detection of target viruses by the RMA is highly sensitive and specific. Each well, containing 20 copies of cDNA of the target genomic regions (Table 2) of the indicated target virus, panel A (RSV A and B; PIV1, -2, -3, -4a, and -4b; InfV A and B; MPV; and HRV1A) and panel B (Ad3 [group B], Ad1 [group C], Ad4 [group E], CoV 229E, CoV NL63, CoV OC43, EV68, EV69, EV70, EV71, CA22 [coxsackievirus A22], and CB1 [coxsackievirus B1]), was analyzed with the RMA. Human genomic DNA (60,000 copies per well) was used as the negative control (NC). Each bar represents the average MFI of triplicate samples. The error bars indicate standard deviations.

trast, RMA detected respiratory virus in 74 samples (71.8% of the total), including 70 samples with one virus and 4 samples with two viruses. RMA had improved rates of detection for RSV, Ad, EnV, and especially HRV. RMA detected HRV in 37 samples, while traditional methods detected it in only 6. Detection rates of InfV A, InfV B, and PIV1, PIV3, and PIV4 were approximately equal for the two assays.

Confirmation of positive RMA results by sequence analysis. To test the possibility that the detection of HRV and EnV by RMA in the 34 culture-negative specimens was a false-positive result due to a nonspecific reaction and/or a laboratory contaminant, we determine the identities of HRV and EnV in these specimens by sequencing their 5′ NCRs amplified from the leftover cDNA for the RMA by seminested PCR. Twenty-

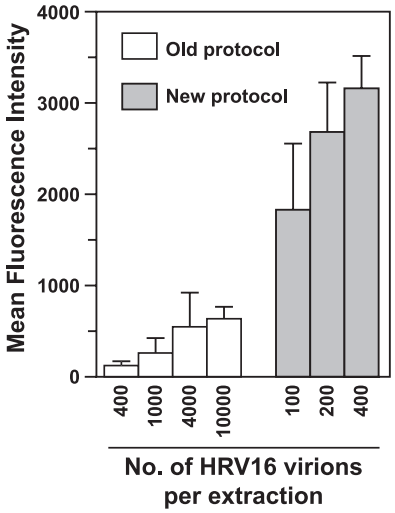

FIG. 3. Improved RNA extraction and cDNA synthesis conditions for the RMA. Samples containing the indicated numbers of HRV16 virions were processed by both the old and new protocols. Each cDNA preparation was subjected to RMA, and each bar represents the average MFI of triplicate samples. The error bars indicate standard deviations.

nine of the 31 HRV samples and all 3 EnV samples produced the predicted 300-base PCR fragments. Each of the 32 PCR fragments was cloned and sequenced. These sequences were compared to the HRV and EnV sequences in the GenBank database and our database of the HRV 5′ NCR sequences (unpublished data) with computer software (Clustal X and BLAST). The results verified that all 29 HRV samples, and an additional 6 samples that were both culture and RMA positive, had HRV and all three EnV samples had EnV. Therefore, the detection of HRV and EnV by RMA in the culture-negative samples was specific. Alignment of these 35 HRV sequences revealed 24 distinct HRV strains (with pairwise nucleotide variations between 9 and 59%); 16 strains were detected once, 5 strains twice, and 3 strains were found in three samples each. None of the sequences identified more than once was from sequential samples, suggesting that none of the repeat isolates was due to cross-contamination.

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

Target virus	No. of WSLH samples	No. RMA positive[a]	Additional viruses (no.) detected by RMA
HRV	20	20	
RSV	21	20[b]	HRV (1), InfV A (2) PIV3 (2), OC43 (1)
InfV A	10	9	
InfV B	10	9	
PIV1	10	9	
PIV3	10	9	EnV (1)
Ad	20	20[c]	HRV (2), InfV A (1) PIV3 (1), MPV (1), OC43 (1)

[a] Samples were called positive when the virus signal was >6 standard deviations from the negative control (~500 MFI in this experiment).
[b] Eleven samples were positive for RSV A, 8 samples had RSV B, and 1 sample had both serotypes.
[c] Six samples had Ad B, 11 samples had Ad C, and 3 samples had both Ad B and Ad E.

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

Virus	No. of viruses 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
Ad	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 from the negative control (~500 MFI in this experiment). A total of 74 samples tested positive for virus; 70 samples had one virus, and 4 samples had two 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.

DISCUSSION

In this report, we describe a fast, multitarget, sensitive, and specific detection assay for many common respiratory viruses (HRV, RSV, InfV, PIV, MPV, Ad, 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-virus 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 (Fig. 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 diagnostic virology laboratories use a combination of viral culture, immunofluorescent 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 to 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). Immunofluorescent 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, the 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 numbers of samples containing multiple respiratory viruses.

The RMA system offers improvements over current multiplex respiratory-virus detection methods in both sample processing and the detection system. It utilizes the unique chemistry of the specific and avid pairing of the nonnatural DNA bases iG and 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 RMA protocol requires only four simple addition steps (PCR, TSE, microspheres, and SAPE reagents), followed by a final step of multiplex signal detection (Fig. 1). All of these steps are carried out in the same 96-well plate and can be completed in <4 h. 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 to 5 weeks when traditional culture and immunofluorescence methods were used. Moreover, reagent costs for RMA are relatively low, and the short processing time translates into substantial savings in technician time compared to traditional techniques or single PCRs.

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 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 (Fig. 2) and detected virus targets in clinical samples specifically and sensitively (Tables 4 and 5). The RMA was more sensitive than culture in detecting HRV, EnV, and Ad and more sensitive than immunofluorescent-antibody techniques in detecting 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 in detecting 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 HRVs 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 the RMA 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 (HRV, EnV, RSV, InfV, PIV, MPV, and CoV) are RNA viruses 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 two primer sites mutating 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 the severe acute respiratory syndrome CoV 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 to our panels.

In conclusion, the RMA is a fast, multitarget, 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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