

## Generic Detection of Coronaviruses and Differentiation at the Prototype Strain Level by Reverse Transcription-PCR and Nonfluorescent Low-Density Microarray<sup>∇†</sup>

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**A nonfluorescent low-cost, low-density oligonucleotide array was designed for detecting the whole coronavirus genus after reverse transcription (RT)-PCR. The limit of detection was 15.7 copies/reaction. The clinical detection limit in patients with severe acute respiratory syndrome was 100 copies/sample. In 39 children suffering from coronavirus 229E, NL63, OC43, or HKU1, the sensitivity was equal to that of individual real-time RT-PCRs.**

Coronaviruses (CoV) (family *Coronaviridae*, order *Nidovirales*) are large enveloped RNA viruses with a 27- to 32-kb genome of positive polarity. They comprise a very diverse spectrum of pathogens of humans and animals (2, 7). The coronavirus etiology of severe acute respiratory syndrome (SARS) and the recent discoveries of the novel human coronaviruses (hCoV) NL63 and HKU1 (5, 13, 15) have triggered intensified efforts in virus identification and diagnostics. Generic reverse transcription (RT)-PCR assays with a very broad detection range are required, but few such assays are available. None of them has been previously validated in a diagnostic setting (9, 12).

The requirement for sequencing in order to achieve strain identification limits the applicability of generic PCR assays in general. Alternative techniques, such as mass spectrometry or complex fluorescent DNA microarrays, have been proposed (10), but these will often be too sophisticated for medical facilities. We describe here a simple and feasible approach to detecting the full spectrum of coronaviruses with diagnostic sensitivity, combining generic RT-PCR and low-cost, low-density (LCD) DNA microarrays which can be read with the naked eye.

Primers for universal RT-PCR for the genus *Coronavirus* were designed after aligning all coronavirus RNA-dependent RNA polymerase genes. RNA-dependent RNA polymerase motifs A and C were targeted because they contain short amino acid patterns that are 100% identical in all coronaviruses (16). Primer binding regions corresponded to patterns LMGWDYPKCD and MMILSDDAV, comprising domains essential for metal ion che-

lation and binding of the primer 3'-end/template complex (11, 16). Reactions (25- $\mu$ l mixtures) were carried out using the QIAGEN (Hilden, Germany) one-step RT-PCR kit, with 200 nM of primer PC2S2 (equimolar mixture of TTATGGGTTGGGAT TATC and TGATGGGATGGGACTATC), 900 nM of primer PC2As1 (equimolar mixture of TCATCACTCAGAATCATCA, TCATCAGAAAGAATCATCA, and TCGTCGGACAAGATC ATCA), 1  $\mu$ l QIAGEN one-step RT-PCR kit enzyme mix, and 5  $\mu$ l RNA extract. The amplification procedure comprised 30 min at 50°C; 15 min at 95°C; 10 cycles of 20 s at 94°C, 30 s starting at 62°C with a decrease of 1°C per cycle, and 40 s at 72°C; and 30 cycles of 20 s at 95°C, 30 s at 52°C, and 40 s at 72°C. To determine the sensitivity of the assay, the target regions including sufficient stretches of flanking sequence were cloned from several coronaviruses (Table 1) and transcribed into RNA (3, 4). Amplification

TABLE 1. Detection of quantified RNA from representative strains of all three coronavirus groups

No. of copies per reaction	Detection of indicated virus (no. of strains correctly detected/total) <sup>a</sup>					
	SARS-CoV	hCoV-OC43	hCoV-229E	hCoV-NL63	hCoV-HKU1	AIBV
90	3/3	3/3	3/3	3/3	3/3	3/3
45	3/3	3/3	3/3	3/3	3/3	3/3
15	1/3	1/3	2/3	0/3	3/3	0/3
5	0/3	1/3	1/3	0/3	3/3	1/3
0	0/3	0/3	0/3	0/3	0/3	0/3

<sup>a</sup> Each datum point summarizes the results of three replicate tests. RNA standards were cloned and transcribed in vitro according to the following genome positions (GenBank accession numbers are shown in parentheses): SARS-CoV, positions 15008 to 15678 (NC\_004718); hCoV-OC43, positions 14942 to 15606 (NC\_005147); hCoV-229E, positions 14118 to 14782 (NC\_002645); hCoV-NL63, positions 14037 to 14701 (AY567487); hCoV-HKU1, positions 15201 to 15865 (AY597011); avian infectious bronchitis virus (AIBV), positions 13976 to 14637 (AJ311317).

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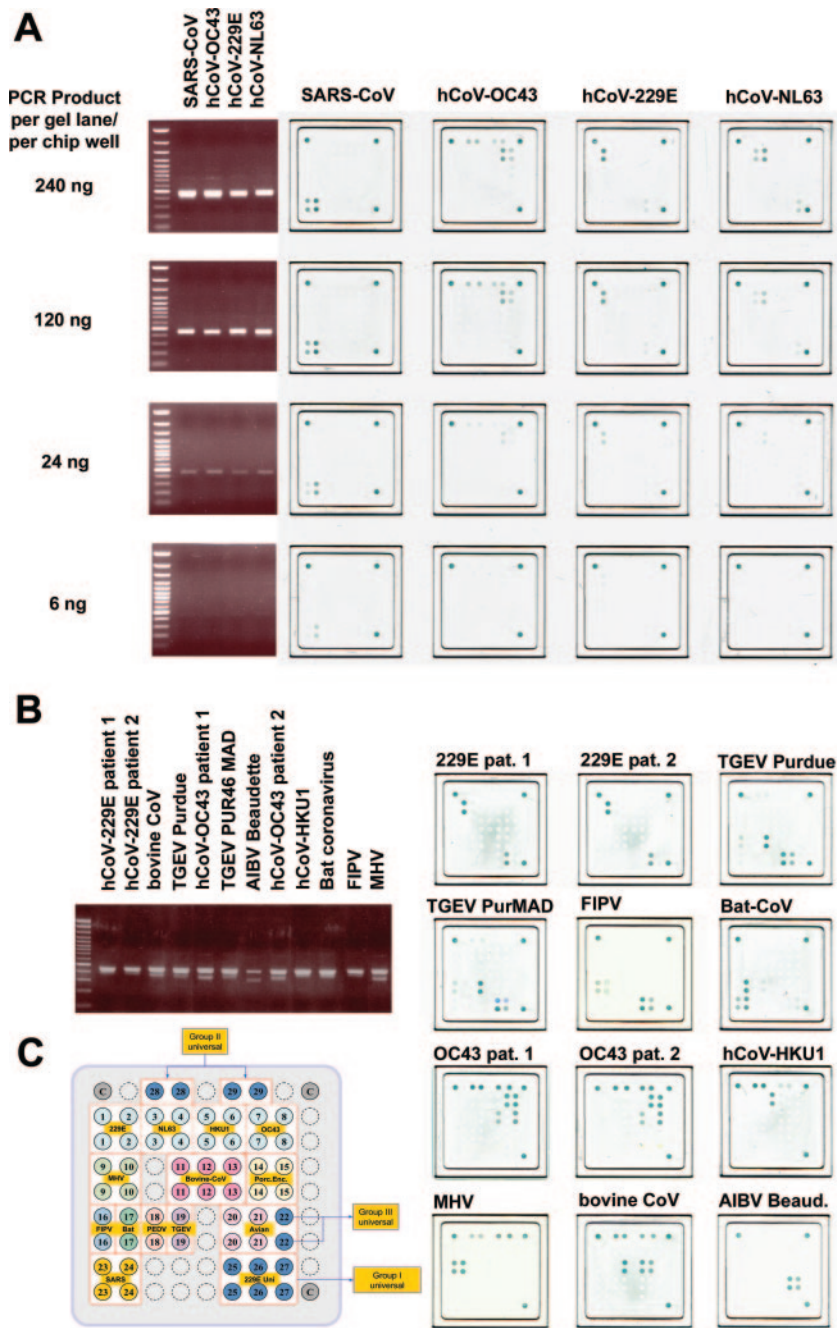


FIG. 1. (A) For four different prototype coronaviruses, as indicated above the four rightmost panels, the amounts of DNA shown in the left column were subjected to gel analysis or array hybridization. Blue dots on the arrays represent hybridization signals. (B) Prototype coronaviruses as indicated above each agarose gel slot were amplified, and the depicted PCR products were hybridized to oligonucleotide arrays as shown in the panel on the right. (C) Spotting pattern of oligonucleotides on array. Each coronavirus group (I to III) is represented by a set of universal probes (blue). Strain-specific probes are depicted in different colors. Each probe is represented at least in duplicate spots. Spots in the upper left and right, as well as in the lower right corner, are staining controls containing biotin. Abbreviations: TGEV, transmissible gastroenteritis virus; AIBV, avian infectious bronchitis virus; FIPV, feline infectious peritonitis virus; MHV, mouse hepatitis virus.

of RNA standards yielded sensitivities in the range of single copies per assay (data not shown). However, when standards were spiked in authentic clinical samples, several  $\log_{10}$ s of sensitivity were lost. Only a nested protocol could recover sensitivity for a broad range of coronavirus RNAs. The protocol was optimized in the presence of a background of nucleic acids as encountered in

routine operation. It used 1  $\mu$ l of first-round PCR product, with 1 $\times$  Platinum *Taq* buffer (Invitrogen, Karlsruhe, Germany), 200  $\mu$ M deoxynucleoside triphosphate, 2.5 mM  $MgCl_2$ , 80 nM of primer PCS (equimolar solutions of CTTATGGGTTGGGATT ATCCTAAGTGTGA and CTTATGGGTTGGGATTATCCC AAATGTGA), 400 nM primer PCNAs (CACACAACACCTT

CATCAGATAGAATCATCA), and 1 U Platinum *Taq* polymerase. The amplification procedure comprised 3 min at 94°C and 30 cycles of 20 s at 94°C, 30 s at 60°C, and 30 s at 72°C. To test the limit of detection of the assay and to challenge its robustness, quantified RNA transcripts were tested in the presence of high levels of background nucleic acids (human DNA, about 50 ng per reaction). As shown in Table 1, constant detection could be achieved with as little as 45 copies of RNA per reaction for all three coronavirus groups. The cumulative hit rates for all viruses were subjected to probit analysis, showing a 50% chance of detection at 15.7 copies per assay (95% confidence interval, 11 to 24 copies per assay). A 95% chance of detection required 34 copies per assay. The specificity of the assay was confirmed on samples tested in an earlier study and determined to contain influenza A virus ( $n = 3$ ), influenza B virus ( $n = 3$ ), human parainfluenza viruses 1 to 3 ( $n = 3$ ), human metapneumovirus ( $n = 3$ ), rhinoviruses ( $n = 3$ ), adenoviruses ( $n = 3$ ), and respiratory syncytial virus ( $n = 1$ ) (8). None of these samples yielded PCR products.

LCD arrays were established next. Oligonucleotide detection probes (see Fig. S1 in the supplemental material) were spotted on plastic microarrays using proprietary technology (Chipron, Berlin, Germany). Primer PCNAs were biotinylated and modified by use of proprietary technology (Chipron) to allow efficient hybridization. PCR products were taken directly from the tube and hybridized to LCD arrays in a 45-min procedure requiring no technical equipment except pipettes and a 37°C incubator (see Fig. S2 in the supplemental material).

To determine whether array hybridization provided the same sensitivity as gel detection, amplification products from hCoV-229E, hCoV-NL63, hCoV-OC43, and SARS-CoV were gel purified and diluted to decreasing concentrations in PCR buffer. The same amount of PCR product was then analyzed by standard gel electrophoresis and LCD array hybridization. As shown in Fig. 1, even faint gel bands yielded corresponding hybridization signals on the array. We thus concluded that the sensitivity of array detection was comparable to that of a gel.

It was also determined how well different coronavirus strains could be discriminated by array hybridization. RNA was extracted from cultured virus or directly from patient material, amplified, and hybridized on the array (Fig. 1). As expected, all PCRs yielded amplification products, and all PCR products gave hybridization patterns on the arrays that matched the expected virus strains. The specificity of array hybridization was confirmed by reactions carried out with mixtures containing about 0.5 µg of background DNA from human leukocytes. PCR inhibition was ruled out by spiking parallel reaction mixtures with low concentrations of feline infectious peritonitis virus or mouse hepatitis virus RNA. They did not generate any unspecific amplification signals or hybridization signals on the arrays (data not shown).

To determine a clinical limit of detection, we retested 11 original RNA preparations from throat swab samples collected from SARS patients during the 2003 epidemic. The material was tested and quantified by a commercial real-time RT-PCR assay as previously described (3). Of the 11 swabs, no detection occurred with only 3, all of which contained less than 100 copies of virus RNA per swab.

The assay was next applied to 39 stored clinical samples as summarized in Table 2. All samples had been determined in

TABLE 2. Results of real-time RT-PCR and universal coronavirus assays in stored clinical samples previously reported positive for human coronaviruses

Assay	No. of positive results/no. of samples			
	hCoV-229E	hCoV-NL63	hCoV-OC43	hCoV-HKU1
Real-time RT-PCR	3/8	8/12	11/13	4/6
Universal coronavirus assay	3/8	7/12	11/13	5/6

other laboratories to contain human coronaviruses by different real-time PCR protocols. Coronavirus types were determined either by sequencing or by separate, virus-specific real-time RT-PCRs (6). Because some of the samples had been stored for a long time, the material was retested in parallel with specific real-time RT-PCRs for hCoV-229E, -OC43, -HKU1, and -NL63 (6, 8). As shown in Table 2, the sensitivity of the universal coronavirus RT-PCR/LCD array was comparable to that of individual virus-specific real-time RT-PCRs. All PCR products were analyzed on LCD arrays and sequenced. All typing results were correct at the group and strain levels.

Though the genetic diversity of coronaviruses is extraordinarily high, this assay provides a simple method of detection and strain identification, obviating the need for sequencing. Its appropriateness on the clinical level has been proven by testing a large panel of virus strains as well as a sufficient number of original patient samples. Sensitivity in clinical samples ranged around 100 copies of RNA per throat swab, which is equivalent to the sensitivities of diagnostic assays which are targeted to one specific virus only, including highly optimized commercial kits (3). It is thus a suitable tool for coronavirus detection, even though enhanced anticontamination measures have to be followed to adapt the nested PCR formulation. Due to the generic features of the LCD array technology, it should be applicable to many other fields in clinical microbiology, e.g., for detecting whole virus families or for differentiating bacteria and fungi. Two examples, for genotyping *Mycobacterium tuberculosis* drug resistance (1) and for subtyping of human papilloma virus in squamous-cell cancer tissue (14), have recently been described. To our knowledge, this is the first application of LCD array technology to a whole viral genus. An expansion of this practical and affordable technology can be expected.

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