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Comprehensive Detection and Identification of Seven Animal Coronaviruses and Human Respiratory Coronavirus 229E with a Microarray Hybridization Assay

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Key Words
Coronavirus · Microarray hybridization · Coronavirus detection

Abstract
Based on microarray hybridization, a diagnostic test for coronavirus infection was developed using eight coronavirus strains: canine coronavirus (CCoV), feline infectious peritonitis virus (FIPV), feline coronavirus (FCoV), bovine coronavirus (BCoV), porcine respiratory coronavirus (PRCoV), turkey enteritis coronavirus (TCoV), transmissible gastroenteritis virus (TGEV), and human respiratory coronavirus (HRCoV). Up to 104 cDNA clones of eight viruses were obtained by reverse transcription PCR with different pairs of primers designed for each virus and a pair of universal primers designed for the RNA polymerase gene of coronavirus. Total RNAs extracted from virus were reverse transcribed, followed by multi-PCR amplification and labeled with Cy3-dCTP. All labeled cDNAs and prepared gene chips were subjected to specific hybridization. The results showed that extensive cross-reaction existed between CCoV, FCoV, FIPV, TGEV and PRCoV, while there was no cross-reaction between BCoV, TCoV and HRCoV. The ultimate specific gene chip was developed with DNA fragments reamplified from the chosen recombinant plasmids without cross-reaction between different coronaviruses. The hybridization results showed that this gene chip could specifically identify and distinguish the eight coronaviruses and the sensitivity of the chip may be 1,000× more sensitive than PCR, indicating that it can be used for the diagnosis of eight coronavirus infections at the same time.

Introduction

Coronaviruses infect a number of different vertebrate species and cause economically important diseases in people, pets, livestock and poultry [1]. Especially with the explosion of SARS in 2003 in several countries, coronavirus research has been becoming more ravishing [2, 3]. These coronaviruses are classified into three groups. One tightly clustered subgroup based on phylogenetic analysis and antigenic cross-reactivity contains canine coronavirus (CCoV), transmissible gastroenteritis virus (TGEV) and feline coronavirus (FCoVs) [4, 5]. In fact, all of these viruses are antigenically so similar that they may be regarded as 'host range mutants' rather than as separate species [6–9]. Coronaviruses are positive-stranded RNA viruses with exceptionally large genome sizes (up to 31 kb). Reverse transcription-PCR (RT-PCR) is widely used for virus identification [10–12]. However, PCR cannot

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distinguish several viruses at the same time from one sample and a positive amplification can be verified only by subsequent assays to elaborate sequence information. By overcoming this limitation, microarrays and biosensors have become valuable tools for viral discovery, detection, and genotyping [13–16]. Microarrays that contain several thousand different DNA sequences (probes) can theoretically identify several thousand different organisms at one time and exhibit more sensitivity and specificity than those tests which employ a single target sequence. However, the high conservation of the coronaviruses represents a design challenge and a main hindrance for the identification. It is only when coronavirus microarrays can provide information for a wide range of viral strains and have no cross-reaction between different coronaviruses that they are then usable and practical for coronavirus detection.

For this purpose, one coronavirus gene chip was developed by using 104 cDNA clones obtained by RT-PCR technique with different primer pairs (4–15 pairs) designed for TCoV, CCoV, FCoV, FIPV, TGEV, PRCoV, BCoV and HCoV, and a pair of universal primers designed for the RNA polymerase gene of coronavirus. Through two times specific hybridization, cross-reaction clones were deserted and DNA fragments reamplified from recombinant plasmids without cross-reaction between different coronaviruses were chosen to produce the ultimate specific gene chip.

Materials and Methods

Virus and Cells

Canine coronavirus (TN449 strain, ATCC No. VR-2068), feline infectious peritonitis virus (WSU 79-1146 strain, ATCC No. VR-2128) and feline coronavirus WSU79-1683 strain, ATCC No. VR-989) were propagated in a monolayer of A72 cells. Porcine respiratory coronavirus (AR310 strain, ATCC No. VR-2384) and transmissible gastroenteritis virus (Purdue strain, kept in our laboratory) were propagated in a monolayer of ST cells. Bovine coronavirus (unknown strain, ATCC No. VR-874) was propagated in a monolayer of MDBK cells. Human coronavirus (229E strain, ATCC No. VR-740) was propagated in a monolayer of MRC-5 cells. Turkey enteritis coronavirus (Minnesota strain, ATCC No. VR-911) was an original stock virus. Above eight viruses and cells were all initially obtained from American Type Culture Collection (ATCC).

Primers

Primers for RT-PCR were selected using Primer Designer (versions 1.01 and 2.01, Scientific and Educational Software, Durham, N.C., USA). Different (4–15) pairs of primers designed for each virus and a pair of universal primers designed for the RNA polymerase gene of coronavirus were used to amplify TGEV, PRCoV, CCoV, FCoV, FIPV, BCoV, TCoV and HRCoV. Sequence analysis and alignments were done with GeneWorks version 2.5.1 (Intelligenetics, Mountain View, Calif., USA). The names and sequences of primers for all viruses used have been listed in table 1.

Construction of cDNA Clones of Eight Viruses

All 104 clones were produced by our laboratory. *Escherichia coli* TGI was offered by Shanghai Veterinary Institute of CAAS and pGEM-T-Easy vector bought from Promaga Co. Viral sequence data were obtained from the GenBank database. A pair of universal primers designed for the RNA polymerase gene of coronavirus and different (4–15) pairs of primers designed for each virus were used. Primers were selected to be exclusive to a given virus, as judged by pairwise BLASTN search. The total RNAs were extracted with a QIAamp Viral RNA Mini Kit (Qiagen, Germany) from TCoV stock virus and CCoV, FCoV, FIPV, TGEV, PRCoV, BCoV and HRCoV purified by sucrose density gradient centrifugation were reverse transcribed (M-MLV Rtase cDNA synthesis kit, Takara Biotechnology (Dalian) Co. Ltd, China) and PCR-amplified with the above primers. The PCR products were purified and then linked with pGEM-T-Easy vector and transfected into *E. coli* TGI [17].

Amplification and Recovery of Probe Fragments

The PCR system was used to amplify probe fragments. The reactions were carried out in a final volume of 100 μl containing 2 μl of each primer (10 pmol), 10× buffer 10 μl, dNTP 8 μl, Taq plus 2 μl, dH_2O 71 μl and 5 μl plasmid template prepared by boiling lysis from different positive clones store at –70°. The amplification reaction was carried out in a DNA Thermal Cycler (PerkinElmer Cetus, USA) for 30 cycles after 94° for 5 min with denaturation at 94° for 45 s, annealing at 52° for 45 s and polymerization at 72° for 90 s. A final extension at 72° for 10 min was carried out before holding the samples at 4°. The amplification reaction of universal primers designed for the RNA polymerase gene was 5 cycles after 94° for 5 min with denaturation at 94° for 45 s, annealing at 40° for 45 s and polymerization at 72° for 60 s, then 30 cycles with denaturation at 94° for 45 s, annealing at 50° for 60 s, polymerization at 72° for 60 s and a final extension at 72° for 10 min. The amplified product was recovered using PCR Kleen™ spin columns (Bio-Rad Laboratories, Inc., USA). The PCR product was cleaned with a Qiagen QIAquick PCR purification kit. These fragments were designed to have similar annealing stabilities.

Preparation of Probes and Spotting on Chips

The concentrations of the above probe fragments were determined by OD_{260} and OD_{280}, then dried and suspended in 300 ng/μl with 50% DMSO. Spotting was completed using a Bio-Rad printer on slides coated with amino saline. The slides were UV cross-linked at 60 mJ for 25 min and baked for 2 h at 80°, bathed for 3 min, immediately put into cold absolute ethanol or a refrigerator for 2 min, centrifuged 8 min at 1,000 rpm, dried, a prehybridization solution was added on the slide, prehybridized at 42° for 1 h, then rinsed twice with distilled water, centrifuged at 1,000 rpm, and stored in a dry dust-free environment. The same virus probes were a designed neighborhood and some QC (quality control, 10 μM HEX), BC (blank control, 50% DMSO), NC (negative control, 127 (SARS)) and EC (PCR product of HLA) were arrayed (see table 2).

Table 1. Names and sequence of primers for all viruses

Primer sets	Sequence of primer	Primer sets	Sequence of primer
FCoV1	Fw primer 5′-TGATTGTGCTCGTAACTTGC-3′ Rw primer 5′-CTGGTGAAGGTGTTATAGTC-3′	FCoV2	Fw primer 5′-TAACACCTTCACCAGCAACC-3′ Rw primer 5′-GAGCAAAGACATTAGTGGCG-3′
FCoV3	Fw primer 5′-GGAGGTTACATACCTGATGG-3′ Rw primer 5′-AGTACATAACAGTACCGTGG-3′	FCoV4	Fw primer 5′-TGCTATTAGTAAGTGGGGCC-3′ Rw primer 5′-ATAACCGCTACGCTTCATAC-3′
FCoV5	Fw primer 5′-CATTACACTACCAATGCAGG-3′ Rw primer 5′-ATACCAACACCAGTTCTACC-3′	FCoV6	Fw primer 5′-ATGGTGTCATCTATTCTGTG-3′ Rw primer 5′-CCTGAAGGTATTCGACTTGC-3′
FCoV7	Fw primer 5′-CACCACACCAGTATCAATAG-3′ Rw primer 5′-CTACACCAGGTAACACCATG-3′	FCoV8	Fw primer 5′-TCTCTTGCAGGTGGTATAAC-3′ Rw primer 5′-GCTTGTCTGGTTAGAGTCTG-3′
FCoV9	Fw primer 5′-GGTTAGGGCTAGTAGACAAC-3′ Rw primer 5′-TGACAAACAACACATCGCAC-3′	FCoV10	Fw primer 5′-GACTGTACCTGAATTGACAC-3′ Rw primer 5′-CTCTAGCAGAAACAACTGTC-3′
FCoV11	Fw primer 5′-CCATAACTATGACGTTCCCC-3′ Rw primer 5′-TGCCAAATAAGATCACCTCC-3′	FCoV12	Fw primer 5′-TTAGCTGGTTCGTGTATGGC-3′ Rw primer 5′-TACATAGTAAGCCCATCCCG-3′
FCoV13	Fw primer 5′-CTCAACAGAAGCACGTACTG-3′ Rw primer 5′-AGACACCGTCAATCTTAGCC-3′	FCoV14	Fw primer 5′-AACATCACTTGGCACTCGTG-3′ Rw primer 5′-GCTGAACTACTTCTAGCACC-3′
FCoV15	Fw primer 5′-AGTGATCTCGTTGCCAATGG-3′ Rw primer 5′-CATGCGTTTAGTTCGTTACC-3′	TGEV1	Fw primer 5′-GTGGTTTTGGTCGTAATGCC-3′ Rw primer 5′-TGGTGGTAGTAGGTGGTGAG-3′
TGEV2	Fw primer 5′-AATTGGGGTAGTGAGTGCAG-3′ Rw primer 5′-CCAACGTGGAGCTATTAGTT-3′	TGEV3	Fw primer 5′-TACAATCAGGTAAGGGTGCC-3′ Rw primer 5′-ACGTCACTATCACCAGTGGT-3′
TGEV4	Fw primer 5′-ACCACTGGTGATAGTGACGT-3′ Rw primer 5′-GGTCAGAACGAATACAGTAC-3′	TGEV5	Fw primer 5′-ACAACATAGTGGGTGTACCG-3′ Rw primer 5′-GCCACGAGTCCTATCATTTG-3′
TGEV6	Fw primer 5′-GTGTTCCATCTGTGTCTAGC-3′ Rw primer 5′-CAACCTGTGTGTCATCAAAC-3′	TGEV7	Fw primer 5′-GGCGATCTTATTTGGCATCT-3′ Rw primer 5′-CCACCAAGACTTAGTCCTTC-3′
TGEV8	Fw primer 5′-GGGAATTTGTACGCTGAAGG-3′ Rw primer 5′-TCTTCCGACCACGGGAATTG-3′	TGEV9	Fw primer 5′-GACACTTTTAGGACCTATGC-3′ Rw primer 5′-TAAGCCACGTATTGCTATGC-3′
TGEV10	Fw primer 5′-TTTTCACAGGAGCCCGTAGT-3′ Rw primer 5′-AGGCATCGTAAGCATGTTGC-3′	BCoV1	Fw primer 5′-CCCCCGTACTGTTATTTTCG-3′ Rw primer 5′-TGGTCTAAGCATCATGCAGC-3′
BCoV2	Fw primer 5′-GGCTTTTGCTGTTATAGGAG-3′ Rw primer 5′-CTTGGTATTTTTGACCTTAGC-3′	BCoV3	Fw primer 5′-GCGTAGAACTATGGCATTGG-3′ Rw primer 5′-ATACGTCGGTAAACATCTGC-3′
BCoV4	Fw primer 5′-TTAATGATAAGTCGGTGCCC-3′ Rw primer 5′-CATCCAATTTACACGGACAG-3′	BCoV5	Fw primer R 5′-GATGGGTCTTTGTGTGTAGG-3′ Rw primer 5′-ACACCTATCCCCTTGTAAAC-3′
BCoV6	Fw primer 5′-TGTTTACAAGGGGATAGGTG-3′ Rw primer 5′-AAGAGTTAGCATGAAAGGCC-3′	BCoV7	Fw primer 5′-GCCTTTCATGCTAACTCTTC-3′ Rw primer 5′-TACAAACCACCTACAGGTTC-3′
BCoV8	Fw primer 5′-ACCTGTAGGTGGTTTGTATG-3′ Rw primer 5′-CCGACATCAGATAACTTTAC-3′	BCoV9	Fw primer 5′-TAATTGTACTGGAGGTGCCG-3′ Rw primer 5′-TCTATCTGAGCTTGCGCTTC-3′
BCoV10	Fw primer 5′-GCGTCTTACCGCTCTTAATG-3′ Rw primer 5′-ACTACCAGTGAACATCCAAG-3′	BCoV11	Fw primer 5′-ATGAATAGGTTACAGGAGGC-3′ Rw primer 5′-CCACTAAACAGCAGGCATTG-3′
BCoV12	Fw primer 5′-ACTGCCATCAACCCAAAAGG-3′ Rw primer 5′-TCTGTACCAGTACCCCTTAG-3′	BCoV13	Fw primer 5′-TACTATCTTGGAACAGGACC-3′ Rw primer 5′-GCTTAGTTACTTGCTGTGGC-3′
BCoV14	Fw primer 5′-CCCCAATAAACAATGCACTG-3′ Rw primer 5′-GTGGTTTTGGACTCATATTC-3′	BCoV15	Fw primer 5′-AATATAAGTGTTGCAGCGCC-3′ Rw primer 5′-TTAACATGCTGGCTCTTCCC-3′
CCoV1	Fw primer 5′-ACGTGGTCGTTCCAATTCTC-3′ Rw primer 5′-GTCCCTTGATTGGTTCACTTC-3′	CCoV2	Fw primer 5′-GCTGCACTCAAAAAGTTAGG-3′ Rw primer 5′-TACAACCTCTTGCTCTACCC-3′
CCoV3	Fw primer 5′-AGGAACATTACCACCCAGTG-3′ Rw primer 5′-AACGAGACCTACTTCACTTG-3′	CCoV4	Fw primer 5′-GGTAGAACTGGTGTTGGTAT-3′ Rw primer 5′-CCACGAGTCCTCTCATTTGT-3′
CCoV5	Fw primer 5′-TGATTGTGCTCGTAACTTGC-3′ Rw primer 5′-TCATCATTCCACTCAAGCCC-3′	CCoV6	Fw primer 5′-CTACATGGCAACACAGTGCT-3′ Rw primer 5′-GCCGAATGGAATTTCACCAT-3′
CCoV7	Fw primer 5′-AGAGCTGCACCGTTTATGAG-3′ Rw primer 5′-TATGACAGTCAATGCCCGAG-3′	FIPV1	Fw primer 5′-GGTTAGGGCTAGTAGACAAC-3′ Rw primer 5′-AACAACACATCACACCCTTC-3′
FIPV2	Fw primer 5′-TGTCAACGCGACTGTAATTG-3′ Rw primer 5′-CAACAACTTCCTAAACAACC-3′	FIPV3	Fw primer 5′-TTATGGTGAACGCTACTGTG-3′ Rw primer 5′-CATCATCCAAAGTGCAAACG-3′
FIPV4	Fw primer 5′-GAAGA(G)ACCAAATCATGGTGG-3′ Rw primer 5′-AAGGTTCATCTCCCCAGTTG-3′	FIPV5	Fw primer 5′-TGATGGA(T)GTCTTCTGGGTTG-3′ Rw primer 5′-TTCCAGGTGTGTTTGTTGGC-3′
FIPV6	Fw primer 5′-AGTGATCTCGTTGCCAATGG-3′ Rw primer 5′-TTACAAGTACAGCATGGACG-3′	FIPV7	Fw primer 5′-ACTGCGAGTGATCTTTCTAG-3′ Rw primer 5′-TTTTGTTTTGGCACAGCAC-3′

Table 1 (continued)

Primer sets	Sequence of primer	Primer sets	Sequence of primer
FIPV8	Fw primer 5′-AGATTAGTTGGTGCTGTGCC-3′ Rw primer 5′-ATAGGGTTGCTTGTACCTCC-3′	FIPV9	Fw primer 5′-TGACAGGGATTTTCAACACC-3′ Rw primer R 5′-ACAATCACTAGATCCAGACG-3′
FIPV10	Fw primer 5′-ACATTACAACACACCAGGTC-3′ Rw primer 5′-GTTGAAAATCCCTGTCATGG-3′	FIPV11	Fw primer 5′-GGTTGAGATGATTGATGAGG-3′ Rw primer 5′-CCTGGTGTGTTGTAATGTAG-3′
PRCoV1	Fw primer 5′-GTGGTTTTGGTT(C)G(A)TAATGCC-3′ Rw primer 5′-CCTTCTTCAAAGCTAGGGAC-3′	PRCoV2	Fw primer 5′-CCGTGGATGT(C)TGTACTTGAC-3′ Rw primer 5′-GGTCTTGTTGTGTATGATGC-3′
PRCoV3	Fw primer 5′-GCATCATACACAACAAGACC-3′ Rw primer 5′-TTCTCTTAAACGGTGCAGCT-3′	PRCoV4	Fw primer 5′-GATTCGCCTACTTAGCATAC-3′ Rw primer 5′-CCTGAGAAAAGGCTGCATTG-3′
TCoV1	Fw primer 5′-CAGATGCTCAGGTTGATAGG-3′ Rw primer 5′-TGATGCCACTTCCAGTCTTG-3′	TCoV2	Fw primer 5′-ACCCTTTCAACCAGCATTGC-3′ Rw primer 5′-CCACACTTACTCATTAGCGG-3′
TCoV3	Fw primer 5′-TCAGTGGCTTGCTAAGTGTG-3′ Rw primer 5′-CCTTCGTAGTAGACTTTTCC-3′	TCoV4	Fw primer 5′-AGTGTAGCAACAGGAGGAAG-3′ Rw primer 5′-CAAAATCGCTCGCCTACTAC-3′
TCoV5	Fw primer 5′-GTAGTAGGCGAGCGATTTTG-3′ Rw primer 5′-AACTTTAGGTGGCTTTGGTC-3′	TCoV6	Fw primer 5′-ACCAAAGCCACCTAAAGTTG-3′ Rw primer 5′-GAGGAATAAAGTCCCAACGG-3′
TCoV7	Fw primer 5′-TTCCTCTGAATCGTGGTAGG-3′ Rw primer 5′-AACACGCCCATCCGTAATAC-3′	TCoV8	Fw primer 5′-ATGGGCGTGTTACAGCAATG-3′ Rw prime 5′-CTACCTCATCATCCTGCTTC-3′
TCoV9	Fw primer 5′-GAAGCAGGATGATGAGGTAG-3′ Rw primer 5′-ATGCCTATCTGCCTTAACTC-3′	HRCoV1	Fw primer 5′-CGGGATCCGAAGGTGTCGTCTGGGTTGC Rw primer 5′-CCGCTCGAGCTGCACTTCCAAAGTTGTG
HRCoV2	Fw primer 5′-CGGGATCCATGTTTGTTTTGCTTGTTG Rw primer 5′-CCGCTCGAGGAAGCTAACGCAACAGTAC	HRCoV3	Fw primer 5′-CGGGATCCACTGGCGTCCCACAACCTG Rw prime 5′-CCGCTCGAGGTGAGCATCTCACTAACATC
HRCoV4	Fw primer 5′-CGGGATCCAGCAAACTTGTTACTTCTGG Rw primer 5′-CCGCTCGAGGTCTGGAAGCACGAACTTC	HRCoV5	Fw primer 5′-CGGGATCCTGTGGAAATGGCACTCACA Rw primer 5′-CGCTCGAGCAACGTCGTAATAAGGAAG
HRCoV6	Fw primer 5′-CGGGATCCCAGAACAATGAAATAATGCC Rw primer 5′-CCGCTCGAGGTCCGGTCACATGTACAGC	HRCoV7	Fw primer 5′-CGGGATCCCACAAAAGGGTGATGCTGC Rw primer 5′-CCGCTCGAGCATCACCAGAAGTTGTACCACC
HRCoV8	Fw primer 5′-CGGGATCCCTATGATGATTCTTTCTGATG Rw primer 5′-CCGCTCGAGTGTGTTACACACATATGGTG	HRCoV9	Fw primer 5′-CGGGATCCGACTACAAGCTTGCTAATG Rw primer 5′-CCGCTCGAGGCACAGAGCGAATCAACAGC
HRCoV10	Fw primer 5′-CGGGATCCGACATTGTTGTCGTGGATG Rw primer 5′-CCGCTCGAGCTTTGCTCTAGTAATGGCAAC	HRCoV11	Fw primer 5′-CGGGATCCGATGTGGAAGGTGCACATG Rw primer 5′-CCGCTCGAGCATATCCACATTACAATTCC
HRCoV12	Fw primer 5′-CGGGATCCGGTGCTGATGGTGAATTACC Rw primer 5′-CCGCTCGAGTCACCATACACAACGTGCTC	HRCoV13	Fw primer 5′-CGGGATCCATCCTCAGTTGCAGAGTGC Rw primer 5′-CCGCTCGAGCCATCTATAAAAGGTCCTTG
HRCoV14	Fw primer 5′-CGGGATCCATGTCAAATGACAATTGTAC Rw primer 5′-CCGCTCGAGGAAAAAATGAAGCAATCTTTC	HRCoV15	Fw primer 5′-CGGGATCCGATCTTAATGCTAATATGTC Rw primer 5′-CCGCTCGAGGCCAAACTTAGCATAATGCC
UP	FW primer 5′-ACTCA(A/G)(A/T)T(A/G)AAT(T/C)TNAAATA(T/C)GC Rw primer 5′-TCACA(C/T)TT(A/T)GGATA(G/A)TCCCA		

Table 2. Array arrangement of primary coronaviruses chip

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A	QC	QC	BCoV1	BCoV1	UP1	UP1	QC	QC	QC	QC	FCoV1	FCoV1	HRCoV1	HRCoV1	QC	QC
B	NC1	NC1	BCoV2	BCoV2	UP2	UP2	FIPV1	FIPV1	PRCoV1	PRCoV1	FCoV2	FCoV2	HRCoV2	HRCoV2	NC2	NC2
C	TGEV1	TGEV1	BCoV3	BCoV3	UP3	UP3	FIPV2	FIPV2	PRCoV2	PRCoV2	FCoV3	FCoV3	HRCoV3	HRCoV3	BC	BC
D	TGEV2	TGEV2	BCoV4	BCoV4	UP4	UP4	FIPV3	FIPV3	PRCoV3	PRCoV3	FCoV4	FCoV4	HRCoV4	HRCoV4	TCoV1	TCoV1
E	TGEV3	TGEV3	BCoV5	BCoV5	UP5	UP5	FIPV4	FIPV4	PRCoV4	PRCoV4	FCoV5	FCoV5	HRCoV5	HRCoV5	TCoV2	TCoV2
F	TGEV4	TGEV4	BCoV6	BCoV6	UP6	UP6	FIPV5	FIPV5	CCoV1	CCoV1	FCoV6	FCoV6	HRCoV6	HRCoV6	TCoV3	TCoV3
G	TGEV5	TGEV5	BCoV7	BCoV7	UP7	UP7	FIPV6	FIPV6	CCoV2	CCoV2	FCoV7	FCoV7	HRCoV7	HRCoV7	TCoV4	TCoV4
H	EC	EC	BCoV8	BCoV8	UP8	UP8	BC	BC	CCoV3	CCoV3	FCoV8	FCoV8	HRCoV8	HRCoV8	EC	EC
I	TGEV6	TGEV6	BCoV9	BCoV9	UP9	UP9	FIPV7	FIPV7	CCoV4	CCoV4	FCoV9	FCoV9	HRCoV9	HRCoV9	TCoV5	TCoV5
J	TGEV7	TGEV7	BCoV10	BCoV10	UP10	UP10	FIPV8	FIPV8	CCoV5	CCoV5	FCoV10	FCoV10	HRCoV10	HRCoV10	TCoV6	TCoV6
K	TGEV8	TGEV8	BCoV11	BCoV11	UP11	UP11	FIPV9	FIPV9	CCoV6	CCoV6	FCoV11	FCoV11	HRCoV11	HRCoV11	TCoV7	TCoV7
L	TGEV9	TGEV9	BCoV12	BCoV12	UP12	UP12	FIPV10	FIPV10	CCoV7	CCoV7	FCoV12	FCoV12	HRCoV12	HRCoV12	TCoV8	TCoV8
M	TGEV10	TGEV10	BCoV13	BCoV13	UP13	UP13	FIPV11	FIPV11	CCoV1	CCoV1	FCoV13	FCoV13	HRCoV13	HRCoV13	TCoV9	TCoV9
N	NC3	NC3	BCoV14	BCoV14	UP14	UP14	UP16	UP16	CCoV2	CCoV2	FCoV14	FCoV14	HRCoV14	HRCoV14	NC4	NC4
O	QC	QC	BCoV15	BCoV15	UP15	UP15	QC	QC	QC	QC	FCoV15	FCoV15	HRCoV15	HRCoV15	QC	QC

Table 3. Hybridization and amplification of multi-PCR of different coronavirus

Group	Primer sets	Cross-reaction gene clones of other viruses
1	BCoV1, BCoV2, BCoV3, BCoV4, BCoV5	NO
2	BCoV6, BCoV7, BCoV8, BCoV9, BCoV10	NO
3	BCoV11, BCoV12, BCoV13, BCoV14, BCoV15	NO
4	TCoV1, TCoV2, TCoV3, TCoV4, TCoV5	NO
5	TCoV6, TCoV7, TCoV8, TCoV9	NO
6	CCoV1, CCoV2, CCoV3, CCoV4, CCoV5	TGEV3, TGEV4, TGEV5, TGEV6, TGEV8, FIPV9, FCoV2, FCoV4, FCoV9, FCoV10, FCoV11
7	CCoV6, CCoV7	FIPV3, FIPV4, TGEV4, TGEV5, TGEV7, TGEV8, TGEV9, TGEV10, FCoV2, FCoV3, FCoV9
8	FIPV1, FIPV2, FIPV3, FIPV4, FIPV5	CCoV1, CCoV2, TGEV7, TGEV8, FCoV7, FCoV9, FCoV10, FCoV11, FCoV12
9	FIPV6, FIPV7, FIPV8, FIPV9, FIPV10, FIPV11	FCoV12
10	FCoV1, FCoV2, FCoV3, FCoV4, FCoV5	CCoV6
11	FCoV6, FCoV7, FCoV8, FCoV9, FCoV10	TGEV7, TGEV10, FIPV1, FIPV3, FIPV4
12	FCoV11, FCoV12, FCoV13, FCoV14, FCoV15	TGEV6, TGEV8, TGEV9, TGEV10, CCoV1, CCoV2, CCoV7, FIPV4, FIPV5, FIPV6, FIPV7, FIPV8
13	TGEV1, TGEV2, TGEV3, TGEV4, TGEV5	CCoV3, CCoV4, FIPV3, FIPV4
14	TGEV6, TGEV7, TGEV8, TGEV9, TGEV10	FIPV4, CCoV1, CCoV2, CCoV7, FCoV10
15	HRCoV1, HRCoV6, HRCoV8, HRCoV13, HRCoV15	UP2, UP7
16	HRCoV2, HRCoV3, HRCoV4, HRCoV5, HRCoV14	NO
17	HRCoV7, HRCoV9, HRCoV10, HRCoV11, HRCoV12	NO
18	PRCoV1, PRCoV2, PRCoV3, PRCoV4	TGEV9, CCoV7

NO = No cross-reaction was observed with gene clones from other viruses.

Samples Processing and Hybridizing

Viral RNAs were extracted from the cryolysate of cell cultures infected with the examined coronavirus strains using a QIAamp Viral RNA Mini Kit (Qiagen, Germany) and reverse transcribed with M-MLV Rtase cDNA synthesis kit (Takara Biotechnology (Dalian) Co. Ltd., China), with oligo-dT as primer, then each cDNA was multi-PCR amplified with different primer sets (see table 3; each virus cDNA with different primer sets, such as CCoV with CCoV1, CCoV2, CCoV3, CCoV4, CCoV5 primer mixture, and CCoV6, CCoV7 primer mixture to amplify separately) and labeled with Cy3-dCTP (PerkinElmer). The labeled fluorescent DNAs were denatured at 96° for 5 min, then placed on a slide and put into a hybridization chamber oven at 42° for 2–3 h. Different multi-PCR samples with Cy3-dCTP were hybridized with the chip. After washing, the slides were scanned on a GenePix 4000B array scanner (Packard Biochip Technologies, Mass., USA) (λex543 nm/λex570 nm) at 10 μm resolution and analyzed with GenePix Pro array analysis software.

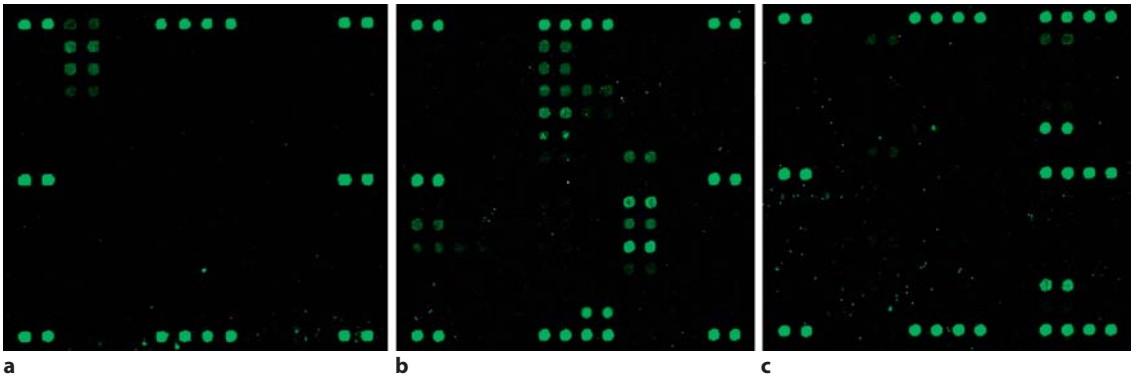
Sensitivity and Application of the Coronavirus Chip

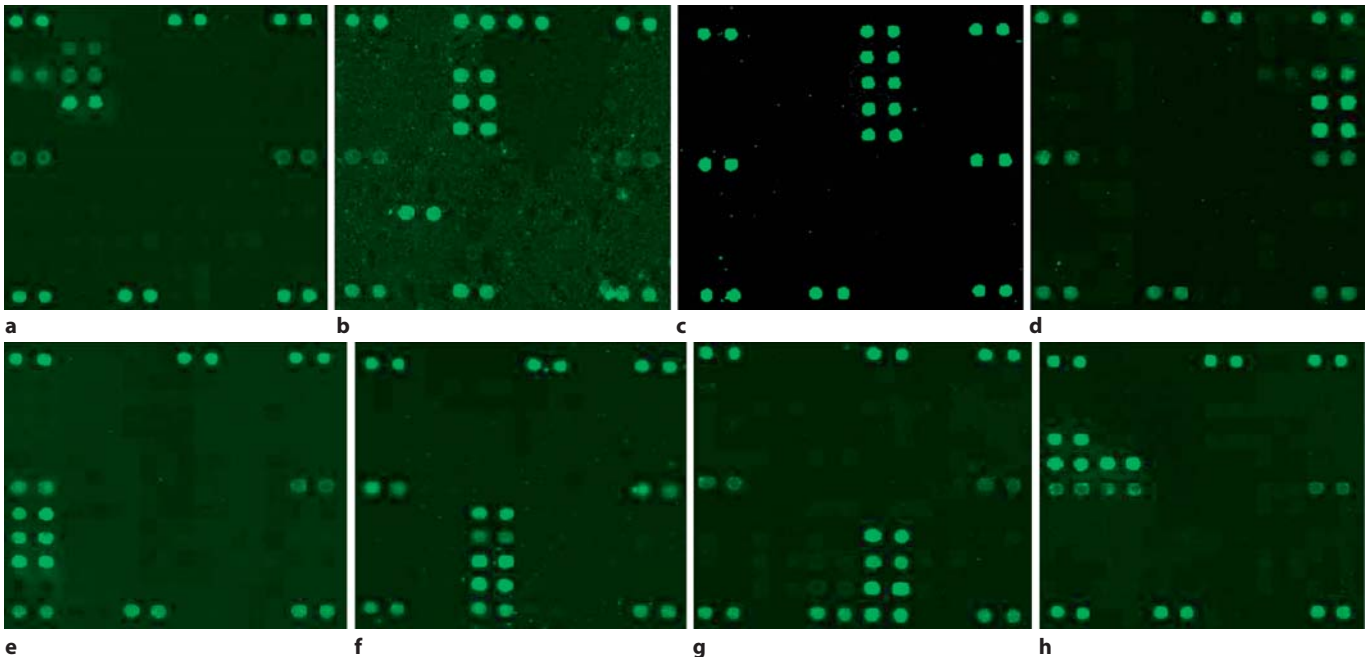
The 10-fold serial BCoV cDNA dilution was detected by multi-PCR and the coronavirus gene chip using the same primer set. The results were shown by electrophoresis in a 1% agarose gel for 1 h at 90 V, visualized by ethidium bromide and a slide reader. Muscle, heart, liver, spleen, lung and kidney were gathered from the pigs infected with live TGEV, PRCoV, canines infected with live CCoV, felines infected with live FCoV, FIPV, and cattle infected with live BCoV, which were detected with RT-PCR, cell culture and microassay, while 10 canine hearts (7 positive and 3 negative to CCoV analyzed by PCR), 5 feline lungs (3 positive and 2 negative to FIPV analyzed by PCR) and 6 pig lungs (5 positive and 1 negative to PRCoV analyzed by PCR) were collected from clinical animals and detected with microassay [12, 13, 15].

Results

Hybridizing to Different Multi-PCR Samples with the Primary Coronavirus Chip

Different multi-PCR samples with Cy3-dCTP were hybridized with the chip. The slides were scanned by a ScanArray 4000c laser scanning system (Packard Biochip Technologies) with a laser for Cy3 dye (λex543 nm/λex570 nm) at 10 μm resolution. Different intense signals appeared. From the hybridization, extensive cross-reactions between CCoV, FCoV, FIPV, TGEV and PRCoV were found, while there were none between BCoV, TCoV and HRCoV. The results are shown in table 3 and figure 1a–c as samples.





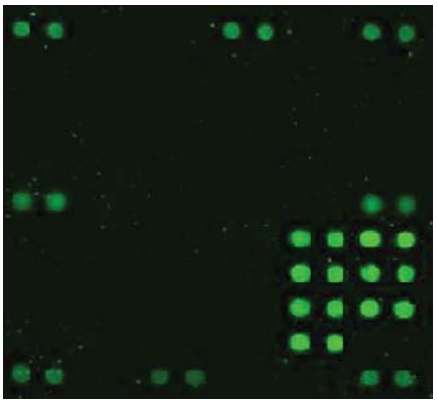


Fig. 1. Detection of gene chip by (**a**) BCoV multi-PCR product (table 3, group 1), (**b**) FIPV multi-PCR product (table 3, group 8), and (**c**) BCoV multi-PCR product (table 3, group 15).
Fig. 2. Hybridization of coronavirus gene chip with (**a**) FIPV, (**b**) BCoV, (**c**) HRCoV, (**d**) TCoV, (**e**) PRCoV, (**f**) CCoV, (**g**) FCoV, and (**h**) TGEV multi-PCR probes.
Fig. 3. Hybridization of coronavirus gene chip with universal primer PCR probes.

Table 4. Array arrangement of ultimate coronaviruses chip

QC	QC	BC	BC	BCoV6	BCoV6	QC	QC	TCoV1	TCoV1	QC	QC
NC1	NC1	FIPV7	FIPV7	BCoV7	BCoV7	HRCoV3	HRCoV3	TCoV2	TCoV2	NC2	NC2
FIPV2	FIPV2	FIPV8	FIPV8	BCoV8	BCoV8	HRCoV4	HRCoV4	TCoV3	TCoV3	TCoV6	TCoV6
TGEV3	TGEV3	FIPV9	FIPV9	BCoV9	BCoV9	HRCoV5	HRCoV5	TCoV5	TCoV5	TCoV7	TCoV7
TGEV4	TGEV4	TGEV5	TGEV5	BCoV10	BCoV10	HRCoV12	HRCoV12	HRCoV13	HRCoV13	TCoV9	TCoV9
EC	EC	TGEV6	TGEV6	BC	BC	HRCoV6	HRCoV6	HRCoV15	HRCoV15	EC	EC
PRCoV1	PRCoV1	BCoV2	BCoV2	CCoV1	CCoV1	HRCoV8	HRCoV8	UP1	UP1	UP10	UP10
PRCoV2	PRCoV2	BCoV3	BCoV3	CCoV2	CCoV2	FCoV6	FCoV6	UP3	UP3	UP12	UP12
PRCoV3	PRCoV3	BCoV12	BCoV12	CCoV5	CCoV5	FCoV7	FCoV7	UP6	UP6	UP14	UP14
NC3	NC3	BCoV13	BCoV13	CCoV7	CCoV7	FCoV8	FCoV8	UP8	UP8	NC4	NC4
QC	QC	BCoV14	BCoV14	QC	QC	FCoV9	FCoV9	BC	BC	QC	QC

QC = Quality control, 10 μM HEX; BC = blank control, 50% DMSO; NC = negative control, 127 (SARS); EC = PCR product of HLA.

Design and Verification of Ultimate Coronavirus Chip

The ultimate specific gene chip was developed with the DNA fragments reamplified from the chosen recombinant plasmids without cross-reaction between different coronaviruses, as shown in table 4. Different multi-PCR samples with Cy3-dCTP using specific primer sets were hybridized with the chip and scanned. Intense signals to the positive and control point and no signal to the negative and blank point were shown. Figure 2a–h shows obvious hybridization in the ultimate gene chip, with which several coronaviruses could be distinguished easily because of no cross-reaction between different coronaviruses.

Hybridization of Coronavirus Gene Chip with Universal Primer PCR Probes

The mixed cDNA of eight coronaviruses was PCR-amplified using a specific universal primer and labeled with Cy3-dCTP, then the PCR product was hybridized with the chip and scanned. The intense signal to the positive and control point and no signal to the negative and blank point, which can determine whether coronavirus exists, are shown in figure 3. It was suggested that this chip can diagnose at least these eight viruses and possibly more, but this still has to be proven.

Sensitivity and Application of the Coronavirus Chip

The 10-fold serial BCoV cDNA dilution was detected by multi-PCR and the coronavirus chip using the same primer sets of BCoV2, BCoV7, BCoV9, BCoV12, BCoV13 and BCoV14. The results showed that 10^{-2} diluted cDNA can be detected by multi-PCR and 10^{-5} diluted cDNA detected by the chip (shown in fig. 4a–h), indicating that the chip may be 1,000× more sensitive than PCR.

Table 5 shows that the sensitivity of cell culture detection was lower, while the microassay was higher and had comparable sensitivities with PCR. At the same time, the results obtained from field samples with microassay detection showed good concordance with PCR methods.

Discussion

Microarrays and biosensors have become valuable tools for viral discovery, detection, and genotyping, which have been commonly used in gene expressions, diagnosis of disease, discovery of new genes and drug screening [18–21]. Here we have constructed a coronavirus-specific DNA microarray. The assay was designed to be broadly reactive with the genome of many coronavirus species. It is demonstrated that it can detect eight coronaviruses including the HRCoV-229E which are well-recognized human pathogens.

In order to make a coronavirus microarray useful, it must provide information for a wide range of viral strains and not have a cross-reaction between different coronaviruses. We constructed 104 cDNA clones of eight viruses including different genes by using different (4–15) pairs of primers designed for TCoV, CCoV, FCoV, FIPV, TGEV, PRCoV, BCoV and HRCoV, and a pair of universal primers designed for the RNA polymerase gene of coronavirus. Probes were compared to the database to ensure each probe was unique to the respective virus. The probe fragment was then obtained through plasmid PCR using

Table 5. Results showing that the sensitivity of cell culture detection was lower, while the microassay was higher and had comparable sensitivities with PCR. At the same time, the results obtained from field samples with microassay detection showed good concordance with PCR methods

Virus infected	Animal n	Tissues	RT-PCR	Chip	Cell culture
CCoV	canine (2)	muscle	–	–	–
		heart	+	+	–
		liver	–	+	–
		spleen	–	–	–
		lung	–	+	–
		kidney	–	–	–
	canine (1)	muscle	–	–	–
		heart	+	+	–
		liver	–	–	–
		spleen	–	+	–
		lung	–	–	–
		kidney	–	–	–
	canine (2)	muscle	–	–	–
		heart	–		–
		liver	–	+	–
		spleen	–	+	–
		lung	–	–	–
		kidney	–	–	–
BCoV	cattle (2)	muscle	+	+	–
		heart	–	–	–
		liver	–	+	–
		spleen	–	–	–
		lung	–	–	–
		kidney	–	–	–
	cattle (2)	muscle	–	–	–
		heart	–	–	–
		liver	–	–	–
		spleen	–	–	–
		kidney	–	–	–
FIPV	feline (3)	muscle	–	+	–
		heart			
		liver	–	+	–
		spleen			
		lung	+	+	–
		kidney	+	+	–
	feline (2)	muscle	+	+	–
		heart	+	+	+
		liver	–	+	–
		spleen			
		lung	+	+	–
		kidney	–	+	–
FCoV	feline (3)	muscle	–	–	–
		heart	–	–	–
		liver	–	+	–
		lung	–	–	–
		kidney	–	+	–
	feline (3)	muscle	–	–	–
		heart	–	–	–
		liver	–	–	–
		spleen	–	–	–
		lung	–	+	–
		kidney	–	–	–
	feline (2)	muscle	–	–	–
		heart	–	–	–
		liver	–	–	–
		spleen	–	–	–
		lung	–	–	–
		kidney	–	–	–
TGEV	pig (3)	muscle	–	–	–
		heart	–	+	–
		liver	–	+	–
		spleen	–	+	–
		lung	–	–	–
		kidney	–	–	–
	pig (3)	muscle	–	–	–
		heart	–	–	–
		liver	–	–	–
		spleen	–	–	–
		lung	–	–	–
		kidney	–	–	–
PRCoV	pig (3)	muscle	–	–	–
		heart	–	–	–
		liver	–	+	–
		spleen	–	–	–
		lung	+	+	–
		kidney	–	–	–
	pig (2)	muscle	–	–	–
		heart	–	–	–
		liver	–	–	–
		spleen	–	–	–
		lung	–	+	–
		kidney	–	–	–

Detection of different tissues with RT-PCR, the chip and cell culture.

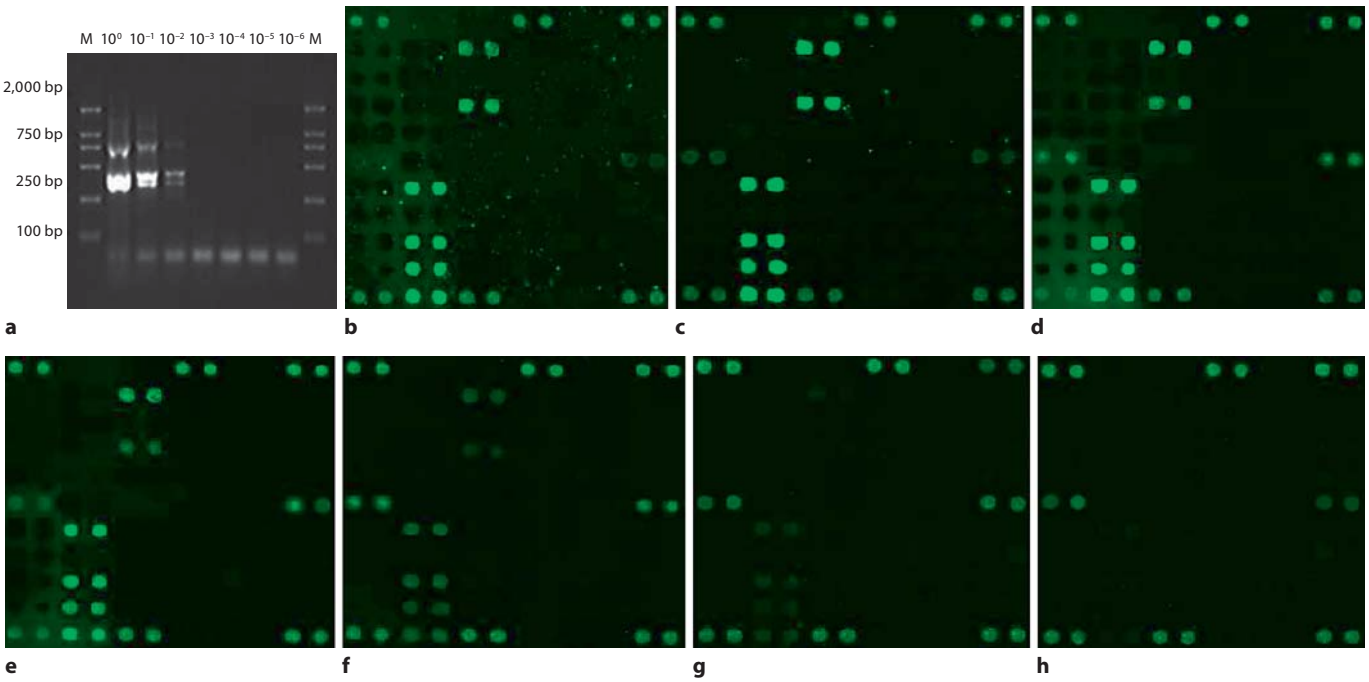


Fig. 4. a Detection of 10^{-0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} BCoV cDNA by multi-PCR. **b–h** Hybridization of coronavirus gene chip with (**b**) 10^{-0} BCoV, (**c**) 10^{-1} BCoV, (**d**) 10^{-2} BCoV, (**e**) 10^{-3} BCoV, (**f**) 10^{-4} BCoV, (**g**) 10^{-5} BCoV, and (**h**) 10^{-6} BCoV cDNA multi-PCR probes.

the same primers as clone construction, precipitated with ethanol and suspended in 300 ng/μl with 50% DMSO for spotting purposes. Viral RNAs were extracted from the cryolysate of cell cultures infected with the examined coronavirus strains, labeled with Cy3 fluorescent dyes during PCR with different primer pair sets, and hybridized to the primary gene chip. Extensive cross-reactions between CCoV, FCoV, FIPV, TGEV and PRCoV were found and these clones may exist in a similar site in genes. Through the screening of hybridization, the ultimate specific gene chip was developed with the DNA fragments reamplified from the chosen recombinant plasmids without cross-reaction between different coronaviruses.

In some studies, the sample cDNA was labeled during reverse transcription and then hybridized [18, 22]. In this study we tried to label the cDNAs of BCoV directly with Cy3-dCTP during reverse transcription and subjected them to specific hybridization, however no positive signal appeared. We think the amount of total vRNAs may be so low that the amount of cDNA labeled cannot meet the chip limit required. The amplification of sample cDNAs with PCR is therefore necessary to obtain satisfactory hybridization results. Secondly, the quality and concentration of probes is another important factor related with the signal intensity [23–25]. We obtained the probes by PCR and then purified and concentrated them to make certain that the OD_{260} was >0.1, the OD_{260}/OD_{280} was >1.4, and the concentration of probes reached 50 μg/ml.

In summary, a new DNA microarray technology is described exhibiting a useful diagnostic method for comprehensive detection of eight coronaviruses – it had a good correlation with PCR and is 1,000× more sensitive than PCR. It is expected to remain effective with possible mutants and to be of value when other new coronaviruses emerge. Because of the same PCR amplification and further Cy3 electrochemistry magnification, the chip method has a more than 1,000× sensitivity than PCR. As we just compare the sensitivity of BCoV detection using the gene chip and PCR methods with the same primer sets, in future we will compare other coronaviruses and different primer sets with these two methods in order to confirm the high sensitivity of DNA microarray technology.

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