

# Novel Rapid Immunochromatographic Test Based on an Enzyme Immunoassay for Detecting Nucleocapsid Antigen in SARS-Associated Coronavirus

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A novel severe acute respiratory syndrome-associated coronavirus (SARS-CoV) has been discovered. The detection of both antigens and antibodies in SARS-CoV from human specimens with suspected SARS plays an important role in preventing infection. We developed a novel rapid immunochromatographic test (RICT) based on the sandwich format enzyme immunoassay (EIA) with an all-in-one device for detecting the native nucleocapsid antigen (N-Ag) of SARS-CoV using monoclonal antibodies (MoAbs), which we produced by immunizing recombinant N-Ag to mice. RICT is a qualitative assay for respiratory aspirates and serum specimens. With this assay, a positive result can be judged subjectively by the appearance of a blue line on the device 15 min

after the sample is applied. RICT with several pairs of MoAbs showed a high sensitivity for the detection of recombinant N-Ag as well as viral N-Ag of SARS-CoV. rSN122 and rSN21-2 were the best MoAbs for immobilized antibody and enzyme labeling, respectively. With regard to analytical sensitivity, RICT detected N-Ag at 31 pg/mL for recombinant N-Ag, and at  $1.99 \times 10^2$  TCID<sub>50</sub>/mL for SARS-CoV. The specificity of RICT was 100% when 150 human sera and 50 nasopharyngeal aspirates (NSPs) were used. RICT based on an EIA using the rSN122/rSN21-2 pair is a sensitive, specific, and reliable rapid assay for detecting N-Ag in SARS-CoV treated with either heat or Triton X-100. *J. Clin. Lab. Anal.* 19:150–159, 2005. © 2005 Wiley-Liss, Inc.

**Key words:** rapid immunochromatographic test (RICT); severe acute respiratory syndrome-associated coronavirus (SARS-CoV); nucleocapsid antigen (N-Ag)

## INTRODUCTION

Since severe acute respiratory syndrome-associated coronavirus (SARS-CoV) (1–4) was first discovered, the need to develop a sensitive, simple assay to detect its antigens and antibodies has become increasingly pressing.

SARS-CoV can be detected by real-time PCR during the early phase of infection (5,6). This technique allows for a highly sensitive determination using nasopharyngeal aspirates (NSPs) of SARS patients, with a sensitivity and specificity of 79% and 98%, respectively

*Abbreviations:* SARS: severe acute respiratory syndrome; SARS-CoV: SARS-associated coronavirus; RICT: rapid immunochromatographic test; EIA: enzyme immunoassay; N-Ag: nucleocapsid antigen; MoAb: monoclonal antibody; TCID<sub>50</sub>: 50 tissue culture infective dose virus; WB: Western blotting; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; POD: peroxidase; BSA: bovine serum albumin; ALP: alkaline phosphatase; NP-40: Nonidet-P40.

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(6). Likewise, a method employing an enzyme-linked immunosorbent assay (ELISA) has also achieved sensitive detection using the blood of SARS patients (7). The detection of antibodies against SARS-CoV by ELISA and Western blotting (WB) in patient serum has also been reported (2,8,9).

A simple device based on the rapid enzyme immunoassay (EIA) format for detecting SARS-CoV antigens that could be used for point-of-care testing near a patient care site (10) has yet to be developed. Recently, an immunochromatographic test using colloidal gold particles serving as detectors, and recombinant N-Ag of SARS-CoV as a source of antigen for capturing the antibodies was described for measuring antibodies to SARS-CoV (11,12). It should be noted, however, that immunochromatographic test methods using gold particles as detectors are likely to be less sensitive for the detection of antigens than EIA or ELISA, which can amplify signal intensity by enzyme catalyzing of the substrate. Thus, a rapid immunochromatographic test (RICT) method based on EIA appears promising for analyte detection with a high sensitivity (10).

The principle behind the RICT assay method (10) is as follows: When a sample is applied onto the sample application pad of the RICT device (as described in Fig. 1), an immune complex is formed between N-Ag of the applied sample and the anti-N-Ag antibody conjugated with ALP in the pad. The immune complex elutes toward the detection zone by lateral flow along the membrane due to capillary force and diffusion by rupturing the developing reservoir in the bottom of the device. Thereafter, N-Ag of the immune complex can react with anti-N-Ag antibodies immobilized at the detection zone. ALP in the complex then catalyzes the dephosphorylation of 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP), resulting in a color

development that can be visualized at the detection zone and judged as positive.

We expressed recombinant N-Ag of SARS-CoV (Urbani strain) based on its genomic sequences. We then developed a novel RICT based on EIA for measuring N-Ag of SARS-CoV, using the monoclonal antibody (MoAb) produced by immunizing the recombinant N-Ag to mice.

## MATERIALS AND METHODS

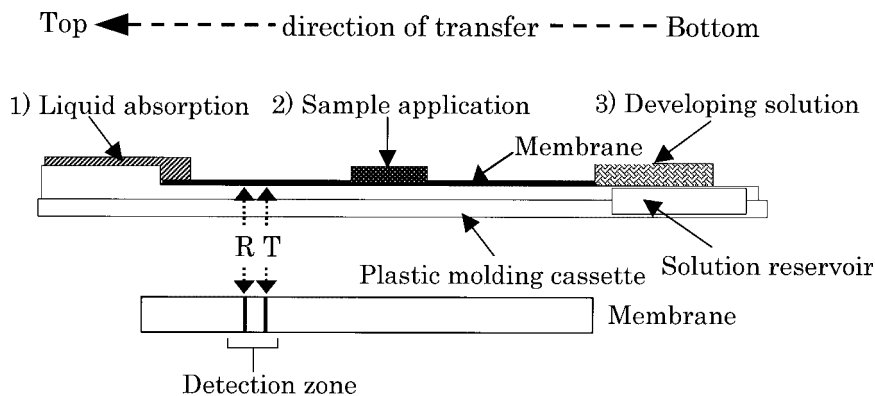
### Preparation of MoAbs

MoAbs rSN150, rSN122, rSN29, rSN21-2, and rSN18 (but not SN5-25) against recombinant N-Ag were prepared in mouse ascites according to a conventional procedure (13,14) and then purified from the ascites by affinity chromatography on protein A agarose (MAPS-II kit; Bio-Rad, Hercules, CA). For the preparation of SN5-25, the synthetic peptide of GQVTKKSAEASKKPR (amino acid sequence: 244–260 of N-Ag), termed SN-5 peptide, was used as the immunizing agent instead of recombinant N-Ag. Polyclonal antibodies used to capture alkaline phosphate (ALP) in the RICT assay were prepared from rabbit serum after immunization with ALP. They were immobilized at the R position on the membrane of the RICT device.

Most of the chemicals used are listed below; all other chemicals used were of reagent-grade quality. The molecular weight marker kit was purchased from Invitrogen (bench mark prestained protein ladder; Invitrogen Japan, K.K, Tokyo, Japan).

### Preparation of Recombinant N-Ag

We constructed cDNA fragments based on genome sequences corresponding to a N-Ag of SARS-CoV virus



**Fig. 1.** Schematic depiction of the RICT device. Three pads for 1) liquid absorption, 2) sample application, and 3) solution development (substrate) were put on the membrane. Anti-ALP antibodies and MoAbs for recombinant N-Ag were dotted on R and T positions in the detection zone, respectively. R and T were the reference and test lines, respectively. The flow of developing solution moves from the reservoir at the bottom to the liquid adsorption pad at the top. This picture was redrawn with some changes from original Fig. 7(10).

(Urbani strain), as obtained from GenBank (access no. AY278741). Thirty-two different primers with a length of 50–54 bases were synthesized based on the sequence of consecutive regions of the entire 1,269-bp sequence (i.e., 1–50, 45–80, ---, 281–330, 321–370, ---, 601–649, 621–670, ---, 901–950, 940–990, ---, and 1219–1269). Each of the primer sequences overlapped the neighboring primer sequence by 10–12 bases, and the entire N-Ag sequence was covered. The primers were synthesized by Sigma Genosis Japan (Ishikari, Hokkaido, Japan).

Two large cDNA fragments (bp 1–649 and 621–1269), both containing the *NheI* restriction site at position 634 of the N-Ag, were prepared stepwise using 16 primers spanning bases 1–649 and 621–1269, respectively, by PCR amplification (model 381A DNA synthesizer; Applied Biosystems, Foster City, CA). For example, the polynucleotide spanning bases 1–649 was synthesized as follows: A solution containing primers spanning bases 281–330 and 321–370 was mixed and PCR amplification was carried out in the presence of Pfu DNA polymerase as described previously (15). This resulted in the formation of an oligonucleotide spanning bases 281–370. The same method using additional neighboring primers at the both ends of the oligomer spanning bases 281–370 was carried out using consecutive primers, which eventually resulted in a cDNA fragment spanning bases 1–649 of N-Ag.

Finally, a full-length DNA polynucleotide containing *EcoRI* and *BamHI* sites was obtained from the two large DNA fragments (1–649 and 621–1269) by clearing and ligation at the site of 634 and was applied to construct a plasmid PCR using primers 5'-CCGCCGG AATTCATGCTCTGATAATGGACCC-3' and 3'-GTT TGCGGATCCTTATGCCTGAGTTGAATC-5'. The obtained recombinant N-Ag DNA was purified using a PCR purification kit following the manufacturer's instructions (Qiagen, Hilden, Germany), and inserted into *EcoRI* and *BamHI* sites of the expression vectors of pW6A (15), resulting in a plasmid named pWSN. *Escherichia coli* (BL21 (DE3) strain) was transformed with the pWSN vector as described elsewhere (15,16). Growth and harvesting of the transformed *E. coli* cells, and the subsequent treatment to obtain the lysate of the cells were performed essentially as described previously (15). The recombinant N-Ag protein from the lysate suspension was also purified as described previously (15). The concentration of purified recombinant N-Ag was determined using a BCA protein assay reagent (Pierce Biotechnology, Inc., Rockford, IL).

### Preparation of SARS-CoV Antigen

SARS-CoV (Urbani strain) was provided by Dr. Koichi Morita of Nagasaki University and prepared at

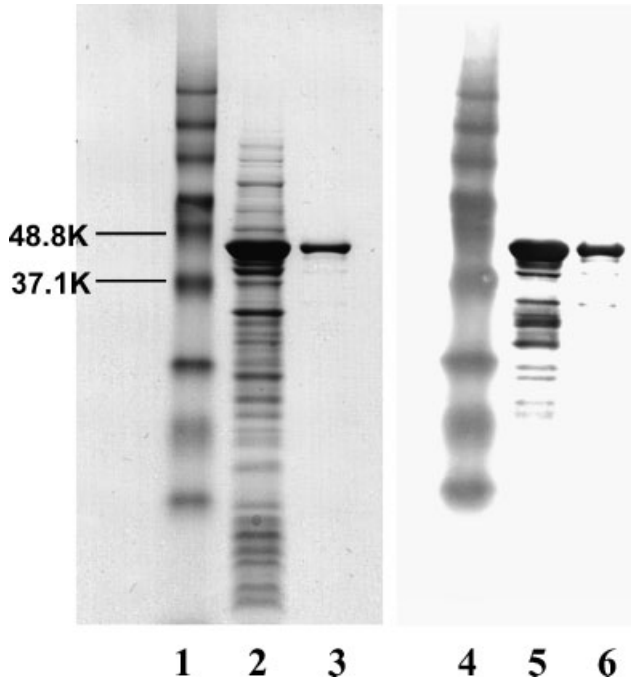
the Biosafety Level 3 Laboratory of the Graduate School of Veterinary Medicine, Hokkaido University. The preparation and treatment of the live viruses were carried out in accordance with the guidelines proposed by Hokkaido University. Briefly, Vero E6 cells were grown to confluence in minimum essential medium containing 10% fetal bovine serum at 37°C. The cells were infected with SARS-CoV at  $10^7$  TCID<sub>50</sub>/mL and then cultured for 42 hr at 37°C. The culture was centrifuged at 4°C and 2,000 rpm for 15 min, and the supernatant was collected. The supernatant solution was heated at 56°C for 90 min and thereafter kept at –80°C until further use. Supernatant from cultured Vero E6 cells that were not infected with SARS-CoV were also prepared as controls.

### WB Analysis

The proteins that were expressed in *E. coli* and then purified were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (15). For WB, the electrophoretic transfer of proteins such as recombinant N-Ag and native N-Ag from gels to nitrocellulose paper (pore size = 0.45 µm; Schleicher and Schnell, Germany) was performed essentially as described previously (15). In brief, the nitrocellulose paper, following incubation with either MoAbs or mouse anti-SN-5 peptide serum and subsequently with the anti-mouse antibody conjugated with peroxidase (POD; Dako Cytomation, Co. Ltd., Kyoto, Japan), was washed with phosphate-buffered saline (PBS) buffer containing 0.05% Tween 20. A solution of 4-chloronaphthol was applied to the paper, and an intense color of catalized 4-chloronaphthol with POD was detected (15). The native N-Ag used for WB analysis was prepared as follows: The precipitate, which was obtained by ultracentrifugation of supernatant from the cultured Vero E6 cells with SARS-CoV, was given 3% SDS in 5 mM Tris buffer pH 7.4, boiled for 5 min, and applied on SDS-PAGE. Mouse anti-SN-5 peptide serum for Fig. 2 or mouse MoAbs against SARS-CoV for Fig. 4 were used as the primary antibody, and rabbit anti-mouse antibodies conjugated with POD were used as the secondary antibody.

### ELISA Procedure

To evaluate the immunoreactivity of MoAbs by ELISA, the wells of a plastic microplate (Falcon 3912, micro test III flexible assay plate; Becton Dickinson, San Jose, CA) were coated with 1 µg/mL of recombinant N-Ag in 10 mM PBS (pH 7.4) for 24 hr at 4°C. The plate was washed with 10 mM PBS (pH 7.4) containing 0.05% Tween 20. The ELISA was carried out as described previously (15). Briefly, 50 µL of solution



**Fig. 2.** Purified recombinant N-antigen expressed in *E. coli*. SDS-PAGE of recombinant N-Ag. Lane 1 represents molecular mass standards in kDa: 181.8 kDa, 115.5 kDa, 82.2 kDa, 63.2 kDa, 48.8 kDa, 37.1 kDa, 25.9 kDa, 19.4 kDa, and 14.8 kDa. Lane 2: N-Ag expressed in *E. coli*. Lane 3: Purified N-Ag. Lanes 1–3 were stained with Coomassie brilliant blue. WB of recombinant N-Ag with mouse anti-SN-5 peptide serum (lanes 5 and 6). Lanes 4–6 are the same as described for lanes 1–3.

containing MoAb at eight different concentrations (1, 3, 10, 30, 90, 280, 830, and 2,500 ng/mL) was added into the wells of the plate and incubated for 1 hr at room temperature. After washing with the PBS buffer containing Tween 20, goat anti-mouse antibodies conjugated with POD (Dako Cytomation, Co. Ltd., Kyoto, Japan) was added and incubated for 1 hr. After the wells were washed with PBS buffer, 50  $\mu$ L of 2,2-azinobis(3-ethyl benzothiazoline-6-sulfonic acid) ammonium salt (ABTS)/H<sub>2</sub>O<sub>2</sub> were added. After the plate was incubated for 5 min, absorbance at 405 nm was measured. The concentrations of MoAb at 50% of the maximum absorbance was used as an index for the apparent affinity of the antibody.

For the competitive ELISA assay of the MoAbs, the wells of a plastic microplate were coated with 1  $\mu$ g/mL of recombinant N-Ag, and essentially the same ELISA procedure as described above was used except that POD was used as the labeling enzyme and the assay buffer was 10 mM Tris buffer (pH 7.4) containing 0.15 M NaCl and 1% BSA. Briefly, a mixture of 1  $\mu$ g/mL of MoAb labeled with ALP and 5  $\mu$ g/mL of free MoAb used as the competitor was added to the wells of a microplate and incubated for 1 hr at room temperature. After the wells

were washed, a 4-nitrophenyl-phosphate (PNPP) solution was added and absorbance at 405 nm was measured.

For detection of the recombinant or native N-Ag of SARS-CoV by a two-step sandwich-format ELISA, the wells of a plastic microplate (Nunc-Immuno™ modules plate; Nunc A/C, Roskilde, Denmark) were coated with MoAbs against recombinant N-Ag or a synthetic peptide in 0.1 M phosphate buffer (pH 7.5) for 24 hr at 4°. The coating concentration of the wells was set at 14  $\mu$ g/mL MoAb. The washing and blocking procedures were the same as described previously (15). The ELISA was carried out in a two-step sandwich format. In brief, MoAbs against N-Ag of SARS-CoV conjugated with ALP were used as the label. Then 100  $\mu$ L of the test sample containing recombinant N-Ag or proteins obtained from supernatant of cultured Vero cells with SARS-CoV were placed in the microplate wells and incubated for 1 hr at ambient temperature. After the plate was washed with 10 mM Tris buffer (pH 8.0) containing 0.15 M NaCl and 0.05% Triton X-100, 100  $\mu$ L of MoAbs conjugated with ALP were added and incubated for 1 hr. After the wells were washed, 100  $\mu$ L of PNPP solution was added and absorbance at 405 nm was measured. The detection limit values were estimated from the standard ELISA curves with a signal-to-noise ratio (SNR) of 2.

### Preparation of the RICT Device, and the RICT Procedure

The basic concept of a rapid immunoassay with an all-in-one device based on the immunochromatographic method was previously described (10). The device used in this study is schematically depicted in Fig. 1. The RICT was designed for a qualitative analysis to detect the nucleocapsid protein of SARS-CoV on the basis of a positive or negative judgment.

Three pads fixed onto sheets of nitrocellulose membrane (0.13 mm, 5 mm, and 50 mm (D  $\times$  W  $\times$  L), respectively) were used for liquid absorption, sample application, and solution development (Millipore Corp., Cork, Ireland). To assemble the assay device, three pads were placed on the entire membrane of the cassette and molded with a plastic cover to make an all-in-one device.

The pads and membrane sheet were pretreated as follows: Pad 2: A 5- $\mu$ L solution containing an anti-N-Ag of SARS-CoV antibody conjugated with ALP in 50 mM Bis-Tris buffer (pH 7.2), 0.15 M NaCl, 0.5% BSA, and 1% Triton X-100 was absorbed onto pads of sizes 0.5 mm, 5 mm, and 9 mm (D  $\times$  W  $\times$  L) made of porous matrix. The pads were subsequently dried by air blowing. The addition of Triton X-100 is essential to avoid interference from an immuno and/or enzyme

reaction from the sample. Pad 3: BCIP (5  $\mu$ L, used for the ALP substrate) was absorbed onto a pad that was then dried. The anti-ALP antibody and anti-N-Ag of SARS-CoV antibody were immobilized at the R and T positions on the membrane sheet in the detection zone. R and T indicated the reference and test lines, respectively. Then 300  $\mu$ L of 0.1 M diethanol amine buffer (pH 9.8) containing 1 mM MgCl<sub>2</sub> (used as the developing solution) was added to the reservoir, which was then sealed with an aluminum film.

The assay procedure for RICT was performed as follows: A 25  $\mu$ L sample containing either recombinant N-Ag or native SARS-CoV antigens was dropped onto the sample application pad, and the developing solution reservoir was then ruptured by pressing it with a finger. The device was incubated at room temperature. At 15 min after sample application, a blue line observed by the naked eye at the T position was judged as positive. When no band was observed after 15 min, the result was judged as negative. In addition to the line at the T position, a blue line appeared at the R position and was used as an index of the validation of test performance.

### Samples to Determine Analytical Sensitivity and Specificity

#### Preparation of SARS-CoV

Most of the viral samples for the assay in this study were prepared from the supernatant of Vero E6 cells inoculated with SARS-CoV from patients. The original viral suspension contained  $7.95 \times 10^6$  TCID<sub>50</sub>/mL of SARS-CoV and was diluted according to test requirements with 0.1 M Tris buffer (pH 8.0) containing 0.15 M NaCl, 3% BSA, and, unless otherwise stated, 0.1% Nonidet P-40 (NP-40). This buffer was also used for dilution of serum and NSP, when necessary. To prevent SARS-CoV infection during the assay procedure, we added NP-40 to the sample buffer to inactivate the SARS-CoV.

#### Samples to Determine Analytical Sensitivity

A 25  $\mu$ L sample was used for each RICT assay. The measurement of analytical sensitivity of the RICT assay with native SARS-CoV was carried out using samples prepared by serial 10-fold dilutions of viral cultures containing  $7.95 \times 10^6$  TCID<sub>50</sub>/mL of SARS-CoV for all MoAb pair systems, and  $1.59 \times 10^5$  TCID<sub>50</sub>/mL for the rSN122 and rSN21-2 pair systems. To obtain a more precise limit of detection, further testing was performed with nine dilutions (1.5-, 2-, 3-, 4-, 5-, 6-, 7-, 8-, and 9-fold dilutions of the positive sample solution with which antigen level was  $7.95 \times 10^2$  TCID<sub>50</sub>/mL for

rSN122/rSN21-2 and rSN122/rSN29 pair system, and  $7.95 \times 10^4$  TCID<sub>50</sub>/mL for rSN150/rSN122 and rSN18/rSN122 pair system).

For the measurement of analytical sensitivity with recombinant N-Ag, we used samples of a serial 2-fold dilution of the solution containing recombinant N-Ag at 2 ng/mL. The highest respective dilutions of the SARS-CoV preparation (TCID<sub>50</sub>/mL) and recombinant N-Ag that resulted in a positive judgment were defined as the limits of analytical sensitivity in the present study.

#### Samples to Determine Specificity and Reproducibility

For the RICT specificity and reproducibility analyses, all tests were performed using the rSN122/rSN21-2 pair system. For the RICT specificity analysis, 150 different human sera (50 normal human sera, 50 sera from pregnant women, and 50 from rheumatoid arthritis patients), as well as 50 human NSPs (including those from 14 patients infected with the influenza virus) were tested. To collect the NSPs we inserted tubing into the subject's nostril parallel to the palate and aspirated the nasopharyngeal secretions, dispensed in 1 mL of sterile saline (pH 7.0). The specimens were kept in sterile vials until they were used in the experiments.

#### Samples for Other Analyses

To prepare positive serum and respiratory aspirates to mimic the patient samples, we added 10  $\mu$ L of the SARS-CoV suspension at  $7.95 \times 10^6$  TCID<sub>50</sub>/mL to 1 mL of human serum or NSP. Most samples were prepared with  $3.3 \times 10$ ,  $3 \times 10^2$ , and  $3 \times 10^3$  fold dilutions of the stock suspension ( $7.87 \times 10^4$  TCID<sub>50</sub>/mL) using normal serum (and NSP) and subjected to RICT assays, in which the lowest concentration of SARS-CoV was  $2.63 \times 10$  TCID<sub>50</sub>/mL at  $3 \times 10^3$  fold dilution.

All samples of Coxsackie virus types A-7 and -9 and B-1-6, parainfluenza virus types 1-4, adenovirus type 3, mumps virus, rubella virus, measles virus, and respiratory syncytial virus were purchased from a manufacturer (Denka-Seiken, Tokyo, Japan). These samples were treated with UV irradiation and/or  $\beta$ -propiolactone by the manufacturer.

## RESULTS

### Expression of Recombinant N-Ag in the *E. coli* System

A recombinant nucleocapsid protein of SARS-CoV, obtained in mostly soluble proteins, was highly expressed in the *E. coli* system (Fig. 2). The expressed proteins were purified and subjected to WB analysis. A major band of molecular mass 47.2 KDa was observed from Coomassie brilliant blue staining of the gels on the

SDS-PAGE, and also on WB using mouse anti-SN-5 peptide serum (Fig. 2, lanes 3 and 6).

### Evaluation of Immunoreactivity of the MoAbs to N-Ag of SARS-CoV by ELISA and WB

MoAbs were produced by immunizing the protein of recombinant N-Ag or synthetic peptide to mice. Six MoAbs were screened for their ability to bind to recombinant N-Ag immobilized on the wells (their titration curves are shown in Fig. 3). Four MoAbs (rSN18, rSN150, rSN122, and SN5-25) gave concentrations of about 70 ng/mL at 50% of the maximum absorbance and had a relatively high apparent affinity, while the corresponding values of rSN21-2 and rSN29 were about 200 ng/mL, indicating nearly three times less affinity compared to that of the above antibodies.

The MoAbs were compared with each other for binding to recombinant N-Ag by a competitive ELISA assay. Typical examples are shown in Table 1, i.e., the absorbance at 405 nm for ALP-labeled rSN122 was significantly reduced by the presence of free SN122 as a competitor, while small changes in absorbance occurred in the presence of other MoAbs. Based on the competitive method described above, MoAbs of rSN18, rSN150, rSN122, and SN5-25 each appeared

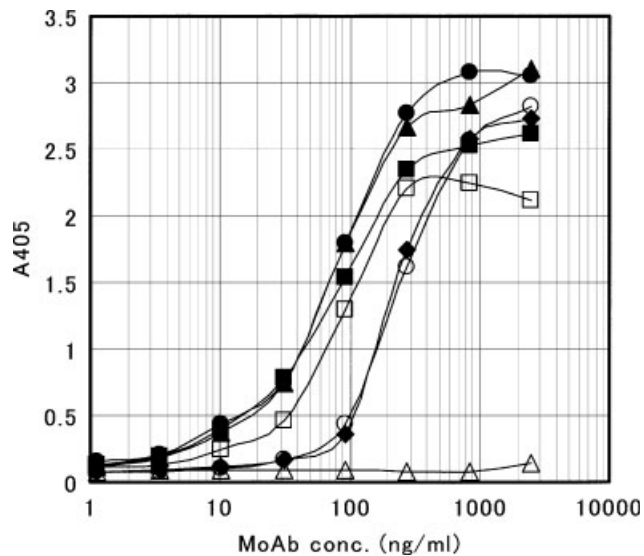
to bind at different epitopes, but rSN21-2 and rSN29 appeared to bind at the same epitope on the recombinant N-Ag (data not shown).

To confirm immunoreactivity with native antigens of SARS-CoV, the six MoAbs were subjected to WB and compared with recombinant N-Ag. Using recombinant N-Ag, we found that all of the MoAbs were reactive to the protein at a molecular mass of 47.2 Kda (Fig. 4a). When native SARS-CoV was used, banding patterns similar to those of recombinant proteins were observed (Fig. 4b). No detectable bands were observed when mouse MoAb E2CT-38, which is not related to SARS-CoV, was applied (lane 1 in Fig. 4a and b).

Preliminary experiments were carried out to evaluate these MoAbs in the sandwich format of the ELISA assay. All possible pair combinations of the ELISA assay using MoAbs were performed. Four pairs of MoAbs (rSN150/rSN122, rSN18/rSN122, rSN122/rSN21-2, and rSN122/rSN29 pairs; the first and second antibodies were for solid-phase and ALP labeling, respectively) were successful, and a linear dose dependency was obtained for recombinant N-Ag up to 5 ng/mL (curves  $\circ$ ,  $\square$ ,  $\bullet$ , and  $\blacksquare$  in Fig. 5).

Qualitatively similar results were obtained for the SARS-CoV up to  $6.8 \times 10^4$  TCID<sub>50</sub>/mL (data not shown).

The detection limits of the ELISA were 0.031–0.107 ng/mL for recombinant N-Ag, and  $6.33 \times 10^2$  –  $2.88 \times 10^3$  TCID<sub>50</sub>/mL for native N-Ag of SARS-CoV.



**Fig. 3.** Immunoreactivity of MoAbs obtained from mouse ascites after immunizing recombinant N-Ag. Microtiter plates were coated with 1  $\mu$ g/mL of recombinant N-Ag per well and used for titration of MoAbs in the buffer, and detection with POD-labeled goat anti-mouse Igs was subsequently performed as described in Materials and Methods. The means of the duplicates are shown. The MoAbs used for titration curves were as follows: rSN18 ( $\bullet$ ), rSN122 ( $\blacksquare$ ), rSN150 ( $\blacktriangle$ ), rSN21-2 ( $\square$ ), rSN29 ( $\circ$ ), and SN5-25 ( $\square$ ). E2CT-38 MoAb ( $\triangle$ ; antibody against estradiol) was used as control.

### Performance of RICT

On the basis of the ELISA results, we further evaluated five MoAbs (rSN122, rSN150, rSN18, rSN21-2, and rSN29) using the optimized conditions for sandwich-format RICT. RICT was developed as a qualitative assay method to detect N-Ag of SARS-CoV, and the appearance of a blue line at the T position of the device 15 min after sample application is judged as positive. All possible pair combinations among these MoAbs were tested, and four sets of MoAb pairs (rSN150/rSN122, rSN18/rSN122, rSN122/rSN21-2, and rSN122/rSN29 (the first MoAb was immobilized and the second was the labeling antibody)) were selected using SARS-CoV as possible candidates for RICT in terms of detectability of N-Ag by a serial 10-fold dilution of samples (Table 2). Table 3 shows the analytical sensitivity of four MoAb pair systems. The highest analytical sensitivity obtained using rSN122/rSN21-2 was 31 pg/mL for recombinant N-Ag, and  $1.99 \times 10^2$  TCID<sub>50</sub>/mL for native antigen of SARS-CoV.

Figure 6 shows the test results for the most sensitive detection of the RICT system using rSN122 and rSN21-2 as solid-phase and ALP labeling, respectively. Prior

TABLE 1. Competitive assay of MoAbs by ELISA\*

	MoAb(A)						Control
	rSN122	rSN18	rSN150	SN5-25	rSN21-2	rSN29	
Labeled A405	0.41	1.43	2.04	1.61	1.36	2.16	1.74
rSN122 %	23.6%	82.2%	117.2%	92.5%	78.2%	124.1%	100%

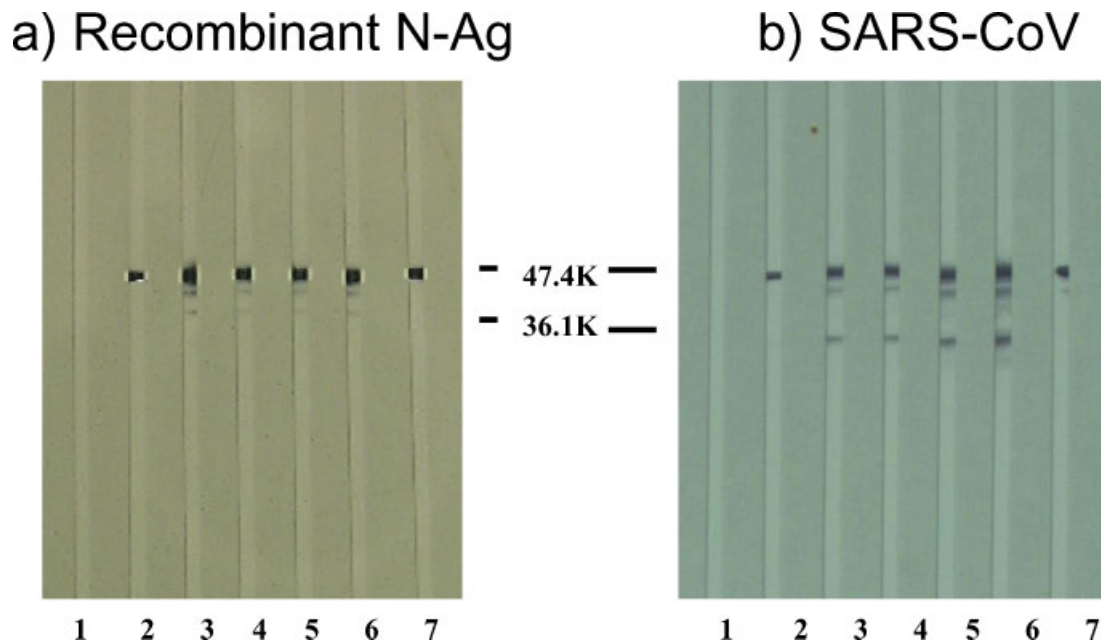
\*rSN122 labeled with ALP was used for detection of recombinant N-Ag immobilized on the wells. Free MoAb(A) used as competitor was added together with ALP-labeled MoAb into the wells as described in Materials and Methods. Absorbance measured at 405 nm is indicated. E2CT-38 MoAb (antibody against estradiol) was used as control. Percentages were indicated using control absorbance as 100%.

to analysis, test samples containing SARS-CoV were heat-treated at 56°C and diluted with a buffer containing NP-40, as described in Materials and Methods. The intensity of the blue line observed at the T position gradually weakened with consecutive 10-fold dilution of applied samples, and could be detected up to a 10-fold dilution, in which the TCID<sub>50</sub>/mL was  $1.59 \times 10^3$  (Fig. 6, lane 3). However, a line could not be observed at the 10-fold dilution ( $1.59 \times 10^2$  TCID<sub>50</sub>/mL) at 15 min (Fig. 6, lane 2). To further probe the limit of analytical sensitivity, we tested nine further dilutions. The highest dilution (8-fold) with a positive judgment was equivalent to the concentrations of  $1.99 \times 10^2$  TCID<sub>50</sub>/mL, while a 9-fold dilution ( $1.77 \times 10^2$  TCID<sub>50</sub>/mL) remained negative. We thus took the value at  $1.99 \times 10^2$  TCID<sub>50</sub>/mL as the limit of analytical sensitivity.

We also performed RICT using samples in the absence of NP-40 without heat treatment, and obtained similar results (data not shown).

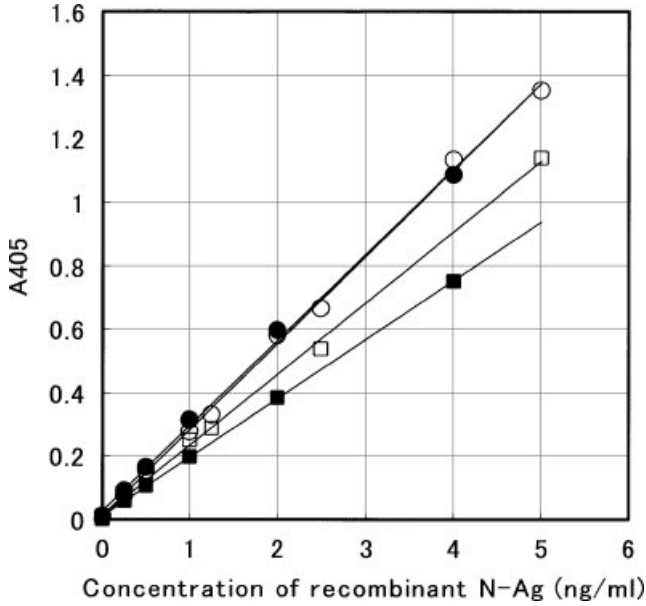
### Specificity of RICT

For the RICT specificity and reproducibility analyses, all tests were performed using the rSN122/rSN21-2 pair system. As positive controls, we prepared mimicked serum and NSPs of SARS patients containing cultured SARS-CoV. The results were all positive for both samples down to  $2.65 \times 10^2$  TCID<sub>50</sub>/mL; however, the result using  $2.65 \times 10$  TCID<sub>50</sub>/mL was negative. The specificity of the RICT assay was 100% (150/150: 50 normal sera, 50 rheumatoid arthritis, and 50 pregnancy sera) for the sera, and 100% (50/50) for the NSPs (Table 4).



**Fig. 4.** WB of recombinant N-Ag and native SARS-CoV antigens from cultured Vero E6 cells using MoAbs. **a:** WB of recombinant N-Ag with MoAbs. **b:** WB of native SARS-CoV antigens with MoAbs. The numbers described in each lane of panels a and b stand for the symbol name of the monoclonal antibodies used. Lane 1: E2CT-38 MoAb (antibody against estradiol) as control. Lanes 2–7: SN5-25, rSN29, rSN21-2, rSN150, rSN122, and rSN18, respectively. 48.8 KDa and 37.1 KDa: molecular mass markers.





**Fig. 5.** Reactivity of recombinant N-Ag with MoAbs on ELISA. Dose-response curves for absorbance measured at 405 nm. Immuno- and enzyme reactions were taken for 1 hr at ambient temperature. The first and second antibodies of the MoAbs used for ELISA were used for immobilization on solid-phase and ALP labeling, respectively. The standard curve gave the following regression equations (each data point in the figure is the average of duplicate experimental points): rSN150/rSN122,  $y = 0.272x + 0.0087$ ,  $r = 0.999$  (○); SN18/rSN122,  $y = 0.224x + 0.0085$ ,  $r = 0.999$  (□); rSN122/rSN21-2,  $y = 0.269x + 0.0288$ ,  $r = 0.998$  (●); and rSN122/rSN29,  $y = 0.185x + 0.0110$ ,  $r = 0.999$  (■).

Of the 50 NSP results, 14 were influenza-infected samples (influenza virus types A (n = 12) and B (n = 2)). Thus, the results from the 14 influenza samples also showed that no cross-reactivity with SARS-CoV occurred. Furthermore, infectious viruses of the respiratory tract, such as Coxsackie virus types A-7 and -9 and B-1-6, parainfluenza virus types 1-4, adenovirus type 3, mumps virus, rubella virus, measles virus, and respiratory syncytial virus, were tested by RICT. None of these samples gave positive results.

**TABLE 2. Detectability of RICT for native SARS-CoV**

Type(MoAbs) <sup>a</sup>	Analytical Sensitivity <sup>b</sup>		SARS-CoV (TCID <sub>50</sub> /mL)	
	$7.95 \times 10^4$	$7.95 \times 10^3$	$7.95 \times 10^2$	$7.95 \times 10^1$
Immobilized/label				
rSN122/rSN21-2	+	+	+	-
rSN122/rSN29	+	+	+	-
rSN150/rSN122	+	+	-	-
rSN18/rSN122	+	-	-	-

<sup>a</sup>Assay combinations were performed with MoAbs used for immobilization and labeling.

<sup>b</sup>Analytical sensitivity was determined as described in Materials and Methods by serial 10-fold dilution of original SARS-CoV. + and - indicate positive and negative, respectively.

**TABLE 3. Analytical sensitivity of RICT for recombinant N-Ag and native SARS-CoV**

Type(MoAbs) <sup>a</sup>	Analytical sensitivity <sup>b</sup>	
	SARS-CoV (TCID <sub>50</sub> /mL)	Recombinant N-Ag(pg/mL)
rSN122/rSN21-2	$1.99 \times 10^2$ /mL	31
rSN122/rSN29	$2.65 \times 10^2$ /mL	125
rSN150/rSN122	$5.30 \times 10^3$ /mL	500
rSN18/rSN122	$7.95 \times 10^4$ /mL	250

<sup>a</sup>Assay combinations were performed with MoAbs used for immobilization and labeling.

<sup>b</sup>Analytical sensitivity was determined as described in Materials and Methods.

**Reproducibility of RICT**

We evaluated the reproducibility of the RICT using five different samples ( $10^4$ ,  $2 \times 10^4$ ,  $3 \times 10^4$ ,  $4 \times 10^4$ , and  $5 \times 10^4$  fold dilutions of the original viral suspension at



**Fig. 6.** Performance of RICT using a sample containing cultured SARS-CoV. Lanes 2-5:  $1.59 \times 10^2$ ,  $1.59 \times 10^3$ ,  $1.59 \times 10^4$ , and  $1.59 \times 10^5$  TCID<sub>50</sub>/mL, respectively. Lane 1: Control with supernatant of cultured Vero E6 without SARS-CoV. Samples were prepared by serial 10-fold dilutions of SARS-CoV suspension at  $1.59 \times 10^5$  TCID<sub>50</sub>/mL. The photograph of the tested RICT device was taken with an Olympus C-990 15 min after sample application. R and T indicate reference and test lines, respectively.



**TABLE 4. Specificity of RICT for serum and nasopharyngeal aspirate**

	Negative
Serum	
Normal	50/50
Pregnancy	50/50
Rheumatoid arthritis	50/50
Total	150/150 (100%)
Nasopharyngeal aspirate	
Normal	36/36
Influenza A	12/12
Influenza B	2/2
Total	50/50 (100%)

$7.95 \times 10^6$  TCID<sub>50</sub>/mL). Each sample was tested three times and the results obtained were excellent, with a positive result in all samples at TCID<sub>50</sub> of  $7.95 \times 10^2$ /mL,  $3.98 \times 10^2$ /mL,  $2.65 \times 10^2$ /mL, and  $1.99 \times 10^2$ /mL. At  $1.59 \times 10^2$  TCID<sub>50</sub>/mL, the results were negative in all three repeats. When we used serum and NSPs at  $7.95 \times 10^2$ ,  $7.95 \times 10^3$ , and  $7.95 \times 10^4$  TCID<sub>50</sub>/mL, mimicking SARS-CoV patient samples, the reproducibilities (n = 3) for RICT were also excellent.

## DISCUSSION

We have demonstrated that our RICT with an all-in-one device using mouse MoAbs (rSN122 for immobilized antibody, and rSN21-2 for enzyme labeling) can be used to detect the N-Ag of SARS-CoV.

Although we could not confirm the sensitivity of RICT due to a lack of available human SARS patient samples, the results were all positive for both samples down to  $7.87 \times 10^2$  TCID<sub>50</sub>/mL of SARS-CoV using mimicked serum and NSPs of SARS patients containing cultured SARS-CoV as positive controls. The specificity of the RICT assay was 100% for sera, and 100% for NSPs (Table 4). However, cross-reactivity using human coronavirus OC43 and 229E remains to be confirmed.

As for the analytical sensitivities of RICT between recombinant and native N-Ag of SARS-CoV (Table 3), the ratio of TCID<sub>50</sub> and concentration of recombinant N-Ag was more or less consistent, except for the rSN18/rSN122 pair system, which resulted in less sensitivity for native SARS-CoV as compared to recombinant N-Ag. These results may indicate that some changes in the reactivity of rSN18 occur only for native N-Ag of SRAS-CoV due to immobilization on the membrane of the device. Despite the lower apparent affinity (Fig. 3), rSN21-2 and rSN29 showed highly sensitive results when used for labeling antibody, indicating that they can bind more strongly to free-form N-Ag than to

immobilized N-Ag. A high analytical sensitivity for RICT using the rSN122/rSN21-2 pair was obtained at 31 pg/mL for recombinant N-Ag and  $1.99 \times 10^2$  TCID<sub>50</sub>/mL for SARS-CoV (Table 2), because the EIA method was employed. The analytical sensitivity for RICT was more or less consistent with the lower limit of detection on ELISA (Fig. 5). The analytical sensitivity was similar to that obtained by ELISA using a mixture of MoAbs for capturing N-Ag and polyclonal antibodies as the detectors (7).

Our results suggest that the presence of Triton X-100 enables the detection of N-Ag of SARS-CoV under conditions in which this would not otherwise be possible. Triton X-100 may cause the destruction of SARS-CoV particles, and the viral N-antigen may thus become detectable by RICT. The effect of Triton X-100 on transmissible gastroenteritis virus, a member of the coronavirus family, was addressed, and it was confirmed that the spherical core structure of the virus is maintained after treatment by Triton X-100 and can react with MoAb specific to N-Ag of transmissible gastroenteritis virus (17).

Given the results obtained by ELISA and RICT that confirmed the detection of N-Ag of SARS-CoV using a heat-treated SARS-CoV sample without Triton X-100 by ELISA, and a sample without heat treatment in the absence of NP-40 by RICT, we conclude that treatment with either heat or Triton X-100 allows the N-Ag of SARS-CoV to be detected.

The molecular mass of N-Ag (46.7 KDa) calculated from its amino acid sequence was consistent with the 47.2 KDa observed by SDS-PAGE and WB (Figs. 2 and 4). It is most likely that the viral antigen that is reactive with MoAbs is a nucleocapsid protein. Also, the immunoreactivity of recombinant N-Ag appeared to be qualitatively similar to that of the native N-Ag. Regardless of the protein configuration used (denatured or intact), the MoAbs reacted not only with the recombinant N-Ag but also with native N-Ag (Fig. 4), indicating that the MoAbs are able to recognize epitopes of N-Ag independently of a higher-order protein configuration.

We constructed DNA based on the Urbani strain for the expression of N-Ag in an *E. coli* system. Genomic N-Ag sequences of SARS-CoV, such as Urbani, CUHK-W1, Tor2, Frankfurt1, HSR1, TWH, BJ01, Shanghai QXC1, etc., have been reported in GenBank, and some changes in the nucleic acids of the sequences have been found (18). However, the putative amino acid sequences for N-Ag were the same in most of the strains, except for a few strains in which one or two amino acids were changed due to nucleic acid mutation, i.e., Frankfurt 1 and FRA (amino acid change; Thr<sup>50</sup>Ile), BJ03 (amino acid change; Asn<sup>154</sup>Tyr), CUHK-Su10 (amino acid

change; Gly<sup>193</sup>Cys), and Shanghai LY (amino acid changes; Val<sup>325</sup>Ala and Thr<sup>326</sup>Ala). Among the four mutated strains, we expressed the recombinant N-Ag of Frankfurt 1, CUHK-Su10, and Shanghai LY strains in the *E. coli* system and detected them by RICT in a preliminary experiment. Further studies are needed to confirm the reactivity of these N-Ag variants by the RICT method described here.

In conclusion, although RICT's analytical sensitivity may be lower than that of PCR, but comparable with the ELISA method, it is nonetheless useful for the detection of N-Ag. In addition, the assay involves a simple manipulation and a short detection time, and there was no cross-reactivity with the other respiratory-related viruses described in this study. Thus, we believe that RICT will be an equally useful method for point-of-care testing to diagnose and treat SARS-suspected patients.

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