Improved detection sensitivity of an antigen test for SARS-CoV-2 nucleocapsid proteins with thio-NAD cycling

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Summary

Antigen tests for infectious diseases are inexpensive and easy-to-use, but the limit of detection (LOD) is generally higher than that of polymerase chain reaction (PCR) tests, which are considered the gold standard. In the present study, we combined a sandwich enzyme-linked immunosorbent assay (ELISA) with thionicotinamide-adenine dinucleotide (thio-NAD) cycling to improve the LOD of antigen tests for coronavirus disease 2019 (COVID-19). For recombinant nucleocapsid proteins of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the LOD of our ELISA with thio-NAD cycling was $2.95 \times 10^{-17}$ moles/assay. When UV-irradiated inactive SARS-CoV-2 was used, the minimum detectable virions corresponding to $2.6 \times 10^4$ RNA copies/assay were obtained using our ELISA with thio-NAD cycling. The assay volume for each test was 100 µL. The minimum detectable value was smaller than that of the latest antigen test using a fluorescent immunoassay for SARS-CoV-2, indicating the validity of our detection system for COVID-19 diagnosis.

Key words

severe acute respiratory syndrome coronavirus 2, antigen test, ultrasensitivity, nucleocapsid protein, thionicotinamide-adenine dinucleotide cycling, coronavirus disease 2019
INTRODUCTION

The emergence of the coronavirus disease 2019 (COVID-19) pandemic has greatly impacted the life and health of people all over the world.\(^1\)–\(^3\)\) A diagnostic method to rapidly and effectively test for COVID-19 infection is in high demand. Real-time reverse transcription polymerase chain reaction (RT-PCR) is generally considered the gold standard test for COVID-19,\(^4\),\(^5\) although the limitations of PCR are widely known.\(^6\)–\(^8\)\) To develop diagnostic methods that are faster and easier to perform than PCR, various state-of-the-art assays based on nucleic acid amplification have been introduced, such as circle-to-circle amplification (C2CA), clustered regulatory interspaced short palindromic repeats (CRISPR), loop-mediated isothermal amplification (LAMP), multiple displacement amplification (MDA), rolling circle amplification (RCA), recombinase polymerase (RPA) assays.\(^9\)–\(^13\)\) As expected, these assays exhibit 100% sensitivity and 100% specificity against PCR.

On the other hand, antigen tests for COVID-19 have attracted much attention for their ease of use, rapidity, and cost-effectiveness.\(^8\),\(^14\),\(^15\)\) A shortcoming of antigen tests, however, is their poor sensitivity for detecting the nucleocapsid proteins of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus strain that causes COVID-19. A meta-analysis of published papers revealed that the mean sensitivity of antigen tests was 56.2% (95% CI: 29.5% to 79.8%) despite a mean specificity of 99.5% (95% CI: 98.1% to 99.9%).\(^16\)\) To overcome this shortcoming, we recently proposed an ultrasensitive enzyme-linked immunosorbent assay (ELISA) for detecting the spike proteins of SARS-CoV-2.\(^17\)\) We aimed to detect the spike proteins because their detection distinguishes SARS-CoV-2 from other SARS coronaviruses (e.g., SARS-CoV).\(^17\)

In our measurement system, we combined a sandwich ELISA and thionicotinamide-adenine dinucleotide (thio-NAD) cycling (Fig. 1).\(^18\)\) Sandwich ELISA uses 2 different antibodies that recognize different epitopes on a target protein, with one of the antibodies (i.e., the detection antibody) labeled with alkaline phosphatase (ALP, EC. 3.1.3.1).\(^19\) When a phosphorylated substrate (e.g., phosphorylated androsterone) is added to the sandwich ELISA system with 3α-hydroxysteroid dehydrogenase (3α-HSD, EC. 1.1.1.50) and its coenzymes (an excess amount of NADH and thio-NAD), 3α-HSD catalyzes the dephosphorylated substrate cycling between the
2 forms of androsterone (3α-hydroxysteroid and 3-ketosteroid). This is called thio-NAD cycling.\(^{20,21}\) In each turn of the cycle, thio-NAD is reduced to thio-NADH, whereas NADH is oxidized to NAD. The absorbance of the accumulated thio-NADH can be measured at 400 nm \((11,900 \text{ M}^{-1} \text{ cm}^{-1})\). A commercially available microplate reader has a 405 nm absorbance measurement, and this absorbance corresponds to the number of target proteins present (Fig. 1).\(^{22−24}\)

Recently, new variants of SARS-CoV-2 have emerged.\(^{25}\) One of these variants was estimated to increase the reproduction number by 0.4 or greater and to increase transmissibility by up to 70%.\(^{26}\) Because these variants are characterized by multiple spike protein mutations,\(^{25,27,28}\) the nucleocapsid proteins should be simultaneously detected with the spike proteins\(^{29}\) in antigen tests for COVID-19. Thus, we must improve the detection sensitivity of an antigen test for the nucleocapsid proteins in SARS-CoV-2. In the present study, we described an ELISA with thio-NAD cycling for SARS-CoV-2 nucleocapsid proteins and estimated the minimum number of detectable virions using inactive SARS-CoV-2.

**MATERIALS AND METHODS**

**Reagents and chemicals** The first antibody \(i.e.,\) capture side) and the second antibody \(i.e.,\) detection side) for SARS-CoV-2 nucleocapsid proteins were purchased from Meridian Life Science, Memphis, TN, USA (Cat #9548 and #9547, respectively). The recombinant antigen for SARS-CoV-2 nucleocapsid proteins was obtained from EastCoast Bio, North Berwick, ME, USA (Cat #LA600). ALP and NADH were purchased from Roche (Basel, Switzerland). 3α-HSD was purchased from Asahi Kasei Pharma (Tokyo, Japan). The thio-NAD was obtained from Oriental East (Tokyo, Japan). 17β-Methoxy-5β-androstan-3α-ol 3-phosphate was synthesized by one of the authors (T.Y.). \(p\)-Nitrophenylphosphate \((p\text{-NPP})\) was purchased from New England Biolabs (Ipswich, MA, USA). SARS-CoV-2 (JPN/TY/WK-521) was propagated in Vero-E6/TMPRSS2 (JCRB1819) cells using Dulbecco’s modified Eagle’s medium (DMEM) solution with 2% fetal calf serum, whose viral infectivity was 5.25 log\(_{10}\) TCID\(_{50}\)/50 μL and RNA amount was 2.6×10\(^7\) copies/μL. The culture supernatant (hereafter referred to as virions) was inactivated by UVB irradiation at 51 μW/cm\(^2\) for 30 min (GL15, Toshiba Lighting & Technology, Yokosuka, Japan).
The other chemicals and the disposable plasticware were of commercial grade.

**ELISA with thio-NAD cycling**  An ELISA with thio-NAD cycling was performed according to the procedures reported previously with slight modification.\textsuperscript{17,30} Briefly, a 100-μL solution of primary antibody, which was adjusted to 1 μg/mL in 50 mM Na\textsubscript{2}CO\textsubscript{3} (pH 9.6), was added to each well of the microplates and incubated at room temperature for 1 h. The microplates were incubated with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) at room temperature for 1 h. Nucleocapsid protein (100 μL) was added to each well and incubated at room temperature for 1 h. The antigen samples were diluted with TBS containing 0.1% BSA. A 100-μL solution of secondary antibody conjugated with ALP and adjusted to 100 ng/mL in TBS including 0.05% Tween 20 and 0.1% BSA was then added to the wells and incubated at room temperature for 1 h. A 100-μL thio-NAD cycling solution was added to each well. This solution contained 1.0 mM NADH, 2.0 mM thio-NAD, 0.4 mM 17β-methoxy-5β-androstan-3α-ol 3-phosphate, and 10 U/mL 3α-HSD in 100 mM Tris-HCl (pH 9.0). Thio-NADH was accumulated in a triangular-number pattern during the cycling reaction, and measured with a microplate reader (Corona Electric SH-1000; Hitachinaka, Ibaraki, Japan) at 405 nm. The 405-nm signals were normalized to those at 660 nm.

For the control experiments, \(p\)-NPP was applied instead of a thio-NAD cycling solution to the sandwich ELISA. The concentration of \(p\)-NPP was 10 mM in Tris-HCl (pH 9.0), which was recommended as the maximum concentration by the manufacturer. The concentration of antigen \textit{(i.e., recombinant nucleocapsid protein)} was 10 ng/mL, which was the maximum value used for an ELISA with thio-NAD cycling. The absorbance was measured with a microplate reader at 405 nm.

To examine whether our ELISA with thio-NAD cycling is applicable to the nucleocapsid proteins in SARS-CoV-2, the UV-inactivated virions were serially diluted with TBS containing 0.1% BSA.

**Statistical analysis**  The experimental data were obtained by subtracting the mean value of the blank signals from each of the corresponding measured datapoints. The limit of detection (LOD) was estimated from the mean of the blanks and the threefold standard deviation (SD) of the blanks.
The limit of quantitation (LOQ) was estimated by the same method used to estimate the LOD, but with the tenfold SD of the blanks. The coefficients of variation (CVs) for nucleocapsid protein antigen were obtained in the examinations of intra-assay and inter-assay reproducibility. The data are expressed as mean ± SD. Significant differences were determined using FreeJSTAT Version 22.0E with \( P < 0.05 \) considered significant.\(^{31)}\)

**RESULTS**

**LOD and LOQ of nucleocapsid proteins using an ELISA with thio-NAD cycling**

Three different experimenters obtained 3 different linear calibration curves for SARS-CoV2 nucleocapsid proteins using an ELISA with thio-NAD cycling in order to estimate the intra-assay CV and inter-assay CV (Fig. 2). One linear calibration curve was expressed as \( y = 1.00 \times 10^{-4}x, R^2 = 1.00 \) (Fig. 2A). This curve was obtained from the absorbance of thio-NADH after 60 min of cycling. The LOD of SARS-CoV2 nucleocapsid proteins, which was obtained statistically using the SD of the blanks and a confidence factor of 3, was \( 2.95 \times 10^{-17} \) moles/assay, and the minimum LOQ with a confidence factor of 10 for SD was \( 6.02 \times 10^{-17} \) moles/assay (n = 3). Because the molecular mass of the antigen was 48 kDa and the assay volume was 100 μL, the LOD and LOQ correspond to 14 and 47 pg/mL, respectively. The intra-assay CV was 5.3% for 50 pg/mL (n = 3).

The linear calibration curve obtained by the second experimenter was expressed as \( y = 7.00 \times 10^{-5}x, R^2 = 1.00 \) (Fig. 2B). This curve was obtained from the absorbance of thio-NADH after 60 min of cycling reaction. The LOD was \( 4.21 \times 10^{-17} \) moles/assay, the minimum LOQ was \( 1.40 \times 10^{-16} \) moles/assay, and the intra-assay CV was 3.5% for 50 pg/mL (n = 3). Finally, the linear calibration curve obtained by the third experimenter was expressed as \( y = 1.00 \times 10^{-4}x, R^2 = 1.00 \) (Fig. 2C). This curve was obtained from the absorbance of thio-NADH after 35 min of cycling reaction. The LOD was \( 5.10 \times 10^{-17} \) moles/assay, and the minimum LOQ was \( 1.70 \times 10^{-16} \) moles/assay. The intra-assay CV was 1.5% for 10,000 pg/mL (n = 3). When the 3 different experimenters obtained the data using 1000 pg/mL, the CV in the inter-assay reproducibility was 15%.

In the control experiments using \( p \)-NPP, we were unable to obtain any signals at 405 nm despite using the maximum concentrations of \( p \)-NPP (i.e., 10 mM) and antigen (i.e., 10 ng/mL).
That is, it was impossible for *p*-NPP to achieve such a highly sensitive detection that can be obtained using an ELISA with thio-NAD cycling.

**Minimum number of virions detected using an ELISA with thio-NAD cycling** Next, 3 different experimenters attempted to detect the minimum number of virions in UV-inactivated SARS-CoV-2 (Fig. 3). For these experiments, the virions were diluted serially from the RNA amount of $2.6 \times 10^7$ copies/μL. The absorbance of thio-NADH was measured after 40 min of thio-NAD cycling. At a concentration over $2.6 \times 10^4$ RNA copies/assay, the 3 experimenters consistently obtained signals higher than those of the blank (n = 3 each). Statistically, there was a significant difference between the results of blank and those of $2.6 \times 10^4$ RNA copies/assay ($P = 0.021$ by paired *t*-test), whereas there was no significant difference between the results of blank and those of $2.6 \times 10^3$ RNA copies/assay ($P = 0.256$ by paired *t*-test) (Fig. 3). Thus, the present results showed that our ELISA with thio-NAD cycling detected SARS-CoV-2 at the level of $10^4$ virions/assay, because a single SARS-CoV-2 virion contains single-stranded RNA.

**DISCUSSION**

In the present study, we succeeded in detecting SARS-CoV-2 at the level of $10^4$ virions/assay using an ELISA with thio-NAD cycling. We compared this result with those of a real-time RT-PCR test for RNA and a fluorescent antigen test for nucleocapsid proteins. Real-time RT-PCR provides a precise assay for calculating the LOD (*e.g.*, a few copies/assay) for various nucleic acids, including SARS-CoV-2 RNA. Many public institutes around the world have offered protocols for detecting as little as a few copies/μL of SARS-CoV-2. However, SYBR Green real-time PCR at threshold cycle (Ct.) 35 indicated $10^4$ RNA copies/μL. On the other hand, a latest antigen test using a fluorescent immunoassay had a LOD of $2 \times 10^3$ copies/μL. Because the detection sensitivity of virions of $10^4$ virions/assay in our ELISA with thio-NAD cycling corresponds to $10^2$ RNA copies/μL, our colorimetric ELISA provides a great advantage for accurate and sensitive testing. In addition, RNA-dependent RNA polymerase (RdRp) may mutate lower frequently than the spike and nucleocapsid proteins, and thus RdRp seems a good target for a marker of diagnosis of COVID-19. In the future studies, we should try to do the
experiments using RdRp.

Our ELISA with thio-NAD cycling is very versatile if the suitable antibodies against target antigens (proteins) are available. For example, for the diagnosis of acquired immunodeficiency syndrome (AIDS), a trace amount of the human immunodeficiency virus (HIV)-1 p24 antigen must be detected, and our ELISA with thio-NAD cycling can detect 0.0065 IU/assay, which is less than the value mandated for a CE-marked HIV antigen/antibody assay.\textsuperscript{38} For the diagnosis of tuberculosis, a culture test is considered the gold standard, but it takes too much time because the bacteria (\textit{Mycobacterium tuberculosis}) grow very slowly. Thus, a rapid and highly sensitive test has long been required. Our ELISA with thio-NAD cycling can provide a diagnosis of tuberculosis in a few hours with almost the same detection sensitivity as that of culture tests due to the measurement of a trace amount of proteins (MPT64) secreted by heating.\textsuperscript{39,40} Similarly, for lifestyle-related diseases, minute quantities of insulin can be precisely measured by our ELISA with thio-NAD cycling,\textsuperscript{18,41} and trace amounts of adiponectin can be detected, not only in serum but also in urine.\textsuperscript{42,43} Thus, our ELISA with thio-NAD cycling eliminates the need for more invasive tests.

Variants of SARS-CoV-2 having mutations in their spike proteins have emerged throughout the world in 2021.\textsuperscript{25,27,28} This situation has required us to improve the detection sensitivity of antigen tests for SARS-CoV-2 nucleocapsid proteins. The present results demonstrated that our ELISA with thio-NAD cycling offers ultra-high detection sensitivity. Previous reports demonstrated that the symptoms of COVID-19 vary from mild flu-like symptoms to very severe respiratory symptoms, and it is not easy to diagnose which infectious disease the patient has contracted.\textsuperscript{44,45} Our ELISA test with thio-NAD cycling using microplates is suitable for these cases, because an ELISA can be used for a multiplex, simultaneous, high-throughput detection.

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Fig. 1. Schematic representation of an ELISA with thio-NAD cycling for SARS-CoV-2 nucleocapsid proteins (N proteins) by coupling a sandwich ELISA with thio-NAD cycling. In addition to the 2 specific types of antibodies in the sandwich ELISA, we used alkaline phosphatase (ALP), the androsterone derivative, 3α-hydroxysteroid dehydrogenase (3α-HSD), and its coenzymes (NADH and thio-NAD). During the thio-NAD cycling reaction, thio-NADH accumulated in a triangular-number fashion. Accumulated thio-NADH was measured directly at an absorbance of 405 nm without interference from the other cofactors. (Color figure can be accessed in the online version.)
Fig. 2. Linear calibration curves obtained from the absorbance for recombinant SARS-CoV-2 nucleocapsid proteins using an ELISA with thio-NAD cycling.

Three datasets measured by 3 different experimenters are presented as (A), (B), and (C). The absorbance was obtained from the cycling reaction time designated in the text. The number of experiments was 3 each. The antigen was applied in the range of 50–10,000 pg/mL.
Fig. 3. Detection of nucleocapsid proteins in UV-inactivated SARS-CoV-2.

Because a SARS-CoV-2 virion contains single-stranded RNA, the RNA copies in the x-axis correspond to the number of virions. Three datasets measured by 3 different experimenters are presented as (A), (B), and (C). The absorbance was obtained from a cycling reaction time of 40 min. The number of experiments was 3 each. The error bars show the standard deviation. There is a significant difference between the results of $2.6 \times 10^3$ RNA copies/assay and those of $2.6 \times 10^4$ RNA copies/assay ($P = 0.013$ by paired $t$-test, $n = 9$ each). The horizontal dashed line indicates the absorbance values of the blank. The 3 experimenters consistently obtained signals that were higher than the blank at concentrations over $2.6 \times 10^4$ RNA copies/assay (see the vertical dashed line). (Color figure can be accessed in the online version.)