NRC·CNRC

NRC Publications Archive
Archives des publications du CNRC

Comparison of the analytical sensitivity of COVID-19 rapid antigen tests in Australia and Canada

Toft, Casey J.; Stocks, Bradley B.; Schaeffer, Patrick M.

This publication could be one of several versions: author's original, accepted manuscript or the publisher's version. / La version de cette publication peut être l'une des suivantes : la version prépublication de l'auteur, la version acceptée du manuscrit ou la version de l'éditeur.
For the publisher's version, please access the DOI link below./ Pour consulter la version de l'éditeur, utilisez le lien DOI ci-dessous.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/j.talanta.2024.126147
Talanta, 275, pp. 1-7, 2024-04-23

NRC Publications Archive Record / Notice des Archives des publications du CNRC :
https://nrc-publications.canada.ca/eng/view/object/?id=607b827a-2678-4af0-8f01-bb05f9aa9298
https://publications-cnrc.canada.ca/fra/voir/objet/?id=607b827a-2678-4af0-8f01-bb05f9aa9298

Access and use of this website and the material on it are subject to the Terms and Conditions set forth at
https://nrc-publications.canada.ca/eng/copyright
READ THESE TERMS AND CONDITIONS CAREFULLY BEFORE USING THIS WEBSITE.

L'accès à ce site Web et l'utilisation de son contenu sont assujettis aux conditions présentées dans le site
https://publications-cnrc.canada.ca/fra/droits
LISEZ CES CONDITIONS ATTENTIVEMENT AVANT D'UTILISER CE SITE WEB.

Questions? Contact the NRC Publications Archive team at PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca. If you wish to email the authors directly, please see the first page of the publication for their contact information.

Vous avez des questions? Nous pouvons vous aider. Pour communiquer directement avec un auteur, consultez la première page de la revue dans laquelle son article a été publié afin de trouver ses coordonnées. Si vous n'arrivez pas à les repérer, communiquez avec nous à PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca.

National Research Council Canada | Conseil national de recherches Canada

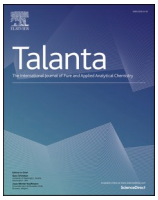
Canada

Talanta 275 (2024) 126147

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta



Comparison of the analytical sensitivity of COVID-19 rapid antigen tests in Australia and Canada

Casey J. Toft [a], Bradley B. Stocks [b,**], Patrick M. Schaeffer [a,*]

[a] College of Public Health, Medical and Veterinary Sciences, James Cook University, Douglas, Queensland, Australia
[b] Metrology, National Research Council Canada, 1200 Montreal Road, Ottawa, Ontario, K1A 0R6, Canada

ARTICLE INFO

Keywords:
COVID-19
Influenza
Analytical sensitivity
Rapid antigen test
Quality control
Nucleocapsid protein

ABSTRACT

Rapid testing has become an indispensable strategy to identify the most infectious individuals and prevent the transmission of SARS-CoV-2 in vulnerable populations. As such, COVID-19 rapid antigen tests (RATs) are being manufactured faster than ever yet lack relevant comparative analyses required to inform on absolute analytical sensitivity and performance, limiting end-user ability to accurately compare brands for decision making. To date, more than 1000 different COVID-19 RATs are commercially available in the world, most of which detect the viral nucleocapsid protein (NP). Here, we examine and compare the analytical sensitivity of 26 RATs that are readily available in Canada and/or Australia using two NP reference materials (RMs) – a fluorescent NP-GFP expressed in bacterial cells and NCAP-1 produced in a mammalian expression system. Both RMs generate highly comparable results within each RAT, indicating minimal bias due to differing expression systems and final buffer compositions. However, we demonstrate orders of magnitude differences in analytical sensitivities among distinct RATs, and find little correlation with the median tissue culture infectious dose ($TCID_{50}$) assay values reported by manufacturers. In addition, two COVID-19/Influenza A&B combination RATs were evaluated with influenza A NP-GFP. Finally, important logistics considerations are discussed regarding the robustness, ease of international shipping and safe use of these reference proteins. Taken together, our data highlight the need for and practicality of readily available, reliable reference proteins for end-users that will ensure that manufacturers maintain batch-to-batch quality and accuracy of RATs. They will aid international public health and government agencies, as well as health and aged care facilities to reliably benchmark and select the best RATs to curb transmission of future SARS-CoV-2 and influenza outbreaks.

1. Introduction

COVID-19 remains a public health challenge. Hospitalizations are rising again for the first time in several months due to the emergence of new variants [1]. The World Health Organization (WHO) has recently published a 'step-by-step' guide for countries to develop a national genomics-based pandemic surveillance strategy [2]. As such, countries will need to step up their preparedness in case the pandemic resurges. However, COVID-19 has transitioned to an endemic phase and the responsibility of detection and isolation is now mostly on individuals. The technical and logistics issues associated with RT-qPCR testing has shifted the majority of COVID-19 testing to be carried out using rapid antigen tests (RATs) [3]. RATs are most useful when the time required post-infection to generate a positive result is minimized. The analytical limit of detection (LOD) of a RAT is one of the most important quantitative measures that can inform end-users on this aspect.

The vast majority of RATs developed to diagnose an active SARS-CoV-2 infection are based on detection of the viral nucleocapsid protein (NP) as the target antigen. However, the preferred method to assess the LOD of COVID-19 RATs remains the median tissue culture infectious dose ($TCID_{50}$) assay despite several concerns highlighted recently [4]. Notably, $TCID_{50}$ is an indirect measure of the antigen being detected, and the laboratory specific conditions used to culture a virus (e.g. cell type, culture conditions and time) affect the relative abundance of both NP and infectious virions making direct comparison of data difficult [5–8]. Furthermore, NP is located inside the virus [9–11] and this encapsidated form is not accessible to the capture or detection antibodies in the RAT and as such unquantifiable. Therefore, RATs

* Corresponding author.
** Corresponding author.
E-mail addresses: bradley.stocks@nrc-cnrc.gc.ca (B.B. Stocks), patrick.shaeffer@jcu.edu.au (P.M. Schaeffer).

https://doi.org/10.1016/j.talanta.2024.126147
Received 23 November 2023; Received in revised form 18 April 2024; Accepted 22 April 2024
Available online 23 April 2024
0039-9140/© 2024 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

exclusively capture and detect 'free' NP that is released from infected cells and viruses upon lysis. This propounds that well-characterized NP reference standards should be most accurate to evaluate the LOD of COVID-19 RATs and demonstrate commutability.

The WHO recognized the importance of accurately assessing the LOD of RATs and proposed a lyophilized preparation of formaldehyde-inactivated cell culture-grown SARS-CoV-2 Omicron variant (sub-variant BA.1) as the WHO International Standard for SARS-CoV-2 antigen to act as a higher order reference and calibrate secondary reference materials [12]. A subsequent multi-center collaborative study further evaluated and confirmed the suitability of the first candidate over seven inactivated virus cultures and one recombinant SARS-CoV-2 NP sample using a range of point of care and laboratory-based antigen detection tests [12]. The concentration of NP was determined by isotope dilution mass spectrometry (IDMS) in all samples. Additionally, the concentration of viral RNA was determined in all inactivated virus culture samples. Surprisingly, the recombinant NP was found to be more variable than the formaldehyde-inactivated virus.

Virus cultures are laborious, time-intensive and hazardous, and require accurate NP quantitation to be informative. Astoundingly, $TCID_{50}$ data have been widely used as an alternative to inform on the LOD of RATs. A recent evaluation highlighted that the $TCID_{50}$ values reported for 10 commercially available RATs were highly discordant against their LOD measured with a green fluorescent protein tagged NP (NP-GFP) as reference material [4]. Stocks et al. have characterized an SI-traceable SARS-CoV-2 NP reference material (NCAP-1) [13], however to our knowledge it has not been employed to evaluate the LODs of COVID-19 RATs.

We selected 26 commercially-available brands of RATs that were approved for personal use in Australia or Canada at the time of this comparative study (Q1 2023). The analytical sensitivity of these RATs was evaluated either in Canada and/or Australia, depending on their availability, with both NCAP-1 and NP-GFP. The results obtained with NCAP-1 and NP-GFP were highly concordant with respect to intra-test reproducibility (NCAP-1/NP-GFP) and ranking of RAT performance. In addition, a GFP-tagged influenza A NP (IAV H5N1 NP-GFP) [14] was also employed for the first time to compare the detection performance of two recently approved influenza A, B and COVID-19 multiplex tests. Taken together with the simplification of logistics and biosafety issues, our data provide support for recombinant NP use as a robust alternative reference material for global benchmarking and reporting of the LOD of antigen detection devices that are based on NP detection.

2. Materials and methods

2.1. Expression and purification of proteins

The SARS-CoV-2 NP-GFP, MERS-CoV NP-GFP and Influenza A virus NP-GFP were produced in *E. coli* BL21(DE3)RIPL as previously described [4,14]. MERS-CoV NP-GFP and Influenza A virus NP-GFP were purified using the same procedure as described in our previous RAT evaluation study [4]. The purification procedure for SARS-CoV-2 NP-GFP was slightly modified to include more stringent conditions for his-tag binding and an extra wash step was included (lysis and wash buffers were supplemented with 40 mM imidazole). Additionally, to streamline protein analyses and lyophilization which was performed post purification, β-mercaptoethanol was not included in the lysis, wash and elution buffers and glycerol (10 % v/v) was replaced with sucrose (10 % w/v). Protein concentrations were determined by Bradford Assay, DC assay (Bio-Rad) and by GFP-fluorescence and the purity assessed by SDS-PAGE. NCAP-1 was from the National Research Council Canada and the reference material production has been described previously [13,15]. Units contained 10 μM NP solutions in 50 mM Tris buffer (pH 8) with 150 mM NaCl.

2.2. Lyophilization of NP-GFP

SARS-CoV-2 NP-GFP and MERS NP-GFP were diluted in PBS +10 % (w/v) sucrose to a final concentration of 10 μM and aliquots of 100 μL were prepared in screw cap tubes. Aliquots (100 μL) were freeze-dried using a SCANVAC CoolSafe (LABOGENE) for 3-h. Lyophilized samples were posted to Canada from Australia at ambient temperature and the pellets dissolved in 95 μL of ultrapure water to reconstitute a 10 μM stock solution (the volume of water required for reconstitution was calculated based on the GFP-fluorescence pre- and post-lyophilization). For consistency, lyophilized NP-GFP samples were also kept at room temperature in Australia for the duration of the transit (7-days) and resuspended in the same conditions. The stability of NP-GFP proteins was determined using fluorescence readings at 1 μM pre-lyophilization and post-reconstitution. Reconstituted proteins were stored at −80 °C.

2.3. Independent evaluation of COVID-19 rapid antigen tests

The LOD of 26 COVID-19 RATs commercially available in Australia and/or Canada was assessed across two laboratories (JCU Australia and NRC Canada). For each RAT, 10 μL of SARS-CoV-2 NP-GFP or NCAP-1 solutions at various concentrations (5, 1, 0.1 and 0.05 nM in PBS) were used to spike the manufacturers' provided buffers. The spiked buffer samples were mixed by inverting five times and used according to the manufacturers' specific instructions. Results were captured after the time indicated and alongside a 'control RAT' printed with a defined colour value line (Hex:#A0A0A0) to normalize band intensities and allow accurate comparison of RAT LOD data. Band intensities were then quantified using ImageJ including the 'control RAT' for normalisation (Fig. S1 for details). To ensure analysis of band intensity was consistent between JCU and NRC, a subset of RAT images from each country were cross analyzed with highly similar values obtained. For each RAT, MERS-CoV NP-GFP at 100 nM was used as a negative control and to assess test specificity. Prior to production of each dilution series, the GFP fluorescence of stock NP-GFP suspensions at 1 μM was quantified as a quality control measure (BMG LABTECH microplate reader, ex485, em520, 1500 gain).

2.4. Evaluation of the influenza A, B and COVID-19 multiplex tests

The analytical sensitivity of two combined influenza A, B and COVID-19 tests were assessed using the same approach as described for the COVID-19 RATs except 10 μL of influenza A virus (H5N1) NP-GFP was spiked into the provided buffers alongside SARS-CoV-2 NP-GFP. The GFP fluorescence of proteins were normalized at 1 μM (BMG LABTECH microplate reader, ex485, em520, 1500 gain) prior to evaluation to ensure the virus specific detection capabilities could be accurately compared.

2.5. Statistical analysis

Statistical analyses and the number of repeats are indicated in the relevant figure legends and methods. Analyses were performed using GraphPad Prism 9. A ratio paired *t*-test was used to evaluate the NP-GFP and NCAP-1 interassay agreements. Spearman's correlation was used to measure the strength of association between band intensities obtained using NP-GFP and NCAP-1 for 44 paired RAT evaluations.

3. Results

3.1. NP-GFP and NCAP-1 reference standards

In this study, 16 COVID-19 RATs approved by the Therapeutic Goods Administration (TGA) Australia and 10 COVID-19 RATs approved by Health Canada were evaluated with two different SARS-CoV-2 NP reference proteins (i.e. SARS-CoV-2 NP-GFP and NCAP-1) using a multi-

center approach. The SARS-CoV-2 NP-GFP was produced with a bacterial expression system and the NCAP-1 with a mammalian expression system (see Fig. S2 for a detailed comparison of the two protein sequences and tags as well as post-translational modifications). The SARS-CoV-2 NP-GFP (produced in Australia) was shipped in lyophilized form at ambient temperature to the NRC Canada, while NCAP-1 (produced in Canada) was shipped to JCU Australia on dry ice. RAT evaluations were performed by spiking known concentrations of these protein suspensions into the provided buffers and run as per the manufacturers' instructions. At the time of this study, only one RAT brand (Panbio) was available and approved for use both in Canada and Australia, and as such could be evaluated in both laboratories. All other brands of RATs could only be assessed either at NRC Canada or JCU Australia. In spite of this limitation, the study was designed to allow a systematic comparison of RAT as well as reference material performances. Of note, using two reference proteins which differ in their design, constituents, and

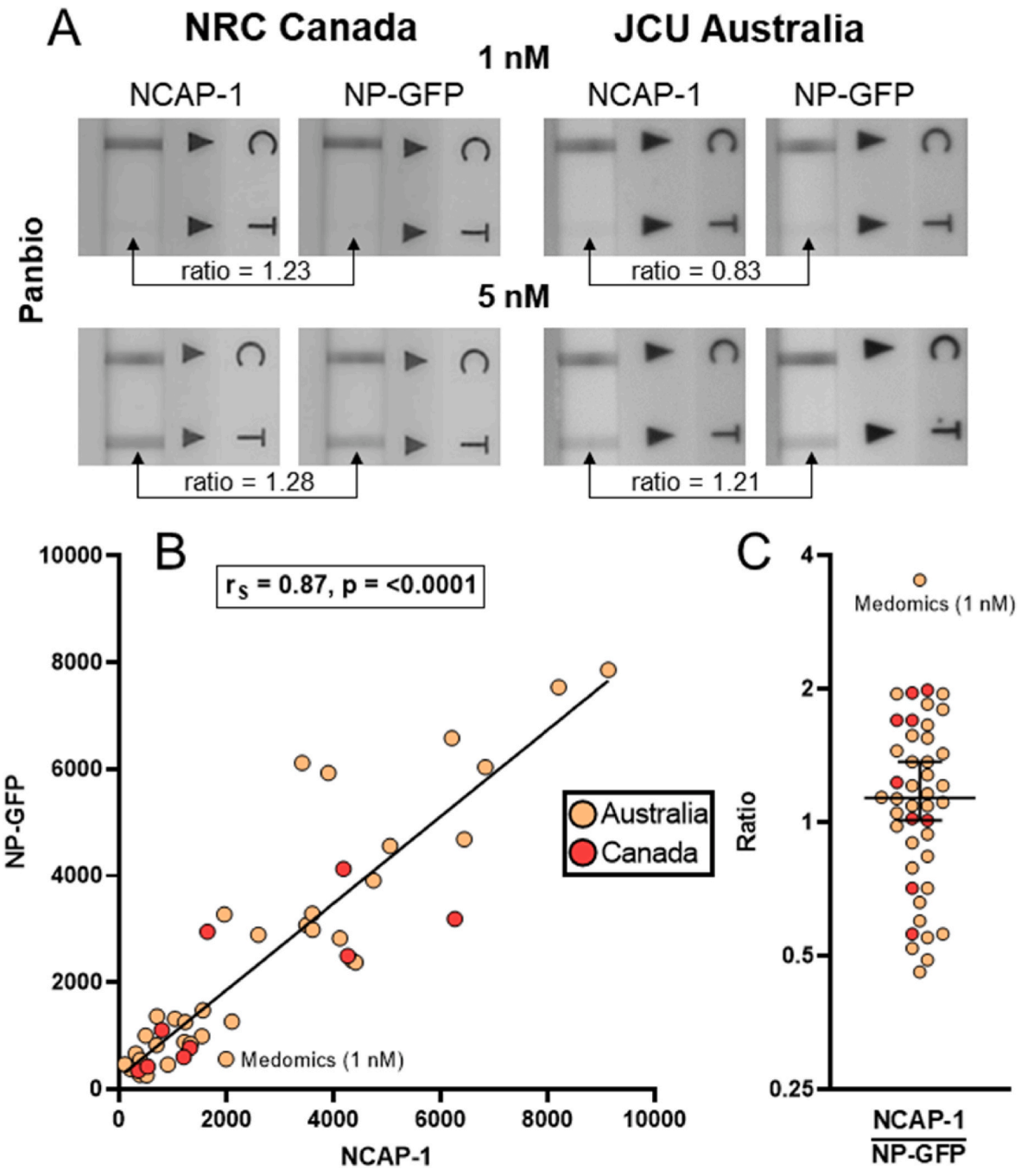


Fig. 1. Comparison of the band intensities obtained using two independently produced SARS-CoV-2 NP reference materials (A) Visual comparison of the Panbio RAT that was independently assessed in Canada and Australia with both NP-GFP and NCAP-1. Protein concentrations are indicated and correspond to the concentrations in the samples used to spike the RAT buffers. Band intensity ratio values are calculated as NCAP-1/NP-GFP (see Fig. S1 for more detail) (B) Scatter plot of the correlation coefficient (spearmen r = 0.87) between band intensities obtained using NP-GFP and NCAP-1 for all 44 paired RAT evaluations tested at various concentrations (0.05 nM–5 nM) (p = < 0.0001). Data points are coloured to indicate if the tests were evaluated at NRC Canada (red) or at JCU Australia (light brown) (C) Band intensity ratios with all paired data (NCAP-1/NP-GFP) plotted on a logarithmic scale to show fold-differences between band intensities obtained for both reference materials. The median with the standard deviation is shown. A ratio paired *t*-test was used to determine if there was a statistically significant difference between band intensity obtained using NP-GFP and NCAP-1 for all paired data (p = 0.43, t = 0.79, df = 43).

production as independent repeats to compare RAT performance, allows evaluation of reproducibility rather than just repeatability.

The Panbio RAT was near its LOD when the buffer was spiked with a 1 nM solution of SARS-CoV-2 NP-GFP and NCAP-1. This is consistent with a lower clinical and analytical sensitivity of the Panbio RAT in comparison with other brands as reported previously [4,16,17]. Importantly, highly similar band intensities were obtained with NP-GFP and NCAP-1 in both laboratories (Fig. 1A). Band intensities were quantitatively assessed using ImageJ integration to eliminate visual bias. The band intensities obtained using NCAP-1 strongly correlated with that of NP-GFP (correlation coefficient = 0.87, $p < 0.0001$) for 25 RATs evaluated at several different concentrations ranging between 0.05 and 5 nM (Fig. 1B). Note here that the CovClear RAT gave a negative result even at 100 nM (Fig. S3) and was therefore excluded from the comparison. The ratio of NCAP-1/NP-GFP was calculated for each RAT and plotted to showcase the limited data variability obtained across both research centers (Fig. 1C). With the exception of the Medomics RAT yielding a 3.5-fold absolute difference when evaluated with SARS-CoV-2 NP-GFP and NCAP-1 (at 1 nM), all other tests produced band intensities within 2-fold of each other. It is important to note that the intra-lot variability of these tests is unknown and could explain the discrepancy obtained with the Medomics RAT at 1 nM as the ratio obtained at 5 nM for the same RAT was only 1.85. Overall, no significant difference between the means of the band intensities obtained using NCAP-1 and NP-GFP was observed in a pairwise comparison (p-value = 0.43, df = 43). Taken together, these data suggest that NCAP-1 and NP-GFP are antigenically identical despite presence of different tags and protein sequences and their different production processes.

3.2. Comparison of RAT LOD

With no discernible difference between RATs evaluated with NP-GFP or NCAP-1, we reasoned the data obtained from both reference materials could be used as independent duplicate measures to compare their analytical performances. As a minimum, RATs were assessed at 0.1 nM followed by an additional assessment at 0.05 nM for tests that were positive, or 1 nM for tests that were negative (Fig. 2A). Analytical specificity was also systematically evaluated using spiked MERS-CoV NP-GFP samples as negative controls and all 26 tests produced a negative result (Fig. S4).

The six best performing RATs produced detectable/visible test lines with buffers spiked with 0.05 nM suspensions of SARS-CoV-2 NP-GFP and NCAP-1. These included the Canadian-approved BTNX Cassette, Flowflex and Medsup as well as the Australian-approved Fanttest, Innoscreen and Juschek. Of note, an additional dilution to failure was deemed unnecessary as the band intensities produced at this concentration were very faint (borderline) and indicative of their LOD (Fig. 2A). The majority of tests (16/26) were unable to produce a visible test line when buffers were spiked with 0.1 nM of either NP reference protein. RATs were further evaluated at 1 nM and 5 nM if they failed to produce a detectable test line at lower concentrations (Fig. 2A).

RAT brands were grouped based on their test line intensities at the three lowest reference protein concentrations (Fig. 2B) to showcase their differences in performance. Test lines were normalized using a 'control RAT line' (see Fig. S1 for details), for quantitative comparison. The least sensitive RATs (Group 4) were near their LOD at 1 nM NP reference protein while RATs in Group 3 (orange) produced significantly more intense test lines (Fig. 2B). The results of the best performing Australian-approved RAT 'Fanttest', is also shown at each concentration to highlight its superior detection capability. A >10-fold higher band intensity was obtained for Fanttest at 1 nM compared to some of the Group 4 RATs which correlates well with its >10-fold increase in analytical sensitivity over some of these tests *i.e.* the LOD of Fanttest is ~0.05 nM compared to ~1 nM for Panbio (Figs. 1A and 2B). Of note, there was no statistical correlation between the reported $TCID_{50}$ values and the lowest reference protein concentration detected for each RAT (r = 0.07, P = 0.76, see

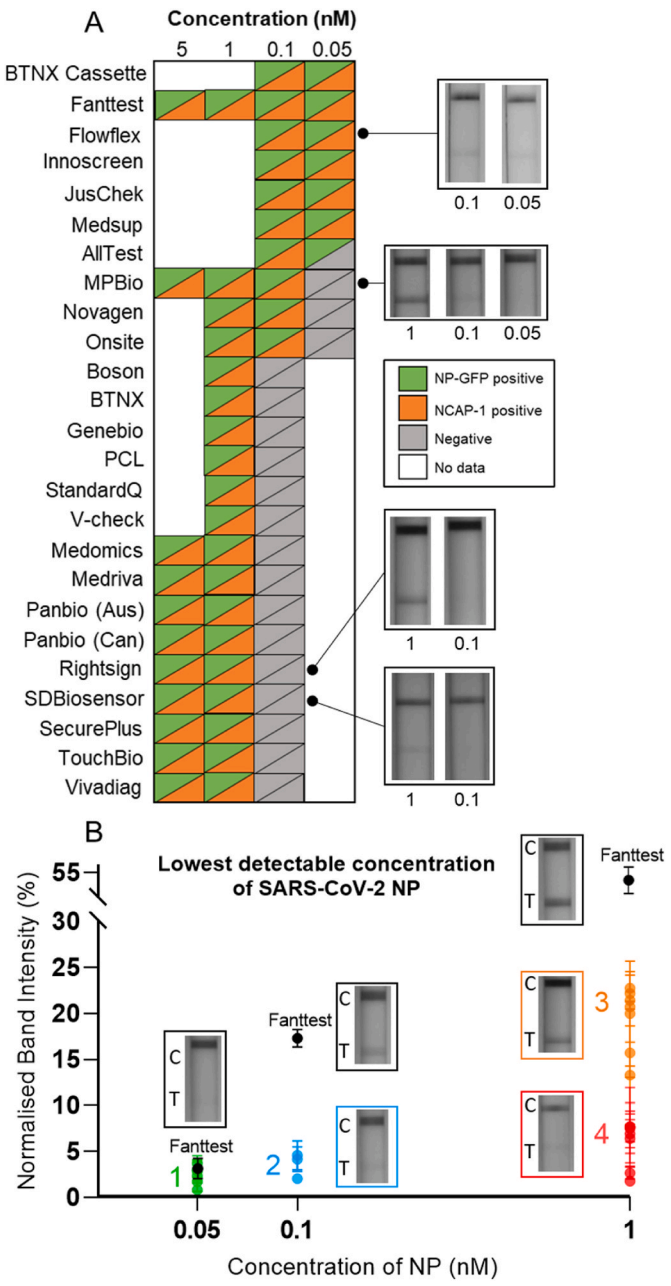


Fig. 2. (A) Comparison of the performance of RATs using known concentrations of NP-GFP and NCAP-1 (as duplicates) spiked into the provided buffers and run as per manufactures instructions. (B) Plotted data is of the lowest detected concentration of NP for each RAT and is normalized by the band intensity of the control RAT (see materials and methods for more details) and grouped according to their similarity in performance (i.e. test line intensity). The best performing RAT (Fanttest) is also plotted at 0.1 nM and 1 nM to showcase its superior performance (black dot). **Group 1** (positive at 0.05 nM): Alltest, BTNX Cassette, Flowflex, Fanttest, Innoscreen, JusChek and Medsup. **Group 2** (positive at 0.1 nM): MPBio, Novagen and Onsite. **Group 3** (positive at 1 nM): Boson, Genebio, Rightsign, SecurePlus, V-chek and VivaDiag. **Group 4** (positive at 1 nM): BTNX, PCL, Medriva, Medomics, PanBio (Aus), PanBio (Can), SDBiosensor, StandardQ and Touchbio. Representative band intensities are shown for each group. See Fig. S3 for CovClear raw data.

Fig. S5).

3.3. Assessment of combined influenza A, B and COVID-19 tests with IAV (H5N1) NP-GFP

While influenza RATs have been available for personal use in some countries, they were not approved in Australia at the time of the study. However, two COVID-19 RAT brands were available in Australia that detect IAV, IBV and SARS-CoV-2 in the same cassette (see Fig. S4 – Fanttest and Touchbio). These multiplex RAT brands, similar to previous influenza A/B RATs, detect the presence of IAV and IBV NP which are the most abundantly expressed protein during infection [18,19]. While NP sequences are highly conserved within a virus type and its subtypes [18,20], they are highly divergent between different types [21]. Here, we used IAV (H5N1) NP-GFP to further evaluate the analytical sensitivity of two influenza A, B and COVID-19 RAT brands that have recently been approved in Australia. Concentrations of SARS-CoV-2 and IAV (H5N1) NP-GFP were matched via fluorescence quantitation (see materials and methods) allowing a direct comparison of the virus specific detection capabilities of these tests. While IAV (H5N1) subtype has limited relevance in humans presently, its NP shares 91 % and 94 % sequence identity with NP from the currently circulating H3N2 and H1N1 strains respectively (Fig. S6). As such, IAV (H5N1) NP-GFP remains a relevant subtype-specific reference standard to compare the performance of IAV RATs, although further assessment with NP from other circulating strains is warranted.

The Touchbio RAT was ~20-fold less sensitive for SARS-CoV-2 NP-GFP than the Fanttest RAT despite identical reported $TCID_{50}$/mL values (Figs. 2A and 3A-B). Regarding IAV detection, both tests produced faint test lines with 1 nM IAV (H5N1) NP-GFP (Fig. 3B). Of note, one batch of Touchbio RATs yielded faint false IBV positive test lines with all concentrations of IAV (H5N1) NP-GFP (cf intensity of IAV test lines at 0.1 nM IAV (H5N1) NP-GFP in Fig. 3B–C). The subsequent batch did not produce detectable IBV test lines at any concentration nor an IAV test line at the lowest concentration (0.1 nM IAV (H5N1) NP-GFP). Analytical specificity was evaluated with 100 nM SARS-CoV-2 NP-GFP (i.e. concentration of spike sample). Here, no RAT produced a positive IAV or IBV test line.

4. Discussion

The production of SARS-CoV-2 NP reference proteins to benchmark RATs is of high global interest. In this study, 26 RATs were used to compare the suitability of two different recombinant NP as reference standards. Both recombinant NP-GFP and NCAP-1 include the original Wuhan SARS-CoV-2 NP amino acid sequence (>98 % identity with the currently circulating Omicron XBB.1.5 and JN.1 proteins - Fig. S7) and were produced in bacterial and mammalian expression systems respectively. NP-GFP and NCAP-1 were independently compared at NRC Canada and JCU Australia with RAT brands that were approved in each respective country. Of note, the low number of overlapping, government-approved tests in both Australia and Canada (Panbio, Flowflex, Carestart and SDBiosensor) and their limited commercial availability precluded a systematic multi-center evaluation. As such, only the Panbio RAT was cross-examined and used for reference. Overall, our combined data showed no significant difference in RAT test line intensities between NP-GFP and NCAP-1. That is, both proteins produced highly agreeable test lines across all RAT brands at each concentration (Fig. 1) supporting their suitability as reference standards for rapid and reliable comparison of RAT brands.

Overall, only 6 out of the 26 COVID-19 RATs yielded faint test lines using buffer spiked with the lowest concentrations of NCAP-1 or NP-GFP suspensions (see 0.05 nM and group 1 in Fig. 2). Four more RATs could detect buffer spiked with 0.1 nM reference protein suspensions (group 2). From here it reasons that any RAT performing below this detection threshold i.e. group 3–4 and especially CovClear (which has been revoked by the TGA Australia in 2022 due to insufficient sensitivity), would be redundant or even undesirable for effective infection control. Of note, both influenza A, B and COVID-19 multiplex RAT brands were poor to detect IAV (H5N1) NP-GFP comparing with the worst performing COVID-19 RATs (group 4). Indeed, both tests yielded only faint test lines using buffer spiked with a 1 nM IAV (H5N1) NP-GFP suspension. In addition, false positive IBV test lines were obtained with one of the Touchbio batches which is concerning as it is already a poor test for COVID-19 (group 4). Additional benchmarking with NP from circulating H3N2 and H1N1 strains are obviously needed for further evaluation of these multiplex RATs.

NP reference standards are essential for manufacturers of laboratory-based immunoassays and RATs, and would also benefit regulatory bodies and other end-users for informed decision making to easily compare, select or revoke RAT brands. Indeed, the production of SARS-CoV-2 viral cultures and their subsequent inactivation is a laborious, time-consuming and costly process that requires appropriate facilities with high biosafety levels, restricting its production and availability for independent testing. Moreover, the complex logistics of transporting bio-hazardous reference material adds additional hurdles and further limits their overall accessibility. The complexity of NP concentration determination in viral cultures has led to indirect measures such as quantitative reverse-transcriptase PCR and even the flawed $TCID_{50}$ assay to be used as proxy methods. Here we demonstrate that NCAP-1 and NP-GFP are robust and reliable as reference proteins and can be safely shipped without biosafety concerns, in frozen solution or at ambient temperature in lyophilized form. As such, NCAP-1 and NP-GFP are safe to be used in any laboratory and non-laboratory settings, significantly expanding their accessibility to potential end-users for independent comparison of the multitude of government-approved RAT brands.

Recently, the WHO selected an inactivated virus culture over recombinant NP as the first reference standard for COVID-19 RATs and laboratory-based immunoassays [12]. The decision stemmed from (a) the possibility that tests could be developed that detect different SARS-CoV-2 antigens than NP and (b) the findings of a multicenter comparative study that showed that inactivated virus cultures standardized by the concentration of NP yielded slightly more homogenous LOD values across assays developed by various manufacturers [12]. Of note, only one sample of NP was compared in that study, while eight viral cultures were cross-compared. We have previously shown that interassay variability was comparable to the variability obtained between two different NP proteins produced in *E. coli* (i.e. NP and NP-GFP) [4]. In the present study we found that both NCAP-1 and NP-GFP produced highly similar test line intensities at all concentrations tested with the 26 RAT brands that were evaluated.

The NP sequences from currently circulating strains XBB.1.5 and JN.1 include five sequence variations compared to the wildtype strain (P13L, E31_S33 deletion, R203K, G204R and S413R). JN.1 NP contains an additional Q226K mutation. As such, these mutations account for less than 2 % change in the NP sequence. Nonetheless, a study exploring the impact of these mutations on the analytical sensitivity of the best performing RATs would be of interest. It would also be interesting to compare the best performing RATs using a selection of identical clinical samples. Taken together, our comparative data, the simplified biosafety and shipment logistics, and the fact that the vast majority of COVID-19 RATs were developed to detect NP analytes, bring forth a strong case for NP-GFP and NCAP-1 and new versions thereof to be included as international reference standards.

Funding

C. J. Toft is supported by a merit-based Research Training Program Scholarship (James Cook University). Sandpit to Seed (S2S) funding (James Cook University) was awarded to PS and CT to perform this study.

A

	Reported $TCID_{50}$/mL for CoV-2	Detection limit# of CoV-2 NP-GFP (nM)	Reported $TCID_{50}$/mL for different IAV subtypes	Detection limit# of IAV NP-GFP (nM)
Fanttest	100	0.05	200-2000	1
TouchBio	100	1	100-50,000	1

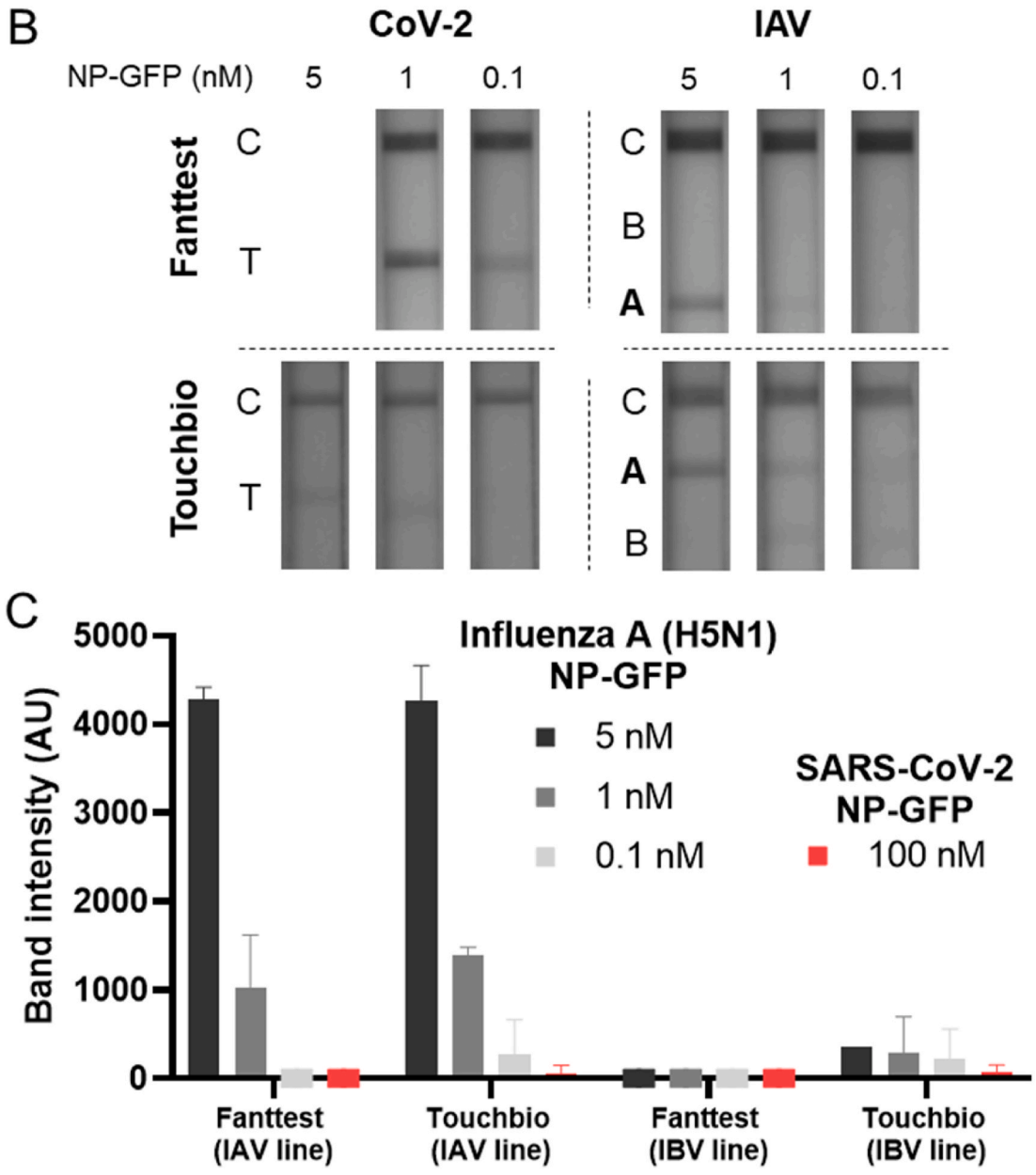


Fig. 3. Assessment of the of the influenza A, B and COVID-19 dual tests using SARS-CoV-2 and IAV (H5N1) NP-GFP. (A) Reported analytical performance of Fanttest and Touchbio using the $TCID_{50}$ assay compared to the detection limit obtained in this study. (B) Comparison of the band intensities obtained at the COVID-19 and IAV test lines between Fanttest and Touchbio using equimolar concentrations of SARS-CoV-2 NP-GFP and IAV NP-GFP. The faint line produced for Touchbio at 0.1 nM of IAV NP-GFP in one of the replicates is a similar intensity as the background IBV test line and thus considered a background response. (C) Quantified band intensities of the IAV and IBV test lines. A 100 nM SARS-CoV-2 NP-GFP 'spike sample' (red) was used to evaluate test specificity and serve as a negative control. #Lowest detectable concentration of NP-GFP in the sample dilution series.

CRediT authorship contribution statement

Casey J. Toft: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Bradley B. Stocks:** Writing – review & editing, Visualization, Validation, Resources, Project administration, Investigation, Formal analysis, Data curation. **Patrick M. Schaeffer:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2024.126147.

References

[1] CDC, Risk Assessment Summary for SARS CoV-2 Sublineage BA.2.86, Centers for Disease Control and Prevention, National Center for Immunization and Respiratory Diseases (NCIRD), August 23, 2023. https://www.cdc.gov/respiratory-viruses/whats-new/covid-19-variant.html.

[2] WHO Global Genomic Surveillance, Research for Health (RFH), Considerations for Developing a National Genomic Surveillance Strategy or Action Plan for Pathogens with Pandemic and Epidemic Potential, World Health Organization (August 18, 2023). https://www.who.int/publications/i/item/9789240076563.

[3] J. Budd, B.S. Miller, N.E. Weckman, D. Cherkaoui, D. Huang, A.T. Decruz, N. Fongwen, G.-R. Han, M. Broto, C.S. Estcourt, J. Gibbs, D. Pillay, P. Sonnenberg, R. Meurant, M.R. Thomas, N. Keegan, M.M. Stevens, E. Nastouli, E.J. Topol, A. M. Johnson, M. Shahmanesh, A. Ozcan, J.J. Collins, M. Fernandez Suarez, B. Rodriguez, R.W. Peeling, R.A. McKendry, Lateral flow test engineering and lessons learned from COVID-19, Nat. Rev. Bioeng. 1 (1) (2023) 13–31.

[4] C.J. Toft, R.A. Bourquin, A.E. Sorenson, P.F. Horwood, J.D. Druce, P.M. Schaeffer, Analytical sensitivity of COVID-19 rapid antigen tests: a case for a robust reference standard, Talanta Open 7 (2023) 100187.

[5] M.I. Zapata-Cardona, L. Florez-Alvarez, D.M. Gomez-Gallego, M.J. Moncada-Diaz, J.C. Hernandez, F. Diaz, M.T. Rugeles, W. Aguilar-Jimenez, W. Zapata, Comparison among plaque assay, tissue culture infectious dose (TCID(50)) and real-time RT-PCR for SARS-CoV-2 variants quantification, Iran. J. Microbiol. 14 (3) (2022) 291–299.

[6] L. Shan, D. Yang, D. Wang, P. Tian, Comparison of cell-based and PCR-based assays as methods for measuring infectivity of Tulane virus, J. Virol Methods 231 (2016) 1–7.

[7] J. Parker, N. Fowler, M.L. Walmsley, T. Schmidt, J. Scharrer, J. Kowaleski, T. Grimes, S. Hoyos, J. Chen, Correction: analytical sensitivity comparison between Singleplex real-time PCR and a multiplex PCR platform for detecting respiratory viruses, PLoS One 13 (10) (2018) e0205483.

[8] T. Lombardo, S. Dotti, S. Renzi, M. Ferrari, Susceptibility of different cell lines to Avian and Swine Influenza viruses, J. Virol Methods 185 (1) (2012) 82–88.

[9] W. Wu, Y. Cheng, H. Zhou, C. Sun, S. Zhang, The SARS-CoV-2 nucleocapsid protein: its role in the viral life cycle, structure and functions, and use as a potential target in the development of vaccines and diagnostics, Virol. J. 20 (1) (2023) 6.

[10] J. Cubuk, J.J. Alston, J.J. Incicco, S. Singh, M.D. Stuchell-Brereton, M.D. Ward, M. I. Zimmerman, N. Vithani, D. Griffith, J.A. Wagoner, G.R. Bowman, K.B. Hall, A. Soranno, A.S. Holehouse, The SARS-CoV-2 nucleocapsid protein is dynamic, disordered, and phase separates with RNA, Nat. Commun. 12 (1) (2021) 1936.

[11] Y.M. Bar-On, A. Flamholz, R. Phillips, R. Milo, SARS-CoV-2 (COVID-19) by the numbers, Elife 9 (2020).

[12] WHO Norms and Standards for Biological Products (NSB), A Collaborative Study to Evaluate the Proposed 1st WHO International Standard for SARS-CoV-2 Antigen, World Health Organization (July 19, 2022). https://www.who.int/publications/m/item/who-bs-2022.2426.

[13] B.B. Stocks, M.P. Thibeault, D. L'Abbe, M. Stuible, Y. Durocher, J.E. Melanson, Production and characterization of a SARS-CoV-2 nucleocapsid protein reference material, ACS Meas. Sci. Au 2 (6) (2022) 620–628.

[14] I. Morin, P.M. Schaeffer, Combining RNA-DNA swapping and quantitative polymerase chain reaction for the detection of influenza A nucleoprotein, Anal. Biochem. 420 (2) (2012) 121–126.

[15] K. Colwill, Y. Galipeau, M. Stuible, C. Gervais, C. Arnold, B. Rathod, K.T. Abe, J. H. Wang, A. Pasculescu, M. Maltseva, L. Rocheleau, M. Pelchat, M. Fazel-Zarandi, M. Iskilova, M. Barrios-Rodiles, L. Bennett, K. Yau, F. Cholette, C. Mesa, A.X. Li, A. Paterson, M.A. Hladunewich, P.J. Goodwin, J.L. Wrana, S.J. Drews, S. Mubareka, A.J. McGeer, J. Kim, M.A. Langlois, A.C. Gingras, Y. Durocher, A scalable serology solution for profiling humoral immune responses to SARS-CoV-2 infection and vaccination, Clin. Transl. Immunol. 11 (3) (2022) e1380.

[16] V.M. Corman, V.C. Haage, T. Bleicker, M.L. Schmidt, B. Muhlemann, M. Zuchowski, W.K. Jo, P. Tscheak, E. Moncke-Buchner, M.A. Muller, A. Krumbholz, J.F. Drexler, C. Drosten, Comparison of seven commercial SARS-CoV-2 rapid point-of-care antigen tests: a single-centre laboratory evaluation study, Lancet Microbe 2 (7) (2021) e311–e319.

[17] C. Mackenzie, M. Batty, G. Papadakis, L. Stevens, Y. Yoga, G. Taiaroa, H. Stefanatos, I. Savic, T. Tran, J. Deerain, J. Prestedge, J. Druce, L. Caly, D. A. Williamson, Analytical sensitivity of lateral flow devices against SARS-CoV-2 Omicron subvariants BA.4, BA.5, and BA.2.75, J. Clin. Microbiol. 60 (11) (2022) e0109722.

[18] R.Y. Kao, D. Yang, L.S. Lau, W.H. Tsui, L. Hu, J. Dai, M.P. Chan, C.M. Chan, P. Wang, B.J. Zheng, J. Sun, J.D. Huang, J. Madar, G. Chen, H. Chen, Y. Guan, K. Y. Yuen, Identification of influenza A nucleoprotein as an antiviral target, Nat. Biotechnol. 28 (6) (2010) 600–605.

[19] A. Portela, P. Digard, The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication, J. Gen. Virol. 83 (Pt 4) (2002) 723–734.

[20] X. Huang, J. Huang, G. Yin, Y. Cai, M. Chen, J. Hu, X. Feng, Identification of NP protein-specific B-cell epitopes for H9N2 subtype of avian influenza virus, Viruses 14 (6) (2022).

[21] L. Sherry, M. Smith, S. Davidson, D. Jackson, The N terminus of the influenza B virus nucleoprotein is essential for virus viability, nuclear localization, and optimal transcription and replication of the viral genome, J. Virol. 88 (21) (2014) 12326–12338.