Journal of Current Science and Technology, May-August 2022 Vol. 12 No. 2, pp. 297-305

ISSN 2630-0656 (Online)

Cite this article: Sripairoj, N., Tunghirun, C., & Chimnaronk, S. (2022, May). Identification of SARS-CoV-2 packaging signals via bacteria-based inhibition assay. *Journal of Current Science and Technology, 12*(2), 297-305. DOI:

Journal of Current Science and Technology

Journal homepage: https://jcst.rsu.ac.th

Identification of SARS-CoV-2 packaging signals via bacteria-based inhibition assay

Nattaporn Sripairoj†, Chairat Tunghirun†, and Sarin Chimnaronk*

Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom 73170, Thailand
†Equal contribution.

*Corresponding author; Email: sarin.chi@mahidol.ac.th

Received 14 April 2022; Revised (Accepted with no Revision); Accepted 18 April 2022
Published online 25 August 2022

Abstract

The COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has damaged global public health. The nucleocapsid (N) protein of SARS-CoV-2 is the major viral RNA-binding protein that recognizes and binds to a specific sequence in the viral RNA genome, designated as a packaging signal (PS), and initiates viral genome packaging. However, the molecular details of the packaging mechanism and consensus on the PS sequence in the SARS-CoV-2 genome remain elusive. This study aims at development of a bacteria-based inhibition assay for measuring the interaction of N protein with viral RNA fragments in order to identify PS from SARS-CoV-2 genome. We initially conducted an unbiased bioinformatic analysis based on the conserved regions in both RNA sequence and secondary structure, and made predictions for three highly plausible packaging signal candidates (PSCs), referred to as PSC1, PSC2, and PSC3, within nucleotides 20,080 to 21,171 in the SARS-CoV-2 genome. These PSC cDNAs were fused with the downstream luciferase gene and introduced, along with the N protein expression plasmid, into the Lemo21 (DE3) *Escherichia coli* system. We carried out extensive optimization of the bacteria-based inhibition system and assessed the N–PS interaction through the translational suppression of luciferase expression. The results showed over 70% inhibition of luciferase expression for PSC1 and PSC2 with both N proteins from SARS-CoV-1 and SARS-CoV-2, supporting our bioinformatic prediction. Our results provide a useful tool for further elucidating of the mechanism of viral genome packaging and for studying other RNA–protein interactions.

Keywords**:** *bacteria-based assay; coronavirus; nucleocapsid protein; packaging signal; SARS-CoV-2.*

1. Introduction

SARS-CoV-2 is a novel coronavirus that has caused the current global pandemic of acute respiratory disease, known as coronavirus disease 2019 (COVID-19). The origin of SARS-CoV-2 was first recorded in a patient from Wuhan province, China in late December 2019 (Wu et al., 2020). Since then, COVID-19 cases have been confirmed in over 400 million individuals and have led to almost six million deaths worldwide (World Health Organization, 2022). COVID-19 is mainly spread through respiratory droplets and aerosols from infected people with SARS-CoV-2 (Meselson, 2020). Patients with COVID-19 present with various degrees of severity, from asymptomatic disease to severe infections, including acute respiratory distress syndrome (ARDS), pneumonia, and kidney failure, resulting in an increased risk of mortality (Cates et al., 2020).

SARS-CoV-2 belongs to the *Betacoronavirus* genus in the Coronaviridae family and is characterized as an enveloped, positive-stranded RNA ((+)ssRNA) virus of approximately 30,000 nucleotides (nt) in size (V'kovski, Kratzel, Steiner, Stalder, & Thiel, 2021). The upstream end of the SARS-CoV-2 genome consists of two open reading frames, ORF1a and ORF1b, which encode sixteen non-structural proteins (nsp1-16) that play

an important role in various functions of viral replication and transcription. In contrast, the downstream end of the SARS-CoV-2 genome encodes four structural proteins that are essential components in the assembly of viral particles, including the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (Naqvi et al., 2020). The function of the S protein facilitates viral entry into host cells by attaching to the host cell's receptor angiotensin-converting enzyme 2 (ACE2) through its receptor-binding domain (RBD), and with the aid of transmembrane serine protease 2 (TMPRSS2) (Hoffmann et al., 2020). The E and M proteins facilitate the virus budding process, as well as contribute to defining the virus's shape (Baudoux, Carrat, Besnardeau, Charley, & Laude, 1998; Mortola & Roy, 2004). The N protein is a critical protein for packaging the viral genome into new viral particles. The process of viral packaging relies on the interaction of the N protein and viral RNA genome. A specific viral RNA sequence, designated as a packaging signal (PS), determines viral RNA–N protein recognition that triggers viral genome packaging. To date, the molecular mechanism of the interaction between the N protein and the PS remains unclear. In addition to its role in viral packaging, the N protein also facilitates viral self-assembly and regulates viral RNA synthesis during the replication/transcription process (Chang, Hou, Chang, Hsiao, & Huang, 2014). Recent studies have shown that the N protein drives the formation of phase-separated condensates that incorporate viral genomic RNA through liquid–liquid phase separation (LLPS), suggesting a key role of the N protein in phase separation to promote ribonucleoprotein (RNP) packaging (Perdikari et al., 2020).

Several studies on the PSs of the coronavirus family have been reported: Molenkamp and Spaan (1997) identified the PS69 (69-nt) fragment, which was sufficient to form a stable secondary structure for the viral RNA encapsidation in mouse hepatitis virus (MHV). Woo, Lee, Lee, Kim, & Cho (2019) investigated the interaction between the N protein and viral RNA PS of SARS-CoV-1 and reported that the PS151 sequence (nt 19,881 to 20,031) was critical for N protein binding. Recently, Syed et al. (2021) developed virus-like particles (VLPs) of SARS-CoV-2 to pack and deliver exogenous RNA transcripts into cells. They found that the PS9 fragment (nt 20,080 to 21,171) in the SARS-CoV-2 genome had the greatest capacity for delivering a reporter mRNA with SARS-CoV-2 VLPs into receiver cells. This finding revealed that the PS9 fragment may contain the critical region necessary for SARS-CoV-2 packaging. Nonetheless, authentication of the sequence and structure of the PS for SARS-CoV-2 remains largely elusive.

Since the emergence of COVID-19, researchers have endeavored to develop effective vaccines against SARS-CoV-2. In particular, the mRNA vaccines developed by Pfizer-BioNTech and Moderna have shown a high level of protection against severe COVID-19 disease (Bajema et al., 2021). Initially, the S protein, which is crucial for the initiation of infection, was exclusively targeted in the development of COVID-19 vaccines; however, the rapid emergence of new SARS-CoV-2 variants with mutations in the S gene, including Alpha (B.1.1.7), Beta (B.1.351), Gamma (B.1.1.28.1), Delta (B.1.617.2), and Omicron (B.1.1.529), raises significant concerns about the utility of targeting the S protein in future strains. Recent evidence suggests that these new variants have all increased transmissibility, in addition to reducing the effectiveness of currently available vaccines, partly by escaping neutralizing antibodies (Planas et al., 2021). Thus, there is an urgent need for the development of new antiviral drugs that are effective against divergent virus strains. Because the N protein in SARS-CoV-2 plays a critical role in viral packaging and assembly, and displays a high homology among the coronavirus family, sharing a 90% similarity to the SARS-CoV-1 N protein (Tilocca et al., 2020), targeting the N protein could present a new strategy for the development of effective, broad therapeutic agents that are resistant to future outbreaks of viral variants. In the present study, we describe a bacteria-based inhibition assay to identify the PS in the SARS-CoV-2 genome and to understand how the N protein recognizes the PS in SARS-CoV-2.

2. Objectives

To assess the RNA packaging signal (PS) of SARS-CoV-2 using bacteria-based inhibition assay.

3. Materials and methods

3.1 Bioinformatic analysis

The multiple sequence alignment of SARS -CoV-2 PS9 (nt 20,080 to 21,171) to corresponding regions among SARS-related CoVs (Bat corona-

virus, accession number MN_996532.2; SARS-CoV-1, accession number NC_004718.3; and SARS-CoV-2, accession number NC_045512.2) was performed using ClustalW2 (Madeira et al., 2019). The conserved RNA secondary structures were predicted using the RNAz webserver (Gruber, Findeiß, Washietl, Hofacker, & Stadler, 2010).

3.2 Construction of reporter and expression plasmids

The packaging signal candidate (PSC) fragments were amplified by PCR with the primers listed in Table 1. The PS9 fragment was synthesized by Twist Bioscience (United Kingdom) and used as a template for amplification. The amplified PSC fragments were cloned into the reporter plasmid encoding the NanoLuc luciferase (Nluc) gene to generate the PSC1-Nluc, PSC2-Nluc, and PSC3-Nluc plasmids. The sequence of PS151 (nt 19,881 to 20,031: accession number AY_291451), which served as a positive control in the present study, was synthesized by Twist Bioscience (United Kingdom), and cloned into the reporter plasmid to generate the PS151-Nluc plasmid. For the protein expression plasmid, the sequence of N proteins of SARS-CoV-1 and SARS-CoV-2 were synthesized by Twist Bioscience (United Kingdom) and cloned into the pET-28a plasmid to generate pET-SCoV-N1 and pET-SCoV-N2, respectively.

Table 1 Primers used in this study

Fragments	**Primer sequences** (5′ - 3′)
PSC1	Forward: ATCACAAGAATTTAAACCCAGGAGTCAAAT Reverse: GGGTAGCTAGTCCAATCAGTAGATGTAAACC
PSC2	Forward: ATCATTCCTATGGACAGTACAGTTAAAAACT Reverse: GGGCAACCTTAGAAACTACAGATAAATCTTGGG
PSC3	Forward: ATCAAGTGTGACCTTCAAAATTATGGTG Reverse: GGGGGTCGTACATATCACTAATAATGAGATCCC

3.3 Transformation

Individual reporter plasmids were co-transformed with the N expression plasmid via the heat shock method at 42°C for 30 sec into Lemo21(DE3)-competent *E. coli* cells. The co-transformants were selected on agar media plates containing 100 µg/ml of ampicillin, 100 µg/ml of chloramphenicol, and 50 µg/ml of kanamycin.

3.4 Optimization of bacteria-based inhibition assay

Transformants were grown in 2 ml 2×YT+P growth media containing antibiotics at 37°C for overnight in a shaking incubator. The overnight cultures were diluted to an optical density (OD) of 600 nm of 0.1 with 1 ml of fresh 2×YT+P growth media and transferred into a 24-well growth plate. The cultures were incubated at 37°C with shaking at 180 RPM until an OD_{600} of 0.4-0.5 was reached. Protein expression was induced by various concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) (0-1 mM), and the cultures were allowed to grow at 37°C with shaking at 180 RPM for 4 hours. The final OD_{600} was measured by a spectrophotometer, and 500 µl of the cultures were collected and stored at –30°C.

To measure the luciferase signals, cultures were diluted with 2×YT+P growth media using a 10-fold serial dilution ($10^0 – 10^{-7}$). Subsequently, 5 µl of diluted cultures were mixed with 5 µl of lysis buffer (2× BugBuster protein extraction reagent, 2× S30-extract buffer, 4 mM DTT) in a 384-well AlphaPlate microplate. The luciferase signals, which appear as relative light units (RLU), were measured immediately with a PerkinElmer plate reader after adding the Nano-Glo luciferase reagent. The workflow for this assay is illustrated in Figure 1.

4. Results and discussion

4.1 Prediction of possible packaging signal candidates (PSCs) in SARS-CoV-2 PS9 (nt 20,080 to 21,171)

We utilized an approach to predict PSCs in the SARS-CoV-2 genome by hypothesizing that such PSCs should be highly conserved in both RNA sequence and structure among SARS-related CoVs. Using ClustalW2 and RNAz webservers, we identified a high probability of PSCs in three regions: PSC1, PSC2, and PSC3, which are located in the nsp15 and nsp16 genes of SARS-CoV-2 (Table 2 and Figure 2). We calculated the nucleotide identity of PSCs among SARS-related CoVs and found that these PSCs had over 70% and 90% of nucleotide identity with SARS-CoV-1 and bat CoV, respectively (Table 3). In addition, these PSCs also possessed high structural preservation, suggesting they might contain the SARS-CoV PS core.

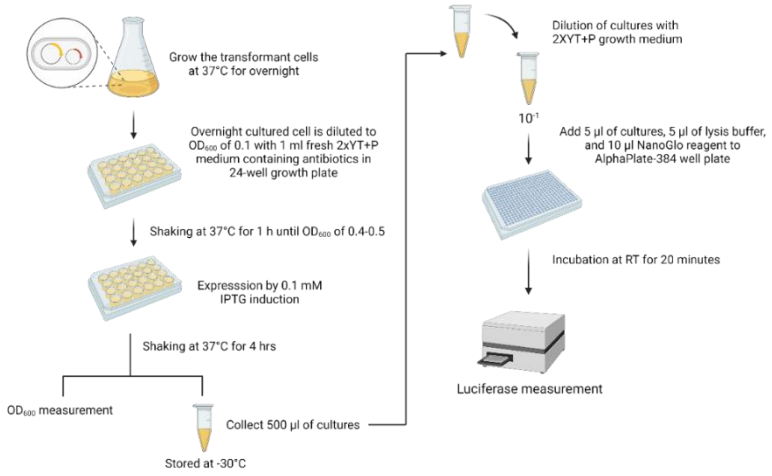

Figure 1 Schematic diagram of bacteria-based inhibition assay established and used in this study

Table 2 The position of PSC sequences identified in this study

Sequence name	Nucleotide positions	Length (nt)
PS9	20080 - 21171	1092
PSC1	20220 - 20386	167
PSC2	20425 - 20572	148
PSC3	20728 - 21058	331

Table 3 The nucleotide identity (%) of PSCs among SARS-CoV related species

	SARS-CoV-1	SARS-CoV-2	Bat CoV RaTG13
PS9	82.69	100	95.79
PSC1	76.05	100	96.41
PSC2	85.14	100	96.62
PSC3	87.01	100	96.98

4.2 Development of bacteria-based inhibition assay for identification of PS in SARS-CoV-2

To investigate the interaction between the N protein and the PS, we developed a bacteria-based inhibition assay based on a previous report (Woo et al., 2019) with some modifications (Figure 3). If the N protein was bound to the target PSC sequence, the translation of Nluc was suppressed due to the interference of ribosomal access to the Shine–Dalgarno (SD) region within the ribosome-binding site (RBS). The degree of N protein binding to the PSC was calculated as a percentage of inhibition on the luciferase translation (see Figure 4A).

To obtain experimental reproducibility and reliable results, we extensively optimized the assay with a pair of previously identified interactions in SARS-CoV-1, between PS151 as the packaging signal and its corresponding N protein. IPTG concentrations were varied, and we found that IPTG at 0.1 mM already provided the maximum inhibition of luciferase translation (Figure 4B). Next, we investigated the time points for harvesting cells after IPTG induction and found that induction for 4 hours reached a plateau of inhibition (Figure 4C). We also determined the amount of cell culture used in our bacteria-based inhibition assay and found that a single 10-fold dilution of cell culture provided an optimal range of detection of the luciferase signal (RLU) by our plate reader (Figure 4D). Finally, the luciferase signals at different time points were monitored after addition of the luciferase substrate (Nano-Glo luciferase reagent). The results showed that the maximum luciferase signal was obtained after 10-20 minutes of incubation (Figure 4E).

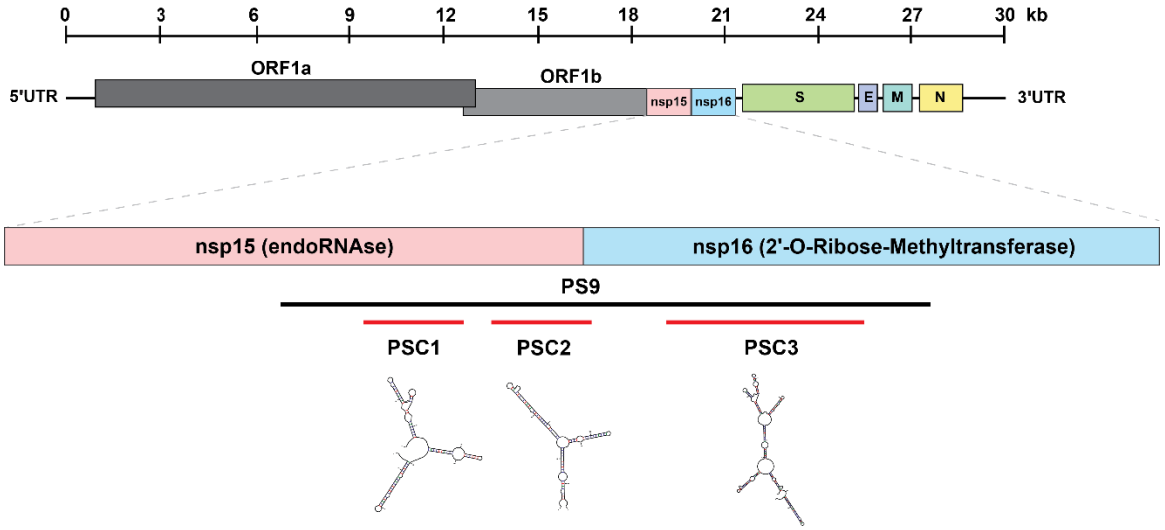

Figure 2 The schematic diagram of the positions of PSCs in the SARS-CoV-2 genome

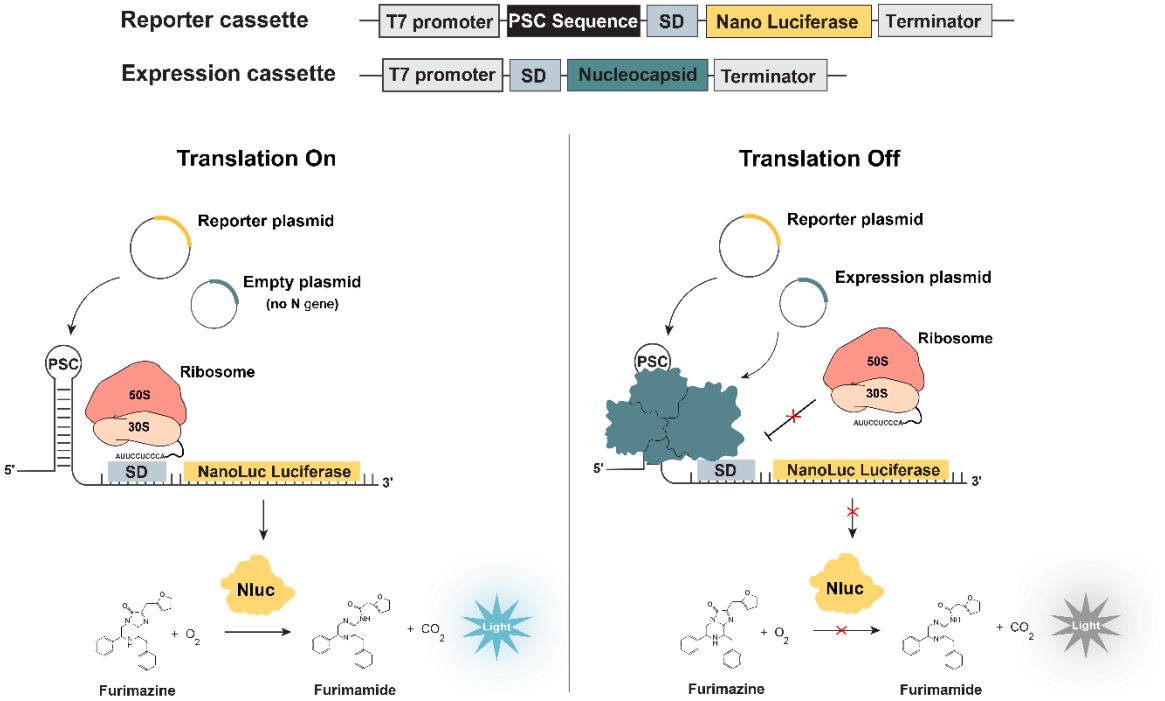

Figure 3 The concept of the bacteria-based inhibition assay used in this study

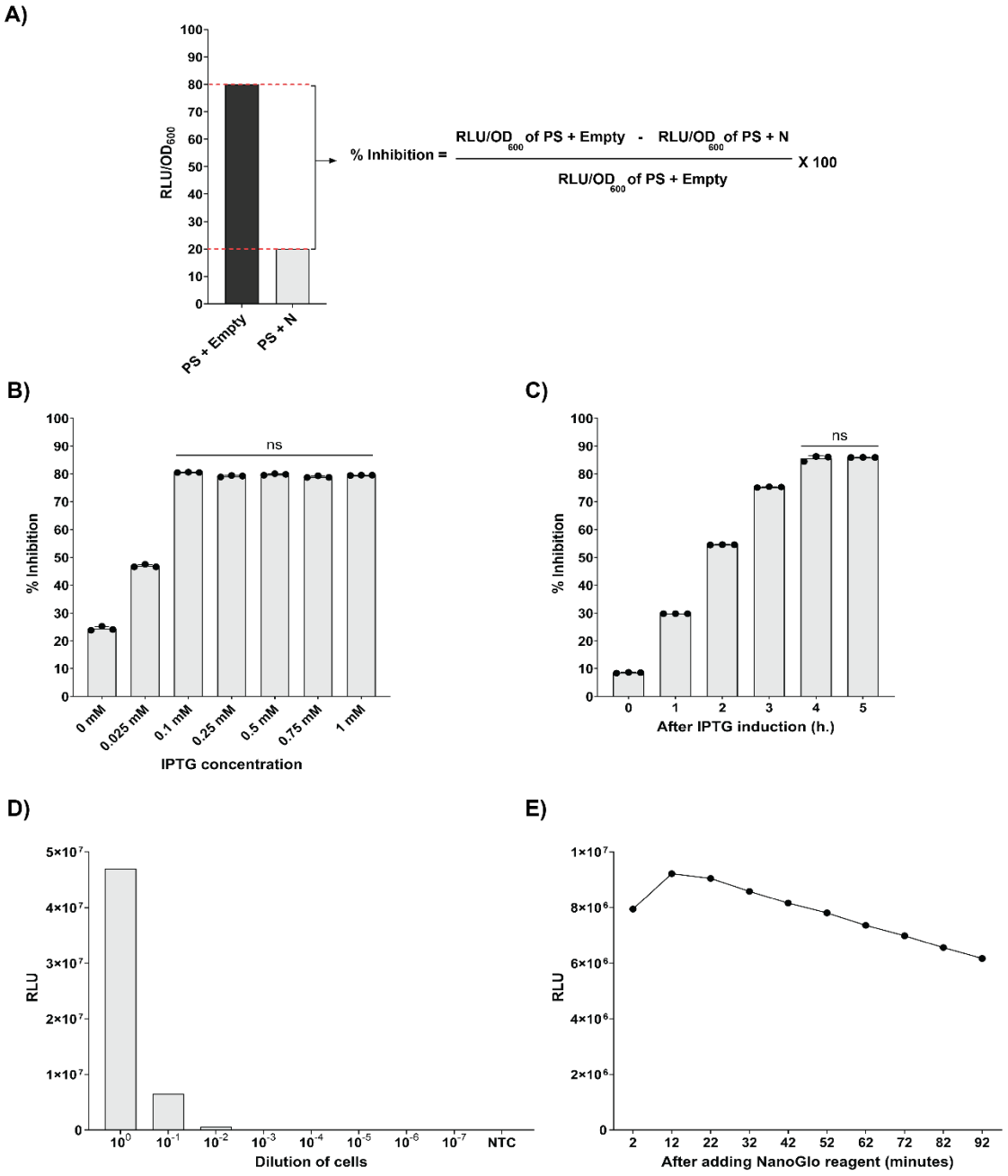

Figure 4 Optimization of the bacteria-based inhibition assay for the PS–N interaction. (**A**) The calculation formula of translational inhibition activity. The assay was optimized by varying different IPTG concentrations (**B**), the periods of cell harvest (**C**), the amount of culture media (**D**), and the reaction time with Nano-Glo luciferase reagent (**E**). Error bars indicate standard deviations for least three biological replicates (n = 3); ns indicates not significant; $P < 0.01$ (*t*-tests and nonparametric tests)

4.3 Assessment of PSCs in SARS-CoV-2

We initially validated our established bacteria-based inhibition assay by a known interaction pair of the SARS-CoV-1 N protein with PS151. The SARS-CoV-1 N protein showed an approximately 80% translational inhibition by binding to PS151, whereas only 10% translational inhibition was observed in the absence of the PS sequence, referred to as the background signal (Figure 5A). This finding demonstrated that the assay could be used to measure the N–PS interaction. The reporter plasmid carrying each PSC (PSC1, PSC2, and PSC3) was subsequently co-transformed with the SARS-CoV-1 N (N1) or SARS-CoV-2 N (N2) expression plasmid. The SARS-CoV-2 N protein showed 75.4%, 72.9%, and 53.9% inhibition for PSC1, PSC2, and PSC3, respectively (Figure 5C). Interestingly, the SARS-CoV-1 N protein gave a result consistent with that of the SARS-CoV-2 N protein, with the highest inhibition for PSC1 (80.4%) and the lowest for PSC3 (65.8%) (Figure 5B). The similarity of SARS-CoV-1 and SARS-CoV-2 N proteins in response to each PSC from the SARS-CoV-2 genome can be attributed to the high amino acid sequence conservation of the N protein between the two viruses (Tilocca et al., 2020). Our results demonstrated that PSC1 and PSC2 could interact with both SARS-CoV-1 and SARS-CoV-2 N proteins, and that the N–PS interactions are likely conserved in SARS-CoVs. Further studies to identify the core of the PS are needed to understand the mechanism of viral packaging.

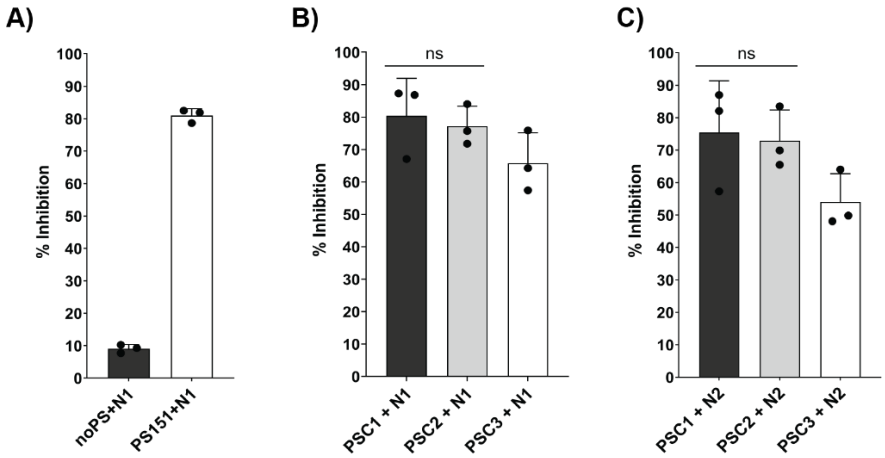

Figure 5 Assay of N–PS interactions by the level of translational inhibition in bacteria. A known interaction of SARS-CoV-1 N protein with PS151 was used as the positive control (**A**). Three predicted PSCs from the SARS-CoV-2 genome were assessed with SARS-CoV-1 N protein (**B**) and SARS-CoV-2 N protein (**C**). Error bars indicate standard deviations for at least three biological replicates (n = 3); ns indicates not significant; $P < 0.01$ (*t*-tests and nonparametric tests)

5. Conclusion

In the present day, we demonstrated the establishment of a bacteria-based inhibition assay to investigate the interaction of the N protein and RNA packaging signals in SARS-CoV-2. We identified three possible PSs in the SARS-CoV-2 genome and showed that PSC1 and PSC2 could interact with N proteins from both SARS-CoV-1 and SARS-CoV-2, a finding that suggests a shared N–PS interaction mechanism. Further experiments are needed to explore the PS core region of the SARS-CoV-2 genome with mutational studies, and by replacing the individual RNA loop structure in PSC1 and PSC2 with the UNAC tetraloops to pinpoint the interaction sites of the N protein (Zhao et al., 2012). Our bacteria-based inhibition assay would be useful not only for the characterization of RNA-binding proteins, but could also serve as a tool for assaying antiviral agents that disrupt viral packaging.

6. Acknowledgements

This work was supported by the Grant-in-Aid for the scientific research from National Research Council of Thailand (NRCT) to SC, and

by Grant for a New Researcher Career path from NRCT to CT. NS was supported by the Development and Promotion of Science and Technology Talents Project (DPST) scholarship.

7. References

Bajema, K. L., Dahl, R. M., Prill, M. M., Meites, E., Rodriguez-Barradas, M. C., Marconi, V. C., Beenhouwer, D. O., Brown, S. T., Holodniy, M., & Lucero-Obusan, C. (2021). Effectiveness of COVID-19 mRNA Vaccines Against COVID-19–Associated Hospitalization—Five Veterans Affairs Medical Centers, United States, February 1–August 6, 2021. *Morbidity and Mortality Weekly Report*, *70*(37), 1294-1299. DOI: https://doi.org/10.15585/mmwr.mm7037e3

Baudoux, P., Carrat, C., Besnardeau, L., Charley, B., & Laude, H. (1998). Coronavirus pseudoparticles formed with recombinant M and E proteins induce alpha interferon synthesis by leukocytes. *Journal of virology*, *72*(11), 8636-8643. DOI: https://doi.org/10.1128/JVI.72.11.8636-8643.1998

Cates, J., Lucero-Obusan, C., Dahl, R. M., Schirmer, P., Garg, S., Oda, G., Hall, A. J., Langley, G., Havers, F. P., & Holodniy, M. (2020). Risk for in-hospital complications associated with COVID-19 and influenza—Veterans Health Administration, United States, October 1, 2018–May 31, 2020. *Morbidity and Mortality Weekly Report*, *69*(42), 1528. DOI: https://doi.org/10.15585/mmwr.mm6942e3

Chang, C.-k., Hou, M.-H., Chang, C.-F., Hsiao, C.-D., & Huang, T.-h. (2014). The SARS coronavirus nucleocapsid protein–forms and functions. *Antiviral research*, *103*, 39-50. DOI: https://doi.org/10.1016/j.antiviral.2013.12.009

Gruber, A. R., Findeiß, S., Washietl, S., Hofacker, I. L., & Stadler, P. F. (2010). RNAz 2.0: improved noncoding RNA detection. In *Biocomputing 2010* (pp. 69-79). World Scientific.

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., ... & Pöhlmann, S. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *cell*, *181*(2), 271-280. e278. DOI: https://doi.org/10.1016/j.cell.2020.02.052

Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., ... & Lopez, R. (2019). The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic acids research*, *47*(W1), W636-W641. DOI: https://doi.org/10.1093/nar/gkz268

Meselson, M. (2020). Droplets and aerosols in the transmission of SARS-CoV-2. *New England Journal of Medicine*, *382*(21), 2063-2063. DOI: https://doi.org/10.1056/NEJMc2009324

Molenkamp, R., & Spaan, W. J. (1997). Identification of a specific interaction between the coronavirus mouse hepatitis virus A59 nucleocapsid protein and packaging signal. *Virology*, *239*(1), 78-86. DOI: https://doi.org/10.1006/viro.1997.8867

Mortola, E., & Roy, P. (2004). Efficient assembly and release of SARS coronavirus-like particles by a heterologous expression system. *FEBS letters*, *576*(1-2), 174-178. DOI: https://doi.org/10.1016/j.febslet.2004.09.009

Naqvi, A. A. T., Fatima, K., Mohammad, T., Fatima, U., Singh, I. K., Singh, A., ... & Hassan, M. I. (2020). Insights into SARS-CoV-2 genome, structure, evolution, pathogenesis and therapies: Structural genomics approach. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1866*(10), 165878. DOI: https://doi.org/10.1016/j.bbadis.2020.165878

Perdikari, T. M., Murthy, A. C., Ryan, V. H., Watters, S., Naik, M. T., & Fawzi, N. L. (2020). SARS-CoV-2 nucleocapsid protein phase-separates with RNA and with human hnRNPs. *The EMBO journal*, *39*(24), e106478. DOI: https://doi.org/10.15252/embj.2020106478

Planas, D., Veyer, D., Baidaliuk, A., Staropoli, I., Guivel-Benhassine, F., Rajah, M. M., ... & Schwartz, O. (2021). Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. *Nature*,

596(7871), 276-280. DOI: https://doi.org/10.1038/s41586-021-03777-9

Syed, A. M., Taha, T. Y., Tabata, T., Chen, I. P., Ciling, A., Khalid, M. M., ... & Doudna, J. A. (2021). Rapid assessment of SARS-CoV-2–evolved variants using virus-like particles. *Science*, *374*(6575), 1626-1632. DOI: https://doi.org/10.1126/science.abl6184

Tilocca, B., Soggiu, A., Sanguinetti, M., Musella, V., Britti, D., Bonizzi, L., ... & Roncada, P. (2020). Comparative computational analysis of SARS-CoV-2 nucleocapsid protein epitopes in taxonomically related coronaviruses. *Microbes and infection*, *22*(4-5), 188-194. DOI: https://doi.org/10.1016/j.micinf.2020.04.002

V'kovski, P., Kratzel, A., Steiner, S., Stalder, H., & Thiel, V. (2021). Coronavirus biology and replication: implications for SARS-CoV-2. *Nature Reviews Microbiology*, *19*(3), 155-170. DOI: https://doi.org/10.1038/s41579-020-00468-6

Woo, J., Lee, E. Y., Lee, M., Kim, T., & Cho, Y.-E. (2019). An in vivo cell-based assay for investigating the specific interaction between the SARS-CoV N-protein and its viral RNA packaging sequence. *Biochemical and biophysical research communications*, *520*(3), 499-506. DOI: https://doi.org/10.1016/j.bbrc.2019.09.115

World Health Organization. (2022). WHO Coronavirus (COVID-19) Dashboard. Retrived from https://covid19.who.int/

Wu, F., Zhao, S., Yu, B., Chen, Y. M., Wang, W., Song, Z. G., ... & Zhang, Y. Z. (2020). A new coronavirus associated with human respiratory disease in China. *Nature*, *579*(7798), 265-269. DOI: https://doi.org/10.1038/s41586-020-2008-3

Zhao, Q., Huang, H. C., Nagaswamy, U., Xia, Y., Gao, X., & Fox, G. E. (2012). UNAC tetraloops: to what extent do they mimic GNRA tetraloops?. *Biopolymers*, *97*(8), 617-628. DOI: https://doi.org/10.1002/bip.22049