DEVELOPMENT OF SURROGATE SARS-CoV-2 VIRUS NEUTRALIZATION TEST METHOD USING PLANT-PRODUCED RECOMBINANT PROTEINS

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จุฬาลงกรณ์มหาวิทยาลัย
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การพัฒนาวิธีทดสอบเซอร์โรเกตซาร์-โควี-2 ไวรัสนิวทรัลไรเซชัน โดยใช้รีคอมบิแนนท์โปรตีนจากพืช

นายพีรวัส จิรโรจน์วัฒน

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	DEVELOPMENT OF SURROGATE SARS-CoV-2 VIRUS NEUTRALIZATION TEST METHOD USING PLANT-PRODUCED RECOMBINANT PROTEINS
By	Mr. Perawat Jirarojwattana
Field of Study	Pharmaceutical Sciences and Technology
Thesis Advisor	Associate Professor WARANYOO PHOOLCHAROEN, Ph.D.

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พีรวัส จิรโรจน์วัฒน : การพัฒนาวิธีทดสอบเซอร์โรเกตซาร์-โควี-2 ไวรัสนิวทรัลไรเซชัน โดยใช้รีคอมบิแนนท์โปรตีนจากพืช. (DEVELOPMENT OF SURROGATE SARS-CoV-2 VIRUS NEUTRALIZATION TEST METHOD USING PLANT-PRODUCED RECOMBINANT PROTEINS) อ.ที่ปรึกษาหลัก : รศ. ดร.วรัญญู พูลเจริญ

การตรวจหาภูมิคุ้มกันต่อไวรัสซาร์-โควี-2 มีความสำคัญในการประเมินประสิทธิภาพของวัคซีนและการตอบสนองต่อการติดเชื้อโดยธรรมชาติ แต่วิธีการทดสอบไวรัสนิวทรัลไรเซชันโดยใช้ไวรัสซาร์-โควี-2 (cVNT) ซึ่งเป็นวิธีที่ได้รับการยอมรับและมีความแม่นยำสูง ต้องใช้ห้องปฏิบัติการที่มีระบบความปลอดภัยทางชีวภาพระดับ3 และใช้ไวรัสก่อโรคในการทดสอบ ส่วนวิธีการทดสอบไวรัสนิวทรัลไรเซชันโดยใช้ไวรัสเทียม จำเป็นต้องใช้อุปกรณ์ที่มีความจำเพาะและผู้เชี่ยวชาญเฉพาะทางในห้องปฏิบัติการที่มีระบบความปลอดภัยทางชีวภาพระดับ2 แต่วิธีการทดสอบเซอร์โรเกตไวรัสนิวทรัลไรเซชัน (sVNT) ได้ถูกพัฒนาขึ้นเพื่อหลีกเลี่ยงข้อจำกัดเหล่านี้ ในการศึกษานี้ใช้โปรตีนตัวรับ (ACE2) บนผิวของเซลล์มนุษย์ที่ผลิตจาก *Nicotiana benthamiana* เพื่อพัฒนาการตรวจหาภูมิคุ้มกันต่อไวรัสซาร์-โควี-2 ผลการศึกษาบ่งชี้ว่า ACE2-His ที่ผลิตจากพืชสามารถจับกับบริเวณส่วนของโปรตีนหนาม (RBD) ของไวรัสซาร์-โควี-2 ได้ และใช้สร้างวิธีการทดสอบเซอร์โรเกตไวรัสนิวทรัลไรเซชันด้วยโปรตีน RBD ที่ผลิตจากพืชได้ ผลการทดสอบเซอร์โรเกตไวรัสนิวทรัลไรเซชัน (sVNT) ที่ใช้โปรตีนที่ผลิตจากพืชพบว่า การทดสอบมีความไวและความจำเพาะต่อการตรวจหาภูมิคุ้มกันเมื่อตรวจสอบด้วยเลือดของหนูที่ได้รับการฉีดวัคซีนด้วย RBD-Fc อีกทั้งผลการทดสอบนี้ยังสอดคล้องกับค่าไตเตอร์ของวิธีการทดสอบไวรัสนิวทรัลไรเซชัน (cVNT) โดยใช้ไวรัสซาร์-โควี-2 กล่าวโดยสรุป การศึกษานี้แสดงให้เห็นว่าพืชอาจมีศักยภาพสูงและเหมาะสำหรับการผลิตโปรตีนเพื่อใช้ในตรวจหาภูมิคุ้มกัน

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Chulalongkorn University

สาขาวิชา เภสัชศาสตร์และเทคโนโลยี ลายมือชื่อนิสิต .....

ปีการศึกษา 2565 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Detecting immunity against SARS-CoV-2 is vital for evaluating vaccine response and natural infection, but a conventional virus neutralization test (cVNT) requires BSL3 and live viruses, and a pseudo-virus neutralization test (pVNT) needs specialized equipment and trained professionals in BSL2. The surrogate virus neutralization test (sVNT) was developed to overcome these limitations. This study explored the use of angiotensin converting enzyme (ACE2) produced from *Nicotiana benthamiana* for the development of an affordable neutralizing antibodies detection assay. The results showed that the plant-produced ACE2-His can bind to the receptor binding domain (RBD) of the SARS-CoV-2, and was used to develop sVNT with plant-produced RBD protein. The sVNT developed using plant-produced proteins showed high sensitivity and specificity when validated with a group of 30 RBD-Fc vaccinated mice sera and the results were correlated with cVNT titer. This preliminary finding suggests that the plants could offer a cost-effective platform for establishing antibody detection assays.

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Chulalongkorn University

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Chulalongkorn University

Perawat Jirarojwattana

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

TABLE OF CONTENTS

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

LIST OF FIGURES

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER I INTRODUCTION

The coronavirus disease 2019 (COVID-19) characterized by respiratory illness and severe pneumonia is caused by a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which was identified in Wuhan, China in 2019. The receptor binding domain (RBD) located in the spike protein of this virus plays an important role in virus binding with the host cell receptor in the respiratory tract, angiotensin converting-enzyme 2 (ACE2) thereby entering the host cell [1]. Recently, several vaccines employing different strategies have been approved for human use against SARS-CoV-2 [2]. The assessment of immune response from convalescent patients and recipients of vaccine candidates is needed for determined and tracked neutralizing antibody (Nab) levels which can wean over time [3]. A neutralization assay is commonly used to quantify Nabs that can bind to a specific epitope of RBD and prevent the binding from ACE2 [4]. Live virus neutralization assay or the conventional virus neutralization test (cVNT) is the gold standard for the measurement of Nabs titers, but it requires biosafety level 3 (BSL3) facilities, intensive skills, high costs, and is time-consuming [1, 5]. The pseudo-virus virus neutralization test (pVNT) is more convenient and can be performed in BSL2, but still needs the use of live viruses and cells [5, 6]. In addition, both tests need 2-4 days to obtain results [5, 6]. These limitations can be overcome with sVNT which can be completed within a few hours and no need for live viruses or cells under BSL2 containment [7]. The principle of this test relies on competitive ELISA binding between the ACE2 receptor and RBD-specific antibodies to the RBD of SARS-CoV-2. To examine the sVNT, the recombinant proteins are produced and purified from several organisms including bacteria, yeast, insect cell, mammalian cell lines, algae, and plants that are used as diagnostic reagents to perform a competitive ELISA [8]. Each expression systems have its own strengths and weaknesses such as production time, cost, protein yield, presence of post-translational modifications (PTMs), and regulatory approval [9].

In the present study, we produced ACE2 and RBD of the SARS-CoV-2 (Wuhan strain) in *Nicotiana benthamiana*, a tobacco plant, using a transient expression system that can rapidly produce eukaryotic proteins with high productivity, low cost, scalability, and safety. This tobacco plant has suitable PTMs such as mammalian-like glycosylation, and protein folding in the endoplasmic reticulum (ER) as reported in many studies [9-14]. We used the plant-produced RBD of the SARS-CoV-2 (Wuhan strain) along with plant-produced ACE2 to detect the Nabs present in the sera of mice vaccinated with RBD-Fc, and we evaluated the performance of the plant recombinant protein in detecting Nabs in comparison with *in vitro* microneutralization (MN) test, which is the cVNT [15].

1.1 Research hypotheses

- The plant-produced ACE2 and RBD of the SARS-CoV-2 (Wuhan strain) can be used to develop the sVNT
- The plant-derived sVNT exhibits high sensitivity, high specificity, and a good correlation with the MN or cVNT when tested with a panel of mice sera.

1.2 Objectives

- To establish the sVNT using plant-produced RBD of SARS-CoV-2 and plant-produced ACE2
- To evaluate the efficiency of the sVNT using plant-produced recombinant protein with vaccinated mice sera

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Chulalongkorn University

CHAPTER II LITERATURE REVIEW

1. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

This virus belongs to the *betacoronavirus* genus that has enveloped and positive-sense single-strand RNA [16]. It consists of 4 structural proteins: spike (S), envelope (E), membrane (M), nucleocapsid (N), and 14 open reading frames (ORFs) in its genome [17, 18]. The SARS-CoV-2 emerged in China in late December 2019 and rapidly spread in several countries becoming global public health. World Health Organization (WHO) announced this coronavirus as a pandemic situation due to its high virulence with more than 700 million confirmed cases and 6 million deaths worldwide as of April 2023 [19]. The virus can cause severe fever, cough, diarrhea, respiratory illness, muscular pain, and pneumonia [20].

The receptor binding domain (RBD), located on the surface of the S protein, can bind to the human lung cell receptor, angiotensin-converting enzyme (ACE2), and fuse into the host cell [21, 22]. Then, the viral genome is loaded into the host cell and translated to synthesize viral particles along with the replication of RNA. Finally, the viral particles and their genome are assembled and burst the host cell to infect other cells [23]. Thus, the RBD sequence or S protein is not only the key target for virus pathogenesis and antigenicity but also for vaccine development, as it can elicit the host immune system [24-27]. Furthermore, the study of monoclonal antibody (mAb) against this virus which has been shown to effectively neutralize SARS-CoV-2, is important for immunotherapies and early diagnosis [10, 28].

Recently, COVID-19, caused by SARS-CoV-2, has been detected using several diagnostic methods. The direct method involves detecting the viral RNA and virus particles using a technique such as reverse transcription polymerase chain reaction (RT-PCR) or clustered regularly interspaced short palindromic repeats (CRISPR). The indirect method involves measuring antibodies against the virus using an enzyme-linked immunosorbent assay (ELISA) test or lateral flow immunoassay (LFIA) [29, 30]. The nucleic acid present in the sample can be detected by the amplification process of PCR within an hour to a couple of days depending on the version of PCR.

However, the pandemic has caused a higher demand for PCR reagents, leading to a global shortage of reagents and further delaying diagnosis results [31]. ELISA and LFIA are commonly used to screen suspicious samples using an antigen-antibody interaction with high throughput but they may produce false negatives during the early stage or in asymptomatic patients, and false positives from the convalescent sample of COVID-19 patients, as well as vaccinated samples [29, 32]. Moreover, they cannot differentiate between the total binding antibodies and the Nabs in patient sera [33-36].

The Nabs play an important role in blocking the infection of the virus into the host at a specific site [37]. Studying Nabs can apply to novel therapeutics and vaccine development along with the investigation of herd immunity, humoral immunity, asymptomatic infection, and case fatality rate [38]. The assessment of Nabs performing with live viruses to detect them in a patient's blood is called the conventional virus neutralization test (cVNT) which requires BSL3 containment, well-trained skills, and is time-consuming [39]. Another test, which is more convenient, is the pseudo-virus neutralization test (pVNT). It can be performed in BSL2, but still requires handling with viruses and cells and complicated procedures [40, 41]. They do not match with the high demand for diagnostic tests or mass surveillance. The serological test has been developed to use as a first-line screening protocol or study of vaccines which is a surrogate virus neutralization test.

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2. Surrogate virus neutralization test (sVNT)

The sVNT is a competitive ELISA assay used to evaluate immunity to infectious diseases. This test coats a recombinant protein in the 96 well plates and incubates it with the Nabs or specific antibodies in sera. The Nabs prevent further binding of the coated protein [7], resulting in low optical density (OD), as shown in Fig.1. Although the sVNT uses the same principle as the cVNT or pVNT, which uses live viruses and cells, this application can be easily performed in BSL2 without the requirement of viruses or cells. The test can provide results in a short time compared with cVNT because it uses only recombinant proteins that mimic the virus-

host interaction in an ELISA plate, making it suitable for implementation in clinical laboratories [7]. The inhibition of binding can be interpreted as the presence of Nabs in the serum sample. The sVNT has several advantages, including measuring vaccine response, assessing the longevity of protective immunity, monitoring neutralizing titers in vaccines and conducting large-scale surveillance [7].

For instance, Tan *et al*. (2020) developed the RBD-specific sVNT, which displayed 99.93% specificity and 95-100% sensitivity in two cohorts of patients. They also demonstrated the correlation between sVNT and cVNT or pVNT with more than R^2 = 0.8 (p<0.0001) [7]. Commercial sVNT kits are developed to assess the immunization with SARS-CoV-2 vaccination [42-45]. Several reports have evaluated the efficacy of these kits and demonstrated not only high sensitivity and sensitivity but also a significant correlation with other tests including cVNT, pVNT, and plaque reduction neutralization test (PRNT) [43-46].

To develop antibody detection kits, recombinant protein coating on ELISA plates or LFIA conjugate pads is necessary. Similarly, the sVNT based on the ELISA technique also requires recombinant proteins. In these days, commercial sVNT kits commonly use mammalian cell lines to produce the recombinant protein and develop the sVNT kits. Moreover, Tan *et al*. (2020) produced the RBD from insect cells along with mammalian cells to develop the sVNT. The performance correlation of these two expression hosts found great performance (R^2 = 0.9736, p<0.0001) [7]. However, there is no report about using plant-produced recombinant proteins in the sVNT technique.

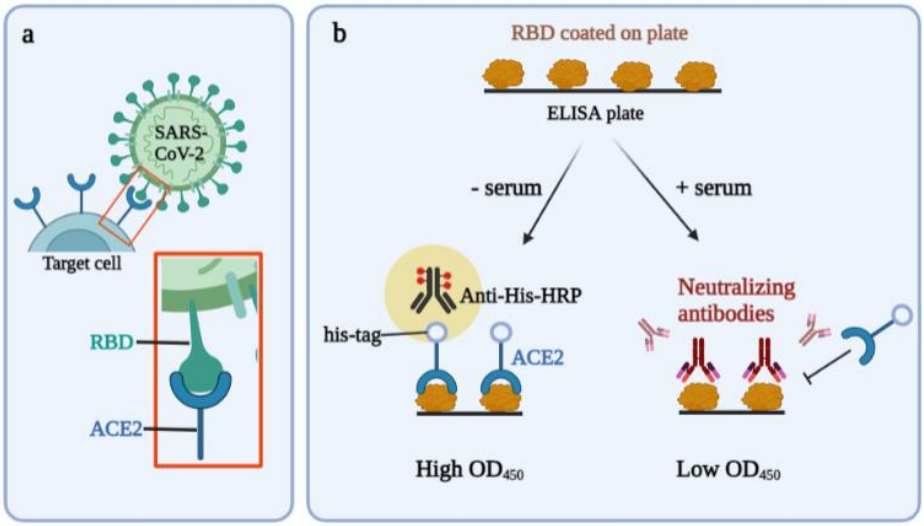

Figure 1 *Diagram presentation of natural infection and surrogate virus neutralization test (sVNT)*

(a) The SARS-CoV-2 spike protein binds to the host cell receptor ACE2 and mediates cell attachment. (b) RBD protein was coated onto the ELISA plate followed by the addition of ACE2-His incubated with serum. The presence of Nabs in serum can block the binding of RBD and ACE2 resulting in a low OD_{450}.

3. Plant produced recombinant protein.

Recombinant proteins have been widely used in several aspects both non-pharmaceutical proteins and pharmaceutical proteins such as industrial enzymes, growth factors, vaccine candidates, mAbs, and diagnostic reagents [9, 47]. These proteins are typically expressed from recombinant DNA or synthetic DNA that has been cloned into an expression vector and transformed into an expression host (prokaryotic organism: bacteria or eukaryotic organism: mammalian cell, yeast, insect cell, or plants cell) depending on their purpose [48].

Each expression host has its own advantages and drawbacks in different aspects. The bacterial expression system e.g., *Escherichia coli* has high productivity because it has a short doubling time and good manufacturing practice (GMP), a list of instructions has been established to make sure that biopharmaceuticals meet appropriate levels of quality and consistency between batches, with the aim of

safeguarding patients from potential harm [49]. However, it lacks PTMs and can produce endotoxins and inclusion bodies while some others eukaryotic organisms have PTMs and do not accumulate bacterial toxins [8]. Some eukaryotic systems, including yeast (*Saccharomyces cerevisiae*), face challenges with hyper-glycosylation that can affect protein immunogenicity [50]. Although *Pichia pastoris* has a higher similarity of glycosylation to mammalian cells, optimizing factors such as methanol and sorbitol concentration, temperature, and incubation time is required to obtain the maximum yield of recombinant proteins [51]. Mammalian cells have the ability to process PTMs, including authentic human N-glycosylation, and can produce large and complex recombinant proteins. However, they present challenges such as high production cost, time consumption, and risk of pathogenic contamination [52].

Plants have emerged as an alternative host for the production of recombinant proteins with PTMs and use in the ELISA technique due to numerous desired traits e.g., high productivity, low-cost medium, rapid scalability, no risk of contamination with human pathogens, and proper PTMs as reported in many studies [9-14].

There are several transformation methods for introducing the gene of interest into a plant cell. Plant transformation can be achieved through stable expression, transient expression, or suspension cultures which involves the incorporation of foreign or interested genes into plants. The stable expression has two targets of transformation which are the nucleus and chloroplast [9, 47]. The foreign gene can be introduced using either *Agrobacterium tumefaciens*-mediated transformation or particle bombardment method to develop transgenic plants [9, 47]. Chloroplast transformation has more attractive features, such as the precise insertion of transgenes through homologous recombinants and the absence of gene silencing, which can overcome the drawback of nuclear transformation with random integration. However, it poses challenges such as unintended homologous recombinant and a lack of species-specific regulatory sequences. Moreover, developing transgenic plant takes time to screen the best transgenic lines, and the expression yield is lower than that of industrial-level protein production [53-56].

4. Transient expression

When producing recombinant proteins from a plant expression system, there are numerous approaches available with a difference in yield, ease of harvest, and PTMs to suit each specific recombinant protein. Genetically modifying plant genomes to create transgenic plants is a conventional method for the production of recombinant proteins [57, 58]. However, this method requires long time-consuming processes that are labor-intensive and non-flexible in dealing with SARS-CoV-2 variations that vary over time [59]. Alternatively, transient expression based on viral vectors provides several advantages [58], such as rapid, cheap, and flexible expression of recombinant proteins for the initial characterization of proteins at the research level [59]. In previous studies, plants and Agrobacterium have been considered as host-microbes for plant genetic engineering. Particularly, the plant pathogen *A. tumefaciens* has been genetically modified and utilized as a tool to transfer the gene of interest into the plant cell and amplify the expression of foreign proteins [60]. Earlier reports have indicated the potential of a plant transient expression system for the rapid production of pharmaceutical proteins. In the study of Rattanapisit *et al*. (2020) the Agrobacteria were used for transient expression of SARS-CoV-2 RBD and mAb in *N. benthamiana* leaves using pBYR2eK, a plant expression vector, to carry the interested genes [11]. This plant has been widely used as a model organism in the field of plant virology due to its large biomass and susceptibility to a broad range of plant-pathogenic agents [61]. Furthermore, it has a high efficiency at the transient expression of various proteins and its protease profile is lower than other plant species [62].

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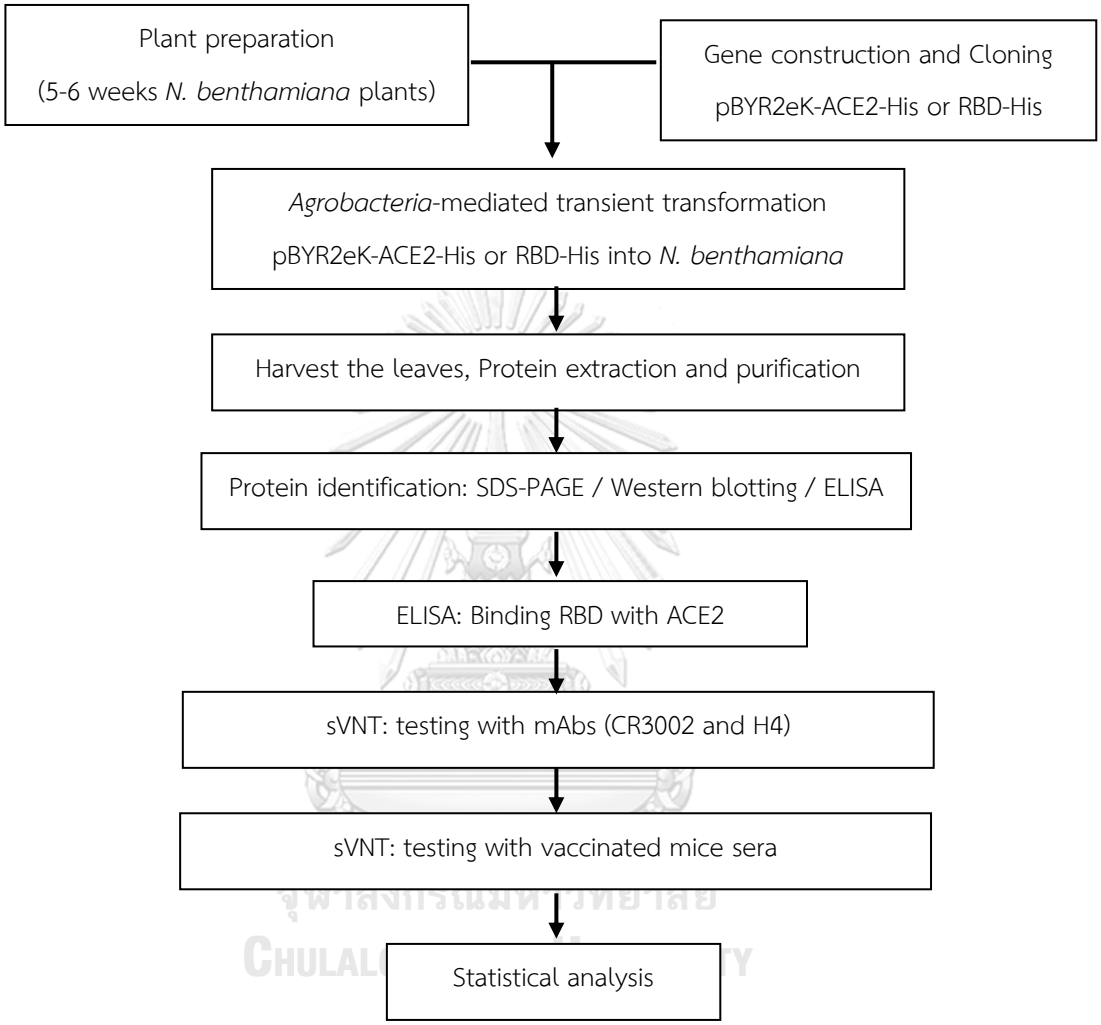
Chulalongkorn University

CHAPTER III METHODOLOGY

1. Experimental design



2. Materials

2.1 Equipment and machines

- 0.45 µm S-Pak membrane filters (Merck, USA)
- 0.22 µm polyethersulfone (PES) syringe filter (Merck, USA)
- Amicon® ultracentrifugal filter 10K (Merck, USA)
- High binding 96-well plate (Greiner Bio-one, Austria)
- 0.45 µm nitrocellulose membrane (Bio-rad, USA)

- T100™ Thermal Cycle (Bio-Rad, USA)
- Mini-PROTEAN® Tetra system (Bio-rad, USA)
- MicroPulser (Bio-Rad, USA)
- 1.5mL Graduated Microcentrifuge tube (Molecular BioProducts, USA)
- 1mL Pipet tips, blue (Molecular BioProducts, USA)
- 1-200 µl Pipet tips, yellow (Molecular BioProducts, USA)
- 0.1-20 µl Pipet tips (Molecular BioProducts, USA)
- Microplate incubator (Hercuvan Lab systems, Malaysia)
- Microplate reader (Hercuvan Lab System, Cambridge, UK)

2.2 Chemical reagents

Agarose (Vivantis, Malaysia), Ampicillin (ITW Reagents, Germany), Kanamycin (Bio Basic, Canada), Rifampicin (Thermo Fischer Scientific, USA), Gentamicin (ITW Reagents, Germany), 2-N-morpholino-ethanesulfonic acid (MES) (ITW Reagents, Germany) , Magnesium Sulphate ($MgSO_4$) (Merck, USA) , Tris (Vivantis, Malaysia) , Glycine (Vivantis, Malaysia), Sucrose (Merck, USA), Enhanced Chemiluminescence (ECL) plus detection reagent (Abcam, UK), color reagent A (stabilized peroxide solution) and color reagent B (stabilized chromogen solution) (R&D Systems, USA) , InstantBlue® coomassie protein stain (Abcam, UK), Ni-NTA affinity resin (Expedeon, Cambridge, UK), Medical X-ray Green/MXG Flim (Carestream, China), β-mercaptoethanol (Merck, USA), Skim milk (BD Difco, USA), 3,3',5,5'–Tetramethylbenzidine (TMB) stabilized substrate (Promega, USA), Sulfuric acid (H_2SO_4) (Merck, 21 USA), Alhydrogel® adjuvant 2% (Aluminium hydroxide gel) (Invivogen, USA), 1X Phosphate buffered saline (PBS) (Hyclone, USA)

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2.3 Enzymes

- *Xba*I (New England Biolabs, USA)

- *Sac*I (New England Biolabs, USA).

- T4 DNA ligase (New England Biolabs, USA).

- *Taq* DNA polymerase (Vivantis, Malaysia)

- Q5 DNA polymerase (New England Biolabs, USA)

2.4 Cloning and Expression vectors

- pGEMT-Easy Vector (Promega, USA) (Appendix A)

- pBYR2eK2Md Vector (Appendix B)

2.5 Molecular Biology kits

- AccuPrep Nano-Plus Plasmid Mini Extraction kit protocol (Bioneer, Korea)

- AccuPrep Gel Purification Kit (Bioneer, Korea)

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2.6 Bacteria

- *Escherichia coli* strain DH10B

- *Agrobacterium tumefaciens* strain GV3101

2.7 Antibodies, serum and recombinant proteins

- HRP-conjugated rabbit polyclonal anti-His antibody (Abcam, UK)

- plant-produced H4 [10] and CR3022 [11] mAb

- plant-produced RBD-Fc (Wuhan strain) [15]

- RBD-Fc immunized mice sera [15]

2.8 Buffers

2.8.1 Buffer for ACE-His purification

Extraction buffer

20 M Tris-HCl pH 7.4, 50 mM NaCl, 5 mM Imidazole

Washing buffer

20 M Tris-HCl pH 7.4, 50 mM NaCl, 5-20 mM Imidazole

Eluting buffer

20 mM Tris-HCl pH 7.4, 50 mM NaCl, 250 mM Imidazole

2.8.2 DNA loading 6x dye

38% (w/v) Glycerol, 0.08% (w/v) Bromophenol blue, 0.08% (w/v) Xylencyanol

2.8.3 Z-buffer non-reducing dye

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125 mM Tris HCl, 12% Sodium Dodecyl Sulphate, 10% Glycerol, 0.001% Bromophenol blue pH 6.8

2.8.4 Z-buffer reducing dye

125 mM Tris HCl, 12% Sodium Dodecyl Sulphate, 10% Glycerol, 0.001% Bromophenol blue, 22% β-mercaptoethanol pH 6.8

2.8.5 1X Phosphate-buffered saline (PBS)

137 mM NaCl, 2.7 mM Potassium Chloride (KCl), 8.1 mM Sodium hydrogen phosphate (Na_2HPO_4), 1.5 mM Potassium dihydrogen phosphate (KH_2PO_4) pH 7.4

2.8.6 Phosphate-buffered saline-Tween (PBST)

1X PBS, 0.05% Tween 20

2.8.7 1X Running buffer (SDS-PAGE)

25 mM Tris, 192 mM Glycine, 1% SDS

2.8.8 1X Transfer buffer (Western blot)

25 mM Tris, 192 mM Glycine, 15% Methanol

2.8.9 1X Infiltration buffer

10 mM MES, 10mM $MgSO_4$ pH 5.5 23

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2.9 Media

2.9.1 Luria Bertani (LB) Broth

1% NaCl, 0.5% Yeast, 1% Peptone

2.9.2 Luria Bertani (LB) Agar

1% NaCl, 0.5% Yeast, 1% Peptone, 1.5% Agar

3. Methods

3.1 RBD-His and ACE2-Fc

3.1.1 RBD-His production

3.1.1.1 Transient expression of RBD-His in *N. benthamiana*

The cultured stock of *A. tumefaciens* harboring pBYR2eK-RBD-His was obtained from the work of my senior and sub-cultured overnight at 28 ºC. The cultured *A. tumefaciens* cells were pelleted and resuspended with infiltration buffer [10mM 2-(N-morpholino) ethanesulfonic acid (MES), 10 mM $MgSO_4$, at pH 5.5]. Then, 6 weeks old *N. benthamiana* plants were used for agroinfiltration, and the leaves were harvested 3 days post infiltration (dpi). For the large-scale infiltration, vacuum infiltration was performed to infiltrate the plant leaves.

3.1.1.2 Extraction and purification of recombinant RBD-His from infiltrated plants

The agroinfiltrated leaves were harvested and proteins were extracted with extraction buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, and 5 mM Imidazole). Then, the crude samples were centrifuged at 15,000 g for 20 min at 4 ºC and the supernatants were filtered using a sterile 0.45 µm filter. The filtered extracts were loaded onto a Ni-NTA affinity column and then the column was washed with washing buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, and 5-20 mM Imidazole). The recombinant protein was eluted with elution buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, and 250 mM Imidazole). The purification profiles were visualized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The concentration of the purified RBD-His protein was determined by the Bradford assay.

3.1.1.4 SDS-PAGE and Western Blotting

The protein samples were analyzed by SDS-PAGE with reducing loading dye (125 mM Tris-HCl pH 6.8, 12% (w/v) SDS, 10% (v/v) glycerol, 22% (v/v), β-mercaptoethanol, and 0.001% (w/v) bromophenol blue). The separated bands in the

gel were visualized by instant blue staining (Expedeon, Cambridge, UK). For Western blotting, the separated proteins were transferred to the nitrocellulose membrane (Biorad, California, USA). The membrane was blocked with 5% skim milk (BD Life Sciences, UK) for 1 h and probed with anti-His antibody conjugated with horse radish peroxidase (HRP) (Abcam, UK) diluted 1:5000 in 3% skim milk for 2 h. Finally, the membrane was washed with 1X PBST (1X PBS with 0.05% Tween) and developed by chemiluminescence using an enhanced chemiluminescence (ECL) detection reagent (Abcam, UK).

3.1.2 Binding of plant-produced RBD-His to plant-produced ACE2-Fc

A 96-well ELISA plate (Corning, USA) was coated with plant-produced ACE2-Fc (4 µg/ml), obtained from a previous study [63], and incubated overnight at 4°C. Then, the plate was washed three times with 1X PBST and blocked with 5% skim milk at 37°C for 1 h. After the plate was washed, it was incubated with 2-fold serial dilution of RBD-His starting at 8 µg/ml and incubated at 37 °C for 1 h. Following the washing step, anti-His-HRP antibody (Abcam, UK) diluted at 1:5000 in 1X PBS was added into each well and incubated at 37 °C for 1 h. Finally, the plate was washed, and the signal was detected using 3,3',5,5'–Tetramethylbenzidine (TMB) substrate (Promega, USA) and stopped with 1M H_2SO_4. The absorbance was measured at 450 nm using a microplate reader (Hercuvan Lab System, Cambridge, UK). The experiment was performed in triplicates.

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3.1.3 Binding of plant-produced H4 and CR3022 mAb with plant-produced RBD-His antigen

ELISA was performed in the same manner as described above. The plate was coated with plant-produced ACE2-Fc. Meanwhile, the plant-produced H4 mAb [10] and CR3022 mAb [11] were serially diluted with 2-fold serial dilution, starting at 40 µg/ml (final concentration), and incubated with plant-produced RBD-His (final concentration 4 µg/ml) in microcentrifuge tube at 37 °C for 1 h. Then, the mixture of RBD and mAbs was incubated on the plate at 37 °C for 1 h. The H4 mAb and CR3022 mAb were used as the positive and negative samples, respectively. The experiment

was performed in triplicates. The OD of RBD-ACE2 binding was used as a negative control for percent inhibition calculation. The percent inhibition was calculated using the formula; % inhibition = (1- OD sample/OD negative control) X100.

3.1.4 Binding of plant-produced RBD-His immunized mice sera with plant-produced RBD-Fc

ELISA was performed in the same manner as described above. The plate was coated with plant-produced ACE2-Fc. The sera of non-immunized and RBD-Fc immunized mice (two each)[15], were incubated on the plate with 2-fold serial dilution starting at 1:20 with PBS at 37 ºC for 1 h in order to identify a suitable dilution for the assay. The experiment was performed by following the steps mentioned in section 3.1.3.

After identifying the suitable dilution, the percent inhibition of 31 samples of sera from immunized mice and 24 samples from non-immunized mice sera was tested. Both RBD-Fc immunized and non-immunized mice sera were used at the dilution of 1:20. Then, a panel of mice sera was tested to evaluate the correlation between sVNT using plant recombinant protein and titers of the *in vitro* MN test [15]. The titer of cVNT from immunized mice sera was transformed into log-10 before computing the coefficients. The sensitivity and specificity of sVNT were calculated using the formula; sensitivity = number of true positives / (number of true positives + false negatives), specificity = number of true negatives / (number of true negatives + false positives).

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3.2 RBD-Fc and ACE2-His

3.2.1 ACE2-His production

3.2.1.1 Transient expression of ACE2-His in *N. benthamiana*

The cultured stock of *A. tumefaciens* harboring pBYR2eK-ACE2-His was obtained from the work of my senior and sub-cultured overnight at 28 ºC. The

cultured *A. tumefaciens* cells were pelleted and resuspended with infiltration buffer [10mM 2-(N-morpholino) ethanesulfonic acid (MES), 10 mM $MgSO_4$, at pH 5.5]. Then, 6 weeks old *N. benthamiana* plants were used for agroinfiltration, and the leaves were harvested 3 dpi. For the large-scale infiltration, vacuum infiltration was performed to infiltrate the plant leaves.

3.2.1.2 Extraction and purification of recombinant ACE2-His from infiltrated plants

The agroinfiltrated leaves were harvested and proteins were extracted with extraction buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, and 5 mM Imidazole). Then, the crude samples were centrifuged at 15,000 g for 20 min at 4 ºC and the supernatants were filtered using a sterile 0.45 µm filter. The filtered extracts were loaded onto a Ni-NTA affinity column and then the column was washed with washing buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, and 5-20 mM Imidazole). The recombinant protein was eluted with elution buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, and 250 mM Imidazole). The purification profiles were visualized using SDS-PAGE and Western blotting. The concentration of the purified ACE2-His protein was determined by the Bradford assay.

3.2.1.3 SDS-PAGE and Western Blotting

The protein samples were analyzed by SDS-PAGE with reducing loading dye (125 mM Tris-HCl pH 6.8, 12% (w/v) SDS, 10% (v/v) glycerol, 22% (v/v), β-mercaptoethanol, and 0.001% (w/v) bromophenol blue). The separated bands in the gel were visualized by instant blue staining (Expedeon, Cambridge, UK). For Western blotting, the separated proteins were transferred to the nitrocellulose membrane (Biorad, California, USA). The membrane was blocked with 5% skim milk (BD Life Sciences, UK) for 1 h and probed with anti-His antibody conjugated HRP (Abcam, UK) diluted 1:5000 in 3% skim milk for 2 h. Finally, the membrane was washed with 1X PBST (1X PBS with 0.05% Tween) and developed by chemiluminescence using an ECL detection reagent (Abcam, UK).

3.2.2 Binding of plant-produced RBD-Fc to plant-produced ACE2-His

A 96-well ELISA plate (Corning, USA) was coated with plant-produced RBD-Fc (4 µg/ml) [15] and incubated overnight at 4°C. Then, the plate was washed three times with 1X PBST and blocked with 5% skim milk at 37°C for 1 h. After the plate was washed, it was incubated with 2-fold serial dilution of ACE2-His starting at 8 µg/ml and incubated at 37 °C for 1 h. Following the washing step, anti-His-HRP antibody (Abcam, UK) diluted at 1:5000 in 1X PBS was added into each well and incubated at 37 °C for 1 h. Finally, the plate was washed, and the signal was detected using TMB substrate (Promega, USA) and stopped with 1M H_2SO_4. The absorbance was measured at 450 nm using a microplate reader (Hercuvan Lab System, Cambridge, UK). The experiment was performed in duplicates.

3.2.3 Binding of plant-produced H4 and CR3022 mAb with plant-produced RBD-Fc antigen

ELISA was performed in the same manner as described above. The plate was coated with plant-produced RBD-Fc. The plant-produced H4 mAb [10] and CR3022 mAb [11] were incubated on the plate with 2-fold serial dilution starting at 10 µg/ml at 37 °C for 1 h before the addition of ACE2-His into the plates. The H4 mAb and CR3022 mAb were used as the positive and negative samples, respectively. The experiment was performed in duplicates. The OD of RBD-ACE2 binding was used as a negative control for percent inhibition calculation. The percent inhibition was calculated using the formula; % inhibition = (1- OD sample/OD negative control) X100.

3.2.4 Binding of plant-produced RBD-Fc immunized mice sera with plant-produced RBD-Fc

ELISA was performed in the same manner as described above. The plate was coated with plant-produced RBD-Fc. The sera of non-immunized and RBD-Fc immunized mice (two each)[15], were incubated on the plate with 5-fold serial dilution starting at 1:20 with PBS at 37 °C for 1 h in order to identify a suitable

dilution for the assay. The experiment was performed by following the steps mentioned in section 3.3.

After identifying the suitable dilution, the percent inhibition of 15 samples each of immunized and non-immunized mice sera were tested. Both RBD-Fc immunized and non-immunized mice sera were used at the dilution of 1:20. Then, a panel of mice sera was tested to evaluate the correlation between sVNT using plant recombinant protein and titers of the *in vitro* MN test [15]. The titer of cVNT from immunized mice sera was transformed into log-10 before computing the coefficients. The sensitivity and specificity of sVNT were calculated using the formula; sensitivity = number of true positives / (number of true positives + false negatives), specificity = number of true negatives / (number of true negatives + false positives).

3.3. Statistical analysis

All graphs were generated using GraphPad Prism 9.3.1 (GraphPad Software, Inc., USA) and Microsoft Office Excel. The difference between the 1:20 dilution of non-immunized and immunized sera was analyzed using an unpaired two-tailed Mann-Whitney test. The correlation between cVNT and sVNT was conducted with Pearson's correlation coefficients.

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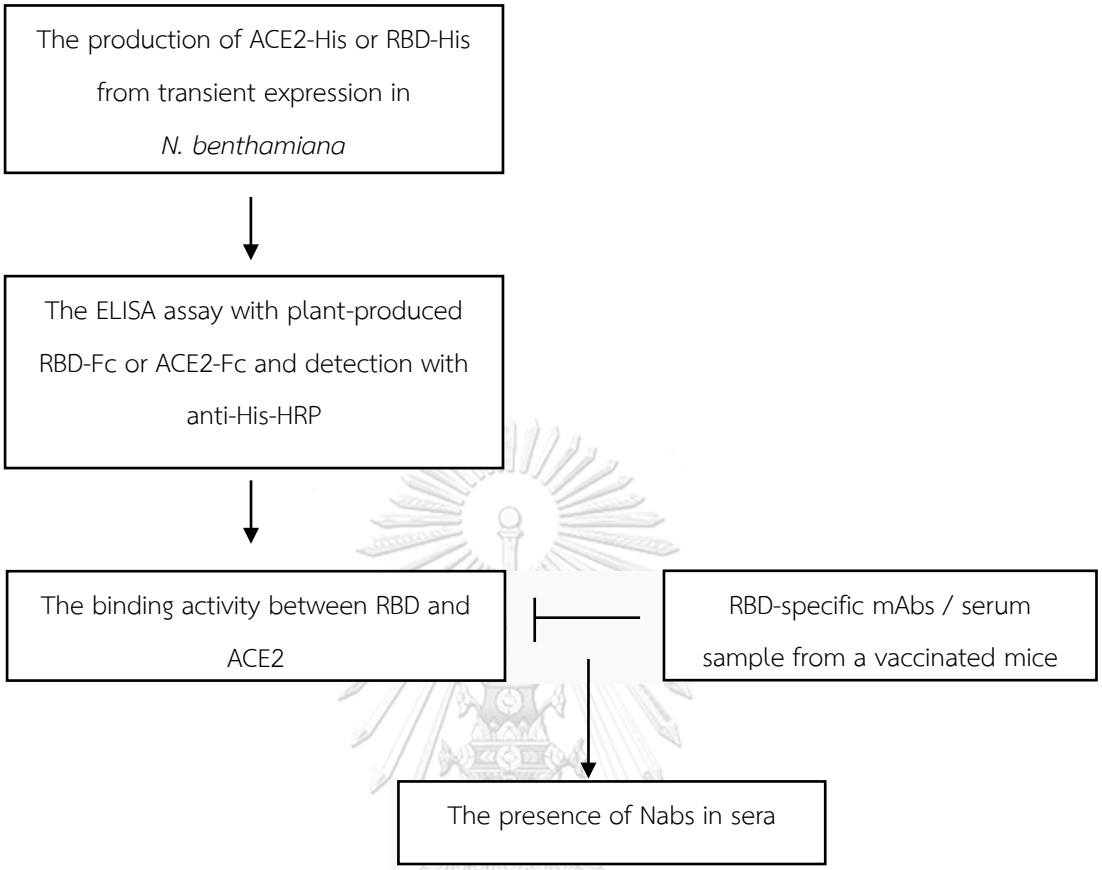
4. Research framework



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CHAPTER IV RESULTS AND DISCUSSION

1. RBD-His and ACE2-Fc

1.1 Expression and purification of plant-produced RBD-His

The cultured *Agrobacteria* harboring pBYR2eK-RBD-His were infiltrated into *N. benthamiana* leaves with a final OD_{600} of 0.2 and the leaves were harvested after 3 dpi. The plant-produced RBD-His was purified using Ni-NTA affinity column chromatography. The expression level of the plant-produced RBD-His accumulated at 117.89 µg/g fresh weight. SDS-PAGE and Western blotting were performed to assess the purity of plant-produced RBD-His. The instant Blue stained SDS-PAGE gel showed the expected band at approximately 40 kDa under reducing conditions (Fig. 2a). For the Western blotting, the bands were detected using anti-His-HRP antibody (1:5000) and the expected major band was detected at 40 kDa. Furthermore, faint bands were detected at about 80 and 120 kDa, which could be protein dimer and trimer, respectively (Fig. 2b). Minor bands below 40 kDa may correspond to fragmented proteins. The signal was developed using ECL detection reagent.

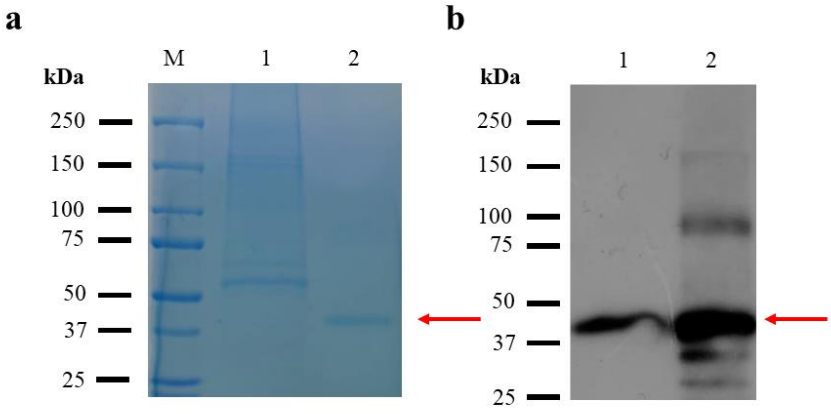

Figure 2 *SDS-PAGE and Western blot analysis of plant-produced RBD-His.*
The crude proteins were extracted from the infiltrated leaves, and the RBD-His protein was purified, analyzed on SDS-PAGE gel, and visualized with InstantBlue (a). Lane M: protein ladder; Lane 1: total soluble protein of *N. benthamiana* agroinfiltrated with plant expression vector harboring the RBD-His; Lane2: purified RBD-His. For Western blot analysis, proteins on the blot were probed with rabbit anti-His antibody conjugated with HRP under reducing condition (b).

Lane 1: total soluble protein of *N. benthamiana* agroinfiltrated with plant expression vector harboring the RBD-His; Lane 2: purified RBD-His. Arrow indicates the expected band.

1.2. Binding of plant-produced RBD-Fc to plant-produced ACE2-His

To evaluate the interaction of ACE2 and RBD, we tested the binding of plant-produced ACE2-Fc and RBD-His (SARS-CoV-2, Wuhan strain) by ELISA. The 96 well plate was coated with plant-produced ACE2-Fc and incubated with serial diluted plant-produced RBD-His. Then, the plate was washed and incubated with 1:5000 anti-His-HRP antibody. The results showed that RBD-His bound to ACE2-Fc which indicated the proper folding of the plant-produced RBD-His recombinant protein (Fig. 3).

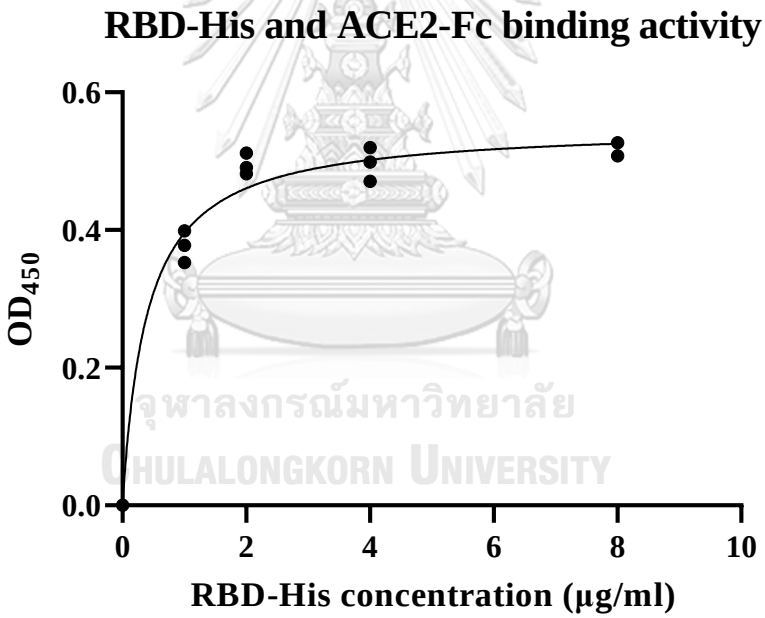

Figure 3 *Binding of plant-produced SARS-CoV-2 RBD-His (Wuhan strain) to plant-produced ACE2-Fc.*

The binding activity was assessed by incubating different dilution of plant-produced SARS-CoV-2 RBD-His (Wuhan strain) on a plate coated with plant-produced ACE2-Fc and detecting it using anti-His antibody conjugated with HRP. The data are represented as the mean of triplicates.

1.3. Binding of plant-produced H4 and CR3022 mAb

After examining the binding between plant-produced ACE2-Fc and RBD-His of SARS-CoV-2 (Wuhan strain), the binding of plant-produced H4 and CR3022 mAbs was performed. Earlier studies showed that the H4 mAb has neutralizing potential that can inhibit the ACE2 binding with RBD and CR3022 mAb can bind to RBD protein but cannot interfere in the RBD-ACE2 interaction *in vitro* [28, 64]. Hence, H4 and CR3022 mAbs were used as positive and negative samples, respectively. The plate was coated with ACE2-Fc and blocked with 5% skim milk. Meanwhile, the plant-produced H4 and CR3022 mAb were serially diluted and incubated with plant-produced RBD-His in microcentrifuge tube. Then, the mixture of RBD and mAbs was incubated on the plate. The plate was further incubated with anti-His-HRP, and the signal was developed using TMB substrate. As shown in Fig. 4, the results were presented as the percent inhibition which indicated the neutralizing potential of the mAb. The H4 mAb showed higher percent inhibition of 55.76% at the highest tested concentration compared with CR3022 (-17.27%). This result suggests that the plant-produced H4 mAb is capable of inhibiting the binding between ACE2 and RBD. However, the concentration of H4 mAb used in this study was still relatively high, resulting in only moderate percent inhibition. The negative values for percent inhibition of CR3022 mAb and certain H4 mAb concentrations can be attributed to the fact that the OD value of CR3022 mAb exceeded the OD value of the negative control (i.e., the ACE2-RBD interaction OD value).

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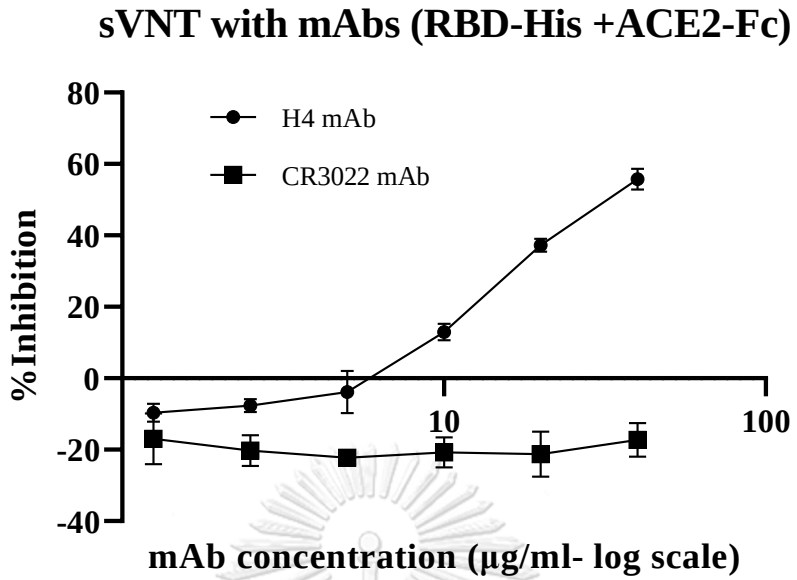

Figure 4 *The percent inhibition of the interaction between plant-produced SARS-CoV-2 RBD-His (Wuhan strain) and ACE2-Fc.*

The percent inhibition of the interaction was determined by performing 2-fold serial dilution of plant-produced mAbs. The H4 and CR3022 mAbs were used as positive and negative sample, respectively. The data are represented as the mean ± SD of triplicates.

1.4. Detection of Nabs in the RBD-Fc immunized mice sera

The sera collected from the RBD-Fc immunized mice showed approximately 75% and 35% inhibition at the maximum tested dilution, whereas the percent inhibition of non-vaccinated sera remained at the same level across the range of dilution and slightly increased at low concentrations (Fig.5). It indicates that the vaccinated sera collected from the mice have Nabs which bound to RBD-Fc and prevented ACE2-His interaction, while non-vaccinated sera have no Nabs specific to the RBD protein and hence it did not interfere with the RBD-ACE2 interaction. The highest dilution (1:20) was used for further experiments to test the Nabs in the panel of 31 immunized and 24 non-immunized sera. The data are shown as the mean ± SD of duplicates.

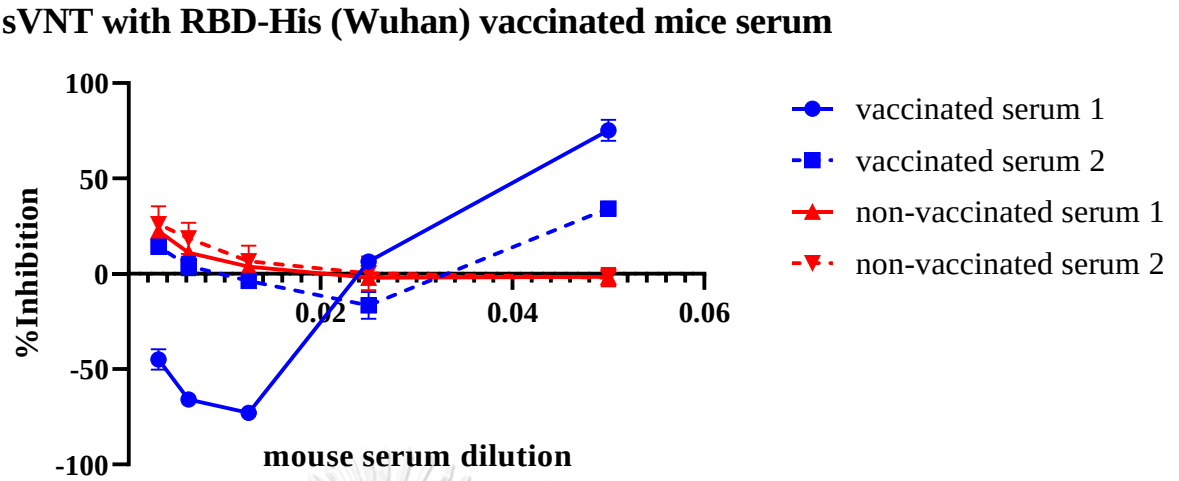

Figure 5 *ACE2-binding percent inhibition of vaccinated mice sera.*

The percent inhibition of ACE2-binding in vaccinated mice sera was determined by measuring the OD value of the interaction between RBD and ACE2, using the OD of a negative control. The percent inhibition was calculated using the formula % inhibition = (1- sample OD/ negative control OD) X 100. The data are represented as mean values of duplicates from each concentration.

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A total of 55 mice sera samples obtained from immunized mice (*n*=31) and non-immunized mice (*n*=24) were tested for the presence of Nabs using developed sVNT. The results screened by sVNT were summarized in Fig. 6. The sensitivity and specificity of sVNT were found to be 22.58% and 95.83%, respectively. The cut-off value for sVNT was determined with an equation: mean OD of negative sera - 3(standard deviation) before percent inhibition calculation. The OD and percent inhibition cut-off were found to be 0.5 ± 0.026 and 9.9%. A sample having a percent inhibition greater than the cut-off was considered to be a positive sample. However, the performance of RBD-His and ACE2-Fc interaction for sVNT still has low sensitivity.

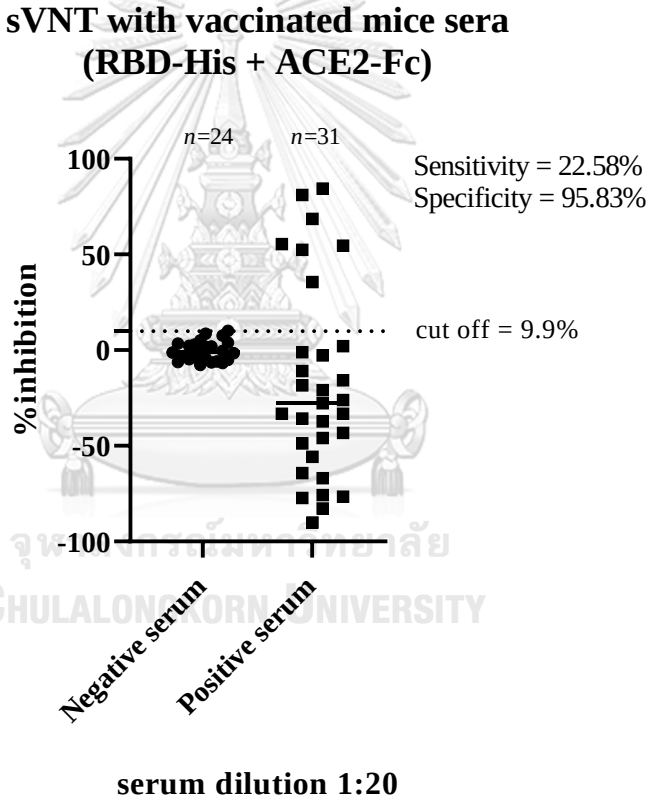

Figure 6 *ACE2-binding percent inhibition of vaccinated and non-vaccinated mice sera*

The percent inhibition of ACE2-binding was determined in each vaccinated (*n*=31) and non-vaccinated (*n*=24) mice sera at the final serum dilution of 1:20. The dotted and horizontal lines represent the cut-off at 9.9% inhibition and median values, respectively. Mann-Whitney test was performed.

To compare the results of sVNT using plant recombinant proteins with other virus neutralization tests, the comparison was performed based on correlations. Correlation analysis was conducted to examine the association between the results from sVNT and neutralizing titers (NTs) from cVNT, a gold standard for virus neutralization test (Fig.7). Among the 55 mice sera obtained from vaccinated and non-vaccinated mice, poor correlation (R^2 = 0.09) between the result of sVNT using plant recombinant proteins and NTs of cVNT was observed. So, we conducted another strategy with RBD-Fc and ACE2-His.

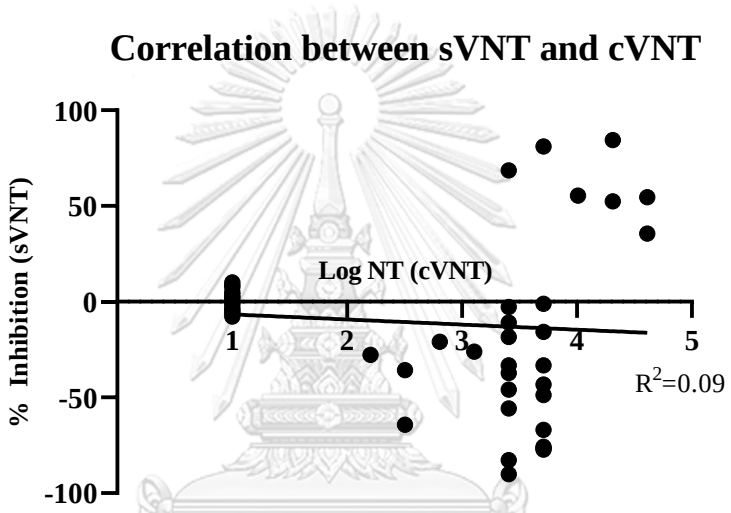

Figure 7 Correlation analysis for 55 mice sera samples with different levels of Nabs by sVNT and cVNT.

Correlation analysis was performed for 55 mice sera samples with varying levels of Nabs as determined by sVNT and cVNT. Pearson's correlation coefficients were used to conduct linear regression and correlations in GraphPad Prism, with statistical significance calculated using a two-tailed test. The data are represented as the log of NTs of cVNT and percent inhibition of sVNT

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2. RBD-Fc and ACE2-His

2.1. Expression and purification of plant-produced ACE2-His

The cultured *Agrobacteria* harboring pBYR2eK-ACE2-His were infiltrated into *N. benthamiana* leaves with a final OD_{600} of 0.2 and the leaves were harvested after 3 dpi. The plant-produced ACE2-His was purified using Ni-NTA affinity column chromatography. The expression level of the plant-produced ACE2-His accumulated

at 91.25 μg/g fresh weight. SDS-PAGE and Western blotting were performed to assess the purity of plant-produced ACE2-His. The instant Blue stained SDS-PAGE gel showed the expected band at approximately 80 kDa under reducing conditions (Fig. 8a). For the Western blotting, the bands were detected using anti-His-HRP antibody (1:5000) (Fig. 8b). The signal was developed using ECL detection reagent.

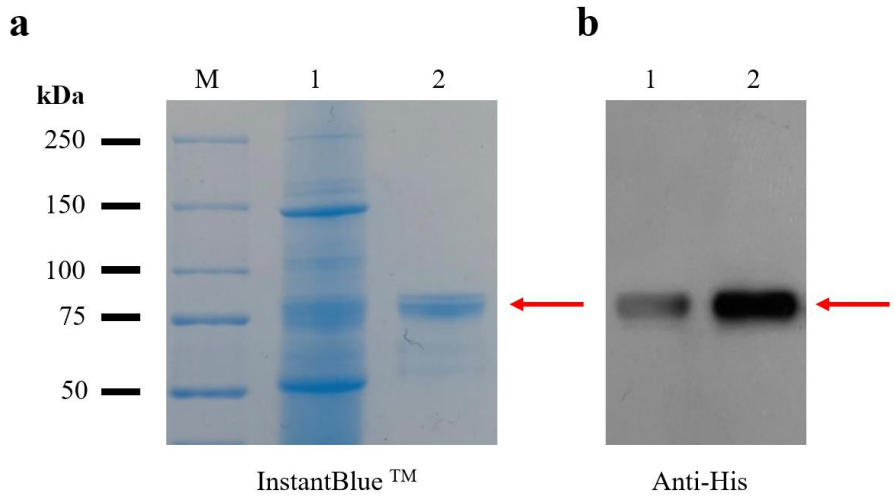

Figure 8 *SDS-PAGE and Western blot analysis of plant-produced ACE2-His.*
The crude proteins were extracted from the infiltrated leaves, and the ACE2-His protein was purified, analyzed on SDS-PAGE gel, and visualized with InstantBlue (a). Lane M: protein ladder; Lane 1: 20 μg of total soluble protein of *N. benthamiana* agroinfiltrated with plant expression vector harboring the ACE2-His; Lane2: 1 μg of purified ACE2-His. For Western blot analysis, proteins on the blot were probed with rabbit anti-His antibody conjugated with HRP under reducing condition (b). Lane 1: 20 μg of total soluble protein of *N. benthamiana* agroinfiltrated with plant expression vector harboring the ACE2-His; Lane 2: 750 ng of purified ACE2-His. Arrow indicates the expected band.

2.2. Binding of plant-produced RBD-Fc to plant-produced ACE2-His

To evaluate the interaction of ACE2 and RBD, we tested the binding of plant-produced ACE2-His and RBD-Fc (SARS-CoV-2, Wuhan strain) by ELISA. The 96 well plate was coated with plant-produced RBD-Fc and incubated with serial diluted plant-produced ACE2-His. Then, the plate was washed and incubated with 1:5000

anti-His-HRP antibody. The results showed that ACE2-His bound to RBD-Fc which indicated the proper folding of the plant-produced ACE2-His recombinant protein (Fig. 9).

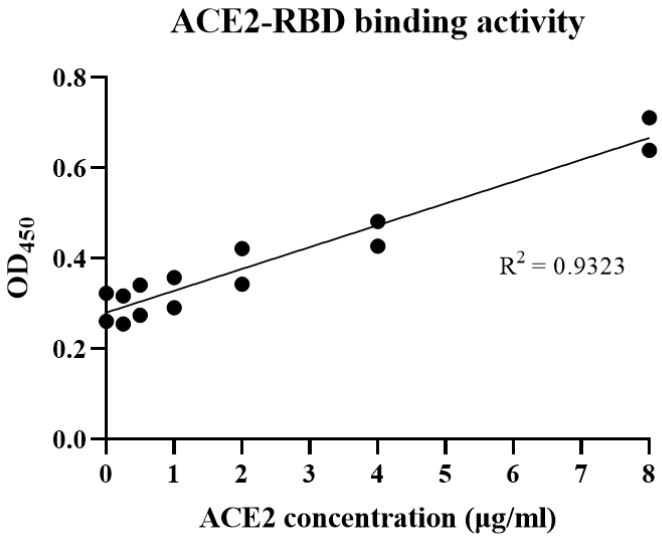

Figure 9 *Binding of plant-produced ACE2-His to plant-produced SARS-CoV-2 RBD-Fc (Wuhan strain).*

The binding activity was assessed by incubating different dilution of plant-produced ACE2-His on a plate coated with plant-produced SARS-CoV-2 RBD-Fc (Wuhan strain) and detecting it using anti-His antibody conjugated with HRP. The data are represented as the mean of duplicates.

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2.3. Binding of plant-produced H4 and CR3022 mAb with plant-produced RBD-Fc

After examining the binding between plant-produced ACE2-His and RBD-Fc of SARS-CoV-2 (Wuhan strain), the binding of plant-produced H4 and CR3022 mAbs was performed. Earlier studies showed that the H4 mAb has neutralizing potential that can inhibit the ACE2 binding with RBD and CR3022 mAb can bind to RBD protein but cannot interfere in the RBD-ACE2 interaction *in vitro* [28, 64]. Hence, H4 and CR3022 mAbs were used as positive and negative samples, respectively. The plate was coated with RBD-Fc and blocked with 5% skim milk. Then, the plant-produced H4 and CR3022 mAb were serially diluted and incubated in the plate followed by

incubation with plant-produced ACE2-His. The plate was further incubated with anti-His-HRP, and the signal was developed using TMB substrate. As shown in Fig. 10, the results were presented as the percent inhibition which indicated the neutralizing potential of the mAb. The H4 mAb showed higher percent inhibition of 64% at the highest tested concentration compared with CR3022 (12.9%). This result indicated that plant-produced H4 mAb can efficiently block the binding of ACE2 and RBD.

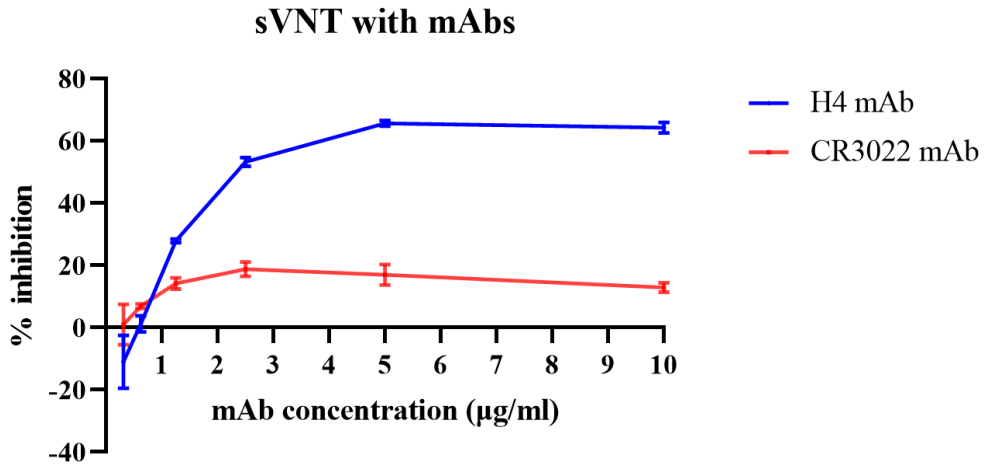

Figure 10 *The percent inhibition of the interaction between plant-produced SARS-CoV-2 RBD-Fc (Wuhan strain) and ACE2-His.*

The percent inhibition of the interaction was determined by performing 2-fold serial dilution of plant-produced mAbs. The H4 and CR3022 mAbs were used as positive and negative sample, respectively. The data are represented as the mean ± SD of duplicates.

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2.4. Detection of Nab in the RBD-Fc immunized mice sera

The sera collected from the RBD-Fc immunized mice showed approximately 60% inhibition at the maximum tested dilution, whereas the percent inhibition of non-vaccinated sera fluctuated over the range of dilution from approximately -20% to 10% (Fig. 11). It indicates that the vaccinated sera collected from the mice have Nabs, while non-vaccinated sera have no Nabs specific to the RBD protein and hence it did not interfere with the RBD-ACE2 interaction. The highest dilution (1:20) was

used for further experiments to test the Nabs in the panel of 15 immunized and 15 non-immunized sera. The data are shown as the mean ± SD of duplicates.

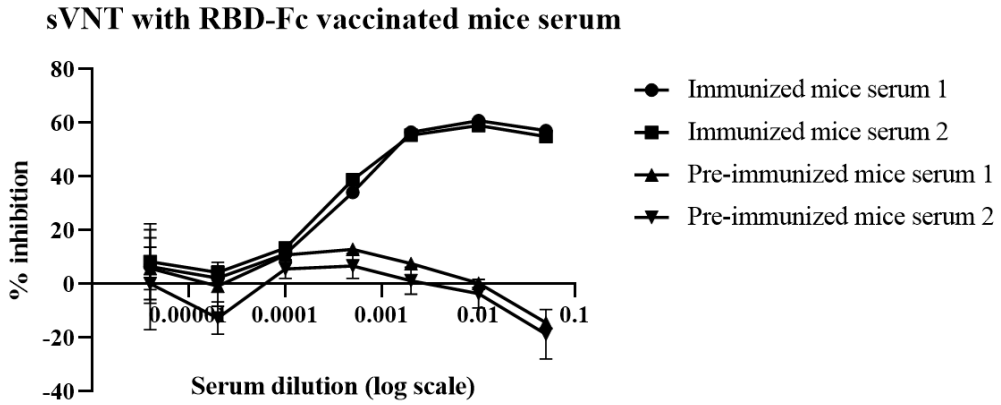

Figure 11 *ACE2-binding percent inhibition of vaccinated mice sera.*

The percent inhibition of ACE2-binding in vaccinated mice sera was determined by measuring the OD value of the interaction between RBD and ACE2, using the OD of a negative control. The percent inhibition was calculated using the formula % inhibition = (1- sample OD/ negative control OD) X 100. The data are represented as mean values of duplicates from each concentration.

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A total of 30 mice sera samples obtained from immunized mice (*n*=15) and non-immunized mice (*n*=15) were tested for the presence of Nabs using developed sVNT. These samples were categorized as positive and negative samples based on the results from *in vitro* MN test which was performed according to the previous study (13). The results screened by sVNT were summarized in Fig. 12. The sensitivity and specificity of sVNT were found to be 100% for both. The cut-off value for sVNT was determined with an equation: mean OD of negative sera - 3(standard deviation) before percent inhibition calculation. The OD and percent inhibition cut-off were found to be 0.77 ± 0.025 and 9.78%. A sample having a percent inhibition greater than the cut-off was considered to be a positive sample.

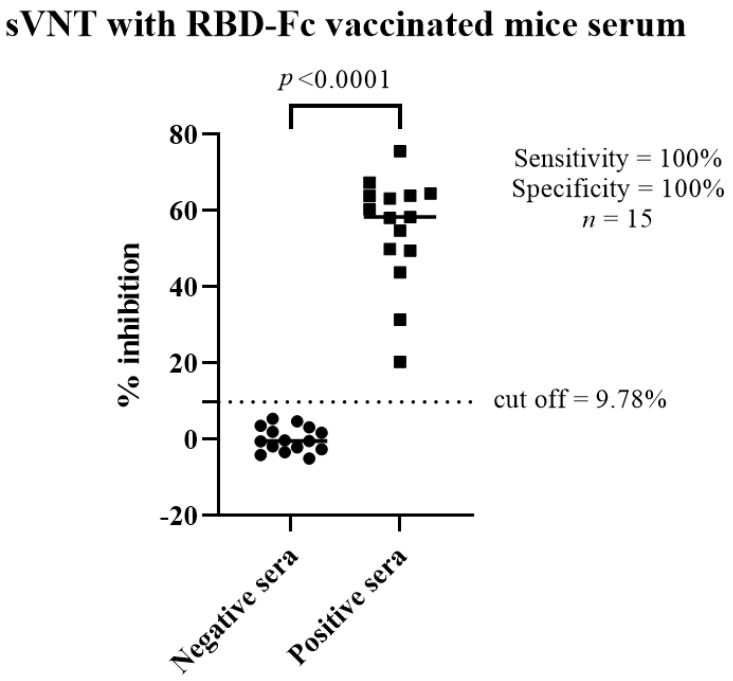

Figure 12 *ACE2-binding percent inhibition of each vaccinated and non-vaccinated (n=15) mice sera*

The percent inhibition of ACE2-binding was determined in each vaccinated (*n*=15) and non-vaccinated (*n*=15) mice sera at the final serum dilution of 1:20. The dotted and horizontal lines represent the cut-off at 9.78% inhibition and median values, respectively. Mann-Whitney test was performed. p <0.0001 was considered significant.

The results of sVNT using plant recombinant proteins were compared with those of cVNT, as in the earlier experiment. Correlation analysis was conducted to examine the association between the results from sVNT and NTs from cVNT, a gold standard for virus neutralization test (Fig. 13). Among the 30 mice sera obtained from vaccinated and non-vaccinated mice, significant correlation between the result of sVNT using plant recombinant protein and NTs of cVNT was observed ($p < 0.001$, r = 0.94, $R^2 = 0.8919$).

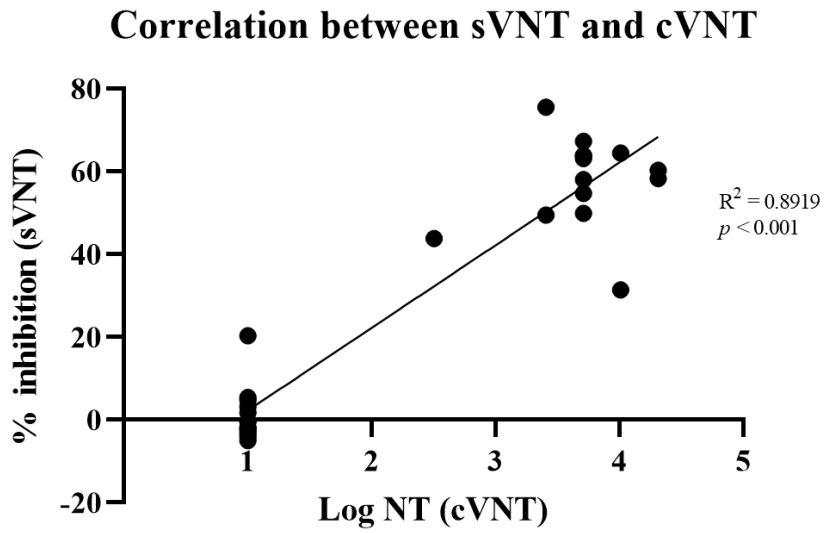

Figure 13 *Correlation analysis for 30 mice sera samples with different levels of Nabs by sVNT and cVNT.*

Correlation analysis was performed for 30 mice sera samples with varying levels of Nabs as determined by sVNT and cVNT. Pearson's correlation coefficients were used to conduct linear regression and correlations in GraphPad Prism, with statistical significance calculated using a two-tailed test. The data are represented as the log of NTs of cVNT and percent inhibition of sVNT

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Discussion

Since 2019, COVID-19 has rapidly spread across the world, threatening global health. This pandemic has also disrupted the national economies, tourism, social activity, merchandise trade, and political systems [65]. The number of infected cases and the death toll has been increasing daily. Recently, some COVID-19 vaccines have been approved for human use [66]. The presence of Nabs prevents re-infection with SARS-CoV-2. However, the humoral immune response to SARS-CoV-2 resulting from vaccination or natural infection reduces significantly over time [3]. Hence, there is a need for a simple and accurate serological test to detect the presence of Nabs in the community or country/region. The sVNT offers a high-throughput assay for evaluating SARS-CoV-2 immunity with a simple protocol and can be conducted in a BSL2 laboratory [7].

Previous reports and other commercial sVNT kits utilized recombinant protein mostly produced from mammalian expression systems [7, 67-70]. Tan *et al*., reported similar performance when using the recombinant proteins produced from the mammalian systems and insect cell lines in sVNT [7]. In addition, there are several reports utilizing mammalian cell-derived proteins to develop sVNT kits that exhibit different sensitivity and specificity [42, 70-73].

In this study, we demonstrated the possibility of using plants as an expression system to produce suitable reagents for the development of a Nab detection assay. Both ACE2 and RBD proteins were effectively produced in the plant expression system [15]. First, we developed the sVNT from plant-produced ACE-Fc and RBD-His. The binding activity of ACE2-RBD showed potential binding of plant-produced RBD-His with initial saturation at 4 µg/ml of plant-produced RBD-His. The sVNT with mAb exhibited moderate percent inhibition of H4 mAb and the negative values in low concentrations may be due to the RBD cross-linkage, as reported in the previous study [74]. Furthermore, the sensitivity of sVNT testing using a panel of mice sera (1:20) for determining vaccinated sera is low, and it has a poor correlation with the MN test. This may be due to the cross-linkage of RBD, as found in sVNT with mAb result. The MN test also showed that log NT values lower than 3.7 (NT=5120), most percent inhibitions lower than zero, indicating a higher OD value than the reference

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OD (RBD-ACE2 interaction OD value). However, at higher NTs, percent inhibition ranged from 35% to 84% (Appendix D). The reverse format of natural binding can overcome this cross-link issue [74]. Thus, the sVNT with plate-bound RBD and soluble ACE2 was performed.

The binding activity of ACE2-His was confirmed with the plant-produced RBD-Fc. The optimal concentration of ACE2-His was found to be 4 µg/ml and the selected concentration was further investigated using plant-produced mAb H4 and CR3022. The results showed that mAb H4 can prevent the binding of RBD-ACE2 up to 60% compared with mAb CR3022 which exhibits low or no inhibition. The results of this investigation exhibited good performance of plant-derived recombinant protein used in the Nab detection assay as well as a correlation (r =0.94, p<0.001) to the gold-standard test cVNT when using mice sera (n=30). A recent study developed the multiplexed sVNT using trimeric spike protein for evaluating Nabs against SARS-CoV-2 variants from patients who received the vaccine (Pfizer-BioNTech or Moderna mRNA vaccine) or were infected with COVID-19 and reported high concordance with PRNT ($PRNT_{50}$, r = 0.8, p<0.001 and $PRNT_{90}$, r = 0.88, p<0.0001) [42]. In another study, 4 different commercial sVNT were evaluated in comparison with cVNT and the results showed that sVNT expressed moderate to good correlation with cVNT (98.01% agreement, p <0.05) [72]. Tan *et al*., validated the sVNT with two cohorts of patients with COVID-19 in two different countries (Singapore and China) that showed 100% specificity and 98% sensitivity with a high correlation between sVNT and MN test [7]. Furthermore, sVNT can be used to detect Nabs in a species-independent manner.

It is important to acknowledge that not all Nabs necessarily bind to RBD, as past studies have reported that antibodies targeting other regions in the S1 and S2 proteins can also contribute to virus neutralization [75]. The trimeric spike protein can mimic the natural confirmations of the native spike protein that has different Nabs-binding sites with RBD and is useful for evaluating Nabs against variants of SARS-CoV-2, as emerging variants of SARS-CoV-2 have mutations or deletions in the spike domain [42]. Even though the full-length S protein and the RBD exhibited well for

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specific antibody detection, recent studies have suggested that RBD-specific Nabs are immunodominant in both SARS-CoV and SARS-CoV-2 infections [76, 77].

Both SARS-CoV and SARS-CoV-2 RBD can recognize ACE2, which means that cross-reactivity with SARS-CoV RBD-specific antibodies may potentially lead to false-positive results in the development of sVNT [78, 79]. However, SARS-CoV seroprevalence are very low in the human population since 2003, and thus it is unlikely that the reactivity of SARS-CoV antibodies would affect the specificity [80]. Furthermore, several published studies have demonstrated the high specificity and sensitivity of the SARS-CoV-2 RBD antigen for antibody detection, with minimal cross-reactivity with other coronaviruses [67, 77, 80, 81]. Premkumar *et al*., have established that most individuals who have been infected with common Human Coronavirus (such as influenza A virus and respiratory syncytial virus) do not have cross-reactive antibodies against the recombinant RBD of SARS-CoVs [77].

There are still limitations in this study such as all the reagents used are plant-produced (ACE2-His, RBD-Fc, H4 mAb, and CR3022 mAb) and we did not use any commercially available SARS-CoV-2 antigen or mAb to compare the binding efficiency. Additionally, we only included a small panel of mice samples to evaluate the assay specificity and did not use the human sera to assess the assay performance.

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CHAPTER V CONCLUSIONS

The SARS-CoV-2, causes COVID-19, a respiratory illness characterized by severe pneumonia. The virus binds to the ACE2 receptor in the respiratory tract via its spike protein, specifically the RBD. Several vaccines have been approved for human use against the virus. To evaluate the immune response of convalescent patients and vaccine recipients, the neutralizing levels of antibodies that prevent the binding of the virus to ACE2 need to be determined and tracked over time. NTs are commonly measured using the cVNT, which requires BSL3 facilities, is labor-intensive, and is costly. The pVNT is a more convenient alternative but still requires live viruses and cells. Both methods take 2-4 days to obtain results. In contrast, the sVNT is a competitive ELISA that can be completed within a few hours, does not require live viruses or cells under BSL2 containment, and is a promising alternative technique to cVNT and pVNT.

Here, we produced RBD-His and ACE2-His in *N. benthamiana*, a tobacco plant, using a transient expression system. The plant-produced RBD-His and ACE2-His were optimally expressed at 117.89 and 91.25 µg/g of leaf fresh weight and had the potential to bind to the plant-produced ACE2-Fc and RBD-Fc of SARS-CoV-2, respectively. We used both plant-produced proteins to evaluate the performance of the sVNT and its correlation with the *in vitro* MN test using serum from vaccinated mice to detect Nabs. The developed sVNT using plate-bound ACE2-Fc and soluble RBD-His demonstrated low sensitivity and correlation with the MN test whereas the sVNT using plate-bound RBD-Fc and soluble ACE2-His exhibited high sensitivity and specificity ($p<0.0001$), and the results were strongly correlated ($R^2 = 0.8919$) with the titer of the cVNT.

In conclusion, we utilized a plant expression platform to produce RBD-His and ACE2-His, which were used as reagents to develop SARS-CoV-2 Nab detection assays. Surrogate VNT utilizing plant-produced reagents could be used for screening vaccinated populations or individuals previously infected with SARS-CoV-2, thus contributing to the mitigation of the pandemic.

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2000421.

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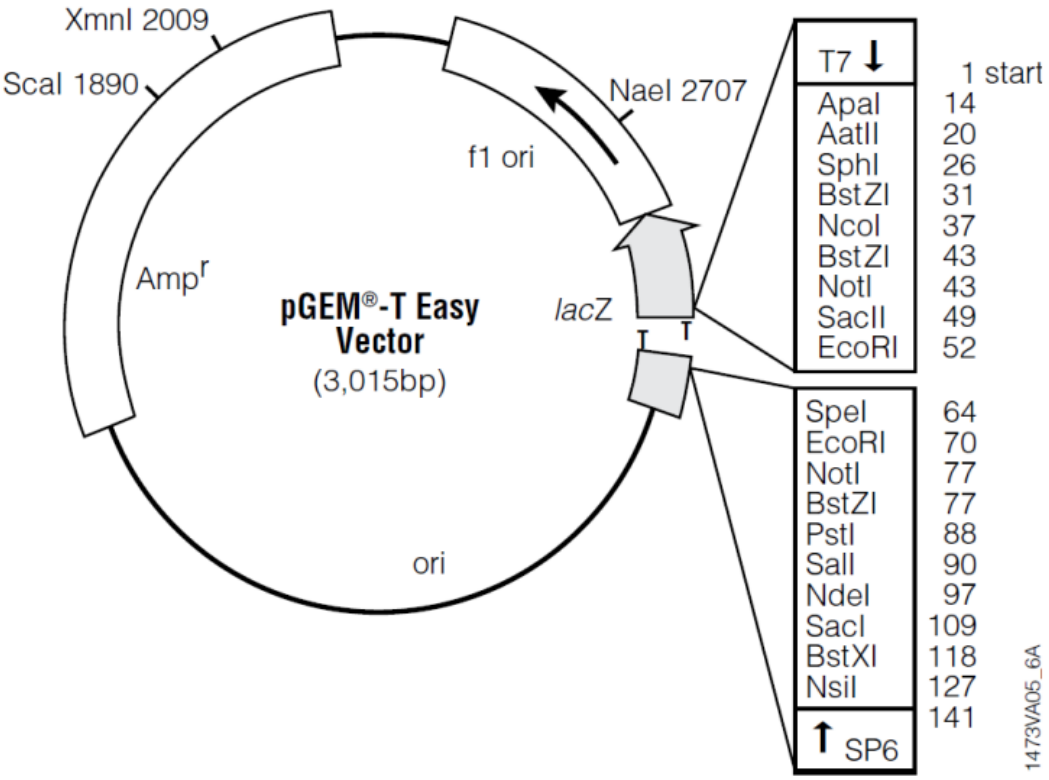
APPENDICES

Appendix A

pGEM®-T Easy Cloning Vector map (Promega, USA)



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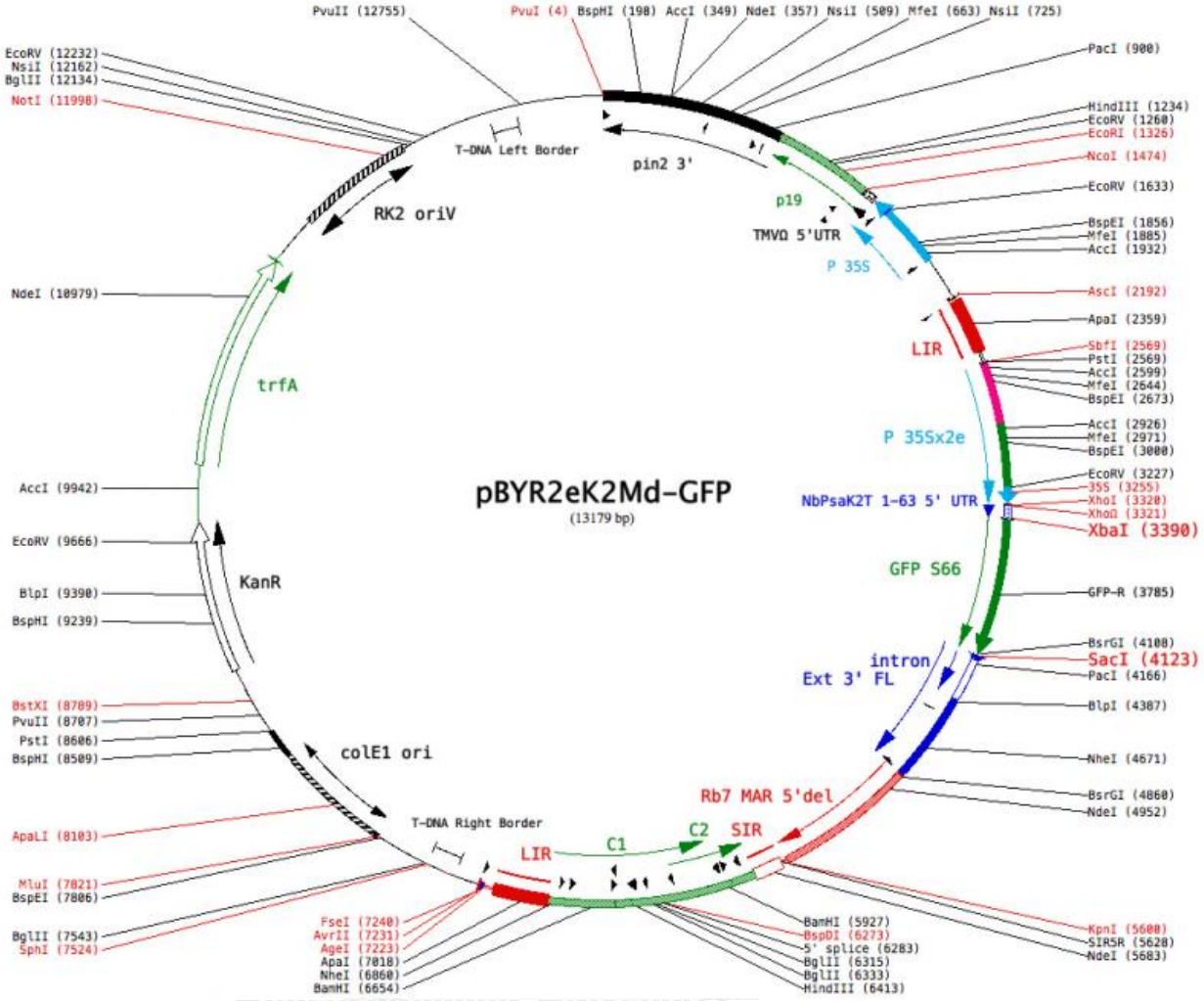
Appendix B

pBYR2eK2Md Plant Expression Vector Map

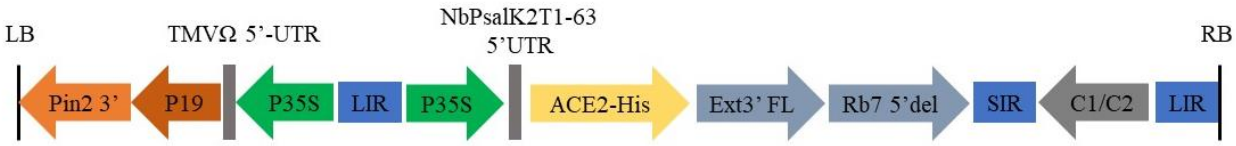


Appendix C

Construction of plant expression vector for ACE2-His production

(GenBank accession number: NP_001358344.)

pBYR2eK-ACE2-His



Schematic representation of ACE2-His gene in plant expression vector.

LB and RB, the left and right border of the T-DNA region in plasmid; PinII 3': the terminator from potato proteinase inhibitor II gene; P19: the RNA silencing suppressor from tomato bushy stunt virus; TMVΩ 5' UTR: untranslated region of tobacco mosaic virus Ω; P35S: Cauliflower Mosaic Virus (CaMV) 35S promoter, LIR and SIR: long and short intergenic region of Bean Yellow Dwarf Virus (BeYDV); NbPsalK2T1-63 5'UTR: 5' untranslated region; ACE2-His; codon-optimized ACE2 gene conjugated with 8X histidine tag; Ext 3' FL: region of tobacco extension gene; Rb7 5'del: tobacco RB7 promotor; C1/C2: BeYDV open reading frame (ORF) C1 and C2 encoding for replication initiation protein (Rep) and RepA.

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ACE2 amino sequence – Topological domain (18-740 amino acid) approximately 81.4 kDa

QSTIEEQAKTFLDKFNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDKWSAFLKEQSTLAQMYPLQEIQNLT
VKLQLQALQQNGSSVLSEDKSKRLNTILNTMSTIYSTGKVCNPDNPQECLLLEPGLNEIMANSLDYNERLWAWES
WRSEVGKQLRPLYEEYVVLKNEMARANHYEDYGDYWRGDYEVNGVDGYDYSRGQLIEDVEHTFEEIKPLYEHLHAY
VRAKLMNAYPSYISPIGCLPAHLLGDMWGRFWTNLYSLTVPFGQKPNIDVTDAMVDQAWDAQRIFKEAEKFFVSV
GLPNMTQGFWENSMLTDPGNVQKAVCHPTAWDLGKGDFRILMCTKVTMDDFLTAHHEMGHIQYDMAYAAQPF
LLRNGANEGFHEAVGEIMSLSAATPKHLKSIGLLSPDFQEDNETEINFLLKQALTIVGTLPFTYMLEKWRWMVFKGEI
PKDQWMKKWWEMKREIVGVVEPVPHDETYCDPASLFHVSNDYSFIRYYTRTLYQFQFQEALCQAAKHEGPLHKC
DISNSTEAGQKLFNMLRLGKSEPWTLALENVVGAKNMNVRPLLNYFEPLFTWLKDQNKNSFVGWSTDWSPYADQ
SIKVRISLKSALGDKAYEWNDNEMYLFRSSVAYAMRQYFLKVKNQMILFGEEDVRVANLKPRISFNFFVTAPKNVSDII
PRTEVEKAIRMSRSRINDAFRLNDNSLEFLGIQPTLGPPNQPPVS

Appendix D

Correlation data for sVNT (RBD-His and ACE2-Fc)

No.	OD	NT	Log NT	Percent inhibition
1	0.829333	5120	3.70927	-66.8679
2	0.877333	5120	3.70927	-76.5258
3	0.739	5120	3.70927	-48.6922
4	0.881333	5120	3.70927	-77.3307
5	0.575	5120	3.70927	-15.6942
6	0.601	640	2.80618	-20.9256
7	0.908	2560	3.40824	-82.6962
8	0.944667	2560	3.40824	-90.0738
9	0.093667	5120	3.70927	81.15359
10	0.156	2560	3.40824	68.61167
11	0.774333	2560	3.40824	-55.8015
12	0.662667	2560	3.40824	-33.3333
13	0.662333	5120	3.70927	-33.2663
14	0.626333	1280	3.10721	-26.0228
15	0.221667	10240	4.0103	55.39906
16	0.674333	320	2.50515	-35.6808
17	0.634667	160	2.20412	-27.6995
18	0.551333	2560	3.40824	-10.9323
19	0.816667	320	2.50515	-64.3192
20	0.486667	10	1	2.079142
21	0.682333	2560	3.40824	-37.2904
22	0.236667	20480	4.31133	52.38095
23	0.725333	2560	3.40824	-45.9423
24	0.077667	20480	4.31133	84.3729
25	0.32	40960	4.61236	35.61368
26	0.226	40960	4.61236	54.52716
27	0.873333	5120	3.70927	-75.721
28	0.712	5120	3.70927	-43.2596
29	0.510667	2560	3.40824	-2.74983
30	0.588333	2560	3.40824	-18.3769
31	0.502667	5120	3.70927	-1.14017

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