Journal of Visualized Experiments

Detection of SARS-CoV-2 Receptor-binding Domain Antibody Using A HiBiT-based Bioreporter

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62488R2
Full Title:	Detection of SARS-CoV-2 Receptor-binding Domain Antibody Using A HiBiT-based Bioreporter
Corresponding Author:	Taha Azad, PhD Ottawa Hospital Research Institute Ottawa, Ontario CANADA
Corresponding Author's Institution:	Ottawa Hospital Research Institute
Corresponding Author E-Mail:	tazad@ohri.ca
Order of Authors:	Taha Azad, PhD
	Reza Rezaei
	Abera Surendran
	Ragunath Singaravelu
	Taylor Jamieson
	Parisa Taklifi
	Joanna Poutou
	Carolina Ilkow
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please specify the section of the submitted manuscript.	Immunology and Infection
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Ottawa , Ontario.
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	This paper is the follow up of another paper that will be published in 1 month. So this paper should not be published before.
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript Click here to access/download;Manuscript;62488_R2.docx

TITLE:

Detection of SARS-CoV-2 Receptor-binding Domain Antibody Using A HiBiT-based Bioreporter

AUTHORS AND AFFILIATIONS:

Reza Rezaei[1,2], Abera Surendran[1,2], Ragunath Singaravelu[1,2], Taylor R. Jamieson[1,2], Parisa Taklifi[3], Joanna Poutou[1,2], Taha Azad[1,2], Carolina S. Ilkow[1,2,*]

[1]Ottawa Hospital Research Institute, Ottawa, ON, K1H 8L6, Canada
[2]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1H 8M5, Canada
[3]Department of Biotechnology, University of Tehran, Tehran, Iran

Email addresses of co-authors:

Reza Rezaei	(rrezaei@ohri.ca)
Abera Surendran	(absurendran@ohri.ca)
Ragunath Singaravelu	(rsingaravelu@ohri.ca)
Taylor R. Jamieson	(tjamieson@ohri.ca)
Parisa Taklifi	(parisa.taklifi@gmail.com)
Joanna Poutou	(joapupo@gmail.com)
Taha Azad	(tazad@ohri.ca)

Corresponding author:

Carolina S. Ilkow (cilkow@ohri.ca)

SUMMARY:

The outlined protocol describes the procedure for producing the HiBiT–receptor-binding domain protein complex and its application for fast and sensitive detection of SARS-CoV-2 antibodies.

ABSTRACT:

The emergence of the COVID-19 pandemic has increased the need for better serological detection methods to determine the epidemiologic impact of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The increasing number of SARS-CoV-2 infections raises the need for better antibody detection assays. Current antibody detection methods compromise sensitivity for speed or are sensitive but time-consuming. A large proportion of SARS-CoV-2-neutralizing antibodies target the receptor-binding domain (RBD), one of the primary immunogenic compartments of SARS-CoV-2. We have recently designed and developed a highly sensitive, bioluminescent-tagged RBD (NanoLuc HiBiT-RBD) to detect SARS-CoV-2 antibodies. The following text describes the procedure to produce the HiBiT-RBD complex and a fast assay to evaluate the presence of RBD-targeting antibodies using this tool. Due to the durability of the HiBiT-RBD protein product over a wide range of temperatures and the shorter experimental procedure that can be completed within 1 h, the protocol can be considered as a more efficient alternative to detect SARS-CoV-2 antibodies in patient serum samples.

INTRODUCTION:

The recent emergence of a new coronavirus, SARS-CoV2[1], has caused more than 2,800,000 fatalities and 128 million infections as of March 30th, 2021[2]. Due to the lack of a reliable and well-established treatment procedure for SARS-CoV-2 clinical therapies, many endeavors have been made to restrict further viral transmission and more importantly, to develop an effective and robust treatment or a vaccine[3]. To date, there are more than 50 COVID-19 vaccine candidates in trials reported by the World Health Organization[4]. Detection of antibodies against SARS-CoV-2 is of paramount importance to determine the long-term stability of humoral response upon administration of the vaccine as well as in recovered patients of COVID-19[5]. Some studies have demonstrated that there is a possibility that recovered SARS-CoV-2 patients lose most of the RBD-binding antibodies after 1 year[5–9]. Further investigation is required to better understand lasting immunity, and more sensitive antibody detection platforms can help further such work. Reports of sustained immunity of mild SARS-CoV-2 infections, which suggest long-term antibody responses, is also an interesting and worthwhile area of study. A fast and accurate method of detection is essential for monitoring antibodies in individuals' sera to provide more information about immunity in the population.

Like other coronaviruses, SARS-CoV-2 uses protruding spike glycoprotein to bind to angiotensin-converting enzyme-2 (ACE2) to initiate a cascade of events that lead to the fusion of the viral and cell membranes[6,7]. Several studies have recently proved the RBD of the Spike protein to have a crucial role in eliciting powerful and specific antibody response against SARS-CoV2[8–11]. In particular, correlations observed by Premkumar et al. between the titer of RBD-binding antibody and SARS-CoV-2 neutralization potency of patients' plasma are consistent with RBD being an immunogenic compartment of the virus structure[9]. With that in mind, many diagnostic tests available for SARS-CoV-2 antibody detection are time and cost-intensive, require a lengthy procedure of incubation and washing (enzyme-linked immunosorbent assay [ELISA]), or lack sensitivity and accuracy (lateral flow immunoassay [LFIA])[12]. Therefore, a quantitative and rapid complementary serological method of COVID-19-derived antibody detection with high sensitivity, fast response, and relatively low cost would serve the need for a reliable serologic test for SARS-CoV-2 epidemiologic surveillance.

Collectively, the limitations of current serological assays prompted the investigation of the bioluminescent reporting system as a potential diagnostic agent in future serosurveys. Bioluminescence is a naturally occurring enzyme/substrate reaction, with light emission. Nanoluc luciferase is the smallest (19 kDa), yet the brightest system compared to *Renilla* and firefly luciferase (36 kDa and 61 kDa, respectively)[13,14]. Further, Nanoluc has the highest signal to noise ratio and stability among the previously mentioned systems. The high signal intensity of Nanoluc supports the detection of even very low amounts of reporter fusions[15]. Nanoluc Binary Technology (NanoBiT) is a split version of the Nanoluc system, which is comprised of two segments: small BiT (11 amino acids; SmBiT) and large BiT (LgBiT) with relatively low-affinity interactions (K_D = 1.5 μM) to form a luminescent complex[16]. NanoBiT is extensively used in various studies involving the identification of protein-protein interactions[15,17–19] and cellular signaling pathways[11,20,21].

Recently, another small peptide with a distinctly higher affinity to LgBiT (K_D = 0.7 nM) was introduced, namely the HiBiT Nano-Glo system, in place of SmBiT. The high affinity and strong signal of the Nano-Glo "add-mix-read" assay makes HiBiT a suitable, quantitative, luminescent peptide tag. In this approach, the HiBiT tag is appended to the target protein by developing a construct imposing minimal structural interference. HiBiT-protein fusion would actively bind to the LgBiT counterpart, producing a highly active luciferase enzyme to generate detectable bioluminescence in the presence of detection reagents (**Figure 1**). Similarly, we developed a HiBiT Nano-Glo-based system to readily measure the neutralizing antibody titer in the sera of SARS-CoV-2 recovered individuals and recently developed a HiBiT-tagged SARS-CoV-2 RBD. This paper describes the protocol for producing the HiBiT-RBD bioreporter using standard laboratory procedures and equipment, and shows how this bioreporter can be used in a fast and efficient assay to detect SARS-CoV-2 RBD-targeting antibodies.

PROTOCOL:

NOTE: The protocol described below adheres to all ethics guidelines according to protocol code 20200371-01H.

1. Production and evaluation of the HiBiT-RBD bioreporter

1.1. Producing a sufficient quantity of HiBiT-RBD bioreporter

1.1.1. Prepare for cell culture

1.1.1.1. Prepare complete Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Then, warm the media in a 37 °C water bath.

1.1.1.2. Turn the biological safety cabinet (BSC) on, and use 70% v/v ethanol for sterilizing the cabinet surface.

1.1.2. Culture the cell line.

1.1.2.1. Take out the cell line from -80 °C or liquid nitrogen, and thaw it in a 37 °C water bath.

NOTE: An appropriate cell line is easy to maintain in culture, has high transfection efficiency, and is suitable for exogenous protein production. Human embryonic kidney, HEK293 cells were used for this protocol.

1.1.2.2. Mix the thawed cells with at least 10 mL of complete medium, pipette the cell suspension to a 10 cm Petri dish, and swirl the plate to distribute cells in the dish uniformly. Place the dish in a cell culture incubator at 37 °C, 5% CO_2, and 85–95% humidity.

1.1.2.3. Observe the cells under the microscope until the confluency level reaches 80–90%. At high confluency, remove the medium, wash the cells with warm phosphate-buffered saline (PBS), and add 1 mL of 0.25% trypsin-ethylenediamine tetraacetic acid to detach the cells from the surface.

NOTE: The HEK293 cells are fairly easily detached. Hence, the washing step should be done very gently to prevent accidental detachment and loss of cells.

1.1.2.4. After approximately 5 min, look at the cells under a microscope. If all cells are floating, add at least 4 mL of medium, and transfer the cell suspension into a new sterile tube. Count the cells using a hemocytometer, and add 1×10^6 cells into each well of a 6-well plate for transfection.

NOTE: After 24 h, cells should be at 80% or more confluent.

1.1.3. Transfection of the HiBiT-RBD plasmid

1.1.3.1. Use 1 µg of the HiBiT-RBD expression plasmid with a suitable transfection reagent. Incubate for 10–15 min at room temperature, and then add the total volume to each well of the plate, drop-wise.

NOTE: Follow the manufacturer's transfection protocol. In this case, a mixture (a specified amount) of the transfection reagent with DMEM was added to the diluted plasmid (1 µg) in DMEM (see the **Table of Materials**). Use a marker containing (e.g., green fluorescent protein [GFP]) plasmid as a control to monitor the transfection efficiency.

1.1.3.2. On the next day, replace the medium containing the transfection mixture with complete medium. Observe the transfection control well 48 h after transfection.

NOTE: If the transfection was positive and efficient (more than 80% GFP-positive), the cells should be ready for harvesting the HiBiT-RBD bioreporter. The construct also contains His-tag, which can be used to obtain purified protein in place of the total supernatant.

1.1.3.3. Collect the supernatant in 1.5 mL microtubes. Add 500 µL of 1x passive lysis buffer (PLB) to the cells; incubate and shake the plate for 15 min at room temperature for cell lysis.

NOTE: The supernatant and the lysate solution can be preserved at -20 °C with minor loss of integrity for at least 6 months. According to Azad et al.[22], the reporter is stable at a wide range of pH (4–12) and temperature (4–42 °C).

1.2. Evaluation of the luminescent signal from the bioreporter by luciferase assay

1.2.1. Preparing the reaction components

1.2.1.1. Use the supernatant as the source of the bioreporter.

NOTE: Both supernatant and lysate contain the HiBiT-RBD bioreporter and can be used for the assay. However, the supernatant is recommended as the source due to reasons explained in the following notes.

1.2.1.2. Dilute the LgBiT and substrate to 1x before use (stock concentration is 100x).

NOTE: See the **Table of Materials** for details about the LgBiT.

1.2.2. Luciferase assay

1.2.2.1. Transfer 50 µL of the supernatant from each well or tube to a 96-well plate, and add 50 µL of 1x LgBiT to each well. Incubate for 5 min at room temperature.

1.2.2.2. Open the luminometer software, add 50 µL of 1x substrate (furimazine) to each well, place the plate in the luminometer, and run the software.

NOTE: Add the substrate immediately before reading the plate to prevent consumption of the substrate by the active enzymes before signal measurement.

2. Detecting anti-RBD antibody with a fast and sensitive assay

2.1. HiBiT-RBD antibody detection assay

2.1.1. Prepare the HiBiT-RBD bioreporter as described in section 1.1 of the protocol.

NOTE**:** It is recommended to use the supernatant for the following assay as it is simpler to collect and contains the mature glycosylated version of the protein. Moreover, the lysate has several other proteins that could interfere with the HiBiT-RBD-antibody interaction.

2.1.2. Combine 50 µL of the HiBiT-RBD-containing supernatant with 1 µg of the commercial SARS-CoV-2-RBD antibody in a 1.5 mL microtube. Add 20 µL of immunoglobulin-binding protein (protein G) to the solution. Bring up the total volume to 300 µL by adding PBS.

NOTE: The total volume of the mixture can be decreased to 150 µL. Lower total volumes are not recommended as it could result in inadequate mixing of antibodies with the bioreporter.

2.1.3. Incubate the tube(s) on a tube shaker or rotator for 30 min. Centrifuge at 12,000 × *g* for 30 s, discard the supernatant, and wash with PBS. Repeat the process three times to remove free HiBiT-RBD. Resuspend in 50 µL of PBS and transfer to a 96-well plate.

2.1.4. Add 50 µL of 1x LgBiT and wait for 5 min. Then, add 50 µL of 1x NanoLuc substrate. Immediately read the luminescent signal with a luminometer.

3. High-throughput detection of the SARS-CoV-2-specific antibodies from patient serum samples

3.1. Prepare larger quantities of the HiBiT-RBD bioreporter for a high-throughput assay by following section 1.1 of the protocol.

3.2. Combine 20 µL of magnetic protein G with 50 µL of the HiBiT-RBD supernatant in a well of 96-well plate for each sample. Add 10 µL of the serum sample to each well and bring up the total volume to 150 µL by adding PBS.

NOTE: Use both nonspecific IgG (negative control) and neutralizing SARS-CoV-2 antibody (positive control) at 1 µg/mL concentration as described in step 2.1.2. Moreover, serum samples from vaccinated mice can also be assessed for antibodies. In this test, serum samples were obtained from Ottawa Hospital General Campus under an approved procedure and with informed consent from individuals.

3.3. Incubate for 30 min on a shaker at room temperature. Place the plate on a magnetic washer to precipitate the protein G–antibody complex.

NOTE: The specific structure of the magnetic washer will precipitate the complex on the side walls of each well.

3.4. Discard the solution in the middle section of each well, and add PBS for washing. Repeat the washing step at least three times to remove excess HiBiT-RBD. Add 50 µL of 1x PBS and 50 µL of 1x LgBiT. Incubate for at least 5 min at room temperature.

3.5. Prepare the luminometer software, and then add 50 µL of 1x substrate. Place the plate in the machine, run the software, and record the signals. Compare the signals from serum samples, control samples, and background (empty wells).

REPRESENTATIVE RESULTS:

The signals from both the HiBit-RBD-containing cell lysate and supernatant of the transfected cells were recorded (**Figure 2**) to evaluate the appropriate protein source. HiBiT-RBD and LgBit were separately used as controls, and the data showed low background compared to a strong signal when both parts were combined. Hence, HiBiT-RBD interaction with LgBiT is necessary to generate active enzyme for substrate digestion and bioluminescence activity (**Figure 1**).

The addition of protein G will help antibody precipitation (**Figure 3**). The assay was used to compare the signal from a commercial SARS-CoV-2-neutralizing antibody with a control IgG. The specific antibody signal was robust, while the control antibody had close to the luminescent background level (**Figure 4A**). Recombinant attenuated oncolytic vesicular stomatitis virus (VSV) with a mutation at position 51 of the M protein (**Δ51)** expressing exogenous RBD was used to vaccinate mice. The serum collected from vaccinated mice produced a robust signal compared to no signal in mice injected with control VSV (**Figure 4B**).

FIGURE AND TABLE LEGENDS:

Figure 1: LgBiT interaction with HiBiT connected to RBD. Upon interaction of the small portion of the nanoluciferase, HiBiT, with the large subunit of the enzyme, LgBiT, the active enzyme complex can produce a luminescent signal after substrate consumption. The RBD does not interfere with this process. Abbreviations: RBD = receptor-binding domain.

Figure 2: Robust reporter activity from both lysate and supernatant of transfected cells. The protein is present in both supernatant and lysate and produces a strong signal. Control groups' luminescent signals were close to the background. Error bars represent Standard Deviation (SD).

Figure 3: Schematic of the HiBiT-RBD interaction with antibodies bound to protein G. The schematic depicts the antibody precipitation by protein G and interaction with HiBiT-RBD. The addition of the LgBit to the mixture will produce a robust signal when the antibody is specific for RBD.

Figure 4: HiBiT-RBD bioreporter generates strong bioluminescence with purified neutralizing antibodies, vaccinated mouse serum, and patient serum samples. (**A**) HiBiT-RBD interacts with RBD-specific neutralizing antibody and generates significantly high signal compared to the negligible signal for nonspecific IgG. (**B**) The bioreporter can detect SARS-CoV-2 antibodies in vaccinated mouse serum. Abbreviations: RBD = receptor-binding domain; IgG = immunoglobulin G; Ab = antibody; VSV = vesicular stomatitis virus; Fluc = firefly luciferase. (**C**) Detection of the SARS-CoV-2 antibodies in patient serum samples, reproduced from Azad et al.[22].

DISCUSSION:

The increasing number of people infected with the SARS-CoV-2 and the ongoing effort for global vaccination necessitates sensitive and fast serologic tests that can be used in large-scale serosurveys. Recent research shows that split nanoluciferase-based bioreporters can be used to develop such assays. We recently developed the HiBiT-RBD bioreporter to design a test that can be used to detect SARS-CoV-2-specific antibodies in patient serum in a fast and reliable fashion (**Figure 4C**).

There are a few critical steps in this assay. Because the system's efficiency depends on RBD protein expression, the protein levels should be validated by western blotting. Moreover, it is necessary to use a positive control, such as a commercial antibody against RBD, and a negative control antibody. Addition of a Nanoluciferase protein is recommended to be used as a positive control for bioluminescence detection. The protein product also contains a His-tag, which can be used for purification for a large number of serum samples.

There are several advantages to using this bioreporter compared to other competing methods. First, an experienced user can perform the complete assay procedure in less than one hour, which is considerably faster than existing tests such as ELISA. Second, the minimum preparation and testing requirements make this test highly valuable for large-scale production at a low cost.

Moreover, the detection limit of the assay is as low as 1 ng of the SARS-CoV-2-neutralizing antibody as described by Azad et al.[22]. A drawback of this approach is the inability to differentiate between different antibody isotypes. Moving forward, the sensitivity of the test should be compared to other routinely used serologic tests.

Azad et al.[22] had used serum samples from patients to evaluate the applicability of the assay. It is also essential that the system is tested for antibody detection in blood samples from patients. This tool could also be very impactful in the assessment of the correlation between the severity of COVID-19 and the presence of SARS-CoV-2-specific antibodies. Overall, such serologic tests could have a substantial impact in estimating the epidemiological impact of the SARS-CoV-2 and can be a convenient substitute for time-consuming and less sensitive detection methods.

ACKNOWLEDGMENTS:

We appreciate and thank the technical assistance of Xiaohong He, Ricardo Marius, Julia Petryk, Bradley Austin, and Christiano Tanese De Souza. We also thank Mina Ghahremani for Graphic Design. We would also like to thank all the individuals who participated and donated their blood samples for this study. DWC is supported in part by uOttawa Faculty and Department of Medicine.

DISCLOSURES:

The authors declare no conflict of interest.

REFERENCES:

1. Ullah, H., Ullah, A., Gul, A., Mousavi, T., Khan, M. W. Novel coronavirus 2019 (COVID-19) pandemic outbreak: A comprehensive review of the current literature. *Vacunas*. doi: 10.1016/j.vacun.2020.09.009 (2020).
2. Coronavirus update (Live) Worldometer. https://www.worldometers.info/coronavirus/.
3. Cacciapaglia, G., Cot, C., Sannino, F. Second wave COVID-19 pandemics in Europe: a temporal playbook. *Scientific Reports*. **10** (1), 15514 (2020).
4. World Health Organization. COVID-19 vaccines. https://www.who.int/emergencies/diseases/novel-coronavirus-2019/covid-19-vaccines.
5. Hueston, L. et al. The antibody response to SARS-CoV-2 infection. *Open Forum Infectious Diseases*. 7 (9), ofaa387 (2020).
6. Lan, J. et al. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature*. **581** (7807), 215–220 (2020).
7. Azad, T. et al. Implications for SARS-CoV-2 vaccine design: fusion of Spike glycoprotein transmembrane domain to receptor-binding domain induces trimerization. *Membranes*. **10** (9), 215 (2020).
8. Piccoli, L. et al. Mapping neutralizing and immunodominant sites on the SARS-CoV-2 Spike receptor-binding domain by structure-guided high-resolution serology. *Cell*. **183** (4), 1024–1042.e21 (2020).
9. Premkumar, L. et al. The receptor-binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. *Science Immunology*. **5** (48), eabc8413 (2020).

10. Walls, A. C. et al. Elicitation of potent neutralizing antibody responses by designed protein nanoparticle vaccines for SARS-CoV-2. *Cell*. **183** (5), 1367–1382.e17 (2020).
11. Azad, T. Nanoluciferase complementation-based biosensor reveals the importance of N-linked glycosylation of SARS-CoV-2 Spike for viral entry. S1525-0016(21)0074-5, doi: 10.1016/j.ymthe.2021.02.007 (2021).
12. Bastos, M. L. et al. Diagnostic accuracy of serological tests for covid-19: systematic review and meta-analysis. *BMJ*. **370**, m2516 (2020).
13. Promega. Bioluminescent Reporters | Reporter Gene Applications | An Introduction to Reporter Genes. https://www.promega.ca/resources/guides/cell-biology/bioluminescent-reporters/#references-6d127eb8-eeae-40b7-86e9-fe300545e8fa.
14. Fleiss, A., Sarkisyan, K. S. A brief review of bioluminescent systems (2019). *Current Genetics*. **65** (4), 877–882, doi: 10.1007/s00294-019-00951-5 (2019).
15. Nouri, K. et al. A kinome-wide screen using a NanoLuc LATS luminescent biosensor identifies ALK as a novel regulator of the Hippo pathway in tumorigenesis and immune evasion. *The FASEB Journal*. **33** (11), 12487–12499 (2019).
16. Boute, N. et al. NanoLuc Luciferase – a multifunctional tool for high throughput antibody screening. *Frontiers in Pharmacology*. **7**, 27 (2016).
17. Nouri, K. et al. Identification of celastrol as a novel YAP-TEAD inhibitor for cancer therapy by high throughput screening with ultrasensitive YAP/TAZ-TEAD biosensors. *Cancers*. **11** (10), 1596 (2019).
18. Azad, T. et al. SARS-CoV-2 S1 NanoBiT: A nanoluciferase complementation-based biosensor to rapidly probe SARS-CoV-2 receptor recognition. *Biosensors and Bioelectronics*. **180**, 113122 (2021).
19. Brown, E. E. F. et al. Characterization of critical determinants of ACE2–SARS CoV-2 RBD interaction. *International Journal of Molecular Sciences*. **22** (5), 2268 (2021).
20. Azad, T. et al. A gain-of-functional screen identifies the Hippo pathway as a central mediator of receptor tyrosine kinases during tumorigenesis. *Oncogene*. **39** (2), 334–355 (2020).
21. Schwinn, M. K. et al. CRISPR-Mediated tagging of endogenous proteins with a luminescent peptide. *ACS Chemical Biology*. **13** (2), 467–474 (2018).
22. Azad, T. et al. A high-throughput NanoBiT-based serological assay detects SARS-CoV-2 seroconversion. *Nanomaterials*. **11** (3), 807 (2021).

Figure

Figure 1

Click here to access/download;Figure;JOVE_Figures.pdf

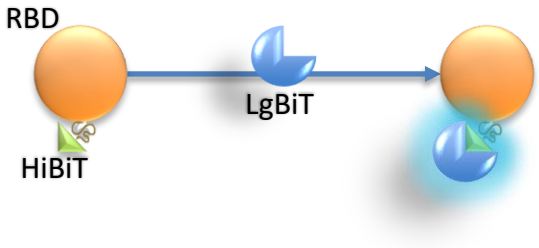

Figure 2

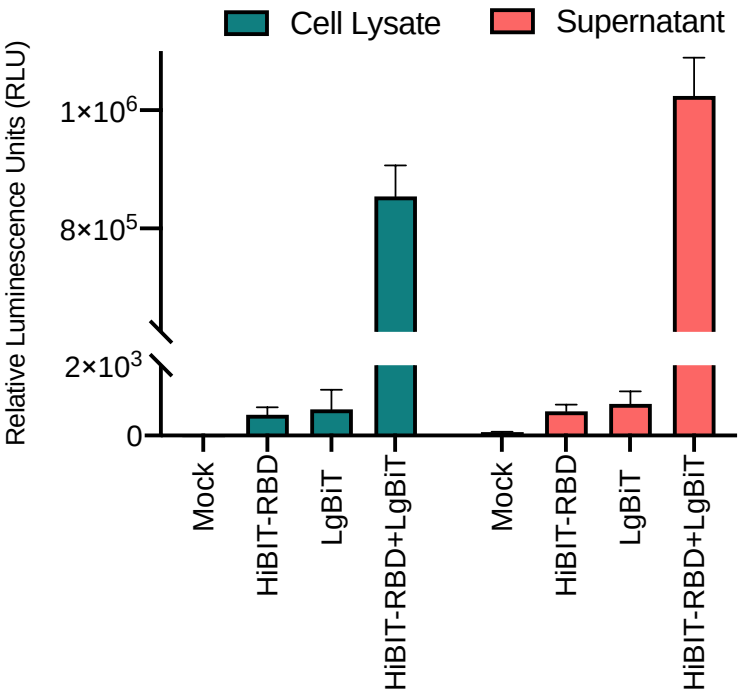
Cell Lysate
Supernatant
Relative Luminescence Units (RLU)
1×10^6
8×10^5
2×10^3
0
Mock
HiBIT-RBD
LgBiT
HiBIT-RBD+LgBiT
Mock
HiBIT-RBD
LgBiT
HiBIT-RBD+LgBiT

Figure 3

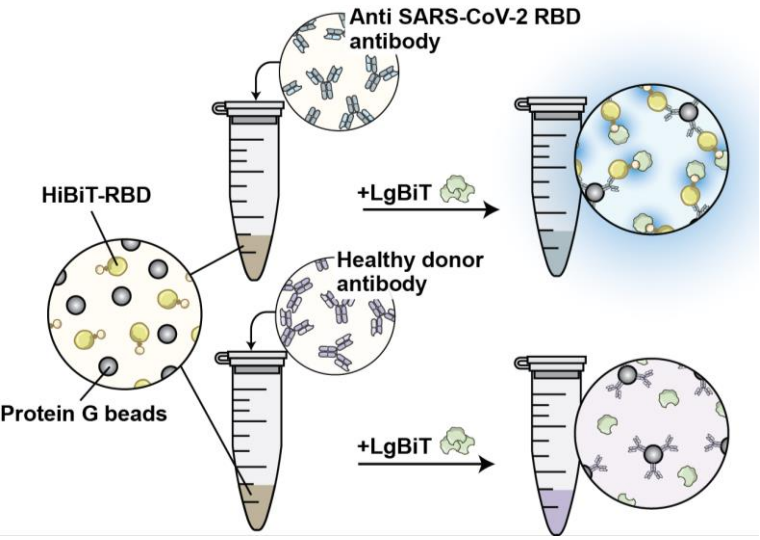
Anti SARS-CoV-2 RBD antibody
HiBiT-RBD
+LgBiT
Healthy donor antibody
Protein G beads
+LgBiT

Figure 4

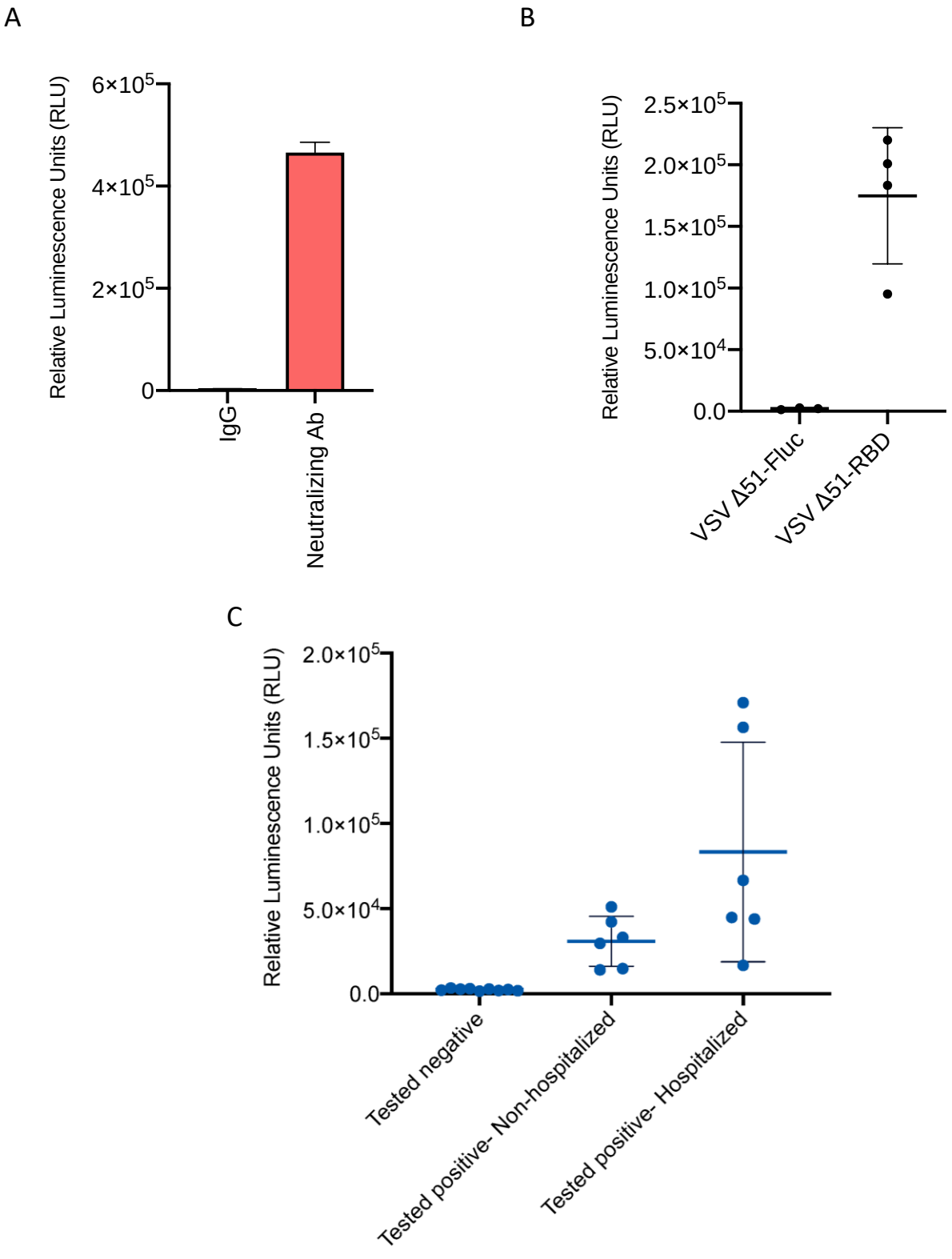
A
Relative Luminescence Units (RLU)
6×10^5
4×10^5
2×10^5
0
IgG
Neutralizing Ab
B
Relative Luminescence Units (RLU)
2.5×10^5
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
VSV Δ51-Fluc
VSV Δ51-RBD
C
Relative Luminescence Units (RLU)
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
Tested negative
Tested positive- Non-hospitalized
Tested positive- Hospitalized

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials (1).xls

Name of Material/ Equipment	Company	Catalog Number
5x Passive Lysis Buffer	Promega	E194A
Bio-Plex Handheld Magnetic Washer	Bio-Rad	171020100
DMEM	Sigma	D6429-500ml
Dual-Glo luciferase Assay System	Promega	E2940
Fetal Bovine Serum (FBS)	Sigma	F1051
HiBiT-RBD Plasmid		
LgBiT	Promega	N3030
penicillin Streptomycin	Thermo Fisher Scientific	15140122
Pierce Protein G Magnetic Beads	Thermo Fisher Scientific	88848
PolyJet In Vitro DNA Transfection Reagent	Signagen	SL100688.5
SARS-CoV-2 (2019-nCoV) Spike Neutralizing Antibody, Mouse Mab	SinoBiological	40592-MM57
Synergy Mx Microplate Reader	BioTek	
Trypsin-EDTA	Thermo Fisher Scientific	2520056

Comments/Description

30 mL

100 mL kit

gacggatcgggagatctcccgatcccctatggtgcactctcagtacaatctgctctgatgccgcatagttaagccagtatctgctccctgcttgtgttggaggtcgctgagtagtgcgcgagcaaaatttaagctacaacaaggcaaggcttgaccgacaattgcatgaagaatctgcttagggttaggcgttttgcgctgcttcgcgatgtacgggccagatatacgcgttgacattgattattgactagttattaatagtaat
caattacggggtcattagttcatagcccatatatggagttccgcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatgggtggagtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgcc
tggcattatgcccagtacatgaccttatgggactttcctacttggcagtacatctacgtattagtcatcgctattaccatggtgatgcggttttggcagtacatcaatgggcgtggatagcggtttgactcacggggatttccaagtctccaccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggactttccaaaatgtcgtaacaactccgccccattgacgcaaatgggcggtaggcgtgtacggtgggag
gtctatataagcagagctctctggctaactagagaacccactgcttactggcttatcgaaattaatacgactcactatagggagacccaagctggctagcgtttaaacttaagcttggtaccgagctcggatccgccaccATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTGACtctggctctagcggctctggctctagcggcggcATGGTGAGC
GGCTGGCGGCTGTTCAAGAAGATTAGCtctagcggcGACTACAAGGACCACGACGGTGACTACAAGGACCACGACATCGACTACAAGGACGACGACGACAAGggcagcggctccggcagcagcggaggaggaggctctggaggaggaggctctagcggcggcaacatcacaaatctgtgcccattcggcgaggtgtttaacgccaccagatttgccagcgtgtatgcctggaa
ccggaagagaatctctaattgcgtggccgactatagcgtgctgtacaatagcgcctccttctctacctttaagtgctatggcgtgtcccccacaaagctgaacgacctgtgcttcaccaacgtgtacgccgactcttttgtgatcaggggcgatgaggtgcgccagatcgcacctggacagacaggcaagatcgccgactacaactataaagctgccagacgatttcaccggctgcgtgatcgcctggaatagcaacaatctgga
ttccaaagtgggcggcaactacaattatctgtaccggctgttcagaaagagcaacctgaagccctttgagcgggatatcagcacagagatctaccaggcaggctccacccctgcaacggagtggagggcttcaattgttatttttcccctgcagagctacggcttccagcctacaaatggcgtgggctatcagccatacagggtggtggtgctgtccttgagctgctgcacgcacctgcaaccgtgtcctctggacacatcga
gggccgccacatgctggagatgggccatcatcaccatcatcaccaccaccaccaccgatagcggccgctcgagtctagagggcccgtttaaacccgctgatcagcctcgactgtgccttctagttgccagccatctgttgtttgcccctcccccgtgccttccttgaccctggaaggtgccactcccactgtccttttcctaataaaatgaggaaattgcatcgcattgtctgagtaggtgtcattctattctgggggggtggggtggg
gcaggacagcaagggggaggattgggaagacaatagcaggcatgctggggatgcggtgggctctatggcttctgaggcggaaagaaccagctggggctctaggggtatcccccacgccgccctgtagcggcgcattaagcgcggcgggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgccctagcgcccgctcctttcgctttcttcccttcctttctcgccacgttcgccggctttccccgtcaagctctaa
atcgggggctccctttagggttccgatttagtgctttacggcacctcgaccccaaaaaacttgattagggtgatggttcacgtagtgggccatcgccctgatagacggtttttcgccctttgacgttggagtccacgttctttaatagtggactcttgttccaaactggaacaacactcaaccctatctcggtctattcttttgatttataagggatttgccgatttcggcctattggttaaaaaatgagctgatttaacaaaaatttaa
cgcgaattaattctgtggaatgtgtgtcagttagggtgtggaaagtccccaggctccccagcaggcagaagtatgcaaagcatgcatctcaattagtcagcaaccaggtgtggaaagtccccaggctccccagcaggcagaagtatgcaaagcatgcatctcaattagtcagcaaccatagtcccgcccctaactccgcccagttccgcccattctccgccccatggctgactaatttttttta
tttatgcagaggccgaggccgcctctgcctctgagctattccagaagtagtgaggaggcttttttggaggcctaggcttttgcaaaaagctcccgggagcttgtatatccattttcggatctgatcaagagacaggatgaggatcgtttcgcatgattgaacaagatggattgcacgcaggttctccggccgcttgggtggagaggctattcggctatgactgggcacaacagacaatcggctgctctgatgccgccgttccg
gctgtcagcgcaggggcgcccggttctttttgtcaagaccgacctgtccggtgccctgaatgaactgcaggacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgcagctgtgctcgacgttgtcactgaagcgggaagggactggctgctattgggcgaagtgccggggcaggatctcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgcaatgcggcggctgcatacgct
tgatccggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagcacgtactcggatggaagccggtcttgtcgatcaggatgatctggacgaagagcatcaggggctcgcgccagccgaactgttcgccaggctcaaggcgcgcatgcccgacggcgaggatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaatggccgctttttctggattcatcgactgtggccggctgggt
gtggcggaccgctatcaggacatagcgttggctacccgtgatattgctgaagagcttggcggcgaatgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttctatcgccttcttgacgagttcttctgagcgggactctggggttcgaaatgaccgaccaagcgacgcccaacctgccatcacgagatttcgattccaccgccgccttctatgaaaggttgggcttcggaatcgttttcc
gggacgccggctggatgatcctccagcgcggggatctcatgctggagttcttcgcccaccccaacttgtttattgcagcttatataatggttacaaataaagcaatagcatcacaaatttcacaaataaagcatttttttcactgcattctagttgtggtttgtccaaactcatcaatgtatcttatcatgtctgtataccgtcgaccctagctagagcttggcgtaatcatggtcatagctgtttcctgtgtgaaattgttatccgctcac
aattccacacaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagtgagctaactcacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcgggagaggcggtttgcgtattgggcgctcttccgcttcctcgctcactgactcgctgcgctcggtcgttcggctgcggcgagcggtatcagctcactcaaaggcggtaat
acggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgtttttccataggctccgcccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttcccctggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgc
ctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacg
gctacactagaagaacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaaccaccgctggtagcggtggtttttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatcttttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaaaaaggatcttcacctagatc
cttttaaattaaaaatgaagttttaaatcaatctaaagtatatatgagtaaacttggtctgacagttaccaatgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatccatagttgcctgactccccgtcgtgtagataactacgatacgggagggcttaccatctggccccagtgctgcaatgataccgcgagacccacgctcaccggctccagatttatcagcaataaaccagccagccggaagggccga
gcgcagaagtggtcctgcaactttatccgcctccatccagtctattaattgttgccgggaagctagagtaagtagttcgccagttaatagtttgcgcaacgttgttgccattgctacaggcatcgtggtgtcacgctcgtcgtttggtatggcttcattcagctccggttcccaacgatcaaggcgagttacatgatcccccatgttgtgcaaaaaagcggttagctccttcggtcctccgatcgttgtcagaagtaagttggccgca
gtgttatcactcatggttatggcagcactgcataattctcttactgtcatgccatccgtaagatgctttttctgtgactggtgagtactcaaccaagtcattctgagaatagtgtatgcggcgaccgagttgctcttgcccggcgtcaatacgggataataccgcgccacatagcagaactttaaaagtgctcatcattggaaaacgttcttcggggcgaaaactctcaaggatcttaccgctgttgagatccagttcgatgtaaccca
ctcgtgcacccaactgatcttcagcatcttttacttcaccagcgtttctgggtgagcaaaaacaggaaggcaaaatgccgcaaaaaagggaataagggcgacacggaaatgttgaatactcatactcttcctttttcaatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaataaacaaatagggggttccgcgcacatttccccgaaaagtgccacctgacgtc

rebuttal letter

Click here to access/download;Rebuttal Letter;Editorial comment_AberaEdits_05Apr21-RezaFinal.docx

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please provide an email address for each author.

3. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the human/animal care guidelines of your institution, with reference to the steps involving serum tests.

4. Use “mL” instead of “ml”, “µL” instead of “uL/uL”. Include a single space between the quantity and its unit. “5 mm” instead of “5mm”.

5. Line 149, 216: Please check the comment.

6. Please clarify what “section 1 of protocol 1”, “section 1 of the previous protocol” refers to.

7. Line 175: How is the serum sample obtained?

8. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and a maximum of 4 sentences per step. Please include a single line space between each step and ensure that that the highlighted section is no more than 3 pages.

9. Please include a Disclosures section, providing information regarding the authors’ competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names. Please include volume and issue numbers for all references.

11. Please revise the table of materials to include all the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

12. Please upload each figure individually to your editorial manager account.

Thanks for the suggestions. We addressed all of them now.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors have applied a commercial detection system based on split luciferase to the detection of antibodies to SARS-CoV-2 receptor binding domain. An example of applying the assay to show qualitatively the presence of antibodies to SARS-CoV-2 RBD in mice immunized with a pseudotyped VSV expressing RBD is described. The assay is relatively rapid compared to standard ELISAs

We thank the reviewer for the insightful comments.

Major Concerns:

The assay is described as highly sensitive but no data are provided. To understand the value of the method the limit of detection would needs to be addressed.

The detailed information on the limit of detection of the assay are provided in the Azad et al. [Nanomaterials -2021] and the current manuscript is the in-depth protocol for that work. The mentioned paper was added to the references and the detection limit data was added to the text (line 327) according to the original publication.

Minor Concerns:

Details and availability of the RBD-HiBIT RBD vector should provided e.g. a plasmid map and vector deposited with Addgene.

The sequence of the plasmid was added to the materials file and the plasmid is available on request from the authors.

The reporter is produced by transient expression and not purified which makes standardization difficult and raises the following questions.

As the referee pointed out, there are some issues with transient expression. Hence, we added a note at line 151 explaining that the construct contains a His tag which can be used for purification of the protein in case of a large scale testing. Moreover, a positive control can be used for transient expression to normalize all samples based on that.

Is the same volume of cell supernatant used for different assays and if so what is it?

The volume of the supernatant was added to the text at lines 188, 203 and 236.

How long can the reporter be stored or does the transfection have to be repeated each time the assay is run?

The information on the stability of the biosensor has been reported in the original publication and also was added to the protocol as a note at line 156-157.

Minor point

According to the Promega website the KDs for SmBiT and HiBiT binding to LgBiT are 1.5 µM and 0.7 nM respectively rather then >10 mM and 700 mM as stated on lines 71 and 74. Presumably these are typographical errors ?

We appreciate reviewer's accurate view of the paper. The typographic error on lines 78 and 81 was revised.

Reviewer #2:

Manuscript Summary:

Rezaei and co-workers describe a method to produce a luciferase-based assay to detect antibodies targeting the receptor binding domain (RBD) of SARS-CoV-2 spike protein. The method exploits a split version of the small Nanoluc that consists of the small peptide HiBiT that, upon binding to the LgBiT larger fragment, produce an active luciferase enzyme. The authors fused the HiBiT tag to the RBD of the spike protein and produced this chimeric protein in HEK293. The conditioned medium from cells expressing the HiBiT-RBD is then incubated with solutions containing anti-RBD antibodies (either patient serum, purified monoclonal antibodies or sera from mice infected with RBD-expressing VSV) and the immunocomplexes are precipitated with protein G-conjugated beads. After washing, the LgBiT is added together with the luciferase substrate and luminescent signal is recorded in a luminometer. The assay principle is very simple, yet elegant and can be easily scaled-up and performed in a high-throughput fashion to test large numbers of patient sera.

We thank the reviewer for their comprehensive feedback and comments on the protocol and results.

Major comments:

The abstract and introduction are well written, the expected results and discussion need to be carefully rephrased and the protocol is quite confusing. The expected results are not described with a level of detail that allows to understand what the authors tested in each experiment. In addition, the expected outcome for part 3 of their protocol (High-throughput detection of the SARS-CoV-2 specific antibodies from patient serum) is missing. Actually, from the discussion it seems that the assay has never been tested on patient samples (line 244-245). If so, why the authors provide a protocol that has never been tested? Please include representative results for part-3 or remove it from the manuscript.

The current manuscript is the detailed protocol for the assay developed in the Azad et al paper. Hence, the results of the high-throughput testing have been reported in the original manuscript. However, as per reviewer's suggestion, a sentence (line 318-320) was added to the discussion about the application of the assay on the patient serum samples which was reported in the original paper.

There are other aspects of this protocol that are not completely clear to me. First, the protocol explains how to produce the HiBiT-RBD protein starting from the HiBiT-RBD plasmid transfection but does not state how to obtain the plasmid. Is it available from the authors on request? Since it is the center of the method, the authors should include how to obtain the plasmid or how to clone it.

The plasmid sequence was added to the supplementary file and it is available upon request from the authors.

Second, there is no mention on how to produce the LgBiT. On line 141 the authors wrote that LgBiT stock is 100x but they did not write how to produce such stock solution. Is it commercially available? Again, this is essential to reproduce the experiment.

The LgBiT protein is commercially available from Promega company and the ordering information was added to the text and the supplementary material table.

On line 133 the authors wrote that supernatant can be stored for some minutes at -20°C. Is it correct? Also, information on stability of the supernatants containing the HiBiT-RDB are missing. Can the HiBiT-RDB containing supernatant be stored at -80°C or at 4°C for long term? If the supernatants are stable for only few minutes, as written by the authors on line 133, the assay has major drawbacks in terms of scale-up since the supernatants must be prepared fresh and differences among batches might introduce variability in the assay. At least, if this is the case, it should be mentioned in the discussion.

The information on the stability of the biosensor has been reported in the original publication and also was added to the protocol as a note at line 131. The part indicating “few minutes of stability” was a typographic error which was revised and replaced with the correct information from the original paper.

Finally, it is not clear how much HiBiT-RDB supernatants should be used in the assay. The authors only suggest to mix the supernatants containing the HiBit-RDB with the 1ug or 10 ul of patient serum. How much HiBiT-RDB must be used? It is not clear to me how the protocol can be reproduced without knowing the amounts of enzyme to be used in the assay. The author should at least include a range of luminescence units (as fold of the controls) they expect to be present in the supernatants of efficiently transfected HEK293 cells and how much of such supernatants should be used for the subsequent assays.

The volume of the supernatant was added to the text at lines 188, 203 and 236.

Minor comments:

SARS-CoV-2 with minus before the 2 (line 51).

Line 83 is missing a reference.

Double check references 5-9 (line 40). At first inspection none of the cited manuscripts state that patients can become re-infected after one year. Piccoli et al. states that 99% of RDB binding abs might decay after 1 year but this is a different message respect that patients can be re-infected one year after first exposure. None of the other references addressed this point. Thus, please include the correct references or rephrase the sentence.

The text at line 40 was rephrased.

While HEK293 cells are in general highly transfectable, the author could specify which and how much transfection reagent they used.

The information for commercially available PolyJet transfection reagent and the amount used was added to the text (line 122).

Line 129 - how much efficient? Give a % of expected transfection efficiency

The expected transfection efficiency was added to the text at line 145.

Line 149 - delete "How to prepare the substrate?". I guess this is a note the authors left for themselves and indeed it might be interesting, if there is a special issue with substrate preparation, to include this step.

The typographic errors were fixed at line 149.

Change the y-axis of the graphs from linear scale to logarithmic so it is clear which is the background signal expected from this assay.

In the representative results, figure 2 appear before figure 1. Please invert them so the order is correct. Or start the paragraph by describing the concept of the assay (Figure 1).

Line 178: double check the sentence. It is not clear if 1 ug total antibody or 1 ug/ml final concentration should be added as control

The typographic errors at line 178 was corrected.

Reviewer #3:

Manuscript Summary:

The manuscript entitled "Detection of SARS-CoV-2 RBD antibody using a HiBiT based bioreporter" dealing with factors

associated with COVID-19 is interesting and potentially represent a significant contribution to this area of research.

I have pointed out the following corrections listed below. The following shortfalls could be addressed to improve

the quality of the study:

We greatly appreciate reviewer's feedback and tried to improve the text accordingly.

Minor Concerns:

1. The introduction section reports disease epidemiology only until January 4 2021,

the status needs to be updated as the current numbers reported are outdated.

2. The authors have not provided any evidence from the literature for their findings

on many occasion. There have been studies on the topic; it would be good to support their

discussion with supportive literature.

The authors could cite one of the latest review paper by Ullah et al. 2020. "Novel coronavirus 2019 (COVID-19)

pandemic outbreak: A comprehensive review of the current literature".

https://doi.org/10.1016/j.vacun.2020.09.009

As reviewer suggested, the epidemiology report was updated and the comprehensive review on SARS-CoV-2 was added to the introduction.

3. The overall discussion is fine, just curiously short in terms of what the findings mean for moving the research field forward and the future perspectives. This would have been interesting to read from someone who has worked on the relevant issue.

A final sentence on the future perspective of the method was added to the discussion.