Analytical Characterization of the SARS-CoV-2 JRC EURM-017 Reference Material

Conklin J, Freeman J.
Siemens Healthcare Diagnostics Inc., Tarrytown, NY, U.S.

siemens-healthineers.com

SIEMENS Healthineers

Analytical Characterization of the SARS-CoV-2 JRC EURM-017 Reference Material

Abstract

Background: COVID-19 results from infection with SARS-CoV-2 virus. The virus spreads from person-to-person or through environmental exposure. Antibodies appear 1–3 weeks postsymptom onset in most patients and are produced in symptomatic and asymptomatic infection, though antibody titer may be higher in symptomatic disease. Unlike typical seroconversion profiles, near-simultaneous production of both IgM and IgG has been observed in symptomatic patients with SARS-CoV-2. Antibodies produced to structural proteins of the virus include spike and nucleocapsid. Data show IgM and IgG antibodies for these structural proteins appear with seroconversion. IgM disappears soon after acute infection, but IgG remains detectable for longer in most patients. Spike is a transmembrane glycoprotein consisting of two regions: S1 and S2. S1 mediates recognition and binding of the viral receptor (ACE2) on host cells, and S2 facilitates viral fusion and entry. The majority of S1 consists of the receptor-binding domain (RBD) that binds directly to ACE2 and is highly immunogenic. The S1 RBD in SARS-CoV-2 contains unique and conserved sequences compared to other beta-coronaviruses. Multiple vaccines that are available or in development target or include the S1 RBD, as initial data indicate antibodies to this region can be neutralizing. The ability to identify specific antibodies associated with neutralization aids in the detection of an immune response to the SARS-CoV-2 virus and identifying patients with an adaptive immune response to SARS-CoV-2, indicating recent or prior infection.

Methods: Characterizing the SARS-CoV-2 JRC EURM-017 reference material requires extracting and purifying five antibody species: S1 RBD, nucleocapsid, S1, S2, and the full-length spike (S1+S2). Affinity purification techniques were performed using biotinylated SARS-CoV-2 antigens coupled to streptavidin-coated latex particles. The particles react with the EURM-017 reference material, are separated by magnetic separation, and washed using a 0.01 M PBS, pH 7.4 + 0.1% TWEEN 20 solution. The particles are then centrifuged, the washing solution removed, and the particles eluted with a 50 mM citrate buffer containing 0.1% TWEEN 20 at a pH of 3.00. The elution is centrifuged again and transferred to another tube, where it is neutralized using a 1 M Tris buffer + 0.1% TWEEN 20 solution at a pH of 11.00. The extracted antibody is concentrated and dialyzed using 30K NMWL centrifugal tube and a 0.01 M PBS, pH 7.4 + 0.1% TWEEN 20 solution. Finally, the protein concentration is measured by A280 analysis.

Results: Protein concentration measured by A280 analysis demonstrated antibody levels of S1 RBD, nucleocapsid, S1, S2, and the full-length spike (S1+S2). The affinity purification of the SARS-CoV-2 JRC EURM017 data is as follows: 17.4, 72.5, 17.7, 29.7, and 34.1 mcg/mL, respectively.

Conclusion: Currently there are no standardized methods for SARS-CoV-2 antibody testing. Understanding and quantifying the SARS-CoV-2 JRC EURM-017 reference material can help produce a standardized method across different assays that use different detection methods for identifying immunocompetence of individuals to the SARS-CoV-2 virus.

Background

COVID-19 (coronavirus disease 2019) is the illness resulting from infection with SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) virus.[1-5] The virus spreads readily from person to person or possibly from environmental exposure.6 Evidence supports spread by both asymptomatic and symptomatic individuals.[7] Antibodies appear approximately 1–3 weeks post-symptom onset in most patients and are produced in both symptomatic and asymptomatic infection.[8,9] Unlike typical seroconversion profiles, near-simultaneous production of both IgM and IgG has been observed in symptomatic patients with confirmed SARS-CoV-2. Titer of antibody may be higher in symptomatic disease, though additional data is needed to confirm this.[10,11] Antibodies produced to structural proteins of the virus include spike antibody and nucleocapsid antibody. Data show both IgM and IgG antibodies for these structural proteins appear with seroconversion. IgM eventually disappears, but IgG remains detectable in most patients. Spike is a transmembrane glycoprotein consisting of two regions: S1 and S2. S1 mediates recognition and binding of the viral receptor (ACE2) on host cells, and S2 facilitates viral fusion and entry.12,13 The majority of S1 consists of the receptor-binding domain (RBD) that binds directly to ACE2 and is highly immunogenic. The S1 RBD in SARS-CoV-2 contains both unique and conserved sequences compared to other beta-coronaviruses.

Multiple vaccines that are available or in development target or include the S1 RBD, as initial data indicate antibodies to this region can be neutralizing which will be an important adjunct to the detection of an immune response to the SARS-CoV-2 virus.[14-23]

Objective

To describe the purification and analytical characterization studies of polyclonal antibodies with specificity to SARS-CoV-2 viral proteins S1, S2, and S1/S2 (full spike); nucleocapsid; and S1 receptor-binding domain (RBD) present in the EURM-017 reference material.

Methods

SARS-CoV-2 biotin conjugate preparation
Each of the five viral proteins was conjugated to NHS-LC-Biotin by reacting 50 milligrams of protein with a 10-molar excess of NHC-LC-Biotin for 1 hour. The unreacted NHS-LC-Biotin was removed from the conjugated protein by gel filtration (G25).

Magnetic particle preparation
Wash 3 grams of INVITROGEN DYNABEADS M-280 Streptavidin-coated microparticles two times with 50 mL Buffer A by centrifugation at 2000 rpm for 10 minutes. Reconstitute to a final concentration of 50 mg/mL particle concentration.

S1 RBD affinity support
React with gentle mixing 10 mL of the 50 mg/mL washed microparticle suspension with 2.5 milligrams of the GenScript SARS-CoV-2 Spike protein biotin conjugate (final concentration = 5 µg of protein conjugate/mg microparticle) for 1 hour at room temperature. Wash the particles by centrifugation at 2000 rpm for 10 minutes three times using 50 mL Buffer A for each wash. Final concentration of microparticles was 50 mg/mL in Buffer A.

Nucleocapsid protein affinity support
React with gentle mixing 10 mL of the 50 mg/mL washed microparticles with 2.5 milligrams of the Meridian SARS-CoV-2 N-Protein biotin conjugate (final concentration = 5 µg of protein conjugate/mg microparticle) for 1 hour at room temperature. Wash the particles by centrifugation at 2000 rpm for 10 minutes three times using 50 mL Buffer A for each wash. Final concentration of microparticles was 50 mg/mL in Buffer A.

S1 affinity support
React with gentle mixing 10 mL of the 50 mg/mL washed microparticles with 2.5 milligrams of the GenScript SARS-CoV-2 S1 protein biotin conjugate (final concentration = 5 µg of protein conjugate/mg microparticle) for 1 hour at room temperature. Wash the particles by centrifugation at 2000 rpm for 10 minutes three times using 50 mL Buffer A for each wash. Final concentration of microparticles was 50 mg/mL in Buffer A.

S2 affinity support
React with gentle mixing 10 mL of the 50 mg/mL washed microparticles with 2.5 milligrams of the Icosagen SARS-CoV-2 Spike S2 protein biotin conjugate (final concentration = 5 µg of protein conjugate/mg microparticle) for 1 hour at room temperature. Wash the particles by centrifugation at 2000 rpm for 10 minutes three times using 50 mL Buffer A for each wash. Final concentration of microparticles was 50 mg/mL in Buffer A.

S1/S2 (full spike) affinity support
React with gentle mixing 10 mL of the 50 mg/mL washed microparticles with 2.5 milligrams of the GenScript SARS-CoV-2 Spike protein biotin conjugate (final concentration = 5 µg of protein conjugate/mg microparticle) for 1 hour at room temperature. Wash the particles by centrifugation at 2000 rpm for 10 minutes three times using 50 mL Buffer A for each wash. Final concentration of microparticles was 50 mg/mL in Buffer A.

Purification of polyclonal antibodies from EURM-017 to each viral protein

Perform the following steps for each of the coupled viral antigen microparticles:

1. Remove buffer A from 50 mg/antigen coupled microparticles by centrifugation at 2000 rpm for 10 minutes. Remove as much liquid as possible.
2. Add 10 mL of EURM-017 reference sera to the microparticles, vortex gently, and rock for 1 hour at room temperature.
3. Centrifuge the microparticle suspension at 2000 rpm for 10 minutes. Decant the serum and store at 2–8°C. Wash the particles by centrifugation at 2000 rpm for 10 minutes three times using 50 mL Buffer A for each wash. Continue to wash the microparticles until the OD 280 nm is <0.05. Remove as much Buffer A as possible after the last wash.
4. Add 1.5 mL Buffer B, vortex, and react for 15 minutes at room temperature. Centrifuge at 2000 rpm for 10 minutes. Remove all liquid from the particles and neutralize with 500 µL of Buffer C. Dialyze the purified antibody into Buffer A. Concentrate to approximately 1.0 mL and determine protein concentration by measuring OD 280 nm.

Results

The analytical results from the five affinity purifications are shown in Table 1.

Table 1.

Antibody Specificity	Volume (mL)	OD-1 (Abs)	OD-2 (Abs)	OD (Abs) Avg	Protein Concentration (µg/mL)	Total Antibody (µg)	IFCC EURM-017 (µg/mL)
S1 RBD	0.825	0.29	0.287	0.289	211	174	17.4
NP	0.943	1.038	1.068	1.053	769	725	72.5
S1	0.932	0.266	0.254	0.260	190	177	17.7
S2	0.935	0.432	0.437	0.435	317	297	29.7
S1/S2 Full Spike	0.963	0.48	0.491	0.486	354	341	34.1

Plaque reduction neutralization test (PRNT)
The plaque reduction neutralization test was performed using all five purified antibodies according to Addendum 1. The protein concentration at each dilution prepared for all purified antibodies is shown in Table 2.

Table 2.

IFCC EURM-017	Antibody Stock (µg/mL)	Antibody Dilution (µg/mL)					
		1:50	1:100	1:200	1:400	1:800	1:1600
S1 RBD	211	4.21	2.11	1.05	0.53	0.26	0.13
NP	769	15.37	7.69	3.84	1.92	0.96	0.48
S1	190	3.80	1.90	0.95	0.47	0.24	0.12
S2	317	6.34	3.17	1.59	0.79	0.40	0.20
S1/S2 Full Spike	354	7.09	3.54	1.77	0.89	0.44	0.22

The percent plaque reduction at each dilution of antibody for the five purified antibodies is shown in Tables 3 and 4.

Table 3.

Antibody Specificity	Dilution Representing 50% Plaque Reduction	Percent Plaque Reduction					
		1:50	1:100	1:200	1:400	1:800	1:1600
S1 RBD	1017	96%	97%	92%	80%	55%	37%
NP	39	32%	11%	–10%	–20%	–4%	NA
S1	1846	100%	100%	94%	97%	69%	58%
S2	355	93%	76%	69%	51%	31%	0%
S1/S2 Full Spike	1234	100%	99%	94%	79%	76%	35%

Table 4.

Antibody Specificity	Dilution Representing 50% Plaque Reduction	Antibody required for 50% PRNT (µg/mL)
S1 RBD	1017	0.207
NP	39	19.7
S1	1846	0.103
S2	355	0.893
S1/S2 Full Spike	1234	0.287

Conclusion

Using affinity purification, the concentration of SARS-CoV-2 protein-specific antibodies present in the EURM-017 reference material is shown in Table 5.

Table 5.

Antibody Specificity	IFCC EURM-017 (µg/mL)
S1 RBD	17.4
NP	72.5
S1	17.7
S2	29.7
S1/S2 Full Spike	34.1

The concentration of each antibody necessary to neutralize the virus to 50% using PRNT is shown in Table 6.

Table 6.

Antibody Specificity	Antibody required for 50% PRNT (µg/mL)
S1 RBD	0.207
NP	19.7
S1	0.103
S2	0.893
S1/S2 Full Spike	0.287

Currently there are no standardized methods for SARS-CoV-2 antibody testing. Understanding and quantifying the SARS-CoV-2 JRC EURM-017 reference material can help produce a standardized method across different assays that use different detection methods for identifying immunocompetence of individuals to the SARS-CoV-2 virus.

Addendum 1. Plaque Reduction Neutralization Test (PRNT) Procedure

Note: Protocol assumes six dilutions for 100 and 300 plaques per well.

I. Preparation of Plates (Day 0)

Purpose: Create the plates needed for the assay.

1. Determine the number of serum samples to be tested.
2. For each sample, seed two 6-well plates at 7 x 105 cells/well. One plate will be used to perform the 100 plaques/well, the other for 300 plaques per well. Seed two additional Control Plates.
   Note: If working from a well-tested Pinpoint Stock, you can use a single plate. See Appendix A for more information.
3. Incubate plates O/N.

II. Preparation of Serum Dilutions (Day 0 or Day 1)

Purpose: Predilute the serum for use in the assay. These dilutions can be prepared the day of the assay or the day before and stored at 2–8°C

1. Prepare Dilution Tube Set 1 by adding 125 µL of PBS to each tube. Prepare five tubes per sample. Prepare one additional dilution tube for your first sample according to the desired first dilution. This will complete Dilution Tube Set 1 with a total of six tubes/sample. Keep in mind that these tubes will be mixed 50:50 with virus to achieve the true final dilution (see Section III). In this step, the serum samples will be diluted by half the desired final dilution.
   - For a 1:50 starting dilution: Add 240 µL PBS and 10 µL serum (1:25).
   - For a 1:20 starting dilution: Add 225 µL PBS and 25 µL serum (1:10).
2. Prepare Dilution Tube Set 2 by adding 125 µL of PBS to each tube. This set of dilution tubes will be serum-free controls for the Control Plates. Prepare six tubes for each control plate.

III. Virus Dilution, Complex Formation, and Infection (Day 1)

Purpose: Perform the actual PRNT.

1. From virus stock (pinpoint or working), dilute the virus (i.e., SARS-CoV-2 WT) to a concentration of 800 pfu/mL (100 pfu/125 µL). Make sure to prepare sufficient volume for all your samples, and mix well. These will be used for your 100 pfu/well dilutions.
2. Repeat Step 1, but dilute to 2400 pfu/mL (300 pfu/125 µL). These will be used for your 300 pfu/well dilutions.
3. Add 125 µL of the virus dilution to each tube in both Dilution Tube Set 1 and Dilution Tube Set 2, as appropriate.
4. Incubate at 37°C for 1 hour so that virus-antibody complex may be formed, i.e., so that the antibody/serum has sufficient time to neutralize the virus.
5. When incubation is finished, decant media from each plate.
6. Pipette 200 µL of each dilution into each well of the 6-well plate. IMPORTANT: Start with the LEAST-diluted serum (which will contain the strongest antibody dose and therefore the least amount of virus) when pipetting. For instance, start with the 1:50 dilution and move forward one by one towards the least diluted.
7. Incubate 45 minutes at 37°C, rocking every 15 minutes.

8. Proceed normally as you would with a plaque assay. Briefly:
   - Melt 1.6% agarose in water and place in a 56°C water bath.
   - Place 2x complete media solutions (2X DMEM, 4% fetal clone serum, 2% antibiotic/antimycotic) in a 37°C water bath.
   - When incubation is complete, return to biosafety cabinet and mix the 1.6% agarose and 2X media solutions and quickly pipette ~2 mL of the mix into each well. Avoid moving the plate for 5–10 minutes so the agarose can solidify.
9. Incubate plates for 2 to 3 days at 37°C, until plaques are visible.
10. Stain with Neutral Red for 3–5 hours, and count every plaque in every well).
11. Calculate percent neutralization for each dilution with the following formula:

$$\% \text{ Neutralization} = \left(1 - \frac{\#\text{ plaques with Ab}}{\#\text{ plaques with no Ab}}\right) + 100$$

Appendix A: Creating a Pinpoint Stock

Purpose: Creating a pinpoint stock saves effort and ensures that your results are more consistent day to day.

1. Using the known titer of your stock, calculate the dilutions need to achieve 8.0 x 104 pfu/mL. You will likely need many intermediate dilutions.
2. Perform these dilutions so that volume is amplified rather than being held constant. For example, when going from 106 to 105, add the entire volume (e.g., 1 mL) to 9 mL PBS to create 10 mL of 105. Do this again 104 to create 100 mL. **Note:** These number are examples. Do what is practical and appropriate.
3. When the dilutions are complete, aliquot and freeze the entirety of your 8.0 x 104 dilution. This is your pinpoint stock.
4. This pinpoint stock can then be diluted 1:100 to create 800 pfu/mL or 1:33 to create 2400 pfu/mL.
5. When the titer of your pinpoint stock is well-characterized, you can do either only the 100-plaque/well or the 300-plaque well dilutions. Both are included above with the expectation that the user is unsure of the final diluted titer.

References:

1. Lai CC, Shih TP, Ko WC, Tang HJ, Hsueh PR. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): the epidemic and the challenges. Int J Antimicrob Agents. 2020;55(3):105924.
2. Guo YR, Cao QD, Hong ZS, Tan YY, Chen SD, Jin HJ, et al. The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak - an update on the status. Mil Med Res. 2020;7(1):11.
3. Li H, Liu SM, Yu XH, Tang SL, Tang CK. Coronavirus disease 2019 (COVID-19): current status and future perspectives. Int J Antimicrob Agents. 2020;29:105951.
4. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72314 cases from the Chinese Center for Disease Control and Prevention. JAMA. 2020;323(13):2648.
5. Pan A, Liu L, Wang C, Guo H, Hao X, Wang Q, et al. Association of public health interventions with the epidemiology of the COVID-19 outbreak in Wuhan, China. JAMA. 2020:6130.
6. Rothan HA, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. J Autoimmun. 2020;109:102433.
7. Lai CC, Liu YH, Wang CY, Wang YH, Hsueh SC, Yen MY, et al. Asymptomatic carrier state, acute respiratory disease, and pneumonia due to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2): facts and myths. J Microbiol Immunol Infect. 2020;S1684-1182(20):30040-2.
8. Sethuraman N, Jeremiah SS, Ryo A. Interpreting diagnostic tests for SARS-CoV-2. JAMA. 2020. doi: 10.1001/jama.2020.8259.
9. Long Q, Liu B, Deng H, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. Nat Med. 2020. doi: 10.1038/s41591-020-0897-1.
10. Zhao J, Yuan Q, Wang H, et al. Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. Clin Infect Dis. 2020. doi: 10.1093/cid/ciaa344.
11. Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LFP. The trinity of COVID-19: immunity, inflammation and intervention. Nat Rev Immunol. 2020;20(6):363-74. doi: 10.1038/s41577-020-0311-8.
12. Ou X, Liu Y, Lei X, et al. Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. Nat Commun. 2020;11(1):1620. doi:10.1038/s41467-020-15562-9.
13. Walls AC, Park JY, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. Cell. 2020;181(2):281-92. doi: 10.1016/j.cell.2020.02.058.
14. Ni F, Ye F, Cheng M, et al. Detection of SARS-CoV-2-specific humoral and cellular immunity in COVID-19 convalescent individuals. Immunity. 2020;S1074-7613. doi: 10.1016/j.immuni.2020.04.023.
15. Li K, Wu M, Huang B, et al. The dynamic changes of antibodies against SARS-CoV-2 during the infection and recovery of COVID-19. Available from: https://doi.org/10.1101/2020.05.18.20105155. Accessed June 9, 2020.
16. Wu F, Wang A, Liu M, et al. Neutralizing antibody responses to SARS-CoV-2 in a COVID-19 recovered patient cohort and their implications. Available from: https://doi.org/10.1101/2020.03.30.20047365. Accessed June 9, 2020.

(References continued on back page)

At Siemens Healthineers, our purpose is to enable healthcare providers to increase value by empowering them on their journey toward expanding precision medicine, transforming care delivery, and improving patient experience, all made possible by digitalizing healthcare.

An estimated 5 million patients globally benefit every day from our innovative technologies and services in the areas of diagnostic and therapeutic imaging, laboratory diagnostics, and molecular medicine, as well as digital health and enterprise services.

We are a leading medical technology company with over 120 years of experience and 18,000 patents globally. Through the dedication of more than 50,000 colleagues in 75 countries, we will continue to innovate and shape the future of healthcare.

Product availability may vary from country to country and is subject to varying regulatory requirements. Please contact your local representative for availability.

References (cont.):

17. Ju B, Zhang Q, Ge J, et al. Human neutralizing antibodies elicited by SARS-CoV-2 infection. Nature. 2020. doi: 10.1038/s41586-020-2380-z.
18. Zhou G, Zhao Q. Perspectives on therapeutic neutralizing antibodies against the Novel Coronavirus SARS-CoV-2. Int J Biol Sci. 2020;16(10):1718-23. doi: 10.7150/ijbs.45123.
19. Jiang S, Hillyer C, Lanying D. Neutralizing antibodies against SARS-CoV-2 and other human coronaviruses. Trends Immunol. 2020;41(5):355-9. Doi: 10.1016/j.it.2020.03.007.
20. Kai-Wang To K, Tsang OT-Y, Leung W-S, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARSCoV-2: an observational cohort study. Lancet Infect Dis. 2020;20(5):565-74. doi: 10.1016/S1473-3099(20)30196-1.
21. Zhu F-C, Li Y-H, Guan X-H, et al. Safety, tolerability, and immunogenicity of a recombinant adenovirus type-5 vectored COVID-19 vaccine: a dose-escalation, open-label, nonrandomized, first-in-human trial. Lancet. 2020;S0140–6736. doi: 10.1016/S0140-6736(20)31208-3.
22. Chen WH, Strych U, Hotez PJ, Bottazzi ME. The SARS-CoV-2 vaccine pipeline: an overview. Curr Trop Med Rep. 2020;1-4. doi: 10.1007/s40475-020-00201-6.
23. Amanat F, Krammer F. SARS-CoV-2 vaccines: status report. Immunity. 2020;52(4):583-9. doi: 10.1016/j.immuni.2020.03.007.

Siemens Healthineers Headquarters
Siemens Healthcare GmbH
Henkestr. 127
91052 Erlangen, Germany
Phone: +49 9131 84-0
siemens-healthineers.com

Published by
Siemens Healthcare Diagnostics Inc.
Laboratory Diagnostics
511 Benedict Avenue
Tarrytown, NY 10591-5005
USA
Phone: +1 914-631-8000

Published by Siemens Healthcare Diagnostics Inc. · Order No. 30-21-DX-592-76 · 05-2021 ·

©Siemens Healthcare Diagnostics Inc., 2021