Received: April 4, 2022
Accepted: April 26, 2022

Acad Med J 2022;2(1):25-40
UDC: 616.98:578.834]-097
DOI:
Original article

PERFORMANCE CHARACTERISTICS OF SNIBE SARS-COV-2 IgM/IgG AND SARS-COV-2 S-RBD IgG SEROLOGICAL ASSAYS

Bogdanska Jasna, Trajkovska Tosheska Katerina, Topuzovska Sonja, Cekovska Svetlana, Petrushevska-Stanojevska Elena, Emin Melda, Ampova Hristina

Institute of Medical and Experimental Biochemistry, Faculty of Medicine, Skopje, Ss. Cyril and Methodius University in Skopje, Republic of North Macedonia
e-mail:jasna.bogdanska@medf.ukim.edu.mk

Abstract

Since corona virus emerged, few tests for diagnosis and follow-up of the disease were approved for urgent use by FDA. Serological tests for the presence of SARS-Cov-2-specific M/G and RBD IgG antibodies manufactured by SNIBE were introduced to the market at the beginning of 2020, with a primary recommendation for monitoring and responding to SARS-Cov-2 infection or vaccines.

According to the Standard ISO 15189, each laboratory should take special actions before implementation of any new analyses as routine ones. Bearing this in mind, a verification of the chemiluminescence method for antibody detection according to the CLSI EP 15-A2 and CLSI EP 15-A3 protocol was done in our laboratory.

Pooled control samples for IgG, IgM and RBG IgG with two levels, as well as serum samples for positive IgG antibodies were used for method verification. As part of the verification procedure, the precision of the method was estimated.

The results of the repeatability and coefficients of variation for SARS-Cov-2 IgM/IgG and SARS-Cov-2 S-RBD IgG were equal or less than the manufacturer’s claims, except for negative control RBD IgG samples. Estimated results for within-laboratory precision (reproducibility) as well as coefficients of variation were less or equal to the manufacturer’s claims, except for positive control samples for IgM.

We can conclude that the estimated performance characteristics of SNIBE SARS-Cov-2 IgM/IgG and SARS-Cov-2 S-RBD IgG serological assays are consistent with the manufacturer’s claim’ and that they can be introduced in our laboratory.

Keywords: SARS-Cov-2, verification of the chemiluminescence immunoassay for SARS-Cov-2 IgM/IgG and SARS-Cov-2 S-RBD IgG antibodies

Introduction

Since a new corona virus emerged at the end of 2019, called Covid-19, the urgent need for an early diagnosis and follow-up of the progression of the disease was one of the main priorities of the health care systems worldwide. As a gold standard for acute infection with SARS-Cov-2, nucleic acid amplification test (NAAT) has been approved by the US Food and Drug Administration (FDA) under emergency use authorization[1]. On the other hand, serological testing for SARS-Cov-2-specific antibodies, especially immunoglobulin M (IgM) and immunoglobulin G (IgG) is not recommended as the primary method for the

diagnosis of acute cases. Antibody tests can detect the presence of these antibodies in serum within days to weeks following acute infection.

Currently, antibody testing is not recommended to assess for immunity to SARS-CoV-2 following COVID-19 vaccination, to assess the need for vaccination in an unvaccinated person, or to determine the need to quarantine after a close contact with someone who has COVID-19.

On the other hand, serological methods have public health value for monitoring and responding to the COVID-19 pandemic and clinical utility in providing care for patients.
Efforts to better understand antibody kinetics, longevity of humoral immune responses, correlation of binding antibody levels to neutralizing antibodies, and serological surrogates of immune protection are dependent on wider availability of quantitative binding antibody assays that are standardized and traceable to an international standard[2].

Currently available antibody tests for SARS-CoV-2 assess IgM/IgG or RBD IgG to one of two viral proteins: S or N. Although current EUA indications do not preclude the use of these tests in vaccinated individuals, none of the currently authorized tests have been specifically authorized to assess immunity or protection of persons who have received a COVID-19 vaccine.

So far, only one test has received an EUA as a quantitative assay (providing a measured and scaled assessment of antibody levels). All other currently authorized tests, to our knowledge, such as those manufactured by SNIBE, are qualitative (providing a result to be positive, negative, or indeterminate) or semi-quantitative that have been validated by the manufacturer. Even then, there is a distinct difference in the quality of testing between the laboratories. The difference can be explained through various confounding variables including quality control procedures, machine maintaining procedures, training-related issues, etc. Having selected an appropriate methodology based on the requirements of the individual laboratory, verification becomes an essential responsibility of each laboratory. According to ISO 15189, the validated examination procedures should be a subject for independent verification before being introduced into routine work.

Bearing this in mind, the aim of our study was to evaluate performance characteristics of 2019-nCov IgM/IgG and SARS-Cov-2 S-RBD IgG serological assays employing CLIA method on SNIBE Maglumi 800 analyser.

CLIA method for IgM/G and S-RBD IgG is an indirect chemiluminescence immunoassay. The sample and magnetic microbeads coated with anti-human IgM monoclonal antibodies, with 2019-nCov recombinant antigen for IgG detection or with S-RBD recombinant antigen are forming complexes with antibodies present in the sample. Following the washing steps, chemiluminescence reaction is performed. The light signal, measured by a photomultiplier as relative luminescent units (RLUs) is proportional to the content of antibodies of interest presented in the sample[3].

The results for 2019-nCoV IgG/M and SARS-Cov-2 S-RBD IgG obtained on MAGLUMI 800 are reported as qualitative and the results are expressed as Absorbance Units/mL (AU/mL). The results are reported to the end user as “Reactive“ and “Non-Reactive”. No AU/ml numerical values are reported to the end user. The samples are considered reactive with a concentration of ≥1.0 AU/mL and for non-reactive <1.0 AU/ml[3,4].

Materials and methods

Pooled control samples with two different levels (negative and positive) for 2019-nCov IgM/IgG and SARS-Cov-2 S-RBD IgG, as well as pooled serum samples for positive 2019-nCov IgG were used for method verification. Control samples were stored at +4 to +8

C^o, and pooled serum samples were frozen on -24 C^o. All measurands were prepared as aliquots of three replicates, each of them represented with three samples.

Precision verification included testing of 45 replicates represented by three replicates for Level 1 (negative) and 45 replicates represented by three replicates of Level 2 (positive) for each immunoglobulin. The additional precision verification of positive IgG immunoglobulin included 45 samples of pooled serum with positive results for the presence of IgG immunoglobulin. Testing was done for 5 consecutive days. This included testing of 3 replicates in triplicate per day under similar operating conditions which were used for estimation of assay precision based on CLSI EP 15-A2 protocol[5,6]. The protocols intended to verify that laboratory's performance is consistent with claims made by the manufacturer. Following the guidelines of the protocols, within-run and within-laboratory precision of the CLIA-based tests on SNIBE 800 was done. The statistical analysis of the results was done using the statistical package Win stat for Windows.

Results

Estimation of repeatability (within-run variance) for negative 2019-nCov IgM/IgG and SARS-Cov-2 S-RBD IgG control samples

Precision for 2019-nCov IgM

Results of the 45 negative and positive control replicates for 2019-nCov IgM were plotted in the tables (Table 1 and Table 2). Visual inspection of the data showed that there were no gross outliers. Significant statistical outliers were ruled out by Grubb's test where the critical value of <0.05 was set as a significant one. According to the Grubb's test, a result qualifies as an outlier if that value lies more than the G ± SDs from the sample mean (Grubb's limit), where G is the Grubb's factor, and SD is the standard deviation of the raw data including the suspected outliers. Grubb's factor was calculated using Grubb's table by the statistical program available online (https://www.graphpad.com/quickcalcs/Grubbs1.cfm). Table 1 presents the data of control IgM negative measurements for 5 consecutive days with mean value of 0.270 AU/ml and standard deviation of 0.039.

Table 1. Compilation of data of negative 2019-nCov IgM

Day	Run	Replicate 1	Replicate 2	Replicate 3
1	Run 1	0.275	0.249	0.396
	Run 2	0.290	0.324	0.093
	Run 3	0.283	0.286	0.244
2	Run 1	0.266	0.283	0.279
	Run 2	0.307	0.266	0.280
	Run 3	0.287	0.274	0.275
3	Run 1	0.262	0.236	0.262
	Run 2	0.271	0.29	0.255
	Run 3	0.267	0.260	0.258
4	Run 1	0.268	0.292	0.253
	Run 2	0.247	0.248	0.262
	Run 3	0.258	0.270	0.257
5	Run 1	0.242	0.253	0.261
	Run 2	0.346	0.286	0.252
	Run 3	0.294	0.269	0.256
Mean value of all measurements	0.270			
SD of the whole data set	0.039			

The results presented in Table 1 show that there were no gross outliers of the measurements. In order to confirm it, the Grubb's test for outliers was done as a step 2 of the Grubb's test. The calculated Grubb's factor (G) was 2.92 for the significance level of 0.05 and the significant outlier was 0.093.

This outlier was found to be present because the absolute difference between the replicates exceeded 5.5 times the SD determined by the preliminary precision test, and this pair was rejected and investigated before repeating the run and performing the statistical evaluation of precision tests.

The calculation of Grubbs limits was done according to the equation 1:

$$\text{Grubb's limits} = \text{mean} \pm G \times SD \qquad \text{Equation 1}$$

Using the equation 1, the Grubb's lower limit was 0.133 and the upper limit was 0.41. Since all the results were within the Grubb's limits, further analysis of the precision was performed.

Results of the measurement of the control positive replicates for IgM are presented in Table 2. The results presented in Table 2 show that there were no gross outliers of the measurements. In order to confirm it, the Grubb's test for outliers was done as a step 2 of the Grubb's test. The calculated Grubb's factor G was 3.08 for the significance level of 0.05.

Table 2. Compilation of data of positive *2019-nCov IgM*

Day	Run	Replicate 1	Replicate 2	Replicate 3
1	Run 1	3.792	3.925	4.117
	Run 2	3.808	3.904	4.136
	Run 3	3.808	3.904	4.136
2	Run 1	5.113	5.087	4.995
	Run 2	5.399	5.214	5.108
	Run 3	5.192	4.952	4.96
3	Run 1	3.971	4.013	4.038
	Run 2	4.197	4.006	3.966
	Run 3	3.819	3.765	3.957
4	Run 1	3.961	4.022	4.069
	Run 2	4.245	4.031	4.067
	Run 3	4.141	4.022	4.093
5	Run 1	4.168	3.904	4.058
	Run 2	4.245	4.178	4.24
	Run 3	3.972	4.014	4.108
Mean value of all measurements	4.24			
SD of the whole data set	0.469			

The calculation of Grubb's limits was done according to the equation 1. For IgM positive control replicates, Grubb's lower limit was calculated to be 2.8 and for the upper limit 5.68. Since all of the results (from Table 2) were within these limits, statistical analyses for the imprecision represented by estimation of the repeatability and within-laboratory precision were ruled out.

Repeatability of the negative and positive control samples for 2019-nCov IgM

According to the EP15-A2 five days protocol, the total number of 45 measurements of each level was made. The results of the negative controls are presented in Table 3. The mean value of the daily means was 0.270 AU/ml ± 0.0139 and the coefficient of variation (CV) was 5.27%.

Table 3. Mean values of the negative control 2019-nCov IgM samples (AU/ml)

Day	Replicate 1	Replicate 2	Replicate 3
1	0.283	0.287	0.271
2	0.280	0.275	0.280
3	0.267	0.263	0.259
4	0.258	0.270	0.257
5	0.294	0.269	0.256

Using the data from Table 3, repeatability of negative control 2019-nCov IgM samples was calculated employing equation 2.

$$s_r = \sqrt{\frac{\sum_{d=1}^{D}\sum_{r=1}^{n}(x_{dr} - x_d)^2}{D(n-1)}}$$ Equation 2

where:
D = total number of days;
N = total number of replicates per day;
$\bar{X}_{dr}$ = result for replicate r on day d;
$\bar{x}_d$ = average of the replicates on day d.

Repeatability (s_r) (employing the equation 2) for negative control 2019-nCov IgM samples was calculated to be 0.0103 AU/ml.

The next step was to calculate the daily variance using equation 3 from the data presented in Table 4.

Table 4. Results from calculations of the variance of the day for negative control 2019-nCov IgM samples (AU/ml)

Day	Mean of the day	(Replicate 1-Mean)^2	(Replicate 2-Mean)^2	(Replicate 3-Mean)^2	Mean of all the results	(Mean of all the results - Mean of the day)^2
1	0.280	0.00001	0.00004	0.00008	0.272	0.000002
2	0.280	0.00004	0.00003	0.00000	0.272	0.000108
3	0.263	0.00002	0.00000	0.00002	0.272	0.000050
4	0.262	0.00001	0.00007	0.00002	0.272	0.000066
5	0.273	0.00043	0.00001	0.00028	0.272	0.000012

$$s^2{}_b = \frac{\sum_d(\bar{x}\,d - \bar{\bar{x}})^2}{D-1}$$

Equation 3

where:
D= total number of days;
$\bar{X}_d$ = average of all replicates on day d;
$\bar{x}$ = average of all results.

Using the presented data (Table 4 and equation 3), the obtained variance of the daily means (s^2_b) for the negative control 2019-nCov IgM samples were 0.00008 AU/ml.

The final step was to calculate the within-laboratory precision using equation 4 from the data presented in Table 4 and employing the equation 4.

$$s_t = \sqrt{\frac{n-1}{n} \times s^2r + s^2b}$$

Equation 4

where:
n = number of replicates per day.

The sum of the squared differences was 0.00107 (from the data presented in Table 4) and since D = 5 and n=3, within-laboratory precision-s_t for negative control 2019-nCov IgM samples was 0.0123 AU/ml.

The same calculations and equations were used for calculation of the repeatability, variance of the day and within-laboratory precision for positive control 2019-nCov IgM samples and the results are presented in Table 5 and 6.

Table 5. Mean values of the positive control 2019-nCov IgM samples (AU/ml)

Run/day	Replicate 1	Replicate 2	Replicate 3
1	3.8	3.915	4.127
2	5.235	5.084	5.021
3	3.996	3.928	3.987
4	4.116	4025	4.076
5	4.128	4.032	4.123

The mean value of the positive control 2019-nCov IgM samples was 4.24 ± 0.463 and the coefficient of variation (CV) was 10.92%.

The data used for the calculation of the repeatability, variance of the day, and within-laboratory precision for the positive control 2019-nCov IgM samples are presented in Table 6.

Table 6. Results from calculations of the variance of the day for positive control 2019-nCov IgM samples

Day	Mean of the day	(Replicate 1-Mean)^2	(Replicate 2-Mean)^2	(Replicate 3-Mean)^2	Mean of all the results	(Mean of all the results - Mean of the day)^2
1	3.947	0.02171	0.00105	0.03228	4.24	0.085
2	5.113	0.01480	0.00086	0.00853	4.24	0.764
3	3.970	0.00066	0.00179	0.00028	4.24	0.072
4	4.072	0.00191	0.00224	0.00001	4.24	0.028
5	4.094	0.00113	0.00389	0.00082	4.24	0.021

Using equations 2 and 3 (and data from Table 6), the repeatability of positive control 2019-nCov IgM samples was calculated to be 0.095 AU/ml and the variance of the day S^2_b was 0.242 AU/ml.

The final step was calculation of the total or within-laboratory SD (s_t) using the equation 4. The sum of the squared differences was 0.09195 and within-laboratory precision for the positive control 2019-nCov IgM samples was estimated to be 0.498 AU/ml.

Precision for 2019-nCov IgG

The results of the 45 negative and positive control replicates from each level for 2019-nCov IgG were plotted in tables (Table 7 and Table 8). There were no gross outliers and there were no statistically significant outliers for both levels (negative and positive control samples). Table 7 presents data of control IgG negative measurements for 5 days with mean value of 0.443 ± 0.022 AU/ml and the coefficient of variation was 4.39%.

Using the equation 1, the Grubb's lower limit for negative control 2019-nCov IgG samples was 0.379 and the upper limit was 0.507. All the measured results were within the Grubb's limit, meaning that statistical evaluation of the performance characteristics of the test could be ruled out.

Table 7. Compilation of data of negative control 2019-nCov IgG samples

Day	Run	Replicate 1	Replicate 2	Replicate 3
1	Run 1	0.440	0.446	0.457
	Run 2	0.447	0.460	0.440
	Run 3	0.457	0.457	0.446
2	Run 1	0.418	0.445	0.448
	Run 2	0.446	0.440	0.457
	Run 3	0.455	0.443	0.445
3	Run 1	0.440	0.448	0.418
	Run 2	0.446	0.443	0.446
	Run 3	0.457	0.440	0.488
4	Run 1	0.445	0.443	0.444
	Run 2	0.444	0.445	0.439
	Run 3	0.439	0.457	0.440
5	Run 1	0.477	0.443	0.434
	Run 2	0.434	0.423	0.324
	Run 3	0.477	0.443	0.434
Mean value of all measurements	0.443			
SD of the whole data set	0.022			

The results of the measurement of the control positive replicates for IgG are presented in Table 8.

Table 8. Compilation of data of positive control 2019-nCov IgG samples

Day	Run	Replicate 1	Replicate 2	Replicate 3
1	Run 1	1.031	0.970	0.947
	Run 2	1.016	1.013	1.026
	Run 3	0.976	1.065	0.995
2	Run 1	1.246	1.226	1.239
	Run 2	1.240	1.213	1.150
	Run 3	1.116	1.220	1.153
3	Run 1	0.980	1.047	1.045
	Run 2	0.914	0.814	1.012
	Run 3	0.987	0.982	0.919
4	Run 1	0.933	0.989	1.024
	Run 2	0.892	0.976	0.931
	Run 3	0.957	0.990	0.966
5	Run 1	1.036	1.062	1.081
	Run 2	0.995	0.990	0.934
	Run 3	1.058	1.029	0.983
Mean value of all measurements	1.030			
SD of the whole data set	0.100			

It can be seen from Table 8 that there were no gross outliers of the measurements. In order to confirm it, the Grubb's test for outliers was done as step 2 of the Grubb's test. The calculated Grubb's factor G was 2.92 with the significance level of 0.05. The Grubb's lower limit for positive control 2019-nCov IgG samples was 0.74 AU/ml and the upper limit was

1.324, i.e., all results were within the limit and the verification of the method could be ruled out. Since the obtained results for repeatability of positive control samples were close to the cut-off value, the verification of the method for positive pooled serum 2019-nCov IgG samples was done.

Table 9. Compilation of data of positive serum 2019-nCov IgG samples

Day	Run	Replicate 1	Replicate 2	Replicate 3
1	Run 1	6.428	6.526	6.588
	Run 2	6.704	6.821	6.681
	Run 3	6.467	6.592	6.520
2	Run 1	6.656	6.484	6.204
	Run 2	6.795	6.652	6.578
	Run 3	6.735	6.658	6.605
3	Run 1	6.762	6.658	6.544
	Run 2	6.611	6.831	6.673
	Run 3	6.856	6.704	6.686
4	Run 1	6.650	6.762	6.537
	Run 2	6.484	6.558	6.595
	Run 3	6.588	6.411	6.641
5	Run 1	6.307	6.564	6.405
	Run 2	6.413	6.590	6.371
	Run 3	6.417	6.404	6.114
Mean value of all measurements	6.740			
SD of the whole data set	0.159			

Table 9 shows that there were no gross outliers of the measurements. In order to confirm it, the Grubb's test for outliers was done as step 2 of the Grubb's test. The calculated Grubb's factor G was 2.92 with the significance level of 0.05. The Grubb's lower limit for positive serum samples for 2019-nCov IgG was 6.276 AU/ml and the upper limit was 7.204, i.e., all results were within the limits, which allowed us to proceed with the verification of the precision of the method.

Repeatability of the negative and positive control samples for 2019-nCov IgG

Total number of 45 measurements of each level was done. The mean values/day per replicate of the negative control samples for 2019-nCov IgG are presented in Table 10. The mean value of the replicates/day was 0.448 AU/ml and the CV was 3.36%.

Table 10. Mean values of the negative control 2019-nCov IgG samples (AU/ml)

Run/day	Replicate 1	Replicate 2	Replicate 3
1	0.440	0.446	0.457
2	0.418	0.446	0.455
3	0.447	0.452	0.471
4	0.445	0.444	0.439
5	0.477	0.443	0.434

As the next step, calculation of repeatability and variance of the day for negative control 2019-nCov IgG samples (employing equation 2 and 3) was performed from the data presented in Table 11.

Data obtained demonstrated that the repeatability was Sr= 0,015 AU/ml and the variance of the day (S^2_b) 0.000045 AU/ml. The last step was calculation of the within-laboratory precision (S_t) using data from Table 11 (and the result of the sum of the squared

differences = 0.00226), which for negative control 2019-nCov IgG samples was estimated to be S_t = 0,014 AU/ml.

Table 11. Results from calculations of the variance of the day for negative control 2019-nCov IgG samples

Day	Mean of the day	(Replicate 1-Mean)^2	(Replicate 2-Mean)^2	(Replicate 3-Mean)^2	Mean of all the results	(Mean of all the results - Mean of the day)^2
1	0.448	0.00006	0.00000	0.00009	0.448	0.000000
2	0.440	0.00047	0.00004	0.00024	0.448	0.000063
3	0.457	0.00009	0.00002	0.00021	0.448	0.000082
4	0.443	0.00001	0.00000	0.00001	0.448	0.000024
5	0.451	0.00066	0.00007	0.00030	0.448	0.000014

The data for the verification of the assay precision for positive control 2019-nCov IgG samples are presented in Table 12.

Table 12. Mean values of the positive control 2019-nCov IgG samples (AU/ml)

Run/Day	Replicate 1	Replicate 2	Replicate 3
1	1.008	1.016	0.989
2	1.217	1.220	1.181
3	0.960	0.948	0.992
4	0.927	0.985	0.974
5	1.030	1.027	0.999

The mean value of all measurements for positive control IgG samples was 1.032 AU/ml, the standard deviation 0.095 and the CV 9.19%.

Using the data from Table 12, the calculated repeatability (Sr) was 0.0220 AU/ml (as calculated from the equation 2).

The data for the calculation of the variance of the day and within-laboratory precision for positive control 2019-nCov IgG samples are presented in Table 13.

Table 13. Results from calculations of the variance of the day for positive control 2019-nCov IgG samples

Day	Mean of the day	(Replicate 1-Mean)^2	(Replicate 2-Mean)^2	(Replicate 3-Mean)^2	Mean of all the results	(Mean of all the results - Mean of the day)^2
1	1.004	0.00001	0.00014	0.00024	1.032	0.00074
2	1.206	0.00012	0.00020	0.00063	1.032	0.03044
3	0.967	0.00004	0.00035	0.00064	1.032	0.00421
4	0.962	0.00123	0.00053	0.00014	1.032	0.00483
5	1.019	0.00013	0.00007	0.00039	1.032	0.00017

Data obtained showed that the variance of the day (S^2_b) was 0.010 AU/ml and within-laboratory precision (S_t) 0.102 AU/ml (as calculated employing equation 3 and 4).

Table 14 presents the data from the measurement of the positive IgG serum samples.

Table 14. Mean values of the positive serum 2019-nCov IgG samples (AU/ml)

Run/Day	Replicate 1	Replicate 2	Replicate 3
1	6.53	6.65	6.60
2	6.73	6.60	6.46
3	6.74	6.73	6.63
4	6.57	6.57	6.59
5	6.37	6.51	6.29

The mean value of all measurements (Table 9) was 6.571 AU/ml, the standard deviation 0.127 and the CV 1.93%.

Using the data from Table 14, the calculated repeatability (Sr) was 0.0866 AU/ml (as calculated from the equation 2).

For the calculation of within-laboratory precision, the variance of the day was calculated and the data are presented in Table 15.

Table 15. Results from calculations of the variance of the day for positive serum 2019-nCov IgG samples

Day	Mean of the day	(Replicate 1-Mean)^2	(Replicate 2-Mean)^2	(Replicate 3-Mean)^2	Mean of all the results	(Mean of all the results - Mean of the day)^2
1	6.59	0.00344	0.00295	0.00002	6.571	0.000
2	6.60	0.01760	0.0000	0.01805	6.571	0.0017
3	6.70	0.00179	0.00092	0.00528	6.571	0.007
4	6.58	0.00002	0.00006	0.00014	6.571	0.000
5	6.39	0.00040	0.01440	0.01000	6.571	0.033

Data obtained showed that the sum of the squared differences was 0.075, the estimated variance of the day (S^2_b) was 0.01267 AU/ml and within-laboratory precision (S_t) 0.132 AU/ml (as calculated employing equations 3 and 4).

Precision for SARS-Cov-2 S-RBD IgG

Following the same protocol, the results for 45 measurements of two different levels (negative and positive controls) were used for evaluation of the assay precision of the test. Table 16 presents the total measurements of negative SARS-Cov-2 S-RBD IgG and Table 17 of positive SARS-Cov-2 S-RBD IgG.

Table 16. Compilation of data of negative SARS-Cov-2 S-RBD IgG

Day	Run	Replicate 1	Replicate 2	Replicate 3
1	Run 1	0.263	0.340	0.316
	Run 2	0.293	0.297	0.325
	Run 3	0.278	0.319	0.320
2	Run 1	0.262	0.261	0.284
	Run 2	0.289	0.281	0.307
	Run 3	0.275	0.271	0.295
3	Run 1	0.331	0.351	0.331
	Run 2	0.335	0.340	0.360
	Run 3	0.333	0.346	0.345
4	Run 1	0.353	0.349	0.329
	Run 2	0.326	0.337	0.349
	Run 3	0.339	0.343	0.339
5	Run 1	0.333	0.34	0.362
	Run 2	0.349	0.359	0.376
	Run 3	0.341	0.350	0.369
Mean value of all measurements	0.324			
SD of the whole data set	0.031			

There were no gross outliers of the measurements. In order to confirm it, the Grubb's test for outliers was done as step 2 of the Grubb's test. The calculated Grubb's factor G was 2.92 for the significance level of 0.05. The Grubb's lower limit for negative controls was 0.233AU/ml and the upper limit was 0.414, i.e., all the results were within the Grubb's limit.

The Grubb's lower limit for positive controls was 3.031AU/ml and the upper limit was 4.57, i.e., all the results were within the Grubb's limits and the verification of the method for this parameter could be performed.

Table 17. Compilation of data of positive SARS-Cov-2 S-RBD IgG

Day	Run	Replicate 1	Replicate 2	Replicate 3
1	Run 1	3.894	3.829	3.689
	Run 2	3.720	3.803	3.811
	Run 3	3.803	3.853	3.865
2	Run 1	4.298	4.309	3.803
	Run 2	4.239	4.085	4.048
	Run 3	4.503	4.339	4.113
3	Run 1	3.702	3.703	3.595
	Run 2	3.379	3.616	3.888
	Run 3	3.573	3.763	3.611
4	Run 1	3.693	3.979	3.888
	Run 2	3.872	3.645	3.804
	Run 3	3.778	3.371	3.897
5	Run 1	3.561	3.657	3.347
	Run 2	3.738	3.673	3.576
	Run 3	3.667	3.756	3.410
Mean value of all measurements	3.803			
SD of the whole data set	0.267			

Repeatability of the negative and positive control samples for SARS-Cov-2 S-RBD IgG

Total number of 45 measurements of each level was done. The results of the negative control samples for SARS-Cov-2 S-RBD IgG are presented in Table 18. The mean value of the replicates measured in 5 days was 0.324 AU/ml and the CV was 9.38%.

Table 18. Mean values of the negative control samples for SARS-Cov-2 S-RBD IgG

Run/day	Replicate 1	Replicate 2	Replicate 3
1	0.2780	0.3190	0.3205
2	0.2755	0.2710	0.2955
3	0.3330	0.3460	0.3455
4	0.3395	0.3430	0.3390
5	0.3410	0.3500	0.3690

Following the protocol, estimated repeatability was 0.0142 AU/ml (S_r= 0.01425 AU/ml) (as calculated employing the equation 2).

The next step was to calculate the variance of the day using equation 3. Using the data from Table 19 and the sum of the differences (0.00203), the variance of the day (s^2b) was calculated to be 0.00091 AU/ml.

Table 19. Results from calculations for the variance of the day for negative control samples for SARS-Cov-2 S-RBD IgG

Day	Mean of the day	(Replicate 1-Mean)^2	(Replicate 2-Mean)^2	(Replicate 3-Mean)^2	Mean of all the results	(Mean of all the results - Mean of the day)^2
1	0.306	0.00077	0.00017	0.00022	0.324	0.00034
2	0.281	0.00003	0.00009	0.00022	0.324	0.00191
3	0.342	0.00007	0.00002	0.00002	0.324	0.00029
4	0.341	0.00000	0.00001	0.00000	0.324	0.00026
5	0.353	0.00015	0.00001	000025	0.324	0.00084

As a final step, the within-laboratory precision for negative control samples for SARS-Cov-2 S-RBD IgG was calculated to be 0.0323 AU/ml (s_t= 0.0323) (from the data presented in Table 16).

The data for the verification of the assay precision for positive control 2019-nCov IgG samples are presented in Table 20.

Table 20. Mean values of the positive control samples for SARS-Cov-2 S-RBD IgG

Day	Replicate 1	Replicate 2	Replicate 3
1	3.807	3.841	3.75
2	4.347	4.244	4.009
3	3.551	3.694	3.698
4	3.781	3.665	3.863
5	3.655	3.695	3.444

The mean value of the positive control samples for SARS-Cov-2 S-RBD IgG was 3.803 AU/ml, and the coefficient of variation 6.32 % (from the data presented in Table 20).

Following the protocol, repeatability was 0.115 AU/ml (as calculated employing the equation 2).

The calculated variance of the day (s^2b), using equation 3 and the data from Table 21 was 0.056 AU/ml.

Table 21. Results from calculations of the variance of the day for positive control samples for SARS-Cov-2 S-RBD IgG

Day	Mean of the day	(Replicate 1-Mean)^2	(Replicate 2-Mean)^2	(Replicate 3-Mean)^2	Mean of all the results	(Mean of all the results - Mean of the day)^2
1	3.799	0.00006	0.00174	0.00243	3.803	0.00001
2	4.200	0.02161	0.00194	0.03648	3.803	0.15766
3	3.648	0.00934	0.00215	0.00253	3.803	0.02411
4	3.770	0.00013	0.01096	0.00871	3.803	0.00111
5	3.598	0.00325	0.00941	0.02372	3.803	0.04200

At the end, within-laboratory precision was calculated employing equation 4 from the data presented in Table 21. Within-laboratory precision (s_t) for positive control samples for SARS-Cov-2 S-RBD IgG was 0.255 AU/ml.

Evaluation of the results

EP15-A2 is generally used to verify that a method is being performed as claimed by the manufacturer, before introducing it into the routine practice. Therefore, the imprecision estimates calculated above must be compared to the manufacturer's claim. If the repeatability and within-laboratory SD are less than that indicated by the manufacturer, then the user has demonstrated precision consistent with the claim and no further calculations are required. In order to compare the estimated repeatability to the claimed value, the critical of the verification value was calculated using the equation 5.

Verification value = σ_r x $\sqrt{C}$ / $\sqrt{v}$
where:
σ_r = claimed repeatability;
C is the 1-α/q percentage point of the Chi-square distribution;
α is the false rejection rate and q is the number of levels tested;
V is the degrees of freedom = D x (n-1).

Equation 5

In our laboratory, the verification values for repeatability of negative and positive control 2019-nCov IgM samples were calculated to be 0.0286 and 0.239 respectively. Since our estimated values for repeatability of negative and positive control 2019-nCov IgM samples (s_r) were 0.0103 and 0.095, which are less than the repeatability verification value, we may say that the data were consistent with the manufacturer's claim.

The calculated repeatability verification values for negative and positive control 2019-nCov IgG samples were 0.034 and 0.284 respectively, which again are higher than the estimated one (0.015 and 0.022), and the verification values were consistent with the manufacturer's claim.

The calculated repeatability verification value for positive serum 2019-nCov IgG samples was 0.227, which again are higher than the estimated one (0.0866) meaning that the verification value was consistent with the manufacturer's claim.

The calculated repeatability verification values for negative and positive control samples for SARS-Cov-2 S-RBD IgG were 0.01 and 0.252, respectively. Since the estimated repeatability for negative and positive control samples for SARS-Cov-2 S-RBD IgG was equal or less than the calculated one (0.01 and 0.115 respectively), the data were consistent with the manufacturer's claim.

For comparison of within-laboratory precision and the manufacturer's claim the equation 6 was used.

Verification value = σ_t x $\sqrt{C}$ / $\sqrt{T}$
where:
σ_t is claimed within-laboratory or total SD;
T is the effective degrees of freedom for the within-laboratory precision estimate.

Equation 6

If the estimated verification value is equal or less than the calculated verification value, than the data are consistent with the manufacturer's claim.

For within-laboratory verification value for negative and positive 2019-nCov IgM control samples, the calculated one was 0.05 and 0.600, respectively. Our estimated values for within-laboratory precision were less than the calculated ones (0.012 and 0.498 respectively) meaning that the data were consistent with the manufacturer's claim.

For within-laboratory verification value for negative and positive control 2019-nCov IgG samples, the calculated one was 0.075 and 0.475, respectively. Our estimated values for within-laboratory precision were less than the calculated ones (0.014 and 0.102 respectively) meaning that the data were consistent with the manufacturer's claim.

For within-laboratory verification value for positive serum 2019-nCov IgG samples the same equation was used and the obtained result was calculated to be 0.473, which was higher than the estimated one (0.132), meaning that the data were consistent with the manufacturer's claim.

The calculated verification values for negative and positive control samples for SARS-Cov-2 S-RBD IgG were 0.110 and 0.358, respectively. The estimated values for within-laboratory precision in our laboratory were less than the calculated ones (0.032 and 0.255), and the data were consistent with the manufacturer's claim.

Finally, the comparison of the estimated coefficients of variation for repeatability and within-laboratory precision was calculated and the data are presented in Table 22. As we can see from the table, only negative controls for RBD IgG had higher estimated coefficients of variation than the claimed one. IgM positive control samples demonstrated higher coefficient of variation for reproducibility (within-laboratory precision) than the claimed one. These

Table 22. Comparison of the imprecision estimates against the manufacturer's claim

Sample	Mean value (AU/ml) (N=45)	Estimated repeatability %CV	Claimed repeatability %CV	Estimated %CV between day	Claimed %CV between day	Estimated reproducibility %CV	Claimed %CV reproducibility	Manufacturer's mean value (N=90)
NQC IgM	0.270	3.81	NA	0,029	NA	5.14	NA	0.293
PQC IgM	4.24	2.24	4.26	5.7	1.58	11.74	8.65	3.920
NQC IgG	0.443	3.38	NA	0.01	NA	3.16	NA	0.293
PQC IgG	1.030	2.13	5.08	0.96	0.82	10	8.68	3.915
Ig G SP	6.740	1.28	6.08	0.187	0.84	2	6.88	9.807
NQC RBD	0.324	4.39	1.77	0.28	9.85	9.9	12.37	0.396
PQC RBD	3.803	3.04	4.49	1.47	1.38	6.71	5.44	3.916

results suggest that the further statistical analyses should be performed. Overall estimated coefficients of variation were equal or lower than the claimed ones, meaning that these parameters can be introduced in the routine ones in our laboratory.

Discussion

Tests issued an EUA by FDA are recommended for clinical and public health purposes. Antibody tests with very high sensitivity and specificity are preferred since they are more likely to exhibit high positive and negative predictive values when administered at least 3 weeks following onset of illness although the clinical and public health applicability of semi-quantitative tests has not been established[1].

Antibody tests can be used in seroprevalence studies to estimate the cumulative incidence of infection (or vaccination) in a community. A negative antibody test does not preclude previous infection. A proportion of persons who are infected with SARS-CoV-2 might not develop measurable antibodies, thereby limiting the sensitivity of any antibody test to detect previous infection in these individuals. In addition, measurable antibodies also might wane over time, and the extent to which seroreversion occurs could vary according to the antibody test used[8] .

To date, to our knowledge, more than 200 different CE marked tests have been identified including manual and automated immunoassays. Various techniques are available such as ELISA (enzyme linked immunosorbent assay), CLIA (chemiluminescence enzyme immunoassays, fluorescence immunoassays, lateral flow immunoassays to detect immunoglobulin G (IgG), A (IgA), M (IgM) as well as different antibodies targets (Spike [S], RBD and/or, nucleocapsid proteins)[1].

It is essential for each laboratory to verify the methods before broad introduction into routine practice. Before proceeding to patient sample testing, each clinical laboratory needs to specify its analytical performance, usually by comparing it with the published data such as manufacturer's claims. Clinical and Laboratory Standards Institute has published guidelines for method verification which were used in this study[3]. Based on CLSI EP15 A2 Guidelines for precision estimation, two levels of control samples for 2019-nCov IgM/G and SARS-Cov-2 S-RBD IgG antibodies from SNIBE diagnostic, China, were used as well as pooled human serum samples for the presence of 2019-nCov IgG. The quantifying of the antibodies was done on SNIBE Maglumi 800 and the repeatability and within-laboratory precision was done on 3 replicates measured three times a day, during five days[6]. The results from our laboratory have shown excellent precision characteristics. In fact, CV of the repeatability for negative control samples of 2019-nCov IgM/G was calculated between 3.38% and 5.26%. The manufacturer has not provided information of the repeatability CVs (%) for negative IgM/G antibodies. The estimated coefficient of variation of negative SARS-Cov-2 S-RBD IgG antibodies was 4.39% that was higher than the claimed one (1.77)[3,4]. The estimated repeatability coefficient of variation was lower than the manufacturer's claim for all positive antibodies. In regard to between-day precision for negative control samples, the manufacturer has not provided data for comparison, except for SARS-Cov-2 S-RBD IgG antibodies, which were higher than the estimated ones (1.77% and 0.28%, respectively)[4]. The estimated between-day coefficient of variation for positive control samples was lower or equal to the manufacturer's claim, except for IgM antibodies (5.7% and 1.58%, respectively).

The estimated reproducibility coefficients of variation compared to manufacturer's claim showed lower or equal value, except for positive IgM/G control samples (11.74 and 8.65, respectively)[3].

Since the results for positive control 2019-nCov IgG did not comply with the manufacturer's claimed concentration (3.92 AU/ml), the action with the manufacturer was

undertaken resulting in the explanation that the control sample for 2019-nCov IgG when used as a sample cannot give the claimed concentration results. Therefore, the pooled positive serum samples (although manufacturer states that the test is suitable for investigating single sample, not pooled samples) were prepared in order to test the manufacturer's claims. The estimated repeatability for pooled positive serum samples for 2019-nCov IgG yielded excellent performance characteristics, which were consistent with the manufacturer's claims and with those reported by other authors[7-10]. Overall data suggest that relatively stable data were provided by this measurement system since a variety of tests were authorised for emergency use by FDA and some manufacturers did not even publish precision data for their products. Regarding the samples showing discrepancies in coefficient of variation between estimated and claimed ones, further actions should be taken, such as calculation of the upper verification limits and verification interval and estimation of the total bias.

The present study has some notable limitations. For example, verification of negative and positive serum samples (except for IgG) was not performed, as well as between lot verification.

In conclusion, the results of this study have shown that Maglumi 800 CLIA may be a reliable immunoassay for assessing the immune response of SARS-Cov2 and has fulfilled the needs of the intended use.

Conflict of interest statement. None declared.

References

1. Interim Guidelines for COVID-19 Antibody Testing in Clinical and Public Health Settings. Available from: https://www.cdc.gov/coronavirus/2019-ncov/lab/resources/antibody-tests-guidelines.html. Visited on February 25th 2022.
2. Gundlapalli AV, Reynolds MS, Brooks JT, et al. SARS-Cov-2 serologic assay needs for the next phase of U.S. COVID-19 pandemic response. *Open forum Infect Dis* 2020; 8: 555.
3. International Standards Organization. Medical laboratories-Requirements for quality and competence International Standard, ISO 15189: 2012(en).
4. Clinical and Laboratory Standards Institute. User verification of performance for precision and trueness; verification of performance for precision and trueness; approved guideline-second edition. CLSI document EP15-A2. Wayne PA, USA: CLSI: 2005.
5. Iglewicz B, Hoaglin DC. How to detect and handle outliers. *ASQC Quality Press* 1994; Wis: Milwaukee; 1993.
6. Chester D. Evaluating Assay Precision. *Clin Biochem Rev* 2008; 29(1): S23-6.
7. Yokoyama R, Kurano M, Morita Y, Takuya Sh, Yuki N, Chungen Q, *et al*. Validation of a new automated chemiluminescent anti-SARS-Cov-2 IgM and IgG antibody assay system detecting both N and S proteins in Japan. *PLOS ONE* 2021; 1-16.
8. Padoan A, Cosma C, Sciacovelli L, Faggian D, Plebani M. Analytical performance of a chemiluminescence immunoassay for SARS-Cov-2 IgM/IgG and antibody kinetics. *Clin Chem Lab Med* 2020; 58(7): 1081-1088.
9. Recipients fact sheets-MAGLUMI 2019-nCov IgG/M. Available from: www.fda.gov. Visited on March 20th 2022.
10. SNIBE-Maglumi SARS-CoV-2 S-RBD IgG II (CLIA). Available from: https://hea.lthcare-in-europe.com. Visited March 18th 2022.