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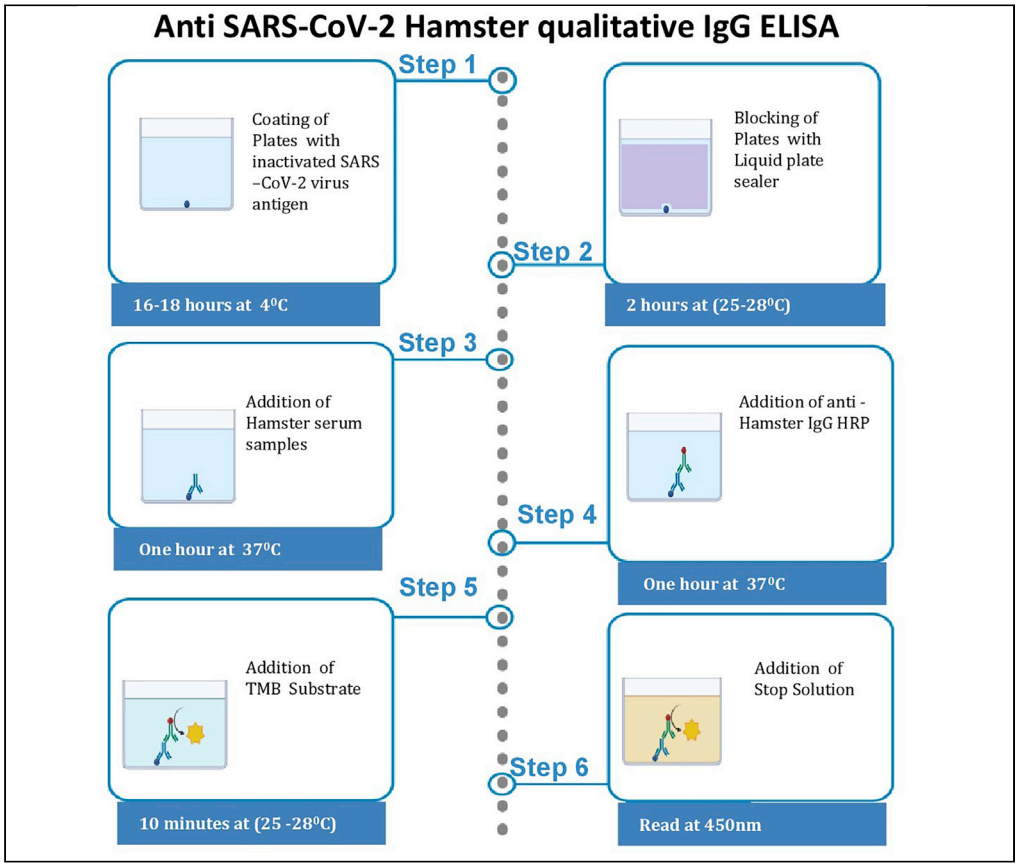
Protocol

A qualitative IgG ELISA for detection of SARS-CoV-2-specific antibodies in Syrian hamster serum samples



This protocol describes an indirect enzyme-linked immunosorbent assay for qualitative detection of IgG antibodies against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in Syrian hamster serum samples. We describe the preparation of inactivated virus antigens and the negative control antigen and the use of antigen-coated microtiter plates to detect SARS-CoV-2-specific antibodies from SARS-CoV-2-infected hamsters, including the criteria for differentiating positive versus negative reaction. The limited batch-to-batch variability of this assay has been verified with two batches of independently prepared antigens.

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Highlights

Inactivated SARS-CoV-2 whole-virion-based IgG ELISA

Qualitative detection of anti-SARS-CoV-2 IgG antibody in infected hamster serum samples

Criteria for determination of cutoff values for the assay

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A qualitative IgG ELISA for detection of SARS-CoV-2-specific antibodies in Syrian hamster serum samples

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SUMMARY

This protocol describes an indirect enzyme-linked immunosorbent assay for qualitative detection of IgG antibodies against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in Syrian hamster serum samples. We describe the preparation of inactivated virus antigens and the negative control antigen and the use of antigen-coated microtiter plates to detect SARS-CoV-2-specific antibodies from SARS-CoV-2-infected hamsters, including the criteria for differentiating positive versus negative reaction. The limited batch-to-batch variability of this assay has been verified with two batches of independently prepared antigens.

For complete details on the use and execution of this protocol, please refer to Mohandas et al. (2021).

BEFORE YOU BEGIN

Ethics statement

The present protocol was approved by Institutional Animal Ethical Committee of ICMR NIV, Pune and all the experiments involving animals were performed as per guidelines laid down by Committee for the Purpose of Control and Supervision of Experiments on animals, Government of India.

Prepare virus stock antigens, gamma irradiation, and confirm inactivation of the virus stock as described below. Prepare Solutions following the recipes mentioned in the Materials and Equipment section. A complete list of reagents and resources required is given in the Key resources table.

SARS-CoV-2 stock preparation

Timing: 5 days

1. Maintain African green monkey kidney cell line (Vero CCL-81) in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL)
2. Infect the cells with SARS-CoV-2 isolate (Sarkale et al., 2020). GISAID:EPI_ISL_420545
   a. Infect the Vero CCL-81 cells (at 80%–90% confluency, in 40 mL MEM, cultured in 225 cm^2 tissue culture flasks) with 1 mL virus isolate with Median Tissue Culture Infectious Dose ($TCID_{50}$) of $10^{6.5}$/mL. Incubate in a CO_2 incubator with 5% CO_2 at 37°C for 4 days (Reed and Muench, 1938).
   b. On day 4 post inoculation, observe for the presence of cytopathic effects (CPE; Figure 1).
   c. Freeze the whole flask in −80°C, and then thaw it and harvest the entire volume of the tissue culture fluid (TCF; the supernatant from the culture)

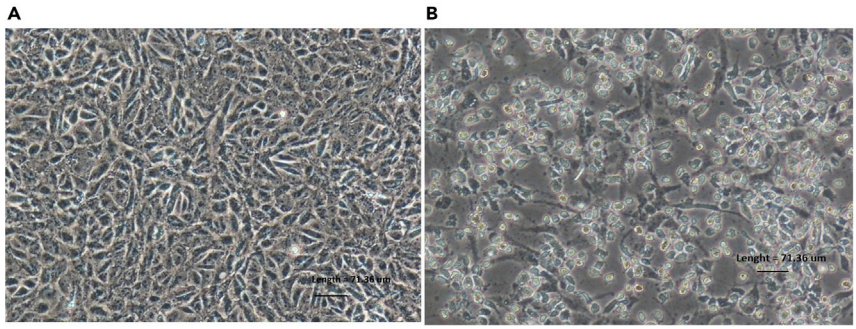

Figure 1. Cytopathic effect of the SARS-CoV-2 isolate demonstrated in Vero CCL-81 cells

(A) Cell control: No cellular changes were observed in the cell control and (B) Post infection day- 4(PID-4): Vero CCL-81 cells that were inoculated with the samples showed evidence of cell rounding. Syncytial cells formed large cell masses that increased in size and number as the infection progressed. Scale bars: 71.36 μm.

d. Centrifuge the TCF at 2490 *g* for 5 min at 4°C
e. After centrifugation, separate and store the clear supernatant at −80°C until further use. This is referred to as virus stock hereafter.
f. Proceed to Gamma irradiation of the stock as described below

3. Preparation of the negative control antigen
   a. Incubate Vero CCL-81 cells in a CO_2 incubator with 5% CO_2 at 37°C for 4 days
   b. Repeat steps 2b–2e to prepare the negative control antigen stock
   c. Proceed to Gamma irradiation of the stock as described below

Gamma irradiation

Timing: 1 day

4. Gamma inactivation of virus stock and Vero CCL-81 control supernatant.
   a. Thaw the virus or the negative control antigen stock and place it on ice in a biohazard bag in the steel container of the gamma chamber
   b. Use a Co-60 source (Gamma Chamber; GC 5000) with a total radiation dose of 8 kiloGray (kGy) for inactivation of SARS-CoV-2, which can be achieved by exposing to gamma dose rate of 6.89 kGy for 1 h 4 min

Confirmation of inactivation

Timing: 10 days

5. Inoculate 100 μL of the irradiated virus stock in Vero CCL-81 cells and observe for 5 days (P1 passage). Inoculate 100 μL supernatant of P1 passage in Vero CCL-81 cells and observe for 5 days (P2 passage) to confirm the inactivation of the virus by checking for absence of CPE.

Concentration of the antigen

Timing: 1 day

6. Add 60 mL of the inactivated antigen in sample reservoir of the Jumbosep centrifugal devices with 30K membrane insert.
7. Centrifuge for 12 min at 177 *g* at 4°C.
8. After centrifugation discard the filtrate collected in the filtrate receiver.

9. Collect approximately 35 mL of the sample retained (now referred as concentrated antigen) in the sample reservoir in a pre-chilled bottle.
10. Aliquot the concentrated antigen 1 mL/vial and store at −80°C for further use.
11. Similarly, for preparation of concentrated negative control antigen, add 60 mL the control supernatant of Vero CCL-81 cells in separate Jumbosep centrifugal devices with 30K membrane insert and repeat steps 7–10.

Note: Estimate the total protein concentration by the Lowry's method (Lowry et al., 1951).

⚠ CRITICAL: Two separate Jumbosep centrifugal devices should be used for SARS-CoV-2 and negative control antigen preparation, respectively.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-hamster IgG horseradish peroxidase	Thermo Fisher Scientific	Cat# PA1-29626
Bacterial and virus strains		
NIV-2020-770	ICMR-National Institute of Virology, Pune	"GISAID: hCoV-19/India/ 770/ 2020 |EPI_ISL_420545|" "GISAID: hCoV19/India/2020 770/ 2020|EPI_ISL_420546|"
Biological samples		
Hamster SARS-CoV-2-positive serum samples	ICMR-National Institute of Virology, Pune	N/A
Hamster SARS-CoV-2-negative serum samples	ICMR-National Institute of Virology, Pune	N/A
Chemicals, peptides, and recombinant proteins		
Minimum Essential Medium	Thermo Fisher Scientific	Cat # 11534466
Fetal bovine serum	Sigma, USA	Cat # F9665
Penicillin streptomycin	HiMedia	Cat # A018
Liquid plate sealer	CANDOR Bioscience GmbH, Germany	Cat # 160902-04
PBS	Sigma-Aldrich, USA	Cat #P4417-100TAB
Tween-20	Sigma-Aldrich, USA	Cat #P1379-500ML
StabilZyme HRP Conjugate Stabilizer	SurModics, Inc., USA	Cat # SZ02-1000
3,3′,5,5′-Tetramethylbenzidine	Clinical Sciences	Cat# 01016-1-1000
Sample diluent powder (skim milk)	Sigma-Aldrich, USA	Cat# 70166-500G
Sulfuric acid	Sigma-Aldrich, USA	Cat # 7664-93-9
Experimental models: Cell lines		
Vero CCL-81	ATCC	Cat#ATCC-CCL-81
Experimental models: Organisms/strains		
Syrian hamster 6–8-week-old female	Indian Council of Medical Research, National Institute of Nutrition, Hyderabad, India	N/A
Others		
ELISA plates	Nunc, Thermo Fisher Scientific, USA	Cat# 469949
Jumbosep Centrifugal Device Membrane Inserts 30K	Pall	Cat # OD030C65
Inverted compound microscope	Olympus	Model: CK2
Biosafety Cabinet	Thermo Scientific	Model: 1376
ELISA reader	Molecular Devices	Model: Versamax microplate reader
ELISA microplate washer	Bio-Rad	Model: 1575

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Co-60 source (Gamma Chamber)	Board of Radiation & Isotope Technology, Mumbai (BRIT)	GC 5000)
Incubator	REMI	CI-10S

MATERIALS AND EQUIPMENT

Alternatives: In this protocol, an Inverted Compound Microscope with camera attachment was used. However, any inverted compound microscope could be used to record the image.

Preparation of wash buffer (1× PBST)		
Reagent	Final concentration	Amount
PBS tablet	0.01M	One tablet in 200 mL deionized water
Tween 20	0.1%	200 µL
One tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer. Once prepared store for one week at 4°C .		

Preparation of sample diluent (5% skim milk)		
Reagent	Final concentration	Amount
Skim milk	5%	5 gm of Skim milk in 100 mL 1×PBST
Prepare freshly do not store		

Preparation of stop solution		
Reagent	Final concentration	Amount
Concentrated H_2SO_4	2M	7.142 mL
Chilled distilled water	-	250 mL

Add 7.142 mL of Concentrated H_2SO_4 in 250 mL chilled distilled water. Mix thoroughly and then store for one month at 4°C .

⚠ CRITICAL: Reagents should be used within the expiry. All the reagents should be opened in aseptic conditions. Wear protective gloves, clothing, and eye and face protection. Wash hands thoroughly after handling Sulfuric acid.

Alternatives: Conjugate diluent (Stablizyme) can be replaced by the sample diluent at step number 8.

STEP-BY-STEP METHOD DETAILS

Coating of microtiter plates

Timing: 1 day

1. Dilute the inactivated SARS-CoV-2 antigen to make a concentration of 2 µg/100 µL with 1× phosphate-buffered saline (PBS) (pH 7.2 to 7.4, 0.01 M).

2. Coat 96-well polystyrene microtiter ELISA plates with 100 µl of the diluted SARS-CoV-2 antigen per well in rows A to D and the negative control antigen in rows E to H
   a. Keep the antigen-coated plates for 16–18 h at 4°C.
3. After coating, block the wells with liquid plate sealer for two hours at room temperature (25°C–30°C). Aspirate and store the plate at 2°C–8 °C.
   a. Wash the plates three times before use with 0.01 M PBS, pH 7.2–7.4 with 0.1% Tween-20 (PBST).

⚠ CRITICAL: It is important to check the pH of the coating buffer.

Assay protocol

Timing: 2 h 30 min

4. Prepare positive and negative controls for the assay
   a. Blood collection from hamsters: Anaesthetize the hamsters prior to blood collection using isoflurane. Perform blood collection (0.25 mL) through retroorbital route. Allow the blood sample to clot at room temperature for 30 min and centrifuge the sample at 177 *g* for 10 min to separate serum. Aliquot the separated serum and store it at −20°C.
   b. Collect positive serum from hamsters infected by SARS-CoV-2 virus and negative control serum from uninfected hamsters. (Mohandas et al., 2021)
5. Dilute hamster IgG positive and negative serum samples to be tested prior to initiating the test.
   a. Dilute the hamster sera to 1:100 with the sample diluent.
6. Add 100 µl of 1:100 diluted serum samples to the coated microtiter plate and incubate at 37°C for one hour.
7. After incubation, wash the plates three times with PBST.
8. Add 100 µl of anti-hamster IgG horseradish peroxidase (HRP; 1:3000 diluted with stabilzyme) per well and incubate the plate for one hour at 37°C.

⚠ CRITICAL: It is important to confirm the dilutions of the serum samples and conjugated antibodies

9. After incubation, wash the plates three times with PBST.
10. Add 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate per well and incubate for 10 min at 25°C–28°C.

Note: Follow the exact incubation time.

11. Stop the reaction using 2M sulfuric acid.
12. Measure the absorbance values at 450 nm using an ELISA reader.
13. Determination of cut-off value for the assay is as follows.
    a. Sample is considered to be positive if optical Density (O.D) of the sample is more than the average O.D of negative control + 0.2 and OD of sample with positive antigen divided by OD of sample with negative antigen.>1.5.
    b. Sample is considered to be negative if O.D of the sample is less than the average O.D of negative control +0.2 and the ratio of OD of sample with positive antigen divided by OD of sample with negative antigen. <1.5.

EXPECTED OUTCOMES

This protocol allows qualitative detection of anti-SARS CoV-2 IgG antibodies in hamster serum samples. In our recent paper (Mohandas et al., 2021), we have applied this IgG ELISA for the detection of SARS-CoV-2 specific antibodies in Syrian hamster serum samples. Three vaccine formulations were

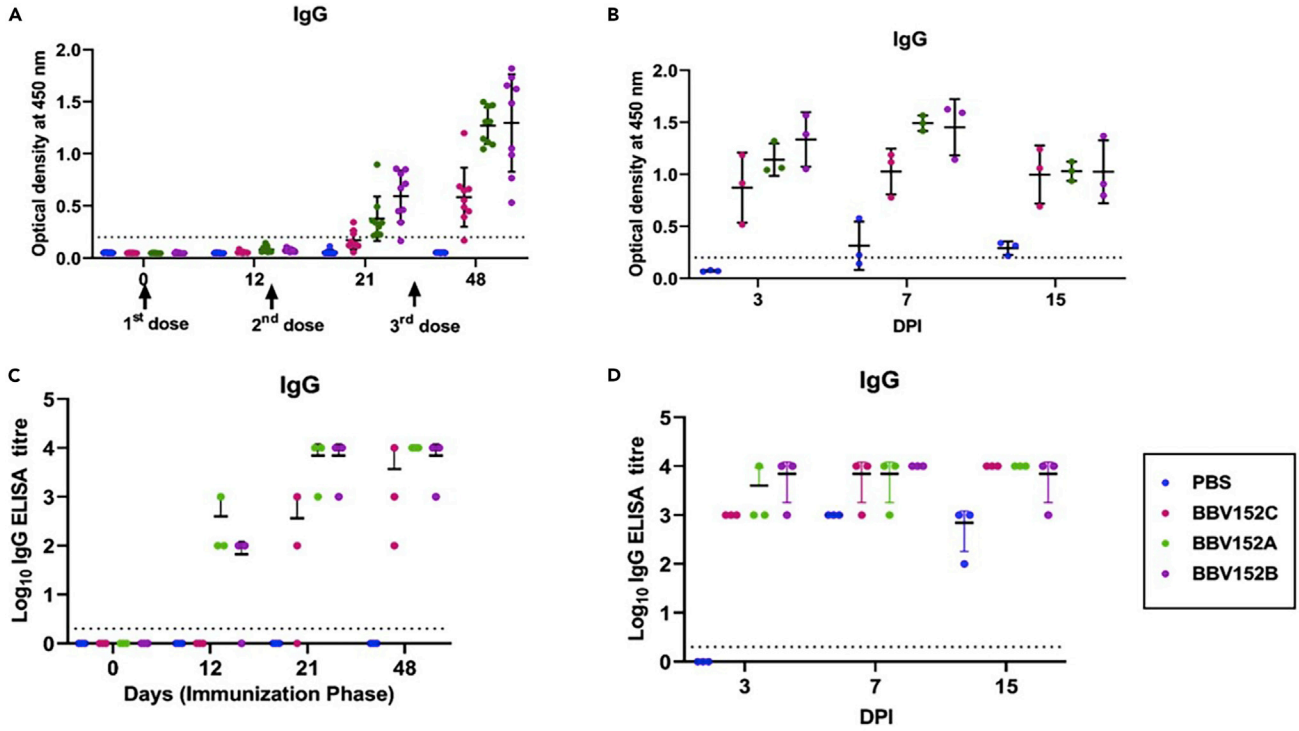

Figure 2. Humoral response in vaccinated hamsters

(A) IgG antibody response during a three-dose vaccine regime in all groups of animals observed from 12, 21 and 48 day post immunization.

(B) IgG antibody response at post-infection (3, 7 and 15 Days post infection[DPI]) for all groups of animals.

(C) Comparison of IgG antibody titers between groups post immunization.

(D) Comparison of IgG antibody titers between groups post-infection. The statistical significance was assessed using the Kruskal-Wallis test followed by the two-tailed Mann-Whitney test between the two groups; p values less than 0.05 were considered to be statistically significant.

Source: page 5 of Mohandas et al. (2021).

evaluated in this study. Serum IgG titers were also determined using this assay by testing 10-fold serial dilutions of each serum sample, starting from 1:100 dilution. Titer values were determined at the highest dilution when the optical density (OD) was more than 0.2 and positive/negative (P/N) ratio above 1.5. Robust anti SARS CoV-2 IgG antibody response was detected in this study against vaccine candidates with an increasing trend of OD of 0.84, 0.97, and 0.91 on 3, 7, and 15 days post infection, respectively (Figure 2).

Reproducibility of the assay has been checked using two different batches of the independently prepared concentrated antigens (Lot 4 vs Lot 7), and the results from the weak positive sera and the negative controls were found to be comparable between these two trials (Figure 3; Table 1).

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Average NC is calculated by taking mean OD of two or more negative controls
   a. Avg of NC = [O.D of NC 1 (0.071)+ O.D of NC 2(0.075)]/2 = 0.073
2. P/N is calculated as OD of sample with positive antigen divided by OD of sample with negative antigen.
   a. O.D of Sample with SARS CoV-2 antigen (0.364)/ O.D of sample with negative control antigen (0.081) = 4.49
3. Cut Off OD is determined as 0.2 added to average NC
   a. Cut off= Avg NC (0.073)+0.2 = 0.273
4. Sample is considered positive if both the following criteria are met:

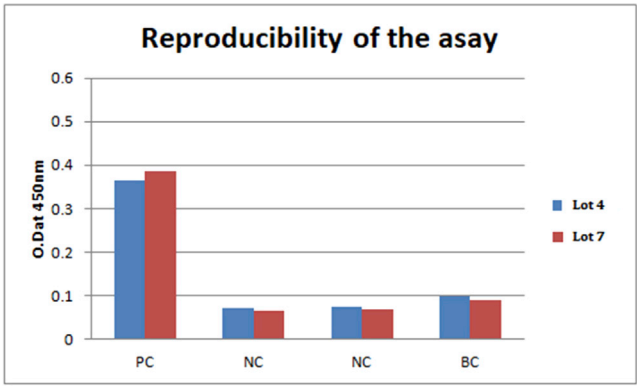

Figure 3. Reproducibility of the assay using two different lots of antigens

Weak positive hamster sera (positive control, PC; n=1) and negative control sera (NC, n=2), and one blank control (BC, n = 1) were used and assay was performed as per the protocol, using two different batches of positive antigens (Lot 4 and Lot 7).

a. OD of the sample is more than cut off
b. P/N of the sample is more than 1.5.
c. Ham-13 Day-10 is considered as positive as O.D is 0.364 which is > cut off (0.273) and P/N is 4.49 which is >1.5

LIMITATIONS

It is an indirect IgG ELISA. The assay has been validated using the limited number of SARS CoV-2 infected Syrian hamsters (Mohandas et al., 2021). The performance of the assay has not been optimized for visual determination.

We have not tested the assay with other species of hamsters. We speculate that there may not be cross reactivity of the Goat anti-hamster IgG horseradish peroxidase (HRP) to other hamsters than Syrian hamsters

TROUBLESHOOTING

Problem 1

No color development (steps 7 and 8)

Reason 1: Improper washing of the antigen coated plates before sample addition.
Reason 2: Loss of activity of the conjugate.
Reason 3: Omission of key reagents

Potential solution

Solution 1: Wash the plate three times with wash buffer before sample addition.
Solution 2: In a glass tube, add 20 μL of conjugate and 20 μL of liquid TMB substrate and check for color development.
Solution 3: Check that all reagents have been added in the correct order

Problem 2 (step 12)

Low optical density (OD) value of ''Positive control''

Reason: OD taken at incorrect wavelength.

Table 1. Verification of batch-to-batch variability

	OD (450 nm)		Sample details	
Positive antigen	0.364	0.385	Ham-13 Day-10	PC
	0.071	0.065	Ham-1 Day-3	NC1
	0.075	0.068	Ham-3 Day-3	NC2
	0.1	0.088	Blank	BC
Negative antigen	0.081	0.07	Ham-13 Day-10	PC
	0.069	0.065	Ham-1 Day-3	NC1
	0.075	0.064	Ham-3 Day-3	NC2
	0.073	0.088	Blank	BC
Plate coated date	October.7, 2020	Feb. 9, 2021		
Concentrated supernatant Lot no	4	7		
P/N	4.49	5.5		
	1.02	1		
	1	1.06		
	1.36	1		
Avg NC	0.073	0.0665		
Cut off	0.273	0.267		

ELISA plates were coated with two different batches of antigens (concentrated supernatant; lot number 4 and 7). PC: positive control (weak positive hamster sera). NC1 and NC2: negative control sera 1 and 2. BC: blank control.

Potential solution

Read OD values at 450 nm.

Problem 3

High OD values of "Negative control" (step 7).

Reason : Improper washing of the antigen coated plates before sample addition.

Potential solution

Solution 1: Follow the protocol meticulously.
Solution 2: Change micropipette tips while addition of negative and positive control.

Problem 4

High background (step 10).

Reason 1: Liquid substrate not properly protected from light.
Reason 2: Contamination of liquid Substrate
Reason 3: Insufficient washing of the plates.
Reason 4: Poor quality of water used for diluting wash buffer concentrate

Potential solution

Solution 1: Incubate the plate in dark after addition of substrate.
Solution 2: Check OD value of substrate blank.
Solution 3: Follow wash protocol meticulously
Solution 4: Glass distilled water is preferred for diluting wash buffer concentrate

Problem 5

Poor reproducibility of test (step 1 and 7).

Reason 1: Uneven coating of the plate

Reason 2: Dispensing errors
Reason 3: Improper washing

Potential solution

Solution 1: Use proper ELISA plate; check coating and blocking volumes
Solution 2: Calibrate micropipettes. Check other dispensing equipment's.
Solution 3: If an automated plate washer is used, check all the ports/manifold for uniform flow of wash buffer. If there are blockages, clean the ports

Problem 6

Uneven color development (step 7).

Reason: Improper washing of wells

Potential solution

Use an automated plate washer, if available.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr Pragya D Yadav (hellopragya22@gmail.com).

Materials availability

All the newly generated materials associated with this protocol will be available, on request to Director, ICMR-National Institute of Virology, subject to fulfillment of institutional criteria for sharing.

Data and code availability

The datasets supporting the current study have not been deposited in a public repository but are available from the corresponding author on request.

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AUTHOR CONTRIBUTIONS

P.D.Y., A.S., and S.M. designed the antigen preparation and ELISA protocol. P.D.Y. monitored the virus propagation and gamma inactivation. A.S. and R.J. performed the ELISA optimization and testing of the hamster immune sera.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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