

# An Optimised Indirect ELISA Protocol for Detection and Quantification of Anti-viral Antibodies in Human Plasma or Serum: A Case Study Using SARS-CoV-2

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## Abstract

Advanced immunoassays are crucial in assessing antibody responses, serving immune surveillance goals, characterising immunological responses to evolving viral variants, and guiding subsequent vaccination initiatives. This protocol outlines an indirect ELISA protocol to detect and quantify virus-specific antibodies in plasma or serum after exposure to viral antigens. The assay enables the measurement of IgG, IgA, and IgM antibodies specific to the virus of interest, providing qualitative and quantitative optical densities and concentration data. Although this protocol refers to SARS-CoV-2, its methodology is versatile and can be modified to assess antibody responses for various viral infections and to evaluate vaccine trial outcomes.

## Key features

- This protocol builds upon previously described methodology [1] explicitly tailored for SARS-CoV-2 and broadens its applicability to other viral infections.
- The protocol outlines establishing antibody responses to SARS-CoV-2 infections by determining optical densities and concentrations from blood plasma or serum.

Keywords: ELISA, SARS-CoV-2, Viral infections, Optical densities, Antibody concentrations

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### **Graphical overview**



**Summary of the conventional ELISA (A) vs. sensitive ELISA (B) procedures.** In both A and B, wells are coated with a capture antigen, such as the spike protein, while in (C) they are coated with human Kappa and Lambda capture antibodies. For the conventional ELISA (A), wells with immobilised capture antigens receive serum/plasma containing the target antibody (A1 and B1). This is followed by an HRP-conjugated detection antibody specific to the captured antibody (A2 and B2) and then a substrate solution that reacts with the HRP, producing a colour proportional to the concentration of the antibody in the serum/plasma (A3 and B3). The reaction is halted, and absorbance is measured. In the sensitive ELISA (B), after the serum/plasma addition (A1 and B1), a Biotin-conjugated primary detection antibody is introduced (A2 and B2). Depending on the target antibody, a secondary streptavidin-HRP conjugated detection antibody is added for IgG or IgM (3a) or a poly-HRP 40 detection antibody for IgA (3b). A substrate is introduced, producing a colour change proportional to the antibody concentration (A4

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and B4). The reaction is then stopped, and absorbance is measured. In Panel C, wells are coated with human Kappa and Lambda capture antibodies. Serial dilutions of a known antibody standard are introduced. After undergoing the standard ELISA steps, a detection antibody is added, specifically binding to the Ig standard antibody. Subsequently, a substrate solution causes a colour change proportional to the antibody concentration in the serum/plasma. The reaction is halted, and the absorbance of each well is measured. The resulting optical densities from the coated wells form the standard curve, plotting the absorbance against concentrations.

### Background

The indirect enzyme-linked immunosorbent assay (ELISA) effectively quantifies and assesses antibody responses, making it invaluable for monitoring public health, evaluating vaccination programs, and testing new vaccines. This has particularly been of importance at the height of the COVID-19 pandemic, during which there was continuous emergence of antigenically distinct variants [2]. It was important to track the dynamics of antigen-specific immune responses to detect immune escape in various populations. Despite similar rates of seropositivity being seen among symptomatic and asymptomatic individuals [3], certain scenarios revealed some differences [4]. A less robust humoral response has been connected to mild COVID-19 cases, prompting concerns about a potentially accelerated decline in immunity. Conversely, severe illness has been linked to a prolonged presence of humoral immunity, enduring for up to 12 months after infection [5, 6]. It is essential to understand the evolution of antibody development within sub-Saharan African (SSA) environments, characterised by distinct disease patterns. Monitoring shifts in IgM, IgG, and IgA levels targeting S, receptor binding domain (RBD), and N antigens becomes crucial in SSA, offering insights into diagnostic approaches, public health strategies, and the immunological aspects relevant to vaccine development. We optimised and validated an in-house indirect ELISA to detect and quantify SARS-CoV-2 spike-, nucleoprotein-, and RBD-directed IgG, IgM, and IgA antibodies in a Ugandan cohort largely characterised by mild and asymptomatic COVID-19. For positive controls, 106 specimens were collected from PCR-confirmed positive study participants; 205 pre-pandemic specimens obtained between October 2012 and November 2017 were used as negative control samples to determine optimal cutoff values, maximizing assay sensitivity and prioritizing assay specificity [1, 7]. Consequently, the determined cutoff values are useful in contexts where mild and asymptomatic SARS-CoV-2 infections are the predominant clinical picture. Commercial standards of known antibody concentrations were used to establish assay limits of detection (LOD) and quantitation (LOQ). Therefore, we present parameters that can be followed to determine optical densities and antibody concentrations in different populations and in regions that had different clinical outcomes and suggest that the same can be done in investigations of antibody responses to other infectious diseases and vaccinations, for example in clinical trials of various vaccine candidates. The COVAC self-amplifying RNA vaccine trial in Uganda [8] has demonstrated the successful application of this protocol.

### Materials and reagents

#### **Biological materials**

Human serum or plasma samples freshly obtained from study participants, heat inactivated at 56 °C for 30 min and centrifuged at  $1,000 \times g$  for 10 min at 18–25 °C. One should expect approximately 1.8 mL of serum/plasma after centrifugation, with a pellet at the bottom and the supernatant at the top in each tube. The supernatant should be collected and aliquoted into different tubes stored at -80 °C. Once an aliquot is picked and thawed, it should be kept in a 2–8 °C fridge and used within only one week.

#### Reagents

- 1. Recombinant SARS-CoV-2 Nucleocapsid protein (R&D Systems, catalog number: 10474-CV-01M)
- 2. Recombinant SARS-CoV-2 Spike protein (R&D Systems, catalog number: 10549-CV-01M)

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## bio-protocol

- 3. Recombinant SARS-CoV-2 Spike S2 Subunit protein (R&D Systems, catalog number: 10594-CV-100)
- SARS-CoV-2 RBD (S1) S-protein obtained from a pHL-sec expression vector in HEK 293T/17 cells [obtained from the American Type Culture Collection (ATCC), catalogue number: CRL-11268]. pHL-sec expression vector was a kind donation from the Doores lab at King's College London
- 5. Goat anti-human Kappa-UNLB antibody (Southern Biotech, catalog number: 2060-01)
- 6. Goat anti-human Lambda-UNLB antibody (Southern Biotech, catalog number: 2070-01)
- 7. Purified human IgG (Sigma, catalog number: I2511)
- 8. Purified human IgA (Sigma, catalog number: I2636)
- 9. Purified human IgM (Sigma, catalog number: I8260)
- 10. Goat anti-human IgG-HRP (Sigma, catalog number: A0170 or AP113P)
- 11. Anti-human IgM-HRP (Sigma, catalog number: A6907)
- 12. Anti-human IgA-HRP (Sigma, catalog number: A0295)
- 13. Goat anti-human Fc IgG-Biotin conjugate (Southern Biotech, catalog number: 2048-08)
- 14. Goat anti-human IgA-Biotin (Southern Biotech, catalog number: 2050-08)
- 15. Goat anti-human IgM-Biotin (Southern Biotech, catalog number: 2020-08)
- 16. PBS/Tween Sachets (PBST) (Sigma, catalog number: P3563)
- 17. Tween-20 (Sigma, catalog number: P2287)
- 18. 1× Dulbecco's phosphate buffered saline (DPBS), CaCl<sub>2</sub> (-) MgCl<sub>2</sub> (-) (Sigma, catalog number: D8537)
- 19. Bovine serum albumin (BSA) (Sigma, catalog number: A3803)
- 20. Goat serum (Sigma, catalog number: G9023)
- 21. Streptavidin-HRP (R&D Systems, catalog number: DY988)
- 22. Tetramethyl benzidine (TMB) substrate (SeraCare, catalog number: 5120-0080 or 52-00-02)
- 23. TMB stop solution (SeraCare, catalog number, 5150-0021 or 50-85-06)
- 24. Casein blocking buffer (Thermo Scientific, catalog number: 37582)
- 25. Poly-HRP-40 (Fitzgerald, catalog number: 65R-S104PHRP)

#### Solutions

- 1. PBST wash buffer (see Recipes)
- 2. BSA assay buffer/sample diluent/blocking buffer (see Recipes)
- 3. Goat serum assay buffer/sample diluent (see Recipes)

#### Recipes

#### 1. Wash buffer

Reagent	Final concentration (%)	Quantity or Volume
1× DPBS pH 7.4	99.95%	999.5 mL
Tween-20	0.05%	0.5 mL
Total	100%	1,000 mL

Store in a capped bottle at room temperature up to seven days.

#### 2. BSA assay buffer/sample diluent/blocking buffer

Reagent	Final concentration (%)	Quantity or Volume
1× DPBS pH 7.4	99.95%	499.75 mL
Tween-20	0.05%	0.25 mL
BSA	1%	5 g
Total	100%	500 mL

Filter the buffer through a 0.22 µm filter unit. Store at 2–8 °C up to seven days.

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3. Goat serum assay buffer/sample diluent

	P	
Reagent	Final concentration (%)	Quantity or Volume
1× DPBS pH 7.4	94%	470 mL
Tween-20	1%	5 mL
Goat serum	5%	25 mL
Total	100%	500 mL

Filter the buffer through a 0.22  $\mu$ m filter unit. Store at 2–8 °C up to seven days.

#### Laboratory supplies

- 1. U-bottom, 96-well dilution plates (Corning, catalog number: 3690)
- 2. Medium binding, flat-well ELISA plates (Greiner Bio-One, catalog number: 655001)
- 3. Sealing tape (Thermo Fisher Scientific, catalog number: 15036)
- 4. Reservoirs (CoStar, catalog number: 4870)
- 5. Pipettes (P2, P5, P20, P100, P200, P300-Multichannel, P1000) (at the discretion of the laboratory)
- 6. Pipette tips (10, 20, and 200  $\mu$ L) (at the discretion of the laboratory)
- 7. 50 mL centrifuge tubes (Sigma, catalog number: T1943)
- 8. Paper towel rolls (at the discretion of the laboratory)
- 9. 2 mL cryovials (Sigma, catalog number: BR114832-1000EA)
- 10. 0.22 µm syringe filters (TISCH Scientific, catalog number: SPEC16251)

## Equipment

- 1. -20 °C or -80 °C freezer (at the discretion of the laboratory)
- 2. Benchtop centrifuge (at the discretion of the laboratory)
- 3. Plate washer (BioTek Washer, ELx405 UCWVSD)
- 4. Plate reader (BioTek Reader, Elx808IU)

### Software and datasets

- Plate reader software: Gen5<sup>TM</sup> Microplate Data Collection and Analysis Software (v3.12, 11/23/2021); license CD purchased through BioTek or provided along with the reader
- Spreadsheet software: Microsoft Office Excel v16.0, 2016 or later (09/22/2015); requires Microsoft Office license.
- 3. Data analysis software: GraphPad Prism v9.3 (GraphPad, 11/15/2021); license obtained through GraphPad
- 4. Software programming language; R Version 4.3.1 (21/04/2023); free to use

## Procedure

The conventional ELISA is effective for detecting and quantifying antibodies in plasma or serum after substantial antigenic exposure such as after natural infection. Conventional ELISA finds its application in scenarios where the target molecule is anticipated to exist in comparatively higher concentrations. On the other hand, sensitive ELISA proves advantageous in early detection of immune responses or diseases, especially when the levels of specific antibodies or antigens are exceedingly low. When low antibody levels are observed in clinical trials, the sensitive version of the ELISA assay is employed, incorporating the addition of a biotin–streptavidin detection step that is particularly useful for amplifying and detecting background responses to antigens that the conventional ELISA

might not discern. Serum should be obtained from blood collected in serum separating tubes (SST) and plasma from blood collected in acid citric dextrose (ACD) tubes to ensure high quality samples that are free of contaminants.

#### A. Preparation of serum or plasma samples and reagents

Serum or plasma samples should be heat inactivated for 30 min at 56 °C and centrifuged at 1,000× g for 10 min at 18–25 °C to harvest the supernatant before testing. If the serum or plasma samples cannot be processed immediately, they should be aliquoted and stored at -80 °C to ensure structural integrity and long-term stability of the antibodies.

**Critical:** The serum/plasma is heat-inactivated to ensure the safety of the operators since this assay is run on the bench and not under a biosafety cabinet. This kills any potential pathogens present in the serum/plasma.

- 2. Unless stated otherwise, all procedures are performed at room temperature (20–25 °C) for the conventional ELISA and at 37 °C for the sensitive ELISA, as described later.
- 3. A plate plan should be drawn to record the position of samples, controls, and standards in each ELISA plate. An example is illustrated in Figure 1.
- Prepare wash buffer as described in Recipe 1 or by dissolving one sachet of PBS/TWEEN in 1 L of distilled water.
- 5. Prepare BSA assay buffer for the conventional ELISA or goat serum assay buffer for the sensitive ELISA as described in Recipes 2 and 3, respectively.
- 6. All prepared and aliquoted reagents, i.e., all standards and detection antibodies, should be kept in a -20 °C freezer. Once an aliquot is picked and thawed, it should be kept in a fridge and used within only one week. It should be clearly labelled with the date of first opened/prepared.
- 7. It is crucial to utilise reagents that are new and still within their specified expiration date.

#### **B.** Coating of medium-binding ELISA plates

**Caution:** Inconsistent pipetting may undermine accuracy in processes such as sample dilution, bubble formation, or protocol deviations. Ensure proper pipetting techniques during the assay, e.g., controlled up and down pipetting to minimise bubbles. Refer to Problem 2 under the Troubleshooting section.

- Coat wells A1–H2 (see Figure 1) of the 96-well medium-binding ELISA plate by adding 50 μL per well
  of a solution containing both anti-Human kappa and anti-Human lambda capture antibodies diluted in
  DPBS, at a final dilution of 1:500 at a 1:1 ratio of anti-Human kappa and anti-Human lambda capture
  antibodies as shown in Table 1. The coated wells will contain the purified antibodies for the standard curve.
- 2. Coat the rest of the wells of the 96-well medium-binding ELISA plate by adding 50 μL of antigen [recombinant SARS-CoV-2 Nucleocapsid protein, recombinant SARS-CoV-2 Spike protein, recombinant SARS-CoV-2 Spike S2 Subunit protein, or SARS-CoV-2 RBD (S1) S-protein] prediluted in DPBS to a working concentration of 3 μg/mL, with a separate plate for each protein. The predilution parameters for the antigen depend on the concentration of the stock solution, which could vary from lot to lot. The demonstrated dilution in Table 2 uses 500 μg/mL as the stock concentration.

Note: Medium-binding plates are more cost effective and suitable for this protocol where non-specific binding might interfere with the assay. They are generally easier to wash and are less likely to retain residual proteins. While they offer strong binding, high-binding plates may be more challenging to wash thoroughly due to their higher binding capacity.

 Cover the plates with sealing tape and incubate for 2 h at 37 °C in a humidified incubator or overnight at 4 °C.

Note: During the 2 h incubation time, proceed to section C.

**Caution:** Do not rock the plates during incubation because rocking can interrupt the formation of bonds between the coating antigen/antibody and the surface of the plate.

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4. After the incubation, wash the ELISA plates with five rounds of 200 μL per well of washing buffer using the plate washer, flick the plates over a container to rid it of excess buffer, and tap gently on paper towel to dry the plate. Then, proceed to section D.

Note: When using the plate washer, set up a protocol by entering the volume of washing buffer required for each well and the number of times each well should be washed on each ELISA plate. Place the plate on the plate holder and run the protocol. If an automated washer is unavailable,  $5 \times$  of manual washing can be done using a multi-channel pipette set at 200  $\mu$ L.

#### Table 1. Predilution of anti-human Lambda and Kappa capture antibodies

Anti-Human Kappa (µL)	Anti-Human Lambda (µL)	DPBS (µL)	Total (µL)
3	3	1494	1500

Note: This 1:500 dilution is for one plate. Multiply volumes by the number of plates in the assay.

#### Table 2. Predilution of SARS-CoV-2 coating antigens

Capture antigen (stock concentration, ug/mL)	Working concentration (ug/mL)	Volume of antigen (uL)	DPBS (µL)	Total (µL)
Spike/Nucleoprotein/RBD	3	36	5,964	6,000
(500 µg/mL)				

Note: This dilution is for one plate. Multiply volumes by the number of plates in the assay.

#### C. Preparation of serum/plasma and control samples

- 1. Sample preparation should be performed on the day of the assay (diluted samples should not be stored).
- 2. Dilute samples in U-bottom dilution plates using BSA assay buffer for the conventional ELISA or goat serum assay buffer for the sensitive ELISA.
- 3. For a 1:100 dilution, add 198  $\mu$ L of assay buffer to the wells of the dilution plate followed by 2  $\mu$ L of test sample in duplicate wells following the plate plan in Figure 1.
- For a 1:50 dilution, add 196 μL of assay buffer to the wells of the dilution plate followed by 4 μL of test sample in duplicate wells following the plate plan in Figure 1.
- 5. Prepare positive and negative control samples at the same dilution used for the test samples; if commercial, prepare according to the manufacturers' recommendations. Positive and negative controls should be prepared using assay buffer in 2 mL cryovials.

Note: For each well of the ELISA plate,  $50 \ \mu L$  of samples or controls will be required. Therefore, the number of wells for each control and number of plates in the experiment should be considered in preparation of the required volume of positive or negative controls.

#### D. Blocking of medium-binding ELISA plates

- Add 200 μL of BSA assay buffer (conventional ELISA) or goat serum assay buffer (sensitive ELISA) to all the wells in each ELISA plate. Seal the plates and incubate for 1 h at room temperature (conventional ELISA) or at 37 °C (sensitive ELISA).
- 2. (Critical) Proceed to section E when there are 20 minutes left to the end of the blocking incubation period.
- 3. After incubation, wash the plates five times with 200  $\mu$ L of wash buffer per well.

#### **E.** Preparation of standards

Use purified human IgG, IgA, or IgM to prepare seven serial dilutions of antibody standards in 2 mL cryovials, as shown in Table 3, Table 4, and Table 5. If more than one plate is being run at a particular time, it is more efficient to prepare the standards in cryovials and then pipette them into their respective ELISA plate wells. Using cryovials also ensures proper mixing as one performs the serial dilutions.

Note: If the stock concentrations of the purified antibodies differ from those provided in the tables, the initial dilutions will have to be adjusted to achieve a starting concentration of 1,000 ng/mL.

The IgG and IgA serial dilutions are 10-fold, while the IgM dilution is 5-fold. These parameters can be 2. adjusted to suit particular experimental conditions.

Table 3. IgG standard serial dilution in assay buffer. Adjust the volumes depending on the plate plan and the number of plates.

Prepare a 1:100 dilution of human IgG in the appropriate buffer prior to preparing the serial dilutions							
	Dilution	Stock (mg/mL)	Stock (µL)	Assay buffe	r (µL)	Total (µL)	
Human IgG	1:100	4.52	2	198		200	
Serial dilution	of human I	gG following the in	itial 1:100 dilut	ion			
Concentration	(ng/mL)	IgG Std (µL)	Assay	Total (µL)	Take over (µL)	Left over	
			buffer (µL)			(µL)	
Std curve	1,000	20	884	904	90	814	
human IgG	100	90	810	900	90	810	
(10-fold	10	90	810	900	90	810	
dilution)	1	90	810	900	90	810	
	0.1	90	810	900	90	810	
	0.01	90	810	900	90	810	
	0.001	90	810	900	-	900	

Initial 1:100 dilution of human IgG

Table 4. IgA stan	dard serial di	lution in assay	buffer.	Adjust the	volumes	depending o	n the plate	plan and
the number of plate	es.							

#### Prepare a 1:100 dilution of human IgA in the appropriate buffer prior to preparing the serial dilutions Dilution Assay buffer (µL) Total (µL) Stock Stock (µL) (mg/mL) Human IgA 1:100 2.0 2 198 200 Serial dilution of human IgA following the initial 1:100 dilution Concentration(ng/mL) Total (µL) Take Left over (µL) IgA Std (µL) Assav over buffer (µL) (µL) Std curve 1,000 45 855 900 90 810 human IgA 100 90 810 900 90 810 10 90 900 (10-fold 810 90 810 90 900 90 dilution) 810 810 1 90 90 0.1 810 900 810 0.01 90 810 900 90 810 0.001 90 810 900 900

Initial 1:100 dilution of human IgA

Table 5. IgM standard serial dilution in assay buffer. Adjust the volumes depending on the plate plan and the number of plates.

Initial 1:100 dilution of human IgM

Prepare a 1:100 dilution of human IgM in the appropriate buffer prior to preparing the serial dilutions									
	Dilution	Stock (mg/mL)	Stock (µ	(μL) Assay buffer (μL)			Total (µL)		
Human IgM	1:100	1.0	2		198			200	
Serial dilution	of human	IgM following the	initial 1:1	00 dilutio	on				
Concentration	(ng/mL)	IgM Std (µL)	Assay	buffer	Total	Take	over	Left	over
			(µL)		(µL)	(µL)		(µL)	
Std curve	1,000	90	810		900	180		720	
human IgM	200	180	720		900	180		720	
(5-fold	40	180	720		900	180		720	
dilution)	8	180	720		900	180		720	

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1.6	180	720	900	180	720
0.32	180	720	900	180	720
0.006	180	720	900	-	900

#### F. Addition of samples, controls, and standards

Add 50 μL of sample/standard/control to the appropriate wells on the plate following the plate plan. Seal plates and incubate for 2 h at room temperature (conventional assay) or for 1 h at 37 °C (sensitive assay). A suggested plate plan for the arrangement of samples, controls, and standards is illustrated in Figure 1.

	Human Standar Curve n	∙d ig/mL	Samples									
	1	2	3	4	5	6	7	8	9	10	11	12
Α	1,000	1,000	SPL1	SPL1	SPL2	SPL2	SPL3	SPL3	SPL4	SPL4	- Control 1	- Control 1
В	100	100	SPL5	SPL5	SPL6	SPL6	SPL7	SPL7	SPL8	SPL8	- Control 3	- Control 3
С	10	10	SPL9	SPL9	SPL10	SPL10	SPL11	SPL11	SPL12	SPL12	+ Control 4	+ Control 4
D	1	1	SPL13	SPL13	SPL14	SPL14	SPL15	SPL15	SPL16	SPL16	-Control 3	-Control 3
Е	0.1	0.1	SPL17	SPL17	SPL18	SPL18	SPL19	SPL19	SPL20	SPL20	+ Control 4	+ Control 4
F	0.01	0.01	SPL21	SPL21	SPL22	SPL22	SPL23	SPL23	SPL24	SPL24	- Control 1	- Control 1
G	0.001	0.001	SPL25	SPL25	SPL26	SPL26	SPL27	SPL27	SPL28	SPL28	+ Control 2	+ Control 2
Η	Blank	Blank	SPL29	SPL29	SPL30	SPL30	SPL31	SPL31	SPL32	SPL32	Blank	Bank

**Figure 1. Representative plate plan to arrange standards, samples, and controls.** "SPL" represents a sample, "control" represents a negative control, and "+ control" represents a positive control.

2. Following incubation, wash the plates five times with 200 µL of wash buffer per well.

#### Notes:

- a Some of the controls (control 1, 3, and 4) are run in more than duplicates as a way of testing operators as they run the assay. Running controls at different positions can show if there is any cross contamination or inconsistency as an operator pipettes the controls into their respective wells.
- b. You can also choose to have different types of controls, where a plate can have two positive controls; one that is an in-house control and another that is a commercially prepared positive control, e.g., WHO seropositive serum.
- c. In-house controls calibrate the assay specifically for the laboratory's equipment, reagents, and techniques, while commercial controls act as a standardised reference point that can be used across different laboratories, making results comparable between different lab settings and experiments.

#### G. Addition of secondary antibody (detection antibody)

- 1. (Critical) The secondary antibody should be prepared 30 min before use. After adding the detection antibody, proceed to section H.
- 2. For the conventional assay:
  - a. For IgG plates, dilute goat α-human IgG-HRP detection antibody 1: 10,000 in assay buffer.
  - b. For **IgM** plates, dilute human anti-IgM-HRP detection antibody 1:1,000 in assay buffer for the Spike protein and 1:5,000 for the N-protein.
  - c. For IgA plates, dilute anti-human-IgA-HRP detection antibody 1:1,000 in assay buffer.
  - d Add 50 μL of the particular detection antibody per well. Seal plates and incubate for 1 h at room temperature. Following incubation, wash the plates five times with PBST using 200 μL per well.
- 3. For the sensitive assay:
  - a Add 50  $\mu$ L of the primary detection antibody, prepared as described below per well. Seal plates and incubate for 1 h at 37 °C.

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i. For **IgG** plates, dilute goat  $\alpha$ -human IgG-Biotin detection antibody 1: 15,000 in goat assay buffer. ii. For **IgM** plates, dilute goat anti-human IgM-Biotin detection antibody 1:20,000 in goat assay buffer. iii. For **IgA** plates, dilute  $\alpha$ -human IgA-Biotin detection antibody 1:20,000 in casein buffer.

b. Following incubation, wash the plates five times with PBST using 200 μL per well. Add 50 μL of the secondary detection antibody prepared as described below per well. Seal plates and incubate for 1 h at 37 °C.

i. For IgA plates, dilute Poly-HRP40 1:20,000 in casein buffer.

Note: For IgA plates, seal plates and incubate at 37 °C in the dark for 40 min.

iii. For IgG and IgM plates, dilute streptavidin-HRP 1:200 in goat assay buffer.

c. Following incubation, wash the plates five times with PBST using 200 µL per well.

#### H. Setting up the plate reader and Gen5 software

- 1. Switch on the reader (Elx808). The reader will perform a system self-test.
- 2. Switch on the PC connected to the reader and double-click the Gen5 3.12 Task Manager icon to open. It will show a window as seen in Figure 2.
- 3. Create a protocol by clicking on *Protocols* > *Create new* as highlighted in Figure 2.

Task Manager		
	Create New	
Read Now	彦 Open	
-	Recent	
<u>=</u>	15-Mar-23_SCREENING_CN54.prt	
Experiments	15-Mar-23_SCREENING_AV.prt	
	20230904_SCREENING -AV.prt	
	20230808_SCREENING SPIKE _IgAprt	
-	20230808_SCREENING N_IgM.prt	
Protocols	20230808 SCREENING S PROTEIN_IgM.prt	
_	20230712 SCREENING N IgG.prt	
	20230719 SCREENING S IgG.prt	
Instrument Cantral	Cosmas and Deborah's Disturbance.prt	
Instrument Control	Cosmas and Deborah's Disturbance.prt	
Setup	FAQ: Protocol vs. Experiment	
	E	kit Gen 5

Figure 2. Open Task Manager window on the Gen5 3.12 software

4. Check Standard protocol and then click OK. The window shown in Figure 3 will be displayed.

🔮 Gen5 3.08 - Protocol1*
File Protocol Take3 Window System Help
E 4 H ] 3 9 H C ] 0 4 ]
File Procedure Create or edit the list of steps to be executed by the reader. Data Reduction Report/Export Builders



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- 5. Click on the icon for creating or editing the list of steps as shown in Figure 3.
- 6. After that, the window shown in Figure 4 will appear. Select the plate type, e.g., 96-well plate. Under *Select* wells, check *At runtime* and indicate if the plate you are reading will have a lid or not.

👌 Procedure - ELx808 (Com3)					×
Select steps Actions	Plate Type:	96 WELL PLATE		✓ Use lid	
Read	Select wells:	O Per step	At runtime		
Shake	Description			Comments	
Kinetic					
Start Kinetic					
Monitor Well					
Pause					
Delay					
Plate Out/In					
Stop/Resume					
Other					
Comment					
Options					
	Validate		ОК	Cancel Help	

#### Figure 4. Procedure layout on the Gen5 software

7. Click on Read, check the desired wavelength (i.e., 450 nm) as shown in Figure 5, and click Ok.

Read Step		Name Trans	818,117		- 11mm	×
Step Label: Detection Method: Read Type: Read Speed:	<default> Absorbance Endpoint Normal</default>	•				Full Plate
Wavelengths		2 🔘 3				
				OK	Cancel	Help

Figure 5. Window where you can enter the desired wavelength for reading the ELISA plate

8. The selected wavelength will be displayed as seen in Figure 6. Click OK after this.

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Deservices El. (000 (Carrol)				<b>—</b> — <b>X</b> —
Procedure - ELX808 (Com8)				
Select steps	Plate Type:	96 WELL PLATE		▼ 🔽 Use lid
Actions		Cuvette		
Read	Select wells:	Per step	At runtime	
Shake	Description			Comments
Kinetic	<b>W</b> Read: (A) 450			
Start Kinetic				
Monitor Well				
Pause				
Delay				
Plate Out/In				
Stop/Resume				
Other				
Comment				
Options				
	Validate		ОК	Cancel Help

Figure 6. Selected wavelength for reading the ELISA plates

9. Double-click *Plate layout* on the tool builder as shown in Figure 7.



Figure 7. Plate Layout step on the Gen 5 software

10. Select the appropriate well types as shown in Figure 8 and click *Next*. Be critical to include the number of assay controls you want in your plate. In this protocol, there are four assay controls used. Click *Next* after this selection.

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## bio-protocol

d pla - la	
used for background signal subtraction	
✓ Assay Controls	
Negative, Positive, Calibrators, or serially dilu	uted controls
Used for assay validation, cut-off analysis, normalizatio curve comparison).	on, or to generate reference curves (toxicology,
Number of different cor	ntrol types: 4
Standard Curves	
Required to generate standard curves and calculate un	nknown concentrations.
Use multiple Standard Curves:	2 🗸
✓ Samples	
Test wells requiring data analysis (calculation of concer	ntration, EC50,)
Sample Controls	
SPLC1 associated with Sample 1, SPLC2 associa	ated with Sample 2,
Can be used as individual sample blanks, spikes	

#### Figure 8. Selection of different well types according to the ELISA plate plan (i.e., Figure 1)

11. Update the blank as appropriate. Enter full name of BLK as BLANK, number of times the blank is repeated (e.g., 2), and the colour code of choice as shown in Figure 9. Click *Next* after entering this information.

III Plate Layout Wiz	ard				×
Blank					
Please define the sett	ings of Blank.				
Plate Layout ID:	BLK BLK BER	Full Name:	BLANK Blank, Buffer		]
Replicates:	2		bany barrer		
Colors					
Text color:	<b>—</b> ~				
Background Color:	~				
_			< Back	Next >	Cancel
Do not show wizard	d again				

#### Figure 9. Information to be entered for the blank wells in the plate layout window

- 12. Repeat step 11 for the assay controls and samples.
- 13. Update the standards as appropriate. As shown in Figure 10, enter full name of STD as the antibody being tested (e.g., IgG), number of times the blank is repeated (e.g., 2), and the colour code of choice. Enter the unit for the standards (e.g., ng) and the dilution factor (e.g., 10) for IgG. Enter the starting concentration

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in STD1 and click in the next boxes to automatically fill in the rest of the standards. Click *Next* after entering this information.

🔳 Pla	te Layout W	lizard	×
Star	idard Ci	ırve #1	
Please	define the se	ttings of Standard Curve #1.	
Plate La	ayout ID:	STD	Full Name: IgG
		STD, REF	Standards, reference curve
Replica	tes:	2	
Conc	Dil. values	Colors	
	Define dilutio	ns/concentrations	
	Type:	Concentrations v	Unit: ng
	STD1	1000	Auto
	STD2	100	Increment:
	STD3	10	
	STD4	1	Factor:
	STD5	0.1	
	STD6	0.01	Ratio:
	STD7	0.001	
	STD8		✓ Clear list
	CTDO		
			< Back Next > Cancel
Do r	not show wize	ard again	

### Figure 10. Information to be entered for the standard wells in the plate layout window

14. Click Ok and the plate layout will be displayed as shown in Figure 11.





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- 15. Allocate the different sample type onto the plate by highlighting the sample type (e.g., BLK for blank) and selecting the appropriate orientation.
- 16. Using the pointer, drag across the required wells for that sample type in the plate layout. Alternatively, click the first well in the series to automatically fill the adjacent wells with the required number of samples for that type in the selected orientation. The plate layout will appear as shown in Figure 12.



Figure 12. Plate layout window, accurately showing the positions of the different well types of the ELISA plate

- 17. After this plate layout has been correctly filled, click OK.
- 18. Double-click *Data reduction* on the tool builder shown in Figure 7. The screen as shown in Figure 13 will be displayed.

ta Reduction Tools	Description	Data Out	Comments
Transformations	Blank Transformation	Blank 450	Blank Subtraction
Blank			
Normalize			
Ratio			
Delta			
Custom			
Data Reduction Variable			
Curve Analysis			
Standard Curve			
Custom - PLA			
🗆 Qualitative Analysis / QC			
Replicate Rejection			
Cutoff			
Validation			
ZPrime			

Figure 13. Data reduction tools in the Gen 5 software protocol

19. Go to Standard curve (Data In) and set the y-axis to 450. Go to Curve Fit and set it to non-linear regression

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(Figure 14).

ank Standard Curve			X
lorm:			
Ratio Curve Name: StdO	urve		
Data In Curve Fit	Data Out		
Custo			
Generate Curve	e(s) from Current Plate		
Well ID:	STD 🗸 Standard Curve		
Custo X Axis Data:	<concentrations dilutions=""></concentrations>	~	
litat Y Axis Data:	450	$\sim$	
Cutoff Ouse Curve from	Calibrator Plate		
/alida Curve Name:		$\sim$	
Prim			
		OK	Cancel Help
	Read and the second second second	Pieck 450	The Labor
nsformations	Blank Transformation	Blank 450	Blank Subtractio
Insformations Iank Standard Curve	Fill Blank Transformation	Blank 450	Blank Subtraction
Insformations Iank Standard Curve Iorma Curve Name: StdCo	urve	Blank 450	Blank Subtractio
Insformations Iank Standard Curve Iormatio Curve Name: StdCurve Ielta Data In Curve Fit	Blank Transformation	Blank 450	Blank Subtractio
Asformations lank Standard Curve form atio Curve Name: StdCi Data In Curve Fit usto	Data Out	Blank 450	Blank Subtractio
Inskormations Iank Standard Curve officiation Data In Curve Fit Usto Curve Fit Method Curve Fit Method	Data Out	Blank 450 Y Axis Data	Blank Subtractio
Inskormations Iank Standard Curve atio elta Usto tata f Curve Name: StdCu Data In Curve Fit Curve Fit Curve Fit Method O Linear Regress tand	Data Out	Blank 450 Y Axis Data Use average of rep	Blank Subtractio
Inskormations Iank Standard Curve atio Curve Name: StdCurve Data In Curve Fit Usto Curve Fit Method Curve Fit Method Curve Fit Method Curve Fit Method O Linear Regress tand () Nonlinear Regress	Blank Transformation	Blank 450 Y Axis Data Use average of rep Weighting:	Blank Subtractio
Inskormations Iank Standard Curve atio Curve Name: StdCurve Data In Curve Fit Usto Curve Fit Method Curve Fit Method Curve Fit Method O Linear Regress Wonlinear Regress Nonlinear Regress O Nonlinear Regress Nonlinear Regress O Nonlinear Regress Nonlinear Regress Nonlinear Regress	i Pill Blank Transformation urve Data Out ion ression 4 Parameter v pression Degree: 2 \$	Blank 450 Y Axis Data Use average of rep Weighting: None	Blank Subtractio
Inskormations Iank Standard Curve atio atio Curve Name: StdCurve Data In Curve Fit Usto Curve Fit Method Curve Fit Method Curve Fit Method O Linear Regress Tand Wonlinear Regress Itand O Polynomial Reg O Point to Point	Blank Transformation	Blank 450 Y Axis Data Use average of rep Weighting: None Extrapolation Factor:	Blank Subtractio
Inskormations Iank Standard Curve Iank Standard Curve Iank Curve Name: StdCu Data In Curve Fit Usto Data In Curve Fit Method Curve Fit Method O Linear Regress Nonlinear Regress Nonlinear Regress Itad O Polynomial Reg Usto O Point to Point Utoff O Spline	Blank Transformation  urve  Data Out  ion ression  4 Parameter  pression  Degree:  2	Blank 450 Y Axis Data Use average of rep Weighting: None Extrapolation Factor: Parameter Constraints	Blank Subtractio
Instormations Instormations Instructions Instructions Curve Name: StaCa Data In Curve Fit Method Curve Fit Method Curve Fit Method Curve Fit Method Curve Fit Method O Linear Regress Nonlinear Regress Nonlinear Regress Nonlinear Regress Point to Point Valida	i Pill Blank Transformation	Blank 450 Y Axis Data Use average of rep Weighting: None Extrapolation Factor: Parameter Constraints () None (Estimate all)	Blank Subtractio
Inskormations Inskormations Inskormations Inskormations Curve Name: StaCo Deta In Curve Fit Intat Insto Curve Fit Method Curve Fit Method Curv	i Pill Blank Transformation	Blank 450 Y Axis Data Use average of rep Weighting: None Extrapolation Factor: Parameter Constraints (a) None (Estimate all) Y Axis Intercent:	Blank Subtractio
Astionations Iank Standard Curve Iank Standard Curve Iank Curve Name: StdCi Data In Curve Fit Uata In Curve Fit Method O Linear Regress Iank O Polynomial Reg Point to Point Ialida Spline Prim Default Axis Prese X: O Linear		Blank 450 Y Axis Data Use average of rep Weighting: None Extrapolation Factor: Parameter Constraints None (Estimate all) Y Axis Intercept:	Blank Subtractio
Astornations Iank Standard Curve Iank Standard Curve Curve Name: StdCd Data In Curve Fit Data In Curve Fit Ourse Fit Method Ourse Fit Method Outse F		Blank 450 Y Axis Data Y Axis Data Y Axis Data Y Axis Data Solution Factor: Parameter Constraints None (Estimate all) Y Axis Intercept: Advanced	Blank Subtraction
Instructions Itank Standard Curve Curve Name: StdCu Data In Curve Fit Data In Curve Fit Method O Linear Regress tand Usto Point to Point O Point to Point O Spline Prim Default Axis Prese X: O Linear Y: © Linear		Blank 450 Y Axis Data Use average of rep Weighting: None Extrapolation Factor: Parameter Constraints None (Estimate all) Y Axis Intercept: Advanced	Blank Subtraction

### Figure 14. Standard curve requirements for the ELISA plate readout

20. Double-click on the Report/Export builder and the window shown in Figure 15 will be displayed.

📑 Rep	oort/Export Builde	ers			×
Nam	e		Scope	Content	New export to Excel
					New export to file
					New report
					New picture export
					New movie export
					Edit
					Delete
Q	uick Export	Clipboard			Close Help



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21. Click on *New export to Excel* and under name enter the name of the report output (e.g., Export 1). Check *Plate, Automatic,* or *Custom* (if you want to customise your output) and click *Ok.* The window in Figure 16 will appear.

🔳 Edit	×
Name: Export1	
Properties	Properties
Content Options	Scope:
Workflow File	<ul> <li>Plate Most common. Results are grouped by plate.</li> </ul>
Format	Patient / Sample For reports only. Results are grouped by patient / sample. This is useful in the case of panelled assays for example, where multiple results are available per patient / sample.
	Content:
	Automatic Content is defined through a list of checkboxes. This feature allows fast, easy creation of report and export files. There is limited control over the formatting.
	O Custom This feature allows full control over the content of the export file or report, as well as extensive formatting options.
	OK Cancel Help

#### Figure 16. Contents of report output, Export 1

22. Check all parameters under Content as shown in Figure 17.

New export to Excel	
Name: Lab report	
Properties	Content
Content Options Workflow File Format	General Information Indude:
	Data Indude:
	< Back Next > OK Cancel Help

#### Figure 17. Different parameters under the context subheading of Lab report

23. Under *Workflow*, check *Auto-execute*, *Each pate in a separate workbook*, and *Save after export* as seen in Figure 18. Click *Next*.

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ame: Lab report	
Properties Content ⊇ Options Workflow File Format	Workflow         Image: Auto-execute on completion of the procedure (reading the plate)         Image: Prompt user to decide for each plate         Export Mode         Image: Export Export Mode         Image: Export Export Mode         Image: Export
	✓ Save after export     Protect workbook     Close after export

#### Figure 18. Workflow parameters

24. Under *File >File name*, enter file name (e.g., <PLATE\_ID>). Check *Folder* and browse the folder where you want your files to be saved, as shown in Figure 19.

New export to Excel	- X-
Name: Lab report	
Properties	File
Content Options	File Naming Convention
Workflow	File name: <plate_id>_</plate_id>
Format	Format: Microsoft Excel 97-2003 Workbook (.xls)
	Example: ID435435xls
	File Location
	🔘 Last folder used
	Folder: C:\Users\beng.MRCUGANDA0\Documents     Browse
	Example: C:\Users\beng.MRCUGANDA0\Documents
	Prompt user to validate pathname
	Before creating a new file
	Before modifying an existing file
	< Back Next > OK Cancel Help

Figure 19. Fields for entering the file name and location where you would like your plate readouts to be stored

- 25. Click Next and Ok.
- 26. To create a customised protocol, click on *New export to Excel*, as seen in Figure 15. Under *Name*, enter the name of the report output (e.g., Export 2), check *Plate* and *Custom* (Figure 20), and click *Next*.



New export to Exce	L
Name: Export2	
Properties Content ⊡ Options Workflow File Format	Properties         Scope:
I	< Back Next > OK Cancel Help

#### Figure 20. Custom parameter when creating a customised protocol

- 27. Click *Edit Template* and this will open an Excel worksheet.
- 28. Click on *Add-Ins* on the toolbar of the Excel spreadsheet. The custom toolbar is displayed as shown in Figure 21.

W 🚽 🤊 - O	Ŧ		_	-	-			SOP	for operation	n and mai	ntenan
File Home	Insert	Page Lay	out R	eferences	Mailings	Review	View	EndNote X	5	_	_
🗶   🚽 🎝 - (°	~   <b>⊋</b> _ W	orksheet in 🕯	<gen5> Tr</gen5>	ial protocol	-12nov20 [Co	ompatibility	Mode] - N	licrosoft Exce	:I _		x
File Hom	e Insert	Page La	ayout	Formulas	Data F	Review	View A	dd-Ins	\$	<b>?</b> -	er 23
X Delete	Protocol Su	ummary + Fie	eld Group	r Matrix *	Table - 🗙 E	ditClose 🤇	0				
Menu Commands			Custo	m Toolbars							
	•	0	$f_{x}$								~
A 1 2 3 4	В	C	D	E	F	G	H		J	K	

#### Figure 21. Custom toolbar in an Excel worksheet

29. Customise your template as appropriate by clicking on the drop-down and dragging into the worksheet.

- a. Go to Field Group and set the category as plate information, field, and label as "plate ID."
- b. Go to *Matrix* and add plate layout.
- c. Go to Matrix and add 450.
- d. Go to *Matrix* and add concentration.
- e. Go to *Table* and then *Statistics* and add 450.
- f. Go to *Table* and then *Statistics* and add concentration.
- g. Go to *Graph* and add standard curve.
- h. Close the Excel and proceed with steps 20-22 above as done in the automatic builder.
- 30. Go to File and save this protocol in a folder labelled "Protocols and Experiments" for easy identification.
- 31. To create an experiment in the program, open the Gen5 Software.
- 32. The task manager box will appear as shown in Figure 22. Choose *Experiments* from options, then *Create using an existing protocol*. Select from the list the protocol that you just saved.

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				$\times$
Task Manager				
		Create New		
Read Now		Create using an existing protocol		
	Þ	Open		
Experiments		Recent		
		20230919_Experiment CN54.xpt	^	
		20230919_Experiment AV.xpt		
		20230915_Experiment CN54.xpt		
Protocols		20230915_Experiment AV.xpt		
		20230912_Experiment CN54.xpt		
		20230912_Experiment AV.xpt		
Tasta ment Castral		20230911_Experiment CN54.xpt		
Instrument Control		20230911_Experiment AV.xpt		
8.6		20230908 Experiment CN54.xpt	v	
×		······		
Setup	?	FAQ: Experiment vs. Protocol		
<b></b>				
			Exit Gen5	

#### Figure 22. Task manager box, highlighting the Experiments section from the options provided

- 33. A new Experiment is created. In tool bar, select *File > Save As* and save with the appropriate name.
- 34. In Task bar select Plate, then Add Plates and add the required number of additional plates to be read.
- 35. Plates appear in the menu tree as shown in Figure 23.
- 36. Double-click *Plate 1*, followed by *Information*. Enter the appropriate plate ID and repeat this to label all the other plates.

File		×														
B-Protocol				😐 Plate 1												
Plate 1				Matrix	Statistics	Graphs										÷8
- Sample IDs				Data:	450				~ 🖹 E	dit Matrix						
Calculation Log					1	2	3	4	5	6	7	8	9	10	11	12
⊞- 🛄 Plate 2				A												
				в												
				С												
				D		_										
👪 Plate 1					×											
Plate Description	Plate Description					-										
Prompts	Reading Date:	<plate not="" read=""></plate>														
	Plate TD:	DI ATTE ID VV7			_											
	Plote 10.	FUNCTION 12			_	-										
	Barcode:															
	Commenta				^	Mar	×									Help
					$\sim$											1.
1																
			OK	C	ancel	à										

Figure 23. Menu tree under a new experiment. The pop-up window is used to enter the plate information for each plate, e.g., in this figure, enter the Plate ID for Plate 1.

- 37. Use Microsoft Excel to create a list of the sample IDs on each plate and paste it into a notepad file. *Note: You cannot import sample ID lists from .xls files into this software.*
- 38. Select Sample IDs from the drop-down menu under each plate, as shown in Figure 24.

## bio-protocol

Plate	ocol 1 (PLA nformat ample l Calculat Calculat 2 2 3 t Trail	TE ID XYZ)* tion Ds tion Log ail		
	Samp	les (Plate 1)		×
	1	Name	^	Auto Numbering
	2	SDD	_	Import
	3	ZSD	_	Export
	4	ZXD		Export
	5	ZZZ		Clear All
	6	OIR		Print List
	7	GFD		
	8	DDE		
	9			Copy From Plate #
	10			1
	11			"Plate 1"
	12			Сору
	13		$\sim$	
	Note: i	nformation displayed in italic with a gray back	iground i	s no longer valid. Cancel Help

#### Figure 24. Sample IDS parameter from the drop-down menu

- 39. Import sample IDs from the notepad file. Alternatively, one can enter the sample IDs manually.
- 40. Add sample ID list to each plate. Save the experiment with the included changes.
- 41. The reader and software are now set up to read the ELISA plates.

#### I. Addition of substrate and determination of absorbencies

- 1. (Critical) TMB substrate solution should be removed from the fridge at least 1 h before use.
- Add 50 µL per well of TMB substrate to the plates and incubate for 3 min in the dark (conventional ELISA) or 5 min (sensitive ELISA) at room temperature in the dark.
- 3. Stop the reaction by the addition of 50  $\mu$ L of stop solution per well.
- 4. Read absorbencies at 450 nm using the plate reader and Gen5 software through study customised protocols.
  - a On your experiment, right-click on the plate you want to read. In *Task* bar, select *Plate* and then *Read Plate 1*.
  - b. Select all the plates to be read by clicking *Select all* as shown in Figure 25.



ell Selection	Well S	Selecti	on											
	Select	a single	group	of conse	ecutive	wells t	o read:							
		1	2	3	4	5	6	7	8	9	10	11	12	Select All
	A	STD1 1000	STD1 1000	SPL1	SPL1	SPL2	SPL2	SPL3	SPL3	SPL4	SPL4	CTL1	CTL1	Chara All
	В	STD2 100	STD2 100	SPL5	SPL5	SPL6	SPL6	SPL7	SPL7	SPL8	SPL8	CTL3	CTL3	Clear All
	С	STD3	STD3	SPL9	SPL9			SP	SP	SP		CTL4	CTL4	
	D	STD4	STD4	SP		SP		SP	SP	SP		CTL3	CTL3	
	E	STD5	STD5			SP		SP	SP	SP		CTL4	CTL4	
	F	STD6	STD6	SP	SP	SP		SP	SP			CTL1	CTL1	
	G	STD7	STD7			SP		SP	SP			CTL2	CTL2	
	н	BLK	BLK	SP		SP		SP	SP			BLK	BLK	

Figure 25. Window where you select the wells to be read using the ELISA reader

c. Place the plate on the carrier and read the plate by clicking OK, as shown in Figure 26.

Load Plate	×
Place the plate on the carrier.	
Press OK when ready.	
OK Cancel	



5. Export the results to an Excel worksheet and store the raw data, which will include a standard curve for each plate read. Examples of these standard curves can be seen in Figure 27, Figure 28, and Figure 29.



Figure 27. Example standard curves produced during measurement of IgG optical densities (450 nm)

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and concentrations in serum/plasma samples. (A) IgG concentrations against the recombinant SARS-CoV-2 Nucleocapsid protein. (B) IgG concentrations against the recombinant SARS-CoV-2 Spike protein. (C) IgG concentrations against the SARS-CoV-2 receptor binding domain (RBD) (S1) S-protein. (D) IgG concentrations against the recombinant SARS-CoV-2 Spike S2 Subunit protein.



Figure 28. Example standard curves produced during measurement of IgM optical densities (450 nm) and concentrations in serum/plasma samples. (A) IgM concentrations against the recombinant SARS-CoV-2 Nucleocapsid protein. (B) IgM concentrations against the recombinant SARS-CoV-2 Spike protein. (C) IgM concentrations against the SARS-CoV-2 receptor binding domain (RBD) (S1) S-protein.





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CoV-2 Nucleocapsid protein. (B) IgA concentrations against the recombinant SARS-CoV-2 Spike protein. (C) IgA concentrations against the SARS-CoV-2 receptor binding domain (RBD)(S1) S-protein.

## Data analysis

In this step, data can be analysed using GraphPad Prism version 9.3 or R Version 4.3.1 or higher. Net optical densities and concentrations are determined.

#### A. Copy the plate reader output onto one Excel sheet

If more than one plate is being run at a time, merge the reader data from each plate and copy onto one Excel sheet, clearly labelling each set of data with its plate information.

#### B. Calculate the net OD values

To obtain the net optical density at 450 nm, subtract the mean OD of the blank wells from the OD of each sample for each plate. This can be done using the code snippet shown in Figure 30. Alternatively, this can be specified in the Gen5 protocol to give net OD values as the raw data.

#### C. Calculate the net concentration values

To obtain the net antibody concentrations, multiply the given concentration from the plate reader data by the sample dilution factor. This can be done using the code snippet shown in Figure 30.



Figure 30. Code snippet showing how to calculate net OD values and net concentration values

Note: Because of assay LOD and LOQ, some samples will not have antibody concentrations in the plate readout and this "?????" might appear instead of their concentrations. However, all samples for each plate should have optical densities.

 Using the R software mentioned above, for the samples without concentrations (i.e., those with "?????"), those whose net OD is below the assay LOD are assigned a "0" and those above the assay LOQ should be titrated to determine their end-point titer/concentration. This can be done using the code snippet shown in Figure 31.

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Figure 31. Code snippet showing R script for handling samples that are below or above the limit of detection (LOD)

2. A detailed description to determine assay cutoff values, as well as assay LOQ and LOD, using the direct capture by coated antigen and indirect estimation by coated anti-kappa and lambda antibodies is published in Oluka et al. (2023) [1].

## Validation of protocol

This protocol has been validated and used in the following research article(s):

- Oluka GK, Namubiru P, Kato L, Ankunda V, Gombe B, Cotten M; COVID-19 Immunoprofiling Team; Musenero M, Kaleebu P, Fox J, Serwanga J. Optimisation and Validation of a conventional ELISA and cutoffs for detecting and quantifying anti-SARS-CoV-2 Spike, RBD, and Nucleoprotein IgG, IgM, and IgA antibodies in Uganda. Front Immunol. 2023 Mar 14; 14: 1113194. doi: 10.3389/fimmu.2023.1113194. PMID: 36999017; PMCID: PMC10045470.
- Serwanga J, Ankunda V, Sembera J, Kato L, Oluka GK, Baine C, Odoch G, Kayiwa J, Auma BO, Jjuuko M, Nsereko C, Cotten M, Onyachi N, Muwanga M, Lutalo T, Fox J, Musenero M, Kaleebu P; COVID-19 Immunoprofiling Team. Rapid, early, and potent Spike-directed IgG, IgM, and IgA distinguish asymptomatic from mildly symptomatic COVID-19 in Uganda, with IgG persisting for 28 months. Front Immunol. 2023 Mar 16; 14: 1152522. doi: 10.3389/fimmu.2023.1152522. PMID: 37006272; PMCID: PMC10060567.
- Nantambi H, Sembera J, Ankunda V, Ssali I, Kalyebi AW, Oluka GK, Kato L, Ubaldo B, Kibengo F, Katende JS, Gombe B, Baine C, Odoch G, Mugaba S, Sande OJ; COVID-19 Immunoprofiling Team; Kaleebu P, Serwanga J. Pre-pandemic SARS-CoV-2-specific IFN-γ and antibody responses were low in Ugandan samples and significantly reduced in HIV-positive specimens. Front Immunol. 2023 Apr 19; 14: 1148877. doi: 10.3389/fimmu.2023.1148877. PMID: 37153598; PMCID: PMC10154590.
- Serwanga J, Baine C, Mugaba S, Ankunda V, Auma BO, Oluka GK, Kato L, Kitabye I, Sembera J, Odoch G, Ejou P, Nalumansi A, Gombe B, Musenero M, Kaleebu P. Seroprevalence and durability of antibody responses to AstraZeneca vaccination in Ugandans with prior mild or asymptomatic COVID-19: implications for vaccine policy. Front Immunol. 2023 May 2; 14: 1183983. doi: 10.3389/fimmu.2023.1183983. PMID: 37205095; PMCID: PMC10187141.

Reliability, reproducibility, and robustness:

- 1. In the validation of the protocol, all samples were run in duplicates and a coefficient of variation  $\leq 25\%$  was ensured. The standard curves conformed to a goodness of fit of  $R^2 \geq 0.9$  (Figure 5, Table 6, Figure 7) [1].
- SARS-CoV-2 specific positive control antibodies CR3022 and CR3009 as well as local plasma samples of known seropositivity were used to monitor the consistency of the assay using Levey-Jennings curves (Figure 8A and 8B). The ODs were consistently within a CV of 25% and two standard deviations of the mean, in line with recommendations of the Westgard-Sigma rules [1].

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- Assays were repeated on different days and by different operators to ascertain inter-assay and inter-operator precision; all tests conformed to a CV ≤ 25% (Figure 5) [1]
- 4. There was inter-site validation of the protocol between the Imperial College London and the Uganda Virus Research Institute, where the same set of samples were tested, and comparable results were obtained as shown in Figure 9 [1].
- The above results from the validation of the protocol are published in the open-access article by Oluka et al. 2023 [1]. <u>https://doi.org/10.3389/fimmu.2023.1113194</u>.

## General notes and troubleshooting

#### Limitations

This ELISA protocol is subject to changes in OD values due to environmental temperature fluctuations in the lab and changes in reagent batches such as the coating antigens and secondary antibodies used in the assay.

- 1. To ensure assay consistency with different reagent batches, coat ELISA plates with different batches of an antigen, e.g., a newly purchased spike antigen being compared with a spike antigen currently in use, following the procedure described above.
- 2. Run a set of both known positive and negative serum/plasma samples on each of these plates to obtain two sets of results from these plates.
- 3. Analyse the data using a scatter plot to determine the correlation coefficient and see if it is significant. One can also use a Wilkson test for matched pairs to give a p-value, which will determine if the distribution is the same in both lots. If the p-value is less than 0.05, we conclude that there is a significant difference between the two groups.

The change in the antigenic determinants of the target pathogen requires new assay validation to ensure consistency and generation of new assay quality controls for use.

#### Troubleshooting

Problem 1: Plates have a high background in the blank wells.

Possible cause(s): Contaminant particles in air or buffer used, plates left too long before reading, or substrate incubation temperature too high.

Solution(s): Coat plates in a biosafety cabinet. Prepare assay buffer in clean environment. Add substrate at room temperature (23 °C) and read plates promptly after adding substrate.

Problem 2: %CV for OD values of sample replicates above 25.

Possible cause(s): Inconsistent pipetting during sample dilution. Bubbles in wells when reading.

Solution(s): Use properly calibrated pipettes and avoid distractions during sample dilution. Ensure controlled up and down pipetting during sample addition. Avoid generating bubbles when adding substrate or stop solution.

Problem 3: Non-sigmoid standard curve.

Possible cause(s): Improper dilution of standards. Contamination of standards. Deterioration of standards. Solution(s): Follow closely the dilution steps performed according to the protocol. Use appropriate diluent as blank. Minimise freeze-thaw cycles for the standards and change aliquots after a week.

Problem 4: Weak signal for standards or strong positive controls. Possible cause(s): Deteriorated antibodies in the sample or standards. Solution(s): Prepare aliquots that should be used within a week and stored at -20 °C.

Problem 5: Unresolved OD values for sample replicate wells. Possible cause(s): High titres of target antibodies in the samples tested above the quantifiable range for the standard.

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Solution(s): Titrate the unresolved sample in triplicates with 2-fold or 3-fold dilution series on a new plate or test unresolved samples at a higher dilution on another plate.

Problem 6: Inconsistent OD values for negative control samples above expected range. Possible cause(s): Contamination of controls or assay buffer during preparation. Solution(s): Prepare controls in clean vials.

Problem 7: Very low or very high signals.

Possible causes: Disregarding the incubation periods and temperature instructions at each step of the assay, contamination, cross-reactivity, poor washing of the plates.

Solution(s): If the high signal is uniform within the whole plate:

- 1. If it is a single sample and not the whole plate showing high signals, one should consider that that particular sample has a high antibody concentration indicated by the high signal.
- 2. One should ensure that the antibodies used in the assay are specific to the target and not cross-reacting with other substances in the sample.
- 3. It is advisable that this protocol be optimised multiple times in the lab to ensure consistency and reproducibility of results before it is implemented.

In case the signal is too low:

- 1. One can increase the enzyme-substrate incubation time.
- 2. One should reevaluate the wash steps to ensure that they are thorough to remove unbound substances, which could interfere with the signal.

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The following reagent was produced under HHSN272201400008C and obtained through BEI Resources, NIAID, NIH: Monoclonal Anti-SARS Coronavirus Recombinant Human Antibody, Clone CR3022 (produced in HEK293 cells), NR-52481. The following reagent was obtained through BEI Resources, NIAID, NIH: Monoclonal Anti-SARS Coronavirus Recombinant Human IgG1, Clone CR3022 (produced in *Nicotiana benthamiana*), NR-52392. The nucleoprotein mAb CR3009 (NIBSC Repository, Product No. 101011) used as a positive control was obtained from the Centre for AIDS Reagents, NIBSC, UK. The first WHO international standard for SARS-CoV-2, RN 20/136, Immunoglobulin, human, S321534 was obtained from the Centre for AIDS Reagents, NIBSC, UK. The first WHO international standard for SARS-CoV-2, Immunoglobulin, human, 20/B770 was obtained from the Centre for AIDS Reagents, NIBSC, UK. A mammalian expression plasmid for the RBD of SARS-CoV-2 was a kind donation by the Doores laboratory at King's College London. Specimens used in the inter-site cross-validation for the protocol were obtained from Dr. Hannah Cheeseman's Laboratory at Imperial College London, United Kingdom. 696. This protocol has been validated as outlined in a prior publication [1] and used in multiple subsequent studies [7, 9–11].

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## **Competing interests**

The authors declare that this protocol was developed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## **Ethical considerations**

The use of human participants to obtain samples used in the development of this protocol was reviewed and approved by the Uganda Virus Research Institute (UVRI) Research and Ethics Committee (Ref: GC/127/833) and the Uganda National Council of Science and Technology (Ref: HS637ES). The patients/participants provided their written informed consent to participate.

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