SARS-CoV-2 S1-RBD ELISA

ELISA for the Determination of SARS-CoV-2 S1-RBD

Instruction Leaflet

THIS PRODUCT IS INTENDED FOR LABORATORY AND RESEARCH USE ONLY. IT IS NOT SUITABLE FOR DIAGNOSTIC OR THERAPEUTIC APPLICATIONS.

1 Intended Use

The critical reagents required to perform the SARS-CoV-2 S1-RBD ELISA (SARS-CoV-2 S1 receptor binding domain-specific enzyme-linked immunosorbent assay) are provided as stock solutions. This specific ELISA method is to be used for the quantitative in-vitro measurement of SARS-CoV-2 S1-RBD in process samples.

2 Test Principle

This quantitative test method (SARS-CoV-2 S1-RBD ELISA) is based on a sandwich enzyme immunoassay to be performed in 96 well microtest plates. The polyclonal antibodies used were raised by rabbit immunisation with SARS-CoV-2 S1-RBD and isolated by a Protein A-based affinity purification to obtain the total IgG fraction. Samples, which potentially may contain SARS-CoV-2 S1-RBD (analyte), are incubated in microtest plate wells which are precoated with the specific capture antibody (anti-SARS-Total-IgG) alongside a standard curve of different analyte concentrations (0.781 – 50 ng/mL).

After incubation and a plate washing step in which unbound components are removed, the specific biotin-conjugated detector antibody (the same as used as capture antibody) is added. After further washing steps, the bound detector antibody in turn reacts with the enzyme conjugate (streptavidin-conjugated peroxidase) as the tracer. After a final washing step, the reaction is developed with tetramethylbenzidine (TMB) resulting in a blue colour. Eventually, the reaction is stopped by addition of sulphuric acid converting the blue colour to yellow and the optical density is measured photometrically at a wavelength of 450 nm (reference wavelength between 620 – 690 nm, recommended 630 nm). The optical density is proportional to the analyte concentration in the wells. The analyte concentration in samples can be calculated based on the corresponding standard curve of known concentrations.

3 Materials and Equipment

3.1 Consumables and Equipment

Consumables and technical devices required to perform the ELISA method are listed below.

- microplate washer (8-channel or 96-channel)
- orbital microplate shaker (400 – 600 rpm) and vortex mixer
- precision pipettes (adjustable volumes, e.g. 10 – 10,000 µL)
- multichannel pipette (8-channel, 100 µL or adjustable volume, e.g. 30 – 300 µL)
- multichannel microplate reader for optical density measurement at 450 nm (reference wavelength adjustable between 620 – 690 nm, recommended 630 nm)
- ELISA microtest plates (MTP), 96 well, 1x8 F-strips, high binding capacity
- pipette tips (volumes of e.g. 10 – 10,000 µL)
- suitable reaction tubes (e.g. 1.7 mL, 2 mL, 5 mL, 15 mL, 50 mL)
- suitable reagent bottles and beakers
- suitable reagent reservoirs for effective multichannel pipetting
- suitable lids/ adhesive foil for covering microtest plates
- absorbent paper towels for removing residual liquid after microtest plate washing

3.2 Reagents

Reagent	Supplier (Cat. No.)	Details
Ultrapure Water	BioGenes (n/a)	to be freshly prepared by the user
Coating Buffer	BioGenes (n/a)	pH 9.6, provided as 10x stock solution, “BlueCap Solutions”, BioGenes GmbH, Cat. No. S207, storage at +2°C to +8°C, shelf life according to product specification
10x Blocking/ Washing/ Assay Buffer	BioGenes (n/a)	Tris-based, provided as 10x stock solution, “BlueCap Solutions”, BioGenes GmbH, Cat. No. S210, storage at +2°C to +8°C, shelf life according to product specification
Master Standard	BioGenes (n/a)	5 vials of 100 µL SARS-CoV-2 S1 (RBD, tag-free) ELISA Standard (lot: PS-130421-01), conc.: 10 µg/mL, storage at below -60°C, single-use vials
Capture Antibody	BioGenes (n/a)	1 vial of 500 µL anti-SARS-Total-IgG (lot: RD-090421-01), conc.: 1.00 mg/mL (BioGenes, UV_{280}), affinity-purified via Protein A, polyclonal rabbit total IgG capture antibody, storage at below -60°C, stable at +2°C to +8°C of up to 2 weeks after thawing once
Detector Antibody	BioGenes (n/a)	1 vial of 100 µL anti-SARS-Total-IgG-Biotin (lot: PS-090421-01), conc.: 0.20 mg/mL (BioGenes, UV_{280}), affinity-purified via Protein A and biotin-conjugated polyclonal rabbit total IgG detector antibody, storage at below -60°C, stable at +2°C to +8°C of up to 2 weeks after thawing once
Enzyme Conjugate	Roche Diagnostics (11089153001)	Streptavidin-POD Conjugate, amount: 500 U, prepared as 100x concentrate (5 U/mL) by BioGenes in POD-Conjugate Stabilizer[1], storage at +2°C to +8°C, shelf life to be defined by the user (at BioGenes: 12 months)
Substrate Solution	KemEnTec Diagnostics (4380A)	TMB ONE substrate solution (ready-to-use), storage at +2°C to +8°C, shelf life according to product specification
Stop Solution	BioGenes (n/a)	0.5 M sulphuric acid, storage at +18°C to +26°C, shelf life to be defined by the user (at BioGenes: 12 months)

[1] **POD-Conjugate Stabilizer:** “BlueCap Solutions”, BioGenes GmbH, Cat. No. S220

4 Warnings and Precautions

- This ELISA is intended for in-vitro laboratory and research use only and should solely be used by qualified personnel.
- Before performing the assay, read the instruction leaflet carefully.
- Note lot numbers and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
- Follow good laboratory practice and safety guidelines. Wear lab coats, disposable gloves and protective glasses when necessary.
- Some reagents of the ELISA contain a mixture of CMIT/ MIT (Methylchloroisothiazolinone/ Methylisothiazolinone) as preservative. These reagents may cause eye and skin irritations and should be handled with care. In case of contact with eyes or skin, flush immediately with water.
- Store the substrate solution protected from light.
- The stop solution consists of 0.5 M sulphuric acid. This reagent is corrosive and may cause eye and skin irritations. It should be handled with care. In case of contact with eyes or skin, flush immediately with water.
- Remaining reagents and solutions have to be treated as potentially hazardous waste according to national safety guidelines and regulations.

5 Procedures

5.1 Reagent Preparation

In general, the 1x coating buffer, 1x washing/ assay buffer, the capture and detector antibody and enzyme conjugate working solutions as well as the different standards and samples should be prepared immediately before use. Each of these solutions should only be used on the day of preparation.

5.1.1 Preparation of the 1x Coating Buffer

Dilute the 10x coating buffer 1:10 with ultrapure water before use (e.g. 100 mL 10x concentrate with 900 mL ultrapure water).

5.1.2 Preparation of the 1x Blocking/ Washing/ Assay Buffer

Dilute the 10x blocking/ washing/ assay buffer 1:10 with ultrapure water before use (e.g. 100 mL 10x concentrate with 900 mL ultrapure water).

5.1.3 Preparation of the Coating Solution (Capture Antibody)

Thaw an aliquot of the capture antibody (1.00 mg/mL) and mix thoroughly. Before diluting the capture antibody, it should be centrifuged for 2 min at ~13,000*g. The capture antibody stock solution should be diluted in 1x coating buffer to 5 µg/mL (1:200).

Example for one MTP:

- 60 µL capture antibody (1.00 mg/mL) + 12 mL 1x coating buffer (final 5 µg/mL)

5.1.4 Preparation of the Standards

The master standard is thawed at 18 – 26°C immediately before use and diluted in 1x assay buffer producing the different standards according to the following scheme. Pure 1x assay buffer is used as assay blank. The volumes can be adjusted depending on the number of microtest plates to be analysed.

Standard ID	Nominal Concentration [ng/mL]	Volume [µL]	of Solution ID	Volume 1x Assay Buffer [µL]
Master Standard	**10 000**	-	-	-
PDS	100	10	**Master Standard**	990
S8	50	500	PDS	500
S7	25	500	S8	500
S6	12.5	500	S7	500
S5	6.25	500	S6	500
S4	3.125	500	S5	500
S3	1.563	500	S4	500
S2	0.781	500	S3	500
S1	0	0	-	2000

PDS: pre-dilution standard; **S:** standard; **S1:** assay blank

5.1.5 Preparation of the Samples

Dilute the samples with 1x assay buffer before ELISA measurement. The minimum required dilutions (MRD) have to be determined by the user for each sample type depending on the analyte content and by taking the assay working range into account. Furthermore, an accurate and precise analyte quantification has to be demonstrated for the selected dilution range.

5.1.6 Preparation of the Detector Antibody Solution

Thaw an aliquot of the detector antibody (0.2 mg/mL) and mix thoroughly. Before diluting the detector antibody, it should be centrifuged for 2 min at ~13,000*g. The detector antibody stock solution should be diluted in 1x assay buffer to 200 ng/mL (1:1000).

Example for one MTP:

- 12 µL detector antibody (0.20 mg/mL) + 12 mL 1x assay buffer (final 200 ng/mL)

5.1.7 Preparation of the Enzyme Conjugate Solution

Dilute the 100x concentrate of the enzyme conjugate (5 U/mL) with 1x assay buffer before use.

Example for one MTP:

- 120 µL of 100x concentrate of enzyme conjugate (5 U/mL) + 12 mL 1x assay buffer (50 mU/mL)

5.2 General ELISA Procedure

All steps of the ELISA are performed at temperatures between 18 – 26°C. Allow all materials and reagents to reach this temperature before opening and using them. During all incubation steps, the plates should be covered with a lid to prevent evaporation and contamination of solutions. All plate washing steps should be performed with an automatic microplate washer and the respective washing programs. Plate shaking is to be performed at 400 – 600 rpm using a suitable microplate shaker.

Step	Reagent	Volume per Well	Incubation Time
Coating	capture antibody, **5 µg/mL**	100 µL	overnight (alternatively up to 72 h at 2 – 8°C)
Washing Step/ Blocking Step	1x washing buffer	4x 250 µL	
Antigen Incubation	standards and samples (cf. microtest plate layout)	100 µL	1 h (continuous shaking)
Washing Step	1x washing buffer	4x 250 µL	
Detector Antibody Incubation	detector antibody, **200 ng/mL**	100 µL	1 h (continuous shaking)
Washing Step	1x washing buffer	4x 250 µL	
Enzyme Conjugate Incubation	enzyme conjugate working solution, **50 mU/mL**	100 µL	20 min (continuous shaking)
Washing Step	1x washing buffer	4x 250 µL	
Substrate Incubation	substrate solution (ready-to-use)	100 µL	15 min (continuous shaking)
Stop Reaction	stop solution (ready-to-use)	100 µL	readout OD_{450}[2] (vs. OD_{630}) within 15 min after stopping

[2]: The optical density at 450 nm (OD_{450}) is measured with a reference wavelength of 630 nm. For further data evaluation (e.g. standard curve regression, (back)calculation of the analyte concentration), the OD_{630} values of each well are subtracted from the corresponding values measured at 450 nm.

5.3 Microtest Plate Layout

The different standards and samples are analysed as exemplarily indicated in the microtest plate layout below for a triplicate well setup.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S8 (50 ng/mL)			SPL1			SPL5			SPL13		
B	S7 (25 ng/mL)			SPL2			SPL6			SPL14		
C	S6 (12.5 ng/mL)			SPL3			SPL7			SPL15		
D	S5 (6.25 ng/mL)			SPL4			SPL8			SPL16		
E	S4 (3.125 ng/mL)			S1			SPL9			SPL17		
F	S3 (1.563 ng/mL)			S1			SPL10			SPL18		
G	S2 (0.781 ng/mL)			S1			SPL11			SPL19		
H	S1			S1			SPL12			SPL20		

S: standard; **S1:** assay blank; **SPL:** sample

5.4 Measurement and Calculations

The optical density at a wavelength of 450 nm is to be measured with a reference wavelength between 620 – 690 nm (recommended 630 nm) using a suitable multichannel microplate reader and the corresponding software. The standard curves should be generated employing a nonlinear regression mode (four-parameter equation). The analyte concentrations are (back)calculated by the microplate reader software for the different standards and samples.

6 System Suitability Test

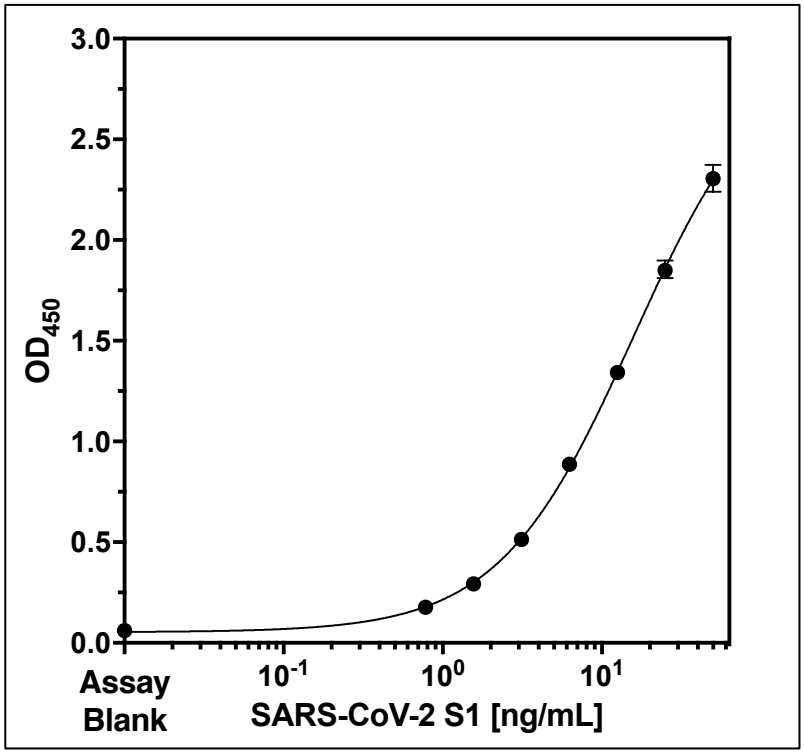
A system suitability test was implemented to demonstrate the integrity of the SARS-CoV-2 S1-RBD ELISA. As presented below, this system suitability test generally includes acceptance criteria for the standard curve and samples. A suitable assay control sample is recommended to be implemented. For example, a process sample containing the analyte can be measured in ELISA in several independent runs ($n \geq 10$). The target range (acceptance range) is then defined as the overall mean ± three times the standard deviation.

Standard Curve	
coefficient of determination R^2:	≥ 0.99
mean OD_{450} S1:	≤ 0.2
mean OD_{450} S8 (50 ng/mL):	1.8 – 3.0
mean OD_{450} S1 vs. mean OD_{450} S2 (0.781 ng/mL):	S1 + 3.3*SD(S1) ≤ S2
CVs OD_{450} S2 to S8 replicates:	≤ 15%
intra-assay recovery** S2 to S8 (based on nominal HCP concentration):	80 – 120%
Samples	
CV OD_{450} SPL replicates (within the assay working range):	≤ 15%

S: standard; **S1:** assay blank; **SPL:** sample**; SD:** standard deviation; **CV:** coefficient of variation; ****:** Intra-assay recovery = (measured HCP concentration / nominal HCP standard concentration) * 100 [%]
The OD_{450} values mentioned above represent the measuring values at a wavelength of 450 nm after subtraction of the OD values obtained at the reference wavelength of 630 nm.

7 Standard Curve

An exemplary SARS-CoV-2 S1-RBD ELISA standard curve is presented below. The suggested set of standards covers a concentration range of 0.781 – 50 ng/mL. The assay working range should be finally defined and confirmed by user's assay qualification according their quality requirements.



8 Troubleshooting

Possible reasons (explanations) for lacking ELISA performance are listed below.

Low reactivity throughout the whole plate

- omission of incubation steps and/ or reagents
- inadequate storage or preparation of ELISA components/ reagents
- reagents were not allowed to reach room temperature before use
- improper wavelength for measuring the optical density

High reactivity and assay background throughout the whole plate

- improper washing steps
- inadequate storage or preparation of ELISA components/ reagents
- overdevelopment of the plate with substrate solution before stopping
- contamination of the substrate solution

Poor intra-assay precision (high CV of replicate wells)

- improper washing steps
- insufficient mixing of solutions
- inhomogeneous samples containing aggregates/ precipitates