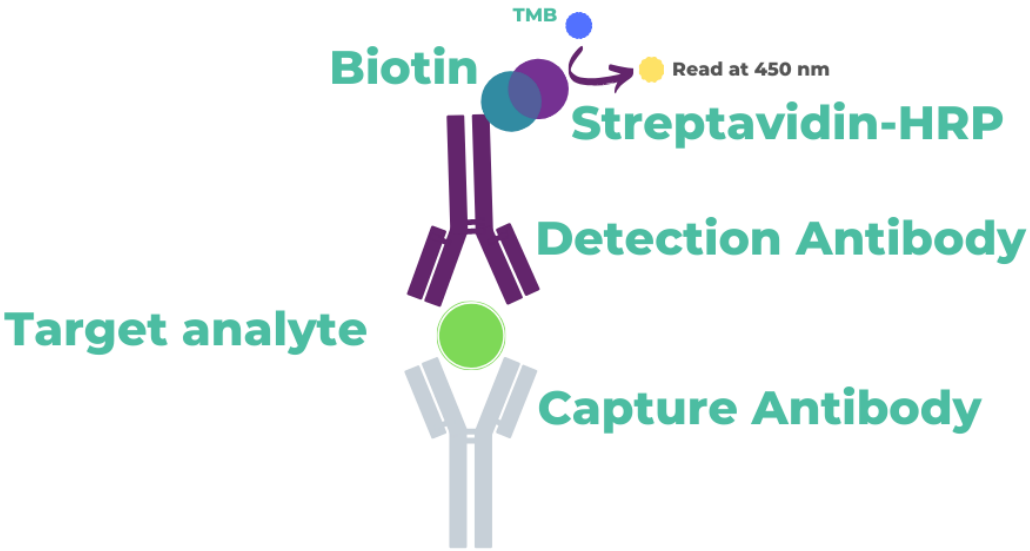

Figure 1: A schematic of a Sandwich ELISA, whereby the capture antibody and detection antibody have bound to the protein of interest.

What is a sandwich ELISA?

Sandwich ELISA (Enzyme-Linked ImmunoSorbent Assay) is a antibody based technique that allows researchers to quantify the amount of protein, hormone or analyte of interest in a sample. Capture and detection antibodies bind to non-overlapping epitopes on the protein to sandwich the protein, hence the name, Sandwich ELISA. Following the addition of the detection antibody, a chemical substrate is added (such as TMB) to produce a colorimetric signal that can be read by an ELISA plate reader.

Sandwich ELISA antibodies

Antibodies used to create a Sandwich ELISA can be either polyclonal or monoclonal antibodies depending on the specificity, sensitivity and analyte being detected.

> Polyclonal antibodies

Polyclonal antibodies are often used to pull down as much analyte as possible in a sample. Polyclonal antibodies can bind to multiple facets of an epitope therefore, provide an increased capture opportunity for detecting proteins of interest.

> Monoclonal antibodies

Monoclonal antibodies allow researchers to pull down a single antigen. Therefore, allow researchers to distinguish between subtle differences in proteins. Monoclonal antibodies also provide increased consistency in data versus polyclonal antibodies.

Advantages of a Sandwich ELISA

Advantage	Description
No sample purification required	Sandwich ELISA assays allow for the measure of proteins/analytes in complex samples without the need for purification.
High specificity	Since capture and detection antibodies are used, a Sandwich ELISA assay has increased sensitivity versus a direct or indirect ELISA assay.
Quantification	Versus other EIA methods such as a Western Blot, the Sandwich ELISA assay allows researchers to quantify the amount of protein in a sample.

Sandwich ELISA Protocols

Sandwich ELISA assays help researchers quantify proteins of interest in samples such as serum, plasma, cell supernatant, tissue and other biological samples. Sandwich ELISA kits can be purchased in two formats, either as a pre-coated ELISA plate, whereby the capture antibody has already be coated on the polystyrene ELISA plate, or antibody pairs can be purchased to develop your own ELISA Sandwich assay. Below we describe both protocols.

Sample preparation & collection

A range of sample types can be used to measure protein/analyte levels by Sandwich ELISA.

According to best practices, extract protein & perform the experiment as soon as possible after sample collection. Alternatively, store the extracts at the designated temperature (-20°C/-80°C) and for optimal results avoid repeated freeze-thaw cycles.

Serum: If using serum separator tubes, allow samples to clot for 30 minutes at room temperature. Centrifuge for 10 minutes at 1,000x g. Collect the serum fraction and assay promptly or aliquot and store the samples at -80°C. Avoid multiple freeze-thaw cycles.

If serum separator tubes are not being used, allow samples to clot overnight at 2-8°C. Centrifuge for 10 minutes at 1,000x g. Remove serum and assay promptly or aliquot and store the samples at -80°C. Avoid multiple freeze-thaw cycles.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples at 4°C for 15 mins at 1000 × g within 30 mins of collection. Collect the

plasma fraction and assay promptly or aliquot and store the samples at -80°C. Avoid multiple freeze-thaw cycles. Note: Over haemolysed samples are not suitable for use.

For further information on different sample types, please see our sample collection guide.

Sandwich ELISA (pre-coated) protocol step-by-step

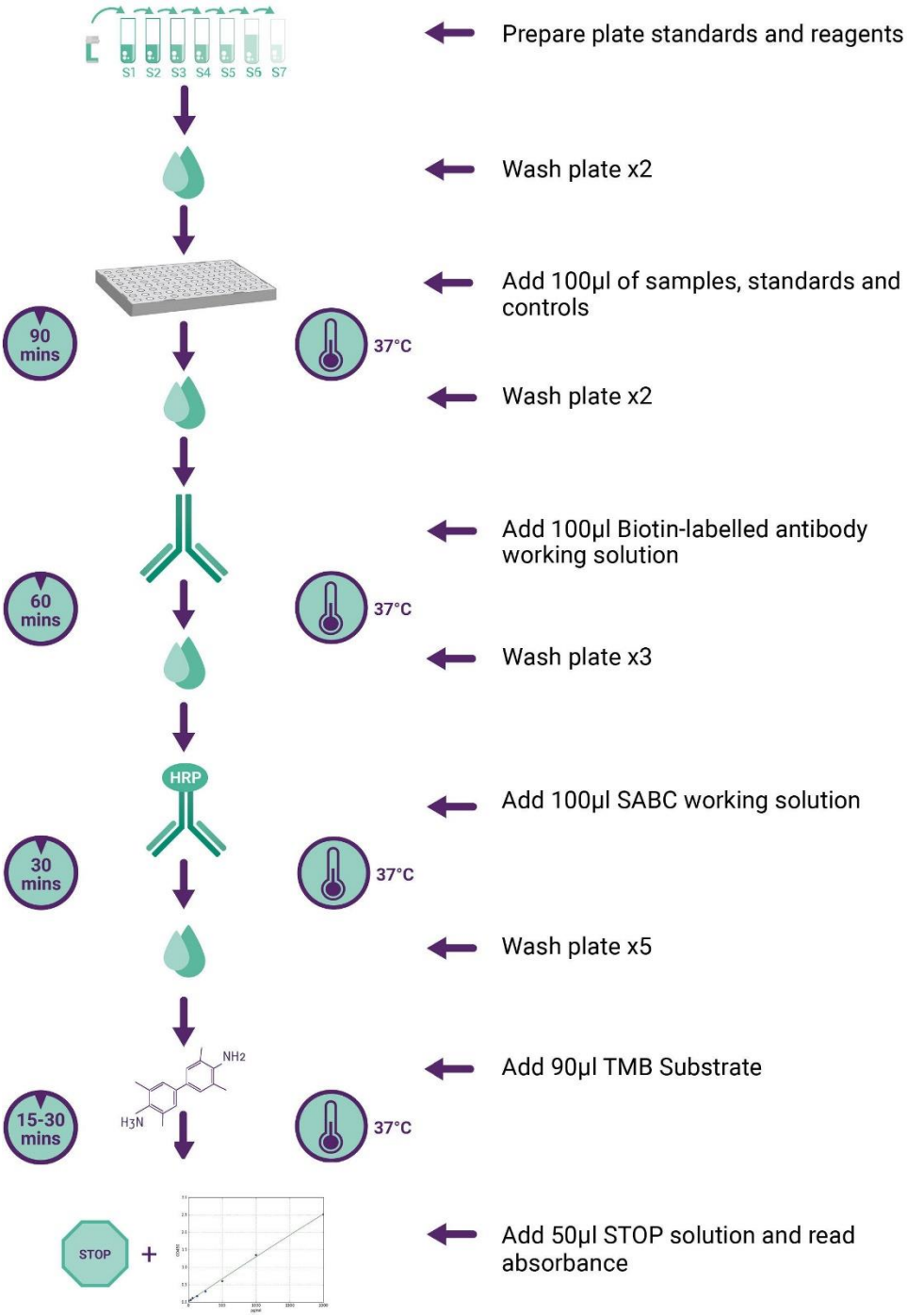

Figure 3: Sandwich ELISA protocol for a pre-coated ELISA plate. Step by step schematic for the steps involved in a sandwich ELISA assay. Firstly, prepare standards, followed by the addition of samples to the ELISA plate & incubate. Once incubated, wash the plate followed by the addition of labelled antibody & incubate. Following incubation, wash the plate and add the SABC working solution. Wash the plate and add the TMB substrate, followed by an incubation. Finally add stop solution and measure.

Step	Procedure
1.	Set standard, test sample and control (zero) wells on the pre-coated plate respectively, and then, record their positions. It is recommended to measure each standard and sample in duplicate. Wash plate 2 times before adding standard, sample and control (zero) wells!
2.	Aliquot 0.1ml standard solutions into the standard wells.
3.	Add 0.1 ml of Sample / Standard dilution buffer into the control (zero) well.
4.	Add 0.1 ml of properly diluted sample (Human serum, plasma, tissue homogenates and other biological fluids.) into test sample wells.
5.	Seal the plate with a cover and incubate at 37 °C for 90 min.
6.	Remove the cover and discard the plate content, clap the plate on the absorbent filter papers or other absorbent material. Do NOT let the wells completely dry at any time. Wash plate X2.
7.	Add 0.1 ml of Biotin- detection antibody working solution into the above wells (standard, test sample & zero wells). Add the solution at the bottom of each well without touching the side wall.
8.	Seal the plate with a cover and incubate at 37°C for 60 min.
9.	Remove the cover, and wash plate 3 times with Wash buffer. Let wash buffer rest in wells for 1 min between each wash.
10.	Add 0.1 ml of SABC working solution into each well, cover the plate and incubate at 37°C for 30 min.
11.	Remove the cover and wash plate 5 times with Wash buffer, and each time let the wash buffer stay in the wells for 1-2 min.
12.	Add 90 µl of TMB substrate into each well, cover the plate and incubate at 37°C in dark within 15-30 min. (Note: This incubation time is for reference use only, the optimal time should be determined by end user.) And the shades of blue can be seen in the first 3-4

Step	Procedure
	wells (with most concentrated standard solutions), the other wells show no obvious color.
13.	Add 50 µl of Stop solution into each well and mix thoroughly. The color changes into yellow immediately.
14.	Read the O.D. absorbance at 450 nm in a microplate reader immediately after adding the stop solution.

Sandwich ELISA (development) protocol step-by-step

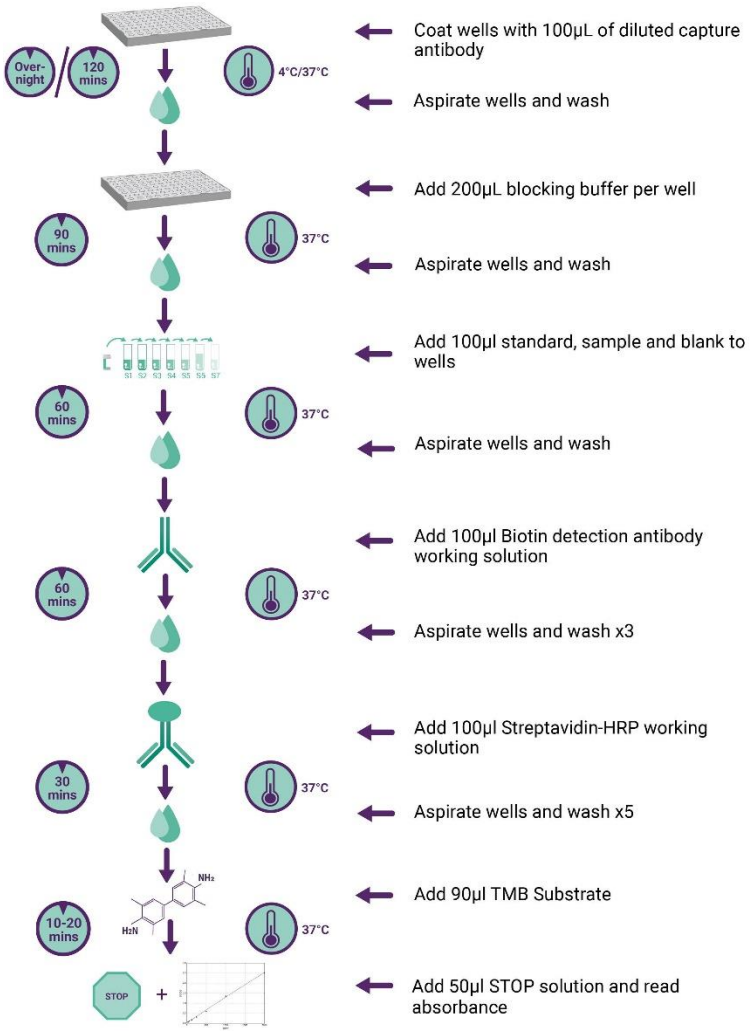

Figure 4: Sandwich ELISA protocol for a development ELISA kit. Step by step schematic for the steps involved in a sandwich ELISA assay.

Coating plate with capture antibody

Step	Procedure
1.	Add 100µl of diluted Capture Antibody to every well.
2.	Cover with a plastic plate cover and incubate at 4°C overnight.
3.	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of washing solution into each well c) Aspirate the contents of each well d) Repeat step b and c
4.	Add 100µl of Blocking Buffer to every well.
5.	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 2 hours.
6.	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of washing solution into each well c) Aspirate the contents of each well d) Repeat step b and c another 2 times
7.	For Immediate use of the plate(s) continue to next section. If you wish to store the coated and blocked plates for future use, bench dry each plate at room temperature (18 to 25°C) for 24 hours. Then store at 2-8°C in a sealed plastic bag with desiccant for up to 12 months.

Development Sandwich ELISA protocol

Step	Procedure
1.	Prepare Standard curve.
2.	Add 100µl of each Standard, Sample, zero (Standard Dilution Buffer) to appropriate wells in duplicate.
3.	Add 50µl of diluted Detection Antibody into all wells.

Step	Procedure
4.	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 1 hour.
5.	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of washing solution into each well c) Aspirate the contents of each well d) Repeat step b and c
6.	Add 100µl of Streptavidin-HRP solution into all wells.
7.	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 30 minutes.
8.	Repeat wash step 5.
9.	Add 100µl of ready-to-use TMB Substrate Solution into all wells.
10.	Incubate in the dark for 5-15 minutes* at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
11.	Add 100µl of Stop Reagent into all wells.
12.	Read the absorbance value of each well (immediately after step 11) on a spectrophotometer using 450 nm as the primary wavelength and optionally 630 nm as the reference wave length (610 nm to 650 nm is acceptable).

Detection and data analysis

Horse Radish Peroxidase (HRP) & Alkaline Phospatase are the most widely used enzymes for the detection of analytes by Sandwich ELISA method and provide different options for researchers depending on the application.

P-Nitrophenyl-phosphate (pNPP)

pNPP is the ALP substrate. Following the addition of pNPP, incubate samples at room temperature for 10-30 mins. Stop the reaction by adding 0.75M NaOH and read samples at 405nm.

Hydrogen Peroxide

Hydrogen peroxide is the substrate for HRP, which allows for a colour change during the reaction.

TMB (3,3’,5,5’-tetramethylbenzidine)

TMB undergoes colour change following it's reduction of hydrogen peroxide in the presence of HRP. To quench the reaction, sulfuric acid is added and the reaction results in a colour change that can be read at 450nm by an ELISA plate reader.

Calculating the results

Calculate using the following equation:

The relative O.D.450 = (the O.D.450 of each well) – (the O.D.450 of Zero well)

The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The concentration of the samples can be determined from the standard curve. It is recommended to use professional software such as curve expert 1.3.

Note: If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.