ELISA Development Guide

a guide for the use of antibodies in ELISA development

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

R&D SYSTEMS

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The ELISA Protocol, as well as the guidelines and tips for building your own ELISA, are based on using R&D Systems' antibody pairs tested for ELISA.

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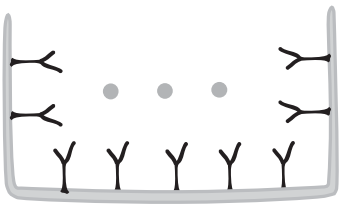
Introduction

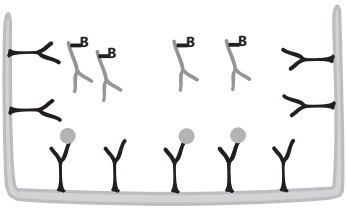
The ELISA (Enzyme Linked Immunosorbent Assay) technique is based on the antibody sandwich principle. First, a capture antibody specific to the analyte of interest is bound to a microtiter plate to create the solid phase. Unbound antibody is removed by washing the plate and a blocking reagent is added. Following a wash, samples, standards, and controls are then incubated with the solid phase antibody, which captures the analyte. After washing away unbound analyte, a conjugated detection antibody (*e.g.* biotin conjugated) is added. This detection antibody binds to a different epitope of the molecule being measured, completing the sandwich. Following a wash to remove unbound detection antibody, a detection reagent (*e.g.* streptavidin-HRP) is added. The plate is washed, a substrate solution (*e.g.* TMB/hydrogen peroxide) is added and color develops in proportion to the amount of bound analyte. Color development is stopped and the intensity of the color is measured.

The assay in Figure 1 involves a detection system which utilizes streptavidin-HRP (R&D Systems, Cat. # DY998) and tetramethylbenzidine (TMB)/peroxide (R&D Systems, Cat. # DY999) as a substrate. TMB/peroxide turns blue when modified by HRP. The final step is to stop the reaction with an acidic solution, which turns the solution yellow. The optical density (O.D.) of the yellow color is read at A_{450} on a microtiter plate reader.

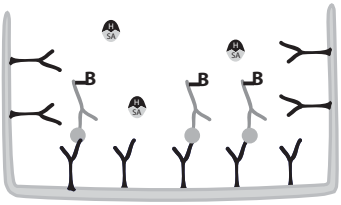
Figure 1.



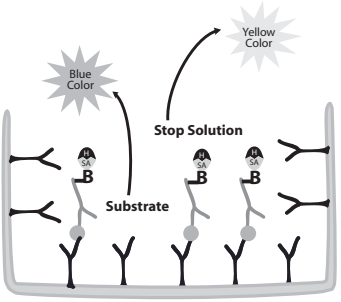
Step 1. Analyte-specific antibody (capture antibody) is pre-coated onto a microplate. The sample is added and any analyte present is bound by the immobilized antibody.



Step 2. A biotin-labeled analyte-specific detection antibody binds to a second epitope on the analyte forming the analyte-antibody complex.



Step 3. Streptavidin-HRP is added and binds to the biotin-labeled detection antibody.



Step 4. TMB/peroxide (substrate) is added and converted by the HRP (enzyme) to a color product (blue) in proportion to the amount of analyte bound (signal increases as analyte concentration increases). The reaction is stopped upon addition of stop solution, changing the solution from blue to yellow.

Legend

Supplies

Materials required but not supplied

- ELISA microtiter plates (Costar, Catalog # 2592 or equivalent)
- Disposable plate sealers (Costar, Catalog # 3095 or equivalent)
- Disposable reagent reservoirs (Baxter, Catalog # 5082-128 or equivalent)
- Assorted graduated cylinders
- Wash bottle and/or automatic plate washer
- Assorted adjustable volume pipettes
- 8 or 12 channel multichannel pipettes
- Pipette tips
- Assorted volume pipettes
- Polypropylene tubes
- ELISA plate reader with optional data reduction software

Solutions required but not supplied

- Wash Buffer - 0.05% Tween 20 in PBS, pH 7.4
- Diluent - refer to the ELISA Protocol on the antibody insert for exact formulation as important differences occur
- Detection System - *e.g.* streptavidin-HRP (R&D Systems, Catalog # DY998), Color Reagent A (H_20_2) and Color Reagent B (TMB) (R&D Systems, Catalog # DY999)
- Stop Solution - based on detection system

ELISA Protocol

Plate Preparation

1. Transfer 100 µL/well of the capture antibody (diluted to the appropriate concentration in PBS, use immediately) to an ELISA plate. Seal plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process for a minimum of 3 washes. Wash by forcefully filling each well with Wash Buffer (400 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating and by inverting the plate and blotting it against clean paper toweling.
3. Block plates by adding 300 µL of recommended Blocking Buffer (see package insert) to each well. Incubate at room temperature for a minimum of 1 hour.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition. Alternatively, the blocking buffer can be aspirated after step 3 and the plates can be dried under vacuum. When sealed with desiccant, the plates can be stored at 4°-8° C for at least 2 months.

Assay Procedure

1. Dilutions of unknowns and standards should be carried out in polypropylene tubes. Add 100 µL of sample or standards in an appropriate diluent, per well. Mix by gently tapping the plate frame for 1 minute. Cover with an adhesive strip and incubate 2 hours at room temperature.
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Add 100 µL of the biotinylated detection antibody, diluted in appropriate diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Add 100 µL Streptavidin-HRP (R&D Systems, Catalog # DY998, dilute according to the directions on the vial label) to each well. Cover the plate and incubate for 20 minutes at room temperature. An alternate detection system may be used. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of Plate Preparation.
7. Add 100 µL Substrate Solution (R&D Systems, Catalog # DY999) to each well. Incubate for 20 - 30 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 µL Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density (O.D.) of each well within 30 minutes. If using R&D Systems Catalog # DY999 or TMB, set the microtiter plate reader to 450 nm. If wavelength correction is available, set to 540 or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plates. Readings made directly at 450 nm without correction may be higher and less accurate.

Calculation of Results

Manual

The values of the unknown samples are assigned in relation to the standard curve. If data reduction software is not available, calculate assay results by averaging the duplicate readings and subtracting the zero standard optical density (O.D.) from the sample O.D. Construct a standard curve by plotting the standard O.D. points by hand and drawing a best fit or point-to-point curve. Plotting the data using log/log or semi-log paper is acceptable. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor. A standard curve should be generated for each set of samples assayed.

Automated

The values of the unknown samples are assigned in relation to the standard curve. Most labs have plate reader software or other software that allows various methods of curve fitting to be tried. It is recommended that various methods (*e.g.*, linear, semi-log, log/log, 4 parameter logistic) be tried to see which curve best fits the data. One way to determine if the curve fit is correct is to backfit the standard curve O.D. values. To do this, first plot the standard curve. Next, treat standards as unknowns and interpolate the O.D. values from your standard curve. They should read close to the expected values (+/-10%). Use the data reduction method that gives the best correlation (r) value and backfit.

Assay Optimization

Once an acceptable standard curve has been obtained using the recommended protocol and reagent concentrations, optimize the assay to meet performance requirements.

There are many parameters which influence the results obtained in an ELISA. These include: antibody quality and concentrations, incubation times, incubation temperatures, detection reagent quality and concentration, and substrate type and quality. For this section, it is assumed that all recommended reagents are being used.

Antibody concentration - the best way to determine the optimal capture and detection antibody concentrations is to perform a grid experiment. A grid experiment provides a method to test many antibody pair concentrations using only one plate. Antibody starting concentrations will vary depending on antibody type (monoclonal versus polyclonal) used for capture and detection, see Table 1. Refer to the product inserts for capture and detection antibody types as well as recommended starting concentrations.

Table 1. Recommended antibody starting concentrations

	Monoclonal Capture/ Polyclonal Detection	Monoclonal Capture/ Monoclonal Detection	Polyclonal Capture/ Polyclonal Detection
Capture Concentration	1, 2, 4 and 8 µg/mL	0.5, 1, 2 and 4 µg/mL	0.2, 0.4 and 0.8 µg/mL
Detection Concentration	50, 100, 200 and 400 ng/mL	0.25, 0.5, 1 and 2 µg/mL	50, 100, 200 and 400 ng/mL

Assay Optimization *cont.*

To form the grid, divide a 96-well plate into 4 quadrants. See Figure 2 for an example of a monoclonal capture-polyclonal detection grid experiment. The 6 columns in each quadrant represent capture antibody concentrations, the 4 rows in each quadrant represent standard curve points, and each of the 4 quadrants represents a different detection antibody concentration. Each quadrant is a "mini-grid", identifying different capture antibody and standard concentrations at one particular detection antibody concentration. In the grid experiment in Figure 2, each quadrant contains all the possible combinations of capture antibody at 1, 2 and 4 µg/mL and standard curve points of ∅ (Diluent stated on the product insert), 1000, 2000, and 4000 pg/mL, at one detection antibody concentration.

From the multiple combinations of antibody pair concentrations illustrated on the grid, select the concentrations that give the best signal to noise ratio. The ∅ standard points give the "noise" or the background value that can be expected at each of the antibody pair concentrations. The 1000, 2000 and 4000 pg/mL standard curve points give the "signal" resulting from each of the many antibody pair concentrations. Select the highest signal to noise ratio that still gives an acceptable background. A signal to noise ratio of at least 10 is excellent, but the ratio should be at least five.

Figure 2. Grid experiment for monoclonal capture-polyclonal detection assay

	50 ng/mL detection						100 ng/mL detection					
	1	2	3	4	5	6	7	8	9	10	11	12
	1 µg/mL capture	1 µg/mL capture	2 µg/mL capture	2 µg/mL capture	4 µg/mL capture	4 µg/mL capture	1 µg/mL capture	1 µg/mL capture	2 µg/mL capture	2 µg/mL capture	4 µg/mL capture	4 µg/mL capture
A	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅
B	1000 pg/mL standard	1000	1000	1000	1000	1000	1000 pg/mL standard	1000	1000	1000	1000	1000
C	2000 pg/mL standard	2000	2000	2000	2000	2000	2000 pg/mL standard	2000	2000	2000	2000	2000
D	4000 pg/mL standard	4000	4000	4000	4000	4000	4000 pg/mL standard	4000	4000	4000	4000	4000
E	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅	∅
F	1000 pg/mL standard	1000	1000	1000	1000	1000	1000 pg/mL standard	1000	1000	1000	1000	1000
G	2000 pg/mL standard	2000	2000	2000	2000	2000	2000 pg/mL standard	2000	2000	2000	2000	2000
H	4000 pg/mL standard	4000	4000	4000	4000	4000	4000 pg/mL standard	4000	4000	4000	4000	4000
	200 ng/mL detection						400 ng/mL detection					

Assay Optimization *cont.*

Background - <0.2 O.D. units. Factors that influence background include: blocking reagent, capture and detection antibody concentrations, detection system, incubation times, diluents, and washing technique.

Curve height - preferably above 1.0, usually between 1.0 and 3.0 O.D. units. Factors that influence curve height include: capture and detection antibody concentrations (see grid experiment in Figure 2), incubation times and temperatures, detection system concentration, avidity of antibodies for antigens, pH, diluents, and quality of reader.

Detection system - assay sensitivity may increase with increasing detection reagent concentration or alternate detection system. However, this may result in higher background readings.

Dilution of serum and plasma samples - serum and plasma samples may require a dilution of at least 2-fold in an appropriate buffer to overcome matrix effects. Empirically determine the dilution of the samples required to result in linearity of dilution. When diluting samples, remember that the diluent used for the standard curve should be the same as that used for samples. If samples are diluted, include the appropriate dilution factor when calculating results.

BSA - bovine serum albumin, used as a blocking and carrier protein. Since different grades of BSA exist and may contribute to background, an ELISA grade BSA should be chosen and validated.

Incubation temperatures - the sample and detection antibody incubations should be performed at room temperature. Sample incubation overnight at 4° C or 1 hour at 37° C may increase assay sensitivity, but may also increase the background.

Incubation times - sensitivity may be increased with a longer incubation time at room temperature. Be aware that the top of the curve may flatten out and become unusable, limiting the assay range. Additionally, background may increase.

Interfering substances - it is important to be aware of the possible presence of interfering substances such as heterophilic antibodies or rheumatoid factors. Please refer to *The Immunoassay Handbook*, edited by David Wild, Nature Publishing Group, copyright 2001, for suggestions on how to control these substances.

Reagent reconstitution and storage conditions - reconstitution and storage instructions provided with each reagent must be followed to ensure proper reagent perfomance.

Sample preparation and storage - while not every analyte has the same stability within a given matrix, there are general precautions which should be followed. Samples that are not used immediately after preparation should be stored in single use aliquots at -70° C. A -20° C freezer may be acceptable, depending on analyte, if it is a manual defrost freezer. It is best if the samples contain carrier protein. Multiple freeze-thaw cycles should be avoided.

Samples/standard volume - use of a larger sample/standard size (200 µL per well vs. 100 µL per well) may increase sensitivity.

Substrate - substrates can vary. However, choosing an alternate substrate will require additional assay condition optimization. Some substrates require a longer incubation time to get the curve to a reasonable height. If the substrate is functioning as expected, sensitivity

Assay Optimization *cont.*

may be enhanced by increasing incubation time. Monitor the plate as it is developing to avoid excessively high backgrounds. Typically, the incubation time ranges from 10 to 30 minutes. Use the correct filters required to read the appropriate wavelength for the substrate chosen. This information is available from the substrate vendor.

Use of a shaker - at room temperature may increase sensitivity. Shakers may be used for some or all of the incubation steps. Incubation times would have to be determined empirically.

Washing - follow washing instructions given in the ELISA Protocol, page 4. Insufficient washing can result in high coefficients of variation (CVs), high background, and poor results.

Sensitivity - varies for each antibody pair. Sensitivity is defined by reliable discrimination from the zero standard. Factors which influence sensitivity include: capture and detection antibody concentrations (refer to the grid experiment shown in Figure 2), incubation times and temperatures, avidity of antibodies for antigens, sample/standard volumes, pH, diluents and wash buffer formulation. However, there is a limit to the sensitivity that can be achieved with each antibody pair.

Troubleshooting Guide

Problem	Possible Cause	Solution
High Background	• Insufficient washing	• See washing procedure on page 4 • Increase number of washes • Add a 30 second soak step inbetween washes
	• Too much streptavidin-HRP or equivalent	• Check dilution, titrate if necessary
	• Insufficient blocking	• Check blocking solution calculations • Increase blocking time
	• Incubation times too long	• Reduce incubation times
	• Interfering substances in samples or standards	• Run appropriate controls
	• Buffers contaminated	• Make fresh buffers

Troubleshooting Guide *cont.*

Problem	Possible Cause	Solution
No Signal	• Reagents added in incorrect order, or incorrectly prepared	• Repeat assay • Check calculations and make new buffers, standards, *etc.* • Review protocol
	• Contamination of HRP with azide	• Use fresh reagents
	• Not enough antibody used	• Increase concentration
	• Standard has gone bad (if there is a signal in the sample wells)	• Check that standard was handled according to directions. • Use new vial
	• Buffer containing FCS used to reconstitute antibodies	• Requalify your reagents of choice
	• Capture antibody did not bind to plate	• Use an ELISA plate (not a tissue culture plate) • Dilute in PBS without additional protein
	• Buffers contaminated	• Make fresh buffers
Too much signal - whole plate turned uniformly blue	• Insufficient washing/washing step skipped - unbound peroxidase remaining	• See washing procedure on page 4
	• Substrate Solution mixed too early and turned blue	• Substrate Solution should be mixed and used immediately
	• Too much streptavidin-HRP	• Check dilution, titrate if necessary
	• Plate sealers or reagent reservoirs reused, resulting in presence of residual HRP. This will turn the TMB blue non-specifically	• Use fresh plate sealer and reagent reservoir for each step
	• Buffers contaminated with metals or HRP	• Make fresh buffers

Troubleshooting Guide *cont.*

Problem	Possible Cause	Solution
Standard curve achieved but poor discrimination between points (low or flat curve)	• Not enough streptavidin-HRP	• Check dilution, titrate if necessary
	• Capture antibody did not bind well to plate	• Use an ELISA plate (not a tissue culture plate) • Dilute in PBS without additional protein
	• Not enough detection antibody	• Check dilution, titrate if necessary
	• Plate not developed long enough	• Increase Substrate Solution incubation time • Use recommended brand of Substrate Solution
	• Incorrect procedure	• Go back to General ELISA Protocol; eliminate modifications, if any
	• Improper calculation of standard curve dilutions	• Check calculations, make new standard curve
Poor Duplicates	• Insufficient washing	• See washing procedures on page 4 • If using an automatic plate washer, check that all ports are clean and free of obstructions, add a 30 second soak step and rotate plate halfway through the wash
	• Uneven plate coating due to procedural error or poor plate quality (can bind unevenly)	• Dilute in PBS without additional protein • Check coating and blocking volumes, times and method of reagent addition. Check plate used • Use an ELISA plate (not a tissue culture plate)
	• Plate sealer reused	• Use a fresh plate sealer for each step
	• No plate sealers used	• Use plate sealers
	• Buffers contaminated	• Make fresh buffers

Troubleshooting Guide *cont.*

Problem	Possible Cause	Solution
Poor assay to assay reproducibility	• Insufficient washing	• See washing procedure on page 4 • If using an automatic plate washer, check that all ports are clean and free of obstructions
	• Variations in incubation temperature	• Adhere to recommended incubation temperature • Avoid incubating plates in areas where enviromental conditions vary
	• Variations in protocol	• Adhere to the same protocol from run to run
	• Plate sealer reused, resulting in presence of residual HRP which will turn the TMB blue	• Use fresh plate sealer for each step
	• Improper calculation of standard curve dilutions	• Check calculations, make new standard curve • Use internal controls
	• Buffers contaminated	• Make fresh buffers
No signal when a signal is expected, but standard curve looks fine	• No cytokine in sample	• Use internal controls • Repeat experiment, reconsider experimental parameters
	• Sample matrix is masking detection	• Dilute samples at least 1:2 in appropriate diluent, or preferably, do a series of dilutions to look at recovery
Samples are reading too high, but standard curve looks fine	• Samples contain cytokine levels above assay range	• Dilute samples and run again

Troubleshooting Guide *cont.*

Problem	Possible Cause	Solution
Very low readings across the plate	• Incorrect wavelengths	• Check filters/reader
	• Insufficient development time	• Increase development time
	• Coated plates are old and have gone bad	• Coat new plates
	• Capture antibody did not bind to the plate	• Use an ELISA plate (not a tissue culture plate) • Dilute in PBS without additional protein
	• Buffer containing FCS used to reconstitute antibodies	• Requalify your reagents of choice
Green color develops upon addition of stop solution when using streptavidin-HRP	• Reagents not mixed well enough in wells	• Tap plate
Edge Effects	• Uneven temperatures around work surface	• Avoid incubating plates in areas where environmental conditions vary • Use plate sealers
Drift	• Interrupted assay set-up	• Assay set-up should be continuous - have all standards and samples prepared appropriately before commencement of the assay
	• Reagents not at room temperature	• Ensure that all reagents are at room temperature before pipetting into the wells unless otherwise instructed in the antibody inserts

Technical Services Troubleshooting Questionnaire

Please consult the ELISA Development Guide to help resolve a problem before submitting this form. Fax the completed Troubleshooting Information Sheets to ***Technical Services at 612-379-6580 in the United States or +44 (0)1235 551129 in the United Kingdom.***

Products Used (fill in the blank spaces)

Plates

Manufacturer:_____

Type: _____

Blocking Buffer

Buffer components: _____

Date made: _____

Time/Temperature:_____

Blocking Agent

Manufacturer:_____

Grade: _____

Secondary Reagent

Manufacturer:_____

Description (enzyme): _____

Substrate

Manufacturer:_____

Description: _____

Expiration Date: _____

Wavelength Used: _____

Wash Buffer

Type: _____

Number of washes: _____

Date buffer was made:_____

Method of washing:

- ❑ wash bottle
- ❑ multi-channel pipette
- ❑ multi-channel manifold
- ❑ automated plate washer

Reconstitution of Reagents

	Capture Antibody	Detection Antibody	Standard
Catalog Number			
Lot Number			
Reconstitution Volume			
Reconstitution Buffer			
Reconstitution Date			
Storage			

Working Concentrations

	Capture Antibody	Detection Antibody	Standard	Secondary Reagent
Diluting Buffer				
Working Concentration				
Time Prior to Addition				
Incubation Time				
Incubation Temperature				

Sample Type:

_____

Were clean pipette tips and buffer reservoirs used at each step of the assay?

❑ Yes

❑ No

Did other assays performed on the same day, using the same secondary/substrate system, work?

❑ Yes

❑ No

Was the procedure followed according to the product insert?

- ❑ Yes
- ❑ No

If not, what was done differently?

_____

_____

_____

_____

_____

Summary of problem:

_____

_____

_____

_____

_____

Please attach a copy of your labeled, raw (non-zero subtracted) O.D. printout.

Glossary

Analyte - the molecule being measured

Antibody - immunoglobulin with specific affinity for a particular antigen

Background - the amount of signal obtained using all reagents except the analyte

Blocking - the use of a reagent to bind non-specific sites on an ELISA plate

BSA - Bovine serum albumin, a commonly used carrier protein

Capture antibody - a primary antibody coated onto the microplate

Cell culture supernates - cell culture medium containing substances produced by cells

Curve height - the optical density (O.D.) of the highest point on the standard curve

Detection antibody - a secondary antibody, often conjugated to biotin or HRP

Detection system - a reporter system usually employing an enzyme and substrate whose end reaction product is detected and correlated to the concentration of analyte being measured

ELISA - Enzyme-Linked-Immuno-Sorbant-Assay, a quantitative assay which utilizes the affinity of antibodies for their antigens and an enzyme which serves as a part of the detection method

FCS - Fetal calf serum, a commonly used reagent to mimic the matrix of serum and plasma samples

HRP - horseradish peroxidase, a commonly used enzyme to modify substrate resulting in color development

Matrix effect - a matrix effect describes an inaccurate result due to a substance in the matrix that prevents full recovery of analyte contained in the sample. All antigens being assayed are contained in a complex solution known as the matrix. The matrix can be simple (PBS) or complex (serum). In general, the more complex the matrix, the more likely a matrix effect may be encountered. Refer to *The Immunoassay Handbook*, edited by David Wild, Nature Publishing Group, copyright 2001, for more information on some types of interfering substances

Microtiter plate - for matched antibody pairs, a 96-well microplate plate with flat-bottomed wells, designed specifically for use in ELISA

Optical density (O.D.) - the absorbance of a particular substance at a specified wavelength

Plasma - a blood component obtained by collection of blood with an anticoagulant (*e.g.* Heparin or EDTA), which is centrifuged to remove red blood cells (RBC), resulting in a sample that has not had the release of clotting factors

Plate sealer - an adhesive backed plastic sheet used to protect plates

Sensitivity - the ability of a kit to discriminate between small differences of concentrations of the cytokine/analyte being measured

Serum - a blood component obtained by allowing the blood sample to clot and removal of that clot

Solid phase - a 96-well microplate to which a capture antibody is bound

Standard - a defined, calibrated sample of the analyte being assayed, which is used to set up a curve of known amounts against which to measure the amount of analyte in the unknown samples

Stop solution - a solution which stops the enzymatic reaction of the detection system (*e.g.* sulfuric acid stops the HRP/TMB-peroxide reaction and changes the blue color to yellow, which is then read optimally at A_{450})

Substrate solution - a solution containing a substance which is cleaved by an enzyme, resulting in a color change

TMB - tetramethylbenzidine, a dye reagent used in conjunction with peroxide resulting in a blue color when oxidized by an enzyme