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Not for Sale or Distribution in the United States of America

DENGUE IgM CAPTURE ELISA

Cat No. E-DEN01M / E-DEN01M05

INTENDED USE

The Panbio Dengue IgM Capture ELISA is for the qualitative detection of IgM antibodies to dengue antigen in serum, as an aid in the clinical laboratory diagnosis of patients with clinical symptoms consistent with dengue fever. The Panbio Dengue IgM Capture ELISA should be used in conjunction with other dengue serology.

INTRODUCTION

Dengue, a flavivirus, is found in large areas of the tropics and sub-tropics. Transmission is by mosquito, principally *Aedes aegypti* and *Aedes albopictus*. Dengue virus infection causes a spectrum of clinical manifestations ranging from unapparent to fatal haemorrhagic disease. Classic dengue or breakbone fever is characterised by the sudden onset of fever, intense headache, myalgia, arthralgia and rash. A diphasic febrile course is common as is insomnia and anorexia with loss of taste or bitter taste. Dengue haemorrhagic fever and dengue shock syndrome are severe complications often associated with a second serotype infection.

Detection of IgM antibodies to dengue virus by ELISA is a valuable procedure, particularly in second and subsequent infections where the occurrence of complications is high. Serum IgM antibodies can be detected from dengue patients as early as three to five days after the onset of fever and generally persist for 30 - 90 days, although detectable levels may be present eight months post-infection.

PRINCIPLE

Serum antibodies of the IgM class, when present, combine with anti-human IgM antibodies attached to the polystyrene surface of the microwell test strips. A concentrated pool of Dengue 1-4 Antigens is diluted to the correct working volume with Antigen Diluent. The antigens are produced using an insect cell expression system and immunopurified utilising a specific monoclonal antibody. An equal volume of the HRP Conjugated Monoclonal Antibody (MAb) is added to the diluted antigen, which allows the formation of antigen-MAb complexes. Residual serum is removed from the assay plate by washing, and complexed antigen-MAb is added to the assay plate. After incubation, the microwells are washed and a colourless substrate system, tetramethylbenzidine / hydrogen peroxide (TMB Chromogen) is added. The substrate is hydrolysed by the enzyme and the chromogen changes to a blue colour. After stopping the reaction with acid, the TMB becomes yellow. Colour development is indicative of the presence of anti-dengue IgM antibodies in the test sample.

MATERIALS PROVIDED
Note: E-DEN01M05 = E-DEN01M x 5

1. *Anti-human IgM Coated Microwells* - (12 x 8 wells) Microwells are coated with anti-human IgM antibodies. Ready for use. Unused microwells should be resealed immediately and stored in the presence of the desiccant. Stable at 2-8ºC until expiry.
2. *Dengue 1-4 Antigens (Recombinant)* - One **Clear**-capped vial, 150 µL **(Blue)** concentrated dengue viral antigens 1, 2, 3 and 4. Unused diluted antigen must be discarded. Concentrated antigen is stable at 2-8ºC until expiry.
3. *Wash Buffer (20x)* - One bottle, 60 mL of 20x concentrate of phosphate buffered saline (pH 7.2-7.6) with Tween 20 and preservative (0.1% Proclin™). Crystallisation may occur at low temperatures. To correct, incubate at 37ºC until clear. Mix well. Dilute one part Wash Buffer with 19 parts of distilled water. Diluted buffer may be stored for one week at 2-25ºC.
4. *Sample Diluent* - Two bottles, 50 mL **(Pink)**. Ready for use. Tris buffered saline (pH 7.2-7.6) with preservatives (0.1% Proclin™) and additives. Stable at 2-8ºC until expiry.
5. *Antigen Diluent* – One bottle, 50 mL **(Clear)**. Ready for use. Phosphate Buffer containing preservatives (0.1% Proclin™ and 0.005% gentamycin). Stable at 2-8ºC until expiry.
6. *HRP Conjugated Monoclonal Antibody Tracer* - One bottle, 7 mL **(Yellow)**. Ready for use. Horseradish peroxidase conjugated monoclonal antibody tracer with preservative (0.1% Proclin™) and protein stabilisers. Stable at 2-8ºC until expiry.
7. *TMB Chromogen (TMB)* - One bottle, 15 mL. Ready for use. A mixture of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide in a citric-acid citrate buffer (pH 3.5-3.8). Stable at 2-8ºC until expiry.
8. *Positive Control* - One **Black**-capped vial, 200 µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8ºC until expiry.
9. *Calibrator* - One **Orange**-capped vial, 400 µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8ºC until expiry.
10. *Negative Control* - One **White**-capped vial, 200 µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8ºC until expiry.
11. *Stop Solution* - One **Red**-capped bottle, 15 mL. Ready for use. 1M Phosphoric acid. Stable at 2-25ºC until expiry.

Proclin™ 300 is a registered trademark of Rohm and Haas Company.

(Xn - Harmful) Control and Calibrator Safety Precaution:
Concentration of sodium azide in these components is classified as harmful and subject to the following risk phrases (R22, R32) "Harmful if swallowed" and "Contact with acids liberates very toxic gas."

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

(1) Accurate adjustable micropipettors with disposable pipette tips (5-1000 µL capacity)
(2) Deionised water
(3) Microplate washing system
(4) Microplate reader with 450 nm filter
(5) Timer
(6) Graduated cylinder
(7) Flask
(8) Test tubes or microtitre plate for serum dilutions
(9) Glass or plastic tubes or vials for diluting antigen

PRECAUTIONS
FOR *IN VITRO* DIAGNOSTIC USE

(i) All human source material used in the preparation of controls has been tested for antibody to human immunodeficiency virus 1 & 2 (HIV 1&2), hepatitis C (HCV) as well as hepatitis B surface antigen and found to be negative. However no test method can offer complete assurance and all human controls and antigen should be handled as potentially infectious material. The Centers for Disease Control and Prevention and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2[1].
(ii) This test should be performed on serum only. The use of whole blood, plasma or other specimen matrix has not been established.
(iii) Icteric or lipaemic sera, or sera exhibiting haemolysis or microbial growth should not be used.
(iv) Do not heat-inactivate sera.
(v) All reagents must be equilibrated to room temperature (20-25ºC) before commencing the assay. The assay will be affected by temperature changes. Do not remove microwells from closed bag until they have reached room temperature (20-25ºC).

(vi) Dispense reagents directly from bottles using clean pipette tips. Transferring reagents may result in contamination.

(vii) Unused microwells should be resealed immediately and stored in the presence of desiccant. Failure to do this may cause erroneous results.

(viii) Substrate System:
- (a) As TMB is susceptible to contamination from metal ions, do not allow the substrate system to come into contact with metal surfaces.
- (b) Avoid prolonged exposure to direct light.
- (c) Some detergents may interfere with the performance of the TMB.
- (d) The TMB may have a faint blue colour. This will not affect the activity of the substrate or the results of the assay.

(ix) Some kit components contain sodium azide, which may react with lead or copper plumbing to form highly explosive metal azide compounds. When disposing of these reagents through plumbing fixtures, flush with a large volume of water to prevent azide build-up in drains.

(x) Sodium azide inhibits conjugate activity. Clean pipette tips must be used for the conjugate addition so that sodium azide is not carried over from other reagents.

FOR FURTHER SAFETY INFORMATION PLEASE REFER TO THE MATERIAL SAFETY DATA SHEET (MSDS) AVAILABLE FROM PANBIO.

SPECIMEN COLLECTION AND PREPARATION

Blood obtained by venipuncture should be allowed to clot at room temperature (20-25ºC) and then centrifuged according to the Clinical and Laboratory Standards Institute (CLSI – Approved Standard - Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture, H3-A5, 2003).

The serum should be separated as soon as possible and refrigerated (2-8ºC) or stored frozen (≤ -20ºC) if not tested within two days. Self-defrosting freezers are not recommended for storage. The use of icteric sera or sera exhibiting haemolysis, lipaemia or microbial growth is not recommended. The CLSI provides recommendations for storing blood specimens, (Approved Standard - Procedures for the Handling and Processing of Blood Specimens, H18-A3, 2004).

TEST PROCEDURE

Note: Ensure all reagents are equilibrated to room temperature (20-25ºC) before commencing assay. Performing the assay outside the time and temperature ranges provided may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.

Serum Predilution

(i) Remove the required number of microwells from the foil sachet and insert into strip holder. Five microwells are required for Negative Control (N), Positive Control (P) and Calibrator (CAL) in triplicate. Ensure the remaining unused microwells are sealed tightly in the foil sachet.

(ii) Using suitable test tubes or a microtitre plate, dilute the Positive Control, Negative Control, Calibrator, and patient samples:
- (a) To 10 µL serum add 1000 µL of Sample Diluent. Mix well.

Alternatively,
- (b) To 10 µL serum add 90 µL of Sample Diluent. Take 20 µL of the diluted serum and add 180 µL Sample Diluent. Mix well.

ELISA PROCEDURE

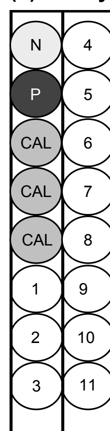
See attached figure for summary of method.

(a) Antigen

(i) Determine the required number of wells for your assay. Dilute the antigen 1/250 using the Antigen Diluent. It is recommended, as a minimum, to dilute 10 µL of antigen into 2.5 mL of Antigen Diluent. This is sufficient for up to five strips (40 wells). A volume of 0.5 mL of diluted antigen is required per strip. Once the antigen is added to the Antigen Diluent, the solution becomes a pale blue colour. Ensure the remaining unused concentrated antigen remains at 2-8ºC.

(ii) Remove the required volume of diluted antigen and mix with an equal volume of MAb Tracer in a clean glass or plastic vial. Gently mix the antigen-MAb Tracer solution and leave at room temperature (20-25ºC) until required. Discard the unused diluted antigen.

(b) Assay Plate



(iii) Within 10 minutes after mixing the MAb Tracer and diluted antigen, pipette 100 µL diluted patient sample and Controls into their respective microwells of the assay plate.

(iv) Cover the plate and incubate for 1 hour at 37ºC ±1ºC.

(v) Wash six (6) times with diluted Wash Buffer. (Refer to washing procedure).

(vi) Mix the antigen-MAb tracer solution before transfer. Pipette 100 µL of antigen-MAb complexes from the antigen vial to the appropriate wells of the assay plate.

(vii) Cover plate and incubate for 1 hour at 37ºC ±1ºC.

(viii) Wash six (6) times with diluted Wash Buffer (refer to washing procedure).

(ix) Pipette 100 µL TMB into each well.

(x) Incubate for 10 minutes at room temperature (20-25ºC), timing from the first addition. A blue colour will develop.

(xi) Pipette 100 µL of Stop Solution into all wells in the same sequence and timing as the TMB addition. Mix well. The blue colour will change to yellow.

(xii) Within 30 minutes read the absorbance of each well at a wavelength of 450 nm with a reference filter of 600-650 nm.

Note: If a dual wavelength spectrophotometer is available, set the reference filter between 600-650 nm. Reading the microwells at 450 nm without a reference filter may result in higher absorbance values due to background.

WASHING PROCEDURE

Efficient washing to remove uncomplexed sample or components is a critical requirement of the ELISA procedure.

A. Automated Plate Washer

(1) Completely aspirate all wells.

(2) Fill all wells to rim (350 µL) during wash cycle.

(3) On completion of six (6) washes, invert plate and tap firmly on absorbent paper towel to ensure all Wash Buffer is removed.

(4) Automated plate washers must be well maintained to ensure efficient washing. Manufacturer's cleaning instructions should be followed at all times.

B. Manual Washing

(1) Discard contents of plate in appropriate waste container.

(2) Fill wells with Wash Buffer using a suitable squeeze bottle. Avoid bubbling of Wash Buffer as this may reduce wash efficiency. Discard Wash Buffer from wells immediately.

(3) Refill wells with Wash Buffer and discard immediately.

(4) Repeat step (3) another four times. This will make a total of six (6) washes with Wash Buffer.

(5) After the final wash, discard contents of wells and tap the plate on absorbent paper towel to ensure all Wash Buffer is removed.

QUALITY CONTROL

Each kit contains Calibrator, Positive and Negative Controls. Acceptable values for these are found on the accompanying specification sheet. The Negative and Positive Controls are intended to monitor for substantial reagent failure. The Positive Control will not ensure precision at the assay cut-off. The test is invalid and should be repeated if the absorbance readings of either the Controls or the Calibrator do not meet the specifications. If the test is invalid, patient results cannot be reported.

Quality Control (QC) requirements must be performed in conformance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard QC procedures.

It is recommended that the user refer to CLSI C24-A and 42 CFR 493.1256 for guidance on appropriate QC practices.

CALCULATIONS

IMPORTANT NOTE: The calibration factor is batch specific and is detailed in the specification sheet. Obtain the calibration factor value before commencing calculations.

(1) Calculate the average absorbance of the triplicates of the Calibrator and multiply by the calibration factor. This is the Cut-off Value.

(2) An index value can be calculated by dividing the sample absorbance by the Cut-off Value (calculated in step (1) above).

Alternatively,

(3) Panbio Units can be calculated by multiplying the index value (calculated in step (2) above) by 10.

Index Value = Sample Absorbance / Cut-off Value

Example: Sample A Absorbance = 0.949
Sample B Absorbance = 0.070

Mean absorbance of Calibrator = 0.802
Calibration Factor = 0.62
Cut-off Value = 0.802 x 0.62 = 0.497

Sample A (0.949/0.497) = 1.91 Index value
Sample B (0.070/0.497) = 0.14 Index value

Panbio Units = Index Value X 10

Sample A 1.91 X 10 = 19.1 Panbio Units
Sample B 0.14 X 10 = 1.4 Panbio Units

INTERPRETATION OF RESULTS

The cut-off has been determined using endemic populations from South East Asia / South America and a local population from Queensland, Australia, of 208 characterised negative (208/409), 91 positive (91/409) and 110 disease controls samples (110/409). The cut-off was determined by two-graph receiver operating characteristic analysis (TG-ROC)[2,3]. A cut-off ratio of 1.0 was selected based on the optimal F Value for sensitivity and specificity.

Diagnosis of Dengue Infection: The Dengue IgM Capture ELISA determines the level of IgM antibodies to dengue in a patient's serum. A positive result (> 11 Panbio Units) is indicative of either an active primary or secondary dengue infection. If differentiation between primary and secondary infection is required, the Dengue Duo (E-DEN01D) ELISA should be used.

INDEX	PANBIO UNITS	RESULT
<0.9	<9	Negative
0.9 – 1.1	9 – 11	Equivocal
>1.1	>11	Positive

RESULT	INTERPRETATION
Negative	No detectable IgM antibody. The result does not rule out dengue infection. An additional sample should be tested in 7-14 days if early infection is suspected. Other dengue assays should be performed to rule out acute infection.
Equivocal	Equivocal samples should be repeated. Samples that remain equivocal after repeat testing should be repeated by an alternative method or another sample should be collected.
Positive	Presence of detectable IgM antibody. Other dengue serology assays should be performed to confirm dengue infection.

The following is a recommended method for reporting the results obtained: "The following results were obtained with the Panbio Dengue IgM Capture ELISA. Values obtained with different methods may not be used interchangeably. The magnitude of the measured result, above the cut-off, is not indicative of the total amount of antibody present." The result should be reported as positive, negative or equivocal, and not as a numerical value.

TEST LIMITATIONS

1. The clinical diagnosis must be interpreted with clinical signs and symptoms of the patient. The results from this kit are not by themselves diagnostic and should be considered in association with other clinical data and patient symptoms.
2. Population seroepidemiology may vary over time in different geographical regions. Consequently, the cut-off may require adjustment based on local studies.
3. Screening of the general population should not be performed. The positive predictive value depends on the likelihood of the virus being present. Testing should only be performed on patients with clinical symptoms or when exposure is suspected.
4. Serological cross-reactivity across the flavivirus group is common (i.e. between dengue 1, 2, 3 & 4, Murray Valley encephalitis, Japanese encephalitis, Yellow fever and West Nile viruses). These diseases must be excluded before confirmation of diagnosis.
5. Heterophilic antibodies are a well-recognised cause of interference in immunoassays[4]. These antibodies to animal IgG may cross-react with reagent antibodies and generate a false positive signal. This must be excluded before confirmation of diagnosis.
6. The performance characteristics have not been established for visual result determination.
7. This assay employs insect expressed proteins. The cross-reactivity or interference of human anti-insect antibodies is unknown with the assay's results.
8. All sera demonstrating a positive result by the Panbio Dengue IgM Capture ELISA test should be referred to a reference laboratory for confirmation of positivity and epidemiological recording.
9. The Panbio Dengue Duo ELISA (E-DEN01D) and Panbio Dengue IgG Capture-ELISA (E-DEN02G) are most convenient for IgG determination. It uses the same capture method as the IgM ELISA, so both IgM and IgG are determined using a common method and common serum dilution.

EXPECTED VALUES

Primary dengue infection is characterised by the presence of significant or rising levels of IgM 3-5 days after the onset of infection, which can persist for 3-5 months. Secondary infection is characterised by elevation of specific IgG 1-2 days after the onset of infection and in the majority of cases (>70%) is accompanied by elevation of IgM. In early infections and some secondary infections detectable levels of IgM antibodies may be low. Some patients may not produce detectable levels of antibody within the first seven to ten days after infection. Where symptoms persist, we recommend that patients be re-tested seven days after the first specimen.

PERFORMANCE CHARACTERISTICS

255 characterised sera were tested on the Panbio Dengue IgM Capture ELISA as part of an in-house study. The sera included 83 endemic seronegative samples, 57 samples from patients with primary dengue infection, and 115 samples from patients with secondary dengue infection. The Panbio Dengue IgM Capture ELISA results were compared to the dengue status of the sera to determine sensitivity, specificity, and agreement of the assay relative to the dengue serological status. The data is summarised in Table 1.

Table 1
Dengue IgM Serological Sensitivity and Specificity of Panbio ELISA versus Dengue Status

Dengue Status	Panbio ELISA Positive	Equivocal[a]	Negative	Total
Seronegative IgM (-) by ELISA	0	0	83	83
Primary Infection IgM (+) by HAI	41	1	1	43
Primary Infection IgM (+) by ELISA	13	0	1	14
Secondary Infection IgG (+) by HAI	41	11	34	86
Secondary Infection IgG (+) by ELISA	23	0	6	29
Total	**118**	**12**	**125**	**255**

			95% CI*
Serological Sensitivity (Primary)	= 54/57	= 94.7%	85.4 – 98.9%
Serological Sensitivity (Secondary)	= 64/115	= 55.7%	46.6 – 64.7%
Serological Specificity (Negative)	= 83/83	= 100%	95.7 – 100%
Serological Agreement (Excluding Secondary sera)	= 137/140	= 97.9%	93.7 – 99.6%

[a] Retesting of equivocal samples was not conducted, as the samples were unavailable.
*Confidence Interval

REPRODUCIBILITY

The reproducibility of the Panbio Dengue IgM Capture ELISA kit was determined by testing 7 sera 3 times each on three Panbio kit batch numbers on three different days. Within-run, between day, between batch and total precision were estimated by analysis of variance (ANOVA Type II) and are presented in Table 2.

Table 2
Panbio Dengue IgM Capture ELISA Precision Measures (Using Index Value*)

			Within		Between Day		Between Batch		Total	
Sample	**n**	***Mean**	***SD**	**CV**	***SD**	**CV**	***SD**	**CV**	***SD**	**CV**
Positive	27	2.87	0.12	4.3%	0.05	1.7%	0.62	21.6%	0.53	18.6%
Cut-off	27	1.00	0.06	5.7%	0.00	0.0%	0.00	0.0%	0.05	5.3%
Negative	27	0.36	0.06	16.7%	0.00	0.0%	0.16	45.2%	0.15	41.0%
#1	27	6.19	0.31	5.1%	0.12	1.9%	0.41	6.7%	0.48	7.7%
#2	27	5.91	0.21	3.6%	0.11	1.9%	0.52	8.8%	0.49	8.3%
#3	27	1.25	0.05	4.1%	0.00	0.0%	0.10	7.9%	0.10	7.7%
#4	27	1.33	0.07	5.4%	0.03	1.9%	0.07	5.4%	0.10	7.2%
#5	27	1.30	0.08	6.1%	0.00	0.0%	0.12	9.3%	0.13	9.8%
#6	27	0.71	0.04	6.0%	0.00	0.0%	0.01	1.7%	0.04	6.0%
#7	27	0.81	0.06	7.7%	0.00	0.0%	0.00	0.0%	0.06	7.3%

All values are calculated from Index values (Cut-off using OD)
SD = Standard Deviation; CV = Coefficient of Variation

Note: Standard Deviation results have been rounded to two decimal places for tabulation purposes.
*Index value is calculated by dividing the sample absorbance by the cut-off value.

CROSS-REACTIVITY

A panel of 115 specimens from patients with confirmed diseases other than dengue fever was tested to establish the analytical specificity of the Panbio Dengue IgM Capture ELISA. The specimens were from patients with diseases that have the potential for cross-reactivity. Each of the specimens included in the study was characterised with respect to disease diagnosis prior to analysis with the Panbio Dengue IgM Capture ELISA. Minimal cross-reactivity was observed for malaria, West Nile virus and rheumatoid factor specimens tested from the disease panel. Refer to Table 3 for a summary of the results.

Table 3
Cross-reactivity Analysis – Panbio Dengue IgM Capture ELISA

Disease Type	Total Specimens	Positive (or Equivocal*) Result
Epstein-Barr virus	10	(0/10)
Malaria	10	(1/10)
Influenza A	7	(0/7)
Influenza B	3	(0/3)
Anti-nuclear antibody	30	(0/30)
Rheumatoid factor	10	(3/10)
Hepatitis A	9	(0/9)
Leptospira	7	(0/7)
Salmonella typhi	9	(0/9)
Scrub typhus	10	(0/10)
West Nile virus	10	(2/10)*
Total	**115**	**(6/115)**

REFERENCES

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3. Greiner, M., Sohr, D. and Gobel, P. (1995). A modified ROC analysis for selection of cutoff values and the definition of intermediate results in serodiagnostic tests. *J. Immunol. Meth.* **185:**123-132.
4. Seth, J. (1991). Standardisation and Quality Assurance. In: Principle and Practice of Immunoassay, Price, C.P. and Newman, D.J. (Eds). MacMillian, London

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DENGUE IgM CAPTURE ELISA
E-DEN01M/E-DEN01M05

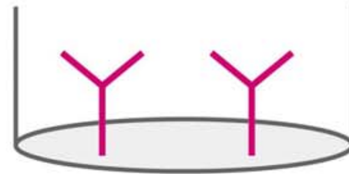
ANTIGEN-VIAL
Stabilised dengue-antigens

1. Add 10 µL of Antigen in 2.5 mL of Antigen Diluent and mix. Unused concentrated antigen should be stored at 2-8°C.

2. Remove required volume of diluted antigen and mix with an equal volume of MAb Tracer in a separate glass vial or test tube. DISCARD UNUSED DILUTED ANTIGEN.

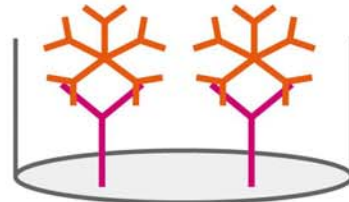
4a. Incubate 1 hour at 20-25°C.

ASSAY PLATE
α-human IgM



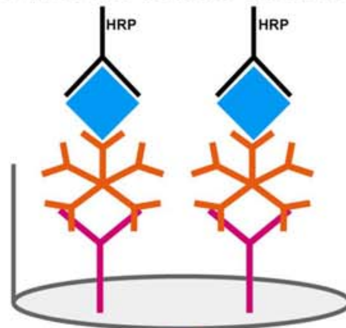
3. Add 100 µL of diluted samples and Controls to assay plate.

4b. Cover plate and incubate 1 hour at 37°C ± 1°C



5. Wash the assay plate x 6. After gentle rotation to mix the antigen-MAb solution, transfer 100 µL per well to the assay plate.

6. Cover plate and incubate 1 hour at 37°C ± 1°C



7. Wash the assay plate x 6. After the final wash, add 100 µL TMB per well and incubate at 20-25°C for 10 minutes. Stop the reaction with 100 µL Stop Solution and read at 450 nm (Reference 600 - 650 nm).