Murine IL-12 ELISA

660 040 096 **1 x 96 tests**
660 040 192 **2 x 96 tests**

INTENDED USE

The mIL-12 ELISA is an enzyme-linked immunosorbent assay for quantitative detection of murine Interleukin-12 in cell culture supernatants, murine serum, plasma or other body fluids. **The mIL-12 ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

SUMMARY

Interleukin-12 (IL-12) is a pleiotropic cytokine, formerly termed cytotoxic lymphocyte maturation factor (CLMF) or natural killer cell stimulatory factor (NKSF) (1,3,4), which is produced primarily by stimulated macrophages. IL-12 has been shown to be a proinflammatory cytokine produced by phagocytic cells (5), B cells (1,3), and other antigen - presenting cells that modulate adaptive immune responses by favoring the generation of T-helper type 1 cells (2).
IL-12 exerts a variety of biological effects on T and natural killer cells. Apart from promotion of Th1 development and its ability to promote cytolytic activity it mediates some of its physiological activities by acting as a potent inducer of interferon (IFN) gamma production and the stimulation of other cytokines from peripheral blood T and NK cells, (6,7). IFN-gamma then enhances the ability of the phagocytic cells to produce IL-12 and other proinflammatory cytokines. Thus, IL-12 induced IFN-gamma acts in a positive feedback loop that represents an important amplifying mechanism in the inflammatory response to infections (2).
Its role in directing development of a Th1 type immune response from naive T cells demonstrates its critical role in regulation of the immune response and strongly suggests its potential usefulness in cancer therapy (4).

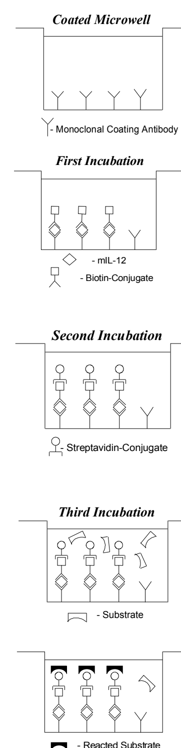
PRINCIPLES OF THE METHOD

An anti-mIL-12 monoclonal coating antibody is adsorbed onto microwells.

mIL-12 present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated monoclonal anti-mIL-12 antibody is added and binds to mIL-12 captured by the first antibody.

Following incubation unbound biotin conjugated anti-mIL-12 is removed during a wash step. Streptavidin-HRP is added and binds to the biotin conjugated anti-mIL-12. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of mIL-12 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven mIL-12 standard dilutions and mIL-12 sample concentration determined.



REAGENTS PROVIDED

REAGENTS (store at 2-8°C)	QUANTITY 1 plate	QUANTITY 2 plates	RECONSTITUTION
96-wells precoated microtiter plate	1	2	Ready-to-use
Plate covers	4	8	
mIL-12 Standard: 4000 pg/ml	2 vials	4 vials	Reconstitute with the volume of distilled water indicated on the vial.
Biotin conjugate anti mIL-12 monoclonal antibody [1,2]	1 vial	2 vials	(100 µl) Dilute 100 times in Assay buffer
Streptavidin-HRP[1,2]	1 vial	2 vials	(150 µl) Make a 1/100 dilution with Assay Buffer before use.
Assay Buffer Concentrate	1 vial	2 vials	(5 ml) 20X concentrate. Dilute in distilled water
Wash Buffer Concentrate	1 bottle	2 bottles	(50 ml) 20X concentrate. Dilute in distilled water
Substrate Solution I	1 vial	2 vials	(7 ml) Dilute with equal volume of Substrate Solution II just prior use.
Substrate Solution II	1 vial	2 vials	(7 ml) Dilute with equal volume of Substrate Solution I just prior use.
Stop Solution (1M Phosphoric acid)	1 vial	2 vials	(12 ml) Ready-to-use
Blue Dye	1 vial	2 vials	(0.4 ml) Make a 1/250 dilution in the appropriate diluent
Red Dye	1 vial	2 vials	(0.4 ml) Make a 1/250 dilution in the Assay Buffer
Green Dye	1 vial	2 vials	(0.4 ml) Make a 1/100 dilution in the Assay Buffer

1) reagents contain preservative

2) It is recommended to spin vial in microcentrifuge before use to collect reagent at the bottom.

MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 10 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis

SAFETY

- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Reagents containing thimerosal as preservative may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.

- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents.The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0 % sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on box front labels.
The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

SPECIMEN COLLECTION, PROCESSING AND STORAGE

Cell culture supernatants, murine serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Clinical samples should be kept at 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactive mIL-12. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

For stability and suitability of samples refer to respective chapter.

PREPARATION OF REAGENTS

1. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

2. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer** Concentrate (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table.

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

3. Preparation of Biotin-Conjugate

Make a 1:100 dilution with **Assay Buffer** in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

4. Preparation of mIL-12 Standard

Reconstitute mIL-12 Standard by addition of distilled water. Reconstitutions volume is stated on the label of the standard vial. Mix gently to ensure complete solubilization. Store reconstituted Standard promptly at –20°C. Discard after one week.

5. Preparation of Streptavidin-HRP

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution in **Assay Buffer** as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (ml)
1 - 6	0.06	6
1 - 12	0.12	12

6. TMB Substrate Solution

Using clean pipettes and containers known to be metal free, dispense an equal volume of **Substrate Solution I** into **Substrate Solution II** and swirl gently to mix. The TMB Substrate Solution may develop a yellow tinge over time. This does not seem to affect product performance. A blue colour present in the TMB Substrate Solution, however, indicates that it has been contaminated and must be discarded. The TMB Substrate Solution must be used within a few minutes after mixing. Warm to room temperature before use. Avoid direct exposure of TMB reagents to intense light and oxidizing agents during storage or incubation.

Substrate preparation according to assay size:

Number of Strips	Substrate Solution I (ml)	Substrate Solution II (ml)
1 - 6	3.0	3.0
1 - 12	6.0	6.0

7. Addition of Colour-giving Dyes

In order to help our customers to avoid any mistakes in pipetting the DIACLONE ELISAs, DIACLONE now offers a new tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.
This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.
Alternatively, the dye solutions from the stocks provided (***Blue-Dye, Green-Dye, Red-Dye***) can be added to the reagents according to the following guidelines:

A. Diluent:

Before sample dilution add the ***Blue-Dye*** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

5 ml Diluent	20 µl ***Blue-Dye***
12 ml Diluent	48 µl ***Blue-Dye***

B. Biotin-Conjugate:

Before dilution of the concentrated conjugate, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet, preparation of Biotin-conjugate.

3 ml Assay Buffer	30 µl ***Green-Dye***
6 ml Assay Buffer	60 µl ***Green-Dye***

C. Streptavidin-HRP:

Before dilution of the concentrated Streptavidin-HRP, add the ***Red-Dye*** at a dilution of 1:250 (see table below) to the Assay Buffer (1x) used for the final Streptavidin-HRP dilution. Proceed after addition of ***Red-Dye*** according to the instruction booklet, preparation of Streptavidin-HRP.

6 ml Assay Buffer	24 µl ***Red-Dye***
12 ml Assay Buffer	48 µl ***Red-Dye***

TEST PROTOCOL

a. Prepare reagents immediately before use and mix them thoroughly without foaming.

b. Determine the number of Microwell Strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra **Microwell Strips coated with Monoclonal Antibody** (rat) to murine IL-12 from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

c. Wash the microwell strips twice with approximately 300 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.
After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

d. Add 100 µl of **Assay Buffer** in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 µl of reconstituted (Refer to preparation of reagents) **mIL-12 Standard**, in duplicate, into wells A1 and A2. Mix the contents of wells A1 and A2 by repeated aspiration and ejection, and transfer 100 µl to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of mIL-12 standard dilutions ranging from 2000 to 31,25 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of mIL-12 standard dilutions:

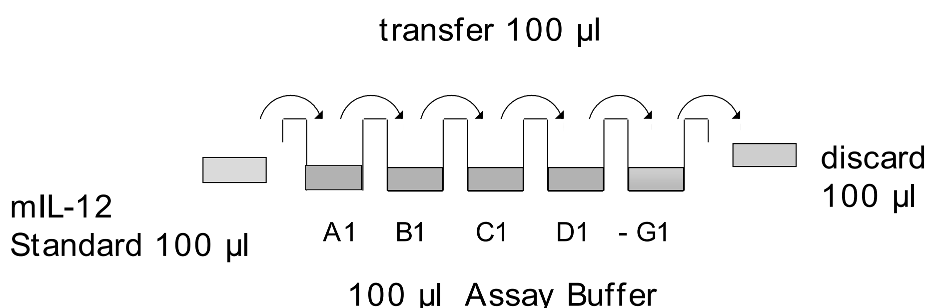


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

Standard Concentrations pg/mL — Sample wells

	1	2	3	4	5	6	7	8	9	10	11	12
A	2000	2000										
B	1000	1000										
C	500	500										
D	250	250										
E	125	125										
F	62.5	62.5										
G	31.25	31.25										
H	Blank	Blank										

e. Add 100 µl of **Assay Buffer,** in duplicate, to the blank wells.

f. Add 50 µl of **Assay Buffer** to the sample wells.

g. Add 50 µl of each **Sample**, in duplicate, to the designated wells.

h. Prepare **Biotin-Conjugate**. (Refer to preparation of reagents)

i. Add 50 µl of diluted **Biotin-Conjugate** to all wells, including the blank wells.

j. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 2 hours on a microplate shaker set at 200 rpm.

k. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.

l. Prepare **Streptavidin-HRP**. (Refer to preparation of reagents)

m. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.

n. Cover with a Plate Cover and incubate at room temperature (18° to 25°C) for 1 hour on a microplate shaker at 200 rpm.

o. Prepare TMB Substrate Solution a few minutes prior to use. (Refer to preparation of reagents).

p. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.

q. Pipette 100 µl of mixed **TMB Substrate Solution** to all wells, including the blank wells.

r. Incubate the microwell strips at room temperature (18° to 25°C) for about **10 minutes** on a microplate shaker at 200 rpm. Avoid direct exposure to intense light. The point at which the substrate reaction need to be stopped is often determined by the ELISA reader being used. Many ELISA readers record absorbance only up to 2.0 O.D. **Therefore the colour development within individual microwells must be watched by the person running the assay. The substrate reaction must be stopped before positive wells are no longer properly recordable.**

s. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

t. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both, the samples and the mIL-12 standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.

- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the mIL-12 concentration on the abscissa. Draw a best fit curve through the points of the graph.

- To determine the concentration of circulating mIL-12 for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding mIL-12 concentration.

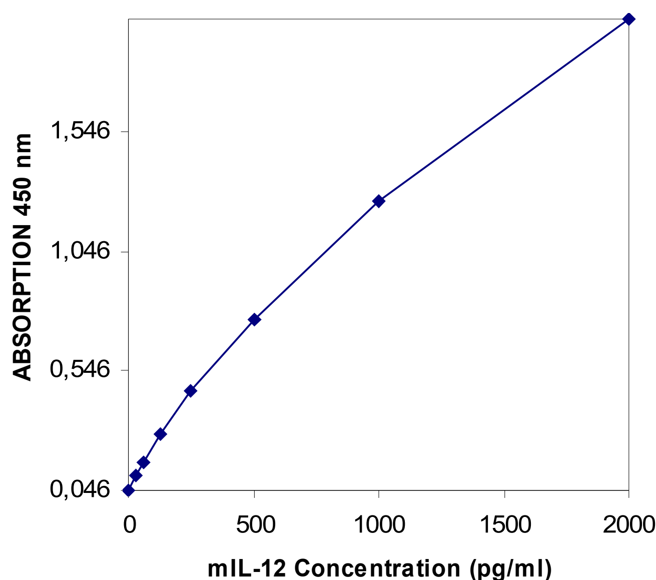
  If samples have been diluted according to the instructions given in this manual (e.g. cell culture supernatants), the concentration read from the standard curve must be multiplied by the respective dilution factor.

  Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low mIL-12 levels. Such samples should be re-analyzed at higher dilution rate in order to precisely quantitate the actual mIL-12 level.

  It is suggested that each testing facility establishes a control sample of known mIL-12 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

- A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for mIL-12 ELISA. mIL-12 was diluted in serial two-fold steps in Assay Buffer, symbols represent the mean of three parallel titrations. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed



Typical data using the mIL-12 ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	mIL-12 Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	2000	2.029	2.019	0.2
	2000	2.020		
2	1000	1.242	1.259	1.8
	1000	1.287		
3	500	0.787	0.762	2.5
	500	0.748		
4	250	0.462	0.466	1.9
	250	0.480		
5	125	0.281	0.281	1.9
	125	0.292		
6	62.5	0.158	0.160	4.5
	62.5	0.173		
7	31.25	0.108	0.109	0.5
	31.25	0.109		
Blank		0.041	0.046	
		0.050		

LIMITATIONS OF THE PROCEDURE

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

PERFORMANCE CHARACTERISTICS

Sensitivity

The limit of detection of mIL-12 defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be 3.96 pg/ml (mean of 6 independent assays).

Reproducibility

a. Intra-assay
Reproducibility within the assay was evaluated in independent experiments. The overall intra-assay coeffiecient of variation has been calculated to be <5%.

b. Inter-assay
Assay to assay reproducibility within one laboratory was evaluated in independent experiments by two technicians. The overall inter-assay coefficient of variation has been calculated to be <10%.

Spike Recovery

The spike recovery was evaluated by spiking four levels of mIL-12 into pooled normal murine serum. Recoveries were determined in two independent experiments with 4 replicates each. Observed values showed an overall mean recovery of 93%.

Dilution Parallelism

Murine serum spiked with different levels of mIL-12 was assayed at four serial two fold dilutions with 4 replicates each. Experiments showed an overall mean recovery of 104 %.

Sample Stability

a. Freeze-Thaw Stability
Aliquots of spiked serum were stored frozen at –20°C and thawed up to 5 times, and mIL-12 levels determined. There was no significant loss of IL-12 by freezing and thawing up to 5 times.

b. Storage Stability
Aliquots of spiked serum were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the mIL-12 level determined after 24 h. There was no significant loss of mIL-12 immunoreactivity during storage at -20°C, 4°C and room temperature. Storage at 37°C gave rise to about 50 % loss of mIL-12 immunoreactivity.

Specificity

The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a mIL-12 positive serum. There was no detectable cross reactivity.

Expected Serum Values

There are no detectable mIL-12 levels found in healthy mice. Elevated mIL-12 levels depend on the type of immunological disorder.

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REAGENT PREPARATION SUMMARY

A. Wash Buffer

Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water

B. Assay Buffer

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. Biotin-Conjugate

Make a 1:100 dilution according to the table.

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

D. Standard

Reconstitute with the volume of distilled water indicated on the vial.

E. Streptavidin-HRP

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (ml)
1 - 6	0.06	6.0
1 - 12	0.12	12.0

F. TMB Substrate Solution

Number of Strips	Substrate Solution I (ml)	Substrate Solution II (ml)
1 - 6	3.0	3.0
1 - 12	6.0	6.0

TEST PROTOCOL SUMMARY

- Wash microwell strips twice with **Wash Buffer**
- Add 100 µl **Assay Buffer**, in duplicate, to all standard wells
- Pipette 100 µl diluted **mIL-12 Standard** into the first wells and create standard dilutions ranging from 2000 to 31,25 pg/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells.
- Add 100 µl **Assay Buffer**, in duplicate, to the blank wells
- Add 50 µl **Assay Buffer**, in duplicate, to the sample wells
- Add 50 µl **Sample**, in duplicate, to designated wells
- Prepare **Biotin-Conjugate**
- Add 50 µl of diluted **Biotin-Conjugate** to all wells
- Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C) on microplate shaker
- Prepare **Streptavidin-HRP**
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of diluted **Streptavidin-HRP** to all wells
- Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C) on microplate shaker
- Prepare **TMB Substrate Solution** few minutes prior to use
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of mixed **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for 10 minutes at room temperature (18°to 25°C) on microplate shaker
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm