# General Vitamin B12 (VB12) ELISA Kit

Catalog No.: RK07421

version: 2.0
This package insert must be read in its entirety before using this product

## Introduction

This kit uses the competitive method to quantitatively determine the content of VB12 in serum, plasma, cell culture supernatant or other biological fluids.

## Principle of the Assay

This assay employs the competitive inhibition enzyme immunoassay technique. An antibody specific to VB12 has been pre-coated onto a microplate. A competitive inhibition reaction is launched between biotin labeled VB12 and unlabeled VB12 (standards or samples) with the pre-coated antibody specific to VB12. After incubation, the unbound conjugate is washed off. Next, avidin conjugated with Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Add substrate TMB. Add acid to stop the reaction and measure the light absorption value. Draw a standard curve according to the light absorption value of the gradient diluted VB12 standard and find the concentration of the sample. The content of VB12 in the sample is negatively correlated with the color depth of TMB.

## Material Provided & Storage Conditions

Unopened kits can be stored at 2-8°C for 1 year, and opened products must be used within 1 month.

| Part | Size | Cat. No. | Storage of opened/reconstituted material |
|---|---|---|---|
| General VB12 Microwell Plate Coated | 8×12 | RM49976 | Put the unused slats back in the aluminum foil bag with the desiccant and reseal them. They can be stored at 2-8℃ for 1 month. |
| General VB12 Standard Lyophilized | 2 vials | RM49977 | It is not recommended to use again after redissolving. |
| General VB12 concentrated Biotin-Conjugate Antigen(100x) | 1 ×60 μL | RM49978 | Store at 2-8℃ for 1 month * |
| Streptavidin-HRP Concentrated (100x) | 1 ×120 μL | RM49979 | Store at 2-8℃ for 1 month * |

| | | | |
|---|---|---|---|
| Standard/Sample Diluent (R1) | 1 ×20 mL | RM00023 | Store at 2-8℃ for 1 month * |
| Biotin-Conjugate Antibody Diluent (R2) | 1 ×10 mL | RM00024 | Store at 2-8℃ for 1 month * |
| Streptavidin-HRP Diluent(R3) | 1 ×10 mL | RM00025 | |
| Wash Buffer(25x) | 1 ×30 mL | RM00026 | |
| TMB Substrate | 1 ×10 mL | RM00027 | |
| Stop Solution | 1 ×10 mL | RM00028 | |
| Plate Sealers | 4 Strips | | |
| Specification | 1 | | |

*Note: The specifications listed in the table are for 96T kit, and the amount of other components in the 48T kit are halved except for the standard, please be aware of this.

## Other Supplies Required

1. Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 630 nm or 570 nm.
2. Pipettes and pipette tips.
3. Deionized or distilled water.
4. Squirt bottle, manifold dispenser, or automated microplate washer.
5. Incubator.
6. Test tubes for dilution of standards and samples.

## Precautions

*For Research use only，not be used for diagnosis.

1. The kit should not be used beyond the expiration date on the kit label.
2. Do not mix or substitute reagents with those from other lots or other sources.
3. If the OD value of the sample obtained from the test exceeds the maximum detection limit of the product, please dilute the sample using the standard/sample diluent (R1) in the product. Therefore, it is recommended to pre-test the sample before formally testing the sample.
4. Sample addition, plate washing, incubation time, incubation temperature and other operations during the experiment will affect the final results, please strictly manage the experimental process and keep good records.
5. Variations in sample collection, processing, and storage may cause sample value differences.
6. Until all factors have been tested in this assay, the possibility of interference cannot be excluded.

7. Reagents may be harmful, if ingested, rinse it with an excess amount of tap water.
8. Stop Solution contains strong acid. Wear eye, hand, and face protection.
9. To ensure the best results, please refer to the labels or instructions for storage of relevant reagent components.
10. Mixing of the reagents after preparation is very important for the results, but some proteins or antibodies may be very sensitive to vigorous vortexing, which may cause loss of activity, so please use vortexing with caution.
11. Please use sterilised consumables for reagent preparation to avoid contamination of the reagents, which may affect the final test results.
12. In order to ensure the best detection effect, it is not recommended to reuse the working solution of the solubilised standard protein and related reagents after freezing.
13. The kit should be away from light when it is stored or incubated.
14. To avoid cross contamination, please use disposable pipette tips.
15. Please prepare all the kit components according to the

Specification. If the kits will be used several times, please seal the rest strips and preserve with desiccants. Do use up within 1 months.

16. The 48T kit is also suitable for the specification.

## Sample Collection & Storage

Cell Culture Supernatant: Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum: Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using EDTA or Heparin as an anticoagulant. Centrifuge for 15 minutes at 1000×g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20℃. Avoid repeated freeze-thaw cycles. (Note: Citrate plasma has not been validated for use in this assay.)

Other biological fluids： Centrifuge samples for 20 minutes at 1,000×g. Collect the supernatants and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze-thaw cycles.

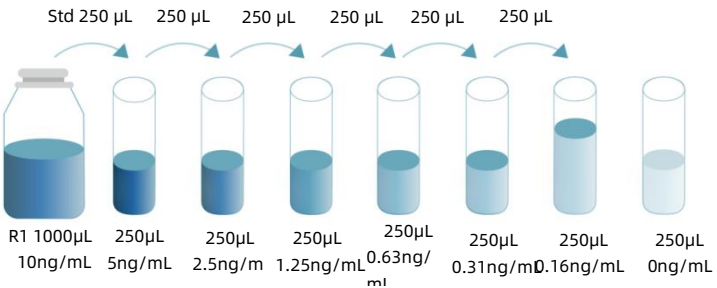
Note: It is suggested that all samples in one experiment be collected at the same time of the day. Avoid hemolytic and hyperlipidemia sample for serum and plasma.

## Reagent Preparation

Bring all reagents to room temperature before use. If crystals have formed in the concentrate, Bring the reagent to room temperature and mix gently until the crystals have completely dissolved.

Standard - Reconstitute the Standard Lyophilized with 1 mL Standard/Sample Diluent(R1). This reconstitution produces a stock solution of 10 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use the 10 ng/mL standard stock to produce a dilution series (below) with Standard/Sample Diluent(R1). Mix each tube thoroughly and change pipette tips between each transfer (recommended concentration for standard curve: 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0 ng/mL). Use diluted standards within 60 minutes of preparation.



Working Biotin Conjugate Antigen - Dilute 1:100 of Concentrated Biotin Conjugate Antigen (100x) with Biotin-Conjugate Antigen Diluent (R2) before use. For example: Add 20 μL of Concentrated Biotin Conjugate Antibody (100x) to 1980 μL Biotin-Conjugate Antigen Diluent (R2) to prepare 2000 μL Working Biotin Conjugate Antigen Buffer.

Working Streptavidin-HRP - Dilute 1:100 of Concentrated Streptavidin-HRP (100x) with Streptavidin-HRP Diluent (R3) before use. For example: Add 20 μ L of Concentrated Streptavidin-HRP (100x) to 1980 μ L Streptavidin-HRP Diluent (R3) to prepare 2000 μL Working Streptavidin-HRP Buffer.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 1:25 with double distilled or deionized water before use, for example : Add 16 mL of Wash Buffer Concentrate to 384 mL of deionized or distilled water to prepare 400 mL of Wash Buffer.

## Sample preparation

For different samples, the appropriate dilution level should be

chosen on a case-by-case basis.Please dilute the sample using the standard/Sample diluent(R1) in the kit.

1. Cell Supernatant: As cell supernatant samples vary considerably depending on the experimental conditions, it is recommended to carry out a pre-test to determine the appropriate dilution.For sample dilution, use standard/Sample diluent (R1) or PBS.

2. Serum/plasma:Due to individual differences, please anticipate the concentration range of the sample in advance and determine the dilution of the sample to be examined by pre-testing.Please refer to the following dilution instructions.For sample dilution, use standard/Sample diluent(R1) or PBS.

Dilution Method

For 100 fold dilution: One-step dilution. Add 5 μL sample to 495 μL sample diluent(R1) to yield 100 fold dilution.

For 1000 fold dilution: Two-step dilution. Add 5 μL sample to 95 μL sample diluent(R1)(1x) to yield 20 fold dilution, then add 5 μL 20 fold diluted sample to 245 μL sample diluent(R1)(1x), after this, the neat sample has been diluted at 1000 fold

successfully.

Each dilution step should be performed at a minimum of 3 μL and at a maximum of 100-fold dilution. Each dilution step should be mixed well to avoid foaming.

## Assay Procedure

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal properly.
2. Prepare the Biotin Conjugate Antigen Concentrated (100x) Working Solution 15 minutes early before use.
3. Add 50 μL Standard/sample Diluent (R1) in blank well, add 50 μL different concentration of standard or sample in other wells. And then add Biotin Conjugate Antigen Working Solution in each well (50μL/well), cover with a new adhesive sealer provided. Incubate for 1 hour at 37°C.
4. Prepare the Streptavidin-HRP Concentrated (100x) Working Solution 15minutes early before use.
5. Add wash buffer 350 μL/well, aspirate each well after holding 40 seconds, repeating the process two times for a

total of three washes.

6. Add Streptavidin-HRP Working Solution in each well (100 μ L/well), cover with a new adhesive sealer provided. Incubate for 45 minutes at 37°C.
7. During the incubation, turn on the microplate reader to warm up for 30 minutes before measuring.
8. Repeat the aspiration/wash as in step 5.
9. Add 90 μL TMB Substrate to each well. Incubate for 15-20 minutes at 37°C. Protect from light.
10. Add Stop Solution (50 μ L/well), determine the optical density of each well within 5 minutes, using a Microplate reader set to 450 nm. If wavelength correction is available, set to 570 nm or 630 nm. If wavelength correction is not available, subtract readings at 570 nm or 630 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may cause higher value and less accurate result.

## Assay Procedure Summary

Prepare the standard and reagents

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Add 50 μL of standards or test samples to each well

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Add 50 μL Working Biotin-Conjugate Antigen

Incubate for 1 hours at 37℃, then wash 3 times

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Add 100 μL Working Streptavidin-HRP

Incubate for 45 minutes at 37℃, then wash 3 times

↓

Add 90 μL Substrate Solution

Incubate for 15-20 min at 37℃ under dark condition

↓

Add 50 μL Stop Solution

↓

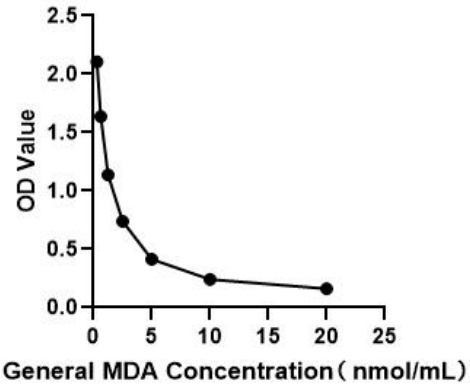
Detect the optical density within 5 minutes under 450nm.

Correction Wavelength set at 570nm or 630nm

## Calculation of Results

1. Calculate the average OD value of the replicate wells for each concentration of standard protein, quality control, sample, etc. The OD value of each test should be subtracted from the OD value of the sub-wavelength.
2. Create a standard curve by reducing the data using computer software capable of generating a four-parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the Y-axis against the concentration on the X-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the EGF concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.
3. If samples have been diluted,the concentration read from the standard curve must be multiplied by the dilution factor.

## Typical Data




The standard curves are provided for demonstration only. A standard curve should be generated for each set of VB12 assayed.

## Detection Range

0.16-10 ng/mL

## Sensitivity

The minimum detectable dose (MDD) of VB12 typically less than 0.06 ng/mL. The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

## Specificity

This assay recognizes both recombinant and natural VB12. The method has high sensitivity and specificity for the detection of VB12.

There is no significant cross-reactivity or interference between VB12 and analogues.

Note:

Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between VB12 and all the analogues, therefore, cross reaction may still exist.

## Precision

Intra-plate Precision

3 samples with low, middle and high level VB12 were tested 20 times on one plate, respectively.

Intra-Assay: CV<10%

Inter-plate Precision

3 samples with low, middle and high level VB12 were tested on 3 different plates, 20 replicates in each plate.

Inter-Assay: CV<15%

| | Intra-Assay Precision | | | Inter-Assay Precision | | |
|---|---|---|---|---|---|---|
| Sample | 1 | 2 | 3 | 1 | 2 | 3 |
| n | 20 | 20 | 20 | 20 | 20 | 20 |
| Mean(ng/mL) | 0.39 | 1.25 | 7.5 | 0.39 | 1.25 | 7.5 |
| Standard deviation | 0.01 | 0.04 | 0.23 | 0.03 | 0.1 | 0.57 |
| CV(%) | 3.78 | 3.25 | 3.07 | 7.59 | 7.84 | 7.56 |

## Recovery

Matrices listed below were spiked with certain level of VB12 and the recovery rates were calculated by comparing the measured value to the expected amount of VB12 in samples.

| Sample | Average Recovery（%） | Range（%） |
|---|---|---|
| Cell Culture Media(n=5) | 94 | 82-103 |
| Serum(n=5) | 98 | 94-101 |

## Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of VB12 and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

| / | / | Cell Culture Media(n=5) | Serum(n=5) |
|---|---|---|---|
| 1:2 | Average of Expected（%） | 95 | 88 |
| | Range（%） | 88-109 | 81-94 |
| 1:4 | Average of Expected（%） | 108 | 92 |
| | Range（%） | 92-118 | 85-97 |
| 1:8 | Average of Expected（%） | 99 | 88 |
| | Range（%） | 95-108 | 82-92 |
| 1:16 | Average of Expected（%） | 101 | 95 |
| | Range（%） | 95-109 | 84-96 |

## Trouble Shooting

| Problem | Possible Cause | Solution |
|---|---|---|
| High Background | Insufficient washing | Sufficiently wash plates as required. Ensure appropriate duration and number of washes. Ensure appropriate volume of wash buffer in each well. |
| | Incorrect incubation procedure | Check whether the duration and temperature of incubation are set up as required. |
| | Cross-contamination of samples and reagents | Be careful of the operations that could cause cross-contamination. Use fresh reagents and repeat the tests. |
| No signal or weak signal | Incorrect use of reagents | Check the concentration and dilution ratio of reagents. Make sure to use reagents in proper order. |
| | Incorrect use of microplate reader | Warm the reader up before use. Make sure to set up appropriate main wavelength and correction wavelength. |
| | Insufficient colour reaction time | Optimum duration of colour reaction should be limited to 15-25 minutes. |

| | | |
|---|---|---|
| | Read too late after stopping the colour reaction | Read the plate in 5 minutes after stopping the reaction. |
| | Matrix effect of samples | Use positive control. |
| Too much signal | Contamination of TMB substrate | Check if TMB substrate solution turns blue. Use new TMB substrate solution. |
| | Plate sealers reused | Use a fresh new sealer in each step of experiments. |
| | Protein concentration in sample is too high | Do pre-test and dilute samples in optimum dilution ratio. |
| Poor Duplicates | Uneven addition of samples | Check the pipette. Periodically calibrate the pipette. |
| | Impurities and precipitates in samples | Centrifuge samples before use. |
| | Inadequate mixing of reagents | Mix all samples and reagents well before loading. |

*For research purposes only. Not for therapeutic or diagnostic purposes.