



**KAMIYA BIOMEDICAL COMPANY**

# Human vitamin B12 (VB12) ELISA

**For the quantitative determination of human VB12 in  
serum and plasma.**

**Cat. No. KU-117**

**For Research Use Only. Not for use in diagnostic procedures.**

**Product Information**  
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**INTENDED USE**

The Human vitamin B12 ELISA is for the quantitative determination of human VB12 in serum and plasma. For research use only. Not for use in diagnostic procedures.

**PRINCIPLE**

This assay employs the direct competitive inhibition enzyme immunoassay technique. Antibody specific for VB12 has been pre-coated onto a microplate. Calibrators and samples are pipetted into the wells with biotin-conjugated VB12. A competitive inhibition reaction is launched between VB12 (Calibrators or samples) and biotin-conjugated VB12 with the pre-coated VB12 antibody. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound reagent, a substrate solution is added to the wells and color develops in opposite to the amount of VB12 bound in the initial step. The color development is stopped and the intensity of the color is measured.

**PERFORMANCE****Detection Range**

1.56 - 100 pg/mL.

**Sensitivity**

The minimum detectable dose of human VB12 is typically less than 1.498 pg/mL.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest human VB12 concentration that could be differentiated from zero. It was determined the mean O.D. value of 20 replicates of the zero calibrator added by their three standard deviations.

**Specificity**

This assay has high sensitivity and excellent specificity for detection of human VB12. No significant cross-reactivity or interference between human VB12 and analogues was observed.

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

**Precision****Intra-assay Precision (Precision within an assay): CV% <8%**

Three samples of known concentration were tested twenty times on one plate to assess.

**Inter-assay Precision (Precision between assays): CV% <10%**

Three samples of known concentration were tested in twenty assays to assess.

**Limitations of the Procedure**

- For research use only. Not for use in diagnostic procedures.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate higher values than the highest calibrator, dilute the samples with Sample Diluent and repeat the assay.
- Any variation in Sample Diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the immunoassay, the possibility of interference cannot be excluded.

## COMPONENTS

Reagents	Quantity
Assay plate	1 (96 wells)
Calibrator	2 x 0.5 mL
Biotin-conjugate (10x concentrate)	1 x 600 µL
HRP-avidin (100x concentrate)	1 x 120 µL
Biotin-conjugate Diluent	1 x 10 mL
HRP-avidin Diluent	1 x 20 mL
Sample Diluent	2 x 20 mL
Wash Buffer (25x concentrate)	1 x 20 mL
TMB Substrate	1 x 10 mL
Stop Solution	1 x 10 mL
Plate sealer for 96 wells	4

## STORAGE

Unopened kit: Store at 4°C. Do not use the kit beyond the expiration date.

Opened kit: May be stored up to one month at 4°C. Keep unused assay plate strips in sealed aluminum foil bag with desiccant. If short-term use is not intended for the HRP-avidin it is advisable to store this reagent at -20°C.

## MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
2. An incubator which can provide stable incubation conditions up to 37°C ± 0.5°C.
3. Squirt bottle, manifold dispenser, or automated microplate washer.
4. Absorbent paper for blotting the microtiter plate.
5. 100 mL and 500 mL graduated cylinders.
6. De-ionized or distilled water.
7. Pipettes and pipette tips.
8. Test tubes for dilution.

## Precautions

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

## SAMPLE COLLECTION AND STORAGE

### Serum

Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1,000 x g. Remove serum and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

## Plasma

Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1,000 x g at 4°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

## SAMPLE PREPARATION

Recommend to dilute serum or plasma samples with Sample Diluent (1:200) before test. The suggested 200-fold dilution can be achieved by adding 5 µL sample to 45 µL of Sample Diluent. Complete the 200-fold dilution by adding 15 µL of this solution to 285 µL of Sample Diluent. The recommended dilution factor is for reference only. The optimal dilution factor should be determined by users according to their particular experiments.



### Note:

1. Kamiya Biomedical Company is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
3. Grossly hemolyzed samples are not suitable for use in this assay.
4. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.
5. Owing to the possibility of mismatching between antigen from other resource and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
6. Influenced by the factors including cell viability, cell number and also sampling time, samples from cell culture supernatant may not be detected by the kit.
7. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.
8. Please predict the concentration before assaying. If values for these are not within the range of the calibration curve, users must determine the optimal sample dilutions for their particular experiments.
9. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.

## REAGENT PREPARATION

### Note:

1. **Kindly use graduated containers to prepare reagent. Please don't prepare the reagent directly in the Diluent vials provided in the kit.**
2. Making serial dilution in the wells directly is not permitted.
3. Bring all kit components to room temperature (18-25°C) before use for 30 minutes.
4. Prepare fresh calibrator for each assay. Use within 4 hours and discard after use.
5. Distilled water is recommended to be used to make the preparation for reagents or samples. Contaminated water or container for reagent preparation will influence the detection result.

### Biotin-conjugate (1x)

Centrifuge the vial before opening. Biotin-conjugate requires a 10-fold dilution. A suggested 10-fold dilution is 100 µL of Biotin-conjugate + 900 µL of Biotin-conjugate Diluent.

### HRP-avidin (1x)

Centrifuge the vial before opening. HRP-avidin requires a 100-fold dilution. A suggested 100-fold dilution is 10 µL of HRP-avidin + 990 µL of HRP-avidin Diluent.

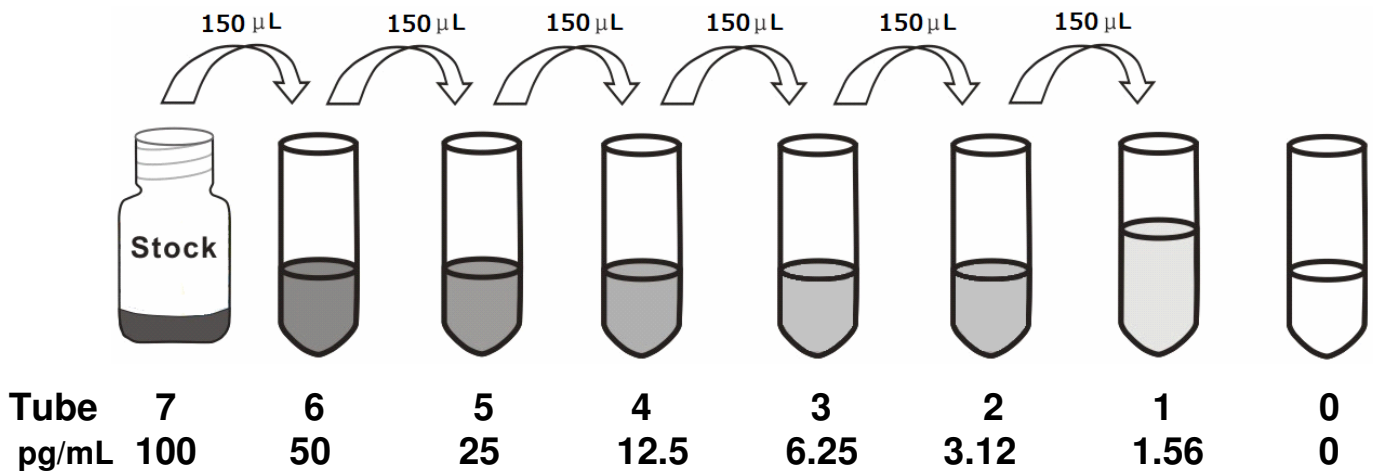
### Wash Buffer (1x)

If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate (25x) into deionized or distilled water to prepare 500 mL of Wash Buffer (1x).

**Calibrator**

Centrifuge the Calibrator vial at 6,000 - 10,000 rpm for 30 seconds. Allow the Calibrator to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 150 µL of Sample Diluent into each tube (0-6). Use the stock solution to produce a 2-fold dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. The undiluted Calibrator serves as the high calibrator (100 pg/mL). Sample Diluent serves as the zero calibrator (0 pg/mL).



**ASSAY PROCEDURE**

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and calibrators be assayed in duplicate.

1. Prepare all reagents, working calibrators and samples as directed in the previous sections.
2. Determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
3. Set a blank well without any solution
4. Add 50 µL of calibrator and sample per well.
5. Add 50 µL of Biotin-conjugate (1x) to each well (not to Blank well). Cover with a new adhesive strip. Incubate for 1 hour at 37°C. (Biotin-conjugate (1x) may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.)
6. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200 µL) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, complete removal of each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
7. Add 100 µL of HRP-avidin (1x) to each well(not to Blank well). Cover the microtiter plate with a new adhesive strip. Incubate for 40 minutes at 37°C.
8. Repeat the aspiration/wash process for five times as in step 6.
9. Add 90 µL of TMB Substrate to each well. Incubate for 20 minutes at 37°C. Protect from light.
10. Add 50 µL of Stop Solution to each well, gently tap the plate to ensure thorough mixing.
11. 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 540 nm or 570 nm. Subtract readings at 540 nm or 570 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 be higher and less accurate.

**\*Samples may require dilution. Please refer to the Sample Preparation section.**

**Note:**

1. The final experimental results will be closely related to operation skills of the end users and the experimental environments.
2. Sample or reagents addition: Please use the freshly prepared Calibrator. Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all calibrators and samples, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of each calibrator level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. Incubation: To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.
4. Washing: The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading. When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
5. Controlling of reaction time: Observe the change of color after adding TMB Substrate (e.g. observation once every 10 minutes), if the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. TMB Substrate is easily contaminated. TMB should remain colorless or light blue until added to the plate. Please protect it from light.
7. Stop Solution should be added to the plate in the same order as the TMB Substrate. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the TMB Substrate.

**ASSAY PROCEDURE SUMMARY**

1. Prepare reagents, samples as instructed;
2. Set a Blank well without any solution;
3. Add 50  $\mu$ L calibrator or sample to each well;
4. Add 50  $\mu$ L Biotin-conjugate (1x) to each well (not to blank well);
5. Incubate 60 minutes at 37°C;
6. Aspirate and wash 3 times;
7. Add 100  $\mu$ L HRP-avidin (1x) to each well (not to blank well);
8. Incubate 40 minutes at 37°C;
9. Aspirate and wash 5 times;
10. Add 90  $\mu$ L TMB Substrate to each well. Incubate 20 minutes at 37°C. Protect from light;
11. Add 50  $\mu$ L Stop Solution. Read at 450 nm within 5 minutes.

**\*Samples may require dilution. Please refer to the Sample Preparation section.**

**CALCULATION OF RESULTS**

Using professional graphing software to make the calibration curve is recommended.

Average the duplicate readings for each calibrator and sample and subtract the average optical density of the blank.

Create a calibration curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct the calibration curve by plotting the mean absorbance for each calibrator on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the VB12 concentrations versus the log of the OD and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the calibration curve must be multiplied by the dilution factor.

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