K-ASSAY®

KAMIYA BIOMEDICAL COMPANY

Nicotinamide Adenine Dinucleotide (NAD) ELISA

For the quantitative determination of NAD in serum, plasma, tissue homogenates, cell lysates, cell culture supernates, and other biological fluids

Cat. No. KT-37157

For Research Use Only. Not For Use in Diagnostic Procedures.

Rev. 37157

Product Information

Nicotinamide Adenine Dinucleotide (NAD) ELISA

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INTENDED USE

The kit is a competitive inhibition enzyme immunoassay technique for the *in vitro* quantitative measurement of NAD in serum, plasma, tissue homogenates, cell lysates, cell culture supernates, and other biological fluids. For research use only. Not for use in diagnostic procedures.

COMPONENTS

	Quantity
Pre-coated, ready to use 96-well strip plate	1
Calibrator (lyophilized)	2 × 0.5 mL
Calibrator Diluent	1 × 20 mL
Detection Reagent A	1 × 120 µL
Detection Reagent B	1 × 120 µL
Assay Diluent A	1 × 12 mL
Assay Diluent B	1 × 12 mL
TMB Substrate	1 × 9 mL
Stop Solution	1 × 6 mL
Wash Solution (30X concentrate)	1 × 20 mL
Plate seal for 96 wells	4

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microplate reader with 450 (± 10) nm filter.
2. Single or multi-channel pipettes with high precision and disposable tips.
3. Microcentrifuge Tubes.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microplate.
6. Container for Wash Solution.
7. 0.01 mol/L (or 1x) Phosphate Buffered Saline (PBS), pH 7.0-7.2.

STORAGE

1. All reagents should be kept according to their labels.
2. For unused kits, the entire kit can be stored at -20°C until the expiration date, while up to one month at 4°C. For experimental convenience, The **Calibrator**, **Detection Reagent A, Detection Reagent B** and the **96-well strip plate** could be stored at -20°C upon receipt while the remaining items could be at 4°C.

3. Once opened, the items must be stored as outlined above. The unused strips should be kept in a sealed bag with the desiccant provided to minimize exposure to damp air. Open test kits will remain stable for 1 month, provided they are stored as described above.

TEST PRINCIPLE

This assay employs a competitive inhibition enzyme immunoassay technique. The microplate provided in this kit has been pre-coated with an antibody specific to NAD. A competitive inhibition reaction is launched between biotin labeled NAD, unlabeled NAD (calibrators or samples) and the pre-coated antibody specific to NAD. After incubation, the unbound conjugate is washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. The amount of bound HRP conjugate is inversely proportional to the concentration of NAD present in the sample. After adding the substrate solution, the intensity of color that develops is inversely proportional to the concentration of NAD present in the sample.

SAMPLE COLLECTION AND STORAGE

Serum

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

Tissue homogenates

The preparation of tissue homogenates will vary depending upon tissue type.

1. Tissues should be rinsed in ice-cold PBS to remove excess blood thoroughly and weighed before homogenization.
2. Mince tissues into small pieces and homogenize them in fresh lysis buffer (different lysis buffer needs to be chosen based on subcellular location of the target protein) (w:v = 1:20 - 1:50, e.g. 1 mL lysis buffer is added in 20-50 mg tissue sample) with a glass homogenizer on ice (Micro Tissue Grinders work as well).
3. The resulting suspension should be sonicated with an ultrasonic cell disrupter until the solution is clarified.
4. Then, centrifuge homogenates for 5 minutes at 10,000 × g. Collect the supernates and assay immediately or aliquot and store at ≤ -20°C.

Cell Lysates

Cells must be lysed before assaying according to the following directions.

1. Adherent cells should be washed with cold PBS gently, then detached with trypsin, and collected by centrifugation at 1,000 × g for 5 minutes (suspension cells can be collected directly by centrifugation).
2. Wash cells three times in cold PBS.
3. Resuspend cells in fresh lysis buffer with concentration of 10^7 cells/mL. If it is necessary, the cells could be subjected to ultrasonication until the solution is clarified.
4. Centrifuge at 1,500 × g for 10 minutes at 4°C to remove cellular debris. Assay immediately or aliquot and store at ≤ -20°C.

Cell culture supernates and other biological fluids

Centrifuge samples for 20 minutes at 1,000 x g. Collect the supernates and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Note:

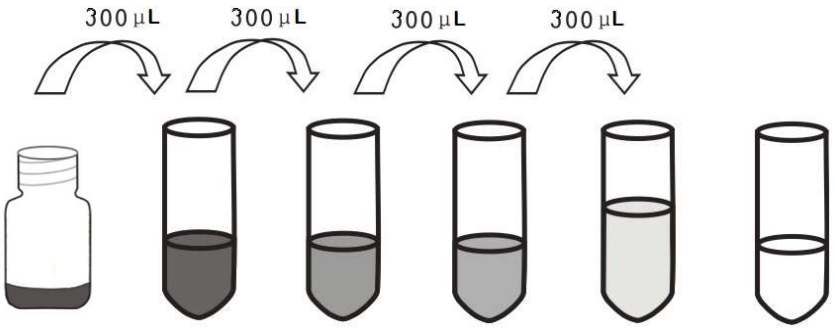
1. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. Sample hemolysis will influence the result, so hemolytic specimens should not be used.
3. Bring samples to room temperature.
4. It is highly recommended to use serum instead of plasma samples for detection based on our in-house data.

REAGENT PREPARATION

Bring all kit components and samples to room temperature (18-25°C) before use. If the entire kit will not be used up in one run, only take out the strips and reagents needed for the present experiment. Leave the remaining strips and reagents in their required condition.

Calibrator

To create a stock solution, reconstitute the **Calibrator** with 0.5 mL of **Calibrator Diluent** kept at room temperature for 10 minutes. Shake gently so as not to create foam. The concentration of the **Calibrator** in the stock solution is 10,000 ng/mL. Prepare 5 tubes containing 0.6mL **Calibrator Diluent** and produce a triple dilution series according to the picture below. Mix each tube thoroughly before the next transfer. Set up 5 points of diluted **Calibrator** such as 10,000 ng/mL, 3,333.3 ng/mL, 1,111.1 ng/mL, 370.4 ng/mL, 123.5 ng/mL. The blank is the **Calibrator Diluent** and is 0 ng/mL



Tube		1	2	3	4	Blank
pg/mL	10,000	3,333.3	1,111.1	370.4	123.5	0

Detection Reagent A and Detection Reagent B

Briefly spin or centrifuge the stock **Detection Reagent A** and **Detection Reagent B** before use. Dilute them to the working concentration 100-fold with **Assay Diluent A** and **Assay Diluent B,** respectively.

Wash Solution

Dilute 20 mL of **Wash Solution Concentrate (30X)** with 580 mL of deionized or distilled water to prepare 600 mL of **Wash Solution (1X).**

TMB substrate

Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Note:

1. Prepare calibrators within 15 minutes before the assay. Do not dissolve reagents directly at 37°C.
2. Do not make serial dilutions directly within the wells.
3. Detection Reagent A and Detection Reagent B are both sticky solutions, slowly pipette them to reduce volume errors.
4. Carefully dilute the **Calibrators** or working **Detection Reagent A and Detection Reagent B** according to the instructions, avoid creating foam and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10 µL for one pipetting.
5. The diluted **Calibrators, Detection Reagent A and Detection Reagent B** can be used only once.
6. If crystals have formed in the **Wash Solution concentrate (30X)**, warm to room temperature and mix gently until the crystals are completely dissolved.
7. Contaminated water or containers for reagent preparation will influence the detection results.

SAMPLE PREPARATION

1. **Kamiya Biomedical Company** is only responsible for the kit itself, not for the samples consumed during the assay. The user should calculate the possible amount or number of samples needed for the entire test and reserve a sufficient amount or number of samples in advance.
2. Predict the concentration of samples before assaying. If the values for these are not within the range of the calibration curve, users must determine the optimal sample dilutions for their particular experiments.
3. If a sample is not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
4. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts from certain chemicals.
5. Due to the possibility of mismatching between antigens from other origins and the antibodies 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 various factors including cell viability, cell number or sampling time, samples from cell culture supernates may not be detected by the kit.
7. Fresh samples are recommended for the test. Otherwise, protein degradation and denaturalization may occur and lead to false results.

ASSAY PROCEDURE

1. Determine which wells will be used for the diluted calibrator, blank and sample. Prepare 5 wells for the calibrators and 1 well for the blank. Add 50 µL of each dilution of calibrator (read Reagent Preparation), blank and samples into the appropriate wells. Next add 50 µL of **Detection Reagent A** to each well **immediately.** Shake the plate gently (using a microplate shaker is recommended). Cover with a **Plate Sealer**. Incubate for 1 hour at 37°C. If **Detection Reagent A** appears cloudy, warm to room temperature and mix gently
2. Aspirate the solution and wash with 350 µL of 1X **Wash Solution** to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeat 3 times. After the last wash, remove any remaining **Wash Solution** by aspirating or decanting. Invert the plate and blot it against absorbent paper.

3. Add 100 µL of **Detection Reagent B** working solution to each well, cover the wells with a plate sealer and incubate for 30 minutes at 37°C.
4. Repeat the aspiration/wash process for a total of 5 times as conducted in step 2.
5. Add 90 µL of **TMB Substrate** to each well. Cover with a new Plate sealer. Incubate for 10 - 20 minutes at 37°C (Do not exceed 30 minutes). Protect from light. The liquid will turn blue with the addition of **TMB Substrate.**
6. Add 50 µL of **Stop Solution** to each well. The liquid will turn yellow with the addition of **Stop Solution.** Mix the liquid by tapping the side of the plate. If the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
7. Remove any drops of water and fingerprints on the bottom of the plate and confirm there are no bubbles on the surface of the liquid. Then, run the microplate reader and conduct a measurement at 450 nm **immediately.**

Note:

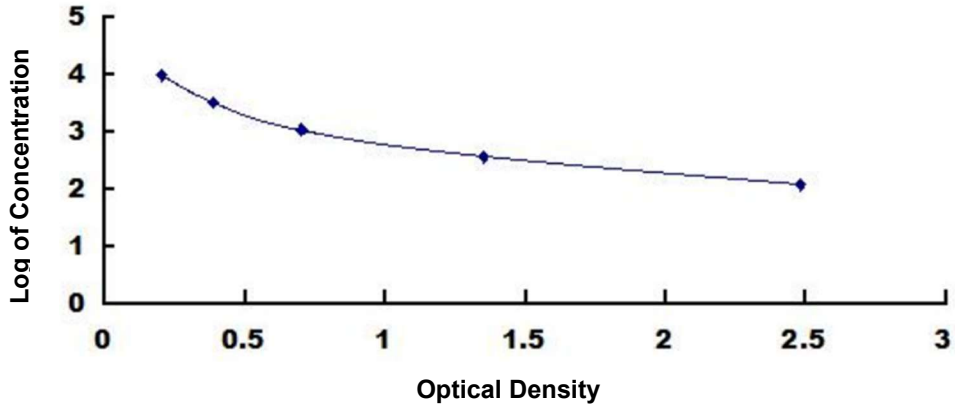
1. **Assay preparation:** Keep the appropriate numbers of wells for each experiment and remove any extra wells from the microplate. The remaining wells should be resealed and stored at -20°C.
2. **Samples or reagents addition: Use freshly prepared Calibrator.** Carefully add samples to the wells and mix gently to avoid creating foam. Do not touch the well walls. For each step in the procedure, total dispensing time for the addition of reagents or samples to the assay plate **should not exceed 10 minutes**. This will ensure that equal elapsed time for each pipetting step, without interruption. Duplication of all calibrators and specimens, although not required, is highly recommended. To avoid cross-contamination, change pipette tips between the addition of calibrators, samples, and reagents. Use separated 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 are added to the well strips, **DO NOT** let the strips **dry out** 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 drops of water and fingerprints on the bottom of the plate. Insufficient washing will result in poor precision and a falsely elevated absorbance reading.
5. **Controlling reaction time:** Observe the change of color after adding the **TMB Substrate** (e.g. observation once every 10 minutes)**,** if the color is too deep, add **Stop Solution** in advance to avoid an excessively strong reaction which will result in inaccurate absorbance reading.
6. **TMB Substrate** is easily contaminated, protect it from light.
7. If the environmental humidity is less than 60%, it may have some effects on the final performance, therefore, a humidifier is recommended in that situation.

CALCULATION OF RESULTS

This assay employs a competitive inhibition enzyme immunoassay technique that shows there is an inverse correlation between NAD concentration in the sample and the assay signal intensity. To find this, average the duplicate readings for each calibrator, control, and sample. Create a calibration curve with the log of NAD concentration on the y-axis and absorbance on the x-axis. Draw a best fit curve through the points using regression analysis. If samples have been diluted, the concentration calculated from the calibration curve must be multiplied by the dilution factor.

Typical Data

To make calculations easier, plot the O.D. value of the calibrator (X-axis) against the log of the concentration of the calibrator (Y-axis), although concentration is the independent variable and O.D. value is the dependent variable. The O.D. values of the calibration curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). The typical calibration curve below is provided for **reference only.**



Typical Calibrator Curve for NAD ELISA

PERFORMANCE

Detection Range

123.5 – 10,000 ng/mL
The calibration curve concentrations used for this ELISA were 10,000 ng/mL, 3,333.3 ng/mL, 1,111.1 ng/mL, 370.4 ng/mL, 123.5 ng/mL.

Sensitivity

The minimum detectable dose of NAD is typically less than 49.3 ng/mL.
The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by subtracting two standard deviations to the mean optical density value of twenty zero calibrator replicates and calculating the corresponding concentration.

Specificity

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

Note:

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

Recovery

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

Matrix	Recovery range (%)	Average (%)
Serum (n=5)	83 - 96	90
EDTA plasma (n=5)	90 - 105	99
Heparin plasma (n=5)	81 - 95	87

Linearity

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

Sample	1:2	1:4	1:8	1:16
serum (n=5)	83 - 95 %	85 - 99 %	92 - 103 %	79 - 89 %
EDTA plasma (n=5)	81 - 96 %	78 - 91 %	90 - 106 %	82 - 98 %
Heparin plasma (n=5)	87 - 102 %	84 - 97 %	89 - 101 %	80 - 94 %

Precision

Intra-assay Precision (Precision with an assay): 3 samples with low, middle and high level NAD were tested 20 times on one plate, respectively.
Inter-assay precision (Precision between assays): 3 samples with low, middle and high level NAD were tested on 3 different plates, 8 replicates in each plate.

CV (%) = SD/mean x 100
Intra-Assay: CV < 10%
Inter-Assay: CV < 12%

Stability

The stability of an ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 5% prior to the expiration date under appropriate storage conditions.
To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, and incubator temperature should be strictly monitored. It is also strongly suggested that the assay be performed by the same operator from beginning to end.

ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and calibrators.
2. Add 50 µL of calibrator or sample to each well. Then add 50 µL of prepared **Detection Reagent A** to each well immediately. Shake and mix. Incubate 1 hour at 37°C.
3. Aspirate and wash 3 times.
4. Add 100 µL of prepared **Detection Reagent B**. Incubate 30 minutes at 37°C.
5. Aspirate and wash 5 times.
6. Add 90 µL of **TMB Substrate**. Incubate 10-20 minutes at 37°C.
7. Add 50 µL of **Stop Solution**. Read at 450 nm immediately.

IMPORTANT NOTE

1. The final experimental results will be closely related to the validity of the products, so kits should be used prior to their expiration date. Store kits according to the instructions.
2. Kits from different batches may vary slightly in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instructions provided within the kit. Electronic instructions obtained from our website are for **reference only.**
3. Do not mix or substitute reagents from one kit lot to another. Only use the reagents supplied by the manufacturer.

4. Protect all reagents from strong light during storage and incubation. All of the bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganisms. The **TMB Substrate** should remain colorless until it has reacted with the enzyme which binds it to the microplate.
5. There may be some foggy substances in the wells when the plate is opened for the first time. It will not have any effect on the final assay results. Do not remove microplates from the storage bag until needed.
6. Incorrect operations completed during the reagents preparation and loading, as well as incorrect parameter setting for the plate reader may lead to incorrect results. A microplate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 O.D. at 450 (± 10) nm wavelength is acceptable for use in absorbance measurement. Please read the instructions carefully and adjust the instrument prior to the experiment.
7. Variations in sample preparation and each step of experimental operation may produce different results. In order to get better reproducible results, the operation of each step in the assay should be controlled.
8. Each kit has passed a strict Q.C test. However, results from end users may be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipment. Intra-assay variance among kits from different batches might arise from the above factors as well.
9. Kits from different manufacturers with the same item might produce different results, since we have not compared our products with other manufacturers.
10. The **Calibrato**r for the kit and the immunogen used for antibody preparation are commonly recombinant proteins. Different fragments, expression systems, and purification methods might be used in recombinant protein preparation. We cannot guarantee the kit will detect recombinant proteins from other companies. It is therefore not recommended to use the kit for the detection of recombinant protein.
11. Predict the concentration of target molecules in the samples or arrange a preliminary experiment. It is a good way to solve a specific problem, e.g. the concentration of samples is beyond the detection range of the kit.
12. The kit may not be suitable for the detection of samples from some special experiments, for instance, knock-out experiments, due to their uncertainty of effectiveness.
13. The **Stop Solution** suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES

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