KAMIYA BIOMEDICAL COMPANY

Human Nuclear Factor Kappa B (NFkB) ELISA

For the quantitative determination of human NFkB in tissue homogenates, cell lysates and other biological fluids

Cat. No. KT-23802

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

Product Information

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INTENDED USE

The kit is a sandwich enzyme immunoassay for the *in vitro* quantitative measurement of human NFkB in tissue homogenates, cell lysates and other biological fluids. For research use only. Not for use in diagnostic procedures.

COMPONENTS

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

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microplate reader with 450 ± 10 nm filter.
2. Precision single and multi-channel pipettes and disposable tips.
3. Eppendorf Tubes for diluting samples.
4. De-ionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.
6. Container for Wash Solution.

STORAGE

All the reagents should be kept according to the labels on vials. The **Calibrator**, **Detection Reagent A, Detection Reagent B** and the **96-well strip plate** should be stored at -20°C upon being received. The unused strips should be kept in a sealed bag with the desiccant provided to minimize exposure to damp air. Opened test kits will remain stable until the expiration date shown, provided it is stored as prescribed above.

PRINCIPLE

The microtiter plate provided in this kit has been pre-coated with an antibody specific to NFkB. Calibrators or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for NFkB. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain NFkB, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The

concentration of NFkB in the samples is then determined by comparing the O.D. of the samples to the calibration curve.

SAMPLE COLLECTION AND STORAGE

Tissue homogenates

The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissues were rinsed in ice-cold PBS (0.02 mol/L, pH 7.0-7.2) to remove excess blood thoroughly and weighed before homogenization. Minced the tissues to small pieces and homogenized them in 5~10 mL of PBS with a glass homogenizer on ice (Micro Tissue Grinders works, too). The resulting suspension was sonicated with an ultrasonic cell disrupter or subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenates were centrifugated for 5 minutes at 5,000 x g. Remove the supernate and assay immediately or aliquot and store at ≤-20℃.

Cell Lysates

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

1. Adherent cells should be detached with trypsin and then collected by centrifugation (suspension cells can be collected by centrifugation directly).
2. Wash cells three times in cold PBS.
3. Resuspend cells in PBS (1X) and the cells was subject to ultrasonication for 4 times (or Freeze cells at ≤-20℃. Thaw cells with gentle mixing. Repeat the freeze/thaw cycle for 3 times.)
4. Centrifuge at 1,500 x g for 10 minutes at 4℃ to remove cellular debris.

Other biological fluids

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

Note:

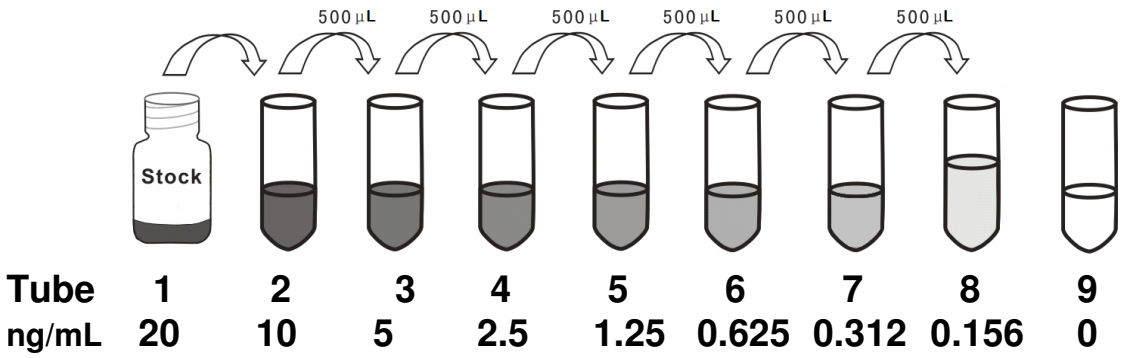
1. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. When performing the assay slowly bring samples to room temperature.

REAGENT PREPARATION

Bring all kit components and samples to room temperature (18-25°C) before use.

Calibrator

Reconstitute the **Calibrator** with 1.0 mL of **Calibrator Diluent,** kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the calibrator in the stock solution is 20 ng/mL. Please firstly dilute the stock solution to 10 ng/mL and the diluted calibrator serves as the highest calibrator (10 ng/mL). Then prepare 7 tubes containing 0.5 mL Calibrator Diluent and use the diluted calibrator to produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted calibrator such as 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.312 ng/mL, and the last EP tubes with **Calibrator Diluent** is the blank as 0 ng/mL.



Assay Diluent A and B
Dilute 6 mL of Assay Diluent A or B Concentrate (2X) with 6 mL of de-ionized or distilled water to prepare 12 mL of Assay Diluent A or B. **The prepared working dilution can't be frozen.**

Detection Reagent A and B
Briefly spin or centrifuge the stock Detection Reagent A and Detection Reagent B before use. Dilute to the working concentration with working **Assay Diluent A or B,** respectively (1:100).

Wash Solution
Dilute 20 mL of Wash Solution Concentrate (30X) with 580 mL of de-ionized 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 calibrator within 15 minutes before assay. Please do not dissolve the reagents at 37°C directly.
2. Making serial dilution in the wells directly is not permitted.
3. Please carefully reconstitute Calibrators or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals have 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 once pipetting.
4. The reconstituted Calibrators, Detection Reagent A and Detection Reagent B can be **used only once**.
5. If crystals have formed in the Wash Solution concentrate (30X), warm to room temperature and mix gently until the crystals have completely dissolved.

ASSAY PROCEDURE

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.

1. Determine wells for diluted calibrator, blank and sample. Prepare 7 wells for calibrator, 1 well for blank. Add 100 µL each of dilutions of calibrator (read Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37°C.

2. Remove the liquid of each well, don't wash.

3. Add 100 µL of **Detection Reagent A** working solution to each well. Incubate for 1 hour at 37°C after covering it with the Plate sealer.

4. 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 it 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 Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

5. Add 100 µL of **Detection Reagent B** working solution to each well. Incubate for 30 minutes at 37°C after covering it with the Plate sealer.

6. Repeat the aspiration/wash process for five times as conducted in step 4.

7. Add 90 µL of **Substrate Solution** to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.

8. Add 50 µL of **Stop Solution** to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by gently tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

⚠ **Note:**

1. **Assay preparation:** Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Removed strips should be resealed and stored at -20℃ until the kit expiration date.
2. **Samples 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 as possible. 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 specimens, 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 observed.
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.
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. Please protect it from light.

CALCULATION OF RESULTS

Average the duplicate readings for each calibrator, control, and samples and subtract the average zero calibrator optical density. 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 a 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 NFkB concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. It is recommended to use some related software to do this calculation. 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.

PERFORMANCE

Detection Range

0.156 - 10 ng/mL.

The calibration curve concentrations used for the ELISA's were 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.312 ng/mL, 0.156 ng/mL.

Sensitivity

The minimum detectable dose of human NFkB is typically less than 0.048 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 the mean O.D. Value of 20 replicates of the zero calibrator plus three standard deviations.

Specificity

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

IMPORTANT NOTES

1. The final experimental results will be closely related to operation skills of the end users and the experimental environments. Please make sure that sufficient samples are available.
2. This assay is designed to eliminate interference by soluble receptors, ligands, 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.
3. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
4. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganism.
5. There may be some foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results. Do not remove microtiter plate from the storage bag until needed.
6. A microtiter plate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 O.D. or greater at 450 nm wavelength is acceptable for use in absorbance measurement.
7. The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

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