Human NF-Kappa B p65 Total ELISA Kit

96T

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FIVEphoton Biochemicals

For research use only.
Not for diagnostics.

Part No. hNFKBp65-ELISA

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Store: 4°C.
Safety: Stop solution contains acid. Avoid contact and inhalation. Wear eye protection.

Human NF-Kappa B P65 Total ELISA Kit (96T)

Part No. hNFKBp65-ELISA

Standard Peptide Concentration: 16ng/ml

Assay Range: 50pg/ml – 15ng/ml

For research only. Not for diagnostic applications.

Storage: 2-8^{o}C, I year shelf life.

Safety: Stop solution contains acid. Avoid eye and skin contact

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Overview: Please familiarize yourself with this protocol before performing the assay. It is recommended that the researcher overviews the literature to identify detailed methods for sample preparation; the sample isolation methods discussed in this protocol serve only as a general guide. Blanks, standards and samples should be measured at least in duplicate to account for pipetting error.

This kit measures p65 Nuclear Factor Kappa B (NFkB) in sandwich ELISA format. The ELISA plate is provided pre-coated with an anti-p65 monoclonal antibody. The experimenter mixes the samples with a provided polyclonal anti-p65 antibody labeled with biotin, and with the streptavidin-HRP solution. This sample-antibody-biotin-streptavidin-HRP mixture is then added to the ELISA plate and incubated for 60 min at 37^{o}C. Unbound biotin-linked antibody is washed away and color reagents are added, resulting in a change in the color of the solution to blue. The blue solution then turns yellow after addition of stop solution. The absorbance of the resulting yellow solution at 450nm corresponds to the concentration of p65 n the samples.

Sample Preparation: The below is provided as a general guide. Prior to performing the assay, the researcher should identify sample preparation methods for their experiment.

Nuclear – Cytoplasmic Fractionation: To follow NF-kappa B activation and measure p65 concentrations, a nuclear – cytoplasmic partitioning protocol is typically performed: A protocol for isolation of nuclear and cytoplasmic fractions is provided with Fivephoton Biochemicals Part no. NPI-1. Measuring p65 in unfractionated cells can be done using a non-denaturing buffer, such as Fivephoton Biochemicals ELISA Lysis and Protein Extraction Buffer (Part ELSP-1). A protocol for isolation of proteins without fractionation is provided with this item. Isolation of p65 in tissues can be performed with these buffers following a tissue homogenization step.

Do not use denaturing cell lysis buffers such as RIPA for protein extraction.

Samples can be aliquoted and stored at -80^{o}C for later use.

1. Materials supplied. Store all materials at 4^{o}C. If particulates are observed in any of the reagents, centrifuge and use the clarified supernatant for the assay.

1	Standard peptide: (16ng/ml)	0.5ml	7	Chromogen Solution A	6ml
2	Standard diluents (1% BSA in PBS pH 7.4	3 ml	8	Chromogen Solution B	6ml
3	Anti-p65 monoclonal antibody coated ELISA plate (96T)	12 well× 8 strips	9	Stop Solution	6ml
4	Streptavidin-HRP	6 ml	10	Instruction Manual	1
5	30×wash solution	20 ml	11	Closure Membrane	2
6	Anti-p65 polyclonal antibody labeled with biotin (detection antibody)	1ml	12	Sealed bags	1

Materials required but not supplied

1. Sample dilution buffer (1% BSA in PBS pH 7.4)
2. 37^{o}C incubator
3. Standard absorbance plate reader
4. Precision pipettes and disposable pipette tips
5. De-ionized water
6. Disposable tubes for sample dilution
7. Absorbent paper

Important notes and preparation for the assay

1. Make a solution that is 1% BSA in PBS pH 7.4 for dilution of samples (i.e. "sample dilution solution").
2. Equilibrate the ELISA plate strips to room temperature for at least 30 minutes. The plate strips should be kept in a zip lock bag to prevent drying of the wells. Store unused sections of the ELISA plate in a sealed bag at 4^{o}C.
3. Use new pipettes tips at each step to prevent contamination.
4. Do not use reagents from other ELISA kits.
5. Substrate B is light sensitive. Limit light exposure.
6. **Manual Wash Method:** Gently aspirate off the liquid in each well. Turn over the plate and pat-dry on absorbent paper. Add 100μl wash solution and let percolate in the wells for 2 minutes prior to aspirating. Repeat the wash steps 5 times with 30 second washes. Therefore 600ul wash solution is needed per well. An automatic washer can also be employed to wash the ELISA wells.
7. **Very Important**: Perform preliminary tests to determine the appropriate dilution of your samples to match the linear assay range of the ELISA kit. For example, remove a 8-well strip, prepare a standard well each at the most concentrated and dilute concentrations of the assay range, set one well as the bank and perform a dilution series of a representative sample at 10X increments, then perform the assay. Identify the dilution factor that provides a measurement at approximately the center of the assay range for most of your samples.

Assay procedures

Blanks, standard and sample preparation: Prepare the blanks, standards and samples in a separate 96-well dish and transfer simultaneously to the ELISA plate. Do not prepare samples in the ELISA plate.

Assay procedure

1. Standard dilutions. Use Table 2 as a guide for dilutions of standards in the standard diluent solution of a standard peptide that is 80ng/ml concentration. Make dilutions in separate tubes or multi-well dishes, not in the

ELISA dish.

Table 2. Standard Dilutions

Standard Concentration	Standard Number	Dilution Instructions
8 ng/ml	7	120µl original standard + 120µl standard diluents solution, mix
4 ng/ml	6	120µl standard No.7 + 120µl standard diluents solution, mix
2 ng/ml	5	120µl standard No.6 + 120µl standard diluents solution, mix
1 ng/ml	4	120µl standard No.5 + 120µl standard diluents solution, mix
500 pg/ml	3	120µl standard No.4 + 120µl standard diluents solution, mix
250 pg/ml	2	120µl standard No 3 + 120µl standard diluents solution, mix
125 pg/ml	1	120µl standard No 2 + 120µl standard diluents solution, mix

2. Blank, standard and sample preparation: (Pre-mix solutions in a separate multiwell dish and transfer the solutions simultaneously to the ELISA dish. Do not pre-mix solutions in the ELISA dish).
   a) Blank wells: Only add chromogen solutions and stop solutions at the appropriate steps of the assay. Otherwise follow the protocol omitting the other solutions.
   b) Standard solution wells: Add 50µl of diluted standard and 50µl Streptavidin-HRP solution to each standard well (detection antibody labeled with biotin has been added in advance to the standards, therefore no biotin antibodies are added to the standards).
   c) Sample well: For each well, prepare 10ul sample (that may have been previously diluted to meet the assay range) in 30µl sample diluent solution (1% BSA in PBS pH 7.4 – made by the experimenter), then add 10µl anti-p65 antibody and 50µl Streptavidin-HRP solution. Mix gently; do not vortex. Accurately transfer 100µl of the premixed sample solution to the ELISA dish. (You may opt to prepare more sample solutions using these reagent ratios to assure 100µl of solution is accurately transferred to each well). Seal the wells with a zip lock bag and rotate the plate gently for 60 minutes at 37^{o}C.
3. Preparation of wash solution: Dilute the 30X wash solution to 1X with dH_20. Prepare 600µl 1X wash solution per well.
4. Wash: Carefully remove the seal plate membrane or zip lock bag that protects the wells from drying: do not cross-contaminate liquid. Gently aspirate off the liquid in each well. Turn over the plate and pat-dry on absorbent paper. Add 100µl wash solution and let percolate in the wells for 2 minutes prior to aspirating. Repeat the wash steps 5 times with 30 second washes. Therefore 600ul wash solution is needed per well. An automatic washer can also be employed to wash the ELISA wells. Blot dry the plate but do not allow the wells to dry.
5. Color development: First add 50µl chromogen solution A to each well and then add 50µl chromogen solution B to each well. Shake gently to mix solutions A and B. Incubate protected from light for 10 minutes at 37^{o}C.
6. Stop: Add 50µl Stop Solution to each well to stop the reaction (the blue color changes into yellow).
7. Read samples at 450nm within 10 min of adding the stop solution: Set the blank well as zero, measure the absorbance (OD) of each well at 450nm.

Data Analysis

1. Compile a standard curve using the blank standard solutions and the corresponding OD values. You may wish to calculate a linear regression equation from the standard curve to determine the concentration of your samples. Take into account dilution factors in your calculation. Alternative methods of data analysis to calculate concentrations of your samples can also be employed.

Flow chart of the procedures

Separately prepare standards, blank and samples with detection antibody and HRP-streptavidin as required

Incubate with the ELISA plate for 1 hr at 37^{o}C.

Wash the plate five times.

Add Chromogen solutions A and B. Incubate for 10 min at 37^{o}C for color development.

Add stop solution

Measure OD value at 450nm within 10 min