NF-{kappa}B RelA p65 Sandwich Elisa General Protocol

FIVEphoton Biochemicals

For research use only.
Not for diagnostics.

This protocol is posted as a reference. Use the protocol shipped with your kit for your experiments

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Store: 4°C. Expires 6 month after arrival.
Safety: Stop solution contains acid. Avoid all contact and inhalation.

BACKGROUND

This ELISA kit targets the RelA (p65) subunit of NF-kappa B, using a 96-well strip plate coated with affinity purified antibody specific for p65, together with a provided p65 peptide to calibrate absorbance to p65 concentration. A horse-radish peroxidase (HRP) conjugated polyclonal antibody and chromogens are employed for colorimetric detection. Simultaneous addition of a dilution series of standard peptide as well as assay samples are applied to individual wells. Following a 30 min – 1 hr incubation period, unbound material is washed, and the HRP conjugated antibody is added to wells. After an additional 30 min – 1 hr incubation, unbound antibody is rinsed and chromogen solutions are added to the wells. The reaction between chromogens and HRP is allowed to proceed for 15 min prior to stopping. An absorbance multiplate reader is employed to measure OD at 450 nm. Correlation of absorbance in the assay samples with the peptide standard, provided at known concentration, is used to determine p65 concentration in the assay samples.

Standards concentrations and assay ranges:

Species	Standard Peptide Concentration	Assay Range
Human	2250 pg/ml	100 – 2000 pg/ml
Mouse	1800 pg/ml	50 – 1500 pg/ml
Rat	2700 pg/ml	30 – 1800 pg/ml

Table 1. Provided Materials in the Kit

Materials provided with the kit	96 determinations	Storage
User manual	1	
Closure plate membrane	2	
Sealed bags	1	
Microelisa strip plate	1	2-8℃
Standard	0.5ml×1 bottle	2-8℃
Standard diluent	1.5ml×1 bottle	2-8℃
HRP-Conjugate reagent	6ml×1 bottle	2-8℃
Sample diluent	6ml×1 bottle	2-8℃
Chromogen Solution A	6ml×1 bottle	2-8℃
Chromogen Solution B	6ml×1 bottle	2-8℃
Stop Solution	6ml×1 bottle	2-8℃
Wash solution (30X)	20ml×1 bottle	2-8℃

Materials required but not supplied

1. 37^{o}C incubator
2. Standard microplate reader
3. Distilled water

4. Disposable tubes for sample dilution
5. Absorbent paper

Important notes and preparation for the assay

1. It is recommended that the experimenter perform preliminary tests using reagents of this kit to identify the sample dilution required to meet the assay range. Perform a preliminary assay with samples, using standards at the high and low dilution. Suspend and dilute experimental samples into the "sample diluent" provided with this kit to meet the requirements of the assay range. A dilution series of several samples may be required to identify the correct concentration that meets the assay range. Concentrate or dilute test samples if adjustment is needed. Set aside sufficient experimental sample for reserve to repeat the assay.
2. Determine whether the sample vehicle reacts with the assay: Dilute the sample in sample diluent or prepare samples in another vehicle to prevent inadvertent experimental readings.
3. The kit should be equilibrated to room temperate for 30 min prior to performing the assay. Store opened microelisa plates in a sealed plastic bag at 4^{o}C. A multi-channel sampler that has been calibrated for accuracy is a preferred method to apply samples. Plates should be sealed during the assay. Wells should not be allowed to dry.
4. Perform dilution of the standards in separate tubes or 96-well plates, not in the ELISA wells. Transfer solutions simultaneously to the ELISA dish.
5. It is recommended that samples are assayed in duplicate to address pipetting error.
6. Use new applicator tips and ELISA plate sealants to avoid cross-contamination.
7. Do not mix reagents from other ELISA kits.
8. Note that sodium azide in samples that is not washed away may inhibit HRP.
9. When calculating concentration of your sample from the assay, make sure to take into account the dilution factor.
10. If wash solution crystallizes during storage at 4^{o}C, heat solution at 37^{o}C and shake until crystals suspend.

Sample preparation for p65(RelA) ELISA assay. Using ELISA to measure NF-κB activation.

p65(RelA) is localized in cells in the cytosol and in the nucleus. It translocates from the cytosol to the nucleus when NF-κB is activated. To isolate a total cellular p65(RelA) pool for ELISA assay, use a non-denaturing detergent lysis buffer such as the Fivephoton Biochemicals ELISA Lysis Buffer (Part ELSP-1). The protocol associated with the lysis buffer can be used to assay the total p65 cellular pool.

This ELISA assay can also be used to measure NF-κB activation by partitioning a cytoplasmic and nuclear p65 pool and assaying the concentration of p65 in the respective cellular compartment. The ratio of nuclear relative to cytoplasmic p65 provides an index of NF-κB activation. The Fivephoton Biochemicals Nuclear Protein Isolation Kit (Part NPI-1), or similar, and the associated protocol, can be employed to isolate nuclear and cytoplasmic fractions for ELISA.

Assay procedures

Standard and Sample Preparation. Standards, samples and blanks should be added simultaneously to wells. Prepare the standards, samples and blanks in a separate 96-well dish and transfer simultaneously to the ELISA dish.

Assay procedure

1. Set aside and mark 12 wells for standard peptide dilutions. Configure six concentrations of standard peptide in duplicate, suspended and mixed as indicated below in separate tubes, or in a separate 96-well dish. Do not use the ELISA wells directly to perform the dilutions: there should be 6 wells in duplicate. The final sample volume in each well should be 50μl.

Table 2. Standard dilutions (Displayed below for rat; mouse and human use a similar dilution protocol).

1800 pg/ml	Standard No. 6	100μl Standard peptide + 50μl standard diluent, mix. Remove 100μl to make Standard No. 5.
1200 pg/ml	Standard No. 5	100μl Standard No. 6 + 50μl standard diluent, mix. Remove 100μl to make Standard No. 4.
600 pg/ml	Standard No . 4	100μl Standard No. 5 + 100μl Standard diluent, mix. Remove 50μl to make Standard No. 3. Remove and discard 100μl.
300 pg/ml	Standard No . 3	50μl Standard No. 4 + 50μl Standard diluent, mix. Remove 50μl to make Standard No. 2.
150 pg/ml	Standard No. 2	50μl Standard No. 3 + 50μl Standard diluent, mix. Remove 50μl to make Standard No. 1.
75 pg/ml	Standard No. 1	50ml Standard No. 2 + 50ml Standard diluents, mix. Remove and discard 50μl.

2. Set up 2 blank wells separately. In blank wells, add 40μl sample diluent and 10μl solution used to solubilize the samples (i.e. vehicle); do not add sample. Perform all other procedures of the assay, except do not add HRP conjugated antibody to the blank wells.

3. For wells with experimental samples, add 40μl sample diluent in each well, then add 10μl of sample diluted in sample diluent. This creates a 5X dilution factor of sample which should be accounted for later when calculating sample concentration.

4. Transfer the Standard Solutions, Blank Well Solutions and Experimental Samples simultaneously to the ELISA dish from the 96-well dish used for solution preparation.

5. Use the closure membrane (or a zip lock bag) to enclose the plate, mix gently with a rotating table, and incubate for 30 min at 37^{o}C, or 1 hr at RT.

6. Dilute the 30X Wash Solution with dH_20. Make 3 ml of diluted wash solution for each assay well.

7. After the first incubation period, discard the liquid in the wells by gently aspirating. Turn the plate upside down and gently pat dry the plate with an absorbent paper. To wash the wells, fill each well with 100 μl of

diluted Wash Solution, oscillate gently with the rocker table for 30 sec, and then aspirate off liquid. Pat dry the microplate with absorbent paper. Repeat the wash steps 5 times.

8. Simultaneously transfer 50 µl HRP-conjugate reagent to each well, except for blank wells. Rotate gently at 37°C for 30 min or R

9. Wash the wells as described in above 5 times. Remove liquid from the wells.

10. Transfer 50 µl of Chromogen Solution A and then 50µl of Chromogen Solution B to each well (prepare these solutions beforehand in a separate 96-well dish). Gently mix for 15 min at 37°C in the dark.

11. Simultaneously add 50 µl of Stop Solution to each well. Upon addition of stop solution, the blue color should immediately change to yellow.

12. Measure the optimal density (OD) at 450 nm within 15 minutes of adding the stop solution. Set the blank wells as zero.
13. If sample readings are higher than the most concentrated standard, you may wish to dilute the samples and assay again.

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. Remember that samples were diluted 5 fold in the assay in your final calculation. Other forms of analysis to calculate concentrations of your samples can also be applied.

Flow chart of the procedures

Prepare standards, blank and samples

Add samples to wells, incubate for 1 hr at RT or 30 min at 37°C.

Wash each well five times.

Add HRP conjugate antibody to each well, incubate for 1 hr at RT or 30 min at 37°C.

Wash each well five times

Add chromogen solutions A and B, 15 min at 37°C

Add stop solution

Measure OD 450 nm within 15 min