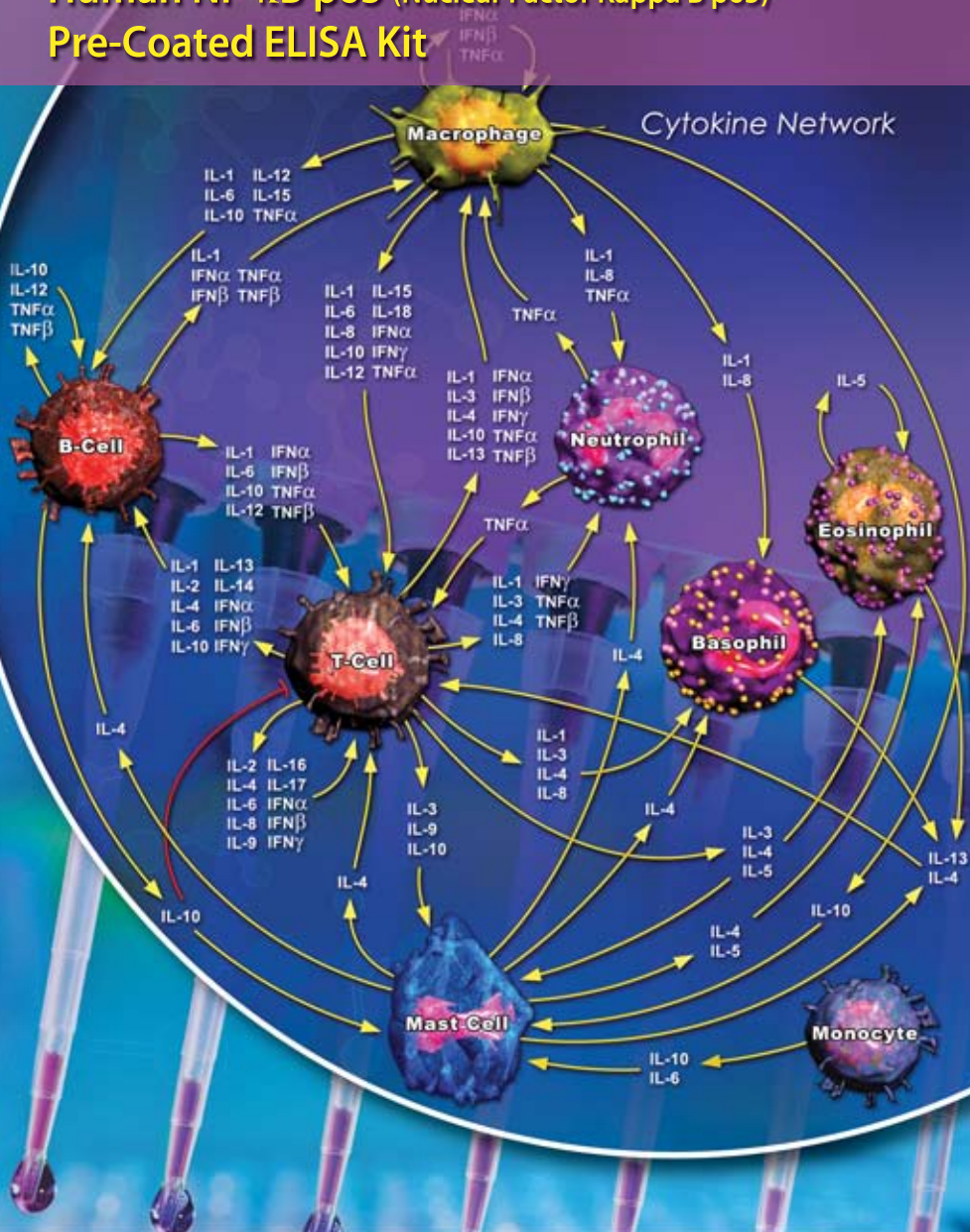


Human NF- κ B p65 (Nuclear Factor Kappa B p65) Pre-Coated ELISA Kit



USER MANUAL

abeomics
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Human NF- κ B p65

(Nuclear Factor Kappa B p65)

Pre-Coated ELISA Kit

Catalog No: 90-2392

1 × 96 well Format (96 tests)

Detection Range: 0.313 – 20 ng/ml

Sensitivity: < 0.188 ng/ml

This immunoassay kit allows for the in vitro quantitative determination of Human NF- κ B p65 concentrations in serum, plasma and other biological fluids. It also detects cytoplasmic, nuclear and total NF- κ B p65 from cells.

This kit is for Research Use Only. Not for use in diagnostic/therapeutics procedures.

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I. BACKGROUND

p65 (RelA) is one of the five members of NF- κ B transcription factor family that forms distinct transcriptionally active homo- and heterodimeric complexes with other members of the family. p65 subunit of NF- κ B is an important regulator of inflammation, proliferation, and apoptosis. p65 consists of a conserved 300 amino acid long amino-terminal Rel homology domain (RHD), and sequences within the RHD are required for dimerization, DNA binding, interaction with I κ Bs, as well as nuclear translocation. The canonical p65/p50 heterodimer is primarily associated with I κ B α and rapid, stimulus-induced degradation of I κ B α is critical for nuclear translocation and DNA binding of NF- κ B p65/p50. Over expression of p65 increases endogenous I κ B protein in both carcinoma and lymphoid cells by two mechanisms: protein stabilization and increased transcription of I κ B mRNA. p65 plays a key role in Treg biology, which may be of critical importance as therapeutic agents that impact upon NF- κ B are increasingly considered for the treatment of a diverse spectrum of diseases.

II. OVERVIEW

This kit was based on sandwich enzyme-linked immune-sorbent assay technology. Anti-Human p65 antibody was pre-coated into 96-well plates. Biotin conjugated anti-human p65 detection antibody was used. Standards, test samples and biotin conjugated detection antibody were added to the wells subsequently. Wash buffer was used to wash any non-specific binding. HRP conjugated Streptavidin was used as secondary antibody. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the Human p65 amount of samples captured in the plate. Optical Density (O.D) can be read at absorbance 450nm in a microplate reader. Concentration of Human p65 can be calculated using the standard curve.

III. ADVANTAGES

Multiple samples can be analyzed in a low volume, high-throughput format. Full analysis can be complete in 4 hours.

IV. STORAGE

Kit can be stored in 4°C, if you are using within a week.

If you are using within 6 months, lyophilized standard can be stored in -20°C and other components at 4°C.

Kit Components

Item	Specifications	Storage
96 well Strip ELISA Plate	8 X 12 well	4°C/-20°C
Lyophilized Standard	2 vials	4°C/-20°C
Sample and Standard Dilution Buffer	20 ml	4°C
Biotinylated Detection Antibody for hp65	120 µl	4°C/-20°C
Antibody Dilution Buffer	10 ml	4°C
HRP Conjugated Streptavidin (SABC)	120 µl	4°C in dark
SABC Dilution Buffer	10 ml	4°C
TMB Substrate	10 ml	4°C in dark
Stop Solution	10 ml	4°C
25X Wash Buffer	30 ml	4°C
Plate Sealer	5 pieces	
Product Manual	1	

Material Required, (Not Supplied)

Microplate Reader

37°C Incubator

Plate Reader

Multi Chanel Pipette and disposable tips

Eppendorf Tubes

Deionized Water

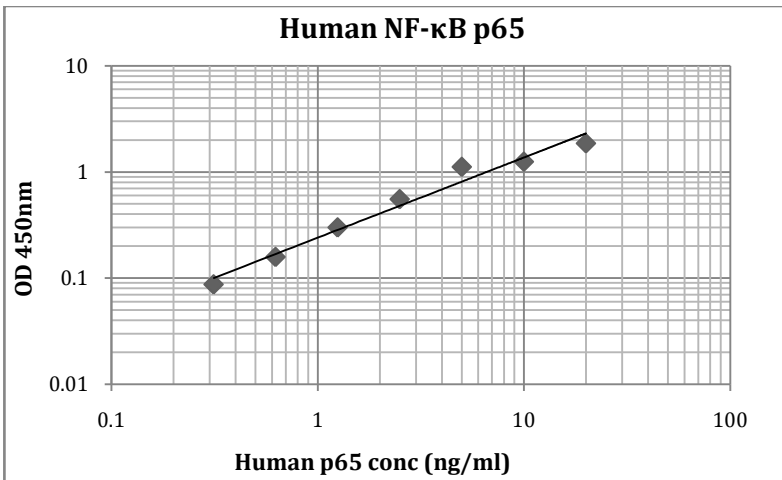
V. PRECAUTIONS FOR USE

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using standards and a small number of samples is recommended.

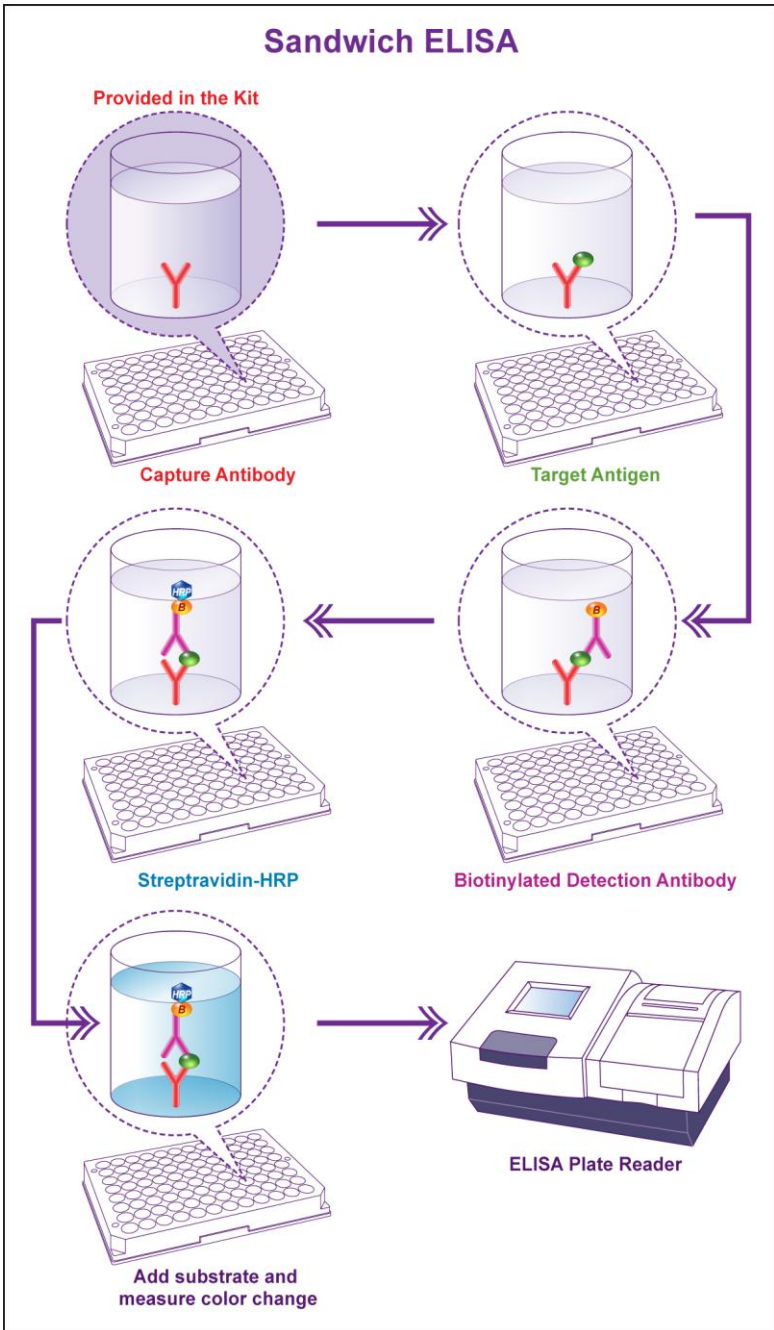
2. After opening and before using, keep plate dry.
3. Before using the Kit, spin tubes and bring down all components to the bottom of tubes.
4. Storage TMB reagents avoid light.
5. Washing process is very important, not fully wash easily cause a false positive.
6. Duplicate well assay is recommended for both standard and sample testing.
7. Don't let Micro plate dry at the assay, for dry plate will inactivate active components on plate.
8. Don't reuse tips and tubes to avoid cross contamination.
9. Avoid using the reagents from different batches together.

VI. STANDARD CURVE

Human NF-κB p65 Standard Curve is shown below.



X	ng/ml	20	10	5	2.5	1.25	0.625	0.313	0
Y	O.D.450	1.901	1.287	1.154	0.590	0.335	0.193	0.122	0.035



VII. REAGENT PREPARATION AND STORAGE

Included buffers and reagents are optimized for use with this kit. Substitution with other reagents is not recommended and may not give optimal results.

1. **Reconstitute the lyophilized Standard:** Standard should be prepared no more than 2 hours before the experiment. Use one tube for each experiment.
 - a. Quick spin down one vial of lyophilized standard. (**DO NOT dilute standard directly on the plate**). Add 1ml of sample/standard dilution buffer into one of the standard tube. Incubate at room temp. for 10 min. Mix thoroughly by vortex. Stock Standard concentration is 20 ng/ml.
 - b. Label 6 eppendorf tubes with 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.62 ng/ml, 0.313 ng/ml respectively. Add 0.3 ml of sample/standard dilution buffer into each tube. Add 0.3 ml of stock standard (500 pg/ml) into 1st tube and mix thoroughly. Transfer 0.3 ml from 1st tube to 2nd tube and mix thoroughly. Transfer 0.3 ml from 2nd tube to 3rd tube mix thoroughly, and so on.

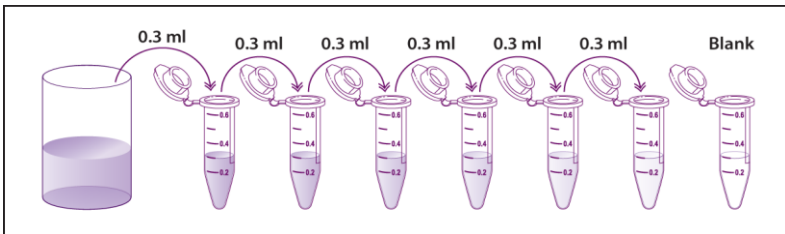


Fig-1: Dilution tubes

Note: Standard Solutions are best used within 2 hrs. Standard solution should be stored at 4°C for up to 12 hrs. or store at -20°C for up to 48 hrs. Avoid repeated freeze-thaw.

2. **Sample Preparation and storage:** Test samples should be collected, analyze immediately (within 2 hrs.) or aliquot and store at -20°C for long term. Avoid multiple freeze-thaw cycles.
 - a. **Cell culture supernatants:** Centrifuge to remove precipitate, analyze immediately or aliquot and store at -20°C.

- b. Serum:** Coagulate the serum at room temp about 1 hr. Centrifuge approximately $1000 \times g$ for 15 min. Analyze serum immediately or aliquot and store at -20°C .
- c. Plasma:** Collect plasma with heparin or EDTA as the anti-coagulant. Centrifuge for 15 min at $2-8^{\circ}\text{C}$ at $1500 \times g$ within 30 min of collection. For eliminating the platelet effect, suggesting that further centrifugation for 10 min at $2-8^{\circ}\text{C}$ at $10,000 \times g$. Analyze immediately or aliquot and store frozen at -20°C .
- d. Tissue Homogenates:** For general information, hemolytic blood may affect the results, you should rinse the tissues with ice cold PBS (0.01M, pH 7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then minced to small pieces. This will be homogenized in PBS in a cold glass homogenizer. (*Volume depends on the weight of the tissue, 1gram of tissue requires 9 ml of ice cold PBS with protease inhibitor*). To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze- thaw cycle. Homogenates are then centrifuged for 5 min. at $5000 \times g$ to get the supernatant.

Note: *Samples to be used within 5 days may be store at 4°C , otherwise sample should be stored at -20°C (<1 month) or -80°C (<2 months) to avoid loss of bioactivity and contamination. Hemolyzed samples are not suitable for use in this Assay.*

- e.** End user should estimate the concentration of the target protein in the test samples first, then select proper dilution factor to make the diluted target protein concentration falls the optimal detection range of the kit. Dilute the samples with the provided dilution buffer. Several trials may be necessary in practice. The test sample should be well mixed with the dilution buffer. Standard curve and sample should be made before the experiment.

High target protein concentration 200-2000 ng/ml:
Dilute 1:100 (add 1 μl of sample into 99 μl of sample/
standard dilution buffer).

Medium target protein concentration 20-200 ng/ml:
Dilute 1:10 (add 10 μ l of sample into 90 μ l of sample/
standard dilution buffer).

Low target protein concentration 0.313-20 ng/ml:
Dilute 1:2 (add 50 μ l of sample into 50 μ l of sample/
standard dilution buffer).

Very low target protein concentration < 0.313 ng/ml:
Do not dilute, use 100 μ l of sample.

- 3. Preparation of Biotin detection antibody working solution:** Prepare within one hour before the experiment. Calculate total volume working solution required. (0.1 ml/well \times number of wells. Add 100-200 μ l extra).

Dilute Biotin detection antibody with antibody dilution buffer at 1:100 and mix thoroughly. (*i.e.* add 1 μ l of Biotin conjugated detection antibody into 99 μ l of antibody dilution buffer).

- 4. Preparation of HRP-Streptavidin Conjugate (SABC) working solution:** Prepare within 30 min before the experiment. Calculate total volume working solution required. (0.1 ml/well \times number of wells. Add 100- 200 μ l extra).

Dilute SABC with SABC dilution buffer at 1:100 and mix thoroughly. (*i.e.* add 1 μ l of SABC into 99 μ l of SABC dilution buffer).

- 5. Preparation of 1 X Wash buffer:** Prepare 1 X Wash buffer by diluting 25X Wash buffer in sterile water. Diluted Wash buffer may be stored at 4°C, however we recommend preparing fresh 1X wash buffer for each experiment.

For example: 10 ml of 25X Wash buffer in 240 ml of sterile water.

VIII. ASSAY PROCEDURE

Before starting the experiment, equilibrate the SABC working solution and TMB substrate for at least 30 min at room temp. When diluting samples and reagents, they should be mixed completely and evenly. It is recommended to plot a standard curve for each test.

** If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.*

1. Set standard, test sample and blank (control zero) wells on the pre-coated plate and then record their position. It is recommended to measure each standard and sample in duplicate. **Note:** Wash plate twice before adding standard, sample and blank into the well.
2. Add 0.1 ml of standard 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml, 15.6 pg/ml, 7.8 pg/ml, and Blank (control zero dilution buffer) into standard well.
3. Add 0.1 ml of diluted samples into test sample wells.
4. Seal plate with a cover and incubate at 37°C for 90 min.
5. Remove the cover and discard samples and standard solution by tapping plate on an absorbent paper. **Note:** DO NOT let the wells completely dry any time. DO NOT wash plate.
6. Add 0.1 ml of Biotin-detection antibody working solution into the above wells (Standards, control zero and samples).
7. Seal plate with cover and incubate at 37°C for 60 min.
8. Remove the cover, and wash plate 3 times with 1X wash buffer.
9. Add 0.1 ml of SABC working solution into each well. Cover the plate and incubate at 37°C for 30 min.
10. Remove the cover and wash plate 5 times with 1X wash buffer. Each time let the wash buffer stay in the well for 1-2 min.
11. Add 90 µl of TMB substrate into each well, cover the plate and incubate at 37°C in dark within 15-30 min. (**Note:** This incubation time is for reference use only. The optimal time should be determined by end user). The shades of blue can be seen in the first 3-4 wells, only on most concentrated standards. Other wells show no obvious color.
12. Add 50 µl of stop solution into each well and mix thoroughly. Color will change into yellow immediately.
13. Read O.D. absorbance at 450 nm in a micro-plate reader immediately after adding the stop solution.

14. Calculation: Relative O.D. 450 = O.D. for each well – O.D. 450 control zero well. The Standard curve can be plotted as the relative O.D. 450 of each standard solution in Y axis vs. the respective concentration of the standard in X axis. Concentration of the samples can be incorporated from the standard curve. If the samples were diluted, multiply the dilution factor to the concentration.

Table-1

	Standard 1	Standard 2	3	4	5	6	7	8	9	10	11	12
A	20ng/ml	20ng/ml										
B	10ng/ml	10ng/ml										
C	5ng/ml	5ng/ml										
D	2.5ng/ml	2.5ng/ml										
E	1.25ng/ml	1.25ng/ml										
F	0.625ng/ml	0.625ng/ml										
G	0.313ng/ml	0.313ng/ml										
H	0 pg/ml	0 pg/ml										

IX. REFERENCES

1. Regulation of RelA (p65) function by the large subunit of replication factor C.
PMID: 12509469
2. The NF-kappaB family of transcription factors and its regulation.
PMID: 20066092
3. The Ubiquitination of NF-κB Subunits in the Control of Transcription.
PMID: 27187478

X. TROUBLE SHOOTING

Problem	Probable Cause	Suggestion
No signal	Forgot to add all components.	Prepare check list and add the components in the correct order.
Low signal	Not enough lysates per well.	Check the protein concentration. Add more lysates.
High background	Washing is not sufficient.	Wash plates thoroughly after incubation with Streptavidin-HRP secondary



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