PRODUCT INFORMATION & MANUAL

NF-κB/p65 (with coated strip plates) ActivELISA™

NBP2-31042

For the Detection of Cytoplasmic, Nuclear and Total NF-B/p65

The NBP2-31042 NF-κB/p65 ActivELISA™ Kit uses pre-coated plates, making it faster (4 hr) and more sensitive than NBP2-29912.

For research use only. Not for diagnostic or therapeutic procedures.

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Novus kits are guaranteed for 6 months from date of receipt

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I. BACKGROUND

Activation of the NF-κB pathway can be triggered by many factors including TNFα, UV, IL-1, lipopolysaccharide (LPS), mitogens, and phorbol esters. NF-κB is controlled by a family of inhibitory proteins called IκBs. IκB proteins are phosphorylated by the IκB kinase complex consisting of at least three proteins, IKK1/α, IKK2/β, and IKK3/γ. External stimuli such as tumor necrosis factor or other cytokines initiate a signal transduction cascade that leads to the activation of the IκB-kinase complex, which then specifically phosphorylates IκBα on Serine-32 and Serine-36. Phosphorylation of these sites leads to ubiquitination of IκBα and subsequent degradation by the 26 S proteasome. Degradation of IκBα results in unmasking of the nuclear localization signal of NF-κB dimers, which subsequently translocates to the nucleus and acts as a transcription factor for genes controlling inflammatory cytokines, adhesion molecules, and other proteins. Thus the nuclear levels of p65 may correlate positively with the activation of NF-κB pathway.

II. OVERVIEW

The NF-κB/p65 ActivELISA™ Kit measures free p65 in the nucleus of either cells or tissues. Standard protocols for detecting NF-κB activity include the electrophoretic mobility shift assay (EMSA), western blot, or reporter genes analysis. These assays are time consuming and may involve the use of radioactivity. The NF-κB ActivELISA™ can be completed in one day using a sandwich ELISA protocol. The p65 antibody coated plate captures free p65 and the amount of bound p65 is detected by adding a second biotin conjugated p65 antibody followed by Streptavidin-HRP using colorimetric detection in an ELISA plate reader at 450 nm. *Specificity: Human, Rat, Mouse*

III. ADVANTAGES

- Multiple samples can be analyzed in a low-volume, high-throughput format.
- Full analysis complete in just hours.
- Allows direct measurement of changes in p65 translocation.
- Allows study of NF-κB activation without gel-shift assay.

IV. EXPERIMENT

To monitor the nuclear translocation of p65, 293 cells were grown to 2 x 10^6 cells/ml and treated with recombinant hTNFα 25 ng/ml. 2 x 10^6 cells were harvested at various time points, nuclear extracts prepared using Novus' Nuclear Extraction Kit (NBP2-29447). NF-κB ActivELISA™ Kit (NBP2-31042) was used to monitor and measure the relative increase of p65 translocation into the nucleus, cytoplasm and total lysate.

EXAMPLE STANDARD

Fig. 1: Following the provided protocol, a p65 standard curve was generated.

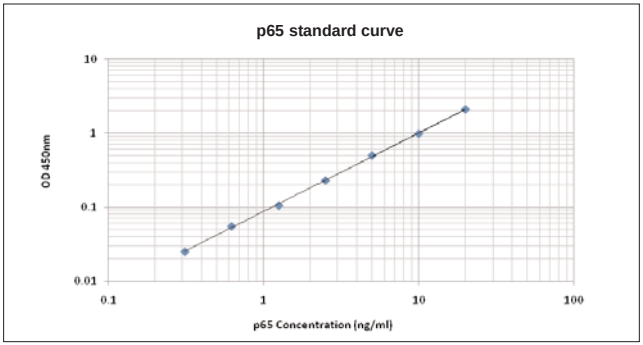


Fig. 2: The level of p65 in the samples were determined with respect to the standard (400 µg of total nuclear extract used).

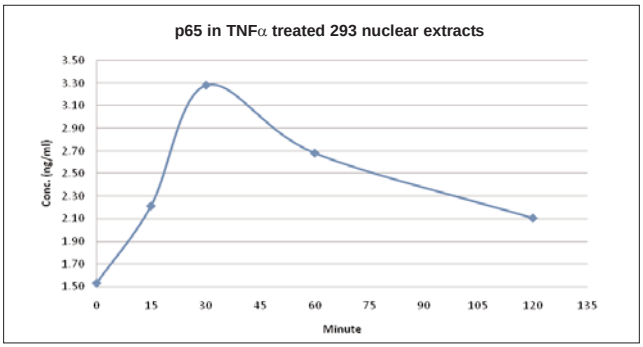
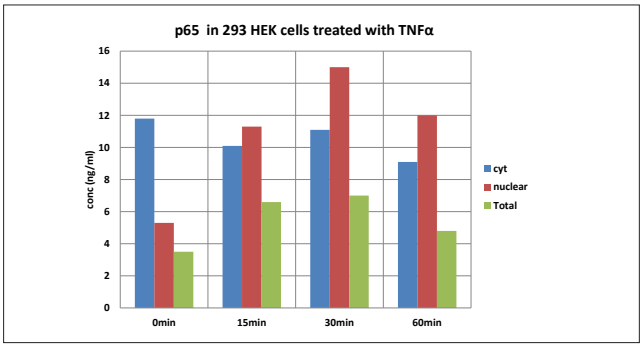
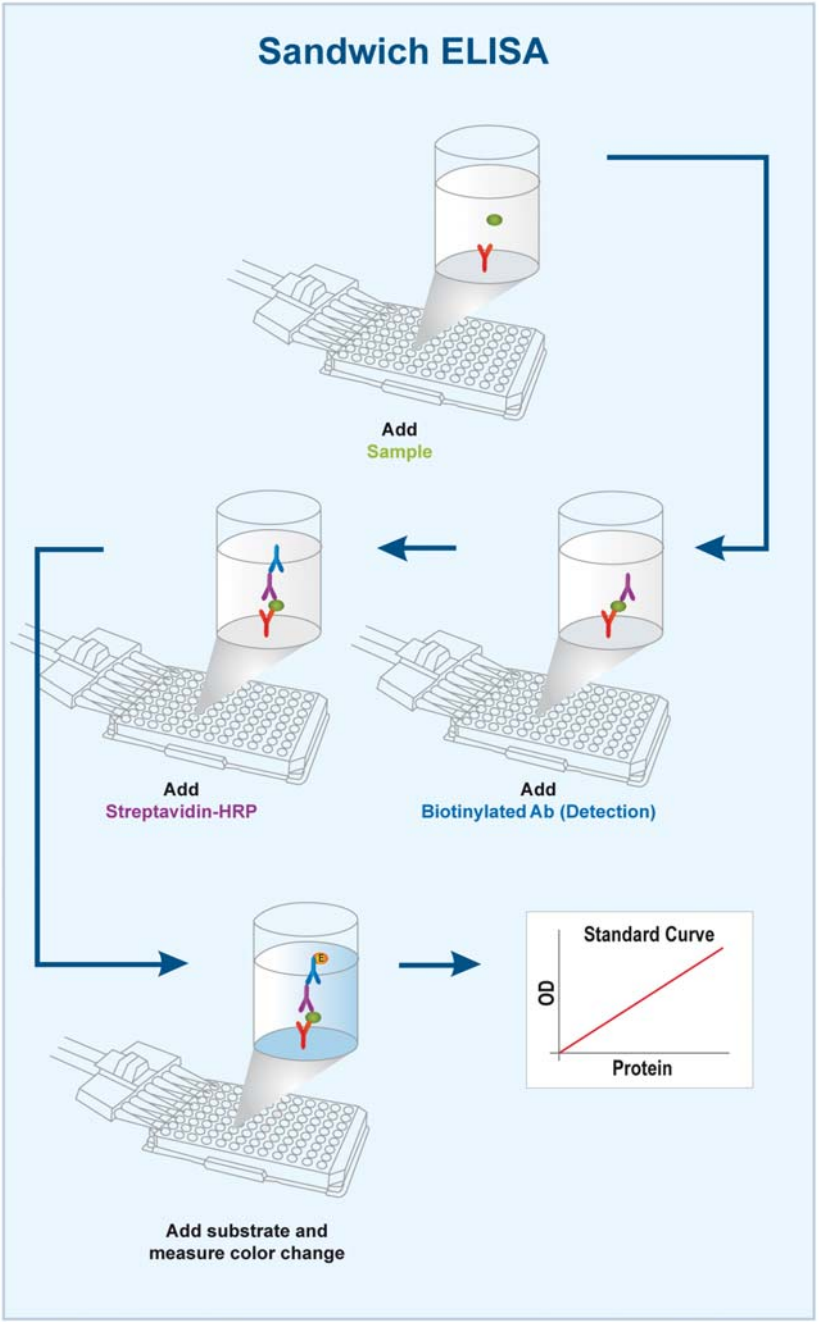


Fig. 3: The level of p65 in the samples were determined with respect to the standard (400 µg of total nuclear and cytoplasmic extracts were used).




Sandwich ELISA
Add
Sample
Add
Biotinylated Ab (Detection)
Add
Streptavidin-HRP
Add substrate and
measure color change
Standard Curve
OD
Protein

V. KIT COMPONENTS AND STORAGE

The NBP2-31042 (NF-κB/p65 ActivELISA™ Kit) contains:

REAGENTS (4°C STORAGE)		
NBP2-31042- 2B	**Detection Antibody***	250 µl
KC-101	**20X Wash Buffer**	50 ml
KC-154	**100X Steptavidin-HRP**	250 µl
KC-153	**5X ELISA Buffer**	30 ml
KC-155	**TMB Substrate Solution**	25 ml
KC-156	**Stop Solution**	25 ml
REAGENTS (-20°C, STORAGE--NON FROST-FREE FREEZER)		
NBP2-31042- 3L	**Recombinant p65 Standard**	2 vials, lyophilized (0.42 µg/vial)
ADDITIONAL ITEMS INCLUDED		
Coated Strip Plates		2
Manual		1

** Contains 0.02 % Sodium azide. Sodium azide is highly toxic.*

Additional items required for the ELISA (not included in the kit):

- Distilled water
- 96-well ELISA plate reader

VI. PREPARATION OF REAGENTS: p65 ActivELISA™

NOTE: *The included buffers and reagents are optimized for use with this kit. Substitution with other reagents is not recommended and may not give optimal results.*

1X Wash Buffer: Prepare 1X Wash Buffer by diluting 20X Wash Buffer (KC-101) in distilled water. The diluted Wash Buffer may be stored at 4°C, however we recommend preparing fresh 1X Wash Buffer for each experiment.

Example:

10 ml of 20X Wash Buffer in 190 ml sterile H_20.

1X ELISA Buffer: Dilute 5X ELISA Buffer to 1X in sterile H_20 by adding 10 ml of 5X ELISA Buffer to 40 ml of sterile H_20.

VII. p65 ActivELISA™ PROTOCOL

This kit allows for the quantitative measurement of p65 activation in a 96-well microtiter format. All 96-wells may be used at one time or you may only use the wells as required by your experimental design. Use of duplicate wells for each time point are recommended to obtain accurate results.

APPROPRIATE CONTROLS TO INCLUDE

Following is a list of suggested controls to include with each analysis:

1. Positive control: use a cell line or tissue known to constitutively express p65 or a recombinantly expressed p65.
2. Negative control: use a cell line or tissue known to not express p65.

ELISA PROTOCOL

1. **Prepare p65 Standard Curve:** Quick spin down the Recombinant p65 Standard vial and add 420 µl of sterile deionized H20. Vortex to dissolve. The stock standard concentration is 1000 ng/ml. Add 10 µl of stock standard (1000 ng/ml) into 490 µl of 1X ELISA Buffer. This makes 20 ng/ml. Set up a standard curve in duplicate using the following concentrations: 20, 10, 5, 2.5, 1.25, 0.62, 0.3 and 0.0 (blank) ng/ml. To obtain an accurate result, we suggest using the test samples in duplicate. (see Table 1 for suggested layout).

   An excessive amount of standard is provided to enhance testing flexibility. The resuspended stock standard should be aliquoted and stored frozen.

   - Wash plate 2X with 200 µl of fresh prepared 1X Wash Buffer. Add 100 µl of 1X ELISA Buffer in each well **B1** through **H1** and **B2** through **H2** for the standard.
   - Pipette 200 µl Recombinant p65 Standard (NBP2-31042-3L) (20 ng/ml) into wells **A1** and **A2**. Transfer 100 µl from wells **A1** and **A2** in to wells **B1** and **B2**.
   - Mix wells **B1** and **B2** by pipetting.
   - Transfer 100 µl from well **B1** to **C1** and **B2** to **C2**.
   - Continue this serial dilution process to wells **G1** and **G2**. After mixing, discard 100 µl of solution from wells **G1** and **G2**.
   - Do not add standard to wells **H1** and **H2**. These will serve as blanks.

2. **Samples:** Pipet 100 µl of positive and negative controls and 100 µl test samples (approx. 400-500 µg of total protein) into the appropriate wells. Incubate plate at RT or 4°C for 2-4 h. Samples may be diluted or serially diluted using ELISA Buffer. Users may need to empirically determine the optimal concentrations of their test samples so that the readings fall on the linear portion of the protein standard curve.

3. **Washing:** Remove samples and controls and wash 4X with 300 µl of 1X Wash Buffer. Tap plate several times upside down to remove residual Wash Buffer after final wash.

4. **Detecting Antibody:** Dilute sufficient Detecting Antibody (NBP2-31042- 2B, 100 µl/well) in 1X ELISA Buffer and add 100 µl of the dilution to each well. Incubate for 1 hr at RT. (For one plate, dilute 120 µl of Detecting Antibody, NBP2-31042- 2B in 12 ml of 1X ELISA Buffer)

Table 1. Set up of a 96-well microtiter plate.

	Standard	Standard	Your Samples									
	1	2	3	4	5	6	7	8	9	10	11	12
A	20 ng/ml	20 ng/ml	-	-	-	-	-	-	-	-	-	-
B	10 ng/ml	10 ng/ml	-	-	-	-	-	-	-	-	-	-
C	5 ng/ml	5 ng/ml	-	-	-	-	-	-	-	-	-	-
D	2.5 ng/ml	2.5 ng/ml	-	-	-	-	-	-	-	-	-	-
E	1.25 ng/ml	1.25 ng/ml	-	-	-	-	-	-	-	-	-	-
F	0.62 ng/ml	0.62 ng/ml	-	-	-	-	-	-	-	-	-	-
G	0.3 ng/ml	0.3 ng/ml	-	-	-	-	-	-	-	-	-	-
H	Blank	Blank	-	-	-	-	-	-	-	-	-	-

5. **Washing:** Remove antibody solution and wash wells 4X with 300 µl of 1X Wash Buffer. Tap plate upside down to remove residual Wash Buffer after final wash.

6. **Streptavidin-HRP:** Dilute sufficient Streptavidin-HRP (KC-154, 100 µl/well) in 1X ELISA Buffer and add 100 µl of the dilution to each well. Incubate for 30 min at RT. (For one plate, dilute 120 µl of KC-154 in 12 ml of 1X ELISA Buffer)

7. Remove the Streptavidin-HRP and wash thoroughly (5X) with 300 µl of Wash Buffer letting the solution sit briefly between each wash. This ensures a thorough wash and lower background. Tap plate upside down several times to remove any residual Wash Buffer.

8. Add 100 µl of TMB Substrate to each well. Incubate the plate at RT for 10-15 min.

9. Add 100 µl of Stop Solution. Read at 450 nm.

VIII. PRODUCT CITATIONS

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12. Whole Body Periodic Acceleration Modifies Experimental Asthma in Sheep. William M Abraham, Ashfaq Ahmed, Irakli Serebriakov, Isabel T Lauredo, Jorge Bassuk, Jose A Adams, and Marvin A Sackner. Am. J. Respir. Crit. Care Med., Jul 2006; 10.1164/rccm.200601-048OC. (Cells harvested from sheep)

IX. TROUBLESHOOTING p65 ActivELISA™

Problem	Probable Cause	Suggestion
No signal	Failure to add all components	Prepare a check-list and add the components in the correct order.
Low signal	Not enough lysate per well.	Check the protein concentration. Add more lysates.
High background	Improper blocking.	Incubate with blocking buffer as recommended in the manual.
	Washing is not sufficient.	Wash plates thoroughly after incubation with Streptavidin-HRP Secondary.