NF-kappa B Activation Assay Kits

Overview: (Cat. Nos. NFKB-1 and NFKB-2). Elicited by extracellular stimuli**,** the inhibitory subunit of NF-kappaB, Iκβ, becomes phosphorylated by Iκβ kinase, ubiquitinated and dissociates from the RelA-p50 complex, which exposes nuclear import signals. The exposure of nuclear import signal targets the RelA-p50 complex through the nuclear pore into the nucleus, where it binds to regulatory elements and modulates gene transcription.

In the FIVEphoton Biochemicals NF-kappa B Activation Assay Kits, cells are fractionated into cytoplasmic and nuclear fractions, which are then subjected to Western blot analysis using an antibody selective to p65 that is provided in kit NFKB-2 (antibody can be added as an option in kit NFKB-1). Observing elevated levels of the transcription factor components of NF-kappa B in nuclear fractions reveals NF-kappa B activation.

Kit Contents (Cat. No. NFKB-1 and Cat. No. NFKB-2)

1. Cytoplasmic Fractionation Reagent (CER-1)
   (Kit NFKB-1, 55 mls)
   (Kit NFKB-2, 55 mls)

2. Nuclear Fractionation Reagent (NER-1)
   (Kit NFKB-1, 3.5 mls)
   (Kit NFKB-2, 3.5 mls)

3. DTT (sufficient to make a 1M solution after addition of 64 μl dH_20)
   For Trial Kit, make a 50 μl 1 M DTT stock solution, and proceed as described in the protocol.

4. p65 antibody is provided in Kit NFKB-2, or can be purchased as an option in kit NFKB-1: 100 μl rabbit polyclonal IgG useable at 1:400 dilution. Reactive for human, mouse, rat, canine and hamster. Store antibody at 4°C.

Materials not provided (or are optional)

1. Protease Inhibitor cocktail, Serine Protease Inhibitor (see Cat. no. PI-1 on Fivephoton.com to purchase protease inhibitor cocktail)
2. Cell Scrapers
3. Refrigerated microcentrifuge for 1.5 ml tubes

Store all components, except antibody, at -20°C. Store antibody to p65 at 4°C.
Safety Precautions: Although contents are not considered hazardous, avoid skin and eye contact and ingestion. Use gloves and eye protection while using this product.

Protocol: NF-kappa B Activation Assay

The recommended reagent volumes detailed below are designated for one confluent (or 80-90% confluent) 10 cm cell culture dish. Adjust reagent volumes accordingly for other sized cell culture dishes based on the surface area of the cell culture dish. Other plates of cells with parallel treatments can be set aside to obtain total cell lysates using a lysis buffer, such as RIPA, for comparison and control to the fractionated materials.

All procedures should be performed rapidly without significant pauses to maintain separation of the cytoplasmic and nuclear fractions. Take note that certain substances employed to coat cell culture dishes and enhance cell adhesion may also activate NF-kappa B.

1. For Kit NFKB-1 and NFKB-2, add 64 µl dH_20 to the provided solid DTT to make a final 1M solution. Vortex until all DTT is suspended.

2. Defrost and prepare 1ml Cytoplasmic Fractionation Reagent (CER-1) and 60 µl Nuclear Fractionation Buffer (NER-1) for each 10 cm dish. Keep both solutions in a tube in ice. Add Protease Inhibitor Cocktail and serine protease inhibitor (such as PMSF) to both the Cytoplasmic and Nuclear fractionation reagents at concentrations recommended by the manufacturer immediately prior to use.

   Add DTT into the Cytoplasmic Fractionation Reagent (CER-1) to make a final concentration of 1 mM DTT immediately prior to use. Keep solutions in ice.

3. Remove media and gently wash the cell culture dish with 3 ml room temperature PBS. Use suction and a Pasteur pipette to remove as much PBS as possible after washing. Place the cell culture dish on a bed of ice.

4. Add 500 µl of Cytoplasmic Fractionation Reagent (CER-1) with protease inhibitors and DTT. Tilt the cell culture dish several times to cover the dish with a film of Cytoplasmic Fractionation Reagent, and keep the dish on a bed of ice for 5 min.

5. Using a cell scraper, scrape cells off the cell culture dish and dispense the cell suspension into a 1.5 ml snap-cap tube. Immediately centrifuge the tube for 3 min at 2500 rpm at 4°C in a refrigerated microcentrifuge.

6. Collect the supernatant (which contains the **cytoplasmic fraction**) in a tube for storage and re-suspend the pellet gently in 500 µl of Cytoplasmic Fractionation Reagent (CER-1) to wash the pellet. You can flick the tube with your fingers several times to re-suspend the pellet. Place the tube in ice for 5 min, and then centrifuge again for 3 min at 2500 rpm, 4° C. This step washes residual cytoplasmic materials from the resulting pellet. Retain the pellet.

   Remove as much supernatant as possible without disturbing the pellet (the pellet contains the nuclear fraction). *Removal of the supernatant wash must be performed immediately after centrifugation.*

7. Add 60 µl of ice cold Nuclear Fractionation Reagent (NER-1) with protease inhibitors to the nuclear pellet, vortex full speed for 1 min and then place the tube in ice for 10 min.

8. Centrifuge at maximum speed (typically 16,000 rpm for a small tube centrifuge) for 10 min. Carefully collect the *supernatant fraction* which corresponds to the **nuclear fraction.**

   Store the nuclear and cytoplasmic fraction (taken from step 6 above) at -80^{o}C prior to Western blot analysis. You may wish to aliquot fractionated materials in separate tubes prior to storage at this step.

9. **Resolving Cytoplasmic and Nuclear Fractions in Western Blots.** We recommend employing a dilution series of isolated materials suspended in Laemmli Sample Buffer to resolve cytoplasmic and nuclear NF-kappa B subunits in Western blots. For both the cytoplasmic and nuclear fractions, the transcription factor components of NF-kappa B may be present at high concentrations, which may result in Western blot band saturation. We recommend using a 1:1000 dilution in Laemmli sample buffer as a reference dilution for both the nuclear and cytoplasmic fractions.

10. **Western blot development with the provided p65 antibody in kit NFKB-2.** A 5-20% gradient gel, or a 10% TRIS-glycine gel is suitable to resolve p65 protein. Use standard TRIS-glycine, 20% methanol transfer buffer to transfer protein bands from the SDS-PAGE gel to the nitrocellulose membrane. Develop the Western blot with the provided p65 antibody at a 1:400 dilution, using anti-rabbit IgG-HRP and ECL for detection. Incubation times for the p65 antibody with the Western blot membrane are: 1 hr at RT or overnight at 4 ^{o}C. A 1 hr incubation time for the anti-rabbit IgG-HRP at RT should be used following exposure of the membrane to the p65 antibody.

Data Interpretation: Cell fractionation can be observed in Western blots by resolving a total cellular lysate, followed by consecutive lanes of the cytoplasmic and nuclear fractions in the absence and presence of conditions that activate NF-kappa B. With successful nuclear/cytoplasmic fractionation, you should observe changes in the relative levels of transcription factor subunits among the cellular fractions.

Troubleshooting

1. Cell Fractionation and NF-kappa B activation were not observed. A potential problem is suspension of the nuclear pellet prior to the removal of the second cytoplasmic fractionation reagent (step 6). Resolution: Perform procedures as rapidly as possible without pauses. Also, make sure protein bands in Western blots are not at saturation levels.

2. The transcription factors in the nuclear fraction resolve poorly in Western blots. Resolution: Dilute samples further in Laemmli Sample Buffer. Heat sample to 60^{o}C for 5 min and vortex for 1 min instead of boiling in Laemmli Sample Buffer.

3. Proteolysis of transcription factors was observed in Western blots. Resolution: Add protease inhibitors freshly into CER and NER reagents immediately prior to use.