NF- kappa B Activation Assay Kits

Part No. NFKB-2, hNFKB-4
Storage: Antibodies: 4°C. Solutions and DTT: -20°C.

Overview: The inhibitory subunit of NF-kappa B, IκB, becomes phosphorylated by IκB kinase, ubiquitinated, and dissociated from the p65(RelA)-p50 complex, which exposes nuclear import signals. The exposure of nuclear import signal targets the NF-kappa B p65(RelA)-p50 complex through the nuclear pore and into the nucleus. p65(RelA) then 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 a provided primary and secondary antibody selective to p65. Observing elevated levels of p65 in the nuclear fraction relative to the cytoplasmic fraction reveals NF-kappa B activation.

Safety Precautions: Although the contents are not considered hazardous, avoid skin and eye contact and ingestion. Use gloves and eye protection while using this product.

Kit Contents

Materials Included With Kit

	Part No.	Description	Volume
1.	CER-1	Cytoplasmic Fractionation Reagent	55 ml
2.	NER-1	Nuclear Fractionation Reagent	3.5 ml
3.	DTT	Dithiothreitol [1]	(solid)
4.	p65Ab	Rabbit IgG antibody to p65 [2]	100 µl
5.	G-HRP	Goat anti-rabbit IgG-HRP (Kit NFKB-2 only) [3]	50 µl

Notes

1. DTT (sufficient to make a 1 M solution after addition of 64 µl dH_20).
2. p65 antibody (Kits NFKB-1, hNFKB-2): 100 µl rabbit polyclonal IgG applicable at 1:400 dilution; reactive to human, mouse, rat, canine, bovine, porcine and hamster p65. Store antibody at 4°C.
3. Goat anti-rabbit IgG-HRP (100 µg/ml). Use at 1:3000 dilution. Provided in kit NFKB-2. Not provided with kit hNFKB-4 Store at 4°C.

Materials not provided and required

1. 1X PBS pH 7.4 to wash cells at ambient temperature.
2. Protease inhibitors, including serine protease Inhibitor.
3. Cell scrapers.
4. Plastic Pasteur pipettes.
5. Refrigerated microcentrifuge for 1.5 ml tubes.
6. ECL Western blot detection kit

Protocol: NF-kappa B Activation Assay

Overview: The reagent volumes detailed below are for one 10 cm cell culture dish with cells at 80-90% confluency. Adjust all reagent volumes for other sized cell culture dishes directly proportional to the surface area of the dish(es). For example, use 3/10 the recommended reagent volumes, for a well in a 6-well dish, or use 4X the recommended reagent volumes for four 10 cm culture dishes.

Parallel plates of cells can be set aside to obtain total cell lysates for comparison with the samples fractionated into nuclear and cytoplasmic components. Use a cell lysis buffer such as RIPA buffer to isolate unfractionated total cell lysate.

All procedures should be performed without significant pauses to maintain the separation of cytoplasmic and nuclear fractions. Take note that certain substances employed to coat cell culture dishes and enhance adhesion may also activate NF-kappa B and stimulate p65 translocation.

Reagent Preparation

Cytoplasmic Fractionation Reagent (CER-1)	1000 (µl)
Nuclear Fractionation Buffer (NER-1)	60 (µl)
DTT (1M)	1/1000 dilution into CER-1

Reagents for one 10 cm cell culture dish with cells grown to 80-90% confluency.

1. Add 64 µl dH_20 to the provided solid DTT to make a final 1 M solution. Vortex until all DTT is suspended. The 1M DTT solution can be aliquoted and stored frozen at -20°C for later use.

2. Defrost and prepare 1000 µl Cytoplasmic Fractionation Reagent (CER-1) and 60 µl Nuclear Fractionation Buffer (NER-1) for each 10 cm cell culture dish. Keep both solutions in a tube in ice. Add a protease inhibitor cocktail and serine protease inhibitor (such as PMSF) to the cytoplasmic (CER-1) and nuclear (NER-1) fractionation reagents at concentrations recommended by the manufacturer prior to use.

3. Add the 1M DTT solution into the Cytoplasmic Fractionation Reagent (CER-1) to make a final concentration of 1 mM DTT prior to use (stock solution is at 1M, therefore dilute DTT 1:1000). Keep solutions in ice.

Fractionation Steps

1. Remove media and gently wash the 10 cm cell culture dish twice with 3 ml of room temperature PBS pH 7.4. Use suction or a Pasteur pipette to remove all PBS. Place the cell culture dish on a bed of ice.

2. Apply 500 µl ice cold Cytoplasmic Fractionation Reagent (CER-1) with protease inhibitors and DTT to each plate of cells. Tilt the cell culture dish several times to cover the dish with a film of Cytoplasmic Fractionation Reagent. Keep the dish on a bed of ice for 5 min.

3. Dislodge cells using a cell scraper and then siphon the cell suspension using a Pasteur pipette with a wide spout into a 1.5 ml centrifuge tube. Centrifuge the tube for 3 min at 2500 rpm, at 4°C (use a refrigerated microcentrifuge or one placed in a cold room).

4. Immediately collect the supernatant which contains the **cytoplasmic fraction**. Re-suspend the pellet gently in 500 µl of ice cold Cytoplasmic Fractionation Reagent (CER-1) prepared with protease inhibitors and DTT, as noted above, 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 minutes, and centrifuge again for 3 min at 2500 rpm, 4° C.

   Remove as much supernatant as possible without disturbing the pellet. *Removal of the supernatant must be performed immediately after centrifugation.* Retain the pellet, which contains the nuclear material. You can discard the supernatant.

5. Add 60 µl of ice cold Nuclear Fractionation Reagent (NER-1) with protease inhibitors to the nuclear pellet; (include protease inhibitors as above). Vortex the tube full speed for 1 minute, and then place the tube in ice for 10 minutes.

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

   Store the **cytoplasmic** (from step 4) and **nuclear fractions** (from step 6) at -80°C. You may wish to aliquot fractionated materials prior to storage, or run samples in gels for Western blotting.

Resolving Cytoplasmic and Nuclear Fractions in Western Blots

An ECL detection kit (not provided) is required for this step.

Resolving cytoplasmic and nuclear proteins in Western blots. p65 protein may be present at high concentrations in the isolated cytoplasmic and nuclear fractions. To avoid Western blot band saturation, use a 1:1000 dilution of cytoplasmic and nuclear fraction solutions in Laemmli sample buffer as a starting reference for samples that will be loaded into gels. (Further dilution adjustments may be required to resolve p65 protein in Western blots in a linear range).

SDS-PAGE gel lanes can be loaded in the following consecutive order: First load lanes of the control, un-stimulated conditions and repeat the same arrangement with samples treated to activate NF-kappa B in the following order: 1) total cell lysate, 2) cytoplasmic fraction, 3) nuclear fraction.

Western blot development with the provided p65 antibody (brief overview).

A 10% TRIS-glycine SDS-PAGE gel or a 5-20% gradient TRIS-glycine SDS-PAGE gel are both suitable to resolve p65 protein (MW approx. 65kD). Load the gel lanes in the arrangement suggested above.

After the SDS-PAGE gel is resolved, transfer the proteins to a nitrocellulose membrane using standard Western blotting procedures. The Western blot membrane can be stored at 4°C or immediately developed with antibodies. Block the Western blot membrane using standard procedures with nonfat milk or protein in PBS-T for 1 hr.

Develop the Western blot with the provided p65 antibody at a 1:400 dilution in PBS-T (you can also add protein block to the antibody solutions). The recommended incubation times for the p65 antibody with the Western blot membrane are 1 hour at room temperature, or overnight at 4°C. (You may also opt to co-develop the Western blot with antibodies to a cytoplasmic marker protein (e.g. GAPDH) or nuclear marker protein (e.g. Histone H1) to calibrate the Western blot). After incubation with anti-p65 antibody, wash the membrane five times with PBS-T with the first wash at 15 minutes, and the remaining washes each at 5 minute intervals afterwards. Remove the wash solution from the membrane.

Dilute the provided goat anti-rabbit IgG-HRP antibody at a 1:3000 dilution in PBS-T, and apply to the Western blot membrane. Incubate the IgG-HRP antibody with the membrane for 1 hour at room temperature. Afterwards, wash with PBS-T five times, with the first wash at 15 min, and the subsequent washes each with 5 minute intervals.

Use an ECL chemiluminescence Western blot development method to detect p65 in the Western blot membrane.

Data Interpretation: Translocation of p65 into the nucleus can be observed and quantified in Western blots by resolving a total cell lysate, followed by consecutive lanes of the cytoplasmic and nuclear fractions. Use densitometry to measure the Western blot band that corresponds to p65. For each treatment, directly calculate the ratio of p65 protein in the nuclear fraction relative to the cytoplasmic fraction. You can also calibrate the data to standard cytoplasmic (e.g.. GAPDH) and nuclear proteins (e.g. histone H1) that are co-detected on the Western blots.

An elevated ratio of p65 protein in the nuclear fraction should be observed with stimuli that activate NF-kappa B.

Troubleshooting

1. Cell fractionation and translocation of p65 were not observed. A potential problem is suspension of the nuclear pellet prior to the removal of the second cytoplasmic fractionation reagent (step 4) due to prolonged and unplanned pauses while performing the isolation. Resolution: Perform all procedures as rapidly as possible. 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 into CER-1 and NER-1 reagents immediately prior to use.