p65(RelA) Translocation Assay Kit

Part No. p65-TRANS

The instructions below are for one well of 80-90% confluent cells in a 6-well dish (9.5cm^2 surface area/well; approximately 9.5 x 10^5 cellls/well). The volumes are scalable to other sized culture dishes.

Overview: The inhibitory subunit of NF-kappa B, Iκβ, becomes phosphorylated by Iκβ kinase, ubiquitinated and dissociated from the p65(RelA)-p50 complex, which exposes nuclear import signals. The exposure of nuclear import signal targets the p65(RelA-p50 complex through the nuclear pore into the nucleus, where it binds to regulatory elements and modulates gene transcription.

In the Fivephoton Biochemicals p65 translocation assay kit, 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 with the kit. Observing elevated levels of p65 in the nuclear fraction reveals NF-kappa B activation.

Kit Contents

1. Cytoplasmic Fractionation Reagent (CER-1): 55 ml
2. Nuclear Fractionation Reagent (NER-1): 3.5 ml.
3. DTT (sufficient to make a 1M solution after addition of 64 μl dH_20).
4. p65 antibody: 100 μl rabbit polyclonal IgG useable at 1:400 dilution. Reactive to human, mouse, rat, canine and hamster. Store antibody at 4^{o}C.

Materials not provided (or are optional)

1. PBS to wash cells (2 ml/well of cells).
2. Protease inhibitor cocktail, serine protease Inhibitor.
3. Cell scrapers
4. Plastic Pasteur pipettes
5. Refrigerated microcentrifuge for 1.5 ml tube
6. Anti-rabbit IgG-HRP
7. ECL Western blot detection kit

Store all components, except antibody, at -20^{o}C. Store antibody to p65 at 4^{o}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: p65 Translocation Assay

Overview: The recommended reagent volumes detailed below are for one well of 80-90% confluent cells in a 6-well culture dish. Adjust reagent volumes accordingly for other sized cell culture dishes based on the surface area of the cell culture dish. Additional 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 for the fractionated materials.

All procedures should be performed rapidly without significant pauses to maintain 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.

Preparation of Reagents

Cytoplasmic Fractionation Reagent (CER-1)	300 (µl)
Nuclear Fractionation Buffer (NER-1)	18 (µl)
DTT (IM)	1/1000 dilution into CER-1

Reagents for 1 well of a 6-well dish

1. 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 300 µl Cytoplasmic Fractionation Reagent (CER-1) and 18 µl Nuclear Fractionation Buffer (NER-1) for each well of cells. Keep both solutions in a tube in ice. Add protease inhibitor cocktail and serine protease inhibitor (such as PMSF) to both the cytoplasmic (CER-1) and nuclear (NER-1) fractionation reagents at concentrations recommended by the manufacturer of the inhibitors immediately prior to use.

3. Add DTT into the Cytoplasmic Fractionation Reagent (CER-1) to make a final concentration of 1 mM DTT immediately prior to use (i.e. 1000X dilution; stock solution 1M). Keep solutions ice cold.

Fractionation Steps

1. Remove media and gently wash the cell culture dish with 2 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.

2. Cover the cells with 150 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.

3. Using a cell scraper, remove cells from the cell culture dish, siphon cells with a wide opening Pasteur pipette, 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.

4. Collect and set aside the supernatant (which contains the **cytoplasmic fraction**) in a tube for storage. Re-suspend the pellet gently in 150 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.

   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. Discard the supernatant.

5. Add 18 µl of ice cold Nuclear Fractionation Reagent (NER-1) with protease inhibitors to the nuclear pellet; include serine protease inhibitor. Vortex full speed for 1 min and then place the tube in ice for 10 min.

6. 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 cytoplasmic (from step 4) and nuclear fractions (from step 6) at -80°C. You may wish to aliquot fractionated materials into separate tubes prior to storage at this step.

Resolving Cytoplasmic and Nuclear Fractions in Western Blots

Anti-rabbit IgG-HRP and an ECL detection kit are required for Western blot development.

Resolving cytoplasmic and nuclear proteins in Western blots. For both the cytoplasmic and nuclear fractions, p65 may be present at high concentrations which may result in Western blot band saturation. Use a 1:1000 dilution of cytoplasmic and nuclear fraction solutions in Laemmli sample buffer as a starting reference dilution prior to loading samples into gels. SDS-PAGE gel lanes can be loaded in the following consecutive order: 1) total cell lysate; 2)cytoplasmic fraction; 3) nuclear fraction under control conditions, and similarly, a repeat of this arrangement under treatment conditions to observe differences in p65 concentration among the fractions.

Western blot development with the provided p65 antibody. A 10% TRIS-glycine SDS-PAGE gel or a 5-20% gradient TRIS-glycine SDS-PAGE gel is suitable to resolve p65 protein (MW approx. 65kD). Load the gel lanes in the arrangement suggested above.

After the SDS-PAGE is resolved, transfer the proteins in the gel to a nitrocellulose membrane using 20% methanol in a TRIS-glycine Western blot transfer buffer using standard Western blot transfer procedures.

Develop the resulting Western blot with the provided p65 antibody at a 1:400 dilution. Use anti-rabbit IgG-HRP and ECL for subsequent detection. The recommended incubation time for the p65 antibody with the Western blot membrane is 1 hr at RT or overnight at 4^{o}C. Use anti-rabbit IgG-HRP and an ECL chemiluminescence Western blot development method to detect p65, which should appear at a migration corresponding to a 65KD protein in the Western blot.

Data Interpretation: p65 translocation 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 in the absence and presence of conditions that activate NF-kappaB. Use densitometry to measure the Western blot bands that correspond to p65. With successful nuclear/cytoplasmic fractionation, you should observe elevated amounts of p65 in the nuclear fraction following exposure to 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). Resolution: Perform procedures as rapidly as possible. 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 into CER and NER reagents immediately prior to use.