PRODUCT INFORMATION & MANUAL

NF-κB/p65 ActivELISA™

NBP2-29661

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

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 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 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 anti-p65 antibody coated plate captures free p65 and the amount of bound p65 is detected by adding a second anti-p65 antibody followed by alkaline phosphatase (AKP) -conjugated secondary antibody using colorimetric detection in an ELISA plate reader.

Specificity: Human, Rat, Mouse

III. ADVANTAGES

- Contains reagents and protocol to prepare whole, nuclear, and cytoplasmic cell fractions.
- 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, Jurkat cells were grown to 2 x 10^6 cells/ml and treated with 15 µg/ml LPS. The cells were harvested at various time points, nuclear extracts prepared, and the NF-κB ActivELISA™ kit was used to monitor and measure the relative increase of p65 translocation into the nucleus.

Nuclear Translocation of NF-κB p65 with LPS in Jurkat cells

Figure 1. Western Analysis

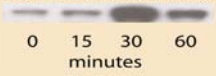
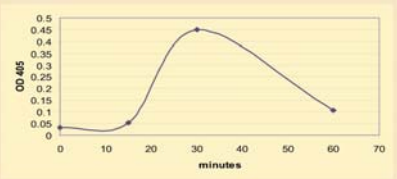


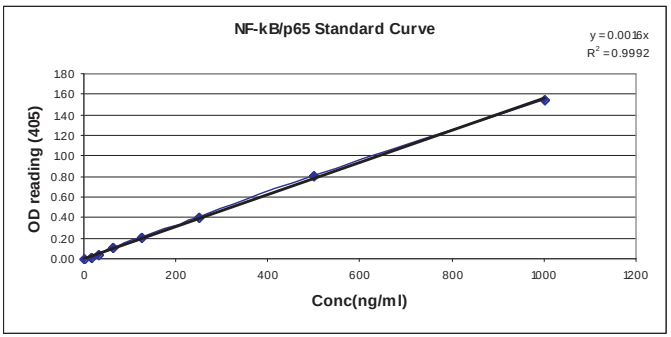
Figure 2. NF-κB p65 ActivELISA™ Assay

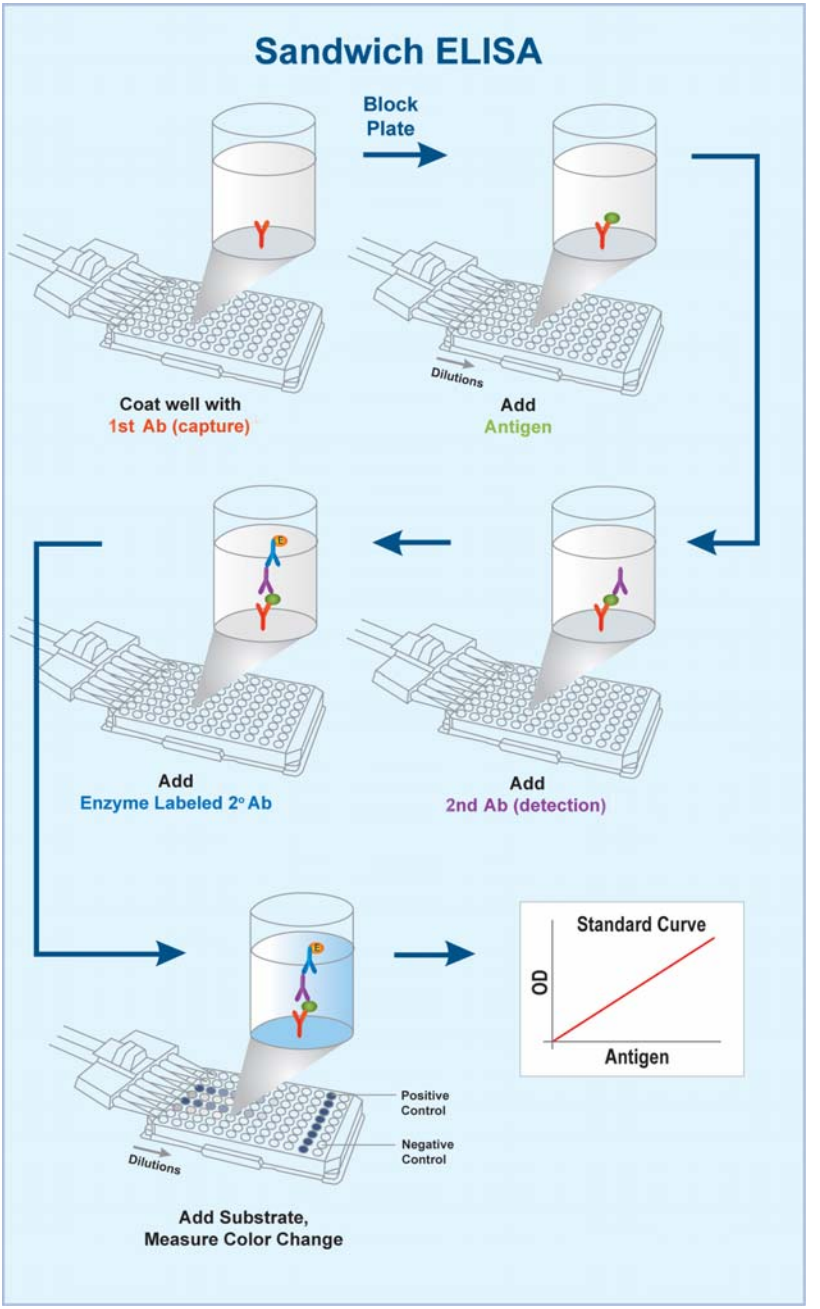


Jurkat cells, grown to a density of 2 X 10^6 cells/ml, were treated with LPS (15 µg/ml). The cells were harvested at various indicated time points, and cell lysates were tested in Western analysis (Fig. 1) using an anti-p65 antibody (Imgenex cat# IMG-150) and in an ActivELISA assay (Fig. 2).

EXAMPLE STANDARD

Following the provided protocol, p65 standard was titrated to determine detectible levels.




Sandwich ELISA
Block Plate
Coat well with 1st Ab (capture)
Dilutions
Add Antigen
Add Enzyme Labeled 2° Ab
Add 2nd Ab (detection)
Standard Curve
OD
Antigen
Positive Control
Negative Control
Dilutions
Add Substrate, Measure Color Change

V. KIT COMPONENTS AND STORAGE

The NBP2-29661 NF-κB/p65 ActivELISA™ Kit contains a NF-κB/p65 ActivELISA™ Module and a Lysate Preparation Module.

Kit Components and storage for the NBP2-29661 NF-kB/p65 ActivELISA™

Module. REAGENTS (4°C STORAGE)		
NBP2-29661-1	**Capture Antibody***	200 µl
NBP2-29661-2	**Detecting Antibody***	200 µl
KC-104	**Coating Buffer**	2 x 10 ml
KC-100	**BSA**	2 x 0.5 g
KC-101	**20X Wash Buffer**	50 ml
KC-132	**AKP-Conjugated Secondary Ab***	10 µl
KC-105	**pNPP Substrate Buffer**	2 x 10 ml
REAGENTS (-20°C, STORAGE--NON FROST-FREE FREEZER)		
NBP2-29661-03L	**Recombinant p65 Standard**	2 vials, lyophilized (0.42 µg/vial)
KC-103	**pNPP**	4 x 5 mg
ADDITIONAL ITEMS INCLUDED		
ELISA Plates		2
NBP2-29661 Manual		1

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

Kit Components and storage for the NBP2-29661 Lysate Preparation Module. *Also described in Appendix A.*

REAGENTS (4°C STORAGE)		
KC-401	**10X Hypotonic Lysis Buffer**	10 ml
KC-402	**1X Nuclear Extraction Buffer**	10 ml
KC-403	**10% Detergent Solution**	10 ml
KC-117	**10X PBS**	2 x 50 ml
REAGENTS (-20°C STORAGE--NON FROST-FREE FREEZER)		
KC-404	**1 M DTT** (for Nuclear Extraction from tissue)	100 µl
KC-405	**10 mM DTT** (for Nuclear Lysis Buffer)	500 µl
KC-406	**100X Protease Inhibitor Cocktail (PIC)**	100 µl
KC-407	**100 mM PMSF**	10 ml

Additional items required for the ELISA (not included in the *NBP2-29661 NF-κB/p65 ActivELISA™ Module*):

- Distilled water
- 96-well ELISA plate reader

Additional items required for lysate preparation (not included in the *NBP2-29661 Lysate Preparation Module*):

- Teflon homogenizer (tissues)
- Cell scraper (cells)
- High-speed cold centrifuge and compatible centrifuge tubes
- Microcentrifuge tubes
- Deionized Water
- Vortex

NOTE: Prior to starting ELISA please see Appendix A for Lysate Preparation.

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.

Blocking Buffer: Dissolve 0.5 g BSA (KC-100) in 50 ml of 1X Wash Buffer in a sterile bottle.

***NOTE:** To prevent microbial growth keep Blocking Buffer and 1X Wash Buffer stored at 4°C when not in use and reduce exposure to contaminants. Sodium azide at a final concentration of 0.02% may be added to prevent bacterial growth. Two aliquots of BSA have been included. Each aliquot contains sufficient BSA for one plate. Prepare Blocking Buffer fresh prior to experiment.*

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. No capture antibody added to well
2. No lysate added to well
3. No capture antibody or lysate added to well
4. Positive control: use a cell line or tissue known to constitutively express p65 or a recombinantly expressed p65.
5. Negative control: use a cell line or tissue known to not express p65.

ELISA PROTOCOL

1. **Coating:** Dilute 100 µl of Capture Antibody (NBP2-29661-1) in 10 ml of Coating Buffer (KC-104). Pipet 100 µl of diluted antibody into each well (**A1** through **H1** and **A2** through **H2 f**or the standard and any of columns 3 through 12 for your samples), cover and incubate the plate overnight (12-24 h) at 4°C. Wash the coated wells twice with 300 µl of 1X Wash Buffer.

2. **Blocking:** Add 200 µl of prepared Blocking Buffer to each well to block the remaining reactive surface. Incubating for 30 min to 1 h at RT.

3. **Prepare p65 Standard Curve:** Quick spin down the Recombinant p65 Standard vial and add 420 µl of sterile deionized H20. Vortex to dissolve. Set up a standard curve in duplicate using the following concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.6 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).
   - Remove Blocking Buffer from wells by flicking into an appropriate waste container and gently tapping the plate face-down on paper towels. Replace with 100 µl of fresh prepared Blocking Buffer in each well **B1** through **H1** and **B2** through **H2** for the standard.

- Pipette 200 µl stock Recombinant p65 Standard (NBP2-29661-3)(1 µg/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.

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	1000 ng/ml	1000 ng/ml	-	-	-	-	-	-	-	-	-	-
B	500 ng/ml	500 ng/ml	-	-	-	-	-	-	-	-	-	-
C	250 ng/ml	250 ng/ml	-	-	-	-	-	-	-	-	-	-
D	125 ng/ml	125 ng/ml	-	-	-	-	-	-	-	-	-	-
E	62.5 ng/ml	62.5 ng/ml	-	-	-	-	-	-	-	-	-	-
F	31.25 ng/ml	31.25 ng/ml	-	-	-	-	-	-	-	-	-	-
G	15.6 ng/ml	15.6 ng/ml	-	-	-	-	-	-	-	-	-	-
H	Blank	Blank	-	-	-	-	-	-	-	-	-	-

4. **Samples:** Pipet 100 µl of positive and negative controls and 100 µl test samples into the appropriate wells*. Incubate plate at 4°C overnight or 4 h at RT. Samples may be diluted or serially diluted using Blocking Buffer. *Users need to empirically determine the optimal concentrations of their test samples so that the readings fall within the curve of the protein standard.

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

6. **Detecting Antibody:** Dilute 100 µl of Detecting Antibody (NBP2-29661-2) in 10 ml of Blocking Buffer and add 100 µl diluted Detecting Antibody to each well. Incubate for 1 h at RT.

7. **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.

8. **Secondary Antibody:** Dilute 5 µl of AKP-Conjugated Secondary Ab (KC-132) in 10 ml of Blocking Buffer (for one plate). Add 100 µl of diluted secondary antibody to each well and incubate for 1 h at RT.

9. Remove the secondary antibody 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. During the last wash, prepare pNPP substrate. Tap plate upside down several times to remove any residual Wash Buffer.

10. **pNPP Substrate**: Dissolve 10 mg pNPP into 10 ml of pNPP Substrate Buffer and mix. **(Note: Prepare substrate mix just before use).** Add 100 µl of pNPP Substrate to each well. Incubate the plate at RT for 30 min. Read the color development at 405 nm.

NOTE: *Incubation time with pNPP Substrate may be increased or decreased depending on the concentration of samples. Most plate readers have a maximum reading of 2.0-3.0.*

VII. PRODUCT CITATIONS

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2. Monomethylfumarate affects polarization of monocyte-derived dendritic cells resulting in down-regulated Th1 lymphocyte responses. Nicolle H. R. Litjens, Mirjam Rademaker, Bep Ravensbergen, Delphine Rea, Mariena J. A. van der Plas, Bing Thio, Andrew Walding, Jaap T. van Dissel, Peter H. Nibbering. Eur J Immunol. 34: 565 - 575 (2004).

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9. The Neuropeptide Vasoactive Intestinal Peptide Generates Tolerogenic Dendritic Cells Mario Delgado, Elena Gonzalez-Rey, and Doina Ganea. J. Immunol., 175: 7311-7324 (2005).

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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)

XI. 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.
	Wells are not washed enough.	Wash plates thoroughly after incubation with detecting antibody.

APPENDIX A: LYSATE PREPARATION

I. OVERVIEW

The Cell and Tissue Lysate Preparation Module provides a simple and convenient method for the isolation of total, nuclear and cytoplasmic extracts from mammalian cells and tissue samples. This procedure is relevant to the monitoring of translocation of cell signaling molecules from cytoplasm to the nucleus. The reagents provided are sufficient for 100 extractions using 100 mm tissue culture plates or 20 extractions using 1 gram of tissue.

Sample Data

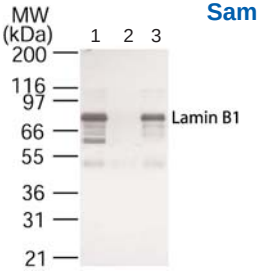

Figure 1. Western blot analysis of Lamin B1 using NB100-56403. 20 ug of HeLa cell proteins were separated on 4-20% SDS-PAGE. Lane 1. Nuclear extract. Lane 2. Cytoplasmic extract. Lane 3. Total cell lysate.

II. COMPONENTS AND STORAGE

REAGENTS (4°C STORAGE)		
KC-401	**10X Hypotonic Lysis Buffer**	10 ml
KC-402	**1X Nuclear Extraction Buffer**	10 ml
KC-403	**10% Detergent Solution**	10 ml
KC-117	**10X PBS**	2 x 50 ml
REAGENTS (-20°C STORAGE--NON FROST-FREE)		
KC-404	**1 M DTT** (for Nuclear Extraction from tissue)	100 µl
KC-405	**10 mM DTT** (for Nuclear Lysis Buffer)	500 µl
KC-406	**100X Protease Inhibitor Cocktail (PIC)**	100 µl
KC-407	**100 mM PMSF**	10 ml

Additional items required for lysate preparation (not provided)

- Teflon homogenizer (tissues)
- Cell scraper (cells)
- High-speed cold centrifuge and compatible centrifuge tubes
- Microcentrifuge tubes
- Deionized Water
- Vortex

III. BUFFER PREPARATION

1X Hypotonic Buffer: Dilute 10X Hypotonic Buffer to 1X in deionized water. 1X Hypotonic Buffer can be stored at 4°C for 1 month.

Buffers Components	60 mm plate ($4x10^6$ cells)	100 mm plate ($10x10^6$ cells)	150 mm plate ($20x10^6$ cells)
10X Hypotonic buffer	50 µl	100 µl	200 µl
Deionized water	450 µl	900 µl	1800 µl
Total Volume required	500 µl	1 ml	2 ml

1X PBS-PMSF: Dilute 10X PBS in deionized water to make 1X PBS. Add 500 µl of 100 mM PMSF to 50 ml of 1X PBS. The 1X PBS-PMSF solution should be used within 24 h (diluted PMSF has a half life less than 24 h).

Buffers Components	60 mm plate ($4x10^6$ cells)	100 mm plate ($10x10^6$ cells)	150 mm plate ($20x10^6$ cells
10X PBS	500 µl	1 ml	2 ml
Deionized water	4.45 ml	8.9 ml	17.8 ml
100 mM PMSF	50 µl	100 µl	200 µl
Total volume required	5 ml	10 ml	20 ml

Nuclear Lysis Buffer: Add 0.5 mM DTT and 1X PIC to Nuclear Extraction Buffer, just prior to use.

Buffers Components	60 mm plate ($4x10^6$ cells)	100 mm plate ($10x10^6$ cells)	150 mm plate ($20x10^6$ cells)
10 mM DTT	2.5 µl	5 µl	10 µl
1X Nuclear Extraction Buffer	47 µl	94 µl	188 µl
100X PIC	0.5 µl	1 µl	2 µl
Total volume required	50 µl	100 µl	200 µl

IV. LYSATE PREPARATION

A. Preparation of Lysates from Cells

i) Cell Culture

1. Grow cells to 70-80% confluency for adherent cells or about 1.5 x 10^6/ml for suspension cells.
2. If necessary, treat cells with desired experimental protocol.

ii) Cell Collection (following protocol is based on 10 x 10^6 HeLa cells grown on 100 mm tissue culture plate):

1. For adherent cells, wash cells with 5 ml of ice cold 1X PBS-PMSF. Aspirate buffer out and add 5 ml of ice cold 1X PBS-PMSF.
2. Dislodge the cells using a cell scraper and transfer into a 15 ml conical tube.
3. To pellet the cells, centrifuge for 5 min at 1000 rpm at 4°C.
4. Aspirate and discard the supernatant. Keep the cell pellet on ice.

iii) Cytoplasmic Fraction Collection:

1. Resuspend cell pellet in 1 ml of ice cold 1X Hypotonic Buffer by pipetting up and down several times and transfer to a pre-chilled microcentrifuge tube.
2. Incubate the cells on ice for 15 min.
3. Add 50 µl of the 10% Detergent Solution and vortex vigorously for 10s (Whole Cell Lysate).
4. Centrifuge the tubes for 30s at 14,000 rpm in a cold microcentrifuge.
5. Carefully remove the supernatant (Cytoplasmic Fraction) into a pre-chilled microcentrifuge tube and store at 4°C. The pellet is the nuclear fraction.

iv) Nuclear Fraction Collection:

1. Resuspend nuclear pellet in 100 µl Nuclear Lysis Buffer by pipetting up and down. Vortex vigorously and incubate suspension at 4°C, for 30 min on a rocking platform.
2. Vortex suspension for 30 s. Centrifuge the suspension at 14,000 rpm for 10 min at 4°C in a microcentrifuge.
3. Transfer the supernatant (Nuclear Fraction) into a pre-chilled microcentrifuge tube. Store the nuclear fraction at -80°C until further use. Avoid multiple freeze/thaw cycles.
4. Determine the protein concentration in the nuclear extract using a detergent compatible assay technique (eg: BioRad DC Protein Assay Method). We recommend using the Nuclear Lysis Buffer as the blank and performing a 1:50 and 1:100 dilution of your sample.

B. Preparation of lysates from Tissue

i) Cytoplasmic Fraction Collection
(Tissue Homogenization based on 1 gram Mouse Spleen).

1. Weigh tissue and cut into small pieces using clean razor blade and wash in 5 ml of cold 1X PBS-PMSF. Collect cut pieces in a clean homogenizer.
2. Add 5 ml of ice cold 1X Hypotonic Buffer supplemented with 1 mM DTT and 1% Detergent Solution. For example add 5 µl of 1M DTT and 500 µl of 10% Detergent Solution to 4.495 ml of ice cold 1 X Hypotonic Buffer per gram of tissue and homogenize. Incubate on ice for 15 to 30 min.(Whole Cell Lysate)
3. Centrifuge for 10 min at 10,000 rpm at 4°C. Transfer the supernatant (Cytoplasmic Fraction) into a 15 ml tube and store at 4°C. The pellet is the nuclear fraction.

ii) Nuclear Fraction Collection

1. Resuspend nuclear pellet in 500 µl Nuclear Lysis Buffer by pipetting up and down. Vortex vigorously and incubate suspension at 4°C, for 30 min on a rocking platform.
2. Vortex suspension for 30 s. Centrifuge the suspension at 14,000 rpm for 10 min at 4°C in a microcentrifuge.
3. Transfer the supernatant (Nuclear Fraction) into a pre-chilled microcentrifuge tube. Store the nuclear fraction at -80°C until further use. Avoid multiple freeze/thaw cycles.
4. Determine the protein concentration in the nuclear extract using a detergent compatible assay technique (eg: BioRad DC Protein Assay Method). We recommend using the Nuclear Lysis Buffer as the blank and performing a 1:50 and 1:10 dilution of your sample.

VI. TROUBLE SHOOTING

Problem	Probable Cause	Suggestion
Low protein concentration in cytosolic fraction.	Cell density is too high for effective lysis of cell membrane.	Larger volume of lysis buffer should be used
Low or no protein yield in cytoplasmic fraction or nuclear fraction	Cell type is not compatible with this extraction procedure	Hypotonic and nuclear extraction buffers should be optimized for this type of cells

If you require additional assistance,
please contact Novus Technical Service:

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