NucBuster™ Protein Extraction Kit

About the Kits

NucBuster Protein Extraction Kit	100 rxn	71183-3

Description

The NucBuster Protein Extraction Kit is designed for rapid, efficient preparation of nuclear extracts from mammalian cell cultures (1). The NucBuster Kit is comprised of two proprietary extraction reagents, protease inhibitors, and the reducing agent dithiothreitol (DTT). The entire procedure is performed in one tube without dialysis and the nuclear proteins are ready for assay within 30 minutes. NucBuster extracts are suitable for activity assays and electrophoretic mobility shift assays (EMSA). The NucBuster Kit is designed to maximize the yield of nuclear proteins from diverse cell lines (e.g., CHO-K1, HEK 293, HeLa) and yields highly consistent results without procedural modification.

Components

- 15 ml NucBuster Extraction Reagent 1
- 7.5 ml NucBuster Extraction Reagent 2
- 1 set Protease Inhibitor Cocktail Set 1 (lyophilized)
- 0.1 ml 100 mM DTT

Storage

Store NucBuster Extraction Reagent 1 and NucBuster Extraction Reagent 2 at 4°C or –20°C. Store Protease Inhibitor Cocktail Set 1 and 100 mM DTT at –20°C.

USA and Canada
Tel (800) 526-7319
novatech@novagen.com

Germany
Tel 0800 100 3496
techservice@merckbiosciences.de

United Kingdom and Ireland
Freefone 0800 622935
Toll Free 1800 409445
customer.service@
merckbiosciences.co.uk

All Other Countries
Contact Your Local Distributor
www.novagen.com
novatech@novagen.com

A Brand of EMD Biosciences, Inc., an Affiliate of Merck KGaA, Darmstadt, Germany
www.novagen.com

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Nuclear Protein Extraction

Prior to use, the lyophilized Protease Inhibitor Cocktail Set 1 should be resuspended in 100 µl sterile water to make a 100X stock. Use immediately or freeze in aliquots at –20°C for long term storage. For every 1–3 × 10^7 cells extracted, 1 µl of the 100X stock is needed.

Note: All extractions should be performed on ice to enhance protein stability.

1. Prepare a single cell suspension using a standard technique (e.g., suspension culture, trypsinization of adherent cells).
2. Count the cells and transfer to a 1.5 ml-tube and centrifuge at low speed (500 × g, 4°C). Remove the supernatant and measure the standard packed cell volume. 1–3 × 10^7 CHO-K1 cells form a packed cell volume of 50 µl. Up to 250 µl packed cell volume can be processed in a single 1.5-ml tube.
3. Resuspend the cell pellet using 150 µl NucBuster Reagent 1 per 50 µl packed cell volume. Adjust extraction reagent volumes proportionately according to the size of the packed cell volume (e.g. for 25 µl packed cell volume, use 75 µl NucBuster Extraction Reagent 1).
4. Vortex 15 sec at high speed, incubate on ice 5 min, and vortex again 15 sec at high speed.
5. Centrifuge at 16,000 × g for 5 min at 4°C.
6. Remove the supernatant (cytoplasmic fraction).
   Optional: A wash with 500 µl of ice-cold 1X PBS (137 mM NaCl, 43 mM Na_2HPO_4, 27 mM KCl, 15 mM KH_2PO_4, pH 7.3) may be used at this step to remove additional cytoplasmic proteins.

Note: The cytoplasmic fraction can be saved for further analysis or may be discarded.

7. Resuspend the pellet in 1 µl of resuspended 100X Protease Inhibitor Cocktail, 1 µl of 100 mM DTT, and 75 µl NucBuster Extraction Reagent 2 per 50 µl packed cell volume.
8. Vortex 15 sec at high speed, incubate on ice 5 min, and again vortex 15 sec at high speed.
9. Centrifuge at 16,000 × g 5 min at 4°C.
10. Transfer the supernatant (nuclear extract) to a separate tube. The extract can be used immediately or stored in aliquots for extended periods at –80°C.

Electrophoretic Mobility Shift Assays (EMSAs) with NucBuster extracts

The following protocols are for traditional EMSA methodology. As an alternative, the NoShift™ Transcription Factor Assay Kit utilizes a microassay plate-based method for analysis of DNA-binding proteins. For more information refer to User Protocol TB403.

Probe Labeling

1. Anneal the two oligonucleotides comprising the desired transcription factor binding site and adjust the final concentration to 10 pmol/µl.
2. Add reagents to a microcentrifuge tube in the following order:

0.5 µl	annealed oligonucleotides (5 pmol)
5 µl	5X Polynucleotide Kinase buffer (500 mM KCl, 350 mM Tris-Cl, 50 mM $MgCl_2$, 5 mM DTT, pH 7.6)
1 µl	T4 Polynucleotide Kinase (10 U/µl, Cat. No. 69248-250UN)
2.5 µl	[γ^{32}P] ATP (25 µCi)
16 µl	sterile water
25 µl total volume	

3. Pipet up and down to mix.
4. Incubate 10 min at 37°C.
5. Incubate 10 min at 65°C to inactivate the kinase.
6. Add 25 µl of STE buffer (100 mM NaCl, 10 mM Tris-Cl, 1mM EDTA, pH 8).

7. Remove unincorporated radioactive nucleotides with a spin column or push column.
8. Bring the final volume to 167 µl with STE buffer (1 µl probe is equal to 0.03 pmol).

DNA binding

Salmon Sperm DNA and Poly(dI-dC)•Poly(dI-dC) are added as non-specific DNA competitors to reduce background.

1. Novagen's EMSA Accessory Kit (Cat. No. 71282-3) provides four key reagents needed for EMSA. The kit contains Poly(dI-dC)•Poly(dI-dC) solution, 4X EMSA Buffer, sonicated Salmon Sperm DNA and 100 mM DTT. Otherwise, prepare 4X binding buffer, sonicated salmon sperm DNA (500 ng/µl), and poly(dI-dC)•poly(dI-dC) (0.01 U/µl) and freeze in small aliquots (–20°C). Add reagents in the following in order to a microcentrifuge tube:

5 µl	4X binding buffer (400 mM KCl, 80 mM HEPES, 2 mM DTT, 0.8 mM EDTA, 80% glycerol, pH 8)
1 µl	sonicated Salmon Sperm DNA (average size ~500 bp)
1 µl	Poly(dI-dC)•Poly(dI-dC) (0.01 U)
x µl	NucBuster extract. Typically 2.5 µl is adequate. Adjust the amount according to the intensity of the shifted band.
y µl	sterile water
17 µl	total volume

Note: If non-specific binding is observed, increasing the amount of sonicated Salmon Sperm DNA and/or Poly(dI-dC)•Poly(dI-dC) may reduce some background binding.

2. Mix by pipetting up and down.
3. Add 1 µl ^{32}P-labeled probe (0.03 pmol) and mix.
4. Incubate on ice 30 min.

Note: If unlabeled specific competitor DNAs are used to establish specificity, mix the "cold" and "hot" DNA prior to adding to the extract. Adjust the total volume with the appropriate amount of water.

5. **Optional**: To identify the complexed protein via an antibody "supershift", add the antibody to the completed protein/DNA complex reaction. Incubate on ice for an additional 20 min before loading on a gel.

Running DNA/protein Complex on Gels

1. Pre-run a 6% non-denaturing DNA retardation gel (29:1 acrylamide to bisacrylamide) in 0.5X TBE (1 L = 5.4 g Tris base, 2.75 g Boric acid, 2 ml 0.5 M EDTA pH 8) for 30 min at 100 V.
2. Add 2 µl 6X DNA Loading Buffer (Cat No. 69180-3) to the 18 µl DNA/protein complex and mix.
3. Load the entire 20 µl reaction in one well of the DNA retardation gel.
4. Run at 100 V for 1 h or until the bottom dye has migrated to the end of the gel.

Note: Running the gel for 1 hr will likely leave uncomplexed probe still on the gel. If background is a problem or if further separation between bands is required, run the gel for an additional 30 min.

5. Dry the gel on DEAE paper using a standard gel dryer.
6. Expose to autoradiographic film for the appropriate length of time (4 h to overnight) at –80°C.

EMSA Troubleshooting Guide

Problem	Probable cause	Solution
No band shift detected on gel	Transcription factor of interest may not be expressed.	Raise expression levels within the cell by induction (e.g., add phorbol ester to increase AP-1 expression) prior to extract preparation.
	Insufficient nuclear extract added.	Increase the amount of nuclear extract added to the binding reaction.
	Too much Salmon Sperm DNA or Poly(dI-dC)•Poly(dI-dC) added.	Lower the concentration of the non-specific competitors.
Too many shifted bands on gel	Transcription factor of interest may form multimeric binding complexes.	Establish specificity with antibody supershift assay.
	Non-specific DNA binding.	Increase the concentration of non-specific competitors to decrease non-specific DNA binding.
Shifted bands appear blurry or faint on autoradiograph	DNA retardation gels may be too old.	Make fresh gels.
	Decayed probe.	Prepare fresh probe.

References

1. Bruggink, F. and Hayes, S. (2002) *in**Nova**tions* **15**, 9–11.

USA and Canada
Tel (800) 526-7319
novatech@novagen.com

Germany
Tel 0800 100 3496
techservice@merckbiosciences.de

United Kingdom and Ireland
Freefone 0800 622935
Toll Free 1800 409445
customer.service@
merckbiosciences.co.uk

All Other Countries
Contact Your Local Distributor
www.novagen.com
novatech@novagen.com

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www.novagen.com