January 2005

Qproteome Nuclear Subfractionation Handbook

For fractionation of nuclear and nucleic-acid binding proteins from eukaryotic cell lysates

Contents

Kit Contents

Qproteome Nuclear Subfractionation Kit	
Catalog no.	**37531**
Number of preps	**6**
Lysis Buffer NL	10 ml
Extraction Buffer NX1	1 ml
Extraction Buffer NX2	1 ml
Nuclear Protein Fractionation Resin	4 ml
Nuclear Protein Fractionation Columns	6
Dilution Buffer ND	5 ml
Elution Buffer NE1	10 ml
Elution Buffer NE2	5 ml
Elution Buffer NE3	5 ml
Detergent Solution NP	0.25 ml
DTT Stock Solution	0.25 ml of a 1 M solution
Benzonase®	2000 Units (25 U/µl)
Protease Inhibitor Solution (100x)	300 µl

Storage

Buffers NL, NX1, NX2, ND, NE1, NE2, and NE3 and Nuclear Protein Fractionation Columns should be stored at room temperature (15–25°C).

Nuclear Protein Fractionation Resin, Detergent Solution NP, and Protease Inhibitor Solution (100x) should be stored at 4°C.

Benzonase® and DTT Stock Solution should be stored at –20°C.

Product Use Limitations

Qproteome Nuclear Subfractionation Kits are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

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At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN® products. If you have any questions or experience any difficulties regarding Qproteome Nuclear Subfractionation Kits or QIAGEN products in general, please do not hesitate to contact us.

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Safety Information

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Introduction

A powerful strategy for proteomics is the combination of classical biochemical fractionation techniques — which enrich particular subcellular structures or classes of protein with distinct functions or characteristics — with mass spectrometric identification of individual proteins.

The functional architecture of the eukaryotic cell nucleus is of great interest to cell biologists. The identification of nuclear proteins — especially nucleic-acid–binding proteins (e.g., transcription factors) — is important for an understanding of genome regulation and function, and provides clues about the molecular function of novel proteins.

The nucleus contains a cell's genetic information and is the site of gene expression. Biological processes involving nucleic acids, such as transcription, replication, recombination, and DNA repair all involve the action of proteins that bind nucleic acids in a sequence-specific manner. These proteins interact with other proteins, which may or may not bind directly to nucleic acids. As a result of these interactions, large functional complexes are formed and anchored to specific nucleic acid sites. Many auxiliary proteins, enzymes, and complexes in the nucleus have important, general functions and are present in small to moderate amounts. However, proteins that bind at selected sites, such as transcription factors bound to specific promoters, represent only 0.01–0.001% of total cellular protein. A fractionation of nuclear proteins, of which over 1000 have been identified, is a prerequisite for analysis of such low-abundance proteins using current methods.

Fractionation of Cell Lysate and Subsequent Subfractionation

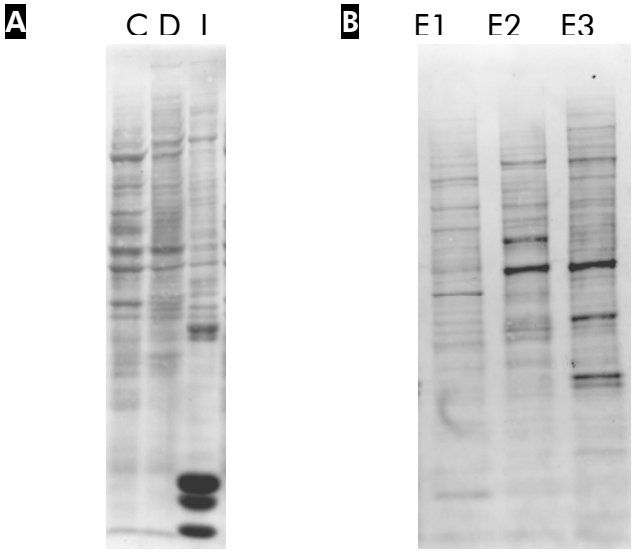
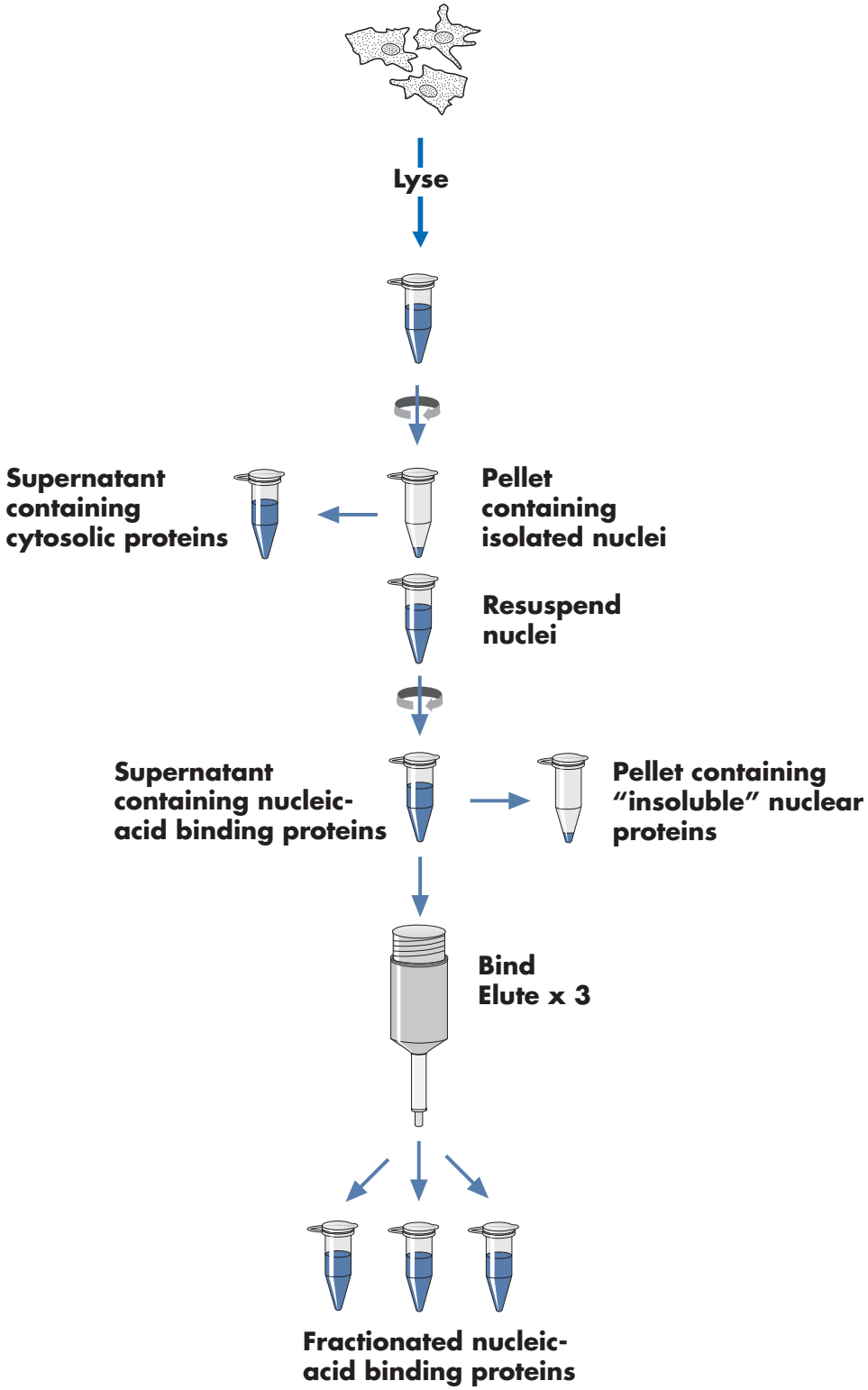


Figure 1 **A** Fractions obtained after processing cell lysates using the Qproteome Nuclear Subfractionation Kit. **C**: Cytosolic fraction; **D**: nucleic-acid–binding protein fraction; **I**: "Insoluble" fraction. **B** Eluates 1–3 after subfractionation of fraction N.

Nuclear Protein Fractionation Procedure



Principle and procedure

The Nuclear Subfractionation Kit is designed for specific enrichment of nuclear proteins and provides efficient subfractionation of nucleic-acid–binding proteins from cultured mammalian cells. The Nuclear Subfractionation Kit provides standardized sample preparation for reliable quantitative and qualitative analysis of proteins for targeted proteomics. Nuclear proteins are separated from complex protein mixtures, allowing detection of low-abundance proteins (e.g., most transcription factors). Starting material for one fractionation procedure is 5×10^6–1×10^7 cells. The procedure has been used successfully with several different mammalian cell lines including HeLa, Jurkat, NIH3T3, HEK293, and Cos (Table 1).

Table 1. Typical Total Protein Yields in Cytosolic, DNA Binding Protein, and Histone Fractions

Cell type	Number of cells processed	Cytosolic fraction	Nucleic-acid–binding proteins	Histones
HeLa	5.4×10^6	1.9 mg	0.34 mg	0.40 mg
HEK293	4.0×10^7	9.4 mg	0.55 mg	0.89 mg
Jurkat	1.5×10^7	2.6 mg	0.23 mg	0.53 mg
Cos	3.7×10^6	2.9 mg	0.14 mg	0.18 mg
NIH3T3	7.9×10^6	1.9 mg	0.28 mg	0.34 mg

Preparation of the cytosolic fraction

Cells are incubated in hypotonic buffer, causing them to swell. Detergent added to the lysis buffer ruptures the plasma membrane and centrifugation is used to separate the cytosolic fraction (supernatant) from the cell nuclei (pellet).

Preparation of the nucleic-acid binding protein fraction

The nuclear pellet is washed to remove cytosolic contaminants from the isolated cell nuclei. Washed cell nuclei are incubated in a buffer containing a high salt concentration. During this incubation step, the nuclei shrink and nucleic-acid–binding proteins (e.g., transcription factors) separate from nucleic acids and diffuse through the nuclear pore to the exterior of the nucleus. A centrifugation step separates the nucleic-acid–binding proteins (which are found in the supernatant) from the nuclear debris, which includes genomic DNA (the pellet). The expected yield from one fractionation procedure is 200–300 µg specific nucleic-acid–binding proteins, at a concentration of approximately 5 µg/µl. This nucleic-acid–binding protein fraction can be used for activity tests (e.g., gel-shift assays,

transcription factor activity assays) and characterization of function without further processing. Alternatively, this fraction can be further fractionated using phosphocellulose columns.

Further fractionation of nucleic-acid–binding proteins

For 2-D PAGE analysis — where a reduction in sample complexity is often desired — fractionation of the nucleic-acid–binding protein sample is recommended. nucleic-acid–binding proteins are fractionated by diluting the nucleic-acid–binding protein fraction and loading it onto a phosphocellulose column, and eluting proteins with increasing salt concentrations (Elution Buffer NE1, 0.1 M NaCl; Elution Buffer NE2, 0.35 M NaCl; Elution Buffer NE3, 1 M NaCl). Subfractionation using stabilized phosphocellulose gravity flow columns reduces the complexity of the nuclear-acid binding protein fraction and allows more sensitive detection and analysis of proteins expressed at low levels (Table 2). For most downstream applications, concentration of the phosphocellulose fractions is required. A protocol for concentration using acetone precipitation is provided on page 16.

Table 2. Typical Protein Yields in Nucleic-Acid–Binding Protein Subfractions

		Subfraction NE1	Subfraction NE2	Subfraction NE3
Cell type	**Number of cells processed**	**Yield (% total)**	**Yield (% total)**	**Yield (% total)**
HeLa	4.0×10^6	70 µg (41%)	50 µg (29%)	50 µg (29%)
HEK293	1.0×10^7	130 µg (52%)	60 µg (24%)	60 µg (24%)
Jurkat	1.3×10^7	60 µg (38%)	50 µg (32%)	45 µg (29%)
NIH3T3	2.6×10^6	50 µg (29%)	60 µg (35%)	60 µg (35%)

Preparation of the "insoluble" nuclear protein fraction

Extraction of "insoluble" nuclear proteins is performed by incubation of the nuclear debris with a buffer containing Benzonase®, a DNase/RNase which digests genomic DNA and releases nuclear proteins intimately associated with DNA (e.g., histones).

Protocol: Fractionation of Nuclear Proteins from Mammalian-Cell Lysates

Important notes before starting

- All steps are performed at 4°C. Use pre-cooled buffers and equipment. Separated protein fractions should be stored at –80°C.
- For downstream applications, such as 2-D gel analysis, phosphocellulose column fractions must be concentrated. This can be achieved by acetone precipitation (see page 16).
- Starting material for one fractionation procedure using the Nuclear Subfractionation Kit protocol is 5×10^6 – 1×10^7 cells.
- Dilute 10 µl of the 1 M DTT Stock solution with 90 µl deionized, sterile water to give a concentration of 0.1 M. Store 0.1 M DTT Solution at –20°C.
- Immediately before starting the protocol, supplement the buffers used in the protocol with Protease Inhibitor Solution, DTT Stock Solutions, and Benzonase® as shown in Table 3.

To be supplied by the user

- PBS
- 15 ml conical tube
- Microcentrifuge tubes
- Parafilm®
- Optional: Acetone stored at –20°C

Table 3. Preparing Buffers for the Nuclear Subfractionation Kit Protocol.

Buffer	Required volume per prep.	Protease Inhibitor Solution	1 M DTT	0.1 M DTT	Benzonase®
Lysis Buffer NL (for protocol steps 6 and 11)	1000 µl	10 µl	–	5 µl	–
Extraction Buffer NX1 (step 14)	50 µl	0.5 µl	–	–	–
Dilution Buffer ND (step 17)	150 µl	1.5 µl	–	3 µl	–
Elution Buffer NE1 (steps 23 and 26)	1300 µl	13 µl	1.5 µl	–	–
Elution Buffer NE2 (step 24)	500 µl	5 µl	4 µl	–	–
Elution Buffer NE3 (step 25)	500 µl	5 µl	1.5 µl	–	–
Extraction Buffer NX2 (step 26)	100 µl	1 µl	–	1 µl	1 µl

Procedure

Cell collection

1. **Aspirate cell-culture medium from culture plate.**
2. **Wash cells twice with 5 ml ice-cold PBS.**
3. **Add 10 ml ice-cold PBS.**
4. **Remove cells from culture plate by gentle scraping with cell-scraper and transfer cells to a pre-chilled 15 ml conical tube.**
5. **Centrifuge cell suspension for 5 min at 450 x *g* in a centrifuge pre-cooled to 4°C. Discard supernatant. Keep cell pellet on ice.**

Cell lysis and isolation of cell nuclei

6. **Gently resuspend the cells collected in step 5 in 500 µl Lysis Buffer NL (supplemented with Protease Inhibitor Solution and 0.1 M DTT, see Table 3) by pipetting up and down several times. Incubate for 15 min on ice.**
7. **Transfer the resuspended cells to a clean pre-chilled microcentrifuge tube.**
8. **Add 25 µl Detergent Solution NP to the cell suspension and vortex for 10 s at maximum speed.**
9. **Centrifuge cell suspension for 5 min at 10,000 x *g* in a microcentrifuge pre-cooled to 4°C.**
10. **Transfer the supernatant (cytosolic fraction) into a new microcentrifuge tube and store at –80°C.**
11. **Resuspend the pellet (which contains cell nuclei) in 500 µl Nuclear Protein Lysis Buffer NL (supplemented with Protease Inhibitor Solution and 0.1 M DTT, see Table 3) by vortexing for 5 s at maximum speed.**
12. **Centrifuge the suspension of nuclei for 5 min at 10,000 x *g* in a pre-cooled microcentrifuge at 4°C.**
13. **Discard the supernatant and save the nuclear pellet.**

Extraction of nucleic-acid–binding proteins

14. **Resuspend the nuclear pellet from step 13 in 50 µl Extraction Buffer NX1 (supplemented with Protease Inhibitor Solution, see Table 3) by pipetting up and down. Incubate suspension for 30 min with gentle agitation (e.g., 750 rpm in a Thermomixer) at 4°C.**
15. **Centrifuge the suspension of nuclei for 10 min at 12,000 x *g* in a microcentrifuge pre-cooled to 4°C.**
16. **Transfer the supernatant (which contains nucleic-acid binding proteins) into a new microcentrifuge tube.**

    Store the supernatant and the pellet at –80°C until you are ready to proceed with further purification steps. Alternatively, this fraction can be used directly for activity assays (e.g., gel-shift assays).

    Continue with step 17 to use the supernatant for fractionation with Nuclear Protein Fractionation Resin. This fractionation is recommended to reduce the complexity of samples for 2-D PAGE analysis.

    Continue with step 26 to use the pellet for extraction of "insoluble" nuclear proteins (e.g., histones).

Subfractionation of nucleic-acid–binding proteins with Nuclear Protein Fractionation Columns

17. **Add 150 µl Dilution Buffer ND (supplemented with Protease Inhibitor Solution and 0.1 M DTT, see Table 3) to a 50 µl aliquot of nucleic-acid–binding protein supernatant from step 16.**

    IMPORTANT: Carry out this and all following steps at 4°C.

18. **Prepare a Nuclear Protein Fractionation Column by detaching the top cap and breaking off the bottom closure.**
19. **Resuspend Nuclear Protein Fractionation Resin by gently vortexing. Pipet 400 µl Nuclear Protein Fractionation Resin into a Nuclear Protein Fractionation Column and allow the resin to settle.**
20. **Pipet 800 µl Elution Buffer NE1 (supplemented with Protease Inhibitor Solution and 1 M DTT, see Table 3) into the Nuclear Protein Fractionation Column.**
21. **Cover the opening of the column with a small square of Parafilm. Press gently on the center of the Parafilm until buffer begins to flow through the column. Remove the Parafilm and allow the buffer to run through the column.**
22. **Pipet the 200 µl diluted nucleic-acid–binding protein supernatant from step 17 onto the equilibrated Nuclear Protein Fractionation Column. Discard the first 200 µl void volume.**
23. **Pipet 500 µl Elution Buffer NE1 (supplemented with Protease Inhibitor Solution and 1 M DTT, see Table 3) onto the column and save the flow-through. Label this fraction as Fraction NE1 and store at –80°C.**
24. **Pipet 500 µl Elution Buffer NE2 (supplemented with Protease Inhibitor Solution and 1 M DTT, see Table 3) onto the column and save the flow-through. Label this fraction as Fraction NE2 and store at –80°C.**
25. **Pipet 500 µl Elution Buffer NE3 (supplemented with Protease Inhibitor Solution and 1 M DTT, see Table 3) onto the column and save the flow-through. Label this fraction as Fraction NE3 and store at –80°C.**

    Note: before 2-D PAGE analysis, elution fractions must be concentrated and desalted. This can be achieved by acetone precipitation (see page 16).

Extraction of "insoluble" nuclear proteins (e.g., histones)

26. **Resuspend the pellet from step 16 in 100 µl Extraction Buffer NX2 (supplemented with Benzonase®, Protease Inhibitor Solution, and 0.1 M DTT Stock Solution, see Table 3) by pipetting up and down. Incubate suspension for 1 h with gentle agitation (e.g., 750 rpm in a Thermomixer) at 4°C.**
27. **Centrifuge pellet suspension for 10 min at 12,000 x *g* in a pre-cooled microcentrifuge at 4°C. Transfer the supernatant to a new microcentrifuge tube and store at –80°C.**

Protocol: Acetone Precipitation of Protein Fractions

This protocol is suitable for concentrating and desalting protein samples for downstream applications such as SDS-PAGE.

1. **Add four volumes of ice-cold acetone to the protein fraction and incubate for 15 min on ice.**
2. **Centrifuge for 10 min at 12,000 x *g* in a pre-cooled microcentrifuge at 4°C. Discard the supernatant and air dry the pellet.**

   Do not overdry the pellet as this may make it difficult to resuspend.
3. **Depending on the application, resuspend the pellet in the required sample buffer.**

Troubleshooting Guide

	Comments and suggestions
Low protein concentration in protein fractions	
Too few cells in starting material	Use a minimum of 5 x 10^6 cells per preparation (see Tables 1 and 2).
Poor protein compartmentalization	
a) Incomplete removal of cytosolic fraction	Ensure that all traces of supernatant are removed after the nuclear pellet wash step.
b) Nuclei are disrupted during nucleic-acid–binding protein extraction	Gently resuspend the nuclear pellet in extraction buffer NX1 by pipetting up and down. Do not vortex.
No or low protein activity	
Fractionated proteins are degraded	Ensure all buffers and equipment are cooled to 4°C during the entire procedure. Ensure that Protease Inhibitor Solution has been added to buffers. Snap-freeze eluted proteins using liquid nitrogen.

Ordering Information

Product	Contents	Cat. no.
Qproteome Nuclear Subfractionation Kit	For 6 nuclear protein preparations: Buffers, Reagents, Nuclear protein Fractionation Columns (6), Nuclear Protein Fractionation Resin, Protease Inhibitor Solution, Benzonase®	37531
Related products		
Qproteome Total Glycoprotein Kit	For 6 total glycoprotein preps: Buffers, Lectin Spin Columns (6), Detergent Solution, Protease Inhibitor Solution, Collection Tubes (6 x 2 ml)	37541
Qproteome Mannose Glycoprotein Kit	For 6 mannose glycoprotein preps: ConA, GNA, and LCH Lectin Spin Columns (2 each); Buffers; Detergent Solution; Protease Inhibitor Solution; Collection Tubes (6 x 2 ml)	37551
Qproteome Sialic Glycoprotein Kit	For 6 sialic acid glycoprotein preps: WGA, SNA, and MAL Lectin Spin Columns (2 each); Buffers; Detergent Solution; Protease Inhibitor Solution; Collection Tubes (6 x 2 ml)	37561
Qproteome O-Glycan Glycoprotein Kit	For 6 O-glycan glycoprotein preps: AIL and PNA Lectin Spin Columns (3 each); Buffers; Protease Inhibitor Solution; Collection Tubes (6 x 2 ml)	37571
Qproteome Albumin/IgG Depletion Kit	For albumin/IgG depletion of 6 serum or plasma samples: Albumin/IgG Depletion Spin Columns (6)	37521
Qproteome Soluble Protein Separation Kit	For 10 soluble protein fractionations: Fractionation Buffer, Precipitation Reagents, Protease Inhibitor Solution, Benzonase®	37512
Qproteome Cell Compartment Kit	For 10 subcellular fractionations: Extraction buffers, Protease Inhibitor Solution, Benzonase®	37502

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