Improved Methods for Nuclear Extract Preparation and Functional Nuclei Isolation

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Abstract

Preparation of nuclei or nuclear extracts is often the first step in studying nuclear components and events. Improved methods of preparing nuclei and nuclear extracts from mammalian cells were developed and characterized. Nuclei were isolated from adherent and non-adherent tissue culture cells and monocytes by using a hypotonic Igepal lysis buffer. Nuclei purity was tested by cytological staining, SDS-PAGE and electron microscopic analysis. The isolated nuclei were functional for synthesis of RNA polymerase II dependent transcripts.

Nuclear extracts were prepared by using hypotonic and isotonic Igepal lysis buffer, as well as physical force, and were tested for purity by western immunoblotting. DNA binding assays for SP1 and Oct-1 transcription factors were performed for cytopasmic and nuclear fractions and confirmed the specificity and quality of the nuclear extract preparations.

Nuclei and nuclear extracts produced by these methods should be useful for many applications in biochemistry and cell biology: as a source of nuclear components, for *in vitro* apoptosis assays, for DNA binding assays and for functional studies on the transcriptional status of cells.

Introduction

Goal - To develop simple, reliable methods for preparing nuclear extracts and functional nuclei from mammalian cells for applications in biochemistry and cell biology.

Approach – To modify and improve existing techniques for:

1) Nuclear extract preparation
2) Functional nuclei isolation

Nuclei Extracts

Gene expression is mediated by interaction of trans-acting, DNA-binding proteins with cis-acting elements in the DNA. Nuclear protein extracts can be used directly in a variety of functional studies such as Electrophoresis Mobility Shift Assay (EMSA), footprinting analysis and transcription assays or as a starting point for purification of regulatory proteins.

We developed a modified version (NuCLEAR™ extract kit) of the conventional methods (1,2,3) for small-scale preparation of functional nuclear protein extracts from various mammalian cell types.

Nuclear Extract Preparation

↓ Collect and wash cells (100 µl - 1 ml packed cells)

↓ Incubate in lysis buffer (15 min., 5x packed cell volume)

↓ Cell disruption (NP-40 detergent or mechanical)

↓ Nuclei purification by centrifugation

↓ Nuclei protein extraction* (15-30 min.)

↓ Centrifuge to collect nuclear extract (supernatant)

* Extraction buffer: 20 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, 1mM DTT and 1:100 protease inhibitor cocktail.

EMSA Analysis

Electrophoresis Mobility Shift Assay (EMSA) was used to test cytoplasmic and nuclear fractions for the presence and the ability of two nuclear proteins (Sp1 and Oct-1) to bind DNA. Fractions were incubated with oligonucleotides composed of the SP1 binding site (5′ATTCGATCGGGGCGGGGCGAGC3′) or the Oct-1 protein binding site octamer motif (5′CGTACTAATTTGCATTTCTA3′) and analyzed by electrophoresis on non-denaturating 4% polyacrylamide gels.

Mechanical Lysis

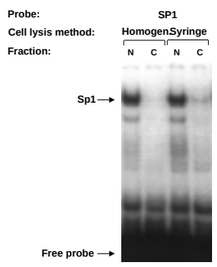

Fig. 1. Sp1 transcription factor is enriched in nuclear fractions prepared after mechanical disruption of CHO cells. Double stranded, ^{32}P-labeled SP1-binding motif oligonucleotide was incubated with 3 µg of CHO cell **cytoplasmic (C) or nuclear (N)** extracts prepared with the Nu-Clear™ extraction kit. Mechanical cell disruption method: **Homogen.** – cells disrupted with a glass homogenizer; **Syringe** – cells disrupted by passage through a syringe. The Sp1 arrow indicates the Sp1-DNA complex.

Detergent Lysis

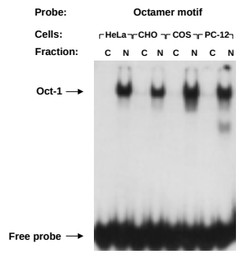

Fig. 2. Oct-1 transcription factor is enriched in nuclear fractions prepared after NP-40 (Igepal CA-630) lysis of cells. Double-stranded, ^{32}P-labeled Octamer motif oligonucleotide was incubated with 4 µg of **cytoplasmic (C)** or **nuclear (N)** fractions prepared from HeLa, CHO, COS and PC-12 cells using the Nu-Clear™ extraction kit. The Oct-1 arrow indicates the Oct-1-DNA complex.

Specificity of Complexes

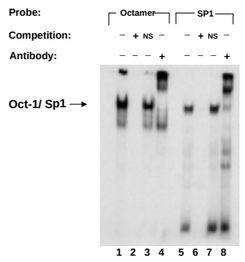

Fig. 3. Complexes formed between nuclear proteins and target DNA sequences are specific. Double stranded, ^{32}P-labeled Octamer motif (lanes 1-4) and SP1-binding motif (lanes 5-8) oligonucleotides were incubated with 2 µg of nuclear extract prepared from CHO cells using the Nu-Clear™ extraction kit. Incubation was performed in the presence **(+)** or absence **(-)** of 150-fold excess of unlabeled oligonucleotide (lanes 2, 3, 6, 7), or in the presence of specific antibody (anti-Oct-1, lane 4; anti-Sp1, lane 8). Antibody binding causes a supershift of the protein-DNA complexes. Lanes 2 and 6 represent competition with a specific oligonucleotide (Oct-1 or SP1, respectively). Lanes 3 and 7 represent competition with a non-specific oligonucleotide **(NS)**. The arrow indicates the Oct-1- or Sp1-DNA complexes.

Fragile Nuclei Extracts

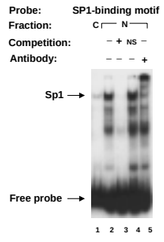

Fig 4. Specific complexes are formed between nuclear proteins and target DNA sequences in nuclear extracts from cells with fragile nuclei. Double stranded, ^{32}P-labeled SP1-binding motif oligonucleotide was incubated with 15 µg of **cytoplasmic (C)** or **nuclear (N)** fractions prepared from Jurkat cells using the Nu-Clear™ extraction kit. Binding reactions with the nuclear extract were performed in the absence **(-)** of competitor (lane 2) or in the presence **(+)** of 200-fold excess of unlabeled Sp1-binding motif oligonucleotide (specific competitor, lane 3) or 200-fold excess of unlabeled Octamer motif oligonucleotide (non-specific **[NS]** competitor, lane 4) or an anti-SP1 antibody (lane 5). Antibody binding causes a supershift of the protein-DNA complex. The Sp1 arrow indicates the Sp1-DNA complex.

Discussion

1) The NuCLEAR™ extract kit was suitable for nuclear protein extraction with three methods of cell lysis from different cell types.
2) The nuclear fractions were significantly enriched in nuclear transcription factors capable of specific binding to target DNA.

Functional Nuclei Isolation

Nuclei isolated from mammalian cells are useful for many applications, e.g. as a source of nuclear components, such as chromatin, genomic DNA, histones and nuclear RNA/RNP, to produce nuclei for *in vitro* apoptosis assays, and for functional studies, such as examination of the transcriptional status of cells.

We developed an improved version (Nuclei EZ Prep™ nuclei isolation kit) of standard methods (4, 5, 6) for small-scale preparation of functional nuclei from mammalian cells. The improved method allows **rapid, simultaneous harvest and lysis** of cells in a hypotonic buffer containing a gentle, nonionic detergent, Igepal CA-630 (NP-40).

Nuclei Isolation Method

Adherent Cells	Suspension Cells
Aspirate medium	Collect cells (centrifuge)
↓	↓
PBS wash/aspirate	PBS wash/spin/aspirate
↓	↓
Scrape in lysis buffer*	Suspend in lysis buffer
↓	↓
Incubate 5 min, 4° C	Incubate 5 min, 4° C
↓	↓
Collect nuclei (5 min, 500 x g)	Collect nuclei (5 min, 500 x g)
↓	↓
Lysis buffer wash	Lysis buffer wash
↓	↓
Collect nuclei (5 min, 500 x g)	Collect nuclei (5 min, 500 x g)
↓	↓
Store in storage buffer (-70° C, >6 months)	Store in storage buffer (-70° C, >6 months)

* Replaces harvesting cells by scraping in PBS.

Harvest and Lysis of Adherent Cells

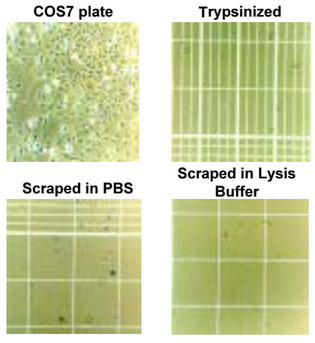

Fig. 5. Scraping adherent cells directly in lysis buffer results in rapid and simultaneous harvest and lysis of cells.

Purified Nuclei

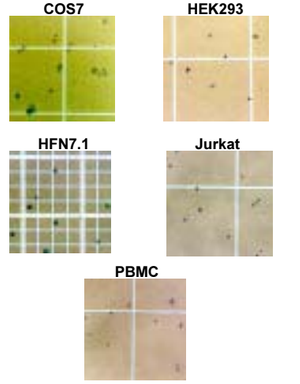

Fig. 6. Morphology of purified nuclei after storage at -70° C.

Electron Micrographs of Purified Nuclei

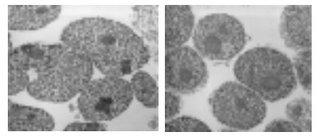

Fig. 7. Nuclei were fixed, sectioned, stained and visualized by transmission EM.

Nuclei Yields

Table 1. Yield of nuclei isolated from commonly used cells using the Nuclei EZ Prep™ Kit (Sigma Product Number KS002).[1]

Cells[2]	Cell Type	Average # Cells [3] x 10^{-7}	Average # Nuclei x 10^{-7}	Average % Yield
CHO	hamster ovary, tissue culture cell line (adherent)	1.0 (±0.2)	1.0 (±0.4)	**98 (±24)**
HEK293	embryonic kidney, tissue culture cell line (adherent)	2.3 (±0.5)	2.1 (±0.6)	**90 (±20)**
HFN7.1	hybridoma, tissue culture cell line (suspension)	3.8 (±1.0)	2.5 (±0.7)	**65 (±2)**
Jurkat	T cell leukemia, tissue culture cell line (suspension)	2.6 (±0.1)	1.6 (±0.1)	**62 (±7)**
PBMC	peripheral blood mononuclear cells	5.0 (±2.1)	3.0 (±1.0)	**63 (±6)**

[1] Data show average results of three separate nuclei isolation experiments for each cell type. Standard errors are indicated in parentheses.
[2] PBMC, peripheral blood mononuclear cells isolated from blood by Histopaque®-1077 gradient centrifugation using the Accuspin™ System (Sigma Product Number A7054).
[3] The data for adherent cells represents preparations each from one 100 mm tissue culture dish of freshly confluent cells. Representative plates were trypsinized and counted for total cell numbers. The data from suspension cells represents preparations each from 15 ml suspension cultures grown in T75 tissue culture flasks. Each PBMC preparation was from about 35 ml freshly donated human blood.

Protein Distribution

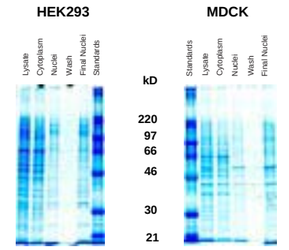

Fig. 8. Fractions taken during nuclei isolation were analyzed by SDS-PAGE and visualized by Coomassie Blue staining.

mRNA Synthesis

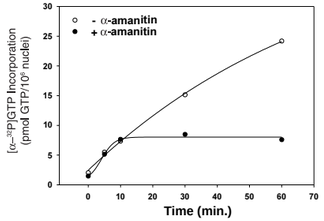

Fig. 9. Nuclei were isolated from Jurkat cells and incubated without (open circles) and with (closed circle) 0.25 ug/ml α-amanitin at 25° C in [α-^{32}P]GTP labeling mix (ref. 6). Samples were taken at times indicated, and TCA precipitable counts were determined.

RNA Isolated from Nuclei

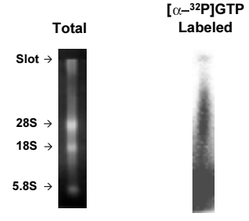

Fig. 10. Nuclei were incubated 1 hr. at 25° C with [α-^{32}P]GTP. The nuclei were lysed in 1% SDS, 10 mM Na_2EDTA, pH 7.0 and extracted with phenol/chloroform. The RNA was ethanol precipitated, dissolved, purified on a G50 spin-column and run on a 1.2% agarose RNA gel. The RNA was visualized by EtBr staining (left) and phosphoimaging (right). The ^{32}P-labeled RNA run-off transcripts can be seen on the right.

Discussion

1) The Nuclei EZ™ Prep nuclei isolation kit was suitable for isolation of nuclei with high yield and purity from mammalian cells.
2) The improved method has the advantage of rapid and simultaneous harvest and lysis of cells.
3) Nuclei isolated by this method are functional for synthesis of run-off mRNA transcripts.

Conclusion

1) Existing techniques were modified and improved to yield simple, reliable methods for preparing nuclear extracts and functional nuclei from mammalian cells.
2) Nuclear extracts and functional nuclei produced by these methods will be useful for a number of applications in cell biology and biochemistry, including studies on the transcriptional status of mammalian cells.

References

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Related Sigma Products

Product Name	Product Number
NuCLEAR™ extract kit	N-XTRACT
Nuclei EZ Prep™ nuclei isolation kit	NUC-101