

Preparation of Nuclear and Cytoplasmic Extracts from Mammalian Cells

UNIT 12.1

Extracts prepared from the isolated nuclei of cultured cells are functional in accurate in vitro transcription and mRNA processing. Thus, such extracts can be used directly for functional studies and as the starting material for purification of the proteins involved in these processes. In Basic Protocol 1 described below, reproducible extracts from different preparations of nuclei are generated. The conditions described are optimized to produce transcriptionally active extracts from HeLa cells. The Support Protocol describes how to optimize Basic Protocol 1 to increase the yield of specific proteins or activities from different cell types. The Alternate Protocol describes an adaptation of Basic Protocol 1 for use with downstream applications such as affinity purification. Basic Protocol 2 describes the preparation of the cytoplasmic (S-100) fraction.

PREPARATION OF NUCLEAR EXTRACTS

**BASIC
PROTOCOL 1**

To prepare nuclear extracts, tissue culture cells are collected, washed, and suspended in hypotonic buffer. The swollen cells are homogenized, and nuclei are pelleted. The cytoplasmic fraction is removed, and nuclei are resuspended in a low-salt buffer. Gentle dropwise addition of a high-salt buffer then releases soluble proteins from the nuclei without lysing the nuclei. Following extraction, the nuclei are removed by centrifugation, the nuclear extract (supernatant) is dialyzed into a moderate-salt solution, and any precipitated protein is removed by centrifugation.

Materials

- Mammalian (i.e., HeLa) cells from spinner cultures or monolayer cultures
- Phosphate-buffered saline (PBS; *APPENDIX 2*)
- Hypotonic buffer (see recipe)
- Low-salt buffer with 0.02 M KCl (see recipe)
- High-salt buffer with 1.2 M KCl (see recipe)
- Dialysis buffer (see recipe)
- Liquid nitrogen
- Beckman JS-4.2 and JA-20 rotors (or equivalent)
- 50-ml graduated, conical, polypropylene centrifuge tubes (or 15-ml tubes for smaller extract volumes)
- Glass Dounce homogenizer with type B (loose) pestle
- Magnetic stirrer *or* tiltboard
- Dialysis membrane tubing ($\leq 14,000$ MWCO; *APPENDIX 3C*)
- Conductivity meter
- Additional reagents and equipment for trypan blue exclusion (*UNIT 11.5*) and Bradford protein assay (*UNIT 10.1A*)

NOTE: Perform this procedure at 0° to 4°C, preferably in a cold room. Use precooled buffers and equipment. All centrifugations are done at 4°C with precooled rotors.

For cells in spinner cultures

- 1a. Centrifuge cells in 1-liter plastic bottles 20 min at $1850 \times g$ (e.g., 3000 rpm in a Beckman JS-4.2 rotor). Set brake on 2. Decant the supernatants and discard. Pool cells by suspending individual cell pellets in a small volume of PBS and combining

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DNA-Protein
Interactions

12.1.1

Supplement 75

into 50-ml graduated conical centrifuge tubes (one tube for every 2 to 3 liters of cells).

The density of the spinner culture should be $5\text{--}10 \times 10^8$ cells/liter.

- 2a. Pellet the cells by centrifuging 10 min at $1850 \times g$ (3000 rpm in a JS-4.2 rotor). Decant the supernatants and discard.
- 3a. Using the graduations on the tube, measure the packed cell volume (pcv). Resuspend the cells in ~ 5 pcv of PBS. Centrifuge the cells again for 10 min.

For cells in monolayer cultures

- 1b. Remove the culture medium from confluent monolayer cultures (about 80% confluency). Wash the cells by pipetting sufficient PBS to cover them, swirling gently, and pouring off the PBS. Scrape the cells into fresh PBS and pool in 50-ml graduated conical centrifuge tubes (one tube for every seven to eight roller bottles with 850 cm^2 of growth surface).

Trypsinization to harvest cells is generally not recommended. Instead, cells can be treated with 0.48 mM EDTA in PBS at room temperature for 5 to 10 min to detach. Alternatively, the cell monolayer can be covered with cold PBS followed by gentle tapping of the vessel to detach.

- 2b. Pellet the cells by centrifuging 10 min at $1850 \times g$ (3000 rpm in a JS-4.2 rotor). Decant the supernatants and discard.
- 3b. Using the graduations on the tube, measure the pcv.
4. Rapidly resuspend the cell pellets in ~ 5 pcv of hypotonic buffer. Centrifuge the cells 5 min at $1850 \times g$ (3000 rpm in a JS-4.2 rotor) and discard supernatant.

Perform this step quickly because proteins can leak out of the cell at this point and be discarded with the supernatant. This step removes salt from the PBS solution so that efficient swelling can occur in the next step; however, some swelling will occur during this step.

5. Resuspend the packed cells in hypotonic buffer to a final volume of 3 pcv and allow to swell on ice 10 min.

Use the original pcv from step 3. If an original pcv of 10 ml in step 3 has swelled to 20 ml in step 4, only 10 ml of additional buffer is required at this step. Note that swollen cell volume varies depending on the cell type. For example, HeLa cells usually swell about 2-fold, while 293 cells may swell very little.

6. Transfer the cells to a glass Dounce homogenizer. Homogenize with 10 to 18 up-and-down strokes using the loose or type B pestle. Monitor cell lysis.

Perform the homogenization slowly, especially the down strokes. After homogenization, check for cell lysis under a microscope. Lysis can be observed by addition of trypan blue to an aliquot of cells (UNIT 11.5). The dye is excluded from intact cells, but stains the nuclei of lysed cells. Lysis should be $>80\%$ to 90% .

7. Transfer cells to graduated, conical, polypropylene centrifuge tubes. Collect the nuclei by centrifuging 15 min at $3300 \times g$ (4000 rpm in a JS-4.2 rotor). Remove the supernatant and save for S-100 cytoplasmic extract preparation (Basic Protocol 2).
8. Using the graduations on the tubes, measure the packed nuclear volume (pnv) from step 7. Resuspend the nuclei in a volume of low-salt buffer equal to $1/2$ pnv.

Resuspension of the nuclei with a small volume of low-salt buffer allows thorough and rapid mixing of the nuclei during the subsequent addition of the high-salt buffer. If necessary, clumps of nuclei can be resuspended with one or two strokes in a Dounce homogenizer using a type B (loose-fitting) pestle.

9. In a dropwise fashion, while stirring gently, add a volume of high-salt buffer equal to 1/2 pnv. Homogenize in a Dounce homogenizer if necessary.

The high-salt buffer must be added dropwise with frequent or continuous mixing. If it is added too quickly, local concentration of salt can become high and some nuclei will lyse. After adding high-salt buffer, the nuclei can be homogenized with two to five up-and-down strokes in a glass Dounce homogenizer (type B pestle) to prevent clumping in large volumes. This is not always necessary for very small volumes (2 to 3 ml).

The final concentration of potassium chloride should be ~300 mM.

10. Allow the nuclei to extract for 30 min with continuous gentle mixing.

Mixing can be done using very gentle stirring on a magnetic stirrer or by tilting on a tiltboard.

11. Pellet the extracted nuclei by centrifuging 30 min at $25,000 \times g$ (14,500 rpm in a Beckman JA-20 rotor). Draw off the resulting supernatant (this is the nuclear extract).

Nuclear pellets can be used as starting material for purification of core histones using hydroxylapatite (UNIT 21.5).

12. Place the nuclear extract in dialysis tubing and seal at least one end of the dialysis bag with a clip.

Using a clip allows the bag to be opened periodically to compare the conductivity of the contents with that of the dialysis buffer to determine when dialysis is complete.

13. Dialyze against 50 vol of dialysis buffer until the conductivities of the extract and buffer are equal (i.e., when the extract reaches 100 mM KCl). To check conductivity, dilute 5 to 10 μ l of the extract to 1 ml with water. Read the conductivity directly with a conductivity meter and compare it to that of an equivalent dilution of dialysis buffer.

Dialyze the extract for the minimum amount of time needed to achieve the desired salt concentration: 75 to 100 ml of nuclear extract require ~5 hr of dialysis, whereas 2 ml of extract require <1 hr. Use the Support Protocol for optimizing nuclear extraction to determine this. To decrease the dialysis time, use tubing with the largest reasonable surface area for the volume.

Dialyzing against dialysis buffer without KCl can also reduce dialysis time. In this case, the conductivity of the extract should be checked frequently and the dialysis stopped when the conductivity equals that of the dialysis buffer with 100 mM KCl. This method requires extreme care as a very rapid decrease in the salt concentration and/or dialysis to <100 mM KCl can result in increased protein precipitation.

14. Remove the extract from the dialysis bag and check the conductivity a final time to ensure that dialysis is complete. Centrifuge the extract 20 min at $25,000 \times g$ (14,500 rpm in a JA-20 rotor). Discard the pellet.

This will remove protein and nucleic acid that precipitate when the potassium chloride concentration is lowered during dialysis.

15. Determine the protein concentration of the supernatant. Dispense aliquots into tubes if desired and rapidly freeze by submerging in liquid nitrogen. Store the extracts at -80°C .

Protein concentration can be determined using the Bradford assay (UNIT 10.1A). If all of the extract is not going to be used at one time (i.e., for protein purification), divide the extract into aliquots before freezing to avoid unnecessary freezing and thawing. In general, avoid more than five cycles of freezing/thawing. The stability of specific proteins may differ. Thaw frozen extracts on ice.

OPTIMIZATION OF NUCLEAR EXTRACTION

This protocol describes a simple method for optimizing the salt concentration during nuclear extraction for specific applications (see Critical Parameters and Troubleshooting). Extracts made with higher or lower potassium chloride concentrations can then be assayed in transcription, splicing, gel-shift analysis (*UNIT 12.2*) to determine the optimum extraction conditions for specific cells and applications.

Additional Materials (also see Basic Protocol 1)

High-salt buffer with 0.8, 1.0, 1.2, 1.4, and 1.6 M KCl

NOTE: As in Basic Protocol 1, perform all steps at 0° to 4°C and use precooled buffers and equipment.

1. Isolate nuclei as described (see Basic Protocol 1, steps 1 to 7).
2. Using the graduations on the tubes, measure the packed nuclear volume (pnv). Resuspend the nuclei in 1/2 pnv of low-salt buffer.
3. Divide the suspension into five aliquots of equal volume.

Before dispensing aliquots, be certain that the nuclei are completely resuspended. Homogenize with one or two strokes in a Dounce homogenizer using a type B (loose-fitting) pestle, if necessary.

4. Add 1/3 aliquot vol of high-salt buffer with frequent or continuous mixing to each aliquot, as follows: add high-salt buffer with the lowest KCl concentration (0.8 M) to the first aliquot and add high-salt buffer with increasing KCl concentrations (up to 1.6 M) to subsequent aliquots.

It is critical to add this buffer in the cold room with gentle continual mixing on a magnetic stirrer.

5. Secure the tubes containing the extracting nuclei on a tiltboard and gently mix 30 min.
6. Remove the nuclei by centrifuging 30 min at 25,000 × *g* (14,500 rpm in JA-20 rotor).
7. Decant the extracts (supernatants) and dialyze against dialysis buffer (see Basic Protocol 1, steps 12 and 13).

Dialyze all the extracts until the one with the highest KCl concentration approaches the conductivity of the dialysis buffer (100 mM KCl).

8. Check the conductivity of each extract, centrifuge the extracts, and determine the protein concentration of the supernatants (see Basic Protocol 1, steps 14 and 15).
9. Assay the extracts directly or dispense aliquots into tubes. Freeze in liquid nitrogen before storing at –80°C.

PREPARATION OF EXTRACTS FOR AFFINITY PURIFICATION

The nuclear and cytoplasmic extracts prepared as described are also suitable for purification of multi-protein complexes by virtue of engineered affinity tags (Sato et al., 2004; *UNIT 16.13*). In this case, a higher concentration of salt (0.42 M) in the nuclear extraction step may be desired since many proteins do not dissociate from chromatin effectively at 0.3 M. Sodium chloride is used instead of potassium chloride to avoid precipitation when crude extracts are analyzed by SDS-PAGE and immunoblotting.

Additional Materials (also see Basic Protocol 1)

- Low-salt buffer without KCl (see recipe)
- 5 M NaCl
- Sterile glycerol (optional)
- Beckman Type 70.1 Ti rotor (or equivalent)

NOTE: As in Basic Protocol 1, perform all steps at 0° to 4°C and use precooled buffers and equipment.

1. Isolate nuclei as described (see Basic Protocol 1, steps 1 to 7).
2. Using the graduations on the tubes, measure the packed nuclear volume (pnv). Resuspend the nuclei in 1 pnv of low-salt buffer without KCl and transfer to a suitable-size Dounce homogenizer. Use a type B pestle to resuspend clumps of nuclei.
3. Calculate the volume of salt needed to make the final concentration 0.42 M ($0.183 \times$ pnv). Add 5 M NaCl to the resuspended nuclei dropwise while gently using a type B pestle to aid mixing.

The concentrated salt solution must be added dropwise and mixed quickly to avoid a transient high local concentration of salt that could lyse nuclei. As the salt concentration increases, the nuclei become progressively more viscous.

4. Transfer the extract to a capped tube and mix gently on a rotating platform for 30 min at 4°C.

Mixing should be gentle to avoid shearing the chromosomes. This step continues to extract the nuclei. Upon completion, the mixture is extremely viscous and has a gooey consistency.

5. Separate the nuclear extract (supernatant) and the chromatin pellet by ultracentrifugation for 1 hr at $100,000 \times g$ (40,000 rpm in a 70.1 Ti rotor), 4°C.

The pellet is composed predominantly of histones and genomic DNA. It can be further processed to purify nucleosomes and histones using hydroxylapatite (UNIT 21.5).

6. If desired, add sterile glycerol at a final concentration of 10% and rapidly freeze the extract by submerging aliquots in liquid nitrogen. Store the extracts at -80°C.

7. To prepare for affinity purification, thaw extracts on ice. Add 1 vol of hypotonic buffer to achieve the desired salt concentration for subsequent affinity binding steps.

Immunoprecipitation by virtue of a FLAG tag as well as GST affinity pull-downs can be achieved efficiently at 0.3 M salt concentration (UNIT 16.13).

8. Before use, ultracentrifuge the diluted extract 30 min at $100,000 \times g$ (40,000 rpm in a 70.1 Ti rotor).

Brief ultracentrifugation removes any precipitate that might have formed due to freeze-thawing or a change in salt concentration.

PREPARATION OF THE CYTOPLASMIC (S-100) FRACTION

Basic Protocol 1 also yields a cytoplasmic extract that may contain proteins of interest (e.g., RNA polymerase III transcription factors). This protocol describes the preparation of the S-100 fraction from this cytoplasmic extract. It is practical to prepare and dialyze the S-100 fraction and the nuclear extract simultaneously.

NOTE: As in Basic Protocol 1, perform all steps at 0° to 4°C and use precooled buffers and equipment.

Additional Materials (also see Basic Protocol 1)

- Cytoplasmic extract (see Basic Protocol 1, step 7)
- 10× cytoplasmic extract buffer (see recipe)
- Beckman Type 50 fixed-angle rotor (or equivalent)

**BASIC
PROTOCOL 2**

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Interactions**

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- Carefully measure the volume of the cytoplasmic extract (supernatant from step 7 of Basic Protocol 1). Add 0.11 vol of 10× cytoplasmic extract buffer and mix thoroughly.
- Centrifuge 1 hr at $100,000 \times g$ (40,000 rpm in a Type 50 rotor).
- Decant the supernatant containing the cytoplasmic (S-100) fraction and place it in dialysis tubing with a clip at one end. Dialyze the S-100 fraction against dialysis buffer until the conductivity of the former reaches that of dialysis buffer (100 mM KCl).
As with the nuclear extract, dialyze for the minimum amount of time necessary (see Basic Protocol 1, step 13).
- Remove the S-100 fraction from the dialysis tubing, and centrifuge 20 min at $25,000 \times g$ (14,500 rpm in a Beckman JA-20 rotor) to pellet precipitated material.
- Decant the supernatant, check the conductivity, and determine the protein concentration (see Basic Protocol 1, steps 14 and 15). Dispense aliquots into tubes, freeze in liquid nitrogen, and store at -80°C .

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

IMPORTANT NOTE: See recipe for PMSF before adding to any solutions.

Cytoplasmic extract buffer, 10×

0.3 M HEPES, pH 7.9 at 4°C
 1.4 M KCl
 0.03 M MgCl_2
 Store up to 2 weeks at 4°C

Dialysis buffer

20 mM HEPES, pH 7.9 at 4°C
 20% glycerol
 100 mM KCl
 0.2 mM EDTA
 Store up to 2 weeks at 4°C
 Immediately before use add:
 0.2 mM PMSF (see recipe)
 0.5 M DTT

High-salt buffer

20 mM HEPES, pH 7.9 at 4°C
 25% glycerol
 1.5 mM MgCl_2
 0.8, 1.0, 1.2, 1.4 or 1.6 M KCl
 0.2 mM EDTA
 Store up to 2 weeks at 4°C
 Immediately before use add:
 0.2 mM PMSF (see recipe)
 0.5 M DTT

Hypotonic buffer

10 mM HEPES, pH 7.9 at 4°C
1.5 mM MgCl₂
10 mM KCl
Store up to 2 weeks at 4°C
Immediately before use add:
0.2 mM PMSF (see recipe)
0.5 M DTT

Low-salt buffer

Prepare high-salt buffer (see recipe) with 0.02 M KCl or without KCl.

PMSF stock, 0.2 M

Dissolve phenylmethylsulfonyl fluoride (PMSF; mol. wt. 174.2) in anhydrous isopropanol as a 0.2 M stock. Store up to 9 months. Add to buffers immediately before use.

PMSF is not stable in aqueous solution, and the stock should be added to solutions drop by drop with vigorous stirring so it will go into solution. Additional PMSF may be added again partway through a long dialysis.

4-(2-Aminoethyl) benzenesulphonyl fluoride (AEBSF) may be a preferable alternative to PMSF due to its high solubility in aqueous solution. It can be made as a 0.1 M stock in water and stored frozen in aliquots. It should be added to buffers immediately before use at a final concentration of 0.5 mM.

COMMENTARY

Background Information

Crude extracts from cultured mammalian cells were first used to demonstrate accurate initiation and termination of transcription from exogenously added purified DNA by RNA polymerase III (Weil et al., 1979a). Subsequently, cell-free soluble systems were developed from cultured cells that could accurately initiate transcription from purified genes by RNA polymerase II. These include a high-speed centrifugation supernatant from a low-salt extraction of cells that was supplemented with purified RNA polymerase II (Weil et al., 1979b) and a high-salt extract of whole cells that contained endogenous polymerase II (Manley et al., 1980).

In order to take advantage of the presumed nuclear localization of transcriptional components, Dignam et al. (1983a) developed a procedure for preparing a soluble extract from isolated mammalian nuclei. This preparation was shown to support accurate transcription initiation from several class II genes by endogenous RNA polymerase II and accessory factors. Although 5S gene transcription by RNA polymerase III required supplementation with the cytoplasmic (S-100) fraction or the gene-specific factor TF IIIA, the nuclear extracts were sufficient for accurate transcription from

tRNA and adenovirus VA genes by RNA polymerase III (Dignam et al., 1983b). Subsequent analyses demonstrated that these extracts were also competent for in vitro pre-mRNA splicing (Krainer et al., 1984).

This protocol represents modifications of the original procedure of Dignam et al. (1983a). It employs KCl instead of NaCl and has the advantage of a normalized salt concentration during the nuclear extraction process. This is achieved by adjusting the buffer volume in the nuclear extraction step to the volume of the nuclear pellet. These modifications result in more reproducible extracts from separate preparations of nuclei.

Nuclear extracts prepared from HeLa cells using this procedure support accurate initiation of transcription by RNA polymerase II and III from purified, cloned promoters. Furthermore, regulatory promoter elements that function in vivo often regulate transcription in nuclear extracts. The presence in these extracts of proteins that bind to such promoter elements can be determined by gel shift, methylation protection, and footprinting assays (UNITS 12.2-12.4). These extracts will also accurately splice pre-mRNA into mature mRNA products. In addition to supplying a rapid functional assay of promoter elements and

sequences required for RNA processing, these extracts provide starting material for purification and mechanistic analysis of the proteins involved in transcription and splicing.

Critical Parameters and Troubleshooting

In the preparation of nuclear extracts it is essential that denaturation of proteins or proteolysis be avoided as this will cause a loss of protein activity. In order to minimize these possibilities the protocols described are performed at 0° to 4°C, preferably in a cold room. In addition, fresh phenylmethylsulfonyl fluoride (PMSF; 0.2 mM final) is added to the buffers just prior to use to further inhibit proteolysis. While these treatments appear adequate for many mammalian cell types, in some instances, additional protease inhibitors may be necessary. For example, transcriptionally active nuclear extracts from yeast cells are prepared in the presence of 1 mM PMSF, 2 μM pepstatin A, and 0.6 μM leupeptin (Lue and Kornberg, 1987).

The approach described here is designed to generate reproducible preparations of extracts; however, there will be some variation in the activity of extracts prepared from separate cultures or prepared at different times. These variations can arise from several factors, including the volume and density of cells, the packed cell and nuclear volumes, and the amount of time needed to perform individual steps and to complete the procedure. Keeping a record of these factors allows one to directly compare the preparation of separate extracts and to correlate these parameters with any variations in activity. A flow chart for this purpose is presented in Figure 12.1.1. The authors recommend that this page be copied, filled in during each extract preparation, and kept in laboratory notebooks.

The preparation of nuclear extracts has two common pitfalls: either the relevant proteins are inefficiently extracted from the nuclei, or an excessive amount of nonspecific inhibitory proteins is also extracted. The significance of these problems depends upon the application for which the extract is intended. For example, if one is trying to purify a particular DNA-binding protein that is much more efficiently extracted with higher salt concentrations, the unwanted proteins also extracted can be removed with additional chromatographic procedures. Of course, if the protein of interest is efficiently solubilized at low KCl concentrations, contamination with proteins extracted at higher salt will be avoided.

If the extracts are to be used for direct functional analysis (i.e., transcription or splicing) where multiple proteins are involved, extraction conditions that achieve the maximum activity should be determined. In the case of transcription analysis, increasing the KCl concentration during extraction of HeLa cells increases the transcriptional potential of the extracts up to the point where the nuclei begin to lyse during extraction. Nuclear lysis results in the release of chromosomal proteins that nonspecifically bind to DNA and thus inhibit transcriptional analysis. The conditions described in Basic Protocol 1 are chosen to give high transcriptional and splicing activity while remaining safely below salt concentrations where nuclei become fragile and lyse. Thus, these conditions are chosen to give reproducible, high-activity extracts. It should be noted that even under these conditions a significant fraction of inhibitory nonspecific DNA-binding proteins are contained in the extracts. These nonspecific proteins inhibit transcription at low template concentrations (Abmayr et al., 1988). Contaminating proteins can be titrated by using an excess of template or nonspecific DNA.

PMSF and DTT should always be added to buffer solutions immediately before use, and PMSF should be added in a dropwise fashion with vigorous stirring to ensure its solubility. Tris buffer can be used as a less expensive alternative to HEPES in all buffer solutions. Note, however, that the pH of Tris buffers is much more sensitive to changes in temperature than that of HEPES buffers due to the large temperature coefficient of Tris (−0.028 pH/°C). For example, a Tris stock solution that is pH 7.9 at 4°C will drop to approximately pH 7.3 at room temperature. Therefore it is essential to adjust the pH of these solutions at the temperature at which they will be used. Additionally, subsequent functional assays using extracts prepared in Tris buffer (e.g., transcription or splicing) must take into account the assay temperature, and the pH must be adjusted as necessary. When used at 30°C, for example, it is necessary to increase the pH of the extract by adding HEPES, pH 8.4 (room temperature), to a final concentration of 40 mM.

Finally, it is important to note that some complexes may be sensitive to disruption by NaCl. Therefore, when preparing extracts for affinity purification (Alternate Protocol, step 3), one may use alternative salts (KCl or KOAc) instead of the recommended NaCl.

Preparation of Nuclear and Cytoplasmic Extracts

Date _____ Cell type _____ Source _____

Cell count _____ Culture volume _____ Total cell number _____

Starting time: _____

Collect and centrifuge spinner cultures 20 min at 1850 *xg* in conical tubes.

Wash monolayer cultures with PBS and collect in conical tubes.

Centrifuge 10 min at 1850 *xg*.

Packed cell volume (pcv): _____

Wash spinner cultures with 5 pcv PBS and centrifuge 10 min at 1850 *xg*.

Resuspend in 5 pcv hypotonic buffer. _____ (vol hypotonic buffer)

Centrifuge 5 min at 1850 *xg*.

Resuspend in hypotonic buffer to 3 pcv. _____ (vol hypotonic buffer)

Swell on ice 10 min.

Homogenize swollen cells 10 strokes.

Pellet nuclei 15 min at 3300 *xg* ; remove cytoplasmic extract.

Nuclear Extract

Packed nuclei volume (pnv): _____

Resuspend nuclei in 1/2 pnv low-salt buffer. _____ (vol low-salt buffer)

Add 1/2 pnv high-salt buffer. _____ (vol high-salt buffer)

Homogenization? _____ (no. strokes)

Extract 30 min: start: _____ stop: _____

Centrifuge 30 min at 25,000 *xg*.

Conductivity of supernatant: _____ = _____ M

Dialysis: start: _____ finish: _____

Conductivity of nuclear extract: _____ = _____ M

Centrifuge 20 min at 25,000 *xg*. _____ (vol nuclear extract)

Aliquots: no. _____ vol. _____

Freeze in liquid nitrogen; store at -80°C.

Ending time: _____

Protein concentration: _____

Cytoplasmic (S-100) Extract

Cytoplasmic extract volume: _____

Add 0.11 vol 10x cytoplasmic extract buffer _____ (vol buffer)

Centrifuge 1 hour at 100,000 *xg*.

Conductivity of supernatant: _____ = _____ M

Dialysis: start: _____ finish: _____

Centrifuge 20 min at 25,000 *xg*. _____ (vol S-100 fraction)

Conductivity of S-100 fraction: _____ = _____ M

Aliquots: no. _____ vol. _____

Freeze in liquid nitrogen; store at -80°C.

Ending time: _____

Protein concentration: _____

Figure 12.1.1 Flow sheet for recording data when preparing nuclear and cytoplasmic extracts.

Anticipated Results

Using this protocol, it is possible to consistently produce extracts of 8 to 12 mg protein/ml. The high protein concentration in the extracts results in a high yield of transcription factors and nonspecific DNA binding proteins.

Time Considerations

The entire procedure should be completed in one day. It will require ~3 to 5 hr to prepare the extracts prior to dialysis, depending on the volume of cells and the amount of time required to centrifuge spinner cultures or scrape monolayer cells. Approximately 4 to 6 hr are required for dialysis. It is important to stop the dialysis and complete the procedure on the same day, rather than dialyze overnight. Dialysis time can be reduced by using dialysis buffer without KCl. In this instance it is necessary to check the conductivity of the extract frequently during dialysis. The dialysis should be stopped promptly when the extract reaches the conductivity of dialysis buffer with 100 mM KCl. After the dialysis is complete, centrifuging, freezing, and storing the extract can be completed in ≤ 45 min.

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Key Reference

Dignam et al., 1983a. See above.

This is the original paper describing the preparation of nuclear extracts from HeLa cells. It also optimizes conditions for transcription from multiple class II promoters in these extracts.

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