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Chapter 8

Preparation of Splicing Competent Nuclear Extracts

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Abstract

Splicing components play an essential role in mediating accurate and efficient splicing. The complexity of the spliceosome and its regulatory networks increase the difficulty of studying the splicing reaction in detail. Nuclear extracts derived from HeLa cells provide all of the obligatory components to carry out intron removal in vitro. This chapter describes the large-scale preparation of nuclear extract from HeLa cells.

Key words Nuclear extracts, In vitro, Splicing competent, mRNA processing, Alternative splicing

1 Introduction

Crude, nuclear, and cytoplasmic extracts can be used to study the regulation of mRNA processing, such as transcription, pre-mRNA splicing, and polyadenylation [1–3]. As such, they can be used to evaluate molecular mechanisms and interactions through the use of immunoassays, mobility shift assays (EMSA), co-immunoprecipitation (Co-IP), and pull-down assays.

Analyzing pre-mRNA splicing cell-free has permitted to characterize the splicing machinery in detail [4]. Whole cell extracts can be used to support splicing in vitro [5]; however, more than 60 % of the reaction volume has to be dedicated to whole cell extracts. Functional nuclear extracts, originally developed to study transcription in a test tube [6], were first applied to in vitro splicing reactions using β-globin minigenes [7]. Several modified nuclear extract methods were reported since then [8–11], all of which contained all the components required for in vitro splicing of short pre-mRNAs synthesized in a separate transcription reaction [4]. Cytoplasmic S-100 extracts, a by-product of nuclear extract preparations, lack serine/arginine (SR)-rich proteins and are therefore unable to support pre-mRNA splicing unless they are supplemented with recombinant SR proteins [12, 13]. HeLa cells are the most commonly used cells for the preparation of nuclear extract. Nevertheless, the following protocol is suitable for extract preparation from other cell lines as well.

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2 Materials

2.1 Cells

Spinner cultured suspension HeLa-S3 cells (National Cell Culture Center) (*see* **Note 1**).

2.2 Reagents (See Note 2)

1. 1 M dithiothreitol (DTT).
2. 100 mM phenylmethanesulfonyl fluoride (PMSF) in isopropanol.
3. 1× phosphate-buffered saline (PBS): 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 8 mM sodium phosphate dibasic (Na_2HPO_4), 1.5 mM monopotassium phosphate (KH_2PO_4), pH 7.4.
4. Hypotonic buffer: 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 1.5 mM magnesium acetate (MgOAc), 10 mM potassium acetate (KOAc), 0.5 mM DTT, 0.2 mM PMSF.
5. Low-salt buffer: 20 mM HEPES, pH 7.9, 25 % glycerol, 1.5 mM MgOAc, 0.02 M KOAc, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF.
6. High-salt buffer: 20 mM HEPES, pH 7.9, 25 % glycerol, 1.5 mM MgOAc, 1.2 M KOAc, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF.
7. Dialysis buffer: 20 mM HEPES, pH 7.9, 20 % glycerol, 100 mM KOAc, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT.
8. Dialysis tubing (10 K MWCO, Fisher Scientific).

3 Methods (*See* Note 3)

1. Wash the cell pellet and determine the total cell number by resuspending the cell pellet with 5 times (X) cell pellet volume of PBS.
2. Determine and record the packed cell volume (PCV) by centrifuging the cells at 1,850 × *g* for 10 min and then remove the supernatant.
3. Wash the cells by resuspending cell pellet with 5× PCV of hypotonic buffer and immediately centrifuging the cells at 1,850 × *g* for 10 min. Discard the supernatant (*see* **Note 4**).
4. Swell the cells by adding hypotonic buffer to a final volume of 3× PCV followed by incubating on ice for 10 min (*see* **Note 5**).
5. Check for cell lysis of pre-dounced cells by staining a small aliquot of cells with trypan blue.

6. Lyse cells by douncing 10–20 plunges in Kontes-B (Wheaton) Dounce homogenizer (Pestle B) and pour into new bottles (*see* **Note 6**).
7. Monitor the douncing cell lysis by staining a small aliquot of the cells with trypan blue.
8. Determine and record the packed nuclear volume (PNV) by centrifuging the cells at 3,300 × *g* for 15 min, then remove the supernatant. The supernatant can be saved for cytoplasmic S100 isolation preparation [10].
9. Resuspend the pellet of nuclei by adding 0.5× PNV of low-salt buffer and transfer to glass beaker. Combine the nuclei into one beaker if there are multiple tubes.
10. Release the soluble proteins from the nuclei by adding 0.5× PNV of high-salt buffer drop-by-drop while gently stirring (*see* **Note 7**).
11. Extract the nuclei on ice while stirring for 30 min.
12. Remove the nuclei by centrifuging at 25,000 × *g* for 30 min, and save the supernatant.
13. Desalt the nuclear extract by dialyzing the supernatant in dialysis tubing with more than 50× supernatant volume of dialysis buffer for 2–2.5 h while stirring.
14. Change the dialysis buffer and dialyze for an additional 2–2.5 h.
15. Remove the precipitate by centrifuging at 25,000 × *g* for 30 min, and then save the supernatant.
16. Aliquot the extract into 1 ml fractions and freeze on dry ice (*see* **Note 8**).
17. Store the extracts at −80 °C (*see* **Note 9**).
18. Validate the activity of the nuclear extracts with in vitro splicing (i.e., β-globin, Chapter 11).

4 Notes

1. This extract prep starts with pelleted HeLa cells, which can either be purchased or grown in the lab.
2. All reagents should be prepared with autoclaved Milli-Q or double-distilled water, followed by sterilization with autoclave or filtration with 0.22 μm filter. DTT and PMSF stock solutions should be stored at −20 °C and added to buffers just prior to use. All other reagents should be stored at 4 °C.
3. To prevent the denature of proteins and RNA, all extraction steps should be carried out on ice in cold room with ice-cold reagents and centrifuge at 4 °C with pre-chilled rotors.

4. This step needs to be carried out quickly because the hypotonic buffer swells the cells and could potentially break them. Consequently, proteins could leak out of the cell and be discarded with the supernatant.
5. The previous washing step using hypotonic buffer may already have initiated the swelling of the cells. Thus, the PCV may have increased. Refer only to the initial PCV that was recorded. For example, the PCV determined in **step 2** is 15 ml, yet after **step 3** it has increased to 25 ml. In **step 4** add hypotonic buffer such that the final volume of cells and buffer is 45 ml.
6. Perform the douncing with gentle strokes and the loose B pestle to ensure only the cell membranes, but not the nuclear membranes are disrupted.
7. The "drop-by-drop" action is important because rapidly increasing the salt concentration may lyse the nuclei. The lysate can be homogenized again by douncing if it is chunky.
8. 30 l of HeLa cell culture with a $4–6 \times 10^5$ cells/ml density would yield about 45 ml of nuclear extract. Every milliliter of nuclear extract should support ~130 (of 25 μl scale) splicing reactions at 30 % NE.
9. The freeze/thaw cycles should be limited to 5 times to avoid compromising extract activity. The non-disturbed extracts can be stored up to years at −80 °C without losing activity; however, the half-life at 4 °C is only 12 h [14].

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References

1. Roca X, Karginov FV (2012) RNA biology in a test tube – an overview of in vitro systems/assays. Wiley Interdiscip Rev RNA 3:509–527
2. Knapp G, Beckmann JS, Johnson PF et al (1978) Transcription and processing of intervening sequences in yeast tRNA genes. Cell 14:221–236
3. Di Giammartino DC, Shi Y, Manley JL (2013) PARP1 represses PAP and inhibits polyadenylation during heat shock. Mol Cell 49:7–17
4. Hicks MJ, Lam BJ, Hertel KJ (2005) Analyzing mechanisms of alternative pre-mRNA splicing using in vitro splicing assays. Methods 37:306–313
5. Kataoka N, Dreyfuss G (2004) A simple whole cell lysate system for in vitro splicing reveals a stepwise assembly of the exon-exon junction complex. J Biol Chem 279:7009–7013
6. Dignam JD, Lebovitz RM, Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489
7. Krainer AR, Maniatis T, Ruskin B et al (1984) Normal and mutant human beta-globin pre-

mRNAs are faithfully and efficiently spliced in vitro. Cell 36:993–1005
8. Pugh BF (1995) Preparation of HeLa nuclear extracts. Methods Mol Biol 37:349–357
9. Mayeda A, Krainer AR (1999) Preparation of HeLa cell nuclear and cytosolic S100 extracts for in vitro splicing. Methods Mol Biol 118:309–314
10. Abmayr SM, Yao T, Parmely T et al (2006) Preparation of nuclear and cytoplasmic extracts from mammalian cells. Curr Protoc Mol Biol. Chapter 12, Unit 12 1.
11. Kataoka N, Dreyfuss G (2008) Preparation of efficient splicing extracts from whole cells, nuclei, and cytoplasmic fractions. Methods Mol Biol 488:357–365
12. Krainer AR, Conway GC, Kozak D (1990) Purification and characterization of pre-mRNA splicing factor SF2 from HeLa cells. Genes Dev 4:1158–1171
13. Fu XD, Mayeda A, Maniatis T et al (1992) General splicing factors SF2 and SC35 have equivalent activities in vitro, and both affect alternative 5′ and 3′ splice site selection. Proc Natl Acad Sci USA 89:11224–11228
14. Carey MF, Peterson CL, Smale ST (2009) Dignam and Roeder nuclear extract preparation. Cold Spring Harb Protoc. pdb prot5330.