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Chapter 2

Preparation of Splicing Competent Nuclear Extract from Mammalian Cells and In Vitro Pre-mRNA Splicing Assay

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Abstract

The ability to perform in vitro splicing assays has paved the way for in-depth studies of the mechanisms and machinery involved in the process of splicing. The in vitro splicing assay is a valuable experimental approach that combines the complexity of the spliceosome and regulatory systems with the flexibility of performing endless splicing and alternative splicing reactions. Through the use of crude nuclear extract and radiolabeled pre-mRNA, spliced mRNAs can be visualized using autoradiography for downstream analysis. This chapter describes the necessary steps to perform an in vitro splicing reaction, including the generation of the key components necessary for the splicing reaction; nuclear extract.

Key words Nuclear extract, HeLa cells, In vitro splicing, Splicing, Alternative splicing, mRNA processing, Pre-mRNA substrate, In vitro transcription, RNA extraction and purification

1 Introduction

RNA splicing is an essential co-transcriptional feature of gene expression in eukaryotes [1]. The spliceosome assembles in a stepwise manner on pre-mRNA transcripts as RNA polymerase elongates the nascent chain. RNA splicing entails the simultaneous excision and ligation of gene coding exons and the eventual degradation of introns [2]. Alternative splicing involves the selective excision and ligation of gene coding exons and is responsible for much of the genetic diversity seen in higher order organisms [2, 3].

In the field of RNA biology, cell-free or in vitro-based assays [4] have played a key role in providing mechanistic insights into the molecular workings of the cell. One such assay has been the in vitro splicing assay [5, 6], which allows for an experimenter controlled method for the investigation of splicing mechanisms, including

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spliceosomal assembly, splicing kinetics, and splicing regulatory processes. In vitro splicing reactions require two critical components: minigene constructs and nuclear extract. Minigene constructs are small fragments of DNA that, at minimum contain two exons, an intron and a phage promoter sequence upstream of the coding strand [7]. Minigene constructs can be transcribed from DNA to RNA using commercial phage polymerases in an in vitro transcription reaction. Nuclear extract preparations were originally developed to study RNA polymerase II transcription in the test tube [8]; however, these extracts were also shown to support intron excision [9]. Over the years many forms of splicing competent nuclear extract have been developed [10–15], all of which contain the essential components required for an efficient splicing reaction of pre-synthesized pre-mRNAs. The extract contains: spliceosomal components, splicing regulatory proteins, ATP, and other components of the nucleus [14, 16]. HeLa cells are the most common cells from which nuclear extract is prepared; however, nuclear extract from other cells can also be prepared in the same manner. In vitro splicing reactions generally use radiolabeled transcription of minigene constructs and subsequent incubation with nuclear extract [6, 16]. Proteins are digested using Proteinase K and the RNA is then separated from the proteins using phenol/chloroform extraction. After RNA precipitation, the spliced RNAs can be electrophoretically separated on a denaturing polyacrylamide gel and analyzed by autoradiography.

In vitro splicing reactions can be used to study many aspects of the splicing reaction such as splice site strength of the exon/intron junctions, influence of splicing regulatory elements, or the molecular interactions during splice site pairing. The ease with which the experimenter can manipulate the spliceosomal assembly pathway renders this assay an invaluable tool.

2 Materials and Reagents

All reagents and materials should be of high quality, RNase free, and molecular biology grade. In vitro splicing steps usually require work with radioactive isotopes; therefore, all necessary precautions must be taken. Carefully follow all local hazardous and radioactive waste disposal regulations when carrying out experiments using radiolabeled RNA.

2.1 Nuclear Extract Components and Reagents

2.1.1 Cells

Spinner cultured suspension HeLa-S3 cells (*see* **Note 1**).

2.1.2 Reagents

1. 1 M dithiothreitol (DTT).
2. 100 mM phenylmethanesulfonyl fluoride (PMSF) in isopropanol (*see* **Note 2**).

3. 1 M 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer, pH 7.9 at 4 °C with KOH. Store at 4 °C (*see* **Note 3**).
4. 1 M magnesium acetate ($Mg(OAc)_2$) (*see* **Note 4**).
5. 2.5 mM potassium acetate (KOAc) (*see* **Note 4**).
6. 0.5 mM EDTA.
7. 10× Phosphate-buffered saline (PBS), pH 7.4. Store at 4 °C.
8. Glycerol (at least 2 L needed for a 30 L culture of cells).
9. Autoclaved or double-deionized water.
10. Trypan blue.

2.1.3 Buffer Recipes

All reagents should be prepared with autoclaved or double-deionized water, followed by filter sterilization with a 0.22 μm filter or sterilized by autoclaving. All reagents should be stored at 4 °C and be cold prior to use. DTT and PMSF should be made fresh and only added to each corresponding buffer prior to use, not ahead of time.

1. Hypotonic buffer: 10 mM HEPES-KOH at pH 7.9, 1.5 mM $Mg(OAc)_2$, 10 mM KOAc, 0.5 mM DTT, 0.2 mM PMSF.
2. Low-salt buffer: 20 mM HEPES-KOH at pH 7.9, 1.5 mM $Mg(OAc)_2$, 20 mM KOAc, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.2 mM PMSF.
3. High-salt buffer: 20 mM HEPES-KOH at pH 7.9, 1.5 mM $Mg(OAc)_2$, 1.4 M KOAc, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.2 mM PMSF.
4. Dialysis buffer: 20 mM HEPES-KOH at pH 7.9, 100 mM KOAc, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.2 mM PMSF.

2.1.4 Equipment

All glassware (including dounce homogenizer) and bottles/tubes should be sterilized and autoclaved ahead of time. Make sure all glassware and plasticware that is used does not contain detergent residue.

1. Dialysis tubing: 10,000 molecular weight cutoff (MWCO).
2. Glass dounce homogenizer with a tight clearance pestle (*see* **Note 5**).
3. Centrifuge with swinging bucket rotor capable of speeds up to 3500 × *g*.
4. Centrifuge bottles: polypropylene, conical bottom with graduations, wide mouth with sealing caps.
5. Ultracentrifuge with fixed angle rotor capable of speeds up to 25,000 × *g*.
6. Centrifuge tubes: polycarbonate with polypropylene screw caps, 30 mL volume.

7. 25 mL serological glass pipettes.
8. 1 L glass bottles.
9. 200 mL glass beaker.
10. 4 L beaker/buckets for dialysis.
11. 1.5 mL tubes.
12. Column to aid in drip addition of high-salt buffer (optional).
13. Glass slide(s).
14. Phase-contrast microscope.
15. Magnetic stir bar.
16. Magnetic stir plate.
17. Ice.
18. Dry ice.
19. −80 °C Freezer.

2.2 In Vitro Splicing Reaction

1. Radiolabeled pre-mRNA generated from an in vitro transcription reaction (*see* **Note 6**).

2.2.1 Splicing Reaction Components

2. Splicing competent nuclear extract (*see* Subheading 3.1).
3. 25 mM adenosine triphosphate (ATP).
4. 0.5 M creatine phosphate (CP).
5. 80 mM $Mg(OAc)_2$ (*see* **Note 4**).
6. RNase inhibitor, 40 units/μL.
7. 100 mM DTT.
8. 13% polyvinyl alcohol (PVA) (optional).
9. 1 M KOAc (*see* **Note 4**).
10. 0.5 M HEPES-KOH, pH 7.9.
11. Wet ice and dry ice.
12. Water bath.

2.2.2 Splicing gel Components

1. Upright vertical gel electrophoresis system.
2. Electrophoresis power supply with temperature probe.
3. 8″ × 8″ glass plates or equivalent.
4. 0.4 mm spacer set.
5. 0.4 mm gel comb.
6. Aluminum plate: 8″ × 8″ or longer and precooled.
7. 1¼″ binder clips (at least four).
8. 10× Tris-Borate-EDTA (TBE): 1 M Tris Base, 1 M boric acid, 20 mM EDTA.
9. 7 M Urea prepared in 1× TBE.
10. 20% (19:1) acrylamide:bis-acrylamide solution: 210.2 g solid urea, 50 mL 10× TBE, 250 mL 40% acrylamide:bis-acrylamide

solution, adjust to 500 mL with sterile water to yield a final concentration of 20% acrylamide/7 M urea/1× TBE.

11. *N,N,N′,N′*-Tetramethylethylenediamine (TEMED).
12. 10% ammonium persulfate (APS).
13. Stop dye: 98% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10 mM EDTA at pH 8.
14. Silicone-based coating solution.
15. 70% ethanol.
16. Putty knife/gel spatula.
17. Filter paper cut in 8″ × 8″ squares.
18. Plastic wrap.
19. Gel dryer.
20. PhosphorImager System. Film may also be used.
21. Lint-free tissue.
22. 30 mL syringe and needles.

2.2.3 RNA Purification

1. Proteinase K at 10 mg/mL.
2. 2× Proteinase K buffer: 20 mM Tris Base, 2% SDS, 200 mM NaCl, 2 mM EDTA, pH 7.5.
3. 100% ethanol.
4. Glycogen.
5. Phenol, chloroform, isoamyl alcohol solution (25:24:1 pH 8.0).

3 Methods

3.1 Nuclear Extract Preparation

To prevent the denaturation of RNA and proteins, all the extraction steps should be carried out on ice (or in a coldroom). All reagents and buffers should be equilibrated to 4 °C and centrifuge rotors should be precooled to 4 °C. Once initiated, this protocol should be carried all the way to completion. *See* Fig. 1 for a quick reference guide to nuclear extract preparation.

3.1.1 Isolation of Nuclei

1. Transfer HeLa cells to a conical centrifuge bottle to pellet the cells. Centrifuge at 1000 × *g* for 5 min at 4 °C. Decant the supernatant carefully so as not to disturb the pellet.
2. Wash the cells by resuspending the pellet with ice-cold 1× PBS. Add PBS at 5× the volume of cells (*see* **Note 7**).
3. Pellet cells post-wash by centrifugation at 1850 × *g* for 10 min at 4 °C. Remove the supernatant carefully. From these pelleted cells, determine the packed cell volume (PCV), using the graduations on the centrifuge bottle.
4. Wash the cells GENTLY by resuspending the pellet with 5× PCV hypotonic buffer. Immediately centrifuge cells to pellet

Nuclear Extract Quick Reference Guide

Date:_____
Start Time:_____
End Time:_____

Cell Type_____ Cell Count_____
Culture Volume_____ Total # of Cells:_____

Isolation of Nuclei

- ☐ Collect and centrifuge culture cells: 1,000x*g*, 5 min, 4°C.
- ☐ Wash cells: 1X PBS – collect in conical tube.
- ☐ Centrifuge: 1,850x*g*, 10 min, 4°C.
- ☐ Determine packed cell volume (PCV):_____
- ☐ Add DTT and PMSF to *hypotonic buffer.*
- ☐ Wash cells: 5x PCV, *hypotonic buffer*. Volume used:_____
- ☐ Centrifuge: 1,850x*g*, 10 min, 4°C.
- ☐ Resuspend to 3x original PCV, *hypotonic buffer.* Volume used:_____
- ☐ Incubate on ice, 10 min.
- ☐ Check for cell lysis. % Lysed:_____
- ☐ Homogenize cells (dounce): 10-20 strokes/plunges.
- ☐ Check for cell lysis. % Lysed:_____

Extraction of Nuclei

- ☐ Pellet nuclei, centrifuge: 3,000x*g*, 15 min, 4°C.
- ☐ Determine packed nuclear volume (PNV):_____
- ☐ Optional: Save supernatant for S-100 preparation.
- ☐ Add DTT and PMSF to *low-salt buffer.*
- ☐ Resuspend pellet to 0.5x PNV, *low-salt buffer.* Volume used:_____
- ☐ Transfer nuclei to glass beaker with stir bar.
- ☐ Add DTT and PMSF to *high-salt buffer.*
- ☐ Add 0.5x PNV, *high-salt buffer*, drop-wise. Volume used:_____
- ☐ Lyse nuclei: stir on ice, 30 minutes.
- ☐ Transfer to tubes and centrifuge: 25,000x*g,* 30min, 4 °C.
- ☐ SAVE Supernatant = Nuclear Extract!

Dialysis and Storage of Extract

- ☐ Rinse dialysis tubing.
- ☐ Add DTT and PMSF to *dialysis buffer.*
- ☐ Dialyze extract (3x, 1.5 hours, 4°C): 50x supernatant volume, *dialysis buffer*.
  Total volume needed per change:_____
  1) Start time:_____ Stop time:_____
  2) Start time:_____ Stop time:_____
  3) Start time:_____ Stop time:_____
- ☐ Centrifuge: 25,000x*g*, 30 min, 4°C.
- ☐ Volume of nuclear extract (supernatant):_____ # of Aliquots:_____
- ☐ Store -80°C.

Fig. 1 Nuclear extract preparation guide for quick referencing and data recording

at 1850 × *g* for 10 min at 4 °C and decant the supernatant (*see* **Note 8**).

5. Resuspend the packed cells in hypotonic buffer to a final volume of 3× the original PCV (the volume of the cells and the hypotonic buffer combined should be 3× PCV). Incubate cells on ice and allow the cells to swell for 10 min (*see* **Note 9**).
   (a) Check for cell lysis of pre-dounced cells. Lysis can be determined by visualizing stained cells under the microscope by the addition of trypan blue. For a dense concentration of cells, dilute with 1× PBS (*see* **Note 10**).
6. Transfer the cells to a dounce homogenizer to aid in cell lysis. Homogenize the cells with 10–20 plunges/strokes using an up and down motion (*see* **Note 11**).
   (a) Monitor cell lysis by checking post-dounced cells as previously described in **step 5a** (*see* **Note 12**).

3.1.2 Extraction of Nuclei

1. Transfer dounced cells to clean centrifuge bottles and spin to pellet by centrifugation at 3300 × *g* for 15 min at 4 °C. Determine and record the packed nuclear volume (PNV), using the graduations on the centrifuge bottle. At this point the supernatant can be saved for cytoplasmic S-100 extract preparation (*see* **Note 13**).
2. Resuspend the pellet (which now contains nuclei) by adding 0.5× PNV low-salt buffer. Transfer the resuspension to a glass beaker with a magnetic stir bar and gently stir on ice (*see* **Note 14**).
3. Gently release the soluble proteins from the nuclei by adding 0.5× PNV high-salt buffer in a dropwise fashion. Continue to stir on ice for 30 min to complete the extraction of the nuclei (*see* **Note 15**).
4. Transfer nuclei to centrifuge tubes to pellet by centrifugation at 25,000 × *g* for 30 min. Save the supernatant, as this is the nuclear extract.

3.1.3 Dialysis and Storage of Extract

1. Prepare the dialysis tubing by rinsing in distilled water while samples are in the centrifuge (*see* **Note 16**).
2. Desalt the nuclear extract by dialyzing the supernatant in the dialysis tubing dialysis buffer at 50× supernatant volume. Dialyze for 1.5 h at 4 °C, while stirring (*see* **Note 17**).
3. Change the dialysis buffer two additional times and dialyze for 1.5 h at 4 °C each time.
4. Transfer the nuclear extract to centrifuge tubes and remove the precipitate by centrifugation at 25,000 × *g* for 30 min at 4 °C. Save the supernatant.

5. Aliquot the supernatant into 1 mL fractions and freeze on dry ice immediately (*see* **Note 18**).
6. Store the nuclear extract aliquots at −80 °C (*see* **Note 19**).
7. Validate the activity of the nuclear extract by performing an in vitro splicing reaction.

3.2 In Vitro Splicing Assay

3.2.1 Splicing Reaction

1. Determine the volume for a master mix. The master mix volume can be determined with the following equation:
   Reaction volume × (# of reactions + 1) = master mix volume
   (a) For example, 25 μL × (4 reactions + 1) = 125 μL. The extra (+1) volume is to account for pipetting errors. Typical splicing reaction volumes range between 10 and 25 μL.
2. Thaw nuclear extract on ice.
3. Allow the following reagents to thaw at room temperature and then immediately place them on ice: ATP, CP, $Mg(OAc)_2$, DTT, HEPES, and KOAc. Thaw radiolabeled RNA and nuclear extract on ice. Keep RNase inhibitor on ice as well (*see* **Note 4**).
4. Combine the following reagents to obtain a final concentration of: 1 mM ATP, 20 mM CP, 3.2 mM $Mg(OAc)_2$, 10 units RNase inhibitor, 1 mM DTT, 3% PVA (optional), 12 mM HEPES, 72.5 mM KOAc, and 10–50% nuclear extract (percent of nuclear extract to use should be optimized for each extract and substrate that will be used). Add sterile water to bring up the master mix volume if necessary (*see* **Notes 20–22**).
5. For each experimental condition, add the following: appropriate volume of master mix, 0.01–0.1 nM RNA (~1000 cpm), experimental variant (such as protein), and/or sterile water to bring up the volume. Add nuclear extract last to initiate the reaction. Gently pipette up and down to mix the reaction. Keep all reaction tubes on ice. Once nuclear extract has been added, quickly take a time 0 ($T = 0$) aliquot from each reaction tube and immediately freeze on dry ice (*see* **Note 23**).
6. Incubate all reactions (except the time 0 control) at 30 °C for 90 min in a water bath (*see* **Note 24**).
7. While the splicing reaction is incubating, prepare the 6% polyacrylamide gel.

3.2.2 Splicing Gel Preparation

1. Prepare a 6% polyacrylamide solution in a 50 mL conical tube. For the gel size specified above, 25 mL is sufficient. Dilute 20% acrylamide/7 M urea/1× TBE with 7 M urea/1× TBE to obtain a 6% acrylamide solution.
2. Carefully clean the glass plates by running them under deionized water (*see* **Note 25**).

3. Place both glass plates on a flat surface with the inside of each plate facing upward. Wipe away any residual water with lint-free paper towels.
4. Spray the glass plates with 70% ethanol and wipe them dry with lint-free paper towels (*see* **Note 26**).
5. Assemble the gel cassette (*see* **Note 27**) and add binder clips to the edges and sides of the glass plates to hold the plates together, taking care to not move the spacers (*see* **Note 28**).
6. Once the gel cassette is ready, add a 1:1000 volume of TEMED and a 1:100 volume of 10% APS to the 6% acrylamide solution (*see* **Note 29**).
7. Aspirate the polyacrylamide solution with a 30 mL syringe that does not have a needle. Hold the gel at a 45° angle and place the syringe tip such that it makes firm and direct contact with the non-siliconized plate. Apply constant pressure to dispense the acrylamide solution. Once the cassette is full, place it on a flat elevated surface such as a test tube rack (*see* **Note 30**).
8. Insert the gel comb (*see* **Note 31**).
9. Leave the cassette flat on a bench top. Flush out any remaining un-polymerized gel from the syringe back into the conical tube. This can be used as a marker to confirm that the gel has polymerized. Let the gel polymerize for ~30 min.
10. Remove the gel comb and bottom spacer. Clamp the gel cassette to the upright gel electrophoresis apparatus using 1¼″ binder clips (*see* **Note 32**).
11. Tilt the electrophoresis apparatus to one side, making a 45° angle between the bench top and the bottom of the electrophoresis apparatus, slowly pour 1× TBE buffer down the raised end while incrementally lowering the apparatus. This will minimize the formation of bubbles between the glass plates (*see* **Note 33**).
12. Make sure the bottom of the gel is submerged in buffer. Fill the top compartment of the electrophoresis apparatus with 1× TBE, until the wells in the gel are filled with buffer.
13. Prerun the gel for 15 min at 30 W. Attach a temperature probe and set the temperature limit to 45 °C to ensure the glass plates do not break.

3.2.3 Proteinase K Digest

1. Once the splicing reaction has reached completion, immediately place the tubes on dry ice.
2. Determine the final volume for the Proteinase K master mix using the following equation:

   Reaction volume × (# of reactions + 1) = master mix volume
3. Mix reagents to yield a final master mix volume: 1× Proteinase K buffer at final desired volume, 0.25 mg/mL glycogen (*see* **Note 34**) and 0.25 mg/mL Proteinase K. Use sterile water to

adjust the volume of the master mix (if necessary) to a final volume of 180 μL per reaction.

4. Add 175 μL of Proteinase K master mix to each tube and incubate at 37 °C for 10–15 min (*see* **Note 35**).

3.2.4 RNA Purification

1. Once the Proteinase K digest is complete, add 200 μL of phenol:chloroform to each reaction tube.
2. Vortex the tubes on high speed for 30s and centrifuge at 16,500 × *g* for 5 min.
3. Carefully remove the aqueous phase (top layer), taking care not to remove any of the organic phase.
4. Pipette the aqueous phase of each tube into a fresh tube and add 3 volumes of 100% ice-cold ethanol. Incubate the tubes at −20 °C for 15 min.
5. Centrifuge the tubes at 16,500 × *g* for 10 min at room temperature (*see* **Note 36**).
6. Remove the ethanol supernatant, taking care not to disturb the pellet. Allow the pellet to air dry for no more than a few minutes. Add a small volume of stop dye (10 μL or less) and pipette up and down to mix. At this point the RNA is ready to be loaded onto the gel.

3.2.5 Electrophoresis and Visualization

1. Load samples onto the gel and run at 30 W (100 V), 45 °C, for 60–90 min or until the bromophenol blue dye reaches the bottom of the gel. The length of time samples that are run on the gel should be optimized based on the size of expected products (*see* **Notes 37** and **38**).
2. Remove the buffer from the top and bottom compartments of the upright electrophoresis apparatus as well as the temperature probe. Carefully detach the 1¼″ binder clips.
3. Cut filter paper into 8″ × 8″ pieces.
4. Pull the vertical spacers out of the gel cassette and use the putty knife to wedge open the glass plates. Remove the siliconized (notched) plate.
5. Place a piece of 8″ × 8″ filter paper on the gel and gently press down. Invert the gel such that the filter paper is on the bench and the glass plate is on top.
6. Use the putty knife to remove the remaining glass plate by slowly lifting one corner. The gel should adhere to the filter paper.
7. Place a piece of plastic wrap on the top of the gel. Take care to avoid creases in the wrap.
8. Dry the gel for 20 min at 80 °C using a gel dryer. Make sure the suction pump is turned on for the duration of drying.
9. Expose a phosphorscreen to the gel for 1 h-overnight (*see* **Notes 39** and **40**).

10. Use a PhosphorImager to obtain an image of the spliced products. This image can be used to quantify parameters such as percentage of splicing or splice site preference in a minigene that contains competitive splice sites.
11. Quantify results using a gel analysis software (*see* **Note 41**).

4 Notes

1. This nuclear extract protocol starts with a large volume (~30 L) of purchased spinner cultured HeLa cells. HeLa cells can also be cultured in the lab.
2. PMSF is dissolved in anhydrous isopropanol and should be prepared fresh and added to corresponding buffers just prior to use (similar to addition of DTT). Store the freshly prepared PMSF solution on ice or at 4 °C during duration of the nuclear extract prep.
3. HEPES solution should be brought to pH 7.9 while at 4 °C or on ice.
4. $MgCl_2$ and KCl can be used as well; however, a previous study has shown that the use of acetate as a counter ion enhances the splicing reaction [17].
5. Use a pestle that has a tight fit in the mortar (approximately 0.025–0.076 mm): Kontes brand homogenizers have a tight clearence in their type-B pestle, alternatively, Wheaton dounce homogenizers have a tighter clearance in their type-A pestle.
6. Pre-mRNAs are generally transcribed with commercially available phage polymerases in the presence of UTP that contains phosphorous-32 at the alpha position (^{32}P α-UTP). The radioactive nucleotide is generally in the 0.3–3 nM range and usually leads to 100,000 cpm/μL incorporation. Other nucleotide triphosphates that have a radioactive α-phosphate can be used instead of ^{32}P α-UTP if needed.
7. It is suggested to use 25 mL serological glass pipettes to wash the cells. Make sure to mix gently and not expel volume from the pipette completely while mixing, so as to prevent the creation of a vacuum.
8. This step should be performed quickly. The hypotonic buffer swells the cells and could potentially cause the cells to leak or burst, leading to loss of protein into the supernatant or cell death. Monitoring cell lysis with trypan blue allows for visualization of lysed cells, which take up the dye, as compared to intact cells, which do not.
9. The previous wash step with hypotonic buffer may have initiated swelling of the cells. Therefore, the PCV may have increased. When determining how much hypotonic buffer to

add in this step, refer only to the initial PCV that was recorded. For example, the PCV determined in **step 4** is 15 mL, yet after **step 5** it has increased to 25 mL. In **step 6** add hypotonic buffer such that the final volume of cells and buffer is 45 mL (3 × 15 mL). Add the hypotonic solution gently while mixing with a serological pipette.

10. Check for the lysis of pre-dounced cells by adding trypan blue, 1:2 dilution. At this point, you want a minimum of 50% of the cells alive. Greater than 80% cell survival is ideal. Use a plastic pipette with a cut tip to check for lysis to prevent shearing of the cells.
11. Perform the dounce homogenization step with gentle strokes maintaining a constant plunging motion. Do not remove the pestle from the dounce until douncing is complete. This will ensure efficient cell breakage.
12. Visualize cell lysis with a 1:2 dilution of cells to trypan blue. 80–90% cell lysis should be expected. A good sampling of the lysed cells can be found from further down the dounce tube, and not from the top.
13. S-100 cytoplasmic fraction extraction can be performed at this step. For further instructions, *see* ref. 11.
14. The lysate can be homogenized again by douncing if the cell solution is chunky. At this point, if there are multiple tubes, combine the nuclei into one beaker.
15. The dropwise addition of the high-salt buffer is vitally important because rapidly increasing the salt concentration may lead to nuclear lysis and precipitation of nuclear components. High-salt buffer permeabilizes the nuclear membrane to allow for release of necessary components. Nuclei can be further homogenized with 5 dounce strokes to prevent clumping when nuclei are in large volumes.
16. Alternatively, dialysis tubing can be rinsed in dialysis buffer.
17. Avoid the presence of bubbles in the dialysis tubing by gently squeezing the bubbles out of the tubing with your fingers. Remember to clamp one end of the tubing prior to addition of sample.
18. 30 L of a HeLa cell culture with a 4–6 × 10^5 cells/mL density should yield about 45 mL of nuclear extract.
19. Freeze/thaw cycles of the nuclear extract aliquots should be limited to avoid compromising extract activity. The non-thawed extracts can be stored up to 2 years at −80 °C without loss of activity. However, the half-life at 4 °C is only 12 h [18].
20. The optimum potassium concentration for an in vitro splicing reaction is around 30 mM; however, splicing can be observed between 2 mM up to 100 mM. Splicing efficiency is reduced

drastically at the high and low extremes. Splicing efficiency may be increased by optimizing the potassium ion concentration [9].

21. When adding KOAc and HEPES it is important to account for the potassium ion and HEPES concentration already present in the nuclear extract. For example, if the nuclear extract already contains 100 mM KOAc and 20 mM HEPES it is possible to calculate how much additional KOAc and HEPES to add to the reaction with the following calculation:

$$\begin{gathered} \textit{For a } 25\,\mu L \textit{ reaction containing } 30\%\ NE: \\ (7.5\ \mu L\ NE)(100\ mM\ KOAc) = (25\ \mu L)(\mathrm{X}) \\ \mathrm{X} = 30\,mM\ KOAc. \end{gathered}$$

The addition of nuclear extract to the reaction yields an initial 30 mM KOAc concentration. This amount must be subtracted from the desired final concentration

$$\begin{gathered} 72.5\ mM\ KOAc - 30\ mM\ KOAc = 42.5\ mM\ KOAc \\ (1000\ mM\ KOAc)(\mathrm{X}) = (25\,\mu L)(42.5\ mM\ KOAc) \\ \mathrm{X} = 1.06\,\mu L\ KOAc. \end{gathered}$$

PVA is a concentration-enhancing polymer that may increase reaction efficiency. It may increase splicing, but is not essential to the reaction [19].

22. Taking a $T = 0$ aliquot and freezing it on dry ice stalls the reaction from proceeding. The $T = 0$ aliquot can be used as a zero time point to mark the initiation of the reaction.
23. Reaction times can be varied and optimized depending on the experimental conditions.
24. If there is residual debris stuck to the plates, water will be forced to flow around the debris. Hold the gel at a 45° angle and run water over it. Move glass plates slowly and scan for debris. Gently wipe debris away if any is present.
25. Cleaning glass plates with 100% ethanol will lead to faster removal of the silicone based coating; therefore, it is best to use 70% ethanol.
26. Place two spacers on the vertical edges of the non-siliconized glass plate. Make sure the spacers are aligned. Place the third spacer horizontally at the bottom of the glass plate. Make sure spacers are flush with each other and place the second (notched) glass plate onto the first. Make sure the siliconized side is facing toward the inside of the gel cassette.
27. If the spacers move during the attachment of 1¼″ binder clips, gaps can appear between them, which will lead to leakage from the gel cassette.

28. Once APS and TEMED have been added it is important to work quickly because the acrylamide will start to solidify. Invert the conical tube three times to ensure mixing.
29. It is critical to avoid bubbles. If a bubble appears inside the gel, tilt the gel cassette to one side and then bring it back to its original position with a rapid and continuous movement.
30. For the best results, press the gel comb flat against the non-siliconized glass plate. Insert the comb by placing thumbs on both sides of the comb and gently pushing down. Do not push the comb down past the top of the wells.
31. To remove the comb, hold the gel at a 45° angle (alternatively, place the gel flat on raised surface such as test tube rack) and place both thumbs at the edges of the comb. Gently push upward, taking care to distribute force evenly.
32. Ensure there are no bubbles on the bottom of the gel cassette. The presence of bubbles will cause uneven current distribution and may cause the gel box to shut off. Take a syringe with a bent needle and draw up buffer. Slide the needle along the bottom of the gel cassette in the opening between the two glass plates and simultaneously dispense buffer with minimal force to move the bubbles out.
33. Glycogen is polysaccharide carrier that aids in the precipitation and visualization of nucleic acids in ethanol precipitation protocols.
34. For 25 μL reactions, add 175 μL of Proteinase K master mix; however, this amount should be scaled up or down depending on the volume of the splicing reaction used.
35. Place tubes in a uniform fashion so that the pellet will appear on the same side for each tube. It is important to minimize loss of the RNA during the ethanol precipitation. If tubes are placed in a uniform manner such that the pellet appears in the same spot, the experimenter can assume the pellet is there even if it is faint or not visible.
36. Wash out the wells of the gel prior to loading samples to remove any residual urea that has settled at the bottom of the wells. Using a syringe with a straight needle attached aspirate some buffer from the top compartment of electrophoresis apparatus and dispense buffer into the wells with mild force to displace the urea.
37. Use 1¼″ binder clips to clamp a precooled aluminum plate to the front of the gel cassette. This will help dissipate heat and prevent the glass plates from breaking.
38. Phosphor storage technology makes use of phosphorscreens that are composed of $BaFBr:Eu^{2+}$ crystals immobilized in an organic matrix. Phosphorscreens can be used for autoradiography and have many advantages over the traditional method of

exposing x-ray film to radioactive gels. For example, phosphorscreens have between 10 and 250 times higher sensitivity and a linear dynamic range that spans five orders of magnitude. High-energy radiation emitted from radioactive atoms, such as ^{32}P, oxidizes Eu^{2+} to Eu^{3+} and leads to an electron being trapped in the BaFBr complex. The reduced $BaFBr^{-}$ has a unique absorbance in the 600 nm range. Exposure of the phosphorscreen to a 633 nm wavelength scanning laser results in the oxidation of the $BaFBr^{-}$ complex and reduction of Eu^{3+} back to Eu^{2+}. The reduction of Eu^{3+} to Eu^{2+} leads to the emission of a photon with a wavelength of 390 nm. These photons can be detected with a photomultiplier instrument as the laser scans the phosphorscreen [20].

39. A 1 h exposure to the phosphorscreen is sufficient for pre-mRNA that has a specific activity of 800 cpm/μL or higher. Longer exposure times may be needed to visualize less abundant splicing intermediates; an optimum exposure time can be found through trial and error.

40. There are many forms of analysis for autoradiograms of in vitro splicing reactions. One of the simplest forms of analysis is to calculate the percent of spliced product (Fig. 2). Calculate the sum of the volume intensity for the spliced band and the unspliced band taking into account background and time 0 (total signal). Divide the intensity of the band corresponding to the spliced product by the total signal intensity and then multiply by 100 to obtain a percentage.

$$\%\,Spliced = \frac{Signal\ from\ final\ spliced\ product}{Total\ signal\ in\ lane}$$

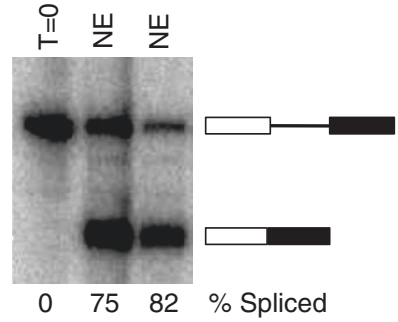

Fig. 2 Autoradiogram of radiolabeled β-globin minigene pre-mRNA splicing reaction (from *left* to *right*) at time 0 ($T = 0$) (*lane 1*) or incubated for 90 min in 30% nuclear extract (NE) from two different extract preparations (*lane 2* and *3*). Reaction products were separated on a 6% polyacrylamide gel. The splicing efficiency (% spliced) was determined using software from Bio-Rad Quantity One® (*see* **Note 41**)

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