Chapter 9

Preparation of Yeast Whole Cell Splicing Extract

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Abstract

Pre-mRNA splicing, the removal of introns from pre-messenger RNA, is an essential step in eukaryotic gene expression. In humans, it has been estimated that 60 % of noninfectious diseases are caused by errors in splicing, making the study of pre-mRNA splicing a high priority from a health perspective. Pre-mRNA splicing is also complicated: the molecular machine that catalyzes the reaction, the spliceosome, is composed of five small nuclear RNAs, and over 100 proteins, making splicing one of the most complex processes in the cell.

An important tool for studying pre-mRNA splicing is the in vitro splicing assay. With an in vitro assay, it is possible to test the function of each splicing component by removing the endogenous version and replacing it (or reconstituting it) with a modified one. This assay relies on the ability to produce an extract—either whole cell or nuclear—that contains all of the activities required to convert pre-mRNA to mRNA. To date, splicing extracts have only been produced from human and *S. cerevisiae* (yeast) cells. We describe a method to produce whole cell extracts from yeast that support splicing with efficiencies up to 90 %. These extracts have been used to reconstitute snRNAs, screen small molecule libraries for splicing inhibitors, and purify a variety of splicing complexes.

Key words Pre-mRNA splicing, Whole cell extract, Liquid nitrogen, Grinding, Reconstitution

1 Introduction

Pre-mRNA splicing was first reported in the late 1970s when several research groups identified regions of genomic sequence that were absent in the corresponding mature mRNA transcripts [1–4]. Over the next several years, these intervening sequences were characterized, and consensus sequences at the splice junctions were identified [5]. It was soon revealed that pre-mRNA splicing was a general phenomenon that was prevalent across eukaryotes and that many eukaryotic genes contained more than one intervening sequence. However, it was not obvious how the correct splice sites were selected since the consensus sequences at the splice sites were short and relatively degenerate. It became clear that in order to understand the mechanism of splicing, it was critical to develop an in vitro splicing system from which splicing intermediates and splicing factors could be isolated, identified, and characterized.

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The first report of an accurate and efficient in vitro pre-mRNA splicing system did not come until 1984, when Krainer et al. successfully spliced approximately 90 % of the in vitro-transcribed, radiolabeled β-globin pre-mRNA transcript that they incubated in a HeLa cell nuclear extract [6]. In this system, the progress of the splicing reaction could easily be followed by separating the splicing intermediates and final products using gel electrophoresis and visualizing them by autoradiography. The following year, Lin et al. published a yeast whole cell in vitro pre-mRNA splicing system that had the added advantage of applying yeast genetics in parallel to study the components and interactions of the spliceosome, the large macromolecular machine responsible for catalyzing splicing [7]. These systems revealed that splicing proceeds via two chemical steps, as well as identifying the basal requirements for pre-mRNA splicing: ATP, Mg^{2+}, and monovalent cations.

A major advantage of an in vitro splicing system is that mutated RNAs can be reconstituted into functional complexes to address very specific questions. In yeast, in vitro reconstitution systems have been developed for four of the five small nuclear RNAs (snRNAs): U2, U4, U5, and U6 [8–11]. In these systems, the endogenous full-length snRNA in the splicing extract is targeted for RNAse H-dependent cleavage using complementary oligonucleotides, resulting in nonfunctional fragmented snRNAs. The depleted snRNA is then replaced with a mutated synthetic or in vitro-transcribed snRNA. While in vivo studies of mutated yeast snRNAs only reveal a defect in cell growth that could be attributable to a defect in splicing, the in vitro assay allows for the direct assessment of the effect of the mutation on splicing and the ability to distinguish first and second step splicing defects. For example, the U6 snRNA mutant, G50A, shows a severe growth defect in vivo, and when studied in vitro, it shows a strong block only in the second step of splicing [12].

Another advantage of reconstitution in splicing extract is that the exogenous RNA can be modified in a variety of ways, including the incorporation of site-specific nucleotide analogs that allow for the covalent attachment of cross-linkable or reactive chemical groups. By changing the length of the cross-linking species at a specific location, one can deduce the proximity of various components of the spliceosome to the modified residue. For example, 4-thiouridine, a structural analog of uridine, will cross-link to anything that is within a covalent bond distance of the reactive thiol group while other cross-linkers such as azidophenacyl have longer reaches and will cross-link over a longer distance [13, 14]. Ryan et al. used a 4-thiouridine- and a 5-iodouridine-based cross-linking strategy to probe for RNAs that are within a covalent bond distance of 26 different residues in U6 snRNA [15]. They used these data to develop a three-dimensional model of the functional spliceosome.

In vitro splicing assays have also been used extensively to study the process of spliceosome assembly and to identify the RNA and protein species found in various splicing complexes blocked at specific steps. Cheng and Abelson [16] and Konarska and Sharp [17, 18] developed these assembly assays using yeast whole cell or HeLa cell nuclear extract, respectively, revealing a striking similarity between yeast and mammalian spliceosome assembly. In both cases, the first splicing complex to form contains U2 snRNA and requires ATP and a 3′ splice site. The second complex contains U2, U4, U5, and U6 snRNAs and requires both a 5′ and a 3′ splice site. Mutations in the 3′ splice site, which allow the first splicing reaction to occur, but block the second reaction, result in accumulation of the second complex [16]. More recently, mass spectrometry has been used to identify proteins present at each step of splicing (reviewed in Will and Luhrmann [19]).

In vitro splicing and assembly assays have been used to screen small molecules for their ability to inhibit the splicing process [20–22]. The discovery of small molecules that block splicing and result in the accumulation of normally transient intermediate splicing complexes should facilitate investigation of the splicing mechanism. Intriguingly, Spliceostatin A exhibits potent antitumor activity, suppressing the growth of various mouse and human tumors and prolonging the life span of affected mice [23].

Despite the development of in vitro pre-mRNA splicing assays over 25 years ago, the splicing mechanism is still very poorly understood. A major reason for this is the asynchronous assembly of the spliceosome and progress of the splicing reactions in whole cell extract. Splicing is a highly dynamic process that involves numerous structural rearrangements within the spliceosome to position the pre-mRNA substrate in an appropriate orientation for each step of the splicing reaction to take place [24]. Consequently, the signals that are observed in whole cell extract are an average of these processes. To overcome this challenge, single molecule fluorescence assays using whole cell splicing extract have recently been developed to monitor in vitro splicing of individual pre-mRNA transcripts as well as assembly of the spliceosome using fluorescently labeled splicing complexes [25, 26]. These studies have already shed some light on the details of the mechanism of splicing by revealing the ordered pathway of spliceosome assembly and the time- and ATP-dependent conformational states of the pre-mRNA during splicing.

Here, we describe a method for preparing yeast whole cell splicing extract from a protease-deficient yeast strain. We also outline the steps required to prepare a radiolabeled pre-mRNA substrate to monitor the splicing reaction. Finally, we describe the procedure for testing the splicing extract for activity and provide the equations that are necessary to determine the splicing efficiency.

2 Materials

Purchase RNase-free plasticware (tubes, tips, etc.). Bake glassware at 250 °C for 2 h to inactivate any RNases. Allow all baked materials to reach room temperature before moving them to 4 °C or −80 °C, as a rapid change in temperature can cause the materials to break. Autoclave all reagents unless otherwise indicated. Filter sterilize indicated buffers through 0.22 μm nitrocellulose (high protein-binding) bottle-top or syringe filters to remove RNases. Chill all centrifuge rotors and tubes prior to use.

2.1 Cell Growth and Harvest

1. Yeast strain BJ2168 (EJ101) (Jones 1991 [27]; available upon request) or strain of your choice.
2. YPD media (4.2 L and one plate): 1 % Bacto™ Yeast Extract, 2 % Bacto™ Peptone, 2 % dextrose. Add 2 % Bacto™ Agar for plates.
3. 4 × 2.8 L Fernbach flasks.
4. Incubator and shaker (30 °C).
5. Beckman Coulter Avanti HP-20 XPI Centrifuge, JA8.1000 rotor, and 4 × 1 L centrifuge bottles (or equivalent).
6. AGK buffer: 10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 200 mM KCl, 0.5 mM DTT (add fresh), 10 % glycerol. Filter sterilize. Store at 4 °C.
7. 4 × 50 mL conical tubes, 1 × 15 mL conical tube.
8. Plastic pitcher, autoclaved.
9. 21-gauge needle and 10 mL syringe.
10. Liquid nitrogen.

2.2 Preparation of Yeast Whole Cell Extract

1. Large (~20 cm diameter) mortar and pestle wrapped in foil, baked, and cooled at −80 °C overnight.
2. 250 mL beaker, small metal spatula, and several Pasteur pipets all wrapped in foil, baked, and chilled at 4 °C.
3. Magnetic stir bar rinsed with ethanol and chilled at 4 °C.
4. 5 mL pipets, autoclaved and chilled at 4 °C, or RNase-free disposable pipets.
5. Liquid nitrogen.
6. Beckman Coulter Avanti HP-20 XPI centrifuge, JA25.50 rotor, 2 Oakridge tubes (or equivalent).
7. 8,000–10,0000 MWCO dialysis membrane tubing.
8. Buffer D: 20 mM HEPES-KOH, pH 7.9, 0.2 mM EDTA, 50 mM KCl, 0.5 mM DTT (add fresh), 20 % glycerol. Store at 4 °C.

2.3 Preparation of Radiolabeled In Vitro-Transcribed Actin Pre-mRNA

1. Plasmid containing the T7 promoter followed by a portion of the actin gene (pJPS149; Vijayraghavan et al. 1986 [28]; available upon request).
2. Microcentrifuge and tubes.
3. Restriction enzyme HindIII, buffer, and BSA.
4. Heat block or water bath (37 °C, 65 °C).
5. Gel extraction kit.
6. 0.8 % agarose gel, gel apparatus, and power supply.
7. 1× TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0.
8. Spectrophotometer (NanoDrop).
9. T7 RNA polymerase and buffer.
10. RNA nucleotides, 100 mM. Make a mixture of ATP, CTP, and UTP 10 mM each in dH_2O. Make a 0.5 mM stock of GTP. Aliquot and store at –80 °C.
11. Superasin RNAse inhibitor
12. α-^{32}P-GTP, 3,000 Ci/mmol, 10 mCi/mL.
13. TE: 10 mM Tris–HCl, pH 7.5, 1 mM EDTA. Filter sterilize.
14. G25 spin column.
15. Scintillation counter.

2.4 Testing the Extract for Splicing Activity

1. Microcentrifuge and tubes chilled at 4 °C.
2. Radiolabeled pre-mRNA transcript.
3. Splicing extract.
4. Splicing buffer components: dH_2O, 25 mM $MgCl_2$, 1 M potassium phosphate, pH 7 (mix 6.15 mL 1 M K_2HPO_4 and 3.85 mL 1 M KH_2PO_4), 30 % PEG 8000, 100 mM ATP. Filter sterilize each component (except ATP) separately, aliquot, and store at –80 °C.
5. Stop solution: 0.30 M NaOAc, 1 mM EDTA, 1 % SDS, 0.034 mg/mL E. coli tRNA (add tRNA when ready to use). Filter sterilize. Store at room temperature.
6. Heat blocks (30 °C, 65 °C)
7. 25:24:1 phenol/chloroform/isoamyl alcohol, pH 6.7 (Sigma).
8. Chloroform.
9. 70 %, 100 % ethanol, –20 °C.
10. 7 M urea loading buffer.
11. Vertical gel apparatus, plates (14.5 × 16.5 cm), spacers (0.75 mm), comb, and power supply.

12. Acrylamide gel components: 40 % (19:1) acrylamide/bis acrylamide, 20× TBE (1.78 M Tris base, 1.78 M boric Acid, 40 mM EDTA, pH 8.0), 10 % APS (in dH_2O, made fresh within the last month), TEMED.
13. Phosphor screen, cassette, and phosphorimager.

2.5 Time Considerations

Cell growth: 4 days from the glycerol stock.

Cell harvest: 1.5 h.

Extract preparation: 7–8 h including dialysis.

Preparation of actin in vitro transcription template: 7–8 h.

Preparation of radiolabeled actin pre-mRNA: 2 h.

Standard splicing assay: 4 h excluding exposure to a phosphorimager screen.

3 Methods

3.1 Cell Growth and Cell Harvest

Splicing extract can be prepared from any yeast strain; however, the presence of proteases can be problematic. The use of a protease-deficient strain results in the most active splicing extract.

1. Grow 4 L of yeast cells in YPD to an OD_{600} of 2.0–2.5 (*see* **Notes 1**, **2**).
2. Harvest the cells at 2,200×*g* for 15 min in a JA 8.1000 rotor (Beckman Coulter Avanti HP-20 XPI Centrifuge). Pour off the YPD and keep the cells on ice from this point on.
3. Resuspend pellets quickly in 50 mL cold dH_2O/2 L cells by vigorous swirling. Spin 12 min at 2,200×*g* in the JA 8.1000 rotor. Gently pour off the dH_2O (*see* **Note 3**).
4. Wash each 2 L cell pellet with 50 mL cold AGK buffer as in the previous step.
5. Resuspend each 2 L cell pellet in 7.5 mL cold AGK buffer and combine in one 50 mL conical tube (*see* **Note 4**).
6. Using a 10 mL syringe with a 21-gauge needle, drip the cell suspension into a 1 L sterile plastic pitcher containing about 200 mL of liquid nitrogen (*see* **Note 5**). Pour off the liquid nitrogen and collect the cell drops in two 50 mL conical tubes (*see* **Note 6**). Store at −80 °C until ready to prepare extract (*see* **Note 7**).

3.2 Preparation of Yeast Whole Cell Extract

1. In the cold room, pour the frozen cell drops into the prechilled mortar holding about 50 mL of liquid nitrogen (*see* **Note 8**). Allow most of the liquid nitrogen to evaporate and then grind the cells into a very fine powder using a chilled pestle. Make sure the cells stay frozen by adding extra liquid nitrogen about

every 4 min. Do not allow the powder to get shiny and sticky. Grind for 30–40 min (*see* **Note 9**).

2. Scrape the powder into the 250 mL beaker, containing the stir bar, using the metal spatula, and thaw at 4 °C for 1 h. Place the beaker in an ice water bath on a stir plate in the cold room and stir for 30 min (*see* **Note 10**).
3. Transfer the thawed powder to an Oakridge tube using a cold 5 mL pipet. Spin in a JLA 25.50 rotor (Beckman Coulter Avanti HP-20 XPI Centrifuge) at 18,000 × *g* for 30 min at 4 °C to remove cell debris.
4. Pipet no more than 8.0 mL of the supernatant from the Oakridge tube into a 70.1 Ti centrifuge tube without disturbing the pellet. Also avoid the floating white lipoproteins at the surface (*see* **Note 11**). Spin at 100,000 × *g* for 1 h in a Beckman XL-70 ultracentrifuge.
5. Use a Pasteur pipet to remove approximately 4–5 mL of the pale yellow aqueous phase from the middle of the supernatant without disturbing the top film or bottom pellet (*see* **Note 11**). Transfer into a 15 mL conical tube on ice.
6. Dialyze twice against 2 L buffer D at 4 °C for 1.5 h each time in 8,000–10,000 molecular weight cutoff dialysis membrane (*see* **Note 12**).
7. Aliquot 75–100 μL of dialyzed extract into chilled microcentrifuge tubes and snap freeze in liquid nitrogen (*see* **Note 13**). Store at –80 °C (*see* **Note 14**).

3.3 Preparation of Radiolabeled In Vitro-Transcribed Actin Pre-mRNA

In order to follow the splicing reaction, radiolabel an appropriate pre-mRNA substrate. Any RNA containing an intron and at least 50 nt of flanking exonic sequences should work. A standard substrate used in yeast splicing assays is a 590 nt segment of actin pre-mRNA downstream of a T7 promoter.

1. Prepare the T7 actin plasmid (pJPS149; Vijayraghavan 1986 [28]) template for run-off in vitro transcription by linearizing 10 μg in a reaction containing 1× restriction enzyme buffer, 0.1 μg/μL BSA, and 20 U HindIII (*see* **Note 15**). Incubate the reaction for 1 h at 37 °C. Run the linearized plasmid on a 0.8 % agarose gel and purify using a gel purification kit, as described by the manufacturer. Quantify the template by reading the A_{260} on a spectrophotometer.
2. In vitro transcribe radiolabeled actin pre-mRNA. Combine, in order, at room temperature: 1 μL 10× T7 RNA polymerase buffer, 0.5 μL 10 mM ATP, CTP, and UTP, 0.5 μL 0.5 mM GTP, 0.5 μL Superasin RNAse inhibitor, 500 ng linearized pJPS149, 2.5 μL α-^{32}P-GTP, 0.5 μL T7 RNA polymerase, and dH_2O to 10 μL (*see* **Notes 16**, **17**). Incubate the transcription reaction for 1.5 h at 37 °C.

3. Remove unincorporated nucleotides from the transcription reaction and quantify the efficiency of incorporation of α-^{32}P-GTP. Dilute the reaction to 50 μL with TE, pH 7.5. Count 1 μL of the diluted reaction in a scintillation counter. Prepare a G25 spin column according to the manufacturer's instructions. Apply the rest of the diluted reaction to the column and elute. Count 1 μL of the eluate in a scintillation counter. Calculate the percent incorporation of α-^{32}P-GTP into the actin transcript and determine the cpm/fmol (*see* **Note 18**). Dilute the actin transcript to 4 fmol/μL in TE, pH 7.5.

3.4 Testing the Extract for Splicing Activity

1. Assemble two splicing reactions on ice by combining 1.4 μL dH_2O, 1 μL 25 mM $MgCl_2$, 0.6 μL 1 M KPO_4, pH 7.0, 1 μL 30 % PEG, 4 μL precleared splicing extract (*see* **Note 19**), 1 μL 100 mM ATP (*see* **Note 20**), and 1 μL radiolabeled actin transcript (4 fmol).
2. Immediately add 200 μL stop solution to one tube and place the reaction on ice. This is a zero-minute negative control reaction to ensure that the actin pre-mRNA is intact. Incubate the second reaction at 30 °C for 30 min to allow splicing to occur, and then add 200 μL stop solution.
3. Phenol/chloroform extract the RNA from both reactions. Add 200 μL phenol/chloroform and invert the tubes 5 times. Incubate at 65 °C for 5 min and then spin the tubes in a microcentrifuge for 5 min at maximum speed. Pipet 170 μL of the aqueous (top layer) phase into a clean microcentrifuge tube and back extract by adding 150 μL chloroform. Invert the tubes 5 times and then spin for 3 min at maximum speed. Remove the chloroform (bottom layer) with a P200 pipetter set to 200 μL. Discard.
4. Ethanol precipitate the RNA. Add 800 μL of cold (−20 °C) 100 % ethanol to each tube, invert 5 times, and then spin for 30 min at maximum speed in a microcentrifuge, at 4 °C. Remove the ethanol with a pipet and add 170 μL of cold (−20 °C) 70 % ethanol. Spin for 3–5 min at maximum speed and then remove all of the ethanol with a pipet. Air-dry the pellet for about 5 min and then resuspend it in 8 μL of 7 M urea loading dye.
5. Resolve the splicing reaction products in a 6 % acrylamide (7 M urea) denaturing gel (*see* **Note 21**). Heat the samples for 3 min at 65 °C and load onto the gel. Run at 400 V for 1 h in 1× TBE. Expose the gel to a phosphorimager screen at −80 °C and visualize the autoradiograph.
6. Quantify the pre-mRNA, splicing intermediates, and products by densitometry and determine the splicing efficiency (*see* Fig. 1, **Note 22**).

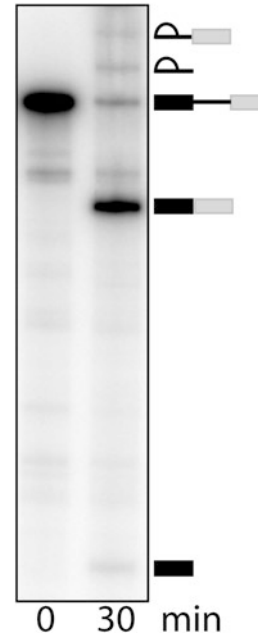

Fig. 1 Testing the activity of splicing extract. Four femtomoles of radiolabeled actin pre-mRNA were incubated in splicing extract and the products of the splicing reaction were separated by electrophoresis in a 6 % polyacrylamide (7 M urea) denaturing gel. The *left lane* is a zero time point showing the location of the pre-mRNA substrate, and the *right lane* shows the splicing products after a 30 min incubation at 30 °C. The identity of the bands is indicated to the right of the gel as follows (*top* to *bottom*): lariat-exon intermediate, lariat intron, pre-mRNA, mRNA, and 5′ exon. Products of the first chemical step of splicing are the free 5′ exon and lariat-exon intermediate, and the final splicing products are mature mRNA and excised lariat intron

4 Notes

1. A minimum of 2 L of culture is recommended in order to prepare a clean extract using this protocol.
2. Streak the yeast strain BJ2168 from a glycerol stock onto a YPD plate and incubate at 30 °C until the colonies have grown to a suitable size (~2 days). Late in the day, inoculate a 2 mL YPD culture with a single colony and grow the culture overnight at 30 °C with shaking (200 rpm). Inoculate 100 mL of YPD with the entire 2 mL culture early the next morning and continue growing. If you do not know the doubling time of your yeast strain, measure the OD_{600} several times during the day to determine it (wait about 3 h before taking measurements to get through the lag phase). The doubling time for BJ2168 is 1.8 h. Later that day, while the culture is still in log

phase, inoculate 4 × 1 L YPD from the 100 mL culture so that the cultures will reach an OD_{600} ~ 2.0–2.5 the next morning. There should be no lag phase as the inoculum is in log phase.

3. Combine 2 pellets so cells are now in two 1 L bottles. Do not transfer to smaller tubes, as the large pellets become much more difficult to resuspend when compacted.
4. If your yeast strain is not protease deficient, add protease inhibitors (e.g., 1 mM AEBSF, 1 mM benzamidine, 1 μg/mL leupeptin, 1 μg/mL aprotinin, or your favorite premade inhibitor cocktail).
5. Remove the needle to fill the syringe with cells. Constantly move the syringe over the beaker while dripping to prevent the drops from aggregating. Be careful not to get the needle too close to the liquid nitrogen, or the cells will freeze in the tip and block it.
6. Poke a hole in the cap of the tube so that the gas from the liquid nitrogen can escape. A nail heated in a Bunsen burner will pierce the cap easily.
7. Cell drops can be stored indefinitely at –80 °C. Alternatively, drip the cell suspension directly into the chilled mortar containing liquid nitrogen and proceed with preparation of the extract.
8. Wear freezer gloves to protect your hands. Grinding is substantially easier if you fashion a holder for the mortar out of a thick piece of Styrofoam (e.g., the lid of a Styrofoam shipping container). Carve out a hollow that snuggly fits the mortar. Line it with foil so that bits of Styrofoam do not get into the extract.
9. Grind slowly at first to prevent the cell drops from popping out of the mortar. Once the cell drops are crushed (5–10 min), grind more vigorously. Grind for about 10 min after the powder is the texture of talcum powder (i.e., very fine—it should feel smooth, not granular, at this stage). Note that excessive grinding will reduce the activity of the extract.
10. Place some ice in the bottom of a 2 L beaker. Add a little water to make a slurry. Add about 1 g of NaCl to lower the temperature of the bath. Nestle the beaker containing the cells into the center, pressing it down to the bottom. The ice should come just part way up the 250 mL beaker so that it does not float.
11. This is the critical step in extract preparation. Be conservative in the amount of supernatant you remove, scrupulously avoiding the pellet and the floating lipids. Although you will end up with less extract, it will be more active and less likely to get hung up in the wells of the splicing gel. Remember: less is more.

12. Buffer D can be reused up to ten times without affecting the quality of the extract. Mark the 2 beakers and always use the same beaker for the first dialysis. Store at 4 °C. Add fresh DTT each time.
13. The protein concentration in the extract should be 15–30 mg/mL, as measured by Bradford.
14. Extract is good for at least a year, probably longer. It can be thawed and refrozen one time without significant loss in activity.
15. We have experienced problems with the pJPS149 plasmid when purified from a glycerol stock of DH5α. Consequently, always freshly transform DH5α cells from the original plasmid stock when more plasmid is needed.
16. The spermidine in the transcription buffer will precipitate nucleic acids at 4 °C or colder, so keep the transcription reagents on ice but assemble the reaction at room temperature.
17. α-^{32}P-UTP can be substituted for the α-^{32}P-GTP. Make up the stocks of ribonucleotides to correspond with the change in the radionucleotide.
18. Calculation for cpm/fmole of actin:

$$\begin{aligned} Total\,moles\,of\,GTP &= Hot\,GTP + Cold\,GTP \\ &= \left(0.835\times10^{-8}\,mmol\right) + \left(2.5\times10^{-7}\,mmol\right) \\ &= 2.58\times10^{-7}\,mmol \end{aligned}$$

$$\%\,\text{incorporation of}\,^{32}\text{P} = \frac{\text{Scintillation count before G 25}}{\text{Scintillation count after G 25}}\times100$$

$$\text{Total m moles of GTP incorporated} = 2.58\times10^{-7}\ \text{mmol}\times\%\,\text{incorporation}$$

$$\text{Moles of full length actin} = \text{Total moles of GTP incorporated}\times\left(1\ \text{mol}/\,1{,}000\ \text{mmol}\right)\times\left(1\ \text{actin}/\,118\ \text{Gs}\right)$$

$$\text{cpm}/\,\text{fmol} = \frac{\left(\text{Scintillation count after G 25}\times50\,\text{mL}\right)}{\text{Moles of full length actin}}\times\frac{1\,\text{mol}}{1.0\times10^{15}\,\text{fmol}}$$

$$\text{fmol}/\,\mu\text{L} = \frac{\text{Scintillation count after G 25}}{\text{cpm}/\,\text{fmole}}$$

19. Thaw all splicing reagents on ice. Once the splicing extract has thawed, spin at maximum speed for 5 minutes in a microcentrifuge. Transfer the cleared extract to a fresh tube on ice.

20. The standard splicing assays in the literature contain 2 mM ATP. However, we find that the splicing efficiency is greatly enhanced with 10 mM ATP.
21. Assemble the gel plate, gasket, and spacers. Dissolve 6.3 g of ultrapure urea in about 6 mL of dH_2O. Add 750 μL 20× TBE, 150 μL 10 % APS, 15 μL of TEMED, and dH_2O to 15 mL. Apply the mixture between the gel plates using a disposable 10 mL pipet and then insert the comb. Allow the gel to polymerize for at least 45 min (can be made the day before, wrapped in plastic with a wet paper towel over the comb, and stored at 4 °C). Remove gasket and comb and place in gel box with 1× TBE. Blow out urea and air bubbles from the wells of the gel using a syringe and needle filled with buffer. Make sure there are no air bubbles trapped under the gel. Pre-run the gel at 400 V for 15–30 min. Blow out the wells again before loading samples.
22.

$$\text{Splicing efficiency} = \frac{(\text{mRNA} + \text{lariat intron})}{(\text{Pre-mRNA} + \text{lariat-3}' \text{ exon} + 5' \text{ exon} + \text{lariat intron} + \text{mRNA})} \times 100$$

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