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Chapter 11

In Vitro Assay of Pre-mRNA Splicing in Mammalian Nuclear Extract

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Abstract

The in vitro splicing assay is a valuable technique that can be used to study the mechanism and machinery involved in the splicing process. The ability to investigate various aspects of splicing and alternative splicing appears to be endless due to the flexibility of this assay. Here, we describe the tools and techniques necessary to carry out an in vitro splicing assay. Through the use of radiolabeled pre-mRNA and crude nuclear extract, spliced mRNAs can be purified and visualized by autoradiography for downstream analysis.

Key words In vitro splicing, Alternative splicing, Splicing analysis, Pre-mRNA substrate, HeLa cell nuclear extract, In vitro transcription, RNA extraction and purification

1 Introduction

The ability to study biochemical changes associated with pre-mRNA splicing in a cell-free-based assay, also referred to as the in vitro splicing assay, has vastly improved our understanding of this complex, key process of gene expression. Not only has it improved our knowledge of the mechanisms and necessary components involved in splicing, but it has also allowed insights into the regulation of alternative splicing as it is mediated by *cis*-acting elements and *trans*-acting factors.

The ease of use, flexibility, and rapid results provided by an in vitro splicing system allows for tailored investigations into various aspects of the splicing reaction. The major benefit, however, lies with the ability to biochemically manipulate the splicing reaction through utilizing two key components: (1) minigene constructs and (2) mammalian crude nuclear extracts. The use of minigene constructs is a common in vitro technique that employs genomic segments from a gene (introns and exons) that include alternatively spliced regions within flanking genomic regions that are cloned

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downstream of efficient promoters. These minigene constructs allow for identification of specific features that control intron and exon usage as well as the characterization of *cis*-acting elements and *trans*-acting factors that interact and modulate regulatory elements necessary for splicing regulation [1]. Crude nuclear extract is another important component of the in vitro splicing reaction that is usually generated from HeLa cells. Importantly, these nuclear extracts contain the necessary proteins and snRNAs for an efficient splicing reaction (*see* Chapter 8). The advantage associated with in vitro biochemical manipulation allows for insights into various factors and processes. These include, but are not limited to, protein regulatory elements and composition, splice site recognition and selection, the influence of RNA elements and their *trans*-acting factors, the characterization of enhancer and silencer elements, and kinetic insights into the splicing pathway. As with all in vitro-based systems, the assay does come with limitations. The rate of intron removal in vitro is slower than rates determined in vivo [2]. The efficiency of in vitro transcription of pre-mRNA, its purification, and subsequent splicing is restricted by the size of the RNA to be used; RNA should be less than 2,000bp [3]. Because of this, the in vitro splicing assay relies heavily on the use of shorter minigenes that are only a subset of a larger gene. The assay also does not take into account the effects of other events associated with splicing, such as transcription, capping, and polyadenylation.

Methods for in vitro splicing reactions have previously been described [4–7]. In general, these protocols employ the use of radiolabeled pre-mRNAs that are incubated for several hours in nuclear extract supplemented with necessary salts and cofactors. The mRNA is then extracted and purified from the nuclear extract, subjected to denaturation on a polyacrylamide gel, and subsequently dried for visualization by autoradiography via film or phosphor imaging. The pre-mRNA, mRNA, and other intermediates are then identified as bands on the autoradiograph.

2 Materials

All reagents should be high quality, molecular biology grade, and RNase-free. Stock solutions should be stored at 4 °C (unless otherwise indicated). Certain reagents can be substituted for their equivalents from other manufacturers or as otherwise stated. The concentrations of chemicals/reagents listed in the materials are stock concentrations, not final concentrations. Since all steps require working with radioactive isotopes, all necessary precautions must be taken. Carefully follow all hazardous and radioactive waste disposal regulations when disposing of waste materials.

2.1 Splicing Reaction Components

1. Radiolabeled pre-mRNA: generated from an in vitro transcription reaction (*see* **Note 1**).
2. Splicing competent nuclear extract (NE) (*see* Chapter 8).
3. 1 mM adenosine triphosphate (ATP). Store at −20 °C.
4. 0.5 M creatine phosphate (CP). Store at −20 °C.
5. 80 mM magnesium acetate ($Mg(OAc)_2$) (*see* **Note 2**).
6. RNase inhibitor (40 U/μl). Store at −20 °C.
7. 100 mM dithiothreitol (DTT). Store at −20 °C.
8. 1 M potassium acetate (KOAc) (*see* **Note 3**).
9. 0.5 M HEPES buffer, pH 7.9 (*see* **Note 3**).
10. 13 % polyvinyl alcohol (PVA): optional (*see* **Note 4**).
11. Wet ice and dry ice (finely ground or small chunks).
12. Water bath.

2.2 6 % Splicing Gel Components

1. Tris-Borate-EDTA (TBE) buffer: 89 mM Tris Base, 89 mM boric acid, 2 mM EDTA.
2. 7 M urea.
3. 40 % (19:1) acrylamide:bis-acrylamide solution: acrylamide is dissolved in 1× TBE/7 M urea.
4. *N,N,N′,N′*-Tetramethylethylenediamine (TEMED).
5. 10 % Ammonium persulfate (APS).
6. Formamide/EDTA stop dye: formamide with 0.1 % bromophenol blue and 0.1 % xylene cyanol and 2 mM EDTA.
7. Radiolabeled RNA ladder/molecular marker.
8. Electrophoresis glass plates: 8″ × 8″ (two): one glass plate should notch to allow for the addition of a comb.
9. 0.4 mm gel plate spacers (three).
10. 0.4 mm comb (same thickness as the spacers).
11. 1¼″ binder clips (four).
12. Aluminum plate: 8″ × 8″ or longer and precooled (*see* **Note 5**).
13. Silicon Gel Slick® Solution (Lonza Rockland) or equivalent.
14. 70 % ethanol.
15. 30–50 ml syringe, with and without a needle (two).
16. Flat gel loading tips.
17. Putty knife/gel spatula.
18. Vertical gel electrophoresis system.
19. Whatman paper, cut into an 8″ × 8″ square.
20. Plastic wrap (such as Saran™ Wrap).
21. Power pack for an electrophoresis system with a temperature probe.

22. Bio-Rad Gel Dryer or equivalent.
23. Bio-Rad Personal Molecular PhosphorImager System or similar. Film may also be used.

2.3 Splicing Digest and RNA Purification

1. Proteinase K 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

Carry out all steps of the reaction on ice unless otherwise stated.

3.1 Splicing Reaction

1. Thaw NE on ice.
2. Thaw ATP, CP, $Mg(OAc)_2$, DTT, HEPES, KOAc, and radiolabeled RNA at room temperature. Once thawed, place them immediately on ice. RNase inhibitor should be kept on ice.
3. Determine the Master Mix reaction volume and reaction size (*see* **Note 6**):
   (a) (# of reactions) + 1 = Master Mix reaction size.
   (b) Reaction volume: 12.5 μl or 25 μl reaction volume total.
4. Mix reagents to a final concentration of 1 mM ATP, 20 mM CP, 3.2 mM $Mg(OAc)_2$, 10 U RNase inhibitor, 1 mM DTT, 10–50 % NE (should be optimized for each extract and substrate used), 72.5 mM KOAc, 12 mM HEPES (*see* **Note 3**), and 3 % PVA (optional). Use sterile water to bring up the Master Mix volume if needed.
5. For each experimental reaction condition: add the appropriate Master Mix volume, 0.01–0.1 nM RNA (~1,000 cpm) (*see* **Note 7**), experimental variant (i.e., protein), and/or sterile water to bring up the volume. Add NE last and pipet carefully to mix (*see* **Note 8**). Keep all reaction tubes on ice. Prepare a time 0 tube as control and immediately place on dry ice after addition of NE (*see* **Note 9**).
6. Incubate all reactions, except the time 0 reaction, at 30 °C water bath for 90 min (*see* **Note 10**).
7. While the splicing reactions are running, prepare the 6 % acrylamide gel.
8. Once the incubation time is complete, immediately place the tubes on dry ice to stop the reactions (*see* **Note 11**).

3.2 Splicing Gel Preparation

1. Prepare a 20 % acrylamide:bis solution: dilute 40 % (19:1) acrylamide:bis-acrylamide solution in 1× TBE with 7 M urea.

2. Prepare a 6 % polyacrylamide mixture from the 20 % acrylamide solution: in a 50 ml conical tube, dilute the 20 % acrylamide solution with the desired amount of 1× TBE/7 M urea buffer to obtain a mixture at the required percentage.
3. Carefully clean the inside face of a siliconized plate (*see* **Note 12**) with 70 % ethanol. Wipe dry with lint-free paper towels.
4. Carefully clean the non-siliconized plate using water and 70 % ethanol. Make sure the gel plates are completely clean, with no small pieces of debris present (*see* **Note 13**). Wipe dry with lint-free paper towels.
5. Place the spacers around the outside edge (bottoms and sides) of a non-siliconized plate. Lay the siliconized notched plate on top and clip the glass plates together using binder clips.
6. Once the gel cassette is ready, add the appropriate amount of 10 % APS and TEMED (*see* **Note 14**) to 20 ml of 6 % acrylamide and mix gently.
7. Using a syringe (without needle), aspirate the acrylamide and gently dispense the mixture between the plates. Once the cassette is filled, lay it flat, place a gel comb with an appropriate well size into the top of the gel, and allow the gel to set at room temperature for approximately 30 min (or until polymerized) (*see* **Note 15**).
8. Pre-run the gel before adding your samples (*see* **Note 16**): clamp the gel cassette onto the vertical gel electrophoresis apparatus, fill the chambers with 1× TBE (*see* **Note 17**), and run the gel at 30 W (100 V), 45 °C, for 15 min.

3.3 Digest

1. Once the last in vitro splicing tube has been placed on dry ice, prepare the Proteinase K digest mix:
   (a) Determine the desired final volume of the Proteinase K Master Mix: reaction volume (µl) × (# of reactions + 1) = Master Mix volume (µl).
   (b) Proteinase K Master Mix: final concentration of 1× Proteinase K buffer, 0.25 mg/ml glycogen, 0.25 mg/ml Proteinase K, and sterile water, for a final volume of 180 µl per reaction.
2. Add 175 µl of Proteinase K Master Mix (*see* **Note 18**) to each reaction tube and incubate at 37 °C for 10–15 min.

3.4 RNA Purification and Precipitation

1. Once the Proteinase K digest has completed, purify the RNA by adding 200 µl of phenol/chloroform, vortex for 30 s, and spin at 16,500 × *g* for 5 min to separate the aqueous and organic layers.
2. To precipitate the RNA: remove the aqueous (top) phase and place into a separate tube (~200 µl). Add 2.5 times the volume of 100 % ice-cold ethanol (for 200 µl of top phase, add 500 µl of ethanol). Incubate at −20 °C or −80 °C for 10–15 min.

3. Centrifuge the tubes at 16,500 × *g* for 10 min at room temperature to pellet.
4. Remove the ethanol supernatant and allow the pellet to air-dry for no more than 5 min (*see* **Note 19**). Resuspend the pellet in a small amount of stop dye within 5–10 min (10 μl or less). Pipet up and down and vortex for 30 s to mix.

3.5 Visualization of Splicing Reaction

1. Load RNA samples onto the pre-run gel. Clamp an aluminum plate to the front glass plate (*see* **Note 20**). Run the gel at 30 W (100 V), 45 °C, for 90 min or until the dye runs off the gel.
2. Remove the gel cassette from the apparatus and dispose of the buffers in appropriate waste containers. Split the plates apart with a putty knife/spatula. The gel should remain attached to the non-siliconized plate.
3. Center the pre-cut Whatman paper on top of the gel and press gently to allow the gel to adhere evenly to the paper. Carefully peel the Whatman paper upward at an angle to allow for the gel to be peeled away from the glass. Cover the gel with plastic wrap, minimizing the presence of any creases (*see* **Note 21**).
4. Dry the gel for 15–20 min using a Bio-Rad Gel Dryer at 80 °C with suction.
5. Expose the gel to film or preferably a phosphor imaging screen (*see* **Note 22**) or similar equipment for the recommended length of time (generally at least 3 h to overnight; *see* **Note 23**).
6. Once the gel has been exposed and imaged, the appearance of spliced product can be used to determine the amount of RNA spliced (% spliced) in each lane (Fig. 1), which can in turn be used to calculate the efficiency of product appearance (*see* Chapter 12 and **Note 24**). Use a suitable computer program to analyze the digital quantitation file (*see* **Note 25**).

4 Notes

1. Generally, for in vitro splicing reactions, DNA is transcribed using T7 polymerase in a reaction containing radiolabeled nucleotides, phosphorus-32 (^{32}P) α-UTP. This reagent is usually in the 0.3–3 nM range, with an incorporation of around 100,000 cpm/μl.
2. Magnesium chloride can also be used; however, chloride has in some cases been shown to inhibit in vitro splicing reactions [8].
3. Potassium chloride or potassium glutamate may also be used although chloride has been shown to inhibit in vitro splicing reactions [8]. KOAc is used in this reaction because the nuclear extract has been prepared in KOAc (*see* Chapter 8). The final volume of KOAc to add to the Master Mix will depend on

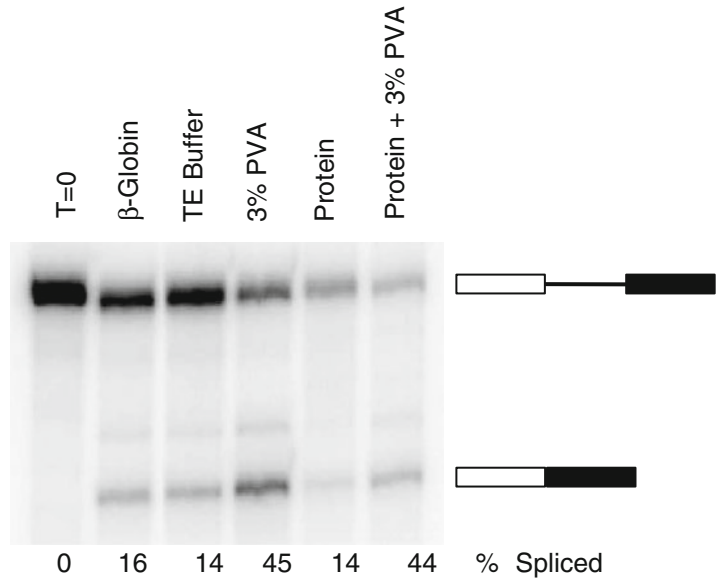

Fig. 1 Autoradiogram of radiolabeled β-globin minigene construct (from *left* to *right*) at time 0, alone, with TE buffer, with 3 % PVA, with 1 μM protein X, with 1 μM protein X with 3 % PVA, run on a 6 % polyacrylamide gel. Analysis of % spliced is performed using Bio-Rad Quantity One (*see* **Note 25**)

how many ions are present in the nuclear extract to begin with. The final volume of HEPES to add will also depend on how many ions are present in the nuclear extract.

4. Addition of PVA is optional but has been shown to potentially increase splicing efficiency in certain reactions [9].
5. Monitor the temperature of the gel using a temperature probe connected to the power pack. It is highly recommended to place a precooled aluminum plate aluminum plate on the front surface the front surface of the gel cassette to keep the cassette cool and prevent it from shattering as well as evenly distribute heat (*see* **Note 19** as well).
6. The extra reaction is to account for pipetting errors. When determining the reaction volume (12.5 μl or 25 μl reaction), consider how many reactions are needed, how much radiolabeled pre-RNA is present, and how radioactive the radiolabeled pre-mRNA is. If the radiolabeled pre-RNA is less than 4,000 cpm/μl, a 12.5 μl reaction may be appropriate with the addition of more pre-mRNA.
7. It is possible to add radiolabeled pre-mRNA to the Master Mix, rather than adding it separately.
8. When adding NE, make sure to prevent any air bubbles from forming. Mix gently by pipetting up and down, and *do not vortex*. Excessive bubbles may reduce splicing efficiency.

9. A time 0 tube should be prepared as a control. Once NE is added to the reaction tube, immediately place the tube on dry ice to prevent the splicing reaction from starting. This time 0 control treatment will be used to adjust for background intensity associated with un-spliced product for all the reactions.
10. The optimal temperature for cleavage at the 5′ splice site is 30 °C [10].
11. The splicing complexes formed on the pre-mRNA will not survive a dry ice freeze/thaw cycle. Therefore, only place the reaction on dry ice if the reaction will not be used to visualize native gel complex formation or for other downstream analyses. In the case of this protocol, only the spliced radiolabeled mRNA products are to be visualized. Therefore, destroying the spliceosomal complexes is not an issue.
12. Coating one of the plates with silicon is not required but highly recommended. A siliconized gel plate allows for easier separation when separating the glass plates. The gel will almost always stick to the uncoated plate, instead of partially sticking to both. Preferably, the notched plate should be siliconized.
13. Both the siliconized and non-siliconized plates should be free of any sort of particles and debris. Make sure to wipe away any debris, as they will form tiny air pockets between the glass plates that will cause leakage when pouring the gel.
14. Altering the amount of APS and TEMED can have different effects on gel polymerization and on how the samples run on the gel [11–13]. Generally a 1:150 dilution of 10 % APS and 1:1,000 dilution of TEMED are used.
15. Avoid the formation of bubbles while making the gel. Hold the clipped gel cassette (with notched plate facing upward) in one hand at a 45° angle, tilted on its corner. Slowly dispense the acrylamide solution. If an air bubble is present, adjust the angle of the cassette to allow the solution to force the air bubble outward. Add the comb immediately before the gel solution has time to harden.
16. Pre-running the gel before adding samples can remove all traces of (APS) and will apply a constant temperature to the gel before use [14].
17. Immediately before loading samples, make sure to flush out the wells with buffer to remove any urea that has leached and deposited into the wells.
18. The spliced RNA solution will be frozen when adding the Proteinase K Master Mix. Pipet the Master Mix up and down slowly in the reaction tube to thaw the spliced RNA. *do not vortex.*
19. Keep track of the orientation of the tubes while centrifuging; the pellet will be very hard to see and sometimes invisible.

If a pellet is not visible, continue to add the stop dye and load samples onto the gel (it is most likely there as the dye will stick to the pellet).

20. As mentioned previously, to prevent the glass plates from cracking and to ensure even conduction of heat, clamp a pre-cooled aluminum plate to the front glass plate using the same binder clips used to hold the gel cassette in place. Make sure the aluminum plate is positioned so that it does not touch any buffer in the lower chamber. Run the gel for an appropriate amount of time; this will differ depending on the splicing products of your reaction and the percent/mix of the gel poured.
21. Make sure there are no creases in the plastic wrap. Remove any extra overhanging plastic wrap using a razor, being careful not to slice the gel. Any extra plastic wrap will bulge and may prevent the gel from being flush with the phosphor imaging screen or film.
22. The PhosphorImager screen is a form of autoradiography that is used to visualize and detect radioactive emission from radiolabeled RNA. Phosphor imaging screens contain BaFBR:Eu^{2+} crystals. When these crystals are exposed to ionizing radiation from radiolabeled RNA, electrons from Eu^{2+} become excited resulting in subsequent oxidation. During screening, the oxidized electrons revert back releasing a photon that can then be detected at certain wavelengths via a photomultiplier system producing a quantitative image [15]. There are many advantages to this method over other methods such as film. These advantages include increased sensitivity over a linear detection range of 5 orders of magnitude, while exposure to film is limited to only 1.5 orders of magnitude, increased exposure time from 10 to 250 times faster than film, easier and faster quantitation of images, and reuse of the phosphor screens indefinitely [16]. Other molecular detection systems similar to the Bio-Rad Molecular Imager are also available.
23. If the radiolabeled pre-mRNA used for the splicing reaction is around 8,000 cpm/μl, 1 h exposure to the PhosphorImager screen or film is sufficient to observe most splicing; however, longer exposures are often needed to see all splicing products or intermediates.
24. Due to the differential rates of decay among some splicing products, not all bands may be suitable for quantification. Depending on the in vitro reaction, lariat formation may be more stable than certain products and can be used as a substitute for calculating % spliced [17]. In addition, certain products may form which will not necessarily be stable in the cell (such as single exons). These RNAs will be degraded in the cell but may persist in an in vitro reaction.

25. % spliced product is obtained by calculating the volume intensity from the digital image of the splicing gel for each band in each lane. Briefly, calculate the sum of the adjusted intensity (taking into account the background of the gel and time 0 reaction) for the spliced and un-spliced band, divide the signal for spliced product by the total signal in the lane, and take the percent:

$$\%\ \text{Spliced} = \frac{\text{Signal from final spliced product}}{\text{Total signal in lane}} \times 100$$

(Total signal in lane = spliced product + un-spliced product)

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