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TITLE:

Complementation of Splicing Activity by a Galectin-3 – U1 snRNP Complex on Beads

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KEYWORDS:

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SUMMARY:

This article describes the experimental procedures for (a) depletion of U1 snRNP from nuclear extracts, with concomitant loss of splicing activity; and (b) reconstitution of splicing activity in the U1-depleted extract by galectin-3 – U1 snRNP particles bound to beads covalently coupled with anti-galectin-3 antibodies.

ABSTRACT:

Classic depletion-reconstitution experiments indicate that galectin-3 is a required splicing factor in nuclear extracts. The mechanism of incorporation of galectin-3 into the splicing pathway is addressed in this paper. Sedimentation of HeLa cell nuclear extracts on 12%–32% glycerol gradients yields fractions enriched in an endogenous ~10S particle that contains galectin-3 and U1 snRNP. We now describe a protocol to deplete nuclear extracts of U1 snRNP with concomitant loss of splicing activity. Splicing activity in the U1-depleted extract can be reconstituted by the galectin-3 – U1 snRNP particle trapped on agarose beads covalently coupled with anti-galectin-3 antibodies. The results indicate that the galectin-3 – U1 snRNP – pre-mRNA ternary complex is a functional E complex leading to intermediates and products of the splicing reaction and that galectin-3 enters the splicing pathway through its association with U1-snRNP. The scheme of using complexes affinity- or immuno-selected on beads to reconstitute splicing activity in extracts depleted of a specific splicing factor may be generally applicable to other systems.

INTRODUCTION:

Production of most eukaryotic messenger RNAs (mRNAs) involves removal of introns and ligation of exons in a nuclear process termed pre-mRNA splicing¹. Two classes of RNA-protein complexes (RNPs) direct the processing of pre-messenger RNA into mature mRNA via spliceosomal complexes. One class, nascent pre-messenger RNPs, is formed co-transcriptionally by the binding of heterogeneous nuclear RNP proteins and other RNA-binding proteins, including some members of the SR family, yielding hnRNP complexes². The second class, uracil-rich small nuclear RNPs (U snRNPs with U1, U2, U4, U5, and U6 snRNAs) is associated with U-specific and core proteins^{3,4}. The U snRNPs interact in an ordered fashion with specific regions of pre-messenger RNPs in a dynamic remodeling pathway as introns are excised and exons are ligated to produce mature mRNPs⁵. Many additional nuclear proteins participate in these processing events⁶.

Galectin-1 (Gal1) and galectin-3 (Gal3) are two proteins that are required factors in the splicing pathway as shown by depletion-reconstitution studies^{7,8}. Removal of both galectins from splicing competent nuclear extracts (NE) abolishes spliceosome assembly and splicing activity at an early step. Addition of either galectin to such a doubly depleted NE restores both activities. Gal1 and Gal3 are components of active spliceosomes as evidenced by specific immunoprecipitation of pre-mRNA, splicing intermediates, and mature mRNA by antiserum specific for either Gal1 or Gal3⁹. Importantly, Gal3 associates with endogenous U-snRNA containing particles in the NE outside the splicing pathway as shown by precipitation of snRNPs by anti-Gal3 antisera¹⁰. Finally, silencing of Gal3 in HeLa cells alters splicing patterns of numerous genes¹¹.

In NE pre-incubated to disassemble preformed spliceosomes¹², snRNPs are found in multiple complexes sedimenting in glycerol gradients from 7S to greater than 60S. Although glycerol gradient fractionation is a common technique for the isolation of spliceosomal complexes and components (see references¹³⁻¹⁵ for example), we have extended this method by characterizing specific fractions using antibody immunoprecipitations. An snRNP sedimenting at 10S contains only U1 snRNA along with Gal3. Immunoprecipitation of the 10S fraction with antisera specific for Gal3 or U1 snRNP co-precipitates both U1 and Gal3 indicating some of the U1 snRNP monoparticles are bound to Gal3¹⁰. As U1 snRNP is the first complex that binds to pre-mRNP in spliceosomal assembly^{1,5}, this step represents a potential entry site for Gal3 into the splicing pathway. On this basis, we showed that 10S Gal3-U1 snRNP monoparticles bound to anti-Gal3 containing beads restored splicing activity to a U1-snRNP depleted NE, establishing this complex as one mechanism by which Gal3 is recruited into the spliceosomal pathway¹⁶. This contrasts with attempts to isolate spliceosomes at specific stages in the splicing reaction and cataloging the associated factors^{17,18}. In such studies, the presence of certain factors at some time point is ascertained but not the mechanism by which they were loaded.

We had previously described in detail the preparation of NE, the splicing substrate, the assembly of the splicing reaction mixture, and the analysis of products in our documentation of the role of galectins in pre-mRNA splicing¹⁹. We now describe the experimental procedures for fractionation of nuclear extracts to obtain a fraction enriched in Gal3 – U1 snRNP complex and for immunoselection of the latter complex to reconstitute splicing activity in a U1-depleted nuclear extract.

[Place **Figure 1** here]

PROTOCOL:

1. Notes on general procedures

1.1. Ensure that all chemicals (buffer components, enzymes, etc.) are kept free of ribonuclease (RNase). Sequester all commercially purchased reagent bottles from general lab use. Wear gloves for all steps of the experimental procedure. Use only glassware and utensils that have been baked (see step 1.2 below) and solutions that have been pre-treated (see step 1.3 below).

1.2. Bake all glassware (beakers, flasks, bottles, pipettes, etc.) for a minimum of 4 h at 177 °C. Wrap other utensils (spatulas, stir-bars, etc.) in aluminum foil before baking under the same conditions.

1.3. Prepare a 0.1% (vol/vol) solution of diethylpyrocarbonate (DEPC) in double-distilled water (ddH₂O). Using a magnetic stir-bar, stir this solution overnight and then autoclave. Use this DEPC-treated H₂O to make all solutions containing Tris; then, filter sterilize using a bottle top vacuum filter. Use regular ddH₂O to prepare all other solutions (without Tris); now, treat with DEPC (0.1%, vol/vol) and autoclave.

NOTE: The buffers used in the following set of experimental procedures are listed alphabetically in **Table 1**.

[Place **Table 1** here]

2. Preparation of NE depleted of U1 snRNP (U1ΔNE)

2.1. Preparation of anti-U1 beads for immunoadsorption

2.1.1. Pre-swell 50 mg Protein A-Sepharose CL-4B beads in excess DEPC-treated H₂O to produce approximately 200 μL of swollen beads and then wash in HEPES wash buffer.

2.1.2. For this wash and all subsequent washes, pellet the beads by centrifugation (1,000 x *g* in a swinging bucket rotor at 4 °C for 10–15 s) and remove the unbound wash using a micropipettor and discard.

2.1.3. Mix 150 μL of washed beads with 150 μL of human autoimmune serum specific for U1 snRNP (volume of antibody to volume of beads in a ratio of 1:1).

2.1.4. Adjust, on the basis of the total volume of (~300 μL from step 2.1.3 above), the mixture to 20 mM HEPES, pH 7.9, corresponding to conditions of HEPES binding buffer; incubate this mixture with continuous rocking at room temperature for 60 min.

2.1.5. Wash the beads bound with antibodies with 1 mL of borate buffer (0.2 M sodium borate, pH 9) and resuspend in 1 mL of the same borate buffer.

2.1.6. To covalently couple the antibody bound to the Protein A-Sepharose beads, add dimethylpimelimidate to a final concentration of 20 mM and incubate at room temperature with rocking for 60 min.

2.1.7. Wash the beads with 1 mL of borate buffer.

2.1.8. To block any unreacted cross-linking reagent, add 1 mL of 0.2 M ethanolamine (pH 8) and incubate at room temperature with rocking for 60 min.

2.1.9. Wash the antibody-coupled beads, hereafter designated as anti-U1 beads, twice with 0.5 mL of TX wash buffer (0.05% Triton X-100 in 60% D).

2.2. Depletion of U1 snRNP from NE (see **Figure 1A**)

NOTE: The procedure for preparing NE from HeLa cells was initially developed by Dignam et al.²⁰. We have described the materials and detailed methods for the preparation of NE for splicing assays¹⁹ (see steps 2.1 and 3.1 of that reference). NE, as initially prepared is in Buffer C and will hereafter be designated as NE(C). NE(C) dialyzed against and equilibrated with Buffer D will be designated as NE(D).

2.2.1. Incubate 200 μ L of NE(C) with 100 μ L of anti-U1 beads from step 2.1.9 above.

2.2.2. Add 5 μ L of RNA inhibitor to the mixture.

2.2.3. Rotate the microtube head-over-tail at 4 °C for 1 h.

2.2.4. Pellet the mixture by centrifugation (1,000 x g in a swinging bucket rotor at 4 °C for 10–15 s) and collect the unbound material (U1 Δ NE) using a Hamilton syringe.

2.2.5. Dialyze the entire volume of U1 Δ NE, along with a separate 50 μ L aliquot of the original, nondepleted NE(C) in separate compartments of a microdialyzer, with stirring, for 75 min against 60% D using a dialysis membrane with 8 K molecular weight cutoff.

2.2.6. Immediately after dialysis, divide these preparations (U1 Δ NE and NE in 60% D) into 20 μ L aliquots; snap freeze in a dry ice/ethanol bath, then store at -80 °C.

2.3. Analysis of the RNA and protein content of U1 Δ NE and material bound on anti-U1 beads

2.3.1. After the removal of the unbound material (U1 Δ NE) (step 2.2.4), wash the material bound to the anti-U1 beads by adding 0.5 mL of TX wash buffer. Pellet the mixture by centrifugation

(1,000 x *g* in a swinging bucket rotor at 4 °C for 10–15 s) and remove the supernatant using a micropipettor and discard.

2.3.2. Repeat the wash steps 2.3.1 twice.

2.3.3. Remove the material bound to the anti-U1 beads by adding 100 µL of 2x SDS sample buffer to 100 µL of the beads and incubating for 10 min at room temperature.

2.3.4. Pellet the mixture by centrifugation (1,000 x *g* in a swinging bucket rotor at 4 °C for 10–15 s); remove the supernatant by Hamilton syringe, and snap freeze in a dry ice/ethanol bath. Store at -80 °C.

2.3.5. Compare the nondepleted NE, the depleted NE (U1ΔNE), and the material bound to the beads (removed from the beads by SDS sample buffer as described in steps 2.3.3 and 2.3.4 above). Follow steps 2.3.6–2.3.8 for RNA analysis or steps 2.3.9–2.3.10 for protein analysis.

2.3.6. For each sample, extract the RNA with 200 µL of phenol-chloroform (50:50, v/v); then extract again with 180 µL of chloroform-isoamyl alcohol (25:1, v/v). Following extraction, add 300 µL of cold 200-proof ethanol, invert to mix, and store the precipitated RNA overnight at -20 °C.

2.3.7. Centrifuge the ethanol-precipitated RNA (12,000 x *g* for 10 min at 4 °C). Wash the pellets with 150 µL of cold 200-proof ethanol. Centrifuge again (12,000 x *g*) at 4 °C for 15 min. Remove the supernatant using a micropipettor and dry the pellets in a speed vac for 10–15 min without heat.

2.3.8. Resuspend the dried RNA pellet in 10 µL of RNA loading buffer, gently vortex, heat to 75–85 °C for 90 s, and then incubate on ice for 2 min. Separate the snRNAs by gel electrophoresis (2 h at 16 mA) through 13% polyacrylamide – 8.3 M urea gels and then either stain with ethidium bromide or subject to northern blotting^{10,16}.

2.3.9. Load the protein samples, in SDS sample buffer from step 2.3.5, onto 12.5% gels and electrophoresis at 200 V for approximately 45–50 min in SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) buffer.

2.3.10. Transfer the separated proteins to nitrocellulose membrane at 400 mA for 2 h in transfer buffer. After transfer, block the membrane by incubating overnight in T-TBS containing 10% non-fat dry milk. Then, immunoblot the membrane to reveal specific proteins^{8,21}.

3. Immunoprecipitation of 10S fractions of glycerol gradients by anti-Gal3

3.1. Preparation of anti-Gal3 beads for immunoadsorption

NOTE: The derivation and characterization of rabbit polyclonal antisera against Gal3 for rabbit #24²¹ and for rabbit #49¹⁰ is described previously.

3.1.1. Use preimmune serum from rabbit #49 as control.

3.1.2. For the preparation of anti-Gal3 beads follow the procedure previously described for the preparation of anti-U1 beads (step 2.1), with the exception that corresponding to step 2.1.3, the ratio of antiserum (e.g., anti-Gal3, #49) to beads is 3:1.

3.1.3. Just before use, wash the antibody-coupled beads, hereafter designated as anti-Gal3 beads twice with 0.5 mL of TX wash buffer. Remove the supernatant, first with a micropipettor to get most of the liquid out and then with a Hamilton syringe to get the liquid out of the beads; discard.

3.2. Immunoprecipitation of glycerol gradient fractions by anti-Gal3 (see **Figure 1B**)

3.2.1. Fractionate NE(D) over a 12%–32% glycerol gradient¹⁰. Combine and mix glycerol gradient fractions 3, 4, and 5 (numbered from the top of the gradient), which are near the 10S region of the gradient.

3.2.2. Prepare two samples, each with 150 μ L aliquot of combined gradient fractions 3–5 (step 3.2.1) and place in 50 μ L of anti-Gal3 beads. Wash as described in step 3.1.3.

3.2.3. In parallel, prepare two samples each with 150 μ L of fraction 1 (containing Gal3 not in complex with U1 snRNP¹⁰; step 3.2.1) and place in 50 μ L of anti-Gal3 beads. Wash as described in step 3.1.3.

3.2.4. As a control, place 150 μ L of 60% D in another microtube of 50 μ L anti-Gal3 beads and wash as described in section 3.1.3.

3.2.4. Mix gently by tapping the tube, then rotate the microtube head-over-tail at 4 °C for 1 h.

3.2.5. Pellet the mixture by gentle centrifugation (1,000 $\times g$ in a swinging bucket rotor at 4 °C for 10–15 s).

3.2.6. Remove the supernatant (unbound material) using a Hamilton syringe. Do not wash the beads and use immediately for the addition of the splicing reactions (section 4.2).

3.3. Analysis of the RNA and protein content in the unbound and bound material from the anti-Gal3 precipitation of 10S gradient fractions

3.3.1. For the analysis of components of the bound and unbound material from anti-Gal3 precipitation of the 10S gradient fractions, collect the unbound material (supernatant after step 3.2.4), transfer into a fresh microtube, and freeze at -20 °C.

3.3.2. Wash the precipitated beads from step 3.2.5 (containing material bound to anti-Gal3) by adding 0.5 mL of TX wash buffer.

3.3.3. Pellet the mixture by gentle centrifugation (1,000 x *g* in a swinging bucket rotor at 4 °C for 10–15 s); remove the supernatant using a micropipettor and discard. Repeat the wash steps twice more.

3.3.4. Add 50 µL of 2X SDS sample buffer to the washed and pelleted anti-Gal3 beads.

3.3.5. Mix the beads gently and incubate for 10 min at room temperature.

3.3.6. Pellet the mixture by gentle centrifugation (1,000 x *g* in a swinging bucket rotor at 4 °C for 10–15 s), collect the supernatant by Hamilton syringe and store in a fresh microtube at -20 °C.

3.3.7. Compare the unbound material (section 3.3.1) and the bound material (step 3.3.6) of the anti-Gal3 precipitation in terms of RNA and protein components, using procedures as described in steps 2.3.6. to 2.3.10, respectively.

4. Assembly of splicing reaction and analysis of products

4.1. Preparation of the splicing substrate

NOTE: The pre-mRNA substrate, designated MINX, contains two exon sequences and one intron sequence from Adenovirus²². The MINX DNA sequence in the plasmid is under the control of T3, T7, or SP6 RNA polymerase promoters. The materials and detailed methods for the linearization of the MINX plasmid DNA with BamHI restriction endonuclease, transcription by SP6 RNA polymerase in the presence of α -³²P[GTP] and the purification of ³²P-labeled MINX for splicing assays are described previously¹⁹ (see steps 2.2 and 3.2 of that reference).

4.1.1. Store the radiolabeled MINX as an ethanol precipitate at -20 °C; use the labeled splicing substrate within 4–6 weeks post transcription.

4.1.2. Just before use, centrifuge the ethanol precipitated ³²P-labeled MINX at 12,000 x *g* for 10 min at 4 °C; remove the supernatant with a micropipettor and discard.

4.1.3. Add 150 µL of 70% ethanol and centrifuge at 12,000 x *g* for 15 min at 4 °C. Discard the supernatant and dry the pellet in speed vac with no heat for 15 min.

4.1.4. Rehydrate the pellet in 50 µL of DEPC water. Spot 2 µL on each of two GF/C filters; immerse the filters in cold 5% trichloroacetic acid (TCA) for 10 min. Rinse with cold 5% TCA, followed by 180-proof ethanol on a vacuum flask. Air dry the filters and subject to scintillation counting in 4 mL of commercially available degreaser.

4.1.5. Dilute ³²P-labeled MINX in 60% D to 10⁴ cpm/µL for the splicing assay.

4.2 Assembly of the splicing reaction (see Figure 1C)

4.2.1. Assemble splicing reactions in a total volume of 24 μL (8 μL U1 ΔNE (from step 2.2.6), 3.5 mM MgCl_2 , 1.5 mM ATP, 20 mM creatine phosphate, 0.5 mM DTT, 20 units RNasin, 4 μL ^{32}P -labeled MINX splicing substrate (10^4 cpm/ μL), 60% D) and add to each tube of beads from section 3.2.5. Assemble an identical set of splicing reactions in a total volume of 24 μL but without U1 ΔNE and add to each tube of beads from step 3.2.6.

4.2.2. Prepare a control splicing reaction in a 12 μL total volume (4 μL NE(D), 3.5 mM MgCl_2 , 1.5 mM ATP, 20 mM creatine phosphate, 0.5 mM DTT, 20 units RNasin, 2 μL ^{32}P -labeled MINX splicing substrate (10^4 cpm/ μL), 60% D).

4.2.3. Mix the tubes gently by tapping and rotate end-over-tail at 30 $^\circ\text{C}$ for 90 min. Pellet the mixture by gentle centrifugation at 1,000 $\times g$ in a swinging bucket rotor at 4 $^\circ\text{C}$ for 10–15 s.

4.2.4. Stop the reaction and elute the proteins off the beads by adding 24 μL of 2x SDS sample buffer to the tubes containing beads, and 12 μL of 2x SDS sample buffer to the control tube containing NE but no beads. Heat the tubes at 100 $^\circ\text{C}$ for 7 min.

4.2.5. Centrifuge the tubes gently at 1,000 $\times g$ in a swinging bucket rotor at 4 $^\circ\text{C}$ for 10–15 s.

4.2.6. Transfer the supernatants (elutions) to fresh microtubes: approximately 48 μL from the bead tubes and 24 μL from the NE control tube.

4.2.7. Add Proteinase K (20 mg/mL) to digest and solubilize the proteins: add 5 μL to the 48 μL elution from beads and add 2.5 μL to the 24 μL NE control.

4.2.8. Incubate the tubes at 37 $^\circ\text{C}$ for 40 min.

4.2.9. Gently centrifuge the tubes at 1,000 $\times g$ in a swinging bucket rotor at 4 $^\circ\text{C}$ for 10–15 s.

4.2.10. Dilute the bead elutions with 39.5 μL TE and 10 μL 3 M sodium acetate. Dilute the NE control with 63.5 μL TE and 10 μL 3 M sodium acetate.

4.2.11. Extract and analyze the RNA as described below (section 4.3).

4.3 Analysis of products of the splicing reaction

4.3.1 Extract the RNAs in each sample with phenol-chloroform, followed by chloroform-isoamyl alcohol; precipitate the RNAs with ethanol, centrifuge, wash the pellets, remove the supernatant and dry the pellets following the same procedure as described previously.

4.3.2. Resuspend the dried RNA pellet in 10 μL of RNA loading buffer, gently vortex, heat to 75–85 $^\circ\text{C}$ for 90 s, and then incubate on ice for 2 min.

4.3.3. Prepare 20 mL of a solution containing 13% polyacrylamide (bisacrylamide:acrylamide, 1.9:50 [wt/wt]) in 8.3 M urea; cast gels 15 cm in length using this solution.

4.3.4. Once the gel is cast, electrophorese it (without any samples loaded) at 400 V for 20 min using TBE as the running buffer. After this step, wash the wells with TBE running buffer.

4.3.5. Load the RNA samples, in RNA loading buffer, and electrophorese with TBE running buffer at 400 V for 3.5 to 4 h. After electrophoresis, remove the urea by immersing and rotating the gel in distilled water for 10 min.

4.3.6. Vacuum dry the gel on 3 M filter paper, first for 2 h 15 min at 80 °C and then for 30 min without heat to slowly cool it. Subject the dried gel to autoradiography on film to detect the positions of migration of the radioactive components.

REPRESENTATIVE RESULTS:

NE depleted of U1 snRNP (U1ΔNE from Section 2.2.6) and Gal3 – U1 snRNP complexes from the 10S region of the glycerol gradient immunoprecipitated by anti-Gal3 (step 3.2.6) were mixed in a splicing reaction.

[Place **Figure 2** here]

This reaction mixture contained U1 snRNA (**Figure 2A**, lane 3), as well as the U1-specific protein, U1-70K (**Figure 2B**, lane 3). As expected, the anti-Gal3 precipitated Gal3 (**Figure 2B**, lane 3). These components (U1 snRNA, U1-70K protein, and Gal3) were not found in the pre-immune (PI) control precipitation (**Figure 2A,B**, lane 2). Compared to a non-depleted NE carried out as a positive control (**Figure 2C**, lane 1), U1ΔNE did not exhibit splicing activity (**Figure 2C**, lane 2). Splicing activity in the U1ΔNE could be reconstituted by the bead-bound Gal3 – U1 snRNP complex (**Figure 2C**, lane 6). Both products of the splicing reaction, ligated exons and excised intron lariat (highlighted by arrows on the right), as well as intermediates, exon 1 and lariat exon 2, were found. The components of fractions 3–5 were critical in restoring splicing to U1ΔNE. When fractions 3–5 were replaced by buffer alone (60% D) in the immunoprecipitation, no splicing activity could be observed on mixing with U1ΔNE (**Figure 2C**, lane 2), indicating that the anti-Gal3 beads were not responsible for the restoration of splicing activity in the latter. More persuasively, when fractions 3–5 were replaced by fraction 1 in the immunoprecipitation procedure and then added to U1ΔNE, no intermediates or products of the splicing reaction were found (**Figure 2C**, lane 4). Previous analysis had documented that the Gal3 in fraction 1 represented free Gal3 protein, not in association with any RNP complex¹⁰ and northern blotting of the material immunoprecipitated from fraction 1 failed to reveal any U1 snRNA¹⁶. Finally, immunoprecipitates from fraction 1 and fractions 3–5 did not exhibit splicing activity when assayed in the absence of U1ΔNE (**Figure 2C**, lanes 3 and 5, respectively).

The results shown in **Figure 2** were obtained with anti-Gal3 (#49). The same results could be observed with another anti-Gal3 (#24) but the pre-immune serum from rabbit #49 failed to reconstitute splicing in U1ΔNE following incubation with fractions 3–5¹⁶. All of these results

strongly suggest that the pre-mRNA substrate can bind to the 10S Gal3 – U1 snRNP particle immobilized on beads and that the ternary complex is functional in the splicing pathway.

FIGURE AND TABLE LEGENDS:

Table 1: Name and Composition of Buffers

Figure 1: Schematic diagram illustrating the complementation of splicing activity in nuclear extract depleted of U1 snRNP by a Gal3-U1 snRNP complex on beads. (A) NE in Buffer C (NE(C)) is incubated with Protein A-Sepharose beads covalently coupled with anti-U1 snRNP (α U1 beads). The unbound fraction is depleted of U1 snRNP (U1 Δ NE). (B) NE in Buffer D (NE(D)) is fractionated over a 12%–32% glycerol gradient by ultracentrifugation. Fractions corresponding to the 10S region (fractions 3–5) are combined and mixed with beads covalently coupled with anti-Gal3 antibodies (α Gal3 beads). The material bound to the beads contains a Gal3-U1 snRNP monoparticle. (C) The Gal3-U1 snRNP complex from Part (B) is mixed with U1 Δ NE from Part (A) in a splicing assay using 32 P-labeled MINX pre-mRNA substrate and the intermediates and products of the splicing reaction are analyzed by gel electrophoresis and autoradiography.

Figure 2: Representative results of the RNA and polypeptide compositions of the anti-Gal3 precipitate of the 10S region of the glycerol gradient and its ability to reconstitute splicing in nuclear extract depleted of U1 snRNP (U1 Δ NE). (A) Northern blotting analysis of U1 snRNA bound to beads coupled with pre-immune serum (PI; lane 2) or to beads coupled with anti-Gal3 (α Gal3; lane 3). Lane 1 represents 20% of the amount of combined glycerol gradient fractions subjected to immunoprecipitation. Lane 2 and lane 3 each represent 25% of the bound material eluted from the respective beads. (B) Western blotting analysis of polypeptides bound to beads coupled with pre-immune serum (lane 2) or anti-Gal3 (lane 3). Protein identities are indicated on the right. Lane 1 represents 20% of the amount of combined glycerol gradient fractions subjected to immunoprecipitation. Lane 2 and lane 3 each represent 75% of the bound material eluted from the respective beads. (C) Analysis of the ability to restore splicing activity to U1 Δ NE by anti-Gal3 (α Gal3) immunoprecipitates of glycerol gradient fraction 1 (Fr 1), fractions 3–5 (Fr 3–5), or 60% Buffer D (60% D). The + or - sign above the bolded solid line indicates the presence or absence of each of these precipitates in the splicing reaction mixture. The + or - sign below the bolded solid line indicates the presence or absence of U1 Δ NE (or NE in Buffer D). Lane 1: 4 μ L NE in a 12 μ L splicing assay, 100% of which were subjected to gel analysis. For lanes 2–6, the total volume of the splicing assay was 24 μ L, 50% of which were subjected to gel analysis. Lane 2: 8 μ L U1 Δ NE plus beads from precipitation of 60% D. Lane 3: beads from precipitation of Fr 1. Lane 4: 8 μ L U1 Δ NE plus beads from precipitation of Fr 1. Lane 5: beads from precipitation of Fr 3–5. Lane 6: 8 μ L U1 Δ NE plus beads from precipitation of Fr 3–5. The positions of migration of the pre-mRNA substrate, the intermediates, and the products (intron lariat and ligated exons, highlighted by arrows) are indicated on the right. The data in Panel C are derived from the same experiment as shown in Panel A, **Figure 2** of Haudek et al.¹⁶.

DISCUSSION:

This report provides the experimental details that document a Gal3 – U1 snRNP complex trapped

on anti-Gal3 coated beads can bind to pre-mRNA substrate and this ternary complex can restore splicing activity to an U1 snRNP-depleted NE. Gal3 is one member of a family of proteins originally isolated on the basis of its galactose-specific carbohydrate-binding activity²³. Early immunofluorescence and subcellular fractionation studies provided the initial hint of an association of Gal3 with components of the splicing machinery: colocalization in nuclear speckles with Sm core polypeptides of snRNPs and the serine- and arginine-rich (SR) family of splicing factors^{24,25} and sedimentation on cesium sulfate gradients at a density (1.3–1.35 g/mL) matching those of hnRNP and snRNP²⁴. Subsequent depletion-reconstitution experiments, in turn, showed Gal3 (as well as another member of the galectin family, galectin-1 (Gal1)) were required, but redundant, factors in cell-free splicing assays^{7,8}.

When NE is fractionated over a glycerol gradient, immunoprecipitation of gradient fractions with anti-Gal3 yielded distinct complexes with varying ratios of different snRNAs and associated proteins, suggesting that Gal3 is associated with multiple snRNPs in the absence of pre-mRNA splicing substrate¹⁰. In particular, Gal3 and U1 snRNP are assembled into a monoparticle that sediments to the ~10S region of the gradient (fractions 3–5 of a 12%–32% glycerol gradient). Since the binding of U1 snRNP to the 5'-splice site of the pre-mRNA represents the initial step in spliceosome assembly^{5,26}, it was possible that Gal3 may gain entry into the splicing pathway via its association with U1 snRNP. This notion is supported by the observation that Gal3, as a part of the 10S Gal3-U1 snRNP complex, could be found to associate with a pre-mRNA substrate but not with control RNA lacking splice sites, suggesting 5'-splice site recognition by U1 snRNP was a key requisite in its assembly into a spliceosome. Moreover, pretreatment of the fractions containing the Gal3-U1 snRNP particle with micrococcal nuclease abolished the loading of Gal3 onto the pre-mRNA¹⁰. The key question, then, is whether this early ternary complex, consisting of Gal3, U1 snRNP, and pre-mRNA, represents a functional E complex committed to the splicing pathway or a dead-end H complex that cannot enter the splicing pathway^{5,26}. We addressed this question by testing whether the Gal3 – U1 snRNP can reconstitute splicing activity in NE depleted of U1 snRNP with concomitant loss of splicing capability (U1ΔNE). The results of our experiments, whose protocol is detailed in the present article, indicate that Gal3 – U1 snRNP binds to pre-mRNA substrate to form a functional E complex and that U1 snRNP is required to incorporate Gal3 into the splicing pathway¹⁶.

Two critical steps within the described experimental protocol need to be noted. First, section 3.2.5 calls for the removal of the supernatant unbound material after anti-Gal3 precipitation of the glycerol fractions. Complete removal of the liquid from the pelleted agarose beads requires that the needle tip of the Hamilton syringe be inserted to the bottom of the beads. This needs to be done carefully such that the beads do not clog up the syringe needle or get lost by adhering to the needle as the latter is pulled out of the tube. Second, it is important that the pelleted beads be used for the assembly of the splicing reactions with as little delay as possible. The coordination of these two steps was critical for the success of the procedure. In this connection, it should also be noted that we have recently improved the recovery of Gal3-U1 snRNP complexes in the pellet fraction by replacing Protein A-Sepharose CL-4B beads with Protein A-magnetic nanoparticles in the covalent coupling of the anti-Gal3 antibody to beads.

The key conclusion is derived from experiments in which U1 Δ NE, devoid of splicing activity, is complemented by the 10S Gal3 – U1 snRNP particle immunoprecipitated by beads covalently derivatized with anti-Gal3 antibodies. Although there was clear evidence of intron removal, the efficiency of exon joining was less than that observed in the reaction conducted in the absence of beads (compare lane 6 versus lane 1 in **Figure 2**). In our cell-free splicing assays, approximately 30% of the radioactivity in the pre-mRNA substrate is typically converted into ligated exons. This deficiency in exon ligation could be due to: (a) some less-than-optimal amount of a critical component, either in U1 Δ NE (**Figure 1A**) or in the Gal3 – U1 snRNP complex (**Figure 1B**); or (b) some structural constraints (e.g., difficulty in undergoing a conformational change) of the Gal3 – U1 snRNP immobilized on the anti-Gal3 beads during the splicing reaction (**Figure 1C**).

Other investigators have reported the use of solid phase immuno-selection to trap specific complexes and document catalytic activity or some ordered progression of spliceosome structures. For example, Chiu et al. showed addition of the yeast splicing factor Cwc25 can chase the pre-mRNA substrate on an antibody-trapped assembled spliceosome into the first catalytic reaction, yielding the intermediates, exon 1 and lariat-exon2²⁷. In the same yeast system, Krishnan et al. used a Protein A-tag to immobilize, via biotinylated-IgG, purified B^{act} spliceosome complex to streptavidin-coated magnetic beads in a biophysical study of pre-mRNA remodeling (distance between the 5'-splice site and branch point) prior to the first splicing step²⁸. Our present article extends these earlier studies in terms of completing both steps of the splicing reaction and, therefore, may carry significance of a technical nature: these same set of procedures could be applied to any number of other splicing factors that associate with snRNPs to examine when and how these factors load onto spliceosomal complexes. The ability to monitor mRNA product formation from the splicing reaction constitutes strong evidence that these assembled spliceosomes are indeed functional. This may further our understanding of how spliceosomes form, as previous reports using proteomic approaches¹⁷ only catalog the presence of some factors in a given complex rather than how the factors may have been loaded onto the complex.

A clear limitation of the general applicability of these procedures to other proteins or snRNPs to evaluate their role in splicing is the availability of a specific antibody directed against the particular factor that will efficiently precipitate its associated complex. For example, polyclonal anti-Gal3 antisera from a number of rabbits (e.g., #24, #49) immunoprecipitated the Gal3-U1 snRNP complex from nuclear extract or glycerol fractions 3-5 (U1 snRNA and U1 70K protein co-precipitated with the cognate antigen, Gal3). In contrast, two commercially available monoclonal antibodies directed against Gal3, Mac-2 and NCL-GAL3, failed to yield the same results. The epitopes of Mac-2 and NCL-GAL3 have been mapped to the amino-terminal domain of the Gal3 polypeptide²⁹ and it is possible that their respective epitopes are not accessible in the Gal3-U1 snRNP complex. Furthermore, an antibody-bound factor (e.g., Gal3) and its associated complex (e.g., U1 snRNP) may no longer be able to form additional interactions with the pre-mRNA or other spliceosomal components. This may be due to physical interference by the antibody or by the matrix to which the antibody is covalently coupled. Therefore, each system of interest needs to be evaluated on a case-by-case basis. Despite these limitations, however, it seems that the scheme of using complexes affinity- or immuno-selected on beads to reconstitute splicing activity in extracts depleted of a specific splicing factor may be generally applicable to other systems.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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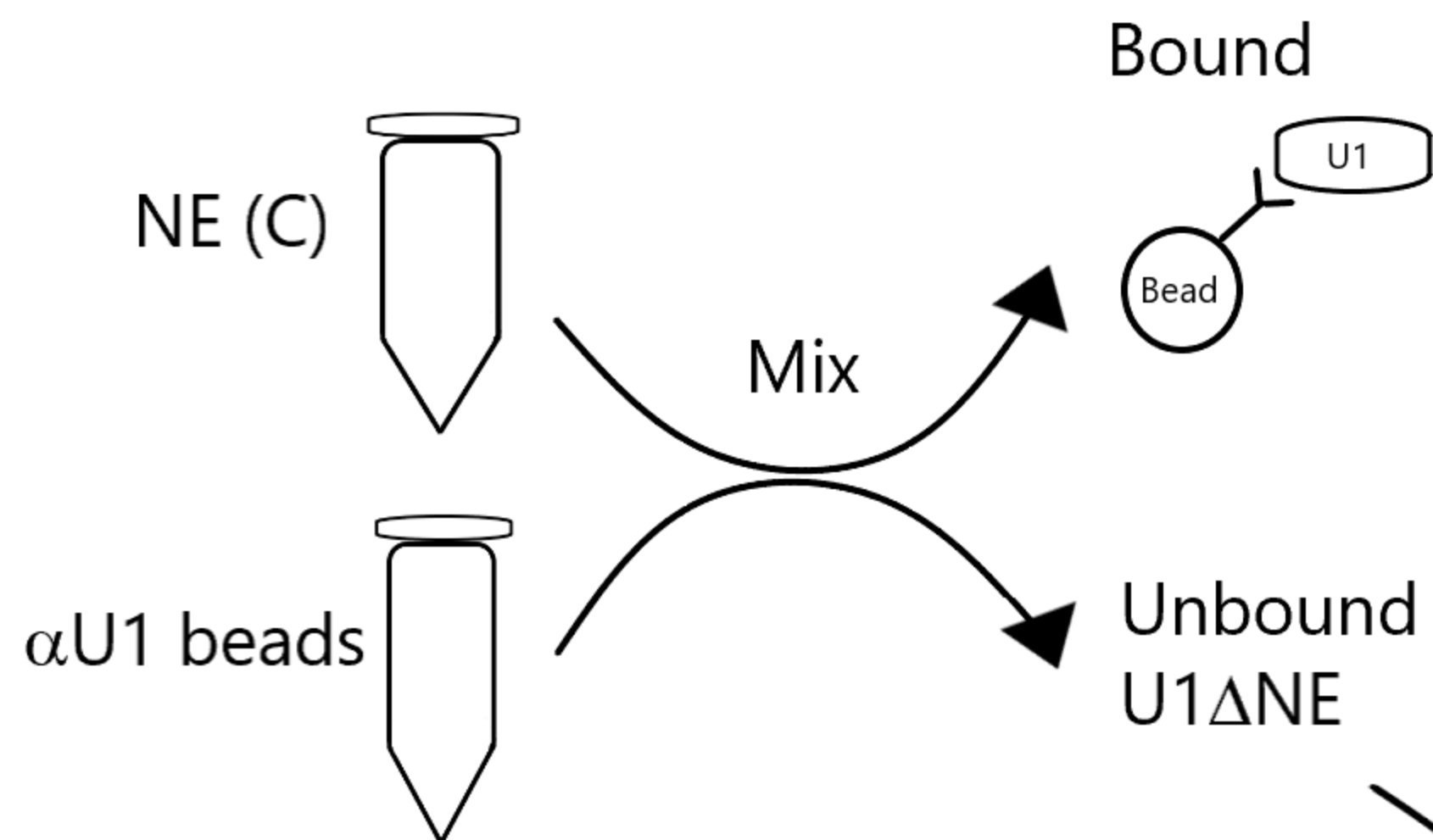
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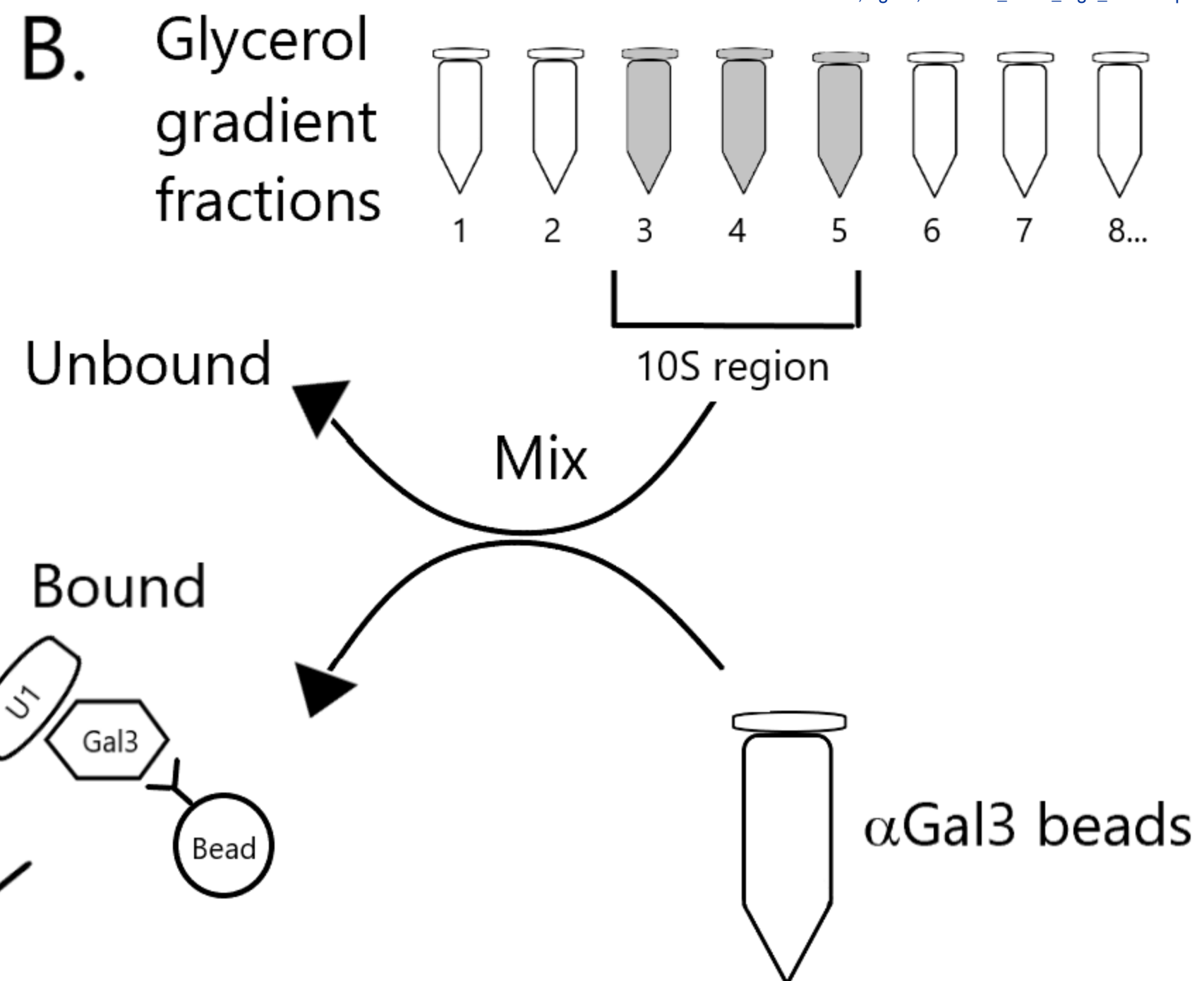
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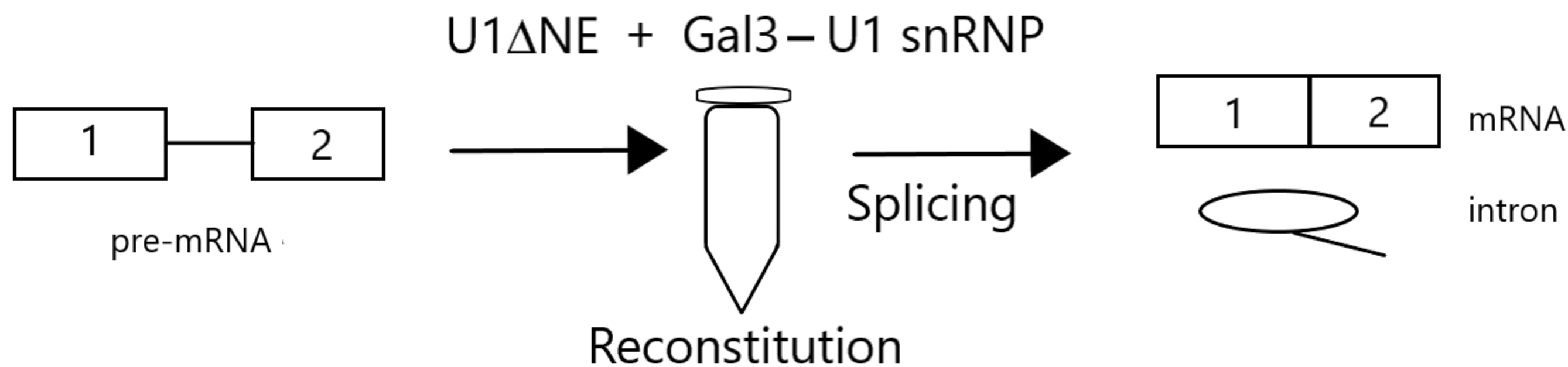
A.



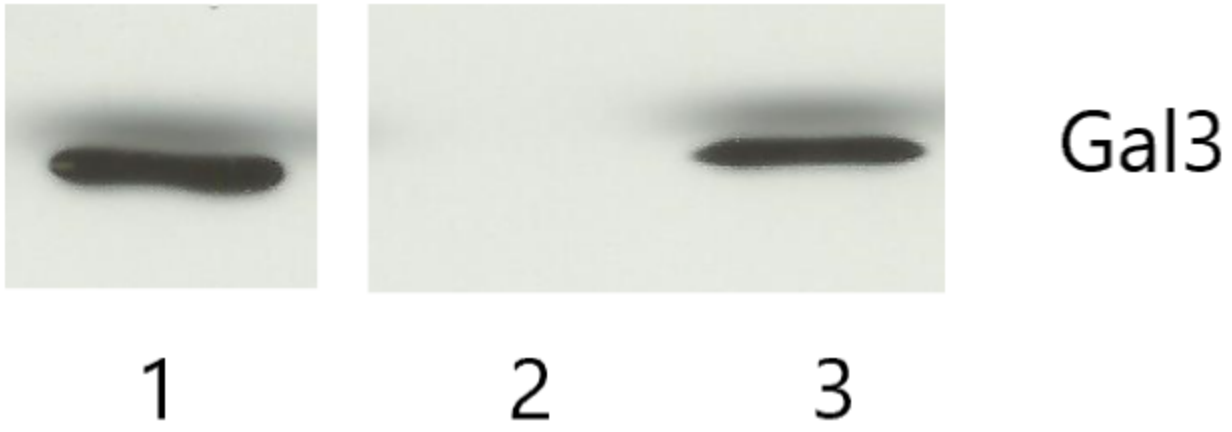
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C.

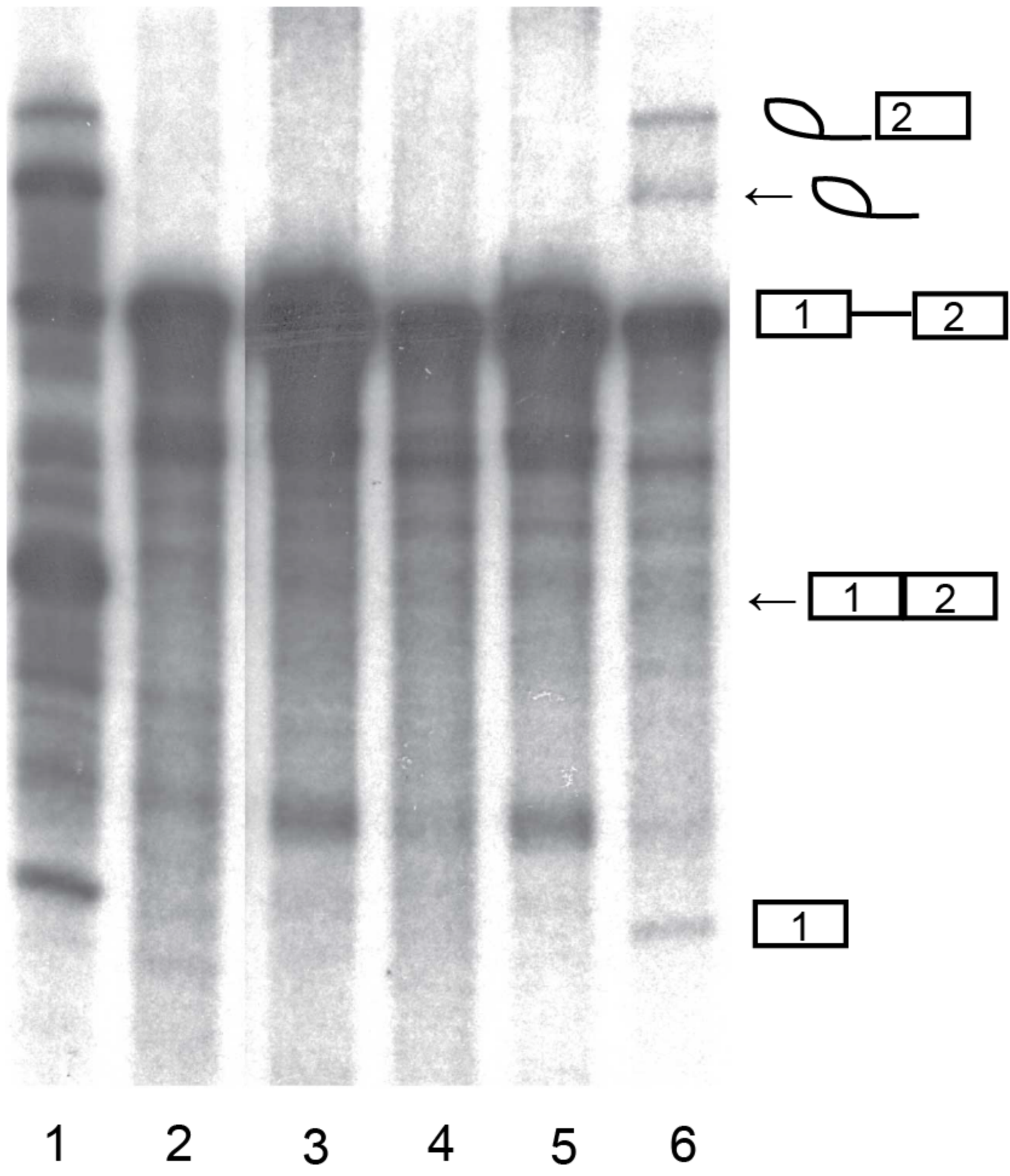


Input PI αGal3



C

Fr 3-5	-	-	-	-	+	+	Preincubated with αGal3 beads
Fr 1	-	-	+	+	-	-	
60%D	-	+	-	-	-	-	
<hr/>							
U1ΔNE	-	+	-	+	-	+	
NE	+	-	-	-	-	-	



Name of buffer
Borate buffer
Buffer C
Buffer D
60%D
Ethanolamine
HEPES binding buffer
HEPES wash buffer
RNA loading buffer
SDS-PAGE buffer
SDS sample buffer
TBE buffer for RNA gels
TE buffer
Transfer buffer
T-TBS buffer
TX wash buffer

Composition

0.2 M sodium borate, pH 9

20 mM HEPES, pH 7.9, 25% (vol/vol) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT)

10 mM HEPES, pH 7.9, 20% (vol/vol) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT

60% Buffer D and 40% H₂O

0.2 M ethanolamine, pH 8

20 mM HEPES, pH 7.9

20 mM HEPES, pH 7.9, 0.5 M NaCl

90% formamide, 20 mM EDTA, pH 8, 0.05% (w/v) bromophenol blue

25 mM Tris, 169 mM glycine, 0.1% sodium dodecyl sulfate (SDS), pH 8.8

62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% (w/v) bromophenol blue

89 mM Tris, 80 mM boric acid, 2.5 mM EDTA, pH 8.3

10 mM Tris, pH 8, 1 mM EDTA

25 mM Tris, 1.92 M glycine, 20% methanol, pH 8.3

10 mM Tris, 0.5 M NaCl, 0.05% Tween 20, pH 7.5

0.05% Triton X-100 (TX) in 60%D

Name of Material/ Equipment	Company	Catalog Number
anti-U1 snRNP	The Binding Site	Hu ENA-RNP #33471
bottle top vacuum filter	Fisher Scientific	Corning 431153 (0.22 µm; PES 150 ml)
centrifuge	International Equipment Company	IEC Model PR-6
diethylpyrocarbonate (DEPC)	Sigma-Aldrich	159220-5G
dimethylpimelimidate (DMP)	Sigma-Aldrich	80490-5G
electrophoresis cell	BioRad Laboratories, Inc	Mini-Protean II
ethanolamine	Sigma-Aldrich	411000-100ml
gel electrophoresis system	Hoefer, Inc	HSI SE 500 Series
gel slab dryer	BioRad	Model 224
Hybond ECL membrane	GE Healthcare	RPN3032D (0.2 µm; 30 cm x 3 m)
microdialyzer (12 x 100 µl sample capacity)	Pierce	Microdialyzer System 100
microdialyzer membranes (8K cutoff)	Pierce	66310
non-fat dry milk	Spartan Stores	Spartan Instant Non-fat Dry Milk
Protein A Sepharose CL-4B	Millipore-Sigma	GE 17-0780-01
Proteinase K	Millipore-Sigma	P2308-5mg
Rnasin	Promega	N2111
rocker/rotator	Lab Industries, Inc	Labquake Shaker 400-110
Safety-Solve	Research Products International Corp.	No. 111177
scintillation counter	Beckman Instruments	LS6000SC
speed vacuum concentrator	Savant	SVC 100H
Transphor electrophoresis unit	Hoefer, Inc	Hoefer TE Series Transphor

Comments/Description

human autoimmune serum specific for U1 snRNP

for filtering solutions containing Tris

for pelleting Sepharose beads in immunoprecipitation

for treatment of water used in preparation of all solutions

for cross-linking antibody to Sepharose beads

for SDS-PAGE separation of proteins

for blocking after the cross-linking reaction

for separating snRNAs by gel electrophoresis

for drying gel slabs for autoradiography

for immunoblotting of proteins on membrane

for exchanging the buffer of nuclear extract

for exchanging the buffer of nuclear extract

for coupling antibody to beads

for stopping the splicing reaction to isolate the RNAs

for inhibiting ribonuclease activity

for mixing protein solutions in coupling reactions and in immunoprecipitation

scintillation counting cocktail for determination of radioactivity in splicing substrate

scintillation counter for determination of radioactivity

for drying ethanol-precipitated RNA pellets

for protein transfer from SDS-PAGE to blotting membrane

Response to Reviewers

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

All four co-authors have read the revised manuscripts and corrected typos as best as possible. All revisions in blue font.

2. Please revise the Introduction to add the following:

a) The advantages over alternative techniques with applicable references to previous studies

b) Information to help readers to determine whether the method is appropriate for their application

Added sections to paragraph 3 of Introduction to address these points.

3. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: 86-98, 141-154, 311-316, 321-336, 392-400, 425-428

All of these sections have been rewritten.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Revised protocol to conform to this, as best as possible.

5. Please move 1.2 Buffers into a separate table of reagents.

Buffers moved to Table 1.

6. 2.1.1: What is the amount of protein A-Sepharose CL-4B beads to be taken? In how much water?

Revised wording to indicate amount.

7. 2.3.5: How do you remove the material bound to the beads with SDS-PAGE sample buffer?

This is actually described in Sections 2.3.3 and 2.3.4. Section 2.3.5 now indicates this explicitly.

8. 2.3.5: Please specify conditions of gel electrophoresis.

This is now described in new sections, 2.3.5.1 and 2.3.5.2.

9. 4.1.1: Please move the information about the plasmid from Dr. Berget to the Acknowledgements.

Done

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11. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Paragraphs 3, 5, and 6 of the Discussion represent additions to address the above.

12. Please sort the Materials Table alphabetically by the name of the material.

The Materials Table is arranged alphabetically by the name of the material.

Reviewers' comments:

Reviewer #1:

Major Concerns:

in section 2.3.5 and 3.3.7. : is there no need to perform an RNA purification (as described in 4.3.1) before sn RNA analysis by gel electrophoresis?

Details of RNA purification, previously described in Section 4.3.1, have been moved forward to Section 2.3.5. This latter section has been extensively revised as a new Section 2.3.5.1. Sections 3.3.7 and 4.3.1 have been rewritten to reflect that the details can be found in the prior section.

Please indicate whether you evaluate any commercial anti-Gal-3 antibody in section 3.1.1.

We have indeed tested commercial anti-Gal3 antibodies. This is now discussed in paragraph 6 of the Discussion.

Minor Concerns:

point 1.2.11 please check Tris concentration in the TE buffer.

Tris concentration corrected

point 4.3.3. did you use heating in the Speed Vac?

The protocol for RNA purification and analysis was moved forward to Section 2.3.5. We did not use heat in the Speed Vac; this is described in Section 2.3.5.1.

point 4.3.9. how long do you vacuum dry your gel ?

Conditions for drying the gel are described in Section 4.3.6.

Reviewer #2:

Major

None

Concerns:

Minor

Concerns:

My only recommendation is to consider reducing the number of acronyms that are used in the introduction. In its present form, the introduction is somewhat difficult to follow.

After a considerable amount of discussion amongst the co-authors, we have decided to keep the acronyms used in the original submission.

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