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SL trans-splicing in a *C. elegans* in vitro extract

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INTRODUCTION

C. elegans is one of a number of organisms in which Spliced Leader (SL) trans-splicing contributes to the maturation of many pre-mRNA transcripts. *C. elegans* and related nematodes have two distinct classes of Spliced Leader RNA, SL1 and SL2, that are used differentially for different classes of pre-mRNAs; namely, SL1 trans-splices non-operon genes and 1st genes in operons, while SL2 splices downstream operon genes. In vitro RNA splicing has previously been used to analyze substrate RNA sequences, required protein and RNA factors, and splicing mechanisms in a variety of systems. This protocol describes an in vitro splicing assay using *C. elegans* extract that promotes both cis- and trans-splicing of substrate RNAs. It has the ability to correctly specify and detect SL1 vs SL2 trans-splicing. Operon-derived substrates splice SL2 while outtron substrates splice SL1. Unlabeled T7 transcribed RNA substrates are incubated with crude embryonic extract and trans-spliced by endogenous SL snRNPs. Spliced RNA is analyzed using RT-PCR. Specific RT and PCR primers ensure detection of trans-spliced substrate RNA and allow a means to distinguish between SL1 and SL2 trans-spliced products. Substrates containing introns are also cis-spliced. All reaction products are ATP dependent.

RELATED INFORMATION

SL trans-splicing was first studied in vitro using extract from the nematode *Ascaris* (Hannon et al. 1990, 1991). Recently, a protocol for in vitro SL trans-splicing in trypanosome extract has been published (Shaked et al. 2010). The *C. elegans* protocol presented here was used to show SL1 vs SL2 specificity and to determine the characteristics and importance of the Ur element in SL2 trans-splicing. See this reference for examples of some of the different RNA substrates used with the protocol below (Lasda et al. 2010).

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MATERIALS

Reagents

2 M sucrose
5X Splicing Buffer +ATP [R]
5X Splicing Buffer –ATP [R]
agarose for gels
Bradford assay reagents
C. elegans worms
dialysis buffer [R]
DNA purification kit (from gel) Qiagen#28604
DNA purification kit (from reaction) Qiagen#28004
dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
E. coli strain NA22, frozen paste, ~14g each unit
ethanol (100%), cold
ethanol (70% to 80%)
ethidium bromide for staining agarose gels
glycogen
homogenization buffer [R]
hypochlorite solution [R]
ice
isopropanol
liquid nitrogen
mMessage mMachine kit with cap analog Ambion/Applied Biosystems #AM1344 PCR reagents
phenol/chloroform/isoamyl alcohol
plasmid DNA (with RNA polymerase promoter sequence and gene of interest)
Poly(A) Tailing kit Ambion/Applied Biosystems #AM1350
primers - for reverse transcription (specific for vector-derived portion of RNA substrate), for PCR-forward (specific for SL sequence), and for PCR-reverse (specific for vector-derived portion of substrate)
Proteinase K (at 10mg/mL)
purification columns - Micro Bio-Spin columns BioRad #732-6250
purification columns - NucAway Spin columns Ambion/Applied Biosystems #AM10070
restriction enzyme (that does not leave a 3' overhang)
RNase OUT Invitrogen #10777-019

S-basal medium
Splicing Stop solution [R]
Superscript II Reverse Transcriptase Invitrogen #18064-022

Equipment

1.5 mL tubes
agarose gel-running equipment
beaker 2 L (or larger) for dialysis
Bradford assay reagents
centrifuge for 1.5 mL tubes
centrifuge for conical tubes
conical tubes (15 mL or 50 mL)
dialysis membrane or tubes
flasks (2 liter)
freezer (-20°C)
freezer (-70°C to -80°C)
glass pipets
ice bucket
PCR machine
shaking incubator for 2 L flasks, set at 20°C
stir bar
stir plate in cold
tight stainless steel dounce homogenizer (such as the Dura-Grind Tissue grinder from Wheaton Science Products)
vortex
water bath at 15°C
water bath at 37°C
water bath or heat block at 42°C
water bath or heat block at 65°C
water bath or heat block at 70°C

METHOD

C. elegans liquid cultures

This section (Steps 1–20) describes the method used to grow large-scale liquid cultures of semi-synchronous worms and isolate large amounts of embryos. Other large-scale culture methods may be used instead.

- 1 Collect mixed stage worms from plates or liquid cultures.

- 2 Add 0.5 mL worms to a 2 L flask with 500 mL S-Basal medium with E. coli strain NA22. Add 1 unit of ~14g frozen E. coli paste per flask.
- 3 Grow at 20°C shaking at 180 rpm for 3 days, or until there are many gravid adults.
- 4 Collect worms in conical tubes by centrifugation for 5 minutes at 4°C.
- 5 Wash pellet of worms 3 times with cold dH₂O.
- 6 Sucrose float to remove bacteria and debris (*Optional. Best done if culture has large amount of bacteria and debris*).
 - a. Resuspend the pellet of worms in 20 mL cold dH₂O.
 - b. Add 20 mL cold 2M sucrose.
 - c. Mix by inversion.
 - d. Centrifuge 1 min at 1000 rpm at room temperature. (*Worms will be at the top of the tube. At the bottom of the tube you will have a small pellet of debris and bacteria.*) Do not use the centrifuge break, as this will disrupt the worm layer.
 - e. Remove worms (top layer) using a glass pipet into a new tube on ice (no more than 10 mL per 50 mL conical tube).
 - f. Wash pellet of worms 3 times with cold dH₂O to remove sucrose.
- 7 Hypochlorite treat to isolate embryos.
 - a. Estimate volume of packed worm pellet.
 - b. Add 10 times volume hypochlorite solution.
 - c. Rotate by hand for up to 10 minutes, until almost all embryos have been released, checking by microscopy every 2–3 minutes.
 - d. Pellet embryos by centrifugation for 5 minutes at 4°C.
 - e. Wash pellet of embryos 2 times (or more) with cold dH₂O until there is no smell of bleach.
- 8 Sucrose float to remove debris (as in Step 6 above).
- 9 Wash pellet of worms 3 times with cold dH₂O to remove sucrose.
- 10 Add 0.5 mL isolated embryos to each 2 L flask with 500 mL S-basal medium (do not add bacteria yet).
- 11 Incubate at 20°C shaking at 180 rpm until all embryos are hatched, usually 12–24 hours (can be kept at this stage for up to 1 week). *Without food, hatched worms will synchronize at the L1 stage.*
- 12 Add E. coli strain NA22. Add 1 unit of ~14g frozen E. coli paste per flask.
- 13 Grow at 20°C shaking at 180 rpm until there are many gravid adults, usually 80–90 hours, adding more E. coli strain NA22 if necessary to ensure the culture does not become starved.
- 14 Collect worms in conical tubes by centrifugation for 5 minutes at 4°C.
- 15 Wash pellet of worms 3 times with cold dH₂O.

- 16 Sucrose float to remove bacteria and debris (as in Step 6 above) (*Optional. Best done if culture has large amount of bacteria and debris*).
- 17 Hypochlorite treat to isolate embryos (as in Step 7 above).
- 18 Sucrose float to remove debris (as in Step 6 above).
- 19 Wash pellet of embryos 3 times with cold dH₂O to remove sucrose.
- 20 Immediately begin extract procedure.

C. *elegans* embryonic extract preparation

This section (Steps 21–33) describes the method required to prepare a high concentration crude embryonic extract active for in vitro assays. A high quality and high concentration extract is **absolutely critical** for the success of the in vitro splicing assay. Other methods of extract preparation and of homogenization were also tested, but only douncing a high concentration of embryos with a tight stainless steel dounce resulted in sufficient quality extract. It is important to work quickly and keep everything cold. Before beginning, prepare and pre-chill all buffers, reagents and equipment.

- 21 Wash pellet of embryos 2 times with cold homogenization buffer.
- 22 Estimate volume of packed embryos.
- 23 Resuspend in equal volume (or less) cold homogenization buffer.
- 24 Transfer to cold tight stainless steel dounce homogenizer.
- 25 Break embryos by douncing at least 30 times on ice. Expect embryo solution to be very viscous and quite difficult to dounce.
- 26 Transfer homogenate to 1.5 mL tubes.
- 27 Centrifuge for 5 min at 6000 rpm (3100×g) at 4°C.
- 28 Transfer supernatant to pre-chilled dialysis membrane or tubes.
- 29 Dialyze against 1 L cold dialysis buffer for 1 hour at 4°C.
- 30 Aliquot on ice. *Small aliquots (25 µL to 100 µL) are best to avoid multiple freeze-thaw cycles*.
- 31 Quick freeze in liquid nitrogen.
- 32 Store at –70°C to –80°C.
- 33 Final concentration should be ~25 µg/µL (Optional: Check protein concentration by Bradford assay).

Splicing substrate design and cloning

This section (Steps 34–36) discusses important considerations required in the design and cloning of the splicing substrates. Any cloning plasmid may be used as a backbone vector, providing it has the desired RNA polymerase promoter sequence (T7, T3, SP6) oriented to direct in vitro RNA transcription of the desired substrate. Use standard cloning techniques.

- 34 Choose the splicing substrate. In our experience, most splicing substrates both cis- splice and trans- splice correctly, with the correct SL specificity in vitro as in vivo. However, some have failed to splice in vitro, some unexpectedly trans-spliced at 3' cis-splice sites, some trans-spliced at cryptic splice sites, and a few failed to show SL specificity. It is therefore worthwhile to test several unrelated substrates to find one that provides optimal splicing efficiency and specificity in

vitro. For examples of substrates that consistently and specifically trans-splice, please see the reference (Lasda et al. 2010).

- 35** Design the splicing substrate. We have obtained robust SL trans-splicing with substrates containing ~100 or more total nucleotides upstream of the 3' trans-splice site (75 nt were endogenous sequence, 22 nt were vector-derived sequence).
- 36** Choose the cloning and linearization restriction enzyme sites of the plasmid backbone. In the transcribed RNA, it is important to have sufficient vector sequence downstream of the cloning site for RT and PCR primers. Choose a linearization enzyme that is sufficiently far downstream and that will not leave a 3' overhang (as that can lead to aberrant transcription products).

Splicing substrate preparation

This section (Steps 37–43) describes the method used to prepare unlabeled RNA to be used as a splicing substrate from plasmid DNA. The plasmid should have a standard in vitro RNA polymerase promoter sequence, the substrate of interest, and a downstream restriction enzyme sequence for DNA linearization. We chose to use Ambion kits for this section. If other manners of RNA preparation are employed, ensure the result is the desired quantity of full-length, highly purified, capped, and polyadenylated sense transcripts. 5' caps and 3' polyA tails are used to increase the stability of the RNA transcripts in extracts during the splicing reaction. (ApppG has also been used successfully in place of m7Gcap analog). However, polyadenylation results in transcript size heterogeneity, so gel purification of the RNA is not recommended. Our substrates ranged in size from 0.5 kb to 1.5 kb, not including the polyA tails.

- 37** Prepare plasmid DNA. Miniprep DNA is not generally recommended by others, since the purity can be lower than in other types of preparations. However we routinely used miniprep DNA without a problem.
 - a.** Linearize 5 µg plasmid DNA using restriction enzyme of choice in a 25 µL digestion reaction. Digest to completion.
 - b.** Purify linearized DNA.
 - i.** Run 2 µL digest reaction and equivalent amount of uncut plasmid DNA on a 1% agarose gel to check completion of restriction enzyme digest reaction.
 - ii.** if digest reactions do not show complete linearization, run remainder of digest on 1% agarose gel, cut out bands, and gel purify with Qiagen MinElute Gel Extraction kit (#28604). Elute with minimum volume H₂O possible.
 - iii.** if digest reactions do show complete linearization, purify remainder of digest with Qiagen MinElute PCR Purification kit (#28004). Elute with 10 µL H₂O.
 - c.** Quantify linearized DNA and determine concentration.
- 38** Transcribe RNA in vitro. For example, use T7 RNA polymerase using the mMessage mMachine kit with cap analog (Ambion/Applied Biosystems #AM1344), according to the manufacturer's instructions. Briefly:
 - a.** Calculate volume of linearized plasmid DNA equal to 1 µg. If greater than 6 µL, just use 6 µL.

- b.** Thaw reagents. Thaw 2X NTP/CAP mix on ice (or thaw and then immediately place on ice), vortex well and quick spin down. Keep on ice. Thaw 10X Reaction Buffer at room temperature, vortex well and quick spin down. Keep at room temperature. Thaw Nuclease-free H₂O and linearized plasmid DNA at room temperature. Keep at room temperature.
- c.** Add together at room temperature: (Refreeze reagents as soon as possible.)

6 μ L linearized plasmid DNA + H ₂ O (1 μ g DNA)
10 μ L 2X NTP/CAP mix
2 μ L 10X Reaction Buffer (for T7)
<u>2 μL T7 RNA polymerase Enzyme Mix</u>
20 μ L TOTAL

- d.** Incubate at 37°C for 2 hours.
 - e.** Add 1 μ L TURBO DNase (included in Ambion kit) and mix well. Incubate at 37°C for 15 minutes. Do not add EDTA to inactivate DNase.
 - f.** (Optional: Can store samples at this stage at -20°C overnight or at -70°C for longer.)
- 39** Polyadenylate RNA in vitro. For example, use the Poly(A) Tailing kit (Ambion/ Applied Biosystems #AM1350), basically according to the manufacturer's instructions. Briefly:

- a.** Add together at room temperature:

21 μ L in vitro RNA reaction (from Step 38 above, all of it)
36 μ L H ₂ O
20 μ L 5X E-PAP Buffer (included in Ambion kit)
10 μ L 25 mM MnCl ₂ (included in Ambion kit)
10 μ L 10 mM ATP (included in Ambion kit)
<u>3 μL E. coli Poly A Polymerase (2U/μL, included in Ambion kit)</u>
100 μ L TOTAL

- b.** Incubate at 37°C for 1 hour.
- 40** Purify RNA by phenol/chloroform extraction and isopropanol precipitation, then by column purification as follows: (This two-part purification is necessary to remove excess ATP used in the polyA tailing reaction.)

- a.** Add together in order:

100 μ L polyA tailing reaction (from Step 39 above, all of it)
--

34 μ L H ₂ O
15 μ L Ammonium Acetate stop solution (included in Ambion transcription kit used in Step 38 above)
1 μ L glycogen
150 μ L phenol/chloroform/isoamyl alcohol
300 μ L TOTAL

- b. Vortex well.
 - c. Spin full speed ~5 minutes (full speed = ~18,000 $\times$ g).
 - d. Take top aqueous layer into new, labeled 1.5 mL tube.
 - e. Add 150 μ L isopropanol and mix by inversion.
 - f. Incubate at -20°C for at least 15 minutes. (Optional: incubate at -20°C overnight)
 - g. Spin full speed, cold, 30 minutes (full speed = ~18,000 $\times$ g).
 - h. Remove supernatant.
 - i. Add 1 mL 70% to 80% ethanol.
 - j. Shake or pipette until pellet floats loose from tube.
 - k. Spin full speed ~5 minutes (full speed = ~18,000 $\times$ g).
 - l. Remove all visible traces of ethanol from pellet.
 - m. Allow to air dry with tops open **only** ~3 minutes. Ensure that there is no visible liquid in the tube. If so, draw it up side of tube with pipette tip until it evaporates.
 - n. Add 25 μ L Nuclease-free H₂O (included in Ambion kits).
 - o. Resuspend RNA gently at room temperature, at least 30 minutes.
 - p. Column purify by either Micro Bio-Spin columns (BioRad #732-6250) or NucAway Spin columns (Ambion/Applied Biosystems #AM10070).
- 41 Quantify RNA and determine concentration.
 - 42 Make several RNA stock tubes at 25 ng/ μ L. Small aliquots (10 μ L to 50 μ L) are best to avoid multiple freeze-thaw cycles).
 - 43 Store at -70°C to -80°C .

In vitro splicing reaction

This section (Steps 44–48) describes the in vitro splicing protocol using the extract and the RNA from the steps above. The 15 μ L reactions with 50% embryonic extract have final concentrations of 60 mM KCl, 4 mM MgCl₂, 2 mM ATP, 20 mM creatine phosphate, 50 μ g/mL creatine kinase, 2 mM DTT, 3 % PEG 8000, 0.25 mM EDTA, 5% glycerol, 10 mM Tris-HCl pH 8.0, 0.5 mM PMSF, 40 U/ μ L RNaseOUT). It is important to set up the splicing reactions quickly and on ice, adding the extract last. Control reactions using the “–ATP” buffer should be set up in parallel. These lack ATP, creatine phosphate, and creatine kinase. The volume of H₂O added may be adjusted to include other reagents without changing the total reaction volume. In some experiments we have included DNA oligonucleotides (the extract has endogenous RNase H activity), additional RNA substrates (substrate competition is apparent), or RNA oligonucleotides. Before beginning, prepare and pre-chill all buffers and tubes.

- 44** Thaw extract and RNA substrate on ice. Thaw 5X Splicing Buffer +ATP and 5X Splicing Buffer –ATP at room temperature.
- 45** Add together on ice: (Refreeze 5X Buffers and RNA substrates as soon as possible. Do not reuse thawed extract.) *Recommended: Optimize amount of RNA substrate.*

2.75 μ L H ₂ O
3 μ L 5X reaction buffer (+ or – ATP)
0.75 μ L RNase OUT (Invitrogen #10777-019)
1 μ L RNA splicing substrate (at 25 ng/ μ L)
<u>7.5 μL <i>C. elegans</i> embryonic extract (at ~25 μg/μL)</u>
15 μ L TOTAL

- 46** Transfer to 15°C water bath directly from ice bucket.
- 47** Incubate in 15°C water bath for 2 hours. *Optional: Perform a time course to determine optimal accumulation of spliced products. In our experience, spliced products continued to accumulate for up to 2 hours.*
- 48** Add 279 μ L Splicing Stop solution (294 μ L TOTAL volume). (Optional: Can store samples at this stage at –20°C overnight or at –70°C for longer.)

In vitro splicing analysis

This section (Steps 49–53) describes the RNA isolation and analysis of the in vitro spliced products from the reactions above. The RT primer and the reverse PCR primer should be specific for a vector-derived region of the RNA substrate downstream of the gene of interest. This ensures detection of substrate RNA rather than endogenous transcripts. For detection of SL trans-spliced products, forward PCR primers should be 23 nt long, including the 22 nt SL + 1 nt within the exon. Longer exon regions (greater than 4 nt) within the primer can result in SL-independent priming.

- 49** Proteinase K digest.
- Add together 294 μ L Splicing Stop reaction (from Step 48 above, all of it) and 6 μ L Proteinase K (at 10 mg/mL) (300 μ L TOTAL volume).
 - Incubate in 37°C water bath for **only** 15 minutes. Longer 37°C incubations can lead to RNA degradation. (Optional: Can store samples at this stage at –20°C overnight or at –70°C for longer.)
- 50** Isolate RNA.
- Add 300 μ L phenol/chloroform/isoamyl alcohol (600 μ L TOTAL volume).
 - Vortex well.
 - Spin full speed ~5 minutes (full speed = ~18,000 $\times$ g).
 - Take top aqueous layer into new, labeled 1.5 mL tube.
 - Add 1 mL cold, 100% ethanol and mix by inversion.
 - Incubate at –20°C overnight.
 - Spin full speed, cold, 30 minutes (full speed = ~18,000 $\times$ g).

- h.** Remove supernatant.
- i.** Add 1 mL 70% to 80% ethanol.
- j.** Shake or pipette until pellet floats loose from tube.
- k.** Spin full speed ~5 minutes (full speed = ~18,000×g).
- l.** Remove all visible traces of ethanol from pellet.
- m.** Allow to air dry with tops open **only** ~3 minutes. Ensure that there is no visible liquid in the tube. If so, draw it up side of tube with pipette tip until it evaporates.
- n.** Add 50 μ L H₂O.
- o.** Resuspend RNA gently at room temperature, at least 30 minutes.
- p.** Optional: Can store samples at this stage at -20°C overnight or at -70°C for longer.

51 Reverse Transcribe RNA using Superscript II RT (Invitrogen #18064-022).

- a.** Add together: (store remaining RNA at -70°C)

10 μ L of the resuspended RNA (20% recovered RNA) 2 μ L RT specific primer (at 1 pmol/ μ L) <u>1 μL dNTP mix (at 10 mM each)</u> 13 μ L TOTAL

- b.** Incubate at 65°C for 5 minutes.
- c.** Incubate on ice for at least 1 minute.
- d.** then add:

4 μ L 5X 1 st strand buffer (from Invitrogen kit) 2 μ L 0.1 M DTT (from Invitrogen kit) 0.25 μ L RNase OUT (Invitrogen #10777-019) <u>0.75 μL SuperScript II Reverse Transcriptase (from Invitrogen kit)</u> 20 μ L TOTAL

- e.** Incubate at 42°C for 60 minutes, then 70°C for 15 minutes.
- f.** Optional: Can store cDNA samples at this stage at -20°C .

52 Amplify by SL specific PCR. *It is necessary to set up SL specific PCR reactions on ice and transfer to a pre-warmed machine.*

- a.** In a 50 μ L PCR reaction, use 2 μ L cDNA (10% of the RT reaction), 5 pmol forward PCR primer and 5 pmol reverse PCR primer.
- b.** Our typical SL cycling parameters for PCR using Taq are 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 60 seconds, for 35 cycles.

- c. Adjust PCR cycle number (or initial substrate amount) to ensure linear range.

53 Visualize and analyze by agarose gel stained with ethidium bromide.

Recommended: verify spliced products by cloning and sequencing.

TROUBLESHOOTING

Problem: Extract concentration is not ~25 µg/µL.

[Step 33]

Solution: The success of the splicing assay depends heavily on the quality and concentration of the extract. Use less homogenization buffer per volume of packed embryos in Step 23. Dounce more times and slower. Monitor embryo breakage by microscopy.

Problem: No visible pellet after splicing and RNA precipitation.

[Step 50]

Solution: Increase amount of glycogen used for co-precipitation, either by adding a greater amount to the Splicing Stop solution used in Step 48 or add additional glycogen directly to the tube in Step 50e.

Problem: Final PCR products resulting from DNA. Test this by including –RT reactions (lacking Reverse Transcriptase) for each sample. This is not a problem for SL trans-splicing analysis, as there is no corresponding DNA template, however it may be a problem during analysis of cis-splicing.

[Step 53]

Solution: DNase treat purified RNA by adding a DNase step either after Step 40 (if the DNA comes from the substrate), after Step 50 (if the DNA comes from the extract), or both. Also ensure that at least one PCR primer is vector-specific to avoid amplifying products of endogenous genes.

Problem: No visible spliced products following final PCR.

[Step 53]

Solution: Unfortunately, this could be due to any number of things. The three most likely causes are low quality/concentration extract, bad RNA substrate (some genes are just not good in vitro splicing substrates and we don't understand why), or splicing below the level of detection.

- Try a different substrate RNA (Step 45).
- Increase amount of substrate RNA used in the splicing reaction (Step 45).
- Optimize PCR conditions, and/or increase number of cycles (Step 52).
- In cis-splicing analysis, the RT-PCR may be overwhelmed by the unspliced substrate. To detect only cis-spliced products, primers spanning the exon-exon junction may be used (as long as the 3' end of the primer corresponds to no more than 4 nt past the splice junction).

- Primers spanning spliced junctions can also be used to specifically detect more rare in vitro splicing events (cis- or SL trans-). Again, ensure that the 3' end of the primer corresponds to no more than 4 nt past the splice junction.

Problem: SL1 PCR product is stronger than expected.

[Step 53]

Solution: Make sure the PCR reactions are being set up entirely on ice and that the tubes are transferred into a pre-warmed machine.

Problem: Unexpected size PCR of product(s).

[Step 53]

Solution: Clone and sequence the product to determine its origin.

- if the product is also visible in the –ATP samples (from Step 45), it may result from products of endogenous genes. Change primer sequences.
- It may result from unexpected or cryptic in vitro splicing. We have observed numerous cases of in vitro splicing events (cis- or SL trans-) that are not detected in vivo.
- It may be the result of RT or PCR artifacts. Change primer sequences and/or PCR conditions.

RECIPES

hypochlorite solution

(20% bleach by volume, 0.5 M KOH)

homogenization buffer

(10mM Tris-HCl pH8.0, 1.5mM MgCl₂, 10mM KCl, 1mM DTT, 50mM sucrose, 0.05% NP-40, 1X complete protease inhibitor tablet, EDTA-free (Roche #1873560), 1mM PMSF) DTT, protease inhibitor and PMSF must be added fresh on the day of homogenization. Chill.

dialysis buffer

(20 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM DTT, 10% glycerol, 0.5 mM EDTA, 1 mM PMSF) DTT and PMSF must be added fresh on the day of homogenization.

5X Splicing Buffer +ATP

(175 mM KCl, 20 mM MgCl₂, 10 mM ATP, 100mM creatine phosphate (Roche 10-621-714-001), 7.5 mM DTT, 250 µg/mL creatine kinase (Roche 10-127-566-001), 15% PEG 8000) Store at –70°C to –80°C.

5X Splicing Buffer –ATP

(175 mM KCl, 20 mM MgCl₂, 7.5 mM DTT, 15% PEG 8000) Store at –70°C to –80°C.

Splicing Stop solution

(10 mM Tris-HCl pH 7.4, 250 mM NaOAc, 1 mM EDTA, 0.25% SDS, 2 µg/mL glycogen) Store at room temperature.

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References

- Hannon GJ, Maroney PA, Denker JA, Nilsen TW. Trans splicing of nematode pre-messenger RNA in vitro. *Cell*. 1990; 61:1247–255. [PubMed: 2163760]
- Hannon GJ, Maroney PA, Nilsen TW. U small nuclear ribonucleoprotein requirements for nematode cis- and trans-splicing in vitro. *J Biol Chem*. 1991; 266:22792–22795. [PubMed: 1835972]
- Lasda EL, Allen MA, Blumenthal T. Polycistronic pre-mRNA processing in vitro: snRNP and pre-mRNA role reversal in trans-splicing. *Genes Dev*. 2010; 24:1645–1658. [PubMed: 20624853]
- Shaked H, Wachtel C, Tulinski P, Yahia NH, Barda O, Darzynkiewicz E, Nilsen TW, Michaeli S. Establishment of an in vitro trans-splicing system in *Trypanosoma brucei* that requires endogenous spliced leader RNA. *Nucleic Acids Res*. 2010; 38:e114. [PubMed: 20159996]