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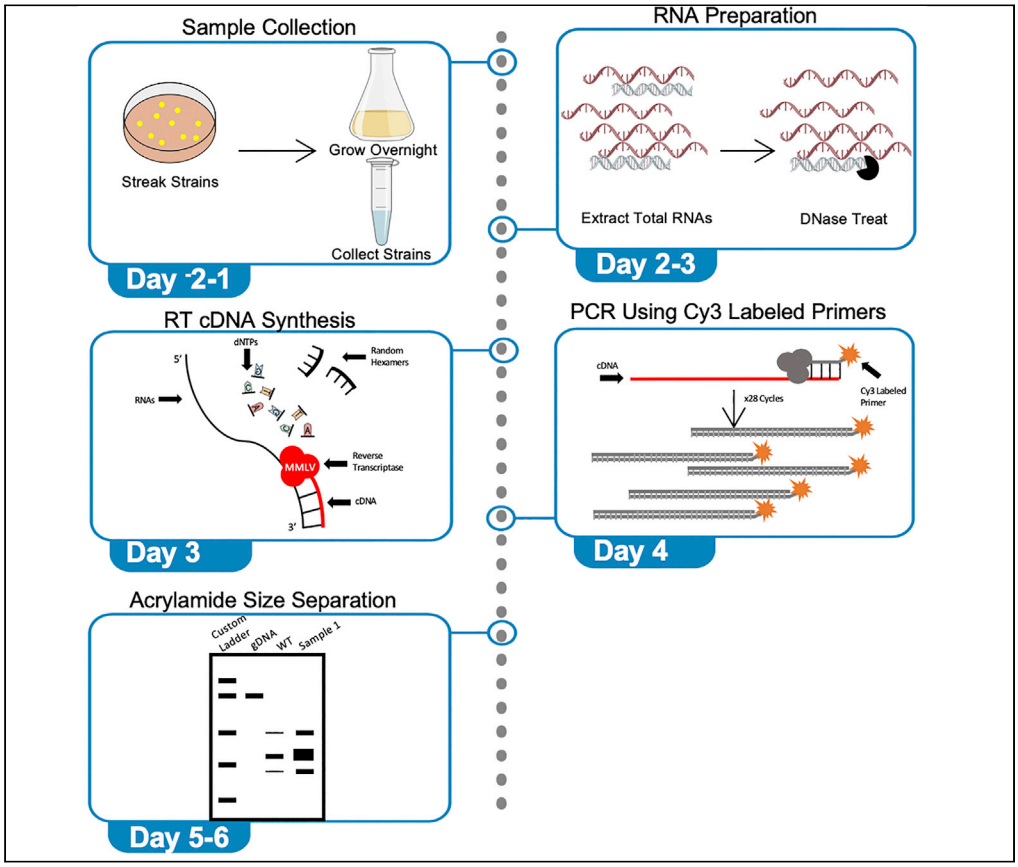
Protocol

Protocol for High-Resolution Mapping of Splicing Products and Isoforms by RT-PCR Using Fluorescently Labeled Primers



We describe an RT-PCR protocol that allows high-resolution mapping of splicing products and isoforms using fluorescently labeled primers. Each species contains one fluorescent group allowing a direct comparison of the different isoforms despite size differences. A custom size ladder enables the precise determination of cDNA lengths and discrimination of isoforms differing by less than five nucleotides on polyacrylamide gels. This protocol also allows the detection of products from *in vitro* splicing reactions, circumventing the need to use radiolabeled transcripts.

Indya Weathers, Jason Gabunilas, Joyce Samson, Kevin Roy, Guillaume F. Chanfreau

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HIGHLIGHTS

RT-PCR protocol to detect spliced and unspliced isoforms at high resolution

Use of Cy3-labeled primers circumvents the use of radioactivity

Includes detailed instructions to prepare a Cy3-labeled custom-size ladder

Protocol can be applied to detect spliced products *in vivo* and *in vitro*

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Protocol for High-Resolution Mapping of Splicing Products and Isoforms by RT-PCR Using Fluorescently Labeled Primers

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SUMMARY

We describe an RT-PCR protocol that allows high-resolution mapping of splicing products and isoforms using fluorescently labeled primers. Each species contains one fluorescent group allowing a direct comparison of the different isoforms despite size differences. A custom-size ladder enables the precise determination of cDNA lengths and discrimination of isoforms differing by less than five nucleotides on polyacrylamide gels. This protocol also allows the detection of products from *in vitro* splicing reactions, circumventing the need to use radiolabeled transcripts.
For complete details on the use and execution of this protocol, please refer to Gabunilas and Chanfreau (2016).

BEFORE YOU BEGIN

Timing: 3 days

1. Prepare the media appropriate for the yeast cultures.

Note: This protocol used Yeast Extract Peptone Dextrose (YPD) medium for the agar plates as well as the liquid growth medium, but the medium can differ depending on the strain of interest.

Yeast extract	10 g
Peptone	20 g
Dextrose	20 g
Agar (for plates)	20 g

Dissolve in 1 L of distilled water and autoclave for 20 min at 121°C and 0.5 bar.

2. 3 days before the start of the experiment (day −2), streak the strains of interest on the appropriate selective media and incubate for 2 days at 30°C.
3. The day before the start of the experiment (day 0), using a sterile 1,000 mL pipette tip, inoculate a single colony of each strain of interest into approximately 20 mL of appropriate media in sterile 50 mL flasks. Grow cells for 8 to 16 h while shaking (200 rpm) at 30°C.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
TURBO DNase Kit including 10× DNase Buffer	Invitrogen by Thermo Fisher Scientific	Cat # AM2239
M-MLV Reverse Transcriptase Kit including 5× First Strand Buffer and 0.1 M DTT	Invitrogen by Thermo Fisher Scientific	Cat # 28025-013
Chemicals, Peptides, and Recombinant Proteins		
Yeast Extract	Fisher Scientific	Cat # BP1422-2
Peptone	Fisher Scientific	Cat # BP1420-2
Dextrose	Fisher Scientific	Cat # D16-500
Agar	Fisher Scientific	Cat # BP1423-2
Random Hexamers	Invitrogen by Thermo Fisher Scientific	Cat # N8080127
RNase H	Invitrogen by Thermo Fisher Scientific	Cat # 18021071
Taq Polymerase	Purified in the lab	N/A
100 μM dNTP Solution Set	New England Biolabs	Cat # N0446S
Tris-HCl (1 M) pH 7.5	Fisher Scientific	Cat # BP1756-100
EDTA	Fisher Scientific	Cat # BP119-500
SDS	Sigma Aldrich	Cat # 11667289001
NaCl	Sigma Aldrich	Cat # S7653
Tris Base	Fisher Scientific	Cat # BP152-500
Boric Acid	Sigma Aldrich	Cat # B6768
EDTA (0.5 M) pH 8.0	Fisher Scientific	Cat # AAJ15694AE
Urea	Fisher Scientific	Cat # U15-500
40% Acrylamide (19:1)	Bio-Rad	Cat #1610144
Bromophenol Blue	Sigma Aldrich	Cat # B5525
Formamide	Sigma Aldrich	Cat # F9037
Phenol: chloroform: iso-amyl alcohol (25:24:1)	Millipore Sigma	Cat# 6810-400ML
GlycoBlue (15 mg/mL)	Fisher Scientific	Cat# AM9515
Ethanol (200 proof)	Sigma Aldrich	Cat# E7023
Isopropanol	Sigma Aldrich	Cat# W292912
TEMED	Thermo Scientific	Cat# 17919
Ammonium Persulfate (APS)	Thermo Scientific	Cat# HC2005
Ambion RNase Inhibitor (40 U/μL)	Thermo Fisher Scientific	Cat# AM2682
Experimental Models: Organisms/Strains		
S. cerevisiae: WT Strain background: BY4741	Open Biosystems	N/A
S. cerevisiae: Strain background: *upf1Δ::kanMX*	Open Biosystems	N/A
Oligonucleotides		
PHO85 40 bp ladder forward primer: 5′-GCTCTATGAACAGATTTAAGCAG-3′	This Paper	N/A

PHO85 50 bp ladder forward primer: 5′-TCAACCTCGAGCTCTATGAAC-3′	This Paper	N/A
PHO85 60 bp ladder forward primer: 5′-CGAGAAAAAATCAACCTCGAGC-3′	This Paper	N/A
PHO85 80 bp ladder forward primer: 5′GATGAATACTAACATATATACGAGA AAAAATCAACC-3′	This Paper	N/A
PHO85 100 bp ladder forward primer: 5′-GAAAGTAAAGAACTAGAAATGAT GAATACTAAC-3′	This Paper	N/A
PHO85 125 bp ladder forward primer: 5′-GTAGTTTTCTAGTCAAGTATCATTGG-3′	This Paper	N/A
PHO85 150 bp ladder forward primer: 5′-ACCAATGTCTTCTTCTTCACAG-3′	This Paper	N/A
PHO85 200 bp ladder forward primer: 5′-ATTGCAGATAAGGGATATATAGCG-3′	This Paper	N/A
PHO85 250 bp ladder forward primer: 5′-TTCTAATTGAAAGATAACAAAGGGAATCC-3′	This Paper	N/A
PHO85 300 bp ladder forward primer: 5′-TTCATTTTCCGTACAAATCACCATAC-3′	This Paper	N/A
PHO85 350 bp ladder forward primer: 5′-ATGCGCTACTTCTATCTATCCTAG-3′	This Paper	N/A
PHO85 400 bp ladder forward primer: 5′-GTCCAGACACGTTAAATGAATTGATAG-3′	This Paper	N/A
PHO85 500 bp ladder forward primer: 5′-TTTTAACAATGCAGAGCAAAAAAGTG-3′	This Paper	N/A
PHO85 Cy3 Reverse ladder primer: 5′-Cy3-TTGCCAAGCTTTTCTAACTGC-3′	This Paper	N/A
PRP5 Forward Primer: 5′-CGAGAAGGGAAATCATAACGC-3′	This Paper	N/A
PRP5 Cy3 Reverse Primer: 5′-Cy3-GGCTTAACCGATACAGGTTCG -3′	This Paper	N/A
RAD14 Forward Primer: 5′-GAACGTAACGCTATGACTCC-3′	This Paper	N/A
RAD14 Cy3 Reverse Primer: 5′-GGTCGCTACTCAGTATTCCC-3′	This Paper	N/A
Software & Algorithms		
Quantity One	Bio-Rad	N/A
Other		
3MM Chromatography Paper	Fisher Scientific	Cat # 057141
Pure Cellulose Chromatography Paper	Fisher Scientific	Cat # 057144
Gel-Running Apparatus	Bio-Rad	Cat # 1653860
Gel Dryer	Bio-Rad	Cat # 1651746
Molecular Imager PharosFX	Bio-Rad	Cat # 1709450
Molecular Imager Sample Tray	Bio-Rad	Cat # 1707811
0.5 mm Glass Disruptor Beads	Fisher Scientific	Cat# 50728198
250 mL Erlenmeyer Flask	Fisher Scientific	Cat# 10040F
50 mL Falcon Tube	Fisher Scientific	Cat# 1495949A
1.5 mL Conical Screw Cap Tubes	Fisher Scientific	Cat# 02707359
250 mL Graduated Cylinder	Thermo Fisher Scientific	Cat# 36620250

MATERIALS AND EQUIPMENT

TE-SDS Buffer	Final Concentration	Amount
Tris-HCl (1 M) pH7.5	10 mM	10 mL
EDTA (0.5 M) pH8.0	1 mM	2 mL
20% SDS	3% (w/v)	150 mL
ddH_2O	n/a	To 1 L
Total	**n/a**	**1 L**

TE Buffer	Final Concentration	Amount
Tris-HCl (1 M) pH7.5	10 mM	10 mL
EDTA (0.5 M) pH8.0	1 mM	2 mL
ddH_2O	n/a	To 1 L
Total	**n/a**	**1 L**

RNA Buffer	Final Concentration	Amount
Tris-HCl pH7.5 (1 M)	50 mM	2.5 mL
NaCl (5 M)	100 mM	1 mL
EDTA pH8.0 (0.5 M)	10 mM	1 mL
ddH_2O	n/a	To 50 mL
Total	**n/a**	**50 mL**

RNA-SDS Buffer	Final Concentration	Amount
Tris-HCl pH7.5 (1 M)	50 mM	2.5 mL
NaCl (5 M)	100 mM	1 mL
EDTA pH8.0 (0.5 M)	10 mM	1 mL
10% SDS	2%	10 mL
ddH_2O	n/a	To 50 mL
Total	**n/a**	**50 mL**

⚠ CRITICAL: EDTA is considered hazardous. Causes serious eye irritation, harmful if inhaled, and may cause damage to organs through prolonged or repeated exposure. Wear gloves and eye protection when handling. Use in well-ventilated area. Do not breathe dust/fume/gas/mist/vapors/spray.

⚠ CRITICAL: SDS (sodium dodecylsulfate) is harmful if swallowed, is toxic when in contact with skin, and causes skin and eye irritation. In powder form, it is hazardous by inhalation. When handling, wear protective gloves, clothing, and face coverings. In powder form, work in a closed fume hood to reduce inhalation.

70% Ethanol	Final Concentration	Amount
200 Proof Ethanol	70% (w/v)	350 mL
ddH2O	n/a	150 mL
Total	**n/a**	**500 mL**

⚠ CRITICAL: Ethanol is a highly flammable liquid and vapor and causes severe eye irritation. Wear protective gloves and eye/face protection while handling and wash skin thoroughly after handling. If exposed or concerned: get medical advice/ attention. If inhaled, remove

victim to fresh air and keep at rest in a position comfortable for breathing. If not breathing, give artificial respiration. If on skin, wash with plenty of soap and water then consult a physician. If in eyes, rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: get medical advice/attention. If swallowed, immediately call a doctor/physician and rinse mouth. In case of fire: use dry sand, dry chemical, or alcohol-resistant foam to extinguish. Store in a well-ventilated place and keep cool.

5× TBE Buffer	Final Concentration	Amount
Tris Base	450 mM	108 g
Boric Acid	450 mM	55 g
EDTA (0.5 M) pH 8.0	10 mM	40 mL
ddH_2O	n/a	Up to 2 L
Total	**n/a**	**2 L**

⚠ CRITICAL: Boric Acid has reproductive toxicity. Do not breathe dust/fume/gas/mist/vapors/spray. Wear protective gloves/ protective clothing/ eye protection/ face protection. If exposed or concerned: Get medical advice/ attention.

6% Acrylamide	Final Concentration	Amount
Urea	8 M	480.48 g
5× TBE	1×	200 mL
40% Acrylamide (19:1)	6%	150 mL
ddH_2O	n/a	Up to 1 L
Total	**n/a**	**1**

⚠ CRITICAL: Acrylamide is considered hazardous. It is toxic if swallowed and harmful if in contact with skin and eyes. Use in a well-ventilated area and wear protective gloves/protective clothing/eye protection/face protection. Do not breathe dust/fume/gas/mist/vapors/spray and do not eat, drink, or smoke when using this product. If exposed or concerned: get medical advice/ attention. If inhaled, remove victim to fresh air and keep at rest in a position comfortable for breathing. If on skin, wash with plenty of soap and water. If in eyes, rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: get medical advice/attention. If swallowed, immediately call a poison center or doctor/physician and rinse mouth.

5% Acrylamide	Final Concentration	Amount
Urea	8 M	480.48 g
5 TBE	1×	200 mL
40% Acrylamide (19:1)	5%	125 mL
ddH_2O	n/a	Up to 1 L
Total	**n/a**	**1 L**

10% APS	Final Concentration	Amount
Ammonium Persulfate	10% (w/v)	1 g
ddH2O	n/a	10 mL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Ammonium Persulfate is an oxidizer and is flammable. It has acute oral toxicity and specific target organ toxicity, causes skin corrosion/irritation as well as eye and respiratory irritation. Do not breathe dust/ fume/ gas/ mist/ vapors/ spray. Wear protective gloves/ protective clothing/ eye protection/ face protection. If exposed or concerned: get medical advice/ attention. If inhaled, remove victim to fresh air and keep at rest in a position comfortable for breathing. If on skin, wash with plenty of soap and water. If in eyes, rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: get medical advice/attention. If swallowed, immediately call a poison center or doctor/physician and rinse mouth.

2× Formamide Dye	Final Concentration	Amount
Bromophenol Blue	0.02% (w/v)	2 mg
EDTA (0.5 M)	50 mM	1 mL
Formamide	n/a	Up to 10 mL
Total	n/a	10 mL

⚠ CRITICAL: Formamide is carcinogenic, has reproductive toxicity, and specific target organ toxicity. Do not breathe dust/ fume/ gas/ mist/ vapors/ spray. Wear protective gloves/ protective clothing/ eye protection/ face protection. If exposed or concerned: get medical advice/ attention.

STEP-BY-STEP METHOD DETAILS

Grow *S. cerevisiae* Strains to Log Phase – Day 1

Timing: 4–10 h

The model organism used in this experiment, *S. cerevisiae*, is typically grown to log phase to assess the effects of mutations of splicing factors and/or cellular conditions on splicing *in vivo* when the cells are actively dividing.

1. Measure the optical density (OD) of the prepared yeast cultures following the 8–16 h (overnight) growth, and dilute the cultures to an OD 600 nm of 0.05 to 0.10 in 50 mL of medium in a 250 mL Erlenmeyer flask. Mix well and confirm the starting OD 600 nm of the cultures.
2. Grow *S. cerevisiae* strains to an OD 600 nm of 0.40 (2–3 doublings). For a wild-type strain, this should take about 4–5 h. Mutants strains will take longer, and approximate incubation times should be calculated according to their doubling time.
3. Once the desired OD 600 nm has been reached, spin down culture in 50mL falcon tubes for 3 min at 2,500 × *g*. The volume of the cell pellet after growth is about 0.25 mL.
   a. Carefully remove the media without disturbing the pellet, while leaving about 1 mL at the bottom of the tube. Use the 1mL to resuspend the pellet and transfer it to a 1.5 mL conical screw cap tube.
   b. Spin down 1.5 mL conical screw cap tube at 18,800 × *g* for 10 s. Remove remaining media and flash freeze in liquid nitrogen. Store at −80°C until ready for RNA extraction.
   c. ***Optional:*** to ensure complete removal of the culture medium, cell pellets can be washed in water prior to flash freezing.

Note: Use ethanol resistant markers to label tubes.

Extract WT Genomic DNA (gDNA) – Day 2

Timing: 1 h

The WT gDNA will be used to determine the size of the cDNA corresponding to unspliced products for the genes of interest. It is also used as a template to amplify fragments of the PHO85 gene of specific sizes, which are used for the custom size ladder preparation.

4. From the WT patch streaked on day −2, with a sterile p1000 pipette tip, scrape off some of the cells (collect approximately the size of a match tip) and dip the tip into 1 mL of sterile water.

Note: Only small amounts of gDNA are needed for this protocol as it is being amplified for the purpose of identifying the size of the unspliced gene products. The final concentration can vary widely from the efficiency of the phenol-chloroform extraction method and how many cells were used. Although, it is not critical that this measurement is precise, the final concentration should lie approximately between 100 ng/μL and 200 ng/μL so that in each PCR reaction, there is approximately between 150 ng and 300 ng of gDNA used.

   a. In safe lock tubes, centrifuge 1 min.
   b. Pipette out water.
5. Add 200 μL TE-SDS buffer. Vortex and incubate at 65°C for 5 min.
6. Add 400 μL of TE buffer and 600 μL DNA phenol-chloroform (pH adjusted to 8.0).
   a. Centrifuge at max speed for 5 min.
7. Take top aqueous layer and add to it 900 μL of 100% isopropanol.
   a. Pipette up and down to mix.
8. Spin at max speed for 10 min, then carefully pipette out the isopropanol without disturbing the cell pellet.
9. Wash the pellets with 200 μL 70%–75% ethanol and vortex briefly.
10. Spin down at max speed for 1 min, then decant the ethanol.
11. Add 100 μL of water.
    a. Warm at 95°C until the pellet dissolves.
12. Cool tubes to approximately 25°C and briefly spin down.
13. Mix until gDNA is fully resuspended.

Prepare the Fluorescently Labeled Custom Size Ladder for Size Determination

Timing: 4 h

The ladder will be used to determine the sizes of the PCR products on the gel. It is generated by amplifying multiple fragments of specific sizes of the PHO85 gene using one reverse Cy3-labeled primer, and multiple forward primers (Figure 1). This results in the production of Cy3 labeled DNA products of specific sizes which can be visualized after electrophoresis using the Bio-Rad imager and the Quantity One software. This strategy can be applied to generate any products of specific size; different genes can also be used to generate the ladder.

14. Prepare a PCR for each size fragment of the ladder. Extension time may be adjusted based on the size of the products.
15. Create a 5 μM forward/reverse primer mix making a 50 μL stock solution for each ladder size.

Note: It is recommended to generate as many reactions as possible such that the ladder samples can be used in multiple experiments.

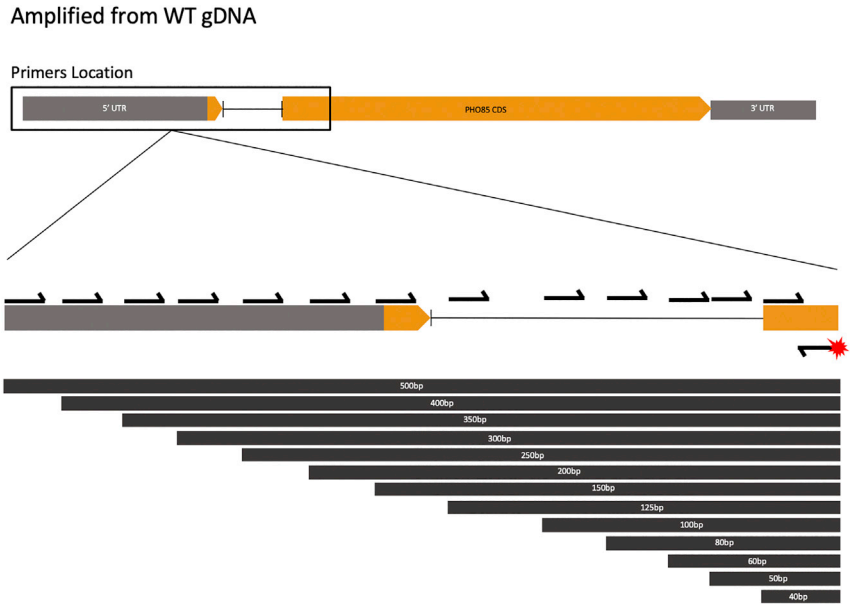

Figure 1. Generating a Size Ladder by Multi-Primer PCR

The size ladder is created using PCR reactions generating various fragments of the *S. cerevisiae PHO85* gene. Ladder DNA sequences ranging in size from 40 to 500 bp were created using 13 different forward primers in combination with one Cy3 labeled reverse primer. The appropriate sizes can be included into the gel analysis depending on the predicted product sizes.

16. For PCR include:

	1 Reaction	10 Reactions	15 Reactions
10× PCR Buffer	2.50 µL	25.0 µL	37.5 µL
25 mM dNTPs	0.20 µL	2.0 µL	3.0 µL
5 µM Primer Mix	2.50 µL	25.0 µL	37.5 µL
cDNA	1.50 µL	15.0 µL	22.5 µL
Taq Polymerase (2 U/µL)	0.50 µL	5.0 µL	7.5 µL
H_2O	17.8 µL	178 µL	267 µL
Total	25 µL		

17. Run the PCR using these conditions:

PCR Cycling Conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 s	35
Annealing	55°C	30 s	
Extension	70°C	1k bases/min or 30 s	
Final Extension	68°C	5 min	1
Hold	4°C	∞	1

Note: The PCR products can be checked on an agarose gel before running it on acrylamide to ensure that the reaction worked efficiently. We typically do not purify the PCR products to remove primer dimers, as these will generally run lower than the smaller fragments we detect compared to our size ladder.

Extract Total RNAs from *S. cerevisiae*

Timing: 4–6 h

This experiment is performed to analyze splicing efficiency *in vivo* and to visualize differently spliced RNA isoforms are produced under certain conditions or in the context of specific RNA processing or splicing factors mutations. RNAs are first extracted, and cDNAs are then generated such that they can undergo amplification via PCR.

18. To the cell pellets that were previously flash-frozen and stored in the 1.5 mL conical screw cap tubes, add 400 mL glass beads, 350 μL of RNA buffer, 350 μL of RNA/SDS buffer, and 700 μL of RNA Phenol-Chloroform (pH 6.7). The final concentration should be between 5 and 10 μg/μL per reaction.
    a. Vortex 1 min. Heat at 65°C for 6 min.
    b. Vortex 1 min. Spin down at 18,800 × *g* for 5 min.

⚠ CRITICAL: Use snap lock tubes for following Phenol-Chloroform steps as well as ethanol resistant markers to label tubes. Phenol/chloroform dissolves latex laboratory gloves, so change gloves if they become soiled with phenol/chloroform.

19. Take top 450 μL aqueous layer and add to 450 μL fresh RNA Phenol-Chloroform (pH 6.7).
    a. Vortex 1 min and spin at 21,400 × *g* for 2 min.
20. Take top 400 μL aqueous layer and add to 1 mL 100% ethanol and 40 μL 3 M NaOAc pH 5.2
21. Cool at −80°C for 30 min

Note: 3 M NaoAc pH 5.2 was made using the Cold Spring Harbor Protocol for Sodium Acetate (Sodium Acetate, 2015).

Pause Point: Samples can be stored at −80°C for several months.

22. Spin down at max speed for 10 min.
23. Remove supernatant and wash pellet in 500 μL 70% ethanol. Spin at max speed for 5 min.
24. Resuspend RNA in 20–50 μL nuclease free water while on ice.
    a. Determine the resuspended RNA concentration (aim for 5–10 μg/μL)

⚠ CRITICAL: Keep samples on ice to prevent RNA degradation.

Note: Best practices for handling RNAs involves maintaining a dedicated RNase-free workspace. Avoid using pipettes that have previously been used for experiments involving the use of Ribonucleases. The combination of high pH and high temperatures promotes RNA degradation, so using close to neutral pH and cold temperatures prevents RNA degradation.

DNase Treatment of Total RNAs – Day 3

Timing: 4–5 h

The extracted total RNAs will be reverse transcribed into cDNAs, so it is important to digest any contaminant DNA so that PCR products derive only from cDNAs and not gDNA.

Note: Reaction can be scaled down as necessary if you do not have enough RNAs.

⚠ CRITICAL: Use snap lock tubes for following Phenol-Chloroform steps as well as ethanol resistant markers to label tubes.

25. Take 40 μg of RNA and add 20 μL of 10× Turbo DNase buffer, 4 μL of 2 U/μL Ambion RNase-free Turbo DNase. Add nuclease free water to 200 μL.
26. Mix and incubate at 37°C for 45 min.
27. Add 200 μL RNA Phenol-Chloroform (pH 6.7) and vortex 1 min.
28. Spin at max speed for 5 min.
29. To your tube, add 200 μL RNA Phenol-Chloroform (pH 6.7).
    a. Vortex 1 min and spin at max speed for 5 min.
30. Take top aqueous layer and add to 1 mL 100% ethanol, 40 μL 3 M NaOAc pH 5.2,

Optional: Add 1 μL GlycoBlue to assist with visualization of the RNA pellet.

31. Cool at −80°C for 30 min
32. Remove supernatant and wash pellet in 500 μL 70% ethanol. Spin at max speed for 5 min. Remove supernatant, air dry RNA pellet.
33. Resuspend RNA in 10–20 μL nuclease free water while on ice.
34. Measure the resuspended RNA concentration.

cDNA Synthesis Using Invitrogen M-MLV Reverse Transcriptase and Random Hexamers

Timing: 1–2 h

cDNAs are prepared from the extracted RNA so that PCR amplification of the desired genes can occur. Random hexamers are used to ensure efficient cDNA synthesis of all RNAs.

35. In PCR tubes, combine 5 μg DNase treated RNA, 0.4 μL dNTPs (25 mM each), 1 μL 50 ng/μL random hexamers, and nuclease free water to 12 μL. The dNTP solution can be added during step 37 to improve efficiency of the cDNA synthesis step as dNTPs are more prone to degradation at high temperatures.
36. Heat at 65°C for 5 min to ensure the denaturation of RNA secondary structures that might prevent efficient annealing of the primers, then put on ice for 2 min.
37. While on ice, add to the PCR tubes 4 μL 5× First Strand Buffer, 2 μL 0.1 M DTT, 1 μL RNase Inhibitor (40 U/μL), and 1 μL (200 U/μL) M-MLV Reverse Transcriptase.
38. Incubate at 25°C for 10 min, then 37°C for 50 min, then 70°C for 15 min.
    a. *Optional:* Add 1 μL RNase H and incubate 37°C for 20 min.

Note: RNase H removes any RNAs that remain bound to the cDNAs after the reverse transcription step. Doing this can improve the sensitivity and efficiency of the PCR in the next step. The data presented in this protocol did not include RNase H in the experiments.

Note: Avoid frequent freezing/ thawing of dNTPs by aliquoting small amounts from the stock solution into flex tubes to be used for experiments. After thawing the aliquots, vortex thoroughly.

PCR Amplification of the cDNAs – Day 4

Timing: 3 h

Gene-specific Cy3-labeled primers are used with Taq Polymerase to amplify the cDNA of interest so that they can be analyzed via Quantity One by Bio-Rad.

39. For PCR include:

	1 Reaction	5 Reactions	10 Reactions
10× PCR Buffer	2.50 µL	12.5 µL	25.0 µL
25 mM dNTPs	0.20 µL	1.0 µL	2.0 µL
5 µM Primer Mix	2.50 µL	12.5 µL	25.0 µL
cDNA	1.50 µL	7.5 µL	15.0 µL
Taq Polymerase (2 U/µL)	0.50 µL	2.5 µL	5.0 µL
H_2O	17.8 µL	89 µL	178 µL
Total	25 µL		

40. Run the PCR using these conditions:

PCR Cycling Conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 s	28–35[a]
Annealing	55°C	30 s	
Extension	70°C	1k bases/min or 30 s	
Final Extension	68°C	5 min	1
Hold	4°C	∞	1

[a]Cycles number may vary based on the concentration and intensity of the PCR product. If 28 cycles result in a weak signal, increase the numbers of cycles up to 35 to improve sensitivity (see below).

Note: The PCR products can be checked on an agarose gel before running it on acrylamide to ensure the reaction worked efficiently.

41. Once the PCR reaction is complete, add 25 µL of 2× formamide dye to the PCR tubes. Heat them at 83°C for 5 min for denaturation.
42. Choose the appropriate ladder size samples for the predicted product.
    a. Take 2 µL of each appropriate size sample and combine in a PCR tube for a total of 10 µL. Add 10 µL 2× formamide dye to the PCR tubes. Heat them at 83°C for 5 min for denaturation.

Set Up the Gel Apparatus and Pour the Gel – Day 5

Timing: 10 min

Note: These instructions were written with reference to the Bio-Rad Sequi-Gen GT System (Bio-Rad #1653860), but any appropriately sized sequencing gel apparatus may be used (outlined in Figure 2).

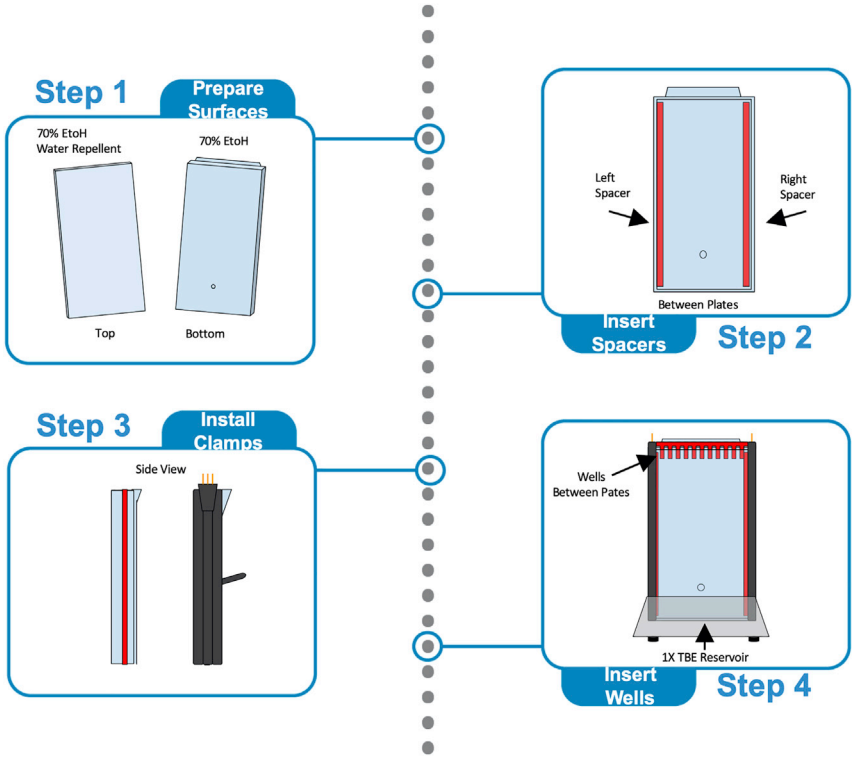

Figure 2. Acrylamide Gel Set-Up

To set up the Bio-Rad Sequi-Gen GT System (Bio-Rad #1653860) gel for RT-PCR gel fractionations, these steps need to be followed.

The surfaces need to be prepared by applying 70% ethanol to the inner sides of both plates and water repellent on the inner side of the top plate. The spacers need to be installed next and the side clamps placed on where the rods face backward. The bottom reservoir trough is then placed on the gel apparatus and the spacers are installed between the plates at the top.

43. Clean the interfaces of the gel plates using 70% ethanol. Treat the inner face of the top plate with a water repellent.

Note: To check for possible leakage before acrylamide is poured, water can be poured into the fully set up gel apparatus prior to the treatment of water repellent.

⚠ CRITICAL: It is important not to treat both plates with water repellent for minimal slipping of the gel when it starts running.

44. To set up the gel-running apparatus, position the spacers on either side of the plates then place the ethanol cleaned sides facing inward.
    a. Stand the plates up and add side clamps with the rods facing backward. Close the clamps tightly.
    b. Place the bottom of the apparatus into the cassette and press firmly onto the foam pad.
45. Insert the well comb in between the two plates with enough room at the top for the acrylamide to empty out between the lanes.

Prepare the Gel

Timing: 2–3 h

The PCR product must be fractionated on 5%–8% sequencing-type acrylamide gels to ensure distinct separation of products down to 1 nucleotide in difference. Larger products between 350 and 500 bp separate better at lower percentage gels while smaller products between 40 and 350 bp separate better at higher percentages.

46. In a 250 mL graduated cylinder, combine: 120 mL of the proper percentage denaturing acrylamide solution, 1 mL fresh 10% APS (or 750 μL for 8%–10% gel), and 200 μL TEMED (or 150 μL for 8%–10% gel).
    a. With parafilm over the top of the cylinder, invert three times to mix.
    b. With the plunger detached from the 300 mL syringe, make sure the tubing is attached on one side to the tip of the syringe and on the other to the tip adapter. Hold the end of the tip adapter side of the tubing above the highest point on the syringe while pinching the end closed to prevent leakage and pour the mixture from the cylinder into the open end of the syringe.
    c. Allow a small volume of the mixture to run out of the tube to clear out any air.

Note: In solution, APS degrades over time and should be stored at 4°C for up to 3 weeks.

⚠ CRITICAL: It is important to move quickly to prevent the gel from solidifying before it is fully poured. 8%–10% percentage gels polymerize faster than 5%.

Note: If air bubbles are forming in the gel, gently tapping the gel while pouring can prevent or remove these bubbles. If there is excessive leaking, dissemble the gel apparatus and thoroughly clean all the components. Ensure that there is no hardened acrylamide anywhere in the side clamps or the bottom reservoir and reassemble pressing everything together tightly.

47. Connect the syringe tube tip adapter into the hole at the bottom stopper to allow the gel mixture to flow into the plates. Gently tap the glass to prevent air bubbles from forming as the gel flows.
    a. Once the gel has polymerized, clean out any leaked or spilled acrylamide that might have fallen into the buffer space.
48. After about 30 min, when the gel has polymerized remove the bottom stopper from the apparatus, add about 1 inch of 1× TBE to the bottom buffer reservoir then carefully place the clamped gel apparatus into the reservoir.
    a. Add the bracer to prevent the plates from swinging.
49. Add 1× TBE to the top of the gel and allow it to flow down and occupy the buffer space behind the rear gel plate.
50. Carefully and evenly remove the well comb.
51. Using a syringe, wash out the wells using 1× TBE and make sure no bubbles remain.
52. Connect the leads to the gel apparatus and place the top cap electrodes onto the gel cassette.
53. Pre-run the gel at 150 W until it reaches 55°C. Once at temperature, reduce the power down to 120 W. Using a syringe, wash out the wells using 1× TBE once more to make sure no bubbles remain and that the wells are free of urea.
54. Using the center wells, load 5 to 16 μL of each sample into the gel.
55. Run the gel at 120 W until the dye front reaches 3/4 of the way down the gel.

Note: Depending on the size of the products and how many alternative products are present, the gel may need to be run longer to ensure proper separation.

Dry the Gel

Timing: 1.5 h

The results of the experiment will be analyzed using the Bio-Rad imager and the Quantity One software, which requires a dried product to prevent distortions and to prevent hardened acrylamide from being inserted in the machine. Drying is also important so that the gel can be preserved.

56. After the gel has finished running, disassemble the apparatus and pour out the buffer. Carefully remove the gel from the running rig, keeping the two gel clamps on the sides of the plate.
    a. Cool the gel to approximately 20°C–25°C by running cold tap water over the gel plates and inside the buffer reservoir in the rear plate.
57. Remove the clamps and gently lift off the top plate. The gel should stick to the bottom plate.
58. Place a sheet of Whatman paper on the gel, smooth side down, and push down gently to ensure good, even contact. Lift the Whatman paper off the plate with the gel stuck to it.

⚠ CRITICAL: Do not allow that Whatman paper to get too wet, or the gel will begin to ripple and distort.

59. Place a sheet of saran wrap over the gel, taking care to minimize the number of wrinkles that form in the saran wrap. Gently press out air bubbles between the saran wrap and the gel.
60. While sandwiched between the Whatman paper and saran wrap, cut off the wells at the top of the gel as well as just above the dye at the bottom of the gel.
61. Place the cut gel still sandwiched between the Whatman paper and saran wrap, between another pair of Whatman papers. Place sandwich onto the mesh surface of the gel dryer.
    a. Dry the gel for 1 h at 80°C.
    b. Place the gel in the Bio-Rad imager for imaging.

EXPECTED OUTCOMES

After gel electrophoresis, gel drying and imaging, the cDNA corresponding to the various isoforms can be visualized. The migration of the cDNAs corresponding the unspliced products is the same as that of the product obtained by the amplification of the WT gDNA. Different spliced products can be viewed which migrate faster than the unspliced cDNA. By comparing the size of the different fragments to the ladder, the size of the PCR products can be verified and compared to the predicted size of the cDNAs arising from the use of different splice sites as shown in Figures 3 and 4. The abundance of each isoform can be directly quantified from the signal obtained for each band because each species amplified by PCR contains a single cyanine-3 fluorescent group which is used for the detection.

Other Applications: *In Vitro* Splicing Products Detection

We have used this protocol extensively to identify splicing isoforms generated *in vivo* from various *S.cerevisiae* strains. However, this protocol can also be used to detect spliced and unspliced RNAs generated after *in vitro* splicing in whole cell extracts. In this case, the protocol can be started at step 35, after *in vitro* splicing and extraction of the RNAs from the extracts, as described in Gabunilas and Chanfreau (2016). The major advantage of this approach is that non-radiolabeled RNA substrates can be used in the *in vitro* splicing reactions, and that the products corresponding to spliced and unspliced RNAs each carry a single fluorescently labeled group. Thus, signal intensity is not correlated to product length, as opposed to typical *in vitro* splicing reactions which usually use RNA internally radiolabeled.

LIMITATIONS

In this experiment the reverse transcriptase used was M-MLV. For more efficient cDNA synthesis, SuperScript III can be used. This protocol has been optimized to amplify products from 40 bp to 500 bp in length. Products larger than 600 bp may not resolve well through the acrylamide gel. Differences in product sizes are optimized around 20–200 bp in length. Larger product size differences can lead to the smaller products running off the gel before the larger ones resolve past the wells.

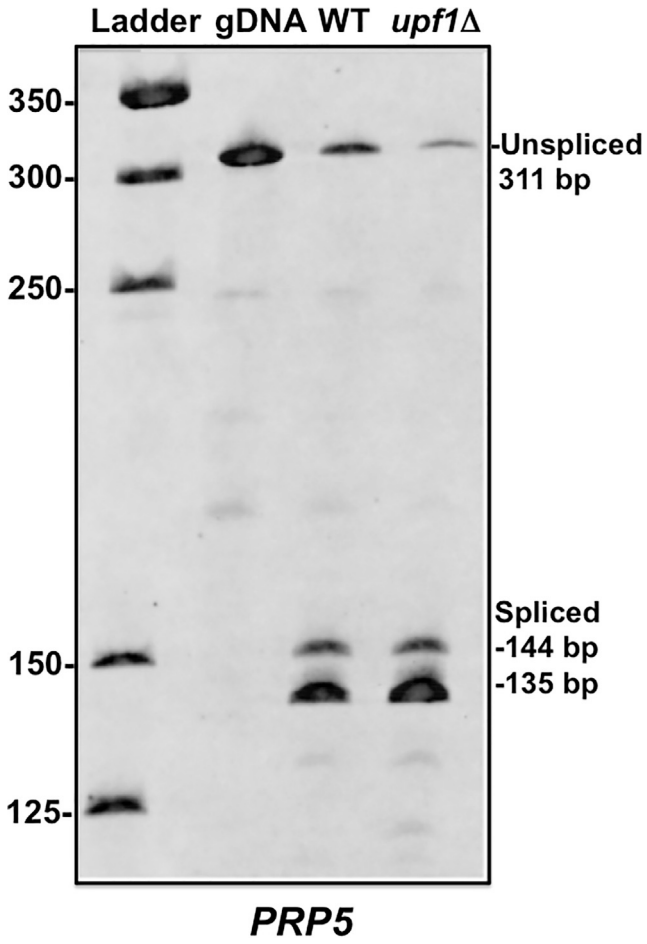

Figure 3. RT-PCR Analysis of the RAD14 mRNAs

Using this protocol, mRNAs encoding the Prp5 protein were reverse transcribed and amplified by PCR (28 Cycles) and two major spliced isoforms identified, one resulting in a cDNA of 144 bp, and another of 135 bp in length.

A WT gDNA sample was included to determine the length of the unspliced product (311 nt) and to also rule out potential PCR artifacts not specific to spliced isoforms obtained in cDNA samples. The unspliced transcript results in a PCR product of 311 bp, so a 5% acrylamide gel gave optimal separation of spliced and unspliced cDNAs. A size ladder (far left lane) was added to identify the sizes of the products. The NMD deficient *upf1Δ* strain typically stabilized unspliced transcripts or spliced isoforms that are NMD-sensitive (Kawashima et al., 2009, 2014). However, in the case of *PRP5*, no major differences were observed compared to the WT strain, except for a slight decrease in accumulation of unspliced transcript, and the increase of minor spliced isoforms correspond to very weak bands migrating at 120 and 130 nt.

TROUBLESHOOTING

Problem

Detection of rare mRNAs or spliced isoforms by RT-PCR can be challenging. When these species are not present abundantly enough to be detected using the standard protocol, the ladder as well as the WT gDNA bands will be detected on the gel, but no cDNA corresponding to the spliced mRNAs will be visible.

Potential Solutions

The easiest possible option to increase the likelihood of detecting rare species is to increase the number of cycles used in the PCR reaction (eg from 28 cycles to 35 cycles; see for instance *RAD14*, Figure 4). This may result in a more efficient amplification of the cDNAs and their detection on the gel. If this is not sufficient, the efficiency of the reverse transcription step can be improved by

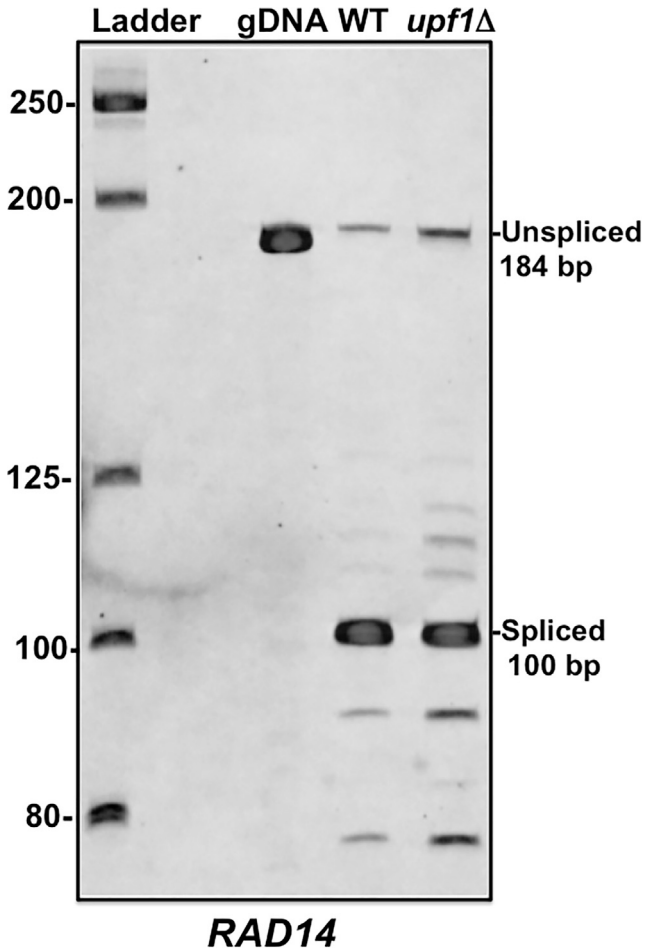

Figure 4. RT-PCR Analysis of the *RAD14* mRNAs

Using this protocol, several splicing isoforms of the *RAD14* mRNA were identified by RT-PCR (35 Amplification Cycles were used as a 28 cycles reaction was insufficient to amplify cDNAs).

The primary spliced and unspliced products generate cDNAs of 100 bp and 184 bp, respectively. In the *upf1Δ* strain, several alternatively spliced products accumulate to a higher extent when compared to the WT strain, most notably spliced isoforms with cDNAs migrating at 75 and 100 nt. A WT gDNA sample was included to determine the length of the unspliced product. A 6% acrylamide gel provided optimal separation of the PCR products corresponding to the alternatively spliced isoforms. Specific ladder products of 80, 100, 125, 200, and 250 bp were included based on the predicted sizes of the products.

using a gene-specific cDNA primer instead of random hexamers in step 35 of this protocol. This will lead to a more efficient cDNA synthesis of the specific mRNA target analyzed and should result in a better PCR amplification of the spliced isoforms for this specific mRNA. The disadvantage of this option is that the cDNAs generated using gene-specific primers cannot be used to amplify other mRNAs, and that single reverse transcription reactions need to be performed for each mRNA analyzed, which is more time consuming and less cost-effective. Another possibility is to use poly(A)-selected RNAs (using oligo-dT purification) instead of total RNAs, which will increase the yield of cDNA synthesis from mRNAs.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Guillaume Chanfreau (guillom@chem.ucla.edu).

Materials Availability

All the materials used in this protocol are commercially available. The yeast strains used to generate the data shown are available from Open Biosystems.

Data and Code Availability

This protocol includes a sample of data obtained from an ongoing study.

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AUTHOR CONTRIBUTIONS

This work was completed by I.W., J.S., K.R., J.G., and G.C. K.R. and J.G. optimized earlier versions of the protocol. I.W. optimized and produced the data for this protocol and wrote the first draft of the manuscript. G.C., J.G., and J.S. created the ladder used for size determination. G.C. acquired funding and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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