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

Indya Weathers,1,2 Jason Gabunilas,1 Joyce Samson,1 Kevin Roy,1 and Guillaume F. Chanfreau1,3,*

1Department of Chemistry and Biochemistry, University of California, Los Angeles, 607 Charles E. Young Drive East, Los Angeles, CA 90095-1569, USA
2Technical Contact
3Lead Contact
*Correspondence: guillom@chem.ucla.edu
https://doi.org/10.1016/j.xpro.2020.100140

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.

   | Ingredient         | Quantity |
   |--------------------|----------|
   | 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.
| 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 # 1167289001 |
| 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’-GCTCTATGAACAGATTAAAGCAG-3’ | This Paper | N/A |
### PHO85 Ladder Forward Primers

| Ladder Size | Primer Sequence | Source |
|-------------|-----------------|--------|
| 50 bp       | 5'-TCAACCTCGAGCTACATGAAC-3' | This Paper, N/A |
| 60 bp       | 5'-CGAGAAAAATCAACCTCGAGC-3' | This Paper, N/A |
| 80 bp       | 5'-GATGATATACATATATGAGAAATAACACC-3' | This Paper, N/A |
| 100 bp      | 5'-GAAAGTAAAGAACTGAAATGATGAATCTAAC-3' | This Paper, N/A |
| 125 bp      | 5'-GTAGTTTTCATAGTAATATGCTTAC-3' | This Paper, N/A |
| 150 bp      | 5'-ACCCACTTCTCTCCTCAG-3' | This Paper, N/A |
| 200 bp      | 5'-ATTGCGATAGGGGATTATACG-3' | This Paper, N/A |
| 250 bp      | 5'-TTCTAATTGAAAGATAACAAAGGGAATCC-3' | This Paper, N/A |
| 300 bp      | 5'-TTATTCCGTAACATAATACCATAC-3' | This Paper, N/A |
| 350 bp      | 5'-ATGGGCTACTTCTATCTCTTCTAG-3' | This Paper, N/A |
| 400 bp      | 5'-GTCCAGACCGTTAAATGATTGAT-3' | This Paper, N/A |
| 500 bp      | 5'-TTTCAAAATGCGAGCAAAAAAGTG-3' | This Paper, N/A |
| Cy3 Reverse | 5'-Cy3-TTGCCAAGCTTTTCTAACTGC-3' | This Paper, N/A |

### PRP5 Forward Primer

| Primer | Source |
|--------|--------|
| 5'-CGAGAAGGGAAATCATACGC-3' | Bio-Rad, N/A |

### PRP5 Cy3 Reverse Primer

| Primer | Source |
|--------|--------|
| 5'-GGCTTAACCGGATACAGGTTC-3' | Bio-Rad, N/A |

### RAD14 Forward Primer

| Primer | Source |
|--------|--------|
| 5'-GAAAGTAAAGAACTGAAATGATGAATCTAC-3' | Bio-Rad, N/A |

### RAD14 Cy3 Reverse Primer

| Primer | Source |
|--------|--------|
| 5'-GCTCGCTACTCAGTATTCCC-3' | Bio-Rad, N/A |

### Software & Algorithms

| Technology | Source |
|------------|--------|
| Quantity One | Bio-Rad, N/A |

### Other

| Item Description | Source |
|------------------|--------|
| 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 |
| ddH2O | 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 |
| ddH2O | 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 |
| ddH2O | 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 |
| ddH2O | 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.

| 5x 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  |
| ddH₂O          | 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 |
| 5x TBE         | 1x                  | 200 mL  |
| 40% Acrylamide (19:1) | 6%               | 150 mL  |
| ddH₂O          | n/a                 | Up to 1 L |
| Total          | n/a                 | 1 L     |

△ 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          | 1x                  | 200 mL  |
| 40% Acrylamide (19:1) | 5%            | 125 mL  |
| ddH₂O          | 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.

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

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 x 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 x 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.
16. For PCR include:

| Component                  | 1 Reaction | 10 Reactions | 15 Reactions |
|----------------------------|------------|--------------|--------------|
| 10x 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       |
| H₂O                        | 17.8 µL    | 178 µL       | 267 µL       |
| Total                      | 25 µL      |              |              |

17. Run the PCR using these conditions:

| PCR Cycling Conditions | 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 10x 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 5x 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 |
|------------|-------------|--------------|
| 10x 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 |
| H₂O | 17.8 µL | 89 µL | 178 µL |
| **Total** | **25 µL** | **25 µL** | **25 µL** |

40. Run the PCR using these conditions:

**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 Seqi-Gen GT System (Bio-Rad #1653860), but any appropriately sized sequencing gel apparatus may be used (outlined in Figure 2).
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, disassemble 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.
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 (e.g., 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...
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).

---

**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.
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.

ACKNOWLEDGMENTS
This work was supported by the National Institute of General Medical Sciences grant R35 GM130370 to G.C. J.G. was supported by the National Research Service Award Training Grant GM007185.

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.

REFERENCES
Kawashima, T., Pellegrini, M., and Chanfreau, G.F. (2009). Nonsense-mediated mRNA decay mutes the splicing defects of spliceosome component mutations. RNA 15, 2236–2247.
Kawashima, T., Douglass, S., Gabunilas, J., Pellegrini, M., and Chanfreau, G.F. (2014). Widespread use of non-productive alternative splice sites in Saccharomyces cerevisiae. PLoS Genet. 10, e1004249.
Gabunilas, J., and Chanfreau, G. (2016). Splicing-Mediated Autoregulation Modulates Rpl22p Expression in Saccharomyces cerevisiae. PLoS Genet. 12, e1005999.
Sodium Acetate (3 m, pH 5.2). Cold Spring Harb. Protoc. 2015, pdb.rec085761.