

# Chapter 2

## Targeted Cloning of Fungal Telomeres

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### Abstract

Telomeres are the sequences that form the ends of eukaryotic chromosomes and are essential structures that confer genome stability and guide chromosome behavior. In addition, the terminal regions of the chromosomes tend to house genes with predicted roles in ecological adaptation. Unfortunately, however, most fungal genome assemblies contain very few telomeres and, therefore, the identities of genes residing near the chromosome ends are often unknown. In an effort to develop a complete understanding of the organization and gene content of chromosome ends in a number of fungi, we developed efficient methods for the identification and targeted cloning of telomeres. This chapter describes the basic steps and shows exemplary results from the targeted cloning of *Epichloë festucae* telomeres.

**Key words:** Genome sequencing, Subcloning, Southern blotting, Colony blotting

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### 1. Introduction

Telomeres protect chromosome ends from degradation caused by normal DNA replication processes and enzymatic activity. They also play important roles in chromosome biology by initiating chromosome pairing (1, 2) and directing chromosome movement (3, 4). The chromosome regions near the telomeres tend to be highly dynamic (5–7), show increased genetic variation (8, 9) and often house genes that enhance an organism's adaptive capabilities (10). Finally, there is evidence that fungi possess specialized mechanisms for regulating the expression of telomere-linked genes (11, 12). However, despite their obvious importance, telomeres are frequently missing from fungal genome sequences (13–15). This is disadvantageous for two reasons. First, the telomeres can be important landmarks for guiding the genome assembly process and verifying the final product. Second, if the telomeres are absent, the sequences that reside at the chromosome ends are unknown and, therefore, information on fungal

genes with potential evolutionary and ecological significance remains elusive.

Analysis of raw sequence data has shown that telomere sequences are frequently captured in genome sequencing efforts but simply escape assembly. Such sequences can be identified and incorporated into genome assemblies through the use of the bioinformatic tool TERMINUS (13). However, even after exhaustive mining of raw sequence data, we find that most genome assemblies still lack sequence information for a number of telomeres. Therefore, in most cases, it is necessary to clone the missing chromosome ends using a targeted approach.

Native telomeres are refractory to cloning because their 3' ends protrude as single stranded tails (16). Therefore, the overhanging nucleotides must be removed before the telomeres can be cloned. Once this has been achieved, however, it is possible to take advantage of the telomere's terminal position to enrich for telomeric restriction fragments. This can be accomplished by using a directional cloning strategy to select for fragments that are blunt at one end and sticky at the other. In this manner, only fragments that are at the ends of DNA molecules are recovered.

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## 2. Materials

### **2.1. Extraction of Genomic DNA**

1. Lysis buffer: 0.5 M NaCl, 1% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8. Store at room temperature. Heat at 65°C before using to dissolve the SDS.
2. Phenol:chloroform:isoamylalcohol. 25:24:1 (PCI): 25 ml phenol equilibrated with Tris-HCl, pH 8; 24 ml chloroform (equilibrated with Tris-HCl, pH 8), 1 ml isoamylalcohol. Store under 0.1 M Tris-HCl, pH 8 in a tightly capped, dark glass bottle at 4°C. Before using, check that the PCI is colorless. Discard and make up a fresh batch if any hint of color is detected.
3. Chloroform:isoamylalcohol 24:1 (CI) 24 ml chloroform (equilibrated with Tris-HCl, pH 8), 1 ml isoamylalcohol. Store in a tightly capped, dark glass bottle at 4°C.
4. T0.1E buffer: 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, pH 8. Store at room temperature.

### **2.2. Agarose Gel Electrophoresis**

1. TBE (10× stock): Add the following to 800 ml of H<sub>2</sub>O: 108 g Tris base, 55 g boric acid and 9.3 g EDTA. Adjust volume to 1 l with additional H<sub>2</sub>O. Make a 0.5× working solution by diluting 20-fold in H<sub>2</sub>O.
2. A 0.7% agarose gel solution (200 ml): add 1.4 g agarose to 200 ml 0.5× TBE. Heat in a microwave at high setting for 4–5 min. Swirl to ensure that all the agarose has dissolved

fully. The agarose solution can be stored molten in a 55°C oven for up to 2 days until needed.

3. Loading Dye (6× stock): 10 mM Tris-HCl, pH 8, 60 mM EDTA, 0.03% bromophenol blue.
4. Kilobase plus DNA size marker (Invitrogen, Carlsbad, CA).
5. Parafilm® (Alcan, Inc., Montreal, QE).
6. Ethidium bromide: Make a stock solution containing 5 mg/ml ethidium bromide.

### **2.3. Electrophoresis**

1. Denaturation solution: 0.5 M NaOH. Store at room temperature.
2. 20× SSC: 3 M NaCl, 0.3 M Na citrate, pH 7. Store at room temperature. Make working solutions by diluting in H<sub>2</sub>O.
3. Jumbo Genie blotting apparatus (Idea Scientific, Minneapolis, MN).
4. Pall Biodyne B Hybridization membrane (Pall Corp., Pensacola, FL).
5. Whatman 3M paper (Whatman, Florham Park, NJ).

### **2.4. Southern Hybridization Analysis**

1. Telomere oligonucleotides: TEL1, 5'-TTAGGGTTAGGGT TAGGGTTAGGG-3' and TEL2, 5'-CCCTAACCCTAACCC TAACCCTAA-3'.
2. ExTaq PCR reagents (Takara, Shiga, Japan).
3. Labeling kit (Promega Corp., Madison, WI).
4. Dye Stop solution: 10 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8, 0.8% dextran blue, 0.04% orange G.
5. Illustra MicroSpin™ G50 columns (GE Healthcare, Piscataway, NJ).
6. Hybridization solution: 0.125 M NaHPO<sub>4</sub> (from a 4× stock consisting of 0.5 M Na<sub>2</sub>HPO<sub>4</sub> that has been adjusted to pH 7.5 with phosphoric acid), 7% SDS, 1 mM EDTA (use 0.5 M EDTA, pH 8 stock).
7. Wash solutions: low stringency, 2× SSC; high stringency, 0.1× SSC, 0.1% SDS.

### **2.5. Plasmid Vector**

1. The vector we use for telomere cloning, pBS-TEL1, is based on the pBluescript KS II<sup>+</sup> (Stratagene, La Jolla, CA) and contains a ~2 kb *Eco*RI “stuffer” fragment inserted into the *Eco*RI site (Fig. 1). The advantage of using this particular plasmid is explained in Note 1.

### **2.6. Molecular Biology Reagents**

1. Restriction enzymes: *Hind*III and *Sma*I (New England Biolabs, Ipswich, MA).
2. Bovine serum albumin (BSA): make a 10× stock (1 mg/ml) by diluting the 100× stock provided by the manufacturer (NEB) tenfold with H<sub>2</sub>O.

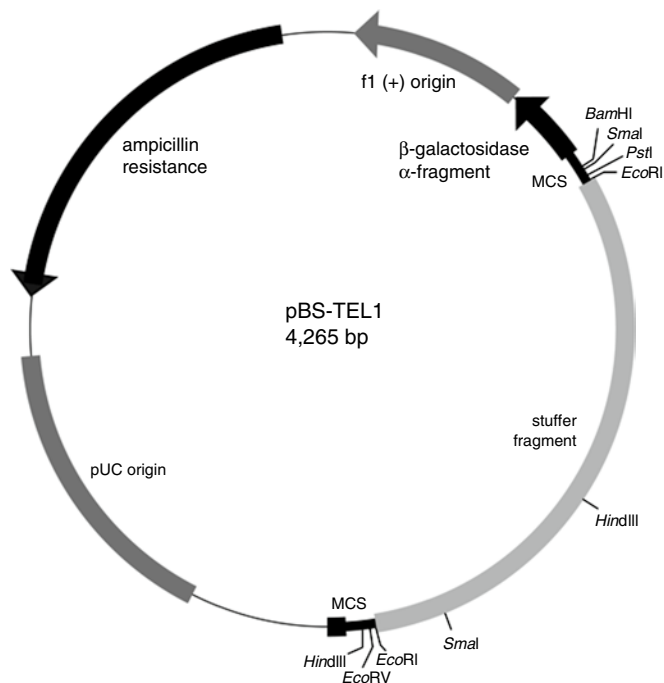


Fig. 1. Plasmid pTEL1. The pBLUESCRIPT KS II + (pBS) vector backbone and 2 kb stuffer fragment are labeled. Ap, ampicillin resistance gene; ORI, origin of replication; f1 ori, F1 origin of replication. Relevant restriction sites in the pBS polylinker and the stuffer fragment are shown.

3. Prime-A-Gene<sup>®</sup> DNA labeling kit (Promega Corp., Madison, WI).
4. End Repair: End-It<sup>™</sup> kit (Epicentre<sup>®</sup> Biotech., Madison, WI).
5. Calf Intestinal Alkaline Phosphatase (CIAP, Promega).
6. LigaFast<sup>™</sup> Rapid DNA Ligation System (Promega).

## 2.7. *Escherichia coli* Transformation

1. Commercially prepared electrocompetent cells: EPI300<sup>™</sup> (Epicentre) or Ecloni<sup>®</sup> 10G (Lucigen Corp., Madison, WI) (see Note 2).
2. Electroporation cuvettes with 2 mm gap.
3. Luria-Bertani (LB) medium: One liter of medium contains 10 g tryptone, 5 g yeast extract and 10 g NaCl. For solid media, add agar (15 g/l). Sterilize by autoclaving at 121°C, 15 p.s.i. for 20 min.
4. Petri plates containing LB agar supplemented with 100 µg/ml ampicillin. Store plates for up to 1 month in the dark at 4°C.
5. Rattler Plating Beads: Zymo Research Corporation (Orange, CA).
6. Slide-A-Lyzer Mini Dialysis tubes (3,500 MWCO): Pierce (Rockford, IL).

### 2.8. Colony Hybridization

1. Whatman 541 paper (Whatman, Florham Park, NJ): cut into 82 mm circles, wrap in foil and sterilize by autoclaving.
2. Colony lysis buffer: 0.5 M NaOH. Store at room temperature.
3. Neutralization solution: 1 M Tris-HCl, pH 7.5. Store at room temperature.
4. 2× SSC: Add 100 ml of 20× SSC (see above) to 900 ml H<sub>2</sub>O.
5. 95% EtOH.
6. Colony blot, prehybridization solution: 5× SSC, 0.1% SDS.

## 3. Methods

### 3.1. Preparation of High Molecular Weight Genomic DNA (see Note 3)

“Cut-off” pipette tips and slow pipetting should be used throughout the following procedures to minimize shearing of the DNA.

1. Place ~200 mg of freeze-dried mycelium in a 15 ml Falcon tube and use a glass rod to grind it against the side of the tube, forming a powder. Add 1.5 ml of lysis buffer that has been preheated to 65°C and mix gently using the glass rod. Place the cap on the tube and incubate in a 65°C water bath for 10 min. Add 1 ml of PCI and mix by gentle inversion. Screw the cap on tightly and return the tube to the water bath. Incubate for 30 min and gently invert the tube several times every 10 min to remix the PCI layer with the aqueous phase.
2. Pellet the cell debris by centrifuging at 3,000 × *g* for 30 min. Use a 1 ml pipette with a cut-off tip to recover 1 ml of supernatant and transfer it to a microfuge tube. Precipitate the DNA by adding 0.54 ml of room temperature isopropanol. If there is a large mass of DNA, spool it onto a glass rod (or sealed Pasteur pipette). Otherwise, pellet the DNA by centrifuging at 18,000 × *g* (see Note 4). Wash the pellet with 70% ethanol and air dry. Redissolve in 100 µl of T0.1E buffer.
3. Quantify the DNA solution using a fluorometer (see Note 5) and adjust DNA concentration to 100 ng/µl.
4. If the DNA solution contains too much polysaccharide, this could interfere with future manipulations. Therefore, if it is very milky in appearance, a differential precipitation procedure (17) should be used to reduce the level of polysaccharide contamination.

### 3.2. Restriction Digestion of Genomic DNA

1. Pipette 500 ng of genomic DNA (100 ng/µl) into a microcentrifuge tube. Add 5 µl of 10× restriction buffer and 5 µl of 10× BSA. Bring the volume up to 49 µl with sterile dH<sub>2</sub>O. Then, add 1 µl (10–20 U) of restriction enzyme and mix well by gently flicking the tube. Incubate at 37°C (or other appropriate temperature) overnight.

2. Remove a 10  $\mu$ l aliquot of the digest and pipette onto a small sheet of Parafilm. Add 2  $\mu$ l of 6 $\times$  loading dye solution.
3. Store the remaining digestion reaction at  $-20^{\circ}\text{C}$  so that if electrophoresis shows the digestion to be not quite complete, additional enzyme can be added and the tube incubated for an additional overnight period.

### **3.3. Agarose Gel Electrophoresis**

1. Prepare a 0.7% agarose gel solution in 0.5 $\times$  TBE buffer and pour into a gel unit that is at least 20 cm long. Use gel combs with teeth that are  $\leq 1$  cm wide but which occupy a volume of at least 50  $\mu$ l.
2. Place the gel in an electrophoresis unit filled with 0.5 $\times$  TBE buffer. Load each restriction digestion reaction into a separate well. Include a DNA size ladder in at least one well. Run the gel overnight using a low voltage (e.g. 30 V, 20 h).
3. Stain the gel by placing it in ethidium bromide staining solution (made by adding 10  $\mu$ l EtBr stock solution to 200 ml 0.5 $\times$  TBE) and incubate for 30 min with gentle shaking.
4. Transfer the gel to a transilluminator and place a ruler alongside it. Switch on the UV lamp and take a photographic/digital image of the gel. Make sure that the gradations on the ruler are visible in the image.

### **3.4. Electroblotting**

1. Presoak two electroblotter pads (supplied with the unit) in a large tray containing 0.5 $\times$  TBE. Use gloved hands to press out air bubbles.
2. Place the cathode into the Genie blotter tray, followed by a plastic grid. Fill the tray to half full with 0.5 $\times$  TBE.
3. Put a single electroblotter pad on top of the plastic grid and press again to expel air.
4. Cut two pieces of Whatman 3M paper and a single sheet of Pall Biodyne B hybridization membrane to the size of the agarose gel. Soak one piece of 3 M paper in 0.5 $\times$  TBE and place it on top of the 3 M blotting pad.
5. Place the gel – open ends of the wells facing downward – on top of the 3 M sheet. Make sure that no air bubbles are trapped underneath the gel.
6. Wet the membrane in 0.5 $\times$  TBE and place on top of the gel, being careful to avoid trapping air bubbles.
7. Wet the second sheet of 3 M paper in 0.5 $\times$  TBE and place on top of the membrane, again being aware of air bubbles. Then, to expel any remaining bubbles, take a 20 mm glass test tube (or similar object), press down firmly and roll from one end the gel to the other.

8. Place the second blotting pad over the top sheet of 3 M paper and add sufficient 0.5× TBE to just cover it. Cover the pad with a second plastic grid, and then insert the anode, followed by the plexiglass top cover.
9. Slide the blotting setup into the blotter housing, stand the whole unit upright and, if necessary, top up with sufficient 0.5× TBE to submerge the gel fully.
10. Apply a voltage of 12 V with constant current of 10 A for 2 h.
11. After the transfer is complete, disassemble the unit and, using a pencil, mark the positions of the wells on the membrane. Cut a notch out of the bottom left of the membrane (which corresponds to the bottom right of the gel, which currently is upside-down). Then, peel the membrane off the gel and place it on a paper towel to wick off excess moisture.
12. Denature the immobilized DNA by floating the membrane on a solution of 0.5 M NaOH for 10 min.
13. Rinse the membrane with 2× SSC and then soak it in fresh 2× SSC for 10 min.
14. Blot the membrane dry with paper towels and then use a cross-linker to fix the DNA. Label the membrane on the top right-hand corner.

### **3.5. Preparation of Telomere Probe (See Note 6)**

1. Pipette the following into a 200 µl thin walled PCR tube: 5 µl 10× ExTaq buffer, 4 µl dNTP mix (2.5 mM each nucleotide), 20 pmol TEL1 primer, 20 pmol TEL2 primer, PCR grade water to 49.8 and 0.2 µl ExTaq enzyme.
2. Mix well by flicking and centrifuge the tube briefly to collect the reagent mix at the bottom.
3. Place in the polymerase chain reaction machine and run the following program: 94°C for 1 min, followed by 35 cycles of 94°, 30 s; 55°C, 30 s; 72°C, 2 min. A final extension step at 72°C for 5 min is provided to complete the synthesis of any incompletely extended molecules.
4. Add 10 µl of 6× loading dye solution, mix by pipetting and load into three wells of a 0.7% agarose mini-gel (10 cm × 7 cm). Load a kilobase plus size marker into an adjacent well, and then run the gel at 20 V for 9.5 h.
5. Stain the gel in an aqueous ethidium bromide solution for 30 min. Then, visualize the DNA on a transilluminator emitting long wavelength UV light (312 nm). The PCR products will appear as a smear. Use a scalpel to excise DNA in the size range from 1.6 to 2 kb and recover the DNA using a commercial gel extraction kit.
6. Quantify the telomere probe using a spectrophotometer.

7. Pipette ~50 ng of telomere probe solution into a microcentrifuge tube and adjust the volume to 16  $\mu$ l with H<sub>2</sub>O. Denature the DNA by placing in a boiling water bath for 10 min. Then, remove the tube from the waterbath and snap cool on ice.
8. To the denatured DNA, add 5  $\mu$ l of 5 $\times$  Prime-A-Gene labeling buffer, 1  $\mu$ l of dNTP mix containing dATP, dGTP and dTTP, 1  $\mu$ l of BSA (100  $\mu$ g/ $\mu$ l), and 1  $\mu$ l of Klenow polymerase. Mix the reagents by flicking the tube and briefly spin in the microcentrifuge. Then, add 2  $\mu$ l of <sup>32</sup>P-dCTP (20  $\mu$ Ci; 3,000 Ci/mmol) and incubate at room temperature for 4 h to overnight.
9. Stop the labeling reaction by adding 75  $\mu$ l of dye stop solution and mix by pipetting up and down.
10. Use a MicroSpin™ G50 column to remove unincorporated nucleotides: First, give the column a “prespin” to compact the sieving matrix. Remove the filter column and discard the buffer that collects in the collection tube. Then, return the filter column to the collection tube and then pipette the labeling reaction on to the top of the sieving matrix. Spin for 15 s at full speed. Retain the collection tube and its contents and discard the spin column in a suitable radioactive waste container.
11. Check <sup>32</sup>P incorporation by pulling 1  $\mu$ l of the column flow up into a pipette tip and holding the tip to a Geiger counter. An adequately labeled probe should emit  $\geq 10,000$  counts/min. Return the test sample to the collection tube.

### **3.6. Detection of Telomeric Restriction Fragments by Hybridization**

1. Place the membrane containing the immobilized DNA in a hybridization bottle with the DNA side facing the inside of the tube. Add 20 ml of hybridization buffer and place in the hybridization oven. Incubate with rolling at 65°C for 10 min.
2. While the membrane is prehybridizing, transfer 50  $\mu$ l of <sup>32</sup>P-labeled telomere probe into a fresh microcentrifuge tube and denature it by adding 5  $\mu$ l of a freshly prepared 2 N NaOH solution. Incubate for 8 min at room temperature.
3. After the denaturation step, neutralize the probe solution by adding 5  $\mu$ l of 1 M Tris-HCl, pH 7.4.
4. Decant off the buffer used for prehybridization and replace it with 5 ml of fresh hybridization solution. Add the probe directly to the hybridization buffer. Be careful not to let any probe touch the membrane directly.
5. Replace the hybridization bottle in the oven and incubate with rolling for 16–24 h.



6. Decant hybridization buffer into a container approved for the disposal of  $^{32}\text{P}$ -dCTP waste. Rinse the membrane by pouring 50 ml  $2\times$  SSC into the hybridization tube and returning it to the chamber for 5 min.
7. Decant the rinse solution into the radioactive waste container and replace it with 50 ml low stringency wash buffer. Return the tube to the chamber and incubate for 20 min.
8. Decant the supernatant into the waste container and replace with 50 ml of high stringency wash buffer. Return the tube to the chamber and incubate for another 20 min.
9. Using forceps, remove the membrane from the hybridization tube and place on a paper towel to wick off excess liquid. *Do not allow the membrane to dry out.*
10. Place the membrane, right-(DNA-side)side up, on a sheet of plastic wrap and cover with a second layer of wrap. Expose to an autoradiographic film or phosphorimage screen overnight.
11. Develop the photographic film or scan the phosphorimage.
12. If necessary, print the phosphorimage at a scale of 1:1. Measure the positions of each telomere-hybridizing band relative to the well. Then, determine the molecular sizes of each band by cross-referencing with the size marker in the image of the original ethidium bromide stained gel (this is where the image of the ruler alongside the gel comes in handy). Alternatively, one can juxtapose the gel image of the size marker with the image of the blot (as shown in Fig. 2).
13. If desired, the membrane can be reused after stripping off the telomere probe. Probe removal is performed by performing two 30 min washes in 0.4 N NaOH at  $50^\circ\text{C}$ . The membrane is then washed with  $2\times$  SSC and allowed to dry before storage.

### **3.7. End Repair of Genomic DNA for Telomere Cloning**

“Cut-off” pipette tips and slow pipetting should be used throughout the following procedures to minimize shearing of the DNA.

1. Place in a microcentrifuge tube 2–4  $\mu\text{g}$  of genomic DNA in a total volume of  $\leq 34 \mu\text{l}$  of T0.1E. Add 5  $\mu\text{l}$  of End-It buffer, 5  $\mu\text{l}$  of nucleotide mix, 5  $\mu\text{l}$  of 10 mM ATP, and then 1  $\mu\text{l}$  of the T4 polynucleotide kinase/T4 polymerase enzyme mix. Allow the reaction to proceed for 45 min at room temperature.
2. Inactivate the enzymes by heating at  $70^\circ\text{C}$  for 10 min.
3. The following PCI/CI extraction steps are critical. Add 50  $\mu\text{l}$  of T0.1E followed by 50  $\mu\text{l}$  of PCI. Vortex briefly and centrifuge at  $18,000\times g$  for 2 min. Recover the aqueous phase (top layer) and transfer to a fresh microcentrifuge tube. Add 50  $\mu\text{l}$  of PCI to this tube and repeat the process (do not add any more T0.1E).

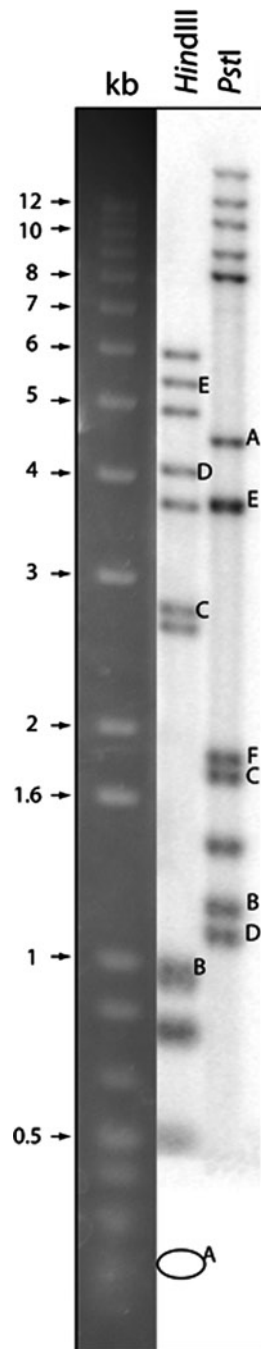


Fig. 2. Identification of telomeric restriction fragments in *Epichloë festucae* isolate E2368. Genomic DNA samples from *E. festucae* were digested separately with *Hind*III and *Pst*I and fractionated by agarose gel electrophoresis alongside a lane containing a 1 kb plus DNA ladder. The gel was then stained with ethidium bromide, imaged and electroblotted to a nylon membrane. After hybridization with the telomere probe, the membrane was exposed to a phosphorimage screen. The figure shows the resulting phosphorimage adjacent to an image of the gel lane that contained the DNA ladder. Counting doubly intense signals as two telomeres, 14 telomeric fragments are visible in the *Pst*I digest and 12 with *Hind*III (two fragments ran off the gel, one – a 0.2 kb fragment was present in the genome sequence and is represented with an oval). Fragments that were present in the *E. festucae* genome assembly are labeled with letters. The unlabeled fragments in the *Hind*III lane were all targets for cloning. Molecular sizes are listed on the left.

4. Recover the aqueous phase from the second PCI extraction and then add 50  $\mu\text{l}$  of CI. Vortex briefly and centrifuge at  $18,000\times g$  for 2 min. Recover the aqueous phase and add  $0.1\times \text{vol.}$  3 M Na acetate, pH 5.2 and 2 vol. room temperature 100% EtOH. Mix gently and precipitate the DNA by centrifugation ( $18,000\times g$ , 10 min).

### **3.8. Restriction Digestion of End-Repaired Genomic DNA**

1. Add 24  $\mu\text{l}$  of  $1\times$  restriction buffer (+100  $\mu\text{g}/\text{ml}$  BSA) to the pellet of end-repaired DNA (*see* Subheading 3.7) and leave on the bench for 30 min to dissolve. Gently flick the tube to disperse the solution and then add 20 U of restriction enzyme. Incubate at  $37^\circ\text{C}$  (or other appropriate temperature) overnight. Add 5  $\mu\text{l}$  of  $6\times$  loading dye solution.
2. Prepare a 0.7% agarose gel solution in  $0.5\times$  TBE buffer and pour into a gel unit that is at least 20 cm long. Use gel combs with teeth that are  $\leq 1$  cm width but which occupy a volume of at least 50  $\mu\text{l}$ .
3. Place the gel in the electrophoresis unit and submerge with  $0.5\times$  TBE buffer. Load each restriction digest(s) into a single well. Load a DNA size ladder into at least one well in the gel.
4. Run the gel overnight using a low voltage (e.g. 30 V, 20 h). Place the gel in ethidium bromide solution for 30 min.
5. UV light causes DNA damage which, in turn, prevents the recovery of subclones. Therefore, *do not photograph the gel before cutting out the DNA fractions*. Also, while cutting out bands, make sure that the DNA is exposed only to LONG wavelength (i.e. low energy) UV light (312 nm).
6. Use a scalpel to cut out gel slices containing DNA fragments of the desired sizes (see Fig. 3a). A good rule of thumb is to recover fragments  $\pm 0.2$  kb for fragments up to  $\sim 4$  kb,  $\pm 0.5$  kb for fragments of 4–8 kb and then  $\pm 1$  kb for larger ones (telomeres of similar sizes can be excised in a single gel slice and resolved after they have been cloned and characterized). Extract the DNA from the gel slices using a commercial kit (e.g. the QiaQuick extraction kit, Qiagen, Valencia, CA). Place the gel in fresh water and put in a refrigerator until it has been confirmed that the correct-sized fragments were excised.
7. Check that the desired telomeres were successfully recovered by running the purified fractions on an agarose gel, alongside a lane of total genomic DNA digested with the same enzyme that was used to produce the fragments. Blot the gel to a membrane and then incubate with the telomere probe (see Subheadings 3.3–3.6). Successful recovery of the desired telomeres is revealed by strong hybridization signals in the lanes containing the different size fractions (see Fig. 3b and c).

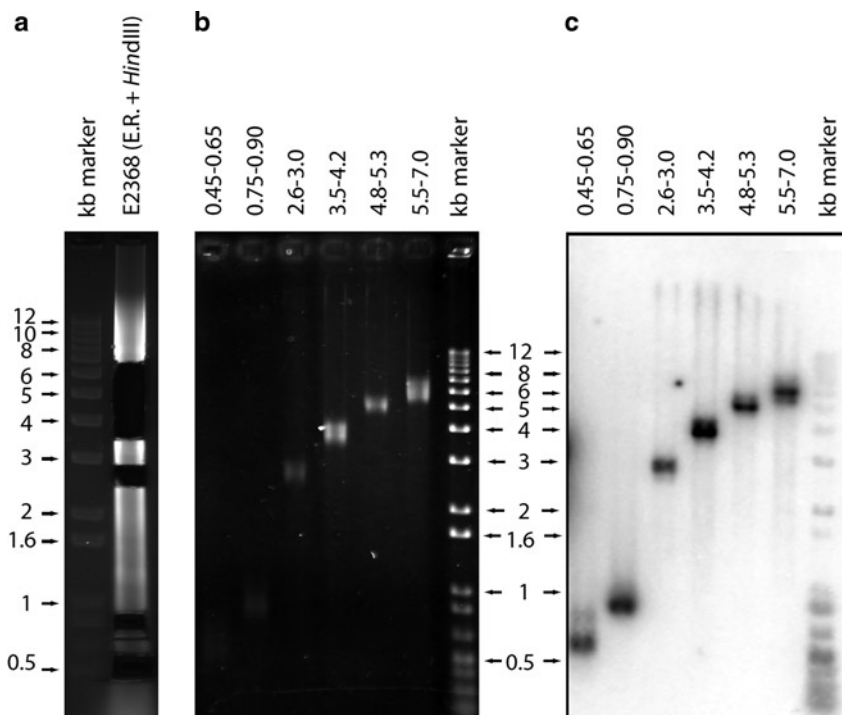


Fig. 3. Purification and verification of DNA fractions containing target telomeres. Approximately 2  $\mu$ g of E2368 DNA was end-repaired and subsequently digested with *Hind*III. After fractionation by electrophoresis, the desired DNA fragments were excised from the gel (a). The fragments were extracted with a Qiagen kit and were then fractionated on a mini-gel to check DNA (and telomere) recovery (b). The mini-gel was electroblotted to a membrane and the immobilized DNAs were then hybridized with the telomere probe. The resulting phosphorimage is shown in (c).

### 3.9. Preparation of Linearized Plasmid Vector

1. Pipette ~2  $\mu$ g of plasmid DNA (100 ng/ $\mu$ l) into a microcentrifuge tube. To this add 10  $\mu$ l of 10 $\times$  reaction buffer, 10  $\mu$ l of 100  $\mu$ g/ml BSA and 59  $\mu$ l of H<sub>2</sub>O. Finally, add 0.5  $\mu$ l (10 U) of each enzyme (see Note 7), mix well and centrifuge briefly to collect the reaction mix at the bottom of the tube. Incubate the tube at 37°C for 2 h.
2. Add 10  $\mu$ l of 10 $\times$  CIAP buffer followed by 0.1 U of CIAP. Mix well and then incubate at 37°C for 1 h. Add another 0.1 U of CIAP and incubate this time at 50°C.
3. Stop the reactions by adding 10  $\mu$ l of 100 mM EDTA, pH 8 and inactivate the enzymes by heating at 70°C for 10 min.
4. *This step is critical:* Extract the reaction two times with PCI followed by one extraction with CI (follow the extraction method described in Subheading 3.7 but omit the addition of T0.1E).
5. Adjust the Na<sup>+</sup> salt concentration of the reaction mix to 100 mM with 5 M NaCl. Then, precipitate the DNA by adding 200  $\mu$ l of room temperature 100% EtOH. Centrifuge immediately for 10 min at 18,000 $\times g$ .

6. Discard the supernatant and then rinse the DNA pellet with 500  $\mu$ l of 70% EtOH. Remove the 70% EtOH and allow the pellet to air dry.
7. Dissolve the pellet in 25  $\mu$ l of T0.1E buffer and add 5  $\mu$ l of 6 $\times$  loading dye solution.
8. Prepare an agarose gel (at least 15–20 cm long and made with 0.5 $\times$  TBE).
9. Immerse the gel in running buffer in the electrophoresis unit. Load the DNA+dye solution into a well that is at least 2 cm in width. Apply a voltage of 30 V for 20 h.
10. Stain the gel by immersing in an ethidium bromide solution for 20 min.
11. *Do not photograph the gel before cutting out the band of interest.* Visualize the gel under LONG wavelength UV light (312 nm) and use a scalpel to cut out a gel slice containing the band that corresponds to the completely cut vector.
12. Extract the vector DNA from the gel slice using a commercial kit and quantify by spectrophotometry.

**3.10. Ligation of  
Telomeric Fragments  
to the Linearized  
Vector**

1. For the negative control: Pipette the following into a microfuge tube: 1  $\mu$ l of plasmid DNA (~50 ng), 2  $\mu$ l of H<sub>2</sub>O, 3.5  $\mu$ l of 2 $\times$  ligation buffer and 0.5  $\mu$ l T4 DNA ligase. Mix by gently flicking the tube and then incubate overnight at 12°C.
2. For the standard reaction: Pipette the following into a microfuge tube: 1  $\mu$ l of plasmid DNA, 2  $\mu$ l of size-fractionated genomic DNA, 3.5  $\mu$ l of 2 $\times$  ligation buffer and 0.5  $\mu$ l T4 DNA ligase. Mix by gently flicking the tube and then incubate overnight at 12°C.
3. Inactivate the ligase by heating at 70°C for 10 min, and then add 13  $\mu$ l of T0.1E buffer to dilute the salt in the ligation buffer.

**3.11. Electrotransfor-  
mation of *E. coli*  
(See Note 2)**

1. Prechill the microcentrifuge tubes (one per ligation) and electroporation cuvettes (one per ligation) on ice.
2. Set the electroporator to 2.5 kV with a resistance of 200  $\Omega$  and a capacitance of 100  $\mu$ F.
3. Quickly thaw the competent cells by rolling the tube between your fingers. As soon as the suspension begins to thaw, transfer as many 10  $\mu$ l aliquots as are needed to the chilled tubes. Remaining cells can be refrozen for later use simply by returning them to the -80°C freezer.
4. Add 1  $\mu$ l of the diluted ligation reaction to the thawed competent cell suspension. Mix thoroughly by pipetting and transfer the entire mix to an electroporation cuvette. Place the cuvette in the chamber and apply a single shock. *As soon*

*as possible*, add 400  $\mu$ l of recovery medium (supplied with the competent cells) and incubate at 37°C for 30 min.

5. While the cells are recovering from the electroschock treatment, place four to five sterile glass beads into the Petri dishes that contain LB + ampicillin selection medium (two plates per transformation). Then, place the plates in a laminar flow hood with their lids off to allow any surface moisture to evaporate.
6. Pipette 200  $\mu$ l of transformation mix into each Petri dish, cover with the lids and then rock the plates back and forth to distribute the mix evenly across the agar surface. Place the dishes on the bench until all moisture is absorbed into the agar surface, dump the glass beads into a waste receptacle and then place the plates upside down in a 37°C incubator. Allow to incubate for 24 h.
7. Count the number of colonies obtained for each transformation. The negative control should produce fewer than ten colonies (usually, we obtain just one or two). In contrast, the “vector plus insert” ligation should yield a total of >500. In this case, screening usually results in the recovery of at least one telomere-containing clone. Conversely, it is usually not worth screening for telomeres unless at least 200 colonies are recovered.

### **3.12. Dialysis of Ligation Reactions**

1. If the transformation frequency is too low (see above), it can be increased up to tenfold by using dialysis to remove salts from the ligation reaction. This can be done very conveniently through the use of microdialysis chambers.
2. Fill a glass beaker with 500 ml T0.1E buffer and drop in a stir bar. Prewet the dialysis membrane by floating the empty chamber in the T0.1E for 10 min with stirring. Be sure to keep the level of the membrane barely below the surface of the buffer to prevent hydrostatic pressure from forcing too much liquid into the chamber.
3. Pipette the ligation mix into the chamber and, again, maintain the chamber’s buoyancy. Incubate with stirring for 2 h.
4. Collect the dialyzed DNA solution with a pipette and transfer into a fresh microfuge tube. It is ready for use immediately.

### **3.13. Colony Lifts (See Note 8)**

1. Use a permanent marker to place orientation marks on Whatman 541 paper disks, as shown in Fig. 4a. In addition, give each disk a label that corresponds to the Petri plate containing the colonies that are to be lifted.
2. With the label side up, use gloved hands to bend a paper disk upward into a U-shape. Touch the bent surface to the middle of the agar and, working outward from the initial point of contact, immediately smooth the sides of the disk evenly over the agar surface. Gently poke any bubbles to ensure good paper-agar contact across the whole plate.

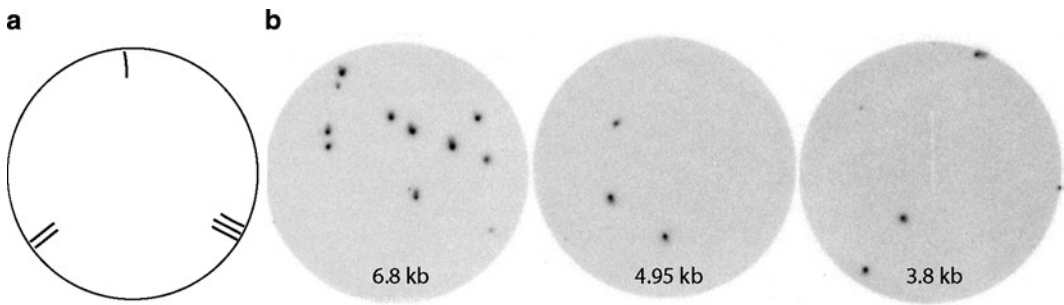


Fig. 4. Colony hybridization. (a) Marking pattern used to record the orientations of Whatman 541 paper disks. (b) Example of colony hybridization results obtained for three different *E. festucae* telomeres (the figure shows only one out of the two disks that were used for each telomere). The sizes of the telomere fragments that were cloned are shown on the respective images.

3. *Before removing the disk*, use a permanent marker to mark the bottom of the Petri dish with lines that correspond with the orientation marks on the disk. This facilitates the eventual alignment of hybridization spots with specific colonies on the agar surface.
4. Use forceps to grasp the edge of the disk. Lift it off the agar surface and place colony-side-up on a paper towel. This should have resulted in transfer of the colonies onto the disk, with only faint spots remaining on the agar. The disk can be processed immediately, or left overnight to air dry.
5. Place the agar plates in a safe location on the bench and allow the colonies to regrow overnight (do not incubate at 37°C, or the colonies will grow too large).
6. Prepare a positive control membrane by spotting 1  $\mu$ l of a 100-fold dilution of a plasmid containing a known telomere. If a positive control plasmid is not available, dilute the (unlabeled) telomere probe 100-fold with T0.1E and spot 1  $\mu$ l on a small square (~1.5 cm  $\times$  1.5 cm) of hybridization membrane. Allow the membrane to dry and then process along with the Whatman paper disks.
7. Pipette 1 ml aliquots of 0.5 M NaOH onto the surface of a clean plexiglass sheet. Place each disk colony-side-up onto an NaOH “puddle.” Allow the solution to soak across the whole disk and incubate for 10 min.
8. Transfer the disks to a tray containing 1 M Tris-HCl, pH 7.5 and incubate with shaking for 10 min.
9. Transfer the disks to a tray containing 2 $\times$  SSC and incubate with shaking for 10 min.
10. Transfer the disks to a tray containing 95% EtOH and incubate with shaking for 10 min.
11. Remove the paper disks from the EtOH and allow to air dry completely.

### **3.14. Colony Hybridization**

1. Place the paper disks and the positive control membrane in a hybridization tube – colony side facing inward. Add colony blot prehybridization solution (~5 ml/disk) and incubate, with rolling, in the hybridization chamber at 65°C for 1 h.
2. Remove the prehybridization solution and replace with a total of 5 ml hybridization buffer. Incubate with rolling for 10 min at 65°C.
3. Pipette 50 µl of denatured telomere probe (~15,000 counts/µl) directly into the hybridization solution, making sure that it does not touch the disks directly. Incubate with rolling for 2–18 h at 65°C.
4. Decant the hybridization buffer into a container approved for the storage/disposal of radioactive waste. Rinse the disks by pouring 50 ml of 2× SSC into the tube and returning it to the chamber for 5 min.
5. Decant rinse solution into approved container and replace with 50 ml 2× SSC. Return tube to the chamber and incubate for 20 min.
6. Decant supernatant into approved container. Using forceps, remove disks from the hybridization tube and place on a paper towel to wick off excess liquid. *Do not allow the disks to dry out.*
7. Place the control membrane and the Whatman paper disks, labels-side-up, on a sheet of plastic wrap and cover with a second layer of wrap. If the disks are sufficiently moist, they should remain firmly in place (this is important for subsequent steps). If they do not stay in place, fix them to the bottom sheet with tape.
8. Expose the disks to autoradiographic film or phosphorimage screens (label-side-up) for 2 h to overnight.
9. Develop the film or scan the phosphorimage. Allow sufficient exposure that the outlines of the paper disks are clearly visible. If telomere-containing clones are present, they will produce spots of very strong hybridization (see Fig. 4b). In this case, *do not remove disks from plastic wrap after exposure*. If no signals are present on the paper disks but the hybridization did work, as indicated by a strong signal on the control membrane, then there will be no telomere-containing clones on the plates (see Note 9).

### **3.15. Identification of Colonies Containing Telomeric Clones** (See Note 10)

1. If the marks on the paper disks faded during hybridization, use the permanent marker to re-mark their positions on the plastic wrap (do not remove the disks).
2. If phosphorimaging was used, print the digital image to paper (or a clear plastic sheet) at a scale of 1:1.
3. Lay the autoradiographic film or printed image over the plastic wrap and use the outlines of the paper disks to register the



photographic/digital disk images on top of the disks (use of a light box allows the outlines of the paper disks and the orientation marks to be seen through plain white paper). Copy the positions of the orientation markers onto the disk images with positive hybridization spots.

4. Once the orientation marks have been copied onto the images, place the corresponding Petri dish on top of the disk image and align the orientation marks. If the disks were accidentally removed from the Petri dishes before the positions of the orientation marks were copied onto them, it will be necessary to stain the paper disks to expose the lysed colonies (see Note 11).
5. Identify the *E. coli* colonies that gave rise to hybridization signals and pick them to liquid LB medium supplemented with 50 µg/ml ampicillin.
6. Grow cultures overnight with shaking at 37°C.
7. Prepare DNA using a commercial plasmid extraction kit and sequence to confirm that a bona fide telomere has been cloned.

### **3.16. Second Round Screening**

If there are several colonies in the region that gave rise to the hybridization signal, it may be necessary to perform second round screening. This can be accomplished in two ways.

1. If the colonies in the region are separated from one another, these can be individually picked (using a dissecting scope, if necessary) and spotted on a fresh LB+ampicillin plate. After overnight growth at 37°C, these can be screened using the colony lift procedure described above.
2. If there are too many colonies in the region of hybridization, it will be difficult, or impossible, to identify the correct one. In this case, a scalpel blade can be used to cut out an agar plug that contains the relevant colonies. Place the plug in a microcentrifuge tube containing 500 µl LB medium, cap the tube and vortex vigorously for a few seconds. Perform serial dilutions of the resulting suspension, plate the suspensions on fresh LB+ampicillin plates and incubate overnight at 37°C. Select plates containing 100–500 colonies and perform colony lifts to screen for telomere-containing clones.

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## **4. Notes**

1. The success of the directional cloning procedure is highly dependent on the quality of the linearized plasmid preparation. The vast majority of fragments in the digested genomic DNA sample have two sticky ends, and it is essential that the vector precludes the cloning of such fragments while allowing

efficient cloning of the telomeric fragments that have one blunt and one sticky end. In addition, because it is necessary to screen for true telomeres among large numbers of clones derived from false ends generated by DNA breakage, it is important that only recombinant plasmids are recovered following transformation of *E. coli*. For this reason, the vector should not be capable of self-ligation.

It is very difficult to generate satisfactory linearized plasmid preparations from standard cloning vectors due to an inability to obtain complete cleavage of restriction sites. To address this issue, we utilize a recombinant pBluescript plasmid that contains a 2 kb stuffer fragment inserted at the *EcoRI* restriction site. To prepare vector samples for cloning telomeres, pBS-TEL1 is cut with either *SmaI* or *EcoRV* and with a second enzyme on the other side of the stuffer fragment. This way, a preparative electrophoretic gel can be used to separate the fully digested plasmid from uncut and singly cut molecules, as well as from the stuffer fragment.

2. Successful recovery of the rare telomeric restriction fragments is highly dependent on obtaining high transformation efficiencies ( $>10^9$  colony forming units  $\mu\text{g}^{-1}$  DNA). For this reason, the use of commercially prepared competent cells is strongly recommended.
3. In an ideal world, only true chromosome ends would be recovered. However, the breakage of molecules during DNA isolation produces “false” ends that are also amenable to cloning by the methods described here. It follows that the more intact the starting DNA, the greater the proportion of clones that contain true telomeres. For this reason, it is important to avoid DNA isolation methods that are overly disruptive, such as the use of bead beaters and excessive pipetting. With careful execution, the following method can produce DNA with an average size of  $\sim 200$  kb. For a fungus, with a genome size of  $\sim 40$  Mb and eight chromosomes, this would mean that  $\sim 1$  in 25 DNA ends should be true telomeres.
4. It is not necessary to use cold temperatures or extended incubation times to obtain essentially quantitative recovery of DNA following precipitation. In fact, the use of cold temperatures simply increases the recovery of undesirable contaminants, such as polysaccharides. Therefore, all precipitations should be performed using room temperature ethanol/isopropanol and centrifugation should be performed immediately after the alcohol has been mixed in.
5. Genomic DNA should be quantified using a fluorometer because the polysaccharides that are usually copurified cause the DNA concentration to be vastly overestimated.

6. Many telomere hybridization studies are performed using end-labeled oligonucleotides as probes. These produce very weak hybridization signals because there is only one labeled nucleotide per probe molecule (~5% of residues). The method for probe production described here generates probes in which 25% of residues are labeled. Consequently, the hybridization signals are at least five times stronger.
7. Depending on the enzymes used for telomere cloning, it may be necessary to perform separate digestion reactions on the plasmid DNA, due to the need for different reaction buffers. In such cases, the DNA should be precipitated between digests.
8. Successful colony lifts require that the agar plates contain just the right amount of moisture. To achieve the desired moisture content, it is important to leave the lids off the Petri plates while the molten agar is setting. Keep the lids off for ~30 min while the plates dry in a laminar flow hood. Colonies should be lifted immediately after removing the plates from the incubator.
9. Under normal circumstances, screening of 500–1,000 colonies should result in the recovery of at least one telomere-containing clone. Failure to do so indicates a problem with the cloning procedure. The most likely cause of failure is inefficient end-repair of the genomic DNA due to the presence of polysaccharides or other impurities. Use of a differential precipitation procedure should help to address these issues (17). However, if the fungus under study frequently yields DNA that is recalcitrant to restriction digestion regardless of what types of “clean-up” methods are used, then it may be necessary to use a DNA isolation kit that uses a column-binding procedure (e.g. Illustra™ Tissue and Cells GenomicPrep Midi Flow, GE Healthcare). Alternatively, growing the fungus under different cultural conditions may reduce the inhibitors to acceptable levels.
10. The method described for aligning hybridization images with the original paper disks requires at least two disks to be imaged on the same film/screen; or one disk and a control membrane. Otherwise, it is difficult to orient the image correctly.
11. If one forgets to mark the bottom of an agar plate before lifting off the Whatman paper disks, this makes it more difficult to match up a positive hybridization spot with a specific colony on the agar plate. In such instances, follow Subheading 3.15 up to the end of step 3. Once the digital image has been labeled with the disk orientation marks, remove the disks from the plastic and incubate them for 2 h

in a dilute solution of gel loading dye (add 100 µl 6× dye to 100 ml TE). The debris from the immobilized colonies stain a faint blue color. The hybridization image can then be placed over the stained disks to identify the specific colony(ies) that produced the hybridization signal(s). The desired clone can then be found on the original agar plate by visual comparison of colony positions.

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