**Submission ID #: 61367**
**Scriptwriter Name: Bridget Colvin**
**Project Page Link:** https://www.jove.com/account/file-uploader?src=18716983

# Title: CRISPR-Cas9-Mediated Genome Editing in the Filamentous Ascomycete *Huntiella omanensis*

**Authors and Affiliations: Andi M. Wilson[1,2] and Brenda D. Wingfield[1,2]**

[1]Department of Biochemistry, Genetics & Microbiology, University of Pretoria
[2]Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria

**Corresponding Author:**
Andi M. Wilson
Andi.wilson@fabi.up.ac.za

**Co-authors:**
Brenda D. Wingfield
Brenda.wingfield@up.ac.za

 

# Author Questionnaire

**1. Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **N**

**2. Software:** Does the part of your protocol being filmed demonstrate software usage? **N**

**3. Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

**Protocol Length**
Shots: 40
Interview statements: 4-6

# Introduction

**1. Introductory Interview Statements**

**REQUIRED:**

1.1. **Andi Wilson**: Our protocol is significant because it gives researchers working on non-model fungi the opportunity to establish the use of cutting-edge genome editing technology in their labs **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

**REQUIRED:**

1.2. **Brenda Wingfield**: As it does not rely on existing techniques, such as expression systems, this protocol offers the advantage of being easier to establish in non-model species **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

**OPTIONAL:**

1.3. **Tuan Duong**: This method can be used across many different fungal species and can be used to elucidate the functions of genes involved in pathways as diverse as mating, growth, and pathogenicity **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

**OPTIONAL:**

1.4. **Vinolia Danki**: When performing this procedure, be sure to set aside enough consecutive days to complete the protocol, as there are only a few points at which the experiment can be paused **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

# Protocol

## 2. Protoplast Extraction

2.1. To harvest the conidia, filter the liquid culture through a layer of sterile laboratory cloth **[1-TXT]** and transfer the conidial suspension into 50-milliliter centrifuge tubes for centrifugation.

2.1.1. WIDE: Talent filtering culture through cloth **TEXT: See text for conidia culture details** NOTE: 2.1.1 and 2.1.2 shot together

2.1.2. Talent adding suspension to tube(s) **TEXT: 10 min, 3220 x g, 4 °C**

2.2. Resuspend the conidia pellet in 5 milliliters of water **[1]** and view a 10-microliter aliquot of the conidia solution under a light microscope at a 40x magnification to confirm that only conidia have been recovered **[2]**.

2.2.1. Shot of pellet, then water being added to tube

2.2.2. Talent pipetting aliquot onto microscope slide

2.3. Next, add 200 milliliters of fresh 1% malt extract broth to a 500-milliliter flask **[1]** and transfer the entire volume of conidia to the flask **[2]**.

2.3.1. Talent adding broth to flask, with broth container visible in frame

2.3.2. Talent adding conidia to flask

2.4. Then incubate the liquid culture for up to 12 hours in a 25-degree Celsius shaking incubator at 120 revolutions per minute **[1]**.

2.4.1. Flask on shaker

2.5. To harvest the germlings, transfer the culture to 50-milliliter centrifuge tubes for centrifugation **[1]** and resuspend the germlings in up to 10 milliliters of 1-molar sorbitol **[2]**.

2.5.1. Talent adding culture to tube(s)

2.5.2. Shot of pellet(s), then sorbitol being added to tube(s), with sorbitol container visible in frame

2.6. Check a 10-microliter aliquot of germling solution under a light microscope to confirm that only germlings have been recovered **[1]** and incubate the spore-enzyme solution for 2-3 hours in the shaking incubator at 80 revolutions per minute **[2-TXT]**.

2.6.1. Talent adding germling solutions to flasks with spore-enzyme solution *Videographer: Difficult step*

2.6.2. Talent placing flask into incubator **TEXT: Check for protoplasts every 30 min by light microscopy** *Videographer: Difficult step*

2.7. To harvest the protoplasts, filter the culture supernatant through a layer of sterile laboratory cloth **[1]** and collect the protoplasts by centrifugation **[2-TXT]**.

2.7.1. Culture being filtered *Videographer: Difficult step*

2.7.2. Talent placing tube(s) into centrifuge *Videographer: Difficult step* **TEXT: 10 min, 1810 x g, 4 °C**

2.8. Then carefully resuspend the protoplast pellet in 200 microliters of STC (S-T-C) buffer **[1-TXT]** and check a 10-microliter aliquot of the solution under a microscope to confirm that only protoplasts have been recovered **[2]**.

2.8.1. Shot of pellet, then buffer being added to tube, with buffer container visible in frame *Videographer: Important step* **TEXT: STC: sorbitol, Tris-HCl, $CaCl_2$**

2.8.2. LAB MEDIA: Figure 3C *Video Editor: please emphasize dotted lines/protoplasts in dotted lines*

## 3. Protoplast and PEG-Assisted Transformation and Transformant Recovery

3.1. To begin the transformation, combine approximately 5 x $10^6$ protoplasts with a single volume of ribonucleoprotein solution **[1]** and approximately 6 micrograms of the donor DNA fragment **[2-TXT]**.

3.1.1. WIDE: Talent added the protoplasts into a 50ml tube, followed by adding RNP to the protoplasts. Note: the RNP and protoplast "containers" were small 2ml tubes and thus may not be entirely visible in the shot.

3.1.2. Talent adding dDNA to tube, with dDNA tube visible in frame **TEXT: See text for dDNA preparation details**

3.2. Next, use a pipette to slowly and evenly drip 1 milliliter of freshly prepared 30% PTC (P-T-C) solution onto the protoplast suspension to create a hydrophobic layer over the protoplasts, and incubate the solution for 20 minutes at room temperature **[1-2 TXT]**.

3.2.1. Talent adding PTC to tube, with PTC container visible in frame *Videographer: Important step* **TEXT: PTC: STC buffer + polyethylene glycol**
3.2.2. Talent setting timer, with tube visible in frame **TEXT: See text for all solution and buffer preparation details**

NOTE: steps 3.2.1 and 3.2.2 combined for a smoother flow.

3.3. At the end of the incubation, add 5 milliliters of osmotic control medium to the protoplast suspension **[1]**, pipetting slowly and gently to thoroughly mix the solution **[2]**.

3.3.1. Talent adding medium to tube, with medium container visible in frame *Videographer: Important step*
3.3.2. Solution being mixed *Videographer: Important step*

3.4. After mixing, incubate the protoplast solution in the shaking incubator at 80 revolutions per minute overnight **[1]**.

3.4.1. Talent placing tube onto shaker

3.5. The next morning, divide the solution between five 60-millimeter culture plates **[1].** Add 10 milliliters of osmotic control medium agar supplemented with 30 micrograms/milliliter of hygromycin B to each plate and slowly rotate each plate to mix **[2 and 3.6.1]**.

3.5.1. Talent adding solution to plate(s)
3.5.2. Talent adding agar to plate(s), with agar and hygromycin B containers visible in frame *Videographer: Important step*

NOTE: 3.5.2 and 3.6.1 coombined.

3.6. Allow the first layer of agar to set, before adding 10 milliliters of osmotic control medium agar supplemented with 40 micrograms/milliliter of hygromycin B to each plate **[2]**.

~~3.6.1. Plate being rotated *Videographer: Important step*~~
3.6.2. Shot of set agar, then agar being added to plate(s), with agar container visible in frame *Videographer: Important step*

3.7. After allowing the second layer of agar to set **[1]**, incubate the cultures at 25 degrees Celsius **[2]** until single isolates can be observed growing through both layers of agar **[3]**.

3.7.1. Shot of set agar
3.7.2. Talent placing plate(s) into incubator
3.7.3. Shot of isolate(s) growing through agar *Videographer: Important step*

3.8. To recover the successfully transformed isolates, transfer the individual, growth-capable isolates to fresh malt extract agar plates supplemented with 50 micrograms/milliliter of hygromycin B **[1]**.

3.8.1. Talent adding isolates to new plate(s)

## 4. Phenotypic Mutant Strain Analysis

4.1. To assess the effects of the targeted gene disruption on the heterothallic capabilities of the fungus, co-inoculate fresh malt extract agar medium with one mutant strain as well as a strain of the opposite mating type. When working with H. omanensis, cover but do not seal the plates. **[1-TXT and 4.2.1]**.

4.1.1. WIDE: Talent adding strain(s) to plate, with strain culture containers visible in frame **TEXT: *e.g. MAT* gene disruption**
NOTE: steps 4.1.1 and 4.2.1 combined.

4.2. Place the plates at room temperature for 7 days **[2]**.

~~4.2.1. Plate being covered~~
4.2.2. Talent stacking plates/placing plates at RT

4.3. At the end of the incubation, visually assess for the production of sexual structures **[1]**.

4.3.1. Shot of 7-day-old plates
NOTE: Two plates were shown here- the one with the light coloured mycelia has not sexual structures. The darker plate has sexual structures.

4.4. To test the homothallic capabilities of the mutant strain, inoculate fresh malt extract agar medium with the mutant strain of interest **[1]** and incubate the plate at room temperature for 1 week as demonstrated **[2]**.

4.4.1. Talent inoculating plate
4.4.2. Talent placing plate at RT

4.5. To assess the effects of the disruption on the growth rate of the fungus being studied, insert the back side of a large, sterile pipette tip into the actively growing edges of the culture of each mutant and wild type strain of interest **[1]** to create mycelial-covered agar plugs **[2]** and inoculate fresh malt extract agar medium with at least three plugs per culture type **[3]**.

4.5.1. Plug being created
4.5.2. Shot of plug
4.5.3. Plug being added to medium

4.6. After 3 days of growth at 20 degrees Celsius, measure the growth in each plate on two perpendicular diameters **[1]**.

4.6.1. Shot of wildtype and mutant strain plates with type of strains indicated, then one growth being measured

# Protocol Script Questions

**A.** Which steps from the protocol are the most important for viewers to see?

2.8., 3.2., 3.3., 3.5.-3.7.

**B.** What is the single most difficult aspect of this procedure and what do you do to ensure success?
2.6., 2.7.: Successfully harvesting protoplasts. To ensure success, I take small aliquots at 30 min intervals and check them under a microscope.

# Results

**5. Results: Representative Protoplast Extraction and Isolate Phenotype Analysis**

5.1. Conidia used as the starting material for the protocol **[1]** are allowed to germinate and grow until they become young germlings **[2]**.

5.1.1. LAB MEDIA: Figure 3A
5.1.2. LAB MEDIA: Figure 3B *Video Editor: please add/emphasize black arrows*

5.2. Note that mature mycelial strands such as these are too mature for degradation and should not be used **[1]**.

5.2.1. LAB MEDIA: Figure 3B *Video Editor: please emphasize strange immediately above left arrow*

5.3. When the cells no longer have cell walls, they become very sensitive to mechanical disruption and release round protoplasts that can be harvested for transformation **[1]**.

5.3.1. LAB MEDIA: Figure 3C *Video Editor: please emphasize protoplasts and/or add dotted circles around protoplasts*

5.4. The success of the protocol can be confirmed upon phenotypic analysis of the mutant strains **[1]**.

5.4.1. LAB MEDIA: Figure 5

5.5. For this mutant *MAT1-2-7* (mat-one-two-seven) isolate, the vegetative radial growth rate was significantly reduced **[1-TXT]**, suggesting a pleiotropic effect for the novel mating gene **[2]**.

5.5.1. LAB MEDIA: Figure 5 *Video Editor: please emphasize bottom left image* **TEXT: MAT: mating type gene**
5.5.2. LAB MEDIA: Figure 5

5.6. Furthermore, the mutant isolate was incapable of completing a sexual cycle, producing only immature sexual structures that did not produce sexual spores **[1]** compared to the wild type isolate, which completed the entire sexual cycle within a few days of incubation **[2]**.

5.6.1. LAB MEDIA: Figure 5 *Video Editor: please emphasize sequentially emphasize 2nd-4th bottom row images*

5.6.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize top row of images*

# Conclusion

**6. Conclusion Interview Statements**

6.1. **Andi Wilson**: RNA is very sensitive and degrades easily. Therefore, a very clean work environment and working quickly on ice are essential to the success of the experiment **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.1.)

6.2. **Tuan Duong**: Once the mutant isolates have been successfully collected, they can be subjected to phenotypic or RNA seq analysis as appropriate for the gene being characterized **[1]**.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*