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Title: Rapid Assembly of Multi-Gene Constructs Using Modular Golden Gate Cloning

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 16
Number of Shots: 36

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Zhen Wang:** This is a detailed protocol for assembling multi-gene plasmids using the yeast MoClo kit.

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

1.2. **Zhen Wang:** This method enables the convenient cloning of a large variety of multi-gene constructs. It is ideal for generating a library of related constructs.

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

Introduction of Demonstrator on Camera

1.3. **Zhen Wang:** Demonstrating the procedure will be Minakshi Mukherjee, a PhD student from my laboratory.

1.3.1. INTERVIEW: Author saying the above.

1.3.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Protocol

2. Cloning parts into the entry vector (pYTK001) to create part plasmids

2.1. Begin by setting up the Golden Gate reaction mix **[1]**. Add 20 femtomoles of each PCR product and the entry vector, 1 microliter of 10X T4 ligase buffer, 0.5 microliters of Esp3I *(pronounce 'E-S-P-3-one')*, and 0.5 microliters of T4 ligase **[2]**. Add double-distilled water to bring the total volume to 10 microliters, then run the cloning reaction **[3-TXT]**. *Videographer: This step is difficult and important!*

2.1.1. WIDE: Establishing shot of talent at the lab bench, preparing the reaction.

2.1.2. Talent adding reagents to the reaction tube.

2.1.3. Talent programming the thermocycler. **TEXT: 25-35 cycles of 37 °C for 5 min and 16 °C for 5 min; 50 °C for 10 min; 80 °C for 10 min**

2.2. Transform the entire reaction mix into the DH5α *(pronounce 'D-H-5-alpha')* strain or equivalent *Escherichia coli* chemically competent cells by heat shock **[1]**. Spread the cells on an LB plate **[2]** with 35 micrograms per milliliter chloramphenicol **[3]**, then incubate the plate at 37 degrees Celsius overnight **[4]**. *Videographer: This step is important!*

2.2.1. Talent adding reaction mix to cells.

2.2.2. Labeled plates with different antibiotics.

2.2.3. Talent spreading the cells on the plate.

2.2.4. Talent putting the plate in the incubator.

2.3. After 16 to 18 hours, take the plate out of the incubator **[1]** and leave it at 4 degrees Celsius for about 5 hours to let the super folder green fluorescent protein, or sfGFP, develop for a more intense green color **[2]**.

2.3.1. Talent taking the plate out of the incubator.

2.3.2. Talent placing the plate in the refrigerator.

2.4. To screen the plate, place it on an ultraviolet or a blue light transilluminator **[1]**. The sfGFP containing colonies will fluoresce under the UV light. The green colonies are negative because they contain the uncut part plasmid. The white colonies are likely positive. The cloning is successful if there are 30 to 100% white colonies **[2]**.

2.4.1. Talent placing the plate on a transilluminator.

2.4.2. Plate with green and white colonies.

3. Assembling part plasmids into "cassette" plasmids

3.1. Assemble an intermediate vector with the left connector, the sfGFP dropout, the right connector, a yeast selection marker, a yeast origin of replication and the part plasmid with an mRFP1*,* an *E. coli* origin and the ampicillin-resistant gene **[1]**. Perform the cloning reaction as described in the text manuscript **[2]**. *Videographer: This step is difficult and important!*

3.1.1. Talent combining the plasmids and ligation reagents.

3.1.2. Talent programming the thermocycler.

3.2. Transform the entire reaction mix into the DH5α strain, then spread the cells on an LB plate with 50 micrograms per milliliter carbenicillin or ampicillin **[1]**. Incubate at 37 degrees Celsius overnight **[2]**. *Videographer: This step is important!*

3.2.1. Talent spreading the transformed cells on a plate.

3.2.2. Talent putting the plate in the incubator.

3.3. After 16 to 18 hours, take the plate out of the incubator. The plate will contain both pale red and pale green colonies **[1]**. Keep it at 4 degrees Celsius for about 5 hours to let the mRFP1 and sfGFP mature **[2]**. Use a UV or a blue light transilluminator to identify the green colonies, which contain the potentially correct intermediate vector **[3]**.

3.3.1. Talent taking the plate out of the incubator.

3.3.2. Talent putting the plate in the refrigerator.

3.3.3. Talent looking at the plate with a transilluminator, with the part of the plate that contains white colonies marked out.

3.4. Streak out the green colonies on an LB-carbenicillin plate **[1]** and incubate at 37 degrees Celsius overnight **[2]**. *Videographer: This step is important!*

3.4.1. Talent streaking out several green colonies

3.4.2. Talent putting the plate in the incubator.

3.5. On the next day **[1]**, streak them out again on a chloramphenicol plate and incubate at 37 degrees Celsius overnight **[2]**. The colonies growing on chloramphenicol plates contain misassembled plasmids **[3]**.

3.5.1. Talent taking the plate out of the incubator.

3.5.2. Talent streaking out the colonies.

3.5.3. Colonies growing on the plate.

3.6. Once the intermediate vector has been successfully assembled, proceed with assembling the transcription units. This 4-piece assembly contains the intermediate vector, a promoter, a CDS, and a terminator. Purify the plasmids, record their

concentrations **[1]**, and dilute each plasmid to 20 femtomoles of DNA per microliter **[2]**.

3.6.1. Talent measuring the DNA concentration.

3.6.2. Talent diluting a plasmid.

3.7. After performing the cloning reaction, transform the entire cloning reaction mix into the DH5α or equivalent *E. coli* competent cells and plate them on LB and carbenicillin **[1]**, then incubate the plate at 37 degrees Celsius overnight **[2]**. *Videographer: This step is important!*

3.7.1. Talent plating the cells.

3.7.2. Talent putting the plate in the incubator.

3.8. After 16 to 18 hours, take the plate out of the incubator **[1]** and keep it at 4 degrees Celsius for about 5 hours to let the sfGFP mature **[2]**. Use a UV or a blue light transilluminator to identify the non-fluorescent white colonies, which contain the potentially correct transcription units **[3]**. *Videographer: This step is important!*

3.8.1. Talent taking the plate out of the incubator.

3.8.2. Talent putting the plate in the refrigerator.

3.8.3. Talent observing the colonies with a transilluminator.

4. Assembling cassette plasmids into "multi-gene" plasmids

4.1. Assemble an intermediate vector for the multi-gene plasmids as described in the text manuscript, then transform the entire cloning reaction mix into DH5α cells **[1]** and plate them on LB with 50 micrograms per milliliter kanamycin **[2]**. Incubate the plate at 37 degrees Celsius overnight **[3]**. *Videographer: This step is important!*

4.1.1. Talent adding the transformation mix to the cells.

4.1.2. Talent plating the cells.

4.1.3. Talent putting the plate in the incubator.

4.2. Perform red and green color-based screening **[1]**, then streak and grow the green colonies on an LB- kanamycin plate to screen for mis-assemblies as previously demonstrated **[2]**. *Videographer: This step is important!*

4.2.1. Talent looking at the plate with a transilluminator.

4.2.2. Talent streaking several green colonies on a plate.

4.3. Next, assemble the multi-gene plasmid by setting up a cloning reaction as described in the text manuscript **[1]**. Transform the entire cloning reaction mix into DH5α cells and plate them on LB-kanamycin **[2]**. Incubate the plate at 37 degrees Celsius overnight

[3], then perform the green and white screening as previously described **[4]**.
Videographer: This step is important!

4.3.1. Talent putting reaction tubes in a thermocycler and programming it.

4.3.2. Talent plating the cells.

4.3.3. Talent putting the plate in the incubator and closing the door.

4.3.4. Talent observing green/white colonies.

Results

5. Results: Multi-gene plasmid cloning in *E. coli* and yeast

5.1. This protocol was used to construct one integrative multi-gene plasmid for disrupting the *ADE2* locus. Four replicative and one integrative multi-gene plasmids were assembled **[1]**.

5.1.1. LAB MEDIA: Table 2.

5.2. The ratio of potentially correct colonies for the intermediate vector cloning was 1.83% **[1]**. Once the intermediate plasmid was cloned, the success rate of assembling multi-gene plasmids from the intermediate was 93.77% **[2]**.

5.2.1. LAB MEDIA: Figure 7 A and B. *Video Editor: Emphasize the green colonies in the inset.*

5.2.2. LAB MEDIA: Figure 7 C and D.

5.3. The negligeable numbers of positive white colonies demonstrate a suboptimal assembly of multi-gene plasmids **[1]**.

5.3.1. LAB MEDIA: Figure 7 E and F.

5.4. After transforming into yeast, colonies producing beta-carotene and lycopene grew on day three. Four colonies from each plate were streaked out onto fresh plates and grown for two more days **[1-TXT]**.

5.4.1. LAB MEDIA: Figure 8 A and B. *Video Editor: Label A "**BTS1-ERG20** fusion TU" and B "**ERG20** TU".*

5.5. The carotenoids were extracted and quantified by UV-Vis spectrophotometry **[1]**. *BTS1-ERG20 (spell out 'B-T-S-E-R-G-twenty')* leads to 35-fold higher production beta-carotene compared to the strain with *ERG20* alone **[2]**. Likewise, the production of lycopene is approximately 16.5-fold higher in the strain with *BTS1-ERG*20 compared to *ERG20* alone **[3]**.

5.5.1. LAB MEDIA: Figure 8 C.

5.5.2. LAB MEDIA: Figure 8 C. *Video Editor: Emphasize the beta-carotene bars.*

5.5.3. LAB MEDIA: Figure 8 C. *Video Editor: Emphasize the lycopene bars.*

5.6. The multi-gene integrative plasmid was used for the disruption of the *ADE2* locus, either with a gRNA and no helper DNA **[1]** or with gRNA and a multi-gene integrative plasmid as helper DNA **[2]**.

5.6.1. LAB MEDIA: Figure 8 D and E. *Video Editor: Emphasize D.*

5.6.2. LAB MEDIA: Figure 8 D and E. *Video Editor: Emphasize E.*

5.7. After 3 to 4 days, red colonies were observed on the YPD plate with nourseothricin, indicating that *ADE2* had been successfully disrupted **[1]**.

5.7.1. LAB MEDIA: Figure 8 E.

Conclusion

6. Conclusion Interview Statements

6.1. **Zhen Wang:** When attempting this protocol, the most important thing to remember is to measure DNA concentrations accurately and pipet carefully while setting up the reaction mix.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.1.1, 3.1.1.*

6.2. **Zhen Wang:** This technique allows researchers in the yeast metabolic engineering field to quickly survey a large number of genes and promoters to maximize product yield.

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