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Title: CRISPR-Based Shuttle Cloning: A High-Throughput Cloning Method

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

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

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

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When you are ready to submit your video files, please contact our China Location Producer, Yuan Yue.

Current Protocol Length

Number of Steps: 10
Number of Shots: 26

Introduction

1.1. **Yutian Peng:** We describe a protocol for a high-throughput cloning method, CRISPRshuttle, which allows the transfer of target DNA fragments between vectors without the need for PCR amplification of the DNA fragments **[1]**.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 3.*

What technologies are currently used to advance research in your field?

1.2. **Yutian Peng:** Existing techniques, such as Gateway, In-Fusion, and Univector cloning, hinge on PCR amplification. This approach necessitates fragment-specific procedures, including primer design and sequencing validation. These steps are labor-intensive and time-consuming **[1]**.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 3*

What advantage does your protocol offer compared to other techniques?

1.3. **Yutian Peng:** CRISPRshuttle eliminates PCR while transferring target DNA fragments between vectors in two sequential test tube reactions. This method bypasses the need for fragment-specific handling, and thus accelerates plasmid library construction **[1]**.

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

Protocol

2. Procedure for the Generation of UAS-cDNA/ORF Plasmids

Demonstrator: Yutian Peng

2.1. To begin, prepare a master mix for a given number of digestion reactions by combining the reagents shown on screen **[1,2]**.

2.1.1. WIDE: Talent pipetting all reagents into a master mix tube.

AND

2.1.2. TEXT on PLAIN BACKGROUND:

Master mix for N number of digestion reactions:

(N+1) x 0.4 µL of 1.22 µM *Streptococcus pyogenes* Cas9

(N+1) x 0.5 µL of 80 ng/µL pLX304-CMV-G1

(N+1) x 0.5 µL of 80 ng/µL pLX304-3'-G1

(N+1) x 0.4 µL of 10x Cas9 Buffer

(N+1) x 1.45 µL of DEPC-treated ultrapure water

2.2. Arrange properly labeled 0.2-milliliter tubes in an aluminum cooling block on ice **[1]**. Prepare the master mix for N number of reactions by mixing appropriate amounts of Cas9 *(Cas-nine)*, sgRNA *(S-G-R-N-A)*, 10x *(ten-ex)* Cas9 Buffer, and DEPC *(D-E-P-C)*-treated water **[2]**. Mix the master mix thoroughly **[3]** and spin it down **[4]**. Aliquot 3.75 microliters of the master mix into each reaction tube **[5]**. Add 0.75 microliters of 0.03 micromolar pLX304-ORF *(P-L-X-three-zero-four O-R-F)* plasmid to each tube, mix thoroughly **[6]**, and incubate the tubes at 37 degrees Celsius for 1 hour **[7].**

2.2.1. Talent placing a couple of labeled tubes in an aluminum cooling block kept on ice.

2.2.2. Talent preparing the master mix by pipetting the mentioned reagents.

2.2.3. Talent mixing the master mix.

2.2.4. Talent vortexing the master mix tube.

2.2.5. Talent pipetting 3.75 microliters of master mix into a tube.

2.2.6. Talent adding 0.75 microliters of pLX304-ORF plasmid to the tube and mixing it.

2.2.7. Talent placing tubes into an incubator.

2.3. Prepare a master mix by combining 0.14 microliters of 3.36 micromolar linearized pBIDC-UASC-pLXvect *(P-B-I-D-C-U-A-S-C-P-L-X-Vect)* and 1.8 microliters of Gibson assembly master mix **[1-TXT]**.

2.3.1. Talent pipetting linearized pBIDC-UASC-pLXvect and Gibson assembly master mix into a master mix tube. **TXT: For N number of Gibson assembly reactions, combine (N+1) x linearized pBIDC-UASC-pLXvect & (N+1) x Gibson assembly master mix**

2.4. Mix the master mix thoroughly **[1]** and spin it down **[2]**. Now, aliquot 1.94 microliters of the master mix into each tube **[3]**. Add 1.66 microliters of Cas9 *(Cas-nine)*-cleaved plasmid into each tube, mix thoroughly **[4]**, and incubate at 50 degrees Celsius for 1 hour **[5]**.

2.4.1. Talent mixing the master mix.

2.4.2. Talent vortexing the Gibson assembly master mix.

2.4.3. Talent pipetting 1.94 microliters into a tube.

2.4.4. Talent adding 1.66 microliters of Cas9-cleaved plasmid into the tube and mixing it.

2.4.5. Talent placing the tube into an incubator.

2.5. Thaw bacterial competent cells on ice **[1]**. Aliquot 10 microliters of the thawed cells into each prechilled 1.5-milliliter tube **[2]**.

2.5.1. Talent thawing bacterial competent cells on ice.

2.5.2. Talent pipetting 10 microliters of cells into a 1.5-milliliter prechilled tube.

2.6. Gently mix 10 microliters of the competent cells with 1 microliter of Gibson assembly product **[1]**. Place the tubes on ice for 30 minutes **[2]**.

2.6.1. Talent adding 1 microliter of Gibson product into competent cells.

2.6.2. Talent placing the tubes on ice.

2.7. Heat-shock the tubes at 42 degrees Celsius for 1 minute **[1]** and then chill on ice for 2 minutes **[2]**.

2.7.1. Talent placing tubes into a 42 degrees Celsius water bath or heater.

2.7.2. Talent transferring tubes onto ice after heat shock.

2.8. Add 100 microliters of prewarmed SOC *(S-O-C)* medium into each tube **[1]** and shake at 250 rpm for 1 hour at 37 degrees Celsius **[2]**.

2.8.1. Talent adding SOC medium into the tube.

2.8.2. Talent placing the tube into a shaker.

2.9. Finally, place the cells onto LB agar plates containing 15 micrograms per milliliter of chloramphenicol **[1]** and incubate them overnight at 37 degrees Celsius **[2]**.

2.9.1. Talent adding cells onto LB agar plates containing chloramphenicol.

2.9.2. Talent placing plates into an incubator.

Results

3. Results

3.1. This figure displays the representative results from the restriction analysis of UAS-cDNA/ORF *(U-A-S-C-D-N-A-O-R-F)* plasmids generated using the CRISPRshuttle *(Crisper-Shuttle)* system **[1]**.

3.1.1. LAB MEDIA: Figure 3.

3.2. In this analysis, restriction digestion of 15 UAS-cDNA/ORF constructs with PvuII *(P-V-U two)* revealed that all samples exhibited the expected fragment patterns at approximately 1,072 base pairs and 1,820 base pairs **[1]**.

3.2.1. LAB MEDIA: Figure 3. *Video Editor: Highlight all the black bands of UH326 to UH341 close to the 1,000 and 2,000 labels.*