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Title: Construction of CRISPR Plasmids and Detection of Knockout Efficiency in Mammalian Cells through a Dual Luciferase Reporter System

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

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

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

NOTE to Video Editor: APV, authors said they followed the script precisely.

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Huaiyuan Qin:** This protocol describes key steps in the generation and optimization of efficient sgRNA vectors. A pre-selection of candidate sgRNA targets will save time and effort.

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

1.2. **Huaiyuan Qin:** The main advantage of this protocol is that it makes it possible to analyse the DNA cutting efficiency for each of the sgRNAs without editing endogenous genes.

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

OPTIONAL:

1.3. **Huaiyuan Qin:** This pre-selection method can also be applied to other gene editing methods through double-stranded breaks.

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

Protocol

2. Oligonucleotide Annealing and sgRNA/CRISPR Vector Digestion

2.1. Begin by diluting the lyophilized oligonucleotides to a final concentration of 10 micromolar in double-distilled water **[1]**. Mix forward and reverse oligonucleotides in a thin wall PCR tube at a one to one ratio without adding any extra buffer **[2]**.

2.1.1. WIDE: Establishing shot of talent diluting the oligos.

2.1.2. Talent mixing the forward and reverse oligos.

2.2. Incubate the mixture at 95 degrees Celsius for 5 minutes **[1]**, then ramp down the temperature to 72 degrees Celsius for 10 minutes **[2]**. Remove the oligonucleotide mix from the PCR machine and allow it to cool at room temperature **[3]**. *Videographer: This step is important!*

2.2.1. Talent putting the tube in the PCR machine and closing the lid.

2.2.2. Talent adjusting the temperature on the machine.

2.2.3. Talent taking the tube out of the PCR machine and setting it on the lab bench.

2.3. To digest the pX330-xCas9 *(pronounce 'p-X-three-three-zero-x-Cas-nine')* vector with *Bbs*I *(pronounce 'B-B-S-one')*, combine the vector, enzyme, and digestion buffer in a 50-microliter volume **[1]** and incubate the reaction at 37 degrees Celsius for 2 hours **[2]**.

2.3.1. Talent combining the reagents. **TEXT: 10 units *Bbs*I per 1 µg of plasmid**

2.3.2. Talent putting the reaction tube in the incubator. Authors: Will you be using the PCR machine for this?

3. Identification of the Correct Recombinant Plasmids by PCR

3.1. After performing cell transformation, choose 5 to 10 bacterial colonies from the LB plate **[1]** and use each of them to inoculate 1 milliliter of LB media with 60 milligrams ampicillin in a 1.5-milliliter tube **[2]**, then incubate the tubes on a rotary shaker for 2 to 3 hours **[3]**.

3.1.1. Talent picking a colony from a plate.

3.1.2. Talent transferring the bacteria to the 1mL of LB.

3.1.3. Talent placing the tubes in the rotary shaker and starting it.

3.2. Perform PCR to screen for recombinant plasmids as described in the text manuscript **[1]**, then run the products on a 2% agarose gel under 10 Volts per centimeter **[2]**. After identifying the positive plasmids, use them along with a reporter vector to co-

transfect an appropriate cell line **[3-TXT]**. *Videographer: This step is difficult and important!*

3.2.1. Talent putting reaction tubes in the PCR machine and closing the lid.

3.2.2. Gel running.

3.2.3. Talent in front of the lab bench preparing to perform transfection, with all reagents set up and the cells on ice. **TEXT: Use an empty vector for negative control**

4. Dual-luciferase Detection

4.1. To lyse the cells, remove the growth medium from the cultured cells **[1]**, gently wash the surface of the culture vessel with PBS **[2]**, and dispense 100 microliters of PLB into each culture well to completely cover the cell monolayer **[3]**.

4.1.1. Talent removing medium from a few wells.

4.1.2. Talent adding PBS to a well, with the PBS container in the shot.

4.1.3. Talent adding PLB to a few culture wells, with the PLB container in the shot.

4.2. Let the plate incubate at room temperature for 20 minutes **[1]**, then transfer the lysate to a tube or vial for further handling or storage **[2]**.

4.2.1. Plate on the lab bench.

4.2.2. Talent transferring lysate to a tube or vial.

4.3. To perform the dual-luciferase assay, program the luminometers to provide a 2-second pre-read delay, followed by a 10-second measurement period **[1]**. Then, dispense 100 microliters of luciferase assay reagent into the appropriate number of luminometer tubes **[2]**. *Videographer: This step is important!*

4.3.1. Talent programming the luminometer.

4.3.2. Talent dispensing the assay reagent into a few tubes.

4.4. Carefully add up to 20 microliters of cell lysate into the luminometer tube and mix by pipetting 2 or 3 times **[1]**. Place the tube in the luminometer and initiate the reading **[2]**. Remove the sample tube from the luminometer **[3]**, add 100 microliters of stop reagent, and vortex briefly to mix **[4]**.

4.4.1. Talent adding cell lysate into a luminometer tube and pipetting to mix.

4.4.2. Talent placing the tube in the luminometer and starting the read. *Videographer: Obtain multiple usable takes, it will be reused in 4.5.1.*

4.4.3. Talent removing the tube from the luminometer.

4.4.4. Talent adding stop reagent to the tube and vortexing it.

4.5. Return the sample to the luminometer and initiate reading **[1]**. Record the renilla luciferase activity normalized to the firefly luciferase activity, specifically the reciprocal of the ratio displayed on screen **[2]**. Discard the reaction tube when finished **[3]**. *Videographer: This step is important!*

4.5.1. *Use 4.4.2.*

4.5.2. Talent looking at the read results on the screen.

4.5.3. Talent discarding the tube.

Results

5. Results: Design of sgRNA to Target Sheep *DKK2* Exon 1

5.1. This method was used to generate 3 sgRNA vectors for sheep *DKK2* exon 1. SgRNA and xCas9 expressing vectors were built by predigesting the vector backbone, followed by ligating it in a series of short double-strand DNA fragments through annealing oligo pairs **[1-TXT]**.

5.1.1. LAB MEDIA: Figure 2. *Video Editor: Label the second lane of the gel "***pX330-xCas9***" and the third lane "***pX330-xCas9 digested with BbsI***".*

5.2. Seven bacterial colonies were screened with specific primer pair-guided PCR and the positive colonies were detected **[1]**.

5.2.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize the bands in lanes 1, 3, 4, 6, and 7.*

5.3. The gene targeting capacities of pX330-xCas9-T1, -T2 and -T3 were simultaneously detected with the dual luciferase assay **[1]**. The last sgRNA vector displayed the highest detection signal and was subsequently used for sheep gene editing research **[2]**.

5.3.1. LAB MEDIA: Figure 5.

5.3.2. LAB MEDIA: Figure 5. *Video Editor: Emphasize the yellow bar.*

Conclusion

6. Conclusion Interview Statements

6.1. **Huaiyuan Qin:** Following this protocol, the T7E1 assay or Surveyor nuclease assay can be performed. These additional methods give more accurate efficiencies of endogenous gene editing.

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