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Title: Functional Assessment of BRCA1 variants using CRISPR-Mediated Base Editors

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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? **Yes, all done**

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**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

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

Current Protocol Length

Number of Steps: 18
Number of Shots: 31

Introduction

1. Introductory Statements

NOTE to VO Talent: Please record the introduction and conclusion statements as well.

1.1. By efficiently inducing point mutations using the CRISPR-mediated cytosine bases editor, the functionality of BRCA1 *('bra-kah-1')* variants, which is important for disease prevention and diagnosis, can be identified.

1.1.1. *Suggested: Figure 1 A.*

1.2. The advantage of this technique is that it directly mutates endogenously expressed BRCA1, which overcomes the limitation of functional evaluation using exogenously expressed BRCA1.

1.2.1. *Suggested: Figure 1 A.*

1.3. Identification of the loss-of-function mutations of BRCA1 can be used to predict the chance of developing cancers associated with BRCA1 mutations such as breast, ovarian, prostate, and pancreatic cancers. It can also be used for finding potential drug targets by searching for essential genes whose viability is reduced through functional depletion.

Protocol

2. Design and construction of *BRCA1* targeting gRNAs

2.1. To begin, obtain the *BRCA1 (pronounce "brah-kuh-one")* genome sequence from GenBank at NCBI and search the 20-base pair target sites with the Protospacer Adjacent Motif sequence "NGG" and "CCN" around the mutation of interest **[1]**.

2.1.1. WIDE: Establishing shot of talent searching for target sites within the sequence.

2.2. The mutation of interest should be located within 4 to 8 nucleotides in the PAM-distal end of the gRNA target sequences **[2]**.

2.2.1. LAB MEDIA: Figure 2 A.

2.3. Order two complementary oligonucleotides per gRNA. For the forward oligonucleotides, add "CACCG" to the 5-prime end of the guide sequence, and for the reverse oligonucleotides, add "AAAC" to the 5-prime end and "C" to the 3-prime end **[1]**.

2.3.1. SCREEN: 61557_screenshot_1.MP4. 0:40 – 0:46. *Video Editor: can also use Figure 2 B.*

2.4. After receiving the oligonucleotides, resuspend them in distilled water to a final concentration of 100 micromolar **[1]**. Mix the two complementary oligonucleotides to a final concentration of 10 micromolar with T4 Ligation buffer **[2]** and heat them to 95 degrees Celsius for 5 minutes **[3]**, then cool them to room temperature for annealing **[4]**.

2.4.1. Talent resuspending the oligos.

2.4.2. Talent mixing the complementary oligos with ligation buffer.

2.4.3. Talent heating the samples.

2.4.4. Talent leaving the samples to cool at room temperature.

2.5. Digest pRG2 using the restriction enzyme Bsa-1 for 1 hour at 37 degrees Celsius **[1]**, then run the digested product on a 1% agarose gel and purify the appropriate-sized band **[2]**.

2.5.1. Digestion reaction incubating.

2.5.2. Gel running.

2.6. Ligate the annealed oligonucleotide duplex to the vector DNA using the purchased DNA ligase according to the manufacturer's protocol and transform them into DH5 alpha-competent cells **[1]**.

2.6.1. Talent adding the ligated vector to the cells.

2.7. Add the transformants to an agar plate containing 100 micrograms per milliliter ampicillin **[1]** and incubate the plate overnight at 37 degrees Celsius **[2]**. Purify plasmid DNA from several transformants and analyze their gRNA sequences by Sanger sequencing with primers that prime at the U6 promoter **[3]**.

2.7.1. Talent adding cells to an agar plate.

2.7.2. Talent putting the plates in the incubator and closing the door.

2.7.3. Talent at the computer, looking at sequencing results.

3. Creation of *BRCA1* Variants using CRISPR-mediated Base Editing Tools

3.1. Seed 5 x 10^5 HAP1-BE3 *(pronounce 'hap-1-B-E-3')* cells per well in 24-well plates 1 day prior to transfection and culture them to reach 70 to 80% confluence for transfection **[1]**. *Videographer: This step is difficult and important!*

3.1.1. Talent seeding cells.

3.2. Transfect *BRCA1*-targeting gRNAs using the purchased transfection reagents according to the manufacturer's protocol **[1]**. Use 1 microgram of *BRCA1*-targeting gRNAs to induce C-G to T-A conversion at *BRCA1* target sites **[2]**, then incubate the cells at 37 degrees Celsius and subculture every 3 to 4 days **[3]**. *Videographer: This step is difficult and important!*

3.2.1. Talent preparing to transfect the cells, with the transfection reagents in the shot.

3.2.2. Talent adding *BRCA1*-targeting gRNAs to the cells.

3.2.3. Talent putting the cells in the incubator and closing the door.

3.3. Harvest the cell pellets 3, 10, and 24 days after transfection to analyze base editing efficiencies. Extract genomic DNA using the genomic DNA purification kit **[1]**.

3.3.1. Talent taking the plates with cells out of the incubator.

4. Sample preparation for Illumina next-generation sequencing (NGS)

4.1. Design the first PCR primers to amplify *BRCA1* target sites. Although there is no restriction on the size of the first PCR product, a product size of less than 1 kilobases is recommended to efficiently amplify a specific region **[1]**.

4.1.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize the top target site with the 1st primers.*

4.2. Design the second PCR primers located inside the first PCR product, taking into consideration the size of the amplicon according to the NGS read length **[1]**. In order to attach essential sequences for NGS analysis, add additional sequences to the 5-prime ends of the second PCR primers **[2-TXT]**.

4.2.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize the 1st amplicon with the 2nd primers.*

4.2.2. LAB MEDIA: Figure 3. *Video Editor: Emphasize the 1st amplicon with the 2nd primers.* **TEXT: Forward Primer: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'; Reverse Primer: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCT-3'**

4.3. Amplify the *BRCA1* target sites on the genomic DNA obtained from the three time points using high-fidelity polymerase. For the first PCR reaction, use 100 nanograms of genomic DNA for amplification over 15 cycles. For the second PCR reaction, use 1 microliter of the first PCR product for amplification over 20 cycles **[1]**. *Videographer: This step is important!*

4.3.1. Talent putting samples in a thermocycler and programming it.

4.4. Run 5 microliters of the second PCR product on 2% agarose gel and confirm the size.

4.4.1. Talent imaging a gel with the PCR product.

4.5. Then, attach the essential sequences for NGS analysis by amplifying the second PCR product using the primers listed in the text manuscript. Amplify each sample using different primer sets **[1]**. Use 1 microliter of the second PCR product for up to 30 cycles of amplification with a high-fidelity polymerase **[2]**.

4.5.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize the 2nd Amplicon and NGS library.*

4.5.2. Talent starting the PCR reaction.

4.6. Run 5 microliters of the PCR product on 2% agarose gel to confirm the size, and purify the amplicon using a commercial PCR clean-up kit **[1]**. Mix each sample in equal amounts to create an NGS library **[2]**.

4.6.1. Talent loading the PCR product onto a gel.

4.6.2. Talent mixing the samples.

4.7. Quantify the NGS library using a spectrophotometer **[1]** and dilute it to a concentration of 1 nanomolar using resuspension buffer or 10 millimolar Tris-HCl at pH 8.5 **[2]**. Prepare 100 microliters of the library diluted to the appropriate loading concentration. As a control, combine phiX *(pronounce it 'fi-X')* with the diluted sample **[3]**.

4.7.1. Talent using the spectrophotometer.

4.7.2. Talent diluting the library.

4.7.3. Prepared NGS samples and control.

4.8. Load the library onto the cartridge and run NGS according to the manufacturer's protocol. Obtain over 10,000 reads per target amplicon for in-depth analysis of base editing efficiency **[1]**.

4.8.1. Talent loading the library onto the cartridge.

Results

5. Results: Functional Study of BRCA1 using CRISPR-Cas9 Systems

5.1. This protocol was used to perform a functional assessment of endogenous *BRCA1* variants generated by CRISPR-based cytosine base editors. Cas9 and gRNAs were transfected into HAP1 cell lines to disrupt *BRCA1* and mutation frequencies were analyzed **[1]**.

5.1.1. LAB MEDIA: Figure 4 A.

5.2. Mutation frequencies decreased significantly over time in HAP1 cell lines, indicating that *BRCA1* is an essential gene for cell viability in these cells **[1]**.

5.2.1. LAB MEDIA: Figure 4 A. *Video Editor: Emphasize BRCA1 #1 and BRCA1 #2 bars.*

5.3. To investigate whether C-G to T-A substituted variants affect the function of *BRCA1*, the DNA plasmids encoding gRNAs that could induce each mutation were transfected to HAP1-BE3 cell lines and the substitution frequencies were analyzed **[1]**.

5.3.1. LAB MEDIA: Figure 4 B.

5.4. The relative substitution frequencies of thirty-five-ninety-eight-C-to-T, a pathogenic variant that induces a nonsense mutation, dramatically decreased **[1]**, whereas those of forty-five-twenty-seven-C-to-T, a benign variant that induces a nonsense mutation, remained similar with time **[2]**.

5.4.1. LAB MEDIA: Figure 4 B. *Video Editor: Emphasize the c.3598C>T (p. Q1200*) bars.*

5.4.2. LAB MEDIA: Figure 4 B. *Video Editor: Emphasize c.4527C>T (p.Y1509Y) bars.*

5.5. It was found that nucleotide substitution frequencies of these three variants decreased in a time-dependent manner **[3]**.

5.5.1. LAB MEDIA: Figure 4 B.

Conclusion

6. Conclusion Interview Statements

6.1. Since it is necessary to obtain a high initial mutation frequency in order to clearly recognize the change in cell viability with the passing of the date, it is a priority to establish optimized transfection conditions.

6.1.1. *Suggested: Shots from 3.1 – 3.2.*

6.2. This procedure can be applied for verifying other unknown genetic variants such as BRCA2 VUS *(spell out 'V-U-S')*. It may provide clues to the pathogenicity of patient-derived unknown variants and their treatment.

6.2.1. *Suggested: Figure 1 B.*