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Dear Nam Nguyen:

We would like to thank the two referees for their helpful comments. We have addressed several points raised by referees as shown in the following pages and in the highlighted text in our manuscript for ease of tracking. In addition, we have revised our manuscript according to the editor's advices.

We believe that our revisions address the reviewers' concerns and look forward to the publication of our manuscript in JoVE. Thank you very much for your help and attention.

Sincerely yours,

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TITLE:

Functional Assessment of *BRCA1* variants using CRISPR-Mediated Base Editors

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KEYWORDS:

CRISPR-Cas; base editing; BE3; *BRCA1*; functional assessment; Genome engineering

SUMMARY:

People with *BRCA1* mutations have a higher risk of developing cancer, which warrants accurate evaluation of the function of *BRCA1* variants. Herein, we described a protocol for functional assessment of *BRCA1* variants using CRISPR-mediated cytosine base editors that enable targeted C:G to T:A conversion in living cells.

ABSTRACT:

Recent studies have investigated the risks associated with *BRCA1* gene mutations using various functional assessment methods such as fluorescent reporter assays, embryonic stem cell viability assays, and therapeutic drug-based sensitivity assays. Although they have clarified a lot of *BRCA1* variants, these assays involving the use of exogenously expressed *BRCA1* variants are associated with overexpression issues and cannot be applied to post-transcriptional regulation. To resolve these limitations, we previously reported a method for functional analysis of *BRCA1* variants via CRISPR-mediated cytosine base editor that induce targeted nucleotide substitution in living cells. Using this method, we identified variants whose functions remain ambiguous, including c.-97C>T, c.154C>T, c.3847C>T, c.5056C>T, and c.4986+5G>A, and confirmed that CRISPR-mediated base editors are useful tools for reclassifying the variants of uncertain significance in *BRCA1*. Here, we describe a protocol for functional analysis of *BRCA1* variants using CRISPR-based cytosine base editor. This protocol provides guidelines for the selection of target sites, functional analysis and evaluation of *BRCA1* variants.

INTRODUCTION:

The breast cancer type 1 susceptibility gene (*BRCA1*) is a widely known tumor suppressor gene. Because the *BRCA1* gene is related to the repair of DNA damage, mutations in this gene would lead to a greater risk of cancer development in an individual[1]. Breast, ovarian, prostate, and pancreatic cancers are linked to inherited loss-of-function (LOF) mutations of the *BRCA1* gene[2]. Functional assessment and identification of *BRCA1* variants may help in preventing and diagnosing the various diseases. To address function of BRCA1 variants, several methods have been developed and broadly used for investigating the pathogenicity of *BRCA1* variants such as embryonic stem cell viability assays, fluorescent reporter assays, and therapeutic drug-based sensitivity assays[3-6]. Although these methods have assessed the function of a lot of BRCA1 variants, the methods involving exogenously expressed *BRCA1* variants pose limitations in terms of overexpression that might affecting downstream regulation, gene dosage, and protein folding[7]. Furthermore, these assays cannot be harnessed to the posttranscriptional regulation such as mRNA splicing, transcript stability, and effect of untranslated region[8,9].

CRISPR-Cas9 system enables targeted genome editing in living cells and organisms[10]. Through a single-guide RNA, Cas9 can induce double-strand breaks (DSBs) in chromosomal DNA at specific genomic loci in order to activate two DNA repair pathways: error-prone nonhomologous end-joining (NHEJ) pathway and error-free homology-directed repair (HDR) pathway[11]. HDR is a precise repair mechanism; however, DSBs induced by Cas9 nuclease for HDR often results in unwanted insertion and deletion (indel) mutation. Additionally, it needs homologous donor DNA templates for repairing DNA damage and has relatively low efficiency. Recently, Cas9 nickase (nCas9) have been fused with cytidine deaminase domains for targeting C:G to T:A substitutions, without the need for homologous DNA templates and DNA double strand breaks[12-15]. Using the cytosine base editor, we developed a new method for functional analysis of BRCA1 variants[16].

In this study, we used CRISPR-mediated cytosine base editor, BE3[14], which induces efficient C:G to T:A point mutations, for implementing the functional assessment of *BRCA1* variants and successfully identified the functions of several *BRCA1* variants (**Figure 1**).

[Place Figure 1 here]

PROTOCOL:

NOTE: Method 1 (generation of HAP1-BE3 cell lines) is optional. Instead of constructing a BE3-expressing cell line, BE3-encoding plasmid DNA can be co-transfected with gRNA-encoding plasmid DNA. Other variants of cytosine base editors, such as BE4max, also can be used for highly efficient base editing.

1. Generation of HAP1-BE3 cell lines

1.1. Construction of plasmid DNA

1.1.1. To construct the lentiBE3-blast plasmid DNA for lentivirus production, amplify the BE3 coding sequences in pCMV-BE3 (**Table of Materials**) by PCR using high-fidelity polymerase. Design the PCR primer to contain overlapping sequences of the digested vector (from step 1.1.2) for isothermal assembly as follows[17]: primer-F: 5'-TTTGCCGCCAGAACACAGGACCGGTTCTAGAGCCGCCACCATGAGCTCAGAG-3', primer-R:5'-CAGAGAGAAGTTTGTTGCGCCGGATCCGACTTTCCTCTTCTTCTTGGG-3'

NOTE: Nucleotides shown in underlined are overlapped with the digested vector and these sequences will be specific to the destination vector chosen by the user.

1.1.2. Digest lentiCas9-blast (**Table of Materials**) plasmid DNA with the restriction enzymes, XbaI and BamHI (1 unit per 1 µg) for 1 h at 37 °C. Run the digested product on a 0.8% agarose gel and purify the appropriate-sized bands (8.6 kb) using a commercial gel extraction kit (**Table of Materials**).

1.1.3. Clone the amplified BE3 PCR product and digested lentiCas9-Blast vector using the isothermal assembly kit (**Table of Materials**). Use a total of 0.02-0.5 pmol of DNA fragments and a 1:3 ratio of vector:insert. Transform the assembly product into DH5 alpha-competent cells[18]. Add the transformants on an agar plate containing ampicillin (100 µg/mL) and incubate the plate overnight at 37 °C.

1.1.4. Pick several colonies and inoculate them in 4 mL of LB (lysogeny broth) medium containing ampicillin (100 µg/mL) and grow the culture in a shaking incubator at 180 rpm.

1.1.5. Purify plasmid DNA using a commercial plasmid DNA purification kit (**Table of Materials**) according to the manufacturer's protocols.

1.1.6. To confirm DNA cloning success, analyze the BE3 sequences of each purified plasmid DNA (from step 1.1.5) by Sanger sequencing using standard and BE3-specific primers (**Supplementary Table 1**) and select the exact cloned lentiBE3-blast construct.

NOTE: To analyze the results of Sanger sequencing, we recommended several tools such as BLAST (https://blast.ncbi.nlm.nih.gov/) and CLUSTALW (https://www.genome.jp/tools-bin/clustalw).

1.2. Cell culture and generation of HAP1-BE3 cell lines

1.2.1. Maintain cells in a healthy condition and in an actively dividing state. Culture HAP1 cells in Iscove's modified Dulbecco's medium (**Table of Materials**) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Culture HEK293T/17 cells in Dulbecco's modified Eagle's medium (**Table of Materials**) containing 10% FBS and 1% penicillin/streptomycin.

NOTE: HAP1 cell is useful for genetic research because of it is nearly haploid cells. However, the cells can spontaneously return to a diploid state in cell culture. Enrichment Hoechst 34580

stained 1*n*-population using flowcytometry will be helpful for maintenance of haploidy of HAP1 cells[19].

1.2.2. Seed 5 x 10^6 HEK293T/17 cells on a 100 nm dish 1 day before transfection. On the day of transfection, transfect the plasmid DNA (15 µg of lentiBE3-blast, 9 µg of psPAX2 for viral packaging, and 6 µg of pMD2.G for vial envelope of 2nd generation lentiviral packaging system) using commercial transfection reagents according to the manufacturer's protocols (**Table of Materials**)[20]. Change the medium at 6 h post-transfection and harvest virus-containing medium at 48 h or 72 h post-transfection. Filter the supernatant using a 0.45 µm filter.

1.2.3. Transduce the lentiviral particles of BE3 into HAP1 cells at a MOI (multiplicity of infection) of 0.1. To adjust for an appropriate MOI, use serially diluted concentrations of virus. Remove the medium and replace it with adjusted viral supernatant and fresh culture medium.

1.2.4. One day after transduction, change the medium to 10 µg/mL of blasticidin (**Table of Materials**)-containing medium and select the transduced cells for 3 days with blasticidin. After blasticidin selection, select a well with ~10% of surviving cells for the next step.

1.2.5. After blasticidin selection, seed the transduced cells on 96-well plates at a density of 0.5 cells/well in order to isolate single clones (e.g., dilute 50 cells in 20 mL (0.5 cells per 200 µL) culture medium and aliquot 200 µL per well in 96 well plate). Incubate the 96-well plates for 2 weeks and pick the single colonies to confirm the BE3 activity.

1.2.6. Divide the single colonies into two sets, one sets for testing BE3 activity and other for maintenance. Transduce the lentiviral particles of gRNAs into one of the sets to confirm the BE3 activity. Analyze the mutation frequency of each colony using T7 endonuclease I (T7E1, **Table of Materials**) assay or by performing the targeted deep sequencing technique (step 4)[21]. Select appropriate single clones that are healthy and have a highly active BE3.

NOTE: To validate exact mutation frequencies, we recommend the targeted deep sequencing than T7E1 assay. HEK2 (5'-GAACACAAAGCATAGACTGCGGG-'3) is well validated target site for BE3.

2. Design and construction of *BRCA1* targeting gRNAs

[Place Figure 2 here]

NOTE: Positive and negative controls of *BRCA1* variants are essential. In this study, c.5252G>A (R1751Q) and c.4527C>T (Y1509Y) are used as benign controls. c.191G>A (C64Y), 81-1G>A, and c.3598C>T (Q1200*) are used as pathogenic controls. Target sequences of each gRNA are listed in **Supplementary Table 1**.

2.1. Obtain the *BRCA1* genome sequence from GenBank at NCBI[22].

2.2. Search the 20-bp target sites with the Protospacer Adjacent Motif (PAM) sequence "NGG"

and “CCN” around mutation of interest. The mutation of interest should be located in 4–8 nucleotides in the PAM-distal end of the gRNA target sequences because of the active window of BE3 is 4–8 nucleotides in the PAM-distal end of the gRNA target sequences[14]. In the case of c.8047C>T (H1283Y)- and c.5252G>A (R1751Q)-targeting gRNAs, 5’-TCA**GGAAC**ATCACCTTAGTG-AGG-3’ and 5’-CCA-CCAAGGTCCAA**AGCGA**CAA-3’, the sequences in bold are active windows. C to T and G to A conversions occur with NGG and CCN PAM, respectively (**Figure 2A**).

NOTE: BE-Designer (http://www.rgenome.net/be-designer/)[23] is useful web-based tools for gRNA design.

2.3. Order two complementary oligonucleotides per gRNA; for the forward oligonucleotides, add “CACCG” to the 5’ end of the guide sequence, and for the reverse oligonucleotides, add “AAAC” to the 5’ end and “C” to the 3’ end. These additional sequences are specific to the destination gRNA expression vector (from step 2.5) used for this protocol, and users should be adjusted for alternative gRNA expression vectors (**Figure 2B**).

2.4. Resuspend the oligonucleotide in distilled water at a final concentration of 100 µM. Mix the two complementary oligonucleotides to a final concentration of 10 µM with T4 Ligation buffer (**Table of Materials**) and heat them at 95 °C for 5 min and cool them at room temperature for annealing.

2.5. Digest pRG2 using the restriction enzyme, BsaI (1 unit per 1 µg) for 1 h at 37 °C (**Table of Materials**). Run the digested product on 1% agarose gel and purify the appropriate-sized band (2.5 kb).

NOTE: Instead of using a classic digestion and ligation method, other cloning method such as Golden Gate cloning can be used.

2.6. Ligate the annealed oligonucleotide duplex to the vector DNA using the purchased DNA ligase (**Table of Materials**) according to the manufacturer’s protocol and transform them into DH5 alpha-competent cells (Other E. coli strains which widely used for subcloning also can be used).

2.7. Add the transformants to an agar plate containing ampicillin (100 µg/mL) and incubate the plate overnight at 37 °C. Purify plasmid DNA from several transformants and analyze their gRNA sequences by Sanger sequencing using primers which prime at U6 promoter (**Table of Materials**).

3. Creation of *BRCA1* variants using CRISPR-mediated base editing tools

NOTE: If HAP1-BE3 cell lines is not used, BE3-encoding plasmid DNA can be co-transfected with *BRCA1*-targeting gRNA. Compared to co-transfection of BE3 and gRNA plasmids, transfection of gRNA plasmid to HAP-BE3 cells induce efficient base editing up to 3-fold at target locus in our hands.

3.1. Seed 5 x 10^5 HAP1-BE3 cells (or HAP1 cells in case of co-transfection methods) per well in 24-well plates 1 day prior to transfection. At the time of transfection, culture cells to reach an appropriate density (70%–80% confluence).

3.2. Transfect *BRCA1*-targeting gRNAs using the purchased transfection reagents (**Table of Materials**) according to the manufacturer's protocol. Use 1 µg of *BRCA1*-targeting gRNAs (with 1 µg of BE3-encoding plasmid DNA in case of co-transfection methods) to induce C:G to T:A conversion at *BRCA1* target sites. Incubate the cells at 37 °C and subculture every 3–4 days.

3.3. Harvest the cell pellets 3, 10, and 24 days after transfection to analyze base editing efficiencies (Samples from 3 days after transfection are analyzed regrading as day 0 samples).

3.4. Extract genomic DNA using the genomic DNA purification kit (**Table of Materials**).

NOTE: We recommend optimizing transfection conditions with variable ratio of reagent to DNA. Optimal condition for HAP1 transfection is 4:1 ration of reagent to DNA in our hands.

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

[Place Figure 3 here]

4.1. Design the 1st PCR primers to amplify *BRCA1* target sites. Although there is no restriction on the size of the 1st PCR product, a product size of <1 kb is recommended to efficiently amplify a specific region (Figure 3).

4.2. Design the 2nd PCR primers located inside the 1st PCR product. Consider the size of the amplicon according to the NGS read length (For example, the size of the amplicon product should be smaller than 300 bp for 2 × 150 bp paired-end run to merge each reads). In order to attach essential sequences for NGS analysis, add additional sequences to the 5' end of the 2nd PCR primers as follows: for the forward primers, add 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3' sequences to the 5' end, and for the reverse primers, add 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCT-3' sequences to the 5' end.

NOTE: We used nested PCR to reduce the amplicon of non-specific binding by preventing primers from attaching to non-target sequence.

4.3. Amplify the *BRCA1* target sites on the genomic DNA obtained from three time points. Use high-fidelity polymerase according to the manufacturer's protocol for minimizing PCR errors. For the 1st PCR reaction, use 100 ng of genomic DNA for amplification over 15 cycles. For the 2nd PCR reaction, use 1 µL of the 1st PCR product for amplification over 20 cycles. Run 5 µL of the 2nd PCR product on 2% agarose gel and confirm the size.

4.4. In order to attach the essential sequences for NGS analysis, amplify the 2nd PCR product

using the primers listed below. For the PCR reaction, 1 µL of the 2nd PCR product is used for amplification up to 30 cycles using high-fidelity polymerase.

4.4.1. To perform multiplex sequencing for large numbers of libraries to be pooled and sequenced simultaneously, use forward (D501 – D508) and reverse (D701 – D712) primers, which have unique index sequence (**Supplementary Table 1**). Amplify each sample using different primers sets to conduct the unique dual indexing strategy.

4.4.2. Run 5 µL of the PCR product on 2% agarose gel to confirm the size, and purify the amplicon using a commercial PCR clean-up kit (**Table of Materials**). Mix each sample in equal amounts to create an NGS library.

4.5. Quantify the NGS library at 260 nm wavelength using spectrophotometers and dilute the NGS library to concentration of 1 nM using resuspension buffer or 10 mM Tris-HCl, pH 8.5. Prepare 100 µL of the library diluted to the appropriate loading concentration depending on the library types (For example, prepare 200 pM of the library for Nextera DNA Flex).

4.5.1. As a control, combine phiX with the diluted sample appropriate for the type of kit.

4.5.2. Load the library onto the cartridge and run NGS according to the manufacturer's protocol. Illumina iSeq 100 or Miseq systems can sequence the amplicons of various length up to 300 bp for single-read or paired end. We recommended over 10,000 reads per target amplicon for in-depth analysis of base editing efficiency.

5. Analysis of base editing efficiency for the functional assessment of *BRCA1* variants

5.1. Analyze the base editing efficiencies using MAUND[24]. The base editing efficiency is calculated as described below.

$$Base\ editing\ efficiency = \frac{Read\ counts\ of\ \boldsymbol{targeted}\ C\ to\ T\ conversions}{Total\ read\ counts\ of\ each\ index}$$

If multiple cytosines are present in BE3 active window, only the C to T conversion that is targeted for evaluating the *BRCA1* variant is considered. For example, if the sequences of BE3 active window are "$C_4A_5T_6C_7T_8$," the possible sequences generated by base editing are "$T_4A_5T_6C_7T_8$", "$C_4A_5T_6T_7T_8$", and "$T_4A_5T_6T_7T_8$". At this time, if position 4 is the targeted position for the desired *BRCA1* variant, then only the ""$T_4A_5T_6C_7T_8$" sequence is considered for calculating base editing efficiency.

NOTE: We also recommend other web-tools for analysis of base editing activity such as CRISPResso2[25], and BE-analyzer[23].

5.2. Verify the results using positive and negative controls of *BRCA1* variants. The base editing efficiencies of the benign control should remain the same, whereas those of the pathogenic control should decrease over time.

5.3. Calculate the relative base editing efficiency of *BRCA1* variants and determine their pathogenicity. The significant differences between Day 0 and Day 21 samples are analyzed appropriate statistical analysis such as *t*-test.

REPRESENTATIVE RESULTS:

The experimental approaches described in this protocol enable the functional assessment of endogenous *BRCA1* variants generated by CRISPR-based cytosine base editors. To select appropriate cell lines for the functional assessment of *BRCA1* variants, researchers should confirm that *BRCA1* is essential gene in the targeted cell lines. For example, we first transfected Cas9 and gRNAs into HAP1 cell lines to disrupt *BRCA1* and analyzed mutation frequencies by targeted deep sequencing. We found that mutation frequencies decreased significantly over time in HAP1 cell lines (**Figure 4A**). These results showed that *BRCA1* is essential gene for cell viability in HAP1 cell lines. To investigate whether C:G to T:A substituted variants affect the function of *BRCA1*, the plasmids DNA encoding gRNAs, which could induce each mutation, were transfected to HAP1-BE3 cell lines and the substitution frequencies were analyzed. The relative substitution frequencies of c.3598C>T (p. Q1200*), a pathogenic variant, dramatically decreased, whereas those of c.4527C>T (p.Y1509Y), a benign variant, remained similar with time (**Figure 4B**). In the ClinVar database, c.154C>T (p. L52F), c.3847C>T (p.H1283Y), and c.5056C>T (p.H1686Y) of *BRCA1* are reported as variants of uncertain significance. We analyzed function of these variants using the methods mentioned above and found that nucleotide substitution frequencies of these three variants decreased in a time-dependent manner (**Figure 4B**). From these results, the three substitutions altered BRCA1 function and could be categorized as pathogenic mutations.

FIGURE AND TABLE LEGENDS:

Figure 1: An overview of the workflow for functional assessment. (**A**) Schematic showing the functional assessment of *BRCA1*. Because the LOF of *BRCA1* affects cell viability, when the *BRCA1* mutation is pathogenic, the cells die as the passage number increases. (**B**) Stages of the functional assessment of *BRCA1*. Dotted box is optional. It can be replaced by co-transfection of gRNA expressing and BE3 expressing plasmids DNA.

Figure 2: An example of a gRNA plasmid DNA. (**A**) To effectively edit the target sequence with BE3, an NGG PAM (CCN PAM) that places the target C (target G) within a five-nucleotide window is required. NGG PAM is shown in red and the base editing window is represented by a gray box. (**B**) The gRNA sequence for c.8047C>T (H1283Y) base editing is indicated and the target C:G pairs are shown in red while the active window is highlighted by a gray box. For gRNA cloning, the overhang sequences indicated in bold are added at both the 5’ ends. Templates for gRNA were generated by annealing the two complementary oligonucleotides.

Figure 3: Preparation for next-generation sequencing. The 1st PCR primer was designed to amplify the *BRCA1* target site on genomic DNA. The 2nd PCR primer was designed such that its sequences are located more inside than the 1st PCR primer sequences. Additional sequences shown as a yellow bar were added at both ends of the 2nd PCR primer to attach the essential sequences for performing next-generation sequencing.

Figure 4: Representative results of functional study of BRCA1 using CRISPR-Cas9 systems. (**A**) BRCA1 disruption affects to the cell viability. HAP1 cells were transfected with plasmid encoding spCas9 and two gRNAs targeting *BRCA1*, respectively, and targeted deep sequencing was performed for cell viability analysis. Mutation frequencies of BRCA1 decreased in a time-dependent manner in cells transfected with two independent gRNAs, and the mutation frequencies of CCR5, which was used as a negative control, remained the same over time. (**B**) Functional assessments of five BRCA1 variants. HAP1-BE3 cells were transfected with gRNAs inducing BRCA1 mutations, respectively, and targeted deep sequencing was performed for cell viability analysis. The relative substitution frequencies decreased in a time-dependent manner in cells of c.3598C>T, c.154C>T, c.3847C>T, and c.5056C>T and those of c.4527C>T remained the same. Error bars show the standard error of mean. Asterisks denote different P values: * $P<0.05$; ** $P<0.005$., n.s: not significant.

DISCUSSION:

This protocol describes a simple method for functional assessments of *BRCA1* variants using CRISPR-meditated cytosine base editor. The protocol describes methods for the design of gRNAs at target locus and construction of the plasmid DNAs from which they are expressed. Cytosine base editors induce nucleotide conversion in an active window (in case of BE3, nucleotides 4–8 in the PAM-distal end of the gRNA target sequences). The researcher should carefully choose target sequences because all cytosines in active window can be substituted to thymines. Furthermore, as described in Step 5, multiple cytosines in an active window should be carefully analyzed to evaluate the function of *BRCA1* variants.

One of the most important steps is transfection in the target cell line, which affects the initial mutation frequency for *BRCA1* functional assessments. To improve the initial mutation frequency, the researchers should optimize the delivery methods to the cell line of interest. As described in Step 1, the generation of BE3 expressing cell lines is useful option to increase the initial mutation frequency. We do not recommend lentiviral transduction of gRNA into the HAP1-BE3 cells, because constitutive expression of BE3 and gRNA could cause accumulative nucleotide conversion, and these results interfere with the functional assessment of *BRCA1* variants.

In addition to the BE3 mediated methods introduced in this protocol, several complementary methods are recommended to further extend the functional assessments of *BRCA1* variants. First, as described above, the mutation frequency in the initial sample is important in order to obtain confident results of *BRCA1* variants. To increase the base editing efficiency, variants of cytosine base editors, such as BE4max, are recommended. Second, the BE3 recognize target DNA sequence through the 5'-NGG-3' PAM sequences, which is a limitation in generating various types of BRCA1 variants. Recently developed Cas9 variants with altered PAM sequences are useful option in this case to extend targetable *BRCA1* variants[26-28]. Third, the BE3 induces substantial base editing at unwanted sites and the off-target effect could influence functional assessment of BRCA1 variants[29-31]. To reduce the off-target effect of BE3, target sites of gRNAs should be carefully chosen without any similar sequences in the genome. SECURE-BE3 or YE1, which has developed for reducing unwanted base editing in genome and transcriptome are useful option[32,33]. Forth, a saturation genome editing (SGE) method based on Cas9-mediated HDR also

great options for functional analysis of BRCA1 variants[19]. The method has no limitation for selecting target sequences and nucleotide positions of BRCA1 variants. However, HDR-based approach is relatively less efficient than the base editors and additionally requires design and synthesis of donor templates[14]. Finally, patient derived *BRCA1* variants include various range of mutations such as point mutations, insertions, and deletions. Of these, point mutations are major population of *BRCA1* variants, which are not only C:G to T:A conversion, but also A:T to G:C , C:G to G:C, and A:T to T:A conversions. To functional assessments of these types of conversions, CRISPR-mediated adenosine base editors and Prime Editors are valuable options[34,35]. Rapidly developing genome engineering technologies will enable functional assessments of more diverse *BRCA1* variants.

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DISCLOSURES:

The authors have nothing to disclose.

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21 Kim, Y. et al. A library of TAL effector nucleases spanning the human genome. *Nature Biotechnology.* **31** (3), 251-258 (2013).
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23 Hwang, G. H. et al. Web-based design and analysis tools for CRISPR base editing. *BMC Bioinformatics.* **19** (1), 542 (2018).
24 Kim, D., Kim, D. E., Lee, G., Cho, S. I., Kim, J. S. Genome-wide target specificity of CRISPR RNA-guided adenine base editors. *Nature Biotechnology.* **37** (4), 430-435 (2019).
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26 Hu, J. H. et al. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature.* **556** (7699), 57-63 (2018).
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28 Walton, R. T., Christie, K. A., Whittaker, M. N., Kleinstiver, B. P. Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. *Science.* (2020).
29 Kim, D. et al. Genome-wide target specificities of CRISPR RNA-guided programmable deaminases. *Nature Biotechnology.* **35** (5), 475-480 (2017).
30 Zuo, E. et al. Cytosine base editor generates substantial off-target single-nucleotide variants in mouse embryos. *Science.* **364** (6437), 289-292 (2019).
31 Jin, S. et al. Cytosine, but not adenine, base editors induce genome-wide off-target mutations in rice. *Science.* **364** (6437), 292-295 (2019).
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628 (2020).
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35 Anzalone, A. V. et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature.* **576** (7785), 149-157 (2019).

Figure 1

Click here to access/download;Figure;1.tif

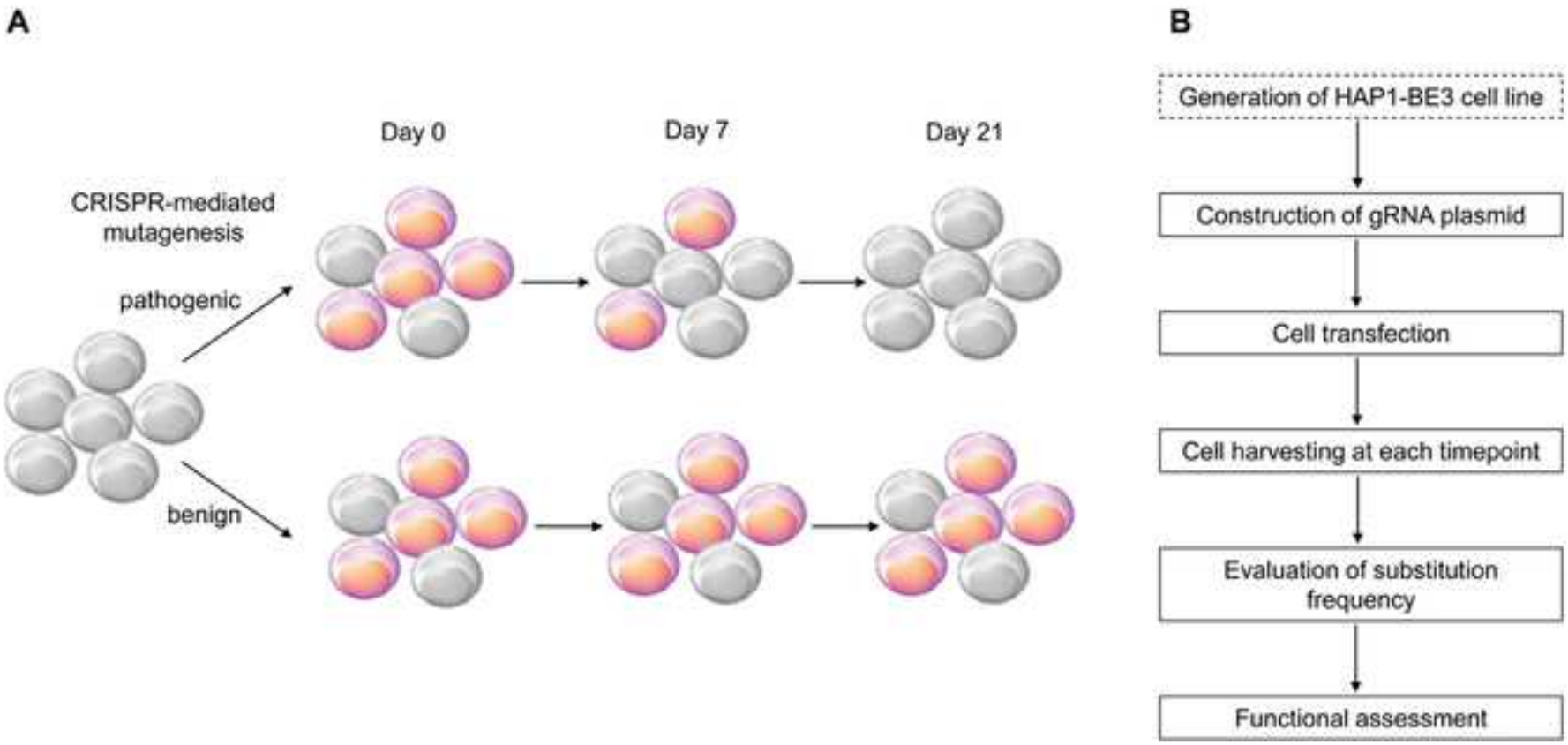

Figure 2

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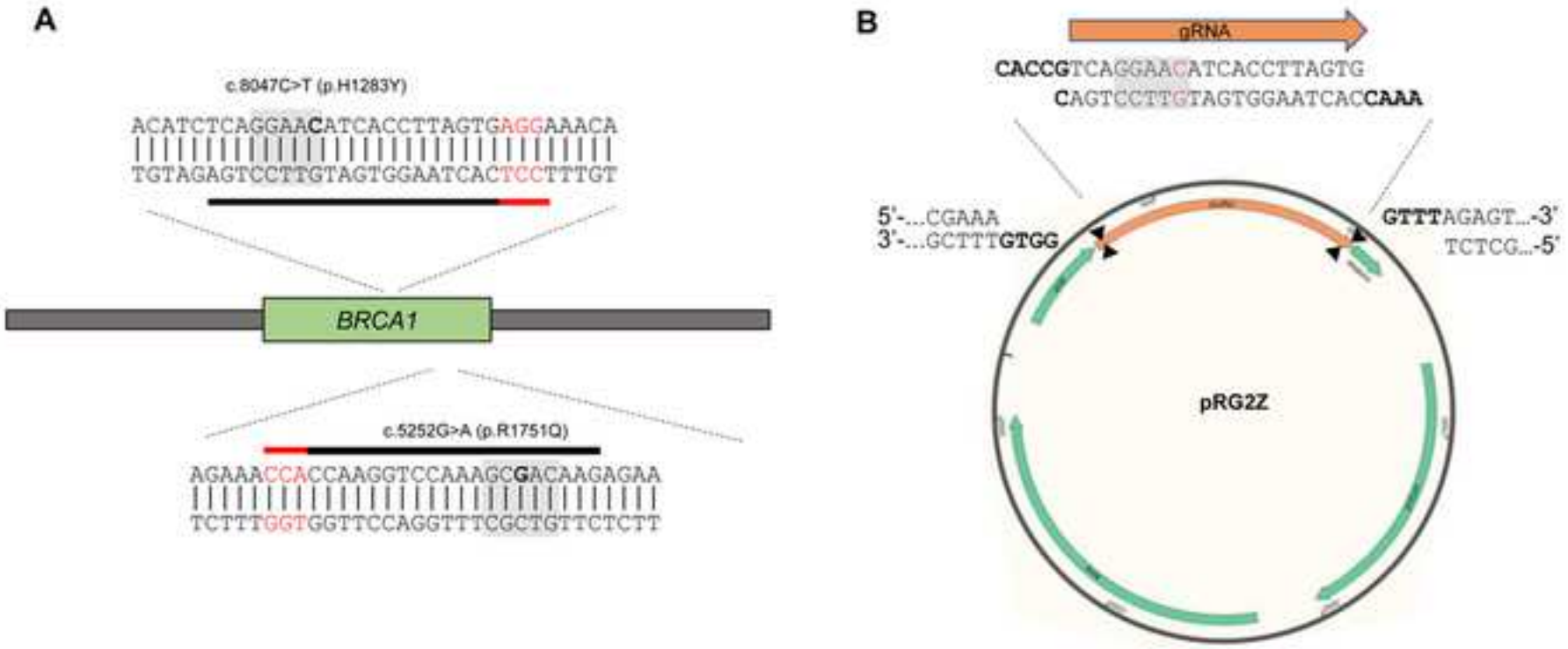

Figure 3

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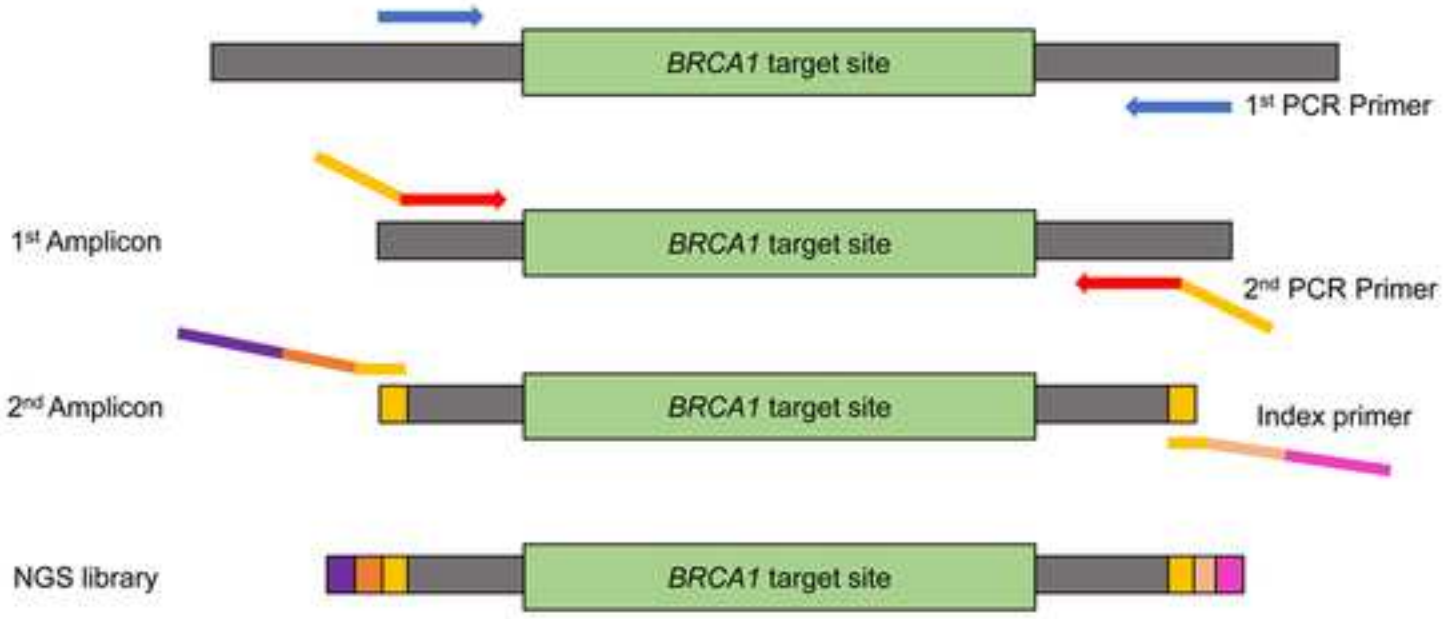

Figure 4

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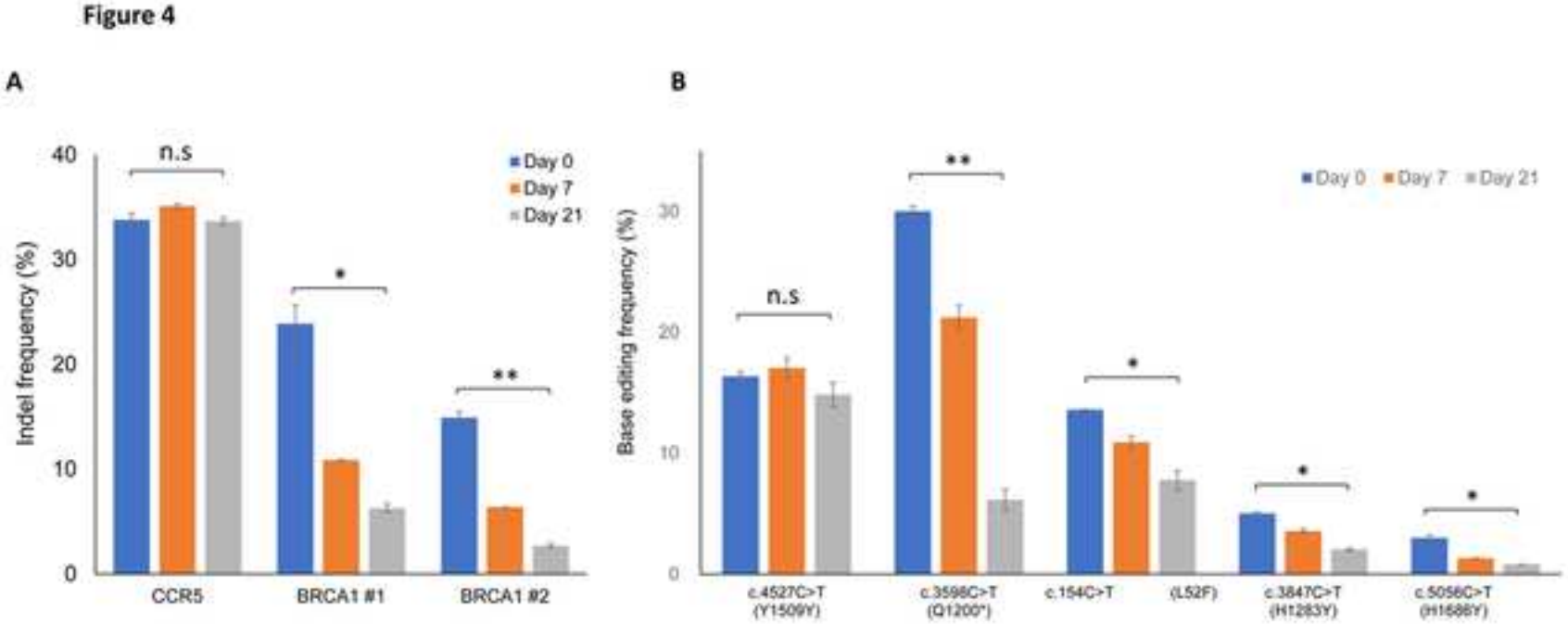

Click here to access/download;Table of Materials;JoVE_Table_of_Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
BamHI	NEB	R3136	Restriction enzyme
Blasticidin	Thermo Fisher Scientific	A1113903	Drug for selecting transduced cells
BsaI	NEB	R0535	Restriction enzyme
DNeasy Blood & Tissue Kit	Qiagen	69504	Genomic DNA prep. kit
Dulbecco's modified Eagle's medium	Gibco	11965092	Medium for HEK293T/17 cells
Fetal bovine serum	Gibco	16000036	Supplemetal for cell culture
FuGENE HD Transfection Reagent	Promega	E2311	Transfection reagent
Gibson Assembly Master Mix	NEB	E2611L	Gibson assembly kit
Iscove's modified Dulbecco's medium	Gibco	12440046	Medium for HAP1 cells
lentiCas9-Blast	Addgene	52962	Plasmids DNA for lentiBE3 cloning
Lipofectamine 2000	Thermo Fisher Scientific	11668027	Transfection reagent
Opti-MEM	Gibco	31985070	Transfection materials
pCMV-BE3	Addgene	73021	Plasmids DNA for lentiBE3 cloning
Penicillin-Streptomycin	Gibco	15140	Supplemetal for cell culture
Phusion High-Fidelity DNA Polymerase	NEB	M0530SQ	High-fidelity polymerase
pMD2.G	Addgene	12259	Plasmids DNA for virus prep.
pRG2	Addgene	104174	gRNA cloning vector
psPAX2	Addgene	12260	Plasmids DNA for virus prep.
QIAprep Spin Miniprep kit	Qiagen	27106	Plasmid DNA prep. Kit
QIAquick Gel extraction Kit	Qiagen	28704	Gel extraction kit
QIAquick PCR Purification Kit	Qiagen	28104	PCR product prep. kit
Quick Ligation Kit	NEB	M2200	Ligase for gRNA cloning
T7 Endonuclease I	NEB	M0302	Materials for T7E1 assay
XbaI	NEB	R0145	Restriction enzyme

Rebuttal Letter

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Editorial comments:

NOTE: Please read this entire email before making edits to your manuscript. Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write the text on lines 55-60, 258-263 to avoid this overlap.

Response: We have revised our manuscript as editor's advice in line 54-61 and line 324-329.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Examples:

1) 1.1.1: Mention primers and list them in the table of materials. Cite a reference for amplification.

Response: We have added primers and references as editor's comments in line 88-96.

2) 1.1.2: how is digestion performed? What are the enzyme concentrations? How is purification performed? Specify the band size.

Response: We have added further information as editor's comments in line 98-101.

3) 1.1.3: cite a reference for transformation.

Response: We have added reference in line 106.

.

4) 1.1.4: Define LB.

Response: We have now mentioned in line 108.

5) 1.1.6: unclear what is done here and how. Please elaborate.

Response: We have now added in line 115-121.

6) 1.2.3: How is transduction done?.

Response: We have now added in line 145-151.

7) 1.2.4: How is cell density evaluated. On line 131, you specify 0.5 cells/well; something is incorrect.

Response: We have now added in line 152-155.

8) 2.2: where and how is the search performed? List any software in the table of materials. Provide a supplementary figure to clarify this step.

Response: We previously added and provided information of gRNA design in Figure 2A. We have now mentioned reference of Figure 2A in 2.2 section.

9) 2.4: Resuspend in PBS?

Response: We have now added in line 198.

10) 2.5: How? Mention Bsal concentration. What is the appropriate band size.

Response: We have now mentioned in line 203 and 205.

11) 4.5: needs more detail. Mention absorbance wavelength.

Response: We have now added in line 280-288.

12) Please review your entire protocol and ensure your steps cover the types details mention above. Please note that we generally cannot film steps that are not described explicitly, such as following a manufacturer's protocol. We also cannot film "design", "order", etc type of steps.

Response: We have now revised in the manuscript.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Response: We have now revised in the manuscript.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response: We have now revised in the manuscript as editor's comments in discussion section.

• **Figures:**
1) Reference Fig 1-3 in-text; the placeholders are not references.
2) Fig 4: define the error bars.

Response: We have now revised.

• **Tables:**
1) Line 147: Number the supplementary table.
2) The list on line 221 should be made into a table submitted as an excel file.

Response: We have now revised as editor's comments.

• **References:** Please spell out journal names.

Response: We have now revised as editor's comments.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Gibson assembly,

Response: We have now revised as editor's comments. We have substituted Gibson assembly to isothermal assembly.

• **Table of Materials:**Sort the list alphabetically.
• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response: We have now revised.

_____

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors present a short protocol to generate and assess mutational variants by CRISPR-CBE. The described protocol uses a lentiviral approach with stable expression of the base editor, combined with the transient transfection of the sgRNA. Functional consequences of the genetic modification is assessed by measuring changes in allele frequency over time by NGS. While the protocol itself is interesting, there are many pitfalls of the method which are not described in detail. Therefore, these points should be added to increase the general applicability of the protocol.

Major/minor Concerns:
1. The authors should mention that off-target editing can occur independent of the sgRNA, by the promiscuous reaction of CBE. This is a particular problem if the base editor is stably expressed. This drawback should be discussed. It should also be mentioned that this problem is less relevant for ABE.
Response: We appreciate the Reviewer's comments and we have now revised our manuscript in discussion section in Discussion section as follows:

Third, the BE3 induces substantial base editing at unwanted sites and the off-target effect could influence functional assessment of BRCA1 variants[27-29]. To reduce the off-target effect of BE3, target sites of gRNAs should be carefully chosen without any similar sequences in the genome. SECURE-BE3 or YE1, which has developed for reducing unwanted base editing in genome and transcriptome are useful option[30,31]

2. The authors use a classic digestion/ligation approach to insert the guide into the RG2 vector. However, more efficient methods using Golden Gate cloning are currently used. The authors should at least

mention this.

Response: We have added as Reviewer's comments in line 206-207 as follows:

NOTE : Instead of using a classic digestion and ligation method, other cloning method such as Golden Gate cloning can be used.

3. An exact PCR protocol for the amplification of genomic DNA should be presented.

Response: We have revised our manuscript according to Reviewer's comments.

4. The NGS protocol is only briefly described. The authors should mention the extend of multiplexing and the exact NGS platform that is recommended (MiSeq?, which sequencing depth per locus needed, recommended read length).

Response: We have now revised in section 4.4 – 4.5 as follows:

4.4. In order to attach the essential sequences for NGS analysis, amplify the 2nd PCR product using the primers listed below. For the PCR reaction, 1 µL of the 2nd PCR product is used for amplification up to 30 cycles using high-fidelity polymerase. To perform multiplex sequencing for large numbers of libraries to be pooled and sequenced simultaneously, forward (D501 – D508) and reverse (D701 – D712) primers, which have unique index sequence, are used (Supplementary Table 1). Each sample should be amplified using different primers sets to conduct the unique dual indexing strategy. Run 5 µL of the PCR product on 2% agarose gel to confirm the size, and purify the amplicon using a commercial PCR clean-up kit (Table of Materials). Mix each sample in equal amounts to create an NGS library.

4.5. Quantify the NGS library at 260 nM wavelength using spectrophotometers and dilute the NGS library to concentration of 1 nM using Resuspension buffer or 10mM Tris-HCl, ph 8.5. Prepare 100 µL of the library diluted to the appropriate loading concentration depending on the library types (For example, prepare 200 pM of the library for Nextera DNA Flex). As a control, combine phiX with the diluted sample appropriate for the type of kit. Load the library onto the cartridge and run NGS according to the manufacturer's protocol. Illumina iSeq 100 or Miseq systems can sequence the amplicons of various length up to 300 bp for single-read or paired end. We recommended over 10,000 reads per target amplicon for in-depth analysis of base editing efficiency.

5. The authors describe the use of MAUND, without explaining what MAUND is and also do not provide a reference for the resource. Also, they should mention others useful tools such as CRISPResso2 to analyse indel frequencies

Response: We have added reference for using MAUND (reference 24) and further methods for base editing analysis as follows:

Note: We also recommend other web-tools for analysis of base editing activity such as CRISPResso2[25], and BE-analyzer[23].

References:

23 Hwang, G. H. et al. Web-based design and analysis tools for CRISPR base editing. BMC Bioinformatics. 19 (1), 542, doi:10.1186/s12859-018-2585-4, (2018).

24 Kim, D., Kim, D. E., Lee, G., Cho, S. I. & Kim, J. S. Genome-wide target specificity of CRISPR RNA-guided adenine base editors. Nat Biotechnol. 37 (4), 430-435, doi:10.1038/s41587-019-0050-1, (2019).

25 Clement, K. et al. CRISPResso2 provides accurate and rapid genome editing sequence analysis. Nat Biotechnol. 37 (3), 224-226, doi:10.1038/s41587-019-0032-3, (2019).

6. The authors should also provide information on how the expected indel/base exchange frequency is for their BRCA variants. The current figures only show relativ changes. Is the indel efficiency the same for all investigated BRCA loci. This is particularly important to understand the efficiency of the method.

Response: We agree with Reviewer's comments and have now revised our manuscript in line 307-309 and figure 4 and legend as follows:

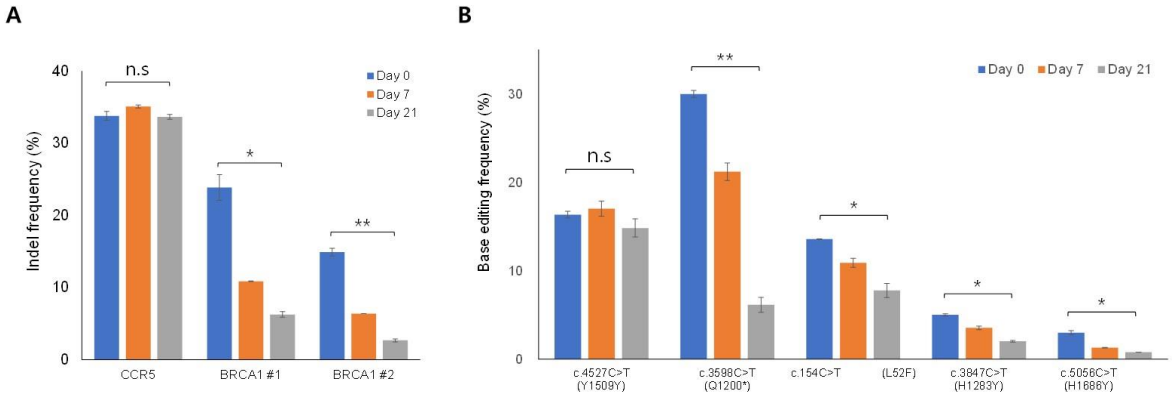
Line 301-303:

5.3. Calculate the relative base editing efficiency of BRCA1 variants and determine their pathogenicity. The significant differences between Day 0 and Day 21 samples are analyzed appropriate statistical analysis such as t-test.

Line 364-365:

Error bars show the standard error of mean. Asterisks denote different P values: * P<0.05; ** P<0.005., n.s: not significant.

Figure 4:



Reviewer #2:
Thank you for the opportunity to review the manuscript entitled "Functional assessment of BRCA1 variants using CRISPR-mediated base editors" by Kim and colleagues. This is a clever application of base editing technology to study the function of BRCA1 variants and should be of great interest to the research community.

While the overall methodology is solid, this protocol is insufficiently detailed and requires major revision in order for experts or non-experts to follow it in full. See below for a non-exhaustive list of sections and items that require attention.

Also, the wording and structure of this protocol lacks clarity. I suggest re-ordering the sections so that they follow the most logical progression of the intended experiments. To this reviewer, that progression would be (1) base editor expression vector and guide RNA expression vector design and construction (2) lentiviral particle generation (3) stable cell line generation and validation (4) guide RNA delivery (5) phenotypic analysis, genomic DNA preparation, and DNA sequencing via the modified cell line. With respect to wording, terms such as "purify the appropriate-sized bands" and "Analyze the BE3 sequences of each plasmid DNA" are overly vague.

Response: We thanks to Reviewer's comments. We also agree with Reviewer's comments the structure of our manuscript, however, we thought that construction of HAP1-BE3 stable cell lines are optional, and primitive step for functional analysis of BRCA1 variants as Figure 1B. In addition, we have now revised our manuscript as Reviewer's comments to clarify our methods.

With respect to the latter, the authors jump immediately from plasmid purification to "analyzing" the plasmid, without giving any information about the specifics of that analysis (Was DNA amplified and by PCR and Sanger sequenced? Was the plasmid sequenced in full using an Oxford Nanopore or other next-generation sequencing approach? What software was used to evaluate the DNA sequence traces?...etc.).

Response: We again thank the reviewer for this advice. To clarify our methods, we have now added more information in line 115-121 as Reviewer's comments as follows:

1.1.6. To confirm DNA cloning success, analyze the BE3 sequences of each purified plsmid DNA (from step 1.1.5) by Sanger sequencing using standard and BE3-specific primers (Table of Materials) and select the exact cloned lentiBE3-blast construct.

NOTE: To analyze the results of Sanger sequencing, we recommended several tools such as BLAST (https://blast.ncbi.nlm.nih.gov/) and CLUSTALW (https://www.genome.jp/tools-bin/clustalw).

Lastly, additional context relating the utility of this method to other approaches should be described in the introduction. In particular, the authors state that artificial over-expression can be problematic for studying the impact of genetic variants. The authors should provide specific references to support this claim. Separately, the authors should contrast their approach to that used by the Shendure lab (Findlay et al., Nature 2018), which elegantly showed how large-scale HDR approaches could be used to introduce many BRCA1 variants in parallel into the HAP1 cell line model. For example, while an HDR approach can facilitate the production of mutants not possible with cytosine base editors, the efficiency may be lower and HDR also requires design and synthesis of donor templates.
Response: We agree with Reviewer's discussion. We have now added reference in line 60 and shortly discussion for comparing with recent study as follows:

Reference:

Gibson, T. J., Seiler, M. & Veitia, R. A. The transience of transient overexpression. Nat Methods. 10 (8), 715-721, doi:10.1038/nmeth.2534, (2013).

Line 390-400:

Forth, a saturation genome editing (SGE) method based on Cas9-mediated HDR also great options for functional analysis of BRCA1 variants[19]. The method has no limitation for selecting target sequences and nucleotide positions of BRCA1 variants. However, HDR-based approach is relatively less efficient than the base editors and additionally requires design and synthesis of donor templates[14].

Non-exhaustive points that should be expanded and/or revised:

1. Please include more detail on and/or rational for the exact methods used in the following sections:

1.1.1. Please note the destination vector used. Include how much overlap with the destination vector is required in the PCR primers, and note that these sequences will be specific to the destination vector chosen by the user.
Response: We have corrected as Reviwer's comments in line 89-96 as follows:

1.1.1. To construct the lentiBE3-blast plasmid DNA for lentivirus production, the BE3 coding sequences in pCMV-BE3 (Table of Materials) are amplified by PCR using high-fidelity polymerase. The PCR primer should be designed to contain overlapping sequences of the digested vector (from step 1.1.2) for isothermal assembly as follows17: primer-F: 5'-TTTGCCGCCAGAACACAGGACCGGTTCTAGAGCCGCCACCATGAGCTCAGAG-3', primer-R:5'-CAGAGAGAAGTTTGTTGCGCCGGATCCGACTTTCCTCTTCTTCTTGGG-3' (nucleotides shown in underlined are overlapped with the digested vector and these sequences will be specific to the destination vector chosen by the user.)

1.1.2. Please include the expected size of the bands, and perhaps a diagram as well.
Response: We have added the expected size of the bands (8.6 kb) in line 100.

1.1.5. Please provide an example of a commercial plasmid DNA purification kit, also please include in the materials section.
Response: We have now added information of a commercial plasmid DNA purification kit in the Table of Materials.

1.1.6. Please provide more detail as to how analysis of BE3 sequences should be performed. If a DNA amplification step is required for this, provide primer sequences and details of the PCR protocol (PCR setup and cycle number, suggested length of product, etc.). How many clones are typically needed?

What is the typical error rate? What software do the authors suggest to visualize/align sequencing reads?

Response: We have now corrected and add information of primer sequences in the Table of Materials.

1.2.1. Please include rationale for using HAP1 cells and emphasize that HAP1 cells have the potential to become diploid, with a note on how to periodically monitor ploidy and select for haploid populations.

Response: We have now added NOTE in line 130-133 as follows:

NOTE: HAP1 cell is useful for genetic research because of it is nearly-haploid cells. However, the cells can spontaneously return to a diploid state in cell culture. Enrichment Hoechst 34580 stained 1n-population using flowcytometry will be helpful for maintenance of haploidy of HAP1 cells[19].

1.2.2. Please note whether this is a 2nd or 3rd generation lentiviral packaging system and provide information on what each of these plasmids are expressing.
Response: We have now added information of lentiviral packaging system in line 135-138 as follows:

1.2.2. Seed 5×10^6 HEK293T/17 cells on a 100-nm dish 1 day before transfection. On the day of transfection, transfect the plasmid DNA (15 µg of lentiBE3-blast, 9 µg of psPAX2 for viral packaging, and 6 µg of pMD2.G for vial envelope of 2nd generation lentiviral packaging system)

1.2.3. Please describe the assay and calculations used to determine the appropriate MOI.
Response: We have now added in line 145-151 as follows:

1.2.3. Transduce the lentiviral particles of BE3 into HAP1 cells at a MOI (Multiplicity Of Infection) of 0.1. To adjust for an appropriate MOI, serially diluted concentrations of virus should be used. Remove the medium and replace it with adjusted viral supernatant and fresh culture medium. One day after transduction, change the medium to 10 µg/mL of blasticidin (Table of Materials)-containing medium and select the transduced cells for 3 days with blasticidin. After blasticidin selection, select a well with ~10% of surviving cells for the next step.

1.2.3. After selecting lentiBE3-blast for "3 days" should the cells be maintained with or without antibiotics?
Response: We have corrected as follows in line 148-151.

One day after transduction, change the medium to 10 µg/mL of blasticidin (Table of Materials)-containing medium and select the transduced cells for 3 days with blasticidin. After blasticidin selection, select a well with ~10% of surviving cells for the next step.

1.2.5. Please specify the control guide RNA sequences to be used for identification of highly active clones. Please provide justification for using lentivirally delivered guide RNAs for the validation of BE3 expressing cells, compared to transient transfection, which is used elsewhere in the protocol.
Response: We have added additional information in line 164-166 as follows:

NOTE: To validate exact mutation frequencies, we recommend the targeted deep sequencing than T7E1 assay. HEK2 (5'-GAACACAAAGCATAGACTGCGGG-'3) is well validated target site for BE3.

We shortly described of lentiviral delivery of gRNAs in Discussion section as follows:

We do not recommend lentiviral transduction of gRNA into the HAP1-BE3 cells, because constitutive expression of BE3 and gRNA could cause accumulative nucleotide conversion, and these results interfere with the functional assessment of BRCA1 variants.

1.2.5. Please clarify that when you say "Divide the single colonies into two sets" what you mean is to expand and split one half of the cell population for testing and one half for maintenance.
Response: We have revised in line 158-159 as follows:

1.2.5. Divide the single colonies into two sets, one sets for testing BE3 activity and other for maintenance.

1.2.5. Provide examples of the T7 assay (gel image?) or deep sequencing readout. Provide rationale for the T7 assay. Would more efficient base editing mask "mutation frequency" in the T7 assay, since the product pool would trend towards uniformity if all bases were edited?
Response: We agree with Reviewer's consideration and we also recommend the targeted deep sequencing than T7E1 assay to analyze mutation frequencies. We have now added note in line 163-165 as follows:

NOTE: To validate exact mutation frequencies, we recommend the targeted deep sequencing than T7E1 assay. HEK2 (5'-GAACACAAAGCATAGACTGCGGG-'3) is well validated target site for BE3.

2.2. What program was used to identify guide RNAs in the BRCA1 gene?
Response: We have added NOTE in line 179-188 as follows:

2.2. Search the 20-bp target sites with the Protospacer Adjacent Motif (PAM) sequence “NGG” and “CCN” around mutation of interest. The mutation of interest should be located in 4–8 nucleotides in the PAM-distal end of the gRNA target sequences because of the active window of BE3 is 4–8 nucleotides in the PAM-distal end of the gRNA target sequences[14]. In the case of c.8047C>T (H1283Y)- and c.5252G>A (R1751Q)-targeting gRNAs, 5'-TCA**GGAAC**ATCACCTTAGTG-AGG-3' and 5'-CCA-CCAAGGTCCAA**AGCGA**CAA-3', the sequences in bold are active windows. C to T and G to A conversions occur with NGG and CCN PAM, respectively (Figure 2A).

NOTE: BE-Designer (http://www.rgenome.net/be-designer/)[23] is useful web-based tools for gRNA design.

2.3. Specify that DNA oligonucleotides are used for cloning, and provide rationale for the addition of the specified sequnces onto the ends of the oligos. Add a note that these sequences are specific to the destination guide RNA expression vector used for this protocol, and they should be adjusted for alternative guide RNA expression vectors.
Response: We have added as Reviewer’s comments in line 191-196 as follows:

2.3. Order two complementary oligonucleotides per gRNA; for the forward oligonucleotides, add “CACCG” to the 5' end of the guide sequence, and for the reverse oligonucleotides, add “AAAC” to the 5’ end and “C” to the 3’ end. These additional sequences are specific to the destination gRNA expression vector (from step 2.5) used for this protocol, and users should be adjusted for alternative gRNA expression vectors (Figure 2B).

2.4. Specify the appropriate buffer to be used for oligo annealing.
Response: We have now added the information of buffer (T4 Ligation buffer) in line 200 and Table of Materials.

2.5. Provide expected size for digested pRG2.
Response: We have added the expected size (2.5kb) in line 205.

2.6. Are there other strains of bacteria that can be used? For example, Stbl3/4 (ThermoFisher)? Please describe how analysis of the gRNA sequence is performed. What primer, sequencing analysis software, etc.?
Response: We have revised as Reviewer’s comments in line 212-213 as follows:

(Other E. coli strains which widely used for subcloning also can be used)
JoVE journal do not permit usage of commercial language such as Stbl3™.

3. NOTE: Please comment on the efficiency of co-transfection of BE3 and gRNA plasmids in HAP1 cells. How well does this work compared to making stable BE3 cell lines?
Response We have revised as Reviewer’s comments in line 220-223 as follows:

NOTE: If HAP1-BE3 cell lines is not used, BE3-encoding plasmid DNA can be co-transfected with BRCA1-targeting gRNA. Compared to co-transfection of BE3 and gRNA plasmids, transfection of gRNA plasmid to HAP-BE3 cells induce efficient base editing up to 2-3 -fold at target locus in our hands.

3.2. How is transfection efficiency optimized for these cell lines? What is the specific transfection reagent recommended for HAP1 cells? Is transfection efficiency calculated or monitored during the experiment?
Response: JoVE journal do not permit usage of commercial language. We previously described the

transfection reagent in Table of Materials. As Reviewer's comments, we have now added note in lie 237-238 as follows:

NOTE: We recommend optimizing transfection conditions with variable ratio of reagent to DNA. Optimal condition for HAP1 transfection is 4:1 ration of reagent to DNA in our hands.

3.2. and Figure 1. In section 3.2, cell pellets are harvested on days 3, 10 and 24 - however in Figure 1, day 0, 7 and 21 are illustrated. Please be consistent or comment on this discrepancy in the methods.
Response: We have now revised in line 234-235 as follows:

(Samples from 3 days after transfection are analyzed regrading as day 0 samples)

4. Provide general considerations for producing and sequencing amplicon libraries. Are the primers/indices noted in this protocol applicable for all sequencing machines (Illumina vs. IonTorrent)? What is the ideal size of the NGS-ready amplicon product?

Response: We have now revised in section 4.

4.2. Please provide justification for the nested PCR approach, as opposed to library preparation from direct genomic DNA amplification.

Response: We have now revised in line 259-260 as follows:

NOTE: We used nested PCR to reduce the amplicon of non-specific binding by preventing primers from attaching to non-target sequence.

4.3. Specify that only a portion of the nested (2nd) PCR product (5uL?) should be run on a 2% gel to confirm size.

Response: We have now revised in line 266 as follows:

Run 5 µL of the 2nd PCR product on 2% agarose gel and confirm the size.

4.4. Please provide additional annotation for the primers listed in the index table and how they should be used. Also, please provide additional experimental details for this PCR reaction: What is the concentration and volume of the primers and template? How many cycles are performed?

Response: We have now revised in line 269-278 as follows:

4.4. In order to attach the essential sequences for NGS analysis, amplify the 2nd PCR product using the primers listed below. For the PCR reaction, 1 µL of the 2nd PCR product is used for amplification up to 30 cycles using high-fidelity polymerase. To perform multiplex sequencing for large numbers of libraries to be pooled and sequenced simultaneously, forward (D501 – D508) and reverse (D701 – D712) primers, which have unique index sequence, are used as below. Each sample should be amplified using different primers sets to conduct the unique dual indexing strategy. Run 5 µL of the PCR product on 2% agarose gel to confirm the size, and purify the amplicon using a commercial PCR clean-up kit (Table of Materials). Mix each sample in equal amounts to create an NGS library.

4.5 What is the expected concentration of the samples after purification? How much product is needed for NGS?
Response: We have now revised in line 280-288 as follows:

4.5. Quantify the NGS library at 260 nM wavelength using spectrophotometers and dilute the NGS library to concentration of 1 nM using Resuspension buffer or 10mM Tris-HCl, ph 8.5. Prepare 100 µL of the library diluted to the appropriate loading concentration depending on the library types (For

example, prepare 200 pM of the library for Nextera DNA Flex). As a control, combine phiX with the diluted sample appropriate for the type of kit. Load the library onto the cartridge and run NGS according to the manufacturer's protocol. Illumina iSeq 100 or Miseq systems can sequence the amplicons of various length up to 300 bp for single-read or paired end. We recommended over 10,000 reads per target amplicon for in-depth analysis of base editing efficiency.

5.3. Please provide statistical methods for determining significance when comparing base editing efficiencies.
Response: We have now revised in line 308-309 and 364-365 as follows:

Line 308-309:

The significant differences between Day 0 and Day 21 samples are analyzed appropriate statistical analysis such as t-test.

Line 364-365:

Error bars show the standard error of mean. Asterisks denote different P values: * P<0.05; ** P<0.005., n.s: not significant.

Figure Legends, Figure 4A/B. Please provide more experimental detail (What Cas9 system was used?)
Response: We have now revised in line 354-365 as follows:

Figure 4: Representative results of functional study of BRCA1 using CRISPR-Cas9 systems. (A) BRCA1 disruption affects to the cell viability. HAP1 cells were transfected with plasmid encoding spCas9 and two gRNAs targeting BRCA1, respectively, and targeted deep sequencing was performed for cell viability analysis. Mutation frequencies of BRCA1 decreased in a time-dependent manner in cells transfected with two independent gRNAs, and the mutation frequencies of CCR5, which was used as a negative control, remained the same over time. (B) Functional assessments of five BRCA1 variants. HAP1-BE3 cells were transfected with gRNAs inducing BRCA1 mutations, respectively, and targeted deep sequencing was performed for cell viability analysis. The relative substitution frequencies decreased in a time-dependent manner in cells of c.3598C>T, c.154C>T, c.3847C>T, and c.5056C>T and those of c.4527C>T remained the same. Error bars show the standard error of mean. Asterisks denote different P values: * P<0.05; ** P<0.005., n.s: not significant.

2. Please fix the following grammatical errors:

Summary, Line 29: "which induce enable to targeted C:G to T:A conversion"

Abstract, Line 44: "using (a) CRISPR-based cytosine base editor."

Introduction, Line 61: "CRISPR-Cas9 system enables"

Introduction, Line 67-68: "has" -> "have" and domain(s)

Introduction, Line 71: "we used (the) CRISPR-mediated cytosine"

Heading, Line 176. Suggest changing to "(Creation) of BRCA1 variants…"

Protocol, 4.5, line 222: "Qualify" -> "Quantify"

Figure legends, Figure 4, Line 288-289: "Representative results of (the) functional study of BRCA1 using (the) CRISPR-Cas9 (and CRISPR-BE3) systems. BRCA1 disruption affects to the cell viability.

Discussion, Line 297-298: "BRCA1 variants using (a) CRISPR-mediated cytosine base editor. (The protocol describes methods for the design of gRNAs at (a) target locus and construction of the plasmid DNAs from which they are expressed.)"

Discussion, Lines 300, 302, 303: "in (an) active window"

Discussion, Line 300: "in (the) case of BE3, nucleotides 4-8 in the PAM-distal"

Discussion, Line 317: "Second, the BE3 recognize(s) target DNA sequence through the 5'-NGG-3' PAM sequences, which is a limitation in generating various types"

Discussion, Line 318: "Cas9 variants with altered PAM sequence are (a) useful"

Discussion, Line 322: "To (perform) functional assessments of these"

Response: We would like to thank reviewer for carefully pointing out our mistake. We have now corrected all grammatical errors as Reviewer's comments. All changes were highlighted.

3. Please insert relevant citations for these statements:

Introduction, Line 56: Reference (3) is from 2012, are there more recent references that can also be included to support this statement?
Response: We have now added additional references as follows:

4 Santos, C. et al. Pathogenicity evaluation of BRCA1 and BRCA2 unclassified variants identified in Portuguese breast/ovarian cancer families. J Mol Diagn. 16 (3), 324-334, doi:10.1016/j.jmoldx.2014.01.005, (2014).

5 Starita, L. M. et al. A Multiplex Homology-Directed DNA Repair Assay Reveals the Impact of More Than 1,000 BRCA1 Missense Substitution Variants on Protein Function. Am J Hum Genet. 103 (4), 498-508, doi:10.1016/j.ajhg.2018.07.016, (2018).

6 Anantha, R. W. et al. Functional and mutational landscapes of BRCA1 for homology-directed repair and therapy resistance. Elife. 6, doi:10.7554/eLife.21350, (2017).

Introduction, Line 58-60: Please include reference for problems with overexpression of BRCA1 variants. Also, please comment on why specifically overexpression is problematic or what exactly these limitations are.
Response: We have now added additional references and comments in line 59-60 as follows:

the methods involving exogenously expressed BRCA1 variants pose limitations in terms of overexpression which might affecting downstream regulation, gene dosage, and protein folding[7]

references:

Gibson, T. J., Seiler, M. & Veitia, R. A. The transience of transient overexpression. Nat Methods. 10 (8), 715-721, doi:10.1038/nmeth.2534, (2013).

Introduction, Line 59-60: Please include references to studies of BRCA1 post-transcriptional regulation.
Response: We have now added references as follows:

8 Quann, K., Jing, Y. & Rigoutsos, I. Post-transcriptional regulation of BRCA1 through its coding sequence by the miR-15/107 group of miRNAs. Front Genet. 6 242, doi:10.3389/fgene.2015.00242, (2015).

9 Saunus, J. M. et al. Posttranscriptional regulation of the breast cancer susceptibility gene

BRCA1 by the RNA binding protein HuR. Cancer Res. 68 (22), 9469-9478, doi:10.1158/0008-5472.CAN-08-1159, (2008).
Introduction, Line 64-65: Reference needed describing NHEJ and HDR based repair.
Response: We have now added references as follows:

11 Sander, J. D. & Joung, J. K. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol. 32 (4), 347-355, doi:10.1038/nbt.2842, (2014).

Introduction, Line 71: Reference needed for "BE3"
Response: We have now added references as follows:

14 Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A. & Liu, D. R. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. 533 (7603), 420-424, doi:10.1038/nature17946, (2016).

Protocol, 5.1, Line 227: Reference for MAUND.
Response: We have now added references as follows:

24 Kim, D., Kim, D. E., Lee, G., Cho, S. I. & Kim, J. S. Genome-wide target specificity of CRISPR RNA-guided adenine base editors. Nat Biotechnol. 37 (4), 430-435, doi:10.1038/s41587-019-0050-1, (2019).
4. Corrections:

Introductions, Line 67: Several base editors, including BE3 which the authors use in this manuscript use Cas9 nickase, not catalytically dead Cas9 (dCas9).
Response: We have now revised as Reviewer's comments

Introduction: Line 64-65: I believe the authors mean to say that introduction of double strand breaks can result in unwanted insertion and deletion mutations. I don't believe it's HDR, but rather NHEJ that causes these indels.
Response: We apologize ~~ and we have now revised in line 66-67 as follows:

however, DSBs induced by Cas9 nuclease for HDR often results in unwanted insertion and deletion (indel) mutation.

5. Other:

Throughout text, Define all acronyms at first mention. Alternatively, this could be done through a separate "glossary" section.
Response: We have now revised as Reviewer's comments in our manuscript

Introduction, Please comment on Hap1 cells and their utility for base editing experiments.

Response: We have now added in line 130-131 as Reviewer's comments.

NOTE: HAP1 cell is useful for genetic research because of it is nearly haploid cells.

Introduction, Please provide additional historical context for this method, including reference to related HDR-based screens that have been performed for BRCA1 in Hap1 cells (for example: Findlay GM, et al. Nature, 2018).

Response: We have now revised in discussion section as follows:
Discussion, Line 312: Suggest recommending alternative base editor BE4max at the beginning of the protocol, perhaps in the NOTE section.
Response: We have now added in line 82-83 as follows:

Other variants of cytosine base editors, such as BE4max, also can be used for highly efficient base editing.

Representative Results. Data shown in Figure 4B is described as the result of co-transfection of BE3 and gRNA plasmids. Please provide representative results comparing this method with the stable BE3 cell line generation method primarily described in this protocol.

Response: We apologize for the inconsistencies in Representative Results section and Figure lengend. We have now corrected in Representative Results section as below. We also noted that transfection of gRNA plasmid to HAP-BE3 cells is more efficient than co-transfection of BE3 and gRNA plasmids up to 3-fold in our hands as below:

Line 220-223:
NOTE: If HAP1-BE3 cell lines is not used, BE3-encoding plasmid DNA can be co-transfected with BRCA1-targeting gRNA. Compared to co-transfection of BE3 and gRNA plasmids, transfection of gRNA plasmid to HAP-BE3 cells induce efficient base editing up to 3-fold at target locus in our hands.

Line 321-322:

the plasmids DNA encoding gRNAs, which could induce each mutation, were transfected to HAP1-BE3 cell lines and the substitution frequencies were analyzed.

Discussion, Line 309-311: Please clarify and expand on the justification for using transiently transfected guide RNAs vs. lentivirally delivered guide RNAs.
Response: We have now revised our manuscript in line 380-382 as below:

We do not recommend lentiviral transduction of gRNA into the HAP1-BE3 cells, because constitutive expression of BE3 and gRNA could cause accumulative nucleotide conversion, and these results interfere with the functional assessment of BRCA1 variants.

Where applicable, please list Addgene plasmid # (e.g. pCMV-BE3 = Addgene #73021 — https://www.addgene.org/73021/).

Response: We described plasmid information in Table of Materials

Supplemental Table1

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Supplementary Table 1. List of oligonucleotides used in this study

Name	gRNA sequence (5' to 3')
c.4527C>T (Y1509Y)	TGGTACATGCACAGTTGCTC
c.3598C>T (Q1200*)	GGCTCAGGGTTACCGAAGAG
c.154C>T (L52F)	AAACTTCTCAACCAGAAGAA
c.3847C>T (H1283Y)	TCAGGAACATCACCTTAGTG
c.5056C>T (H1686Y)	ACTCATGTTGTTATGAAAAC
Name	**primer sequence (5' to 3')**
CMV-F	CGCAAATGGGCGGTAGGCGTG
BE_1F	AAGCCCACTGGCCTAGGTAT
BE_2F	CCCAACGATTTATCACCTCA
BE_3F	AAAAACGGGTACGCAGGTTA
BE_4F	TGAATGCTTCGATTCTGTCG
BE_5F	AAAGGAGCATCCTGTGGAAA
BE_6F	GCGAACAGGAGATAGGCAAG
BE_7F	GCGTCCCATTACGAGAAGTT
BE_1R	TAATCCCGTGATGGATTGGT
BE_2R	CCTTTTTACGAGCGATGAGC
BE_3R	TGCCGCCAATAGTTCTTCAT
BE_4R	ATAAGTTTCCGCGACAATCG
BE_5R	AGCATGCAATTCGCCTAAGT
BE_6R	AAGAATAGCCTTCGCATCCA
BE_7R	GGAATTAGTGCCGATGGCTA

Supplemental Table2

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Supplementary Table 2. index sequences in bold

Name	Primer sequence (5' to 3')
D501	AATGATACGGCGACCACCGAGATCTACAC**TATAGCCT**ACACTCTTTCCCTACACGAC
D502	AATGATACGGCGACCACCGAGATCTACAC**ATAGAGGC**ACACTCTTTCCCTACACGAC
D503	AATGATACGGCGACCACCGAGATCTACAC**CCTATCCT**ACACTCTTTCCCTACACGAC
D504	AATGATACGGCGACCACCGAGATCTACAC**GGCTCTGA**ACACTCTTTCCCTACACGAC
D505	AATGATACGGCGACCACCGAGATCTACAC**AGGCGAAG**ACACTCTTTCCCTACACGAC
D506	AATGATACGGCGACCACCGAGATCTACAC**TAATCTTA**ACACTCTTTCCCTACACGAC
D507	AATGATACGGCGACCACCGAGATCTACAC**CAGGACGT**ACACTCTTTCCCTACACGAC
D508	AATGATACGGCGACCACCGAGATCTACAC**GTACTGAC**ACACTCTTTCCCTACACGAC
D701	CAAGCAGAAGACGGCATACGAGAT**CGAGTAAT**GTGACTGGAGTTCAGACGTGT
D702	CAAGCAGAAGACGGCATACGAGAT**TCTCCGGA**GTGACTGGAGTTCAGACGTGT
D703	CAAGCAGAAGACGGCATACGAGAT**AATGAGCG**GTGACTGGAGTTCAGACGTGT
D704	CAAGCAGAAGACGGCATACGAGAT**GGAATCTC**GTGACTGGAGTTCAGACGTGT
D705	CAAGCAGAAGACGGCATACGAGAT**TTCTGAAT**GTGACTGGAGTTCAGACGTGT
D706	CAAGCAGAAGACGGCATACGAGAT**ACGAATTC**GTGACTGGAGTTCAGACGTGT
D707	CAAGCAGAAGACGGCATACGAGAT**AGCTTCAG**GTGACTGGAGTTCAGACGTGT
D708	CAAGCAGAAGACGGCATACGAGAT**GCGCATTA**GTGACTGGAGTTCAGACGTGT
D709	CAAGCAGAAGACGGCATACGAGAT**CATAGCCG**GTGACTGGAGTTCAGACGTGT
D710	CAAGCAGAAGACGGCATACGAGAT**TTCGCGGA**GTGACTGGAGTTCAGACGTGT
D711	CAAGCAGAAGACGGCATACGAGAT**GCGCGAGA**GTGACTGGAGTTCAGACGTGT
D712	CAAGCAGAAGACGGCATACGAGAT**CTATCGCT**GTGACTGGAGTTCAGACGTGT