

Using the CRISPR-Cas9 system with paired Dharmacon Edit-R synthetic crRNAs for functional knockout of microRNA hsa-miR-221.

Authors

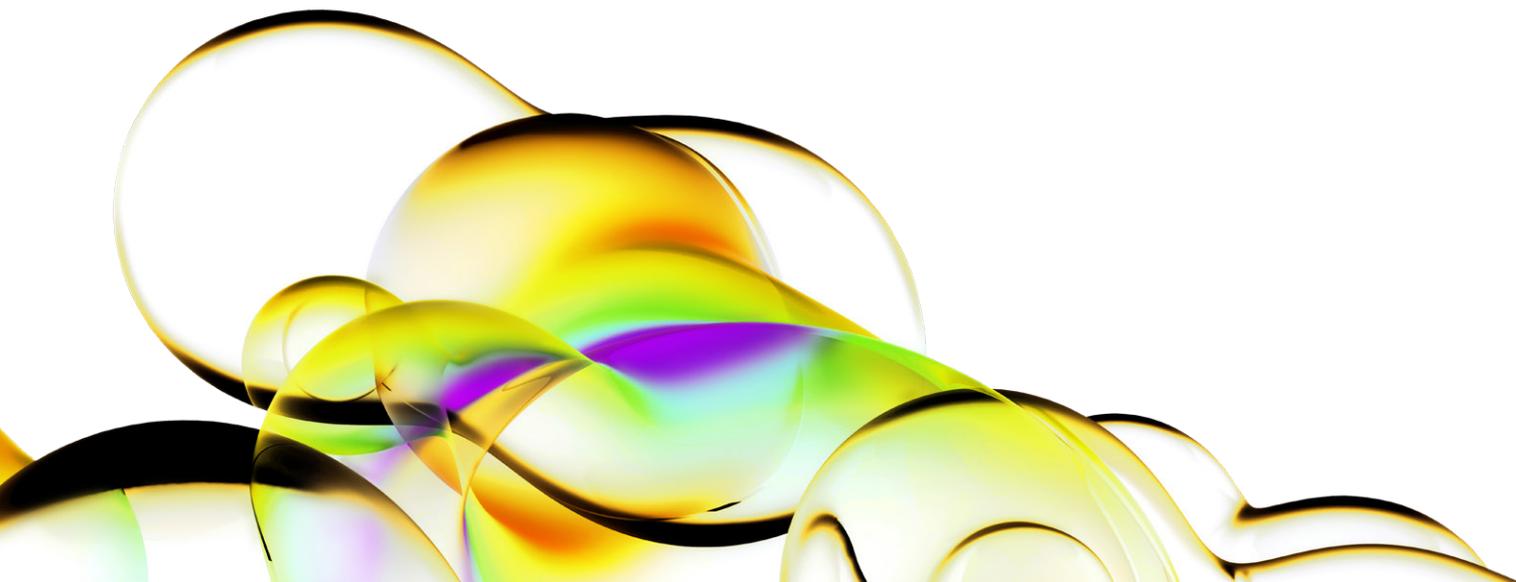
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Introduction

The CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated 9) system derived from *Streptococcus pyogenes* uses a Cas9 nuclease directed by a guide RNA (gRNA) to create a DNA double-strand break (DSB) at the target site. The gRNAs can be dual synthetic molecules, like the native bacterial system containing a CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA), or a chimeric single guide RNA (sgRNA). The DSB is most often repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR) through endogenous mechanisms within mammalian cells. NHEJ can result in insertions or deletions (indels) that produce functional gene knockouts through nonsense mutations or introduction of a stop codon. When using CRISPR-Cas9 components targeting coding genes, there are typically multiple protospacer adjacent motif (PAM) sequences (NGG for *S. pyogenes*) to choose from along the gene to design a gRNA. For most CRISPR-Cas9 genome engineering experiments, one targeting gRNA is sufficient to generate the desired functional gene knockout. However, for some applications, it may be advantageous to use two gRNAs to generate a larger deletion and ensure gene knockout or to remove an exon, long non-coding RNA (lncRNA), or transcriptional regulatory element¹.

Targeting microRNAs is another application where paired gRNAs can be beneficial to ensure complete functional knockout of the microRNA.



Endogenous microRNAs regulate levels of mRNA(s) involved in many cellular pathways². They are transcribed as stem-loop structures and subsequently processed by several enzymes into functional, mature microRNAs with one or two mature (targeting) strands. These stem-loop structures are known to have secondary structure due to bulges or mismatches and are not typically complementary. To knockout a microRNA using the CRISPR-Cas9 system, the gRNA must disrupt either the mature microRNA(s) or the stem-loop region, which has a limited design space (60-90 bp)³ in which to find PAM sequences for gRNA recognition.

Additionally, it is unknown if Cas9-induced small or variable indels within the stem-loop of the microRNA will be disruptive enough to effectively knockout the function of the microRNA⁴. To overcome these limitations, it has been shown that the use of paired gRNAs result in larger deletions of the genomic region encoding the mature microRNA than the use of a single gRNA⁵.

Here we evaluate the efficiency of various synthetic crRNAs to determine the best pair to excise the genomic sequence encoding hsa-miR-221 to generate a knockout clonal cell line.

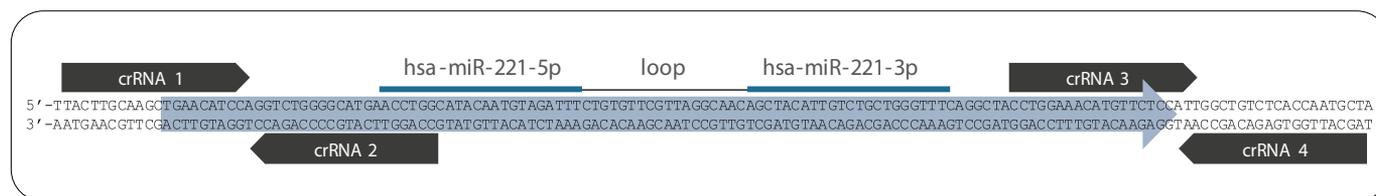


Figure 1: miR-221 genomic sequence with annotated mature miRNAs and crRNA target sequences.

Results and summary

When working with CRISPR-Cas9 reagents to create a clonal cell line, the transfection of Cas9 nuclease and a crRNA:tracrRNA need to be optimized to ensure the maximum number of cells are transfected, thereby decreasing the total number of cell clones to be characterized for the desired genome modification. We, and others⁶, find that many NHEJ-based repairs of CRISPR-Cas9 DSBs can be relatively small indels in the genome near or within target sequence, which may not be sufficient to knockout the function of a microRNA. To generate a deletion in the genomic DNA (gDNA), paired crRNAs were designed to each end of the annotated hsa-miR-221 precursor sequence using the Dharmacon™ CRISPR Design Tool (Figure 1). U2OS cells stably expressing Cas9 nuclease were transfected with the different crRNA:tracrRNA pairs, harvested 72 hours post-transfection and a mismatch detection assay was performed on the cell population to estimate the editing efficiency and determine which crRNA:tracrRNA pair generated the highest percentage of indels (Figure 2). Similar gene editing efficiencies were estimated from the four pairs of crRNA:tracrRNA (Figure 3), indicating that crRNA directionality and DNA target strand did not have much of an impact upon the experimental outcome.

Thus, the pair of crRNAs 2 and 4, which generate a 102 bp deletion, were chosen for single colony expansion into 48 wells of a 96-well plate, nine of which were randomly chosen for further analysis. gDNA spanning the Cas9 cut location was amplified, and PCR amplicons were separated on an agarose gel alongside U2OS cells transfected with a non-targeting crRNA negative control (Figure 4). Five of the nine cell lines displayed a single lower molecular weight PCR band (Figure 4, blue asterisks) suggestive of a homozygous deletion of all alleles present in this cell line, while an additional three hsa-miR-221 cell lines displayed heterozygous deletions of varying lengths (Figure 4, blue question marks). All nine clonal cell lines were Sanger sequenced to determine the exact genomic deletion; clonal cell lines #1, #4, #5, #6 and #9 contained the expected 102 bp deletion (Figure 5). Furthermore, clonal cell line #3 displayed heterozygous indels between the two crRNA locations, while clonal cell line #8 contained small deletions around the PAM of both crRNA 2 and crRNA 4 (Figure 5).

To assess the level of functional knockout of hsa-miR-221, the nine hsa-miR-221 cell lines were transfected with a dual luciferase reporter plasmid containing a hsa-miR-221 response element. When the mature microRNA is present within the cell, it binds to the microRNA response element and suppresses the translation of luciferase. Upon knockout and depletion of the target microRNA, luciferase expression is not suppressed and its intensity can be measured.

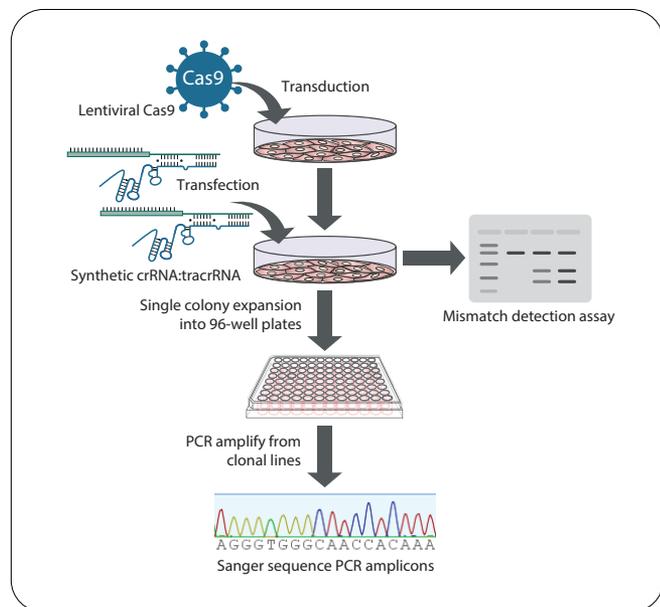


Figure 2: CRISPR-Cas9 experimental workflow for generating clonal cell lines using paired gRNAs.

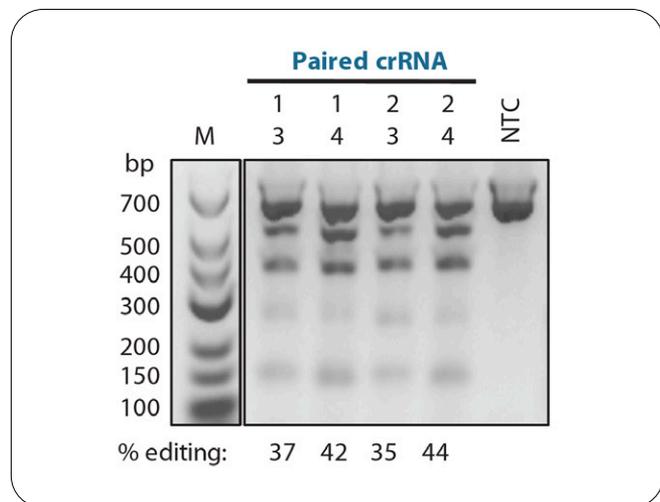


Figure 3: Estimated gene editing of hsa-miR-221 with paired crRNAs Mismatch detection assay of U2OS-Cas9 cells 72-hours post-transfection with paired crRNAs designed to target each strand of the genomic DNA. NTC = Dharmacon Edit-R Non-targeting crRNA control #1.

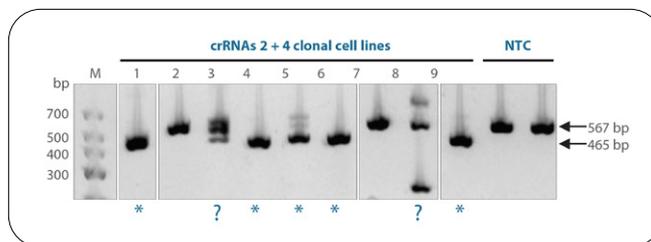


Figure 4: Clonal U2OS cells lines isolated from paired crRNA transfection to delete hsa-miR-221. PCR primers were designed to span the Cas9 cleavage region and resulting PCR amplicons from each individual has-miR-221 clonal cell line were ran out on a 1.2% agarose gel. Homozygous deletions are marked by a blue asterisk. Heterozygous deletions of varying lengths are marked by a blue question mark. NTC = Dharmacon Edit-R Non-targeting crRNA control #1.

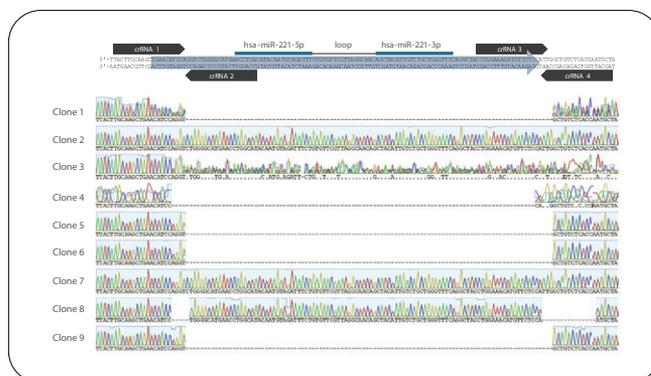


Figure 5: Sanger sequencing of U2OS hsa-miR-221 paired gRNA clonal cell lines. Sanger sequencing of eleven clonal cell lines. (-) represents homozygous deletions while (.) represents the occurrence of different nucleotides at the respective nucleotide position.

In the hsa-miR-221 clonal cell lines with homozygous deletions, clonal cell lines #1, #4, #5, #6 and #9, a significant level of luciferase was measured compared to untransfected cells (Figure 6). Cell lines with either heterozygous indels or having both edited and wild type alleles showed lower levels of luciferase indicating knockdown, but not complete knockout, of the endogenous hsa-miR-221 function, while wild-type cells were the same as negative controls (Figure 6).

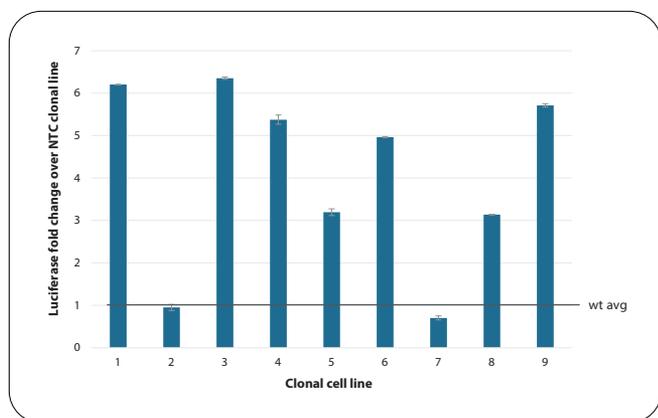


Figure 6: Use of a luciferase reporter plasmid to detect hsa-miR-221 knockout. U2OS hsa-miR-221 paired gRNA clonal cell lines were transfected with an hsa-miR-221 luciferase reporter plasmid and luciferase expression was measured (72-hours) after transfection. Unedited, wild-type U2OS cells were also transfected and are indicated by the wt avg solid line on the graph.

Conclusions

microRNAs can be more difficult to knockout with CRISPR-Cas9 technology than coding genes due to limited design space, and small indels may be tolerated by microRNAs due to the imperfect complementarity of the native structure. Here we demonstrated that microRNA knockout clonal cell lines can be effectively generated with Dharmacon Edit-R CRISPR-Cas9 reagents; in this case, with a Cas9 lentiviral-integrated cell line and a pair of synthetic crRNAs. Sanger sequencing verified clonal cell lines, displayed a range of deletions for the hsa-miR-221 target including several large, homozygous deletions as expected for the paired crRNAs used in these experiments. In this workflow (Figure 2), seven of the nine randomly chosen clonal cell lines contained a hsa-miR-221 precursor deletion or disruption in the gDNA. Furthermore, by using the CRISPR RNA Configurator, we were able to quickly design highly functional synthetic crRNAs that were effective in functional knockout of microRNA hsa-miR-221 in U2OS cells. The successful generation of hsa-miR-221 knockout clonal cell lines with CRISPR-Cas9 and paired synthetic crRNA:tracrRNA shows an extension of the utility of CRISPR-based genome engineering to enable researchers to disrupt noncoding genomic targets.

Materials and methods

Cell culture: U2OS-CAG-Cas9 cells were generated with Dharmacon Edit-R Lentiviral Blast-Cas9 Nuclease particles (Revvity, Cat #VCAS10129) and maintained in standard growth medium per ATCC U2OS recommendations (ATCC, Cat #HTB-96).

crRNA:tracrRNA transfection: U2OS-CAG-Cas9-integrated cells were seeded in a 96-well plate at 10,000 cells per well one day prior to transfection. Dharmacon Edit-R synthetic crRNAs (Dharmacon, custom synthesis), NTC synthetic control (Revvity, Cat #U-007501-01-05) and tracrRNA (Revvity, Cat #U-002000-20) were individually resuspended in 10 mM Tris-HCl pH7.5 to a concentration of 100 μ M. crRNA and tracrRNA were combined at equimolar ratio and diluted to 2.5 μ M using 10 mM Tris-HCl pH7.5. A final concentration of 50 nM crRNA:tracrRNA complex was used for transfection. Cells were transfected using 0.4 μ L/well of DharmaFECT Duo transfection reagent (Revvity, Cat #T-2010-03). hsa-miR-221 targeting sequences:

crRNA 1: TACTTGCAAGCTGAACATCC,

crRNA 2: CCAGGTTTCATGCCCCAGACC,

crRNA 3: CCTGGAAACATGTTCTCCAT, and

crRNA 4: AGCATTGGTGAGACAGCCAA

Clonal cell line isolation: Cell isolation was done by dilution; cells were grown and diluted to one cell per well in a 96-well plate and were monitored for single cell growth.

Genomic DNA isolation, DNA mismatch detection assay and Sanger sequencing: Genomic DNA was isolated 72 hours post-transfection by direct lysis of the cells in Phusion™ HF buffer (Thermo Scientific, Cat #F-518L), proteinase K (Thermo Scientific, Cat #EO0491) and RNase A (Thermo Scientific, Cat #EN0531) for 1 hour at 56 °C followed by heat inactivation at 96 °C for 5 minutes. PCR was performed with primers flanking the cleavage sites Forward 5'-GCCCACGTACCTACCAGTTT-3', Reverse 5'-TCAGCTTTCTTGCAGTCCCTT-3'. PCR products (500 ng) were treated with T7 endonuclease I (T7EI; NEB, Cat #M0302L) for 25 minutes at 37 °C and the samples were separated on a 2% agarose gel. Percent editing in each sample was calculated using ImageJ software National Institutes of Health, Bethesda, MD). Purified PCR products were sequenced by Eurofins.

Luciferase plasmid transfection and detection: A dual-luciferase vector, psiCHECK™-2, containing Firefly Luciferase (Fluc) and Renilla Luciferase (hRluc) (Promega, Cat #C8021) was used to clone a fully complementary target site for miR-221 microRNA between the XhoI-NotI restriction sites in the multiple cloning region in the 3' UTR of the hRluc gene. Characterized, clonal cells were transfected with 100 ng/well of the reporter plasmid using 0.2 µL/well DharmaFECT Duo transfection reagent.

Firefly and Renilla luciferase expression was measured 72-hours after transfection using the Dual-Glo™ Luciferase Assay System (Promega, Cat #E2980) and expression levels of Renilla Luciferase/normalized to Firefly Luciferase. Normalized Renilla Luciferase expression was then compared to that of the negative controls (fold change).

References

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