APPLICATION NOTE

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Edit-R™ Lethal crRNA controls for transfection optimization and ongoing monitoring of CRISPR-Cas9 experimental conditions

Edit-R Lethal crRNA Control #1 and #2 were developed to assist researchers in optimization of crRNA delivery conditions and monitoring transfection efficiency during CRISPR-Cas9 experiments, including arrayed screens. These crRNA controls are designed to kill cells by targeting up to thousands of locations in the genome at once, causing double-strand breaks which have been shown to result in apoptosis[1,2]. Since the observed cell death is Cas9- and guide RNA-dependent, the controls can be used to monitor both Cas9 functionality and crRNA delivery efficiency. The cell death phenotype is easily assessed by visual inspection, staining using a standard viability indicator (trypan blue or propidium iodide), or a metabolic indicator assay (resazurin). Furthermore, this cell death phenotype can develop in as few as 48 hours post-delivery, allowing for assessment of successful delivery before downstream assays need to be performed.

Edit-R Lethal crRNA control #1 induces very potent cell death, while Edit-R Lethal crRNA control #2 induces moderate cell death (Figure 1). These two controls span a large dynamic range and allow for the optimization of CRISPR-Cas9 delivery for observation of both strong and moderate CRISPR-Cas9 phenotypes.

Optimal conditions for delivery (for example, the ideal amount of transfection reagent), can easily be determined by visual inspection of the cell populations (Figure 1) with the use of Edit-R Lethal crRNA crRNA controls. Unlike gene editing assays (such as mismatch detection), these controls do not require additional sample preparation and analysis.

The cell viability effects of Edit-R Lethal controls can be quantified and compared to other positive and negative controls to refine experimental conditions. Cell viability as determined by resazurin assay shows robust and moderate effects of Lethal crRNA control #1 and #2, respectively (Figure 2A). The graph shows that 0.13 µg/well of DharmaFECT™ 4 transfection reagent gives the most robust cell death phenotype without any viability effects in the non-targeting control (NTC).

A proteasome-related phenotype was also examined to determine whether the optimal conditions identified by the lethal controls would correlate with a robust phenotype in a specific cellular assay (Figure 2B). Briefly, U2OS proteasome cells express a mutant human ubiquitin fused to EGFP where

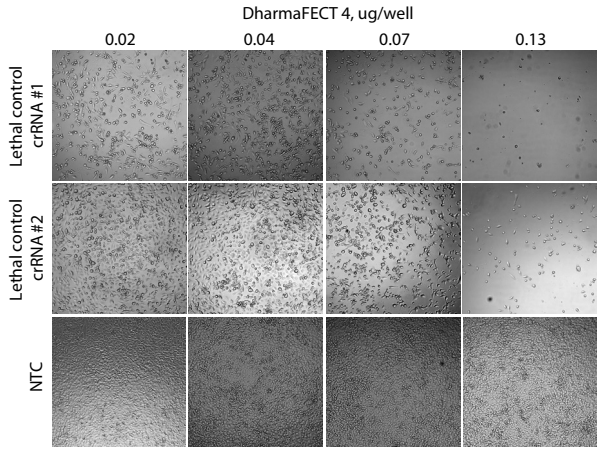

Figure 1. Phase contrast images indicate clear loss of cell viability in Cas9-expressing cells treated with Edit-R Lethal crRNA controls. Cas9-expressing U2OS-Proteasome cells[3] were plated in 96-well plates at 10,000 cells per well. 24 h after plating, cells were transfected with 25 nM crRNA:tracrRNA using 0.02-0.13 µg/well of DharmaFECT 4 Transfection Reagent. Cells were visually assessed for cell viability. NTC = Non-targeting control

the EGFP fusion is degraded by the proteasome. When the proteasome pathway is disrupted by functional knockout of a key gene, such as PSMD7, the ubiquitin-EGFP fusion accumulates and EGFP fluorescence is high. Comparison of the proteasome phenotype with PSMD7 crRNA and the cell viability phenotype with Edit-R Lethal controls reveals the utility of this approach for optimization. A weak phenotype of proteasome disruption (low EGFP expression) at 0.02 and 0.04 µg/well of DharmaFECT 4 transfection reagent corresponds to weak cell viability effect of Edit-R Lethal controls. While using 0.07 µg/well of DharmaFECT 4 improves the phenotype, the strongest phenotype is only achieved with 0.13 µg/well of DharmaFECT 4 and PSMD7 crRNA (which disrupts cellular proteasome function) and corresponds to the highest amount of cell death with Edit-R Lethal controls. PPIB and Non-targeting control do not exhibit reduced cell viability with this amount of DharmaFECT 4 transfection reagent, indicating that the cell death is specific to the action of the lethal controls.

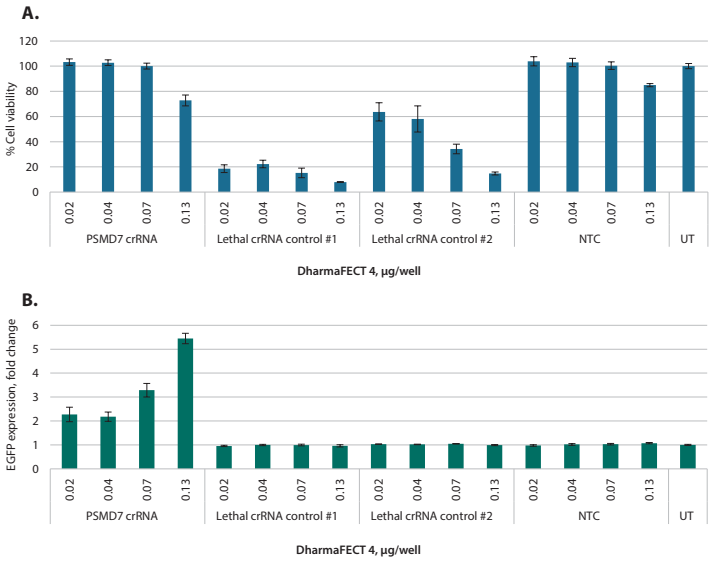

Figure 2. Cell death from lethal controls and proteosome-dependent phenotype correlate with optimal transfection conditions. A. Recombinant U2OS Ubi[G76V]-EGFP-Cas9 cells were analyzed for cell viability using a resazurin assay under a range of transfection conditions. Lethal controls show a dose-dependent level of cell death. **B.** Cells were analyzed for EGFP expression (proteasome disruption) using fluorometry. Optimal conditions indicated by cell viability reproduced the optimal conditions for high EGFP expression. Recombinant U2OS Ubi[G76V]-EGFP-Cas9 cells were plated in 96-well plates 10,000 cells per well. 24 h after plating, cells were transfected with 25 nM crRNA:tracrRNA using 0.02-0.13 µg/well of DharmaFECT 4 Transfection Reagent. All results were normalized to UT, 72 h after transfection. UT = untreated cells, NTC = Non-targeting control

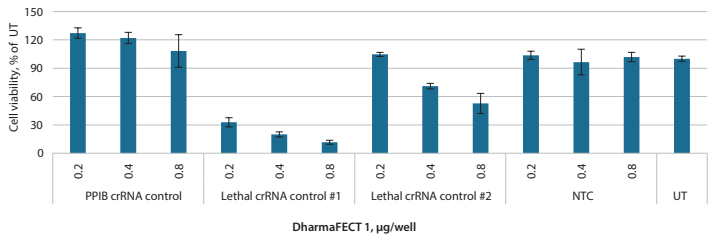

Figure 3. Optimization of RNP delivery in HUVEC cells with Edit-R lethal crRNA controls. HUVEC cells were plated in 96-well plates at 5,000 cells per well. 24 h after plating, cells were transfected with 50 nM crRNA:tracrRNA and 25 nM Cas9 NLS protein, using 0.2, 0.4 and 0.8 µg/well of DharmaFECT 1 transfection reagent. Cell were analyzed for cell viability using resazurin assay 72 h after transfection. UT = untreated cells, NTC = non-targeting control

Edit-R Lethal crRNA controls can also be used to optimize guide RNA delivery with Cas9 mRNA and protein. Edit-R Cas9 Nuclease mRNA and Cas9 Nuclease protein NLS in conjunction with Edit-R synthetic sgRNA or crRNA:tracrRNA, enables researchers to perform genome engineering experiments in a completely DNA-free manner.

In HuVECs in which crRNA:tracrRNA was delivered as an RNP with Edit-R Cas9 Nuclease protein NLS, a robust lethal phenotype occurred with Edit-R lethal controls #1 and #2 using 0.8 µg/well of DharmaFECT 1 transfection reagent (Figure 3). Importantly, no significant reduction in viability in the PPIB or Non-targeting control crRNA was observed at this dose of DharmaFECT 1.

Conclusions

Edit-R Lethal crRNA controls are universal positive controls that result in cell death via CRISPR-Cas9-mediated double-strand breaks in thousands of multiple locations across the genome. These controls allow for rapid and easy assessment of a viability phenotype, without the need for laborious and time-consuming downstream assays, like DNA mismatch detection assays or sequencing.

The viability phenotype observed with the Edit-R lethal controls correlates with the delivery of the Cas9 and guide RNA components. These easy-to-implement controls can be used just like gene-targeting crRNAs, and are compatible with a variety of Cas9 sources, making them ideal for virtually every CRISPR-Cas9 gene knockout application.

References

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3. Anderson, E.M; Haupt, A. *et al*; Systematic analysis of CRISPR–Cas9 mismatch tolerance reveals low levels of off-target activity; *Journal of Biotechnology* **2015**; 211:56–65 DOI: 10.1016/j.jbiotec.2015.06.427

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