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APPLICATION NOTE

Fluorescent Cas9 mRNA for enrichment of CRISPR-mediated knockout and knock-in using synthetic guide RNA.

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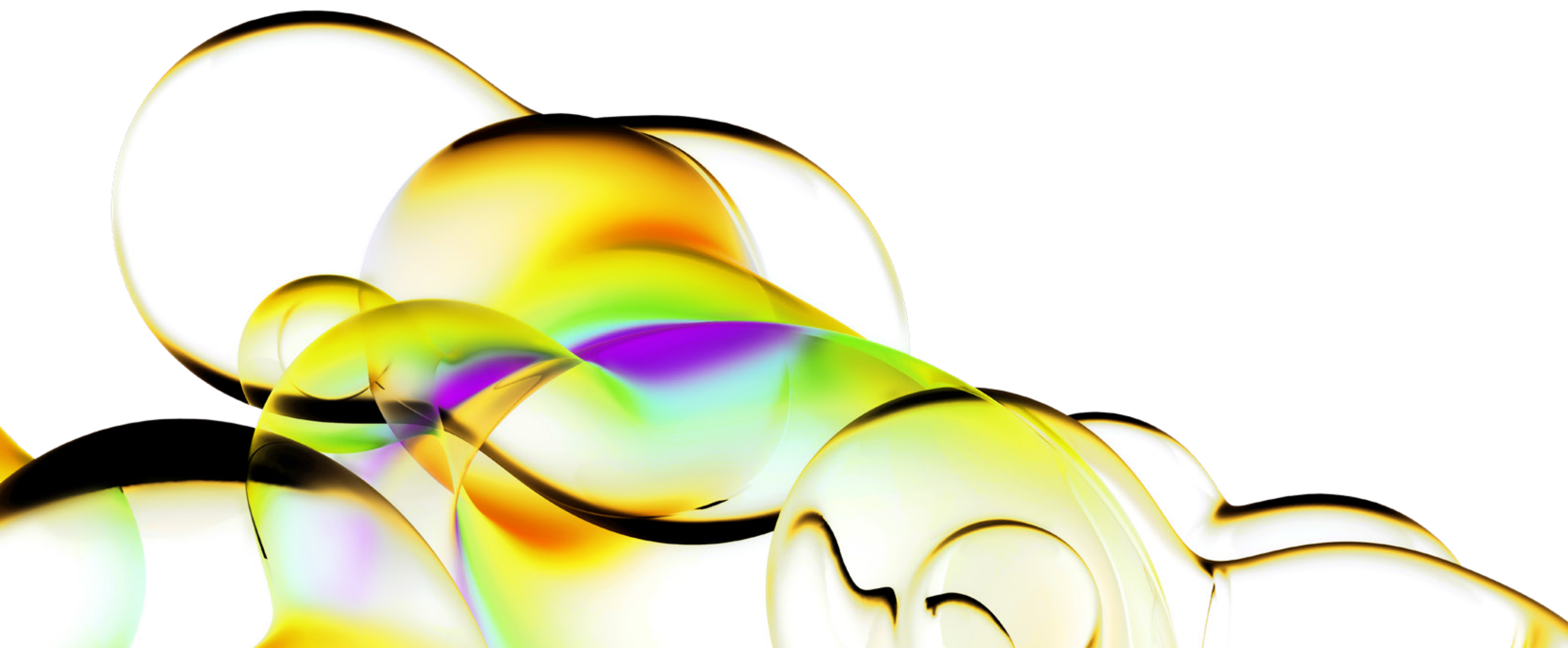
Introduction

The CRISPR-Cas9 gene editing system is a powerful tool for making changes in a cell's genomic DNA. Co-delivery of *S. pyogenes* Cas9 nuclease and a DNA-targeting guide RNA, causes a targeted double-strand break that may be imperfectly repaired by the endogenous cellular machinery resulting in a mutation at the desired locus [1, 2, 3, 4]. By combining CRISPR-Cas9 with the ability to select edited cells, either through antibiotic markers or fluorescent reporters, enrichment of gene-edited populations is possible. Multiple reports have demonstrated using the CRISPR-Cas9 gene-editing platform with fluorescence activated cell sorting (FACS) for enrichment of gene editing events [5, 6, 7]. By including a fluorescent reporter in the Cas9 vector, transfected cells are collected and sorted based on fluorescence intensity, resulting in enriched gene editing in the sorted populations. However, the use of plasmid-based gene editing reagents includes the risk of unintended integration events as well as longer timepoints for cell sorting.



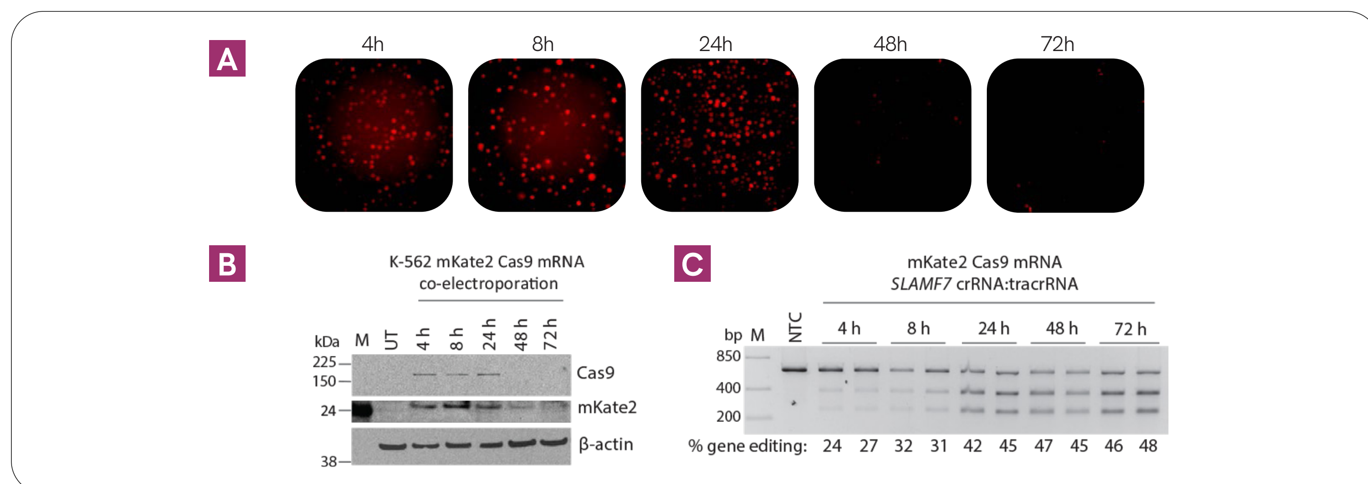


Figure 1: **mKate2 and Cas9 protein expression and gene-editing activity peak at 24 hours. A.** mKate2 fluorescence over time, in K-562 cells, after co-electroporation of mKate2 Cas9 mRNA with *SLAMF7* crRNA:tracrRNA. **B.** Western blot analysis of Cas9 and mKate2 over time with β-actin as a loading control. **C.** DNA mismatch detection assay of *SLAMF7*-targeting crRNA and mKate2 Cas9 mRNA over time.

Here we present a DNA-free enrichment system utilizing a modified version of Dharmacon Edit-R™ Cas9 Nuclease mRNA that includes a fluorescent reporter, either EGFP or mKate2. By utilizing a 2A self-cleaving peptide, both the fluorescent reporter and Cas9 are delivered within the same mRNA strand and translated into two separate proteins.

By combining this with synthetic guide RNA we demonstrate multiple uses of this fluorescent Cas9 mRNA, including:

- observation of mRNA translation of Cas9 and fluorescent protein over time
- enrichment of transfected cells with mKate2 and EGFP reporters for gene knockout
- enrichment of cells undergoing the homology-directed repair (HDR) process with the mKate2 reporter

Results

Determination of fluorescent Cas9 mRNA expression and gene editing

To understand the dynamics of both fluorescent and Cas9 proteins translated from mRNA over time, K-562 cells were electroporated with Dharmacon Edit-R mKate2 Cas9 nuclease mRNA and crRNA:tracrRNA targeting SLAMF7. Because of the immediate and efficient delivery by electroporation, timepoints started at 4 hours after electroporation and continued to 8, 12, 24, 48 and 72 hours later. At each timepoint, cells were observed using fluorescent microscopy for mKate2 fluorescence and an aliquot of cells was removed for both western blotting and a DNA mismatch detection assay (T7EI). Peak fluorescence was observed 24 hours after electroporation (Figure 1A), which also correlated with the highest protein levels of both Cas9 nuclease and mKate2 by western blot (Figure 1B). At 4 hours, gene editing was detectable (~ 26%) and almost doubled at 24 hours (~ 44%), and no significant increase was observed at the 72 hour timepoint.

Fluorescent mRNA enrichment using FACS

Based on the time course experiment above, it was determined that 24 hours after transfection is the ideal time to sort cells using fluorescence. K-562 cells were electroporated with mKate2 Cas9 mRNA, Dharmacon Edit-R synthetic *PPIB* control crRNA and tracrRNA. Twenty-four hours after electroporation, a portion of the total cell population was sorted into different mKate2 fluorescence intensity levels (negative, dim, and top 10%). These individual cell populations were then cultured for an additional 48 hours and assayed for gene editing. The control unsorted population resulted in ~ 36% gene editing, while cells that were negative for fluorescence produced negligible levels of editing, and the dim sorted cell population produced similar levels of editing (~ 33%) to unsorted (Figure 2A). However, when the cell population with the top 10% of mKate2 fluorescence was analyzed, an increase in gene editing was observed (~ 45%; Figure 2A).

To further determine the ability to enrich for gene editing events with fluorescent Cas9 mRNA using different delivery methods, lipid transfections were performed under less-than-optimal and optimal conditions with

EGFP Cas9 Nuclease mRNA. In less-than-optimal conditions, a higher percent of the cell population is negative for EGFP compared to optimal transfection conditions (Figure 2B). The optimal transfection conditions show a shift in the fluorescent cell population from negative to higher levels (dim and top 10% fluorescent bins; Figure 2B). When unsorted and sorted populations in the less-than-optimal conditions are examined for gene editing, low levels of editing are observed in the unsorted population (~ 9%) and gene editing is increased by sorting into the dim EGFP positive population (18%) and the top 10% EGFP positive population (38%) (Figure 2C). When optimal lipid transfection conditions are sorted based upon EGFP fluorescence, editing of the dim EGFP population is almost doubled (30%) compared to the unsorted cell population (16%) (Figure 2C). Gene editing is further enriched when sorted into the top 10% EGFP positive population (38%; Figure 2C).

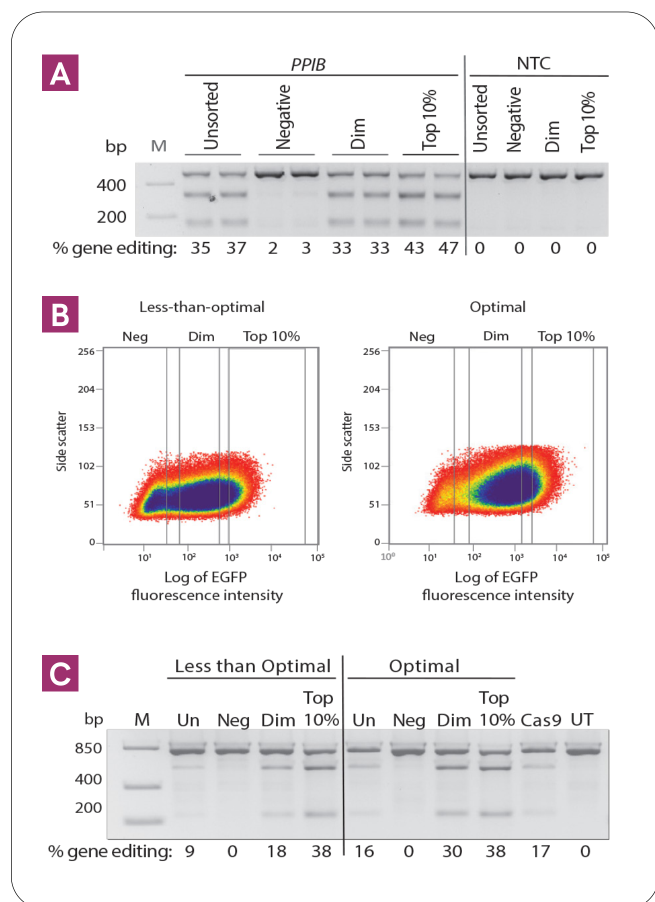


Figure 2: **Enrichment of gene knockout with fluorescent Cas9 mRNA A.** Mismatch detection assay on K-562 cell populations co-electroporated with mKate2 Cas9 mRNA and positive control *PPIB* crRNA:tracrRNA and sorted based on mKate2 fluorescence. **B.** FACS data from EGFP Cas9 mRNA sorted U2OS cell populations in less-than-optimal and optimal lipid transfection conditions. **C.** Mismatch detection assay on EGFP Cas9 mRNA sorted U2OS cell populations

HDR enrichment using fluorescent mRNA

U2OS cells were transfected with DharmaFECT™ Duo transfection reagent, mKate2 Cas9 mRNA, *EMX1* crRNA, tracrRNA, and a donor oligo. The donor oligo was designed to insert a single FLAG tag and NheI restriction enzyme recognition sequence into the C-terminus of EMX1 by using 30 nucleotide (nt) homology arms and including two phosphorothioates on each end of the oligo (Figure 3A). Twenty-four hours after transfection, the cells were enriched for mKate2 expression using FACS, plated onto a 24-well culture dish, and grown for an additional 48 hours. Transfected cells were assayed for knock-in of the inserted sequence by a restriction fragment length polymorphism (RFLP) assay (Figure 3B). The highest amount of HDR knock-in was observed in the conditions corresponding to the sorted cells expressing the top 10% highest mKate2 (30% knock-in) compared to unsorted (15% knock-in), low mKate2 expression (9% knock-in) and medium mKate2 expression (16% knock-in; Figure 3B).

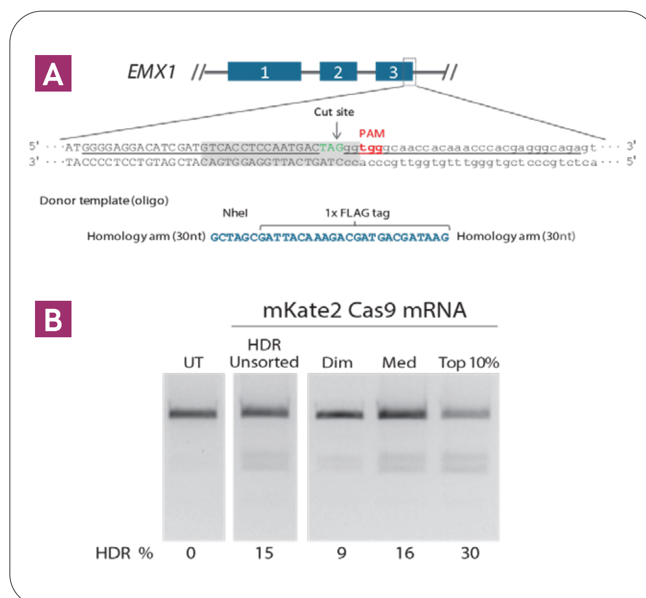


Figure 3: **Enrichment of HDR edited cells with mKate2 Cas9 mRNA A.** HDR donor design to insert a NheI and FLAG tag at the C-terminus of the EMX1 gene. **B.** RFLP analysis of HDR edited cells sorted based on mKate2 fluorescence.

Discussion

Here, we have demonstrated how Dharmacon Edit-R Fluorescent Cas9 Nuclease mRNA can be used to enrich transfected or electroporated cells for both gene knockout and knock-in experiments. By using fluorescent Cas9 mRNA, translation and protein levels of Cas9 can be examined over time through observing the fluorescent protein. This correlates with Cas9 protein translation, and suggests that protein levels peak at 24 hours after electroporation. When using fluorescent Cas9 mRNA in electroporation (a highly optimized delivery method with few variables to change), enrichment by collecting the top 10% fluorescent cells is modest. Suboptimal lipid transfection conditions were used to simulate results that may be observed in cells that are difficult to transfect. Under these conditions, enrichment of the top 10% fluorescent cells restores gene editing to levels observed with similar sorting following transfection with optimal conditions. The data presented here suggest that using Dharmacon Edit-R Fluorescent Cas9 mRNA, followed by FACS enrichment, can isolate a population of cells with higher gene editing efficiency for a more robust experimental outcome.

In addition to selecting the top 10% fluorescent population, single cells can be sorted for clonal isolation and screening for the desired mutation. By using fluorescent Cas9 mRNA, this enables selection for cells with Cas9 nuclease present, likely increasing the chances of finding the desired mutation. This is also evident with delivering a ssDNA oligo with fluorescent Cas9 mRNA and guide RNA for HDR experiments. When we selected for the top 10% we could see a 2-fold improvement in oligo insertion through RFLP. If sorting for single cells, this can increase the chances of finding the desired gene correction, reducing the number of clones that need to be screened.

Conclusions

Dharmacon Edit-R Fluorescent Cas9 Nuclease mRNA enables enrichment for transfected cells expressing Cas9. Two fluorescent options, mKate2 and EGFP, provide flexibility for different wavelengths depending on experimental variables and microscopy capabilities. With Dharmacon Edit-R Fluorescent Cas9 Nuclease mRNA, expression of the fluorescent protein, and therefore Cas9 nuclease, can be observed over time using microscopy as well as enrichment of fluorescent cells. By selecting the top 10% fluorescent positive population, both gene knockout and gene correction/insertion can be enriched.

Materials and methods

Tissue culture: K-562 cells (ATCC, CCL-243) were maintained in RPMI 1640 medium (GE Healthcare Hyclone, Cat #SH30096.01) and supplemented with 10% fetal bovine serum (FBS; GE Healthcare Hyclone, Cat #SH30071.03), 2 mM L-glutamine (GE Healthcare Hyclone, SH3003401), 1 mM sodium pyruvate (GE Healthcare Hyclone, Cat #SH30239.01), non-essential amino acids (NEAA) (GE Healthcare Hyclone, Cat #SH30238.01) and 10 mM HEPES (GE Healthcare Hyclone, Cat #SH30237.01). U2OS (ATCC, HTB96) cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 2 mM L-glutamine.

Electroporation: At the time of electroporation, 2×10^6 K-562 cells were collected per reaction and centrifuged at 500 × g for 2 minutes. Cell pellets were washed with DPBS (GE Healthcare Hyclone, Cat #SH30264.01) and re-centrifuged at 500 × g for an additional 2 minutes. Cells were resuspended in 100 µL Buffer V (Lonza, Cat #VCA-1003) as described in the Dharmacon Edit-R Cas9 Nuclease mRNA Electroporation – Protocol. Cells were then mixed with Dharmacon Edit-R mKate2 Cas9 mRNA (5 µg, Dharmacon Cat #CAS11859) and 5.36 µM tracrRNA (Dharmacon Cat #U-002000-120) with 5.36 µM predesign Dharmacon Edit-R *SLAMF7* crRNA (Dharmacon Cat #CM-017122-02-0020) or 5.36 µM Dharmacon Edit-R crRNA Non-targeting Control #1 (Dharmacon Cat # U-007501-01-20). Electroporations were carried out with either the Lonza Nucleofector™ 2b according to the manufacturer's protocol.

DNA mismatch detection assay: Cells (1×10^5 per sample) were lysed in Phusion™ GC buffer (Thermo Scientific, Cat #F-549S) with 5 µL each of Proteinase K (Thermo Scientific, Cat #EO0492) and RNase A (Thermo Scientific, Cat #EN0531), and incubated for 1 hour at 56 °C then heat inactivated at 98 °C for 10 minutes. Fifty µL PCR reactions were carried out using 0.5 µL Phusion Hot Start II DNA Polymerase (Thermo Scientific, Cat #F-549S), 5X Phusion HF buffer (Thermo Scientific, Cat #F-549S), 200 µM each dNTP (Thermo Scientific, Cat #F-549S), 0.5 µM forward and reverse primers (*SLAMF7*: Forward 5′-GCTGATGAAGATGGGGAGCA-3′, Reverse 5′-GCCCTCAAAAGGAGAGAAGGC-3′; *PPIB*: Forward 5′-ACCGTGTATTTTGACCTACGAAT-3′, Reverse 5′-AAACATTCGTAGGTCAAAATACA-3′) and 5 µL direct cell lysis template. Touchdown PCR and an annealing program with the following thermal cycling steps were run for each sample: Denature/enzyme activation at 98 °C for 3 minutes followed by 10 cycles of 98 °C for

10 seconds, 72 °C for 15 seconds -1 °C/cycle, and 72 °C for 30 seconds then 25 cycles of 98 °C for 10 seconds, 62 °C for 15 seconds and 72 °C for 30 seconds and final extension at 72 °C for 10 minutes. Samples were heated to 95 °C for 10 minutes and slowly cooled to re-anneal. Then, 10 µL of annealed PCR products were combined with 5 units of T7EI enzyme and NEBuffer 2 (New England Biolabs, Cat #M0302L) and incubated at 37 °C for 25 minutes. Three µL of 6X Orange Loading Dye (Thermo Scientific, Cat #R0631) was added to the T7EI reactions and the entire volume was loaded and ran on a 2% agarose gel. For more details see the Dharmacon Edit-R Synthetic crRNA Positive Controls Protocol. The level of editing was calculated using densitometry (% gene editing) in Image J and gene editing was estimated with the equation $(1-\sqrt{(1-(a+b)/(a+b+c))})*100$ (9).

Immunoblotting: Cells (5×10^5 per sample) were lysed on ice with 50 µL of a RIPA based lysis buffer supplemented with 1x Protease Inhibitor Mix (GE Healthcare, Cat #80-6501-23). Samples were frozen at -80 °C until gel electrophoresis. Prior to sample prep, samples were centrifuged at max speed (13,300 rpm) for 10 minutes at 4 °C. Supernatants were transferred to pre-chilled tubes and denatured in NuPAGE™ 4X LDS sample buffer and NuPAGE Sample Reducing Agent (10X) (Thermo Scientific, Cat #NP0008, #NP0009). Samples were then heated to 70 °C for 5 minutes before running on a Novex™ 4-20% Tris Glycine Mini Protein Gel (Thermo Scientific, Cat #EC6025BOX) at 125 V for 85 minutes. The protein was wet transferred to a 0.2 µM Amersham Protran nitrocellulose membrane (GE Healthcare, Cat #10600104) using the Invitrogen™ Xcell II Blot Module (Thermo Scientific, Cat #EI0002). After transfer, the membranes were blocked for 30 minutes in SuperBlock™ (PBS formulation; Thermo Scientific, Cat #37515). The membrane was cut into strips based on the protein size and probed with the respective primary antibody. Primary antibody [mouse anti- Cas9 polyclonal 1:1000 dilution (Novus Biologicals, Cat #NBP2-36440), mouse anti-TagRFP 1:1000 dilution (Thermo Scientific, Cat #MA5-15257) or mouse anti-beta-actin 1:2000 dilution (Abcam, Cat #6276)] was diluted in SuperBlock and incubated with the respective membrane overnight at 4 °C. Membranes were washed four times for 5 minutes in 0.05% Tween 20 diluted in PBS. Secondary antibody [goat anti-mouse IgG (H+L) Secondary Antibody, HRP conjugate (Thermo Scientific, Cat #32430)] was diluted 1: 20,000 in SuperBlock™ (PBS formulation) with 0. 05% Tween 20 and the membrane incubated for 2 hours at room temperature. The membranes were then submerged in SuperSignal™ West Dura Substrate (Thermo Scientific, Cat #34016) solution for beta-actin blots and West Femto Maximum Sensitivity Substrate (Thermo Scientific, Cat #34095) for Cas9 and mKate2 blots, shaken for 5 minutes and exposed to film.

FACS analysis: For sorting both U2OS and K-562 cells, U2OS cells were trypsinized, and 3/4 of each well plate was collected for both cell lines. Collected cells were centrifuged and resuspended in cell sorting medium (400 µL), and stored on ice until sorting. Cells were sorted into tubes using a Moflo XDP 100 cell sorting instrument by the Flow Cytometry Core, University of Colorado Cancer Center [Cancer Center Support Grant (P30CA046934)], using FBS-enriched medium (50% FBS and appropriate base medium). After sorting, cells were plated in the appropriate well size for the number of cells collected. Cells were assessed for gene editing 48 hours after sorting using a DNA mismatch detection assay. For more details on the complete protocol see Using Dharmacon Edit-R Fluorescent Cas9 Nuclease mRNA for enrichment of transfected cells - Protocol.

Lipid transfection for gene editing: U2OS cells were plated at 300,000 cells/well in a 6-well plate one day before transfection. At the time of transfections, 5 µg of Dharmacon Edit-R EGFP Cas9 Nuclease mRNA (Dharmacon Cat #CAS11160) with 25 nM tracrRNA (Dharmacon Cat #U-002000-120), with 25 nM Dharmacon Edit-R *PPIB* Synthetic crRNA Control (Cat #U-007000-01-20) or Dharmacon Edit-R crRNA Non-targeting Control #1 (Dharmacon Cat # U-007501-01-20) was combined with 3 µL (less-than-optimal) or 9 µL (optimal) DharmaFECT™ Duo transfection reagent (Dharmacon Cat #T-2010-02) in serum-free medium for 20 minutes. Following incubation, fullserum medium was added to the lipid complex. Medium from cell plates was removed and replaced with the lipid complex. Cells were placed into incubators at 37 °C with 5% CO_2 for 24 hours and then sorted. HDR lipid transfections were performed as above with a final donor oligo concentration of 10 nM (see Transfection of ssDNA donor oligonucleotides for HDR-mediated gene modifications – Protocol for more details.)

ssDNA donor oligos: DNA oligos were synthesized using standard solid-phase DNA synthesis and desalting procedures. Each DNA oligo used contained two phosphorothioate linkages between the first two and last two DNA bases. Oligos were ethanol precipitated and resuspended (100 µM) in 10 mM Tris-HCl (pH 7.5) and further diluted to 1 µM prior to transfection.

Restriction fragment length polymorphism assay: Genomic isolation was performed as described above, 72 hours post-transfection. PCR was performed with primers flanking the restriction enzyme knock-in site and outside of the homology arms used for each donor DNA oligo (Forward 5′-GGAGCAGCTGGTCAGAGGGG-3′, Reverse 5′-GGGAAGGGGGACACTGGGGA-3′). PCR products were further purified and eluted in water. PCR products (500 ng) were digested in FastDigest™ Buffer with 1 U of FastDigest NheI enzyme (Thermo Scientific Cat FD0974) for 30 minutes at 37 °C and the samples were separated on a 2% agarose gel. HDR knock-in for each sample was calculated using ImageJ software.

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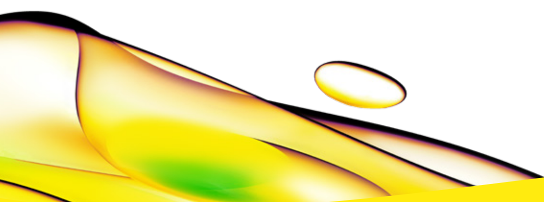

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