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Transfection of ssDNA donor oligonucleotides for HDR-mediated gene modifications using the Dharmacon™ Edit-R™ system

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Guidelines for HDR-mediated gene modification co-transfection using the Edit-R platform and ssDNA donor oligonucleotides (oligos)

The following are example protocols for co-transfection with Edit-R components (Cas9 nuclease, synthetic guide RNA and donor oligo) required for HDR-mediated gene modification (insertion, removal, or replacement) in a 96-well format. The first protocol is for Cas9 nuclease mRNA, synthetic guide RNA, nd DNA donor oligo and the second protocol is for Cas9 nuclease protein, synthetic guide RNA, and DNA donor oligo. See the Appendix for additional resources for general recommendations on cell plating, optimizing lipid co-transfection conditions and donor oligo design.

We recommend DNA-free reagents, Cas9 protein or Cas9 mRNA and synthetic guide RNA, to reduce the potential for unwanted sequence integrating into the genomic DNA instead of your specific donor oligo.

Materials required

Edit-R CRISPR-Cas9 materials for gene editing can be ordered at dharmacon.horizondiscovery.com.

- Cas9 Nuclease (choose one):
  - Edit-R Cas9 Nuclease mRNA
    a. Edit-R Cas9 Nuclease mRNA, 20 µg (1 µg/µL; Cat #CAS11195)
    b. Edit-R mKate2 Cas9 Nuclease mRNA, 20 µg (1 µg/µL; Cat #CAS11859)
    c. Edit-R EGFP Cas9 Nuclease mRNA, 20 µg (1 µg/µL; Cat #CAS11860)

    or
  - Edit-R Cas9 Nuclease protein NLS, 500 pmol (20 µM; Cat #CAS11200)
- Synthetic targeting guide RNA (choose one):
  - Edit-R synthetic crRNA and tracrRNA oligos
    a. crRNA, predesigned for your gene of interest in a variety of sizes, or designed and ordered using the Dharmacon CRISPR Design Tool
    b. tracrRNA, 5, 20 or 50 nmol (Cat #U-002005-XX)

    or
  - Edit-R synthetic sgRNA, custom ordering using the Dharmacon CRISPR Design Tool

We recommend testing three to five guide RNA designs per experiment to identify the one with highest function. It has been demonstrated that HDR-mediated gene modification is most efficient when the double-strand break (DSB) is less than 10 bp from the desired insertion site, so choosing a guide RNA close to the DSB with highest function is desirable.

- Donor oligo designed and ordered using the Edit-R HDR Donor Designer
- Dharmacon™ DharmaFECT Duo transfection reagent [Cat #T-2010-01 (0.2 mL); T-2010-02 (0.75 mL); T-2010-03 (1.5 mL); or T-2010-04 (1.5 mL × 5 tubes)]

Reagents to be supplied by user

Transfection experiments require standard cell culture reagents and instruments appropriate for maintenance of cells. The following additional materials are required but not supplied:

- Multi-well tissue culture plates or tissue culture dishes
- Antibiotic-free complete medium: Cell culture medium (including serum and/or supplements) recommended for maintenance and passaging of the cells without antibiotic
- Serum-free medium: Cell culture medium without serum or antibiotic but containing supplements recommended for maintenance of the cells such as HyClone™ HyQ-RS medium (Cat #SH30564.01)
- Assay for assessing cell viability (such as Resazurin cell viability reagent)
- Assay(s) for detecting gene editing events in a cell population
- 10 mM Tris pH 7.4 nuclease-free buffer (Tris buffer) solution (Dharmacon, Cat #B-006000-100)

Co-transfection of donor oligos with Edit-R Cas9 Nuclease mRNA and Edit-R synthetic guide RNAs

The following is a general protocol using DharmaFECT Duo transfection reagent to deliver Cas9 mRNA, synthetic guide RNA, and donor oligo into cultured mammalian cells. Exact reagent amounts and parameters for co-transfection should be empirically determined through careful optimization in your cells of interest prior to experimentation (See the Transfection optimization section of the Dharmacon Edit-R CRISPR-Cas9 genome engineering with Cas9 nuclease mRNA and synthetic guide RNAs technical manual). The protocol below describes delivery conditions in U2OS cells in one well of a 96-well plate and is given for illustrative purposes only. Reagent volumes should be calculated to include replicate samples and pipetting overage as necessary.

Day 1

1. Plate cells in 96-well plates using growth medium at a cell density so that the cells are 70 to 90% confluent the next day.
2. Incubate cells at 37 °C in a humidified CO_2 incubator overnight.

Day 2

3. Prepare a 100 ng/µL Cas9 mRNA working solution by thawing Cas9 mRNA on ice and adding 2 µL of 1 µg/µL stock solution of Cas9 mRNA to 18 µL of Tris buffer. Verify the Cas9 mRNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 100 ng/µL.

On initial use, aliquot remaining Cas9 mRNA stock or working solution into convenient volumes and store all aliquots at -80 °C to minimize the number of freeze-thaw cycles.

4. Prepare 1 µM donor oligo working solution by resuspending in the appropriate volume of Tris buffer. Verify the ssDNA concentration using UV spectrophotometry at 260 nm and adjust the volume if necessary to obtain 1 µM concentration.

If the tube of donor oligo has 5 nmoles, resuspend in 50 µL Tris buffer to obtain a stock concentration of 100 µM. Next dilute the stock concentration another 100 fold to obtain a 1 µM working concentration (for example dilute 2 µL of 100 µM stock with 198 µL Tris buffer).

5. Prepare 2 µM synthetic guide RNA transfection complex from previously prepared 10 µM stocks (See Appendix).
   - For crRNA:tracrRNA add 2 µL of 10 µM crRNA and 2 µL of 10 µM tracrRNA to 6 µL of Tris buffer (total volume is 10 µL).
   or
   - For synthetic sgRNA add 2 µL of 10 µM synthetic sgRNA to 8 µL of Tris buffer (total volume is 10 µL).
6. In a 1.7 mL tube (or deep well plate) prepare for each sample to be transfected as described in Table 1 (columns 2-5) for a 25 nM concentration of the guide RNA, 200 ng/well of Cas9 mRNA and 10 nM concentration of the donor oligo in the final transfection mixture.

We strongly recommend including three controls: 1) an untransfected control as a negative control and to assess cell viability, 2) a gene editing control to confirm NHEJ-mediated editing and transfection efficiency and 3) a donor oligo-only control as an additional negative control.

7. Prepare a 30 µg/mL DharmaFECT Duo working solution by diluting 3 µL of 1 mg/mL stock DharmaFECT Duo transfection reagent in 97 µL serum-free medium and mix gently; this volume is sufficient for 10 wells with 0.3 µL/well in a 96-well format. Incubate for 5 minutes at room temperature.

Table 1. Preparing transfection samples for gene editing experiments in a 96-well plate format.

Sample	Serum-free medium	Synthetic guide RNA transfection complex (2 µM)	Cas9 mRNA working solution (100 ng/µL)	Donor oligo (1 µM)	DharmaFECT Duo working solution (30 µg/mL)	Antibiotic-free complete medium	Total volume per well
Cas9 mRNA with donor oligo and gene-specific synthetic guide RNA **(HDR-edited)**	5.75	1.25	2	1	10	80	100
Cas9 mRNA with gene-specific synthetic guide RNA **(gene editing control)**	6.75	1.25	2	0	10	80	100
Donor oligo-only (No Cas9 mRNA, or synthetic guide RNA (negative control)	9	0	0	1	10	80	100
Untransfected **(negative control)**	20	0	0	0	0	80	100

Volumes (µL) are for a single well of a 96-well plate of U2OS cells to be transfected. It is recommended to prepare sufficient sample volumes for the total number of replicates and to account for pipetting error. Exact reagent amounts for co-transfection in other cell lines of interest should be empirically determined through careful optimization prior to experimentation (See the Transfection optimization section in the Edit-R Cas9 Nuclease mRNA and synthetic guide RNAs technical manual).

8. Add 10 µL DharmaFECT Duo working solution to each sample tube as shown in Table 1 (column 6); this will result in 0.3 µL/well final concentration. DO NOT add DharmaFECT Duo working solution to the untransfected control, which should contain serum-free medium only. This brings the total volume to 20 µL in each tube. Mix by pipetting gently up and down and incubate for 20 minutes at room temperature.
9. Prepare transfection mixture by adding 80 µL antibiotic-free complete medium to each sample to bring the total volume in each tube to 100 µL as shown in Table 1 (columns 7 and 8).

If using Edit-R Fluorescent Cas9 mRNA, we suggest enriching for positive fluorescent cells using FACS 24 hours after transfection. Refer to the protocol Using Dharmacon Edit-R Fluorescent Cas9 mRNA for enrichment of transfected cells for more information.

10. Remove medium from the wells of the 96-well plate containing cells and replace with 100 µL of the appropriate transfection mixture to each well.
11. Incubate cells at 37 °C with 5% CO_2 for 72 hours before proceeding with gene editing analysis.
12. At 72 hours split cells and retain an aliquot of the cell samples for analysis of HDR-mediated gene modification (See Gene editing assay recommendations in the Appendix) and expand remaining cells for clonal cell line generation using cell sorting or serial dilution methods.

Co-transfection of donor oligos with Edit-R Cas9 Nuclease protein NLS and Edit-R synthetic guide RNAs

The following is a general protocol using DharmaFECT Duo transfection reagent to deliver Cas9 protein, synthetic guide RNA, and donor oligo into cultured mammalian cells. Exact reagent amounts and parameters for co-transfection should be empirically determined through careful optimization in your cells prior to experimentation (See the Transfection optimization section of the Dharmacon Edit-R CRISPR-Cas9 Genome Engineering with Cas9 protein and synthetic guide RNAs technical manual). The protocol below describes delivery conditions in U2OS cells in one well of a 96-well plate and is given for illustrative purposes only. Reagent volumes should be calculated to include replicate samples and pipetting overage as necessary.

Day 1

1. Plate cells in 96-well plates using growth medium at a cell density so that the cells are 70 to 90% confluent the next day.
2. Incubate cells at 37 °C in a humidified CO_2 incubator overnight.

Day 2

3. Prepare a 2.5 µM Cas9 protein working solution by adding 0.5 µL of Cas9 protein (20 µM stock solution) to 3.5 µL of serum-free medium.
4. Prepare 1 µM donor oligo working solution by resuspending in the appropriate volume of Tris buffer. Verify the ssDNA concentration using UV spectrophotometry at 260 nM and adjust the volume if necessary to obtain 1 µM concentration.

If the tube of donor oligo has 5 nmoles, resuspend in 50 µL Tris buffer to obtain a stock concentration of 100 µM. Next dilute the stock concentration another 100 fold to obtain a 1 µM working concentration (for example dilute 2 µL of 100 µM stock with 198 µL Tris buffer).

5. Prepare 2 µM synthetic guide RNA transfection complex from previously prepared 10 µM stocks (See Appendix)
   - For crRNA:tracrRNA add 2 µL of 10 µM crRNA and 2 µL of 10 µM tracrRNA to 6 µL of Tris buffer (total volume is 10 µL).
   or
   - For synthetic sgRNA add 2 µL of 10 µM synthetic sgRNA to 8 µL of Tris buffer (total volume is 10 µL).
6. In a 1.7 mL tube (or deep well plate) prepare for each sample to be transfected as described in Table 2 (columns 2-5) for a 50 nM concentration of the guide RNA, 25 nM concentration of Cas9 protein and 10 nM concentration of the donor oligo in the final transfection mixture.

We strongly recommend including three controls: 1) an untransfected control as a negative control and to assess cell viability, 2) a gene editing control to confirm NHEJ-mediated editing and transfection efficiency and 3) a donor oligo-only control as an additional negative control.

7. Prepare a 6 µg/mL DharmaFECT Duo working solution by diluting 3 µL of 1 mg/mL stock DharmaFECT Duo transfection reagent in 497 µL serum-free medium and mix gently; this volume is sufficient for 10 wells with 0.3 µL/well in 96-well format. Incubate for 5 minutes at room temperature.
8. Add 50 µL DharmaFECT Duo working solution to each sample tube as shown in Table 2 (column 6); this will result in 0.3 µL/well final concentration. DO NOT add DharmaFECT Duo working solution to the untransfected control, which should contain serum-free medium only. This brings the total volume to 100 µL in each tube. Mix by pipetting gently up and down and incubate for 20 minutes at room temperature.

Table 2. Preparing transfection samples for gene editing experiment in a 96-well plate format.

Sample	Serum-free medium	Synthetic guide RNA transfection complex (2 µM)	Cas9 protein working solution (2.5 µM)	Donor oligo (1 µM)	DharmaFECT Duo working solution (6 µg/mL)	Total volume per well
Cas9 Nuclease protein with donor oligo and gene-specific synthetic guide RNA **(HDR-edited)**	45.5	2.5	1	1	50	100
Cas9 Nuclease protein with gene-specific synthetic guide RNA **(gene editing control)**	46.5	2.5	1	0	50	100
Donor oligo-only (No Cas9 protein,or synthetic guide RNA) **(negative control)**	49	0	0	1	50	100
Untransfected **(negative control)**	100	0	0	0	0	100

Volumes (µL) are for a single well of a 96-well plate of U2OS cells to be transfected. It is recommended to prepare sufficient sample volumes for the total number of replicates and to account for pipetting error. Exact reagent amounts for co-transfection in other cell lines of interest should be empirically determined through careful optimization prior to experimentation (See the Transfection optimization section in the Edit-R Cas9 Nuclease protein NLS and synthetic guide RNAs technical manual).

9. Remove medium from the wells of the 96-well plate containing cells and replace with 100 µL of the appropriate transfection mixture to each well.
10. Incubate cells at 37 °C with 5% CO_2 for 14-18 hours; replace transfection mixture on the cells with regular growth medium without antibiotics.
11. Continue to incubate cells at 37 °C with 5% CO_2 until 48-72 hours post-transfection.
12. At 48-72 hours post-transfection split cells and retain an aliquot of the cell samples for analysis of HDR-mediated gene modification (See Gene editing assay recommendations in the Appendix) and expand remaining cells for clonal cell line generation using cell sorting or serial dilution methods.

Appendix

Additional references

For general recommendations on cell plating and optimizing lipid co-transfection conditions, see the Dharmacon Edit-R CRISPR-Cas9 Genome Engineering with Cas9 Nuclease mRNA and Synthetic RNAs technical manual; and/or the Dharmacon Edit-R CRISPR-Cas9 Genome Engineering with Cas9 protein and synthetic guide RNAs technical manual. We recommend optimizing lipid co-transfections with Edit-R components (Cas9 nuclease and synthetic guide RNA) before performing lipid co-transfection with a donor oligo. This maximizes the number of double-strand breaks available to the cell for repair through the HDR pathway.

For general HDR DNA donor oligo design recommendations see this useful guide. The tips included in the guide are used in the Edit-R HDR Donor Designer.

For a protocol to make Tris buffer see this protocol 10 mM Tris-HCL pH 7.4 buffer (Tris buffer).

For a protocol to make 10 µM concentrated stock solutions of synthetic guide RNA see the Dharmacon Edit-R synthetic guide RNA resuspension protocol.

Gene editing assay recommendations

Detect NHEJ-mediated gene editing with mismatch detection assay

The most commonly used method for detection of indels in a cell population is a DNA mismatch detection assay (Guschin, 2010; Reyon, 2012; Cong, 2013). This assay can be performed on either purified genomic DNA or whole cell lysate using T7 Endonuclease I. Mismatch detection should be run on the gene editing control (Cas9 nuclease + synthetic guide RNA) and compared to the negative control (untransfected) sample to obtain the NHEJ-mediated editing of the transfection experiment. This is typically done 48 - 72 hours post-transfection. We recommend first determining the most functional guide RNAs (from 3-5 ordered guide RNAs) using a DNA mismatch detection assay in a preliminary editing experiment without the donor oligo, and subsequently performing the HDR transfection (including donor oligo) with the most functional guide RNA.

For HDR-mediated gene modification experiments, including the analysis of the gene editing control (Cas9 nuclease + synthetic guide RNA) is useful to confirm efficient editing in the transfection. If the NHEJ-mediated editing on the control sample is significantly less than the previously optimized transfections then the experiment may need to be repeated or further optimized, because the efficiency of HDR-mediated changes are always lower than NHEJ-mediated editing (and might not be detectable with low transfection efficiency).

Detect integration of a DNA donor oligo

The most commonly used methods for detecting HDR-mediated integration in a population of cells are PCR-based assays [e.g. restriction fragment length polymorphism (RFLP) assay] and Sanger sequencing. For more details about gene editing assays to detect HDR-mediated modifications on a population level, please see the following application note Homology-directed repair with Dharmacon Edit-R CRISPR-Cas9 reagents and single-stranded DNA oligos.

After analysis at a population level, we recommend isolating several clonal lines and performing Sanger sequencing across the modification site to verify correct genomic changes. For more details about clonal cell line generation, please see the following application note A CRISPR-Cas9 gene engineering workflow: generating functional knockouts using Edit-R Cas9 and synthetic guide RNA.

Frequently asked questions

Why do I need to test several guide RNAs for functionality?

Effective HDR-mediated modifications require high functionality and specificity, as well as very close proximity to the gene modification site. Our CRISPR Design Tool takes these factors into consideration and weights functionality and specificity scoring with proximity to the desired modification site for each guide RNAs sequence. However, the highest ranking guide RNAs for specificity and functionality may not always be in the optimal genomic location, increasing the importance of functionally testing several guide RNAs.

How do I enrich HDR-edited cells without antibiotic selection or a fluorescent reporter?

We recommend optimizing transfection conditions of NHEJ-mediated editing prior to including the donor oligo (Cas9 nuclease + highest functional guide RNA). After efficient NHEJ-mediated editing is achieved we recommend optimizing the amount of the donor template. Using this method, we found 2.5 nM to 10 nM concentrations of the donor oligo were optimal for U2OS cells.

What is the benefit to using Cas9 protein or Cas9 mRNA instead of Cas9 plasmid or a Cas9-integrated cell line for HDR experiments?

Specifically for HDR-mediated modifications Cas9 protein and Cas9 mRNA are preferred as they typically give higher gene editing efficiencies compared to Cas9 plasmid. This is important because HDR-mediated modifications will only occur in a fraction of DSBs at the target site. Additionally, Cas9 protein and Cas9 mRNA are DNA-free options so there is no concern of DNA integration resulting from the Cas9 source. Similarly, a Cas9-integrated cell line is not ideal for HDR-mediated modifications because continuous expression of the Cas9 nuclease increases potential for undesired off-targets and mutations.

Can the ploidy of my cell line affect the results of my CRISPR-Cas9 gene editing experiment?

When using a CRISPR-Cas9 system to disrupt gene function, it is important to know as much as you can about the gene you are targeting and your cells. In particular, the ploidy of your cells, the gene copy number and the presence of any SNPs are important considerations. In normal diploid cells, obtaining mutations on both (or all) alleles may be required for a complete knockout and an observable phenotype. Virtually all cancer lines and many immortalized cell lines exhibit aneuploidy, and as such, mutation of multiple alleles may be necessary. The presence of SNPs and multiple genomic locations should be taken into account when designing guide RNAs, as they can affect whether a complete knockout or knock-in can be achieved. Expansion of clones from single cells followed by sequence validation to verify the desired mutation on all alleles is the most rigorous approach for confirming complete gene knockout.

What is the stability of the Edit-R synthetic guide RNAs?

Dried RNA oligonucleotide pellets are stable at room temperature for two to four weeks, but should be placed at -20 °C or -80 °C for long-term storage. Under these conditions, the dried crRNAs, tracrRNA and sgRNA will be stable for at least one year. Maintaining sterile, RNase- and DNase-free conditions is always recommended as a critical precaution.

How many freeze-thaw cycles can the Edit-R Cas9 Nuclease mRNA be subjected to?

We recommend not exceeding two to three freeze-thaw cycles for Cas9 mRNA. On initial use, thaw the Cas9 mRNA on ice and aliquot remaining stock into convenient volumes and store all aliquots at -80 °C to minimize the number freeze-thaws cycles prior to each experiment.

How many freeze-thaw cycles can the synthetic sgRNA, crRNA, or tracrRNA be subjected to?

We recommend not exceeding four to five freeze-thaw cycles for synthetic guide RNA to ensure RNA integrity.

How should I store my HDR DNA oligo?

DNA oligonucleotides should be stored at -20 °C or -80 °C in a non-frost free freezer, either as a dried pellet or resuspended in an DNase-free solution buffered to pH 7.4-7.5 to help with stability during freeze-thaw cycles. We recommend that DNA oligonucleotides be resuspended to a convenient stock concentration and stored in small aliquots to avoid multiple freeze-thaw cycles.

How should I store my synthetic guide RNA?

RNA oligonucleotides should be stored at -20 °C or -80 °C in a non-frost free freezer, either as a dried pellet or resuspended in an RNase-free solution buffered to pH 7.4-7.5 to help with stability during freeze-thaw cycles. We recommend that RNA oligonucleotides be resuspended to a convenient stock concentration and stored in small aliquots to avoid multiple freeze-thaw cycles. RNA oligonucleotides should not go through more than four to five freeze thaw cycles. If degradation is a concern, the integrity of the RNA oligonucleotides can be evaluated on an analytical PAGE gel. For resuspension, see the Dharmacon crRNA and tracrRNA Resuspension protocol.

What is the formula for spectrophotometric quantification of RNA and/or DNA?

To quantify RNA, use Beer's Law: Absorbance (260 nm) = (ε)(concentration)(path length in cm), where ε, epsilon, is the molar extinction coefficient (provided on the Product Transfer Form supplied with the HDR DNA oligo or crRNA order; the extinction coefficient for tracrRNA is 757400). When solved for the unknown, the equation becomes: Concentration = (Absorbance, 260 nm) / [(ε)(path length in cm)]. When a standard 10 mm cuvette is used, the path length variable in this equation is 1. If a different size of cuvette is used, e.g., a 2 mm microcuvette, then the path length variable is 0.2.

I see a fair amount of cell death after transfection of my cells. What can I do about this?

Extensive cell death following transfection is an indication that delivery conditions need to be further optimized. Basic parameters to consider when optimizing transfection include transfection reagent and cell-specific conditions such as the amount of transfection reagent, duration of transfection, cell passage number, cell density at transfection, and the lot/batch of transfection reagent. Often decreasing the amount of lipid present during transfection and/or the total duration of transfection will help minimize the toxic effect to the cells. If the problem persists, we recommend contacting Technical Support (dharmacon.horizondiscovery.com/service-and-support) for additional troubleshooting help.

Can I use a transfection reagent other than DharmaFECT Duo to deliver the Edit-R components into my cells for HDR?

We have validated our protocols and demonstrated successful gene editing using DharmaFECT Duo transfection reagent to efficiently deliver the Edit-R components for HDR (both ssDNA donor oligos and donor plasmids). We cannot predict the co-transfection ability of other transfection reagents, nor can we troubleshoot experiments performed with any reagent other than DharmaFECT Duo for this application. However, other suitable transfection reagents could be utilized provided co-transfection conditions are carefully optimized for each cell line of interest.

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