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Simplified CRISPR tools for efficient genome editing and streamlined protocols for their delivery into mammalian cells and mouse zygotes

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ABSTRACT

Genome editing using the CRISPR/Cas9 system requires the presence of guide RNAs bound to the Cas9 endonuclease as a ribonucleoprotein (RNP) complex in cells, which cleaves the host cell genome at sites specified by the guide RNAs. New genetic material may be introduced during repair of the double-stranded break via homology dependent repair (HDR) if suitable DNA templates are delivered with the CRISPR components. Early methods used plasmid or viral vectors to make these components in the host cell, however newer approaches using recombinant Cas9 protein with synthetic guide RNAs introduced directly as an RNP complex into cells shows faster onset of action with fewer off-target effects. This approach also enables use of chemically modified synthetic guide RNAs that have improved nuclease stability and reduces the risk of triggering an innate immune response in the host cell. This article provides detailed methods for genome editing using the RNP approach with synthetic guide RNAs using lipofection or electroporation in mammalian cells or using microinjection in murine zygotes, with or without addition of a single-stranded HDR template DNA.

1. Introduction

CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) is a bacterial/archaeal immune system that can be adapted to perform sequence-specific genome engineering in mammalian cells and to make novel model organisms [1]. The CRISPR toolbox consists primarily of two components; a guide RNA and a Cas9 nuclease, with a repair DNA template as an optional third component. The guide RNA provides sequence specificity and targets the Cas9 nuclease to a complementary site in the genome where the nuclease creates a double-stranded break. The double-stranded break is healed by cellular repair machinery (non-homologous end joining, or NHEJ), which is often imprecise and can disrupt the amino acid coding sequence if the guide targets a coding exon. The guide RNA occurs naturally as a 2-molecule complex comprising a target-specific crisprRNA (crRNA) bound to a trans-activating crRNA (tracrRNA) that directs binding of the RNAs to Cas9. Alternatively, the guide can comprise a single molecule that is a fusion between the crRNA and the tracrRNA, called a single guide RNA (sgRNA) [2]. Furthermore, novel genetic material can be inserted at the cleavage site by supplying a DNA template, which can be as simple as single-base mutagenesis or insertion of kilobases of new DNA content via homology-directed repair (HDR).

The CRISPR toolbox is being constantly improved. Early methods expressed Cas9 and the guide RNA from plasmid or viral templates, but overexpression of these components from such sources can lead to a high incidence of undesired off-target effects. In this case, double-stranded breaks occur at sites in the genome that are

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not identical to the guide sequence but that have sufficient homology to enable Cas9-mediated cleavage [3]. Direct delivery of the Cas9 nuclease with guide RNA as a ribonucleoprotein (RNP) complex limits the amount of the critical components and gives a "fast on/fast off" character to the genome editing machinery, resulting in a significant reduction in off-target effects [4–6]. Two different versions of Cas9 RNP complexes can be employed: (1) combination of sgRNA and Cas9 protein, and (2) combination of crRNA, tracrRNA (two separate strands to form a complete guide RNA) and Cas9 protein. Cas9, the common component of the two versions, is used as a recombinant protein; the RNA components in the first version (sgRNA) are typically synthesized using *in vitro* transcription and the RNA components in the second version (crRNA and tracrRNA) are chemically synthesized. To distinguish the two types of RNPs, we recently proposed the terms sgRNP and ctRNP for the complexes containing sgRNA or crRNA + tracrRNA as RNA components respectively [7].

Until recently, only two types of DNA repair templates have been used: (1) a single-stranded synthetic oligodeoxynucleotide (ssODN) if the aim is to insert or modify a short sequence (up to 200 bases, usually with 30–60 base homology arms) [8–10], or (2) a double-stranded DNA (dsDNA) with much longer homology arms (500–1000 bases) that supports insertion of up to several thousand bases [11]. However, recent reports have demonstrated that long single-stranded DNAs (ssDNAs) enzymatically generated from cloned sources can be used as repair templates that do not require as long of homology arms yet can show higher efficiency of insertion than similar templates in dsDNA form [12,13]. The same RNP protocols can be used for both sgRNP and ctRNP complexes, with the exception that the crRNA and tracrRNA must be annealed before final complex formation for the ctRNPs.

In this report, we describe methods and protocols related to use of CRISPR RNPs containing chemically-modified crRNA + tracrRNA complexed with Cas9 protein for direct delivery into cells and mouse zygotes. Specifically, we provide protocols for (1) lipofection of ctRNPs into mammalian cells, (2) electroporation of ctRNPs into mammalian cells, (3) general outline of genotyping and screening for mutations, and (4) microinjection of ctRNPs and long ssDNA donors into mouse zygotes for creating knock-in alleles. These streamlined protocols are suitable for delivering either ctRNPs or sgRNPs with optional repair DNAs.

2. Methods

2.1. Ribonucleoprotein complex lipofection

All methods described herein employ a CRISPR system that uses two synthetic RNA oligonucleotides, a crRNA and a tracrRNA, that must be annealed prior to mixing with Cas9 protein and subsequent delivery as a ctRNP complex. Further, the RNAs employed are chemically-modified and length optimized variants of the native guide RNAs (Alt-R™ CRISPR crRNAs and tracrRNA, Integrated DNA Technologies, Coralville, IA, USA). The optimized lengths of crRNA and tracrRNA are 36 and 67 bases respectively (Fig. 1). Lipofection is the least expensive method for introducing Cas9 RNP into cell lines amenable to lipofection. The present protocol has been optimized for delivery into HEK293 cells. Electroporation (Section 2.2) may be considered to introduce RNP into cell lines or cell types where lipofection is not efficient. Cas9 ctRNP lipofection can be coupled with co-transfection of ssODNs as HDR templates. When a ssODN HDR donor is included, we suggest use of high-fidelity Ultramer® DNA oligonucleotides (Integrated DNA Technologies) for templates of up to 200 bases and suggest using desalted oligonucleotides (PAGE purification adds cost and, in some settings, toxicity from residual acrylamide or urea with this

Fig. 1. Aligned crRNA and tracrRNA sequences in the guide RNA complex. The crRNA is shown (blue) aligned with the tracrRNA (red). The variable target-specific protospacer domain of the crRNA is indicated with "N" bases.

method of preparation). We recommend adding 30–50 base homology arms on either side of the predicted crRNA cleavage site. The basic protocol involves 3 steps: 1) annealing the crRNA and tracrRNA to form a complete guide RNA, 2) forming a complex between Cas9 and the guide RNAs, and 3) delivery into cells (Fig. 2).

2.1.1. Lipofection of ctRNP complexes for NHEJ into HEK293 cells

1. Form guide RNA complexes by combining the crRNA and tracrRNA in equal molar amounts in IDT Duplex Buffer (30 mM HEPES, pH 7.5, 100 mM Potassium Acetate) at 1 μM concentration by heating the oligos to 95 °C and slowly cooling to room temperature. We typically keep working stocks of crRNAs and tracrRNA at 10 μM concentration in TE (10 mM Tris, pH 7.5, 0.1 mM EDTA), in which case mix 1 μL of crRNA and 1 μL of tracrRNA with 8 μL of Duplex Buffer. While not always necessary, the heat/cool step improves performance for approximately 10% of target sites. Excess of the 1 μM crRNA:tracrRNA complex can be stored for later use at 4 °C, −20 °C or −80 °C for at least 3 months. Aligned sequences of the crRNA:tracrRNA complex after annealing are shown in Fig. 1.
2. Dilute Alt-R™ 3NLS Cas9 Nuclease (Integrated DNA Technologies) from stock 61 μM (10 mg/mL) to 1 μM in Opti-MEM (Thermo Fisher Scientific, Carlsbad, CA USA). Final transfections will employ 10 nM ctRNP complex.
3. The following preparation of the ctRNP complex is intended for biological triplicates in 96-well culture format (3.5× of the required solution for 1 well is made using this protocol):
   a. The ctRNP complex is prepared by combining 5.25 μL of the 1 μM crRNA:tracrRNA complex with 5.25 μL of the 1 μM diluted stock of Cas9 protein. (Note: excess of the 1 μM RNP complex can be made and stored for later use at 4 °C or −80 °C for at least 3 months.)
   b. Add 77 μL of Opti-MEM medium, bringing the final volume to 87.5 μL, yielding a final 60 nM concentration of RNP complex.
4. Incubate this mixture at room temperature for 5 min.
5. Mix 4.2 μL of Lipofectamine® RNAiMAX (Thermo Fisher Scientific) with 83.3 μL of Opti-MEM and add this mixture to each sample of ctRNP complex (87.5 μL), resulting in a final volume of 175 μL with an RNP concentration of 30 nM.
6. Incubate RNP-lipid complexes at room temperature for 20 min. The ctRNP transfection solution is now ready for use.
7. Trypsinize and count HEK293 cells cultured in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific). Pellet HEK293 cells. Due to the frequent presence of RNases in trypsin, which