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Genome editing via delivery of Cas9 ribonucleoprotein

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ABSTRACT

The CRISPR-Cas genome editing system is very powerful. The format of the CRISPR reagents and the means of delivery are often important factors in targeting efficiency. Delivery of recombinant Cas9 protein and guide RNA (gRNA) as a preformed ribonucleoprotein (RNP) complex has recently emerged as a powerful and general approach to genome editing. Here we outline methods to produce and deliver Cas9 RNPs. A donor DNA carrying desired sequence changes can also be included to program precise sequence introduction or replacement. RNP delivery limits exposure to genome editing reagents, reduces off-target events, drives high rates of homology-dependent repair, and can be applied to embryos to rapidly generate animal models. RNP delivery thus minimizes some of the pitfalls of alternative editing modalities and is rapidly being adopted by the genome editing community.

1. Introduction

1.1. Genome editing

Genome editing is a family of technologies that allows efficient targeted DNA sequence alterations within the genomes of eukaryotic cells. The use of genome editing has transformed many domains of biological research, and holds great promise for medicine and agriculture in the near future. All genome editing platforms – ZFNs, TALENs, meganucleases, and CRISPR-Cas – operate by making targeted breaks in chromosomal DNA [1]. Cellular repair processes then convert the breaks into local insertion and deletion mutations (indels) by nonhomologous end joining (NHEJ) or incorporate sequences from a donor DNA to modify the target locus by homology-dependent repair (HDR).

Genome editing nucleases must be introduced efficiently into cells and ultimately into the nucleus. Many years of experience with delivering DNAs, RNAs and proteins to cells have left researchers with a host of options, and many variations have been employed for editing components. In the case of CRISPR, this involves introduction of a Cas effector (*e.g.*, Cas9) and one or more guide RNAs (commonly single guide RNAs, or sgRNAs) that direct cleavage of specific genomic targets [2].

Cas9 protein can be produced in cells by delivering the corresponding coding sequences as purified DNA (*e.g.*, in a plasmid), as mRNA, or as part of a viral genome. Direct DNA or RNA delivery can be accomplished in cultured cells with good efficiency by a number of procedures, including electroporation, lipid transfection, or chemical transduction [3,4]. Viral delivery requires more prior engineering, but can be very effective, particularly in whole organisms. When the delivery vehicle is DNA, whether purified or part of a viral genome, appropriate regulatory elements must be provided.

When coding sequences for sgRNAs are delivered on DNA vectors, it is typical to put them under the control of an RNA polymerase III promoter (*e.g.*, U6 or H1), since the capping, tailing and other processing enjoyed by RNA polymerase II transcripts are neither necessary nor desirable. Purified sgRNAs can also be introduced directly into cells already expressing Cas9 by RNA transfection (similar to siRNAs). Delivering the RNA independently of Cas9 allows control over the timing of its presence, but exposes it to degradation by cellular RNases. Delivering Cas9 along with the sgRNA as a preformed sgRNA ribonucleoprotein (RNP) complex avoids many of these limitations [5,6].

1.2. Advantages of RNP delivery

RNP delivery avoids many of the pitfalls associated with mRNA, DNA, or viral delivery. The RNP, when paired with a DNA donor, comprises a "total package" that does not require the cellular environment to synthesize Cas9 and sgRNA, and ensures temporal coordination of the editing reagents. Cas9 RNP delivery was first reported for direct injection into *C. elegans* gonads as a means of avoiding RNA interference [7], and this approach is still commonly used [8].

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In cultured mammalian cells, RNP editing is very effective in generating targeted indels, and it has several advantages over other methods (Fig. 1). First, editing is very rapid: indels are measurable very shortly after electroporation of cells, with robust editing within three hours and reaching a plateau by 24 h [5]. Cas9 protein is rapidly cleared from cells and is largely absent within 24 h. In contrast, Cas9 delivered by plasmid electroporation persists in cells for at least 72 h [5]. Since on-target activity during plasmid editing reaches a plateau while Cas9 protein is still being expressed, the only remaining effect would be to continue undesirable cleavage at off-target sites. Accordingly, RNP-mediated editing exhibits reduced off-target mutagenesis and cell death as compared with plasmid-mediated editing [6,8,9]. DNA delivery also risks insertional mutagenesis by integration of the vector at random into the genome.

The rapid timing of RNP editing also enables advances in on-target editing efficacy in multiple cellular contexts, including embryonic stem cells and induced pluripotent stem cells [6,9]. Delivery of RNP coupled with cell cycle arrest [6] or modified donor DNA design [10] can further be used to alter the balance of NHEJ and HDR products in human cells, leading to HDR frequencies greater than 30%. In some cases, RNP editing lends itself well to direct mechanical injection of fertilized eggs, and this has been applied to zebrafish, rats and mice [11–14]. Cas9 RNPs have even been efficacious when delivered to mouse zygotes by electroporation, achieving high frequencies of indel formation and HDR without cumbersome manual injection [15–17]. This greatly reduces the time and labor required to generate knockout mice, and may reduce (but not eliminate) mosaicism in the embryo as well. Reports also demonstrate the utility of Cas9 RNP delivery for in utero [18] and postnatal [19] targeted gene disruption.

In this article, we describe methods to assemble and deliver pre-formed Cas9 RNPs to human cells in culture. The following procedures are based on our experience as related in published studies [10,20], unpublished experiments, and at the IGI CRISPR Workshop, held in Berkeley, California, July 11–15, 2016.

2. Methods

Detailed protocols for Cas9 expression and purification, sgRNA transcription, RNP assembly, electroporation of cultured cells, analysis of editing by T7 endonuclease digest, and quantification of genome editing by next-generation sequencing can be found at https://www.protocols.io/groups/innovative-genomics-institute.

2.1. Cas9 protein

Our experience has been largely with the *Streptococcus pyogenes* Cas9 expressed in *E. coli* from a bacterial expression vector that is available from Addgene (https://www.addgene.org/69090/) [6]. The construct includes two nuclear localization signals (NLSs), an HA epitope tag (for Western blot and/or immunofluorescence) and a His_6 tag (for purification). We have used Cas9 purified by the method described by Lin et al. [6]. We have also used a less highly purified preparation [5] successfully in *C. elegans* and in several human cell lines (D.C., unpublished observations). Several companies now supply purified Cas9 protein with some or all of the tags discussed above (NLS and HA tags). Care should be taken to obtain Cas9 protein of high quality and purity, as activity can vary between suppliers.

2.2. sgRNA

We typically make our own sgRNA by *in vitro* transcription (IVT) in two steps: template synthesis by assembly PCR followed by vitro transcription and purification of the sgRNA (Fig. 2). The template is produced from four DNA oligonucleotides, 3 of which are constant and one that carries a T7 RNA polymerase promotor along with the guide sequence that is unique for each new sgRNA. We use a promotor with a single terminal guanine, resulting in sgRNAs with a 5′ guanine in addition to the 17–20 nt guide sequence, which has been shown to have little effect on editing efficiency [21]. The PCR mixture includes two long oligos, one encoding the promotor and the variable guide sequence, the other representing the constant 3′ end of the sgRNA. Two short oligos (T7 FP, T7 RP; Fig. 2) are used for amplification. The reaction is run for 15 cycles. The DNA need not be purified, but can be used directly for sgRNA synthesis. We often check the product by running an aliquot of the reaction on a 2% agarose gel (Fig. 3).

We transcribe sgRNA from this template using the NEB HiScribe T7 High Yield RNA Synthesis Kit, in a 20 μL total transcription volume, of which 8 μL is unpurified assembly PCR product. We have found that purification of the assembly PCR does not substantially improve the transcription reaction. The reaction is incubated at 37° overnight. We often purify the sgRNA with a QIAgen RNeasy mini kit, using the modifications for small RNAs, and elute it in 20 μl of RNAse-free water. Fluorometric quantitation (e.g. using a Qubit instrument) of purified sgRNA is preferred, as impurities may confound analysis using a spectrophotometer. For high-throughput applications, we purify sgRNAs using AMPure SPRI beads (5X ratio of SPRI beads to IVT reaction), following the manufacturer's protocols, and elute in 20 μL of water. Yields of 100–200 μg of sgRNA are common. An aliquot of 0.5–1.0 μg of the sgRNA is run on a 10% polyacrylamide TBE-urea gel or Agilent Bioanalyzer. to check its integrity (Fig. 3).

Synthetic sgRNAs are now available commercially, often incorporating non-hydrolyzable linkages or non-natural bases [22,23]. Inhibiting degradation of the sgRNAs in cells by introducing nuclease-resistant terminal phosphorothioates, for example, has less of an influence with RNP delivery than in cases where Cas9 is produced separately in cells from DNA or mRNA templates

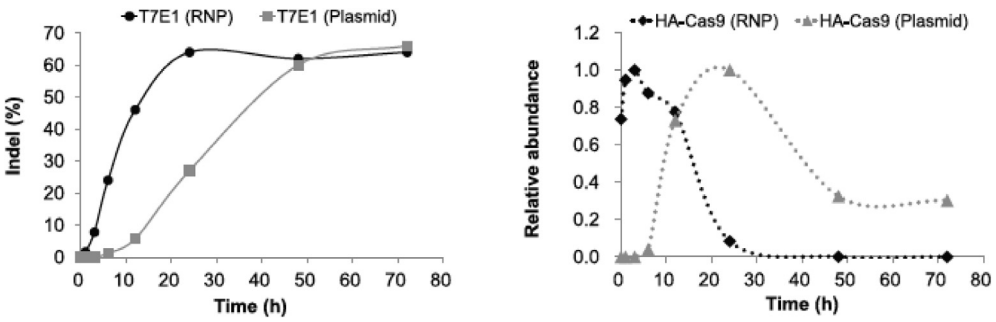

Fig. 1. Time course of indel formation (left) and Cas9 persistence (right) after delivery of Cas9 by RNP or plasmid. From Kim [5] Genome Res 24: 1012.

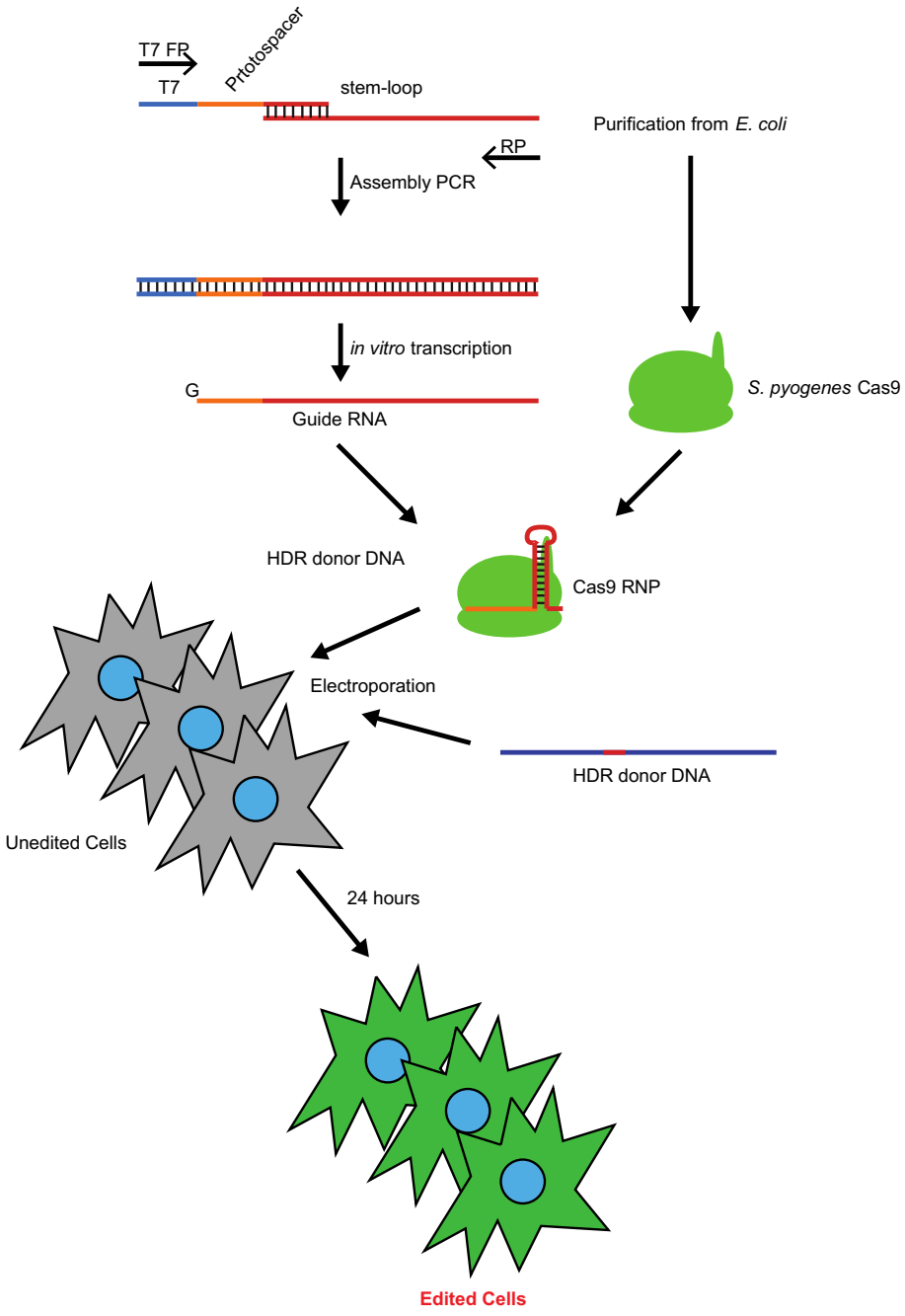

Fig. 2. Scheme for production of sgRNA and assembly of Cas9 RNP. The double-stranded DNA template for sgRNA synthesis is assembled by PCR from 4 oligonucleotides (top left). One oligo carries the desired guide (protospacer) sequence (orange) and the sequence of the T7 promoter (blue), one represents the constant portion of the sgRNA (red), and two shorter ones are used for amplification. That template is transcribed with T7 RNA polymerase, generating the sgRNA. This is combined with purified Cas9 protein and, a donor DNA (blue) that carries a change in the target sequence (red). The mixture is then delivered to cells by electroporation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[22]. Several companies offer guides as variable crRNA/invariant tracrRNA pairs as well. These pairs, as well as synthetic sgRNAs, are much more expensive than guides produced by IVT. We anticipate that pricing could change in the future as more suppliers enter this growing market.

2.3. ssDNA HDR donor

For short, precise genomic edits (e.g. single-nucleotide mutations) with high efficiency, we prefer a short single-stranded DNA HDR donor. Work in our laboratories has demonstrated that design of the HDR donor can have a significant impact on the efficiency [10]. An optimal HDR donor is designed with a specific mutation and sgRNA in mind. The donor should incorporate the desired genomic edit, mutations that destroy the PAM of the guide RNA (to prevent cutting of the edited sequence), and homology arms that match the targeted sequence. In a coding region, PAM mutations must be incorporated through silent mutations that do not alter the amino acid sequence. The donor should be complementary to the strand displaced by sgRNA binding, i.e., not the strand that binds the sgRNA. We further found that the donor should have a longer 5′ homology arm (~80–90 bp), and a shorter

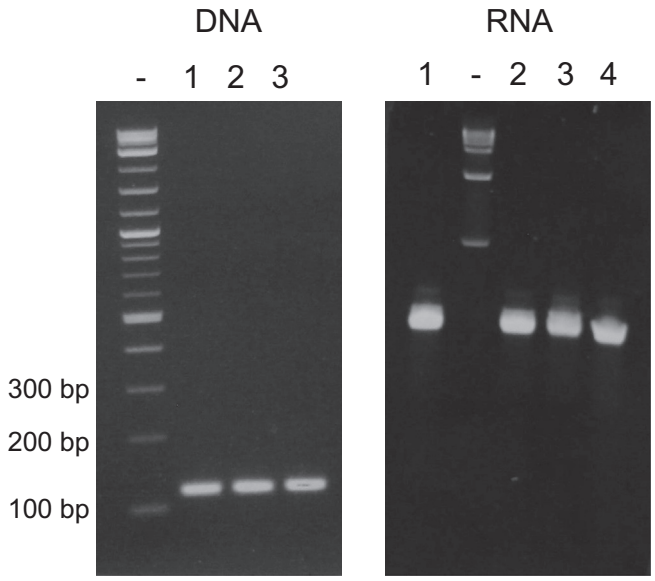

Fig. 3. Electrophoretic analysis of sgRNA transcription templates in a 2% agarose gel (left) and RNA transcripts in a 10% polyacrylamide TBE-urea gel (right). The numbers above the lanes identify different sgRNAs; – indicates marker lanes.

3′ homology arm (~30–40 bp), measured from the sgRNA cut site (3–4 nt away from the PAM within the protospacer region). Once the sequence has been designed, the donor can be purchased commercially. We purchase our HDR donors as DNA Ultramers from IDT, which are affordable and of good quality. The donor is dissolved in sterile nuclease-free water to 100 μM before use.

2.4. RNP assembly and electroporation

The RNP complex is produced simply by mixing Cas9 and one or more sgRNAs in an appropriate buffer. To avoid solubility problems that arise if there is an excess of protein over RNA during mixing, we use a final 1.2- to 1.5-fold molar excess of sgRNA and add Cas9 to the sgRNA slowly with manual stirring [6]. This is typically done with 100 pmol of Cas9 and 120–150 pmol of sgRNA in a total of 10 μl of Cas9 buffer [6]. For HDR editing, 100 pmol ssDNA HDR donor is added at this step. This mixture is incubated for 5 min at room temperature before electroporation.

We use the Lonza 4D Nucleofector to electroporate mammalian cells. Other laboratories have successfully used electroporators from other manufacturers (*e.g.*, Harvard BTX and Life Technologies Neon). Lonza has optimized the composition of their reagents and pulse protocols for a wide variety of cell types. For electroporation, a cell pellet (typically 2×10^5 cells) is resuspended in 20 μl of the appropriate solution: Solution SF for HEK293T and K562 cells, Solution SE for Jurkat cells, and Solution P3 for human CD34+ HSPCs. The 10 μl RNP solution is added to the cells (generating a 3.3 μM final RNP concentration), and the mixture is transferred to an electroporation cuvette. Electroporation programs are specified by the manufacturer for various cell types. After electroporation, cells are returned to culture for an appropriate time before analysis, 48 h for genomic analysis, and 5–10 days for alterations in protein expression. For established cell lines, we incubate the electroporation reaction for 5–15 min after electroporation before transfer to culture.

In our laboratory, we exclusively rely on electroporation to deliver Cas9 RNP to mammalian cells. However, other groups have reported successful delivery via cationic lipid transfection [24]. The Cas9 RNP may also be amenable to delivery within a nanoparticle, which may enable *in vivo* delivery of the Cas9 RNP to selected tissues [25].

2.5. T7 endonuclease (T7E1) assay

For routine analysis of the levels of editing, the frequency of indel mutations can be estimated by T7 endonuclease I (T7E1) digest. This requires three steps: genomic DNA extraction, PCR amplification of the edited region, and digestion with T7E1. We extract genomic DNA from cultured cells with Quick Extract solution (Epicentre Genomics). 5×10^5 edited cells should yield 3–5 μg of genomic DNA (3.2 pg per haploid genome). Commonly used column-based genomic extraction kits, such as the Blood and Tissue Kit (Qiagen) or the Nucleospin kit (Machery-Nagel) can also be used, although purified DNA is not required for PCR. The targeted region is amplified by PCR using appropriate primers to generate an amplicon containing the targeted region, ideally <1000 bp. If a donor template has been used, care should be taken to not amplify with primers that can anneal to the donor, since that can greatly confound the results (see Fig. 5 and below). We use 50–100 ng of genomic DNA in a 50 μl reaction and amplify using PrimeSTAR GXL polymerase (Takara Clontech, Inc.), following manufacturer's instructions. Other polymerase systems can be used, such as NEB Phusion or Q5, Invitrogen Platinum Taq, or Applied Biosystems Amplitaq. The cycling conditions depend on the primers used. For a typical target, we amplify for 30 cycles. Over-amplification of PCR products can result in unwanted side products.

An aliquot (200–500 ng) of the resulting PCR product is mixed with water and NEB buffer 2. This is subjected to a denaturation/annealing protocol to generate heteroduplexes between wild-type and indel-containing (edited) DNA. 10 μl is removed to serve as an undigested sample. To the remainder, 1 μl of T7 Endonuclease (NEB, 10 u/μl) is added and the sample is incubated for 15 min at 37°. The digested and undigested samples are run in adjacent lanes in a 2% agarose gel or a 10% polyacrylamide gel (Fig. 4). The polyacrylamide gel shows heteroduplexes in the undigested samples running as bands above homoduplexes (Fig. 4A). In the less dense agarose gel matrix, they are not resolved from the homoduplexes (Fig. 4B). T7E1 digestion is the most commonly employed method for estimating the rates of gene editing, and is well-suited to routine applications, such as sgRNA optimization [20]. The TIDE method, which is based on deconvolution of Sanger sequences of mixed PCR products using an on-line analysis tool, is also popular [26].

2.6. Analysis of HDR by restriction digest or droplet digital PCR

The T7E1 assay reports qualitatively on the amount of editing at the target, but not on their identities. If a DNA template for HDR is included in the experiment, it will contribute to those sequence changes. The level of HDR can be assessed in the PCR product from the preceding section, if it generates a specific signature – *e.g.*, a novel restriction site. In this case the level of HDR can be estimated by simply digesting the PCR product with the restriction enzyme using standard methods, along with suitable positive and negative controls (*e.g.*, IDT gBlocks for the edited and unedited alleles).

We have also used two-color droplet digital PCR (ddPCR) to quantify HDR [20]. ddPCR offers reliable quantitation down to 1% of alleles. To design a ddPCR assay, reliable qPCR primers must developed and tested that amplify the edited region. Next, pairs of FAM- and HEX-labeled probes to the unedited and edited sequences, respectively, should be tested at small scale. We use IDT g-blocks matching the unedited and edited sequences as standards for optimization. Once a primer and probe set have been developed on standard samples, the same assay can be used to

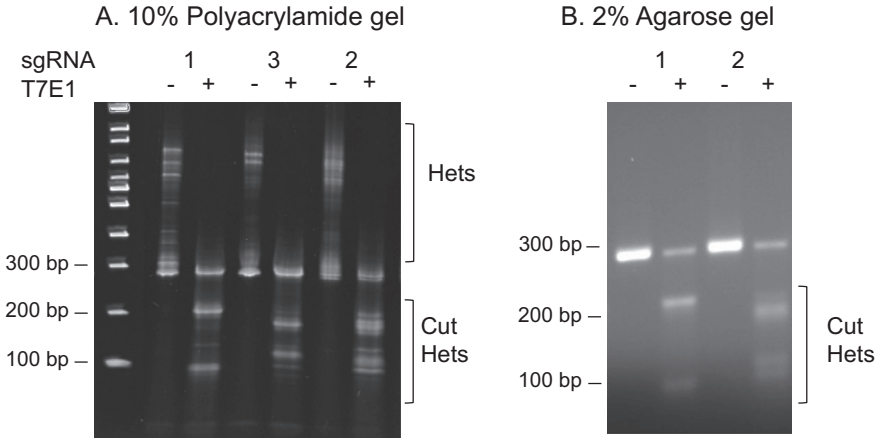

Fig. 4. Analysis of a T7 endonuclease assay on a 10% polyacrylamide TBE-urea gel (A) and a 2% agarose gel (B). After denaturation and annealing, pairs of samples were run without (–) or with (+) T7 endonuclease (T7E1) treatment. The numbers indicate the identities of the sgRNAs; marker lanes are unlabeled.

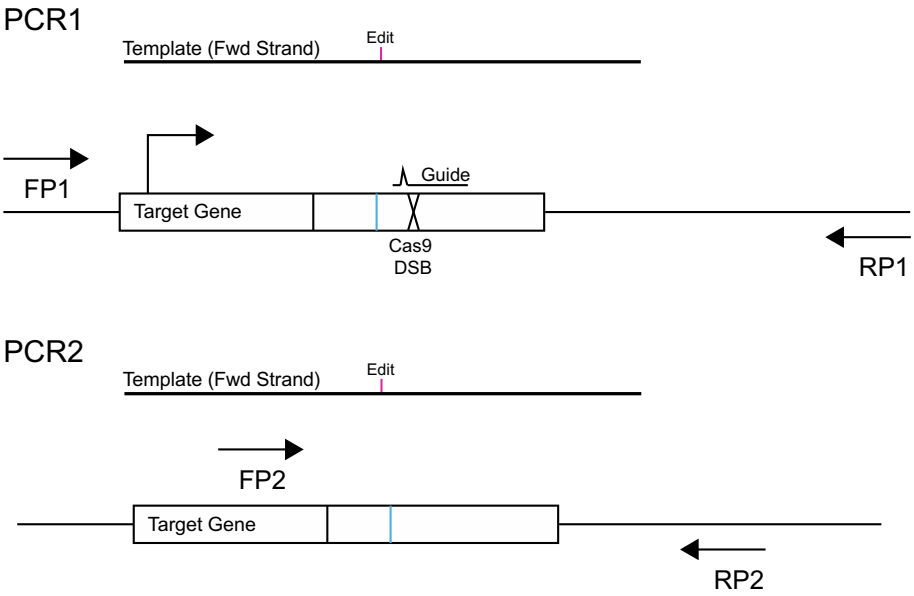

Fig. 5. Analysis of edited cells by next-generation sequencing. Preparation of PCR amplicons for sequencing. When a single-stranded donor DNA is used, no primer should anneal to the donor, so the first PCR will amplify outside the region matching the donor. The second PCR should not have many cycles to avoid over-amplification artifacts. The resulting PCR product can be sequenced using paired-end sequencing to achieve exceptional limits of detection (~0.01% in many cases).

assess editing in genomic DNA extracted from edited cells, with no modification. The extensive optimization and higher cost of ddPCR (compared to qPCR) make this best-suited to precision analysis of many samples, all edited at the same target.

2.7. Analysis by next generation sequencing

In principle, determining the sequences of all genomic targets in a population of cells will reveal the complete range of Cas9-mediated sequence modifications and their abundance. In practice, the choice of PCR primers to amplify the target and the length of sequence reads will lead to missing some products, such as large deletions and translocations. Still, deep sequencing provides far more detail about editing outcomes than the other procedures mentioned above. Therefore, we ultimately rely on next-generation sequencing to confirm our editing results.

We prepare amplicons for next generation sequencing by two rounds of PCR, followed by Illumina library preparation (Fig. 5). We begin with an initial purified PCR product (identical to that used above for T7 endonuclease digest). We purify this PCR product using SPRI beads (1.8X ratio of beads to PCR according to the manufacturer's instructions), followed by quantification using a Qubit fluorimeter and the DNA HS kit. We next perform a second PCR to generate a smaller amplicon compatible with Illumina sequencing, typically 150–200 bp. If a DNA template for HDR has been used, ensure that the forward primer cannot anneal to the template. Avoid using too many PCR cycles (fewer than 10) for the second PCR, to avoid artifacts due to over-amplification. The resulting products are prepared for sequencing using the Illumina TruSeq DNA library preparation kit and manufacturer's instructions. Kits from other manufacturers work as well. Since the PCR amplicons generated by PrimeSTAR GXL are blunt-ended, we start NGS library preparation with the adenylation step.

Sequencing is done on an Illumina MiSeq instrument, with paired-end reads (we typically use 2 × 150 cycles, but 2 × 250 kits are now available). Ideally, the reads from each end can be compared to limit the contribution of sequencing errors. To analyze the resulting data, we constructed a custom analysis pipeline, but

online tools are available, such as CRISPResso [27]. Following the above guidelines, we achieve excellent sensitivity: indel frequencies down to 0.01% can be detected, and single-nucleotide substitutions by HDR can be detected down to 0.05% [20,28].

3. Some results with RNP delivery

To test the efficacy of RNP delivery in cultured human cells, we created a reporter line carrying a single-copy BFP gene in HEK293T cells using a lentiviral vector and isolated stable transformants [10]. This vector is available on addgene. The cell line was generated using standard lentiviral transduction methods, followed by single-cell cloning, and selection of a clone with stable expression of BFP by FACS. The difference in coding sequence between BFP and GFP is a single codon at position 67: CAT (His) in BFP, TAC (Tyr) in GFP (Fig. 6A). We made an sgRNA to target a Cas9 cut one bp from that codon and generated the corresponding RNP. The complex was introduced into the BFP + cells by electroporation, along with a 196-nt single-stranded DNA donor (ssODN) carrying the GFP codon and a change designed to disrupt the PAM. After 6 days in culture to allow for alterations in protein expression, the edited cells were sorted by FACS for blue and green fluorescence (Fig. 6B). Before electroporation, 94% of the cells were BFP+; after treatment, only 1% remained BFP+, 25% had been converted to GFP+ via HDR from the ssODN template, and 74% showed no fluorescence, indicating BFP knockout by NHEJ. Thus, both delivery to the cells and induced cleavage were very efficient, and the level of HDR was quite high.

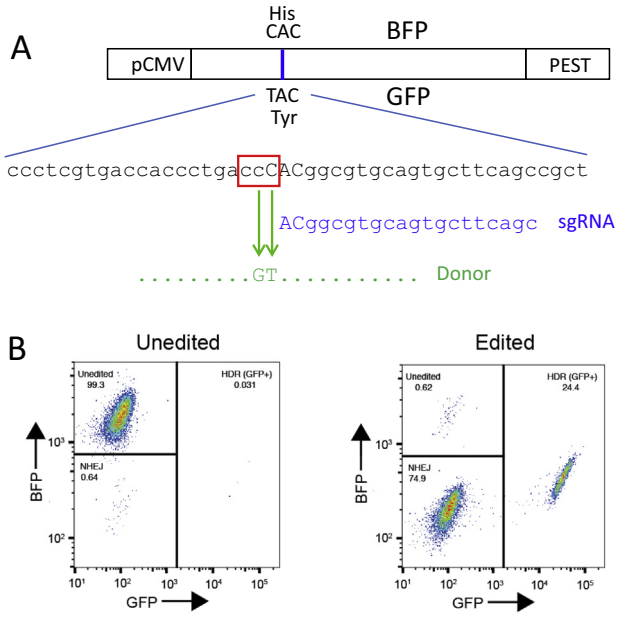

Fig. 6. Experiment illustrating the efficiency of the Cas9 RNP-mediated NHEJ and HDR at a BFP transgene. (A) Diagram of the BFP gene driven by a eF1a promoter. The sequence of the BFP target is shown in black; the PAM is in a red box and the expected site of Cas9 cleavage is indicated with a red arrow. The guide sequence of the sgRNA targeting BFP is in blue; the changes encoded in the single-stranded donor DNA in green. The changes are roughly centered in the 196-nt donor, and they serve to destroy the PAM, as well as convert BFP to GFP. (B) FACS analysis of 293T cells before and 6 days after electroporation with the Cas9 RNP and donor DNA.

In another application, we achieved levels of HDR and NHEJ almost as high at the sickle cell disease site at the adult β-globin locus (HBB) in the K562 erythroleukemia cell line (Fig. 7A) and in primary human HSPCs (hematopoietic stem and progenitor cells, as determined by next-generation sequencing (Fig. 7B) [20].

The efficiency of delivery and the balance between HDR and NHEJ varies among cell types. As an illustration of this issue, efficiencies of HDR in mixed HSPCs are considerably higher than in the long-term repopulating stem cells that persisted in immunocompromised mice [20,23]. Schumann et al. reported ~20% HDR in primary human T cells [29]. Lin et al. found levels of HDR up to ~35% at several loci in HEK293T cells, but much lower levels in other cell types [6,30]. By optimizing various parameters, Richardson et al. [10] and Liang et al. [9,31] were able to push HDR frequencies a bit higher, but only in established cell lines.

4. Conclusions

RNP delivery has several advantages over alternative methods for introducing CRISPR reagents into cells. As described here, once the Cas9 protein is available, the method involves no cloning and can be completed to the point of initial analysis in a matter of days. It is applicable to a wide range of cell types in culture, including primary cells and stem cells, although the detailed outcomes will vary depending on the inherent repair capabilities of the cells. In each of these contexts the hit-and-run character of the method avoids long-term expression of the nuclease, which has been shown to reduce off-target effects. The RNP can be co-delivered or sequentially delivered with donor DNAs of any chosen type, including single-stranded DNA (ssODNs), plasmid DNAs, PCR prod-

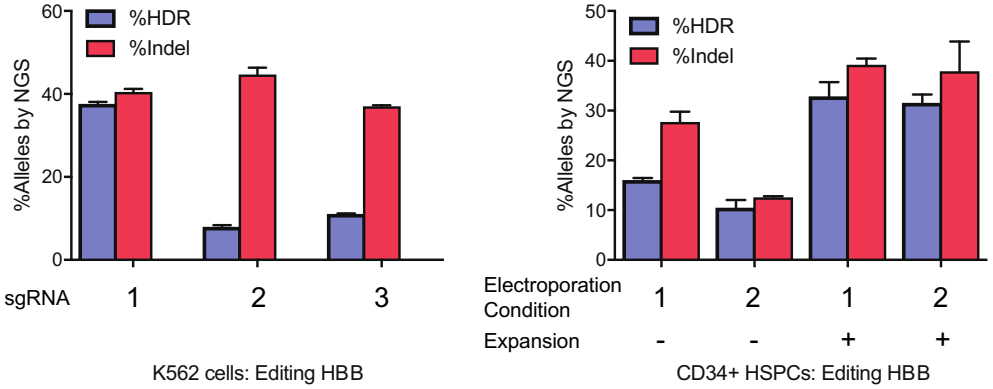

Fig. 7. Representative next-generation sequencing results of cells edited with the Cas9 RNP and a single-stranded donor DNA, based on data published in DeWitt et al. [20]. A) Sequencing results depicting editing K562 erythroleukemia cells at the adult β-globin gene (HBB) using three different sgRNAs. B) Sequencing results depicting editing of human CD34+ hematopoietic stem/progenitor cells at HBB using two different electroporation protocols and the optimal sgRNA discovered in K562 cells, before and after expansion in erythroid differentiation conditions.

ucts, or viral genomes. Experience in many labs has shown the method to yield both indels and HDR changes at levels as high or higher than other approaches.

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