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Comparative Analysis of Lipid-Mediated CRISPR-Cas9 Genome Editing Techniques

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Abstract

CRISPR-Cas technology has revolutionized genome engineering. While Cas9 was not the first programmable endonuclease identified, its simplicity of use has driven widespread adoption in a short period of time. While CRISPR-Cas genome editing holds enormous potential for clinical applications, its use in laboratory settings for genotype-phenotype studies and genome-wide screens has led to breakthroughs in the understanding of many molecular pathways. Numerous protocols have been described for introducing CRISPR-Cas components into cells, and here we sought to simplify and optimize a protocol for genome editing using readily available and inexpensive tools. We compared plasmid, ribonucleoprotein (RNP), and RNA transfection to determine which was method was most optimal for editing cells in a laboratory setting. We limited our comparison to lipofection-mediated introduction because the reagents are widely available. To facilitate optimization, we developed a novel reporter assay to measure gene disruption and the introduction of a variety of exogenous DNA tags. Each method efficiently disrupted endogenous genes and was able to stimulate the introduction of foreign DNA at specific sites, albeit to varying efficiencies. RNP transfection produced the highest level of gene disruption and was the most rapid and efficient method overall. Finally, we show that very short homology arms of 30 base pairs can mediate site-specific editing. The methods described here should broaden the accessibility of RNP-mediated lipofection for laboratory genome-editing experiments.

Keywords

DNA damage/repair; Molecular genetics/cloning; Oxidative Stress

Key phrase

Cas9; CRISPR; Genome editing

1. Introduction

Programmable site-specific endonucleases have transformed genomic research (Barrangou, 2014). Traditionally, the functional characterization of proteins and RNAs was accomplished by over expression or knocking-down expression of a desired gene. The ability to modify

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genes in their genomic context permits studying their function under physiologic conditions. Programmable endonucleases, such as Zinc-finger nucleases (ZNFs)(Bibikova et al, 2001, Kim et al, 1996), transcription activator-like effector nucleases (TALENs)(Boch et al, 2009, Miller et al, 2011, Moscou et al, 2009), and the RNA-guided nuclease CRISPR associated protein 9 (Cas9)(Cong et al, 2013, Jinek et al, 2012) introduce double strand breaks that are repaired by one of two highly conserved DNA repair pathways; non-homologous end joining (NHEJ) or homology directed repair (HDR)(Chapman et al, 2012). Non-homologous end joining often results in small deletions or insertions that can disrupt the coding sequence of targeted genes (Chang et al, 2017). Homology directed repair is much less error prone and can be used to promote homologous recombination at desired locations to enable genome editing (Bibikova et al, 2001, Jasin et al, 2013). The ability to edit genomes has exciting implications for both research and clinical interventions for genetic diseases.

The clustered regularly interspaced short palindromic repeats (CRISPR) and associated RNAs and proteins (CRISPR-Cas) constitute an adaptive immune mechanism present in bacteria and archaea (Barrangou et al, 2007, Makarova et al, 2011, Makarova et al, 2015). This system enables bacteria to incorporate small pieces of invading viral genomes into their own genome rendering the bacteria resistant to future infection (Barrangou et al, 2007). The CRISPR-Cas system is similar to ZNFs and TALENs in that an endonuclease can be directed to specific locations in the genome, however the Cas system relies on guide RNAs (gRNAs) to direct the nuclease to its intended target (Deltcheva et al, 2011). Further simplification of the type II CRISPR-Cas system from *Streptococcus pyogenes* and adaptation to eukaryotic cells has driven its widespread adoption and adaptation for many uses in eukaryotic cells from diverse species (Bhaya et al, 2011, Sander et al, 2014, Terns et al, 2011).

A number of methods have been described for introduction of Cas9 and gRNAs into cells. Transfection, electroporation, and microinjection of plasmids or RNA encoding Cas9 and gRNAs have been reported to induce genome modification (Kim et al, 2014, Mali et al, 2013, Wang et al, 2013). In addition, Cas9 and gRNAs have been introduced as ribonucleoproteins (RNPs) using similar methods (Kim et al, 2014, Liang et al, 2017). Cas9 Lentiviral vectors are available for generation of cell lines stably expressing Cas9 (Cong et al, 2013) and transgenic animals, cell lines, and induced pluripotent stem cells that express Cas9 under the control of conditional promoters have been created (Bisht et al, 2017, Dow et al, 2015, Wang et al, 2017). Each of the methods described above has advantages and disadvantages associated with its use. For example, while lentiviral vectors are efficient transducers of a wide variety of cell lines, cells infected with a lentivirus will express Cas9 and gRNAs indefinitely, which has the potential to contribute to non-specific effects or insertional mutagenesis. Furthermore, some of the above methods require specialized equipment or expertise (e.g., microinjection). The specific methods selected to introduce CRISPR-Cas components will likely depend on the target cell and goal of the experiment.

The generation of knockout cell lines and introduction of exogenous sequences at specific loci are rapidly becoming common approaches used to investigating the role of specific proteins (Roy et al, 2015). Given the simplicity of generating of CRISPR-Cas components and their importance in exploring genotype-phenotype relationships, we compared

conditions and methods for introduction of genome editing components into cell lines. We elected to not use lentiviral-mediated delivery, due to the additional biosafety requirements, or methods that require specialized equipment (i.e., nucleofector), because they not universally available. We chose to compare plasmid and ribonucleoprotein (RNP) transfection into two commonly used cell lines. We also examined the efficiency of gRNA transfections into cells stably expressing Cas9, acknowledging that this approach may not be useful in a clinical context. We developed a novel reporter system for detecting HDR and optimized conditions for genome editing. In contrast to previously developed EGFP-based reporter systems (Bialk et al, 2015, Richardson et al, 2016, Rivera-Torres et al, 2017), the reporter described here allows for the introduction of various exogenous sequences while still expressing EGFP exclusively with successful locus modification. We also found that short homology arms could be used in single- and double-stranded donors templates as others have shown (Bialk et al, 2015, Bialk et al, 2016, Liang et al, 2017). In the experimental conditions utilized here, our findings suggest that RNP transfection may be the most rapid and efficient method of gene disruption and genome editing.

2. Materials and Methods

2.1 Plasmid Construction

pSpCas9(BB)-2A-Puro (PX459) V2.0, a gift from Feng Zhang (Addgene #62988), was used to construct Cas9 and gRNA expressing vectors according to previous published protocols (Ran et al, 2013). The lentiCas9-blast plasmid, a gift from Feng Zhang (Addgene #52962), was used to stably express Cas9 in cell lines according to previous published protocols (Sanjana et al, 2014). pCDNA5/FRT-EGFP and – ATG-EGFP plasmids were constructed using Gibson Assemblies following standard procedures. A puromycin-expression plasmid was generated by cloning the puromycin *N*-acetyl-transferase gene in an SV40-expression cassette into the pBluescript II KS(+) vector. See supplementary table 1 for a list of all oligonucleotides used in this paper.

2.2 Cell Culture and Transfection

HeLa cells were purchased from the ATCC (Cat #CCL-2). HEK 293 Flp-In cells were purchased from Invitrogen. All cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 0.29 mg/ml L-Glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). Cells were grown in a 37°C humidified incubator with 5% CO₂. HEK 293 Flp-In cells were transfected with the EGFP or ATG-EGFP plasmids and a plasmid encoding FLP recombinase according to manufacturer's protocols and selected with hygromycin. Cas9 stable cell lines were generated by infecting cells with a Cas9-expressing lentivirus (Addgene plasmid #52962) followed by isolation of individual clones of cells stably expressing high levels of Cas9. All px459v2, RNP, and gRNA transfections were done in 24 well plates using 1 µL of Lipofectamine 2000 (Invitrogen) as the transfection reagent. Plasmid transfections were done using 0.5 µg of plasmid. RNP transfections used 250 ng pBS-Pur, 100 ng gRNA and 0.5 µg of Cas9 purified protein. Cas9 and the gRNA were incubated prior to the addition of pBS-pur and donor template for 5 minutes at room temperature. In RNA only transfections, 250 ng pBS-Pur and 100 ng of gRNA were used. For knock-in experiments, we optimized

conditions for the highest efficiency of knock-in using each method (not shown). The amount of donor template used for each method are as follows; 500 ng for plasmid transfections, 50 ng for RNP transfections, and 10 ng for gRNA only transfection. 24 hours after transfection 2 µg/ml of puromycin was added to the cells. The cells were incubated in puromycin for 48 hours after which the selection reagent was removed from the cells. Individual clones of cells were isolated by limiting dilution.

2.3 Western blots, screening, and sequencing of modified clones

Western blots were carried out using standard procedures. Briefly, 20–40µg of cell lysate was separated on a 4–12% SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blotted with Flag (M2; Sigma), GFP (Aves Laboratories), KEAP1 (Cell Signaling Technologies), GAPDH (Santa Cruz Biotechnology), and secondary antibodies (Licor) and imaged on an infrared scanner (Licor). Genomic DNA was isolated from individual clones by suspending cells in lysis buffer (300µl; 10mM Tris-HCL, 25mM EDTA, 0.5% SDS, pH 8), precipitating protein by addition of salt (100µl; 5M Ammonium Acetate), and extraction of DNA with ethanol. PCR was performed using primers flanking the Cas9 cut site and amplicons were cloned using zero-blunt cloning according to the manufacture's protocol (Invitrogen). Several colonies from each cloned amplicon were Sanger sequenced to characterize individual mutations.

2.4 Flow Cytometry and microscopy

Cells were trypsinized and then neutralized using DMEM containing 10% FBS. Cells were washed and resuspended in PBS containing 2% FBS. The percentage of cells expressing EGFP was assessed using a BD Accuri Flow cytometer. Viable cells were gated using morphology and EGFP percentages of the viable cells were measured. Imaging of fluorescent proteins was performed on an Olympus IX70 inverted microscope equipped with a MicroFire Color camera (Olympus) and 20× objective (numeric aperture 0.4).

2.5 Donor Template Creation

Single stranded donors for ATG and ATG-FLAG were synthesized by Integrated DNA Technologies with 30 base pairs of homology on the 5' and 3' ends. Double stranded template for ATG-RFP knock in was generated by PCR amplification and incorporated a short linker on the C-terminus of RFP and 35 base pairs of homology on either end.

2.6 Protein purification

Cas9 was expressed in BL21(DE3) cells using pET-28b-Cas9-His, a gift from Alex Schier (Addgene plasmid # 47327)(Gagnon et al, 2014). Following overnight induction with IPTG at 18°C, bacterial were harvested and lysed in lysis buffer (20mM Tris pH 8, 500mM NaCl, and protease inhibitors [Sigma]). Bacteria were lysed using sonication or high-pressure microfluidic shearing, and the lysate was centrifuged at 15,000g for 20 minutes. Clarified supernatants were mixed with 2ml of nickel resin (BioRad) and mixed for 1 hour at 4°C. The suspension was poured into a gravity column and washed with wash buffer (20mM Tris pH8, 50mM NaCl, 25mM imidazole). Cas9 was eluted (20mM Tris pH 8, 500mM NaCl,

250mM Imidazole) and dialyzed into storage buffer (20mM Tris pH 8, 500mM KCL, 10mM MgCl₂, 50% glycerol).

2.7 In Vitro Transcription and In Vitro Cutting with Cas9

We constructed a primer containing a T7 transcription site as well as a gRNA sequence (see supplemental table of primers). We used the plasmid pSpCas9(BB)-2A-Puro as a template for PCR to create a product with a T7 transcriptional start site, gRNA sequence, and gRNA scaffold. *In vitro* transcription was performed with a MEGAshortscript T7 Transcription kit (Invitrogen) per the manufacturer's protocol. RNA was isolated using a phenol chloroform extraction and diluted in water to 1µg/µl. The RNA was then stored in aliquots at -80°C. To test the functionality of the RNP we performed an *in vitro* digestion with 200 ng target DNA, 100 ng gRNA, 1µl BSA (1 mg/ml, NEB), 1µl NEB buffer 3, .25–1 µg Cas9, and water assembled into a 10 µl reaction. This mixture was incubated at 37°C for 1 hour followed by a 10-minute incubation at 65°C. Digestion products were separated on a .75% agarose gel.

2.8 Hydrogen peroxide challenge

KEAPI-targeted and parental HeLa cells were cultured in a 96-well plate and exposed to increasing concentrations of H₂O₂ (0–250 µM) for 24 hours. Following exposure, cell viability was measured using an 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) using previously described methods (van de Loosdrecht et al, 1994).

2.9 Data analysis and statistics

We used GraphPad Prism v7 for analysis and generation of graphs. Means were compared using Student's *t*-test. *P* values < 0.05 were considered significant and all values shown are two-sided.

3. Results

3.1 Comparison of methods for Cas9-mediated gene disruption

We sought to compare the various methods of Cas9 and gRNA introduction into cells to determine optimal conditions for gene disruption by non-homologous end joining (NHEJ). We chose to focus on cationic lipid-mediated transfection (lipofection hereafter) methods as the reagents are widely available and do not require specialized equipment. We tested various platforms for the introduction of Cas9 and gRNA including transfection of 1) a single plasmid expressing both Cas9 and gRNA, 2) Cas9 protein and *in vitro* transcribed RNA preassembled into a ribonucleoprotein (RNP) transfection, and 3) gRNA transfection into cells stably expressing Cas9 protein. To measure the efficiency of gene disruption, we created a novel reporter system in HEK 293 cells. Using the FRT site, we introduced a single copy of EGFP creating an isogenic HEK 293 cell line stably expressing a single copy of EGFP (293-GFP) or EGFP and Cas9 (293-GFP-Cas9; Figure 1A). Prior to transfection of RNPs, we functionally tested them by digesting target sequences *in vitro* (Figure 1B). We tested various commercially available lipofection reagents including Lipofectamine 2000, Lipofectamine CRISPRMAX, and Lipofectamine RNAiMAX and obtained the most favorable results using Lipofectamine 2000 (data not shown). Cells were co-transfected with a plasmid expressing puromycin *N*-acetyl-transferase (PAC) for selection of cells that had

been successfully transfected (the Cas9 plasmid co-expresses Cas9 and PAC genes separated by the self-cleaving 2A peptide). We optimized conditions for each transfection and measured the efficiency of EGFP disruption five days post transfection. All of the methods tested produced some fraction of cells in which GFP was disrupted; however, RNP transfection was the most efficient method of disrupting the target gene (Figure 1C–D). In all cases, selecting cells with puromycin increased the fraction of cells with successful gene-disruptions, suggesting that transfection efficiency is a significant limiting factor to editing cells *in vitro*. Given the simplicity of introduction and the high level of gene disruption, we selected RNP transfection of Cas9/gRNA for further analyses.

3.2 Efficient disruption of endogenous genes using RNP transfection

We tested whether RNP transfection could efficiently disrupt endogenous genes at other loci. We selected two genes for targeting; the catalytic component of telomerase, telomerase reverse transcriptase (*TERT*), and kelch like ECH associated protein 1 (*KEAP1*), and designed 1–2 gRNAs per gene. We used the Broad GPP portal design tool to select appropriate gRNAs (<http://portals.broadinstitute.org/gpp/public/>) and targeted the coding region of genes biased toward the 5' end of the coding sequence or regions of known functional importance (Doench et al, 2016). gRNAs were tested *in vitro* prior to transfections (Figure 2A–B). We focused our analysis on HeLa and HEK 293 cells as these are widely used. In each case, cells were transfected with RNPs directed to the gene of interest and a selection plasmid to enrich for transfected cells. Following selection, cells were cloned by limiting dilution and individual lines (~10) were expanded. Genomic DNA was isolated from each clone and the region around the Cas9 target site was amplified and examined for evidence of length heterogeneity (Figure 2C). Any PCR product that appeared to have multiple bands was sub-cloned so that individual alleles could be sequenced. We identified small insertions or deletions in a majority of all isolated clones sequenced (Figure 2D). Interestingly, we were unable to disrupt *KEAP1* in HeLa cells by RNP transfection despite numerous attempts and turned to plasmid transfection approaches. Clones containing mutant *KEAP1* were readily isolated from plasmid transfection (Figure 2E–G) and western blots confirmed absence of the protein (Figure 2F). *KEAP1* is a key regulator of oxidative stress response system by binding and promoting the proteasomal degradation of nuclear factor (erythroid-derived 2)-like 2 (NRF2) (Hayes et al, 2001, Katoh et al, 2005, Sihvola et al, 2017). Accumulation of NRF2 promotes the increased expression of key antioxidant and phase II detoxification enzymes yielding a cytoprotective effect against oxidants. Disruption of *KEAP1* is predicted to render cells resistant to oxidative stress due to constitutive NRF2 activation. We functionally tested cells that we had targeted *KEAP1* disruption by challenging cells with hydrogen peroxide (H₂O₂). *KEAP1*-targeted cells showed an increased resistance to the cytotoxic effects of increasing concentrations of H₂O₂, demonstrating that *KEAP1* function had been disrupted (Figure 2H).

3.3 Comparison of methods for Cas9-mediated genome editing

Many experiments require site-specific modification to test the consequences of specific nucleotide changes. We therefore compared three methods of introducing Cas9 and a gRNA into cells using lipofection. Transfection of a plasmid encoding both components, Cas9 and gRNA as preassembled RNP, and an *in vitro* transcribed gRNA into cells stably expressing

Cas9 were compared for their ability to introduce a three nucleotide insertion into a specific loci. We modified our above system by removing the ATG start codon from EGFP and introducing this construct into the Flp-In locus of HEK 293-Flp-In cells. These cells are GFP-negative, but become GFP-positive if an ATG sequence is knocked-in at the 5' end of the truncated GFP (Figure 3A–B). We designed a donor single stranded oligonucleotide (ssDNA) that consisted of an ATG flanked by 30 base pairs of homology on either side. 30 base pairs of homology were selected based on recent findings that single stranded templates of 72 base pairs worked well in cell lines (Bialk et al, 2015, Bialk et al, 2016). We selected a new gRNA sequence that directed Cas9 to cut the target DNA 5 base pairs from the ATG insertion site to stimulate recombination at this site with our donor template (supplementary table 1). We validated that our selected gRNA was functional *in vitro* (Figure 3B) and transfected HEK 293 Flp-In ATG EGFP cells with donor DNA plus plasmid or RNP and HEK 293 Flp-In ATG EGFP Cas9-blast cells with donor DNA plus *in vitro* transcribed gRNA under optimal conditions (optimization not shown). Similar to our previous experiments, we included a plasmid encoding resistance to puromycin for selection of cells that were successfully transfected. Very few cells survived puromycin selection after transfection with gRNA and puromycin plasmid, suggesting that the presence of significant amounts of RNA may decrease transfection efficiency of plasmid transfection. Since very few cells survived, these cells were left to proliferate for 7 days prior to measuring the fraction of GFP-positive cells by flow cytometry. All methods induced a significant number of modified cells (Figure 3C–D) with RNA transfection resulting in the highest fraction of modified cells. However, given that few cells survived following selection with puromycin, RNA transfection may not be as compatible with co-transfection of a selectable marker.

3.4 Introduction of large tags with short homology arms using RNP transfection

To examine if larger tags could be introduced at our reporter locus, we examined the efficiency of knocking in epitope- and fluorescent protein-tags (Figure 4A). The ATG EGFP reporter system has a distinct advantage over previously used mutant EGFP cell lines (Bialk et al, 2015, Richardson et al, 2016, Rivera-Torres et al, 2017) in that we are able to knock in a variety of tags as opposed to only correcting the mutated base pair. In each case, the insert was flanked by 30–35 base pairs of homology on each side of the targeted locus. ATG- and ATG-FLAG-donors were ssDNA, whereas the ATG-RFP donor was generated by PCR and was therefore double stranded DNA. In each case, cells were transfected with an RNP, donor template, and puromycin selection cassette. Modified GFP-positive cells were readily detected in each case, however, as the size of the insert increased, the efficiency of editing decreased (Figure 4B). Interestingly, when cells were treated with RNP alone, a band corresponding to GFP was visible by western blot (Figure 4C), potentially due to formation of start codon by mutation or small deletion. Indeed, we noted that an 8 base pair deletion at the putative Cas9 cut site would generate an in-frame start codon. In cells targeted with an ATG-RFP donor, all RFP-positive cells were GFP positive, but we did identify a significant fraction of GFP only cells (Figure 4D) suggesting that a significant number of cells had taken up the RNP, but had not undergone HDR and incorporated donor sequence. These data suggest that both ssDNA and dsDNA templates with short homology arms can be used to insert tags into the genome, however the frequency of editing will likely decrease as the insert size increases.

4.0 Discussion

Since its adaptation to eukaryotic cells (Cong et al, 2013), the number of applications and uses of the CRISPR-Cas system have expanded to a wide array of tools to knock-out, modify expression, or visualize specific loci within cells. The rapid adoption of this technique is likely driven by its relative simplicity (only two components), rapid generation time of targeting components, and relative cost effectiveness. Numerous protocols and methods have been reported to modify various cell types and here we sought to identify an inexpensive, rapid, and simple method for modifying cells. We developed a novel reporter system to rapidly evaluate gene disruption and a system that is useful for examining the efficiency of larger knock-ins. By limiting our approach to lipid mediated transfection, CRISPR-Cas components can be delivered to a wide range of cell types at a wide range of scales. Furthermore, inexpensive alternatives to commercial lipid-mediated transfection reagents are available (e.g. Polyethylenimine), further making this method widely applicable (Hsu et al, 2012). We showed that introduction of Cas9 and gRNA in all forms was effective at disrupting targeted genes. Our data suggest that transfection efficiency significantly affects the fraction of gene edited cells and that including a selectable marker to exclude non-transfected cells will significantly increase the fraction of targeted cells. RNP and RNA transfection were superior to plasmid transfection for the introduction of exogenous DNA. While all forms were effective at creating modified cells, in our experiments, RNP lipofection was the most rapid and efficient overall.

RNP transfection has a number of advantages over plasmid or RNA transfection. First, the generation of an RNP is rapid and does not require cloning. Once oligonucleotides for the generation of the gRNA have been obtained, RNPs can be assembled within hours, whereas standard oligonucleotide cloning requires a minimum of 3–4 days. Second, RNP transfections are economical. A single preparation of Cas9 protein is sufficient for thousands of transfections. The remaining reagents are generally available and affordable. Third, RNPs can be tested functionally *in vitro* prior to transfection. A target DNA is required but can usually be generated simultaneously with the RNP. We typically obtain PCR oligonucleotides that flank the target site and generate products ranging from 300–1000 base pairs. Verifying that the RNP is functional and will cut the target DNA prior to transfection increases the likelihood of experimental success. Finally, linear double- or single-stranded DNA is often used as a donor template to stimulate homologous recombination or gene conversion. The half-life of these DNAs is short within in cells, on the order of 50–90 minutes (Lechardeur et al, 1999, Woolf et al, 1990). Delivering a pre-assembled functional RNP will generate double strand breaks faster than any other method and will increase the probability that template DNA is still present in the cell while repair is taking place.

The selection of specific gRNA may determine which transfection method is most appropriate. Unexpectedly, we were unable to disrupt the *KEAPI* gene in human cells using RNP transfection after several attempts. However, *KEAPI*-disrupted clones were more readily generated from cells transfected with plasmids encoding Cas9 and the same gRNA. These results are potentially due to differences in gRNA stability. Recent studies have shown that the sequence content of gRNAs can affect both transcription and stability of the gRNA in cells (Haeussler et al, 2016, Moreno-Mateos et al, 2015). Potentially, the gRNA we

designed was degraded rapidly in cells, yet when expressed from the strong U6 promoter, it was able to accumulate. A recently developed gRNA design tool, CRISPOR, scores each gRNA based on delivery method, RNP or plasmid, and takes into account gRNA transcription efficiency and stability (Haeussler et al, 2016). Selection of RNP or plasmid transfection reagent will likely depend on the specific cells type and setting of each experiment.

Finally, our data suggest that large inserts can be introduced with short homology arms to specific sites within the genome. Effective knock in was found using both single stranded and double stranded DNA templates with short homology arms. Our findings replicated the finding recently reported by others (Liang et al, 2017, Natsume et al, 2016, Song et al, 2017). Additionally, these templates with short homology arms can be created through PCR, which greatly facilitates targeting experiments. Adding homology arms using PCR, rather than cloning each arm individually, can save days or even weeks of laborious cloning. We noted a significant decrease in the efficiency of introducing exogenous DNA as the size of the insert increased. Potentially, the decrease in the copy number of the template was responsible for the change in efficiency. Future optimization of transfection may permit the introduction of very large pieces of DNA with short homology arms. The ability to rapidly edit genomes and introduce exogenous DNA holds enormous potential for research and medical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TALENs	transcription activator-like effector nucleases
ZNFs	zinc-finger nucleases
HDR	Homology-directed repair
NHEJ	Non-homologous end joining
gRNA	Guide RNA

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Figure 1. Comparison of methods to introduce Cas9 and guide RNAs

A) Western blot of HEK 293 Flp-In lines expressing GFP reporter and Cas9. GFP was introduced at Flp-In locus to ensure that only a single copy of the target gene was present; Cas9 was introduced by infecting cells with a lentivirus encoding flag-tagged Cas9 (see materials and methods). B) *In vitro* digestion of target DNA by RNP complex. A 1000 base pair (bp) amplicon was generated by PCR amplification of a GFP-containing plasmid. Digestion of the amplicon results in two 500 bp segments. C) Representative histogram of flow cytometry data demonstrating disruption of GFP by Cas9. The histogram shows viable cells five days post transfection with a GFP-specific RNP. The green line represents control cells (GFP+) and the grey line represents GFP+ cells that had been transfected with a GFP-specific RNP. D) Quantification of the fraction of GFP positive cells remaining after treatment with Cas9 and gRNA using different methods of introduction (n=3/group). Data are expressed as mean $\pm$ SEM. ** $P < 0.02$, *** $P < 0.001$, and **** $P < 0.0001$; Student's t -test.

Figure 2. RNP mediated disruption of endogenous genes

A) *In vitro* digestion of DNA fragment containing sequence complementary to TERT gRNA sequence. A target substrate was generated by digestions of a plasmid containing TERT cDNA to yield a 3.6 kb product. Digestion of the product with RNP yields a 2.8 kb and 800 bp band. B) *In vitro* digestion of amplicon containing KEAP1 cDNA sequence. A substrate was generated by PCR amplification of a 250 bp fragment containing the KEAP1-targeting sequence. Digestion with KEAP1-specific RNP yields a 150 and 100 bp products. C) PCR of genomic DNA from *TERT*-targeted clones showing amplicon size heterogeneity in several clones. Approximate primer positions shown in D. D) Schematic and sequencing results from one clone showing frame shifting mutations in 2 out of 2 alleles detected. PCR products from C were sub-cloned using zero-blunt cloning, so that individual alleles could be sequenced. 16–18 subclones were sequenced for each independent clone. The number of alleles is inferred from the number of unique sequences that were detected. E) PCR of genomic DNA from *KEAP1*-targeted clones showing amplicon size heterogeneity in several clones. Approximate primer positions shown in G. F) Western blot for showing loss of protein in targeted cells. G) Schematic of *KEAP1* gene and location of gRNA. Protospacer adjacent motif (PAM) sequence (green) and Cas9 cut site (arrow) are depicted. Note, the gRNA target sequence was located on the opposite strand of DNA. Frameshifting mutant alleles are shown from sequencing sub-cloned PCR products. H) *KEAP1*-deficient cells were functionally tested by challenging with an oxidant (H₂O₂). Cell viability was measured using MTT assay 24 hours post exposure to increasing concentrations of H₂O₂. Error bars show SEM for each measurement. Data are expressed as mean $\pm$ SEM. **** $P < 0.0001$; Student's *t*-test.

Figure 3. Comparison of methods for inserting exogenous sequences

A) A Reporter system for detection of HDR-mediated introduction of exogenous sequences.

A single ATG-EGFP cassette was inserted at the Flp-In locus in HEK 293 cells.

Introducing an ATG at the 5' end of the cassette results in GFP positive cells. B) Schematic of target and donor DNA and *in vitro* digestion with RNP. The exogenous ATG (red) is shown in the donor. Kozak sequence (underlined), Cas9 cut site (arrow) and PAM (box) is shown in the target gDNA. *In vitro* digestion was done using a 1000 bp PCR product of

ATG-EGFP. Digestion with RNP results in two 500bp segments. C) Representative histogram of control and edited cells showing the shift in GFP fluorescence five days post transfection. D) Quantification of the fraction of GFP positive cells after treatment with

Cas9, gRNA, and donor DNA using different methods of introduction (n=3/group). Data are expressed as mean ± SEM. *P < 0.05; Student's t-test.

Figure 4. Introduction of epitope tags and fluorescent-protein tags using RNP transfection

A) Schematic of donor templates that were inserted into our reporter construct. Size of the inserted DNA is shown on the right. Kozak sequence (underlined), Cas9 cut site (arrow) and protospacer adjacent motif (PAM; box) is shown in the target gDNA. B) Fraction of GFP positive cells after RNP transfection with different donors (n=3/group). C) Western blot with anti-GFP antibody showing GFP and modified-GFP after editing. D) Representative immunofluorescence images of edited cells showing co-localization of GFP and RFP fluorescence. Scale bar is 100 μ m. Data are expressed as mean $\pm$ SEM. * P < 0.05; Student's t -test.