



Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection



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ABSTRACT

CRISPR-Cas9 systems provide a platform for high efficiency genome editing that are enabling innovative applications of mammalian cell engineering. However, the delivery of Cas9 and synthesis of guide RNA (gRNA) remain as steps that can limit overall efficiency and ease of use. Here we describe methods for rapid synthesis of gRNA and for delivery of Cas9 protein/gRNA ribonucleoprotein complexes (Cas9 RNPs) into a variety of mammalian cells through liposome-mediated transfection or electroporation. Using these methods, we report nuclease-mediated indel rates of up to 94% in Jurkat T cells and 87% in induced pluripotent stem cells (iPSC) for a single target. When we used this approach for multigene targeting in Jurkat cells we found that two-locus and three-locus indels were achieved in approximately 93% and 65% of the resulting isolated cell lines, respectively. Further, we found that the off-target cleavage rate is reduced using Cas9 protein when compared to plasmid DNA transfection. Taken together, we present a streamlined cell engineering workflow that enables gRNA design to analysis of edited cells in as little as four days and results in highly efficient genome modulation in hard-to-transfect cells. The reagent preparation and delivery to cells is amenable to high throughput, multiplexed genome-wide cell engineering.

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1. Introduction

CRISPR-Cas9 mediated genome engineering enables researchers to modify genomic DNA *in vivo* directly and efficiently (Cho et al., 2013a; Mali et al., 2013; Jiang et al., 2013; Wang et al., 2013). Three components (Cas9, mature crRNA and tracrRNA) are essential for functional activity. Although the mature crRNA and tracrRNA can be synthesized chemically, the quality of the synthetic RNA is not sufficient for *in vivo* cell engineering due to the presence of truncated by-products (data not shown). Therefore, templates for the mature crRNA and tracrRNA or a combined single gRNA are often cloned into a Cas9 expression plasmid or built into separate plasmids driven by either U6 or H1 promoters for transcription after transfection of mammalian cells (Cong et al., 2013; Mali et al., 2013). However the plasmids have been shown to have toxicity in some

cell lines (Kim et al., 2014). Recently, the use of Cas9 delivered as mRNA has led to increases in the rate of genomic cleavage in some cells. For example, a mixture of Cas9 mRNA and a single species of gRNA were co-injected into mouse embryonic stem (ES) cells resulting in biallelic mutations in 95% of newborn mice (Wang et al., 2013). To make guide RNA, often a linearized plasmid containing the T7 promoter and the gRNA sequence is used directly or a linear template is created via PCR amplification of the targeting sequence from a plasmid. If a 5' T7 promoter does not appear in the plasmid, it is often added at this step and the resulting PCR product can be used in an *in vitro* transcription reaction (Jinek et al., 2012; Wang et al., 2013). Alternatively, a synthetic DNA fragment containing a T7 promoter, crRNA and tracrRNA can be used as a template to prepare a gRNA by *in vitro* transcription. Overall, these represent a labor-intensive and time-consuming workflow, which led us to seek a simpler method to synthesize high quality gRNA. To that, we describe here a streamlined modular approach for gRNA production *in vitro*. Starting with two short single stranded oligos, the gRNA template is assembled in a 'one pot' PCR reaction. The product is then used as template in an *in vitro* transcription (IVT) reaction which is followed by a rapid purification step, yielding transfection-ready gRNA in as little as four hours.

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; CAS9, CRISPR associated protein; gRNA, guide RNA; crRNA, CRISPR RNA; tracrRNA, trans-activating crRNA.

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To streamline the cell engineering workflow further, we sought to eliminate any remaining cellular transcription or translation by directly introducing Cas9 protein/gRNA ribonucleoprotein (RNP) complexes directly to the cells. Microinjection of Cas9 protein and gRNA complexes into *C. elegans* was first described in 2013 (Cho et al., 2013b) and was subsequently used to generate gene-knockout mice and zebrafish with mutation rates of up to 93% in newborn mice (Sung et al., 2014). Following that report, Cas9 protein/gRNA RNP complexes were delivered into cultured human fibroblasts and induced pluripotent stem cells (iPSC) via electroporation with high efficiency and relatively low off-target effects (Kim et al., 2014). In that study, a large amount of Cas9 protein (4.5–45 µg) and gRNA (6–60 µg) were necessary for efficient genome modification (up to 79% indel efficiency). Another recent study delivered Cas9/gRNA RNPs along with donor DNA for homology directed repair into HEK293 T, human primary neonatal fibroblasts and human ESCs cells via electroporation (Lin et al., 2014). Here also, large amounts of Cas9 protein (4.8–16 µg) were necessary for efficient modification. Most recently, delivery of Cas9 protein-associated gRNA complexes via liposomes was reported, in which RNAiMAX was used to deliver Cas9:sgRNA nuclease complexes into cultured human cells and into the mouse inner ear *in vivo* with up to 80% and 20% genome modification efficiency, respectively (Zuris et al., 2015).

The CRISPR/Cas system has been demonstrated as an efficient gene-targeting tool for multiplexed genome editing (Wang et al., 2013; Kabadi et al., 2014; Sakuma et al., 2014; Cong et al., 2013). For example, co-transfections of mouse ES cells with constructs expressing Cas9 and three sgRNAs targeting Tet1, 2, and 3 resulted in 20% of cells having mutations in all six alleles of the three genes based on restriction fragment length polymorphism (RFLP) assay (Wang et al., 2013). Lentiviral delivery of a single vector expressing Cas9 and four sgRNAs into primary human dermal fibroblasts resulted in about 30% simultaneous editing of four genomic loci among ten clonal populations based upon genomic cleavage detection assays (Kabadi et al., 2014). In one recent study, 'all-in-one' expression vectors containing seven guide RNA expression cassettes and a Cas9 nuclease/nickase expression cassette were delivered into 293T cells with genome cleavage efficiency ranging from 4 to 36% for each individual target (Sakuma et al., 2014). In general, the efficiency of editing multiple genes in the human genome using plasmid-based delivery methods remains relatively low which subsequently increases the workload for downstream clonal isolation.

In this study, we developed an *in vitro* gRNA production system and used a systematic approach to optimize the conditions for delivery of Cas9:gRNA complexes via lipid-mediated transfection or electroporation. A variety of mammalian cell lines were tested, including primary cells and other hard-to-transfect cells. Plasmid DNA, mRNA and Cas9 protein transfections were evaluated side by side. Using Cas9 protein transfection via electroporation, we achieved superior genome editing efficiencies even in hard-to-transfect cells. In addition, we explored the genome editing of multiple targets simultaneously using the Cas9 RNPs delivery system described here. We found that delivery of Cas9 RNPs not only led to high indel production at single locus, but supports highly efficient biallelic modulation of at least two genes in a single transfection.

2. Materials and methods

2.1. Materials

293FT cells, Gibco® Human Episomal iPSC line, mouse E14Tg2a.4 embryonic stem cells, primary human keratinocytes

cells neonatal, inactivated embryonic fibroblasts, DMEM medium, RPMI 1640 medium, IMDM, McCoy 5A modified medium, DMEM/F-12, KnockOut™ DMEM, Fetal Bovine Serum (FBS), Knockout™ Serum Replacement, Non-Essential Amino Acid solution, basic fibroblast growth factor, Collagenase IV, TrypLE™ Express Enzyme, Geltrex, Opti-MEM Medium, Essential 8™ medium, StemPro®-34 SFM Complete Medium, FluoroBrite™ DMEM, recombinant human leukemia inhibitory factor, GeneArt® Genomic Cleavage Detection Kit (GCD), Lipofectamine® 2000, Lipofectamine® 3000, Lipofectamine® RNAiMAX, Lipofectamine® MessengerMAX, GeneArt® CRISPR Nuclease Vector with GFP Reporter, 2% E-Gel® EX Agarose Gels, PureLink® PCR Micro Kit, TranscriptAid T7 High Yield Transcription Kit, MEGAclean™ Transcription Clean-Up Kit, Zero Blunt® TOPO® PCR Cloning Kit, PureLink® Pro Quick96 Plasmid Purification Kit, Qubit® RNA BR Assay Kit, TRA-1-60 Alexa Fluor® 488 conjugated antibodies, SSEA4 Alexa Fluor®647, and Phusion Flash High-Fidelity PCR Master Mix were from Thermo Fisher Scientific. Jurkat T cells and K562 cells were obtained from the American Type Culture Collection (ATCC). CD34+ cord blood cells were purchased from AllCells. A549 cells, U-2 OS cells, Neuro-2a (N2A) cells were purchased from ATCC. MEF feeder cells and ROCK inhibitor Y-27632 were purchased from EMD Millipore. Monoclonal Cas9 antibody was ordered from Diagenode. Recombinant Cas9 protein with a NLS was initially purified as described (Kim et al., 2014) and later obtained from Thermo Fisher Scientific. All oligonucleotides used for gRNA synthesis and genomic cleavage detection were from Thermo Fisher Scientific (Table S1).

2.2. One-step synthesis of gRNA template

The 80 bp cr/tracrRNA constant region was PCR amplified from the GeneArt® CRISPR Nuclease Vector (1 ng) using the Constant Forward and Universal Reverse oligos (10 µM) and purified via agarose gel extraction. The concentration of PCR product was measured by Nanodrop (Thermo Fisher Scientific) and the molarity was calculated based on the molecular weight of 49.6 kDa. To prepare a mixture of oligonucleotides, the 80 bp cr/tracrRNA PCR product (0.15 µM) was mixed with universal forward and reverse oligos (10 µM) as well as target-specific forward and reverse oligos (0.3 µM).

For each target locus, 2 oligonucleotides that recreate the target sequence and share complementarity with the bordering T7 promoter and 80 bp cr/tracr constant region were designed (Fig. 2A). The forward oligo (Target F1) contains the 18 base T7 promoter sequence as well as the first 16 bases of the target and the reverse oligo (Target R1) contains the reverse complement of the first 15 bases of the cr/tracr constant region and the last 19 bases of the target (Table 1s). To set up the synthesis of gRNA template, aliquots of the pooled oligonucleotides were added to a Phusion Flash High-Fidelity PCR Master Mix and amplified using manufacturer's recommended reaction conditions. The PCR product was analyzed by a 2% E-Gel® EX Agarose Gel, followed by purification using Purelink® PCR micro column. The gRNA template was eluted with 13 µl water and the concentration was determined by Nanodrop instrument. To determine the error rate, the PCR product was cloned into Zero Blunt® TOPO® vector, followed by plasmid DNA isolation and sequencing with a 3500xl DNA analyzer (Thermo Fisher Scientific).

2.3. In vitro transcription

The *in vitro* transcription of gRNA template was carried out using TranscriptAid T7 High Yield Transcription Kit using the manufacturer's recommended conditions. The gRNA product was purified using MEGAclean™ Transcription Clean-Up kit as described in the

Table 1
Comparison of plasmid DNA, Cas9 mRNA/gRNA and Cas9 RNP transfection and resulting editing efficiencies as measured by GCD assay in a variety of cell lines.

Cell lines	Plasmid		mRNA		Protein	
	Lipid	Electro	Lipid	Electro	Lipid	Electro
HEK293FT	49	49	70	40	51	88
U2OS	15	50	21	24	18	70
Mouse ESCs	30	45	45	20	25	70
Human ESCs (H9)	0	8	20	50	0	64
Human iPSCs	0	20	66	32	5	87*
N2A	66	75	66	80	66	82
Jurkat	0	63	0	42	0	94*
K562	0	45	0	27	0	72
A549	15	44	23	29	20	66
Human Keratinocytes (NHEK)	0	30	0	50	0	35
Human Cord Blood Cells CD34+	n/a	0	n/a	0	n/a	24

Note:

- (1) gRNA targets are HPRT for human cell lines and Rosa 26 for mouse cell lines.
(2) *Confirmed by sequencing.

manual. The concentration of RNA was determined using Qubit® RNA BR Assay Kit.

2.4. Mammalian cell culture

HEK 293FT, A549 and N2A cells were maintained in DMEM medium supplemented with 10% FBS. U2OS cells were maintained in McCoy 5A modified medium supplemented with 25 mM HEPES and 10% FBS. Jurkat T cells were propagated in RPMI medium containing 10% FBS. K562 cells were cultured in IMDM medium supplemented with 10% FBS. Human ESCs (H9) were cultured in Essential 8™ medium on tissue culture dishes coated with Geltrex® hESC-qualified reduced growth factor basement membrane matrix. After thawing, cells were passaged 2–3 times before using for transfection. Feeder-dependent human episomal iPSC were cultured on mitotically inactivated MEF feeder cells in human ESC (hESC) media containing 20% Knockout™ Serum Replacement, 10 μM Non-Essential Amino Acid solution, 55 μM 2-Mercaptoethanol, and 4 ng/ml basic fibroblast growth factor in DMEM/F-12. iPSC cultures were maintained with daily media changes and were passaged regularly using Collagenase IV. Mouse E14Tg2a.4 embryonic stem cells were cultured on mouse (strain ICR) inactivated embryonic fibroblasts in the presence of recombinant human leukemia inhibitory factor (LIF) in mouse ESC medium consisting of KnockOut™ DMEM 15% embryonic stem cell-qualified Fetal Bovine Serum, 1 × MEM Non-Essential Amino Acids Solution, 1 × GlutaMAX™ Supplement, 1 × 2-mercaptoethanol, and 10 ng/ml LIF. Before transfection, cells were adapted to feeder-free conditions and maintained on attachment-factor-coated plates in mouse ESC-conditioned medium. When setting up the experiments for transfections, 1 × 10⁵ cells were plated per well in a 24-well tissue culture dish coated with attachment factor. CD34+ cord blood cells were cultured using StemPro®-34 SFM Complete Medium supplemented with 100 ng/ml of SCF, 50 ng/ml of IL-3 and 25 ng/ml of GM-CSF. Primary human keratinocytes cells neonatal (HEKn) were grown in a tissue culture dish treated with Coating Matrix Kit in EpiLife medium containing 60 μM of calcium chloride. Cells were grown for three passages before using for experiments. All cultures were maintained in 5% CO₂ at 37 °C in a humidified incubator. Prior to transfection, adherent cells were detached with Gibco TrypLE Select Enzyme and then resuspended in the appropriate growth media.

2.5. Lipid-mediated cell transfection

One day prior to transfection, the cells were seeded in a 24-well plate at a cell density of 1–2 × 10⁵ cells per well.

For plasmid DNA transfection, 0.5 μg DNA was added to 25 μl of Opti-MEM medium, followed by addition of 25 μl of Opti-MEM containing 2 μl of Lipofectamine 2000. The mixture was incubated at room temperature for 15 min and then added to the cells.

For Cas9 mRNA transfection, 0.5 μg Cas9 mRNA (Thermo Fisher Scientific) was added to 25 μl of Opti-MEM, followed by addition of 50–100 ng gRNA. Meanwhile, 2 μl of either Lipofectamine 3000, MessengerMax or RNAiMAX was diluted into 25 μl of Opti-MEM and then mixed with mRNA/gRNA sample. The mixture was incubated for 15 min prior to addition to the cells.

For Cas9 protein transfection, 500 ng of purified Cas9 protein was added to 25 μl of Opti-MEM medium, followed by addition of 120 ng gRNA. The molar ratio of gRNA to Cas9 protein was kept at approximately 1 to 1.2: 1. The sample was mixed by gently tapping the tubes a few times and then incubated at room temperature for 10 min. To a separate test tube, 2 μl of Lipofectamine RNAiMAX, MessengerMax, or 3000 was added to 25 μl of Opti-MEM medium. The diluted transfection reagent was transferred to the tube containing Cas9 protein/gRNA complexes, followed by incubation at room temperature for 15 min and then added to the cells.

In each case, the entire solution was added to the cells in a 24-well plate and mixed by gently swirling the plate. The plate was incubated at 37 °C for 48 h in a 5% CO₂ incubator. The percentage of locus-specific indel formation was measured by GeneArt® Genomic Cleavage Detection Kit (GCD). The band intensities were quantitated using the Alpha Imager software (Bio-Rad). Each cell line was tested with each version of Lipofectamine (3000, RNAiMAX, and MEssengerMAX). The lipid that resulted in highest cleavage efficiency is listed in Table S2.

2.6. Electroporation

For suspension cells, such as Jurkat T cells, K562 cells or CD34+ human cord blood cells, 1–2 × 10⁵ cells were used per electroporation using Neon® Transfection System 10 μL Kit (Thermo Fisher Scientific). For adherent A549, U2OS and N2A cells, 5 × 10⁴ cells were used per electroporation. For adherent HEK293FT, mESC, hESC, human iPSC, A549, and NHEK cells, 1 × 10⁵ were used per electroporation.

To maximize the genome cleavage efficiency, the Neon 24 optimization protocol was applied according to the manufacturer's instruction. To set up a master mix, 24 μg of purified Cas9 protein was added to 240 μl of Resuspension Buffer R provided in the kit, followed by addition of 4.8 μg of gRNA. The mixture was incubated at room temperature for 10 min. Meanwhile, 4.8 × 10⁶ cells were transferred to a sterile test tube and centrifuged at 500 × g for 5 min. The supernatant was aspirated and the cell pellet was resuspended in 1 ml of PBS without Ca²⁺ and Mg²⁺. Upon centrifugation, the supernatant was carefully aspirated so that almost all the PBS buffer was removed with no or minimum loss of cells. The Resuspension Buffer R containing the Cas9 protein/gRNA complexes was then used to resuspend the cell pellets. A 10 μl cell suspension was used for each of the 24 optimization conditions, which varied in pulse voltage, pulse width and the number of pulses. The electroporated cells were transferred immediately to a 24 well containing 0.5 ml of the corresponding growth medium for each cell line and then incubated for 48 h in a 5% CO₂ incubator. The cells were harvested by centrifugation and then washed once with PBS, followed by GCD assay. Upon optimization of electroporation condition, a higher amount of Cas9 protein (1.5–2 μg) and gRNA (300–400 ng) could be applied to further increase the genome editing efficiency if needed.

Each cell line was tested for with the Neon 24 optimization protocol. Specific electroporation conditions that resulted in the highest cleavage efficiency are listed in Table S2.

For each target in the multiplexing assays, 1–2 µg of Cas9 protein and 200–400 ng of gRNA (maintaining a 1:1 ratio) were pre-incubated separately in Resuspension Buffer R for 10 min at room temperature prior to mixing with the cell pellet for electroporation.

For clonal isolation, the cell number of transfected cells was counted upon 48 h incubation, followed by a serial of dilution to 96 well plates with a cell density of 10–20 cells per plate based on the cell count. After clonal expansion for three weeks, cells from each individual well were harvested, followed by PCR amplification of the target locus. The PCR fragments were then cloned using a TOPO vector and transformed into TOP10 competent cells. Approximately 8 *E. coli* colonies were randomly picked for sequencing for each individual target locus. The single cell population was determined by the homogeneity of sequences for each allele. Single cells containing bi-allelic mutations on all desired targets were considered homozygotic indels. Downstream sequence analysis to confirm frame-shift induced stop codon introduction was not done.

For transfection of feeder free adaptation of iPSC, feeder dependent iPSC were grown to 80% confluence prior to harvest with collagenase. Following removal of the cell clusters from the feeder layer, they were gravity sedimented to prevent MEF contamination. The cell clusters were then seeded on to tissue culture dishes coated with Geltrex® in MEF conditioned media supplemented with 4 ng/ml bFGF. MEF conditioned media was produced using inactivated feeder cells, which was harvested on 7 continuous days, sterile filtered and frozen until usage. The cultures were allowed to reach 80–90% confluence. The day prior to transfection, the cultures were pretreated with 5 µM ROCK inhibitor Y-27632. On the day of harvest the cultures were inspected for signs of differentiation and any contamination differentiated cells were removed via microdissection. The cultures were washed once with DPBS and then harvested using TrypLE™ Express Enzyme. Single cells suspensions were counted using the Countess® automated cell counter. Following transfections, the cells were seeded onto multi-well (24 well) tissue culture dish coated with Geltrex® and incubated overnight with MEF conditioned media containing 5 µM ROCK. Media was replaced daily, without ROCK inhibitor, prior to analysis.

2.7. Cell surface immunostaining

To ensure maintenance of pluripotency post transfection and genome editing, iPSC cells were tested for expression of cell surface markers of self-renewal. The wells to be probed were washed with DMEM/F12 basal media. TRA-1-60 Alexa Fluor® 488 conjugated antibodies and SSEA4 Alexa Fluor®647 were multiplexed in basal DMEM/F-12 media. Both antibodies were added at a concentration of 2 µl of each antibody into 0.5 ml of pre-warmed DMEM/F-12 media and incubated at 37 °C for 45 min. Following the incubation, the antibody solution was removed and the wells were washed twice with DMEM/F-12. Prior to observation the media was exchanged with pre-warmed FluoroBrite™ DMEM. Images were taken using a Zeiss Axiovision microscope using a FITC and Cy5 laser/filter combination.

2.8. Analysis of pluripotency markers

Cultures were detached and dissociated using TrypLE™ Select and trituration. Single cell suspensions were incubated with TRA-1-60 Alexa Fluor® 488 conjugated antibodies and SSEA4 Alexa Fluor®647 for 1 h at room temperature with gentle agitation. Two microliters (50× concentration, as supplied) of each antibody were added to 0.5 mL of DMEM/F-12. Following the incubation, the cells were centrifuged and washed once with Dulbecco's

Phosphate-Buffered Saline (DPBS). After the removal of the DPBS wash, the pelleted cells were gently re-suspended in 1 ml of DPBS and stained through a strainer capped tube. The cells were then measured for the expression of both markers using the Attune® Acoustic Focusing Cytometer and the data was analyzed using FlowJo software.

2.9. Western Blot analysis

HEK293FT cells were transfected with either Cas9 plasmid DNA, mRNA or protein as described above. Mouse ESCs were electroporated with Cas9 RNPs as described above. Cells were harvested at indicated times to perform both GCD assay and Western Blot analysis. The cell lysate was fractionated using a 4–12% Novex Bis-tris gel. The proteins were transferred to a PVDF membrane using an iBlot following the manufacturer's protocol. Upon blocking, the membrane was incubated for 2 h with monoclonal mouse Cas9 antibody at 1:3000 dilution. After washing, the membrane was incubated for 1 hour with rabbit anti-mouse antibody-HRP conjugate at 1:2000 dilution. Upon extensive washing, the membrane was developed with Pierce ECL reagent, followed by imaging using a Fuji imager LAS 4000 instrument.

3. Results

3.1. Three day cell engineering workflow

To streamline the genome engineering workflow, we sought to simplify the gRNA synthesis procedure and shorten the time from experimental design to initial analysis as much as possible. We present a process where on day 1, the researcher designs and orders short DNA oligonucleotides and seeds the cells of interest for next day transfection (Fig. 1). Upon receiving the oligonucleotides on day 2, the researcher assembles the gRNA template in less than 1 h by 'one pot' PCR. The resulting PCR product is then subjected to *in vitro* transcription to synthesize gRNA in approximately 3 h. Upon association of gRNA with purified Cas9 protein, the Cas9 RNPs are used to transfect cells via lipid-mediated delivery or electroporation. As early as day 3 (24 h post transfection), the cells can be harvested for analysis of locus-specific genome modification efficiency.

We used an online web tool to identify candidate 20 nt gRNA target sequences for each loci (Beta Testing Version, Thermo Fisher Scientific). A pair of 34 nt forward and reverse oligonucleotides comprising each target sequence were designed as described. To assemble the DNA template for gRNA production, we combined the 2 target specific oligos (Target F1/R1) with a mixture of the 2 universal oligos (Universal Forward/Reverse) and a purified PCR product representing the constant non-targeting region of the full gRNA (Fig. 2A). The oligonucleotide pool concentrations as well as the PCR conditions were optimized such that the template was amplified in less than 40 min in a single tube with (Fig. 2B). The gRNA template was used directly to prepare gRNA via *in vitro* transcription (IVT). The resulting gRNA was purified, yielding high levels of gRNA with low amounts of detectable by-products (Fig. 2C). This approach was validated by synthesis of more than 96 distinct gRNAs (unpublished results). To determine the error rate in the synthetic gRNA DNA template, we cloned and sequenced the PCR fragments and found that using this design approximately 7% of gRNA templates harbored mutations, mainly small deletions occurring at the extreme 3' end and 5' ends of the mature template compared to 21% mutants with an alternative design of using long overlapping oligonucleotides (Fig. 2D). Since all the mutations observed using our design appeared to be due to errors in the oligonucleotides, we next tried HPLC-purified universal forward and reverse primers which further decreased the error rate to 3.6% with no mutations

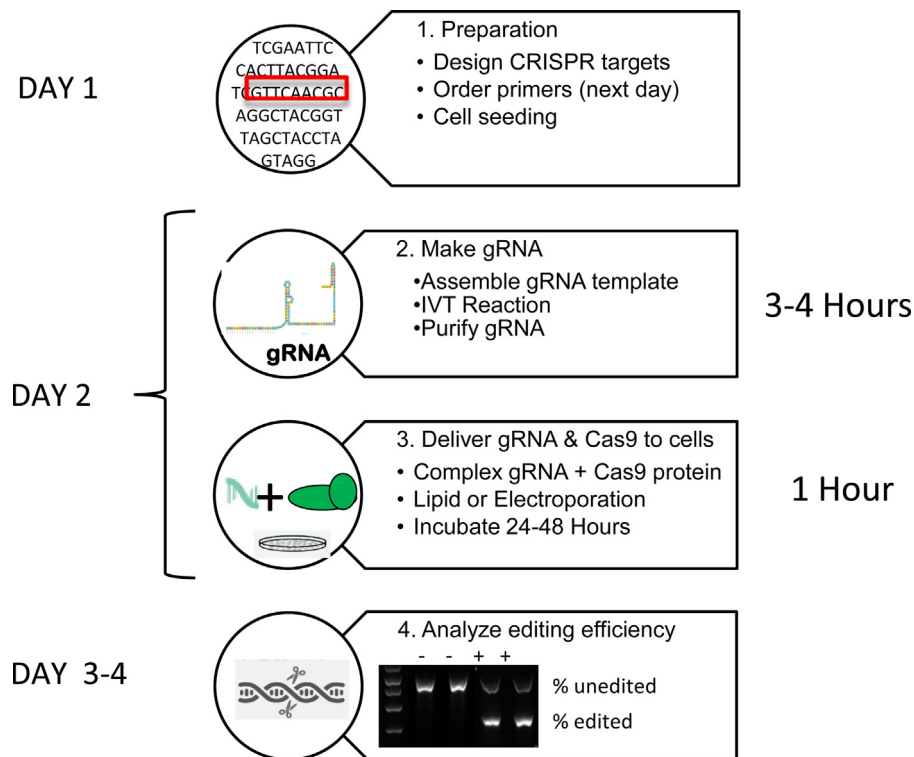


Fig. 1. Cell engineering workflow. On day 1, the researcher designs CRISPR targets and seeds cells. Synthesis of gRNA and cell transfection with Cas9 protein/gRNA complex (Cas9 RNP) are performed on day 2. Genome cleavage assays carried out on days 3–4.

detected in the target region, which was similar to the 2% error rate observed with the control template prepared from an ‘all-in-one’ plasmid. Taken together, this optimized process facilitates the conversion of a small set of DNA oligonucleotides into purified gRNA in approximately 4 h with an accuracy of up to 96% and no errors detected in the targeting or Cas9 complexing (cr/tracrRNA) regions. This level of accuracy should be sufficient for routine screening of gRNAs, but if a sequence verified gRNA is required the gRNA template can easily be Topo cloned. Given that the process consists solely of liquid handling PCR, transcription, and RNA isolation steps, it is well suited for high throughput gRNA production and screening.

3.2. Liposome-mediated Cas9 protein transfection

To examine the activity of synthetic gRNA, we pre-complexed purified synthetic IVT gRNA with Cas9 protein, hypothesizing that creating complexes of purified gRNAs with Cas9 protein prior to delivery to the cells might lead to higher genome editing efficiency due to the protection of the gRNA as it transits to the nucleus during the transfection process. To examine *in vivo* functionality of the system, human embryonic kidney (HEK293FT) cells were transfected with pre-complexed Cas9/gRNA ribonucleoproteins (Cas9 RNPs) using a set of cationic lipid reagents, followed by a GCD assay. The commonly-used plasmid DNA or RNA lipofectamine transfection reagent had been shown to be able to deliver many proteins into cells, so we tested several lipofectamine variants for their ability to efficiently deliver Cas9 RNPs (Sells et al., 1995). Lipofectamine 3000 and RNAiMAX outperformed Lipofectamine 2000 in HEK 293 cells (Figure S1), which is in agreement with the recent finding that RNAiMAX performed better than Lipofectamine 2000 for delivery of Cas9 RNPs with low cell toxicity (Zuris et al., 2014). We tested the molar ratio of cas9 protein to gRNA and generally observed that cleavage activity plateaued at a 1:1 ratio (data not shown). For protein transfection, serum-free medium is generally used to avoid

serum protein inference. In this study however, we observed that the complete medium containing 10% FBS could facilitate increased protein transfection and genome modification (Fig. 3A, panel a). The efficiencies of genome editing via plasmid DNA, mRNA and Cas9 RNP transfection were evaluated using three different target loci, HPRT, AAVS and RelA. Plasmid DNA and mRNA were delivered into HEK293 cells by Lipofectamine 3000, whereas Cas9 RNPs were delivered with RNAiMAX. The efficiencies of genome modification were similar among three target loci in DNA, mRNA and Cas9 protein-transfected cells (Fig. 3A).

Next we examined the kinetics of genome cleavage by transfecting cells with either plasmid DNA, mRNA, or Cas9 RNPs, followed by GCD assays and western blot analysis of cell lysates. We observed similar cleavage kinetics between Cas9 delivered as plasmid DNA, mRNA and protein with efficient cleavage seen at 24 h and plateauing at 48–72 h post-transfection in HEK293 cells (Fig. 3B). However, the kinetics of Cas9 RNP and mRNA encoded Cas9 appearance and turnover inside the transfected cells was quite different from that seen with Cas9 delivered via plasmid DNA. Measuring by western blot (Fig. 3C), we found that Cas9 protein accumulated over time in plasmid DNA-transfected cells, whereas the relatively low expression of Cas9 in mRNA-transfected cells seemed to peak as early as four hours post transfection and remained relatively stable for approximately 48 h before diminishing. In the Cas9 RNP transfected cells, the level of Cas9 protein peaked in at the first time point, then rapidly decreased and was barely detectable in our assay at 48 h. As a control, the blot membrane was stripped and re-probed with anti-actin antibody. Similar levels of actin expression were observed among samples (data not shown).

Because of the observed faster protein depletion, we hypothesized that the off-target cleavage activity for Cas9 RNP and mRNA/gRNA transfection would be lower than that of plasmid DNA transfection (Fu et al., 2013; Pattanayak et al., 2013; Hsu et al., 2013). To test this, we targeted a locus (target site 3) in the VEGFA gene which has been identified as having several high activity

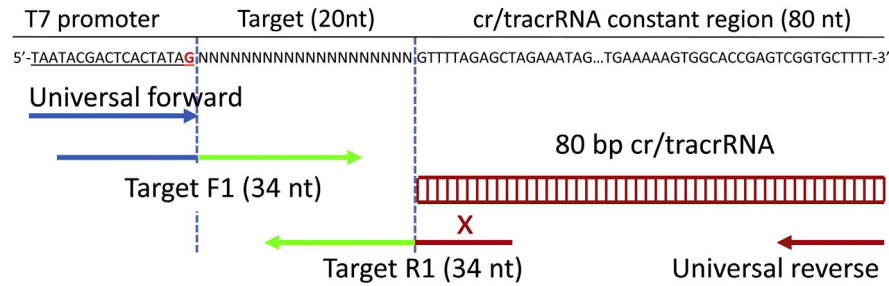
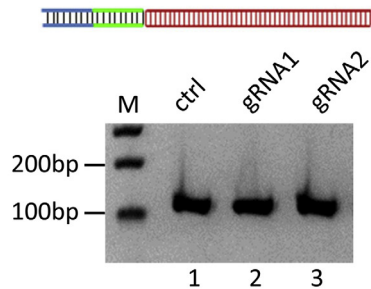
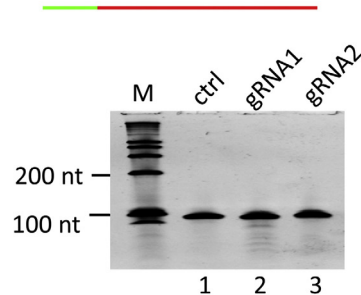
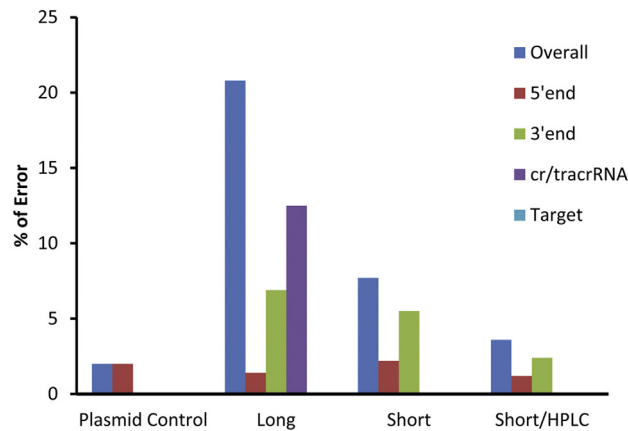
(A) Oligo design for gRNA template**(B) gRNA template by PCR****(C) synthetic gRNA by IVT****(D) Error rates in synthetic gRNA templates**

Fig. 2. Design and synthesis of gRNA. (A) Design of gRNA DNA template mix. The mixture consists of an 80 bp PCR product encoding the cr/tracrRNA constant region, universal forward and reverse primers, and 2 overlapping target-specific 34 nt DNA oligonucleotides (Target F1 and R1). (B) One-step PCR synthesis of gRNA DNA template. The mixture of oligonucleotides and PCR product were assembled in a single tube by PCR and the product was analyzed by agarose gel electrophoresis (Lanes 2 and 3). A gRNA DNA template amplified from a plasmid served as control (Lane 1). (C) Aliquots of PCR product (Lanes 2 and 3) along with the plasmid derived control (Lane 1) were subjected to *in vitro* transcription. The resulting product was analyzed by denaturing gel. (D) Analysis of gRNA DNA template sequence error rate. The gRNA DNA templates were synthesized using the standard gene synthesis approach with a set of long oligonucleotides (Long). Alternatively, the oligonucleotide mixture described above was used for PCR assembly. The two standard desalted universal forward and reverse primers (Short) were compared to HPLC or PAGE-purified primers (Short/HPLC). The synthetic gRNA DNA templates, as well as a control gRNA DNA template PCR amplified from a DNA control (Plasmid) were cloned into a TOPO vector. For each individual template, 96 colonies were randomly picked for sequencing and the errors grouped by location.

off-target sites (Fu et al., 2014) via Cas9 DNA, mRNA, and RNP transfection into HEK293FT cells followed by genome cleavage and locus sequencing analysis. Among the six potential off-target sites that have been studied previously (OT3-1, OT3-2, OT3-4, OT3-9, OT3-17 and OT3-18), only OT3-2 and OT3-18 were detected to harbor off-target mutation based on GCD analysis. Further analysis of locus OT3-2 by sequencing indicated that the ratio of indel mutation of OT3-2 over on-target in mRNA and Cas9 RNP transfected cells was 2 fold and 2.5 fold lower than that in DNA-transfected cells, respectively. The ratio of indel mutation of OT3-18 over on-target was 1.6 fold and 28 fold lower in mRNA or Cas9 RNP-transfected cells,

respectively than in DNA-transfected cells (Fig. 3D). Next we tested the effect of increased Cas9 RNP (maintaining a 1:1 molar ratio of gRNA to protein) on off-target levels and observed that while on-target editing efficiency increased with an increased dose of Cas9 RNP, reaching plateau at around 2 μ g of Cas9 protein, the off-target modification at the OT3-T2 loci increased at a lower rate (Figure S2). Taken together, these data suggest that Cas9 delivery as mRNA and pre-complexed protein supports increased genomic cleavage specificity compared with standard DNA plasmid transfection. In a survey of 11 cell lines (Table 1, Table S2), Lipofectamine 3000 worked best for Cas9 plasmid delivery, while depending on cell

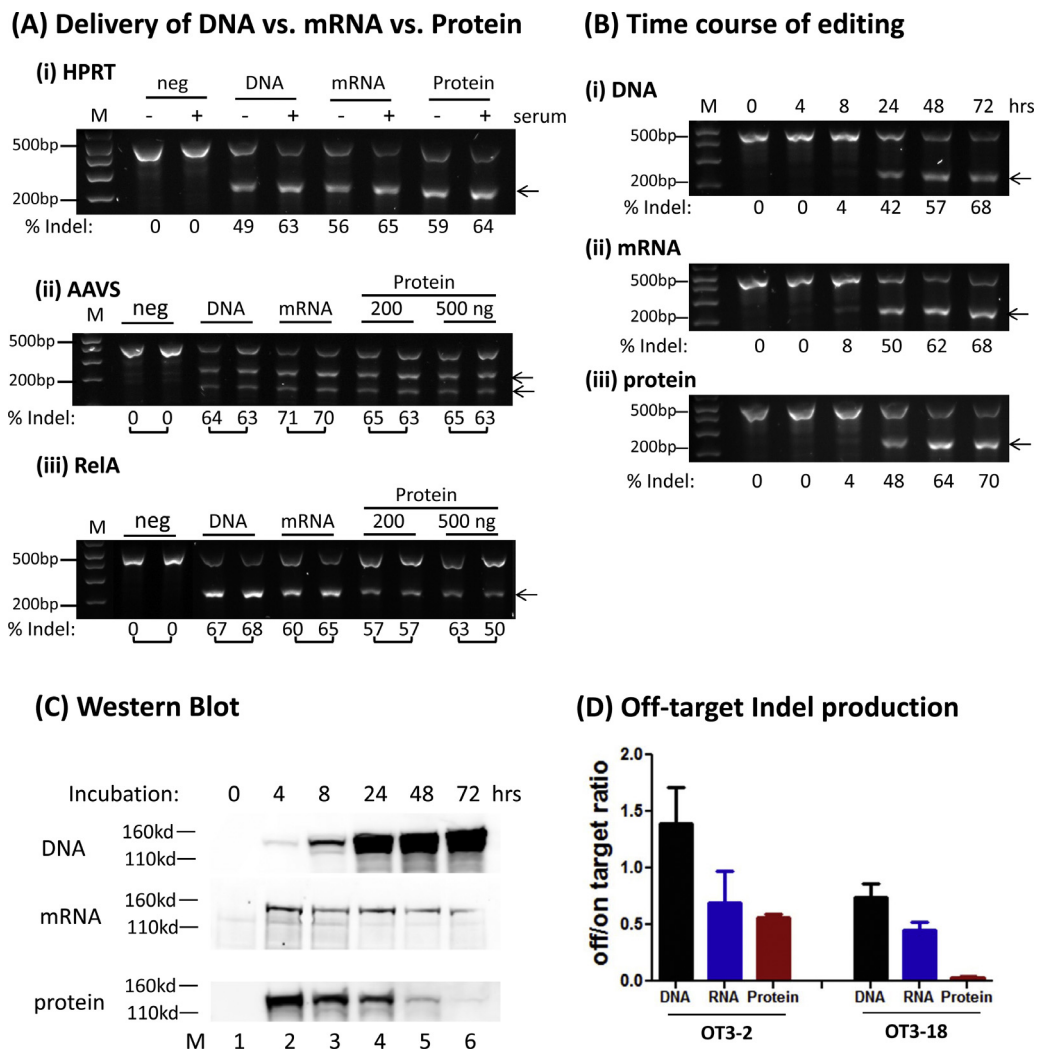


Fig. 3. Lipid-mediated transfection. (A) Three separate genomic loci (HPRT, AAVS or RelA) were edited via Cas9 plasmid DNA, mRNA or protein using lipid mediated transfection of HEK293FT cells. For the HPRT target, transfection was performed in the presence or absence of serum. For AAVS and RelA, serum was used and 2 concentrations of cas9 and RelA protein were tested. The percentage of edited cells (% indel) that resulted in an indel was determined using the GCD assay by quantitating the amount of uncut vs cut DNA (arrow). (B) HEK293FT cells were transfected with either plasmid DNA, Cas9 mRNA/gRNA or Cas9 RNPs directed to the HPRT loci. Cell samples were taken at different time points and analyzed by GCD assays. (C) Western Blot analysis of samples taken at different time points. (D) Off-target mutation of VEGFA T3 target caused by Cas9 plasmid DNA, mRNA or protein transfection. Percentages of on-target mutation as well as OT3-2 and OT3-18 off-target mutations were determined by sequencing.

line either Lipofectamine RNAiMAX or MessengerMAX worked well for Cas9 mRNA or RNP delivery. However, with some cell lines no conditions were found for efficient Cas9 delivery.

3.3. Electroporation-mediated Cas9 protein transfection

Many biologically and physiologically relevant cell lines, such as patient derived iPSC and progenitor cells, are refractory to efficient transfection by lipid-based reagents. Any improvement in the efficiency of genome modulation would facilitate isolation of appropriately engineered cells for experimentation and therapy so we explored alternate means of delivering Cas9 RNPs and Cas9 mRNA/gRNA formulations and their effect on indel generation. Using Jurkat T cells as an initial model, we compared the delivery of Cas9 and gRNA plasmid DNA, Cas9 mRNA/gRNA formulations and Cas9 RNPs using electroporation with a Neon Transfection system and a 24 condition optimization protocol (Fig. 4, Figure S3). Our results showed that, compared with plasmid DNA and mRNA deliveries, superior genome editing efficiency was achieved via delivery of Cas9 RNPs with ~90% HPRT locus-specific modification under

several electroporation conditions (Fig. 4A). The cleavage efficiency was dose-dependent, reaching a maximum of 88% at approximately 1.5 μg Cas9 protein and 300 ng gRNA per transfection (Fig. 4B). After sequencing the cell pools, we found that actually 94% of target loci harbored mutations near the expected cleavage site located around 3 bases upstream of the NGG PAM sequence. The majority of mutations were distinct from each other with 75% insertion and 16% deletion. When we tested the same optimization protocol with human iPSCs, the optimal conditions varied significantly from the Jurkat T settings (Figure S4A). The cleavage efficiency of the Cas9 RNP, was improved from 62% at the standard 1 $\mu\text{g}/\mu\text{l}$ Cas9 RNP concentration to 89% at 3 $\mu\text{g}/\mu\text{l}$ Cas9 RNP (Figure S4B). We checked the transfected iPSCs pluripotency markers SSEA4 and TRA-1-60 by flow cytometry (Figure S4C) and observed only a small change from 93% dual positive in the control to 85% after transfection with Cas9 RNP. When we sequenced the cell pools at the HPRT locus, we saw an 88% indel rate and a different indel distribution from Jurkat T of 32% insertion and 52% deletion with more large deletions and smaller insertions (Table S3). Using the optimization protocol, we surveyed the same 11 cell lines used previously for delivery of Cas9

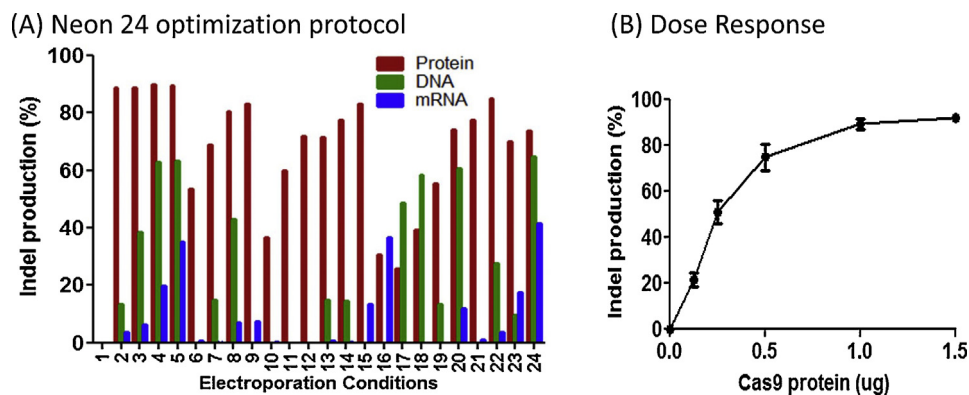


Fig. 4. Electroporation-mediated transfection. (A) Mastermix of plasmid DNA, Cas9 mRNA/gRNA or Cas9 protein/gRNA were used to electroporate Jurkat T cells using the Neon 24 optimization protocol. The numbers 1–24 refer to different conditions which vary pulse voltage, pulse width and number of pulses (Figure S3). The percentage of locus-specific genome cleavage was estimated 48-hour post transfection using a GCD assay. (B) Dose-dependent effect of genome editing. While keeping the ratio of Cas9 protein/gRNA constant, different amounts of Cas9 RNPs were used for electroporation using protocol 5. Experiments were done in triplicate. The percentage of cleavage was confirmed by sequencing.

plasmid, mRNA or RNPs and saw efficient delivery in all cell lines except for cord blood cells where only RNPs resulted in measurable indel formation (Table 1, Table S2). The specific pulse conditions varied widely depending on cell line and construct. Overall, we saw best results with Cas9 RNPs. Finally we measured the cleavage rate and turnover kinetics of the Cas9 RNPs in mouse ESCs and observed that cleavage could be detected within 4 h, plateaued after 8 h and the protein appeared to be almost depleted by 12 h (Figure S5).

3.4. Targeting multiple loci in a single transfection

Given the high single-locus cleavage efficiency measured with the Cas9 RNP system, we next wanted to test the ability to efficiently lesion multiple genes in a single transfection. Here we examined the capability of multiplexing Cas9 RNP transfection at three loci (AAVS1, RelA and HPRT). After pooling and delivering multiple species of Cas9 RNP (differing only by gRNA target), we found that the efficiency of simultaneous editing of AAVS1/HPRT or AAVS1/RelA/HPRT loci was significantly greater at all loci compared with either plasmid or mRNA delivery of Cas9 (Fig. 5A and C). To gain insight into the molecular level of multiplexing, we performed one round of clonal isolation by serial dilution. After clonal expansion each of the loci was PCR amplified, followed by DNA cloning and sequencing. In the case of two gene editing, we found that all of 16 isolated clonal cell lines harbored bi-allelic indel mutations on single AAVS1 loci and 93.7% (15 of 16) of clonal cells harbored one allelic indel mutation at the HPRT locus as the HPRT target was located on the X chromosome of a male Jurkat T cell line. Overall, 93.7% of the clonal cell populations carried indel mutations on both the AAVS1 and HPRT loci (Fig. 5B). For multiplexing of three genes, we performed three individual cell transfections and clonal isolation with a total of 53 single cell lines analyzed. In this experiment, 90% and 65% of the clonal cell lines analyzed harbored bi-allelic indel mutations at the AAVS1 and RelA loci, respectively, whereas 80% of the clonal cells carried indel mutations at the single HPRT locus. Overall, 65% of the clonal cell lines harbored indel mutations on all three targets at all 5 alleles (Fig. 5D). Further, 100% of the Jurkat T cell clones were edited at least once, suggesting that the transfection efficiency reached nearly 100%. Taken together, Cas9 RNP delivery via electroporation under the conditions used here achieved exceptionally high mutagenesis frequencies. This represents a substantial improvement in Cas9-mediated genome editing and significantly reduces the workload needed for clonal isolation by significantly reducing the number of cells that must be screened in order to identify and isolate the desired cell line.

4. Discussion

The ability to easily modulate the sequence specificity of the Cas9 nuclease by simply changing the 20 nucleotide targeting sequence of the gRNA offers significant versatility in delivery options over other nucleases that have been utilized for genome editing, such as zinc finger nucleases and TAL effectors. Now, researchers are able to choose from cost-effective and rapid design options by formulating the nuclease as either plasmid DNA, pre-made mRNA or purified protein. The design versatility is enabled by rapid production of the guide RNA component. Until recently, the gRNA was generally produced via cloning of a template sequence into a plasmid vector or vectors and expressing the Cas9 and gRNA *in vivo*. We describe a streamlined protocol where gRNA design and template construction is facilitated by synthesis of two short single stranded oligonucleotides. The oligonucleotides are incorporated into gRNA templates via a short PCR reaction followed by conversion to gRNA by *in vitro* transcription. Target-specific oligos can be designed, ordered, and converted to purified gRNA in as little as two days. On the second day, the gRNA is formulated with either Cas9 mRNA or protein, and immediately used to transfect cells. The entire process consists completely of liquid handling and enzymatic reaction steps, which make it amenable to higher throughput gRNA production and transfection in multi-well plates.

We compared the streamlined gRNA workflow across the three delivery options and found that in general, Cas9 RNPs offered superior indel production efficiency in most of the cell lines we used as a test bed. It is currently not clear why Cas9 RNP and total RNA formulations perform as they do but a factor could be overall size of the lipid complexes, the controlled complexing of the Cas9 protein and gRNA *in vitro*, the ability of Cas9 protein to protect the gRNA from cellular degradation, and the elimination of DNA-based cellular toxicity. In relation to plasmid delivery, Cas9 introduced as a Cas9 RNP or mRNA appears in the cell at low but evidently functional levels and is cleared rapidly. The rate of protein cas9 clearance and cleavage kinetics appears to be related to the cell lines used as we saw faster evidence of cutting and faster clearance in mouse ESCs than in HEK293FT cells. This variation in cleavage kinetics is supported by previous results in K562 where >20% cleavage activity was observed within 8 h (Kim et al., 2014) and U2OS where 7.8% cleavage activity was observed with the first 3 h (Zuris et al., 2014). However there are differences in the amount of protein used and the delivery method (electroporation or lipid-mediated) and further study is needed. We also saw variations in how double strand breaks at the same HPRT loci were repaired between Jurkat

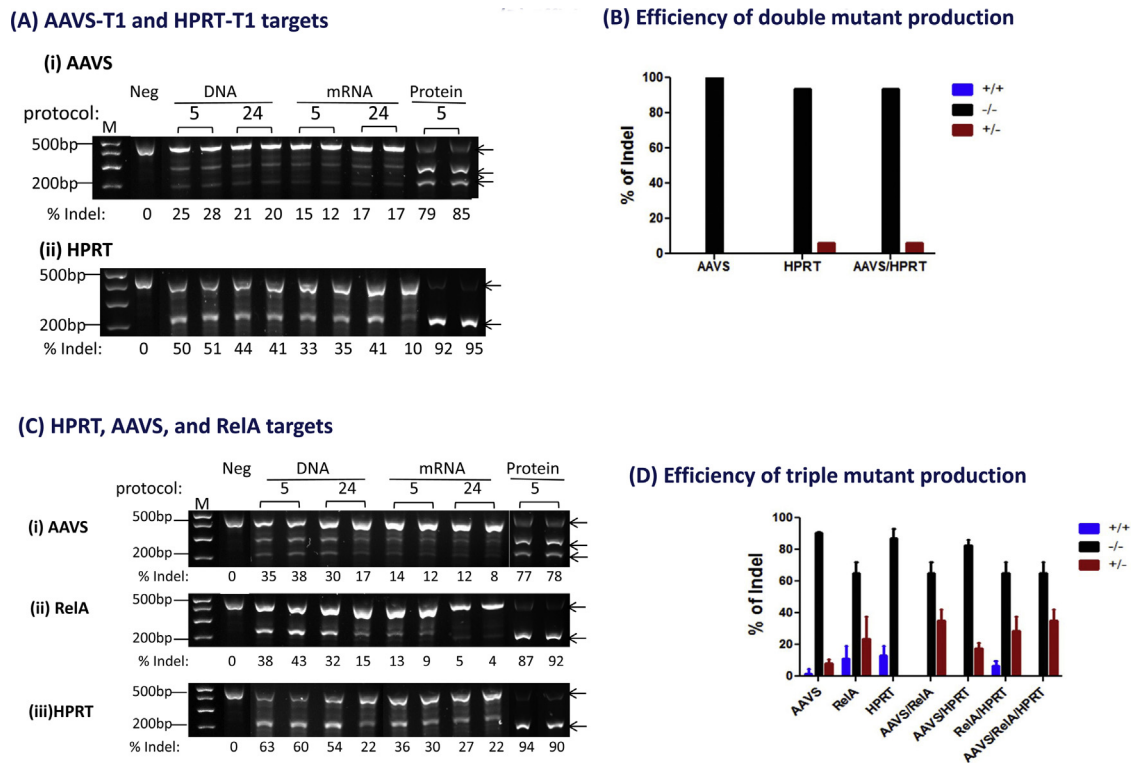


Fig. 5. Multiple gene editing in the human genome. Jurkat T cells were cotransfected with either a Cas9 plasmid pool, a Cas9 mRNA/gRNA pool or Cas9 RNP complexes targeting AAVS1 and HPRT targets (A) or AAVS, RelA and HPRT targets (C). GCD assays were performed for each locus at 48 h post transfection. Cell aliquots were then subjected to clonal isolation by serial dilution. After clonal expansion, each locus was PCR-amplified from each clonal cell line. The PCR product was then cloned into a plasmid vector and the percentage of indel mutation was determined by sequencing of eight individual *E. coli* colonies. Quantitation of double mutants for AAVS1 and HPRT was based on 16 clonal cell lines (B), whereas quantitation of triple mutants of AAVS, RelA and HPRT was based on a total of 53 clonal cell lines derived from three independent experiments (D). For individual loci, ++ indicates homozygote wildtype, +/- heterozygote, and -/- indicates heterozygote knock out where each allele has a different indel. For multiple loci, such as AAVS/HPRT, +/- indicates homozygote wildtype for all alleles targeted, +/- indicates heterozygote knock out of at least one allele, and -/- indicates heterozygote knock out of all alleles.

T and human iPSCs. Our results along with previous work (Mali et al., 2013; Wang et al., 2014), suggests that different cell lines may have variations in how they conduct NHEJ. The fast turnover of cas9 RNP and mRNA compared to the long persistence of cas9 expressed from plasmid could reduce the opportunity for off-target binding and cleavage. In addition, many off-target sites are cut with lower efficiency than the on-target site (Jinek et al., 2012). Previous research (Kim et al., 2014; Zuris et al., 2014) also saw reduced off-target cleavage for Cas9 RNP compared to plasmid. Much progress has been made to reduce or eliminate off-target cleavage in CRISPR systems, such as use of paired Cas9 nickases and dimeric ‘dead Cas9’ FokI fusions, which has been shown to reduce off-target activity by 50–1500-fold (Guilinger et al., 2014; Tsai et al., 2014). Perhaps delivery of these tools via Cas9 RNPs would lead to even higher specificity while retaining high activity levels.

In this work, we were also able to multiplex three Cas9 RNP species targeting separate loci in Jurkat T cells while achieving high levels indel production at all three loci. Further, we observed high rates of biallelic modification at two diploid alleles (AAVS1 and RelA) in these experiments even when also modifying a third haploid locus (HPRT) at similarly high levels. Taken together, the high rates of biallelic modification in cell populations suggest that employing Cas9 RNP delivery would significantly simplify the workflow by facilitating the selection of multigene knockout cell lines from a single experiment. Additionally, the system could be adapted by varying the dosages of individual Cas9 RNP species to better interrogate multigenic disorders.

We performed a survey of eleven commonly used mammalian cell lines comparing CRISPR delivery via plasmid, Cas9

mRNA/gRNA, and Cas9 RNP (Table 1) and found that Cas9 mRNA/gRNA or Cas9 RNPs were superior to plasmid delivery in all cell lines tested. Delivery of these reagents via electroporation offered the highest target-specific indel production under the conditions tested. In all but one case (NHEK cells), Cas9 RNP outperformed Cas9 mRNA/gRNA and in human CD34+ cord blood cells, Cas9 RNP delivered via electroporation was the only method that yielded a significantly robust editing solution. With both lipid-mediated transfection and electroporation, optimization of transfection conditions for each cell line is necessary for achieving the best cleavage efficiency.

5. Conclusion

We describe here a streamlined approach to the mammalian genome engineering workflow that takes as few as three days to modify mammalian genomes from CRISPR target design through evaluation of genome editing. To achieve high mutagenesis efficiencies in hard-to-transfect cells, we used a systematic approach to optimize transfection conditions where we compared delivery of CRISPR editing tools via plasmid DNA, Cas9 mRNA/purified gRNA formulations, and pre-complexed Cas9 RNPs. We found Cas9 mRNA/gRNA and Cas9 RNP performance superior to ‘all-in-one’ plasmid DNA constructs in the variety of cell lines analyzed in this work. Most likely due to the high efficiency of Cas9 RNP delivery, we were able to efficiently modify the genome at multiple loci simultaneously, thereby reducing the workload for downstream clonal isolation in schemes where more than one gene knock-out is desired. Further, we found that delivery of Cas9 RNPs to cell lines

considered hard to transfect (Jurkat, iPSC, CD34+) via electroporation could yield high levels of locus specific modification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jbiotec.2015.04.024>

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