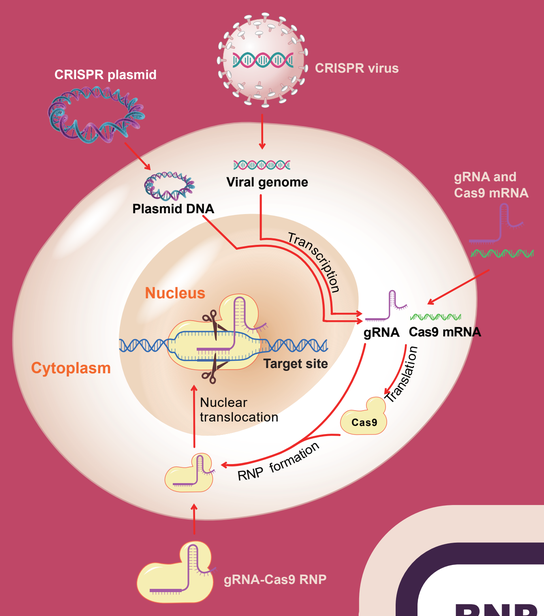
VectorBuilder

Optimizing approaches for CRISPR delivery

Abstract

The breadth and complexity of options for CRISPR approaches is ever-growing, and knowing which approach and delivery option is most appropriate is increasingly difficult. We have developed and optimized approaches for viral and non-viral delivery of CRISPR components in both in vitro and in vivo systems. By increasing efficiency of lentivirus packaging, mRNA production and encapsulation, and RNP/donor vector electroporation, we have made delivery of CRISPR components more dynamic and powerful. This allows researchers to utilize CRISPR approaches that best address their study question in a time- and cost-effective manner.

Introduction



CRISPR/Cas9 systems allow for targeted editing at the genomic level for a variety of applications. While gene knockouts are one of the most commonly used CRISPR applications, a variety of both forward and reverse genetics approaches are available, including up-regulation, down-regulation, library construction, and stable cell line generation.

Additionally, multiple options have been developed for introduction of CRISPR components to target cells, including viral transduction, IVT mRNA transfection, and electroporation of the ribonucleoprotein (RNP). Which delivery method is most appropriate depends on the identity of the target cells, their location (in vitro or in vivo), and the type of application (knockout, knockin, knockdown, or point mutation).

Methods

Cas9 and gRNA cloning, preparation, and packaging: Single or dual gRNAs are designed using VectorBuilder's independent gRNA database and cloned into vector backbones based on delivery system. Cas9 may be cloned into the same vector or a separate vector. Cas9 vectors may then be packaged into virus (lentivirus, AAV, etc), transcribed into mRNA and packaged into lipid nanoparticles, or translated into protein for RNP electroporation. gRNAs are introduced via viral transduction or non-viral transfection, and cells are screened for genome editing.

Transduction of lentiviral vectors for knockout, knockdown, or up-regulation

Advantages
- Efficient transduction in most cell types
- Effective in vivo and in vitro
- High efficiency integration

Limitations
- Technically complex
- Potential of continued activity of Cas9

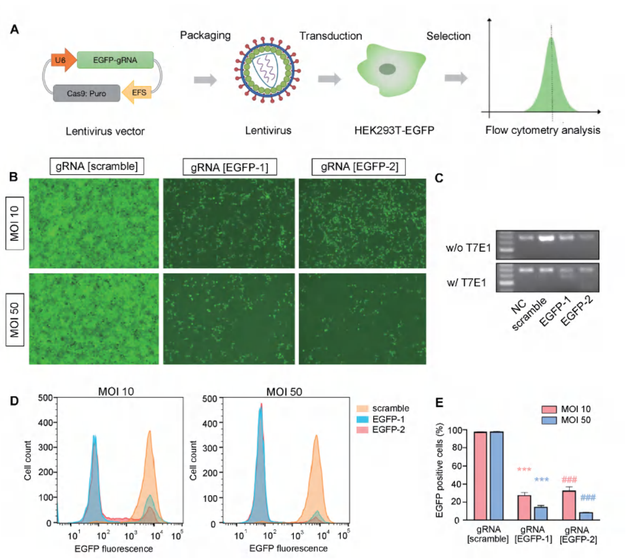


Figure 1. Gene editing with the all-in-one lentivirus-based CRISPR system. (A) Lentivirus vectors expressing Cas9:T2A:Puro and EGFP-targeting or scramble gRNA were packaged into the corresponding lentiviral particles and transduced into HEK293T cells stably expressing EGFP at MOI 10 or 50. Antibiotic selection with puromycin (Puro) was performed to isolate positively transduced cells. (B) EGFP in the transduced cells was observed under microscopy (100X). (C) The gRNA-targeted region was PCR amplified from the genomic DNA of non-transduced cells (NC), cells transduced with scramble gRNA, gRNA [EGFP-1], or gRNA [EGFP-2]. The gene editing was confirmed using the T7E1 assay. (D) The EGFP positive cells were quantified using flow cytometry. (E) The ratios of EGFP positive cells were decreased to 28-32% or 8-14% after editing using lentivirus at MOI 10 or 50, respectively. ***P<0.001, gRNA [EGFP-1] vs gRNA [scramble], ###P<0.001, gRNA [EGFP-2] vs gRNA [scramble], ANOVA with Dunnett's post hoc test.

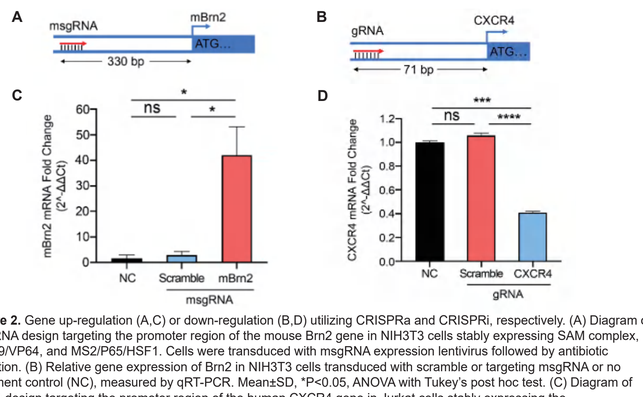


Figure 2. Gene up-regulation (A,C) or down-regulation (B,D) utilizing CRISPRa and CRISPRi, respectively. (A) Diagram of msgRNA design targeting the promoter region of the mouse Brn2 gene in NIH3T3 cells stably expressing SAM complex, dCas9/VP64, and MS2/P65/HSF1. Cells were transduced with msgRNA expression lentivirus followed by antibiotic selection. (B) Relative gene expression of Brn2 in NIH3T3 cells transduced with scramble or targeting msgRNA or no treatment control (NC), measured by qRT-PCR. Mean±SD, *P<0.05, ANOVA with Tukey's post hoc test. (C) Diagram of gRNA design targeting the promoter region of the human CXCR4 gene in Jurkat cells stably expressing the dCas9/KRAB/MeCP2 transcriptional repressor complex. Cells were transduced with gRNA expression lentivirus followed by antibiotic selection. (D) Relative CXCR4 gene expression in Jurkat cells transduced with scramble or targeting gRNA or no treatment control (NC), measured by qRT-PCR. Mean±SD, ***P<0.001, ****P<0.0001, ANOVA with Tukey's post hoc test.

IVT mRNA transfection in vitro and in vivo

Advantages
- Optimizations for high level expression
  - UTR, Kozak, Codon optimization
- Capability of tissue targeting
- Transient expression of Cas9

Limitations
- Linearized plasmid required
- Sense and antisense not possible from single vector

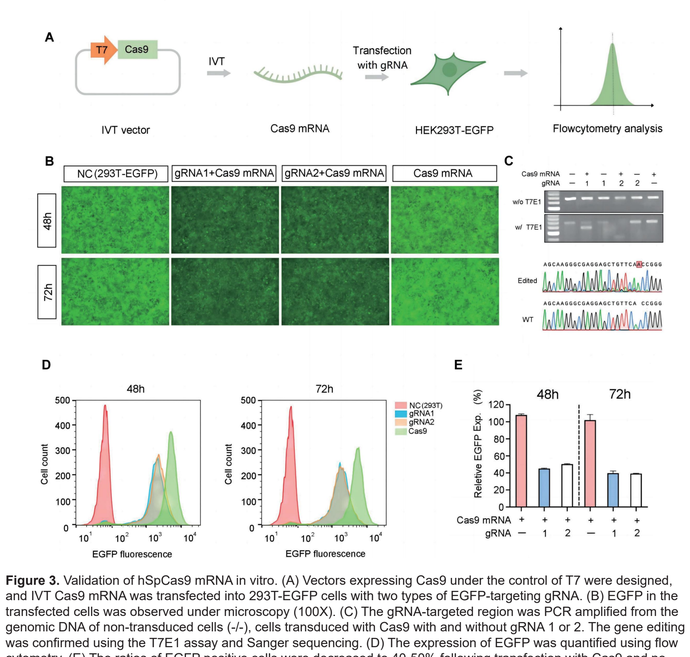


Figure 3. Validation of hSpCas9 mRNA in vitro. (A) Vectors expressing Cas9 under the control of T7 were designed, and IVT Cas9 mRNA was transfected into 293T-EGFP cells with two types of EGFP-targeting gRNA. (B) EGFP in the transfected cells was observed under microscopy (100X). (C) The gRNA-targeted region was PCR amplified from the genomic DNA of non-transduced cells (-/-), cells transduced with Cas9 with and without gRNA 1 or 2. The gene editing was confirmed using the T7E1 assay and Sanger sequencing. (D) The expression of EGFP was quantified using flow cytometry. (E) The ratios of EGFP positive cells were decreased to 40-50% following transfection with Cas9 and no gRNA (control) or gRNA 1 or 2.

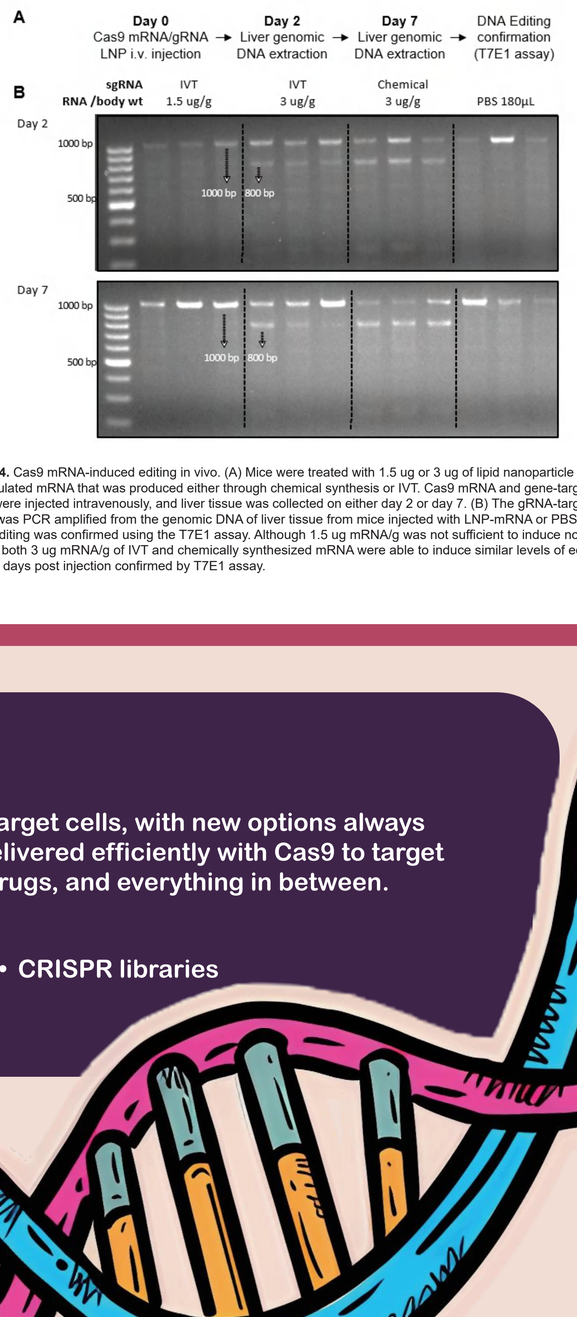


Figure 4. Cas9 mRNA-induced editing in vivo. (A) Mice were treated with 1.5 ug or 3 ug of lipid nanoparticle (LNP) encapsulated mRNA that was produced either through chemical synthesis or IVT. Cas9 mRNA and gene-targeting gRNA were injected intravenously, and liver tissue was collected on either day 2 or day 7. (B) The gRNA-targeted region was PCR amplified from the genomic DNA of liver tissue from mice injected with LNP-mRNA or PBS (control). Gene editing was confirmed using the T7E1 assay. Although 1.5 ug mRNA/g was not sufficient to induce noticeable editing, both 3 ug mRNA/g of IVT and chemically synthesized mRNA were able to induce similar levels of editing both 2 and 7 days post injection confirmed by T7E1 assay.

RNP-mediated knockout or knockin for stable cell lines

Advantages
- HDR efficiency of 30-50% with proprietary donor vector design and delivery
- Fast Cas9 activity independent of promoter
- Non-viral electroporation with minimal off-target effects and low cytotoxicity

Limitations
- Requires expensive equipment
- Limited use in vivo

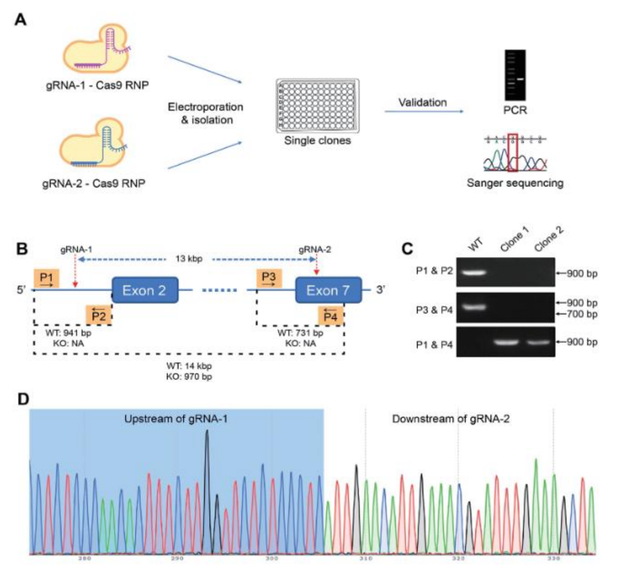


Figure 5. Generating homozygous CD274 knockout (KO) mutants using the gRNA-Cas9 ribonucleoprotein (RNP) approach. (A) The editing RNP is electroporated into target cells, and single clones are isolated and screened. The genotypes of the candidates are validated using PCR and Sanger sequencing. (B) In this case study of editing a murine colon adenocarcinoma cell line, cells were electroporated with RNP binding to two sites on the targeted gene to KO a 13-kbp region. Four primers, P1 to P4, were used in three PCR to differentiate KO and WT clones. Based on the (C) PCR results, clone 1 are validated to be homozygous KO mutants, which is also confirmed by (D) sequencing results.

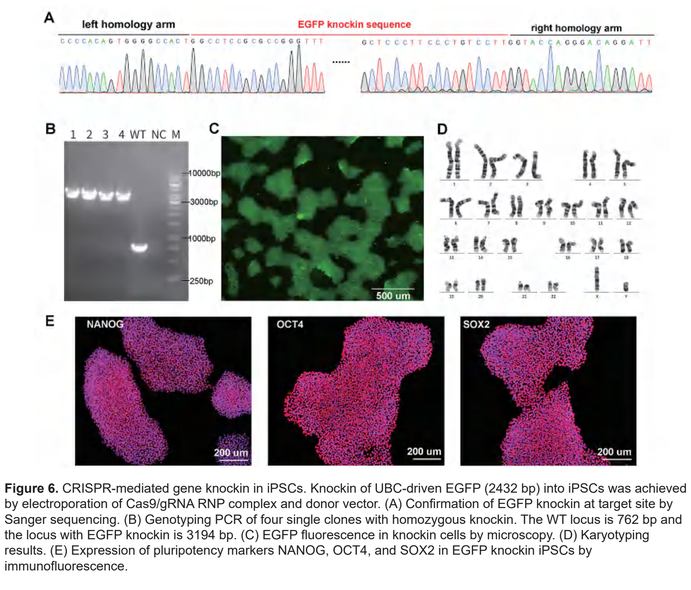


Figure 6. CRISPR-mediated gene knockin in iPSCs. Knockin of UBC-driven EGFP (2432 bp) into iPSCs was achieved by electroporation of Cas9/gRNA RNP complex and donor vector. (A) Confirmation of EGFP knockin at target site by Sanger sequencing. (B) Genotyping PCR of four single clones with homozygous knockin. The WT locus is 762 bp and the locus with EGFP knockin is 3194 bp. (C) EGFP fluorescence in knockin cells by microscopy. (D) Karyotyping results. (E) Expression of pluripotency markers NANOG, OCT4, and SOX2 in EGFP knockin iPSCs by immunofluorescence.

Conclusions

Researchers can utilize the best CRISPR approach for their system and target cells, with new options always being developed. Ensuring gRNA is designed with high specificity and delivered efficiently with Cas9 to target cells is imperative for the study of gene function, development of novel drugs, and everything in between.

VectorBuilder offers a variety of CRISPR related products and services:

- Vector design and cloning
- Virus packaging
- IVT mRNA and LNP production
- Stable cell line engineering
- CRISPR libraries

Want to learn more?
Design your own custom vectors in minutes or visit us to talk all things CRISPR!