Optimized Methods for Gene Disruption Using CRISPR/Cas9 Editing in Cell Culture With IDT gBlocks® Gene Fragments

Ashley M Jacobi, Garrett R Rettig and Mark A Behlke
Integrated DNA Technologies, Coralville, IA, USA

Introduction
Effective gene editing using the CRISPR/Cas9 system requires efficient expression of both the Cas9 protein and targeting RNAs (in the simplest form, a single fusion guide/tracr ‘sgRNA’). In spite of optimizing a variety of transfection reagents, we consistently observed poor transfection efficiency of large plasmids which express both HEK293 cell line expresses constant low levels of Cas9 protein and the targeting sgRNA (efficiencies of 5-40% were seen, even in easy-to-transfect HEK293 cells where short plasmids can give 90% efficiency). The Cas9 plasmid was decreased in size by removing the sgRNA expression cassette and used to create a HEK293 cell line that stably expresses Cas9. The target-specific guide was expressed in a short linear gBlocks® Gene Fragment. This system simplifies gene targeting experiments and addresses the observed problem of low transfection efficiency. An optimized protocol was developed to transfect U6-sgRNA gBlocks Gene Fragments for CRISPR/Cas9-mediated editing of genomic DNA. Editing efficiency can be rapidly and quantitatively assessed using PCR heteroduplex product degradation by a mismatch endonuclease (such as T7 endonuclease 1) followed by analysis with semi-automated capillary electrophoresis (Fragment Analyzer™, Advanced Analytical).

Experimental Setup
A sgRNA targeting human HPRT1 was made as a CRISPR expression cassette using gBlocks® Gene Fragments (dsDNA) or as a phosphatase-treated in vitro transcribed RNA (i.e., ssRNA). To obtain a high yield of dsDNA for optimization studies, the HPRT sgRNA gBlocks fragment was PCR amplified using KAPA HiFi Polymerase (KAPA Biosystems) and amplicons were purified using Qiagen PCR Purification Spin Columns (Qiagen). For IVT synthesis, Ultramer® DNA oligos containing the T7 promoter region and a fused HPRT guide/tracr sequence were synthesized by IDT. Complementary strands were annealed and in vitro transcribed using T7 RNA Polymerase from the MEGAClear Kit (Ambion/Thermo Fisher Scientific) then purified using the MEGAClear Kit (Ambion/Thermo Fisher Scientific). Antarctic phosphatase (NEB) was used to remove the 5' triphosphate and the final IVT product was verified via electrospray ionization mass spectrometry (ELI-MS). The HPRT1 targeting gBlocks fragments and IVT constructs were reverse transfected at 30, 3, and 0.3 nM into HEK293 cells stably expressing wild-type Cas9. Transfections were done in a 96-well plate using 0.75 µL RNAiMAX (Thermo Fisher Scientific) per well. Genomic DNA was isolated after 48 hrs by lysing cells with QuickExtract DNA Extraction Solution (Epicentre) and heating extracts at 65°C for 15 min followed by 95°C for 15 min. 15 nM of genomic DNA (gDNA) isolated from control and transfected cells was amplified using KAPA HiFi Polymerase to make a 692 bp amplicon spanning the region of interest. 300 ng of each PCR product was denatured by incubation at 95°C for 10 min then slowly cooled to room temperature to permit heteroduplex formation between the wild-type and edited gDNAs present. Annealed dsDNAs were incubated with T7 endonuclease 1 (T7E1, NEB) at 37°C for 30 min, resulting in cleavage fragments of approximately 251 bp and 441 bp from the heteroduplexes present. A 12-fold dilution of the digestion reaction was visualized by capillary electrophoresis on the Fragment Analyzer™ (Advanced Analytical) and percent cleavage was calculated. Total RNA was also isolated from parallel transfections at 24 hrs, converted to cDNA using Superscript® II Reverse Transcriptase (Thermo Fisher Scientific), and expression levels of innate immune system targets (PSF, ITIFM1, TRL3, RIGI, OAS2, AIM2 & ILI16) were quantified using qPCR (IDT).

Results
We can reliably detect and quantify the efficiency of CRISPR gene-editing in a mixed cell population. Using the HEK293-Cas9 cell line and transfection of target-specific U6-driven sgRNA gBlocks® Gene Fragments, gene editing events are detectable at 24 hrs and become abundant at 48 hrs post-transfection with little additional increase seen at 72 hrs. In one experiment, 30% cleavage in the T7E1 PCR amplicon heteroduplex assay correlated with a 74% mutation rate seen in Sanger sequencing of the same amplicons. Maximum activity was obtained with transfection of 3 nM of a dsDNA U6-driven gBlocks CRISPR cassette. In contrast, gene editing events directed by a ssRNA IVT sgRNA required >10 times more transfected material and was limited by toxicity, a common problem encountered when transfecting any long unmodified RNA. RT-qPCR showed evidence for stimulation of the innate immune system by the dephosphorylated IVT RNA at 24 hrs with up-regulation of PSF, IFITM1, TRL3, RIGI, OAS2, AIM2 & ILI16 mRNAs. gBlocks Gene Fragments are inexpensive, have a rapid delivery time of 2-4 days, and are effective triggers for Cas9-mediated gene editing using the protocol described herein.

Corresponding author: ajacobi@idtdna.com