A Streamlined Method for the Production, Screening, and Application of sgRNAs for CRISPR/Cas Gene Editing

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Abstract

The very recent development of CRISPR/Cas technology has opened new opportunities for genome editing, allowing a previously unattainable level of targeting flexibility, efficiency, and ease of use. The Type II CRISPR/Cas system has been engineered to perform robust genome modifications using an endonuclease (Cas9) and a single guide RNA (sgRNA) for target cleavage-sequence recognition. We have developed a streamlined method and reagent set for: 1) producing the sgRNA *in vitro*, 2) testing its *in vitro* cleavage efficiency in combination with the CRISPR/Cas9 complex, and 3) monitoring the efficiency of genome editing in cultured cells using the chosen sgRNA. We developed a very fast, comprehensive, and efficient method to transcribe sgRNA *in vitro*: Using a premade high-yield lyophilized PCR master mix, we were able to generate a PCR amplicon for use in an *in vitro* transcription reaction to produce any sgRNA in less than 3 hours. Once the sgRNA transcripts were produced, we utilized a highly optimized *in vitro* cleavage assay consisting of the *in vitro*-transcribed sgRNA, recombinant Cas9 protein and a PCR amplicon containing the sgRNA target sequence. The efficiency of target sequence cleavage was assessed through densitometric analysis of agarose gels. For those sgRNAs that produced >50% cleavage, the *in vitro*-transcribed sgRNA itself (or a mammalian expression vector encoding the selected sgRNA) and a mammalian expression vector encoding rCas9 were transfected into a number of different cell lines for modification of a genomic target sequence. We then assayed each transfected cell population for the presence of indel mutations that resulted from the CRISPR/Cas9 targeted cleavage event and subsequent NHEJ-based repair of the site. Our protocol, which includes a direct PCR/heteroduplex-cleavage assay of the cell pellet, reduced assay time from the 6 hr required by existing mismatch detection protocols to just 3.5 hr. In addition, this protocol provided increased mutation detection efficiency and demonstrated less sensitivity to buffers used in the PCR reaction than existing protocols. Taken together, our sgRNA production protocol, cleavage efficiency, and mismatch detection assays exactly follow the experimental flow of genomic editing using CRISPR/Cas technology, and should increase the success rate of this new and exciting technology.

Introduction

CRISPR/Cas9 gene editing technology has revolutionized the field of genome modification. This system uses a relatively robust RNA-guided endonuclease for targeting and cleaving desired genomic loci. In practice, only two elements are required, the Cas9 protein and an sgRNA consisting of a 23-base pair region complementary to the target locus (crRNA) linked to a canonical tracrRNA sequence. Currently, the crRNA sequence is chosen using one of several available web-based tools that analyze sequence specificity and assess the potential for off-target cleavage (1). Even with these types of tools, not every sgRNA will cleave the intended target sequence with equivalent efficiency. Because of this lack of consistency, it is necessary to screen a number of different sgRNAs for the desired activity. Screening, which includes vector construction, transfection of cells, and analysis of cleavage efficiency in cells, typically takes several days to perform. The focus of this study was to design an approach to expedite the sgRNA-testing procedure and create a protocol that can be completed in as little as one day. Our streamlined approach utilizes *in vitro* production and testing of sgRNAs prior to transfection, and subsequent PCR-based confirmation of genomic cleavage in cultured cells.

1. Hsu, P. *et al.* (2013) *Nature Biotechnology* **31**(9):827–832.

Conclusions

- We developed a robust *in vitro* system and reagents that provide a rapid method for the production and cleavage analysis of multiple sgRNAs.
- Our method produced high yields of intact sgRNA, amounts sufficient for multiple *in vitro* screening reactions and/or cell transfections.
- This sgRNA production method does not require cloning of the sgRNA into a vector prior to use.
- Compared to the Surveyor assay, our mutation detection protocol is several hours shorter, more sensitive, and less prone to non-specific cleavage.

Abbreviations

CRISPR—Clustered Regularly Interspaced Short Palindromic Repeat
sgRNA—single guide RNA
indel—insertion and deletion
NHEJ—non-homologous end joining
crRNA—CRISPR RNA
tracrRNA—trans-activating crRNA

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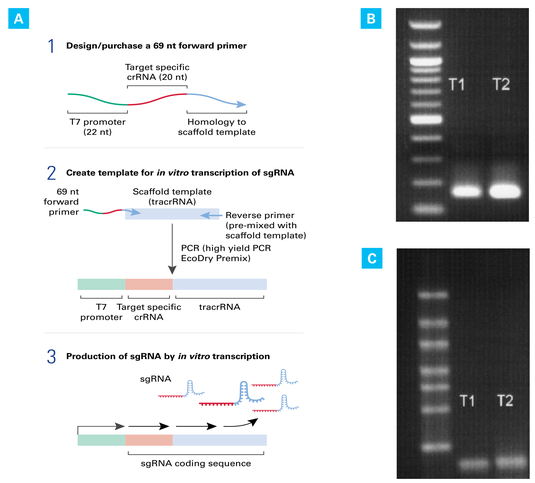
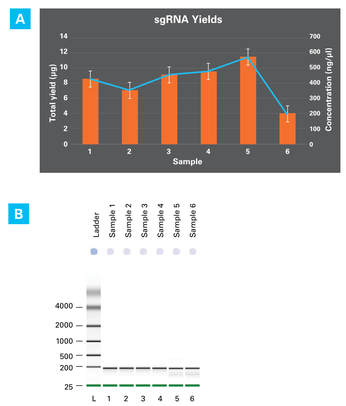
1 An Efficient System for sgRNA Production



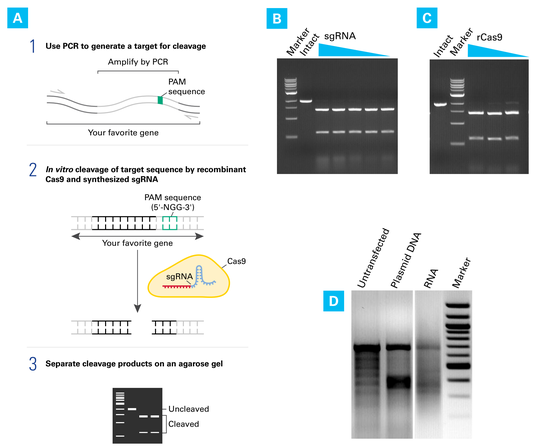
The Guide-it™ sgRNA *In Vitro* Transcription System provides a cloning-free method for sgRNA production. The Guide-it scaffold template is amplified using a 69-nt sgRNA-specific primer and a scaffold template-specific primer with EcoDry™ Premix, a high-yield lyophilized PCR master mix. This protocol allows an sgRNA template for *in vitro* transcription to be generated in <4 hr (Steps 1 & 2; **Panel A**). **Panel B.** For two different sgRNAs targeting AcGFP (T1 and T2), PCR amplification using this system yielded a single ~140 bp band. This PCR product was purified and 50 ng was used as a template for T7 polymerase-mediated *in vitro* transcription (**Panel A,** step 3). **Panel C.** RNA integrity and yield was confirmed for both templates (T1 and T2) by agarose gel electrophoresis; the *in vitro* transcription reaction yielded >4 µg sgRNA.

2 High Yields of Intact sgRNA Generated by *In Vitro* Transcription



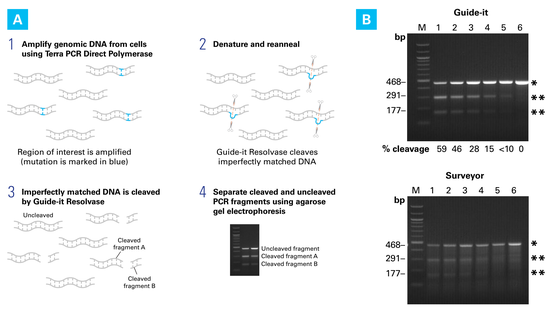
High yields of high quality sgRNA can be obtained with the Guide-it sgRNA *In Vitro* Transcription System. Several sgRNA preparations were produced (samples 1—6) according to the Guide-it *In Vitro* Transcription Kit protocol. Following *in vitro* transcription, the sgRNAs were treated with DNaseI and then purified by phenol/chloroform/isoamyl alcohol precipitation. **Panel A**. RNA yield was determined using a NanoDrop 2000. **Panel B.** RNA integrity was analyzed using an Agilent Bioanalyzer 2100. For all samples, >4 µg of intact sgRNA of the expected size was obtained.

3 An *In Vitro* Assay for Estimation of Cleavage Efficiency



***In vitro* analysis of cleavage efficacy of an sgRNA prior to use for Cas9-mediated gene editing. Panel A.** With the Guide-it sgRNA Screening system, a PCR template containing an sgRNA target site is combined with several candidate sgRNAs and recombinant Cas9 (rCas9) nuclease (Step 1). The cleavage efficiency of Cas9 nuclease is assessed and quantified by agarose gel electrophoresis (Steps 2 & 3). **Panels B & C.** A 2-kb fragment of human chromosome 10 was PCR-amplified and an sgRNA designed to cleave a target sequence within the fragment was generated. The target fragment (100 ng) was combined with varying amounts of sgRNA (1 µg to 0.25 µg) and rCas9 (500 ng), or varying amounts of rCas9 (1 µg to 0.25 µg) and the sgRNA (10 ng). Agarose gel analysis showed ~100% cleavage of the target fragment for almost all of the tested conditions, illustrating the robustness of the assay. **Panel D.** *In-vitro* transcribed sgRNA can be directly transfected into cells to modify a desired sequence. In this example, HT1080 cells containing a single copy of the AcGFP1 gene were transfected with an AcGFP1-specific sgRNA (1 µg) and mRNA for Cas9 (1 µg). Mutation detection analysis was performed 48 hr after transfection. Agarose gel electrophoresis of cleavage products indicated that transfection with RNA components resulted in genomic modification. Using RNA transfection rather than plasmid transfection minimizes the chance of random sequence integration into the genome.

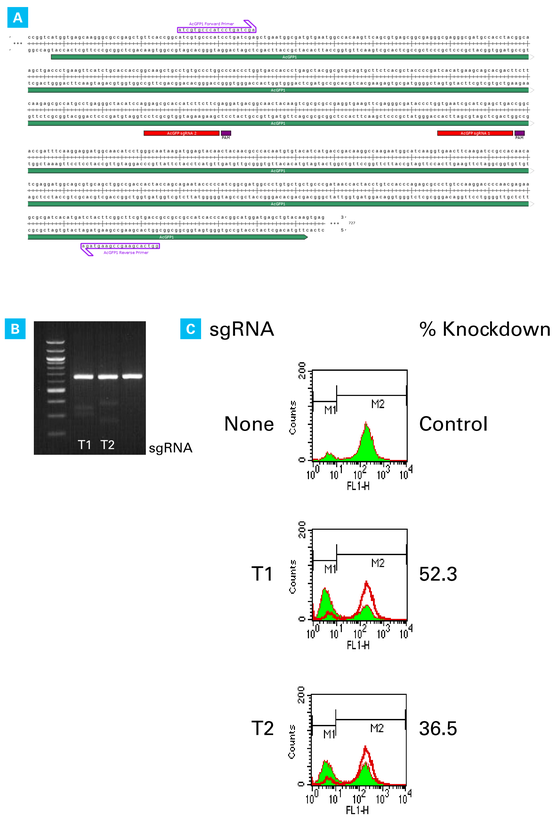
4 A PCR-Based Method to Confirm the Presence of Mutations



Comparison of mutation detection assays for detecting NHEJ-based repair in mammalian cells. Panel A. With our method, the target sequence is amplified directly from cells, without genomic DNA extraction/purification, using Terra™ PCR Direct Polymerase (Step 1). Then, the PCR products are melted and rehybridized, forming mismatched targets that can be cleaved by the Guide-it Resolvase (Steps 2 and 3). **Panel B.** To compare the Guide-it system and the Surveyor assay for detecting CRISPR/Cas9-introduced mutations in mammalian cells, 293T cells were transfected with plasmids encoding Cas9 and an sgRNA specific for the *AAVS1* locus. Transfected cells harvested 48 hr post-transfection were mixed with untransfected cells at varying ratios (refer to table). A DNA fragment containing the *AAVS1* locus was generated by PCR using Terra Direct Polymerase, and the products were purified and cleaved with either Guide-it Resolvase (Guide-it Mutation Detection Kit) or the Cel1 enzyme (Surveyor assay). Mutations were easily discernible when using the Guide-it kit (* : uncleaved; ** : cleavage products). In contrast, the Surveyor assay showed considerable smearing, making it difficult to determine cleavage efficiency and reducing the ability to detect lower levels of mutation.

	1	2	3	4	5	6
transfected	100	80	60	40	20	0
Non-transfected	0	20	40	60	80	100

5 Efficient Knockout of Single Copy AcGFP1 using CRISPR/Cas Gene Editing



Successful knockout of AcGFP1 in HT1080 cells using the CRISPR/Cas9 system. Panel A. Schematic of the AcGFP DNA sequence and the location of sgRNAs tested and primer placement for the mutation detection assay. HT1080 cells containing a single copy of AcGFP1 were transfected with 1.5 µg of plasmid DNA for Cas9 expression and 1.5 µg of a plasmid harboring one of two sgRNAs (T1 or T2) using Xfect™ Transfection Reagent. The cell population was assayed 6 days post-transfection for cleavage efficiency and loss of fluorescence. **Panel A.** Using the Guide-it Mutation Detection Kit, cleavage products were detected for both sgRNAs, indicating that both CRISPRs successfully disrupted the AcGFP1 locus. **Panel B.** The AcGFP1 disruptions were functionally relevant, as a subpopulation of non-fluorescent cells could be detected by FACS.

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