Nov 26, 2019

In vitro testing of guide RNA efficiency for CRISPR-mediated genome editing

Srinidhi Holalu[1], Angela Fang[1], Benjamin Blackman[1]

[1]Department of Plant and Microbial Biology, University of California, Berkeley

1 *Works for me* dx.doi.org/10.17504/protocols.io.8zshx6e

Mimulus

Benjamin Blackman
University of California, Berkeley

ABSTRACT

In CRISPR-mediated genome editing, guide RNAs (gRNAs) serve to target the Cas9 nuclease to an intended target genomic locus. Many gRNAs are not efficient in facilitating Cas9-mediated cleavage by introduction of double-strand breaks. Therefore, after designing possible gRNA sequences via web-based tools, it is critical to assess their cleavage efficiency at the target locus and identify the most efficient gRNA(s) *in vitro* before deploying in binary plasmids for genome editing *in planta*.

Invitro_test_gRNA_090419.pdf

MATERIALS

NAME	CATALOG #	VENDOR
HiScribe T7 Quick High Yield RNA Synthesis Kit - 50 rxns	E2050S	New England Biolabs
Cas9 Nuclease, S. pyogenes - 70 pmol	M0386S	New England Biolabs

MATERIALS TEXT

Reagents necessary:

- Zymo/Bioneer DNA cleanup kit
- CTAB extraction buffer for genomic DNA extraction
- Nuclease free water
- Equipment and reagents to run DNA products through standard agarose gel electrophoresis
- Phusion DNA polymerase kit or any high-fidelity DNA polymerase

SAFETY WARNINGS

For safety information and warnings, please refer to the SDS (Safety Data Sheet).

Cloning a template fragment from genomic DNA

1 Perform DNA extraction using CTAB method or any other methods that you may use routinely for your plant species.

2 Design primers flanking your target PAM sites (NGG). PCR to amplify around 500-600 bp of genomic/gene fragment around the target. Large size templates are easy to resolve on gel electrophoresis.

3 Clean PCR product using Zymo/Bioneer DNA cleanup kit and Sanger sequence to confirm that your PCR amplifications are intended gene. This will also help later for screening indel polymorphisms at PAM site.

Designing gRNA and synthesis of guide RNA using *in vitro* T7 kit: **Oligo Design**

4 Design targets for editing target exon(s) using gRNA design tool (http://crispr.mit.edu/ or any other portal). The following primers are then used to synthesize the DNA template to prepare gRNA for each target.

Oligo design

a. Primer 1 (specific for each guide):

a) TAATACGACTCCTATAGGG-(20 bp guide sequence)-gtttAagagctaTGCTGgaa

b) The three bolded G's are included in the transcript and required for transcription. If the guide-specific sequence starts with one or more G's, the same number of bolded G's can be deleted from the primer.

b. Primer 2 (always the same):

a) Reverse-gRNA Template:

aaaagcaccgactcggtgccacttttcaagttgataacggactagccttatttAaacttgctaTGCTGtttc CAGCAtagctctTaaac

Designing gRNA and synthesis of guide RNA using in vitro T7 kit: **PCR Double-stranded DNA synthesis for gRNA**

5

Set up the following PCR reaction:

- **30.5 µl** nuclease free water
- **10 µl** 5X Phusion buffer
- **1 µl** **10 Milimolar (mM)** dNTP
- **4 µl** primer 1 (**10 Micromolar (µM)**)
- **4 µl** primer 2 (**10 Micromolar (µM)**) Always Primer 2 (Reverse-gRNA Template)
- **0.5 µl** Phusion polymerase

Total **50 µl**

6

Program for PCR:

1. **98 °C** **00:01:00**
2. **98 °C** **00:00:30**
3. **55 °C** **00:00:15**

8X-10X cycles of these three steps

4. **72 °C** **00:01:00**
5. **72 °C** **00:05:00**
6. **4 °C** hold

7 Nano-drop to quantify the PCR product. Run a small aliquot of the PCR product out on a gel to check the bands. The resulting band should be **90 bp** (very small) and so it is necessary to run a **≥ 3 % agarose gel**.

8 Purify the PCR product using Zymo/bioneer DNA cleanup kit.

gRNA synthesis

9

Use HiScribe T7 High Yield RNA Synthesis Kit (NEB E2050S)

10

Set up reaction in PCR tube:

- 7 µl water (nuclease free)
- 5 µl NTP mix [M]10 Milimolar (mM) each
- 2 µl Template DNA (~ 100 ng – 500 ng)
- 1 µl T7 RNA Polymerase Mix

Total 15 µl

11

Incubate at 37 °C for 02:00:00 .

12

Move the reaction into 1.5 ml eppendorf tube.

13

Add 435 µl DNAase/RNAse free water.

14 Measure gRNA concentration on nanodrop. Note it down. (May not be accurate, as NTPs may inflate the nanodrop readings).

15

Optional to cleanup using any RNA clean-up kit. Dilute to [M]10 ng / µL by adding nuclease free water.

Cas9 nuclease assay and gel electrophoresis: **Incubation of template DNA with gRNA and Cas9 nuclease (NEB Cas9 M0386S)**

16

Set up reaction (include a negative RNA control):

- 7.5 µl water (nuclease free)
- 1.5 µl 10X Cas9 reaction buffer
- 1.5 µl [M]300 Nanomolar (nM) sgRNA ([M]10 ng / µL)
- 0.5 µl Cas9 Nuclease

Total 11 µl

17 

Pre-incubate for 00:10:00 at 37 °C .

18 

Add 4 µl substrate DNA (~ 50 ng – 100 ng total).

19 

Incubate 01:00:00 at 37 °C (or longer up to 01:30:00).

20 

Denature Cas9 by incubation at 80 °C for 00:10:00 .

21 Set up a control reaction including all the above ingredients without gRNA. In addition, different ratios of gRNA to Cas9 nuclease can also be setup to replicate the efficiency tests.

Cas9 nuclease assay and gel electrophoresis: **Gel Electrophoresis**

22 Prepare [M]1.1 % volume agarose gel.

23 

Mix the Cas9 incubated reaction with 2 µl of loading dye. Load the mixture on gel. If stain was not added during gel preparation, soak gel in staining buffer.

24 Run gel electrophoresis until the ladder resolves and check the cleavage of template DNA under UV illumination.