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In vitro Cleavage and Electrophoretic Mobility Shift Assays for Very Fast CRISPR

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[Abstract] CRISPR-Cas9 has transformed biomedical research and medicine through convenient and targeted manipulation of DNA. Time- and spatially-resolved control over Cas9 activity through the recently developed very fast CRISPR (vfCRISPR) system have facilitated comprehensive studies of DNA damage and repair. Understanding the fundamental principles of Cas9 binding and cleavage behavior is essential before the widespread use of these systems and can be readily accomplished *in vitro* through both cleavage and electrophoretic mobility shift assays (EMSA). The protocol for *in vitro* cleavage consists of Cas9 with guide RNA (gRNA) ribonucleoprotein (RNP) formation, followed by incubation with target DNA. For EMSA, this reaction is directly loaded onto an agarose gel for visualization of the target DNA band that is shifted due to binding by the Cas9 RNP. To assay for cleavage, Proteinase K is added to degrade the RNP, allowing target DNA (cleaved and/or uncleaved) to migrate consistently with its molecular weight. Heating at 95°C rapidly inactivates the RNP on demand, allowing time-resolved measurements of Cas9 cleavage kinetics. This protocol facilitates the characterization of the light-activation mechanism of photocaged vfCRISPR gRNA.

Keywords: CRISPR-Cas9, *In vitro*, Cleavage, Electrophoretic mobility shift assay, EMSA, Genome editing

[Background] Endonucleases, especially Cas9, from clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (CRISPR-Cas) adaptive immune systems (Marraffini *et al.*, 2015) have found widespread use as highly versatile tools in the biomedical sciences from genome editing, functional screening, to imaging (Adli *et al.*, 2018). Recently, highly resolved spatiotemporal control over Cas9 activity via very fast CRISPR (vfCRISPR) has enabled detailed studies of the DNA damage response through highly synchronized activation of Cas9 in space and time (Liu *et al.*, 2020). For all uses, characterization of these nucleases is essential and can be most readily accomplished *in vitro* (Singh *et al.*, 2016). Electrophoretic mobility shift assays (EMSA) are powerful tools to characterize protein-DNA binding that can be directly applied to determine the binding affinity of Cas9 to nucleic acids (Hellman *et al.*, 2007). In contrast, *in vitro* cleavage assays allow characterization of the Cas9 DNA cleavage mechanism. In this manuscript, we outline detailed protocols for both strategies to characterize the properties of the vfCRISPR light-inducible Cas9 system. We demonstrate how both EMSA and *in vitro* cleavage assays characterize the binding, speed, and efficiency of vfCRISPR, which paves the

way for its use in biological systems.

Materials and Reagents

1. 10 mg/ml SpCas9 (purified in-house from BL21 CodonPlus (DE3)-RIL cells [Agilent 230245], but can also be purchased from Integrated DNA Technologies) (Alt-R®, catalog number: 1081058, storage temperature: -80°C)
2. tracrRNA (Integrated DNA Technologies) (Alt-R®, catalog number: 1072533, storage temperature: -80°C)
3. Photocaged crRNA (cgRNA) from vfCRISPR targeting *PPP1R2* (BioSynthesis, sequence: GAC**UU**CC**U**CUAUGGUGGCGUGUUUUAGAGCUAUGCUGUUUUG; **U** corresponds to replacements of uracil with NPOM-dT photocaged nucleotides, storage temperature: -80°C)
4. HEK293T cells (ATCC, catalog number: CRL-3216, storage temperature: liquid nitrogen vapor)
5. Nuclease-Free Duplex Buffer (Integrated DNA Technologies, catalog number: 11-01-03-01, storage temperature: 4°C)
6. *PPP1R2* Fwd PCR primer (Integrated DNA Technologies, storage temperature: -20°C, sequence: 5'-GTTTCCGAGGCAGCAGTTG-3')
7. *PPP1R2* Rev PCR primer (Integrated DNA Technologies, storage temperature: -20°C, sequence: 5'-GCATGATAAACGTCATCGCCC-3')
8. DNeasy Blood & Tissue Kit (Qiagen, DNeasy, catalog number: 69504)
9. QIAquick PCR purification kit (Qiagen, QIAquick, catalog number: 28104)
10. Q5® High-Fidelity 2X Master Mix (New England BioLabs, Q5®, catalog number: M0492, storage temperature: -20°C)
11. NEBuffer™ 3.1 (New England BioLabs, catalog number: B7203S, storage temperature: -20°C)
12. Proteinase K, Molecular Biology Grade (New England BioLabs, catalog number: P8107S, storage temperature: -20°C)
13. E-Gel™ Agarose Gels with SYBR™ Safe DNA Gel Stain, 4% (Thermo Fisher, E-Gel™, catalog number: A45206, storage temperature: room temperature)
14. 100 bp DNA ladder (New England BioLabs, catalog number: N3231S)
15. HEPES (Sigma-Aldrich, catalog number: H3375)
16. KCl (Sigma-Aldrich, catalog number: P3911)
17. Glycerol (Sigma-Aldrich, catalog number: G5516)
18. NaOH (Sigma-Aldrich, catalog number: 221465)
19. Aluminum foil (Reynolds Wrap)
20. Nuclease-Free Water (not DEPC-treated) (Invitrogen™, catalog number: AM9932)

Equipment

1. C1000 Touch™ Thermo Cycler (Bio-Rad, model number: 1851148), or any alternative

thermocycler that can perform polymerase chain reaction (PCR)

2. E-Gel® iBase™ Power System (Thermo Fisher, E-Gel™, catalog number: G6465), or alternative gel electrophoresis system
3. JAXMAN 365 nm LED flashlight (Amazon, JAXMAN, https://www.amazon.com/JAXMAN-Ultraviolet-365nm-Detector-Flashlight/dp/B06XW7S1CS/)
4. Typhoon™ FLA 9000 (GE Healthcare), or any alternative gel imager that can image ethidium bromide or SYBR Gold agarose gels

Procedure

A. Generation of target DNA at *PPP1R2* for *in vitro* cleavage or EMSA

1. Purify genomic DNA (gDNA) from HEK293T cells using the DNeasy Blood & Tissue Kit following manufacturer's instructions. Elute in 200 µl AE provided by the kit. Store gDNA in -20°C.
2. Prepare PCR primer mixture by mixing 10 µM of Fwd primer with 10 µM of Rev primer, both diluted in water.
3. Set up PCR reaction.

Component	**Volume**
Nuclease-Free Water (NFW)	3 µl
Genomic DNA (10-50 ng)	1 µl
Fwd/Rev primer mixture (10 µM)	1 µl
Q5® High-Fidelity 2× Master Mix	5 µl
Total	**10 µl**

4. Start thermocycling protocol on the thermocycler.

Step	**Temp**	**Time**
Initial Denaturation	98°C	30 s
35 cycles	98°C	10 s
	68°C (*PPP1R2*)	10 s
	72°C	20 s
Final extension	72°C	2 min
Hold	4°C	Inf

5. Extract PCR-amplified target DNA with the QIAquick PCR Purification Kit following manufacturer instructions. Elute in 30 µl EB provided by the kit.

B. Prepare 10 ml of Cas9 Dilution Buffer

1. Make solution composed of 20 mM HEPES, 500 mM KCl, and 20% glycerol.
2. Add sufficient 1 M NaOH to bring pH to 7.5.

C. Prepare 10 µM Cas9

Dilute 5 µl of 10 mg/ml Cas9 with 25 µl of Cas9 Dilution Buffer to make 30 µl of 10 µM SpCas9.

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D. Anneal light-activatable crRNA with tracrRNA to form cgRNA

1. Resuspend photocaged crRNA and tracrRNA separately to 100 µM with Duplex Buffer.
2. Mix 3 µl of 100 µM photocaged crRNA with 3 µl of 100 µM tracrRNA in PCR tube.
3. Heat at 95°C for 3 min in the thermocycler with heated lid.
4. Cool on benchtop for 5 min.
5. Mix in 24 µl of Duplex Buffer to make 30 µl of 10 µM annealed cr/trRNA.

E. *In vitro* cleavage to test light activation capability of cgRNA

1. Mix the following components together in order. Thoroughly mix NFW and NEBuffer™ 3.1 before adding cgRNA and Cas9 and mix again. Prepare 11 identical volumes, all in separate PCR tubes.

Component	**Volume**
NFW	8.1 µl
NEBuffer™ 3.1	1 µl
10 µM cgRNA targeting *PPP1R2*	0.5 µl
10 µM Cas9	0.4 µl
Total	**10 µl**

2. Leave on benchtop (room temperature) for 30 min to form RNP complex. Cover with aluminum film because the photocaged gRNA are light-sensitive.
3. Add 2 µl (~60 fmol) of PCR-amplified *PPP1R2* target DNA to each RNP tube (11 in total). Mix well with pipette. For a highly efficient reaction, Cas9/cgRNA should be in at least 100-fold excess relative to target DNA. The target DNA should also be easily visible on an agarose gel with a gel imager.
4. Set up two thermocyclers – one to 37°C and the other to 95°C.
5. Move one tube to the 37°C thermocycler, open lid, and wait for 1 min. Turn on 365 nm LED flashlight 10 cm above tube for 1 s; then, immediately transfer tube to 95°C for 5 min and then to ice (which corresponds to '1 s' sample) (Figure 1).

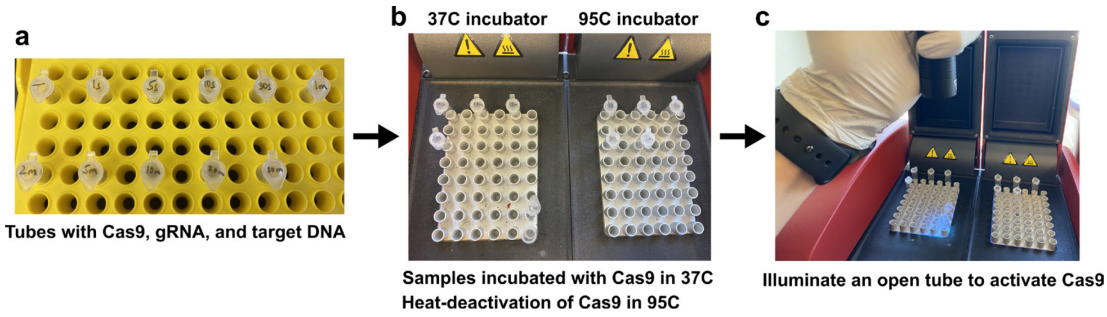

Figure 1. *In vitro* cleavage setup. (a) Cas9, gRNA, and target DNA are added to tubes labeled with the appropriate incubation times. (b) Tubes are first placed in the 37°C incubator. After light exposure and 37°C incubation for the specified time, each tube is transferred to the 95°C

incubator for heat-deactivation of Cas9. (c) Light exposure using a commercially available LED flashlight. The tube is first opened, the flashlight is held 10 cm above the tube, and the light is turned on for 30 s.

6. Repeat using three other tubes for 5 s, 10 s, and 30 s of light illumination (which corresponds to '5 s', '10 s', and '30 s' samples, respectively).
7. Move another tube to the 37°C thermocycler, open lid, and wait for 1 min. Turn on 365 nm LED flashlight 10 cm above tube for 30 s, turn off flashlight and incubate tube with cap closed for another 30 s; transfer to 95°C for 5 min and then to ice (which corresponds to '1 min' sample).
8. Repeat using five other tubes with 30 s light illumination, followed by 1.5 min, 4.5 min, 9.5 min, 19.5 min, and 29.5 min incubations at 37°C; transfer to 95°C for 5 min, and then to ice (which corresponds to '2 min', '5 min', '10 min', '20 min', and '30 min' samples, respectively).
9. The one remaining sample should not be exposed to light but directly moved to 95°C for 5 min and then to ice (which corresponds to 'no light' sample).
10. Add 0.5 µl of Proteinase K to all 11 tubes and incubate at 55°C for 15 min.
11. Load 5 µl of each tube (in ascending order of time-duration) mixed with 15 µl of water into each well of a 4% agarose E-Gel and run for 10 min. Add 100 bp ladder to the left-most lane. An alternative agarose gel electrophoresis system may also be used.
12. Visualize with Typhoon™ FLA 9000 using the SYBR Gold setting. An alternative gel imager may also be used (Figure 2).

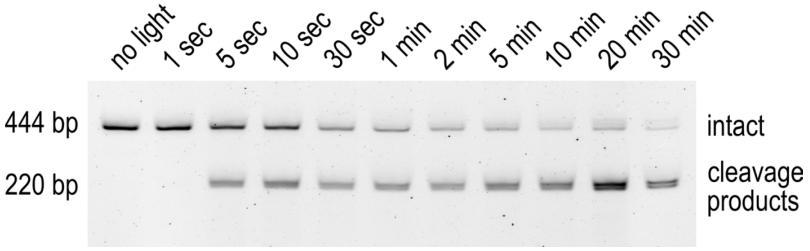

Figure 2. *In vitro* cleavage results. The top band migrating at 444 bp corresponds to the uncleaved PCR product of *PPP1R2*. The bottom band is formed by two unresolved bands, both migrating around 220 bp, which correspond to the cleavage products of the 444 bp top band. Each lane corresponds to either no light exposure or a different light exposure/incubation condition.

F. EMSA

1. Mix the following components together in order. Thoroughly mix NFW and NEBuffer™ 3.1 before adding cgRNA and Cas9 and mixing again. Prepare 2 identical volumes, both in PCR tubes as samples {B} and {C}.

Component	**Volume**
NFW	8.1 µl

NEBuffer™ 3.1	1 µl
10 µM cgRNA targeting *PPP1R2*	0.5 µl
10 µM Cas9	0.4 µl
Total	**10 µl**

2. Prepare sample {A} by replacing Cas9 and cgRNA with water (negative control).
3. Leave on benchtop (room temperature) for 30 min to form RNP complex. Cover with aluminum film because photocaged gRNAs are light-sensitive.
4. Add 2 µl (~60 fmol) of PCR-amplified *PPP1R2* target DNA to each of the three tubes. Mix well with pipette. For a highly efficient reaction, Cas9/cgRNA should be in at least 100-fold excess relative to target DNA. The target DNA should also be easily visible on an agarose gel with a gel imager.
5. Add 0.5 µl of Proteinase K to {C}, incubate at 55°C for 15 min.
6. Load 5 µl of each tube (in ascending order of time-duration) mixed with 15 µl water into each well of a 4% agarose E-Gel and run for 10 min. Add 100 bp ladder to the left-most lane. An alternative agarose gel electrophoresis system may also be used.
7. Visualize with Typhoon™ FLA 9000 using SYBR Gold setting. An alternative gel imager may also be used (Figure 3).

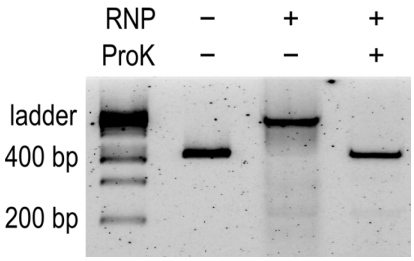

Figure 3. EMSA results. Lane 1 is the ladder; lane 2 is sample {A} (negative control without RNP); lane 3 is sample {B} (EMSA); lane 4 is sample {C} (Proteinase K digested sample). There is no cleavage product because the cgRNA was not exposed to any light, demonstrating that Cas9 with cgRNA binds to DNA even in the absence of light activation.

Acknowledgments

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Competing interests

The authors and Johns Hopkins University have filed patent application PCT/US20/57256 on the method of spatiotemporal control of Cas9 activities via cgRNA.

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