Identifying Anti-CRISPR Small Molecules via High-throughput Assay

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Method Article

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Abstract

There is a high demand for anti-CRISPR molecules to precisely control CRISPR-associated nucleases. Small molecule anti-CRISPRs can be easily delivered to cells and improve CRISPR editing outcomes by their fast action and temporal control of nuclease activity, though no assays currently exist for the high-throughput identification of these compounds. Here we report a high-throughput assay platform to rapidly identify multiple small-molecule inhibitors of SpCas9.

Introduction

SpCas9 is a programmable RNA-guided endonuclease used to induce site-specific DNA double-strand breaks, though their sometimes non-specific and excessive activity has been associated with poor editing outcomes.[1,2] The precision control of intracellular enzymes is accomplished using small molecules, as they are cell-permeable, fast-acting, easy to use and inexpensive.[3] Though a reliable enzyme assay is key to the successful identification of small-molecule inhibitors, assays for SpCas9 inhibitors are currently unavailable due to the low enzyme turnover that prevents signal amplification.[4,5] Their often-deemed chemically intractable DNA-binding domains, novel protein folds and extensive conformational changes complicate the rational design of small-molecule inhibitors.[6,7]

Here we report the construction of a Förster resonance energy transfer (FRET)-based cumulative activity assay (CAA) for SpCas9. This CAA can report all the catalytic steps of SpCas9, such as binding of the protospacer adjacent motif (PAM), conformational change, and cleavage of the target and non-target strand. This assay is based on the observation that while Cas9 is bound to the double-stranded DNA substrate following the DNA cleavage, the 5' distal non-target strand is weakly held by Cas9 and can be displaced via excess complementary single-stranded DNA.[4] In the CAA, the 5' terminus of the non-target strand in the DNA substrate is labeled with Alexa-Fluor 647 while the 3' terminus of the displacing single-stranded DNA is appended with a quencher. Following cleavage of the DNA substrate by active nuclease, an excess of the single-stranded DNA outcompetes the 3' strand of the substrate to anneal to the 5' strand, which quenches the fluorescence and provides an optical readout for SpCas9 activity. Thus, inhibitory compounds give rise to high fluorescence signals. This FRET assay is amenable to high-throughput screening to test a large number of chemical libraries.

Reagents

- SpCas9 substrate forward oligonucleotide (Table 1, synthesized by Integrated DNA Technologies)
- SpCas9 substrate reverse oligonucleotide (Table 1, synthesized by Integrated DNA Technologies)
- SpCas9 displacer oligonucleotides (Table 1, synthesized by Integrated DNA Technologies)
- SpCas9 prepared in-house

- sgRNA prepared in-house (Table 1)
- 10x assay buffer (200 mM Tris-HCl, pH 7.5, 1 M KCl, 50 mM $MgCl_2$)
- 500 mL conical tubes (Corning 431123)
- 384-well microplate, low flange black flat bottom, non-binding surface (Corning 3575)

Equipment

- Thermocycler
- Multidrop Combi Reagent Dispenser (Thermo Scientific)
- Compound pin transfer robot (optional, custom-designed by ICCB-Longwood Screening Facility)
- Envision Multimode Plate Reader (PerkinElmer)
- Humidified incubator set at 37°C.

Procedure

1. The general conditions for high-throughput screening are as follows, which are described in detail in the subsequent points:

- Final concentrations in assay buffer: SpCas9 substrate dsDNA (0.5 nM), displacer ssDNA (2.5 nM), SpCas9 (5 nM), and gRNA (6 nM) in 1x assay buffer (20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 5 mM $MgCl_2$)
- A compound plate is tested in duplicate, such that compounds inhibiting SpCas9 in both tests are identified as hits.

2. Prepare SpCas9 substrate dsDNA solution by annealing two complementary SpCas9 substrate oligonucleotides.

- Mix 5 µL of SpCas9 substrate forward oligonucleotide solution (100 µM), 5 µL of SpCas9 substrate reverse oligonucleotide solution (100 µM), 5 µL of 10x assay buffer, and 35 µL of water in a PCR tube.
- Anneal the two complementary oligonucleotides with a thermocycler. Heat to 95°C for 5 min, then cool to 25°C at a rate of 0.1°C/s.
- Dilute the resulting 10 µM substrate dsDNA solution to 1 µM using 1x assay buffer and store at −20°C.

3. Prepare SpCas9 displacer ssDNA solution.

· Mix 5 µL of SpCas9 displacer ssDNA solution (100 µM) with 95 µL of 1x assay buffer. Store this 5 µM solution at −20°C.

4. Prepare 2x RNP solution and 2x apo SpCas9 solution.

· In a single tube, dilute SpCas9 stock solution to 1 µM and gRNA stock solution to 1.2 µM in 1x assay buffer to form SpCas9:gRNA RNP complex. Incubate for 5 min at 4°C, then dilute the RNP solution to 10 nM with 1x assay buffer and store at room temperature. Conical tubes (500 mL) can be used for storing large amounts of solution.

· For the apo SpCas9 positive control (no SpCas9 activity), dilute the SpCas9 stock solution to 10 nM in 1x assay buffer.

5. Add RNP solution or apo SpCas9 solution to 384-well assay plates using a Multidrop Combi Reagent Dispenser.

· Transfer 25 µL of 2x RNP solution to wells for test compounds and negative controls.

· Transfer 25 µL of 2x apo SpCas9 solution to wells for positive controls.

· Prepare two assay plates for each compound, as the screening is done in duplicate.

6. Transfer compound libraries into the assay plates via pinning and perform the 1st counter-screen.

· Note: For the pinning, we used an Epson Compound Transfer Robot with the assistance of the ICCB-Longwood Screening Facility, though the pinning process should be modified based on the setup of each laboratory.

· Pin compounds or vehicle (stocked in 384-well plates) into the 384-well assay plate. To each well, 100 nL of compound solution (usually 10 mM or 5 mg/mL stocks in DMSO) is added.

· Cover and incubate the pinned plates at room temperature for at least 30 minutes.

· Measure Alexa-Fluor 647 fluorescence to rule out compounds that auto fluorescence in the presence of RNP (1st counter-screen).

7. Add dsDNA substrate and displacer ssDNA solution to the assay plates and perform the 2nd counter-screen.

· Prepare the 2x solution of substrate dsDNA and displacer ssDNA by diluting 1 μM substrate dsDNA solution from step 2 to 1 nM and diluting 5 μM displacer ssDNA solution from step 3 to 5 nM in 1x assay buffer. Conical tubes (500 mL) can be used for storing large amounts of solution.

· Using a Multidrop Combi Reagent Dispenser, transfer 25 μL of the solution to every well.

· Immediately after addition, measure the Alexa-Fluor 647 fluorescence to identify compounds that are auto fluorescent in the presence of all the assay components (2nd counter-screen).

8. Perform the SpCas9 reaction and obtain the primary screen result.

· Incubate the covered plates at 37 °C for 2.5 h in a humidified incubator to prevent the vaporization of liquids. Minimize plate stacking to more rapidly reach the target temperature.

· Measure the Alexa-Fluor 647 fluorescence (primary screen).

9. Analyze the data.

· Export the data from Envision. Using Microsoft Excel, calculate the Z-score using $[x - \mu] / \sigma$, where x is the signal from the sample, μ and σ are mean and standard deviation of the negative controls.

· Identify compounds with Z-score > 3 as potential hits for further testing in cell-based assays.

· Analyze counter-screen results to exclude auto fluorescent compounds.

Troubleshooting

Troubleshooting

· Insufficient amounts of the solutions: The solutions should be prepared in 10–20% excess to account for the void volumes arising from the use of Multidrop Combi Reagent Dispenser.

· RNP precipitation: Precipitates may be observed during the RNP formation. To avoid this, perform the initial RNP (1 μM) formation step for 5 min at 4°C and immediately dilute the solution to 10 nM. The RNP solution should be prepared just before use.

· Insufficient reaction: Avoid insufficient reactions by allowing the plates to reach the target incubation temperature as quickly as possible. Thus, minimize plate stacking inside the incubator. After the 2.5 h

reaction, the fluorescence intensities from the negative controls usually reach 30–40% of the positive controls. If this is not the case, extend the incubation time of the assay plates.

Time Taken

Times are given for the preparation of 20 assay plates, corresponding to the screening of 10 compound plates, and with the use of automated plat loading on the plate reader.

· Preparation of 2x RNP solution, 2x apo SpCas9 solution, and 2x substrate dsDNA and displacer ssDNA solution: approximately 20 min when starting from premade DNA stock solutions.

· Transfer of 2x RNP solution and 2x apo SpCas9 solutions to assay plates: approximately 30 min

· 1st counter-screen: approximately 30 min

· Pin transfer of compounds: approximately 30 min

· Transfer of 2x substrate dsDNA and displacer ssDNA solution: approximately 25 min

· 2nd counter-screen: approximately 30 min

· SpCas9 reaction time: 2.5 h

· Primary fluorescence measurement: approximately 30 min

· The overall experimental time can be reduced because the above steps can be performed simultaneously. For example, fluorescence reading can be done while performing liquid transfers for other plates.

Anticipated Results

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Supplementary Files

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