High-Affinity Protein-RNA Interactions

Determination of low-picomolar affinities of sgRNAs and crRNA/tracrRNAs for Cas9

Tatjana Theer#, Jochen Deckert#, Philipp Hadwiger#, Ingo Röhl#, Dennis Breitsprecher*, Nuska Tschammer*

*NanoTemper Technologies GmbH, Floessergasse 4, 81369 Munich

#Axolabs GmbH, Fritz-Hornschuch-Straße 9, 95326 Kulmbach

Abstract

Recent advances in genome engineering technologies based on the RNA-guided CRISPR endonuclease Cas9 are enabling systematic manipulation of genome function in a variety of organisms, ranging from bacteria and archaea to humans. Cas9 is guided to specific locations within a genome by a short RNA search string. Since genome editing leads to permanent modifications within a genome, the targeting specificity of Cas9 nucleases is of particular importance, especially for clinical application and gene editing.

In this work, we demonstrate the versatility of MicroScale Thermophoresis (MST) to determine the binding affinities of various single-guide RNA (sgRNA), duplex of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) constructs with Cas9. The MST assay is superior to classical methods like electrophoretic mobility shift assay (EMSA) because it allows effortless Kd determination free in solution. Additionally, MST provides excellent sensitivity while consuming a small amount of non-hazardous fluorescent-labeled oligos or protein. We analyzed several modified RNAs, some of which were labeled with the fluorophore Cy5. Using NanoTemper Technologies Monolith NT.115Pico instrument, we determined the affinities of Cas9 with RNAs differing in length and chemical modification pattern. All interactions were in the lower picomolar range, the highest measured affinity was 1.0 pM.

Introduction

CRISPR/Cas9 genome editing technologies are one of the most significant discoveries of this decade with a vast potential in human therapeutics, food science and other fields. Recently, the US Department of Agriculture approved the cultivation and commercialization of CRISPR/Cas9 gene-edited button mushroom (Agaricus bisporus).[1]

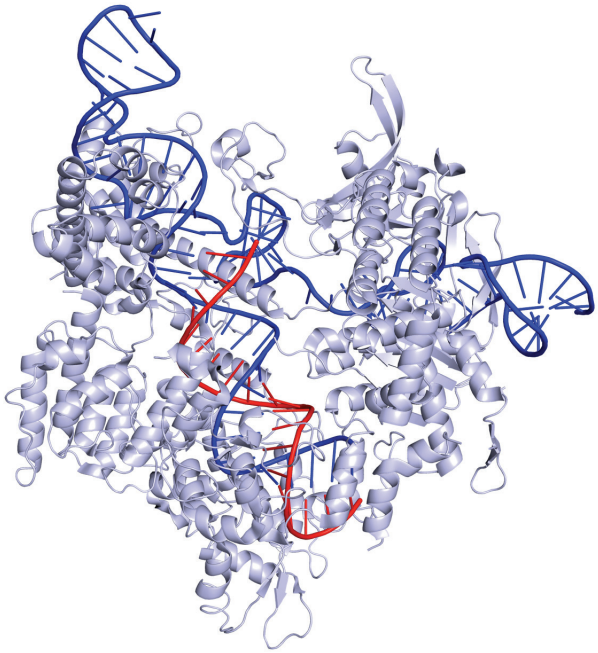

Figure 1. Crystal structure of Streptococcus pyogenes *Cas9 in complex with guide RNA (blue) and target DNA (red) (PDB ID: 4OO8)*[2]

Furthermore, an advisory committee at the US National Institutes of Health (NIH) approved a proposal to use CRISPR-Cas9 to help augment cancer therapies that rely on enlisting a patient´s T cells. Clustered regularly interspaced short palindromic repeats (CRISPRs) are segments of prokaryotic DNA with short repeating segments. The CRISPR-Cas systems, as exemplified by CRISPR-Cas9 (Cas9 = CRISPR associated protein 9), are adaptive immune systems used by bacteria and archaea to defend against viral infections. Cas9 is an RNA-guided DNA endonuclease from Streptococcus pyogenes, which utilizes Cas9 to memorize[3] and later interrogate and cleave foreign DNA.[4] The combination of Cas9 from S. pyogenes and a synthetic single-guide RNA (sgRNA) containing the guide region, in addition to a duplex of CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA) has been used as a two-component programmable system for genetic manipulation in various organisms.[5-7] The process of optimizing the CRISPR/Cas9 system for therapeutic approaches requires accurate quantification of the binding affinities of diverse RNA constructs to Cas9.

In this Application Note, we demonstrate that MST offers unprecedented advantages over classical methods for DNA/RNA/protein interaction studies, such as immuno-precipitation or electrophoretic mobility shift assay (EMSA). Immunoprecipitation studies are laborious and require the use of antibodies. Classical radioisotope or biotin/streptavidin-based EMSA approaches require several steps, the use of hazardous material, problematic waste disposal and inconvenient detection. Additionally, a chemical equilibrium cannot be reached in an EMSA assay, and RNA dissociation from protein during electrophoresis can prevent detection of an interaction. The MST assay offers excellent sensitivity with minute amounts of non-hazardous fluorescent-labeled oligonucleotides or proteins, the protein/RNA complexes are detected

free in solution, and a chemical equilibrium is reached. Unlike other approaches, a Kd value can be measured in less than 10 min using NanoTemper Technologies Monolith NT.115^{Pico}, or less than 2 min using the Monolith NT.Automated. Importantly, binding affinities in picomolar range of either modified sgRNA or hybridized crRNA/tracrRNA (so-called dual guide RNA (dgRNA)) are easily and precisely determined using MST.

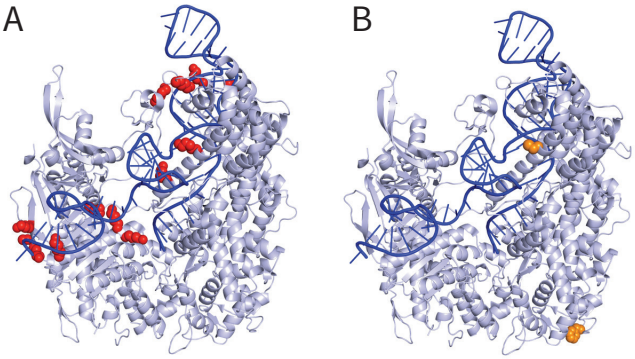

Figure 2: Crystal structure of catalytically-active S. pyrogene *Cas9 in complex with single-guide RNA[8]. A) Lysines (red spheres) in the contact with the sgRNA; other lysines are not shown for clarity. B) Position of cysteines (orange spheres).*

Results and Discussion

In order to measure the binding affinities of various RNAs with Cas9, we followed two different approaches. The first approach was the direct fluorescent labeling of Cas9. Cas9 has 150 lysine residues, some of which are involved in RNA/DNA binding (Figure 2A).[8]

Hence, lysine-directed NHS labeling was not amenable to our study. Instead, two cysteine residues, one residing on the outer rim of the binding crevice[8] (Figure 2B), were chosen as suitable targets for fluorescent labeling using maleimide chemistry.

We fluorescently labeled Cas9 using the cysteine-directed maleimide-NT647 dye. Since we anticipated very high affinities of the RNA for Cas9 protein, we performed MST measurements on the Monolith NT.115^{Pico} instrument, which enables determination of K_d values in the picomolar range. We combined 50 pM labeled protein with RNA in a serial dilution series, with 10 nM as the highest RNA concentration. Using this approach, we were able to successfully measure the affinities of various sgRNA for Cas9 (Figure 3, Table 1).

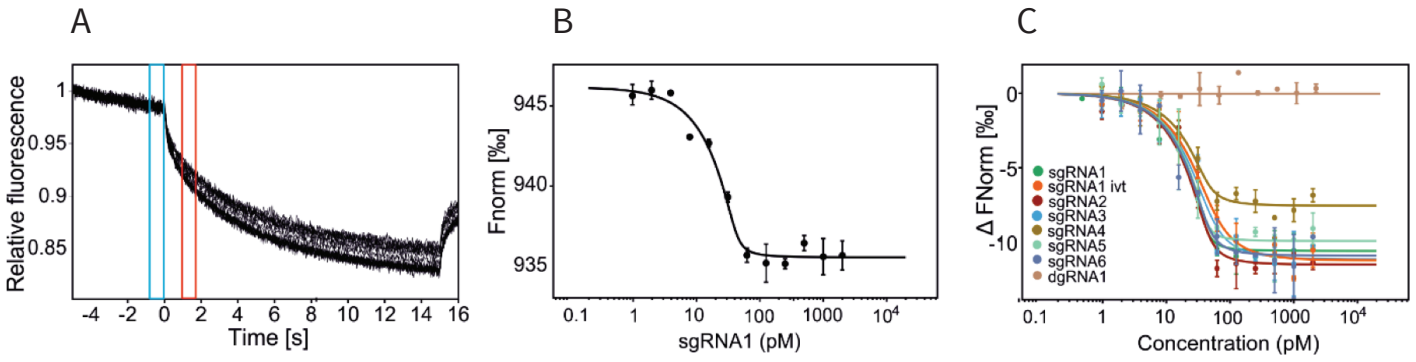

Figure 3: The binding of sgRNAs to fluorescently labeled Cas9. MST traces (A) and dose-response curve (B) for the sgRNA1 interaction with Cas9. C) Dose-response curves for the interaction of various sgRNAs and a dgRNA with Cas9. The resulting dose-response curves were fitted to a one-site binding model to extract K_d values. MST experiments were performed with 50 pM of Cas9-NT647 at 80 % LED and 40 % MST power. F_{norm} = normalized fluorescence

Importantly, we noticed an abrupt loss of Cas9 binding for the probe containing crRNA hybridized with the corresponding tracrRNA (Table 1).

sgRNA ID (> 90 nt)	crRNA/ tracrRNA ID (42 nt, 68 nt)	K_d (pM)
sgRNA1	—	1.1 ± 1.3
sgRNA1 ivt	—	10.7 ± 3.9
sgRNA2	—	2.4 ± 2.3
sgRNA3	—	6.7 ± 4.0
sgRNA4	—	2.8 ± 2.4
sgRNA5	—	1.0 ± 1.4
sgRNA6	—	2.4 ± 2.5
—	dgRNA1	No binding

Table 1: Affinities of various sgRNAs and a crRNA/tracrRNA hybrid as determined by MST measurements using the Monolith™ NT.115Pico instrument. MST experiments were performed with 50 pM Cas9-NT647 at 85 % LED and 40 % MST power.

We recognized that the proximity of Cys808 to the binding pocket might influence the binding of RNA to Cas9 (Figure 3, Table 1). To determine if the loss of RNA binding is the result of our labeling strategy, we hybridized a Cy5-labeled probe to crRNA and measured its affinity for Cas9. The same Cy5-crRNA was then hybridized with various tracrRNAs. In this manner, we were able to label different dgRNAs with a single fluorescent probe. Additionally, we hybridized the same Cy5 probe to a sgRNA.

Throughout the assays, the Cy5-labeled RNA was used at concentrations of 100 pM, and the highest Cas9 concentration in the serial dilutions was 10 nM. The affinity measurements of the Cy5-labeled RNA constructs yielded excellent MST traces and subsequent signal-to-noise ratio (Figure 4).

Overall, 10 dgRNA constructs displayed affinities in the lower picomolar range (between 1.3 and 12.9 pM, Figure 4 and 5, Table 2); sgRNA/Cas9 binding affinity was 7.3 pM. As shown in Figure 5, all MST experiments are characterized by an excellent signal-to-noise ratio that enables reliable determination of picomolar affinities for RNA-Cas9 interactions (Table 1). Although cysteine labeling might be a valid approach for monitoring sgRNA/Cas9 affinity, this study suggests that the unintended labeling of the binding-pocket-proximal cysteine could affect the binding of hybridized dgRNA. We therefore recommend using a Cy5-labeled probe for hybridization with an array of different RNA constructs. By using such probes, MST allows for a rapid and straightforward determination of affinities between RNA and Cas9 protein.

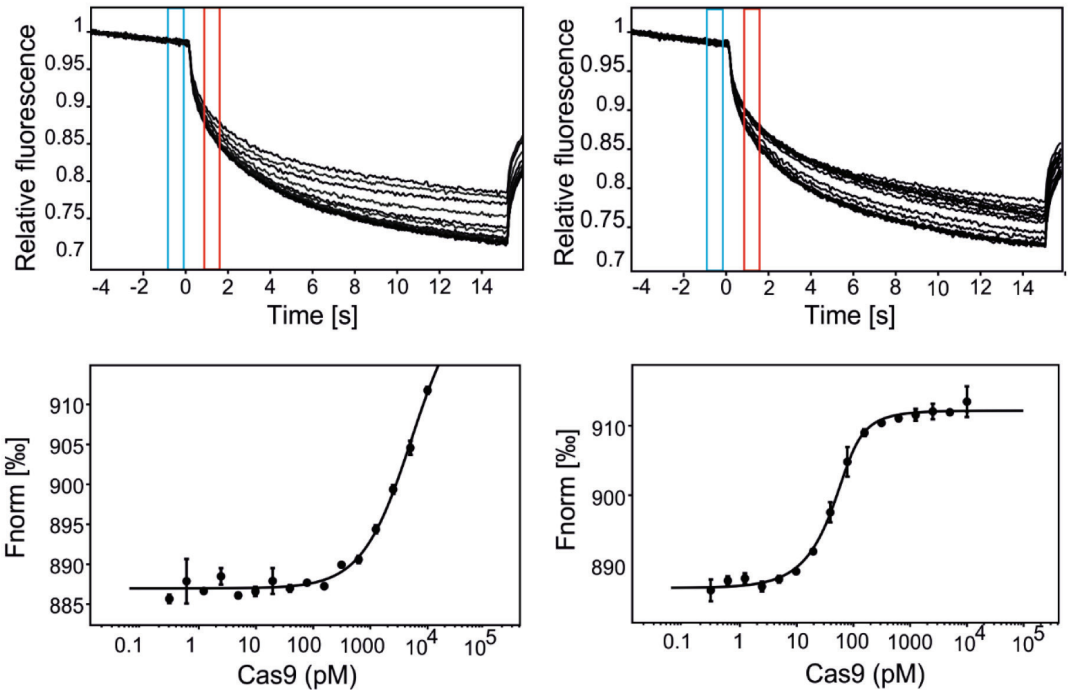

Figure 4: MST traces (top) and dose-response curves (bottom) for the dgRNA interaction with Cas9. dgRNA2 (A) and dgRNA4 (B) differ significantly in length. The resulting dose-response curves were fitted to a one-site binding model to extract Kd values. MST experiments were performed with 100 pM Cy5-RNA at LED and 60 % MST power. Fnorm = normalized fluorescence

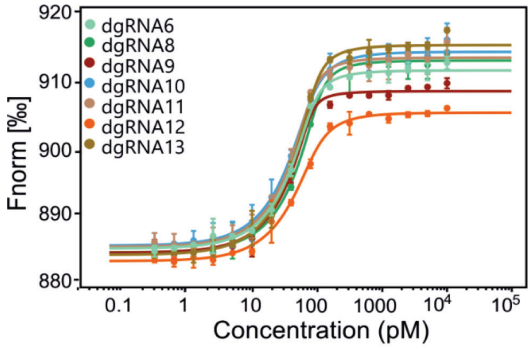

Figure 5: Dose-response curves for the interaction of various dgRNAs with Cas9. MST experiments were performed with 100 pM Cy5-RNA at 50 % LED and 60 % MST power. Extracted K_d values are summarized in the Table 1.

Conclusion

Genome editing technologies based on CRISPR/Cas9 provide a plethora of applications in human therapeutics, food science and other fields. To characterize high-affinity interactions between various RNA constructs and Cas9, MST offers unprecedented advantages and delivers precise K_ds even in the low picomolar range. Unlike other approaches, K_d values can be obtained in less than 10 min.

The high information content of MST data enables straightforward assay development including selection of the optimal labeling strategy. For this interaction system we recommend using a Cy5-labeled probe, which can be hybridized with an array of different RNA constructs providing highest flexibility and most convenient assay set-up.

dgRNA ID (crRNA, 42 nt; varying tracrRNA length, < 90 nt)	sgRNA ID (> 90 nt)	K_d (pM)
dgRNA2	—	5170 ± 906
dgRNA3	—	5181 ± 975
dgRNA4	—	12.9 ± 3.9
dgRNA5	—	7.2 ± 3.0
dgRNA6	—	6.1 ± 2.4
dgRNA7	—	6.6 ± 5.5
dgRNA8	—	6.6 ± 3.3
dgRNA9	—	1.3 ± 2.0
dgRNA10	—	8.8 ± 3.9
dgRNA11	—	5.5 ± 3.0
dgRNA12	—	12.6 ± 4.5
dgRNA13	—	6.1 ± 2.7
	sgRNA7	7.3 ± 3.5

Table 2: Affinities of various crRNA/tracrRNA hybrids and a sgRNA as determined in MST measurements using the Monolith™ NT.115Pico instrument. MST experiments were performed with 100 pM Cy5-RNA at 50 % LED and 60 % MST power.

Material and Methods

RNA Synthesis, Cy5 labeling and hybridization

All RNAs were provided by Axolabs GmbH (Kulmbach, Germany) and were made by automated chemical solid-phase synthesis using commercially available phosphoramidites. After cleavage from the solid support and deprotection, the synthesized RNAs were purified by high performance liquid chromatography (HPLC). For labeling of guide RNAs, a Cy5-conjugated probe (Axolabs GmbH; Kulmbach, Germany) complementary to the target binding site of the crRNAs (in dgRNAs) or the sgRNAs was used. This hybridization was performed using a 10 % molar deficiency of the Cy5-labeled probe. The Cy5-labeled crRNAs were finally hybridized to the tracrRNAs in an equimolar ratio. In general, hybridizations were carried out in 1 x phosphate buffered saline (PBS; pH 7.4) by heating samples to 70 °C followed by slow cooling down to room temperature.

Cas9 labeling

The maleimide-NT647 dye (Cat.# MO-L004) was used for the labeling of Cas9. For buffer exchange and removal of the residual dye, the Antibody Labeling Kit columns were used (Cat.# MO-L007) according to the manufacturer's instructions. For labeling, 3 µM of Cas9 NLS from S. pyogenes (M0641, New England Biolabs) were mixed with 15 µM of maleimide-NT647 dye and incubated in the dark at room temperature for 30 min. After removal of residual dye, labeled protein was centrifuged at 10 000 g for 10 min at 4 °C.

MST experiments

The interactions of RNA with the Cas9 were measured in MST-T buffer, pH 7.8, containing 50 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl2 and 0.05 % Tween-20. The Cy5-labeled RNAs were each used at a concentration of 100 pM, Cas9-NT647 was used at 50 pM. The highest concentration of either unlabeled RNA or Cas9 used was 10 nM.

Instrumentation and data analysis

Measurements were performed on a NanoTemper Monolith™ NT.115^{Pico} instrument. Final Cy5-RNA concentration of 100 pM yielded fluorescence intensities of ~ 4000 counts at an LED power of 50 %. The samples were measured at 60 % MST power with a MST-on time of 15 s and a laser-off time of 1 s. The parameters were deduced in T-Jump and the resulting dose-response curves fitted to a one-site binding model to extract K_d values.

References

1. Walzt, E. Gene-edited CRISPR mushroom escapes US regulation. Nature News 532, 293 (2016).
2 Nishimasu, H. et al. Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell 156, 935-949 (2014).
3 Heler, R. et al. Cas9 specifies functional viral targets during CRISPR-Cas adaptation. Nature 519, 199-202 (2015).
4 Jinek, M. et al. A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 337, 816-821, doi:10.1126/science.1225829 (2012).
5 Jiang, W. & Marraffini, L. A. CRISPR-Cas: New tools for genetic manipulations from bacterial immunity systems. Annual review of microbiology 69, 209-228 (2015).
6 Sternberg, S. H. & Doudna, J. A. Expanding the biologist's toolkit with CRISPR-Cas9. Molecular cell 58, 568-574 (2015).
7 Hsu, P. D., Lander, E. S. & Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. Cell 157, 1262-1278 (2014).
8 Jiang, F., Zhou, K., Ma, L., Gressel, S. & Doudna, J. A. A Cas9–guide RNA complex preorganized for target DNA recognition. Science 348, 1477-1481 (2015).