

Scope

This application note highlights case studies where the label-free Octet® BLI platform has been used to measure and characterize CRISPR/Cas mediated binding and Gene activation.

Keywords or phrases:

Octet®, BLI, CRISPR, CRISPR/Cas, Cas9, Cas-gRNA, Cas activity, DNA binding, RNA binding, Gene activation, CRISPR Characterization

Applying Label-Free Insights to Decipher CRISPR/Cas Mechanisms and Workflows

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Abstract

The use of prokaryotic biological tools to edit and regulate genetic information has been commonplace since the discovery of bacterial restriction enzymes in the mid-1900s. In addition to restriction endonucleases, meganucleases, zinc-finger nucleases, DNA recombinases and transcription activator-like effector nucleases, are some of the other prokaryotic genetic elements have been serving as invaluable tools to study and uncover genetic and biological mechanisms. The discovery of the prokaryotic adoptive immunity machinery CRISPR/Cas technology revolutionized the ability to manipulate genetic information, providing a powerful method to induce deletions, insertions and specific sequence changes at defined target sites in a wide variety of biological systems and live organisms. Multi-subunit CRISPR/Cas systems are prevalent across a variety of bacterial and archaea species with varying levels of complexities and mechanism of actions. All CRISPR/Cas systems discovered thus far rely on CRISPR RNAs (crRNA) or guide RNAs (gRNAs) for guidance on target recognition and specificity. A multitude of interactions and recognition events direct the CRISPR/Cas machinery towards target DNA cleavage. The hybridization of the spacer region of the gRNA to target DNA sequence located next to a protospacer adjacent motif (PAM) initiates DNA processing. Thus, specific genomic regions can be targeted for processing by using a custom spacer sequence specific to a target DNA sequence adjacent to PAM sequences offering unprecedented re-programmability.

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While best characterized CRISPR/Cas systems such as the Class 2 *Streptococcus pyogenes* Cas9 are widely applied in basic and translational research, tremendous strides have been made to further improve this machinery and expand its targeting efficiencies and applications. For example, how increased targeting flexibility can be achieved by altering PAM specificities and improved targeting fidelity using Cas9 variants have been studied¹⁻⁵. Use of Cas inhibitors and engineering novel interaction motifs in gRNAs have been proposed to reduce off-targets effects⁶. New applications utilizing impaired Cas proteins as RNA guided DNA binding mechanisms have been adopted, taking advantage of the precise target recognition capabilities of the Cas-gRNA complex to introduce additional accessory domains together with the gRNA to upregulate or downregulate gene expression⁷, deaminate DNA⁸ and modify the epigenome⁹.

To further understand and explore the intricate molecular mechanisms of these systems, tools are required that can deconvolute the various CRISPR/Cas interactions and their consequences towards gene editing. Bio-Layer Interferometry (BLI) provides a convenient and rapid way to

investigate CRISPR-Cas recognition elements including inhibitor and modulator molecules and CRISPR-Cas mediated gene expression through *in vitro* studies: all in one platform. The Octet® platform is highly versatile and can analyze a variety of analytes ranging from small molecules¹¹⁻¹⁶, nucleic acids¹⁷⁻²³, proteins and peptides²⁴⁻²⁹, nanoparticles³⁰⁻³⁴, membrane proteins³⁵⁻³⁹ and cells⁴⁰⁻⁴² further expanding their analytical capabilities. The operation is simple and easy as it is a fluidics-free sample delivery platform that enables the analysis of samples in crude or unpurified matrices hence reducing sample preparation times. BLI is an optical method where binding interactions are monitored in real-time on biosensors. The availability of a wide-range of biosensor chemistries provides you with flexibility in reagent design and assay formats. The Octet® platform provides fast time to results stemming from its capability to parallel process data from 1-96 biosensors simultaneously. Here we describe a few examples of how the Octet® BLI platform was used not only to decipher CRISPR/Cas related interactions, but also to quantitate and validate downstream genetic manipulations mediated by the CRISPR/Cas machinery.

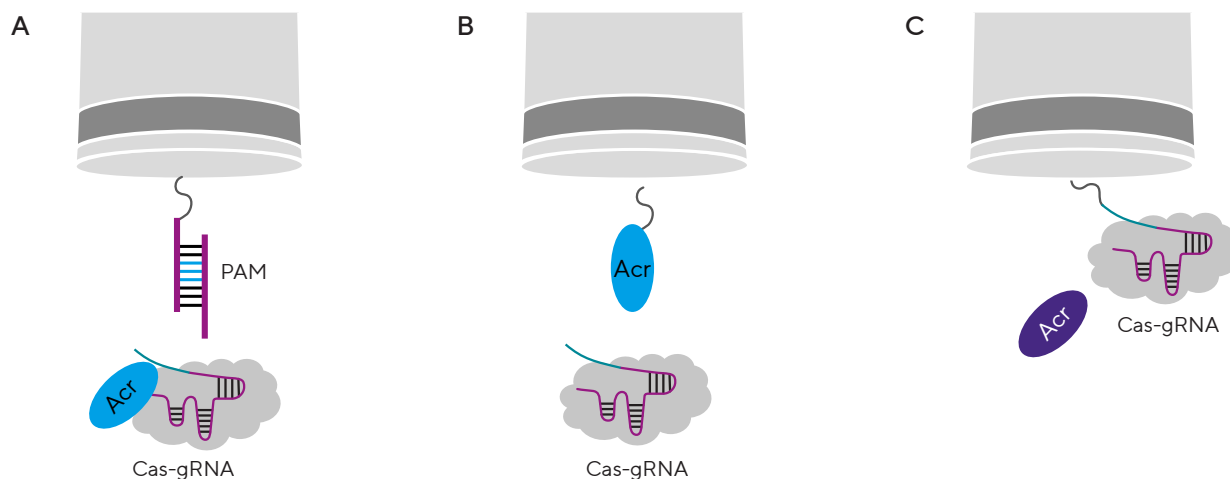
Characterizing CRISPR/Cas Inhibitors

Anti-CRISPRs (Acrs) are a class of proteins that are evolved to block CRISPR/Cas immunity in bacteriophages. These naturally occurring regulatory mechanisms are currently being investigated as a potential tool that can be used to regulate and exert more control on CRISPR/Cas functions. One of the early studies by Shin et al. showed that AcrIIA4 from *Listeria monocytogenes* prophages binds to the assembled Cas9-single guide RNA (sgRNA) target surveillance complex and not to *Streptococcus pyogenes* Cas protein alone⁴³. In this study, an Octet® BLI kinetic assay was used to confirm the non-equilibrium mode of inhibition where AcrIIA4 requires access to Cas9-sgRNA before formation of the Cas9-sgRNA-DNA complex. The assay was constructed using Streptavidin biosensors where a biotinylated double-stranded target DNA was immobilized and its binding with Cas9-sgRNA-AcrIIA4 complexes with varying levels of AcrIIA4 was measured (Figure 1A). The data obtained confirmed that AcrIIA4 binding to Cas9-sgRNA complex inhibited target DNA recognition. A separate Octet® competition assay indicated that AcrIIA4 was unable to compete with a pre-formed Cas9-sgRNA-DNA complex validating the non-equilibrium mode of inhibition. This indicated that AcrIIA4 proteins primarily engage the Cas-sgRNA target surveillance complex. Cryo-EM data further indicated AcrIIA4 binding to Cas9

PAM recognition regions. They further indicated that controlled expression of AcrIIA4 in cells exhibited reduced off-target effects exerting more precise gene editing functions. Similar observations were also recorded by Hynes et al. and Fuchsbaauer et al. on Acr proteins from *Streptococcus thermophilus* phages that inhibited St1Cas9 activity^{44,45}. Octet® kinetic assays indicated that AcrIIA6 also recognizes the Cas-sgRNA complex but not the Cas9 protein. Here the Acr proteins were immobilized onto Streptavidin biosensors and Cas9 protein and Cas9-sgRNA complex binding was assessed (Figure 1B). Similar to AcrIIA4, Octet® assays confirmed that AcrIIA6 reduces DNA target recognition by binding to the Cas9-sgRNA surveillance complex.

In an effort to discover Cas9 inhibitors, Uribe et al., developed a high-throughput approach to screen for type 2 CRISPR/Cas inhibitors in metagenomic libraries⁴⁶. After several rounds of filtering, 11 putative Acr proteins identified from the screens were tested for *in vitro* target DNA cleavage inhibition and CRISPR/Cas complex binding. An Octet® binding assay was established to measure comparative binding affinities of different putative Acr proteins to Cas-gRNA complexes. In this work, Cas-gRNA complexes were immobilized onto Streptavidin

Figure 1: Assay orientations for Anti-CRISPR (Acr) protein binding analysis.



Note: (A) Pre-complexed Cas-gRNA-Acr binding to target PAM-DNA sites. The DNA oligonucleotide is biotinylated and immobilized onto Streptavidin biosensors. (B) Evaluation of Acr protein binding to Cas-gRNA complex. Biotin-labeled Acr protein is immobilized onto Streptavidin biosensors. (C) Acr protein binding assessment to Cas-gRNA complex. The Cas-gRNA complex was immobilized using a biotinylated Cas protein and various Acr proteins were evaluated for binding.

biosensors using biotinylated Cas9 proteins (Figure 1C). Data indicated a wide range of affinities to Cas-gRNA complexes implying varying mechanisms of discovered Acr proteins.

These studies establish a possible common theme as to how Acr proteins exert its primary mechanism of action of inhibiting PAM engagement to the Cas-gRNA complex. More importantly, evidence from these studies show controlled titration of Acr proteins in cells can reduce CRISPR/Cas off-target effects implying the utility of these proteins in genomic editing applications. Octet® assays were critical in evaluating and characterizing Acr interactions while also noting different assay orientations that were used for the determination of Acr interactions with Cas-gRNA complexes (Figure 1).

Small molecule inhibition of Cas-gRNA complexes could be another strategy to exercise control on CRISPR/Cas systems. The usual advantages of small molecule inhibitors over protein-based molecules such as ease of manufacturability, cell permeability, reversible inhibition, resistance to proteolysis and non-immunogenic behaviors can benefit CRISPR/Cas related applications as well. A novel high-throughput (HT) assay was developed by Maji et al. to screen for Cas9 small molecule inhibitors⁴⁷. The HT assay was designed to capture small molecules that can inhibit the Cas9 recognition of PAM. The PAM binding site has been the mode of action for most naturally evolved Acr proteins and the lower binding affinity between PAM-Cas interactions can favor the small molecule

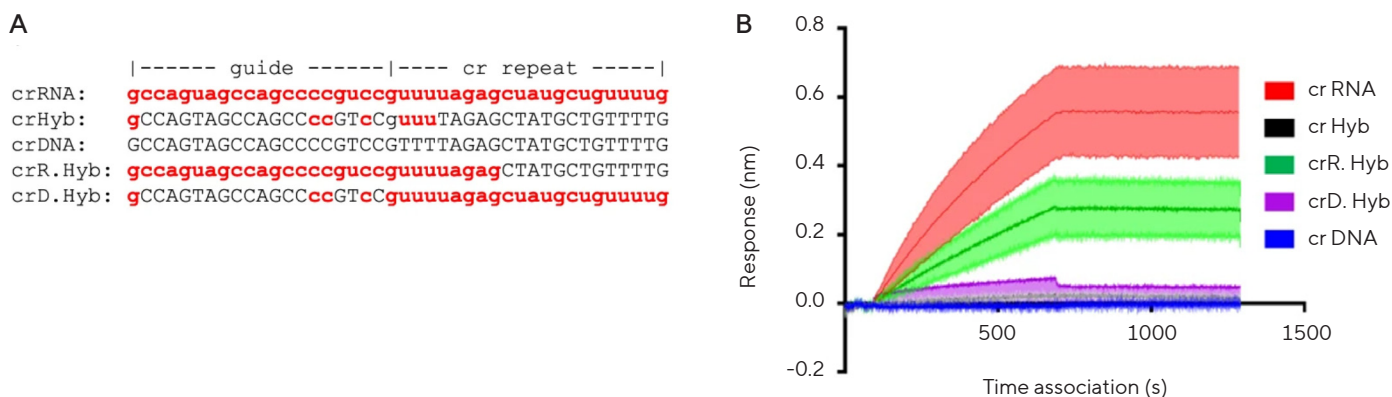
intervention strategy. A fluorescent polarization (FP) assay was developed for primary screening of small molecules. A fluorescently labeled PAM-containing oligonucleotide binding to Cas9 was measured using FP where binding of the relatively small oligonucleotide to Cas proteins would reduce DNA tumbling rates increasing fluorescent anisotropy signals. The lower affinity between the DNA-PAM oligonucleotide and Cas9 proteins had resulted in anisotropy signals that did not show measurable differences between bound and unbound states. To overcome this challenge and construct a more stable binding complex, the authors sought to add additional PAM binding sites on the test DNA oligonucleotide to strengthen the complex and reliably distinguish anisotropy signals between bound and unbound states. An Octet® BLI assay was used to confirm the improved affinity upon the addition of PAM sequences thus optimizing the FP assay for screening. In this BLI assay, DNA oligonucleotides with different PAM densities (0-8 additional sites) were immobilized onto Streptavidin biosensors (SA) and binding levels were assessed with Cas9-gRNA complex to identify PAM densities to be used in the FP assay that would respond reliably to small molecule inhibition. Small molecules identified with this HT assay were validated using a variety of biochemical, binding and cell-based assays to show target engagement and Cas9 inhibition by lead compounds. *In vitro* target binding validation was also performed using an Octet® assay where the small molecule lead compound was immobilized onto Streptavidin biosensors followed by titrating varying concentrations of Cas-gRNA complexes.

Mapping Sugar Dependency of crRNAs for *in Vitro* and *in Vivo* Activity

Understanding the specificity requirements and features of crRNA and tracerRNA can be helpful to delineate the mechanism of Cas9 functions and can allow manipulation to extend its capabilities and improve the usability of the system. Furthermore, sequence alterations to RNA molecules have shown to improve both transcription and stability compared to natural systems⁴⁸. A study by Rueda et al. investigated the requirement of RNA bases in crRNA and tracerRNA towards Cas protein activity⁴⁹. Key differences between RNA and DNA are 2'-hydroxyl (2'-OH) on the ribose sugar moiety, preferential RNA C3'-endo sugar pucker and the extra methyl group at the thymine base that converts to an uracil in RNA. These differences drive distinctions in key structural properties and stability between the two molecules. The single 2'-OH groups increase the susceptibility towards hydrolysis, reducing overall stability of RNA compared to DNA. In this work, attempts were made to identify critical RNA requirements on the crRNA/tracerRNAs by carefully manipulating the nucleotide backbone that would improve specificities, stability and activity. Data showed that a DNA CRISPR molecule was unable to direct Cas nuclease activity both *in vitro* and *in vivo*. However, a hybrid DNA/RNA molecule (crHyb, Figure 2A) constructed with seven conserved RNA

residues that allowed critical hydrogen bond formation with the 2'-OH, was able to exert Cas9 nuclease activity *in vitro*. An Octet® BLI binding assay was used to determine Cas9 target engagement with crXNA (DNA/RNA hybrids). Here a short DNA oligonucleotide was immobilized onto Streptavidin biosensors and dCas9: crRNA/target DNA binding with several crXNAs was measured (Figure 2). Octet® data indicated favorable binding with natural crRNAs, but not with crDNA that also correlates with measured *in vitro* Cas9 nuclease activities. Interestingly, crHyb that showed *in vitro* Cas9 activity was not able to detect significant binding to target DNA. Both crR.Hyb and crD.Hyb that consisted of a majority of RNA nucleotides in guide and cr-repeat regions respectively exhibited binding to target DNA with varying levels (Figure 2B). Octet® data indicated that RNA residues in the guide region is critical for target binding. While the lack of binding of the crHyb was intriguing due to the presence of *in vitro* Cas9 activity, Octet® data coincided well with cellular Cas9 activity with these constructs. Insights from this work can be used with Cas9 for *in vitro* site-specific nuclease purposes and provide a novel platform for cellular manipulation and allow the development of more

Figure 2: DNA residues in the crRNA affects target DNA binding.

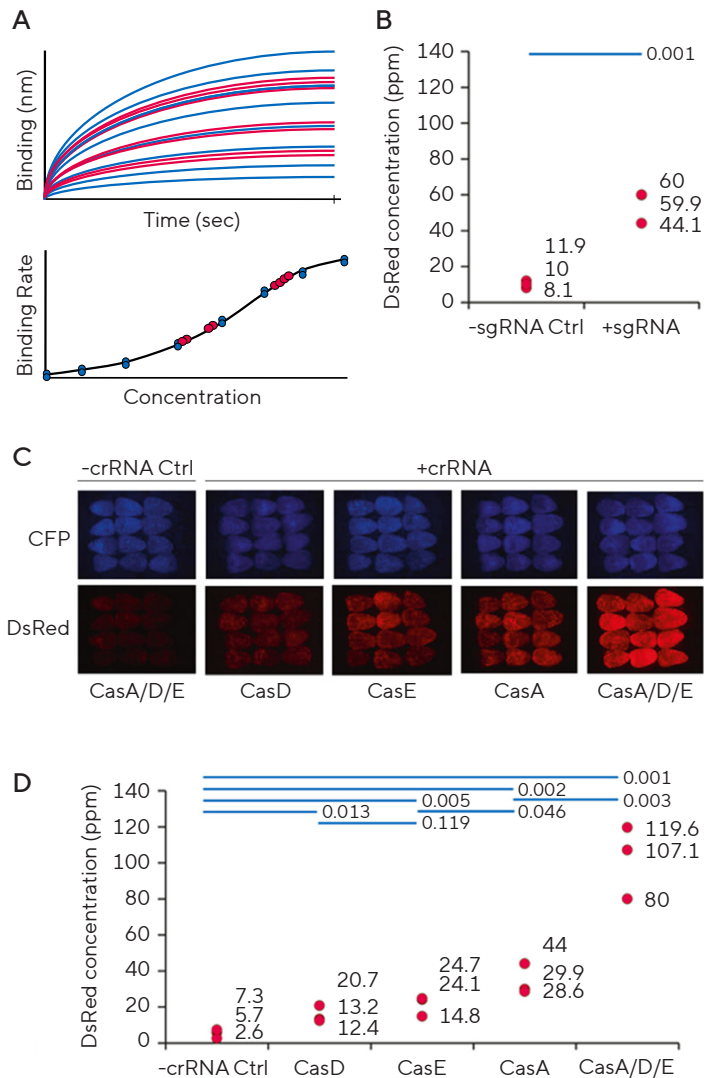


Note: (A) Nucleoside composition of crXNA molecules; RNA shown in lowercase red, DNA in uppercase black. (B) BLI sensogram showing association kinetics to immobilized target DNA of Cas9:tracrRNA complexed with crRNA (red), crHyb (black), crDNA (blue), crR.Hyb (green) or crD.Hyb (purple). Shaded areas show SD of three independent measurements. (Image reproduced with permission from ref. 49)

Measuring CRISPR/Cas Mediated Gene Activation Levels

In contrast to type 2 CRISPR/Cas systems that operate with a single effector protein, type 1 CRISPR/Cas systems rely on a cascade of proteins for function. In a recent study, Young et al. utilized the type I CRISPR/Cascade to control gene expression in cells⁵⁰. Here, type I-E CRISPR cascade proteins (CasA, CasB, CasC, CasD) from *Streptococcus thermophilus* were utilized and fused to a plant transcriptional activation domain from the Arabidopsis cold binding factor 1 (CBF1) to the 3' end of the cascade genes (casA, casB, casC and casD). The CRISPR/Cas machinery was implemented as an RNA-guided DNA binding platform that can deliver external effector proteins, in this case a transcriptional activator to elevate gene expression to a specific genomic locus. The system was tested in *Zea mays* cells using a DsRed reporter system co-delivered with the cascade-CBF1 cassette and sgRNA constructs capable of directing Cas-CBF1 binding to a region upstream of the promoter in the reporter construct. After 48 hours, post-delivery gene activation was quantified using an Octet[®] assay (Figure 3). Octet[®] platforms are fully automated, require simplified workflows with low user intervention and are an excellent replacement for more complicated quantitative immunoassays. In this work, DsRed protein expression levels were quantified using a DsRed antibody captured onto Anti-Human Fc Capture (AHC) biosensors. The Octet[®] workflow for a typical quantitation assay is depicted in Figure 3A, where a standard curve is constructed using known concentrations of recombinant DsRed proteins by plotting DsRed binding rates vs. concentration. The standard curve is then utilized to measure DsRed expression products as response to CRISPR/Cas mediated gene activation. As indicated in Figure 3B, the amount of DsRed protein generated was above the negative control (reactions assembled without the sgRNA). While it is evident that all possible cascade combinations produced DsRed protein expression (Figure 3C), CBF1 fusions to CasA produced more DsRed than analogous CBF1 fusions to CasD and CasE (Figure 3D). Furthermore, it was evident that the recruitment of multiple Cas domains (A/D/E) produced the highest level of gene activation conforming an additive effect. These results establish the potential utilization of class 1 type I Cascade complexes as viable tools for RNA guided DNA binding applications and also as a method to systematically enhance gene activation..

Figure 3: Octet[®] assay to measure CRISPR/Cas mediated gene activation levels.



Note: (A) Overview of Octet[®] quantitation assay. Anti-DsRed antibody is immobilized onto AHC biosensors. A DsRed standard curve is generated using purified DsRed protein followed by measuring DsRed gene expression levels in *Zea mays* cells. Binding responses (top) and binding rates (bottom) of known DsRed concentration (blue) and unknown gene expression levels (red) are indicated. (B) DsRed concentration in dCas9-CBF1 transformed 48 hr post-transformation determined by Octet[®] assays. dCas9 is a mutant form of Cas9 whose endonuclease activity is removed. (C) CFP and DsRed images of Cas-CBF1 transformed cells. CFP fluorescence served as a control for transformation efficiency. Experiments performed with CasA-CBF1, CasB, CasC, CasD-CBF1, and CasE-CBF1 expression cassettes in the absence of a crRNA expression construct served as the negative control (-crRNA Ctrl). (D) Quantification of DsRed protein resulting from Cascade transcriptional activation 48 hr after transformation in three independent experiments (image 3B, 3C, and 3D reproduced with permission from ref. 50).

Conclusions

Tools derived from the bacterial inert immunity machinery CRISPR/Cas systems provide an unprecedented range of applications in research ranging from basic science to translational medicine. All CRISPR/Cas systems discovered thus far rely on a number of interactions between effector proteins and crRNA for target engagement and activity. Interrogation of CRISPR/Cas components and interactions have helped further improve the usability and extend into new application areas. The Octet® label-free interaction analysis platform is a highly versatile tool that can be utilized to evaluate binding interactions across a wide range of analytes and application areas. Here we described the use of label-free binding assays to evaluate CRISPR/Cas

inhibitor proteins, develop high-throughput assay conditions for CRISPR/Cas small molecule inhibitor development, and target validation and assessing of crRNA-DNA interaction moieties. In addition, we showed how the label-free biomolecular interaction analysis can be used as a quantitative immunoassay platform to determine CRISPR/Cas induced gene activation levels and to quantitate protein expression levels using a simple one-step assay. Overall, the Octet® BLI interaction analysis platform can serve as an invaluable tool that can not only be utilized to analyze a wide range of analytes, but also in a variety of applications spanning from binding kinetic and affinity analysis, quantitation and as a screening tool.

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