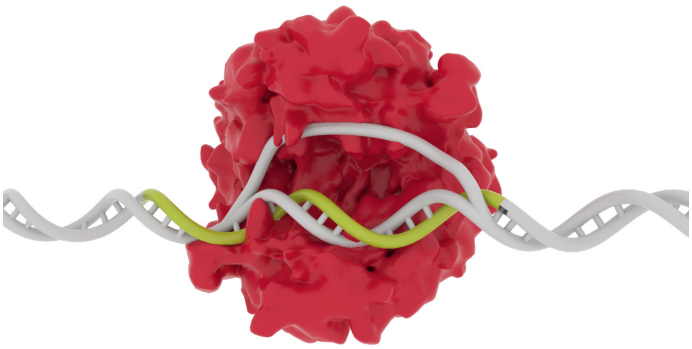
CRISPR-Complete: An *In Vitro* Engine for Optimizing CRISPR-Cas Performance



Abstract

Clustered regularly interspaced short palindromic repeat (CRISPR)-Cas systems have been widely applied to basic research questions and biotechnology, enabling the launch of a new class of powerful therapeutics: cell and gene therapies. This activity has given rise to easily accessible tools and workflows that can accelerate pre-clinical R&D of CRISPR-based therapeutics. Yet, the most commonly used CRISPR-Cas editing workflows rely heavily on time-consuming and expensive methods. *In silico* guide RNA (gRNA) design algorithms are imperfect at predicting editing outcomes. On- and off-target editing is determined using cell-based assays and costly PCR- and NGS-based methods. In addition, failed or inefficient editing is challenging to troubleshoot, as cell-based assays provide little biochemical validation of Cas protein (Cas)-gRNA complex formation or DNA target recognition. *In vitro* assays, though not commonly used in pre-clinical development, can help fill this knowledge gap, offering new insights into CRISPR-Cas function. Here, we present CRISPR-Complete, a rapid, highly-sensitive platform for validating *in vitro* Cas-gRNA performance and verifying cleavage using amplicons representing loci of interest. The CRISPR-Complete platform is built on Cardea Bio's graphene transistor technology – the Biosignal Processing Unit (BPU™) – that enables label-free translation of biological activity into digital information. By using CRISPR-Complete, cell and gene therapeutics developers can gain valuable and unprecedented insights into CRISPR-Cas function *in vitro* before investing in cost-, time-, and labor-intensive downstream assays.

Introduction

CRISPR-based technologies have enabled the application of genome editing to nearly every biological field. Currently, there is a treasure trove of well-studied, validated natural and engineered CRISPR-Cas systems, expanding the sensitivity, selectivity, and scope of genome engineering and positioning these easily-programmable tools as platforms for elevating the human condition.[1,2] With this now-massive toolbox of CRISPR-Cas systems and the ability to edit, knock-in, knock-out, activate, or repress genes in eukaryotic and prokaryotic genomes, the biotechnology community has untangled once-elusive questions about human biology and pioneered the development of promising first-in-class cell and gene therapies.[2]

The adoption of CRISPR-Cas systems in therapeutics development has led to the rapid emergence of several clinical candidates for treating rare and challenging diseases, including sickle cell anemia, ß-thalassemia, Duchenne muscular dystrophy, and ocular indications. Translation into the clinic has been facilitated by a robust pre-clinical framework of genome editing tools, empowering nearly any R&D team to streamline gRNA design and synthesis, select the most desirable Cas protein for their application, and characterize on- and off-target editing in live cells.

These tools form the foundation for a typical pre-clinical CRISPR editing workflow. Generally, they involve the following steps: 1) CRISPR-Cas system selection, 2) gRNA design (and synthesis, in the case of ribonucleoprotein (RNP) transfection), 3) transfection into live cells, 4) single-cell cloning, 5) screening, and 6) on-/off- target analysis.[3-5]

While seemingly straightforward, there are drawbacks to this now-routine, entrenched workflow. The typical CRISPR editing workflow can be labor-, time-, and cost-intensive, often applied to a broad matrix of experimental conditions that can include many Cas-gRNA combinations, various transfection or cell culture conditions, and PCR- or NGS-based techniques for assessing on-/off--target editing.

Notably, several *in silico* algorithms and frameworks have made gRNA design and selection easy and streamlined, taking into account many parameters, including PAM positioning, GC content, secondary structures, and mismatches.[3] Yet, gRNAs that appear perfect on a computer can often fail, lead to minimal editing, or result in undesirable off-target effects in cells. Troubleshooting these issues can be challenging and time-consuming, focusing on *in silico* gRNA optimization and redesign, tweaking of transfection conditions, or switching strategies for Cas-gRNA delivery or expression. Furthermore, conventional workflows don't include *in vitro* assays for Cas-gRNA complex

formation or target binding, forcing many researchers to operate and optimize with incomplete information.

In Vitro Insights: The Missing Link in CRISPR-Cas Editing Workflows

In principle, the issues encountered in the traditional "straight-to-cell" approach can be addressed using *in vitro* methods, commonly used in small molecule and biopharmaceutical development pipelines. Though not often used in CRISPR-Cas editing workflows, in vitro assays can provide indisposable insights into gRNA binding affinity, target or non-target DNA binding affinity, and cleavage efficiency, bridging the growing knowledge gap between the *in silico* and cell-based worlds.

Currently, there are several reconstituted CRISPR-Cas systems for assessing in vitro activity. Gel-based cleavage assays help determine the cleavage efficiency of Cas-gRNA RNPs but can be low-throughput and low-sensitivity, further slowing already cumbersome editing workflows.[6,7] More rapid, sensitive, and specific assays have been developed, namely SHERLOCK and HOLMES, which rely on isothermal T7 RNA polymerase-mediated and PCR-based amplification, respectively, and use a fluorescent reporter to detect CRISPR-Cas cleavage.[8,9]

By providing a better understanding of cleavage activity, such assays would be valuable to conventional CRISPR-Cas editing workflows. However, an ideal *in vitro* assay would also provide insight into other biochemical steps, such as Cas-gRNA complex formation and RNP recognition of target DNA sequence upstream of target cleavage. In addition, reliance on fluorescence-based assays and optical assays, in general, requires amplification, resulting in additional time, reagents, and instrumentation.

What is a BPU™?

The BPU™ is the first mass-produced chip that creates a gateway between the live signals in biology and the digital world. It offers direct communication with biology by translating real-time streams of multiomics signals into digital information, removing the limitations of optical and static measurements used in the life sciences.

To learn more about the BPU™, visit cardeabio.com.

For a more in-depth description of the physics that powers the BPU™, check out Goldsmith BR et al. Sci Rep. 2019;9(1):434.

Pioneering Label-Free, Rapid, Electrical Biosensing for CRISPR-Cas Systems

Recently, the detection of biological activities has gone through a transformation, trading optics-based methods for chip-based electrical methods. Novel technology pioneered by Cardea Bio – the BPU™ – enables this leap, facilitating rapid, highly sensitive translation of biological activity into digital information. The BPU™ is a graphene transistor that can be functionalized with a wide range of biomolecules. Performing biochemical reactions, such as a binding interaction or catalysis, near the graphene surface of the BPU™ alters its electrical characteristics resulting in real-time electrical signal output.[10] All of this happens without amplification, which is usually required with optical methods. Because measurements are in real-time, kinetics data can also be collected, providing robust insights into the molecular dynamics of DNA, RNA, protein, or any other type of biomolecule.

First reported in 2019, the CRISPR-Chip applies the CRISPR-Cas system to the BPU™ platform to detect target sequences of interest within a genomic DNA context (Figure 1).[11] At CRISPR QC, we use CRISPR-Chip to understand and optimize CRISPR-Cas performance, including gRNA interaction, target recognition, and cleavage.[11] The graphene surface of the BPU™ is functionalized with pyrenebutyric acid (PBA), which electrostatically interacts with the graphene and can be covalently coupled via

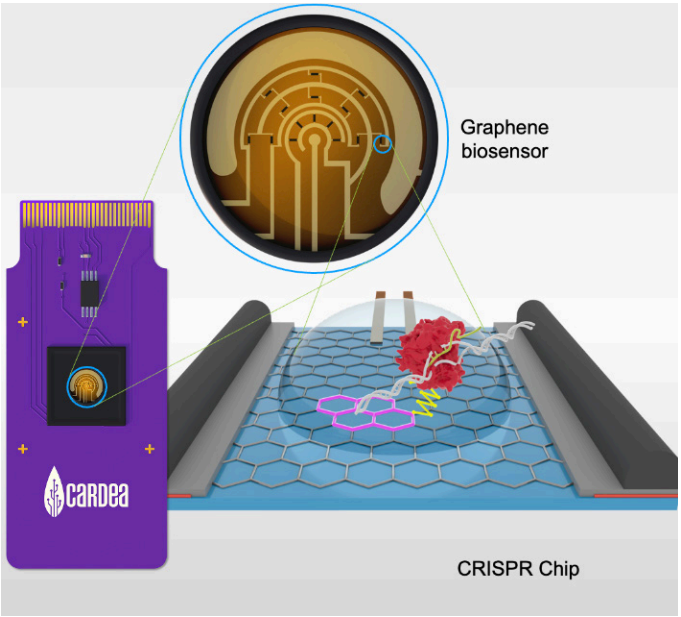


Figure 1. The CRISPR-Chip contains multiple transistors arranged into three separate channels. One transistor is magnified to show how a Cas protein can be linked to the graphene surface of the BPU™.

carbodiimide crosslinking to any Cas protein. Unfunctionalized areas of the PBA are blocked by coupling to an inert molecule, amino-polyethylene glycol 5-alcohol, that doesn't interfere with any downstream biochemical reaction or electrical detection.

Following the blocking reaction, gRNA can be introduced to measure Cas-gRNA RNP formation near the surface of the graphene chip. Biological activity that occurs near the surface of the graphene results in a current change between the drain and source electrodes compared to a baseline signal (Figure 1). Finally, additional reagents, such as PCR-amplified target DNA or reaction components, can be added to measure binding interactions or cleavage reactions.

The CRISPR-Complete Workflow: Insights for CRISPR-Cas Optimization

Our team at CRISPR QC has developed a workflow using the CRISPR-Chip – called CRISPR-Complete – to help optimize your CRISPR-Cas designs before embarking on expensive and time-consuming cell-based assays (Figure 2). CRISPR-Complete can help rank, order, and prioritize your most promising Cas-gRNA candidates, effectively derisking cell-based editing assays.

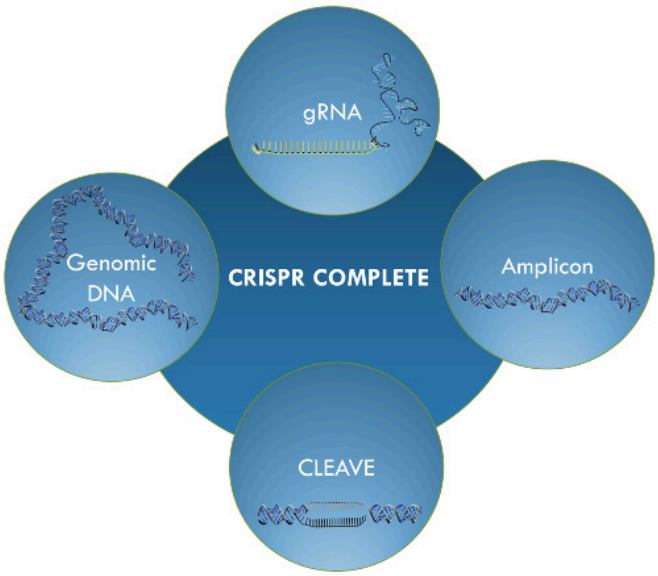


Figure 2. CRISPR-Complete measures binding between gRNA and your Cas protein-of-choice and binding of Cas-gRNA complexes to PCR-amplified target DNA sequences (i.e., amplicons) or unamplified genomic DNA. In addition, CRISPR-Complete can measure the cleavage of target amplicons by Cas-gRNA complexes.

Here's what the CRISPR-Complete workflow can do:

- Measure binding between candidate gRNAs and your Cas proteins-of-interest
- Determine binding interaction between Cas-gRNA complexes and target amplicons
- Demonstrate cleavage of Cas-gRNA complexes at target amplicons
- Confirm binding of Cas-gRNA complexes to unamplified DNA sequences in a genomic context

The CRISPR-Complete workflow offers unprecedented insight into which gRNAs to select, Cas protein to use, and DNA sequence to target, all of which cannot be deconvoluted using conventional CRISPR-Cas editing workflows or data from cell-based assays. Unlike other *in vitro* assays, cleavage can also be correlated with detailed gRNA or target DNA binding data and measured sensitively, without amplification and the reagents or instruments required for optics-based methods.

We've established an automated CRISPR-Complete system that offers reproducible, precise data and provides the flexibility to accommodate our partner's unique questions. Figure 3 shows both raw and processed data generated from a representative CRISPR-Complete workflow. This experiment measured the cleavage of a Cas-gRNA complex at target and non-target amplicons. As various components of our reconstituted Cas-gRNA system are added stepwise, there is a real-time change in the electrical characteristics of the graphene chip (Figure 3; left panel). This data is calibrated to a baseline and processed to generate an *I* response, which measures the change in current between the baseline and end state (Figure 3; right panel). Triplicate reactions are run on distinct transistors to ensure the validity of the observed results.

A reduced *I* response is synonymous with amplicon cleavage in this cleavage experiment. As shown in the right panel of Figure 3, the RNP using gRNA #1 cleaves its target amplicon (purple box) and, as expected, does not cleave a non-target amplicon (orange box).

This experiment demonstrates the power of CRISPR-Complete. In the case of gRNA #1, we validated Cas-gRNA complex formation, RNP cleavage at a target amplicon, and discrimination against cleavage at a non-target amplicon. If we had not seen cleavage of the target amplicon, we could use CRISPR-Complete to further investigate the upstream steps of RNP formation and target amplicon binding to further optimize or redesign this Cas-gRNA complex.

This analysis is just one example of a CRISPR-Complete use case. We've used our platform to help small and large biotechnology companies rapidly identify high-affinity gRNAs, determine target amplicon binding, compare cleavage activity across different Cas9 vendors, and much more.

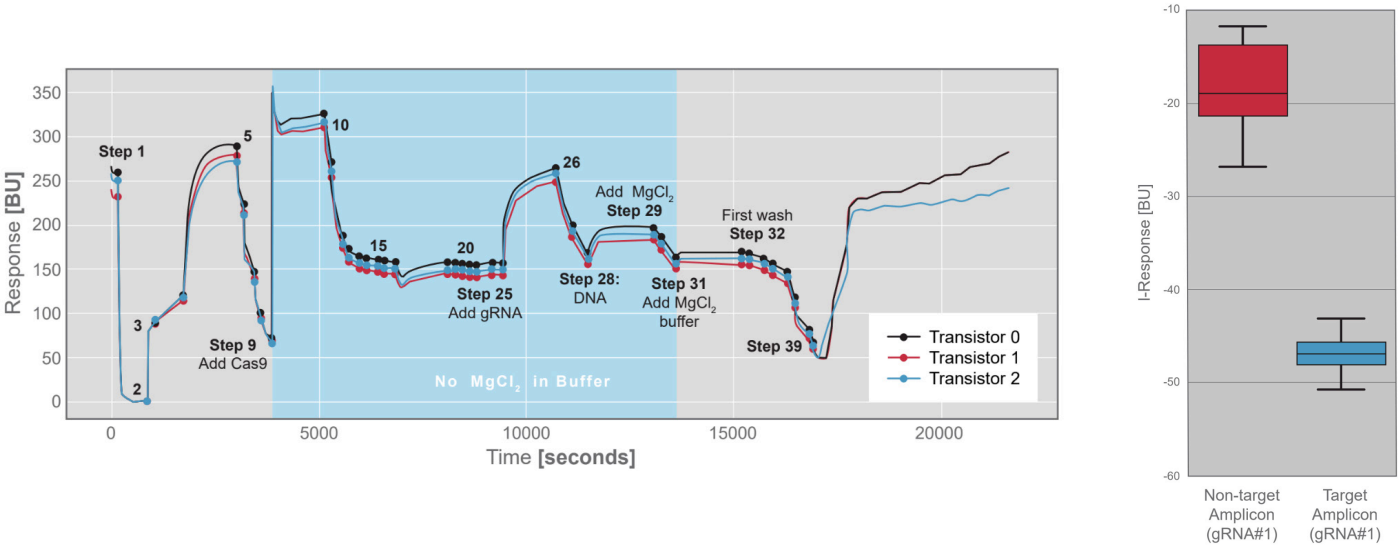


Figure 3. CRISPR-Complete can detect CRISPR-Cas function on target and non-target amplicons. A. *I* response is measured in real-time as Cas9 (step 9), gRNA #1 (step 25), amplified target DNA (step 28), $MgCl_2$ (step 31), and wash buffer (step 32) are added. The reaction buffer does not contain $MgCl_2$ before step 31 to inhibit Cas9 nuclease activity prior to the addition of Cas9, gRNA, and amplified DNA. Reactions were set up and measured in triplicate on different transistors. B. *I* response (BU) was measured by subtracting the *I* response at step 33 from the *I* response at step 29 in panel A and plotted in the blue box. Another reaction, gRNA #1 + non-target amplicon (red box), was tested in parallel and processed similarly.

Conclusion

As demonstrated above, CRISPR-Complete provides unprecedented biological insights into cleavage efficiency, the upstream process of Cas-gRNA RNP formation, and target DNA binding. These insights enable gRNA optimization for Cas-gRNA complex formation and confirmation of binding to and cleavage of target amplicons sequences. In addition, CRISPR-Complete can detect the binding of Cas-gRNA complexes to unamplified genomic DNA. Ultimately, this is a crucial validation step, saving R&D teams significant time and money on downstream cell-based assays that may not generate valuable data. CRISPR-Complete provides new knowledge to shape your CRISPR-Cas genome editing efforts, a more streamlined workflow, and more promising outcomes at the cellular level.

Need more insights into your CRISPR-Cas experiments? Visit crisprqc.com or email us at info@crisprqc.com.

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