Methods for characterizing, applying, and teaching CRISPR-Cas systems

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1 Bacteria and archaea possess fascinating defense systems comprised of Clustered
2 Regularly Interspaced Short Palindromic Repeats (CRISPR) arrays and CRISPR-associated
3 (Cas) proteins whose characterization is driving a rapid and widespread technological
4 revolution. In their natural form, CRISPR-Cas systems confer adaptive immunity against
5 mobile genetic elements through a rich diversity of mechanisms and functions. This
6 functional diversity and the role of easy-to-design guide RNAs (gRNAs) led to the facile
7 translation of these systems into versatile tools impacting applications ranging from genome
8 editing and gene regulation to diagnostics, antimicrobials, and imaging agents. Ongoing
9 progress in applications such as human gene therapy and gene drives in turn has spurred
10 heated ethical debates, underscoring an urgent need to inform the wider population about
11 these technologies and their ramifications.

12 This special issue in *Methods* comprises two broad reviews and seven original
13 methods representing a broad sampling of current methodologies. Below is a brief overview
14 of each article grouped based on their primary relevance to the fundamental characterization
15 of CRISPR-Cas immune systems, implementation of CRISPR technologies specifically in
16 bacteria, and teaching CRISPR through hands-on modules intended for the classroom.

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18 **Methods for characterizing the biology of CRISPR-Cas systems.** Five articles in the
19 special issue entail methodologies for identifying and characterizing the biomolecular
20 properties of CRISPR-Cas systems and their related components. Alkhnbashi and
21 coworkers provide a comprehensive review of available bioinformatics tools [ref1]. The
22 review principally describes tools for identifying and annotating CRISPR-Cas immune
23 systems, including finding CRISPR arrays and *cas* genes within genomic sequences,
24 properly orienting the arrays, identifying targets of the encoded CRISPR RNAs (crRNAs, the
25 natural form of gRNAs), and classifying the type and sub-type of each system. Furthermore,
26 available design tools for gRNAs relevant to CRISPR technologies are also described. The
27 bioinformatics tools for identifying CRISPR-Cas systems are particularly useful given the

28 ubiquity of these systems in prokaryotes and their extensive diversity that is still being
29 understood and mined for technologies.

30 In the second review, Behler and Hess describe common approaches for interrogating
31 the mechanisms underlying crRNA biogenesis, with resulting insights across CRISPR-Cas
32 types and subtypes [ref2]. Prior work has shown that these mechanisms can vary widely,
33 requiring a broad set of techniques to pinpoint each mechanism and the responsible genetic
34 factors. The authors describe how these techniques helped reveal the different ways in
35 which a transcribed CRISPR array can be converted into individual crRNAs and aspects of
36 crRNA biogenesis that remain to be explored.

37 The first of the methods articles transitions the special issue from crRNA biogenesis to
38 target cleavage by the CRISPR effector nuclease responsible for immune defense.
39 Creutzburg and coworkers report a medium-throughput method to evaluate DNA cleavage
40 activity *in vitro* by the Type V-A nuclease Cas12a [ref3]. This single-effector nuclease offers
41 unique advantages over canonical Cas9 nucleases, such as staggered DNA cleavage or the
42 ability to generate crRNAs without accessory factors. As part of the method, the authors
43 targeted linear DNA with biotin on one end and a fluorophore on the other end, allowing the
44 facile measurement of the solution's fluorescence following the addition and removal of
45 streptavidin beads. The method was used to detect nuclease concentration and cleavage
46 rates, although it could be further applied to elucidate potential protospacer-adjacent motifs
47 (PAMs) or the impact of guide-target mismatches.

48 The next method from Gramelspacher and coworkers addresses a unique feature of
49 some Cas9 nucleases: the capacity to target RNA [ref4]. The authors detail how to
50 characterize RNA cleavage activity under *in vitro* conditions, with a focus on evaluating the
51 extent of cleavage, mapping the cleavage site, and determining whether the cleaved target
52 remains bound to Cas9. They also describe the purification of an anti-CRISPR protein (Acr)
53 and testing its ability to inhibit RNA cleavage by Cas9 *in vitro*. The method should be useful
54 when evaluating the extent to which other Cas9 nucleases exhibit this behavior as well as
55 the impact of different Acrs.

56 The final method devoted to CRISPR biology takes a unique approach to the
57 characterization of Acrs. Acrs are thought to exist against virtually every CRISPR-Cas
58 system, yet Acrs have only been associated to-date with a small fraction of all CRISR-Cas
59 subtypes. Wandera and coworkers report the use of an *E. coli*-based transcription-
60 translation system (TXTL) to rapidly and scalably characterize different Acrs, using Acrs
61 against Cas9 as a case study [ref5]. In particular, they show how to modify a previously
62 published assay to minimize non-specific inhibition of gene expression associated with some
63 Acrs.
64
65 **Methods for implementing CRISPR technologies in bacteria and archaea.** The special
66 issue then turns from CRISPR biology to CRISPR technologies—specifically, programmable
67 gene editing and repression in prokaryotes [ref6]. Diallo and coworkers developed an
68 inducible two-plasmid system for genome editing with Cas9 for industrial strains of the
69 bacterial genus Clostridia. One of the plasmids encoded the recombineering template,
70 allowing for repair in coordination with Cas9 cleavage. The authors used this strategy to
71 delete a sporulation gene or insert a fungal cellobiohydrolase gene in one strain of
72 *Clostridium beijerinckii* followed by phenotypic characterization. The strategy was further
73 applied in another strain of *C. beijerinckii* to delete a gene conferring thiamphenicol
74 resistance and remove an endogenous plasmid, together allowing greatly improved
75 transformation of a plasmid selected with thiamphenicol.
76 Next, Depardieu and Bikard detail how to achieve programmable gene repression
77 with a catalytically-dead Cas9 (dCas9) in the bacteria *Escherichia coli* and *Staphylococcus*
78 *aureus* [ref7]. This mode of gene repression has been extremely effective for single or
79 multiplexed gene knock-down and has been easier to implement across bacterial species
80 than genome editing. Beyond showing how to generate and apply the basic constructs, the
81 authors incorporated origins-of-replication with broad host ranges. They also devised a
82 clever high-throughput screen to determine the optimal expression level of dCas9

83 expression that maintains silencing activity without inducing a cytotoxic “bad seed” effect
84 observed previously by the authors.
85 Finally, Albers and coworkers describe an entirely distinct approach for achieving
86 CRISPR-based gene repression in archaea [ref8]. Rather than relying on dCas9 or another
87 heterologous nuclease, the authors co-opted the CRISPR-Cas system endogenous to the
88 archaeon *Haloferax volcanii.* The system was devoid of the nuclease-encoding subunit Cas3
89 as well as the processing enzyme Cas6; as a result, expressing a mature version of a
90 designed crRNA allowed the authors to achieve targeted gene silencing without competition
91 by endogenous crRNAs. Through this work, the authors provided basic design rules for
92 efficient gene silencing and demonstrated silencing of chromosomal and plasmid-encoded
93 genes, providing a strong foundation for CRISPR-based repression in other archaea.
94
95 **Methods for using CRISPR in the classroom.** The final article of the special entails how to
96 communicate the basic properties of genome editing with CRISPR technologies in a
97 classroom setting. Here, Ziegler and Nellen report a set of experiments to illustrate DNA
98 cleavage in *E. coli* [ref9]. They provide detailed protocols and instructions as well as lecture
99 materials, and the entire module can be completed in a day using simple and low-cost
100 materials and equipment. They also provided one round of student assessments indicating
101 positive reception as well as feedback to further improve the module.
102
103 Collectively, this special issue covers available methods for characterizing the rich diversity
104 of CRISPR biology, implementing a cross-section of current CRISPR technologies, and
105 porting CRISPR into the classroom. I hope you enjoy this issue and find it useful for your
106 own interests and pursuits.
107
108 **References**
109 1. Voss [review] – Bioinformatics tools
110 2. Hess [review] – Characterizing crRNA biogenesis and resulting insights

111 3. van der Oost [method] – Characterizing cleavage by Cas12a in vitro

112 4. Zhang [method] – Characterization of RNA targeting by Cas9

113 5. Beisel [method] – Acr characterization using TXTL

114 6. Diallo [method] – Inducible editing with Cas9 in Clostridia

115 7. Bikard [method] – dCas9 in Strep, E. coli

116 8. Marchfelder [method] – CRISPRi in archaea

117 9. Ziegler [method] – CRISPR module for the classroom