A Highly Adaptable Screening System for Directed Evolution of Cas Nucleases

Introduction

Cas nucleases offer a tool for gene editing that is highly specific and very versatile. These nucleases recognize a short protospacer adjacent motif (PAM) and a target sequence in DNA. The subsequent double-stranded cleavage can result in insertions and/or deletions; see Figure 1. Alternatively, new genetic material can be incorporated by homologous recombination at the site of the cleaved gene.

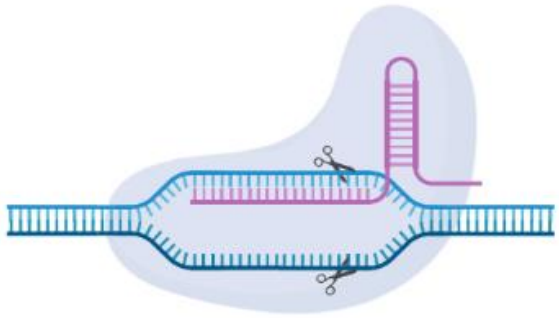

Figure 1. Schematic of Cas nuclease:RNA:DNA complex

Numerous Cas nucleases with a variety of PAM requirements and other properties have been isolated from a plethora of microorganisms. In spite of this diversity, few naturally occurring Cas nucleases are suitable for practical applications in eukaryotes. Maxygen has developed methods for optimizing Cas nucleases for specific applications.

Directed Evolution

Directed evolution is a powerful method for modifying proteins and adapting them to specific requirements of use. This approach involves creating libraries of protein variants (diversity), followed by the selection or screening of such libraries to find variants that satisfy the specifications. Maxygen's molecular breeding technology for diversity creation is one of the most effective ways of generating libraries in which a high proportion of variants are functional.

Analysis of function is unique to each protein under study. For Cas nucleases, an efficient selection method uses *E. coli*; only those Cas variants that can recognize and cleave a conditionally toxic plasmid will persist in the bacterial survivors of the assay.

Such selection assays in eukaryotic cells are less robust. An alternative is to use a screening assay, in which a phenotypic result is indicative of the Cas nuclease activity. Yeast is an excellent candidate organism for this purpose since these eukaryotic cells can be readily manipulated by existing genetic methods. Yeast cells can be grown over range of temperatures, allowing evaluation of Cas nuclease activity under conditions suitable for plants (~25°C) or mammalian cells (37°C).

Yeast Screening Assay

Maxygen scientists have developed a yeast-based phenotypic assay for evaluation of Cas nuclease activity.

The *ADE2* gene in yeast encodes a protein that catalyzes a step in purine nucleotide biosynthesis. If this gene is not functional (e.g., by mutation), the adenine biosynthetic pathway is blocked. Yeast cells grown in optimized media will accumulate a red pigment. If ADE2 gene function is restored, normal white colonies will appear.

The *ADE2* gene can be split into two halves using standard yeast genetic methods, such that the end of the first half contains sequences overlapping those found in the first part of the second half (see hashed regions in Figure 2, top). A fragment of non-*ADE2* DNA containing a PAM site and target sequence can be inserted between the two partially overlapping halves. When the Cas target is cleaved, the yeast cells respond by undergoing homologous recombination and reconstitute an intact functional *ADE2* gene (Figure 2, bottom).

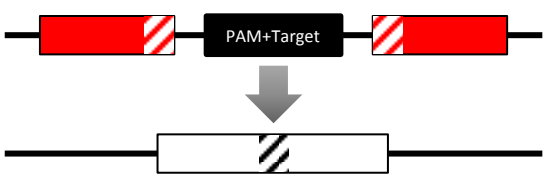

Figure 2. Split *ADE2* gene and the intact product of recombination

A Sensitive and Highly Tunable Phenotypic Assay

Cleavage of the Cas nuclease target site in the split *ADE2* gene is achieved by introducing two plasmids. One

plasmid expresses the guide RNA needed to direct the Cas nuclease to the target site in the split *ADE2* gene. The second plasmid expresses the nuclease coding sequence under the control of a promoter whose activity can be modulated by inducers in the media.

Varying the concentration of such inducers allows exquisite control of Cas nuclease expression. In a population of yeast cells with a split *ADE2* gene and expressing a nuclease at different levels, yeast colonies show a red, white, or variegated phenotype depending on the level of Cas activity. The result of a typical experiment is shown in Figure 3 below.

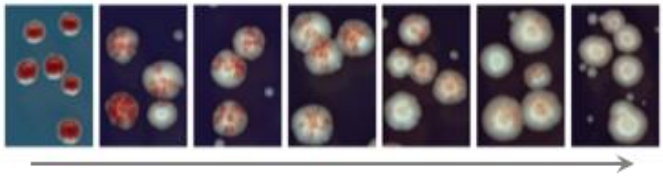

Figure 3. Increasing concentrations of inducer in the culture medium lead to higher levels of Cas nuclease cleavage of the *ADE2* gene resulting in a white phenotype

In the absence of a Cas nuclease, the yeast colonies are entirely red (leftmost panel in Figure 3). As the concentration of inducer in the media is increased for cells expressing both a nuclease and guide RNA, DNA cleavage occurs at the target site and homologous recombination creates an intact *ADE2* gene. At intermediate concentrations of inducer, only a proportion of cells in a colony become white. At the highest concentrations, colonies are almost entirely composed of white cells.

Applications of the Yeast Assay

The assay described above is a valuable tool for the study of Cas nucleases. The conversion of the red to white phenotype depends strictly on: (i) the expression of a Cas nuclease, (ii) localization to the nucleus, (iii) an appropriate PAM sequence, and (iv) a target sequence determined by the co-expressed guide RNA. Each of these parameters can be studied with this yeast assay.

Because yeast is a eukaryotic organism, the results obtained in the study of Cas nucleases can be extrapolated to other eukaryotes. Yeast can be grown at temperatures ranging from an optimum of 25-30°C up to 37°C or higher. Evaluation of Cas nucleases in yeast can therefore be a precursor to studies in plants or mammalian cells.

Libraries of Cas nuclease variants can be screened in the yeast assay to identify variants that recognize a non-canonical PAM or have a higher activity on a specific target sequence. Evaluation of activity can be performed by using inducer concentrations in the media that give intermediate activity for the wild-type nuclease (see the variegated phenotypes in Figure 3). These conditions will allow the identification of variants that produce entirely white colonies.

With the increasing number of Cas nucleases available for study, the directed evolution approach and yeast screening assay offer many opportunities to refine and optimize the properties of these novel proteins to accommodate many applications.

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