ACCURATE QC TO OPTIMIZE CRISPR/CAS9 GENOME EDITING SPECIFICITY

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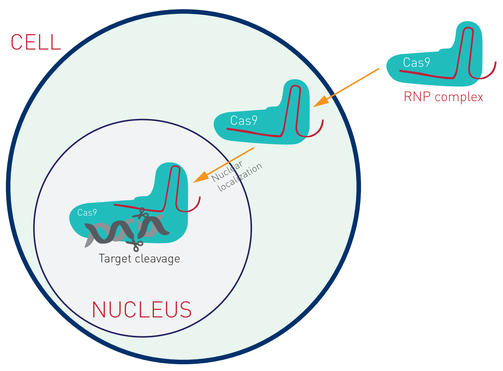
Introduction

In recent years, CRISPR has evolved from the "curious sequences of unknown biological function" into a highly functional genome editing tool. The CRISPR/Cas9 technology is now delivering superior genetic models for fundamental disease research, drug screening, therapy development, rapid diagnostics, and transcriptional modulation. Although CRISPR/Cas9 enables rapid genome editing, several aspects affect its efficiency and specificity including guide RNA design, delivery methods, and off-targets effects. Diagenode has developed strategies to overcome these common pitfalls and has optimized CRISPR/Cas9 genome editing specificity. Here we present a cloning-free CRISPR method for rapid and specific genome editing. Diagenode has also developed highly validated anti-Cas9 antibodies that can be easily used to verify Cas9 delivery in the target cells. We have used these ChIP-grade antibodies to detect where Cas9 binds in the genome and to determine the quality of the guide RNA design. In addition, the ChIP-Cas9 method is an innovative tool to isolate specific genomic regions from cells for their biochemical characterization.

Cas9 Nuclease protein NLS for cloning-free CRISPR

1. Cloning-free CRISPR Workflow

Cas9 Nuclease protein NLS can be combined with guide RNA to form an ribonucleoprotein (RNP) complex to be delivered to cells for rapid and highly efficient genome editing. Benefits of RNP-based genome editing include:



- Rapid and user friendly: RNPs can be delivered directly to cells
- DNA-free assay, no cloning required
- Go from design of gRNA to validation of genome edit in as little as 3-4 days
- Reduced off-targets effects in comparison with vector-based systems
- N-Term and C-term Nuclear Localization Signal enable rapid and efficient nucleus delivery

2. Cloning-free CRISPR Results

Using Cas9 Nuclease protein with in vitro transcribed (IVT) sgRNA allows a completely DNA-free workflow and efficient gene editing. The DNA-free system reduces the concern of unwanted integration as well as potential off-targets.

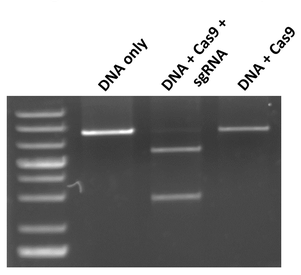


Figure 1. A fast and sensitive in vitro Cas9 cleavage assay.
Experimental validation of sgRNAs before practical application helps minimizing wasted experiments on sgRNAs with poor activity.

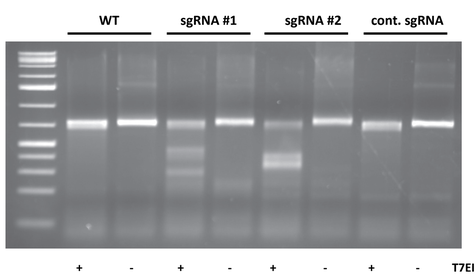


Figure 2. Validation of genome editing in cell culture
HEK293T cells were transfected by lipofectamine with Cas9 protein NLS and sgRNA targeting EZH2. Untransfected cells and a control sgRNA were used as controls. T7EI was performed to estimate gene editing efficiency. As can be seen, Cas9 Nuclease Protein NLS, when combined with specific sgRNAs (#1 or #2) provides consistent and effective gene editing.

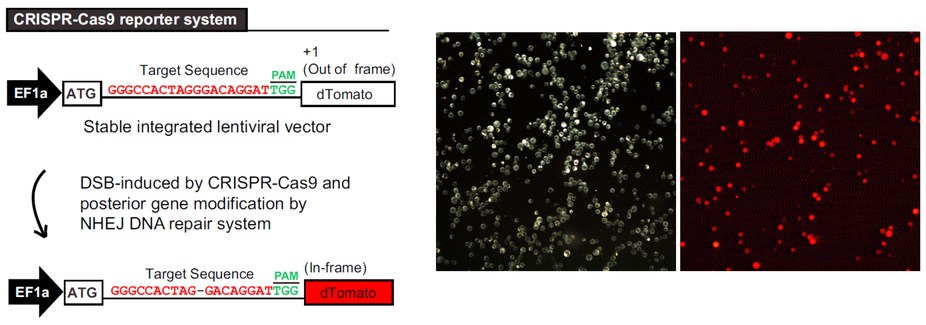


Figure 3. Verifying Cas9 activity with a dtTomato reporter assay.
The reporter is composed of an out-of-frame dtTomato coding sequence. Upon delivery, Cas9 and the specific sgRNA can induce double strand break at the target site in front of dtTomato, leading to frameshift mutations and expression of dtTomato.

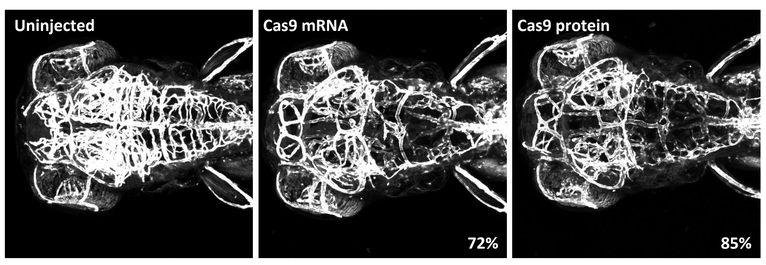


Figure 4. Cas9 Nuclease NLS protein outperforms Cas9 mRNA in mutating GPCR124 in zebrafish. *Tg(krdl:GFP) embryos were injected (one-cell stage) with 300pg Cas9 protein or 300pg of Cas9 mRNA and 30pg guide RNA. Embryos at 4 days post fertilization are shown. The % reflects the number of embryos showing a loss of cranial vasculature.*

CRISPR/Cas9 antibodies

1. Four critical reasons to use anti-CRISPR/Cas9 antibodies

#1 Check the transfection success of the Cas9 protein

#2 Check that the Cas9 protein was delivered to the nucleus

#3 Check the Cas9 expression level

#4 Check the binding specificity of Cas9

Diagenode has developed the largest collection of anti-CRISPR/Cas antibodies, validated in different applications (WB, IF, IP and ChIP):

- *S. pyogenes* Cas9 antibodies
- *S. aureus* Cas9 antibodies
- *Acidaminococcus sp.* Cpf1 antibody
- *L. bacterium* Cpf1 antibody

2. Detection of *S. pyogenes* Cas9 protein using antibodies

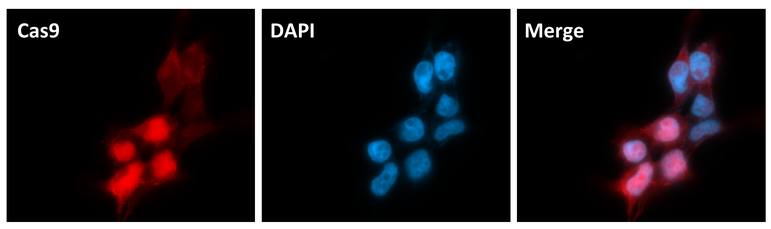


Figure 5. Cas9 is detected in the nucleus of transfected HEK293T cells.
Immunolocalization of Cas9 (red) in transfected cells using monoclonal Cas9 antibody.

3. Determine the binding specificity of a sgRNA by ChIP-Cas9

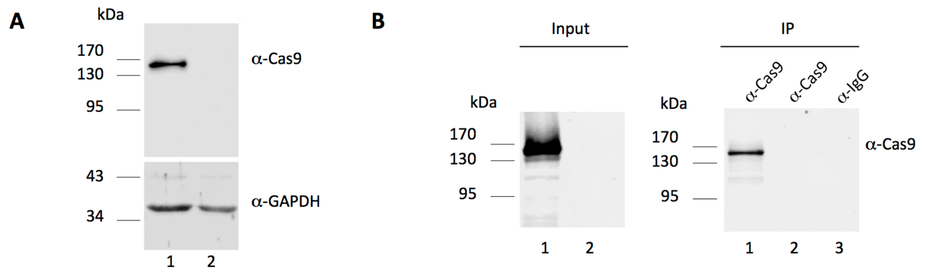
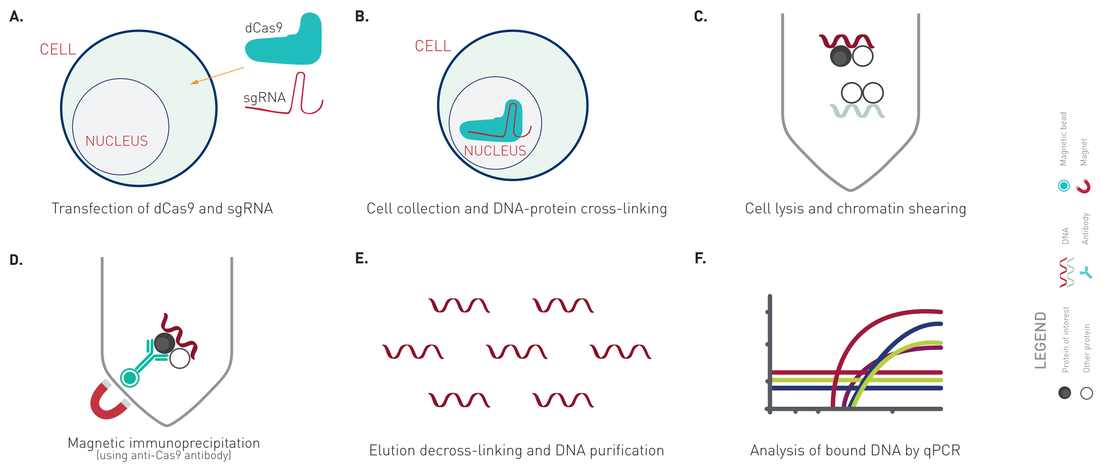


Figure 6. Anti-Cas9 antibodies are validated in Western blot and in immunoprecipitation.
A. Western blot analysis using a monoclonal antibodies directed against Cas9. B. Immunoprecipitation using monoclonal antibodies directed against Cas9. The proteins were analyzed by western blot with the polyclonal Cas9 antibody.

ChIP-Cas9 Workflow



ChIP-Cas9 Results

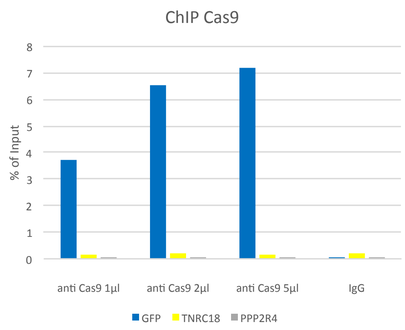


Figure 7. Confirmation of sgRNA binding specificity by ChIP-Cas9

ChIP was performed on NIH3T3 cells stably expressing GFP- H2B, nuclease dead Cas9, and a GFP-targeting gRNA. 50 µg chromatin was incubated with either 1, 2 or 5 µl of the polyclonal antibody against Cas9. IgG was used as negative IP control. Then qPCR was performed with primers specific for the GFP gene, and for two non-targeted regions: TNRC18 and PPP2R4, used as negative controls. This figure shows the recovery, expressed as a % of input.

Conclusion

Cas9 RNP can be rapidly delivered to cells using standard transfection techniques, which reduces off-target events and allows embryos to rapidly generate animal models. Specific anti-Cas9 antibodies can be used to measure Cas9 expression level, to confirm Cas9 presence in the nucleus and to determine the quality of the guide RNA design. Together, these optimized tools and proper controls are essential to the assessment of CRISPR/Cas9 genome editing experiments.