A ZEBRAFISH-BASED PIPELINE FOR *IN VIVO* QUALITY CONTROL OF NEW CAS9 VARIANTS

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INTRODUCTION

The **field of gene editing** is rapidly moving towards the implementation of CRISPR/Cas9–based approaches to tackle human disease. The development of novel Cas9 variants is a crucial step to achieve **precise and effective gene therapy**.

To assess the safety of modified Cas9 versions, *in vivo* standardized strategies for evaluation of on-target endonuclease efficacy and potential toxicity are necessary, since *in vitro* approaches are not predictive of the impact of such complex molecules on a living organism.

Here, we propose a **zebrafish-based pipeline for quality control of two Cas9 protein variants**, by evaluating, in a single assay, Cas9-induced toxicity, teratogenicity and on-target mutagenesis.

EXPERIMENTAL RATIONALE

To perform a comparative analysis of Cas9 variants, we established an **experimental multistep approach** composed by:

1. Dose-range finding of Cas9/sgRNA complexes;
2. Assessment of teratogenic phenotypes to uncover putative developmental defects induced by injection of complexes;
3. High-throughput analysis of loss-of-function phenotypes *via* a fully automated microfluidic system;
4. Evaluation of Cas9 mutagenesis efficacy based on INDEL analysis of each analyzed larva for accurate correlation between phenotype and genotype.

As proof of principle, we targeted the tyrosinase locus, since biallelic disruption of this gene results in loss of pigmentation, thus providing a direct readout of Cas9/sgRNA efficacy.

METHODS

0-1 hpf — **Injection**
- Negative control
- Wild type Cas9
- Cas9 variant

24 hpf — **Mortality evaluation**
First time-point

96 hpf — **Mortality evaluation**
Second time-point
High-throughput raw data collection
- Teratogenic phenotypes
- Pigmentation

Individual genotyping

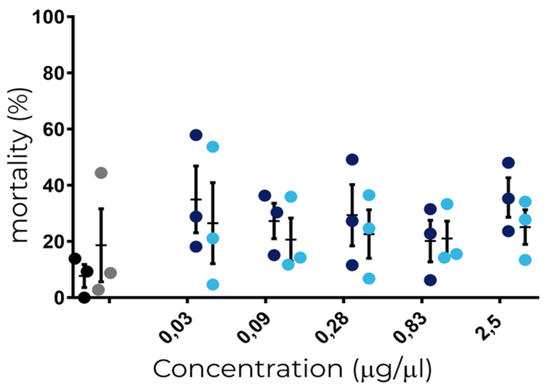
7 dpf — **Data analysis**
- Quantification of **teratogenic phenotypes**
- Evaluation of **phenotypic efficiency** (percentage of embryos with lost pigmentation)
- Quantification of **mutagenesis rates**

RESULTS

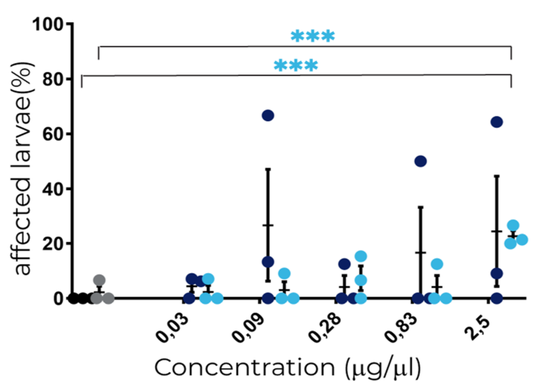
1. The cas9 variant tested has a toxicity profile similar to the one of wild-type Cas9

- Not injected
- Cas9 buffer
- Wild-type Cas9
- Cas9 variant

A Cumulative mortality



B Body axis defects

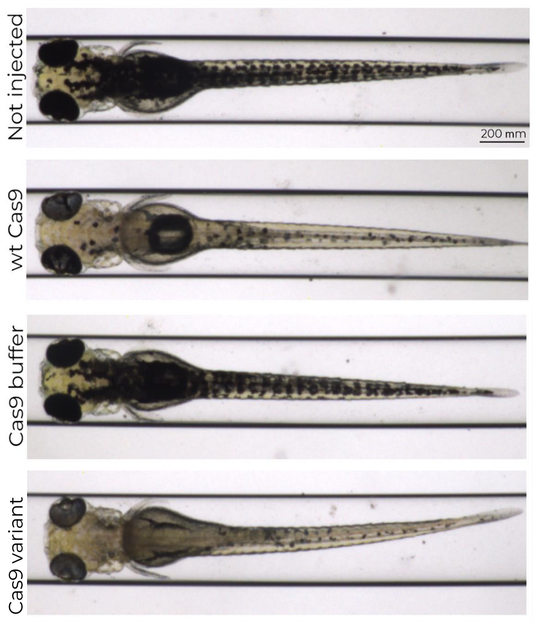


C Heart edema

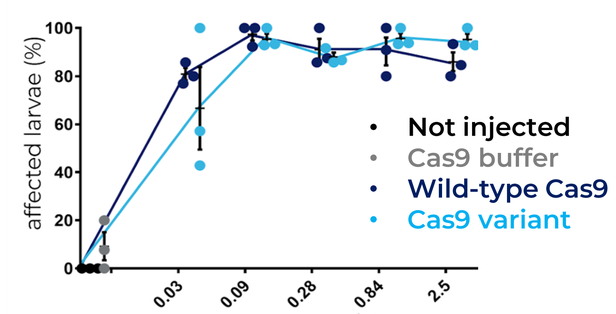
In the first part of this study, we compared the toxicity of a new Cas9 variant to the one of the wild-type Cas9. Upon injection of the two proteins, we evaluated the mortality rates (A) and the potential teratogenic effect of the two nucleases. Both proteins did not induce high mortality and only caused a mild increase of two teratogenic phenotypes: body axis defects (B) and heart edema (C). These observations suggest that **both Cas9 have a very low toxicity.**

2. The Cas9 variant tested has an on-target efficacy profile similar to the one of wild-type Cas9

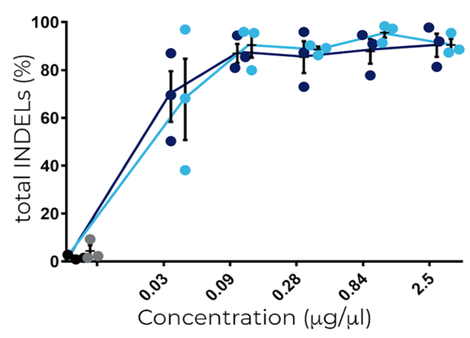
A



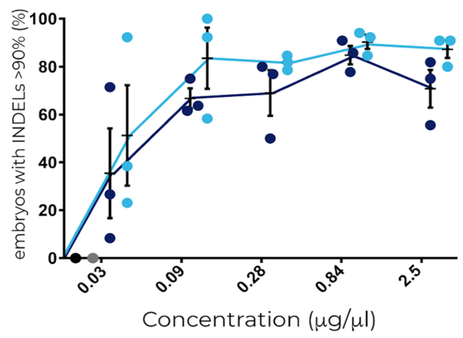
B Pigmentation loss



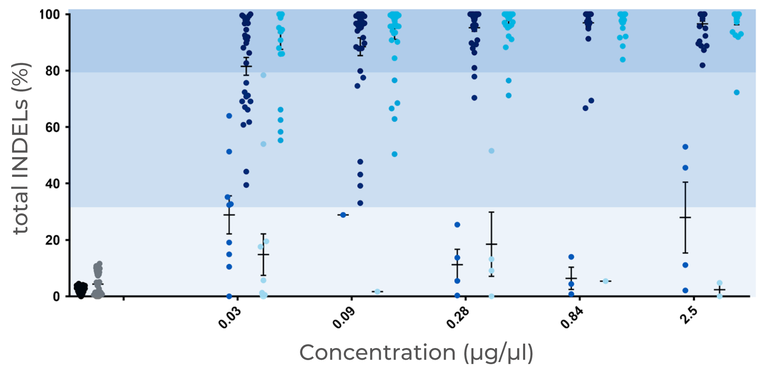
C Mutation rate



D Mutation rate >90%



To perform a comparative analysis of the efficiency of the two Cas9 variants, we evaluated the proportion of injected larvae displaying the expected loss-of-function phenotype (A,B,E) and we measured the mutation rates carried by each individual (C,D,E). **Both Cas9 demonstrated to be able to induce *tyr*-mutations with high efficiency**, already at the lowest concentration tested. Interestingly, we noticed that, at lower concentration, the mutation rates induced have higher variability while higher concentrations resulted in a more robust INDELs and phenotypic outcome (E). Overall, our data demonstrate that both Cas9 are extremely efficient, with the best performance detected at the concentration of 0.84 µg/µl.



- Not injected
- Wild-type Cas9 pigmented
- Cas9 buffer
- Cas9 variant not pigmented
- Wild-type Cas9 not pigmented
- Cas9 variant pigmented

CONCLUSIONS

The comparative assay performed in this study has revealed similar efficiency profiles for the two Cas9 variants used. More importantly, our data provide evidence supporting the use of **zebrafish as a robust system to perform medium to high-throughput Quality Control of new Cas9 proteins** and pave the ground to the use of this model to test other preclinical CRISPR/Cas9-based applications.

Finally, thanks to the possibility of large scale toxicity, efficacy and phenotypic assessment in a single assay, our experimental pipeline can be used to perform comparative studies for validation of innovative gene-therapy tools targeted to different therapeutic areas.