SeekRNA: comparative and multiplex studies of a novel CRISPR/Cas9 alternative

Grant proposal – László Szalai | BIO555 HT-2024

Summary and general aims

There are only a few known methods that are nearing the potential in genetic engineering of CRISPR/Cas9, which itself faces bottlenecks regarding speed and efficiency. In 2024 a novel method for inserting genetically engineered sequences into target DNAs emerged in a study led by Ruth Hall and Sandro Ataide at the University of Sydney [1]. The researchers found that specific insertion sequence (IS) families present in multiple bacteria contain the necessary elements to be able to transport their encoded genes to in between other specific DNA sequences. This process is aided by a self-encoded transposase enzyme and guiding RNA termed SeekRNA. The method has not yet been thoroughly experimented with.

The aim of this proposal is to produce statistical data and supposedly direct evidence that using SeekRNA has significantly increased speed and efficiency compared to CRISPR/Cas9 while also exploring the possibility of applying multiple sequence insertion at once, further improving efficiency.

Survey of the field

Introduction to CRISPR/Cas9

The CRISPR/Cas9 system, derived from a bacterial adaptive immune mechanism, has revolutionized genetic engineering by enabling clear and precise genome editing. The method utilizes a programmed or synthesized single-guide RNA (sgRNA) to direct the Cas9 nuclease to specific DNA sequences, facilitating targeted double-stranded breaks. These breaks are repaired by cellular mechanisms, allowing for gene disruption or the insertion of new genetic material. Despite its transformative impact, CRISPR/Cas9 faces challenges such as off-target effects, variable efficiency caused by the gene disruption, and limitations in multiplex editing.

Emergence of SeekRNA

In 2024, researchers at the University of Sydney introduced SeekRNA, a new gene-editing tool offering enhanced accuracy, efficiency and flexibility over CRISPR/Cas9. SeekRNA incorporates a programmable RNA strand that directly identifies insertion sites within genetic sequences, simplifying the editing process and reducing errors. This method eliminates the need for additional proteins like Cas9, streamlining gene editing.

Mechanism of SeekRNA

SeekRNA operates by utilizing a short RNA molecule derived from noncoding regions within some specific insertion sequences (IS) like IS1111 and IS110 found in bacteria. The SeekRNA guides a transposase enzyme to precise DNA targets, facilitating the insertion of genetic material coded within the IS coding region without introducing double-stranded breaks. The programmability of SeekRNA allows for targeting a wide spectrum of genomic sites, potentially enabling multiplex editing.

Comparative Analysis with CRISPR/Cas9

While CRISPR/Cas9 has been instrumental in advancing genetic engineering, its limitations include off-target mutations and challenges in achieving high efficiency, particularly in multiplex editing scenarios (Fig 1.). Many scientific researches tried to improve on the efficiency of CRISPR/Cas9 [2] but extreme results are yet to be achieved. SeekRNA addresses these issues by providing a more streamlined and accurate approach. Its ability to target multiple sites simultaneously without the need for additional proteins positions it as a promising alternative for complex genetic modifications.

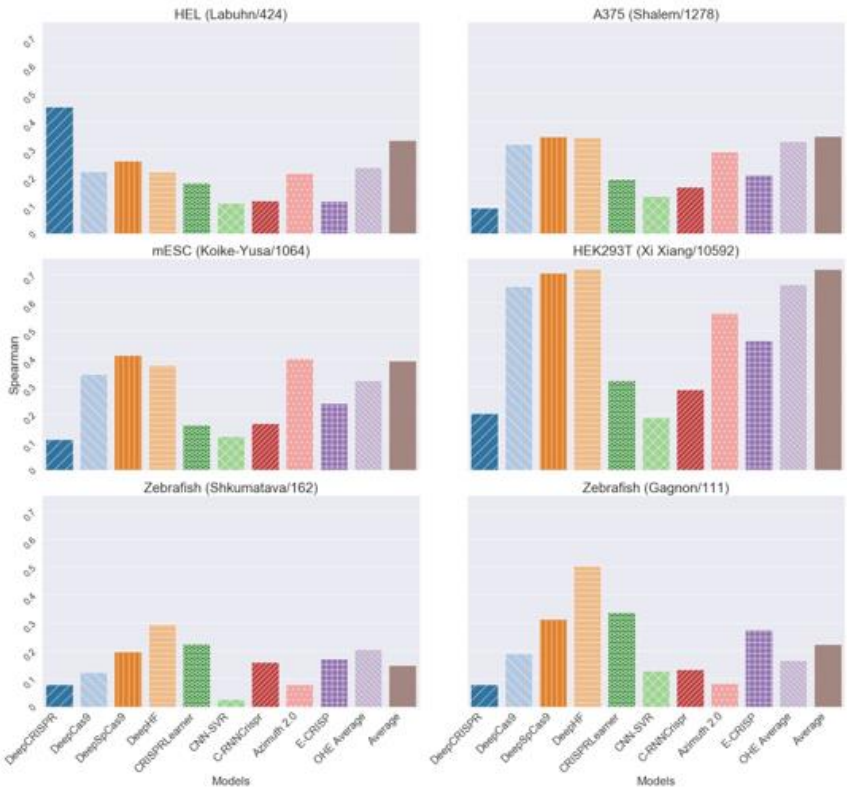

Figure 1. Comparison of gRNA efficiency predictions using Spearman correlation. The name and number of instances for each dataset are shown in parentheses [3]. The highest efficiency is about 70%, however it is limited to certain organisms and the majority of tests resulted in less than 40% successful insertions.

Current Research and Applications

The development of SeekRNA is in its early stages, with ongoing research focused on validating its efficiency and safety. Preliminary studies have demonstrated its potential in precise gene insertion and the ability to reprogram SeekRNAs to direct transposition to new target sequences, showcasing its versatility [4]. Future applications may extend to various fields, including medicine, agriculture, and biotechnology, where precise and efficient gene editing is favorable or even crucial.

Project description

In many studies the viability of genetical engineering of CRISPR/Cas9 has already been tested in multiple species. Among many, C. Elegans could potentially be a good candidate for comparative and multiplex testing with CRISPR/Cas9 and SeekRNA.

Origin of the idea

In a study conducted in 2013 in C. Elegans [5] researchers were curious if CRISPR/Cas9 can be used to alter genetically heritable mutations like Unc-119 and Dpy-13. By inserting fluorophore containing complex near the site of the mutations and then starting a nonhomologous end joining (NHEJ) with CRISPR/Cas9 they were able to check which worms had the mutations in advance. After checking their progenies, the fluorescent marker showed extreme precision in preliminarily determining which worms had and did not have the mutations.

Implementation – Preparation

A commonly used protocol [6] describes what must be done prior to beginning of the research. It is noted that consulting the IDT Custom Alt-R® CRISPR-Cas9 guide RNA website is of great help in preparation. The SeekRNA and the sgRNA for the CRISPR/Cas9 should be created with the following practicalities mind.

The guide RNAs should be centered at about 35 bp from the target gene while using 120 bp homology arms. The CRISPR/Cas9 guide RNAs can be synthesized but it is labor intensive and requires an increased number of materials, therefore an alternative can be used which is ordering specific sequences with integrated homology arms with plasmid integration from companies working within the industry. A 2nmol scaled and a sufficient amount of guide RNAs might be needed. For a lot of the experiments with C. Elegans 5 microliters of sgRNA is used therefore depending on the extensivity of the research 1-5 mL would be required.

For acquiring the SeekRNA we would need to consult the University of Sydney research group currently working on SeekRNA development for exact instructions on reprogramming the sequence into the IS's SeekRNA sequence.

It is important to note that the duplex and multiplex tests should only be performed if the comparative tests are completed and show significant likelihood to be valuable to continue the research with multiple fluorescent proteins.

Implementation – Comparative test

The test itself would be conducted in homozygous mutation carrying C. Elegans as most of its genomes are well researched and widely understood while also experimenting on the species are quite simple. For the analytical data on how well SeekRNA performs compared to CRISPR/Cas9 we will use Green Fluorescence Protein (GFP) coding region to be inserted into a genetic marker with a well-defined sequence like Dpy-10 or Dpy-13 via CRISPR/Cas9 and SeekRNA within plasmid vector to 2 different groups of worms called CC (CRISPR/Cas9) and SR (SeekRNA). After the microinjection we separate the hermaphrodite worms one by one to different, at least 100 plates each group while also noting their life cycle stage as these might add extra information on how different stages tolerate both methods.

In all F1 progenies we check for measurable fluorescence presence and transfer each positive progeny to a separate plate, letting them self-fertilize creating F2 progenies. Then we measure how many plates of the F2 progenies have only GFP positive worms and the plates where non GFP producing worms are present. This would indicate how many times the GFP successfully insert into both chromosomes and how many times it is into only one chromosome. Statistical data would be set by how many times an insertion was created, counting the double insertion as two instances.

This method is the first step as to seeing the borders and the efficiency drop of both methods we would need to measure with different volume of inserted complexes. As basic methods suggest about 5µl of CRISPR/Cas9 we would start with 5µl of both CRISPR/Cas9 and SeekRNA with the same complex density and redo the experiments with gradually lower volume of 3µl, 1µl, 0.5µl and 0,1µl microinjections.

Implementation – Duplex test

Gene editing would be extremely efficient if all the desired insertions could be done at the same time. It may be far to do hundreds of editing at once, but the potential exists in SeekRNA to do so. In this experiment we would like to discover if SeekRNA is capable of successfully completing 2 insertions within the same injection.

The core of the experiment is the same as the comparative test. Using C. Elegans microinjections would be used to insert a plasmid vector integrating GFP coding region to the genome but this time we only use SeekRNA and incorporate another fluorescent protein coding gene called mCherry.

After the initial self-fertilization of separated P1s, the F1 progenies will possibly show green or red or both red and green fluorescence. In this case we separate once more the F1s so that any fluorescently positive worms are selected and check how many of the F2s show only homozygosity for any fluorescence.

Similarly to the comparative test we calculate the efficiency of the method for both insertions and repeat the experiments with the already stated decrease in volume with regards that for the double injection we use 5-5µls for both fluorescent proteins.

Implementation – Multiplex

If the Duplex test was a success, it would be informative to test with more and different fluorescent protein coding genes to see the limitations of the SeekRNA.

Significance

This project will determine with high accuracy if SeekRNA is an advancement compared to CRISPR/Cas9 and CRISPR in general in the means of efficiency, speed and multiplexity. Gene editing is already an enormous breakthrough especially with CRISPR, but if multiple insertions or deletions could be made at the same time and with more efficiency it would exponentially decrease the time it takes and could present unique, never before seen advancements in genetic engineering and could lead the development of gene therapies.

Personnel, infrastructure and other funding

The principal investigator (László Szalai) will be overseeing the project and perform key experiments. Two PhD students (Jakab Gipsz) and (Elek Beviz) will be responsible for the maintaining of the C. Elegans strains and the administration works while an undergraduate student (Kálmán Beka) will help with the day-to-day laboratory works.

A possible place for the experiment is the University of Gothenburg's Natrium laboratory section of biochemistry but further discussions on the schedule are required. The laboratory contains basic equipment however every listed equipment and material is required from the 2.1-2.2 paragraphs of the protocol by C. J. Martin et al. [6] although every equipment to my knowledge can be found in other departments and laboratories in Natrium therefore shared access would be a viable option.

This grant aims to secure funding from organizations focused on advancing genetic engineering and precision medicine. This will support the purchase of reagents (e.g., Cas9 protein, sgRNAs, repair templates), consumables (e.g., pipette tips, plates, culture media), expenses regarding the collaboration with the University of Sydney and access to specialized facilities for genome sequencing and analysis with an approximate total cost of 200.000€.

The estimated timeline of the project would incorporate 3 months of preparation, 6 months of comparative test, 6 months of duplex test, 6 months of multiplex test and 3 months of finalization, data analysis and publication.

Therefore, with proper funding the project should last 24 months and give a definite result. In the future the project could be continued to test insertions of significant genes, even including tests regarding human cell cultures.

References

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