International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

CRISPR Based Gene Knock Out Study in Western Clawed Frog

Dibyajyoti Dwari

Scientist, University of Portsmouth

Abstract

The advancement of biotechnology has contributed a revolutionary change in field of gene editing for the medical research and industrial biotechnology. There are different types of technology developed in recent era but the contributions of CRISPR/Cas 9(Clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9) has the major role for the advancement of gene editing[1]. Anciently it was known as acquired immunity that protects the bacteria from plasmid infection. It switches the bacterial immune system to eliminate the odd genetic material[2]. The technology has enormous potential to edit the genomes very accurate and efficient manner. It also provides various applications in the field of medical research. There are several elements involved in the CRISPR process involved in the process of gene editing. The developments of the process increased its potential for various model organisms. The major two components of the process are generally the sgRNA(Synthetic Single guide RNA) and the Cas 9[3]. The sgRNA is associated with genome specific side targeting and the Cas 9 has the ability to form the side specific DNA cleavage. There are several advantages of this technology over the disease like cystic fibrosis, sickle cell anemia, hemophilia and single nucleotide based genetic disorders[4]

1. AIM OF THE EXPERIMENT

TO perform CRISPR/cas9 based gene knock out study in Xenopus tropicalis

2. OBJECTIVE

The experiment was performed for fulfillment of following objectives.

- Identification of target gene from database associated with kidney development the species
- Construction of sg RNA along with cas9 for the selected region of gene
- Injection of the sample mixture inside the embryo
- Extraction of genomic DNA from the species and observe the mutagenesis
- Phenotyping of the species to know whether the gene were involved in the kidney disease or not?

3. INTRODUCTION

The advancement of biotechnology has contributed a revolutionary change in field of gene editing for the medical research and industrial biotechnology. There are different types of technology developed in recent era but the contributions of CRISPR/Cas 9(Clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9) has the major role for the advancement of gene editing[1]. Anciently it was known as acquired immunity that protects the bacteria from plasmid infection. It switches the bacterial immune system to eliminate the odd genetic material[2]. The technology has enormous

potential to edit the genomes very accurate and efficient manner. It also provides various applications in the field of medical research. There are several elements involved in the CRISPR process involved in the process of gene editing. The developments of the process increased its potential for various model organisms. The major two components of the process are generally the sgRNA(Synthetic Single guide RNA) and the Cas 9[3]. The sgRNA is associated with genome specific side targeting and the Cas 9 has the ability to form the side specific DNA cleavage. There are several advantages of this technology over the disease like cystic fibrosis, sickle cell anemia, hemophilia and single nucleotide based genetic disorders[4].

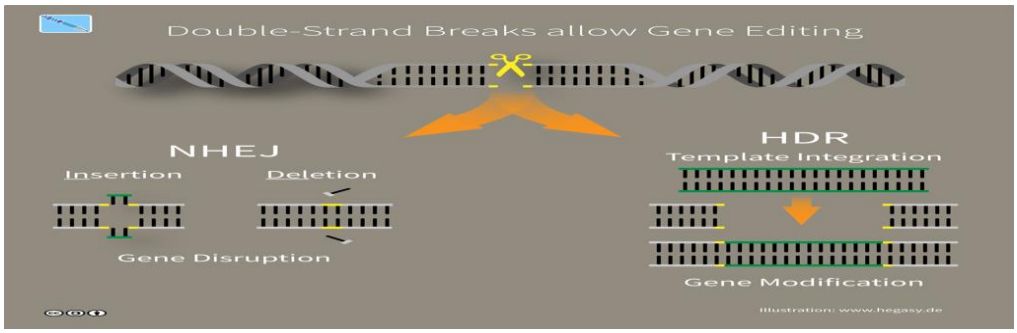
3.1 Principle of CRISPR Process

The designed CRISPR system contains two components: a guide RNA (gRNA or gRNA) and a CRISPR-binding endonuclease (Cas protein). gRNA is a short synthetic RNA consisting of the scaffold sequence required for Cas binding and a user-defined buffer of approximately 20 nucleotides that identifies the genomic target to be modified[4]. Thus, one can alter the genomic target of the Cas protein simply by altering the target sequence present in the gRNA[5].

The CRISPR mechanism involved in DNA repair. While the job of the bacteria's defenses is done after it cuts through the foreign DNA, genome editing tools have to put the pieces back together[6]. Fortunately, cells that can rely on repair machinery automatically attempt to repair DNA double-strand breaks. If a cell can access a genetic sequence similar to or "homologous" to the damaged DNA, such as a guide RNA carried by a Cas9 protein, the cell uses this corresponding sequence as a template. to fill in what it thinks is the missing segment by a process known as homology-directed repair (HDR)[7]. If a template is not available because a Cas9 tool is designed to cut but not edit, the cell will repair the damage by simply gluing the ends of the DNA together through "non-terminal splicing" (NHEJ) is error prone.

The resulting DSB is then repaired by one of two general repair pathways:

1. The efficient but error-prone non-homologous end joining (NHEJ) pathway
2. The less efficient but high-fidelity homology directed repair (HDR) pathway



(Figure.1 Showing Gene disruption and template integration by the Double strand break allowing effective gene editing)

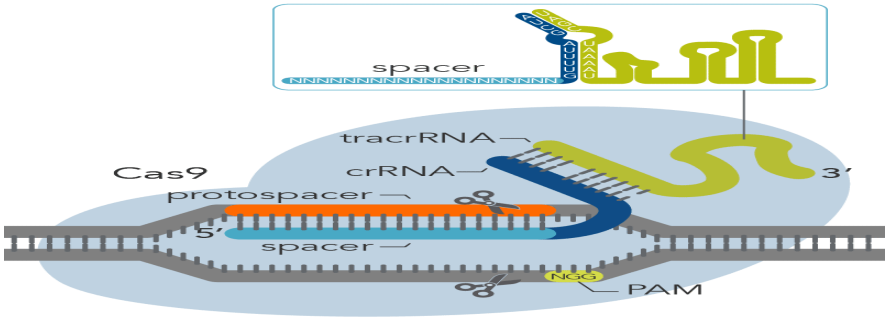
3.3 Role of PAM sequences

The CRISPRCas9 machine recognizes complementary DNA targets for a short CRISPR RNA sequence. The part of the cRNA(CRISPR RNA) sequence that complements the target sequence is called the spacer. For Cas9 to work, it also requires a specific protospacer-adjacent motif (PAM) that varies depending on the bacterial species of the Cas9 gene[8,9]. The most commonly used Cas9 nuclease, derived from S.

pyogenes, recognizes the NGG PAM sequence located directly below the target sequence in genomic DNA, on the non-target strand.

CRISPR to induce knockout cells or animals by co-expressing an endonuclease such as Cas9 or Cas12a (also known as Cpf1) and a targeted gene-specific gRNA[8]. A genome target can be any DNA sequence of ~20 nucleotides, as long as it meets two conditions given below

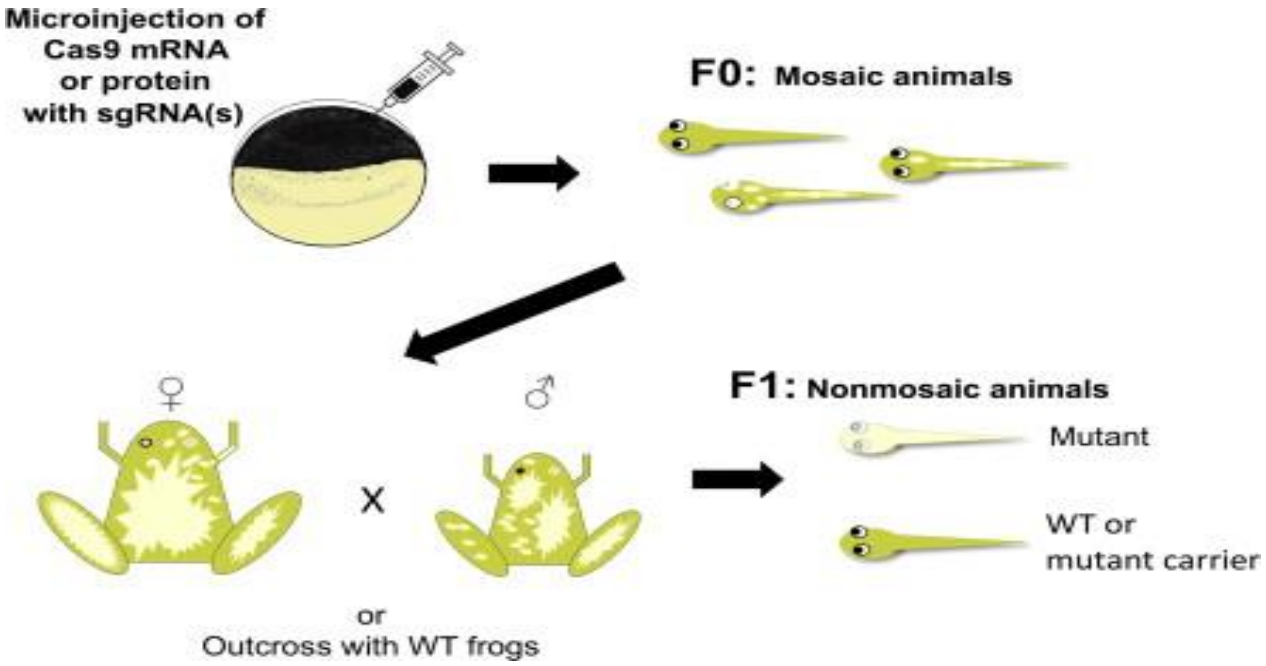
1. The sequence is unique from the genome
2. Target is present right next to the Protospacer Adjacent Model (PAM)
3.



(Figure.2 As with the sequenced knockdowns of morpholino antisense, the effects of multiple gene knockouts could be investigated using CRISPR/Cas. Simultaneous injection of multiple sgRNAs, each targeting a different site or gene, would likely enable such analyses. This is particularly useful in situations where redundancy, possibly due to gene duplication, makes phenotypic analyzes of gene function problematic[8].)

3.4 Xenopus as a favorable model for this technology

The functions of the genes which are associated with various type of disease in humans can be easily studied by the most relevant model organisms. Some factors like high proliferation, genetic and molecular pathways need to be considered for identifying the model organism[10]. Xenopus has the major characteristics like the formation of large number of eggs which are necessary to perform different process of the experiment in laboratory. Every model organism has its own advantages and limitations. The significant advantages of the model which is called as Xenopus tropicalis(Western clawed frog) is the survival at 24-26 ℃[11]. The species has the higher fertilization capacity with formation of large number of eggs. The complete genome sequencing of the species has provided a high degree of similarity with the human genome[10,11]. The eggs can be stored in refrigeration without losing its fertilization capacity. The size of the eggs of this species are nearly around 0.8 to 1.2 mm which is beneficial for the process of microinjection[12].



(Figure.3 Representation of CRISPR/cas9 based mutagenesis process in Xenopus tropicalis. Cas9 and PAM combinedly recognize the target cleavage site. Cleavage creates a double strand break using NHEJ mechanism).

4. METHODOLOGY

The genome manipulation technology had been proved as the best technology for the genome associated study and in the field of gene therapy. The large utilization of this technology had overcome various challenge and also improved a lot in recent era. The whole experiment required as the general molecular biology techniques and manual handing of the xenopus species like engineering of embryos and the technique of microinjection inside the laboratory. The test was performed using the general lab based instruments which were already available in the molecular biology laboratory previously[10].

4.1 Construction of sg RNA

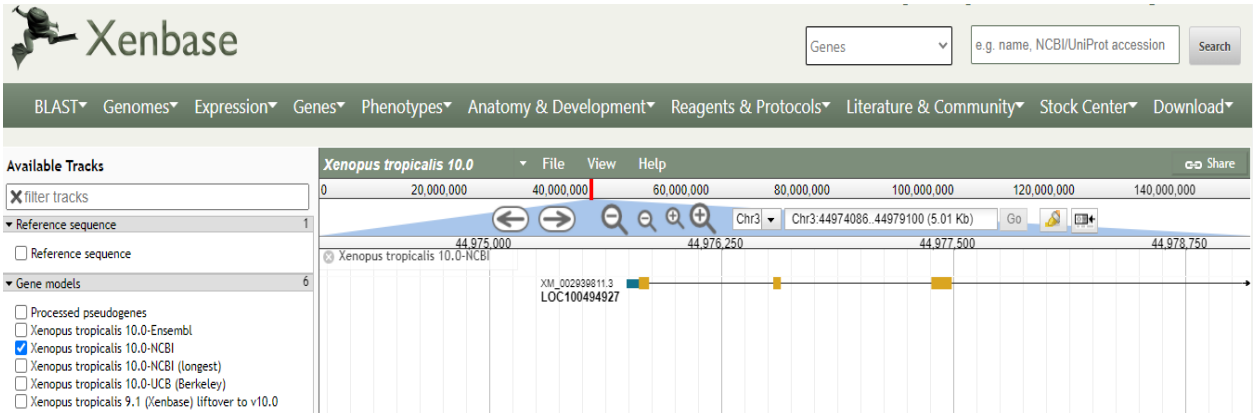
For the construction of guide RNA the first step was taken as the identification of Specific target inside on the gene of interest. The step involved selective targeting of the region of gene which need to be considered for the whole experiment. Various factors like the PAM sequences were considered very carefully for the construction of guide RNA. It plays an important role in the process of mutagenesis.

4.1.1 Identification of target gene

The overall success rate of a gene targeting always be the fact that where the mutation really caused inside the gene. The targets can be easily analyzed by the selective targeting of the PAM sequences with that region. The nucleotide called as Guanine nearest to 18-20 base pairs above the region will possibly the most favorable for CRISPR target[13]. The fact taken into consideration that any sequence in that region can be easily targeted by the adding of G/ GG not associated with the genomic sequence of the target. The next step of this process involves the determination of targeted sequence having any identity towards the other regions of the gene which may cause the different type of mutations which was not exactly the target[13].

The whole identification of target performed by using the various tools of bioinformatics. There were various web based platforms to do this type of work. But they were specific for selective species. Xenbase

(https://www.xenbase.org) database was majorly used to identify the regions of the target gene in this experiment. For identification of the specific target side and the off-target sides another type of computational tool was used called as CRISPR Scan(https://www.crisprscan.org). There were also various tools same as this having various functions and they can be used as different purpose. The main advantage for using this web platform has made the process more easy and reliable for analysis. As the experiment was based on invitro synthesis of sg RNA. The overall information about the target gene was cep290 was extracted by Xenbase. The flexibility of this database was it has the direct link with the CRISPR Scan. The gene expression and the exon which the guide RNA was extracted from the database as shown in the figure.4



(Figure.4 cep290 exon containing genome structure selected region)

For determination of best sg RNA a specialized data tool was selected called as CRISPR Scan. The predicted sequences by this web based platform has high accuracy and flexibility with minimized error rate. By using this site the off- targets were avoided**[14]**. When the double strand break happens then the mutations caused due to the insertion and deletion were very sort. Sometimes there may be a chance that the mutations will not be the sufficient enough to terminate the protein sequences. For avoiding this fact the protien folded domains need to be given as first priority.

The predicted sg RNA data for the gene cep290 knock out was as follows

The best suited guide RNA was the seventh translated exon Xtr sgRNA has a score of 46, no off targets. The position of the target was Chr03:44962166-44962189 (+): Upstream position have the Tm 57C amplicon 401bp.

Cep290 sg1: taatacgactcactataGGATTTGTAATCCTGTCCCCgttttagagctagaa

Cep 290 Upstream primer: CAACATGCTCACATTCACTAGC

Cep 290 Downstream primer: TTTCTTTTGCTTCTAATGTTTCTTT

4.1.2 Construction of DNA template

There were different types of methods for generating the sg RNA templates. But the simplest method was to directly synthesize the sg RNA templates. Generally two types of template can be taken into consideration like the plasmid based and the PCR based. Here the PCR based template construction was followed to make the process more flexible. It involves the process of making the T7- guide RNA transcribed templates and using them directly to synthesize the template DNA through the PCR process**[15]**.

The sequence arrangement was the first step for template construction. The 5' end of the primer is always identical to the site of interest.

5'-CAACATGCTCACATTCACTAGC G(N)16-20 TTTCTTTTGCTTCTAATGTTTCTTT-3'

The underlined portion refers as the site for T7 promoter

Where G(N)16-20 was the specified target sequence of interest

The universal oligonucleotides were used to perform reaction of the specific sequence of the gene. They were annealed and extended by following the standard protocol of PCR for 100 µL reaction mixture.

The PCR reaction setup was performed as following

100 µL PCR Reaction

46 µL Nuclease Free Water

50µ L GoTaq® G2 Green Master Mix

2µL Specific target Oligonucleotide(100µM)

2µL Universal CRISPR Oligonucleotide(100µM)

The reaction was prepared to a PCR tube containing reaction mixture of 100µ L of total sample. 3µL sample from the reaction mixture was taken out from the tube and transferred to another PCR tube for the negative control to run on a agarose gel. 3µL reaction kept inside the ice bucket.

Conditions for Thermal Cycler

Step	No of Cycle	Temperature	Time(Min:Sec)
1	1	95°C	05:00
2	13	95°C	00:20
		65°C	00:20
		68°C	00:15
3	30	94°C	00:20
		58°C	00:20
		68°C	00:15
4	1	68°C	05:00
5	1	4°C	

Reaction was performed by following the above conditions. After the reaction was completed 3µL double standard DNA template was taken to run on the Agarose gel electrophoresis using 1.2 % Of agarose and 100 BP ladder sequence. Results obtained from the reaction described in Figure.6

4.1.3 sg RNA Synthesis

A specialized kit which is named as MEGAshortscript™ T7 Transcription kit(Invitrogen- ThermoFisher Scientific) for the invitro synthesis of sg RNA. According to the manufacturer instruction 8 µL template DNA used for getting the guide RNA(0.25- 0.6 µL PCR generated DNA template). There were some safety measure followed for getting the exact result described below.

- The T7 Reaction Buffer, Four ribonucleotide solutions, and the nuclease free water were taken to room temperature as they were frozen.
- Once it was on the normal temperature ribonucleotide solutions was mixed and kept inside the ice for avoiding denaturation
- The T7 Reaction and the ribonucleotide solutions mixed properly to avoid any error result.
- The reactions was microfuge to avoid any losses and contaminations of the sample.
- T7 Enzyme mix always kept on the ice as they were temperature sensitive

- The reaction was assembled in an room temperature with RNase- Free conditions. All nucleotides were properly mixed.

The PCR Reaction setup performed by following components

20µL PCR Reaction

2µL 10X Reaction Buffer
2µL ATP Solution(75mM)
2µL CTP Solution(75mM)
2µL GTP Solution(75mM)
2µL UTP Solution(75mM)
8µL Template DNA
2µL T7 Enzyme Mix

- The contents were mixed properly by propelling the tube, So that the reaction mixture was collected bottom of the tube.
- Then the solution was incubated overnight at 37℃ On a specialized incubator
- After the overnight storage 1 µL DNase added to the solution to conform that no DNA leftover in the reaction mixture
- Then the solution was incubated again for 15 minutes

After the process was completed the final solution was the mixture of buffer solution, Some DNA fragments, And the RNA solution. But the pure form of guide RNA is required for the CRISPR process. For getting the pure form, a specialized technique was followed which is called chromatography. It is the separation technique of mixtures based on the relative amount of each solute distributed between moving stream of fluid. Here the guide RNA were column purified by using the commercial kit called SigmaSpin™ and the pure sample was visualized under 2 % agarose gel electrophoresis[16].

According to the manufactures instruction Some instructions followed to perform the Sigma Spin™ column chromatography[17]. They were as follows.

- The column was pre spin at 750xg for 2 minutes to removes the extra buffer
- Cap of the column was half closed so that the extra air inside the column was removed.
- After that the samples are loaded into the middle of the column.
- The sample was centrifuge for 4 minutes at 750xg to get the purified product at the bottom of the column.

The purity of the product was analyzed by using highly accurate spectrophotometer[18]. It is the instrument which is used for measuring the concentration of a sample by the wavelength of light.

4.2 CRISPR/Cas9 construction

For the successful mutagenesis a proper proportion of guide RNA and cas9 doses are required. Many of the cases it was observed as amount of 50 to 500 pg of the sample of sg RNA were sufficient enough to cause the changes[19]. But sometimes there will be a chance due to toxicity which would result ultimate lethality in the embryo. Here the concentration of guide RNA was taken in between 50 to 200 pg. Effective guide RNA doses was really an important factor for the success of the experiment. Researchers has found that the optimal range doses for the embryo was 3-4 ngm. The cas9 NLS protien was pre synthesized by 2.6ng EnGen®(New England Biolab).

The dosing was prepared by following this calculation

Total solution for embryo injection= 4µL(3.2 µL sample + 0.8µL cas9 NLS protien)

4.3 Procedure of Microinjection

Microinjection is a standard technique of insertion of Foreign DNA into living cell through a glass pipette[20]. In this procedure the glass pipette was heated up to the it will generate the fine tip of 0.5 mm diameter which was suitable for the embryo injection. The process of microinjection is same for all the experiment but the amount of sg RNA and the cas9 differs according to which type of targeting inside the embryo needs to be done. Sometimes the results were not consisted in this case. Embryos were collected at and the sample containing the mixture of guide RNA and cas9 was injected to the embryo. It was injected early stage of embryo fertilization for observing all possible phenotypes. The general doses are performed by using the amount of 1-4 nl of sample to the hemisphere region of the embryo.

(Figure.5 Microinjection of CRISPR with the mixture prepared by the mixture of cas9 and sg RNA)

4.4 Genotyping of Embryo

Genotyping is the technology which can detect the genetic differences which are responsible for the major changes in the phenotypes. It may be both physical changes or any other form of changes[21]. Here the main aim was to observe the knock out of cep290 gene worked properly or not which was assumed to be associate with kidney diseases. There are various methods for genotyping but here the T7 endonuclease essay was taken for the experimentation. Here two different process were followed. At the initial stage the genomic DNA was extracted from the embryo and then the possible mutations were observed through T7 endonuclease essay.

4.4.1 Extraction of Genomic DNA

The embryos of the Xenopus were transferred to PCR tubes and frozen at -80℃. For the embryo lysis the whole samples was digested at 56 ℃ in 60µL lysis buffer:50mM Tris(pH 8.5), 1mM EDTA, 0.5% Tween-20(fisher scientific) containing the proteinase K for 2 hours. The proteinase K was inactivated at 95℃ for 15 minutes and stored at -20℃. Samples were centrifuged at 1000* g for 1 minute for the better result. Here the process followed by avoiding contamination by spinning procedure. It was able to generate the pure product.

4.4.2 Amplification Of selective region

The sample was amplified using the PCR process. The genomic DNA extracted from the previous process were amplified using the GoTaq Master mix with the help of a thermal cycler. The PCR primers were taken which will amplify the 300-400 Bp of the genomic region. The Thermocycler reaction was as follows

20µL Reaction

10µL GoTaq G2 Green Master Mix
1µL Forward Primer(10µM)
1µL Reverse Primer(10µM)

50ng Template(1-2 µL)
The volume made 20µL by using nuclease free water
Thermal cycler conditions for the PCR was as follows

Step	Cycle	Temperature	Time(Min:Sec)
1	1	95℃	05:00
2	40	95℃	00:30
		58℃	00:30
		72℃	01:00
3	1	72℃	07:00
4	1	4℃	

After the reaction was completed the amplified product was visualized under 1.2 % agarose gel electrophoresis.

4.4.3 T7 Endonuclease Assay

T7 endonuclease assay is known as the best method for knowing the genome editing success rates in the process of CRISPR[22]. This process detects the duplex DNA generated through the genome lysis process was modified or not. This assay gives an idea whether the experimentation was successful or not. There were also different assays. But this assay was more flexible because it can be performed by using the general lab instruments. The this process the modified embryos were digested with 1 µL T7E1 and the fragmented DNA samples were observed through 2 % of gel.

20µL reaction

2µL NEB Buffer 2
3µL PCR amplicon of target region
14µL Nuclease free water
1µL T7E1(10U)

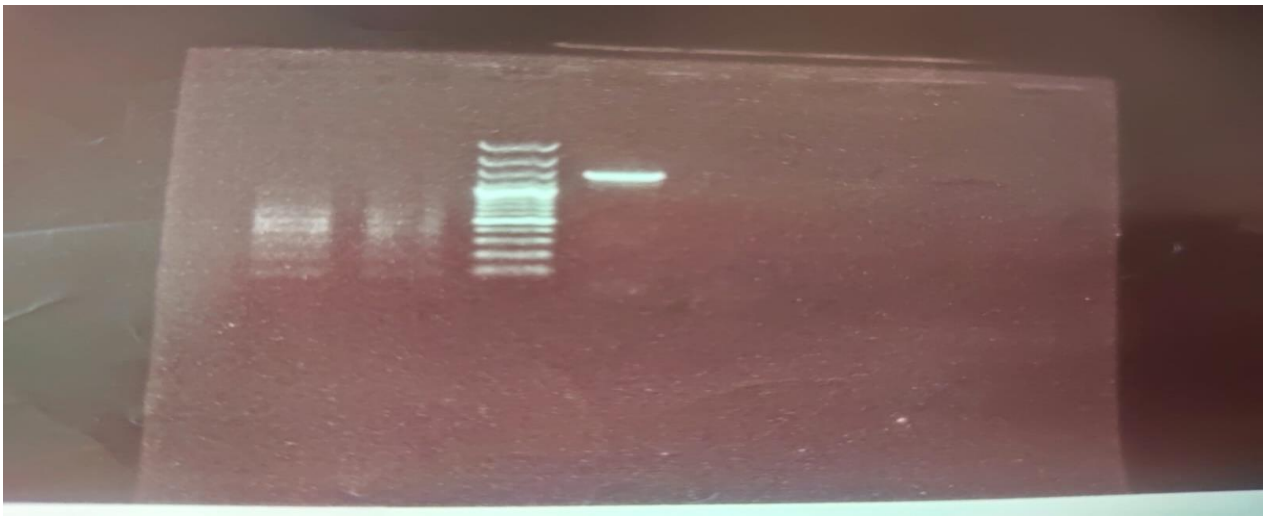
The thermal cycler conditions are as follows

Step	Cycle	Temperature	Time(Min:Sec)
1	1	95℃	05:00
2	1	85℃	05:00
3	1	25℃	05:00
		4℃	05:00
Add T7 Endonuclease to Sample			
4	1	37℃	15:00
		4℃	

5. RESULTS

5.1 PCR based construction of DNA template was more effective in case of Xenopus tropicalis

By following the procedure of oligonucleotide annealing and extension was more effective in this experiment. High fidelity DNA polymerase was used to generate the single product. Commercially synthesized reaction mixture had made the process more easy. Here Mgcl2 was not used as the nuclease free water contained the mineral in it. The green color master mix also contains tracking dye in it so that separately it was not added. A typical result obtained from the process of Agarose gel electrophoresis was shown In Figure 6.

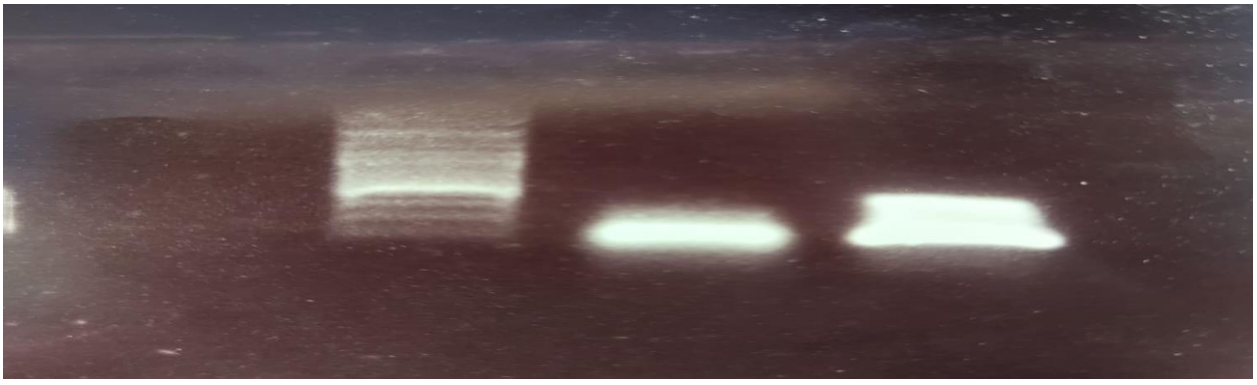


(Figure.6 PCR based template DNA synthesis. Oligonucleotide were kept inside the thermocycler for annealing and extension with specified temperature conditions. Results showing the gel bands between control vs amplified product using 1oo bp ladder sequence. After the reaction was completed the product was visualized under Ultra violet transilluminator showing strong band of DNA synthesized)

The experiment followed by following standard procedure of Polymerase Chain Reaction. From the above Fig.1 it was illustrated that PCR product was generated from the reaction. The band at the last showing the product of amplified template DNA was generated throughout the process. This result conformed that the DNA template strand which was required for the synthesis of guide RNA sample was successfully generated through the process.

5.2 Invitro transcription of sg RNA was more flexible by using the commercially manufactured Transcription kit

The double stranded DNA generation was successful from the previous result. The ds DNA was then transcribed to RNA by the commercially manufactured kit called MEGAshortscript™. The major flexibility by using this kit was all the necessary nucleotide solutions and other materials were separately available and it was ready for use by mixing with proper quantity. A significant result obtained from the Gel electrophoresis was shown in Figure 7.



(Figure.7 Guide RNA construction through MEGAshortscript™. The reaction setup done using a thermocycler in DNA free condition. Results was visualized in UV transilluminator showing

strong bands of synthesized guide RNA sample. Results was obtained through control vs sample as shown)

The real concentartion of pure RNA sample was determined by using a specialized spectrophotometer. The exact quantity and purity of RNA obtained from the spectroscopic analysis was shown in Figure.8

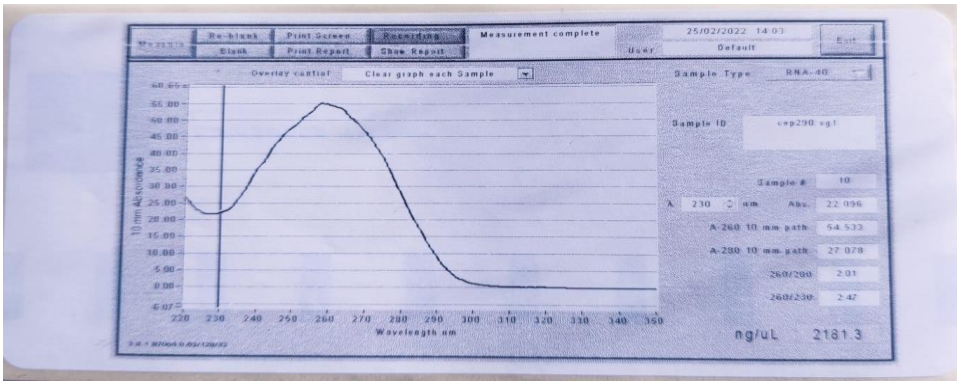


Figure.8 Spectroscopic analysis of RNA sample and results obtained from Nanodrop version 3.8.1

5.3 Genomic DNA extraction showing the limited result(Genotyping)

Guide RNA with the cas9 mix was injected to the embryo. Embryos were collected at the later stage for genotyping analysis. Here the T7 endonuclease assay was performed after genomic DNA extraction which was helpful for detecting the mismatches between the wild type and mutant DNA. The genomic DNA extraction was assumed to be successful but when the amplified product was observed through the UV transilluminator it was showing no bands in it. The possible region of the experiment was identified may be the survival of the embryo. Also there was a fact that the sgRNA along with the cas9 was not properly triggered the mutation inside the embryo. The band was not generated that refers that the amplified product was not generated itself which can be demonstrated from the figure.9

Figure.9 Amplified genomic DNA product generated through the PCR process was analyzed through UV transilluminator. No signs of amplified product was found on the gel. Gel electrophoresis was showing no bands means the product was not generated properly during the process

T7 endonuclease assay was not performed due to the negative genomic result DNA extraction. But the process was followed by following other genes in which the genomic DNA extraction was successful. The result obtained from the process suggests that there are no possible amplified product was generated from the genomic DNA. From this it was clear that the specific gene was not associated with kidney disease. By the result of knockout either some other genes switched on or off causing the lethality and abnormal growth in the species.

5.4 Phenotyping Study gives a clear idea about the gene expression

Genotyping study was failed to give any clear cut idea about the mutations inside the embryo. But the phenotyping studies were performed by observing the embryos under stereo microscope[23]. Quite interesting facts were collected by showing the different phenotypical structure of the embryo. The development of the embryos were not normal at all and they were developed badly at the gastrula stage. Because they faced difficulty to swim on the petri plate. There were a group of phenotypical abnormalities found on the developing embryo. Some of the cases the gene may be caused the mutations leading towards the lethality. The overall growth of the embryo was affected due to the mutation may be the possible cause the functioning of another gene or switch off some genes which was not necessary at all. Some cases it was found to be suffering from oedema can be observed from figure.10

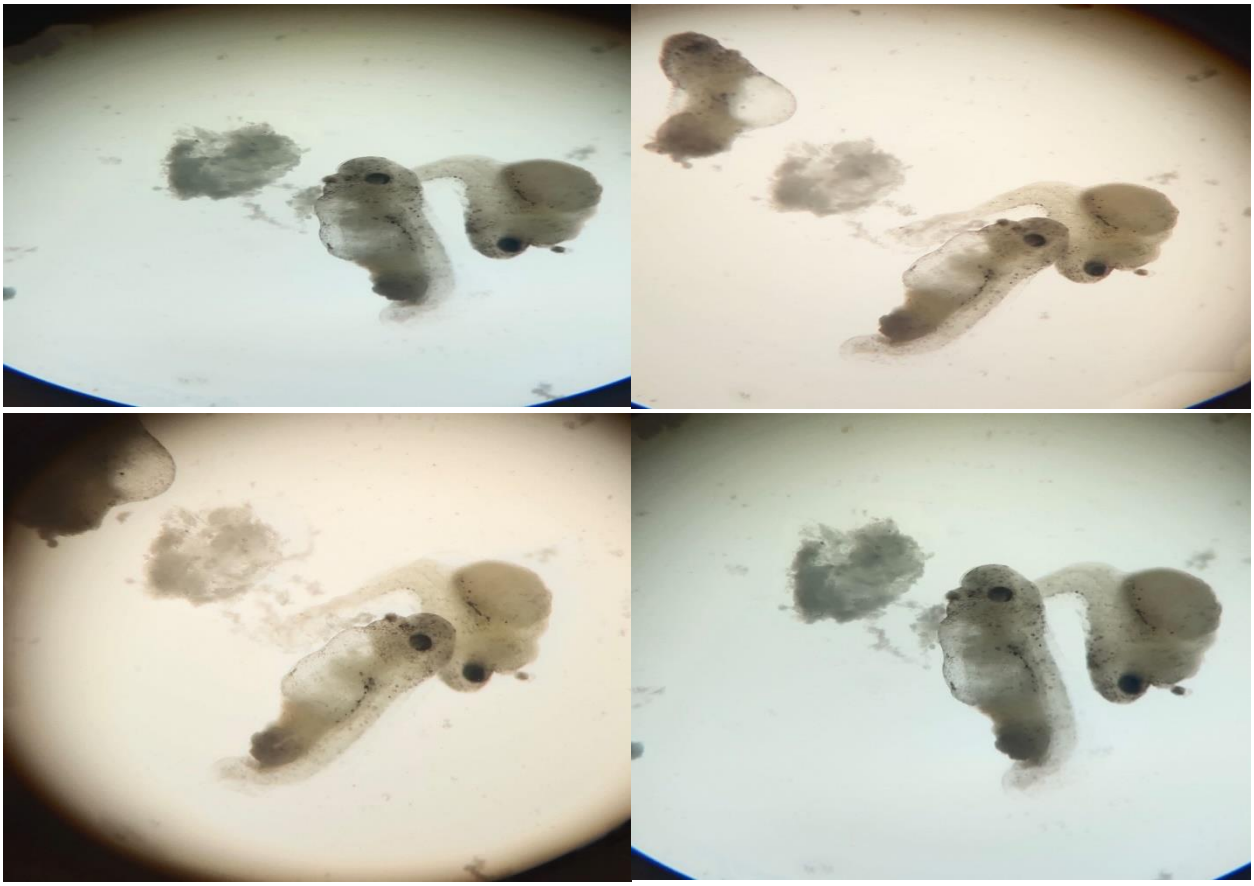


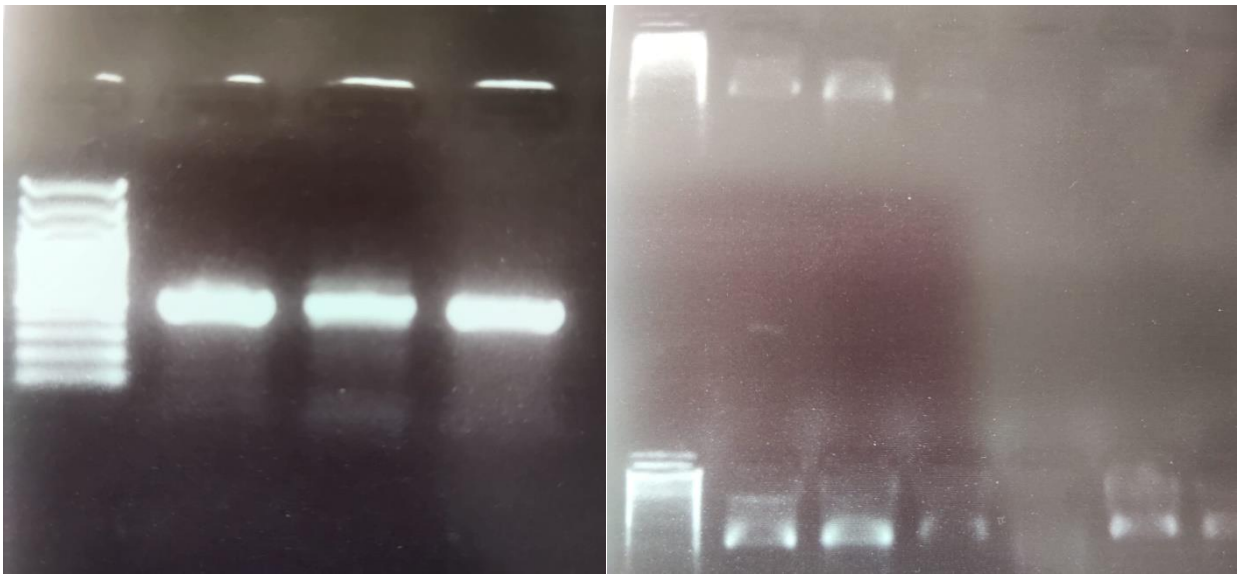
Figure.10 Phenotyping study of the Xenopus embryo under stereo microscope for the phenotypic changes occurred due to the gene editing. Some are found abnormal growth at gastrula stage and some were suffering from oedema. Some embryos were suffering from poor growth and difficulty to swim over the petri plate

6. DISCUSSION

The whole experiment was performed by following the standard protocol of CRISPR technology. These includes the basic steps which involves the gene editing of xenopus species. But there are also some other aspects of the experiment to make it more effective and error free. The applications of this technology are many more.

6.1 Genotyping result Analysis limitations

For the specific gene which is called as cep290 the experiment was not successful for this time. But genomic studies are not limited to its mode of action and application. Genotyping can be performed by following another type of gene. The results were quite straight forward to go for mutagenesis studies with in the embryo. If the product was amplified in the genome extraction phase then it gives a clear indication that our experiment was successful and can be forwarded to phenotypic studies. If the experiment was successful then the bands were expected to come like the below Figure 11.



1.Genotyping bands **2. T7 endonuclease assay**

Figure.11 Genotyping and T7 endonuclease assay performed using another gene called as pkd1 showing positive amplified result with possible changes in mutation due to the genome editing. The first figure bands showing bands for the amplified genomic DNA and second figure showing the conform results of T7 endonuclease assay

6.2 Avoiding off Targets through multiple targeting

Mismatches are identified within the target nucleotides that are distal to the parent seed sequence, which could lead on to off-target targeting of the species. There are variety of Web-based search tools are developed that predict both target and off-target sites for sgRNAs in Xenopus tropicalis[24]. When choosing between potential sites to focus on, one should avoid sequences with off-target mismatches that are largely located distal to the seed sequence. Also, one should detain mind that sequences followed by the PAM can even be off-target sites, therefore off-target analysis should include both PAMs site[25]. If the study of various mosaic animals isn't desirable, then various breeding strategies for creating non-mosaic animals could also be employed. Ideally, the experiment can be constructed by a mutant that contains the identical lesion at both alleles; however, since multiple generations are needed to realize this, a time consuming process, analysis of phenotypes is also significantly delayed.

6.3 Applications of Mutagenesis in Xenopus species

The CRISPR-mediated mutagenesis strategy described here may provide a means to analyze the various parameters in first stage of animals. However, the mosaicism of the final F0 embryos may be interfere with some analyzes and the thus improvements in CRISPR/Cas efficiency in Xenopus species would be valuable[26]. As with the sequenced knockdowns of morpholino antisense, the effects of multiple gene knockouts could be investigated using CRISPR/Cas. Simultaneous injection of multiple sgRNAs, each targeting a different site or gene, would likely enable such analyses. This is particularly useful in situations where redundancy, possibly due to gene duplication, makes phenotypic analyzes of gene function problematic[27]. Another application is homology-directed recombination (HDR)-dependent gene targeting, i.e. introducing point mutations or introducing larger genetic elements such as sequence tags or transgenes into any position in the genome.

References

1. CRISPR Service - Creative Biolabs. Accessed March 20, 2022. https://www.creative-biolabs.com/stem-cell-therapy/crispr-service.htm?msclkid=223ffc44e63814e79a329e73c11a0793&utm_source=bing&utm_medium=cpc&utm_campaign=YL-ST-Service&utm_term=crispr%20method&utm_content=1-1-4-1%20CRISPR%20Service
2. What is CRISPR? | New Scientist. Accessed March 20, 2022. https://www.newscientist.com/definition/what-is-crispr/
3. Components of CRISPR/Cas9. Accessed March 20, 2022. https://blog.addgene.org/components-of-crispr/cas9-our-new-crispr-101-ebook
4. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014;346(6213). doi:10.1126/SCIENCE.1258096
5. What is CRISPR-Cas9? | Facts | yourgenome.org. Accessed March 20, 2022. https://www.yourgenome.org/facts/what-is-crispr-cas9
6. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014;346(6213). doi:10.1126/SCIENCE.1258096
7. Gupta RM, Musunuru K. Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. Journal of Clinical Investigation. 2014;124(10):4154-4161. doi:10.1172/JCI72992
8. What is the PAM sequence for CRISPR and where is it? | IDT. Accessed March 20, 2022. https://eu.idtdna.com/pages/support/faqs/what-is-a-pam-sequence-and-where-is-it-located
9. CRISPR: A game-changing genetic engineering technique - Science in the News. Accessed March 20, 2022. https://sitn.hms.harvard.edu/flash/2014/crispr-a-game-changing-genetic-engineering-technique/
10. Tandon P, Conlon F, Furlow JD, Horb ME. Expanding the genetic toolkit in Xenopus: Approaches and opportunities for human disease modeling. Developmental Biology. 2017;426(2):325-335. doi:10.1016/J.YDBIO.2016.04.009
11. Nakayama T, Blitz IL, Fish MB, et al. Chapter Seventeen Cas9-Based Genome Editing in Xenopus tropicalis. Methods in enzymology. 2014;546(C):355. doi:10.1016/B978-0-12-801185-0.00017-9
12. Pattanayak S, Vázquez-Maldonado LA, Deiters A, Chen JK. Combinatorial control of gene function with wavelength-selective caged morpholinos. Methods in Enzymology. 2019;624:69-88. doi:10.1016/BS.MIE.2019.04.007

13. sgRNA Template Construction for Cas9 Gene Editing | NEB. Accessed March 20, 2022. https://international.neb.com/applications/genome-editing/sgrna-template-construction-for-cas9-gene-editing
14. CRISPRscan : CRISPRs in vivo. Accessed March 20, 2022. https://www.crisprscan.org/
15. Cheng S, Fockler C, Barnes WM, Higuchi R. Effective amplification of long targets from cloned inserts and human genomic DNA. Proceedings of the National Academy of Sciences of the United States of America. 1994;91(12):5695-5699. doi:10.1073/PNAS.91.12.5695
16. Biotechnology 101 Protocol: Gel Electrophoresis | Bento Lab. Accessed March 20, 2022. https://bento.bio/protocol/biotechnology-101/gel-electrophoresis/
17. Moldoveanu SC, David V. Equilibrium Types in HPLC. Essentials in Modern HPLC Separations. Published online 2013:85-114. doi:10.1016/B978-0-12-385013-3.00003-3
18. Spectrophotometer- Principle, Instrumentation, Applications. Accessed March 20, 2022. https://microbenotes.com/spectrophotometer-principle-instrumentation-applications/
19. Pickerill ES, Bernstein DA. CRISPR mediated genome editing, a tool to dissect RNA modification processes. Methods in Enzymology. 2021;658:435-452. doi:10.1016/BS.MIE.2021.06.013
20. Hartmann N, Englert C. A microinjection protocol for the generation of transgenic killifish (Species: Nothobranchius furzeri). Developmental Dynamics. 2012;241(6):1133-1141. doi:10.1002/DVDY.23789
21. PCR genotyping protocol – Zhuang Laboratory. Accessed March 20, 2022. https://sites.duke.edu/zhuanglab/genotyping/genotyping-protocol/
22. Determining Genome Targeting Efficiency using T7 Endonuclease I | NEB. Accessed March 20, 2022. https://international.neb.com/protocols/2014/08/11/determining-genome-targeting-efficiency-using-t7-endonuclease-i
23. Shacham-Silverberg V, Wells JM. Generation of esophageal organoids and organotypic raft cultures from human pluripotent stem cells. Methods in Cell Biology. 2020;159:1-22. doi:10.1016/BS.MCB.2020.04.009
24. Carbery ID, Ji D, Harrington A, et al. Targeted genome modification in mice using zinc-finger nucleases. Genetics. 2010;186(2):451-459. doi:10.1534/GENETICS.110.117002
25. Wang H, Yang H, Shivalila CS, et al. One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering. Cell. 2013;153(4):910-918. doi:10.1016/J.CELL.2013.04.025
26. Nakayama T, Fish MB, Fisher M, Oomen-Hajagos J, Thomsen GH, Grainger RM. Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in Xenopus tropicalis. genesis. 2013;51(12):835-843. doi:10.1002/DVG.22720
27. Olson DJ, Hulstrand AM, Houston DW. Maternal mRNA Knock-down Studies: Antisense Experiments Using the Host-Transfer Technique in <Emphasis Type="Italic">Xenopus laevis</Emphasis> and <Emphasis Type="Italic">Xenopus tropicalis</Emphasis>. Methods in Molecular Biology. 2012;917:167-182. doi:10.1007/978-1-61779-992-1_10