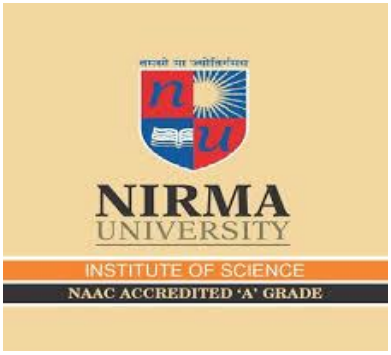
# CRISPR Cas9 mediated Knock-In of fluorescent tag to track GATA6 in Mouse Embryonic Stem Cells

A Thesis submitted to
**NIRMA UNIVERSITY**

In partial fulfillment for the award of the Degree of
**MASTERS OF SCIENCE**
**IN**
**BIOTECHNOLOGY**





by

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***I would like to dedicate all the work that I have done to my parents.***

# ACKNOWLEDGMENTS

I would like to express my deep and sincere gratitude to my **parents and siblings** for their continuous and unparalleled love, help and support. I am thankful to my **best friend** for his continuous motivation, moral support and love.

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I would like to thank **Madhavi Joshi** for correcting my thesis.

# CERTIFICATE

This is to certify that the thesis entitled "***CRISPR Cas9 mediated Knock-In of fluorescent tag to track Gata6 in mouse Embryonic Stem Cells***" submitted to **Institute of Science, Nirma University** in partial fulfillment of the requirement for the award of the degree of M.Sc. in Biotechnology, is a record of research work carried out by **Shah Hiral Shailesh (18MBT028)** under the guidance of **Dr. Amee K. Nair**. No part of the thesis has been submitted for any degree of diploma.

Prof. Sarat K. Dalai
(Director)

Dr. Amee K. Nair
(Dissertation Guide)
Assistant Professor

## DECLARATION

I hereby declare that the project report entitled "***CRISPR Cas9 mediated Knock-In of fluorescent tag to track Gata6 in mouse Embryonic Stem Cells***" is a record of original work carried out by me under the supervision of **Dr. P Chandrashekar** at **Centre for Cellular and Molecular Biology**, Hyderabad-500 007. This project has not been submitted earlier in part or full for the award of any degree, diploma, associate ship or fellowship.

Dr. P Chandrashekar

Hiral Shailesh Shah

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## LIST OF ABBREVATIONS

| | |
|---|---|
| **Amp** | Ampicillin |
| **BSD** | Blasticidin |
| **DNA** | Deoxyribonucleic acid |
| **ESCs** | Embryonic Stem Cells |
| **ET** | Water for Embryo Transfer |
| **FACS** | Fluorescence Activated Cell Sorting |
| **FP** | Forward primer |
| **GMEM** | Glasgow Minimum Essential Media |
| **HF** | High Fidelity |
| **h** | Hours |
| **ICM** | Inner Cell Mass |
| **IRES** | Internal Ribosomal Entry Site |
| **LAF** | Laminar Air Flow |
| **LB** | Luria Bertani |
| **LIF** | Leukaemia inhibitory factor |
| **MEFs** | Mouse Embryonic Fibroblasts |
| **MG** | Molecular Graded |
| **Mins** | Minutes |
| **MM** | Master Mix |
| **MN** | Macherey Nagel |
| **NEAA** | Non-Essential Amino Acids |
| **NEB** | New England Biolabs |
| **OptiMEM** | Optimum Minimal Essential Media |

| | |
|---|---|
| **PBS** | Phosphate Buffer Saline |
| **PCR** | Polymerase Chain Reaction |
| **PEG** | Polyethylene Glycol |
| **RA** | Retinoic Acid |
| **RNA** | Ribonucleic acid |
| **RP** | Reverse primer |
| **Secs** | Seconds |
| **UV** | Ultra Violet |

## Abstract

The embryonic stem cells (ESC) are pluripotent cells that are derived from the inner cell mass of the blastocyst and have the ability to differentiate to almost all cell types, *in-vitro and in-vivo*. Cells respond to the extracellular environment with a set of receptors which is altogether known as mechanical signaling. Recent studies reveal that the mechanical environment affect the behaviour and function of different types of cells, including stem cells. However, signaling pathways involved in the mechanical regulation of stem cell properties remain largely unknown. During early mammalian development, once the inner cell mass is formed, next cell fate choice is formation of Epiblast and Primitive Endoderm (PE). Nanog is an epiblast marker and marker for PE is GATA-6. In this study, an endogenous *GATA-6* BFP ESC line is created by CRISPR-CAS9 mediated Knock-In. This reporter cell line will be used to detect/track primitive endoderm cells during ES cell differentiation in EBs and blastocyst.

# 1. Introduction

Totipotency is the ability of a single cell to divide and produce all of the differentiated cells in an organism. When a sperm fertilizes an egg, it forms zygote, a single totipotent cell. (Figure1 Saiz N, and Plusa B Reproduction 2013; 145: R65-R80). The zygote undergoes 3 cleavage divisions from 1 cell to 2 cells, 2 cells to 4 cells and the 4 cells to 8 cells stage (Morula). During early embryo development the blastomeres adhere to each other to form a cluster of cells called the Morula by Day 2.5. The embryo remains totipotent until the morula stage. Once the blastocyst is formed they lose their totipotency and assume pluripotency.

The blastocyst is formed by the Day 3.5. The pluripotent cells give rise to the progenitors, which are multipotent and hence gives rise to multiple cell types. The multipotent cells show restricted potency and hence assume unipotency or bipotency giving rise to specific cell types. The blastocyst consists of three layers- the inner cell mass which are pluripotent; the primitive endoderm that form the yolk sac and the Trophoectoderm that give rise to the placenta.

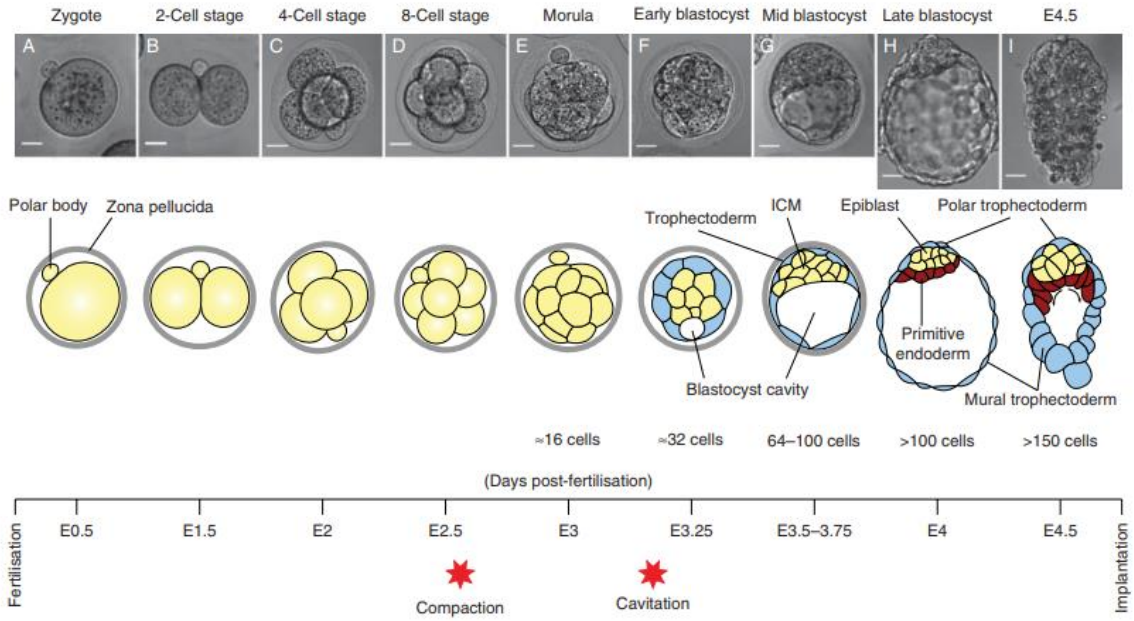


**Figure 1:** Stages of mouse preimplantation development.

**(A) Zygote (fertilized egg)**; **(I) E4.5 (peri-implantation blastocyst)**. The timeline indicates the time elapsed since fertilisation in embryonic days (E); ICM- inner cell mass. Timeline not to scale. Scale bars =20 mm.

The embryonic stem cells (ESCs) are pluripotent stem cells that are derived from the inner cell mass (ICM) during the pre- implantation stage. The ESCs are cultured in labs with the help of a substratum. They are either cultured on feeder layers like mouse embryonic fibroblasts (MEFs) or on gelatin for the cells to adhere. ESCs are cultured with the assistance of cytokines like LIF (Leukaemia inhibitory factor) and BMP4 (Bone morphogenetic proteins). LIF and BMP4 helps to maintain the pluripotency of ESCs.

The embryonic genome activation or the transcription activation is evident during day 3, that is: the 4 cell to 8 cell stage of the development. There are certain factors in the genome which regulate the pluripotency state in ESC. Shinya Yamanaka discovered that adult cells can be reprogrammed back to stem cell like state by putting the transcription factors Oct4, sox2, klf4 and c-Myc which are highly expressed in ESC. They are also called as the Yamanaka factors. They help ESCs maintain pluripotency, preventing the cells from differentiating into different cell types.

Ian chambers co- discovered another core transcription factor that regulates ESC pluripotency – Nanog. It plays a key role in maintaining pluripotency in ESC. Several extrinsic signals such as LIF, BMP and Wnt can support the self-renewal and pluripotency of ESC through regulating the pluripotent genes like Oct4 and Sox2. Nanog is one of the key downstream effectors of these signals. Elevated levels of Nanog helps mESC maintain self – renewal independent of LIF. Nanog works with Oct4 and Sox2 to control a set of target genes and form a regulatory network to support or limit each other's expression level which is crucial in maintaining the properties of ESC.

It has been reported that soluble factors play an important role in the maintenance of stem cells. Recent studies show that mechanical factors influence various cell properties. Externally applied mechanical stimulation can be transduced to intracellular biochemical signals through conformational changes in focal adhesion-related proteins (Friedland JC et.al 2009). Notably, these molecules regulate cell behaviour when grown on substrates with different elasticities (Jiang G et al. 2006).

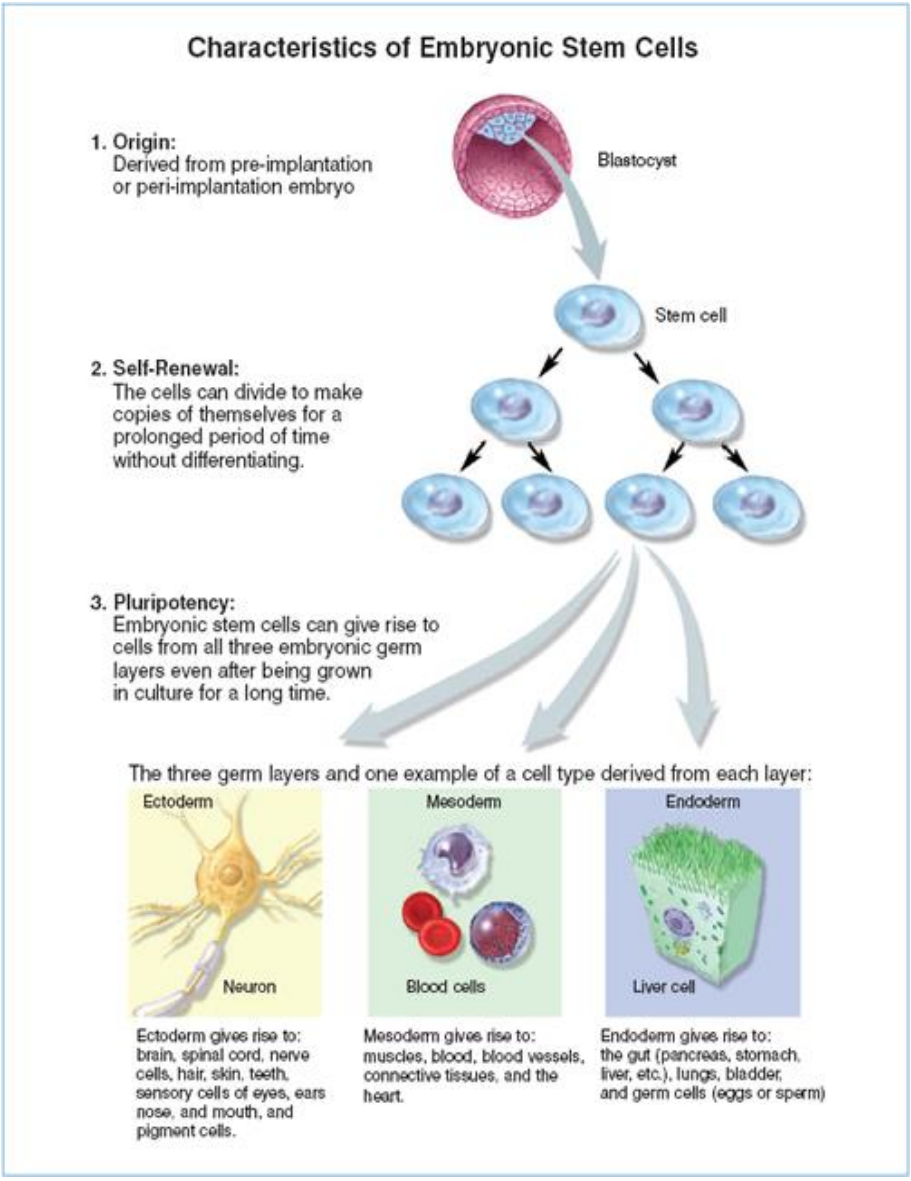


**Figure 2.** Characteristics of Embryonic Stem Cells.

Embryonic stem cells are derived from ICM of blastocyst. They have two unique characteristics – Self renewal and Pluripotency. ESCs can differentiate into almost all cell types and give rise to all three germ layers – Ectoderm, Mesoderm and Endoderm. Terese Winslow, 2006

## 1.3 NANOG:

Nanog is a homeobox-containing transcription factor with a key role in maintaining the pluripotency of the inner cell mass and in the derivation of embryonic stem cells (ESCs) from these. It is a protein composed of 305 amino acids with a conserved homeodomain motif. It is located on Chromosome 12 in Human and Chromosome 6 in Mouse (Jauch, et al, 2008).


**Figure 3**: Representation of Nanog protein

Nanog is a transcription factor composed of 305 amino acids. **ND** – N- terminal domain, **HD** – Homeodomain, **CD1**- C- terminal domain 1, **WR** – Tryptophan repeats, **CD2**- C terminal domain 2

NANOG possesses 3 functional domains:

1. The N-Terminal Domain

2. The C- Terminal Domain

3. The Conserved Homeodomain

The N-Terminal Domain is rich in Proline, Threonine and Serine residues and the C- terminus contains a Tryptophan rich domain. The Homeodomain in Nanog ranges from 95 to 155 residues and enables DNA binding (Mullin et al. 2008).

**FUNCTIONS:**

Nanog is an essential transcription regulator which maintains pluripotency and self-renewal of inner cell mass and embryonic stem cells. It maintains pluripotency of ESCs by preventing their differentiation towards extraembryonic endoderm and trophectoderm lineages (Oh J.-H. et al., 2005). Elevated levels of Nanog can maintain mouse ESC self –renewal independent of LIF. Nanog forms a regulatory network with Oct4 and Sox2 to control a set of target genes and support or limit each other's expression level which is fundamental in maintaining the properties of ESC (Kim JS, et al. 2005).

## 1.4 Review of literature:

### 1.4.1 Introduction to Mechanical Signaling:

ESCs can self-renew and differentiate depending on the various culture conditions and signaling pathways activated by it. They self-renew continuously in media containing LIF and 2i (Takahasi and Yamanaka, 2006). But most of the mechanisms responsible for this self-renewal and cell fate choice are unknown. A recent branch in the field, studies about the ability of cells to sense the stiffness of the environment, and accordingly make cell fate choices. This is known as mechanical signaling. Depending on the substrate stiffness and and geometry the ESCs behaviour will change.

Recent studies indicate that the mechanical environment influences the behavior and function of various types of cells, including stem cells. However, signaling pathways which are involved in the mechanical regulation of stem cell properties are unknown. The extracellular matrix prevents common form of apoptosis and control the integrin signaling (Saunders et al., 2017). The activation of integrin signaling further activates various downstream pathways affecting cell growth, differentiation and metabolism. This further increases the complexity of understanding the mechanisms and extrapolating the previously known findings to an entirely new level.

### 1.4.2 Key players in mechanical signalling - Focal Adhesion Kinase (FAK) and SRC Kinase:

It is known that that seven of the eight mammalian Src family kinases are expressed in self-renewing murine ES cells. c-Src, c-Yes, Fyn, and Hck are active in cycling ES cells cultured in the presence of LIF and serum. The first study relating c-Src and ES cell was done in 1995 where Matthias et.al showed that aberrant c-Src expression in ES cells can lead to differentiation. (Martin, G.R., 1981). The later papers which came in the field had shown that different SRC kinases have divergent effect on ES cell self-renewal and differentiation. Although it is known that an increased SRC activity can lead to ES cell differentiation, the mechanism underlying this process is unknown. The most interesting fact about this particular field is that only a hand full of publication is available on the topic and none of them explains the mechanism or to the extent to which this pathway influences pluripotency. So, we decided

to peruse with the question ‘How Src pathway affects mouse ES cell pluripotency? What are its implications with respect to mechanical signaling?

The major pathway which is activated during mechanical signalling is Integrin Signaling Pathway. Integrin signaling helps the cells to sense the attachment and movement. (Vitillo and Kimber, 2017)

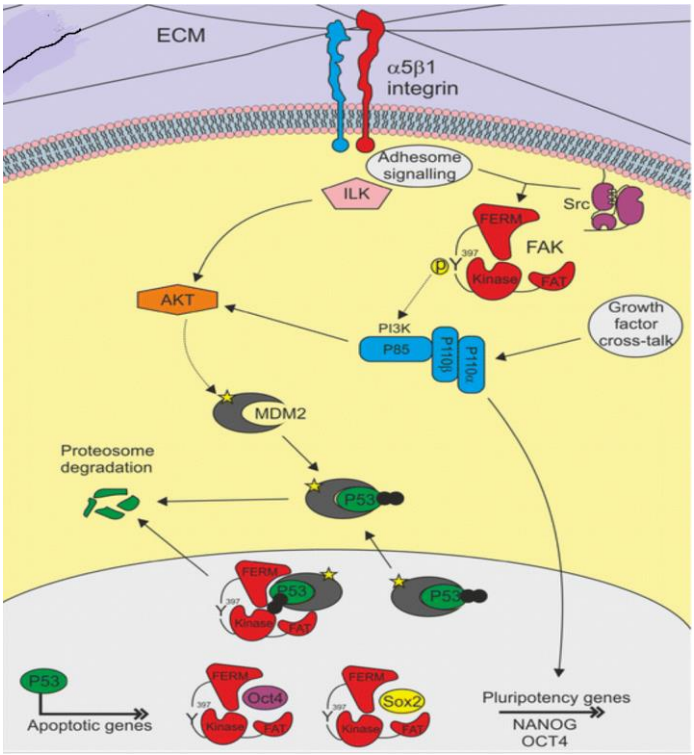


**Figure 4:** FAK signalling in ESCs

Integrin and FAK have been consigned to the conventional role of cell adhesion receptor systems in PSCs. FAK- Focal Adhesion Kinase, ILK- Linked Integrin Kinase, FAT- Focal Adhesion Targeting domain, FERM- Ezrin Radixin Moesin domain, Mdm2- Mouse double minute 2 homolog, AKT- Protein Kinase B

### 1.4.3 Steps involved in FAK signaling pathway:

1. Interaction between ECM and integrins induces activation of FAK by phosphorylation at Y397 residue, at the cell surface. This initiates the transduction of attachment cues to the cell.
2. In turn, FAK Y397 triggers the PI3K survival cascade by phosphorylation of AKT and its downstream target MDM2.
3. MDM2 continuously ubiquitinate p53 targeting it for proteosomal degradation. Therefore, it maintains low levels so that it cannot induce differentiation or apoptosis.

4. In turn, FAK is localized in the nucleus where it binds pluripotency factor, leading to activation of Nanog which maintains cell in pluripotency state (Vitillo and Kimber, 2017).

Although it has been identified that Nanog is phosphorylated at multiple residues, the role of these phosphorylation in development is poorly understood, and no information exists regarding its functions (Brumbaugh, 2004). Among the known phosphorylation, serine and threonine are the most studied ones. No studies with respect to tyrosine phosphorylation in ES cells have been done before. The only evidence of Nanog tyrosine phosphorylation came in SW620 cancer cells where it was discovered that Focal Adhesion Kinase (FAK) phosphorylates Y35 and Y175 in humans. Since mechanical signaling was considered to be a key player in spatiotemporal organization and a prerequisite for lineage specification in the pre implantation embryo we speculate that the tyrosine phosphorylation of Nanog during early development is a key player in it (Ho, Baotran et al. 2012).

## 1.5 Objectives:

**To study Spatio-temporal regulation of GATA-6 during early differentiation in blastocyst.**

This will be achieved by-

i. Construction of targeting vector for *GATA6* tagging.
ii. Constructing the CRISPR-Cas 9 vector for *GATA6* to target last exon.
iii. Tagging endogenous *GATA6* gene with Blue fluorescence protein in mouse embryonic stem cell line (Tg2a) by CRISPR-Cas9 mediated Knock-In.
iv. Detect/track primitive endoderm cells during ES cells to ESCs differentiation using the reporter cell line.

## 2. Materials and methods

### 2.1 Materials required:

#### (a) Molecular cloning:

Template Plasmid: pAC154-dcas9-BFP-KRAB-T2A-NeoPA, pFDTA-BSD, Cdx2 2A mCherry fusion TV, Enzymes: ClaI, NotI, BamHI, Restriction enzyme buffer: Cutsmart buffer, 3.1 buffer, Primers, Master Mix: Phusion MM, Emerald MM, Gibson MM, MG water, 0.8% ,1% and 1.5% Agarose, DH5- α E. coli cells, LB agar, LB broth, Ampicillin 100mg/ml, MN plasmid isolation kit miniprep, MN Plasmid isolation kit Midi, 5M NaCl, PEG, Isopropanol, Absolute Ethanol and 70% ethanol, Micropipettes, tips, Microcentrifuge tubes.

**Instruments:** Centrifuge, PCR, Gel electrophoresis units, UV transilluminator, NanoDrop, Thermocycler mixer, vortex, LAF and an incubator, FACS facility.

#### (b) Tissue culture:

1. Tissue culture flasks, dishes, 96, 24 and 6 well plates, Multichannel pipettes, aspirator and FACS polysterene tubes, 0.1% gelatin, PBS, PBS + serum, GMEM media, Differentiation media, TrypLE and OptiMEM
2. Antibiotics: 1X Blasticidin , 1X PS –Penicillin and streptomycin.

**(c) Softwares** - Snapgene, Flowjo

# Cloning strategy of Gata-6-LA-T2A-BFP-BSD TV

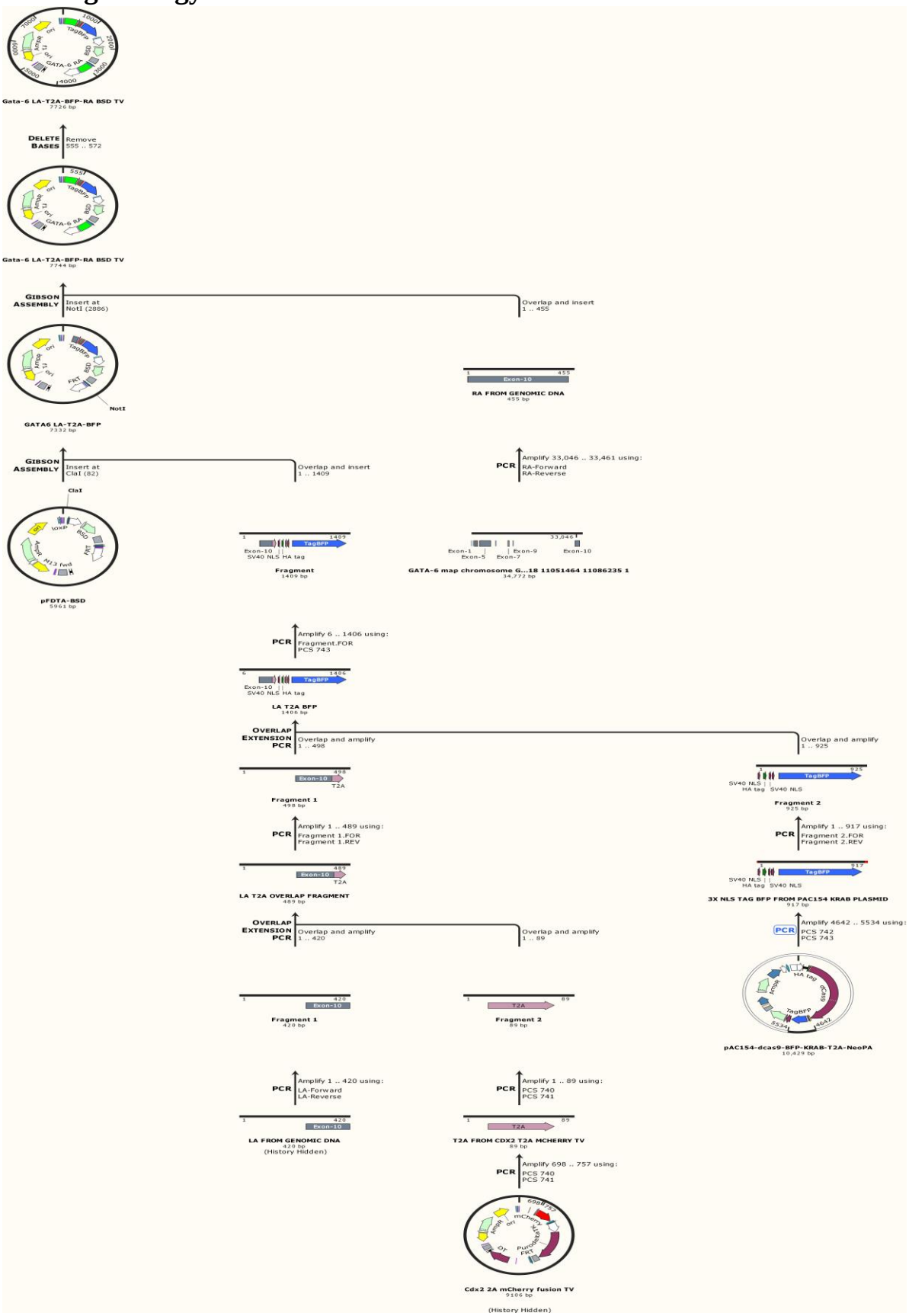


**Figure 5:** Cloning strategy of Gata-6-LA-T2A-BFP-BSD TV

Summary of cloning strategy of GATA-6 TV. The final construct consists of LA-T2A-BFP-RA-BSD cassette.

## Cloning strategy:

1) PCR Amplification of left arm and right arm from gDNA
2) Cloning of T2A from Cdx2 2A mCherry fusion TV
3) Overlap extension PCR – LA and T2A fragment
4) Amplification of tag BFP from pAC 154- dcas9-BFP-KRAB-T2A-NeoPA
5) Overlap extension PCR – LA-T2A and BFP fragment
6) ClaI digestion for pFDTA-BSD
7) Gibson assembly for insertion of LA-T2A-BFP in pFDTA-BSD
8) NotI digestion of Gata6 LA-T2A-BFP
9) Gibson assembly for insertion of RA in Gata6 LA-T2A-BFP

## 1) PCR Amplification of left arm and right arm from gDNA:

Genomic DNA was Mouse embryonic cell line - Tg2a was isolated.

The Primers to be used were diluted in 1:10 ratio by taking 10 µl of primer from 100picomoles stock and 90 µl of MG water. The working primer concentration is 10 picomoles/ µl.

**A. Amplification of Gata6 left arm:**

**Forward Primer (PCS 738)**

TCGAGGTCGAGACGGTATCTGGGTGGTCACTGGGTTCCGAAG

**Reverse Primer (PCS 739)**

GGCCAGGGCCAGAGCACACCAA

**PCR Reaction mixture:**

| | |
|---|---|
| Emerald Mastermix (2X) | 20 µl |
| Primer (Forward) | 1 µl |
| Primer (Reverse) | 1 µl |
| Plasmid Template | 1 µl |
| MG water | 17 µl |
| Total volume | 40 µl |

**PCR reaction conditions:**

**Table 1**: PCR reaction conditions for Gata6 left arm amplification

Heated Lid: 110°C

| Reaction | Temperature (°C) | Duration |
|---|---|---|
| Initial Denaturation | 95 | 3 min |
| Denaturation | 95 | 30 sec |
| Annealing | 55 | 30 sec |
| Extension | 72 | 30 sec |
| Final Extension | 72 | 5 min |
| Hold | 4 | ∞ |

The PCR sample was run on 0.8% Agarose gel electrophoresis and were eluted in 20µl of MG water.

The concentration obtained was 30 ng/ µl

**B. Amplification of Gata-6 right arm:**

**Forward Primer (PCS 744)**

GAAGTTATCCTGCAGGGCGCTCTGAAGGCCTCATACCACTTG

**Reverse Primer (PCS 745)**

GGGATCCACTAGTTCTAGAGCCTCCACGAACGCTTGTGAAATGTG

**PCR Reaction mixture:**

| | |
|---|---|
| Emerald mastermix (2X) | 20 µl |
| Primer (Forward) | 1 µl |
| Primer (Reverse) | 1 µl |
| Plasmid Template | 1 µl |
| MG water | 17 µl |
| Total volume | 40 µl |

**PCR reaction conditions:**

**Table 2**: PCR reaction conditions for Gata-6 right arm amplification

Heated Lid: 110°C

| Reaction | Temperature (°C) | Duration |
|---|---|---|
| Initial Denaturation | 95 | 3 min |
| Denaturation | 95 | 30 sec |
| Annealing | 56 | 30 sec |
| Extension | 72 | 30 sec |
| Final Extension | 72 | 5 min |
| Hold | 4 | ∞ |

The PCR sample was run on 0.8% Agarose gel electrophoresis and were eluted in 20µl of MG water. The concentration obtained was 26 ng/ µl

**2) Cloning of T2A from Cdx2 2A mCherry fusion TV**

The Primers to be used were diluted in 1:10 ratio by taking 10 µl of primer from 100picomoles stock and 90µl of MG water. The working primer concentration is 10 picomoles/ µl.

**Forward Primer (PCS740)**

GGTGTGCTCTGGCCCTGGCCGAGGGCAGAGGAAGTCTGC

**Reverse Primer (PCS741)**

CTTCTTGGGGCTAGCAGGGCCGGG

**PCR Reaction mixture:**

Emerald mastermix (2X) 20 µl

Primer (Forward) 1 µl

Primer (Reverse) 1 µl

Plasmid Template 1 µl

MG water 17 µl

Total volume 40 µl

**PCR reaction conditions:**

**Table 3**: PCR reaction conditions for T2A amplification

Heated Lid: 110°C

| Reaction | Temperature (°C) | Duration |
|---|---|---|
| Initial Denaturation | 95 | 3 min |
| Denaturation | 95 | 30 sec |
| Annealing | 55 | 30 sec |
| Extension | 72 | 20 sec |
| Final Extension | 72 | 5 min |
| Hold | 4 | ∞ |

The PCR sample was run on 1% Agarose gel electrophoresis and were eluted in 20µl of MG water.

The concentration obtained was 35 ng/ µl

**3) Overlap extension PCR – LA and T2A fragment:**

Overlap extension was done by setting up PCR with LA and T2A fragment without primers. The PCR reaction was done for 10 cycles as per the condition given below:

**Table 4** – OE PCR for LA and T2A fragment

Heated Lid: 110°C

| Reaction | Temperature (°C) | Duration |
|---|---|---|
| Initial Denaturation | 95 | 3 min |
| Denaturation | 95 | 30 sec |
| Annealing | 55 | 30 sec |
| Extension | 72 | 30 sec |
| **10 cycles** | | |
| Final Extension | 72 | 5 min |
| Hold | 4 | ∞ |

Once the reaction is over the tubes were taken and forward and reverse primer was added on to the reaction. The tubes were again set for PCR reaction with same

condition mentioned above except for the number of cycles and extension time of 45sec. The reaction was run for 40 cycles.

The PCR sample was run on 1% Agarose gel electrophoresis and were eluted in 20µl of MG water.

The concentration obtained was 15 ng/ µl

**4) Amplification of tag BFP from pAC 154- dcas9-BFP-KRAB-T2A-NeoPA:**

The Primers to be used were diluted in 1:10 ratio by taking 10 µl of primer from 100picomoles stock and 90µl of MG water. The working primer concentration is 10 picomoles/ µl.

**Forward Primer (PCS742)**

CCTGCTAGCCCCAAGAAGAAGAGAAAGGT

**Reverse Primer (PCS743)**

TTTTGAATTCCGCGCCATATCCCTCCGCCACCG

**PCR Reaction mixture:**

| | |
|---|---|
| Emerald mastermix (2X) | 20 µl |
| Primer (Forward) | 1 µl |
| Primer (Reverse) | 1 µl |
| Plasmid Template | 1 µl |
| MG water | 17 µl |
| Total volume | 40 µl |

**PCR reaction conditions:**

**Table 5**: PCR reaction conditions for BFP amplification

Heated Lid: 110°C

| Reaction | Temperature (°C) | Duration |
|---|---|---|
| Initial Denaturation | 95 | 3 min |
| Denaturation | 95 | 30 sec |
| Annealing | 55 | 30 sec |
| Extension | 72 | 30 sec |
| Final Extension | 72 | 5 min |
| Hold | 4 | ∞ |

The PCR sample was run on 1% Agarose gel electrophoresis and were eluted in 20µl of MG water.

The concentration obtained was 25 ng/ µl

**5) Overlap extension PCR – LA-T2A and BFP fragment**

Overlap extension was done by setting up PCR with LA-T2A and BFP fragment without primers. The PCR reaction was done for 10 cycles as per the condition given below:

**Table 6** – OE. PCR for LA-T2A and BFP fragment

Heated Lid: 110°C

| Reaction | Temperature (°C) | Duration |
|---|---|---|
| Initial Denaturation | 95 | 3 min |
| Denaturation | 95 | 30 sec |
| Annealing | 55 | 30 sec |
| Extension | 72 | 40 sec |
| **10 Cycles** | | |
| Final Extension | 72 | 5 min |
| Hold | 4 | ∞ |

Once the reaction is over the tubes were taken and forward and reverse primer was added on to the reaction. The tubes were again set for PCR reaction with same condition

mentioned above except for the number of cycles and extension time of 45sec. The reaction was run for 40 cycles.

The PCR sample was run on 1% Agarose gel electrophoresis and were eluted in 20µl of MG water.

The concentration obtained was 15 ng/ µl.

6) **Restriction Digestion for pFDTA-BSD**

The plasmid was digested with enzymes ClaI to give fragments of size 6 Kb.

| | |
|---|---|
| Template (3 µg) | : 3.3µl |
| Enzyme Cla1 | : 1µl |
| 3.1 Buffer(10X) | : 3 µl |
| MG water | : 21.2µl |
| Total volume | : 30 µl |

**7) Gibson assembly**

The respective PCR product LA-T2A-BFP was inserted into ClaI digested pFDTA-BSD vector by gibson assembly. The insert concentration is calculated using NEBiocalculator:

Insert length: 1.4Kb

Vector length: 6Kb

Vector mass: 100ng

Insert mass (1:1): 23.33ng

**Reaction Mixture:**

| | |
|---|---|
| Gibson Master Mix | 5 µl |
| Vector (100 ng) | 0.7 µl |
| Insert (23.33) ng | 0.3 µl |
| MG water | 4 µl |
| Total volume | 10 µl |

**PCR program:**

Heated lid: 110°C

Ligation temperature: 50°C for 20 minutes

Hold : 4°C for ∞

**A. Transformation**

Gibson assembled plasmid was then transformed in ultra- competent DH5- α E. coli cells.

**PCR program:**

No Heated Lid

4°C for 20 minutes

Heat Shock: 42°C for 1.30 minutes

4°C for 10 minutes

The above-transformed cells were plated on LB + Ampicillin plates and incubated at 37°C for 12- 14 hours.

**B. Colony PCR**

The insertion of the insert would be confirmed by the colony PCR. The primers are designed in such a way that one of the primers comes from the insert and the other from the vector. Thirty two colonies were picked up for colony PCR.

**PCR reaction mixture:**

| | |
|---|---|
| Emerald Master Mix(2X) | 5 µl |
| Primer (Forward) | 1 µl |
| Primer (Reverse) | 1 µl |
| Plasmid Template | a picked up colony |
| MG water | 3 µl |
| Total volume | 10 µl |

**PCR reaction conditions:**

**Table 7**: Colony PCR reaction for insertion of LA-T2A-BFP

Heated Lid: 110°C

| Reaction | Temperature (°C) | Duration |
|---|---|---|
| Initial Denaturation | 95 | 3 min |
| Denaturation | 95 | 30 sec |
| Annealing | 56 | 30 sec |
| Extension | 72 | 30 sec |
| Final Extension | 72 | 5 min |
| Hold | 4 | ∞ |

The 4 positive clones on the basis of colony PCR were then picked and inoculated for plasmid isolation in 50ml falcons with 10ml LB broth + 10 µl (100µg/µl) Ampicillin. The clones were incubated at 37°C for 12-14 hours. The plasmids from the culture were isolated with MN plasmid Isolation kit.

**Procedure:**

1. The cells were harvested and centrifuged at 4000 rpm for 10 minutes. The supernatant was carefully discarded.
2. The pellet obtained was re-suspended in 300 µl of A1 (Resuspension buffer). The re-suspended pellet is then taken in 1.5 ml micro centrifuge tubes.
3. 300 µl of A2 (Lysis buffer with pH indicator) was added next. The solution turns blue due to the indicator. The tubes were mixed and incubate for 4 minutes.
4. 300 µl of A3 (Neutralisation buffer) was added and gently mixed by inverting the tubes. The solution turned white indicating cell lysis.
5. The tubes were centrifuged at 11,000 rpm for 10 minutes.
6. 600 µl of the supernatant was added to a silica column and centrifuged at 11,000 rpm for 1.00 minute.
7. The leftover supernatant was added into the column and centrifuged again.
8. A4 wash buffer with ethanol was added and centrifuged at 11,000 rpm for 1 minute.
9. The columns were then dry spun at 11,000 rpm for 2 minutes.
10. The Plasmids were then eluted with 20 µl MG water (50°C).

11. The DNA was quantified using a Nano Drop.

The concentration of the plasmids was

C1 - 700 ng/µl

C8 -1000 ng/µl

C11 – 834 ng/µl

C30- 1200ng/µl

**C. Restriction digestion confirmation:**

The isolated plasmids were then digested with BamHI to confirm the insertion of LA-T2A-BFP into the vector. The digestion of the plasmid will give fragments of size 4.6Kb, 2.1Kb and 500bp confirming successful Gibson Assembly.

| | |
|---|---|
| Template (0.5µg) | 1.0 µl |
| Cutsmart Buffer (10X) | 1.0 µl |
| BamHI enzyme | 1.0 µl |
| MG water | 7.0 µl |
| Total volume | 10.0 µl. |

**D. Sequencing confirmation**

The plasmid C30 was sent for sequencing with primers (PCS-542, PCS435).
The sequencing result had confirmed the insertion of LA-T2A-BFP into the plasmid pFDTA- BSD.

**8) Restriction digestion of Gata6 LA-T2A-BFP**

The plasmid was digested with enzyme NotI to give fragments of size 7.3Kb

| | |
|---|---|
| Template (3 µg) | : 3.3µl |
| Enzyme Cla1 | : 1µl |
| CutSmart Buffer(10X) | : 3 µl |
| MG water | : 21.2µl |
| Total volume | : 30 µl |

**9) Gibson assembly for insertion of RA in Gata6 LA-T2A-BFP**

The right arm was inserted into NotI digested Gata6 LA-T2A-BFP vector by Gibson assembly. The insert concentration is calculated using NEBiocalculator:

Insert length: 455bp

Vector length: 7.3Kb

Vector mass: 100ng

Insert mass (2:1): 12.47ng

**Reaction Mixture:**

| | |
|---|---|
| Gibson Master Mix | 5 µl |
| Vector (100 ng) | 0.7 µl |
| Insert (12.47) ng | 0.3 µl |
| MG water | 4 µl |
| Total volume | 10 µl |

**PCR program:**

Heated lid: 110°C

Ligation temperature: 50°C for 20 minutes

Hold : 4°C for ∞

**A. Transformation**

Gibson assembled plasmid was then transformed in ultra- competent DH5- α E. coli cells.

**PCR program:**

No Heated Lid

4°C for 20 minutes

Heat Shock: 42°C for 1.30 minutes

4°C for 10 minutes

The above-transformed cells were plated on LB + Ampicillin plates and incubated at 37°C for 12- 14 hours.

**B. Colony PCR**

The insertion of the insert would be confirmed by the colony PCR. The primers are designed in such a way that one of the primers comes from the insert and the other from the vector. Twenty One colonies were picked up for colony PCR.

**PCR reaction mixture:**

Emerald Master Mix(2X) 5 µl

Primer (Forward) 1 µl

Primer (Reverse) 1 µl

Plasmid Template a picked up colony

MG water 3 µl

Total volume 10 µl

**PCR reaction conditions:**

**Table 8**: Colony PCR reaction for insertion of RA

Heated Lid: 110°C

| Reaction | Temperature (°C) | Duration |
|---|---|---|
| Initial Denaturation | 95 | 3 min |
| Denaturation | 95 | 30 sec |
| Annealing | 56 | 30 sec |
| Extension | 72 | 30 sec |
| Final Extension | 72 | 5 min |
| Hold | 4 | ∞ |

The 3 positive clones on the basis of colony PCR were then picked and inoculated for plasmid isolation in 50ml falcons with 10ml LB broth + 10 µl (100µg/µl) Ampicillin. The clones were incubated at 37°C for 12-14 hours. The plasmids from the culture were isolated with MN plasmid Isolation kit and the DNA was quantified using a Nano Drop.

The concentration of the plasmids was

C8 – 444.3 ng/ µl

C15 -397.3 ng/ µl

C17- 832.3 ng/ µl

### C. Sequencing confirmation

The plasmid C8 and C15 was sent for sequencing with primers (PCS-542, PCS435). The sequencing result had confirmed the insertion of LA-T2A-BFP into the plasmid pFDTA-BSD.

## Primary and secondary inoculation for scale up:

1. For primary inoculation, around 5-10 colonies are inoculated in 5ml of LB broth with ampicillin and incubated at 37°C for 6 hours.
2. 1ml of the above culture is inoculated in 250ml flask of LB broth with ampicillin.

The culture flask was inoculated at 37°C for 12-14 hours.

**Plasmid scale up:**

1. The 250ml culture was poured into GSI bottles and weighed to check balance. They were centrifuged at 4000rpm for 10 minutes at room temperature.
2. The supernatant was discarded carefully such that no media is left over in the pellet.
3. The pellet was completely re-suspended in 10ml of resuspension buffer (A1) + RNase by pipetting the cells up and down and transferred to a 50ml falcon.
4. To the re-suspended pellet 10ml of Lysis buffer (A2) was added and gently inverted. It was incubated for 4 minutes.
5. 10ml of Neutralization buffer (A3) was next added and the falcon was inverted gently until the blue solution turned white.
6. The NucleoBond® Xtra Column was placed in its holder and was equilibrated with 12ml of equilibration buffer. The buffer was added 1ml at a time onto the rim of the inserted filter column.
7. The lysed culture was added to the filter column and the elute was collected. This was again added into the column to ensure maximum plasmid absorption by the silica resin.
8. The filter column is again washed with 8ml of equilibration buffer added to the rim 1ml at a time.
9. The filter column is discarded and the NucleoBond® Xtra Column is washed with 8ml of Wash buffer. The wash buffer was added to the sides of the column thereby washing it.
10. Once the wash buffer has completely flown through the plasmid DNA is eluted in 5ml of warm Elution buffer.
11. Initially 4ml of elution buffer was added and the flow through was collected. Lastly 1ml of elution buffer was added and collected in a 15ml falcon tube.
12. The amount of elute collected X 0.7 = isopropanol was added and gently mixed.
13. The tubes were centrifuged at 12000rpm at 4°C for 30 minutes.

14. The supernatant was carefully discarded and 2ml of ice cold ethanol was added to the pellet.
15. The tubes were centrifuged at 12000rpm for 15 minutes.
16. The pellet was warmed in a thermocycler at 50°C until all traces of ethanol had evaporated.
17. The pellet was dissolved in 150-200µl of ET water. It was resuspended to ensure complete dissolution of Plasmid DNA in ET water. 18. The Plasmid DNA was quantified using a Nano Drop.
18. Concentration of the plasmids was –
    Targeting vector- 3.215 µg/µl
    Cas9 vector - 4.187 µg/µl

**PEG purification:**

1. 100 µg of Plasmid DNA was transferred to a 1.5ml microcentrifuge tubes.
2. 500 µl of endotoxin free 5M NaCl and 500 µl of PEG was added to the tubes.
3. The vials were mixed well and vortexed completely.
4. The mixture was incubated in ice for 1 hour.
5. The vials were then centrifuged at 15000rpm for 15 minutes.
6. The supernatant was discarded and the pellet was resuspended in 1ml of ethanol.
7. The tubes were centrifuged at 15000rpm for 15 minutes.
8. The pellet was warmed in a thermocycler at 50°C until all traces of ethanol had evaporated.
9. The pellet was dissolved in 200µl of ET water. The Plasmid DNA was quantified using a Nano Drop.

The concentration of plasmid was –

Targeting vector – 2.832 µg/µl

Cas9 vector- 3.383 µg/µl

## Strategy of guide RNA cloning:

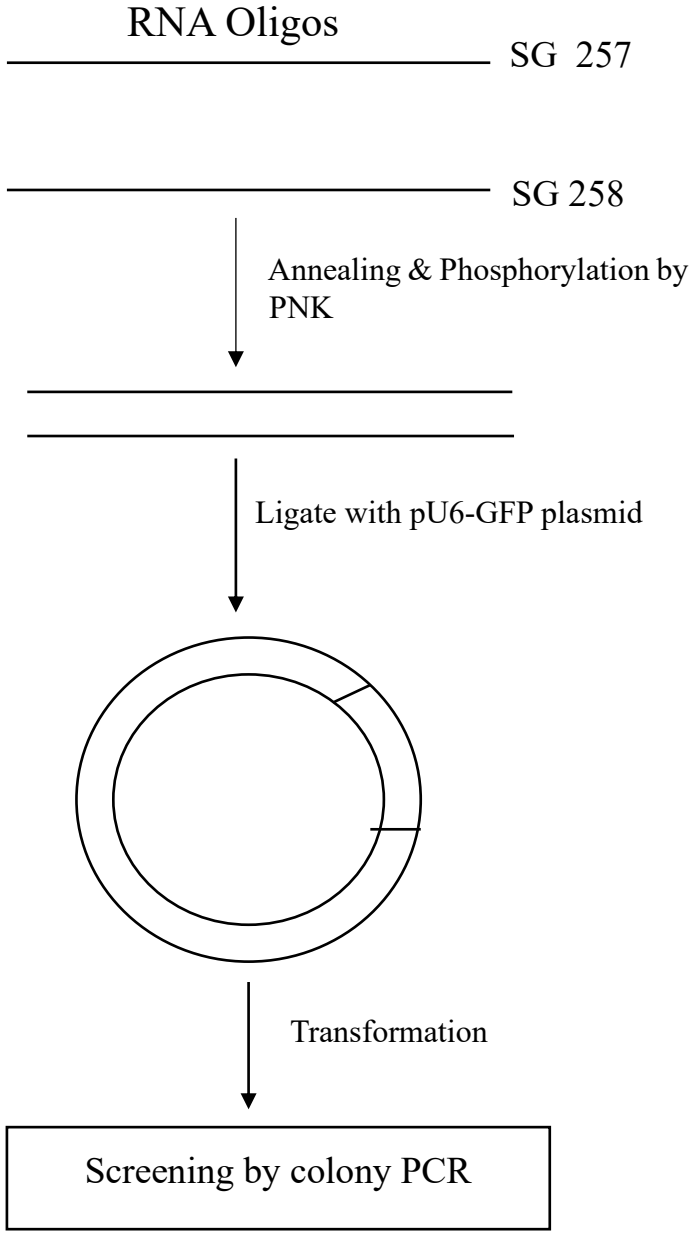


**Figure 6:** Outlined representation of guide RNA cloning

gRNA pairs were annealed and phosphorylated by Polynucleotide Kinase. It was ligated into pU6-GFP vector. This was transformed in *E.coli* DH5α and colonies were screened by colony PCR.

### (I) Phosphorylation & annealing of each pair of oligos:

The guide RNA pairs are complementary to each other. gRNA pairs are incubated together with Polynucleotide Kinase which adds 5' phosphate group to both this oligos and are further ligated into the vector by T4 DNA Ligase.

**Table 9:** Reaction mixture for annealing of guide oligos

| | |
|---|---|
| Oligo-1 (SG 257) | 1µl |
| Oligo-2 (SG 258) | 1µl |
| (10X) DNA ligase buffer | 1µl |
| T4 PNK | 0.5 µl |
| M.G. water | 6.5 µl |
| Total volume | 10µl |

**Conditions:**

**PCR conditions used were –**

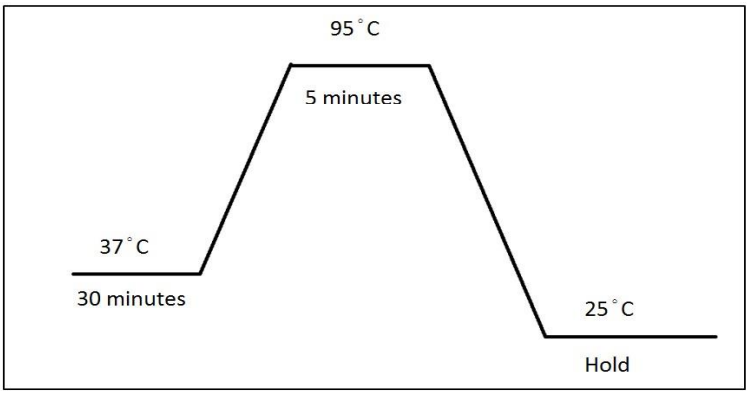


**Figure 7**: Temperature scale of annealing

PCR reaction condition used for annealing of guide oligos were 37ºC for 30 mins, Denaturation at 95ºC for 5 mins and hold at 25ºC.

**The oligos were diluted in 1:10 ratio. And diluted oligos were used as template for ligation reaction with pU6 vector.**

**II. Restriction digestion of pU6 vector with Bpi1:**

pU6 GFP vector was digested with Bpi1 enzyme to linearize it.

**III. Ligation of oligos with linearized pU6 GFP vector:**

**Table 10:** Reaction mixture for ligation of oligos

| | |
|---|---|
| Plasmid vector pU6-GFP (77ng/µl) | 1.3µl |
| Annealed oligos (1:10 diluted) | 1.5µl |
| (10X) DNA ligase buffer | 2µl |
| T4 DNA ligase enzyme | 0.5µl |
| M.G. water | 14.7µl |
| Total volume | 20 µl |

**Condition: 22ºC for 1hr (No heated lid)**

**IV. Transformation**

The ligated plasmid sample was added to 100 µl ultra competent DH5 alpha *E coli* cells.

**PCR program:**

No Heated Lid

Cold shock: 4°C for 20 minutes

Heat shock: 42°C for 1 minute

Hold: 4°C for ∞

After that the transformed cells were spread plated on LB agar and incubated for 12-14hrs.

**V. Colony PCR**

The insertion of the insert would be confirmed by the colony PCR. The primers are designed in such a way that one of the primers comes from the insert and the other from the vector. Four colonies were picked up for colony PCR.

**PCR reaction:**

Emerald Master Mix (2X) 5 µl

Primer (Forward) 1 µl

Primer (Reverse) 1 µl

Plasmid Template a picked up colony

MG water 3 µl

Total volume 10 µl

**PCR reaction conditions:**

**Table 11**: Colony PCR condition for insertion of oligos

Heated Lid: 110°C

| Reaction | Temperature (°C) | Duration |
|---|---|---|
| Initial Denaturation | 95 | 3 mins |
| Denaturation | 95 | 30 sec |
| Annealing | 55 | 30 sec |
| Extension | 72 | 15 sec |
| Final Extension | 72 | 5 min |
| Hold | 4 | ∞ |

The 2 positive clones on the basis of colony PCR were then picked and inoculated for plasmid isolation in 50ml falcons with 10ml LB broth + 10 µl(100µg/µl) Ampicillin. The clones were incubated at 37°C for 12-14 hours. The plasmids from the culture were isolated with MN plasmid Isolation kit and the DNA was quantified using a Nano Drop.

The concentration of the plasmids was

C3 - 870.3 ng/ µl

C4 - 635.9 ng/ µl

**VI. Sequencing confirmation**

The plasmid C3 and C4 were sent for sequencing along with PCS-127. The sequencing result had confirmed insertion of guide oligo in pU6 Vector.

## Tissue culture

**E14Tg2a**:

- This cell line was developed by M. Hooper in 1987. It is a one of the derivative of embryonal stem cell (ES) lines.
- The cells lack HGPRT (HPRT) and are resistant to 0.06 mM 6-thioguanine.
- The cells do not undergo differentiation when cultured on feeder layers (embryonic fibroblasts or STO cells). It requires substratum for attachment.
- In absence of feeder layer, the cells spontaneously differentiate and form embryonal structures.
- When injected into blastocysts, the cells can colonize the germline. They may be used to reconstitute mouse embryos after modifying it using conventional molecular gene techniques
- This cell line is a suitable transfection host. It can be used to study early post implantation structures.

**Maintenance of Cell Lines:**

Embryonic stem cells are cultured in lab with the help of certain factors that are capable of self-renewal and inhibit differentiation like LIF and BMP4. GMEM is the media that is used for growing ESCs. It is a modification of Eagle's Minimal essential media. GMEM uses a sodium bicarbonate buffer system and hence requires supplementation of 5% CO2.

Phosphate buffered saline is used to wash the media from the wells. It maintains the pH in a constant state.

OptiMEM is a modification of Eagle's Minimum essential media. It is buffered with HEPES and sodium bicarbonate, and complemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth factors. OptiMEM is used during nucleofection of cells.

**Composition of the GMEM media (1000ml):**

**Table12**: Composition of GMEM media

| COMPONENTS | VOLUME |
|---|---|
| GMEM | 12.5g |
| 15% FS (Fetal Serum) | 116ml |
| Sodium bicarbonate | 2.75g |
| Sodium pyruvate | 1ml |
| Non-essential aminoacids | 11.2ml |
| Leukemia Inhibitory Factor | 400µl |
| Milli Q water | Made upto 1000ml |

**(I) Gelatinizing the tissue culture plates:**

- The tissue culture plates were gelatinized under the LAF Hood.
- For the growth of Tg2A cells, T75 tissue culture flask was taken and it was coated with 5ml of 0.1% gelatin such that it covered the entire floor of the tissue culture-treated vessel.
- The flask was incubated at room temperature for 2-12 hours.
- After incubation, the gelatin was aspirated out and 8ml GMEM media was added.

**(II) Revival of the cell line:**

- Passage 16 of Tg2a cells were taken out from liquid nitrogen storage containers and thawed until they were at room temperature.
- A sterile microcentrifuge vial with 250µl of GMEM media was taken.
- The Tg2A cells were added to the tube and gently resuspended.
- The tube was centrifuged at 1000 rpm for 5 minutes.
- The supernatant was discarded and the pellet was re suspended in 500µl of fresh GMEM media.
- The cells were then added into the gelatinized culture flask and gently spread laterally.
- The culture flask was then incubated at 37°C with 5% CO2

### (III) Maintaining the Tg2a cells:

- After 24 hours the media was aspirated out, replaced with fresh media.
- The cells were observed under the microscope every day and the above process was repeated until the cells were 70% confluent and ready for nucleofection.

### (IV) Preparation for Nucleofection

- The media from the Tg2a cells was aspirated out and gently washed with PBS buffer.
- TryplE reagent was added to the cells and incubated at 37°C for 4 minutes to trypsinize the cells.
- Around 3ml of GMEM media was added to the culture flask and the cells were gently resuspended to detach the cells from the bottom of the culture flask.
- The culture was then transferred to a 15ml falcon and centrifuged at 1000rpm for 10 minutes. The supernatant was carefully aspirated out.
- The pellet was resuspended in OptiMEM media for nucleofection.

**Nucleofection:**

**Aim:** To transfect Tg2A cell lines with Gata6 LA-T2A-BFP-RA-BSD TV plasmid and pu6 gRNA GFP by Nucleofection.

**Principle:** Nucleofection is physical method of gene transfer. It is used for transfection of nucleic acid – DNA or RNA in a cell by applying a specific voltage and reagents. Like electroporation, nucleofection uses a combination of electrical parameters, generated by a device called Nucleofector, with cell-type specific reagents. It is a powerful tool for transfecting large DNA fragments. It has a good transfection efficiency.

**Requirements:**

1. Tg2a Cell lines
2. Gata6 LA-T2A-BFP-RA-BSD TV and pU6 gRNA GFP plasmid
3. OptiMem Media

4. Tryple

5. PBS

6. Nucleofection Cuvette

7. GMEM Media

**Procedure:**

1. Nucleofection cuvette was sterilized initially with 70% ethanol and washed with PBS buffer. The step was repeated twice.
2. 200µl of the cell suspension was added to the cuvettes.
3. To this cell suspension, 1.5µg of each of the plasmids targeting vector and vector was added.
4. Select the appropriate Nucleofector Program on the Lonza 4D-Nucleofector™ System.
5. Insert the cuvette with cell/DNA suspension into the Nucleofector Cuvette Holder and apply the selected program.
6. Take the cuvette out of the holder once the program is finished.
7. Immediately transfer the nucleofected cells into 5ml of GMEM medium in a 15ml falcon.
8. The 5ml volume of nucleofected cells of each cell line were split into two dilutions: 2ml and 3ml and added into the gelatinized P100 petri dishes separately.
9. The P100s were gently spread laterally and incubated at 37°C with 5% CO2.

**Processing the cells for FACS:**

1. After 20h, the media was aspirated out and washed with PBS buffer.
2. The cells were trypsinized with 1 ml of TryplE and incubated at 37°C for 4 minutes.
3. 2ml of GMEM + LIF media was added to neutralize TryplE activity.
4. The cells were spinned down and pellet was resuspended in 1% serum + PBS.
5. The cells positive for GFP were sorted using the FACS machine BD- LSR Fortessa$^{TM}$.

The GFP positive cells were seeded into gelatinized P100 plate with GMEM media and 2X Penicillin and Streptomycin and incubated at 37°C with 5% CO2.

**Blasticidin drug Selection**

1. After 24 hours of incubation, the cells were started with Blasticidin treatment in order to screen them for positive colonies.
2. The GMEM media was treated with 0.5X BSD (100mg/ µl concentration). The media was aspirated out every 24 hours and fresh media with BSD was added.
3. The selection was done for 4-5 days until all the negative cells died and positive colonies were visible.

**Colony Picking:**

1. Drug selection was continued until the colonies were big enough to be picked with the naked eye.
2. Two 96 well plates were taken.
    1. 96 well plates
    2. Gelatinized 96 well plates with 100 µl of GMEM media
3. The GMEM media was aspirated out from the P100 plate and washed with PBS once. The PBS was aspirated out and replaced with fresh PBS buffer just to cover the surface of the petri plate.
4. The plates were visualized under the microscope and 96 colonies were picked with a pipette into the non- gelatinized 96 well plate.
5. 20 µl of trypsin was added to each of the 48 wells.
6. 50µl of GMEM media was added to each of the wells and the cells were gently resuspended. The processed 96 clones were transferred into the gelatinized plate immediately.
7. The 96 well plate was then incubated at 37°C with 5% CO2.
8. The media was changed every 24 hours until they reached 80% confluency and were ready to be split.

**Splitting of cells:**

1. Once the cells were 80% confluent, the cells were split into duplicates.
2. The media was aspirated out and washed with PBS buffer.
3. 20µl of TryplE was added to each well and incubated at 37°C for 4 minutes
4. 45.0µl of GMEM was added to each of the wells and the cells were gently resuspended.

5. The above culture was split into two gelatinized 96 well plates with 100 µl GMEM.
7. The 96 well plates were then incubated at 37°C with 5% CO2.
8. The media was changed every 24 hours until the cells reached 80% confluency.

**Genotyping of Tg2a Gata6 cell line.**

1. Once the confluency has reached 80% the media was aspirated and washed with PBS.
2. 40µl of TryplE was added to each of the well and incubated for 4minutes at CO2 incubator.
3. After that the wells were added with GMEM media and the cells were gently resuspended.
4. The suspension was transferred to 15ml Falcon tubes and centrifuged at 1000 rpm for 5mins.
5. After centrifugation, the media was removed and fresh media was added. In that 1/10th part was taken for genotyping, rest was cryopreseved.
6. To that $1/10^{th}$ part 100µl of tail lysis buffer added with 1X proteinase K(20µg/ µl) was added.
7. Then it was incubated overnight at 37°C with 5% CO2.
8. To the tube 70% by volume Isopropanol was added.
9. The tube was incubated at 4°C for 5 mins.
10. It was centrifuged at 14,000 rpm for 30 mins at 4°C.
11. The supernatant was removed and 100 µl of 70% ethanol was added.
12. It was centrifuged at 14,000 rpm for 30 mins at 4°C.
13. The ethanol was removed and air dried for 5 mins.
14. To the tube 30 µl of molecular graded water preheated to 70°C was added.
15. The tubes were kept in thermomixer at 50°C for 2Hrs.
16. The concentration of the DNA was quantified using the Nanodrop.

**PCR amplification**

The Primers to be used were diluted in 1:10 ratio by taking 10 µl of primer from 100picomoles stock and 90 µl of MG water. The working primer concentration is 10 picomoles/ µl.

The following pairs of primers were used for standardizing genotyping using gradient PCR:

1. **PCS 654 + PCS 745**
2. **DNG 141 + PCS 276**
3. **DNG 141 + PCS 394**

Once the genotyping primers were standardized, genotyping for all 96 clones was carried out.

**96 well genotyping**

1) Emerald master mix was used for genotyping the clones. The master mix was made as mentioned below:

**Forward primer (DNG 141)**

GGCTGAAGTTTCGCGGAAGCCTTG

**Reverse primer** (**PCS 276)**

GGCGATTCTCTTTTGCATGCTAGCAGGGCCGGGATTC

**Table 13**: Reaction mixture for genotyping of Tg2a Gata-6 KI clones

| | |
|---|---|
| Genomic DNA from each clone | 1µl |
| Forward primer (DNG 141) | 1µl |
| Reverse primer (PCS 276) | 1µl |
| Emerald Master mix | 7.5µl |
| M.G. water | 5 |
| Total volume | 15 µl |

The reaction was set on a 96 well PCR plate with the help of multichannel pipette and Easypet Explorer pipettes.

2) PCR reaction was set for the whole plate with Biorad 96 well format PCR as per the conditions mentioned below:

**Table 14**: PCR reaction conditions for genotyping of clones

Heated Lid: 110°C

| Reaction | Temperature (°C) | Duration |
|---|---|---|
| Initial Denaturation | 95 | 3 mins |
| Denaturation | 95 | 30 sec |
| Annealing | 62 | 30 sec |
| Extension | 72 | 15 sec |
| Final Extension | 72 | 5 min |
| Hold | 4 | ∞ |

# 3. RESULT AND DISCUSSION:

## 3.1 Construction of targeting vector

### 3.1.1 PCR Amplification of left arm and right arm from gDNA:

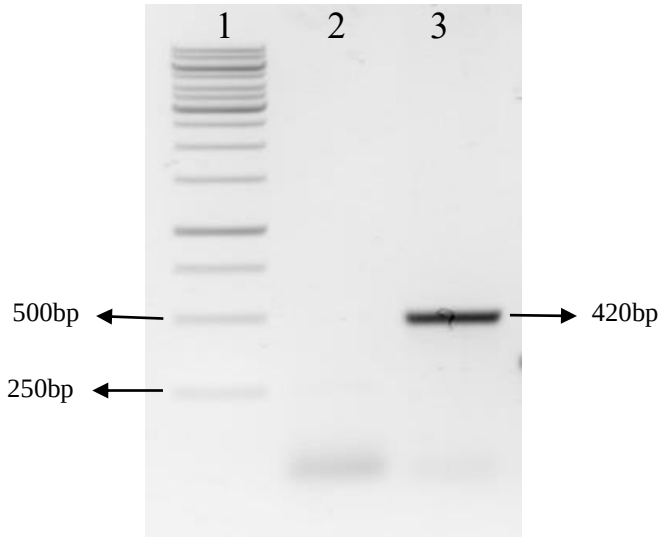


**Figure 8**: PCR amplification of left arm

1 – 1Kb DNA ladder

2 – Negative control- no amplification

3 – Left arm amplicon (420bp)

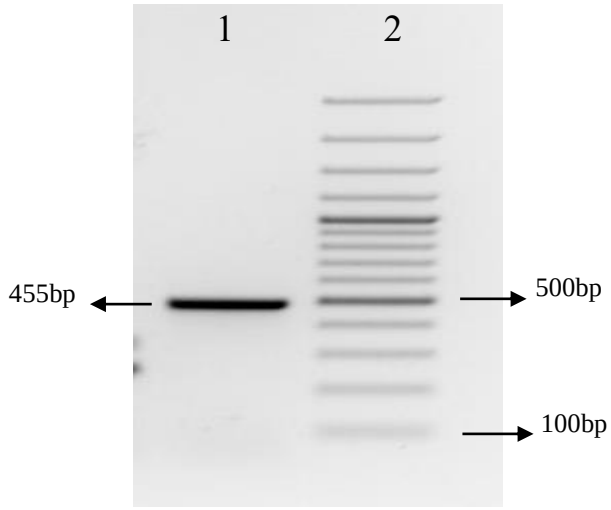


**Figure 9**: PCR amplification of right arm

1 – Right arm amplicon (455bp)

2 – 100bp DNA ladder

### 3.1.2 Cloning of T2A from Cdx2 2A mCherry fusion TV:

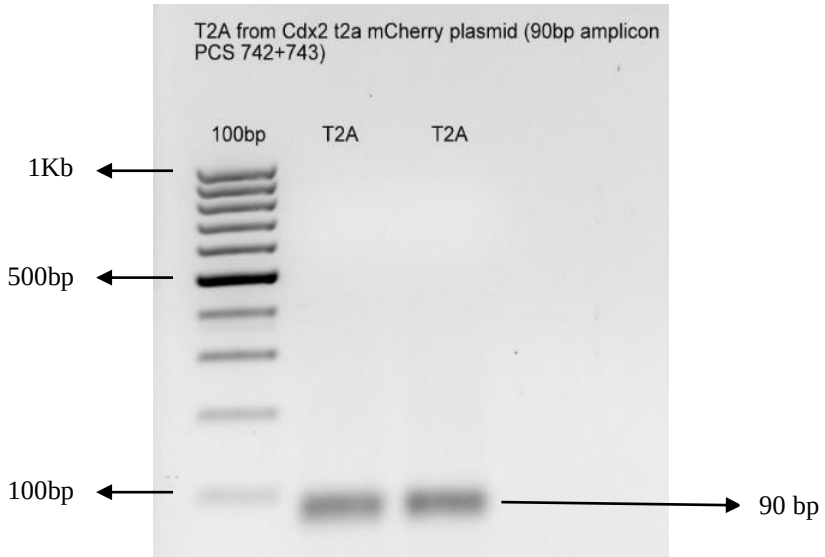


**Figure 10**: PCR amplification of T2A

T2A was cloned from Cdx2 2A mCherry fusion TV using PCS 742+743.100 bp- DNA ladder, T2A amplicon size was 90bp.

### 3.1.3 Overlap extension PCR – LA and T2A fragment:

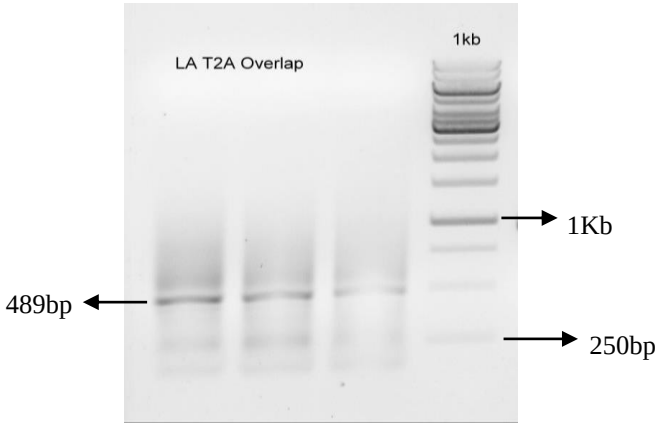


**Figure 11**: OE PCR – LA and T2A

OE PCR was done to overlap LA and T2A fragment. LA T2A overlapped amplicon had size of 489bp.

### 3.1.4 Amplification of tag BFP from pAC 154- dcas9-BFP-KRAB-T2A-NeoPA:

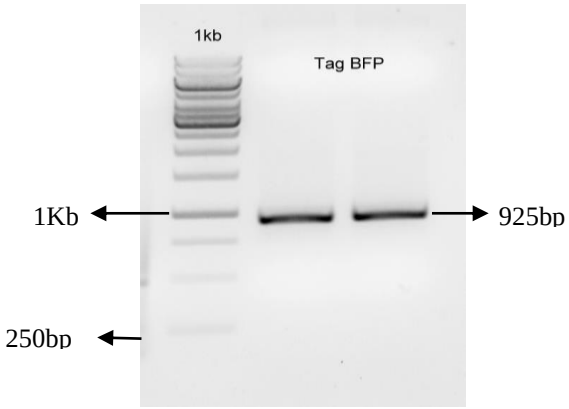


**Figure 12**: PCR amplification of BFP tag

BFP tag was amplified from pAC 154- dcas9-BFP-KRAB-T2A-NeoPA vector using PCS742+743. Positive amplification of BFP gave 925 bp amplicon.

### 3.1.5 Restriction Digestion of pFDTA-BSD:

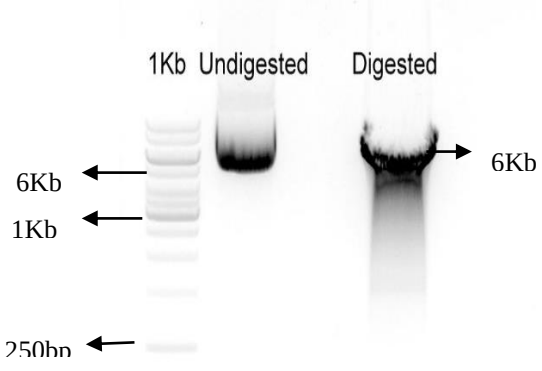


**Figure 13**: ClaI digestion of pFDTA – BSD

pFDTA-BSD was digested with ClaI enzyme to give fragment of size 6 Kb.

### 3.1.6 GATA-6 LA T2A BFP GIBSON ASSEMBLY COLONY PCR

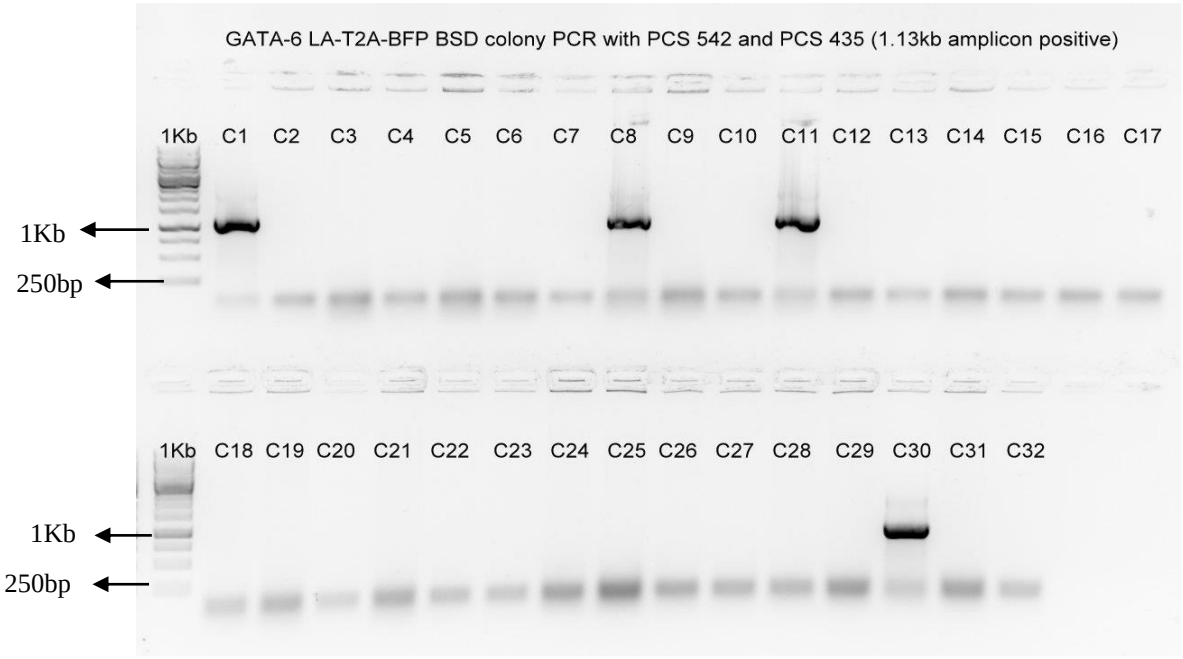


**Figure 14**: Colony PCR GATA-6 LA T2A BFP

Gibson assembly was done for pFDTA-BSD (ClaI digested) and LA-T2A-BFP fragment. 1Kb – 1Kb DNA ladder, C1-C32 are different colonies used for screening by colony PCR with primers PCS 542 and PCS 435. C1, C8, C11 and C30 shows positive amplification of 1.13 Kb.

### 3.1.7 Restriction digestion confirmation of GATA-6 LA T2A BFP clones

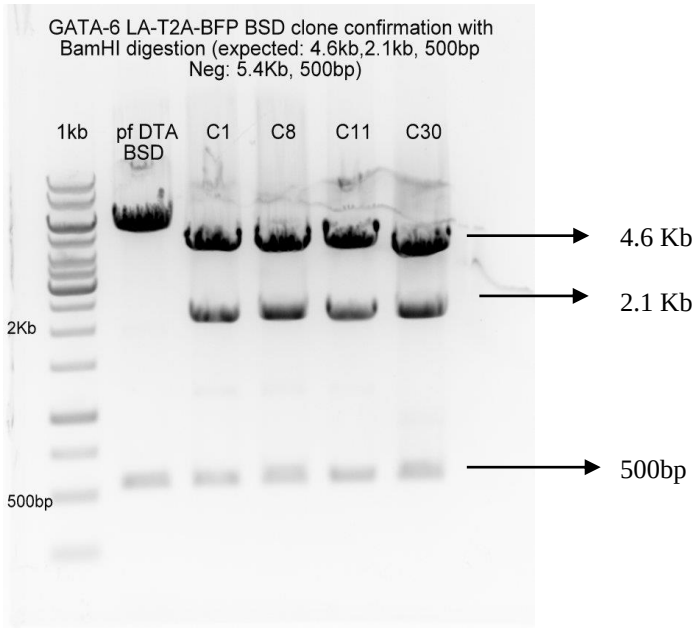


**Figure 15**: Bam HI digestion of Gata-6 LA T2A BFP clones

Restriction digestion was used to confirm insertion of LA-T2A-BFP. Insert has BamHI restriction site. pf DTA BSD – BamHI digested vector, gave fragment size of 5.4Kb and 500bp, C1, C8, C11 and C30 are BamHI digested clones. Expected band size for positive clone is 4.6Kb. 2.1Kb and 500 bp.

### 3.1.8 Linearization of Gata6 LA-T2A-BFP with NotI:

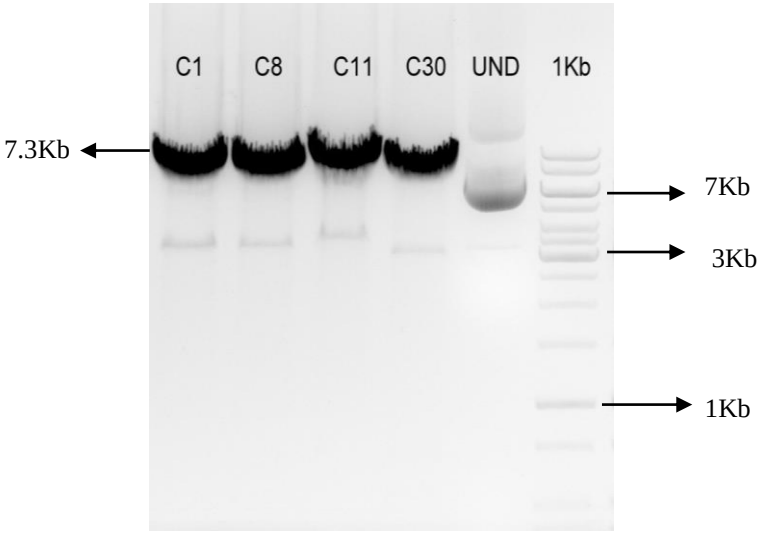


**Figure 16**: NotI digestion of Gata6 LA-T2A-BFP

GATA-6 LA-T2A-BFP was digested with NotI to linearize it. Digested clones: C1, C8, C11 and C30. UND: Undigested plasmid. All three isoforms were observed for UND, 1Kb: 1Kb DNA ladder. Expected size of fragment is 7.3 Kb.

### 3.1.9 Gibson assembly for insertion of RA in Gata6 LA-T2A-BFP:

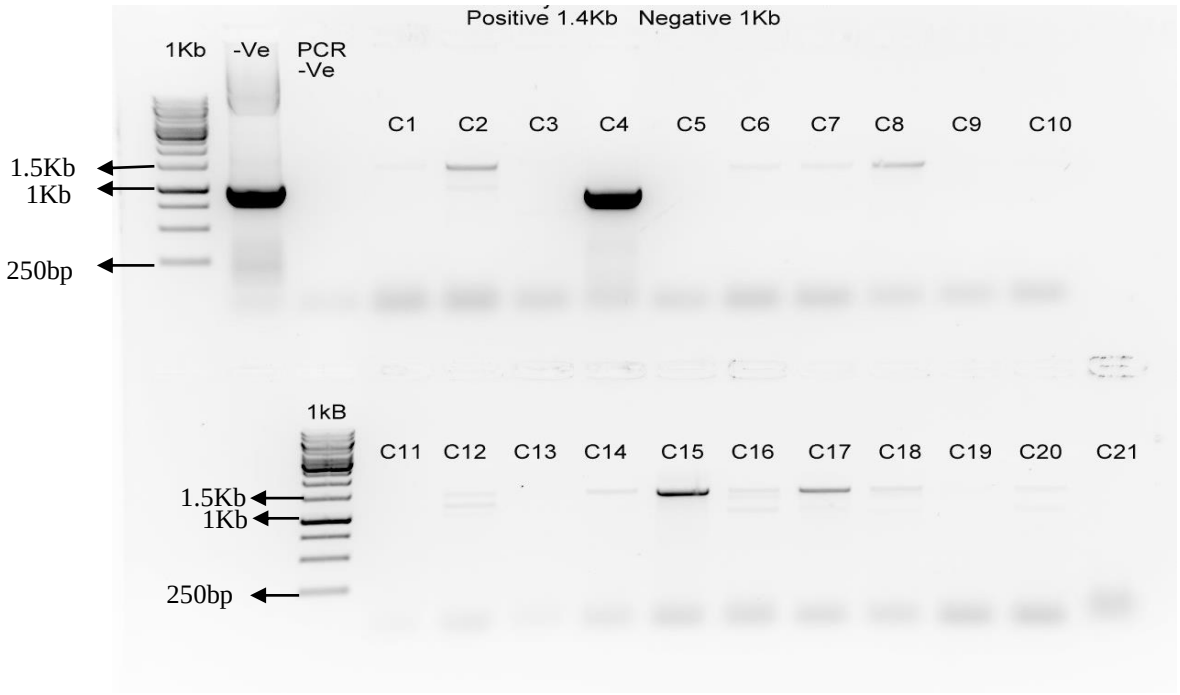


**Figure 17**: Colony PCR for RA insertion

Insertion of RA in Gata6 LA-T2A-BFP was done by Gibson assembly. Total 21 colonies were screened. -ve: Negative control, PCR -ve: Non-template control, Positive clones: C2, C8, C15, C17 –1.4Kb amplicon, Negative clone will give 1Kb amplification. C4 is a negative clone.

## 3.2 Sequence confirmation of Gata-6 LA-T2A-BFP-BSD TV:

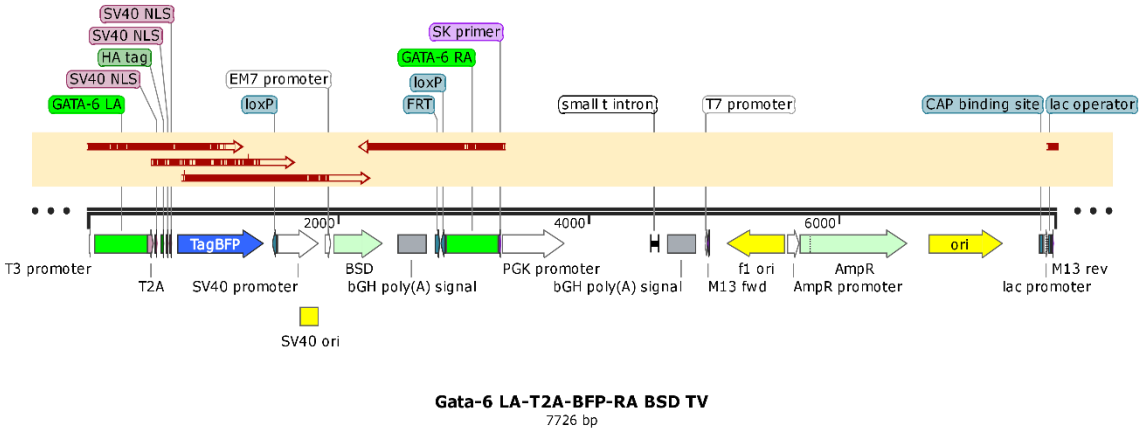


**Figure 18**: Sequencing result for Gata-6 TV

Gata-6 LA-T2A-BFP-BSD TV was sent for sequencing along with primers PCS-542, PCS435. The sequencing result had confirmed the insertion of LA-T2A-BFP into the plasmid pFDTA-BSD.

### 3.2.1 Final construct of targeting vector:

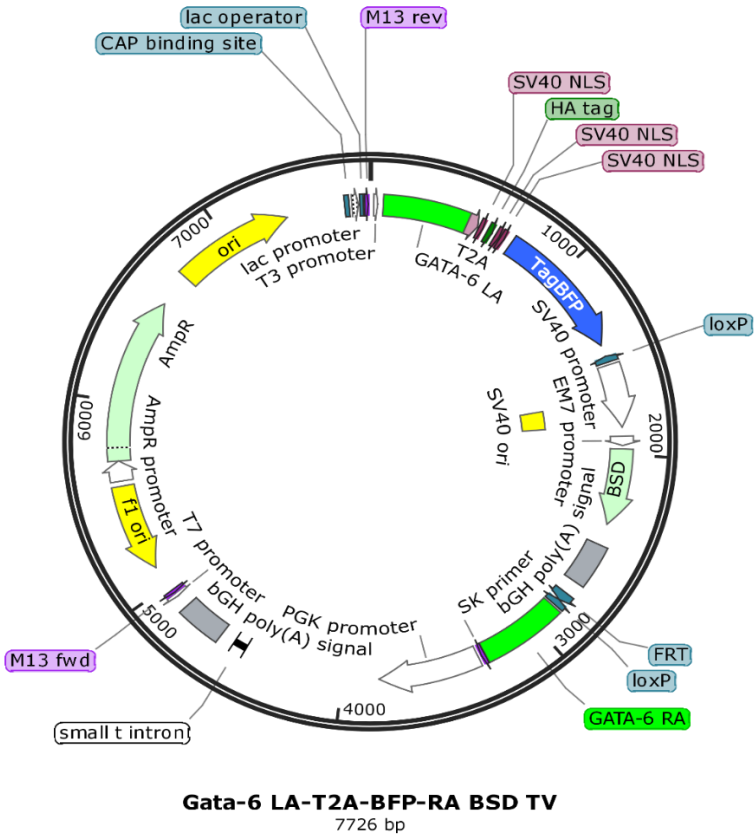


**Figure 19**: Map of targeting vector

Gata-6 TV consists of T2A sequence - self cleaving peptide, BFP tag, Blasticidin selection cassette expressed under PGK promoter. It has Ampicillin resistance used as selectable marker for selection in bacteria.

## 3.3 Construction of guide vector:

### 3.3.1 Colony PCR for pU6 GFP gRNA anneal with SG 258+PCS 127

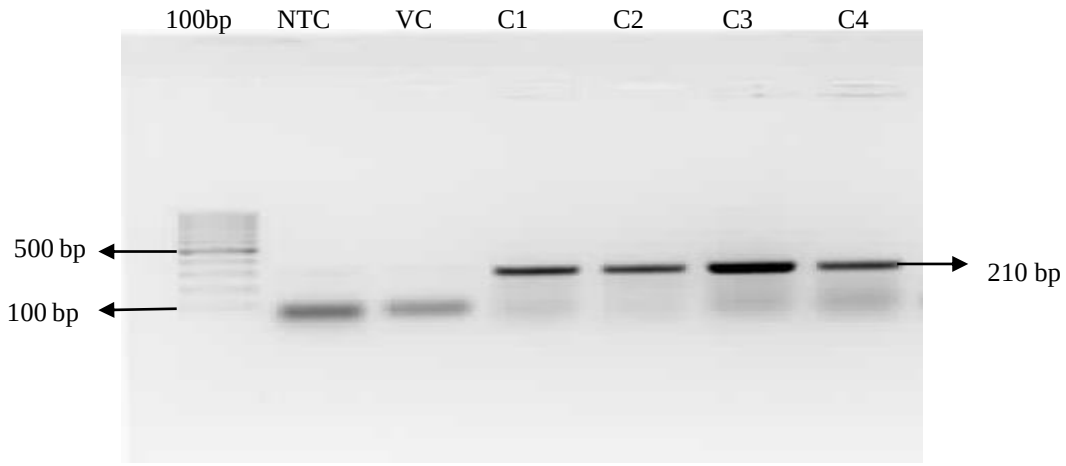


**Figure 20**: Colony PCR for guide anneal

Colony PCR for guide anneal was done using primers SG258 + PCS 127. Expected amplicon size is 210 bp. NTC- Non template control, VC – Vector control, Positive clones: C1, C2, C3, C4, NTC and VC do not show any amplification.

### 3.3.2 Sequence confirmation of pU6 - GFP gRNA vector:

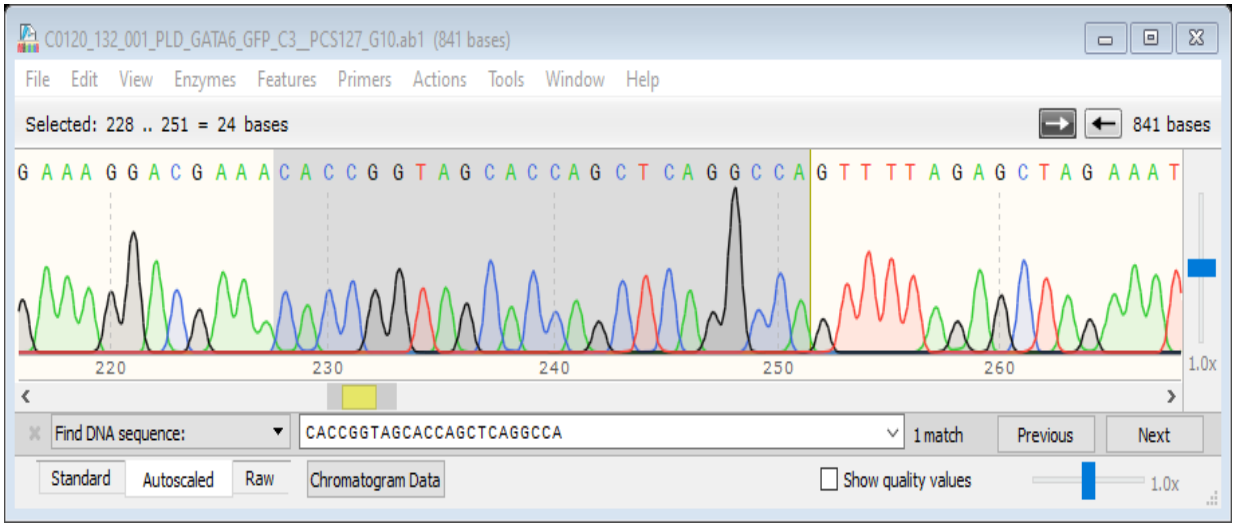


**Figure 21:** Sequence confirmation of C3 clone

The C3 clone of pU6 – GFP vector was sent for sequencing along with PCS 127. Sequencing result confirmed insertion of gRNA in the pU6 – GFP vector.

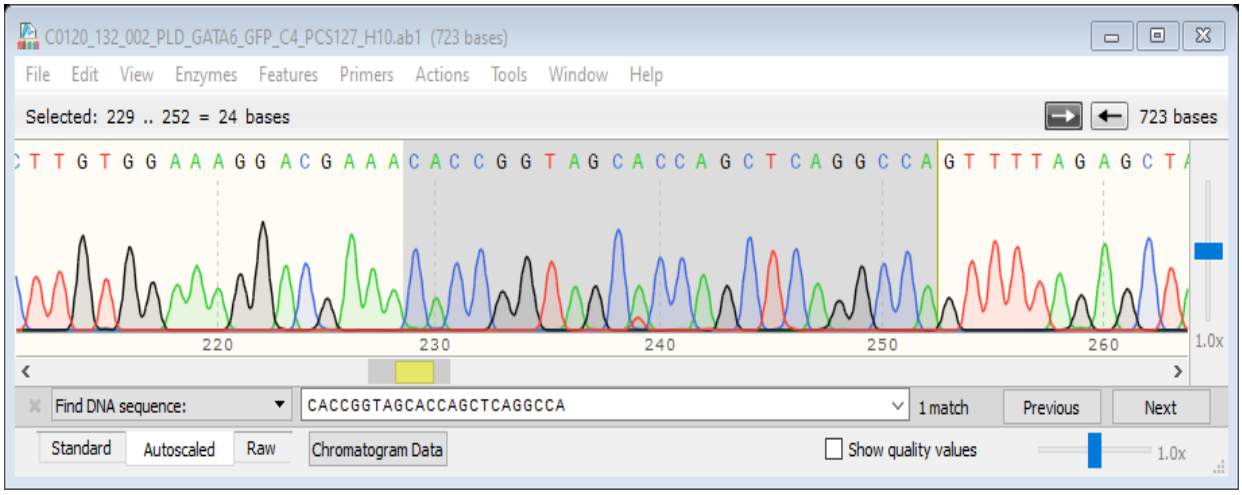


**Figure 22: Sequence confirmation of C4 clone**

The C4 clone of pU6 – GFP vector was sent for sequencing along with PCS 127. Sequencing result confirmed insertion of gRNA in the pU6 – GFP vector.

## 3.3.3 Final Construct of guide vector

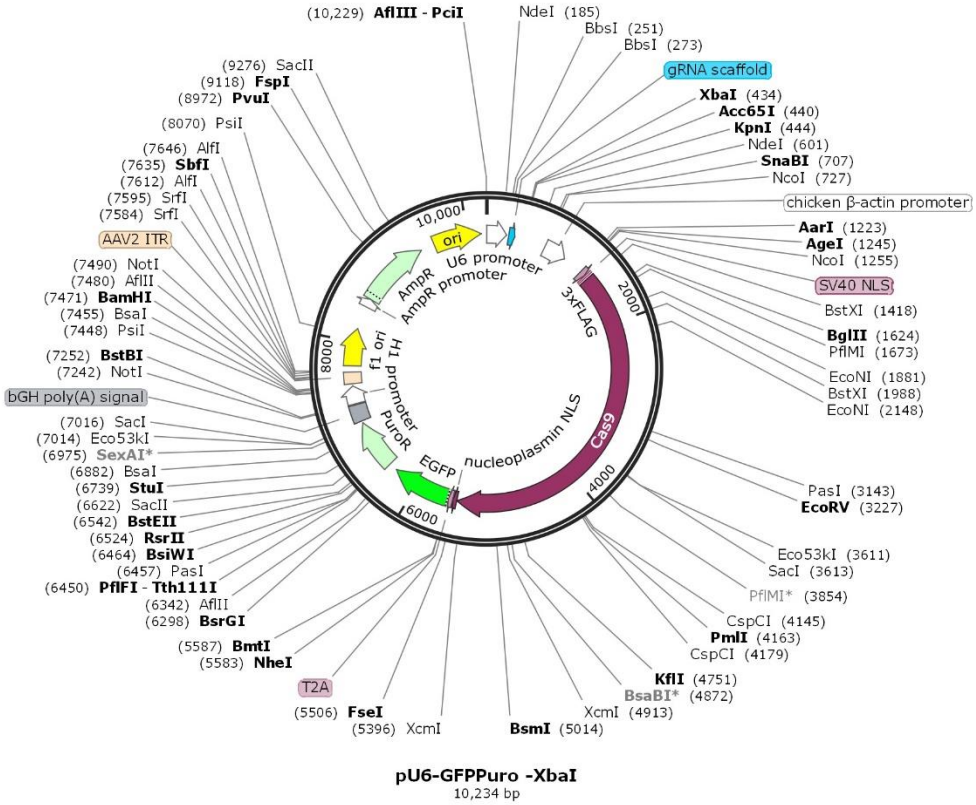


**Figure 23:** Final construct of pU6-GFP gRNA

Guide vector i.e., pU6-GFPPuro – XbaI comprise of guide RNA and GFP fluorophore expressed under U6 promoter. Nucleofected clones were sorted based on expression of GFP using MoFlo™ XDP cell sorter. It has ampicillin resistance gene which serve as a selectable marker in bacteria.

## 3.4 Genotyping standardization

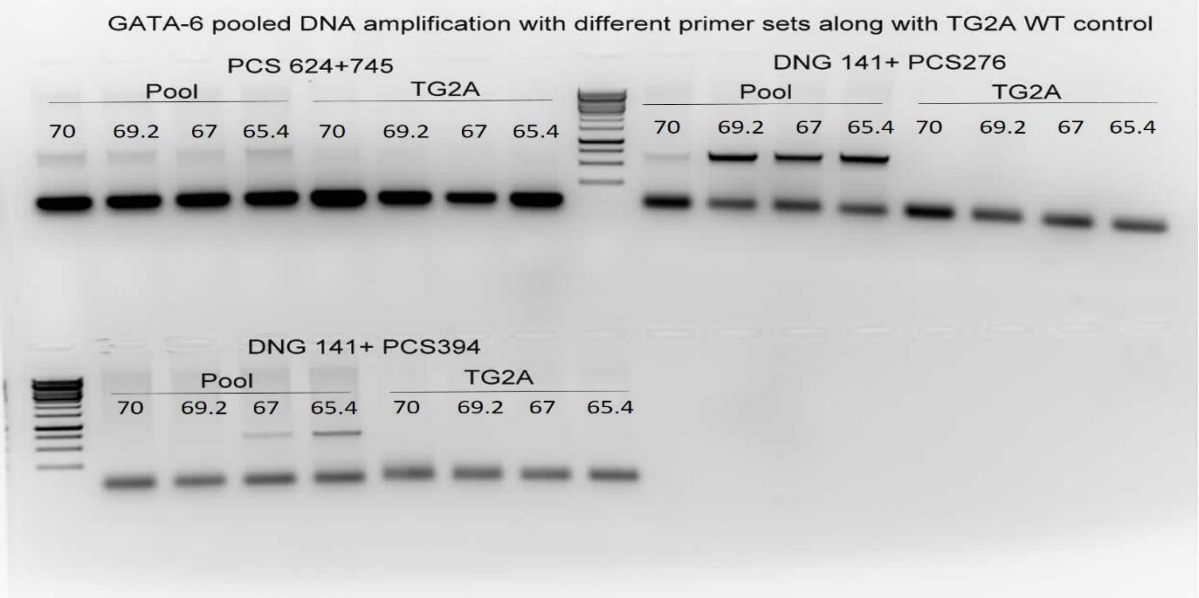


**Figure 24:** Standardization of genotyping for GATA-6 pooled DNA

Genotyping was standardized using pooled DNA along with wild type Tg2A with 3 different sets of primer – PCS 624+745, DNG 141+PCS 276 and DNG 141 + PCS 394. Gradient PCR was performed in order to determine specific temperature of annealing. Good annealing was observed at temperature 69.2ºC, 67ºC and 65.4ºC with DNG 141+ PCS 276.

## 3.5 Genotyping of 96 clones:

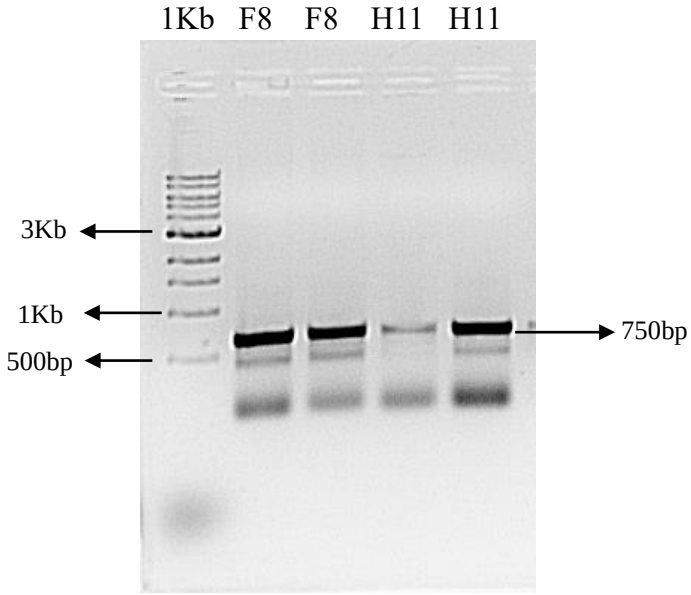


**Figure 25:** Genotyping for Tg2A GATA-6 KI clones

Genotyping of all 96 clones was carried out with DNG141 + PCS276. The annealing temperature used was 61.9ºC. Amplicon size of 750bp indicates positive clones. Two positive clones F8 and H11 were obtained.

During early embryo development, once the blastocyst is formed at day 3.5, cells lose their totipotency and assume pluripotency. Blastocyst can give rise to Epiblast or Primitive Endoderm. Mechanical Signaling is considered to be a key player in spatio-temporal organization and pre-requisite for lineage specification. So here in this study we have created a GATA-6 Knock-IN in Mouse Embryonic cell line where endogenous GATA-6 is tagged with BFP. For this we have constructed targeting vector i.e., **Gata-6 LA-T2A-BFP-BSD TV** and a guide vector **– pU6-GFP gRNA.** The wild type **E14Tg2a cell line** was co-nucleofected with both vectors. Cells positive for GFP were sorted using MoFlo™ XDP cell sorter. Sorted cells were maintained in GMEM media until confluent. Cells were under Blasticidin selection for 5 days. 96 colonies were picked up. Clones were maintained until confluent. Genotyping was standardized using pooled DNA. Genotyping was carried out for all 96 clones and positive clones were sent for sequencing.

The cell line Tg2a GATA-6-*BFP*, that we had generated is a novel model for understanding the mouse cell development and lineage specification. In order to study the developmental stages, this reporter cell line will be differentiated into embryoid bodies. The expression of the endogenous GATA-6 tagged with BFP would be analysed by Fluorescence Activated Cell Sorting (FACS). The cells will be differentiated and grown on different substrates having varying stiffness. This will help to determine how cells respond to extracellular matrix and undergo cell fate choice. This cell line will be used to detect/track primitive endoderm cells during ES cells to ESCs differentiation using Light Sheet Microscopy.

Although extensive studies have been made to study the early lineage commitment of ES cells in blastocyst there were numerous limitations in it. The first and foremost was that for immunostaining of blastocyst. The whole blastocyst is fixed and cells are dead. This will only show the presence of the marker one is looking for and not the lineage commitments. The Tg2a GATA-6 BFP cell line which we have made provide insight in identifying the exact time point of development where GATA-6 or endoderm is formed and from which cells and its spatiotemporal organization.

## 4. Summary and Conclusion:

### Summary:

Recent studies in the field indicate that the mechanical environment influences the behaviour and function of various types of cells. Interactions involving cell−cell and cell− extracellular matrix (ECM) contacts regulate self-renewal in ESCs. In this study, an endogenous *GATA-6* BFP ESC line is created by CRISPR-CAS9 mediated Knock-In. The cells will be differentiated and expression of the endogenous GATA-6 tagged with BFP would be analysed by Fluorescence Activated Cell Sorting (FACS). Cells will be grown on different substrates and hence how cell respond to ECM-Cell interactions will be determined. The cells differentiating into primitive endoderm will be tracked using Light sheet microscopy. Thus, exact time point of development where PE is formed can be determined.

### Conclusion:

CRISPR technology is the simplest, most versatile and powerful method of gene editing. Using this unique technology, we have created a knock-in Mouse Embryonic cell line where an endogenous GATA-6 is tagged with Blue Fluorescent Protein. This novel cell line will help to study Spatio-temporal regulation of GATA-6 during early differentiation in blastocyst and embryoid bodies.

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