Heterologous Expression of Fluorescent Protein Gene in *E. Coli* DH5α

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Abstract: Gene editing has opened a new field of molecular genetics research and the door for humans to understand, identify, isolate and modify genes to create new species. This project aims to produce heterologous fluorescent protein in E. coli cells by gene editing. By PCR amplification, the fluorescent protein gene has been obtained from the plasmid carrier; the target gene has been connected to the carrier, and finally transformed into the clone vector. The results of this project show that the fluorescent protein expressed by E. coli can be obtained, and the heterologous protein gene can be expressed in E. coli.

1 INTRODUCTION

Gene editing is an emerging technology that modifies specific target genes in an organism's genome. It can efficiently carry out site-specific genome editing, showing great potential in gene research, gene therapy and genetic improvement. The rapid growth of the human population increases the demand for meat and dairy products speedily, which drives the demand for crops, biofuels and livestock. It is an urgent problem to consider how to improve the effective yield of major crops The gene-editing technology can significantly improve crop yield by increasing plant resistance and improving crop quality to meet human needs (Ahmar, 2020).

Genome editing to correct disease-causing mutations is a promising way to treat human diseases. Gene editing-based therapy has the potential to treat more than 10,000 human single-gene diseases such as sickle cell disease (Park, 2021), and benefit many more complex diseases (Memi, 2018). Research shows that gene editing has also achieved results in treating cancer and AIDS.

As a simple and programmable nuclease-based genome-editing tool, the clustered regularly spaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system dramatically improves the ability to make precise changes in the human genome. In recent years, rapid advances in CRISPR-based technologies have expanded their reach and promoted CRISPR-based therapies in preclinical trials (Cui, 2018). The number of gene therapy drugs has increased rapidly, showing significant therapeutic effects on some cancers, genetic diseases and infectious diseases, especially after the emergence of the CRISPR technology in 2013.

E. coli is an essential tool in gene-editing technology. It can be transformed into a variety of functions and uses of cell factories, to achieve enzymes production, monoclonal antibodies, etc. It can also be used to catalyze biological and chemical reactions, synthesize complex compound molecules difficult to be synthesized before, and accelerate the creation and development of new drugs.

Researchers have isolated a variety of fluorescent proteins from living organisms, using molecular biology techniques to evolve mutants that cover almost the entire fluorescence spectrum. The red fluorescent protein mCherry and green fluorescent protein GFP are widely used. They have good characteristics in fluorescence intensity, light stability, acid-base resistance, maturity rate and other aspects. They can be used as fusion protein labels and applied in multi-fluorescent labelling imaging systems.

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This project is based on gene-editing technology. In this project, the fluorescent protein gene is integrated onto prSFDUET-1 plasmid by the homologous recombination technique, and the recombinant plasmid is transformed into E. coli BL21(DE3) cells. The fluorescent protein gene is expressed in E.coli by induction, and the size of the expressed protein is identified by SDS-PAGE.

2 MATERIALS AND METHODS

2.1 PCR Amplification of the Target Gene

2.1.1 Reagents

Plasmids:

pRSFDuet-1, pET30a-mCherry, YEp181-TEF1-GFP-FBA1.

Polymerases:

PrimeSTAR® Max DNA Polymerase enzyme, Hieff® Taq DNA Polymerase.

Primers:

GY-mCherry-F: 5'-TAATAAGGAGATATACCATGGTTTCCAAGGGCGAGGAG-3';

GY-mCherry-R: 5'-TCTGTTCGACTTAAGCATTACTTGTAGAGTTCGTCCATG-3';

GY-GFP-F: 5'-TAATAAGGAGATATACCATGCGTAAAGGAGAAGAACTT-3';

GY-GFP-R: 5'-TCTGTTCGACTTAAGCATTATTTGTATAGTTCATCCATGCCAT-3';

GY-bb-F: 5'-TAATGCTTAAGTCGAACAGAAAGTAAT-3';

GY-bb-R: 5'-CATGGTATATCTCCTTATTAAAGTTAAA-3'.

Others:

ddH_2O, agarose, 1×TAE buffer, 5×loading marker, DNA marker.

2.1.2 Materials

PCR tube (sterilized);

1.5 mL centrifuge tube (sterilized).

2.1.3 Apparatus

PCR machine, horizontal electrophoresis

2.1.4 Procedures

1) Miniprep plasmid (templates of PCR)
2) Single digestion of plasmid
3) Agarose gel detection of plasmid
4) PCR Reaction

Table 1: Reaction system: PCR Mixture in PCR tube.

Primestar polymerase	15 μL
Primer F	1.2 μL
Primer R	1.2 μL
plasmid template	<10ng
ddH_2O	Up to 30 μL
total volume	30 μL

Calculate the number of volumes to be added for pET30a-mCherry, pRSFDuet-1 and YEp181-TEF1-GFP-FBA1 according to each reagent in the system (ng/μL). Dosages are added to the PCR tubes in descending order. Mix the reactants in a centrifuge tube. Centrifugate for 5 s. Place the reaction tube into the sample hole in the base of the PCR instrument, set up the program, and conduct the PCR reaction:

Table 2: PCR Reaction Steps.

Preheat	94 ℃	2 min
Denaturation	98 ℃	10 s
Annealing	49 ℃	5 s
Stretching	72 ℃	40 s (10 s/kb)
Renaturation	72 ℃	10 min
End	16 ℃	forever

Perform the denaturation, annealing and extension cycles for 30 times. Then identify the products of the PCR reaction.

The PCR products are identified whether amplified or not by the pre-prepared 2% agarose gel electrophoresis. Record the sequence of dot sampling at 120 V and for 20 min as below.

Sampling: 4 μL PCR product + 1 μL 5× loading buffer;

DNA marker: 5 mL;

2% agarose gel: 0.2 g agarose + 20 mL 1× TAE buffer

The amplified product sizes: GFP (751 bp), mCherry (711 bp), backbone (3745 bp).

The PCR products are purified and then stored in a refrigerator at 4 ℃.

2.2 Double Fragment Homologous Recombination

2.2.1 Recombination System Configuration

Carrier backbone=0.02×3745 bp=75 ng.

Fragment mCherry=0.04×711 bp=32 ng, GFP=0.04×718 bp=32 ng

Table 3: ①pRSF-mCherry recombination.

System	20 μL
carrier backbone	75 ng
fragment mCherry	32 ng
5×CE II Buffer	4 μL
Exnase II	2 μL
ddH_2O	up to 20 μL
total volume	20 μL

Table 4: ②pRSF-GFP recombination.

System	20 μL
carrier backbone	75 ng
fragment GFP	32 ng
5×CE II Buffer	4 μL
Exnase II	2 μL
ddH_2O	up to 20 μL
total volume	20 μL

2.2.2 Recombination Reaction

The reaction takes place in a metal bath at 37 ℃ for 30 min, then the reagents are immediately placed on ice for 5 min.

2.3 Transformation of the Recombinant DNA Mixture into E. Coli DH5A Receptor Cells

2.3.1 Procedures

20 μL E. coli DH5α competent cells are removed from a refrigerator at -80 ℃ and placed on an ice box. Defrost at room temperature and are marked.

Add 5 μL of the recombinant system to the competent cells, mix well, and take an ice bath for 30 min.

Recovery: operate heat shock for 90 s in a water bath at 42 ℃, and then immediately place on the ice box for 3 min.

Add 800 μL LB medium to each tube and culture at 180 rpm, at 37 ℃, for 1 h.

Add 150 μL conversion solution to the corresponding resistant (50 ng/μL kanamycin) plate and evenly coat it with the liquid.

Place the plate upside-down in a constant-temperature incubator at 37 ℃ for overnight culturing.

2.4 Screening of Positive Clones by Colony PCR

2.4.1 Materials

Conversion board, Hieff® Taq DNA Polymerase, ddH_2O, agarose, 1×TAE buffer, 5×loading marker, DNA marker, PCR tubes (sterilized).

Primers:

GY-mCherry-F:5'-TAATAAGGAGATATACCATGGTTTCCAAGGGCGAGGAG-3'

GY-GFP-F:5'-TAATAAGGAGATATACCATGCGTAAAGGAGAAGAACTT-3'Carrier (0.5 μL)

Seq-GY(-R):5'-TTCGATTATGCGGCCGTG-3'

2.4.2 Procedures

Remove the plate transformed the night before from the 37℃ incubator and draw a pat line.

Patch and amplify the bacterium colony.

Single colonies are enriched and cultured at 37 ℃ for 5-7 h after 20 μL ddH_2O is added into the PCR tube, and the bacteria coating is scraped into the PCR tube at a ultra-clean table, with one tube for each bacterium. After the thallus is fully dissolved in ddH_2O, the thallus DNA is released into the water by lysis at 98 ℃ for 10 min. Centrifuge for 5 min, with the supernatant used as the template DNA of the colony PCR.

The following PCR systems are prepared.

Table 5: PCR Systems for Screening.

2×Hieff premix DNA Polymerase	5 μL
Primer F	0.4 μL
Primer R	0.4 μL
Template	2 μL
ddH_2O	up to 10 μL
total volume	10 μL Mix

Mix and centrifuge the reactants for 5 s.

Place the reaction tube into the sample hole in the base of the PCR instrument, set up the program, and conduct the PCR reaction.

Table 6: PCR Reaction Steps.

Preheat	94 ℃	5 min
Denaturation	94 ℃	30 s
Annealing	58 ℃	30 s
Stretching	72 ℃	30 s (30 s/kb)
Renaturation	72 ℃	10 min
End	16 ℃	forever

Perform the denaturation, annealing and extension cycles for 30 times. The PCR products are subjected to the pre-prepared 2% agarose gel electrophoresis to identify whether the target products are amplified. Record the sequence of the spot sampling at 120 V and for 20 min.

Spot sample: 5 μL colony PCR product, DNA marker 5 μL.

2% agarose gel: 0.2 g agarose + 20 mL 1× TAE buffer.

The amplified product sizes: GFP positive bacteria (785 bp), mCherry positive bacteria (779 bp).

2.5 Miniprep of Plasmid DNA

The colony PCR-verified positive strains are inoculated into LB/K+ medium for overnight culturing at 220 rpm at 37 ℃. Plasmid DNA is extracted.

2.6 Enzyme Validation

The plasmid DNA is cleaved by a restriction endonuclease.

2.6.1 Enzyme Digestion System

At 37 ℃ enzyme for 30 min.

Table 7: Enzyme digestion system.

system	10 μL
10×FastDigest buffer	1 μL
NcoI	0.2 μL
XhoI	0.2 μL
Template	500 ng
ddH_2O Up to	10 μL

2.6.2 Identification of Enzyme Digestion Results

The cleavage product bands of pRSF-GFP are 745+3711bp.

The cleavage product bands of pRSF-mCherry are 906+3544bp.

2.7 Plasmid DNA Transformed into E. Coli BL21(DE3) Competent Cells

When the sequencing results are correct, the plasmid DNA is transformed into E. coli BL21(DE3) competent cells expressing the host.

2.8 mCherry/GFP Expression in E. Coli BL21 (DE3) Induced by IPTG

2.8.1 Materials and Instruments

E. coli BL21 (DE3) containing pRSF-GFP plasmid, E. coli BL21 (DE3) containing PRSF-mCherry plasmid, LB/K+ solid and liquid medium, 1M IPTG storage solution, kanamycin storage solution, 20mM Tris-HCl buffer, 50ml, 2ml, 1.5ml centrifuge tubes, pipette gun and head, hood spectrophotometer, low-temperature centrifuge, ultra-clean table, spectrophotometer.

2.8.2 Procedures

Strains containing recombinant plasmid are streaked on LB plates containing kanamycin and cultured overnight.

Single colonies are selected into about 5 mL LB medium containing kanamycin and cultured overnight at 37 ℃ and 220 rpm to obtain seed liquid.

The seeds are inoculated at 1:50 in 50 mL LB medium containing kanamycin and cultured at 37 ℃ and 220 rpm for 1-1.5 h.

When OD600 is between 0.5 and 0.7, 25 μL IPTG is added and induced at 180rpm, at 30 ℃ for 7 h. Observe the colour of the solution.

After the induction, the bacterial solution OD is measured, and the induced bacterial solution is transferred to a 50 mL centrifuge tube. After being centrifuged at 1340rpm for 30 min at low temperature, the supernatant is discarded, and the colour of the thallus is observed and photographed. Resuspend with the buffer until 10OD/mL. Place 1 ml of the sample on ice to be treated with an ultrasonic crusher for 7 min (power 20%, 3 s each time. Interval 3 s).

Centrifuge at 12000 rpm for 10 min, transfer the supernatant to another centrifuge tube, and precipitate 1 mL phase. Resuspend with the buffer. Add 80μL supernatant or precipitate solution into the 1.5 mL centrifuge tube, add 20μL 5× protein loading buffer

and mix evenly. Boil for 5 min at 100 ℃. Cool and set aside at -20 ℃.

2.9 SDS-PAGE

2.9.1 Procedures

Preparation of protein gel.

Table 8. SDS-PAGE Gel formula

12% separation gel		5% stacking gel	
Reagent	Volume	Reagent	Volume
ddH_2O	3.3 mL	ddH_2O	2.2 mL
30%(w/v) polyacrylamide	4 mL	30%(w/v) polyacrylamide	0.67 mL
1.5 M Tris-HCl(pH8.8)	2.5 mL	1.5 M Tris-HCl(pH8.8)	1.0 mL
10% SDS	100 μL	10% SDS	40 μL
10% APS	100 μL	10% APS	40 μL
TEMED	6 μL	TEMED	4 μL

Fix the gel glass plate on the electrophoresis device and add trIS-glycine electrophoresis buffer in the upper and lower tanks. Add the samples in sequence, 10 μL solution for each aperture. Add an equal volume of 1×SDS gel loading buffer to the unused sample well.

Connect the electrophoresis device with the power supply (positive electrode slot) and apply 90 V on the gel. After the dye front enters the separation glue, the voltage is increased to 120 V. The electrophoresis continue until the bromophenol blue reaches the bottom of the separation glue, and then the power is turned off.

Remove the glass plate from the electrophoresis device and carefully pry the glass plate.

Soak the gel in at least 5 times the volume of dyeing solution and gently shake it on the decolorizing shaker for at least 0.5 h.

Remove the dye. Soak the gel in the decolorization solution, gently shake for 1-2 hours, changing the decolorization solution 4 times.

3 RESULTS

3.1 Recombinant DNA

After the recombinant DNA is transformed into the DH5α cells, the transformants on the plate are tested by colony PCR.

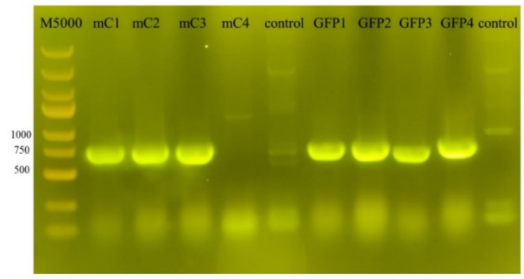


Figure 1: Colony PCR gel map.

Figure 1 shows that the colony is a positive colony containing the target gene.

3.2 Plasmid Verification

To further verify whether the extracted plasmid DNA is a positive clone, we cut the plasmid DNA using restriction endonuclease to detect whether the target band size is correct.

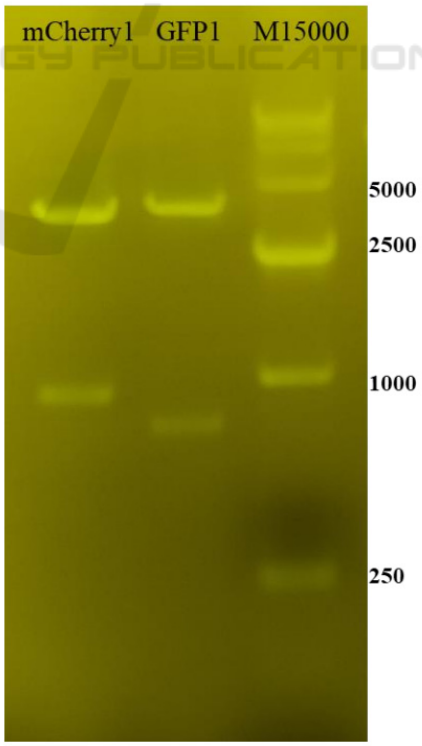
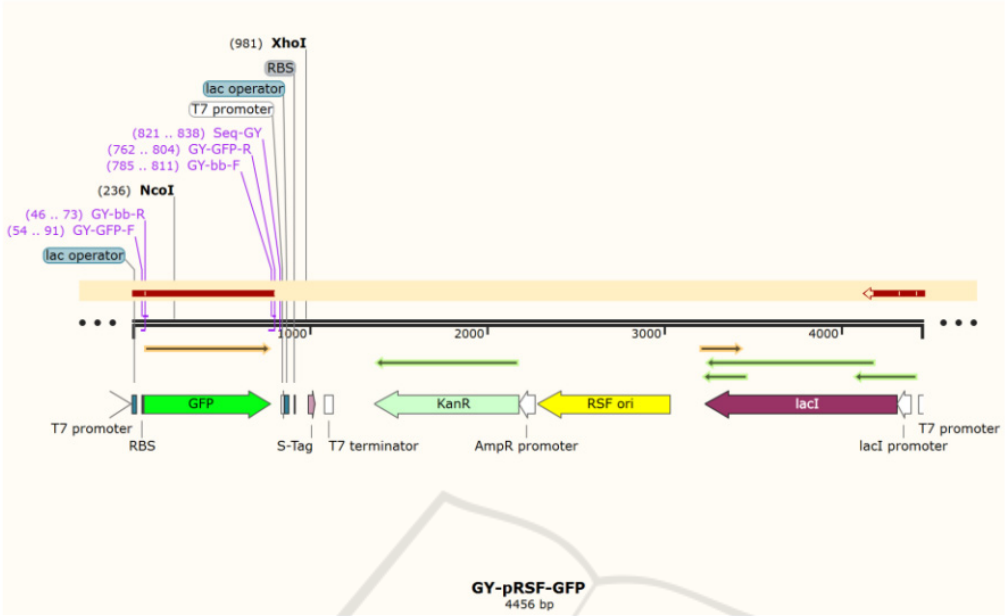
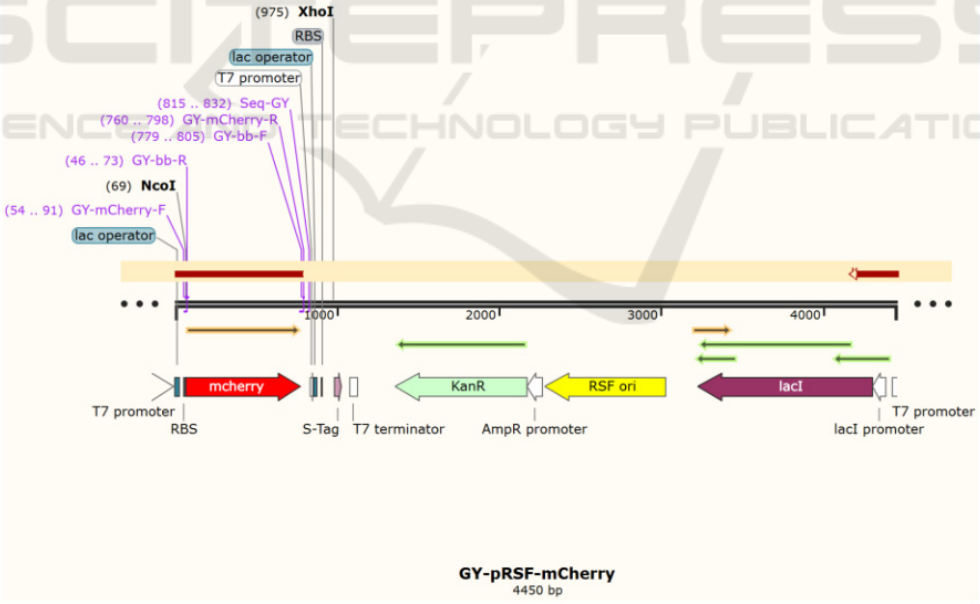


Figure 2: Enzyme digestion validation.

The results of enzyme digestion are correct (Figure 2); the plasmid and primer (SEQ-Gy) are sequenced and each identified by 4μL.



(a) dhwefhohc.



(b) fhrjfh.

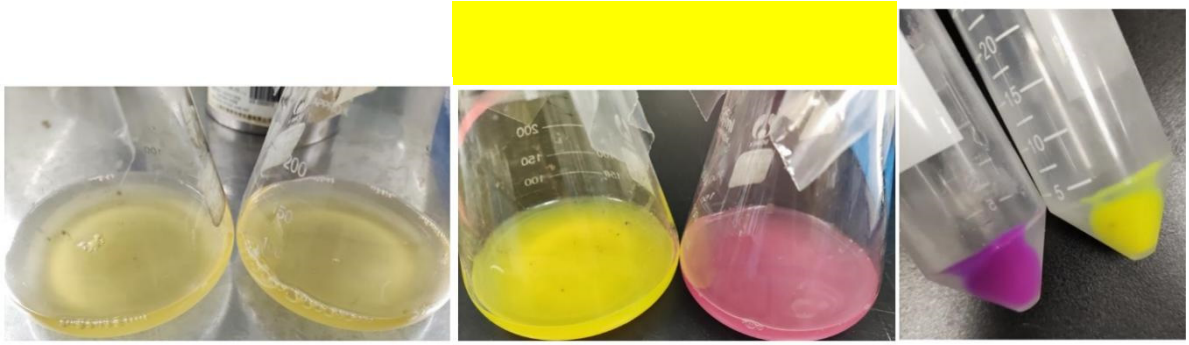
Figure 3: Sequencing analysis.

Sequencing results show that mCherry is correct, and there is a base mutation after the GFP starts codon ATG. A) dhwefhohc. B) fhrjfh.

3.3 Fluorescence

IPTG is used as an inducer to induce the promoter controlled by lactose operon to ensure stable

expression of heterologous protein in the condition of good growth of E.coli and avoid the influence of a large amount of heterologous protein expression on the growth of E.coli.



(a) GFP and mCherry before induction (b) mCherry after induction (c) bacteria mCherry and GFP

Figure 4: Results of fluorescence.

Figure 5: Results of SDS-PAGE.

3.4 SDS-PAGE of mCherry and GFP protein

The molecular weights of the products are verified by SDS-PAGE protein polyacrylamide gel electrophoresis.

4 CONCLUSION

This project demonstrates the basic operation model of genetic engineering. The fluorescent protein gene is integrated into the pRSFDuet-1 plasmid by homologous recombination technology, and the resulting recombinant plasmid is transformed into E.coli BL21(DE3) cells, and the fluorescent protein gene is expressed in E.coli by induction.

4.1 The Future of Gene Editing

The number of mutations in somatic cells increases dramatically with age, some of which can lead to cancer. In the future, gene-editing technology may help modify specific regions of the genome to prevent disease development.

In addition, the development of efficient genome-editing tools, including base editors with high specificity and no off-target effects, will open the possibility of using them in human embryos to avoid the spread of disease-causing mutations. Similarly, newly developed CRISPR-free mitochondrial base-editing methods are expected to correct pathogenic mutations in mtDNA present in unfertilized oocytes or embryos in the future and prevent transmission of mitochondrial disease to the next generation (Li, 2020).

Genetic diseases are not only caused by mutations in genes but also caused by low expression of the said genes, thus affecting the function of tissues and organs. During ageing, dysregulation of epigenetic markers results in reduced expression of several different genes that are important for the proper functioning of cells and tissues, ultimately leading to disease.

Since customizable nucleases have been shown to regulate the expression of target genes without modifying the genome sequence, they could be used to treat a variety of diseases, improve cellular characteristics of ageing, and restore tissue functions (Reddy, 2020).

One of the major challenges in cancer immunotherapy is targeting cancer cells specifically while keeping healthy cells unharmed. Targeting immune checkpoints through gene editing has been shown to be a possible strategy (Ernst, 2020). In treating primary tumors, targeted knockout can be applied to viral infections such as HPV, which means efficient gene knockout is required to reduce tumors effectively. Targeting viral sequences rather than endogenous genomic locations may be an advantage to reduce the risk of unwanted genomic changes due to gene editing. This could also be the potential advantage of strategies to eradicate pre-HIV viruses from the genome. HIV infection causes the body to build up a dormant reservoir of proviruses, making it much more difficult to cure (Wayengera, 2011). The removal or destruction of HIV genes in vivo by vector transport endonuclease can eliminate the innate adaptive replication and survival of HIV and provide an opportunity to prevent HIV gene replication.

The way genome editing works presents a double-edged sword: while it offers unprecedented therapeutic benefits, it also has safety concerns.

Because a gene-editing therapy works by producing DSBs in genomic DNA, the risk of missing the target at an unintended site is higher than that of another therapy that does not induce chromosome insertion or genomic changes (Shim, 2017). Because therapeutic gene targeting strongly relies on the creation of DSB at specific targets, in vitro selection libraries (Guilinger, 2014), mismatch detection nuclease assays (Vouillot, 2015), and whole-genome sequencing (Gabriel, 2011) have been developed to assess the targeting specificity of nucleases. Studies have shown that base editors (BEs) composed of a cytidine deaminase fused to CRISPR/Cas9 enables efficient RNA-guided base editing (Kim, 2017), which improve the efficiency of nuclease editing. There is also the challenge of developing efficient and safe methods to deliver gene-editing elements to cells in the body. Existing delivery methods, such as lipid nanoparticles (Gilleron, 2013), are widely used. This method is safe and simple but inefficient. In contrast, viral vectors such as adenoviruses (Holkers, 2013) have higher delivery efficiency, but there are unintentional mutations and safety issues (Silva, 2011).

Moreover, unlike chemicals and antibody drugs, gene-editing therapies require people to select relevant animal models for safety studies. For in-vivo gene-editing therapies, the binding specificity of the designed nuclease is controlled by specific sequences in the genome. Because the mouse genome is substantially different from the human genome, preclinical safety studies should instead be conducted in animal models that mimic the human genome.

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