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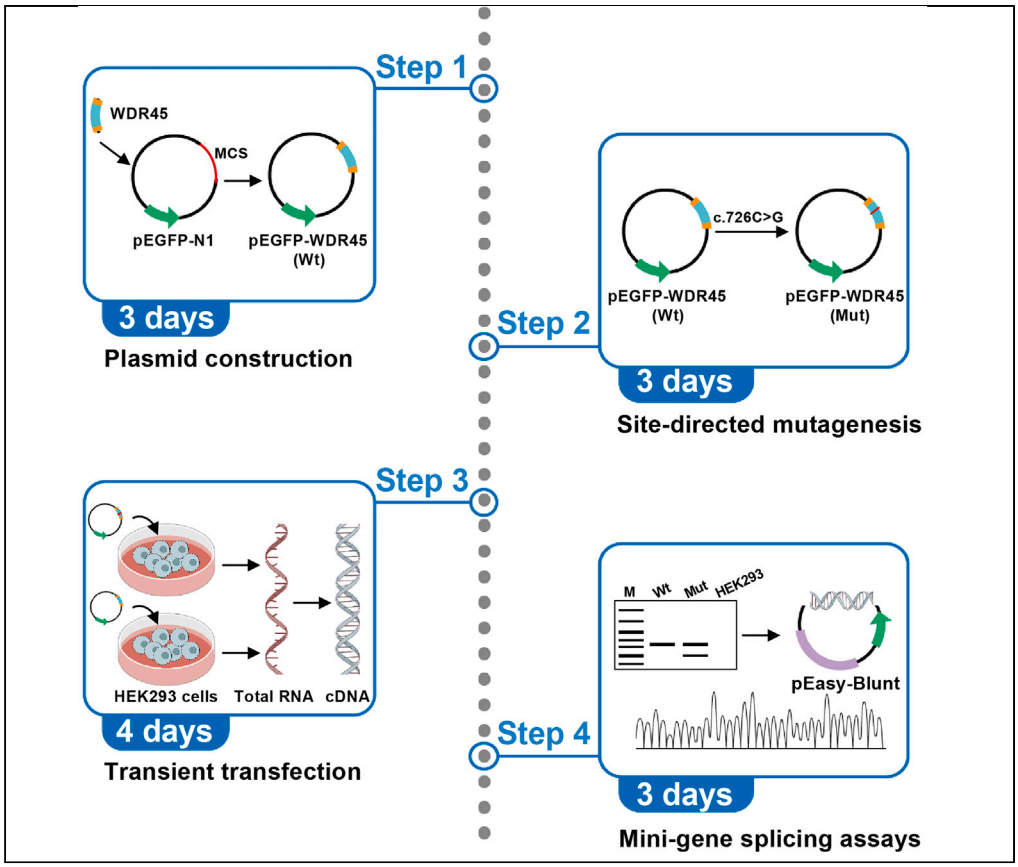
Protocol to study the effects of mutations near splicing sites on pre-mRNA splicing



Mutations at RNA splicing sites or regulatory elements can alter splicing efficiency or patterns, affecting RNA functionality and tissue-specific expression. Here, we present a protocol to study the impact of mutations near splicing sites on precursor mRNA (pre-mRNA) splicing. We describe steps for constructing plasmids by cloning the target gene into the pEGFP-N1 vector, performing site-directed mutagenesis, and transiently transfecting HEK293 cells. We then detail procedures for conducting mini-gene splicing assays to analyze splicing patterns influenced by mutations.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for generating mini-gene reporters via seamless cloning

Procedure for introducing single-nucleotide mutations using overlapping PCR

Instructions for analyzing pre-mRNA splicing patterns in the mini-gene assay

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Protocol to study the effects of mutations near splicing sites on pre-mRNA splicing

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SUMMARY

Mutations at RNA splicing sites or regulatory elements can alter splicing efficiency or patterns, affecting RNA functionality and tissue-specific expression. Here, we present a protocol to study the impact of mutations near splicing sites on precursor mRNA (pre-mRNA) splicing. We describe steps for constructing plasmids by cloning the target gene into the pEGFP-N1 vector, performing site-directed mutagenesis, and transiently transfecting HEK293 cells. We then detail procedures for conducting mini-gene splicing assays to analyze splicing patterns influenced by mutations.

For complete details on the use and execution of this protocol, please refer to Peng et al.[1]

BEFORE YOU BEGIN

In eukaryotes, precursor mRNAs (pre-mRNAs) with multi exons are maturated by RNA splicing, which is tightly regulated by different *cis*-acting regulatory regions, such as exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), intronic splicing silencers (ISSs), splicing acceptor site, splicing donor site, branch point sequences, and polypyrimidine tract.[2] And by trans-acting splicing factors such as SF2/ASF (SRSF1), SC35 (SRSF2), SRp40 (SRSF5), and SRp55 (SRSF6).[3–5] Mutations at splicing regulatory regions could affect pre-mRNA splicing by different mechanisms, such as activation or silencing of splice sites, aberrant splice site usage, inclusion of intronic sequences, exon skipping or insertion. Therefore, understanding the impact of mutations on RNA splicing is crucial for elucidating their role in regulation of gene expression and development of various diseases.

Mutations at RNA splicing sites, including splicing regulatory elements such as enhancers and silencers, can disrupt normal splicing patterns, leading to exon exclusion or intron retention.[6] These alterations can significantly influence the functionality and tissue-specific expression of the resultant RNAs.[7] In this protocol, we present a mini-gene reporter assay designed to analyze the effects of specific mutations on splicing patterns. This assay allows for the systematic investigation of splicing efficiency and the identification of altered splicing outcomes associated with the mutations of interest. Following the outlined steps will enable you to effectively assess how these mutations influence RNA splicing, providing insights into their potential biological implications.

Plasmid construction

Timing: variable

1. Synthesize target gene.

Synthesize the genomic DNA fragment of the target gene, covering at least more than one exon upstream and downstream of the mutation site.

Note: Since mutations around splicing site not only affects the splicing of the exon where the mutation is located but can also influence adjacent exons. Therefore, it is better to add a few additional exons. As for small genes with fewer than 3 exons, the whole genomic region containing the open reading frame should be cloned in the mini-gene reporter assay. It has been reported that some cryptic splicing regulatory elements is located in introns, which potentially leads to aberrant intron-derived exons.[8,9]

⚠ CRITICAL: To analyze authentic splicing events affected by mutations, include the entire intronic regions in the assay. In our experiment involving the WDR45 (WD repeat domain 45) gene, where the mutation is located in the exon 9, we used a synthetic genomic sequence containing three exons upstream and downstream of exon 9, along with the corresponding introns (chrX: 48,934,391–48,932,465, hg19). The plasmid containing the WDR45 gene was named pUC57-amp-WDR45(WT).

⚠ CRITICAL: It is important to notice that the target gene should be cloned into an ampicillin-resistant plasmid vector (pUC57-amp vector). This is because the WDR45(WT) gene needs to be cloned into a kanamycin-resistant plasmid vector (pEGFP-N1). This allows us to plate the transformation on kanamycin-containing plates for positive clone selection without growth of the original pUC57-amp-WDR45(WT) plasmid, thereby reducing false-positive clones. Additionally, using an ampicillin-resistant vector enables direct ligation of the PCR product into the pEGFP-N1 plasmid without the need for gel extraction of the PCR product, simplifying the workflow.

Cell culture

Timing: 1 week

These steps provide general guidance for thawing and passaging human embryonic kidney 293 (HEK293) cells and are not required to be repeated throughout the protocol. Ideally, HEK293 cells should be thawed and passaged at least twice before proceeding with transfection.

2. Quickly thaw HEK293 cells in a 37°C water bath and gently transfer them to a 15 mL conical tube.
3. Centrifuge the thawed cells (approximately 1×10^6 cells) at 300 g for 5 min at 20°C–25°C.
4. Carefully remove the freezing medium and resuspend the cell pellet in 5 mL of Dulbecco's Modified Eagle Medium (DMEM)+ medium containing 10% fetal bovine serum (FBS).
5. Plate the entire cell suspension (approximately 1×10^6 cells) in a T25 culture flask and incubate at 37°C with 5% CO_2.
6. When HEK293 cells reach 80% confluency. Aspirate the culture medium and wash cells twice with sterile 1× PBS.
7. Add 2 mL of 0.25% Trypsin-EDTA to the T25 flask and incubate for 3–5 min at 37°C.
8. To neutralize Trypsin-EDTA, add 4 mL of DMEM+ medium, then collect the detached cells into a 15 mL conical tube.
9. Plate approximately 0.5 mL of the cell suspension with 10 mL of fresh medium in a T75 flask.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
DH5α competent *E.coli* strain	Vazyme	Cat#C502-03
Chemicals, peptides, and recombinant proteins		
Penicillin-Streptomycin	Gibco	Cat# 15140122
PBS	Corning	Cat# 21031-CV
DMEM, high glucose	Thermo Fisher Scientific	Cat# C11995500BT
Fetal bovine serum (FBS)	ExCell Bio	Cat# FSP500
Trypsin-EDTA (0.25%), phenol red	Gibco	Cat# 25200072
Opti-MEM	Thermo Fisher Scientific	Cat# 31985062
1× TE buffer (pH 8.0)	Biosharp	Cat# BL531A
Phenol:chloroform:isoamyl alcohol (25:24:1)	Acmec	Cat# AP1012
10 M NH_4OAc	Beyotime	Cat# ST354
Glycogen (20 mg/mL)	Beyotime	Cat# D0812
DEPC water	Beyotime	Cat# R0021
2×EasyTaq PCR Super Mix	TransGen	Cat# AS111-11
TransZol	TransGen	Cat# ET101-01
Critical commercial assays		
ClonExpress MultiS One Step Cloning Kit	Vazyme	Cat# C113
Fast Mutagenesis System	TransGen	Cat# FM111
TransIntro PL Transfection Reagent	TransGen	Cat# FT301-01
TransScript Reverse Transcriptase [M-MLV,$RNaseH^-$]	TransGen	Cat# AT101-02
EasyPure Quick Gel Extraction Kit	TransGen	Cat# EG101
pEASY-Blunt Cloning Kit	TransGen	Cat# CB101-01
Plasmid Mini Kit	Omega	Cat#D6950-02
EcoRI-HF	NEB	Cat# R3101S
SalI-HF	NEB	Cat# R3138S
PrimeSTAR Max DNA polymerase	Takara	Cat# R045A
Experimental models: Cell lines		
HEK293 cells	ATCC	CRL-1573
Oligonucleotides		
WDR45 forward primer: ctcgagctcaagctt CAGTGCTGATCTGGGACGATGC	This paper	N/A
WDR45 reverse primer: cgggcccgcggtacc AAAGTCATCATCATCACAGATGTCAAG GTACACG	This paper	N/A
Mutagenic forward primer: CCCTGCCA CCCTCTACTGGTGAGCAC	This paper	N/A
Mutagenic reverse primer: GTAGAGGG TGGCAGGGTCAGTGCCTCG	This paper	N/A
Exon7 forward primer: ATGTGTACTCCTTCCCCGAC	This paper	N/A
GFP reverse primer: GAACTTGTGGCCGTTTACGT	This paper	N/A
M13 forward primer: GTAAAACGACGGCCAGT	This paper	N/A
M13 reverse primer: CAGGAAACAGCTATGAC	This paper	N/A
Recombinant DNA		
pUC57-amp vector	This paper	N/A
pEGFP-N1 cloning vector	Addgene	Cat# 172281
pEGFP-WDR45(Wt)	This paper	N/A
pEGFP-WDR45(Mut)	This paper	N/A
pEASY-Blunt cloning vector	TransGen	Cat# CB101-01
pEASY-Blunt-WDR45(Wt)	This paper	N/A
pEASY-Blunt-WDR45(Mut)L	This paper	N/A
pEASY-Blunt-WDR45(Mut)S	This paper	N/A
Software and algorithms		
SnapGene	Dotmatics	https://www.snapgene.com/

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Image Lab	Bio-Rad	https://www.imagelab.co/
Adobe Illustrator	Adobe	https://www.adobe.com/hk_zh/products/illustrator.html
Other		
Biological Safety Cabinets	Suzhou Antai Airtech Co., Ltd	BSC-13041IA2
Carbon-dioxide incubator	Thermo Fisher Scientific	BB15
Microcentrifuge	Thermo Fisher Scientific	Legend Micro 21R
Benchtop centrifuge	Thermo Fisher Scientific	Multifuge X1
Shaker	Shanghai Zhichu General Equipment Development Co., Ltd	ZQTY-70
Constant temperature incubator	Shanghai Zhichu General Equipment Development Co., Ltd	CT-150i
PCR System	Thermo Fisher Scientific	ProFlex
Electrophoresis system	Tanon	HE-90
Gel imaging system	Bio-Rad	GelDoc Go
Microvolume spectrophotometers	Thermo Fisher Scientific	NanoDrop One

MATERIALS AND EQUIPMENT

Dulbecco's Modified Eagle Medium (DMEM)+ (Supplemented with FBS and penicillin-streptomycin at a final concentration of 10% and 1%, respectively). Store at 4°C for up to 1 month.

STEP-BY-STEP METHOD DETAILS

Plasmid construction: Cloning the target gene into the *pEGFP-N1* plasmid

Timing: 3 days

These steps describe the steps for cloning the target gene into the multiple cloning site (MCS) of the pEGFP-N1 plasmid to perform a mini-gene splicing assay. Using the ClonExpress MultiS One Step Cloning Kit, the amplified fragment of target gene (e.g., WDR45 gene) is inserted into the MCS of the pEGFP-N1 plasmid according to the manufacturer's instructions: https://www.vazymeglobal.com/product-center/seamless-cloning/clonexpress-multis-one-step-cloning-kit. The main steps are summarized below.

1. Preparation of linear vector.
   a. Prepare the following reaction system on ice in 2.0 mL microcentrifuge tube (Table 1).
   b. Incubate in a 37°C incubator for 2 h.

Note: Double endonuclease digestion is recommended to achieve complete linearization and low transformation background (false positive clone). If single digestion is necessary, extend the digestion time to minimize residual circular plasmid and further decrease background during transformation.

Table 1. Restriction enzyme digestion mix solution

Reagent	Amount	Final
pEGFP-N1 plasmid (200 ng/μL)	250 μL	50 μg
10× NEB Cutsmart buffer	40 μL	1×
EcoRI-HF (20 units/μL)	16 μL	160 units
SalI-HF (20 units /μL)	16 μL	160 units
ddH_2O	78 μL	N/A
Total	400 μL	N/A

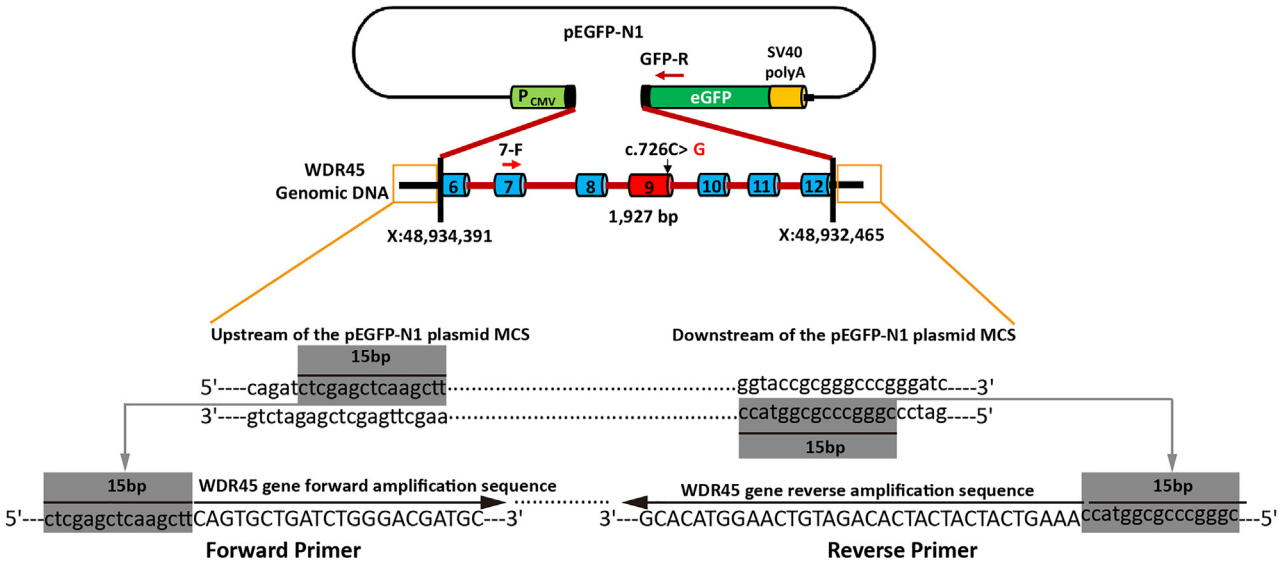

Figure 1. Design scheme of upstream forward amplification primer and downstream reverse amplification primer for the WDR45 gene

Alternatives: For linearizing a vector through reverse PCR amplification, use a high-fidelity polymerase to minimize amplification mutations (PrimeSTAR Max DNA Polymerase). In a 50 μL PCR reaction, use 0.1–1 ng of circular plasmid or pre-linearized plasmid as the template to reduce residual circular plasmid's impact on clone positivity.

c. Purify the linear vector by following these steps.
   i. Add 600 μL 1× Tris-EDTA(TE) Buffer (pH 8.0) and 1 mL phenol: chloroform: isoamyl alcohol (25:24:1) to the sample, and vortex gently.
   ii. Centrifuge at 18,000 × g for 5 min at 2°C–8°C to separate the phases.
   iii. Carefully transfer the upper aqueous layer to a clean, sterile 1.5 mL microcentrifuge tube, discarding the interface and lower phase.
   iv. Add 400 μL of 95% ethanol, 25 μL of 10 M NH_4OAc (or 1/10 volume 3 M sodium acetate anhydrous (NaOAc)), and 1 μL glycogen (20 mg/mL). Vortex gently.
   v. Centrifuge at 18, 000 × g for 5 min at 2°C–8°C.
   vi. Discard the supernatant and wash the pellet with 300 μL of 70% ethanol.
   vii. Centrifuge again at 18, 000 × g for 2 min.
   viii. Carefully aspirate off the supernatant and air-dry the pellet for ∼15 min at 20°C–25°C.
   ix. Dissolve the DNA pellet in 10 μL of sterile 1× TE Buffer (pH 8.0) and store at −20°C.

2. Insert fragment acquisition.
   a. Introduce homologous sequences at the 5′ end of the primers to ensure that the amplified products and the linearized cloning vector share identical sequences (15–20 bp, excluding the restriction site) for *in vitro* homologous recombination. Use *pEGFP-WDR45* (WT) as an example (Figure 1).

⚠ CRITICAL: The gene-specific forward/reverse primers refer to the sequences used to amplify the insert, with a preferred Tm value between 60°C and 65°C.

⚠ CRITICAL: The homologous sequences at the upstream and downstream ends of the vector should be the terminal sequences of the linearized vector (for homologous recombination), with a GC content ideally between 40% and 60% (Figure 1).

Table 2. PCR reaction master mix

Component	Volume	Final concentration
PrimeSTAR Max DNA Polymerase	25 μL	1×
WDR45 Forward Primer (10 μM)	1 μL	0.2 μM
WDR45 Reverse Primer (10 μM)	1 μL	0.2 μM
Template (*pUC57-amp-WDR45(WT)*)	10 ng	N/A
Nuclease-free Water	Up to 50 μL	N/A

⚠ CRITICAL: If the final primer length exceeds 40 bp, it is advisable to purify the primers using polyacrylamide gel electrophoresis (PAGE). This purification step can enhance the cloning success rate by removing any incomplete or undesired primer products.

b. Insert fragment PCR amplification: Amplify the insert fragment using any PCR enzyme (Taq enzyme or high-fidelity enzyme) (Tables 2 and 3).

Note: It does not matter whether the product has an A tail, as this will be removed during the recombination process and will not appear in the final vector.

⚠ CRITICAL: To minimize the introduction of amplification mutations, it is recommended to use a high-fidelity polymerase for amplification, such as PrimeSTAR Max DNA Polymerase, strictly following the manufacturer's protocol: https://www.takarabio.com/documents/User%20Manual/R045A_e.v2102Da.pdf.

c. Gel recovery PCR fragments: Run the PCR product on a 1.5% agarose gel at 120 V for 25 min. Use the EasyPure Quick Gel Extraction Kit to extract the target DNA band, strictly following the manufacturer's protocol: https://www.transgenbiotech.com/pdf/271. The essential steps are outlined below.
   i. Cut the target DNA band from the gel, transfer it to a clean centrifuge tube, and weigh it (e.g., assume 100 mg ≈ 100 μL).
   ii. Add Gel Solubilization Buffer (GSB, yellow) solution at three times of the gel volume, then place the tube in a 55°C water bath for 6–10 min, gently mixing every 2–3 min until fully melted.

Note: If the melted gel solution appears purple, add an appropriate amount of 3M NaOAc (pH 5.2) to adjust it to the GSB solution color (yellow).

   iii. Once the solution cools to 20°C–25°C, transfer it to a centrifuge column, let it sit for 1 min, then centrifuge at 10,000g for 1 min and discard the flow-through.
   iv. Add 650 μL of Wash Buffer (WB) solution, centrifuge at 10,000g for 1 min, and discard the flow-through.
   v. Centrifuge again at 10,000g for 2 min to remove any remaining WB solution.
   vi. Place the column in a clean tube, open the lid, and let it sit for 1 min to evaporate any residual ethanol.

Table 3. PCR cycling conditions

Steps	Temperature	Time	Cycles
Denaturation	98°C	10 s	30–35
Annealing	55°C	5 s or 15 s[a]	30–35
Extension	72°C	10 s (5 s/kb)	30–35
Final extension	72°C	2 min	1
Hold	4°C	Forever	1

[a]When the Tm value is above 55°C, set the annealing time to 5 s; when the Tm value is below 55°C, set the annealing time to 15 s.

Table 4. Recombination reaction master mix

Reagent	Recombination reaction	Negative control	Positive control
Linear vector	X μL (pEGFP-N1 plasmid)	X μL	1 μL (pUC19 control vector)
Insert fragment	Y μL	0 μL	1 μL (Control insert Mix)
5× CE MultiS Buffer	4 μL	0 μL	4 μL
Exnase MultiS	2 μL	0 μL	2 μL
ddH_2O	Up to 20 μL	Up to 20 μL	Up to 20 μL

vii. Add 30–50 μL of pre-warmed Elution Buffer (EB) buffer (60°C–70°C) to the column center, let it sit for 1 min, and centrifuge at 10,000g for 1 min to elute the DNA.
viii. Measure the concentration using an absorbance-based instrument like NanoDrop One. Store the DNA at −20°C.

Alternatives: Takara MiniBEST Agarose Gel DNA Extraction Kit v.4.0 (Takara) may also be used as an alternative to EasyPure Quick Gel Extraction Kit for gel recovery of PCR fragments.

3. Recombination reaction.
   a. Calculate the amount of DNA required for the recombination reaction according to the formula below. In order to ensure the accuracy of the sample addition, the linearized vector and the inserted fragment can be appropriately diluted before preparing the recombination system, and the amount of each component added should not be less than 1 μL.
   The optimal amount for each fragment = [0.02 × number of base pairs of the fragment] ng.
   b. Prepare the following reaction system on ice (Table 4).

⚠ CRITICAL: The amount of linearized cloning vector used should be between 50 and 200 ng. The amount of each insert fragment used should be greater than 10 ng. When the optimum dosage calculated using the above formula is lower than this value, 10 ng can be used directly.

Note: The amount of vector(X) and inserted fragment(Y) is calculated according to the formula.

Note: Negative control: Check for any remaining circular plasmids in the linearized cloning vector.

Note: Positive control: Use to control for experimental materials and procedural factors.

   c. Mix the components gently with a pipette (avoid vortexing) and briefly centrifuge to collect the reaction mix at the bottom of the tube.
   d. Incubate at 37°C for 30 min, then cool to 4°C or place on ice immediately.

Note: For best results, use a precise temperature control device, like a PCR instrument, as recombination efficiency peaks at around 30 min. Excessive or insufficient incubation time may reduce cloning efficiency.

Note: The recombination product can be stored at −20°C for up to a week, to be thawed for transformation as needed.

Alternatives: In-Fusion Snap Assembly cloning kits (Takara) may also be used as an alternative to ClonExpress MultiS One Step Cloning Kit for recombination reaction. However, the cloning must be done at 50°C for 15 min as opposed to 37°C for ClonExpress MultiS One Step Cloning Kit.

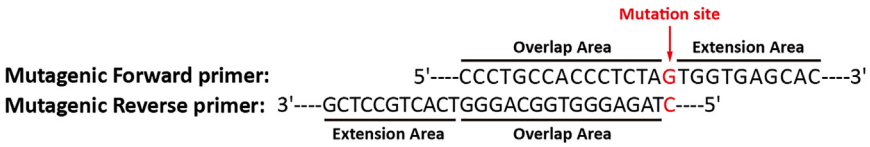

Figure 2. Primer design example

4. Transformation of Recombinant Product.
   a. Thaw chemically competent cells (e.g., DH5α, Vazyme #C502) on ice.
   b. Add 10 μL of the recombinant product to 100 μL of competent cells, mix by gently tapping the tube wall (avoid vortexing), and incubate on ice for 30 min.
   c. Perform a 45 s heat shock at 42°C, then cool on ice for 2–3 min.
   d. Add 900 μL of Super Optimal Broth (SOC) or Luria-Bertani (LB) medium (without antibiotics), and shake at 37°C for 1 h (220 rpm).
   e. Pre-warm LB agar plate with kanamycin in a 37°C incubator.
   f. Centrifuge the culture at 2,400 × g for 5 min, discard 900 μL of the supernatant, resuspend the cells in the remaining medium, and spread onto kanamycin- plates using a sterile spreader.
   g. Invert the plates and incubate at 37°C for 12–16 h.
5. Identification of recombinant products.

Note: After 12–16 h incubation, hundreds of colonies should appear on the recombination reaction plate. In comparison, the negative control plate should show significantly fewer colonies.

   a. Pick several colonies from the recombination reaction plate for colony PCR.

Note: Use at least one universal sequencing primer on the vector; successful clones should show a band slightly larger than the inserted fragment.

   b. For PCR-positive colonies, inoculate the remaining bacterial culture in liquid LB medium containing ampicillin and grow 12–16 h.
      i. Extract the plasmid and perform Sanger sequencing.
      ii. Name the successfully constructed plasmid *pEGFP-WDR45*(WT).

Site-directed mutagenesis of the *pEGFP-WDR45*(WT) plasmid

Timing: 3 days

This section outlines the process of site-directed mutagenesis on the *pEGFP-WDR45*(WT) plasmid using Fast Mutagenesis System, strictly following the manufacturer's protocol: https://www.transgenbiotech.com/pdf/215. The steps are summarized below.

6. Primer design.

Primer length: Each primer should be approximately 25–30 nucleotides long, excluding the mutation site. The 5′ overlapping region must contain 15-20 bases, while the 3′ extension region should have at least 10 bases.

Mutation primers: The mutation sites are incorporated into each primer.

⚠ CRITICAL: The forward mutation primer includes the mutation site just downstream of the overlapping region. The reverse mutation primer positions the mutation site at the 5′ end,

Table 5. PCR reaction master mix

Component	Volume	Final concentration
Plasmid (*pEGFP-WDR45*(WT))	1–10 ng	N/A
Mutagenic Forward Primer (10 μM)	1 μL	0.2 μM
Mutagenic Reverse Primer (10 μM)	1 μL	0.2 μM
2× *TransStart FastPfu* Fly PCR Super Mix	25 μL	1×
Nuclease-free Water	Up to 50 μL	N/A

adjacent to the overlap. For example, consider the mutation in *pEGFP-WDR45*(Mut) (c.726C > G) (Figure 2).

7. PCR amplification (Tables 5 and 6).
8. Electrophoresis detection: Take 10 μL of the PCR product and perform 1.5% agarose gel electrophoresis.

Note: If multiple amplified bands are present, proceed with the DMT (an improved version DpnI restriction enzyme) digestion and transformation reaction as long as the target band is of the correct size.

9. Digestion of PCR products: Add 1 μL of DMT Enzyme (10 units/μL) to the PCR product. Mix well and incubate at 37°C for 1 h.
10. Transformation.
    a. Add 2–5 μL of the DMT enzyme -digested product to 50 μL of DMT Competent Cells. Gently tap the tube wall to mix (do not oscillate) and place on ice for 20–30 min.
    b. Heat shocks the mixture in a 42°C water bath for 45 s, then immediately cool on ice for 2–3 min.
    c. Add 250 μL of SOC or LB medium (without antibiotics) and shake at 37°C for 1 h (220 rpm).
    d. Preheat LB -agar plate containing kanamycin in a 37°C incubator.
    e. Take 100–200 μL of the bacterial solution and spread it evenly on the plate. Invert and incubate in a 37°C incubator for 12–16 h.

⚠ CRITICAL: If no clones grow or the number of clones is low, use the EasyPure Quick Gel Extraction Kit to purify the DMT enzyme digestion product. Then take 2–5 μL of the purified product for transformation.

11. Identification of recombinant products.

Select 4–6 single clones and inoculate them into LB medium containing kanamycin. Culture 12–16 h, then extract plasmids for first-generation sequencing. The successfully constructed plasmid is named *pEGFP-WDR45*(Mut).

⏸ Pause Point: The prepared *pEGFP-WDR45*(WT) and *pEGFP-WDR45*(Mut) plasmids can be stored at −20°C while preparing HEK293 cultures.

Table 6. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	2–5 min	1
Denaturation	94°C	20 s	20–25
Annealing	55°C	20 s	20–25
Extension	72°C	20 s (6 kb/min)	20–25
Final extension	72°C	10 min	1
Hold	4°C	Forever	1

Alternatives: Mut Express II Fast Mutagenesis Kit V2 (Vazyme) may also be used as an alternative to Fast Mutagenesis System for site-directed mutagenesis.

HEK293 transient transfection

Timing: 4 days

These steps describe the transfection of HEK293 cells with *pEGFP-WDR45*(Wt) and *pEGFP-WDR45*(Mut) plasmids. Following transfection (TransIntro PL Transfection Reagent is used, strictly following the manufacturer's protocol: https://www.transgenbiotech.com/pdf/422), total RNA is extracted from the cells and reverse transcribed into complementary DNA (cDNA). This process establishes a foundation for the subsequent analysis of how point mutations affect RNA splicing.

12. Seed HEK293 cells into 24-well plates at a density of 4×10^4 cells/well. Culture the cells at 37°C with 5% CO_2 for 12–24 h.

⚠ CRITICAL: Ensure that cells reach 70–80% confluence before proceeding to transfection.

13. Before transfection, replace the culture medium with 500 μL of serum-free Opti-MEM medium.
14. Dilute 0.4 μg of plasmid DNA in 100 μL of serum-free Opti-MEM medium. Mix gently.
15. Add 1.5 μL of TransIntro PL to the diluted plasmid DNA and mix gently. Allow the mixture to stand at 20°C–25°C for 10–20 min.

Alternatives: Transfection reagents such as Lipofectamine 3000 (Thermo Fisher) may also be used as an alternative to TransIntro PL Transfection Reagent.

16. Add the plasmid DNA-TransIntro PL complex evenly to the cells. Incubate at 37°C with 5% CO_2.
17. Replace the complete medium 4–6 h after transfection and continue to culture the cells for an additional 24 h.
18. Extract total RNAs using *TransZol* by strictly adhering to the manufacturer's protocol: https://www.transgenbiotech.com/pdf/276. Follow these brief steps:
    a. Aspirate the medium and wash the cells once with 1x PBS.
    b. Add 200 μL of TransZol and horizontally position the plate for a moment to distribute the lysate evenly. Use a pipette to gently detach the cells off the plate.
    c. Transfer the lysate containing cells to a 1.5 mL centrifuge tube. Pipette repeatedly until no visible clumps remain in the lysate.
    d. Shake the tube at 20°C–25°C for 5 min.
    e. Add 40 μL of RNA Extraction Agent, shake vigorously for 15 s, and incubate at 20°C–25°C for 3 min.

    Note: After adding the RNA Extraction Agent, shake thoroughly to ensure effective extraction. Chloroform can also be used instead of the RNA Extraction Agent.

    f. Centrifuge at 10,000 x g for 15 min at 2°C–8°C.

    Note: At this point, the sample separates into three layers: the colorless aqueous phase (upper layer), the middle layer, and the pink organic phase (lower layer). RNA primarily resides in the aqueous phase, which comprises about 60% of the total volume.

    g. Transfer the colorless aqueous phase to a new centrifuge tube, add 100 μL of isopropanol, mix by inversion, and incubate at 20°C–25°C for 10 min.
    h. Centrifuge at 10000 x g for 10 min at 2°C–8°C and remove the supernatant.

Table 7. Reverse transcription mix solution

Component	Volume
Total RNA	1 μg
Anchored Oligo(dT)18 Primer (0.5 μg/μL)	1 μL
10 mM dNTPs	1 μL
5x TS RT Buffer	4 μL
Ribonuclease Inhibitor (50 units/μL)	0.5 μL
RNase-free Water	Up to 20 μL

Note: A gelatinous precipitate forms on the tube's sides and bottom.

i. Add 0.5 mL of 75% ethanol (prepared with DEPC water) and vortex vigorously.
j. Centrifuge at 7500 x g for 5 min at 2°C–8°C.
k. Discard the supernatant and air-dry the precipitate at 20°C–25°C for about 5 min.

⚠ CRITICAL: To control the salt ion content in RNA, remove as much ethanol as possible.

l. Dissolve the precipitate in 50 μL RNA dissolution solution.
m. Incubate at 55°C–60°C for 10 min.

Note: The extracted RNA can be used immediately or stored at −70°C for long-term use.

⚠ CRITICAL: It is important to assess the efficiency of transfecting the relatively large plasmids. Since the genes are cloned into the pEGFP-N1 vector, a fluorescent microscopy image of the transfected cells or a Western blot analysis using a GFP antibody should be performed before total RNA extraction to demonstrate plasmid transfection efficiency.

⚠ CRITICAL: Ensure that organic reagents (isopropanol, 75% ethanol, etc.) are free from RNase contamination. Consumables such as centrifuge tubes and pipette tips must also be RNase-free.

19. Reverse transcription of total RNAs.
Use TransScript Reverse Transcriptase[M-MLV,RNaseH$^-$] according to the manufacturer's protocol: https://www.transgenbiotech.com/pdf/86. Follow these steps.
a. Prepare the reaction system on ice (Tables 7 and 8).

Note: For complex RNA templates or to obtain higher synthesis efficiency, it is recommended to mix the RNA template, primers and RNase-free water, incubate at 65°C for 5 min, and then place on ice for 2 min before adding other components.

b. Store the cDNA product at −20°C.

Mini-gene splicing assays

Timing: 3 days

Table 8. PCR cycling conditions

Steps	Temperature	Time	Cycles
Reverse transcription	42°C	30 min	1
Inactivation	85°C	5 s	1
Hold	4°C	Forever	1

Table 9. PCR reaction master mix

Component	Volume	Final concentration
PrimeSTAR Max DNA Polymerase	25 μL	1×
Exon7 Forward Primer (10 μM)	1 μL	0.2 μM
GFP Reverse Primer (10 μM)	1 μL	0.2 μM
cDNAs	X μL (Equivalent to 125 ng total RNA)	N/A
Nuclease-free Water	Up to 50 μL	N/A

cDNAs were amplified via PCR and analyzed using 1.5% agarose gel electrophoresis. The resulting distinct bands were ligated into the *pEasy*-Blunt cloning vector. Subsequent sequencing of these constructs allowed for the assessment of how mutations impact RNA splicing. This approach provides insight into the molecular mechanisms underlying splicing alterations associated with specific mutations.

20. Amplification of cDNAs by PCR (Tables 9 and 10).

⚠ CRITICAL: Ensure that the Forward Primer is on exon 7 and the Reverse Primer is on the eGFP gene. This ensures that the PCR product is derived from *pEGFP-WDR45*(Wt) or *pEGFP-WDR45*(Mut).

Note: The PrimeSTAR Max DNA Polymerase is from a commercial kit (see key resources table for catalog information).

21. Run the PCR amplification product on a 1.5% agarose gel at 120 V for 25 min.
22. Use a gel imaging system for visualization (Figure 3).

Note: If the mutation site affects pre-mRNA splicing, two or more bands will appear in the gel for the plasmid containing the mutation.

23. Collect all the PCR electrophoresis bands into separate 1.5 mL centrifuge tubes respectively. Use a 1 mL pipette tip to crush the gel, making the pieces as small as possible.
24. Centrifuge at 10,000 x g for 10 min at 2°C–8°C. Transfer the supernatant to a new 1.5 mL centrifuge tube.

⚠ CRITICAL: Do not use a gel extraction kit for recovery, as the amount of product is minimal.

25. Ligate all the PCR products to *pEASY*-Blunt Cloning Vector respectively, strictly adhering to the manufacturer's protocol: https://www.transgenbiotech.com/pdf/246. The steps are summarized below.
    a. Prepare the reaction system at 20°C–25°C (Table 11).
    b. Mix gently and incubate at 25°C for 5 min. Place the centrifuge tube on ice afterward.

Table 10. PCR cycling conditions

Steps	Temperature	Time	Cycles
Denaturation	98°C	10 s	30–35
Annealing	55°C	5 s or 15 s[a]	30–35
Extension	72°C	10 s (5 s/kb)	30–35
Final extension	72°C	2 min	1
Hold	4°C	Forever	1

[a]When the Tm is above 55°C, set to 5 s; When the Tm is below 55°C, set to 15 s.

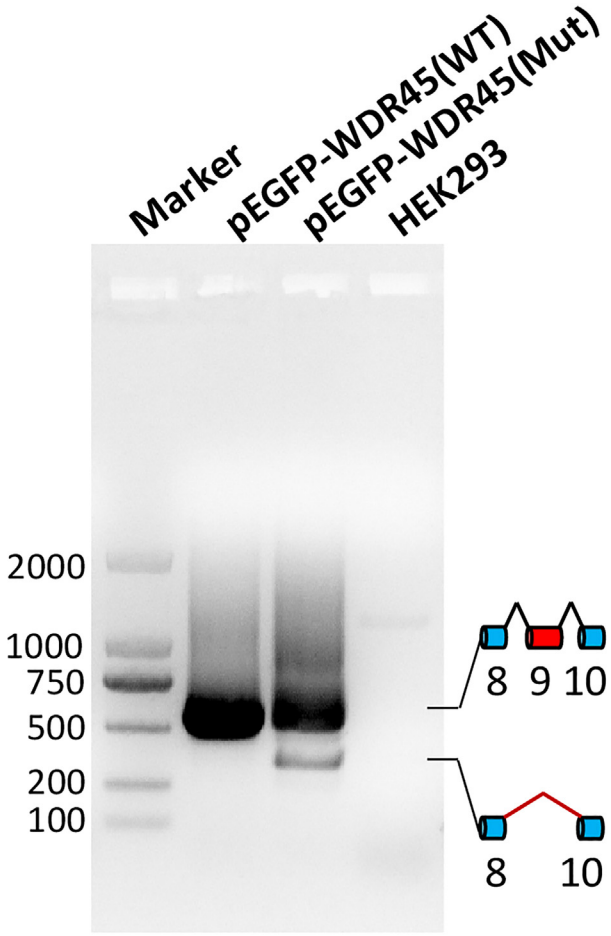

Figure 3. Agarose electrophoresis of the PCR products

A single band of 600 bp in the wild-type (WT) sample and two distinct bands in the Mut sample. An unclear smear above the 600 bp band in Mut samples was attributed to unspecific amplification. Figure reprinted with permission from Peng, Qiongling et al., 2024.

Note: Optimum reaction time varies with fragment length: 0.1–1 kb, 5 min; 1–2 kb, 10 min; 2–3 kb, 15 min; >3 kb, 20 min.

c. Transformation.
   i. Add the ligation product to 50 μL Trans1-T1 competent cells. Flick to mix, then place on ice for 20–30 min.
   ii. Heat shock in a 42°C water bath for 45 s, then immediately cool on ice for 2–3 min.
   iii. Add 250 μL of SOC or LB medium (without antibiotics) and shake at 37°C for 1 h (220 rpm).
   iv. Prepare IPTG and X-gal mix: Take 8 μL of 500 mM IPTG and 40 μL of 20 mg/mL X-gal, mix, and apply evenly to the LB agar plate containing ampicillin. Incubate at 37°C for 30 min.
   v. After absorption, take 200 μL of bacterial solution and evenly apply it on the plate, incubating 12–16 h at 37°C.

Note: For more clones, centrifuge at 1500 x g for 1 min, discard some supernatant, retain 100–150 μL, and plate all the suspended bacteria.

Note: The Trans1-T1 competent cells are from a commercial kit (see key resources table for catalog information).

Table 11. Recombination reaction master mix

Component	Volume
PCR Product	1 μL
pEASY-Blunt Cloning Vector	1 μL

d. Identify positive clones.
   i. Select white monoclonal clones and add 10 μL of sterile water. Vortex to mix.
   ii. Take 1 μL of the mixture and add it to a 25 μL PCR system, using M13 Forward and Reverse Primers for identification (Tables 12 and 13).

Note: The 2× *EasyPfu* PCR SuperMix is from a commercial kit (see key resources table for catalog information).

   iii. Electrophorese the PCR amplification product on a 1.5% agarose gel at 120 V for 25 min. Use a gel imaging system for visualization.

Note: If the vector self-ligates, the product length will be 200 bp; if ligation is successful, the product length will be greater than 200 bp.

e. For PCR-positive colonies, inoculate the remaining bacterial culture in liquid LB medium containing ampicillin and grow 12–16 h.
   i. Extract the plasmid use Plasmid Mini Kit.
   ii. Perform Sanger sequencing use M13 forward primer and M13 reverse primer.

Note: As shown in Figure 3, the *pEGFP-WDR45(Mut)*displayed a 600 bp band, nearly identical to the *pEGFP-WDR45(WT)* transcript except for the mutated nucleotide c.726G, and a shorter 400 bp band, indicating intragenic splicing between exon 8 and 10, resulting in the exclusion of exon 9. As shown in Figure 4, upon aligning all Sanger-sequenced bands against the WDR45 reference, three types of isoforms were identified.

EXPECTED OUTCOMES

It had been reported that variants around RNA splicing sites, splicing regulatory elements (splicing enhancers or silencers) or branching points could affect the splicing efficiency or splicing patterns of pre-mRNA.[10] The abnormally generated mRNAs could lose their proper function or tissue-specific expression. It's very necessary to unveil the possibly-affected transcripts and the underlying molecular mechanisms, which is very important for genetic counselors to have a comprehensive understanding of the patient's pathogenic factors. Mutations in splicing regulatory elements can disrupt normal splicing and lead to alternative splicing outcomes, such as exon skipping or the inclusion of cryptic intronic-exons. These disruptions can result in the production of multiple transcript variants from the same mutant allele.[11–15] As for the mutation in this protocol, it might produce three types of abnormal transcripts of WDR45, such as transcripts containing the nonsense mutant (726G), transcripts with an exon deleted or an intron retained.

Table 12. PCR reaction master mix

Component	Volume	Final concentration
Template	1 μL	N/A
M13 Forward Primer (10 μM)	1 μL	0.2 μM
M13 Reverse Primer (10 μM)	1 μL	0.2 μM
2× *EasyPfu* PCR SuperMix	25 μL	1×
Nuclease-free Water	Up to 50 μL	N/A

Table 13. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	10 min	1
Denaturation	94°C	30 s	30
Annealing	55°C	30 s	30
Extension	72°C	30 s (1–2 kb/min)	30
Final extension	72°C	10 min	1
Hold	4°C	Forever	1

LIMITATIONS

This protocol involves fusing genomic DNA fragments containing mutations into the multiple cloning site (MCS) of the pEGFP-N1 plasmid, but several limitations must be considered. Gene size limitations pose a challenge when amplifying regions with large introns, especially due to the presence of short or interspersed nuclear elements (SINE or LINE) and simple low-complexity repeats. Additionally, the pEGFP-N1 vector has a limited capacity, typically under 10 kb, which can hinder the insertion of larger genes or long fragments containing multiple regulatory elements, potentially omitting crucial regulatory regions.

Other limitations include fluorescence signal interference caused by in-frame GFP fusion, which may lead to false-positive or false-negative results due to issues such as improper folding, positional errors, or spontaneous fluorescence. Variability in transfection efficiency between different cell types, particularly primary cells, may affect the reproducibility of results. The lack of an internal reference, such as co-transfection with RFP, YFP, or luciferase, could also lead to deviations when comparing experimental groups. Furthermore, regulatory elements may only be active in specific cell types, meaning mini-gene results obtained in commonly used cell lines like HEK293 may not fully reflect physiological conditions.

TROUBLESHOOTING

Problem 1

No clones grew or very few clones appeared on the plate (related to Step 5).

Potential solution

- Check recombination reaction system: Ensure the amounts and ratios of the linearized cloning vector and insert amplification product match the recommendations in the protocol.
- Purity of vector and insert: The volume of unpurified DNA should not exceed 4 μL (1/5 of the total reaction volume). Gel-recover and purify the linearized vector and PCR products, dissolving the purified products in ddH_2O at pH 8.0 to prevent potential inhibitors from affecting the reaction.
- Evaluate competent cell efficiency: Confirm that the transformation efficiency of your competent cells exceeds 10^7 CFU/μg. You can verify this by transforming 1 ng of plasmid and plating 1/10 of the transformation volume. If you observe about 1,000 colonies, your transformation efficiency is acceptable. Ensure the volume of the recombinant product added does not exceed 1/10 of the volume of the competent cells, as larger volumes can reduce transformation efficiency. Use competent cells specifically designed for cloning (e.g., DH5α or XL10) rather than expression-competent cells, as these are optimized for plasmid propagation.

Problem 2

No bands in colony PCR (related to Step 5a).

Potential solution

- Incorrect primers: It is recommended to use universal primers of the vector for bacterial testing, or at least use one universal primer.

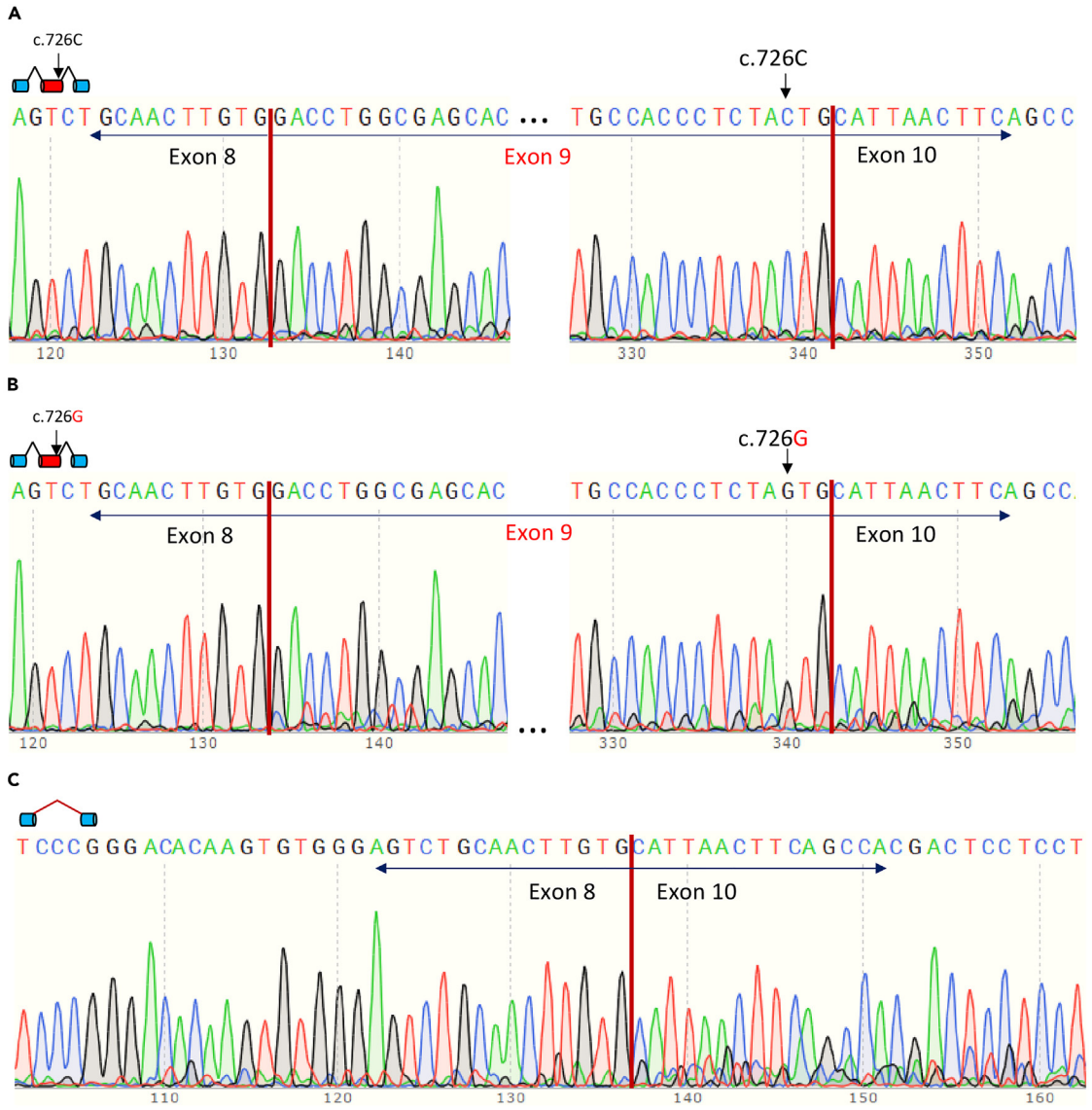

Figure 4. Sanger-sequenced bands

(A) Sanger sequencing for the wild-type transcripts (*pEASY*-Blunt-WDR45(Wt)).

(B) Sanger sequencing for the long Mut transcripts (*pEASY*-Blunt-WDR45(Mut)L).

(C) Sanger sequencing for the short Mut transcripts (*pEASY*-Blunt-WDR45(Mut)S). Figure reprinted with permission from Peng, Qiongling et al., 2024.

- Inappropriate PCR system or procedure: There is no target band or empty plasmid band. It is recommended to optimize the PCR system and procedure; or extract plasmid and use plasmid as template for PCR verification; or perform enzyme digestion verification.
- Recombination failure: There is only an empty plasmid band, indicating that the recombination is unsuccessful and the vector linearization is incomplete. It is recommended to optimize the enzyme digestion system. Check recombination reaction system: Insufficient or excessive amounts of the linearized cloning vector and the amplified insert fragment, or an improper ratio between them, may affect the recombination efficiency. It is recommended to prepare the recombination reaction system according to the amounts and ratios suggested in the manufacturer's instructions: https://www.vazymeglobal.com/product-center/seamless-cloning/clonexpress-multis-one-step-cloning-kit.

Table14. The amount of culture medium, DNA and TransIntro PL used in transfection in different cell culture plates

		Amount of culture medium		DNA transfection	
Cell culture plate	Area of single hole	Plating medium	Diluting medium	DNA	TransIntro PL
96-well	0.3 cm^2	100 μL	20 μL	0.1 μg	0.2–0.5 μL
48-well	1 cm^2	250 μL	55 μL	0.2 μg	0.4–1 μL
24-well	2 cm^2	500 μL	100 μL	0.4 μg	0.8–2 μL
12-well	4 cm^2	1 mL	200 μL	0.8 μg	1.6–4 μL
6-well	10 cm^2	2 mL	400 μL	2 μg	4–10 μL
35 mm	10 cm^2	2 mL	400 μL	2 μg	4–10 μL
60 mm	20 cm^2	5 mL	1 mL	4 μg	8–20 μL
10 cm	60 cm^2	10 mL	2 mL	12 μg	24–60 μL
T25	25 cm^2	6 mL	1.2 mL	5 μg	10–25 μL
T75	75 cm^2	13 mL	2 mL	15 μg	30–75 μL

Problem 3

Low plasmid transfection efficiency (related to Step 17).

Potential solution

- To optimize transfection efficiency while minimizing cytotoxicity, it's essential to adjust the ratio of DNA to TransIntro PL and the initial cell density. Below is a suggested optimization range (Table 14).
- The cell confluency should be within the recommended range (70%–80%) at the time of transfection, as suggested by the TransIntro PL Transfection Reagent protocol.

Problem 4

No amplification product or poor amplification efficiency (related to Step 20).

Potential solution

- Use an appropriate amount of cDNAs, ranging from 25 ng to 125 ng of total RNA, and improve the purity of the DNA template.
- The extension time can be set at the speed of 10–60 s / kb.
- Attempt a 25 μL reaction system.

Problem 5

The recombinant clones appear light blue or "Fish eye" (related to Step 25d).

Potential solution

This is because the inserted fragment does not affect the LacZ gene reading frame, or the inserted fragment is too short, in this case the clone appears light blue or "Fish eye" and can be identified normally.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Guanting Lu (guantlv@126.com).

Technical contact

Questions regarding the technical specifics of performing this protocol should be directed to and will be answered by the technical contact, Daoyuan Xie (xiedaoyuan@alu.scu.edu.cn)

Materials availability

The materials used and generated in this study are available from the lead contact upon reasonable request with a completed Materials Transfer Agreement. However, there are restrictions to the availability of plasmid reagents

generated for this study due to the cloning of the WDR45 gene into an expression vector and the point mutations performed on the WDR45 gene.

Data and code availability

No original code or any dataset has been generated in this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.L.; methodology and investigation, D.X., Q.P., and G.L.; validation, Y.T.; writing – original draft, D.X. and Q.P.; writing – review and editing, G.L.; resources and funding acquisition, D.X. and G.L.; supervision, Y.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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