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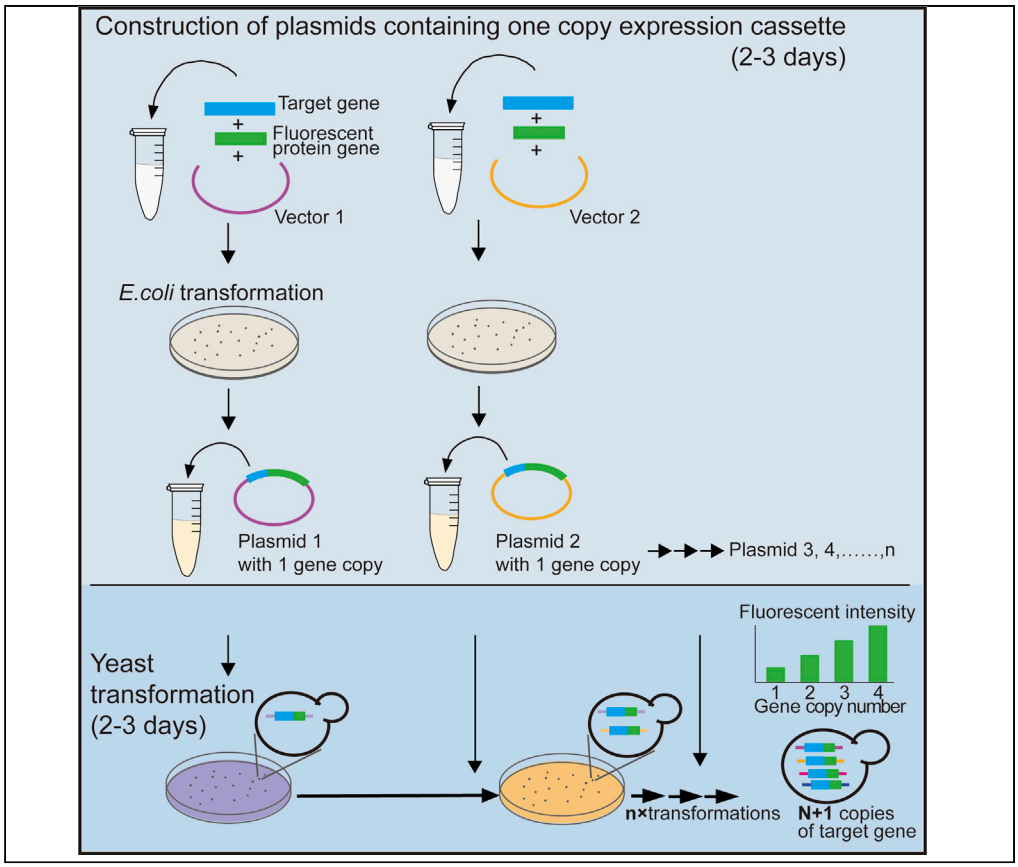
Protocol for Titrating Gene Expression Levels in Budding Yeast



The biological phenotype is affected by the level of gene expression. Here, we provide a step-by-step protocol for precisely titrating and quantitatively observing the target gene expression level in budding yeast by manipulating its copy number in the genome. Using this method, we construct various strains with different gene copy numbers of the cell cycle inhibitor Whi5. This protocol enables stable and inherent control of gene expression at the expected level with fluorescent intensity as the quantitative readout.

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HIGHLIGHTS

Precise manipulation of gene copy number in budding yeast.

Stable and inherent control of gene expression level.

Quantitative readout of gene expression level.

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Protocol for Titrating Gene Expression Levels in Budding Yeast

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SUMMARY

The biological phenotype is affected by the level of gene expression. Here, we provide a step-by-step protocol for precisely titrating and quantitatively observing the target gene expression level in budding yeast by manipulating its copy number in the genome. Using this method, we construct various strains with different gene copy numbers of the cell cycle inhibitor Whi5. This protocol enables stable and inherent control of gene expression at the expected level with fluorescent intensity as the quantitative readout.

For complete details on the use and execution of this protocol, please refer to Qu et al. (2019).

BEFORE YOU BEGIN

Primer Design

Timing: 0.5–1 h

1. Before the start of the experiment, design the primers for a recombinant plasmid, which is to be used to add one copy of the target gene into the yeast strain. The recombinant plasmid is expected to contain an expression cassette of target gene tagged with a fluorescent protein driven by the endogenous promotor of the target gene and ending by its endogenous terminator (Figure 1). Here we use Gibson assembly protocol to insert all 3 DNA inserts (i.e. gene with its endogenous promoter, fluorescent protein, gene's endogenous terminator) into the plasmid vector (Gibson et al., 2010; Gibson et al., 2009). The primers are designed to amplify the target gene with its promoter from the yeast genome (Forward primer 1 and Reverse primer 1), a fluorescent protein (Forward primer 2 and Reverse primer 2) and the terminator of the target gene (Forward primer 3 and Reverse primer 3) from the corresponding DNA template and insert them into a plasmid backbone. We amplified 1,000 bp before the ATG of WHI5 ORF to include its promoter and 1,000 bp since after the stop codon of WHI5 ORF to include its terminator. The 5′ end of the primer of each DNA insert should contain 15 bases that are homologous to 15 bases at 3′ end of the DNA insert to which it will be joined (i.e., the linearized vector or another DNA insert). The 3′ end of the primer should contain sequence that is specific to the target DNA insert, and only the 3′end of the Reverse primer 3 should contain 15 bases that are homologous to the 15 bases at 3′end of the linearized vector (Figure 2). Therefore, 5′end and 3′end of each DNA insert contains 15 bases that are homologous to the DNA to which it will be joined. The fluorescent protein is

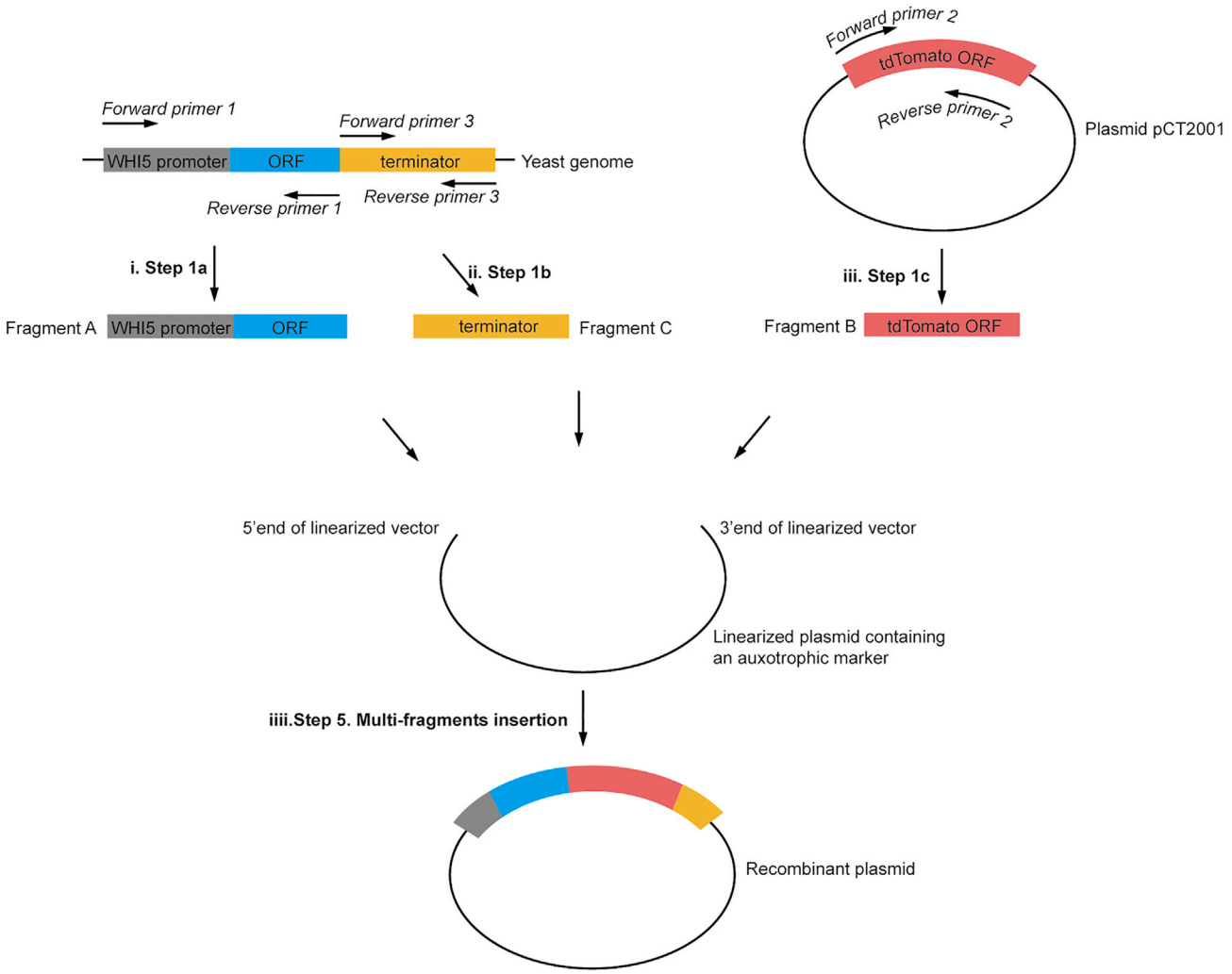

Figure 1. Schematic of the Construction of the Plasmid Containing One Copy of the Target Gene Tagged with a Fluorescent Protein

fused to the C-terminus of the target protein by a linker. The DNA sequence of the linker is added to the 5′end of the fluorescent protein ORF.

2. Design primers for verifying recombinant plasmids by performing colony PCR, as depicted in Figure 3A. Design primers to check if the gene copy has been integrated to the corresponding marker locus in the yeast transformants by PCR, as depicted in Figure 3B.

Note: Try to design primers of colony PCR that amplify DNA fragments around 500 bp for verifying recombinant plasmids. DNA fragments >1,000 bp will reduce the efficiency of colony PCR. For DNA fragments amplified by the primers longer than 1,000 bp, one should extract the genome of the colony to perform PCR.

Genome Extraction

Timing: 14 h

3. 1 day before the start of the experiment (day −1), extract the wildtype yeast genome. Pick a single colony of wildtype W303 strains from an agar plate stored at 4°C in a test tube containing 10 mL of YPAD media and grow at 30°C at 220 rpm for 12 h.

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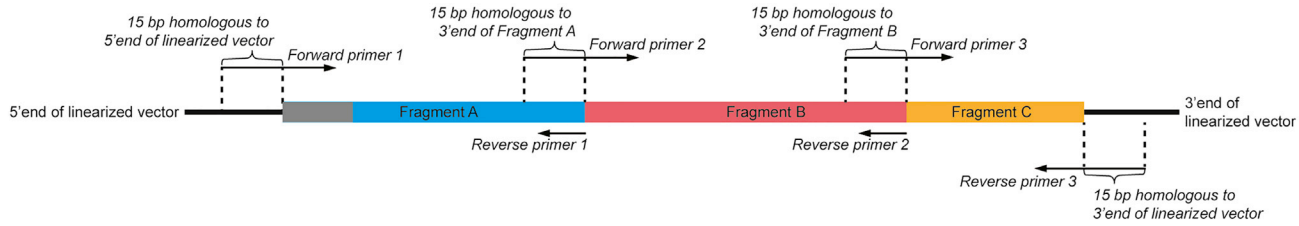

Figure 2. Schematic of Designing Primers to Amplify the DNA Inserts

Alternatives: If the strains are stored on frozen glycerol at −80°C or −70°C, scrape off a small amount of frozen glycerol stock with a sterile inoculator or pipette tip and patch onto a YPAD plate. Keep the stock frozen. Incubate the plate at 30°C for 2–3 days. When single colonies are visible, streak out on a YPAD plate and incubate at 30°C for 1–2 days. Pick a single colony on this plate in a test tube containing 10 mL of YPAD media and grow at 30°C at 220 rpm for 12 h. Store this plate at 4°C for future use.

Alternatives: Here we use YPAD medium, but other appropriate media such as SC medium can be used as well. See Materials and Equipment for recipes.

Pause Point: The culture can be stored at 4°C for several hours.

4. In the morning, use the TIANamp Yeast DNA kit to extract the yeast genome. Resuspend the yeast genome extracted from the 10 mL YPAD culture to ~500 μL in a microcentrifuge tube.

Alternatives: The *S. cerevisiae* genomic DNA is also commercially available.

⚠ CRITICAL: Use pH 7.5–8.0 double-distilled water (ddH_2O) to elute the yeast genome for the subsequent molecular cloning steps.

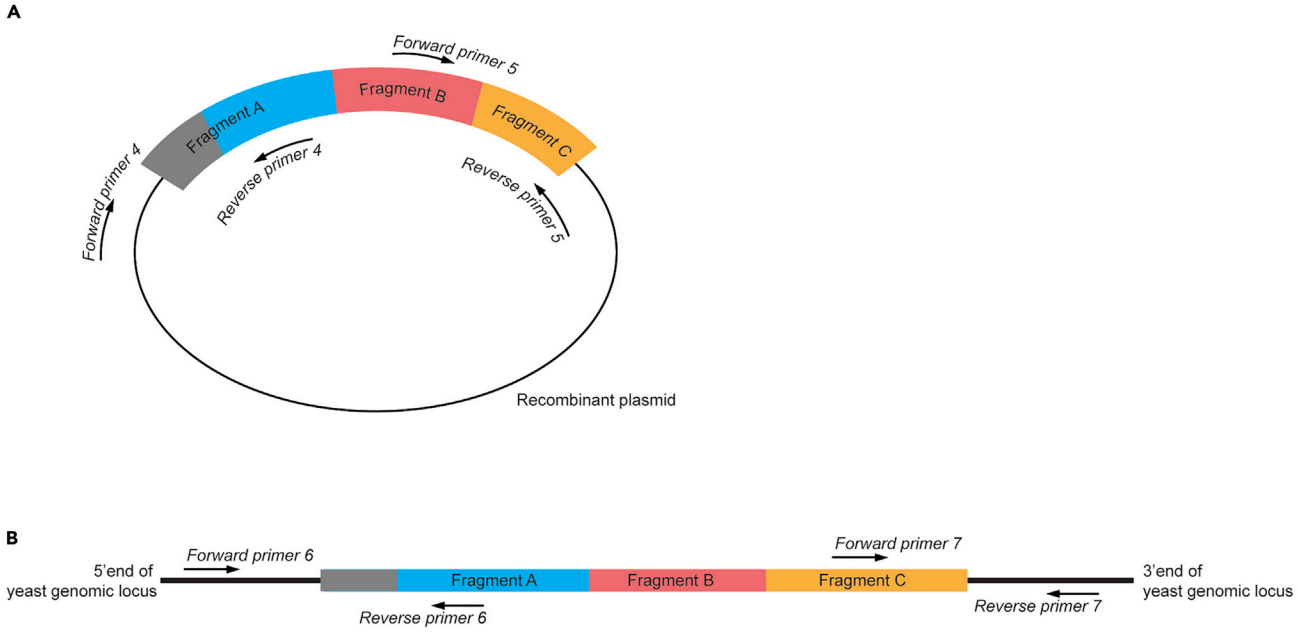

Figure 3. Schematic of Designing Primers for Colony PCR

(A) Schematic of designing primers to verify the recombinant plasmid by colony PCR.

(B) Schematic of designing primers for verifying yeast transformants by colony PCR.

Pause Point: The yeast genome can be frozen and stored at −20°C for 1 year.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
PEG3350	Sigma-Aldrich	Cat#P4338
Lithium acetate	Sigma-Aldrich	Cat#517992
salmon sperm DNA	Sigma-Aldrich	Cat#15632-011
Yeast extract	Sigma-Aldrich	Cat#Y1625
Peptone	Sigma-Aldrich	Cat# P5905
Glucose	Sigma-Aldrich	Cat#G7021
Yeast nitrogen base without amino acids	Sigma-Aldrich	Cat#Y0626
L-arginine	Sigma-Aldrich	Cat#A8094
L-methionine	Sigma-Aldrich	Cat#M5308
L-tyrosine	Sigma-Aldrich	Cat#T8566
L-isoleucine	Sigma-Aldrich	Cat#I7403
L-Lysine	Sigma-Aldrich	Cat#L5501
L-phenylalanine	Sigma-Aldrich	Cat#P5482
L-glutamine	Sigma-Aldrich	Cat#G7513
L-aspartic acid	Sigma-Aldrich	Cat#A7219
L-valine	Sigma-Aldrich	Cat#V0513
L-threonine	Sigma-Aldrich	Cat#T8441
L-serine	Sigma-Aldrich	Cat#S4311
Leucine	Sigma-Aldrich	Cat#L6914
Histidine	Sigma-Aldrich	Cat#H3911
Tryptophan	Sigma-Aldrich	Cat#T8941
Uracil	Sigma-Aldrich	Cat#U1128
Adenine	Sigma-Aldrich	Cat#A2786
Agar	Sigma-Aldrich	Cat#L2897
Critical Commercial Assays		
TIANamp Yeast DNA Kit	TIANGEN Biotech (Beijing)	Cat#DP307
QIAquick PCR Purification Kit	QIAGEN	Cat#28104
Gibson Assembly Master Mix	NEW ENGLAND Biolabs Inc.	Cat# E2611
Phusion® High-Fidelity DNA Polymerase	NEW ENGLAND Biolabs Inc.	Cat#M0530S
QIAprep Spin Miniprep Kit	QIAGEN	Cat#27104
QIAquick Gel Extraction Kit	QIAGEN	Cat#28706
2× Taq PCR MasterMix	TIANGEN Biotech (Beijing)	Cat#KT201
Trans5α Chemically Competent Cell	TransGen Biotech	Cat#CD201-01
Experimental Models: Organisms/Strains		
Saccharomyces cerevisiae strain W303 (MATa leu2-3/112 ura3-1 trp161 his3-11/15 can1-100 GAL SUC2 mal0)	(Qu et al., 2019)	N/A
Saccharomyces cerevisiae strain QYJS002 (W303 WHI5-tdTomato::URA3)	(Qu et al., 2019)	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Saccharomyces cerevisiae strain 2 × WHI5 (W303 WHI5-tdTomato::URA3 WHI5pr-ORF-tdTomato::HIS3)	(Qu et al., 2019)	N/A
Saccharomyces cerevisiae strain 3 × WHI5 (W303 WHI5-tdTomato::URA3 WHI5pr-ORF-tdTomato::HIS3 WHI5pr-ORF-tdTomato::LEU2)	(Qu et al., 2019)	N/A
Saccharomyces cerevisiae strain 4 × WHI5 (W303 WHI5-tdTomato::URA3 WHI5pr-ORF-tdTomato::HIS3 WHI5pr-ORF-tdTomato::LEU2 WHI5pr-ORF-tdTomato::TRP1)	(Qu et al., 2019)	N/A
Recombinant DNA		
Plasmid pNH603 (Selectable marker, *Candida Glabrata* HIS3; Bacterial Resistance, Ampicillin)	A gift from Ping Wei's lab	(Mitchell et al., 2015)
Plasmid pNH604 (Selectable marker, *Candida Glabrata* TRP1; Bacterial Resistance, Ampicillin)	A gift from Ping Wei's lab	(Mitchell et al., 2015)
Plasmid pNH605 (Selectable marker, *Candida Glabrata* LEU2; Bacterial Resistance, Ampicillin)	A gift from Ping Wei's lab	(Mitchell et al., 2015)
Software and Algorithms		
ImageJ	https://imagej.net/Welcome	N/A
Other		
NanoDrop 2000/2000c Spectrophotometers	Thermo Fisher Scientific	Cat#ND-2000
Eppendorf Centrifuge 5418 R	eppendorf	Cat#5418707005
Eppendorf Centrifuge	eppendorf	Cat#5942000393
C1000 Touch Thermal Cycler	Bio-Rad Laboratories	Cat#1851148
Eclipse Ti-E microscope	Nikon	N/A
Magnetic stirrer	Jiangsu Jinyi Instrument Technology Company Limited	Cat#78-1
Precision™ General Purpose Baths	Thermo Fisher	Cat#TSGP02
Vortex	N/A	N/A
Glass Test tube (20 × 150 mm, 36 mL)	N/A	N/A
Spectrophotometer	N/A	N/A

MATERIALS AND EQUIPMENT

Reagents of Synthetic Complete (SC) Media for Yeast Transformation

Ingredient	Final Concentration
Glucose	20 g/L
Yeast nitrogen base without amino acids	6.7 g/L
L-arginine	0.01 g/L
L-methionine	0.01 g/L
L-tyrosine	0.15 g/L
L-isoleucine	0.015 g/L
L-Lysine	0.015 g/L

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Ingredient	Final Concentration
L-phenylalanine	0.025 g/L
L-glutamine	0.05 g/L
L-aspartic acid	0.05 g/L
L-valine	0.075 g/L
L-threonine	0.1 g/L
L-serine	0.2 g/L
Leucine	0.02 g/L
Histidine	0.004 g/L
Tryptophan	0.004 g/L
Uracil	0.004 g/L
Adenine	0.004 g/L
Agar	20 g/L

Reagents of YPAD Plate

Ingredient	Final Concentration
Yeast Extract	10 g/L
Peptone	20 g/L
Adenine	120 mg/L
Agar	20 g/L

STEP-BY-STEP METHOD DETAILS

Recombinant Plasmid Construction

Timing: 2–3 days

Construct the recombinant plasmid to add one copy of target gene into the yeast genome. Insert one copy of target gene (including its promoter), fluorescent protein ORF and target gene terminator into single-integrating plasmids pNH603 (HIS3), pNH604 (TRP1) and pNH605 (LEU2), respectively. These plasmids are specifically designed for single copy insertion, and were kind gifts from the Wei Laboratory (Mitchell et al., 2015). Use the auxotrophic marker as a selection marker, transform the yeast strain that bears the corresponding auxotrophic mutation with this recombinant plasmid, and insert the expression cassette of target gene tagged with the fluorescent protein into the genomic site of the auxotrophic mutation. But other appropriate plasmids that provide selection markers can be used as well.

1. Day 0: Prepare a 50 μL PCR (polymerase chain reaction) in a PCR tube to amplify each DNA insert from the corresponding template. PCR as depicted in Table 1. Mix the reaction and run following Table 2.
   a. Run PCR to amplify the target gene promoter together with its open reading frame (ORF) from the extracted yeast genome (fragment A, Figure 1i).
   b. Run PCR to amplify the target gene terminator from the yeast genome extracted before the you begin (fragment C, Figure 1ii).
   c. Run PCR to amplify the fluorescent protein ORF from the corresponding plasmid (fragment B, Figure 1iii).
2. Linearize the plasmid pNH603. Set up a microcentrifuge tube and add 50 μL of restriction enzyme digestion reaction as depicted in Table 3, mix and incubate for 3 h or for ~12 h at the incubation temperature using the corresponding restriction enzymes.

Table 1. Reagents of a 50 μL PCR

Reaction Component	Component Volume	Final Concentration
5× Phusion HF or GC Buffer	10 μL	1×
10 mM dNTPs	1 μL	200 μM
10 μM Forward Primer	2.5 μL	0.5 μM
10 μM Reverse Primer	2.5 μL	0.5 μM
DMSO (optional)	1.5 μL	3%
Template DNA	Variable	<250 ng
Phusion DNA Polymerase	0.5 μL	1.0 units/50 μL PCR
ddH2O (as needed)	to 50 μL	

Pause Point: The PCR products and the restriction enzyme digestion mixture after heat inactivation can be frozen and stored at −20°C for several days.

3. Load the PCR products and restriction enzyme digestion mixture in 1%–2% agarose gel. Run DNA electrophoresis.
4. Isolate the corresponding DNA (i.e., DNA inserts and the linearized plasmid vector)
   a. Cut out the gel band and put into a microcentrifuge tube.
   b. Use QIAquick PCR Purification Kit to purify the DNA inserts.
   c. Use QIAquick Gel Extraction Kit to purify the linearized cloning vector.
   d. Resuspend the DNA in elution buffer in a microcentrifuge tube.
   e. Use the NanoDrop Spectrophotometer to measure the concentration of the purified DNA.

Pause Point: The purified PCR products and the linearized vector can be frozen and stored at −20°C for several months.

⚠ CRITICAL: To verify the concentration of the corresponding DNA solution, elute the 50 μL PCR product and restriction enzyme reaction to a smaller volume (e.g., 30 μL). Then adjust the concentration to a final concentration 50–100 ng/μL for DNA inserts and 20–50 ng/μL for vectors for a high efficiency of the subsequent DNA ligation.

5. Ligate the 3 DNA inserts with the linearized cloning vector all at once using the Gibson assembly protocol.
   a. Set up a PCR tube and add a 10 μL of Gibson assembly reaction as depicted in Table 4. The appropriate final concentration for the DNA inserts and linearized vector should be adjusted according to their lengths as depicted in Table 4. Mix the reaction.
   b. Incubate the reaction mixture at 50°C for 15 min and then immediately place it on ice.
6. Transform competent *Escherichia coli* (*E.coli*) with the Gibson assembly reaction mixture.

Table 2. Conditions for an Amplification PCR

PCR Cycling Conditions			
Steps	**Temperature**	**Time**	**Cycles**
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	5–10 s	25–35 Cycles
Annealing	55–65°C	10–30 s	
Extension	72°C	15–30 s per kb	
Final Extension	72°C	5–10 min	1
Hold	4–10°C	forever	

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Table 3. Reagents of a 50 μL Restriction Enzyme Reaction

Reaction Component	Component Volume
Restriction Enzyme	10 units is sufficient, generally 1 μL is used
DNA	1 μg
10× Restriction reaction buffer	5 μL (1×)

a. Take the competent cells out of −80°C. Add 50 μL in each microcentrifuge tube for one transformation and thaw the cells on ice for 20–30 min.
b. Add 5 μL cloning reaction mixture to 50 μL competent cells and place the mixture on ice for 30 min.
c. Incubate the mixture at 42°C for 45 s and then immediately place it on ice for 2 min.

Alternatives: A recovery step can be performed to increase the efficiency of transformation. Add 900 μL SOC or LB media to the mixture and incubate at 37°C for 30–60 min. This step is important for the cells to build ampicillin expression before getting transferred to the selection plate.

d. Plate the mixture onto a 10 cm selection plate. Here we use LB agar plate containing the 100 μg/mL antibiotic ampicillin for the selection of colonies containing the recombinant plasmid selection.
e. Incubate the transform plate at 37°C for ~12 h.

Alternatives: If recover step is performed, the ~1 mL mixture should be plated on 2 different selection plates in 1:10 and 9:10 dilutions to prevent bacterial lawn.

Pause Point: The transform plate on which colonies grow out can be stored at 4°C for at least several days.

7. Day 1: Isolate the correct recombinant plasmid in which all the three DNA inserts are correctly inserted by performing colony PCR.
   a. Set up a PCR tube and add 20 μL fresh selection liquid medium (LB medium containing 100 μg/mL ampicillin) in each tube.
   b. Set up the colony solution for the colony PCR. Use a 10 μL autoclaved pipette tip to pick a colony from the selection plate and pipette the selection liquid medium in the PCR tube.
   c. Repeat for as many colonies as desired.
   d. Prepare a 20 μL PCR for each colony PCR as depicted in Table 5. Add 2 μL colony solution to each PCR. Mix the reaction.
   e. Run the colony PCR following Table 6.
   f. Load 5–10 μL of each PCR mix into in 1%–2% agarose gel and run electrophoresis to visualize the colony PCR products for each colony on the gel. Look for the DNA bands showing the expected size and distinguish the positive colonies.

Pause Point: The PCR mixture can be stored at −20°C for several days.

Table 4. Reagents of a 10 μL Gibson Assembly Reaction for DNA Ligation

Reaction Component	Component Volume/Amount
Purified PCR fragment	10–200 ng (<0.5 kb, 10–50 ng; 0.5–10 kb, 50–100 ng; >10 kb, 50–200 ng)
Linearized vector	50–200 ng (<10 kb, 50–100 ng; >10 kb, 50–200 ng)
Gibson Assembly Master Mix (2X)	2 μL
ddH_2O	to 10 μL

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Table 5. Reagents of a 20 μL Colony PCR

Reaction Component	Component Volume
Colony solution, genome solution or transformants picked from the agar plate	Variable
10 μM Forward Primer	1 μL
10 μM Reverse Primer	1 μL
2× Master Mix 12.5 μL (Taq DNA polymerase, dNTP, PCR buffer and agarose gel electrophoresis loading dye)	10 μL
ddH2O	up to 20 μL

8. Recombinant plasmid extraction.
   a. Pipette the rest colony solution of the positive colony and put it into 10 mL selection liquid medium in a test tube. Shake the culture in the incubator at 250 rpm at 37°C for ~12 h.
   b. Day 2: In the morning, use the QIAprep Spin Miniprep Kit to extract the plasmid from the culture. Elute the plasmid in a smaller volume of elution buffer (e.g., 50 μL) in a microcentrifuge tube from each 10 mL culture. Then adjust its final concentration to about 100 ng/μL. Use the NanoDrop Spectrophotometer to measure the concentration of the extracted plasmids.

Pause Point: The extracted plasmids can be stored at −20°C for several months.

9. Take some of the plasmid solution for sequencing and store the rest at −20°C.
10. Distinguish the correctly constructed recombinant plasmid according to the sequencing results. The recombinant plasmid should contain one copy of the target gene tagged with a fluorescent protein gene and an auxotrophic marker. This plasmid is to be used to integrate one copy gene tagged with a fluorescent protein gene into the corresponding auxotrophic marker locus in the yeast genome.

Note: As the junction points between DNA inserts are prone to mutations, primers that read through these regions should be designed for sequencing.

11. Repeat this construction procedure for constructing other two recombinant plasmids using other two plasmid backbones containing different auxotrophic markers. Obtain 3 recombinant plasmids with 3 different auxotrophic markers (e.g., His3, Leu2 and Trp1), namely, plasmid 1, plasmid 2 and plasmid 3. Performing the subsequent steps to sequentially transform these recombinant plasmids to the corresponding yeast strain can generate yeast strains with 2, 3 and 4 copies of the target gene.

Note: Using the Gibson assembly protocol to ligate multiple DNA fragments into one vector may induce mutations. Thus, sequencing the positive plasmid according to the colony PCR results is necessary for the subsequent yeast transformation.

Table 6. Conditions for a Colony PCR

PCR Cycling Conditions			
Steps	Temperature	Time	cycles
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	30 s	30 cycles
Annealing	55°C	0.5–2 min (variable according to the length of the amplified DNA fragment)	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	4–10°C	forever	

Table 7. Reagents of Transformation Mix for One Transformation

Reaction Component	Component Volume
50% (w/v) PEG (polyethylene glycol) MW 3350	100 μL
1 M Lithium acetate	15 μL
2 mg/mL carrier DNA (salmon sperm DNA)	20 μL
DMSO	18 μL

Yeast Transformation

Timing: 2–3 days

Transform the yeast strain with the recombinant plasmid (plasmid 1) to add one copy of the target gene into a 1-copy strain (background strain for transformation). The yeast transformation protocol used in this protocol is based on the previous one (Gietz and Woods, 2006). Here we have made 3 changes: 1) we use 100 mM Lithium acetate to wash the yeast culture instead of sterile water to increase the permeability of yeast cell (step 18), 2) we include a step of incubating the mixture at 30°C for 30 min to increase the exposure of cells to the transformation mixture (step 23), and 3) we reduce the time of heat-shock at 42°C from 20 min to 15 min to decrease the heat-shock damages to the yeast cell (step 23).

12. Day 3: Use an autoclaved 10 μL pipette tip to pick a single colony of the yeast strain which contains one copy of the target gene, pipette gently into 5 mL YPAD medium in a test tube and incubate at 30°C at 220 rpm for ~12 h.]
13. Day 4: In the morning, dilute the culture 1/20 into 10 mL YPAD medium in a flask. Incubate the culture at 30°C until it reaches at the mid-log phase ($OD_{600} \approx 0.6$) (The number of yeast cells of 1 OD is $\sim 2 \times 10^7$/mL). The time for incubating varies dependent on the doubling time of the strain. A 3 mL culture is used for each transformation.

Note: After diluting the culture, use the spectrophotometer to measure the OD_{600}. The OD_{600} should be 0.05–0.2.

14. Take salmon sperm DNA out of −20°C, thaw and dilute to 2 mg/mL. Boil salmon sperm DNA at 100°C for 5 min, immediately put it on ice, cool for at least 15 min before use.

Note: The salmon sperm DNA is boiled every time for yeast transformation.

15. Prepare the transformation mixture as depicted in Table 7. Vortex until the components are thoroughly mixed.

Note: PEG is a hygroscopic chemical and absorbs a lot of moisture. To make a 50% w/v concentration of the PEG solution, the volume of water should be increased slightly. We suggest place 10 g PEG3350 in a 100 mL glass beaker and add 12 mL ddH_2O to appropriately provide a 50% w/v concentration. Stir for 1–2 h with a magnetic stirring bar until dissolved. Sterilize the PEG solution by filtration and store at ~25°C.

16. Linearize the recombinant plasmid in the restriction enzyme reaction as depicted in Table 3.

Note: The cut site of the recombinant plasmid should be chosen appropriately to make sure that the gene copy can be integrated at the specific marker locus in the yeast genome. Take one recombinant plasmid that we constructed to integrate WHI5 copy gene for example. The

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integrating plasmid should have 5′ UTR of HIS3, *Candida Glabrata* HIS3 (including promoter and terminator), WHI5 gene, tdTomato ORF, WHI5 terminator and 3′ UTR of HIS3 after linearization. So that the WHI5 gene copy can be inserted into the HIS3 locus in the yeast genome locus by homologous recombination.

17. Set up a 15 mL centrifuge tube and add 3 mL cell culture. Pellet cells by centrifugation at 2,500 × *g* at ~25°C for 5 min. Use the pipette to remove the supernatant.
18. Use 300 μL 100 mM Lithium acetate (sterilized by filtration) to wash cell pellet from 3 mL cell culture. Gently pipette the cells using Lithium acetate and centrifuge at 2,500 × *g* at ~25°C for 5 min. Use the pipette to remove the supernatant. Repeat this step once.

Note: Use a 200 μL pipette tip to remove all the supernatant. Leftover of the supernatant can reduce the efficiency of yeast transformation.

19. Resuspend cells in 30 μL 100 mM Lithium acetate. Gently pipette the cells.
20. Set up a microcentrifuge tube and add the cell solution.
21. Add 153 μL transformation mixture into each microcentrifuge tube. Mix by gently pipetting.
22. Add ~400 μg digested plasmid into each microcentrifuge tube and mix by vortexing briefly.
23. Incubate the mixture at 30°C for 30min and then at 42°C for 15min.
24. Centrifuge the mixture at 2,500 × *g* for 1min. Use a 200 μL pipette tip to discard the supernatant. Resuspend cells into 100 μL YPAD medium by gently pipetting.
25. Incubate the resuspended cells at 30°C for 3–4 h to increase the transformation efficiency.
26. Use an autoclaved glass spreader to plate the cells on the selective plate. Incubate the plate at 30°C for 2–3 days. See Materials and Equipment for recipe for the selective plate.

⚠ CRITICAL: Be sure to make selection plates suitable for the plasmid being transformed (e.g., transformation with the plasmid constructed based on pNH603, omit Histidine in the medium when making the selection plate.)

Pause Point: The transform plate on which colonies grow out can be stored at 4°C for at least several days.

Note: Make liquid stocks of the corresponding ingredients needed for making synthetic complete (SC) media and sterilize them by filtration. Dissolve 20 g agar in ddH_2O, autoclave, then add the stocks of rest ingredients, and fully mixed, cool to 60°C, then pour the plates. Omit the amino acid for the transformation of plasmid that bears the corresponding auxotrophic marker. Recipe for SC media is included in Materials and Equipment.

27. Day 5–6: Verify the transformation of the recombinant plasmid into the yeast genome by performing PCR.
    a. Set up a PCR tube that contains a 20 μL colony PCR in each tube as depicted in Table 5.
    b. Use a 10 μL autoclaved pipette tip to gently pick trace amount of colony from the selection plate and briefly swirl it in the PCR tube.
    c. Repeat for as many colonies as desired. Store the transform plate at 4°C.
    d. Run the colony PCR following Table 6.
    e. Load 5–10 μL of each colony PCR products into 1%–2% agarose gel and run DNA electrophoresis to visualize the colony PCR products for each colony on the gel. Check correct insertion by looking for the DNA bands showing the expected size and distinguish the positive colonies.

Note: To amplify DNA fragments >1,000 bp, one should extract the genome of the corresponding colony to prevent the failure of PCR. The genome extraction step as depicted in the "Genome Extraction" section.

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Alternatives: The plasmid integration locus can be verified by DNA sequencing instead of using PCR.

Pause Point: The PCR products can be stored at −20°C for several days.

Measurement of Fluorescent Intensity Level

Timing: 1–2 days

Use the fluorescent microscope and ImageJ to measure the fluorescent intensity level of each positive colony. Use the fluorescent intensity level as a quantitative readout of the expression level of the target gene in each strain.

28. Inoculate the positive colonies from the transformation plate (stored at 4°C). Inoculate the control strain which contains 1 copy of the target gene fused with the same fluorescent protein from the plate stored at 4°C. Each single colony is inoculated from the corresponding agar plate with a pipette tip into a test tube containing 4 mL selective liquid medium and incubate at 30°C at 220 rpm for ~12 h.

Alternatives: If the strain is stored on glycerol at −80°C or −70°C, scrape off a small amount of frozen glycerol stock with a sterile inoculator or pipette tip and patch onto a YPAD plate. Keep the stock frozen. Incubate at 30°C for 2–3 days. When single colonies are visible, streak out on YPAD plate and incubate at 30°C for 1–2 days. Inoculate a single colony from this plate. This plate can be stored at 4°C for future use.

29. Day 7–8: In the morning, dilute the culture 1/20 into 10 mL YPAD medium in a flask. Incubate the culture at 30°C until it reaches at the mid-log phase (OD_{600} ≈ 0.6). The time for incubating varies dependent on the doubling time of the strain.
30. Acquire images of the fluorescent cells by the fluorescence microscope.
    a. Drop about 10 μL cell culture on a glass slide and apply a cover slip.
    b. Acquire images of the fluorescent cells using a Nikon Eclipse TiE microscope with an Apo 100×/1.49 oil TIRF objective. Exposure times of 50–100 ms are typically used in the fluorescent channel. A 540–580 nm excitation and a 600–660 nm barrier filter is typically used to detect tdTomato signals. A 465–495 nm excitation and a 515–555 nm barrier filter is typically used to detect GFP signals.
31. Use ImageJ to quantify the fluorescent level of fluorescent cells.
    a. Select the cell and measure its area and total fluorescent intensity.
    b. Select 3 regions in the fluorescent image that has no fluorescence. These regions are the background. Measure the mean fluorescent intensity of the 3 regions and calculate the mean value (V_m). V_m is used to correct the total fluorescent intensity for each cell on the same fluorescent image.
    c. Correct the total fluorescent intensity for each cell by this formula: corrected total fluorescent intensity = total fluorescent intensity – (area of selected cell × V_m).
32. Quantify multiple cells of each strain. Calculate the averaged value of the mean fluorescent intensity of each strain (V_a). V_a is the quantitative readout of the fluorescent intensity level of the target gene in the corresponding strains.
33. Distinguish transformants which shows a V_a ~1 fold higher than the V_a of the control strain. Strain contains 2 copies of the target gene is obtained.
34. Add the third copy of the target gene to the 2-copy strain to generate 3-copy strain.
    a. Repeat steps 12–27 to transform the 2-copy strain with another recombinant plasmid (plasmid 2) to generate the 3-copy strain.

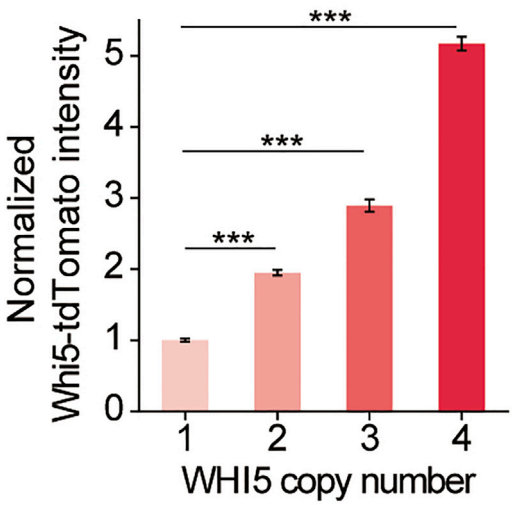

Figure 4. The Fluorescent Intensity of Strains Containing the Indicated WHI5 Gene Copy Number

Whi5-tdTomato intensities were normalized by the mean Whi5-tdTomato intensity of 1× WHI5 strain. Mean ± SEM. ***p < 0.001.

Note: Perform PCR to check after each transformation if the previous copy that has been integrated before is still in the corresponding marker locus in the yeast genome.

b. Repeat steps 28–33 to measure the fluorescent intensity of the positive transformants. Distinguish the transformant which shows a higher V_a compared to the V_a of the 2-copy strain. The strain that contains 3 copies of the target gene is obtained.

35. Add the fourth copy of the target gene to the 3-copy strain to generate 4-copy strain.
    a. Repeat steps 12–27 to transform the 3-copy strain with plasmid 3 to generate the 4-copy strain. Use the recombinant plasmid that contains a different auxotrophic marker to perform the yeast transformation.
    b. Repeat steps 28–33 to measure the fluorescent intensity of the positive transformants. Distinguish the transformant which shows a V_a ~1 fold higher than the V_a of the 3-copy strain. The strain that contains 4 copies of the target gene is obtained. Then 3 different strains that contain 2, 3 and 4 copies of the target gene are constructed.

EXPECTED OUTCOMES

Using this step-by-step protocol, we constructed a series of recombinant plasmids that contain one copy of WHI5 gene together with its promoter and terminator (Qu et al., 2019). We then transformed yeast with these plasmids sequentially and thus got a series of yeast strains that contain 2,3 and 4 copy numbers of WHI5 gene. In our hands, this step-by-step protocol routinely achieve high efficiency of constructing strains containing multi-copies of the target gene. The Gibson assembly protocol of inserting multi DNA fragments into the plasmid vector highly reduces the time of construction. By inserting one WHI5 gene copy into three kinds of plasmid vectors and then transform the yeast sequentially, we have constructed strains containing 4 WHI5 copy numbers. As the target gene is tagged with fluorescent protein, we can not only manipulate the target gene expression but also observe its expression level quantitatively using microscope or FACS. The fluorescence intensity which represents the gene expression level significantly increases with the WHI5 gene copy number across the strains shown as Figure 4 and Table 8. Thus, this protocol provides a method for accurately manipulating the gene expression level.

LIMITATIONS

Generally, our protocol is applicable to overexpress genes whose known functional form is a translated protein. Although overexpression is a commonly used method in studying the function of a

Table 8. Statistics of Fluorescent Intensities

Strain	Number	Mean	Standard Deviation	T Value	P Value
1×WHI5	135	140.7	37.6		
7WHI5	169	267.2	71.8	−18.5	4.5E−52
3×WHI5	12	390.7	138.2	−20.2	9.8E−56
4×WHI5	259	722.1	225.9	−29.7	1.4E−102

protein, it may have limitations in some situations. Overexpression of certain genes may lead to deleterious effects to the cell and compromise cell viability. This may limit the maximum expression level of the target gene that can be introduced into the cell. For instance, Whi5 is an inhibitor of G1/S transition in budding yeast, therefore excessive overexpression of Whi5 protein could significantly inhibit cell proliferation. To prevent this situation, these gene copies could be introduced under the control of an inducible promoter. So that the expression of the target gene can be switched off manually if needed.

Technically, the copy number of the target gene that can be inserted into the yeast genome is limited by the number of selection marker that can be used to transform the yeast strain. To introduce more gene copies into the yeast genome, one can construct a recombinant plasmid containing more than one copy of target gene. To use the fluorescent protein to quantify the expression level of the target gene, each copy should be tagged with a fluorescent protein gene. In this situation, Gibson assembly is also useful for inserting multiple DNA fragments into the plasmid vector. In this situation, the packaging capacity of plasmid backbone limits the copy number of the target gene that can be inserted into the yeast genome. Another possible solution is the CRISPR-Cas9 approach, which can allow marker-free integration of DNA fragment in yeast (Ryan et al., 2016).

Tagging the gene copy with a fluorescent protein gene provides a way to quantify the expression level of the target gene. It would not be necessary if the user only would like to insert multiple gene copies into the yeast genome. However, due to the complex process of gene transcription and translation process, the protein product of the target gene may not have a proportional correlation to its gene copy number. Therefore, methods such as tagging a fluorescent protein may be needed if the user would like to detect the expression level of the target gene. In practice, there are some limitations for tagging a fluorescent protein to the target gene in some situations. For instance, it may interfere with the folding of the target gene and thus affect its function. It may change the turnover of the mRNA of the target gene and thus affect its expression level. In some situations, C-terminal tagging may affect the function of the target protein. Then a potential solution could be tagging the fluorescent protein to the N-terminal of the target gene. In this situation, Gibson assembly used in our protocol could also be useful for inserting multiple DNA fragments into the plasmid vector.

TROUBLESHOOTING

Problem

Few or no colonies after *E.coli* transformants.

Potential Solution

Few or no transformants may be due to the invariable competent cells or inefficiency of ligation. First, transform 100 pg–1 ng uncut vectors to check competent cell viability. Competent cells with high efficiency of transformation should be used. Second, for Gibson assembly protocol, low concentration of the DNA insertion (~10 ng/μL) can decrease the ligation efficiency. To increase the concentration of DNA inserts, one can use a decreased volume of elution

buffer to elute the PCR products or use one volume of buffer to elute two volumes of PCR products.

Problem

Smears instead of separated colonies grow on the selective plate after *E.coli* transformation. Normally, separated colonies will grow on the plate. Here, smears mean that instead of separated colonies, the *E.coli* spreads thinly on the surface of the agar plate and grows to a "lawn" on the plate surface.

Potential Solution

Smears may be due to low concentration of antibiotic or inefficiency of vector linearization. First, confirm the antibiotic and increase its concentration. Take ampicillin for example, its final concentration in the selection plate can be increased to 200 μg/mL. Second, more efficient the vector linearization is, better will be the transformation. Non-linearized vectors may lead to many false positive colonies. Therefore, transform the same amount of linearized vector to check its efficiency of linearization. If smears still appear on the selection plate, this means the linearization of vector is not efficient. Moreover, efficiency of linearization can also be checked by agaroses gel electrophoresis of the digested and undigested products. Third, confirm the restriction enzyme digestion reaction.

Problem

Few or no colonies after yeast transformation. Few or no colonies may be due to low efficacy of yeast transformation.

Potential Solution

First, confirm the selection plate. Second, make new PEG solution which is important for yeast transformation. Third, incubating the yeast cells for 2–3 h before plating on the selection plate (step 25). Fourth, increase the volume of cell culture for each transformation from 3 mL to 6 mL.

RESOURCE AVAILABILITY

Lead Contact

Further information requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Chao Tang (tangc@pku.edu.cn).

Materials Availability

This study did not generate any new materials.

Data and Code Availability

This study did not generate a dataset or code.

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AUTHOR CONTRIBUTIONS

C.T., Y.Q., and J.J. conceived the study. Y.Q. and J.J. designed and performed the experiments. C.T. and X.Y. supervised the whole project; Y.Q., J.J., X.Y., and C.T. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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