Protocol

A.Polymerase Chain Reaction

Q5® Hot Start High-Fidelity 2X Master Mix is used in polymerase chain reaction.

1.Defrost template, 2x Master Mix(Q5 Hot Start High-Fidelity 2X Master Mix) and primers

2.Determine desired total working volume (~25uL)

3.Label reaction tubes

4.Create below reaction mix

COMPONENT	25 µl REACTION	50 µl REACTION	FINAL CONCENTRATION
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	25 µl	1X
10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM
Template DNA	Genomic:1 ng – 1 µg Plasmid:1 pg – 1 ng	Genomic:1 ng – 1 µg Plasmid:1 pg – 1 ng	<1,000 ng
Nuclease-Free Water	to 25 µl	to 50 µl	

5.Place mixture in thermal cycler at settings shown below

STEP	TEMPERATURE	TIME
Initial Denaturation	98° C	30 seconds
25 – 35 Cycles	98° C	10 seconds
	50 – 72° C	30 seconds
	72° C	20 – 30 seconds/kb
Final Extension	72° C	2 minutes
Hold	4 – 10° C	

B. PCR Cleanup

E.Z.N.A.® Cycle Pure Kit was used to purify the thermal cycler product and remove remaining polymerases and nucleotides.

1. Transfer the PCR product into a clean 1.5 mL microcentrifuge tube.
2. Add 4-5 volumes CP Buffer.
3. Vortex to mix thoroughly. Briefly centrifuge to collect any drops from the inside of the lid.
4. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube.
5. Add the sample to the HiBind® DNA Mini Column.
6. Centrifuge at maximum speed (≥13,000 x g) for 1 minute at room temperature.
7. Discard the filtrate and reuse collection tube.
8. Add 700 µL DNA Wash Buffer.
9. Centrifuge at maximum speed for 1 minute.
10. Discard the filtrate and reuse collection tube.
11. Repeat Steps 10-12 for a second DNA Wash Buffer wash step.
12. Centrifuge the empty HiBind® DNA Mini Column at maximum speed for 2 minutes to dry the column.
13. Transfer the HiBind® DNA Mini Column into a clean 1.5 mL microcentrifuge tube.
14. Add 30-50 µL Elution Buffer or sterile deionized water directly to the center of column matrix.
15. Let sit at room temperature for 2 minutes.

16. Centrifuge at maximum speed for 1 minute.
17. Get the DNA sample and store it at -20° C.

C. DNA Restriction Digest

1. Gather DNA sample, digestion buffer, and restriction enzymes from cold storage.
2. Label tubes.
3. Create the below reaction mixture. (Assuming these two digestion can function in the same buffer)

Component	Volume for 25uL reaction
DNA Sample	15uL
10x FastDigest Digestion Buffer	2.5uL
Restriction Enzyme 1	1uL
Restriction Enzyme 2	1uL
H2O	5.5uL

4.Place mixture in 37° C heat block for 10 minutes.

D. Gel Extraction

Zymoclean™ Gel DNA Recovery Kit is used to extract DNA fragment from agarose gel.
1. Excise the DNA fragment from the agarose gel using a razor blade, scalpel or other device and transfer it into a 1.5 ml microcentrifuge tube.
2. Add 3 volumes of ADB to each volume of agarose excised from the gel.
3. Incubate at 37-55 C for 5-10 minutes until the gel slice is completely dissolved.
4. Transfer the melted agarose solution to a Zymo-Spin™ Column in a Collection Tube.
5. Centrifuge for 30-60 seconds. Discard the flow-through.
6. Add 200 µl of DNA Wash Buffer to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat the wash step.
7. Add ⩾ 6 µl DNA Elution Buffer 4 or water directly to the column matrix. Place column into a 1.5 ml tube and centrifuge for 30-60 seconds to elute DNA.

E.Ligation

1. Retrieve digested samples(insert and vector DNA), ligase, and ligation buffer from cold storage.
2. Determine a desired ratio of insert:vector molecules solution volume in reaction (3:1 to 9:1).
3. Set up the ligation mixture, including vector, insert, ligase, ligase buffer, H2O.
4. Create a negative control with vector DNA but no insert DNA.
5. Mix via flicking the tube. Incubate at room temperature for 2 hours.

F.Transformation

1. Retrieve ligation sample, and enough plates for all samples and a control.
2. Warm up plates in 37°C.
3. Retrieve competent cells from freezer and store in ice.
4. Mix total ligation mixture with 50uL of cells in a tube. Mix via pipetting.
5. Incubate on ice for 30 minutes.
6. Place tubes in 42°C heat block for 45 seconds to heat shock.

7. Put them back on ice for 5 minute.
8. Add 500 µl LB media to tubes.
9. Tape tubes to 37°C shaking incubator for 45 minutes to recover.
10. Remove from incubator and pellet in centrifuge. Discard the supernatant.
11. Add 100uL of LB broth with antibiotic that matches selected plates.
12. Pipette total mixture onto plates with antibiotic. Spread with glass beads.
13. Incubate overnight at 37°C.

G. Miniprep

We used QIAprep ® Spin Miniprep Kit to miniprep our plasmid.

1. Pellet 1-5 ml bacterial overnight culture by centrifugation at >8000 rpm(6800 x g) for 3 min at room temperature (15-25° C).
2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. If using LyseBlue reagent, the solution will turn colorless.
5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.
6. Apply the supernatant from step 5 to the QIAprep spin column. Centrifuge for 30-60 s and discard the flow-through.
7. Wash the QIAprep spin column by adding 0.5 ml Buffer PB. Centrifuge for 30-60 s and discard the flow-through.
8. Wash the QIAprep spin column by adding 0.75 ml Buffer PE. Centrifuge for 30-60 s and discard the flow-through. Transfer the QIAprep spin column to the collection tube.
9. Centrifuge for 1 min to remove residual wash buffer.
10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB or water to the center of the QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

H. Inverse PCR individual inserts

1. Isolate genomic DNA: we use PureLink Genomic DNA Mini Kit from invitrogen. In this procedure, we followed manufacturer's protocol.
2. Digest 150 ng of genomic DNA with the DpnII enzyme in 25 ul volume for 3 hours. The mixture of DpnII digestion is shown below

Component	1x
DNA sample (150 ng - add water to 10ul)	10 ul
Restriction buffer DpnII (10x)	2.5 ul
Restriction enzyme (DpnII 10U/ul)	1.0 ul
H_20	11.5 ul
Total	25 ul

Reaction conditions: Digest at 37ºC for three hours to overnight.
Heat inactivate the enzyme after restriction diges at 80ºC for 20 min.
3. Ligate the digested DNA for 2 hours at room temperature

The ligation mixture is shown below

Component	1x
Digested DNA from step 2	2.5 ul
10x ligation buffer	2.5 ul (Enzymatics ligase buffer)
T4 ligase	1.0 ul (Enzymatics ligase)
H_20	19.0 ul
Total	25 ul

4. First round of inverse PCR

Set up a **10** ul PCR reaction with the following components:

Component	1x
Ligation mix from step 3	2.0 ul
Primer oCF1587 (10 uM)	1.0 ul
Primer oCF1588 (10 uM)	1.0 ul
dNTPs (10 mM)	0.2 ul
Phusion 5x GC buffer	2.0 ul
NEB Phusion Polymerase	0.1 ul
H_20	3.7 ul
Total	10 ul

oCF1587 ATAGTTTGGCGCGAATTGAG

oCF1588 GGTGGTTCGACAGTCAAGGT

PCR settings:

Initial denaturation: 2 minutes @ 98C

PCR cycles: 30x

Annealing temperature: **64ºC**

Elongation time: 1 min

5. Second round of inverse PCR

Dilute the first round of PCR product 100 fold.

Set up a 25 ul PCR reaction with the following components:

Component	1x
Diluted PCR from step 4	1.0 ul
Primer oCF1589 (10uM)	2.5 ul
Primer oCF1590 (10uM)	2.5 ul
dNTPs (10 mM)	0.5 ul
Phusion 5x GC buffer	5.0 ul
NEB phusion polymerase	0.2 ul
H_20	13.0 ul
Total	24.7 ul

oCF1589 AGAGCAAACGCGGACAGTAT

oCF1590 CGATAAATATTTACGTTTGCGAGAC

PCR settings:

Initial denaturation: 2 minutes @ 98C

PCR cycles: 30x

Annealing temperature: **64ºC**

Elongation time: 1 min

6. Run the PCR products on a 1% agarose gel, excise clear bands from gel and gel purify.

7. Determine insertion site on wormbase.

8. If there is no clear bands, redo the PCR reactions with oligos that anneal at the other end of the transposon. Start with the ligated DNA from step3.

3' end Primer

oCF1591 AAAAATGGCTCGATGAATGG

oCF1592 TAAGAATCGAAGCGCTGCTC

oCF1593 AGCTAGCGACGGCAAATACT

oCF1594 CATCGAAGCGAATAGGTGGT