Rolling circle amplification-mediated hairpin (hp)-RNA libraries

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Abstract

A sequence-independent method to turn genomic DNA into libraries of hairpins.

An efficient way to get a different adapter on each end of a DNA fragment is to clone it and digest the fragment back out. Design the vector to have unique restriction sites outside the unique regions you want to put on each end. After digestion, your DNA fragments will have two different "adapters" on each end.

Adapted from:

Wang L., Zheng J., Luo Y., Xu T., Zhang Q., Zhang L., Xu M., Wan J., Wang MB., Zhang C., Fan Y. 2013. Construction of a genomewide RNAi mutant library in rice. *Plant Biotechnology Journal* 11:997–1005. DOI: 10.1111/pbi.12093.

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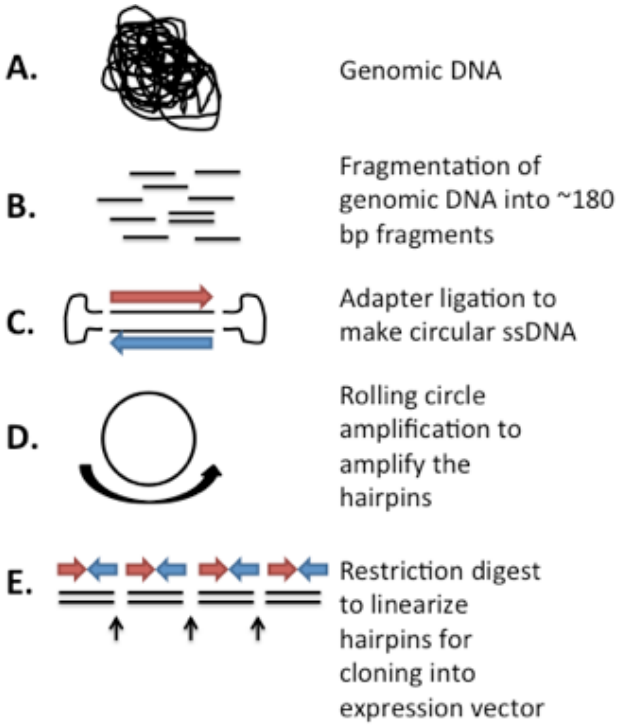
Before start

Overview of the procedure:

1. Preparation of DNA fragments (in this example we'll use a fragment of YFP DNA, but this could be random sheared, end repaired DNA, too) (A-tail and kinase to add phosphate).
2. Prepare vector to clone DNA fragment (T-tail)
3. Ligate vector and insert (T-A cloning)
4. Restriction digest to get fragments with unique ends
5. 'Bell' adapter ligation to make the fragment circular. Unique ends on the DNA fragment allow different adapters to ligate to each side.
6. Rolling circle amplification
7. Restriction digestion to get single hairpin fragments (inverted repeats of the initial sequence). This can then be cloned and used in whatever downstream application is needed (e.g. RNAi, etc.)



Protocol

Generation of DNA fragments

Step 1.

GOAL: Make DNA fragments, A-tail and kinase.

In genomic library protocol, genomic DNA could be sheared with covaris, and end repaired.

In this example of the protocol, we will use a fragment of the YFP gene that could be used to knock down YFP in a test case. Use primers;

RMHR-YFP-1: 5'-gtgaaccgcatcgagctgaa

RMHR-YFP-3: 5'-tagtggtcggcgagctgcac

Reagent	Amount
NEB OneTaq 2x master mix with standard PCR buffer (OneTaq adds the desired A-tail)	25 µL
Water	22 µL
10 µM each primer mixed	2 µL
YFP template	1 µL

Cycle: 98 C for 2 min, 30 rounds: (98 C for 10 s, 60 C for 20 s, 72 C for 30 s).

Treat with DpnI (add 1 ul enzyme, NEB, and incubate reaction at 37 C for 1-2 hr)

Cleanup with Qiagen PCR cleanup column.

Kinase reaction:

Reagent	Amount
Purified DNA from above	39 µL
T4 PNK buffer	5 µL
100 mM ATP	5 µL
T4 PNK	1 µL

Incubate 30 min at 37 C. Inactivate at 65 C for 5 min.

Cleanup with Qiagen PCR cleanup column.

Prepare vector

Step 2.

GOAL: T-tail the vector for efficient T-A cloning.

Amplified pACYC184 with primers below using PCR cycle in Step 1. These primers will add a TypeIIS restriction site, BsaI. Redigest with BsaI in a later step will leave unique overhangs on each end that will be compatible with the bell adapters for rolling circle amplification.

Primers:

BsaI-ACYC-F: 5'- CaggtTGAGACCTTAACGACCCTGCCCTGAAC

BsaI-ACYC-R: 5'- CgggaTGAGACCTAGCCGCTTATGTCTATTGCTG

T-Tail reaction:

Reagent	Amount

DNA (purified PCR product)	30 µL
NEB buffer 2	5 µL
dTTP (100 mM)	0.5 µL
Klenow fragment (NEB)	3 µL
water	11.5 µL

37 C for 30 min. Purify with Qiagen PCR cleanup column.

T-A ligation of vector and insert

Step 3.

LIgate approximately 50-100 fmoles insert and vector.

Reagent	Amount
A-tailed Insert (37 fmol/µL)	3 µL
T-tailed Vector (14 fmol/µL)	5 µL
T4 ligase buffer	1 µL
T4 ligase	1 µL

Incubate overnight at 16 C.

Cleanup with SPRI beads (Ampure, Beckman Coulter).

Follow manufacturer's instructions, add 40 µL beads to 50 µL DNA (dilute ligation).

Elute in 28 µL Qiagen Elution Buffer (EB, 10 mM TRIS, pH 8).

Digest out fragments with unique ends

Step 4.

GOAL: Digest ligated fragments with BsaI to regenerate linear ends that each have a different overhang.

Reagent	Amount
DNA	26 µL
NEB cutsmart buffer	3 µL
BsaI enzyme (NEB)	1 µL

incubate 2 hr at 37 C.

Cleanup with SPRI beads.

30 µL DNA + 15 µL SPRI beads (Ampure, Beckman Coulter).

Remove supernatant and transfer to new tube.

Add 45 µL more SPRI beads

bind and wash according to manufacturer's protocol.

Elute in 10 ul QIagen EB buffer.

Ligate Adapters

Step 5.

GOAL: to ligate 'bell' adapters to make dsDNA fragment into a closed ssDNA circle (see step C in before start instructions).

Primers to make 'bell' adapters (i.e. they form a hairpin with an overhang compatible with the DNA fragment ends of generated in step 4.

Minihairpin-1

5′- GGGAGCGATCTGCAAGGATCCATTTCCTCTTTAGGTGAGCTCCGATCCTTGCAGATCGC

Minihairpin-2

5′-
GGTGTGGCCAAGTAGGCCATGGTACGTTTACATGTTTTTTTTCCCTTCATATCGATTTTTTTTTGGCGCAGCATG
GCCTACTTGGCCAC

Dilute each to 1 µM.

Reagent	Amount
DNA fragment (digested and purified from step 4)	6 µL
Minihairpin-1	1 µL
Minihairpin-2	1 µL
10X T4 Ligase buffer	1 µL
T4 ligase	1 µL

Incubate overnight at 16 C.

Precipitate ligation:

add 1 µL glyco blue.

Add 10 µL 3M sodium acetate pH 7.

Add 250 µL ethanol.

After mixing, spin 10 min at 4 C. Wash 1x in 70% ethanol and resuspend in 5 µL RCA sample buffer (IllustraPhi, GE).

Rolling circle amplification

Step 6.

GOAL: to amplify the hairpin as a concatamer of inverted repeats using rolling circle.

Use the GE TempliPhi kit for rolling circle amplification.

To DNA resuspended in 5 µL sample buffer (step 5), denature at 95 C, 3 min.

Add 5 µL reaction buffer to 0.2 µL enzyme and add to denatured DNA.

Incubate overnight (18 h) at 30 C.

Heat inactivate 10 min at 65 C.

Digest to unit length of hairpin

Step 7.

GOAL: digest concatamer to unit length (i.e. single inverted repeat hairpin). The BamHI restriction site is found in one of the minihairpin bell adapters.

Digest 5 ul of the RCA reaction with BamHI. Reserve the remaining 5 ul as control.

RESULTS: see gel photo below. 500 bp band after BamHI digest that is not found in control, undigested reaction.