Carrington Lab Protocols
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Protocol to clone amiRNAs or syn-tasiRNAs in *Bsa*I/*ccd*B-based ('B/c') vectors

1. Oligonucleotide annealing

-Dilute sense oligonucleotide and antisense oligonucleotide in sterile H_2O to a final concentration of 100 μM.

-Prepare Oligo Annealing Buffer:

60 mM Tris-HCl (pH 7.5)

500 mM NaCl

60 mM $MgCl_2$

10 mM DTT

Note: *Prepare 1 ml aliquots of Oligo Annealing Buffer and store at -20^{0}C.*

-Assemble the annealing reaction in a PCR tube as described below:

Forward oligonucleotide (100 μM)	2 μL
Reverse oligonucleotide (100 μM)	2 μL
Oligo Annealing Buffer	46 μL
Total volume	50 μL

The final concentration of each oligonucleotide is 4 μM.

-Use a thermocycler to heat the annealing reaction 5 min at 94°C and then cool down (0.05ºC/sec) to 20°C.

-Dilute the annealed oligonucleotides just prior to assembling the digestion-ligation reaction as described below:

Annealed oligonucleotides	3 μL
dH_2O	37 μL
Total volume	40 μL

The final concentration of each oligonucleotide is 0.15 μM.

Note*: Do not store the diluted oligonucleotides.*

2. Digestion-ligation reaction

-Assemble the digestion-ligation reaction as described below:

B/c vector (x ug/uL)	Y µL (50 ng)
Diluted annealed oligonucleotides	1 µL
10x T4 DNA ligase buffer	1 µL
T4 DNA ligase (400 U/µL)	1 µL
*Bsa*I (10U/ µL, NEB)	1 µL
dH_2O	to 10 µL
Total volume	10 µL

Note: *Prepare a negative control reaction lacking BsaI.*

-Mix the reactions by pipetting. Incubate the reactions for 5 minutes at 37°C.

3. *E.coli* transformation and analysis of transformants

-Transform 1-5 ul of the digestion-ligation reaction into an *E. coli* strain that doesn't have *ccd*B resistance (e.g. DH10B, TOP10, …) to do counter-selection.

-Pick two colonies/construct, grow LB-Kan (100 mg/ml) cultures and purify plasmids.

-Sequence 2 clones per construct with appropriate primers: M13-F (CCCAGTCACGACGTTGTAAAACGACGG) and M13-R (CAGAGCTGCCAGGAAACAGCTATGACC) for *pENTR*-based vectors; attB1 (ACAAGTTTGTACAAAAAAGCAGGCT) and attB2 (ACCACTTTGTACAAGAAAGCTGGGT) primers for *pMDC32B*-, *pMDC123SB*-, *pFK210B*- or *pH7WG2B*-based vectors).

Eudicot amiRNA vectors: *Bsa*I/*ccd*B-based ('B/c') vectors for direct cloning of amiRNAs to use in eudicot species.
CaMV, *Cauliflower mosaic virus*; nos, nopaline synthase; *rbcS,* Rubisco small subunit.

Vector	Bacterial antibiotic resistance	Plant antibiotic resistance	GATEWAY use	Promoter	Terminator	Plant species tested	Addgene number
pENTR-AtMIR390a-B/c	Kanamycin	-	Donor	-	-	-	51778
pFK210B-AtMIR390a-B/c	Spectinomycin	BASTA	-	*CaMV 35S*	*rbcS*	*A.thaliana*	51777
pMDC123SB-AtMIR390a-B/c	Kanamycin	BASTA	-	*CaMV* 2x35S	*nos*	*A. thaliana* *N. benthamiana*	51775
pMDC32B-AtMIR390a-B/c	Kanamycin Hygromycin	Hygromycin	-	*CaMV* 2x35S	*nos*	*A. thaliana* *N. benthamiana*	51776

Monocot amiRNA vectors: *OsMIR390-Bsa*I/*ccd*B ('B/c') vectors for direct cloning of amiRNAs to use in monocot species.
CaMV, *Cauliflower mosaic virus*; nos, nopaline synthase; *Os*, *Oryza sativa*.

Vector	Bacterial antibiotic resistance	Plant antibiotic resistance	GATEWAY use	Promoter	Terminator	Plant species tested	Addgene number*
pENTR-OsMIR390-B/c	Kanamycin	-	Donor	-	-	-	-
pMDC123SB-OsMIR390-B/c	Kanamycin	BASTA	-	*CaMV* 2x35S	*nos*	*N. benthamiana*	-
pMDC32B-OsMIR390-B/c	Kanamycin Hygromycin	Hygromycin	-	*CaMV* 2x35S	*nos*	*N. benthamiana* *B. distachyon*	- -
pH7WG2-OsMIR390-B/c	Spectinomycin	Hygromycin	-	*Os Ubiquitin*	*CaMV*	*B. distachyon*	-

*These vectors are not yet available at Addgene. Please, contact us at administrator@carringtonlab.org to request any of them.

syn-tasiRNA vectors: *Bsa*I/*ccd*B-based ('B/c') vectors for direct cloning of syn-tasiRNAs to use in *Arabidopsis thaliana* and closely related species*.
CaMV, *Cauliflower mosaic virus*; nos, nopaline synthase.

Vector	Bacterial antibiotic resistance	Plant antibiotic resistance	GATEWAY use	Promoter	Terminator	Plant species tested	Addgene number
pENTR-AtTAS1c-B/c	Kanamycin	-	Donor	-	-	-	51774
pMDC123SB-AtTAS1c-B/c	Kanamycin	BASTA	-	*CaMV* 2x35S	*nos*	*A. thaliana* *N. benthamiana**	51772
pMDC32B-AtTAS1c-B/c	Kanamycin Hygromycin	Hygromycin	-	*CaMV* 2x35S	*nos*	*A. thaliana* *N. benthamiana**	51773

*As miR173 is not conserved In *N. benthamiana* and other species not closely related to *Arabidopsis thaliana*, a construct expressing miR173 has to be co-expressed with the syn-tasiRNA construct to trigger syn-tasiRNA biogenesis.