ART-sRNA CLONING IN B/c VECTORS

Updated on 12/01/2021 by Alberto Carbonell.

Notes:

-Available B/c vectors are listed in Table I at the end of the section.

-AtMIR390-B/c- *and* AtTAS1c-B/c-*based vectors must be propagated in a* ccd*B resistant* E. coli *strain such as DB3.1.*

-Alternatively, Bsa*I digestion of the B/c vector and subsequent ligation of the amiRNA oligonucleotide insert can be done in separate reactions*

1. OLIGONUCLEOTIDE ANNEALING

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

-If necessary, prepare 1 ml of Oligo Annealing Buffer by mixing the following

Stock	Volume to add	Final Conc.
H_2O	613.4 µL	-
1 M Tris-HCl (pH 7.5)	60 µL	60 mM Tris-HCl pH7.5
3 M NaCl	166.6 µL	500 mM NaCl
1 M $MgCl_2$	60 µL	60 mM $MgCl_2$
0.1 M DTT	100 µL	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

-Prepare a negative control reaction lacking *Bsa*I or diluted annealed oligo.

-Mix the reactions by pipetting. Incubate the reactions for 5-30 minutes at 37°C.

***Note**: Cleaning up the reactions with Zymo columns usually increases the number of colonies.*

3. *E. COLI* TRANSFORMATION AND ANALYSIS OF TRANSFORMANTS

-Transform 1-4 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 with appropriate primers: M13-F (CCCAGTCACGACGTTGTAAAACGACGG) and M13-R (CAGAGCTGCCAGGAAACAGCTATGACC) for *pENTR*-based vectors; attB1 (ACAAGTTTGTACAAAAAAGCAGGCT) and attB2 (ACCACTTTGTACAAGAAAGCTGGGT) primers for *pMDC32B*-, *pMDC123SB*- or *pFK210B*-based vectors).

Table I: *Bsa*I/*ccd*B-based ('B/c') vectors for direct cloning of amiRNAs and syn-tasiRNAs.

Vector	Small RNA class	Bacterial antibiotic resistance	Plant antibiotic resistance	GATEWAY use	Backbone	Promoter	Terminator	Plant species tested
pENTR-AtMIR390a-B/c	amiRNA	Kanamycin	-	Donor	*pENTR*	-	-	-
pFK210B-AtMIR390a-B/c	amiRNA	Spectomycin	BASTA	-	*pGreen III*	*CaMV* 35S	*rbcS*	*A. thaliana*
pMDC123SB-AtMIR390a-B/c	amiRNA	Kanamycin	BASTA	-	*pMDC123*	*CaMV* 2x35S	-	*A. thaliana* *N. benthamiana*
pMDC32B-AtMIR390a-B/c	amiRNA	Kanamycin Hygromycin	Hygromycin	-	*pMDC32*	*CaMV* 2x35S	*nos*	*A. thaliana* *N. benthamiana*
pENTR-AtTAS1c-B/c	syn-tasiRNA	Kanamycin	-	Donor	*pENTR*	-	-	-
pMDC123SB-AtTAS1c-B/c	syn-tasiRNA	Kanamycin Hygromycin	BASTA	-	*pMDC123*	*CaMV* 2x35S	*nos*	*N. benthamiana*
pMDC32B-AtTAS1c-B/c	syn-tasiRNA	Kanamycin Hygromycin	Hygromycin	-	*pMDC32*	*CaMV* 2x35S	*nos*	*A. thaliana* *N. benthamiana*
pENTR-AtTAS1c-D2-B/c	syn-tasiRNA	Kanamycin	-	Donor	*pENTR*	-	-	-
pMDC32B-AtTAS1c-D2-B/c	syn-tasiRNA	Kanamycin Hygromycin	Hygromycin	-	*pMDC32*	*CaMV* 2x35S	*nos*	*N. benthamiana*