Carrington Lab Protocols
Donald Danforth Plant Science Center
St Louis, MO (USA)
Updated 02/28/2014
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Supplemental Protocol S1

Protocol to design and clone amiRNAs or syn-tasiRNAs in *Bsa*I/*ccd*B-based ('B/c') vectors containing *AtMIR390a* or *AtTAS1c* precursors, respectively.

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1. Selection of the amiRNA or syn-tasiRNA(s) sequence(s)

A link to a web tool for automated design of the amiRNA or syn-tasiRNA sequence(s) will be available at http://p-sams.carringtonlab.org/

2. Design of amiRNA or syn-tasiRNA oligonucleotides

A link to a web tool for automated design of the amiRNA or syn-tasiRNA oligonucleotide sequences will be available at http://p-sams.carringtonlab.org/

2.1 Design of amiRNA oligonucleotides

2.1.1 Sequence of the *AtMIR390a* cassette containing the amiRNA

The following FASTA sequence includes the amiRNA sequence inserted in the *AtMIR390a* precursor sequence:

>amiRNA in *AtMIR390a* precursor

TATAGGGGGGAAAAAAAGGTAGTCATCAGATATATATTTTGGTAAGAAAATATAGAAATGAATA

ATTTCACGTTTAACGAAGAGGAGATGACGTGTGTTCCTTCGAACCCGAGTTTTGTTCGTCTATA

AATAGCACCTTCTCTTCTCCTTCTTCCTCACTTCCATCTTTTTAGCTTCACTATCTCTCTATAA

TCGGTTTTATCTTTCTCTAAGTCACAACCCAAAAAAACAAA**GTAGAGAAGAATCTGTA**$X_1X_2X_3X_4$

$X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}$**ATGATGATCACATTCGTTATCTATTTTT**

T$X_1X_2X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}$**CATTGGCTCTTCTTAC**TACAAT

GAAAAAGGCCGAGGCAAAACGCCTAAAATCACTTGAGAATCAATTCTTTTTACTGTCCATTTAA

GCTATCTTTTATAAACGTGTCTTATTTTCTATCTCTTTTGTTTAAACTAAGAAACTATAGTATT

TTGTCTAAAACAAAACATGAAAGAACAGATTAGATCTCATCTTTAGTCTC

Where:

- **X** is a DNA base of the amiRNA sequence, and the subscript number is the base position in the amiRNA 21-mer
- **X** is a DNA base of the amiRNA* sequence, and the subscript number is the base position in the amiRNA* 21-mer
- **X** is a DNA base of the *AtMIR390a* foldback
- **X** is a DNA base of the *AtMIR390a* foldback included in the oligonucleotides required to clone the amiRNA insert in B/c vectors
- **X** is a DNA base of the *AtMIR390a* foldback that may be modified to preserve the authentic *AtMIR390a* duplex structure

-X is a DNA base of the *AtMIR390a* precursor

In the sequence above:

-Insert the amiRNA sequence where you see

$X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}$

-Insert the amiRNA* sequence that has to verify the following base-pairing:

$X_1\ X_2\ X_3\ X_4\ X_5\ X_6\ X_7\ X_8\ X_9\ X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}\ X_{19}\ X_{20}X_{21}$

$X_{19}X_{18}X_{17}X_{16}X_{15}X_{14}X_{13}X_{12}X_{11}X_{10}X_9\ X_8\ X_7\ X_6\ X_5\ X_4\ X_3\ X_2\ X_1\ X_2\ X_1$

Note that:

-In general, X_1=T for amiRNA association with AGO1. In this case, X_{19}=A

-Bases X_{11} and X_9 DO NOT base-pair to preserve the central bulge of the authentic *AtMIR390a* duplex. The following base-pair rule applies:

-If X_{11}=G, then X_9=A

-If X_{11}=C, then X_9=T

-If X_{11}=A, then X_9=G

-If X_{11}=U, then X_9=C

2.1.2. Sequence of the amiRNA oligonucleotides

The sequences of the two amiRNA oligonucleotides are:

-Forward oligonucleotide (75 b),

TGTA$X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}$**ATGATGATCACA**

TTCGTTATCTATTTTTT$X_1X_2X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}$

-Reverse oligonucleotide (75 b),

AATG$Y_{19}Y_{18}Y_{17}Y_{16}Y_{15}Y_{14}Y_{13}Y_{12}Y_{11}Y_{10}Y_9Y_8Y_7Y_6Y_5Y_4Y_3Y_2Y_1Y_2Y_1$**AAAAAATGATAACG**

AATGTGATCATCAT$Y_{21}Y_{20}Y_{19}Y_{18}Y_{17}Y_{16}Y_{15}Y_{14}Y_{13}Y_{12}Y_{11}Y_{10}Y_9Y_8Y_7Y_6Y_5Y_4Y_3Y_2Y_1$

Where:

-$X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}$=amiRNA sequence

-$X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}$=partial amiRNA* sequence

-$Y_{21}Y_{20}Y_{19}Y_{18}Y_{17}Y_{16}Y_{15}Y_{14}Y_{13}Y_{12}Y_{11}Y_{10}Y_9Y_8Y_7Y_6Y_5Y_4Y_3Y_2Y_1$=amiRNA reverse- complement sequence

-**TGY$_{19}$Y$_{18}$Y$_{17}$Y$_{16}$Y$_{15}$Y$_{14}$Y$_{13}$Y$_{12}$Y$_{11}$Y$_{10}$Y$_9$Y$_8$Y$_7$Y$_6$Y$_5$Y$_4$Y$_3$Y$_2$Y$_1$**=amiRNA* reverse-complement sequence

-**X$_1$X$_2$** = *AtMIR390a* sequence that may be modified to preserve authentic *AtMIR390a* duplex structure.

-**Y$_2$Y$_1$** = reverse-complement of **X$_1$X$_2$**

Example:

The sequences of the two oligonucleotides to clone the amiRNA 'amiR-Trich' (TCCCATTCGATACTGCTCGCC) are:

-Sense oligonucleotide (75 b),

TGTATCCCATTCGATACTGCTCGCC**ATGATGATCACATTCGTTATCTATTTTTTGGCG**AGCAGTCTCGAATGGGA

-Antisense oligonucleotide (75 b),

AATGTCCCATTCGAGACTGCTCG**CCAAAAAATAGATAACGAATGTGATCATCAT**GGCGAGCAGTATCGAATGGGA

Note: *the 75 b long oligonucleotides can be ordered PAGE-purified, although oligonucleotides of 'Standard Desalting' quality worked well.*

2.2 Design of syn-tasiRNA oligonucleotides

2.2.1 Sequence of the *AtTAS1c* cassette containing the syntasiRNA(s)

The following FASTA sequence includes two syn-tasiRNA sequences inserted in the *AtTAS1c* precursor sequence:

>syn-tasiRNA-1 and syn-tasiRNA-2 in *AtTAS1c*

AAACCTAAACCTAAACGGCTAAGCCCGACGTCAAATACCAAAAAGAGAAAAACAAGAGCGCCGT

CAAGCTCTGCAAATACGATCTGTAAGTCCATCTTAACACAAAAGTGAGATGGGTTCTTAGATCA

TGTTCCGCCGTTAGATCGAGTCATGGTCTTGTCTCATAGAAAGGTACTTTCGTTTACTTCTTTT

GAGTATCGAGTAGAGCGTCGTCTATAGTTAGTTTGAGATTGCGTTTGTCAGAAGTTAGGTTCAA

TGTCCCGGTCCAATTTTCACCAGCCATGTGTCAGTTTCGTTCCTTCCCGTCCTCTTCTTTGATT

TCGTTGGGTTACGGATGTTTTCGAGATGAAACAGCATTGTTTTGTTGTGATTTTTCTCTACAAG

CGAATAGACCATTTATCGGTGGATCTTAGAAA**ATTA**X$_1$X$_2$X$_3$X$_4$X$_5$X$_6$X$_7$X$_8$X$_9$X$_{10}$X$_{11}$X$_{12}$X$_{13}$X$_{14}$X$_{15}$

X$_{16}$X$_{17}$X$_{18}$X$_{19}$X$_{20}$X$_{21}$X$_1$X$_2$X$_3$X$_4$X$_5$X$_6$X$_7$X$_8$X$_9$X$_{10}$X$_{11}$X$_{12}$X$_{13}$X$_{14}$X$_{15}$X$_{16}$X$_{17}$X$_{18}$X$_{19}$X$_{20}$X$_{21}$**GAAC**TAGA

AAAGACATTGGACATATTCCAGGATATGCAAAAGAAAACAATGAATATTGTTTTGAATGTGTTC

AAGTAAATGAGATTTTCAAGTCGTCTAAAGAACAGTTGCTAATACAGTTACTTATTTCAATAAA

TAATTGGTTCTAATAATACAAAACATATTCGAGGATATGCAGAAAAAAAGATGTTTGTTATTTT
GAAAAGCTTGAGTAGTTTCTCTCCGAGGTGTAGCGAAGAAGCATCATCTACTTTGTAATGTAAT
TTTCTTTATGTTTTCACTTTGTAATTTTATTTGTGTTAATGTACCATGGCCGATATCGGTTTTA
TTGAAAGAAAATTTATGTTACTTCTGTTTTGGCTTTGCAATCAGTTATGCTAGTTTTCTTATAC
CCTTTCGTAAGCTTCCTAAGGAATCGTTCATTGATTTCCACTGCTTCATTGTATATTAAAACTT
TACAACTGTATCGACCATCATATAATTCTGGGTCAAGAGATGAAAATAGAACACCACATCGTAA
AGTGAAAT

Where:

-**X** is a DNA base of the syn-tasiRNA-1 sequence, and the subscript number is the base position in the syn-tasiRNA-1 21-mer

-**X** is a DNA base of the syn-tasiRNA-2 sequence, and the subscript number is the base position in the syn-tasiRNA-2 21-mer

-**X** is a DNA base of the *AtTAS1c* precursor included in the oligonucleotides required to clone the syn-tasiRNA insert in B/c vectors

-X is a DNA base of the *AtTAS1c* precursor

Note that in general, X_1=T and X_1=T for syn-tasiRNA association with AGO1.

In the sequence above, replace the sequences $X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}$ and $X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}$ by the sequences of syn-tasiRNA_1 and syn-tasiRNA_2, respectively.

2.2.2. Sequence of the syn-tasiRNA oligonucleotides

The sequences of the two syn-tasiRNA oligonucleotides are:

-Sense oligonucleotide (46 b):

ATTA$X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}$

-Antisense oligonucleotide (46 b):

GTTC$Y_{21}Y_{20}Y_{19}Y_{18}Y_{17}Y_{16}Y_{15}Y_{14}Y_{13}Y_{12}Y_{11}Y_{10}Y_9Y_8Y_7Y_6Y_5Y_4Y_3Y_2Y_1Y_{21}Y_{20}Y_{19}Y_{18}Y_{17}Y_{16}Y_{15}Y_{14}Y_{13}Y_{12}Y_{11}Y_{10}Y_9Y_8Y_7Y_6Y_5Y_4Y_3Y_2Y_1$

Where:

-$X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}$=syn-tasiRNA-1 sequence

-$X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}$=syn-tasiRNA-2 sequence

-$Y_{21}Y_{20}Y_{19}Y_{18}Y_{17}Y_{16}Y_{15}Y_{14}Y_{13}Y_{12}Y_{11}Y_{10}Y_9Y_8Y_7Y_6Y_5Y_4Y_3Y_2Y_1$=syn-tasiRNA-1 reverse-complement sequence

-$Y_{21}Y_{20}Y_{19}Y_{18}Y_{17}Y_{16}Y_{15}Y_{14}Y_{13}Y_{12}Y_{11}Y_{10}Y_9Y_8Y_7Y_6Y_5Y_4Y_3Y_2Y_1$=syn-tasiRNA-2 reverse-complement sequence

Example

The sequences of the two oligonucleotides to clone syn-tasiRNAs 'syn-tasiR-Trich' (**TCCCATTCGATACTGCTCGCC**) and 'syn-tasiR-Ft' (**TTGGTTATAAAGGAAGAGGCC**) in positions 3'D3[+] and 3'D4[+] of *AtTAS1c*, respectively, are:

-Sense oligonucleotide (46 b):

ATTATCCCATTCGATACTGCTCGCCTTGGTTATAAAGGAAGAGGCC

-Antisense oligonucleotide (46 b):

GTTCGGCCTCTTCCTTTATAACCAAGGCGAGCAGTATCGAATGGGA

3. Cloning of the amiRNA/syn-tasiRNA sequences in *Bsa*I/*ccd*B (B/c) vectors

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*

3.1. 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.*

3.2. 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.

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

3.3. *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 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*