**Supplemental Protocol 1**

Protocol to design and clone syn-tasiRNAs downstream the 3’D1[+] position in *AtTAS1c-D2*-*Bsa*I/*ccd*B-based (‘B/c’) vectors containing *AtTAS1c* precursor.

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

Use the Syn-tasiRNA Designer app from the P-SAMS webtool at <http://p-sams.carringtonlab.org/syntasi/designer>.

**2. Design of syn-tasiRNA oligonucleotides**

Use the Syn-tasiRNA Designer app from the P-SAMS webtool at <http://p-sams.carringtonlab.org/syntasi/designer>.

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 downstream position 3’D1[+]:

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

AAACCTAAACCTAAACGGCTAAGCCCGACGTCAAATACCAAAAAGAGAAAAACAAGAGCGCCGTCAAGCTCTGCAAATACGATCTGTAAGTCCATCTTAACACAAAAGTGAGATGGGTTCTTAGATCATGTTCCGCCGTTAGATCGAGTCATGGTCTTGTCTCATAGAAAGGTACTTTCGTTTACTTCTTTTGAGTATCGAGTAGAGCGTCGTCTATAGTTAGTTTGAGATTGCGTTTGTCAGAAGTTAGGTTCAATGTCCCGGTCCAATTTTCACCAGCCATGTGTCAGTTTCGTTCCTTCCCGTCCTCTTCTTTGATTTCGTTGGGTTACGGATGTTTTCGAGATGAAACAGCATTGTTTTGTTGTGATTTTTCTCTACAAGCGAATAGACCA**TTTAX1X2X3X4X5X6X7X8X9X10X11X12X13X14X15X16X17X18X19X20X21X1X2X3X4X5X6X7X8X9X10X11X12X13X14X15X16X17X18X19X20X21TCGG**TGGATCTTAGAAAATTATTCTAAGTCCAACATAGCGTATTCTAAGTTCAACATATCGACGAACTAGAAAAGACATTGGACATATTCCAGGATATGCAAAAGAAAACAATGAATATTGTTTTGAATGTGTTCAAGTAAATGAGATTTTCAAGTCGTCTAAAGAACAGTTGCTAATACAGTTACTTATTTCAATAAATAATTGGTTCTAATAATACAAAACATATTCGAGGATATGCAGAAAAAAAGATGTTTGTTATTTTGAAAAGCTTGAGTAGTTTCTCTCCGAGGTGTAGCGAAGAAGCATCATCTACTTTGTAATGTAATTTTCTTTATGTTTTCACTTTGTAATTTTATTTGTGTTAATGTACCATGGCCGATATCGGTTTTATTGAAAGAAAATTTATGTTACTTCTGTTTTGGCTTTGCAATCAGTTATGCTAGTTTTCTTATACCCTTTCGTAAGCTTCCTAAGGAATCGTTCATTGATTTCCACTGCTTCATTGTATATTAAAACTTTACAACTGTATCGACCATCATATAATTCTGGGTCAAGAGATGAAAATAGAACACCACATCGTAAAGTGAAAT

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, **X1=T** and **X1=T** for syn-tasiRNA association with AGO1.

In the sequence above, replace the sequences **X1X2X3X4X5X6X7X8X9X10X11X12X13X14X15X16X17X18X19X20X21** and **X1X2X3X4X5X6X7X8X9X10X11X12X13X14X15X16X17X18X19X20X21** 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):

**TTTAX1X2X3X4X5X6X7X8X9X10X11X12X13X14X15X16X17X18X19X20X21X1X2X3X4X5X6X7 X8X9X10X11X12X13X14X15X16X17X18X19X20X21**

-Antisense oligonucleotide (46 b):

**CCGAY21Y20Y19Y18Y17Y16Y15Y14Y13Y12Y11Y10Y9Y8Y7Y6Y5Y4Y3Y2Y1Y21Y20Y19Y18Y17 Y16Y15Y14Y13Y12Y11Y10Y9Y8Y7Y6Y5Y4Y3Y2Y1**

Where:

-**X1X2X3X4X5X6X7X8X9X10X11X12X13X14X15X16X17X18X19X20X21**=syn-tasiRNA-1 sequence

-**X1X2X3X4X5X6X7X8X9X10X11X12X13X14X15X16X17X18X19X20X21**=syn-tasiRNA-2 sequence

-**Y21Y20Y19Y18Y17Y16Y15Y14Y13Y12Y11Y10Y9Y8Y7Y6Y5Y4Y3Y2Y1**=syn-tasiRNA-1 reverse-complement sequence

-**Y21Y20Y19Y18Y17Y16Y15Y14Y13Y12Y11Y10Y9Y8Y7Y6Y5Y4Y3Y2Y1**=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):

**TTTATCCCATTCGATACTGCTCGCCTTGGTTATAAAGGAAGAGGCC**

-Antisense oligonucleotide (46 b):

**CCGAGGCCTCTTCCTTTATAACCAAGGCGAGCAGTATCGAATGGGA**

**3. Cloning of the syn-tasiRNA sequence(s) in AtTAS1c-D2-B/c-based vectors**

*Notes:*

*-Available AtTAS1c-D2-B/c vectors are listed in Table I at the end of the section.*

*-*AtTAS1c-D2-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 H2O to a final concentration of 100 μM.

-Prepare Oligo Annealing Buffer:

60 mM Tris-HCl (pH 7.5)

500 mM NaCl

60 mM MgCl2

10 mM DTT

***Note:*** *Prepare 1 ml aliquots of Oligo Annealing Buffer and store at -200C.*

-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

dH2O 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

dH2O 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*-based vectors).

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| **Table I**: *Bsa*I/*ccd*B-based (‘B/c’) vectors for direct cloning of syn-tasiRNAs downstream position 3’D1[+] in *AtTAS1c* precursor. | | | | | | | | | | | |
| Vector | | Small RNA expressed | Bacterial antibiotic resistance | | Plant antibiotic resistance | | GATEWAY use | Backbone | Promoter of syn-tasiRNA cassette | Terminator of  syn-tasiRNA cassette | Plant species tested |
| *pENTR-AtTAS1c-D2-B/c* | – | | Kanamycin | – | | Donor | | *pENTR* | – | – | – |
| *pMDC32B-AtTAS1c-D2-B/c* | syn-tasiRNA(s) | | Kanamycin  Hygromycin | Hygromycin | | – | | *pMDC32* | *CaMV* 2x35S | *Nos* | *A. thaliana*  *N. benthamiana* |
| *pMDC32B-AtTAS1c-D2-B/c-*  *AtMIR173* | syn-tasiRNA(s)  miR173 | | Kanamycin  Hygromycin | Hygromycin | | – | | *pMDC32* | *CaMV* 2x35S | *nos* | *A. thaliana*  *N. benthamiana* |