

## **Additional file 4**

### **Methods S3**

#### **Golden Gate assembly method for the assembly of three or four gRNAs**

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## Simplified protocol

1. Manually search for 23-bp target sites (5'-N<sub>20</sub>NGG-3') within exons of genomic DNA sequences of genes of interest, and then evaluate target specificities on the website of potential off-target finder (<http://www.rgenome.net/cas-offfinder/>). Users can also search for target sites on the website of genome-wide prediction of plant CRISPR/Cas9 target sites (<http://www.genome.arizona.edu/crispr/CRISPRsearch.html>).
2. Design primers:
  - a) Find names of PCR fragments and primers according to plant species (monocots or dicots) and gRNA numbers in Table S3-1.
  - b) Find the sequences of the primers according to the names.
  - c) Replace 19-nt N in the forward primers with your 19-nt target sequences in front of PAM (NGG), and 19-nt N in the reverse primers with reverse complement sequences of your 19-nt target sequences in front of PAM (NGG).
3. Carry out PCR reactions according to information provided under the sequences of the PCR fragments. Refer to Additional file 3: Methods S2.
4. Set up Golden Gate reactions. Refer to Additional file 3: Methods S2.
5. Transform *E.coli* competent cells, select positive clones on kanamycin LB agar plates.
6. Identify correct clones by colony PCR and verify them by sequencing.

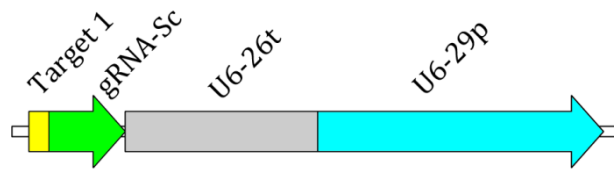
**Table S3-1 Nomenclature of PCR products and the primers for Golden Gate cloning**

<b>For dicots</b>			
	DT1-PCR DT1-BsF/DT1-F0 DT0-BsR2	DT2-PCR DT2-BsF2/DT2-F0 DT0-BsR3	Number of gRNAs
DT2T3-PCR2 DT2-BsF2/DT2-F0 DT3-R0/DT3-BsR	+	-	3
DT3T4-PCR2 DT3-BsF3/DT3-F0 DT4-R0/DT4-BsR	+	+	4
<b>For monocots</b>			
	MT1-PCR MT1-BsF/MT1-F0 MT0-BsR2	MT2-PCR MT2-BsF2/MT2-F0 MT0-BsR3	Number of gRNAs
MT2T3-PCR2 MT2-BsF2/MT2-F0 MT3-R0/MT3-BsR	+	-	3
MT3T4-PCR2 MT3-BsF3/MT3-F0 MT4-R0/MT4-BsR	+	+	4

**Notes:**

1. The primer names are under the PCR names and highlighted.
2. “+” or “-” indicates whether or not the PCR fragment in the column is mixed with the PCR fragment in the row of Column 1 for Golden Gate cloning.
3. When using more than two PCR primers, dilute the F0/R0 primers to 20 times of the F/R primers.
4. BsF/BsR, forward/reverse primer with *BsaI* site. The BsR2 and BsF2 have compatible *BsaI* sites, so do the BsR3 and BsF3. The BsR2 and BsR3 are universal primers and can be used repeatedly. F0 represents no target sequence added to the primer.

**Sequence of DT1-PCR with Target 1 for dicots**



**(Target-1)-(gRNA-Sc)-(U6-26t)-(U6-29p)**

ATATATGGTCTCGATTGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAAT  
AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCCAGTCCGGTGCTTTTTTTTGCAAATTTCCAGATCG  
ATTTCTTCTTCTCTGTTCTTCGGCGTTCAATTTCTGGGGTTTTCTCTTCGTTTTCTGTAACGAAACCTAAAAT  
TTGACCTAAAAAAATCTCAAATAATATGATTCAGTGGTTTTGTACTTTTCAGTTAGTTGAGTTTTGCAGTTCC  
GATGAGATAAACCAATATAATCCAAACTACTGCAGCCTGACAGACAAATGAGGATGCAAACAATTTTAAAG  
TTTATCTAACGCTAGCTGTTTTGTTCTCTCTGGTGCACCAACGACGGCGTTTTCTCAATCATAAAGAG  
GCTTGTTTTACTTAAGGCCAATAATGTTGATGGATCGAAAGAAGAGGGCTTTAATAAACGAGCCCGTTTA  
AGCTGTAACGATGTCAAAAACATCCCACATCGTTCAGTTGAAAATAGAAGCTCTGTTTATATATTGGTAG  
AGTCGACTAAGAGATTGAGACCAATAATAT

**Primers:**

DT1-BsF: ATATATGGTCTCGATTGNNNNNNNNNNNNNNNNNNNNGTT  
DT1-F0: TGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC  
DT0-BsR2: 5' ATATTATTGGTCTCAATCTCTTAGTCTGACTCTACCAAT

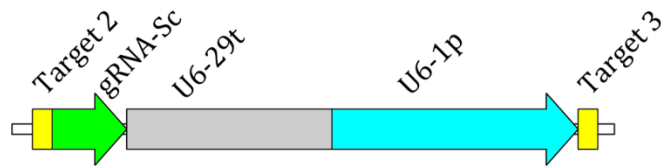
**Template:** pCBC-DT1T2

**Length:** 603-bp

**Notes:**

1. The 19-nt N in primers represent any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers) in front of PAM (NGG).
2. Enlarged and boxed letters indicate 5' protruding ends produced by *Bsa*I digestion.
3. When using DT1-BsF/DT1-F0/DT0-BsR2 three-primer mixture, dilute DT1-F0 to 20 times of DT1-BsF or DT0-BsR2.

**Sequence of DT2T3-PCR2 with Targets 2 and 3 for dicots**



**(Target-2)-(gRNA-Sc)-(U6-29t)-(U6-1p)-(Target-3)**

ATATTATTGGTCTCAAGATGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTT  
 AAAATAAGGCTAGTCCGTTATCAACTGAAAAAGTGGCACCAGTCGGTGTCTTTTTGGATAGAATTTCCC  
 AGCTTTTTGCGTGTTTCAGCTCTCATGATCCTTGGCCAATGGGTGTAGTAAATTTCTGCACATTCATTGG  
 ATGGAATAATGGTTTTAGCTTAGGGAATAAGAAAAGTGTATAGGAAGGGGATTTTTGTACAATCACA  
 TTTGAATTAGGTCTTTGAAATGACAGGGAATGAGGACATATGATGAGACGGTCATTGTTTTAGTTCCACCA  
 CGATTATATTGAAATTTACGTGAGTGTGAGTGAGACTGCATAAGAAAATAAAATCTTTAGTTGGGAAA  
 AAATTCAATAATAAATGGGCTTGAGAAGGAAGCGAGGGATAGGCCTTTTTCTAAAATAGGCCATTTA  
 AGCTATTAACAATCTTCAAAAGTACCACAGCGCTTAGGTAAGAAAAGCAGCTGAGTTTATATATGGTTAG  
 AGACGAAGTAGTGATTGNNNNNNNNNNNNNNNNNNNNGTTTCGAGACCAATAAT

**Primers:**

DT2-BsF2: 5' ATATTATTGGTCTCAAGATGNNNNNNNNNNNNNNNNNNNNGTT 3'  
 DT2-F0: 5' TNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC 3'  
 DT3-R0: 5' AACNNNNNNNNNNNNNNNNNNNNCAATCACTACTTCGTCTTAACCAT 3'  
 DT3-BsR: 5' ATTATTGGTCTCGAAACNNNNNNNNNNNNNNNNNNNNNC 3'

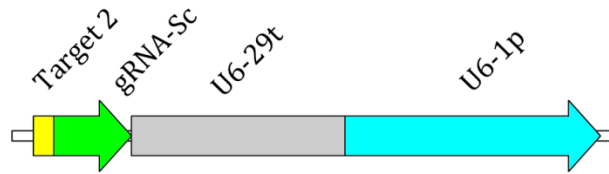
**Template:** pCBC-DT2T3

**Length:** 613-bp

**Notes:**

1. The 19-nt N in primers represent any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers) in front of PAM (NGG).
2. Enlarged and boxed letters indicate 5' protruding ends produced by *BsaI* digestion.
3. When using DT2-BsF2/DT2-F0/DT3-R0/DT3-BsR four-primer mixture, dilute DT2-F0 and DT3-R0 to 20 times of DT2-BsF2 or DT3-BsR.

**Sequence of DT2-PCR with Target 2 for dicots**



**(Target-2)-(gRNA-Sc)-(U6-29t)-(U6-1p)**

ATATTATTGGTCTCAAGATGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTT  
 AAAATAAGGCTAGTCCGTTATCAACTGAAAAAGTGGCACCAGTCGGTGTCTTTTTGGATAGAATTTCCC  
 AGCTTTTTGCGTGTTTCAGCTCTCATGATCCTTGGCCAATGGGTGTAGTAAATTTCTGCACATTCATTGG  
 ATGGAAAATAATGGTTTTAGCTTTAGGGAATAAGAAAAGTGTATAGGAAGGGGATTTTTGTACAATCACA  
 TTTGAATTAGGTCTTTGAAATGACAGGGAATGAGGACATATGATGAGACGGTCATTGTTTTAGTTCCACCA  
 CGATTATATTTGAAATTTACGTGAGTGTGAGTGAGACTTGATAAGAAAATAAAATCTTTAGTTGGGAAA  
 AAATTCATAATAATAAATGGGCTTGAGAAGGAAGCGAGGGATAGGCCTTTTCTAAAATAGGCCATTTA  
 AGCTATTAACAATCTTCAAAAGTACCACAGCGCTTAGGTAAAGAAAGCAGCTGAGTTTATATATGGTTAG  
 AGACGAAGTGTGATGAGACCAATAATAT

**Primers:**

DT2-BsF2: 5' ATATTATTGGTCTCAAGATGNNNNNNNNNNNNNNNNNNNNGTT 3'  
 DT2-F0: 5' TNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC 3'  
 DT0-BsR3: ATATTATTGGTCTCATCTACTTCGTCTCTAACCAT

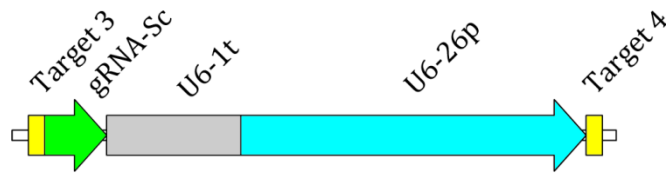
**Template:** pCBC-DT2T3

**Length:** 589-bp

**Notes:**

1. The 19-nt N in primers represent any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers) in front of PAM (NGG).
2. Enlarged and boxed letters indicate 5' protruding ends produced by *BsaI* digestion.
3. When using DT2-BsF2/DT2-F0/DT0-BsR3 three-primer mixture, dilute DT2-F0 to 20 times of DT2-BsF2 or DT0-BsR3.

**Sequence of DT3T4-PCR2 with Targets 3 and 4 for dicots**



**(Target-3)-(gRNA-Sc)-(U61-t)-(U6-26p)-(Target-4)**

ATATTATTGGTCTCA**GTGA**TTGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGT  
TAAATAAGGCTAGTCCGTTATCAACTGAAAAAGTGGCACCGAGTCGGTGC TTTTTTTTGGCAAAAATTTTC  
AGATTTTTTCTTCATCTGTAGATTTCTGGGTTTTTTTTCCGTTTCGTGAATCATAAGTGAAGTTTTGGATGC  
AAATCTGCGCGAAAAAAGTTGGACCTGCAATGAGCTTATTTAGATAGCTAAGACAAAGTGATTGGTCCGT  
TCGACTTGCCTCCGCACAATACATCATTCTTCTTAGCTTTTTTCTTCTTCTTCGTTCCATACAGTTTTTTTTG  
TTTATCAGCTTACATTTCTTGAACCGTAGCTTCGTTTTCTTCTTTTAACTTCCATTCGGAGTTTTTGATCT  
TGTTTTCATAGTTTGTCCCAGGATTAGAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTT  
CATTCTAAGATATGAAGATAATCTTCAAAGGCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCATTATA  
TGGGAAAGAACAATAGTATTTCTTATATAGGCCATTTAAGTTGAAAACAATCTTCAAAGTCCCACATCGCTT  
AGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTGATTGNNNNNNNNNNNNNNNNNN  
NNN**GTTT**AGAGACCAATAAT

**Primers:**

DT3-BsF3: 5' ATATTATTGGTCTCA**GTGA**TTGNNNNNNNNNNNNNNNNNNNNGTT  
DT3-F0: 5' TGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC 3'  
DT4-R0: 5' AACNNNNNNNNNNNNNNNNNNNNCAATCACTACTTCGACTCTAGCTGTAT  
DT4-BsR: 5' ATTATTGGTCTT**AAAC**NNNNNNNNNNNNNNNNNNNN 3'

**Template:** pCBC-DT3T4

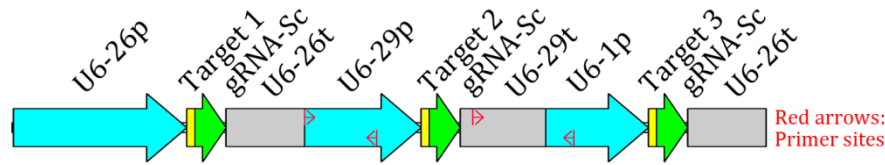
**Length:** 743-bp

**Notes:**

1. The 19-nt N in primers represent any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers) in front of PAM (NGG).
2. Enlarged and boxed letters indicate 5' protruding ends produced by *BsaI* digestion.
3. When using DT3-BsF3/DT3-F0/DT4-R0/DT4-BsR four-primer mixture, dilute DT3-F0 and DT4-R0 to 20 times of DT3-BsF3 or DT4-BsR.

## Sequence of three gRNA expression cassettes for dicots

DT1-PCR + DT2T3-PCR2 + pHSN401 et al.



(U6-26p)-(Target-1)-(gRNA-Sc)-(U6-26t)-(U6-29p)-(Target-2)-(gRNA-Sc)-(U6-29t)-  
(U6-1p)-(Target-3)-(gRNA-Sc)-(U6-26t)

CGACTTGCCTTCCGCACAATACATCATTTCTTCTTAGCTTTTTTCTTCTTCTTCGTTTCATACAGTTTTTTTTTGTATTATCAGCTTAC  
ATTTTCTTGAACCGTAGCTTTCGTTTTCTTCTTTTTAACTTCCATTCCGGAGTTTTGTATCTTGTTCATAGTTTTGCCAGGATTA  
GAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGATATGAAGATAATCTTCAAAAAGCCCC  
TGGGAATCTGAAAAGAAGAGAAGCAGGCCCATTTATATGGGAAAAGAACAATAGTATTTCTTATATAGGCCCATTTAAGTTGAAAAC  
AATCTTCAAAAAGTCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGT**ATTG**NN  
NNNNNNNNNNNNNNNNNNNN**G**TTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCA  
CCGAGTCGGTGCTTTTTTTTGCAAAATTTCCAGATCGATTCTTCTCTCTGTTCTCGGCGTTC AATTTCTGGGGTTTTCTCTT  
CGTTTTCTGTAACCTGAAACCTAAAATTTGACCTAAAAAAATCTCAAATAATATGATTCAGTGGTTTTGTACTTTTCAGTTAGTTGA  
GTTTTGCAGTTCGGATGAGATAAAACCAATA**TTAATCCAACTACTGCAGCCTGAC**AGACAAATGAGGATGCAAAACAATTTTAA  
GTTTATCTAACCGTAGCTGTTTTGTTTCTTCTCTGGTGCACCAACGACGGCGTTTTCTCAATCATAAAGAGGCTGTTTTACT  
TAAGCCAATAAT**GTTGATGGATCGAAAGAAGAGGGCT**TTAATAAACGAGCCGTTAAGCTGTAACGATGCAAAAAC  
ATCCACATCGTTTCAGTTGAAAATAGAAGCTCTGTTTTATATATTGGTAGAGTCGACTAAG**AGAT**TGNNNNNNNNNNNN  
NNNNNNNNNN**G**TTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGT  
GTTTTTTTTGGATAGAATTTCCAGCTTTTTT**CGTGTTTCAGCTCTCATGATCCTTG**GCCAATGGGTGTAGTAAATTTCTGC  
ACATTCATTGGATGAAAATAATGGTTTTAGCTTTAGGGAATAAGAAAAGTGATAGGAAGGGGATTTTTGTACAATCACAT  
TTGAATTAGTCTTTGAAATGACAGGGAATGAGGACATATGATGAGACGGTCATTGTTTTAGTTCCACCACGATTATATTTGA  
AATTTA**CGTGAGTGTGAGTGAGACTTGCATA**AGAAAATAAAATCTTAGTTGGGAAAAAATCAATAATATAAATGGGCTTG  
AGAAGGAAGCGAGGGATAGGCCTTTTTCTAAAATAGGCCATTTAAGCTATTAACAATCTTCAAAAAGTACCACAGCGCTTAG  
GTAAAGAAAGCAGCTGAGTTTATATATGGTTAGAGACGAAGTAGTATTGNNNNNNNNNNNNNNNNNNNN**GTTT**TA  
GAGCTAGAAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTGCAAAA  
TTTTCCAGATCGATTTCTTCTTCTCTGTTCTTCGGCGTTCAATTTCTGGGGTTTTCTTCTCGTTTTCTGTAACCTGAAACCTAAAAT  
TTGACCTAAAAAAATCTCAAATAATATGATTCAGTGGTTTTGTACTTTTCAGTTAGTTGAGTTTTGCAGTTCGGATGAGATAAAC  
CAATA

### Notes:

- Underlined letters come from binary vectors, while the others come from PCR fragments.
- Red letters indicate primer sites.
- Enlarged and boxed letters indicate 5' protruding ends produced by *BsaI* digestion.
- Primer sequences are as follows:

#### Colony PCR primers (5'→3')

U6-29p-F: TTAATCCAACTACTGCAGCCTGAC  
U6-1p-R: TATGCAAGTCTCACTCACACTCAG  
(U6-29p-F + U6-1p-R = 659 bp)

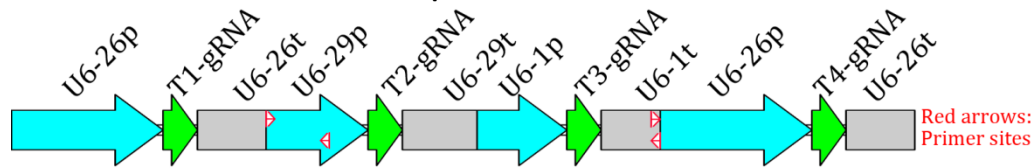
#### Sequencing primers (5'→3'):

U6-29p-F: TTAATCCAACTACTGCAGCCTGAC  
U6-29p-R: AGCCCTCTTTTCGATCCATCAAC  
U6-29t-F: CGTGTTTCAGCTCTCATGATCCTTG



## Sequence of four gRNA expression cassettes for dicots

DT1-PCR + DT2-PCR + DT3T4-PCR2 + pHSN401 et al.



(U6-26p)-(Target-1)-(gRNA-Sc)-(U6-26t)-(U6-29p)-(Target-2)-(gRNA-Sc)-(U6-29t)-  
(U6-1p)-(Target-3)-(gRNA-Sc)-(U6-1t)-(U6-26p)-(Target-4)-(gRNA-Sc)-(U6-26t)

CGACTTGCCTCCGCACAATACATCATTCTCTTAGCTTTTTCTCTCTCTCGTTCATACAGTTTTTTTTTGTTTATCAGCTTAC  
ATTTCTTGAACCGTAGCTTTCGTTTTCTCTTTTAACTTCCATTCGGAGTTTTGTATCTGTTCATAGTTTGTCCAGGATTA  
GAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGATATGAAGATAATCTCAAAAAGGCCCC  
TGGGAATCTGAAAAGAAGAGAAGCAGGCCATTATATGGGAAAAGAACAATAGTATTTCTTATATAGGCCATTAAAGTTGAAAAC  
AATCTTCAAAAGTCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGT**ATTG**NN  
NNNNNNNNNNNNNNNNNN**GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCA**  
**CCGAGTCGGTGC**TTTTTTTGGCAAATTTCCAGATCGATTTCTCTCTCTGTTCTCGGCGTTCAATTTCTGGGGTTTTCTCT  
CGTTTTCTGTAACCTGAAACCTAAAATTTGACCTAAAAAAATCTCAAATAATATGATTCAGTGGTTTTGTACTTTTCAGTTAGTTGA  
GTTTTGCAGTTCGGATGAGATAAACCAATA**TAATCCAAACTACTGCAGCTGAC**AGACAAATGAGGATGCAAACAATTTTAA  
GTTTATCTAACGCTAGCTGTTTTGTTTCTCTCTCTGGTGACCAACGACGGCGTTTTCTCAATCATAAAGAGGCTGTTTTACT  
TAAGGCCAATAAT**GTTGATGGATCGAAAGAAGGGCT**TTTAAATAACGAGCCGTTAAGCTGTAAACGATGTCAAAAAC  
ATCCACATCGTTCAGTTGAAAATAGAAGCTCTGTTTATATATTGGTAGAGTCGACTAAG**AGAT**TGNNNNNNNNNNNN  
NNNNNNNN**GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGT**  
**GC**TTTTTTTGGATAGAATTTCCAGCTTTTTGCGTGTTCAGCTCTCATGATCCTTGCCCAATGGGTGTAGTAAATTTCTGCA  
CATTATTGGATGGAAAATAATGGTTTTAGCTTTAGGGAATAAGAAAAGTGTATAGGAAGGGGATTTTTGTACAATCACATT  
TGAATTAGGCTTTGAAATGACAGGGAATGAGGACATATGATGAGACGGTCATTGTTTGTAGTCCACCAGATTATATTGGA  
AATTTA**CGTGAGTGAGTGAGACTTCATA**AGAAAATAAAATCTTATAGTTGGGAAAAAATCAATAATATAAATGGGCTTG  
AGAAGGAAGCGAGGGATAGGCCTTTTTCTAAAATAGGCCATTTAAGCTATTAACAATCTCAAAAAGTACCACAGCGCTTAG  
GTAAAGAAAGCAGCTGAGTTTATATATGGTTAGAGACGAAGTA**GTGA**TTGNNNNNNNNNNNNNNNNNN**GTTTT**  
**AGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC**TTTTTTTGGCAA  
AAATTTTCAGATTTTTCTCATCTGTAGATTTCTGGGTTTTTTTTCCGTTTCGTGAATCATAAGTGAAGTTTTGGATGCAAAT  
CTGCGCGAAAAAAGTTGGACCTGCAATGAGCTTATTTAGATA**GCTAAGACAAAGTGATTGGTCCGTT**CGACTTGCCTCCGC  
ACAATACATCATTCTCTTAGCTTTTTCTCTCTCTCGTTCATACAGTTTTTTTTTGTTTATCAGCTTACATTTCTTGAACCGTA  
GCTTTCGTTTTCTTTTTAACTTCCATTCGGAGTTTTGTATCTGTTCATAGTTTGTCCAGGATTAGAATGATTAGGCATC  
GAACCTTCAAGAAATTTGATTGAATAAAACATCTTCATTCTAAGATATGAAGATAATCTCAAAAAGGCCCTGGGAATCTGAAG  
AAGAGAAGCAGGCCATTATATGGGAAAAGAACAATAGTATTTCTTATATAGGCCATTAAAGTTGAAAACAATCTCAAAAAGTC  
CCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTGATTGNNNNNNNNNNNNNNNNNN  
NN**GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC**T  
TTTTTTGGCAAATTTCCAGATCGATTTCTCTCTCTGTTCTCGGCGTTCAATTTCTGGGGTTTTCTCTCGTTTTCTGTAAC  
GAAACCTAAAATTTGACCTAAAAAAATCTCAAATAATATGATTCAGTGGTTTTGTACTTTTCAGTTAGTTGAGTTTTGCAGTTCC  
GATGAGATAAACCAATA

### Notes:

- Underlined letters come from binary vectors, while the others come from PCR fragments.
- Red letters indicate primer sites.
- Enlarged and boxed letters indicate 5' protruding ends produced by *BsaI* digestion.
- Primer sequences are as follows:

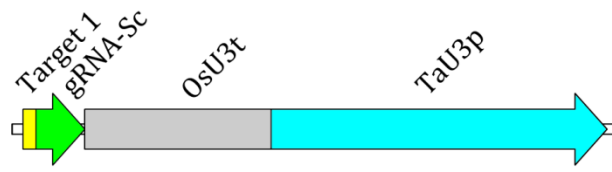
#### Colony PCR primers (5'→3'):

U6-29p-F: TTAATCCAACTACTGCAGCCTGAC'  
U6-1t-R: AACGGACCAATCACTTTGTCTTAGC'  
(U6-29p-F + U6-1t-R = 1.1 kb)

#### Sequencing primers (5'→3'):

U6-29p-F: TTAATCCAACTACTGCAGCCTGAC  
U6-29p-R: AGCCCTCTCTTCGATCCATCAAC  
U6-1t-F: GCTAAGACAAAGTGATTGGTCCGTT  
U6-1t-R: AACGGACCAATCACTTTGTCTTAGC

**Sequence of MT1-PCR with Target 1 for monocots**



(Target-1)-(gRNA-Sc)-(OsU3t)-(TaU3p)  
 ATATATGGTCTCTGGCGNNNNNNNNNNNNNNNNNNNNGTTTGTAGAGCTAGAAATAGCAAGTTAAAA  
 TAAGGCTAGTCCGTTATCAACTGAAAAAGTGGCACCAGTCCGGTCTTTTTTTTTTCGTTTTGCATTGAGTT  
 TTCTCCGTCGCATGTTTGCAGTTTTATTTCCGTTTTGCATTGAAATTTCTCCGTCTCATGTTTGCAGCGTGTTCC  
 AAAAAGTACGCAGCTGTATTCACTTATTTACGGCGCCACATTTTCATGCCGTTTGTGCCAATATCCCGAGCT  
 AGTGAATACAGCTTGCTTCACACAACACTGGTGACCCGCTGACCTGCTCGTACCTCGTACCCTGTACCGC  
 ACAGCATTGGAATTAAGGGTGTGATCGATACTGCTTGCTGCTCATGAATCCAAACCACACGGAGTTCAAA  
 TTCCCACAGATTAAGGCTCGTCCGTCGCACAAGGTAATGTGTGAATATTATATCTGTCGTGCAAAATTGCCTG  
 GCCTGCACAATTGCTGTTATAGTTGGCGGCAGGGAGAGTTTAACATTGACTAGCGTGTGATAATTTGTGA  
 GAAATAAATGACAAGTAGATACTGACATTTGAGAAGAGCTTCTGAAGTGTATTAGTAACAAAAATGGAA  
 AGCTGATGCACGGAAGGAAAAGAAAAGCCATACTTTTTTTAGGTAGGAAAAGAAAAGCCATACGA  
 GACTGATGTCTCTCAGATGGCCGGGATCTGTCTATCTAGCAGGCAGCAGCCCAACCTCACGGGCCAG  
 CAATTACGAGTCTCTAAAAGCTCCCGCCAGGGGGCGCTGGCGCTGCTGTGCAGCAGCAGCTCTAACATTA  
 GTCCACCTCGCCAGTTTACAGGGAGCAGAACCAGCTTATAAGCGGAGGCGCGCACCAAG**AAGC**  
 AGAGACCAATAAT

**Primers:**

MT1-BsF: ATATATGGTCTCTGGCGNNNNNNNNNNNNNNNNNNNNGTT  
 MT1-F0: TGNNNNNNNNNNNNNNNNNNNNNGTTTGTAGAGCTAGAAATAGC  
 MT0-BsR2: ATTATGGTCTCTGCTTCTTGGTGCCGC

**Template:** pCBC-MT1T2

**Length:** 940-bp

**Notes:**

1. The 19-nt N in primers represent any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers) in front of PAM (NGG).
2. Enlarged and boxed letters indicate 5' protruding ends produced by *BsaI* digestion.
3. When using MT1-BsF/MT1-F0/MT0-BsR2 three-primer mixture, dilute MT1-F0 to 20 times of MT1-BsF or MT0-BsR2.

**Sequence of MT2T3-PCR2 with Targets 2 and 3 for monocots**



(Target-2)-(gRNA-Sc)-(TaU3t)-(U6-26p)-(Target-3)

ATATATGGTCTCT**AAGC**GNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAA  
ATAAGGCTAGTCCGTTATCAACTGAAAAAGTGGCACCGAGTCGGTGC TTTTTTTTTTGCTTCTGTTTTTT  
AGTCAGTCTCTTTTTTCAGAAGTACAACATCTTTTTTTGTCCTTCTGTTTTTTAGTCAGTCTCTTTTTTCAGA  
AGTACAACATCTTTTTTTGCTTCTGTTTTTTAGTCAGTCTCTTTTTTCAGAAGTACAACATCTTTTTTTG  
TCCTTCTGTTTTTTAGTCAGTCTCTTTTTTCAGAAGTACTCTATGATGATCGTTCTGGGAAATGTCTGTCTGT  
CTACAACCCATAATCTATATTGCAATCACACATCTAATATTCTCTGTGACAAGACAGCCGAACA**CGACTTGC**  
**CTTCCGCACAATACATCATTTCTTCTAGCTTTTTTCTTCTTCTCGTTCATACAGTTTTTTTTGTTTATCAGC**  
TTACATTTCTTGAACCGTAGCTTTCGTTTTCTTTTTAACTTCCATTCGGAGTTTTGTATCTGTTTCATA  
GTTTGTCCCAGGATTAGAATGATTAGGCATCGAACCTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAA  
GATATGAAGATAATCTTCAAAGGCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCATTATATGGGAAA  
GAACAATAGTATTTCTATATAGGCCATTAAAGTTGAAAACAATCTTCAAAGTCCACATCGCTTAGATAAG  
AAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTGATT**GNNNNNNNNNNNNNNNNNNNN****G**  
**TTT**AGAGACCAATAAT

**Primers:**

MT2-BsF2: ATATATGGTCTCT**AAGC**GNNNNNNNNNNNNNNNNNNNNGTT  
MT2-F0: T**GNNNNNNNNNNNNNNNNNNNN**GTTTTAGAGCTAGAAATAGC  
MT3-R0: AAC**NNNNNNNNNNNNNNNNNNNN**CAATCACTACTTCGACTCTAGC  
MT3-BsR: ATTATTGGTCTCT**AAAC**NNNNNNNNNNNNNNNNNNNNC

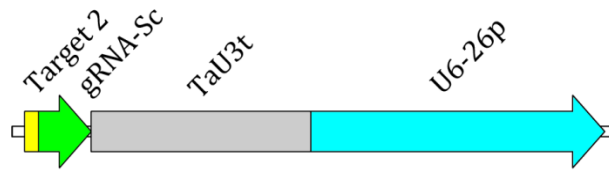
**Template:** pCBC-MT2T3

**Length:** 891-bp

**Notes:**

1. The 19-nt N in primers represent any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers) in front of PAM (NGG).
2. Enlarged and boxed letters indicate 5' protruding ends produced by *BsaI* digestion.
3. When using MT2-BsF2/MT2-F0/MT3-R0/MT3-BsR four-primer mixture, dilute MT2-F0 and MT3-R0 to 20 times of MT2-BsF2 or MT3-BsR.

## Sequence of MT2-PCR with Target 2 for monocots



(Target-2)-(gRNA-Sc)-(TaU3t)-(U6-26p)

```

ATATATGGTCTCTAAGCGNNNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAA
ATAAGGCTAGTCCGTTATCAACTGAAAAAGTGGCACCGAGTCGGTGC TTTTTTTTTGTCCTTCTGTTTTTT
AGTCAGTCTCTTTTTTCAGAAGTACAACATCTTTTTTTGTCCTTCTGTTTTTTAGTCAGTCTCTTTTTTCAGA
AGTACAACATCTTTTTTTGTCCTTCTGTTTTTTAGTCAGTCTCTTTTTTCAGAAGTACAACATCTTTTTTTG
TCCTTCTGTTTTTTAGTCAGTCTCTTTTTTCAGAAGTACTCTATGATGATCGTTCTGGGAAATGTCTGTCTGT
CTACAACCCATAATCTATATTGCAATCACACATCTAATATTCTCTGTGACAAGACAGCCGAACACGACTTGC
CTTCCGCACAATACATCATTTCTTCTAGCTTTTTTCTTCTTCTTCGTTCATAACAGTTTTTTTTGTTTATCAGC
TTACATTTCTTGAACCGTAGCTTTCGTTTTCTTTTTAACTTTCATTTCGGAGTTTTGTATCTGTTTTCATA
GTTTGTCCCAGGATTAGAATGATTAGGCATCGAACCTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAA
GATATGAAGATAATCTTCAAAGGCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCATTATATGGGAAA
GAACAATAGTATTTCTTATATAGGCCATTAAAGTTGAAAACAATCTTCAAAGTCCCACATCGCTTAGATAAG
AAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTATTGAGAGACCAATAAT
    
```

### Primers:

MT2-BsF2: ATATATGGTCTCT**AAGC**GNNNNNNNNNNNNNNNNNNNNNNGTT

MT2-F0: TGNNNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC

MT0-BsR3: ATTATTGGTCTCT**CAAT**CACTACTTCGACTCTAGC

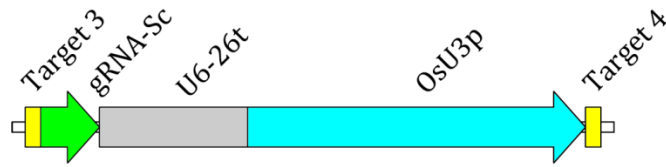
**Template:** pCBC-MT2T3

**Length:** 868-bp

### Notes:

1. The 19-nt N in primers represent any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers) in front of PAM (NGG).
2. Enlarged and boxed letters indicate 5' protruding ends produced by *BsaI* digestion.
3. When using MT2-BsF2/MT2-F0/MT0-BsR3 three-primer mixture, dilute MT2-F0 to 20 times of MT2-BsF2 or MT0-BsR3.

**Sequence of MT3T4-PCR2 with Targets 3 and 4 for monocots**



(Target-3)-(gRNA-Sc)-(U6-26t)-(OsU3p)-(Target-4)  
 ATATATGGTCTCT**ATTG**NNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAT  
 AAGGCTAGTCCGTTATCAACTTAAAAAGTGGCACCGAGTCGGTGCCTTTTTTTGCAAAATTTCCAGATCG  
 ATTTCTTCTTCTCTGTTCTTCGGCGTTCAATTTCTGGGGTTTTCTCTCGTTTTCTGTAAGTAAACCTAAAAT  
 TTGACCTAAAAAATCTCAAATAATGATTCAAGTGGTTTTGTACTTTTCAGTTAGTTGAGTTTTGCAGTTCC  
 GATGAGATAAACCAATAAGTAATTCATCCAGGTCACCAAGTTCTAGGATTTTCAGAAGTCAACTTATTTTATC  
 AAGGAATCTTTAAACATACGAACAGATCACTTAAAGTTCTTCTGAAGCAACTTAAAGTTATCAGGCATGCATG  
 GATCTTGGAGGAATCAGATGTGCAGTCAGGACCATAGCACAAGACAGGCGTCTTCTACTGGTGCTACCAG  
 CAAATGCTGGAAGCCGGGAACACTGGGTACGTTGGAACCACGTGATGTGAAGAAGTAAGATAAACTGTAG  
 GAGAAAAGCATTTCTAGTGGCCATGAAGCCTTTCAGGACATGTATTGCAGTATGGCCGGCCATTACGC  
 AATTGGACGACAACAAGACTAGTATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTTAAAGAG  
 TTGTGCAGATGATCCGTGGCGNNNNNNNNNNNNNNNNNN**GTTT**AGAGACCAATAAT

**Primers:**

MT3-BsF3: ATATATGGTCTCT**ATTG**NNNNNNNNNNNNNNNNNNNNGTT  
 MT3-F0: T**NNNNNNNNNNNNNNNNNNNN**GTTTTAGAGCTAGAAATAGC  
 MT4-R0: AAC**NNNNNNNNNNNNNNNNNNNN**CGCCACGGATCATCTGCACAAC 3'  
 MT4-BsR: ATTATTGGTCTCT**AAAC**NNNNNNNNNNNNNNNNNNNNC

**Template:** pCBC-MT3T4

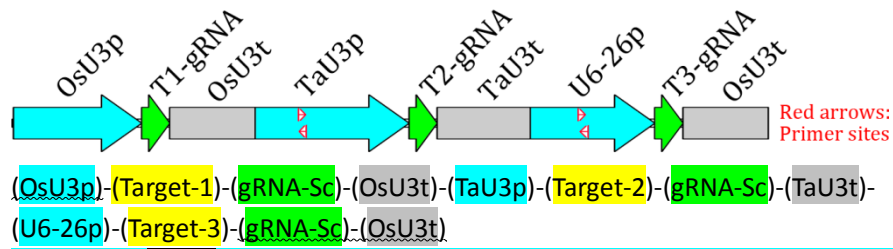
**Length:** 778-bp

**Notes:**

1. The 19-nt N in primers represent any 19-nt target sequence (forward primers) or reverse complement sequence of any 19-nt target sequence (reverse primers) in front of PAM (NGG).
2. Enlarged and boxed letters indicate 5' protruding ends produced by *BsaI* digestion.
3. When using MT3-BsF3/MT3-F0/MT4-R0/MT4-BsR four-primer mixture, dilute MT3-F0 and MT4-R0 to 20 times of MT3-BsF3 or MT4-BsR.

## Sequence of three gRNA expression cassettes for monocots

MT1-PCR + MT2T3-PCR2 + pBUN411 et al.



```

AGTAATTCATCCAGGTCTCCAAGTTCAGGATTTTCAGAAGTCAACTTATTTATCAAGGAATCCTTAAACATACGAACAGATCACTTAAAGTTCCTGAA
GCAACTTAAAGTTATCAGGCATGCATGGATCTTGGAGGAATCAGATGTGCAGTCAGGGACCATAGCACAAAGACAGGCGCTTCTACTGGTGCTACCAGCA
AATGCTGGAAGCCGGGAACACTGGGTACGTTGGAAACCAGTGATGTGAAGAAGTAAGATAAAGCTAGGAGAAAAGCAITTCGTAGTGGGCCATGAAG
CCTTTCAGGACATGTATTGCAGTATGGCCGCGCCATTACGCAATTGACGACAACAAAGACTAGTATTAGTACCACCTCGGCTATCCACATAGATCAAAGC
TGATTAAAGAGTTGTGCAGATGATCCGTTGGCGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
CGTTATCAACTTGA AAAAGTGGCACCAGTTCGGTGC TTTTTTTTTTCGTTTTGCATTGAGTTTTCTCCGTCGCATGTTTCAGTTTTATTTCGGTTTTGCATT
GAAATTTCTCCGTCATGTTTTCAGCGGTTCACAAAAGTACGCAGCTGATTTCACTTATTACGGCGCCACATTTTCATGCCGTTTTGTGCCAACTATCCCG
AGCTAGTGAATACAGCTTGGCTTACACAACACTGGTGACCCGCTGACCTGCTGACCTCGTACCGTCGTACGGCACAGCATTGGAAATTAAGGGGTGTG
ATCGATACGCTTCTGCTGCTCATGAATCCAAACCACACGGAGTTCAAATTCACAGATTAAGGCTCGTCCGTCGCACAAGGTAATGTGTGAATATTATATCTG
TCGTGCAAAATTCCTGCGCTGCACAATGCTGTATAGTTGGCGCAGGAGAGTTTTAACAATTGACTAGCGTCTGATAATTTGTGAGAAAATAAATT
GACAAGTAGATACTGACATTTGAGAAGAGCTTCTGAACCTGTATTAGTAAACAAAATGGAAGCTGATGCACGAAAAAGGAAAAGAAAAGCCATATCTT
TTTTAGGTAGGAAAAAGAAAAGCCATACGAGACTGATGTCTCAGATGGCCGGGATCTGTCTATAGCAGGACAGCCACCACTCACGGGCC
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CAGAACCAGCTTAAAGCGAGGCGCGGCACCAAGAAAGC GNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
GGCTAGTCCGTTATCAACTTGA AAAAGTGGCACCAGTTCGGTGC TTTTTTTTTTGCTCTCTGTTTTTTAGTCAGTCTCTTTTTCAGAAGTACAACATCTT
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GCTTAGATAAAGAAAACGAAGCTGAGTTTATATACAGCTAGTCAAGTAGTGATTGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
GCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA AAAAGTGGCACCAGTTCGGTGC TTTTTTTTTTCGTTTTGCATTGAGTTTTCTCCGTCGCATGTTG
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AGCATTTGGAATTAAGGGGTGTGATCGATACTGCTTCTGCT
    
```

### Notes:

1. Underlined letters come from binary vectors, while the others come from PCR fragments.
2. Red letters indicate primer sites.
3. Enlarged and boxed letters indicate 5' protruding ends produced by *Bsa*I digestion.
4. Primer sequences are as follows:

#### Colony PCR primers (5'→3'):

TaU3p-F2: TTGACTAGCGTGCTGATAATTTGTG

U6-26p-R2: AGGTTCGAT GCCTAATCATTCTAATCTT

(TaU3p-F2 + U6-26p-R2 = 987 bp)

#### Sequencing primers (5'→3'):

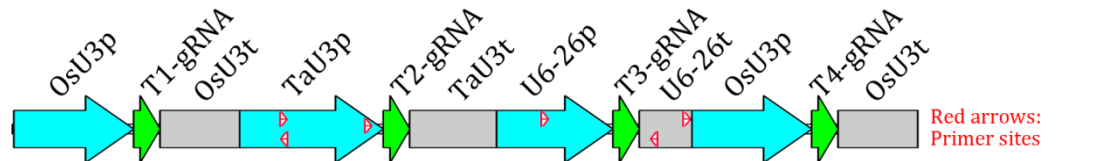
TaU3p-F2: TTGACTAGCGTGCTGATAATTTGTG

TaU3p-R: CTCACAAATTATCAGCACGCTAGTC

U6-26p-F: TGTCCAGGATTAGAATGATTAGGC

## Sequence of four gRNA expression cassettes for monocots

MT1-PCR + MT2-PCR + MT3T4-PCR2 + pBUN411 et al.



```
(OsU3p)-(Target-1)-(gRNA-Sc)-(OsU3t)-(TaU3p)-(Target-2)-(gRNA-Sc)-(TaU3t)-  
(U6-26p)-(Target-3)-(gRNA-Sc)-(U6-26t)-(OsU3p)-(Target-4)-(gRNA-Sc)-(OsU3t)  
AGTAATTCATCCAGGTCTCCAAGTTCAGGATTTTCAGAAGTGCACCACTTATTTATCAAGGAATCTTTAAACATACGAACAGATCACTTAAAGTCTCTGAA  
GCAACTTAAAGTATCAGGCATGCATGGATCTTGGAGGAATCAGATGTGCAGTCAGGGACCATAGCACAAGACAGGCCTTCTACTGTTGCTACCAGCA  
AATGCTGGAAGCCGGGAACACTGGGTACGTTGGAAACCACGTGATGTGAAGAAGTAAGATAAATCTGAGGAGAAAAGCAITTCGTAGTGGGCCATGAAG  
CCTTTCAGGACATGTTGTCAGTATGGCCGGCCATTACGCAATTCGACGACAACAAGACTAGTATTAGTACCACCTCGGCTATCCACATAGATCAAAGC  
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CGTTATCAACTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTCGTTTTGTCATTGAGTTTTCTCCGTCGCATGTTGTCAGTTTATTTCCGTTTTGCATT  
GAAATTTCTCCGTTTCATGTTTGCAGCGTGTCAAAAAGTACGCAGCTGTATTTCACTTATTTACGGCGCCACATTTCATGCCGTTTTGTGCCAACTATCCCG  
AGCTAGTGAATACAGCTTGGCTTTCACACAACACTGGTAGCCCGTACCTGCTGACTGCTGACCTCGTACCGTCAGTACCGCACAGCATTGGAAATTAAGGGTGTG  
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CCTCGTACCGTCTGACGGCACAGCATTTGGAATTAAGGGTGTGATGCATCTGCTTTGCTGCT
```

### Notes:

- 1. Underlined letters come from binary vectors, while the others come from PCR fragments.
- 2. Red letters indicate primer sites.
- 3. Enlarged and boxed letters indicate 5' protruding ends produced by *Bsa*I digestion.
- 4. Primer sequences are as follows:

**Colony PCR primers (5'→3'):**  
TaU3p-F: **TTAGTCCCACCTCGCCAGTTTACAG**  
U6-26t-R: **CCCAGAAATTGAACGCCGAAGAAC**  
(TaU3p-F + U6-26t-R = 1.2 kb)

**Sequencing primers (5'→3'):**  
TaU3p-F2: **TTGACTAGCGTCTGATAATTTGTG**  
TaU3p-R: **CTCACAATATCAGCAGCTAGTC**  
U6-26p-F: **TGTCAGGATTAGAATGATTAGGC**  
U6-26t-F2: **GAGTTTTGCAAGTTCCGATGAGATAAACC**