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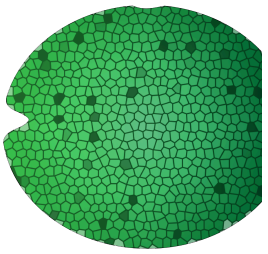
Generation of multiplex tRNA-gRNA constructs for Marchantia polymorpha CRISPR



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Abstract

This protocol describes a *Marchantia polymorpha* specific modification of the Xie et al 2015 protocol (https://doi.org/10.1073/pnas.1420294112) for the synthesis of multicomplex tRNA-gRNA modules by Golden Gate Assembly/Loop Assembly. It has 3 main steps:

A. Primer design and PCR amplification of tRNA-gRNA fragments using the pGTR plasmid as the template.
B. Loop assembly cloning of the amplified tRNA-gRNA fragments into an L1 vector,
to combine them with the Mp*U6* promoter
C. Loop assembly cloning into the L2 pCsA acceptor vector to combine the *MpU6*::tRNA-gRNA unit with the transcription unit for Cas9 expression

Materials

L1_lacZgRNA-Ck2 or L1_lacZgRNA-Ck3 vectors (Addgene #136136 or Addgene #136137).

T4 DNA ligase buffer (NEB),
1 mg/mL bovine serum albumin (NEB, #B9200S)
T4 DNA ligase 400 U/µL (NEB, #M0202S),
1.5 µL of BbsI-HF 10 U/µL (NEB, #R3539),

50 µg/mL kanamycin (MELFORD, #K22000–1.0)
40 µg/mL X-Gal
(5-bromo-4-chloro-3-indolyl-β-D-galactoside) (ThermoFisher, #15520018).

The pGTR plasmid (Addgene #63143)
Phusion polymerase (ThermoFisher, #F534S)
QIAquick Gel Extraction Kit (QIAGEN, #28704)

SapI (10 U/µL, NEB, #R0569S),
pCsA vector (Addgene #136067)
spectinomycin (MERCK, #S4014-5G)

L1 vectors: L1_Cas9-Ck4 containing the transcription unit for the Cas9 expression, (Addgene #136135 **(Sauret-Güeto et al. 2020)**); L1_CsR-Ck1 (Plasmid #136124) or L1_HyR-Ck1 (Addgene, #136125) containing the transcription unit for chlorsulfuron or hygromycin selection respectively; and if necessary the L1 spacer vectors, pCk2_spacer (Addgene, #136072) or pCk3_spacer (Addgene, #136073).

Troubleshooting

A. Primer design and amplification of tRNA-gRNA parts using the pGTR plasmid as template

1 The tRNA-gRNA spacer specific primers with 4 bp overlapping overhangs for BbsI Golden Gate/Loop Assembly should have the following sequences:

BbsI enzyme recognition sequence highlighted with orange.
gRNA sequence shown with blue letters.
Overhang sequences for cloning into the acceptor vector, highlighted with red.
Sequence that is part of the gRNA scaffold or the tRNA highlighted with grey.

G-primer-F

5′ AGgaagacTACTCGAACAAAGCACCAGTGG 3′

“gRNA c Primer R” in the main Figure 1:

5′ GAgaagacTATAAAAC-LAST-gRNA-REVcompl-TGCACCAGCCGGGAATC 3′

“gRNA a Primer F” and “gRNA b Primer F” in the main Figure 1:

5′ GAgaagacATxxxxxxxxxGTTTTAGAGCTAGAA 3′

“gRNA a Primer R” and “gRNA b Primer R” in the main Figure 1:

5′ GAgaagacTAxxxxxxxxxTGCACCAGCCGGGAA 3′

Primer combination is:
1 - G-primer-F & gRNA a Primer R
2 - gRNA a Primer F & gRNA b Primer R
3 - gRNA b Primer F & gRNA c Primer R

Use
the pGTR plasmid as template

B- Loop assembly cloning of tRNA-gRNA parts into the L1 vector to combine tRNA-gRNA parts with the MpU6 promoter

2

Plasmid concentrations should be according to **(Sauret-Güeto et al. 2020)**. Aliquots of
the DNA part were prepared at a concentration of 15 nM and of the acceptor vector at a concentration of 7.5 nM
To calculate the concentration in ng/µL:
- For a final concentration of 15 nM, the concentration in [ng/µL] equals N (the length in bp of the plasmid) divided by 110. This is an approximation of the formula:
15·10^(-9)mol/L
x ((607.4 x N) + 157.9)g/mol x 10^(-6)L/µL x 10^9ng/g = concentration (ng/µL)
- For a final concentration of 7.5 nM, the concentration in [ng/µL] equals N divided by 220.
Prepare reaction master mix (in µL):

	MilliQ H2O	Up to 20
	BSA (1 mg/mL)	1.5
	10x T4 DNA Ligase buffer (NEB)	2
	T4 DNA Ligase at 400 U/µL (NEB, #M0202S)	1.5
	BbsI-HF 10 U/µL (NEB, #R3539)	1.5
	OP-074 or OP-075 plasmids	1
	tRNA-gRNA parts (Gel extracted)	1 µL per part

-Place samples on the thermocycler and incubate using the following program: Loop Assembly: [3 minutes at 37^{o}C and 4 minutes at 16^{o}C] x26, Termination: 5 minutes at 50^{o}C and 10 minutes at 80^{o}C

-Transform chemically competent using 7-10 µL
of reaction and plate on LB agar plates with 50 µg/mL kanamycin and 40 µg/mL
X-gal. Incubate at 37^{O}C for 16 h.

-Confirm with Sanger sequencing

C- Loop assembly cloning into the L2 pCsA acceptor to combine of tRNA-gRNA with the transcription unit for Cas9 expression

3

Prepare reaction master mix (in µL), plasmid concentrations
should be as above and according to **(Sauret-Güeto et al. 2020)**:

	MilliQ H2O	Up to 20
	10x T4 DNA Ligase buffer (NEB)	2
	T4 DNA Ligase at 400 U/µL (NEB, #M0202S)	1.5
	SapI 10 U/µL (NEB, #R0569S)	1.5
	OP-074 or/and OP-075 plasmids (or appropriate pCk spacers)	1 each
	Selection marker plasmid	1
	pCsA	1

-Place samples on the thermocycler and
incubate using the following program: Loop Assembly: [3 minutes at 37^{O}C
and 4 minutes at 16^{O}C] x26, Termination: 5 minutes at 50^{O}C
and 10 minutes at 80^{O}C

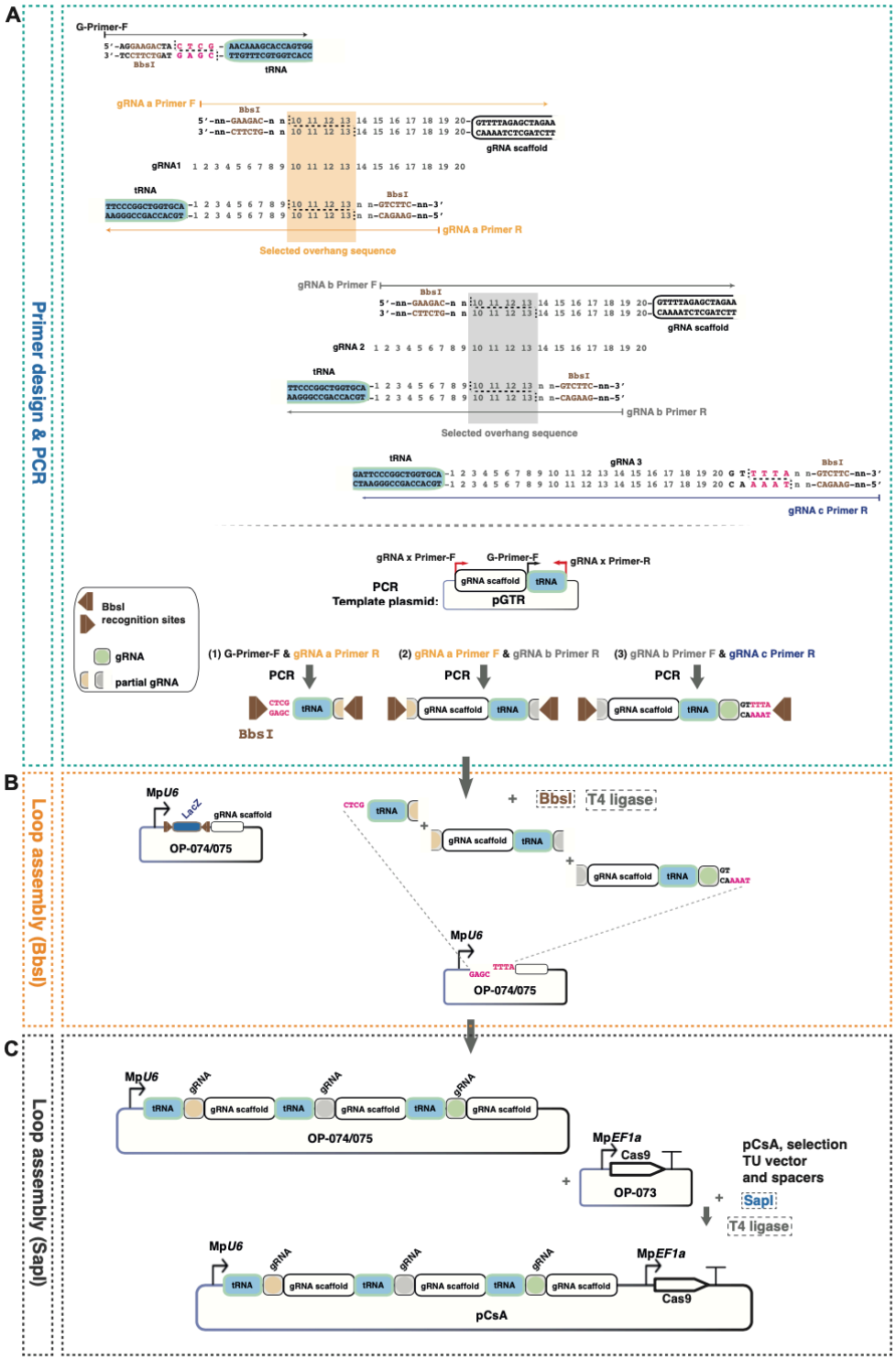
-Transform chemically competent using 10-12 µL
of reaction and plate on LB agar plates with 100 µg/mL spectinomycin and 40

μg/mL X-gal. Incubate at 37^{o}C for 16 h.

-Confirm with Sanger sequencing

Schematic diagram of the workflow for generating a tRNA-mediated multiplex gRNA expression construct

4


A
Primer design & PCR
G-Primer-F
tRNA
gRNA a Primer F
gRNA scaffold
gRNA1
gRNA a Primer R
Selected overhang sequence
gRNA b Primer F
gRNA 2
gRNA b Primer R
Selected overhang sequence
gRNA 3
gRNA c Primer R
gRNA x Primer-F
G-Primer-F
gRNA x Primer-R
PCR
Template plasmid:
pGTR
BbsI recognition sites
gRNA
partial gRNA
(1) G-Primer-F & gRNA a Primer R
(2) gRNA a Primer F & gRNA b Primer R
(3) gRNA b Primer F & gRNA c Primer R
PCR
BbsI
B
Loop assembly (BbsI)
MpU6
LacZ
gRNA scaffold
OP-074/075
BbsI
T4 ligase
C
Loop assembly (SapI)
gRNA
OP-074/075
MpEF1a
Cas9
OP-073
pCsA, selection
TU vector
and spacers
SapI
T4 ligase
pCsA

(A)
Top: The first primer includes a portion of the tRNA sequence along with a BbsI recognition site and four-base overhang sequences for Loop assembly. The middle primers contain BbsI recognition sites and four-base overlapping overhang sequences for Loop assembly, which can correspond to any four consecutive nucleotides (highlighted with colored rectangles) within the 20-nucleotide gRNA sequence (shown with numbers from 1 to 20). Forward middle primers also contain a portion of the gRNA scaffold sequence, whereas reverse middle primers contain a portion of the tRNA sequence. The final primer includes part of the tRNA sequence, the full reverse-complement of the last gRNA, and a BbsI recognition site plus four-base overhang sequences for Loop assembly. Overhangs for cloning into the acceptor vectors shown with pink letters. Bottom: The pGTR plasmid should be used as the
template for the PCR reactions. The primer combinations used for amplification are as follows: (1) G-primer-F & gRNA a Primer R, (2) gRNA a Primer F & gRNA b Primer R, and (3) gRNA b Primer F & gRNA c Primer R, n: any nucleotide .
(B)
After PCR amplification and gel extraction, all fragments are combined with the OP-074 or OP-075 vector (Sauret-Gueto et al., 2020) in a Loop Assembly/Type IIS cloning reaction. LacZ: lacZα cassette for blue-white screening of colonies (negative blue colonies
contain undigested L1 vectors, while positive white colonies contain tRNA-gRNA parts inserted into the L1 vectors.
(C)
Finally, the tRNA-gRNA-OP-074 or OP-075 vector is combined with the OP-073 vector, which contains the Mp*EF1a::Cas9*
transcription unit, an appropriate L1 vector for plant selection, in a Loop Assembly/Type IIS cloning reaction.

An example

5 An
example:

G-primer-F
5′ AGgaagacTACTCGAACAAAGCACCAGTGG 3′

gRNA14

Forward sequence: 5′ TTGCAGCAGTGGAGAAAAGA 3′
Reverse complement sequence: 5′ TCTTTTCTCCACTGCTGCAA 3′

GLK14-a-F
5′ GAgaagacATGTGGAGAAAAGAGTTTTAGAGCTAGAA3′
GLK14-a-R
5′ GAgaagacTACCACTGCTGCAATGCACCAGCCGGGAA3′

gRNA3
Forwardsequence: 5′ GGGCGAGGGCTTCAAGATAC 3′
Reverse complement sequence: 5′ GTATCTTGAAGCCCTCGCCC 3′

GLK3-b-F
5′ GAgaagacATGCTTCAAGATACGTTTTAGAGCTAGAA3′
GLK3-b-R
5′ GAgaagacTAAAGCCCTCGCCCTGCACCAGCCGGGAA3′

gRNA7

Forward sequence: 5′ AGGATATGGAGTGGGTTGCT 3′
Reverse complement sequence: 5′ AGCAACCCACTCCATATCCT 3′

Final-c-GLK7
5′ GAgaagacTATAAAACAGCAACCCACTCCATATCCTTGCACCAGCCGGGAATC 3′

Amplify
using PCR from pGTR plasmid, Gel extract and then directly clone into L1
plasmids using BbsI GG reaction

For the PCR, use Phusion (Thermo) or a similar proofreading polymerase

PCR
cycling conditions were: 98^{O}C (1:30), [98^{O}C (30sec), 55^{O}C (30sec), 72^{O}C (45sec)] x35, 72^{O}C (5min)

Primer combination is:

1 - G-primer-F & GLK3-b-R
2 – GLK3-a-F & GLK14-b-R
3 - GLK14-b-F & Final-c-GLK17

After cloning in the L2 acceptor the tRNA-gRNA
construct will be:

Protocol references

Sauret-Güeto S, Frangedakis E, Silvestri L, Rebmann M, Tomaselli M, Markel K, Delmans M, West A, Patron NJ, Haseloff J. 2020. Systematic Tools for Reprogramming Plant Gene Expression in a Simple Model, Marchantia polymorpha. ACS Synthetic Biology

Xie K, Minkenberg B, Yang Y. 2015. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proceedings of the National Academy of Sciences of the United States of America