

Supplemental Protocol: Golden Gate assembly for CRISPR-TSKO

INTRODUCTION

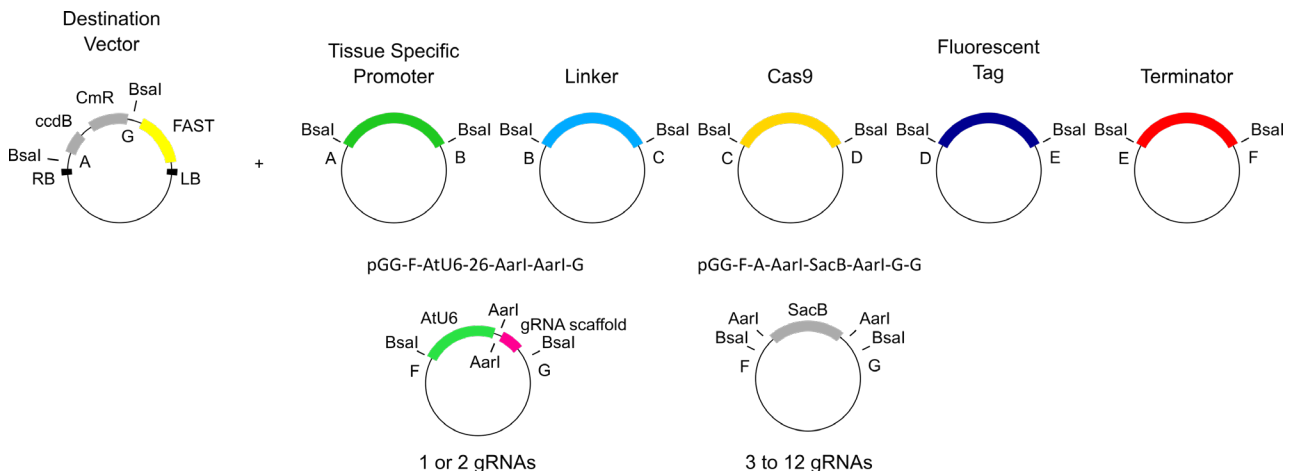
This cloning protocol makes use of the GreenGate cloning system (Lampropoulos et al., 2013), which is based on the Golden Gate method (Engler et al., 2008). Six DNA elements in entry vectors are assembled into a destination vector using a Golden Gate reaction with the type II restriction enzyme BsaI and T4 DNA ligase. This assembly is fast, efficient, and inexpensive.

CRISPR-TSKO is a technique to efficiently mutate genes in specific cell types, tissues, or organs using a tissue-specific promoter controlling Cas9 expression. We use two Golden Gate cloning steps to generate new CRISPR-TSKO destination vectors. In the first step (as described in **I**), a new cloning vector is assembled by combining a promoter, Cas9, N- and C-tags, and a terminator in entry vectors. Different F-G entry vectors allow the creation of two types of destination vectors that can accept either one or two gRNAs or multiple gRNAs (up to 12). The addition of one or two gRNAs requires a single Golden Gate reaction and is detailed in section **II**. The cloning of multiple or multiple gRNAs requires two Golden Gate steps and is described in section **III**.

I. GOLDEN GATE ASSEMBLY OF NEW TSKO DESTINATION VECTORS

A. Golden Gate

In the first step, six Golden Gate entry vectors are combined into a destination vector. There are various Golden Gate destination vectors containing different plant selectable and/or visual markers that can be used (see Golden Gate destination vector (*ccdB*⁺) in **Supplemental Data Set 1**). The first entry vector (AB) contains the promoter for tissue-specific expression. The third entry vector (CD) contains the nuclease and can be combined with N-terminal (BC) or C-terminal tags (DE). Alternatively, linker sequences are used if no tag is desired. The fifth entry vector (EF) contains the plant terminator. The sixth Golden Gate entry vector (FG) chosen depends on the final goal. To clone a vector compatible with one or two gRNAs, make use of the unarmed gRNA entry vector pGG-F-AtU6-26-AarI-AarI-G (see Unarmed gRNA entry vectors in **Supplemental Data Set 1**). To clone a vector compatible with multiple gRNAs, make use of the variable linker pGG-F-A-AarI-SacB-AarI-G-G (see Variable Linkers in **Supplemental Data Set 1**). As our cloning strategy uses the restriction enzymes BsaI and AarI, there is the requirement that all vectors need to be BsaI- and AarI-free (apart from the cloning sites).



The following components are mixed in a PCR tube:

Component		Amount	Volume (μL)
A-B entry vector	(Tissue-specific) Promoter	100 ng	1
B-C entry vector	N-tag or Linker	100 ng	1
C-D entry vector	Nuclease	100 ng	1
D-E entry vector	C-tag or Linker	100 ng	1
E-F entry vector	Terminator	100 ng	1
F-G entry vector	Variable	100 ng	1
Destination vector		100 ng	1
10X CutSmart Buffer		1X	1.5
10 mM ATP		1 mM	1.5
T4 DNA ligase		200 U	0.5
Bsal-HF [®] v2		10 U	0.5
MQ water			4
			15

Golden Gate reaction conditions:

37°C	3 min	30 x
16°C	3 min	
50°C	5 min	
80°C	5 min	
16°C	∞	

Golden Gate reaction conditions are flexible. We regularly use 2 minute steps for the 37°C and 16°C steps with 20 cycles. This reduces the reaction time to ca. 1 hour and 45 minutes. For difficult reactions, the step time and number of cycles can be increased.

Five μL of the reaction mixture is transformed into 50 μL DH5α *E. coli* cells via heat shock. The transformed cells are plated on LB medium containing 100 μg mL⁻¹ spectinomycin. The vector should be validated using colony PCR and restriction digestion. Primers and enzyme(s) vary depending on destination vector used.

Note: The TSKO Destination Vectors for multiple gRNAs contain the *SacB* gene. This gene encodes an enzyme that converts sucrose to levans, which accumulate in the periplasm and are toxic to *E. coli* (Gay et al., 1985). This allows for counter selection in subsequent cloning on LB medium containing 10% sucrose.

B. AarI restriction digestion

The TSKO destination vectors made in step **I.A**, or those available from our collection (**Supplemental Data Set 1**), have two AarI restriction sites. Upon digestion with AarI, these vectors contain four base pair overhangs that are used to directly load one or two gRNAs (as described in **II**) or multiple gRNAs (as described in **III**). More conveniently however, we replace the two AarI sites with two BsaI sites flanking *ccdB* and *CmR* expression cassettes (as described in **I.C**).

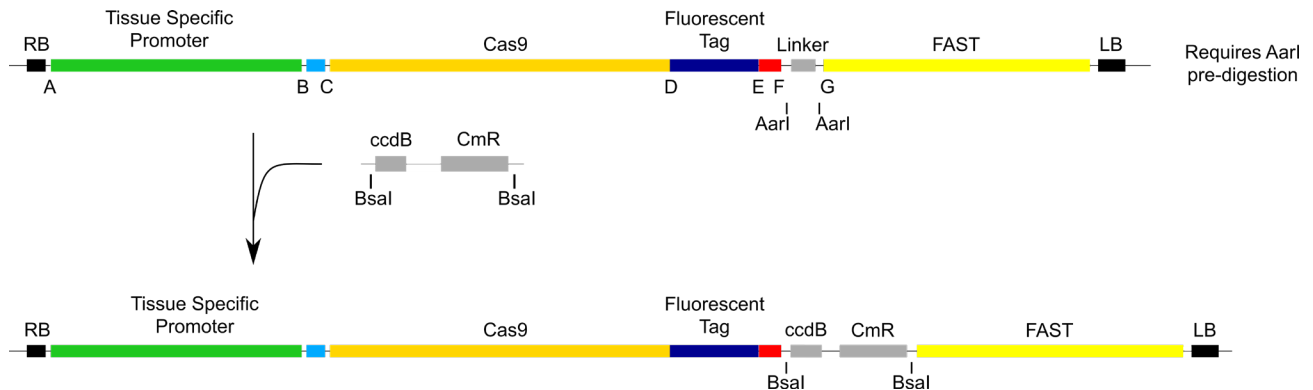
AarI digestion is performed according to the manufacturer's recommendations.

Component	Amount	Volume (μL)
Plasmid DNA (100 ng/μL)	1 μg	10.0
MQ water		7.1
Buffer AarI	1x	2.0
Oligonucleotides	0.5 μM	0.4
AarI	1 U	0.5
		20.0

Mix gently and spin down for a few seconds. Incubate at 37°C for 1-16 hours. Heat inactivate the AarI enzyme for 20 min at 65°C and confirm digestion by running ~100-200 ng on a 0.8% agarose gel. Alternatively, the digest can be gel purified. Include the undigested vector as a control and excise the digested fragment when it has separated from the uncut band.

Expected bands:

- The TSKO destination vectors compatible with cloning one or two gRNAs generate two fragments. The smallest fragment (32 bp) is too small to be visualized. The largest fragment is shifted upwards compared to the undigested vector.
- The TSKO destination vectors compatible with cloning multiple gRNAs generate two fragments. The fragment containing the *SacB* selectable marker is 1,898 base pairs long. The largest fragment is > 10 kb.

C. Replacement of AarI restriction sites for BsaI restriction sites

A fragment containing the *ccdB* and *CmR* selectable markers flanked by BsaI sites is PCR amplified with Q5® High-Fidelity DNA Polymerase from the vector pEN-L4-A-G-R1. The primers for this reaction depend on the vector that you are cloning.

- To clone a one-step vector compatible with 1 or 2 gRNAs, the PCR is performed with oligos 1436 & 1437 (resulting in a 1451 bp fragment). Following BsaI digestion, this fragment will have the ATTG and AAAC overlaps corresponding to the AtU6-26 promoter and SpCas9 scaffold sequences, respectively.
- To clone a vector compatible with multiple gRNAs, the PCR is performed with oligo 23 & 24 (resulting in a 1834 bp fragment). Following BsaI digestion, this fragment will have the ACCT and ATAC overhangs corresponding to the A and G sites in the GreenGate cloning system.

Mix the following components in a PCR tube:

Component	Amount	Volume (μL)
pEN-L4-A-G-R1 (100 ng/μL)	50 ng	0.5
FW primer (10 μM)	0.5 μM	2.5
REV primer (10 μM)	0.5 μM	2.5
dNTPs	200 μM	1
Q5® Reaction Buffer	1X	10
Q5® High-Fidelity DNA Polymerase	1U	0.5
MQ water		33
		50*

Reaction conditions:

98°C	30 sec	34 x
98°C	10 sec	
T _a **	30 sec	
72°C	1 min	
72°C	2 min	
16°C	∞	

* The manufacturer's recommended final volume is 50 µL. This can however, be reduced to 10 µL.

** 1436 + 1437 (T_a = 67°C) and 23 + 24 (T_a = 57°C), as calculated by the NEB Tm Calculator (<http://tmcalsculator.neb.com/#!/main>)

Purify from gel and digest with BsaI

Component	Amount	Volume (µL)
PCR product	1 µg	
10X CutSmart buffer	1X	5
BsaI-HF [®] v2	10 U	0.5
MQ water		
		50

Mix gently and spin down for a few seconds. Incubate at 37°C for at least 1 hour, and heat inactivate the BsaI-HF[®]v2 enzyme for 20 min at 80°C.

The following components are mixed in a microcentrifuge tube:

Component	Amount	Volume (µL)
AarI digested vector		1
BsaI digested PCR product		1
T4 DNA Ligase	400 U	1
10X T4 DNA Ligase buffer	1X	1
MQ water		6
		10

Gently mix the reaction and microfuge briefly. According to the manufacturer's recommendation, incubate at 16°C overnight or at room temperature for 10 minutes. We, however, generally incubate for at least 1 h (at room temperature). Heat inactivate at 65°C for 10 minutes. Chill on ice and transform 1-5 µL of the reaction mixture into 50 µL One Shot[™] ccdB Survival[™] 2 T1R Competent Cells via heat shock. Transformed cells are selected on LB medium containing 25 µg µL⁻¹ chloramphenicol. Alternatively, for destination vectors containing a SacB selectable marker, negative selection can be performed on LB medium containing 10% sucrose. The inserted fragment is verified via Sanger sequencing, and the vector should be validated by colony PCR and restriction digestion (NheI is recommended).

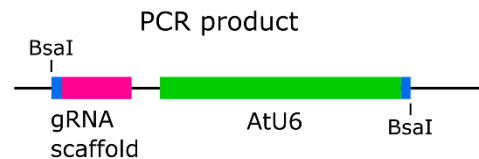
B. Two gRNAs: PCR product

To clone two gRNAs in the destination vector, we use the following approach as described by (Xing et al., 2014) with some modifications. **BsaI sites** in bold.

- FW: 5'-TTTT**GGTCTCA**ATTG-N₂₀-GTTT**AGAGCTAGAAATAGC** with N₂₀ being the protospacer of the first gRNA
- REV: 5'-TTTT**GGTCTCA**AAAC-N₂₀-CAAT**CACTACTTCGACTC** with N₂₀ being the reverse complement of the protospacer of the second gRNA

Component	Amount	Volume (μL)
pEN-2xAtU6 template	50 ng*	0.5
10 μM FW primer	0.5 μM	2.5
10 μM REV primer	0.5 μM	2.5
10 mM dNTPs	200 μM	1
5X Q5® Reaction Buffer	1X	10
Q5® High-Fidelity DNA Polymerase	0.02 U/μl	0.5
MQ water		33
		50**

98°C	30 sec	34 x
98°C	10 sec	
55°C***	15 sec	
72°C	15 sec	
72°C	2 min	
16°C	∞	



* This is the amount of plasmid used for this report. We routinely use less (≤ 1 ng).

** The manufacturer's recommended final volume is 50 μL. However, we routinely scale this down to 10 μL.

*** As calculated by the NEB Tm Calculator (<http://tmcalculator.neb.com/#!/main>) using the invariable 3' sequence of the primer.

The products are run on a 0.8% agarose gel and the 575 bp band is gel purified. Gel purification is highly recommended to ensure that no primer dimers are incorporated.

The following components are mixed in a PCR tube:

Component	Amount	Volume (μL)
PCR product (575 bp band)	~10 ng	1
Destination vector	100 ng	1
10X CutSmart Buffer	1X	1.5
10 mM ATP	1 mM	1.5
T4 DNA ligase	200 U	0.5
Bsal-HF®v2	5 U	0.5
MQ water		9
		15

37°C	3 min	30 x
16°C	3 min	
50°C	5 min	
80°C	5 min	
16°C	∞	

Five μL of the reaction mixture is transformed via heat shock into 50 μL DH5α *E. coli* cells. The cells are plated on LB medium containing 100 μg mL⁻¹ spectinomycin. Plasmids are validated by restriction digestion and the inserted fragment verified via Sanger sequencing. Primers and enzyme(s) vary depending on destination vector used.

The armed gRNA entry vectors are combined in a destination vector via a Golden Gate reaction. One or more linkers (see Linkers in **Supplemental Data Set 1**) can be used to span the unused positions. The following components are mixed in a PCR tube:

Component	Amount	Volume (μL)			
A-B armed gRNA	100 ng	1	37°C	3 min	30 x
B-C armed gRNA	100 ng	1		3 min	
C-D armed gRNA	100 ng	1		5 min	
D-E armed gRNA	100 ng	1		5 min	
E-F armed gRNA	100 ng	1		5 min	
F-G armed gRNA	100 ng	1		∞	
Destination vector	100 ng	1	80°C	5 min	
10X CutSmart Buffer	1x	1.5	16°C		
10 mM ATP	1 mM	1.5			
T4 DNA ligase	200 U	0.5			
Bsal-HF®v2	10 U	0.5			
MQ water		4			
		15			

Five μL of the reaction mixture is transformed into 50 μL DH5α *E. coli* cells via heat shock. The transformed cells are plated on LB medium containing 100 μg mL⁻¹ spectinomycin. The vector should be validated using colony PCR and restriction digestion. Sanger sequencing is not recommended, as the repetitive arrays of gRNAs usually cause the reactions to fail.

MATERIALS

<u>Product</u>	<u>Concentration</u>	<u>Provider</u>
CutSmart Buffer	10X	New England Biolabs
T4 DNA ligase	400,000 U/mL	New England Biolabs
Bsal-HF®v2	20,000 U/mL	New England Biolabs
BbsI-HF	20,000 U/mL	New England Biolabs
ATP	10 mM	ThermoFisher Scientific
NheI	10 U/mL	Promega Corporation
Buffer B	10X	Promega Corporation
Acetylated BSA	10 µg/µL	Promega Corporation
AarI	2 U/mL	ThermoFisher Scientific
Buffer AarI	10X	ThermoFisher Scientific
Oligonucleotides	50X (0.025 mM)	ThermoFisher Scientific
Q5® High-Fidelity DNA Polymerase	2,000 U/mL	New England Biolabs
Q5® Reaction Buffer	5X	New England Biolabs
10 mM dNTPs	10 mM	New England Biolabs
One Shot™ ccdB Survival™ 2 T1R Competent Cells		ThermoFisher Scientific
Zymoclean™ Gel DNA Recovery Kit		Zymo Research

Primers

Oligo 23	GTAAAACGACGGCCAG
Oligo 24	CAGGAAACAGCTATGAC
Oligo 1436	TTTTATTGTGAGACCGCGGCCGCATTAG
Oligo 1437	TTTAACTGAGACCGTCTGACTTATATTCCC
Oligo 62	CGACGGCCAGGTAATACGACT

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