ENABLE® Gene Editing *in planta* toolkit

Quick protocol for experienced users

This quick protocol is designed for experienced users of the ENABLE® Gene Editing *in planta* toolkit. The user must have ordered suitable oligos for subcloning their sgRNA sequences into monocot- or dicot-specific vectors as well as prepared vector stocks from vectors pGMF1 – pGMF6. For a more detailed protocol for beginners and further information, see:

https://www.growmorefoundation.org/enable-crispr-kits

PROTOCOL

Step 1: Subcloning of target sequences

- Adjust the concentration of your four oligos encoding your sgRNA target sequences to 10 µM with ddH_2O.
- In **two separate** tubes, for each sgRNA target sequence mix 2 µL of the corresponding complementary oligos and incubate for 5 minutes at room temperature.
- Choose either pGMF1-D and pGMF2-D for CRISPR experiments in **D**icot species or pGMF1-M and pGMF2-M for work in **M**onocot species
- Set up **two** restriction-ligation reactions in **two separate** PCR tubes, resulting in two vectors: pGMF1::annealed_oligo_pair1 and pGMF2::annealed_oligo_pair2.

Reaction 1:	
1 µL	pGMF1 (-M **or** -D, adjusted to 100 ng/ µL)
1 µL	Annealed Oligo Pair 1 (10 µM)
1.5 µL	T4 DNA Ligase buffer (10 x)
1.5 µL	Recombinant BSA (1 mg/ mL)
1 µL	BsaI-HF® (20 U/ µL)
1 µL	T4 DNA Ligase (400 U/ µL)
8 µL	Double distilled water

Reaction 2:	
1 µL	pGMF2 (-M **or** -D, adjusted to 100 ng/ µL)
1 µL	Annealed Oligo Pair 2 (10 µM)
1.5 µL	T4 DNA Ligase buffer (10 x)
1.5 µL	Recombinant BSA (1 mg/ mL)
1 µL	BsaI-HF® (20 U/ µL)
1 µL	T4 DNA Ligase (400 U/ µL)
8 µL	Double distilled water

- Place the tubes in a thermocycler and run the following program:

Step	Temperature	Time	
First restriction	37 ˚C	20 s	
Restriction	37 ˚C	3 min	50 x
Ligation	16 ˚C	4 min	
Heat inactivation I	50 ˚C	5 min	
Heat inactivation II	80 ˚C	5 min	
Hold	16 ˚C	until use	

- Transform 2 µL of each restriction-ligation reaction **separately** in **two** aliquots of competent *E. coli*.
- Grow transformed cells overnight at 37 °C on LB plates supplemented with **Ampicillin** (100 µg/mL), **IPTG** (0.5 mM), **X-Gal** (80 µg/mL).
- Inoculate a white *E. coli* colony from each transformation plate in LB media supplemented with **Ampicillin** (100 µg/mL). Grow overnight at 37 °C/300 rpm and extract plasmid DNA using standard methods.
- Verify successful subcloning using full plasmid sequencing **or** Sanger sequencing using primer GMF001 **or** restriction digest of plasmids using BbsI-HF®v2 (expected pattern for pGMF1/2-M based constructs: if positive 3147, 390 bp, if negative: 3147, 962 bp. Expected pattern for pGMF1/2-D based constructs: if positive 2370, 592 bp, if negative: 2370, 1164 bp) **or** with a PCR using primer pairs GMF001/GMF002 (expected product size for pGMF1/2-M based constructs: 670 bp if successful, 1242 bp if no integration occurred. Expected product size for pGMF1/2-D based constructs: 872 bp if successful, 1444 bp if no integration occurred).
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Step 2: Assembling the binary T-DNA vector

- Set up the following restriction-ligation reaction. Choose between adding pGMF5 (confers Hygromycin resistance *in planta*) or pGMF6 (confers eGFP expression for protoplast/transient expression experiments).

1 µL	pGMF1::annealed_oligo_pair1 (adjusted to 100 ng/ µL)
1 µL	pGMF2::annealed_oligo_pair1 (adjusted to 100 ng/ µL)
1 µL	pGMF3 (adjusted to 100 ng/ µL)
1 µL	pGMF4 (also known as pFH54, adjusted to 100 ng/ µL)
1 µL	pGMF5 (also known as pICSL11059) **or** pGMF6 (adjusted to 100 ng/ µL)
1.5 µL	T4 DNA Ligase buffer (10 x)
1.5 µL	Recombinant BSA (1 mg/ mL)
1 µL	BbsI-HF®v2 (20 U/ µL)
1 µL	T4 DNA Ligase (400 U/ µL)
5 µL	Double distilled water

- Place the tubes in a thermocycler and run the following program:

Step	Temperature	Time	
First restriction	37 ˚C	20 s	
Restriction	37 ˚C	3 min	50 x
Ligation	16 ˚C	4 min	
Heat inactivation I	50 ˚C	5 min	
Heat inactivation II	80 ˚C	5 min	
Hold	16 ˚C	until use	

- Transform 2 µL of the restriction-ligation reaction into competent *E. coli* following standard protocols.
- Grow cells overnight at 37 °C on LB plates supplemented with **Kanamycin** (50 µg/mL).
- Inoculate a white colony in LB media supplemented with **Kanamycin** (50 µg/mL). Grow overnight at 37 °C/300 rpm and extract plasmid DNA using standard methods.
- Verify successful assembly using full plasmid sequencing (recommended) **or** Sanger sequencing using primers GMF001, GMF002, GMF003 **or** restriction digest of plasmid using HincII (Expected pattern for final vector containing pGMF1/2-D and pGMF5 if positive 436, 678, 1537, 2128, 2206, 3227, 4520 bp. Expected pattern for final vector containing pGMF1/2-M and pGMF5 if positive 436, 678, 1537, 2128, 2206, 2823, 4520 bp. Expected pattern for final vector containing pGMF1/2-D and pGMF6 if positive 678, 1744, 2128, 2206, 3227, 4520 bp. Expected pattern for final vector containing pGMF1/2-M and pGMF6 if positive 678, 1744, 2128, 2206, 2823, 4520 bp).