pMGE vectors for genome editing in monocots [02/2018]

System overview

Vector description

| name          | name (other) | description                                                                 | resistance [plant]         |
|---------------|--------------|-----------------------------------------------------------------------------|----------------------------|
| **recipient (genome editing) vectors**                              |                                            |                             |
| pMGE598       | pLH_recipient-Hygro | LB_35S:hpt-t35S_pZmUbi:Cas9-tocs; ACTA-Bsal_ccdB-Cm\textsuperscript{R}_BsaI-GGGA_RB | Spec, Cm [Hygro]            |
| pMGE599       | pLH_recipient-Hygro 2xNLS | LB_35S:hpt-t35S_pZmUbi:Cas9(2xNLS)-tocs; ACTA-Bsal_ccdB-Cm\textsuperscript{R}_BsaI-GGGA_RB | Spec, Cm [Hygro]            |
| **sgRNA shuttle vectors – Oryza sativa U6 promoter (OsU6)** |                                             |                             |
| pMGE501       | pUC M1-OsU6   | Bsal-ACTA-link(M13)-pOsU6-GTTG-Bpil_ccdB-Cm\textsuperscript{R}_BsaI-GTTT-sgRNA scaff.-CGGT-Bsal | Amp, Cm                    |
| pMGE503       | pUC M2-OsU6   | Bsal-CGGT-pOsU6-GTTG-Bpil_ccdB-Cm\textsuperscript{R}_BsaI-GTTT-sgRNA scaff.-GCAC-Bsal | Amp, Cm                    |
| pMGE505       | pUC M2E-OsU6  | Bsal-CGGT-pOsU6-GTTG-Bpil_ccdB-Cm\textsuperscript{R}_BsaI-GTTT-sgRNA scaff.-GGGA-Bsal | Amp, Cm                    |
| pMGE509       | pUC M3-OsU6   | Bsal-GCAC-pOsU6-GTTG-Bpil_ccdB-Cm\textsuperscript{R}_BsaI-GTTT-sgRNA scaff.-AGCG-Bsal | Amp, Cm                    |
| pMGE507       | pUC M4-OsU6   | Bsal-AGCG-pOsU6-GTTG-Bpil_ccdB-Cm\textsuperscript{R}_BsaI-GTTT-sgRNA scaff.-CCAG-Bsal | Amp, Cm                    |
| pMGE508       | pUC M4E-OsU6  | Bsal-AGCG-pOsU6-GTTG-Bpil_ccdB-Cm\textsuperscript{R}_BsaI-GTTT-sgRNA scaff.-GGGA-Bsal | Amp, Cm                    |
| pMGE516       | pUC M1E-OsU6  | Bsal-ACTA-link(M13)-pOsU6-GTTG-Bpil_ccdB-Cm\textsuperscript{R}_BsaI-GTTT-sgRNA scaff.-GGGA-Bsal | Amp, Cm                    |
| pMGE512       | pUC M5-OsU6   | Bsal-CCAG-link(JS838)-pOsU6-GTTG-Bpil_ccdB-Cm\textsuperscript{R}_BsaI-GTTT-sgRNA scaff.-CCAG-Bsal | Amp, Cm                    |
| pMGE513       | pUC M6-OsU6   | Bsal-CCAG-pOsU6-GTTG-Bpil_ccdB-Cm\textsuperscript{R}_BsaI-GTTT-sgRNA scaff.-GGGA-Bsal | Amp, Cm                    |
| pMGE514       | pUC M7-OsU6   | Bsal-GTGT-pOsU6-GTTG-Bpil_ccdB-Cm\textsuperscript{R}_BsaI-GTTT-sgRNA scaff.-CGAA-Bsal | Amp, Cm                    |
| pDGE515       | pUC M8E-OsU6  | Bsal-CGAA-pOsU6-GTTG-Bpil_ccdB-Cm\textsuperscript{R}_BsaI-GTTT-sgRNA scaff.-GGGA-Bsal | Amp, Cm                    |
All vectors contain a ccdB cassette and must be propagated in DB3.1 or ccdB survival cells.
shuttle vectors:

pMGE501-516

\[ \text{BsaI} \] → \[ \text{pOsU6} \] → \[ \text{ccdB} \ → \text{Cm}^R \] → \[ \text{sgRNA} \] → \[ \text{BsaI} \]

\text{gL} = \text{GTG}^N_{19,20}
\text{gR} = \text{AAAC}^N_{19,20}

pMGE556-570

\[ \text{BsaI} \] → \[ \text{pTaU6} \] → \[ \text{ccdB} \ → \text{Cm}^R \] → \[ \text{sgRNA} \] → \[ \text{BsaI} \]

\text{gL} = \text{CTTG}^N_{19,20}
\text{gR} = \text{AAAC}^N_{19,20}

pMGE584-589

\[ \text{BsaI} \] → \[ \text{pHVU3} \] → \[ \text{ccdB} \ → \text{Cm}^R \] → \[ \text{sgRNA} \] → \[ \text{BsaI} \]

\text{gL} = \text{AGCA}^N_{19,20}
\text{gR} = \text{AAAC}^N_{19,20}

pMGE590-595

\[ \text{BsaI} \] → \[ \text{pOsU3} \] → \[ \text{ccdB} \ → \text{Cm}^R \] → \[ \text{sgRNA} \] → \[ \text{improved} \ → \text{BsaI} \]

\text{gL} = \text{GGCA}^N_{19,20}
\text{gR} = \text{AAAC}^N_{19,20}

pMGE624-629

\[ \text{BsaI} \] → \[ \text{pHVU3} \] → \[ \text{ccdB} \ → \text{Cm}^R \] → \[ \text{sgRNA} \] → \[ \text{BsaI} \]

\text{gL} = \text{AGCA}^N_{19,20}
\text{gR} = \text{AAAC}^N_{19,20}

genome editing vectors:

pMGE598

[Spec]

\[ \text{LB} \] → \[ 35S:hpt-t35S \] → \[ pZmUbi:Cas9-tocs \] → \[ \text{BsaI-L} \] → \[ \text{ccdB} \ → \text{Cm}^R \] → \[ \text{RB} \]

pMGE599

[Spec]

\[ \text{LB} \] → \[ 35S:hpt-t35S \] → \[ pZmUbi:Cas9(2xNLS)-tocs \] → \[ \text{BsaI-L} \] → \[ \text{ccdB} \ → \text{Cm}^R \] → \[ \text{RB} \]

The arrow below the ccdB cassette indicates the direction of the sgRNA array after cloning.
Selection and design of sgRNAs

goRNA coding sequences are introduced to the pMGE system as 23-24 nt long, hybridized oligonucleotides (oligos). Any PAM sequence (NGG) present in a target region can potentially serve as “anchoring point” for design of an sgRNA, although GC content, on target activity and off-targets should be considered. Note that the PAM sequence is not part of the actual sgRNA, but only present in the target sequence. See for example the following review for a list of tools for selecting target sites: In Silico Meets In Vivo: Towards Computational CRISPR-Based sgRNA Design (Chuai GH, Wang QL, Liu Q; Trends Biotechnol. 2017 Jan;35(1):12-21. doi: 10.1016/j.tibtech.2016.06.008). Below, examples of (randomly chosen) target sites on plus and minus strands, and design of respective oligos to produce sgRNAs with the pMGE vector system, are given:

sgRNA and oligonucleotide design for U6 promoter shuttle vectors:

sgRNA1:
Oligo 1 (g/c)ttgCTTGTCTAACCATCAATTTG
Oligo 2 aaacCAAATTGATGGTTAGACAAG

sgRNA2:
Oligo 1 (g/c)ttgTTGTGCTGTCCACAAATTGA
Oligo 2 aaacTCAATTTGTGGACAGCACAA

sgRNA3:
Oligo 1 (g/c)ttgCTTGTCTAACCATCAATTTG
Oligo 2 aaacCAAATTGATGGTTAGACAAG

sgRNA4:
Oligo 1 (g/c)ttgCTTGTCTAACCATCAATTTG
Oligo 2 aaacCAAATTGATGGTTAGACAAG

Oligo 1 has to start with gttg for pOsU6 vectors

Oligo 1 has to start with cttg for pTaU6 vectors

The “G” within the “gttg” or “cttg” used as cloning overhang (Oligo 1) is the transcription start site. sgRNA3 and sgRNA4 are examples for target sites G(N)19NGG. In this case, 23 nt oligos may be used, and the variable part of the sgRNA will be 20 nt in length with perfect complementarity. sgRNA1 and sgRNA2 are examples for target sites (N)20NGG. In this case, 24 nt oligos should be used. This will result in a sgRNA variable part of 21 nt in length, starting with a non-complementary “G” followed by 20 nt complementary to the target site. To our experience, the “dangling G” when addressing (N)20NGG target sites does not have negative effects on Cas9 efficiency, but this was not evaluated in detail.
sgRNA and oligonucleotide design for U3 promoter shuttle vectors:

sgRNA5:
Oligo 1 (a/g)gcaGGAAGGGCCGAGCGCAGAAG
Oligo 2 aaacCTTCTGCGCTCGGCCCTTCC

sgRNA6:
Oligo 1 (a/g)gcaCTGCGCTCGGCCCTTCCGGC
Oligo 2 aaacGCCGGAAGGGCCGAGCGCAG

sgRNA7:
Oligo 1 (a/g)gcAACTTTATCCGCCTCCATCC
Oligo 2 aaacGGATGGAGGCGGATAAAGT

sgRNA8:
Oligo 1 (a/g)gcATGGAGGCGGATAAAGTTGC
Oligo 2 aaacGCAACTTTATCCGCCTCCA

Oligo 1 has to start with agca for pHvU3 vectors

Oligo 1 has to start with ggca for pOsU3 vectors

The “A” within the “agcA” or “ggcA” used as cloning overhang (Oligo 1) is the transcription start site. sgRNA7 and sgRNA8 are examples for target sites A(N)19NGG. In this case, 23 nt oligos may be used, and the variable part of the sgRNA will be 20 nt in length with perfect complementarity. sgRNA5 and sgRNA6 are examples for target sites (N)20NGG. In this case, 24 nt oligos should be used. This will result in a sgRNA variable part of 21 nt in length, starting with a non-complementary “A” followed by 20 nt complementary to the target site. To our experience, the “dangling A” when addressing (N)20NGG target sites does not have negative effects on Cas9 efficiency, but this was not evaluated in detail.

Not allowed within sgRNA sequences:
Bsal sites [GGTCTC], BpiI sites [GAAGAC], polyT stretches [≥ 5 Ts; transcriptional termination]

Note for sgRNA cloning procedures:
The overhangs used for cloning of hybridized oligos in vectors containing the OsU6 promoter are not ideal. The sticky ends from BpiI digestion (GTTG/GTTT) can re-ligate at a certain frequency, as there is only one nt mismatch. Normally, ~ 10 % of clones on a plate will be the empty vector without the ccd8 cassette. Increasing amount of hybridized oligo may help. We advise use of polyclonal plasmid preparations (see below) when working with shuttle vectors.
**Cloning and utilization of “one step” vectors**

If desired, M1E modules (containing any U3/U6) promoters can be transferred into “recipient” vectors by BsaI cut/ligation to create “one step, one nuclease” vectors as previously described (pDGE62-65 in Ordon et al., 2017, Plant Journal: Generation of chromosomal deletions in dicotyledonous plants employing a user-friendly genome editing toolkit). BsaI cut/ligation reactions are essentially prepared as described (page 8), but should be terminated by a final cycle of BsaI digestion (after denaturation) to ligate any remaining “recipient” vector. The following steps should be subsequently conducted to load one step vectors with oligonucleotides to generate functional nucleases:

1. **Hybridization of oligos**
   - oligonucleotide stock concentration: 100 µM
   - mix oligos at 10 µM (for example 5 µl of each oligo + 40 µl H2O)
   - denature oligos by heating to 98 °C for 5 min
   - let cool down slowly (leaving tube @ RT for several minutes is sufficient)
   - prepare a 1:100 dilution (100 fmol / µl) of the hybridized oligos

2. **Loading of oligos into recipient vectors**

   cut/ligation reaction:

   | 20 fmol = 230 ng | pMGE574/575/596/597 | 37 °C | 2 min | 10-30 cycles |
   |------------------|---------------------|-------|--------|--------------|
   | 100 fmol = 1 µl  | hybridized oligos   | 16 °C | 5 min  |              |
   | 1 µl             | 10 x Ligation buffer| 50 °C | 10 min |              |
   | 1 µl             | 10 x BSA (1mg/ml)   | 80 °C | 10 min |              |
   | 0,5 µl           | BpiI                |       |        |              |
   | 0,5 µl           | T4 DNA Ligase (1 u/µl)|     |        |              |
   | 10 µl            | H2O                 |       |        |              |
   | 10 µl            | Total               |       |        |              |

   - transform cut/ligation reaction into Dh10b/TopTen cells
   - plate on LB-Spec media
   - start liquid cultures from 1-2 colonies for plasmid preparation
   - sequence sgRNA fraction (Primer JS1057)
   - transform into your favorite *Agrobacterium* strain
Cloning of multiplex genome editing constructs (pMGE598, pMGE599)

The cloning of multiplex genome editing constructs necessitates first the loading of sgRNA shuttle vectors with hybridized oligos by BpiI cut/ligation to generate the desired sgRNA transriptional units (sgRNA TUs). Subsequently, 2, 4, or 8 sgRNA TUs are assembled in a genome editing vector by BsaI cut/ligation to generate a final construct for plant transformation. Modules are named according to their position within the final sgRNA array, and modules for ending the array (circularization of the vector) are named with an additional “E”. Modules containing different U3/U6 promoters may be combined, if desired. In the following, compatible combinations using only one type of U3/U6 promoter are listed:

| number of sgRNA TUs | Pol III promoter | derivatives of pMGE for sgRNA array assembly |
|---------------------|------------------|---------------------------------------------|
| 2 sgRNA TUs         | OsU6             | pMGE501, 503, 505                           |
|                     | TaU6             | pMGE566, 568                                |
|                     | HvU3             | pMGE585, 587                                |
|                     | OsU3             | pMGE591, 593                                |
|                     | HvU3 (impr. sgRNA scaffold) | pMGE625, 627                         |
| 4 sgRNA TUs         | OsU6             | pMGE501, 503, 509, 508                      |
|                     | TaU6             | pMGE566, 567, 569, 570                      |
|                     | HvU3             | pMGE585, 586, 588, 589                      |
|                     | OsU3             | pMGE591, 592, 594, 595                      |
|                     | HvU3 (impr. sgRNA scaffold) | pMGE625, 626, 628, 629                   |
| 8 sgRNA TUs         | OsU6             | pMGE501, 503, 509, 507, 512, 513, 514, 515 |

1. Hybridization of oligos
   - oligo stock concentration: 100 µM
   - mix oligos at 10 µM (for example 5 µl of each oligo + 40 µl H₂O)
   - denature oligos by heating to 98 °C for 5 min
   - let cool down slowly (leaving tube @ RT for several minutes is sufficient)
   - prepare a 1:100 dilution (100 fmol / µl) of the hybridized oligos

2. Loading of oligos = guide sequences into sgRNA shuttle vectors

| cut/ligation reaction: | shuttle vector | 37 °C | 2 min | 10-30 cycles | 16 °C | 5 min | Hydrolase | 50 °C | 10 min | H₂O | 80 °C | 10 min |
|-----------------------|----------------|-------|-------|--------------|-------|-------|-----------|-------|--------|------|-------|--------|
| 20 fmol = 60 ng       |                 |       |       |              |       |       |           |       |        |      |       |        |
| 100 fmol = 1 µl       |                 |       |       |              |       |       |           |       |        |      |       |        |
| 1 µl                  |                 |       |       |              |       |       |           |       |        |      |       |        |
| 1 µl                  |                 |       |       |              |       |       |           |       |        |      |       |        |
| 0,5 µl                |                 |       |       |              |       |       |           |       |        |      |       |        |
| 0,5 µl                |                 |       |       |              |       |       |           |       |        |      |       |        |
| 10                    |                 |       |       |              |       |       |           |       |        |      |       |        |

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monoclonal approach:
- transform cut/ligation reaction into Dh10b/TopTen cells
- plate on selective media (Amp)
- start liquid cultures from 1-2 colonies for plasmid preparation
- sequence using M13r primer
  [Do not use M13f for M1/M1E modules]

polyclonal approach *(advised)*:
- transform cut/ligation reaction into Dh10b/TopTen cells
- directly inoculate liquid cultures with transformed cells in selective media
- use liquid cultures for polyclonal plasmid preparation

3. Assembly of sgRNA TUs in genome editing vector

cut/ligation reaction:

| 20 fmol = 230 ng | recipient vector | 37 °C | 2 min | 30-50 cycles |
|------------------|------------------|-------|-------|--------------|
| 20 fmol = 40 ng  | sgRNA TU shuttle vectors | 16 °C | 5 min | 50 °C | 10 min |
| 2 µl             | 10 x Ligation buffer | 50 °C | 10 min |
| 2 µl             | 10 x BSA (1mg/ml) | 80 °C | 10 min |
| 1 µl             | BsaI | |
| 1 µl             | T4 DNA Ligase (1-5 u/µl) | |
| H₂O              | | |
| 20               | Total | |

- transform cut/ligation reaction into Dh10b/TopTen cells
- plate on selective media (Spec)
- 2 sgRNA TUs: Start liquid cultures from 2 clones
- 4 sgRNA TUs: Start liquid cultures from 2-4 clones
- 8 sgRNA TUs: Start liquid cultures of 4-6 colonies directly or do colony PCR on 8-16 clones.
- verify plasmids by restriction digest
- confirm sgRNA array by DNA sequencing (Primers JS1057 and/or M13f)
- transform plasmid into your favorite *Agrobacterium* strain

Previously used oligos for DNA sequencing:

| name   | sequence          | location         |
|--------|-------------------|------------------|
| M13f   | GTTTTCCAGTCAGAC   | sgRNA TU 1, fwd  |
| JS838  | GCCAGCTTCTAGTACTGA | sgRNA TU 3 or 5, fwd |
| JS1057 | CATCAGACAAACCGCCAG| vector, rev     |

Additional oligos can be designed in linker sequences included in shuttle vectors.