pDGE Dicot Genome Editing vectors: Cloning Manual

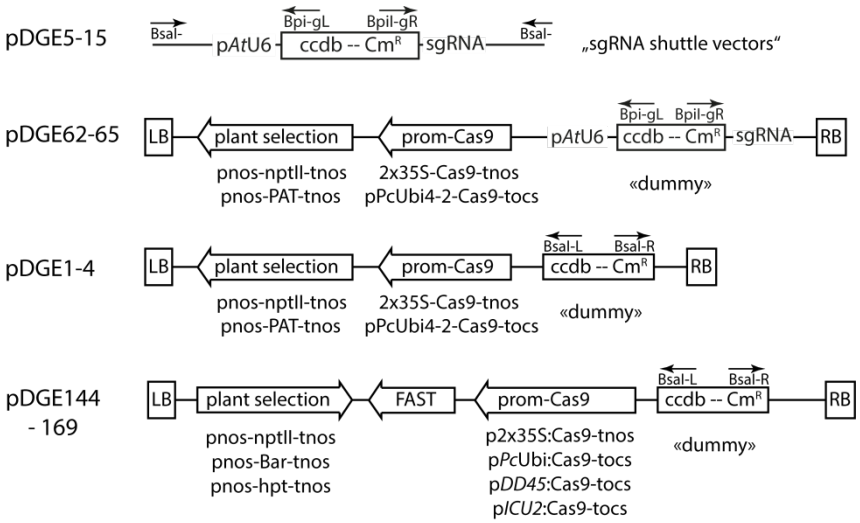
Vector description

name	name (other)	description	resistance [plant]
pDGE5	pUC M1	BsaI-TTGC-link-pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.-CCCT-BsaI	Amp, Cm
pDGE6	pUC M1E	BsaI-TTGC-link-pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.-GCTT-BsaI	Amp, Cm
pDGE7	pUC M2	BsaI-CCCT-pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.-CGGT-BsaI	Amp, Cm
pDGE8	pUC M2E	BsaI-CCCT-pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.-GCTT-BsaI	Amp, Cm
pDGE9	pUC M3	BsaI-CGGT-pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.-ATGG-BsaI	Amp, Cm
pDGE10	pUC M4	BsaI-ATGG-pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.-GACT-BsaI	Amp, Cm
pDGE11	pUC M4E	BsaI-ATGG-pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.-GCTT-BsaI	Amp, Cm
pDGE12	pUC M5	BsaI-GACT-link-pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.-TCCG-BsaI	Amp, Cm
pDGE13	pUC M6	BsaI-TCCG-pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.-CCAG-BsaI	Amp, Cm
pDGE14	pUC M7	BsaI-CCAG-pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.-CGAA-BsaI	Amp, Cm
pDGE15	pUC M8E	BsaI-CGAA-pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.-GCTT-BsaI	Amp, Cm
pDGE1	35SCas_nptII_ccdB	pnos:nptII-tnos; 2x35S:Cas9-tnos; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [Kan]
pDGE2	35SCas_PAT_ccdB	pnos:PAT-tnos; 2x35S:Cas9-tnos; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [BASTA]
pDGE3	pUbiCas_nptII_ccdB	pnos:nptII-tnos; pPcUbi:Cas9-tocs; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [Kan]
pDGE4	pUbiCas_PAT_ccdB	pnos:PAT-tnos; pPcUbi:Cas9-tocs; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [BASTA]
pDGE62	1sg_35SCas_nptII	pnos:nptII-tnos; 2x35S:Cas9-tnos; pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.	Spec, Cm [Kan]
pDGE63	1sg_35SCas_PAT	pnos:PAT-tnos; 2x35S:Cas9-tnos; pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.	Spec, Cm [BASTA]
pDGE64	1sg_pPcUbiCas_nptII	pnos:nptII-tnos; pPcUbi:Cas9-tocs; pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.	Spec, Cm [Kan]
pDGE65	1sg_pPcUbiCas_PAT	pnos:PAT-tnos; pPcUbi:Cas9-tocs; pAtU6-ATTG-BpiI_ccdB-Cm^R_BpiI-GTTT-sgRNA scaff.	Spec, Cm [BASTA]
pDGE144	Bar_FAST_35S:Cas9	pnos:Bar-tnos; FAST; 2x35S:Cas9-tnos; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [BASTA]
pDGE146	Bar_FAST_Ubi:Cas9	pnos:Bar-tnos; FAST; pPcUbi:Cas9-tocs; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [BASTA]
pDGE148	Bar_FAST_DD45:Cas9	pnos:Bar-tnos; FAST; pDD45:Cas9-tnos; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [BASTA]
pDGE151	Bar_FAST_ICU2:Cas9	pnos:Bar-tnos; FAST; ICU2:Cas9-tnos; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [BASTA]
pDGE160	nptII_FAST_35S:Cas9	pnos:nptII-tnos; FAST; 2x35S:Cas9-tnos; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [Kan]
pDGE161	nptII_FAST_Ubi:Cas9	pnos:nptII-tnos; FAST; pPcUbi:Cas9-tocs; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [Kan]

pDGE164	nptII_FAST_DD45:Cas9	pnos:nptII-tnos; FAST; pDD45:Cas9-tnos; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [Kan]
pDGE165	nptII_FAST_ICU2:Cas9	pnos:nptII-tnos; FAST; ICU2:Cas9-tnos; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [Kan]
pDGE166	hpt_FAST_35S:Cas9	pnos:hpt-tnos; FAST; 2x35S:Cas9-tnos; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [Hyg]
pDGE167	hpt_FAST_Ubi:Cas9	pnos:hpt-tnos; FAST; pPcUbi:Cas9-tocs; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [Hyg]
pDGE168	hpt_FAST_DD45:Cas9	pnos:hpt-tnos; FAST; pDD45:Cas9-tnos; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [Hyg]
pDGE169	hpt_FAST_ICU2:Cas9	pnos:hpt-tnos; FAST; ICU2:Cas9-tnos; TTGC-BsaI_ccdB-Cm^R_BsaI-GCTT	Spec, Cm [Hyg]

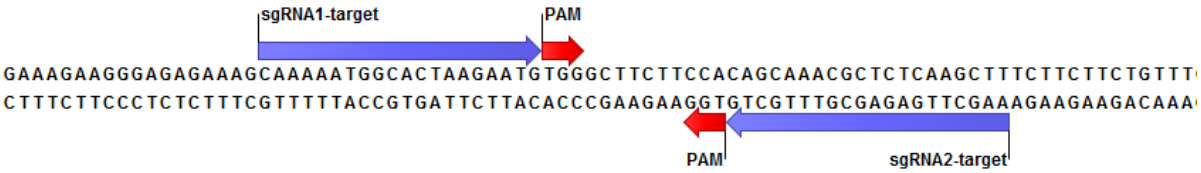
All empty vectors contain a ccdB cassette and must be propagated in DB3.1 or ccdB survival cells.

Overall vector architecture:



Selection and design of guide RNAs

sgRNA coding sequences are introduced to the pDGE system as 24 nt long, hybridzed oligos. Any PAM sequence (NGG) present in a target region can potentially serve as "anchoring point" for design of a guide RNA, 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: Editing plant genomes with CRISPR/Cas9 (Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V; Curr Opin Biotechnol. 2015 Apr; 32:76-84.). Below, examples of target sites on plus and minus strands, and design of respective oligos to produce sgRNAs with the pDGE vector system, are given::



sgRNA1:

Oligo 1	attgCAAAAATGGCACTAAGAATG
Oligo 2	aaacCATTCTTAGTGCCATTTTTG

sgRNA2:

Oligo 1	attgAAGCTTGAGAGCGTTTGCTG
Oligo 2	aaacCAGCAAACGCTCTCAAGCTT

The "G" within the "ATTG" used as cloning overhang (Oligo 1) is the transcription start site. Resulting guide sequences will be 21 nt in length, starting with an, in most cases, non-complementary G residue. This 5' extension will generally not affect efficiency. Alternatively, $G(N)_{19}NGG$ target sites may be chosen, and 23 nt oligos are then used for generation of guide sequences.

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

Cloning of simple nuclease constructs (pDGE62-65)

1. Hybridization of oligonucleotides
   - oligonucleotide stock concentration: 100 µM
   - mix oligos at 10 µM (for example 5 µl of each oligo + 40 µl H_2O)
   - denature oliogos by heating to 98 °C for 5 min
   - let cool down slowly (leaving tube @ RT for several minutes is sufficient)
   - prepare a 1:200 dilution (50 fmol / µl) of the hybridized oligos

2. Loading of oligonucleotides = guide sequences into genome editing recipient vectors

cut/ligation reaction:

20 fmol ≈ 200 ng	pDGE62/63/64/65	37 °C	2 min	10-30 cycles
50 fmol = 1 µl	hybridzed 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)			
	H2O			
10	Total			

- Transform cut/ligation reaction into Dh10b/TopTen cells
- Plate on LB-Spec media
- Start liquid cultures from 1-2 colonies for plasmid preparation
  Note: Clonings are essentially free of background. Verification of a single clone will normally be sufficient
- Sequence sgRNA with pPMR primer
- Transform into your favorite *Agrobacterium* strain

Cloning of multiplex genome editing constructs (pDGE1-4)

The cloning of multiplex genome editing constructs necessitates first the loading of sgRNA shuttle vectors (pDGE5-15) with hybridized oligos by BpiI cut/ligation to generate the desired sgRNA transcriptional units (sgRNA TUs). Subsequently, 2, 4, or 8 sgRNA TUs are assembled in a genome editing recipient vector (pDGE1-4, pDGE144-169) by BsaI cut/ligation to generate a final construct for plant transformation. The following modules and their derivatives, respectively, have compatible overhangs for assembly in recipient vectors:

2 sgRNA TUs: pDGE5, pDGE8
4 sgRNA TUs: pDGE5, pDGE7, pDGE9, pDGE11
8 sgRNA TUs: pDGE5, pDGE7, pDGE9, pDGE10, pDGE12, pDGE13, pDGE14, pDGE15

1. Hybridization of oligonucleotides
   - oligonucleotide stock concentration: 100 µM
   - mix oligos at 10 µM (for example 5 µl of each oligo + 40 µl H_2O)
   - denature oligos by heating to 98 °C for 5 min
   - let cool down slowly (leaving tube @ RT for several minutes is sufficient)
   - prepare a 1:200 dilution (50 fmol / µl) of the hybridized oligos

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

cut/ligation reaction:

20 fmol ≈ 60 ng	pDGE5-15	37 °C	2 min	10-30 cycles
50 fmol = 1 µl	hybridzed 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)			
	H2O			
10	Total			

- Transform cut/ligation reaction into Dh10b/TopTen cells
- Plate on LB-Amp or LB-Carb media
- Start liquid cultures from 1-2 colonies for plasmid preparation
  Note: Clonings are essentially free of background. Verification of a single clone will normally be sufficient
- Perform test digest if desired; e.g. using PvuII
  Note: PvuII digestion will yield a 2350 bp backbone fragment and a 550 bp insert fragment in most cases. Digestion of pDGE12 derivatives will yield a 700 bp insert fragment. The linker sequence in pDGE5 contains an additional PvuII site, yielding 160 bp and 530 bp insert fragments. Fragment sizes will be different if cloned guide sequences contain PvuII sites.
- Confirm guide sequences by DNA sequencing if desired; M13f or M13r primers may be used.
  Note: Sequence verification of the final sgRNA array after assembly in a genome editing recipient vectors is generally sufficient.

3. Assembly of sgRNA TUs in genome editing recipient vectors (pDGE1-4, pDGE144-169)

cut/ligation reaction:

20 fmol ≈ 220 ng	pDGE1-4 or pDGE144-169	37 °C	2 min	30-50 cycles
20 fmol ≈ 40 ng	sgRNA TU shuttle vectors	16 °C	5 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 LB-Spec media
- 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 using oligos JS838/pPMR (pDGE1-4) or oligos JS838/JS1132 (pDGE144-169); start liquid cultures from 2 positive clones
- verify plasmids by restriction digest (see table below)
- confirm sgRNAs by DNA sequencing:
  2 sgRNA TUs: Primer pPMR (pDGE1-4); JS1132 (pDGE144-169)
  4 sgRNA TUs: Primer pPMR and/or JS811 (pDGE1-4); JS1132 and/or JS811 (pDGE144-169)
  8 sgRNA TUs: Primers pPMR and JS811 (pDGE1-4); JS1132 and JS811 (pDGE144-169). Primers JS838 and JS839 may additionally be used, if necessary.
- transform plasmid into your favorite *Agrobacterium* strain

Useful restriction digests:

plasmid	sgRNA TUs	Enzyme(s)	Buffer (Thermo)	fragments***
pDGE1	2	PstI	O	**717**, 7941, 953, 308, 1216, 238, 1972, 1137
pDGE1	4	HincII, BamHI	Y	**1914**,1402, 8123*, 564, 1392, 75, 1380
pDGE1	8	PstI, SmaI	Y, 30°C	**2033**, 7941, 953, 308, 1216,238, 847, 1125, 1137
pDGE2	2	PstI	O	**717**, 308, 1216, 238, 1972, 1137, 8263
pDGE2	4	PstI, EcoRV		**1113**, 308, 1216, 238, 1972, 218, 919, 8263
pDGE2	8	PstI, SmaI	Y, 30°C	**2033**, 308, 1216, 2378, 847, 1125, 1137, 8263
pDGE3	2	PstI	O	**717**, 1404, 308, 1216, 238, 1972, 1266, 7941
pDGE3	4	HincII, BamHI	Y	**2034**, 564, 1392, 75, 607, 782, 1402, 8574*
pDGE3	8	PstI, SmaI	Y, 30°C	**2033**, 1404, 308, 1216, 238, 847, 1125, 1266, 7941
pDGE4	2	PstI	O	**717**, 308, 1216, 238, 1972, 1266, 8714
pDGE4	4	PstI	O	**1113**, 308, 1216, 238, 1972, 1266, 8714
pDGE4	8	PstI, SmaI	Y, 30°C	**2033**, 308, 1216, 238, 847, 1125, 1266, 8714

* an additional HincII annotated in maps (flanking the Spect resistance) is apparently not present

** XbaI site in pMGE500 blocked by methylation

*** The fragment depicted in bold corresponds to the sgRNA array. Sizes considering that no additional restriction sites are present within cloned sgRNA sequences

useful oligonucleotides:

name	sequence	location
pPMR	AGCGAAACCCTATAAGAA	vector, rev (pDGE1-4)
JS811*	GAATATCATCCGGTGCAGC	sgRNA TU 1, fwd
JS838**	GCCAGCTTCTATGAGTACTGA	sgRNA TU 5, fwd
JS839**	TTTCGCCAAAATCTCTTCCG	sgRNA TU5, rev
JS1132	AACGCTCTTTTCTCTTAGGT	vector, rev (pDGE144-169)

* oligo might not work for sequencing depending on company/protocol. Additional oligos more suitable may be designed within the linker region included in the first sgRNA TU (pDGE5)

** oligos not verified for sequencing