

A Golden Gate-based Protocol for Assembly of Multiplexed gRNA Expression Arrays for CRISPR/Cas9

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[Abstract] The CRISPR (clustered regularly interspaced short palindromic repeats)-associated protein 9 (Cas9) has become the most broadly used and powerful tool for genome editing. Many applications of CRISPR-Cas9 require the delivery of multiple small guide RNAs (gRNAs) into the same cell in order to achieve multiplexed gene editing or regulation. Using traditional co-transfection of single gRNA expression vectors, the likelihood of delivering several gRNAs into the same cell decreases in accordance with the number of gRNAs. Thus, we have developed a method to efficiently assemble gRNA expression cassettes (2-30 gRNAs) into one single vector using a Golden-Gate assembly method (Vad-Nielsen *et al.*, 2016). In this protocol, we describe the detailed step-by-step instructions for assembly of the multiplexed gRNA expression array. The gRNA scaffold used in our expression array is the gRNA 1.0 system for the Cas9 protein from *Streptococcus pyogenes* driven by the human U6 promoter.

Keywords: CRISPR, SpCas9, Golden-Gate assembly, Multiplexed gRNA array, Simultaneously genetic manipulation

[Background] The broadened CRISPR toolbox based on wild-type Cas9 or nuclease-deficient Cas9 (dCas9) has greatly facilitated genome/epigenome editing and regulation in all organisms. Multiplexed gene editing or regulation requires simultaneous expression of several gRNAs in the same cell. The traditional way of delivering several gRNAs into cells is based on either co-transfection of individual gRNA expression vectors or generation of a vector carrying multiple gRNA expression cassettes using traditional cloning; a process which is extremely time consuming. Another way of generating a vector containing multiple gRNA expression cassettes is based on gene synthesis, which is costly and only applicable when working with a very limited number of gRNA expression cassettes. The current protocol is based on Golden Gate cloning which can be used to assemble up to 30 individual gRNA expression cassettes into a single vector within 7 days (Figure 1), with each cassette being driven by an individual human U6 promoter. In our study, we have validated the applicability of this system in both human and porcine cells, but it is in principle compatible with applications in any other organisms that can utilize the human U6 promoter. Compared with existing methods, our method is cost effective, rapid (7 days) and flexible (applicable with any gRNAs that do not contain a *BbsI*, *BsaI* or *BsmBI* recognition site). Many applications of CRISPR/Cas9 may benefit from using our system, including multiplexed gene knockout

by CRISPR/SpCas9, multiplexed gene inhibition by CRISPRi, and multiplexed gene activation by CRISPRa.

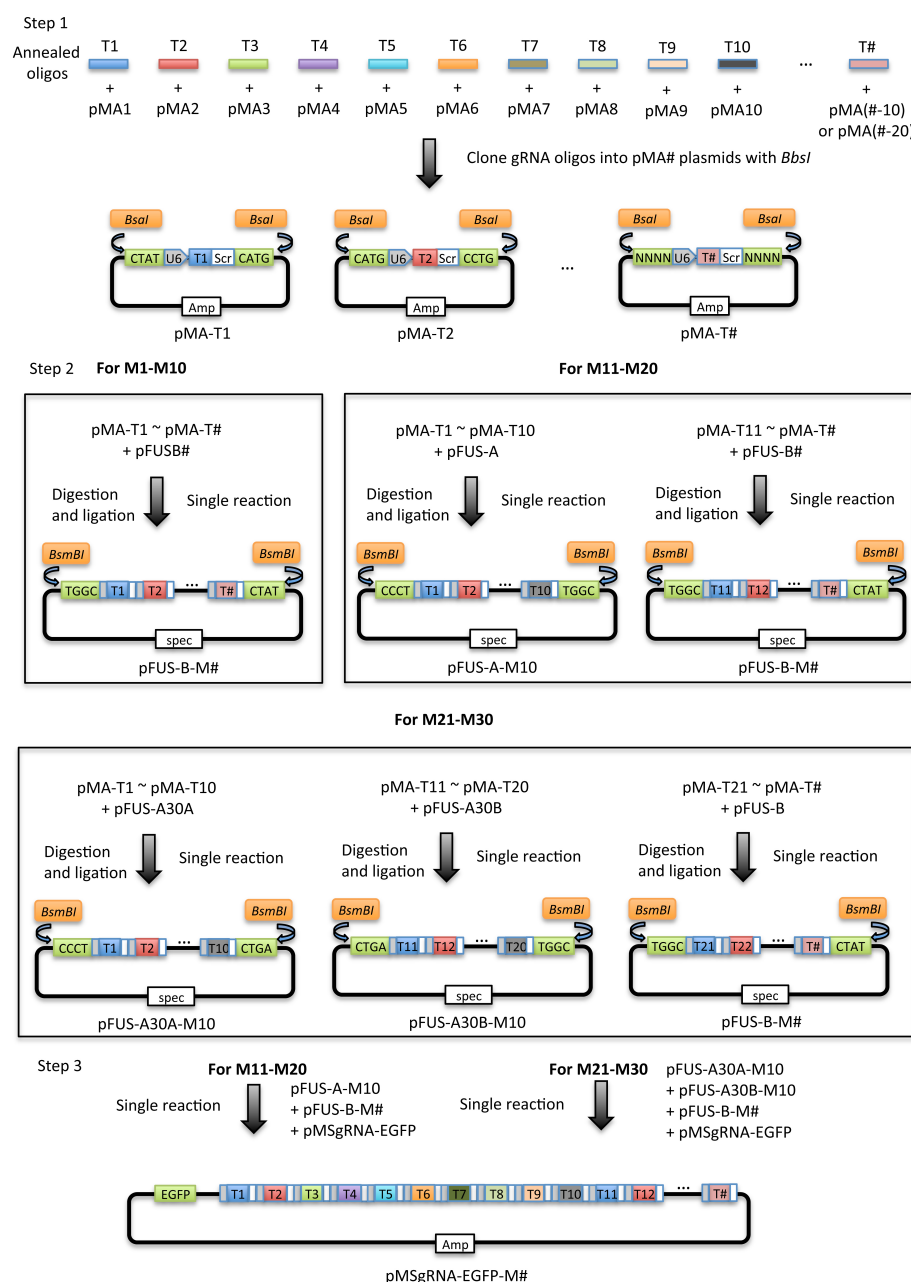


Figure 1. Schematic illustration of the principle of the current protocol. The current protocol is carried out in 2-3 major steps which vary depending on the number of gRNA expression cassettes to be assembled. Step 1: The gRNA oligonucleotides (T#) are cloned into individual modular single gRNA expression vectors (pMA-SpCas9-g#). Step 2: The individual gRNA expression vectors (pMA-T#) are assembled into 1-3 array vectors depending on the total number of gRNAs. Step 3: For assembly of 11-30 gRNA expression cassettes, 2 to 3 individual array vectors are subjected to a second round of assembly to yield the final EGFP expressing vector (pMSgRNA-EGFP).

Materials and Reagents

1. 200 µl PCR tubes
2. 1.5 ml Eppendorf tubes
3. 10 or 100 µl pipette tips
4. Competent *E. coli* cells (No particular preference, but should be recombination deficient)
5. The modular gRNA plasmids (available from Addgene, see Table 1 for corresponding Addgene plasmid IDs)
6. The pFUS-B1 to pFUS-B10, pFUS-A, pFUS-A30A and pFUS-A30B plasmids (available from Addgene, Golden Gate TALEN and TAL Effector Kit 2.0) (Addgene, catalog number: 1000000024)
7. NEB buffer 2 (New England BioLabs, catalog number: B7002S)
8. *Bbs*I (FastDigest) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: FD1014)
9. T4 DNA ligase (5 U/µl) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: EL0014)
10. Distilled H₂O
11. Ampicillin (Sigma-Aldrich, catalog number: A1593)
12. LB medium
13. dNTP
14. DreamTaq or other equivalent DNA polymerase (Thermo Fisher Scientific, Thermo Scientific™, catalog number: K1072)
15. Agarose
16. Plasmid prep mini kit (No particular preference, we used the Nucleo Spin plasmid easy pure kit from MACHEREY-NAGEL, catalog number: 740727)
17. *Bsa*I (BpiI) (FastDigest) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: FD0293)
18. Plasmid-Safe™ ATP-Dependent DNase (Epicentre, catalog number: E3101K, ATP is included in this kit)
19. Spectinomycin (Sigma-Aldrich, catalog number: PHR1441)
20. X-gal (Sigma-Aldrich, catalog number: B4252)
21. IPTG (dissolved IPTG in water) (Sigma-Aldrich, catalog number: I6758)
22. *Afl*II (BspTI) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: FD0834)
23. *Xba*I (Thermo Fisher Scientific, Thermo Scientific™, catalog number: FD0684)
24. *Bsm*BI (Esp3I) (FastDigest) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: FD0454)
25. Universal primers for PCR screening (see Table 1)

Table 1. Plasmids and primers. Plasmids developed for this protocol are available from Addgene with corresponding Addgene IDs. Single modular plasmids (pMA-SpCas9-g1 to pMA-SpCas9-10) for cloning gRNA oligonucleotides into individual gRNA expression plasmids. Array plasmid (pMA-MsgRNA-EGFP) for assembly of gRNA expression array containing 11-30 gRNA expression cassettes. Primer sequences used in this protocol for screening of assembled array plasmids.

Plasmid	Addgene ID
pMA-MsgRNA-EGFP	80794
pMA-SpCas9-g10	80793
pMA-SpCas9-g9	80792
pMA-SpCas9-g8	80791
pMA-SpCas9-g7	80790
pMA-SpCas9-g6	80789
pMA-SpCas9-g5	80788
pMA-SpCas9-g4	80787
pMA-SpCas9-g3	80786
pMA-SpCas9-g2	80785
pMA-SpCas9-g1	80784
Primers	Sequences (5'-)
Universal U6 Forward	ATAAGGATCCGGTCTCGCTATGAGGGCCTATTTCCCATG
Universal Scr Reverse	ATAATGTACAGGTCTCCCATGTAAGTTGCTATTTCTAGCTC

Note: The Universal U6 and Scr primers have been modified to suit the PCR conditions in this protocol.

Equipment

1. Heating block (Grant Instruments, model: QBD2)
2. Microcentrifuge (Eppendorf, model: Centrifuge 5424)
3. 37 °C Thermo incubator (Labnet International, model: 211DS)
4. 37 °C shaking incubator (environmental incubator shaker G24) (Eppendorf, New Brunswick Scientific, model: G24)
5. Thermal cycler (Thermo Fisher Scientific, Applied Biosystems™, model: Veriti® 96 well thermal cycler)
6. DNA electrophoresis apparatus (Bio-Rad Laboratories, model: Wide mini-sub cell GT)
7. Nanodrop (Thermo Fisher Scientific, model: Nanodrop 1000 Spectrophotometer)

Procedure

A. Preparation: design and order CRISPR gRNA oligos

1. Design target gRNA oligonucleotides using any of the online designing tools such as the optimized CRISPR design tool (<http://crispr.mit.edu/>).

Notes:

- a. *Because the gRNA expression cassette utilizes a human U6 promoter, the first nucleotide of the CRISPR target site should preferably be a 'G' to ensure efficient transcription.*
 - b. *It is also very important to exclude gRNA oligonucleotides that contain any of the following type IIS restriction sites: BbsI, BsaI, and BsmBI.*
2. For target sites starting with a 'G', add the following four nucleotide overhangs to the sense (SS) and antisense (AS) gRNA oligonucleotides:
SS: 5'CACC(N₂₀)
AS: 5'AAAC(N₂₀)
 3. For target sites not starting with a 'G', add the following overhangs:
SS: 5'CACCG(N₂₀)
AS: 5'AAAC(N₂₀)C
 4. Order oligonucleotides from any qualified company, dilute the oligonucleotides to a stock concentration of 100 pmol/μl (μM) and store at -20 °C. (Standard desalted purification of the oligos is sufficient).

B. Annealing of gRNA oligos

1. Anneal the sense and antisense gRNA oligonucleotides for each gRNA in separate 1.5 ml EP tubes:
Sense gRNA oligo: 1 μl (100 pmol/μl)
Antisense gRNA oligo: 1 μl (100 pmol/μl)
10x NEB buffer 2: 2 μl
Add ddH₂O to a final volume of 20 μl.
2. Denature the mixture at 95 °C for 5 min in a heating block.
3. Place the heating block on the lab bench and slowly anneal oligonucleotides until mixture is at room temperature (which takes approximately 1-2 h).
4. Quick-spin the annealed gRNA oligonucleotides to the bottom of the tubes and store at -20 °C for later use or proceed with the following experimental procedure.

C. Vector cloning

Using this protocol, the generation of the gRNA expression array vector is accomplished in 5 days (for 1 to 10 gRNA expression cassettes) to 7 days (for 11 to 30 cassettes). See schematic in Figure 2 for an overview. In the protocol, the location of a target site in the CRISPR expression array is referred to as T# (#, an integer between 1 and 30 denoting the order of the gRNAs). The protocol

utilizes 10 individual gRNA expression cassettes that are tandemly assembled into a set of ‘array’ vectors depending on the number of cassettes, where each array vector contains up to 10 cassettes. When assembling 11-30 cassettes, multiple array vectors are finally assembled into a single vector. The selection of plasmids for assembly is described in step C3c, Table 3. A schematic illustration is given in Figure 1.

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Anneal oligos	Colony screening by PCR	Miniprep of positive colonies	Colony screening by PCR	Miniprep of positive colonies	Colony screening by PCR	Miniprep of positive colonies
Assemble pMA# plasmid	Culture positive colonies	Second round of assembling (Step 2)	Culture positive colonies	Third round of assembling (for M11-M30, step 3)	Culture positive colonies	Digestion validation
Transformation		Transformation		Transformation (For M11-M30)		

Figure 2. Protocol overview. Day to day overview of the protocol. For assembly 1 to 10 gRNA expression cassettes, the protocol is completed after miniprep of positive colonies at day 5. For assembling 11 to 30 gRNA expression cassettes, an additional round of assembly is performed at day 5 and completed after day 7.

1. Day 1. Ligation of gRNA oligonucleotides into individual modular single gRNA expression vectors
 - a. Select pMA plasmids for annealed gRNA oligonucleotides T1-TN ($N \leq 30$) generated in step B4 according to the table below (pMA-SpCas9-g1 for T1, pMA-SpCas9-g2 for T2, ..., pMA-SpCas9-g1 for T11, pMA-SpCas9-g2 for T12, ..., pMA-SpCas9-g1 for T21, pMA-SpCas9-g2 for T22, *etc.*) (Table 2).

Table 2. Assembly scheme of modular single gRNA expression vectors and annealed gRNA oligonucleotides. This table illustrates how to choose the right single modular plasmids (pMA-SpCas9-g1 to pMA-SpCas9-10) for cloning of the gRNA oligonucleotides into individual gRNA expression plasmids (named pMA-T# after successful cloning). The number of each gRNA represents the position of the gRNA in the tandem gRNA expression array.

Plasmid Name	Addgene ID	Description	Restriction enzymes for individual gRNA cloning	Selection for cloning
pMA-SpCas9-g1	80784	For cloning T1, T11 or T21	<i>BbsI</i>	Ampicillin
pMA-SpCas9-g2	80785	For cloning T2, T12 or T22	<i>BbsI</i>	Ampicillin
pMA-SpCas9-g3	80786	For cloning T3, T13 or T23	<i>BbsI</i>	Ampicillin
pMA-SpCas9-g4	80787	For cloning T4, T14 or T24	<i>BbsI</i>	Ampicillin
pMA-SpCas9-g5	80788	For cloning T5, T15 or T25	<i>BbsI</i>	Ampicillin
pMA-SpCas9-g6	80789	For cloning T6, T16 or T26	<i>BbsI</i>	Ampicillin
pMA-SpCas9-g7	80790	For cloning T7, T17 or T27	<i>BbsI</i>	Ampicillin
pMA-SpCas9-g8	80791	For cloning T8, T18 or T28	<i>BbsI</i>	Ampicillin
pMA-SpCas9-g9	80792	For cloning T9, T19 or T29	<i>BbsI</i>	Ampicillin
pMA-SpCas9-g10	80793	For cloning T10, T20 or T30	<i>BbsI</i>	Ampicillin

- b. Prepare assembly reaction in individual PCR tubes containing:
 - i. 100 ng modular single gRNA expression plasmid (pMA-SpCas9-g#).
 - ii. 1 µl of annealed gRNA oligonucleotide from step B4 (T#).
 - iii. 1 µl *BbsI* restriction enzyme.
 - iv. 1 µl T4 DNA ligase.
 - v. 2 µl 10x T4 ligase buffer (to a final concentration of 1x).
 - vi. Nuclease-free water up to 20 µl total reaction volume.
- c. Incubate the reactions in a thermal cycler with the following conditions:

10 cycles	{	5 min at 37 °C
		10 min at 22 °C
Hold for		30 min at 37 °C
Hold for		15 min at 75 °C
Keep at		4 °C

- d. Transform competent *E. coli* using 2 µl of the ligation product.
Note: Protocols for generation of competent E. coli and transformation are available from the authors upon request.
- e. Plate (1/10) transformed cells on a 10 cm LB agar plate containing 50 µg/ml ampicillin.
Note: Do not over-plate the cells.
- f. Incubate agar plates at 37 °C overnight.

2. Day 2. 1st colony screening and culture

- a. Pick two to three colonies from each transformation using a 10 or 100 µl pipette tip.
- b. Dip the pipette tip in 100 µl LB medium (50 µg/ml ampicillin) to start initial culture. Transfer the pipette tip to a PCR tube containing 30 µl nuclease-free water and pipette up and down to release cells.

Note: The initial culture and PCR tube should be labeled with the same ID. If working with over 10 gRNAs, we normally use two sets of 200 µl PCR tubes, one set for the initial culture and the other set for the cell lysate.

- c. Place the initial culture tubes in a 37 °C incubator.
- d. Place the PCR tubes in a thermal cycler at 98 °C for 10 min to lyse cells.
- e. Perform gRNA PCR screenings of cell lysates. Add to each reaction:
 - i. 1 µl transformed *E. coli* lysate.
 - ii. 5 pmol universal U6 Forward primer (Table 1).
 - iii. 5 pmol antisense oligonucleotide of the gRNA in question.
 - iv. 0.4 µl dNTP (10 mM).
 - v. 0.1 µl DreamTaq polymerase (or similar).
 - vi. 2 µl 10x DreamTaq buffer (to final concentration of 1x).
 - vii. Nuclease-free water to 20 µl total reaction volume.

Example: For colony screening of pMA-SpCas9-T1, PCR is performed for 2-3 picked colonies using the universal U6 promoter as the forward primer and T1-antisense oligonucleotide as the reverse primer.

- f. Incubate the PCR reactions in a thermal cycler with the following conditions:

Hold for		5 min at 94 °C
35 cycles	{	30 sec at 94 °C
		30 sec at 58 °C
		30 sec at 72 °C
Hold for		7 min at 72 °C
Keep at		4 °C

- g. Visualize PCR product on a 1% agarose gel by electrophoresis. The expected amplicon size is approximately 270 bp.
- h. Select one positive colony from each transformation and transfer 50 µl of initial culture to individual tubes containing 5 ml LB medium with 50 µg/ml ampicillin.
- i. Incubate overnight at 37 °C in an orbital shaker.
3. Day 3. Plasmid prep and assembly of individual gRNA expression cassettes into the 'array' plasmids
 - a. Isolate the plasmid DNA (named pMA-T#) from the overnight cultures using a commercial miniprep kit according to the manufacturer's instructions.
 - b. Validate the single gRNA expression plasmids by Sanger sequencing using the universal U6 primer (recommended).

- c. Select array plasmids for 1st assembly of single gRNA expression plasmids according to Table 3:

Table 3. Selection of array plasmids for assembly of pMA-T# vectors. Selection of array plasmids depends on the number of gRNA expression cassettes to be assembled. For assembly of 1-10 gRNA cassettes (Left), one of the pFUS-B# array plasmids is selected depending on the number of gRNA cassettes. When assembling 11-20 gRNA cassettes (Middle), pFUS-A is selected for the assembly of gRNA cassettes 1 to 10. For the remaining cassettes (≤ 10), one of the pFUS-B# array plasmids is selected depending on the number of gRNA cassettes. For assembly of 21-30 gRNA cassettes (Right), pFUS-A30A is used for assembly of the first 10 gRNA expression cassettes. pFUS-A30B is used for assembling gRNA cassettes 11 to 20. The remaining cassettes (≤ 10) are cloned into a pFUS-B# array plasmid depending on the number of gRNA cassettes.

Assembly of 1-10 gRNA cassettes (M1-M10)		Assembly of 11-20 gRNA cassettes (M11-M20)		Assembly of 21-30 cassettes (M21-M30)	
The pMA-T1 to pMA-T# (# ≤ 10) are assembled into the pFUS-B# array plasmid in a single reaction. The final plasmid is named pFUS-B#-M#.		The pMA-T1 to pMA-T10 are assembled into the pFUS-A array plasmid in one reaction. The final plasmid is named pFUS-A-M10.		The pMA-T1 to pMA-T10 are assembled into the pFUS-A30A array plasmid in one reaction. The final plasmid is named pFUS-A30A-M10.	
<i>Note: It is important to maintain the correct order of the array and pMA plasmids. pFUS-B3 will not assemble any 3 single gRNA expression cassettes (i.e., pMA-T6 to pMA-T9), but will only assemble pMA-T1 to pMA-T3. This applies to all the array plasmids.</i>		The pMA-T11 to pMA-T# (11 ≤ # ≤ 20) are assembled into the pFUS-B# array plasmid in another reaction (as for the assembly of 1-10 gRNA cassettes). The final plasmid is named pFUS-B#-M#.		The pMA-T11 to pMA-T20 are assembled into the pFUS-A30B array plasmid in another reaction. The final plasmid is named pFUS-A30B-M10.	
		The gRNA expression cassettes in the two array plasmids will be combined in a second round of assembly.		The pMA-T21 to pMA-T# (21 ≤ # ≤ 30) are assembled into the pFUS-B# array plasmid in a third reaction (as for assembly of 1-10 gRNA cassettes). The final plasmid is named pFUS-B#-M#.	
				The gRNA expression cassettes in the three array plasmids will be combined in a second round of assembly.	
Array plasmid name	Description		Restriction enzyme for assembly	Selection for cloning	
pFUS-B1	For cloning of pMA-T11 or pMA-T21		Bsal	Spectinomycin	
pFUS-B2	For cloning of pMA-T1 to pMA-T2, pMA-T11 to pMAT-12 or pMA-T21 to pMA-T22		Bsal	Spectinomycin	
pFUS-B3	For cloning of pMA-T1 to pMA-T3, pMA-T11 to pMAT-13 or pMA-T21 to pMA-T23		Bsal	Spectinomycin	
pFUS-B4	For cloning of pMA-T1 to pMA-T4, pMA-T11 to pMAT-14 or pMA-T21 to pMA-T24		Bsal	Spectinomycin	
pFUS-B5	For cloning of pMA-T1 to pMA-T5, pMA-T11 to pMAT-15 or pMA-T21 to pMA-T25		Bsal	Spectinomycin	
pFUS-B6	For cloning of pMA-T1 to pMA-T6, pMA-T11 to pMAT-16 or pMA-T21 to pMA-T26		Bsal	Spectinomycin	
pFUS-B7	For cloning of pMA-T1 to pMA-T7, pMA-T11 to pMAT-17 or pMA-T21 to pMA-T27		Bsal	Spectinomycin	
pFUS-B8	For cloning of pMA-T1 to pMA-T8, pMA-T11 to pMAT-18 or pMA-T21 to pMA-T28		Bsal	Spectinomycin	
pFUS-B9	For cloning of pMA-T1 to pMA-T9, pMA-T11 to pMAT-19 or pMA-T21 to pMA-T29		Bsal	Spectinomycin	

Table 3. Continued

pFUS-B10	For cloning of pMA-T1 to pMA-T10, pMA-T11 to pMAT-20 or pMA-T21 to pMA-T30	Bsal	Spectinomycin
pFUS-A	For cloning of pMA-T1 to pMA-T10. The final plasmid can be assembled into M11-M20 with any of the pFUS-B based plasmids (M1-M10) in the second assembly	Bsal	Spectinomycin
pFUS-A30A	For cloning of pMA-T1 to pMA-T10. The final plasmid can be assembled into M21-M30 with pFUS-A30B and any of the pFUS-B based plasmids (M1-M10) in the second assembly	Bsal	Spectinomycin
pFUS-A30B	For cloning of pMA-T11 to pMA-T20. The assembled plasmid can be assembled into M21-M30 with pFUS-A30B and any of the pFUS-B based plasmids (M1-M10) in the second assembly	Bsal	Spectinomycin

d. Set up Golden Gate assembly reactions in individual PCR tubes containing:

i. 30 ng array plasmid.

Note: The plasmid amount has been optimized.

ii. 50 ng of each individual modular single gRNA plasmid (maximum of 10 plasmids).

iii. 1 µl Bsal restriction enzyme.

iv. 1 µl T4 DNA ligase.

v. 2 µl 10x T4 ligase buffer (to a final concentration of 1x).

vi. Nuclease-free water to 20 µl total reaction volume.

e. Incubate the reactions in a thermal cycler with the following conditions:

15 cycles	{ 5 min at 37 °C 10 min at 22 °C
Hold for	30 min at 37 °C
Hold for	15 min at 75 °C
Keep at	4 °C

f. Add 1 µl 25 mM ATP and 1 µl plasmid safe DNase to the reaction.

Note: Plasmid safe DNase treatment digests all unligated linear dsDNA fragments, incomplete assembly products and linearized vectors. This step minimizes the risk of in vivo recombination of linearized dsDNA fragments in the competent cell. This step is very important when assembling more than five gRNA expression cassettes.

g. Incubate the reactions in a thermal cycler for 1 h at 37 °C.

h. Transform competent *E. coli* cells using 2 µl of the assembled product.

i. Plate (1/20) of the transformed cells on 10 cm LB agar plates containing 50 µg/ml spectinomycin as well as 8 µl 100 mg/µl X-gal and 8 µl 0.5 M IPTG for blue/white colony screening.

j. Incubate agar plates at 37 °C overnight.

4. Day 4. 2nd colony screening and culture

- a. Inspect plates for positive white colonies. The expected ratio of white to blue colonies is at least 25% for successful assembly of 10 gRNA expression cassettes and will increase gradually with a decreased number of gRNA expression cassettes (Figure 3).

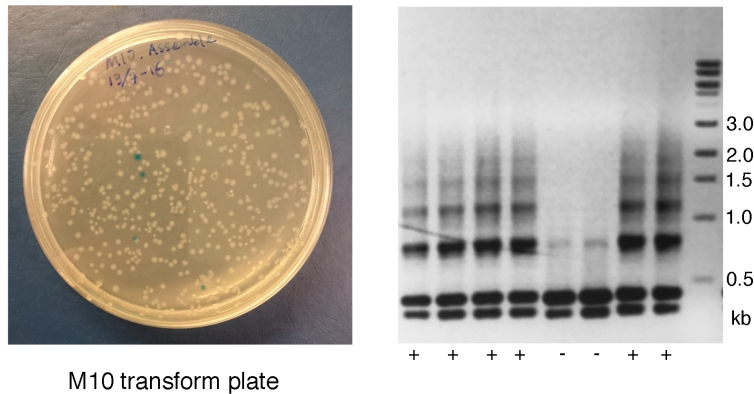


Figure 3. Inspection and screening of first round of assembling. Left: The transformation plate for M10. White colonies contain vectors with insertions. Blue colonies contain empty vectors. Right: Universal PCR for M10 screening of 8 white colonies. Lanes 1-4 and 7-8 show multiple bands resembling a ladder indicative of a positive colony. Lanes 5 and 6 only show the two dominant bands without a ladder indicative of an incorrect insertion.

- b. Pick two to three white colonies from each transformation as described for Day 2.
- c. Set up PCR screening reactions for all picked colonies in individual PCR tubes containing:
 - i. 1 μ l transformed *E. coli* lysate.
 - ii. 5 pmol universal U6 Forward primer (Table 1).
 - iii. 5 pmol universal Scr Reverse primer (Table 1).
 - iv. 0.4 μ l dNTP.
 - v. 0.1 μ l DreamTaq polymerase (or similar).
 - vi. 2 μ l 10x DreamTaq buffer (to a final concentration of 1x).
 - vii. Nuclease-free water to 20 μ l total reaction volume.
- d. Incubate the PCR reactions in a thermal cycler with the following conditions:

Hold for	5 min at 94 °C
35 cycles	30 sec at 94 °C
	30 sec at 58 °C
	2 min at 72 °C
Hold for	7 min at 72 °C
Keep at	4 °C

- e. Visualize PCR product on a 1% agarose gel by electrophoresis. The correct amplification pattern should be a 'ladder' with a dominant band at approximately 400 bp and an increase

of 392 bp for each 'step' corresponding to each additional gRNA expression cassette included in the amplification (Figure 3B).

- f. Select one positive colony from each transformation and transfer 50 µl of initial culture to individual tubes containing 5 ml LB medium with 50 µg/ml spectinomycin.
- g. Incubate overnight at 37 °C in an orbital shaker.
5. Day 5. Plasmid DNA prep and 2nd assembly for plasmids containing 11 to 30 expression cassettes
 - a. Isolate the plasmid DNA from the overnight cultures using a commercial miniprep kit according to the manufacturer's instructions.
 - b. Validate the assembled array plasmids by restriction enzyme digestion. In a 1.5 ml EP tube, add:
 - i. 0.5 µg assembled array plasmid DNA.
 - ii. 1 µl *Afl*I restriction enzyme.
 - iii. 1 µl *Xba*I restriction enzyme.
 - iv. 2 µl 10x reaction buffer (to a final concentration of 1x).
 - v. Nuclease-free water to 20 µl total reaction volume.
 - c. Visualize digestion product on a 1% agarose gel by electrophoresis.
Note: The expected size of the released expression cassette is approximately 4 kb for 10 cassettes (~400 bp per gRNA expression cassette). We strongly recommend generating a theoretical sequence map for each multiplex vector. The RFLP results will be different if there are AflI or XbaI recognition sites in any of the gRNA guide sequences.
 - d. For further validation of the assembled array plasmid, set up guide specific PCRs for all gRNA expression cassettes present in the array vector in individual PCR tubes.
Add to each PCR reaction:
 - i. 1 ng array plasmid DNA.
 - ii. 5 pmol sense oligonucleotide of the specific gRNA in pMA-TN as the forward primer.
 - iii. 5 pmol antisense oligonucleotide of the next specific gRNA in pMA-TN+1 as the reverse primer.
 - iv. 0.4 µl dNTP.
 - v. 0.1 µl DreamTaq polymerase (or similar).
 - vi. 2 µl 10x DreamTaq buffer (to a final concentration of 1x).
 - vii. Nuclease-free water to 20 µl total reaction volume.

Example: For validation of assembled pFUS-B4-M4, three individual PCR reactions should be performed. The first one is with the SS oligonucleotide of T1 as the forward primer and the AS oligonucleotide of T2 as the reverse primer. The second one is with the SS oligonucleotide of T2 as the forward primer and the AS oligonucleotide of T3 as the reverse primer. The third one is with the SS oligonucleotide of T3 as the forward primer and the AS oligonucleotide of T4 as the reverse primer. The total number of PCR reactions per

assembled array plasmid is N-1 (N is the number of gRNA expression cassettes contained in the array plasmid) (Figure 4).

- e. Incubate the PCR reactions in a thermal cycler with the following conditions:

Hold for	5 min at 94 °C
35 cycles	30 sec at 94 °C
	30 sec at 58 °C
	30 sec at 72 °C
Hold for	7 min at 72 °C
Keep at	4 °C

- f. Visualize PCR product on a 1% agarose gel by electrophoresis. The expected amplicon sizes are approximately 400 bp.

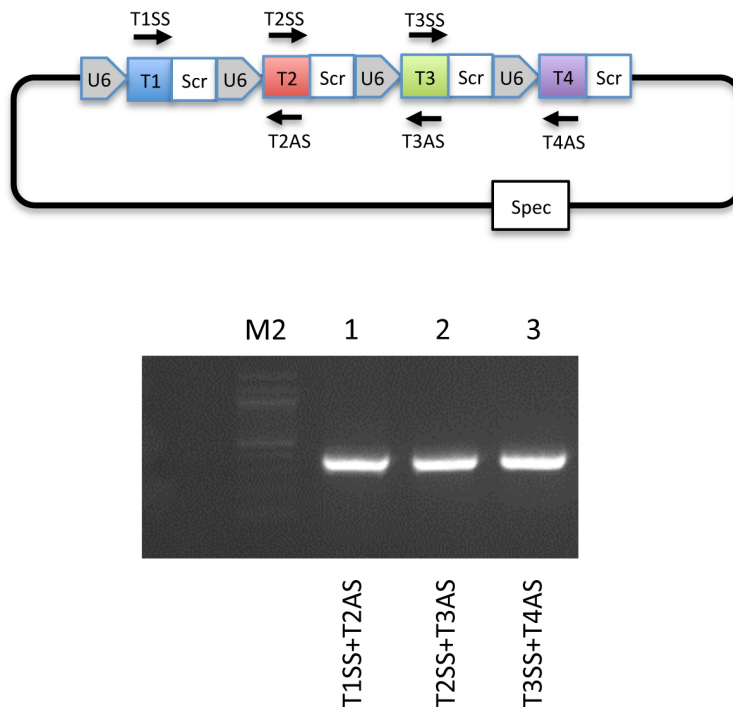


Figure 4. Guide specific PCR validation. Top: Schematic illustration of primers used for guide specific PCR. Bottom: An example of a guide specific PCR using a pFUS-B4-M4 plasmid visualized on a 1% agarose gel.

- g. Set up 2nd assembly of array plasmids in a PCR tube (only for assembly of 11-30 cassettes) containing:
- 50 ng DNA of each assembled array vector (pFUS-A-M10 + pFUS-B#-M# OR pFUS-A30A-M10 + pFUS-A30B-M10 + pFUS-B#-M#).
 - 50 ng DNA of array plasmid pMsgRNA-EGFP (Table 1).
 - 1 μ l *Bsm*BI restriction enzyme.

- iv. 1 µl T4 DNA ligase.
- v. 2 µl 10x T4 ligase buffer (to a final concentration of 1x).
- vi. Nuclease-free water to 20 µl total reaction volume.
- h. Incubate the reaction in a thermal cycler with the following conditions:

10 cycles	{	5 min at 37 °C
		10 min at 22 °C
Hold for		30 min at 37 °C
Hold for		15 min at 75 °C
Keep at		4 °C

- i. Transform competent *E. coli* cells using 2 µl of the ligation product.
- j. Plate (1/10) of the transformed cells on a 10 cm LB agar plate containing 50 µg/ml ampicillin as well as 40 µl of 20 mg/ml X-gal and 40 µl of 0.1 M IPTG for blue/white colony screening.
- k. Incubate agar plate at 37 °C overnight.
- 6. Day 6. 3rd colony screening and culture
 - a. Inspect plate for positive white colonies. The expected ratio of white to blue colonies is > 80% for successful assembly.
 - b. Pick two to three white colonies as described for Day 2.
 - c. Set up overlap PCR screening reactions for each assembled section of gRNA expression cassettes for all picked colonies. To individual PCR tubes, add:
 - i. 1 µl transformed *E. coli* lysate.
 - ii. 5 pmol sense oligonucleotide of the last gRNA (T10 or T20) in the first or second expression cassette array as the forward primer.
 - iii. 5 pmol antisense oligonucleotide of the first gRNA (T11 or T21) in the second or third expression cassette array as the reverse primer.
 - iv. 0.4 µl dNTP.
 - v. 0.1 µl DreamTaq polymerase (or similar).
 - vi. 2 µl 10x DreamTaq buffer (to a final concentration of 1x).
 - vii. Nuclease-free water to 20 µl total reaction volume.

Example: For validation of assembled pMsgRNA-EGFP-M30 (array plasmid containing 30 gRNA expression cassettes generated from the assembly of pFUS-A30A – pFUS-A30B – pFUS-B10), two individual overlap PCR reactions are performed. The first reaction uses the sense (SS) oligonucleotide of T10 as the forward primer and the antisense (AS) oligonucleotide of T11 as the reverse primer. The second uses the SS oligonucleotide of T20 as the forward primer and the AS oligonucleotide of T21 (Figure 5).
 - d. Incubate the PCR reactions in a thermal cycler with the following conditions:

Hold for	5 min at 94 °C
35 cycles	30 sec at 94 °C
	30 sec at 58 °C
	1 min at 72 °C
Hold for	7 min at 72 °C
Keep at	4 °C

- e. Visualize PCR product on a 1% agarose gel by electrophoresis. The expected amplicon size is approximately 460 bp.
- f. (Optional) Perform overlap PCRs for all neighboring gRNAs.

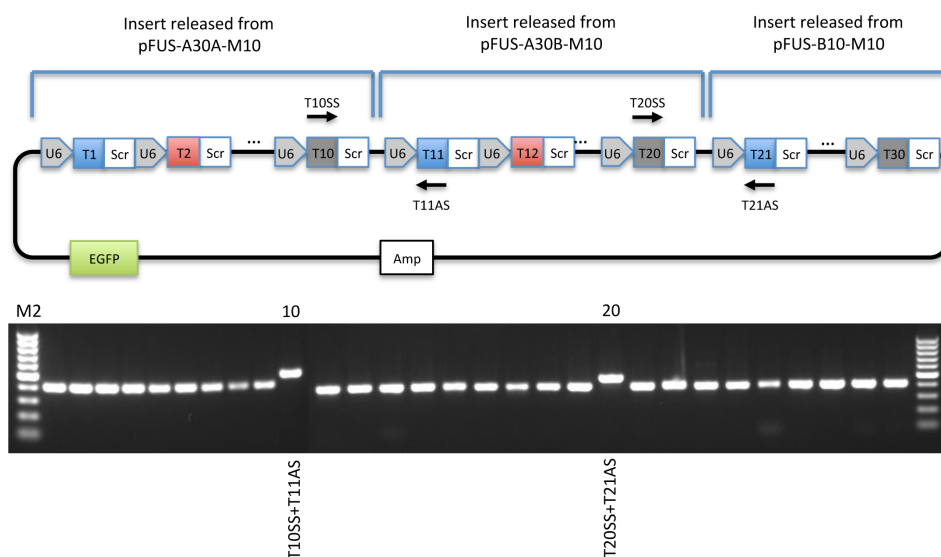


Figure 5. Overlap PCR screening. Top: Schematic illustration of overlap PCR screening of pMsgRNA-EGFP-M30. Bottom: Example of guide specific PCR validation of pMsgRNA-EGFP-M30 visualized on a 1% agarose gel. Lane 10 and lane 20 contain the overlap PCR reactions described above. Note that the overlap PCR produces a slightly larger band than the guide specific PCRs. Generating a theoretical plasmid sequence map is highly recommended as a guideline for assembly, PCR validation, and restriction enzyme digestions.

- g. (Optional) Sanger sequencing using T# as primers.
- h. Select one positive colony and transfer 50 µl of initial culture to individual tubes containing 5 ml LB medium with 50 µg/ml ampicillin added.
- i. Incubate overnight at 37 °C in an orbital shaker.
7. Day 7. Plasmid DNA preparation and validation
 - a. Isolate the plasmid DNA from the overnight culture using a commercial miniprep kit according to manufacturer's instructions.
 - b. Validate the assembled pMsgRNA-EGFP-M# array plasmid by restriction enzyme digestion to release the combined gRNA expression array cassette. Add to a PCR tube:

- i. 0.5 µg assembled array plasmid DNA.
 - ii. 1 µl *Sfi*I restriction enzyme.
 - iii. 2 µl 10x reaction buffer (to a final concentration of 1x).
 - iv. Nuclease-free water to 20 µl total reaction volume.
- c. Visualize digestion product on a 1% agarose gel by electrophoresis. The expected size of the released expression cassette is approximately 1662 + N x 379 bp (N is the total number of gRNA expression cassettes contained in the final array plasmid).

Notes

1. For the efficiency of cloning in each assembly reaction, we generally experience higher than 95% positive rates for the single gRNA expression vectors. This positive rate is based on PCR screening. The positive rate for the assembly of gRNA expression arrays is based on the ratio of white (positive) to blue (negative) colonies. Please note that all blue colonies are negative. However, the white colonies may also be negative if they contain incorrect ligation or recombination products. It is therefore important to perform PCR screenings.
2. If a high blue colony rate is observed, it is recommended to check the expiry date of your enzymes as the FastDigest enzymes have a relatively short storage time. Other measures such as lowering the amount of the pFUS plasmid in assembly reactions, increasing cycle numbers, and increasing the amount of enzyme can increase the white colony rate as well.

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