

Golden Gate TALEN assembly with **GG compatible pJDS expression vectors**

*****This protocol is modified from original Voytas/Cermak protocol. Please see **Day3 and Day4 for modifications, which are highlighted yellow.**

This is an expanded and slightly modified TAL assembly protocol published in The original form in Cermak, et al., 2011 (<http://dx.doi.org/10.1093/nar/gkr218>) Modifications to the published protocol by Michelle Christian, Colby Starker and other

members of Dan Voytas' lab.

Reagents :

- A) Set of 60 library vectors (see the file TALEN_golden_gate_library_stock_plate_sheet)
- B) 10X T4 DNA ligase buffer (NEB)
- C) Quick ligase or T-4 DNA ligase (NEB)
- D) restriction endonuclease BsaI (NEB)
- E) restriction endonuclease Esp3I (Fermentas or Fisher [FERER0452])
- F) Plasmid-Safe nuclease (Epicentre Biotechnologies E3110K)
- G) 10mM ATP
- H) chemically competent cells
- I) SOC
- J) LB plates and liquid media with Tetracycline (10mg/l), Spectinomycin (50mg/l), Ampicillin (50mg/l)
- K) X-gal/IPTG
- L) Miniprep kit (Qiagen)

For screening/sequencing of transformants:

Primers

pCR8_F1: ttgatgcctggcagttccct

pCR8_R1: cgaaccgaacaggcttatgt

pJDS-GG screen F : CTGCTGAAGATCGCGAAGA

pJDS-GG screen R : GACCCTTTTTGACTGCATCG

AND/OR restriction endonucleases – for restriction screening (NEB)

AflII

XbaI

BstAPI or StuI

AatII

BspEI

DAY1

1. Choose your TALEN RVD sequence: N = number of RVDs (12-31)

If the TALEN length is 12-21:

2. Pick plasmids for the RVDs 1-10 (e.g. pNI1, pNN2, pHD3, pHD4....) + destination vector pFUS_A

3. Pick plasmids 11 up to N-1 + destination vector pFUS_B #N-1 (pFUS_B plasmids are labeled B1-B10 but they are used for RVDs 11-30 – if the RVD #N-1 is 19 or 29, use the same destination vector pFUS_B9)

If the TALEN length is 22-31:

2. Pick plasmids for the RVDs 1-10 + destination vector pFUS_A30A, pick plasmids for the RVDs 11-20 + destination vector pFUS_A30B

3. Pick plasmids 21 up to N-1 + destination vector pFUS_B#N-1
Note: pFUS_B plasmids are labeled B1-B10 but they are used for RVDs 11-30 – if the RVD #N-1 is 19 or 29, use the same destination vector pFUS_B9)

4. Mix golden gate reaction **#1** – for each set of vectors separately:

1-10 + pFUS_A;

11-(N-1) + pFUS_B(N-1)

or

1-10 + pFUSA30A

11-20 + pFUSA30B

21-(N-1) + pFUS_B(N-1)

a) 150ng of each module vector + 150ng of pFUS vector.

b) 1µl Bsal

c) 1µl Quick ligase or T-4 DNA ligase (QL is higher efficiency, but T-4 is much cheaper and works fine)

d) 2µl 10X T4 DNA ligase buffer (to final concentration of **1X**)

e) H2O up to 20µl total reaction volume

Note: Published protocol indicates using 20µL reactions, but we find 10µL reactions are reliably effective (same concentrations as in published protocol). have done ½ reactions (same concentrations, only 10µL total volume). If a particular cloning reaction is somewhat difficult (failed more than once), it may be useful to use a 20µL reaction.

5. Run cycle: **10x** (37°C/5min + 16°C/10min) + 50°C/5min + 80°C/5min
With this cycle you will get hundreds of white colonies with 90-100% efficiency.

6. Plasmid-Safe nuclease treatment: this destroys all unligated linear dsDNA fragments including incomplete ligation products with lower number of repeats fused; and cut and linearized vectors. The incomplete, shorter fragments would be cloned into the destination vector *in vivo* by recombination in the bacterial cell, if not removed (the start of the first repeat and the end of the last repeat are in the destination vector backbone, so the backbone has homology to each repeat module as they differ only in RVDs)

To each of your golden gate #1 reactions add:

a) 1ml 10mM ATP

b) 1ml Plasmid-Safe nuclease

Incubate at 37°C/1h

Note: The Plasmid-Safe nuclease manual says you should inactivate the enzyme

by heating the reaction to 70°C for 30 minutes, but our experience, for bacterial transformation, inactivation is not necessary

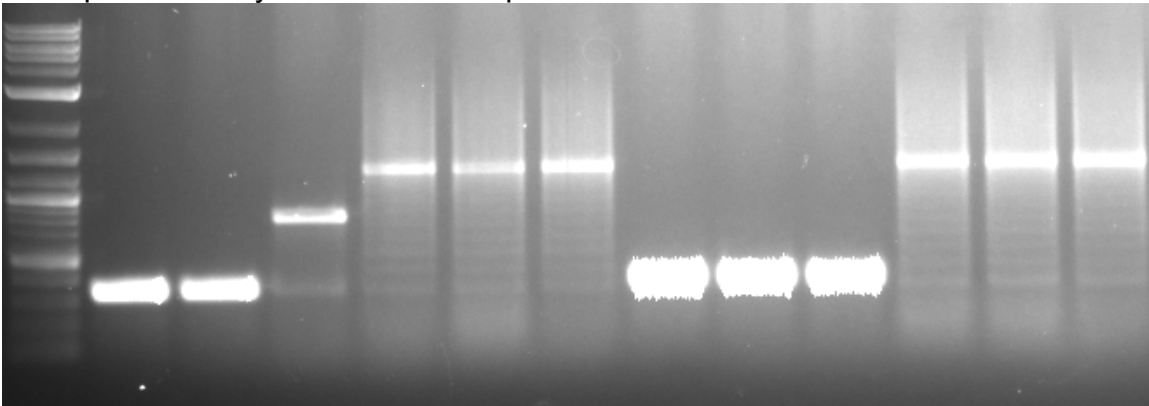
7. Transform your chemically competent cells (we use 5ml of the GG reaction)

8. Plate on Spec50 plates + 40µL of 20mg/mL X-gal +40µL of 0.1M IPTG. When plating transformations of the pFUSB vectors that have fewer repeats (especially less than 6 repeats), be careful to not plate all the cells as the efficiency is so high you can't pick single colonies on day 2.

DAY2

9. Pick 1-3 white colonies from each plate and check by **colony PCR** using primers pCR8_F1 and pCR8_R1 (primers are the same for each pFUSA, pFUSA30A, pFUSA30B, pFUSB1-10 vector). PCR program: Anneal at 55°, extend 1.75min, cycle 30-35X. You should get a band around your expected size (~1.2KB for vectors with 10 repeats), but you will also get smearing and a 'ladder' of bands starting at ~200bp and every 100bp up to ~500bp. This is the sign of a correct clone and is the result of the repeats in the clones.

Example of Colony PCR results for pFUS vectors:



Note: Lanes 2 and 3 are negative pFUS clones (empty). Lane 4 contains the 'correct' clone for this pFUS. pFUS clones that only contain 1 or 2 repeats are very similar in size to empty pFUS clones – check the size carefully. Lanes 5,6,7,11,12,13 show the 'laddering' effect well. DNA ladder is NEB's 2Log

10. Start the over-night cultures with the correct clones

DAY3

11. Miniprep the plasmids: pFUS_A with first 10 repeats cloned (**A**)

pFUS_B with 11-(N-1) repeats cloned (**B**)

or

pFUS_A30A with first 10 repeats cloned (**A1**)

pFUS_A30B with second 10 repeats cloned (**A2**)

pFUS_B with 21-(N-1) repeats cloned (**B**)

12. Optional restriction digestion testing/sequencing:
Use enzymes AflIII and XbaI (same for all destination vectors) to cut out the array of fused repeats: 1048bp for pFUS_A vectors, different sizes depending on number of repeats cloned for pFUS_B vectors
and/or sequence with primers pCR8_F1, pCR8_R1

13. Mix golden gate reaction #2

a) 150ng of each vector **A** and **B** (or **A1**, **A2** and **B**)

b) **No pLR vector!**

c) 75ng of destination vector "**pJDSXX-GG**" (see below)

d) 1µl Esp3I

e) 1µl Quick ligase or T-4 Ligase (this reaction is so efficient that T-4 ligase is always sufficient)

f) 2µl 10X T4 DNA ligase buffer or Tango buffer (the buffer for the Esp3I enzyme)

g) H2O up to 20µl

Do not put the pLR vectors came with the kit in this reaction. Expression vectors were designed so that "half-repeat" is already in the vector. You have to use specific destination vector depending on your last repeat.

**If your last repeat is "NI", use pJDS70-GG-SNI,
if your last repeat is "HD", use pJDS71-GG-SHD,
if your last repeat is "NN", use pJDS74-GG-NNN,
if your last repeat is "NG", use pJDS78-GG-SNG.**

14. Run cycle: 10x(37°C/5min + 16°C/10min) + 37°C/15min + 80°C/5min for hundreds of white colonies, OR 37°C/10min + 16°C/15min + 37°C/15min + 80°C/5min for tens of white colonies* This 1 cycle reactions is sufficient for the second GG reaction, and this is what the Voytas lab usually does.

15. Transform your competent cells (use 5ml of the reaction)

Note: Plasmid-Safe nuclease treatment is not necessary in this case, because the final destination vector has no homology with the inserted repeats

16. Plate on Carb(Amp)50 plates **(No X-gal and IPTG).**

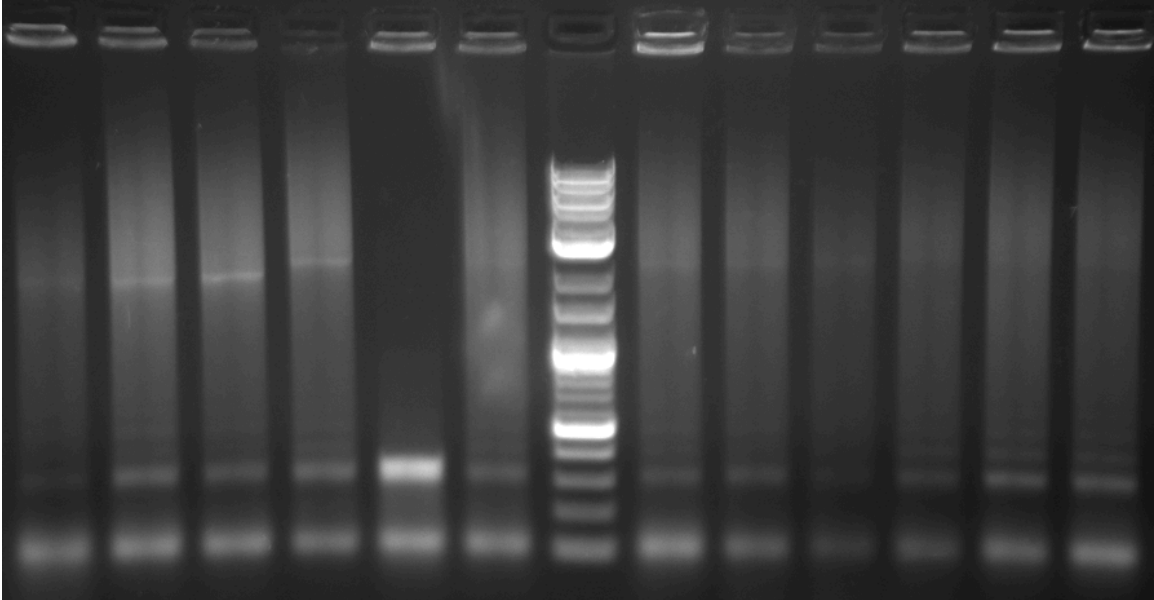
DAY4

17. Pick 1-3 white colonies and check by colony PCR using primers **pJDS-GG screen F and R** using these conditions:

a. Anneal at 55°, extend 3 minutes, cycle 30-35X

b. Very often, you can't see the band of the size you expect, but instead see a smear and the 'ladder' effect – again, this is the sign of a correct clone.

18. Run on a gel, choose a correct clone and start an over-night culture
Example of Colony PCR results:



Note that in lane 5, you don't see very much 'smear' around 3KB (Ladder is NEB's 2Log), which indicates that this clone is NOT correct. In most other cases in the above gel you can see faint bands around 2-3 KB, which are the correct length for the completed TALs in the picture above. For TALs with >22 repeats, it is common to fail to amplify enough full-length TAL to see on a gel, however if you can see the 'smear' those clones are almost always correct. The 'ladder effect' is evident in some of the lanes (4, 11,12,13).

DAY5

19. Miniprep the pTAL vectors containing your final full-length TALEN and sequence with primers **pJDS-GG screen F and R.**