Golden Gate TALEN assembly

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 (those ==highlighted== are often not found in most labs and need to be ordered specifically for this protocol):

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
TAL_F1: ttggcgtcggcaaacagtgg
TAL_R2: ggcgacgaggtggtcgttgg
SeqTALEN_5-1 catcgcgcaatgcactgac (use this for sequencing in place of TAL_F1)

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 BsaI
    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) H_2O up to **20µl total reaction volume**

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 transformatin, inactivation is not necessary

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

8. Plate on Spec^{50} 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 55º, 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:
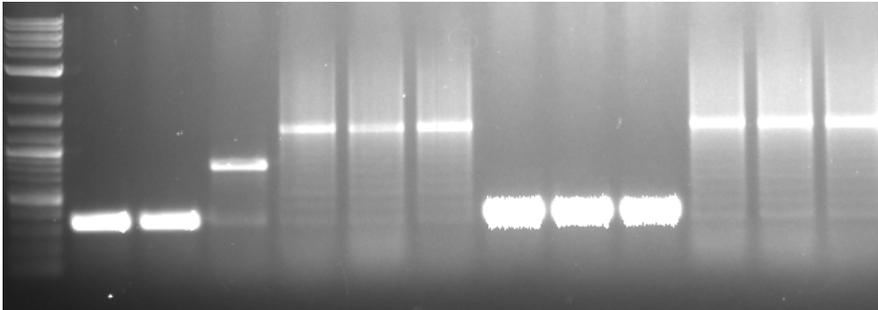
Note: Lanes 2 and 3 are negative pFUS clones (empty). Lane 4 contains the 'correct' clone for this pFUS. pFUS clones that only contain 1 or 2 repeats are very similar in size to empty pFUS clones – check the size carefully. Lanes 5,6,7,11,12,13 show the 'laddering' effect well. DNA ladder is NEB's 2Log

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 AflII 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) 150ng of respective **pLR vector** – this is the vector containing the last "half-repeat" including the last RVD, choose it according to your TALEN sequence – it is the last RVD in the sequence (there are 5 pLR vectors – pLR-HD, pLR-NG, pLR-NI, pLR-NN, pLR-NK)
c) 75ng of destination vector pTAL1, 2, 3, or 4
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) H_2O up to 20µl

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 + X-gal and IPTG (see above). After you gain confidence/experience with the GG cloning, it's reasonable to skip the IPTG/X-gal for the pTAL cloning.

DAY4

17. Pick 1-3 white colonies and check by colony PCR using primers TAL_F1 and TAL_R2 using these conditions:
    a. Anneal at 55º, 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:
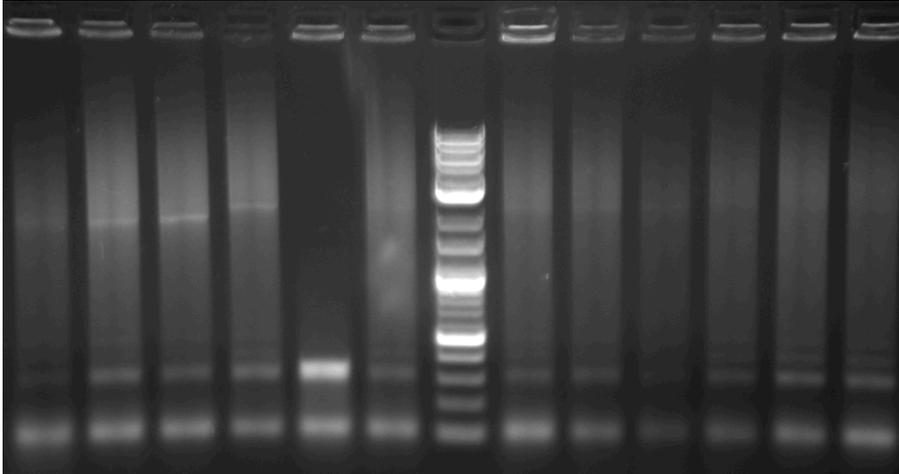
Note that in lane 5, you don't see very much 'smear' around 3KB (Ladder is NEB's 2Log), which indicates that this clone is NOT correct. In most other cases in the above gel you can see faint bands around 2-3 KB, which are the correct length for the completed TALs in the picture above. For TALs with >22 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

20. Optional restriction digestion testing/sequencing:
Use enzymes BstAPI (or StuI) and AatII to cut out the final array of repeats – check on a gel
You can use BspEI enzyme, which cuts only in HD repeats (except HD1 repeats) – on the gel you'll get a pattern resembling your TALEN sequence and HD repeats position

Note: there is no BspEI site in the first (i.e. #1) and the last (i.e. #N) HD repeat

and sequence with primers SeqTALEN 5-1 and TAL_R2

21. Your TALEN is ready to use in a yeast-based DNA cleavage assay. Or further cloning, you can cut the TALE domain out using BamHI or combination of XbaI and EcoRV (blunt)