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 86 library vectors (Available from: http://www.addgene.org/TALeffector/goldengateV2/)
B) 10X T4 DNA ligase buffer (NEB: included with ligase)
C) T-4 DNA ligase (NEB:M0202)
D) restriction endonuclease BsaI (NEB: R0535)
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
TAL_Seq_5-1 catcgcgcaatgcactgac (use this for sequencing in place of TAL_F1)
TAL_R3: GGCTCAGCTGGGCCACAATG

restriction endonucleases – for restriction screening – all optional (NEB)
AflII
XbaI
BstAPI or StuI

AatII
BspEI

Hints, Tips and Troubleshooting

PLASMIDS: There are known issues with several plasmids in our TAL assembly kit.

A. E. coli containing pHD2 grow slowly. The basis for this phenotype is unknown.
B. Plasmids grown in DH5-alpha yield significantly more plasmid DNA than those grown in DH10B. Plasmids obtained from Addgene are in one of these two E. coli strains. The basis for this plasmid yield difference is unknown.
C. Several plasmids are known to have re-arrangements in the backbones. These rearrangements do not affect their use in the Golden Gate assembly protocol. The plasmids with these known problems are pNK1, pNK2, pNK3, pNK4, pNK5, pNK6, pNK7, pNK8, pNK9, pNK10, pNG9.
D. Because of the high efficiency digestion/ligation required in DAY1, we recommend using Qiagen minipreps with a maximum culture volume of 5mL for the plasmids containing the repeats and the pFUS vectors (e.g. pHD1-pHD10). We have found that prepping these plasmids with kits from other users seems to reduce efficiency of the reaction in DAY1, and this is supported by notes from other users. For reactions on DAY3, we routinely use Miniprep kits from other manufacturers with excellent success.
E. We highly recommend sequencing each plasmid prep of the Golden Gate reagents. This includes each time your re-prep a plasmid simply because you may have run out of one. After sequencing, carefully look at the sequencing trace from each of the repeat-containing plasmids; specifically the bases that encode the RVD. If you have a mix of two of the same number of plasmid (e.g. pNN1 and pNG1), you can see double peaks at the bases that encode RVD.

DAY1, Step 1: The Voytas lab generally chooses DNA-binding arrays of lengths of 14-21 repeats. Not only does this make the assembly easier, but also makes many of the downstream applications easier. If the precise site selection is critical for an application, we will choose longer DNA-binding arrays if the target requires. Further, though a recent publication (Reyon, et al., 2012) has shown that most of the original design parameters described in Cermak, et al (2011) are not required, our experience, and several publications, show that TALENs with these design parameters do work well. If TAL binding location is paramount and the TALENT software doesn't identify a TAL in the desired location, we remove selection criteria one at a time, in the following order:

1. Do not allow sites to end in G and Require a T at position N
2. Require C, G, or T at position 2 (not an A)
3. Require A, C, or G at position 1 (not a T)
4. Percent Composition

Additionally, data from our group (Christian, et al., 2012. PLoS One 7(9)) show that spacer lengths as short as 13bp can be used with the Delta152,+63 architecture (see DAY3 Step10).

DAY1, Step 4: Make sure to use BsaI (NEB # R0535), NOT BsaI-HF. Other users have reported that BsaI-HF doesn't work well in the T4 DNA Ligase buffer. In the Voytas lab, we currently do only 10µL reactions. The protocol below is written for 20µL reactions as described in Cermak, et al., 2011. We have also found that normal T4 Ligase from NEB (M0202) is sufficient for the reaction and that Quick Ligase is not required. The most common problem that users have with the protocol is poor efficiency of the ligation/digestion reaction with large number of repeats. The most common cause is old/bad T4 Ligase Buffer. We aliquot our T4 Ligase buffer into ~25uL aliquots and freeze/thaw them only a few times before discarding. One should also (carefully) smell the buffer – if you can't smell DTT, throw it out and get a new tube. We dilute all of our plasmids that are used over and over again to a standard concentration (usually 150ng/µL). This greatly simplifies the assembly procedure.

Note about additions to Golden Gate TALEN and TAL Effector kit

pZHY500 and pZHY501 are Golden Gate compatible yeast expression vectors with deletions in the coding sequence that result in truncations of both the amino and carboxyl portions of the TAL portion of the protein. These plasmids are derived from the pTAL3 and pTAL4 vectors. Details can be found in Plant Physiol. 2013 Jan;161(1):20-7. doi: 10.1104/pp.112.205179. Epub 2012 Nov 2.

pCP5b is an unpublished plasmid that allows one to test TALEN activity in yeast. This plasmid is very similar to the published pCP5 (see Proc Natl Acad Sci U S A. 107(26):12028-33.), differing in that the origin of replication and the antibiotic resistance has been changed for convenience.

The final additions are the NH RVD module plasmids. Recent data show that the NH RVD binds to guanine more specifically than NN RVDs, however NH RVDs do not bind as strongly as NN RVDs. For more information on this type of RVD, see *Nature Biotechnology* **30**, 593–595 (2012).

The NH RVD module plasmids and pCP5b plasmid are provided pre-publication. The creators of these plasmids would appreciate acknowledgements to AJ Bogdanove (Cornell University) and C Schmidt (Iowa State University).

DAY1

1. Choose your TALEN RVD sequence: N = number of RVDs (12-31). Visit https://tale-nt.cac.cornell.edu/ for sequence analysis tools.

If the TALEN length is 12-21:

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

3a. 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:

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

3b Pick plasmids 21 up to N-1 + destination vector pFUS_B#N-1

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 T4 DNA ligase
d) 2µl 10X T4 DNA ligase buffer (to final concentration of **1X**)
e) 2µL 10X Bovine Serum Albumen (final concentration of 0.1mg/mL)
f) H_2O 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). We 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.

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.

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) 1µl 10mM ATP
b) 1µl 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

Transform your chemically competent cells (we use 5µl of the GG reaction)

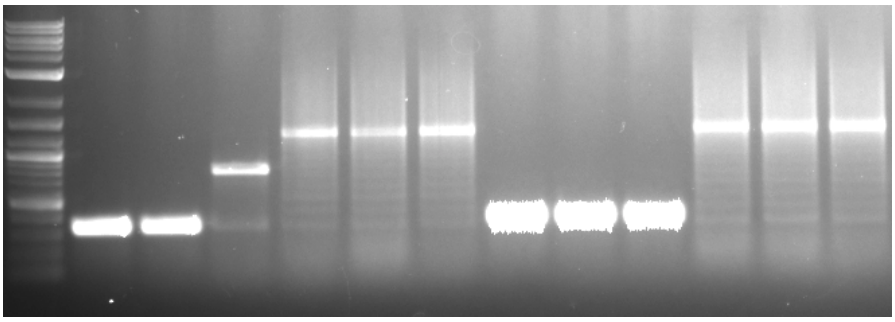
5. Plate on $Spec^{50}$ plates + 40µL of 20mg/mL X-gal +40µL of 0.1M IPTG. When plating transformations of the pFUSB vectors that have fewer repeats (especially less than 6 repeats), be careful to not plate all the cells as the efficiency is so high you can't pick single colonies on day 2.

DAY2

6. 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. A detailed explanation of the cause of this can be found in Hommelsheim, et al., 2014; doi:10.1038/srep05052.

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

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

DAY3

8. 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**)

9. 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

10. Mix golden gate reaction **#2**
    a) 150ng of each vector pFUS vector
    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 4 pLR vectors – pLR-HD, pLR-NG, pLR-NI, pLR-NN).

c) 75ng of destination vector pTAL1, 2, 3, or 4
d) 1µl Esp3I restriction enzyme
e) 1µl T4 Ligase
f) 2µl 10X T4 DNA ligase buffer (the buffer for the Esp3I enzyme)
g) H_2O up to 20µl

11. 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.**

12. Transform your competent cells (use 5µl of the reaction)

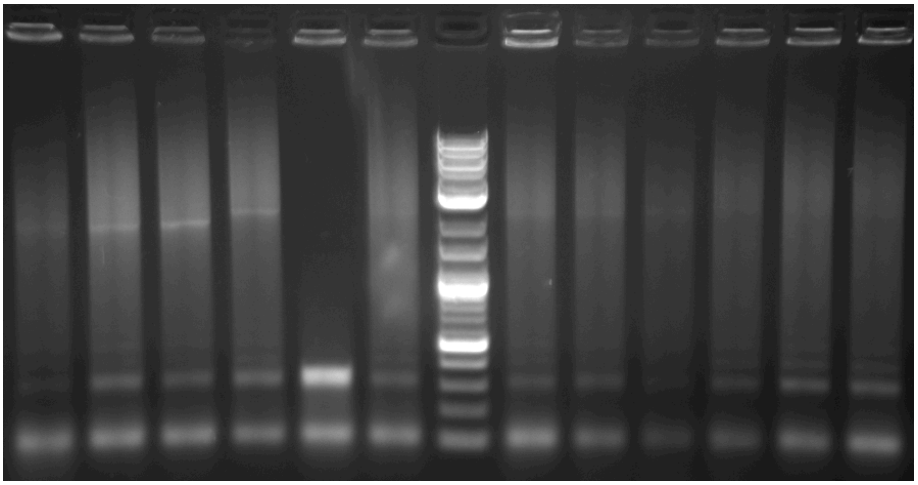
Note: Plasmid-Safe nuclease treatment is not necessary in this case, because the final destination vector has no homology with the inserted repeats

13. 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

14. 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.

15. 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

16. Miniprep the pTAL vectors containing your final full-length TALEN

17. 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. See the document: Sequencing_TALENs.doc for more information.

18. 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)