LIC-TALE assembly protocols

Based on the technology published in Schmid-Burgk and Schmidt et al.

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Reagents

Enzymes	1. Mva1269I 10U/µl (Fermentas)
	2. PstI FastDigest (Fermentas)
	3. KpnI FastDigest (Fermentas)
	4. T4 DNA polymerase 3U/µl (Enzymatics)
Buffers	1. 10x FastDigest buffer (Fermentas)
	2. 10x buffer R (Fermentas)
	3. 10x NEB2 buffer (NEB)
	4. BSA solution 10g/l (NEB)
	5. dATP and dTTP solution 100mM (Fermentas)
Plasmids	1. 2-mer fragment library (available from Addgene)
	2. level 1 backbone plasmids (available from Addgene)
	3. level 2 backbone plasmids (available from Addgene)
Bacteria	1. Chemically competent DH10b bacterial cells
	2. LB medium
	3. Ampicillin stock solution100mg/ml
	4. Kanamycin stock solution 30mg/ml
	5. Silica-based plasmid purification kit (e.g. Promega)

Equipment:

1. Water bath or standard thermocycler (20°C – 80°C)
2. Standard pipettes

Master mixes:

1.1 Digestion master mix for fragments

0.3µl 10x Buffer R
0.1µl Mva1269I (10U/µl)
2.6µl H_2O

1.2 Digestion master mix for backbones

6µl 10x buffer FastDigest
2µl KpnI FastDigest
(For BBL1 only: add 2µl PstI FastDigest)
30µl H_2O

2.1 Chew back mix A

1µl 10x NEB2 buffer
0.1µl BSA (10g/l)
0.1µl dATP 100mM
0.33µl T4 DNA polymerase (3U/µl)
5,47µL H_2O

2.2 Chew back mix T

1µl 10x NEB2 buffer
0.1µl BSA (10g/l)
0.1µl dTTP 100mM
0.33µl T4 DNA polymerase (3U/µl)
5,47µl H_2O

A. LIC TALE assembly protocol using 2-mer fragments - manual mode

Protocol:

1. Preparation of 2-mer fragments and level 1 backbones for LIC

a. Digest 7µl 2-mer fragment plasmids (100 ng/µl, eluted in 1x Buffer R) by adding 3µl digestion master mix 1.1 at 37°C for 60 minutes.

b. Digest 20µl backbone plasmids (1µg/µl, eluted in water) by mixing with 40µl digestion master mix 1.2 at 37°C for 60 minutes.

c. Gel-purify level 1 backbone digestions.

d. Add 3µl of the digested DNA solution to 7µl of chewback master mix containing the appropriate STOP dNTP:

- *2-mer ID 1-2 & ID 3-4 / Level 1 Backbone ID 4-3:* *Chew back mix A*
- *2-mer ID 2-3 & ID 4-1/ Level 1 Backbone ID 1-4 & ID 3-2:* *Chew back mix T*

e. Incubate at 27°C for 5 minutes, put on ice and then heat up to 75°C for 20 minutes.

f. Dilute 2-mers and backbones 20-fold using 1 x NEB2 buffer. *(These dilutions can be stored at -20°C for further use. Indeed, it is recommended to generate a pre-digested and pre-chewed fragment library for all subsequent assembly reactions based on the available Addgene library plate. Such a library contains 2-mer fragments that can be used in up to 80 assembly reactions. Of note, our assembly calculator that can be found at www.hornunglab.de/Tassembly.html uses the coordinates of the 96-well Addgene library plate.*

2. Assembly reaction of 6-mer fragments

a. Combine 2.5µl of the appropriate three 2-mer fragments (7.5µl in total) and 2.5µl of the appropriate predigested and chewed level 1 backbone to a total volume of 10µl.

b. Incubate at 55°C for 30 minutes and then at 25°C for 3 hours.

3. Transformation

a. Mix 2µl of the assembly reaction with 10µl of ice-cold chemically competent DH10b *E. coli*

b. Heat shock at 37°C for 3 minutes

c. Incubate on ice for 2 minutes

d. Add 100µl LB medium

e. Incubate at 37°C for 1h at 900rpm

f. Add 1ml LB medium supplemented with 30 µg/ml Kanamycin

g. Incubate at 37°C for 12-16 hours at 900rpm

4. Plasmid purification

Purify plasmid DNA using a standard silica spin-column based purification protocol and elute DNA in 100µl 1x Buffer R.

5. Preparation of 6-mer fragments for LIC

a. Digest 7µl 6-mer fragment plasmids (100 ng/µl, eluted in 1x Buffer R) by adding 3µl digestion master mix 1.1 at 37°C for 60 minutes.

b. Digest 20µl level 2 backbone plasmids (1µg/µl, eluted in water) by mixing with 40µl digestion master mix 1.2 at 37°C for 60 minutes.

c. Gel purify level 2 backbone digestions.

d. Add 3µl of the digested DNA solution to 7µl of chewback master mix containing the appropriate STOP dNTP:

- *6-mer ID 1-4 and ID 3-2* *Chew back mix A*
- *6-mer ID 4-3 / Level 2 Backbones ID 1-2* *Chew back mix T*

e. Incubate at 27°C for 5 minutes, put on ice and then heat up to 75°C for 20 minutes.

f. Dilute backbones 20-fold using 1 x NEB2 buffer.

6. Assembly of 18-mer fragments

a. Combine 2.5µl of the appropriate three 6-mer fragments (7.5µl in total) and 2.5µl of the desired predigested and chewed level 2 backbone to a total volume of 10µl.

b. Incubate at 55°C for 30 minutes and then at 25°C for 3 hours.

7. Transformation

a. Mix 2µl of the assembly reaction with 10µl of ice-cold chemically competent DH10b *E. coli*

b. Heat shock at 37°C for 3 minutes.

c. Incubate on ice for 2 minutes.

d. Add 100µl LB medium.

e. Incubate at 37°C for 30 minutes at 900rpm.

At this point three different options can be pursued: (8a-9a) growth in polyclonal culture, (8b-9b) growth at limiting dilution (recommended) or (8c-9c) growth on a bacterial plate.

8a. Growth in polyclonal culture

a. Add 1ml LB medium supplemented with 100 µg/ml Ampicillin to the transformation mix.

b. Incubate at 37°C for 12-16 hours at 900rpm.

9a. Plasmid purification

Purify plasmid DNA using a standard silica spin-column based purification protocol and elute DNA in 100µl H_2O.

8b. Growth at limiting dilution

a. Transfer 1µl* of the transformation mix to 1000µl LB medium supplemented with 100 µg/ml Ampicillin in quintuplicates.

b. Incubate at 37°C for 12-16 hours at 900rpm.

c. Harvest 1-2 grown cultures for plasmid purification.

** 1µl: the amount of bacteria being transferred has to be determined experimentally. As outlined in Suppl. Fig. 5, we recommend an amount that leads to 50% growth in the inoculated cultures.*

9b. Plasmid purification

Purify plasmid DNA using a standard, silica spin-column based purification protocol and elute DNA in 100µl H_2O.

8c. Growth on bacterial plate

a. Streak out the entire transformation mix onto an LB agar plate supplemented with 100 µg/ml Ampicillin.

b. Incubate plates at 37°C overnight.

c. Pick 1-2 single colonies and inoculate 1000µl LB medium supplemented with 100 µg/ml Ampicillin.

d. Incubate at 37°C for 12-16 hours at 900rpm.

9c. Plasmid purification

Purify plasmid DNA using a standard, silica spin-column based purification protocol and elute DNA in 100µl H_2O.

B. LIC TALE assembly protocol using 5-mer fragments - manual mode

In order to generate 15.5 RVD TALENs using the 5-mer library, steps 5-9 from the above protocol have to be followed with the following chew back mix combinations:

- *5-mer ID 3-2 / Level 2 Backbone ID 4-1 Chew back mix A*
- *5-mer ID 4-3 and ID 2-1 Chew back mix T*

C. LIC TALE assembly protocol using 2-mer fragments - automated mode

Reagents:

As above

As plastic material we recommend the following material:

- 384-well plates for assembly reactions (Costar, 3657)
- 96 deep-well plates for bacterial cultures (Abgene, Ab-0661)
- 96-well plates for plasmid DNA storage (Greiner Bio One, 650101)
- 96-well silica column binding plates (Promega, Wizard SV96)

Equipment:

1. Liquid handling workstation equipped with single channel pipettes and a 96-channel pipette head
2. 96- or 384-well reagent dispenser
3. Waterbath and standard 96-well thermocycler

Master mixes:

As above

Protocol:

The main structure of the protocol for automated assembly of TALENs using LIC is the same as the above described protocol for manual assembly. However, various steps can be automated as follows:

1. Preparation of 2-mer fragments for LIC

We recommend preparing a pre-cut and pre-chewed 2-mer fragment library arrayed in a 96-well plate. This fragment library can be stored at -20°C in between uses. Also, pre-digested, pre-chewed stocks of the backbones can be prepared in advance and stored at -20°C.

2. Assembly reaction of 6-mer fragments

Using the 2mer fragment library as the source, assembly reactions can be performed in 384-well plates using a standard pipetting robot. We recommend to pre-aliquot the level 1 backbone into the assembly plate using a reagent dispenser. For the required temperature step we employ a water bath.

3. Transformation

We recommend adding the competent bacteria directly to the 2µl aliquot of the reaction using a reagent dispenser. All liquid handling steps of the transformation protocol can be performed using a reagent dispenser / pipetting robot and bacteria are grown in 96 deep-well plates.

4. Plasmid purification

Plasmid DNA preparation can be performed using a 96 well spin column purification kit. We recommend eluting the resulting 6-mer fragment plasmids and 18.5 TALEN plasmids in 100µl 1xBuffer R or 100µl H_2O, respectively. This typically results in 200 ng/µl DNA (within a range of ±50 ng/µl DNA it is not required to adjust the concentration of DNA).

5. Preparation of 6-mer fragments for LIC

7µl of plasmid DNA containing the 6-mer fragments are transferred to a 384-well plate using a 96-channel pipetting head. To this plate we add digestion master mix using a reagent dispenser and subsequently transfer 3µl of the digested DNA using a 96-channel pipetting head to a new 384-well plate. 7µl chewback mix is spotted on top of the digested DNA using a reagent dispenser. For the required temperature step we employ a water bath.

6. Assembly of 18-mer fragments

*As described above (*2. Assembly reaction of 6-mer fragments*), all LIC assembly steps can be performed using a liquid handling robot.*

7. Transformation

*As described above (*3. Transformation*).*

8. Plasmid Purification

We highly recommend using the limiting dilution protocol for automated processing of the fully assembled TALEN constructs. To this effect we transfer the required amount of transformation mix to a standard 96 well plate already containing 100µl LB medium supplemented with 100µg/ml Ampicillin. The next day, 20 µl of cultures grown under limiting dilution conditions can be used to inoculate cultures of 1000µl LB medium containing 100µg/ml Ampicillin in a 96 deep-well plate using a liquid handling robot. After 4-6 hours the cultures can be processed for subsequent plasmid DNA preparation.

D. LIC TALE assembly protocol using 5-mer fragments - automated mode

In order to generate 15.5 RVD TALENs using the 5-mer library, steps 5-9 from the above protocol have to be followed with the following chew back mix combinations:

- *5-mer ID 3-2 / Level 2 Backbone ID 4-1 Chew back mix A*
- *5-mer ID 4-3 and ID 2-1 Chew back mix T*