**Detailed STAR protocol**

***Library preparation***

Primers used: pTAL3: CGCGACGTAATACGACTCAC; pTAL4: CACGACAGGTTTCCCGACT.

Template input: 1 ng at 1 ng/µl (= 1µl)

Using Herculase II DNA Polymerase (Agilent Technologies) two reactions are enough to use the 1mer for 32 assemblies; this protocol is for quadruplicate PCRs.

Master Mix:

|  |  |  |
| --- | --- | --- |
|  | Per reaction [µl] | 70x MasterMix [µl] |
| H2O | 64 | 4480 |
| Buffer | 20 | 1400 |
| PrimerMix (10µM each) | 5 | 350 |
| dNTPs (10mmM) | 10 | 700 |
| Herculase II | 1 | 70 |
| *Total* | *100* | *7000* |

Cycling conditions: 98˚C - 3 min; 35 cycles of 98˚C - 10 sec, 60˚C - 10 sec, 72˚C - 30 sec; 72˚C - 5 min; 4˚C – forever.

3 µl of each PCR are run for QC on 1% agarose gel. PCR products are pooled and purified using the QIAquick PCR Purification Kit (Qiagen) and eluted in 40 µl (2x 20 µl) preheated ddH2O (to 60˚C) to maximise yield.

DNA amount can be measured here and should range between 15-20µg in total.

Digest is optimised for complete digest of 4mers. 40 units of BciVi will accomplish this in most cases and is still cost-effective. CutSmart buffer and 4 µl BciVi (= 40 units) per reaction are added to a total volume of 50 µl and fragments are digested for 12 hours at 37˚C. Heat inactivation of the enzyme is performed for 20min at 80˚C. Samples are run on 2.5-3% agarose gels using SybrSafe.

Gels have to be as thin as possible to visualise bands of high DNA content and to minimize the amount of agarose in gel purification.

Expected Band sizes:

1 (Longest): 444bp +~80bp

4, 5, 8, 9, 12, 13, 16 (Long): 393bp +~80bp

2, 3, 6, 7, 10, 11, 14, 15 (Short): 145bp+~75-80bp

gel purify with Qiagen kit

DNA is eluted in 30µl (20+10µl) pre-heated ddH2O. For 4mer assembly, 90 ng of the large fragments (1, 4, 5, 8, 9, 12, 13, 16) and 30 ng of the small fragments (2, 3, 6, 7, 10, 11, 14, 15) are needed, with 1.25 µl of each fragment, therefore it is convenient to adjust the 1mers to the according concentrations (72 ng/µl for big fragments, 24 ng/µl for small fragments).

***4mer assembly***

A total volume of 1mers of 5µl is mixed with 15 µl Gibson mix and incubated at 50˚C for 30 minutes. In the meanwhile, PlasmidSafe (Epicentre Plasmid-safe Cat No E3105K) Master Mix is prepared as follows:

|  |  |
| --- | --- |
|  | Per reaction [µl] |
| H2O | 13.9 |
| buffer | 4 |
| ATP | 1.6 |
| enzyme | 0.5 |
| *Total* | *20* |

PlasmidSafe is added and incubated for 15 minutes at 37˚C before heat-inactivating immediately at 72˚C for another 15 minutes.

***Amplification and digest of 4mers***

Use of 0.5 µl 4mer as PCR input with HerculaseII (Agilent Technologies).

MasterMix:

|  |  |
| --- | --- |
|  | Per reaction [µl] |
| Buffer | 10 |
| dNTPs (10mM) | 5 |
| PrimerMix (10µM each) | 2.5 |
| Herculase II | 0.5 |
| H2O | 31.5 |
| *Total* | *49.5* |

Cycling parameters: 98°C for 2 minutes, 30 cycles of 98°C for 20 seconds, 60°C for 20 seconds and 72°C for 30 seconds, 72°C for 3 minutes.

For blunt end generation, restriction digest using FastDigest SchI (Life Technologies) is performed directly in PCR buffer.

|  |  |
| --- | --- |
|  | Per reaction [µl] |
| DNA | 50 |
| Buffer (10x) | 10 |
| SchI | 1.5 |
| H2O | 88.5 |
| *Total* | *150* |

Incubation for 1 h at 37°C with an additional 5 minutes at 80 °C for heat inactivation of the enzyme.

***4mer purification using Agencourt AMPure XP (Beckman-Coulter)***

Samples are mixed 1:1 with 15% PEG-8000 (in 1.8 M NaCl; final PEGconc. of 7.5%). It is important that samples are mixed exactly with a 1:1 ratio. Beads are warmed up to RT for 30 min. 20 µl of beads/sample are added and mixed. Incubation for 20 min at RT and before leaving on magnet for 5 min and discarding supernatant. Samples are washed twice with 50 µl of 70% EtOH (left on magnet for washes): incubate for 1 min, take off EtOH and repeat washing step. After wash steps, beads are dried for 2 min on magnet. It is important that there is no remaining alcohol, but beads should not ‘overdry’, either. DNA is eluted in 40ul H2O / sample. For this, samples are taken off the magnet, incubated with water for 2 min, returned to magnet for 5 min and samples are collected.

Concentration measurement of clean 4mers is performed on Agilent TapeStation or Qubit Fluorometric Quantitation (Thermo Fisher Scientific). Qubit can overestimate concentration by 3-fold, therefore all 4mers should be measured with the same device to guarantee equimolar ratios in 16mer assembly.

***TALE assembly***

Prior to the assembly, 5 µg of the respective destination vector are digested with 20 units of BciVI for 12 hours and gel purified. Open backbone needs to be aliquoted to prevent numerous freeze and thaw cycles.

In case of BciVI sites present in the functional domain of choice (e.g. KRAB or p300core), an alternative strategy can be employed to insert DBDs into TALE destination backbones with the STAR method. Instead of linearising the backbone with BciVI, 2µg of the backbone are digested with 10U SbfI-HF (NEB) for 1h at 37˚C with subsequent heat-inactivation at 80˚C for 20 min. For degradation of 3’ overhangs, the open vector is incubated with 2U Mung Bean Endonuclease (NEB) directly in CutSmart buffer for 30 min at 30˚C. The reaction is directly loaded onto an agarose gel and gel extraction of the open backbone is performed.

A total of 51.5 ng of 4mers is assembled with an equimolar ratio, i.e. 14 ng of first 4mer and 12.5 ng each of the three other 4mers with 50 ng backbone of choice. If 4mers are too diluted for a final reaction volume of 5 µl, i.e. in more than 4µl with the backbone at 50ng/µl, they need to be vacuum-concentrated with caution not to overdry them. The backbone is added to the concentrated samples, before addition of 15 µl Gibson mix. Mixture is pipetted up and down several times to mix and wash tube and transfered back to a PCR tube for Gibson assembly of 1h at 50˚C.

1 µl of the Gibson reaction are directly transformed into competent bacteria and grow on Amp resistance plates ON at 37˚C.

***Colony PCR*** is performed with DreamTaq:

|  |  |  |
| --- | --- | --- |
|  | Per reaction [µl] | 50x |
| buffer | 2 | 100 |
| dNTPs | 0.4 | 20 |
| primer mix (STAR2 seq) | 2 | 100 |
| DreamTaq | 0.25 | 12.5 |
| H2O | 14.35 | 717.5 |
| *Total* | *19 µl* | *950* |

For colony PCR, master mix is prepared in 96 well plate together with a second 96 well plate with 50 µl ddH2O / well. Bacterial colonies are directly picked with pipette set to 1µl. Tip is first washed in H2O plate and subsequently 1 µl of H2O is transferred to the PCR mix plate with the same tip. Products are run on 1% agarose gels and analysed for ~1.77 kb product.