ddRAD protocol (EcoRI-MseI)

Last update: 13/10/2017

This protocol is based on a combination of the original ddRAD SE protocol of Parchman et al (2012) used previously in Patrik Nosil’s Lab and the ddRAD protocol of Peterson et al (2012) (basically adding a common MseI Y/fork adaptor). It was adapted for PE by Jill Olofsson (postdoc at Pascal-Antoine Christin Lab) and slightly modified afterwards by Clarissa Ferreira de Carvalho (PhD student at Patrik Nosil’s Lab). Changes to the original ddRAD SE protocol are highlighted.

# 1. Preparing adaptors and primers

## 1.1 EcoRI (barcoded)

EcoRI\_P2.1: 5’ CTCTTTCCCTACACGACGCTCTTCCGATCT+barcode+C 3’
EcoRI\_P2.2: 5’ AATTG+barcode+AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT 3’

Same EcoRI adaptor as in the original protocol is used. Barcodes are 8-10 bp long and differ by at least 4 bases. They are located between the adaptor sequence and the restriction site. Order as single stranded => they come in pairs

1. 1 µl of each oligo (100 µM stock) + 98 µl H2O => 100 µl of 1 pmol/µl (1 µM) of double-stranded adaptor stock.
2. On PCR machine heat to 95ºC for 5 min and then slowly cool to room temperature (leave it in the machine for 20-30 min and then take it out and let it cool completely)

## 1.2 MseI (common adaptor)

Use the MseI adaptor from Peterson et al (2012) (P2.2 modified for cutting site):

MseI\_P2.1: 5’ GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3’
MseI\_P2.2: 5’ TAAGATCGGAAGAGCGAGAACAA 3’

but method for annealing is:

1. 10 µl of each oligo (100 µM stock) + 80 µl H2O => 100 µl of 10 pmol/µl (10 µM)
2. On PCR machine heat to 95ºC for 5 min and then slowly cool to room temperature (leave it in the machine for 20-30 min and then take it out and let it cool completely)

## 1.3 Primers

Forward primer is the same than previous SE protocol, reverse is different:

Illpcr1 (Forward):
5’ A\*A\*TGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3’

PCR2\_Idx\_1\_ATCACG (Reverse):
5’ CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGC 3’

1. Mix 2.5 µl of each primer (100 µM stock) with 95 µl H2O => working stock

# 2. Double digestion

1. 7 µl DNA in each well (20-150 ng/µl = 120-900 ng), keep on ice, semi-skirted plate
2. Prep master mix (vortex and spin)

|  |  |  |
| --- | --- | --- |
|  | **1 sample (µl)** | **x10 samples (50% overhang) (µl)**  |
| 10X T4 Buffer | 1.15 | 17.25 |
| 1 M NaCl | 0.60 | 9 |
| 1 mg/mL BSA | 0.60 | 9 |
| H2O | 0.25 | 3.75 |
| MseI enzyme | 0.12 | 1.8 |
| EcoR1 enzyme | 0.28 | 4.2 |

1. Add 3 µl MM to each sample (seal, vortex, spin)
2. Incubate at 37º C for 8 h (exactly) with a heated lid

# 3. Adaptor ligation

1. Thaw the adaptors (very slowly, in the fridge, takes about 3h)
2. Make master mix, flick/invert to mix

|  |  |  |
| --- | --- | --- |
|  | **1 sample (µl)** | **x10 samples (50% overhang) (µl)**  |
| MseI adaptor | 1 | 15 |
| H2O | 0.072 | 1.08 |
| 10X T4 buffer | 0.1 | 1.5 |
| 1M NaCl | 0.05 | 0.75 |
| 1mg/µL BSA | 0.05 | 0.75 |
| T4 ligase | 0.1675 | 2.5125 |

1. Add 1.4 µl MM to digested DNA
2. Add 1 µl EcoRI adaptor to correct digested DNA according to plate set-up
3. Cover, seal, vortex and centrifuge
4. Incubate at 16 ºC overnight.
5. Dilute the digest-ligation products up to 120 µl with 0.1X TE (the original SE protocol was to up to 200 µl)
6. Store at 4 ºC for a month or -20 ºC for longer

# 4. PCR amplification

Run two separated 20 µl PCR reactions per sample and pool together later.

1. Make master mix

|  |  |
| --- | --- |
|  | **20 µl rxn/ sample** |
| H2O | 9.67 |
| 5X Iproof buffer | 4.0 |
| dNTP (10 mM) | 0.4 |
| MgCl2 (50 mM) | 0.4 |
| Primers (2.5 µM for each) | 1.33 |
| Iproof taq | 0.2 |
| DMSO | 0.15 |

1. Add 16.15 µl MM to each well of a plate, non-skirted
2. Add 4 µl R/L product to each well

98 ̊C

30s

98 ̊C

60 ̊C

72 ̊C

72 ̊C

20s

X 20

30s

40s

10min

# 5. Verification of libraries

On a 0.8% agarose gel load 5 µl of each PCR product to check for success of library prep.

# 6. Size selection

Pool 5µl of each PCR product (=10 µl for each sample/library) and then size select for ~300-500 bp fragments. Size selection was done by the sequencing service (NCGR) using beads. There are multiple methods for in-house size selection including cutting gel bands, beads and BluePippin.