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Single-strand library preparation protocol

Tomasz Suchan[1]

[1]W. Szafer Institute of Botany, Polish Academy of Sciences

1 Works for me dx.doi.org/10.17504/protocols.io.q5ydy7w

Tomasz Suchan
W. Szafer Institute of Botany, Polish Academy of Sciences

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Tin MM-Y, Economo EP, Mikheyev AS (2014) Sequencing Degraded DNA from Non-Destructively Sampled Museum Specimens for RAD-Tagging and Low-Coverage Shotgun Phylogenetics. PLoS ONE 9(5): e96793. https://doi.org/10.1371/journal.pone.0096793

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GUIDELINES

This library preparation protocol can be easily adapted for different indexing strategies. Combinations of indexed primes can be used for generating single- or double-indexed libraries. In the first case, PCR_F_universal primer is used in combination with PCR_R_**** primers. For double indexed libraries, the combinations of PCR_F_**** and PCR_R_**** can be used for up to 384 combinations with the primers listed below.

Additionally, libraries can be prepared by adding inline index (barcode) to the adapters and amplified either with unindexed illumina primers, or single- or double-indexing strategy. This sequence is then present at the begining of the reads and can be used for sample demultiplexing.

Finally, by combining inline barcode with indexed forward and reverse primers, triple-indexed libraries can be constructed. This design is especially useful for the libraries used for the sequence-capture protocols. In such experiments, the reads are captured on DNA or RNA baits and reamplified afterwards. If performed on pooled

samples, the post-capture PCR can cause chimeric reads formation among hopmologous sequences from different specimans. Triple-indexing strategy allows for controlling these by comparing the index combination used with the inline barcode.

Adapter sequences:

Unindexed adapters:
Tin_P1_lower: [PHO]AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
Tin_P1_upper: CCCTACACGACGCTCTTCCGATCT

Inline indexed (barcoded) adapters:
Tin_P1_lower_barcoded: [PHO]nnnnnnnnAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
Tin_P1_upper_barcoded: CCCTACACGACGCTCTTCCGATCTnnnnnnnn

The barcode sequences (nnnnnnnn) can be designed using published scripts (https://bioinf.eva.mpg.de/multiplex/). To prepare 25 µM working solution, mix 50 µl of the upper and lower oligos (100 µM stock) with 40 µl of water and 10 µl of the 10x annealing buffer to obtain 100 µL of working solution. Adapters have to be annealed as follows: heat to 95°C for 1 minute and slowly bring to 20°C with a ramp of 0.1°C/s.

Oligo used in the second-strand synthesis step:

Tin_P2-C5: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCCCC

Primer sequences used:

forward: AATGATACGGCGACCACCGAGATCTACACnnnnnnnnACACTCTTTCCCTACACGACGC
reverse: CAAGCAGAAGACGGCATACGAGATnnnnnnnnGTGACTGGAGTTCAGACGTGTGC
nnnnnnn = index sequence

PCR_F_A501: AATGATACGGCGACCACCGAGATCTACAC**TGAACCTT**ACACTCTTTCCCTACACGACGC
PCR_F_A502: AATGATACGGCGACCACCGAGATCTACAC**TGCTAAGT**ACACTCTTTCCCTACACGACGC
PCR_F_A503: AATGATACGGCGACCACCGAGATCTACAC**TGTTCTCT**ACACTCTTTCCCTACACGACGC
PCR_F_A504: AATGATACGGCGACCACCGAGATCTACAC**TAAGACAC**ACACTCTTTCCCTACACGACGC
PCR_F_A505: AATGATACGGCGACCACCGAGATCTACAC**CTAATCGA**ACACTCTTTCCCTACACGACGC
PCR_F_A506: AATGATACGGCGACCACCGAGATCTACAC**CTAGAACA**ACACTCTTTCCCTACACGACGC
PCR_F_A507: AATGATACGGCGACCACCGAGATCTACAC**TAAGTTCC**ACACTCTTTCCCTACACGACGC
PCR_F_A508: AATGATACGGCGACCACCGAGATCTACAC**TAGACCTA**ACACTCTTTCCCTACACGACGC

PCR_F_D501: AATGATACGGCGACCACCGAGATCTACAC**TATAGCCT**ACACTCTTTCCCTACACGACGC
PCR_F_D502: AATGATACGGCGACCACCGAGATCTACAC**ATAGAGGC**ACACTCTTTCCCTACACGACGC
PCR_F_D503: AATGATACGGCGACCACCGAGATCTACAC**CCTATCCT**ACACTCTTTCCCTACACGACGC
PCR_F_D504: AATGATACGGCGACCACCGAGATCTACAC**GGCTCTGA**ACACTCTTTCCCTACACGACGC
PCR_F_D505: AATGATACGGCGACCACCGAGATCTACAC**AGGCGAAG**ACACTCTTTCCCTACACGACGC
PCR_F_D506: AATGATACGGCGACCACCGAGATCTACAC**TAATCTTA**ACACTCTTTCCCTACACGACGC
PCR_F_D507: AATGATACGGCGACCACCGAGATCTACAC**CAGGACGT**ACACTCTTTCCCTACACGACGC
PCR_F_D508: AATGATACGGCGACCACCGAGATCTACAC**GTACTGAC**ACACTCTTTCCCTACACGACGC

PCR_F_universal: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG

PCR_R_A701: CAAGCAGAAGACGGCATACGAGAT**GTCGTGAT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A702: CAAGCAGAAGACGGCATACGAGAT**ACCACTGT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A703: CAAGCAGAAGACGGCATACGAGAT**TGGATCTG**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A704: CAAGCAGAAGACGGCATACGAGAT**CCGTTTGT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A705: CAAGCAGAAGACGGCATACGAGAT**TGCTGGGT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A706: CAAGCAGAAGACGGCATACGAGAT**GAGGGGTT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A707: CAAGCAGAAGACGGCATACGAGAT**AGGTTGGG**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A708: CAAGCAGAAGACGGCATACGAGAT**GTGTGGTG**GTGACTGGAGTTCAGACGTGTGC

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PCR_R_A709: CAAGCAGAAGACGGCATACGAGAT**TGGGTTTC**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A710: CAAGCAGAAGACGGCATACGAGAT**TGGTCACA**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A711: CAAGCAGAAGACGGCATACGAGAT**TTGACCCT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_A712: CAAGCAGAAGACGGCATACGAGAT**CCACTCCT**GTGACTGGAGTTCAGACGTGTGC

PCR_R_D701: CAAGCAGAAGACGGCATACGAGAT**CGAGTAAT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D702: CAAGCAGAAGACGGCATACGAGAT**TCTCCGGA**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D703: CAAGCAGAAGACGGCATACGAGAT**AATGAGCG**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D704: CAAGCAGAAGACGGCATACGAGAT**GGAATCTC**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D705: CAAGCAGAAGACGGCATACGAGAT**TTCTGAAT**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D706: CAAGCAGAAGACGGCATACGAGAT**ACGAATTC**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D707: CAAGCAGAAGACGGCATACGAGAT**AGCTTCAG**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D708: CAAGCAGAAGACGGCATACGAGAT**GCGCATTA**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D709: CAAGCAGAAGACGGCATACGAGAT**CATAGCCG**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D710: CAAGCAGAAGACGGCATACGAGAT**TTCGCGGA**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D711: CAAGCAGAAGACGGCATACGAGAT**GCGCGAGA**GTGACTGGAGTTCAGACGTGTGC
PCR_R_D712: CAAGCAGAAGACGGCATACGAGAT**CTATCGCT**GTGACTGGAGTTCAGACGTGTGC

Demultiplexing:

For index 1 (i7) - use the reverse complementary sequence in bold from reverse primers.
For index 2 (i5) for NovaSeq, MiSeq, HiSeq 2000/2500 systems - use the sequence of the one in bold from forward primers.
For index 2 (i5) for iSeq 100, MiniSeq, NextSeq 550, NextSeq 500, HiSeq 4000, and HiSeq 3000 systems - use the reverse complementary sequence in bold from forward primers.

BEFORE STARTING

Primers: prepare 5 µM solutions.

Oligo used in the second-strand synthesis step (Tin_P2-C5): prepare 15 µM solution

Adapters: prepare 25 µM solutions:

- mix 50 µl of each pair of barcoded Tin_P1_lower and Tin_P1_upper (100 µM stock), 10 µl of 10x annealing buffer and 40 µl of Tris 10 mM,
- incubate both tubes in a PCR cycler: 10 s at 95°C, bring down to 12°C 0.1 C per sec; you have the annealed adapters at the concentration of 40 µM,
- adapter is now at 25 µM in 100 µl.

Make sure that you anneal the proper adapter oligos together (from the same pair)! Make sure not to crosscontaminate your barcoded adapters (use filter tips, clean bench with bleach, ideally wotk in a PCR or laminar-flow hood).

Dephosphorylation

1 Prepare master mix 1:
- **1.3 µl NEBuffer 4 (10x)**
- **0.7 µl water**
- **1 µl FAST alkaline phosphatase (1 U/µl)**

2 Add 3 µl of master mix 1 to 10 µl of DNA (total volume = 13 µl).

3 Incubate for 1 h at 37°C, denature the DNA for 5 min at 95°C, put immediatly on ice.

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Prepare ice at the and of incubation. Move sample immediatelly form 95°C to the ice.

Guanidine tailing

4 Prepare master mix 2 (assemble at room temperature, can precipitate when on ice):

- **2.5 µl water**
- **0.7 µl NEBuffer 4 (10x)**
- **2 µl CoCl2 (2.5 mM)**
- **0.8 µl GTP (100 mM)**
- **1 µl TdT (20 U/µl)**

5 Add 7 µl of the master mix 2 to the denatured DNA (total volume = 20 µl).

6 Incubate for 30 min at 37°C, heat-kill the enzymes for 10 min at 70°C. Spin down.

Second strand synthesis

7 Prepare master mix 3:

- **5.4 µl water**
- **1 µl NEBuffer 4 (10x)**
- **0.6 µl dNTP mix (25 mM each)**
- **1 µl P2-CCCCC oligo (15 mM)**
- **2 µl Klenow exo- (5 U/µl)**

8 Add 10 µl of the master mix 3 to 20 µl of the DNA (total volume = 30 µl).

9 Incubate at room temperature for 3 hours, heat-kill enzymes for 20 min at 75°C. Spin down.

Blunt-end reaction

10 Prepare master mix 4:

- **3.95 µl water**
- **0.5 µl NEBuffer 4 (10x)**
- **0.35 µl BSA (10 mg/ml)**
- **0.2 µl T4 DNA polymerase (3 U/µl)**

11 Add 5 µl of the master mix 4 to 30 µl of the DNA (total volume = 35 µl).

Keep both DNA and the mix on the ice when assembling the reaction.

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12 Incubate for 15 min at 12°C. Hold at 4°C and proceed directly to the next step.

13 Perform AMPure cleanup with the beads:sample ratio 2:1 (70 µl of the beads and 35 µl of the reaction) according to the manufacturer's instructions. Resuspend in 11 µl of 10 mM Tris or water.

Ligation of P1 adapter

14 Transfer 10 µl the purified DNA to the plate/tubes.

15 Prepare barcoded P1 adapters (25 µM concentration).

> Each sample should be mixed with a different adapter in case the barcoded adapters are used.

16 Prepare master mix 5:

- **3.5 µl water**
- **2.5 µl PEG-4000 (50%)**
- **2 µl T4 ligase buffer (10x)**
- **1 µl T4 DNA ligase (400 U/µl)**

17 Add 1 µl of the adapter (25 µM working solution) to 10 µl of the DNA. Briefly vortex and spin.

18 Add 9 µl of the master mix 5 to each sample (total volume = 20 µl).

19 Incubate at 16°C for 3h. Spin down.

20 Perform AMPure cleanup with the beads:sample ratio 1:1 (20 µl of the beads and 20 µl of the reaction) according to the manufacturer's instructions. Resuspend in 11 µl of 10 mM Tris or water.

PCR amplification

21 Prepare master mix 4:

- **24.1 µl water**
- **10 µl Q5 polymerase buffer (5x)**
- **0.4 µl dNTP mix (25 mM each)**
- **0.5 µl Q5 hot-start polymerase**

22 Add 35 µl of the master mix 3 to each tube/well.

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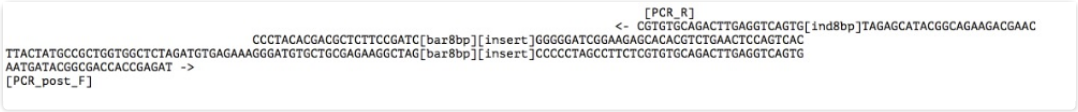
Add 2.5µl of each primer (5 µM working solutions).
Add 10 µl of the template.

23 Run PCR program:

30 s at 98°C
15 cycles of:
- 10 s at 98°C
- 20 s at 60°C
- 25 s at 72°C

5 min at 72°C
hold at 4°C

Determine experimentally the required number of PCR cycles.



24 Check reaction on a gel or Tapestation/Fragment Analyzer. Quantify using Qubit fluorimeter.

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