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Protocol of shotgun metagenomics library construction

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We use this protocol and it's working

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Abstract

This protocol is an optimization for metagenomic library construction from peat bog and arable soil samples. It is suitable for DNA inputs ranging from 20 pg to 10 ng, with a fragment size of 250 bp for Illumina sequencing.

Materials

QIAseq® FX DNA Library Core Kit Cat. No. 1120146
QIAseq® UDI Y-Adapter Kit B (96) Cat. No. 180314
AMPure XP Beckman CoμLter Cat. No. A63880
FastGene Gel/PCR Extraction Kit Cat. No. FG-91302
Magnetic bead separation DynaMagTM-2 Magnet Life technologiesTM Cat. No. 12321D
Labtop cooler TrueNorth®
Quant-iT™ PicoGreen™ Cat. No. P7589
Ethanol (100% molecular grade) Sigma Aldrich Cat. No. E7023
QX200™ ddPCR™ EvaGreen Supermix Bio-Rad Cat. No. 186-4036
Tween 20 Sigma Aldrich Cat No. P1379-25ML
PCR tubes Axygen Cat. No. 321-02-051
qPCR tubes
Filter tips and pipettes

Equipment

Vortex Genie
Microcentrifuge Legend Thermo Scientific
Rotor-Gen Q Qiagen
Mastercycler Nexus Gradient Eppendorf
NanoDrop™ 3300 Fluorospectrometer

Before start

1. The workspace should be kept clean and sterile, using UV radiation for sterilization.
2. Library preparation needs to be carried out in a laminar flow hood.
3. Ensure pipettes are clean and calibrated.
4. A negative control should be included throughout the process to confirm the absence of reagent contamination.
5. To prevent batch effects, the same person should conduct the library construction.
6. Do not dissolve DNA in 1x TE or any solution containing EDTA.
7. Quantify DNA concentration using Quant-iT™ PicoGreen™ assay kit before starting the fragmentation process.
8. Each DNA sample must be normalized to 1 ng µL-1 using Tris 10 mM or water.

Thermocycler programs

1 Set up the thermocycler with the following programs:

Table 1. Fragmentation reaction program

	Step	Temperature	Time
	Pre-chilled	4ºC	1 min
	Fragmentation	32ºC	24 min*
	Enzyme inactivation	65ºC	30 min
	Hold	4ºC	Hold

*20 pg of input DNA, we used 14 min

Table 2. Adapter ligation reaction program

	Step	Temperature	Time
	Ligation	20ºC	30 min
	Hold	4ºC	Hold

Table 3. Library amplification program

	Step	Temperature	Time	Cycles
	Initial denaturation	98ºC	2 min	1
	Denaturation	98ºC	20 s	
	Annealing	60ºC	30 s	10
	Extension	72ºC	30 s	
	Final extension	72ºC	1 min	1
	Hold	4ºC		Hold

* 20 pg of input DNA, we used 16 cycles

Fragmentation

2 1. Thaw the reagents on ice: Fx buffer 10x, FX Enzyme Mix and Fx Enhancer from QIAseq ® FX DNA Library Core Kit.
2. Keep the reactions on ice during setup.
3. Prepare the following reaction mix for 10 ng or 20 pg of input DNA.

Table 4. Fragmentation reaction mix for samples containing 10 ng of input DNA

	Component	Volume
	Fx buffer 10x	5.0 µL
	DNA (1 ng/µL)*	10.0 µL
	Nuclease free-water	25.0 µL
	Total	40.0 µL

*DNA dissolved in Tris 10 mM or water, do not contain EDTA

Table 5. Fragmentation reaction mix for samples containing 20 pg of input DNA

	Component	Volume
	Fx buffer 10x	5.0 µL
	DNA (20 pg)*	20.0 µL
	Nuclease free-water	12.5 µL
	Fx Enhancer	2.5 µL
	Total	40.0 µL

*DNA dissolved in Tris 10 mM or water, do not contain EDTA

4. Add 10 µL of FX Enzyme Mix to each reaction tube and mix thoroughly by pipetting up and down.
5. Place de PCR tubes into a pre-chilled thermocycler set to 4 ºC.
6. Use the thermocycler program from Table 1, ensuring the fragmentation time matches the input DNA.
7. Proceed directly with adapter ligation.

Adapter ligation

3 1. Prepare the adapter's dilutions according to Table 6.

Table 6. Adapter ligation factor

	DNA amount	Adapter dilution
	10 ng	1:15
	20 pg	1:300

2. Add 5 µL of diluted adapters to each fragmentation tube.
3. Add 45 µL of adapter ligation mix.

Table 7. Adapter ligation mix

	Component	Volume
	DNA ligase buffer 5x	20 µL
	DNA ligase	10 µL
	Nuclease free-water	15 µL
	Total	45 µL

4. Mix by pipetting up and down.
5. Incubate 20 ºC for 30 min (thermocycler program Table 2).
Note: do not use thermocycler with heated lid
6. Purify the adapter ligation tube immediately.

Adapter ligation cleanup

1. Use FastGene Gel/PCR Extraction Kit following the manufacturer instructions.
2. Elute the DNA with 40 µL of elution buffer.

Library amplification

4 1. Thaw reagents on ice: HiFi PCR master mix 2x and Primer mix from QIAseq® FX DNA Library Core Kit.
2. Use the following library amplification mix.

Table 8. Library amplification mix

	Component	Volume
	HiFi PCR master mix 2x	25.0 µL
	Primer mix (10 µM)	1.5 µL

	Component	Volume
	Library purified	23.5 µL
	Total	50.0 µL

3. Mix all the components by pipetting up and down.
4. Use the library amplification program from table 3.

Library cleanup

Take 50 µL of the amplicon library and cleanup with FastGene Gel/PCR Extraction Kit following the manufacturer instructions.

Library quantification with qPCR

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1. Prepare a 1:4000 dilution of the samples that used adapter 1:15, and a 1:500 dilution for those with adapter 1:300, using Tween 20 (0.1%).
Note: To prepare Tween 20 (0.1%), mix 10 mL of H_2O with 10 µL of Tween-20.
2. Thaw the reagents on ice: QX200™ ddPCR™ EvaGreen Supermix.
3. Use the following library amplification mix.

Table 9. Library quantification mix

	Component	Volume
	QX200™ ddPCR™ EvaGreen Supermix 2x	4.50 µL
	Primer forward (10 µM)	0.18 µL
	Primer reverse (10 µM)	0.18 µL
	Diluted library	1.00 µL
	Nuclease free-water	4.14 µL
	Total	10.00 µL

4. Mix all components thoroughly by pipetting up and down
5. Open the lid of the Rotor-Gen Q, place the samples, and follow the instructions in the equipment's operation manual to start the quantification process.

Library pooling

6

1. Normalize each sample to 4 ng μL^{-1} use the data obtained from qPCR.
2. Add 4 µL of each sample (4 ng μL^{-1}) to a tube and proceed with cleanup with AMPure XP Beckman Coulter beads.

Library pool cleanup

7 1. Add 80 µL of AMPure XP Beckman Coulter beads to each 75 µL of pooled library and mix by pipetting.
2. Incubate for 5 minutes at room temperature.
3. Place the tube on a DynaMag™-2 Magnetic Separator (Life Technologies™) for a few minutes until a brown pellet forms.
4. Carefully remove the supernatant with a micropipette, ensuring the magnetic bead pellet is not disturbed.
5. Wash the beads with 200 µL of 80% ethanol and remove the supernatant.
6. Repeat the 80% ethanol wash once more.
7. Incubate the beads on the DynaMagTM-2 for 5-10 min, or until dry. NOTE: Do not overdry the beads, as this reduces DNA recovery efficiency.
8. Remove the tube from the DynaMagTM-2 . Add 37.5 µL of elution buffer (1x TE) and mix using the micropipette.
9. Place the tube back on the DynaMagTM-2 to separate the magnetic beads from the solution.
10. Transfer the eluate to a new sterile tube.