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Adapter Ligation Mediated Emulsion PCR Amplification of Nanogram quantities of DNA for Illumina Sequencing

INTRODUCTION

This protocol is designed for DNA sequencing from **nanogram** or even **sub-nanogram** quantities of DNA by using Illumina technologies. It has been applied many low-DNA-content samples including permafrost, sea water, ancient DNA, ChIP-DNA, and cDNA. Briefly, DNA is fragmented to 500 bps or less. The sheared DNA is then end-repaired and ligated Illumina adaptors. PCR is set up in water-in-oil emulsion droplets. Each droplet contains no more than one DNA template. This helps to minimize the PCR bias found to be associated with the conventional PCR, and preserve the sequence complexity level in the starting material. Frequently, we obtain 500-fold to 1000-fold increase of template DNA through emulsion PCR amplification. The amplified DNA is gel purified and ready for sequencing by using Illumina sequencers.

PROCEDURE

1. Quantification of DNA

1.1 For low-DNA-content sample, we quantify the DNA using Quant-iT ds DNA HS Assay kit (Q32851, Invitrogen). Please refer to the company's manual for detailed procedure.

2. DNA fragmentation, end-repair and adapter ligation

There are two options for shearing DNA described in 2.1.a and 2.1.b.

2.1.a In 100ul glass tube provided by Covaris, adjust volume of DNA sample to 100ul in TE buffer. Shear DNA in Covaris S-series machine. Settings will depend on input DNA and desired fragment size.

2.1.b The volume of DNA sample is adjusted to 100 μl in a 1.5 ml Eppendorf tube. The tube is placed in a microtube holder, which is placed in a cup horn (Misonix, Inc. NY) containing 150 ml of ice-water mixture (50:100, v/v). The bottom of the tube is in touch with the horn. The DNA is sonicated indirectly for 10 minutes in the tube. The output of the generator is set to 50-60% of the capacity. The ice-water bath is changed after every two minutes of sonication to keep the temperature at 4 °C.

2.2. Concentrate the DNA by adding 10% of 3M NaOAc, 4x volume of absolute ethanol and 10 μg GlycoBlue. Place the tube at -80 °C for 1 hour. Spin down the tube at 4 °C for 15 minutes at 14,000 rpm. Wash the pellet with 70% ethanol. Spin down the tube at 4 °C for 5 minutes at 14,000 rpm. Dry the pellet for 10 minutes in a fume hood. Dissolve the pellet in 20 μl of water.

2.3. Repair the DNA ends using End-It DNA End-Repair Kit (see manufacturer's manual)

2.4. Phenol/chloroform extraction and ethanol precipitation.

2.5. Re-suspend the DNA in 5 µl H_2O.

2.6. Set up reaction for adapter ligation:

Repaired DNA	5 µl
Adapter-1 (10x)	1 µl
Adapter-2 (10x)	1 µl
10x ligation buffer	1 µl
PEG (50%)	1 µl
Ligase (5 u/ul)	1 µl
Total volume	10 µl

Incubate at 22 °C for 1 hour.

2.7. Clean up ligation reaction using MiniElute Reaction Cleanup Kit (see manufacturer's manual). Elute in 20 µl of elution buffer. The DNA is now ligated with adaptors and ready for emulsion PCR.

3. Emulsion PCR

3.1. Prepare the oil-surfactant mixture by thoroughly mixing the following components in a 50-ml centrifuge tube at 25 °C:

Span 80	2.25 ml
Tween 80	200 µl
Triton X-100	25 µl
Mineral oil	to 50 ml

3.2. Transfer 400 ul of the pre-made oil-surfactant mixture to a 1.8 ml CryoTube vial (Nunc, #347627), and add a stir bar (8 mm x 1.5 mm, Chemglass, #G-2003-15). Begin stirring the mixture at the maximum speed (~1,000 rpm), on the magnetic stirrer.

3.3. Prepare the aqueous phase for the emulsion by mixing the following components in a 1.5 ml tube:

DNA template (<10^9 molecules or 1.66 fmol)	1 to 20 µl
10x Pfu PCR buffer	26 µl
BSA (100 mg/ml)	26 µl
dNTP (10 mM)	5.2 µl
Forward primer (10 µM)	13 µl
Reverse primer (10 µM)	13 µl
H2O	to 260 µl

3.4. Set up a heat block at 72 °C. Incubate the tube at 72 °C for 3 minutes. Add 5.2 µl of Pfu Turbo DNA polymerase into the tube. Immediately mix the content by tapping the tube a few times. Continue to incubate the tube at 72 °C for 5 minutes. Remove the tube from the heat block and leave it on bench for 5 minutes.

3.5. Transfer 50 µl PCR mixtures in one PCR tube as control. Overlay with one droplet of mineral oil. Proceed to step 8 for control PCR. For emulsion PCR, continue to the next step.

3.6. Transfer the remaining 200 µl of the aqueous phase to the oil-surfactant mixture in a drop by drop. In each round of pipetting, 7 µl of PCR mixture are added into the oil-surfactant mixture. After the addition is complete, continue to stir for 5 minutes. A water/oil emulsion is generated containing approximately 10^8–10^9 PCR-competent compartments per milliliter of emulsion.

3.7. Cut a p200 tip at the sharp end. Use this blunt tip to gently transfer 50 µl into wells of a PCR plate. Each sample will occupy 10 wells. Add one drop of mineral oil to each well.

3.8. Subject the PCR tubes to the following program of temperature cycling:

94 °C 4 min,
(94 °C 1 min, 72 °C 3 min) x40,
72 °C 6 min,
16 °C forever.

3.9. Add 50 µl of H_2O into the non-emulsified reaction and go to step 12. For emulsion PCR product, continue to the next step.

3.10. Pool the emulsified PCR reaction in a 1.5-ml microcentrifuge tube and spin at 14,000 rpm for 5 min at 25 °C. Dispose of the upper (oil) phase.

3.11. Add 100 µl of H2O into the tube and vortex the tube for 30 seconds. Keep the tube in -80 °C for 30 minutes. Warm up the tube and repeat the following steps twice:

a) Add 0.5 ml of water-saturated diethyl ether in a fume hood

b) Vortex the tube for 10 seconds
c) Spin down the tube at 14,000 rpm for 5 minutes at room temperature
d) Carefully remove and dispose of the upper (diethyl ether) phase.

3.12. Add 2x volume of water saturated chloroform into the tubes. Vortex the tubes for 10 seconds. Spin down the tubes at 14,000 rpm for 5 minutes at room temperature. Carefully transfer the top aqueous phase into a clean tube.

3.13. Clean up the PCR products by using PCR mini-elute column (Qiagen). Elute in 30 µl of elution buffer (Qiagen).

3.14. Quantify PCR product and check size using the bioanalyzer DNA 1000 kit or by electrophoresis using 5% PAGE. Load ~30 ng PCR products per lane. Stain the gel with Syber-Gold. Record the image using Dark Reader and Fluor-S MultiImager (Bio-Rad).. The size of PCR products should be 100-500 bps. Sometimes, the adapter dimmers are also amplified together with the sample DNA.

3.15. Run the rest of the PCR products in 2% agarose low melt gel. Cut out fragments corresponding to desired size and extract DNA using MiniElute Gel Extraction Kit (Qiagen). Elute in 15 µl of elution buffer (Qiagen).

3.16. Quantify the gel purified material using Quant-iT ds DNA HS Assay kit (Q32851, Invitrogen) or using bioanalyzer DNA 1000 chip.

MATERIALS

1	End-It DNA End-Repair Kit (Epicentre, #ER0720)
2	T4 DNA ligase (with PEG; Fermentas, #EL0334)
3	Mineral oil (for molecular biology; Sigma, #M5904)
4	Span 80, a surfactant (Fluka, #09569)
5	Triton X-100 (Sigma, T8787)
6	Tween 80 (Sigma, #P5188)
7	Bovine serum albumin (BSA) (for molecular biology, powder; Sigma, #B4287)
8	Pfu Turbo DNA polymerase (2.5 U/ul; Stratagene, #600252-52)
9	Diethyl ether (water-saturated; greater than or equal to 99.9%; Sigma, #309966)
10	CryoTube vials (1.8 ml, round bottoms, star-feet; Nunc, #347627)
11	Magnetic stirrer with speed controller (Corning stirrer/hot plate, #PC-420)
12	Stir bars (8x1.5 mm, polytetrafluoroethylene; Chemglass, #G-2003-15)
13	5% Ready Gels (TBE; Bio-Rad, #161-1109)
14	MiniElute PCR Purification Kit (Qiagen, #28004)
15	MiniElute Reaction Cleanup Kit (Qiagen, #28204)
16	MiniElute Gel Extraction Kit (Qiagen, #28604)

Primers and Adapters:

454 Adapter-A	Forward primer	5'-CCATCTCATCCCTGCGTGTCCCATCTGTTCCCTCCCTGTCTCAG-3'
	Reverse primer	5'-CTGAGACAGGGA-3'
454 Adapter-B	Forward primer	5'-CCTATCCCCTGTGTGCCTTGCCTATCCCCTGTTGCGTGTCTCAG-3'
	Reverse primer	5'-CTGAGACACGCA-3'
454 PCR primers	Forward primer	5'-CCATCTCATCCCTGCGTGTCCCATCTGTTCCCTCCCTGTCTCAG-3'
	Reverse primer	5'-[BioTEG]CCTATCCCCTGTGTGCCTTGCCTATCCCCTGTTGCGTGTCTCAG-3'
Illumina P1 Adapter	Forward primer	5'-TGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'
	Reverse primer	5'-AGATCGGAAGAG-3'
Illumina P2 Adapter	Forward primer	5'-CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT-3'
	Reverse primer	5'-AGATCGGAAGAG-3'
Illumina PCR primers	Forward primer	5'-TGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'
	Reverse primer	5'-CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT-3'
Illumina Paired End Adapter 1	Forward primer	5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
	Reverse primer	5'-AGATCGGAAGAG-3'
Illumina Paired End Adapter 2	Forward primer	5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT
	Reverse primer	5'-AGATCGGAAGAG-3'
Illumina Paired End Primers	Forward primer	5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
	Reverse primer	5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

Preparation of 10x adapters:

Set up 100 µl annealing reaction as following in a PCR tube.

Forward primer (100 µM)	20 µl
Reverse primer (100 µM)	20 µl
10xPCR buffer (as salt)	10 µl
H2O	50 µl
Total	100 µl

Anneal the primers using the following program. Keep the adapters in -20°C.

95°C	5 min
0.1°C/sec to 85°C	
85°C	2 min
0.1°C/sec to 75°C	
75°C	2 min
0.1°C/sec to 65°C	
65°C	2 min
0.1°C/sec to 55°C	
55°C	2 min
0.1°C/sec to 45°C	
45°C	2 min

0.1°C/sec to 35°C	
35°C	2 min
0.1°C/sec to 25°C	
25°C	2 min
0.1°C/sec to 10°C	
10°C	10 min
4°C	forever

References:

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