Preparing Samples for Sequencing Genomic DNA - Illumina GA II

HTSF UNC at Chapel Hill

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Fragment the Genomic DNA

Perform End Repair

Add ‘A’ Bases to the 3' End of the DNA Fragments

Ligate Adapters to DNA Fragments

Purify Ligation Products

Enrich the Adapter-Modified DNA Fragments by PCR

Validate the Library

Essential Components:

QIAquick PCR Purification Kit (QIAGEN, Cat # 28104)
MinElute PCR Purification Kit (QIAGEN, Cat#28004)
QIAquick or MinElute Gel Extraction Kit (QIAGEN, Cat # 28004)
Low molecular weight DNA ladder (NEB, Cat # N3233L)
Purified DNA (1–5 µg, 5 µg recommended)
DNA should be as intact as possible, with an OD260/280 ratio of 1.8–2
TE Buffer (10 mM Tris, 1 mM EDTA)
Certified low-range Ultra Agarose (BIO-RAD, Cat # 161-3106)

Prepare:

1mM dATP
10mM dNTP Mix

Enzymes (NEB):

T4 DNA Polymerase
M0203S 150 units 3,000 units/ml $58.00
T4 Polynucleotide Kinase
M0201S 500 units 10,000 units/ml $53.00
DNA Polymerase I, Large (Klenow) Fragment
M0210S 200 units 5,000 units/ml $53.00
Klenow Fragment (3´→5´ exo–)
M0212S 200 units 5,000 units/ml $56.00
Phusion High Fidelity DNA Polymerase
F-530S 100 units 2 units/µl $100.00

Kits (NEB):

Quick Ligation Kit
M2200S 30 reactions $95.00

Additional (not required) components [for validation constructed libraries]:

4-20% TBE Gels 1.0 mm, 10 well
EC6225BOX 1 box (10gels) $115.20 (Invitrogen)
SYBR® Green I nucleic acid gel stain *10,000X concentrate in DMSO
SKU# S-7563 500ul $197.00 (Invitrogen)

We can perform validation of your library in Facility

Genomic DNA oligonucleotide sequences

Adapters

5' /Phos/-GATCGGAAGAGCTCGTATGCCGTCTTCTGCTTG

5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT

PCR Primers

5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT

Paired End DNA oligonucleotide sequences

PE Adapters

5' /Phos/-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG

5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT

PE PCR Primers

5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

5' CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

Caution!!

The genomic DNA adapters and PCR primers are additionally modified, treated and purified in a proprietary manner. We cannot guarantee efficient cluster generation or sequencing if customer does not use adapters and PCR primers purchased from Illumina in the genomic DNA sample prep kit.

We can provide original (Illumina) adapters for ligation step in 10ul/ per sample. We can provide also original (Illumina) or custom made primers for amplification.

Cost of adapters will be added to sequencing service.

Preparation of Adapters (optional)

Oligos for adapters and primers should be ordered as PAGE or HPLC purified.

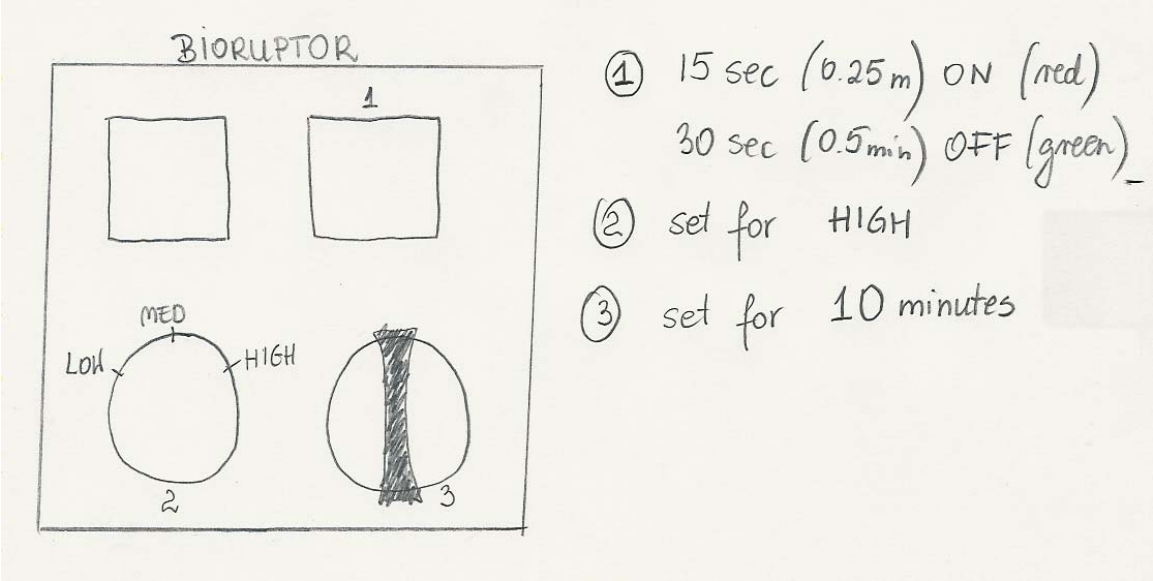
Tris-HCl (25 µl) (20mM), pH 7.9 + 37.5 µl oligo 1 (100 uM stock), and 37.5 µl of oligo 2 (100 uM stock) were mixed, and 50-µl aliquots were placed in Eppendorf tubes at 95°C heat block for 5 min. The samples were transferred to a 70°C heater block for 5 min and the block was placed at RT and was allowed to cool to 25°C. The block was then transferred to 4°C and allowed to stand overnight. The linkers were stored at -20°C. When you are using adapters keep them on ice to avoid melting.

Fragment the Genomic DNA

For DNA fragmentation we recommend bioruptor (for now located in Fordham Hall Jason Lieb Lab). For all question concerning availability and operation contact Asia from LiebLab.

Settings:
The starting points are the sonication parameters which should give the expected results for a maximum of applications.

Water level in the tank:	Precisely to the blue line
Temperature:	**4^{O}C** (see above for details)
Power:	Set the Output selector switch on High (H)
Cycling parameters:	Set the Multitimer on 15 seconds "ON" & 30 seconds "OFF" (Red and green needles set on 0.5 Min.)



Manual

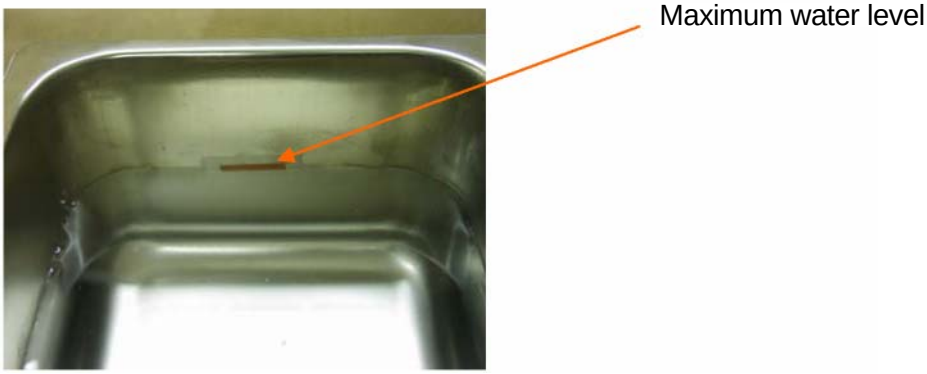
2. THE BIORUPTOR

Read entirely this user manual to get familiar to all the components of the Bioruptor.

2.1. Water bath:

2.1.1. Water level in the tank

The transfer of the ultrasounds from the generators located below the tank to the samples is done through a water bath.
- The level of the water has been optimized and should always reach the **blue line** (sticker on the wall of the tank)
- Tap water or distilled water can be used to fill the tank.



Automatic temperature control

A refrigerated circulation bath can be used to guarantee the automatic temperature control of the water bath during the whole sonication process.
The optional circulation bath RTE-7 features two pumps ("IN" and "OUT") and produces a regular water flow with a constant water level in the tank. An additional regulating valve is adapted on the water circuit going from the refrigerating unit to the Bioruptor. In this way, the water flow can be reduced to an optimal level.
Keep the water flow tiny to not interfere with the resonance process in the water bath (Flow around 500 ml/ minute).
This instrument can be ordered directly through Diagenode with all the required tubing (See price list).

2.3.2. **1.5 ml microtube unit (MAT-1 5)** See above.
To ensure reproducibility, always use the same brand of Eppendorf tubes.
The 2 ml Eppendorf tubes should not be used with the Bioruptor.
All Eppendorf tubes are generally in polypropylene. Special Eppendorf tubes in TPX plastic with a slightly better ultrasound transfer rate are available from Diagenode (See price list).

Perform End Repair

1. Purify the DNA with a QIAquick PCR spin column (QIAGEN, #28106), and elute in 31µL of EB solution.

2. Prepare the following reaction mix in order:

▪ Eluted DNA	30µL
▪ H_2O	45µL
▪ T4 DNA ligase buffer with 10mM ATP (NEB)	10µL
▪ dNTP mix (10mM)	4µL
▪ T4 DNA polymerase (3U/µL)	5µL
▪ Klenow DNA polymerase (5U/µL)	1µL
▪ T4 PNK (10U/µL)	5µL

2. **Mix well** using pipettor !!! Avoid foam and bubbles.
3. Incubate the sample at 20°C for 30 minutes.
4. Purify the DNA with a QIAquick PCR spin column (QIAGEN, #28106), and elute in 33µL of EB solution.

Add 'A' Bases to the 3' End of the DNA Fragments

1. Prepare the following reaction mix:

▪ Eluted DNA	32µL
▪ Klenow buffer	5µL
▪ dATP(1 mM)	10µL
▪ Klenow 3' to 5' exo- (5U/µL)	3µL

2. **Mix well** using pipettor !!! Avoid foam and bubbles.
3. Incubate the sample at 37°C in for 30min.
4. Purify the DNA with a QIAquick MinElute column (QIAGEN, #28006), and elute in 11µL of EB solution.

Ligate Adapters to DNA Fragments

Caution!!

This procedure uses a 10:1 molar ratio of adapter to genomic DNA insert, based on a starting quantity of 5 µg of DNA before fragmentation. If you started with less than 5 µg, titrate the volume of adapter reagent accordingly to maintain the 10:1 ratio of DNA.

1. Prepare the following reaction mix:

▪ Eluted DNA	10µL
▪ H_2O	X – if needed
▪ 2x Quick Ligation buffer	25µL
▪ Adaptor oligo mix	10µL
▪ Quick DNA Ligase (1U/µL)	2.5µL

2. **Mix well** using pipettor !!! Avoid foam and bubbles.
3. Incubate the sample at RT for 25min.
4. Purify the DNA with a QIAquick MinElute column (Qiagen, #28006), and elute in 10µL of EB solution.

Purify Ligation Products

1. Prepare a 60 ml, **2% agarose gel** with distilled water and TAE. Final concentration of TAE should be 1X at 60 ml.
2. Add ethidium bromide (EtBr) after the TAE-agarose has cooled to avoid ethidium bromide inhalation. Final concentration of EtBr should be 400 ng/ml (i.e., add 24 µg EtBr to 60 ml of 1X TAE).
3. Add 3 µl of loading buffer to 8 µl of the ladder.
4. Add 3 µl of loading buffer to 10 µl of the DNA from the purified ligation reaction.
5. Load all of the ladder solution to one lane of the gel.

6. Load the entire sample in another lane of the gel, leaving at least a gap of one empty lane between ladder and sample.
7. Run the gel at 120 V for 60 minutes.
8. View the gel on a Dark Reader transilluminator, which is a safer alternative to a UV transilluminator [minimize exposure to UV (shield with bottom tray if possible)].
9. Excise a region of gel with a clean scalpel. The gel slice should contain the material in the 150–200 bp range [Cut the band of interest with clean razor blade - keep the volume as low as possible (around 100 µl)].
10. Use a QIAGEN Gel Extraction Kit (QIAGEN, part # 28704) to purify the DNA from the agarose slices. If the gel slice is large, you may need two columns.
    - Use **6x volume of QG** buffer to 1x volume of gel and incubate in 50^0C.
    - After gel slice has dissolved completely, add **2 gel volumes of isopropanol** to the sample and mix.
    - From this step follow qiagen protocol.
    - For one column, elute in 30 µl EB.
    - For two columns, use the minElute columns, elute each one in 15 µl EB, and pool.

Enrich the Adapter-Modified DNA Fragments by PCR

1. Set up PCR master mix, make 10% extra reagent for multiple samples.
   We recommend using 10-50ng of DNA for PCR reaction.

▪ DNA	4ul (50ng)
▪ H2O	33µL
▪ 5 × Phusion Buffer HF	10µL
▪ PCR primer 1.1	1µL
▪ PCR primer 2.1	1µL
▪ 25 mM dNTP mix	0.5µL
▪ Phusion polymerase	0.5µL
	Total volume 50ul

2. Run following PCR cycle:

▪ 98°C	30 sec	
▪ 98°C	10 sec	←
▪ 65°C	30 sec	16-18×
▪ 72°C	30 sec	—
▪ 72°C	5 min	
▪ 4°C	hold	

3. Purify the DNA with a QIAquick column (Qiagen, #28106) and elute in 30µL of EB solution.