Illumina BS Genomic Library Construction Protocol

Revised by Min Chen and Jer-Young Lin (12.15.2011)

Reference: Tzung-Fu Hsieh et al (2009) Genome-Wide Demethylation of Arabidopsis Endosperm. Science 324:1451-1454.

This protocol is suitable to make BS DNA sequencing library with 50ng to 1000ng of genomic DNA.

Input DNA Amount:

1) Genomic DNA

DNA Sample	DNA Conc. (ng/ul)	Vol. of 1ug DNA (ul)

2) Unmethylated cl857 Sam7 Lambda DNA (GenBank®/EMBL Accession Number J02459; Promega cat# D1521). We will add 0.3% (w/w) lambda DNA into the genomic DNA. For example, 3ng of lambda DNA will be added into 1000ng genomic DNA.
Note: *The purpose to adding unmethylated Lambda DNA is to monitor the bisulfite conversion rate. With 2 rounds of sodium bisulfite treatment, over 99% of cytosines in unmethylated Lambda DNA should be converted to thymines.*

Equipment:

Low retention tubes (Fisherbrand 02-681-331 works fine for us)
Thermal cycler with heated lid
Microcentrifuge
Heating block

AMPure XP Handling:

The following indicates the appropriate handling methods when working with Agencourt AMPure XP beads:

- Prior to use, allow the beads to come to room temperature.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous.
- After adding the beads to the reaction, mix the solution thoroughly by pipetting up and down 10 times.

A. Shearing genomic DNA (~ 45 min)

Reagents:

EB buffer
SPRI beads (Beckman AMpure XP beads, cat#A63880)

Procedure:

i) Add EB buffer to get 52.5ul of total volume, and transfer it to Covaris microTUBE. Spin at low speed (~8k rpm) to remove the bubbles.

Sample	Vol. of 1ugDNA (ul)	Lambda (ul)	EB (ul)	Total Vol. (ul)
				52.5
				52.5
				52.5

ii) Sonication using Covaris s-series machine.

Covaris parameter: duty 10%; intensity 5; burst 200;

Total time: non-LCM DNA for 2 min; LCM DNA for 1.5 min.

A. Turn on cooling system
   1. Fill Covaris tank with ddH2O. There are two marks on the tank:
      Left: WATER LEVEL, fill the water to this mark.
      Right: RUN, Do not use this one.
   2. Turn on the cooler (on the right side of the laptop) to 5'C.

B. Turn on Covaris system (Note: Turn on the hardware first, then the software)
   1. Turn on the Covaris machine (on the left side of the laptop).
   2. Turn on the laptop (need password, ask Chris or Suhua).
   3. Run the software by clicking on the icon on the desktop.
   4. Run degas by clicking on "DEGAS" button. (**Note: degas takes at least 30 min before sonication**). Always turn on degas.

C. Set the parameter for sonication
   1. Click "open" and choose the file "HMW 2min.pro" (this is the parameter for sonication genomic DNAs isolated from fresh seed). Then click "OK".
   2. To Change the parameters, click "CONFIGURE".
      i. Sonication parameter. There are four parameters under within the box "TREATMENT": DUTY, INTENSITY, BURST, and TIME. Change these parameters regarding your target size.
      ii. Sonication time. Under the "TREATMENT" box, there are two parameters: "NUMBER OF CYCLE" and "TOTAL PROCESS TIME".
      iii. Save the changed parameter into a new file.
   3. Click "RETURN TO MAIN PANEL".

D. Run sonication
   1. Get the pivot assembly out from the cabinet (left side of the door).
   2. Put microTUBE with your sample into the pivot assembly.
   3. Insert pivot assembly into Covaris machine.
   4. Click "START". A window "USER ALERT" pops out. There are six issues in the window. Make sure every issue has been done.
   5. Click "START" in the pop-out window. The sonication starts!
   6. After the sonication is done, there is a pop-out window "DONE". Click "OK" in the pop-out window.
   7. Take the pivot assembly out of the machine. Get microTUBE out of the pivot assembly.
   8. Repeat above procedure (2 to 7) to other samples.

E. Turn off Covaris system
   1. Turn off "DEGAS".
   2. Click on "EXIT".
   3. Follow the guide on the screen. For instance, "RAISE the pivot assembly".
   4. Close the software.

5. Turn off the cooler, Covaris machine, the computer in order.

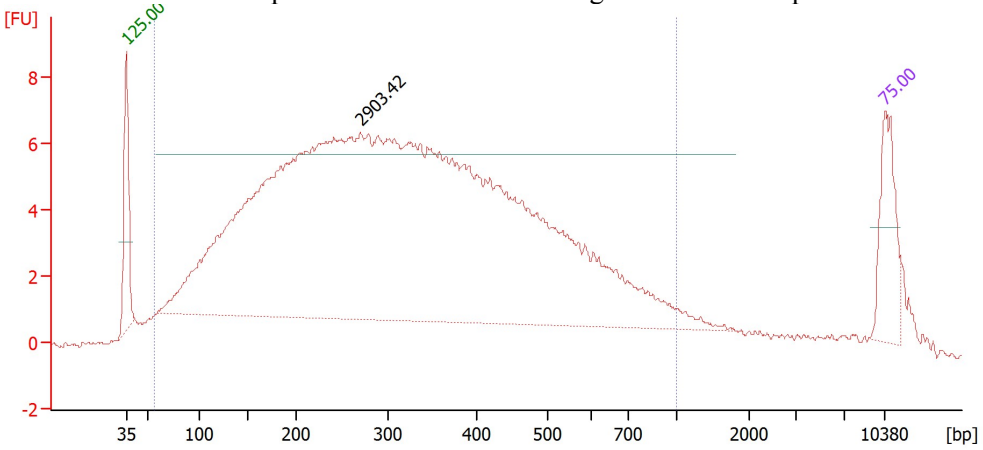
F. Clean the system
   1. Dry the pivot assembly, and put back to cabinet.
   2. Dump the water, put the tank back. The tank can hold the water drops from pivot. Keep it for hours.
   3. When Covaris is back to RT, wipe covaris with kimwipe, then put down pivot.

iii) Save 1 ul for checking DNA concentration and running on BioAnalyzer. The other 51 ul goes to ***B.*** DNA should be smeared around 100 bp - 500bp.

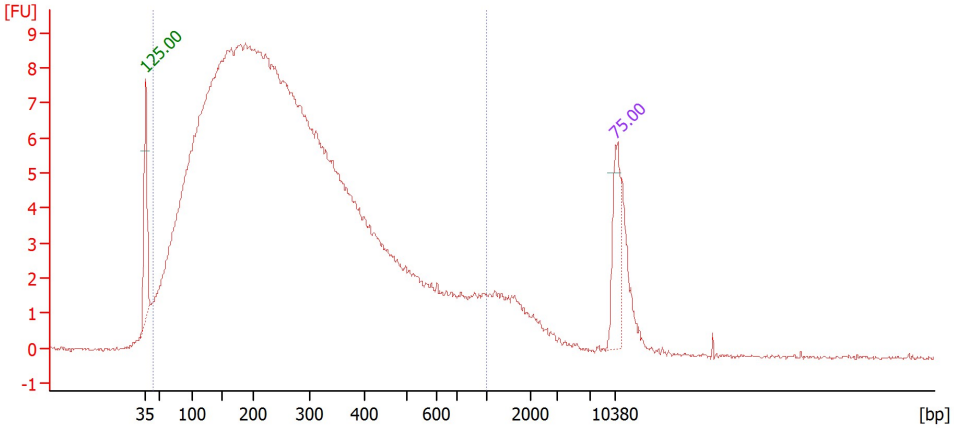
NOTE: For starting with 50ng genomic DNA:

- ***It is optional to check DNA concentration and size.***
- ***All sheared DNA goes to B.***

Example A: BioAnalyzer trace profile of sheared DNA from fragmented DNA, which was isolated from LCM captured tissues. The size range is 60 ~ 1000 bp.



Example B: BioAnalyzer trace profile of sheared DNA from intact genomic DNA. The size range is 50 ~ 1000 bp.



B. Repair Ends (~ 35 min)

Reagents:

T4 DNA Ligase buffer (NEB B0202S)
10mM dNTPs-dCTP (2.5mM each)
T4 DNA Polymerase (NEB M0203S)
Klenow DNA Polymerase (NEB M0210S)
T4 PNK (NEB M0201S)

Procedure:

In 0.2ml PCR tube, add the following reagents:

51 ul	gDNA
10 ul	T4 DNA Ligase buffer (NEB B0202S)
4 ul	10mM dNTPs-dCTP (2.5mM each)
1 ul	T4 DNA polymerase (NEB M0203S)
1 ul	Klenow DNA polymerase (NEB M0210S)
1 ul	T4PNK (NEB M0201S)
32 ul	ddH2O
Total volume: 100 ul	

NOTE: For starting with 50ng genomic DNA:

- ***Adjust the ddH2O volume based on genomic DNA. Total volume of genomic DNA and ddH2O is 83 ul.***

Mix thoroughly with pipetting and centrifuge briefly. Incubate at 20'C for 30 min in thermal cycler.

C. Clean Up End Repair Products with 1.8x SPRI beads (~ 75 min)

Reagents:

SPRI beads (Beckman AMpure XP beads, cat#A63880)
80% EtOH (fresh prepared)
EB buffer or 10mM Tris pH8.0

Procedure:

i. Take the SPRI beads out of fridge. Vortex it. Take 180ul of beads (per reaction) into 1.5 microcentrifuge tube. Let stand at RT for 5 min.
ii. Add 180 uL of beads to 100 uL of reaction. Mix thoroughly (pipetting more than 10 times) and let stand at RT for 15 min.
iii. Place tubes on a Magnet separator for 15 minutes to separate beads from solution.
iv. Aspirate 275 ul cleared solution from the reaction tubes and discard (leave ~5 ul in the tube). This step is performed while the reaction tubes are situated on the Magnet separator.
v. Dispense 200 µL of 80% ethanol to each tubes, and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of

two washes.

vi. Place the reaction tubes on bench top to air-dry for 15 min. Be sure to allow beads to dry completely (if is dry, you may some cracks on the bead pellet).

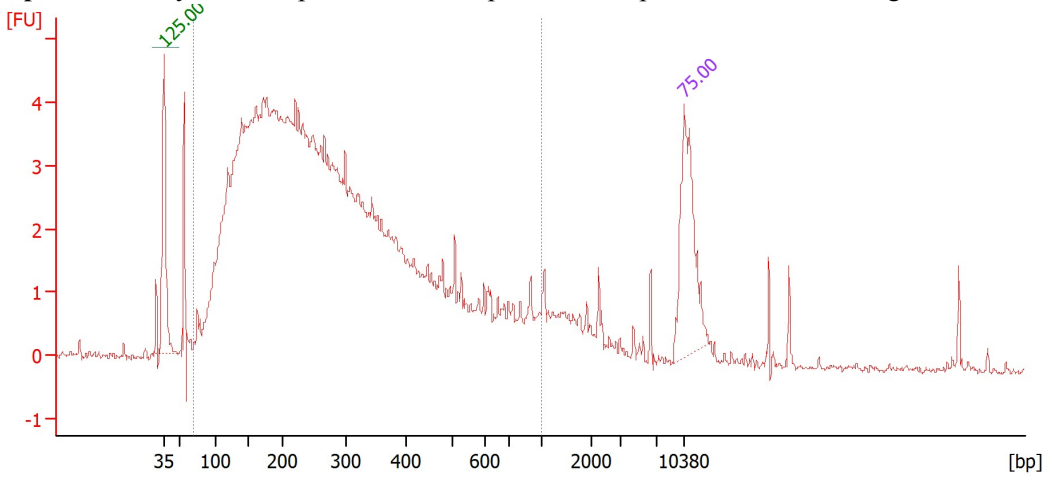
vii. Add 42.0 uL of elution buffer (EB or 10 mM Tris pH8) to each tube. Pipette to mix beads 10 times and EB thoroughly and let stand RT for 5 mins.

viii. Place tubes on Magnet separator for 5 min, and transfer clear liquid ~41.0 ul to new tube. Keep 1 ul for checking DNA concentration and running on BioAnalyzer. The other 40 ul goes to ***D.***

NOTE: For starting with 50ng genomic DNA:

- ***Add 41.0ul of elution buffer at step vii.***
- ***It is optional to check DNA concentration and size.***
- ***All end repair product DNA goes to D.***

Example: BioAnalyzer trace profile of end repaired DNA product. The size range is 70 ~ 1000 bp.



D. Add A bases to 3'end (Adenylation) (~ 35 min)

Reagents:

Klenow buffer (NEB)
10mM dATP (NEB)
Klenow exo minus (NEB M0212S)

Procedure:

In 0.2ml PCR tube, add the following reagents:

40 ul	end-repaired DNA
5 ul	Klenow buffer (NEB buffer 2)
4 ul	25 mM dATP
1 ul	Klenow exo minus (NEB M0212S)

Total volume: 50 ul

Mix thoroughly with pipetting and centrifuge briefly. Incubate at 37C for 30 min in thermal cycler.

E. Clean Up Adenylation Products with 1.8x SPRI beads (~ 75 min)

Reagents:

SPRI beads (Beckman AMpure XP beads, cat#A63880)
80% EtOH (fresh prepared)
EB buffer or 10mM Tris pH8.0

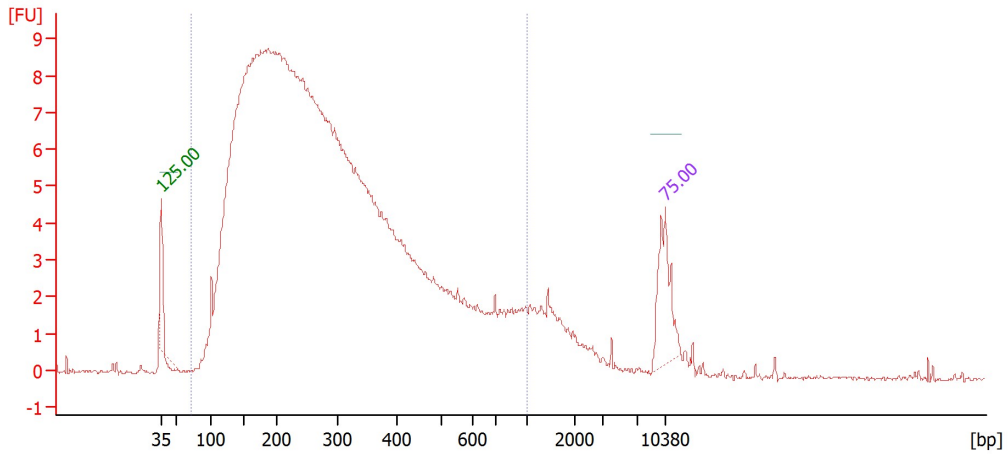
Procedure:

i. Take the SPRI beads out of fridge. Vortex it. Take 90ul of beads (per reaction) into 1.5ml microcentrifuge tube. Let stand at RT for 5 min.
ii. Add 90 uL of beads to 50 uL of reaction. Mix thoroughly and let stand at RT for 15 mins.
iii. Place tubes on a Magnet separator for 15 minutes to separate beads from solution.
iv. Aspirate 135 ul cleared solution from the reaction tubes and discard (keep ~5 ul). This step is performed while the reaction tubes are situated on the Magnet separator.
v. Dispense 200 µL of 80% ethanol to each tubes, and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of two washes.
vi. Place the reaction tubes on bench top to air-dry for 15 min. Be sure to allow beads to dry completely (if is dry, you may some cracks on the bead pellet).
vii. Add 23.5 uL of elution buffer (EB or 10 mM Tris pH8) to each tube. Pipette to mix beads 10x and EB thoroughly and let stand RT for 5 mins.
viii. Place tubes on Magnet separator for 5 mins, and transfer clear liquid 23.0 ul to new tube. Keep 1 ul for checking DNA concentration and running on BioAnalyzer. The other 22 ul goes to ***F.***

NOTE: For starting with 50ng genomic DNA:

- ***Add 22.5.0ul of elution buffer at step vii.***
- ***It is optional to check DNA concentration and size.***
- ***All adenylation product DNA goes to F.***

Example: BioAnalyzer trace profile of Adenylation product. The size range is 70 ~ 1000 bp.



F. Ligate Adapters (~ 30 min)

Reagents:

DNA Quick Ligase buffer (NEB M2200S)
100uM Methylated Adapter
DNA Quick Ligase (NEB M2200S)

Procedure:

In 0.2ml PCR tube, add the following reagents:

22 ul	A-base-added DNA
25 ul	DNA Quick Ligase buffer (NEB)
2 ul	Methylated Adapter (100 uM) (totally 16.0 ul from TFH)
1 ul	DNA Quick Ligase (NEB M2200S)
50ul	total

Mix thoroughly with pipetting and centrifuge briefly. Incubate at RT for 15 min.

G. Clean Up Ligation Products with 1.8x SPRI beads (~ 75 min)

Reagents:

SPRI beads (Beckman AMpure XP beads, cat#A63880)
80% EtOH (fresh prepared)
EB buffer or 10mM Tris pH8.0

Procedure:

i. Take the SPRI beads out of fridge. Vortex it. Take 90ul of beads (per reaction) into 1.5 microcentrifuge tube. Let stand at RT for 5 min.
ii. Add 90 uL of beads to 50 uL of reaction. Mix thoroughly and let stand at RT for 15 mins.
iii. Place tubes on a Magnet separator for 15 minutes to separate beads from

solution.

iv. Aspirate the cleared solution 135 ul from the reaction tubes and discard. This step is performed while the reaction tubes are situated on the Magnet separator.

v. Dispense 200 µL of 80% ethanol to each tubes, and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of two washes. Do a quick spin and remove residual EtOH.

vi. Place the reaction tubes on bench top to air-dry with lid opened for 15 min. Be sure to allow beads to dry completely (if is dry, you may some cracks on the bead pellet).

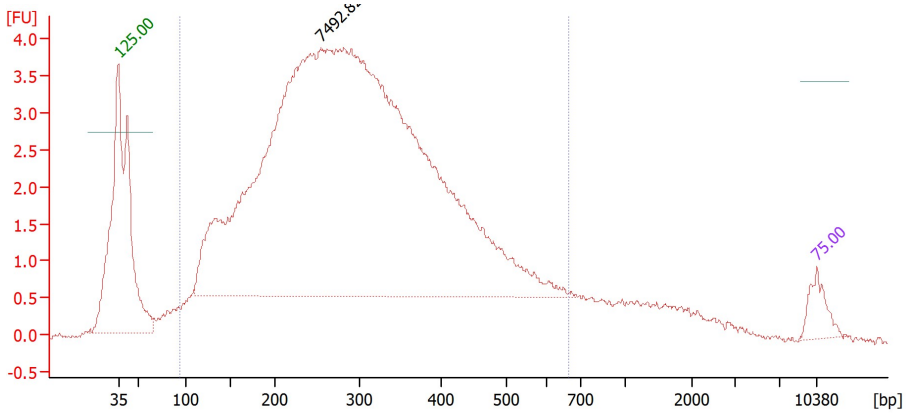
vii. Add 23.0 uL of elution buffer (EB or 10 mM Tris pH8) to each tube. Pipette to mix beads and EB thoroughly and let stand RT for 2 mins.

viii. Place tubes on Magnet separator for 5 mins, and transfer 22.0 ul clear liquid to new tube. Keep 1 ul for further analysis (0.5 ul for PCR to check the ligation reaction result; take 1 ul to dilute 4x to check in a nanodrop and run on BioAnalyzer). Take 20 ul for BS reaction.

NOTE: For starting with 50ng genomic DNA:

- ***Add 21.0ul of elution buffer at step vii.***
- ***It is optional to check DNA concentration and size.***
- ***All end repair product DNA goes to BS reaction.***

Example: BioAnalyzer trace profile of ligation product. The size range is 100 ~ 670 bp.



H1. Quantify DNA Product from step A, by NanoDrop Fluorospectrometer with Quant-iT PicoGreen dsDNA Kit.

NOTE: For starting with 50ng genomic DNA:

- ***It is optional to check DNA concentration and size.***

***H2. PCR Amplification for Library* (~ 50 min)**

(This step does not necessarily tell you how well adaptor ligation work. I usually do PCR at this step to make sure library can be amplified by PE primers.)

NOTE: For starting with 50ng genomic DNA:

- ***It is optional.***

Reagents:

10x ExTaq Buffer (Fisher TAK RR001B)
ExTaq DNA Polymerase (Fisher TAK RR001B)
10mM dNTP (2.5mM each)
10uM PE1 primer
10uM PE2 primer

Procedure:

In 0.2ml PCR tube, add the following reagents:

0.5 ul	gDNA lib (from step **G**)
1.25 ul	10x ExTaq Buffer
1.0 ul	10mM (2.5mM each) dNTPs
0.25 ul	PE 1 (10uM)
0.25 ul	PE 2 (10uM)
0.1 ul	Extaq
9.15 ul	water to 12.5 ul total volume.

Mix thoroughly with pipetting and centrifuge briefly.

PCR cycle

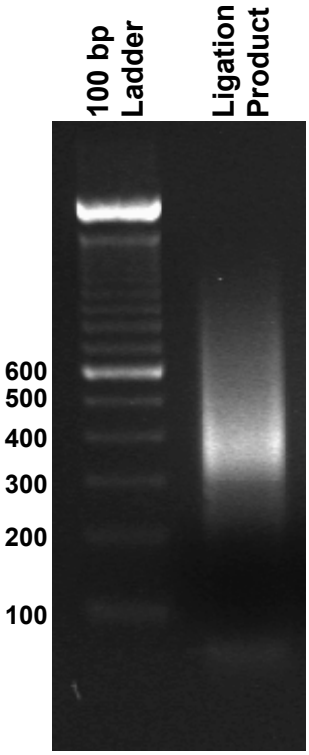
1. 95C 2’

40 cycles of

2. 95C 30”
3. 65C 30”
4. 72C 1”

Take all PCR products and run them on 2% agarose gel. You should see smearing pattern similar to sheared DNA.

Example of PCR product (right).



I. Bisulfite Conversion Using **QIAGEN EpiTect Bisulfite Conversion Kit (Cat#59104)**

- Convert unmethylated cytosines with Sodium Bisulfite.
- This protocol is designed for processing DNA isolated from formalin-fixed, paraffin-embedded (FFPE) tissue samples (e.g., using QIAamp, DNeasy, or EZ1® Kits). An optimized binding step in the cleanup stage facilitates binding of DNA from fixed tissues. Using this protocol, 1 ng – 2 µg DNA in a volume of up to 20 µl can be processed.
- We follow the FFPE instructions of Qiagen EpiTect Kit (59104), but do not use carrier RNA during the clean-up steps.
- In addition, we use external control system from Human Genetic Signature (Randwick, Australia) to work parallel with our samples and monitor the bisulfite conversion process. Control sample 1 is untreated DNA. Control sample 2 is bisulfite treated DNA served as positive control. Control sample 3A and 3B are PCR primers and can be used to check the integrity of bisulfite conversion. Nested PCR primers are used to further improve the sensitivity of the detection.
- Also two contiguous rounds of bisulfite conversions will be performed with purification between the two rounds.
  Note*: with two rounds of conversion, the conversion rate of unmethylated cytosines in ummethylated Lambda genome is over 99%.*

Important points and things to know before starting

- **BS mix (sulfanation and deamination)**
  1. Capacity of each aliquot: Each aliquot of Bisulfite Mix is sufficient for 8 conversion reactions.
  2. Before using: Dissolve ONE aliquot by adding 800 µl RNase-free water. Vortex until the Bisulfite Mix is completely dissolved. This can take up to 5 min. If necessary, heat the Bisulfite Mix–RNase-free water solution to 60°C and vortex again.
  3. Working condition:
     Equilibrate buffers to room temperature
     When using it, do not place dissolved Bisulfite Mix on ice.
  4. Storage condition: store at –20°C for up to 4 weeks without any loss of performance.

- **DNA Protect Buffer**
  1. When using: after addition it to DNA–Bisulfite Mix (step 2), the color should turn from green to blue indicating sufficient mixing and correct pH for the bisulfite conversion reaction.
  2. Storage condition: store at 4°C

- **Buffer BW (adjust column condition for BD and wash BD)**
  1. First time using: If using the kit at the first time, add 30 ml ethanol (96–100%) to Buffer BW
  2. Storage condition: at room temperature (15–25°C).
  3. Working condition:
     Before using it, invert the bottle several times.

- **Buffer BD (for desulfanation)**
  1. First using: If using the kit at the first time, add 27 ml ethanol (96–100%) to Buffer BD
  2. Storage condition: at 2–8°C.
  3. Working condition:
     Equilibrate buffer to room temperature
     Before using it, invert the bottle several times
     When aspirating it, avoid precipitates in BD. (White precipitates may form in the Buffer BD–ethanol mix after some storage time. These precipitates will not affect the performance of Buffer BD.)
     After using it, make sure to close the bottle immediately.

- **Buffer BL (bind DNA to column)**
  1. Before using: If Buffer BL contains precipitates, dissolve by heating (maximum 70°C) with gentle agitation.
  2. Storage condition: at RT.
  3. Working condition:
     Equilibrate buffer to room temperature
     Before using it, invert the bottle several times

- Optional: Set a heating block to 60°C for use in step 1.
- **Equilibrate samples and buffers to room temperature**
- Perform all centrifugation steps at room temperature (15–25°C).
- **Test BS mix solution with control DNA (Human Genetics Signature) before using it on your samples.**

I1 1st Bisulfite DNA conversion procedure (BS conversion: ~ 7hr; nested PCR: ~ 5hr)

1a. Prepare BS mix solution.

I. If there is BS mix solution in -20C fridge and which is not older than 2 weeks, take 1 aliquots (3 BS reactions per aliquots) of BS mix solution. Let them stand at RT for 10 min. Make sure that the solution is clear.

II. If you need to make a fresh BS mix solution, take one tube of aliquot of Bisulfite Mix powder, and add 800 µl RNase-free water to the tube containing BS mix. Invert the tube every 5 min and check if is completely dissolved. If you don't see obvious particles, spin down the down briefly, then check if there is any particle until the Bisulfite Mix is completely dissolved. It may take up to 30 min. The total volume of the BS solution is ~900ul (910ul – 950ul). Then aliquot 180 ul of the BS solution into 1.5 microcentrifuge tubes. Write the date on the tube. Store the rest of aliquot in -20C.

NOTE:

- ✓ ***Do not weight the powder and make bisulfite mix solution.***
- ✓ ***Do not place dissolved Bisulfite Mix on ice.***
- ✓ ***You can use external control system from Human Genetic Signature to test the fresh prepared BS mix solution before treating your sample with it. This would add one extra day for the whole procedure.***

1b. Thaw DNA to be used in the bisulfite reactions:

i) Control sample1 from MethylEasy (Human Genetics Signature) 4 ul (140 ng) is used for control in BS rxn.

ii) 20ul sample DNA from step G, purified adapter-ligated DNA

1c. Take DNA protection buffer aliquot from 4'C fridge and keep it at RT for 10 min.

2. Prepare the bisulfite reactions in 200µl PCR tubes according to **Table 6**.

NOTE: ORDER for adding reagents is important.

Add each component in the order listed. Total volume for DNA and water is 20 ul.

Table 6. Bisulfite reaction components

Component	Volume per Reaction (ul)
DNA Solution from Step G (After ligation)	4 ul of control sample 1; 20 ul of adapter-ligated sample
RNase-free water	16 ul for control sample 1; no water for adapter-ligated sample
Bisulfite Mix (dissolved)	85
DNA Protect Buffer	35
Total Volume	**140**

3. Close the PCR tubes and mix the bisulfite reactions thoroughly. Store tubes at room temperature (15–25°C).
   NOTE: DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.

4. Perform the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to Table 7. The complete cycle should take approximately 5 h.
   NOTE: If using a thermal cycler that does not allow you to enter the reaction volume (140 µl), set the instrument to the largest volume setting available.

Table 7. Bisulfite conversion thermal cycler conditions
Converted DNA can be left in the thermal cycler overnight without any loss of performance.

Step	Time	Temperature (°C)
Denaturation	5 min	95
Incubation	25 min	60
Denaturation	5 min	95
Incubation	85 min (1 h 25 min)	60
Denaturation	5 min	95
Incubation	175 min (2 h 55 min)	60
Hold	Indefinite*	20

5. Place the PCR tubes containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation.
   NOTE: Since the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure. Converted DNA can be left in the thermal cycler overnight without any loss of performance.

Cleanup of bisulfite converted DNA
Before go to step 6,

- **Set the heatblock to 56'C for step 17.**
- Take spin columns and buffer **BD** bottle from **4'C** fridge. BD buffer is for step 12, not step 9, 10 and 11. For BD, **invert the BD buffer bottle for several times**. Take 510 ul into a 1.5 ml tube; close the bottle immediately (IMPORTANT) and take it back to the 4'C fridge. Keep the tube with buffer BD at RT for 10 minutes.

6. Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes.
   Note: Transfer of precipitates in the solution will not affect the performance

or yield of the reaction.

7. **Before using BL: If Buffer BL contains precipitates, dissolve by heating (maximum 70°C) with gentle agitation.**
   Working condition: Equilibrate buffer to room temperature
   Before using it, invert the bottle several times
   Add 310 µl Buffer BL **containing no carrier RNA** to each sample. Mix the solutions by vortexing for 5 sec and then centrifuge briefly.

8. Add 250 µl ethanol (96–100%) to each sample. Mix the solutions by pulse vortexing for 15 s, and centrifuge briefly to remove the drops from inside the lid.

9. Transfer the entire mixture from the tubes in steps 7 and 8 into the corresponding EpiTect spin columns.

10. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes. There is a little bit solution left on the rim of the opening outside/ above the membrane and inside the column. Use pipette to take it back to the middle of the membrane and centrifuge the spin column at maximum speed for 10 sec.

11. Before using buffer **BW**, **invert the bottle for several times**. Add buffer BW 500 µl supernatant into the spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

12. If there are precipitates in buffer BD, centrifuge the tube for 1 min at maximum speed. Take 500 µl supernatant carefully without disturbing the pellet to the spin columns and close the lid (IMPORTANT) to incubate for 15 min at room temperature (15–25°C).

13. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

14. Before using buffer **BW**, **invert the bottle for several times**. Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

15. Repeat step 14 once.

16. Place the spin columns into new 2 ml collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.

17. Place the spin columns with open lids into a clean 1.5 ml microcentrifuge tube and incubate the columns for 5 min at 56°C in a heating block to evaporate any remaining liquid.

18. Place the spin columns into clean 1.5 ml microcentrifuge tubes (not provided). Dispense 25 µl Buffer EB onto the center of each membrane. Elute the purified DNA by centrifugation for 2 min at approximately 15,000 x g (12,000 rpm).
RECORD the elution volume HERE (1st BS/ 1st elution)

19. Add another 20 ul Buffer EB onto the center of each membrane for 2nd elution. Elute the purified DNA by centrifugation for 2 min at approximately 15,000 x g (12,000 rpm).
RECORD the elution volume HERE (1st BS/ 2nd elution)

20. Take 20 ul 1st elution for another round of BS conversion. Keep rest of 1st elution for checking ssDNA concentration using Quant-iT OligoGreen ssDNA Kit

NOTE: For starting with 50ng genomic DNA:
- ***Add 21.0ul of elution buffer at step 18.***
- ***It is optional to check ssDNA concentration.***
- ***All 1st BS treated DNA goes to 2nd BS reaction.***

20. Nested PCR to check BS reaction result (~5hr)
(i) Materials
Control sample1 35 (ng/ul) (MethyalEasy, Human Gentetics Signature)
Control sample2 20 (ng/ul) (MethyalEasy, Human Gentetics Signature)
control primer 3A (MethyalEasy, Human Gentetics Signature)
control primer 3B (MethyalEasy, Human Gentetics Signature)
ExTaq (Takara)
10x ExTaq buffer
25 mM dNTP (25 mM for each dNTP)

(ii) Make master mix for 1st PCR reaction

Reagents	PCR mix for 1 rxn	PCR mix for 4.2 rxn
ExTaq (Takara)	0.125 ul	0.525 ul
10x buffer	2.5 ul	10.5 ul
2.5 mM dNTP	2 ul	8.4 ul
ddH_2O	7.875 ul	33.075 ul

Primer 3A	2 ul	8.4 ul
total	14.5 ul	60.9 ul

Mix thoroughly with pipetting and spin briefly.

(iii) 1st PCR (volume: ul)

PCR rxn	1	2	3	4
	BS Treated Control sample 1	Untreated Control sample 1	Control sample 2	No template control
PCR mix	14.5	14.5	14.5	14.5
Template	4	1	1	-
ddH_2o	6.5	9.5	9.5	10.5
Total	25	25	25	25

Mix thoroughly with pipetting and spin briefly.

Thermal cycle

temp.	time	cycle number
95'C	3m	x1
95'C	30s	x35
50'C	1m	
72'C	1m	
72'C	10m	x1

(iv) Make master mix for 2nd PCR reaction

Reagents	PCR mix for 1 rxn	PCR mix for 5.2 rxn
ExTaq (Takara)	0.125 ul	0.65 ul
10x buffer	2.5 ul	13.0 ul
2.5 mM dNTP	2 ul	10.4 ul
ddH_2O	7.875 ul	40.95 ul
Primer 3B	2 ul	10.4 ul
total	14.5 ul	75.4 ul

(v) 2nd PCR (Volume: ul)

PCR rxn	1	2	3	4	5
	BS treated Control sample 1	Untreated Control sample 1	Control sample 2	No template of 1st PCR	No template
PCR mix	12.5	12.5	12.5	12.5	12.5
Template (1st PCR product)	10.5	2	2	2	-
ddH_2O	0	8.5	8.5	8.5	10.5
Total	25	25	25	25	25

Mix thoroughly with pipetting and spin briefly.

Thermal cycle (same as 1st PCR)

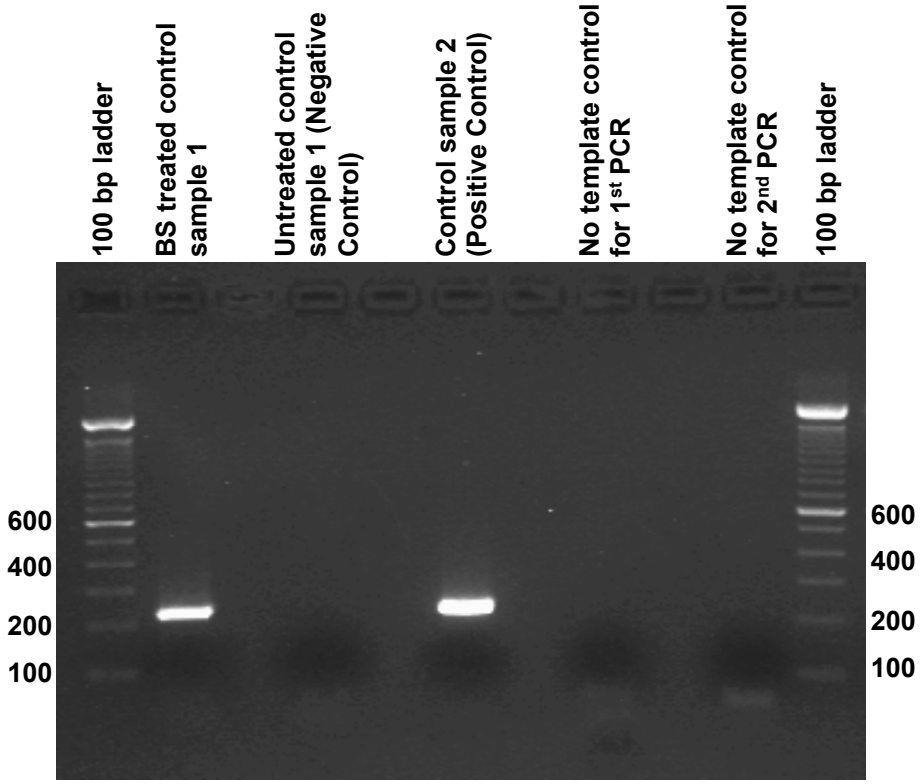
temp.	time	cycle number

95'C	3m	x1
95'C	30s	x35
50'C	1m	
72'C	1m	
72'C	10m	x1

(v) Gel electrophoresis

- Prepare 2% agarose gel and load 20 ul to check the PCR result.
- Load 250 ng100 bp ladder per lane as DNA marker.

Example: if the BS treatment/conversion worked, you should expect the result shown below.



I2 2nd Bisulfite DNA conversion procedure (BS conversion: ~ 7hr; nested PCR: ~ 5hr)

1a. If there is BS mix solution in -20C fridge and which is not older than 2 weeks, take 1 aliquots (3 BS reactions per aliquots) of BS mix solution. Let them stand at RT for 10 min. Make sure that the solution is clear.

1b. Thaw DNA to be used in the bisulfite reactions:
i) Control sample1 from MethylEasy (Human Genetics Signature)
4 ul (140 ng) is used for control in BS rxn.
ii) Control sample DNA from 1st BS reaction elution1. Add elution2 to get 20 ul. Record the volume from elution1 and 2, repectively so that the accurate input DNA amount is available for caculating the retention rate of the 2nd BS reaction product.
ii) Sample DNA from 1st BS reaction elution1 20 ul

1c. Take DNA protection buffer aliquot from 4'C fridge and keep it at RT for 10 min.

2. Prepare the bisulfite reactions in 200µl PCR tubes according to **Table 6**.
NOTE: ORDER for adding reagents is important.
Add each component in the order listed. Total volume for DNA and water is 20 ul.

Table 6. Bisulfite reaction components

Component	Volume per Reaction (ul)
DNA Solution	4 ul for control; 20 ul from 1st BS product
RNase-free water	16 ul for control; no water for 1st BS product
Bisulfite Mix (dissolved)	85
DNA Protect Buffer	35
Total Volume	**140**

3. Close the PCR tubes and mix the bisulfite reactions thoroughly. Store tubes at room temperature (15–25°C).
Note: *DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.*

4. Perform the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to Table 7. The complete cycle should take approximately 5 h.
Note: *If using a thermal cycler that does not allow you to enter the reaction volume (140 µl), set the instrument to the largest volume setting available.*

Table 7. Bisulfite conversion thermal cycler conditions
Converted DNA can be left in the thermal cycler overnight without any loss of performance.

Step	Time	Temperature (°C)
Denaturation	5 min	95
Incubation	25 min	60
Denaturation	5 min	95
Incubation	85 min (1 h 25 min)	60
Denaturation	5 min	95
Incubation	175 min (2 h 55 min)	60
Hold	Indefinite*	20

5. Place the PCR tubes containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation.
   NOTE: *Since the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure. Converted DNA can be left in the thermal cycler overnight without any loss of performance.*

Cleanup of bisulfite converted DNA
Before go to step 6, set the heatblock to 56'C for step 17.

6. Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes.
   Note: Transfer of precipitates in the solution will not affect the performance or yield of the reaction.

7. **Before using BL: If Buffer BL contains precipitates, dissolve by heating (maximum 70°C) with gentle agitation.**
   Working condition: Equilibrate buffer to room temperature
   Before using it, invert the bottle several times
   Add 310 µl Buffer BL **containing no carrier RNA** to each sample. Mix the solutions by vortexing for 5 sec and then centrifuge briefly.

8. Add 250 µl ethanol (96–100%) to each sample. Mix the solutions by pulse vortexing for 15 s, and centrifuge briefly to remove the drops from inside the lid.

9. Take spin columns and buffer **BD** bottle from **4'C** fridge. BD buffer is for step 12, not step 9, 10 and 11.
   For BD, i**nvert the BD buffer bottle for several times**. Take 510 ul into a 1.5 ml tube; close the bottle immediately (IMPORTANT) and take it back to the 4'C fridge. Keep the tube with buffer BD at RT for 10 minutes.
   For columns, place the necessary number of EpiTect spin columns and

collection tubes in a suitable rack. Transfer the entire mixture from the tubes in steps 7 and 8 into the corresponding EpiTect spin columns.

10. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes. There is a little bit solution left on the rim of the opening outside/ above the membrane and inside the column. Use pipette to take it back to the middle of the membrane and centrifuge the spin column at maximum speed for 10 sec.

11. Before using buffer **BW**, **invert the bottle for several times**. Add buffer BW 500 µl supernatant into the spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

12. If there are precipitates in buffer BD, centrifuge the tube for 1 min at maximum speed. Take 500 µl supernatant carefully without disturbing the pellet to the spin columns and close the lid (IMPORTANT) to incubate for 15 min at room temperature (15–25°C).

13. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

14. Before using buffer **BW**, **invert the bottle for several times**. Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

15. Repeat step 14 once.

16. Place the spin columns into new 2 ml collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.

17. Place the spin columns with open lids into a clean 1.5 ml microcentrifuge tube and incubate the columns for 5 min at 56°C in a heating block to evaporate any remaining liquid.

18. Place the spin columns into clean 1.5 ml microcentrifuge tubes (not provided). Dispense 25 µl Buffer EB onto the center of each membrane. Elute the purified DNA by centrifugation for 2 min at approximately 15,000 x g (12,000 rpm).
    RECORD the elution volume HERE (2nd BS/ 1st elution)

19. Add another 20 ul Buffer EB onto the center of each membrane for 2nd elution. Elute the purified DNA by centrifugation for 1.5 min at approximately 13,200 rpm.
RECORD the elution volume HERE (2nd BS/ 2nd elution)

NOTE: For starting with 50ng genomic DNA:

- ***Add 21.0ul of elution buffer at step 18.***
- ***It is optional to check ssDNA concentration.***
- ***All 2nd BS treated DNA goes to J.***

20. Nested PCR to check BS reaction result
Because the primer in the PCR is not sufficient for all reaction, the PCR reaction for the control using 1st BS reaction product as template will not be done here.

(i) Materials

Control sample1 35 (ng/ul) (MethyalEasy, Human Gentetics Signature)
Control sample2 20 (ng/ul) (MethyalEasy, Human Gentetics Signature)
control primer 3A (MethyalEasy, Human Gentetics Signature)
control primer 3B (MethyalEasy, Human Gentetics Signature)
ExTaq (Takara)
10x ExTaq buffer
25 mM dNTP (25 mM for each dNTP)

(ii) Make master mix for 1st PCR reaction

Reagents	PCR mix for 1 rxn	PCR mix for 4.2 rxn
ExTaq (Takara)	0.125 ul	0.525 ul
10x buffer	2.5 ul	10.5 ul
2.5 mM dNTP	2 ul	8.4 ul
ddH_2O	7.875 ul	33.075 ul
Primer 3A	2 ul	8.4 ul
total	14.5 ul	60.9 ul

Mix thoroughly with pipetting and spin briefly.

(iii) 1st PCR (Volume ul)

PCR rxn	1	2	3	4
	BS treated Control sample 1	Untreated Control sample 1	Control sample 2	No template control
PCR mix	14.5	14.5	14.5	14.5
Template	4	1	1	-
ddH_2o	6.5	9.5	9.5	10.5
Total	25	25	25	25

Mix thoroughly with pipetting and spin briefly.

Thermal cycle

temp.	time	cycle number
95'C	3m	x1

95'C	30s	x35
50'C	1m	
72'C	1m	
72'C	10m	x1

(iv) Make master mix for 2nd PCR reaction

Reagents	PCR mix for 1 rxn	PCR mix for 5.2 rxn
ExTaq (Takara)	0.125 ul	0.65 ul
10x buffer	2.5 ul	13.0 ul
2.5 mM dNTP	2 ul	10.4 ul
ddH_2O	7.875 ul	40.95 ul
Primer 3B	2 ul	10.4 ul
total	14.5 ul	75.4 ul

Mix thoroughly with pipetting and spin briefly.

(v) 2nd PCR (Volume: ul)

PCR rxn	1	2	3	4	5
	BS treated Control sample 1	Untreated Control sample 1	Control sample 2	No template of 1st PCR	No template
PCR mix	12.5	12.5	12.5	12.5	12.5
Template (1st PCR product)	10.5	2	2	2	-
ddH_2O	0	8.5	8.5	8.5	10.5
Total	25	25	25	25	25

Mix thoroughly with pipetting and spin briefly.

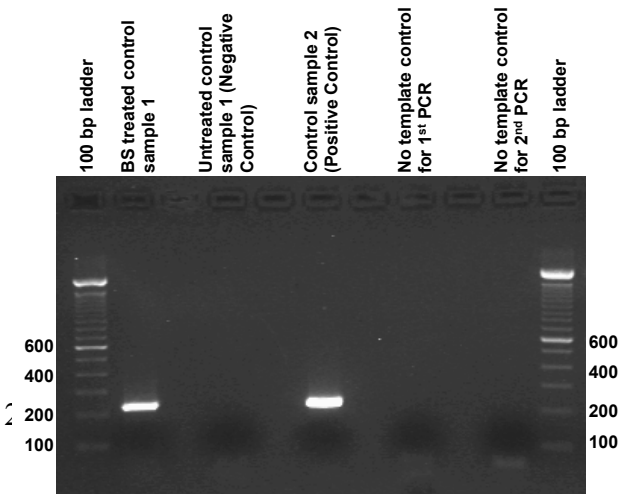
Thermal cycle (same as 1st PCR)

temp.	time	cycle number
95'C	3m	x1
95'C	30s	x35
50'C	1m	
72'C	1m	
72'C	10m	x1

(v) Gel electrophoresis

- Prepare 2% agarose gel and load 20 ul to check the PCR result.
- Load 250 ng 100 bp ladder per lane as DNA marker.

Example: if the BS treatment/conversion worked, you should expect the result shown below.



J1. PCR Amplify the library using Uracil tolerant ExTaq DNA Polymerase (Fisher TAK RR001B) (~ 2 hr)

In step J, BS lib will be amplified and size-selected by AMPure beads (Beckman) method. Multiple PCR amplifications can be performed. For each PCR reaction, take ~30 ng of 2nd BS product as PCR template.

Reagents:

10x ExTaq Buffer (Fisher TAK RR001B)
ExTaq DNA Polymerase (Fisher TAK RR001B)
10mM dNTP (2.5mM each)
10uM PE1 primer
10uM PE2 primer

Procedure:

In 0.2ml PCR tube, add the following reagents:

5 ul	10x ExTaq buffer
4 ul	10mM (2.5mM each) dNTPs
1 ul	PE 1 (10uM)
1 ul	PE 2 (10uM)
0.3ul	Extaq
x ul	2nd BS product (depending on the concentration of 2nd BS product)
add water to 50 ul	

NOTE: For starting with 50ng genomic DNA:

- ***You can divide the 2nd BS production into two batches and perform two individual PCR amplifications.***

Mix thoroughly with pipetting and spin briefly.

PCR cycle

1. 95C	2 min	
2. 95C	30 sec	
3. 65C	30 sec	10 cycle for step 2-4
4. 72C	1 min	
5. 72C	5 min	
6. 4C		

J2. Cleanup PCR product with 0.8x SPRI beads (~ 75 min)

i. Take the SPRI beads out of fridge. Vortex it. Take 36 ul of beads (per reaction) into 1.5 micro-centrifuge tube. Let stand at RT for 5 min.
ii. Add 36 uL of beads to 45 uL of reaction (volume ratio 0.8: 1). Mix thoroughly and let stand at RT for 15 mins.
iii. Place tubes on a Magnet separator for 15 minutes to separate beads from

solution.

iv. Aspirate 71 ul cleared solution from the reaction tubes and discard (leave 5 ul in tube). This step is performed while the reaction tubes are situated on the Magnet separator.

v. Dispense 200 µL of 80% ethanol to each tubes, and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of two washes.

ix. Place the reaction tubes on bench top to air-dry for 15 mins. Be sure to allow beads to dry completely (if is dry, you may some cracks on the bead pellet).

vi. Add 16.5 uL of elution buffer (EB or 10 mM Tris pH8) to each tube. Pipette to mix beads and EB thoroughly and let stand RT for 5 mins.

vii. Place tubes on Magnet separator for 5 mins, and transfer 16.0 ul clear liquid to new tube.

RECORD the recovery volume (Beads Size Selection)

J3. Measuring Concentration by NanoDrop Fluorospectrometer Quant-iT PicoGreen dsDNA Kit.

J4. Check library size distribution and concentration using Agilent Bioanalyzer System.

Example: BioAnalyzer trace profile of size-selection product (sequencing library). The size range is 170 ~ 580 bp. Average size is 364 bp.