MEDIP-SEQUENCING PROTOCOL

MAGMEDIP KIT Cat. No. C02010020

- The GenDNA module provides you with an excess of buffer for the preparation of DNA. Sufficient buffer is given for the preparation of several genomic DNA batches, each obtained from 1 to 3.10^6 cultured cells (see Step 1 and scale volumes accordingly based on your starting material).
- From about 3 million cells, 20 to 30 µg of DNA can be expected (see Table 1).
- It is also possible to start with less cells, keeping in mind that 1 µg of DNA is needed per IP (Table 1).
- Scale volumes accordingly based on the starting material that is available. Then, the number of IPs that can be done will also depend on the amount of prepared DNA that is available (Table 1).
- After DNA preparation, most of the DNA is then sheared to be used in the IP experiment, but remember that some of the DNA and sheared DNA are needed as control for:
    1. DNA preparation efficiency
    2. Shearing efficiency
    3. The IP experiment efficiency: input sample

Table 1

DNA preparation	Cell number needed	DNA amount expected	DNA to be used in IP
For 1 Methyl DNA IP	0.3x 10e6	2.0-3.0µg	1µg
For 10 Methyl DNA IPs	1-1.5x10e6	8-12µg	10 µg/10 IPs
For 20 Methyl DNA IPs	3x10e6	20-30µg	20 µg/20 IPs

DNA preparation for Methyl DNA IP

The goal of this first step is to get high molecular weight genomic DNA.

The GenDNA module form Diagenode has been optimized for the preparation of genomic DNA from cultured cells.

Note: The quality of the DNA to be used in IP is very important, we therefore highly recommend the use of our GenDNA module (Cat.No. C03030020).

STEP 1. Cell collection and lysis

Starting material: culture cells

1. Pellet suspension culture out of its serum-containing medium or trypsinize adherent cells and collect cells form the flask. Centrifuge at 300 g for 5 minutes at 4°C.
2. Discard the supernatant. Resuspend cells in 5 to 10 mL ice-cold PBS. Centrifuge at 300 g for 5 minutes. Discard the supernatant. Repeat this resuspension and centrifugation step once more. This step is to wash the cells.
    - Meanwhile, place the GenDNA Digestion Buffer at room temperature and the GenDNA Proteinase K on ice (to be used at Point 3. below).
3. Prepare the complete Digestion Buffer by adding 5 µL GenDNA Proteinase K to 1 mL GenDNA Digestion Buffer.
4. Resuspend cells in complete Digestion Buffer.
    - For 1.0 to 1.5 million cells, use up to 500 µL complete Digestion Buffer.
    - It might be necessary to use more buffer to avoid viscosity when performing the extractions.
5. Incubate the samples with shaking at 50°C for 12 to 18 hours in tightly capped tubes

STEP 2. Nucleic acid extraction and DNA purification

6. Thoroughly extract the samples with an equal vloume of phenol/chloroform/isoamyl alcohol (work under a fume hood).
    - Add 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1).
    - One volume is 500 µL (see Point 4. above).
    - Incubate the samples at RT for 10 minutes on a rotating wheel before centrifugation. Use gentle rotation and do not vortex.
7. Centrifuge at 1,700 g for 10 minutes.
    - If the phases do not resolve properly, add another volume of GenDNA Digestion Buffer omitting proteinase K, and repeat the centrifugation.
    - If there is a thick layer of white material at the interface between the phases, repeat the extraction.
8. Transfer the aqueous (top) layer to a new tube.
    - Increase volume to avoid viscosity if necessary and pipette slowly.
9. Thoroughly extract the samples with an equal volume of chloroform/isoamyl alcohol.
10. Incubate for 10 minutes at room temperature.
11. Centrifuge at 1,700 g for 10 minutes.
12. Transfer aqueous layer to a new tube.
13. Add ½ volume of GenDNA precipitant and 2 volumes of 100% ethanol.
    - One volume corresponds to the original amount of top layer.
14. Recover DNA by centrifugation at 1,700 g for 5 minutes.
    - Do not use higher speed to avoid genomic DNA fragmentation.
    - This brief precipitation in the presence of an optimized high salt precipitant (GenDNA Precipitant) reduces the amount of RNA in the DNA sample. For long-term storage it is convenient to leave DNA in the presence of ethanol.
15. Rinse the pellet with 70% ethanol. Decant ethanol and air-dry the pellet.
    - It is important to rinse extensively to remove any residual of salt and phenol.
16. Resuspend the pellet of DNA at ~1 mg/mL in GenDNA TE until dissolved. Shake gently at room temperature or at 65°C for several hours to facilitate solubilization. Store at 4°C.
    - From 1-1.5 million cells, ~8 to 12 µg of DNA can be expected (in a volume of 8 to 12 µL).
    - From 3 million cells, ~20 to 30 µg of DNA can be expected (in a volume of 20 to 30 µL).
    - If possible, it is recommended to get at least 10 µg of DNA (when enough materials is available) to be able to work with 30 µg of DNA : see Step 3. DNA shearing protocol).
17. Residual RNA has to be removed at this step by adding 2 µL of GenDNA RNAse (DNAse-free) per mL of DNA sample and incubating 1 hour at 37°C, followed by phenol/chloroform extraction and ethanol precipitation.

STEP 3. DNA shearing

Genomic DNA must be randomly sheared by sonication to generate fragments around 200 bp (see example below). To perform the MeDIP-Seq at least 1 µg of sheared DNA is needed in a volume smaller than 55 µl.

To choose the best protocol for the sonication with Bioruptor use our online DNA Shearing Guide:
https://www.diagenode.com/en/dna-shearing-guide

Only use the recommended tubes!

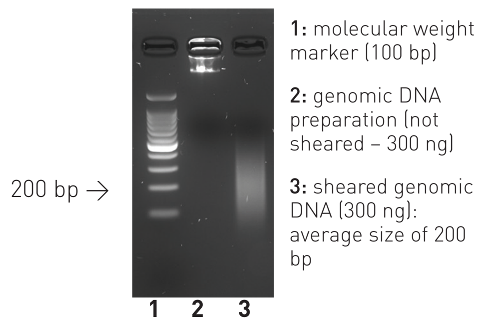
Example of shearing for MeDIP-seq using the Bioruptor® Pico

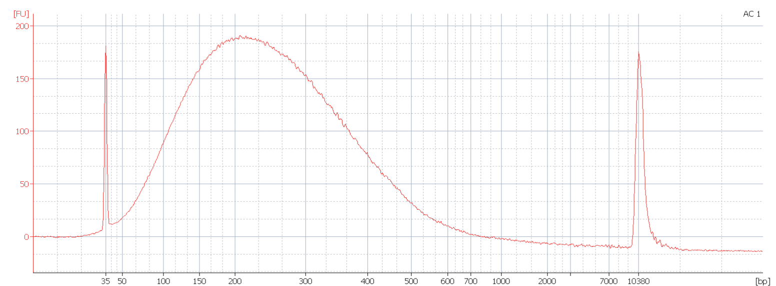
The genomic DNA was diluted in GenDNA TE to reach a concentration of 100 ng/µL and 100 µL were sheared in a 0.65 mL Bioruptor® Microtube (Cat. No. C30010011).

The following program was used:

- Cycles: [30 seconds "ON" & 30 seconds "OFF"]
- 13 cycles

Analysis of the genomic DNA after shearing:





Agilent High Sensitivity DNA chip profile of sheared genomic DNA: peak at 200 bp

STEP 4. End preparation

18. Mix the following components in a nuclease-free 0.2 mL tube:

iDeal Library End Repair/dA-Tailing Enzyme Mix (green)	3.0 µL
iDeal Library End Repair/dA-Tailing Buffer (green)	6.5 µL
Fragmented DNA	10 µL*
Nuclease-free water	45.5 µL*
Total volume	**65 µL**

*If DNA concentration is different from 0.1 µg/µL adapt the DNA and water volumes to obtain 1 µg DNA in 55.5 µL of water.

19. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

20. Place in a thermocycler, with the heated lid on, and run the following program:

- 30 minutes at 20°C
- 30 minutes at 65°C
- Hold at 4°C

STEP 5. Adaptor ligation

21. Add the following components directly to the iDeal Library End Preparation mixture and mix well:

iDeal Library Ligation Master Mix (red)	15 µL
iDeal Library Adaptor for Illumina (red)	2.5 µL
iDeal Library Ligation Enhancer (red)	1 µL
Total volume	**83.5 µL**

22. Incubate at 20°C for 15 minutes in a thermal cycler.

23. Add 3 µL of iDeal Library Uracil Excision Reagent (red) to the ligation mixture.

24. Mix well and incubate at 37°C for 15 minutes.

STEP 6. Size selection of adaptor-ligated DNA

The following size selection protocol is for libraries with 200 bp inserts only. For libraries with different size fragment inserts, refer to Table 2 for the appropriate volume of beads to be added. The size selection protocol is based on a starting volume of 100 µL.

25. Vortex AMPure XP beads to resuspend.

26. Add 13.5 µL of nuclease-free water to the ligation reaction for a 100 µL total volume.

27. Add 55 µL of resuspended AMPure XP beads to the 100 µL ligation reaction. Mix well by pipetting up and down at least 10 times.

28. Incubate for 5 minutes at room temperature.

29. Quickly spin the tube and place it on the DiaMag02-magnetic rack (Cat. No. B04000001) to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully transfer the supernatant containing your DNA to a new tube.Discard the beads that contain the unwanted large fragments.

Do not discard the supernatant.

30. Add 25 µL resuspended AMPure XP beads to the supernatant, mix well and incubate for 5 minutes at room temperature.

31. Quickly spin the tube and place it on the magnetic rack to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA targets

Do not discard the beads.

32. Add 200 µL of 80% freshly prepared ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

33. Repeat step 36 twice for a total of three washes.

34. Air dry beads for 10 minutes while the tube is on the magnetic rack with the lid open.

35. Elute the DNA target from the beads into28 µL of 10 mM Tris-HCl or 0.1X TE, pH 8.0. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and place it on a magnetic stand. After the solution is clear (about 5 minutes), transfer 23 µL to a new PCR tube for amplification.

Note: Be sure not to transfer any beads. Trace amounts of bead carry over may affect the optimal performance of the polymerase used in the iDeal Library PCR Master Mix in the subsequent PCR step.

Table 2: Recommended conditions for bead based size selection

Library parameters	**Approximate insert size**	**150 bp**	**200 bp**	**250 bp**	**300-400 bp**	**400-500 bp**	**500-700 bp**
	Total library size (insert+adaptor)	270 bp	320 bp	400 bp	400-500 bp	500-600 bp	600-800 bp
Volume to be added (µL)	1st Bead Selection	65	55	45	40	35	30
	2nd Bead Selection	25	25	25	20	15	15

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STEP 7. Methylated DNA immunoprecipitation

Beads preparation

36. Prepare the Bead Wash Buffer by diluting 1:5 the MagBuffer A with water. The volume of Bead Wash Buffer needed per IP reaction is 100 µL.

37. Resuspend the provided Magbeads and transfer 11 µL of Magbeads per IP into a new tube.
    - Keep beads in liquid suspension during storage at 4°C and at all handling steps, as drying will result in reduced performance.

38. Place the tube on a magnet or centrifuge to get rid of the supernatant. Keep the beads.

39. Wash the provided Magbeads twice with ice-cold Bead Wash Buffer as follows: resuspend the beads in buffer, then centrifuge for 5 minutes at 1.300 rpm (if volume is small, use the magnetic rack instead), discard the supernatant and keep the bead pellet.
    - For 2 IPs: add a volume of 55 µL Buffer to 22 µL stock solution of beads.
    - For 8 IPs: add a volume of 220 µL Buffer to 88 µL stock solution of beads.
    - For 16 IPs add a volume of 440 µL Buffer to 176 µL stock solution of beads.

Keep the beads homogenously in suspension at all times when pipetting. Variation in the amount of beads will lead to lower reproducibility.

40. After washing, resuspend the beads in wash buffer as indicated below, keep on ice.
    - For 1 IP: add 22 µL of bead wash buffer.
    - Do not freeze the beads.

Resuspend the beads before each use

IP mix preparation

41. Prepare the IP incubation mix as described in Table 3:
    - When preparing the IP incubation mix, take into account that the input sample must also be used as a control next to the IP. Note that this mix includes the 2 DNA internal IP controls (unmethylated and methylated DNA).

(I) If performing 1 IP and input add the volumes indicated in Table 3 for 1IP + Input, directly at your sample tube. The total volume is 90 µL corresponding to 1 IP (75 µL), 10% input (7.5 µL) and excess (7.5 µL).

(II) If performing 2 IPs and input,add to a new tube: 130 µL of IP incubation mix prepared using the volumes indicated in Table 3 for 2 IPs + Input, and the pool of two DNA samples of 23 µL each (46 µL of Adaptor-ligated DNA). The total volume is 180 µL corresponding to 2 IPs (150 µL), 10% input (7.5 µL) and excess (22.5 µL).

Table 3. IP incubation mix

Reagent	Volume per 1 IP + Input (I)	Volume per 2 IPs + input (II)
Water	34 µL	64 µL
MagBuffer A*	24 µL	48 µL
MagBuffer B	6 µL	12 µL
Positive meDNA control	1.5 µL	3 µL
Negative unDNA control	1.5 µL	3 µL
Adaptor-ligated DNA	23 µL	46 µL
Total volume	**90.00 µL**	**180.00 µL**

*Contains detergent; if its appearance is cloudly and cristallized please warm gently prior to use.

42. Incubate at 95°C for 3 minutes.
43. Quickly chill sample on ice (it is best to use ice-water).
44. Perform a pulse spin to consolidate your sample.
45. Take out 7.5 µL (that is 10% input) and transfer to a new labeled tube. Keep the input samples at 4°C. The input sample is to be used as a control of starting material and it is therefore not to be used in IP.

46. hen, transfer from what is left: 75 µL per tube into 200 µL tubes using the provided 200 µL tube strips (or individual 200 µL tubes that can fit in our Magnetic Rack). Keep at 4°C.

47. In a new tube, dilute the antibody 1:2 with water.

48. Then use the 1:2 freshly diluted antibody to prepare the Diluted Antibody mix as shown in Table 4 below. Add to the antibody, the MagBuffer A and water first. Add the MagBuffer C afterwards.

Table 4. Diluted Antibody Mix and volume to prepare for different IP numbers.

Reagents	For 1 IP	For 2 IPs	For 4 IPs	For 8 IPs
Antibody 1:2	0.30 µL	0.75 µL	1.50 µL	3.00 µL
MagBuffer A	0.60 µL	1.50 µL	3.00 µL	6.00 µL
Water	2.10 µL	5.25 µL	10.50 µL	21.00
MagBuffer C	2.00 µL	5.00 µL	10.00 µL	20.00 µL
Final volume	**5.00 µL**	**12.50 µL**	**25.00 µL**	**50.00 µL**

49. Add 5 µL of Diluted Antibody Mix per 200 µL IP tube (from Point 50. Above).
    - Diluted Antibody Mix is added to the IPs, which contain IP incubation mix and DNA sample.
    - The Diluted Antibody Mix that is left must be discarded.

50. Mix and add 20 µL of washed beads to each 200 µL IP tubes (final volume: 100 µL)

51. Place on a rotating wheel at 4 °C for 4 hours or overnight.

STEP 8. Washes

52. Place the MagWash Buffers and Magnetic Rack on ice. It is best to perform the washes on ice or in a cold room.

53. Spin down and place the IP tubes in the ice-cold Magnetic Rack and work on ice, wait 1 minute and discard the buffer.

54. Wash the DNA IP samples three times as follows. Add per tube, 100 µL ice-cold MagWash Buffer-1, close the tube caps, invert the 8-tubes strip to resuspend the beads, incubate for 4 minutes at 4°C on a rotating wheel (40 rpm), spin, place in the Magnetic Rack, wait 1 minute and discard the buffer. Keep the captured beads.
    - Do not disturb the captured beads attached to the tube wall.
    - Always briefly spin the tubes to bring down liquid caught in the lid prior to positioning in the Diagenode Magnetic Rack.
    - Use 150 µL Wash Buffer for each wash if working in a 1.5 mL tube-Magnetic Rack.

55. Wash the beads once with 100 µL ice-cold MagWash buffer-2.

56. After the last wash, discard the last traces of Wash buffer (using a P200 pipet). Keep the bead pellets on ice and proceed to the next step.

From the washed beads, the bound DNA can now be purified (proceed to the next point).

STEP 9. DNA elution

57. Prepare the Elution buffer by mixing Buffer A and B as follows:

Elution Buffer	1 rxn
Buffer A	115.4 µL
Buffer B	4.6 µL
Total volume	**120 µL**

- Place Buffer A at 25°C during 30 min. before use.
- 100 µL of Elution buffer are needed per IPure reaction (20 µL excess).
- 1 IPure reaction corresponds to the purification of 1 MeDIP or 1 input sample.

Make sure when working with Buffer A, that there are no crystals left in solution. Otherwise heat up gently and mix until complete disappearance of such crystals.

58. Add 50 µL of Elution Buffer to the bead pellets (tube strip).

59. Take your input sample back from 4°C and perform a pulse spin. Add 92.5 µL of Elution buffer and 7.5 µL of input sample to a new 200 µL tube (8-tubes strip).

- Input sample corresponds to 10% of the IP sample.

60. Incubate samples and input DNA for 15 minutes at room temperature on a rotating wheel (40 rpm).

61. Spin the 8-tubes strip and place it into the DiaMag02 (magnetic rack). After 1 minute, transfer the supernatants to a new labelled 8-tubes strip.

62. Repeat the incubation of the bead pellets for 15 minutes at room temperature on a rotating wheel (40 rpm) in 50 µL Elution buffer.

- For input DNA samples: 1 elution in 100 µL.
- For MeDIP samples: 2 elutions in 50 µL (total volume 100 µL per sample).

63. Spin the 8-tubes strip. Place the 8-tubes strips into the DiaMag02, wait 1 minute and transfer the supernatants to new 8-tubes strips.

- Elutions of IP and input samples are now completed in 100 µL and are in the same 8-tubes strip.

STEP 10. DNA binding

64. Add 2 µL of carrier to each IP and input sample. Vortex briefly and perform a short spin.

65. Add 100 µL of 100% isopropanol to each IP and input sample. Vortex briefly and perform a short spin.

Note: Following the addition of isopropanol the solution may become cloudy. This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.

66. Resuspendthe provided Magnetic beads and transfer 15 µL to each IP and input sample.

- Keep Magnetic beads in liquid suspension during storage at 4°C and at all handling steps, as drying will result in reduced performance.
- The final volume is now 217 µL per IPure reaction.

67. Incubate IP and input samples for 10 min at room temperature on a rotating wheel (40 rpm).

STEP 11. Washes

68. Prepare the Wash buffer 1 containing 50% isopropanol as follows:

Wash buffer 1	24 rxns	100 rxns
Wash buffer 1 w/o isopropanol	2 mL	8 mL
Isopropanol (100%)	2 mL	8 mL
Total volume	**4 mL**	**16 mL**

- Never leave the bottle open to avoid evaporation.

69. Briefly spin the tubes, place in the DiaMag02, wait 1 minute and discard the buffer. Keep the captured beads and add per tube, 100 µL Wash buffer 1. Close the tubes, invert the 8-tubes strip to resuspend the beads and incubate for 5 minutes at room temperature on a rotating wheel (40 rpm).

- Do not disturb the captured beads attached to the tube wall.
- Always briefly spin the tubes to bring down liquid caught in the lid priori to positioning into the Diagenode Magnetic Rack.

70. Prepare the Wash buffer 2 containing 50% isopropanol as follows:

Wash buffer 2	24 rxns	100 rxns
Wash buffer w/o isopropanol	2 mL	8 mL
Isopropanol (100%)	2 mL	8 mL
Total volume	**4 mL**	**16 mL**

- Never leave the bottle open to avoid evaporation.

71. Wash the IP and input sampleswith the Wash buffer 2 as follows. Briefly spin the tubes, place into the DiaMag02, wait 1 minute and discard the buffer. Keep the captured beads and add per tube, 100 µL Wash buffer 2. Close the tubes, invert the 8-tubes strip to resuspend the beads and incubate for 5 minutes at room temperature on a rotating wheel (40 rpm).

- Do not disturb the captured beads attached to the tube wall.
- Always briefly spin the tubes to bring down liquid caught in the lid prior to positioning in the DiaMag02.

STEP 12. DNA elution

72. Briefly spin the tubes, place them into the DiaMag02, wait 1 minute and discard the buffer. Keep the captured beads and add per tube 25 µL Buffer C (alternatively, a higher volume can be used if necessary). Close the tubes, invert the 8-tubes strip to resuspend the beads and incubate for 15 minutes at room temperature on a rotating wheel (40 rpm).

73. Spin the 8-tubes strip and place it into the DiaMag02, wait 1 minute and transfer the supernatants to new labelled 1.5 mL tubes. Keep the bead pellets on ice.

74. Place the DNA on ice and proceed to the PCR amplification.

<u>Notes:</u>

- You can stop the experiment at this step and store the DNA at -20°C or -80°C until further use.
- At this step it is possible to confirm the enrichment by MeDIP by performing qPCR.

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STEP 13. PCR amplification

75. Mix the following components in sterile strip tubes:

IPDNA samples	23 µL
iDeal Library PCR Master Mix (blue)	25 µL
iDeal Library Index Primer* (blue)	1 µL
iDeal Universal PCR Primer (blue)	1 µL
Total volume	**50 µL**

*These primers (Index Primer Set 1) are included. Index Primer Set 2 may be purchased separately (Cat. No. C05010021).

76. Follow the PCR cycling conditions described below:

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	15*
Annealing	65°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	5 minutes	1
Hold	4°C	∞	

*Further optimization of PCR cycle number may be required.

STEP 14. Cleanup of PCR amplification

77. Vortex AMPure XP beads to resuspend.

78. Add 50 µL of resuspended AMPure XP beads to the PCR reactions(~50 µL). Mix well by pipetting up and down at least 10 times.

79. Incubate for 5 minutes at room temperature.

80. Quickly spin the tube and place it onDiaMag02 to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

Do not discard beads.

81. Add 200 µL of 80% ethanol to the PCR tube while in the DiaMag02. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

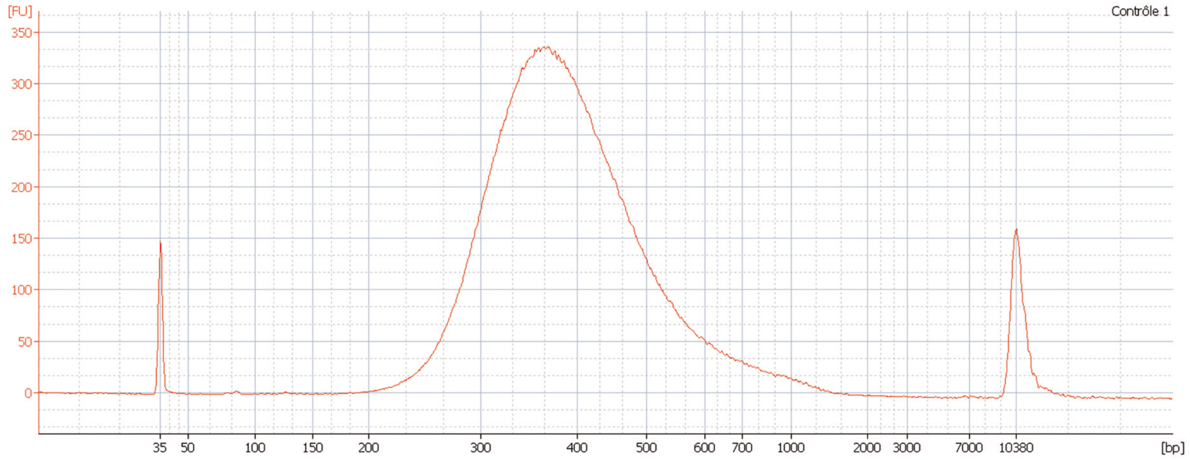
82. Repeat step 96 once.

83. Air dry the beads for 10 minutes while the PCR tube is on the magnetic stand with the lid open.

84. Elute DNA target from beads into 33 µL 10 mM Tris-HCl, pH 8.0 or TE Buffer pH 8.0. Mix well by pipetting up and down at least 10 times. Quickly spin the tube and place it on the magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully transfer 28 µL supernatant to a new PCR tube. Store libraries at -20°C.

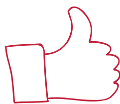
85. Dilute the library 5 fold with nuclease-free water and check the size distribution on an Agilent high sensitivity chip.

Library of methylated DNA



Agilent High Sensitivity DNA chip profile of library obtained with Diagenode's MeDIP-Seq protocol

One microgram of starting DNA was used, 15 amplification cycles were applied and a 1/5 dilution was performed before Bioanalyzer run.



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