GENE-SWitCH

The regulatory GENomE of SWine and CHicken: functional annotation during development

Protocol WP1
PIG and CHICKEN RRBS Library Preparation and NovaSeq Sequencing

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1 Summary

GENE-SWitCH aims at identifying functional elements located in the genomes of pig and chicken working on seven different tissues at three different developmental stages. One of the important marks of the functional genome elements is DNA methylation which is generally reverse correlated with gene expression at the functional genome elements. The objective were to achieve high quality DNA methylation data from the tissues sampled from the different developmental stages (D1.1) and make the DNA methylation data available to the GENE-SWitCH/FAANG community and to make it public available.

Details about DNA extraction is available on the FAANG data portal.

Pig:
https://data.faang.org/api/fire_api/assays/INRA_SOP_GENESWITCH_WP1_PIG_EXTRACTION_DNA_RNA_20201111.pdf

Chicken:
https://data.faang.org/api/fire_api/assays/ROSLIN_SOP_GENESWITCH_WP1_CHICK_EXTRACTION_DNA_RNA_20201111.pdf

2 Protocol description

2.1.1 Construction of RRBS libraries

Construction of libraries and sequencing on the Illumina NovaSeq 6000 was performed at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. Reduced Representation Bisulfite Sequencing (RRBS) libraries were constructed with the Ovation RRBS Methyl-Seq kit from Tecan. Briefly, 100ng of high molecular weight DNA was digested with MspI, ligated to sequencing adaptors, treated with bisulfite and amplified by PCR. The final libraries were quantitated with Qubit (ThermoFisher, MA) and the average size was determined on a Fragment Analyzer (Agilent, CA). The libraries were diluted to 10nM and further quantitated by qPCR on a CFX Connect Real-Time qPCR system (Biorad, Hercules, CA) for accurate pooling of barcoded libraries and maximization of number of clusters in the flowcell.

2.1.2 Sequencing of libraries in the NovaSeq 6000

The pooled barcoded shotgun libraries were loaded on three NovaSeq 6000 S1 lanes for cluster formation and sequencing. They were sequenced for 151nt from one side of the DNA fragments. The demultiplexed fastq and unique molecular identifier (UMI) read files were generated with the bcl2fastq v2.20 Conversion Software (Illumina, San Diego, CA).

The detailed protocol for library production can be downloaded using the following link:
https://www.nugen.com/sites/default/files/M01394_v6_User_Guide%3A_Ovation_RRBS_Methyl-Seq_System_5912.pdf

and is also reproduced below:

For each section of the protocol, remove reagents from storage as listed. Thaw and place reagents at room temperature or on ice as instructed. After each section, continue immediately to the next section of the protocol unless otherwise directed.

Important: Ensure a working stock of desulfonation buffer is prepared fresh at least 2 hours before use of the oxidant.

A. Sample Preparation

This protocol includes an integrated oxidative bisulfite workflow using the TrueMethyl oxBS Module.

- For each genomic DNA sample to be analyzed for 5hmC, two independent NGS libraries should be prepared. For each sample, one aliquot (100 ng) will be processed through oxBS and the other aliquot (100 ng) will be processed through a parallel MOCK oxBS workflow. The MOCK oxBS workflow excludes the oxidant solution.
- In order to multiplex the oxBS and MOCK oxBS processed samples, unique indexes are required.
- For bisulfite conversion without oxidation, follow the MOCK oxBS workflow.
- For alternate workflows without the TrueMethyl oxBS Module, substitute the 3rd party bisulfite treatment for steps V.E. - V.H. in the protocol. Step V.J. Library Amplification is designed to accommodate a volume of 20 µL of bisulfite converted, desulfonated DNA for PCR enrichment.

1. Aliquot each input sample (100 ng) into a 0.2 mL PCR strip tube or plate.
2. Adjust each sample to 8.5 µL with nuclease-free water.

B. MspI Digestion

Table 4. MspI Master Mix

REAGENT	MspI BUFFER MIX (BLUE)	MspI ENZYME MIX (BLUE)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	1.0 µL	0.5 µL

1. Spin down the MspI Enzyme Mix and place on ice.
2. Thaw MspI Buffer Mix at room temperature. Mix MspI Buffer Mix by vortexing, spin and place on ice.
3. Prepare a master mix by combining MspI Buffer Mix and MspI Enzyme Mix in a 0.5 mL capped tube, according to the volumes shown in Table 4. Mix by pipetting, spin down briefly and immediately place on ice.
4. Add 1.5 µL of MspI Master Mix to each sample tube for a total of 10 µL. Mix by pipetting, spin down and place on ice.
5. Place the tubes in a thermal cycler programmed to run Program 1 (MspI Digestion; see Table 3):

   37 °C – 60 min, hold at 4 °C

6. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

C. Adaptor Ligation

Table 5. Ligation Master Mix

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	LIGATION BUFFER MIX (YELLOW: L1)	LIGATION ENZYME MIX (YELLOW: L3)
STORAGE	—	–20 °C	–20 °C
1X REACTION VOLUME	2 µL	4 µL	1.0 µL

1. Spin down L3 and place on ice.
2. Thaw Adaptor Mixes (L2V20DR) on ice, spin down, and return to ice.

Important: Do not warm Ligation Adaptor Mixes above room temperature. Heating will severely degrade performance.

3. Thaw L1 at room temperature. Mix by vortexing, spin and place on ice.

Note: L1 is extremely viscous. Pipet this reagent slowly and mix thoroughly. Ensure it is well mixed after thawing, and that the Ligation Master Mix and ligation reactions are well-mixed.

4. Add 3 µL of the appropriate L2 Ligation Adaptor Mix to each sample. Mix thoroughly by pipetting.
5. Just prior to use, prepare a master mix by combining D1, L1 and L3 in a 0.5 mL capped tube, according to the volumes shown in Table 5. Mix by pipetting slowly, without introducing bubbles, spin and place on ice. Use the master mix immediately.
6. Add 7 µL Ligation Master Mix to each reaction tube for a total of 20 µL. Mix thoroughly by pipetting slowly and gently, spin down and place on ice. Proceed immediately with the incubation.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 2 (Ligation; see Table 3):

   25 °C – 30 min, 70 °C – 10 min, hold at 4 °C
8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

D. Final Repair

1. Remove the TrueMethyl oxBS Module Magnetic Bead Solution, Binding Buffer 1 and Binding Buffer 2 from 4 °C and place at room temperature for use in the next step.

Table 6. Final Repair Master Mix

REAGENT	FINAL REPAIR BUFFER MIX (PURPLE: FR1 VER 4)	FINAL REPAIR ENZYME MIX (PURPLE: FR2)	NUCLEASE-FREE WATER (GREEN: D1)
STORAGE	–20 °C	–20 °C	—
1X REACTION VOLUME	6 µL	0.5 µL	13.5 µL

2. Spin down FR2 and place on ice.
3. Thaw FR1 at room temperature. Mix by vortexing, spin down and place on ice.
4. Prepare a master mix by combining FR1, FR2 and D1 in a 0.5 mL capped tube according to the volumes shown in Table 6.
5. Add 20 µL of the Final Repair Master Mix to each sample for a total of 40 µL. Mix by pipetting, spin down and place on ice.
6. Place the tubes in a thermal cycler pre-heated to 60 °C and programmed to run Program 3 (Final Repair; see Table 3):

   60 °C – 10 min, 70 °C – 10 min, hold at 4 °C
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
8. If using the True Methyl Module, continue with section E. DNA Purification and Denaturation. For alternative bisulfite conversion methods, perform bisulfite conversion according to the manufacturer recommendations and proceed to protocol section I. Library Amplification Optimization with qPCR.

Important: Bisulfite conversion is critical for successful library amplification.

E. DNA Purification and Denaturation

1. Remove the TrueMethyl oxBS Module Oxidant Solution from –20 °C and thaw on ice for use in the next step.

Note: The Oxidant Solution is light-sensitive. Keep protected from light.

Table 7. Magnetic Bead Binding Solution 1 Master Mix

REAGENT	BINDING BUFFER 1	MAGNETIC BEAD SOLUTION
STORAGE	4 °C	4 °C
1X REACTION VOLUME	120 µL	2.4 µL

2. Remove Acetonitrile, Ultra Pure water, and Denaturing Solution from storage and place on bench top.
3. Prepare a fresh stock of 80% acetonitrile, using the Ultra Pure water provided with the kit. Mix by vortexing or inversion and place at room temperature.
4. Ensure Magnetic Bead Solution and Binding Buffer 1 have reached room temperature before proceeding.
5. Mix Binding Buffer 1 by inversion until homogenized.
6. Vortex Magnetic Bead Solution until homogenized.
7. Prepare a master mix of Magnetic Bead Binding Solution 1 (MBBS1) as directed in Table 7.

Note: MBBS1 should be prepared fresh on the day of use. Do not store for longer than 1 week.

8. Vortex MBBS1 master mix thoroughly to ensure the beads are homogenized in solution.
9. At room temperature, add 10 µL of Ultra Pure water to each sample for a total of 50 µL.
10. Add 100 µL of MBBS1 master mix to each 0.2 mL tube containing 50 µL sample for a total of 150 µL. Mix by pipetting and centrifuge briefly.
11. Incubate at room temperature for 20 min.
12. Transfer tubes to a magnetic separation plate and incubate at room temperature for 5 minutes to completely clear the solution of beads.
13. Keeping the tubes on the magnet, carefully remove the supernatant and discard it.
14. With the tubes still on the magnet, carefully add 200 µL of 80% Acetonitrile wash to the tubes without disturbing the bead pellet.
15. Remove and discard the 200 µL 80% Acetonitrile wash, carefully avoiding aspiration of the bead pellet.

16. Repeat Steps 13 and 14 twice to perform 3 x 200 µL 80% Acetonitrile washes in total. Remove as much of the final wash as possible.
17. Air dry the bead pellets for 5 minutes at room temperature, leaving the lids of the tubes open.

Note: Ensure the tubes are dry before continuing the protocol. If the tubes aren't dry after 5 minutes, incubate for a longer period of time.

18. Remove the tubes from the magnet.
19. Add 10 µL of Denaturing Solution directly onto the bead pellet. Mix thoroughly to ensure all beads are resuspended.
20. Centrifuge briefly to collect solution at bottom of the tubes.
21. Place the tubes in a pre-warmed thermal cycler programmed to run Program 4 (Denaturation; see Table 3):

    37°C – 5 min, hold at 25 °C
22. Remove the tubes from the thermal cycler, spin to collect condensation and transfer to the magnet.
23. Incubate at room temperature for 2 minutes.
24. Carefully remove 9 µL of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh 1.5 mL microcentrifuge tube and place at room temperature.

F. DNA Oxidation

1. Remove Ultra Pure water from storage and place on bench top.
2. Set a heat block to 40 °C.
3. Prepare individual oxidation and mock oxidation reactions as follows:
   - For each sample to be processed through the oxBS workflow, add 1 µL of oxidant solution to 9 µL of DNA for a total of 10 µL.
   - For each sample to be processed through the MOCK oxBS workflow, add 1 µL of Ultra Pure water to 9 µL of DNA for a total of 10 µL.
4. Mix reactions by vortexing and centrifuge briefly.
5. Place tubes in heat block and incubate for 10 min at 40 °C.
6. Centrifuge reactions at 14000 x g for 10 minutes at room temperature to pellet any black precipitate. MOCK oxBS treated samples will remain clear and will not have any black precipitate.

Important: In samples treated with oxidant solution, the color of the oxidation reaction should remain orange after the 10 minute centrifugation, indicating a successful oxidation. If the solution turns any color other than orange, please see Appendix C.

7. Transfer the orange supernatant to a fresh 0.2 mL PCR tube and place at room temperature. Proceed immediately to the next step.

Note:
- Take care not to carry any black precipitate over as this could inhibit downstream steps.
- Do not place the oxidized samples on ice to cool as this may cause the solution to precipitate.

G. Bisulfite Conversion

1. Set a heat block or heated orbital incubator to 60 °C.
2. Remove Bisulfite Diluent and Bisulfite Reagent aliquots from storage and place on bench top. Remove 1 aliquot of Bisulfite Reagent for every 20 reactions to be processed.
3. Prepare Bisulfite Reagent Solution by adding 700 µL of Bisulfite Diluent to each aliquot of Bisulfite Reagent.

Note: Each aliquot of Bisulfite Reagent Solution is sufficient for up to 20 samples. A fresh aliquot of solution should be prepared each time the kit is used and disposed of immediately after use.

4. Seal the lid of each aliquot with Bisulfite Reagent Solution tightly.
5. Incubate the aliquots of Bisulfite Reagent Solution for 15 min at 60 °C. Vortex regularly until the Bisulfite Reagent Solution is completely dissolved.
6. Spin down Bisulfite Reagent Solution briefly and place at room temperature.
7. Ensure oxidized DNA samples from previous step are at room temperature before proceeding.
8. Prepare Bisulfite Conversion Reaction mix by adding 30 µL of Bisulfite Reagent Solution to each 10 µL of DNA for a total of 40 µL. Ensure that each sample pair being processed through the oxBS and BS workflow is treated with the same aliquot of Bisulfite Reagent Solution.
9. Mix by pipetting, spin down and place at room temperature.

Note:
- If the Bisulfite Reagent Solution precipitates, return to 60 °C until dissolved.
- Samples treated with the oxidant solution may turn light gray in color.

10. Place the tubes in a pre-warmed thermal cycler programmed to run Program 5 (Bisulfite Conversion, see Table 3):

    95 °C – 5 min, 60 °C – 20 min, 95 °C – 5 min, 60 °C – 40 min, 95 °C – 5 min, 60 °C – 45 min, hold at 20 °C

Optional stopping point: You may hold samples at room temperature (+20 °C) for up to 16 hours. Do not store below +20 °C.

11. Once the bisulfite conversion is complete, centrifuge samples briefly to collect solution at bottom of the tubes.
12. Transfer samples to 1.5 mL tubes and centrifuge for 10 min at 14000 x g.
13. Continue to H. Bisulfite-Converted DNA Desulfonation and Purification while the samples are in the centrifuge.

H. Bisulfite-Converted DNA Desulfonation and Purification

1. Remove Desulfonation Buffer, Binding Buffer 2, Magnetic Bead Solution and Elution Buffer from storage and place at room temperature for a minimum of 30 minutes before use.

Table 8. Magnetic Bead Binding Solution 2 Master Mix

REAGENT	BINDING BUFFER 2	MAGNETIC BEAD SOLUTION
STORAGE	4 °C	4 °C
1X REACTION VOLUME	200 µL	2.4 µL

2. Prepare a fresh stock of 70% Ethanol. Mix by vortexing or inversion.
3. Mix Binding Buffer 2 by inversion until homogenized.
4. Vortex Magnetic Bead Solution until homogenized.
5. Prepare a master mix of Magnetic Bead Binding Solution 2 (MBBS2) as directed in Table 8.

Note:

- MBBS2 should be prepared fresh on the day of use. Do not store for longer than 1 week.
- MBBS2 is a viscous solution. Pipet this reagent slowly and mix thoroughly. Ensure that MBBS2 and the MBBS2-sample mix are well-mixed.

6. Transfer 40 µL of the supernatant to a fresh set of 0.2 mL PCR tubes. Avoid disturbing the pellet in the oxidant solution-treated samples.
7. Vortex MBBS2 thoroughly to ensure the solution is homogenous before aliquoting.
8. Carefully add 160 µL of MBBS2 to each tube containing 40 µL bisulfite converted sample for a total of 200 µL. Mix thoroughly by pipetting slowly and gently, spin down and place at room temperature.
9. Incubate at room temperature for 5 minutes.
10. Centrifuge briefly to collect solution at bottom of the tubes.
11. Place the tubes onto the magnet and incubate at room temperature for at least 5 minutes to completely clear the solution of beads.
12. Carefully remove the supernatant and discard it.
13. Remove the tubes from the magnet.
14. Add 200 µL of 70% Ethanol to each sample tube. Resuspend the beads completely by pipetting.

15. Place the tubes on the magnet and incubate at room temperature for 5 minutes to completely clear the solution of beads.
16. Carefully remove the 70% Ethanol wash and discard it. Remove as much of the wash as possible.
17. Remove samples from the magnet.
18. Add 200 µL of Desulfonation Buffer with EtOH added directly onto the bead pellet. Resuspend the beads completely by pipetting.

Note: Be sure that the ethanol has been added to the desulfonation buffer, as described in Section III. B. Preparation of Desulfonation Buffer for the TrueMethyl oxBS Module.

19. Close lids of sample tubes securely and place the tubes into the magnetic separation rack. Incubate at room temperature for 5 minutes to completely clear the solution of beads.
20. Remove the tubes from the magnet, open the tubes, and return to the magnet.
21. Carefully remove 200 µL of the Desulfonation Buffer and discard it. Remove as much of the Desulfonation Buffer as possible without disturbing the bead pellet.
22. Remove the tubes from the magnet.
23. Add 200 µL of 70% Ethanol to each sample tube. Resuspend the beads completely by pipetting.
24. Place the tubes onto the magnet and incubate at room temperature for 5 minutes to completely clear the solution of beads.
25. Remove the 200 µL 70% Ethanol wash and discard it.
26. Repeat Steps 23–25 to perform 2 x 200 µL 70% Ethanol washes in total. Remove as much of the final wash as possible.
27. Air-dry the the beads on the magnet for 15 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated.
28. Remove the tubes from the magnet.
29. Add Elution Buffer directly onto the bead pellet and resuspend completely by pipetting:
    - For Library Amplification Optimzation with qPCR (recommended), resuspend beads in 23 µL Elution Buffer.
    - If qPCR optimization is not required, resuspend beads in 21 µL Elution Buffer.
30. Incubate at room temperature for 5 minutes to elute the TrueMethyl converted DNA from the beads.
31. Centrifuge briefly to collect sample at bottom of the tubes.
32. Place the tubes onto the magnet and incubate at room temperature for 5 minutes to completely clear the solution of beads.
33. Carefully transfer eluate into a fresh 0.2 mL tube:
    - For Library Amplification Optimzation with qPCR (recommended), transfer 22 µL eluate.
    - If qPCR optimization is not required, transfer 20 µL eluate.

I. Library Amplification Optimization with qPCR

Note: qPCR optimization should be performed when running the kit for the first time, when using a new sample type or input, and any time degraded or low input samples are used.

Table 9. Library Amplification qPCR Master Mix

REAGENT	AMPLIFICATION PRIMER MIX (RED: P2 VER 8)	AMPLIFICATION ENZYME MIX (RED: P3 VER 3)	20X EvaGreen
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	1.0 µL	4.75 µL	0.5 µL

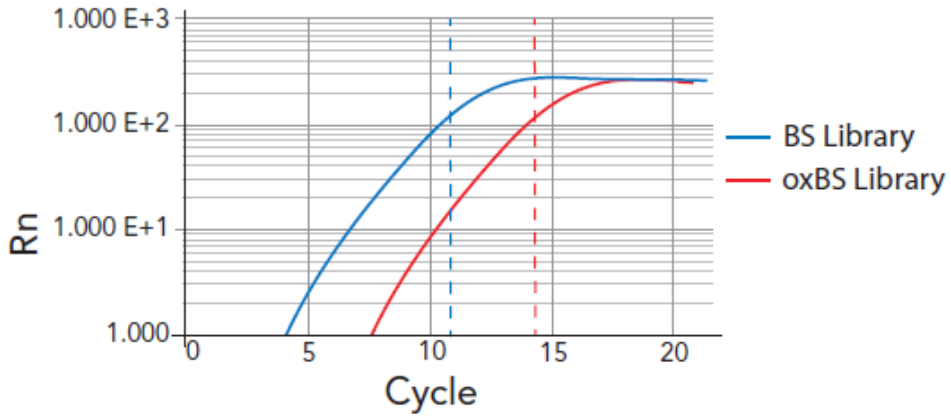
1. Aliquot 14 µL of DR1 into a fresh set of 0.2 mL tubes (one tube for each sample).
1. Add 2 µL of each sample to each tube for a total of 16 µL. Reserve the remaining 20 µL of sample on ice. Mix well by pipetting, spin down and place on the bench top.
2. Aliquot 3.75 µL of each diluted sample into an appropriate PCR plate or optically clear strip tubes, in triplicate. Mix well by pipetting, spin down and place on ice.
3. Prepare a master mix by combining P2, P3 and 20x EvaGreen in an appropriately sized capped tube according to the volumes shown in Table 9. Add P3 at the last moment and mix well by pipetting, taking care to avoid bubbles. Spin down and place on ice.
4. On ice, add 6.25 µL of Library Amplification qPCR Master Mix to each 3.75 µL of sample, in triplicate, for a total of 10 µL per replicate.
5. Mix well by pipetting, spin down and place on ice.
6. Perform qPCR with the following cycling conditions:

   95 °C – 2 min, 35x(95 °C – 15 s, 60 °C – 1 min, 72 °C – 30 s)
7. Examine the log fluorescence vs. cycle number plot from the qPCR system to determine the appropriate number of library amplification cycles.

   a. First, select a cycle number within the late exponential phase of the amplification plot. In the example in **Figure 8**, this is 11 cycles the 'BS Library' (blue dotted vertical line, left) and 14 cycles in the 'oxBS Library' (red dotted vertical line, right). Note the cycle number determined experimentally may differ from this stylized plot in **Figure 8**.

   b. Next, subtract 3 from the selected cycle to determine the number of PCR cycles to perform in the next step (J. Library Amplification). This compensates for the 1:8 dilution of sample used in the qPCR reaction. In the example in **Figure 8** below, this is 11-3 = 8 cycles of PCR for the 'BS Library', and 14-3=11 cycles of PCR for the 'oxBS Library'.

Figure 8. Stylized qPCR amplification plot



J. Library Amplification

1. Remove Agencourt Beads from 4 °C and DR1 from –20 °C and place at room temperature for use in the next step.

Table 10. Library Amplification Master Mix

REAGENT	AMPLIFICATION PRIMER MIX (RED: P2 VER 8)	AMPLIFICATION ENZYME MIX (RED: P3 VER 3)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	5.0 µL	25.0 µL

Note: When starting with degraded DNA or significantly less than 100 ng of high-quality DNA, consult section I. Library Amplification Optimization with qPCR.

2. Spin down P3 and place on ice.
3. Thaw P2 at room temperature. Mix by vortexing, spin down and place on ice.
4. Prepare a master mix by combining P2 and P3 in a 0.5 mL capped tube according to the volumes shown in Table 10. Mix well by pipetting, taking care to avoid bubbles, spin down and place on ice.
5. On ice, add 30 µL of Amplification Master Mix to each sample for a total of 50 µL.
6. Place tubes in a pre-warmed thermal cycler programmed to run Program 6 (Library Amplification; see Table 3):

   95 °C – 2 min, N(95 °C – 15 s, 60 °C – 1 min, 72 °C – 30 s), hold at 10 °C
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

Optional stopping point: Store samples at –20 °C.

K. Amplified Library Purification

1. Ensure the Agencourt beads and DR1 have completely reached room temperature before proceeding.
2. Resuspend the beads by inverting and tapping the tube. Ensure the beads are fully resuspended before adding to samples. After resuspending, do not spin the beads.
3. Add 50 µL (1 volume) of the bead suspension to each reaction. Mix thoroughly by pipetting 10 times.
4. Incubate at room temperature for 10 minutes.
5. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
6. Carefully remove 90 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the final yield, so ensure beads are not removed with the binding buffer or the wash.

7. With samples still on the magnet, add 200 µL of freshly prepared 70% ethanol.
8. Remove the 70% ethanol wash using a pipette.
9. Repeat steps 7 and 8 for a total of two washes.

Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

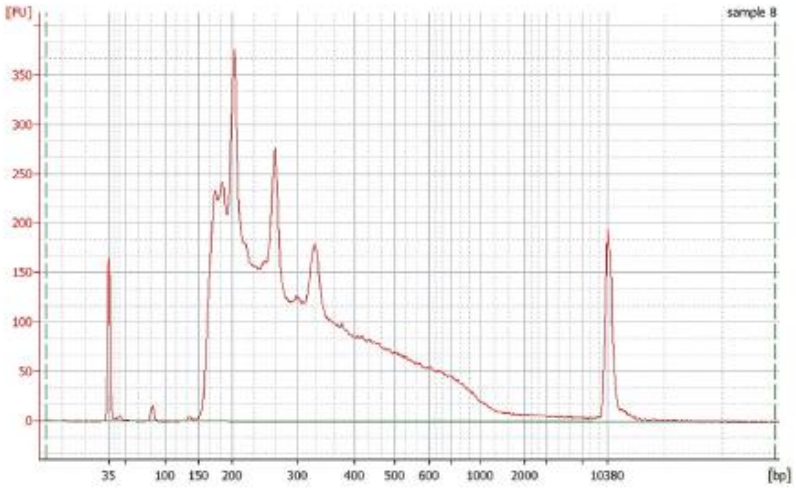
10. Air dry the beads on the magnet for 10 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
11. Remove the tubes from the magnet.
12. Add 20 µL DR1 to the dried beads. Mix thoroughly to ensure all beads are resuspended.
13. Transfer the tubes to the magnet and let stand for 5 minutes to completely clear the solution of beads.
14. Carefully remove 18 µL of the eluate, ensuring as few beads as possible are carried over and transfer to a fresh set of PCR tubes.

Optional stopping point: Store samples at –20 °C.

L. Quantitative and Qualitative Assessment of the Library

1. Measure library concentration using 2 µL of each library with the Qubit® Fluorometer and dsDNA HS Assay Kit (Life Technologies). Dilute an aliquot to 5 ng/µL using DR1 buffer and load 1 µL on the Bioanalyzer High Sensitivity DNA Chip. Fragment distribution should be as shown in **Figure 9**. The three peaks at 200 bp, 265 bp, and 330 bp are due to MspI-containing micro-satellite repeats, and are characteristic of RRBS libraries made from human DNA.
2. Quantify the library using a qPCR-based method. Use 250 bp as the library size for calculations.

Figure 9. Fragment distribution when 1 µL of 5 ng/µL library is loaded into a High Sensitivity DNA Chip from 100 ng human DNA.



3. Normalize and pool libraries following the Illumina guidelines "Best practices for manually normalizing library concentrations" and the "Low-Diversity Sequencing" guidelines for your specific sequencer. See Appendix A. of this guide for guidelines on color balancing and multiplexing of Tecan libraries.