GENE-SWitCH

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

Protocol WP1
PIG and CHICKEN WGBS Library Preparation and NovaSeq Sequencing

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Table of contents

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 WGBS libraries and sequencing, general overview:

Construction of libraries and sequencing on the Illumina NovaSeq 6000 was performed at the Novogene (UK) Company Limited, 25 Cambridge Science Park, Milton Road Cambridge CB4 0FW United Kingdom. Genomic DNA spiked with lambda DNA was fragmented by sonication to 200-400 bp with Covaris S220 (Covaris, Inc., Woburn, MA, USA), followed by end repair and A-ligation. Cytosine-methylated barcoded adapter was then ligated to sonicated DNA for different samples. The DNA bisulfite conversion was performed using the EZ DNA Methylation Gold Kit (Zymo Research, Irvine, USA). Then DNA fragments were size selected and amplified using the KAPA HiFi HotStart ReadyMix (2X) (Kapa Biosystems, Wilmington, USA). The library concentration was quantified using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, USA) and qPCR (iCycler, BioRadLaboratories, Hercules, USA), and the insert size was checked. Library was then sequenced on NovaSeq 6000 S4 flow cell with PE150 strategy.

2.1.2 General overview of the flow chart for WGBS library construction and sequencing

A. Library Construction and Sequencing

From samples' DNA to final data, each step(including sample testing, library construction and sequencing) would influence the data's quality. The quality of data would have direct impacts on the subsequent analysis results. Therefore, obtaining of high quality of sequencing data is necessary to ensure the reliability of the data.In order to keep accuracy and reliability of the data, quality control was performed on each step of the procedure.

1 Samples DNA Testing

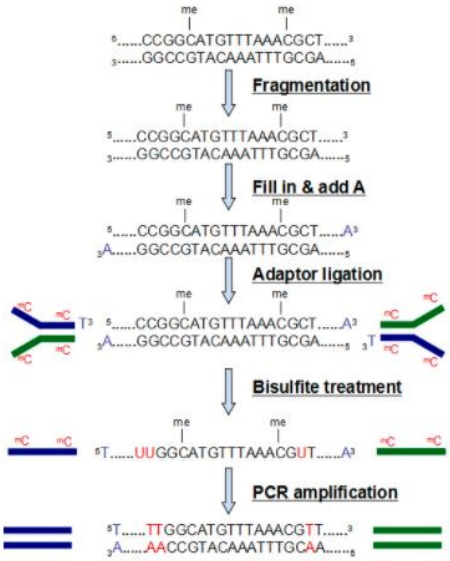
There are three methods to test samples' DNA:

(1) Agarose Gel Electrophoresis: to test DNA degradation and potential RNA contamination

(2) Qubit: to quantify DNA concentration

2 Library Construction

After samples DNA testing, positive control DNAs were added into the DNAs, and then they were fragmented into 200-400bp using Covaris S220. Next, teriminal repairing, A-ligation, methylation sequencing adapters ligation were performed to the DNA fragments. And the final DNA library was ready after Bisulfite treatment (EZ DNA Methylation Gold Kit, Zymo Research. After BS treatment, unmethylated Cytosine will change into Uracil, while methylated Cytosine will stay unchanged), size selection and PCR amplification steps.



3 Library Testing

Library concentration was firstly quantified by Qubit2.0, and then was diluted to 1ng/ul before checking insert size on Agilent 2100 and was quantified with more accuracy by Q-PCR(effective concentration of library >2nM).

4 Sequencing

After passing Library testing, different libraries would be pooled together and then fed into Illumina devices according to effective concentration and expected data volume. The sequencing strategy was paired-end sequencing.

2.1.3 Bisulfite conversion (EZ DNA Methylation-Gold™ Kit) protocol

Specifications:

- **DNA Input:** Samples containing 500 pg - 2 µg of DNA. For optimal results, the amount of input DNA should be from 200 to 500 ng.
- **Conversion Efficiency:** > 99% of non-methylated C residues are converted to U; > 99% protection of methylated cytosines.
- **DNA Recovery:** > 75%

Reagent Preparation:

- **Preparation of CT Conversion Reagent**

  The **CT Conversion Reagent** supplied within this kit is a solid mixture and must be prepared prior to first use. Prepare as follows:

  1. Add 900 µl water, 300 µl of **M-Dilution Buffer**, and 50 µl **M-Dissolving Buffer** to a tube of **CT Conversion Reagent**.
  2. Mix at room temperature with frequent vortexing or shaking for 10 minutes.

  Note: It is normal to see trace amounts of undissolved reagent in the **CT Conversion Reagent**. Each tube of **CT Conversion Reagent** is designed for 10 separate DNA treatments.

  Storage: The **CT Conversion Reagent** is light sensitive, so minimize its exposure to light. For best results, the **CT Conversion Reagent** should be used immediately following preparation. If not used immediately, the **CT Conversion Reagent** solution can be stored overnight at room temperature, one week at 4°C, or up to one month at -20°C. Stored **CT Conversion Reagent** solution must be warmed to 37°C, then vortexed prior to use.

- **Preparation of M-Wash Buffer**

  Add 24 ml of 100% ethanol to the 6 ml **M-Wash Buffer** concentrate (D5005) or 96 ml of 100% ethanol to the 24 ml **M-Wash Buffer** concentrate (D5006) before use.

Protocol:

*For DNA volumes >20 µl, an adjustment needs to be made during the preparation of the **CT Conversion Reagent**. The amount of water is decreased 100 µl for each 10 µl increase in DNA sample volume. For example, for a 40 µl DNA sample, 700 µl of water is added to make the **CT Conversion Reagent**. The volume of **CT Conversion Reagent** added to the sample must also be decreased by the same volume as the sample is increased, total reaction volume remains 150 µl. The maximum DNA sample volume to be used for each conversion reaction is 50 µl. Do not adjust the volumes of either the **M-Dissolving Buffer** or **M-Dilution Buffer**.

The capacity of the collection tube with the column inserted is 800 µl. Empty the collection tube whenever necessary to prevent contamination of the column contents by the flow-through.

1. Add 130 µl of the **CT Conversion Reagent** to 20 µl* of your DNA sample in a PCR tube. If the volume of the DNA sample is less than 20 µl, make up the difference with water. Mix the sample by flicking the tube or pipetting the sample up and down, then centrifuge the liquid to the bottom of the tube.

2. Place the sample tube in a thermal cycler and perform the following steps:

    1. 98°C for 10 minutes
    2. 64°C for 2.5 hours
    3. 4°C storage for up to 20 hours

    Note: The 4°C storage step is *optional*. For some samples, alternative parameters may yield improved results (see Appendix). If you have been using this kit with good results using different reaction conditions than described above, you can continue using those same conditions.

3. Add 600 µl of **M-Binding Buffer** to a **Zymo-Spin™ IC Column** and place the column into a provided **Collection Tube**.

4. Load the sample (from Step 2) into the **Zymo-Spin™ IC Column** containing the **M-Binding Buffer**. Close the cap and mix by inverting the column several times.

5. Centrifuge at full speed (≥10,000 x *g*) for 30 seconds. Discard the flow-through.

6. Add 100 µl of **M-Wash Buffer** to the column. Centrifuge at full speed for 30 seconds.

7. Add 200 µl of **M-Desulphonation Buffer** to the column and let stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at full speed for 30 seconds.

8. Add 200 µl of **M-Wash Buffer** to the column. Centrifuge at full speed for 30 seconds. Add another 200 µl of **M-Wash Buffer** and centrifuge for an additional 30 seconds.

Alternatively, water or TE (pH ≥ 6.0) can be used for elution if required for your experiments.

9. Place the column into a 1.5 ml microcentrifuge tube. Add 10 µl of **M-Elution Buffer** directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA.

    The DNA is ready for immediate analysis or can be stored at or below -20°C for later use. For long term storage, store at or below -70°C. We recommend using 1-4 µl of eluted DNA for each PCR, however, up to 10 µl can be used if necessary. The elution volume can be > 10 µl depending on the requirements of your experiments, but small elution volumes will yield more concentrated DNA.

2.1.4 Library protocol (KAPA Library Amplification Kit)

KAPA Library Amplification Kit

Illumina® Platforms — Technical Data Sheet

Library Amplification Protocol

1. Library Amplification

Library Amplification Primer Mix (10X) (KK2623), sold separately, is designed to eliminate or delay primer depletion during library amplification reactions performed with KAPA HiFi HotStart ReadyMix (2X). The primer mix is suitable for the amplification of all Illumina libraries flanked by the P5 and P7 flow cell sequences. Primers are supplied at a 10X concentration of 20 µM each, and have been formulated as previously described. User-supplied primer mixes may be used in combination with incomplete or custom adapters. For guidelines on the formulation of user-supplied library amplification primers, please contact Technical Support at kapabiosystems.com/support.

1.1 Assemble each library amplification reaction as follows:

Component	Volume
KAPA HiFi HotStart ReadyMix (2X)	25 µL
Library Amplification Primer Mix (10X)*	5 µL
Adapter-ligated library DNA	20 µL
Total volume:	50 µL

*Or another suitable 10X library amplification primer mix. The recommended final concentration of each primer in the library amplification reaction is 0.5 – 2 µM.

1.2 Mix thouroughly and centrifuge briefly.

1.3 Amplify using the following cycling protocol:

Step	Temp.	Duration	Cycles
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	Minimum required for optimal amplification
Annealing*	60°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	1 min	1
HOLD	4°C	∞	1

*Optimization of the annealing temperature may be required for non-standard (i.e., other than Illumina TruSeq®) adapter/primer combinations.

†The optimal cycling number will depend upon the volume and concentration of adapter-ligated, size separated, purified library DNA added to each enrichment PCR reaction.

1.4 Proceed directly to Post-amplification Cleanup (step 2).

2. Post-amplification Cleanup

2.1 In the library amplification plate/tube(s), perform a 1X bead-based cleanup by combining the following:

Component	Volume
Library amplification reaction product	50 µL
KAPA Pure Beads	50 µL
Total volume:	100 µL

2.2 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

2.3 Incubate the plate/tube(s) at room temperature for 5 – 15 min to bind DNA to the beads.

2.4 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

2.5 Carefully remove and discard the supernatant.

2.6 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

2.7 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

2.8 Carefully remove and discard the ethanol.

2.9 Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.

2.10 Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

2.11 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

2.12 Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated. *Caution: over-drying the beads may result in reduced yield.*

2.13 Remove the plate/tube(s) from the magnet.

2.14 Thoroughly resuspend the beads in an appropriate volume of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) or PCR-grade water. *Always use PCR-grade water if proceeding to target capture.*

2.15 Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

2.16 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

2.17 Transfer the clear supernatant to a new plate/ tube(s). Store purified, amplified libraries at 4°C for 1 – 2 weeks, or at -20°C.