GENE-SWitCH

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

Protocol WP1 T1.6
Promoter Capture Hi-C

Authors: Marine Beinat and Hervé Acloque (INRAE)

Workpackage: WP1

Version: 1.0

Protocol associated with Deliverable(s):	D1.4
Submission date to FAANG:	25/03/2021, month M21

Research and Innovation Action, SFS-30-2018-2019-2020 Agri-Aqua Labs
Duration of the project: 01 July 2019 – 30 June 2023, 48 months

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 817998

Table of contents

1 Summary

Promoter Capture Hi-C allows identifying by Next Generation Sequencing all the genomic regions in interactions with a defined set of promoters. It is indeed a key approach to identify enhancer/promoter interactions and allow to add spatial information on genome annotations.

The aim is to provide sequencing raw data of capture Hi-C libraries produced from muscle and liver of chicken and pigs samples at three developmental stages. For each species (pig and chicken), each developmental stage (early and late organogenesis, newborn) and each tissue (liver and muscle), 2 biological replicates (1 male and 1 female) were processed. It represents a total of 24 libraries.

Hi-C libraries are usually performed on single cell suspension. Single cell suspensions were performed by INRAE (https://data.faang.org/api/fire_api/samples/INRA_SOP_GENESWITCH_Capture HiC_Cell_Dissociation_20210331.pdf) for each sample by enzymatic dissociation of primary tissues. Unfixed cell pellets were then snapfrozen in liquid nitrogen and stored at -80°C

To perform Promoter Capture Hi-C, we used the ARIMA Hi-C+ kit for its strong reproducibility and efficiency, coupled with the SureSelect XT HS kit from Agilent using a custom design of probes for pig promoters.

We designed the probes necessary for the Promoter Capture Hi-C in close collaboration with Arima Genomics and Agilent technical and bioinformatic supports. We designed a total of 25994 probes, covering a total of 14017 different promoters and an average coverage of 3 probes per promoter.

2 Protocol description

2.1 Instruments, kits and solutions required

2.1.1 Instruments

- Covaris fragmentation system
- Qubit fluorometer
- QuantStudio real-time PCR system (Applied Biosystem) (or any other device for quantification by qPCR)
- BioAnalyzeur (Agilent Technologies)
- Thermomixer

2.1.2 Kits and reagents

- Hi-C+ kit (ARIMA Geomics)
- Qubit dsDNA HS and BR assay kit
- AMPure XP purification beads (Beckman Coulter)

- KAPA library amplification kit +primers (Roche)
- KAPA hyper prep PCR-free (Roche)
- TruSeq DNA single indexes i7 (Illumina)
- Library quantification kit (Illumina)
- Dynabeads MyOne Streptavidin T1 (Invitrogen)
- SureSelect XT HS reagent kit, target enrichment baits (custom design) (Agilent Technologies)

  Design is on the SureSelect website (https://earray.chem.agilent.com/suredesign/)

  Design ID: 3225281 (available under request to Hervé Acloque: herve.acloque@inrae.fr)

  Design contains 45218 probes covering 14017 promoters

2.1.3 Solutions

- PBS 1X
- Ethanol
- 2% formaldehyde solution (prepare fresh, keep at RT)
- 1M Glycine

2.2 Procedure

2.2.1 In-situ crosslinking

Preparation: cool down centrifuge for Eppendorf tubes and 15mL Falcon tubes

1. Resuspend the isolated nucleus pellet in 5 mL PBS 1X
2. Transfer each batch of cells to a 15 mL Falcon tube
3. Add 5 mL of freshly made 2% formaldehyde solution (1% final)
4. Crosslink cells tumbling for exactly 10 min at RT
5. Take 10 µL and determine the number of cell nucleus (with Kova counting blades-slide or any other counting system)
6. Add 2,5 mL 1M Glycine to quench the formaldehyde and put immediately on ice
7. Centrifuge 5 min 500g at 4°C
8. Remove the supernatant
9. Wash 2 times with 5mL PBS 1X
10. Centrifuge 5 min, 500g at 4°C

11. Remove supernatant
12. Resuspend in the appropriate volume of PSB 1X and transfer to Eppendorf Safe lock tubes to make 1 *10^6 aliquots
13. Freeze cells in liquid N_2 and store at -80°C

2.2.2 Lysis, restriction digest and proximity ligation

14. Resuspend one reaction of 1 * 10^6 crosslinked cells in 20 µL of **Lysis Buffer** and incubate at 4°C for 15 min
15. Add 24 µL of **Conditioning Solution**, mix gently by pipetting and incubate at 62°C for 10 min in shaker.
16. Add 20 µL of **Stop Solution 2**, mix gently by pipetting and incubate at 37°C for 15 min in shaker.
17. Add 12 µL of a master mix containing the following reagents:

Reagent	vol. per reaction	extra 10%
Buffer A	7 µL	7,7 µL
Enzyme A1	1 µL	1,1 µL
Enzyme A2	4 µL	4,4 µL
TOTAL	**12 µL**	

18. Mix gently by pipetting and incubate as follow:

Temperature	Time
37°C	45 min
65°C	20 min
25°C	10 min

19. Add 16 µL of a master mix containing the following reagents:

Reagent	vol. per reaction	extra 15%
Buffer B	12 µL	13,8 µL
Enzyme B	4 µL	4,6 µL
TOTAL	**16 µL**	

20. Mix gently by pipetting and incubate at RT for 45 min in shaker
21. Add 82 µL of a master mix containing the following reagents:

Reagent	vol. per reaction	extra 15%
Buffer C	70 µL	80,5 µL

Enzyme C	12 µL	13,8 µL
TOTAL	**82 µL**	

22. Mix gently by pipetting and incubate at RT for 15 min in shaker
23. Warm **Enzyme D** at RT to prevent precipitation
24. Add 35,5 µL of a master mix containing the following reagents:

Reagent	vol. per reaction	extra 15%
Buffer D	10,5 µL	12,1 µL
Enzyme D	25 µL	28,8 µL
TOTAL	**35,5 µL**	

25. Add 20 µL of **Buffer E**, mix gently by pipetting and incubate as follow:

Temperature	Time
55 °C	30 min
68 °C	90 min
25 °C	10 min

Size selection

Warm sample and AMPure XP beads to room temperature 30 min before use.
Vortex Ampure beads well!

26. Add 100µL of AMPure XP beads, mix thoroughly, and incubate at RT for 5 min
27. Place sample against magnet for 2 min
28. Discard supernatant
29. While sample is still against magnet, add 300µL of fresh 80% ethanol
30. Incubate at RT for 1 min
31. Discard supernatant
32. While sample is still against magnet, add 300µL of fresh 80% ethanol
33. Incubate at RT for 1 min
34. Discard supernatant
35. While sample is still against magnet, incubate beads at RT for 5 min to air-dry the beads
36. Remove sample from magnet and resuspend beads thoroughly in 100µL of Elution Buffer
37. Incubate at RT for 5 min
38. Place sample against magnet for 2 min, and transfer supernatant to a new tube
39. Quantify sample using Qubit (2 µL with HS assay)
40. Transfer 75ng of sample into a new tube labelled “Arima-QC1”, and add Elution Buffer to Arima-QC1 to bring the volume to 50µL
41. Proceed to ***Arima-QC1 Quality Control*** protocol or store at -20°C
42. Store all remaining sample at -20°C until ready to proceed to library preparation

43. Add 50µL of **QC Beads** and mix thoroughly by pipetting
44. Incubate at RT for 15 min
45. Place sample against magnet until solution is clear
46. Discard supernatant, and remove sample from magnet
47. Resuspend beads in 200µL of Wash Buffer
48. Incubate at 55°C for 2 min
49. Place sample against magnet until solution is clear
50. Discard supernatant, and remove sample from magnet
51. Resuspend beads in 200µL of Wash Buffer
52. Incubate at 55°C for 2 min
53. Place sample against magnet until solution is clear
54. Discard supernatant, and remove sample from magnet
55. Resuspend beads in 100µL of Elution Buffer
56. Place sample against magnet until solution is clear
57. Discard supernatant, and remove sample from magnet
58. Resuspend beads in 7µL of Elution Buffer
    Proceed to next step with resuspended beads

    The following step involves the Qubit quantification of the **bead-bound** DNA

59. Quantify the total amount of bead-bound DNA using Qubit (2µL with HS assay)
60. Determine the Arima-QC1 value by following the Arima-HiC QC Worksheet. High quality Arima-QC1 values are expected to be **>10%.**

2.2.3 Fragmentation

Prepare Covaris for use at least 40 min before us (add MQ to fill level 15)

61. Add Elution Buffer to your sample to bring the volume to **100 µL**
62. Transfer to a Covaris millitube and shear using the following parameters:

Duty factor	15%
Peak incident	500
Cycles/burst	200
Duration	30 seconds

63. Transfer sample to fresh 1.5 mL Lo-bind Eppendorf tube. If necessary, add Elution Buffer to your sample to bring the volume to **100 µL**

Size selection

Warm sample and AMPure XP beads to room temperature 30 min before use.
Vortex Ampure beads well!

64. Add 60µL of AMPure XP beads, mix thoroughly, and incubate at RT for 5 min
65. Place sample against magnet for 2 min
66. Transfer 160 µL of supernatant to a new Eppendorf tube
67. Add 40µL of AMPure XP beads, mix thoroughly, and incubate at RT for 5 min
68. Place sample against magnet for 2 min
69. Discard supernatant
70. While sample is still against magnet, add 200µL of fresh 80% ethanol
71. Incubate at RT for 1 min
72. Discard supernatant
73. While sample is still against magnet, add 200µL of fresh 80% ethanol
74. Incubate at RT for 1 min
75. Discard supernatant
76. While sample is still against magnet, incubate beads at RT for 5 min to air-dry the beads
77. Remove sample from magnet and resuspend beads thoroughly in 100µL of Elution Buffer
78. Incubate at RT for 5 min
79. Place sample against magnet, incubate until for 2 min, and transfer supernatant to a new Lo-Bind Eppendorf tube
80. Quantify sample using Qubit (2 µL with HS assay)
81. Sample can be stored at -20°C for up to 3 days.

2.2.4 Biotin enrichment

82. Ensure that the size-selected sample (0,125-2 µg) is in 100 µL. If not, add Elution Buffer to your sample to bring the volume to 100 µL
83. Add 100 µL of **Enrichment beads** and mix thoroughly by pipetting
84. Incubate at RT for 15 min
85. Place sample against magnet until solution is clear
86. Discard supernatant
87. Remove sample from magnet and resuspend beads thoroughly in 200µL of Wash Buffer at 55°C for 2 min
88. Place sample against magnet until solution is clear
89. Discard supernatant
90. Remove sample from magnet and resuspend beads thoroughly in 200µL of Wash Buffer at 55°C for 2 min
91. Place sample against magnet until solution is clear
92. Discard supernatant
93. Remove sample from magnet and resuspend beads thoroughly in 100µL of Elution Buffer
94. Place sample against magnet until solution is clear

95. Discard supernatant
96. Remove sample from magnet and resuspend beads thoroughly in 50µL of Elution Buffer

2.2.5 End-repair, dA-tailing, and Adapter ligation (KAPA Hyper Prep kit)

97. Add 10 µL of a master mix containing the following reagents to the bead-bound biotin-enriched DNA:

Reagent	vol. per reaction	extra 10%
End-Repair and A-Tailing Buffer	7 µL	7,7 µL
End-Repair and A-Tailing Enzyme	3 µL	3,3 µL
TOTAL	**10 µL**	

98. Mix thoroughly by pipetting and incubate as follow in a thermal cycler with the lid temperature set to >75°C:

Temperature	Time
20°C	30 min
65°C	30 min
4°C	∞

99. Add 1 µL of a unique Illumina TruSeq sequencing Adapter (15 µM)
100. Add 49 µL of a master mix containing the following reagents:

Reagent	vol. per reaction	extra 10%
PCR-grade water	9 µL	9,9 µL
Ligase Buffer	30 µL	33 µL
DNA Ligase	10 µL	11 µL
TOTAL	**49 µL**	

101. Mix thoroughly by pipetting and incubate as follow in a thermal cycler with the heated lid turned off

Temperature	Time
20°C	15 min
4°C	∞

102. Place sample against magnet until solution is clear
103. Discard supernatant

104. Remove sample from magnet and resuspend beads thoroughly in 150µL of Wash Buffer at 55°C for 2 min
105. Place sample against magnet until solution is clear
106. Discard supernatant
107. Remove sample from magnet and resuspend beads thoroughly in 150µL of Wash Buffer at 55°C for 2 min
108. Place sample against magnet until solution is clear
109. Discard supernatant
110. Remove sample from magnet and resuspend beads thoroughly in 100µL of Elution Buffer
111. Place sample against magnet until solution is clear
112. Discard supernatant
113. Remove sample from magnet and resuspend beads thoroughly in 22µL of Elution Buffer
114. Proceed to ***Arima-QC2 Quality Control*** protocol or store at 4°C.
115. Sample can be stored at 4°C for up to 3 days

QC2 Quality Control

Use the KAPA Library Quantification Kit to determine the Arima-QC2 values and estimate the appropriate number of PCR cycles needed to amplify the library

116. Vigorously mix each bead-bound Hi-C library and add 1 µL of it in 999 µL of water, to prepare a 1:1000 dilution

Plate layout: triplicate for each of the 6 KAPA Standards + triplicate for water + triplicate for each Hi-C library

117. Prepare this following master mix plus excess:

Reagent	vol. per reaction
qPCR Master Mix	10 µL
Illumina Primer Mix	2 µL
Water	4 µL
TOTAL	**16 µL**

118. Add 16 µL of this master mix to each well of the qPCR plate
119. Add 4 µL of each KAPA Standard, 1:1000 library or water to each well containing the 16 µL master mix
120. Run the following qPCR cycling protocol:

1x	95°C	5 min
35x	95°C	30 sec
	60°C	45 sec
Melting curve	65°C - 95°C	

121. N.B.: KAPA Master mix contain a **FAST**-SYBR. Hence we can run a **FAST** qPCR program (40 min)

122. Extract the Cq values and fill the Arima-HiC QC Worksheet to calculate the Arima-QC2 values and estimate the required number of PCR cycles for library amplification. High quality Arima-QC2 values are expected to be **>0,2%.**

2.2.6 Library amplification

123. Split the bead-bound Arima-HiC library into 4 PCR tubes containing each 5 µL
124. Add to each of the 4 PCR tube 15 µL water to bring the volume to 20 µL
125. Add 30 µL of a master mix containing the following reagents to each 20 µL of bead-bound Arima-HiC library:

Reagent	vol. per reaction	extra 10%
2X HiFi HotStart Ready Mix	25 µL	27,5 µL
10X Primer Mix	5 µL	5,5 µL
TOTAL	**30 µL**	

126. Mix thoroughly by pipetting and run the following PCR program in a thermal cycler with the lid temperature set to 105°C:

1x	98°C	45 sec
Nx	98°C	15 sec
(cf. Arima-QC2)	60°C	30 sec
	72°C	30 sec
1x	72°C	1 min
1x	10°C	∞

Size selection

Warm AMPure XP beads to room temperature 30 min before use.
Vortex Ampure beads well!

127. Place the PCR tubes against magnet until solution is clear
128. Transfer each supernatant to a fresh Lo-bind Eppendorf tube
129. Add 45µL of AMPure XP beads for each aliquot, mix thoroughly, and incubate at RT for 5 min
130. Place the tubes on a magnet for 5 min at RT
131. Discard the supernatant

132. While sample is still against magnet, add 150µL of fresh 80% ethanol
133. Incubate at RT for 1 min
134. Discard supernatant
135. While sample is still against magnet, add 150µL of fresh 80% ethanol
136. Incubate at RT for 1 min
137. Discard supernatant
138. While sample is still against magnet, incubate at RT for 5 min to air-dry the beads
139. Remove sample from magnet and resuspend beads thoroughly in 50µL total of Elution Buffer (12,5 µL each aliquot) for 5 min
140. Pool the 4 aliquots of the same sample to obtain 50µL
141. Place sample against magnet until solution is clear
142. Transfer sample to a fresh Lo-bind Eppendorf tube
143. Add 45µL of AMPure XP beads, mix thoroughly, and incubate at RT for 5 min
144. Place the tubes against magnet for 5 min at RT
145. Discard the supernatant
146. While sample is still against magnet, add 150µL of fresh 80% ethanol
147. Incubate at RT for 1 min
148. Discard supernatant
149. While sample is still against magnet, add 150µL of fresh 80% ethanol
150. Incubate at RT for 1 min
151. Discard supernatant
152. While sample is still against magnet, incubate at RT for 5 min to air-dry the beads
153. Remove sample from magnet and resuspend beads thoroughly in 25µL of Elution Buffer
154. Incubate at RT for 5 min
155. Place sample against magnet until solution is clear
156. Transfer sample to a fresh Lo-bind Eppendorf tube
157. Determine concentration of the sample with Qubit (2 µL with BR assay)

Sample can be stored at -20°C prior to NextSeq sequencing (2x 50 bp PE)

Optional: Assess the quality by doing a low-depth PE sequencing (1 Million reads)

2.2.7 Hybridization and capture

158. Place 1 µg of each library in a total volume of 12 µL in PCR tubes
159. Add 5 µL of **SureSelect XT HS and XT Low Input Blocker mix**
160. Mix thoroughly by vortexing, spin and run the following PCR program in a thermal cycler with the lid temperature ON (105°C) :

1x	95°C	5 min
1x	65°C	10 min
HOLD		

1x	65°C	1 min
60x	65°C	1 sec
	37°C	3 min
1x	65°C	hold

161. During the program, prepare a 25% SureSelect RNase Block solution :

Reagent	vol. per reaction	extra 10%
SureSelect RNase Block	0.5 µL	0.55 µL
RNase-free water	1.5 µL	1.65 µL
TOTAL	**2 µL**	

162. Prepare the amount required plus excess. Mix well and keep on ice
163. Prepare at RT the Capture Library hybridization mix for **Capture libraries >3Mb**:

Reagent		vol. per reaction	extra 10%
25%SureSelect RNase Block solution	2 µL	2.2 µL	
Capture library > 3 Mb		5 µL	5.5 µL
SureSelect Fast Hybridization Buffer		6 µL	6.6 µL
TOTAL		**13 µL**	

164. Once the thermal cycler starts the ***HOLD*** step, open the thermal cycler
165. While keeping the samples in the cycler, transfer 13 µL of the RT Capture library Hybridization mix from step 153 to each sample
166. Mix well by pipetting up and down slowly 10 times
167. Seal the wells with fresh strip caps.
168. Vortex briefly then spin and immediately return the tubes to the thermal cycler
169. Press the ***OK*** button to resume the thermal cycler program to allow hybridization of the prepared DNA samples to the Capture Library.

During the hybridization step, prepare the Dynabeads MyOne Streptavidin **T1** :

170. Vigorously mix the beads and add 50 µL of the resuspended Dynabeads MyOne Streptavidin **T1** to an Eppendorf tube
171. Wash the beads by adding 200 µL of SureSelect Binding Buffer
172. Mix by pipetting up and down 20 times
173. Put the tube against magnet until solution is clear then remove supernatant
174. Repeat steps 170 to 172 two more time for a total of **3 washes**
175. Resuspend the beads in 200 µL of SureSelect Binding Buffer

176. After the hybridization step is completed and the thermal cycler is at 65°C hold step, transfer the samples to RT
177. Immediately transfer the entire volume (30 µL) to the tubes containing 200 µL of washed Streptavidin **T1** beads
178. Pipette up and down 5-8 times
179. Incubate the samples at RT 30 min in a thermomixer at 1800 rpm
180. During the 30 min incubation time, prewarm 6x 200 µL of SureSelect Wash Buffer 2 for each DNA sample and incubate at 70°C in a thermomixer
181. After incubation, put the samples against magnet until solution is clear
182. Remove the supernatant
183. Resuspend the beads in 200 µL of SureSelect Wash Buffer 1
184. Mix by pipetting up and down 20 times
185. Put the samples against magnet until solution is clear
186. Remove supernatant
187. Resuspend beads in 200 µL of prewarmed Wash Buffer 2.
188. Pipette up and down 20 times then vortex briefly
189. Incubate the samples for 5 min at 70°C in a thermomixer
190. Put the samples against magnet for 1 min until solution is clear
191. Remove supernatant
192. Repeat steps 186 to 190 5 more times for a total of **6 washes**
193. After the last wash, be sure that all the Wash Buffer has been removed
194. Resuspend the beads in 25 µL of nuclease-free water. Keep the samples on ice

195. Add 25 µL of a PCR mix containing the following reagents to each 25 µL of bead-bound target-enriched DNA:

Reagent		vol. per reaction	extra 10%
5X Herculase II Reaction Buffer	10 µL	11 µL	
Herculase II Fusion DNA Polymerase		1 µL	1.1 µL
100 mM dNTP Mix		0.5 µL	0.55 µL
SureSelect Post-Capture Primer Mix		1 µL	1.1 µL
Nuclease-free water		12.5 µL	13.75 µL
TOTAL		**25 µL**	

196. Mix thoroughly by pipetting up and down and run the following PCR program in a thermal cycler with the lid temperature ON. Put the samples into the thermocycler only when the lid as reached the temperature.

1x	98°C	2 min
11x	98°C	30 sec
	60°C	30 sec

(lib. 3-5 Mb)	72°C	1 min
	72°C	5 min
1x	4°C	hold

Size selection

Warm AMPure XP beads to room temperature 30 min before use.
Vortex Ampure beads well!

197. Put the samples against magnet 2 min until solution is clear
198. Transfer each supernatant (50 µL) to a fresh Lo-bind Eppendorf tube
199. Add 50µL of AMPure XP beads, mix thoroughly, and incubate at RT for 5 min
200. Place the tubes against magnet for 5 min at RT
201. Discard the supernatant
202. While sample is still against magnet, add 200µL of fresh 80% ethanol
203. Incubate at RT for 1 min
204. Discard supernatant
205. Repeat steps 201 to 203 2 more times for a total of **3 washes**
206. While sample is still against magnet, incubate at RT for 5 min to air-dry the beads
207. Remove sample from magnet and resuspend beads thoroughly in 25µL of nuclease-free water for 5 min
208. Place sample against magnet until solution is clear
209. Transfer each supernatant (25 µL) to a fresh Lo-bind Eppendorf tube

Assess DNA library quantity and quality with Bioanalyzer