Standard operating procedure for Chromatin isolation by RNA precipitation sequencing (ChIRP-seq) of lncRNA in bovine cells and tissue

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1 Overview

ChIRP-seq of MDBK and MAC-T cells and bovine liver is performed essentially according to the procedure developed by Chu and colleagues (2011). Briefly, the chromatin of the MDBK and MAC-T cells and bovine liver tissue nuclei is extracted, cross-linked and sonicated. Biotinylated anti-sense tiling oligonucleotides (biotinylated) are designed specific to the selected lncRNA and hybridised to the target chromatin regions. The resulting biotin-labelled complexes consisting of lncRNAs, RNA-binding proteins and genomic DNA (RNA interactome) are captured with streptavidin magnetic beads. After separating the interacting DNA from the complexes, the eluted DNA is used for DNA library preparation and subjected to DNA sequencing. Bioinformatic analysis of ChIRP-seq data is performed to identify chromatin interaction sites targeted by the lncRNA. Based on the original methological description of the ChIRP procedure, a commercial analysis kit for application on cell samples is available from Merck-Millipore (Magna ChIRP RNA Interactome Kit, #17-10494). Some modifications have to be developed for the application to liver tissue samples, which are indicated accordingly in the detailed method description section below.

1.1. Design of the ChIRP probes

Before starting with ChIRP-probe (anti-sense tiling oligonucleotides) design, validation of the lncRNA locus model according to the annotated sequence is performed based on the RNA-seq data complemented by PCR amplification and resequencing as described above. For ChIRP probe design, the ChIRP probe designer (https://www.biosearchtech.com/support/tools/design-software/chirp-probe-designer, LGC Biosearch Technologies) is used. The following parameters for probe design are set: length: 20 nt, GC content: 40-50 %, distance between two adjacent probes: about 80 bp. Regions of the targeted lncRNA containing repetitive motifs are identified and masked using the RepeatMasker program, https://www.repeatmasker.org/). These regions and those of previously known or further experimentally verified exon-intron boundaries (splice junctions) of the lncRNA should be excluded from ChIRP probe design. Finally, ChIRP probes are designed for the target lncRNA and are synthesised with biotin TEG labelling (HPLC-purified, Sigma-Aldrich).

1.2. Cell culture

MDBK (Madin-Darby bovine kidney, ATCC- CCL-22) cells are cultured in EMEM (Minimum Eagle's Essential Medium, Merck) containing 10 % FCS (foetal calf serum), 2 % L-glutamine, 1 % NEAA (non-essential amino acids) and 1 % penicillin-streptomycin). MAC-T (bovine mammary alveolar epithelial) cells [31] are cultured in complete DMEM (Dulbecco's Modified Eagle's Media, Merck) supplemented with 10 % FCS, 2 % L-glutamine, 0.1 mM L-methionine, 0.4 mM L-Lysine, 0.01 mM sodium pyruvate, 1 µg/ml prolactin, 1 µg/ml hydrocortisone, 1 µg/ml insulin, and 1 % penicillin-streptomycin. After reaching 80-90 % confluence, the cells are trypsinized. About 10 million cells (1×10^7) are pelleted (1000 rpm, 5 min, 15 °C), washed in PBS and used for one ChIRP experiment each.

1.3. Liver tissue samples

Fresh liver tissue is collected from a slaughter house. Aliquots of 200 mg fresh liver tissue are minced and crushed using a sterile scalpel, mixed thoroughly with 5 ml of ice-cold PBS supplemented with Protease inhibitor cocktail III (PCI III, Merck-Millipore) and homogenized using a pre-cooled Dounce homogenizer (loose pestle, 10 strokes) on ice. The tissue suspension is

diluted with 15 ml of ice-cold PBS supplemented with PIC III (PBS-PIC III, Merck-Millipore) and centrifuged for 5 min with 800 x g at 4°C. The pellet is subjected to ChIRP procedure.

1.4 Chromatin crosslinking

Chromatin crosslinking of cell and tissue samples is performed according to the ChIRP procedure (Chu *et al.* 2011) and the manufacturer's instructions (Magna ChIRP RNA Interactome Kit, Merck-Millipore). Whereas cell pellets are resuspended in 10 ml 1% glutaraldehyde-PBS, liver pellet samples are resuspended in douple volume of the solution followed by cross-linking with incubation at room temperature for 5 min for cell samples and 8 min for liver samples. Quenching is performed as suggested with 1 mL of 10X glycine for cell samples and 2 ml are used for liver samples. After pelleting the suspensions (2,000 x g centrifugation at 4°C for 5 min), the supernatants are aspirated and the pellets are resuspended in 20 ml ice-cold PBS-PIC III followed by an additional centrifugation step. The cell samples are treated according to the manufacturer's protocol (Magna ChIRP RNA Interactome Kit, Merck-Millipore). For liver chromatin samples, an additional washing step with 20 ml ice-cold PBS-PIC III is performed and after centrifugation and removing of the supernatant, the pellet is resuspended (wide bore tips) in 5 ml ice-cold PBS-PIC III. In contrast to cell samples, the liver chromatin suspensions are homogenized again using a pre-cooled Dounce homogeniser (loose pestle A) on ice (10 strokes). After centrifugating the homogenates (2,000 x g at 4°C for 5 min) and removing the supernatants, the washed pellets of fixed chromatin are resuspended in 2 ml ice-cold PBS-PIC III. The suspension is divided into 1mL aliquots and transferred to a 2 ml tube, followed by a final centrifugation step (2,000 x g for 3 min at 4°C) and aspiration of the supernatant.

1.5 Sonication

For one ChIRP experiment three cross-linked chromatin pelletsper biological matrix (cell lines, tissue) are required. The sonication is preceded by a resuspension of the samples in 1 ml swelling buffer (0.1 M TrisCl pH7.0, 10 mM potassium acetate, 15 mM magnesium acetate, 0.1 % IGEPAL CO-630 (Sigma Aldrich), 5 µl PIC III (Merck Millipore), 50 U Superase-In RNase Inhibitor (Thermo Fisher), 1 mM PMSF (Sigma Aldrich) incubated on ice, using 10 min for cell samples and 20 min for liver samples to allow swelling and lysis of the cells while keeping the cell nuclei intact. Subsequent sonication is carried out using the S220 Focused Ultrasonicator (Covaris). Processing of cell samples after centrifugation (1,700 x g for 5 min at 4°C) and aspiration of the supernatants is performed according to the Magna ChIRP RNA Interactome Kit (Merck-Millipore). Each cell pellet sample (100 mg) is resuspended in 1 ml complete lysis buffer (1ml Lysis buffer completed with 5 µl PIC III, 50 U Superase-In RNase Inhibitor using a Dounce homogeniser (loose pestle A, 5 strokes) and incubated for 10 min on ice. The sonication procedure is performed using 1 ml AFA Fiber tubes (milliTube-1ml, Covaris). The sonication conditions for MDBK and MAC-T cells, which obtained the desired fragment size range of 100 to 600 bp after previous time course experiments are listed in Table 1 (MDBK, MAC-T cells). After sonication the chromatin samples are centrifuged at 16,200 x g at 4°C for 10 min. the supernatants are transferred into a 2 ml tube and the volume of the each supernatant is made up to 1 ml with complete lysis buffer, shock frozen and stored in liquid nitrogen at -80°C.

The sonication protocol is specifically adapted for liver samples. The liver chromatin suspension samples incubated in swelling buffer are subjected to a two-stage sonication protocol. First, each 1 ml sample is divided into two 500 µl aliquots, each of which is transferred into a 500 µl

sonication micro tube (microTube-500 AFA Fiber Cap, Covaris). The samples are subjected to the first sonication step with the performance conditions given in Table 1 (Liver step I). Sonicated samples are transferred into a fresh 2 ml tube each, the sonication micro tube is rinsed with 500 µl swelling buffer and both 500 µl volumes are pooled and centrifuged for 5 min at 2,000 x g (4°C). After removal of the supernatant, each pellet sample is resuspended in 1 ml complete lysis buffer as described for cell samples (according to the Magna ChIRP RNA Interactome Kit, Merck-Millipore). After incubation of the suspensions for 15 min on ice, the second sonication step is performed using 1 ml AFA Fiber tubes (milliTube-1ml, Covaris). The sonication conditions that produced the desired fragment size ranging from 100 to 600 bp are summarised in the Table below (Liver step II). After sonication the chromatin sample is processed as has been described for cell samples.

	Liver step I (Swelling buffer)	**Liver step II (Complete lysis buffer)**	**MDBK and MAC-T cells (Complete lysis buffer)**
Target Size (bp)		150 - 600 bp	150 - 600 bp
Maximum power (PIP)	140	120	140
Duty Factor (%)	10	2	5
Cycles per burst (CPB)	300	200	200
Treatment Time (min)	5	50	25
Temperature (°C)	4°C	4°C	4°C
Sample Volume (ml)	0.5	1	1

Table 1 Sonication conditions for chromatin crosslinking

1.6 Hybridisation

Prior to hybridization the target lncRNA anti-sense tiling oligonucleotides consecutively numbered in order of position along the target lncRNA sequence, are mixed equimolar into two separate pools with only odd (probe pool 1) and only even numbers (probe pool 2), respectively, for a final concentration of 50 µM. For each experiment with MDBK, MAC-T cells or liver, each of the three 1 mL of cross-linked, sonicated cell lysates is diluted with 2 mL complete hybridisation buffer according to the Magna ChIRP RNA Interactome Kit (Merck-Millipore). Two of the lysate replicates are mixed with a pool of biotin-labelled target lncRNA ChIRP probes (2 µL, probe pool 1 or 2). Both target lncRNA ChIRP probe pools thus serve as internal controls for each other during the parallel hybridisation of the sample replicates. The third sonicated lysate replicate is mixed with ChIRP probes specific for LacZ (LacZ probe pool) serving as a negative control (Magna ChIRP negative control probe set, Merck-Millipore). Hybridisation at 37 °C is carried out for 4 h for MDBK and MAC-T cells and overnight for liver samples, respectively. Complexes consisting of the target lncRNA, RNA-binding proteins and genomic DNA (target lncRNA interactome in chromatin regions) that hybridise to biotin-labelled ChIRP probes were captured with streptavidin-coated magnetic Beads according to the Magna ChIRP RNA Interactome Kit (Merck-Millipore) and the lncRNA-bound chromatin (DNA) is eluted from the beads using a combination of RNase A and H. After separating the lncRNA-interacting DNA from these complexes, the eluted DNA is used for DNA library preparation and subsequent DNA sequencing.

1.7 DNA sequencing

Sequencing is carried out on the eluted DNA samples after hybridisation with the two lncRNA-specific probe pools (odd and even) and the LacZ control pool, as well as the DNA of the respective input samples isolated from the chromatin extracts before hybridisation (as a reference genome for the evaluation of the ChIRP signals). For the preparation of corresponding DNA libraries, the RNA-associated chromatin DNA, extracted by ChIRP, the Qiaseq Ultralow Input Library Kit (Qiagen) is used. DNA sequencing is performed as a 2x 100 bp paired-end approach on the HighSeq 2500 platform (Illumina).