Chromosome Conformation Capture (3C) Assay

3C assay was performed in HC11 cells based on protocol described in Vakoc et al. (2005 Mol Cell 17: 453-462) with some modifications.

1. Cells (3x10^7) were dissolved in 45 ml of fresh culture media

2. 1.35 ml of 37% Formaldehyde (1% final) was added to cells with pipetting up and down few times and incubated 10 min at room temperature

3. 6.25ml of 1M Glycine was added to stop the reaction with incubation at room temperature for 5 min

4. Incubate on ice for 15 min

5. Cells were centrifuged at 2000 rpm at room temperature for 10 min and the pellet was re-suspended in 1ml of ice-cold lysis buffer (10mM Tris, pH 8.0, 10mM NaCl, 0.2% NP-40), supplemented with 0.1ml protease inhibitor cocktail. Incubation on ice for 15 min

6. Nuclei were released by Dounce homogenization with 20 strokes (Pestle B) on ice

7. Cells were centrifuged at 5000 rpm for 5 min at room temperature and washed with 0.5 ml of 1x restriction enzyme buffer. Then they were re-suspended in 125 ml of the same buffer

8. The nuclei suspension was divided into 5 individual tubes (each tube contained 25 ml which is approx. 6x10^6 cells), centrifuged 5 min at 5000 rpm and re-suspended in 362 ml of 1x restriction enzyme buffer each

9. 38 ml of 1% SDS was added to each tube and incubated 10 min at 65^0C

10.44 ml of 10% Triton X-100 was added to each tube to sequester SDS

11.400 U of HindIII was added per tube and incubated at 37^0C for overnight

12.86 ml of 10% SDS was added to each tube and incubated 30 min at 65^0C to inactivate the restriction enzyme

13. The reactions were diluted in 8 ml of ligation buffer (1% Triton X-100, 50 mM Tris, pH 7.5, 10 mM MgCl2, 10mM DTT, 0.1 mg/ml BSA, 1mM ATP, 4000U of T4 DNA Ligase) and incubated 4 hrs at 16^0C
14. 100 mg of Proteinase K was added to each reaction and overnight incubation at 65°C was performed to reverse the crosslinks.

15. Reactions from 5 individual tubes were extracted with phenol:chloroform:isoamylalcohol (25:24:1, v/v) twice, combined together and ethanol precipitated.

16. To remove extra salt, DNA was washed five times with 70% ethanol.

17. Pellet was dissolved in TE buffer (pH 8.0) and treated with 0.5 mg of DNase-free RNase A for 15 min at 37°C.

18. 3C templates were stored at -20°C.

We used BAC RP23-457P20 to generate a PCR template that consists of all possible ligation products in the region under investigation in equimolar amounts. This template was used to normalize for differences in primer efficiency between the used primer pairs. BAC DNA (20 mg) was digested with HindIII and DNA was made as described in (Ausubel et al., 2006, Current protocols in molecular biology).

Analysis of the ligation products was made by qPCR. The linear range of amplification was determined for the experimental 3C templates and control 3C template by making serial dilutions (1:10, 1:50, 1:100, 1:250, 1:500, 1:1000 and 1:2000).