

Chromosome Conformation Capture (3C) Protocol set up for *Drosophila melanogaster* embryonic tissue culture line *Schneider*.

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Generation of crosslinked template

Cells:

I used *Drosophila* Schneider S2 cells grown in serum free insect culture medium (HyQ SFX, Hyclone)+1% penicillium-streptomycin, or *Drosophila* Schneider S3 cells grown in *Drosophila* medium (Gibco) supplemented with 12.5% fetal bovine serum (FBS) and 1% penicillium-streptomycin.

Crosslinking:

The following steps are performed at room temperature.

- Spin down $\sim 4 \times 10^7$ cells (1600 rpm, 10 min) and resuspend in 45 ml of fresh medium.
- As a control, one tube can be treated without crosslinking.
- Add different amounts of formaldehyde solution (from 0.1 to 2%, I usually use a final concentration of 1%, but optimal concentrations may depend on cell lines and medium composition).
- Mix by pipetting up and down; incubate 10 minutes at room temperature, shake every now and then.
- Add 2.5 ml of 2.5 M Glycine. Mix by pipetting up and down; incubate 5 minutes at room temperature. Glycine will quench the formaldehyde and thus stop the crosslinking.

Store on ice for at least 15 min.

- Spin down (10 min, 2000 rpm), resuspend in 1 ml ice-cold lysis buffer, consisting of 10 mM Tris, pH 8, 10 mM NaCl, 0.2% Igepal (NP-40) and protease inhibitors (2 μ g/ml leupeptin, 2 μ g/ml aprotinin and 2 μ g/ml pepstatin). Incubate on ice for \sim 15 min.
- Lyse cells on ice using a dounce homogenizer with pestle A.
- Spin down (5 min, 5000 rpm), wash with 0.5 ml 1 times appropriate Restriction Buffer (the non-crosslinked sample will not dissolve very well). Spin down again and resuspend in 0.5 ml 1 times appropriate Restriction Buffer, 0,01% TRITON X-100.

Digestion:

- Use $\sim 4 \times 10^6$ cells per tube (50 μ l of 0.5 ml solution), spin down and resuspend pellet in 362 μ l 1 times appropriate Restriction Buffer. Add 38 μ l 1% SDS, and incubate for 15 minutes at 37°C.
- Add 44 μ l 10% Triton X-100, 0,01% TRITON X-100. Mix by pipetting up and down, preventing the formation of bubbles.
- Add 20 μ l restriction enzyme. I usually use 400 U HindIII/tube. Mix and incubate for 1.5 hrs at 37 °C.
- Add 86 μ l 10% SDS and incubate for 30 minutes at 65 °C in order to inactivate the restriction enzyme.

Ligation:

- Transfer reactions to 15 ml plastic tubes.
- Add 745 μ l 10% Triton X-100, 745 μ l 10 times Ligation Buffer, 80 μ l 10 mg/ml BSA, 80 μ l 100 mM ATP, 5800 μ l dH₂O and 10 μ l T4 DNA Ligase (4000 U). Incubate for 2 hours at 16 °C.
- Stop the reaction by adding 160 μ l 0.5 M EDTA pH 8.0 (10 mM final concentration).

Purification of DNA:

- Add 25 μ l 20 mg/ml Proteinase K (in TE). Incubate 5h at 50°C and O/N at 65 °C. During this incubation the crosslinks are reversed and most of the proteins are degraded.
- Add 10 μ l of 10 mg/ml RNase A per tube and incubate 30' at 37°C.
- Transfer solutions to sterile polypropylene tubes and then extract once with 4 ml Phenol-Chloroform 1:1 (vortex and spin 10 min at 10000 rpm).
- Transfer supernatant to fresh tubes.
- Precipitate DNA by addition of 0.8 ml 3M NaAc pH 5.2 and 20 ml Ethanol. Incubate at -80 °C for 20 minutes.
- Spin tubes 20 minutes at 10000 rpm.
- Dissolve DNA in 50 μ l TE.
- Store DNA at -20 °C.

Generation of control template

To prepare a control template we used BACs spanning the region of interest. The DNA from each BAC was quantified by real time PCR using primers that anneal in the backbone of the BAC. I used equimolar amount of each BAC.

- Isolate BAC DNA from bacterial strain.
- Digest in 4 reactions each containing 10 μ g genomic DNA in 400 μ l (using the same restriction enzyme as used for the nuclei). Incubate for 2 hours at 37 °C.

- Extract each reaction once with 400 µl Phenol-Chloroform 1:1.
- Precipitate by addition of 40 µl 3 M NaAc pH 5.2 and 1 ml Ethanol.
- Dissolve DNA in 20 µl dH₂O per tube.
- Add to each tube: 3 µl 10 times Ligation Buffer, 3 µl 1 mg/ml BSA, 3 µl 10 mM ATP and 2 µl T4 DNA ligase (800 U). Incubate for 1 hour at 16 °C.
- Pool the 4 reactions. Stop the reaction by addition of 3 µl 0.5 M EDTA pH 8.0.
- Extract once with 100 µl Phenol-Chloroform 1:1.
- Precipitate by addition of 10 µl 3 M NaAc pH 5.2 and 300 µl Ethanol.
- Dissolve in 100 µl dH₂O.

Analysis of ligation products by PCR

Templates:

- Control Template. This template is required to correct for differences in primer efficiencies
- Experimental Templates. This is the DNA obtained with 3C (with and without crosslinking).

PCR:

- PCR primers should be designed to give ~150-200 bp PCR products, and all ideally should have a similar T_m.
- I use 200-500 ng of the Cross-linked Template and 300-400 ng of the Control Template per PCR reaction. This may vary, dependent on the efficiency of digestion and ligation. PCR is performed in a 50 µl volume, in buffer containing 10 mM Tris-Cl pH 8.4, 70 mM KCl, 2.25 mM MgCl₂, 0.5 mM dNTPs and 0.4 mM of each primer.
- The following PCR program gives quantitative results:
35 times: 1 minute 95 °C, 45 seconds 65 °C, 2 minutes 72 °C. Followed by 1 minute 95 °C, 45 seconds 65 °C, 8 minutes 72 °C. Test varying amounts of each template to find the concentration that gives quantitative PCR product.
- PCR products are analyzed on 1.5% agarose gels and subsequently stained with an 0.75 mg/ml Ethidium bromide solution. A loading buffer without bromo-phenol blue is recommended, since this dye will run close to the PCR products and will interfere with the quantification. PCR products are quantified using a BioRad phospho-imager, using Quantity One software. Note: any gel imaging equipment with digital camera can be used.
- Using the Control Template, different primer combinations should yield comparable amounts of PCR product. Using the Non-crosslinked Template, no or only very low amounts of PCR products should be obtained. Using the Crosslinked Template, a variable amounts of PCR products should be obtained, depending on the nuclear proximity of the two restriction fragments involved. The ratio of the amount of PCR product obtained with the Crosslinked Template and the amount of product obtained with the Control Template is a measure for the interaction frequency of the two restriction fragments in the cell. In general interaction frequencies decrease with increasing

genomic site separation of the two restriction fragments. The sequences that are more represented (overlapping region of two contiguous BAC) should be taken in consideration in the final calculation of the value of crosslinking frequencies.

Further readings:

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