Chromosome Conformation Capture (3C) (PROT05)

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Introduction

An alternative protocol (PROT31) for 3C has been adopted by the author's lab. Much of it is identical to this version. The major differences are in the amounts of DNA used at different steps. We reliably get the same results as with this version, but also a greater yield of 3C material. Yields from this version are ample for real-time PCR analysis, but greater yields are required if the 3C material is going to be processed further.

The 3C (Chromosome Conformation Capture) technique generates a population average measurement of juxtaposition frequency between any two genomic loci, thus providing information on their relative proximity in the nucleus (Dekker *et al*., 2002). Cells are fixed with formaldehyde which forms DNA-protein and protein-protein cross-links between regions of the genome in proximity (see figure 1). Subsequent restriction enzyme digestion and intra-molecular ligation produces novel junctions between restriction fragments in proximity in the nucleus. Novel ligation products can be detected by PCR. We adapted the 3C assay (Dekker *et al*., 2002) to determine the conformation of mouse chromosome 7 and in particular the co-localization of actively transcribed genes in transcription factories (Osborne *et al*., 2004). The 3C assay can also be used to reveal proximity between active genes and distal genomic elements (Tolhuis *et al*., 2002).

An important consideration in the interpretation of 3C data is the understanding that not all pairs of restriction fragments that provide a positive result (i.e. generate a novel PCR product) are necessarily engaged in a functional interaction in the nucleus. For example, compare results of Tolhuis *et al*., 2002, with Carter *et al*., 2002 in which the higher order structure of the mouse hbb locus was assayed by two different methods, 3C and RNA TRAP. Clearly, distal fragments can be cross-linked by formaldehyde simply because they are near each other in the nucleus, and presumably can "bump into" each other during the fixation process (Osborne *et al*., 2004). Therefore fixation conditions are critical in the 3C assay since increased fixation leads to greater cross-linking resulting in the detection of chromatin fragments that may be in proximity in the nucleus but not necessarily engaged in a specific intermolecular interaction with implied function (see comment 1).

Procedure

Fixation

Cells are fixed in 2% formaldehyde to generate DNA-protein and protein-protein cross-links. This results in the cross-linking of physically close chromatin segments throughout the genome.

1. Take $4x10^7$ cells and make up to 50ml with DMEM (supplemented with 10% foetal calf serum) (see note 1);
2. Add 2.7ml of 37% formaldehyde (final conc. 2%);
3. Fix for 5 minutes at room temp with mixing (see note 2);
4. Quench with 3ml of 2M glycine (0.125M final);
5. Spin down for 15 minutes at 3500rpm.

Lysis

The cells are lysed to isolate nuclei.

1. Resuspend cells in 50ml of lysis buffer
   with freshly added protease inhibitors. Add a small magnetic stirrer and incubate on ice for 90 minutes on a stirrer plate to ensure good stirrage;
2. Remove the magnetic flea, spin down for 15 minutes at 2500rpm.

Digestion

The DNA is completely digested with a restriction enzyme. The restriction enzyme used depends on the region of interest and the resolution required. It is important to check the ability of the chosen enzyme to digest cross-linked chromatin. I have used Bgl II.

1. Resuspend the nuclei in 2ml of 1X NEB buffer 3 + 0.3% SDS. Incubate at 37°C for 1 hour with shaking;
2. Add Triton-X to a final concentration of 1.8% (180µl of 20% Triton-X) to sequester the SDS. Incubate 1 hour at 37°C with shaking;
3. Count the nuclei using a haemocytometer;
4. Use a $1x10^6$ nuclei aliquot (~15µg) for digestion. Digest overnight at 37°C with shaking (see note 3).

Ligation

The DNA is extensively diluted to favour intra-molecular ligations i.e. only restriction fragments that are cross-linked together will be ligated.

1. To inactivate the restriction enzyme, add SDS to a final volume of 1.6% and heat to 65°C for 20 minutes;
2. Take a 2µg aliquot of chromatin, make up to 800µl with ligation buffer (final conc. 2.5ng/µl);
3. Add Triton-X to a final conc. of 1% (40µl of 20% triton). Incubate for 1 hour at 37°C;
4. Lower temperature to 16°C and add desired amount of T4 DNA ligase. Incubate for 4 hours (see note 4).

DNA Purification

Cross-links are reversed and the DNA is purified prior to PCR analysis.

1. Add proteinase K (100µg/ml final) and incubate at 65°C overnight. Prolonged incubation at high temperature reverses formaldehyde cross-links;
2. Treat with RNase A (0.5µg/ml final) for 30 minutes at 37°C;
3. Phenol extract and ethanol precipitate the DNA.

PCR amplification

Ligation products are detected by PCR. Nested primers can be used with two rounds of PCR to increase sensitivity (see comment 2). Primers should be tested on a DNA sample that contains equimolar amounts of all expected or possible ligation products (see comment 3). For example BAC DNA encompassing the genomic region to be assessed should be digested and randomly ligated *in vitro*
to generate every possible combination of ligation products. This DNA can then be used to test the efficiencies of various primer pairs. Where the genomic region(s) to be analyzed are too distal to be contained within a single large DNA clone, multiple clones can be used. Alternatively, the sequences around the individual restriction sites to be analyzed (for example genomic regions encompassing Bgl II sites) can be PCR'd up and then mixed in equimolar amounts followed by restriction digestion and ligation to provide a DNA sample with all possible combinations of ligation products.

1. Quantify DNA using Picogreen dsDNA Quantitation Kit (Molecular Probes).
2. Use 300ng DNA in two rounds of PCR with nested primers. PCR primers should be designed to give rather small PCR fragments (less than 500bp) to maintain efficient amplification (see comment 4).

Materials & Reagents

lysis buffer	10mM Tris.HCl pH 8.0 10mM NaCl 0.2% NP-40 Add protease inhibitors fresh before each lysis: 0.1mM PMSF, 1:500 protease inhibitor cocktail (Sigma).
ligation buffer	30mM Tris.HCl pH 8.0 10mM $MgCl_2$ 10mM DTT 1mM ATP

Authors Notes

1. Work quickly to ensure that nuclear structure is preserved before fixation.
2. Fixation can be extended to 10 or 20 minutes to increase the range of detection.
3. Good digestion is required, 600 units of high conc. Bgl II (in a total volume of 70µl) digests more than 80% of "inactive" or closed chromatin and a similar amount of active chromatin areas. The extent of digestion should be verified, either by Southern blotting or quantitative PCR across selected restriction sites. Do not digest for more than 24 hours as DNA-protein cross-links can be reversed with prolonged incubation at 37°C.
4. I have used 30 weiss units (2000 cohesive end units) of T4 DNA ligase in 800 µl of buffer (final conc. = 0.0375U/µl). The concentration of chromatin in the ligation is important (2.5ng/µl) to ensure that intra-molecular ligations are favoured).

Reviewer Comments

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1. In our hands, interactions between loci separated by up to 100-150kb can usually be detected. These interactions are in most cases not functional: they reflect the highly flexible and random organization of the chromatin fiber. In general the frequency of these non-functional interactions is inversely related to the genomic distance that separates the two loci and can, to some extend, be described by a random walk model (Dekker *et al.*, 2002). It is very important to emphasize that detection of an interaction by 3C is therefore by itself not sufficient to conclude that the two loci are engaged in a functional interaction. On the other hand, failure to detect these non-functional interactions can mean that the 3C experiment did not perform optimally, or PCR detection was not sensitive enough.

   Functional interactions between elements are evident when their interaction frequency is higher than you would expect. Expected interaction frequency can be inferred by analysis of interactions with a number of adjacent fragments. Functional interactions will stand out as local peaks in interaction frequency. This approach has been used to identify several functional interactions, e.g. by Tolhuis *et al.*, 2002, and Vakoc *et al.*, 2005, for the beta-globin locus, by Spilianakis and Flavell, 2004 for the T helper type 2 cytokine locus and by Dekker *et al.*, 2002, for yeast chromosomes.
2. For accurate analysis of 3C experiments, one needs to carefully quantify the frequency with which specific ligation products are formed. Nested PCR has been used successfully by Osborne *et al.*, 2004, to convincingly detect very long-range interactions. However, in most cases nested PCR is not necessary, and a single round of PCR (with up to 35 cycles) is usually sufficient. In our hands, using fewer cycles for PCR detection results in quantitatively more reliable results.
3. To normalize for differences in primer efficiency, interaction frequencies are calculated by dividing the amount of PCR product obtained with the 3C DNA by the amount of PCR product obtained with the control DNA.
4. DNA concentration must be titrated to ensure that PCR detection is quantitative.

Figures

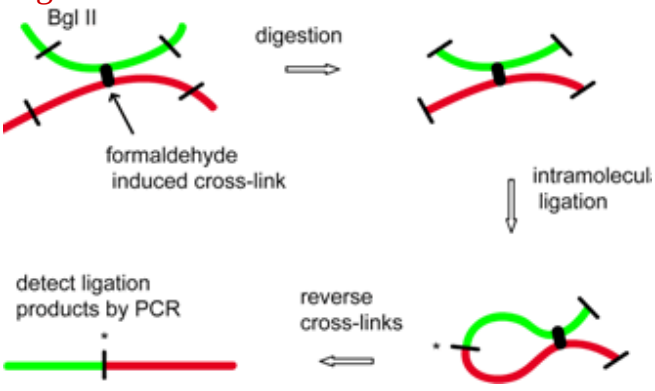

Figure 1.
An overview of the 3C technique. Fixation with formaldehyde is followed by BglII digestion and intra-molecular ligation. Cross-links are reversed and novel ligation products are detected by PCR (adapted from Figure 1A. Dekker *et al.*, 2002).

References

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