Quantitative Chromosome Conformation Capture (3C-qPCR)

Vuthy Ea[1], Thierry Forné[1] and Franck Court[1,2]

[1] Institut de Génétique Moléculaire, UMR5535 CNRS University of Montpellier, France
[2] Present address: Inserm UMR1103, CNRS UMR6293, F-63001 Clermont-Ferrand, France and Clermont Université, Université d'Auvergne, Laboratoire GReD, BP 10448, F-63000 Clermont-Ferrand, France.

Email feedback to: franck.court1@udamail.fr

Key words: 3C-qPCR, chromosome conformation capture

Introduction

Here we describe a modified 3C-qPCR protocol with four-fold increased efficiency of the 3C assay compared to the initially published protocols (Ref.1-4). Classical SybrGreen technique can then be used, instead of TaqMan probes, to quantify by quantitative PCR relevant 3C chimerical ligation products. It is optimized for nuclei preparation as starting material, coming from fresh tissues or cultured cells. In our hands, it was successfully used on human and mouse samples.

Procedure

1. The 3C library

Step 1 Crosslinking reaction (Figure 1A)

1. Defrost nuclei on ice (frozen nuclei can be stored at -80°C in a glycerol buffer for several months).

2. Carefully resuspend 5.10^6 nuclei into a tube containing 700 µl of 3C buffer and leave 5 min at Room Temperature (RT).

3. Add 19.7 µl of formaldehyde (final conc. 1%) and leave precisely 10 min at RT, but mix by inverting the tube several times during this incubation.

4. Block the crosslinking reaction by adding 80 µl of 1.25 M Glycin (125 mM final), mix by inverting the tube several times and leave 2 min at RT. Stop the reaction by putting the tube on ice during 5 min.

5. Centrifuge 3min/5000rpm/RT, remove Supernatent (SN) and carefully resuspend the pellet with 1 ml of 3C buffer (to remove Glycin and formaldehyde).

6. Centrifuge 3min/5000rpm/RT, remove SN and carefully resuspend the pellet with 100 µl of 3C buffer.

Step 2 Permeabilisation and digestion (Figure 1A)

7. Add 1 µl SDS 20% (final conc. 0.2%), mix with the pipet and incubate at 37°C for 35 min while shaking at 200 r.p.m. (horizontal shaker) then 35 min more at 37 °C and 120 r.p.m.

8. Add 16.8 µl of 10 % TritonX-100 (diluted in 1X ligase buffer) (final conc.1.2%), mix softly by pipetting up and down and incubate at 37°C for 35 min while shaking at 200 r.p.m. (horizontal shaker) then 35 min more at 37 °C and 120 r.p.m.

9. Take a 10 µl aliquot by pipetting on the wall of the tube (do not mix before). Store this sample at -20°c until point 19 of step 4 below (this sample corresponds to undigested DNA Figure 1A).

10. DNA digestion will be done by adding serially 150 units (U) of restriction enzyme for a total of 450 U. For example with *Hind*III: add gently 3 µl of *Hind*III HC (50U/µl), incubate at 37°C for 2h shaking at 120 rpm, then add gently for a second time 3 µl of *Hind*III and incubate at 37°C for 2h shaking at 200 rpm, then add again 3 µl of *Hind*III for an overnight digestion at 37°C shaking at 200 rpm.

11. Take a 4.5 µl aliquot by pipetting on the wall of the tube (do not mix before). Store this sample at -20°c until point 19 of step 4 below (this sample corresponds to digested DNA Figure 1A).

Step 3 Restriction enzyme inactivation and ligation of chimerical products (Figure 1A)

12. Add 12 µl of 20% (v/v) SDS and mix with the pipet and incubate 30 min at 37°C shaking at 120 rpm.

13. Transfer the reaction mixture into a 12ml tube (Greiner). Caution: Do not rinse the wall of the tube. Triton is neutralizing the SDS by forming miscells.

14. Add 3.28 ml 1X ligase buffer.

15. Add 390 µl of 10 %(v/v) Triton X-100 diluted in 1X ligase buffer (final conc. Triton 1%) then incubate 2h at 37°C shaking at 200 rpm.

16. Centrifuge 1min/2200g/4°C.

17. Put the sample on ice then take off 3.27 ml of SN such as 500 µl remain in the tube.

18. On ice, add 6.5 µl of ligase HC (30U/µl) and 3 µl of ATP. Mix carefully and resuspend the pellet, then incubate overnight at 16°C (after incubation this sample corresponds to 3C library, Figure 1A).

Step 4 Reversal of crosslinking (Figure 1A)

19. Add 2ml of 2X PK buffer and 1.5 ml of H_2O to the 3C library. Add 500 µl of 1xPK buffer to the undigested and digested samples (aliquots from points 9 and 11 of step 2 above).

20. Add 5 µl of proteinase K (PK 20mg/ml) to the 3C library mixture and 1 µl of PK to the undigested and digested DNA.

21. Incubate all samples during 1 h at 50°C.

22. Incubate all samples 4 h at 65°C to reverse the crosslinking reaction.

Step 5 DNA purification (Figure 1A)

23. Make a phenol chloroform purification: Add 1 volume of phenol to each sample (i.e.4ml to 3C library and 500 µl to the undigested and digested samples). Mix vigorously.

24. Centrifuge 15 min/2200g/RT for the 3C library and 10 min/16100g/RT for the undigested and digested fractions.

25. Transfer the SN in a new tube (i.e. 12 ml for the 3C library and 1.5ml for the undigested and digested fraction) add 1 volume of chloroform to each sample (i.e. 4ml and 500 µl).

26. Centrifuge the 3C library during 15 min/2200g/RT and the undigested and digested fractions for 10 min/16100g/RT.

27. Transfer the SN in a new tube (i.e. a 12 ml tube for the 3C library and a 1.5ml tube for the undigested and digested fractions). Add 200 µl of 5M NaCl to the 3C library and 25 µl of 5M NaCl to the undigested and digested samples then add 1 µl of glycogen to each sample.

28. Add 2 volume of absolute ethanol to each samples (i.e. 8ml or 1ml).

29. Mix and put the samples at -20°C overnight.

30. Centrifuge the 3C library tube for 45 min/2200g/4°C and centrifuge the undigested and digested tubes for 20 min/16100g/4°C.

31. Remove the SN and wash the pellets, with 2ml of ethanol 70% for the 3C library, or with 500 µl of ethanol 70% for the undigested and digested pellets.

32. Centrifuge the 3C library tube for 15 min/2200g/4°C and centrifuge the undigested and digested tubes for 10 min/16100g/4°C.

33. Remove the SN and dry the pellets at RT.

Step 6 Secondary digestion (Figure 1A)

34. Resuspend all the pellets (i.e. 3C library, undigested and digested samples) with 500 µl of 1x restriction buffer (use commercial buffer compatible for digestion with the secondary restriction enzyme).

35. Add 5 µl of 1mg/ml RNAse A and 100 U of the secondary restriction enzyme and incubate 2h at 37°C.

36. Repeat the phenol chloroform purification follow by the ethanol precipitation with a 500 µl volume as in points 23 to 33 above (i.e. + 500 µl of phenol / centrifuge / SN + 500 µl chloroform / centrifuge / SN + 25µ NaCl + 1 µl glycogen + 1 ml absolute ethanol / Mix / ON at -20°C / centrifuge / remove SN/ + 500 µl ethanol 70% /centrifuge/ remove SN and dry at RT).

37. Resuspend the 3C library pellet with 150 µl of 10mM Tris-HCl pH 7.5 and resuspend the undigested and digested pellets into 60 µl of water.

Step 7 Concentration adjustments (Figure 1A)

38. Determine total DNA concentration of all samples by quantitative PCR. This can be done by comparing sample concentrations to a standard curve performed on a genomic DNA with a well-known concentration. This comparison is made with PCR primers that do not amplify across the restriction sites used during the 3C experiment. qPCR reactions are as follow: 1 µl of DNA, 1 µl of primer pair (5µM each),1 µl of 10X qPCR Mix (ref.5, 6) and 7 µl of H_2O. qPCR parameters on a 480 LightCycler Roche: 3 min at 95° C of denaturation followed by 45 cycles of PCR: 1 sec at 95°C, 5 sec at 60°C, 15 sec at 72° C, then a cycle for a dissociation curve: 30 sec at 45°C then increase the temperature to 95°C at a rate of 0.2°C/sec.

39. Adjust the original 3C assays (point 37) with H_2O to 25 ng/µl +/-10% and Digested and Undigested fractions to 2ng/µl. Verify this concentration by repeating the measurements as described in point 38 above. These last values will be used as a "loading control" (see below, point 2, step 5 of "Standard curves and qPCR").

2. Standard curves and qPCR

Step1 Digested DNA for dilutions of 3C libraries (Figure 1B).

1. Digest several µg of genomic DNA with the restriction enzyme 1 and 2 (if the buffer is compatible both digestion can be done at the same time, otherwise make sequential digestions).

2. Perform on the DNA a phenol chloroform purification followed by an ethanol precipitation (points 23 to 33 above, adjust volumes to that of your reaction).

3. Resuspend the DNA in 200 µl of Tris-HCl pH 7.5.

4. Determine the DNA concentration by quantitative PCR (or NanoDrop apparatus).

5. Store this digested DNA at -20°C. This DNA will be used at a concentration of 25ng/ul to dilute the chimerical products for the 3C-qPCR standard curve.

Step 2 Production of chimerical product for 3C-qPCR standard curve (Figure 1B)

6. Digest several µg of BAC DNA that cover all the locus that you will study with restriction enzyme 1 (2h at 37°C with 500 units).

7. Purify the digested BAC DNA with a phenol/chloroform extraction, and precipitate with ethanol (points 23 to 33 above, adjust volumes to that of your reaction).

8. Resuspend the digested BAC with 500 µl of 1 X ligation buffer and perform an ON ligation at 16°C with 5 µl of ligase HC (30U/µl).

9. Purify the digested/ligated BAC DNA with a phenol/chloroform extraction, and precipitate with ethanol (points 23 to 33 above, adjust volumes to that of your reaction).

10. Resuspend the digested/ligated BAC DNA with 500 µl of 1 X digestion buffer for the restriction enzyme 2 and digest during 2h at 37°C with 500 units of restriction enzyme 2.

11. Purify the double-digested/ligated BAC DNA with a phenol/chloroform, and precipitate with ethanol (points 23 to 33 above, adjust volumes to that of your reaction).

12. Resuspend the double-digested/ligated BAC DNA into 100 µl of Tris-HCl pH 7.5.

Step 3 3C-qPCR standard curve adjustments (Figure 1B).

13. To determine the dilutions of the double-digested/ligated BAC to use for the standard curve, make serial 10X dilutions from $1/10^{-2}$ up to $1/10^{-10}$ in a 25ng/µl of digested DNA (from

step 1, point 5 above) and dilute some 3C library to 25ng/µl. Perform a qPCR for several chimerical products (Use some chimerical products close to the constant primer and some others more distant).

14. For proper quantifications, the less diluted point used for the standard curve will be the dilution that is just better amplified compared to the most strongly amplified chimerical products in 3C library.

Step 4 Standard curve for digestion efficiency (Figure 1C).

15. Dilute several µg of genomic DNA in 500 µl of 1 X digestion buffer for the restriction enzyme 2 and digest with restriction enzyme 2 (2h at 37°C with 500 units).

16. Purify the digested genomic DNA with a phenol/chloroform extraction, and precipitate with ethanol (points 23 to 33 above, adjust volumes to that of your reaction).

17. Resuspend the digested genomic DNA in 200 µl of Tris-HCl pH 7.5.

18. Determine the DNA concentration by quantitative PCR (or NanoDrop apparatus).

19. Store this digested DNA at -20°C. This DNA will be used to make the standard curve for digestion efficiency measurements. This standard curve is composed of 5 points made by 5X serial dilutions starting at 20 ng/µl of total DNA.

Step 5 Quantitative PCR (Figure 1D)

20. Digestion efficiencies are determined by doing quantifications through relevant restriction sites in the undigested and digested samples. Perform real-time PCR quantifications of the "adjusted" undigested and digested samples ([C] ~ 2ng/µl from step 7 of 3C assay), use the standard curve for digestion efficiency (from step 4 of Standard curves and qPCR part) to determine the amplification efficiencies of each couple of primers (slope and intercept). The quantification has to be done at all restriction site (R) used in the 3C analysis and also for loading control primers (C).

Digestion efficiency = $[1-(qR_{Digested}/qC_{Digested})/(qR_{Undigested}/qC_{Undigested})]*100$

with qR=10^[(standard curve intercept R – CtR_{sample})/ - slope R] primer R

qC=10^[(standard curve intercept C – CtC_{sample})/ - slope C] primer C

qPCR reaction conditions are as follows (10 µl final reaction volume): 1 µl of sample, 1 µl of primer pair (5 µM each), 1 µl of qPCR mix and 7 µl of H_2O. 3C products can be quantified on a LightCycler 480 II apparatus (Roche) (10 min. at 95°C followed by 45 cycles 10 sec. at 95°C / 8 sec. at 69°C / 14 sec. at 72°C) using the Hot-Start Taq Platinium Polymerase from Life Technologies or the GoTaq G2 Hot Start from Promega and a standard 10X qPCR mix (ref.5-6).

21. Perform real-time PCR quantifications of ligation products on 1 µl (containing ~25 ng of DNA) of the "adjusted" 3C samples (from point 39 of step 7 of 3C assay), use the standard curve for chimerical product (step 3 of "Standard curves and qPCR" part) to determine the amplification efficiencies of each couple of primers (slope and intercept). The quantification has to be done for the ligation product (L) and for loading control primers (C). Normalized quantification of ligation product is obtained by doing =qL/qC

with qL=10^[(standard curve intercept L – CtL_{sample})/ - slope L] primer L

qC=10^[(standard curve intercept C – CtC_{sample})/ - slope C] primer C

A more powerful normalization process was described in Braem et al. 2008 (ref.7).

qPCR reaction conditions are as follows (10 µl final reaction volume): 2 µl of sample, 1 µl of primer pair (5 µM each), 1 µl of qPCR mix and 6 µl of H_2O. 3C products can be quantified on a LightCycler 480 II apparatus (Roche) (10 min. at 95°C followed by 45 cycles 10 sec. at 95°C / 8 sec. at 69°C / 14 sec. at 72°C) using the Hot-Start Taq Platinium Polymerase from Life Technologies or the GoTaq G2 Hot Start from Promega and a standard 10X qPCR mix (ref.5-6) where the usual 300 µM dNTP have been replaced by 1500 µM of CleanAmp 3'THF dNTP.

Author notes

Primers used in quantitative PCR are typically 21-23 mers with a Tm in the range 55-65°C with a 2°C maximum difference between primers used in one experiment. They should be designed close (50bp) to the restriction sites (enzyme 1 used for the 3C assays) but the amplicon should not contain the restriction sites for the secondary enzyme (enzyme 2). Gapdh primers can be used for loading controls (step 7 above). Their sequences (human/mouse) are as follow: forward primer: acagtccatgccatcactgcc, reverse primer: gcctgcttcaccaccttcttg.

Lists of materials and reagents

-37% (v/v) formaldehyde.
-Glycine 1 M.
-20% w/v Sodium Dodecyl Sulfate (SDS).
-Triton X-100.
-3C buffer (50 mM Tris-HCl pH 8.0; 50 mM NaCl; 10 mM $MgCl_2$; 1 mM DTT).
-High concentration restriction enzyme (40U/µl, *Fermentas*).
-T4 DNA ligase high concentration (20U/µl, *Promega*).
-10x Ligation buffer (*Fermentas*) (40 mM Tris-HCl pH 7.8; 10 mM DTT; 10 mM MgCl2; 5 mM ATP).
-ATP (*Fermentas* R0441).

-Ribonuclease A (RNase A) 1 mg/ml.
-Proteinase K (PK) 10 mg/ml.
-PK buffer (5 mM EDTA pH 8.0; 10 mM Tris-HCl pH 8.0; 0.5% SDS).
-UltraPure™ buffer saturated Phenol (pH 7.5 - 7.8).
-Chloroform.
-Ethanol absolute.
-BAC (*Invitrogen*).
-Genomic DNA (purified from relevant samples).
-CleanAmp 3'THF dNTP (*Tebu-Bio* ref. 040N-9501-10).
-SYBR® Green PCR Mix as described in Ref.5 with modifications given in Ref.6.
-Hot-Start Taq *Platinium* Polymerase (10966-34 *Life Technologies*).
-Centrifuge (for 15ml or 50ml Falcon tubes) and Microcentrifuge (for 1.5ml tubes).
-Orbital shaker.
-qPCR apparatus (LightCycler®480 *Roche*).

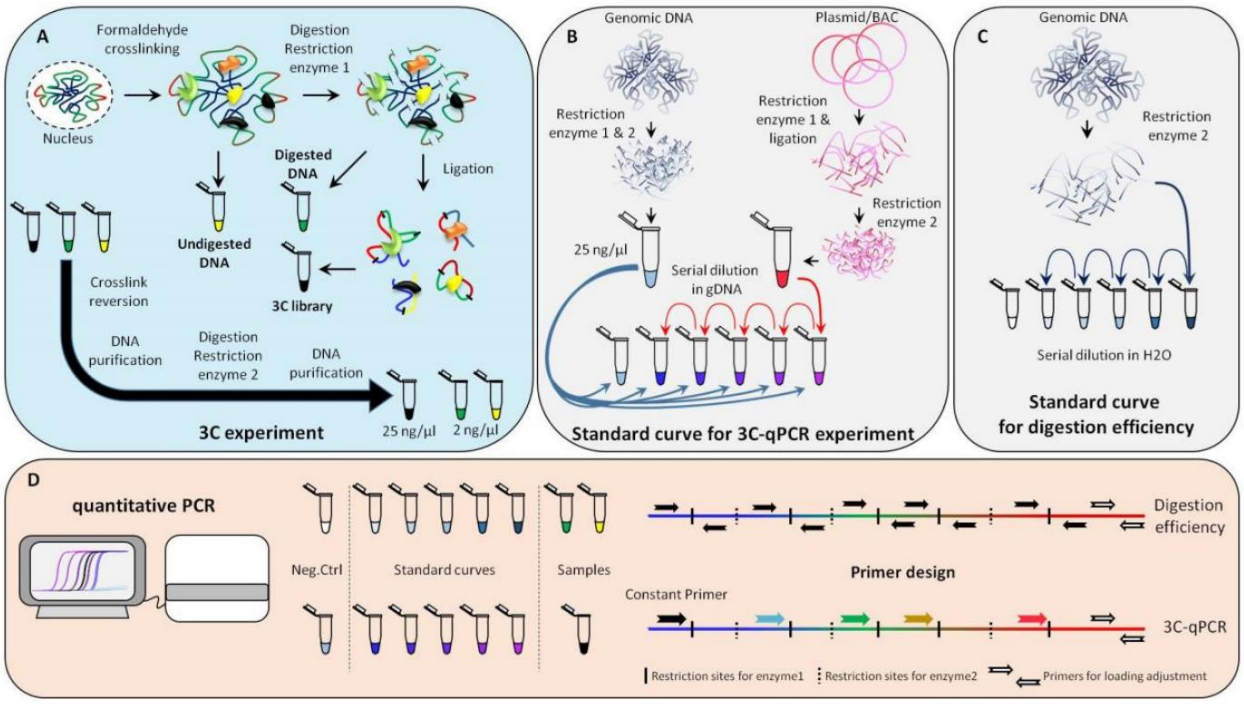

Figure 1: Overview of the 3C-qPCR protocol.

References

1- Dekker J, Rippe K, Dekker M, Kleckner N (2002) Capturing Chromosome Conformation. *Science* **295**:1306-11.

2- Tolhuis B, Palstra RJ, Splinter E, Grosveld F, de Laat W (2002) Looping and Interaction between Hypersensitive Sites in the Active β-globin Locus. *Molecular Cell* **10**:1453-1465.

3- Horton A, Sexton T and Fraser P (2005) Chromosome Conformation Capture (3C) *Epigenesys Protocols* Prot. 31.

4- Hagège H, Klous P, Braem C, Splinter E, Dekker J, Cathala G, de Laat W and Forné T (2007) Quantitative analysis of chromosome conformation capture assays (3C-qPCR). *Nature Protocols* **2**: 1722-1733.

5- Lutfalla G and Uzé G (2006) Performing quantitative reverse-transcribed polymerase chain reaction experiments. *Methods Enzymol*. **410**:386-400.

6- Court F, Miro J, Braem C, Lelay-Taha M-N, Brisebarre A, Atger F, Gostan T, Weber M, Cathala G and Forné T (2011) Modulated contact frequencies at gene-rich loci support a statistical helix model for mammalian chromatin organization. *Genome Biology* **12**:R42.

7- Braem C, Recolin B, Rancourt RC, Angiolini C, Barthès P, Branchu P, Court F, Cathala G, Ferguson-Smith AC and Forné T (2008) High-salt Recovered sequences and 3C-qPCR assays identify novel putative regulatory elements at the imprinted Dlk1/Gtl2 locus *J. Biol. Chem.* **283**:18612-18620.