Chromatin Conformation Capture (3C)

Materials:

37% formaldehyde solution (Calbiochem #344198)
Protease inhibitor cocktail (Roche Applied Sciences, Complete, #11697498001)
T4 DNA ligase, high concentration (Promega M1794, 20 Weiss units/μl; NEB M0202M, 2000 cohesive units/μl; 1 Weiss unit = 66.7 cohesive unit)
RNaseA, DNase-free (0.5 mg/ml, Roche #11119915001)
Proteinase K (20 mg/ml, Roche)
Restriction enzymes (high concentration):
EcoRI HF™ (NEB # R3101L, 20 U/μl); HindIII (Roche #10798983001, 40 U/μl)

Protocol I: Generation of 3C Template from Mammalian Cells

1. ***Cell preparation:*** Obtain single-cell preparations from mouse tissues or culture cells. Filter through 40μm cell strainer. Prepare 10~20 x 10^6 cells.

2. Formaldehyde cross-linking:

a. Per 10 x 10^6 cells, add 10 ml of ***2%*** formaldehyde/10% FCS/PBS (8.44 ml PBS + 1ml FCS + 0.54 ml 37% formaldehyde). Incubate for ***10 min*** at RT with shaking.

Note: 1% formaldehyde is also routinely used. Formaldehyde concentration may affect digestion efficiencies; if digestion efficiency is likely to be a problem, use 1% instead of 2% formaldehyde.

b. Transfer the reaction tubes to ice and add 0.5 ml of 2.5 M glycine (1/20 vol. F.C. 0.125 M) to quench the crosslinking reaction.

c. Spin for 8 min at 225g (1000 rpm) at 4°C and carefully remove all supernatant.

3. Lyse cells:

a. Wash cells twice with ice-cold PBS.

b. Resuspend cells in 5 ml cold ***Lysis Buffer (w/ or w/o NP-40***) and incubate 10 min on ice.

Note: It is important to obtain a homogenous preparation of nuclei, which may be facilitated by gently pipetting the mixture up and down. Depending on the cell type, more stringent lysis buffer may be needed to prepare nuclei.

c. Centrifuge for 5 min at 400g (1500 rpm) at 4°C and remove all supernatant. The pelleted nuclei can be frozen in liquid nitrogen and store at -80°C for several months.

Note: *The cells can also be crosslinked with Formaldehyde directly in cell culture dishes. But cells need to be stroked with D-dounce after incubation with lysis buffer to get mononulei.*

4. Digestion:

a. Resuspend nuclei in 0.5 ml of 1.2x restriction enzyme buffer and transfer to safe-cap tube.

b. Place the tube at 37°C and add 7.5 μl of 20% SDS (F.C. 0.3%).

Note: If the digestion efficiency is still low after decrease concentration of formaldehyde of crosslinking, reduce SDS to 0.1% by adding 2.5ul 20% SDS and then sequestered with 1% TritonX100 by adding 25ul of 20% Tritonx-100.

c. Incubate for 1 h at 37°C with vigorous shaking (900 rpm on temperature-controlled shaker). *Note: Nuclei form aggregates after adding SDS, but will separate again after adding TritonX-100*

d. Add 50 μl of 20% Triton X-100 (F.C. 2%) to sequester the SDS.

e. Incubate for 1 h at 37°C with vigorous shaking at 900 rpm.

Note: Save a 5 μl aliquot of the sample as undigested genomic DNA control (UND). Store at -20°C .

f. Add ***400~1,500 U*** of the selected restriction enzyme to the remaining sample and

incubate overnight at 37°C while shaking at 900 rpm.

Note: Save a 5 µl aliquot of the sample as digested genomic DNA control (D). Store at -20°C.

5. DNA ligation:

a. Add 40 µl of 20% SDS (F.C. 1.6%) to the samples from overnight digestion.
b. Incubate for 20~25 min at 65°C while shaking at 900 rpm.
c. Transfer the digested nuclei to a 50 ml falcon tube.
d. Add 6.125 ml of 1.15x ligation buffer (0.7 ml of 10xligation buffer + 5.425 ml water).
e. Add 375 µl of 20% Triton X-100 (F.C. 1%).
f. Incubate for 1 h at 37°C while shaking gently (400 rpm at a bacterial incubator).
g. Add T4 DNA ligase (Wouter de Laat et al used 100 weiss units or 6,667 cohesive units; Job Dekker et al used 4000 weiss units (?) for 2 h at 16°C). Incubate for 4~5 h at 16°C followed by 30 min at RT.
h. Add 15 µl of 20 mg/ml Protease K (300 µg final).
i. Incubate at 65 °C overnight to de-crosslink the sample.

6. DNA purification:

a. Add 30 µl of 10 mg/ml RNaseA (300 µg final).
b. Incubate for 30~45 min at 37°C.
c. Add 7 ml of phenol/chloroform and mix vigorously.
d. Centrifuge for 15 min at 2,200g at RT.
e. Transfer the supernatant in a new 15 ml tube, extract once with 7 ml chloroform.
f. Transfer the supernatant into a new 50 ml tube and add 7 ml of distilled water, 1.5 ml (1/10 vol.) of 3M NaAc pH 5.2, and 35 ml of ethanol.

Note: Increasing the volume before precipitation will dilute the DTT present in the ligation buffer and prevent it from precipitating (white pellet).

g. Mix and place on dry ice for ~1 h.
h. Centrifuge for 45 min at 2,200g at 4°C.

Note: There is big white pellet come out after this step, which will not solved a lot into 70% ethanol.

i. Remove the supernatant and add 10 ml of 70% ethanol.
j. Centrifuge for 15 min at 2,200g at 4°C.
k. Repeat 70% ethanol wash twice to remove extra salt in the template.
l. Remove the supernatant and briefly dry the pellet at RT.
m. Dissolve the DNA pellet in 150 µl of 10 mM Tris pH 7.5. The 3C template is now ready for qPCR analysis. 3C template may be kept at -20°C for several months.

Note: The concentration of 3C template from $10x10^6$ HUVEC cells is around 10ug/ul. It is not correct to directly measure DNA concentration by Nanodrop because of high concentration of salt. But it will give you a linear concentration after dilution of 3C template in 1:10 and 1:30.

Protocol II: Generation of 3C Control Template from Mammalian DNA

This protocol outlines the method to obtain a control template from mammalian genomic DNA. Due to the complexity of the mammalian genome, a control template generated from whole genomic DNA does not contain detectable levels of any specific ligation product. Thus, a control template is generated from the genomic region of interest only. In this prot°C ol, a bacterial artificial chromosome (BAC) clone or set of clones containing the genomic region of interest is purified, digested, and randomly ligated. This procedure generates a control template, ie. a collection of all possible ligation products that are present in equimolar amounts. This control template is used in combination with the 3C template to determine interaction frequencies

1. Select, amplify, and purify BAC clone(s):

a. Select one or more BAC clones covering the genomic region of interest. If more than one clone is needed, there should be minimal overlap and possibly no gaps between the clones.
b. Purify the BAC DNA from 500 ml overnight cultures by the modified alkaline lysis method. Resuspend BAC DNA in 1 ml of TE.
c. Estimate DNA concentration by running 1 µl of BAC DNA on a 0.8% agarose beside a molecular weight standard of known concentration.

Note: The concentration of the BAC DNA should be between 50 and 100 µg/ml. If more than one BAC clone is required to cover the genomic region of interest, the clones should be mixed in equimolar ratios before digestion. For this, the BAC preparations should be quantified by qPCR using universal primers that amplify part of the BAC vector backbone.

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2. Digest BAC DNA:

a. Transfer ~20 µg of BAC DNA in a 2 ml tube. If more than one BAC is required, add a total of 20 µg DNA. The total DNA volume should represent 10% of final digestion volume (ie. ~100 µl).
b. Add an appropriate amount of water, 10x restriction enzyme buffer, and BSA (if needed), 8.75% (v/v) of restriction enzyme (high concentration ie. 40U/µl). For example, per 1 ml digestion, add 87.5 µl (3,500 U) of restriction enzyme (40 U/µl).
Note: BAC DNA is often difficult to digest to completion. This protocol has been optimized and standardized for digestion efficiency.
c. Incubate overnight at 37°C .
d. Add an equal volume of phenol/chloroform, vortex, and centrifuge 5 min at 14,000 rpm.
e. Transfer aqueous phase to a fresh tube and add 1/10 vol of 3M NaAc (pH 5.2), 2.5vol of 100% ethanol.
f. Incubate on dry ice for 15 min and centrifuge at 14,000 rpm at 4°C for 20 min.
g. Discard supernatant and wash pellet with 1 ml 70% ethanol.
h. Air dry DNA pellet and resuspend in 161 µl of water. Incubate 15 min at 37°C to dissolve the DNA completely.

Note: Save 5 µl of digested DNA to run on an agarose gel later.

3. DNA ligation:

a. Combine the following and incubate overnight at 16°C in 200 µl final volume:
157 µl of digested BAC DNA
20 µl of 10x ligation buffer
2 µl of 10 mg/ml BSA
100 Weiss units T4 DNA ligase

Note: Job Dekker et al used 7600 weiss units (?)

b. Incubate DNA 15 min at 65°C to inactivate the ligase.

4. Purify BAC DNA control template:

a. Add 200 µl of phenol/chloroform, vortex and centrifuge 5 min at 14,000 rpm.
b. Transfer aqueous phase to a new tube and add 200 µl of chloroform, vortex and centrifuge 5 min at 14,000 rpm.
c. Transfer aqueous phase to a new tube and add 1/10 vol of 3M NaAc (pH 5.2), 2.5vol of 100% ethanol. Incubate on dry ice for 15 min and centrifuge 14,000 rpm at 4°C for 20 min.
d. Discard supernatant and wash once with 1 ml 70% ethanol

e. Air dry pellet and resuspend in TE buffer to final concentration ~100 ng/µl.
f. Incubate 15 min at 37°C to completely dissolve DNA. This is the 3C control template.
g. Run 1 µl of uncut BAC DNA, 4 µl of cut BAC DNA, and 1 µl of ligated BACDNA side by side on a 0.8% agarose gel.

Protocol III: Determination of digestion efficiency

Digestion efficiency should be carefully assessed for each restriction site involved in the analysis. Indeed, a two fold drop in digestion of a given site causes a two fold reduction in the available amount of a given restriction end, which could affect the number of ligation product it can form. Therefore, digestion efficiencies should be in the same range for each of sites interest (>65%).

a. Add 500ul of 1x PK buffer and 1ul of 20mg/ml PK (20ug final) to the control aliquots saved in before (UND) and after digestion (D).
b. Incubate for 30min at 65 °C (or overnight at 65 °C if performed with 3C DNA)
c. Equilibrate for a few minutes at 37 °C, then add 1ul of 1mg/ml RNaseA (1ug final) and incubate for 2h at 37 °C .
d. Add 500ul of phenol-chloroform, mix vigorously
e. Centrifuge for 20min at 16,000g at RT
f. Transfer the supernatant (300ul) into a new tube and add 30ul of 3M sodium acetate (pH5.6), then 900ul of ethanol.
g. Mix and place on dry ice until frozen (about 45min)
h. Centrifuge for 20min at 16,100xg at 4 °C.
i. Remove the supernatant and add 500ul of 70% ethanol.
j. Centrifuge for 4min at 16,000g at RT
k. Remove the supernatant and dry the pellet at RT
l. Resuspend the pellet in 60ul of water
m. Perform realtime-PCR quantification (Sybgreen) on both samples (UND and D) using regular qPCR conditions. To correct the differences in the amount of template added to the PCR, also amplify the GAPDH control region without restriction sites of interest.
n. Use the cycle thresholds to calculate the restriction efficiency according to the following formula:

% restriction= $100-100/2^{((Ct_R-Ct_c)D-(Ct_R-Ct_c)UND)}$

Note: The efficiency of the restriction enzyme digestion should be above 60-70%, but ideally >80% should be digested. Samples with lower digestion efficiencies should be discarded.

3C Solutions

Lysis Buffer (w/o NP40)

Final Conc.	*Stock Solution*	*For 10 ml*	*For 150 ml*
10 mM Tris-HCl (pH 8.0)	1M Tris-HCl (pH8.0)	0.1 ml	1.5 ml
10 mM NaCl	5 M NaCl	0.02 ml	0.3 ml
5 mM $MgCl_2$	1 M $MgCl_2$	0.05 ml	0.75 ml
0.1 mM EGTA	0.1 M EGTA	0.001 ml	0.015 ml
ddH_2O		9.83 ml	147.44 ml

Lysis Buffer (w/ NP40)

Final Conc.	*Stock Solution*	*For 10 ml*	*For 150 ml*
10 mM Tris-HCl (pH 8.0)	1M Tris-HCl (pH8.0)	0.1 ml	1.5 ml
10 mM NaCl	5 M NaCl	0.02 ml	0.3 ml
0.2% (v/v) NP-40	20%	0.1 ml	1.5 ml
ddH_2O		9.78 ml	146.7 ml

Note: Make fresh on day of experiment and add Protease Inhibitor Cocktail freshly.

Proteinase K Buffer

Final Conc.	*St°C k Solution*	*For 10ml*	*For 150 ml*
5mM EDTA (PH8.0)	500mM (PH8.0)	0.1ml	1.5ml
10mM TrisCl (PH8.0)	1M TrisCl	0.1ml	1.5ml
0.5% SDS	20% SDS	0.25ml	3.75ml
ddH2O		9.55ml	143.25ml

10xLigation Buffer

Final Conc.	*St°C k Solution*	*For 10ml*	*For 150 ml*
50mM DTT	1M DTT	0.5ml	7.5ml
500mM TrisCl (PH7.5)	1M TrisCl	5ml	75ml
50mM $MgCl_2$	1M $MgCl_2$	0.5ml	7. 5ml
10mM ATP	100mM ATP	1ml	15ml
ddH2O		3ml	45ml

References:

1. Helene H, Klous P, Braem C, Splinter E, Dekker J, Cathala G, de Laat W, and Forne T. (2007) Nature Protocol 2:1722-1733
2. Miele A, Gheldof N, Tabuchi TM, Dostie J, Dekker J. (2006) Current Protocols in Molecular Biology 21.11.1-21.11.20
3. Palstra R, Tolhuis B, Splinter E, Nijmeijer R, Grosveld F, de Laat W. (2003) Nature Genetics 35:190-194
4. Tolhuis B, Palstra R, Splinter E, Grosveld F, de Laat W. (2002) Molecular Cell 10:1453-1465