4C Protocol

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Protocol adapted from Naumova et al. 2012, van de Werken et al. 2012, and Demare et al. 2013.

Day 1: Harvest of embryonic tissues and crosslinking. (Collect ~50-100ug of chromatin)

Dissect pregnant mother and place embryos in Petri dish with PBS on ice.
Remove the tissues of interest from embryo and transfer to fresh tube and keep on ice in PBS.
Disrupt tissue with blue plastic pestle.
Add ice cold PBS to final volume of 1mL.
Add 37% formaldehyde to final of 1% (27 uL in this case) and invert quickly several times.
Place on rotator and incubate at room temp for 15 minutes.
Add 2.5 M glycine to 150 mM final (63 uL in this case) and invert quickly several times.
Place on rotator and incubate at room temp for 10 minutes.
Harvest cells by centrifugation (2000g, 5 min, 4C).
Remove supernatant and resuspend pellet in 1mL of fresh, cold PBS by flicking the tube.
Harvest cells by centrifugation as above.
Wash 2 more times with PBS and harvest by centrifugation.
Remove all buffer from pellet.
Flash freeze pellet in liquid nitrogen and store at -80C until further use.
** for cultured cells collect 10^7 cells and follow and the above crosslinking protocol

Day 2: Digest chromatin

Resuspend pellet in 6 volumes of pelleted cells (vpc) of Lysis buffer I (+protease inhibitor) by pipetting up and down (~500uL).
Transfer cells to dounce and homogenize (if necessary, i.e. very clumpy) with pestle A (5-20 strokes).
Incubate on ice for 15 to 20 minutes.
Homogenize 20 to 30 strokes with pestle B.
Transfer solution to fresh tube. Rinse dounce with fresh swelling buffer and add to tube.
Harvest nuclei by centrifugation (2500g, 5min, 4C).
Make up 5mL of 1X NEB restriction buffer.
Wash in 500 ul of 1X restriction nuclease buffer and harvest by centrifugation.
Resuspend in 500 ul 1X NEB restriction nuclease buffer and add 15uL of 10%SDS.
Shake for 10 min at 65C at 800 rpm.
Add 150uL of 10% TritonX-100
Mix for 10 mins at 37C on nutator.
Add 16.5uL 10X NEB buffer and split in to two tubes.
Save 5uL in 40uL water for undigested control
Add 200U 4-cutter restriction enzyme to each tube and place on nutator at 37 O/N.
*****Make sure this enzyme can digest intact chromatin as can be deactivated by heat*****

Day 3: Ligate and reverse crosslinking

Next morning add 150U of enzyme and place on 37C nutator for 2 hours.
Heat inactivate enzyme at 65C stationary for 30' (SDS can also be used
Save 5ul in 40uL water for digestion control

Set up ligation reactions on ice (3 tubes per sample):

745 ul 10X T4 ligase buffer (NEB)
745 ul 10% TritonX-100
8 ul 100mg/ml BSA (or 80 uL BSA 10mg/ml)
5.5 ml H20

Add 200ul heat inactivated digestion reaction to each ligation mix.
Add 1ul of Thermo T4 DNA ligase (30 Weiss U/ul) and mix gently.
Incubate at 16C for 2 hr. (O/N ok too)
Add 50ul 25mg/ml Proteinase K and incubate O/N at 65C.

For undigested and digested controls:
Add 1 ul 10mg/ml RNase A and incubate for 1 hr at 37C.
Add 10 ul 25mg/ml Proteinase K and incubate O/N at 65C.

Day 4: Purify DNA

Add additional 25ul 25 mg/ml proteinase K and incubate 2 hr at 65C
Prepare 6 MaxTract tubes by centrifuging 1-2 min at 1500g.
Add ligation reactions to MaxTract tubes and add equal volume of phenol:chloro:isoamyl alcohol, mix by inverting several times
Spin 5' at 1500g. Decant aqueous phase into 30 ml glass centrifuge tubes
Add 1/10 vol 3M sodium acetate pH 5.2 and mix by inverting (cover with parafilm)
Add 0.7 vol isopropanol, cover tubes with aluminum foil, and place in -80C > 1 hr.
Let tubes thaw ~30 at RT, then spin 14000rpm at 4C for 45'
Resuspend pellets and combine into 1ml TE total
Add 10ul 10mg/ml RNAase A and incubate at 37C for 1 hour.
Prep 2 x 2ml phase lock tubes by spinning 30 seconds at 16000g
Split sample into 2 x 500 ul and add to phase lock tube and add equal volume of phenol:chloro:isoamyl alcohol, mix by inverting several times and spin 5' at 16000g
Add equal volume of chloroform, mix by inverting, spin 5' at 16000g
Pipet off aqueous phase into fresh tube, add 1/10 vol sodium acetate and 2.5 vol cold ethanol
Mix and place at -80C for > 1 hr.
Spin 45' max speed at 4C.
Wash 5 x 1ml 70% EtOH
Resuspend pellet in TE (100ul -1 ml depending on expected yield)
Save 5uL in 40uL water for ligation control

Day 5: Secondary RE digestion

Set-up digestion reaction:

150ul 3C library
50uL 10X RE Buffer

50 Units of secondary 4-cutter restriction enzyme
295uL water

Incubate o/n at 37C

Day 6: Secondary Ligation

Heat inactivate enzyme at 65C for 25 min
Take 5ul in 40uL water to serve as secondary digestion control
Transfer sample to a 50mL falcon tube on ice and add:
1.4mL 10X T4 ligase buffer (NEB)
12.6mL Cold Water
Add 3.3ul of Thermo T4 DNA ligase (30 Weiss U/ul) and mix gently.
Incubate for >2hrs (O/N ok) at 16C

Day 7: Purify DNA

Split sample in to 2 glass tubes and add to each tube:
0.7 mL of 3M NaAc pH 5.2
7 uL glycogen
17.5 mL of EtOH

Store at -80C until completely frozen.
Let thaw at RT and spin for 45 min at 9000 rpm (8300g) at 4C.
Remove supernatant and wash with 15 mL of cold 70% EtOH.
Spin for 15 min at 3650 rpm (3300g) at 4C.
Remove supernatant and allow pellet to dry.
Dissolve pellet in 150uL of TE at 37C.

Purify with 3 qiaquick pcr purification columns and re-suspend each column in 50uL TE, pool, and quantify on nanodrop.

Day 8: PCR and DNA Size Selection

Divide 1ug of purified 4C template over 10 (100ng/rxn) concurrent individual 50ul PCR reactions:
Use previously designed 4C primers and a long-template polymerase.
Run the reaction for 28 cycles.
Pool the 10 reactions and PCR purify on a qiagen column.

Combine 75uL of purified PCR product DNA and combine with .45x ampure bead solution (33.75ul).
Vortex and allow to sit for ~5 mins.
Place tube on magnet for 2 mins.
Collect 100ul of supernatant and mix with an additional 38ul of ampure bead solution in a new tube.

Follow ampure bead protocol as normally directed:

Vortex solution and allow to sit for ~5 mins.
Place tube on magnet for 2 mins.
Remove supernatant and wash beads twice (while still on magnet) with 200uL 80% EtOH.
Air dry beads until majority of EtOH is gone, but pellet not entirely dry, ~15 mins.
Remove from magnet and elute DNA by vortexing in 50uL TE.
Place back on magnet for 2 mins and remove supernatant.
Quantify DNA concentration with nanodrop and check for size selection on an agarose gel.

This size selected product can be submitted for library prep and sequencing.

*During sequencing, phiX DNA doping may be required if all submitted samples utilize the same primer set.