Adapted 4C protocol

Date: 2023-05-25
Tags: 4C
Created by: Lara Holtes

Changes/differences to the Krijger et al. protocol are highlighted in blue

4.1 Crosslink and collect samples

Isolation buffer: 10% vol/vol FBS/PBS (prepare fresh)
Fixation buffer: 4% vol/vol formaldehyde/isolation buffer (prepare fresh)

Resuspend cells as single-cell suspension in isolation buffer at $2x10^6$ cells/ml at room temperature.
Add an equal volume of freshly prepared 4% fixation buffer (2% final conc formaldehyde) and mix by inverting.
Incubate at room temperature for 10 min on tube roller.
Immediately add cold 1 M glycine to a final concentration of 0.13 M to quench the reaction.
Immediately centrifuge for 5 min at 500g (4°C) and remove all supernatant.
Resuspend pellet in 1 ml of cold PBS and transfer to 1.5ml Safe-lock tubes and centrifuge for 5 min at 500g (4°C).
Discard supernatant and flash-freeze pellets in liquid nitrogen. Store in -80°C.

4.2 Lysis

Reagents	Initial conc	Final conc
Tris-HCl	1.5 M	50 mM
Igepal	20%	1%
TX-100	20%	1%
NaCl	1 M	150 mM
EDTA	0.5 M	5 mM
Protease inhibitor	25X	1X
Nuclease-free water		

*Pipette detergents slowly and use cut tips

Take cell pellets out to thaw on ice
Gently resuspend the pellet in 1 ml of freshly prepared cold cell lysis buffer. Incubate for 20 min on ice.
Ensure the pellet is properly broken up

Centrifuge for 5 min at 600g (4 °C).
Carefully remove the supernatant and resuspend the nuclei in 450 µl 1.2X RE1 buffer (DpnII buffer).
Centrifuge for 5 min at 600g (4 °C).
Carefully remove supernatant and resuspend the nuclei in 500 µl 1.2X RE1 buffer (DpnII buffer).
Warm up sample to 37˚C in a thermomixer and add 15 µl 10% SDS (final, 0.3%).
    Ensure that any aggregates are properly broken up. Check during the incubation periods too.
Incubate 1 h at 37 °C while shaking at 900 rpm.
Add 75 µl 20% Triton X-100 (final, 2.5%). If nuclear aggregates are present resuspend sample by gentle pipetting until most of the aggregates disappear.
Incubate 1 h at 37 °C while shaking at 900 rpm.
Take a 5 µl aliquot of the sample and store as the “undigested” control at 4 °C

4.3. First restriction enzyme digestion

Add the primary restriction enzyme (100U DpnII = 2 µl) and incubate for ~3 h at 37 °C while shaking at 900 rpm.
Add a second round of primary restriction enzyme and incubate **overnight** at 37 °C while shaking at 900 rpm.
Take a 5 µl aliquot of the sample as the digested control.
    Leave the remaining sample at 37°C if possible, otherwise at room temperature while checking the efficiency
Determine the digestion efficiency:
    Add 42.5 µl 10mM Tris-HCl pH 7.5 and 2.5 µl Prot K (20 mg/ml) to 5 µl of both the undigested and digested control.
    Incubate for 1 h at 65 °C while shaking at 500 rpm.
    Add loading dye and load 20 µl of each control alongside each other on a 0.6% (wt/vol) agarose gel.
        50 ml gel: 0.3 g agarose, 50 ml TAE buffer, 5 µl SYBR safe
        Make 20 µl ladder: 1 µl of GeneRuler 1kb Plus, 3.3 µl of 6X loading dye, 15.7 µl nuclease-free water
        Mix 10 µl of 6X loading dye with 50 µl sample (put leftover sample at 4°C for subsequent gels)
        100V for 30-40 min
If digestion is of good quality, proceed with the first ligation. Otherwise add more restriction enzyme.

4.4 First ligation

Heat inactivate enzyme as recommended in the manufacturer’s instructions. For DpnII, NlaIII and Csp6I inactivate the enzyme by incubating for 20 min at 65 °C.
Transfer the samples to a 50 ml centrifugation tube and add 700 µl 10X ligation buffer, nuclease-free water to 7 ml and 50 U Ligase (10 µl) and incubate **overnight** at 17 °C.
    Room temperature fluctuates too much, there is an incubator in the other lab.
Determine the ligation efficiency:
    Take a 42.5 µl aliquot of the sample as the “ligated control”.
    Add 2.5 µl Prot K (20 mg/ml) and incubate for 1 h at 65 °C while shaking at 500 rpm.
    Add loading dye and load 20 µl of the digested control and the ligated control alongside each

other on a 0.6% (wt/vol) agarose gel.
Mix 9 µl of 6X loading dye with 45 µl sample (put leftover sample at 4 °C)
100V for 30-40 min
A clear upwards shift in molecular weight should be visible.
If ligation is of good quality, proceed with de-crosslinking. Otherwise add fresh ATP and repeat ligation step.

4.5. Reversal of cross-links and purification

Add 15 µl Prot K (20 mg/ml) and incubate **overnight** at 65 °C.
Purify the 3C template using an ethanol precipitation.
Adjust the concentration of the sample to 0.2M NaCl (final) and incubate for 5-15 min on ice.
To 7 ml add 280 µl of 5 M NaCl
Check centrifuge is at 4°C, place absolute EtOH on ice and prepare any centrifuge balances
Add 2.5X cold absolute EtOH to the sample and ensure thorough mixing.
To 7.280 µl add 18.2 ml
Incubate for 30 min on ice
Centrifuge 4000g at 4°C for 30 min
Aspirate supernatant. Wash pellet with ~30 ml 70% EtOH
Centrifuge 4000g at 4°C for 5 min
Repeat wash step (5 & 6)
Aspirate supernatant. Airdry sample for 10 min on its side and aspirate any residual ethanol
Add 450 µl of 10mM Tris-HCl pH 7.5 buffer and resuspend by incubating at 55°C until dissolved (15-30 min)
(Optional) Quantify the amount of template using the Qubit dsDNA HS Assay Kit, following the manufacturer's instructions.

4.6 Second RE digestion

To ~450 µl of the 3C template add 50 µl 10× RE2 buffer (CutSmart buffer) and 50 units secondary RE (NlaIII).
Incubate **overnight** at 37 °C while shaking at 500 rpm.
Determine digestion efficiency:
Take a 2.5 µl aliquot of the sample as the "2nd digestion control" and add 15 µl 10mM Tris-HCl pH 7.5.
Add loading dye and load 20 µl of the 2nd digested control and the ligated control alongside each other on a 0.6% (wt/vol) agarose gel.
Mix 3.5 µl of 6X loading dye with 17.5 µl sample
100V for 30-40 min
A clear downward shift in molecular weight should be visible. The majority of the smear should be <2.5 kb.
If digestion is of good quality, proceed with the second ligation (Section 4.7). Otherwise repeat digestion.

4.7 Second ligation

Heat inactivate enzyme as recommended in the manufacturer's instructions. For DpnII, NlaIII and Csp6I inactivate the enzyme by incubating for 20 min at 65°C.
Quantify the amount of template using the Qubit dsDNA HS Assay Kit, following the manufacturer's instructions.
Transfer the samples to a 50 ml centrifugation tube and perform a ligation reaction with a final DNA concentration of 5 ng/µl. For 25 µg of template, add 500 µl 10X ligation buffer, 50 U Ligase and nuclease-free water up to 5 ml. Incubate **overnight** at 17°C.

4.8 Purification of 4C template

First, purify the 4C template with an ethanol precipitation

- Check centrifuge is at 4°C, place absolute EtOH on ice and prepare any centrifuge balances
- Adjust the concentration of the sample to 0.2M NaCl (final) and incubate for 5-15 min on ice.
- Add 2.5X cold absolute EtOH to the sample and ensure thorough mixing.
- Incubate for 30 min on ice
- Centrifuge 4000g at 4°C for 30 min
- Aspirate supernatant. Wash pellet with an appropriate volume of 70% EtOH
- Centrifuge 4000g at 4°C for 5 min
- Repeat wash step (5 & 6)
- Aspirate supernatant. Airdry sample for 10 min on its side and aspirate any residual ethanol
- Add 350 µl of 10mM Tris-HCl pH 7.5 buffer and resuspend the pellet by incubating at 55°C until dissolved (30 min - 1 hr). If the pellet is not dissolving then it is possible that the quantity of DNA is too high. Try adding progressively more buffer (up to 450 µl) and/or gentle pipette mixing.
- Transfer sample to a 1.5 ml tube (ideally a DNA LoBind tube)

Second, purify the 4C template using the AMPure XP beads

- Allow AMPure XP beads to come to room temperature and vortex prior to use.
- Add 2:1 of AMPure XP beads (e.g. 700 µl to 350 µl sample), vortex and spin down tubes briefly.
- Incubate 10 min at room temperature.
- Place sample on a magnetic separation rack and remove the supernatant when the solution becomes clear.
- Gently add ~1200 µl 80% ethanol without disturbing the beads.
- Remove the supernatant when the solution becomes clear.
- Gently add ~1200 µl 80% ethanol without disturbing the beads.
- Remove the supernatant and shortly centrifuge using a tabletop centrifuge (a few seconds at 1000g).
- Place the tube back on the magnet rack. When the beads have moved from the bottom of the tube, remove any residual ethanol.
- Air-dry sample for 30 sec/until beads go from shiny to matt
- Remove the tube from the magnetic rack. Resuspend the beads in 130 µl 10mM Tris-HCl pH 7.5.
- Pipette over the beads repeatedly to remove from the side of the tube.
- Incubate 10 min at room temperature.
- Shortly centrifuge the bead solution (a few seconds at 1000g) and place back on the magnet rack.
- When the solution is clear, transfer 125 µl of the supernatant to a new 1.5 ml tube.

Quantify the amount of template using the Qubit dsDNA HS Assay Kit, following the manufacturer's

instructions.
Continue with the inverse PCR or store samples at -20°C.

5. 4C-seq PCR

5.1 Primer test (only when testing new VP primers)

VP specific PCR primers designed according to our recommendations (Sections 3.3 and 3.4) should first be tested using a single PCR to determine their efficiency and to test the 4C template quality (see note 11). Ideally, primers with proven efficiency are included in this test as a positive control for comparison.

1. Perform the PCR using the mix and program indicated below.

PCR mix:

	Reaction mix (µl)
10X PCR buffer I	2.5
dNTP (10 mM)	0.5
Reading primer (25 µM)	0.5
Non-reading primer (25 µM)	0.5
Expand Long Template Polymerase mix	0.35
100 ng of 4C template	
Nuclease-free water	
Total	25

PCR program: 94°C for 2 min; 30X (94°C for 10 sec, [primer specific]°C for 1 min, 68°C for 3 min); 68°C for 5 min; hold 4°C (Total time = ~2 hr 30 min)

2. Run 15 µl of the PCR product on a 1.5% (wt/vol) agarose gel. If primers give a strong DNA smear (in addition to the prominent “self-ligation” and “undigested” bands (see note 12) and show minimal primer dimers continue with the sequence library preparation.

5.2. Sequencing library preparation

The first PCR step is performed to amplify the fragments ligated to the VP. We generally perform 4 PCR reactions with 200 ng 4C template per reaction.

1. Perform the first PCR step using the reaction mix and program indicated below.

	Reaction mix (µl)
10X PCR buffer I	5

dNTP (10 mM)	1
Reading primer (25 µM)	1
Non-reading primer (25 µM)	1
Expand Long Template Polymerase mix	0.7
200 ng of 4C template	
Nuclease-free water	
Total	50

Primers:

	Forward	Reverse
VP2	234140495	234140496
VP3	234140497	234140498
VP6	234140499	234140500

PCR program: 94°C for 2 min; 16X (94°C for 10 sec, 54°C for 1 min, 68°C for 3 min); 68°C for 5 min; hold 4°C (Total time = ~1 hr 30 min)

Note: 54°C is appropriate for VP2, VP3 and VP6

2. Pool all PCR reactions, mix well and transfer a 50 µl aliquot to a new 1.5 ml DNA LoBind tube. Store remaining PCR product at -20°C.

3. Perform a 0.8X AMPure XP purification on the 50 µl aliquot.

Allow AMPure XP beads to come to RT and vortex prior to use.
Add 40 µl of AMPure XP beads to the sample, vortex and spin down tubes briefly.
Incubate 5 min at room temperature.
Place sample on a magnetic separation rack and remove the supernatant when the solution becomes clear.
Gently add ~500 µl 80% ethanol without disturbing the beads.
Remove the supernatant when the solution becomes clear.
Gently add ~500 µl 80% ethanol without disturbing the beads.
Remove the supernatant and shortly centrifuge using a tabletop centrifuge (a few seconds at 1000g).
Place the tube back on the magnet rack.
When the beads have moved from the bottom of the tube, remove the residual ethanol.
Air-dry sample for 30 sec.
Remove the tube from the magnetic rack. Resuspend the beads in 50 µl 10mM Tris-HCl pH 7.5. Pipette over the beads repeatedly to remove from the side of the tube.
Incubate 10 min at room temperature.
Shortly centrifuge the bead solution (a few seconds at 1000g) and place back on the magnet rack.
When the solution is clear, transfer 45 µl of the supernatant to a new 1.5 ml tube.

A second round of PCR is performed on the purified PCR product obtained after round 1. In this second PCR, universal primers are used that contain the Illumina adapters required for flow cell binding of the amplicons, a 6-nt index sequence and the Illumina sequencing primer sequences for single and pair-end sequencing. Select indexes carefully to ensure optimum base calling and demultiplexing by having different bases at each cycle of the index read.

4. Perform the second PCR step using the reaction mix and program indicated below.

	Reaction mix (µl)
10X PCR buffer I	5
dNTP (10 mM)	1
Universal Fw primer (25 µM)	1
Rv Index primer (25 µM)	1
Expand Long Template Polymerase mix	0.7
Purified round 1 PCR	5
Nuclease-free water	36.3
Total	50

*Take note of which reverse index primer is used for each sample

PCR program: 94°C for 2 min; 20X (94°C for 10 sec, 60°C for 1 min, 68°C for 3 min); 68°C for 5 min; hold 4°C (Total time = ~1 hr 45 min)

5. Perform a 0.8X AMPure XP purification on 50 µl PCR.

Allow AMPure XP beads to come to RT and vortex prior to use.
Add 40 µl of AMPure XP beads to the sample, vortex and spin down tubes briefly.
Incubate 5 min at room temperature.
Place sample on a magnetic separation rack and remove the supernatant when the solution becomes clear.
Gently add ~500 µl 80% ethanol without disturbing the beads.
Remove the supernatant when the solution becomes clear.
Gently add ~500 µl 80% ethanol without disturbing the beads.
Remove the supernatant and shortly centrifuge using a tabletop centrifuge (a few seconds at 1000g).
Place the tube back on the magnet rack. When the beads have moved from the bottom of the tube, remove the residual ethanol.
Air-dry sample for 30 sec.
Remove the tube from the magnetic rack. Resuspend the beads in 50 µl 10mM Tris-HCl pH 7.5. Pipette over the beads repeatedly to remove from the side of the tube.
Incubate 10 min at room temperature.

Shortly centrifuge the bead solution (a few seconds at 1000g) and place back on the magnet rack. When the solution is clear, transfer 45 µl of the supernatant to a new 1.5 ml tube.

6. Determine the sample quantity using the Qubit dsDNA HS Assay Kit, following the manufacturer's instructions.

7. Bioanalyzer. Make a 1 ng/µl dilution of each 4C library to see if profile of fragments is suitable and ready to be sent for sequencing.

8. 4C-seq libraries can be stored at −20°C and can be directly sequenced using an Illumina high-throughput sequencing equipment.

Steps

- Crosslink and collect samples
- Cell lysis and digestion 1
- Check digestion and ligation 1
- Check ligation and reversal of crosslinks
- Clean-up
- Digestion 2
- Check digestion and ligation 2
- Clean-up
- PCR1 and clean-up
- PCR2 and clean-up
- Bioanalyzer
- Submit samples for sequencing

Attached file

Krijger-et-al.-2020-4C-seq-from-beginning-to-end-A-detailed-protocol.pdf
sha256: 9be6015751bc1128b4fd2bce462f2db878bcf64808c54073be612c5e221a1060

Unique eLabID: 20230525-b30d0769422ef6a518b708f95abbaa482b744829
Link: https://elabftw.science.ru.nl/experiments.php?mode=view&id=5438