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Enzymatic fragmentation of plant chromatin for Hi-C libraries V.1

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We use this protocol and it's working

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Abstract

The aim of this protocol is to learn how to prepare, evaluate and optimize a plant chromatin sample to produce a proximity ligated sample ready for NGS library preparation. We introduce several quality checks along the process to avoid wasting precious material or time working with a suboptimal sample (quality and quantity-wise).

Image Attribution

New Plymouth Coastal Walkway, Taranaki, New Zealand (Elena Hilario, 2021)

Guidelines

Every sample brings a different challenge and no chromatin protocol is universal. It is recommended to practice with a less precious sample before attempting the preparation of the final Hi-C library. There are a few commercial kits available to prepare Hi-C libraries and perform well under most circumstances, especially since often they have good technical support to help you along the way. This protocol describes an in vitro enzymatic fragmentation of plant chromatin and aims to help understand the importance of QC steps along the process.

Ideally, the chromatin fragmentation should be random, using DNase I, but in practice, this approach can take some time to optimize and if your sample size is limited you may consider using a 4-base **restriction enzyme**, selected based on the genome's GC content. Or you could also use a mix of two or more 4-base restriction enzymes to target as many sites as possible, either by having a different restriction site or a different methylation sensitivity. Although it is easier to control a restriction enzyme digestion, beware that the genome coverage is reduced, compared to a randomly fragmented chromatin sample. We strongly recommend to perform digestion tests on nuclear gDNA preps with several restriction enzymes until you find a combination that will produce an even digestion profile.

Using a biotinylated bridge to mark the contact ends adds extra steps and some assurance that the contacts detected are real, but you could also omit this step and fill in the 5'-end overhangs with biotinylated dNTPs. Beware the length of the carbon chains that links the biotin to the nucleotide (the standard is 6 carbon atoms, used in this protocol) has an impact on how well it will bind to the streptavidin molecule. The longer the carbon chain, the more efficient the binding.

If you have trouble isolating a clean nuclei pellet, you could purify it with a Percoll gradient, as described **here**. The wash volumes of the chromatin bound to magnetic beads can be increased if the sample is hard to clean, or extra washes can be implemented too. The lysis buffer volume can also be increased to reduce the amount of contaminants even before starting the chromatin washes.

Materials

~ 3 g of leaves **ground with liquid nitrogen** used immediately or previously ground and stored in a 50 mL Falcon tube at -80°C

Sorbitol Wash Buffer: 100 mM Tris-HCl pH 8.0, 0.35 M Sorbitol, 5 mM EDTA pH 8.0, 1 % (w/v) Polyvinylpyrrolidone Molecular wt. 40,000 (PVPK40). Autoclave and store at 4°C, it will last for at least 6 months. Add β-mercaptoethanol (1 % v/v) before the extraction

NEB complete buffer: 0.5 M Mannitol, 10 mM PIPES-KOH pH 6, 10 mM $MgCl_2$ $6H_2O$, 2% PVP K40, 200 mM L-lysine monohydrochloride, 6 mM EGTA. Add Triton X-100 (final concentration 0.5% v/v), sodium bisulfite (final concentration 18 mM) and β-mercaptoethanol (final concentration 0.04% v/v) just before use

NEB-βME: 0.5 M Mannitol, 10 mM PIPES-KOH pH 6, 10 mM $MgCl_2$ $6H_2O$, 2% PVP K40, 200 mM L-lysine monohydrochloride, 6 mM EGTA. Add sodium bisulfite (final concentration 18 mM) just before use

1X PBS buffer, sterile. 50 mL

10X Wash Buffer: 100 mM Tris-HCl pH 8, 1 M NaCl, sterile. Add Tween 20 to a final concentration of 0.5% after autoclaving and store at 4°C

1X Wash Buffer: Prepare at least 50 mL by diluting the 10X stock with sterile deionized water and store at 4ºC

Quenching Solution: 1X Wash Buffer

FA buffer 50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS. Prepare the buffer without the detergents, autoclave, and when cooled to room temperature, add the detergents. Store at 4°C, in the dark

1X CutSmart 1% SDS: Prepare 1 mL with sterile deionized water

100X TE pH 7.5: 1 M Tris pH 7.5, 100 mM EDTA, autoclave and keep at room temperature

80% ethanol, freshly prepared, 10 mL

Deionized sterile water (ddH_2O)

TE Buffer: Dilute 100X TE pH 7.5 1:100 with sterile deionized sterile water in a 50 mL sterile Falcon tube

10 mM Tris-HCl pH 8.0, sterile

0.5 M EDTA pH 8.0, sterile

5 M NaCl, sterile

DBBB: 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 20% PEG 8000, 2.5 M NaCl, 0.025% Tween 20. Prepare this solution from sterile stocks and store at 4ºC

1% agarose gel and 1XTAE buffer

Bridge adaptor,: Biotinylated bridge adaptor (+) (5′ /5Phos/GCTGAGGGA/iBiotin-dT/C) and Bridge adaptor (-) (5'/ 5Phos/CCTCAGCT). The biotin hapten is connected to the oligonucleotide by a 6-carbon atom chain.

- HEPES Sodium salt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H7006
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8787-50ML
- Sodium deoxycholate **Catalog #**D6750
- Tween 20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9416-50ML
- Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
- DNase I, RNase free **Thermo Fisher Scientific Catalog #**EN0525 supplied with **$MnCl_2$**
- NEB 10X CutSmart Buffer **New England Biolabs Catalog #**B7204S
- SDS, 10% Solution **Life Technologies Catalog #**AM9822
- Proteinase K (2 ml) **Qiagen Catalog #**19131 20 mg/mL
- RNase A **Qiagen Catalog #**19101 100 mg/mL
- Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q32854
- HS Genomic DNA Assay 75 - 20000 bp **Agilent Technologies Catalog #**DNF-488-0500
- HS NGS Fragment Assay 1-6000 bp 500 reactions **Agilent Technologies Catalog #**DNF-474-0500
- 1 Kb Plus DNA Ladder **Invitrogen - Thermo Fisher Catalog #**10787018
- Lambda DNA **Thermo Fisher Catalog #**SD0011
- SYBR SAFE DNA stain **Invitrogen - Thermo Fisher Catalog #**S33102
- NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #**E7546S
- NEBNext FFPE DNA Repair Mix - 24 rxns **New England Biolabs Catalog #**M6630S
- T4 DNA Ligase, LC (1 U/µL) **Thermo Fisher Catalog #**EL0016
- NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 3) **New England Biolabs Catalog #**E7710L
- NEBNext Ultra II Q5 Master Mix - 250 rxns **New England Biolabs Catalog #**M0544L
- Dynabeads™ M-270 Streptavidin **Thermo Fisher Scientific Catalog #**65305
- Polyethyleneglycol 8000 50% w/v **Jena Bioscience Catalog #**CSS-256

Equipment

Bench top centrifuge with swing bucket rotor, refrigerated
Fume hood
Disposable 1 µL inoculation loops, sterile
Ice bucket
Orbital shaker
Miracloth square (10 × 10 cm) with small funnel
50 mL Falcon tubes, sterile with rack
100 and 40 µm cell strainers
Dounce homogenizer with B pestle, 7 mL size
1.5 mL Eppendorf tubes, screw capped tubes and microcentrifuge
0.2 mL PCR tubes
Magnet for 1.5 mL tubes
0.5 mL microcentrifuge tubes compatible with Qubit fluorimeter
FA-WASTE container, β-ME-WASTE container and $MnCl_2$-WASTE container
1 mL Wide Bore tips
200 µL Wide Bore tips
Mini gel box and powerpack
Water bath
Timer

Equipment

ThermoMixer® C	NAME
Eppendorf	BRAND
Catalog No. 2231000680	SKU
https://online-shop.eppendorf.us/US-en/Temperature-Control-and-Mixing-44518/Instruments-44519/Eppendorf-ThermoMixerC-PF-19703.html	LINK

Equipment

Gel Doc XR+ Gel Documentation System	NAME
Gel Documentation System	TYPE
Bio-rad Laboratories	BRAND
1708195	SKU
https://www.bio-rad.com/en-us/product/gel-doc-xr-gel-documentation-system?ID=O494WJE8Z	LINK

Equipment

Fragment Analyzer	NAME
capillary based nucleic acid fragment size separation	TYPE
Agilent	BRAND
M5311AA	SKU
https://www.agilent.com/en/product/automated-electrophoresis/fragment-analyzer-systems/fragment-analyzer-systems/5300-fragment-analyzer-system-365721	LINK

Troubleshooting

Safety warnings

Prepare waste containers for the formaldehyde and manganese solutions labelled with the proper warning signs:

FA-WASTE container, β-ME-WASTE container and $MnCl_2$-WASTE container

Before start

Characterize your sample

The protocol is developed for plant leaf tissues that will yield at least **5 µg nuclear gDNA per gram of leaf sample**. It is crucial that you characterize your biological sample before starting. This will give you a good understanding of the expected yields along the process and make decisions if you fall below the minimum amount of DNA to safely continue to the next stage.
Adjust the amount of starting leaf tissue to obtain the desired amount of ngDNA and make sure you have at least 6 tubes of nuclei before starting because it will take at least two tries before you get the optimal digestion conditions. Each tube should contain at least 2 µg of ngDNA.

How to anneal the bridge adaptor

- Dissolve the oligos in TE pH 7.5, at 1 mM. Store at -20°C after use
- Heat up a water bath to 65°C, containing at least 3 L of water
- Prepare the following mix in a 1.5 mL screw capped tube:

	A	Volume, µL
	TE pH 7.5	82
	25 mM MgCl2	8
	1 mM Biotinylated bridge adaptor (+)	5
	1 mM Bridge adaptor (-)	5
	Total volume	**100**

- Vortex briefly and do a quick spin
- Incubate the adaptor mix at 65°C for 5 min
- Transfer ~1.5 L from the water bath to a shallow plastic container (2 L capacity) on the lab bench
- Transfer the adaptor mix tube to the container, and place a thermometer
- Let it cool down to ~ 25°C, it should take about 2.5 h, but you can leave it overnight
- When ready, do a quick spin and store at -20°C
- This solution is enough for five bridge ligation reactions

Crosslinking Solution

0.5 mL 1X PBS + 27 µL
Formaldehyde solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F8775-25ML , freshly prepared

Streptavidin magnetic bead binding buffers

The following buffers can be prepared ahead of time and stored at 4 °C for at least 6 months

	A	1X B/W + T20	1X B/W	2X PEGBB
	ddH2O	39.25 mL	39.75 mL	3 mL
	100X TE pH 7.5	250 µL	250µL	200 µL
	5 M NaCl	10 mL	10 mL	8 mL
	50% w/v PEG 8000	--	--	8 mL
	2.5% v/v Tween 20	500 µL	--	800 µL
	Total volume	**50 mL**	**50 mL**	**20 mL**

Abbreviations:

B/W + T20 = Bind and Wash plus Tween 20 buffer (0.5X TE pH 7.5, 1 M NaCl, 0.025% v/v Tween 20)
B/W = Bind and Wash Buffer (0.5X TE pH 7.5, 1 M NaCl)
PEGBB = Polyethylene glycol 8000 Binding Buffer (1X TE pH 7.5, 2 M NaCl, 20% v/v PEG 8000, 0.1% v/v Tween 20)

Nuclei isolation and integrity check

1

Safety information

Work in the fume hood

Add 20 mL sorbitol wash buffer to the ground tissue by gently dislodging it with an inoculation loop. Stir gently and drag the lump up against the tube wall until it is resuspended

2 Centrifuge 3500 rpm, 10°C, 00:05:00 . Pour off the supernatant into designated β-ME-WASTE container. The supernatant will have the consistency of light syrup and it might be light green with fine sediment

3 go to step #1 at least another 2 times. It can take up to 4 rounds of washing to obtain a supernatant that pours off like water and is clear. Place the tube on ice

4 Add 20 mL NEB complete buffer and gently resuspend the ground tissue by tapping the bottom of the tube and mixing by inversion. Add another 20 mL NEB complete buffer and mix by inversion. Place the tube horizontally over an ice bucket, and on the orbital undetermined, Room temperature , 00:10:00 Slow speed

5 Assemble the funnel over a 50 mL Falcon tube and place the Miracloth. Pre-wet the miracloth with some NEB complete buffer

6 Filtrate the extract through one sheet of Miracloth

7 Filtrate the extract through a **100 µm cell strainer** assembled over a 50 mL Falcon tube

8 Filtrate the extract through a **40 µm cell strainer** assembled over a 50 mL Falcon tube. Lift the cell strainer slightly and press the mesh against the tube inner wall to speed up the filtration

Note

The flow through the 40 µm cell strainer can be very slow. If it clogs, continue with a new 40 µm cell strainer or stir the liquid very gently with a sterile 1 µL inoculation loop

9 Collect the nuclei by centrifugation at 1800 x g, 10°C, 00:15:00

9.1 Discard the supernatant in the designated β-ME-WASTE container

10 Add 25 mL NEB –βME and resuspend the pellet gently by tapping the bottom of the tube and mixing by inversion

11 go to step #9 go to step #9.1

12 Add 1 mL NEB –βME and detach the pellet gently by tapping the bottom of the tube. Resuspend the nuclei using a 1 mL pipette set at 500 µL and wide bore tips by drawing the liquid in and out very slowly. Avoid introducing air bubbles. It is very important the nuclei suspension is **homogeneous**

Note

A Dounce homogenizer with a B pestle can be used for this task

12.1 Transfer 50 µL resuspended nuclei to a 1.5 mL tube to check the DNA integrity (see below)

13 Split the nuclei suspension in **eight** 1.5 mL screw capped tubes, spin down 1 min at maximum speed, discard the buffer and the nuclei pellet tubes at -80 °C

Note

- *To defrost a nuclei sample, place it on ice and flick the bottom of the tube gently until the pellet is resuspended*
- *If the cell density of the leaf sample is low, split the nuclei suspension in 5 or 6 tubes instead*

13.1 It is recommended to check the **DNA integrity** before preparing the library

1. Add 50 µL 1X CutSmart Buffer + 1% SDS (prewarmed @ 55ºC) to the nuclei go to step #12.1 vortex briefly
2. Add 1 µL Proteinase K and vortex 2 seconds
3. Place the tube in the thermomixer programmed as follows:
   55°C 15 min 1250 rpm → 68°C 45 min 1250 rpm
1. Spin down the tube briefly and add 100 µL AMPure XP beads mix and let stand 00:05:00 room temperature
2. Place 00:02:00 magnet or until the solution is clear. Discard the supernatant
3. While the tube is on the magnet, add 200 µL 80% ethanol over the beads and remove it 00:00:30 exactly. Repeat this step one more time
4. Quick spin the tube, place it on the magnet and remove all ethanol
5. Remove tube from magnet and add 50 µL TE buffer. Tap the bottom of the tube to resuspend the beads and let the DNA elute at least 00:10:00 room temperature
6. Place the tube on the magnet and transfer the eluted DNA to a new tube
7. Quantify 1 µL eluted DNA with the HS dsDNA Qubit kit
8. Calculate the amount of nuclear genomic DNA in 50 µL and then extrapolate to go to step #12 1 mL of resuspended nuclei
9. Calculate how much ngDNA is per tube (~118 µL if split in 8 tubes, or ~158 µL if split in 6 tubes). Ideally you will have ≥3 µg of ngDNA per tube
10. Analyze 200 ng nuclear genomic DNA in a 1% agarose gel against the 1 kb+ ladder and 200 ng Lambda DNA (48.5 kbp). The extracted DNA should be >20 kbp. If the DNA is not intact, **do not proceed** with crosslinking and DNase I test. Prepare a new nuclei prep and QC the sample again

Chromatin Crosslinking

14 Add 0.5 mL 1X PBS to one of the defrosted nuclei tubes go to step #13 . Tap gently the bottom of the tube, or use an inoculation loop to dislodge the nuclei from the tube walls. The nuclei should be fully resuspended

15 Add 0.5 mL crosslinking solution to the nuclei pellet, and mix by inversion

16 Incubate at 00:10:00 room temperature with gentle mixing using the orbital shaker by placing the tube horizontally 10m

Note

If you can't find an orbital shaker, a rotisserie oven set at room temperature or a hula mixer are good alternatives

17 Centrifuge at 3500 rpm, 00:05:00 , at room temperature and discard the supernatant in designated FA-WASTE container

Note

Some plant nuclei might not form a solid pellet at this buffer/centrifugal speed/time combination. You can increase the speed to 6000 or 13000 rpm instead.

18 Quench the crosslinking reaction by adding 1 mL 1X Wash buffer and resuspend the pellet gently by tapping the bottom of the tube, or by using an inoculation loop, and mix by inversion

19 Centrifuge at 3500 rpm, 00:05:00 , (6000 or 13000 rpm) and discard the supernatant in designated FA-WASTE container

Safety information

Move back to your lab bench

Lysate characterization

20 Resuspend nuclei in 500 µL FA buffer and incubate on the thermal mixer set at 1250 rpm, 37°C, 00:15:00

Note

Optional: RNA can be removed after lysis by adding 5 µL RNase A (100 mg/uL) *and incubating* Room temperature 5 min

21 Take 50 µL lysate and go to step #13.1 DNA QC to extract the lysate DNA and run 100 or 200 ng in a 1% agarose gel. The DNA should be > 20 kbp. **Do not proceed** if the DNA is degraded.

Keep the rest of the extracted lysate DNA at 4ºC for quality check in the Fragment Analyzer at a later stage, together with the DNase I titration experiment (see below).

Note

After the incubation, the lysate can be stored at -80 °C *if you can't continue with the next section*

Chromatin Capture

22 Calculate the volume of lysate to have **2 µg** of DNA go to step #21 and add 2 volumes of AMPure XP beads, mix and let it stand 00:05:00 at room temperature

Note

The chromatin lysate in the presence of magentic beads becomes messy. The beads might stick to the inside and outside of the pipette tip and won't come off easily by pipetting in and out. Instead, dispense the beads above the lysate and mix the beads/lysate by tapping the bottom of the tube to avoid losing captured chromatin every time you add any liquid to the tube.

23 4m

- Place the tube on the magnet for at least 00:02:00 or until the solution is clear.
- Discard the supernatant, and wash the beads with 1 mL 1X Wash buffer .

- Place the tube on the magnet again for at least 00:02:00 or until the solution becomes clear and discard it

24 go to step #23 at least one more time, but it might take 4 or more rounds of washing until the solution becomes clear when the beads are on the magnet. The total number of washes depend on the plant species.

Note

If you can't proceed with the rest of the steps, add 200 µL 1X Wash buffer *to the beads and store at* 4 °C *overnight*

DNase I Digestion Dilution Series

25

This section explains how to determine the optimal condition to digested chromatin. You will use one of the the nuclei tubes go to step #13 **.**

Once you have found the optimal condition, you will apply to them to a new nuclei tube (section DNase I Digestion and onwards)

Prepare the following solutions:

	A	DNase I solution	Beads solution
	Sterile ddH2O	450 µL	400 µL
	10X DNase I reaction buffer	50 µL	50 µL
	100 mM MnCl2	--	50 µL
	Total volume	**500 µL**	**500 µL**

25.1 Set the thermomixer at 1250 rpm, 37°C ∞ min

26
- Mix the **DNase I 1 U/µL stock** by inversion 20 times, quick spin
- Mix by inversion again 20 times, and quick spin

26.1
- Transfer 20 µL of DNase I solution to a new tube and add 5 µL **DNase I 1 U/µL stock**

- Mix by inversion 20 times and quick spin
- The **DNase I** concentration of this dilution is **0.2 U/µL**
- Place tube on ice

26.2

- Prepare the dilution series as follows:

	A	DNase I solution	DNase I 0.2 U/µL	U/µL
	Dilution A	94 µL	6 µL	0.012
	Dilution B	95 µL	5 µL	0.01
	Dilution C	96 µL	4 µL	0.008
	Dilution D	97 µL	3 µL	0.006

- Mix the dilution by tapping the bottom of the tube 20 times and quick spin
- Place on ice

27 After removing the last 1X Wash buffer solution from go to step #24 captured chromatin add 500 µL Beads solution go to step #25 and resuspend by vortexing briefly

28 Prepare one tube labelled **T = 0 h** and four tubes as follows:

	A	Dilution A	Dilution B	Dilution C	Dilution D
	T = 1 h	A-1 h	B-1 h	C-1 h	D-1 h

29

- Transfer 50 µL captured chromatin from go to step #27 to the tube labelled **T = 0 h**
- Transfer 100 µL captured chromatin to each tube **A-1 h to D-1 h**
- Place the 5 tubes on ice

30

- Start the digestion by adding **10 µL** of its corresponding dilution to tubes **A-1 h to D-1 h** and flick the bottom of the tube to mix the beads
- Transfer the 5 tubes to the thermomixer
- 01:00:00 TIMER

1h

31

- Stop the reaction by adding 25 µL 0.5 M EDTA pH 8.0 to tubes **A-1 h** to **D-1 h** and vortex 3 s
- Add 12.5 µL 0.5 M EDTA pH 8.0 to tube **T = 0 h** and vortex 3 s

32 Add 200 µL 1X Wash buffer to each tube, vortex briefly and place them on the magnet until the solution is clear

32.1 Discard the supernatant in the $MnCl_2$ -WASTE container and go to step #32 to repeat the wash step

33 After removing the supernatant, add 200 µL 1X Wash buffer and 2 µL 0.5 M EDTA pH 8 to each tube, vortex briefly and store at 4 °C overnight

STEP CASE

Time series option From 83 to 84 steps

1. Prepare **100 µL DNase I solution**
2. Mix the DNase I stock solution (1 U/ µL) by inversion and do a quick spin to collect the solution at the bottom of the tube
3. Add **1 µL DNase I 1 U/uL** to 100 µL DNase I solution and mix by tapping the bottom of the tube and do a quick spin. **The DNase I is now diluted at 0.01 U/uL. Place the tube on ice**
4. After removing the last 1X Wash buffer solution from the captured chromatin add **500 µL Beads solution** and resuspend by vortexing briefly
5. Prepare the tubes for the digestion time course. Label 9 tubes at 10 min intervals from T= 10 min to T = 1 h 30 min and add 12.5 µL 0.5 M EDTA pH 8.0 to each tube. The EDTA will stop the DNase I digestion.
6. Remove **50 µL captured chromatin** and transfer to a tube labelled ZERO and place it in the thermomixer
7. Add **2 µL DNase I 0.01 U/uL** to the **450 µL remaining captured chromatin**, vortex gently and place the tube in the thermomixer.
8. START THE TIMER
9. Sample **50 µL captured chromatin digestion** every 10 min. Transfer the bead sample to the tube labelled with the time point. Vortex briefly and leave at room temperature while you finish sampling the rest of the beads. ***Note**: The bead lysate mixture is messy. Avoid dipping the pipette tip too far into the solution so you don't carry too much mixture outside the tube.*
10. Once the time course sampling is finished, add **200 µL 1X Wash buffer** to each tube, mix and place them on the magnet until the solution is clear
11. Discard the supernatant in the $MnCl_2$-WASTE container and repeat the wash step one more time
12. After removing the supernatant, add **200 µL 1X Wash buffer plus 2 µL 0.5 M EDTA pH 8.0** mix and store at 4 °C overnight

***Note**: If the digestion is incomplete, repeat the experiment but add **6 µL of DNase I at 0.01 U/µL** in step 7 and continue with the rest of the time course.*

Chromatin Quality Check

34

- Incubate 1 mL 1X CutSmart Buffer NEB + 1% SDS for 55 °C 10 min before use
- Vortex and quick spin before opening the tube

34.1 Set the thermomixer program as follows:

55°C 15 min 1250 rpm → 68°C 45 min 1250 rpm

35
- Equilibrate the tubes go to step #33 at room temperature ~ 5 minutes
- Place the tubes on the magnet for at least 00:02:00 until the solution becomes clear
- Discard the supernatant and place the tubes on a rack

36 Add 100 µL prewarmed 1X CutSmart Buffer NEB + 1% SDS to each tube

36.1 Add 1 µL Proteinase K (20 mg/mL) to each tube, vortex briefly and quick spin

Note

Most samples will be fully digested with 20 µg of Proteinase K under these conditions, but you could increase it to 40 or 50 µg if you have experienced problems digesting your sample with this enzyme in other protocols (total genomic DNA extraction, for example).

37 Place the tubes in the thermomixer go to step #34.1 and start the program

38 When the Proteinase K digestion is finished, quickly spin down the tubes and place them on the magnet for at least 00:02:00

39 Transfer the **supernatant containing the DIGESTED CHROMATIN to a new tube** and discard the beads

40
- Add 180 µL AMPure XP beads, mix by flicking the bottom of the tube until the solution is homogeneous
- Let it stand 00:05:00 at room temperature

40.1 Capture the beads with the magnet for at least 00:02:00 and discard the supernatant in the $MnCl_2$-WASTE container

40.2
- Without removing the tube from the magnet add 200 µL 80% ethanol over the beads and wait for **exactly** 00:00:30 before removing the ethanol
- Repeat the ethanol wash one more time

40.3 Spin down the tubes 00:00:02 and place them on the magnet again and remove **all** the liquid

41
- Add 50 µL TE buffer directly onto the beads, close the tube and remove from the magnet. Mix the beads by gently flicking the bottom of the tube
- Do a quick spin (~ 1 second) to collect the beads at the bottom
- Let the DNA elute at least 00:10:00 at room temperature

41.1 Place the tube on the magnet for 00:02:00 until all the beads are against the magnet and transfer the eluted DNA to a new tube. Keep at Room temperature

Note

If you can't proceed with the DNA quantification step, store the tubes at 4 °C overnight

42 Quantify 1 µL eluted DNA with the High Sensitivity dsDNA Qubit kit and estimate the total amount of DNA for each time point (see table below)

43 Prepare the samples for the Fragment Analyzer run. Use the HS NGS kit 1-6000 bp:

- If the concentration is **> 5 ng/µL**, dilute it to 2.5 ng/µL with TE buffer and use 2 µL of 2.5 ng/uL dilution
- If the concentration is **< 2.5 ng/µL** use 2 µL undiluted .
- Open the Fragment Analyzer outfile with ProSize 3.0 and perform the Smear Analysis by selecting the following fragment ranges, and fill out the table below:

44

	A	B	C	D	E	F	G	H	I	J
		% Smear (bp)	**% Smear (bp)**	**% Smear (bp)**	**% Smear (bp)**	**Qubit**	**Qubit**	**Qubit**	**Sample (1 nuclei tube)**	**Sample (1 nuclei tube)**
		100-2500	**300-2000**	**100-300**	**2000-2500**	**ng/µL**	**Vol µL**	**Yield ng**	**Vol µL**	**Yield ng**
		Optimal	Middle	Low end	High end					
	Nuclei sample						50		equals 1 mL by number of nuclei tubes (step 13)	Aim for 2000

	A	B	C	D	E	F	G	H	I	J
	Lysate						50		500 (step 20)	Expect 1900-2000
	T = 0 h						50			
	Diltuion A						50			
	Dilution B						50			
	Dilution C						50			
	Dilution D						50			

Note

*The nuclei, lysate and T = 0 h samples will run over the upper marker (6000 bp), this is fine. It is more important that the resolution is higher between 1 and ~ 3000 bp. But if you prefer, use the HS Genomic DNA kit instead. However, the ProSize 3.0 software can't combine data from two different methods in the "*Project*" option. The amplified Hi-C library (see below) is best analyzed with the HS NGS method because the resolution between 1 and 500 bp is higher. If you want to compare this data with the DNase I digest and later the intramolecular ligated chromatin sample, use the HS NGS method.*

45

- The amount of DNA in the nuclei sample allows you to estimate the DNA yield per tube and per gram of tissue
- The lysate should produce intact DNA, with same profile as your nuclei QC sample described in go to step #13 Nuclei prep. The lysate QC step allows you calculate how much chromatin to expect per lysis event. The lysate and T = 0 h are internal controls to monitor if DNA degradation occurred during sample manipulation
- The goal of the DNase I dilution series is to determine the optimal amount of DNase I where most of the DNA is within the 100-2500 bp range

≥ 50% of the input DNA is within 100-2500 bp

- If you have at 0.9-1 µg of optimally digested chromatin you can now take another nuclei tube and process it in the same way (Section DNase I onwards) to produce either a Nanopore or an illumina library

- If you have ~ 150 ng of optimally digested chromatin you can only prepare an illumina library under these condition
- If you have 80-100 ng of optimally digested chromatin you are at the lower limit for preparing an illumina library, but it is till possible to produce one

< 50% of the input DNA is within 100-2500 bp

- If most of the smear is **≤ 300 bp** for all dilutions (or time points - see step case option), the sample was **over digested**. Repeat the DNase I dilution series but incubate the digestion for 30 min only. If you did a time series go to step #33 step case use a 0.005 U/µL dilution of DNase I

- If most of the smear is **≥ 2500 bp**, the sample was **under digested**. Before repeating or modifying the chromatin prep, check that the DNase I stock is working correctly. Perform a control digestion of 1 µg of Lambda DNA, 20 min only, in 1X DNase I buffer + 10 mM $MnCl_2$, 0.02 U DNase I, in a total volume of 20 µL, 37ºC. Stop the reaction with 1 µL 0.5 M EDTA pH, add 4 µL 6X gel loading buffer and load it **all** in a 1% agarose gel and include an aliquot of undigested Lambda DNA as control (1X TAE, 140 V, 30 min, stain the gel with SybrSafe after the run or add it directly on the gel). If the test shows no digestion, buy a new DNase I stock (with $MnCl_2$)

- If the under digested result is accompanied by a high DNA yield (**>5 µg**), QC the lysate again and make sure you only use **2 µg** for the chromatin capture step

DNase I Digestion

46 This section describes how to process one nuclei tube from crosslinking to the DNase I digestion step once the optimal reaction conditions have been established.

You will collect samples of lysate, T = 0 h and optimal digestion time for QC later. This is a precaution to ensure the same results observed earlier are reproduced

46.1
- Select the time point that contains **≥50% of the DNA within 100-2500 bp** to prepare your library
- Take one tube go to step #13 Nuclei prep from the -80ºC freezer and perform the steps from go to step #14 Chromatin crosslinking up to go to step #24 Chromatin capture

46.2 Carry out the **DNase I digestion** with two time points only: **T = 0 h** and **Optimal QC** as follows:

- Transfer 50 µL captured chromatin resuspended in 500 µL Beads solution to a tube labelled **T = 0 h**
- Start the reaction by adding the go to step #26.2 Optimal DNase I dilution to the remaining 450 µL captured chromatin and place both tubes in the thermomixer

For example: if the optimal digestion was observed with dilution B

	A	Captured chromatin	Dilution B @ 0.01 U/µL	U/captured chromatin (µL)
	Dilution series	100 µL	10 µL	0.001
	Library prep	450 µL	45 µL	0.001

- When the time is up, transfer 50 µL DNase I digested captured chromatin to a tube labelled **Optimal QC** and add 12.5 µL 0.5 M EDTA pH 8.0, mix and leave it on a rack at room temperature
- Add 12.5 µL 0.5 M EDTA pH 8.0 to the **T = 0 h**, mix and leave it on the rack
- Add 100 µL 0.5 mM EDTA pH 8.0 to the remaining **400 µL** of digested chromatin, mix and leave it on the rack

46.3 2m

- Add 800 µL 1X Wash Buffer to each tube, mix and place on the magnet for at least 00:02:00 until the solution is clear
- Discard all the solution in the $MnCl_2$-WASTE container
- Remove the tubes from the magnet and add 1 mL 1X Wash buffer to each tube, mix and place on the magnet again
- When the solution is clear transfer it to the $MnCl_2$-WASTE container

46.4

- Extract the DNA from **T = 0 h** and **Optimal QC** tubes as described before go to step #34 Chromatin Quality Check

These samples, together with the leftover lysate (go to step #21) and the intra molecular ligated chromatin will be quantified and loaded in the Fragment Analyzer later (see Chromatin Reverse Crosslinking Section and Quality Check section below)

46.5
- Add 200 µL 1X Wash Buffer and 2 µL 0.5 M EDTA pH 8.0 to the **Digested chromatin** tube and store at 4 °C overnight
- This is the sample to be used for preparing your library (see section **End Repair**)

End Repair

47
- From the Optimal QC sample determine how much DNA is digested and captured in the AMPure XP beads
- The NEBNext Ultra II End repair can only repair up to **1 µg** of DNA. If there is more than 1 µg of digested chromatin needs to be repaired, up-scale the End Repair according to the table shown below
- The **NEBNext FFPE DNA repair reaction** is only needed for Nanopore library preparation and can only repair up to 1 µg of DNA. If you are preparing an Illumina library, omit this reagent
- Make sure the buffers are fully defrosted and dissolved. Vortex and quick spin all the reagents

48 Place the **digested captured chromatin** from go to step #46.5 on the magnet and discard the clear supernatant. Wash the beads twice with 200 µL 1X Wash buffer and discard all the liquid

49 Add the following reagents of the desired library to be prepared to the washed beads in the order shown below. Mix the contents before and after adding the enzymes:

	Reagent	Nanopore library	Illumina library
		Volume µL	**Volume µL**
	Total volume	**60**	**60**
	Deionized sterile water	48	50
	NEBNext End Repair buffer	3.5	7
	NEBNext FFPE DNA Repair buffer	3.5	
	End repair enzyme mix	3	3
	FFPE enzyme mix	2	

49.1 Place the tube in the thermal mixer set at 1250 rpm, 20°C, 01:00:00

49.2 Quick spin the tube and place it on the magnet for ~ 00:02:00 magnet and discard all the liquid 2m

49.3
- Remove the tube from the magnet and add 1 mL 1X Wash buffer and gently resuspend the beads by tapping with a pen
- Quick spin the tube and place it for at least 00:02:00 magnet and discard all the liquid

2m

49.4 Repeat go to step #49.3 one more time and proceed to the **Bridge Ligation** step immediately

Bridge Ligation

50 Defrost the ligase buffers and vortex until no solid precipitate (DTT) and no syrupy pellet (polyethylene glycol) are visible. Keep the buffers on ice

51 Add the following reagents to the beads in the **order specified**:

	Reagent	Volume µL
	Deionized sterile water	50
	Annealed Biotinylated Bridge 50 µM	20
	Mix beads gently	
	10X T4 DNA ligase buffer	10
	50% PEG 4000	10
	Mix beads gently	
	T4 DNA ligase 1 U/µL	10
	Mix beads genlty	
	Final volume	**100**

51.1 Place the tube in the thermomixer set at 1250 rpm, 22°C, 01:00:00

51.2 Quick spin the tube and place it for at least 00:02:00 magnet and discard all the liquid 2m

51.3 2m

- Remove the tube from the magnet and add 500 µL 1X Wash buffer and gently resuspend the beads by tapping with a pen
- Quick spin the tube and place it for at least 00:02:00 magnet and discard all the liquid

51.4

- Repeat go to step #51.3 one more time making sure all the liquid is discarded
- Place the tube on a rack and continue with **Proximity Ligation** section

Proximity Ligation

52 While performing the Bridge ligation, defrost the ligase buffers and vortex it until no solid precipitate (DTT) and no syrupy pellets are visible. Keep the buffers on ice

53 Add the following reagents to the beads go to step #51.4 in the **order specified**:

	A	B
	Reagent	**Volume µL**
	Deionized sterile water	390
	10X T4 DNA ligase buffer	50
	50% PEG 4000	50
	Mix beads gently	
	T4 DNA ligase 1 U/µL	10
	Mix beads gently	
	Final volume	**500**

53.1 Place the Proximity Ligation reaction tube in the thermomixer set at 1250 rpm, 22°C, 16:00:00

Chromatin Reverse Crosslinking and Quality Check

54

- Program the thermomixer as follows: **55°C 15 min 1250 rpm → 68°C 45 min 1250 rpm**
- Prewarm the thermomixer at **55°C** for ~ 15 min without shaking

55
- Incubate 1 mL 1X CutSmart (NEB) + 1% SDS for 00:10:00 at 55 °C before use
- Vortex and quick spin before opening the tube

55.1 Quick spin the go to step #53.1 Proximity Ligation reaction tube, place it for at least 00:02:00 magnet and discard all the liquid

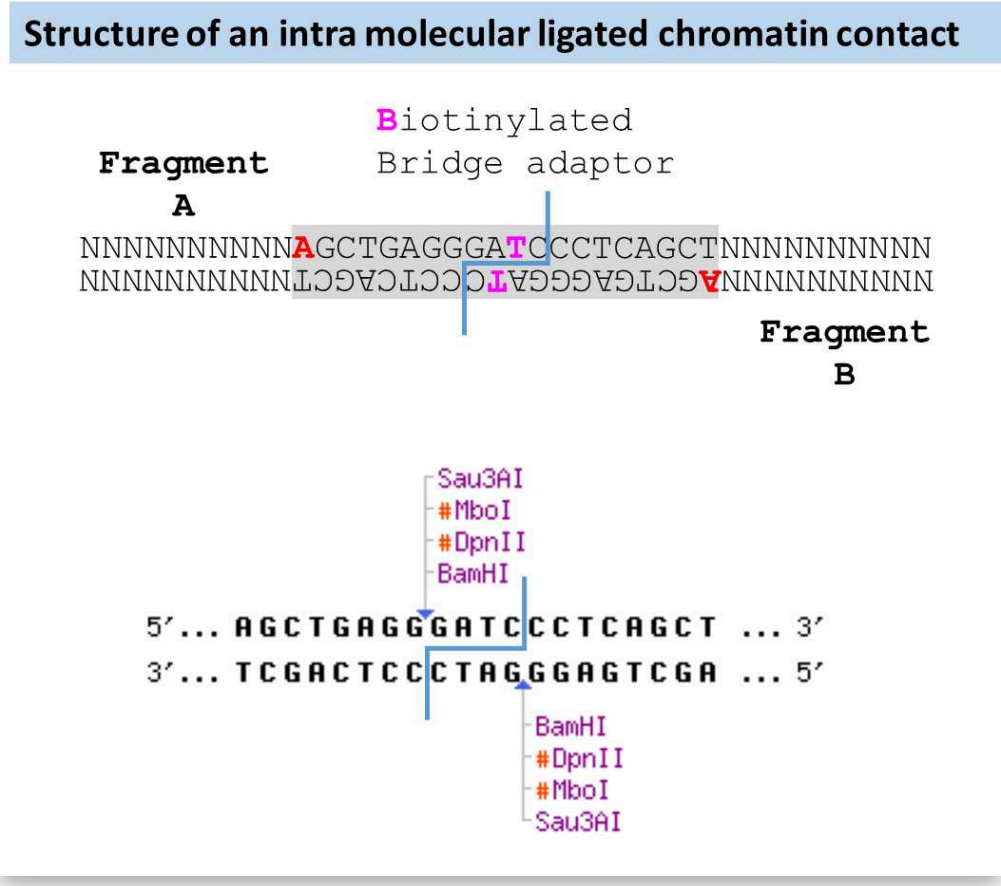
55.2 Add 100 µL 1X CutSmart buffer (NEB) + 1% SDS and vortex briefly

55.3 Add 1 µL Proteinase K, vortex briefly and quick spin the tube (see note go to step #36.1)

55.4 Place the tube in the thermomixer and start the program go to step #54

56 Quick spin the tube and **TRANSFER THE LIQUID TO A NEW TUBE**

THIS IS YOUR INTRA-MOLECULAR LIGATED CHROMATIN (IMLC)



56.1 Add 50 µL TE buffer pH 7.5 to the beads, vortex gently, place for 00:02:00 magnet and transfer the liquid to the rest of the IMLC from the previous step

The total volume is **150 µL IMLC.** Discard the beads

Note

If you can't continue with the DNA purification, store the IMLC at -20 °C .
Defrost the tube on the bench and warm it up at 30 °C ~ 5 min *until the solution is clear*

57 Add 105 µL AMPureXP beads to the **150 µL IMLC** and vortex gently until the sample is homogeneous

Note

The bead to sample ratio at 0.7 will select fragments above 200 bp. See Left Side Selection *graph* ***here***

57.1 Incubate 00:05:00 at room temperature

57.2 Place the tube on magnet and let it stand for at least 00:02:00 magnet or until all the beads are against the magnet and discard the liquid 2m

57.3
- While the tube is still on the magnet, add 200 µL 80% ethanol 30s
- Remove it **exactly** after 00:00:30 ethanol wash

57.4 Repeat step go to step #57.3 one more time

57.5
- Place the tube on the centrifuge and do a quick spin (~ 00:00:02) and place on magnet again Remove **all** traces of ethanol 2s

57.6
- Add 54 µL 10 mM Tris-HCl pH 8.0 directly onto the beads, vortex gently and do a quick spin 10m
- Let the DNA elute at room temperature for **at least** 00:10:00 DNA elution

57.7
- Place the tube on the magnet for ~ 00:02:00 magnet until all the beads are collected 2m
- Transfer the eluted DNA to a new tube

58
- Quantify 1 µL IMLC and also 1 µL of **T = 0 h** and **Optimal QC** samples go to step #46.4 with the HS dsDNA Qubit kit
- Run 2 µL at 2.5 ng/uL each sample on the Fragment Analyzer (HS NGS kit 1-6000 bp)

58.1 Store the IMLC at -20 °C until ready to prepare the sequencing library

Nanopore library preparation

59 To prepare a Nanopore library you will need a least 1 µg IMLC . Check the **Nanopore Community page** to select the kit and best conditions for sequencing

Illumina library preparation

60 **IMLC End Prep and Illumina adaptor ligation**

The universal illumina adaptor and USER enzyme can be found in the NEBNext Multiplex Oligos for Illumina (Index Primers Set 3) kit

Set up the following reaction in a 200 µL PCR tube:

	A	Volume µL
	IMLC	**51**
	NEBNext Ultra II End prep Buffer	7
	NEBNext Ultra II End prep Enzyme	3
	Total volume	**61**

Place the tube on a thermocycler programmed as follows: **20°C 30 min → 65°C 30 min → 12°C ∝**

61 Add 2.5 µL NEB Universal Illumina adaptor (15 µM) , vortex and quick spin

62 Add the following reagents to the End prep IMLC + illumina adaptor tube in the **order specified**:

	Reagent	Volume µL
	Deionized sterile water	11.5
	10X T4 DNA ligase buffer	10
	50% PEG 4000	10
	Mix beads gently	
	T4 DNA ligase 1 U/µL	5
	Mix beads gently	
	Final volume	**100**

62.1 Incubate at 20 °C 30 min . Or leave it in the refrigerator overnight

62.2 Add 3 µL USER enzyme mix , vortex, quick spin and incubate at 37 °C 15 min

62.3 Spin down the tube and transfer all the sample to a 1.5 mL tube

62.4
- Add 50 µL TE pH 7.5 to the PCR tube, vortex, quick spin and transfer all to the 1.5 mL tube
- Total volume: **153 µL**

63 Add 120 µL AMPure XP beads and vortex gently until completely homogeneous

63.1
- Incubate Room temperature at least 5 min
- Place the tube on magnet 00:02:00 or until the solution becomes clear and discard the solution

63.2
- Add 180 µL 80% ethanol to the tube while on the magnet
- Discard all liquid after 00:00:30 exactly
- Repeat this wash one more time
- Quick spin the tube, place it on the magnet and remove **all** liquid

63.3
- Add 100 µL TE pH 7.5 to the tube, remove from the magnet, and resuspend the beads by gently vortexing the tube
- Incubate Room temperature for at least 00:10:00
- Return the tube to the magnet and when the solution becomes clear transfer it to a new 1.5 mL tube

This is now your **IMLC-illumina library**

64 Quantify 1 µL IMLC-illumina library with the Qubit HS dsDNA kit

Note

If you can't continue with the Streptavidin magnetic bead capture or for long term storage , keep the IMLC-illumina library at -20 °C

65 The following steps describe how to prepare the streptavidin magnetic beads for binding an IMLC-illumina library at a concentration of 2.5 ng/µL or higher. 4m

If your IMLC-illumina library is less concentrated, resuspend the SAMB in a different volume. For example: The IMLC-illumina library is at 1.5 ng/µL. To have 100 ng for SAMB capture you need 66 µL IMLC-illumina . Resuspend the prepared SAMB beads in 66 µL 2X PEGBB instead of 40 µL as described here:

Streptavidin magnetic bead (SAMB) preparation

1. Coat one 1.5 mL screw-capped tube by adding 1 mL 1X B/W + T20 , mix and remove all liquid
2. Add 1 mL 1X B/W to the tube
3. Vortex the SAMB until fully resuspended and transfer 20 µL SAMB to the tube, mix by pipetting. This volume contains **200 µg** of beads
4. Place the tube on the magnet for at least 00:02:00 and discard **half** of the liquid
5. Quick spin the tube and place it on the magnet
6. When the solution is clear, discard it **all**
7. Remove the tube from the magnet and add 20 µL 1X B/W and mix by pipetting
8. Place the tube on the magnet for 00:02:00 and discard the liquid
9. Repeat this wash step two more times
10. Add 40 µL 2X PEGBB and mix by pipetting

66 **SAMB capture** 36m

1. Calculate the volume required of **IMLC-illumina library** go to step #64 to have **100 ng** and adjust it to a final volume of 40 µL 1X TE pH 7.5 . Mix and quick spin the tube
2. Transfer the IMLC-illumina solution to the prepared **SAMB** go to step #65 , vortex briefly and place in the thermomixer 1250 rpm, 25°C, 00:30:00
3. Quick spin the tube and place it on the magnet for at least 00:02:00 and discard the solution*
4. Remove the tube from the magnet and add 120 µL 1X B/W + T20 and mix by pipetting
5. Place the tube on the magnet for at least 00:02:00 and discard the solution*

Note

** The clear solution from the unbound and the washes contain IMLC-illumina library, about 50% of the input material. You could discard it, but it is recommended to keep it in case you need to prepare another SAMB capture sample. See instructions on how to recover the unbound IMLC-illumina from these fractions in the last step-case of the* Expected Results *section*

6. Repeat steps 4 and 5 one more time
7. Remove the tube from the magnet and add 120 µL 1X B/W and mix by pipetting
8. Place the tube on the magnet for at least 00:02:00 and discard the solution*
9. Repeat steps 8 and 9 one more time
10. Resuspend the beads in 40 µL 10 mM Tris-HCl pH 8.0

The bead concentration is **~ 5 µg beads/µL.** Store at 4 °C
The sample is stable at this temperature for at least 2 weeks

67 **Library amplification and size selection**

Set up the following PCR master mix:

	A	5 reactions, µL
	Sterile ddH20	100
	NEBNext Ultra II Q5 master mix	125
	Universal PCR primer 10 µM	12.5
	NEBNext index primer 10 µM	12.5
	IMLC-illumina-SAMB ~5 µg/µL	10
	Total volume	**250**

- Vortex until the beads are mixed evenly
- Aliquot 50 µL PCR master mix per PCR tube and place them in the thermocycler

PCR profile:

98ºC 30 sec → (98ºC 10 sec - 65ºC 75 sec) x 12 → 65ºC 5 min → 12ºC ∞

67.1 Spin down the tubes briefly and transfer all the solution to one 1.5 mL tube

This tube contains the amplified Hi-C library plus IMLC-illumina-SAMB

67.2 Wash the PCR tubes:

- Add 100 µL 1X TE pH 7.5 to one of the PCR tubes and set the pipette volume to **150 µL**
- Pipette the solution several times and transfer to the next tube. Repeat this step on all the PCR tubes
- Transfer the solution to the 1.5 mL tube go to step #67.1

67.3 Recover the SAMB-illumina bound library: 4m

1. Place the 1.5 mL tube go to step #67.2 on the magnet for at least 00:02:00
2. Transfer the clear solution to a **new 1.5 mL tube** and set aside. **This is your amplified Hi-C library to be sequenced**
3. Add 50 µL 1X TE pH 7.5 to the SAMB beads, remove it from the magnet, vortex gently and place it in the magnet again for at least 00:02:00
4. Transfer the solution to the 1.5 mL tube set aside earlier (step 67.3.2)
5. Add 10 µL 10 mM Tris-HCl pH 8 to the SAMB-illumina bound library and store at 4 °C This sample can be used again to prepare more amplified library if needed, but use it within 1 week
6. Estimate the total volume of amplified library using a 1000 µL pipette . Expect ~ 400 µL (250 µL PCRs + 100 µL PCR tubes wash + 50 µL beads wash)

67.4

- Add 0.8 - volumes of AMPure XP beads to the PCR reaction (step 67.3.2). For example: if 400 µL were recovered, add 320 µL of AMPure XP beads
- Vortex briefly until the solution is homogeneous

Note

The amount of AMPure XP beads will remove unused primers only. Once the amplified library has been analyzed, you will need to do a double size selection (0.5X/0.3X) before sequencing to select for fragments with an average size of 670 bp. See instructions described in the last step case in Expected Results *section below.*

67.5

- Incubate at Room temperature 5 min
- Place the tube on the magnet for at least 00:02:00 or until the solution is clear
- Discard the solution

67.6
- While still on the magnet, add 500 µL 80% ethanol to the beads and remove it exactly after 00:00:30
- Repeat this step one more time

67.7
- Quick spin the tube and place it on the magnet again
- Remove **all** traces of ethanol
- Remove the tube from the magnet and place it on a rack
- Add 100 µL 1X TE pH 7.5 and gently vortex the tube until the beads are resuspended
- Incubate Room temperature at least 10 min
- Place the tube on the magnet and when the solution is clear, transfer it to a new 1.5 mL Eppendorf tube

This is the amplified Hi-C library cleaned with 0.8X AMPure XP beads

67.8
- Qubit 1 µL amplified Hi-C library with Qubit HS dsDNA kit
- Prepare 10 µL of a 2.5 ng/µL dilution of the amplified library and run it in the Fragment Analyzer (HS NGS 1-6000 bp method)

68 We have tested this protocol on several species and although not all of them showed ideal digestions and IMLC profiles (see step case below) the MiSeq QC run produced usable libraries.

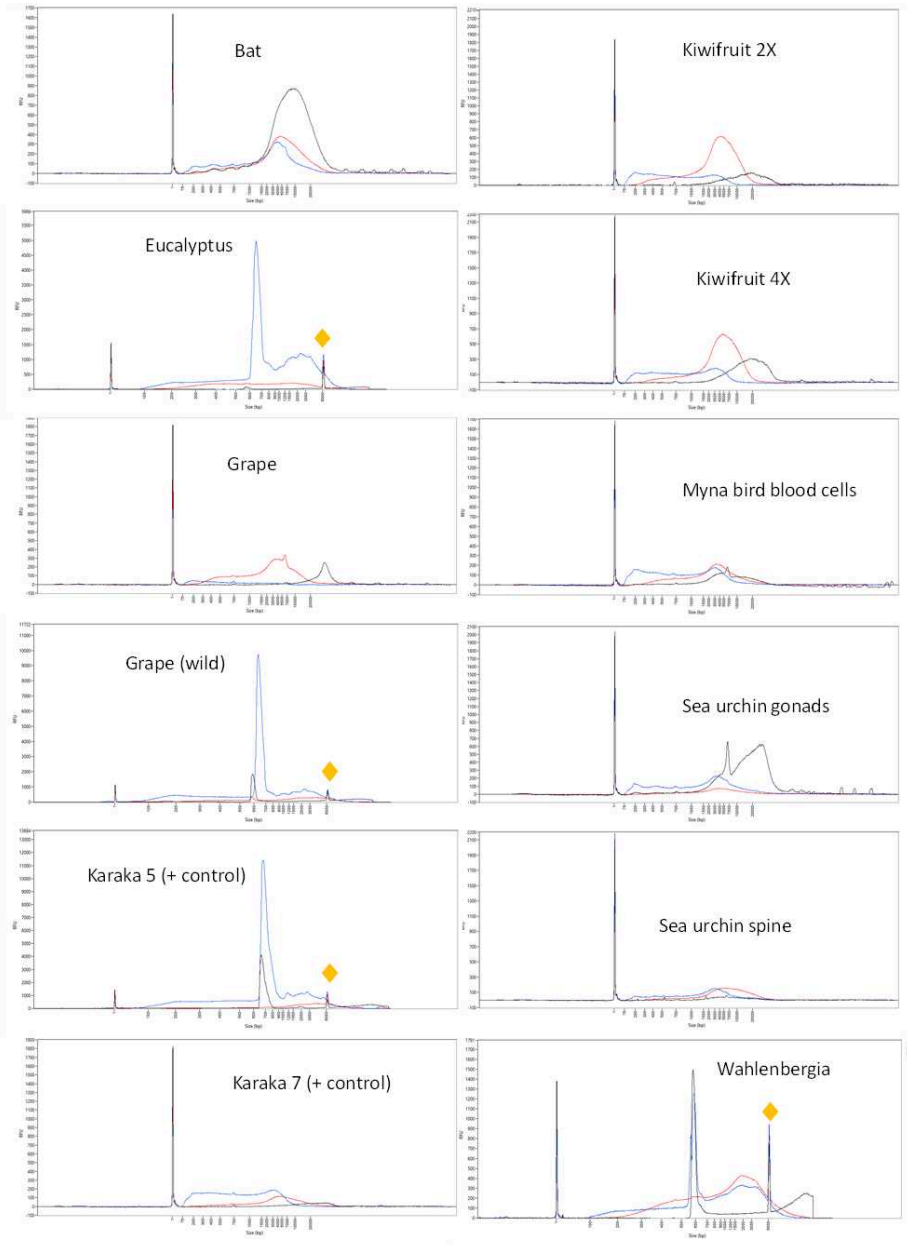


Fig. 1 Fragment size distribution of the lysate, DNase I digestion, and Intramolecular ligated chromatin (IMLC).
The samples were characterized with either the HS NGS method or the HS Genomic DNA method on the Fragment Analzyer. The upper marker of the NGS method is denoted with a yellow rhomboid. The sharp peak at ~ 600 bp shown in some samples could be RNA or aggregated DNA. The peak disappears after the reverse crosslinking of the IMLC.

The sea urchin and bat samples included in this section were grounded in liquid nitrogen, washed and resuspended in 1X PBS before crosslinking. After quenching the formaldehyde with 1X Wash buffer, the cells were filtered through a 200 µm cell strainer and collected by centrifugation. The myna bird blood cells were defrosted in 1X TE pH

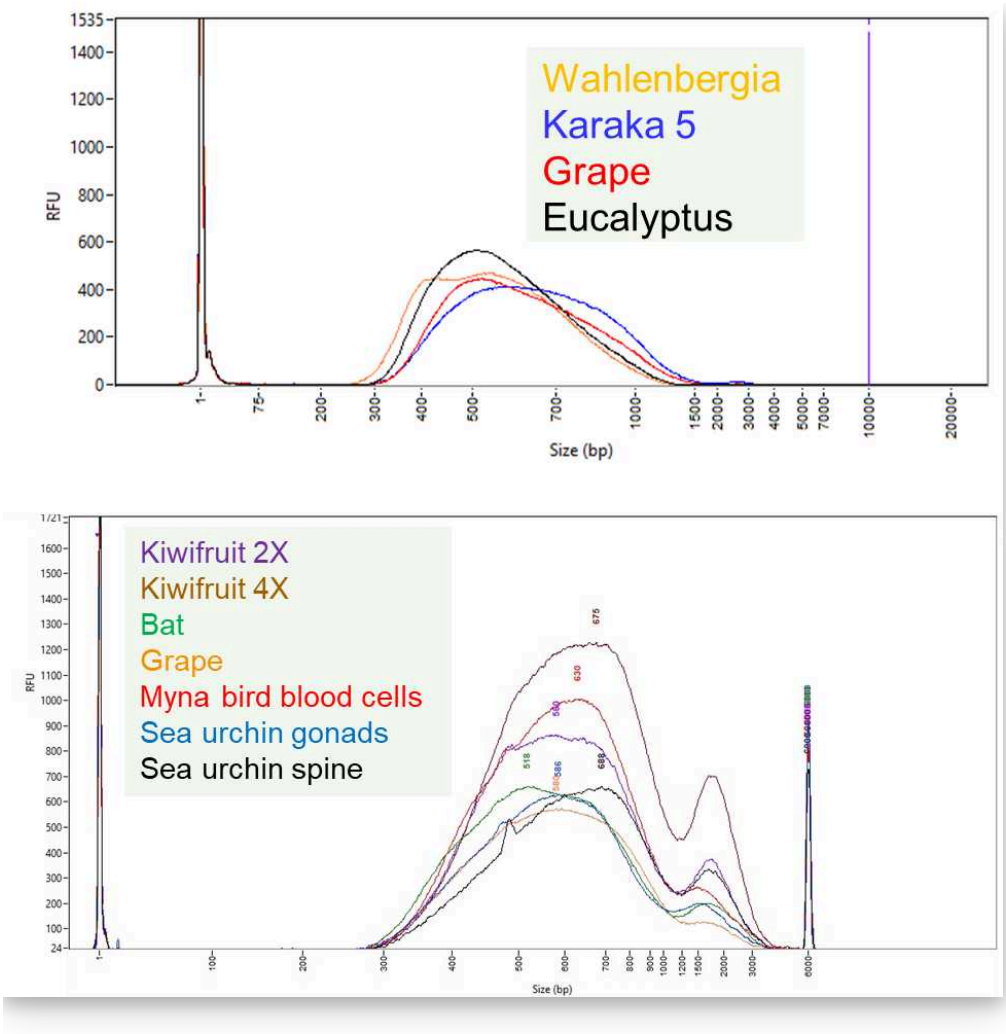
7.5, 100 mM EDTA as soon as they were taken out of the -80ºC freezer, spun down, washed with 1X PBS twice before crosslinking and processed as the other animal samples.

The table below shows the expected yields along the protocol across 5 different plants.

		Diploid kiwifruit (*Actinidia chinensis*)	*Eucalyptus sp.*	Karaka (*Corynocarpus laevigatus*)	Grape (*Vitis vinifera*)	*Wahlenbergia albomarginata*
Nuclei prep stage	**Unit**					
Leaf sample	g	3	3	4	4	10
# Nuclei tubes	tube	8	8	8	8	6
Hi-C Library prep stage		**Total per nuclei tube**				
Lysate	ng DNA	2200	1960	2380	2285	5000
Intramolecular ligated chromatin (IMLC)	ng DNA	825	83	906	142	398
IMLC-illumina library	ng DNA	581	61	644	100	290
# Streptavidin-magnetic bead (SAMB) capture (100 ng IMLC-illumina each)	captures	5	1	6	1	3
# Library amplification reactions		100	20	120	20	60
Library amplification stage (2 µL SAMB-IMLC-illumina per reaction)		**10 PCRs**				
Amount amplicon after 0.8X AMPure XP clean up	ng DNA	2060	206	756	970	3640
After 0.5X/0.3X size selection	ng DNA	664	108	308	432	1408
% amplicon to be sequenced		32%	52%	41%	45%	39%

It is crucial to use no more than 100 ng of IMLC-illumina sample for the streptavidin magnetic bead capture because not all of it will be captured. Under the conditions described in this method, about 50 ng of the initial 100 ng of IMLC-illumina library is bound to the streptavidin beads. It is possible that by using a longer linker connecting the biotin hapten to the Bridge (+) strand, the steric hindrance might be avoided.

The Hi-C library amplification double size selection might look a bit lumpy:

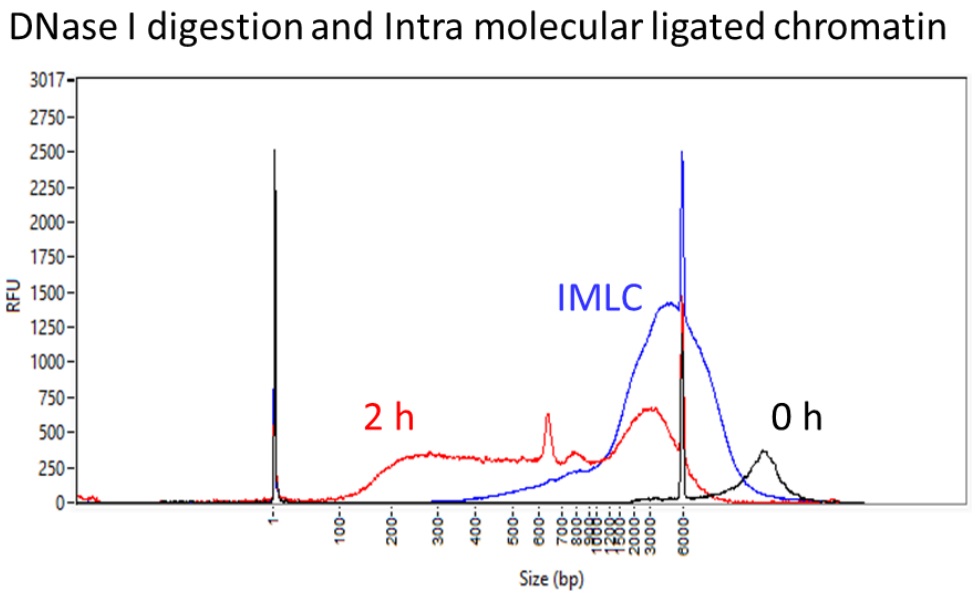


And the next table shows the metrics obtained with the MiSeq QC run:

Hi-C MiSeq comparisons.xlsx

STEP CASE

Ideal DNase I and IMLC profiles 1 step



The sharp peak at ~650 bp observed on the digested chromatin might be an artifact produced when the crosslinked chromatin binds to the AMPure XP beads. We have observed this peak in two plants and on a bird blood library prep. The peak disappears after the intramolecular ligation and reverse crosslinking steps.

Acknowledgements

69 We would like to thank Ashley Jones (Australian National University, Canberra) for joining us in our first Hi-C marathon in 2022, and our colleagues for providing samples to test: Susan Thomson, Wendy Hall, Ed Morgan (Plant and Food Research, New Zealand), Anna Santure (University of Auckland, New Zealand), Annabel Whibley and Darrell Lizamore (Bragato Institute), Jessie Prebble (Landcare Research, New Zealand), Niel Gimmel and Joanne Gillum (University of Otago, New Zealand).

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