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Pore-C Protocol for Plant Samples

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High molecular weight DNA extraction from all kingdoms
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We use this protocol and it's working

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Abstract

The following protocol represents an end-to-end Pore-C workflow tested on the water lily *Victoria cruziana*. This workflow is an adapted version based on several previously published protocols.

Materials

Note: Material list is intended for one sample.

Day 1: Crosslinking

	Buffer / Chemicals	Materials	Devices
	2.5 M glycine solution	1× 500 ml Round-bottom flask	Rotovapor or other vacuum device
	Vacuum infiltration buffer (400 ml)	Double gloves	Fume hood
	10% ECOSURF EH-9 solution	1x Büchner funnel	
	Formaldehyde	Sintered filter or paper filter	
	ddH20	1× 500 ml filtering flask	
		Spatula	
		1× 50 ml tubes	
		Liquid nitrogen (in case of snap freezing)	
		Paper towel	

Day 1: Cryogrinding

	Buffer / Chemicals	Materials	Devices
		Liquid nitrogen	
		Mortar and pestle (Pre-cooled at -80°C)	
		Spatula	
		1x chilled 50 ml tube	
		Ice	
		Styrofoam pad	

Day 1: Nuclei isolation

Note: Homogenisation buffer, nuclei isolation buffer and washing buffer do not require autoclaving.

	Buffer / Chemicals	Materials	Devices
	1x PBS (chilled)	1x Pre-cooled 50 ml tube (4°C)	Centrifuge (4°C) for 50 ml
	10x Homogenisation buffer (pH 9.0 - 9.4)	Ice	Centrifuge (4°C) for 2 ml
	Nuclei isolation buffer (chilled, with β-mercaptoethanol)	Cell strainer (40 µm)	
	Washing buffer (chilled)	Wide-bore pipette tips	
		1x pre-cooled 2 ml tubes	

Day 1: Chromatin denaturation

	Buffer / Chemicals	Materials	Devices
	0.5% SDS	Wide-bore pipette tips	Thermomixer (Pre-heated to 62°C, later 37°C)
	10% ECOSURF EH-9	Cut reaction: NEB CutSmart buffer, NEB NIaII	
		Nuclease free water	

Day 2: Proximity ligation (Variant A)

	Buffer / Chemicals	Materials	Devices
		Ligation reaction: Nuclease free water, NEB ligase buffer, recombinant albumin, T4 DNA ligase	Thermomixer (Pre-heated to 65°C, later 16°C)
		Wide-bore pipette tips	

Day 2: Protein degradation and de-crosslinking

	Buffer / Chemicals	Materials	Devices
	10% SDS stock solution	Protein degradation reaction: Nuclease free water, Tween-20, SDS (10%), Proteinase K	Thermomixer (Pre-heated to 56°C)

Day 3: DNA extraction

Note: 5 M NaCl and 3 M sodium acetate solution requires autoclaving. If TE buffer is selfprepared, autoclaving is also required.

	Buffer / Chemicals	Materials	Devices
	phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 mM Tris-HCL pH 8.0 1 mM EDTA (chilled)	4x Pre-cooled 50 ml (or 5 ml tubes)	Centrifuge (15°C and 4°C)
	5 M NaCl	Ice	Qubit (dsDNA BR Assay)
	3 M sodium acetate (pH 5.5)	Nuclease free water	
	EtOH 100%	1× 1.5 ml tube	
	EtOH 80%		
	EtOH 70%		
	TE buffer		

Custom buffer for size selection

Note: 0.5 M EDTA pH 8.0, PEG 8000 (40% (w/v)) solution, Tris-HCl 1M and NaCl 5 M requires autoclaving.

	Component	Stock conc.
	Tris-HCl	1 M
	EDTA (pH 8.0)	0.5 M
	NaCl	5 M
	PEG 8000	40% (w/v)
	Nuclease-free water	/

Size selection

	Buffer / Chemicals	Materials	Devices
	Custom buffer with AMPure XP beads	1.5 ml tube	Magnetic rack
	70% EtOH		Thermomixer (Pre-heated to 50°C)
	TE buffer pH 8.0		Hula mixer
			Qubit (dsDNA BR Assay)

Day 1: Crosslinking: *Preparations*

1 Prepare 10 ml filtered 2.5 M glycine.

2 Prepare 400 ml fresh vacuum infiltration buffer, store at 4°C prior to use.

	Component	Stock conc.	Final conc.	Volume
	PBS pH 7.4	10x	1x	40 ml
	Sucrose	/	100 mM	13.69 g
	ddH2O	/	/	Up to 400 ml
	Total			**400 ml**

Vacuum infiltration buffer for one sample.

3 Prepare 10% v/v ECOSURF^{TM} EH-9 solution

Day 1: Crosslinking: *Procedure*

4 Collect ~2 g plant material. Young, fresh material is recommended. If necessary, material can be disected into smaller pieces.

4.1 Wash material with ddH_2O and carefully dry with paper towel.

5 Preload 100 ml of vacuum infiltration buffer in a 500 ml round-bottom flask.

6 Suspend 2 g of plant material in the preloaded buffer.

7 Add 100 µl of 10% ECOSURF^{TM} EH-9 to the plant suspension to get a final concentration of 0.01% v/v (Reduces surface tension and prevent plant material from clumping). Perform all subsequent steps inside a fume hood with double gloves.

8 Add 2910 µl of 36.5% v/v formaldehyde to plant suspension for a final concentration of 1% v/v.

9 Connect round-bottom flask to a rotovapor and apply 160 mBar (16 kPa) vacuum with 100 rpm for 15 minutes at room temperature.

10 After 15 minutes, release vacuum to atmospheric pressure and reapply 160 mBar (16 kPa) vacuum with 100 rpm for 15 minutes at room temperature.

11 Release vacuum to atmospheric pressure and decant suspension into Büchner funnel with filter (sintered filter or paper filter).

12 Vacuum filter plant suspension over 500 ml filtering flask. Discard filtrate.

13 Resuspend plant material in 100 ml of fresh vacuum infiltration buffer in the round-bottom flask.

14 Add 5270 µl of 2.5 M glycine to plant suspension for a final concentration of 1% w/v gylcine and mix by swirling.

15 Connect round-bottom flask to a rotovapor and apply 160 mBar (16 kPa) vacuum with 100 rpm for 10 minutes at room temperature.

16 Release vacuum to atmospheric pressure and decant suspension into Büchner funnel with filter (sintered filter or paper filter).

17 Rinse out round-bottom flask with ddH_2O and also decant into Büchner funnel.

18 Vacuum filter plant suspension over 500 ml filtering flask. Discard filtrate.

19 Wash plant material twice with 50 ml vacuum infiltration buffer for ~3 minutes in Büchner funnel. Vacuum filter and discard filtrate.

20 Wash plant material in Büchner funnel with 50 ml distilled water. Filter again. Discard filtrate.

21 Transfer plant material using a spatula into a fresh 50 ml centrifuge tube.

21.1 Now, crosslinked plant material can be removed from the fume hood.

22 Directly continue to cryogrinding OR snap freeze material in liquid nitrogen and store as -80°C for later use.

Day 1: Cryogrinding: *Preparations*

23 Pre-cool mortar and pestle at -80°C for at least 30 minutes.

24 Get liquid nitrogen.

25 Cool 50 ml tube.

Day 1: Cryogrinding: *Procedure*

26 Place chilled mortar and pestle on styrofoam pad.

27 Add liquid nitrogen into mortar and then add cross-linked plant material.

28 Grind material briefly until liquid nitrogen evaporates.

29 Grind cross-linked plant material into fine powder. Minimize thawing! If material starts thawing, add small volume of liquid nitrogen.

30 Collect cryo-ground powder into chilled 50 ml tube on ice using a spatula.

31 Directly continue to nuclei isolation OR snap-freeze powder in liquid nitrogen and then store at -80°C for later use.

32 NOTE: Do not proceed futher unless it is possible to complete the remaining protocol until size selection.

Day 1: Nuclei isolation: *Preparations*

33 Pre-cool 50 ml centrifuge to 4°C

33.1 Pre-cool 2 ml centrifuge to 4°C

34 Cool 1× 50 ml tubes on ice.

35 Prepare 10 ml of 1x PBS and store at 4°C prior to use.

36 Prepare fresh 10x homogenization buffer:

	Component	Stock conc.	Final conc.	Volume
	Trizma-base	/	100 mM	0.61 g
	KCl	/	800 mM	3.00 g
	EDTA	0.5 M	100 mM	10 ml
	Spermidine trihydrochloride	/	10 mM	0.13 g
	Spermine tetrahydrochloride	/	10 mM	0.17 g
	ddH2O	/	/	Up to 50 ml
	Total			**50 ml**

10x homogenization buffer for one sample. NOTE: Adjust pH to 9.0 - 9.4 with NaOH or acetic acid and store at 4°C prior to use.

37 Prepare fresh nuclei isolation buffer:

	Component	Stock conc.	Final conc.	Volume
	10x homogenization buffer	10x	1x	10 ml
	Sucrose	/	500 mM	17.12 g
	PVP-40	/	1% w/v	1.00 g
	ECOSURF EH-9	100%	0.5% v/v	500 µl
	ddH2O	/	/	Up to 100 ml
	β-mercaptoethanol	100% (14.3 M)	0.25% v/v (35.75 mM)	250 µl
	Total			**100 ml**

Nuclei isolation buffer for one sample. NOTE: Do not add β-mercaptoethanol until ready to use. Store at 4°C prior to use.

38 Prepare fresh washing buffer:

	Component	Stock conc.	Final conc.	Volume
	10x homogenization buffer	10x	1x	1 ml
	Sucrose	/	500 mM	1.71 g
	ddH2O	/	/	Up to 10 ml
	Total			**10 ml**

Washing buffer for one sample. NOTE: Store at 4°C prior to use.

Day 1: Nuclei isolation: *Procedure*

39 Resuspend cryo-ground plant material in 20 ml of chilled nuclei isolation buffer in a 50 ml tube. Note: Buffer has to be supplemented with β-mercaptoethanol!

40 Carefully inverting the tube by hand for 15 minutes.

41 Pass the plant suspension through a 40 µm cell strainer into a fresh chilled 50 ml tube on ice. This step might take a while. If cell strainer is clogged, transfer samples to a new cell strainer and continue.

42 After the sample has passed the strainer, rinse the original sample tube with further 5 ml of chilled nuclei isolation buffer and filter through the (same) 40 µm cell strainer.

43 Centrifuge for 20 minutes at 4°C with a centrifugation force recommended for the genome size:

	Genome size (Mbp)	Centrifugation force (g)
	25	4800
	50	4370
	100	3970
	250	3500
	500	3190
	1 000	2900
	2 500	2560
	5 000	2330
	10 000	2120
	25 000	1870
	50 000	1700

Centrifugation forces according to genome size. Based on: *"Restriction enzyme Pore-C protocol for plant samples" v5, 02nd December 2024.*

44 Carefully discard supernatant.

45 Using a wide-bore pipette tip, gently resuspend the pellet in 1 ml chilled nuclei isolation buffer.

46 Add further 19 ml chilled nuclei isolation buffer and mix by swirling.

47 Centrifuge for further 10 minutes at 4°C using a centrifugation force appropriate to genome size (see table).

48 Repeat step 44 - 47 from this section.

49 Pre-cool 2 ml centrifuge to 4°C.

50 Carefully discard supernatant.

51 Using a wide-bore pipette tip, carefully resuspend the pellet in 1 ml cold washing buffer.

52 Transfer sample to fresh 2 ml on ice.

53 Rinse original tube with further 1 ml of cold washing buffer and transfer to the same tube as the transferred sample.

54 Centrifuge for 5 minutes at 4°C using a centrifugation force recommended for the genome size (see table).

55 Discard as much supernatant as possible without disturbing the pellet.

56 Gently resuspend the pellet in 2 ml of chilled 1x PBS using a wide-bore pipette tip.

57 Centrifuge for 5 minutes at 4°C at 3000 g.

58 Discard supernatant.

Day 1: Chromatin denaturation: *Preparations*

59 Pre-heat thermomixer to 62°C, later to 37°C.

60 Prepare 0.5% SDS dilution.

Day 1: Chromatin denaturation: *Procedure*

61 Resuspend pellet of crosslinked nuclei in 100 µl of 0.5% SDS and mix by gentle pipetting with a wide-bore pipette tip.

62 Incubate at 62°C for 6 minutes without agitation. Then cool to room temperature.

63 Pre-heat thermomixer to 37°C.

64 Directly add 250 µl nuclease-free water and 50 µl of 10% v/v ECOSURF^{TM} EH-9 to the sample.

65 Mix gently by pipetting with wide-bore pipette tip.

66 Incubate at 37°C for 15 minutes without agitation. Chromatin is now ready for digestion.

67 Add following reagents to the sample for final concentration of 1 U/µl for the selected restriction enzyme (NIaIII):

	Component	Stock conc.	Final conc.	Volume
	Plant nuclei suspension	/	/	400 µl
	NEB CutSmart buffer	10x	1x	50 µl
	NEB NIaII	10 U/µl	1 U/µl	50 µl
	Total			**500 µl**

Restriction enzyme mix for one sample.

68 Mix by gentle inversion and incubate sample in the thermomixer at 37°C for 18 hours without agitation.

Day 2: Proximity ligation: *Preparations*

69 NOTE: Proximity ligation is depending on the previous chosen restriction enzyme for digestion (enzyme heat-denaturable or not). The following steps are applicable for NlaIII. This enzyme is recommended by ONT as it is suitable for many species and generates high density contact outputs with optimal fragment length.
However, an *in silico* restriction digest can be performed to identify potential areas of reduced cleavage within the genome or repeat rich genomic regions to determine other restriction enzyme candidates.

70 Pre-heat thermomixer to 65°C, later 16°C.

Day 2: Proximity Ligation: *Procedure*

71 Heat-denature the restriction enzyme by incubating the sample in the thermomixer at 65°C for 20 minutes at 300 rpm.

72 Pre-cool thermomixer to 16°C.

73 Allow sample to cool to room temperature.

74 Set up proximity ligation by adding the reagents in the following order directly to the sample:

	Component	Stock conc.	Final conc.	Volume
	Plant nuclei suspension (Digestion reaction)	/	/	500 µl
	Nuclease-free water	/	/	345 µl
	NEB ligase buffer	10x	1x	100 µl
	Recombinant albumin	20 µg/µl	0.1 µg/µl	5 µl
	T4 DNA ligase	400 U/µl	20 U/µl	50 µl

	Component	Stock conc.	Final conc.	Volume
	Total			**1000 µl**

Proximity ligation set up for one sample.

75 Mix by gentle pipetting with wide-bore pipette tip and incubate the sample in a thermomixer at 16°C for 6 hours without agitation. Note: Avoid prolonged ligation to minimize trans-chromosomal contacts.

Day 2: Protein degradation and de-crosslinking: *Preparations*

76 Pre-heat thermomixer to 56°C.

77 Prepare 10% SDS stock solution.

Day 2: Protein degradation and de-crosslinking: *Procedure*

78 Set up protein degradation reaction by adding the following reagents to the ligation reaction:

	Component	Stock conc.	Final conc.	Volume
	Plant nuclei suspension (Ligation reaction)	/	/	1000 µl
	Nuclease-free water	/	/	300 µl
	Tween-20	20%	5%	500 µl
	SDS	10%	0.5%	100 µl
	Proteinase K	20 µg/µl	1 µg/µl	100 µl
	Total			**2000 µl**

Protein degradation mix for one sample.

79 Mix through gentle inversion.

80 Incubate sample in a thermomixer at 56°C for 18 hours with periodic <1000 rpm for <30 seconds every 15 minutes (to prevent condensation).

Day 3: DNA extraction: *Preparations*

81 Pre-cool centrifuge to 15°C.

82 Cool 50 ml tubes (if available 5 ml tubes).

83 Prepare 5 M NaCl solution.

84 Prepare 3 M sodium acetate (pH 5.5).

85 Prepare EtOH dilutions: 100%, 80%, 70%

86 Prepare TE buffer.

Day 3: DNA extraction: *Procedure*

87 Cool sample on ice.

88 Transfer entire volume to a 50 ml tube (or 5 ml tube).

89 Rinse original tube with 200 µl of nuclease-free water and transfer to the 50 ml tube (or 5 ml tube). Total volume should be ~2200 µl.

90 Add the same volume of chilled phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 mM Tris-HCL pH 8.0 1 mM EDTA. Adjust volume accordingly to equal the sample volume.

91 Mix through gentle inversions for 5 minutes. A homogenous emulsion should be obtained.

92 Centrifuge sample at 15 000 g at 15°C for 15 minutes.

93 Carefully collect ~2000 µl of the upper aqueous phase and transfer to a fresh chilled 50 ml tube (or 5 ml tubes).

94 Add the same volume of chilled phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 mM Tris-HCL pH 8.0 1 mM EDTA. Adjust volume accordingly to equal the sample volume.

95 Mix through gentle inversion for 5 minutes. A homogenous emulsion should be obtained.

96 Centrifuge sample at 15 000 g, 15°C for 15 minutes.

97 Carefully transfer the aqueous phase to a fresh chilled 50 ml tube (or 5 ml tube) and **note the recovered volume** (~1000 µl). Note: Do not take any of the interphase layer.

98 Split the recovered aqueous phase to a second 50 ml tube (or 5 ml tubes) to get two equal aliquots in two seperate tubes.

99 Add 0.02 volumes of 5 M NaCl (0.1 M final) and 0.1 volumes of 3 M sodium acetate pH 5.5 (0.3 M final) based on the volume of the recovered aqueous phase in step 97 to both 50 ml tubes (or 5 ml tubes).

100 Mix through gentle agitation.

101 Add 3 volumes of 100% EtOH based on the volume of the recovered aqueous phase in step 97 to both 50 ml tubes (or 5 ml tubes).

102 Mix thorugh gentle inversion and precipitate at -80°C for 3h - 6h. This time span resulted in positive results for *Victoria cruziana*. However, ONT recomments at least >1h or overnight at -20°C.

103 Pre-cool centrifuge to 4°C.

104 Centrifuge the samples in the 50 ml tubes (or 5 ml tubes) at 16 000 g at 4°C for 30 minutes.

105 Discard the supernatant before washing the pellets in 4 ml 80% EtOH.

106 Centrifuge both samples at 16 000 g at 4°C for 5 minutes.

107 Discard supernatant before washing the pellets in 2 ml 70% EtOH.

108 Centrifuge both samples at 16 000 g at 4°C for 5 minutes.

109 Discard the supernatant.

110 Briefly spin down the tubes and remove any residual supernatant.

111 Air-dry the pellets for 5 minutes.

112 Gently resuspend each samples in 75 µl TE or LTE buffer and incubate for 5 minutes at toom temperature. Mix through gentle agitation every few minutes.

113 Briefly spin down the tubes and pool both samples into a fresh 1.5 ml tube.

114 Quantify the DNA concentration by using a Qubit Assay Kit and measure dsDNA. Samples might be diluted 1/10 as Qubit reading could be affected by high salt concentrations.

115 Store the samples at 4°C until library preparation.

Size selection for Pore-C samples

116 NOTE: The following part is based on a protocol from Miriam Schalamun and Benjamin Schwessinger, with some modifications.
To enrich >2 kb fragments for optimal Pore-C data, it is recommended to use 0.85x volume of custom SPRI beads (AMPure XP Beads).

Size selection: *Preparation of custom buffer with AMPure XP beads*

117 Prepare PEG 8000 Stock solution 40% w/v: 40g / 100 ml for 1 ml buffer. Take 0.4 g of PEG 8000.

118 Pipette custom buffer for size selection:
NOTE: Accuratly pipette 548 µl of 40% (w/v) PEG 8000 using a wide-bore pipette tip.

	Component	Stock conc.	Final conc.	Volume
	Tris-HCl	1 M	10 mM	20 µl
	EDTA pH 8.0	0.5 M	1 mM	4 µl
	NaCl	5 M	1.6 M	640 µl
	PEG 8000	40% (w/v)	11% (w/v)	548 µl
	Nuclease-free water	/	/	780 µl
	Total			**1992 µl**

Preparation of custom buffer for size selection.

119 Transfer mixed AMPure XP beads into two 1.5 ml tubes (each containing 1 ml).

120 Place tubes on magnetic rack until solution is clear. The tube lid should be opened before placing the tube on the magnetic rack to avoid disturbing the sedimented beads at a later stage.

121 Discard supernatant.

122 Remove tubes from magnetic rack. Wash beads by resuspending the pellet in 1 ml of nuclease-free water.

123 Place tubes on the magentic rack again with open lid and allow beads to pellet.

124 Pipette off the supernatant.

125 Repeat step 122 and 124.

126 Spin down and place the tubes back on the magnetic rack. Remove any residual water using a pipette.

127 Resuspend the bead pellets in both tubes with 200 µl custom buffer.

128 Pool both resuspended pellets into the remaining custom buffer.

129 If not used immediatly, store at 4°C. After storing the custom buffer at 4°C bring the bead suspension back to room temperature before use.

Size selection: *Procedure*

130 Pre-heat thermomixer to 50°C.

131 Dilute sample DNA to 60 ng/µl in a final volume of 50 µl TE buffer pH 8.0.

132 Add 0.85x (42.5 µl) of the custom bead suspension to the DNA sample.

133 Mix by flicking the tube and incubate for 10 minutes on a Hula mixer at room temperature.

134 Spin down briefly and pellet on a magnetic rack.

135 Keep tube on the magnet and pipette off the supernatant.

136 Keep tube on the magnetic rack and wash beads with 200 µl 70% EtOH (prepared fresh) without disturbing the pellet.

137 Remove the 70% EtOH and discard.

138 Repeat step 136 and 137 again.

139 Spin down the tube and place it back on the magnetic rack. Pipette off any residual volume.

140 Air-dry the pellet for 30 sec.

141 Remove the tube from the magnetic rack and resuspend the pellet in 40 µl TE buffer.

142 Incubate for 1 minute at 50°C and afterwards for 5 minutes at room temperature.

143 Pellet the beads on the magnetic rack until the eluate is clear.

144 Pipette off 40 µl of eluate into a clean 1.5 ml tube.

145 Quantify 1 µl of size-selected DNA using a Qubit fluorometer. Expect around 50% loss of DNA through size selection.

Information: Library preparation

146 For library preparation of pore-c DNA, the protocol of the ligation sequencing kit V14 (SQK-LSK114) has been used with the following deviations:
- Step 6: Incubation in thermal cycler at 20°C for 15 minutes then 65°C for 5 minutes (instead of 20°C for 5 minutes and 65°C for 5 minutes).

Information: Bioinformatic analysis

147 For further informations regarding bioinformatic analysis see:
https://doi.org/10.1101/2024.06.15.599162

Protocol references

This workflow is an adapted version based on several previously published protocols:

"Restriction enzyme Pore-C protocol for plant samples" v5, 02nd December 2024 (https://nanoporetech.com/document/extraction-method/plant-pore-c)

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"SPRI size selection protocol for >1.5-2 kb DNA fragments"; based on the work by Miriam Schalamun and Benjamin Schwessinger, with some modifications (https://nanoporetech.com/document/extraction-method/spri-size-selection)

For further detailed information on alternative applications, please consider the original protocol: “Restriction enzyme Pore-C protocol for plant samples” (ONT).

Nowak et al. 2024, Genome sequence and RNA-seq analysis reveal genetic basis of flower coloration in the giant water lily Victoria cruziana
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