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From low cost plant HMW DNA extraction to MinION sequencing

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ABSTRACT

This low cost protocol is efficient to extract high molecular weight DNA from several tropical plants to prepare LSK109 libraries for Nanopore sequencing. Pulsed-field gel electrophoresis reveals a DNA size range from 40 to 350 Kb for four species of rice, Paspalum, date palm and *Ocotea obtusata*. Sequencing on MinION Nanopore flow cells yielded from 3.3 to 25.3 Gb with reads N50 values ranging from 15.4 to 31.3 Kb.

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HMW DNA extraction, tropical plant, Nanopore

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Plant materiel sampling 10s

1 Collect up to **5 g** of fresh young leaves in a 50ml clean tube.
Grind sample with liquid nitrogen, mortar and pestel to a fine powder.
An analytical grinder (A15, IKA, Germany) can also be used for 2x **00:00:10** .

Tissue lysis 3h

2 Add **15 mL** of freshly prepared MATAB lysis buffer (100mM Tris pH8, 1.4M NaCl, 20mM EDTA ph8, 1% PEG6000, 3h
2% MATAB, 1mM DTT).
Homogenize slowly by inversion.

Incubate at 65 °C for 01:30:00 and mix by gentle inversion every 00:15:00 .

Add 2.5 µl of proteinase K (20 mg/ml; eg from Terra PCR Kit) and incubate at 55 °C for 01:30:00 with homogeneisation by inversion every 00:15:00 .

DNA purification 1h 30m

3 1h 30m

Add 15 mL of Chloroform:Isoamyl alcohol 24:1 **Sigma Aldrich Catalog #C0549** and homogenize slowly manually or with horizontal shaker 5 rpm, Room temperature , 00:12:00 .

Centrifuge 4000 rpm, 20°C, 00:20:00 and carefully transfer the supernatant to a clean 50 ml tube by using a 1000µl pipette tip cut at the extremity.

Optionnal:

If the supernatant is not clear, add 14 mL *of* Chloroform:Isoamyl alcohol 24:1 **Sigma Aldrich Catalog #C0549** *and agitate* slowly manually or with horizontal shaker 5 rpm, Room temperature , 00:12:00 .

Centrifuge 4000 rpm, 20°C, 00:20:00 *and carefully transfer the supernatant to a clean 50 ml tube by using a* 1000µl pipette tip cut at the extremity..

Add 16 µl of RNase A Solution, 4mg/ml **Promega Catalog #A7973** , slowly homogenize and incubate 00:30:00 at Room temperature .

Add 13 mL of Chloroform:Isoamyl alcohol 24:1 **Sigma Aldrich Catalog #C0549** and agitate slowly manually or with horizontal shaker 5 rpm, Room temperature , 00:12:00 .

Centrifuge 4000 rpm, 20°C, 00:20:00 and carefully transfer the supernatant to a clean 50 ml tube by using a 1000µl pipette tip cut at the extremity..

Clear supernatant after centrifugation

DNA precipitation 15m

4 15m

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Add [M]**10 % volume** of 3M Sodium acetate pH5.2 and [M]**70 % volume** of

2-Propanol **Sigma**

Aldrich Catalog #190764 .

Homogenize by very slowly inverting the tube until DNA precipitate appears.

DNA precipitate appears after homogeneization

Glass hook DNA and transfer into a 2 ml Eppendorf tube containing 1 ml 70% EtOH.
Then, air dry the DNA precipitate **00:02:00** .

Transfer the DNA precipitate into **300 µl** of ultrapure water in a 1.5ml Eppendorf tube.

Let the DNA resuspend **Overnight** at **4 °C**

DNA clean-up 15m

5 15m

To avoid DNA shearing, always cut tip extremities using a clean razor blade before pipetting.

Resuspend the AMPure XP magnetic beads equilibrated at room temperature by vortexing.

In a 2ml Eppendorf tube, add **150 µl** of AMPure XP magnetic beads to **150 µl** of DNA.

Homogenize by gently flicking the tube and incubate **00:10:00** at **Room temperature** .
Spin down and pellet on the magnet.
Carrefully pipette off the supernatant and wash twice with **500 µl** of fresh 70% ethanol.

Remove the tube from the magnet and add **150 µl** of ultrapure water.

Incubate at **65 °C** until the beads are completely resuspended (approximately 2x **00:00:30**).
Help resuspension by gently flicking the tube.

DNA quantification

6 Quantify 1µl of DNA using a Qubit fluorometer.

Check HMW using Pulsed-Field Gel Electrophoresis

7 Molecular weight of extracted DNA was assessed by PFGE by loading **20 µl** of DNA (**1.5 µg** to **5.5 µg** of

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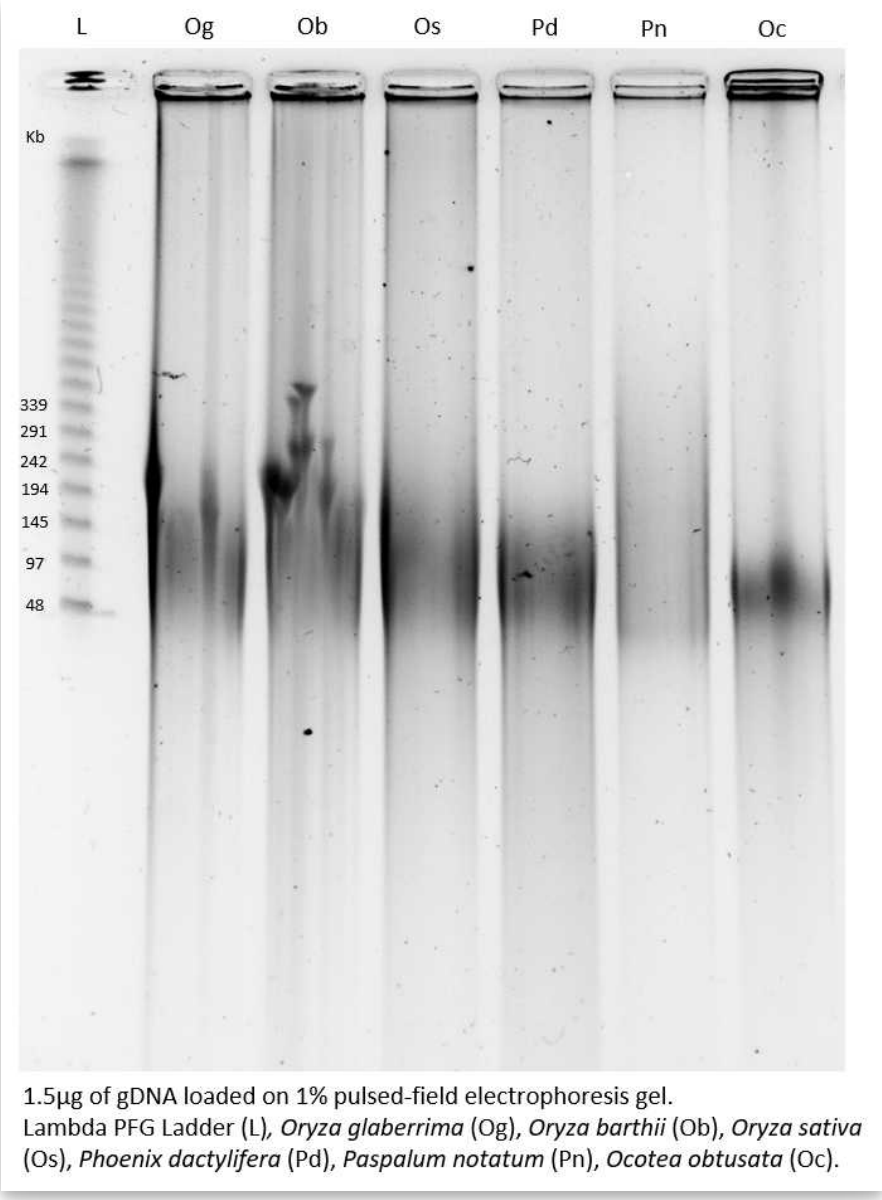
DNA, **5 µl** of 6x loading buffer) and

Lambda Ladder PFG Marker - 50 gel lanes **New England Biolabs Catalog #N0340S** into 1% agarose gel

and using the following parameters for migration:

Pulse time : initial = 5, final = 120
Run time = 18 h
V/cm = 5
Angle = 120
Temp = 14°
mA end of run = 255



1.5µg of gDNA loaded on 1% pulsed-field electrophoresis gel.
Lambda PFG Ladder (L), *Oryza glaberrima* (Og), *Oryza barthii* (Ob), *Oryza sativa* (Os), *Phoenix dactylifera* (Pd), *Paspalum notatum* (Pn), *Ocotea obtusata* (Oc).

Short read elimination

8 Perform short read elimination with a 25Kb cut-off by following the Circulomics SRE kit (SKUSS-100-101-01) instructions.

Incubate **Overnight** at **4 °C** in elution buffer before Qubit quantification and library preparation.

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SQK-LSK109 library preparation 23m

9 SQK-LSK109 library preparation is performed according to Ligation Sequencing Kit **Oxford Nanopore Technologies Catalog #SQK-LSK109** protocol with several modifications :

9.1 DNA repair & end-prep

25m 45s

In a 1.5ml Eppendorf DNA LoBind tube mix:

47 µl DNA sample (9µg of extracted DNA with volume adjusted with 50/50 Ultrapure water/TE pH8)

3.5 µl NEBNext FFPE DNA Repair Buffer

NEBNext FFPE DNA Repair Mix - 24 rxns **New England Biolabs Catalog #M6630S**

2 µl

3.5 µl Ultra II End-prep Reaction Buffer

3 µl Ultra II End-prep Enzyme Mix

Mix by gently flicking the tube, and spin down.

Using a thermal cycler, incubate at **20 °C** for **00:08:00** and **65 °C** for **00:05:00** .

Resuspend the AMPure XP magnetic beads equilibrated at room temperature by vortexing.

Add **50 µl** of AMPure XP magnetic beads to the end-prep reaction and mix by gently flicking the tube.

Incubate **00:10:00** at **Room temperature** .

Meanwhile, prepare **500 µl** of 70% ethanol using nuclease-free water.

After the incubation, spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

Wash the beads twice with **200 µl** of freshly-prepared 70% ethanol.

Spin down and place the tube back on the magnet to pipette off the residual ethanol.

Remove the tube from the magnet and resuspend the pellet in **62 µl** nuclease-free water.

Incubate **00:02:00** at **Room temperature** and **00:00:30** at **65 °C** . Gently flick the tube and incubate **00:00:15** more at **65 °C** .

Pellet the beads on a magnet until the eluate is clear and colourless.

Remove and retain **62 µl** of eluate into a clean 1.5ml Eppendorf DNA LoBind tube.

Quantify **1 µl** of eluted sample using a Qubit fluorometer.

9.2 Adapter ligation

38m

In a 1.5ml Eppendorf DNA LoBind tube, mix:

61 µl DNA sample from the previous step

25 µl Ligation Buffer (LNB)

10 µl NEBNext Quick T4 DNA Ligase

5 µl Adapter Mix (AMX)

Mix by gently flicking the tube, and spin down.

Incubate the reaction for **00:18:00** at **20 °C**

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Resuspend the Room temperature equilibrated AMPure XP magnetic beads by vortexing.

Add 50 µl of AMPure XP magnetic beads to the end-prep reaction and mix by gently flicking the tube.

Incubate 00:10:00 at Room temperature .

Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

Wash the beads by adding 250 µl of Long Fragment Buffer (LFB) at room temperature. Remove the supernatant and repeat the wash step.

Spin down and place the tube back on the magnet to pipette off the residual LFB.

Remove the tube from the magnet and resuspend the pellet in 16 µl of Elution Buffer (EB).

Incubate 00:05:00 at Room temperature and 00:05:00 at 37 °C .

Pellet the beads on a magnet until the eluate is clear and colourless.

Remove and retain 16 µl of eluate into a clean 0.5ml Eppendorf DNA LoBind tube.

Quantify 1 µl of eluted sample using a Qubit fluorometer.

MinION sequencing 5m

10

Split the 16µl library into two 8µl in 0.5ml Eppendorf DNA LoBind tube. Store one tube at 4 °C until use.

10.1 Day 1 - First Load

5m

In the second tube, add to the 8µl library:

4 µl Elution buffer (EB)

37.5 µl Sequencing buffer (SQB)

25.5 µl Loading beads (LB), mixed immediately before use.

Using MinION mk1C device, perform the flow cell check. (FLO-MIN106D - Spot-ON Flow Cell R9 version)

Prepare the priming mix by adding 30 µl of Flush tether (FLT) directly into a fresh Flush buffer (FB) tube.
Mix by vortexing.

After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to
remove any bubbles (a few µl):
- Set a P1000 pipette to 200 µl
- Insert the tip into the priming port
- Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the
pipette tip.

Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air
bubbles. Wait for 00:05:00 .

Remove again any bubbles :
- Set a P1000 pipette to 200 µl

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- Insert the tip into the priming port
- Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the
pipette tip.

Gently open the SpotON sample port.
Load an additional 200 µl of the priming mix into the still open priming port.

Mix the prepared library gently by pipetting up and down just prior to loading.

Slowly, add **75 µl** of the library into the SpotON sample port.

Make sure the priming port is still open during this step and the library should easily fall into the sample port.
Close the priming port and replace the SpotON sample port cover.
Close the lid of the MinION device.
Start the sequencing experiment by setting a 96h run.

10.2 Day 3 - Second Load

After 48h running, pause the sequencing run.

Perform the flow cell washing step according to the ONT protocol (EXP-WSH003).

The wash kit allows to revert "unavailable pores" into "active pores".
This procedure provides the opportunity to maximise the flow cell yield.

At the end of this protocol, re-prime the flow cell as indicated in the 10.1 step.

In the 8µl library tube stored at 4°C, add the following:

4 µl Elution buffer (EB)

37.5 µl Sequencing buffer (SQB)

25.5 µl Loading beads (LB), mixed immediately before use.

Load on the flow cell as the 10.1 step.
Resume the sequencing run.

10.3

After 72h running, if translocation speed falls below 250 bases per seconde, pause the run and refuel:

After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to
remove any bubbles (a few µl):
- Set a P1000 pipette to 200 µl
- Insert the tip into the priming port
- Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip.

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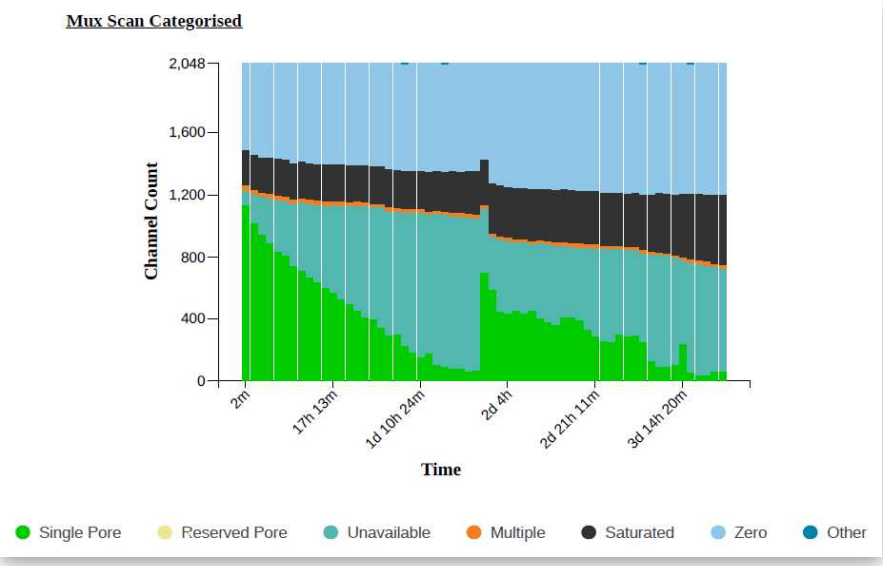
Load **150 µl** of Flush Buffer (FB). Close the priming port and resume the run.



Mux scan result.
Visualisation of the reverting of unvailable pores into single pores after performing the wash step after 2 days.

Results

11

Species	Sample	Flow cell ID	SRE treatment	Number of reads	N50 reads	Median reads	Mean quality	Number of nucleotides sequenced
Oryza barthii	B88	FC1	Yes	4,90E+05	23 613,00	14 779,00	10,70	8,26E+09
Oryza barthii	TB65	FC2	No	2,09E+06	17 226,00	1 805,00	9,80	1,21E+10
Oryza barthii	Ob546	FC3	Yes	3,72E+05	28 222,00	15 865,00	10,60	7,15E+09
Oryza barthii	Ob569	FC4	No	1,22E+06	23 393,00	3 728,00	10,10	1,20E+10
Oryza glaberrima	Og20	FC5	Yes	4,77E+05	29 040,00	15 192,00	10,90	9,23E+09
Oryza glaberrima	Og132_MG12	FC6	Yes	4,92E+05	28 339,00	14 483,00	10,70	8,96E+09
			Yes	1,25E+05	25 522,00	14 119,00	10,40	2,15E+09
Oryza glaberrima	Tog5672	FC7	No	1,87E+06	15 467,00	857,00	10,00	6,22E+09
			No	7,40E+05	16 967,00	864,00	9,10	2,69E+09
Oryza glaberrima	Tog7291	FC8	No	7,05E+05	21 544,00	3 438,00	10,00	6,12E+09
			No	5,31E+05	16 276,00	3 010,00	10,20	3,75E+09
Oryza sativa	ChomrongDanh	FC9	No	1,09E+06	28 860,00	2 356,00	10,40	1,01E+10
Oryza sativa	DJ123	FC10	Yes	7,25E+05	23 913,00	13 554,00	10,60	1,17E+10
			Yes	2,95E+05	17 083,00	10 385,50	10,40	3,57E+09
Oryza sativa	IUPLR17	FC11	Yes	3,56E+05	31 321,00	13 871,00	10,40	6,59E+09
Oryza sativa	Llanura11	FC12	Yes	8,57E+05	25 609,00	14 692,00	10,80	1,52E+10
Oryza sativa	Nerica4	FC13	No	1,51E+06	24 898,00	2 618,00	9,90	1,29E+10
Oryza sativa	NericaL19	FC14	No	5,81E+05	24 116,00	1 281,00	9,20	3,36E+09
		FC15	Yes	5,46E+05	26 490,00	13 457,00	10,70	9,18E+09
Oryza sativa	Sahel108	FC16	No	1,86E+06	18 414,00	5 169,00	10,10	1,75E+10
Oryza sativa	Tequing	FC17	Yes	5,77E+05	28 130,00	14 673,00	10,60	1,07E+10
Paspalum notatum	Q4117	FC18	Yes	2,02E+06	21 655,00	8 170,00	10,90	2,53E+10

Output illustration of different species sequecing.
(SQK-LSK109, Flow cell R9 version, minION mk1c)

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