STANDARD OPERATING PROCEDURE (SOP)

SOP Title:	**HIGH MOLECULAR WEIGHT NUCLEAR DNA EXTRACTION FROM PLANT TISSUES**
SOP Number:	SOP201-0
Effective Date:	10 May 2019
Current Review Date:	10 May 2021
Replaces SOP Number:	
Group:	GIH

I have read this document and approve its contents.

	Name	**Team**	**Signature**	**Date**
Written By:	Subash Rai	Long Read Sequencing		10 May 2019
Reviewed By:				
Reviewed By:				
Reviewed By:				
Reviewed By:				
Authorised By:				

SOP Number	**Author**	**Date Originated or Revised**
SOP201-0	Subash Rai	10 May 2019

A. PURPOSE AND APPLICATION

This SOP covers required materials, procedures, and expected result for nuclei isolation from plant tissues and high molecular weight DNA extraction using Nanobind Plant Nuclei Big DNA Kit. The procedures described herein are adopted from Workman, W. *et al* (2018) and manufacturer's Handbook for kit (v0.18) with minor modification. The method described in this SOP is suitable for extracting DNA from plant materials where the classical DNA extraction methods are not optimal for getting good quality HMW DNA compatible for long read sequencing technologies such as Nanopore. However, young tissue materials are required to get higher yield of isolated nuclei as well as easiness of grinding materials into a fine powder. This method requires working with liquid nitrogen for at least 30 minutes.

B. BRIEF SUMMARY OF METHOD

In this method, nuclei are isolated from young tissue materials to enrich nuclear DNA and get rid of pigments that interfere in the sequencing. In the next step, Nanobind Plant Nuclei Big DNA Kit (Circulomics) helps to extract intact HMW DNA.

C. DEFINITIONS AND ABBREVIATIONS

HB: Homogenisation Buffer
NIB: Nuclei Isolation Buffer
PVP-360K: Polyvinylpyrolidine average molecular weight 360,000
EDTA: Ethylenediaminetetraacetic acid
RT: Room Temperature
HMW DNA: High Molecular Weight DNA
DAPI: 4',6-diamidino-2-phenylindole

D. OCCUPATIONAL HEALTH AND SAFETY

No OH&S risk is identified with this SOP.

E. CAUTIONS

Use young tissues such as young leaves sprout or shoot or root etc. to get better yield of nuclei. Grind the tissues in liquid nitrogen into a fine powder to get better yield.

F. PERSONNEL QUALIFICATIONS, TRAINING AND RESPONSIBILITIES

Training Requirements:

[X] Read and Understand Document [X] Training Required [Author to delete X in relevant box]

G. EQUIPMENT AND MATERIALS

Equipment

a. Temperature controlled Centrifuge
b. Mortar and pestle
c. HulaMixer
d. Magnetic rack
e. Microscope (Florescence &/bright field)
f. Bucket for transporting liquid nitrogen
g. Magnetic rod and stirrer or equivalent
h. Nanodrop
i. Qubit

Materials and Reagents

a. Trizma Base (T6066-500G, Sigma Aldrich)
b. Potassium Chloride (P5405-500G, Sigma Aldrich)
c. EDTA (EDS-500G, Sigma Aldrich)
d. Spermidine trihydrochloride (S2501-1G, Sigma Aldrich)
e. Spermine tetrahydrichloride (S1141-1G, Sigma Aldrich)
f. Sodium hydroxide (1064980500, Merck)
g. Sucrose (S0389-500G, Sigma Aldrich)
h. Triton X-100 (T8787-100ML, Sigma Aldrich)
i. β-mercaptoethanol (M6250-100mL, Sigma Aldrich)
j. PVP-360K (PVP360-100G, Sigma Aldrich)
k. Nanobind Plant Nuclei Big DNA Kit (SKU NB-900-801-01, Circulomics)
l. Miracloth (US1475855-1R, Merck)
m. Wide bore pipette tips
n. Falcon tubes (50 ml and 15 ml)
o. Spatula
p. Beaker

H. PROCEDURE

A. Nuclei Isolation

Prepare following buffers before starting experiment

Homogenisation buffer (HB) stock 10× 100 ml

Reagent	Quantity
Trizma Base	1.21 gm
KCl	5.96 gm
0.5 M EDTA	20 ml
Spermidine trihydrochloride	0.255 gm
Spermine tetrahydrichloride	0.348 gm
ddH_2O	up to 100 ml

Note: Please adjust pH to 9-9.4 with 10N NaOH drops and can be stored at 4 °C in glass bottle for up to 1 year

1×HB (1000 ml)

Reagent	Quantity
10×HB	10 ml
Sucrose	171.2 gm
ddH_2O	up to 1000 ml

Note: Solution can be stored at 4 °C in glass bottle for 3 months

Triton X-100 (20% v/v, 100 ml) mix

Reagent	Quantity
Triton X-100	20 ml
10×HB	10 ml
Sucrose	17.15 gm
ddH_2O	up to 100 ml

Note: Solution can be stored at 4 °C in glass bottle for up to 1 year

Nuclei Isolation Buffer (NIB) (100ml for 5gm tissue materials and prepare this buffer on the day of experiment or stir all the reagent except β-mercaptoethanol overnight at cold room)

Reagent	Quantity
Triton X-100 mix	2.5 ml
1×HB	97.5 ml
β-mercaptoethanol	250 µL – should add immediately before use
PVP-360k	1 gm

Note: Stir ~1 h at room temperature to dissolve PVP

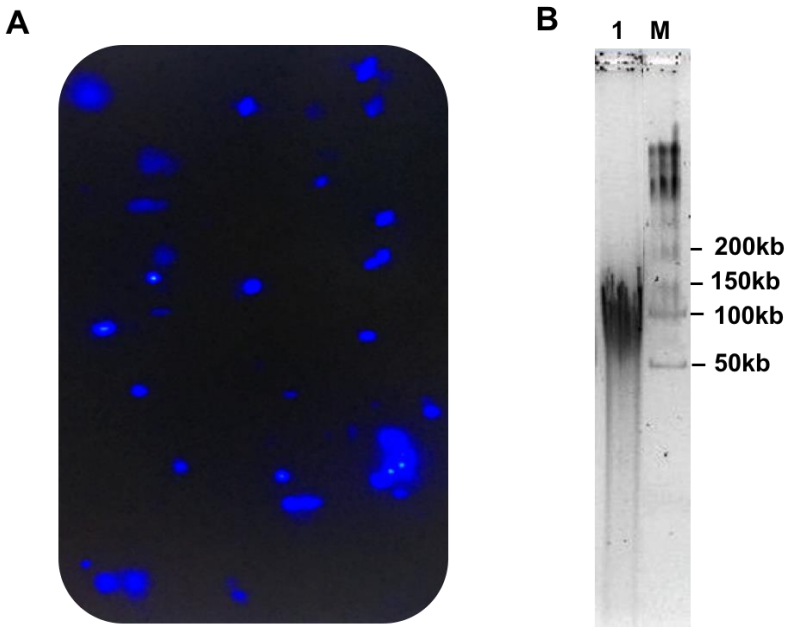
1. Grind 5 gm of healthy leaves (fresh or fresh snap freeze) in liquid nitrogen into fine powder using mortar and pestle. Grinding takes 20-30 min to make fine powder and topping up of liquid nitrogen 4-5 times.
2. Transfer the grinded leaves powder in a 500 ml beaker containing 50 ml (for 5 gm) ice-cold NIB and stir with magnetic stirrer for 15 min. Complete disruption of leaves powder clumps is essential for the better recovery of nuclei.
3. Gravity filter (20 min) the leaves powder solution through 5-layers of Miracloth in a cold room and collect the filtrate in 50 ml falcon tube.
4. Spin the filtrate at 3000×g for 20 min at 4 °C and discard the supernatant. Resuspend the pellet in 1-2 ml ice-cold NIB buffer using wide bore 1ml pipette tips and adjust the final volume of 45 ml with ice-cold NIB buffer.
5. Spin the resuspended solution at 200×g for 15 min at 4 °C and transfer the supernatant in a fresh 50 ml falcon tube. Spin the transferred supernatant at 3000×g for 15 min at 4 °C and discard the supernatant.
6. Resuspend the pellet in 1 ml of ice-cold NIB with the help of 1 ml wide bore pipette tips. Transfer the resuspended solution in a 15 ml falcon tube and make the final volume of 15 ml with ice-cold NIB. Mix the solution by inverting the tubes 5-6 times in cold room.
7. Spin the solution in 3000×g for 10 min at 4 °C. Discard the supernatant and resuspend the pellet with 15 ml ice-cold NIB.
8. Spin the solution at the same condition in step 7 and repeat this step 3-4 times or until the supernatant appears as a clear solution.
9. Resuspend the pellet with 1 ml ice-cold 1×HB buffer. Keep 20 µl to observe isolated nuclei under microscope (20× and 40×). Spin the remaining solution at 5000×g for 5 min and discard the supernatant. Snap freeze the pellet in liquid nitrogen and store at -80°C.

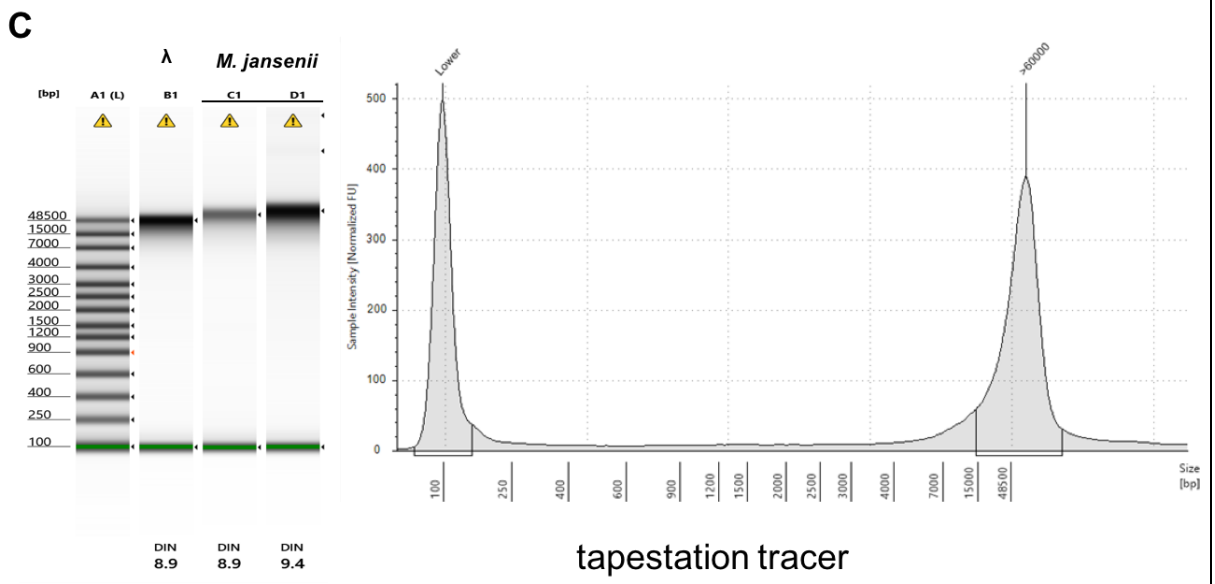
B. DNA extraction using Nanobind Plant Nuclei Big DNA Kit

1. Resuspend the nuclei pellet directly in 60 µl of proteinase-K and vertex until it becomes homogenous mixture. Vertexing in this step is very important for proper lysis of nuclei and getting pure DNA.
2. Add 15 µl of RNase and vertex 10× and incubate at RT for 5 min to remove RNA.
3. Add 160 µl of AP1 buffer and vertex 5× each for 2 sec. Vertexing of sample is necessary for the proper lysis and getting pure DNA.
4. Incubate the sample in a thermomixer at 55 °C, 900×g for 2 h. Check every 30 min and mix the sample by inverting the tubes 5-6 times and a quick spin down for 2 sec and continue incubation.
5. Spin the sample at 13,000×g for 5 min at RT and transfer the supernatant into a 1.5 ml Eppendorf tube containing Nanobind disk. Use wide bore pipette tips from this step. Adding Nanobind disk prior transferring the supernatant help to merge disc in the solution.
6. Add equal volume of 100% Isopropanol and spin the tube in Hulamixer for 20 min. Set the parameters of Hulamixer as: 9 rpm rotation, tiling for 70° for 12 sec and vibration 2° for 1 sec.
7. Place the tube in magnetic rack as recommended *Nanobind Plant Nuclei Big DNA Kit Handbook v0.18*, Page-11.
8. Discard the supernatant and avoid contacting Nanobind disk.
9. Add 500 µl of Buffer PW1, inversion mix 4×, replace the tubes in the magnetic rack and discard the supernatant. Repeat this step once.
10. Remove any residual liquid from cap if tube by spinning the tube on a mini centrifuge for 2 sec. Repeat this step. If the Nanobind disk is blocking the bottom of the tube, gently push it aside with the tip of the pipette. At this stage, DNA is tightly bound to the disk.
11. Add 50-200 µl Buffer EB and spin the tube on a mini centrifuge for 2 sec. Incubate at RT for 10 min. Confirm the entire Nanobind disk is fully immersed in the Buffer EB during incubation.
12. Collect DNA by transferring eluate to a new 1.5 ml Eppendorf tube. Use standard P200 pipette tip to remove residual liquid after much of the eluate has been removed with a side-bore pipette.
13. Spin the tube containing the Nanobind disk on a mini centrifuge for 5 sec and transfer any additional liquid that comes off the disk to the previous eluate. Repeat it if necessary. A small amount of white or gel like material may remain on the Nanobind disk after transferring the eluate in a step 12. This is HMW DNA that has not fully solubilised and this spin step will allow DNA to slide off the Nanobind disk into the bottom of the tube, after which it can be pipetted out and combined with the previously transferred eluate.
14. if the extracted HMW DNA is heterogenous and insolubilised, pipette mix 1-5 times with a standard P200 pipette tip and let the sample rest for overnight at 4 °C.
15. Analyse the recovery and purity of the DNA by NanoDrop and Qubit. The ratio A260/A280 and A260/A230 should be 1.6-2.0 and >1.8 respectively. Analyse the DNA integrity in TapeStation and the distribution of DNA size in Pulse-Field-Gel-Electrophoresis.

I. WORKED EXAMPLE

HMW DNA extraction from this SOP has successfully tested in young and mixed (young and mature) leaves tissue of Macadamia plant (both cultivar and wild type).





A: DAPI stained isolated nuclei under Fluorescence microscope (20xmagnification)

B: High Molecular Weight Nuclear DNA in Pulse Field Gel Electrophoresis; 1: HMW nuclear DNA extracted from *Macadamia jansenii* leaves and M: Lambda PFG Ladder (N0341S, NEB)

C: Tapestation result showing Highly Intact HMW DNA (DIN=8.9 – 9.4) of *M. jansenii*

J. SOP VALIDATION DETAILS

This SOP has been tested with two Macadamia species and sweet potato and the quality of HMW DNA has assessed through NanoDrop, Qubit, tapestation, PFGE and Nanopore sequencing. The result obtained from this SOP is comparable with the result shown in Workman et al (2018) and manufacturer's Handbook for kit (v0.18).

K. WASTE MANAGEMENT AND DISPOSAL

All the chemicals used in this SOP is very low amount and do not use any carcinogenic substances. The chemical wastes are disposed in clinical waste bin and buffers such as NIB and HB are poured through basin.

L. DATA RECORDS MANAGEMENT

There is no requirement of specific data management system. Users can save Quality assessment data such as microscopic pictures of isolated nuclei, TapeStation result, PFGE pictures according to their available data management system.

M. REFERENCE DOCUMENTS

1. *CIRCULOMICS 2018. Nanobind Plant Nuclei Big DNA Kit Handbook v0.18*
2. *WORKMAN, R., TIMP, A., FEDAK, R., KILBURN, D., HAO, S. & LIU, K. 2018. High Molecular Weight DNA Extraction from Recalcitrant Plant Species for Third Generation Sequencing. Protocol Exchange.*

N. QUALITY CONTROL (QC) AND QUALITY ASSURANCE (QA) SECTION

Following points are required to be considered for getting better performance of this SOP:

1. Tissue materials should be young and fresh (or snap freeze).

2. While grinding tissues materials, never let it be dried keep adding liquid N2 until the tissue turns into fine powder.

3. Observed isolated nuclei under microscope before moving into DNA extraction step. The quality and quantity of isolated nuclei determine performance of *Nanobind Plant Nuclei Big DNA Kit.*