PURIFICATION OF HIGH MOLECULAR WEIGHT DNA FROM CANNABIS SATIVA

GET HIGH MOLECULAR WEIGHT DNA COMPATIBLE WITH YOUR NGS WORKFLOWS

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CONTEXT

Long-read sequencing techniques such as Oxford Nanopore and PacBio require very long and very pure DNA. Because small fragments are preferentially sequenced, it is important to limit their presence to increase downstream contiguity. Plant cells are encased in a defensive cell wall and are frequently 80% vacuole. Isolation of nuclei removes the large fraction of nucleases and other hydrolytic components that can attack DNA upon lysis.

Cannabis tissues are rich in polyphenols, and so the use of reducing agents such as PVP (Polyvinylpyrrolidone) and BME (β-mercaptoethanol) are essential. Because important genes, such as the cannabinoid synthases, are highly duplicated and separated by large repetitive regions, long-read sequencing is necessary to resolve their structure with high fidelity.

Here the **Precellys Evolution homogenizer** has been used to **isolate nuclei** from *Cannabis sativa* **plant cells** and obtain **high molecular weight DNA**, compatible with NGS long-read sequencing techniques .

MATERIALS

- **Precellys Evolution** (Bertin Technologies, Montigny-le-Bretonneux, France)
- For 1g of sample: 6 empty reinforced **2mL tubes** (P000943-LYSK0-A) filled with **2.8mm stainless steel beads** (P000925-LYSK0-A) or 6 tubes of Precellys lysing kit **MK28R 2mL** (P000917-LYSK0-A). Alternatively, one **7mL tube** (P000944-LYSK0-A) filled **2.8mm stainless steel beads** (P000944-LYSK0-A)
- Buffers: As described in Circulomics' Nuclei Isolation Protocol (Circulomics, Baltimore, United States) and in Edwards 1991. Rather than preparing 10x Homogenization Buffer (HB), it was found that 1L of 1x is sufficient for tens of extractions.

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PROTOCOL

I. Nuclei Isolation (modified from Circulomics' protocol, (Circulomics, Baltimore, United States))

A. Tissue Collection and homogenization

1. Divide 1g of fresh tissue among six 2mL tubes with 2.8mm metal beads, or use one 7mL tubes with 2.8mm metal beads (see **Materials)**
    a. Young shoot tips are preferred.
    b. It may be beneficial to incubate in the dark for 3 days prior to extraction, to reduce carbohydrate content.
2. Snap-freeze in liquid nitrogen .
3. Disrupt frozen samples at 6200 RPM for 5s with the **Precellys Evolution**

B. Nuclei Purification

1. Add 1.6 mL of Nuclei Isolation Buffer (NIB) to each tube with a disposable pipet and resuspend.
    a) The buffer will likely freeze on contact with the matrix.
    b) Disruption liberates abundant nucleases. It is important to saturate the cells with buffer as soon as they thaw, so that the EDTA can chelate metal ions and inactivate them.
2. Incubate with gentle shaking at room temperature for 15m.
3. Filter at first 60 um and then 30 um into new 2 mL tubes.
    a) This recommendation is based on pour-through filters.
    b) With suction, as in a Steriflip, a single filtration at 25 uM may be possible.
4. Pellet nuclei at 1500g for 1m at 4C and remove supernatant.
    a) Smaller genomes *may* require stronger centrifugation.
    b) If the pellet includes green flecks, try to spin more softly.
5. Resuspend pellet in 1 mL ice-cold NIB by pipetting.
6. Repeat steps 4 and 5 until the supernatant is absolutely clear.
    a) This will require 2-5 washes depending on the state of the starting material.
    b) Wide-bore tips may be preferred but appear to not be essential.
7. Resuspend pellet in 1x ice-cold HB (Homogenization Buffer) and spin down at 1500g for 1m at 4C.
8. Remove HB ((Homogenization Buffer) and proceed to DNA purification, or store pellet at -80C.

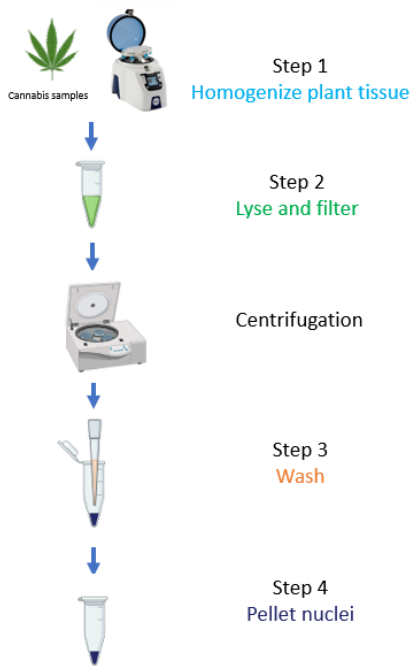

***Figure 1**. Workflow for nuclei isolation*

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PROTOCOL

II. DNA purification

A. Lysis

1. Resuspend pellet in 30 uL Proteinase-K and vortex strongly for 20s.
    a) Thorough resuspension is critical. Try to ensure that each nuclei is surrounded by proteinase prior to lysis.
2. Add 400 uL of Edward's buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25mM EDTA, 0.5% SDS) and vortex briefly to mix.
    a) Beyond this point the DNA is fragile as it is no longer condensed around histones.
    b) Wide-bore tips and gentle movements are encouraged.
3. Incubate at 65°C for 30m-2h.
4. Pellet cellular debris at 12000g for 5m.
5. Remove 400 uL supernatant to a new tube.

B. Purification

1. Add 200 uL of 1M KCl, mix by inversion, and incubate at room temperature for 5m.
    a) KCl is used in place of KOAc here to minimize acid hydrolysis.
    b) Potassium dodecyl sulfate seems to be sufficiently insoluble at both acid and neutral pH.
2. Spin down precipitate at 12000g for 5m and remove 500 uL supernatant to a new tube.
3. Add 2.5 uL RNAse, mix by inversion, and incubate at RT for 5m.
4. Add 500 uL $CHCl_3$ and incubate at room temperature for 10m with occasional inversion.
5. Separate phases at 5000g for 1m.
6. Transfer 400 uL of aqueous layer to new tube. There may be a precipitate at the interphase layer. Be sure to not disturb it.
7. Add 200 uL of saturated NaCl and mix by inversion.
8. Add 600 uL of 95% EtOH and mix by inversion. DNA gel should soon be visible.
9. Hook out the DNA with a plastic inoculation loop or p200 tip and transfer to a tube containing 1 mL 70% EtOH.
10. Incubate at room temperature for 10m.
11. Hook out into another tube with 1 mL 70% EtOH.
12. Spin down at 1000g for 1m.
13. Remove alcohol and air dry the gel at RT for 30m. It should be clear.
14. Resuspend in 100 uL EB (10 mM Tris-8.0, no EDTA).
15. Incubate at room temperature overnight to allow DNA to relax prior to QC.

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RESULTS

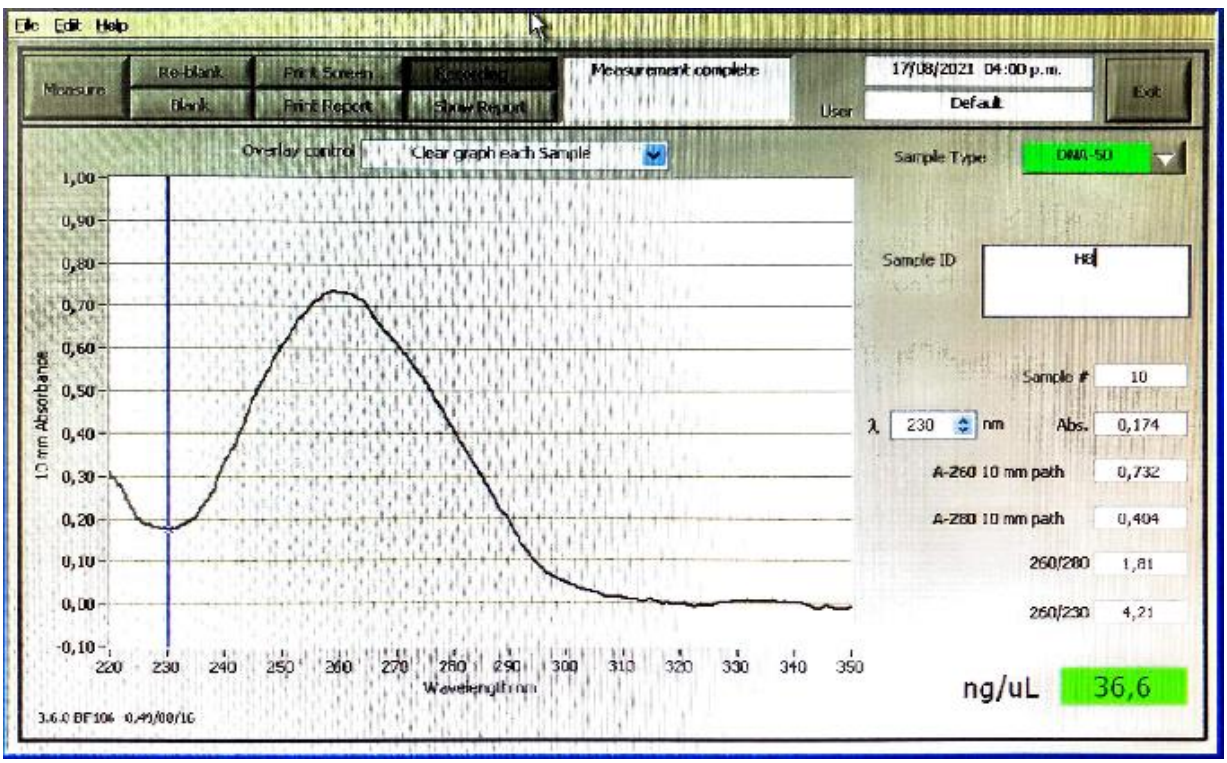

Figure 2. *Nanodrop results for* Cannabis sativa *samples.*

These results indicate that the proposed method leads to a high DNA yield. The 260/230 ratio shows that the DNA obtained is very pure.

Yield: For 1g of young *Cannabis sativa* shoots, the average yield is 4 ug.

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CONCLUSION

The Precellys Evolution homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) can be used to isolate nuclei from *Cannabis sativa* plant samples. Here, the nonpsychoactive variety 'Cherry Pie #16' was processed in accordance with Resolution 1164 of 2021, issued by the Ministry of Justice of the Republic of Colombia. After homogenization with the Precellys, the resulting sample can then be used to perform DNA extraction and obtain high-quality DNA, compatible with NGS long-read sequencing techniques, such as Oxford Nanopore and PacBio. These results should guide future studies of the locus encoding THC and CBD synthases in *Cannabis sativa.*

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